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FIELDS **VIROLOGY**

SIXTH EDITION

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Jeffrey I. Cohen
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Robert A. Lamb
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VOLUME ONE

VOLUME I

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Stephen E. Straus, 1946–2007

Steve Straus was the consummate physician–scientist with broad interests in the basic science and clinical aspects of viral and immunological diseases and therefore was an ideal person to serve as clinical virology editor for *Fields Virology*. We were fortunate to work with him in his role as associate editor for the third through fifth editions of *Fields Virology*. However, unfortunately, with Steve's premature death in 2007, we lost our friend, colleague, and fellow editor. Steve's medical training and accomplishments are detailed elsewhere (*J Infect Dis* 2007;196:963–964). His research interests were broad and included the molecular biology and pathogenesis of varicella-zoster and herpes simplex viruses, acyclovir suppression of oral and genital herpes simplex viruses, antiviral drug resistance, clinical testing of herpes simplex virus and varicella zoster virus vaccines, chronic active Epstein–Barr virus, chronic fatigue syndrome, and autoimmune lymphoproliferative syndrome. Steve was one of the leading scientists in the National Institutes of Health intramural program, serving as chief of the Laboratory of Clinical Investigation at the National Institute of Allergy and Infectious Diseases and the founding director of the National Center for Complementary and Alternative Medicine.

Steve cowrote the chapter on varicella zoster virus, and additionally worked effectively as an associate editor, for the third to fifth editions of *Fields Virology*. He seemed to read and edit the chapters immediately upon their submission, amazing us with his ability to do all of this on top of his other responsibilities. Steve was diagnosed with brain cancer in 2004 but insisted on editing chapters for the fifth edition right through the compiling of the chapters. The book was published in early 2007, not long before his death in May 2007.

On behalf of everyone who contributed to the sixth edition of *Fields Virology*, we dedicate this book to the memory of Stephen E. Straus, MD.

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In the early 1980s, Bernie Fields originated the idea of a virology reference textbook that combined the molecular aspects of viral replication with the medical features of viral infections. This broad view of virology reflected Bernie's own research, which applied molecular and genetic analyses to the study of viral pathogenesis, providing an important part of the foundation for the field of molecular pathogenesis. Bernie led the publication of the first three editions of *Virology* but unfortunately died soon after the third edition went into production. The third edition became *Fields Virology* in his memory, and it is fitting that the book continues to carry his name.

We are pleased that the printed book of the sixth edition of *Fields Virology* contains four-color art throughout and that an e-book version accompanies the printed book as well. We have increased the numbers of figures in each chapter, and with the color and availability of the figures from the e-book for use as slides, most chapters should have sufficient figures for slides for one lecture. There have been continued significant advances in virology since the previous edition 6 years ago, and all of the chapters have been updated to reflect these advances. Our increased knowledge of virology has caused us to use shortened lists of key references (up to 200 in most cases) in the printed book to save space, whereas complete reference lists appear as part of the e-book. We have retained the general organization of the earlier editions for the sixth edition of *Fields Virology*. Section I contains chapters on general aspects of virology, and Section II contains chapters on replication and medical aspects of specific virus families and specific viruses of medical importance. In Section I, we have added a new emphasis on virus discovery in the Diagnostic Virology chapter and emerging viruses in the Epidemiology chapter to address the interest in discovery of new viruses and emerging viruses. In Section II, we have added new chapters on circoviruses and mimiviruses and have added a new section on Chikungunya virus to the alphavirus chapter.

Numerous chapters have been updated to include the latest information on outbreaks during the past 5 years, including pandemic H1N1 influenza, new adenovirus serotypes, noroviruses, human polyomaviruses, the re-emergence of West Nile virus in North America, novel coronaviruses, novel Coxsackie and rhino viruses, and other emerging and re-emerging viruses. Important advances in antivirals, including new hepatitis C virus protease inhibitors and HIV integrase inhibitors, have been described. As with the previous edition, we have continued to combine the medical and replication chapters into a single chapter to eliminate duplication and to present a more coherent presentation of that specific virus or virus family. The main emphasis continues to be on viruses of medical importance and interest; however, other viruses are described in specific cases where more is known about their mechanisms of replication or pathogenesis. Although not formally viruses, prions are still included in this edition for historical reasons and because of the intense interest in the infectious spongiform encephalopathies.

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Lynn W. Enquist • Vincent R. Racaniello

Virology: From Contagium Fluidum to Virome

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Pathogen Discovery

Arnold Levine's Afterword: d'Herelle's Dream and Koch's Postulates (see eBook)

Virology has had a remarkable history. Even though humans did not realize viruses existed until the late 1880s, viral diseases have shaped the history and evolution of life on the planet. As far as we know, all living organisms, when studied carefully, are infected by viruses. These smallest microbes exert significant forces on every living thing, including themselves. The consequences of viral infections have not only altered human history, they have powerful effects on the entire ecosystem. As a result, virologists have gone to extraordinary lengths to study, understand, and eradicate these agents. It is noteworthy that just as the initial discovery of viruses required new technology (porcelain filters), uncovering the amazing biology underlying viral infections has gone hand in hand with new technology developments. Indeed, virologists have elucidated new principles of life processes and have been leaders in promoting new directions in science. For example, many of the concepts and tools of molecular biology and cell biology have been derived from the study of viruses and their host cells. This chapter is an attempt to review selected portions of this history as it relates to the development of new concepts in virology.

THE CONCEPT OF VIRUSES AS INFECTIOUS AGENTS

A diverse microbial world of bacteria, fungi, and protozoa had been widely accepted by the last half of the 19th century. An early proponent of the germ theory of disease was the noted German anatomist Jacob Henle of Gottingen (the discoverer of Henle's loop and the grandfather of 20th-century virologist Werner Henle). He hypothesized in 1840 that specific diseases were caused by infectious agents that were too small to be observed with the light microscope. However, he had no evidence for such entities, and consequently his ideas were not generally accepted. It would take the work of Louis Pasteur and Henle's student, Robert Koch, before it became evident that microbes could cause diseases.



Three major advances in microbiology came together to set the stage for the development of the concept of a submicroscopic agent that would come to be called a virus (e-Table 1.1). The first advance concerned spontaneous generation of organisms, which for years had been both supported and refuted by a variety of experiments. Louis Pasteur (1822–1895) used his swan-neck flasks to strike a mortal blow to the concept of spontaneous generation. Afterward Pasteur went on to study fermentation by different microbial agents. From his work he concluded that “different kinds of microbes are associated with different kinds of fermentations,” and he soon extended this concept to diseases. Pasteur’s reasoning strongly influenced Robert Koch (1843–1910), a student of Jacob Henle and a country doctor in a small German village. Koch developed solid media to isolate colonies of bacteria to produce pure cultures, and stains to visualize the microorganisms. With these tools in hand, Koch identified the bacterium that causes anthrax (*Bacillus anthracis*, 1876) and tuberculosis (*Mycobacterium tuberculosis*, 1882). Joseph Lister (1827–1912), a professor of surgery in Glasgow, had heard about Pasteur’s work, and he surmised that a sterile field should be maintained during surgery. Although many other scientists of that day contributed tools and concepts, it was principally Pasteur, Lister, and Koch who put together a new experimental approach for medical science.

These observations led Robert Koch to formalize some of Jacob Henle’s original ideas for defining whether a microorganism is the causative agent of a disease. Koch’s postulates state that (a) the organism must be regularly found in the lesions of the disease, (b) the organism must be isolated in pure culture, (c) inoculation of such a pure culture of organisms into a host should initiate the disease, and (d) the organism must be recovered once again from the lesions of the host. By the end of the 19th century, these concepts outlined an experimental method that became the dominant paradigm of medical microbiology. It was only when these rules broke down and failed to yield a causative agent that the concept of a virus was born.

THE BIRTH OF VIROLOGY

Pathogen Discovery, 1886–1903 (e-Table 1.1)

Adolf Mayer (1843–1942) was a German agricultural chemist and director of the Agricultural Experiment Station at Wageningen in The Netherlands when he was asked to investigate a disease of tobacco. He named the affliction tobacco mosaic disease after the dark and light spots that appeared on infected leaves (e-Fig. 1.1). To investigate the nature of the disease, Mayer inoculated healthy plants with the juice extracted from diseased plants by grinding up the infected leaves in water. Mayer reported that, “in nine cases out of ten (of inoculated plants), one will be successful in making the healthy plant... heavily diseased”.¹³¹ Although these studies established the infectious nature of the tobacco mosaic disease, neither a bacterial agent nor a fungal agent could be consistently cultured or detected in these extracts, so Koch’s postulates could not be satisfied. In a preliminary communication in 1882,¹³⁰ Mayer speculated that the cause could be a “soluble, possibly enzyme-like contagium, although almost any analogy for such a supposition is failing in science.” Later Mayer concluded that the mosaic disease “is bacterial, but that the infectious forms have not yet been isolated, nor are their forms and mode of life known”.¹³¹

A few years later, Dimitri Ivanofsky (1864–1920), a Russian scientist working in St. Petersburg, was commissioned by the Russian Department of Agriculture to investigate the cause of a tobacco disease on plantations in Bessarabia, Ukraine, and the Crimea. Ivanofsky repeated Mayer’s observations by showing that the sap of infected plants contained an agent that could transmit the disease to healthy plants. But he added an important step—before the inoculation step, he passed the infected sap through a Chamberland filter (e-Fig. 1.2). This device, made of unglazed porcelain and perfected by Charles Chamberland, one of Pasteur’s collaborators, contained pores small enough to retard most bacteria. Ivanofsky reported to the Academy of Sciences of St. Petersburg on February 12, 1892, that “the sap of leaves infected with tobacco mosaic disease retains its infectious properties even after filtration through Chamberland filter candles”.⁹⁴

Ivanofsky, like Mayer before him, failed to culture an organism from the filtered sap and could not satisfy Koch’s postulates. Consequently he suggested that a toxin (not a living, reproducing substance) might pass through the filter and cause the disease. As late as 1903, when Ivanofsky published his thesis,⁹⁵ he still believed that he had been unable to culture the bacteria that caused this disease. Bound by the dogma of Koch’s postulates, Ivanofsky could not make a conceptual leap. It is therefore not surprising that Pasteur, who worked on the rabies vaccine¹⁴⁵ at the same time (1885), never investigated the unique nature of the infectious agent.

The conceptual leap was provided by Martinus Beijerinck (1851–1931), a Dutch soil microbiologist who collaborated with Adolf Mayer at Wageningen. Unaware of Ivanofsky’s work, in 1898 Beijerinck independently found that the sap of infected tobacco plants could retain its infectivity after passage through a Chamberland filter. But he also showed that the filtered sap could be diluted and regain its “strength” after replication in living, growing tissue of the plant. This observation showed that the agent could reproduce (therefore, it was not a toxin) but only in living tissue, not in the cell-free sap of the plant. Suddenly it became clear why others could not culture the pathogen outside its host. Beijerinck called this agent a *contagium vivum fluidum*,¹⁰ or a contagious living liquid. He sparked a 25-year debate about whether these novel agents were liquids or particles. This conflict was resolved when d’Herelle developed the plaque assay in 1917³⁶ and when the first electron micrographs were taken of tobacco mosaic virus (TMV) in 1939.¹⁰⁴

Mayer, Ivanofsky, and Beijerinck each contributed to the development of a new concept: a novel organism smaller than bacteria—an agent defined by the pore size of the Chamberland filter—that could not be seen in the light microscope, and could multiply only in living cells or tissue. The term *virus*, from the Latin for slimy liquid or poison,⁸⁹ was at that time used interchangeably for any infectious agent, and so the agent of tobacco mosaic disease was called tobacco mosaic virus, or TMV. The literature of the first decades of the 20th century often referred to these infectious entities as filterable agents, and this was indeed the operational definition of viruses. Sometime later, the term *virus* became restricted in use to those agents that fulfilled the criteria developed by Mayer, Ivanofsky, and Beijerinck, and that were the first agents to cause a disease that could not be proven by using Koch’s postulates.

Shortly after this pioneering work on TMV, the first filterable agent from animals was identified by Loeffler and Frosch—foot-and-mouth disease virus.¹²² The first human virus discovered was yellow fever virus (1901), by Walter Reed and his team in Cuba.¹⁵⁴

The years from 1930 to 1956 were replete with the discovery of a plethora of new viruses (e-Table 1.2). In fact, in this short time, virologists found most of the viruses we now know about. More fascinating perhaps is that these studies laid the groundwork for the birth of molecular virology.

Plant Viruses and the Chemical Period: 1929–1956

For the next 50 years, TMV played a central role in research that explored the nature and properties of viruses. With the development of techniques to purify proteins in the first decades of the 20th century came the appreciation that viruses were proteins and so could be purified in the same way. Working at the Boyce Thompson Institute in Philadelphia, Vinson and Petre (1927–1931) precipitated infectious TMV—using an infectivity assay developed by Holmes⁸⁸—from the crude sap of infected plants using selected salts, acetone, or ethyl alcohol.¹⁹³ They showed that the infectious virus could move in an electric field, just as proteins did. At the same time, H. A. Purdy-Beale, also at the Boyce Thompson Institute, produced antibodies in rabbits that were directed against TMV and could neutralize the infectivity of this agent.¹⁵¹ This observation was taken as further proof of the protein nature of viruses, although it was later realized that antibodies recognize chemicals other than proteins. With the advent of purification procedures for viruses, both physical and chemical measurements of the virus became possible. The strong flow birefringence of purified preparations of TMV was interpreted (correctly) to show an asymmetric particle or rod-shaped particle.¹⁸⁰ Max Schlesinger,¹⁶⁷ working on purified preparations of bacteriophages in Frankfurt, Germany, showed that the virions were composed of proteins and contained phosphorus and ribonucleic acid. This observation led to the first suggestion that viruses were composed of nucleoproteins. The crystallization of TMV in 1935 by Wendell Stanley,¹⁷³ working at the Rockefeller Institute branch in Princeton, New Jersey, brought this infectious agent into the world of the chemists. Within a year, Bawden and Pirie^{8,9} had demonstrated that crystals of TMV contained 0.5% phosphorus and 5% RNA. The first “view” of a virus came from x-ray crystallography using these crystals to show rods of a constant diameter aligned in hexagonal arrays containing RNA and protein.¹⁶ The first electron micrographs of any virus were of TMV, and they confirmed that the virus particle is shaped like a rod¹⁰⁵ (e-Fig. 1.3).

The x-ray diffraction patterns¹⁶ suggested that TMV was built from repeating subunits. These data and other considerations led Crick and Watson³³ to realize that most simple viruses had to consist of one or a few species of identical protein subunits. By 1954–1955, techniques had been developed to dissociate TMV protein subunits, allowing reconstitution of infectious TMV from its RNA and protein subunits⁶⁴ and leading to an understanding of the principles of virus self-assembly.²⁵

The concept that viruses contained genetic information emerged as early as 1926, when H. H. McKinney reported the isolation of “variants” of TMV with a different plaque

morphology that bred true and could be isolated from several geographic locations.^{132,133} Seven years later, Jensen confirmed McKinney’s observations¹⁰¹ and showed that the plaque morphology phenotype could revert. Avery’s DNA transformation experiments with pneumococcus⁵ and the Hershey-Chase experiment with bacteriophages,⁸³ both demonstrated that DNA was genetic material. TMV had been shown to contain RNA, not DNA, and this nucleic acid was shown to be infectious, and therefore comprise the genetic material of the virus, in 1956^{64,72}—the first demonstration that RNA could be a genetic material. Studies on the nucleotide sequence of TMV RNA confirmed codon assignments for the genetic code, added clear evidence for the universality of the genetic code, and helped to elucidate the mechanisms of mutation by diverse agents.⁶³ Research on TMV and related plant viruses has contributed significantly to both the origins of virology and its development as a science.

BACTERIOPHAGES

Early Years: 1915–1940

Frederick W. Twort was superintendent of the Brown Institution in London when he discovered viruses of bacteria in 1915. In his research, Twort was searching for variants of vaccinia virus (the smallpox vaccine virus), which would replicate in simple defined media outside living cells. In one of his experiments, he inoculated nutrient agar with an aliquot of the smallpox vaccine. The virus failed to replicate, but bacterial contaminants flourished on the agar medium. Twort noticed that some of these bacterial colonies changed visibly with time and became “watery looking” (i.e., more transparent). The bacteria within these colonies were apparently dead, as they could no longer form new colonies on fresh agar plates. He called this phenomenon glassy transformation. Simply adding the glassy transforming principle could rapidly kill a colony of bacteria. It readily passed through a porcelain filter, could be diluted a million-fold, and when placed upon fresh bacteria would regain its strength, or titer.^{188–190}

Twort published these observations in a short note¹⁹⁰ in which he suggested that a virus of bacteria could explain glassy transformation. He then went off to serve in World War I, and when he returned to London, he did not continue this research.

While Twort was puzzled by glassy transformation, Felix d’Herelle, a Canadian medical bacteriologist, was working at the Pasteur Institute in Paris. When a *Shigella* dysentery infection devastated a cavalry squadron of French soldiers just outside of Paris in August 1915, d’Herelle readily isolated and cultured the dysentery bacillus from filtered fecal emulsions. The bacteria multiplied and covered the surface of his agar plates, but occasionally d’Herelle observed clear circular spots devoid of growth. He called these areas *taches vierges*, or plaques. He followed the course of an infection in a single patient, noting when the bacteria were most plentiful and when the plaques appeared.^{35,36} Plaques appeared on the fourth day after infection and killed the bacteria in the culture dish, after which the patient’s condition began to improve.

d’Herelle found that a filterable agent, which he called a bacteriophage, was killing the *Shigella* bacillus. In the ensuing years he developed fundamental techniques in virology that are utilized to this day, such as the use of limiting dilutions to

determine the virus titer by plaque assay. He reasoned that the appearance of plaques showed that the virus was particulate, or “corpuscular,” and not a liquid as Beijerinck had insisted. d’Herelle also found that if virus was mixed with a host cell and then subjected to centrifugation, the virus was no longer present in the supernatant fluid. He interpreted this to mean that the first step of a virus infection is attachment, or adsorption, of virus to the host cell. Furthermore, viral attachment occurred only when bacteria sensitive to the virus were used, demonstrating that host specificity can be conferred at a very early step in infection. Lysis of cells and the release of infectious virus were also described in startlingly modern terms. d’Herelle clearly established many of the principles of modern virology.^{34,35}

Although d’Herelle’s bacteriophages lysed their host cells, by 1921 it had become apparent that under certain situations the virus and cell existed peacefully—a condition called lysogeny. In some experiments it became impossible to separate the virus from its host. This conundrum led Jules Bordet of the Pasteur Institute in Brussels to suggest that the transmissible agent described by d’Herelle was nothing more than a bacterial enzyme that stimulates its own production.²² Although incorrect, the hypothesis has remarkable similarities to modern ideas about prion structure and replication (see Chapter 77).

During the 1920s and 1930s, d’Herelle sought ways to use bacteriophages for medical applications, but he never succeeded. Furthermore, the basic research of the era was frequently dominated by the interpretations of scientists with the strongest personalities. Although it was clear that there were many diverse bacteriophages, and that some were lytic while some were lysogenic, their interrelationships remained ill defined. The highlight of this period was the demonstration by Max Schlesinger that purified phages had a maximum linear dimension of 0.1 micron and a mass of about 4×10^{-16} grams, and that they were composed of protein and DNA in roughly equal proportions.^{166,167} In 1936, no one quite knew what to make of that observation, but over the next 20 years it would begin to make a great deal of sense.

Phages and the Birth of Molecular Biology: 1938–1970 (e-Table 1.3)

Max Delbrück was trained as a physicist at the University of Göttingen, and his first position was at the Kaiser Wilhelm Institute for Chemistry in Berlin. There he joined a diverse group of individuals who were actively discussing how quantum physics related to an understanding of heredity. Delbrück’s interest in this area led him to develop a quantum mechanical model of the gene, and in 1937 he moved to the biology division at the California Institute of Technology to study genetics of *Drosophila*. Once there, he became interested in bacteria and their viruses, and teamed up with another research fellow, Emory Ellis,⁵¹ who was working with the T-even group of bacteriophages, T2, T4, and T6. Delbrück soon appreciated that these viruses were ideal for the study of virus replication, because they allowed analysis of how genetic information could determine the structure and function of an organism. Bacteriophages were also viewed as model systems for understanding cancer viruses or even for understanding how a sperm fertilizes an egg and a new organism develops. Together with Ellis, Delbrück showed that viruses reproduced in one step, in contrast to the multiplication of other organisms by binary

fission.⁵² This conclusion was drawn from the elegant one-step growth curve experiment, in which an infected bacterium liberates hundreds of phages synchronously after a half-hour period during which viral infectivity was lost (e-Fig. 1.4). The one-step growth curve became the experimental paradigm of the phage group.

When World War II erupted, Delbrück remained in the United States (at Vanderbilt University) and met an Italian refugee, Salvador E. Luria, who had fled to America and was working at Columbia University in New York (on bacteriophages T1 and T2). After their encounter at a meeting in Philadelphia on December 28, 1940, they went to Luria’s laboratory at Columbia where they spent 48 hours doing experiments with bacteriophages. These two scientists eventually established the “phage group,” a community of researchers focused on using bacterial viruses as a model for understanding life processes. Luria and Delbrück were invited to spend the summer of 1941 at Cold Spring Harbor Laboratory, where they pursued research on phages. The result was that a German physicist and an Italian geneticist joined forces during the war years to travel throughout the United States and recruit a new generation of biologists (e-Fig. 1.5).

When Tom Anderson, an electron microscopist at the RCA Laboratories in Princeton, New Jersey, met Delbrück, the result was the first clear pictures of bacteriophages.¹²⁶ At the same time, the first phage mutants were isolated and characterized.¹²⁵ By 1946, the first phage course was being taught at Cold Spring Harbor, and in March 1947, the first phage meeting attracted eight people. From these humble beginnings grew the field of molecular biology, which focused on the bacterial host and its viruses.

Developing the Modern Concept of Virology (see e-Tables 1.3 to 1.5)

The next 25 years (1950–1975) was an intensely productive period of bacteriophage research. Hundreds of virologists produced thousands of publications that covered three major areas: (a) lytic infection of *Escherichia coli* with the T-even phages; (b) the nature of lysogeny, using lambda phage; and (c) the replication and properties of several unique phages such as ϕ X174 (single-stranded circular DNA), the RNA phages, and T7. This work set the foundations for modern molecular virology and biology.

The idea of examining, at the biochemical level, the events occurring in phage-infected cells during the latent period had come into its own by 1947–1948. Impetus for this work came from Seymour Cohen, who had trained first with Erwin Chargaff at Columbia University, studying lipids and nucleic acids, and then with Wendell Stanley working on TMV RNA. His research direction was established when after taking Delbrück’s 1946 phage course at Cold Spring Harbor, Cohen examined the effects of phage infection on DNA and RNA levels in infected cells using a colorimetric analysis. The results showed a dramatic alteration of macromolecular synthesis in infected cells. This included cessation of RNA accumulation, which later formed the basis for detecting a rapidly turning-over species of RNA and the first demonstration of messenger RNA (mRNA).⁴ DNA synthesis also halted, but for 7 minutes, followed by resumption at a 5- to 10-fold increased rate. At the same time, Monod and Wollman showed that the synthesis of a cellular enzyme, the inducible β -galactosidase, was inhibited

after phage infection.¹³⁴ Based on these observations, the viral eclipse period was divided into an early phase, prior to DNA synthesis, and a late phase. More importantly, these results demonstrated that a virus could redirect cellular macromolecular synthetic processes in infected cells.³²

By the end of 1952, two experiments had a critical effect on virology. First, Hershey and Chase asked whether viral genetic information is DNA or protein. They differentially labeled viral proteins ($^{35}\text{SO}_4$) and nucleic acids ($^{32}\text{PO}_4$), and allowed the “tagged” particles to attach to bacteria. When they sheared the viral protein coats from the bacteria using a Waring blender, only DNA was associated with the infected cells.⁸³ This result proved that DNA had all the information needed to reproduce new virus particles. A year later, the structure of DNA was elucidated by Watson and Crick, a discovery that permitted full appreciation of the Hershey-Chase experiment.¹⁹⁵ The results of these two experiments formed a cornerstone of the molecular biology revolution.²⁶

While these blockbuster experiments were being carried out, G. R. Wyatt and S. S. Cohen were quietly making another seminal finding.²⁰⁷ They identified a new base, hydroxymethylcytosine, in the DNA of T-even phages, which replaced cytosine. This began a 10-year study of how deoxyribonucleotides were synthesized in bacteria and phage-infected cells, and it led to the critical observation that the virus introduces genetic information for a new enzyme into the infected cell.⁶⁰ By 1964, Mathews and colleagues had proved that hydroxymethylase does not exist in uninfected cells and must be encoded by the virus.³² These experiments introduced the concept of early enzymes, utilized in deoxypyrimidine biosynthesis and DNA replication,¹⁰⁹ and provided biochemical proof that viruses encode new information that is expressed as proteins in an infected cell. At the same time, phage genetics became extremely sophisticated, allowing mapping of the genes encoding these viral proteins. Perhaps the best example of genetic fine structure was done by Seymour Benzer, who carried out a genetic analysis of the rII A and B cistrons of T-even phages with a resolution of a single nucleotide (without doing any DNA sequencing!).¹³ Studies on viral DNA synthesis, using phage mutants and cell extracts to complement and purify enzyme activities *in vitro*, contributed a great deal to our understanding of DNA replication.¹ A detailed genetic analysis of phage assembly, utilizing the complementation of phage assembly mutants *in vitro*, revealed how complex structures are built by living organisms using the principles of self-assembly.⁴⁷ The genetic and biochemical analysis of phage lysozyme helped to elucidate the molecular nature of mutations,¹⁷⁶ and the isolation of phage amber mutations (nonsense mutations) provided a clear way to study second-site suppressor mutations at the molecular level.¹⁴ The circular genetic map of the T-even phages¹⁷⁶ was explained by the circularly permuted, terminally redundant (giving rise to phage heterozygotes) conformation of these DNAs.¹⁸⁶

The remarkable reprogramming of viral and cellular protein synthesis in phage-infected cells was dramatically revealed by an early use of sodium dodecyl sulfate (SDS)–polyacrylamide gels,¹¹² showing that viral proteins are made in a specific sequence of events. The underlying mechanism of this temporal regulation led to the discovery of sigma factors modifying RNA polymerase and conferring gene specificity.⁷⁵ The study of gene regulation at almost every level (transcription, RNA

stability, protein synthesis, protein processing) was revealed from a set of original contributions derived from an analysis of phage infections.

Although this remarkable progress had begun with the lytic phages, no one knew quite what to make of the lysogenic phages. This situation changed in 1949 when André Lwoff began his studies with *Bacillus megaterium* and its lysogenic phages at the Pasteur Institute. By using a micromanipulator, Lwoff could show that single lysogenic bacteria divided up to 19 times without liberating a virus particle. No virions were detected when lysogenic bacteria were broken open by the investigator. But from time to time a lysogenic bacterium spontaneously lysed and produced many viruses.¹²⁸ Ultraviolet light was found to induce the release of these viruses, a key observation that began to outline this curious relationship between a virus and its host.¹²⁹ By 1954, Jacob and Wollman^{97,98} at the Pasteur Institute had made the important observation that a genetic cross between a lysogenic bacterial strain and a non-lysogenic recipient resulted in the induction of the virus after conjugation, a process they called zygotic induction. In fact, the position of the lysogenic phage or prophage in the chromosome of its host *E. coli* could be mapped by interrupting mating between two strains.⁹⁸ This experiment was crucial for our understanding of lysogenic viruses, because it showed that a virus behaved like a bacterial gene on a chromosome in a bacterium. It was also one of the first experimental results to suggest that the viral genetic material was kept quiescent in bacteria by negative regulation, which was lost as the chromosome passed from the lysogenic donor bacteria to the nonlysogenic recipient host. This conclusion helped Jacob and Monod to realize as early as 1954 that the “induction of enzyme synthesis and of phage development are the expression of one and the same phenomenon”.¹²⁸ These experiments laid the foundation for the operon model and the nature of coordinate gene regulation.

Although the structure of DNA was elucidated in 1953¹⁹⁵ and zygotic induction was described in 1954, the relationship between the bacterial chromosome and the viral chromosome in lysogeny was still referred to as the attachment site and literally thought of in those terms. The close relationship between a virus and its host was appreciated only when Campbell proposed the model for lambda integration of DNA into the bacterial chromosome,²⁷ based on the fact that the sequence of phage markers was different in the integrated state than in the replicative or vegetative state. This model led to the isolation of the negative regulator or repressor of lambda, a clear understanding of immunity in lysogens, and one of the early examples of how genes are regulated coordinately.¹⁵⁰ The genetic analysis of the lambda bacteriophage life cycle is one of the great intellectual adventures in microbial genetics.⁸² It deserves to be reviewed in detail by all students of molecular virology and biology.

The lysogenic phages such as P22 of *Salmonella typhimurium* provided the first example of generalized transduction,²¹⁰ whereas lambda provided the first example of specialized transduction.¹³⁷ The finding that viruses could not only carry within them cellular genes, but transfer those genes from one cell to another, provided not only a method for fine genetic mapping but also a new concept in virology. As the genetic elements of bacteria were studied in more detail, it became clear that there was a remarkable continuum from lysogenic phages to episomes,

transposons and retrotransposons, insertion elements, retroviruses, hepadnaviruses, viroids, and prions. Genetic information moves between viruses and their hosts to the point where definitions and classifications begin to blur. The genetic and biochemical concepts that emerged from the study of bacteriophages made the next phase of virology possible. The lessons of the lytic and lysogenic phages were often relearned and modified as the animal viruses were studied.

ANIMAL VIRUSES

Cell Culture Technology and Discovery: 1898–1965 (see e-Tables 1.1 to 1.3)

Once the concept of viruses as filterable agents took hold, many diseased animal tissues were subjected to filtration to determine if a virus were involved. Filterable agents were found that were invisible in a light microscope, and replicated only in living animal tissue. There were some surprises, such as the transmission of yellow fever virus by a mosquito vector,¹⁵⁴ specific visible pathologic inclusion bodies (virions and subviral particles) in infected tissue,^{95,142} and even viral agents that can “cause cancer”.^{50,159}

Throughout this early time period (1900–1930), a wide variety of viruses were found (see e-Tables 1.1 and 1.2) and characterized with regard to their size (using the different pore sizes of filters), resistance to chemical or physical agents (e.g., alcohol, ether), and pathogenic effects. Based on these properties alone, it became clear that viruses were a very diverse group of agents. Some were even observable in the light microscope (vaccinia in dark-field optics). Some were inactivated by ether, whereas others were not. Viruses were identified that affected every tissue type. They could cause chronic or acute disease; they were persistent agents or recurred in a periodic fashion. Some viruses caused cellular destruction or induced cellular proliferation. For the early virologists, unable to see their agents in a light microscope and often confused by this great diversity, their studies certainly required an element of faith. In 1912, S. B. Wolbach, an American pathologist, remarked, “It is quite possible that when our knowledge of filterable viruses is more complete, our conception of living matter will change considerably, and that we shall cease to attempt to classify the filterable viruses as animal or plant”.²⁰⁴

The way out of this early confusion was led by the plant virologists and the development of techniques to purify viruses and characterize both the chemical and physical properties of these agents (see previous section, The Plant Viruses and the Chemical Period: 1929–1956). The second path out of this problem came from the studies with bacteriophages, where single cells infected with viruses in culture were much more amenable to experimental manipulation than were virus infections of whole animals. Whereas the plant virologists of that day were tethered to their greenhouses, and the animal virologists were bound to their animal facilities, the viruses of bacteria were studied in Petri dishes and test tubes. Nevertheless, progress was made in the study of animal viruses one step at a time: from studying animals in the wild, to laboratory animals, such as the mouse⁶⁶ or the embryonated chicken eggs,²⁰⁵ to the culture of tissue, and then to single cells in culture. Between 1948 and 1955, a critical transition converting animal virology into a laboratory science came in four important steps: Sanford and colleagues

at the National Institutes of Health (NIH) overcame the difficulty of culturing single cells¹⁶³; George Gey at Johns Hopkins Medical School cultured and passaged human cells for the first time and developed a line of immortal cells (HeLa) from a cervical carcinoma⁷¹; and Harry Eagle at the NIH developed an optimal medium for the culture of single cells.⁴⁶ In a demonstration of the utility of all these advances, Enders and his colleagues showed that poliovirus could replicate in a nonneural human explant of embryonic tissues.⁵⁴

These ideas, technical achievements, and experimental advances had two immediate effects on the field virology. They led to the development of the polio vaccine, the first ever produced in cell culture. From 1798 to 1949, all the vaccines in use (smallpox, rabies, yellow fever, influenza) had been grown in animals or embryonated chicken eggs. Poliovirus was grown in monkey kidney cells that were propagated in flasks.^{84,117} The exploitation of cell culture for the study of viruses began the modern era of molecular virology. The first plaque assay for an animal virus in culture was done with poliovirus,⁴³ and it led to an analysis of poliovirus every bit as detailed and important as the contemporary work with bacteriophages. The simplest way to document this statement is for the reader to compare the first edition of *General Virology* by S. E. Luria in 1953¹²⁴ to the second edition by Luria and J. E. Darnell in 1967,¹²⁷ and to examine the experimental descriptions of poliovirus infection of cells. The modern era of virology had arrived, and it would continue to be full of surprises.

The Molecular and Cell Biology Era of Virology (see e-Tables 1.4 to 1.6)

The history of virology has so far been presented chronologically or according to separate virus groups (plant viruses, bacteriophages, animal viruses), which reflects the historical separation of these fields. In this section, the format changes as the motivation for studying viruses began to change. Virologists began to use viruses to probe questions central to understanding all life processes. Because viruses replicate in and are dependent on their host cells, they must use the rules, signals, and regulatory pathways of the host. By using viruses to probe cells, virologists began to make contributions to all facets of biology. This approach began with the phage group and was continued by the animal virologists. The recombinant DNA revolution also took place during this period (1970 to the present), and both bacteriophages and animal viruses played a critical and central role in this revolution. For these reasons, the organization of this section focuses on the advances in cellular and molecular biology made possible by experiments with viruses. Some of the landmarks in virology since 1970 are listed in e-Tables 1.4 to 1.6.

The Role of Animal Viruses in Understanding Eukaryotic Gene Regulation

The closed circular and superhelical nature of polyomavirus DNA was first elucidated by Dulbecco and Vogt⁴² and Weil and Vinograd.¹⁹⁷ This unusual DNA structure was intimately related to the structure of the genome packaged in virions of simian vacuolating virus 40 (SV40). The viral DNA is wound around nucleosomes⁷⁰; when the histones are removed, a superhelix is produced. The structure of polyoma viral DNA served as an excellent model for the *E. coli* genome²⁰⁶ and the mammalian

chromosome.¹¹³ Viral genomes have unique configurations not found in other organisms, such as single-stranded DNA (ssDNA),¹⁷¹ plus or minus strand RNA, or double-stranded RNA (dsRNA) as modes of information storage.

Many elements of the eukaryotic transcription machinery have been elucidated with viruses. The first transcriptional enhancer element (acts in an orientation- and distance-independent fashion) was described in the SV40 genome,⁷⁶ as was a distance- and orientation-dependent promoter element observed with the same virus. The transcription factors that bind to the promoter, SP-1,⁴⁴ or to the enhancer element, such as AP-1 and AP-2,¹¹⁶ and which are essential to promote transcription along with the basal factors, were first described with SV40. AP-1 is composed of fos and jun family member proteins, demonstrating the role of transcription factors as oncogenes.²¹ Indeed, the great majority of experimental data obtained for basal and accessory transcription factors come from *in vitro* transcription systems using the adenovirus major late promoter or the SV40 early enhancer–promoter.¹⁹⁶ Our present-day understanding of RNA polymerase III promoter recognition comes, in part, from an analysis of the adenovirus VA gene transcribed by this polymerase.⁶²

Almost everything we know about the steps of messenger RNA (mRNA) processing began with observations made with viruses. RNA splicing of new transcripts was first described in adenovirus-infected cells.^{15,31} Polyadenylation of mRNA was first observed with poxviruses,¹⁰² the first viruses shown to have a DNA-dependent RNA polymerase in the virion.¹⁰³ The signal for polyadenylation in the mRNA was identified using SV40.⁵⁹ The methylated cap structure found at the 5′ end of most mRNAs was first discovered on reovirus mRNAs.⁶⁷ What little is known about the process of RNA transport out of the nucleus has shown a remarkable discrimination of viral and cellular mRNAs by the adenovirus E1B-55 Kd protein.¹⁴⁷

Most of our understanding of translational regulation has come from studies of virus infected cells. Recruitment of ribosomes to mRNAs was shown to be directed by the 5′ cap structure first discovered on reovirus mRNAs. The nature of the protein complex that allows ribosomes to bind the 5′ cap was elucidated in poliovirus-infected cells, because viral infection leads to cleavage of one of the components, eIF4G. Internal initiation of translation was discovered in cells infected with picornaviruses (poliovirus and encephalomyocarditis virus).^{99,146} Interferon, discovered as a set of proteins that inhibits viral replication, was subsequently found to induce the synthesis of many antiviral gene products that act on translational regulatory events.^{92,93} Similarly, the viral defenses against interferon by the adenovirus VA RNA has provided unique insight into the role of eIF-2 phosphorylation events.¹⁰⁸ Mechanisms for producing more than one protein from a eukaryotic mRNA (there is no “one mRNA one protein” rule in bacteria) were discovered in virus-infected cells, including polyprotein synthesis, ribosomal frameshifting, and leaky scanning. Posttranslational processing of proteins by proteases, carbohydrate addition to proteins in the Golgi apparatus, phosphorylation by a wide variety of important cellular protein kinases, or the addition of fatty acids to membrane-associated proteins have all been profitably studied using viruses. Indeed, a good deal of our present-day knowledge of how protein trafficking occurs and is regulated in cells comes from the use of virus-infected cell

systems. The field of gene regulation has derived many of its central tenets from the study of viruses.

Animal Viruses and the Recombinant DNA Revolution

The discovery of the enzyme reverse transcriptase,^{6,185} not only elucidated the replication cycle of retroviruses, but also provided an essential tool to convert RNA molecules to DNA, which could then be cloned and manipulated. The first restriction enzyme map of a chromosome was done with SV40 DNA, using the restriction enzymes HindII plus HindIII DNA,^{37,38} and the first demonstration of restriction enzyme specificity was carried out with the same viral DNA cleaved with EcoRI.^{136,138} Some of the earliest DNA cloning experiments involved insertion of SV40 DNA into lambda DNA, or human β -hemoglobin genes into SV40 DNA, yielding the first mammalian expression vectors.⁹⁶ A debate about whether these very experiments were potentially dangerous led to a temporary moratorium on all such recombinant experiments following the scientist-organized Asilomar Conference. From the earliest experiments in the field of recombinant DNA, several animal viruses had been developed into expression vectors to carry foreign genes, including SV40,⁷⁴ the retroviruses,¹⁹⁸ the adenoviruses,^{69,78} and adeno-associated virus.¹⁶² which has the remarkable property of preferential integration into a specific genomic site.¹¹⁰ Modern-day strategies of gene therapy rely on some of these recombinant viruses. Hemoglobin mRNA was first cloned using lambda vectors, and the elusive hepatitis virus C (non-A, non-B) viral genome was cloned from serum using recombinant DNA techniques, reverse transcriptase, and lambda phage vectors.³⁰

Animal Viruses and Oncology

Much of our present understanding of the origins of human cancers is a consequence of work on two major groups of animal viruses: retroviruses and DNA tumor viruses. Oncogenes were first discovered in the genome of Rous sarcoma virus, and subsequently shown to exist in the host cell genome.¹⁷⁴ Since those seminal studies, virologists have identified a wide variety of oncogenes that have been captured by retroviruses (see Chapter 8). Additional oncogenes were identified when they were activated by insertion of the proviral DNA of retroviruses into the genomes of cells.⁷⁷ The second group of genes that contribute to the origins of human cancers, the tumor suppressor genes,¹¹⁸ has been shown to be intimately associated with the DNA tumor viruses. Genetic alterations at the p53 locus are the single most common mutations known to occur in human cancers—they are found in 50% to 80% of all cancers.¹¹⁹ The p53 protein was first discovered in association with the SV40 large T-antigen.^{115,120} SV40, the human adenoviruses, and the human papillomaviruses all encode oncoproteins that interact with and inactivate the functions of two tumor suppressor gene products, the retinoblastoma susceptibility gene product (Rb) and p53.^{40,44,115,120,164,200,201} Our understanding of the roles of cellular oncogenes and the tumor suppressor genes in human cancers would be far less significant without the insight provided by studies with these viruses. Curiously, none of the four human polyoma viruses central to these studies was associated with human cancers. However, in 2008, a new polyomavirus associated with Merkel cell carcinoma was discovered.⁵⁷

Viruses that cause cancers have provided some of the most extraordinary episodes in modern animal virology.¹³⁵ The recognition of a new disease and the unique geographic distribution of Burkitt's lymphoma in Africa²⁰ set off a search for viral agents that cause cancers in humans. From D. Burkitt²⁴ to Epstein, Achong, and Barr⁵⁶ to W. Henle and G. Henle,⁸¹ the story of the Epstein-Barr virus and its role in several cancers, as well as in infectious mononucleosis, is a science detective story without rival. Similarly, the identification of a new pathologic disease, adult T-cell leukemia, in Japan by K. Takatsuki^{181,191} led to the isolation of a virus that causes the disease by I. Miyoshi and Y. Hinuma²⁰⁸ and the realization that this virus (human T-cell leukemia virus type 1 [HTLV-1]) had been identified previously by Gallo and his colleagues.¹⁴⁹ Even with the virus in hand, there is still no satisfactory explanation of how this virus contributes to adult T-cell leukemia.

An equally interesting detective story concerns hepatitis B virus and hepatocellular carcinoma. By 1967, S. Krugman and his colleagues¹¹¹ had strong evidence indicating the existence of distinct hepatitis A and B viruses, and in the same year B. Blumberg²⁰ had identified the Australia antigen. Through a tortuous path, it eventually became clear that the Australia antigen was a diagnostic marker—the coat protein—for hepatitis B virus. Although this discovery freed the blood supply of this dangerous virus, Hilleman at Merck Sharp & Dohme and the Chiron Corporation (which later isolated the hepatitis C virus) went on to produce the first human vaccine that prevents hepatitis B infections and very likely hepatocellular carcinomas associated with chronic virus infections (see Chapter 69). The idea of a vaccine that can prevent cancer—first proven with the Marek's disease virus and T-cell lymphomas in chickens,^{18,49}—comes some 82 to 85 years after the first discoveries of tumor viruses by Ellerman, Bang, and Rous. An experiment is under way in Taiwan, where 63,500 newborn infants have been inoculated to prevent hepatitis B infections. Based on the epidemiologic predictions, this vaccination program should result in 8,300 fewer cases of liver cancer in that population in 35 to 45 years.

Vaccines and Antivirals

Among the most remarkable achievements of our century is the complete eradication of smallpox, a disease with a greater than 2,000-year-old history.⁷⁹ In 1966, the World Health Organization began a program to immunize all individuals who had come into contact with an infected person. This strategy was adopted because it simply was not possible to immunize entire populations. In October 1977, Ali Maolin of Somalia was the last person in the world to have a naturally occurring case of smallpox (barring laboratory accidents). Because smallpox has no animal reservoir and requires person-to-person contact for its spread, most scientists agree that we are free of this disease, at least as a natural infection.⁷⁹ As a consequence, most populations have not maintained immunity to the virus and the world's populations are becoming susceptible to infection. Many governments now fear the use of smallpox virus as a weapon of bioterrorism, and the debate continues over whether to destroy the two known stocks of smallpox virus in the United States and Russia.⁸⁰ As a consequence, the development of new, more effective vaccines and safe anti-smallpox virus drugs has risen high on the list of priorities for some countries, and such vaccines have already been stockpiled in

the United States. It is paradoxical that humankind's most triumphant medical accomplishment is now tarnished by the spectre of biowarfare.

The Salk and Sabin poliovirus vaccines were the first products to benefit from the cell culture revolution. In the early 1950s in the United States, just before the introduction of the Salk vaccine, about 21,000 cases of poliomyelitis were reported annually. Today, thanks to aggressive immunization programs, polio has been eradicated from the United States (see Chapters 18 and 19).¹⁴¹ As of this writing, only three countries have seen interruption of wild-type poliovirus circulation: Nigeria, Afghanistan, and Pakistan. With the substantial financial support of the Gates Foundation, there is hope that global immunization campaigns can lead to eradication of poliomyelitis from the planet.

The first viral vaccines deployed included infectious vaccines, attenuated vaccines, inactivated virus vaccines, and subunit vaccines. Both the Salk inactivated virus vaccine and the recombinant hepatitis B virus subunit vaccine were products of the modern era of virology. Today many new vaccine technologies are either in use or are being tested for future deployment.^{3,23,168} These include recombinant subunit vaccines, virus-like particle vaccines, viral antigens delivered in viral vectors comprising vaccinia virus or adenovirus, and DNA plasmids that express viral proteins from strong promoters. Therapeutic vaccines boost the immune system using specific cytokines or hormones in combination with new adjuvants to stimulate immunity at specific locations in the host or to tailor the production of immune effector cells and antibodies. Considering that the first vaccines for smallpox were reported in the Chinese literature of the 10th century,⁵⁸ vaccinology has clearly been practiced well before the beginning of the field of virology.

Although vaccines have been extraordinarily successful in preventing specific diseases, up until the 1960s, few natural products or chemotherapeutic agents that cured or reduced viral infections were known. That situation changed dramatically with the development of Symmetrel (amantadine) by Dupont in the 1960s as a specific influenza A virus drug. Soon after, acyclovir, an inhibitor of herpesviruses, was developed by Burroughs-Wellcome. Acyclovir achieves its remarkable specificity because to be active, it must be phosphorylated by the viral enzyme thymidine kinase before it can be incorporated into viral DNA by the viral DNA polymerase. This drug blocks herpes simplex virus type 2 (HSV-2) replication after reactivation from latency and stopped a growing epidemic in the 1970s and 1980s (Chapter 14). The development of other nucleoside analogs has led to many compounds effective against DNA viruses. Until the human immunodeficiency virus (HIV) epidemic, few drugs effective against RNA viruses other than the influenza A virus were known. As natural products, the interferons (Chapter 9) are used successfully in the clinic for hepatitis B and C infections, cancer therapy, and multiple sclerosis. The interferons, novel cytokines found in the course of studying virus interference,^{23,92,93} modulate the immune response and continue to play an increasing role in the treatment of many clinical syndromes.

Virology and the Birth of Immunology

Edward Jenner was a British surgeon who is credited with making the first smallpox vaccine in 1796, and has also been called

the “father of immunology.” Jenner began a long tradition of virology providing seminal discoveries about the immune response. Two examples will serve to illustrate this pattern.

Alick Issacs and Jean Lindenmann, while working at the National Institute for Medical Research in London, found that addition of heat-inactivated influenza virus to the chorioallantoic membrane of chicken eggs interfered with the replication of influenza virus. When they published this observation in 1957, they coined the term *interferon* (IFN).⁹² In the 1970s the protein was purified from cells by Sidney Pestka and Alan Waldman,¹⁶¹ and subsequently the genes encoding the proteins were cloned.⁷³ This allowed formal proof that IFN—by that time known to comprise a variety of different proteins—could interfere with viral replication. Extensive work with viruses showed that IFNs bind to cell-surface receptors, and through the JAK-STAT signal transduction pathway, induce the synthesis of more than 1,000 mRNAs that establish an antiviral state.³⁹ IFNs protect against both viral and bacterial infections, and also play a role in tumor clearance.

While working at the John Curtin School of Medical Research in Australia, Rolf Zinkernagel and Peter Doherty provided seminal insight into how cytotoxic T cells (CTLs) recognize virus-infected cells. They were studying infection of mice with lymphocytic choriomeningitis virus (LCMV). Because this virus is noncytopathic, they hypothesized that brain damage in infected mice was a consequence of CTLs attacking virus-infected cells. They made the observation that CTLs isolated from LCMV-infected mice lysed virus-infected target cells *in vitro* only if both cell types had the same major histocompatibility complex (MHC) haplotype. This requirement was termed MHC restriction.²¹¹ In other words, a CTL must recognize two components on a virus-infected cell: one virus specific and one from the host. Subsequent research revealed that CTLs recognize a short viral peptide bound to MHC class I (MHC-I) proteins on the surface of target cells. These observations revolutionized our understanding of T-cell-mediated killing, thereby establishing a foundation for understanding the general mechanisms used by the immune system to recognize both foreign microorganisms and self-molecules. The results have had wide implications for clinical medicine, not only in infection but also in areas such as cancer and autoimmune reactions in inflammatory diseases.

Emerging Viruses

In general, emerging viruses cause human infections that have not been seen or reported before. They usually attract the public's attention, often by media sound bites like “killer viruses emerge from the jungle.” The fact is that spread of infections through different hosts is well known in virology. Most so-called emerging infections represent zoonotic infections: infection of humans by a virus that normally exists in an animal population in nature.¹⁸⁷

Perhaps the most infamous emerging virus infection of the 20th century is the human immunodeficiency virus type 1, HIV-1, a retrovirus.⁸⁵ Progenitor HIV viruses exist in primates, and we now believe they infected humans as a result of hunting and slaughter for food.¹⁷⁰ HIV was first recognized as a new disease entity by clinicians and epidemiologists in the early 1980s, and they rapidly tracked down the venereal mode of virus transmission. The virus was detected in blood products

and transplant tissue. The immune system of HIV-infected individuals is severely compromised, which results in a variety of infections by usually benign microbes. The first published report of acquired immunodeficiency syndrome (AIDS) was in June 1981. Possible causative agents were first suggested in 1983,⁷ and then 1984.⁶⁸ Had this pandemic occurred in 1961 instead of 1981, neither the nature of retroviruses nor the existence of its host cell (CD4 helper T cell) would have been understood. HIV is a lentivirus (*lenti* is Latin for slow) and despite its recent appearance in humans, lentiviruses have been around for a long time. In fact, one of the first animal viruses to be identified in 1904 was the lentivirus that causes infectious equine anemia.

Many other examples of emerging viruses have attracted global concern and an exceptional rapid response of scientists and health officials.¹⁸⁷ The severe acute respiratory syndrome (SARS) and West Nile virus epidemics revealed the presence of a new human coronavirus (SARS), identified with unprecedented speed, and the invasion of an Old World virus into the Western hemisphere (West Nile virus).^{90,140} In 2006, chikungunya virus (an endemic virus infection in Africa) spread explosively to several countries where it was hitherto unknown.¹⁶⁹ On La Reunion Island, more than 40% of the population of 800,000 people was infected. The first appearance of avian influenza A (H5N1) virus in humans in 1997 produced fears of a pandemic of serious proportions because humans had no immunological history of infection by this avian strain.¹⁸² Soon thereafter, the emergence of the pandemic H1N1 influenza virus in 2009 produced similar worries because of the relationship of the virus to the deadly 1918 influenza epidemic.¹⁸⁴ The mobilization of world health networks, public health officials, vaccine producers, veterinarians, clinicians, and molecular virologists marked a new chapter in dealing with emerging diseases.

Epidemiology of Viral Infections

The study of the incidence, distribution, and control of disease in a population is an integral part of virology. The technology advancements of the last 50 years have provided epidemiology with a terrific boost. The discovery of specific molecular reagents (e.g., recombinant DNA technology, antibodies, polymerase chain reaction [PCR], rapid diagnostic tests, high volume DNA and RNA sequencing) now enables detection of virions, proteins, and nucleic acids in body fluids, tissue samples, or in the environment. Moreover, we now can compare and classify viral isolates rapidly, determine the relationships between virus strains, and track the spread of infections around the world. The marriage of behavioral, geographic, and molecular epidemiology made this a most powerful science.⁸⁷

The understanding of epidemics and pandemics of our most common viral infections such as influenza requires the perspectives of ecology, population biology, and molecular biology.^{106,182} G. Hirst and his colleagues (1941–1950) developed the diagnostic tools that permitted both the typing of the hemagglutinin (HA protein) of influenza A strains and the monitoring of the antibody response to this antigen in patients (see Chapters 42 and 43). These observations have been expanded, with more and more sophisticated molecular approaches, to prove the existence of animal reservoirs for influenza viruses, the reassortment of viral genome segments between human and

animal virus strains (antigenic shift), and a high rate of mutation (antigenic drift) caused by RNA-dependent RNA synthesis with no known RNA editing or corrective mechanisms.^{153,184} These molecular events that lead to episodic local epidemics and worldwide pandemics are understood in broad outline. Many viruses are now known to evolve at high rates following basic Darwinian principles in a time frame shorter than that of any other organism. Indeed, we now understand that RNA virus populations exist as a quasispecies or a swarm of individual viral genomes where every member is unique. Influenza viruses are successful because they have evolved to carry the very engines of evolution: mechanisms of mutation and recombination (reassortment). Influenza A virus has not been eliminated even with effective vaccines and antiviral drugs. Variants always arise that escape effective immune responses thorough high mutation (drift), and when co-infection occurs with viruses spreading from nonhuman hosts, new reassortants regularly arise. Expression of these new combinations of viral genes can change the pattern of infection from local to pandemic via an antigenic shift of its HA and NA subunit proteins. These studies (Chapters 42 and 43) have revealed an extraordinary lifestyle that reverberates around the planet in birds, farm animals, and humans. The study of the mechanisms of viral pathogenesis and modulation of the immune system have led to new insights in the virus–host relationship.

New technology discovered and developed over the last 35 years is changing the way viral infections are studied in the laboratory and in the field, and is changing our appreciation of epidemiology and virus ecology.¹⁸³ Amplification technologies such as PCR permit rapid sampling of viral nucleic acids without growth in culture or plaque purification. Microarray technology where discriminatory DNA sequences from all sequenced viral genomes are put on a single array enables rapid classification of PCR-amplified nucleic acids.¹⁹⁴ Rapid genome sequencing has revealed hitherto described viral genomes, relationships among viruses, and sequence heterogeneity within a virus population.¹²³ Mutations can be detected rapidly, documented, and localized in the viral genome. Importantly, the biological consequences can be monitored quickly. For example, in the late 1970s, viral epidemiologists were confronted with a highly transmissible, lethal infection of puppies.¹⁴⁴ In record time, scientists found that just two mutations in the capsid gene of feline parvovirus altered the host range such that the mutant could infect dogs. In less than a year, a completely new, highly pathogenic virus called canine parvovirus spread all around the world. Its evolution has continued to be monitored, and a highly effective vaccine was developed. A similar type of molecular archeology enabled scientists to analyze serum samples collected from patients in the 1950s in efforts to understand the origins of HIV.⁸⁵ Sequence analysis of the HIV genome from one sample (ZR 1959) suggested that the virus may have emerged in the 1940s to 1950s. Field studies in Africa of viruses present in primate feces indicated that HIV most likely derived from a chimpanzee lentivirus in Africa.¹⁷⁰ After the initial human infection, rapid mutation and selection established the first human variants of this lentivirus that replicated and continued to evolve as they spread through their new human hosts.

The advances in our understanding of the viral etiology of tumors pay tribute to the modern epidemiology strategy by D. Burkitt and K. Takatsuki, leading to the identification of Epstein-

Barr virus (EBV) and HTLV-1. Similarly, the recombinant DNA revolution overcame the problems of propagating human papillomaviruses. The human papillomaviruses (see Chapter 56) differ in transmission, location on the body, their nature of pathogenesis, and persistence. New technology permitted the identification of new virus serotypes, triggering epidemiologic correlations for high- or low-risk cancer viruses.²¹² The same technology enabled the development and use of an effective vaccine against cervical cancer. We cannot forget the considerable impact of veterinary virus epidemiology on our understanding of complicated human diseases. For example, careful epidemiologic work by Sigurdsson and colleagues on unusual diseases of sheep¹⁷⁵ provided the first understanding of slow infections in sheep (Visna-Maedi virus; a lentivirus) and infectious proteins (prions), which cause spongiform encephalopathies (Chapter 78).

As we describe in the next section, molecular epidemiology is reaching new levels of sophistication, not only in detecting new viruses, but also taking inventory of the viral ecosystem. Whether the next human epidemic will result from a novel variant of Ebola virus, coronavirus, or Norwalk virus, or the more likely possibility of a new pandemic variant of influenza virus, remains to be seen. The new technologies also enable analysis of virus populations in natural communities of nonhuman animals. For example, we can now monitor pandemic spread of avian influenza virus in wild birds and other nonhuman hosts.¹⁵³ These alternative hosts have never been sampled for virus populations in such molecular detail. New insights into the selection pressures and bottlenecks are emerging almost faster than the viruses. What is abundantly clear, however, is that the demographics of the human population on earth are changing at unprecedented rates (Table 1.1). Even as birth rates slow, our planet will house 8 to 10 billion people by 2050 to 2100. For the first time, there will be three to four times more people older than the age of 60 than younger than 3 to 4 years of age. Not only are we an aging population, we are moving to urban environments, with more than 20 to 30 cities containing more than 10 million people. Clearly, patterns of human behavior (increased population density, increased travel, increased ages of the population) will provide the environment for the selection of emerging viruses and the challenges to the new field of molecular epidemiology.

HOST–VIRUS INTERACTIONS AND VIRAL PATHOGENESIS

The technologies that contributed most to the modern era of virology (1960 to present), were advances in cell culture and molecular biology.⁵⁵ Virologists were able to describe the replicative cycles of viruses in great detail under well-defined conditions, and they demonstrated the elaborate interactions between viral genomes, viral proteins, and the cellular machinery of the host. As indicated previously, these advances resulted in an extraordinary inquiry into the functions of infected or uninfected host cells using the tools of both molecular biology and cell biology. As this approach matured, it became more reductionist in nature, and the questions became more detailed. However, some virologists used the new knowledge to move back to more complicated *in vivo* systems to study previously difficult problems in host–virus

TABLE 1.1 Advances and Challenges

Vaccines	<p>Yellow fever virus vaccine, live attenuated</p> <p>Salk and Sabin vaccines for poliovirus, killed and live attenuated</p> <p>Recombinant hepatitis B vaccine, subunit</p> <p>Vaccinia virus vaccine to eradicate natural smallpox virus from the planet</p> <p>Influenza virus vaccines, inactivated and live attenuated</p> <p>Varicella-zoster virus vaccines, live attenuated</p> <p>Rotavirus vaccines, live attenuated</p> <p>Measles vaccines, live attenuated</p> <p>Recombinant human papillomavirus vaccine, subunit; prevents cancers and virus infections</p>
Antiviral drugs	<p>Acyclovir against herpes simplex type 1 and type 2</p> <p>Combination therapy: Protease, reverse transcriptase, and integrase inhibitors against HIV</p> <p>Interferon therapy for hepatitis B and C</p> <p>Amantadine against influenza A virus</p> <p>Neuraminidase inhibitors against influenza virus</p>
Epidemiologic advances	<p>Understanding the molecular basis of antigenic shift and drift in influenza viruses</p> <p>Identification of the causes of AIDS and SARS</p> <p>Prion diseases recognized and mechanisms elucidated</p> <p>Deep sequencing, genome analysis; pathogen discovery, uncovering the molecular nature of epidemic and pandemic infections</p> <p>Recognition of the role of zoonotic infections in the emergence of new viral diseases</p> <p>Recognition of specific viruses as causative agents in human cancers</p> <p>Elucidation of the concept of viral quasispecies and the molecular biology of viral populations</p>
Viral pathogenesis	<p>Identification of viral virulence genes</p> <p>Identification of host genes affecting virus replication and spread</p> <p>Identification of the molecular bases for antiviral immune defenses (adaptive immunity)</p> <p>Identification of the molecular basis of front-line cellular defenses (intrinsic and innate immunity) including apoptosis and induction of defensive cytokines</p> <p>Understanding of the molecular basis for viral tropism</p> <p>Elucidation of the mechanisms involved in viral quiescence and persistence</p>
The challenges (societal)	<p>Population explosion: more people now live on the planet than at any time in our existence (predicted to be 8 to 10 billion in the next few decades)</p> <p>Population concentration: world populations are concentrating in large urban centers of 10 to 20 million people or more</p>
The challenges (scientific)	<p>Population demographics: for the first time there are more people older than the age of 60 than younger than the age of 4</p> <p>Population interactions: world populations interact physically at rates and extents never before possible</p> <p>Pandemic viral diseases and bioterrorism provide continuing challenges for human survival</p> <p>Research costs money: how do we alleviate the pressures on funding and support of fundamental research</p> <p>Discoveries cannot be predicted: how to balance true discovery research with applied (translational) research</p> <p>Public support: how do we develop support and advocacy for virology research</p> <p>Policy makers need to understand virology: more engagement of scientists with lawmakers and the general public</p> <p>Public education about vaccination and other public health issues</p> <p>Discovering an effective vaccine against HIV</p> <p>Developing vaccines against persistent viruses</p> <p>Discovering and developing new antiviral drugs</p> <p>Development of rapid viral diagnostic and identification strategies</p> <p>Coupling new technology with established procedures</p> <p>Balancing risks and benefits of dangerous pathogen research</p> <p>Developing surrogates for Koch's postulates in modern pathogen discovery programs</p> <p>Defining and understanding the composition and interplay of microbial communities inside and outside hosts (natural versus unnatural flora)</p>

AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; SARS, severe acute respiratory syndrome.

interactions involving the natural host or animal models of infection. Chief among these new questions was, how does a virus cause disease processes in the animal? How do we quantify viral virulence and what is the genetic basis of an attenuated virus? These studies have identified, in selected viruses, a set of genes and functions that broadly influence our understanding of pathogenesis.

Despite an abundance of data, we have distilled six general categories relating to viral pathogenesis. Four of these involve viral gene products and two involve the hosts.

1. Mutations in genes that impair virus replication in the host, lower the threshold of pathogenesis by reducing the number of progeny produced. These mutations are found in essential genes (essential for life) *in vivo*.
2. A second class of mutations impairs virulence (reduces the degree of pathogenicity), but does not alter normal virus replication (at least in some cell or tissue types). Here, host- or tissue-range mutations are most common. Mutations can change the pattern of virion adsorption to a particular cell type and so prevent viral entry into a cell. Mutations in viral enhancer elements can alter viral transcription in selected cell types. In some viral genomes, mutations affect rates of translation such that virulence is reduced. A classic example comes from analysis of the attenuated strains of poliovirus in the Sabin vaccine. All three strains of the Sabin poliovirus vaccine contain mutations in the 5' untranslated region of the viral RNA genome, which impair translation of these RNAs, and as a consequence virus yields are reduced. As a result, after infection, viral replication occurs, the host is immunized, but disease does not occur.
3. A third class of genes affecting virulence is involved in producing products that modify the host defenses. Intrinsic host defenses depend on receptors inside and on the surface of cells that detect viral gene products. When these receptors are activated, cytokines can be produced to alert more global innate immune defenses, the cell may die by apoptosis, or autophagy may be induced to engulf virus particles. It is likely that every successful virus can bypass or modulate these most fundamental cell-autonomous defenses. Mutations in these primary defense systems or viral proteins that block them affect virulence and spread. Some viruses encode genes that produce viral homologs of host cytokines (virokines). These proteins are secreted from infected cells and modify the immune response to infection. Other viruses encode decoy receptors that bind host-produced cytokines and reroute the immune response as a result. Many viral genomes encode genes whose products block infected cells from undergoing apoptosis in response to a virus infection. Some viruses, such as African swine fever virus, secrete a pro-apoptotic factor that kills lymphocytes and enhances its virulence. Many viruses produce proteins that alter the MHC proteins (MHC-I and MHC-II; also known as human leukocyte antigens or HLA proteins). These complex proteins display on the cell surface, short peptides derived from newly made or newly ingested proteins inside the cell. T cells detect these complexes and respond if non-self-peptides are detected. Many viral infections alter the expression or function of these MHC proteins. Other viruses encode superantigens that stimulate or eliminate lymphoid cells of a selected specificity or with a class of receptors. HIV infection kills CD4 T cells and disrupts the immune response.
4. A fourth class of viral virulence genes enhances the spread of a virus in the host. Some viruses are released from infected cells at the apical or basolateral surface, permitting selected spread *in vivo*. Some RNA viruses acquire infectivity (maturation) only after specific proteolytic cleavage of their structural proteins. In some cases, maturation is accomplished by a viral protease and in others by a cellular protease, each with a specific amino acid sequence required for proper cleavage and resulting spread of the virus. Altering this sequence will affect virulence and overall transmissibility of the infection in a host population.
5. A fifth class involves host gene products. A wide variety of polymorphisms or mutations in the host result in modulated resistance or virulence of a virus. These host mutations can even be selected during viral epidemics, changing the gene pool of the surviving host population. In humans, polymorphisms in a chemokine receptor gene (a co-receptor) impart resistance to HIV infection at the level of viral absorption. New antiviral drugs have been designed to target this viral-cytokine interaction. Variations in the immune responses of diverse hosts in a population will result in large variations in viral virulence. The host mechanisms that minimize viral diseases after infection are certainly major topics in viral pathogenesis.
6. The final class involves the society and interaction of hosts. Changes in population density, lifestyles, cultural traditions, and economic factors all play a major role in viral virulence. Poliovirus was a minor endemic virus infection for 3,000 years before the introduction of improved sanitation in the last century. As a result, human populations were infected for the first time at a later age and large poliovirus epidemics resulted. It may not have been a coincidence that the worst influenza epidemic in the century, killing 20 to 40 million people, started in about 1918 toward the end of World War I, with so many people dislocated and moving about the world in very crowded and poor conditions. If there is a general lesson from history it is that cultural and environmental changes will surely play a role in the virulence of viruses in the future.

THE FUTURE OF VIROLOGY? (E-TABLE 1.7 AND TABLE 1.1)

The future of virology is unpredictable, but it is guaranteed to be exciting. Who knows what discoveries remain? Certainly, the number of astounding and groundbreaking discoveries in biology over the last 50 years is remarkable.⁵⁵ Most could not have been predicted or even imagined, prior to their discovery. That virologists participated in making many of these discoveries is no accident: Viral gene products have evolved to engage all the key nodes of biology ranging from the atomic to the organismal. We only have to be smart enough to figure out how to identify these nodes. The forces that will drive our field are technology development, public health, information processing, and, of course, personal curiosity. Indeed new life science technologies invariably

will give rise to new, unexpected insights in virology to meet our current challenges. That has been, and continues to be, the future of virology (see Table 1.1).

Despite a cloudy crystal ball, three general trends are likely to rise to the forefront of virology research over the next 10 years.

1. **The detailed understanding of the systems biology inherent in virus–host interactions.** Although virus particles are inanimate, it is the living, infected cell that delivers the phenotype promoted by the viral genome. The change of state of a cell or tissue from uninfected to infected is fertile ground for modern systems biology. The constellation of new gene products (viral and host) and altered host pathways produced in an infected cell give rise to biological outputs that go far beyond the single cell in the laboratory. Viruses offer useful modalities for the systems biologist. One can synchronize an infection and go from the uninfected to infected state within minutes, or use the same virus to produce an acute or a quiescent infection. Regulatory circuits, modulation of host defenses, emergence of pathogenesis, and modes of efficient transmission in a hostile environment, are all inherent in the nanobiology of viruses. How can a viral genome with so few genes relative to the host, dominate a cell and the host so quickly and dynamically? How does it all work? How has evolution produced such diversity of infected cell phenotypes? Microarrays, PCR, mass spectroscopy, microfluidics, large-scale nucleic-acid sequencing, massive database assembly, and computer modeling are what toothpicks and Petri dishes were to the students of the Delbrück phage school 60 years ago.
2. **The understanding of viruses as integral participants in the ecosystem.** Such knowledge means uncovering the multiple interrelationships and interactions of all viruses and their hosts. This is ecology, but on a scale that has hitherto been unimaginable for virologists. Viruses exist wherever life is found, and they are the most abundant entities on the planet. Indeed their biomass rivals that of the prokaryotes. Estimates are that we know less than 1% of the viral genomes on the planet, but first principles inform us that there can be only a limited number of genome strategies for replication and expression of information. Therefore, despite what appears to be incredible diversity, we will be able to identify new viruses by the unique signatures of a viral genome. The viral ecology problem, therefore, is one of knowing what is out there and why. The powerful techniques of interrogating virus populations in the wild for their RNA, DNA, proteins, and unique small molecules have changed the worldview of ecologists and molecular biologists alike. The new biology will require the intellectual firepower of computer scientists, engineers, chemists, and physicists, as well as biologists. As part of this growing knowledge of the viral ecosystem, virologists will come to be more ecumenical in their studies and not balkanize the field into animal and plant virology or viruses of single cell hosts.
3. **Health of humans and the world.** The fundamental need for public health measures is unprecedented, as the human population is now greater than ever before. However, despite all attempts to prove otherwise, humans are not the

top of the food chain. Every living thing ultimately engages every other entity directly or indirectly—and, as far as we know, every living thing is infected with viruses. These infections shape human existence on the planet. A human centric view of public health is short-sighted. First principles tell us that all successful viruses today carry a collection of genes that have survived the best defenses that hosts can muster. Our knowledge of the microbial world must be used to inform our national and international health policies. The bedrock of old-fashioned public health policies cannot be ignored: clean water, sewage treatment, proper nutrition, and management of epidemic childhood disease by vaccines. However, the continuing divide between rich and poor nations, the conflicts among ethnic and religious groups, the changing climate, and resulting calamities of drought and other natural disasters stress even these most basic attempts at maintaining public health. Certainly the high-tech approach to public health of developed counties will find no purchase in those countries where the basics of survival are lacking.

Intrinsic and Extrinsic Defenses Against Viral Infections

It is likely that considerable work in the future will be directed to the host defenses that meet viral infections in the first minutes to hours. All viral infections begin as individual, single-cell events that either are resolved or expand to produce the characteristic phenotypes of the persistent or acute infection. Ancient single-cell pathways of response to external stimuli have been honed over millions of years to provide cells and communities of cells, a repertoire of defensive actions that are now being revealed. Every cell is capable of responding to infection immediately (so-called intrinsic resistance) by processes whose nature and actions will fuel discovery research in the near future.^{17,61} These processes act immediately upon infection, before the so-called innate and adaptive immune responses are called into action. We understand some of these processes, such as apoptosis in some detail, but others, including RNA interference (RNAi), autophagy, DNA repression, and the restriction factors first defined by retrovirologists, remain fertile ground for discovery.^{28,29,41} The interaction between signals of early warning from single cells with the local multicellular innate immune response and the global adaptive immune response are likely to be key to recognizing and responding to the various patterns of viral infections that arise in nature. Primary questions concerning the molecular biology and cellular biology of persistent and latent infection cannot be answered without knowledge of early defense responses of single cells and local tissues.

DNA microarray technology has enabled the measurement of the whole genome responses of single cells exposed to a wide variety of viral infections.¹⁰⁰ The systematic profiling of gene-expression changes has provided an exceptionally rich database from which we now are learning of cell-common and cell-specific responses to infection. The differences and similarities are proving to be the proverbial gold mine of information on the definition of evolutionarily conserved host-defense components and viral gene products that counter them. Understanding the relationship of common cell-stress responses and

pathogen-specific responses and counter-responses will certainly provide insights into potential diagnostic and therapeutic targets for viral infections.¹⁰⁰

Viruses and Cancer

Since the 1960s, seven different human viruses have been isolated, identified, and shown to be associated with the etiology of human cancer.¹³⁵ Surprisingly, even after 50 years, we have only a rudimentary understanding of the oncogenic pathogenesis of these infectious agents.¹³⁵ The first cancer-associated virus was discovered in 1964 when Epstein, Achong, and Barr⁵⁶ detected herpesvirus particles in cells obtained from a Burkitt's lymphoma.²⁴ The DNA episomes of the Epstein-Barr virus (or EBV) have been consistently found to be associated with some types of B-cell lymphomas. Despite this 40-year period, it remains unclear how or even if this virus actually causes this lymphoma. Although it is certain that the EBV genome contains one or more oncogenes (latent membrane protein 1, LMP-1), they are not expressed in the lymphoma cells. The only viral gene product expressed in these lymphoma cells is Epstein-Barr nuclear antigen 1 (EBNA-1), and its possible role of contributing to lymphomas is still controversial. Similarly the HTLV-1 viral genome does not contain a cellular oncogene, and it does not integrate into the host-cell DNA near a cellular proto-oncogene in a consistent fashion. Therefore, HTLV-1 does not employ the two most common mechanisms for tumor formation observed with the retroviruses. There is no clear association of any hepatitis B or C gene products in the causation of liver cancers. Rather it appears that immune destruction of liver cells followed by the regeneration of this tissue activates several growth factors made by the surrounding tissue resulting in fibrosis. The local milieu of inflammation and the positive feedback loop for growth drives the division of liver cells and hepatocellular carcinoma. This complex mix of infection, immune-mediated cell death, and chronic inflammation in a tissue with regenerative capacity is challenging to analyze. Although Kaposi's sarcoma herpesvirus also encodes potential oncogenes, no clear mechanism of how it initiates or propagates cancer is available. On the other hand, studies of the human papillomaviruses⁴⁵ have provided a mechanistic understanding of how these viruses transform cells. The viral E7 protein binds to the cellular retinoblastoma protein and inactivates its function, thereby initiating entry of the cell into the cell cycle and division. The viral E6 protein binds the cellular p53 protein and promotes its ubiquitylation and proteolytic degradation, thereby preventing cellular apoptosis.¹⁶⁵ More research is needed to fully understand the mechanisms that lead to cancers after infection by these viruses.¹⁹⁹

A Role for Systems Biology in Virology

Not too long ago, molecular virology was limited to studies of one virus and one gene or gene product at a time. More complex studies often were seen as "descriptive." Times have changed! New technology enables virologists to interrogate simultaneously many viruses and large groups of genes or gene products in ever-expanding environments and biological networks. In this context, a network is defined as the interconnected intracellular processes that control everything within a cell, for example, DNA replication, processes of gene expression, organelle bio-

genesis, and metabolism to name a few.¹³⁹ The definition also encompasses networks of intercellular communication at the tissue, organ, and whole-organism level. Virologists are beginning to embrace a tenet of systems biology where information flows through these networks and disease arises when these networks are perturbed. Viral gene products cause changes in network architecture and thereby alter the dynamics of information flow. Future studies of viral pathogenesis are likely to involve identification and understanding of specific viral signatures of network imbalance that do not affect just one pathway but alter the fundamental homeostatic balance.^{19,55,152,179}

Genomics and the Predictive Power of Sequence Analysis

The development of technologic advances in biology often drives new approaches and permits one to ask novel questions that could not even be framed in the past. In the last decade of the 20th century, rapid and inexpensive DNA-sequencing methods paved the way to sequence the genomes of many viruses and their hosts. This created large databases containing information about the variation of DNA or RNA sequences within a single virus (e.g., HIV, influenza) and permitted predictions about the nature of the mutations that were driving selective changes, mutation frequencies of different viruses, and evolutionary changes from isolates around the world. The correlations of these sequence variations with drug resistance, changes in the genetic background of the host, and virulence have been informative. By combining this information with the three-dimensional structure of the influenza A hemagglutinin (HA) protein, J. Plotkin and colleagues have examined codon use in this gene and suggested that the degeneracy of codon use was being optimized to permit changes in amino acids at critical positions in this protein, so as to reduce the impact of the immune response to this virus.¹⁴⁸ Although this concept has been controversial, it has permitted a set of predictions of the direction of future changes in these codons as the host develops its immune response and immunity of the population. Predicting the future changes in influenza strains provides a testable hypothesis and might then impact how we prepare for genetic drift in virus populations by designing vaccines.¹⁸⁴

The degeneracy of the genetic code means that there are different codons that encode the same amino acid. As a result, many sequences can encode the same protein. This choice of sequences is constricted by several selective forces such as restrictions on transfer RNA (tRNA) availability in a host, giving rise to preferential codon use, the overall G-C content of a genome, the frequency in which two or three amino acids appear next to each other in proteins encoded by the virus, or the avoidance of some sequence contexts due to a high mutational load.¹⁵⁸ The low level of CpG dinucleotides in some genomes may result because a C-residue can be methylated. This change is mutagenic because methyl-C will pair with a T residue, causing a C to T transition in the genome. Once these restrictions on the frequency of certain dinucleotide to septanucleotide sequences are appreciated, they can be factored into a calculation of whether certain nucleotide sequences are over-represented or under-represented in a genome despite these selected pressures observed in a particular genome.

Algorithms have been designed to accomplish this, and it is clear from an analysis of 209 prokaryotic genomes and 90 bacteriophages that replicate in these hosts, that selected sequences of di-septanucleotides are over-represented and others are under-represented in these viral and bacterial genomes.¹⁵⁷ Having factored out the genetic codon preferences in this algorithm, these preferences represent a second code of under- or over-represented frequencies of nucleotide sequences, and the available data indicate that these sequences are functional and are selected for over evolutionary time scales. First, coding regions of a genome have been shown to have different over- or under-represented sequences in a genome. Second, if these coding regions sequences are employed to assemble a phylogenetic tree, these sequences do an excellent job in reconstructing the known evolutionary relationships of these 209 prokaryotic genome sequences (done originally by aligning the ribosomal gene sequences). Third, about 80% of the viruses in these databases can be correctly assigned to their hosts by matching the over- and under-represented sequences in their viral and host genomes. The same selection pressure acting upon this second code in a host genome also acts upon the genomes of their parasites. We now await the application of this algorithm to the more complex genomes and viruses of eukaryotes. Host genomes contain an amazing number of viral or viral-related sequences. More than 50% of the DNA sequences found in the human genome were derived from retroviruses, retrotransposons, DNA transposons and randomly amplified sequences of genes (short interspersed nuclear element [SINES] and the 7S RNA gene), pseudogenes, and repetitive DNA sequences.^{114,192} Viruses certainly have left a major mark upon the evolution of their host's genomes in addition to the selective pressures they exert via virus infections and deaths. During the evolution of humans from their ancestral line, retroviruses and retro-transposons (the long interspersed nuclear element [LINE-1]) have entered the germ line, amplified their copy numbers, and integrated at various sites in the genome. This process introduces mutations, alters patterns of gene expression, and creates new interactions of viruses with their hosts. This is clearly one of the drivers of host evolution. Over time these retroviruses (human endogenous retroviruses, or HERVs) accumulate mutations in their genes, and some recombine out of the genome leaving only the long terminal repeats (LTRs) as a remnant marking their past insertion. Although humans no longer contain viable HERVs, the multiple copies of HERV-H or HERV-K viruses when transcribed in cells, produce functional viral proteins from different copies of these viruses, and the viral particles that are produced are defective and very poorly transmitted. Cellular transcription factors regulate the expression of the HERVs, and the p53 transcription factor (activated by stress and DNA damage) transcribes the HERV-H genome and produces particles in response to such stress.²⁰⁹ Similarly the LINE-1 retrotransposons, which have about 300 viable and movable elements in the human genome today, are responsible for about 1% of the mutations found in each generation. LINE-1 transposons also contain p53 DNA response elements⁸⁶ and thus are also regulated by stress responses recorded by the host. Although it is clear that retroviruses and transposons can shape the host genome, it is equally clear that the host genome is a place for new viral genomes to evolve, recombine with

exogenous viral genomes, and possibly produce a new agent optimized for replication in its host. Understanding of the dynamics of these vestiges of viruses that reside in our genome is a challenge for the future.

With many host-genome sequences representing all kingdoms of life in the databases, it has been possible to do some rather eye-opening analyses. For example, the resurrection of endogenous retroviruses from inactive sequences in host DNA has allowed the investigation of interactions between extinct pathogens called paleoviruses and their hosts that occurred millions of years ago.⁵³ By cloning these sequences, it has been possible to identify the cellular receptor of these extinct retroviruses.¹⁷² Perhaps more amazing is that similar "viral genome fossils" representing DNA copies of filoviruses and bornaviruses as well as parvoviruses and circoviruses have been found in a variety of host genomes.^{11,12} When the evolutionary history of various host genomes harboring these viral sequences were compared, it was possible to deduce that ancestors of modern viruses were in existence millions of years ago. What is even more curious is that these genome-insertion events seemed to happen around the same time in a wide variety of mammals. What global event could have stimulated such activities?

The Virome: How Many Viruses Are There? Where Are They? Why Are They There?

Virus ecology, as a result of modern virus discovery technology, is posing many questions (see 106,183). In 1977, when Fred Sanger sequenced the DNA genome of coliphage phiX174, many virologists were impressed with the wealth of information contained in a "simple" DNA sequence and the congruence of genetic and biochemical data with the genome structure. In fewer than 25 years, sampling, sequencing, and computer technology now provide the wherewithal to identify and sequence entire viral communities from their natural environment without the intervention of time-held techniques of isolation and characterization of individual viruses.^{48,178,194} In early 2003, a novel viral DNA microarray was used to reveal and partially sequence a previously uncharacterized coronavirus in a viral isolate cultured from a patient with SARS. This chip technology has advanced to the point that essentially all the known viral genomes can be represented on a single microarray. New techniques for discovery and analysis of viral populations are certain to be found. As can be expected in this "omics" era, the identification and study of an entire community of viruses in their natural habitat has been called metagenomics.^{2,156,202} The diversity of viruses in the environment is essentially unknown, as we have been limited to studying only those viruses that are easy to work with in the laboratory or those that have major impact on human health. The first metagenomic studies on viruses have revealed stunning diversity of genes and gene products that remain to be understood even in principle.^{178,203} The combination of host and bacteriophage genome sequencing in the bacteria has proved to be an exceptional window on genome evolution and gene transfer. The practical value of identifying new gene products with novel functions cannot be overestimated. The repertoire of tactics for gene control and regulation is far more extensive than any of us imagined before the era of metagenomics. We can only expect that as the metagenomics of animal and plant viruses advances,

the effect of knowing everything that is out there and the resulting knowledge of the dynamics of host–parasite interactions will be mind-boggling.¹⁷⁷

Pathogen Discovery

Historically, discovery of new viral pathogens followed identification of diseases of consequence to humans, animals, and plants. Field biologists, clinicians, veterinarians, and the lay public noted syndromes, unusual behaviors, or drastic changes of animal and plant populations, which motivated scientists to discover the cause. The early days of virology were all “translational research.” Koch’s postulates were developed to identify the causative agent for a given disease. Advances in virus identification were driven in large part by technology developments such as porcelain filters, animal models, tissue and cell culture, microscopic visualization of cytopathic effect, serology, immunoassays, hybridization, western blotting, PCR, sequencing, microarrays, and imaging technology. These advances paved the way to our current understanding of viral pathogens and provided the data to advance our current understanding of mechanisms of pathogenesis. Modern pathogen discovery has entered a new phase where via sequencing technology, virologists can detect and identify viral nucleic acids with unprecedented sensitivity in essentially any sample.¹²³ We no longer need to be able to grow a virus stock to be able to identify it and develop diagnostic reagents, vaccines, or antiviral drugs.

The discovery of new viral genomes is proceeding at an amazing pace.¹⁴³ Although the discovery process is straightforward, understanding what these viruses are doing is a serious challenge.^{91,155} If one finds novel viral genomes in samples from patients with disease, are these viruses the cause of the disease? Is it possible that they may be part of the normal flora of an individual (the microbiome;¹⁰⁷)? There are many populations of microbes in and on various parts of the body. Just identifying the microbiome differences in body sites of a single individual is challenging enough; cataloging the microbiome variation from individual to individual is even more difficult.¹⁵⁶ What functions does the microbiome have? There is evidence that our normal microbial flora stimulates local and systemic immune responses that protect against or suppress responses that contribute to pathogenesis by more-virulent microbes. Future virologists will have to unravel these heretofore unknown microbial relationships, and to do so we will need new technology. Whatever we find will undoubtedly reveal unanticipated insights about viruses and their hosts. Modern pathogen discovery will require the interaction of infectious disease specialists, epidemiologists, and bioinformatics specialists; virologists will have to be professionally “multilingual”.¹²¹

Perhaps of fundamental importance is that proof of causation can no longer rely on the time-honored Koch’s postulates.⁹¹ This assertion is made not only because it may be difficult to propagate new viruses and find models to test their pathogenicity, it also is likely that many diseases will involve the interaction of multiple microbial communities (viruses, bacteria, fungi) that will be difficult to reproduce in the laboratory. Pathogen discovery will require new biomarkers of health and disease, methods to improve sampling and stability of samples, technology to record relevant data, and

capacity to associate all this data with the sample. In the past, pathogen identification methods were slow and tedious, and working with multiple samples was difficult if not impossible. It is now possible to collect and analyze serial samples over time as patients move from health to disease. Assembling data, maintaining databases, and providing access for analysis will also involve advances in software and bioinformatics. In the end, the fundamental challenge will be how one moves from correlation of the presence of an agent or agents in disease to proof of causation.

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Principles of Virology

Virus Taxonomy

History and Rationale

The International Committee on Taxonomy of
Viruses Universal System of Virus Taxonomy

Virus Cultivation and Assay

Initial Detection and Isolation

Hosts for Virus Cultivation

Recognition of Viral Growth in Culture

Virus Cultivation

Quantitative Assay of Viruses

Quantitative Considerations in Virus Assay,
Cultivation, and Experimentation

One-Step Growth Experiment

Virus Genetics

Mutants

Genetic Analysis of Mutants

Reverse Genetics

Defective Interfering Particles

Phenotypic Mixing and Pseudotypes

Viruses are unique in nature. They are the smallest of all self-replicating organisms, historically characterized by their ability to pass through filters that retain even the smallest bacteria. In their most basic form, viruses consist solely of a small segment of nucleic acid encased in a simple protein shell. Viruses have no metabolism of their own but rather are obliged to invade cells and parasitize subcellular machinery, subverting it to their own purposes. Many have argued that viruses are not even living,¹²⁸ although to a seasoned virologist, they exhibit a life as robust as any other creature.

The apparent simplicity of viruses is deceptive. The truth is that as a group, viruses infect virtually every organism in nature, they display a dizzying diversity of structures and lifestyles, and they embody a profound complexity of function.

The study of viruses—virology—must accommodate both the uniqueness and the complexity of these organisms. The singular nature of viruses has spawned novel methods of classification and experimentation entirely peculiar to the discipline of virology. The complexity of viruses is constantly challenging scientists to adjust their thinking and their research to describe and understand some new twist in the central dogma revealed in a *simple* virus infection.

This chapter explores several concepts fundamental to virology as a whole, including virus taxonomy, virus cultivation

and assay, and virus genetics. The chapter is not intended as a comprehensive or encyclopedic treatment of these topics, but rather as a relatively concise overview with sufficient documentation for more in-depth study. In addition to primary resources and practical experience, the presentation draws heavily on previous editions of *Fields Virology*^{35–37} for the taxonomy and genetics material, plus several excellent texts for material on virus cultivation and assay.^{20,34,41,59,70,76,81} It is hoped that this chapter will be of value to anyone learning virology at any stage: a novice trying to understand basic principles for the first time, an intermediate student of virology trying to understand the technical subtleties of virological protocols in the literature, or a bewildered scientist in the laboratory wondering why the host-range virus mutant received from a colleague does not seem to manifest the described host range.

VIRUS TAXONOMY

A coherent and workable system of classification—a taxonomy—is a critical component of the discipline of virology. However, the unique nature of viruses has defied the strict application of many of the traditional tools of taxonomy used in other disciplines of biology. Thus, scientists who concern themselves with global taxonomy of organisms have traditionally either ignored viruses completely as nonliving entities or left them scattered throughout the major kingdoms, reasoning that viruses have more in common with their individual hosts than they do with each other.^{82,90} By contrast, for practical reasons at least, virologists agree that viruses should be considered together as a separate group of organisms regardless of host, be it plant, animal, fungus, protist, or bacterium, a philosophy borne out by the observation that in several cases viruses now classified in the same family—for example, family *Reoviridae*—infect hosts from different kingdoms. Interestingly, the discipline of virus taxonomy brings out the most erudite and thought-provoking, virtually philosophical discussions about the nature of viruses, probably because the decisions that must be made to distinguish one virus from another require the deepest thought about the nature of viruses and virus evolution. In the end, all of nature is a continuum, and the business of taxonomy has the unfortunate obligation of drawing boundaries within this continuum, an artificial and illogical task but necessary nevertheless. The execution of this obligation results today in a free-standing virus taxonomy, overseen by the International Committee on Taxonomy of Viruses (ICTV), with rules and tools unique to the discipline of virology. The process of virus taxonomy that has evolved

uses some of the hierarchical nomenclature of traditional taxonomy, identifying virus species and grouping these into genera, genera into families, and families into orders, but at the same time, to cope with both the uniqueness and diversity of viruses as a group, the classification process has been deliberately nonsystematic and thus is “based upon the opinionated usage of data”.⁹²

Most importantly, the virus taxonomy that has been developed works well. For the trained virologist, the mention of a virus family or genus name, such as “family *Herpesviridae*” or “genus *Rotavirus*” immediately conjures forth a set of characteristics that form the basis for further discussion or description. Virus taxonomy serves an important practical purpose as well, in that the identification of a limited number of biological characteristics, such as virion morphology, genome structure, or antigenic properties, quickly provides a focus for identification of an unknown agent for the clinician or epidemiologist and can significantly impact further investigation into treatment or prevention of a virus disease. Virus taxonomy is an evolving field, and what follows is a summary of the state of the art, including important historical landmarks that influenced the present system of virus taxonomy, a description of the system used for virus taxonomy and the means for implementation of that system, and a very brief overview of the taxonomy of viruses that infect humans and animals.

History and Rationale

Virology as a discipline is scarcely 100 years old, and thus the discipline of virus taxonomy is relatively young. In the early 1900s, viruses were initially classified as distinct from other organisms simply by virtue of their ability to pass through unglazed porcelain filters known to retain the smallest of bacteria. As increasing numbers of filterable agents became recognized, they were distinguished from each other by the only measurable properties available, namely the disease or symptoms caused in an infected organism. Therefore, animal viruses that caused liver pathology were grouped together as hepatitis viruses, and viruses that caused mottling in plants were grouped together as mosaic viruses. In the 1930s, an explosion of technology spawned a description of the physical properties of many viruses, providing numerous new characteristics for distinguishing viruses one from another. The technologies included procedures for purification of viruses, biochemical characterization of purified virions, serology, and perhaps most importantly, electron microscopy, in particular negative staining, which permitted detailed descriptions of virion morphology, even in relatively crude preparations of infected tissue. In the 1950s, these characterizations led to the distinction of three major animal virus groups, the myxoviruses, the herpesviruses, and the poxviruses. By the 1960s, because of the profusion of data describing numerous different viruses, it became clear that an organized effort was required to classify and name viruses, and thus the ICTV (originally the International Committee on Nomenclature of Viruses [ICNV]) was established in 1966. The ICTV functions today as a large, international group of virologists organized into appropriate study groups, whose charge it is to develop rules for the classification and naming of viruses and to coordinate the activities of study groups in the implementation of these rules.

Early in its history, the ICTV wrestled with the fundamental problem of developing a taxonomic system for classification and naming of viruses that would accommodate the unique properties of viruses as a group and that could anticipate advancements in the identification and characterization of viruses. Perhaps the most critical issue was whether the classification of viruses should consider virus properties in a monothetical, hierarchical fashion or a polythetical, hierarchical fashion. A *monothetic* system of classification is defined as a system based on a single characteristic or a series of single characteristics. *Polythetic* is defined as sharing several common characteristics without any one of these characteristics being essential for membership in the group or class in question. Thus, a monothetical, hierarchical classification, modeled after the Linnaean system used for classification of plants and animals, would effectively rank individual virus properties, such as genome structure or virion symmetry, as being more or less important relative to each other and use these individual characteristics to sort viruses into subphyla, classes, orders, suborders, and families.⁷⁹ Although the hierarchical ordering of viruses into groups and subgroups is desirable, a strictly monothetical approach to using virus properties in making assignments to groups was problematic because both the identification of individual properties to be used in the hierarchy and the assignment of a hierarchy to individual properties seemed too arbitrary. A polythetic approach to classification would group viruses by comparing simultaneously numerous properties of individual viruses without assigning a universal priority to any one property. Thus, using the polythetic approach, a given virus grouping is defined by a collection of properties rather than a single property, and virus groups in different branches of the taxonomy may be characterized by different collections of properties. One argument against the polythetic approach is that a truly systematic and comprehensive comparison of dozens of individual properties would be at least forbidding if not impossible. However, this problem could be avoided by the adoption of a nonsystematic approach, namely, using study groups of virologists within the ICTV to consider together numerous characteristics of a virus and make as rational an assignment to a group as possible. Therefore, the system that is currently being used is a nonsystematic, polythetical, hierarchical system. This system differs from any other taxonomic system in use for bacteria or other organisms; however, it is effective, useful, and has withstood the test of time.⁹¹ As our understanding of viruses increases, and as new techniques for characterization are developed, notably comparison of gene and genome sequences, the methods used for taxonomy will undoubtedly continue to evolve.

As a consequence of the polythetic approach to classification, the virus taxonomy that exists today has been filled initially from the middle of the hierarchy by assigning viruses to genera, and then elaborating the taxonomy upward by grouping genera into families and, to a limited extent, families into orders. By 1970, the ICTV had established two virus families each containing 2 genera, 24 floating genera, and 16 plant groups.¹³³ A rigorous species definition,¹²⁶ discussed later, was not approved by the ICTV until 1991 but has now been applied to the entire taxonomy and has become the primary level of classification for viruses. As of this writing, the currently accepted taxonomy recognizes 6 orders, 87 families, 19 subfamilies, 348 genera,

TABLE 2.1 Summary Characteristics of Vertebrate Virus Families

Family	Nucleocapsid morphology	Envelope	Virion morphology	Genome ^a	Host ^b
dsDNA viruses					
<i>Adenoviridae</i>	Icosahedral	No	Icosahedral	1 ds linear, 26–48 kb	V
<i>Alloherpesviridae</i>	Icosahedral	Yes	Spherical, tegument	2 ds linear, 135–294 kb	V
<i>Asfviridae</i>	Icosahedral	Yes ^c	Icosahedral	1 ds linear, 165–190 kb	V, I
<i>Herpesviridae</i>	Icosahedral	Yes	Spherical, tegument	1 ds linear, 125–240 kb	V
<i>Iridoviridae</i>	Icosahedral	No ^d	Icosahedral	1 ds linear, 140–303	V, I
<i>Papillomaviridae</i>	Icosahedral	No	Icosahedral	1 ds circular, 7–8 kb	V
<i>Polyomaviridae</i>	Icosahedral	No	Icosahedral	1 ds circular, 5 kb	V
<i>Poxviridae</i>	Ovoid	Yes	Ovoid	1 ds linear, 130–375 kb	V, I
ssDNA viruses					
<i>Anellovirus</i>	Icosahedral	No	Icosahedral	1 – circular, 2–4 kb	V
<i>Circoviridae</i>	Icosahedral	No	Icosahedral	1 – or ± circular, 2 kb	V
<i>Parvoviridae</i>	Icosahedral	No	Icosahedral	1 +, – or ± linear, 4–6 kb	V, I
dsDNA reverse transcribing viruses					
<i>Hepadnaviridae</i>	Icosahedral	Yes	Spherical	1 ds circular, 3–4 kb	V
ssRNA reverse transcribing viruses					
<i>Metaviridae</i>	Spherical	Yes	Spherical	1 + linear, 4–10 kb	F, I, P, V
<i>Retroviridae</i>	Spherical, rod or cone shaped	Yes	Spherical	1 + linear dimer, 7–13 kb	V
dsRNA viruses					
<i>Birnaviridae</i>	Icosahedral	No	Icosahedral	2 ds linear, 5–6 kb	V, I
<i>Picobirnaviridae</i>	Icosahedral	No	Icosahedral	3 ds linear, 4 kb	V
<i>Reoviridae</i>	Icosahedral	No	Icosahedral, layered	10–12 ds linear, 19–32 kb	V, I, P, F
Negative sense ssRNA viruses					
<i>Bornaviridae</i>	ND ^e	Yes	Spherical	1 – linear, 9 kb	V
<i>Deltavirus^f</i>	Isometric	Yes	Spherical	1 – circular, 2 kb	V
<i>Filoviridae</i>	Helical filaments	Yes	Bacilliform, filamentous	1 – linear, 19 kb	V
<i>Orthomyxoviridae</i>	Helical filaments	Yes	Pleomorphic, spherical	6–8 – linear, 10–15 kb	V
<i>Paramyxoviridae</i>	Helical filaments	Yes	Pleomorphic, spherical, filamentous	1 – linear, 13–18 kb	V
<i>Rhabdoviridae</i>	Coiled helical filaments	Yes	Bullet shaped	1 – linear, 11–15 kb	V, I, P
Positive sense ssRNA viruses					
<i>Arteriviridae</i>	Linear, asymmetric	Yes	Spherical	1 + linear, 13–16 kb	V
<i>Astroviridae</i>	Icosahedral	No	Icosahedral	1 + linear, 6–8 kb	V
<i>Caliciviridae</i>	Icosahedral	No	Icosahedral	1 + linear, 7–8 kb	V
<i>Coronaviridae</i>	Helical	Yes	Spherical	1 + linear, 26–32 kb	V
<i>Flaviviridae</i>	Spherical	Yes	Spherical	1 + linear, 9–13 kb	V, I
<i>Hepevirus^g</i>	Icosahedral	No	Icosahedral	1 + linear, 7 kb	V
<i>Nodaviridae</i>	Icosahedral	No	Icosahedral	2 + linear, 4–5 kb	V, I
<i>Picornaviridae</i>	Icosahedral	No	Icosahedral	1 + linear, 7–9 kb	V
<i>Togaviridae</i>	Icosahedral	Yes	Spherical	1 + linear, 10–12 kb	V, I
Ambisense ssRNA viruses					
<i>Arenaviridae</i>	Filamentous	Yes	Spherical	2 ± linear, 11 kb	V
<i>Bunyaviridae</i>	Filamentous	Yes	Spherical	3 – or ± linear, 11–19 kb	V, I, P
Subviral agents: prions					
Prions	—	—	—	—	V, F

^aNumber of segments, polarity (ds, double stranded; +, mRNA like; –, cRNA like; ±, ambisense), conformation, size.

^bV, vertebrate; P, plant; I, insect; F, fungus.

^cContains both an outer envelope plus a lipid membrane internal to the capsid.

^dContains a membrane internal to the capsid.

^eND, not determined.

^f*Deltavirus* represents an unassigned genus.

and 2,290 species. The complete virus taxonomy is far too extensive to relate here; however, examples of the results of the taxonomy are offered in Tables 2.1 and 2.2. Table 2.1 lists the distinguishing characteristics of the vertebrate animal virus families, whereas Table 2.2 provides an example of the entire taxonomic classification of one virus order, namely order *Mononegavirales*.

The International Committee on Taxonomy of Viruses Universal System of Virus Taxonomy

Structure and Function

The ICTV is a committee of the Virology Division of the International Union of Microbiological Societies. The objectives of the ICTV are to develop an internationally agreed taxonomy

TABLE 2.2 Taxonomy of the Order *Mononegavirales*

Order	Family	Subfamily	Genus	Type species	Host	
Mononegavirales	Bornaviridae		Bornavirus	Borna disease virus	V	
			Rhabdoviridae	Vesiculovirus	Vesicular stomatitis Indiana virus	V, I
	Lyssavirus		Rabies virus	V		
	Ephemerovirus		Bovine ephemeral fever virus	V, I		
	Novirhabdovirus		Infectious hematopoietic necrosis virus	V		
	Cytorhabdovirus		Lettuce necrotic yellows virus	P, I		
	Nucleorhabdovirus		Potato yellow dwarf virus	P, I		
	Filoviridae		Marburgvirus	Lake Victoria marburgvirus	V	
			Ebolavirus	Zaire ebolavirus	V	
			Paramyxoviridae	Paramyxovirinae	Rubulavirus	Mumps virus
	Avulavirus		Newcastle disease virus		V	
	Respirovirus		Sendai virus		V	
	Henipavirus		Hendra virus		V	
	Morbillivirus		Measles virus		V	
	Pneumovirinae		Pneumovirus		Human respiratory syncytial virus	V
	Metapneumovirus		Avian metapneumovirus	V		

V, vertebrate; I, insect; P, plant.

and nomenclature for viruses, to maintain an index of virus names, and to communicate the proceedings of the committee to the international community of virologists. The ICTV publishes an update of the taxonomy at approximately 3-year intervals.^{32,33,39,85,86,92,133} At the time of this writing, the ninth report is being completed. The official taxonomy is also available on line at the ICTV website: <http://www.ictvonline.org>.

Virus Properties and Their Use in Taxonomy

As introduced previously, the taxonomic method adopted for use in virology is polythetic, meaning that any given virus group is described using a collection of individual properties. The description of a virus group is nonsystematic in that there exists no fixed list of properties that must be considered for all viruses and no strict formula for the ordered consideration of properties. Instead, a set of properties describing a given virus is simply compared with other viruses described in a similar fashion to formulate rational groupings. Characters such as virion morphology, genome organization, method of replication, and the number and size of structural and nonstructural viral proteins are used for distinguishing different virus families and genera. Characters such as genome sequence relatedness, natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, physicochemical properties of virions, and antigenic properties of viral proteins are used for distinguishing virus species within the same genus.¹²⁷

The Hierarchy

The ICTV has adopted a universal classification scheme that employs the hierarchical taxonomic levels of order, family, subfamily, genus, and species. Because the polythetic approach to classification introduces viruses into the middle of the hier-

archy, and because the ICTV has taken a relatively conservative approach to grouping taxa, levels higher than order are not currently used. Interestingly, groupings above the level of order may prove to be inappropriate: Higher taxons imply a common ancestry for viruses, whereas multiple independent lineages for viruses now seems the more likely evolutionary scenario.³² Taxonomic levels lower than species, such as clades, strains, and variants, are not officially considered by the ICTV but are left to specialty groups.

A virus species is defined as “a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche”.¹²⁶ The formal definition of a polythetic class is “a class whose members always have several properties in common although no single common attribute is present in all of its members”.¹²⁷ Thus, no single property can be used to define a given species, and application of this formal definition of a polythetic class to species accounts nicely for the inherent variability found among members of a species. The qualification of a replicating lineage implies that members of a species experience evolution over time with consequent variation, but that members share a common ancestor. The qualification of occupation of an ecological niche acknowledges that the biology of a virus, including such properties as host range, pathogenesis, transmission, and habitat, are fundamental components of the characterization of a virus. A *type species* has been identified for each genus. The type species is not necessarily the best characterized or most representative species in a genus; rather, it is usually the virus that initially necessitated the creation of the genus and therefore best defines or identifies the genus.

Taxonomic levels higher than species are formally defined by the ICTV only in a relative sense, namely a genus is a group of species sharing certain common characters, a subfamily is a group of genera sharing certain common characters, a family is a group of genera or subfamilies

sharing certain common characters, and an order is a group of families sharing certain common characters. As the virus taxonomy has evolved, these higher taxa have acquired some monothetic character. They remain polythetic in that they may be characterized by more than one virus property; however, they violate the formal definition of a polythetic class in that one or more defining properties may be required of all candidate viruses for membership in the taxon. Not all taxonomic levels need be used for a given grouping of viruses, thus whereas most species are grouped into genera and genera into families, not all families contain subfamilies, and only a few families have been grouped into orders. Consequently, the family is the highest consistently used taxonomic grouping, it therefore carries the most generalized description of a given virus group, and as a result has become the benchmark of the taxonomic system. Most families have distinct virion morphology, genome structure, and/or replication strategy (see Table 2.1).

Nomenclature

The ICTV has adopted a formal nomenclature for viruses, specifying suffixes for the various taxa, and rules for written descriptions of viruses. Names for genera, subfamilies, families, and orders must all be single words, ending with the suffixes -virus, -virinae, -viridae, and -virales, respectively. Species names may contain more than one word and have no specific ending. In written usage, the formal virus taxonomic names are capitalized and written in italics, and preceded by the name of the taxon, which is neither capitalized nor italicized. For

species names that contain more than one word, the first word plus any proper nouns are capitalized. As an example, the full formal written description of human respiratory syncytial virus is as follows: order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus *Pneumovirus*, species *Human respiratory syncytial virus*. The ICTV acknowledges that vernacular (informal) taxonomic names are widely used; however, they should not be italicized or capitalized. For example, the vernacular name “herpesvirus” refers to a member of the family *Herpesviridae*.

Informal Groupings and Alternate Classification Schemes

For convenience in presenting or tabulating the virus taxonomy, informal categorical groupings of taxa are often used. The criteria applied for such groupings typically include nature of the viral genome (DNA or RNA), strandedness of the viral genome (single stranded or double stranded), polarity of the genome (positive sense, negative sense, or ambisense), and reverse transcription. Separate categories accommodate subviral agents (including viroids, satellites, and prions) and unassigned viruses. The Baltimore classification system, named after its creator David Baltimore, is a widely used scheme based on the nature of the genome packaged in virions and the pathway of nucleic acid synthesis that each group takes to accomplish messenger RNA (mRNA) synthesis.¹ This classification divides viruses into seven categories as depicted in Figure 2.1. Most usages of this system group ambisense virus families (family *Arenaviridae*

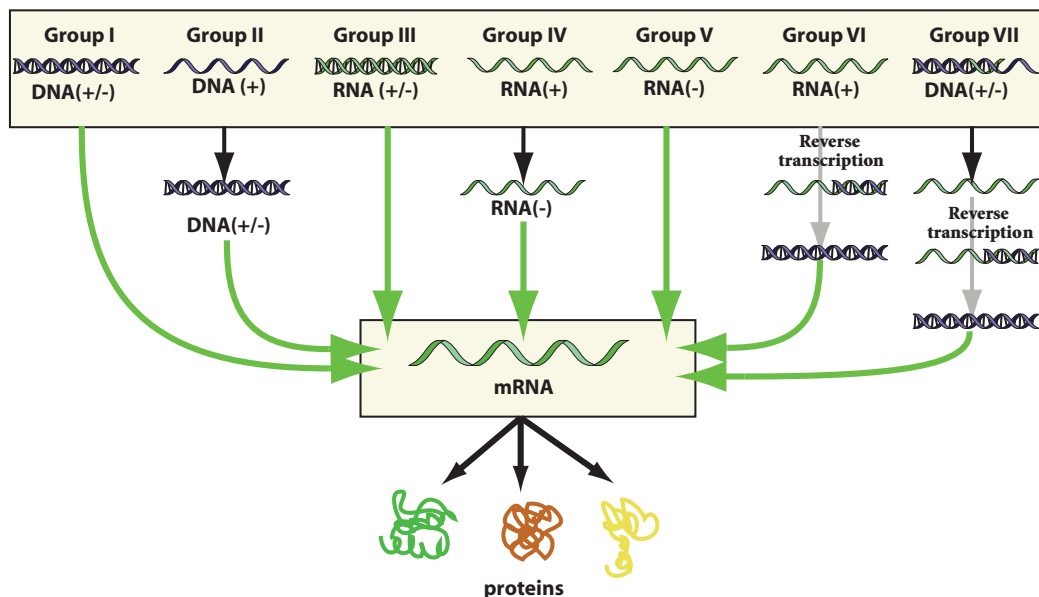


FIGURE 2.1. The Baltimore classification, a virus classification scheme based on the form of nucleic acid present in virion particles and the pathway for expression of the genetic material as messenger RNA.¹

The original scheme contained groups I through VI and has been expanded to accommodate DNA-containing, reverse transcribing viruses. Viruses containing ambisense single-stranded RNA genomes are grouped under negative sense single-stranded RNA viruses. (Reprinted from Hulo C, de Castro E, Masson P, et al. ViralZone: a knowledge resource to understand virus diversity. *Nucleic Acids Res* 2011;39 (Database issue):D576–D582; ViralZone, Swiss Institute of Bioinformatics, <http://www.expasy.ch/viralzone/>, with permission.)

and family *Bunyaviridae*) along with negative sense, single-stranded RNA (ssRNA) viruses. The families of vertebrate viruses listed in Table 2.1 have been grouped according to the Baltimore classification, with ambisense viruses split into an eighth genome category.

Universal Virus Database

To facilitate the management and distribution of virological data, the ICTV has established the universal virus database of the ICTV (ICTVdB). The ICTVdB is accessible on the Internet at <http://www.ictvdb.org>. Constructed from virus descriptions in the published reports of the ICTV, the database comprises searchable descriptions of all virus families, genera, and type species, including microscopic images of many viruses. The ICTVdB is a powerful resource for management of and access to virological data, and promises to considerably extend the reach and capability of the ICTV.

VIRUS CULTIVATION AND ASSAY

Different branches of science are defined in large part by their techniques, and virology is no exception. Whereas the study of viruses uses some general methods that are common to other disciplines, the unique nature of viruses and virus infections requires a unique set of technical tools designed specifically for their investigation. Conversely, what we know and *can* know about viruses is delimited by the techniques used; therefore, a genuine understanding of virology requires a clear understanding of virological methods. What follows is a summary of the major techniques essential and unique to all of virology, presented as fundamental background for understanding the discipline.

Initial Detection and Isolation

The presence of a virus is evidenced initially by effects on a host organism or, in the case of a few animal viruses, by effects on cultured cells. Effects on animal hosts obviously include a broad spectrum of symptoms, including skin and mucous membrane lesions; digestive, respiratory, or neurological disorders; immune dysfunction; specific organ failure such as hepatitis or myocarditis; and death. Effects on cultured cells include a variety of morphological changes in infected cells, termed *cytopathic effects* and described in detail later in this chapter and in Chapter 15. Both adenovirus¹⁰⁸ and the polyomavirus SV40¹²¹ were discovered as cell culture contaminants before they were detected in their natural hosts.

Viruses can be isolated from an infected host by harvesting excreted or secreted material, blood, or tissue and testing for induction of the original symptoms in the identical host, or induction of some abnormal pathology in a substitute host or in cell culture. Historically, dogs, cats, rabbits, rats, guinea pigs, hamsters, mice, and chickens have all been found to be useful in laboratory investigations,⁷⁰ although most animal methods have now been replaced by cell culture methods.⁸¹ Once the presence of a virus has been established, it is often desirable to prepare a genetically pure clone, either by limiting serial dilution or by plaque purification.

Viruses that are cultivated in anything other than the natural host may adapt to the novel situation through acquisition

of genetic alterations that provide a replication advantage in the new host. Such adaptive changes may be accompanied by a loss of fitness in the original host, most notably by a loss of virulence or pathogenicity. Whereas this adaptation and attenuation may present problems to the basic scientist interested in understanding the replication of the virus in its natural state, it also forms the basis of construction of attenuated viral vaccines.

Hosts for Virus Cultivation

Laboratory Animals and Embryonated Chicken Eggs

Prior to the advent of cell culture, animal viruses could be propagated only on whole animals or embryonated chicken eggs. Whole animals could include the natural host or laboratory animals such as rabbits, mice, rats, and hamsters. In the case of laboratory animals, newborn or suckling rodents often provide the best hosts. Today, laboratory animals are seldom used for routine cultivation of virus; however, they still play an essential role in studies of viral pathogenesis.

The use of embryonated chicken eggs was introduced to virology by Goodpasture et al⁴⁴ in 1932 and developed subsequently by Beveridge and Burnet.⁴ The developing chick embryo, 10 to 14 days after fertilization, provides a variety of differentiated tissues, including the amnion, allantois, chorion, and yolk sac, which serve as substrates for growth of a wide variety of viruses, including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, herpesviruses, and poxviruses.⁷⁰ Members of each of these virus families may replicate in several tissues of the developing egg, or replication may be confined to a single tissue. Several viruses from each of the previously mentioned groups cause discrete and characteristic foci when introduced onto the chorioallantoic membrane of embryonated eggs, thus providing a method for identification of virus types, or for quantifying virus stocks or assessing virus pathogenicity (Fig. 2.2). Although embryonated eggs have been almost wholly replaced by cell culture techniques, they are still the most convenient method for growing high titer stocks of some viruses and thus continue to be used both in research laboratories and for vaccine production.

Cell Culture

The growth and maintenance of animal cells *in vitro*, described generally (albeit incorrectly) as tissue culture, can be formally divided into three different techniques: organ culture, primary explant culture, and cell culture. In *organ culture*, the original three-dimensional architecture of a tissue is preserved under culture conditions that provide a gas-liquid interface. In *primary explant culture*, minced pieces of tissue placed in liquid medium in a culture vessel provide a source for outgrowth of individual cells. In *cell culture*, tissue is disaggregated into individual cells prior to culturing. Only cell culture will be discussed in detail here, because it is the most commonly used tissue culture technique in virology.

Cultured cells currently provide the most widely used and most powerful hosts for cultivation and assay of viruses. Cell cultures are of three basic types—primary cell cultures, cell strains, and cell lines—that may be derived from many animal species and that differ substantially in their characteristics. Viruses often behave differently on different types of cultured cells; in addition, each of the culture types possess technical

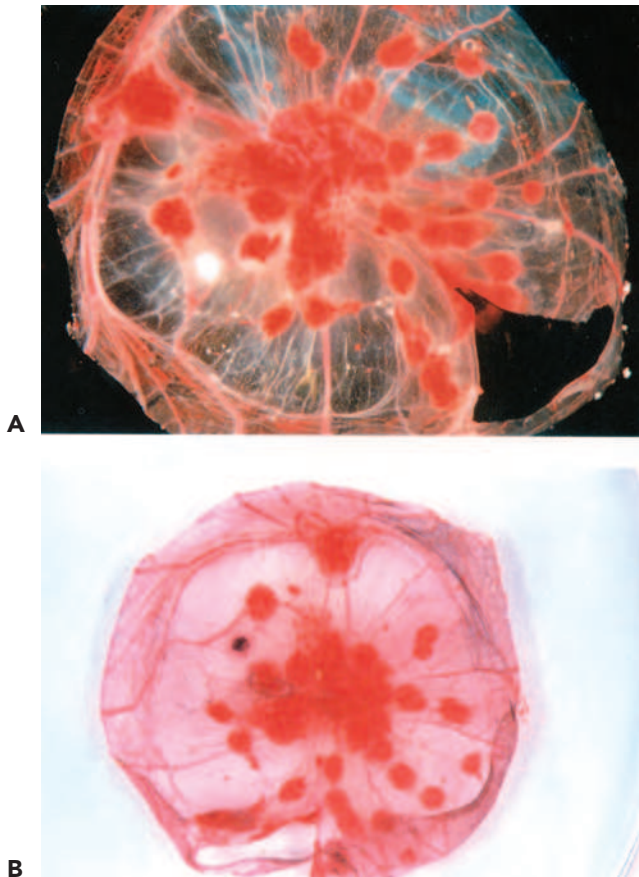


FIGURE 2.2. Cowpox-induced pock formation on the chorioallantoic membrane of chick embryos. The chorioallantoic membrane of intact chicken embryos, 11 days old, were inoculated with cowpox, and the eggs were incubated for an additional 3 days at 37.5°C. Chorioallantoic membranes were then dissected from the eggs and photographed. The membrane shown in **A** was untreated, whereas the membrane in **B** was stained with NBT, an indicator of activated heterophils.⁴⁰ Wild-type cowpox forms red hemorrhagic pocks on the membrane (**A** and **B**). Spontaneous deletion mutants of cowpox virulence genes occur at a high frequency, resulting in infiltration of inflammatory cells into the pock. The infiltration of inflammatory cells causes the pocks to appear white in unstained membrane preparations or dark blue on NBT-stained membranes. The unstained membrane preparation (**A**) contains a single white pock, whereas the NBT-stained preparation (**B**) contains a single blue pock. NBT, nitroblue tetrazolium. (Courtesy of Dr. R. Moyer.)

advantages and disadvantages. For these reasons, an appreciation of the use of cultured cells in animal virology requires an understanding of several fundamentals of cell culture itself. A detailed description of the theory and practice of cell and tissue culture is provided by Freshney,⁴¹ and several additional texts provide excellent summaries of cell culture as it specifically applies to virology.^{20,34,59}

PRIMARY CELL CULTURE

A primary cell culture is defined as a culture of cells obtained from the original tissue that have been cultivated *in vitro* for the first time and that have not been subcultured. Primary

cell cultures can be established from whole animal embryos or from selected tissues from embryos, newborn animals, or adult animals of almost any species. The most commonly used cell cultures in virology derive from primates, including humans and monkeys; rodents, including hamsters, rats, and mice; and birds, most notably chickens. Cells to be cultured are obtained by mincing tissue and dispersing individual cells by treatment with proteases and/or collagenase to disrupt cell–cell interactions and interactions of cells with the extracellular matrix. With the exception of cells from the hemopoietic system, normal vertebrate cells will grow and divide only when attached to a solid surface. Dispersed cells are therefore placed in a plastic flask or dish, the surface of which has been treated to promote cell attachment. The cells are incubated in a buffered nutrient medium in the presence of blood serum, which contains a complex mixture of hormones and factors required for the growth of normal cells. The blood serum may come from a variety of sources, although bovine serum is most commonly used. Under these conditions, cells will attach to the surface of the dish, and they will divide and migrate until the surface of the dish is covered with a single layer of cells, a monolayer, whereupon they will remain viable but cease to divide. If the cell monolayer is “wounded” by scraping cells from an isolated area, cells on the border of the wound will resume division and migration until the monolayer is reformed, whereupon cell division again ceases. These and other observations lead to the conclusion that the arrest of division observed when cells reach confluency results from cell–cell contact and therefore is called *contact inhibition*. Primary cultures may contain a mixture of cell types and retain the closest resemblance to the tissue of origin.

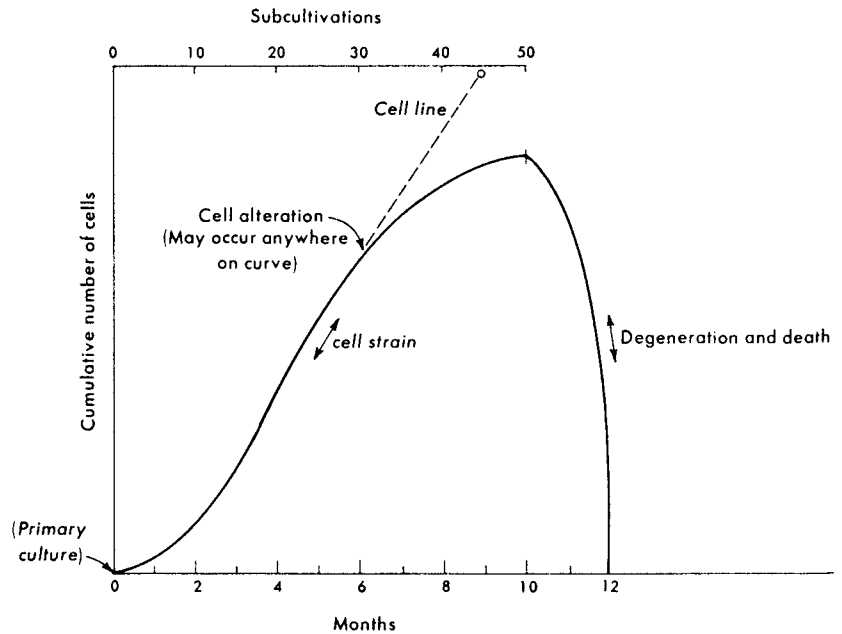
SUBCULTIVATION

Cells from a primary culture may be subcultured to obtain larger numbers of cells. Cells are removed from the culture dish and disaggregated by treating the primary cell monolayer with a chelating agent, usually EDTA, or a protease, usually trypsin, or both, giving rise to a single cell suspension. This suspension is then diluted to a fraction of the original monolayer cell density and placed in a culture dish with fresh growth medium, whereupon the cells attach to the surface of the dish and resume cell division until once again a monolayer is formed and cell division ceases. Cultures established in this fashion from primary cell cultures may be called *secondary cultures*. Subsequently, cells may be repeatedly subcultured in the same fashion. Each subculturing event is called a *passage*, and each passage may comprise several cell generations, depending on the dilution used during the passage. Most vertebrate cells divide at the rate of approximately one doubling every 24 hours at 37°C. Thus, a passage performed with an eightfold dilution will require three cell doublings over 3 days before the cells regain confluency.

CELL STRAINS

Normal vertebrate cells cannot be passaged indefinitely in culture. Instead, after a limited number of cell generations, usually 20 to 100 depending on the age and species of the original animal, cultured normal cells cease to divide, then degenerate and die, a phenomenon called *crisis* or *senescence*⁵¹ (Fig. 2.3). Starting with the establishment of a secondary culture and until cells either senesce or become transformed as described later, the culture is termed a *cell strain* to distinguish it from a primary culture

FIGURE 2.3. Growth of cells in culture. A primary culture is defined as the original plating of cells from a tissue, grown to a confluent monolayer, without subculturing. A cell strain (*solid line*) is defined as a euploid population of cells subcultivated once or more *in vitro*, lacking the property of indefinite serial passage. Cell strains ultimately undergo degeneration and death, also called *crisis* or *senescence*. A cell line (*dashed line*) is an aneuploid population of cells that can be grown in culture indefinitely. Spontaneous transformation or alteration of a cell strain to an immortal cell line can occur at any time during cultivation of the cell strain. The time in culture and corresponding number of subcultivations or passages are shown on the abscissas. The ordinate shows the total number of cells that would accumulate if all were retained in culture. (Reprinted from *Animal cells: cultivation, growth regulation, transformation*. In: Davis BD, Dulbecco R, Eisen HN, et al, eds. *Microbiology*. 4th ed. Philadelphia: J. B. Lippincott Company.)



on the one hand, or a transformed, immortal cell line on the other hand. During culture, cells in a strain retain their original karyotype and are thus called *euploid*; however, culturing induces profound changes in the composition and characteristics of the cell strain, which are manifested early during the passage history and may continue during passage. Whereas primary cell cultures may contain a mixture of cell types that survive the original plating of cells, only a few cell types survive subculturing; thus, by

the second or third passage, typically only one cell type remains in the cell strain. Cell strains are usually composed of one of two basic cell types—fibroblast-like or epithelial-like—characterized based on their morphology and growth characteristics (Fig. 2.4). Fibroblasts have an elongated, spindle shape, whereas epithelial cells have a polygonal shape. Although after only a few passages only one cell type may remain in a cell strain, continued passage may select for faster-growing variants, such that the

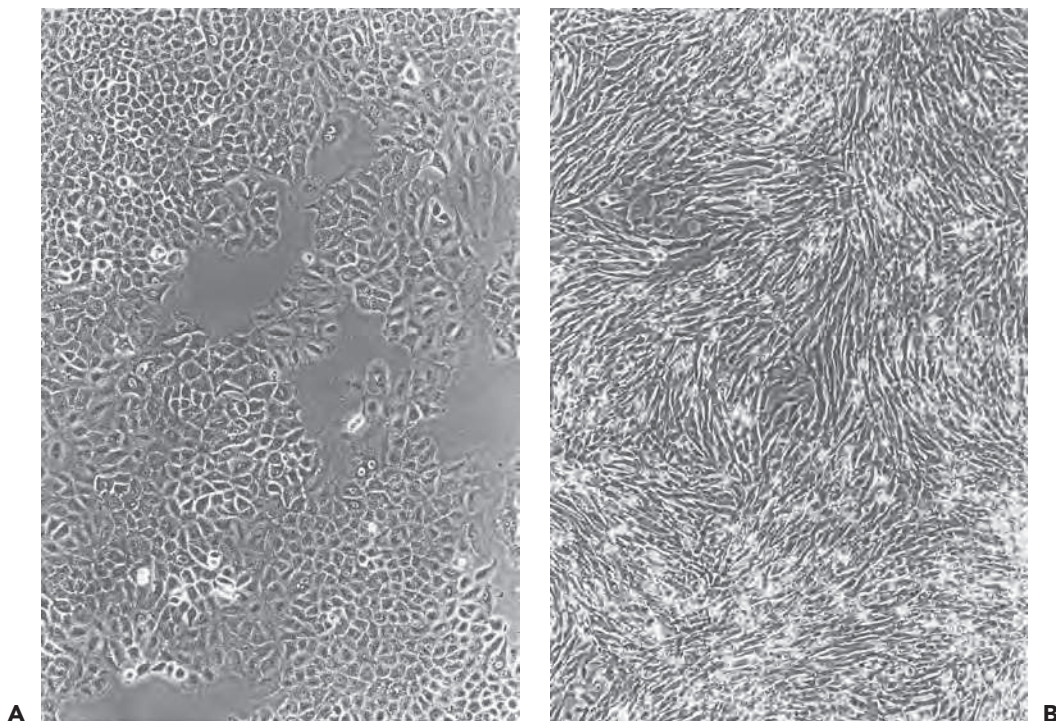


FIGURE 2.4. Cultured cell types. Phase contrast photomicrographs are shown. **A:** Epithelial-like cells, A549, a human lung carcinoma cell line, a slightly subconfluent monolayer. **B:** Fibroblast-like cells, BHK, a baby hamster kidney cell line. (A549 cell culture courtesy of J. I. Lewis. BHK cell culture courtesy of D. Holmes and Dr. S. Moyer.)

characteristics of a cell strain may change with increasing passage number. Despite the fact that normal cell strains experience senescence in culture, they may be maintained for many years by expanding the culture to a large number of cells early during the passage history and storing numerous small samples of low passage cells by freezing. Therefore, as a given strain approaches high passage number and senescence, low passage cells of the same strain may be thawed and cultured.

CELL LINES

At any time during the culture of a cell strain, cells in the culture may become *transformed* such that they are no longer subject to crisis and senescence but can be passaged indefinitely. Transformation is a complex phenomenon, discussed in more detail later and in Chapter 7; however, in the context of cell culture, the most important characteristic of transformation is that the transformed cells become immortalized. Immortal cell cultures are called *cell lines*, or sometimes *continuous cell lines*, to distinguish them from primary cultures and cell strains. Immortalization can occur spontaneously during passage of a cell strain, or it can be induced by treatment with chemical mutagens, infection with tumorigenic viruses, or transfection with oncogenes. In addition, cells cultured from tumor tissue frequently readily establish immortal cell lines in culture. Spontaneous immortalization does not occur in cultured cells from all animal species. Thus, immortalization occurs frequently during culture of rodent cells (e.g., in mouse and hamster cell strains), and it has been observed in monkey kidney cells, although it occurs rarely, if at all, during the culture of chicken or human cells. Immortalization is typically accompanied by genetic changes such that cells become aneuploid, containing abnormalities in the number and structure of chromosomes relative to the parent species, and not all cells in a culture of a continuous cell line necessarily display the same karyotype. Like cell strains, cell lines are usually composed of cells that are either fibroblast-like or epithelial-like in morphology.

As with the propagation of cell strains, continued culture of a cell line may result in selection of specific variants that outgrow other cells in the culture over time, and thus with passage the character of a cell line may change substantially, and cell lines of the same origin cultured in different laboratories over a period of years may have significantly different characteristics. It is prudent, therefore, to freeze stocks of cell lines having specific desirable properties so that these cells can be recovered if the properties disappear during culture. Likewise, it makes sense to obtain a cell line showing certain desired characteristics directly from the laboratory that described those characteristics, because cells from alternate sources may differ in character.

TRANSFORMATION

Transformed cells are distinguished from normal cells by myriad properties that can be grouped into three fundamental types of changes: immortalization, aberrant growth control, and malignancy. *Immortalization* refers simply to the ability to be cultured indefinitely, as described previously. *Aberrant growth control* comprises a number of properties, several of which have relevance to experimental virology, including loss of contact inhibition, anchorage independence, and tumorigenicity. Loss of contact inhibition means that cells no longer cease to grow as soon as a monolayer is formed, and cells will now grow on top of one another. Anchorage independence means that the cells no longer need to attach to a solid surface

to grow. Anchorage independence is often assayed as the ability to form colonies suspended in a semisolid medium such as agar, and a practical consequence of anchorage independence is the ability to grow in liquid suspension. Tumorigenicity refers to the ability of cells to form a tumor in an experimental animal, and *malignancy* refers to the ability to form an invasive tumor *in vivo*. While malignancy is obviously of vital importance as a phenomenon in its own right, it has limited application in virology except within the specific discipline of tumor virology (Chapter 7). Importantly, the many properties of transformed cells are not necessarily interdependent, and no one property is an absolute prerequisite for another. Thus, transformation is thought to be a multistep genetic phenomenon, and varying degrees of transformation are measurable. Tumorigenicity is often regarded as the most stringent assay for a fully transformed cell and is most closely correlated with anchorage independence.

The fact that the various characteristics of transformed cells are not interdependent has important consequences for experimental virology, especially in the assay of tumor viruses. Specifically, a transformed cell line that is immortalized but still contact inhibited may be used in a viral transformation assay that measures the further transformation to loss of contact inhibition. When cells in a monolayer are transformed by a tumor virus and lose contact inhibition, they grow on top of a confluent monolayer, forming a *focus*, literally a pile of cells, which is readily distinguishable from the rest of the monolayer. This property forms the basis for quantitative biological assay of tumor viruses,¹²⁹ described in more detail later.

ADVANTAGES AND DISADVANTAGES OF DIFFERENT CULTURED CELL TYPES

The various types of cultured cells described previously have specific application to different problems encountered in experimental virology. For most applications, an adherent cell line provides the most useful host cell. Cell lines are relatively easy to maintain because they can be passaged indefinitely, and adherence is a prerequisite for a plaque assay, described later. A distinct technical advantage of adherent cells is that the culture medium can easily be changed for the purposes of infection or metabolic labeling by simply aspirating and replacing fluid from a monolayer, a process that requires repeated centrifugations with suspension cells. By contrast, relative to adherent cell lines, suspension cell lines are easier to sample than adherent cells, and they produce large numbers of cells from a relatively small volume of medium in a single culture vessel, which has significant advantages for some high-volume applications in virology. Unfortunately, not all viruses will grow on a cell line, and often under these circumstances, a primary cell culture will suffice. This may reflect a requirement for a particular cell type found only under conditions of primary cell culture, or it may reflect a requirement for a state of metabolism or differentiation closely resembling the *in vivo* situation, which is more likely to exist in a primary culture than it is in a cell line.

Lastly, some viruses do not grow in cell culture at all. In such cases, investigators are reliant either on the old expedients of natural hosts, laboratory animals, or embryonated eggs, or on some more modern advances in tissue culture and recombinant DNA technology. The papillomaviruses, which cause warts, provide an enlightening example of this situation (Chapter 54). Although the viral nature of papillomatosis was

demonstrated more than 90 years ago, progress on the study of papillomaviruses was seriously hampered in the virology heyday of the mid 20th century because the viruses grow well only on the natural host; they do not grow in culture. The inability to grow in culture is now reasonably well understood, and results from a tight coupling of the regulation of viral gene expression with the differentiation state of the target epithelial cell, which in turn is tightly coupled to the three-dimensional architecture of the epidermis, which is lost in culture. Specialized tissue culture techniques have now been developed that result in the faithful reconstruction of an epidermis by seeding primary keratinocytes on a “feeder” layer composed of an appropriate cell line and incubating these cells on a “raft” or grid at a liquid–air interface. On these raft cultures, the entire replication cycle of a papillomavirus can be reproduced *in vitro*, albeit with difficulty.⁷ In the meantime, it is significant that a large fraction of the genetics and biology of papillomaviruses was determined primarily through the use of recombinant DNA technology, without ever growing virus in culture. Thus, the genetic structure of both the model bovine papillomavirus and many human papillomaviruses has been determined by cloning genomic DNA from natural infections, and regulation and function of many genes can be gleaned from sequence alone, from *in vitro* assays on individual gene products expressed *in vitro*, and from cell transformation assays that use all or parts of a papillomavirus genome. In summary, the inability to grow a virus in culture, although it increases the challenge, no longer presents an insurmountable impediment to understanding a virus.

Recognition of Viral Growth in Culture

Two principal methods exist for the recognition of a virus infection in culture: cytopathic effect and hemadsorption. *Cytopathic effect* comprises two different phenomena: (a) morphological changes induced in individual cells or groups of cells by virus infection that are easily recognizable under a light microscope, and (b) inclusion bodies, which are more subtle alterations to the intracellular architecture of individual cells. *Hemadsorption* refers to indirect measurement of viral protein synthesis in infected cells, detected by adsorption of erythrocytes to the surface of infected cells. Cytopathic effect is the simplest and most widely used criterion for infection; however, not all viruses cause a cytopathic effect, and in these cases, other methods must suffice.

Morphological changes induced by virus infection comprise a number of cell phenomena, including rounding, shrinkage, increased refractility, fusion, aggregation, loss of adherence or lysis. Morphological changes caused by a given virus may include several of these phenomena in various combinations, and the character of the cytopathic effect may change reproducibly during the course of infection. Morphological changes caused by a given virus are very reproducible and can be so precisely characteristic of the virus type that significant clues to the identity of a virus can be gleaned from the cytopathic effect alone (Chapter 15). Figure 2.5 depicts different cytopathic effects caused by two viruses—measles and vaccinia. Most important to the trained virologist, a simple microscopic examination of a cell culture can reveal whether an infection is present, what fraction of cells are infected, and how advanced the infection is. In addition, because cytopathology results directly from the action of virus gene products, virus mutants can be obtained that are

altered in cytopathology, yielding either a conveniently marked virus or a tool to study cytopathology *per se*.

The term *inclusion bodies* refers generally to the observation of intracellular structures specific to an infected cell and discernible by light microscopy. The effects are highly specific for a particular virus type so that, as with morphological alterations, the presence of a specific type of inclusion body can be diagnostic of a specific virus infection. Electron microscopy, combined with a more detailed understanding of the biology of many viruses, reveals that inclusion bodies usually represent focal points of virus replication and assembly, which differ in appearance depending on the virus. For example, Negri bodies formed during a rabies virus infection represent collections of virus nucleocapsids⁸⁴ (Chapter 31).

Hemadsorption refers to the ability of red blood cells to attach specifically to virus-infected cells.¹¹¹ Many viruses synthesize cell attachment proteins, which carry out their function wholly or in part by binding substituents such as sialic acid that are abundant on a wide variety of cell types, including erythrocytes. Often, these viral proteins are expressed on the surface of the infected cell—for example, in preparation for maturation of an enveloped virus through a budding process. Thus, a cluster of infected cells may be easily detectable to the naked eye as areas that stain red after exposure to an appropriate preparation of red blood cells. Hemadsorption can be a particularly useful assay for detecting infections by viruses that cause little or no cytopathic effect.

Virus Cultivation

From the discussion presented previously, it may be obvious that ultimately the exact method chosen for growing virus on any particular occasion will depend on a variety of factors, including (a) the goals of the experiment, namely whether large amounts of one virus variant or small amounts of several variants are to be grown; (b) limitations in the *in vitro* host range of the virus, namely whether it will grow on embryonated eggs, primary cell cultures, continuous adherent cell lines, or suspension cell lines; and (c) the relative technical ease of alternative possible procedures. Furthermore, the precise method for harvesting a virus culture will depend on the biology of the virus—for example, whether it buds from the infected cell, lyses the infected cell, or leaves the cell intact and stays tightly cell associated. As a simple example, consider cultivation of a budding, cytopathic virus on an adherent cell line. Confluent monolayers of an appropriate cell line are exposed to virus diluted to infect a fraction of the cells, and progress of the infection is monitored by observing the development of the cytopathic effect until the infection is judged complete based on experience with the relationship between cytopathic effect and maximum virus yield. A crude preparation of virus can be harvested simply by collecting the culture fluid; it may not even be necessary to remove cells or cell debris. Most viruses can be stored frozen indefinitely either as crude or purified, concentrated preparations.

Quantitative Assay of Viruses

Two major types of quantitative assays for viruses exist: physical and biological. *Physical* assays, such as hemagglutination, electron microscopic particle counts, optical density measurements, or immunological methods, quantify only the presence of virus particles whether or not the particles are infectious. *Biological* assays, such as the plaque assay or various endpoint

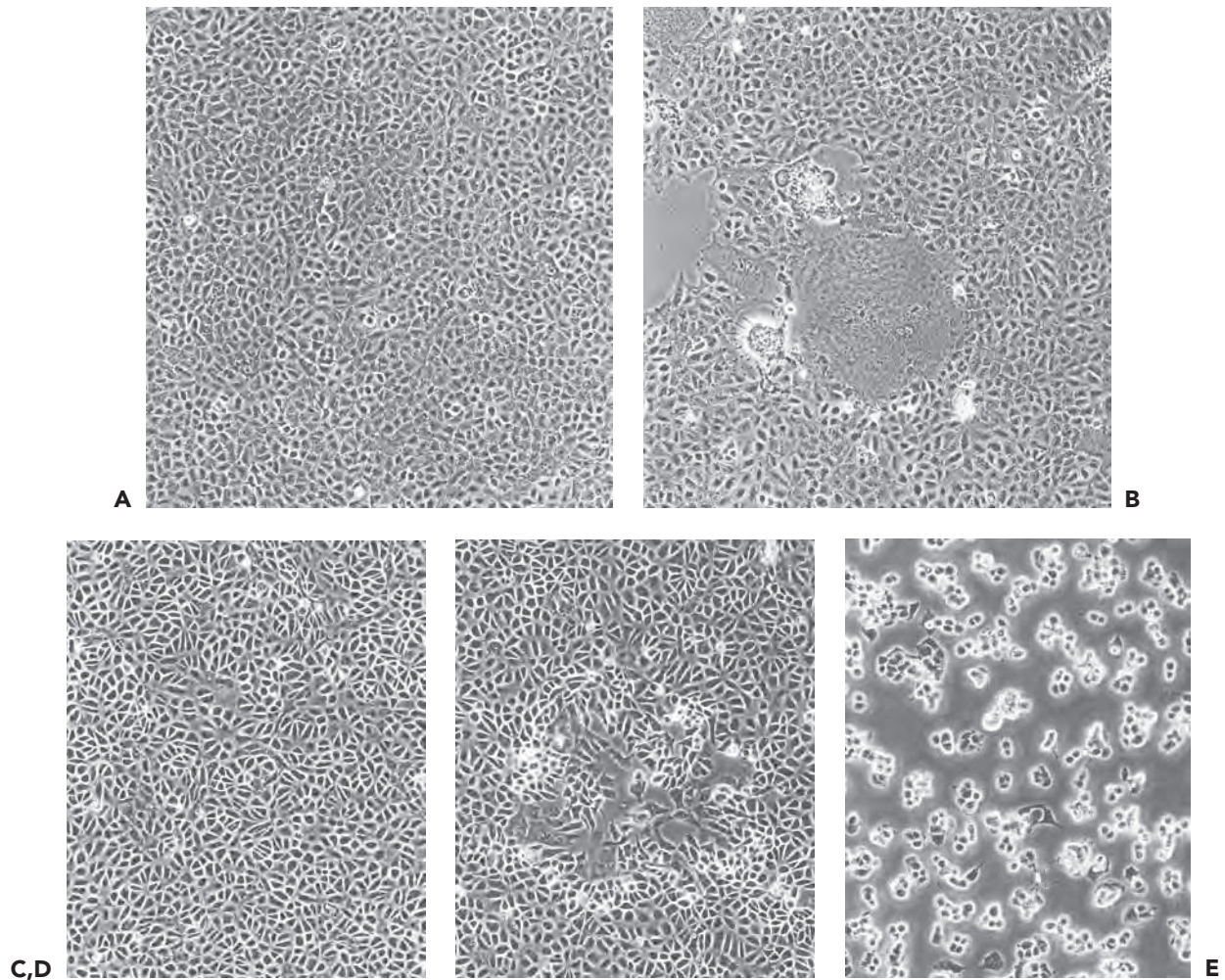


FIGURE 2.5. Virus-induced cytopathic effects. Phase contrast photomicrographs are shown. **A:** Uninfected A549 cells, a human lung carcinoma cell line. **B:** A549 cells infected with measles virus at a moi of less than 0.01 pfu/cell. Individual plaques can be discerned. Measles fuses cells, causing formation of syncytia. In mid field is a large syncytium containing multiple nuclei. Surrounding this area are additional syncytia, including two that have rounded and are separating from the dish. **C:** Uninfected BSC40 cells, an African green monkey cell line. **D:** BSC40 cells infected with vaccinia virus at a moi of less than 0.01 pfu/cell. A single plaque is shown in the middle of the field. **E:** BSC40 cells infected with vaccinia virus at a moi of 10 pfu/cell, 48 hours after infection. All cells are infected and display complete cytopathic effect. (Cultures of vaccinia infections courtesy of J. I. Lewis. Cultures of measles infections courtesy of S. Smallwood and Dr. S. Moyer.)

methods that have in common the assay of infectivity in cultured cells or *in vivo*, measure only the presence of infectivity and may not count all particles present in a preparation, even many that are in fact infectious. Thus, a clear understanding of the nature and efficiency of both physical and biological quantitative virus assays is required to make effective use of the data obtained from any assay.

Biological Assays

THE PLAQUE ASSAY

The plaque assay is the most elegant, the most quantitative, and the most useful biological assay for viruses. Developed originally for the study of bacteriophage by d'Herelle¹⁸ in the early 1900s, the plaque assay was adapted to animal viruses by Dulbecco and Vogt²⁸ in 1953, an advance that revolutionized animal virology by introducing a methodology that was

relatively simple and precisely quantitative, which enabled the cloning of individual genetic variants of a virus, and which permitted a qualitative assay for individual virus variants that differ in growth properties or cytopathology.

The plaque assay is based simply on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathology on an otherwise normal monolayer of cultured cells. Specifically, if a single cell in a monolayer is infected with a single virus particle, new virus resulting from the initial infection can infect surrounding cells, which in turn produce virus that infects additional surrounding cells. Over a period of days (the exact length of time depending on the particular virus), the initial infection thus gives rise through multiple rounds of infection to an area of infection, called a *plaque*. Photomicrographs of plaques are shown in Figure 2.5, and stained monolayers containing plaques are shown in Figure 2.6.

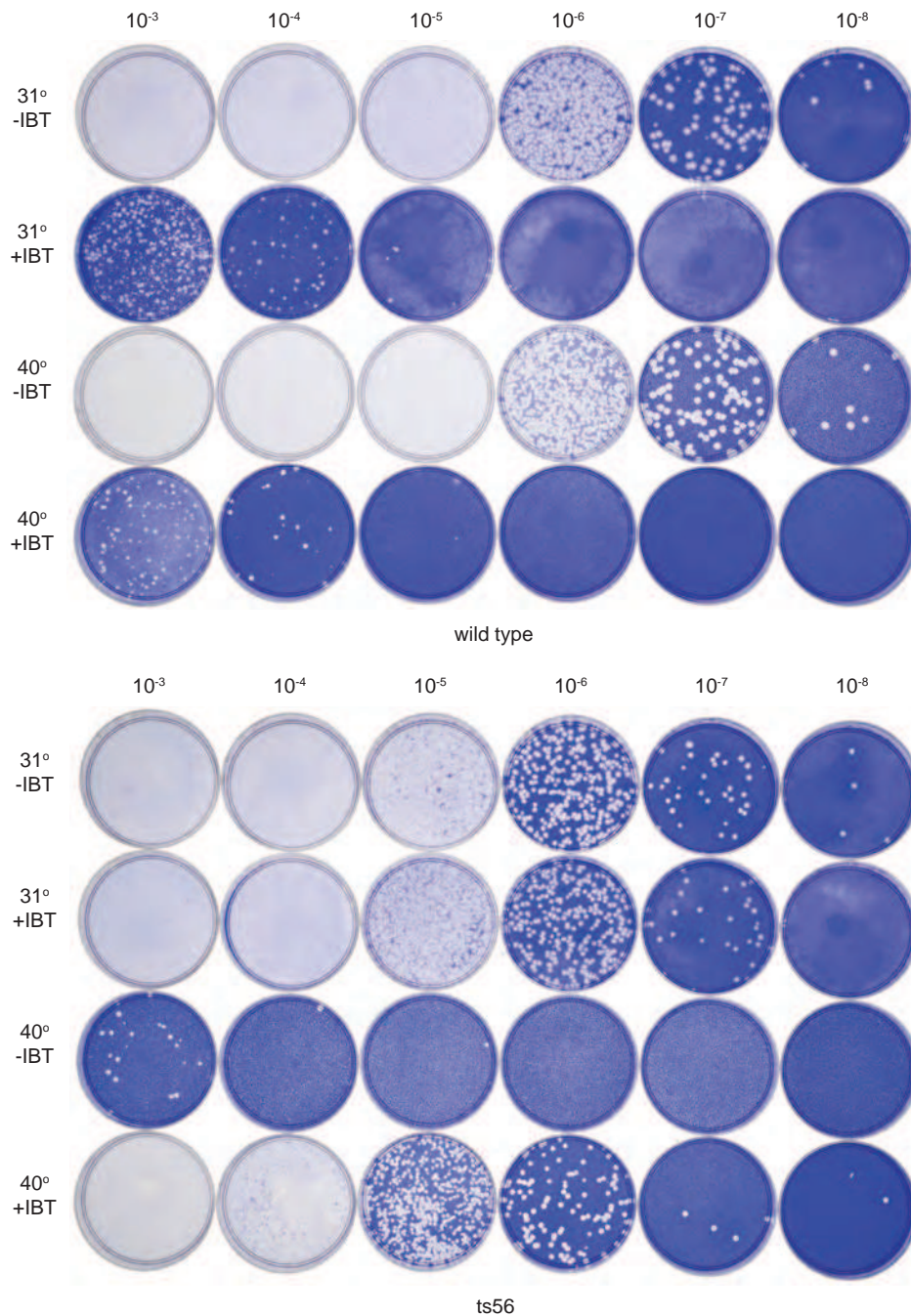


FIGURE 2.6. Plaque assay. Monolayers of the African green monkey kidney cell line BSC40 were infected with 0.5-mL portions of 10-fold serial dilutions of wild-type vaccinia virus or the temperature-sensitive vaccinia mutant, *ts56*, as indicated. Infected monolayers were overlaid with semisolid medium and incubated at 31°C or 40°C, the permissive and nonpermissive temperatures for *ts56*, in the presence of 45 μ M isatin- β -thiosemicarbazone (IBT) or in the absence of drug as indicated, for 1 week. Overlays were removed, and monolayers were stained with crystal violet. Wild-type vaccinia virus forms plaques at both 31°C and 40°C; however, plaque formation is inhibited by IBT. Spontaneous IBT-resistant mutants in the wild-type virus stock are revealed as plaques forming at 10^{-3} , 10^{-4} , and 10^{-5} dilutions in the presence of IBT. *ts56* carries a single-base missense mutation in the vaccinia gene *G2R*.⁸⁷ *G2R* is an essential gene that when completely inactivated renders virus dependent on IBT; hence, *ts56* is not only temperature sensitive, forming plaques at 31°C but not at 40°C in the absence of IBT, but it is also IBT dependent at 40°C, forming plaques in the presence but not the absence of IBT. *ts56* is slightly defective at 31°C; it forms smaller than wild-type plaques and is IBT resistant, forming plaques both in the presence and absence of IBT, a phenotype intermediate between the wild-type IBT-sensitive phenotype and the null *G2R* mutant IBT-dependent phenotype. Wild-type, temperature-insensitive revertants present in the *ts56* stock are revealed as plaques growing on the 10^{-3} plate at 40°C. Based on this assay, the titer of the wild-type stock is 2.0×10^9 pfu/mL, and the titer of the *ts56* stock is 6.0×10^8 pfu/mL. IBT, isatin- β -thiosemicarbazone.

The plaque assay can be used to quantify virus in the following manner (see Fig. 2.6). A sample of virus of unknown concentration is serially diluted in an appropriate medium, and measured aliquots of each dilution are seeded onto confluent monolayers of cultured cells. Infected cells are overlaid with a semisolid nutrient medium usually consisting of growth medium and agar. The semisolid medium prevents formation of secondary plaques through diffusion of virus from the original site of infection to new sites, ensuring that each plaque that develops in the assay originated from a single infectious particle in the starting inoculum. After an appropriate period of incubation to allow development of plaques, the monolayer is stained so that the plaques can be visualized. The precise staining technique depends on the cytopathology; however, vital dyes such as neutral red are common. Neutral red is taken up by living cells but not by dead cells; thus, plaques become visible as clear areas on a red monolayer of cells. In cases where the virus cytopathology results in cell lysis or detachment of cells from the dish, plaques exist literally as holes in the monolayer, and a permanent record of the assay can be made by staining the monolayer with a general stain such as crystal violet, prepared in a fixative such as formalin. The goal of the assay is to identify a dilution of virus that yields 20 to 100 plaques on a single dish—that is, a number large enough to be statistically significant yet small enough such that individual plaques can be readily discerned and counted. Usually, a series of four to six 10-fold dilutions is tested, which are estimated to bracket the target dilution. Dishes inoculated with low dilutions of virus will contain only dead cells or too many plaques to count, whereas dishes inoculated with high dilutions of virus will contain very few, if any, plaques (see Fig. 2.6). Dishes containing an appropriate number of plaques are counted, and the concentration of infectious virus in the original sample can then be calculated taking into account the serial dilution. The resulting value is called a *titer* and is expressed in plaque-forming units per milliliter (pfu/mL) to emphasize specifically that only viruses capable of forming plaques have been quantified. Titers derived by serial dilution are unavoidably error prone, owing simply to the additive error inherent in multiple serial pipetting steps. Errors of up to 100% are normal; however, titers that approximate the real titer to within a factor of two are satisfactory for most purposes.

A critical benefit of the plaque assay is that it measures infectivity, although it is important to understand that infectivity does not necessarily correspond exactly to the number of virus particles in a preparation. In fact, for most animal viruses, only a fraction of the particles—as few as 1 in 10 to 1 in 10,000—may be infections as judged by comparison of a direct particle count, described later, with a plaque assay. This low *efficiency of plating*, or high particle to infectivity ratio, may have several causes. First, to determine a particle to infectivity ratio, virus must be purified to determine the concentration of physical particles and then subjected to plaque assay. If the purification itself damages particles, the particle to infectivity ratio will be increased. Second, some viruses produce empty particles, or particles that are for other reasons defective during infection, resulting in a high particle to infectivity ratio. Lastly, it is possible that not all infectious particles will form plaques in a given plaque assay. For example, infectious virus may require that cells exist in a specific metabolic state or in a specific stage of the cell cycle; thus, if not all cells in a culture are identical in this regard, only a fraction of the potentially

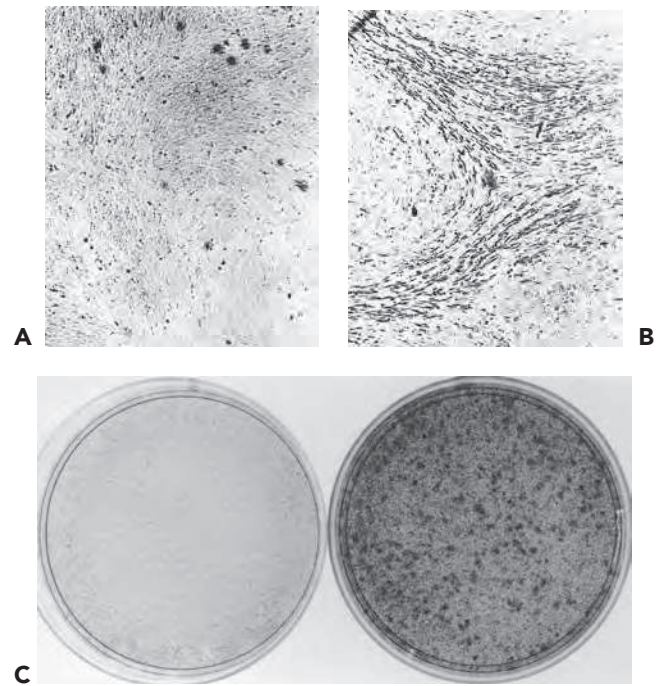


FIGURE 2.7. Focus assay. Monolayers of the NIH3T3 mouse fibroblast cell line were infected with Maloney murine sarcoma virus. **A, B:** Photomicrographs of uninfected cells (**A**) and a single virus-induced focus (**B**). **C:** Stained dishes of uninfected (**left**) and infected (**right**) cells. Foci are clearly visible as darker areas on the infected dish. (Courtesy of Dr. D. Blair.)

infectious virions may be able to successfully launch an infection and form a plaque.

In addition to its utility as a quantitative assay, the plaque assay also provides a way to detect genetic variants of a virus that possess altered growth properties, and it provides a very convenient method to clone genetically unique variants of a virus (see Fig. 2.6). Genetic variants are considered in detail in the Virus Genetics section; in brief, they may comprise viruses that plaque only under certain conditions of temperature or drug treatment, or form plaques of altered size or shape. Because each plaque results from infection with a single infectious virus particle, unique genetic variants of a virus can be cloned simply by picking plaques—that is, literally excising a small plug of semisolid medium and infected cells from a plaque using a Pasteur pipette.

THE FOCUS ASSAY

Some tumor viruses, most notably retroviruses, normally transform cells rather than killing them but can nevertheless be quantified by taking advantage of the transformation cytopathology.^{116,129} For example, retrovirus transformed cells may lose contact inhibition and therefore grow as foci, literally piles of transformed cells, on top of a contact-inhibited cell monolayer. Dense foci of transformed cells stain more darkly than cells in a monolayer and thus can be quantified on treatment of an infected monolayer with an appropriate stain. Otherwise, the focus assay is similar to the plaque assay in both technique and function. Photomicrographs of foci and stained monolayers containing foci are shown in Figure 2.7.

POCK FORMATION

As mentioned previously in the discussion of embryonated eggs, many viruses will cause focal lesions on the chorioallantoic membrane of eggs. While cumbersome, this assay can be used to quantify virus in a fashion similar to a plaque assay. The pock assay found utility before the adaptation of the plaque assay to animal virology, although now it has largely been replaced with other assays utilizing cultured cells and is used only for specialized purposes as noted in Figure 2.2.

THE ENDPOINT METHOD

Viruses that cannot be adapted to either a plaque or a focus assay but nevertheless cause some detectable pathology in cultured cells, embryonated eggs, or animals can be quantified using an endpoint method. Briefly, virus is serially diluted, and multiple replicate samples of each dilution are inoculated into an appropriate assay system. After a suitable incubation period, an absolute judgment is made as to whether or not an infection has taken place. The dilution series is constructed such that low dilutions show infection in all replicate inoculations, and high dilutions show infection in none of the inoculations, although some dilutions result in infection in some but not all inoculations. Statistical methods, described in more detail later, have been devised to calculate the dilution of virus that results in infection in 50% of replicate inoculations, and titers are expressed as the infectious dose 50 (ID_{50}). Assay systems are various and include, for example, observation of cytopathic effect in cultured cells, yielding tissue culture infective dose 50 ($TCID_{50}$); cytopathology or embryonic death in inoculated embryonated chicken eggs, yielding egg infective dose 50 (EID_{50}); or death of an experimental laboratory animal, yielding lethal dose 50 (LD_{50}). As with the plaque assay, the focus assay, and the pock assay, the endpoint method has the advantage of measuring infectivity; however, importantly, the unit of infectivity measured by the endpoint method may require more than one infectious particle. A sample determination of a $TCID_{50}$ is provided in the eBook.



Physical Assays

DIRECT PARTICLE COUNT

The concentration of virus particles in a sample of purified virus can be counted directly using an electron microscope.^{78,131} Briefly, a purified preparation of virus is mixed with a known concentration of microscopic marker particles such as latex beads, which can be easily distinguished from virus particles in the electron microscope. Samples of the solution containing virus and beads are then applied to an electron microscope grid and visualized following shadowing or staining. The volume of liquid applied to a given area of the grid can be determined by counting the beads. The virus particles in the same area can then be counted, resulting in an accurate determination of the concentration of virus particles in the original solution. An example of an electron microscopic count of vaccinia virus is shown in Figure 2.8. Given a solution of virus with a known concentration determined by microscopic particle count, the same solution can be subjected to any number of chemical or spectrophotometric analyses to yield a conversion from protein, nucleic acid, or simply absorbance at a fixed wavelength to a concentration of virus in particles per unit volume.

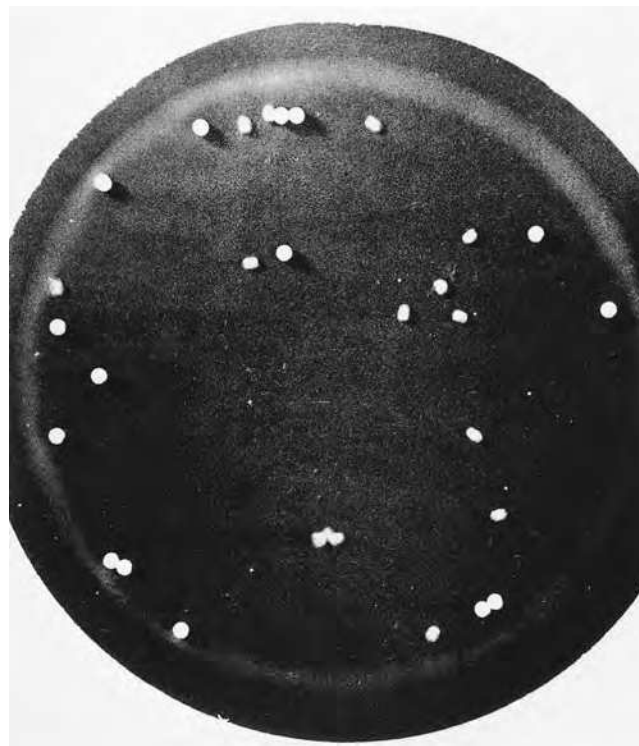


FIGURE 2.8. Direct electron microscopic particle count. An electron micrograph of a spray droplet containing 15 latex beads (spheres) and 14 vaccinia virus particles (slightly smaller brick-shaped particles). (Reprinted from Dumbell KR, Downie AW, Valentine RC. The ratio of the number of virus particles to infective titer of cowpox and vaccinia virus suspensions. *Virology* 4(3):467–482, © 1957 with permission from Elsevier.)

Thus, once a microscopic particle count has been performed, future quantitative assays of purified virus are greatly simplified. Importantly, the direct particle count does not distinguish infectious from noninfectious particles.

HEMAGGLUTINATION

As noted previously in the discussion of hemadsorption, many viruses express cell attachment proteins, which carry out their function wholly or in part by binding substituents such as sialic acid that are abundant on a wide variety of cell types, including erythrocytes. Because these cell attachment proteins decorate the surface of the virion, virions may bind directly to erythrocytes. Because both the virions and the erythrocytes contain multiple binding sites for each other, erythrocytes will agglutinate, or form a network of cells and virus, when mixed with virus particles in sufficiently high concentration. Agglutinated erythrocytes can be easily distinguished from cells that are not agglutinated, and thus hemagglutination can be used as a simple quantitative assay for the presence of a hemagglutinating virus.

In practice, a hemagglutination assay is carried out as follows (Fig. 2.9). Virus is serially diluted, mixed with a fixed concentration of erythrocytes, and the mixture is allowed to settle in a specially designed hemagglutination tray, containing wells

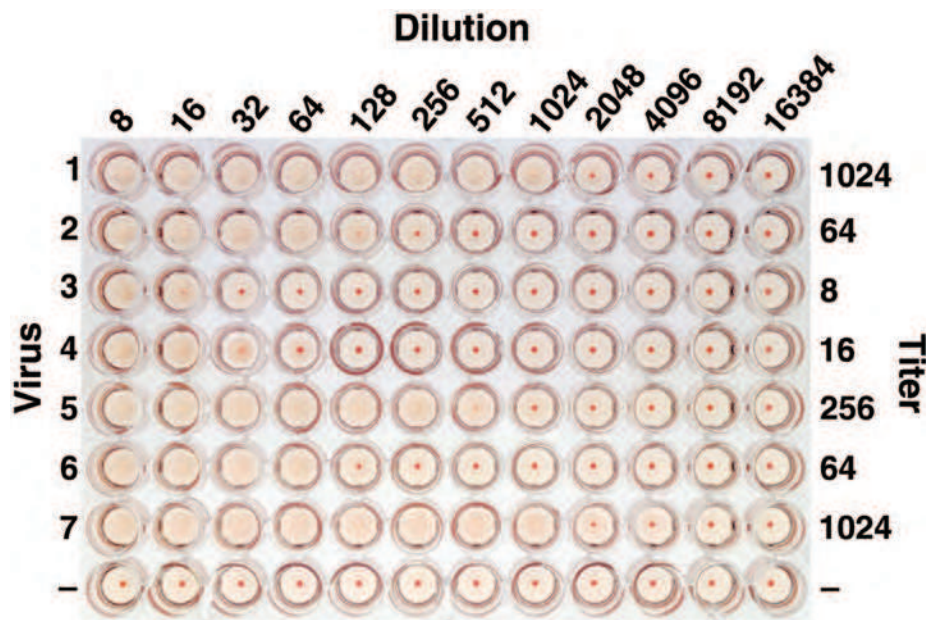


FIGURE 2.9. Hemagglutination assay. Seven different samples of influenza virus, numbered 1 through 7 at the **left**, were serially diluted as indicated at the **top**, mixed with chicken RBCs, and incubated on ice for 1 to 2 hours. Wells in the **bottom row** contain no virus. Agglutinated RBCs coat wells evenly, in contrast to nonagglutinated cells, which form a distinct button at the bottom of the well. The hemagglutination titer, shown at the **right** is the last dilution that shows complete hemagglutination activity. RBCs, red blood cells. (Courtesy of Drs. J. Talon and P. Palese.)

with rounded bottoms. Erythrocytes that are not agglutinated are free to roll to the bottom of the well, forming a dense, easily recognizable button, or cluster of cells. Erythrocytes that are agglutinated are not free to roll to the bottom of the well but instead evenly coat the bottom surface of the well to form what is called a *shield*. One hemagglutination unit is defined as the minimum amount of virus required to cause agglutination, and the titer of the virus solution, expressed as hemagglutination units per milliliter (HA units/mL), can be calculated taking into account the serial dilution. It is noteworthy that, such as with the direct particle count assay, the hemagglutination assay does not distinguish infectious from noninfectious particles. In addition, because it may require many particles to cause a detectable hemagglutination, one HA unit may represent many physical particles.

Quantitative Considerations in Virus Assay, Cultivation, and Experimentation

Dose Response in Plaque and Focus Assays

With few exceptions, the number of infectious units observed on a given plate in a plaque assay is a linear function of the dilution of the virus; thus, the development of plaques follows single-hit kinetics, proving that each plaque results from infection with a single virus particle. Exceptions include the murine sarcoma viruses, assayed in a focus assay, which require co-infection with both a defective transforming virus and a nondefective helper virus, in which case the number of foci observed relative to the dilution used follows two-hit kinetics.⁴⁶

Comparison of Quantitative Assays

As noted in the previous individual descriptions, the various quantitative assays of viruses measure different physical and biological properties, and a one-to-one correlation between assays cannot be assumed. Table 2.3 summarizes the titers of an influenza virus preparation as measured by several different

assays and thus provides an example the magnitude of differences that might be expected in the various assays. Hence, relative to a direct particle count, the efficiency of virus detection in the influenza sample shown in Table 2.3 is 10^{-1} as assayed in eggs, 10^{-2} as assayed in a plaque assay, and 10^{-7} as assayed in a hemagglutination assay. As indicated in the foregoing discussion, some differences result from different properties being measured (e.g., physical particles versus infectivity), and some differences result from differences in the sensitivity of the assay (e.g., direct particle count versus assay of particles by hemagglutination).

Multiplicity of Infection

Multiplicity of infection, often abbreviated “moi,” measures the average amount of virus added per cell in an infection. Multiplicity of infection can be expressed using any quantitative measure of virus titer—for example, particles/cell, HA units/cell, TCID₅₀/cell, or pfu/cell. Because the efficiency of plating varies depending on the method of quantitation used, some knowledge of the infectivity of the sample or the efficiency

TABLE 2.3 Comparison of Quantitative Assay Efficiency

Method	Amount (per mL)
Direct electron microscope count	10^{10} EM particles
Quantal infectivity assay in eggs	10^9 egg ID ₅₀
Quantal infectivity assay by plaque formation	10^8 pfu
Hemagglutination assay	10^3 HA units

EM, electron microscopy; ID₅₀, infective dose 50; pfu, plaque-forming unit; HA, hemagglutination assay.

Reprinted from Fenner F, McAuslan BR, Mims CA, et al. *The Biology of Animal Viruses*. New York: Academic Press, © 1974, with permission from Elsevier.

of plating is required to correctly anticipate the consequences of the use of a particular moi. The multiplicity of infection used in different protocols can have a profound outcome on the procedure. For example, some viruses, if serially passaged at a moi of greater than 1 infectious unit/cell, will accumulate spontaneously deleted defective particles that are maintained during passage by the presence of complementing wild-type helper virus.¹³⁰ Passage of the same virus at very low moi (e.g., 0.01 infectious units/cell) discourages the accumulation of defective particles because few cells will be co-infected with an infectious and a defective particle, and defective particles cannot replicate in the absence of a wild-type helper. Conversely, most metabolic labeling experiments are done at a high moi (e.g., 10 infectious units/cell) to ensure that all cells in the culture are infected and that the infection is as synchronous as possible. For such experiments, use of too low a moi may result in an apparently asynchronous infection and a high background owing to the presence of uninfected cells in the culture.

The Poisson distribution can be used to predict the fraction of cells in a population infected with a given number of particles at different multiplicities of infection. As applied to virus infections, the Poisson distribution can be written as:

$$P(k) = e^{-m} m^k / k!,$$

where $P(k)$ equals the probability that any cell is infected with k particles, m equals moi, and k equals the number of particles in a given cell.

To determine the fraction of uninfected cells in any experiment—that is, when $k = 0$ —the equation simplifies to:

$$P(0) = e^{-m}$$

For practical purposes, solution of this equation for given values of m and k (other than 0) is most easily accomplished using published tables.¹⁴² Sample solutions are shown in Table 2.4 for commonly used multiplicities of infection. Inspection of this table and consideration of the error inherent in any virus titration involving a serial dilution leads to some significant practical guides in experimental design. Note first that in a culture infected at a moi of 1 pfu/cell, 37% of cells remain

uninfected—an unacceptably high number for an experiment designed to measure a single round of synchronous infection. A moi of at least 3 is required to infect 95% of the cells in culture. Given that titers can easily be inaccurate by a factor of two, the use of a calculated moi of 10 ensures that 99% of the cells in a culture will be synchronously infected even if the measured titer is twofold higher than the actual titer.

One-Step Growth Experiment

A classic experiment developed initially for bacteriophage²⁹ and still frequently used to determine the essential growth properties of a virus is the one-step growth experiment. The goal of this experiment is to measure the time course of virus replication and the yield of virus per cell during a single round of infection. The experiment is carried out as follows. Several dishes containing confluent monolayers of an appropriate cultured cell are infected simultaneously with virus at a high moi (e.g., 10 pfu/cell). After an adsorption period, monolayers are washed to remove unabsorbed virus and then incubated in culture medium. At various times after infection, virus from individual dishes is harvested, and at the completion of the experiment, the virus titer in samples representing each time point is determined. The virus yield at each point can be converted to pfu/cell (also called *burst size*) by dividing the total amount of virus present in the sample by the number of cells originally infected in the sample.

The results from one example of a one-step growth experiment, in this case comparing growth of wild-type vaccinia virus and a temperature-sensitive mutant at permissive and nonpermissive temperatures, are shown in e-Figure 2.1. Several features of the growth curve are noteworthy. First, during the first several hours of the wild-type infection or the $\Delta 56$ infection at the permissive temperature, the titer in the cultures decreases and then increases. This dip in the growth curve is called *eclipse* and results from the fact that early during the experiment, virus attached to the cell surface but not uncoated remains infectious; however, infectivity is lost following uncoating during the first few hours of infection, and infectivity is recovered only after new virus is produced. The infection then enters a rapid growth phase, followed by a plateau. The plateau results from the fact that all infected cells have reached the maximum yield of virus, or have died or lysed, depending on the type of virus infection. The time interval from infection to plateau represents the time required for a single cycle of growth, and the yield of virus at plateau shows the amount of virus produced per cell. The experiment in e-Figure 2.1 demonstrates the utility of the one-step growth experiment. As judged by this experiment, wild-type virus grows with identical kinetics and to the identical yields at both 31°C and 40°C, which are permissive and nonpermissive temperatures for the temperature-sensitive mutant, respectively. The temperature-sensitive mutant, $\Delta 56$,⁸⁷ grows more slowly than wild-type virus at 31°C, indicating some defective character even at the permissive temperature, although at plateau the yields of mutant virus at 31°C are equivalent to wild-type virus. The experiment demonstrates conclusively that the mutant does not grow at all at the nonpermissive temperature of 40°C.

Multiplicity of infection is a critical factor in the design of a virus growth experiment. A true one-step growth experiment can only be done at high moi. If the moi is too low and a large fraction of cells are left uninfected, then virus produced during

TABLE 2.4 The Poisson Distribution: Values of $P(k)$ for Various Values of m and k

# / cell (k)	moi (m)			
	1	3	5	10
0	0.37	0.05	0.01	0.00
1	0.37	0.15	0.03	0.00
2	0.18	0.22	0.08	0.00
3	0.06	0.22	0.14	0.01
4	0.02	0.17	0.18	0.02
5	0.00	0.10	0.18	0.04
6	0.00	0.05	0.15	0.06
7	0.00	0.02	0.10	0.09
8	0.00	0.00	0.07	0.11
9	0.00	0.00	0.04	0.13
10	0.00	0.00	0.02	0.13

moi, multiplicity of infection.

the first round of infection will replicate on previously uninfected cells, and thus multiple rounds of infection rather than one round will be measured. A growth experiment done at low *moi* has utility in that it measures both growth and spread of a virus in culture; however, the time from infection to plateau does not accurately reflect the time required for a single cycle of infection. It is also noteworthy that some mutant phenotypes are multiplicity dependent.⁶

VIRUS GENETICS

Viruses are subject to the same genetic principles at work in other living systems, namely mutation, selection, complementation, and recombination. Genetics impacts all aspects of virology, including the natural evolution of viruses, clinical management of virus infections, and experimental virology. For example, antigenic variation, which is a direct result of mutation and selection, plays a prominent role in the epidemiology of influenza virus and human immunodeficiency virus (HIV) in the human population, and mutation to drug resistance offers a significant challenge to the clinical management of virus infections with antiviral drugs. This section deals primarily with the application of experimental genetic techniques to basic virology.

The ultimate goal of experimental virology is to understand completely the functional organization of a virus genome. In a modern context, this means determination of the structure of a virus genome at the nucleotide sequence level, coupled with isolation of mutational variants of the virus altered in each gene or control sequence, followed by analysis of the effects of each mutation on the replication and/or pathogenesis of the virus. Thus, genetic analysis of viruses is of fundamental importance to experimental virology.

Before the advent of modern nucleic acid technology—that is, during a *classical* period of *forward* genetics—genetic analysis of viruses consisted of the random, brute force isolation of large numbers of individual virus mutants, followed first by complementation analysis to determine groupings of individual mutants into genes, then recombination analysis to determine the physical order of genes on the virus genome, and finally the phenotypic analysis of mutants to determine gene function. This approach, pioneered in the 1940s through the 1960s in elegant studies of several bacteriophage, notably lambda, T4, and T7 (Chapter 75), was the primary method for identifying, mapping, and characterizing virus genes. The application of cell culture techniques to animal virology opened the door to classical genetic analysis of animal viruses, resulting in a flurry of activity in the 1950s through the 1970s, during which time hundreds of mutants were isolated and analyzed in prototypical members of most of the major animal virus families.³⁸ Modern nucleic acid technology introduced in the 1970s brought with it a variety of techniques for physical mapping of genomes and mutants, including restriction enzyme mapping, marker rescue, and DNA sequence analysis, which together replaced recombination analysis as an analytic tool. Mutants and techniques from the classical period continue to be of enormous utility today; however, recombinant DNA technology has brought with it *reverse* genetics, in which the structure of the genome is determined first using entirely physical methods, then the function of individual genetic ele-

ments is determined by analyzing mutants constructed in a highly targeted fashion.

The genetic approach to experimental virology, or any field of biology for that matter, has the profound advantage of asking of the organism under study only the most basic question—What genes do you need to survive, and why do you need them?—without imposing any further bias or assumptions on the system. Happily, organisms often respond with surprises that the most ingenious biochemist or molecular biologist would never have imagined. What follows is a summary of the critical elements of both the classical and modern approaches to virus genetics as applied to experimental virology.

Mutants

Wild-type Virus

It is important to understand that in the context of experimental virus genetics, a virus designated as *wild-type* can differ significantly from the virus that actually occurs in nature. For example, virus genetics often relies heavily on growth and assay of viruses in cell culture, and as noted previously, natural isolates of viruses may undergo significant genetic change during adaptation to cell culture. In addition, viruses to be designated as wild-type should be plaque purified before initiating a genetic study to ensure a unique genetic background for mutational analysis. Lastly, viruses may be specifically adapted for use in genetic analysis—for example, by passage under conditions that are to be restrictive for conditionally lethal mutants so that the analysis can be initiated with a preparation free from spontaneous mutants.

Fundamental Genetic Concepts

Concepts fundamental to genetic analysis of other organisms apply to genetic analysis of viruses, and a clear understanding of these concepts is essential to understanding virus genetics. The most important of these concepts, including distinctions between genotype and phenotype, a selection and a screen, and essential versus nonessential genes, are briefly summarized next.

GENOTYPE AND PHENOTYPE

Genotype refers to the actual genetic change from wild-type in a particular virus mutant, whereas *phenotype* refers to the measurable manifestation of that change in a given assay system. This distinction is emphasized by the fact that a single genotype may express different phenotypes depending on the assay applied. Thus, for example, the same missense mutation in a virus gene may cause temperature sensitivity in one cell line but not another, or a deletion in another virus gene may have no effect on the replication of virus in culture but may alter virulence in an animal model.

SELECTION AND SCREEN

Selection and screen refer to two fundamentally different methods of identifying individual virus variants contained in a mixed population of viruses. *Selection* implies that a condition exists where only the desired virus will grow, and growth of unwanted viruses is suppressed. Thus, a drug-resistant virus can be identified by plating a mixture of wild-type, drug-sensitive, and mutant, drug-resistant viruses together on the same cell monolayer in the presence of the inhibitory drug, thereby selecting for drug-resistant viruses that grow,

and selecting against wild-type viruses that do not grow (see Fig. 2.6). A *screen* implies that both the desired virus variant and one or several other unwanted virus types grow under a given condition, such that many viruses must be analyzed individually to identify the desired variant. For example, in searching for a temperature-sensitive mutant (i.e., a virus whose growth is inhibited relative to wild-type virus at an elevated temperature), no condition exists under which the mutant alone will grow. Therefore, virus must be plated at a low temperature where both wild-type and mutant virus will grow, and plaques tested individually for temperature sensitivity. Sometimes a screen can be streamlined by introducing a phenotypic marker into the variant of choice. For example, a knockout virus might be constructed by inserting the β -galactosidase gene into the virus gene to be inactivated. In the presence of an appropriate chromophoric substrate, viruses containing the insertional knockout produce blue-colored plaques and can therefore be distinguished from unmodified viruses, which form clear plaques, growing on the same plate.¹³⁹ This latter example is still a screen, because both wild-type and mutant viruses grow under the conditions used; however, the screen is simplified because mutant viruses can be readily identified by their color, obviating the need to pick and test individual plaques. Selections have considerable advantages over screens but are not always possible.

ESSENTIAL AND NONESSENTIAL

The terms *essential* and *nonessential* describe phenotypes, specifically whether a given gene is required for growth under a specific condition. Most viruses are finely tuned through selection to fit a specific niche. Not all viral genes are absolutely required for virus replication in that niche; some may simply confer a subtle selective advantage. Furthermore, if the niche is changed—such as from a natural animal host to a cell line in a laboratory—some genes that may have been essential for productive infection in the animal may not be required for replication in cell culture. Genes that are required for growth under a specific condition are termed *essential*, and those that are not required are termed *nonessential*. Because as a phenotype essentiality may be a function of the specific test conditions, the test conditions need to be specified in describing the mutation. As an example, the herpesvirus thymidine kinase gene is nonessential for virus replication in cell culture. Genes that are either essential or nonessential under a given condition present unique characteristics for analysis. Thus, mutants in nonessential genes may be easy to isolate because the gene can be deleted, although the function of the gene may be difficult to determine because, by definition, nonessential genes have no phenotype. Conversely, genes that are essential can be used to study gene function by characterizing the precise replication defect caused by a mutation in the gene; however, acquiring the appropriate mutant is confounded by the necessity for identifying a condition that will permit growth of the virus for study.

Mutation

SPONTANEOUS MUTATION

Spontaneous mutation rates in viruses are measured by fluctuation analysis,⁶⁰ a technique pioneered by Luria and Delbrück⁷⁷ for analysis of mutation in bacteria, and later adapted to viruses

by Luria.⁷⁵ Fluctuation analysis consists of measuring the proportion of spontaneous mutants with a particular phenotype in many replicate cultures of virus and applying the Poisson distribution to these data to calculate a mutation rate. Importantly, because spontaneous mutations occur at random and may occur only rarely, the raw data in a fluctuation analysis displays enormous scatter, with some cultures containing a high proportion of mutants and some containing no mutants. Thus, from a practical perspective, although the proportion of mutants in a single culture of virus may reflect the mutation rate, it does not necessarily provide an accurate measure of mutation rate.

Both DNA and RNA viruses undergo spontaneous mutation; however, the spontaneous mutation rate in RNA viruses is usually much higher than in DNA viruses. In general, the mutation rate at a specific site in different DNA viruses ranges from 10^{-8} to 10^{-11} per replication, whereas in RNA viruses it is at least hundred-fold higher, between 10^{-3} and 10^{-6} per replication. The difference in mutation rate observed between RNA and DNA viruses is thought to result primarily from differences in the replication enzymes. Specifically, the DNA-dependent DNA polymerases used by DNA viruses contain a proofreading function, whereas the reverse transcriptases used by retroviruses and RNA-dependent RNA polymerases used by RNA viruses lack a proofreading function. The difference in spontaneous mutation rate has profound consequences for both the biology of the viruses and for laboratory genetic analysis of viruses. Specifically, RNA viruses exist in nature as *quasispecies*²⁵—that is, populations of virus variants in relative equilibrium with the environment but capable of swift adaptation owing to a high spontaneous mutation rate (Chapter 11). Conversely, DNA viruses are genetically more stable but less adaptable. In the laboratory, the high mutation rate in RNA viruses presents difficulties in routine genetic analysis because mutants easily revert to wild-type virus that can outgrow the mutant virus.

It is noteworthy that whereas the actual mutation rate at a single locus is probably relatively constant for a given virus, the apparent mutation rate to a given phenotype depends on the nature of the mutation(s), which can give rise to that phenotype. For example, spontaneous mutation to bromodeoxyuridine (BrdU) resistance in vaccinia virus may occur at least 10 to 100 times more frequently than spontaneous reversions of temperature-sensitive mutations to a wild-type, temperature-insensitive phenotype. In the case of BrdU resistance, any mutation that inactivates the thymidine kinase causes resistance to BrdU, and thus there are literally hundreds of different ways in which spontaneous mutation can give rise to BrdU resistance. By contrast, a temperature-sensitive mutation is usually a single-base missense mutation, in which may exist only one possible mutational event that could cause reversion to the wild-type phenotype; thus, the apparent spontaneous mutation rate for the revertant phenotype is lower than the apparent spontaneous mutation rate to the BrdU-resistant phenotype. From a practical perspective, the apparent spontaneous mutation rate for specific selectable phenotypes may be sufficiently high such that induction of mutants is unnecessary for their isolation. Note, for example, that the wild-type vaccinia virus culture titered in Figure 2.6 contains numerous spontaneous isatin- β -thiosemicarbazone (IBT)-resistant viruses that could easily be plaque purified

from assays done in the presence of IBT. However, for most mutants (e.g., temperature-sensitive mutants), where the desired mutational events are rare and a screen must be used rather than a selection, induced mutation is required for efficient isolation of mutants.

INDUCED MUTATION

Under most circumstances, the incidence of spontaneous mutations is low enough so that induction of mutation is a practical prerequisite for isolation of virus mutants. It is usually desirable to induce limited, normally single-base changes, and for this purpose, chemical mutagens are most appropriate. Commonly used chemical mutagens are of two types: *in vitro* mutagens and *in vivo* mutagens.²⁶ *In vitro* mutagens work by chemically altering nucleic acid and can be applied by treating virions in the absence of replication. Examples of *in vitro* mutagens include hydroxylamine, nitrous acid, and alkylating agents, which through chemical modification of specific bases cause mispairing leading to missense mutations. *In vivo* chemical mutagens comprise compounds such as nucleoside analogs that must be incorporated during viral replication and thus must be applied to an infected cell. One of the most effective mutagens is the alkylating agent nitrosoguanidine, which although is capable of alkylating nucleic acid *in vitro* is most effective when used *in vivo*, where it works by alkylating guanine residues at the replication fork, ultimately causing mispairing.

The effectiveness of a mutagenesis is often assayed by observing the killing effect of the mutagen on the virus, the assumption being that many mutational events will be lethal and thus an effective mutagenesis will decrease a virus titer relative to an untreated control. However, killing does not always correlate precisely with mutagenesis, especially with an *in vitro* mutagen that can damage virion structure without necessarily causing mutation. An alternative method for assessing mutagenesis is to monitor an increase in the mutation frequency to a selectable phenotype where possible. For example, in vaccinia virus, mutagenesis causes a dose-dependent increase in resistance to phosphonoacetic acid, a drug that prevents poxvirus replication by inhibiting the viral DNA polymerase.¹² In summary, the use of mutagens can increase the mutation frequency several hundred-fold, such that desired mutants may comprise as much as 0.5% of the total virus population.

DOUBLE MUTANTS AND SIBLINGS

The existence of *double mutants* and *siblings* can theoretically complicate genetic analysis of a virus. A double (or multiple) mutant is defined as a virus that contains more than one mutation contributing to a phenotype. Theoretically, because the probability that a double mutant will be created increases as the dose of a mutagen is increased, there is a practical limit to the amount of induced mutation that is desirable. Double mutants are usually revealed as mutants that are noncomplementing with more than one mutant or are impossible to map by recombination or physical methods. Siblings result from replication of mutant virus either through amplification of a mutagenized stock or during an *in vivo* mutagenesis. The only completely reliable method to avoid isolation of sibling mutants is to isolate each mutant from an independently plaque-purified stock of wild-type virus.

Mutant Genotypes

There exist two basic categories of mutation: base substitution and deletion/insertion mutations. Both mutation types can occur with consequence in either a protein coding sequence or in a control sequence, such as a transcriptional promoter, a replication origin, or a packaging sequence. *Base substitution* mutations consist of the precise replacement of one nucleotide with a different nucleotide in a nucleic acid sequence. In coding sequences, base substitution mutations can be silent, causing no change in amino acid sequence of a protein; they can be missense, causing replacement of the wild-type amino acid with a different residue; or they can be nonsense, causing premature translation termination during protein synthesis. *Deletion and insertion* mutations comprise deletion or insertion of one or more nucleotides in a nucleic acid sequence. In a coding sequence, deletion or insertion of multiples of three nucleotides can result in precise deletion or insertion of one or more amino acids in a protein sequence. In a coding sequence, deletions or insertions that do not involve multiples of three nucleotides result in a shift in the translational reading frame, which almost invariably results in premature termination at some distance downstream of the mutation. In general, nonsense mutations, frameshift mutations, or large in-frame insertions or deletions are expected to inactivate a gene, whereas missense mutations may cause inactivation or much more subtle phenotypes such as drug resistance or temperature sensitivity.

Mutant Phenotypes

In the context of experimental virology where the goal is to understand the function of individual virus genes, the most useful mutants are those that inhibit virus replication by inactivating a virus gene. The nonproductive infections with these lethal mutants can be studied in detail to determine the precise aspect of virus replication that has been affected, thus providing information about the normal function of the affected gene. However, one must be able to grow the mutant to conduct experiments. Thus, a condition must be found where the mutation in question is not lethal—hence, the general class of mutant phenotypes, *conditional lethal*. Conditional lethal mutants comprise by far the largest and most useful class of mutant phenotypes, consisting of host-range, nonsense, temperature-sensitive, and drug-dependent phenotypes, described individually in the next section. Two additional classes of mutant phenotypes—resistance and plaque morphology—have very specific application to genetic analysis of viruses and are also described.

HOST RANGE

A host-range virus mutant is broadly defined as a mutant that grows on one cell type and not on another, in contrast to wild-type virus, which grows on both cell types. Two general subcategories of host-range mutants exist: natural and engineered. *Natural* host-range virus mutants are relatively rare, primarily because they must be identified by brute force screen or serendipity, in many cases in the absence of a viable rationale for the targeted host range. The existence of a host-range phenotype implies that a specific virus–host interaction is compromised, which also implies that for any specific host-range phenotype, only one or a limited number of virus genes will be targeted. A classic example of a natural host-range mutant would be the host range-transformation (hr-t) mutants of mouse polyoma

virus, which affect both small and middle T antigens and grow on primary mouse cells but not continuous mouse 3T3 cell lines.³ *Engineered* host-range mutants are constructed by deleting an essential gene of interest in the virus while at the same time creating a cell line that expresses the gene. The engineered cell line provides a permissive host for growth of the mutant virus because it complements the missing virus function, whereas the normal host lacking the gene of interest provides a nonpermissive host for study of the phenotype of the virus. This technology has been useful for study of a variety of viruses, notably adenovirus and herpes simplex virus, where it has facilitated study of several essential virus genes.^{21,61}

NONSENSE MUTANTS

Nonsense mutants contain a premature translation termination mutation in the coding region of the mutant gene. They are formally a specific class of conditionally lethal, host-range mutants. Specifically, the permissive host is one that expresses a transfer RNA (tRNA) containing an anticodon mutation that results in insertion of an amino acid in response to a nonsense codon, thus restoring synthesis of a full-length polypeptide and suppressing the effects of the virus nonsense mutation. The nonpermissive host is a normal cell in which a truncated, nonfunctional polypeptide is made. In practice, most nonsense mutants in existence have been isolated by random mutagenesis followed by a brute force screen for host range. Nonsense mutants have three distinct advantages for the conduct of virus genetics: (a) mutants can be isolated in virtually any essential virus gene using one set of permissive and nonpermissive hosts and one set of techniques; (b) the mutations result in synthesis of a truncated polypeptide, thereby facilitating identification of the affected gene; and (c) virus mutants can be engineered relatively easily because the exact sequence of the desired mutation is predictable. Nonsense mutants have provided the single most powerful genetic tool in the study of bacteriophage, where efficient, viable nonsense suppressing bacteria are readily available. Unfortunately, attempts to isolate nonsense-suppressing mammalian cells have met with only limited success, probably because the nonsense-suppressing tRNAs are lethal in the eukaryotic host.¹¹⁰

TEMPERATURE SENSITIVITY

Temperature sensitivity is a type of conditional lethality in which mutants can grow at a low temperature but not a high temperature, in contrast to wild-type virus, which grows at both temperatures (see Fig. 2.6). Genotypically, temperature-sensitive mutations result usually from relatively subtle single amino acid substitutions that render the target protein unstable and hence nonfunctional at an elevated or nonpermissive temperature while leaving the protein stable and functional at a low, permissive temperature. In practice, temperature-sensitive mutants are usually isolated by random mutagenesis followed by brute force screening for growth at two temperatures. Screening can be streamlined by a plaque enlargement technique in which mutagenized virus is first plated at a permissive temperature, then stained and shifted to a nonpermissive temperature after marking the size of plaques, to screen for plaques that do not increase in size at the nonpermissive temperature.¹¹² Replica plating techniques that permit relatively straightforward screening of thousands of mutant candidates in yeast and bacteria have not been successfully adapted to virology; thus, a screen

for temperature sensitivity, even when streamlined with plaque enlargement, ultimately depends on the laborious but reliable process of picking and testing individual plaques. Temperature-sensitive mutants have the profound advantage of theoretically accessing any essential virus gene using a single set of protocols. Temperature-sensitive mutants have proved enormously useful in all branches of virology but have been particularly useful for the study of animal viruses, where nonsense suppression has not been a viable option. Cold-sensitive mutants (i.e., mutants that grow at a high but not a low temperature) comprise a relatively rare but nevertheless useful alternate type of temperature-sensitive mutants.

Temperature-sensitive mutants can actually be divided into two subclasses: thermolabile and temperature sensitive for synthesis (tss) mutants.¹⁴⁰ *Thermolabile mutants* are those in which the gene product can be inactivated following synthesis by a shift from the permissive to the nonpermissive temperature. *Ts* mutants display gene dysfunction only if the infection is held at the nonpermissive temperature during synthesis of the mutant gene product; if the gene product is made at the permissive temperature, it cannot be inactivated by raising the temperature. Clearly, the two mutant types can be distinguished by performing appropriate temperature shift experiments. Thermolability obviously implies that a protein preformed at the permissive temperature is directly destabilized by raising the temperature. Tss mutations commonly involve multisubunit structures or complex organelles, where theoretically the quaternary structure of a complex formed correctly at the permissive temperature stabilizes the mutant protein, making the mutation resistant to temperature shift. If a tss mutant protein is synthesized at the nonpermissive temperature, it may be degraded before assembly or may not assemble properly because of misfolding. For most purposes, the thermolabile and tss mutant types are equally useful.

DRUG RESISTANCE AND DEPENDENCE

Several antiviral compounds have now been identified, and virus mutants that are resistant to or depend on these compounds have found utility in genetic analysis of viruses. A few compounds have been identified that target similar enzymes in different viruses, including phosphonoacetic acid, which inhibits DNA polymerases^{50,114} and BrdU, which targets thymidine kinases.^{27,119} More often, however, antiviral drugs are highly specific for a gene product of one particular virus—for example, guanidine, which targets the polio 2C NTPase^{98,99}; acyclovir, which targets the herpes simplex virus thymidine kinase and DNA polymerase^{9,109}; amantadine, which targets the influenza virus M2 virion integral membrane ion channel protein⁴⁹; or isatin- β -thiosemicarbazone, which is highly specific for poxviruses and targets at least two genes involved in viral transcription.^{11,17,87} The most useful drugs are those that inhibit wild-type virus growth in a plaque assay without killing cells in a monolayer, such that resistant or dependent viruses can be selected by virtue of their ability to form plaques on a drug-treated monolayer. Examples of both drug resistance and drug dependence are shown in Figure 2.6.

Drug-resistant or drug-dependent virus mutants have two general uses in virus genetics. First, they can be useful in identifying the target or mechanism of action of an antiviral drug. For example, studies of influenza virus mutants resistant to amantadine were of importance in characterizing both the M2

gene and the mechanism of action of amantadine.¹⁰⁰ Second, resistant or dependent mutants provide selectable markers for use in recombination mapping, for the assessment of specific genetic protocols, or for selection of recombinant viruses in reverse genetic protocols. For example, guanidine resistance has been used as a marker for use in three-factor crosses in recombination mapping of poliovirus temperature-sensitive mutants¹⁶; phosphonoacetic acid resistance and isatin- β -thiosemicarbazone dependence has been used in vaccinia virus to assess the efficiency of marker rescue protocols^{31,47}; and acyclovir resistance and BrdU resistance, resulting from mutation of the herpesvirus or poxvirus thymidine kinase genes, has been used in both herpesviruses and in poxviruses to select for insertion of engineered genes into the viral genome.^{10,80,94}

PLAQUE MORPHOLOGY

Plaque morphology mutants are those in which the appearance of mutant plaques is readily distinguishable from wild-type plaques. Most commonly, the morphological distinction is plaque size (i.e., mutant plaques may be larger or smaller than wild-type plaques); however, other morphological distinctions are possible, such as formation of clear versus turbid bacteriophage plaques. Most plaque morphology mutants affect very specific virus functions, which in turn affect the virus–host relationship in a fashion that impacts on the appearance of a plaque. Notable examples from bacteriophage research include clear plaque mutants of bacteriophage lambda and rapid lysis mutants of the T-even bacteriophage. Wild-type lambda forms turbid plaques because some percentage of cells are lysogenized and thus survive the infection, leaving intact bacteria within a plaque. Clear mutants of lambda typically affect the lambda repressor such that lysogeny is prevented and all infected bacteria lyse, resulting in a clear plaque.⁶³ Wild-type T-even phages produce small plaques with a turbid halo because only a fraction of infected bacteria lyse during a normal infection, a phenomenon called *lysis inhibition*. Rapid lysis mutants, which affect a phage membrane protein, do not display lysis inhibition and as a result form large, clear plaques.⁵³ Examples from animal virus research include large plaque mutants of adenovirus and syncytial mutants of herpes simplex virus. The large plaque phenotype in adenovirus results from faster than normal release of virus from infected cells.⁶⁸ Syncytial mutants of herpesvirus express altered virus surface glycoproteins and result in fusion of infected cells, whereas wild-type virus causes cells to round and clump without significant fusion. Thus, syncytial mutants form large plaques readily distinguishable from the smaller dense foci caused by wild-type virus.¹⁰⁷ All of these specific plaque morphology mutants have value either in the study of the actual functions affected or as specific phenotypic markers for use in recombination studies, where they can be used in the same fashion as drug resistance markers, described previously.

In addition to the existence of specific plaque morphology loci in several viruses, it is noteworthy that any mutation that affects virus yield or growth rate may result in production of a smaller than wild-type plaque, which can be useful in genetic experiments. Thus, many temperature-sensitive mutants form smaller than wild-type plaques even at the permissive temperature because the mutant gene may not be fully functional even under permissive conditions, and this property is often useful in mutant isolation or for distinguishing wild-type from mutant virus in plaque assays involving several virus variants. Note,

for example, in Figure 2.6 that the vaccinia virus temperature-sensitive mutant *ts56* forms smaller than wild-type plaques at the permissive temperature of 31°C. Lastly, intragenic or extragenic suppressors of conditional lethal virus mutants may grow poorly relative to wild-type virus and form small plaques as a result, facilitating their isolation from a mixture containing true wild-type revertant viruses.¹⁴

NEUTRALIZATION ESCAPE

Neutralization escape mutants are a specific class of mutants selected as variant viruses that form plaques in the presence of neutralizing antibodies. Such mutants affect the structure or modification of viral surface proteins and have been of value in studies of virus structure, antigenic variation, and virus–cell interactions.^{43,55}

Reversion

Reversion may be defined as mutation that results in a change from a mutant genotype to the original wild-type genotype. Accordingly, revertants in a stock of mutant virus are revealed as viruses that have acquired a wild-type phenotype. For example, Figure 2.6 shows that when the vaccinia virus temperature-sensitive mutant *ts56* is plated at the nonpermissive temperature, plaques with wild-type morphology, probably revertants, are detectable at low dilutions of virus. Spontaneous reversion of missense mutations probably results from misincorporation during replication, because the reversion frequency of different viruses often reflects the error rate of the replication enzyme. Spontaneous reversion of significant deletion mutations occurs rarely, if at all, because reversion would require replacement of missing nucleotides with the correct sequence. Reversion impacts on viral genetics in two ways. First, in any genetic experiment involving mixed infections with two genetically different viruses, wild-type viruses can arise either through reversion or recombination; in most cases, it is important to be able to distinguish between these two processes. This is discussed in more detail in the later sections describing complementation and recombination. Second, as described earlier in the description of spontaneous mutation, if the spontaneous reversion rate is extremely high, revertants can easily come to dominate a mutant virus stock, thus obscuring the mutant phenotype and causing serious difficulties in both genetic and biochemical analysis of mutants.

Leakiness

Not all conditionally lethal mutants are completely defective in replication under nonpermissive conditions, and leakiness is a quantitative measure of the ability of a mutant virus to grow under nonpermissive conditions. Leakiness can be quantified with a one-step growth experiment. To quantify leakiness of a temperature-sensitive mutant, for example, cells are infected at a high moi with wild-type or mutant virus, infected cells are incubated at either permissive or nonpermissive temperatures, and maximum virus yields are then determined by plaque titration under permissive conditions so that the growth of mutant and wild-type virus can be quantitatively compared. Ideally, for wild-type virus, the ratio of the yield for infections done at the nonpermissive temperature relative to the permissive temperature should be one—that is, the virus should grow equally well at both temperatures. For mutant viruses, the ratio of the

yield for infections done at the nonpermissive temperature relative to the permissive temperature may range from less than 10% to as much as 100%, even for mutants that are clearly defective in plaque formation under nonpermissive conditions. Mutants that are *tight*—or grow poorly under nonpermissive conditions—are desirable for phenotypic characterization relative to leaky mutants, because leaky mutants will logically display considerable wild-type phenotypic behavior. Special cases exist where extreme leakiness is an expected and desirable trait. Specifically, virus mutants that are wild-type for replication and production of infectious virions but defective in cell-to-cell spread have a phenotype characterized by defective plaque formation, which requires spread, but 100% leakiness, which does not require spread if assayed in a high moi one-step growth protocol.⁵

Genetic Analysis of Mutants

Complementation

Complementation analysis provides a general method for determining whether two different virus mutants affect the same or different genes. The quantitative test to determine complementation is a two-step procedure in which co-infections are first done to induce an interaction between two mutants, and the results of those infections are quantitatively assessed by plaque titration. The test compares the ability of two mutants to grow in mixed relative to single infections done under nonpermissive conditions. Specifically, cells are first infected with two different virus mutants at high moi so that all cells are co-infected with both mutants, and infected cells are incubated under nonpermissive conditions where neither mutant alone can replicate, for an interval sufficient to achieve maximum virus yield. Single high moi infections under nonpermissive conditions are performed as controls. Virus is then harvested, yields are quantified by plaque titration under both permissive and nonpermissive conditions, and a complementation index (CI) is calculated according to the following formula:

$$\frac{\text{yield}(A+B)_p - \text{yield}(A+B)_{np}}{\text{yield}(A)_p + \text{yield}(B)_p} = \text{CI},$$

where A and B represent individual virus mutants, and the subscripts p and np represent the conditions, either permissive or nonpermissive, under which the virus yields were plaque titrated. Because both mutant and wild-type viruses will be counted in plaque titrations done at the permissive temperature, the first term in the numerator, $\text{yield}(A+B)_p$, measures the yield of all viruses, both mutant and wild-type, from the initial high moi mixed infections done under nonpermissive conditions. The second term in the numerator, $\text{yield}(A+B)_{np}$, measures the yield of wild-type viruses, mostly recombinants, from the high moi mixed infections done under nonpermissive conditions, because only wild-type viruses will be counted in plaque titrations done at the nonpermissive temperature. Subtraction of the wild-type viruses from the total viruses leaves a count of only the mutant viruses in the numerator. The denominator measures the ability of each of the mutants to grow in single high moi infections done initially under nonpermissive conditions. If the two mutants, A and B, are in different virus genes, then in the mixed infection done under nonpermissive

conditions, mutant A can contribute wild-type B gene product and mutant B can contribute wild-type A gene product. Thus, the mutants can help or complement each other, resulting in a high yield of mutant virus in the mixed infection compared to the single infections, and a CI significantly greater than one. If the two mutants, A and B, affect the same gene, then the wild-type gene product will be lacking in the mixed infection. In this case, the yield from the mixed infection will be equivalent to the yield from the single infections, and the CI should not exceed one. In practice, owing to error in plaque assays and from other sources, mixed infections with mutants in the same viral gene will often yield CIs of slightly greater than one, and the practical cutoff must be determined empirically for a given viral system. An example of complementation analysis is provided in the eBook.



Qualitative complementation tests have also been devised for use with both bacterial and mammalian viruses.^{8,12,71,118}

These qualitative tests are much easier to perform than quantitative tests and in practice are just as reliable. In general, the tests are designed such that bacterial lawns or eukaryotic cell monolayers are infected either singly or with two viruses under nonpermissive conditions and at relatively low moi. The moi must be high enough so that numerous cells are doubly infected in the mixed infection, although low enough so that most cells are uninfected and a lawn or monolayer is maintained. Complementing mutant pairs produce plaques or cleared areas under nonpermissive conditions, whereas noncomplementing mutant pairs do not. An example of a qualitative complementation test is shown in Figure 2.10. A theoretical disadvantage of the qualitative test is that it does not discriminate between complementation and recombination. In some cases, recombination between mutants in the same complementation group under nonpermissive conditions is sufficiently rare, thus the qualitative test is reliable.¹³ If recombination does occur under nonpermissive conditions, false positives occur in the qualitative test and the number of complementation groups is overestimated.⁶⁵ Nevertheless, negative tests are still a reliable measure of noncomplementation.

Complementation analysis has been of tremendous benefit in sorting mutants in most, but not all, viral systems. A notable exception is poliovirus, where complementation between temperature-sensitive mutants *in vivo* is not observed. The lack of complementation in picorna viruses may be related to the unique mechanism of viral gene expression, in which all protein products are produced from a polyprotein precursor by proteolytic cleavage. If individual temperature-sensitive mutants affect structure, synthesis, or cleavage of the polyprotein precursor, they may behave as if they all belong to a single complementation group, even though they may map to different protein end products.

As a concept, complementation impacts broadly on virology and is not limited simply to the grouping of conditionally lethal mutants into genes. For example, the growth of engineered host-range deletion mutants in essential virus genes, discussed previously, relies on complementation of the missing viral function by an engineered cell line that expresses the wild-type viral gene product. In addition, the accumulation of defective virus genomes at high multiplicity passage, also discussed earlier, results from a complementing helper function provided by wild-type virus.

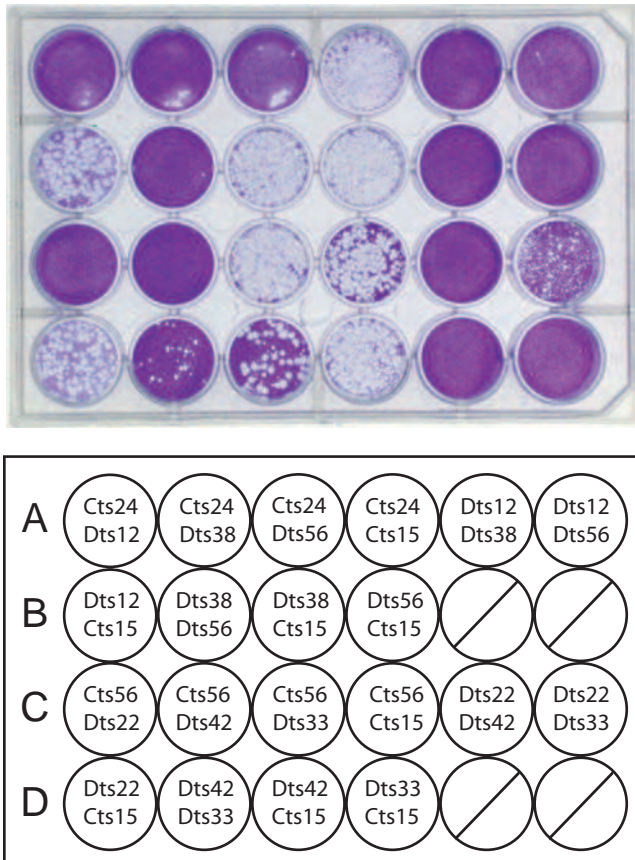


FIGURE 2.10. Qualitative complementation test. This test was done to confirm the composition of two different complementation groups in vaccinia virus, one in gene *D5* (rows *A* and *B*) and one in gene *G2* (rows *C* and *D*). Candidate mutants in gene *D5* are *Cts24*, *Dts12*, *Dts38*, and *Dts56*. Candidates in gene *G2* are *Cts56*, *Dts22*, and *Dts42*. *Cts15* and *Dts33* were known to map to different genes and were used as positive controls for complementation. Monolayers of the African green monkey kidney cell line BSC40 grown in a 24-well dish were infected at very low moi (~ 0.03 pfu/cell) with individual mutants or mutant pairs. The dish was incubated at a nonpermissive temperature (40°C) for 3 days and stained with crystal violet. The stained dish is shown at the **top**, and a key to the infections is shown at the **bottom**. Mixed infections in rows *A* and *B* represent all possible pairwise combinations of gene *D5* temperature-sensitive mutants along with the *Cts15* positive control, and mixed infections in rows *C* and *D* represent all possible pairwise combinations of gene *G2* temperature-sensitive mutants along with *Cts15* and *Dts33* positive controls. The absence of plaques confirms that mutants reside in the same complementation group. Control single infections produced no plaques (not shown). (Reprinted from Lackner CA, D'Costa SM, Buck C, et al. Complementation analysis of the dales collection of vaccinia virus temperature-sensitive mutants. *Virology* 2003;305:240–259, © 2003, with permission from Elsevier.)

Recombination and Reassortment

Recombination describes a process by which nucleic acid sequences from two genotypically different parental viruses are exchanged so that the progeny contain sequences derived from both parents. In viral systems, there exist three distinct mechanisms of recombination, dictated by the structures of

the viral genomes. For DNA viruses, recombination occurs by the physical breakage and rejoining of parental DNA molecules through regions of sequence homology, in a fashion similar or identical to the same process in bacteria or higher organisms. For RNA viruses containing segmented genomes, gene exchange occurs primarily through reassortment of individual parental genome segments into progeny viruses, although intragenic recombination has been reported for orthomyxoviruses, reoviruses, and bunyaviruses.^{97,101,103,115,120} For most nonsegmented RNA viruses, recombination appears to be a much less frequent event compared with DNA viruses. Recombination has been observed in several ssRNA virus families representing both positive and negative sense genomes; picornaviruses, coronaviruses, togaviruses, and retroviruses display relatively efficient recombination.^{2,45,56,66,67,73,74,136} Recombination in RNA viruses is thought to occur during replication via *copy choice*, namely switching templates during replication such that the newly synthesized genome contains sequence from two different parental molecules.¹⁶ Historically, recombination has been used to construct genetic maps of virus mutants and to construct novel virus genotypes. Although recombination mapping has been largely replaced by physical mapping techniques such as marker rescue, a technical knowledge of recombination mapping can contribute to an appreciation of the complexity of genetic interactions between viruses.

The methods used to determine recombination frequencies are the same regardless of genome structure or mechanism of recombination. As with complementation, the quantitative test to determine recombination frequency between two mutants, called a *two-factor cross*, is a two-step procedure, but in this case co-infections are first done under conditions permissive for replication, then the fraction of recombinants relative to the total virus yield is quantitatively assessed by plaque titration. Specifically, cells are first infected with two different virus mutants at high moi so that all cells are co-infected with both mutants, and infected cells are incubated under permissive conditions so that both mutants have maximum opportunity for interaction, for an interval sufficient to achieve maximum virus yield. Single high moi infections under permissive conditions are performed as controls. Virus is then harvested, yields are quantified by plaque titration under both permissive and nonpermissive conditions, and a recombination frequency (RF) is calculated according to the following formula:

$$\frac{\text{yield}(A+B)_{np} - \text{yield}(A)_{np} - \text{yield}(B)_{np}}{\text{yield}(A+B)_p} \times 2 \times 100\% = \text{RF},$$

where *A* and *B* represent individual virus mutants, and the subscripts *p* and *np* represent the conditions, permissive or nonpermissive, under which the virus yields were plaque titrated. The first term in the numerator, $\text{yield}(A+B)_{np}$, quantifies wild-type virus emerging from the mixed infection, including both recombinants and revertants, because only wild-type virus will grow in the plaque assay done under nonpermissive conditions. The second and third terms in the numerator, $\text{yield}(A)_{np}$ and $\text{yield}(B)_{np}$, quantify wild-type virus emerging from the control single infections, providing a measure of reversion in each of the two mutants. Subtraction of the revertants from the total yield of wild-type virus leaves a

measure of recombinants only in the numerator. The denominator, $\text{yield}(A + B)_p$, quantifies the total virus yield from the mixed infection including both wild-type and mutant virus, because all input virus types will grow in the plaque assay done under permissive conditions. The quotient is multiplied by a factor of two to account for unscored progeny representing the reciprocal of the wild-type recombinants, namely double mutants, and converted to a percent.

Recombination mapping in DNA viruses relies on the assumption that the frequency of recombination between two genetic markers is proportional to the distance between the two markers. For several DNA viruses, observed recombination frequencies comprise a continuous range from less than 1% up to a theoretical maximum of 50%, allowing for construction of linear genetic maps.³⁸

In viruses with segmented genomes, recombination between markers on the same segment is rare but reassortment of segments is extremely efficient; thus, recombination is effectively an all or none phenomenon, with markers on the same segment displaying no recombination, and markers on different segments displaying very high levels of recombination.¹⁰² For these reasons, genetic exchange in segmented RNA viruses is commonly referred to as reassortment rather than recombination. Reassortment analysis for segmented viruses is useful for determining whether or not two mutants map to the same genome segment but cannot be used to determine the order of markers on a given segment. Mutants can be mapped to individual RNA segments by performing *intertypic* crosses between virus types that differ in the electrophoretic mobility of each RNA segment. Specifically, if crosses are performed between a wild-type virus of one type and a mutant virus of another type and numerous wild-type progeny analyzed, one segment bearing the wild-type allele will be conserved among all the progeny, whereas all other segments will display reassortment.¹⁰²

Marker Rescue

Marker rescue is a physical mapping technique that measures directly whether a given virus mutation maps within a specific subfragment of a virus genome. The use of marker rescue is confined to DNA viruses where homologous recombination takes place and has been of enormous value in these systems. The application of the technique varies somewhat depending on the virus system under study; however, the general principles are the same. Specifically, full-length mutant viral genomic DNA plus a wild-type DNA genomic subfragment, either a cloned DNA molecule or a PCR product, are introduced into cells under conditions permissive for recombination and for wild-type virus replication. For viruses that contain infectious DNA, such as herpesviruses,¹¹⁷ adenoviruses,⁴² and polyomaviruses,^{72,89} the mutant genomic DNA and the wild-type genomic subfragment may be co-transfected into cells. For viruses containing noninfectious genomic DNA, such as poxviruses,¹²² the mutant DNA must be introduced into cells by infection with the mutant virus, which is then followed by transfection with the wild-type DNA subfragment. In either case, the protocol allows for homologous recombination between the mutant genome and the wild-type DNA subfragment. If the wild-type DNA subfragment contains the wild-type allele for the mutation, the recombination can exchange the wild-type for the mutant sequence in the

mutant genome, creating wild-type virus. Conversely, if the wild-type fragment does not contain the wild-type allele for the mutation, no wild-type virus, above a background of revertants, will be created in the experiment. The presence of wild-type virus can be assayed using either a two-step or a one-step protocol. In the two-step protocol, depending on the nature of the mutation being rescued, infected and/or transfected cells are incubated under permissive conditions to facilitate recombination and replication, or nonpermissive conditions to select for wild-type recombinants, then wild-type virus yields are quantified by plaque titration under nonpermissive conditions. In the one-step protocol, the infection and/or transfection is done so that only a small fraction of the cells in a monolayer are infected, and cells are then incubated under nonpermissive conditions such that wild-type virus formed during a successful rescue will form plaques on the monolayer.¹²² In short, regardless of the precise method used, conversion or *rescue* of mutant virus to wild-type with a given wild-type DNA fragment means that the mutation maps within that fragment. Initial marker rescue mapping experiments may be facilitated by the use of a few large but overlapping wild-type DNA fragments, and fine mapping may be accomplished with fragments as small as a few hundred nucleotides. Marker rescue mapping has completely replaced recombination mapping as a method for mapping mutations in DNA viruses, and precise genetic maps of several DNA viruses have now been constructed.

Reverse Genetics

Prior to the advent of recombinant DNA and DNA sequencing technologies, classical genetic analysis, namely random isolation and characterization of virus mutants, was one of the few effective methods for identifying, mapping, and characterizing virus genes, and the only method for obtaining virus mutants. With the current ready availability of genomic sequences for virtually all prototypical members of each virus family and a versatile package of genetic engineering tools, the experimental landscape has changed completely. One can now conduct a genetic analysis with a reasonably complete foreknowledge of the genetic structure of the virus, focus attention on individual genes of interest, and deliberately engineer mutations in genes to study their function. Termed *reverse genetics*, this process has come to dominate the genetic analysis of viruses. Reverse genetics covers a broad range of activities ranging from engineering a single nucleotide substitution in a target gene to engineering chimeric viruses to be used as gene therapy vectors, oncolytic vectors, or vaccines. Currently, virtually every significant human viral pathogen can be engineered using reverse genetic approaches. Perhaps one of the most impressive feats in reverse genetics is the resurrection of the deadly 1918 pandemic strain of influenza using genome sequences derived from archived formalin-fixed lung autopsy materials and from frozen, unfixed lung tissues from an Alaskan influenza victim who was buried in permafrost.¹²⁵

Reverse genetic analysis involves two distinct considerations: strategies for design of a given mutation and strategies for incorporation of mutations into virus. The principles governing these strategies highly depend on the structure of a given viral genome and the strategy of virus replication, and thus vary in the extreme. However, some general principles can

be identified, which are discussed next, accompanied by a few specific examples to illustrate the general principles.

Incorporation of Mutations into Virus

The methods used for incorporation of mutations into a virus depend on several features of the individual virus under consideration, including genome size, whether or not the nucleic acid is infectious, whether the genome is composed of DNA or RNA or replicated via reverse transcription, and whether replication is nuclear or cytoplasmic.

DNA VIRUSES AND REVERSE TRANSCRIBING VIRUSES

With the exception of poxviruses, which because of their cytoplasmic site of replication must carry virion-encapsidated transcription enzymes into cells during infection, virtually all DNA virus genomes (see Table 2.1) are infectious. Likewise, double-stranded DNA (dsDNA) comprising the genomic sequences of reverse transcribing viruses that package RNA (retroviruses) or DNA (hepadnaviruses) genomes are also infectious. Thus, in these cases, the incorporation of a mutation into the virus genome is essentially an exercise in molecular cloning, and pure mutant virus is produced by transfection of the cloned mutant genome into cultured cells. In practice, the herpesvirus genome is sufficiently large such that manipulation as a full-length genomic clone presents

some difficulties, and therefore incorporation of mutations into the viral genome is often done by co-transfecting cells with full-length genomic viral DNA along with a DNA fragment containing the desired mutant allele flanked by wild-type DNA sequences. Replication is launched from the transfected infectious wild-type genomic DNA, and homologous recombination between the co-transfected mutant DNA fragment and the wild-type genome incorporates the mutant allele into a fraction of the replicating wild-type genomes¹³⁹ (Fig. 2.11). A similar protocol is applied to engineering poxviruses; however, because poxvirus DNA is noninfectious, virus replication must be initiated by infection with intact virus. In its simplest form, this protocol entails infection with virus bearing the wild-type target genome followed by transfection with a DNA fragment containing the desired mutation flanked by wild-type DNA sequences⁹⁵ (Fig. 2.12; identical to the protocol for marker rescue described earlier). Similar to the herpesvirus co-transfection protocol just described, homologous recombination catalyzed by viral enzymes results in incorporation of the mutant allele into a fraction of the wild-type infecting genomes. An alternate protocol for constructing poxvirus recombinants involves first infecting cells with a replication defective, nonhomologous helper poxvirus, followed by transfection with either a cloned full-length mutant genome or a mixture of fragments comprising the desired engineered genome.^{23,105,137} The helper virus provides

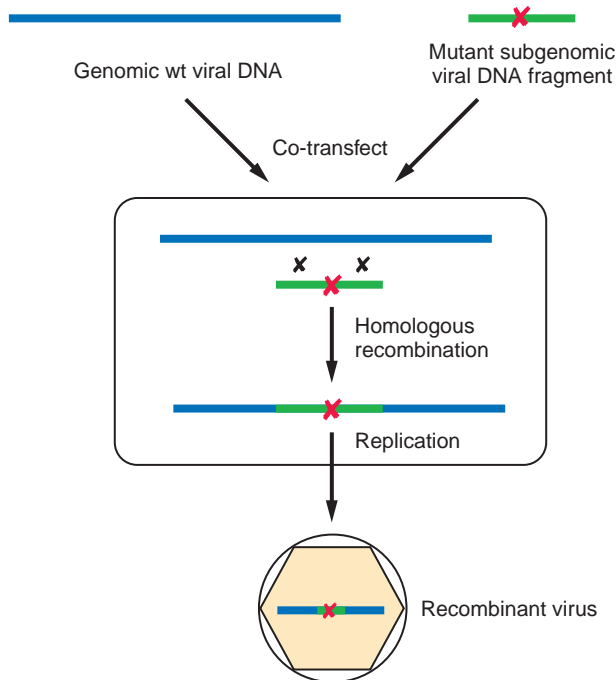


FIGURE 2.11. Reverse genetics with large double-stranded DNA viruses containing infectious genomes. Wild-type full-length infectious genomic viral DNA (blue line) is co-transfected into cells along with a subgenomic viral DNA fragment (green line) containing the desired mutation (red X). Homologous recombination between the co-transfected viral DNAs takes place within the cell catalyzed by viral and/or cellular enzymes. The recombinant genome is replicated and packaged to yield recombinant virus (mixed with wild-type virus replicated from unrecombined genomes).

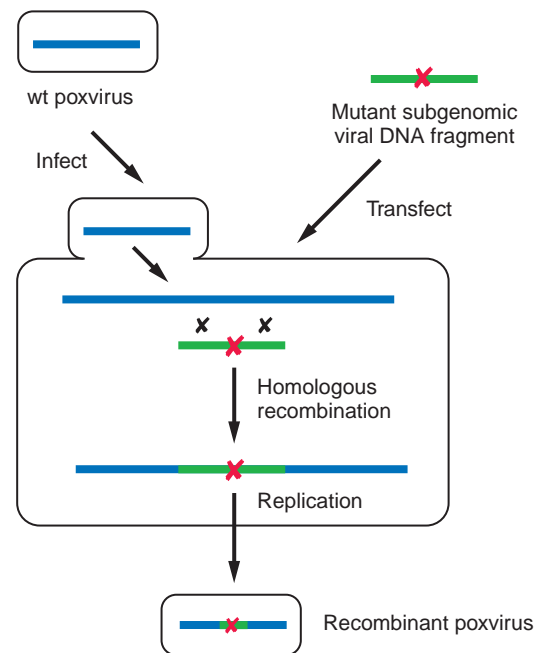


FIGURE 2.12. Reverse genetics with large double-stranded DNA viruses containing noninfectious genomes (poxviruses). Cells are infected with virus containing a wild-type DNA genome (blue line) and transfected with a subgenomic viral DNA fragment (green line) containing the desired mutation (red X). Homologous recombination between the infecting viral DNA and co-transfected fragment takes place within the cell catalyzed by viral enzymes. The recombinant genome is replicated and packaged to yield recombinant virus (mixed with wild-type virus replicated from unrecombined genomes).

enzymes critical for launching replication of the transfected full-length mutant viral DNA genome, or for homologous recombination among transfected subgenomic fragments to assemble and launch replication of the desired virus.

When mutants are engineered in cloned, infectious genomes, only the mutant allele of the target gene is present in the construction, all virus recovered from the transfection will be mutant, and no selection or screen for mutants is required. However, in situations where *in vivo* homologous recombination has been used to incorporate the cloned mutation into a wild-type genome, such as in poxviruses or herpesviruses, both mutant and wild-type viruses emerge from the mutant construction protocol, and thus a screen or selection is required to identify the mutant of interest. For mutations in nonessential genes, this may be a relatively straightforward matter of inserting into the target gene a color marker such as β -galactosidase¹³⁹ or green fluorescent protein¹⁹ to facilitate a screen, or inserting a dominant selectable marker such as *Escherichia coli* guanine phosphoribosyltransferase,³⁰ to facilitate a selection. For conditionally lethal phenotypes such as temperature sensitivity, although techniques exist that enrich for recombinant viruses, mutant isolation ultimately relies on a screen of individual mutants for differential growth under permissive and nonpermissive conditions.⁴⁷ The use of full-length clones of herpesvirus and vaccinia virus obviates the need for *in vivo* recombination, and thus only mutant virus will be recovered after transfection or reactivation of an engineered, mutant genome, and no mutant screen or selection is necessary.^{24,88}

RNA VIRUSES

The genomes of positive sense, ssRNA viruses (see Table 2.1) are infectious; therefore, as with most DNA viruses, the engineering of mutant viruses is largely an exercise in molecular cloning, yet formidable for large RNA viruses such as coronaviruses.¹³⁸ To recover infectious virus, complementary DNA (cDNA) clones of mutant genomes may be transfected directly into cultured cells or transcribed *in vitro* into positive sense RNA that is then transfected into cells. Virus replication is launched by translation of the transfected RNA *in vivo*, resulting in recovery of only mutant virus. A good example of the application of this technology is the directed construction of temperature-sensitive mutants in poliovirus.²²

Negative sense ssRNA viruses must package in the virion a virus-coded RNA-dependent RNA polymerase so that the genome, in the form of a nucleocapsid, can be transcribed into mRNA immediately following infection. Thus, negative sense ssRNA virus genomes are not infectious, and engineering these viruses becomes more of a challenge. Generally, the strategy consists of transfection of cells with multiple plasmids, some of which are transcribed into genome-length RNAs (encoding the desired genotype) and some of which direct expression of proteins required for genome replication, specifically a nucleocapsid protein and proteins comprising the viral RNA-dependent RNA polymerase. The expressed nucleocapsid protein encapsidates the transcribed genomic RNA, and this nucleocapsid can then be transcribed into mRNA by the expressed viral RNA polymerase, thus launching the infection and ultimately yielding pure virus of the desired genotype. Historically, the requirements for “rescue” of virus from cloned fragments are different for the segmented negative sense ssRNA orthomyxo-

viruses compared to most nonsegmented negative sense ssRNA viruses, namely rhabdoviruses, paramyxoviruses, and filoviruses, comprising most of the order *Mononegavirales*. The differences are attributable, at least in part, to the fact that the orthomyxoviruses replicate in the nucleus, whereas most of the viruses in the order *Mononegavirales* replicate in the cytoplasm. Specifically, for the orthomyxoviruses,⁹³ genome segments are cloned so that they are transcribed from a polymerase I promoter to yield the negative sense genomic RNA, and the replication proteins are cloned so that they are transcribed from a polymerase II promoter to yield mRNA. Both polymerases are expressed in the cell nucleus so that after transfection of the plasmids, the viral RNAs are synthesized in the appropriate cellular compartment. The number of plasmids required for rescue can be minimized by flanking each genome segment with a polymerase I promoter at the 3' end and a polymerase II promoter at the 5' end so that each plasmid yields both a negative sense genomic RNA and a positive sense mRNA (Fig. 2.13). For most *Mononegavirales* viruses,¹⁵ rescue is best achieved if synthesis of both the genomic RNA and the replication proteins are driven by the bacteriophage T7 RNA polymerase, which localizes efficiently to the cell cytoplasm. The T7 RNA polymerase can be supplied either by infection with a poxvirus expressing T7 RNA polymerase, by using a cell line containing a stably integrated copy of the T7 RNA polymerase gene, or by transfection of an additional plasmid designed to express the enzyme. An additional (counterintuitive) requirement for rescue of *Mononegavirales* viruses is that the plasmid encoding the genomic RNA is configured so that it is initially transcribed to yield positive sense, antigenomic RNA, which is then encapsidated with expressed nucleocapsid protein, and replicated into encapsidated negative sense genomic RNA, which is in turn transcribed into mRNAs to launch the infection (Fig. 2.14). The use of a plasmid that expresses the negative sense genomic RNA compromises the rescue, presumably because the negative sense genomic RNA will hybridize in the cytoplasm with the positive sense mRNAs for replication proteins, thus repressing their expression. Despite these generalities, examples exist of rescue of cytoplasmic *Mononegavirales* viruses using polymerase I or polymerase II promoters.⁸³

The segmented, double-stranded (dsRNA)-containing, cytoplasmic reoviruses can be rescued using a protocol similar to that used for *Mononegavirales*.⁶⁹ Specifically, cDNAs of individual segments are cloned downstream from a bacteriophage T7 promoter, each yielding an RNA product that doubles as mRNA and the positive strand template for genomic dsDNA. Transfection of these plasmids into cells expressing T7 RNA polymerase results in synthesis of genomic segments and replication proteins, ultimately yielding pure virus of the desired genotype. T7 RNA polymerase can be supplied by any of the previously mentioned methods: co-transfection of an expression plasmid, expression from a stably integrated chromosomal gene, or infection with a poxvirus expressing the enzyme. Interestingly, this protocol does not work for all reoviruses, most notably the important human pathogen rotavirus, which must still be engineered using more complex helper-mediated protocols.¹²⁴

Mutation Design

Design of mutations for use in virology is problematic only if the gene in question is essential, necessitating isolation of

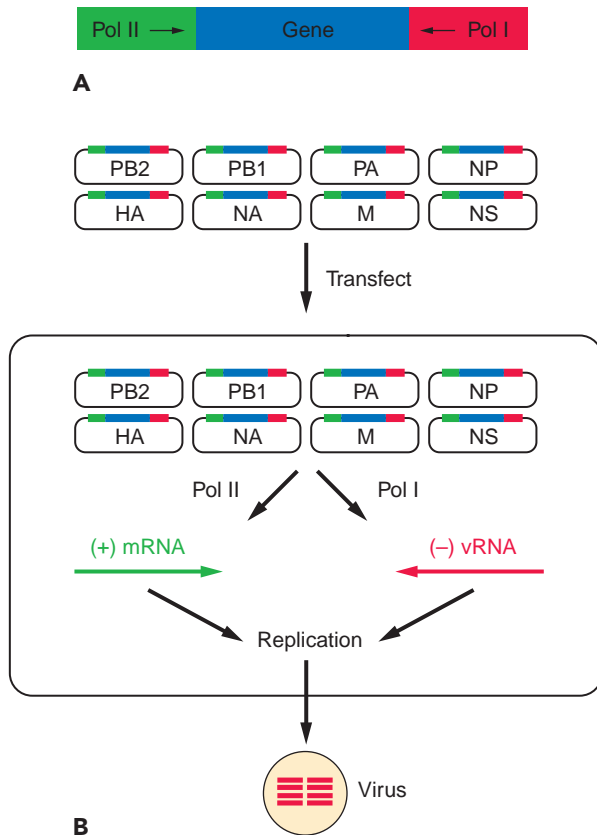


FIGURE 2.13. Reverse genetics with orthomyxoviruses (influenza).

A: Detailed structure of cloned viral genes. Coding sequences for each viral gene (blue) are cloned flanked by an RNA polymerase II transcriptional promoter (green) at the upstream (5') end and an RNA polymerase I transcriptional promoter (red) at the downstream (3') end. **B:** Reverse genetic protocol. Cells are transfected with eight plasmids together representing the entire complement of virus genes. Transcription in the nucleus with polymerase II produces positive sense messenger RNAs (green) that are translated into viral proteins including the polymerase (PB1, PB2, PA) and nucleocapsid protein (NP). Transcription from the polymerase I promoter produces negative sense genomic viral RNAs (red), which are then replicated by the viral replication proteins. Further transcription, replication, and encapsidation produces virus.

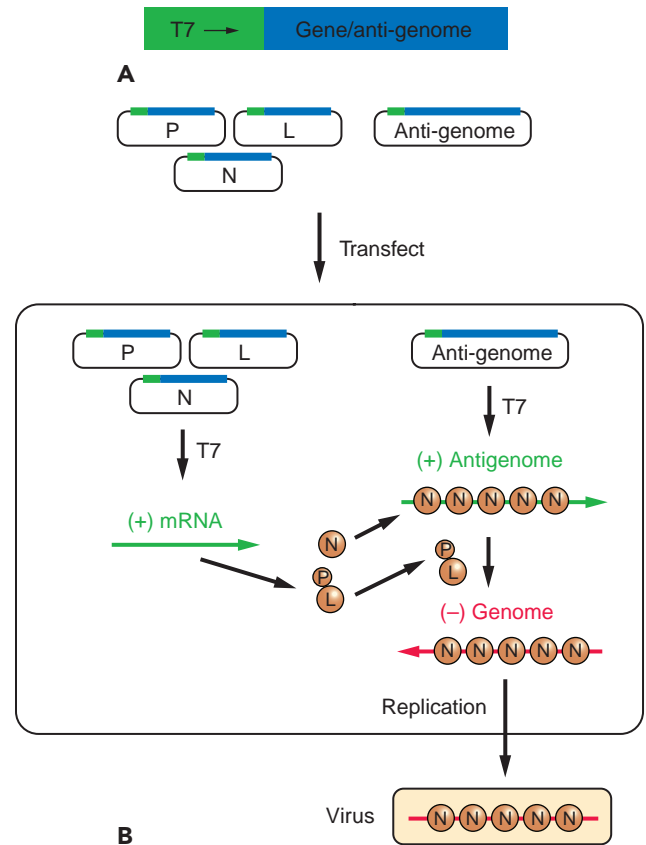


FIGURE 2.14. Reverse genetics with viruses in the order *Mononegavirales*.

A: Detailed structure of cloned genes. Coding sequences for replication proteins or the full-length viral genome (blue) are cloned downstream from a bacteriophage T7 transcriptional promoter (green). **B:** Reverse genetic protocol. Cells are transfected with four plasmids, three of which encode replication proteins (P, L, and N) and one of which contains the full-length viral genome oriented such that transcription yields a positive sense antigenomic RNA. Transcription by T7 RNA polymerase (usually encoded by an engineered stably integrated copy in the cell nucleus) yields messenger RNA for the replication proteins (green) plus positive sense antigenomic viral RNA (green). Translation of the messenger RNAs provides nucleocapsid protein (N), which encapsidates the antigenomic RNA. Antigenomic nucleocapsid is replicated by the viral polymerase (L and P) to yield negative sense genomic viral nucleocapsid (red viral RNA encapsidated with brown N protein), which can be further transcribed and replicated to yield recombinant virus.

a conditionally lethal mutation. For genes that are nonessential, mutation design is a simple matter of engineering a null mutation (e.g., a deletion, insertion, or nonsense mutation) into the cloned gene sequence. Three basic types of engineered conditionally lethal mutations are currently in use: host-range deletion mutants, which rely on the availability of a complementing host cell; temperature-sensitive mutants constructed by clustered charge to alanine scanning; and artificially induced gene regulation. For host-range deletion mutants, the primary problem is construction of a host cell that expresses the target gene in a fashion appropriate for complementation of a null mutant in the virus. Once a cell line has been isolated, construction of the cloned mutation in the virus gene follows the same principles governing construction of a null mutation in a nonessential gene. The fundamental problem in

creating temperature-sensitive mutations is that it is currently impossible to predict from primary amino acid sequence or even from three-dimensional protein structure what type of mutation will render a protein temperature sensitive. This difficulty has been partially overcome with the use of clustered charge to alanine scanning mutagenesis, in which clusters of three or more charged residues in the primary amino acid sequence of a protein are all changed to alanine.¹³² In theory, charge clusters are likely to reside on the surface of the protein where they may facilitate protein–protein interactions, and neutralization of the charge by replacement with alanine may weaken such interactions without seriously disrupting the three-dimensional conformation of the protein. In practice, as

much as 30% of clustered charge to alanine scanning mutants prove to be temperature sensitive *in vivo*, and this mutagenesis technique has been successfully used to construct temperature-sensitive mutants of both picornaviruses and poxviruses.^{22,48} Lastly, conditionally lethal mutants have been constructed in poxviruses by placing essential genes under bacterial operator-repressor control in the viral genome.^{52,106,123}

Defective Interfering Particles

Interference refers generally to a phenomenon whereby infection by one virus results in inhibition of replication of another virus.³⁴ Defective interfering (DI) particle-mediated interference was first described by von Magnus,¹³⁰ who noted that serial undiluted passage of influenza virus resulted in a dramatic decrease in infectious titer while the number of particles remained constant. Essentially the same phenomenon was subsequently observed in a wide variety of RNA and DNA animal viruses, as well as in plant and bacterial viruses.⁵⁷ The mechanism of interference in each case is similar, namely virus stocks accumulate *DI particles*. DI particles are virus particles that contain genomes that are grossly altered genetically, usually by significant deletion of essential functions, but nevertheless retain critical replication origins and packaging signals, allowing for amplification and packaging in co-infections with complementing wild-type *helper* virus. DI particles usually display a replication advantage relative to wild-type virus, resulting from increases in the copy number or efficiency of replication origins. DI particles actively inhibit replication of wild-type virus, presumably by competing for limiting essential replication factors. Study of DI particles has provided significant insight into the viral replication, particularly structure and function of replication origins.

Phenotypic Mixing and Pseudotypes

If two heterologous viruses infect the same cell, then depending on the relatedness of the two viruses, the opportunity exists for packaging of either virus genome into a nucleocapsid or envelope comprised wholly or in part of structural proteins encoded by the heterologous virus. This phenomenon, termed *phenotypic mixing*, has been observed in mixed infections in a laboratory setting using both nonenveloped and enveloped viruses. Phenotypic mixing is a transient phenomenon, because infection of a cell with a single phenotypically mixed particle will result in replication and assembly only of viruses that reflect the infecting genome. In mixed infections with nonenveloped viruses, phenotypic mixing has been observed between closely related adenoviruses, reoviruses, and picornaviruses.^{54,103,135} Phenotypic mixing has been observed between picornaviruses as distantly related as echovirus 7 and coxsackievirus A9.⁵⁸ In the case of enveloped viruses, phenotypic mixing consists of packing the nucleocapsid of one virus within an envelope of a heterologous virus, and the resulting viruses are called *pseudotypes*. Pseudotype formation among enveloped viruses is relatively promiscuous, especially among retroviruses and rhabdoviruses.¹⁴¹ For example, pseudotypes have been formed that contain retrovirus envelope antigens combined with genomes from rhabdoviruses, paramyxoviruses, orthomyxoviruses, or herpesviruses. Conversely, pseudotypes have been formed that contain a rhabdovirus genome combined with envelope antigens from togaviruses,

retroviruses, bunyaviruses, arenaviruses, paramyxoviruses, orthomyxoviruses, herpesviruses, or poxviruses. Historically, phenotypic mixing experiments have contributed to understanding virus structure and assembly. Currently, the concept of phenotypic mixing and pseudotype formation is of critical utility in packaging and delivery of virus vectors, particularly because phenotypic mixing permits the tropism of a virus particle to be manipulated.⁶² Lastly, there has been some speculation that phenotypic mixing may actually play a role in a natural setting, serving to maintain otherwise unfit genomes within a quasispecies over time.¹³⁴

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Principles of Virus Structure

How Virus Structures Are Studied

Symmetry of Viruses

Structures of Closed Shells

- Quasiequivalent Icosahedral Arrangements
- Nonequivalent Icosahedral Surface Packings
- Frameworks and Scaffolds
- Elongated Shells
- Multishelled Particles
- Rearrangements in Surface Lattices
- Two Recurring Globular Domains in Icosahedral Capsid Proteins

Self-Assembly and Cleavage Steps

Genome Packaging

- Positive-Strand RNA Genomes
- dsDNA Genomes
- dsRNA Genomes
- Negative-Strand RNA Genomes

Viral Membranes

- Budding
- Internal Structures
- Surface Envelope Proteins

Structural Biology of Virus Entry

- Receptor Binding
- An Irreversible Step Between Assembly and Entry
- Membrane Fusion
- Penetration by Nonenveloped Viruses

Virus particles are carriers of genetic material from one cell to another. They are, in effect, extracellular organelles. They contain most or all of the molecular machinery necessary for efficient and specific packaging of viral genomes, escape from an infected cell, survival of transfer to a new host cell, attachment, penetration, and initiation of a new replication cycle. In many cases, the molecular machinery works in part by subverting more elaborate elements of a host cell's apparatus for carrying out related processes.

A number of organizational modes have evolved to perform the functions just outlined. The most critical distinction, from a structural perspective, is between *enveloped* viruses—those with lipid-bilayer membranes—and *nonenveloped* viruses—those without such membranes. Both categories include well-known human pathogens. Examples of the former are human immunodeficiency virus (HIV) and

influenza virus; examples of the latter, poliovirus and papillomavirus. Enveloped viruses have, in their lipid bilayer, an impermeable barrier between their genomes and the outside environment, reducing the need for continuity of any protein layer. Nonenveloped viruses require a tightly packed shell to exclude nucleases or other sources of genomic damage.

For the structure of any virus particle, a central constraint is that the information needed to specify its macromolecular components must not exhaust the genetic capacity of the packaged genome. This requirement for genetic economy is in practice quite stringent. For example, consider a very simple genome of 5 kb, enough to encode about 1,600 amino acid residues, if reading frames do not overlap. A tightly condensed single-stranded RNA or DNA of this size will occupy a spherical volume about 90 Å in radius. To protect it with a gap-free protein shell, 30 Å thick, would require roughly 25,000 amino acid residues—far more than the viral nucleic acid can encode. The shell of a nonenveloped virus with even a very small genome must therefore contain a large number of identical protein subunits—at least 60, if the coat-protein gene is to use up less than 25% of the coding capacity in the enclosed nucleic acid. As explained later, an important consequence of this observation (first made by Crick and Watson⁵⁶ even before a triplet code had been established) is that virus particles, or their substructures, are usually highly symmetric.

HOW VIRUS STRUCTURES ARE STUDIED

Electron microscopy is the most direct way to determine the general morphology of a virus particle. Traditional thin-sectioning methods are useful for examining infected cells and larger, isolated particles. The thickness of a section and the coarseness of staining methods limit resolution to about 50 to 75 Å, even in the best cases. (*Resolution* means the approximate minimum size of a substructure that can be separated in an image from its neighbor. Recall that one atomic diameter is 2.3 Å; an α -helix, 10 Å; and a DNA double helix, 20 Å.) Negative staining, with uranyl acetate, potassium phosphotungstate, or related electron-dense compounds, gives somewhat more detailed images of isolated and purified virus particles. Viruses embedded in negative stain are often relatively well preserved. The electron beam destroys the particle itself very rapidly, but it leaves the dense “cast” of stain undamaged for much longer. If the particle is fully covered by the negative stain, the image contains contrast from both the upper and the lower surface of the particle, and visual interpretation of finer aspects of the image can be difficult.⁵⁷

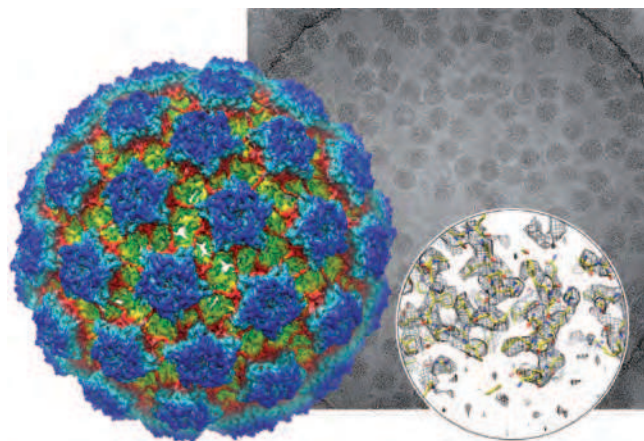


FIGURE 3.1. Bovine papillomavirus (BPV), as seen by electron cryomicroscopy (cryoEM). In the foreground is a color rendering of the three-dimensional image reconstruction, based on the kinds of micrographs shown in the background picture. The circular inset at **lower right** illustrates that this reconstruction provides information that extends to a nearly atomic level of detail (*resolution*); it shows a small part of the density map that resulted from the image analysis and the fit to that map of parts of the L1 polypeptide chain. (See Grigorieff and Harrison⁹⁴ and Wolf et al.²⁴⁶)

Methods for preserving viruses and other macromolecular assemblies by rapid freezing to liquid nitrogen or liquid helium temperatures have permitted visualization of electron-scattering contrast from the structures in the particle itself and not just from the cast created by a surrounding layer of negative stain.¹⁰ Moreover, quantitative methods for image analysis, originally developed for studying negatively stained particles, have been applied effectively to such images. An advantage of such electron cryomicroscopy (cryoEM) is that regular images can be selected from a heterogeneous field, allowing study of unstable or relatively impure preparations. Advances during the decade preceding the current revision of this chapter have enabled cryo-EM three-dimensional density maps at resolutions that reveal molecular details—the tracing of a polypeptide chain and the orientations of large amino acid side chains.⁹⁴ One example is illustrated in Figure 3.1.²⁴⁶ Such *image reconstructions* are obtained by combining information from hundreds or thousands of different images of individual particles. The combination is possible because the particles of these viruses are all the same. When such uniformity is not present, for example, as in the case of a complete herpesvirus particle rather than an isolated nucleocapsid, then information from different particles cannot be combined. A *tomographic* tilt series of images from a single particle can be obtained (analogous to a computed tomography [CT] scan in medical radiography), but the resulting three-dimensional image is of much lower resolution, as electron damage limits its quality, even when the data are taken at liquid nitrogen or liquid helium temperatures (electron cryotomography, or cryoET). Tomographic reconstructions can nonetheless be very useful, as illustrated in Figure 3.2. In some cases, averaging the images of defined substructures within a tomogram or among many tomograms (e.g., the “spikes” on the surface of certain enveloped viruses) can yield a more detailed representation.

The information obtained from even the most elegant of electron microscopy methods still falls short of the atomic detail that often can be obtained by x-ray diffraction methods, if single crystals of the relevant structure can be prepared. It has been known since the 1930s that simple plant viruses, such as tomato bushy stunt virus (TBSV), can be crystallized,¹³ and the first x-ray diffraction patterns of such crystals were recorded as early as 1938.¹⁷ Crystallization of poliovirus and other important animal viruses showed that the approach could be extended to human pathogens.²¹³ The first complete high-resolution structure of a crystalline virus was obtained from TBSV in 1978,¹⁰⁷ and since then the structures of a number of animal, plant, and insect pathogens have been determined (for a compilation, see the VIPER website: <http://viperdb.scripps.edu>). Only very regular structures can form single crystals, and in order to study the molecular details of larger and more complex virus particles, it is necessary to “dissect” them into well-defined subunits or substructures. This dissection was originally done with proteases, by disassembly, or by isolation of substructures from infected cells. For example, the structure of the influenza virus hemagglutinin²⁴⁴—the first viral glycoprotein for which atomic details were visualized—was obtained from crystals of protein cleaved from the surface of purified virions²⁴³; the structure of the adenovirus hexon was obtained from excess unassembled protein derived from adenovirus-infected cells.¹⁸⁹ In the past two decades, this dissection has more commonly been carried out using recombinant expression (e.g., of a fragment of gp120 from HIV-1¹³¹). Most of the high-resolution structures of enveloped virus components described in this chapter—both surface glycoproteins and internal proteins—come from x-ray crystallographic analysis of recombinant gene products, often suitably truncated or otherwise modified to enable crystallization. A handful of atomic-level structures of virus components have come from nuclear magnetic resonance (NMR) spectroscopy,^{178,200} but application of that technique is limited to relatively small proteins or protein complexes.

SYMMETRY OF VIRUSES

Virus particles must assemble specifically and rapidly in an infected cell, as directed by the mutual interactions among their component protein subunits. Specificity requires a defined stereochemical relationship between contacting proteins. Because there are many copies of the same subunit, there must also be many repeating instances of the same kind of contact. This repetition—a consequence of the requirement for genetic economy described in the introductory section of this chapter—implies symmetry.

A rigorous definition of symmetry involves an operation, such as a rotation, that brings an object into self-coincidence. For example, if the ring of three commas in Figure 3.3A is rotated by 120 or 240 degrees, it will not be possible to recognize that a rotation has occurred (assuming that the commas are truly indistinguishable). The full symmetry of an object is defined by the collection of such operations that apply to it. In the case of protein assemblies, these operations can be rotations, translations, or combinations of the two. A symmetry axis that includes rotation by 180 degrees is called a *twofold axis* or a *dyad*; one with a 120-degree rotation (and, of course,

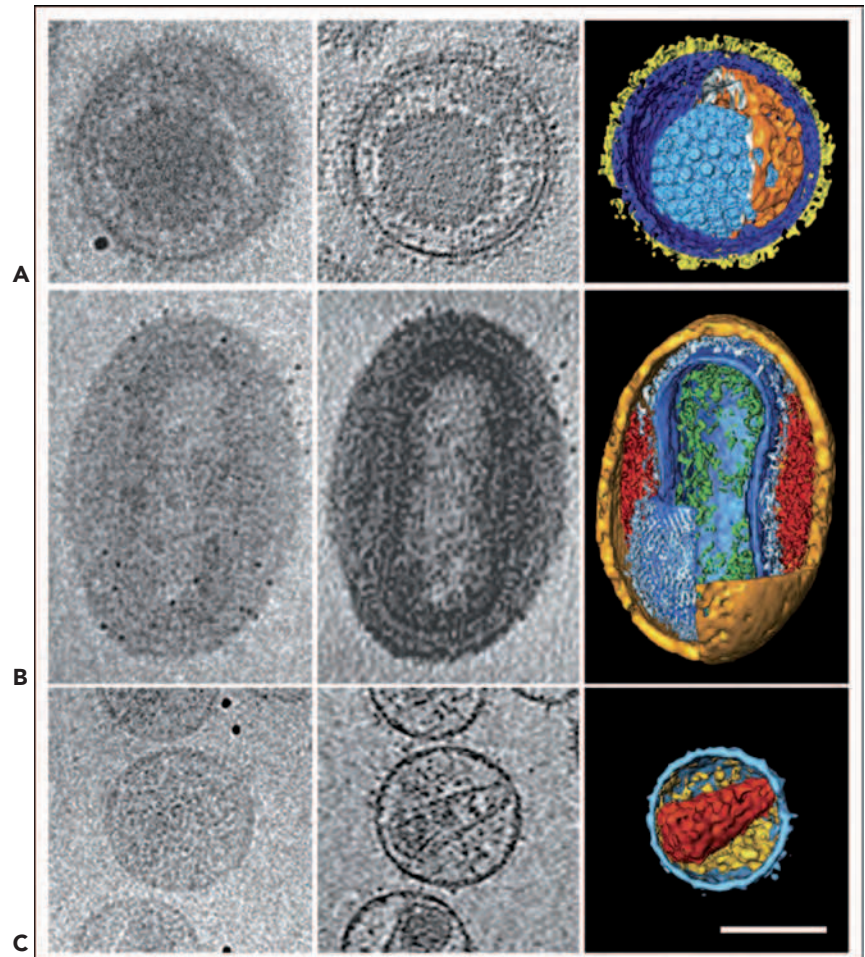


FIGURE 3.2. Electron cryotomography (cryoET) of herpes simplex virus type 1 (A),⁹⁸ vaccinia intracellular mature virion (B),⁵⁸ and HIV-1 (C).²³ Images in the **left-hand column** are single, projected images; those in the **middle column**, slices through the reconstructed tomogram; those on the **right**, cut-away surface renderings of the three-dimensional tomographic reconstructions. (Adapted from Cyrklaff M, Risco C, Fernandez JJ, et al. Cryo-electron tomography of vaccinia virus. *Proc Natl Acad Sci U S A* 2005;102:2772–2777.)

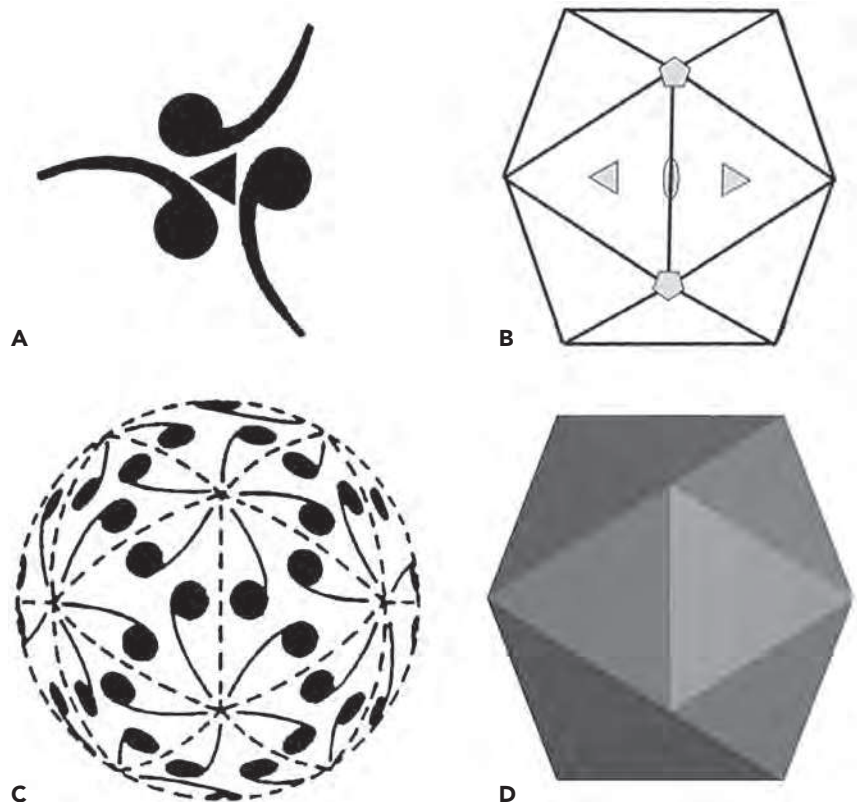


FIGURE 3.3. Icosahedral symmetry. A: Threefold symmetry: the three commas are related to each other by 120-degree rotations about the central axis, marked by a small triangle. **B:** Outline of an icosahedron, showing positions of some of the symmetry axes (imagined to extend from the center of the icosahedron to the point on the surface marked by the symbol): fivefold, threefold, and twofold axes are marked by pentagons, triangles, and an oval, respectively. **C:** An icosahedrally symmetric arrangement of commas on the surface of a sphere. For locations of symmetry axes, compare with panel B. **D:** Shaded surface view of an icosahedron.

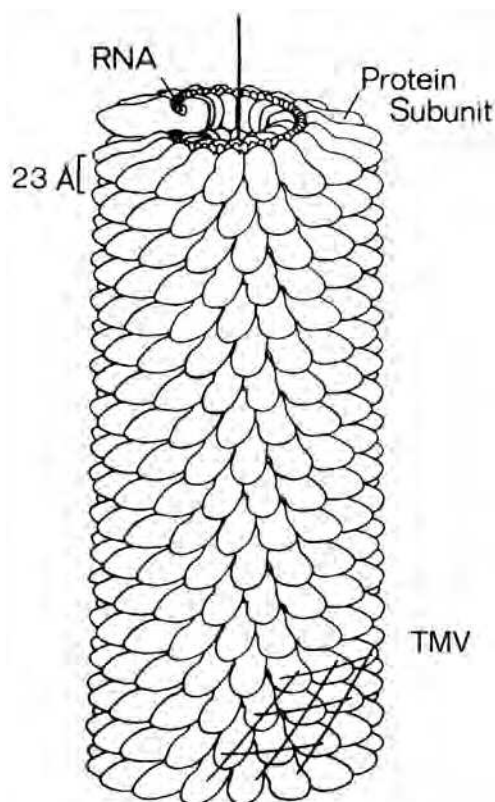


FIGURE 3.4. Diagram of the tobacco mosaic virus (TMV) particle.

The elongated “loaves,” with a groove for the RNA, represent the protein subunits. Three RNA nucleotides fit into the groove on each subunit. There are $16\frac{1}{3}$ subunits per turn of the right-handed helix (i.e., 49 subunits in three turns), with a rise of 23 Å as indicated. At the **lower right**, the surface lattice is drawn onto the outer particle. (Adapted from Caspar DL. Assembly and stability of the tobacco mosaic virus particle. *Adv Protein Chem* 1963;18:37–121.)

a 240-degree rotation as well) is called a *threefold axis*; and so forth. Note the distinction between shape and symmetry: the *shape* of an object refers to the geometry of its outline, whereas its *symmetry* refers to the operations that describe it. The set of commas in Figure 3.3A has threefold symmetry; so does an equilateral triangle, the beer-company symbol with three interlocked rings, and countless other objects with unrelated shapes.

As a first example, consider the rod-like coat of tobacco mosaic virus (TMV)¹²⁵ (Fig. 3.4). The helical arrangement of its protein subunits illustrates that symmetry is an important consequence of its assembly from many identical building blocks. If we look at the model of TMV, we find that a rotation of 22 degrees and a translation of 1.4 Å along the particle axis will superpose subunit 1 on subunit 2. But if the surfaces of subunit 2 are the same as those of subunit 1, the same rotation and translation must superpose subunit 2 on subunit 3, and so forth. The combination of rotation and translation that effects this superposition is a *screw axis*. Strictly speaking, the screw axis of TMV would only be an ideal symmetry operation if the helix were infinite. In practice, it is so long that we can neglect end effects.

In TMV, and probably in the nucleocapsids of negative-strand RNA viruses such as influenza and vesicular stomatitis

virus (VSV), the RNA winds in a helical path that follows the protein.¹²⁵ That is, the tubular package does not simply contain the RNA; it co-incorporates it. There are exactly three nucleotides per subunit in TMV, and they fit into a defined groove between the helically arrayed proteins. By contrast, the protein coat of a filamentous, single-stranded DNA (ssDNA) phage, such as M13, forms a sleeve that surrounds and constrains the closed, circular genome, without there being a specific way in which each subunit contacts one or more nucleotides.⁸⁷ Thus, there can be a nonintegral ratio of nucleotides to protein monomers.

The length of the packaged nucleic acid determines the length of virus particles such as TMV or M13. Structures such as the tail of bacteriophage lambda or T4 have a protein component that extends from the initiating structure at the base of the tail to the end connected to the head.³ The number of such polypeptide chains corresponds to the rotational symmetry of the tail.

Rod-like structures are not very efficient ways to package long genomes. At least one dimension of a helical assembly such as TMV grows linearly with the length of the packaged viral DNA or RNA, leading to awkwardly elongated particles. The number of subunits is likewise proportional to length. *Isometric* (i.e., essentially spherical) particles are more compact and more economical: if the nucleic acid condenses into the interior of the particle, then the diameter increases as the cube root of the genome length, and the number of required subunits as the genome length to the two-thirds power. Most animal viruses are roughly isometric.

Closed, isometric shells composed of identical subunits that interact through conserved, specific interfaces can have one of only three symmetries: the symmetry of the regular tetrahedron, the cube, or the regular icosahedron. These shells will accommodate 12, 24, or 60 subunits, respectively. The icosahedral shells are obviously the most efficient of the three designs: they use the largest number of subunits to make a container of a given size, and hence they use subunits of the smallest size and the smallest coding requirement. Tetrahedral and cubic symmetries have not appeared in any naturally occurring virus assemblies. Note the distinction between icosahedral symmetry and icosahedral shape. Not all objects with icosahedral symmetry have even the vague outline of an icosahedron; conversely, painting a single asymmetric object, such as a comma, on each face of an icosahedron, rather than three such objects related by the threefold axis through the middle of the face, would destroy the symmetry of the decorated object but would not affect its shape.

The diagram in Figure 3.3B shows the operations that belong to an icosahedrally symmetric object. They are a collection of twofold, threefold, and fivefold rotation axes. Placement of a single, asymmetric object on a surface governed by this symmetry leads to the generation of 59 others, when the various rotations are applied (Fig. 3.3C). One such object, one-sixtieth of the total shell, can therefore be designated as an *icosahedral asymmetric unit*, the fundamental piece of structure from which all the rest can be produced by the operations of icosahedral symmetry.

STRUCTURES OF CLOSED SHELLS

With a typical, compact protein domain of 250 to 300 amino acid residues, close to the upper limit for most single-protein

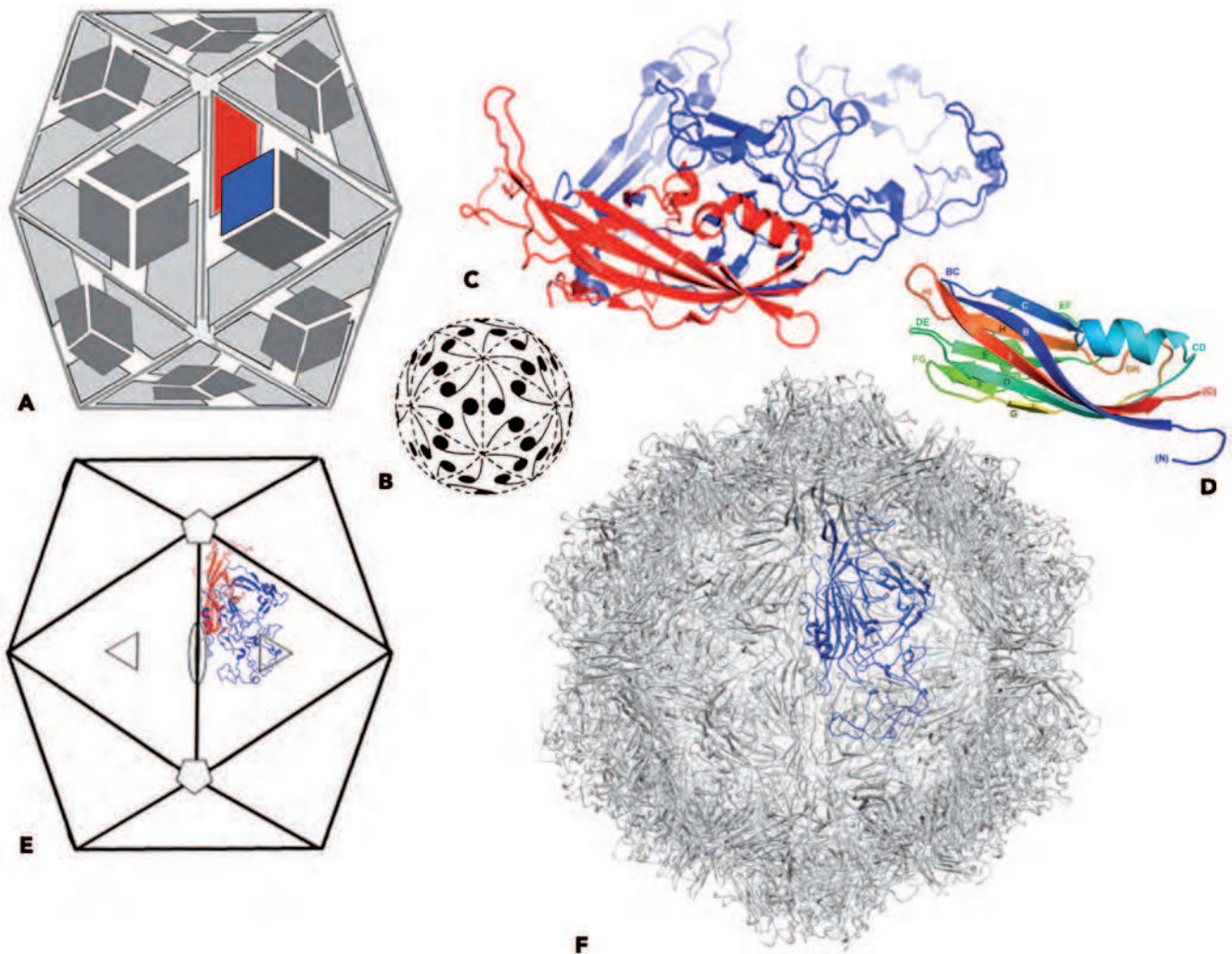


FIGURE 3.5. Canine parvovirus (CPV): a simple, icosahedrally symmetric virion. **A:** Icosahedron, viewed along a twofold axis, with diagrammatic representations of a protein subunit with a core domain (colored red on one of the subunits) and a projecting region (blue). Compare the subunits with the representation of commas in **B**, repeated from Figure 3.3 **C**. **C:** Ribbon diagram of the CPV protein subunit; the core domain (red) is a β -jelly-roll, from which emanate several loops that cluster to form a complex projecting region (blue). The simplified representation of the β -jelly-roll in **D** is in rainbow coloring, from blue at its N-terminus to red at its C-terminus. The eight strands are lettered B–I; the loops have the letters of the strands they connect. The projecting region of the CPV subunit comprises loops BC, EF, and GH. **E:** Icosahedron, as in **A**, but with a ribbon representation of one subunit; symbols for symmetry axes as in Figure 3.3B. **F:** Ribbon representation of all 60 subunits, with the subunit from **E** in blue and all others in gray.

domains, what sort of icosahedrally symmetric container can we construct? Suppose that the protein is so shaped that 60 copies fit together into a 30-Å thick shell with no significant gaps. Then the cavity within that shell will have a radius of about 80 Å, which can contain a 3- to 4-kb piece of single-stranded DNA or RNA, tightly condensed. A few, very simple virus particles indeed conform to this description. The parvoviruses (see Chapter 57) contain a 5.3-kb ssDNA genome, and their shells have 60 copies of a protein of approximately 520 residues (Fig. 3.5). The capsid protein therefore uses up about one-third of the genome. (“Capsid,” from the Latin *capsa*, “box,” designates the protein shell that directly packages DNA or RNA; “nucleocapsid” refers to the shell plus its nucleic acid contents.) Likewise, the satellite of tobacco necrosis virus (STNV) con-

tains 60 copies of a 195-residue subunit and a 1,120-bp single-stranded RNA (ssRNA) genome, of which over half is used for the coat protein.¹⁴¹ As the name implies, however, STNV is actually a defective virus, and it requires tobacco necrosis virus co-infection to propagate.

More complex viruses have evolved ways to make larger, icosahedrally symmetric shells without expending unnecessary genetic resources. The simplest, but least economical, is just to use several different subunits, each of “garden variety” size, to make up one icosahedral asymmetric unit. The picornaviruses (polioviruses, rhinoviruses, etc.) have 60 copies of three distinct proteins, VP1, VP2, and VP3, each between 230 and 300 amino acid residues, as well as 60 copies of a small internal peptide, VP4 (see Fig. 3.6). The shell has a cavity about 95 Å

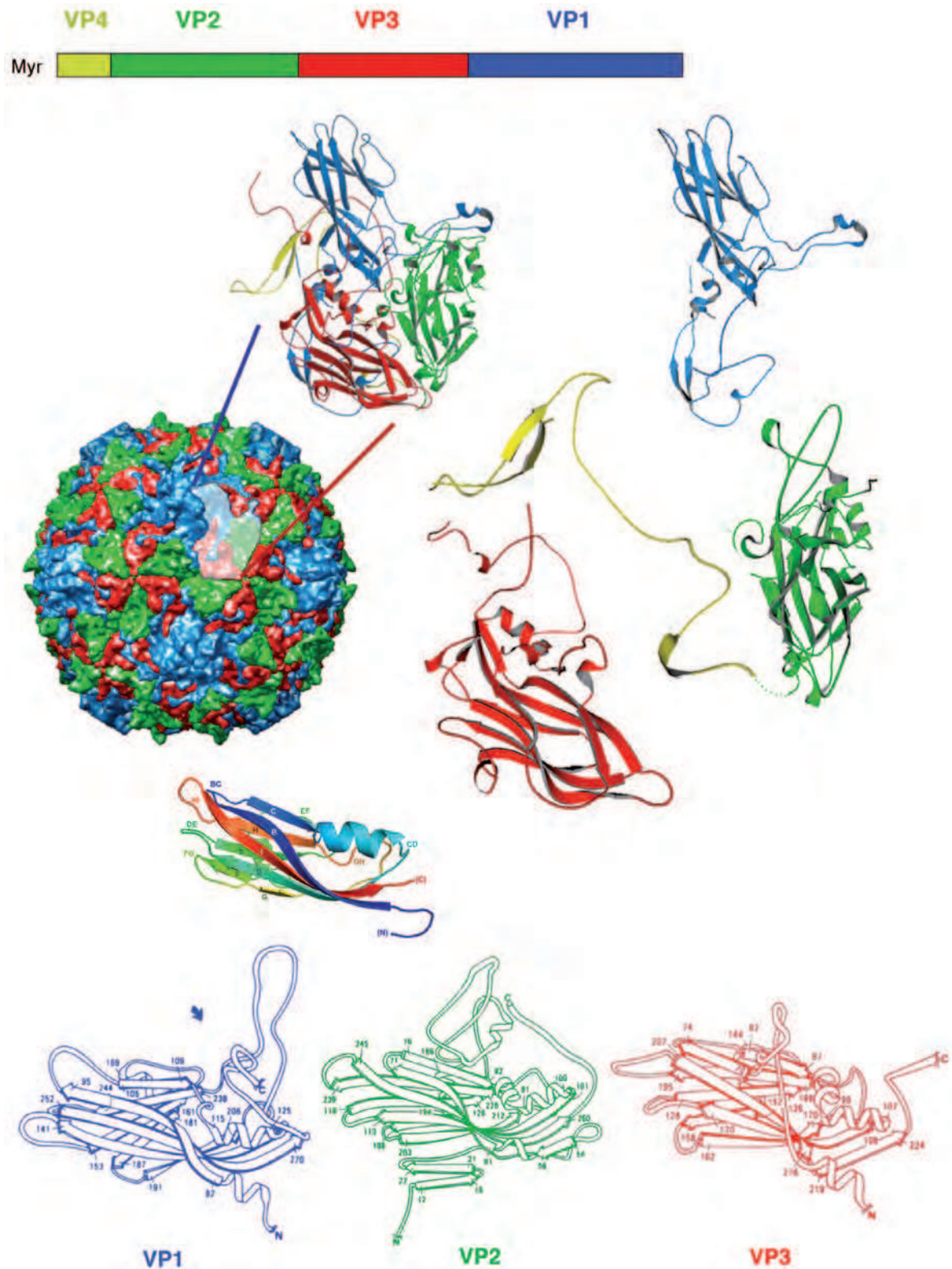


FIGURE 3.6. Poliovirus. **Top:** The order of structural proteins in the polyprotein encoded by the viral RNA. These domains are at the N-terminal end of the polyprotein, which is modified by myristoylation (Myr). The viral protease that cleaves between VP0 (= VP4 + VP2) and VP3 and between VP3 and VP1 is encoded by a region 3' to the region that encodes the structural proteins; the VP4-VP2 cleavage is autolytic and occurs only after assembly of the virion precursor. **Middle:** Surface representation of the virus particle, with colors as in the diagram at the top. Two successively "exploded" views of an icosahedral asymmetric unit (*protomer*) are shown next to the surface rendering. VP1, VP2, and VP3 each have a central β -jelly-roll, with variable interstrand loops and variable N- and C-terminal extensions. The rainbow-colored β -jelly-roll below the surface view is repeated from Figure 3.5D. **Bottom:** Side-by-side views of the β -jelly-roll domains of VP1, VP2, and VP3 to illustrate their congruence.

in radius, which holds an RNA genome of 7.5 to 8 kb. The picornaviruses thus expend about one-third of their genome to encode the structural proteins of the virion. (The term *virion* means *virus particle*, generally implying the mature, infectious structure.) We note here two other important features of picornavirus molecular architecture. First, the folded structures of VP1, VP2, and VP3 all have the same kernel—a domain known as a *jelly-roll β -barrel* (Figs. 3.5 and 3.6). The single subunits of the parvoviruses and of STNV have the same basic fold. It is a module particularly well suited to the formation of closed, spherical shells because of its block-like, trapezoidal outline, but its prevalence among viral subunits may be evidence of a deeper evolutionary relationship. A second noteworthy feature of picornavirus design is that arm-like extensions of the subunits tie together the assembled particle (Fig. 3.6). The importance of scaffold-like intertwining of subunit arms was first discovered in the simple plant viruses.¹⁰⁷ In effect, folding of part of the subunit and assembly of the shell are concerted processes.

Quasiequivalent Icosahedral Arrangements

A more economical way to build shells from more than 60 average-sized, identical subunits was described by Caspar and Klug³⁵ in 1961. It is illustrated by the diagram of 180 commas in Figure 3.7. The commas have similar interactions (head-to-head in pairs; neck-to-neck in rings of three; tail-to-tail in rings of five or six), but they fall into three sets, designated A, B, and C. If the commas are taken to represent proteins, then the conformational differences between A and B positions, for example, involve the differences between rings of five and rings of

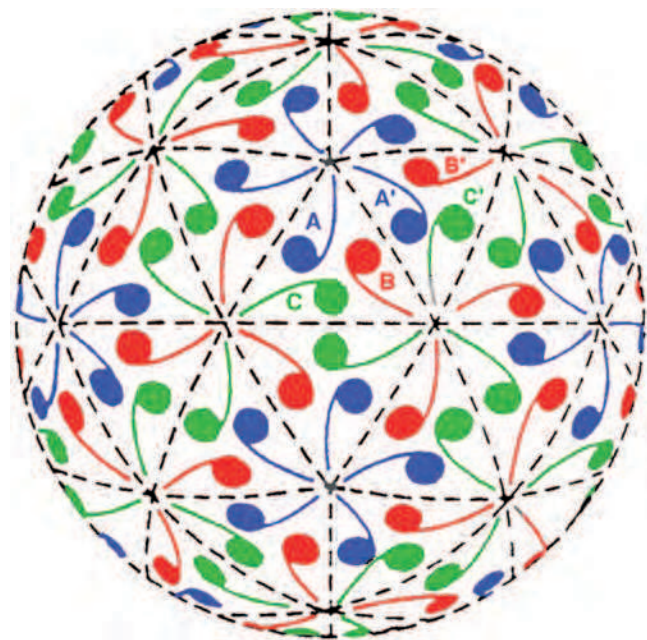


FIGURE 3.7. Quasiequivalent arrangement of 180 commas, in a $T = 3$ icosahedral surface lattice on a sphere. Compare Figure 3.3C, a $T = 1$ arrangement of 60 commas with icosahedral axes oriented similarly. The three quasiequivalent positions within a single icosahedral asymmetric unit are shown in blue, red, and green and labeled A, B, and C, respectively, in two of the asymmetric units.

six, for contacts involving the parts of the proteins symbolized by the tails. Caspar and Klug³⁵ suggested that protein subunits might have the sort of flexibility or capacity for conformational switching needed to accommodate somewhat different packing environments without sacrificing specificity. They postulated that viruses with more than 60 chemically and genetically identical subunits might exhibit the sort of near equivalence seen in the A, B, and C conformers in the comma illustration. They called this sort of local distortability, which might conserve much of the specificity and character of the protein contacts, *quasi-equivalence*.

A number of plant and animal viruses, such as TBSV¹⁰⁷ and Norwalk virus,¹⁸² conform to this description of quasiequivalent arrangements (Fig. 3.8). In TBSV and Norwalk virus, there are 180 genetically and chemically identical subunits in the capsid. The subunits are actually larger than those of the picornaviruses, but most of the extra size comes from a second, projecting domain that serves functions other than the construction of a closed shell. The size of the *shell domain* (S domain) in both cases is just about 200 residues, and the folded structure of the domain is again a jelly-roll β -barrel. The important feature of the packing of these 180 S domains is illustrated by the TBSV diagram in Figure 3.8. The contents of an icosahedral asymmetric unit can be described as three chemically identical subunits, with somewhat different conformations. These conformers are denoted A, B, and C, echoing the designation of commas in Figure 3.7. The differences among the conformers reside principally in an ordered or disordered conformation for part of the N-terminal arm and in the angle of the hinge between the S domain and the projecting, *P domain*. The A and B conformations are nearly identical, with disordered arms and similar hinge angles. The C conformation has an ordered arm and a different hinge angle from A and B. The ordered arms extend along the base of the S domain and intertwine with two others around the icosahedral threefold axis. Thus, the whole collection of 60 C-subunit arms forms a coherent inner scaffold.

How equivalent or nonequivalent are the actual inter-subunit contacts in TBSV and related structures? Most of the interfaces are well conserved, with very modest local distortions that do not significantly change the way individual amino acid side chains contact each other. The interfaces between conformers that do exhibit noteworthy differences are those that include the ordered arms in one of the quasiequivalent locations (the C-conformer). At these interfaces, there is a discrete switch between two states, with ordering and disordering of the arm as the toggle. Nonetheless, many side chain contacts are conserved around the fulcrum that relates an A/B dimer to a C/C dimer (Fig. 3.8).

Only certain multiples of 60 subunits can pack with quasiequivalent contacts; they are given by the formula $T = h^2 + hk + k^2$, where h and k are any integer or zero.¹²⁵ The multiple T is known as the *triangulation number*, because, as illustrated by comparison of the 60- and 180-comma structures in Figures 3.3 and 3.7, they correspond to subtriangulations of an icosahedral net on the surface of a sphere. Such nets are known as *surface lattices*. If we think of an icosahedrally symmetric structure as a folded-up hexagonal net (Fig. 3.9), then 12 uniformly spaced sixfold vertices are transformed into fivefold vertices.

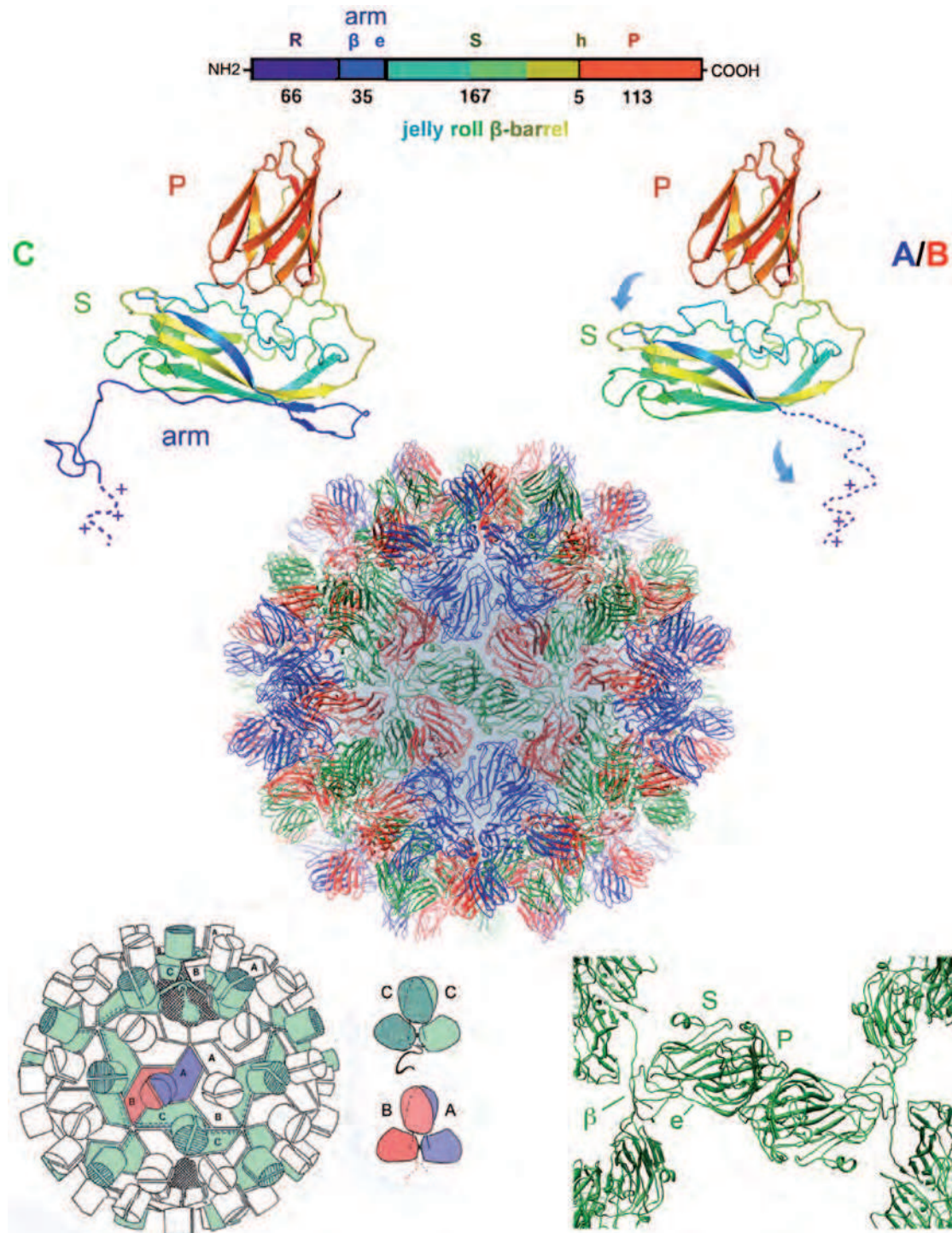


FIGURE 3.8. Tomato bushy stunt virus (TBSV), a T = 3 icosahedral structure. **Top:** Modular organization of the TBSV coat-protein polypeptide chain. R: unstructured, positively charged N-terminal region. β , e: segments of the “arm,” ordered on the C-conformation subunits and unstructured on the A- and B-conformation subunits; when ordered, the β segment forms an interdigitated β -annulus with corresponding segments from two other chains, and the e segment extends along the base of the subunit (see panel at **bottom, left**). S: shell domain, a β -jelly-roll. P: projecting domain, a β -sandwich of somewhat different fold from the jelly-roll S domain. h: hinge between the S and P domains. The color coding in the bar representation of the chain is repeated in the ribbon diagrams of the C (**left**) and A/B (**right**) conformations. Note that the two conformations differ in two respects: the ordering of the arm and the hinge angle between S and P domains (curved arrows on the **right-hand ribbon diagram**). **Center:** Ribbon representation of the entire protein coat of the virus; the colors of the A-, B-, and C-conformation subunits are as in Figure 3.7. **Bottom left:** Schematic figure, showing that the arms of the C-subunits (green) interdigitate around threefold axes of the icosahedral symmetry, forming a coherent inner framework. **Bottom right:** Magnified view of some of the C-subunits from the coat seen in the central part of the figure, illustrating the β -annulus (β) and the extended part of the arm (e). In the **bottom center** are schematic views of the C-C and A-B dimers, showing how the hinge between S and P domains correlates with the ordering of the arms (inserted into the slot between S domains, which have rotated away from the contact that they have when the arms are unfolded into the particle interior).

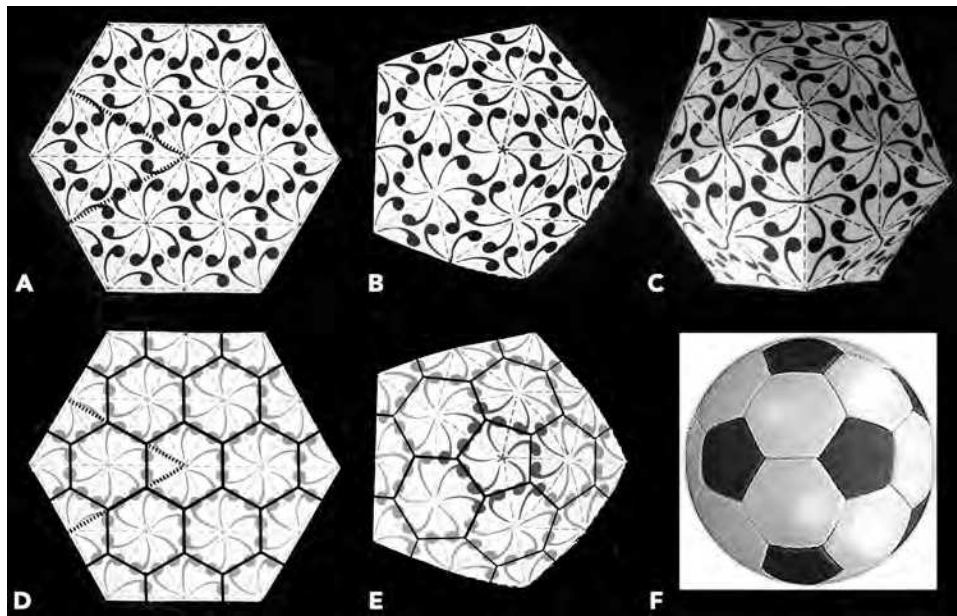


FIGURE 3.9. Generation of curved structures from planar lattices. **A:** Portion of a hexagonal lattice. Six triangular cells of the lattice meet at each lattice point, and each triangular cell contains three “subunits” (commas). Thus, there is a sixfold symmetry axis at each lattice point, a threefold symmetry axis at the center of each triangle, and a twofold axis at the midpoint of each edge. Imagine that the lattice extends indefinitely in all directions. **B:** Curvature can be introduced by transforming one of the sixfold positions into a fivefold (**center**). A 60-degree “pie slice” has been removed from the object in **A** by cutting along the heavy dotted lines, and the cut edges have been joined to generate the curved lattice shown here. **C:** If further cuts are made at regular intervals in an extended lattice, such as the one in **A**, and the edges joined as in **B**, a closed solid can be produced. In the case of the icosahedral solid shown here, vertices of the lattice separated by two cell edges have been transformed into fivefolds, while the intervening lattice points have been left as local sixfolds, producing a $T = 4$ ($h = 2, k = 0$) structure. Notice that the local sixfolds are actually only approximately sixfold in character; they correspond strictly to the twofold axes of the icosahedral object. **D:** Lines joining the centers of the triangular cells in **A** create a pattern of hexagons. **E:** When a sixfold is transformed into a fivefold, a hexagon becomes a pentagon. **F:** If second nearest-neighbor lattice points are all transformed into pentagons, a soccer-ball figure results. This is a $T = 3$ structure. A description of the lattice as a network of hexagons and pentagons is complementary to its description as a network of triangles. The representations in Figures 3.3, 3.5, 3.13, and 3.16 (**left**) use triangles. The representation in Figure 3.16 (**right**) uses hexagons and pentagons. One representation for a given lattice can easily be derived from the other.

Nonequivalent Icosahedral Surface Packings

Hexagonal packing is an efficient way to tile a surface (think of hexagonal floor tiles), even if the building blocks themselves do not have sixfold symmetry and hence do not interact identically with their neighbors. In many larger, icosahedrally symmetric virus particles, the outer-shell building blocks are centered at the vertices of an icosahedral surface lattice, subtriangulated as anticipated by Caspar and Klug, but the oligomeric building blocks themselves are not hexamers. In some cases, for example, adenoviruses (Fig. 3.10), they are trimers, with a chemically distinct, pentameric building block on the fivefold vertices; in other cases, for example, the polyoma- and papillomaviruses, the building blocks are all identical pentamers (Fig. 3.1). Viewed at low resolution (e.g., by negative-stain electron microscopy), all of these viruses have globular “lumps” at the vertices of a lattice with one of the allowed triangulation numbers ($T = 25$ for the adenoviruses: Fig. 3.10; $T = 7$ for the polyoma- and papillomaviruses: Fig. 3.1), but when seen at higher resolution, the six-coordinated lumps are actually trimers or pentamers, and in the former case, the five-coordinated lumps are pentamers of a related but distinct polypeptide chain.

Special mechanisms (either involving other structural proteins or flexible intersubunit connections) are needed to hold the particle together because a single set of repeating, quasiequivalent intersubunit contacts is not possible. Before the molecular principles of virus structure were fully understood, the globular lumps seen by low-resolution electron microscopy were called *capsomeres*, meaning the *structural units* of the *capsid*. This word is still used when referring to apparent morphologic units on the surface of a virus shell, but it is best reserved for cases where all capsomeres are the same and hence represent a defined oligomer, as in the pentameric units of papovaviruses (see later).

The flaviviruses and picobirnaviruses illustrate yet another adaptation to icosahedral packing. As illustrated in Figure 3.7, the asymmetric unit of an icosahedral surface lattice can be represented by a (spherical) triangle with a fivefold axis and two adjacent threefold axes as its vertices. The flavivirus envelope protein (E) is a flat, elongated dimer; three such dimers neatly fill a twofold-related pair of asymmetric-unit triangles, with the dyad of the central dimer coincident with the icosahedral twofold (Fig. 3.11).¹²⁸ The shell contains 180 subunits, but not in a $T = 3$ arrangement. The picobirnavirus coat protein

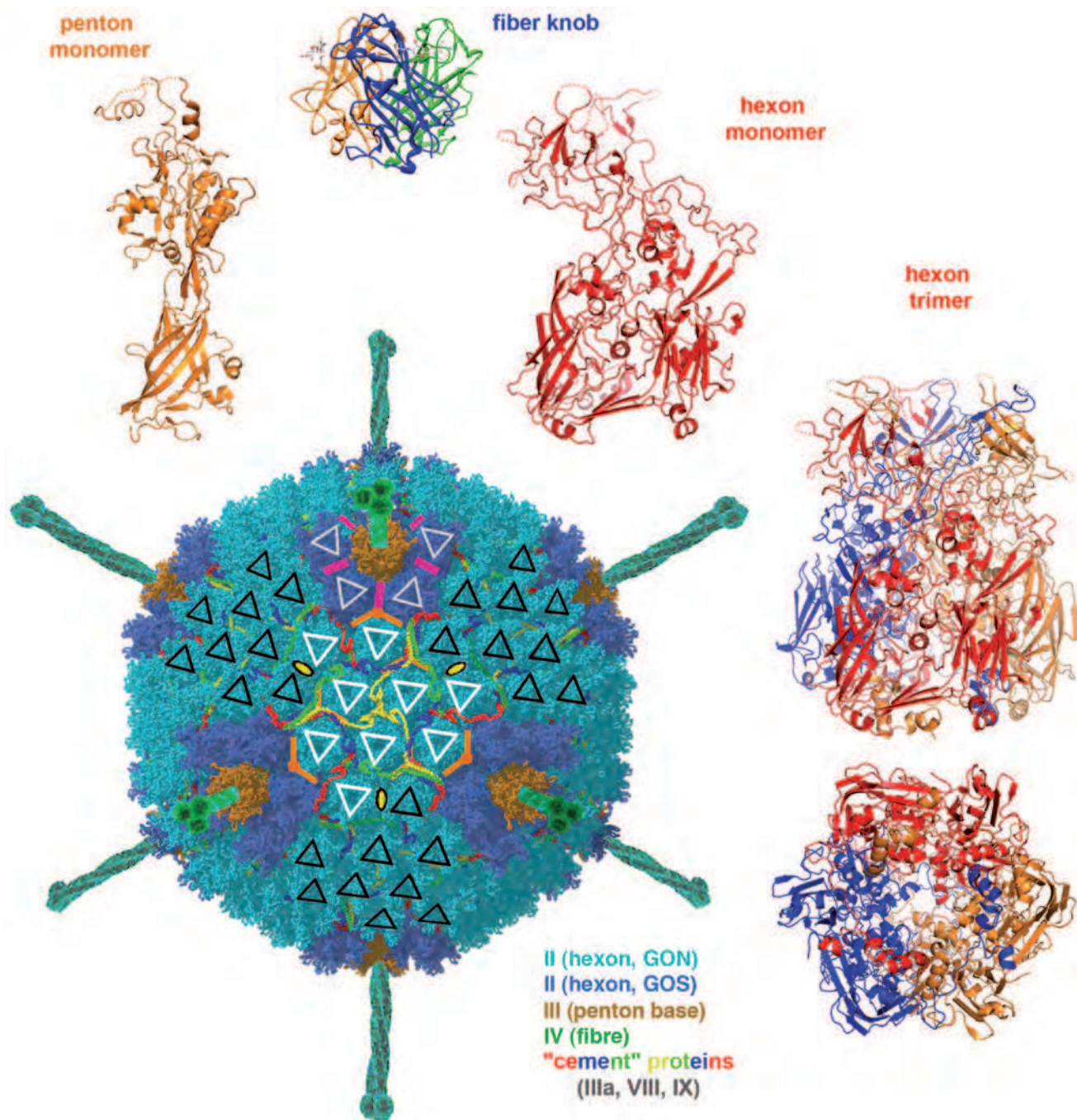


FIGURE 3.10. Adenovirus structure. A representation of the complete particle, based on a high-resolution electron cryomicroscopy (cryoEM) image reconstruction,¹⁴² is at the **lower left**, surrounded by ribbon representations of a number of the component proteins. The view of the particle is along a threefold symmetry axis. The hexons (light and medium blue) and the pentons (brown) lie on vertices of a $T = 25$ icosahedral lattice, but the hexons are actually trimers with a pseudohexameric character, as illustrated by the “bottom view” (as if from the particle interior) at the **lower right**. Three species of so-called cement proteins (IIIa, VIII, and IX) retain the hexons and pentons in the shell and determine its fixed geometry. One of them (various chains in red, dark blue, yellow, and light green) fits into the crevices between the hexons and organizes them into *groups of nine* (GON)—as shown by the sets of white and black triangles on the hexon surfaces. The other two are on the inner surface of the hexon–penton shell and cement five “peripentonal” hexons and the penton base into a *group of six* (GOS); locations of some of them are shown here simply as magenta and orange lines, because they are not visible from the outside of the particle. The trimeric fibers project from each penton base, with a receptor-binding knob (**top of figure**) at their tip. Each hexon monomer (see red ribbon diagram, **upper right**) has two jelly-roll β -barrels, in parallel orientation, imparting a pseudohexagonal character to the trimer. The penton base (**upper left**) has a single β -jelly roll. (Image reconstruction courtesy Z. H. Zhou; see also Harrison¹⁰³).

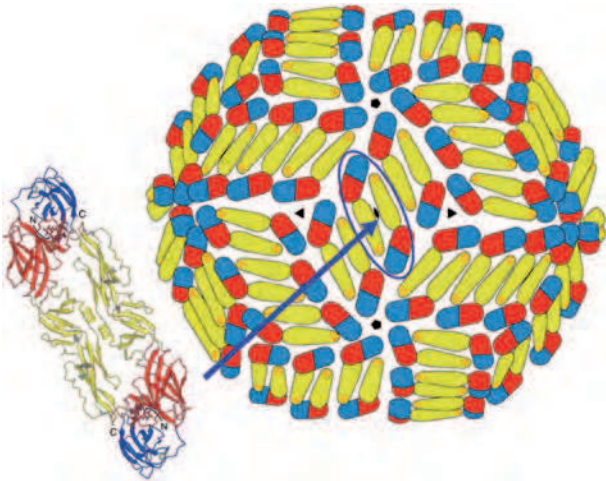


FIGURE 3.11. Organization of a flavivirus particle. Ninety dimers of the E protein tile the surface as shown. E is an elongated, three-domain protein (**lower left**), oriented with its long axis parallel to the surface of the virion. At the tip of domain II (yellow) is a hydrophobic *fusion loop* (orange, shown also as an asterisk on the larger schematic).

is so shaped that two dimers can fill a similar (smaller) rhombic unit; the icosahedral twofold lies between the two dimers, and the complete coat contains 120 subunits.⁶⁶ Recombinant brome mosaic virus coat-protein dimer, expressed in yeast cells, packs in a closely related way when it assembles into 120-subunit virus-like particles.¹²⁶

The arrangement of 120 copies of the inner- (core-) shell protein in double-stranded RNA (dsRNA) viruses is a particularly striking example of nonequivalent packing (Fig. 3.12). There are two completely distinct environments for this protein (designated A and B in Fig. 3.12, center): two is not a permitted triangulation number, and quasiequivalent packing of 120 proteins in an icosahedral array is not possible. The amino acid side chains on the lateral surface of the core-shell protein have different partners, depending on the interface in which they lie. The distortion of the subunit itself, when the two environments are compared, is quite small.

Frameworks and Scaffolds

The protein subunits of TBSV or picornaviruses have extended N- or C-terminal arms augmenting a central jelly-roll β -barrel. These arms are essential for building a stable coat. They form an internal framework, such as the one illustrated for TBSV in Figure 3.9. In TBSV, the *assembly unit*—the oligomer of the coat subunit that forms spontaneously in solution (and by inference, in the cell following its synthesis)—is a dimer, which can have two conformations: an “A/B” dimer, with disordered N-terminal arms, and a “C/C” dimer, with folded arms.¹⁰⁵ The local curvature of those two conformations is different, and the framework of C/C arms fixes the overall diameter of the particle. Removal of the N-terminal arms of TBSV-like subunits leads to self-assembly of a small, 60-subunit icosahedrally symmetric particle that cannot package RNA.⁸⁸ That is, without the arms, there is no mechanism for a conformational switch.

In the papilloma- and polyomaviruses, N- and C-terminal extensions (principally the latter) of the subunit globular

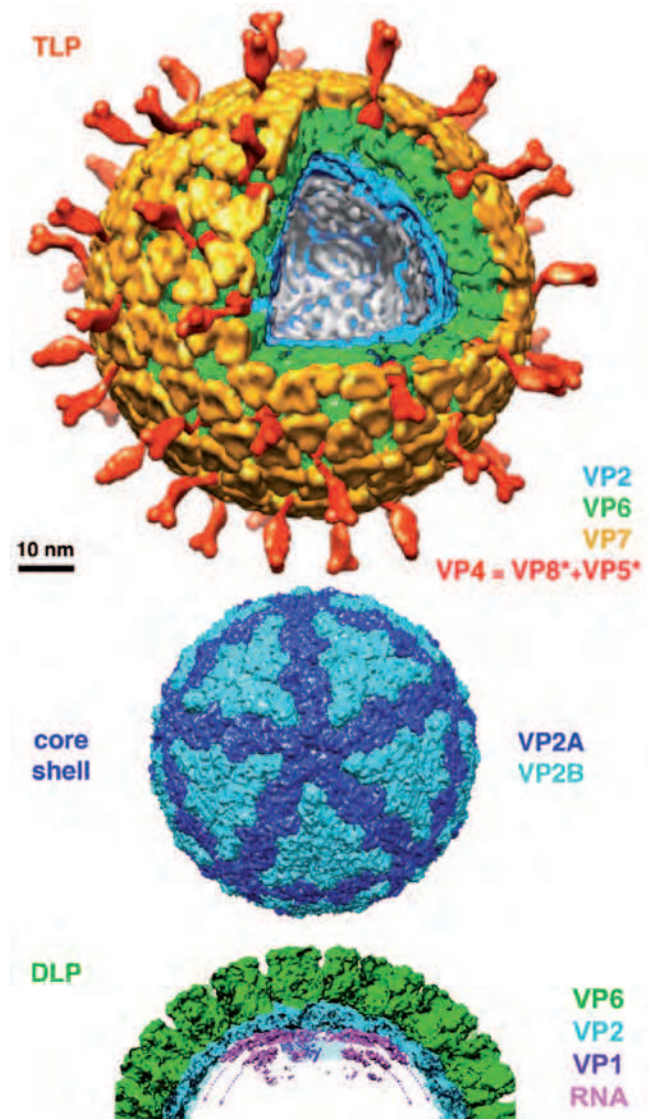


FIGURE 3.12. Molecular organization of a rotavirus particle, illustrating the multiple concentric protein shells.^{42,202,259} The complete virion (**top**) or *triple-layered particle* (TLP) has an outer layer composed of VP7 (yellow) and VP4 (red: cleaved during maturation into two parts, VP8* and VP5*, which remain associated). The *double-layered particle* or DLP (**bottom**) has a core shell (**center**) with 120 VP2 subunits (blue) surrounded by a layer of 290 VP6 trimers (green) in a $T = 13$ icosahedral lattice. The VP6 layer in turn dictates the organization of the VP7 layer, which clamps into place 60 VP4 trimers projecting from a particular set of six-coordinated positions. The locations of the VP1 polymerase (purple, ribbon representation)⁷² and of tightly wound, double-stranded RNA (dsRNA) (magenta)¹⁵¹ are also shown in the bottom cutaway. The icosahedrally symmetric core shell has 120 VP2 subunits in two sets (designated A and B, dark blue and light blue, respectively), with completely nonequivalent contacts and only slightly different conformations. This type of shell is characteristic of many groups of dsRNA viruses.

domains tie together the pentameric building blocks, which have almost no contacts except through these extensions (Fig. 3.13).^{139,246} Flexibility of the arms allows formation of the different kinds of contacts required to surround a pentamer with six other pentamers (i.e., to position a pentamer at the

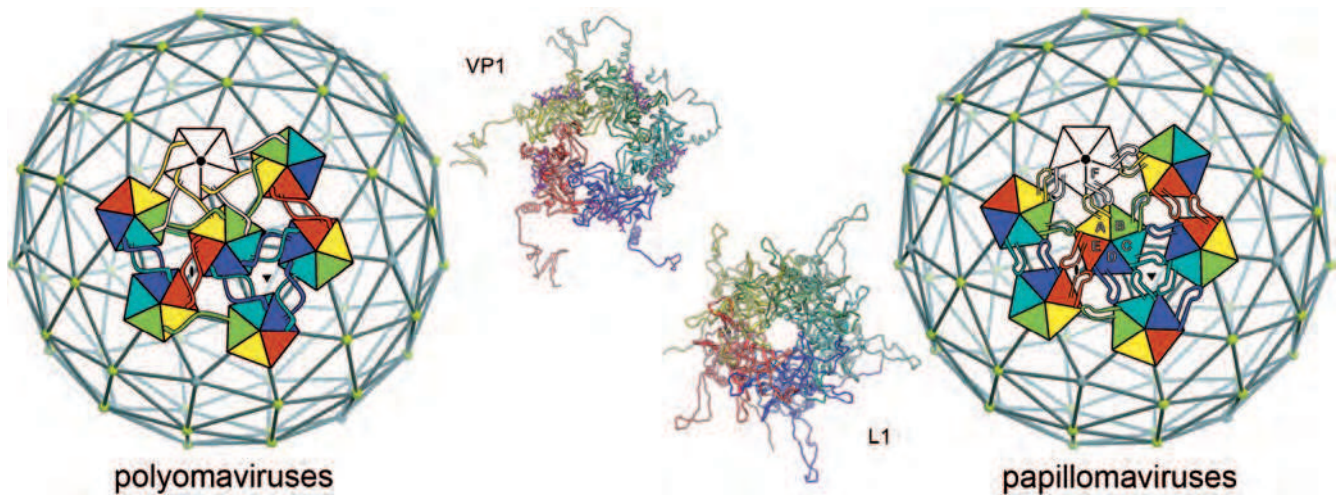


FIGURE 3.13. Packing of pentamers in the capsids of polyoma- and papillomaviruses. The ribbon diagrams in the center show pentamers of VP1 (polyomaviruses) and L1 (papillomaviruses), viewed from their outward-facing surfaces. Note the C-terminal arms of the subunits, which extend away from the pentamers in VP1 but loop back to it in L1. The schematic diagrams to the left and right illustrate the packing of these pentamers in the virion shell. The framework shows a $T = 7$ icosahedral lattice; VP1 or L1 pentamers are centered on both six- and five-coordinated positions.

six- as well as at the five-coordinated vertices of a $T = 7$ subtriangulated icosahedral lattice). The C-terminal arms emanate from one pentamer and dock into another. The way they dock is the same for all 360 arms, with identical interactions locking them in place; their configurations differ, however, between the point at which they emerge from the globular domain of their subunit of origin and the point at which they dock into their target subunit.

Larger and more complex structures, such as adenoviruses, have separate framework proteins. The principal outer-shell components of adenoviruses are *hexons* (trimers of a subunit with two similar jelly-roll β -barrel domains) and *pentons* (pentamers of a subunit with a single jelly-roll β -barrel domain); a set of additional proteins cement the structure together and determine its size (Fig. 3.10).^{80,214,215} The elaborate interaction patterns of these *cement* proteins stabilize a *group of nine* hexons, centered on the icosahedral threefold axis, and a *group of six* (five hexons and a penton), centered on the icosahedral fivefold axis.^{134,142} The structure of an adenovirus-like bacteriophage, PRD1,¹⁶ shows a somewhat simpler size-determining and stabilizing framework: a *tape-measure protein* extends from the penton toward the icosahedral twofold axis, where it interacts with an identical protein running toward it from the twofold-related penton (Fig. 3.14).² Unlike adenoviruses, PRD1 has a lipid-bilayer membrane between the P3 layer and the internally coiled DNA.^{11,49}

During assembly of the heads of most double-stranded DNA (dsDNA) bacteriophages, an internal scaffold protein directs formation of a *prohead*.³³ Signals related to initiation of DNA packaging trigger release and recycling (P22) or degradation (T4) of the scaffold, accompanied by a reorganization and expansion of the head (Fig. 3.15A,B).^{68,119} DNA is pumped into the empty head until it reaches a tightly coiled state, as illustrated in Figure 3.15C.^{69,70,211} In these examples, *scaffold* is a good description of the internal protein, because it is removed once the structure is complete.

The fundamental principle embodied in all the various structures just described is one of mass production. One or more standard building blocks assemble into the larger structure. In simple ($T = 1$) cases, such as the parvoviruses and picornaviruses, a repeating set of identical interactions determines the final structure. Even in many of these cases, however, extended arms form an interconnecting framework. In more elaborate cases, framework elements, either permanent or transient, ensure a unique outcome.

Elongated Shells

The examples in Figure 3.16 illustrate elongated particles with caps at either end. In many of the dsDNA bacteriophages, the shell looks like a familiar icosahedral design at the poles. As the lattice approaches the equator, however, the regular interspersion of fivefolds and local sixfolds gives way to local sixfolds only, so that there is a tubular region around the middle of the particle (Fig. 3.16A–C).²²⁴ The tubular region can be of varying extent; in extreme cases, it can be much longer than the caps themselves. A further variation on this theme is found in the shells formed by the CA fragment of the lentivirus Gag protein. Conical structures seen within HIV-1 particles have been shown to be based on the sort of arrangement shown in Figure 3.16D, where one cap has more than six fivefolds and the other has less, so that the diameters of the two caps are different.⁸³ (Note that if there are only sixfold and fivefold vertices in a closed surface lattice, there will always be exactly 12 of the latter.)

Multishelled Particles

Most dsRNA viruses have a genuinely multishelled icosahedral organization, with some common features and some variation from group to group (see Chapters 44–46). In virions of the mammalian dsRNA virus groups (reoviruses, rotaviruses, and orbiviruses), the innermost protein shell contains 120 copies of

FIGURE 3.14. Bacteriophage PRD1. **Left:** Side and bottom views of the *hexon* protein, P3. The colors correspond to those in the ribbon diagrams of the adenovirus hexon trimer in Figure 3.10. Like the adenovirus hexon, P3 has two jelly-roll β -barrels, but the loops that project outward are much less elaborate.¹⁶ (The variable adenovirus hexon loops probably evolved as a means of immune evasion, not relevant for a bacteriophage.) The image on the **upper right**, based on a crystal structure of the intact phage particle,² is a view along a twofold axis. One three-fold set of P3 trimers is highlighted by triangles. The pentons (P31) are in red. At the **lower right** is a view with the outer layer stripped away, to show the extended *tape-measure* protein, P30, which helps determine the size of the shell, and the lipid bilayer just beneath it. There are 60 copies of P30; each chain extends from a twofold axis (N-terminal end, blue) to the inner surface of a penton (C-terminal end, red). At the twofold axis, one P30 associates with a second, twofold-related P30, which projects toward the opposite icosahedral vertex. (Courtesy D. Stuart, Oxford University.)

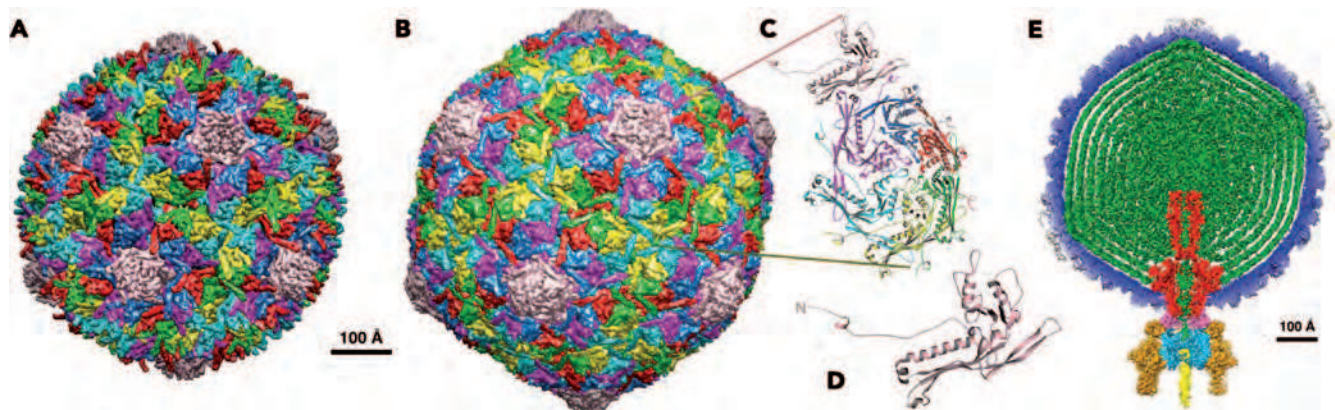
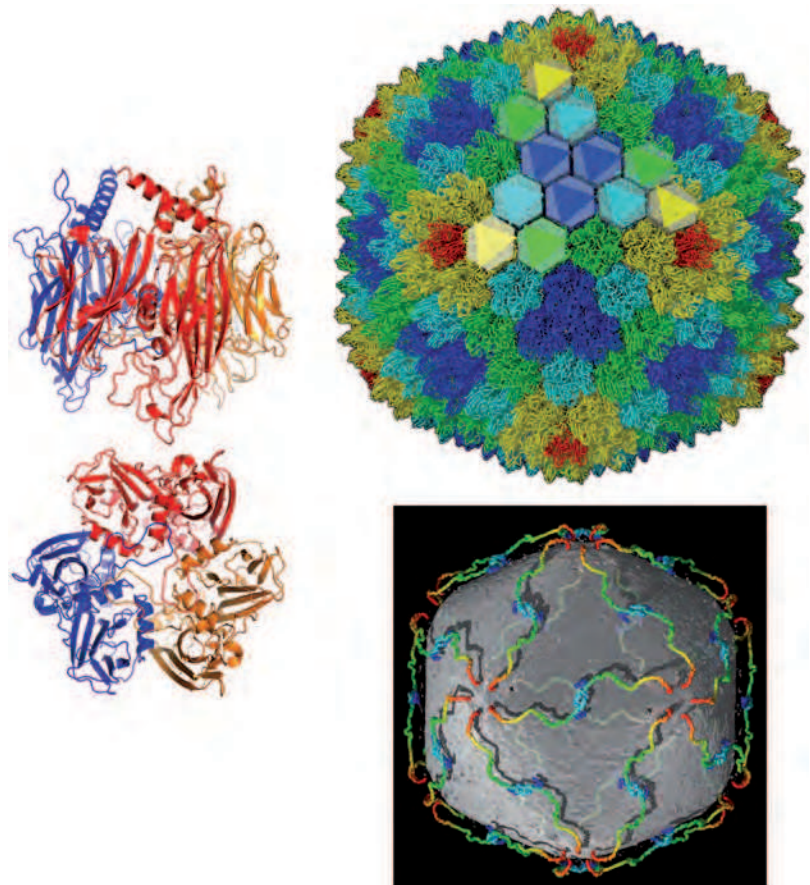


FIGURE 3.15. Capsid reorganization and DNA packaging in tailed bacteriophages.¹¹⁹ **A:** Surface of the HK97 procapsid. The surface organization is a locally distorted $T = 7$ arrangement, with fivefold symmetric association of the subunit at the fivefold positions (beige) but a skewed arrangement in the rings of six subunits that surround a local six-coordinated position (colored in magenta, blue, red, green, yellow, and cyan, in clockwise order).⁵³ An N-terminal extension of the head subunit is the scaffold for prohead assembly; its cleavage by a co-assembled protease triggers rearrangement of the subunits into the expanded, thinner, more angular shell illustrated in **B**.⁶⁵ **B:** Capsid (head) of the mature HK97 particle; molecular surface, based on crystallographic model, colored as in **A**.²⁴² This view is oriented so that a fivefold axis is vertical. The image is derived from the structure of an empty capsid with 420 subunits in a $T = 7$ icosahedral lattice. In a wild-type bacteriophage particle, one of the rings of five subunits is replaced with a portal protein connected to a tail (see **E**). **C:** Expanded view in ribbon representation of one icosahedral asymmetric unit (i.e., one of the five subunits in the pentameric ring and one each of the quasisymmetric subunits in the hexameric ring). All subunits are chemically identical. In HK97, but not in many related bacteriophages, an intersubunit isopeptide bond, which forms during maturation, crosslinks the entire coat.⁶⁵ **D:** A further enlarged view of a single, 31-kD subunit. The 105-residue N-terminal extension that functions as an assembly scaffold is indicated schematically by a dotted line. **E:** Cutaway representation of a three-dimensional electron cryomicroscopy (cryoEM) image reconstruction of bacteriophage P22. Its assembly is formally similar to that of HK97, but there is a distinct, recycled scaffold protein³³ and no covalent crosslinking of the head.¹⁷³ The packaged DNA (green) winds tightly around an internal extension of the portal protein (red).^{169,222} The axis of DNA winding is vertical in this view; averaging of many particles in the reconstruction produces concentric shells of density, because the exact register of the DNA coils varies from particle to particle. (Images in **A–D** from Vlrus Particle Explorer [VIPERdb] Web Site, <http://viperd.b.scripps.edu/>.)

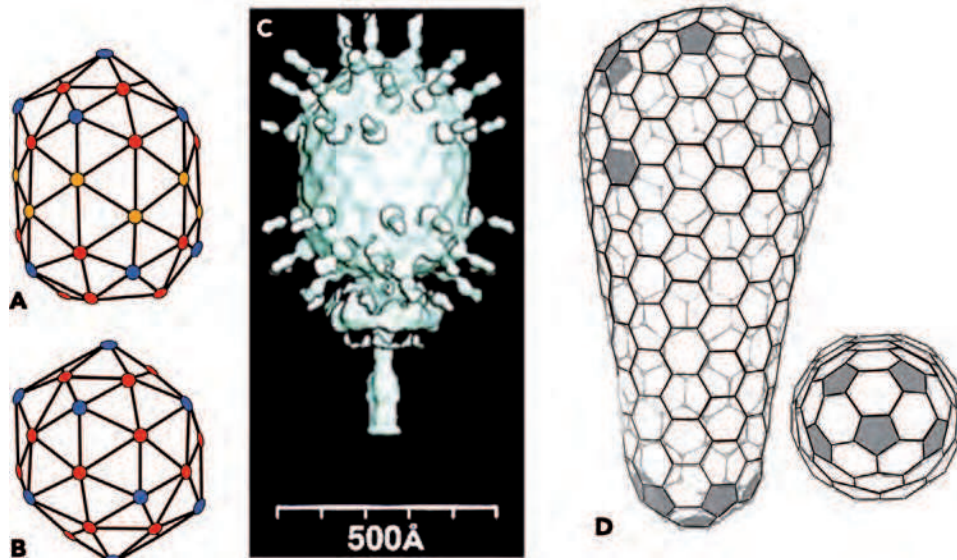


FIGURE 3.16. Elongated shells. **A–C:** Bacteriophage $\phi 29$.²²⁴ The surface lattice of the $\phi 29$ capsid (**A**) has the equivalent of a $T = 3$ icosahedral cap (**B**) at either pole with an equatorial insertion of two rows of six-coordinated positions (i.e., six, locally sixfold-related, coat-protein subunits). The blue dots are at five-coordinated positions (five, locally fivefold-related, coat-protein subunits); the red dots are at the six-coordinated positions of a $T = 3$ lattice in the cap; the orange dots are at the inserted six-coordinated positions. The cap at the “south pole” is further modified by replacement of the axial pentameric cluster of coat subunits with the collar and tail structure, as shown in the surface view in **C**. **D:** The conical structure of the mature capsid of HIV-1.⁸³ The capsid subunit, CA, cleaved from the Gag precursor, forms a structure with two unequal caps, one with seven five-coordinated positions and one with five. In the former, the five-coordinated positions have more intervening six-coordinated lattice points than in the latter, so that the radius of the one is larger than the radius of the other. The shaft of six-coordinated positions is wrapped in such a way that a circumference includes increasing numbers of subunits as one traces from the “bottom” to the “top” of the conical capsid, as illustrated here. The two caps have a five-coordinated lattice point at the apex, but immediately deviate from an icosahedral arrangement, as shown in the end-on view of the lower cap (**bottom left**).

a large, rather plate-like protein^{96,151,186} (Fig. 3.12). Surrounding the inner shell is a second characteristic layer. In most cases, it contains 780 copies of a trimeric protein with a radially directed jelly-roll β -barrel and inwardly directed N- and C-termini, which together form an extensive and largely α -helical “base” domain.^{95,140,149} This second layer corresponds closely to a “classical,” quasiequivalently packed, $T = 13$ icosahedral shell—all the interactions between adjacent trimers are variations on the same set of contacts.

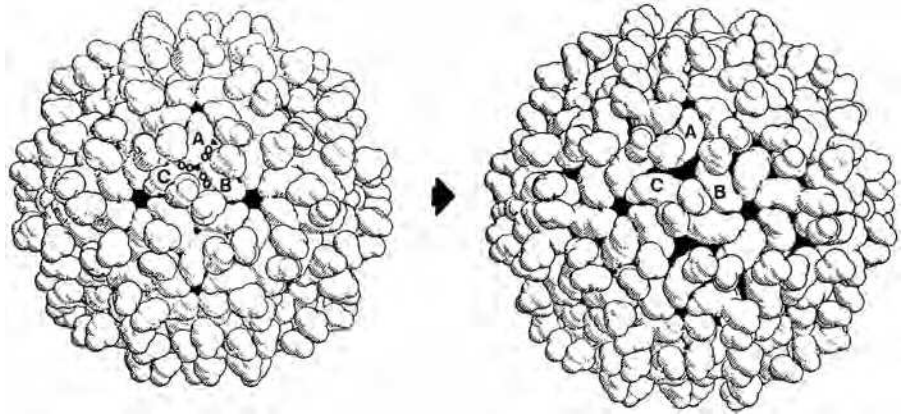
Various elaborations and simplifications of the two-layer design just described differentiate the families of dsRNA viruses. For example, in the reoviruses, the $T = 13$ layer has gaps, through which pentameric “turrets” of yet another protein, anchored on the inner shell, project; only 600 of the potential 780 subunits are actually present.^{27,64,186} The birnaviruses lack the 120-subunit layer altogether and have instead 780 copies of a single major capsid protein, with a shell domain that resembles those of plant and insect viruses and a trimer-clustered projecting domain that resembles the jelly-roll β -barrel in the $T = 13$ shell of rotaviruses and orbiviruses.⁵⁴ The $T = 13$ packing of the shell domain so closely recalls that of its counterparts in $T = 3$ and $T = 4$ positive-strand RNA virus structures that a bridge between the two families seems plausible. Similarities in the RNA-dependent RNA polymerases of these viruses also suggest some common ancestry. The dsRNA bacteriophages such as $\phi 6$ contain the 120-subunit, inner-shell

layer and a fenestrated, $T = 13$ layer (rather like reoviruses), contained within a lipid-bilayer membrane.^{27,112,114,236}

Rearrangements in Surface Lattices

Icosahedral surface lattices can undergo rearrangements, which preserve the overall symmetry of the structure but change the pattern of specific intersubunit contacts. There can be an accompanying change in the diameter of the shell. These rearrangements are cooperative—that is, they occur more or less simultaneously across the whole structure. As illustrated in Figure 3.14, when dsDNA bacteriophages such as P22 insert their genomic DNA into a preformed prohead, the outer shell of the prohead expands as its subunits shift around to form the mature structure.^{33,53,117,133} Another well-characterized example is expansion of the $T = 3$ plant viruses, which occurs when the calcium ions that stabilize a particular set of subunit interfaces are removed¹⁹⁰ (Fig. 3.17). This swelling is believed to be the first step in disassembly; plant viruses are injected by their vectors directly into the cytoplasm of the recipient cell, where they are exposed to a low Ca^{2+} environment. A similar, but transient, expansion occurs when poliovirus binds its receptor.¹⁵ In both the $T = 3$ plant viruses and the picornaviruses, internally directed “arms” of the protein subunits move outward from the interior as expansion creates gaps in the shell. Exposure of the arms may be part of the uncoating process in the case of the plant viruses or of the penetration

FIGURE 3.17. Expansion of tomato bushy stunt virus (TBSV).¹⁹⁰ The mature, compact particle (**upper left**) expands when Ca^{2+} ions (small circles) are removed. The expanded form (**upper right**) is reached by a smooth transition, in which many of the intersubunit contacts are conserved. The contacts that included the ions in the compact state have separated substantially, creating a fenestrated shell.



process in the case of the picornaviruses. Cooperativity of these rearrangements implies that a few points of inhibition can prevent the change. For example, only a few intersubunit crosslinks from bound neutralizing antibodies are sufficient to block infection by a picornavirus particle.⁷¹ The same may be true of small molecules that inhibit the subunit conformational changes needed for the receptor-triggered expansion of picornaviruses.^{8,177,212}

Helical surface lattices can also rearrange without dissociating. Contraction of bacteriophage tail sheaths is a good example.

Two Recurring Globular Domains in Icosahedral Capsid Proteins

The icosahedrally symmetric shells of nearly all well-characterized, nonenveloped viruses contain one of two types of globular domain. (The known exceptions at the time of writing this chapter are the RNA bacteriophages—R17, Q β , and their relatives²²⁹—and the dsRNA picobirnaviruses.⁶⁶) One is the jelly-roll β -barrel in viruses of animals and plants, which we have described in various examples of viruses of animals and plants; it is also the principal component of icosahedral ssDNA bacteriophage capsids (e.g., ϕ X174).¹⁵² The various ways this module can form a coat are quite different, of course, and we have emphasized earlier the importance of framework components (either as extensions of the polypeptide chain of the β -barrel or as separate protein species) in directing or regulating coat assembly. What sort of evolutionary parsimony resulted in such widespread appearance of a single kind of protein module is not evident. Viruses can jump from plants to insects and from insects to vertebrates, so the recurrence of the jelly-roll β -barrel is unlikely to reflect a common origin for all these viruses that antedates host divergence, but rather the result of more recent selection and genetic exchange. Cellular fusion proteins acquired from viral fusion proteins through retrotransposons illustrate one way in which such exchange can occur.

Figure 3.14 shows the second basic building block, discovered initially in the coat of dsDNA bacteriophages such as HK97 and subsequently found in most other dsDNA bacteriophages (T4, lambda, P22, etc.). This *HK97 fold* is also the core of the herpesvirus capsid subunit.⁹ Like their bacteriophage cousins, herpesviruses pump their genome DNA into a preformed shell through a specialized icosahedral

vertex and a dodecameric *portal protein*.^{39,163,164} Adenoviruses, and probably their bacteriophage cousins like PRD1, with hexon-like capsid subunits, are also thought to insert DNA into a preassembled empty capsid, but the motors that effect the insertion seem to be different from those in the herpesviruses.^{113,170,256,257} Thus, the structures of the coat proteins of two major classes of dsDNA viruses appear to correlate with the machinery by which members of each of these classes package DNA.

SELF-ASSEMBLY AND CLEAVAGE STEPS

Some of the simplest virus particles can assemble spontaneously from their dissociated or recombinant components, in the absence of any further modifications or scaffolds. These particles are said to *self-assemble*, because they do not require additional activities (encoded either by the virus or by the host cell) to form. In an infected cell, however, host chaperones, such as Hsc70 and its paralogs, may enhance efficiency of subunit folding or subunit assembly, even when they are not absolutely essential.

Most viruses, and nearly all viruses that infect animal cells, cannot reassemble from dissociated particles, because one or more irreversible steps intervene in forming the mature, infectious virion. The picornaviruses, already described, illustrate one kind of irreversible step. In an infected cell, the principal structural proteins are cleaved from a polyprotein precursor (by a viral protease) before particle assembly, but one final, autocatalytic cleavage step occurs after assembly—the scission of a peptide bond between VP4 and VP2 (see Chapter 16 and caption to Fig. 3.7). The cleavage depends on the three-dimensional arrangement of the scissile bond, as found in a newly assembled precursor particle. Rearrangements of parts of the subunits following the cleavage stabilize the now mature, infectious virion. Proteolytic cleavages by cellular or extracellular host proteases are critical steps in the maturation of many types of virus particles, even when processing of a precursor polyprotein is not involved. For example, many of the surface glycoproteins that facilitate membrane fusion during entry of enveloped viruses require activation by a furin-like protease late in the secretory pathway.

Specific, postassembly proteolytic cleavage usually has two consequences. First, as in poliovirus or many viral fusion

proteins (see later), it leads to a local rearrangement of polypeptide chains that stabilizes the structure. Second, it allows the structure to undergo a much larger reorganization when “triggered” by binding of a specific ligand. Thus, when a mature poliovirus particle binds its receptor, an expansion occurs that allows VP4 to escape and to interact with adjacent membrane—a critical first step for translocating the particle (or its genome) from an endocytic compartment into the cytosol.^{21,100,157} Likewise, many fusion proteins of enveloped viruses undergo large-scale, fusion-promoting conformational changes when they bind protons in acidic endosomes—but again, only if the critical cleavage has occurred.²⁰⁹ In physicochemical terms, the cleaved structure is *metastable*: a large kinetic barrier separates it from its true energy minimum. The barrier can be so large that the virus remains infectious for many weeks or months. Ligand binding (receptors, protons, etc.) lowers the kinetic barrier, leading to a rapid conformational rearrangement, coupled in most cases to an important step in viral entry.

GENOME PACKAGING

Incorporation of viral nucleic acid must be specific, but it must also be independent of most of the base sequence of the genome. Therefore, viral genomes generally have a *packaging signal*—a short sequence or set of sequences that directs encapsidation. Recognition of the packaging signal depends on the nature of the genome and on the complexity of the assembly mechanism. In many cases, there is a direct interaction between the packaging signal and the capsid protein. Some complex viruses insert genomic nucleic acid into a preformed shell, and genome recognition is a property of the packaging system. If replication and packaging are closely coupled, as they are in picornaviruses,¹⁶⁸ flaviviruses,¹²¹ and at least some RNA plant viruses,⁵ a specific packaging signal may be less essential.

Positive-Strand RNA Genomes

Viruses with ssRNA genomes (e.g., most icosahedral plant viruses; picornaviruses; alphaviruses; flaviviruses) require no definite overall secondary or tertiary fold for the genomic RNA, aside from the restriction that it fit within the shell. This restriction is actually quite severe, and the RNA is packed very tightly, approximating the density of RNA in crystals.¹⁶⁰ Even random-sequence RNA contains about 60% to 70% of its nucleotides in base-paired stems,⁹¹ and to fit efficiently within the interior of a capsid, these RNA stems must pack tangentially, not radially, with many of the stems in contact with the inward-facing surface of the shell. Such packing can be achieved by assembly around the RNA, without definite capsid–RNA interactions, other than those of a few subunits with a packaging signal (if present). In some viruses, segments of partially ordered RNA can be detected, tucked into shallow grooves on the inner capsid surface.^{44,74} The ordered positions of these segments probably result just from the shapes of the grooves and the possible structures that a tightly packed polynucleotide chain can adopt; there do not appear to be any base-specific contacts.

In a few cases, we know the molecular details of RNA packaging-signal recognition. A translational regulatory sequence

that is probably also the packaging signal of RNA bacteriophages such as MS2 and R17 folds into a stem-loop structure (Fig. 3.18A), recognized by a dimer of the coat protein (the assembly unit for this T = 3 particle).^{30,230,231} Bases in the loop and a looped-out base in the stem fit into a groove on the inward-facing surface of the subunit dimer; conserved bases make defined protein contacts.

Many nonenveloped, positive-stranded RNA viruses of eukaryotes recognize their genomic RNA, not through a groove-like site on the protein, but rather through a flexibly extended, positively charged protein arm, often at the N-terminus of the coat protein. There is an interaction of this kind between the coat protein of alfalfa mosaic virus and a 3′-terminal segment of RNA.⁹⁹ A bound coat-protein dimer is essential for replication—probably to recruit the RNA-dependent RNA polymerase. The same interaction is also likely to initiate packaging. The dimer contacts a pair of tandem RNA hairpins through a 26-residue, N-terminal arm. The two arms and the two RNA stem loops fold together into a well-defined structure, cross-strutted by base pairing between conserved AUCG sequences that follow each stem (Fig. 3.18B). There are six such stem loops in the 3′ segment of the viral genome; it is possible that binding of three coat-protein dimers initiates shell assembly.

The enveloped alphaviruses also have a multi-stem-loop packaging signal, recognized (with structural details not yet determined) by an extended N-terminal arm of the nucleocapsid subunit.¹²² There appears to be some redundancy in the stem loops, all of which contain a GGG trinucleotide in the loop, as deletion of several of the eight stem loops does not compromise the efficiency of RNA packaging and virion assembly.

Retroviral packaging signals, known as *psi* sequences, are recognized by the nucleocapsid (NC) domain of the Gag protein. The HIV-1 *psi* element has a stem-loop structure that associates with two *zinc-knuckle* modules in HIV-1 NC⁶⁰ (Fig. 3.18C). The two zinc modules are flexibly linked in unbound NC, but they adopt a defined, three-dimensional organization in complex with the RNA. Thus, the structure of the RNA imparts additional order to the protein element with which it binds (just as in alfalfa mosaic virus).

Assembly of helical structures requires unwinding of any base-paired stems in the RNA genome being packaged. TMV has evolved an assembly-driven helix-breaking feature into its packaging pathway. Viral assembly begins at an internal origin sequence, about 1 kb from the 3′ end of the genome.^{227,261} A 75-base sequence containing a presumptive stem-loop structure is sufficient to initiate specific encapsidation (Fig. 3.18D), which proceeds by a mechanism that requires the 5′ end of the RNA to be drawn through a channel along the axis of the assembling particle.²⁸

The mechanism of overall condensation of a viral genome is in general distinct from the specific recognition just discussed, unless, as in TMV, there is a regular, repeated interaction between coat protein and genomic RNA. There are various strategies for neutralizing the net negative charge on the nucleic acid. Those icosahedral viruses with inwardly projecting, positively charged arms use most of their arms for nonspecific interactions with RNA and only a few for specific recognition. In the picornaviruses, polyamines are incorporated to achieve charge neutralization.

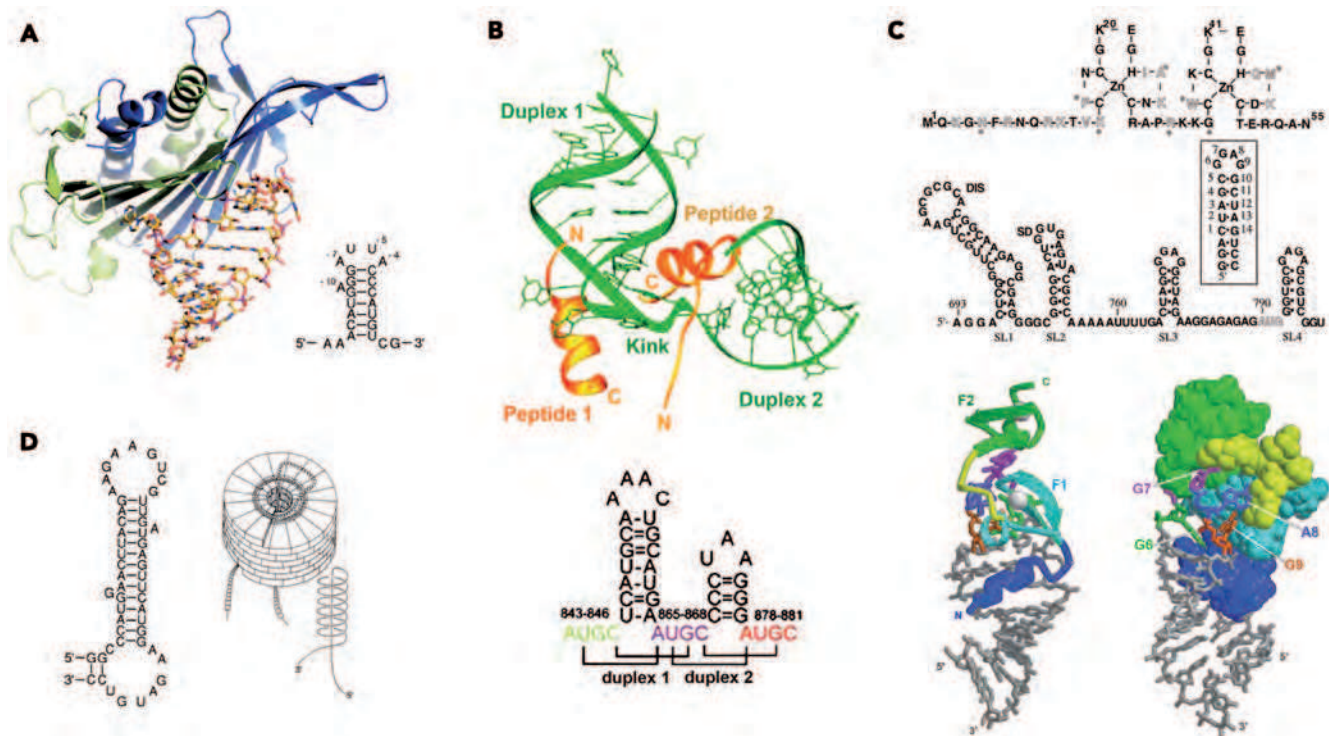


FIGURE 3.18. Various modes of single-stranded RNA (ssRNA) recognition and packaging. **A:** RNA bacteriophage. A stem-loop (sequence shown as inset) packs against the inward-facing surface of a protein-subunit dimer; there are specific contacts between residues in the protein and four unpaired bases (-4, -5, -7, -10). **B:** Alfalfa mosaic virus. A folded, stem-loop, RNA structure (green) is a docking site for two N-terminal subunit arms (gold). The arms are unstructured until they associate with the RNA. **C:** HIV-1. Two zinc-knuckle domains (labeled F1 and F2 in the ribbon representation at the **lower left**), near the C-terminal end of the Gag polypeptide (sequences shown at the **top**), bind a stem-loop structure in the packaging signal of the genomic RNA (**center and bottom**). Purine bases that have conserved stacking interactions are labeled in the surface representation at the **lower right**. **D:** Tobacco mosaic virus.^{28,227,261} The sequence at which RNA packaging initiates, shown on the **left**, is roughly 500 nucleotides from the 3' end of the genome, and assembly of the helical particle proceeds by addition of subunits at one end of the growing helix, drawing the 5' end up through the center of the particle, as shown on the **right**. Coating of the 3' overhang proceeds more slowly at the other end of the particle. (**A** adapted from Valegard K, Murray JB, Stonehouse NJ, et al. The three-dimensional structures of two complexes between recombinant MS2 capsids and RNA operator fragments reveal sequence-specific protein-RNA interactions. *J Mol Biol* 1997;270:724–738. **B** modified from Guogas LM, Filman DJ, Hogle JM, et al. Cofolding organizes alfalfa mosaic virus RNA and coat protein for replication. *Science* 2004;306:2108–2111.) **C** adapted from De Guzman RN, Wu ZR, Stalling CC, et al. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. *Science* 1998;279:384–388.

dsDNA Genomes

The best-understood dsDNA packaging mechanisms are those of the tailed bacteriophages.⁹⁷ DNA inserts into a preformed prohead, from which the scaffold has been lost by triggered release or by proteolysis.^{14,33} Removal of the scaffold leads in most cases to a substantial expansion of the head, accomplished through conformational rearrangements in the major capsid protein.^{68,119} The head itself is either an isometric icosahedral shell (e.g., lambda, P22, or HK97; Fig. 3.15) or a prolate one (e.g., T4 or ϕ 29; Fig. 3.16). In the latter cases, the scaffold protein directs the elongation. DNA packaging depends on adenosine triphosphatase (ATP) hydrolysis by a multicomponent motor. The *connector* or *portal protein*, which connects head to tail in the completed particle, is part of the motor complex, but the ATPase itself is shed from the prohead after DNA packaging is complete.⁹⁷ The ϕ 29 connector is a dodecameric ring attached at a fivefold symmetric vertex.²⁰⁷ The substantial

internal pressure of the packaged DNA²¹¹ may help drive injection into a target bacterium.

Various models have been proposed for the coupling of ATP hydrolysis by the five ATPase subunits that surround the connector with the concomitant transport of DNA into the head.^{1,156,207,217–219} To avoid entanglement, it is possible that the leading end of the DNA attaches to the head interior.¹⁰⁴ In P22, a tube-like, inward-projecting extension of the portal may also help direct coiling and prevent tangles²²²; closely related viruses lack the prominent tube, however. DNA insertion leads to formation of a tight, uniform coil (Fig. 3.15C). Because the DNA is tightly wound, the side-to-side spacing of adjacent segments is very regular; the value of this spacing is determined by the precise volume of the head and by the length of the inserted genome.⁶⁹ Viruses such as bacteriophage lambda that replicate their DNA in a rolling-circle mode couple DNA packaging with cleavage of the replicated concatemer. Others, such as

φ29, have a virally encoded protein that primes synthesis of both DNA strands and that remains attached to the ends of the encapsidated genome.

DNA packaging into herpesvirus capsids resembles the process just described for the tailed bacteriophages (see Chapter 75). Not only does the shell-forming domain of the major capsid protein appear to have the HK97 fold,⁹ as described earlier, but also the portal protein, attached to a unique vertex, likewise resembles its tailed-phage counterpart.^{113,164} Rolling-circle DNA replication late in infection yields a concatemer, and cleavage of the DNA into a single “head-full” accompanies encapsidation.

Adenoviruses have, near the left-hand end of their linear genome, a set of AT-rich repeats that determines DNA incorporation into virions (see Chapter 55). Virions contain about 1,000 copies of a protein (VII) with strong positive charge, and it is believed that this protein condenses the viral DNA within the virion core and that it may remain associated with the DNA after uncoating. In the adenovirus-like bacteriophage PRD1, there is a unique vertex defined by the presence of proteins required for DNA packaging and injection.⁹⁰ One of these, the ATPase, is distantly related to protein IVa2 of adenoviruses and more closely related to candidate packaging ATPases for other dsDNA viruses with internal membranes.¹¹⁵

Papovaviruses incorporate cellular histones, so that the closed, circular DNA comprises about 20 to 25 nucleosomes (see Chapters 53 and 54). This minichromosome is further condensed as the capsid assembles around it. Packaging appears to be directed by sequences in a histone-free region.

dsRNA Genomes

RNA packaging by dsRNA viruses presents several puzzles, the most important of which is selection of RNA segments (see Chapters 44–46). Reoviruses have 10 RNA segments, and rotaviruses, 11. Random incorporation would lead to a vanishingly small proportion of fully infectious particles. Moreover, the range of segment sizes is substantial, and a capsid-full of RNA accommodates just one of each size. The RNA must wind tightly into nonentangled spools to enable the many rounds of transcription of each gene segment that occur when the inner capsid particle is released into the cytoplasm of an infected cell.²²³

Some molecular details of assembly have been worked out for the dsRNA bacteriophage, φ6, and its relatives.^{153,180} A procapsid assembles, into which each of the three positive-stranded RNA segments inserts sequentially, in a specific order. Minus-strand synthesis occurs inside the shell. The procapsid includes the major shell protein (similar to that of reovirus or rotavirus, Fig. 3.12), the polymerase, an ATPase, and a protein thought to serve as an assembly “clamp.” One copy of the hexameric ATPase, which may be a packaging helicase analogous in function to the packaging proteins of dsDNA bacteriophages, lies at each fivefold vertex⁶¹; RNA insertion appears to occur at only 1 of these 12 positions, even though all are occupied by an ATPase.

Assembly of other dsRNA viruses probably exhibits some similar features, but it seems likely that the inner shell co-assembles with the polymerase and the various plus-strand RNA segments.¹⁷⁵ The rotavirus polymerase, VP1, recognizes a conserved sequence at the 3′ end of the plus strand (the template for dsRNA synthesis); this interaction may direct specific

packaging of viral RNA.¹⁴⁶ VP1 requires association with the shell protein, VP2, for activity, and it is plausible to infer that one copy of VP1 and 10 copies of VP2 (together with one copy of the capping enzyme, VP3) make up the inner-core assembly unit. The ssRNA template could extend away from the incomplete particle, with dsRNA synthesis as the driving force to reel it into the shell,^{82,151,174} or it could condense into the interior of the assembling shell, as in the ssRNA viruses. Packaging of the genome as ssRNA, rather than as completed dsRNA segments, has an attractive feature: the tightly wound dsRNA spools required to fit the full genome into the shell could be generated readily during synthesis²²³ (Fig. 3.12). The presence of an RNA cap-binding site on the surface of the polymerase provides a mechanism for associating a particular polymerase molecule with a particular gene segment during subsequent rounds of transcription, which occurs without disassembly.²²³

Reoviruses and rotaviruses have a nonstructural protein, designated σNS and NSP2, respectively, that appears to have a role in RNA packaging. NSP2 is an octamer with a central channel that could accommodate ssRNA.^{116,118,129,225} A second nonstructural protein, NSP5, appears to compete with RNA for binding to NSP2, suggesting it may have some sort of co-chaperone-like activity.¹¹⁸

The central question remains: how does packaging of the *n*th RNA segment lead to selection of segment *n* + 1? The most likely mechanism involves RNA–RNA recognition: for example, when the *n*th RNA has been partially packaged, a single-strand region near its trailing edge will be exposed and perhaps unwound from internal secondary structural interactions with regions already packaged. This trailing segment could then recognize some feature—base sequence or three-dimensional structure—of segment *n* + 1. An allosteric mechanism involving protein conformational changes has been proposed for packaging the three segments of φ6,¹⁵³ but extending such a picture to 10 or 11 distinct states seems unlikely.

Negative-Strand RNA Genomes

The nucleocapsid proteins (N) of three negative-strand RNA viruses with single-segment genomes (VSV and rabies virus, both rhabdoviruses, and borna disease virus) all have closely related structures.^{4,93,197} Recombinant N proteins from VSV and rabies viruses bind nonspecific RNA from the expression host and form rings of 10 to 14 subunits, with an N-terminal arm that embraces one neighbor in the ring and a loop near the C-terminus that extends into the other neighbor. The subunits have two lobes with a groove between them that faces the center of the ring and binds the RNA—nine bases per subunit. The VSV-N ring is evidently a more tightly wound and circularized version of the helical ramp that the nucleocapsid forms in the bullet-shaped rhabdoviruses (see Fig. 3.19). Sequences at the 5′ end of the negative-strand RNA, not present in the crystal structures, participate in specific packaging, but the structures do not indicate any preferential base recognition in the RNA grooves. One possibility is that there are distinct recognition events at the 5′ end, which can contact the lateral surface of the initial N-protein subunit in the ribonucleoprotein complex (RNP), and at the 3′ end, for polymerase entry.⁴ The polymerase must withdraw the RNA from the groove in the RNP.^{4,93}

Influenza, like other orthomyxoviruses, has an eight-segment genome. The eight RNPs resemble rods folded back on themselves and coiled.⁵¹ The rod lengths, from 300 to 1,200 Å,

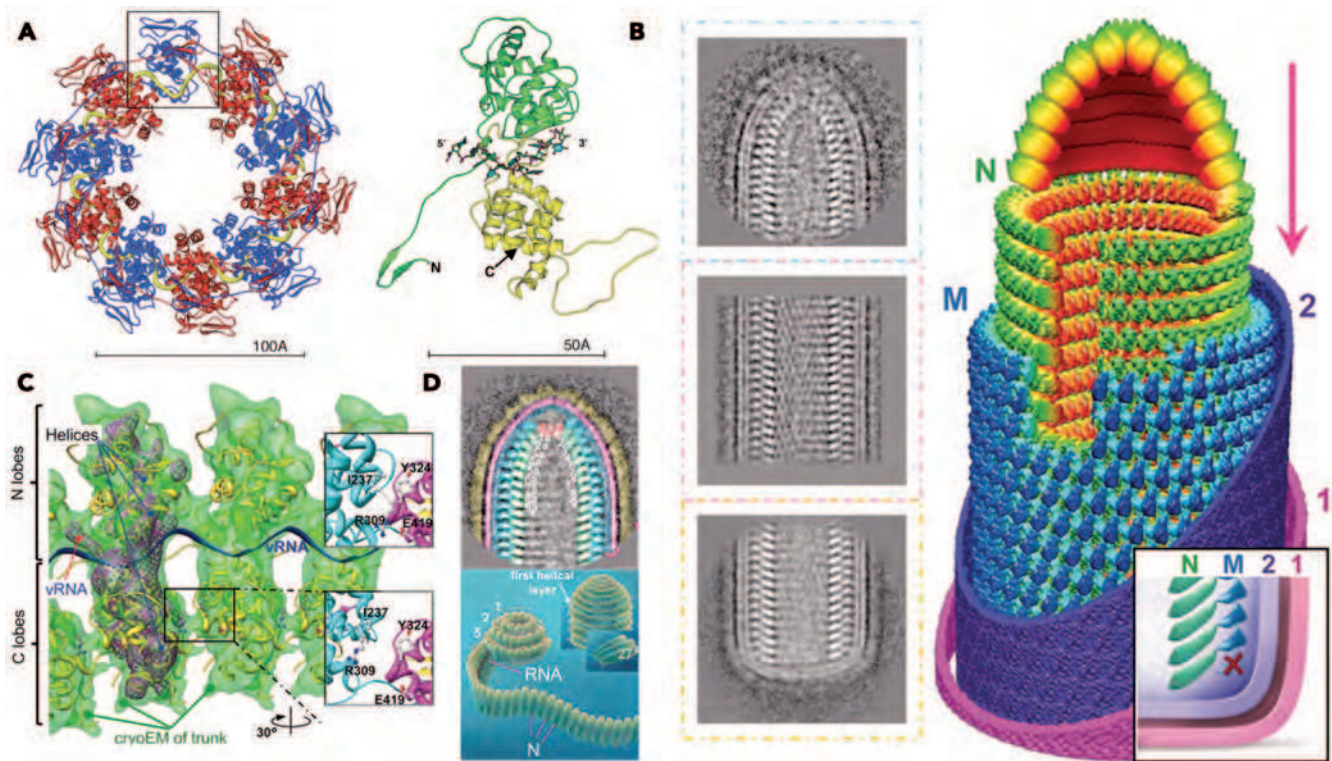


FIGURE 3.19. RNA binding and organization of the ribonucleoprotein complex (RNP) in vesicular stomatitis virus. A: Binding of RNA by N. **Left:** A ring of 10 recombinant nucleocapsid (N) protein subunits (alternating red and blue) binds a 90-nucleotide RNA segment (yellow). Recombinant N forms rings of various sizes, which take up random fragments of cellular RNA tightly enough to withstand purification. The view of the ring is from the “bottom” (C-terminal lobe) of the subunit; this lobe has been removed from one of the subunits (boxed), to show the (yellow) RNA more clearly. **Right:** One subunit from the ring, with a nine-nucleotide RNA segment in the groove between the two lobes of the protein. N- and C-terminal extensions project laterally and interact with neighboring subunits: the radius of the ring can vary, because these links are flexible. **B:** Image reconstruction, from electron cryomicroscopy (cryoEM) images (averaged projections of which are shown on the **left**), of the bullet-shaped vesicular stomatitis virus (VSV) particle. The outer glycoprotein (G) layer is not well-enough ordered to appear as discrete density in the map, but a fuzzy “halo” on the surface of the particle is evident in the projections. The nucleocapsid (green) winds into a shallow helix, guided by association with the matrix protein (M, blue), which in turn contacts the membrane (purple and magenta for the inner and outer headgroup layers, respectively). **C:** View from the inside of the RNP helix. The two insets illustrate the relationship between the subunits seen from the inside of the 10-subunit ring (**upper box**) and as they “unwrap” to form the larger-diameter helix in the virion (**lower box**). **D:** Color-coded interpretation of the upper projection in **B**, with colors as in the surface representation in **B**, and diagram showing wrapping of the RNP into the particle. The inner diameter of the helical coil formed by the RNP is about 450 Å and the rise per turn, about 50 Å. (**A** adapted from Green TJ, Zhang X, Wertz GW, et al. Structure of the vesicular stomatitis virus nucleoprotein-RNA complex. *Science* 2006;313:357–360. **B–D** adapted from Ge P, Tsao J, Schein S, et al. Cryo-EM model of the bullet-shaped vesicular stomatitis virus. *Science* 2010;327:689–693.)

correspond to the various genome segment lengths, when coiled as described; their diameter is about 120 Å.¹⁶⁷ A super-helical organization of the RNP probably determines the dimensions. Partially complementary sequences in the 5′ and 3′ noncoding regions probably dictate the folded-back arrangement; sequences at either end of the coding region also contribute to specific packaging.¹⁶¹ Serial transverse sections through elongated buds of the WSN strain of influenza A show eight rods, seven around one, about 120 Å in diameter, extending for variable distances from the tip of the bud toward its proximal end; the distances correspond to the lengths of the various genome segments.¹⁶⁷ Tomographic reconstructions of purified filamentous influenza virus particles show that this internal organization is retained in the budded particle.²⁹ Interfering with packaging of one segment reduces packaging of others.¹⁶¹

Thus, there appears to be a sequential recognition mechanism to ensure a proper complement of genome segments, perhaps formally (although not structurally) analogous to the selection mechanism in dsRNA viruses. The RNA that forms the central element in the seven-around-one arrangement may have a particularly critical role; some evidence suggests that this segment is the one that encodes polymerase subunit PB2.¹⁶¹

The influenza N-protein, like those of the single-segment negative-strand RNA viruses, has two lobes with an RNA-binding groove between them, but the folded structures are not identical.²⁴⁸ The recombinant N forms trimers in a tight association determined by a loop, toward the C-terminus of the polypeptide chain, that inserts into a neighboring subunit as two antiparallel strands. The groove likely to accept RNA faces away from the threefold axis of the trimer. The relationship

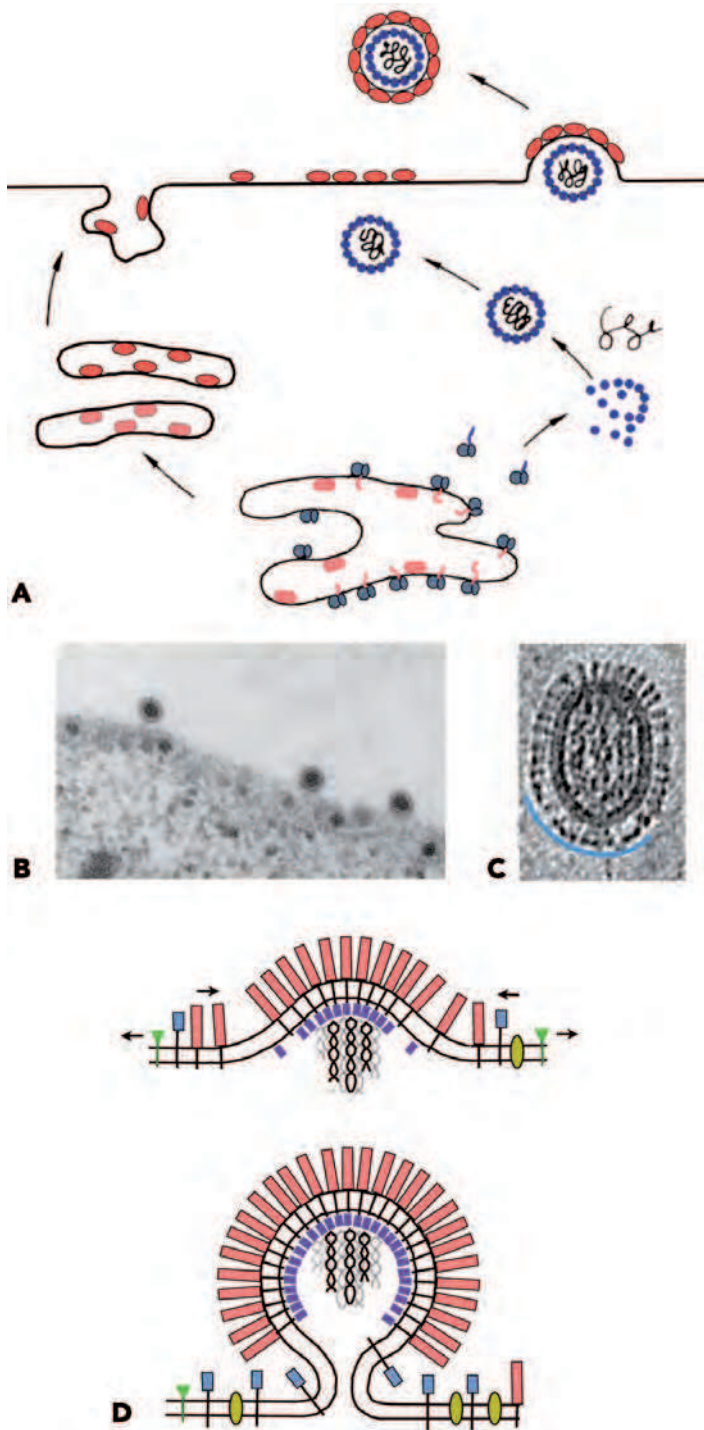


FIGURE 3.20. Budding of enveloped viruses. **A:** Schematic of alphavirus maturation and budding. The core protein, C (blue), synthesized on cytosolic ribosomes, assembles with viral RNA into T = 4 nucleocapsids. The envelope proteins, E1 and pE2 (red), synthesized on endoplasmic reticulum (ER)-bound ribosomes, mature as they pass through the ER and Golgi, and pE2 is cleaved as it passes the trans-Golgi network to the cell surface. The nucleocapsid organizes the E1-E2 heterodimers into a T = 4 lattice as the particle buds. **B:** Electron micrograph of budding Semliki Forest virus. Arrow: complete virus particle just released. Arrowhead: nucleocapsid in the cytosol. Bar = 1,000 Å. **C:** Section through a tomogram of an isolated influenza virus particle. The hemagglutinin (HA) and neuraminidase (NA) spikes, which project from the membrane bilayer, have distinct outlines, and the blue curve at the bottom of the figure illustrates that the NA concentrates at one end of the particle. The M1-protein lines the inner membrane surface; the ribonucleoprotein complexes (RNPs) pack in longitudinal orientation inside the particle. Bar = 1,000 Å. **D:** Diagram of influenza virus budding. The HA (red), NA (blue), M2 proton channel (yellow), M1 protein (purple), and eight RNPs (gray and black) co-assemble at the site of budding. Host cell proteins (green) are excluded. NA and M2 enter the particle late in the process and cluster toward one end. (**B** adapted from Sjöberg M, Garoff H. Interactions between the transmembrane segments of the alphavirus E1 and E2 proteins play a role in virus budding and fusion. *J Virol* 2003;77:3441–3450. **C** adapted from Calder LJ, Wasilewski S, Berriman JA, et al. Structural organization of a filamentous influenza A virus. *Proc Natl Acad Sci U S A* 2010;107:10685–10690.)

of the trimer configuration to N-protein interactions in the double-helical nucleocapsid has not yet been determined.

VIRAL MEMBRANES

Budding

Most enveloped viruses (except for the large and very complex poxviruses and probably some of the membrane-containing bacteriophages) acquire their membrane, a lipid bilayer

with associated proteins, by budding through some cellular membrane—the plasma membrane in many cases, the endoplasmic reticulum (ER), Golgi, or nuclear membrane in others (Fig. 3.20). These viruses direct insertion of their surface glycoproteins into the relevant membrane of the cell, through the usual cellular compartmentalization pathways. The driving force for budding then comes either from interactions between cytoplasmic tails of the glycoproteins and assembling or preassembled internal structures, from lateral interactions between glycoprotein subunits, or from a

combination of both. Retroviruses can bud without any glycoprotein at all: interactions between the myristoylated Gag protein and the lipid bilayer are sufficient to induce formation of a bud. Pinching off from the cell surface, or into the lumen of the ER or Golgi, appears in some cases (alphaviruses and flaviviruses) not to require a cellular pinching activity; assembly of viral components provides the force needed to distort the membrane bilayer. In many other cases, however, completion of budding requires that the virus recruit components of a cellular budding machinery.¹⁸¹ In the examples that have been studied in some detail (retroviruses, especially HIV), the virus redirects to the cell surface a set of protein complexes (the ESCRT machinery) that normally function at late endosomes, where they generate pinched-off invaginations into the endosomal lumen to create *multivesicular bodies*.^{85,216} The topology of viral budding from the cell surface is the same as the topology of luminal vesicle formation (away from the cytosol).

The lipids in the viral membrane derive from the cell, whereas the viral genome encodes the proteins. To a first approximation, the incorporated lipids represent a sample of those in the membrane through which the virus budded.¹²⁴ Viruses that emerge through the plasma membrane contain phospholipid and cholesterol in characteristic proportions, whereas those that emerge into the lumen of the ER contain almost no cholesterol. Cholesterol tends to increase the thickness of a bilayer by restricting free rotation about single bonds in the fatty acid chains of adjacent phospholipids.¹⁸³ The lengths of α -helical transmembrane segments in viral glycoproteins vary accordingly: from about 26 residues in flu (which buds at the cell surface) to 18 to 20 in yellow fever (which buds into the ER). Viral envelope proteins can also specify detailed specificities in lipid incorporation. For example, when present on a cell surface, the influenza virus envelope proteins (hemagglutinin [HA] and neuraminidase [NA]) associate with lipids characteristic of cholesterol-rich microdomains, and the lipid composition of the virus reflects this bias.^{199,221,254} The differential lipid composition of the viral membrane may contribute to membrane scission, which is ESCRT independent.⁴⁰ The viral M2 protein, a small, tetrameric ion channel that does not partition into microdomain lipids, incorporates at the base of the bud; an amphipathic helix in M2 appears to participate in pinching off the nascent virion.¹⁹⁴ The N protein also incorporates selectively at the base.²⁹

The two examples in Figure 3.20 illustrate features of the budding process. The structure of the nucleocapsid varies with virus type. It is a compact, spherical particle in the alphaviruses; a filamentous, helical nucleocapsid in paramyxoviruses and rhabdoviruses; and a multisegmented helical nucleocapsid in the orthomyxoviruses. The viral glycoproteins are anchored in the cellular membrane by a transmembrane hydrophobic segment (in some cases, a hydrophobic hairpin), and there is a small cytoplasmic domain. In alphaviruses (Fig. 3.20A), a core particle (nucleocapsid) assembles independently in the cytoplasm. Interactions between the core and the cytoplasmic tail of the glycoproteins then determine the location of budding. Lateral interactions of the glycoproteins probably assist the budding process. In orthomyxoviruses such as influenza (Fig. 3.20C), the M (*matrix*) protein associates with the nucleocapsid segments and with the inner face of the membrane, presumably by interaction with the cyto-

plasmic domains of the glycoproteins. M organizes both the glycoproteins and the nucleocapsids. Budding then proceeds by co-assembly of structures on both surfaces of the membrane. The two patterns of budding shown in Figure 3.20 are not fundamentally different; rather, they depend on the relative strength of core–core, envelope–envelope, and core–envelope contacts. In at least one case, a mutation has been shown to convert budding from one mode to the other.¹⁸⁸ Absolute specificity is sometimes violated in viral budding, leading to cases of phenotypic mixing, in which, for example, simian virus type 5 (SV5) glycoproteins can be found in the membrane of VSV. HIV buds with only a few copies of its glycoprotein on the virion surface, and some host–cell membrane proteins tend to incorporate nonspecifically into the particle.

The simplest enveloped virus particles—those of the alphaviruses and the flaviviruses—are icosahedrally symmetric.^{45,128,179,232,233} In these positive-strand RNA viruses, one-to-one interactions between the envelope glycoprotein and the nucleocapsid subunit appear to ensure coherence between external and internal structures (Figs. 3.20A and 3.21). The larger, negative-strand RNA bunyaviruses also have an icosahedrally symmetric envelope (a $T = 12$ lattice), but their internal structures are probably not icosahedrally organized, and the outer lattice is the major determinant of symmetry and stability.²³⁴ The rhabdoviruses have a helically organized shaft with a (probably hemi-icosahedral) cap at one end (Fig. 3.19). The orthomyxoviruses, like influenza A, bud out as variable, round or elongated structures with no overall symmetry, although there is probably considerable local order.¹⁶⁷ Retrovirus particles also appear to have no global symmetry.⁷⁹ The fluid character of a lipid bilayer means that the virus can form a closed structure without a perfect surface lattice. Defects in a protein layer that would produce unacceptable holes in a nonenveloped virus are tolerable if the barrier protecting the genome is a lipid membrane rather than a protein shell.

Internal Structures

The proteins on the internal side of viral envelopes are significantly more varied in design than those in the shells of nonenveloped viruses. The alphaviruses have subverted a serine protease to serve as the principal domain of the capsid subunit (C)⁴⁶ (Fig. 3.21). The protease is functional in the single step required to cleave C from the nascent polyprotein of these positive-strand RNA viruses. The core, sealed within the bilayer, can afford to be fenestrated. The hexamer and pentamer clusters of the protease domains do not contact each other, and interacting N-terminal arms maintain coherence of the $T = 4$ icosahedral lattice.²⁵⁸ These positively charged arms, like those of TBSV (Fig. 3.7), appear to knit the core together as well as to recognize and neutralize RNA. The hepatitis B capsid is also an open, almost lattice-like, structure, formed by a largely α -helical subunit that can assemble into either $T = 3$ or $T = 4$ shells.²⁰ The retroviral Gag precursor is usually anchored by an N-terminal myristoyl group to the membrane bilayer, and successive domains are separated by cleavage into radially organized layers^{84,247} (see Chapter 47). The structures of the various domains from HIV-1 Gag are known,⁷⁷ as are those of certain domains from a few other retroviruses.

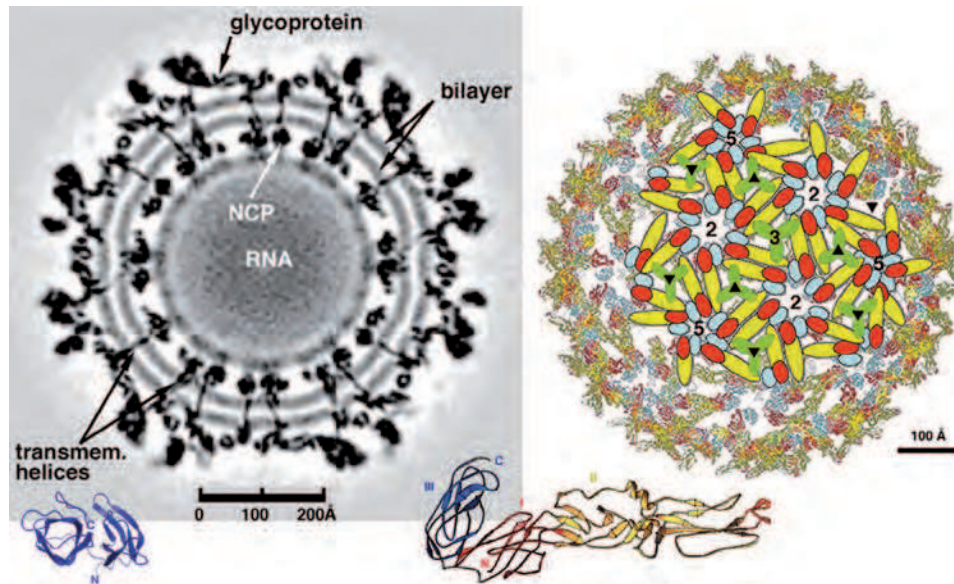


FIGURE 3.21. Molecular organization of alphavirus particles. **Left:** Cross-section through an electron cryomicroscopy (cryoEM) three-dimensional image reconstruction of Sindbis virus.²⁵⁸ The labels point out the glycoprotein layer (E1 and E2) on the outside, anchored into the lipid bilayer through C-terminal transmembrane helices, with a short segment at the C-terminus of E2 in contact with the nucleocapsid protein (NCP). **Right:** The $T = 4$ glycoprotein surface lattice of the closely related Semliki Forest virus.¹³⁶ Superposed on a ribbon representation of the lattice of 240 E1 subunits is a more schematic diagram of the packing of E1 and E2. The E1 subunits are in red, yellow, and blue, representing respectively the three subdomains of the E1 ectodomain (see ribbon representation at **bottom, center**, in the same colors; the three subdomains are labeled I, II, and III). The approximate location of the E2 ectodomain is in green. The numerals 5, 3, and 2 designate positions of fivefold, threefold, and twofold icosahedral symmetry axes; black triangles designate local threefold positions in the $T = 4$ surface lattice. The E2 subunits clamp the E1 subunits in place; exposure to low pH releases the clamp. At the bottom left is a ribbon representation (blue) of the nucleocapsid protein (an autoprotease with a trypsin-chymotrypsin-like fold; the cleavage generates its C-terminus, which remains bound in the catalytic cleft as an inhibitor); N and C designate its termini.⁴⁶

Surface Envelope Proteins

Most viral envelope proteins are so-called type I membrane proteins, with a single transmembrane α -helix linking an N-terminal ectodomain and a C-terminal tail inside the membrane. Some (e.g., the influenza virus neuraminidase) have the opposite polarity (type II). In flavivirus structural proteins, which derive from a polyprotein precursor (as in most positive-strand RNA viruses), the anchor is an α -helical hairpin that traverses the membrane twice.³⁶ The transmembrane helices have been resolved in cryoEM studies of alphaviruses and flaviviruses^{159,255,258} (Fig. 3.21). Contacts between the cytoplasmic tails of viral envelope glycoproteins and target sites on the underlying core of matrix subunits generally determine specificity of envelope protein incorporation (Figs. 3.20D and 3.21). These interactions often involve a short segment of envelope polypeptide, fitting against a site on the internal protein.^{19,135,171} Individual interactions are weak, and bilayer disruption by nonionic detergents readily dissociates them.

The proteins on the outer surface of an enveloped virus must carry out at least two functions: receptor binding and fusion. In addition, there may be a receptor-destroying enzyme (e.g., the influenza virus neuraminidase or the coronavirus esterase) to promote viral release. The membrane of influenza A contains a fourth activity: a proton channel that assists uncoating and transcriptase activation (M2). In certain cases (e.g., rhabdoviruses and retroviruses), the receptor-binding and

fusion activities are combined in a single protein; in others (e.g., paramyxoviruses), there are two distinct proteins to carry out these functions. Structures of viral fusion proteins are described later in the subsection on membrane fusion.

STRUCTURAL BIOLOGY OF VIRUS ENTRY

Receptor Binding

There are no simple generalizations about virus receptors and how they bind with viral surfaces (Fig. 3.22). We note two points here. The first is that most viruses have evolved a mechanism to avoid “getting stuck” at the cell surface when emerging from an infected cell. Many viruses simply bind weakly to their receptors, and thus can dissociate in a reasonable time. The virulence of polyomavirus in mice is inversely related to viral affinity for its sialoglycoconjugate receptor (Fig. 3.22A), demonstrating that spread in the animal host, rather than entry into cells, is the principal correlate of pathogenesis.¹² Like polyoma, influenza virus recognizes a sialic acid-containing carbohydrate for cell attachment¹⁷⁶ (Fig. 3.22B). A receptor-destroying enzyme (neuraminidase) is present on the surface of the virion; its activity allows release of newly assembled virions from the cell surface through which they have budded.¹⁷² The neuraminidase is thus required for effective spread of the virus, and the enzyme is the target of anti-influenza drugs, developed

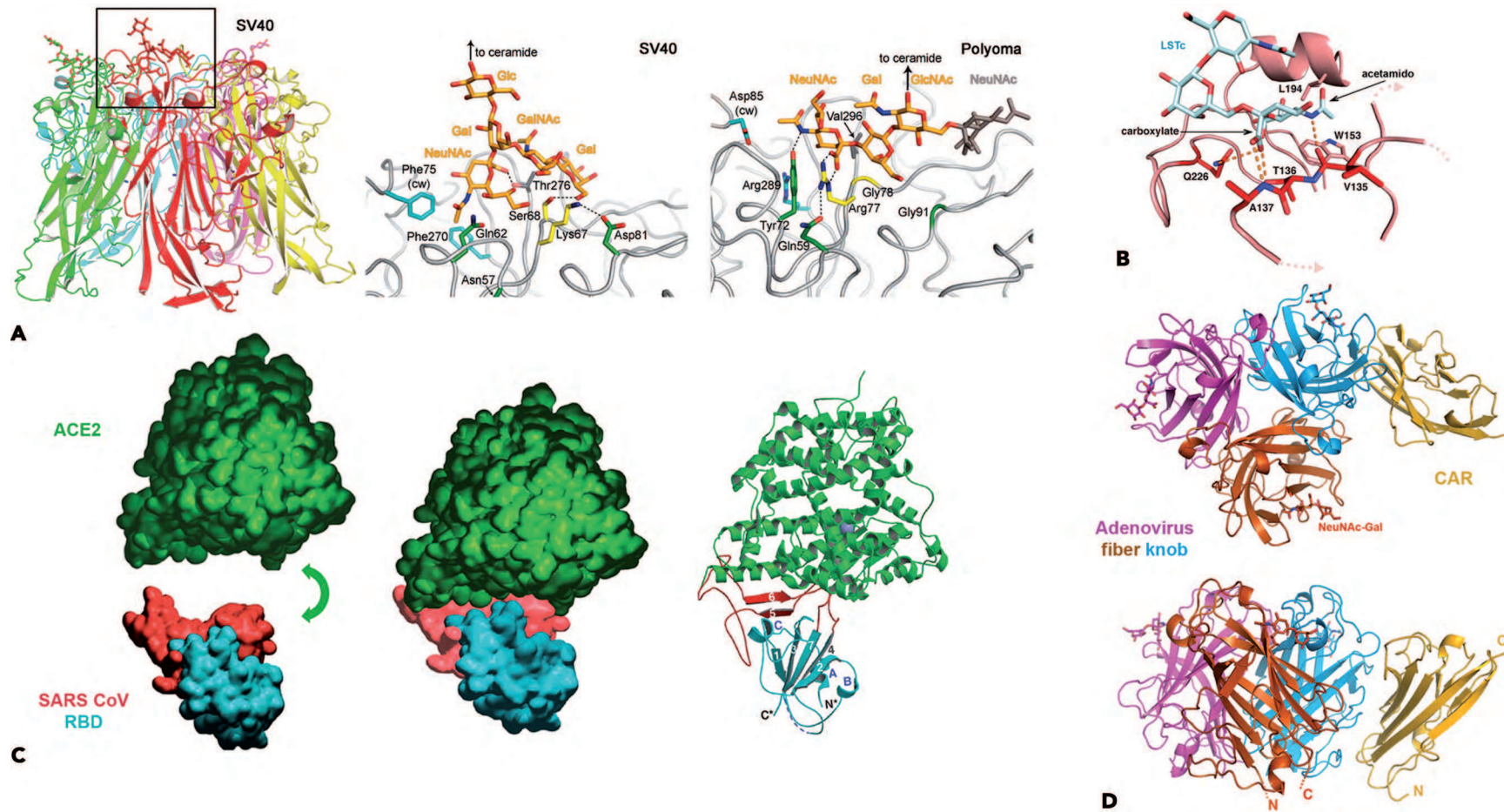


FIGURE 3.22. Examples of virus–receptor interactions. **A:** Simian virus type 40 (SV40) and polyomavirus. **Left:** Pentamer of SV40 VP1, showing location of the interaction on the outward-facing surface of the subunit. **Right:** Detailed views of the receptor-binding sites for the two viruses (boxed region in the **left-hand panel**), showing interactions of distinct glycan structures (from glycolipid headgroups) with homologous sites on the VP1 subunits. **B:** Influenza virus: sialic acid–binding site on the HA1 “head.” **C:** Severe acute respiratory syndrome (SARS) coronavirus: docking of the ACE2 receptor with the receptor-binding domain (RBD) of the viral glycoprotein spike.¹³⁷ **D:** Adenovirus penton-fiber knob (**top view**), bound with domain 1 of the multi-Ig domain receptor, CAR (coxsackievirus-adenovirus receptor), and with sialic acid (**side view**), which helps recruit type 2 adenovirus to cell surfaces.²⁰¹ (**A** adapted from Neu U, Woellner K, Gauglitz G, et al. Structural basis of GM1 ganglioside recognition by simian virus 40. *Proc Natl Acad Sci U S A* 2008;105:5219–5224. **B** adapted from Whittle JR, Zhang R, Khurana S, et al. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc Natl Acad Sci U S A* 2011;108:14216–14221.)

in part by exploiting knowledge of the NA structure.²³⁵ HIV-1 has several mechanisms for down-regulating its receptor (CD4) after infection, both to avoid envelope–receptor interactions within the secretory pathway and to facilitate viral release after budding (see Chapter 49).

The second general point is that some viruses require a cascade of at least two distinct receptors—one for initial cell attachment and a second for triggering fusion or penetration. The receptor used for initial attachment may be a widely distributed molecule, such as sialic acid or other glycans (heparan sulfate for herpes simplex virus-1), or it may be a quite specific protein, such as the adenovirus receptor, CAR, or the HIV-1 receptor, CD4 (Fig. 3.22C). The molecule that triggers fusion or penetration is sometimes called a *co-receptor*—for example, the chemokine receptors for HIV-1. In the case of HIV-1, CD4 primes the envelope glycoprotein to bind the co-receptor, which in turn induces fusion activation. An obligate order of this sort may turn out to be relatively common.

Viruses that mutate to avoid recognition by the immune system (e.g., influenza, HIV) have sometimes evolved structural features to sequester their conserved, receptor-binding surface from interaction with antibodies. The footprint of an antibody-combining site is substantial, and thus even if a receptor site is exposed, it rarely matches the full extent of the surface within which amino acid residue changes will lower antibody affinity and hence escape neutralization.⁵⁰ While some viral receptor sites (e.g., those on certain picornaviruses: Fig. 3.4) lie within a groove or pocket too narrow to admit the antigen-combining end of an antibody (sometimes called a “canyon”^{195,196}), others are fully exposed or even protruding (Fig. 3.22D).¹³⁷ Moreover, antibodies with unusually long or prominent heavy-chain CDR3 loops can penetrate relatively tight cavities.

An Irreversible Step Between Assembly and Entry

Assembly of TMV protein and RNA into infectious particles was among the key observations that triggered thinking about viral symmetry.⁵⁶ *In vitro* self-assembly of components from the mature virion into complete infectious particles is, however, an exceptional characteristic of the simplest plant and bacterial RNA viruses. A far more general property of virus assembly pathways is a modification, often a simple proteolytic cleavage, that “primes” the particle for large-scale, irreversible events accompanying entry. Loss of a scaffolding protein is a particularly extreme example of such a modification. Poliovirus and other picornaviruses assemble from VP0, VP1, and VP3, but autolytic cleavage of VP0 into VP4 (an internal peptide) and VP2 accompanies assembly (see Chapter 16). When receptor binding triggers expansion of the viral shell, exit of VP4 renders the rearrangement irreversible.^{15,100} The receptor is a catalyst that lowers the energy barrier to an irreversible reorganization.²²⁶ The function of this reorganization is viral entry, and the triggering mechanism has evolved to occur only in an appropriate location. Reoviruses have an outer protein, $\sigma 3$, that caps the penetration protein, $\mu 1$.^{64,140} Proteolytic removal of $\sigma 3$ is required to render the particle competent to attach and penetrate (see Chapter 44). There is, in addition, an essential autolytic cleavage of $\mu 1$.¹⁶⁵ The HA of influenza virus folds in the ER into a stable, trimeric structure. Cleavage of one peptide bond in HA by the protease furin in a compartment late in the secretory pathway primes the

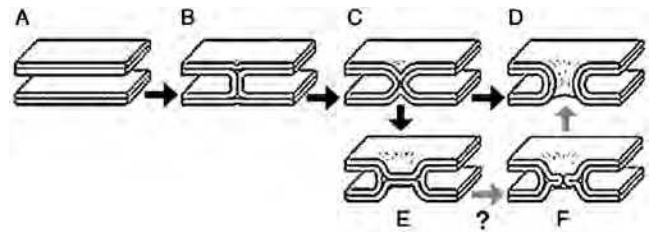


FIGURE 3.23. Fusion of two lipid bilayers. **A:** Two parallel bilayer membranes. There is a substantial barrier to close approach. **B:** Hemifusion stalk. **C:** Proposed transition structure. **D:** Fusion pore (before lateral expansion). **E:** Hemifusion diaphragm. **F:** Some models include perforation of the hemifusion diaphragm as a productive step toward fusion-pore formation, but diaphragm formation is more often considered a dead end. (Adapted from Jahn R, Lang T, Sudhof TC. Membrane fusion. *Cell* 2003; 112:519–533.)

protein to undergo a dramatic, low-pH-triggered rearrangement, which mediates fusion of viral and target cell membranes. In effect, cleavage renders the virion form of HA metastable, but the barrier to rearrangement is so great at neutral pH that no conformational change occurs. Proton binding in the low pH environment of the endosome removes this barrier and triggers a refolding of the HA protein. Protons have the role taken in other cases by a co-receptor (e.g., the chemokine receptors for HIV-1). The expression “spring loaded” has been used to describe the state of HA at neutral pH after cleavage to HA₁ and HA₂.³² *Jack-in-the-box* might be a comparable image for poliovirus after cleavage of VP0.

Membrane Fusion Bilayer Fusion

The bilayer fusion reaction common to all the enveloped viral entry pathways is shown schematically in Figure 3.23. It is believed to pass through an intermediate known as a *hemifusion stalk* (Fig. 3.23, top center), in which the two apposed leaflets have fused, but not the distal ones.^{130,147,184,205} Hemifused bilayers can then form either a *fusion pore* (Fig. 3.24, right) or a structure in which the two distal leaflets create a single bilayer. This state, which can spread laterally, is called a *hemifusion diaphragm* (Fig. 3.23, bottom center). Bilayers do not fuse spontaneously (e.g., concentrated liposomes are quite stable), because the reaction in Figure 3.23 has a high activation barrier, both at the step between the precursor bilayers and the hemifusion stalk and at the step between the hemifusion stalk and the fusion pore. A newly opened pore may revert to a hemifusion structure (*flickering*), and the largest kinetic barrier may be for the step in which the pore dilates rather than reverts.^{148,184}

Conformational Rearrangements in Viral Fusion Proteins

Viral fusion proteins must lower the kinetic barriers to fusion of viral and cellular membranes. They do so by undergoing dramatic conformational rearrangements that lead to tight apposition of the two membranes.¹⁰⁶ We can thus distinguish *prefusion* and *postfusion* conformations, as well as potential intermediates. The viral fusion proteins analyzed in detail at the time this

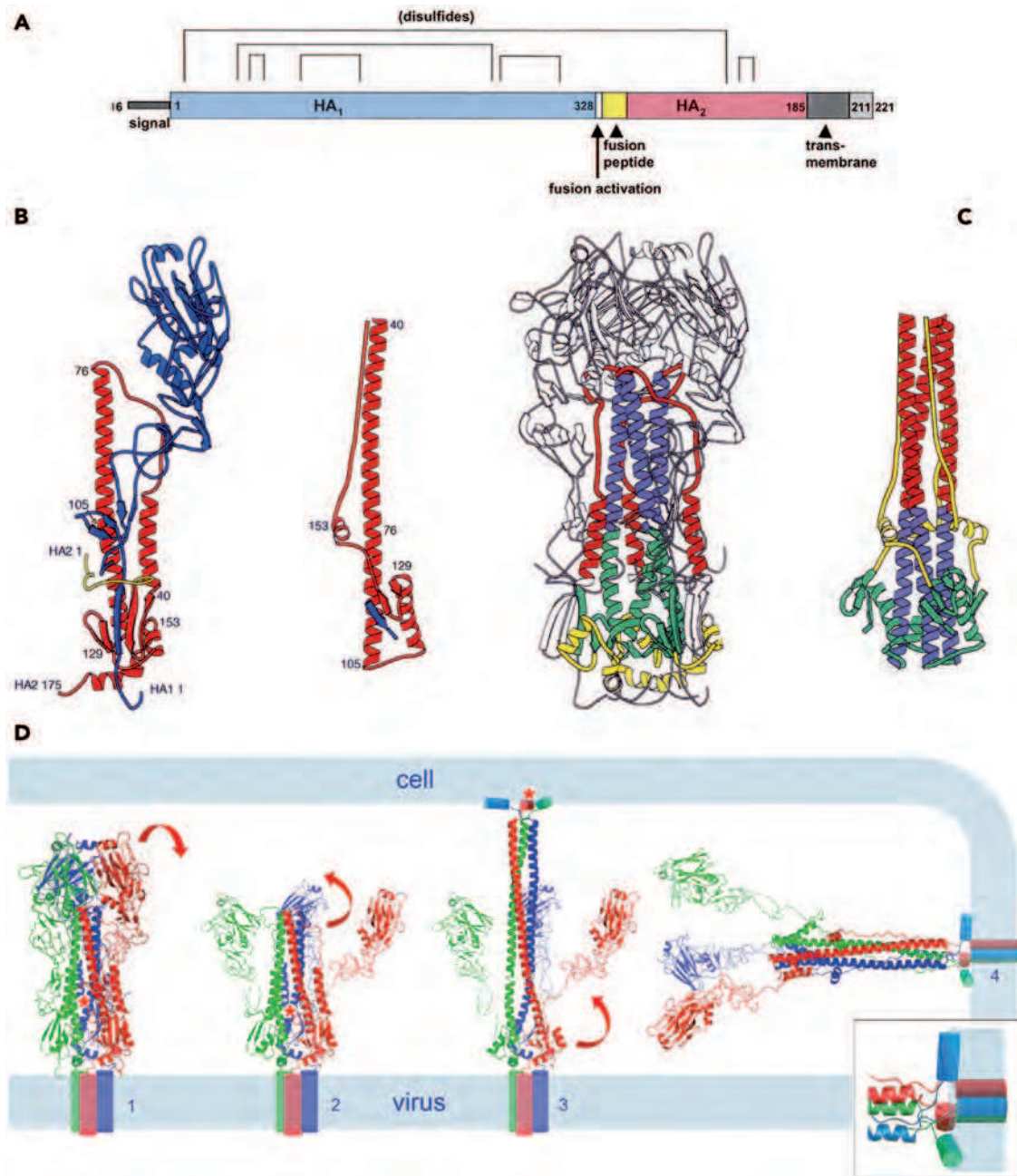


FIGURE 3.24. Influenza virus hemagglutinin (HA): structure and fusion-promoting conformational change. **A:** The HA polypeptide chain. HA₁ in blue; HA₂ in yellow (fusion peptide), red (remainder of ectodomain), and gray (transmembrane and internal segments). The position of the fusion activation cleavage between HA₁ and HA₂ is a narrow white stripe, indicating excision of a single residue in many cases. The location of disulfide bonds is shown schematically above the bar. Residue numbers correspond to positions in the HA of strain X-31. **B:** The HA ectodomain monomer, in prefusion and postfusion (the latter, HA₂ only) conformations to the left and right, respectively. HA₁ in blue; HA₂ in red. The postfusion HA₂ structure illustrated here lacks the fusion peptide as well as additional residues at both ends of the chain. **C:** The HA ectodomain trimer, in prefusion and postfusion (the latter, HA₂ only) conformations, to the left and right, respectively. HA₁ in black and white; HA₂ in colors showing various segments of the ectodomain, so that their reconfiguration during the transition from the pre- to the postfusion structure is evident. Note the loop-to-helix transition in the C-terminal portion of the red segment. **D:** Model for the coupling of the fusion-promoting conformational change in HA to the fusing membranes. **Stage 1:** Prefusion conformation. Red asterisk shows position of fusion peptide at N-terminus of HA₂. Engagement with a sialic acid receptor in the target membrane is not shown. **Stages 2 and 3:** Transition to an extended intermediate, in which the three fusion peptides of the trimer associate tightly with the target cell membrane. The fusion peptide is shown schematically as an amphipathic helix in the membrane surface—the actual structure is probably an amphipathic helical hairpin.^{143,144} Proton binding at low pH dissociates HA₁ from HA₂, although the two fragments remain tethered by a disulfide bond. **Stage 4:** During the transition of HA₂ from intermediate to postfusion conformation, the fusion peptide and transmembrane segment come together, thereby bringing the two membranes close enough to fuse. The final, postfusion conformation of HA₂ is locked in place by the “cap,” shown in the **inset**, in which residues near the C-terminus of the HA₂ ectodomain interact with residues between the fusion peptide and the long, central α -helix.⁴¹

chapter went to press fall into three structural classes, generally designated I, II, and III. Common characteristics of fusion by all three classes are insertion of a segment of the fusion protein into the target membrane and refolding of the protein so that this inserted segment and the transmembrane anchor are adjacent, thereby bringing together cell and viral membranes. The three structural classes probably represent meaningful evolutionary categories, as a cellular representative of at least one of the classes very closely resembles its viral orthologs.

Influenza Virus Hemagglutinin, a Class I Viral Fusion Protein

The defining characteristics of class I fusion proteins are synthesis as a precursor that requires a proteolytic cleavage for activation (often, but not always, by a furin-like enzyme in the trans-Golgi network); trimeric oligomerization in both pre- and postfusion conformations, based on a central, three-chain α -helical coiled-coil in the postfusion conformation; and presence of a hydrophobic *fusion peptide* near the N-terminus created by the activating cleavage. The fragment C-terminal to the cleavage, with the viral transmembrane segment, is the fusogen; the fragment N-terminal to the cleavage, in many but not all cases, is a receptor-binding structure, which generally dissociates when suitably triggered, releasing its grip on the fusogenic fragment. The final, postfusion structure is a *trimer of hairpins*, as described later.

Influenza virus HA is the best-studied class I fusion protein.²⁴⁴ HA₀, the uncleaved precursor, and HA₁/HA₂, the cleavage product, are almost identical in structure, except for a local shift that tucks the fusion peptide (the N-terminus of HA₂) between the played helices of the central coiled-coil (Fig. 3.24B). Unless induced to refold by lowered pH or by heat, HA₁/HA₂ is very stable. HA₁ forms a globular domain at the “top” of the molecule, with a binding pocket (Fig. 3.24B,C) for the receptor, sialic acid.²³⁷ Proton-induced rearrangement of HA (Fig. 3.24B–D) has two essential features. The first is ejection of the protected hydrophobic fusion peptide (Fig. 3.24D, transition from stage 2 to stage 3). The second is folding back of the fusion protein (HA₂) so that the N-terminus (the fusion peptide) and the C-terminus (the viral membrane anchor) come together (Fig. 3.24D, transition from stage 3 to stage 4).^{26,41,210} A likely intermediate state, for which there is experimental evidence in the case of HIV-1 gp41,^{62,81,108} is an extended structure with the fusion peptide buried in one membrane and the anchor in the other (Fig. 3.24D, stage 3). Zipping up of the C-terminal part of the HA₂ ectodomain along the core of this *prefusion intermediate* will cause the transmembrane anchor and the fusion peptide to approach each other. Formation of an intricate “cap” on the three-helix core snaps the refolded structure in place (Fig. 3.24D, inset).⁴¹ Note that the zipping-up process cannot be symmetrical, because the trimer would otherwise encase itself in lipid. The C-terminal outer-layer segments are long enough, in their unfolded state, to reach around the core of the refolding trimer. Contacts between the three outer-layer chains in the refolded structure are minimal, so that the three can zip up independently and at different rates. Several rearranged fusion proteins might be required to surround and induce a hemifusion stalk. Estimates from measurements of fusion by HA expressed on a cell surface and of fusion of virions with a supported bilayer *in vitro* indicate that on average, three HA trimers participate in fusion pore formation.^{59,76}

Other Class I Fusion Proteins

The postfusion conformation of HA is a trimer of hairpins. The N- and C-termini of each subunit lie at the same end of the elongated protein, and the polypeptide chain traverses the length of the molecule just twice—once from the N-terminus to the distal end, and once back to the C-terminus—with some modest complexity in the distal loop. The *inner core* is a trimeric coiled-coil; the *outer layer* of each subunit is largely extended chain, with a short helical segment. Other class I fusion proteins have the same postfusion characteristics (Fig. 3.25), the structure in the case of HIV-1 being particularly simple, as both inner core and outer layer are helical.^{18,37,145,239} The two helices are sometimes designated HR1 or HRA and HR2 or HRB (*helical region 1* or *A* and *helical region 2* or *B*, respectively), but the postfusion HA structure illustrates that identification of two helical regions may not always be informative. Moreover, a major part of the central coiled-coil in postfusion HA₂ is not even helical in the prefusion trimer—another reason why “HR1” and “HR2” are partly misleading designations. Note further that in influenza HA, the N-terminal parts of HA₂ are on the outside of the spike in the prefusion conformation and on the inside in the postfusion conformation (Fig. 3.24). The protein turns itself inside out during the refolding.

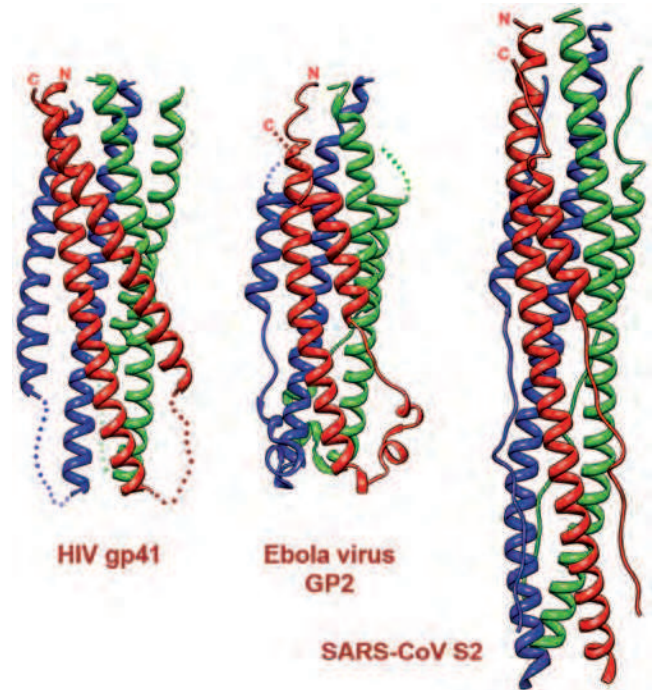


FIGURE 3.25. Postfusion conformations of three class I fusion-protein ectodomains.^{37,220,238,239,260} Only the folded-back cores of the proteins are shown. The fusion peptide extends from the N-terminus of the trimeric bundle; the transmembrane segment is at its C-terminus. Compare with the postfusion conformations of influenza virus HA₂ in Figure 3.24C and of the paramyxovirus human parainfluenzavirus 3 (hPIV3) F2 protein in Figure 3.26B. The HIV gp41 structure is particularly simple: a six-helix bundle with a relatively short loop (dotted lines) between the inner (N-terminal) and outer (C-terminal) helices (HR1 or HRA and HR2 or HRB, respectively). A 200-residue domain intervenes between the postfusion inner and outer layers of severe acute respiratory syndrome virus-coronavirus (SARS-CoV) S2.

The postfusion conformation of a class I fusion protein is the most stable one it can adopt when constraints such as the covalent linkage between the two fragments have been removed, and proteins in this conformation have therefore been easier to prepare and study than have prefusion conformers or protein models for intermediate structures. It is important to emphasize, however, that no inferences can be drawn about the prefusion structures of these proteins from their trimer-of-hairpins postfusion conformers.

One other class I fusion protein for which both pre- and postfusion structures have been determined is paramyxovirus F^{43,52,132,249,250} (Fig. 3.26). It has a cleavage site just N-terminal to a fusion peptide, which resembles (in being hydrophobic and relatively glycine-rich) the fusion peptides in gp160 and HA₀. Cleavage is essential for fusion activity, but not for the fusion-promoting conformational change. In the mushroom-like, prefusion conformation, a three-strand α -helical coiled-coil, the stem of the mushroom, is the C-terminal part of the ectodomain. It connects directly (in the intact protein) to the transmembrane segment. The strap between the coiled-coil stem and the head of the mushroom is an ordered, but very extended, stretch of polypeptide chain, which wraps around the outside of the globular cap. The cap also presents a groove

to accommodate the fusion peptide. A separate protein (designated HN, H, or G in various paramyxoviruses) binds receptor and triggers the conformational rearrangement of F (see Chapter 33). In the refolded state, no parts of the protein dissociate (as they do from HA and gp120/gp41), but a long, three-strand coiled-coil forms from segments (all C-terminal to the cleavage site) that are part of the globular “cap” in the prefusion structure.²⁴⁹ The C-terminal coiled-coil comes apart so that the C-terminal helices can fold back up along the outside of the newly formed coiled-coil. The length of the strap between the globular domains and these helices, which probably dissociate, unfold, translocate, and refold as they zip along the coiled-coil core, allows sufficient flexibility for this transition. None of the trimer contacts in the prefusion state are fully conserved in the postfusion structure, raising the possibility of a monomeric intermediate, but the overall geometry does permit the assembly to refold as a trimer without such dissociation.

The Flavivirus Envelope Subunit (E), a Class II Fusion Protein

Class II fusion proteins have been found only on alphaviruses, flaviviruses, and bunyaviruses, all of which have compact, icosahedrally symmetric virions. The defining characteristics of fusion

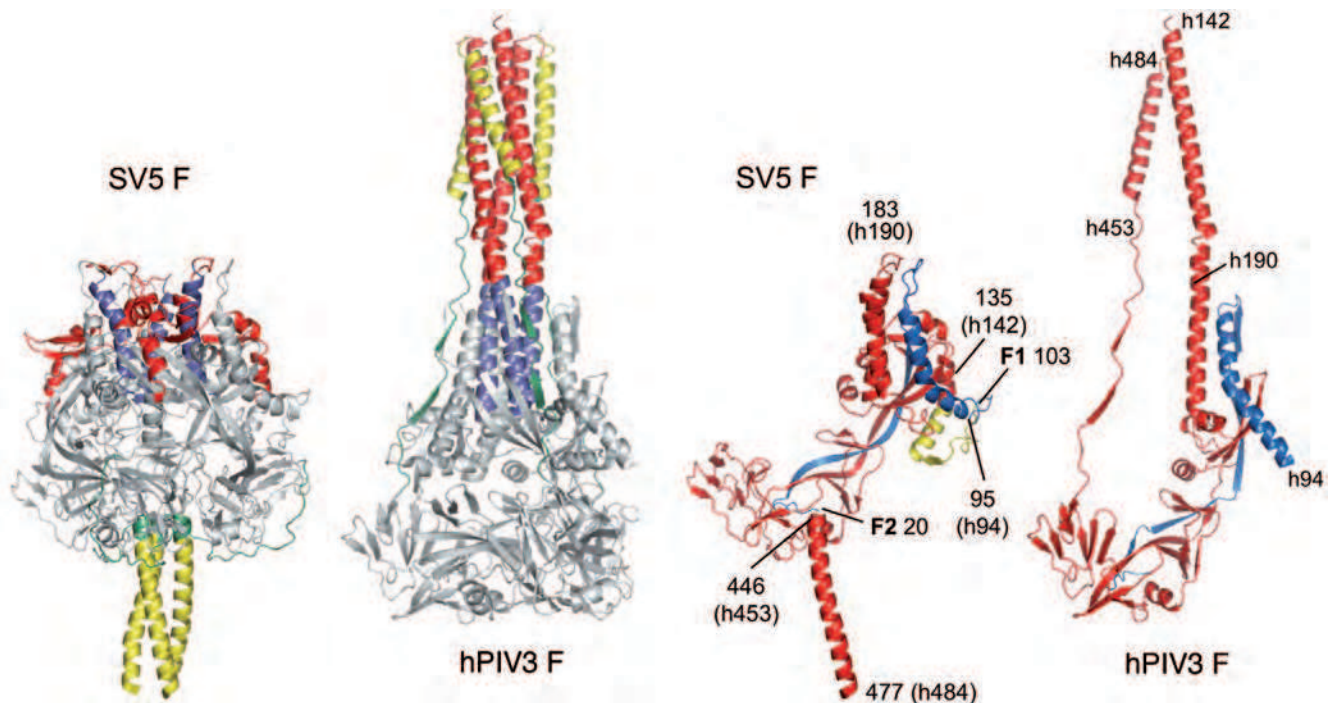


FIGURE 3.26. The paramyxovirus fusion protein (F). **A:** The ectodomain trimer of simian virus type 5 (SV5) F in its prefusion conformation. F1 is in black and white; F2, in color. The order of colors corresponds to the order of colors in influenza HA₂ in Figure 3.24C. The viral membrane would be at the bottom of the figure: the polypeptide chain of F2 enters the membrane immediately following the yellow segment, which forms a three-chain coiled-coil in the prefusion conformation. **B:** The postfusion conformation of F from another paramyxovirus, human parainfluenzavirus 3 (hPIV3). Color scheme as in **A**. Note that the red and blue segments toward the N-terminus of F2 have refolded into a three-chain coiled-coil, projecting the fusion peptide (not shown) toward the “top” of the trimer. Compare these segments with those of corresponding color in Figure 3.24 C. Also note that the yellow segments at the C-terminus of the F2 ectodomain no longer form a coiled-coil, but rather align along the outside of the coiled-coil generated by the (red and blue) N-terminal region. **C, D:** Pre- and postfusion conformations of monomers, with F1 in blue, F2 in red, and fusion peptide (N-terminus of F2) in yellow. Numbers in **C** correspond to SV5 F; numbers with “h” in **C** and **D**, to hPIV3 F. (Courtesy of Ted Jardetzky, Stanford University.)

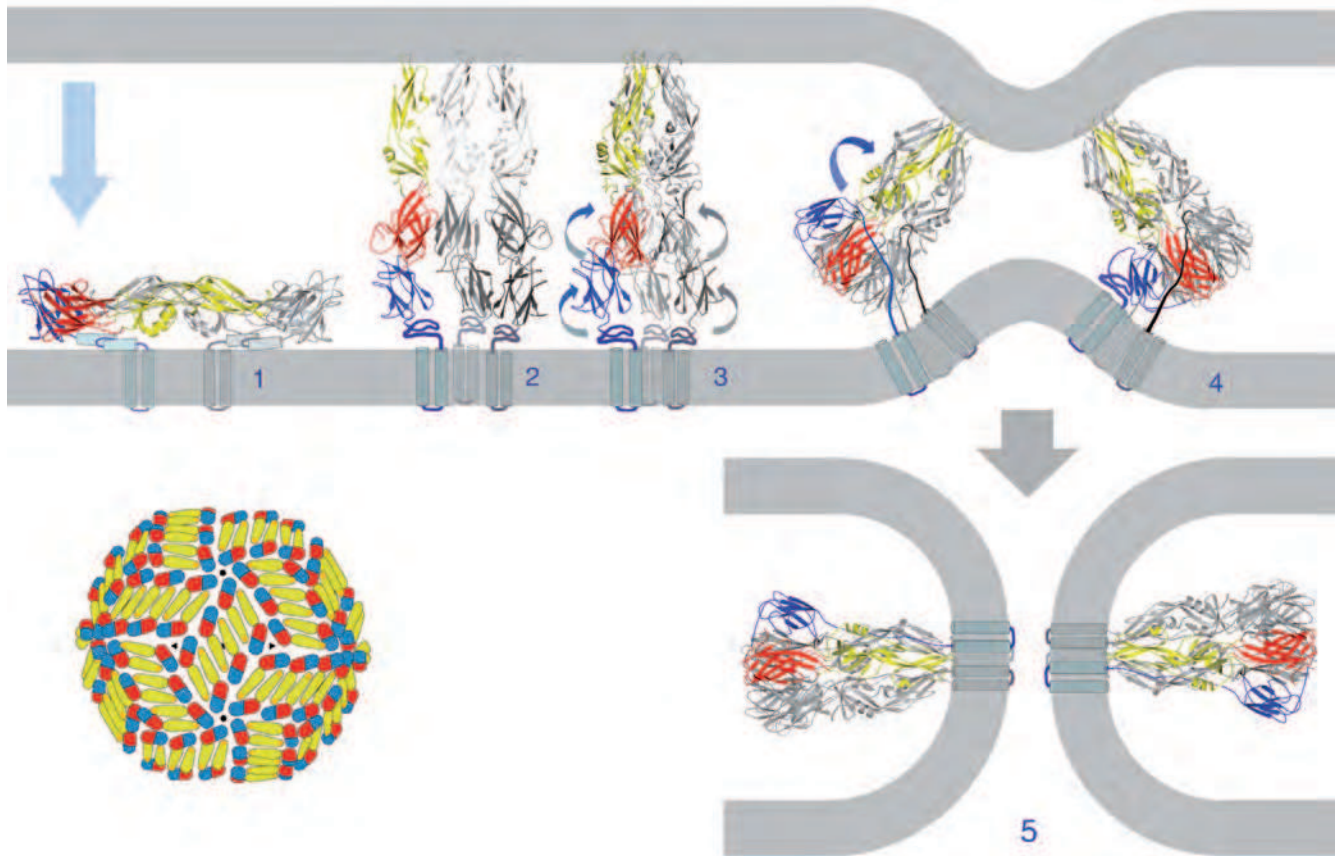


FIGURE 3.27. Membrane fusion induced by the flavivirus envelope protein, E. **Diagram lower left:** Dimer-clustered packing of E on the virion surface. The three domains of each protein ectodomain are in red, yellow, and blue. **Numbered sequence:** likely series of conformational states of E and their links to viral and cellular membranes. Structures for states 1 and 5 are known; those for states 2 to 4 are inferred from indirect data. One subunit in each dimer or trimer is colored as in the schematic; its partner(s) are in gray. **1:** E dimer on the virion surface. The ectodomain terminates in a helical hairpin called the *stem* (light blue) on the surface of the viral membrane (lower gray bar) and connects with a transmembrane helical hairpin. Blue arrow from above symbolizes a receptor interaction with domain III (blue). **2:** Exposure to low pH (in endosomes) dissociates the E dimer, allowing the subunits to project outward, so that the fusion loops (tip of the yellow domain II) encounter the endosomal membrane (upper gray bar). **3:** Initial trimer association, requiring some rearrangement across the surface of the virion. Arrows show presumed pattern of folding back; upper arrows: domain III (blue) flips over against domain I (red); lower arrows: the stem refolds to “zip up” alongside the trimer clustered domain II (yellow). **4:** Stem continues to reorganize (asymmetrically), pulling the two membranes together. The fusion loops must be firmly anchored in the target membrane. **5:** Formation of a fusion pore allows the refolding to finish, so that all three stems and all three fusion loops cluster together, restoring full threefold symmetry.

proteins in this class are a three-domain subunit, with an internal, hydrophobic *fusion loop* at the tip of the elongated second domain; association with a viral “chaperone” protein, which must be cleaved to prime the fusion process; and formation of a stable trimer in the postfusion state, with the three fusion loops and the three C-terminal, transmembrane anchors clustered at one end (Fig. 3.27). The fusion proteins are known, respectively, as E1 and E in alphaviruses and flaviviruses; the chaperones, as pE2 and prM. Cleavage of the latter proteins generates E2 and M, with release (at some point in the fusion process) of a “pre” fragment, which covers the fusion loop of E1 or E in the unprimed state.

The flavivirus E protein tiles the surface of the virion as a tightly associated dimer¹²⁸ (Fig. 3.11). There are 90 such dimers; their packing is not a quasiequivalent, T = 3 arrangement, but a herringbone-like pattern. On an immature particle,

before cleavage of prM (the chaperone), E forms heterodimers with prM rather than homodimers with itself.¹²⁸ Substantial structural rearrangements accompany maturation and dissociation of the “pre” fragment.^{138,252,253} Likely steps in the fusion process, deduced from comparison of the pre- and postfusion conformations,^{22,154,155,187} are illustrated in Figure 3.27. The underlying similarity of class I- and class II-mediated fusion should be evident. The fusion loops insert only partway into the outer leaflet of the target membrane.

VSV-G, a Class III Fusion Protein

Class III fusion proteins mediate penetration of particles as distinct as the rhabdoviruses^{191,193} and herpesviruses,^{7,109} as well as the insect baculoviruses.^{6,120} There appears to be no proteolytic cleavage, either of the fusion protein itself or of a chaperone, required for priming, and the fusion-inducing conformational

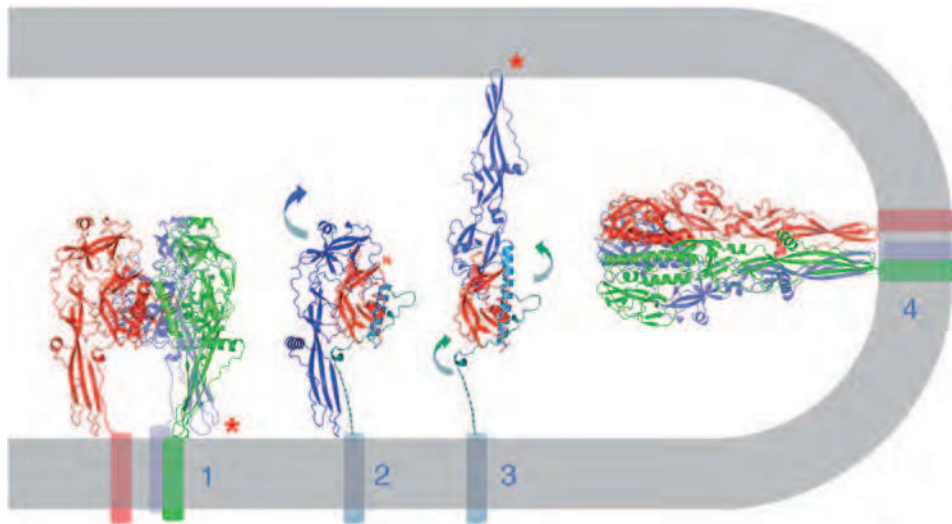


FIGURE 3.28. Membrane fusion induced by the vesicular stomatitis virus (VSV) glycoprotein (G). Sequence of conformational events as in Figures 3.24 and 3.27. **1:** Prefusion trimer of VSV G on the virion surface. Subunits colored in red, blue, and green. Red asterisk: fusion loops of the green and blue subunits. **2:** Conformation of one subunit of the trimer in **1**, now colored to show a central, red domain and a set of peripheral, blue domains. An axial helix (lighter blue) joins part of the central domain to the set of peripheral domains. In the low-pH-triggered rearrangement, the blue regions reorient with respect to the red domain (arrow). **3:** Presumed initial rearrangement, in which the domain bearing the fusion loops projects toward the cellular (endosomal) membrane, into which the fusion loops insert. The axial helix remains, augmented by a segment derived from its connection to the peripheral domains. Arrows indicate likely reorganization that follows: the central domain flips over and the *stem* at the C-terminus of the ectodomain zips up, bringing together the two membranes. **4:** Formation of a fusion pore (not shown explicitly—compare the last two stages in Figure 3.27) allows the three sets of fusion loops and the membrane-proximal segments of the ectodomain to cluster. Part of the stem rearrangement includes formation of a helical segment, which forms a six-helix bundle with the central-region helix. The conformational details thus have features of both class I fusion proteins (formation of a six-helix bundle) and of class II fusion proteins (preconfigured, internal fusion loops that insert into the target membrane).

change is in at least some cases reversible.¹⁹² That is, virions inactivated by prolonged incubation at pH less than 6 can be reactivated by raising the pH to neutral or above, and both conformations of the multidomain, trimeric protein can be obtained from the same protein preparation.

VSV-G, the only protein on the surface of the virion, has two hydrophobic loops that can interact with membrane lipids⁶⁷ (Fig. 3.28). The connectivity of the strands joined by these fusion loops is different from the connectivity in domain II of the class II proteins (i.e., the domains themselves have different folds), but the general picture is quite similar: hydrophobic residues (including at least one tryptophan) are displayed on tightly structured loops at the tip of an elongated domain. In the prefusion conformation of VSV-G, these domains face the viral membrane around the periphery of the trimer.¹⁹³ In the postfusion conformation, they cluster around the three-fold axis¹⁹¹ (Fig. 3.28; compare the “inside-out” transition in influenza HA, Fig. 3.25).

In the rhabdovirus G protein, a core domain contains residues from the N-terminal segment of the polypeptide chain and residues from near the C-terminal part of the chain: it is a framework around which the rest of the molecule reorients. Two other domains form a jointed, two-part fusion machinery. The result of their rotations relative to the core domain (and to each other) is to move the fusion loops away from the viral membrane and toward the target membrane. In a likely extended intermediate conformation (shown in Fig. 3.29, but

for which there are no direct structural data), the C-terminal segment still connects toward the viral membrane in one direction, while the fusion loops interact with the target membrane in the opposite direction. In the fully rearranged, low-pH conformation, the C-terminal segment has zipped up along the fusion domains, much like in the flavivirus fusion transition.

The herpesvirus fusion protein, gB, looks like an elongated version of VSV-G.¹⁰⁹ This unexpected similarity between fusion proteins of a DNA virus and a negative-strand RNA virus has allowed information about one protein (e.g., the identification of the rhabdovirus fusion loops) to be carried over to the other.¹⁰² Only the postfusion structure of gB has been determined so far. The gB conformational transition is triggered not by changes in pH, but rather by receptor binding to another surface protein, gD.^{31,127} A binding-induced conformational change in gD leads to the reorganization of gB, with participation of yet another protein, the gH/gL heterodimer.^{48,150}

Penetration by Nonenveloped Viruses

Nonenveloped viruses must breach a membrane to access the cytoplasm or nucleus of a cell, but unlike their enveloped cousins, they cannot do so by membrane fusion. One can imagine two classes of models by which a nonenveloped particle, bound at the surface of a cell or taken up into an endosome or other internal compartment, translocates itself (or its genome) across the intervening lipid bilayer (i.e., *penetrates*). Models of one

class (*pore formation*) invoke creation of a pore, through which the viral genome is drawn into the cell. Those of the other class (*membrane perforation*) postulate a more extensive, transient disruption of a cellular membrane (e.g., the membrane of an endosome), in order to admit the virion (in altered form) into the cytosol. Either of these models is consistent with a variety of distinct molecular mechanisms. In all well-studied cases, binding of a receptor, co-receptor, or some other ligand induces a conformational change in the virus particle, with consequent exposure of previously buried, hydrophobic structures. Examples of the exposed components are a pore-forming peptide or protein, frequently N-terminally myristoylated; a protein with membrane-interacting, hydrophobic loops; and a lipase. Certain bacterial viruses, such as the T-even bacteriophages, have much more elaborate injection structures that couple the induced conformational change to mechanical force generation.

Released or exposed virion components that bear an N-terminal myristoyl group include VP4 of picornaviruses,⁴⁷ VP2 of polyomaviruses,¹⁹⁸ and μ 1N of reoviruses.¹⁶⁶ Myristoyl groups target proteins to membranes, and it is logical to suppose that exposure of the myristoylated peptide protein leads it to associate with membranes and ultimately to contribute to penetration. In at least one case (reovirus μ 1N), pore-forming activity has been shown directly.

Receptor binding by picornaviruses triggers a rearrangement or destabilization of the virion, exposing the myristoylated VP4 as well as a hydrophobic N-terminal segment of VP1.^{24,78,89,100,101} Evidence from electron microscopy suggests that a poliovirus particle, bound to membrane-anchored receptors and therefore altered in this way, interacts closely with the receptor-bearing membrane.^{25,228} In one proposed model, the exposed hydrophobic segments form a pore in the endosomal membrane, through which the genomic RNA passes.^{24,110} This model requires a mechanism for destabilizing secondary-structural elements in the RNA in order to make translocation possible. One candidate helicase would be a ribosome or ribosome-associated factor, by analogy with an uncoating mechanism established (*in vitro*) for certain positive-strand RNA plant viruses. With those viruses, exposure of the 5' end of the RNA (e.g., through expansion of the virion induced by intracellular ionic conditions) leads to association of ribosomes with the still largely packaged RNA genome, and progress of the ribosome along the message-sense genome appears to uncoat the particle.^{203,204,245} A similar mechanism could, in principle, draw RNA through a membrane pore as well as through an opening in the viral shell. An alternative model for picornavirus penetration would involve membrane disruption (a "large" pore). If receptor binding and subsequent endocytosis caused the shell to dissociate, rather than just to expand or reorganize, components of the dissociated shell could be the agents of membrane disruption, and concomitant RNA unwinding would not be required.

For adenoviruses, the entry route is endosomal uptake; penetration proceeds by disruption of the endosome containing the virion.^{75,92} The subviral particle admitted to the cytoplasm lacks pentons as a result of events triggered by receptor and co-receptor binding. Exposure of an internal viral protein, pVI, which depends on the activity of a packaged viral protease, leads to perforation of the endosomal membrane.^{206,241} The membrane-disrupting properties of pVI

may come from an N-terminal amphipathic α -helix.¹⁵⁸ Following penetration, the partially stripped virion migrates to a nuclear pore, where it disassembles and liberates its DNA for nuclear import.

Like adenoviruses, the dsRNA viruses release into the cytoplasm an intact, roughly 700-Å-diameter subviral particle (called the *core* in the case of reoviruses and the *double-layered particle* in the case of rotaviruses). This inner capsid particle never uncoats, however, as it contains all the enzymes necessary for messenger RNA (mRNA) synthesis and modification (see Chapters 44–46). The penetration protein of reoviruses is the outer-shell trimer, μ 1.^{38,111,165} On the virion, this protein is associated with a "chaperone" subunit, σ 3; degradation of σ 3 (by proteases in the gut or by cathepsins in endosomes) and autocleavage of μ 1 allows μ 1 to release a myristoylated, N-terminal peptide (μ 1N). The released peptide, up to 600 copies of which could emerge from a single virion, forms membrane pores. The penetration protein of rotaviruses is VP4, which must also be cleaved (by intestinal trypsin) to activate entry. VP4 is not a homolog of μ 1, although rotaviruses do have such a homolog, VP6, which appears to have a purely structural role.¹⁴⁹ The conformation of VP4 changes quite dramatically when cleaved to VP8* and VP5* by trypsin: the initially disordered "spikes" of this protein become rigid projections,^{55,202} which rearrange further in subsequent, penetration-inducing steps.^{63,251} VP5* presents a set of hydrophobic loops, noticeably similar to the fusion loops of class II and class III fusion proteins, that direct membrane association.¹²³ The observed conformational transitions of VP5* resemble the folding back of fusion proteins, but there is yet no evidence for a direct coupling of these rearrangements to membrane breakage.

Parvoviruses have a single kind of coat subunit (see Fig. 3.5), but a few of the 60 copies of this protein have an extra, N-terminal domain, which is sequestered within the virion. This domain is a phospholipase A₂. During entry, it moves to the outside of the particle, to which it nonetheless remains tethered. Its lipase activity is essential for entry.⁷³

Disruption of the membrane of an endosome or other intracellular compartment is a relatively nonspecific process, in the sense that other particles within the same compartment can accompany the active particle into the cytosol, once the membrane is breached. Thus, several of the viruses described earlier mediate penetration of bacterial toxins that lack their own cell-entry mechanism, and other viruses (e.g., adenoviruses or nondefective parvoviruses) can complement a phospholipase-deficient parvovirus.

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Virus Entry and Uncoating

The Barriers

Virus Binding to the Cell Surface

Role of Carbohydrates

Mobility of Cell-Associated Viruses

Virus-Induced Signals

Endocytic Pathways of Infection

Clathrin-Mediated Endocytosis

Macropinocytosis

Caveolar and Lipid Raft-Mediated Endocytosis

The Endocytic Pathway

Penetration by Membrane Fusion

Penetration by Nonenveloped Viruses

Intracellular Trafficking

Uncoating

Triggering the Uncoating Program

Nuclear Import

Transmission Directly from Cell to Cell

Perspectives

Viral particles have a single mission: to transport the viral genome from an infected host cell to a noninfected host cell and to deliver it into the cytoplasm or the nucleus in a replication-competent form. The target can be a neighboring cell, a cell elsewhere in the host organism, or a cell in another organism. The process starts in an infected cell with the packaging of the viral genome and accessory proteins into a new virus particle, which is released into the extracellular space. When the virus contacts the surface of a new host cell, a complex series of events ensues tightly coordinated in time and space. These events include binding to receptors and signaling, often followed by endocytic internalization, vesicular trafficking, membrane penetration, cytosolic transport, and nuclear import (Fig. 4.1). Uncoating is an integral part of the process; the virus particle is modified, destabilized, disassembled, and eventually the genome, present in a protected and condensed form in the virion, is decondensed and exposed in a replication- or transcription-competent form. The progression of a virus particle through its entry program depends critically on cellular functions. The *Trojan horse strategy* that is used is necessary because the particles are simple and capable of limited independent functions.

This chapter describes some of the general concepts that govern cellular entry of animal viruses. For information about the entry of specific viruses and virus families, the reader is referred to the virus chapters. Information relevant to the

topics covered here also can be found in numerous reviews that cover early virus cell interactions.^{43,81,100,109,110,124,150,167,212}

THE BARRIERS

The first barrier that incoming viruses must overcome is the glycocalyx, a layer of glycoconjugates that covers the external surface of cells. It is composed of glycoproteins, glycolipids, and proteoglycans. The composition and thickness of this layer is variable. By binding to oligosaccharides, many viruses make use of the glycocalyx for initial attachment.

The next barrier is the plasma membrane. Responsible for the cell's exchanges with the environment, it is the most complex and most dynamic of all cell membranes. The composition and properties are regulated by the endocytic and secretory pathways and by a continuous association and disassociation of proteins that interact with the cytosolic leaflet. The plasma membrane is a highly sensitive organ for recognizing and responding to external stimuli. Viruses take advantage of this during entry.

After clearing the plasma membrane by direct penetration or by exploiting endocytic pathways, viruses and viral capsids have to reach sites deeper in the cytoplasm. The cortical actin network underneath the plasma membrane and extreme crowding constitute major barriers to movement within the cytoplasm.¹⁸⁴ Finally, because many viruses replicate in the nucleus, the genome and accessory proteins must travel to the nucleus and cross the nuclear envelope. This requires cooperation between the incoming virus and the nuclear import machinery.²¹¹

VIRUS BINDING TO THE CELL SURFACE

Viruses can only infect cells to which they can bind. Binding occurs to *attachment factors* and *virus receptors* on the surface of the cell. To a large extent, the identity, distribution, and behavior of these cellular components determine which cell types, tissues, and organisms a virus can infect. The receptors also define, in part, the pathogenic potential of a virus as well the nature of the disease that it causes.

Virus receptors can be defined as cell surface molecules that bind the incoming viruses to the cell, and, in addition, promote entry by (a) inducing conformational changes in the virus that lead to priming, association with other receptors, membrane fusion, and penetration; (b) transmitting signals through the plasma membrane that lead to virus uptake or penetration and prepare the cell for the invasion; or (c) guiding bound virus particles into a variety of endocytic pathways.¹²⁴ *Attachment factors* help to concentrate the particles on the cell's

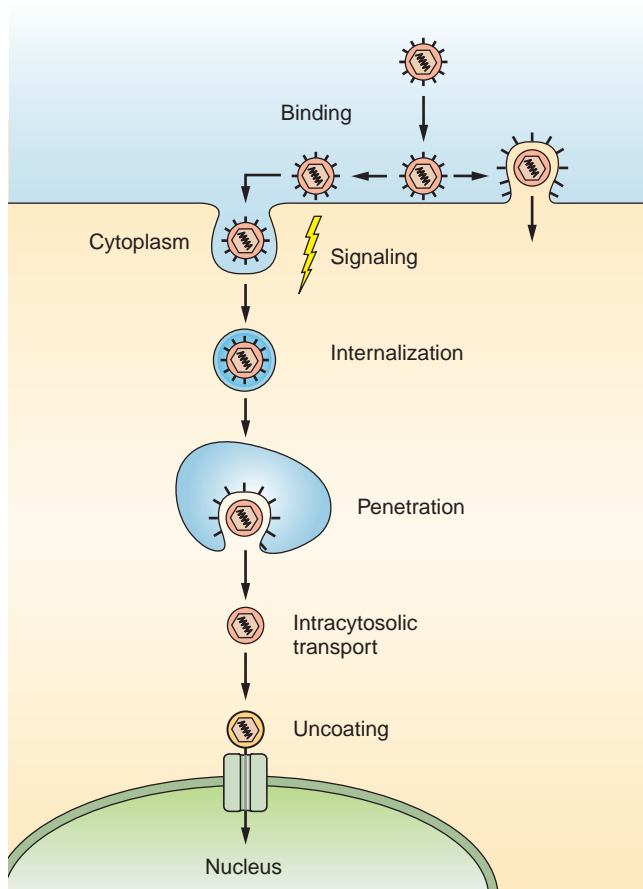


FIGURE 4.1. Stepwise entry of animal viruses. The entry of animal viruses involves a series of steps that start with virus binding to the cell surface. Binding is multivalent and involves cell surface molecules serving as passive attachment factors or receptors that are more active in that they activate signaling pathways, induce conformational changes in the virus, or mediate endocytic internalization. Although some enveloped viruses are able to fuse with the plasma membrane, the majority of viruses rely on internalization by different endocytic mechanisms. Internalization is followed by transport to secondary organelles (usually endosomes), where the virus receives cues to trigger the penetration process. After reaching the cytosol, the viruses or viral capsids are transported to the site of genome uncoating and replication. For most DNA viruses and a few RNA viruses, this site is the nucleus. Many viruses have evolved mechanisms that allow them to deliver their genome and accessory proteins through the nuclear pore complexes.

surface, thus enhancing entry and infection. Unlike receptors, however, they do not actively promote entry and mediate signals. Often, the interactions with attachment factors are not highly specific. In practice, the distinction between a receptor and an attachment factor is not always straightforward because the consequences of virus binding to a given surface component are difficult to assess experimentally and can vary depending on cell type and conditions.

Receptors and attachment factors constitute a diverse collection of proteins, carbohydrates, and lipids with physiologic functions unrelated to pathogen interaction. Ranging from abundant and ubiquitous to rare and species specific, they differ from one virus to the next. In the past few decades, an

impressive number have been identified in different virus and host cell systems. As shown in Table 4.1, which gives a partial list, receptors include ligand-binding receptors, glycoproteins, ion channels, gangliosides, carbohydrates, proteoglycans, and so on. Some families of surface molecules seem to be favored; the immunoglobulin-G superfamily of transmembrane proteins, proteoglycans, and glycoconjugates with terminal sialic acid residues, belong to these. In addition, a large group of viruses use integrins as their receptors.¹⁹⁰

Many viruses use multiple attachment factors and receptors. They interact with them in parallel or in series, or they use different receptors for entry into different cell types. When multiple receptors are required for productive entry, it is the convention to call the one contacted first a *receptor* and the subsequent ones *coreceptors*. A good example is human immunodeficiency virus type 1 (HIV-1), which makes use of heparan sulfate proteoglycans as attachment factors, CD4 as a receptor,²⁵ and CXCR4 and CCR5 (or a related chemokine receptor) as coreceptors.^{13,127} In this case, the two receptors are used to induce stepwise conformational changes in the spike glycoprotein. In other cases (e.g., adenovirus 2 and coxsackie B virus), two receptors seem to be needed to overcome anatomic and topological barriers.^{39,131} Herpes viruses are able to infect a variety of cell types, probably in part because they possess proteins that can bind to several different receptors.¹⁸⁵ Cases are also seen where unrelated viruses make use of the same receptors. One well-studied example is coxsackie and adenovirus receptor (CAR) (Table 4.1), which is used as a receptor both by adenovirus 2 and 5 and coxsackie B viruses.²¹⁹

The interaction between a viral surface protein and a receptor can be highly specific, but the affinity is often low. However, the presence of multiple, closely spaced binding sites on the surface of the virus particle allows multivalent binding, and the avidity is therefore frequently high. The affinity of influenza hemagglutinin for sialic acid containing glycoconjugates is, for example, in the millimolar range, but virus binding to cells is virtually irreversible.¹⁸⁰ That most receptor molecules are laterally mobile in the plasma membrane allows, moreover, the formation of a local *microdomain* rich in receptors under the bound virus with a composition and properties different from that in the surrounding membrane.⁵² The consequences can be the inclusion of the virus in lipid rafts, or entrapment in caveolae, coated pits, and other membrane specializations. Receptor clustering can also lead to transmembrane signaling, changes in the actin cytoskeleton, and recruitment of cytosolic factors such as a clathrin coat to the plasma membrane.

Although the interaction between a virus and its receptors is generally direct, there are cases where adaptor proteins are involved. For example, binding of antibodies to dengue virus allows infection of macrophages via Fc receptors on the surface of these cells.¹⁴³ In this case, the virus particle is internalized as an immune complex. Instead of neutralizing the virus, the antibodies mediate expansion of the host cell repertoire.

In enveloped viruses, the spike glycoproteins are responsible for receptor binding. Typically, these are oligomeric type 1 integral membrane proteins that have the bulk of their mass outside the membrane with the receptor-binding domain exposed. Other external domains in the same protein may be responsible for membrane fusion and receptor destruction. In some spike proteins (e.g., the HIV-1 glycoprotein), the receptor-binding moiety is not covalently connected to the rest, which allows its dissociation

TABLE 4.1 Receptor Proteins for Some Viruses

Virus	Family	Receptor	Function	References
G-protein-coupled receptors				
HIV	<i>Retroviridae</i>	CXCR4, CCR3, CCR2b, CCR8 CCR5	Chemokine receptors	2,32,47,163
HIV/SIV	<i>Retroviridae</i>	CCR5, Bonzo/STRL-33/ TYMSTR, BOB/GPR15, GPR1	Chemokine receptors	3,56,99
Proteins with multiple membrane-spanning domains				
GALV/FelV-B/SSAV	<i>Retroviridae</i>	PiT-1	Phosphate transport	137,196
MLV-E	<i>Retroviridae</i>	MCAT-1	Cationic amino acid transport	1
MLV-A	<i>Retroviridae</i>	PiT-2	Phosphate transport	125,205
MLV-X/MLV-P	<i>Retroviridae</i>	XPR1/Rmc1/SYG1	Transporter	8,195
HCV	<i>Flaviviridae</i>	CD81	Tetraspanin membrane protein	147
Immunoglobulin-related proteins				
Poliovirus	<i>Picornaviridae</i>	PVR (CD155)	Adhesion receptor	121
PRV/BHV-1	<i>Herpesviridae</i>	PVR (CD155)	Adhesion receptor	67
HSV-1/HSV-2/PRV	<i>Herpesviridae</i>	Prr2/HveB/nectin-2	Adhesion	55
HSV-/HSV-2/	<i>Herpesviridae</i>	Prr1/HveC/nectin-1	Adhesion	67
Coxsackie B	<i>Picornaviridae</i>	CAR	Homotypic cell interaction	9,198
Ad-2/Ad-5	<i>Adenoviridae</i>	CAR	Homotypic cell interaction	10,198
MHV-A59	<i>Coronaviridae</i>	MHVR/Bgp1 (a)	Biliary glycoprotein	49
Human rhinoviruses (type B, and A major group)	<i>Picornaviridae</i>	ICAM-1	Cell adhesion/signaling	71,188
HIV/SIV	<i>Retroviridae</i>	CD4	T-cell signaling	106
HHV-7	<i>Herpesviridae</i>	CD4	T-cell signaling	104
Low-density lipoprotein receptor–related proteins				
Rous Sarcoma virus (type A)	<i>Retroviridae</i>	LDLR	Lipoprotein receptor	7
Human rhinoviruses (type A, minor group)	<i>Picornaviridae</i>	LDLR/ α 2MR/LRP	Lipoprotein receptors	80
Integrins				
Adenovirus	<i>Adenoviridae</i>	α v β 3	Vitronectin binding	213
Coxsackie A9	<i>Picornaviridae</i>	α v β 3	Vitronectin binding	159
Adenovirus	<i>Adenoviridae</i>	α v β 5	Vitronectin binding	214
Echoviruses-1/-8	<i>Picornaviridae</i>	α 2 β 1	Collagen/laminin binding	12
Foot-and-mouth-disease virus	<i>Picornaviridae</i>	α 2 β 1, α v β 3, α v β 6	Vitronectin binding	14,84
Hantaan virus	<i>Bunyaviridae</i>	α 3 integrins		65
Rotavirus	<i>Reoviridae</i>	α 4 β 1, α v β 3, α 2 β 1		78
Cytomegalovirus	<i>Herpesviridae</i>	α v β 3, α 2 β 1, α 6 β 1		58
Tumor necrosis factor receptor–related proteins				
ALV-B/D/E	<i>Retroviridae</i>	TVB	Apoptosis-inducing receptor	17
Herpes simplex virus 1	<i>Herpesviridae</i>	HveA	LIGHT receptor	26,115
Small consensus repeat–containing proteins				
Epstein-Barr virus	<i>Herpesviridae</i>	CR2	C3d/C3dg/iC3b binding	59,60
Measles	<i>Paramyxoviridae</i>	CD46	Complement inhibition	48
Echoviruses	<i>Picornaviridae</i>	CD55	Complement inhibition	9
Coxsackie B-1/-3/-5	<i>Picornaviridae</i>	CD55	Complement inhibition	11,173
Miscellaneous				
Coronavirus-229E/TGEV	<i>Coronaviridae</i>	Aminopeptidase-N	Metalloproteinase	11,217
LCMV/Lassa fever virus	<i>Arenaviridae</i>	α -Dystroglycan	Laminin/agrin binding	24

TABLE 4.2 pH-Dependence of Virus Families

Low pH-dependent	pH-independent
Adeno	Corona (majority)
Alpha	Retroviruses (majority)
Borna	Herpes (majority)
Bunya	Paramyxo
Corona (some)	Hepadna
Filo	Pox (some)
Flavi	Rota
Orthomyxo	Picorna (most)
Parvo	Noro
Papilloma	
Picorna (some)	
Pesti	
Pox (some)	
Rhabdo	
Arena	
Arteri	
Hepaci	

once receptor interaction has occurred. X-ray crystal structures of spike glycoprotein–receptor complexes exist for several enveloped viruses (see Chapter 3).

In nonenveloped viruses, the structures that bind receptors are projections or indentations in the capsid surface. Adenoviruses have trimeric fiber proteins with globular knobs that project from the vertices.¹⁵ The penton base protein of many adenovirus subfamilies contains in addition an exposed Arg-Gly-Asp (RGD) sequence that associates with integrins.¹⁸⁹ Many enterovirus receptors bind in a cleft in the capsid surface called the *canyon*, the molecular features of which have been analyzed in great detail.¹⁶¹

ROLE OF CARBOHYDRATES

Glycoconjugates on the cell surface have an important role during entry of many viruses as receptors and attachment factors. Glycoproteins and glycolipids, with terminal sialic-acid residues, serve as specific receptors for a variety of viruses, including orthomyxo-, paramyxo-, and polyoma viruses. The HA1 subunits of influenza A virus hemagglutinin (HA) bind terminal sialic acid residues associated with galactose through either a Neu5Ac $\alpha(2,3)$ -Gal or Neu5Ac $\alpha(2,6)$ -Gal bond.¹⁸⁰ Human influenza recognizes the $\alpha(2,6)$ linkage; avian and equine viruses, the $\alpha(2,3)$ linkages, whereas porcine viruses appear to recognize both. These specificities reflect the structure of the glycans expressed in the different species and play a central role in limiting cross-species transmission. The tetrameric hemagglutinin-neuraminidase (HN) proteins of parainfluenza virus 5, has specificity for $\alpha(2,3)$ -sialyllactose,²¹⁸ and polyoma-viruses bind to specific saccharide residues in the glycan moieties of various gangliosides.²⁰¹ A difference limited to a single atom in sialic acids plays a major role in species specificity of simian virus 40 (SV40), because it binds better to the simian GM1 ganglioside, which has a *N*-glycolylneuraminic acid, than to the human, which has a *N*-acetylneuraminic acid.²³

The list of viruses recognized as binding to glycosaminoglycan (GAG) chains (e.g., heparan sulfate) is steadily growing.⁶ It now includes several herpes-, alpha-, flavi-, retro-, parvo-, picorna-, and papillomaviruses. Binding often involves positively charged patches in viral surface proteins. In some cases, viruses adopt GAGs as receptors when grown in tissue culture; their surface proteins mutate and express more basic residues.^{22,181} In contrast to tissue culture-adapted strains, natural isolates do not necessarily bind to heparan sulfate. Indeed, adaptation of different glycan receptors is likely part of the age-old war against pathogens, including viruses and their hosts, a war in which the diversity of surface carbohydrates plays an important role.

In most cases, it is the viruses that recognize host cell glycans. However, the reverse is true when cell surface lectins bind to glycans present in the envelope proteins of incoming viruses. One such lectin is DC-SIGN, a tetrameric, C-type lectin present on the surface of immature dendritic cells. It binds *N*-linked glycans of the high-mannose type,⁵⁷ such as in glycans that have failed to undergo terminal glycosylation in the Golgi complex of the infected cells. Because glycoproteins synthesized in insect cells have exclusively high-mannose glycans, viruses introduced into the skin via insect bites are often recognized by DC-SIGN, resulting in the infection of dendritic cells. Viruses that bind to these lectins include HIV-1, Sindbis, human cytomegalovirus, dengue, and severe acute respiratory syndrome (SARS) viruses.^{66,91,103,149,178,197} Thus cells that our body uses in the front-line defense against pathogens end up serving the interests of viruses instead of spreading the infection.

MOBILITY OF CELL-ASSOCIATED VIRUSES

The encounter between individual viruses and the cell can be visualized live by light microscopy using fluorescent viruses. What happens depends on the virus, the receptor, and the host cell. Parvovirus particles undergo rapid binding and release events that eventually result in permanent attachment and endocytic internalization.¹⁷¹ Polyomavirus particles bind firmly and diffuse laterally in the membrane for 5 to 10 seconds, after which they are arrested in confinement zones defined by the cortical actin network and eventually internalized.⁵⁴ Reoviruses do not show lateral motion after binding.⁵⁰ In the case of a bunyavirus, Uukuniemi virus, the rapid clustering of receptor molecules (GFP-tagged DC-SIGN) can be seen to occur at the site of virus binding.¹⁰²

Filopodia have been shown to play an active role by providing directed transport of surface-associated virus particles toward the cell body.⁹⁶ They are thin, mobile extensions of the plasma membrane stabilized by an actin filament bundle. Such “virus surfing” occurs at a rate of 1 to 2 $\mu\text{m}/\text{min}$, mirroring the rate of retrograde actin flow from the tip of the filopodia inward.¹⁶⁸ It is actin dependent and inhibited by inhibitors of myosin II. Although such motility of viruses is not essential for infection in tissue culture cells, it may play a role in tissues.

After endocytosis, the actin- and microtubule-dependent movement of intracellular vacuoles, viruses, and naked capsids inside the cell can also be visualized.^{50,77,95,164,207} This is illustrated by fluorescent influenza A viruses, which after a slow period of actin-restricted motion in the cell periphery, undergo rapid microtubule-mediated transport toward the perinuclear space where penetration by membrane fusion occurs.^{95,164} In

the case of adenovirus 2, the transport of capsids along microtubules is both plus- and minus-end directed, but net transport in the minus-end direction allows the virus to reach the nucleus.¹⁹³ The entry of parvovirus adeno-associated virus 2 has been traced all the way to the nucleus, inside of which it moves unidirectionally along well-defined pathways.¹⁷¹

VIRUS-INDUCED SIGNALS

Many viruses use the host cell's signaling systems to promote entry and optimize infection.^{68,124,131} Viruses take advantage of the fact that cells are exquisitely sensitive to ligands that bind to the plasma membrane, particularly if they induce clustering of surface components. More specifically, signaling is used to trigger access to coreceptors, to induce endocytic responses, to reprogram endocytic pathways, and to induce favorable intracellular conditions for infection.

Signaling starts at the plasma membrane after binding of the virus to receptors and formation of receptor clusters. Depending on the virus, receptors, and host cells, initial binding can lead to activation of tyrosine or other kinases, which, in turn, trigger cascades of downstream responses at the plasma membrane, in the cytoplasm, and, in some cases, in the nucleus. Virus-induced signaling depends on the usual panel of second messengers (phosphatidylinositides, diacylglycerides, and calcium), and on numerous regulators of membrane trafficking and actin dynamics.

One well-studied case that demonstrates the complexity of virus-induced signaling is provided by adenoviruses 2 and 5, which use CAR and integrin $\alpha\beta 3$ as receptors.^{68,131} Endocytic internalization occurs via clathrin-coated vesicles, and penetration takes place in endosomes. The interaction with the integrin triggers activation of p85/p110, a PI(3) kinase. The synthesis of PI(3,4)P₂ and PI(3,4,5)P₃ activates protein kinase C. Small GTPases (e.g., Rab and Rho family members) are also activated. One of the downstream responses is the transient activation of macropinocytosis, an actin-dependent process that results in a rapid increase in internalization of fluid.¹¹⁸ This response seems to promote subsequent penetration of adenovirus from endocytic vacuoles by virus-induced rupture.

Another example is SV40, which is entirely dependent on signaling for entry. After binding to GM1 gangliosides, the virus induces local activation of tyrosine kinases, which results in actin filament reorganization, activation of caveolar dynamics, internalization of the virus in caveolar or lipid raft vesicles, and induction of long-distance transport of the virus-containing vesicles.^{44,135,145,187} More than 50 different kinases were shown to regulate the entry and early steps in the infection of HeLa cells by this virus.¹⁴⁴

A final example involves Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8).²⁸ The glycoprotein gB of this virus possesses an RGD sequence in the ectodomain that allows it to bind to the integrin $\alpha 3\beta 1$. Binding activates focal adhesion kinase (FAK) and Src kinases, which, in turn, activate PI(3) kinases and Rho GTPases. Furthermore, via the PI(3) K-PKCzeta-mitogen activated or extracellular regulated kinase (MEK) pathway, the virus induces the extracellular signal-regulated kinase 1 and 2 (ERK1/2). Activation of these pathways leads to major alterations in the actin cytoskeleton, and the virus is internalized by macropinocytosis in human fibroblasts.

ENDOCYTIC PATHWAYS OF INFECTION

Whether viruses penetrate into the cytosol directly through the plasma membrane or after endocytosis has been a hotly debated issue from the beginning of animal virology. It is now recognized that a majority of animal viruses—whether enveloped or nonenveloped—make use of endocytosis for productive infection. They exploit one or more of several endocytic mechanisms offered by cells (Fig. 4.2), and most of them enter endocytic vacuoles where penetration into the cytosol occurs often triggered by low pH. Enveloped virus families (e.g., paramyxoviruses, herpesviruses, and retroviruses) that can penetrate directly through the plasma membrane because they do not require endocytosis for fusion may still depend on endocytosis for productive infection at least in some cell types.^{92,133} Here, the reason may be that fusion at the plasma membrane remains nonproductive because it does not ensure passage of the capsids through further barriers such as the actomyosin cortex.¹⁰⁸

The main reason why endocytosis is a preferred mode of entry is most likely that endocytic vesicles offer viruses a free ride through the cortical cytoskeleton and other barriers that encumber movement of virus-sized particles in the cytoplasm. By delaying their penetration, viruses can in this way get a ride to the perinuclear region of the cell. In endocytic vacuoles, viruses can, moreover, count on receiving specific cues such as a drop in pH and exposure to proteases to trigger penetration

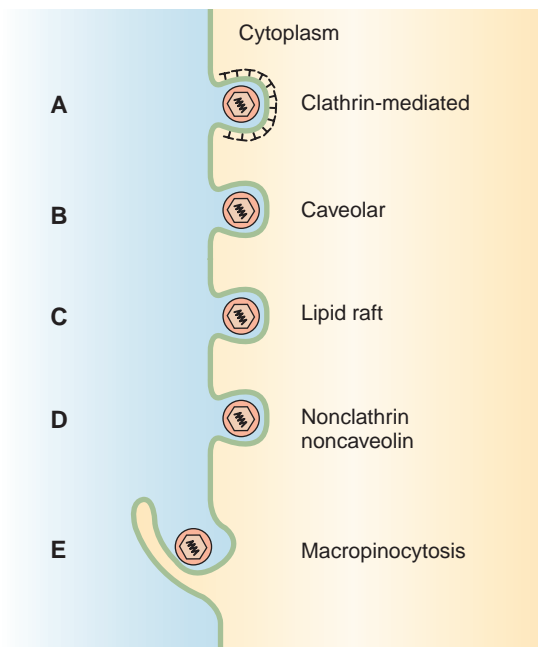


FIGURE 4.2. Mechanisms of endocytosis used for virus entry. Viruses can use different mechanisms of endocytosis. The majority of animal viruses enter cells by endocytosis. The mechanisms include (A) clathrin-mediated endocytosis, the most commonly used mechanism for virus entry; (B) caveolar endocytosis, a caveolin and lipid raft-dependent mechanism mainly used by polyomaviruses; (C) a caveolin-independent, lipid raft-mediated mechanisms with many similarities to the caveolar mechanism; (D) various caveolin- and clathrin-independent mechanisms often with similarities to macropinocytosis; and (E) macropinocytosis, a transient, ligand-induced, actin-dependent mechanism.

and uncoating. Because no trace of the virus is left exposed on the plasma membrane, immunorecognition of infected cell is delayed. Moreover, for nonenveloped viruses that use membrane lysis or pore formation for penetration, it may be essential to cross membranes of intracellular organelles to minimize damage to the cell.

In recent years, the landscape of endocytosis research has expanded dramatically beyond phagocytosis and the classic clathrin-mediated endocytosis pathway. New mechanisms include caveolar or lipid raft-mediated endocytosis, macropinocytosis, and several other clathrin- and caveolae-independent pathways (Fig. 4.2).^{46,116,123,141,203} The situation is often confusing because the mode of uptake of a virus can vary between cell types and strains, and many viruses can make use of multiple receptors and parallel routes of endocytosis in the same cell. In addition to caveolae, SV40 can, for example, utilize a related, noncaveolar pathway.⁴⁴ Influenza A uses both clathrin-mediated and clathrin-independent pathways,^{112,164,175} and HIV-1, which can fuse with the plasma membrane, can in some cell lines also make efficient use of an endocytic pathway for entry.⁴² The use of multiple receptors and redundant endocytic pathways provides viruses with a degree of flexibility and adaptability that make entry a difficult step for host organisms to protect themselves against.

The cell biology of endocytosis and its regulation is complex.^{35,98} Not surprisingly, the analysis of virus entry by high throughput siRNA silencing screens have led to the identification of hundreds of genes involved as critical factors in early infection of tissue culture cells.^{31,79,144} Entry studies are often further complicated by the fact that only a small fraction of the cell-associated viruses enter productively. Because most morphological and biochemical methods fail to distinguish between particles that enter productively and those that do not, studies using these methods must be complemented with readouts based on infection (i.e., the biological outcome of successful entry). This involves the use of inhibitors, dominant negative mutants, small interfering RNAs, mutant viruses, and mutant cell lines. Only a combination of methods allows pathways of productive entry to be charted with confidence.

CLATHRIN-MEDIATED ENDOCYTOSIS

The clathrin-mediated endocytic pathway is used by many viruses (Figs. 4.2 and 4.3). It is a process that cells use to internalize a spectrum of receptor-bound ligands, fluid, membrane proteins, and lipids for recycling or degradation. By binding to receptors that have the internalization signals necessary for inclusion in clathrin-coated pits, viruses make use of this pathway as opportunistic ligands. Uptake is characterized by rapid kinetics (viruses are generally internalized within a few minutes after binding) and by high capacity (3,000 virus particles or more per minute).¹⁰⁹ With a diameter up to 120 nm, coated vesicles are large enough for the endocytosis of most animal viruses. Sometimes larger particles (e.g., vesicular stomatitis virus [VSV]) can be accommodated.¹¹³

Although clathrin-mediated endocytosis is a continuously ongoing process, it is under stringent control. Interestingly, when the uptake of VSV, influenza, and reovirus particles has been followed in live cells, it has been observed that most are

internalized by clathrin-coated pits that form *de novo* under the virus particles.^{41,85,164} Only a few enter via pre-existing clathrin-coated pits. Exactly how the virus induces a transbilayer signal to direct the assembly of the clathrin coat remains to be defined.

A role for clathrin-coated pits in internalization and infection can be demonstrated by inhibiting clathrin function using dominant negative mutants or depletion of adaptors such as epsin, eps15, AP2, or the clathrin chains themselves.^{37,175} Inhibition of dynamin 2, a scission factor in clathrin vesicle formation, is not a sufficient indicator for clathrin involvement because dynamin 2 is also involved in other forms of endocytosis.

MACROPINOCYTOSIS

Among the clathrin-independent mechanisms, macropinocytosis and related processes are commonly used by larger viruses such as vaccinia, herpes, adeno 3, and Ebola virus, but evidently also in some cases by smaller viruses such as HIV-1 and influenza A.^{4,87,107,122,165} Macropinocytosis is ligand triggered, transient, actin dependent, and regulated by a complex signaling pathway.^{123,194} The physiological cargo is mainly composed of extracellular fluid that is trapped in large vacuoles, the formation of which depends on plasma membrane ruffling. The process differs from phagocytosis in the signaling pathways used and in that it can be activated in most cell types, not only in specialized cells.¹⁹⁴ In addition, by serving as a major mechanism in the elimination of apoptotic debris in tissues, macropinocytosis differs from phagocytosis by failing to activate innate immune responses and inflammation.

In macropinocytosis, the interaction of viruses with the plasma membrane induces a rapid activation of receptor tyrosine kinases or integrins. This leads to a signaling cascade that usually involves the activation of GTPases Rac1 or cdc42, the p21-activated kinase (PAK1), myosin II, and numerous other kinases and signaling factors.¹²³ A change in the dynamics of cortical actin leads to ruffling of the plasma membrane, where the ruffles can take the form of lamellipodia, filopodia, and blebs. In the case of vaccinia virus and Kaposi's sarcoma virus, internalization by macropinocytosis occurs during bleb retraction, and the viruses enter macropinosomes from which they escape by membrane fusion.^{122,202} As more is learned about the mechanisms underlying macropinocytosis, it is becoming increasingly clear that there are variations of the general themes. Differences between cell lines and signaling pathways lead to a complex spectrum of related activities.

CAVEOLAR AND LIPID RAFT-MEDIATED ENDOCYTOSIS

The caveolar and lipid raft-mediated pathways of endocytosis were first observed for SV40 and mouse polyomavirus^{3,44,158,187} (Figs. 4.2 and 4.3). They are cholesterol dependent, tyrosine kinase activated, cargo induced, and involve small endocytic vesicles. The cholesterol dependence reflects a central role of lipid rafts. Three variants of caveolar or lipid raft endocytosis are currently recognized⁹⁴: (a) endocytosis via classical caveolae, dynamin 2 dependent; (b) noncaveolar, lipid raft-mediated

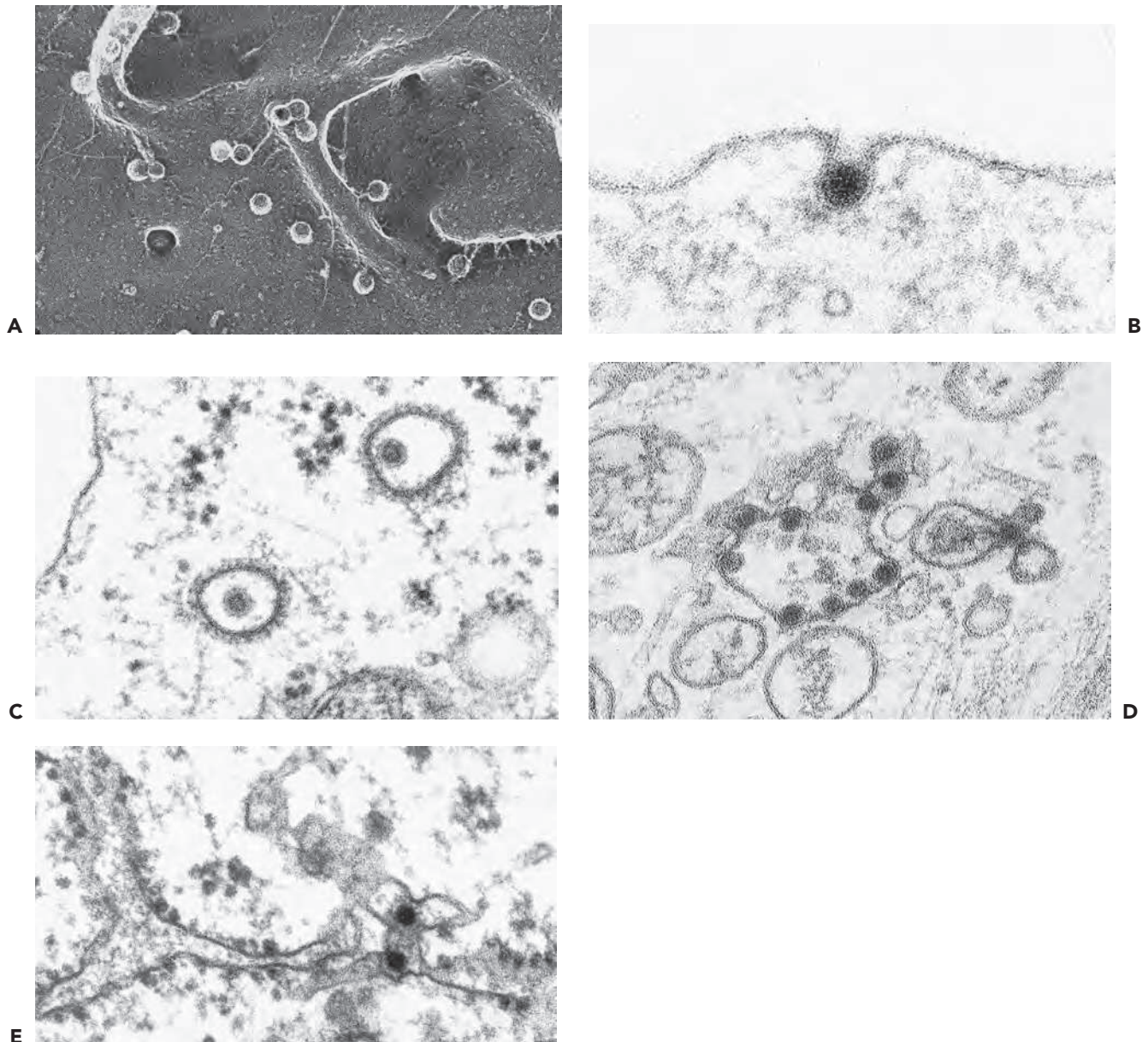


FIGURE 4.3. Electron microscopy of virus endocytosis. **A:** A surface replica of a BHK21-cell with Semliki Forest virus (SFV) particles attached. Some particles are bound to microvilli, and one is about to be endocytosed inside a coated vesicle (Courtesy of J. Heuser and A. Helenius). **B:** Internalization of a SV40 particle by caveolar- or raft-mediated endocytosis. The tight fitting vesicle in which the virus is internalized has a diameter of about 60 to 70 nm, and it has no visible coat (Courtesy of J. Kartenbeck and A. Helenius). **C:** SFV particles in clathrin-coated vesicles (Courtesy of *J Cell Biol*). **D:** SV40 particles in an early endosome. **E:** Incoming SV40 particles in a smooth membrane section of the endoplasmic reticulum, which they reach via the endocytic pathway. (From Kartenbeck J, Stukenbrok H, Helenius A. Endocytosis of simian virus 40 into the endoplasmic reticulum. *J Cell Biol* 1989;109(6 PE1): 2721–2729, with permission.)

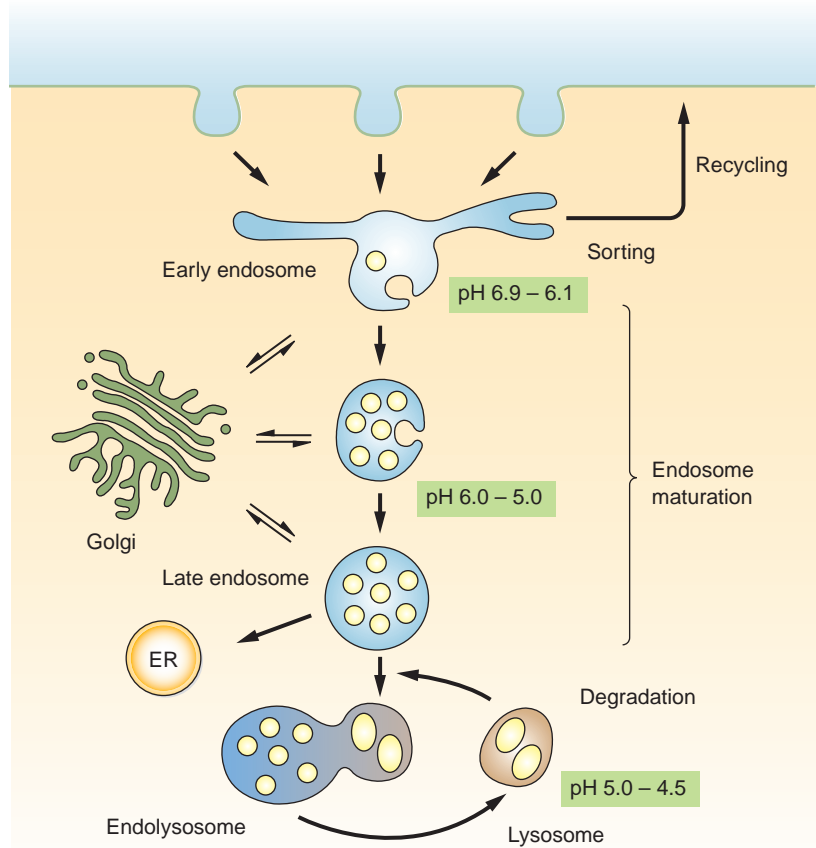
endocytosis, dynamin 2 dependent; and (c) noncaveolar, lipid raft-mediated endocytosis, dynamin independent.

Caveolae constitute 70-nm flask-shaped indentations that contain caveolins and cavins as major protein components and a membrane enriched in cholesterol and sphingolipids.^{141,156} Most cell surface caveolae are stationary, with a minority population undergoing a local cycle of fission and fusion with the plasma membrane.^{146,204} When local tyrosine phosphorylation is activated by a virus such as SV40, caveolae become more dynamic.^{94,141}

With a virus particle trapped inside, the caveolae pinch off and move into the cytoplasm where they fuse with endosomes.

SV40 and other polyomaviruses also enter in vesicles devoid of caveolar proteins.⁴⁴ After association with lipid rafts in the plasma membrane or artificial liposomes, the binding of SV40 to multiple receptor gangliosides, GM1, leads to the induction membrane curvature following the shape of the virus and the formation of tight-fitting indentations of variable depth.⁵³ For detachment of a vesicle, these inward-oriented,

FIGURE 4.4. The endosomal pathway. The pathway functions as two interconnected cycles of membrane trafficking. One involves the plasma membrane, early endosomes, and a variety of carrier vesicles. Its major role is the sorting and recycling of incoming membrane components, ligands, and fluid via the endosome back to the cell surface. In this pathway, the pH does not drop below about 6.0, and the cargo is not exposed to a spectrum of lysosomal enzymes. The main function of the second cycle, the lysosome cycle, is degradative (i.e., the down-regulation of receptor-ligand complexes, degradation, and processing of incoming nutrients and their carriers, digestion of autophagic substrates, elimination of incoming pathogens, etc.). The endocytic cargo to be degraded, including viruses, is sorted from early endosomes into late endosomes, and these deliver the cargo to lysosomes 10 to 40 minutes after formation by fusing with them to form endolysosomes. The late endosomes undergo a complex maturation process, acquire intraluminal vesicles, and move along microtubules to the perinuclear region of the cells. Degradation occurs in the endolysosomes through the action of soluble hydrolases. Endolysosomes and lysosomes keep fusing with new late endosomes in a continuous cycle. Early and late endosomes communicate via vesicle trafficking with the Golgi complex, and late endosomes and endolysosomes have a poorly understood connection to the endoplasmic reticulum.



virus-containing “buds” require the activation of kinases and other cellular factors. The process shares many features with the endocytic mechanism triggered by certain bacterial toxins, such as shiga and cholera.¹⁶⁰

THE ENDOCYTIC PATHWAY

The early endosomes in the periphery of the cytoplasm receive incoming viruses a few minutes after internalization (Figs. 4.4 and 4.5). Early endosomes constitute complex, heterogeneous organelles with tubular and vacuolar elements. They are mildly acidic (pH 6.6 to 6.0), which is enough to activate viruses with a high pH threshold for penetration, and these viruses are thought to penetrate from early endosomes.¹¹⁰ Most viruses are not activated in this pH range and continue deeper into the degradative arm of the endocytic pathway in order to penetrate from late endosomes or endolysosomes. In exceptional cases, such as some of the polyomaviruses, viruses continue even farther, using a poorly characterized transport system that brings them to the endoplasmic reticulum (ER).^{51,152} Their penetration occurs through the ER membrane. In the case of macropinocytosis, the penetration is likely to occur in macropinosomes, but there is not much information available about macropinosome maturation and fate.

To understand virus entry, it is important to understand the cell biology of endosomes. There are numerous reviews that provide insights into this important topic.^{63,73,120,166,170} One of the central topics is the maturation of late endosomes, a program

of changes that prepares them for fusion with lysosomes.⁸² The changes include a further drop in pH, a switch of predominant Rabs from Rab5 to Rab7, a switch from phosphatidylinositides (PI(3)P to PI(3,5)P₂, exchange of tethering factors for fusion, exchange of microtubule-dependent motors, formation of intraluminal vesicles, and accumulation of lysosomal membrane proteins and hydrolases. It is a complex process where the various alterations are coordinated and interdependent. The key factors include small GTPases of the Rab, Arf, and Rho families and their effectors, phosphatidylinositides and their kinases and phosphatases, protein ubiquitination and the endosomal sorting complex required for transport machinery responsible for the formation of intraluminal vesicles in endosomes, the vacuolar ATPase responsible for acidification, and various soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors and tethering factors required for selective fusion events.

Late penetrating viruses such as influenza virus, minor group rhinoviruses, polyomaviruses, and bunyaviruses depend on a smoothly functioning maturation program. They require the formation of late endosomes, the reduction in pH, and transport of the endosome to the perinuclear region.^{63,72,101,174} Infection can be blocked by interfering with the maturation program using inhibitors, dominant negative mutants, and siRNA depletion of endocytosis factors.

The significance of low pH in endosomes as a cue for the activation of virus penetration was discovered a long time ago.⁷⁶ It is now clear that for the majority of animal viruses, low pH is needed to trigger conformational changes in metastable viral particles and fusion proteins, thus activating membrane

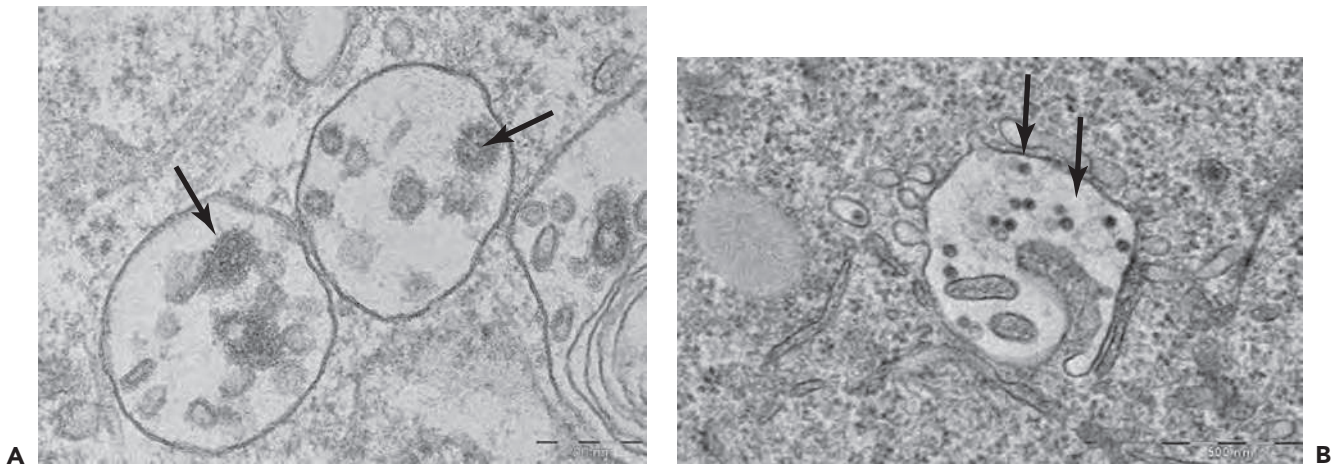


FIGURE 4.5. Viruses enter endosomes. Endosomes are cytoplasmic vacuoles with complex and quite heterogeneous morphology. Thin section electron microscopy reveals that many of them are filled with intracellular vesicles and membrane lamellae. **A:** Influenza A viruses (arrow heads) are here seen in multivesicular endosomes closely connected to microtubules. **B:** Human papilloma-16 pseudovirus particles are here seen in an endosome with tubular extensions. (Courtesy of Roberta Mancini.)

penetration mechanisms (see Chapter 3). Viruses with a relatively high pH threshold (pH 6.5 to 6.0) such as VSV are activated 3 to 10 minutes after internalization in early endosomes.¹⁰⁹ Viruses with a lower pH threshold are sorted from early endosomes into the degradative branch and penetrate later (10 to 50 minutes or even longer after infection) and less synchronously in late endosomes or endolysosomes that have a pH of 6.0 to 4.9. For example, influenza A virus, with a pH threshold of 5.6 to 4.9, passes via early endosomes to perinuclear late endosomes before membrane fusion and penetration occurs.¹⁷⁶

PENETRATION BY MEMBRANE FUSION

The membrane of an enveloped virus is a *de facto* transport vesicle designed for intercellular membrane traffic (Fig. 4.6).

Like intracellular transport vesicles, the transport process relies on budding, fission, and fusion. The cargo is the viral capsid, which does not have to cross the hydrophobic barrier of a membrane. The fusion reaction during entry can occur with the plasma membrane or with the limiting membrane of an endosome. On the basis of studies with VSV, it has been proposed that a virus can also fuse with luminal membrane vesicles inside multivesicular endosomes followed by a delayed second fusion event between the vesicle and the limiting membrane of late endosomes.^{72,162} The second fusion would have to depend on a cellular rather than viral fusion machinery.

As described in Chapter 3, viral fusion proteins are integral membrane proteins, with the bulk of their mass external to the viral envelope. They are usually glycoproteins and occur as homo- or hetero-oligomers. Many of them combine fusion and receptor-binding activities in the same molecule. To become

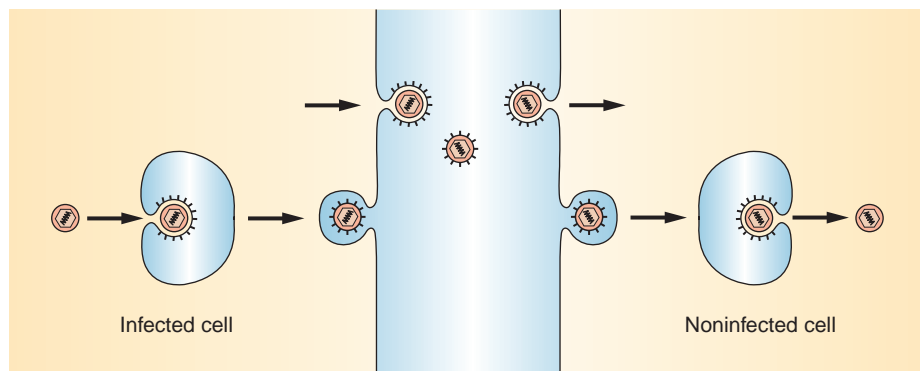


FIGURE 4.6. Enveloped viruses use a vesicle shuttle mechanism. They transport the capsids and the viral genome from cell to cell using a vesicle transport strategy based on membrane fission and fusion. The viral envelope constitutes the transport vesicle, and the capsid is the cargo. The vesicle is formed after capsid loading and glycoprotein sorting in the infected cell by budding and membrane fission at the plasma membrane or internal membranes. The viral envelope membrane protects the capsid during the voyage through the extra cellular space. After associating with a new host cell, the virus delivers the capsid into the cytosol by membrane fusion, either at the plasma membrane or after endocytosis, at the limiting membranes of intracellular organelles. The advantage of this strategy is the viral genome, and accessory proteins can be transferred from cytosol to cytosol without the need of translocation directly across the hydrophobic barrier of any membrane.

fusion competent after folding and assembly in the ER, they are often primed by proteolytic cleavage during transit through the trans-Golgi network (TGN). Typically, the proteases responsible for priming are furin family convertases that cleave either the chains involved in fusion directly (i.e., in myxo-, retro-, and paramyxoviruses) or the companion proteins in the spike protein complex (i.e., alpha- and flaviviruses).^{90,186,208} If virus assembly and budding occurs intracellularly, as for flaviviruses, the activating cleavages take place as the assembled viral particles pass through the TGN.⁷⁵ In some cases, such as Ebola virus, proteolytic activation can occur as part of the entry program by proteases present in endocytic vacuoles.³⁰

Proteolytic priming renders fusion proteins metastable and, thus, competent to undergo large, irreversible conformational changes without added energy.²⁷ This is important because, to elicit fusion, they must undergo changes that dramatically alter their quaternary or tertiary structure. As a result, they expose previously hidden hydrophobic peptide segments (fusion peptides) that interact with the target membrane, and in doing so allow the proteins to be hydrophobically anchored in both membranes.²¹⁰ The conformational change is thought to provide the energy required to overcome the so-called *hydration force* that prevents biological membranes from fusing spontaneously.¹⁵⁵

The changes in conformation are triggered either by low pH in endosomes or by interaction between viral proteins and receptors in the plasma membrane. Sometimes receptor binding followed by low pH is needed. This type of receptor-mediated priming, observed for some retroviruses,¹²⁸ may allow the virus particle to limit conversion of spike glycoproteins to the site most intimately in contact with the target membrane. The priming and activation of viral membrane fusion proteins as well as the mechanisms of fusion are discussed in greater detail in Chapter 3.

PENETRATION BY NONENVELOPED VIRUSES

Nonenveloped viruses penetrate into the cytosol through the limiting membranes of internal organelles (e.g., endosomes, lysosomes, the ER). The cues that trigger penetration are similar to those used by enveloped viruses (e.g., receptor-binding, low pH, redox environment). These viruses must, however, transfer their capsids, genomes, and associated proteins through a cellular membrane without the convenience of the membrane-fusion mechanism.

The mechanisms involved have proved challenging and remain incompletely understood. It seems, however, that non-enveloped viruses can use three general strategies:

- (a) *Membrane puncture.* The virus particle generates a pore in the membrane through which the genome is selectively released into the cytosol. The viral capsid does not enter the cytosol, and release of fluid phase markers from the lumen of the organelle is either undetectable or limited to small molecular weight compounds.
- (b) *Perforation.* The entire capsid is transferred through the membrane without major lysis of the membrane and little loss of luminal fluid markers.
- (c) *Lysis.* The virus particles induce breakage of the membrane of cytoplasmic organelles, allowing the virus and other luminal contents to be released into the cytosol.

The puncture or pore mechanism is favored for picornaviruses.^{64,81} Some rhinoviruses and foot-and-mouth disease virus are acid-activated and penetrate from endosomes, whereas others (e.g., polio, coxsackie B, and echovirus 1) are pH independent but penetrate from endosomes or other intracellular organelles. In the case of poliovirus, which is one of the best characterized, penetration is induced by binding of a cluster of poliovirus receptor (PVR) molecules to “canyons” in the capsid surface. This triggers a large, concerted, irreversible change in the particle, the so-called *eclipse*, which leads to the formation of a penetration competent conformation. An internal protein, VP4, is released, and the myristylated *N*-terminus of VP1 inserts into the endosomal membrane.^{16,61} The RNA is most likely released to the cytosolic side of the membrane through a narrow pore.¹⁹⁹ According to this view, penetration and uncoating occur simultaneously, and the capsid does not enter the cytosol. Recently, cryo-electron microscopy (cryoEM) studies have shown that one of the icosahedral vertices interacts with five receptors that connect the modified particle intimately with the membrane.¹⁹

Adenoviruses make use of a lytic mechanism.¹⁷² The best studied are adenoviruses 2 and 5, which penetrate by acid-activated rupture of the endosomal membrane.^{117,119} The lytic effect is thought to involve a change in the penton base and exposure of an amphipathic helix in protein VI, but the mechanism is unclear.¹³⁰ Altogether, the process is complex; it depends on low pH, the integrin receptors, cleavages in structural proteins induced by the L3/p23 viral protease, the release of fiber proteins, activation of macropinocytosis, and signaling through protein kinase C.^{117,119,131}

For parvoviruses, evidence is accumulating that the *N*-terminal domain of VP1 possesses a phospholipase 2 domain activated by low pH.^{38,74} It is likely that this promotes membrane penetration of the intact virus by modifying the permeability of endosomal and lysosomal membranes. Lysis of the membrane is not detected.

INTRACELLULAR TRAFFICKING

Before they can replicate, viruses and capsids delivered into the cytosol must be transported to the correct location before uncoating and replication can take place. Within the nucleus, replication usually occurs in defined foci. In the cytosol, it is often associated with specific membrane organelles (e.g., the ER or the ER-Golgi intermediate compartment) or with virus factories in the perinuclear space.^{136,215}

Given the extreme crowding in the cytoplasm that prevents diffusion of virus- and capsid-sized particles as well as uncondensed forms of DNA or RNA, it is not surprising that viruses rely on cytoplasmic transport systems offered by the cell.^{45,70,105,182} For long-distance transport, viruses mainly exploit microtubule-mediated mechanisms. When actin filaments play a role, it is usually in short-distance movement close to the plasma membrane. Although viruses can undergo partial disassembly *in transit* through the cytosol, they postpone final uncoating of the condensed genome until they have reached their final destination. Viruses travel variable distances. To reach the cell body, neurotropic viruses that enter via axons may have to move in a retrograde direction over the full length of axons, which can be more than 1 m in length.

To move through the cytoplasm, incoming viruses have two options. They can postpone penetration into the cytosol and move as cargo in endocytic vesicles and thus benefit from the motor-driven transport of vesicles and organelles through the cytoplasm. Alternatively, they can penetrate early into the cytosol, in which case the viruses or their capsids are themselves responsible for associating with molecular motors and adaptors (e.g., dynein and kinesins). The former strategy is used by viruses that enter by endocytosis, the latter by viruses that prefer to penetrate through the plasma membrane. Many viruses make use of both; part of the journey is mediated by vesicular traffic, the rest by cytosolic transport. Thus, viruses (e.g., adeno- and parvoviruses) that enter by endocytosis have been shown to use microtubule-mediated transport after penetration into the cytosol.^{171,192,193} Although transport of capsids along microtubules is often bidirectional and characterized by stops, restarts, and changes in direction, net transport generally occurs in the minus-end direction toward the microtubule organizing center, where viruses and capsids are often found to accumulate before transport to the nucleus.¹⁵⁴ Whether they switch to plus-end directed motors for the final leg of transport is not known.

UNCOATING

The entry of viruses includes partial or full disassembly as an essential, integrated part of the program. For enveloped viruses, uncoating involves loss of the envelope during membrane fusion. Often, the capsid thus released undergoes further stepwise uncoating steps. Once the capsids have reached the correct location within the cell, then, and only then, they release the replication competent form of the genome. In some cases (e.g., retro-, reo-, and poxviruses), the cytosolic capsids serve as a protected site for reverse transcription of the genome or transcription of messengers following entry into the cytosol.^{88,134}

In the case of nonenveloped viruses, the uncoating process involves conformational changes, progressive loss of structural proteins, proteolytic cleavages, isomerization of intermolecular disulfide bonds, and weakening of intermolecular interactions.^{69,81,117,132,169} For adenovirus 2, disassembly starts already at the cell surface with loss of some of the fibers followed by activation of a viral protease (the L3/p23 protease, located within the virion), proteolytic cleavage of capsid proteins, and loss of stabilizing capsid components. Final disassembly of the particle and DNA release occurs at the nuclear pore complex (NPC).

The conformational changes that accompany penetration and uncoating of polio and other picornaviruses have been extensively analyzed.^{64,81,100} Depending on the virus, the initial uncoating event is triggered by receptor association, low pH, or both. Conversion from a 150S to 160S particle to a slower sedimenting 135S particle occurs with elimination of the internal VP4 protein and externalization of the myristylated *N*-terminus of VP1. This leads to membrane association, followed by the release of the RNA, resulting in the RNA-free 80S particle. The single-stranded viral RNA is likely to escape through one of the 12 vertices, possibly aided by the VPg protein covalently linked to the 5' end of the viral RNA.^{18,89}

The capsids of viruses with a double-stranded RNA genome (e.g., reoviruses) undergo many alterations in transit into the cell, but instead of releasing their genomic RNA in free

form into the cytosol, they retain it in a modified capsid, which serves as an RNA-replication and transcription factory.^{29,177}

TRIGGERING THE UNCOATING PROGRAM

Penetration brings many viruses and viral capsids for the second time into a cytosolic environment. The first time is when they assemble in the cytosol of an infected cell or when they pass through the cytosol on their way from the nucleus to the extracellular space. During entry into a new host cell, the agenda involves disassembly and uncoating instead of assembly. This means that, in the entry phase, something must be profoundly different either about the virus itself or the cell.

Usually, the difference is in the virus or the capsid because it has undergone structural alterations *in transit*. After release from the infected cell or during earlier stages of entry, the viruses are structurally *reset* so they can respond to cellular cues according to requirements of the uncoating program. The best illustration of this is provided by retroviruses, in which the viral protease induces a series of cleavages in Gag and Gag-Pol proteins during and after virus budding. The capsid is reorganized and ready for reverse transcription and for the formation of functional preintegration complexes (PICs) in the cytosol of a new target cell.¹³⁴

Another example is influenza A, in which the switch involves a change in the properties of the matrix protein (M1). M1 serves as an adaptor between the virus ribonucleoproteins (vRNPs) and the viral membrane as well as between the vRNPs, and it plays a crucial role during assembly of these components during virus assembly and budding.^{157,220} During entry, dissociation of these interactions is induced by an irreversible conformational change in M1 triggered by acid exposure in endosomes.²⁰ To acidify the internal space of the virus, where the M1 and the vRNPs are located, the viral membrane possesses acid-activated proton channels in the form of M2 protein complexes.^{148,191} If the M2 proton channel is blocked using amantadine, a specific M2 channel blocker used as an anti-influenza drug; HA-mediated fusion occurs normally in endosomes, but the vRNP and M1 fail to dissociate from each other, and transport of vRNP to the nucleus is inhibited.^{21,111}

Alphaviruses seem to use an altogether different strategy. Here the *switch* seems to involve a change in the cell rather than in the viral capsid. A cellular factor required for uncoating of incoming capsids is inactivated during the course of infection, thus allowing assembly of progeny capsids. The factor in question is the 60S ribosomal subunit, which has high affinity binding sites for the viral capsid protein.^{179,209} Incoming capsids rapidly lose capsid proteins to ribosomal subunits, and the viral RNA is thus liberated. When synthesis of structural protein starts later in an infection, newly synthesized capsid proteins bind to the ribosomal subunits and the ribosomal subunits can no longer interfere with assembly of progeny capsids.

NUCLEAR IMPORT

Most DNA viruses and a few negative-stranded RNA viruses replicate in the nucleus. To enter the nucleus, they can make use of the NPC for transport of the genome and accessory proteins into the nucleoplasm.^{34,40,69,211,212} (Fig. 4.7). Alternatively, the viruses may enter by rupturing the nuclear envelope, a process for which there is some evidence in the parvovirus field. These

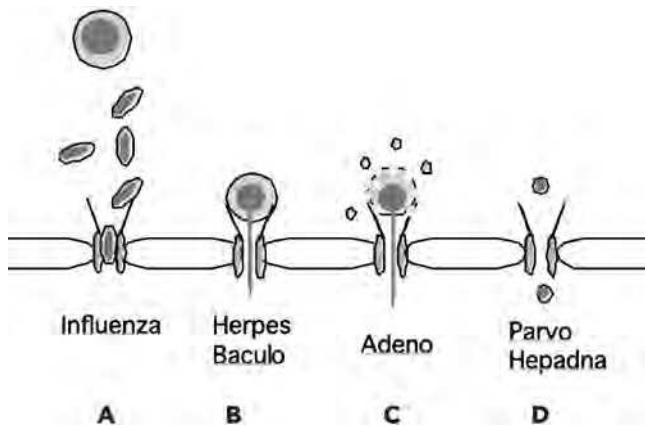


FIGURE 4.7. Import of viruses and subviral particles through the nuclear pore complex. To circumvent the size limitation (diameter 35 to 40 nm) of particle transport through the nuclear pore complex (NPC), viruses have evolved different strategies. **A:** The genome of a virus can be divided in multiple subgenomic particles with an elongated shape thin enough for individual entry (e.g., influenza virus). **B:** Limited uncoating takes place in the cytosol with the generation of an opening in the capsid wall that allows the DNA to escape, leaving an empty capsid at the NPC (e.g., herpes simplex virus 1). **C:** The virus disassembles after association with the NPC, allowing the genome and accessory proteins to pass through the NPC (e.g., adenoviruses). **D:** The virus particles or capsids are small enough to enter as spherical particles without uncoating or major deformation with uncoating occurring in the nucleoplasm.

two entry routes allow infection of nondividing, terminally differentiated, interphase cells in which the nuclear envelope represents a permanent barrier. Finally, viruses and viral capsids may wait in the cytosol for the dissolution of the nuclear envelope during cell division. This mechanism is used by most retroviruses with the exception of lentiviruses (and possibly papilloma viruses)¹⁵¹ and restricts infection to cell populations that undergo division. In principle, a fourth possibility would be penetration directly from the lumen of the ER through the inner nuclear membrane because the lumen of the ER is continuous with the space between the membranes in the nuclear envelope. Although some incoming viruses do pass through the ER and incoming viral particles have been occasionally seen between the two membranes of the nuclear envelope,¹¹⁴ no evidence currently indicates that any viruses use this pathway.

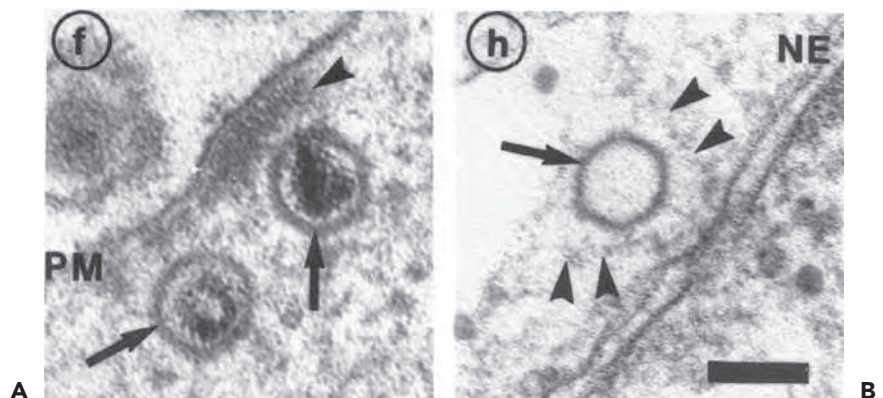
Nuclear import via the NPC involves several steps: binding of import receptors, transport through the cytosol, association with the NPC, and transfer of the intact virus, a subviral complex, or a nucleic acid through the pore. To be recognized by the cellular import machinery, viruses and viral capsids make use of nuclear localization signals (NLSs) similar to those present on cellular proteins and ribonucleoprotein complexes. These signals in viral proteins are recognized by soluble receptor proteins (importins or karyopherins) that mediate recognition, transport, and docking of the viral capsids to the NPC. In some cases, the NLS and the importins involved have been identified. Exposure of the NLS is sometimes modulated by phosphorylation-induced conformational modifications to avoid premature capsid import in the infected cell,⁸⁶ and some viruses are thought to be processed by proteasomes.²⁰⁶ It is also possible that viruses bind directly to the NPC without interaction with importins. This seems to be the case for adenovirus 2, which binds directly to the CAN/Nup214 nucleoporin.²⁰⁰

The size limitation for transport through the NPC is an obvious problem. Although estimates of the functional pore diameter have been adjusted upward to 39 nm,¹⁴⁰ only the smallest viruses and capsids are likely to enter intact without modifications. These include parvoviruses and the capsids of hepatitis B virus (HBV).^{153,206} When nuclear import of injected HBV capsids through the NPC are imaged by electron microscopy, the capsids can be seen to line up on the cytosolic fibers of the NPC and inside the central channel (Fig. 4.8). Uncoating of these capsids occurs in the basket, a structure located on the nucleoplasmic side of the NPC.¹⁵³

Being too large, most viruses and capsids must undergo shape changes or disassembly before passage of the genome through the NPC. Partially uncoated and modified adenovirus 2 particles bind to the CAN/Nup214 nucleoporins on the outer surface of the NPC, where they break apart, releasing the linear, double-stranded viral DNA for transport through the NPC.²⁰⁰ A histone protein, H1, has been implicated as a disassembly factor and a trans-NPC *guide* for the released DNA.

Binding of herpes simplex virus capsids to the NPC is mediated by capsid and tegument proteins.^{139,142} After association with CAN/Nup214 and another NPC protein, hCG1, through the minor capsid protein pUL25, and after opening of the portal structure at one of the vertexes of the capsid, the viral DNA escapes into the nucleus, leaving an empty capsid behind at the mouth of the NPC (Fig. 4.9). Influenza A viruses deal with the problem of size limits by having a segmented

FIGURE 4.8. Herpes simplex virus 1 (HSV-1) entry at the plasma membrane level and the nuclear envelope. **A:** In HSV-1, virus can fuse with the plasma membrane and release the capsid and the tegument into the cytosol. A large part of the tegument can be seen separating from the capsid. **B:** After binding to the cytosolic fibers attached to the nuclear pore complex (NPC), the viral capsid releases its DNA genome through one of the pentameric facets, and an intact-looking empty capsid shell remains bound to the NPC for some time. PM, plasma membrane; NE, nuclear envelope. Space bar, 100 nm. (Courtesy of B. Sodeik and A. Helenius.)



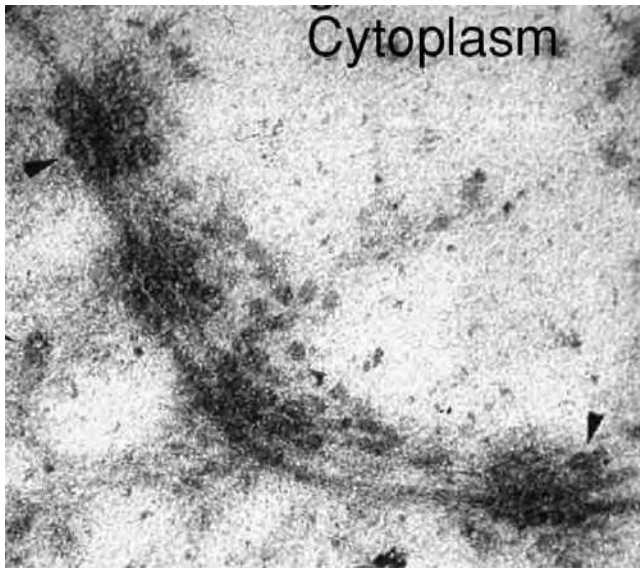


FIGURE 4.9. Import of hepatitis B virus (HBV) capsids through the nuclear pore complex (NPC). After injection into *Xenopus oocytes*, isolated HBV cores can be seen binding to fibers at the mouth of the NPC, and to line up in a row inside the channel of the pore. Uncoating of this capsid occurs in the basket on the nucleoplasmic side of the NPC. (Courtesy of N. Pante and M. Kann.)

genome. The eight subgenomic RNA are individually packaged into viral vRNP complexes. When interactions with the M1 protein are dissociated after exposure of the virus to low pH in endosomes, the vRNP can interact with importins, travel to the nucleus, and enter individually through the NPC (Fig. 4.7).^{20,111,138} The vRNPs are rod shaped and, although variable in length, have a thickness of only 10 to 20 nm.³⁶

The PIC of retroviruses has been reported to be about 50 to 60 nm in diameter.¹²⁶ Too big to enter the nuclei without conformational adjustments, they remain in the cytosol until the breakdown of the nuclear envelope occurs during cell division.⁹⁷ Being capable of entering interface nuclei, the PIC of HIV-1 and other lentiviruses are exceptions. The details of how and in which form lentivirus PICs are imported into the nucleus remain controversial.

TRANSMISSION DIRECTLY FROM CELL TO CELL

Discussion so far has focused on the mechanisms of entry by free viral particles attaching to the surface of cells. It is increasingly clear that there are situations in which infection occurs from cell to cell without participation of free virus particles^{129,167} (Fig. 4.10). One mechanism involves fusion of an infected cell's plasma membrane with the membrane of a neighboring cell. In infected tissues and cultures, the result is the formation of multinucleated cells (i.e., syncytia). Fusion in this case is mediated by viral fusion proteins expressed on the surface of the infected cell with receptors present on the noninfected cell. Commonly observed with paramyxovirus, herpesvirus, and other viruses with pH-independent fusion proteins, cell

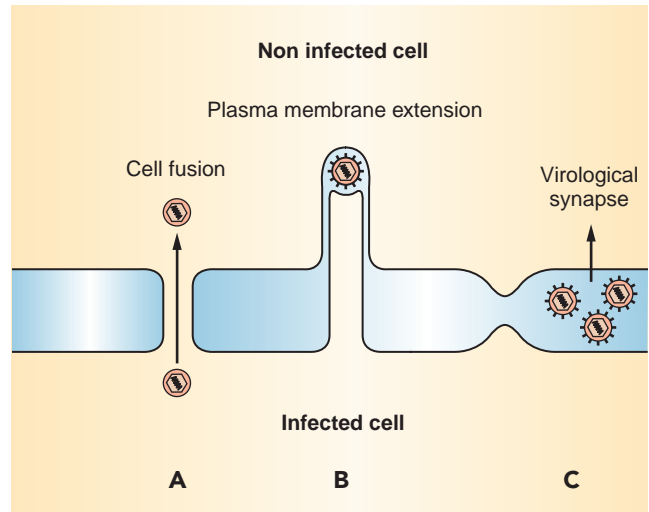


FIGURE 4.10. Direct cell-to-cell transmission. Several mechanisms allow infection to be transmitted via cell contacts without the release of free virus particles into the extracellular space. **A:** Due to the presence of viral fusion proteins on the surface of the infected cells and virus receptors on the noninfected cells, fusion of two cells can occur without producing virus particles. This results in the formation of syncytia. This mechanism is often seen with enveloped viruses and with fusion proteins that do not require low pH for fusion. **B:** In the case of poxviruses, extracellular viruses belonging to the so-called extracellular class of virions remain attached to the surface of the infected cell, where they trigger the formation of a motile, actin-containing, extension of the plasma membrane. This extension is thought to promote contact of the virus with the surface of a neighboring cells. **C:** The infected cells can undergo polarization so that progeny viruses are selectively released into a region of the cell periphery intimately in contact with another cell. Such specialized regions are called virological synapses.

fusion provides a mechanism for transmitting infection independently of virus particle assembly.³³

Other mechanisms of cell-to-cell transmission require the formation of virus particles, but these are not released freely into the extracellular space. Poxviruses such as vaccinia virus generate infectious particles called external enveloped viruses, most of which not only remain attached to the cell surface after formation, but also induce the formation of a motile surface extensions through the polymerization of actin inside the cytoplasm.^{62,70,183,216} The actin polymerization reaction helps to form membrane extensions that push the virus into contact with the neighboring cell, thus generating an efficient mechanism for local dissemination in infected tissues.

Structures termed infectious or virological synapses were first described for the transmission of the human T-cell leukemia virus type 1 (HTLV-1).⁸³ These are areas of intimate contact between infected and uninfected cells reminiscent of immunological synapses. They provide a domain where virus assembly and release are focused with efficient targeting of the virus particles to the next host cell without access of antibodies from the outside.¹²⁹ Similar means of transfer have been described for herpesviruses and for the T-cell to T-cell transfer of HIV. Dendritic cells that bind HIV-1 via DC-SIGN help to transport the virus to lymph nodes where they present the virus to T cells, providing an efficient system for targeted infection of host cells through virological synapses.^{66,93}

PERSPECTIVES

The entry and uncoating mechanisms and strategies are highly variable depending on the virus and the target cell. They have in common that the viruses depend critically on the host cell and its activities. To extract the necessary assistance from the cell, viruses make use of the detailed *insider information* they have about the host organisms, their tissues, the cells, and a variety of molecular processes. This information has been acquired during millions of years of coevolution. Thus, although exceptionally simple in structure and composition, viruses are able to elicit exceedingly complex cellular responses. The induction of signals, the activation of endocytic pathways, the exploitation of intracellular targeting systems and molecular motors, and the cell-assisted mechanisms of uncoating provide examples of the ways in which viruses make use of cellular machinery during entry. As details unfold, interesting and important insights about the viruses and their hosts continue to emerge.

The entry program involves the transport of the incoming viruses and capsids through the membranes and the compartments of the cell, the stepwise dismantling of the virus particle, and the release of the genome. Entry and uncoating involve *switches* in particle structure and properties that have to occur in the right place at the right time. It is remarkable how little seems to be left to chance. In the future, these switches need more attention because they provide powerful targets for therapeutic and prophylactic intervention. Also, the cellular defenses against virus entry, ranging from degradation of incoming viruses to interferon-induced expression of interception proteins in the cytosol, deserve thorough study. After all, it is clear that, of the incoming cell-associated viruses, only a small fraction generally reach the intended goal.

It will be important to focus on the cellular factors involved in infection (i.e., on the Trojans and not only on the Trojan horses). They represent new targets for antiviral strategies. How viruses enter tissues and cells in intact organisms remains for the most part territory uncharted. New technologies and model systems are emerging that allow work at the whole animal level. A multidisciplinary approach that combines cell and molecular biology, structural biology, biochemistry, physiology, systems biology, and medicine as central disciplines is required. As important as knowing the pathogens, it will also be important in the spirit of an ancient greek aphorism to know ourselves (i.e., to understand the cell and molecular biology of the host cells).

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Viral Replication Strategies

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Summary and Perspectives

Acknowledgment

exploit the biology of their hosts. Despite their comparatively limited genetic repertoire, viruses encode the information necessary to rewire their hosts to become viral factories. The intimacy of this relationship and the co-evolution of virus and host continue to provide unique mechanistic insights into host biology at the molecular, cellular, organismal, and population levels. Understanding this interplay enriches our understanding of the biosphere in general and virus–host relationships in particular, but also creates opportunities for the rational development of antiviral drugs, and for domesticating viruses as expression vectors, live-attenuated vaccines, and pesticides. This chapter provides an overview of the replication strategies of the major virus families that infect vertebrates, attempting where possible to emphasize the general principles that guide and constrain virus replication and evolution.

Viral Genome Diversity and Replication Strategies

Perhaps the most striking aspect of viruses at the molecular level is the diversity of their genome structures and replication strategies. Unlike cellular genomes, which consist uniformly of double-stranded DNA (dsDNA), viral genomes provide examples of almost every structural variation imaginable. As shown in Table 5.1, different families of viruses have genomes made of either double-stranded (ds) or single-stranded (ss) DNA or RNA; of either positive, negative, or ambisense polarity; of either linear or circular topology; and comprising either single or multiple segments. Each variation has consequences for the pathways of genome replication, viral gene expression, and virion assembly. This diversity argues strongly that viruses had several different evolutionary origins and can be thought of in D. J. McGeoch's evocative phrase as “mistletoe on the tree of life.” Accordingly, viral taxonomy above the family level is patchy, with only 22 of 87 families assigned to the six orders that are currently recognized.⁵⁷ However, it is likely that more distant phylogenetic relationships will emerge as the number of genome sequences and protein structures increase, and as more powerful comparison algorithms become available.

INTRODUCTION

Replication of genetic information is the single most distinctive characteristic of living organisms, and nowhere in the biosphere is replication accomplished with greater economy and apparent simplicity than among viruses. To achieve the expression, replication, and spread of their genes, different virus families have evolved diverse genetic strategies and replicative cycles to

Unique Biology of Virus Replication

As obligate intracellular parasites, all viruses depend heavily on functions provided by their host cells. This dependence, as well as the extensive metabolic overlap between host and parasite, limits the number of possible targets for antiviral therapy. Nevertheless, almost all viruses encode and express unique proteins, including enzymes, and many viruses exploit pathways of information transfer that are unknown elsewhere in the biosphere. This is particularly evident among the RNA

Updated from the previous text by L. Andrew Ball.

TABLE 5.1 Families and Genera of Viruses that Infect Vertebrates

Virus family or genera	Genome				Genome replication	
	Type	Polarity ^a	Topology ^b	Segments	Enzyme	Intracellular site
<i>Adenoviridae</i>	dsDNA	Both	Linear	1	Viral DdDp	Nucleus
<i>Anelloviridae</i>	ssDNA	Negative	Circular	1	Cellular DdDp	Nucleus
<i>Asfarviridae</i>	dsDNA	Both	Linear	1	Viral DdDp	Cytoplasm
<i>Circoviridae</i>	ssDNA	Negative or ambisense	Circular	1	Cellular DdDp	Nucleus
<i>Hepadnaviridae</i>	dsDNA	Both	Linear	1	Virion RdDp	Nucleus/cytoplasm
<i>Herpesviridae</i>	dsDNA	Both	Linear	1	Viral DdDp	Nucleus
<i>Iridoviridae</i>	dsDNA	Both	Linear	1	Viral DdDp	Nucleus/cytoplasm
<i>Papillomaviridae</i>	dsDNA	Both	Circular	1	Cellular DdDp	Nucleus
<i>Parvoviridae</i>	ssDNA	Either	Linear	1	Cellular DdDp	Nucleus
<i>Polyomaviridae</i>	dsDNA	Both	Circular	1	Cellular DdDp	Nucleus
<i>Poxviridae</i>	dsDNA	Both	Linear	1	Viral DdDp	Cytoplasm
<i>Arenaviridae</i>	ssRNA	Ambisense	Linear	2	Virion RdRp	Cytoplasm
<i>Arteriviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
<i>Astroviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
<i>Birnaviridae</i>	dsRNA	Both	Linear	2	Virion RdRp	Cytoplasm
<i>Bornaviridae</i>	ssRNA	Negative	Linear	1	Virion RdRp	Nucleus
<i>Bunyaviridae</i>	ssRNA	Negative or ambisense	Linear	3	Virion RdRp	Cytoplasm
<i>Caliciviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
<i>Coronaviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
Deltavirus genus	ssRNA	Negative	Circular	1	RNA pol II	Nucleus
<i>Filoviridae</i>	ssRNA	Negative	Linear	1	Virion RdRp	Cytoplasm
<i>Flaviviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
<i>Hepeviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
<i>Nodaviridae</i>	ssRNA	Positive	Linear	2	Viral RdRp	Cytoplasm
<i>Orthomyxoviridae</i>	ssRNA	Negative	Linear	6–8	Virion RdRp	Nucleus
<i>Paramyxoviridae</i>	ssRNA	Negative	Linear	1	Virion RdRp	Cytoplasm
<i>Picornaviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
<i>Reoviridae</i>	dsRNA	Both	Linear	10–12	Virion RdRp	Cytoplasm
<i>Retroviridae</i>	ssRNA	Positive	Linear	2 identical	Virion RdDp	Nucleus/cytoplasm
<i>Rhabdoviridae</i>	ssRNA	Negative	Linear	1	Virion RdRp	Cytoplasm
<i>Togaviridae</i>	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm

DdDp, DNA-dependent DNA polymerase; ds, double-stranded; RdDp, RNA-dependent RNA polymerase; ss, single-stranded.

^aPolarity of the encapsidated genome.

^bTopology of the encapsidated genome—note that some circularize during replication.

viruses, which are the only organisms that are known to store their genetic information in the form of RNA. They accomplish this by replicating their genomes via one of two unique biochemical pathways—either by RNA-dependent RNA synthesis (RNA replication), or, among the retroviruses, by RNA-dependent DNA synthesis (reverse transcription) followed by DNA replication and transcription. Both pathways require enzymatic activities that are not usually found in uninfected host cells and must therefore be encoded by the viral genome and expressed during infection. Furthermore, in some families of RNA-containing viruses those unique synthetic processes are required right at the start of the infectious cycle. This necessitates co-packaging of the corresponding polymerase and other associated enzymes with the viral genome during the assembly of viral particles in preparation for the next round of infection.

Whatever the structure and replication strategy of their genomes, all viruses must express their genes as functional messenger RNAs (mRNAs) early in infection in order to direct the cellular translational machinery to make viral proteins. The various genomic strategies employed by viruses can therefore be organized around a simple conceptual framework centered on viral mRNA (Figs. 5.1 and 5.2). By convention, mRNA is defined as positive-sense and its complement as negative-sense. The pathways leading from genome to message vary widely among the different virus families and form the basis of viral taxonomy. Although it is generally believed that viruses originated from cellular organisms, perhaps fairly recently in evolutionary times, it remains possible that some RNA viruses are descended directly from a primordial “RNA world” or “ribonucleoprotein world,” which may have predated the emergence of DNA and cells.

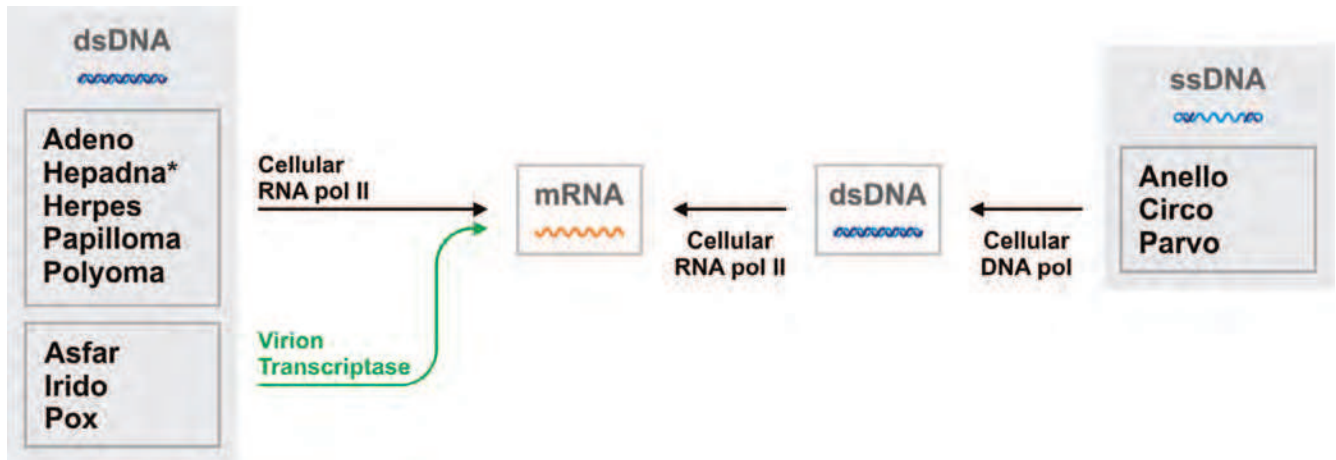


FIGURE 5.1. Pathways of primary mRNA synthesis by DNA viruses of animals. Hepadnaviruses replicate via reverse transcription of an ssRNA intermediate.

Subcellular Sites of Viral Replication

Most DNA viruses of eukaryotes transcribe and replicate their genomes and assemble progeny in the nucleus, the site of cellular DNA transcription and replication. The exceptions are the poxviruses, iridoviruses, and African swine fever virus, which replicate their DNA genomes partly or completely in the cytoplasm. In contrast, most RNA viruses replicate their genomes in the cytoplasm. However, in addition to the retroviruses that integrate DNA copies of their genomes into the host chromosomes, other notable excep-

tions to this generalization are the orthomyxoviruses, bornaviruses, and many plant-infecting rhabdoviruses, whose linear negative-sense RNA genomes replicate in the nucleus. The circular RNA genome of hepatitis delta virus (HDV), also replicates in the nucleus (Table 5.1). Each site of replication presents distinct opportunities and challenges in terms of which cellular components and pathways are available to be co-opted, and how the synthesis and trafficking of viral proteins, genome replication, virion assembly, and the release of progeny can be coordinated. For example, RNA splicing occurs only in the nucleus, so among the RNA viruses,

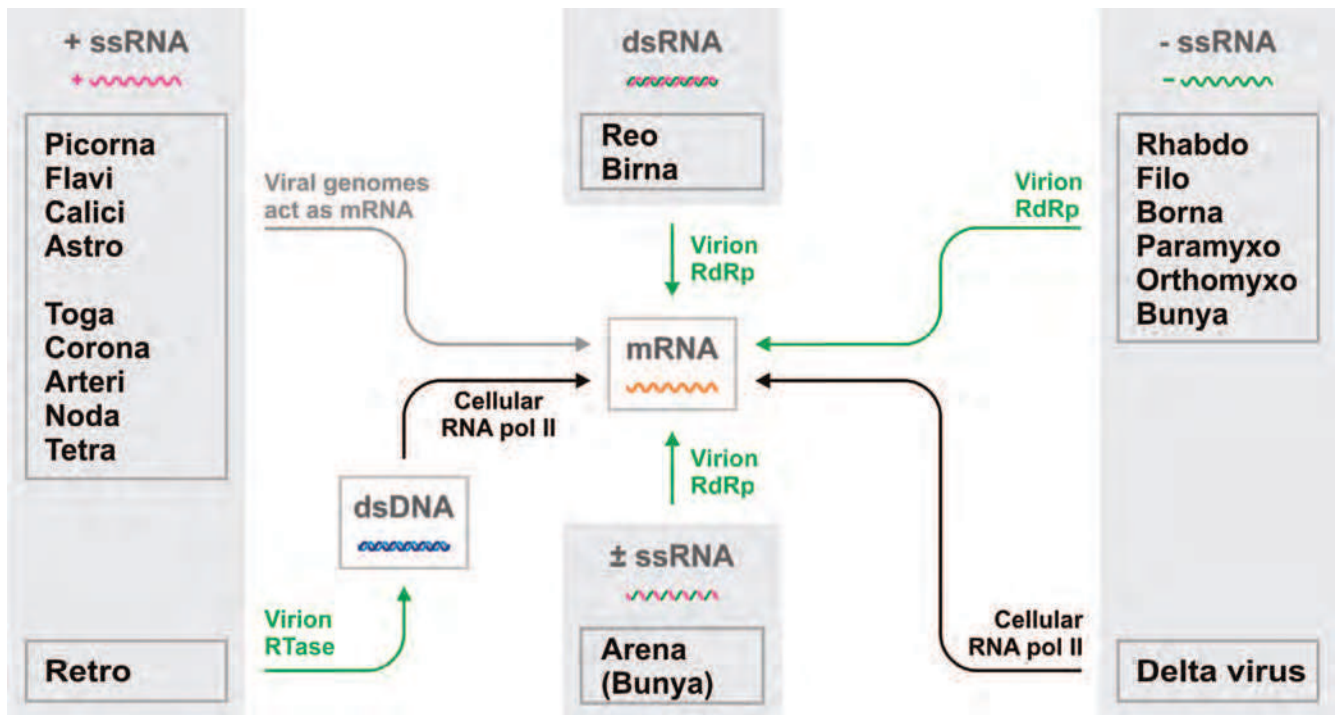


FIGURE 5.2. Pathways of primary mRNA synthesis by RNA viruses of animals. How RNA viruses produce mRNA at the start of infection depends upon the nature of the viral genome.

this mechanism of accessing more than one open-reading frame in a single transcript can be employed by only the retro-, orthomyxo-, and bornaviruses that transcribe there. It is remarkable that the paramyxoviruses that replicate in the cytoplasm have evolved a transcriptional editing mechanism that achieves a similar result.⁹⁹ Irrespective of the site of replication (nuclear or cytoplasmic) the viral replication machinery itself is frequently compartmentalized within specific structures or viral-induced organelles. For example, herpesviruses form replication compartments within the nucleus at nuclear speckles,^{16,88} and many RNA viruses that replicate in the cytoplasm do so in association with membranes or an inclusion-like structure that contains the viral replication machinery.²⁵

Evasion of Host Response to Infection

To ensure their survival, host organisms have evolved a variety of responses to combat viral infection. In turn, many viruses express specific gene products that act to circumvent one or more of those antiviral defense mechanisms. Examination of these measures and countermeasures provides a revealing glimpse into the heart of the host–parasite relationship as it plays out in nature. Host-defense mechanisms can be categorized as innate or adaptive. Among the former, which operate at the cellular level, are apoptosis (programmed cell suicide that limits the spread of infection, see Chapter 8), the induction and action of interferons in vertebrates (inducible cytokines that render cells resistant to infection by inducing a multifaceted antiviral state, see Chapter 8), and RNA interference in plants and invertebrates (a sequence-specific mechanism of RNA degradation, see Chapter 8). Adaptive immune mechanisms operate at the organismal level, and include the cell- and antibody-mediated immune response (see Chapter 9). Increasingly, specific restriction factors have been identified that limit the replication of subsets of viruses. Such factors include tripartite motif containing protein 5 (TRIM5), which appears to trigger the premature disassembly of the incoming human immunodeficiency virus type 1 (HIV-1) capsid to limit the establishment of infection; the apolipoprotein B mRNA editing, enzyme catalytic (APOBEC) family, which induces a biased hypermutation in RNA through its cytidine deaminase activity that converts C to U; and bone marrow stromal antigen 2 (BST2)/Tetherin, which is incorporated into the membranes of some enveloped viruses resulting in a linking together of budding viral particles. Although a number of other cellular proteins have been termed “restriction factors,” they are distinguished by their dependence on induction by interferon and are therefore not considered here. In different viruses, mechanisms and gene products have been identified that inhibit apoptosis, intercept interferons or suppress their activities, obstruct RNA interference, either evade or suppress different arms of the adaptive immune response, or block intrinsic restriction factors.^{4,36,46,47,63,70,90,92,93,100}

Viruses are sensed by the host in ways that appear to involve recognition of unique signatures present in viral genomes or gene products. Such signatures are termed pathogen-associated molecular patterns (or PAMPS), and are recognized by an array of host pathogen-recognition receptors (or PRRs). Those PRRs include the toll-like receptors (TLRs), which are membrane-associated molecules that sense invading pathogens directly at the plasma membrane or during endosomal transit. The retinoic acid inducible gene (RIG)-

like receptors (RLRs), which are cytoplasmic RNA helicases that recognize the products of RNA viral nucleic-acid replication, and the absent in melanoma 2-like receptors that recognize cytoplasmic DNA. Although viral ligands have not been defined, the NOD-like receptors (NLRs), which sense bacterial peptidoglycan, appear to also detect some viruses. A striking example of such PRR function is the detection of off-pathway products of replication such as abortive initiation products, dsRNA, and defective viral genomes, which can serve as ligands for the cytoplasmic sensors RIG-I and melanoma differentiation association protein 5 (MDA-5) to engage in a signaling cascade that leads to the activation of interferon.⁴⁰ The net result of interferon activation is both the blocking of infection within the cell, and the preactivation of defense mechanisms in neighboring cells to render them less susceptible to infection. The latter is accomplished by the transcription of interferon (IFN)–stimulated genes (ISG), which themselves act to block various steps in the replication cycle of DNA and RNA viruses⁹³ (see Chapter 8). In turn, viruses themselves have evolved countermeasures to such host-defense mechanisms that act to block the induction of IFN itself, or to interfere with specific ISG function.^{22,43} The elaborate arms race between viruses and their hosts is described in more detail in Chapter 8 and Chapter 9, as well as within the specific chapters dealing with individual virus families. Molecular signatures of this arms race throughout evolution are also visible in the sequences of virus and host genes. Retroviruses provide a unique insight into this, since they integrate into the host genome. Evidence for integration of portions of other viral genomes into the host chromosome including RNA viruses such as bornavirus, and lymphocytic choriomeningitis virus, has also emerged.^{33,80}

Error Prone Nature of RNA Replication

The polymerases that catalyze RNA replication and reverse transcription have minimal proofreading activities. The polymerase error rate of such RNA-dependent RNA polymerases (RdRp's) and reverse transcriptases is approximately three orders of magnitude higher than that of DNA-dependent DNA polymerases, and approaches the reciprocal of their genome length.^{28,52,74} The net result is that the genomes of RNA viruses evolve at a much faster rate than those of their hosts. Biologically, RNA viruses therefore represent a swarm of sequences around a consensus sequence or master sequence.^{31,62} This molecular swarm provides a fertile source of phenotypic variants that can respond rapidly to changing selection pressures by shifting its composition. As a consequence, RNA viruses can evolve up to 1 million times faster than DNA-based organisms. The error prone nature of RNA virus replication is also critical for pathogenesis in infected hosts. The diversity of viral sequences regenerated following bottleneck transmission of HIV in humans,⁸⁹ and experimental poliovirus infection of mice,^{84,101} provide striking examples of this *in vivo*. In the case of HIV, the resulting sequence variation achieved following transmission of a limited number of genomes is enormous and accounts for—among other phenotypes—the rapid escape of the virus from neutralizing antibody, and the escape from antiviral monotherapy.

Such rapid rates of evolution are not without cost for the RNA viruses, however, because higher polymerase error rates impose upper limits on genome size. The combination

of replicative error rate and genome size defines an “error threshold” above which a virus cannot maintain even the sequence integrity of its quasispecies.³¹ As a result, few RNA virus genomes contain more than 30 kilobases (kb) and most have between 5 and 15 kb. RNA genomes of this size are poised just below their error thresholds, and although their genetic diversity inevitably wastes individual progeny that carry deleterious mutations, the cost is offset by the potential for rapid evolutionary response to changing selective pressures. This positioning of RNA viruses—just below their error threshold—may also present an opportunity for antiviral development. Specifically, therapeutics that lead to an increase in error rate can shift the balance beyond the error threshold toward “error catastrophe.” Indeed evidence has accumulated that this is one such mechanism by which ribavirin, an adenosine analog, may inhibit the replication of some RNA viruses.²¹ The largest RNA virus genomes currently recognized are those of the coronaviruses, which approach a size of 30 kb. Strikingly, it appears that for coronaviruses the non-structural protein nsp14 functions as an RNA exonuclease that may function as a proofreading mechanism that could help maintain genome integrity.²⁷

Levels of Segmentation

Another distinctive feature of eukaryotic cells—besides their partitioning into nuclear and cytoplasmic compartments—has a profound influence on the biology of their viruses. On most mRNAs, eukaryotic ribosomes require a methylated mRNA cap structure at the 5′ end that plays a critical role in signaling the initiation of protein synthesis. As a result, eukaryotes typically conform to the “one mRNA one polypeptide chain” rule; with very few exceptions, each message operates as a single translational unit. Similarly, viral RdRp’s generally appear somewhat restricted in their ability to access internal promoter elements on RNA templates, and this creates a problem of how an RNA virus can derive several separate protein products from a single genome.

Through evolution, different RNA virus families have found three different solutions: fragmentation at the level of proteins, mRNAs, or genes, with some viruses using more than one of those solutions. For example, RNA viruses in the picorna- toga-, flavi-, and retrovirus families rely on extensive proteolytic processing of polyprotein precursors to derive their final protein products.²⁹ Others (in the orders *Mono- negavirales* and *Nidovirales*) depend on complex transcriptional mechanisms to produce several monocistronic mRNAs from a single RNA template.^{1,91} Still others (in the reo-, orthomyxo-, bunya-, and arenavirus families, among others) have solved the problem by fragmenting their genomes and assembling virions that contain multiple genome segments, each often representing a single gene.^{34,69,76} Among plant viruses, such RNA genome segments are often packaged into separate virions, necessitating co-infection by several virus particles to transmit infectivity,¹⁰⁷ but the genome segments of animal viruses are typically co-packaged into single virions. In contrast, DNA viruses seldom use either genome segmentation or polyprotein processing. This is likely due to the relative ease with which monocistronic mRNAs can be transcribed from internal promoter elements of dsDNA, and the extensive use of differential splicing of nuclear transcripts to express promoter-distal open-reading frames.

Host Cell Components for Replication

Viruses depend on their host cells to support their replication, and this degree of dependency—to some extent—reflects their genome size. Although all viruses depend on the host translational machinery, large DNA viruses, such as mimivirus, may encode specific initiation factors that may provide a translational advantage for viral genes.¹⁸ Entry of viruses into cells usually requires specific host-cell factors, and can require co-opting of cellular endocytic pathways.⁷¹ The end point of entry is the release of the minimal viral replication machinery into the host-cell cytoplasm to initiate infection. How viruses establish infection in the hostile environment of the host cell remains one of the least understood steps of the viral replication cycle. The input genomes must either associate directly with ribosomes in the case of positive-strand RNA viruses, or be copied into mRNA, in the case of the negative-strand RNA viruses, dsRNA viruses, and DNA viruses. Because the particle-to-infectivity ratio of some viruses approaches 1:1, this process must be highly efficient despite its inherent challenges. Our knowledge of the subsequent viral rewiring of host-cell structures to establish replication compartments, traffic viral proteins and nucleic acids, and assemble viral particles is also far from complete, but has yielded a wealth of information into host biology as well as that of the viruses themselves. Indeed, study of viruses has contributed enormously to our understanding of promoters, transcriptional enhancers, the mRNA cap structure, RNA splicing, and mechanism of translation. Similarly, critical discoveries in host-cell transport and trafficking pathways including endocytosis, exocytosis, and secretory transport were achieved because of the ability to synchronize infections with viruses. Although systematic approaches including RNA interference (RNAi), proteomics, gene-knockout studies, and microarrays are helping to further transform our understanding of the virus–host interaction at the molecular level, we have yet to understand fully the complexities of the interactions of any virus with its host. Zoonotic viruses must strike a balance for optimal replication in often quite disparate hosts, likely adding further complexity to this intimate relationship. Striking examples of this are provided by members of the *Flaviviridae*, such as Dengue virus (which replicates in both its mosquito host and animals), and experimentally with many viruses including vesicular stomatitis virus (which replicates in virtually all eukaryotic cells in culture).

STRUCTURES AND ORGANIZATION OF VIRAL GENOMES

DNA versus RNA Genomes

Among families of viruses that infect vertebrates, those with RNA genomes outnumber those with DNA genomes by about 2 to 1 (Table 5.1); among viruses infecting plants the disparity is even greater. Indeed, no dsDNA viruses of plants are known except for those that like the hepadnaviruses of vertebrates, replicate via reverse transcription (see Chapter 68). This remarkable observation remains to be explained, but it may suggest that non-RT dsDNA viruses arose only after animals and plants diverged. Be that as it may, the prevalence of RNA viruses attests to the evolutionary success and versatility of RNA as genetic material for smaller genomes. As discussed

previously, the high error rates of RNA replication restrict RNA genome sizes to 30 kb or less, whereas proofreading and error repair ensure sufficiently accurate replication of DNA virus genomes as large as that of the 1200-kb megaviruses.³ In addition, the fact that DNA is more chemically stable than RNA likely explains why all known viruses of thermophilic hosts have dsDNA genomes.⁵⁷

Single- and Double-Stranded Genomes

Although all viral genomes replicate via conventional Watson-Crick base pairing between complementary template and daughter strands, viruses that belong to different families encapsidate and transmit different molecular stages of the genome replication cycle. Families of ssRNA viruses outnumber families of dsRNA viruses by almost 10 to 1, roughly the inverse of the ratio between ssDNA and dsDNA viruses. In view of the greater chemical stability of double-stranded nucleic acids of both types, this difference calls for an explanation. Two possibilities seem plausible: First, dsRNA viruses must somehow circumvent the translational suppression that can result from the coexistence of equimolar amounts of the sense and antisense RNAs. How the dsRNA reoviruses solve this problem is addressed in Chapter 44. Second, dsRNA is widely recognized by the cells of higher eukaryotes as a signal for the induction of defense mechanisms that act to suppress viral replication, such as the IFN system in vertebrates (see also Chapter 8), gene silencing in plants, and RNAi in a variety of organisms.^{15,40,93,106} These effects probably suffice to explain the relative scarcity of dsRNA virus families.

For these same reasons, it is important even for ssRNA viruses to limit the accumulation of replicative intermediates that contain regions of dsRNA, and the strategies to ensure this differs between the positive- and negative-sense RNA viruses. All known positive-strand RNA viruses synthesize disproportionately low amounts of the negative-strand RNA—typically 1% to 5% of the levels of the positive-strand—and thereby minimize the potential for dsRNA accumulation. Moreover, because the replication of these viruses appears to universally occur in sequestered membranous compartments, there appears to be a physical separation of the replicative intermediates from the host-cell cytoplasm, likely reducing the chances of detection.²⁵ In contrast, negative-strand RNA viruses, which need substantial amounts of both positive- and negative-sense RNAs to use as messages and progeny genomes, respectively, prevent the complementary RNAs from annealing to one another by encasing the genomic and antigenomic RNAs with a viral nucleocapsid protein.^{2,44} Here, RNA synthesis also appears confined at some stages of infection to specific subcellular compartments that may help serve to limit detection of viral products of RNA synthesis by the innate immune system.

Positive, Negative, and Ambisense Genomes

The differences between positive- and negative-strand RNA viruses extend beyond the polarity of the RNA assembled into virions. Positive-sense RNA genomes exchange their virion proteins for ribosomes and cellular RNA binding proteins at the onset of infection. Once synthesized and assembled the virus-specified RdRp and other nonstructural proteins replace the ribosomes to accomplish RNA replication. Virion structural proteins are reacquired during the assembly of progeny

virions. In contrast, negative-strand RNA genomes and their antigenomic complements remain associated with their nucleocapsid proteins, both within the viral particles and throughout the viral replication cycle, even during RNA replication. These fundamentally different adaptations can be attributed to the fact that whereas positive-sense RNA genomes must satisfy criteria for translation that are dictated by the host cell, negative-sense RNA genomes must only satisfy the template requirements for the virus-specified RdRp because they are replicated but never translated. Although the precise mechanism by which the protein-coated templates of negative-strand RNA genomes are copied by their cognate polymerases is not fully understood, short naked RNAs that correspond to the terminal promoters can be copied by their viral polymerases.^{26,59,73} Such experimental evidence is consistent with a model for RNA synthesis in which the nucleocapsid protein is transiently displaced from the template RNA during copying of the genome.

The dsRNA virus genomes are intermediates between the two. The parental genome remains sequestered within a subviral particle during the synthesis of the unencapsidated positive-sense mRNA transcripts, which are replicated to produce progeny dsRNAs only after being assembled into subviral core particles.⁸¹ Although the core RdRp's of each of these viruses are structurally as well as functionally analogous, the distinctions in the genomic structure likely place additional structural constraints on the viral polymerase complexes.

Linear and Circular Genomes

Genome replication not only requires an acceptable error rate as described previously, but must also avoid the systematic deletion or addition of nucleotides. Genome termini are particularly troublesome in this respect, a fact that has been dubbed “the end problem.” For DNA replication, the end problem is exacerbated by the fact that DNA polymerases cannot initiate the synthesis of daughter strands and must therefore use primers, thus creating additional complications of replicating the primer-binding site(s). Among several known solutions, the most economical and widespread in nature is to eliminate the ends altogether by covalently circularizing the genomic DNA, as occurs in the genomes of prokaryotes. Polyoma-, papilloma-, circo-, and anellovirus genomes follow this model, and the dsDNA genomes of herpes and hepadnaviruses, although linear, in virions are covalently circularized before replication. Poxviruses and asfiviruses also have linear dsDNA genomes, but in these cases the individual complementary strands are covalently continuous at the termini of the duplex, which provides another solution to the end problem. A similar close-ended duplex DNA is generated during the replication of the ssDNA genomes of parvoviruses (Chapter 57). Terminal redundancy (iridoviruses), inverted terminal repeats (adenoviruses), and the use of protein primers that do not occlude the binding site (adenoviruses and hepadnaviruses) represent the other ways that DNA viruses have evolved to ensure accurate and complete replication of their genome termini.

Unlike DNA polymerases, most RNA polymerases do not require primers, so RNA genomes are less susceptible to the end problem. Accordingly, most RNA genomes are linear molecules. Covalently closed circular RNAs are found only in HDV in animals (Table 5.1) as well as among the viroids and other subviral RNA pathogens that infect plants. Nevertheless the termini of linear RNA genomes are vulnerable to degra-

dation, and their replication is likely to be particularly error prone. Consequently, every family of RNA viruses has features designed to preserve the termini of the genome.⁶ For example, many positive-strand RNA viruses have a 5' cap structure and 3' polyadenylate tail that serve to protect eukaryotic RNAs against degradation, and a similar role is likely played by the VPg that is covalently linked to the 5' end of the picornavirus genomes,⁶⁴ and by the stable RNA secondary structures present at the 3' end of the flaviviral RNA and other genomes. The 3' ends of many plant virus RNAs form clover leaf structures that resemble transfer RNAs (tRNAs) so closely that they are recognized by the cellular tRNA charging and modifying enzymes.³⁰ In addition to playing protective roles, terminal modifications of positive-sense RNAs may also serve to bring their ends together by binding to interacting cellular proteins such as the poly(A) binding protein and cap-binding complex, thereby forming noncovalent functionally circular complexes that may promote repetitive translation by ribosomes and repetitive replication by RdRp's.^{49,102}

Unlike the genomes of positive-sense RNA viruses, negative-sense and ambisense RNA virus genomes rarely carry covalent terminal modifications. Those RNA genomes show some degree of terminal sequence complementarity that is thought to lead to the formation of a panhandle type of structure that, in the case of the segmented viruses, favors RNA replication. Because the templates are encapsidated by the viral nucleocapsid protein, it is not clear how the RNA bases can engage in base-pairing interactions between the termini. However, complementarity between the genomic termini favors replication and likely promotes polymerase transfer during RNA synthesis to ensure efficient reinitiation of replication. In other solutions to the end problem among the RNA viruses, retroviral genomes are terminally redundant and have direct repeats of 12 to 235 nucleotides at each end that maintain and restore the integrity of the termini during reverse transcription and virus replication (see Chapter 47).

Segmented and Nonsegmented Genomes

As discussed previously, segmentation of RNA genomes is one way to facilitate the production of multiple gene products in eukaryotic cells, but it also means that the various segments must each contain appropriate *cis*-acting signals to mediate their expression, replication, and assembly into virions. In some virus families whose members have segmented genomes (e.g., the orthomyxoviruses and some reoviruses), these signals comprise conserved sequences at the RNA termini, but in others (e.g., the bipartite nodaviruses and tetraviruses) sequence conservation between the segments is minimal. In these latter cases, the specificity of RNA replication and assembly is presumably dictated by conserved RNA secondary or tertiary structures. Moreover, segmentation of the viral genome requires a level of coordination to ensure that the correct amounts of viral gene products are expressed and to ensure the packaging of multiple genome segments to form infectious virus particles. How such coordination is achieved is not understood. Furthermore, in the case of the negative-sense, ambisense, and dsRNA viruses that have segmented genomes, a mechanism is required to ensure that the polymerase is packaged into the virus particle so that the incoming segments can be transcribed into mRNA. For the dsRNA viruses the polymerase is an integral structural component of the core

transcribing particle ensuring that the polymerase and capping machinery are present within the incoming particle. In the case of the arenavirus, Machupo, this is a function of a small viral protein Z, which locks the polymerase on the promoter in an inactive form.⁶⁰

Evidently, the evolutionary barrier between viruses with segmented and nonsegmented RNA genomes is readily transversed because both genome types occur in members of the alphaviruslike supergroup, a taxonomic cluster based on phylogenetic comparisons of nonstructural protein sequences. Indeed, among the tetraviruses, segmented and nonsegmented genomes can even be found in the same family. Furthermore, the genomes of some togaviruses, rhabdoviruses, and paramyxoviruses, which are naturally nonsegmented, have been experimentally divided into segmented genomes without destroying viral infectivity,^{38,96} thus confirming the flexibility of RNA genomes in this regard. Nevertheless, genome segmentation has major effects on the biology of a virus because individual segments can reassort between dissimilar strains in co-infected cells, which enables segmented genome viruses to make substantial evolutionary leaps by horizontal gene transfer. This mechanism underlies the antigenic shifts that produce new pandemic strains of the orthomyxovirus influenza virus (see Chapter 40 and Chapter 41).

As discussed previously, genome segmentation is almost unknown among DNA viruses, most likely because internal initiation of transcription and alternative splicing provide more facile ways to access multiple open-reading frames. Only the polydnviruses, a family of dsDNA viruses that infect parasitic wasps and participate in a complex and unusual host-parasite relationship, show extensive DNA segmentation.⁷

Cis-Acting RNA Signals and Specificity

Replication and packaging of viral RNAs display striking specificity; both processes unerringly pick the correct viral molecules from among thousands of cellular RNAs that may be much more abundant. This is generally attributed to the presence of *cis*-acting signals that selectively channel the viral RNAs into replication and assembly complexes, but in most RNA virus genomes these signals remain to be clearly identified. Those that have been characterized most, comprise not linear nucleotide stretches, but RNA secondary structures such as bulged stem-loops, tRNA-like cloverleaves, and pseudoknots, which are believed to create distinctive three-dimensional molecular shapes that interact specifically with the viral enzymes and structural proteins. Although high-resolution structures have been determined for some RdRp's, reverse transcriptases, and several viral capsids, our understanding of the molecular basis of specificity in RNA replication and virus assembly is limited by the scant knowledge of the three-dimensional structures of viral RNA and its *cis*-acting signals. However, the structural basis of RNA specificity during replication and assembly has often proved elusive, perhaps because the specificity determinants can be redundant, dispersed, or global properties of the viral genome. Furthermore, in both RNA replication and assembly, specific interaction is followed by less-specific RNA-protein interactions that propagate the reactions. The transitions between these different stages are largely unexplored, and much remains to be learned concerning the recognition of *cis*-acting RNA signals and how they promulgate RNA replication and assembly. In the case of some viruses, the products of

replication are selectively channeled into the assembly pathway, thereby diminishing the need for separate assembly signals.

Promising advances in our understanding of *cis*-acting regions of RNA viral genomes have recently come from the application of a chemical probing methodology termed selective 2' hydroxyl acetylation analyzed by primer extension (SHAPE), first applied to provide an overview of the complete genome of HIV-1.¹⁰³ In addition to correctly identifying known structures within the HIV-1 genome, several structures were identified within the coding regions at regions close to the positions of polyprotein processing. Such structured elements are thought to lead to a slowing of ribosomes to facilitate the correct folding of the preceding region of the polypeptide chain, although data proving this are lacking. Whatever the function of such structured elements, application of this methodology promises to improve our definition of the *cis*-acting elements within RNA virus genomes as well as the overall structure of viral genomes.

Satellite, Dependent, and Defective Genomes

Occasionally, subviral genomes arise that are neither independently infectious nor essential for infectivity, but nevertheless contain *cis*-acting signals that promote their own replication and/or packaging by the proteins encoded by another virus. Such satellite nucleic acids are parasitic on the parental virus and can modulate its replication and virulence.⁹⁴ Most commonly, they are ssRNAs, but dsRNA and ssDNA satellites are also known. Among the RNA viruses of animals, a prime example is hepatitis delta virus (or HDV), which packages its ssRNA genome in virion proteins encoded by the hepadnavirus hepatitis B virus and can severely exacerbate its pathogenicity.⁹⁷ Dependence of an RNA satellite on a DNA virus parent is unusual; more commonly satellite RNAs are replicated and encapsidated by the proteins of an RNA virus parent with which they share at least some sequence homology. In some instances, satellite RNAs encode their own distinct capsid proteins, or proteins required for RNA replication (as in the case of HDV), but in others they are translationally silent. Satellite RNAs are much more common among the viruses of plants than those of animals (see Chapter 72), perhaps because the transmission of animal viruses between hosts generally involves narrower bottlenecks that select against the spread of satellites. Dependence of one virus on another is also found occasionally among viruses with DNA genomes. For example, adenovirus-associated virus (family *Parvoviridae*, genus *Dependovirus*) requires coinfection of host cells by adenoviruses or herpesvirus to provide helper functions necessary for its replication.

In contrast to the transmission of viral infection between hosts, the spread of infection within a single animal usually involves successive episodes of localized viral replication that resemble the conditions of plaque formation and serial high multiplicity passage in cell culture. These conditions favor the generation and amplification of defective viral genomes, which can arise from a simple internal deletion of genes as well as more complex genome rearrangements that occur during RNA replication. Like satellite RNAs, defective RNAs parasitize the parent virus and usually interfere with its replication, but because they also depend upon it for their own survival, they typically establish a fluctuating coexistence. Most families of animal RNA viruses readily generate defective interfering (DI) RNAs in cell culture, but their influence on viral disease and evolution is less well understood.

EXPRESSION AND REPLICATION OF DNA VIRUS GENOMES

DNA Virus Genome Strategies

Viral DNA genomes range in size from the 1.8-kb circoviruses to the 1,200-kb genomes of the *Megaviridae*.³ This difference in the coding capacity means that viruses from different families vary widely in how many of the functions necessary for viral replication they can encode themselves. For example, DNA viruses with small genomes such as the polyoma-, papilloma-, and parvoviruses use host-cell enzymes for transcription and replication (Figs. 5.3 and 5.4). Those with intermediate-size genomes (up to 35 kb) such as adenoviruses, encode much of their DNA replication machinery including a DNA polymerase, terminal protein and ssDNA binding protein, but they employ cellular RNA polymerase II and III for transcription (Fig. 5.5). Those with larger genomes (150 to 350 kb), such as the herpesviruses and poxviruses, encode DNA polymerases and binding proteins. In the case of herpesviruses, multiple specific transcription factors serially modify the promoter specificity of RNA polymerase II (Fig. 5.6), or in the case of the poxviruses multi-subunit transcriptase complexes perform all the functions of capping and polyadenylation as well as RNA transcription (Fig. 5.7). Hepadnaviruses buck this general trend in that they are small genomes (3 kb) but encode the DNA polymerase/reverse transcriptase that executes their unique mechanism of DNA replication via an ssRNA intermediate (Fig. 5.8).

Because cellular DNA synthesis occurs during the S phase of the cell cycle and not at all in terminally differentiated G0 cells, viruses that depend on the host DNA polymerase must either wait for the infected cell to enter S phase spontaneously, as in the case of parvoviruses, or early in infection, they must express one or more viral oncogenes to override the regulation of the cell-cycle control proteins p53 or pRb and thereby stimulate infected cells to enter S phase, as in polyomaviruses and papillomaviruses. Inactivation of pRb releases cellular transcription factor E2F, which induces expression of the cellular DNA polymerase α primase, DNA polymerase δ , ssDNA binding protein, and several critical cellular enzymes that are involved in both the *de novo* and the salvage pathways of deoxynucleotide triphosphate (dNTP) biosynthesis, including ribonucleotide reductase, thymidylate synthetase, dihydrofolate reductase, deoxyuridine triphosphate nucleotidohydrolase (dUTPase), and thymidine and thymidylate kinases. Viruses with large DNA genomes (e.g., herpesviruses and poxviruses) encode some of those enzymes themselves and can thus replicate in nondividing cells and other environments that would not normally support DNA replication, such as terminally differentiated cells of the nervous system (some herpesviruses) or even the cytoplasm (poxviruses). Although these viral genes are often dispensable for virus replication in actively dividing cells in culture, they can exert a profound influence on viral virulence in infected organisms and thus provide targets for chemotherapeutic intervention. For example, the thymidine kinase gene of some herpesviruses (but not the host enzyme) phosphorylates the prodrug acyclovir to generate a dNTP analog that terminates nascent strands during DNA synthesis.³²

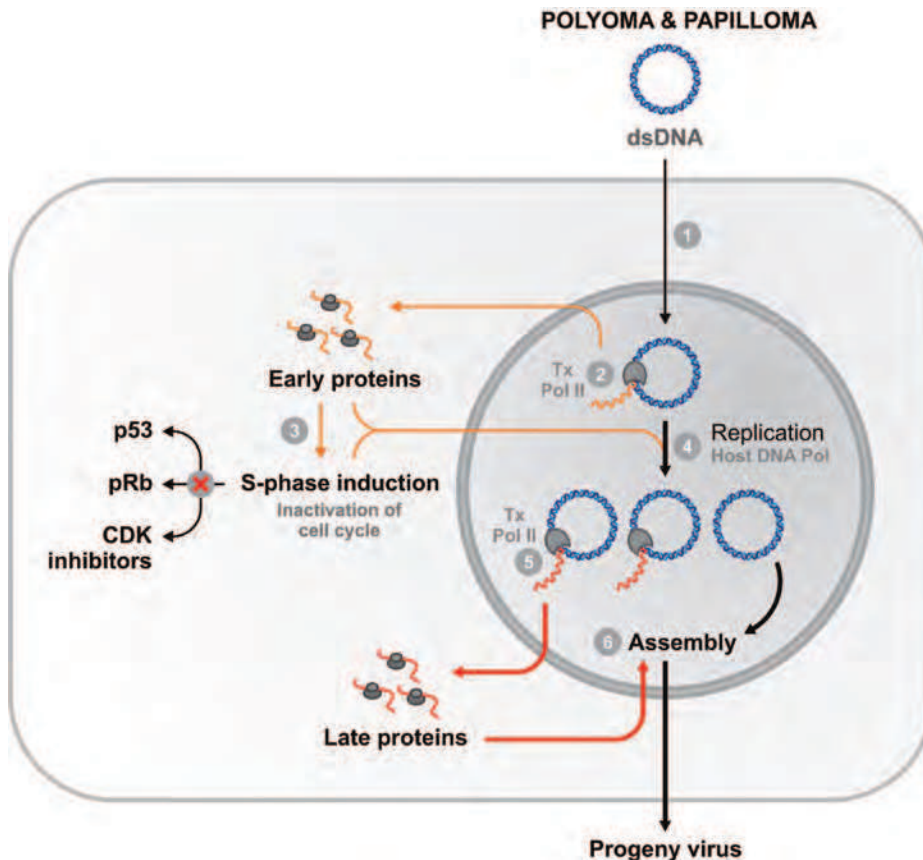


FIGURE 5.3. Simplified view of the replication scheme of *Polyomaviridae* and *Papillomaviridae*. The circular dsDNA genome is transported to the nucleus (step 1) where cellular RNA polymerase II transcribes the early genes (step 2) that encode the viral oncoproteins or transforming (T) antigens. The products of step 2 interfere with the host-cell cycle control proteins p53 and pRb or interact with inhibitors of cyclin-dependent kinases (CDKs) to stimulate cellular DNA replication (step 3). In nonpermissive cells that cannot support the vegetative replication cycles and therefore survive the infection, these early events can lead to neoplastic transformation. In permissive cells, the viral DNA is replicated by the host-cell DNA polymerase (step 4), following which cellular RNA pol II can transcribe the late genes that encode viral structural proteins (step 5). The assembly of viral particles occurs in the nucleus (step 6). The thickness and color intensity of the arrows signifies the predominant events.

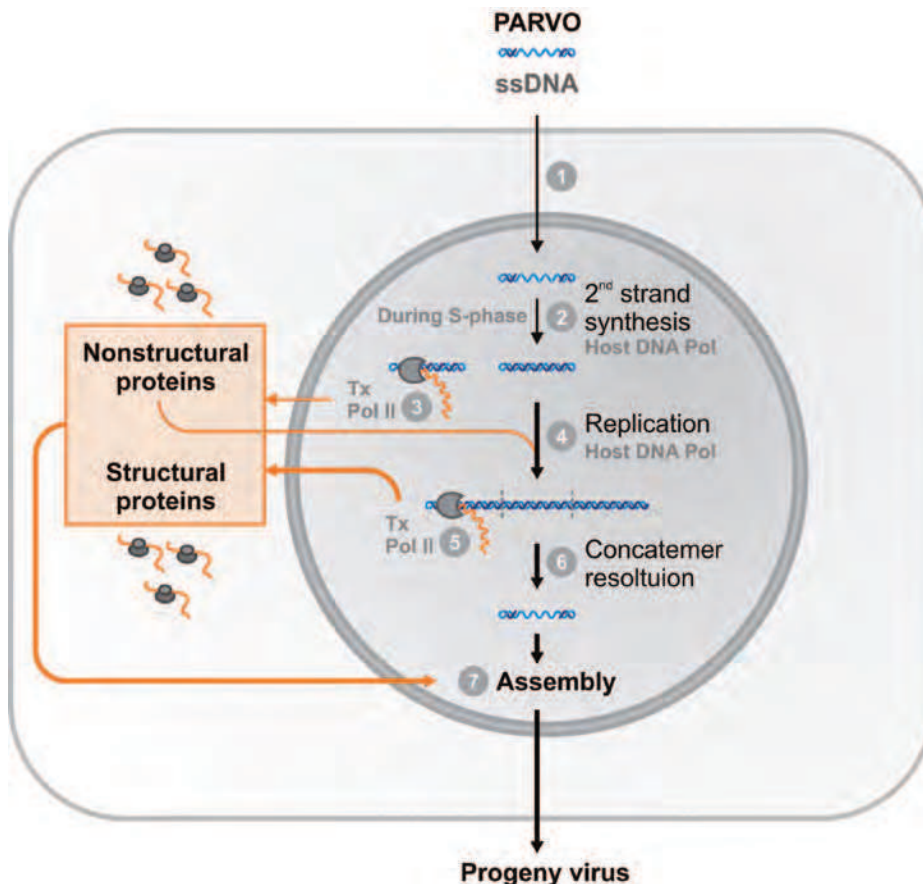


FIGURE 5.4. Simplified view of the replication scheme of *Parvoviridae*. Following entry, the linear ssDNA genome is delivered to the nucleus (step 1) where self-primed second strand synthesis is mediated by the host DNA polymerase during the S phase of the cell cycle (step 2). The resulting dsDNA hairpin is transcribed by the cellular RNA polymerase II (step 3) to produce mRNAs that encode viral nonstructural and structural proteins, and is ligated to form a covalently continuous duplex. The nonstructural proteins promote further DNA replication by the host DNA polymerase, which occurs via a rolling hairpin mechanism to produce double-stranded concatamers of the viral genome (step 4). The concatamers are templates for transcription by host RNA polymerase II to produce further viral proteins (step 5), and they are resolved (step 6) prior to assembly (step 7) into viral particles.

FIGURE 5.5. Simplified overview of the replication scheme of *Adenoviridae*.

The linear dsDNA genome is delivered into the nucleus (step 1), where it is transcribed by the host cell RNA polymerase II (step 2) to produce the early gene products including oncoproteins and the viral DNA polymerase. In adenoviruses of primates, the host RNA polymerase III also transcribes the genome to produce VA RNAs that act as interferon antagonists (step 3). The early gene products override cell cycle controls and inhibit apoptosis (step 4) as well as provide the essential viral polymerase components for genome replication (step 5). Following DNA replication, the late genes are transcribed by the host RNA polymerase II from a single major late promoter, and following extensive differential splicing provide the viral structural proteins (step 6). Virus assembly (step 7) occurs in the nucleus.

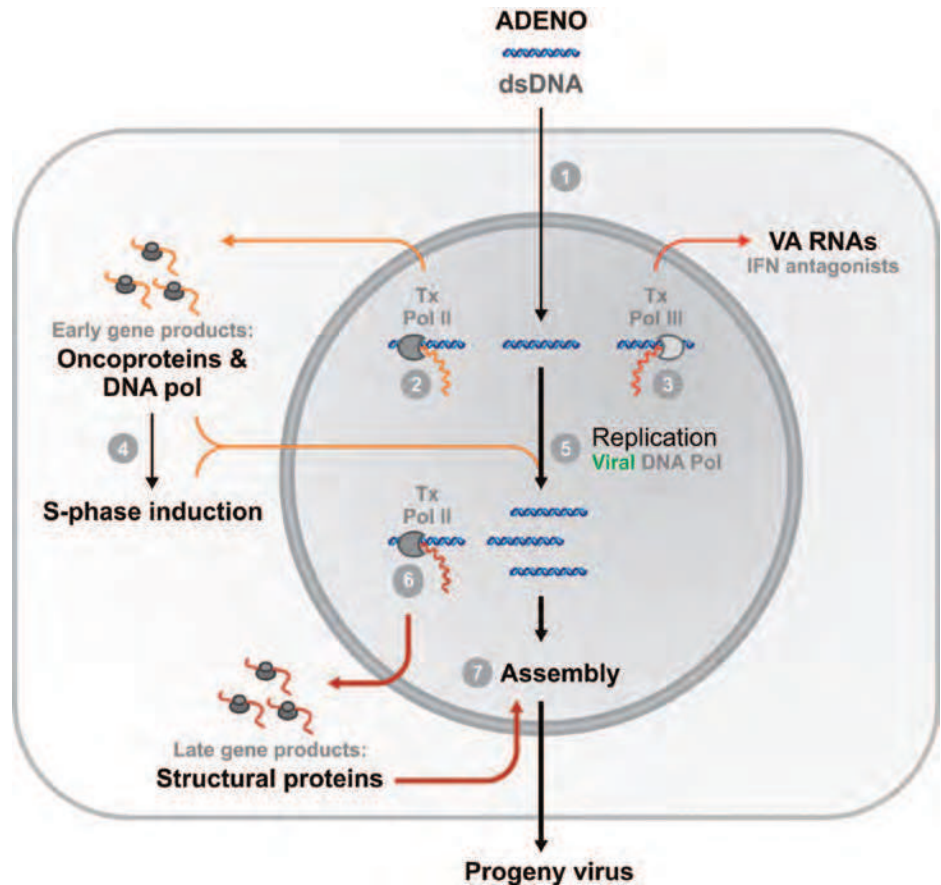
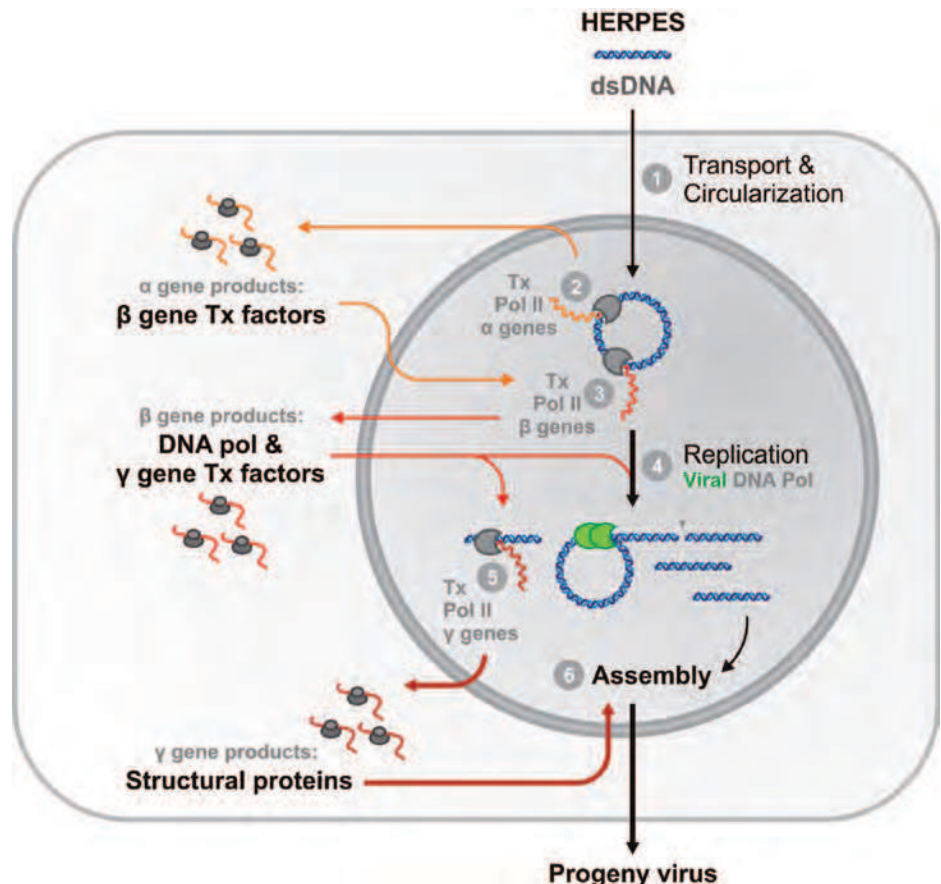


FIGURE 5.6. Simplified overview of the replication scheme of *Herpesviridae*.

The linear genomic dsDNA genome is delivered to the nucleus, where it undergoes covalent circularization (step 1). Cellular RNA polymerase II transcribes the immediate-early α genes (step 2) that encode transcription factors that mediate the host RNA polymerase II recognition of the promoters for the delayed-early β genes (step 3). Those genes encode the viral DNA polymerase and other proteins required for genome replication (step 4) as well as transcription factors required for the pol II-mediated expression of the late γ genes that encode most of the structural proteins (step 5). Virions assemble in the nucleus (step 6) and exit through the nuclear pore. The assembled virion contains the necessary transcription factors for expression of the immediate-early α genes on infection of the next cell. Note that this scheme represents only the vegetative cycle of *Herpesviridae* replication. Readers are referred to the individual *Herpesviridae* chapters regarding the establishment, maintenance of, and reactivation from latency.



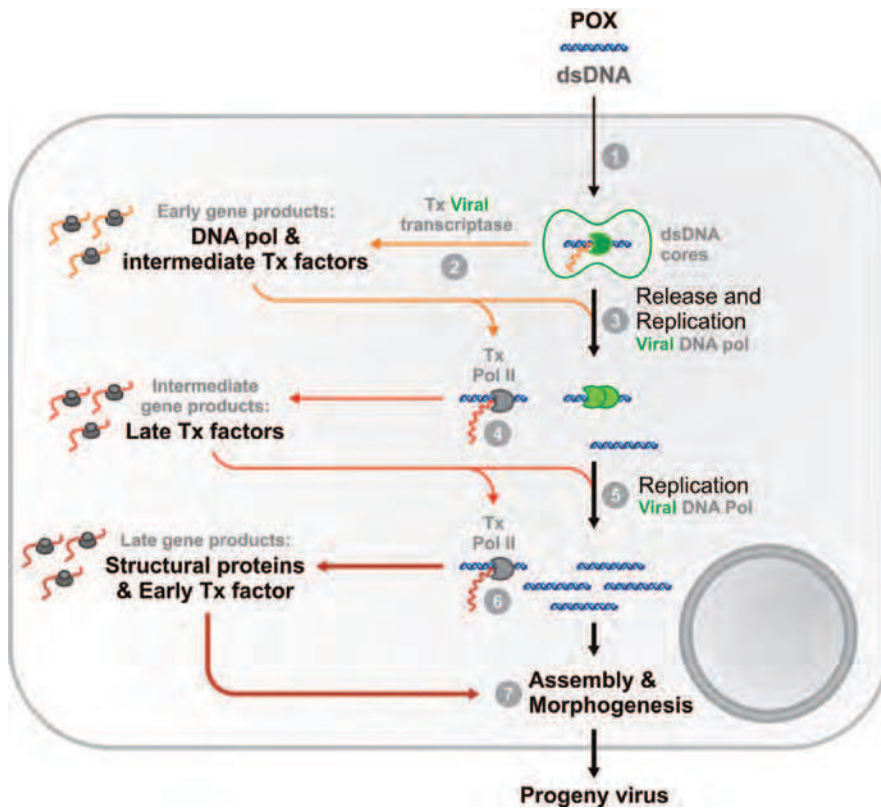


FIGURE 5.7. Simplified scheme of the replication of *Poxviridae*. Entry delivers the viral core containing the dsDNA genome into the cytoplasm—the site of viral RNA synthesis (step 1). In the cytoplasm the multisubunit virion DNA-dependent RNA polymerase transcribes the early viral genes (step 2), which comprise approximately 50% of the genome. The early gene products include factors that mediate the release of the dsDNA into the cytoplasm (second stage uncoating), the viral DNA polymerase and associated enzymes required for replication (step 3), and transcription factors that direct the viral RNA polymerase to transcribe a limited number of intermediate genes (step 4). DNA replication proceeds via a rolling hair-pin mechanism (step 3 and 5) similar to that for parvoviruses, and is concurrent with the expression of the intermediate gene products (step 4). The intermediate gene products include transcription factors required for late gene expression (step 6). The products of late gene expression encode most of the structural proteins as well as the viral transcriptase and associated factors that will be required at the start of a new infection. Progeny genomes, viral structural proteins, and membranes of the host cell participate in the assembly of viral particles that undergo extensive morphogenesis and maturation (step 7) prior to release.

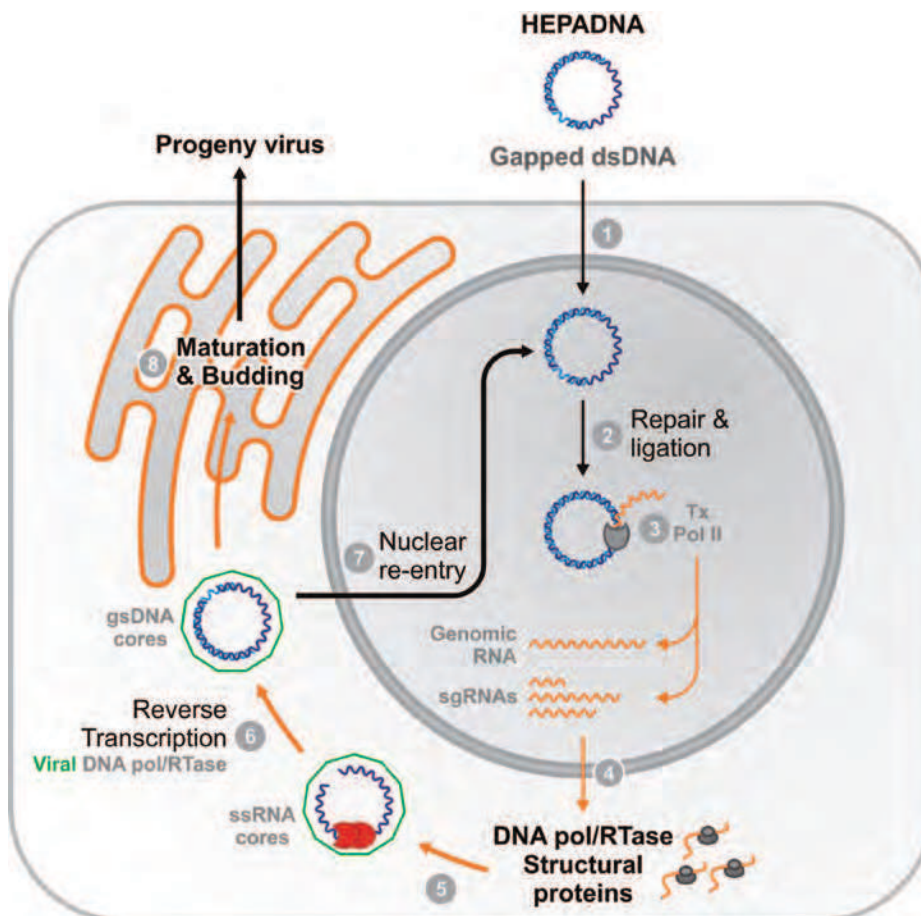


FIGURE 5.8. Simplified replication scheme for hepadnaviruses. Following entry and partial uncoating (step 1), viral cores containing the gapped dsDNA genome migrate to the nucleus where the dsDNA genome is repaired and ligated into a covalently closed circle (steps 1 and 2). This repaired genome is transcribed by the host-cell RNA polymerase II (step 3) to yield terminally redundant pregenomic RNA and subgenomic RNAs for the viral structural and nonstructural proteins (step 4). In the cytoplasm, the viral DNA polymerase, reverse transcriptase (RTase), and core proteins associate with the pregenomic RNA to form immature cores (step 5) that mediate polymerase-primed reverse transcription of the pregenomic RNA to yield gapped dsDNA genomes (step 6). The progeny cores either then enter the nucleus (step 7) to repeat the cycle or can bud through the endoplasmic reticulum to yield viral particles (step 8).

Regulation of Viral Gene Expression

During the early, prereplicative phase of the infectious cycle of a typical DNA virus, a subset of so-called immediate early viral genes is expressed to produce mostly catalytic quantities of nonstructural proteins required for DNA replication and host-cell manipulation. After DNA replication, a different set of genes is expressed (late genes) that direct the synthesis of stoichiometric amounts of the structural proteins required for viral assembly. Expression of the early genes is often concomitantly repressed. This early/late switch, which is a common feature of many DNA virus families, can be understood as an evolutionary adaptation that gives viruses an advantage in competing with the host cell for gene expression: Early gene expression is modest, whereas later during the postreplicative stage of the infectious cycle, increased gene copy numbers enable the virus to dominate the protein synthesis capacity of the cell. In addition to the early and late genes, sets of intermediate genes have been identified in the poxviruses and herpesviruses, with each temporal class encoding factors that switch on the next in a transcriptional cascade (Figs. 5.6 and 5.7). For the viruses in these families, transcription factors for immediate early genes are expressed late in infection and assembled into progeny virions in preparation for the next infectious cycle.

At the start of infection, immediate early viral promoters face stiff competition from overwhelming numbers of cellular promoters. To successfully recruit RNA pol II and other components of the transcriptional machinery, many DNA viral genomes contain enhancers: *cis*-acting regulatory elements that facilitate the assembly of transcription complexes by binding multiple cellular transcription factors and other accessory proteins. A defining feature of enhancers, which were first identified in the genome of the polyomavirus simian vacuolating virus 40 (SV40), is that they exert their effects from either upstream or downstream of promoters and can act over distances of several kilobases.

Efficient transcription from late promoters usually requires one or more early gene products, as well as cellular transcription factors that may differ from those used by the early promoters. Dependence on specific cellular transcription factors can limit the expression of late genes to particular cells or tissues where the necessary factors are naturally expressed. For example, transcription of late papillomavirus genes requires a specific transcription factor that is expressed only in fully differentiated skin cells. As a result the replication cycle is stalled after DNA replication (Fig. 5.3, step 4) until the cell differentiates.

Infection of cells with herpesviruses and poxviruses increases the rate of mRNA degradation.¹⁹ In some herpesviruses, that is mediated by a protein component of the infecting virions called vhs (virion host shutoff). Although enhanced turnover is not specific for cellular mRNAs, viral mRNAs are readily replenished by robust transcription of the viral genome so that the net result is the selective suppression of host protein synthesis. In addition, the rapid turnover of viral mRNAs accelerates the transitions in the transcriptional cascade.

Mechanisms of DNA Replication and Transcription

Most DNA viruses produce functional viral mRNAs by usurping the transcriptional machinery of the cell (Fig. 5.1). This

machinery includes RNA pol II, multiple transcription factors, poly(A) polymerase, guanylyltransferase, methyltransferases, and the pathway of mRNA export from the nucleus. Even viruses with unusual genome structures such as parvoviruses and hepadnaviruses use these cellular components because their genomes are rendered into dsDNA before transcription (Figs. 5.4 and 5.8). Only DNA viruses that replicate in the cytoplasm (pox-, irido-, and asfiviruses) use virus-specific enzymes for transcription and posttranscriptional modification of their mRNAs (Fig. 5.7). Because these enzymes are virion structural components, they can often be purified more readily than their cellular counterparts, and in the case of the vaccinia poxvirus and Chlorella virus, their reactions and structural properties have been well studied.^{45,51,53} The majority of RNA viruses also replicate in the cytoplasm and employ virus-specific enzymes to synthesize and modify their mRNA.²⁴

Viral DNA genomes replicate by at least five different mechanisms, which are summarized as follows (for more details readers are referred to the chapters that describe each viral family).

1. The circular dsDNA genomes of polyomaviruses and papillomaviruses (Fig. 5.3) replicate bidirectionally from a single AT-rich origin via the RNA-primed synthesis of continuous leading strands and discontinuous lagging strands at both replication forks. Circularity of the genome aside, the reactions at the replication forks closely resemble how the host chromosome is replicated.^{35,68}
2. In stark contrast, the linear dsDNA genome of adenoviruses (Fig. 5.5) is replicated by a protein-primed synthesis of only the leading strand, resulting in displacement of ssDNA from each end of the parental duplex. The termini of the displaced strands anneal via inverted terminal repeats, creating duplex panhandle structures that serve as secondary origins of replication. The primer (preterminal protein) is the product of an early gene, and a copy of this protein is covalently bound to the 5' end of each of the daughter strands.^{23,67}
3. The linear dsDNA of herpesvirus genomes is first circularized and then replicated from one or more internal origins, most likely by an RNA-primed mechanism that eventually produces dsDNA concatamers (Fig. 5.6). Progeny DNA can undergo isomerization by homologous recombination between internal and terminal repeated sequences, and unit length genomes are resolved from the concatamers during packaging into virions.⁷⁵
4. Despite their different structures and sizes, poxvirus (Fig. 5.7) and parvovirus (Fig. 5.4) genomes replicate by similar mechanisms. The close-ended duplex poxvirus genome (or the closed-ended duplex intermediate in parvovirus replication) is nicked near its terminus, and the newly generated 3' end serves to prime DNA synthesis using the complementary strand of the duplex as template. This initial self-priming event is reproduced by partially base-paired hairpin structures located at each end of the duplex genome, resulting in so called "rolling hairpin" replication. For both poxviruses and parvoviruses, the product is a dsDNA concatamer from which unit length genomes are excised by resolution of concatamer junctions.^{9,20,104}
5. Finally, in the most tortuous mechanism of all, hepadnaviruses (Fig. 5.8) replicate their dsDNA genome by a

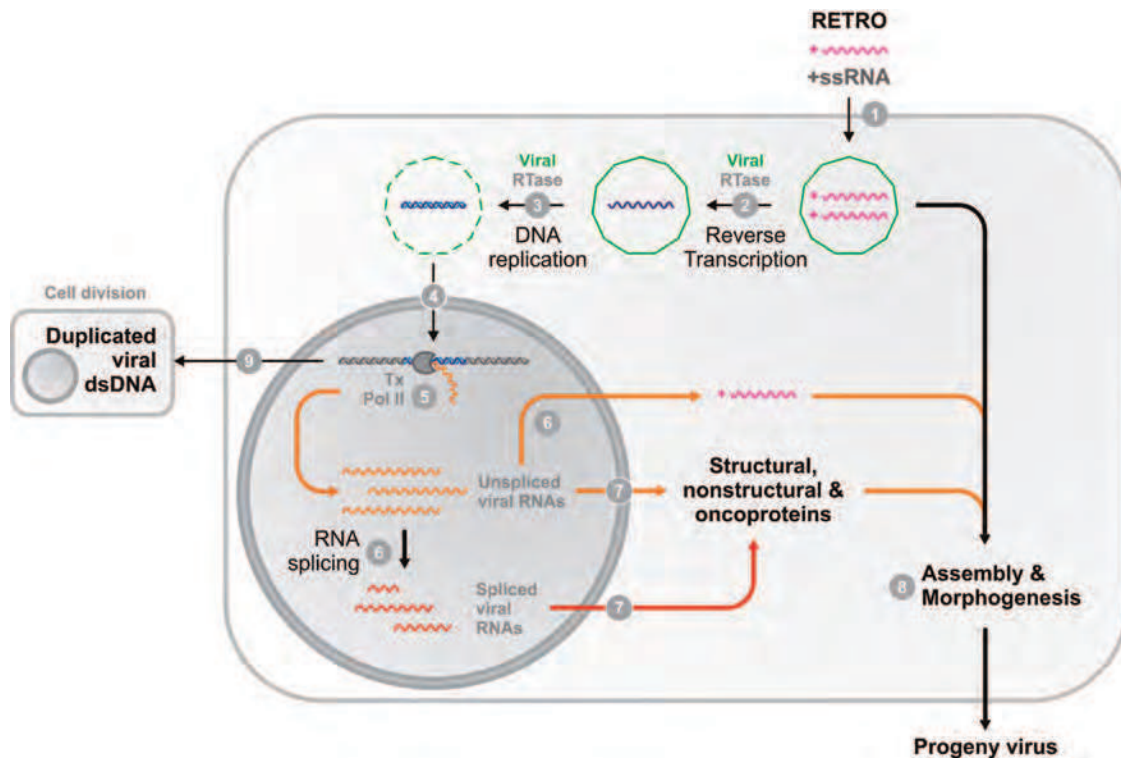


FIGURE 5.9. Simplified replication scheme of *Retroviridae*. Following entry and partial uncoating (step 1), the viral genome is copied into dsDNA by the reverse transcriptase (step 2 and 3) and integrated into the host chromosome by the virion DNA integrase (step 4). The integrated viral genome (provirus) is transcribed by the host-cell RNA polymerase II (step 5) to produce viral transcripts that function as precursors to the mRNA for the viral proteins (steps 6 and 7) as well as progeny genomes for assembly into infectious particles (step 8).

full-length pregenomic ssRNA transcript made by RNA polymerase II. Pregenomic RNA is then reverse transcribed by the viral encoded DNA polymerase/reverse transcriptase to produce dsDNA progeny. In contrast to retroviruses (Fig. 5.9), DNA integration is not required for hepadnavirus replication, the genome being maintained as a circular episome in the nucleus of infected cells. Caulimoviruses—the only dsDNA viruses that infect plants—use a similar reverse transcriptase (RT)-mediated replication strategy. To prime first strand DNA synthesis, hepadnavirus RTase uses a domain of the polymerase itself. This differs from the tRNA-primed strategy employed by retroviruses and caulimoviruses.

The polymerases employed for these strategies of replication are structurally and functionally homologous—yet they accomplish replication via very distinct mechanisms. Thus the evolutionary origin of such disparate mechanisms of replication remains uncertain.

Remarkably protein-primed replication is discontinuous. In the three known examples—adenovirus, poliovirus, and hepadnaviruses—the first few nucleotides of the genome are templated from an internal motif rather than at the very 3' end of the parental genome. This necessitates a jump or realignment of the protein-primer product together with the polymerase to the 3' end of the parental genome to complete

synthesis of the daughter strand. In the case of adenovirus, the first templated nucleotides added to the primer are positions 4–6 of the genome, which then realigns with the 3' end of the genome to complete daughter-strand synthesis following annealing of the nascent strand to the first three nucleotides of the parental genome.²³ For hepadnaviruses, the first four nucleotides are added to the RTase from a stem loop positioned at the 5' end of the pregenomic RNA. The RTase nascent strand RNA product leaps almost 3 kb and then continues processively to complete synthesis of the daughter strand. Among the RNA viruses, the picornaviruses employ an internal stem loop termed the *cis*-acting replication element (*cre*) within the parental strand to template the uridylation of a protein primer, VPg.⁸² This primer is then repositioned together with polymerase at the 3' end of the genome to prime synthesis of the new strand. Such protein-primed mechanisms likely aid in maintaining the integrity of the genome ends, which contain vital signals for replication.

Latent and Persistent Infections

In addition to the typical vegetative replication cycles illustrated in Figures 5.3 through 5.8, many DNA viruses establish latent or persistent infections of their hosts. Several distinct mechanisms of persistence have been identified with different viruses, but they all involve suppression of viral cytopathic effects,

long-term maintenance of the viral genome, and evasion of the cellular and organismal defences. For example, herpesviruses typically establish latent infections in which the viral genome is maintained as a circular episome in the nucleus, expressing at most only a few viral genes and yielding no infectious virus. Such latent herpesvirus infections persist throughout the life of the host, successfully evading host immune surveillance, yet able to reemerge at intervals as productive lytic infections. Because of its importance for human health, understanding the establishment and maintenance of herpesvirus latency and the mechanisms that regulate the reemergence of infectious virus are the subject of intense study (see Chapters 59 to 65). In the case of human herpesvirus 4 (Epstein-Barr virus), which infects B lymphocytes and causes mononucleosis, latent infections can be established and maintained in cell culture; this has greatly facilitated experimental study of the mechanisms involved. For other herpesviruses, the establishment and maintenance of latency occur in less-accessible cell types and are much less well understood.

Viral Oncogenes and Neoplastic Transformation

In cells that somehow survive DNA virus infection, such as nonpermissive cells that express early genes but cannot replicate viral DNA or produce infectious progeny, the expression of viral oncogenes and the consequent loss of cell cycle control can lead to neoplastic transformation and the formation of tumors in infected animals (see Chapter 7). Unlike typical retrovirus-induced tumors, where the entire viral genome is integrated into the host chromosome as an essential step of

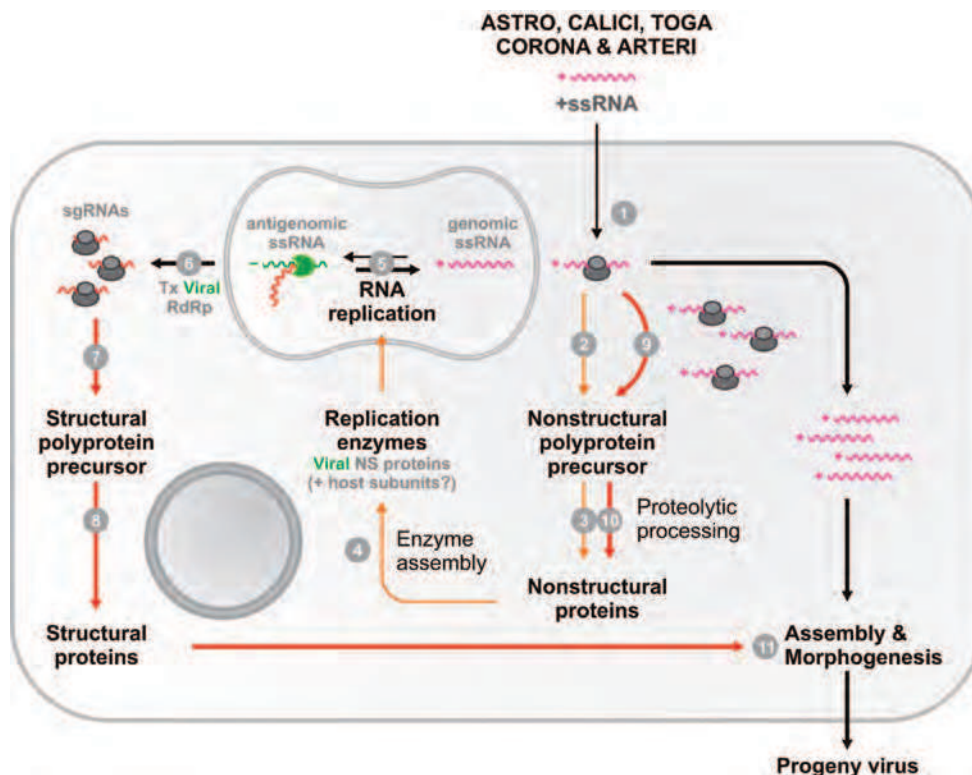
the viral replication cycle, tumors induced by DNA viruses rarely contain a complete viral genome or produce infectious virus. Instead, they typically express only the viral oncogenes from integrated copies, disrupting cell cycle control by inactivating p53/pRb or by activating cyclin-dependent kinases, for example. Alone among the DNA viruses that replicate in the nucleus, parvoviruses do not induce tumors because they are unable to override cell cycle controls. Some poxviruses induce the formation of self-limiting benign tumors when they secrete a virus-encoded growth factor that induces surrounding cells to divide.

EXPRESSION AND REPLICATION OF RNA VIRUS GENOMES

RNA Virus Genome Strategies

The type of RNA genome dictates the first biosynthetic steps following infection. For example, the message sense positive-strand RNA viruses, excluding the retroviruses, all initially deliver their genomes to ribosomes to ensure the synthesis of essential proteins to establish viral replication (Figs. 5.10 and 5.11). Consequently the viral genomic RNA alone is infectious once delivered into a host cell—a fact that greatly facilitated the genetic manipulation of such viruses. That the RNA alone was infectious was first shown for tobacco mosaic virus, in experiments that helped establish that genes were comprised of nucleic acids.⁴¹ By contrast to the genomes of positive-sense RNA viruses, those of the negative-strand RNA viruses, retroviruses, and double-stranded RNA viruses all must deliver into

FIGURE 5.10. Simplified replication scheme of positive-strand RNA viruses that produce subgenomic RNA. Following entry and uncoating (step 1), the genomic RNA is engaged by the host-cell ribosome to produce the nonstructural proteins including the RdRp (steps 2 and 3). The viral replication enzymes together with host components form replication compartment in which the genomic RNA is replicated into an antigenome and progeny genomes (steps 4 and 5). The viral RdRp also transcribes one or more subgenomic RNAs (step 6) that encode viral structural proteins (steps 7 and 8). Replicated genomes are translated to amplify the production of viral proteins (steps 9 and 10) and may be used as templates for further replication. The genomes are assembled with viral structural proteins (step 11) to yield progeny virions. The scheme by which the *Coronaviridae* and *Arteriviridae* synthesize their subgenomic RNA is different from that employed by the *Toga*-, *Astra*-, and *Caliciviridae*.



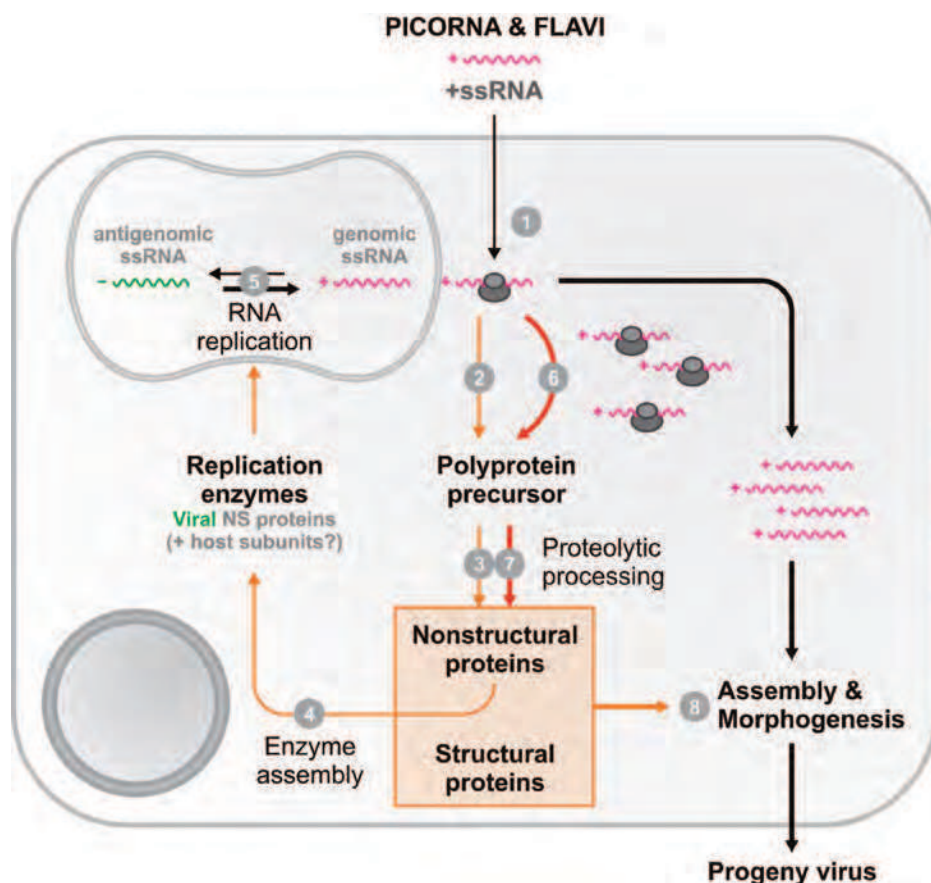


FIGURE 5.11. Schematic of the replication cycle of positive-strand RNA viruses that do not make subgenomic RNA. Following entry and uncoating (step 1), the genomic RNA is used directly as an mRNA to synthesize both structural and nonstructural proteins (steps 2 and 3). The nonstructural proteins, including the RdRp and host components, establish a membrane-associated replication compartment in which the genomic RNA is copied into antigenomic RNA and progeny genomes (steps 4 and 5). Replication produces additional genomic RNA that can be used for further translation and genomic amplification (steps 6, 7, and 5) as well as assembly with the viral structural proteins (step 8) to yield progeny virions.

the cell a protein RNA complex that contains within it the viral polymerase.

The positive-sense RNA viruses fall into two general groups, those that transcribe subgenomic RNAs (Fig. 5.10) and those that do not (Fig. 5.11). Viruses that do not transcribe subgenomic RNA are translated by the host ribosomes to produce one or more polyprotein precursors that undergo a set of *in cis* and *in trans* cleavage reactions by viral encoded proteases.²⁹ Those cleavage reactions result in the production of the individual non-structural proteins essential for the replication of the viral genome, including the establishment of the site of viral replication. For viruses that produce a single polyprotein the precursors of the catalytic components are generated in equimolar amounts to the structural proteins. In some cases, control over the abundance of the catalytic components is provided by the accumulation of intermediates in the processing cascade in which one or more of the catalytic activities are absent.⁹⁵ Such components can play crucial structural or regulatory roles in the establishment of replication compartments. Viruses that produce two polyprotein precursors always employ an internal ribosome entry site (IRES) to drive the expression of the second polyprotein. Because the translation of the second polyprotein is independent of the first, the proteins are not produced in equimolar amounts, allowing for some regulation of the abundance of the different proteins.⁵⁰ For viruses that produce subgenomic RNAs, the input genome is first translated into a precursor of the nonstructural proteins that includes the RdRp. The genome is then subsequently copied into one or more subgenomic mRNAs that encode the

structural proteins. The production of subgenomic mRNAs facilitates the synthesis of distinct amounts of the structural and nonstructural proteins—such that the structural proteins are often produced in vast molar excess of the catalytically essential nonstructural proteins.

In contrast to the naked RNA of the positive-sense RNA viruses, that of the negative-sense, ambisense, and dsRNA viruses is noninfectious (Figs. 5.12–5.14). This is because the infectious unit is a ribonucleoprotein complex comprising the genomic RNA associated with the necessary viral polymerase components for synthesis of mRNA.^{76,105} In the case of the negative-sense and ambisense RNA viruses, the input genomic RNA is copied by the viral polymerase complex into mRNA (Figs. 5.12 and 5.13). The input genomes are then replicated to yield antigenomes, a process that requires newly synthesized viral nucleocapsid protein to coat the nascent RNA strand.^{76,105} For the negative-sense RNA viruses (Fig. 5.12), the antigenomes are positive sense, but they do not serve as templates for translation, rather they function exclusively as templates for genomic replication.^{76,105} The negative-sense orthomyxoviruses and bornaviruses replicate within the host-cell nucleus, whereas the mammalian infecting rhabdoviruses as well as the paramyxoviruses and filoviruses replicate in the cytoplasm. A further important distinction is that the rhabdo-, filo-, paramyxo-, and bornaviruses sequentially transcribe a series of 5 to 10 monocistronic mRNAs from a single genomic template, whereas the orthomyxoviruses synthesize only a single mRNA from each segment. In the case of the ambisense RNA viruses, the genomic and antigenomic RNAs have both message and

FIGURE 5.12. Simplified replication scheme for negative-sense RNA viruses.

Following entry and partial uncoating (step 1), the encapsidated viral genomic RNAs are transcribed by the virion RdRp into mRNAs (step 2) that encode the viral structural and nonstructural proteins (step 3). For the *Orthomyxoviridae* and *Bornaviridae*, transcription occurs in the nucleus (reflecting their need for splicing), whereas for the *Rhabdo*, *Filo*, *Paramyxo*, and *Bunyaviridae* this occurs in the cytoplasm. Replication is concomitant with encapsidation of the genomic and antigenomic RNAs (steps 4 and 5), and the newly produced genomic RNAs serve as templates for further mRNA production (step 6) as source of further viral proteins (step 7) as well as for assembly of progeny virions (step 8). The segmented negative-sense RNA viruses transcribe each genomic segment into a single transcript (which can be alternately spliced in the case of the orthomyxoviruses). The non-segmented negative-sense RNA viruses sequentially transcribe a series of 5 to 10 monocistronic mRNAs from the genomic template.

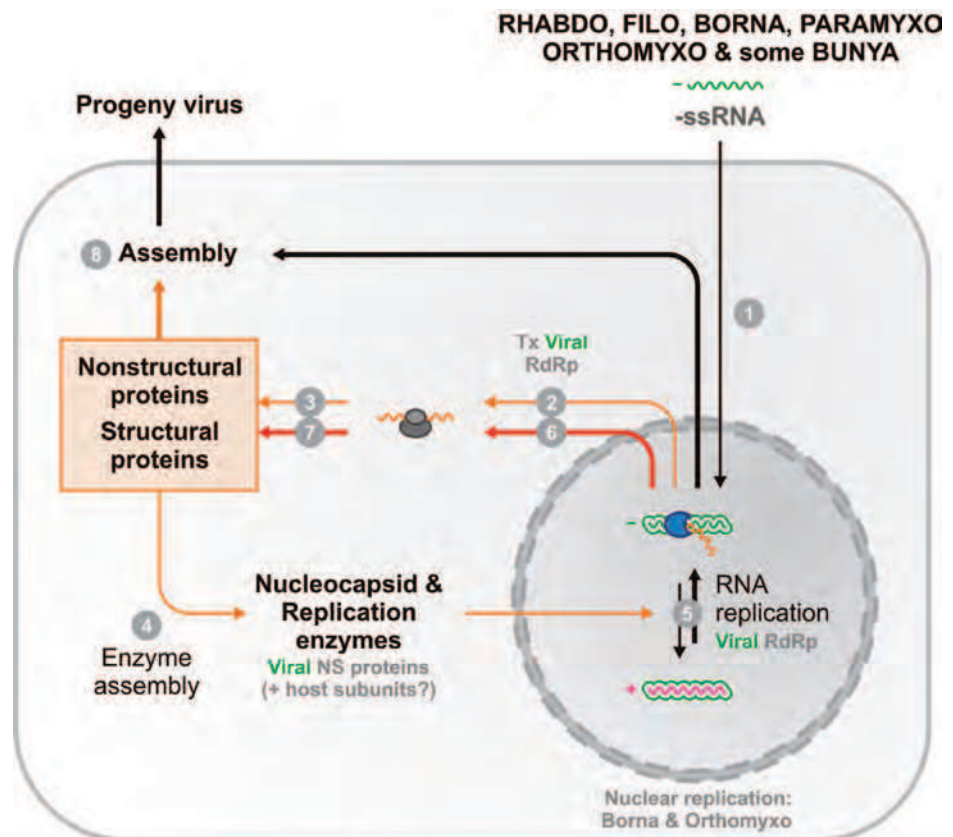
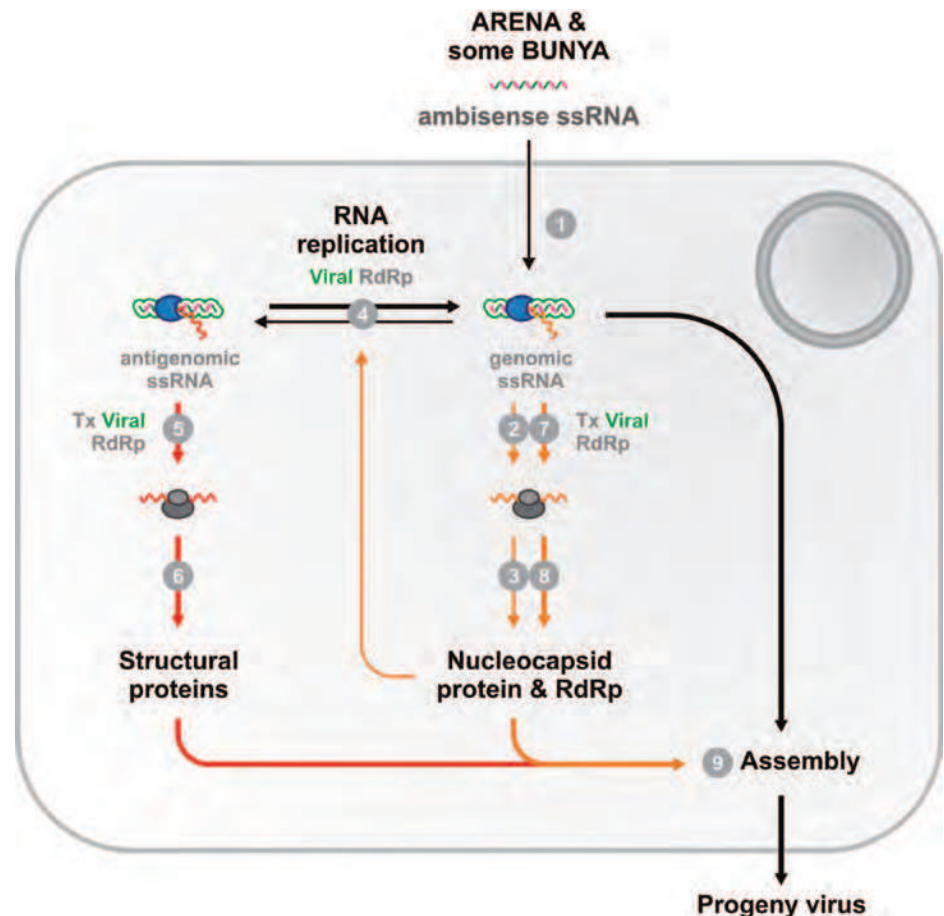


FIGURE 5.13. Replication scheme for the ambisense RNA viruses.

Following entry and partial uncoating (step 1), the encapsidated genomic RNAs are transcribed by the virion RdRp to yield mRNAs (step 2) that encode the viral nucleocapsid protein and RdRp (step 3). These proteins catalyze the synthesis of antigenomic RNA (step 4), which serves both as templates for transcription of additional mRNAs (step 5) that encode the remaining viral proteins (step 6), as well as templates for production of more genomic RNA (step 4). The replication products (both genomic and antigenomic) can serve as further templates for transcription of mRNA that encode both viral structural and nonstructural proteins (steps 5 through 8). The genomic RNAs are also assembled together with the structural proteins into infectious viral particles (step 9). Note some bunyaviruses are also simply negative-sense.



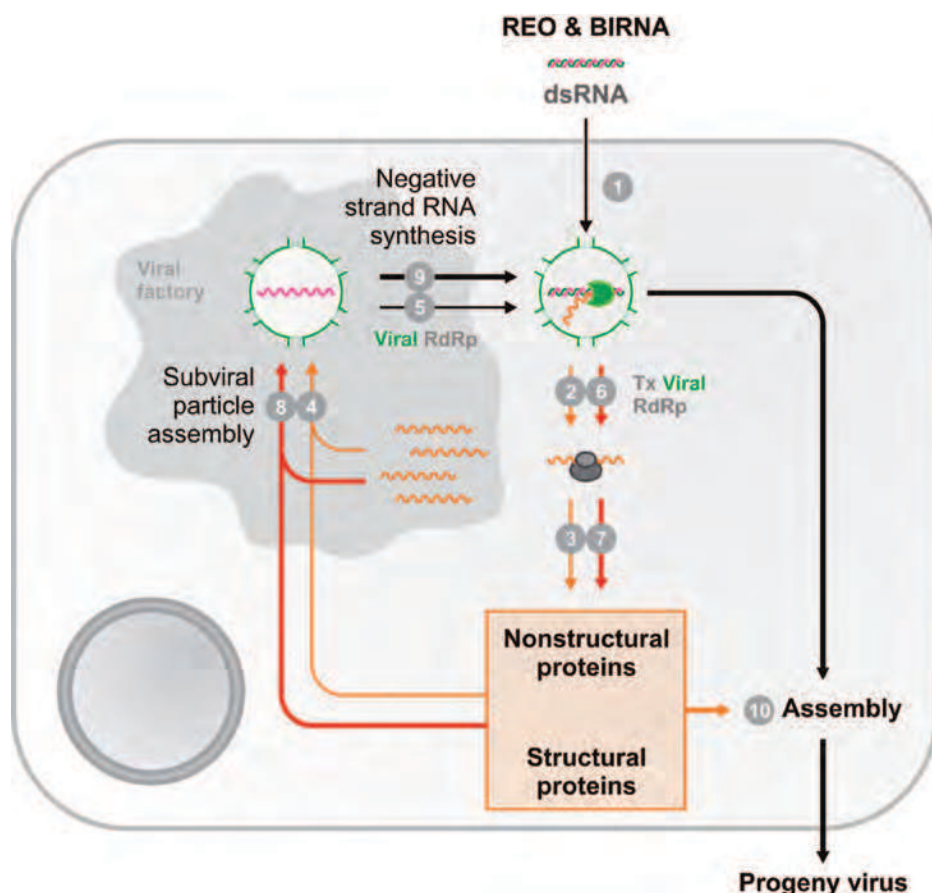


FIGURE 5.14. Simplified replication scheme for dsRNA viruses. Following entry and partial uncoating (step 1), the dsRNA segments within viral cores are transcribed by the core associated RdRp to produce mRNA (step 2) for the viral proteins (step 3). These form subviral particles around the mRNA (step 4), which are then copied to produce genomic dsRNAs (step 5). RNA synthesis and replication likely occur within a specific cytoplasmic factory established by the viral proteins. Progeny subviral particles contribute to viral gene expression (steps 6 and 7) and replication (steps 8 and 9) and assemble with outer shell proteins to form progeny virus particles (step 10).

anti-message polarity. However, those RNAs serve exclusively as templates for both mRNA transcription and replication (Fig. 5.13) rather than translation.³⁴ In contrast to all other negative-strand RNA viruses, the circular ssRNA genome of HDV does not require a specialized viral polymerase for copying. Rather, the HDV genome is transcribed and replicated by the host DNA-dependent RNA polymerase II in a unique RNA-templated reaction.⁹⁷

For the dsRNA viruses, the segmented genomes are delivered into the cell as a subviral particle that remains intact for transcription of the mRNA (Fig. 5.14). The polymerase and RNA-modifying enzymes form a structural element through which the nascent ssRNA strand passes and is cotranscriptionally modified. The ssRNA and their encoded proteins reassemble into new subviral particles that can direct the synthesis of antigenome RNA and form a dsRNA genome. Those progeny subviral particles can serve as templates for the production of further mRNA, and the viral protein or can be packaged into new virions.⁶⁹

The positive-sense RNA genomes of retroviruses (Fig. 5.9) use a distinct mechanism for replication. Here, the incoming ssRNA is copied into a dsDNA provirus using the virally encoded reverse transcriptase—an essential component of the incoming virion.^{5,98} This dsDNA is integrated into the host genome, where it is copied into differentially spliced transcripts that either serve as template for production of viral proteins or for production of viral progeny.

Regulation of Gene Expression

In contrast to DNA viruses, which exhibit clearly demarked early/late-phase gene expression, most RNA viruses show little differentiation between the pre- and postreplicative phases of the infectious cycle, and express their genes at roughly the same relative levels throughout infection. In cases such as the togaviruses and orthomyxoviruses (e.g., the influenza viruses) where some temporal regulation of gene expression occurs, the differences are subtle and mostly accompanied at the transcriptional level by modulation of mRNA levels.

Although DNA viruses utilize alternate translational control mechanisms as well as RNA viruses, translational control is especially prevalent among RNA viruses. The rate-limiting step for translation of cellular mRNA by the ribosome is at the process of initiation, and more specifically recognition of the mRNA cap structure by the cap-recognition protein eIF-4E, part of a multi-subunit complex. The cap-recognition complex then facilitates the recruitment of the small subunit of the ribosome in complex with other initiation factors. This complex then scans to the first AUG on the template RNA, typically localized within 100 or so nucleotides of the mRNA cap structure. Following this scanning to the AUG, the small subunit is joined by the large ribosomal subunit, and polypeptide chain synthesis is initiated. The poly(A) tail present at most cellular mRNA structures stimulates translation, as it is bound by the poly(A)-binding protein that bridges interactions with

the eIF-4G component of the cap-recognition complex. This may serve to functionally circularize the mRNA to facilitate ongoing translation. There are many notable exceptions to this general mechanism that have been exploited by RNA viruses. This reflects the fact that although superficially many RNA virus positive-sense transcripts resemble cellular mRNAs, there are distinctions at both the 5' and 3' ends of the RNA that are accompanied frequently by an altered mechanism of translational initiation.

One of the best known examples of an altered mechanism of translational initiation was provided by studies of how the genomes of picornaviruses—which lack a 5' mRNA cap structure and instead contain a genome-linked protein VPg—are expressed. The 5' untranslated regions of picornavirus genomes are unusually long (several 100s of nucleotides, including several AUG-specifying triplet) and are highly structured. Those structured elements termed internal ribosome entry sites (or IRES) serve to recruit the ribosome directly to the viral RNA,⁸³ without the need for a full complement of initiation factors for translation. Indeed in some viruses, such as cricket paralysis virus, the ribosome is recruited to the IRES without the need for any of the initiation factors that are essential for conventional translation. Such altered mechanisms are exploited by viruses to facilitate the efficient translation of the viral mRNA, thereby outcompeting cellular transcripts for translation ensuring the robust synthesis of viral proteins.

There are also many RNA viruses that produce mRNAs that lack a polyadenylate tail, the dsRNA reoviruses and rotaviruses, many of the ambisense arenaviruses and bunyaviruses, as well as numerous positive-strand RNA viruses. On most cellular mRNAs, the polyadenylate tail is generally thought to function as an element that stabilizes the mRNA and additionally favors translation by facilitating recycling of ribosomes through a protein-mediated bridging mechanism that brings the 5' and 3' ends of the mRNA together. Such circularization is likely achieved by direct RNA–RNA interactions in the case of some positive-strand RNA plant viruses—such as the luteovirus, barley yellow dwarf virus.⁵⁸ A similar mechanism has been postulated to function for the flavivirus, Dengue virus, although direct evidence for this is lacking.

A further mechanism of regulation of translation initiation exploited by RNA viruses is a translation termination–reinitiation strategy. This strategy involves the termination of translation of an upstream open-reading frame followed by the reinitiation at a proximal (<40 nt away) downstream open-reading frame. The stop and start codons are frequently found in an overlapping arrangement, but in distinct reading frames to one another, and termination is essential for the subsequent reinitiation. This strategy is exploited by the positive-strand RNA calciviruses, such as feline calicivirus and murine norovirus; as well as the negative-strand RNA viruses influenza B virus and respiratory syncytial virus.⁸⁶ Although the mechanism is not well understood, evidence from studies of calcivirus translation implicates the presence of a secondary structure in the mRNA that may function to “tether” the small ribosomal subunit to the transcript before reengagement of the large subunit.

Suppression of termination is also exploited during the translation of many viral mRNAs, notably those of the alphavirus and retrovirus families, as a mechanism of producing a protein with an extended C-terminus. In the case of some

retroviruses, a *cis*-acting regulatory element at the Gag-Pol junction functions to suppress termination of translation by leading to the misreading of the UAG termination codon to form the Gag-Pol polyprotein. Processing of this polyprotein then provides the viral reverse transcriptase and integrase. A second strategy is used by Rous sarcoma virus as well as other retroviruses, termed ribosomal frameshifting. A slippery sequence in the template leads to a realignment of the tRNA and the subsequent accessing of an altered reading frame. Such a mechanism is also exploited by other positive-strand viruses (notably the *Coronaviridae*) as well as several DNA viruses.

Structural and Nonstructural Proteins

By definition, virus-specified structural proteins are incorporated into virus particles, whereas nonstructural proteins are found only in infected cells. However, negative-sense, ambisense, and dsRNA viruses assemble their RdRp's and associated enzymes into progeny virions and therefore encode predominantly or exclusively structural proteins. In addition to the polymerase, virus-encoded enzymes often include one or more proteases, an RNA helicase, guanylyltransferase and methyltransferases, poly(A) polymerase, sometimes a nuclease, and in the case of retroviruses, a DNA integrase. However, for several RNA viruses, the evidence that these enzymes are virally encoded is only circumstantial or based on inconclusive sequence homologies.

The proteases process the primary translation products of which they are a part by cleaving them at highly specific target sequences, and in some picornavirus-infected cells they also selectively inhibit host-cell protein synthesis by cleavage of the eIF-4G component of the cap-recognition complex. Particularly among the larger RNA viruses, RNA helicases may be required to disrupt intermolecular or intramolecular base pairing during RNA synthesis, although some RdRp's are capable of melting RNA duplexes without help. Guanylyl and methyltransferases construct the 5' caps found on the mRNAs of most eukaryotic RNA viruses,^{24,37} except for the picornaviruses, which are uncapped, and the orthomyxoviruses, arenaviruses and bunyaviruses, which acquire their caps by stealing them from the host using a virus-encoded cap-dependent endonuclease.^{72,85} The cap structures of the *Mononegavirales* are formed by an altered mechanism involving a guanosine diphosphate (GDP)—polyribonucleotidyltransferase that is found within the same polypeptide chain that contains the polymerase and mRNA cap methylase activities.^{65,66,77,78} At their 3' ends, most animal virus mRNAs carry untemplated poly(A) tails, although 3' tRNA-like structures are common among plant RNA viruses (see Chapter 72). Polyadenylation is usually ascribed to a stuttering side reaction of the viral RdRp rather than to a separate poly(A) polymerase, as found in poxviruses, although the enzymology of the reaction remains to be clearly defined.^{76,105}

Comparisons among the amino acid sequences of viral RdRp's establish clear phylogenetic relationships that also reflect other differences in viral genome structures and strategies. Moreover, the x-ray crystal structures of the polio-, hepatitis C, reo-, rota-, phi6- and other RdRp's show clear resemblance to one another as well as to reverse transcriptases and DNA-dependent RNA and DNA polymerases. Although

the overall levels of amino acid sequence homology among these diverse polymerases are statistically insignificant, RdRp's share many of the characteristic polymerase motifs that occupy critical positions in the three-dimensional architecture of the polymerase active site. Taken together the structural similarities of their RdRp's firmly anchor the RNA viruses to the rest of the biosphere, and suggest that despite their diversity these viruses probably escaped from cellular origins to establish a horizontally transmissible parasitic existence using polymerases that radiated from a common ancestor.

Host Cell Factors

Proteins provided by the host undoubtedly play essential roles in RNA virus replication, although different cellular proteins have been implicated in different virus systems. The clearest example comes from the RNA replicase enzymes of the RNA bacteriophages Q β and MS2, which, in addition to the single phage-specified polypeptide that provides the polymerase active site, contain four cell-specified subunits: the *Escherichia coli* ribosomal protein S1, two translation elongation factors (EF-Tu and EF-Ts), and a strand-specific RNA-binding protein, host factor 1.⁸ In view of the similarity of their host components, the distinct specificities of the Q β and MS2 replicases for their cognate RNA templates must be determined by their unique viral subunits. The unexpected recruitment of translation factors to assist RNA replication—despite the evident differences between these two processes—may reflect underlying biochemical similarities in the RNA–protein interactions involved.

Translation factors have also been implicated in the replication of RNA viruses of eukaryotes. For example, a subunit of the initiation factor eIF-3 binds to the brome mosaic virus RdRp and increases its activity,⁸⁷ and several other host proteins have been found to associate with viral RNAs in infected cells. One of the difficulties in definitively assigning whether such proteins are just passengers, or active players in the replication of the viral genome, is often the lack of *in vitro* replication assays and/or genetically tractable host systems. Substantial progress has been made in this regard with the use of genetic, chemical, RNAi, as well as proteomic screens to hunt for host factors that are required for viral replication. Reconstructing the replication cycle of viruses in yeast,^{54,79} has permitted screens in collections of gene knockouts in *Saccharomyces cerevisiae*, and the application of RNAi has extended such screens to invertebrate and vertebrate cells.¹⁷ More recently the use of haploid human cells and gene-trap retroviruses has allowed the discovery of host-cell factors that are essential for the entry into cells of a number of viral pathogens.^{13,14} Although in some cases the overlap of “hits” between seemingly similar screens is low—likely reflecting technical difficulties of such large scale screens—the convergence on specific pathways exploited by viruses for their replication is generally high.⁴² Such systematic analyses provide a potentially fertile source for understanding the virus–host relationship; however, the detailed mechanistic understanding of how specific host proteins mediate viral replication lags behind.

In addition to host proteins, host nucleic acids also appear to participate directly in the replication of some RNA viruses. One such example is provided by host micro RNA's that function to titrate cellular gene expression by promoting transcript turnover or by sequestration. Work with hepatitis

C virus (HCV) identified a liver specific microRNA-122—which regulates cholesterol biosynthesis—that binds to two sites at the 5' end of the viral genome and enhances HCV replication.⁵⁵ Such control of viral replication only further serves to underscore the intimate relationship between viruses and their host cells.

Compartmentalization of Replication Sites

Unlike the phage replicases, the RdRp's of eukaryotic viruses are invariably found associated with some type of supramolecular assembly: host-cell membranes for positive-sense RNA viruses, nucleocapsids frequently found as inclusion-like structures for the negative-sense RNA viruses, and subviral particles arranged as factories for the dsRNA viruses. The positive-strand RNA viruses (Figs. 5.10 and 5.11) represent the best-characterized examples of membrane-associated replication complexes, with most information being available for the picornavirus and nodavirus families.²⁵ In most cases, the membranes are extensively rearranged, often—although not exclusively—derived from the endoplasmic reticulum, and invariable topologically arranged as an invagination of the membrane of the endoplasmic reticulum (ER) to form an open-necked crucible. Within each vesicle are a relatively small number of genomic templates—possibly as few as one, along with the viral replication machinery and cellular proteins that appear to play a role in the formation of the vesicular structure. The prevailing model is that such vesicular structures represent the sites of viral RNA replication, and that the nascent RNA chains are rapidly exported to the cytoplasm. In many cases, virion assembly sites are juxtaposed to such compartmentalized replication sites, which likely facilitates the coordination of the replication and assembly of viral particles. In the case of some positive-strand RNA viruses, notably the coronaviruses, the vesicles that are formed by the replication machinery appear to be closed from the cytoplasm. Whether specific viral protein(s), or a complex of viral and cellular proteins, are required to exchange nucleoside triphosphates and progeny genomes is not yet certain.

In the case of the negative-sense RNA viruses that replicate in the cytoplasm (Fig. 5.12), the viral replication machinery is typically found in inclusion-like structures that are often rich in cellular chaperones such as heat shock protein (hsp)70.⁶¹ Although such inclusion-like structures are not essential for RNA synthesis—as they are absent at the onset of infection and at a time at which the intracellular environment is likely most hostile for viral replication—such structures have been shown to be active sites of RNA transcription and seem to be sites of genome replication.^{48,61} Likewise, dsRNA viruses establish factories or viroplasms (Fig. 5.13) in the infected cell that are sites of RNA synthesis and assembly.¹⁰ Such compartmentalization of the replication machinery is therefore a conserved theme across a spectrum of viruses. Although it is clear that replication can occur in such sites, and this sequestration may both facilitate catalysis and sequester the viral replication machinery from surveillance by host defense machinery, it is largely uncertain what events lead to the establishment of such sites. Although there is a strong correlation between compartment formation and viral replication, examination of the very initial replication events including establishment of such sites remains challenging.

Mechanisms of RNA Replication and Transcription

The simplest mechanism of RNA replication is that used by HDV in which rolling circle synthesis by RNA polymerase II makes multimeric RNAs of both positive and negative polarity. *cis*-Acting ribozymes then cleave linear RNA monomers from these concatamers and covalently circularize them to produce mature antigenomes and genomes, respectively⁹⁷ (see Chapter 69). Although this simple mechanism is shared by several viroids and other subviral RNA pathogens of plants, HDV is the only RNA pathogen of vertebrates known to replicate by a rolling circle mechanism. Far more commonly, ssRNA genomes are replicated via the synthesis of complementary RNA monomers produced by successive rounds of strand displacement during end-to-end copying of linear templates.¹¹ However, both ends of the RNA are generally required for template activity, suggesting that even linear RNAs may be functionally circularized, perhaps to facilitate reiterative replication and hinder erosion of the termini.

The relative abundance of the genomic and antigenomic RNAs is regulated in different ways in different virus families. In the case of Q β , negative-strand RNA synthesis is limited by the requirement for an additional host subunit that is not required for positive-strand RNA synthesis.⁸ In other virus systems, distinct *cis*-acting RNA signals are largely responsible for determining the relative template activities of the complementary strands, although it is possible that different host factors are involved here as well. For the togavirus Sindbis, negative-sense RNA is synthesized only by a short-lived version of the RdRp, which is an intermediate in the proteolytic processing pathway of the viral nonstructural proteins.⁵⁶ As infection proceeds, increased viral protease activity cleaves this transient intermediate and thereby switches the template specificity of the RdRp to the synthesis of positive-strand RNA (see Chapter 22).

There are two general mechanisms of initiation by RdRp's: primer dependent and primer independent mechanisms. The majority of RNA viruses appear to employ a primer independent mechanism. Some of the best studied examples of this are provided by the bacteriophages Q β , MS2, and ϕ 6, as well as by eukaryotic viruses HCV, and reo-, rota-, and vesicular stomatitis virus. The general mechanism by which *de novo* initiation occurs is that the RdRp initiates synthesis by forming a phosphodiester bond between the initiation nucleoside triphosphate (iNTP) and a second NTP.¹² Atomic structures of polymerases engaged in the steps of initiation have provided remarkable insights into this process. The second general mechanism is a primer-dependent mechanism of initiation. The primers themselves can come in different flavors, for example, the picornavirus RdRp's use a small viral-encoded protein, VPg, that is covalently uridylylated by the polymerase on an internal RNA structure at a single A residue.⁸² This primer, polymerase complex then relocates to the terminus to prime the initiation reaction. Nucleic acids can also serve as primers for RNA synthesis, and here there are two currently known variations of this mechanism of primed initiation. The first of these is exploited by the RdRp's of the segmented negative-strand RNA viruses: *Orthomyxo*-, *Bunya*-, and *Arenaviridae* to prime messenger RNA synthesis. Here a virally encoded endonuclease cleaves a short capped primer from either host-cell pre-mRNA in the case of the *Orthomyxoviridae*, or mature cytoplasmic mRNA in the case of the *Arena-*

and *Bunyaviridae*.⁸⁵ Such primers then base pair with the ends of the template to prime synthesis of the viral mRNA. Remarkably the polymerases of those viruses also initiate "*de novo*". However, the available evidence supports that this reaction occurs not by initiation of synthesis opposite the terminal nucleotide, but by initiation at the penultimate nucleotide followed by a subsequent realignment of the nascent strand to "prime" the process of replication.³⁹ This strategy, termed "prime and realign" may help ensure the integrity of the termini are maintained during copying.

SUMMARY AND PERSPECTIVES

Mechanistic studies of viral replication have led us to some of the most fundamental discoveries in biology as well as provided critical knowledge to facilitate the development of vaccines and antiviral therapeutics to combat disease. The global burden of infectious disease caused by existing viruses and the ongoing threat of emerging viral infections further emphasizes the need to fully understand the mechanisms by which viruses replicate. Such studies promise to continue to uncover strategies to combat disease, and to harness viral replication for beneficial purposes both in the laboratory as expression vectors, gene knockout tools, neural circuit tracers, and probes of cellular, organismal, and population biology, but also in the clinic as potential vaccines, delivery vehicles, and imaging tools. The industrial applications of viruses are equally profound—as polymers, as well as tools to make microcircuits and even as potential fuel cells. All these applications of viruses have depended on our current understanding of the replication machinery and the strategies of viral replication. With the ability to simply manipulate viral genomes combined with the new burst in discovery of viruses from large scale sequencing projects, the future of fundamental studies of viral replication promises to be even more informative than the exciting past.

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Virus Assembly

Partitioning of Proteins Within the Cell

- Nuclear Import and Export of Proteins and Nucleic Acids

- The Secretory Pathway of the Cell

Intracellular Targeting and Assembly of Virion Components

- Assembly of Nonenveloped Viruses in the Nucleus

- Assembly of Enveloped Viruses in the Nucleus

- Assembly of Viruses in the Cytoplasm

- Assembly of Enveloped Viruses at Cellular Membranes

- Complex Interactions with the Secretory Pathway

- Modification of the Secretory Pathway

Incorporation of the Nucleic Acid Genome During the Assembly Process

- DNA Viruses

- RNA Viruses

Postassembly Modifications and Virus Release

- Proteolytic Cleavage and Virus Maturation

- Budding: Role of Viral and Cellular Proteins in Membrane Extrusion

- Mechanisms to Facilitate the Release of Nascent Particles

Virus assembly, a key step in the replication cycle of any virus, involves a process in which chemically distinct macromolecules are transported, often through different pathways, to a point within the cell where they are assembled into a nascent viral particle. A diversity of strategies and intracellular assembly sites are employed by members of the various virus families to ensure the efficient production of fully infectious virions. Nevertheless, a virus, irrespective of its molecular structure (membrane enveloped or nonenveloped) or the symmetry with which it assembles (icosahedral, spherical, or helical), must be able to take advantage of the intracellular transport pathways that exist within the cell if it is to achieve this goal. The end product of this selection process is assembly of each virus at a defined point within the cell. This chapter will focus on the cell biology of these intracellular targeting events, the intermolecular interactions that mediate targeting, and the assembly steps themselves.

Most viruses encode a very limited number of gene products. They therefore depend on the cell not only for biosynthesis of the macromolecules that constitute the virus particle but also for the pre-existing intracellular sorting mechanisms that

the virus utilizes to achieve delivery of those macromolecules to the sites of virion assembly. Because these are the same sorting mechanisms that the cell uses to delineate its subcellular organelles, the viral macromolecules must possess targeting signals similar to those of the components of those organelles. For a virus such as adenovirus, which assembles its nonenveloped capsids in the nucleus, this means that, following translation in the cytoplasm, each of the structural proteins of the mature virus must have the necessary protein-targeting information to be efficiently routed through the nuclear membrane to the assembly site. The situation is more complicated for a membrane-enveloped virus, such as influenza virus. For this virus, which assembles and releases virions from the apical surface of the epithelial cells that it infects, there is a necessity to ensure that the surface glycoproteins of the virus are correctly sorted by the secretory pathway of the cell to apical membranes. In addition, nucleocapsids, assembled in the nucleus, must be transported into and through the cytoplasm to the same location. As will be discussed later, the intracellular site at which the final phase of assembly and budding of an enveloped virus takes place is most often defined by the accumulation of the viral glycoproteins at a specific point in the secretory pathway of the cell. This also implies that there is specific molecular recognition of the virally encoded, membrane-spanning envelope components by the cytoplasmic nucleocapsids for a productive budding process to occur. Thus, interactions between proteins of viral and cellular origin, between viral proteins and nucleic acids and lipids, and between the viral proteins themselves are at the heart of the assembly process.

PARTITIONING OF PROTEINS WITHIN THE CELL

For an actively growing eukaryotic cell, there is a constant need to transport proteins and nucleic acids from their site of synthesis to the specific intracellular domains where they must function (Fig. 6.1). Proteins synthesized on cytosolic ribosomes, for example, must be transported to specific regions of the cytoplasm, to mitochondria and into the nucleus, whereas those synthesized on membrane-bound ribosomes will enter the secretory pathway and be targeted to specific organelles along the way. At the same time, messenger RNAs (mRNAs) and integral RNA components of the ribosome, or ribosomal RNAs (rRNAs), must be transported out of the nucleus. Proteins that participate in these intracellular trafficking processes have evolved to contain specific motifs that ensure the correct localization of the protein within the cell. Viruses have similarly evolved to take advantage of these pre-existing

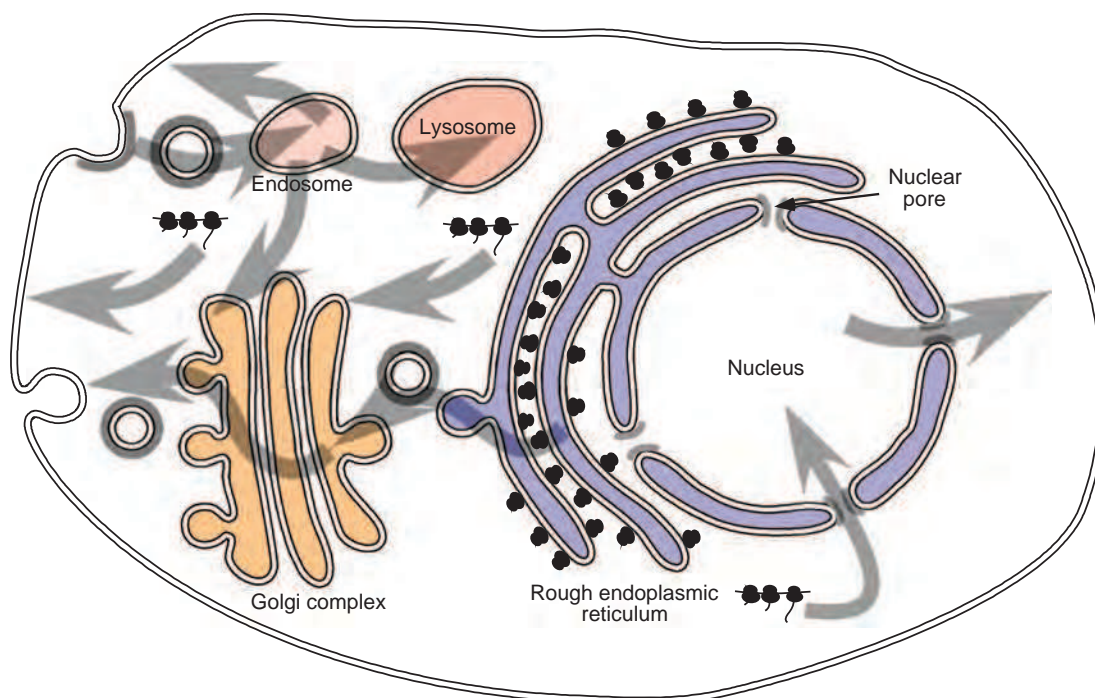


FIGURE 6.1. Protein localization in a mammalian cell. Proteins destined for the plasma membrane traverse the secretory pathway of the cell. They associate with the endoplasmic reticulum co-translationally and are translocated across the ER membrane via a proteinaceous pore—the translocon. Proteins are transported from the ER to the Golgi complex and on to the plasma membrane unless they contain specific amino acid motifs that localize or retain them at an intermediate location. Transport from one compartment to the next is via coated vesicles. Some membrane-spanning (integral membrane) proteins contain endocytosis motifs in their cytoplasmic domain that facilitate incorporation into clathrin-coated endocytic vesicles. Such proteins can be sorted back to the plasma membrane, to the trans-Golgi compartment of the secretory pathway, or to a lysosome for degradation. Proteins destined for the nucleus contain nuclear localization signals. These are short amino acid sequences that allow interaction with the nuclear pore machinery for transport across the nuclear membrane. Some proteins shuttle between the nucleus and cytoplasm and contain in addition a nuclear export signal. ER, endoplasmic reticulum.

pathways to accumulate, in specific locations, the necessary components for assembly of a nascent virus.

Nuclear Import and Export of Proteins and Nucleic Acids

The Nuclear Pore Complex

The nucleus is segregated from the cytoplasm by an inner and outer membrane; thus, access to and egress from this subcellular domain is mediated by specialized structures termed *nuclear pore complexes* (NPCs).²⁰² There are approximately 3,000 NPCs on the nuclear envelope of an animal cell, and each provides a proteinaceous channel between the nucleus and cytosol. The NPC itself is a very large structure, with a molecular mass exceeding 50 mDa that exhibits eightfold symmetry and is constructed of multiple copies of approximately 30 different proteins called *nucleoporins* (Nups). Negative stain and cryo-electron microscope reconstructions of these complexes reveal a 125-nm diameter core structure in which eight spokes in a radially symmetrical arrangement join to form three main rings surrounding a central channel of approximately 35 nm.³ Attached to both faces of the central framework are peripheral structures, cytoplasmic filaments, and a nuclear basket assembly, which interact with molecules that transit the NPC.⁵⁸ The

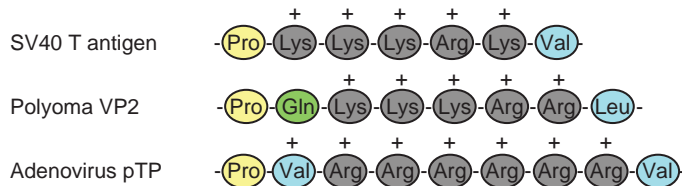
channel is filled with flexible, filamentous FG-Nups, which are characterized by regions of multiple Phe-Gly repeats and form a *virtual gate* restricting transport into and out of the nucleus.¹⁸¹ Small molecules and proteins may be able to passively diffuse through the NPC; however, it acts as a molecular sieve for macromolecules. Larger proteins and macromolecular assemblages must be actively moved through what is clearly a dynamic, malleable transporter structure that has the capacity to accommodate macromolecular complexes with diameters of up to nearly 35 nm.¹⁵⁴

Nuclear Localization Signals

Proteins that are actively transported into or out of the nucleus are characterized by the presence of amino acid motifs that allow them to interact with the nuclear transport machinery. For import into the nucleus, these motifs are termed *nuclear localization signals* (NLS) and for export, *nuclear export signals* (NES). NLS motifs, such as that first identified in the SV40 T antigen,⁹⁵ are not only necessary for nuclear localization of the proteins in which they are present but are also sufficient to actively direct large foreign proteins, such as β -galactosidase, into the nucleus. Although there is no conservation of sequence in different NLS motifs, they are generally short (<20 amino acids), rich in basic amino acids, and

A. Nuclear localization signals:

Simple:



Bipartite:

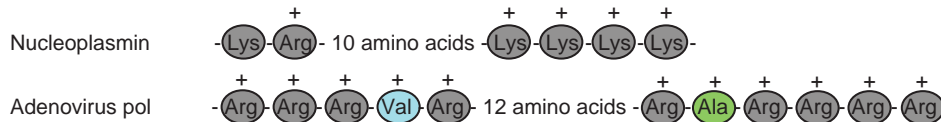
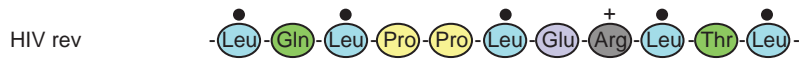
**B. Nuclear export signals:**

FIGURE 6.2. Nuclear localization and export signals. A. Nuclear localization signals (NLS) are the amino acid motifs that direct proteins into the nucleus. Simple NLS sequences, such as those present in the SV40 virus T antigen or polyoma virus VP2, often contain a proline residue followed by a stretch of basic residues. This short sequence can relocate large proteins such as β -galactosidase into the nucleus. Bipartite NLS sequences, such as those present in nucleoplasmin or the adenovirus polymerase, are characterized by two stretches of basic amino acids separated by a variable spacer sequence. These NLS sequences are recognized by import receptor molecules such as importin- α . **B.** Proteins that shuttle into and out of the nucleus, such as Rev, possess a second motif, the nuclear export signal. These motifs are characterized by a pattern of conserved leucine residues.

frequently preceded by proline residues (Fig. 6.2A). Some NLS, such as that in the adenovirus DNA-binding protein, are bipartite and require two separate short clusters of basic residues to be functional.²³⁸

To be exported from the nucleus, proteins contain an NES. This nuclear transport signal is also short (~10 amino acids) and contains a pattern of conserved leucines¹³⁴ (Fig. 6.2B). Some proteins, such as Rev of human immunodeficiency virus type 1 (HIV-1), possess both an NLS and an NES and appear to shuttle back and forth between the cytoplasm and the nucleus.¹²⁴

Nuclear Transport Pathways: In and Out

Nuclear import is a two-stage process. In the first stage, the newly synthesized NLS-containing protein interacts with cytosolic receptor proteins that then bind to phenylalanine-glycine (FG) repeat containing Nups (FG-Nups) that make up the filaments on the cytoplasmic side of the NPC - a process referred to as docking. This complex is then translocated, in an energy-independent process, through the nuclear pore into the nucleus, where the complex is disassembled, allowing the transported protein to become functional.

The best-characterized protein import receptor is importin- α (also named karyopherin- α). After binding its NLS-containing cargo, importin- α interacts with importin- β , which then mediates docking with the NPC.⁹⁸ Most nuclear transport receptors belong to one large family of proteins (karyopherins), all of which share homology with importin- β (also named karyopherin- β). Members of this family have been classified as importins or exportins on the basis of the direction that they carry their cargo. Importins and exportins are regulated

by the small guanosine triphosphatase (GTPase), Ran, which is highly enriched in the nucleus in its GTP-bound form. Importins recognize their substrates in the cytoplasm and transport them through nuclear pores into the nucleus. In the nucleoplasm, RanGTP binds to importins, inducing the release of their cargoes. In contrast, exportins interact with their substrates only in the nucleus in the presence of RanGTP and release them after GTP hydrolysis in the cytoplasm, causing disassembly of the export complex (Fig. 6.3).²⁰⁴ Thus, the directionality of transport is regulated by whether Ran is complexed with guanosine diphosphate (GDP) or GTP.

Active transport of large molecules in either direction across the nuclear pore involves interaction with the FG-Nups. The FG repeats in these filamentous proteins provide binding sites for the nuclear transport receptors as well as other molecules, such as nuclear transport factor 2 (NTF2), involved in this process. The exact mechanism by which the importin-cargo complex is carried through the nuclear pore is not known. It is likely, however, that the process involves a series of docking and release cycles with the FG-Nup proteins that make up the transporter machinery within the nuclear pore channel.^{199,202}

The Secretory Pathway of the Cell

Proteins of both viral and cellular origin that are destined for the outer membrane of the cell travel along a highly conserved route known as the secretory pathway. This complex series of membrane-bound subcellular compartments, through which proteins pass sequentially, includes the endoplasmic reticulum (ER), an intermediate membrane compartment, and the cis-, medial-, and trans-compartments of the Golgi apparatus

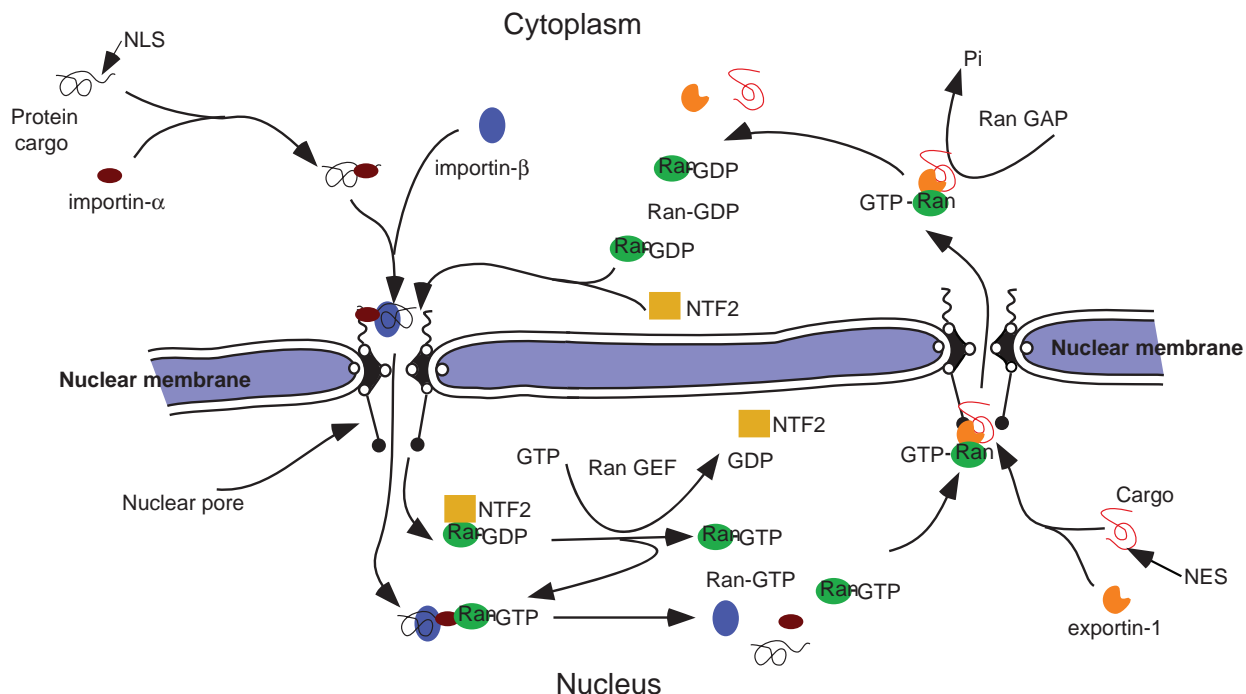


FIGURE 6.3. Nuclear import and export pathways. A protein bearing a nuclear localization signal is recognized and bound by importin- α . Importin- β binds to this complex and carries it to the cytoplasmic filaments of the nuclear pore, where together they mediate translocation of the protein complex into the nucleus in an energy-independent process. In the nucleus, Ran-GTP binds to the importin complex, and it dissociates delivering the protein cargo into the nucleus. Proteins destined for export out of the nucleus bind to exportin-1 via their nuclear export signal. This complex, together with Ran-GTP, binds to nucleoporins localized to the nuclear basket of the pore complex and initiates translocation into the cytoplasm. Once there, Ran-GTP is converted to Ran-GDP by a Ran-GTPase-activating protein (RanGAP-1), causing the cargo-exportin-1-Ran complex to dissociate, thereby delivering the cargo to the cytoplasm. GTP, guanosine triphosphate; GDP, guanosine diphosphate.

(see Fig. 6.1). Proteins that traverse this pathway, such as the envelope glycoproteins of viruses, enter via the ER. Insertion of proteins into the ER occurs during translation through a process termed *translocation*. The ER network of tubules and sacs defines a unique environment in which protein modification and folding can occur isolated from the cytoplasm. Because polyribosomes, in the process of translating proteins that are translocating into the secretory pathway, are bound tightly to the ER membrane, regions containing them are known as the rough ER.

Translocation

Translating ribosomes are directed to the ER membrane by a short sequence in the nascent polypeptide known as the signal sequence. In most proteins, this 15 to 30 amino acid sequence, which contains a core of hydrophobic amino acids, is located at the N-terminus of the protein. Shortly after the signal peptide emerges from the ribosome, it is bound by a ribonucleoprotein (RNP) complex known as the signal recognition particle (SRP). Binding of SRP transiently arrests any further translation and directs the ribosome to an SRP-receptor on the ER (Fig. 6.4). Both SRP and its receptor have GTP-binding components, and the presence of this nucleotide is essential for efficient targeting.¹⁸⁷

Following the initial docking of the translationally arrested complex, the ribosome becomes tightly associated with the ER membrane via the translocon—a gated, aqueous, protein

channel that spans the ER membrane. Concomitant with this process, the SRP and its receptor are released, the signal peptide is introduced into the channel, and translation resumes. The components of the translocon have been identified through biochemical approaches in mammalian cells and genetic approaches in yeast and are comprised of a heterotrimeric complex known as the Sec61p (composed of Sec61 alpha, beta, and gamma chains). Based on the crystal structure of the archaeobacterial Sec61 homolog, SecY $\beta\gamma$, this complex forms a 40 Å × 40 Å structure with a pore-like central cavity and a single potential lateral opening to allow transition of membrane-spanning domains into the lipid bilayer.^{195,239} Additional proteins (e.g., the translocating chain-association membrane or TRAM protein) are necessary for optimal translocation. It is unlikely that the central pore of the translocon ever allows free diffusion between the cytosol and the lumen of the ER, because these compartments are chemically distinct. A constriction in the channel appears to be plugged by a short helix, and widening of the constriction as well as displacement of the plug have been linked to conformational changes induced by SRP binding to the complex.²¹⁹ GRP78 (BiP), a member of the Hsp70 family of chaperone proteins located in the lumen of the ER, plays multiple roles in gating the channel and facilitating translocation. The chaperone is then poised to facilitate the folding of the nascent polypeptide chain as it emerges from the pore, although

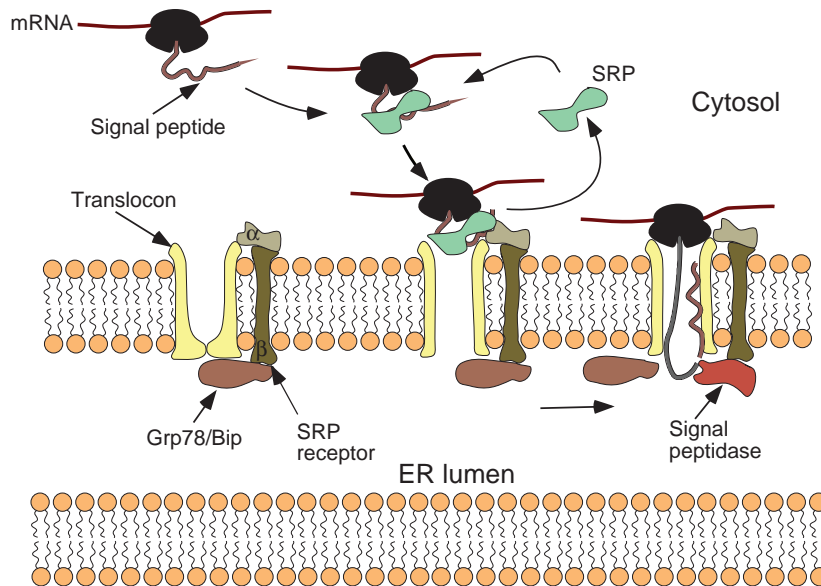


FIGURE 6.4. Protein translocation into the secretory pathway. Translation of a protein destined for the secretory pathway proceeds until the signal peptide exits the ribosome. The SRP binds to the signal peptide and the ribosome and arrests translation. The ribosome-SRP complex moves to the ER membrane, where SRP binds to its receptor (SRP receptor). This interaction, with concomitant hydrolysis of bound GTP, releases SRP and mediates a tight interaction between the ribosome and a proteinaceous channel—the translocon. The release of SRP and binding of the signal peptide to components of the translocon induces a conformational change that widens a constriction in the channel and allows resumption of translation to occur. The chaperone protein, Grp78 (BiP), present in the lumen, is poised to facilitate the folding of the nascent polypeptide chain as it emerges from the pore. The signal peptidase complex removes the signal peptide co-translationally from those proteins that have a cleavable signal peptide. Secreted proteins will continue to traverse the translocon until they are completely located in the lumen of the ER. In contrast, for integral membrane proteins, translocation will stop following introduction of the hydrophobic anchor domain into the translocon, and transition into the lipid bilayer occurs through a single lateral opening in the pore. SRP, signal recognition particle; ER, endoplasmic reticulum; GTP, guanosine triphosphate.

this binding is not essential for translocation to proceed. The signal peptide, in those proteins with a transient N-terminal sequence, is cleaved from the rest of the polypeptide by a complex of five proteins called the *signal peptidase* shortly after it enters the lumen of the ER (see Fig. 6.4).

For secreted proteins, translocation of the polypeptide through the translocon continues until the entire protein is present in the lumen of the ER. In contrast, integral membrane proteins, such as the envelope glycoproteins of viruses, contain a stop-transfer, membrane-anchor sequence that is generally located toward the C-terminus of the protein. Following the translation of this short (~25 amino acids) and mostly hydrophobic sequence, the translocon undergoes a conformational change that allows the membrane-spanning domain to be associated directly with the lipid bilayer. The product of this process is a type I integral membrane protein (Fig. 6.5), such as the influenza virus hemagglutinin (HA), in which the N-terminal ectodomain is in the lumen of the ER and the C-terminus is in the cytoplasm. In some proteins, such as the influenza virus neuraminidase (NA), a longer N-terminal signal peptide also functions as the membrane anchor. In this case, the signal peptide is not cleaved from the polypeptide chain and the sequences C-terminal to it are translocated into the ER lumen, resulting in a type II orientation. Multiple membrane-spanning proteins, such as the M protein of the coronaviruses, appear

to possess hydrophobic sequences that are alternatively recognized as signal and stop-transfer sequences, and translocation of such proteins may involve multiple Sec61 heterotrimers.¹⁹⁵

Posttranslational Modifications

Protein Folding and Quality Control. Proteins enter the lumen of the ER in an unfolded state and the process of folding into a transport-competent conformation is facilitated by interactions with molecular chaperones and folding enzymes located there. This collection of proteins includes BiP, calnexin (Cnx), calreticulin (Crt), GRP94, and protein disulfide isomerase (PDI). In addition to assisting in folding the nascent molecules, these proteins retain incompletely folded molecules in the lumen and act as a quality control system for the secretory pathway.²⁰ Oligomeric proteins, such as the receptor/fusion proteins of enveloped viruses, also assemble into their quaternary conformation in this compartment. For many of these molecules, oligomerization appears to be a prerequisite for transport out of the ER.

Glycosylation. Most proteins that traverse the secretory pathway are modified by the addition of oligosaccharide side chains either to the amino group of asparagines (N-linked glycosylation) or through the hydroxyl group of serines or threonines (O-linked glycosylation). N-linked moieties are added co-translationally in the lumen of the ER, where mannose-rich

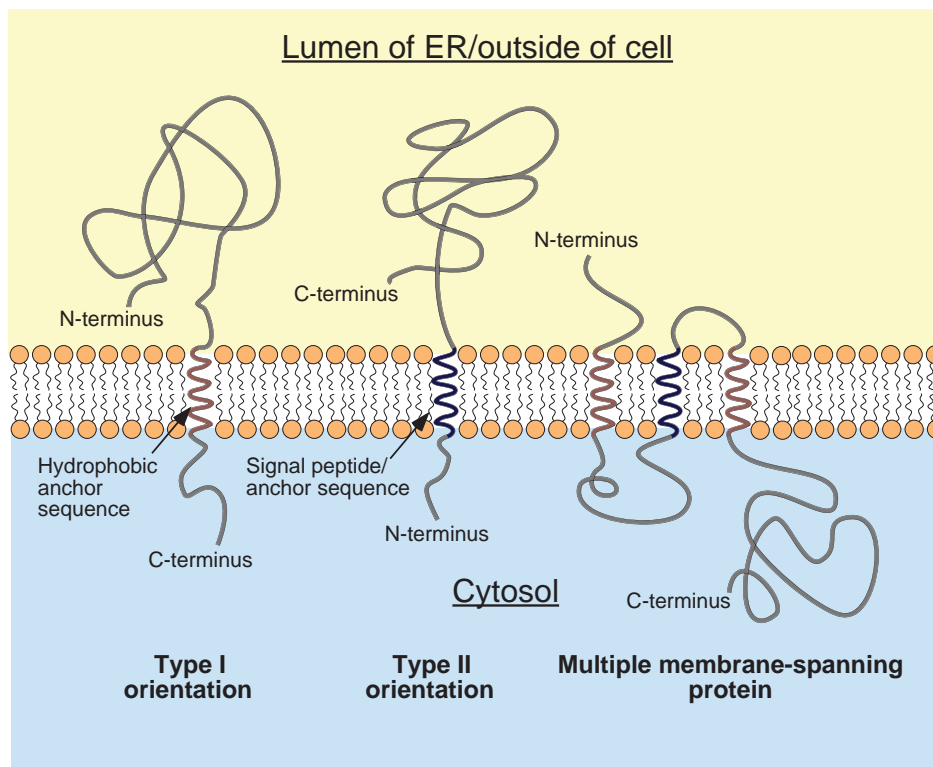


FIGURE 6.5. Protein topology. Proteins with type I orientation generally have a cleavable N-terminal signal peptide that is removed co-translationally from the nascent protein. The protein continues to be transferred into the lumen of the ER until a hydrophobic anchor sequence is translated and enters the translocon. Translocation then stops, and the protein transitions from the protein pore into the lipid bilayer. Thus, type I integral membrane proteins, such as the hemagglutinin of influenza virus, have their C-terminus in the cytoplasm and their N-terminus in the lumen of the ER (topologically equivalent to outside the cell). In type II proteins, such as the influenza virus neuraminidase, the signal peptide forms the membrane anchor domain; thus, at the end of translation, the C-terminal sequences are translocated into the lumen of the ER, leaving the N-terminus in the cytoplasm. For multiple membrane-spanning proteins, such as the M protein of the coronaviruses, translocation is initiated at the first signal peptide sequence and continues until the first anchor domain. It is reinitiated following translation of a subsequent signal sequence and stopped again following translation of a second anchor. The exact mechanism by which this is accomplished without dismantling the translocon at intermediate steps in the process is not understood. ER, endoplasmic reticulum.

oligosaccharides are transferred by oligosaccharyltransferase from a lipid (dolichol) carrier to asparagine residues present in NXS/T motifs (where X is any amino acid but proline) within the protein. Trimming of terminal glucose and mannose residues from the branched oligosaccharide occurs in the ER and is closely linked with the Cnx- and Crt-mediated quality control process.²⁰ Further trimming of mannose residues followed by addition of other sugars (N-acetylglucosamine, galactose, fucose, and sialic acid), to yield complex oligosaccharide structures, occurs in the Golgi complex. O-linked oligosaccharides are also added in this organelle.

Transport Through the Secretory Pathway

Transport of soluble and membrane-spanning proteins from one compartment of the secretory pathway to the next is mediated by the formation of coated membrane vesicles that travel to and fuse with the target organelle. Thus, once the process of protein folding and quality control has been completed in the ER, proteins are sequestered into these transport vesicles prior to transit to the Golgi complex. The processes of cargo protein selection, budding, targeting, and fusion are probably

all mediated by specific protein constituents that define the different transport vesicles involved in shuttling proteins between components of the secretory pathway.^{86,210} In the case of ER-to-Golgi transport, the vesicles have coat protein complex II (COPII),⁴³ whereas retrograde transport of vesicles from the Golgi to the ER, as well as anterograde transport through the Golgi, is mediated by COPI coats.¹⁴⁹ Budding is initiated at specialized regions of the ER (transitional ER) when a small myristoylated protein (SAR1) is converted to the GTP-bound form, allowing it to bind to the membrane and recruit coat proteins (Sec23, Sec24, Sec13, and Sec31) in a stoichiometric manner.⁴³ Formation of the coat itself induces membrane curvature and vesicle budding. Sorting signals displayed on the cytosolic surfaces of transmembrane protein cargo direct it into COPII vesicles, in some instances through physical association of the Sec23/Sec24 components in a sorting signal-dependent manner.¹⁰⁰ Soluble proteins and transmembrane proteins lacking COPII sorting signals depend on a diversity of transmembrane adaptor proteins that can link them to the budding machinery. These include the endoplasmic reticulum–Golgi intermediate compartment-53 (ERGIC-53) and p24 family

of receptor proteins, as well as a set of multiple membrane-spanning ER vesicle (Erv14p, 26p, and 29p) proteins that can facilitate concentration of soluble proteins in COPII vesicles.⁴³

Membrane receptors that mediate docking of the transport vesicle with the target organelle are also incorporated into the coat and appear to define the specificity with which the cargo protein is delivered. Rab-GTPases and tethering proteins appear to play an important role in defining the initial vesicle-target interactions,^{79,210} whereas soluble N-ethylmaleimide-sensitive factor (Nsf) attachment protein receptors (SNAREs) are generally accepted to mediate the final stage of vesicle docking and the subsequent membrane fusion events that are critical to transport.^{16,79} A vesicle-specific SNARE (v-SNARE) interacts with a target-membrane-specific SNARE (t-SNARE) complex (generally comprised of three peptides) during this process. Two additional proteins, the Nsf and soluble Nsf attachment proteins (SNAPs), act to disassemble the complex following fusion, allowing the SNARE components to be recycled.^{79,86}

Protein Localization

Subcellular localization of proteins within the secretory pathway appears to be determined by a combination of sorting/targeting signals that mediate interactions with the coat complex for inclusion in a transport vesicle and retention signals that localize the protein to a specific compartment within the secretory pathway. Localization is enhanced by the interplay of anterograde and retrograde transport that allows retrieval of proteins inadvertently transported beyond their target location. The classical example of this is the KDEL peptide sequence found on soluble proteins that are localized to the lumen of the ER.¹³¹ Proteins containing this sequence are efficiently retrieved from the cis-Golgi by the KDEL receptor, which is incorporated into COPI vesicles for trafficking back to the ER. Similarly, membrane-spanning proteins localized to the ER have, at the C-terminus of the cytoplasmic domain, a dilysine (KKXX) COPI-binding motif, which ensures their efficient retrieval from the Golgi complex.⁸⁴ This type of motif is utilized by the primate foamy viruses to concentrate the envelope glycoprotein complex (gp80/gp48) in the ER/intermediate compartment (IC), where virus budding occurs.⁶⁶ For integral membrane proteins, retention signals often appear to be associated with the membrane-spanning domain(s) of the protein, as is the case for the coronavirus M protein, and may reflect preferred association with specific lipid compositions of the membrane within a particular component of the secretory pathway.

The Golgi Complex

The Golgi complex represents a unique organelle within the secretory pathway in that it is comprised of a series of membrane-bound compartments that are the sites for specific biochemical modifications to proteins and oligosaccharides, as well as locations where specific protein-sorting decisions are made. Proteins transported from the ER enter the Golgi complex via the cis-Golgi network and, after traversing the cis-, medial-, and trans-cisternae, exit via the trans-Golgi network.¹⁷⁹ Each of the compartments provides a spatially distinct site for maintaining an ordered set of enzymes involved in the process of oligosaccharide maturation. They are also the sites at which proteins undergo O-linked glycosylation, through the addition, at certain serines and threonines, of monomeric sugar residues. It is in the trans-Golgi

cisternae and trans-Golgi network where viral glycoprotein precursors, such as the Env polypeptide of the retroviruses, are cleaved to their mature forms through the action of members of the furin family of proteinases—enzymes that normally function to process cellular substrates such as polypeptide hormone precursors. This cleavage event is critical for the generation of a biologically functional glycoprotein and thus for virus infectivity.

INTRACELLULAR TARGETING AND ASSEMBLY OF VIRION COMPONENTS

Viruses can be nominally divided into two groups based on the presence or absence of a lipid bilayer envelope. The nonenveloped viruses can assemble in the cytoplasm or nucleus and generally, for those that propagate in animal cells, exhibit icosahedral symmetry (Chapter 3). For these viruses, the viral structural proteins and genomic nucleic acid must be targeted to or retained at the subcellular domain at which assembly occurs. Enveloped viruses, by their very nature, must acquire a lipid bilayer from one of the cell's membranes during the process of assembly. In some viruses, such as the herpesviruses and some retroviruses, this envelopment step takes place after the assembly of an intact capsid shell, whereas for others the processes of envelopment and capsid assembly occur concomitantly. Some viruses undergo transient envelopment and in some cases re-envelopment during the process of assembly.

For nonenveloped viruses, the tightly assembled structure of the icosahedral shell forms a protective coat that prevents degradation of the genome by environmental factors. For enveloped viruses, the integrity of the nucleocapsid structure is less critical because the membrane provides a barrier to external degradative enzymes.

Assembly of Nonenveloped Viruses in the Nucleus

Adenoviruses are nonenveloped icosahedral viruses, 70 to 100 nm in diameter, that have a protein shell surrounding a DNA core. The protein shell (capsid) is composed of 252 capsomeres, of which 240 are hexons and 12 are pentons. Each penton consists of a five-subunit base (polypeptide III) and a trimeric fiber (polypeptide IV) that extends out and away from the shell. The hexon capsomeres are comprised of trimers of three tightly associated molecules of polypeptide II (Fig. 6.6).

For a nonenveloped virus such as adenovirus, which replicates exclusively in the nucleus, there is a strong dependence on nuclear targeting/transport pathways to export newly synthesized mRNAs out of the nucleus and to import structural proteins back into the nucleus. Nuclear import of the major capsid protein—hexon or polypeptide II—depends on the involvement of a second adenovirus protein, the pVI (precursor) polypeptide, which acts as a nucleocytoplasmic shuttling adapter and provides the necessary NLS for transporting the hexon into the nucleus.²²⁹ Trimer formation, in turn, depends on yet another virus-encoded, chaperone-like protein—L4 100K—which transiently binds to the newly synthesized hexon monomer and mediates its association with two additional monomers.²⁴ Thus, the most abundant structural protein of the adenovirus capsid needs to interact with two additional virus-encoded factors to attain the correct tertiary structure and subcellular location for assembly.

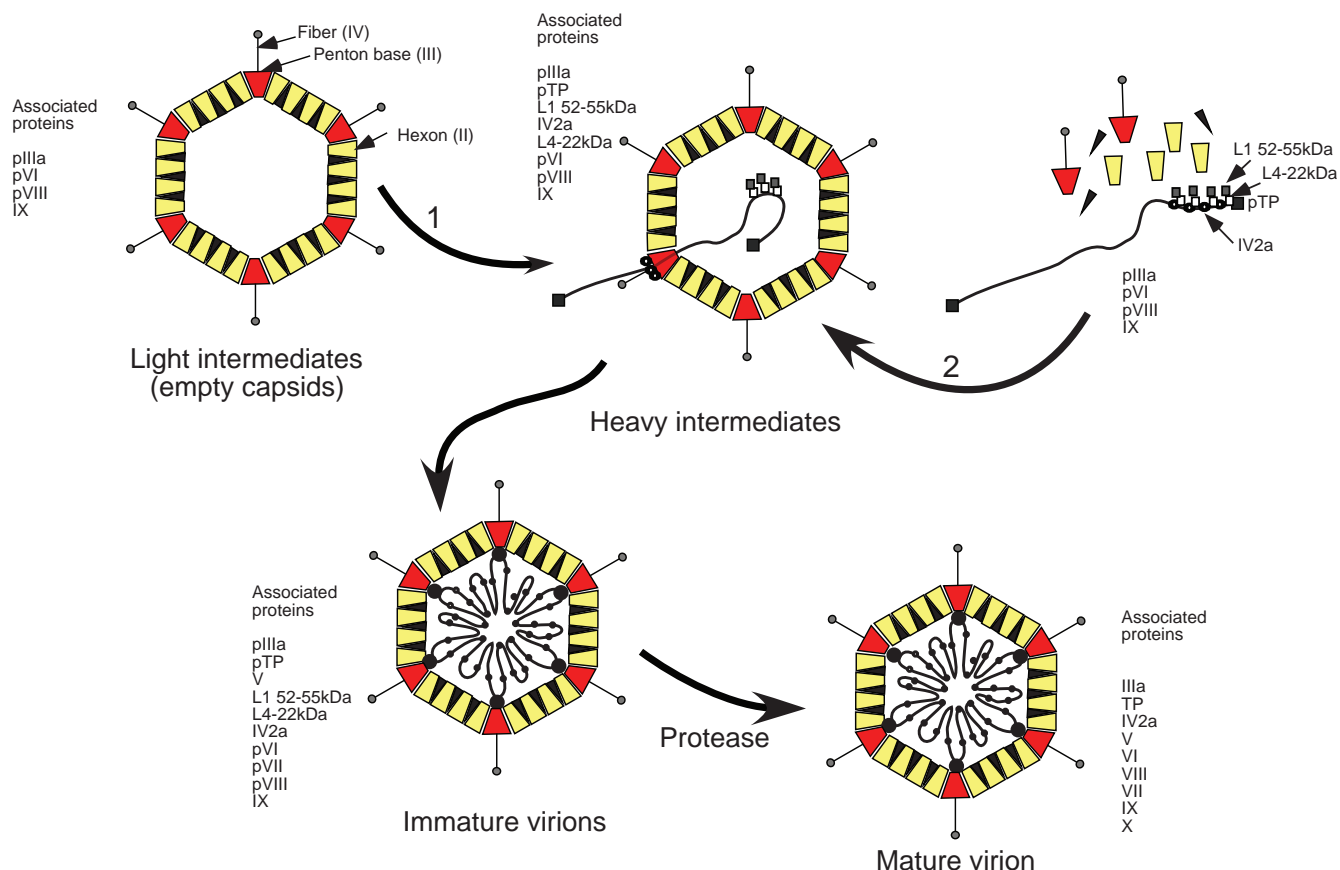


FIGURE 6.6. Assembly pathway for adenoviruses. Following transport into the nucleus, the hexons and pentons are proposed to assemble into empty capsids (previously known as light intermediates of assembly) around scaffolding proteins. These nonstructural scaffolding proteins are lost on packaging of viral DNA, which is inserted into this structure via a packaging sequence at the left end of the genome. The mechanism of insertion appears to be similar to DNA phages and involves a portal complex. The pIV2a protein may represent the ATP-hydrolyzing component required to drive DNA through the portal structure in the procapsid. Heavy intermediate forms of the capsid probably represent those in which DNA packaging is incomplete and the DNA is fragmented. Precursor core proteins would be packaged into the empty capsid along with the genome to form immature virions. Proteolytic cleavage of the precursor proteins by the viral proteinase yields the mature virion. ATP, adenosine 5'-triphosphate.

The two other proteins that form the 12 vertices of the capsid—penton and fiber—appear to assemble independently in the cytoplasm. Mutations in the C-terminus of the penton that block assembly into pentamers do not prevent transport into the nucleus,⁹⁶ indicating that each monomer has an active NLS. In contrast, the fiber must form trimers to be efficiently transported to the nucleus,¹⁴⁶ even though this protein has an active NLS located at its N-terminus. It seems likely that the penton base and fibers are transported independently into the nucleus and assemble into the intact penton at the site of assembly.

Although two distinct pathways for adenovirus capsid assembly have been postulated from a large body of work in this area, it now appears that an empty procapsid is first formed around scaffolding proteins, in a manner similar to that observed with DNA phages. Results from experiments that combined kinetic labeling with temperature-sensitive replication mutants and additional site-directed mutants have yielded the assembly scheme shown in Figure 6.6. Several viral products appear to act as scaffolding proteins, around which shells are assembled and that facilitate the encapsidation process. These proteins are present in the intermediate capsid-like

particles but are absent once DNA has been encapsidated.³⁹ Disruption of adenovirus capsids with denaturants results in the release of groups of nine hexons that are associated with the faces of the icosahedron and lack the peripentonal hexons. Under acidic conditions, these nanomers can reassemble to form icosahedral shells that lack the 12 vertices, which would normally be composed of the penton and the five peripentonal hexons, raising the possibility that hexon nanomers are intermediates of adenovirus capsid assembly. Following procapsid assembly, DNA and associated core proteins are then subsequently packaged into these empty shells to yield immature virions that then undergo proteolytic maturation. Recent studies have demonstrated that the approximately six to eight copies of the pIV2a protein, which binds to the packaging sequence, are present at a single apex of the mature virion.²⁹ This, and the fact that the pIV2a protein contains motifs (the Walker A and B boxes) associated with the binding and hydrolysis of adenosine 5'-triphosphate (ATP), suggests that it may represent the ATP-hydrolyzing component required to drive DNA through a portal structure in the procapsid. Consistent with this, mutations that either prevent synthesis of pIV2a or prevent binding

of ATP to the protein block genomic packaging and result in the assembly of empty procapsids.¹⁵⁰

Assembly of Enveloped Viruses in the Nucleus

Two enveloped animal virus families—the herpesviruses and the orthomyxoviruses—utilize cell components that are located within the nucleus in their replication and initiate their assembly within that compartment. In addition to importing into the nucleus the necessary components for assembly, these viruses must also export large nucleoprotein complexes back out into the cytoplasm.

In some respects, the herpesviruses represent a hybrid between a nonenveloped virus, such as adenovirus, and a more conventional enveloped virus, such as a retrovirus, in that they utilize the compartments of the secretory pathway to transport large capsid structures from the nucleus to the outside of the cell. Members of this group of large viruses assemble, within the nucleus, an icosahedral capsid shell that is 160 Å thick and 1,250 Å in diameter. The major component of this protein shell is pUL19, which forms both the pentameric and hexameric capsomeres necessary to assemble the icosahedral structure. Associated with the outer surface of the hexamers is the abundant small protein pUL35. Two additional proteins—pUL38 and pUL18—in a 1:2 ratio, form heterotrimeric triplexes that fit between and link together adjacent capsomeres.¹⁴⁰ Scaffolding proteins are essential for herpes simplex virus type 1 (HSV-1) capsid assembly/maturation; in their absence, incomplete and aberrantly shaped capsids are assembled. As with adenovirus, the major capsid protein lacks a nuclear targeting signal, and its transport into the nucleus requires an interaction with either the scaffolding protein (pUL26.5) or the triplex protein pUL38, which presumably provide the necessary NLS. pUL18 similarly requires pUL38 for nuclear localization, whereas pUL35 appears to be directed there via its interaction with pUL19.¹⁷⁰

Studies of virus-infected cells together with *in vitro* assembly studies have provided valuable insights into the assembly process.^{123,142} These studies point to a pathway in which pUL19, pUL38, and pUL18 assemble around a scaffold to form an icosahedral but predominantly spherical procapsid—the *B-capsids* identified by electron microscopy. Although not required for capsid formation, in its presence the portal complex apparently initiates capsid formation leading to its incorporation into the nascent capsid.¹⁴¹ Cleavage of the scaffolding protein at a site near its C-terminus by the viral protease removes a 25 amino acid sequence that is necessary for binding to pUL19. The resulting disassociation and release of scaffold allows for packaging of the viral DNA genome and induction of maturation of the capsid into a more angular icosahedral structure, the previously identified *C-capsids*. DNA enters the procapsid through a unique vertex composed of the portal protein pUL6, which is assembled into rings composed of 12 subunits to form the portal complex.¹⁴³ The pUL6 portal resembles the connector or portal complexes employed for DNA encapsidation by double-stranded DNA bacteriophages such as ϕ 29, T4, and P22. In the absence of an active proteinase, the scaffolding proteins remain associated with the protein shell, preventing packaging of the viral DNA, and the procapsid is unable to mature.¹⁴⁴

Unlike adenoviruses, which accumulate in the nucleus and are released on lysis of the cell, herpesviruses exit the nucleus by budding into the lumen of the nuclear membrane. This process depends on the products of two highly conserved genes—UL31 and UL34—that encode a phosphoprotein and

a type II membrane protein, respectively. Nuclear localization of pUL31 depends on its interaction with pUL34.⁹⁷ Although both proteins are present in the primary enveloped virions present in the lumen of the nuclear membrane, they are absent from mature virions, consistent with a model in which herpesvirus virions are first enveloped at the inner nuclear membrane, de-envelop by budding through the outer nuclear membrane and are re-enveloped by Golgi membranes in the cytoplasm. The complexities of this interaction and subsequent steps in assembly will be discussed later in this chapter.

For influenza virus to take advantage of its unusual capacity to “steal” the capped 5′ ends of host cell mRNAs to initiate its own mRNA synthesis, transcription and viral RNA replication must occur in the nucleus (Chapters 5, 40 and 41). Genomic (minus sense) RNAs are replicated by a different mechanism to yield templates for mRNA synthesis as well as progeny viral genomes. The eight viral RNA segments of this virus are packaged into individual RNPs containing the four proteins of the transcriptase complex (PB1, PB2, PA, and NP; each containing a functional NLS sequence) and the nuclear export protein (NEP; previously NS2), but are not exported into the cytosol until late in infection when the viral matrix protein begins to be synthesized.¹¹³ Under conditions where matrix synthesis is inhibited, the viral ribonucleoproteins (vRNPs) accumulate in the nucleus, tightly associated with the nuclear matrix. The block to export can be relieved by expression of matrix from an independent vector.²² It has also been shown that the vRNPs remain in the nucleus of cells if NEP is not encoded by the virus. NEP does not interact directly with vRNPs; rather, it mediates (RanGTP-dependent) formation of a bridge between the cellular export receptor Crm1 and the N-terminal domain of M1, which in turn binds to the vRNP via its C-terminal domain. This *daisy-chain* complex of (Crm1–RanGTP)–NEP–M1–vRNP is likely what mediates the export of vRNP across the nuclear envelope.^{2,137} Matrix association with the vRNPs also appears to be important for preventing their re-entry into the nucleus, because conditions such as acidification or mutations that promote dissociation of M1 allow the RNPs to be reimported.²²⁸ Thus, the matrix protein of influenza virus is a key modulator of vRNP transport into and out of the nucleus.

Assembly of Viruses in the Cytoplasm

Targeting and import of proteins into the nucleus or secretory pathway involves well-characterized motifs on the proteins involved; therefore, the processes by which proteins are targeted to destinations within the cytoplasm remains for the most part obscure. Nevertheless, most viruses, even those that are nonenveloped, initiate or complete their assembly in association with membranes of the secretory or endocytic pathways, although the intracellular pathways that function to transport their capsid components and genomes to these sites have not been defined. Reoviruses are the only animal viruses that appear to complete their assembly entirely in the cytoplasm without the involvement of membranes. Genome replication and virus assembly both occur in specialized areas of the cytoplasm known as virus factories or viroplasm. The virus nonstructural proteins NSP2 and NSP5 appear to play a critical role in establishing these sites of virus replication and can form morphologically similar structures when expressed in the absence of other viral proteins.¹⁵⁸ It is likely that they are responsible for recruitment of the other viral proteins and viral

nucleic acid to these sites, thereby avoiding the complexities of transporting virion components to multiple separate cytoplasmic assembly sites following translation.

Intracytoplasmic Transport and Assembly of Retroviral Capsids

Retroviruses are enveloped viruses that, for the most part, complete their assembly by budding through the plasma membrane of the infected cell. For these viruses, the immature capsid of the virus is assembled from polyprotein precursors that must be transported through the cytoplasm to the inner leaflet of the membrane. The viral glycoproteins, on the other hand, must be transported through the secretory pathway of the cell to the cell surface, where they co-localize with the nascent,

membrane-extruding capsid (Fig. 6.7). All replication competent retroviruses contain four genes that encode the structural and enzymatic components of the virion. These are *gag* (capsid protein), *pro* (aspartyl proteinase), *pol* (reverse transcriptase and integrase enzymes) and *env* (envelope glycoprotein) (Chapter 47). However, the product of the *gag* gene has been shown to possess the necessary structural information to mediate intracellular transport, to direct self-assembly into the capsid shell, and to catalyze the process of membrane extrusion known as budding.¹⁸³ For most retroviruses, the nascent Gag polyproteins are transported to the plasma membrane, where assembly of the capsid shell and envelopment occur simultaneously (see Fig. 6.7, Pathway 1). Viruses that undergo this *type C* form of morphogenesis include members of the alpha- and

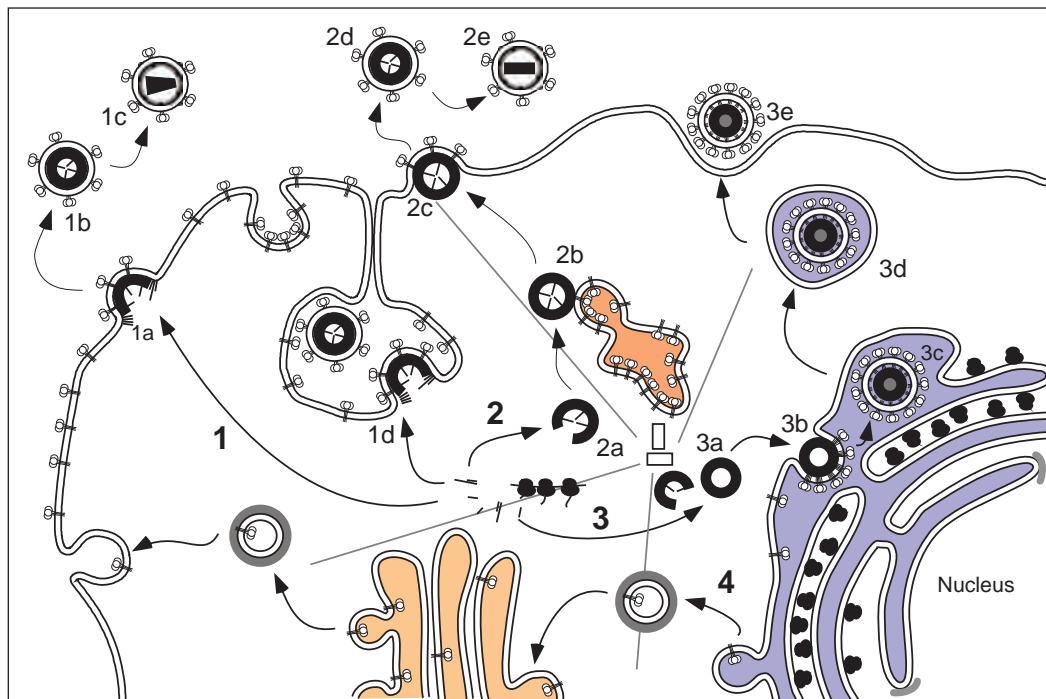


FIGURE 6.7. Assembly of retroviruses. The assembly pathways of retroviruses that exhibit C type morphogenesis (*Pathway 1*), B-/D-type morphogenesis (*Pathway 2*), and that of the foamy viruses (*Pathway 3*) are shown. The envelope glycoproteins are translated on membrane-bound polysomes and, for most retroviruses, are transported to the cell surface through the cell's secretory pathway (*Pathway 4*). For all morphogenic classes, the Gag proteins are synthesized on free polysomes. In the case of the C-type morphogenic viruses (i.e., RSV and HIV), the Gag and Gag-Pol proteins migrate either individually or in small multimers to the plasma membrane, where immature capsid assembly and envelopment occurs concurrently (1a). At some point in this pathway, the viral genomic RNAs associate with the Gag and Gag-Pol precursors and are incorporated into the developing capsid. For HIV in macrophages, assembly can occur on deep invaginations of the plasma membrane to which Env has been targeted (1d). In the case of the B-/D-type viruses, polysomes translating Gag and Gag-Pol precursors are first transported via microtubules to an intracytoplasmic, pericentriolar assembly site (2a), where they assemble into immature capsids. The immature structures are then transported, most likely in association with endosomal vesicles (2b), to the plasma membrane (2c), where they associate with the envelope glycoproteins and induce viral budding. For both classes of retroviruses, the capsids of the nascent immature particles appear as doughnut-shaped structures and contain unprocessed Gag and Gag-Pol precursors (1b and 2d). The mature virus particles contain electron-dense cores with morphologies characteristic of the virus (1c and 2e). The maturation step is required for infectivity and is the result of the activation of the viral protease, which cleaves the Gag and Gag-Pol precursors into the internal structural and enzymatic proteins of the virus. For the foamy viruses, Gag and Pro-Pol precursors also assemble into immature capsids in a pericentriolar site (3a); however, budding primarily occurs at the ERGIC compartment, where the viral glycoproteins are retained (3b). The enveloped virion is presumably transported to the plasma membrane by transport vesicles (3d). Maturation cleavage of the immature core is limited to removal of 4kd from the C-terminus of the precursor; the mature infectious virion maintains an immature morphology (3e). RSV, Rous sarcoma virus; HIV, human immunodeficiency virus; ERGIC, endoplasmic reticulum–Golgi intermediate compartment.

gammaretroviruses. Lentiviruses and deltaretroviruses assemble their capsids in a similar fashion in most cell types. In the second morphogenic class of retroviruses, the *type B/D* class, the Gag precursors are targeted first to an intracytoplasmic site, where capsid assembly occurs. These assembled immature capsids are then transported to the plasma membrane, where they undergo budding and envelopment (see Fig. 6.7, Pathway 2). Viruses that undergo this process of assembly and release include members of the betaretroviruses. Members of the spumavirus family also assemble immature capsids in the cytoplasm but are targeted to the ER or ERGIC for envelopment (see Fig. 6.7, Pathway 3).

Whereas the size and protein content of the precursor varies between different retroviral families, at least three *gag*-encoded proteins are found in all retroviruses: the matrix protein (MA), the capsid protein (CA), and the nucleocapsid protein (NC). In addition to these functionally conserved domains, the Gag precursor can, depending on the virus encoding it, contain additional peptide sequences (Fig. 6.8) whose functions in virus assembly and future cycles of infection are only now being resolved.

The detailed mechanisms by which the capsid precursor proteins are directed to the site of assembly are only now starting to be elucidated in molecular detail; however, the process is mediated primarily by the MA domain of the Gag precursor. In most retroviruses, the matrix protein contains two elements involved in plasma membrane targeting. The first of these is

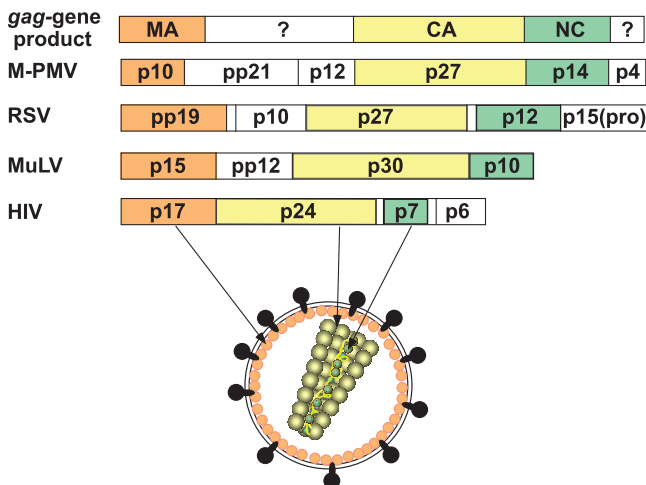


FIGURE 6.8. Organization of the retroviral Gag precursor. The Gag precursor polyproteins of all retroviruses contain, beginning at the N-terminus, matrix (MA), capsid (CA), and nucleocapsid (NC) domains linked in this order. The *gag* gene products of the betaretrovirus Mason-Pfizer monkey virus (M-PMV), the alpharetrovirus Rous sarcoma virus (RSV), the gammaretrovirus murine leukemia virus (MuLV), and the lentivirus human immunodeficiency virus (HIV) are shown. The unshaded boxes represent regions of the Gag precursor for which no common functions or locations in the mature virion have been established. However, for the three representatives of the alpha-, beta-, and gammaretroviruses, a *late domain* function required for pinching off of the virus particle from the cell is located between the MA and CA domains. The specific name associated with the Gag cleavage products is derived from their respective apparent molecular weights ($\times 10^{-3}$).

an N-terminal myristic acid, which is thought to insert into the hydrophobic lipid bilayer. The second is a surface patch of basic amino acids that are hypothesized to mediate the initial interaction of Gag with the negatively charged, phospholipid head groups of the membrane. Mutations that interfere with either myristoylation or the charged residues can abrogate plasma membrane targeting, and in some instances, the mutated Gag precursors are targeted to internal membranes.⁵⁷

In the betaretroviruses, where capsid assembly and virus budding are discrete events, a genetic dissection of this process has shown that Gag-containing precursors express a dominant sorting signal (the cytoplasmic targeting/retention signal [CTRS]) that targets the proteins to the initial assembly site.²⁸ Recent studies suggest that the CTRS interacts with Tctex-1, a light chain of the microtubule-associated dynein motor, and directs nascent Gag proteins and translating polysomes to the centriolar region of the cell, where capsid assembly occurs.^{194,222} A point mutation within the Mason-Pfizer monkey virus (M-PMV) CTRS domain can abrogate intracytoplasmic targeting and results in the type C-like transport of precursors to the plasma membrane, where efficient capsid assembly occurs.⁸¹ Efficient transport of wild-type capsids out of the assembly site depends on both the presence of the M-PMV Env protein and endosomal trafficking, and appears to reflect a requirement for Gag-Env interactions at the pericentriolar recycling endosome.¹⁹³ Interestingly, studies in murine leukemia virus (MuLV), in which the viral RNA was tagged for visualization by fluorescence microscopy, have suggested that prebudding complexes of Env, Gag, and RNA associate with late endosomes and are routed in this way to the plasma membrane.⁸ It is likely that similar preassembly complexes also participate in the intracellular transport of HIV-1 Gag proteins, because their transport is modulated by the cellular adaptins AP-1, AP-2, and AP-3, and intracellular interactions with Env direct Gag assembly to specific plasma membrane regions of the cell.^{5,49} Although in macrophages HIV-1 Gag was initially thought to be targeted to a late endosomal compartment for intracellular assembly and budding, more recent studies suggest that these compartments are derived from deep invaginations of the plasma membrane^{5,11} (see Fig. 6.7, Pathway 1d). Targeting of the plasma membrane by HIV-1 Gag for assembly in part reflects specific MA domain recognition of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], which is enriched there. Depletion of this lipid component by overexpression of the cognate phosphatase (5-phosphatase IV) redirects HIV-1 assembly away from the plasma membrane to internal membranes.⁵

Irrespective of the assembly site, Gag precursor proteins must associate in a reproducible fashion to assemble into the nascent capsid. Mutational analyses of *gag* genes, as well as *in vitro* assembly studies, have shown that the CA and NC domains of Gag play a critical role in assembly; MA is dispensable for this process.^{36,57} NC binding to RNA may act to nucleate the capsid assembly process, whereas CA forms a symmetrical hexameric network of proteins. *In vitro*, the CA protein alone can assemble into tubes that exhibit local sixfold symmetry (hexamers), and cryo-electron tomography studies of immature HIV-1 capsids have revealed a similar but distinct arrangement of the CA domain of Gag. A striking observation in the context of HIV-1 is that released immature virus particles have an incomplete protein shell, with the ordered

Gag lattice covering, on average, only two-thirds of the membrane surface.^{19,231}

For most retroviruses, capsid assembly drives the process of membrane extrusion known as budding. As we will discuss later, for an infectious virus to be formed, the Gag precursors (or for other viruses, NPs) and surface glycoproteins of the virus must be targeted to the same region of the same membrane. In this way, during virus budding, a proper complement of glycoproteins can be incorporated into the nascent virion.

Assembly of Enveloped Viruses at Cellular Membranes

For most enveloped viruses, the location within the cell at which envelopment takes place is determined by the targeting to or retention of the viral glycoproteins at that site. Indeed, with the exception of the retroviruses, the efficiency of virus budding and particle release highly depends on the presence of the envelope glycoprotein(s), and in its absence, few particles are produced. Because the glycoproteins define the site of virus budding, specific interactions between the viral NP and the glycoproteins, sometimes mediated by a matrix protein, must take place to ensure that the genome of the virus is incorporated. For each of the viruses that assemble at intermediate points within the secretory pathway, fully assembled viruses must traverse the remainder of the pathway to be released from

the cell (Fig. 6.9). Glycoproteins on these released viruses have complex oligosaccharides and are most likely modified by the Golgi-localized enzymes on the way to the cell surface.

Assembly at the Endoplasmic Reticulum–Golgi Intermediate Compartment

The coronaviruses are positive-stranded RNA viruses with large (30-kb) genomes packaged in a helical nucleocapsid. The nucleocapsid acquires its envelope by budding into the lumen of the ERGIC, a pre-Golgi compartment of the secretory pathway. Coronaviruses invariably encode three envelope proteins. The spike protein (S), which determines the host range of the virus, is a type I glycoprotein that forms the distinct bulbous peplomers of the virus. Expressed independently of the other glycoproteins, infectious bronchitis virus (IBV) S is transported to the plasma membrane, although it does contain both ER retention and endocytosis signals that can redirect it to the ERGIC.¹⁰⁶ The most abundant virion protein is the membrane (M) glycoprotein. M spans the lipid bilayer three times, exposing a short N-terminal domain outside the virus and a long C-terminus inside the virion. Because of its abundance and because M is transported to the Golgi complex but not to the surface, it was initially thought to define the site at which this family of viruses was enveloped. Studies, however, have shown that the small envelope protein (E), which is only

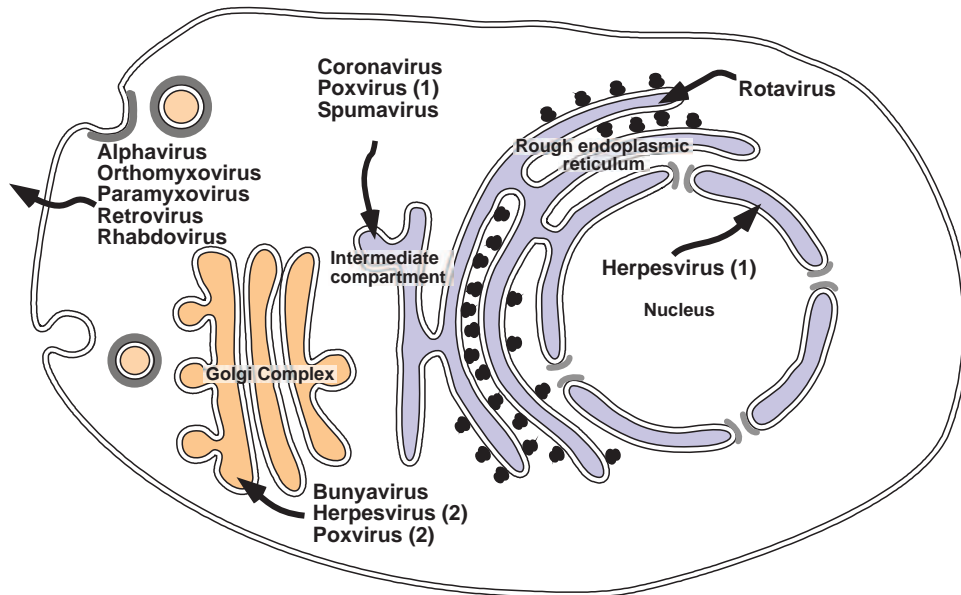


FIGURE 6.9. Viral assembly at cellular membranes. Schematic representation shows the intracellular locations at which enveloped virus assembly takes place. For each virus that is enveloped at organelles within the secretory pathway, the virions must traverse the remainder of the pathway to be released from the cell. Rotaviruses appear to utilize the ER membrane as a scaffold for assembly of virion proteins—the capsids that form during the assembly process are only transiently enveloped, and nonenveloped particles accumulate in the lumen of the ER. Coronaviruses localize the three membrane proteins (E, M, and S) in the ER–Golgi intermediate compartment, and virus budding occurs into the lumen of this compartment of the secretory pathway. In contrast, the vaccinia virus nucleocapsid appears to be wrapped by a double membrane derived from this compartment [Poxvirus (1)] but remains free in the cytoplasm so that it can then be further enveloped by Golgi-derived membranes [Poxvirus (2)]. Similarly, herpesviruses initially bud into the lumen of the nuclear membrane [Herpesvirus (1)]; however, after fusion and release of the capsid into the cytoplasm, it is re-enveloped by Golgi membranes [Herpesvirus (2)]. The G1 and G2 glycoproteins of bunyaviruses co-localize in the trans-Golgi compartment to direct budding of nucleocapsids at this location. For the retroviruses, which can assemble and release virus particles in the absence of envelope glycoproteins, the envelope glycoproteins appear to direct virus budding to the basolateral plasma membrane of polarized epithelial cells. ER, endoplasmic reticulum.

a minor component of virions, is the key to defining the site and nature of coronavirus envelopment.⁷⁷ E is a hydrophobic type I membrane protein that localizes to the ERGIC and induces the formation of tubular, convoluted membrane structures characteristic of virus infection.^{34,166} The E protein of IBV appears to be retained in the ERGIC by a novel ER retrieval signal (RDKLYS-COOH) and localizes M to this site through intermolecular interactions.¹⁰² Co-expression of E, M, and S results in the assembly and release from cells of virus-like particles (VLPs) containing these three viral membrane proteins. The enveloped particles produced by this system form a homogeneous population of spherical particles indistinguishable from authentic virions in size and shape.²²¹ Only M and E are required for efficient particle formation, and expression of E alone can mediate particle release.¹¹⁰ The S glycoprotein is thus dispensable for virus particle assembly but is retained in the Golgi by the M protein, which appears to direct its assembly into virions. M protein also directs the incorporation of nucleocapsids containing the genome-length RNA into virions, and nucleocapsids associated with newly synthesized M protein have been localized to the budding site in the ERGIC.¹³⁵

Although most retroviruses are enveloped at the plasma membrane of the cell, for most members of the spumavirus genus, this occurs on internal membranes. In human fibrosarcoma cells, primate foamy virus (FV) Gag and Env appear to co-localize predominantly in the trans-Golgi region of the cell;²³⁵ however, in other cells, immature capsids appear to bud into the ERGIC region of the secretory pathway. This is consistent with the observation that expression of the primate FV Env complex, gp80/gp48, in the absence of other structural proteins, results in its localization to the ER. A di-lysine ER retrieval motif at the C-terminus of gp48 is responsible for this localization, and mutation of either lysine results in efficient transport of the protein to the plasma membrane. Although a greater fraction of capsids bud from the plasma membrane in mutant virus-infected cells, envelopment of capsids at the ER is still observed.⁶⁶ A second distinguishing feature of foamy viruses is the dependence of capsid envelopment on Env expression.⁶ As with the betaretroviruses, assembly of immature FV capsids is targeted to the pericentriolar region of the cell by a CTRS located around an arginine residue at position 50 in Gag.²³⁵ They are then transported to the ER (or plasma membrane) for envelopment. In the absence of FV Env, these preassembled capsids do not associate with membranes or initiate budding. Recent experiments have shown that it is the posttranslationally cleaved (148 amino acids long), membrane-spanning, signal peptide domain of the FV Env that mediates capsid membrane association/envelopment; they have also shown that this protein is incorporated into the virus in the process.¹⁰³

Assembly in the Golgi Complex

Bunyaviruses are negative-stranded, enveloped viruses with a segmented genome that assembles in tube-like virus factories that are built around the Golgi complex and are connected to mitochondria and rough ER²²⁶ (see Fig. 6.9). By physically juxtaposing viral RNA replication and assembly, these factories appear to allow accumulation of RNPs that can associate with viral glycoproteins and bud into the lumen of swollen Golgi stacks. The glycoprotein spikes of the best-characterized member of this family, Uukuniemi virus, are comprised of

two type I glycoproteins—Gn (previously G1) and Gc (G2)—that determine the site of virus budding. Gn and Gc are co-translationally cleaved from a single precursor protein by signal peptidase, which cleaves after the internal signal sequence that mediates translocation of Gc. The two proteins have been shown to fold with distinctly different kinetics, but once properly folded, they form a Gn-Gc heterodimer that is transported to the Golgi complex. Gc expressed in the absence of Gn is retained in the ER, whereas Gn expressed alone is targeted to the Golgi.⁷¹ The Uukuniemi virus Golgi localization signal of Gn has been mapped, through analysis of mutations and glycoprotein chimeras, to the membrane proximal half of the 98 amino acid long cytoplasmic tail of the protein. Glycoprotein retention in this case appears, therefore, to depend on interactions between the cytoplasmic tail of Gn in the Gn-Gc heterodimer with components residing on the cytoplasmic side of the Golgi membrane. However, although all bunyavirus Gn-Gc complexes accumulate in the Golgi, the exact location and nature of the signal(s) that ensure this do appear to differ among the genera.²²⁶

During assembly of bunyaviruses, the helical nucleoproteins, consisting of the three single-stranded genomic RNA segments and the associated nucleocapsid (N) protein, accumulate in the Golgi component of the virus factories and through interactions with the Gn and Gc cytoplasmic domains initiate the budding of virus particles into the Golgi lumen. The region of the secretory pathway at which Gn-Gc heterodimers accumulate clearly defines the budding site, because in the presence of brefeldin, a drug that redistributes Golgi components to the ER, virus budding occurs into the ER.⁷¹

Assembly at the Plasma Membrane

Members of several virus families undergo their envelopment at the plasma membrane. These families include the togaviruses, the rhabdoviruses, the para- and orthomyxoviruses, and the retroviruses. In each of these cases, the viral glycoproteins, either as hetero-oligomeric complexes or homo-oligomers, have traversed the entire secretory pathway to be delivered to the plasma membrane of the cell. Assembly at the plasma membrane obviates the need for the assembled virus to navigate additional compartments of the secretory pathway, because virions are released directly into the external milieu of the cell. Alphaviruses are among the best characterized of these different viral systems, because a combination of biochemical, genetic, and structural information has been amassed to shed light on the complexity of this assembly process. The major glycoproteins E1 and E2 of the alphaviruses are translated from a subgenomic 26S RNA as a pE2, 6K, E1 precursor complex. The 6K and E1 proteins are released from the precursor by signal peptidase but remain in a complex with pE2. Following transport to the Golgi, pE2 is processed to E2 and E3. Stable trimers of E1-E2 heterodimers are then transported to the plasma membrane, where they associate with nucleocapsids formed from the capsid (C) protein and genome-length RNA. The 6K protein travels to the plasma membrane with the E1-E2 complex but is inefficiently incorporated into virions.^{62,89} Cryo-electron microscopy analyses of mature alphavirus particles have revealed a detailed structure of this enveloped virion (Chapters 3 and 23). These studies have shown that both the envelope and the core display icosahedral symmetry. Surprisingly, however, the trimers of E1 and E2 are located at the

threefold and quasi-threefold symmetry axes of the pentameric and hexameric order of the nucleocapsid. Moreover, the heterodimers of each spike splay out above the membrane in a skirt-like fashion, traverse the lipid bilayer individually, and interact with three underlying capsid (C) proteins that belong to three separate capsomeres. This creates a complex network of molecular interactions where the glycoprotein–capsid protein interactions mediate not only the binding of the nucleocapsid to the spikes but also stabilize the connections between the capsomeres.⁸⁹ Above the membrane, the skirts formed by the E1-E2 heterodimers form lateral connections that mimic the pentameric and hexameric arrangement of the capsid. These interactions may facilitate the process of budding by providing a multivalent binding site for the capsid, as well as by providing a force for membrane bending. Budding, however, requires a cooperative interaction between the glycoproteins and the capsid protein, because in the absence of either, budding does not occur. Moreover, mutations in the cytoplasmic tail of the E2 protein alone can abrogate budding. Two models have been proposed for assembly.⁶² In the first, glycoprotein trimers assemble around a preformed nucleocapsid that is associated with the inner leaflet of the plasma membrane. The second hypothesizes that it is through interactions between E1-E2 heterotrimers that the icosahedral structure of the virus is established and that interactions between the E1-E2 heterodimers and C proteins organize the nucleocapsid similarly. Evidence for the latter model came from deletion mutants in C that are defective in the formation of intracellular capsids but that nevertheless can associate with E1-E2 to facilitate the release of $T = 4$ icosahedral capsids.⁵⁶

For the negative-strand RNA viruses, orthomyxoviruses, paramyxoviruses, and rhabdoviruses, an additional protein, the matrix protein, mediates the interactions between the viral glycoproteins and the RNP and appears to play a key role in envelopment. These proteins are able to bind to membranes through hydrophobic domains or, as with the retroviruses, through a cluster of positively charged residues that initiate electrostatic interactions with the plasma membrane. Cross-linking studies have demonstrated that these M proteins form homo-oligomers in the virus and can self-associate *in vitro* or when expressed at high levels in cells. As discussed earlier, the M1 protein of influenza virus acts to mediate the transport of RNPs from the nucleus to the site of virus assembly on the plasma membrane, and it seems likely that the intracytoplasmic transport pathway for this M1-vRNP complex involves interactions with cytoskeletal components of the cell.⁴ The importance of the M protein in the paramyxovirus budding process was inferred from the defective measles viruses found in subacute sclerosing pan-encephalitis that are unable to assemble virus particles and that have mutations in the M protein coding region.¹³ However, the development of reverse genetics systems for the negative-stranded viruses, in which mutations can be reintroduced into the viral genome, has allowed a more rational approach to examining these questions. Construction of rhabdovirus genomes that lack the M coding region resulted in a dramatic (more than 10^7 -fold) decrease in the release of virus particles, and those that were released lacked the characteristic bullet-shape morphology. The defect could be complemented by expression of M *in trans*.¹²⁰ Thus, in the rhabdoviruses, the M protein condenses the helical RNP into its characteristic shape and mediates the envelopment process. Similarly, for the orthomyxoviruses, M1 can define

whether spherical or filamentous forms of the virus particle are produced¹⁷; although in the absence of M1 particles can be released, this is a much less efficient process.¹⁷⁸

Reverse genetics approaches have also shed light on the role of the G glycoprotein in rhabdovirus assembly and of the HA and NA in orthomyxovirus assembly. Rhabdoviruses that lack the G-protein coding domain do assemble and release bullet-shaped particles but at only 3% to 10% the efficiency of wild-type.^{119,173} Initial studies with G proteins lacking a cytoplasmic tail have suggested that this domain might be important in the budding process; however, more recent studies have shown that although there is a general requirement for a short cytoplasmic tail, it is amino acid sequence independent, and that G proteins with large C-terminal extensions can be efficiently incorporated into virions, thereby leaving the basis for M protein interactions with G unresolved.⁸⁸ Although foreign glycoproteins can be efficiently incorporated into rhabdovirus particles (creating pseudotype particles), they do not stimulate budding.¹⁷¹ This is consistent with experiments that have identified a domain within the extracellular membrane-proximal stem (GS) of vesicular stomatitis virus (VSV) G that is required for efficient VSV budding. Recombinant viruses encoding glycoprotein chimeras with 12 or more membrane-proximal residues of the GS, as well as the G protein transmembrane and cytoplasmic tail domains, produced near-wild-type levels of particles. In contrast, those with shorter regions produced 10- to 20-fold fewer particles. It is possible, therefore, that this region of the G protein membrane-proximal domain modifies the membrane to facilitate the budding process.¹⁷³ Recent cryo-electron microscopy studies of VSV suggest that assembly begins with the formation of an RNA-N protein nucleocapsid ribbon, which, after forming a tight ring that represents the tip of the bullet-shaped particle, is forced to curl into larger rings that eventually tile the helical trunk. The assembling structure would be stabilized by the binding of M protein to the outside of the nucleocapsid, which in turn forms a triangular platform for binding G-trimers and the lipid membrane.⁶⁴

In influenza virus, the fact that the cytoplasmic domains of all three membrane proteins (HA, NA, and M2) are highly conserved in all isolates of the virus pointed to a role for these domains in assembly of enveloped virus. Early studies indicated that HA proteins with foreign cytoplasmic tails are not incorporated into virions, although HAs lacking this domain can be incorporated at levels 50% that of wild-type.¹³³ Studies using reverse genetics have confirmed these findings and have shown that the short (six amino acid) cytoplasmic domain of NA also plays an important role in morphogenesis. Viruses encoding the truncated NA protein were released less efficiently and were larger and more filamentous. However, in viruses encoding tailless versions of both HA and NA, a 10-fold reduction in virus release was observed and morphogenesis was drastically altered. Virus particles released from these cells were greatly elongated with an extended irregular shape and a reduced level of viral RNA. Similarly, truncation of the cytoplasmic tail of M2, which binds to M1, results in much reduced virus infectivity, coupled with reduction in the amount of packaged RNA and budding efficiency.¹¹⁸ Thus, it appears that for influenza virus, the interactions between M1 and the viral membrane-spanning proteins are so important for envelopment and morphogenesis that the virus has developed redundant interaction domains in these proteins.^{136,178}

With the exception of the spumaviruses, glycoprotein–capsid interactions are not absolutely required for the assembly and release of enveloped retrovirus particles; the Gag precursor alone contains the information necessary to be specifically targeted to the plasma membrane and to drive budding. Nevertheless, for members of the lentivirus family, such as HIV and simian immunodeficiency virus (SIV), that encode envelope glycoproteins with a long (150+ amino acid) cytoplasmic domain, a specific interaction between this region of the transmembrane component of Env and the MA domain of the Gag precursor appears to be necessary for Env incorporation. For these viruses, mutations in MA or in the cytoplasmic tail can abrogate Env incorporation.²⁶ Whereas viral glycoproteins are not required for assembly and budding of most retroviruses, in the case of M-PMV, Env appears to facilitate the intracellular transport of Gag molecules to the plasma membrane.¹⁹³ An understanding of the process by which Gag is transported to the plasma membrane remains incomplete; however, there is a growing body of evidence that Gag and Env may interact at intracellular sites and utilize components of the endocytic pathway to target these structural gene products, as well as genomic RNA, to an assembly site on the plasma membrane. Further evidence for an Env–Gag interaction is derived from polarized epithelial cells, where retroviral glycoproteins define the plasma membrane domain at which virus budding and release occur (see later discussion).

Several viruses appear to make use of cholesterol- and sphingolipid-rich domains on the plasma membrane known as lipid rafts to assist in the concentration and organization of viral components for assembly.²⁵ These rafts are characterized by their insolubility in detergent at low temperatures (4°C), and proteins associated with them remain in the insoluble fraction following extraction from cells. Both HA and NA of influenza virus cluster in lipid rafts via targeting signals that have been mapped to both their transmembrane and cytoplasmic domains, and it is hypothesized that through interactions with the clustered glycoproteins, M1 and the vRNPs are organized for budding.¹³⁷ Consistent with these observations, the lipid bilayer of influenza virions is enriched in cholesterol and sphingolipids and appears also to be in ordered domains.¹⁸⁹ Similarly, the glycoproteins and capsid precursors of HIV-1 have been shown to associate with lipid rafts, and depletion of cholesterol from the plasma membrane impairs HIV-1 particle production.²²⁵ Gag–Env interactions appear to be required for Env association with lipid rafts, as mutations in either MA or the cytoplasmic domain of the gp41 transmembrane protein that interfere with Gag–gp41 interactions prevent Env association with rafts.¹⁰ Although it was initially assumed that VSV assembled independently of rafts, it has been shown that G clusters in microdomains and can co-localize on the plasma membrane with proteins known to be located in rafts.¹⁶³ Thus, it is possible that VSV also utilizes rafts to organize its components for assembly.

Targeting of Viral Glycoproteins in Polarized Epithelial Cells Defines the Site of Budding

Many viruses initiate their infection of a host by interacting with cells at an epithelial surface. Individual cells within an epithelial layer are tightly connected by junction complexes that form a barrier to diffusion of molecules throughout the cell membrane and divide the cell surface into two distinct plasma membrane domains: the apical domain, which faces the exterior,

and the basolateral domain, which faces the interior. As a result of differential targeting of lipids and protein components to apical and basolateral membranes, epithelial cells in tight monolayers are highly polarized with each plasma membrane domain having a distinct lipid and protein composition.¹⁷⁵ The assembly and release of many viruses from epithelial cells is also highly polarized, occurring selectively at either the apical or basolateral surface. Influenza virus releases newly assembled virions from the apical surface of polarized epithelial cells, whereas VSV and many retroviruses are released from the basolateral membrane. In the absence of other viral proteins, VSV G protein and several retroviral Env proteins are transported to the basolateral surface, whereas HA, NA, and M2 are targeted to the apical membrane.^{32,136} Sorting of proteins to one or the other domain occurs in the trans-Golgi network, and recent evidence suggests that proteins destined for the basolateral surface contain within their cytoplasmic domain motifs that direct the protein to that membrane. In the VSV G protein, a tyrosine-based motif within the cytoplasmic tail appears to be critical for basolateral targeting.²¹⁴ Similarly, tyrosine-based endocytosis motifs in the cytoplasmic domain of both the MuLV and HIV Env proteins are important for their polarized expression and for basolateral budding of their cognate viruses.

Apical transmembrane proteins appear to contain two signals that probably act cooperatively in targeting to the apical surface. Glycosylation in the ectodomain and a signal in the membrane-spanning domain function together to ensure association with sphingolipid-cholesterol-enriched membrane domains or rafts, which have been proposed to mediate apical transport in polarized epithelial cells.

In the absence of a basolateral targeting signal, retroviral Env proteins are delivered to and virus buds from both membranes with equal efficiency, arguing that interactions with Env guide Gag to the basolateral surface.¹⁰⁵ The rhabdoviruses differ in this regard, in that even when the basolateral targeting signal for G has been mutated such that G is transported to the apical surface, virus assembly still occurs predominantly at the basolateral surface. Similarly, under conditions where the influenza virus HA is directed to the basolateral surface, virus budding remains predominantly apical. In these viruses, therefore, it appears that the internal components of the virion possess independent targeting signals that direct them to their respective membranes.¹³⁶ Polarized virus assembly and release may be important in determining the pathogenesis of viral infections, as it can influence, in a major fashion, the pattern of virus spread in the infected host.³²

Complex Interactions with the Secretory Pathway

Whereas most enveloped viruses undergo assembly and envelopment at a single site within the secretory pathway, some viruses have a more complex interaction that can involve de-envelopment or re-envelopment as part of their assembly pathway.

Rotavirus Assembly Within the Endoplasmic Reticulum

Rotaviruses are nonenveloped viruses that undergo transient envelopment at the ER as an essential step in the formation of the mature double-shelled (triple-layered) virus that is retained within the lumen of the ER until cell lysis. As with the reoviruses, the nucleocapsid, in this case containing

11 double-stranded RNA segments, is assembled in electron-dense areas of the cytoplasm, located close to the ER membrane, known as viroplasm. This assembly domain depends on expression of two nonstructural proteins—NS2 and NS5—and mutations or silencing of either gene product results in its abrogation.^{55,107,159} The nucleocapsids that assemble have an outer icosahedral shell assembled from VP6, the most abundant protein of the virus, surrounding an inner core (Chapters 44 and 45). They appear to bud directly into regions of the ER that contain the two outer shell proteins VP7 and VP4. A nonstructural protein, NSP4, which forms hetero-oligomers with the two outer shell proteins, mediates the interaction of the immature particle with the ER membrane.^{125,147,212}

The exact topology of VP7 in the ER is not known. The mature protein has a cleaved signal peptide but is retained in the ER as if it were an integral membrane protein.^{94,200} VP4 is thought to associate with VP7 and NSP4 just prior to budding of the nucleocapsid into the ER. Enveloped particles can be observed in the lumen of the ER, which then undergo a process of calcium-dependent de-envelopment, forming in the process an outer icosahedral shell of VP7 and VP4.¹⁶⁵ This process seems to be directed by VP7 because the silencing of this protein has not blocked the budding of double-layered particles into the ER but instead has arrested maturation at the membrane-enveloped particle stage in this compartment.¹⁸² The rapid association of VP4 with lipid rafts and the nonlytic release of rotaviruses from the apical plasma membrane of gut epithelial cells has led to the suggestion that the site of budding might be the ERGIC and that rafts may play a role in transport of virus directly from this region to the plasma membrane.^{37,47} Thus, this nonenveloped icosahedral virus appears to transiently utilize membranes of the secretory pathway as a scaffold on which to assemble an icosahedral shell and a nontraditional vesicle-mediated route to the plasma membrane.

Herpesvirus Transport from the Nucleus

As we described previously, the end product of herpesvirus capsid assembly is a large icosahedral structure that is too large to transit the nuclear pore. Members of this family have therefore evolved to utilize a complex envelopment/de-envelopment/re-envelopment strategy that allows the final acquisition of a lipid envelope to occur at a late compartment of the secretory pathway¹²¹ (Fig. 6.10). The first evidence for this initially controversial pathway came from electron microscopic observations of infected cells.^{71,169} Capsids that have assembled in the nucleus

acquire an envelope derived from the inner leaflet of the nuclear membrane as they bud into the luminal space (termed *primary envelopment*). Products of the UL31 and UL34 genes are involved in this process—the former encoding a nuclear phosphoprotein and the latter encoding a type II membrane protein. Both proteins are located on the nuclear membrane; however, the UL31 protein requires UL34 for nuclear targeting. Absence of either protein abrogates primary envelopment and results in capsid accumulation in the nucleus.¹⁶⁷ Although these proteins are components of the primary enveloped virions, they are absent from mature virions. Primary envelopment does not occur in the absence of the pUL25 capsid-associated protein and thus appears to be required for the budding process.⁹⁷ The initially enveloped virions lack proteins that are abundant in mature virus particles (e.g., pUL47 or pUL49), indicating that the most prominent components of the viral tegument have to be added during later steps of virion morphogenesis.¹²³

In the de-envelopment step, the newly enveloped virus fuses with the outer nuclear membrane to release the capsid into the cytoplasm. It is here that tegument proteins of the mature virus, a collection of proteins including transcription factors located between the viral capsid and its lipid bilayer in the mature particle, are proposed to associate with the capsid prior to its re-envelopment by Golgi-derived membrane vesicles.¹²³ For herpes simplex virus and pseudorabies virus, glycoproteins of the gE/I complex together with gM appear to be essential for the re-envelopment step, and in their absence, intracytoplasmic aggregates of tegument-associated capsids are formed.¹⁸

Some of the clearest evidence for this complex assembly pathway comes from studies of human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6). In these viruses, the tegument is a dense structure that can be observed in electron micrographs. Enveloped virions in the lumen of the nuclear membrane display no evidence of tegument but acquire this layer following loss of the nuclear-derived envelope and subsequent release into the cytoplasm.^{176,216} Studies of the glycoproteins of the gamma herpesvirus, Epstein-Barr virus, also support this model. Distribution of the viral glycoproteins gp110 and gp350/220 is consistent with the former being incorporated in virions at the nuclear membrane and then lost on budding into the cytoplasm, whereas the latter is acquired only during re-envelopment in the Golgi.⁶⁷ Similarly, studies of the lipid composition of extracellular HSV-1 virions indicate that it is similar to that of the Golgi complex and distinct from that of the nucleus.²²⁰ Why would viruses evolve to utilize such

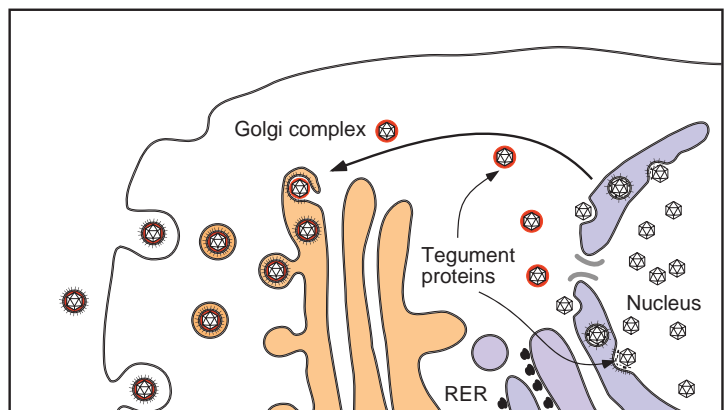


FIGURE 6.10. Herpesvirus assembly. In the re-envelopment pathway, nascent assembled capsids bud into the lumen of the nuclear membrane utilizing a subset of virally encoded glycoproteins. These enveloped particles then fuse with and are released from the outer nuclear membrane into the cytoplasm. There they associate with the tegument proteins and are transported through the cytoplasm to be enveloped by Golgi-derived membranes that contain the full complement of virion glycoproteins.

a complex assembly strategy? Perhaps the most persuasive argument comes from the perspective of the neurotropic alphaherpesviruses. These viruses infect nerve endings in the periphery; however, DNA replication and capsid assembly must occur in the cell body. To initiate a second round of infection at the nerve synapse, viral components must be transported down the axon. Thus, by separately transporting the viral capsids on microtubules and viral glycoproteins by the vesicular pathway, the site at which infectious virus is assembled can be much more accurately controlled.^{120,215}

Poxvirus Acquisition of Multiple Membranes

Poxviruses, exemplified by vaccinia virus, exhibit an equally complex interaction with the secretory pathway. These large DNA viruses, which encode all of the machinery necessary for genomic replication and transcription, propagate entirely in the cytoplasm in specialized areas designated as virus factories (Chapters 66 and 67). The assembly and envelopment of these viruses is particularly complex because they apparently can be enclosed by multiple lipid bilayers.^{33,172} The first recognizable structure is a crescent-shaped membrane that appears to be comprised of a single lipid bilayer with an external protein lattice constituted of trimers of the D13 protein.^{76,209} Initial electron microscopy studies suggested that the crescent membranes that wrap the immature virions (IV) are formed *de novo* within the virus factories,⁴² and additional high-resolution electron microscopy experiments have been interpreted as support for the conclusion that the crescents are formed from a single membrane.⁷⁸ Nevertheless, the origin, mode of formation, and composition of these crescent membranes remains controversial. Several investigators have provided evidence that these envelopes are composed of two closely apposed membranes, which are derived from the IC of the secretory pathway.^{168,198} Co-localization of IC resident cellular proteins with several vaccinia virus membrane proteins (A17L, A14L, and A13L) known to be critical for the formation of IV membranes have supported this model.^{99,185} However, subsequent studies have shown that transport of proteins from the ER to the IC and Golgi is not necessary for IV formation and that there is a transport pathway from the ER to the assembling IV.⁸² Moreover, epitope tagging of the L2 protein, an early poxvirus protein essential for crescent formation, showed that it was associated with the ER tubules throughout the cytoplasm and in some instances appeared to be continuous with the membrane crescents.¹¹⁶ The D13 protein, which forms a honeycomb lattice on the outer surface of the IVs, is critical for crescent formation and appears to act as a scaffold for their formation through its interaction with the N-terminus of the membrane-spanning A17 protein.¹⁴ Interestingly, Rouiller et al¹⁸⁰ have reported that the virus core of the related African swine fever virus core is also wrapped by specialized regions of the ER.

The process by which the nucleoprotein/transcription machinery is targeted into the immature envelopes is unclear; however, a multiprotein complex of seven viral proteins—A15, A30, D2, D3, F10, G7, and J1—appears to be critical. Repression of several of these viral products yields the same phenotype—the failure of viral membranes and viroplasm to associate with each other.²⁰⁸ Maturation of the IV to the infectious intracellular mature virus (IMV) involves a series of proteolytic cleavages of vaccinia structural proteins.³³ The mature virus particles are transported out of the assembly areas toward the periphery of the cell, where additional membranes that are derived from the

trans-Golgi or early endosomal compartments wrap them to form the intracellular enveloped virus (IEV).^{196,197} The membranes of these compartments contain vaccinia proteins that will be present in the external enveloped virus (EEV). Wrapping requires the participation of at least one protein present on the IMV and two EEV membrane proteins. It thus appears to be driven by interactions between vaccinia-encoded membrane proteins.^{15,174} It is likely then that the IEV contains at least three concentric membranes, one derived from the ER and two from the Golgi/endosome. These particles are then transported via a reorganized actin cytoskeleton (so-called actin-tails) to the plasma membrane,³⁸ where membrane fusion releases the infectious EEV form of the virus.

Modification of the Secretory Pathway Transcription and Assembly of Poliovirus on a Disassembled Secretory Pathway

Poliovirus is a nonenveloped positive sense RNA virus that modifies the host cell extensively during its replication. Once introduced into a target cell, the genomic RNA is translated into a long polyprotein precursor that contains both structural and replicative proteins of the virus (Fig. 6.11). Early in the replication of the virus, there is a massive rearrangement of the intracellular membranes into clusters of vesicles that are 200 to 400 nm in diameter.¹⁸⁶ The altered membranes appear to have characteristics of autophagosomal vesicles; they have two membranes and contain, in addition to the viral RNA replication machinery, several autophagosomal proteins, including LAMP1 and LC3. Moreover, stimulation of autophagy has been shown to increase poliovirus yield, and inhibition of the autophagosomal pathway decreased virus yield, suggesting subversion of this pathway by poliovirus.^{85,213} Electron microscopy and inhibitor studies have suggested that the induced vesicles are derived from the host cell secretory pathway, as they appear to be connected to ER membranes and both their formation and poliovirus replication are blocked by Brefeldin A.⁸³ Viral RNA transcription occurs in close association with these vesicles, and both viral RNA and viral proteins known to be required for RNA replication have been shown by electron microscopy to be associated with their cytoplasmic surface.^{12,217} Similarly, priming of RNA synthesis by the protein primer VPg appears to require membranes.²¹¹ For poliovirus, the viral precursor protein 2BC, when expressed alone, was shown to cause both membrane vesiculation and the formation of multilamellar structures²⁷; however, it is in combination with poliovirus protein 3A, which causes the inhibition of ER-to-Golgi protein traffic,⁵⁰ that membrane alterations most consistent with the pattern in poliovirus-infected cells are observed.²⁰⁶

The formation of vesicular platforms for transcription may also be necessary for efficient capsid assembly. The poliovirus capsid appears to be assembled in a sequential process in which the 5S protomers containing VP1, VP3, and VP0 assemble to form 14S pentamers, which in turn are assembled into virus capsids (see Fig. 6.11). Pentamers may associate with newly synthesized genomic RNA on intracytoplasmic vesicles and then assemble to form RNA-containing capsids on completion of the RNA. This assembly pathway is supported by immunoelectron microscopy studies showing that pentamers associated with the replication complex can, if released by detergent, rapidly assemble into empty capsids—arguing that the vesicle

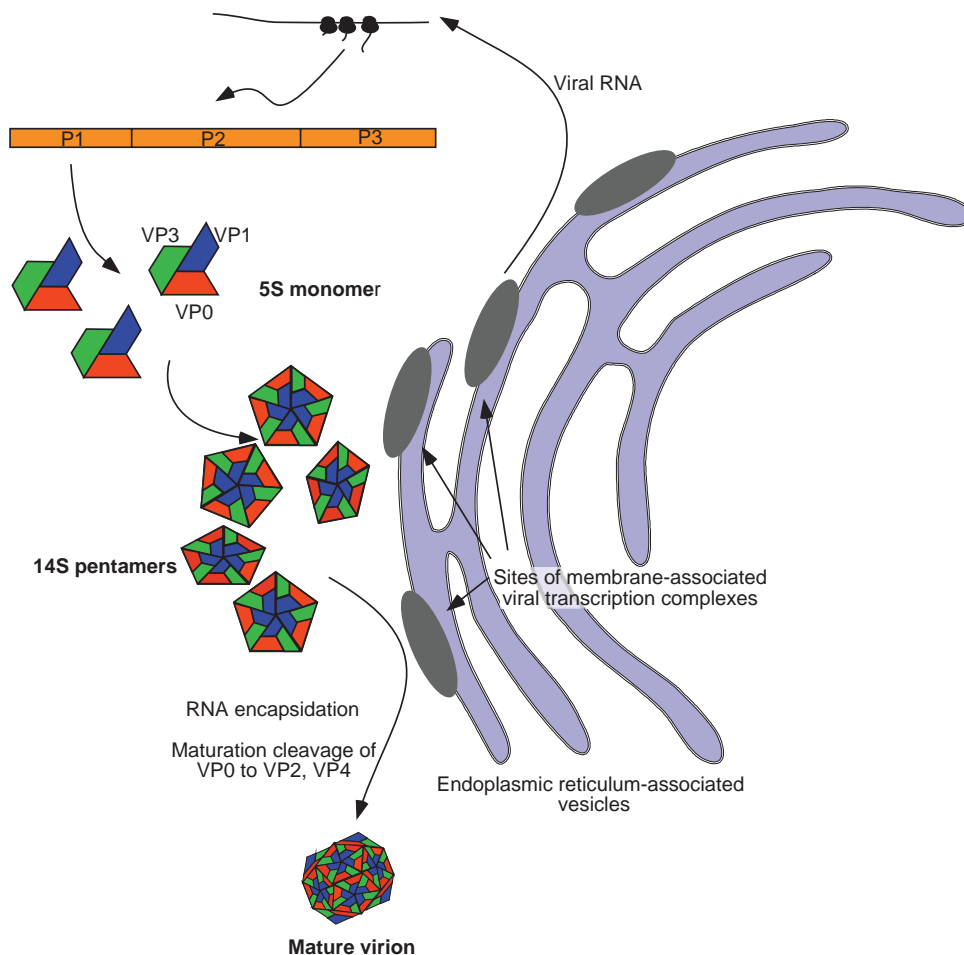


FIGURE 6.11. Poliovirus assembly. The P1 precursor protein is cleaved via an autocatalytic event from the nascent polypeptide and is then cleaved twice by the 3CD-protease to yield VP1, VP2, and VP0. This cleavage is essential for assembly of the 5S monomers into 14S pentamers. It is likely that 12 of these pentamers associate directly with nascent genomic RNA that is being transcribed from membrane-bound transcriptional complexes to assemble an immature 150S provirion. Maturation cleavage of VP0 to yield VP2 and VP4 results in conversion of these immature provirions into the 160S mature virion.

prevents assembly of the capsid and keeps the pentamer in an appropriate configuration for association with the RNA.¹⁶² In this instance, poliovirus appears to have circumvented the problems of intracytoplasmic localization of replicative and structural components by utilizing a membranous organizing center.

Modification of Post-Golgi Vesicle pH by the Influenza Virus M2 Protein

The influenza HA is synthesized as a precursor protein (HA0) that in the case of the avian influenza viruses is cleaved in the trans-Golgi by furin-like proteinases to the biologically functional HA1-HA2 heterodimer, three of which in turn form the trimeric HA complex (Chapters 3, 40 and 41). The influenza virus HA present on an infecting virion is activated to its fusogenic state by the low pH of the endosome. Thus, the similarly low pH of the trans-Golgi network and post-Golgi vesicles poses a problem for the virus during the assembly phase of its life cycle, if it is to maintain HA in a functional prefusogenic form. This is apparently solved by the action of the M2 protein, a small tetrameric protein that forms cation-selective ion channels that can reduce the pH of the trans-Golgi network by pumping out hydrogen ions.^{164,205}

INCORPORATION OF THE NUCLEIC ACID GENOME DURING THE ASSEMBLY PROCESS

The processes involved in the assembly of structural components of a virus are complex and interplay intimately with those of the host cell; thus, they merely provide a mechanism by which the genomic information of the virus can be packaged into a protective environment for transfer to additional host cells. As such, the assembling virus must have evolved to efficiently select its genomic nucleic acid out of the pools of RNA and/or DNA that are present at the assembly site. Packaging of viral genomes generally involves a cis-acting sequence in the nucleic acid—the so-called packaging sequence—and a structural component(s) of the virus that can recognize and bind this element.

DNA Viruses

As described previously, for adenovirus, an empty procapsid is first formed around scaffolding proteins in a manner similar to that observed with DNA phages. Following procapsid assembly, DNA and associated core proteins are then subsequently

packaged into these empty shells to yield “immature virions” that then undergo proteolytic maturation.

Early studies indicated that *light* virus particles contain subgenomic-length viral DNA with an overrepresentation of left-end sequences. Subsequent studies showed that a cis-acting packaging sequence that is absolutely required for encapsidation of viral DNA is located within the left 390 bp of the genome. This region is highly conserved between different adenovirus subtypes and, in Ad 5, overlaps with two distinct enhancer elements for E1A transcription. Deletion of the packaging domain abrogates viability; however, infectivity can be restored by substitution of the left-end sequences at the right end of the genome.^{73,74} The packaging sequence only functions if it is within 600 bp of the inverted terminal repeat (ITS) but does not depend on the ITS itself.¹⁵² Mutational analyses showed that this packaging region contains at least seven functional AT-rich units called *A-repeats*. These repeats are functionally redundant but not functionally equivalent. Moreover, a sequence homology shared by the most important A-repeats, 5′-TTTG-N₈-CG-3′, is essential for packaging domain function. Overexpression of an A-repeat multimer competes *in trans* resulting in a dramatic decrease in viral yield without affecting DNA replication or late transcription, suggesting the involvement of limiting viral protein components in packaging.^{69,191} It seems likely from a variety of genetic analyses, *in vitro* DNA-binding studies, and chromatin immunoprecipitation approaches that the viral IVa2 protein together with the L4-22K protein bind directly to the A-repeat sequences to initiate packaging.^{54,151,153} The L1 52/55K protein, which is also critical for DNA packaging, is a part of this complex perhaps through interactions with IVa2 or L4-22K.^{153,161}

Packaging of the herpesvirus genome is equally complex. As with the large DNA phages, herpesvirus DNA replicates to yield concatemers of genomes in a head-to-tail arrangement, thus packaging must be linked to the generation of genomic-length units of DNA. In this virus, two packaging sequences—*pac1* and *pac2*—located within the terminally repeated *a* sequences appear to be important for both recognition and cleavage of the DNA (Chapters 59 and 60). As with the DNA phages, a combination of cleavage recognition sequences and *head-full* packaging appears to ensure the incorporation of unit-length genomes into the procapsid. Studies utilizing a temperature-sensitive protease mutant of HSV-1, which accumulates procapsids (containing an uncleaved scaffolding protein) at the nonpermissive temperature, although can remove the scaffold and package DNA on temperature shift to the permissive temperature, have shown that ATP is necessary for DNA packaging to occur but DNA synthesis is not.^{30,44} At least seven viral proteins—products of the genes UL6, UL15, UL17, UL25, UL28, UL32, and UL33—are required for cleavage and packaging of HSV-1 DNA. When cells are infected with HSV-1, mutants lacking the function of any of the seven genes, capsid formation, and DNA replication occur normally; however, no packaging takes place. By analogy with the DNA bacteriophages such as P22, the portal vertex, which is assembled from 12 copies of the *UL6* gene product, likely forms the docking site for these packaging proteins. Sequence analysis reveals that another critical component, encoded by the *UL15* gene, shares homology with gp17, the large catalytic subunit of the bacteriophage T4 terminase. Thus, the product of this gene, together with that of UL28, which can specifically recognize

the *pac* site, may play a direct role in the cleavage of viral DNA replication intermediates into monomers.^{1,234}

RNA Viruses

For retroviruses, genomic packaging represents a particularly difficult problem because the assembling capsid must select genomic RNAs from a large pool of viral and cellular mRNAs that are also present in the cytoplasm. Viral RNAs to be specifically packaged are identified by the presence of an RNA sequence named the packaging signal or ψ and are selected from the cytoplasmic pool by a specific interaction with the zinc knuckle motif(s) of the NC domain of the Gag precursor.^{41,108} The cis-acting RNA element is located at the 5′ end of the viral genome, has the capacity to mediate the packaging of heterologous RNAs, and appears to be comprised of a series of stem-loop structures that each contribute to the strength and specificity of the signal. One of the best characterized, although perhaps most complex, ψ -sequence is that of HIV-1, which is now known to span the 5′ untranslated region of the genome, as well as downstream nucleotides in the Gag region for optimal packaging efficiency. This 5′ untranslated region, which can confer packaging specificity on foreign RNAs, includes the TAR sequence that is important in Tat-mediated transcriptional control, the adjacent poly-A loop, and four stem loops that incorporate the primer binding site (PBS), the dimerization sequence (DIS), the 5′-splice donor (SD) and the packaging signal (ψ), respectively. Gag has been shown to bind specifically to this region, with PBS, DIS, and ψ stem loops each providing independent high-affinity binding sites. Disruptive and compensatory mutations within the TAR, Poly(A), PBS, DIS, and ψ elements indicate that the structure of the stem loops is important for packaging.^{31,117} NMR analyses of SL3 in the presence and absence of the NC protein have shown that tight binding is mediated by specific interactions between the amino- and carboxyl-terminal CCHC-type zinc knuckles of the NC protein and nucleotide bases of the RNA loop.^{45,108,237}

Regions of retroviral genomes that promote genome packaging often overlap with segments that promote the RNA dimerization, which is necessary for packaging of the diploid genome. Moreover, there is mounting evidence that dimerization and packaging events are intimately coupled. An elegant structural analysis by D’Souza and Summers⁴⁰ showed that dimerization of the MuLV genome results in an altered secondary structure in the ψ region of the RNA, which exposes conserved UCUG elements that bind NC with high affinity. These elements are base paired and do not bind NC in the monomeric structure. Thus, for MuLV, the problem of ensuring the encapsidation of two genomic RNAs has been solved by linking exposure of the packaging recognition sequence to dimer formation itself. It seems likely that HIV and other retroviruses will utilize similar switches for this purpose.¹⁴⁸

Given its role in specifying genomic RNA packaging, one might expect the ψ -sequence to be located downstream of the SD site for subgenomic mRNAs. This is the case for the MuLV and reticuloendotheliosis virus ψ -sequences but not the case for HIV-1, as described earlier, or for Rous sarcoma virus (RSV). The major RNA packaging signal for HIV-2 is present on all RNA species, although this virus appears to overcome the lack of packaging signal location specificity by two novel

mechanisms—co-translational packaging and competition for limiting Gag polyprotein.⁷⁰

The cellular location in which Gag:RNA recognition occurs remains an unanswered question. Parent et al¹⁵⁶ have provided strong evidence that for RSV, genome packaging depends on Gag trafficking through the nucleus, a process that is mediated by NLS and NES signals in the MA domain of Gag.^{60,72} However, the role of nuclear trafficking of HIV-1 Gag in RNA packaging remains unresolved and controversial.^{108,156} Recent experiments by Moore et al suggest that HIV-1 RNAs form dimers preferentially in the cytoplasm and that dimerization, mediated by the DIS sequence in the genome, is critical to efficient packaging into virions.^{126,127} The incorporation of genomic RNA into assembling virions was recently imaged in live cells and showed that in the absence of Gag, genomic RNA molecules were highly dynamic and did not localize at specific sites at or near the plasma membrane. In contrast, in the presence of Gag, genomes were targeted to specific sites on the plasma membrane, where after initially exhibiting slow lateral movement they became fixed and were the site for accumulation of additional Gag molecules.^{90,92}

Genomic RNA packaging signals in other RNA viruses are less well defined. In the alphaviruses, this appears to involve direct binding of genomic RNA by the capsid protein. A short (132-nt) segment of RNA in the region of the genome encoding the nonstructural protein NS1 is critical for this interaction and for packaging of genomic RNA. As with the retroviral psi-sequence, it has also been predicted to form a series of stem-loop structures. The N-terminal third of the capsid protein is basic and unstructured in the current crystal structure. Nevertheless, a 32 amino acid region (residues 76 to 107) has been shown to be essential for RNA binding in a gel shift assay.¹⁹⁰

For segmented viruses, such as the orthomyxoviruses or the reoviruses, genomic packaging requires that a complete set of segments be packaged together in a single virus particle. For influenza virus, reverse genetic approaches have shown that the signals for transcription and replication of an RNA segment are located in the 22 5′ terminal and the 26 3′ terminal nucleotides of the RNA.¹⁰⁹ These studies do not, however, address how a full complement of RNAs is selected during assembly. Two models have been proposed to accomplish this. The random incorporation model assumes a common structural feature in all vRNPs, which enables them to be incorporated randomly into virions. Support for this model comes from the observation that an influenza A virion can possess more than eight vRNPs.^{7,52} The selective-incorporation model predicts the presence of specific structures in each vRNA segment, leading to their individual incorporation into virions. Two lines of evidence support this hypothesis: the first was data showing that defective RNA segments could inhibit the packaging of the parental segment into virions,⁵¹ and the second was the finding that viruses grown in the presence of bacterial NA and antibody to NA retained an extensively deleted NA vRNA, suggesting that the altered NA segment participates in viral replication and carries structural features required for its incorporation into virions.^{80,232} Experiments utilizing reverse genetics approaches have further supported the selective-incorporation model by demonstrating that all eight vRNA segments must be present for efficient virion formation and that sequences within the coding region of the NA vRNA encode a signal that drives incorporation of this

segment into virions.^{59,101,132} Electron microscopic studies of serially sectioned influenza virus A particles, which showed the RNPs organized in a distinct pattern (seven segments of different lengths surrounding a central segment), also support a model where the eight segments are specifically packaged.¹⁴⁵

POSTASSEMBLY MODIFICATIONS AND VIRUS RELEASE

Proteolytic Cleavage and Virus Maturation

Virus-encoded proteolytic enzymes play important roles in the process of assembly for many viruses and in a postassembly maturation step that is required for the development of an infectious particle for others. These cleavage events can act as molecular switches that introduce flexibility, as well as irreversibility, into the assembly process.

Proteolytic cleavage in the case of the alphaviruses, for example, is used to allow protein domains, translated from a single mRNA, to enter different transport pathways in the cell. The C protein, which is encoded at the 5′ end of the subgenomic 26S mRNA, folds co-translationally into an active serine proteinase that cleaves itself from the growing polypeptide chain. This releases the C protein into the cytoplasm for capsid assembly while freeing up the N-terminus of the signal peptide for the p62 glycoprotein precursor, which can then bind SRP and direct the ribosome to the ER.⁶²

In the herpesviruses, proteolytic cleavage of the scaffolding protein occurs after assembly of the procapsid is complete and is a prerequisite for DNA packaging. The scaffolding proteins of HSV-1 are encoded by a pair of overlapping genes—UL26 and UL26.5—in which the open reading frame of UL26 is an in-frame N-terminal extension of that of UL26.5. The product of the smaller gene is the abundant scaffolding protein pre-VP22a, whereas the larger gene encodes a protease precursor, which cleaves itself internally to give the proteins VP24 (protease) and VP21. Temperature-sensitive mutants in UL26 that have an inactive proteinase at the nonpermissive temperature are blocked at the procapsid stage of assembly.¹⁶⁹

A somewhat more complex proteolytic pathway is utilized by the picornaviruses to regulate the release of both structural and replicative protein components from the single polyprotein precursor that is translated from its positive-stranded RNA genome (see Fig. 6.11). The initial event in this proteolytic cascade is the primary cleavage in which the P1 structural protein precursor is separated from the P2-P3 precursor. This reaction is catalyzed by the 2A proteinase, which hydrolyzes a Tyr-Gly bond at its own amino-terminus. Whereas cleavage of the P2-P3 region is carried out by the 3C proteinase at Gln-Gly bonds, the efficient cleavage of the structural protein precursor at the VP0-VP3 and VP3-VP1 boundaries is catalyzed specifically by the 3CD precursor that includes the viral polymerase. Cleavage of the P1 precursor appears to be a prerequisite for entry of the capsid proteins into the assembly pathway. The final processing step is cleavage of VP0 to VP4 and VP2. This autocatalytic, maturational cleavage occurs late in the assembly pathway and appears to be linked to encapsidation of the viral RNA genome. It is likely that the structural alterations that accompany the cleavage event both stabilize the capsid and irreversibly commit the virus to the conformational changes

induced on receptor binding that are necessary for productive infection of cells.⁷⁵

A similar maturational cleavage is required following assembly and envelopment of retroviral capsids. As we described earlier, the retroviral capsid is assembled from polyprotein precursors that are encoded by the *gag* gene. The viral aspartyl proteinase is encoded by the *pro* region of the genome and is translated from unspliced, genome-length RNA by a ribosomal frameshift mechanism as a Gag-Pro or Gag-Pro-Pol precursor protein (Chapters 47 and 49). The presence of the Gag sequences ensures that this key enzyme is targeted to and incorporated into the assembling capsid. The mechanism by which the protease is activated late in the assembly process (most probably after envelopment is complete) is not known. Because the active enzyme is a homodimer of two protease subunits, it has been postulated that dimerization of proteinase precursors in the nascent capsid might allow a functional enzyme to form. However, in the betaretroviruses, the immature capsid contains a full complement of proteinase precursors, which are not activated until late in envelopment at the plasma membrane. Immature capsids of the betaretrovirus M-PMV are stable when isolated from infected cells; however, the protease can be activated to cleave the assembled precursors to their mature products by treatment with the reducing agent dithiothreitol, suggesting that modification of cysteine residues might act to regulate the initiation of proteolysis.¹⁵⁷

Cleavage of the Gag precursors in the immature capsid is accompanied by a major morphological rearrangement in which the mature NC protein condenses the genomic RNA inside a CA shell (see Fig. 6.7)—the electron dense core structure of the virus seen in electron micrographs.⁶⁵ Cleavage at the N-terminus of CA induces a conformational change in the protein that allows the newly freed N-terminal proline to form a charge pair with a nearby aspartic acid, redirecting assembly from a *spherical* immature capsid to assembly of the cylindrical or cone-like core shell structure.^{139,223} Maturational cleavage, as in the case of poliovirus, results in an irreversible commitment to the entry pathway. It prevents a simple reversal of the assembly process and presumably, through the formation of the core, allows the rearrangement of RNA and the reverse transcriptase into a conformation that is optimal for reverse transcription of the genome. In the absence of cleavage, reverse transcription cannot occur and the virions are noninfectious.²⁰⁷

Adenoviruses, like retroviruses, undergo a maturational cleavage after assembly that is essential for infectivity. The immature virions formed after DNA packaging contain five precursor proteins that must be processed before the mature infectious virion is produced. These include components of the core and the preterminal protein (pTP) used to initiate DNA synthesis. The 23kD adenovirus proteinase, which is encoded by the L3 region of the genome, is inactive in its purified form. It requires an 11 amino acid peptide present at the carboxy-terminus of pVI and viral DNA as co-factors for its activity. This unique use of DNA as a co-factor ensures that proteolytic maturation of the virus cannot take place until packaging of DNA is complete.^{39,112}

Budding: Role of Viral and Cellular Proteins in Membrane Extrusion

It is clear from the discussions in previous sections that various strategies are utilized by different enveloped viruses to mediate

the process of membrane extrusion that we have termed *budding*. Envelopment by a lipid bilayer fulfills two functions for a virus: it provides a protective outer layer into which can be embedded the necessary machinery for target cell attachment and entry, and, in most cases, it releases the virus into an environment that is topologically equivalent to the exterior of the cell. The strategies used to drive the budding process can be classified into three general mechanisms.⁶¹ In the first, membrane extrusion is driven by envelope glycoproteins alone. This is exemplified by the E protein of the coronaviruses that is capable of inducing the release of VLPs in the absence of an RNP. The second strategy is that used by the retroviruses where the capsid precursors, in the absence of the viral glycoproteins, are able to efficiently induce membrane extrusion and particle release. In this instance, it is hypothesized that the tight interaction between the MA domain of Gag and the lipid bilayer, coupled with the force of Gag-Gag interactions in assembly, drives the budding process. The third mechanism is that of the alphaviruses, which involves interactions between the viral glycoprotein spikes and the assembled capsid. In this case, neither the glycoproteins nor the capsid alone can mediate budding. It appears that lateral interactions between the glycoproteins, coupled with cytoplasmic domain-capsid connections, progressively bend the membrane to form the spherical particle.⁶²

For all of these mechanisms, the final step of budding—the process of pinching off—requires a membrane fusion event. For several viruses, it is now clear that this is a separable event that requires the involvement of both viral and host components. Experiments in HIV have shown that deletion of the p6 domain at the C-terminus of Gag results in a block to virus release at the pinching off stage,⁶⁸ and it is now understood that diverse retroviruses encode functionally equivalent Gag *late function* or L domains that are required for efficient release of virions.^{48,130} Viral L domains fall into three classes based on their characteristic tetrapeptide sequence motifs—Pro-Thr/Ser-Ala-Pro (PTAP or PSAP), Pro-Pro-X-Tyr (PPXY), and Tyr-Pro-(X)_n-Leu [YP(X)_nL]. In many instances, these viral L domains are functionally interchangeable and can mediate pinching off of the virion even when positioned at different locations in Gag.^{155,236} Evidence has accumulated over the past several years that these motifs interact with components of a complex cellular machinery that is used in the biogenesis of an endosomal compartment known as the multivesicular body (MVB). Unlike other mechanisms used by the cell to form and pinch off vesicles into the cytoplasm, this machinery facilitates the pinching off into the MVB of exosomes that have the same topology as virus. This process is mediated by a complex and highly conserved protein network—the endosomal sorting complex required for transport (ESCRT) machinery—which exists in all eukaryotes and consists of five hetero-oligomeric complexes (ESCRT-0, ESCRT-1, ESCRT-II, ESCRT-III, and VPS-4).^{11,114,227} Components of this complex also appear to be involved in the last stage of cytokinesis, termed *abscission*, where, as with virus release, a membranous stalk must undergo membrane fission to release the two daughter cells.^{23,128} Recent studies have shown that the ESCRT-0 complex functions to concentrate ubiquitinated cargo on endosomal membranes; the ESCRT-I and -II complexes together induce bud formation while remaining outside the vesicle; and the ESCRT-III complex

mediates membrane scission from the cytosolic side of the bud.²³⁰ Mammalian ESCRT-III is formed by charged multivesicular body proteins (CHMPs), a family of structurally related but highly divergent α -helical proteins that has 12 known members in humans.^{114,227}

The initial indication that retroviral budding and this machinery were linked came from the observation that the ESCRT-I component Tsg101 interacted with p6 and that this interaction depended on the PTAP motif.⁶³ A Tsg101 interacting protein, AIP1, which can also interact directly with ESCRT-III, has been shown to interact with the YP(X)_nL motif in both equine infectious anemia virus and HIV.^{115,201} It is less clear how Gag proteins, which contain the PPXY motif, enter the pathway. These proteins interact with Nedd4 family members that are ubiquitin E3 ligases involved in the covalent attachment of ubiquitin to membrane-associated proteins.^{184,233} Monoubiquitination has been shown to direct the modified protein to the MVB and likely functions to link PPXY L-domain proteins to the ESCRT machinery.¹³⁰

In addition to those L-domains that play a major role in virus release, it is becoming clear that in several viruses, multiple auxiliary L-domains are present that can augment the process. In HIV-1, for example, there is evidence that the PTAP motif acts synergistically with ALIX-binding sequences at the C-terminus of p6 to optimize release.¹¹⁴ Through these disparate and still incompletely understood mechanisms^{114,227} viruses recruit components of ESCRT-III to the site of budding to mediate the process of membrane scission. Only a subset of the 12 proteins known to make up the ESCRT-III complex may be involved, as a recent study demonstrated that the co-depletion of CHMP2 or CHMP4 family members profoundly impairs HIV-1 release, whereas other CHMPs are not required.¹²⁹

The ATPase vacuolar protein sorting 4 (VPS4) acts at a late stage of ESCRT function, providing energy for ESCRT disassembly. Recent live cell imaging studies reveal that ESCRT-III and VPS4 are rapidly and transiently recruited before HIV-1 particle release, supporting the notion that VPS4 has a direct role in HIV-1 budding.^{9,93} Overexpression of a dominant negative mutant of this protein inhibits the budding of several retroviruses that encode each of the tetrapeptide motifs, arguing that divergent retroviruses all depend on the ESCRT sorting complex.^{115,224}

What is now clear is that retroviruses are not the only virus family that interact with this cellular pathway. Late domain motifs that facilitate release of virions have been identified in the structural proteins of the filoviruses,⁸⁷ rhabdoviruses,³⁵ arenaviruses,^{160,203} and paramyxoviruses,¹⁹² suggesting that divergent virus families have adopted a common mechanism for this late stage in enveloped virus budding.

Influenza virus is an exception to this widespread dependence on the ESCRT machinery.²¹ Rossman et al.¹⁷⁷ showed that for this virus, it is the viral M2 protein, rather than a host component, that mediates virus membrane scission. During morphogenesis, the M2 protein localizes to the neck of budding virions, and a highly conserved amphipathic helix in the cytoplasmic tail appears to mediate a cholesterol-dependent alteration in membrane curvature. Mutation of the amphipathic helix results in failure of the virus to undergo membrane scission and virion release.^{177,178} Thus, it is possible that viral proteins such as M2 are able to mimic the functions of the

ESCRT III machinery by constricting the viral membrane and inducing the scission events required for virus release.

Mechanisms to Facilitate the Release of Nascent Particles

If viruses are to efficiently travel to an uninfected target cell, they face one last hurdle following envelopment and release. This is the need to avoid rebinding to receptors on the producing cell. For viruses such as the ortho- and paramyxoviruses, which utilize sialic acid as a receptor on the cell surface, this problem is circumvented by the incorporation of a receptor-destroying enzyme, a sialidase, which can remove these residues during transit through the secretory pathway. For influenza virus, the NA is critical for virus release because, in its absence, nascent virus binds to both the cell surface and other virions in large aggregates.¹⁰⁴

Retroviruses have similarly evolved mechanisms to facilitate release and thereby reduce superinfection of a cell. Cells infected with one retrovirus are generally resistant to subsequent infection by a second virus of the same receptor class. The presumed mechanism for this viral interference is the synthesis of viral glycoproteins in large excess over the levels that are incorporated into virions with the effect that cellular receptors that are transported to the cell surface are already occupied by their ligand. This results in removal or down-regulation of the receptor from the cell surface. For HIV-1, down-regulation of the primary receptor CD4 seems to be crucial, because the virus has evolved two additional mechanisms to ensure this. Two of the accessory proteins encoded by HIV—Vpu and Nef—act by binding CD4 in the ER and at the cell surface, respectively, and targeting the receptor for degradation (Chapter 49).

Independent of this need to down-regulate viral receptors, recent work has demonstrated that virus escape from the cell surface, the last step of the replication cycle, is effectively targeted by a host cell restriction factor, tetherin (also known as BST2 or CD317). This protein was initially identified as the factor responsible for the block to virion release from certain cell types infected by HIV-1 mutants lacking the accessory gene *vpu*.^{11,114,138,218} Tetherin can be induced by type I interferon and other proinflammatory stimuli and has an unusual topology in which the extracellular domain forms an extended parallel coiled-coil that is anchored at the N-terminus by a protein transmembrane domain and at the C-terminus by a glycosyl phosphatidylinositol GPI anchor. Biochemical and structural data favor a model where parallel tetherin dimers crosslink virions to the plasma membrane or the membrane of other virions.^{11,114} Tetherin can restrict the release of all retroviral particles tested to date, including those of the spumavirus and betaretrovirus families, which preassemble capsids in the cytoplasm. In addition, mammalian tetherins block release of a variety of membrane viruses, including filoviruses, arenaviruses, rhabdoviruses, and at least one herpesvirus.^{53,91} In turn, viruses have evolved countermeasures that have allowed them to overcome this inhibition of release. The Vpu protein of HIV-1 is the prototypical antagonist to tetherin and interacts through the transmembrane region to mediate down-regulation and lysosomal degradation of the host restriction factor. In the case of SIVs, this function is primarily carried out by the Nef protein, although in some instances, as in HIV-2 or Ebola virus, it can be mediated by the viral glycoprotein.⁵³ Given the

diversity of mechanisms utilized to counteract tetherin, it is clear that this last step of the life cycle is critical to the virus. Indeed, the evolution of primate lentiviruses, particularly pandemic HIV-1, has been shaped by species-specific differences in this host cell restriction factor.¹⁸⁸

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Viruses, Cell Transformation, and Cancer

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Why Do Viruses Transform Cells?

GENERAL PRINCIPLES OF VIRAL TRANSFORMATION

In 1908, Ellerman and Bang¹⁴³ reported that cell-free filtrates from chickens with leukemia could transmit the disease to healthy birds. The ability of the causative agent to pass through a fine filter identified it as a virus, providing the first evidence that viruses could cause cancer as well as acute contagious diseases. Three years later, Peyton Rous³⁸² showed that a solid tumor in chickens, sarcoma, could also be transmitted by cell-free filtrates. In the 100 years since these discoveries, tumor viruses have been studied intensely in the belief that thorough understanding of these relatively simple agents

would provide mechanistic insight into carcinogenesis, identify the causes of some human malignancies, and suggest novel strategies to prevent and treat cancer. All of these expectations have been met. Tumor viruses dysregulate or exploit crucial regulatory nodes in cells in order to replicate and persist in their host. Because these nodes regulate cell proliferation and survival, these viruses can cause cancer. Furthermore, because of their intimate association with this central cellular machinery, tumor viruses have proven to be prime tools for unraveling the complexities of all human cancers, not just those induced by viruses themselves. For example, studies of tumor viruses led to the discovery of cellular oncogenes and tumor suppressor proteins and to the elucidation of many important aspects of signal transduction, cell cycle control, cellular biochemistry, and carcinogenesis. Indeed, the concept that cancer is a genetic disease emerged largely from studies of tumor viruses. We have also learned that tumor viruses are associated with specific types of human cancer and that tumor virus infection is responsible for approximately 15% of human cancer deaths.³¹⁰ Vaccines have been developed and deployed that prevent infection by certain tumor viruses and inhibit the formation of precancerous lesions or cancers.⁴⁰³ The remarkable success of these endeavors is due to the efforts of generations of virologists, biochemists, molecular biologists, epidemiologists, and clinicians, and to the favorable properties of the viruses themselves.

Cell Transformation

Early studies of tumor viruses focused on isolation of viruses from naturally occurring tumors in animals and description of the effects of these viruses in experimental animals. These studies revealed that a wide variety of viruses can induce tumors (Table 7.1). Diverse taxonomic groups of viruses with DNA genomes can cause tumors: polyomaviruses, papillomaviruses, adenoviruses, herpesviruses, hepadnaviruses, and poxviruses. Among the RNA viruses, a subset of retroviruses cause tumors in animals, and Hepatitis C virus has been implicated in human cancer. Thus, the ability to induce tumor formation is not associated with a particular class of virus or mode of virus replication. The types of tumors induced by viruses are also diverse and include sarcomas (tumors of mesenchymal cells), leukemias and lymphomas (tumors of hematopoietic cells), and carcinomas (tumors of epithelial cells).

Although such experimental pathogenesis studies revealed many fascinating and complex features of viral tumorigenesis, experiments in animals are cumbersome, slow, expensive, and often poorly suited for biochemical and mechanistic analysis. Therefore, cultured cell models of tumorigenesis are widely used. Cells explanted from tumors display many properties that

TABLE 7.1 **Oncogenic Viruses**

Taxonomic grouping	Examples	Primary tumor types
RNA viruses		
<i>Flaviviridae</i>	Hepatitis C virus (HCV)	Hepatocellular carcinoma
<i>Retroviridae</i>		
Alpharetroviruses	Avian leukosis and sarcoma viruses Rous sarcoma virus (RSV) Rous-associated viruses (RAV)	Sarcoma B-cell lymphoma, erythroleukemia
	Avian acute leukemia viruses Avian myeloblastosis virus (AMV) Avian erythroblastosis virus (AEV) Myelocytoma virus MC29	Myeloid and/or erythroid leukemia Erythroid leukemia Myeloid leukemia
Betaretroviruses	Mouse mammary tumor virus (MMTV) Jaagsiekte sheep retrovirus	Mammary carcinoma Lung carcinoma
Gammaretroviruses	Murine leukemia viruses (MuLVs) Moloney MuLV Murine sarcoma viruses (MuSVs) Harvey MuSV Feline leukemia viruses Feline sarcoma viruses Simian sarcoma virus Gibbon ape leukemia virus Koala retrovirus	Leukemias and lymphomas T lymphoma, also B-lymphoma and myeloid leukemia Sarcoma Leukemias and lymphosarcomas Sarcomas Sarcomas Leukemia T-cell leukemia
Deltaretroviruses	Human T-lymphotropic virus (HTLV) Bovine leukemia virus	Adult T-cell leukemia B-cell leukemia
Epsilonretroviruses	Walleye dermal sarcoma virus	Sarcoma
DNA viruses		
<i>Adenoviridae</i>	All types	Various solid tumors
<i>Hepadnaviridae</i>	Hepatitis B virus (HBV)	Hepatocellular carcinoma
<i>Herpesviridae</i>	Epstein-Barr virus (EBV)	Burkitt lymphoma (African) Nasopharyngeal carcinoma
	Kaposi sarcoma herpesvirus (KSHV)	Kaposi sarcoma
<i>Polyomaviridae</i>	SV40, polyomavirus	Various solid tumors (parotid gland tumors, mammary carcinoma)
<i>Papillomaviridae</i>	Human papillomavirus (HPV), bovine papillomavirus	Papillomas, carcinomas
<i>Poxviridae</i>	Shope fibromavirus	Myxomas, fibromas

distinguish them from normal cells. Collectively, these properties are referred to as the transformed phenotype (Table 7.2), and cells displaying these properties are called transformed cells. Infection of normal cells in culture with many (but not all) tumor viruses can cause the rapid acquisition of the transformed phenotype, a process called transformation. The most commonly used cell types for transformation studies are rodent fibroblasts, which in general are easier to transform *in vitro* than human cells.

The most striking property of transformed cells is their ability to form tumors when inoculated into animals. Experiments in animals have classically been used to determine whether a virus can cause a tumor in a living organism and to identify the viral gene(s) responsible for this activity. Although mice are used for the vast majority of such experiments, other commonly used hosts are rats, hamsters, rabbits, and monkeys. Immunodeficient animals (e.g., athymic nude mice) are frequently used in order to avoid immune rejection of the inoculated cells.

In addition to tumorigenicity, transformed cells display a variety of features in culture that distinguish them from normal cells. These include changes in growth, morphology, metabolism, and intracellular and cell surface biochemistry. Normal cells have very stringent requirements for growth. To proliferate in culture, normal cells require peptide growth factors, commonly supplied as fetal bovine serum. When plated on plastic, normal adherent cells typically divide to fill the available space on the surface of the culture vessel until they become a confluent monolayer of cells and then cease proliferation, even if supplied with adequate amounts of nutrients and growth factors. The cessation of growth at confluence is known as density-dependent growth inhibition or contact inhibition. Most normal cells also require attachment to a solid substrate and are unable to grow when suspended in semisolid medium such as agarose or methylcellulose, a property known as anchorage dependence. Finally, normal cells usually display a limited life span in culture after isolation from an animal and can be serially passaged for no more than a few months before they

TABLE 7.2 Properties of Transformed Cells

	Normal cells	Transformed cells	Behavior of transformed cells in the laboratory
Growth	Nontumorigenic	Tumorigenic	Form tumors upon inoculation into susceptible animal hosts
	Finite life span	Immortal	Indefinite life span in culture
	Contact inhibition	Loss of contact inhibition	Increased saturation density
	Anchorage dependent	Anchorage independent	Focus formation
	Growth factor dependent	Growth factor independent	Colony formation in semisolid medium
Biochemical	Normal nutrient transport	Increased nutrient transport	Growth in reduced concentration of growth factors or serum
	Oxidative respiration	Aerobic glycolysis (Warburg effect)	DNA synthesis despite nutrient deprivation
Acidification of culture medium			
Other changes in transformed cells include:			
<ul style="list-style-type: none"> • Decreased levels of fibronectins, decreased adhesion to solid substrates, and loss of stress fibers • Increased agglutination by lectins and synthesis of cell surface proteases • Redistribution of microfilaments and altered morphology 			

cease proliferation and adopt a permanently growth-arrested state called senescence.

Transformed cells display strikingly different growth properties than normal cells (Table 7.2). Transformed cells have minimal requirements for growth factors and can proliferate in low concentrations of serum (or even in its absence). Transformed cells continue to proliferate after reaching confluence and display a multilayered, piled-up appearance and higher saturation densities than normal cells (Fig. 7.1). If a transformed cell is present in an excess of normal cells in a culture, it will override contact inhibition at confluence and continue to proliferate locally, forming a discrete, clonal group of transformed cells known as a focus (Fig. 7.1).¹⁹⁸ Because each focus originates from a single transformed cell, the number of foci formed provides a convenient measure of the number of transforming viruses in an inoculum. In addition, because foci are readily apparent on a background monolayer of cells, focus formation provides a simple means of identifying and isolating transformants. Transformed cells also display anchorage independence and can form colonies in semisolid medium without adhering to a solid surface.²⁹⁴ Anchorage independence is the property of transformed cells that is most closely associated with the ability to form tumors in animals. Finally, in contrast to normal cultured cells, transformed cells that divide indefinitely in culture are said to be immortal.

Transformed cells can also display striking morphologic differences from normal cells due to changes in the cellular cytoskeleton, extracellular matrix, and cell surface.^{226,371} Transformed cells also switch from oxidative respiration to aerobic glycolysis, a phenomenon known as the Warburg effect. The resulting lactic acid production and acidification of the culture medium (and the tell-tale yellow of medium containing phenol red, a pH indicator) allows the rapid identification of transformed cultures.

It should be noted that transformation is not an all-or-nothing phenomenon. Some transformed cells display only a subset of these properties. For example, in some cases cells can form foci but fail to display anchorage independence or tumorigenicity, or they can display reduced serum requirements

but not form foci. It is therefore important to specify the assay used to assess transformation. “Normal” established cell lines, such as murine NIH3T3 cells, are immortal but do not display other features of transformed cells and thus can be regarded as being minimally transformed.

Identification of Viral Oncogenes

To determine whether a virus has tumorigenic or transforming activity, host animals or cells are infected and the appearance of tumors or the acquisition of a transformed phenotype in culture is measured. Acute transforming retroviruses cause the rapid formation of tumors in animals and rapidly transform cells in culture because they carry genetic information known as oncogenes. In contrast, retroviruses that lack oncogenes cause tumors in animals many months after infection and do not transform cells in culture. Most DNA tumor viruses contain oncogenes and can transform cells in culture and induce tumors in animals.

For viruses that contain oncogenes, cell transformation typically occurs within a few days or weeks of infection, although some measures of transformation, such as cell immortalization, take months to assess. When infected at high multiplicity of infection (moi), the entire culture of cells may undergo transformation, which can allow biochemical characterization of cell transformation. Infections at low moi require the use of quantal measures of transformation, such as focus formation or colony formation in agarose, to score transformation because most cells in the culture remain normal.

Although acute properties of transformation are fairly easy to elicit, for some viruses stable transformation that persists in culture is a very rare event. In addition, for lytic DNA viruses, where replication is cytocidal, transformation can only occur when the virus life cycle cannot go to completion. Transformation by these viruses is commonly studied following infection of nonpermissive cells that cannot support a full virus life cycle. In these cells, the initial steps (the early phase) of virus infection take place—virus attachment to cells, penetration into the cells, virion uncoating, and expression of the early proteins—but then

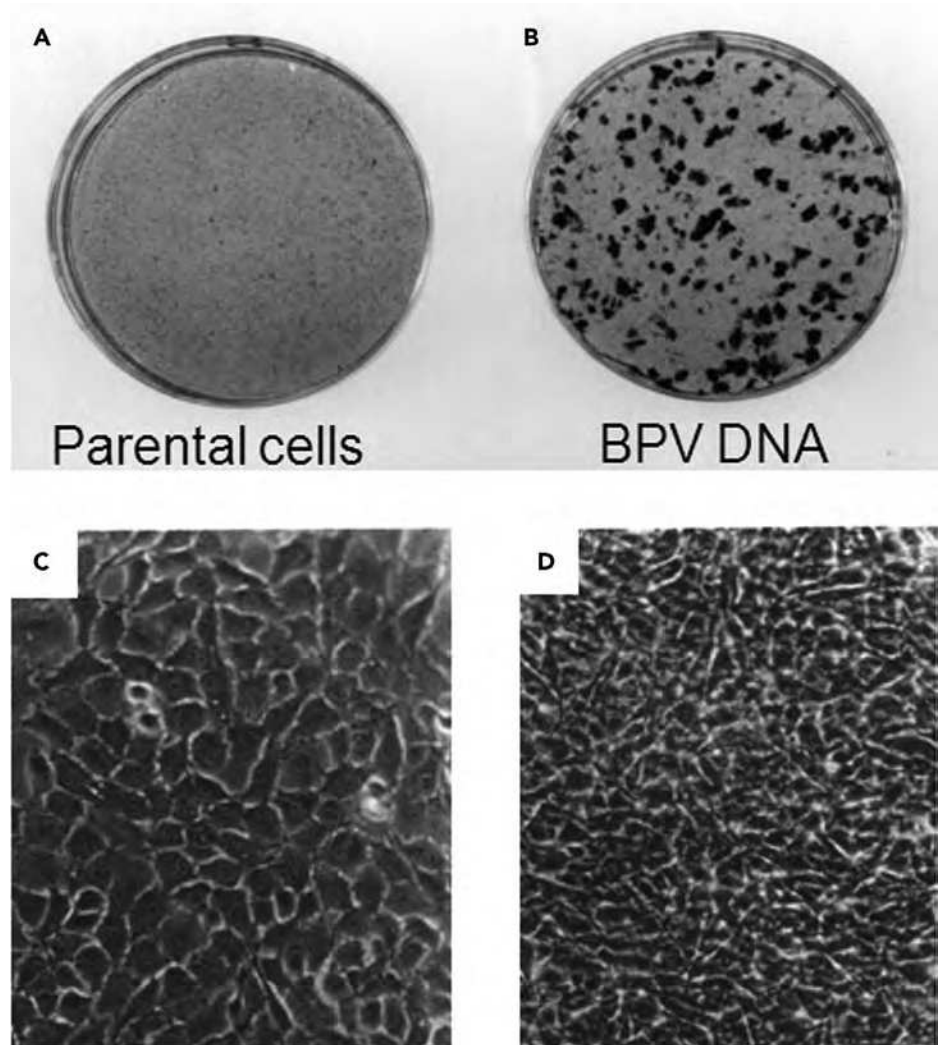


FIGURE 7.1. Appearance of transformed cells. **A:** Plate showing a monolayer of normal murine C127 fibroblasts. **B:** Plate of fibroblasts showing transformed foci induced by transfection with bovine papillomavirus DNA. **A** and **B** were stained 12 days after transfection. **C:** Micrograph of a monolayer of normal murine fibroblasts, showing flat, spread-out morphology. **D:** Culture of murine fibroblasts transformed by the bovine papillomavirus E5 protein. Note the piled-up, refractile appearance of small, transformed cells.

productive infection is aborted before the onset of vegetative viral DNA replication. For example, rat and mouse cells are nonpermissive for replication by simian virus 40 (SV40), a monkey virus, so many transformation assays are conducted in these cells. Alternatively, permissive cells, in which the wild-type virus can complete its life cycle, can be transformed if viral replication is blocked. Infection with replication-defective viral mutants or transfection with nonreplicating, subgenomic viral DNA fragments that contain viral oncogenes can transform permissive cells.²⁹⁹ In addition, some tumor viruses (such as herpesviruses) can establish latency in permissive cells, and in some cases expression of a subset of viral genes in the latently infected cells can lead to transformation. Infection by acute transforming retroviruses usually does not result in cell death, and stable transformation is very efficient, with virtually every infected cell becoming transformed.

An oncogene can display potent activity in one transformation assay but be totally devoid of activity in another assay, even in the same host cell, because it may modulate multiple independent cellular signaling pathways. Host-specific differences can also greatly influence transforming activity, so a variety of host animals or cells are commonly tested to assess transforming activity. Some oncogenes have potent transforming

activity in numerous assays but are unable to cause cell immortalization. Therefore, transformation is often studied in established cell lines that are already immortal.

Viral mutants can be used to identify specific viral genes with transforming activity. In early experiments, mutagenized viral stocks were tested for loss of transforming activity or for the acquisition of temperature-sensitive transforming activity. The generation and analysis of conditional mutants were particularly useful for identifying and studying transforming genes, especially those that are essential for virus replication.^{380,467} In these situations, the virus is first propagated under permissive conditions, and then the virus is used to infect cells under nonpermissive conditions and transformation is assessed. Genetic mapping approaches can then be used to identify the mutant viral gene responsible for the transformation defect. More recently, specific mutations were constructed in individual viral open reading frames, and the resulting virus mutants were assayed for transforming activity. Alternatively, individual viral genes or subgenomic segments can be introduced into cells or transgenic animals and assessed for transforming activity or tumorigenicity.^{192,420} This approach is complicated when more than one viral oncogene is required for transformation, because no single gene displays

activity. In these situations, combinations of viral genes can be transferred into cells, or the ability of viral genes to cooperate with cellular oncogenes can be tested.

The analysis of murine polyomavirus transforming functions illustrates some of the power and pitfalls of these approaches. Murine polyomavirus (hereafter called polyomavirus) is related to SV40. SV40 and polyomavirus express the early protein, large T antigen (LT), which is required for viral DNA replication. Certain mutations in the SV40 *LT* gene caused temperature-sensitive defects in cell transformation as well as viral DNA replication, identifying the *LT* gene as the major oncogene of SV40.² However, the equivalent polyomavirus LT mutants were temperature sensitive for virus replication but, at least under some conditions, able to transform cells at the nonpermissive temperature. In addition, the polyomavirus *LT* gene was often disrupted in transformed cells. These findings suggested that, in contrast to SV40, LT was not the major transforming protein of polyomavirus. Indeed, Benjamin²⁶ isolated transformation-defective polyomavirus mutants that could replicate in mouse cells that had been transformed by wild-type polyomavirus, but not in normal mouse cells, so-called *host-range-restricted* and *transformation-defective* (hr-T) mutants. Genetic mapping revealed that the hr-T mutant phenotype resulted not from mutations in the *LT* gene but rather from mutations in an alternative open reading frame in the early region of polyomavirus, which encoded a distinct protein, middle T antigen (mT) (e-Fig. 7.1). SV40 does not encode mT. These findings indicated that SV40 and polyomavirus, though genetically similar, used different mechanisms to transform cells.

To determine the activities of the individual early proteins of polyomavirus, a panel of complementary DNA (cDNA) clones that each expressed a single viral protein was assayed in rat1 cells, an established cell line. The clone expressing polyomavirus mT antigen alone was the only one that displayed transforming activity: cells expressing mT antigen formed foci, appeared morphologically transformed, and formed tumors in animals.⁴⁶⁸ These results implied that mT is the major transforming protein of polyomavirus. However, cells transformed by mT antigen were unable to grow in low serum and were thus only partially transformed. On the other hand, cells expressing polyomavirus LT alone were able to grow in low serum, even though they did not display other properties of transformed cells.³⁷³ By using a different assay to study polyomavirus transformation, namely, the ability of the viral genes to convert primary rat embryo fibroblasts into immortalized cells, Rassoulzadegan et al³⁷⁴ found that LT was active while mT was not. Furthermore, sequential transfer of the *LT* and *mT* genes could convert primary cells into immortal, fully transformed cells. Thus, the individual oncogenes of polyomavirus displayed different transforming activities. Together, these important experiments identified the polyomavirus oncogenes, revealed that they had different effects on cell growth, demonstrated that oncogenes can cooperate to generate fully transformed cells, and highlighted the critical importance of the particular assays used to assess transformation.

Another approach to identify viral oncogenes rests on the finding that genes responsible for transformation are frequently retained in transformed cells, whereas the other viral genes can be lost. Thus, DNA virus genes that are uniformly expressed in transformed cells are likely to be viral oncogenes.

Experiments searching for viral DNA and RNA in transformed cells led to the presumptive identification of E1A and E1B as the primary adenovirus oncogenes.^{123,167} As the roster of viral and cellular oncogenes has expanded, it is now often possible to identify viral oncogenes by comparing the sequences of viral genes to sequence databases, because novel viral oncogenes are often homologous to validated oncogenes.

The mapping experiments outlined previously demonstrated that DNA tumor viruses typically contain multiple oncogenes. In contrast, acute transforming retroviruses usually contain one oncogene, although there are examples of retroviruses with two (e.g., avian erythroblastosis virus [AEV] ES4 contains both *ErbA* and *ErbB* oncogenes). Nonacute retroviruses lack oncogenes.

Maintenance of Transformation

Is continuous expression of viral oncogenes required to maintain the transformed phenotype, or do tumor virus oncogenes only initiate cell transformation, after which they are not required (a hit-and-run-type mechanism)? If viral genes are required to maintain transformation, then they will be expressed in stably transformed cells. Biochemical and immune-based analysis can detect the presence of viral genes and proteins in cells transformed by many viruses, but often only a subset of viral genes is expressed in these cells. The production of infectious virus by transformed cells would provide definitive evidence for the presence of an intact, functional viral genome in the cells, but one of the striking properties of virally transformed cells is that they often do not produce infectious virus. This is not surprising for viruses, including many of the DNA tumor viruses, whose transforming activity is typically studied in nonpermissive cells unable to support virus replication. In contrast, cells transformed by Rous sarcoma virus or some other retroviruses produce infectious virus.

The presence of wild-type virus genomes in transformed cells was demonstrated by virus rescue experiments. In a typical experiment, SV40-transformed mouse cells (which are nonpermissive for replication of SV40) were fused to nontransformed, permissive monkey cells (e-Fig. 7.2). The fused cells produced infectious wild-type SV40, which could be detected by plaque formation assays in permissive cells.²⁶⁶ This demonstrated that transformed cells could contain full-length, infectious virus genomes in a latent state, which could be activated by replication factors present in permissive cells. We now know that the inability of mouse cells to support SV40 replication is due to the inability of murine DNA polymerase- α /primase to interact with SV40 LT.⁴⁰⁸

The study of conditional viral mutants and expression constructs proved that viral genes expressed in transformed cells are required to maintain the transformed state. In these experiments, cells transformed under the permissive condition when the viral oncoprotein was active often reverted to normal under nonpermissive conditions that inactivated the oncoprotein. This was initially shown with temperature-sensitive mutants of viral oncogenes, where transformation was extinguished at the higher, nonpermissive temperature.^{27,302,416} More recently, individual oncogenes driven by inducible/repressible promoters or maneuvers such as RNA interference were used to show that viral oncogene expression was required to maintain the transformed phenotype. Ongoing oncogene expression is not required in cells transformed by all viruses, but it is commonly

observed for cells transformed by a variety of DNA viruses and acute transforming retroviruses.

Continuous oncogene expression is also required for cells derived from some virally induced human tumors. For example, repression of human papillomavirus (HPV) oncogene expression in cervical cancer cells causes the rapid cessation of proliferation or the induction of apoptosis (e.g., reference [225]). Thus, even though mutations presumably accumulated in these cells during the extended period of human carcinogenesis, these mutations are not sufficient to maintain the transformed phenotype in the absence of viral oncogene expression. This finding raises the hope that it will be possible to treat virally induced cancers by interfering with the expression or action of viral oncogenes.

To continuously provide viral oncogene products in stably transformed cells, the viral genome must persist. Most commonly, the viral DNA is covalently integrated into the cellular DNA and replicates passively with the host genome. For retroviruses, integration of an essentially intact viral genome is an integral step in the viral life cycle, but for most DNA viruses, integration is incidental (and indeed inimical) to normal viral DNA replication. For these viruses, integration does not occur at specific sites in the cellular genome, and often only portions of the viral genome containing the viral oncogenes are present in the cell. Integration of DNA virus genomes is a rare event, which may explain at least in part why stable transformation by these viruses is so inefficient. Papillomavirus and herpesvirus genomes stably replicate as plasmids in transformed cells, but the DNA of these viruses can be integrated in cancer cells.

Origin of Viral Oncogenes

Where do viral oncogenes come from? Initial studies with retroviruses were conducted with Rous sarcoma virus (RSV). Cells transformed by infection with a mutant RSV carrying a temperature-sensitive *src* gene rapidly reverted to a nontransformed phenotype when shifted to the nonpermissive temperature, but the mutant was replication competent at both high and low temperatures.^{74,302} Similarly, transformation-defective RSV deletion mutants were isolated that retained the ability to replicate.²⁷ These important results indicated that a specific gene (*src*) was responsible for RSV transformation but not for virus replication. Stehelin, Varmus, and Bishop⁴⁴¹ later used molecular hybridization to show that *src* sequences were actually derived from chicken cell DNA. We now know that acute transforming retroviruses such as RSV incorporate altered versions of cellular genes into the viral genomes, a process known as transduction. These transduced genes typically encode constitutively active versions of cellular signal transduction components, such as growth factor receptors and other signaling proteins (see next section). The cellular versions of these genes (designated by the prefix *c*, e.g., *c-src*) are known as the proto-oncogenes, whereas the active viral versions (e.g., *v-src*) are called oncogenes.⁸⁶ In contrast, the nonacute retroviruses induce tumors by integrating near-cellular proto-oncogenes and activating their expression.

The discovery that cellular proto-oncogenes can be activated by viruses to generate cancer suggested that nonviral cancers might also arise by activation of proto-oncogenes. Indeed, spontaneously arising human tumors frequently contain altered or overexpressed versions of the proto-oncogenes targeted by oncogenic retroviruses. In several cases, drugs have been developed that specifically inhibit activated proto-oncogene products and provide substantial clinical benefit to

cancer patients (e.g., in chronic myelogenous leukemia and lung cancer). Thus, the practical legacy of tumor virology extends far beyond virally induced tumors.

In contrast to most retroviral oncogenes, the oncogenes of the small DNA tumor viruses are intrinsic viral genes required for virus replication, and mutations that interfere with transforming activity usually also inhibit viral replication. In some cases, phylogenetic analysis shows that these genes have been essential for virus replication for millions of years. DNA virus oncogenes usually encode proteins that bind and modulate cellular proteins that regulate cell growth. Most commonly, nuclear oncoproteins of a number of DNA tumor viruses bind to the p53 and retinoblastoma (Rb) tumor suppressor proteins and neutralize their growth inhibitory activity, but the oncoproteins of DNA tumor viruses can modulate other cellular targets as well. Some DNA virus oncoproteins mimic constitutively active forms of a cellular regulatory protein.

p53 and Rb are not only targets of viral tumorigenesis. Most sporadic human cancers, as well as some inherited cancers, contain inactivating mutations in the *p53* and *Rb* genes and in genes in the pathways they control.²⁸⁴ As was the case with the discovery of cellular proto-oncogenes, studies of DNA tumor viruses identified cellular proteins and pathways crucial for understanding the genetic basis of naturally occurring, nonviral tumors.

Although retroviral oncogenes usually have cellular origins and DNA tumor virus oncogenes typically have viral origins, these distinctions are not absolute. The Jaagsiekte sheep retrovirus envelope protein is required for both virus replication and cell transformation, and the gp55P oncoprotein of the Friend erythroleukemia retrovirus is a mutant retroviral envelope protein that contributes to erythroleukemia in mice.^{285,296} On the other hand, the genome of Kaposi sarcoma herpesvirus (KSHV), a large DNA-containing virus, contains a number of genes essential for viral replication and cell transformation that appear to have been acquired from the cellular genome in relatively recent evolutionary times.^{328,389}

Cellular Targets of Viral Oncogenes

The great majority of viral oncogenes stimulate cell proliferation (most of the others inhibit cell death). In untransformed cells, the cell division cycle is regulated by intrinsic oscillators, which ensure the orderly progression of the cell cycle, and by external growth factors, which indirectly affect the activity of the cell cycle machinery. Like serum growth factors, many viral oncogenes can stimulate DNA synthesis in normal cells made quiescent by serum starvation. Thus, the signal transduction pathways activated by growth factors provide a useful framework to understand the function of viral oncogenes (Fig. 7.2).

Serum contains polypeptide growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), which bind to specific cell surface receptors to initiate mitogenic signaling. These growth factor receptors are often transmembrane proteins with an extracellular ligand binding domain, a membrane-spanning domain, and an intracellular signaling domain. Ligand binding induces dimerization and/or reorientation of the receptors, initiating intracellular signaling cascades.⁴⁰⁷ Many of these receptors are tyrosine kinases or associate with tyrosine kinases. The molecular events induced by ligand binding result in activation of the intrinsic tyrosine kinase activity of the receptors (or of the associated kinases) and in tyrosine phosphorylation of the receptor itself. Phosphorylation on

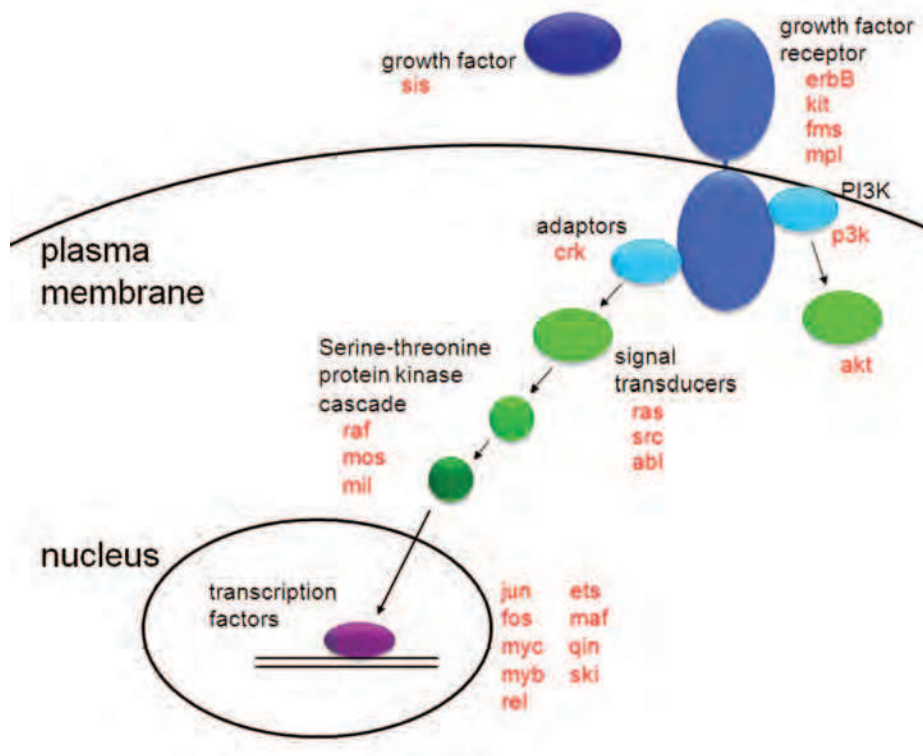


FIGURE 7.2. Mitogenic signal transduction pathways and retroviral oncogenes. This figure shows a schematic representation of mitogenic signal transduction. Binding of a growth factor to a growth factor receptor initiates signal transduction pathways involving adaptor proteins, signal transducing proteins, and serine/threonine kinases. This results in the phosphorylation of transcription factors to alter gene expression and execute pro-proliferative or antiapoptotic signals. Components of this pathway that were transduced by acute transforming retroviruses are listed in red.

tyrosine in turn generates specific binding sites for a variety of signaling proteins that contain SH2 (*src* homology region 2) or other protein–protein interaction domains, which themselves may be tyrosine phosphorylated after binding the activated receptor.³⁸⁶ Among the targets of these signaling proteins are intracellular guanine nucleotide exchange factors (GEFs) that activate signal transducing molecules such as p21^{ras}.³⁹ This then initiates a cytoplasmic signaling cascade involving a series of serine/threonine kinases (the mitogen-activated protein [MAP] kinase cascade) that ultimately results in the phosphorylation and activation of nuclear transcription factors, such as AP1 (composed of jun and fos subunits) and c-myc, allowing them to stimulate the expression of various cellular genes.^{34,36,80} Growth factor treatment also activates phosphatidylinositol 3-kinase (PI3K) signaling, which generates antiapoptotic and pro-growth signals mediated by the downstream kinase, akt.

A major target of transcriptional activation following growth factor treatment is the cyclin D gene.³⁰⁶ Cyclins, including cyclin D, are regulatory subunits of the cyclin-dependent kinases (cdks), which directly regulate the cell cycle by allowing cells to move past specific cell cycle checkpoints (e.g., the G1/S boundary) (Fig. 7.3). Cyclin concentrations vary periodically throughout the cell cycle, and association of cyclins with cdks stimulates the activity of the cdk complexes, which catalyze the phosphorylation of nuclear substrates. For example, association of cyclin D with cdk4 results in phosphorylation of members of the Rb family (Fig. 7.3). Phosphorylation of Rb proteins causes their release from E2F proteins, a family of transcription factors that was first identified because E2F is required for activation of the adenovirus E2 promoter.²⁶⁸ Rb release increases transcription of a set of E2F-dependent cellular genes that encode proteins required for cellular DNA synthesis, including DNA polymerases, topoisomerases, and

enzymes involved in nucleotide synthesis.¹¹⁴ Thus, growth factor treatment triggers a signal transduction cascade that results in the inactivation of the Rb family and stimulation of the G1 to S phase transition and cellular DNA synthesis.

Cdks are also regulated by the p53 pathway.²⁸⁴ The p53 protein is a transcription factor that can stimulate or repress the expression of various genes. Typically present at low levels

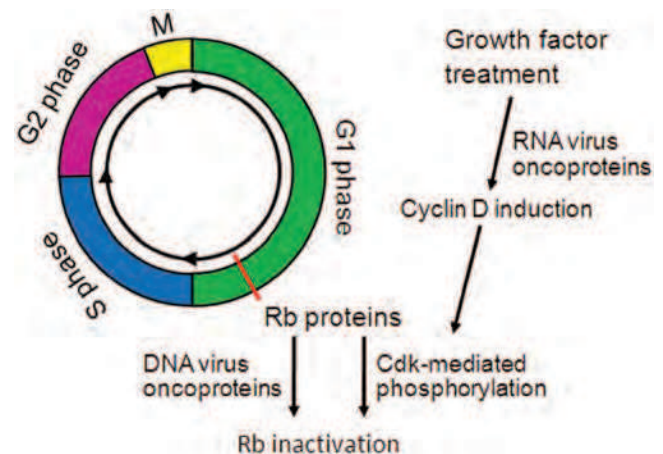


FIGURE 7.3. The cell cycle. The figure shows a simplified version of the cell cycle. The active form of the retinoblastoma family members prevents progression through the G1 phase. Rb-mediated inhibition is abrogated by growth factor treatment, which results in accumulation of cyclin D, stimulation of cyclin-dependent kinases (cdks), and Rb phosphorylation. Rb can also be inhibited by the expression of several DNA virus oncoproteins, such as adenovirus E1A, human papillomavirus (HPV) E7, or simian virus 40 (SV40) large T antigen (see Fig. 7.12).

and inactive, the expression and activity of p53 is induced by a variety of stresses, including DNA damage. An important target of p53-mediated transcriptional activation is p21^{WAF1/CIP1}, an inhibitor of cdk activity and cell cycle progression.¹³⁹ Therefore, a direct consequence of p53 activation is p21^{WAF1/CIP1} induction and the inhibition of cdk activity. This causes the retention of the Rb family in the underphosphorylated form, which inhibits cell cycle progression by preventing E2F-mediated gene activation. Conversely, inhibition of p53 reduces the levels of p21^{WAF1/CIP1}, and the resulting increase in cdk activity and Rb phosphorylation dissociates E2F and Rb, allowing DNA synthesis to occur.

According to this simplified view of cell cycle control, proliferation can be stimulated by activation of mitogenic signaling or by inhibition of tumor suppressor proteins. Not surprisingly, most viral oncogenes function in just this way, either by activating signal transduction cascades or by inhibiting tumor suppressor pathways.^{31,47} Indeed, much of our molecular understanding of mitogenic signal transduction and the role of tumor suppressor proteins in cell proliferation and cancer is derived from genetic and biochemical analysis of viral oncoproteins.

Cells not only respond to mitogenic signals but also possess intrinsic pathways that limit cell proliferation in response to a variety of stresses. DNA damage elicits cell cycle arrest (via p53 activation) to allow cells time to repair the damage. If the damage is extensive, apoptosis will result. Virus infection or oncogene action can also activate checkpoints that block cell proliferation. For example, replicating viral DNA itself can be recognized as foreign and trigger a DNA damage response.⁴⁸⁸ In addition, the aberrant activation of mitogenic signaling pathways by viral oncoproteins can cause unscheduled DNA synthesis, which in turn induces apoptosis or senescence.¹³⁷ Because such premature cell death or inhibition of proliferation will reduce virus yield, these cellular responses can be viewed as antiviral defense mechanisms. In many cases, the p53 and the Rb pathways are required for the execution of these antiviral processes. Neutralization of these tumor suppressor pathways by viral oncoproteins will not only directly stimulate cell proliferation but also interfere with these checkpoints and remove these barriers to transformation and tumorigenesis. Furthermore, when the checkpoint fails, the resulting inaccurate DNA repair or unscheduled DNA synthesis allows the accumulation of mutations in cellular regulatory genes or global genomic instability, which further contribute to the erosion of cell growth control.

TRANSFORMATION BY RETROVIRUSES

Retroviruses were discovered because they caused cancers in animals. The first transmissible tumors associated with retroviruses were avian myeloblastosis in 1908¹⁴³ and avian sarcomas in 1911,³⁸² caused by avian myeloblastosis virus (AMV) and RSV, respectively. Other early retroviruses associated with cancers include murine mammary tumor virus (MMTV, mammary carcinomas)⁶⁰ and murine leukemia viruses (MuLVs, lymphomas and leukemias).¹⁹⁵ Of the seven retrovirus genera, viruses from five (alpha, beta, gamma, delta and epsilon) are associated with cancers. Only lentiviruses and foamy viruses have not been found to directly induce cancer—and for human immunodeficiency virus type 1 (HIV-1), a lentivirus, a major clinical manifestation of acquired immunodeficiency syndrome (AIDS) is development of cancer.

Most oncogenic retroviruses can be divided into two categories, based on the rapidity with which they induce tumors. *Acute transforming* retroviruses induce tumors rapidly (often within weeks in experimental animals) and can often transform cells in culture. As mentioned earlier, acute transforming retroviruses carry additional genetic information beyond the standard genes for retroviral replication (*gag*, *pol*, and *env*; see Chapter 47). These additional sequences, the viral oncogenes, are responsible for rapid and efficient tumorigenesis and cell transformation. *Nonacute* retroviruses induce tumors in animals more slowly, do not carry oncogenes, and usually do not transform cells in culture. Nevertheless, these viruses often induce tumors with high efficiency, typically by causing transcriptional activation of cellular proto-oncogenes.

Acute Transforming Retroviruses and Viral Oncogenes

Acute transforming retroviruses belong to the alpha- and gammaretrovirus genera (Table 7.1). The first acute transforming retroviruses to be studied were avian alpharetroviruses, beginning with RSV and avian acute leukemia viruses such as AMV. Acute transforming gammaretroviruses include murine and rat sarcoma viruses, murine acute leukemia viruses, and feline and simian sarcoma viruses. Study of acute transforming retroviruses and their cognate proto-oncogenes has provided numerous important insights into pathways of growth regulation. Indeed, some of the most important proteins in signal transduction and cancer were first identified through study of retroviral oncogenes, including *Ras*, *Src*, *Abl*, *Myc*, *Fos*, and *Jun*.

RSV and *src*. Infection of chick embryo fibroblasts with RSV results in cell transformation; a focus-formation assay can be used to quantify the amount of transforming virus present.⁴⁶³ After the original isolation of RSV, several different strains of related transforming viruses were developed. Some of these strains (e.g., Schmidt-Ruppin [SR-RSV] and Prague [Pr-RSV]) are replication competent. Others, such as the Bryan high-titer strain, consist of a replication-defective RSV genome, which carries the viral oncogene, as well as a related replication-competent “helper” virus genome (e.g., Rous-associated virus [RAV]). In a mixed infection, this helper virus encodes viral replication proteins *in trans* to allow the production of infectious virus.²⁰⁴

Passage of replication-competent RSV occasionally results in loss of transforming capacity without affecting viral replication—such variants are termed *transformation-defective* RSV (tdRSV).¹³⁰ Thus, oncogenesis and transformation are not required for RSV replication. The size of the tdRSV genome was smaller than wild-type RSV, suggesting that viral genomic sequences responsible for transforming activity were missing from tdRSV. These deleted sequences were mapped to the 3′ end of the RSV genome,⁴⁸⁴ and they were eventually termed *src*,⁸⁶ providing the first example of a retroviral oncogene (Fig. 7.4). *Src* is expressed via a spliced messenger RNA (mRNA) from the splice donor site at the 5′ end of the genome and an acceptor site at the 5′ end of the *src* gene.²⁰²

Antisera from RSV-infected rabbits were used to identify the protein product of the *src* oncogene as a 60-kD phosphoprotein designated pp60^{src}.⁵⁰ When an anti-*src* antibody was incubated with a cell lysate from RSV-transformed cells and

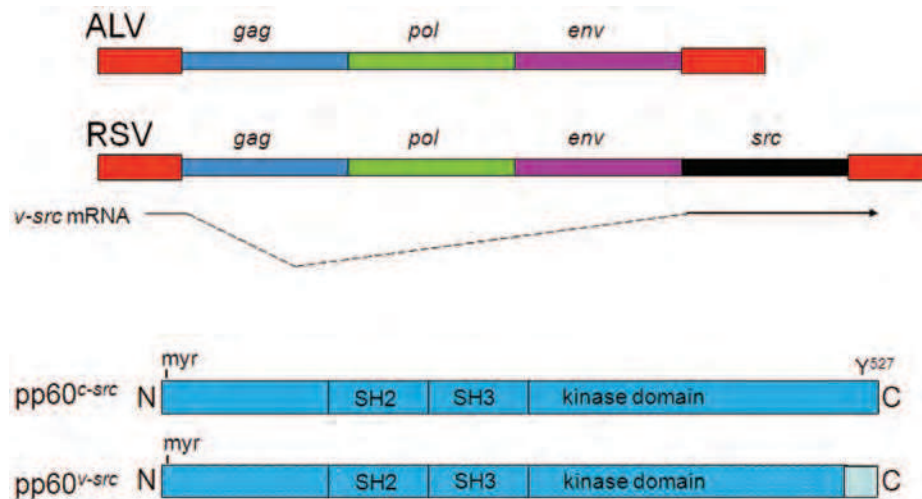


FIGURE 7.4. Structure of Rous sarcoma virus (RSV) and pp60^{src}. The **top part** of the figure shows the proviral structure of avian leukosis virus containing the three retroviral genes required for virus replication, *gag*, *pol*, and *env*. The long terminal repeats (LTRs) are shown in red. The second provirus shows RSV with the *src* gene located at the 3' end of the viral genome. A schematic picture of spliced *v-src* messenger RNA (mRNA) is shown below the RSV provirus, with spliced-out introns depicted as dashed lines. The **bottom part** of the figure shows schematic diagrams of pp60^{v-src} and pp60^{c-src}. Both proteins contain conserved SH2 and SH3 protein–protein interaction domains, tyrosine kinase domains, and an amino-terminal myristal (myr) group. A regulatory tyrosine at position 527 in pp60^{c-src} (Y⁵²⁷) is also shown. pp60^{v-src} contains several amino acid substitutions compared to pp60^{c-src}, represented by the stippling, as well as a frameshift mutation that replaces the 19 carboxyl-residues of pp60^{c-src}, including the Y527, with 12 unrelated amino acids.

γ -³²P-adenosine triphosphate (ATP) as a phosphate donor, ³²P was transferred to the antibody heavy chain, thus identifying pp60^{v-src} as a protein kinase.⁸⁸ Subsequently it was shown that pp60^{v-src} is a tyrosine-specific protein kinase; that is, it phosphorylates tyrosine residues in its substrate proteins.^{89,223} Phosphorylation of proteins on tyrosines is a relatively rare modification (compared to phosphorylation on serine and threonine), and pp60^{v-src} was the first tyrosine kinase discovered. Tyrosine phosphorylation is now known to be a major mechanism to regulate protein activity.

Another key discovery was the determination of the origins of the RSV *src* gene. Hybridization experiments with radioactive *src*-specific DNA probes revealed the presence of homologous sequences in DNA from uninfected chickens.⁴⁴¹ The *src*-specific DNA also hybridized with DNA of other species, and the extent of hybridization correlated with the evolutionary distance from chickens. Thus, the *src* gene of RSV, *v-src*, was derived by capture of a normal cell gene, *c-src*, referred to as a proto-oncogene.

The domains of pp60^{v-src} are shown in Figure 7.4. The protein is modified by myristoylation at the N-terminus, which anchors it to the inner surface of the plasma membrane.⁵² Membrane attachment is necessary for transformation, although not for kinase activity.²⁴³ The kinase domain is located in the internal region of pp60^{v-src}. Two adjacent domains are “*src* homology” domains—SH2 and SH3. These domains were originally identified as regions of homology between *v-src* and *src*-related oncogenes of other avian acute transforming retroviruses.³⁹³ The SH2 and SH3 domains mediate the specific interaction of pp60^{v-src} (and other proteins) with cellular proteins, including regulatory proteins and substrates for phosphorylation.^{261,319} SH2 and SH3 domains are present in many cellular proteins, and they are important

protein–protein interaction motifs. SH2 domains bind phosphotyrosine residues in specific sequence contexts in their target proteins, while SH3 domains bind proline-rich regions.²⁶¹

The *c-src* proto-oncogene encodes a similar protein, pp60^{c-src}, which differs from pp60^{v-src} by several amino acid substitutions throughout the protein and by a different C-terminal sequence due to a frameshift mutation in *v-src* (Fig. 7.4).^{454,460} The C-terminus of pp60^{c-src} is a regulatory domain that contains a crucial tyrosine residue at position 527 (Y⁵²⁷). When Y⁵²⁷ is phosphorylated, it binds the SH2 domain on the same molecule of pp60^{c-src}, resulting in a closed conformation in which the kinase is inactive.⁵⁰³ Dephosphorylation of Y⁵²⁷ displaces the SH2 domain, resulting in activation of *src* kinase.⁹⁴ Of note, the viral protein pp60^{v-src} lacks the regulatory Y⁵²⁷ and is thus constitutively in the open, kinase-active form where it signals for cell transformation by catalyzing high-level phosphorylation of substrates.

Substrates phosphorylated by pp60^{v-src} provide the key to understanding transformation and oncogenesis by RSV. Various substrates have been identified by searching for proteins that show enhanced levels of tyrosine phosphorylation in RSV-transformed cells compared to normal cells.^{91,97,367,381,398,415} pp60^{v-src} substrates whose phosphorylation is important for RSV transformation include the mitogen-activated protein kinase (MAPK) Erk-1/2,³⁸¹ focal adhesion kinase (FAK),³⁹⁸ and Connexin43, a tight junction protein.⁹⁸

Overview of Other Retroviral Oncogenes. The basic principles elucidated by analysis of RSV also apply to the other acute transforming retroviruses and are summarized here. With a few exceptions described below, acute transforming retroviruses contain viral oncogenes resulting from capture of cellular proto-oncogenes (Table 7.3), although the location

TABLE 7.3 Oncogenes Transduced by Acute Transforming Retroviruses

Oncogene	Retrovirus	Oncoprotein ^a	Identity
Growth factor <i>sis</i>	Simian sarcoma virus	p28 ^{env-sis}	Platelet-derived growth factor-B
Receptor tyrosine kinases			
<i>erbB</i>	AEV-ES4, AEV-R, AEV-H	gp65 ^{erbB}	Epidermal growth factor receptor
<i>fms</i>	McDonough FeSV	gp180 ^{gag-fms}	Colony stimulating factor-1 receptor
<i>sea</i>	S13 AEV	gp160 ^{env-sea}	Macrophage-stimulating protein receptor
<i>kit</i>	Hardy-Zuckerman-4 FeSV	gp80 ^{gag-kit}	Stem cell factor receptor
<i>ros</i>	UR2 ASV	p68 ^{gag-ros}	Ligand unknown
<i>eyk</i>	Avian retrovirus RPL30	gp37 ^{eyk}	Gas6 receptor
Cytokine receptor <i>mpl</i>	Mouse myeloproliferative leukemia virus	p31 ^{env-mpl}	Thrombopoietin receptor
Hormone receptor <i>erbA</i>	AEV-ES4, AEV-R	p75 ^{gag-erbA}	Thyroid hormone receptor
Lipid kinase p3k	ASV16	p150 ^{gag-p3k}	Catalytic subunit of PI3K
Ubiquitin ligase <i>cbl</i>	Cas NS-1 MuLV	p100 ^{gag-cbl}	
G proteins			
<i>H-ras</i>	Harvey MSV	p21 ^{H-ras}	
<i>K-ras</i>	Kirsten MSV	p21 ^{K-ras}	
Adaptor protein <i>crk</i>	CT10, ASV-1	p47 ^{gag-crk}	
Nonreceptor tyrosine kinases			
<i>src</i>	Rous sarcoma virus	pp60 ^{src}	
<i>abl</i>	Abelson MuLV	p160 ^{gag-abl}	
<i>fps^b</i>	Fujinami ASV	p130 ^{gag-fps}	
	PRC 11 ASV	p105 ^{gag-fps}	
<i>fes^b</i>	Snyder-Theilen FeSV	p85 ^{gag-fes}	
	Gardner-Arnstein FeSV	p110 ^{gag-fes}	
<i>fgr</i>	Gardner-Rasheed FeSV	p70 ^{gag-actin-fgr}	
<i>yes</i>	Y73 ASV	p90 ^{gag-yes}	
	Esh ASV	p80 ^{gag-yes}	
Serine-threonine kinases			
<i>akt</i>	AKT8 MuLV	p105 ^{gag-akt}	
<i>mos</i>	Moloney MSV	p37 ^{env-mos}	Cytostatic factor component
<i>raf^c</i>	3611-MSV	p75 ^{gag-raf}	
<i>mil^c</i>	MH2 avian myelocytoma virus	p100 ^{gag-mil}	
Transcription factors			
<i>jun</i>	ASV17	p65 ^{gag-jun}	AP1 component
<i>fos</i>	Finkel-Biskis-Jenkins MSV	p55 ^{fos}	AP1 component
<i>myc</i>	MC29 avian myelocytoma virus	p100 ^{gag-myc}	
	CMII avian myelocytoma virus	p90 ^{gag-myc}	
	OK10 avian leukemia virus	p200 ^{gag-pol-myc}	
	MH2 avian myelocytoma virus	p59 ^{gag-myc}	
<i>myb</i>	AMV BAI A	p45 ^{myb}	
	AMV-E26	p135 ^{gag-myb-ets}	
<i>ets</i>	AMV-E26	p135 ^{gag-myb-ets}	
<i>rel</i>	Avian reticuloendotheliosis virus T	p64 ^{rel}	NF-κB component
<i>maf</i>	Avian retrovirus AS42	p100 ^{gag-maf}	
<i>ski</i>	SKV ASV	p110 ^{gag-ski-pol}	
<i>qin</i>	ASV31	p90 ^{gag-qin}	

AEV, avian erythroblastoma virus; AMV, avian myeloblastoma virus; ASV, avian sarcoma virus; FeSV, feline sarcoma virus; MSV, murine sarcoma virus; MuLV, murine leukemia virus; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase.

^aThe nomenclature of viral oncoproteins refer to basic structural data: p, protein; gp, glycoprotein; pp, phosphoprotein; the numbers designate the molecular weight in kilodaltons; the superscript indicates the genes from which the coding information is derived in a 5' to 3' direction.

^b*fps* and *fes* are the same oncogene derived from the avian and feline genomes, respectively.

^c*raf* and *mil* are the same oncogene derived from the murine and avian genomes, respectively.

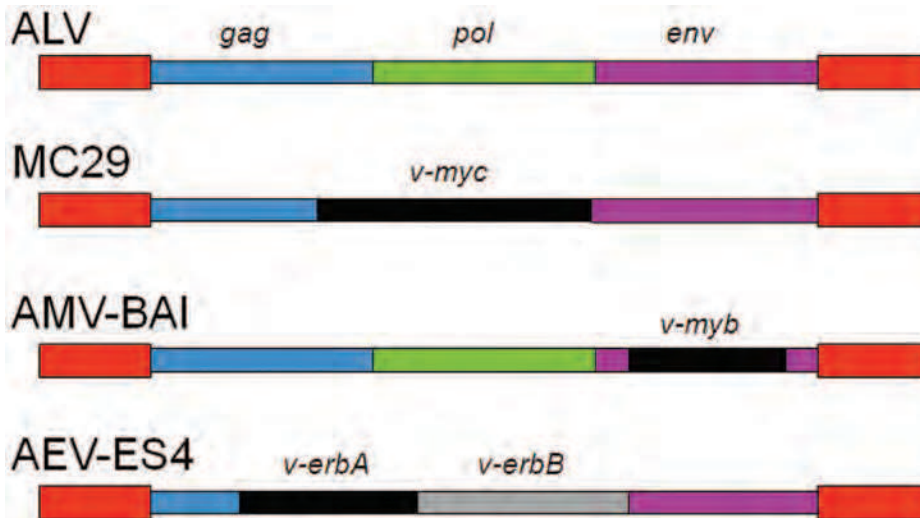


FIGURE 7.5. Genome maps of avian acute transforming retroviruses. The genome structures of the following avian acute transforming viruses are shown: ALV, avian leukosis virus; MC29, avian myelocytoma virus MC29; AMV-BAI, avian myeloblastosis virus BAI; AEV-ES4, avian erythroblastosis virus ES4. The proviral long terminal repeats (LTRs) are shown in red, and the retroviral structural genes *gag*, *pol*, and *env* are shown in blue, green, and lavender, respectively. Transduced oncogenes are shown in black or gray.

of the oncogene in the viral genome can vary (Fig. 7.5). In some cases the same proto-oncogene has been captured independently by several different viruses. For example, the avian retroviruses Fujinami sarcoma virus and PRC-II both captured the *c-fps* proto-oncogene from the chicken genome, and the Snyder-Theilen strain of feline sarcoma virus captured the homologous gene (*c-fes*) from the cat genome.¹⁹⁴ Proto-oncogene proteins, which generally play positive roles in stimulation of cell growth or division, are typically tightly regulated. The viral oncogene proteins often differ from their parent proteins by amino acid substitutions, deletions, and/or fusion to an intrinsic viral replication protein, most commonly *gag*. These differences lead to activation of the viral oncogene proteins compared to the proto-oncogene proteins by allowing the viral proteins to escape from the tight regulatory control imposed on the normal cellular homologs. For example, deletions may lead to loss of regulatory regions present in the normal proto-oncogene protein (e.g., pp60^{v-src} vs. pp60^{c-src} as described earlier). Amino acid substitutions can also result in loss of regulatory mechanisms (e.g., the *v-ras* proteins), and fusion of viral sequences to the oncogene protein can enhance activity (e.g., the *v-abl* protein). In other cases, the viral oncogene product may be identical to the proto-oncogene product but delivers a sustained or enhanced signal because it is overexpressed. In either case, the viral oncogene proteins signal for cell proliferation by the same molecular mechanisms as the proto-oncogene proteins, but in a constitutive or uncontrolled manner (see Fig. 7.2).

Most acute transforming retroviral genomes are replication defective, because capture of the proto-oncogene sequences generally results in loss of part of the viral genome (Fig. 7.5). Therefore, in order to produce infectious virus, these viruses require co-infection with a helper virus that supplies replication functions. Infection of cells with low concentrations of an acute transforming virus stock can generate foci of transformed cells harboring only the acute transforming viral genome (and not the helper virus genome). Cells from these foci express the viral oncogene, but they do not produce infectious virus—they are called nonproducer cells. Infectious transforming virus can be recovered from these nonproducer cells by infecting them

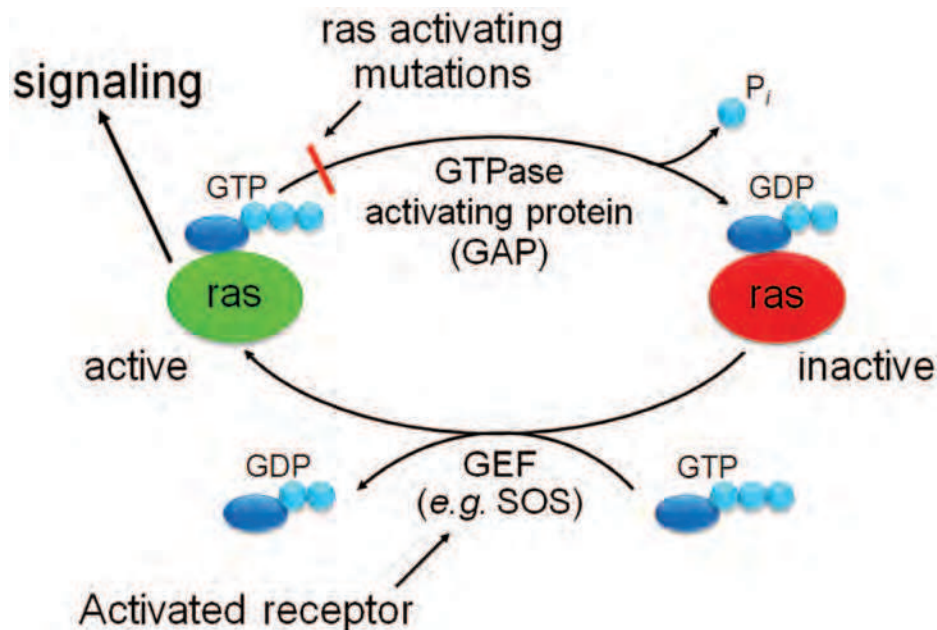
with a replication-competent helper virus to provide replication functions.

Retroviral oncogenes also differ from their corresponding proto-oncogenes because they lack introns. This likely reflects the mechanism of oncogene capture, which is thought to be initiated by integration of a retroviral provirus upstream from a cellular proto-oncogene.⁴⁵⁴ Read-through transcription from the retrovirus into the proto-oncogene causes the production of a fusion transcript containing 5′ retroviral sequences and 3′ proto-oncogene sequences. The fusion transcript is then processed by mRNA splicing to remove the proto-oncogene introns, followed by packaging into virions. In a subsequent infection, recombination during reverse transcription presumably results in recovery of the 3′ end of the viral genome and the generation of an acute transforming retroviral genome containing a captured oncogene lacking introns.

Mechanism of Action of Retroviral Cytoplasmic and Membrane-Associated Oncogene Products (see Fig. 7.2).

Many oncogenes encode membrane-associated proteins that are derived from RTKs and other growth factor receptors. For example, the *v-erbB* oncogene of AEV ES4 is a transduced version of the EGF receptor.¹²⁵ In comparison to the wild-type EGF receptor, the *v-erbB* protein of AEV lacks the extracellular growth factor binding domain, as well as a tyrosine-containing inhibitory autoregulatory domain at the C-terminus (analogous to Y⁵²⁷ of pp60^{c-src}).²¹⁰ As a result, the truncated *v-erbB* protein delivers downstream signals in an EGF-independent manner. Other RTKs captured and constitutively activated by acute transforming retroviruses include the stem cell factor receptor (*v-kit*) and the colony-stimulating factor 1 receptor (*v-fms*).^{219,425,517} The *v-mpl* oncogene encodes a constitutively active version of the thrombopoietin receptor, which lacks intrinsic kinase activity but associates with intracellular tyrosine kinases.^{109,249,291} The *v-cbl* oncogene encodes a ubiquitin ligase that can regulate receptor abundance and activity.²³⁹ On the other hand, the *v-sis* oncogene of simian sarcoma virus is a transduced version of a growth factor itself, the platelet-derived growth factor B chain (PDGF-B) gene.^{79,338} Thus, *v-sis* can transform only cells that express the PDGF receptor.

FIGURE 7.6. The ras cycle. Cellular ras proteins exist in two different forms, active guanosine triphosphate (GTP)-bound ras (green) and inactive GDP-bound ras (red). Inactive ras is activated by guanine nucleotide exchange factors (GEFs, such as SOS) that load GTP onto ras; GEFs are activated by growth factor receptor signaling. Active ras is inactivated by its intrinsic GTPase activity, which hydrolyzes bound GTP to guanosine diphosphate (GDP). GTPase activity is stimulated by GTPase activating proteins (GAPs). Activating point mutations at particular positions in v-ras cause the accumulation of ras in the active form, usually by preventing GAP action. Active ras induces signaling by binding and activating effector proteins such as raf.



The *src* family constitutes a class of membrane-associated tyrosine kinases that function in the cytoplasm and lack a ligand binding domain. Thus, *src* family members are not RTKs. In addition to *v-src* itself, other avian acute transforming retroviruses contain the *v-fps/v-fes* oncogene and the *v-yes* oncogene.¹⁹⁴ These oncogene proteins share substantial homology with pp60^{v-src} in the kinase domain as well as in other domains.^{261,393} There are other cellular tyrosine protein kinases in the *src* family that function in different signal transduction pathways (e.g., *lck*, whose protein is important for signaling in B lymphocytes⁴⁷⁸), but they have not been captured by acute transforming retroviruses. The genes encoding other cytoplasmic tyrosine kinases unrelated to *src* have been transduced and activated by other acute transforming retroviruses, such as the *v-abl* oncogene of Abelson murine leukemia virus.^{496,497}

The cytoplasmic products of other retroviral oncogenes are kinases that phosphorylate protein substrates on serines or threonines, rather than tyrosines. Three oncogenes in this class are *v-raf* (of murine sarcoma virus 3611 [and the homologous *v-mil* from an avian virus]),^{234,372} *v-mos* (of Moloney murine sarcoma virus),⁴¹³ and *v-akt* (of Akt murine leukemia virus).²⁴ The corresponding cellular proto-oncogene proteins function in different signaling pathways. Cellular Raf protein is a mediator in signal transduction from RTKs and Ras (see later), initiating a cascade of protein phosphorylations that ultimately leads to phosphorylation and nuclear translocation of MAPKs of the extracellular signal-regulated kinase (ERK) family.^{262,514} Cellular Mos protein is required for meiosis and is a component of cytoskeletal factor (CSF).⁵⁰⁸ *v-mos* is activated primarily by overexpression from the viral long terminal repeat (LTR).³³ Cellular Akt protein is a key intermediate in downstream signaling from PI3 kinase, which is also coupled to RTK signaling (see Fig. 7.2). Akt activation delivers survival and proliferative signals to cells. In addition, PI3 kinase itself has been captured by an avian acute transforming retrovirus (AS-16) as the *v-p3k* oncogene.⁶⁴ For the viral oncogenes that encode protein kinases, constitutive, unregulated enzyme activity causes over-

active signaling through the corresponding signal transduction pathways, leading to transformation and tumorigenesis.

Other important cytoplasmic oncogenes are the *ras* oncogenes, *v-Hras* and *v-Kras*, identified in Harvey and Kirsten murine sarcoma viruses, respectively.^{86,145} These viruses were generated by the capture of two closely related cellular proto-oncogenes, *c-Hras* and *c-Kras*, when MuLVs were passaged through rats. The cellular Hras and Kras proteins, which play critical roles in signal transduction, are guanosine triphosphatases (GTPases) (known as G proteins) that bind guanine nucleotides (guanosine diphosphate [GDP] or guanosine triphosphate [GTP]). In the GTP-bound form, Ras proteins induce signals that result in cell proliferation (e.g., by binding and activating Raf kinase), but they are inactive when bound to GDP (Fig. 7.6).^{39,44} Guanine nucleotide exchange factors (GEFs), which stimulate replacement of GDP with GTP, cause inactive Ras to cycle to GTP-bound, active Ras. GEFs include adaptor proteins such as Grb-2 or Sos, which are mobilized by RTK activation.^{136,292} The active GTP-bound Ras cycles back to the inactive form by hydrolysis of the bound GTP to GDP by the intrinsic GTPase activity of the Ras protein, which is stimulated by GTPase activating proteins (GAPs).³⁵ The *v-Hras* and *v-Kras* proteins contain missense mutations at key residues (e.g., amino acids 12 and 59 for *v-Hras*)⁴⁵⁹ that reduce the ability of GAP to stimulate GTP hydrolysis.¹ Therefore, the *v-Ras* proteins accumulate in the active, GTP-bound form because they are unable to cycle to the inactive form, and they signal constitutively for cell growth.

The avian sarcoma virus CT10 and its transduced oncogene, *v-crck*,³⁰⁷ have provided insight into a family of cellular proteins known as adaptor proteins. The proto-oncogene protein *c-crck* (*crckI*), along with proteins from related proto-oncogenes *crckII* and *crckL*, contain SH2 and SH3 domains, but they do not have enzymatic activity. Rather, they function as adaptor proteins that assemble proteins into signal transduction complexes by way of interactions via the SH2 and SH3 domains. Cellular signaling proteins that interact with Crk family proteins include Abl, Sos, and FAK.³⁰⁴

Mechanism of Action of Retroviral Nuclear Oncogene Products (see Fig. 7.2).

The nuclear oncogenes of retroviruses are largely derived from proto-oncogenes that encode DNA binding proteins, many of which act as transcription factors. The *v-myc* oncogene, originally identified in the avian acute leukemia virus MC29 (but also captured by other avian acute transforming viruses), was one of the first studied.^{421,476} The *c-myc* protein (Myc) is involved in early responses to mitogenic stimuli and binds E-box motifs in promoters/enhancers of target genes.¹³⁸ Myc exists as a homodimer or as a heterodimer with a related protein, Max. The Myc-Myc homodimers bind E-boxes with low affinity, while Myc-Max heterodimers bind with high affinity.³² Early after a mitogenic stimulus, levels of Myc transiently rise, leading to activation of E-box-containing genes such as those encoding E2F and cyclin D and repression of genes including those encoding cdk inhibitors.¹³⁸ *v-myc* appears to transform cells because of its constitutive overexpression of the retroviral LTR.

The *v-myb* oncogene was also discovered in two different avian acute leukemia viruses (AMV BAI A and AMV E26).²⁵⁸ It is derived from *c-myb*, which encodes a cellular DNA binding protein that acts as a transcription factor important in hematopoietic cell development.²⁸ Structural alterations in *v-myb* appear to be responsible for activation.^{28,193}

The oncogene of acute transforming viruses of the Finkel-Biskis-Jenkins (FBJ) murine osteosarcoma virus complex is *v-fos*,⁴⁷² and *v-jun* is the oncogene of avian sarcoma virus S17 (“jun” is 17 in Japanese).²⁹⁷ The *c-fos* and *c-jun* proto-oncogenes encode nuclear DNA binding proteins that are subunits of the transcription factor, activator protein-1 (AP1).³⁷⁵ Jun can homodimerize, or it can heterodimerize with Fos. Jun-Jun homodimers bind AP1 sites in DNA with low affinity, while Fos-Jun heterodimers bind these sites with high affinity. Activation of Fos transcription from undetectable levels is one of the earliest events after mitogenic stimulation—levels of Fos protein rise within 15 minutes of bombesin stimulation in fibroblasts.³²⁴ In contrast, Jun protein is present in the absence of mitogenic stimulation, and Jun levels do not change substantially in response to growth factor treatment. As a result, early after mitogenic stimulation, there is a shift from Jun-Jun homodimers to Fos-Jun heterodimers, resulting in activation of promoters/enhancers containing AP1 sites.³²⁹ Expression of these target genes (e.g., *c-myc*)²²⁷ is important for cell division and growth. Both the *v-jun* and the *v-fos* proteins contain structural alterations responsible for oncogenic activation. For example, *v-jun* contains missense mutations and a deletion that removes an auto-inhibitory segment, resulting in constitutive activation.⁴¹

Another nuclear oncogene is *v-erbA* of AEV. The *c-erbA* proto-oncogene encodes the thyroid hormone receptor- α (THR- α), a sequence-specific DNA binding protein.^{395,487} In contrast to wild-type THR- α , which binds DNA and activates transcription of target genes, the *v-erbA* protein consists largely of the DNA binding domain.^{105,395} As a result, it binds to DNA nonproductively and acts as a dominant negative form by inhibiting transcription of THR- α -responsive genes. This leads to a block in erythroid cell differentiation, which in turn results in enhanced proliferation of undifferentiated erythroid progenitors.

The *v-rel* oncogene was initially identified in avian reticuloendotheliosis virus.^{73,443} The *c-rel* proto-oncogene encodes

a member of the nuclear factor- κ B (NF κ B) transcription factor family and is important for B-cell development.¹⁸⁰ C-Rel can homodimerize or heterodimerize with the p50 NF κ B protein and bind NF κ B sites in target genes. Point mutations and amino acids derived from env are required for v-rel activation.^{172,457}

Tumorigenesis by Nonacute Retroviruses

Oncogenic nonacute retroviruses belong to the alpha-, beta-, and gammaretrovirus genera. Prototypic nonacute retroviruses include avian leukosis virus (ALV), MMTV, and various MuLVs. These viruses are replication competent, lack viral oncogenes, and do not transform cultured cells. In contrast to tumors induced by acute transforming retroviruses, which are often polyclonal, tumors induced by nonacute retroviruses are monoclonal or oligoclonal (i.e., derived from one or a few transformed cells). Another feature of these viruses is that high-level infection in animals typically occurs many months before development of tumors. Thus, infection is not synonymous with oncogenic transformation for nonacute viruses. The common mechanism by which most nonacute retroviruses induce tumors is insertional activation of cellular proto-oncogenes by the integrated retroviral genome.

B Lymphomas Induced by ALV and Activation of *c-myc*.

Pioneering experiments investigating tumorigenesis by nonacute retroviruses were conducted on chicken B-cell lymphomas induced by ALV. Neel et al³³¹ analyzed DNA from these tumors and found that many of them did not contain intact integrated ALV proviruses, although each tumor contained at least some segment of viral DNA. They studied tumor RNA by northern blot analysis by using two different radioactive viral cDNA hybridization probes, one representing the entire viral coding region and another representing only the ends of the viral RNA including portions of the viral LTR. The full-length probe did not detect viral RNAs in all tumors; however, the LTR probe invariably detected transcripts in tumors, and different tumors gave transcripts of similar sizes. This led Neel et al³³¹ to propose the *promoter insertion* model of ALV tumorigenesis, in which transcription from the downstream ALV LTR continues into adjacent cellular sequences (Fig. 7.7). Furthermore, the similar sizes of the transcripts suggested that the ALV proviruses were inserted into the same chromosomal locus in different tumors. Further experiments showed that the ALV LTR was indeed inserted into the same chromosomal region in independent tumors, adjacent to the *c-myc* proto-oncogene.²¹¹ This placed *c-myc* under control of the strong ALV promoter/enhancer, resulting in read-through transcription into the proto-oncogene. Thus, *c-myc* can be activated either by transduction by the acute transforming retroviruses or by proviral insertion by the nonacute retroviruses. Many ALV-induced tumors contain a deletion of a portion of integrated proviral ALV DNA, resulting in loss of the upstream LTR, so that most of the viral DNA is not transcribed. Deletion of the upstream LTR likely stimulates the activity of the downstream LTR, which drives enhanced transcription of *c-myc*.

In some ALV-induced B lymphomas overexpressing *c-myc*, the relative transcriptional orientations of ALV DNA and *c-myc* were not consistent with the promoter insertion model.³⁵² For example, some tumors contained proviral DNA

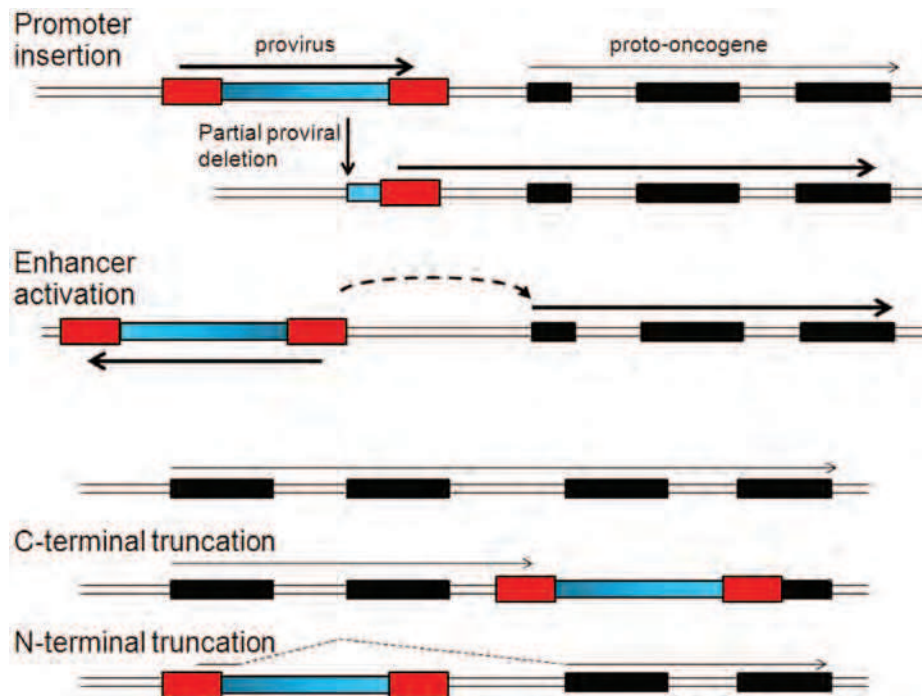


FIGURE 7.7. Insertional activation of cellular proto-oncogenes by retroviruses. The **top two diagrams** show proto-oncogene activation by promoter insertion. The **top diagram** shows a provirus integrated upstream of a proto-oncogene in the same transcriptional orientation. The thickness of the horizontal arrows indicates the relative level of expression. Partial deletion of the provirus activates the downstream long terminal repeat (LTR), which results in read-through transcription into the proto-oncogene. The **third diagram** shows proto-oncogene activation by enhancer activation. In the example shown, the provirus integrates farther upstream from the proto-oncogene in the opposite transcriptional orientation, and the enhancer in the viral LTR activates expression from the proto-oncogene promoter (dashed arrow). Proviruses inserted downstream of a proto-oncogene can also enhance expression. The **bottom three diagrams** show a proto-oncogene with four exons. Proviral insertion in the same transcriptional orientation in the 3' portion of the proto-oncogene could result in synthesis of a C-terminally truncated protein product due to cleavage/polyadenylation sequences in the LTR. A provirus inserted in the 5' portion of the proto-oncogene could be transcribed into downstream proto-oncogene exons followed by splicing from a viral splice donor site to a proto-oncogene splice acceptor site. This could result in the deletion or splicing out of proto-oncogene exons and expression of an N-terminally truncated protein product. If the deleted portions regulate the activity of the wild-type protein, these deletions might result in proto-oncogene activation.

inserted upstream of *c-myc* but in the opposite orientation, whereas in other cases the provirus was inserted downstream of *c-myc* (Fig. 7.7). Activation of *c-myc* expression in these cases can be explained by activation of the endogenous *c-myc* promoter by the enhancer sequences in the ALV LTR, which can activate adjacent promoters in an orientation- and position-independent manner. Thus, activation of proto-oncogenes by nonacute retroviruses can be considered as LTR activation, either by promoter insertion or by enhancer activation. Insertional activation of proto-oncogenes has proven to be a common mechanism of tumorigenesis by nonacute retroviruses. Other variations of these mechanisms are described in the next sections.

MMTV Tumorigenesis. MMTV (a betaretrovirus) also employs insertional activation. Common proviral insertion sites (CISs) were identified³⁴⁰ in independently arising MMTV-induced mammary tumors. For MMTV, the first CIS did not correspond to any proto-oncogene known at the time, but by analogy to ALV-induced B lymphomas, it was hypothesized that the CIS marked a cellular gene that was activated by the MMTV provirus. This gene was named *int-1*

(integration site-1), and *int-1* RNA was overexpressed in tumors. Subsequently *int-1* was renamed *wnt-1* because the *Drosophila* homolog had been identified by the developmental mutation *wingless*.³⁷⁸ *Wnt-1* is an important molecule in the *Wnt*- β -catenin signal transduction pathway, which is mutated in a wide variety of human cancers, most notably colorectal cancer.²⁵⁴ Thus, CISs initially identified in nonacute retroviral tumors led to discovery of new proto-oncogenes (Table 7.4). In contrast to ALV-induced B lymphomas where the provirus was integrated quite near *c-myc*, the MMTV provirus in most cases was inserted farther from *Wnt-1*.³⁴⁰ Activation of *Wnt-1* likely occurs by enhancer activation, because transcriptional enhancers can work over relatively long distances (kilobases). Other CISs in MMTV-induced tumors include *int-2* (FGF-3),¹²⁰ *int-3*, and *int-4*.²⁴⁷

MuLV Leukemogenesis. MuLVs are gammaretroviruses and include Moloney MuLV (M-MuLV), SL3-3 MuLV, and radiation leukemia virus (RadLV), which induce T lymphoma, and Friend MuLV (F-MuLV), which induces erythroid or myeloid leukemia. Activation of *c-myc* commonly occurs in leukemias induced by M-MuLV.^{417,440} Other M-MuLV

TABLE 7.4 Proto-Oncogenes Identified As Common Insertion Sites

Virus	Disease	Common insertion site or activated proto-oncogene
Moloney MuLV	T lymphoma In mice	<i>c-myc</i> , <i>pim-1</i> , <i>pvt-1/mis-1/mlvi-1</i> , <i>lck</i> , <i>pim-2</i> , ^a <i>n-myc</i> , ^a <i>bmi-1</i> , ^a <i>frat-1</i> , ^a <i>pal-1/gfi-1</i> ^a
	In rats	<i>c-myc</i> , <i>pvt-1/mis-1/mlvi-1</i> , <i>mlvi-2</i> , <i>mlvi-3</i> , <i>mlvi-4</i> , <i>dsi-1</i> , <i>lck</i> , <i>tpl-1/ets-1</i> , ^a <i>tpl-2</i> , ^a <i>gfi-1/pal-1</i> , ^a <i>gfi-2/IL-9R</i>
AKR MuLV/Gross Virus; SL3-3 MuLV	Myeloid leukemia	<i>c-myb</i> , <i>mml-1</i>
	T lymphoma	<i>c-myc</i> , <i>gin-1</i> , <i>n-ras</i>
RadLV	T lymphoma	<i>c-myc</i> , <i>pim-1</i> , <i>vin-1/cyclinD2</i> , <i>notch1</i> , <i>kis-1</i> , <i>kis-2</i>
Friend MuLV	Erythroleukemia	<i>fli-1</i> , <i>fre-2</i>
Endogenous MuLV (AKXD, BXH-2 recombinant inbred mice)	Myeloid leukemia	<i>fis-1</i> , <i>fim-1</i> , <i>evi-1/fim-3</i> , <i>c-fms/fim-2</i>
	Myeloid leukemia	<i>evi-1/fim-3</i> , <i>evi-2</i> , <i>meis-1</i> , and others
Abelson MuLV ^b	B-lymphoma	<i>ahi-1</i> , <i>ahi-2</i> ^c
Friend SSFV ^d	Erythroleukemia	<i>Spi-1</i> , <i>p53</i> ^e

MuLV, murine leukemia virus; RadLV, radiation leukemia virus.

Data from retroviral tagging of mice genetically predisposed to cancer (e.g., *myc* transgenic mice) are not included here. They can be found in the Mouse Retrovirus Tagged Cancer Gene (RTCG) database at <http://RTCGD.ncifcrf.gov>.

^aInsertions associated with tumor progression or that collaborate with other proto-oncogene activations.

^bAlso contains v-abl oncogene.

^cCommon proviral insertion site of helper virus.

^dAlso contains gp55 oncogene.

^eInsertion at *p53* inactivates its function.

tumors contained novel CISs that led to the discovery of new proto-oncogenes such as the cytoplasmic serine/threonine protein kinases, *pim-1* and *pim-2*^{48,99} (Table 7.4). A number of other CISs have been identified in T lymphomas induced by M-MuLV or RadLV, some of which correspond to known cellular genes such as cyclin D2.²⁰⁵ F-MuLV-induced tumors show activation of a different set of proto-oncogenes than those activated by M-MuLV. Proto-oncogenes commonly activated by F-MuLV include *fli-1* (a nuclear DNA binding protein of the *ets* family), *fim-1*,⁴³⁵ and *fim-2* (M-CSF).¹⁸² In rapidly arising erythroid leukemias induced by the Friend virus complex, a provirus activates the *spi-1* proto-oncogene, which encodes another *ets* family transcription factor.^{320,350} In addition, an acute transforming retrovirus has also been identified in the Friend virus complex: spleen focus-forming virus (SFFV), which contains an oncogene encoding gp55. Gp55 is unusual in that it is not derived from a cellular proto-oncogene, but rather is a deleted form of an endogenous (genetically transmitted) MuLV *env* gene that recombined into the F-MuLV genome.⁴⁹⁸

The different activated proto-oncogenes in leukemias induced by M-MuLV and F-MuLV likely reflect the fact that different signaling pathways are important for transformation of lymphoid versus erythroid/myeloid cells. Studies of MuLV leukemogenesis revealed that the viral LTRs played critical roles in leukemogenicity. First, exchange of the U3 regions of the LTRs between an oncogenic MuLV (e.g., M-MuLV or SL3-3) and a nononcogenic virus (e.g., an endogenous MuLV) demonstrated that leukemogenicity was lost when the U3 region of the LTR was derived from the nononcogenic MuLV.^{118,281}

Furthermore, substitution of the enhancer region of the F-MuLV LTR into M-MuLV shifted the disease spectrum from T-lymphoid to erythroid/myeloid tumors.⁶⁸ This finding reflects the fact that enhancers are often tissue specific; that is, they specifically bind transcription factors present in a subset of differentiated cells. Indeed, in reporter assays, the M-MuLV LTR is most active in T-lymphoid cell lines and less so in erythroid/myeloid cells, while F-MuLV LTR is most active in erythroid/myeloid cells.⁴²⁸ Taken together, these results indicate that in order for an MuLV to induce tumors, it must have LTRs that efficiently activate cellular proto-oncogenes in the target cells.

Recombinant inbred strains of mice have also been used to study MuLV leukemogenesis. Certain strains of mice genetically transmit replication-competent endogenous MuLVs to their offspring. Crossing such strains with other mouse strains, followed by in-crossing, yields recombinant inbred lines. Some of these lines have high rates of leukemia development due to activation of the endogenous virus, which in turn infects cells and activates cellular proto-oncogenes. Leukemias in such lines have been used to identify new proto-oncogenes that are activated in the tumors, such as *evi-1* (endogenous virus insertion site-1) and *evi-2*, associated with myeloid leukemias.⁹²

Another feature of leukemogenesis by MuLVs is the formation of recombinants between these viruses and endogenous MuLV proviruses present in the mouse germline. The genomes of most higher eukaryotes carry retroviral DNA that was introduced by germline infection by retroviruses sometime during evolution. This endogenous retroviral DNA is transmitted vertically from parents to offspring, and multiple endogenous proviruses have accumulated over time. Indeed, approximately 8%

of the human genome is derived from endogenous retroviruses. Many endogenous retroviruses are replication defective, perhaps reflecting evolutionary selection against genetically transmitted, replication-competent viruses, but some can still be expressed. When mice are infected with MuLVs, recombination between the incoming MuLV and endogenous MuLVs can occur to generate mink cell focus-inducing (MCF) recombinant viruses.²⁰⁷ MCFs express an endogenous MuLV-derived envelope protein that allows them to infect cells by using a different cellular receptor than the original MuLV. Most MuLVs that infect mice or mouse cells are ecotropic and bind to the ecotropic receptor murine cationic amino acid transporter 1 (MCAT-1) on mouse cells; in contrast, MCFs infect via the xenotropic and polytropic retrovirus receptor (XPR-1) receptor. MCFs have been suggested to be the *proximal leukemogens* for some MuLVs (e.g., in AKR mice that develop spontaneous leukemia by activation of an ecotropic endogenous MuLV [Akv-MuLV]),²⁰⁷ although for other MuLVs MCF recombinants may play early roles in disease.¹⁴⁸ For MuLVs with weak LTR enhancer activity (e.g., Akv-MuLV), MCF recombinants also acquire a stronger LTR by recombination with a second endogenous provirus.⁴⁴⁸

Other Mechanisms of Insertional Oncogenesis. While the predominant mechanisms of oncogenesis by nonacute retroviruses are LTR-mediated activation of proto-oncogenes by promoter insertion or enhancer activation, several related mechanisms have also been identified, all of which involve integration of proviral DNA into the genome at specific places, leading to changes in cellular gene expression and development of tumors.

In one such mechanism, a provirus is inserted upstream of a proto-oncogene or within a proto-oncogene in the same transcriptional orientation as the cellular gene. The viral genome is transcribed beginning in the upstream LTR, but transcription continues past the poly(A) signal in the downstream LTR into the proto-oncogene (Fig. 7.7). Splicing of the fusion transcript from the viral splice donor into downstream proto-oncogene splice acceptor sites can generate mRNAs that encode truncated proto-oncogene proteins. This has been observed in erythroid leukemias induced by ALV in line 15₁ chickens, where a truncated c-ErbB (EGF receptor) protein is produced,¹⁶⁴ and in myeloid leukemias induced by M-MuLV in pristane-primed Balb/c mice, where a truncated c-Myb protein is generated.³³⁰ Similarly, transcription of a proto-oncogene could terminate at the LTR of a provirus inserted into a downstream portion of the proto-oncogene, resulting in a C-terminal truncation (Fig. 7.7). In these cases, the truncations remove regulatory domains from the proto-oncogene proteins, leading to the synthesis of unregulated, growth stimulatory proteins.

As mentioned earlier, inoculation of chicken embryos with ALV leads to rapid development of B lymphomas harboring proviral activation of *c-myc*. In addition, some ALV-induced lymphomas contain proviruses at both *c-myc* and a novel CIS, *bic-1*.⁸⁴ Although *bic-1* RNA was overexpressed in these tumors, presumably due to provirus insertion, *bic-1* does not contain protein-coding sequences. Rather, *bic-1* encodes the precursor for microRNA (miRNA) miR155—a so-called onco-miR that is overexpressed in a variety of human cancers.¹⁴⁹ miR155 causes the down-regulation of growth inhibitory genes, which presumably accounts for its oncogenic potential.³⁸ Insertional activation of other miRNAs has also been observed in retrovi-

rally induced tumors. The miR-103-363 cluster is activated in RadLV-induced T lymphomas,²⁷⁵ and miR-106 a is activated in SL3-3 MuLV-induced T lymphomas.²⁹³

Another mechanism of insertional oncogenesis involving miRNAs has been described for T lymphomas induced by SL3-3 MuLV. In these tumors, the provirus is inserted into downstream noncoding sequences of the *gfi-1* proto-oncogene.¹⁰⁰ These sequences contain binding sites for several miRNAs, including miRNA-155. This results in overexpression of a truncated *gfi-1* mRNA, presumably because the inserted provirus eliminates binding sites for inhibitory miRNAs.

Finally, insertions of the SFFV provirus into the p53 tumor suppressor gene occur in erythroleukemia cell lines established from tumors induced by the acute transforming Friend SFFV/F-MuLV complex.^{25,323} The insertions inactivate p53, and in these tumors the normal *p53* gene is generally lost. Similarly, in BXH2 recombinant inbred mice, proviral insertion occurs in *evi-2*, which encodes the NF-1 tumor suppressor.²⁷⁷ In these cases, then, rather than activating proto-oncogenes, proviral insertion inactivates tumor suppressor genes.

Multiple Changes in Nonacute Retrovirus-Induced Tumors.

Multiple genetic changes may be involved in some tumors induced by nonacute retroviruses. Proviral insertions next to more than one proto-oncogene have been observed in some MMTV-induced mammary tumors and M-MuLV-induced T lymphomas (e.g., *wnt-1* and *int-2* for MMTV and *c-myc* and *pim-1* for M-MuLV).^{311,417} Although in some cases this reflects two independent tumors, each containing an insertion event near a different proto-oncogene, in other cases the same tumor cell harbors both of the activated proto-oncogenes, suggesting that the proto-oncogenes cooperate in tumorigenesis. This notion has been further developed by studying leukemogenesis in transgenic mice. For example, mice with a *pim-1* transgene under expression of a T-cell-specific promoter develop lymphomas very inefficiently. However, if these mice are infected with M-MuLV, tumors develop extremely rapidly, and analysis of the tumors revealed proviral activations of both *c-myc* and *n-myc*.⁴⁷³ This result provided direct evidence that overexpressed *pim* family members and *myc* family members can cooperate in T lymphomagenesis. In later experiments, several additional pairs of cooperating proto-oncogenes have been identified.⁴⁷⁴

Secondary events may also be important in tumors induced by acute transforming retroviruses. Abelson MuLV (carrying the *v-abl* gene) induces B-cell lymphomas when it is co-infected with an M-MuLV helper virus but not with other helper MuLVs, suggesting that M-MuLV also contributes to tumor development. The critical region for this activity was localized to the M-MuLV LTR, and a CIS for the M-MuLV helper in Abelson MuLV tumors was identified adjacent to the *ahi-1* gene.³⁶¹ Thus, expression of the *v-abl* oncogene along with *ahi-1* protein²³⁸ is required for efficient lymphomagenesis.

Another approach to identify activated proto-oncogenes that contribute to later steps in tumorigenesis involves *in vitro* or *in vivo* passage of tumors. For example, *in vitro* passage of rat T lymphomas induced by M-MuLV results in acquisition of additional proviral CISs, which have been termed tumor progression loci (e.g., *tpl-1* and *tpl-2*).^{20,349} Likewise, *in vitro* infection of growth factor-dependent M-MuLV-induced

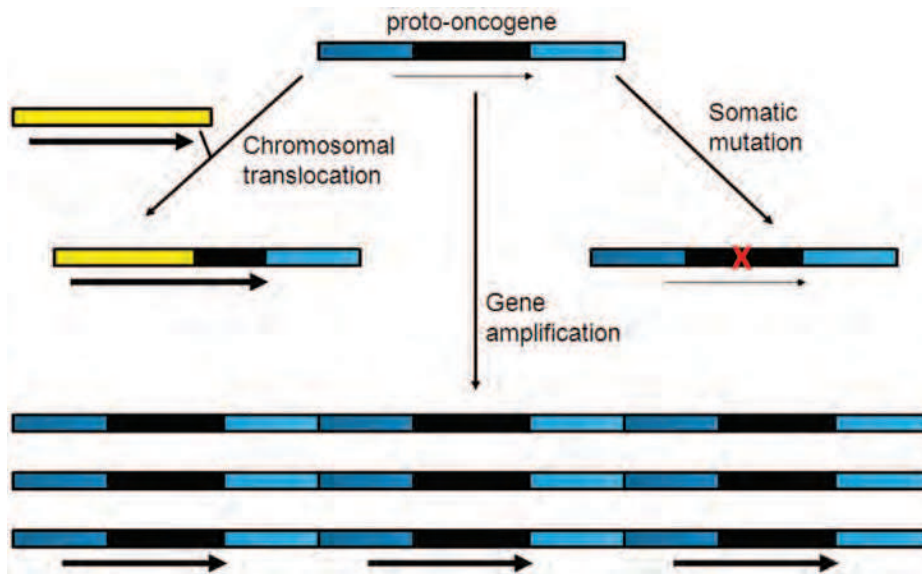


FIGURE 7.8. Proto-oncogene activation in nonviral tumors. The figure shows three mechanisms of proto-oncogene activation in tumors of nonviral origin: tandem amplification of the oncogene and flanking cellular DNA, resulting in overexpression of the oncogene; chromosomal translocation resulting in overexpression and/or structural alteration of the oncogene, leading to its constitutive activation; and point mutations in the coding region of an oncogene, resulting in constitutive activation. The thickness of the horizontal arrows indicates the relative level of expression. Black, proto-oncogene; blue, flanking normal cell DNA; yellow, translocated chromosome. Red X indicates mutation.

lymphomas with a second MuLV (MCF-MuLV) resulted in the generation of factor-independent cells, which contained proviral activation of new proto-oncogenes (e.g., *gfi-1*).^{159,179} Finally, as described earlier, insertional inactivation of p53 occurs during *in vitro* passage of erythroleukemia cell lines from Friend virus complex-infected mice, so it is a late step in the development of these lines.²⁵

Proto-Oncogene Discovery in the Age of Genomics. The initial discovery of CISs and activated proto-oncogenes was very labor intensive and typically required identifying virus-induced tumors harboring a small number of proviruses, followed by generating a lambda phage library of tumor DNA and cloning all of the proviruses. Adjacent cell sequences from each provirus were then used as hybridization probes on Southern blots of DNA from other virus-induced tumors to identify additional tumors that harbored proviral insertions in the same cellular site.^{211,352} The availability of whole genome sequences substantially increased the pace of CIS discovery, particularly in mice.⁴²² New cloning techniques such as inverse polymerase chain reaction (PCR) facilitated the cloning of proviral DNA and viral–host junctions.⁴²² Sequencing of the adjacent cellular DNA in these clones, followed by alignment of the cellular DNA sequence with the host genome, allowed rapid localization of each proviral insertion site within host cell DNA. Common insertion sites could be readily identified by insertions into similar chromosomal locations in different tumors.

The advent of high-throughput DNA sequencing has further enhanced the efficiency of CIS discovery. In a recent approach, linker-ligated PCR techniques are used to amplify viral–cellular junctions from tumor samples, and then the entire reactions are analyzed by deep sequencing.²⁶⁵ This provides high-resolution analysis of all of the proviral insertions in a tumor, and then comparison with other tumors induced by the same virus is used to identify CISs. In a recently published study, over 9,000 insertion sites were analyzed in a collection of 476 murine tumors, and multiple new CISs were identified.²⁶⁵

Activation of Proto-Oncogenes in Nonviral Tumors

Studies of retroviral oncogenes and their corresponding proto-oncogenes led to the first discoveries of molecular genetic alterations in human cancers, namely, activation of proto-oncogenes. Genetic alterations in cancer are now major subjects of study in cancer research. The basic mechanisms of proto-oncogene activation in tumor cells—gene amplification, chromosomal translocation, and missense mutations—mirror the consequences of retroviral activation of oncogenes (Fig. 7.8).

Gene amplification in tumor cells is manifested by tandem head-to-tail duplication of chromosomal regions (up to several hundred kilobases in length), resulting in as many as ~10 additional copies of the amplified region of the chromosome. Amplified regions typically contain a proto-oncogene (as well as flanking genes) and were originally detected on Southern blots by cross-hybridization with viral oncogene probes. For example, *c-myc* is amplified in several kinds of tumors such as lung cancer and neuroblastoma.^{410,411} Amplification leads to overexpression of the protein product of the proto-oncogene and excessive stimulation of growth. Early studies on *c-myc* amplification led to the discovery of *c-myc*–related genes that were also amplified in tumors (e.g., *n-myc* in neuroblastomas and *l-myc* in lung cancers). These findings expanded the concept of proto-oncogenes beyond those that had been captured by acute transforming viruses: proto-oncogenes are cellular genes that are activated in tumors and stimulate growth or division.

Chromosomal translocations have also been observed in tumors; for example, the 9:22 translocation (the Philadelphia chromosome) is pathognomonic for chronic myelogenous leukemia (CML).³⁸⁵ The first molecular description of such translocations was in Burkitt lymphoma (of B-cell origin), where *c-myc* is characteristically present at an 8:14 translocation breakpoint,¹⁰³ which moves *c-myc* on chromosome 8 next to the immunoglobulin heavy chain gene. In other cases, Burkitt lymphoma cells harbor a translocation of the *c-myc* gene into the immunoglobulin light chain locus on chromosome 22. In B lymphocytes, whose function is to produce antibodies, the immunoglobulin genes are highly expressed. Therefore, translocation

of *c-myc* into these regions leads to *c-myc* overexpression and growth stimulation. Similarly, the 9:22 translocation in CML juxtaposed *c-abl* from chromosome 9 next to the Bcr (breakpoint cluster region) on chromosome 22.¹⁰⁸ This translocation results in a novel Bcr-abl fusion protein, which exhibits elevated tyrosine kinase activity compared to the normal Abl protein.²⁶⁴ Thus, chromosomal translocations can have the same consequences as proviral insertion and proto-oncogene transduction. Characteristic translocations have been detected in various additional tumors (particularly of hematopoietic lineage, but also in prostate and lung carcinoma), and genes at the translocation junctions either are overexpressed or have altered signaling properties.²⁵⁷ These genes are now considered proto-oncogenes as well.

Missense mutations in proto-oncogenes in tumors were first detected by DNA transfection experiments. When naked DNA from certain tumor cell lines or primary tumors was introduced into mouse NIH-3T3 fibroblasts, foci of transformed cells appeared, whereas DNA from normal cells was, in general, devoid of transforming activity.⁴²⁶ Thus, these tumor cells contained a genetic change that could be transferred to other cells to cause transformation. Molecular cloning from the EJ bladder carcinoma cell line identified the transforming gene as cellular *H-ras*.³⁴⁶ The *H-ras* gene in the EJ cells had a missense mutation at residue 12 (G12V) of the protein,⁴⁵⁸ resulting in inhibition of GTPase activity and constitutive signaling, similar to changes in the *v-ras* proteins described earlier. Other tumors had missense mutations in the *K-ras* gene¹¹⁷ or in a third homolog, *N-ras*.¹⁶⁸ *Ras* mutations are some of the most frequent genetic alterations in human tumors (e.g., they are present in greater than 90% of pancreatic cancers), but this assay has identified other transforming genes in human tumor DNA as well. Activating mutations have also been found by direct sequencing of cellular proto-oncogenes (e.g., EGF receptor mutations in lung cancer, c-kit mutations in gastrointestinal stromal tumors, and B-raf mutations in melanoma).

Other Mechanisms of Retroviral Oncogenesis

While transduction of oncogenes by acute transforming retroviruses and insertional activation of proto-oncogenes by nonacute retroviruses are the most common mechanisms of retroviral oncogenesis, a few retroviruses induce tumors by other mechanisms. These mechanisms involve replication-competent retroviruses, which carry genes that function as oncogenes, although they are not derived from cellular genes.

HTLV-I and BLV. Human T-cell leukemia virus type I (HTLV-I) and bovine leukemia virus (BLV) induce adult T-cell leukemia (ATL) in humans and bovine B-cell leukemia, respectively. These deltaretroviruses are covered in Chapter 48 in detail. *In vitro* infection of T lymphocytes with HTLV-I leads to immortalization, suggesting that the virus carries an oncogene.²⁴⁵ Deltaretroviruses contain additional genes not found in other retroviruses, which encode regulatory proteins such as Tax, encoded by alternatively spliced mRNA within the *X* region of the virus, downstream of *env*. The Tax protein is a transcriptional transactivator of the HTLV-I LTR.^{57,153} It can also transactivate or inhibit cellular promoters by affecting the activity of the NF- κ B pathway, various transcriptional co-activators, and interleukin-2 (IL-2) signaling,^{72,283,333,430} and it induces defects in DNA replication and repair by nontranscriptional mechanisms.^{288,300} Tax has many properties

of an oncogene; for instance, it can transform rodent fibroblasts in culture, and mice harboring a *tax* transgene develop tumors of mesenchymal origin.³³³ Tax can also up-regulate telomerase expression, inactivate p53, and immortalize human T cells in the presence of IL-2.

WDSV and Dermal Sarcomas. Walleye dermal sarcomavirus (WDSV) is an epsilonretrovirus that induces dermal sarcomas in walleye pike.³⁸³ Related viruses (WEHV-1 and -2) induce epidermal hyperplasia in these fish, and similar viruses induce tumors in other fish. Tumors arise only during the winter; during spring spawning the tumors are shed, where the released virus presumably infects new hatchlings. WDSV and other epsilonretroviruses carry three additional reading frames besides the standard retroviral genes—*orf A*, *orf B*, and *orf C*. During the winter, the tumors do not express infectious virus, and the only transcripts present are for *orf A* and *orf B*, so the proteins encoded by these genes are likely involved in tumorigenesis.³⁸³ *Orf A* encodes a viral cyclin, rvCyclin, which binds cellular cdk8 and cdk3 and affects viral transcription and transformation. rvCyclin in complex with cdk8 negatively regulates transcription from the WDSV LTR and may be involved in inhibiting viral expression in the winter. rvCyclin can also affect expression of cellular genes, and it may positively affect cell transformation and tumorigenesis. Mice transgenic for rvCyclin develop squamous epithelial hyperplasia and dysplasia,²⁷⁴ but tumors do not form in transgenic rvCyclin fish.³⁵¹ *Orf B* may also contribute to tumorigenesis. This gene appears to have an antiapoptotic function, and *orf B* can transform cells in culture.³⁸³

JSRV and Env as an Oncogene. Jaagsiekte sheep retrovirus (JSRV) and the related enzootic nasal tumor virus (ENTV-1 and -2) are betaretroviruses. JSRV causes a transmissible lung cancer (ovine pulmonary adenocarcinoma) in sheep, and ENTV-1 and -2 cause adenocarcinoma of nasal epithelial cells in goats.²¹⁶ Interestingly, the native envelope protein of these viruses not only mediates viral entry but also functions as an oncogene. Expression of Env protein can transform various fibroblast and epithelial cells *in vitro*²⁹⁶ and induce tumors in mice or sheep.^{59,78,102,500}

As is the case for all retroviruses, there are two Env proteins, which are derived by cleavage from a polyprotein precursor—the SU (surface) protein on the exterior of virions and the TM (transmembrane) protein that spans the lipid bilayer of the viral envelope. In JSRV-transformed cells, the SU protein is presumably extracellular and the TM spans the plasma membrane. The intracellular cytoplasmic tail (CT) of TM is necessary for transformation, and a specific required tyrosine residue in CT might serve as a docking site for PI3K.³⁴³ Signal transduction pathways important for JSRV transformation include PI3K/Akt/mammalian target of rapamycin (mTOR) and Ras/Raf-MEK/MAPK.²⁹⁵ In addition to TM, the SU region of JSRV Env is also important for transformation.

Studies in a human lung epithelial line (BEAS-2B) suggested another mechanism for JSRV transformation. In these cells, JSRV Env binds hyaluronidase 2 (Hyal2), which itself binds and inactivates the Ron RTK (also known as STK).¹⁰⁶ Expression of JSRV Env leads to binding and degradation of Hyal-2, releasing Ron from inhibition. Signaling downstream from Ron involves the PI3K/Akt/mTOR and Ras/Raf-

MEK/MAPK pathways. However, JSRV Env is able to transform cells such as mouse fibroblasts, whose Hyal-2 does not bind Env and where Ron is not expressed.²⁸⁹ Thus, while binding and inactivation of Hyal-2 may participate in transformation of lung epithelial cells, this interaction is not required for transformation of other cell types.

As described earlier, MMTV induces mammary tumors by insertional activation of proto-oncogenes. However, MMTV envelope may also have oncogenic properties. MMTV-infected cell lines are not transformed when grown in monolayer culture. However, MMTV-infected MCF10 mammary epithelial cells show enhanced and disorganized growth when cultured in three dimensions, whereas normal MCF10 cells form well-organized spheres consisting of a single layer of polarized epithelial cells.²⁴⁸ This transforming activity requires an immunoreceptor tyrosine-based activation motif (ITAM) in the MMTV Env TM protein, which leads to signaling through pathways used for ITAM signaling in lymphocytes.²⁴⁸

Finally, as mentioned earlier, the oncogene of the acute transforming Friend SFFV virus encodes an internally deleted form of an endogenous MuLV Env protein recombined into F-MuLV, designated gp55.⁸⁵ Gp55 protein binds two growth factor receptors on the surface of erythroid cells: the erythropoietin receptor (EpoR)²⁸⁵ and the short form of STK (sfSTK)/Ron.³³⁹ Binding leads to constitutive downstream signaling from these receptors through signaling molecules such as Stat 3, Ras/Raf-MEK/MAPK, and PI3K/Akt.⁸⁵ Signaling through sfSTK appears to be more important for oncogenic transformation in some cell types, although in others EpoR signaling is sufficient.

MECHANISMS OF DNA VIRUS ONCOGENE ACTION

Overview

As shown in Table 7.1, several families of DNA-containing viruses induce transformation in cell culture or tumors in animals or humans. The DNA tumor viruses with the smallest genomes and clearly defined oncogenes are the polyomaviruses, including murine polyomavirus and SV40 (a polyomavirus of African green monkeys). Polyomaviruses have circular double-stranded DNA genomes of ca. 5,200 base pairs (bp) that encode two or three oncoproteins—the tumor (T) antigens (large T [LT], small T [sT], and in the case of murine polyomavirus, middle T [mT]). Papillomaviruses have slightly larger circular double-stranded genomes (ca. 8,000 kb) and cause warts in various species. Major oncogenic papillomaviruses include bovine papillomavirus (BPV) and oncogenic strains of HPV. Papillomaviruses encode three oncoproteins: E6, E7, and E5. Adenoviruses are common respiratory viruses with intermediate-sized, linear double-stranded DNA genomes (ca. 30 kb). Certain strains of human adenoviruses can induce tumors in rodents and transform rodent cells in culture. The major oncoproteins of adenovirus are encoded by the E1 region. In addition, adenoviruses express some less well-studied oncogenes, including adenovirus type 9 E4-orf1, which is a major determinant of estrogen-dependent mammary tumor formation in female rats.²³⁵ Herpesviruses are enveloped viruses with large double-stranded DNA genomes (100–150 kb). Several viruses

of the gammaherpesvirus genus induce tumors in their host species including humans. Oncogenic human herpesviruses include Epstein-Barr virus (EBV—primarily lymphomas and nasopharyngeal carcinomas) and KSHV (primarily Kaposi's sarcoma). Herpesviruses encode multiple oncoproteins. Another virus with a small DNA genome, hepatitis B virus, can cause tumors, but its mode of action remains poorly defined.

The mechanisms of action of many DNA tumor virus oncogene products are known in considerable detail, and several common features of DNA virus transformation have emerged. DNA virus oncoproteins are required for normal virus replication, typically acting during the early phase of infection prior to the onset of viral DNA replication or active in replication itself. Most DNA viruses encode more than one oncoprotein, which often cooperate to transform cells, in some cases because one protein negates the deleterious effects of the other one. Most commonly, these oncoproteins function by binding to cellular proteins and modulating their activities (Table 7.5). Often, a single viral oncoprotein can bind to numerous cellular targets. As is the case for transduced retroviral oncogenes, some DNA tumor virus oncoproteins stimulate cellular mitogenic

TABLE 7.5 DNA Virus Oncoproteins and Their Major Targets

Adenovirus E1A	Rb family members, p300/CBP, CtBP
Adenovirus E1B 19K	Bak, Bax
Adenovirus E1B 55K	p53
Adenovirus E4orf6	p53
Bovine papillomavirus E5	PDGF- β receptor
Epstein-Barr virus EBNA2	RBP-J κ /CBF1, glycogen synthetase kinase
Epstein-Barr virus LMP1	Tumor necrosis factor signaling components, PI3K
Epstein-Barr virus LMP2	<i>Src</i> family members
Hepatitis B virus X protein	p53
Herpesvirus saimiri STP	Tumor necrosis factor signaling components
Herpesvirus saimiri Tip	<i>Src</i> family members
Human papillomavirus E5	<i>Src</i> family members
Human papillomavirus E6	EGF receptor
Human papillomavirus E7	p53, PDZ proteins, E6-AP, DNA repair and apoptosis machinery
KSHV K-bZIP	Rb family members, p21, p27, p600
KSHV ORF 50	p53
KSHV vCyclin	p53
Polyomavirus large T antigen	Cyclin-dependent kinase 6
Polyomavirus middle T antigen	p53 (not murine polyomavirus), Rb family members
Polyomavirus small T antigen	<i>Src</i> family members, PI3K, PP2A, shc
	PP2A

EBNA, Epstein-Barr virus nuclear antigen; EGF, epidermal growth factor; KSHV, Kaposi sarcoma herpesvirus; LMP, latent membrane protein; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PP2A, protein phosphatase 2A.

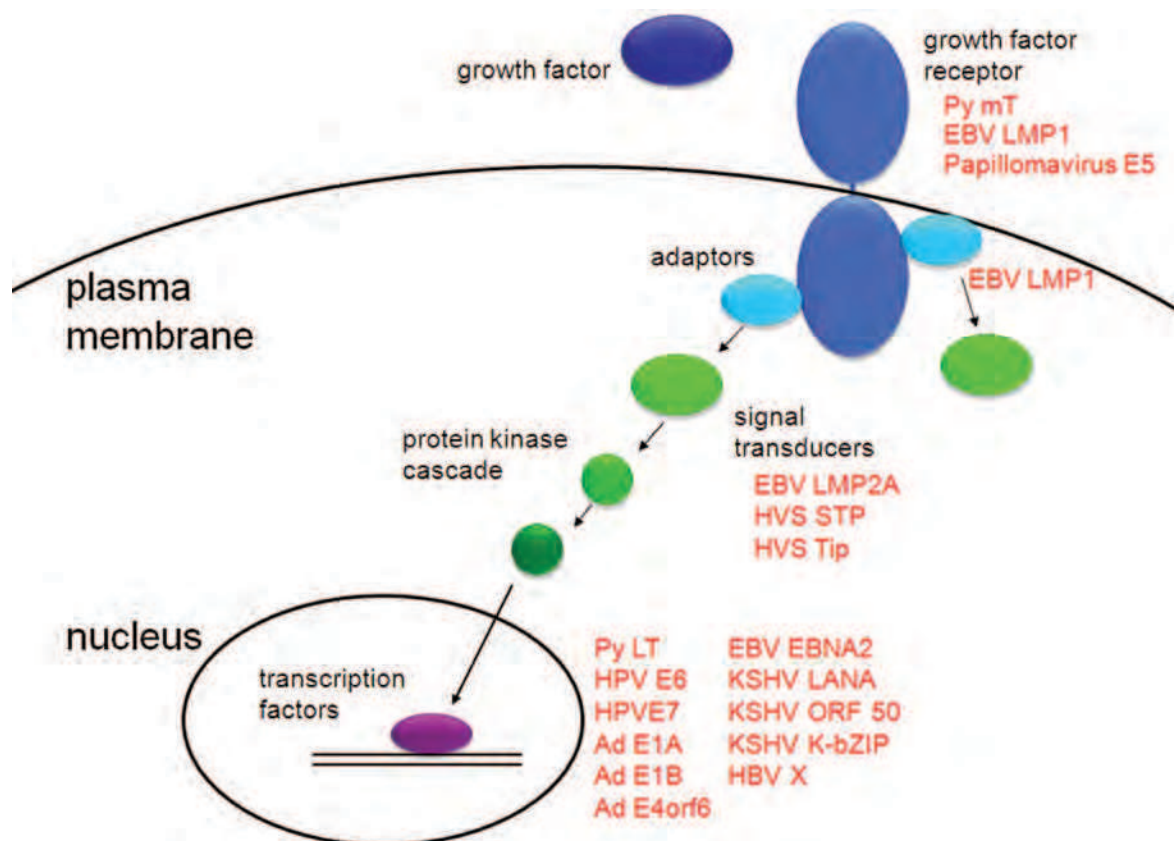


FIGURE 7.9. Mitogenic signal transduction pathways and DNA virus oncogenes. In this diagram of mitogenic signal transduction, DNA tumor virus oncoproteins that mimic or modulate cellular signaling components are listed in red.

signal transduction pathways (Fig. 7.9), and these examples are described first. In a mechanism of action that is shared among several DNA viruses, other oncoproteins inactivate the Rb and p53 cellular tumor suppressor pathways (Fig. 7.9). It is striking that diverse DNA tumor viruses modulate a limited number of common cellular targets, implying that there are relatively few crucial nodes that regulate cell proliferation (Table 7.6).

DNA Virus Oncoproteins That Stimulate Mitogenic Signaling Pathways

Polyomavirus Middle T Antigen. mT is responsible for the major transforming activity of murine polyomavirus in established lines of rodent cells and can induce a variety of cancers in animals.⁴⁶⁸ mT is also important for productive polyomavirus infection by regulating viral DNA replication, transcription, and virion assembly.¹⁶⁰

mT is a 55-kD, largely cytoplasmic protein that is anchored in the plasma membrane by a C-terminal transmembrane segment (Fig. 7.10). Membrane association is required for mT activity, but the native mT transmembrane domain can be replaced by certain other membrane-targeting sequences.¹⁴⁴ The first clue to the mechanism of mT function came from biochemical studies showing that mT associates with protein kinase activity.¹³⁴ Further analysis revealed that mT is not an enzyme, but rather associates with a cellular nonreceptor tyrosine kinase. This cellular enzyme turned out to be none

TABLE 7.6 Common Targets of DNA Virus Oncogenes

<i>p53</i>	SV40 large T antigen High-risk HPV E6 Adenovirus E1B 55K and E4orf6 KSHV K-bZIP, LANA, and ORF 50 Hepatitis B virus X protein
<i>Rb</i> family members	Polyomavirus large T antigens (e.g., murine polyomavirus, SV40, Merkel cell polyomavirus) High-risk HPV E7 Adenovirus E1A KSHV LANA
<i>Src</i> family members	Middle T antigen EBV LMP2A HVS STP and Tip
<i>PI3K</i>	Polyomavirus middle T antigen, EBV LMP1
<i>PP2A</i>	Polyomavirus small T antigen and middle T antigen

EBV, Epstein-Barr virus; HPV, human papillomavirus; HVS, *Herpesvirus saimiri*; KSHV, Kaposi sarcoma herpesvirus; LANA, latency-associated nuclear antigen; LMP1, latent membrane protein 1; PI3K, phosphatidylinositol 3-kinase; PP2A, protein phosphatase 2A; SV40, simian virus 40.

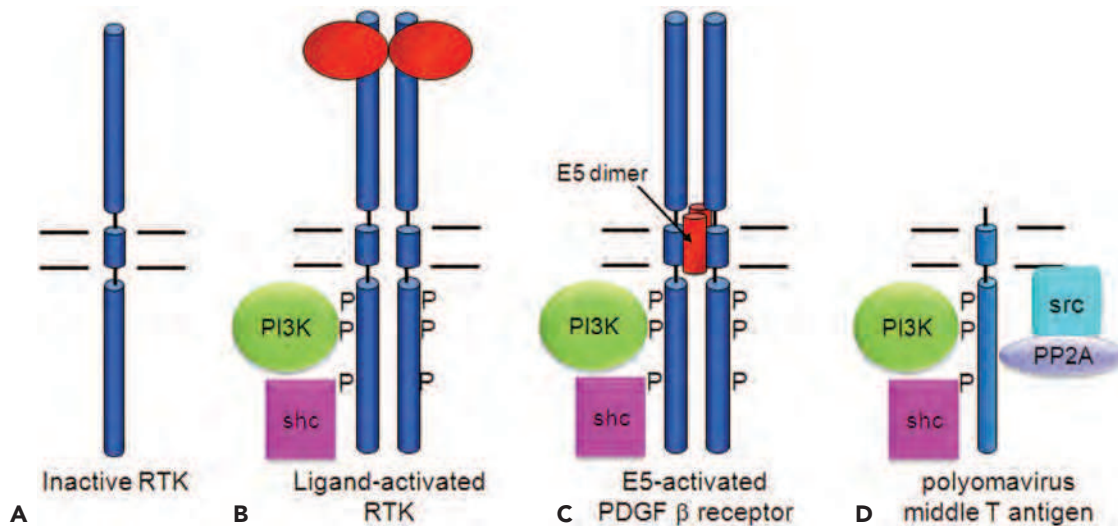


FIGURE 7.10. DNA virus oncoproteins and receptor tyrosine kinases. **A:** An unstimulated receptor tyrosine kinase (RTK) exists as unphosphorylated monomer with an extracellular ligand binding domain, a membrane-spanning segment, and an intracellular catalytic domain. Horizontal lines represent the plasma membrane. **B:** Treatment with ligand induces receptor dimerization and tyrosine phosphorylation, which recruits various signaling proteins to the receptor. To simplify the drawing, only a single molecule of two substrates is shown. **C:** A dimer of the bovine papillomavirus E5 oncoprotein interacts the transmembrane domain of two molecules of the platelet-derived growth factor- β (PDGF- β) receptor, resulting in receptor dimerization, autophosphorylation, and recruitment of signaling proteins. **D:** Murine polyomavirus middle T antigen (mT) is anchored into the plasma membrane by a carboxyl-terminal transmembrane domain. Protein phosphatase 2A (PP2A) binds to the cytoplasmic domain of mT and recruits pp60^{c-src} (src), which catalyzes mT tyrosine phosphorylation and generates binding sites for signaling proteins.

other than pp60^{c-src}, the product of the *c-src* proto-oncogene!⁹⁶ mT also associates with some other members of the *src* kinase family.¹⁶⁰ Biochemical studies showed that mT activated the tyrosine kinase activity of pp60^{c-src} by preventing an inhibitory phosphorylation event at Y^{527,37,94}. These experiments drew the first connection between RNA and DNA tumor viruses and suggested that DNA viruses transformed cells, at least in this case, by causing biochemical activation of a cellular proto-oncogene product. This, of course, is what happens during oncogene transduction by the acute transforming retroviruses, but in the case of mT, activation was induced by binding to a viral protein and not by structural alterations in the proto-oncogene product itself. Nevertheless, these results provided a satisfyingly unified view of tumor virus action.

The importance of *c-src* for mT transformation was illustrated by experiments showing that down-regulation of *c-src* expression by RNA interference attenuated mT-mediated transformation.³ Similarly, knockout of *src* family members can inhibit tumor formation in mice expressing mT, but the interpretation of these experiments is complicated by redundancy between different members of the *src* family.^{160,464}

mT also binds to protein phosphatase 2A (PP2A), an important regulatory serine/threonine kinase.^{342,480} The major role of PP2A in mT function appears to be as a scaffolding factor that recruits *src* family members to the mT/PP2A complex. Consequently, mT mutants unable to bind PP2A are defective for pp60^{c-src} binding and transformation. Once bound to wild-type mT, activated pp60^{c-src} phosphorylates mT on several cytoplasmic tyrosine residues, thereby generating specific binding sites for cellular signaling proteins including

PI3K, phospholipase C γ , and shc, many of which are then tyrosine phosphorylated and activated by the *src* family kinase.^{95,462} Although the results are complex and influenced by the cell type studied, mutating these phosphorylation sites on mT often caused transformation and tumor-forming defects, suggesting that these binding partners are important for transformation.¹⁶⁰ For example, mutation of an asparagine-proline-threonine-tyrosine (NPTY) sequence in mT prevented transformation, but insertion of an ectopic NPTY motif elsewhere in mT restored the activity to the original transformation-defective mutant, suggesting that this motif functions as a modular protein-protein interaction motif.¹²⁶ Biochemical studies demonstrated that phosphorylation of the tyrosine in the NPTY motif generates a binding site for the adaptor protein shc.^{56,121} Once bound to mT, shc is tyrosine phosphorylated by pp60^{c-src}, allowing it to bind the SH2 domain of Grb2. Grb2 then recruits Sos to the mT signaling complex, and ras-MAPK signaling is activated.

Another major phosphorylation-dependent binding partner of mT is the p85 regulatory subunit of PI3K.^{246,493} Indeed, it was through studies of mT that PI3K was identified as a lipid kinase that phosphorylated phosphatidylinositol bisphosphate (PIP2) to generate the second messenger, PIP3. In mouse models of mT-mediated tumorigenesis, genetic knockout of the p110 α catalytic subunit of PI3K prevented tumor formation, highlighting the importance of PI3K signaling in mT transformation.⁴⁷¹ The main role of PIP3 is to activate proteins that contain pleckstrin homology (PH) domains by recruiting them to the plasma membrane. Targets of this mechanism include the kinases PDK1 and Akt,

which in turn phosphorylate their own substrates and regulate numerous important cellular processes, including apoptosis and cell growth.¹⁰¹

On the basis of these experiments, the following view of mT action has emerged. Membrane-anchored mT binds to PP2A, which allows it to bind and activate pp60^{c-src}, which in turn catalyzes tyrosine phosphorylation of the cytoplasmic domain of mT. This results in the recruitment and activation of a constellation of signaling molecules, which activate ras-MAPK signaling and block apoptosis. mT, then, can be viewed as a mimic of a constitutively active growth factor receptor, which assembles a phosphotyrosine-dependent signaling complex containing many of the same proteins at the cell membrane (Fig. 7.10).

Papillomavirus E5 Proteins. The BPV E5 gene can cause morphologic and tumorigenic transformation of cultured fibroblasts, reflecting the ability of BPV to cause fibroblastic tumors in animals. Only 44 amino acids long, E5 is the smallest autonomous oncoprotein. Even more strange is its extremely hydrophobic amino acid composition, resembling a membrane-anchoring domain of a transmembrane protein. Indeed, the E5 protein is essentially an isolated transmembrane domain that exists in the intracellular membranes of transformed cells as a disulfide-linked dimer.^{51,406,461} The role of E5 in the BPV life cycle is not known.

Analysis of transformed cells revealed that the E5 dimer simultaneously binds to the transmembrane domains of two molecules of the PDGF- β receptor, causing ligand-independent receptor dimerization and activation^{185,273,356,357} (Fig. 7.10). This results in tyrosine phosphorylation of the cytoplasmic domain of the receptor, recruitment and activation of cellular signaling proteins, and mitogenic signaling.¹²⁷ Thus, like *v-sis*, the oncogene of the simian sarcoma retrovirus, the E5 protein causes constitutive activation of the PDGF receptor. However, E5 does not resemble the natural ligand and uses entirely different biochemical interactions to drive receptor activation.

Numerous genetic, biochemical, and pharmacologic studies demonstrated the importance of PDGF- β receptor activation for transformation by the E5 protein.⁴⁶¹ Most notably, cells lacking PDGF- β receptor expression are not susceptible to E5-mediated transformation unless the receptor gene is introduced into the cells.^{127,187,337,461} Interestingly, the E5 protein activates an immature, intracellular form of the PDGF- β receptor, suggesting that signaling occurs from an intracellular location.^{51,357}

The ability of the BPV E5 protein to activate the PDGF- β receptor by transmembrane interactions suggested that it might be possible to reprogram the E5 protein to bind to other transmembrane protein targets by changing the sequence of its transmembrane domain.¹⁶¹ Indeed, by screening a library expressing several hundred thousand E5-like proteins with randomized transmembrane domains, Cammett et al⁵⁵ isolated an artificial 44-amino acid transmembrane protein that caused ligand-independent activation of the human erythropoietin receptor, a cytokine receptor unrelated to the PDGF- β receptor. Even though this small transmembrane activator bears no biochemical resemblance to erythropoietin, it is able to stimulate erythroid differentiation of primary human hematopoietic cells *in vitro*. It may be possible to extend this approach to

isolate artificial small transmembrane proteins that interact with many other transmembrane targets, providing a new approach to modulate cell activity.

The BPV E5 protein also associates with a 16-kD transmembrane subunit of the vacuolar H⁺-ATPase and appears to inhibit its ability to acidify intracellular organelles.^{186,399} It has not been firmly established if this E5 activity, which is shared with HPV E5 proteins,⁹⁰ is required for E5-mediated cell transformation.

The high-risk HPV E5 proteins display weak transforming activity in cultured cells and can induce epithelial hyperplasia and contribute to the development of cancer in transgenic mice. These proteins are also hydrophobic and short (about twice the size of their BPV counterpart). The HPV E5 proteins appear to increase the sensitivity of the EGF receptor to EGF, but the biochemical mechanism for this activity remains obscure.⁴⁴⁹ A functional interaction of the HPV16 E5 protein and the EGF receptor is responsible for the growth-promoting effects of the HPV16 E5 protein in transgenic mice.¹⁷⁵ The B-cell-associated protein, Bap31, has also been implicated in the proliferative effects of the HPV16 E5 protein.³⁷⁷ Although the role of E5 in HPV replication is poorly defined, it appears to be required for efficient, differentiation-dependent viral DNA replication and expression of the late viral genes.^{150,174}

Epstein-Barr Virus and Herpesvirus Saimiri. Although herpesviruses normally grow lytically, they can also establish a state of viral latency in which a limited subset of genes (the latency genes) are expressed. Latently infected cells do not produce progeny virus, but the viral genome is maintained in the cells as an extrachromosomal plasmid. In some circumstances, latently infected cells can reactivate lytic viral gene expression and produce infectious virus. The cells in which lytic infection takes place can be different from those where latency is established. For example, EBV replicates lytically in epithelial cells of the oropharynx, whereas latency is established in infected lymphocytes. Tumors induced by the gammaherpesvirus, such as EBV and KSHV, often express the latency genes.

EBV can convert primary B lymphocytes into long-term lymphoblastoid cell lines.^{213,362,451} It can also transform rodent fibroblasts growing in culture. Like the small DNA tumor viruses, EBV contains multiple oncogenes. The first EBV oncogene identified was latent membrane protein 1 (LMP1), which can transform established lines of rodent fibroblasts.⁴⁸¹ LMP1 is also essential for EBV-mediated transformation of human B lymphocytes.^{250,354} In transgenic mice, LMP1 can induce epithelial and B-lymphocytic hyperplasia, which can progress to lymphoma.²⁷¹

LMP1 is a dimeric, integral membrane protein with six membrane-spanning domains and a 200-amino acid cytoplasmic, carboxyl-terminal tail (Fig. 7.11). Sequences in the carboxyl-terminal tail of LMP1 that are required for lymphocyte transformation bind to several proteins that mediate signaling by the tumor necrosis factor- α (TNF- α) receptor.^{230,231,322} These proteins include several TNF receptor-associated factors (TRAFs), receptor interacting protein (RIP), and a TNF receptor-associated death domain (TRADD) protein.^{229,230,231,322} Constitutive association of these proteins with LMP1 activates a number of cellular transcription factors, including NF- κ B, c-Jun N-terminal kinase (JNK), and p38, resulting in up-regulation of several antiapoptotic proteins

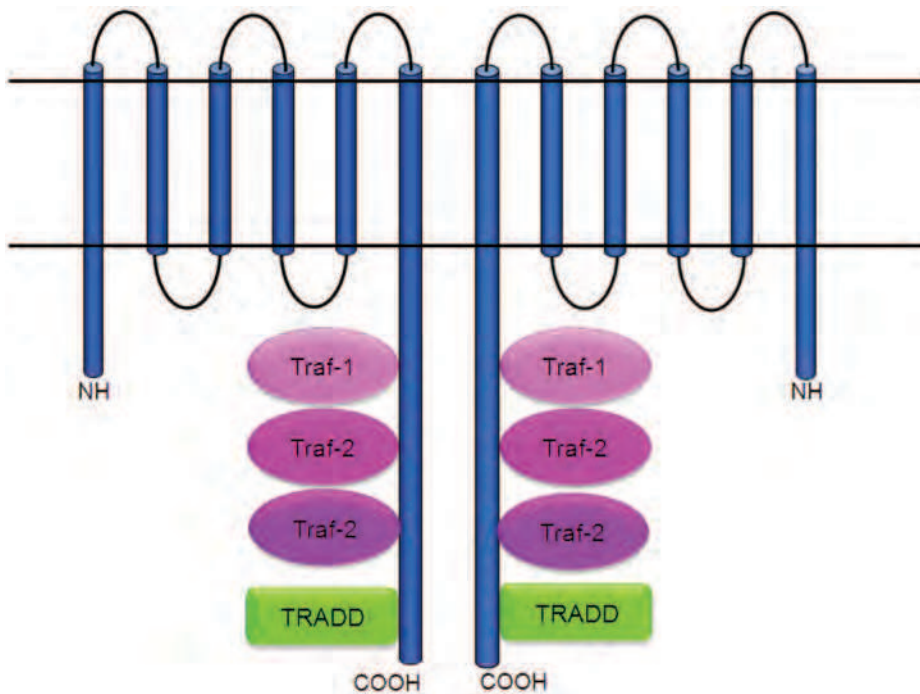


FIGURE 7.11. Structure of latent membrane protein 1 (LMP1). The figure shows a schematic diagram of a dimer of Epstein-Barr virus LMP1 protein, a multi-pass, transmembrane protein (blue). The carboxyl-terminal segment of LMP1 binds to a number of cellular signal transduction proteins in the tumor necrosis factor- α pathway. Horizontal lines represent the plasma membrane.

including Bcl-2 and survivin.^{140,141,384} The LMP1 sequences bound by the TNF signaling proteins are homologous to binding sites in the pro-survival CD40 form of the TNF receptor active in B lymphocytes. Thus, LMP1 mimics a constitutively active CD40 TNF receptor to deliver a pro-survival signal.⁴⁶⁹ The cytoplasmic tail of LMP1 also binds the p85 subunit of PI3K, which may contribute to its pro-survival effects by activating akt.¹⁰⁷

EBV also expresses a structurally related protein, LMP2A, which contains 12 transmembrane segments. In epithelial cells, LMP2A can induce features of transformation, but most studies of LMP2A have been carried out in lymphocytes. LMP2A binds to the *lyn* tyrosine kinase, a member of the *src* family, resulting in inhibition of B-cell antigen receptor signaling.^{54,313,314} Because B-cell receptor signaling induces EBV to enter the lytic replication cycle, LMP2A can inhibit viral reactivation and allow the persistence of latently infected cells. LMP2A also mediates B-lymphocyte survival by activating akt signaling.^{363,456}

Studies of replication-competent EBV strains unable to transform lymphocytes revealed that the Epstein-Barr virus nuclear antigen 2 (*EBNA2*) gene is required for lymphocyte transformation.^{87,190,203} The EBNA2 protein is a nuclear phosphoprotein that transactivates the expression of several EBV genes, including LMP1, as well as numerous cellular genes involved in cell proliferation, including *c-Myc* and *CD23*.⁴⁸² EBNA2 acts by forming a complex with the cellular site-specific DNA binding protein, RBP-J κ /CBF1.^{197,286} Once bound to DNA, EBNA2 recruits additional transcription factors to promoters to mediate gene induction.²⁴⁰ The activated cellular receptor for Notch also stimulates transcription through RBP-J κ , so EBNA2 can be regarded as a mimic of a constitutively active Notch receptor. Consistent with this view, activated Notch can partially restore B-cell-transforming activity to EBV lacking EBNA2.¹⁸⁹

EBV expresses additional antiapoptotic proteins that can contribute to tumorigenesis. For example, the *BHRF1* gene product is homologous to the cellular antiapoptotic protein, Bcl-2, and binds and inhibits the pro-apoptotic activities of a number of cellular pro-apoptotic proteins including Bim, Bak, and Puma.²¹⁴

Herpesvirus saimiri (HVS) infects nonhuman primates and causes T-cell lymphomas in new world monkeys. The STP oncogene of HVS, like EBV LMP1, transforms lymphocytes by activating NF κ B via TNF receptor signaling molecules.¹⁰⁴ The STP protein of subgroup A HVS also associates with pp60^{src}. The HVS Tip oncoprotein interacts with *lck*, another *src* family kinase, and inhibits T-cell receptor signaling.²⁹

DNA Virus Oncoproteins That Inactivate Rb or p53

As described in this section, several DNA tumor viruses inactivate the Rb pathway. This induces a p53-dependent stress response that can result in growth arrest or apoptosis, either of which would limit virus production.^{137,445} There are multiple pathways that lead to p53 activation. In one such pathway, E2F1 released by Rb neutralization increases expression of p19^{ARF}.⁵¹⁵ p19 in turn stabilizes p53 by interfering with mdm2, a ubiquitin ligase that would otherwise destabilize p53.⁴⁴⁷ To inhibit the antiviral response elicited by Rb inactivation, viruses engage mechanisms to block p53 function. Thus, many DNA tumor viruses inactivate p53 as well as Rb to neutralize the pro-apoptotic signals initiated by Rb inactivation. The one known apparent exception to this shared strategy among the small DNA tumor viruses is murine polyomavirus. Polyomavirus LT binds Rb but does not bind p53. mT also lacks p53 binding. Therefore, this virus evidently has developed a strategy to overcome the deleterious effects of Rb neutralization without expressing a protein that directly inactivates p53. Viral oncogenes that target Rb and p53 are described in the following sections.

Adenovirus Oncogenes. Early studies revealed that adenovirus-transformed cells invariably retained only the *E1A* and *E1B* genes at the left end of the viral genome, suggesting that these were the major viral oncogenes.^{123,167} Indeed, transfection studies demonstrated that these genes had transforming activity and that full transformation required both *E1A* and *E1B*.²⁴² The 12S *E1A* gene product can stimulate cellular DNA synthesis and immortalize cells on its own, but it requires a cooperating oncogene such as *E1B* or activated *ras* to cause full transformation of primary rat embryo fibroblasts.³⁸⁸ As is the case for most DNA tumor virus oncogenes, *E1A* and *E1B* are required for normal productive infection.

Adenovirus *E1A*. Comparison of the amino acid sequences of *E1A* proteins from various strains of adenoviruses revealed that they shared a number of conserved motifs including conserved region 2 (CR2), which contains the sequence leucine-X-cysteine-X-glutamic acid, where X can be any amino acid (the LXCXE motif) (e-Fig. 7.3). Mutational analysis demonstrated that CR2 was required for transformation. Strikingly, SV40 LT and HPV E7 proteins also contain required LXCXE motifs,^{242,495} even though they are otherwise unrelated to *E1A* or each other (e-Fig. 7.3). Substitution of the LT CR2 region into a transformation-defective *E1A* mutant lacking its own CR2 restored transforming activity, suggesting that CR2 might constitute a discrete binding site for a cellular protein required for transformation.³¹⁸ Based on the precedent of the association of mT and *src*, it seemed plausible that the CR2 motif of *E1A* bound to the product of a cellular oncogene and activated it, resulting in transformation.

To identify cellular proteins that bind to *E1A* and possibly mediate transformation, *E1A* antibodies were used to immunoprecipitate *E1A* and associated cellular proteins from infected cell extracts.^{206,507} A 105-kD *E1A*-associated protein was soon identified as the product of the *RB1* gene.⁴⁹⁴ *RB1* encodes the retinoblastoma tumor suppressor protein, p105^{Rb}, and had been previously implicated in retinoblastoma, a hereditary cancer of the eye. Notably, in retinoblastoma, *RB1* acts not as an oncogene, but rather as a tumor suppressor gene, whose loss predisposes individuals to tumor development. Mutations in *E1A* CR2 that blocked p105^{Rb} binding also inhibited transformation.³⁷⁶ Biochemical and gene transfer experiments showed that the association of *E1A* and p105^{Rb} freed E2F from repressive Rb/E2F complexes, resulting in increased transcription of E2F-regulated genes^{16,71,376} (Fig. 7.12). These seminal and at the time surprising findings showed that viral oncogenes can transform cells not only by activating the products of cellular proto-oncogenes but also by inactivating cellular proteins that inhibit cell proliferation. The ability of *E1A* to inactivate p105^{Rb} was foreshadowed by the observation that injection of adenovirus type 12 into the eyes of newborn rats caused retinoblastoma.²⁶⁰

Two additional members of the retinoblastoma tumor suppressor family, p107 and p130, also bind *E1A*. Because all three Rb family members evidently share a structural pocket that binds to the LXCXE motif on various viral oncoproteins, these Rb family members are sometimes referred to as the pocket proteins.

In addition to CR2, conserved sequences in the amino-terminus of *E1A* (CR1) are also required for induction of DNA synthesis and transformation (but not for Rb binding),

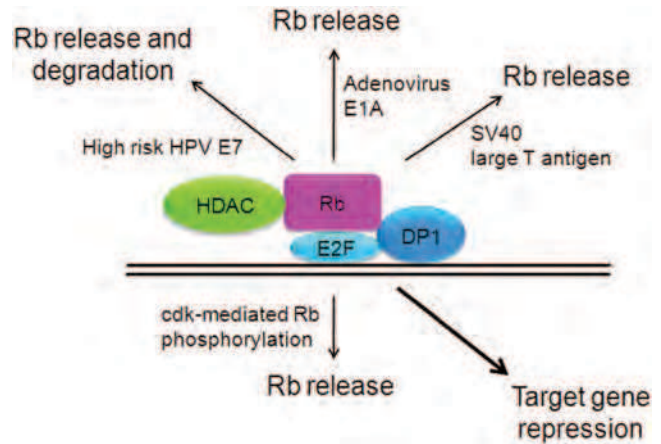


FIGURE 7.12. Interaction between retinoblastoma proteins and DNA tumor virus oncoproteins. Retinoblastoma (Rb) family members in complex with an E2F/DP1 heterodimer recruit histone deacetylases (HDACs) to promoters, resulting in their repression. Horizontal lines represent double-stranded DNA. Repressive complexes are disrupted in uninfected cells by cdk-mediated phosphorylation of Rb, allowing E2F to stimulate transcription. The effects of oncoproteins from three unrelated DNA tumor viruses are shown above the DNA.

implying that there are additional cellular targets of *E1A* involved in transformation.⁴⁹⁵ These sequences bind to p300 and CBP, closely related histone acetyl transferases that are co-activators for the CREB transcription factor.⁶ These proteins presumably contribute to transformation by directly regulating the activity of cellular genes. SV40 LT and high-risk HPV E7 also bind p300/CBP, as well as Rb. The extreme C-terminus of *E1A* binds a cellular protein, CtBP, a transcriptional co-repressor.³⁹⁷ Deletion of this binding site affects the ability of *E1A* to cooperate with other oncogenes.⁷⁷

Adenovirus *E1B*. Co-expression of adenovirus *E1A* and *E1B* genes can cause cell transformation.²⁴² However, in the absence of *E1B*, acute expression of *E1A* causes a burst of cellular DNA synthesis followed by a dramatic cellular phenotype involving degradation of viral and cellular DNA and cell death.^{8,359,370,491} This phenotype is now recognized as apoptosis and is a response in large part to the unscheduled DNA synthesis elicited by the inactivation of Rb by *E1A*. Thus, a major function of *E1B* is to counter *E1A*-induced apoptosis.^{110,370} How does *E1B* accomplish this? There are two unrelated *E1B* protein products encoded by alternatively spliced mRNAs, each of which can independently cooperate with *E1A*. The 55-kD *E1B* product of adenovirus binds to p53, which is required for *E1A*-induced apoptosis, and neutralizes its activity.^{110,390,391,396} The importance of p53 inactivation for transformation was shown by the ability of dominant-negative p53 (see next section) to cooperate with *E1A* in inducing transformation of primary cells.³⁸⁸ The adenovirus E4orf6 protein can also cooperate with *E1A* to transform cells by inhibiting p53 function, and in cooperation with *E1B*-55K causes the degradation of p53.⁴³⁸ The complete absence of amino acid sequence homology between *E1B*-55K, E4orf6, SV40 LT, and HPV E6, proteins that all inactivate p53, is a striking example of convergent evolution and highlights the need of diverse DNA tumor viruses to interfere with p53 activity.

The smaller E1B gene product, E1B-19K, also inhibits E1A-mediated apoptosis.^{370,492} E1B-19K is homologous to the cellular *Bcl-2* gene family that regulates apoptosis.⁴²³ E1B-19K inhibits apoptosis by sequestering the pro-apoptotic family members Bak and Bax and preventing the formation of pores in the mitochondrial outer membrane and the release of pro-apoptotic proteins such as cytochrome c.⁴⁹²

SV40 Large T Antigen. LT is a multifunctional protein required for SV40 DNA replication, late gene expression, and other aspects of the viral lytic life cycle.² It is also the major oncoprotein of SV40, and expression of LT in transgenic mice can give rise to a number of tumors including choroid plexus tumors, retinoblastoma, pancreatic carcinoma, and intestinal hyperplasia. At least three segments of LT are required for transforming activity: a J domain and a conserved LXCXE motif in the amino-terminal half of the protein, and a distinct segment in the carboxyl-terminal half.² The relative importance of these segments depends on the transformation assay employed, but in some assays the amino-terminal half of LT was sufficient for transformation. In addition, a second early protein of SV40, small t antigen, which shares amino acid sequences with the amino-terminus of LT, can contribute to transformation under some conditions, apparently by inhibiting PP2A.^{30,392,505,510}

Soon after it was discovered that adenovirus E1A bound p105^{Rb}, LT was also found to bind the Rb family.¹¹² Furthermore, assay of a series of point mutations in the LT LXCXE motif demonstrated that Rb binding was required for transformation. The molecular chaperone function of the J domain of LT is also required for Rb inactivation by recruiting an Hsp70 activity to remodel the Rb/E2F complexes and release free E2F to activate cellular genes required for cell proliferation.^{111,424,450} Thus, two otherwise unrelated transforming proteins, adenovirus E1A and SV40 LT, shared a common property, namely, the ability to bind to and neutralize Rb proteins (Fig. 7.12).

Rb family members are not the only cellular proteins bound by LT. In fact, the well-known tumor suppressor protein, p53, was first discovered as an LT binding protein. The analysis of p53 was initially confusing and is a tale worth telling. Antibodies that recognized LT also co-immunoprecipitated a cellular protein called p53.^{276,287} In general, p53 levels were high in LT-transformed cells. p53 binding mapped to the C-terminal segment of LT required for transformation, and LT mutants unable to bind p53 were transformation defective.^{2,516} In addition, molecular clones of the *p53* gene from immortalized mouse cell lines displayed transforming activity in cooperation with validated oncogenes such as *v-ras*, and the *p53* gene was amplified in human sarcomas.^{142,236,341,345} These results strongly suggested that p53 was an oncogene product. This led to the conclusion that LT bound and activated the product of a cellular oncogene (namely, p53), resulting in transformation.

Results soon accumulated that challenged this apparently straightforward interpretation. First, some proviral integration sites in retrovirus-induced leukemias actually disrupted the *p53* gene, rather than activated it.²⁴¹ Second, inactivating p53 mutations were frequently found in naturally arising sporadic human tumors, as well as in some familial cancers, where mutant p53 displayed a pattern of inheritance consistent with tumor suppressor function.^{284,298} Mice engineered to lack p53 displayed a higher incidence of spontaneous tumor forma-

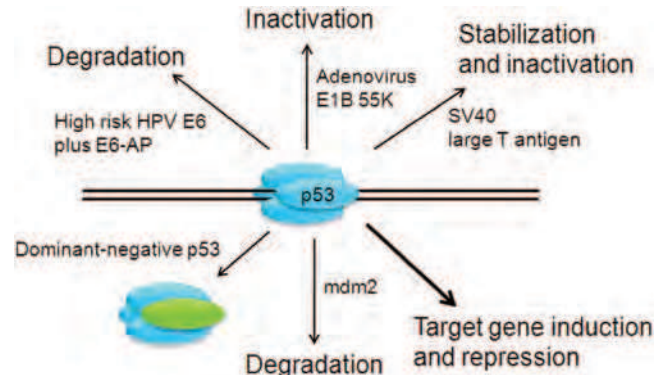


FIGURE 7.13. Interaction between p53 and DNA tumor virus oncoproteins. A tetramer of p53 bound to double-stranded DNA is shown in blue at the middle. The ability of p53 to regulate gene expression can be inactivated in uninfected cells by mdm2-mediated p53 degradation or by expression of a dominant negative mutant of p53, which forms an inactive tetramer. The effects of three oncoproteins from unrelated DNA tumor viruses are shown above the DNA.

tion.¹²⁴ In addition, the interaction with LT did not stimulate the transcriptional activity of p53 but rather blocked it.³¹² Finally, independent molecular clones of the *p53* gene from primary, nonimmortalized cells did not display transforming activity, but inhibited cell growth.^{11,156} The role of p53 was finally resolved with the discovery that the original cloned *p53* genes that displayed transforming activity were actually mutants. The wild-type *p53* gene acts not as an oncogene but as a tumor suppressor gene.²⁸⁴ Thus, as was the case with E1A or LT with Rb, LT transforms cells in part by inactivating the tumor suppressor activity of p53 (Fig. 7.13).

Certain p53 mutants with transforming activity are said to be dominant negative, because they hetero-oligomerize with the wild-type version of p53 and neutralize its activity.²⁸⁴ Expression of such a mutant effectively titrates active p53 from the cell and is functionally equivalent to the loss of both p53 alleles, as is the case for a classic inherited tumor suppressor gene. Such p53 mutations are some of the most common genetic alterations in human cancer.

Human Papillomavirus Nuclear Oncogenes. E6 and E7 encode short nuclear proteins (~100 amino acids), which are required for productive infection.¹⁵⁸ The high-risk HPV *E6* and *E7* genes cooperate to immortalize primary human keratinocytes, the progenitor cells of cervical and other squamous cell cancers; block the terminal differentiation of these cells; and disrupt genomic integrity.^{22,209,325,405,490} The ability of HPV to immortalize cultured cells correlates with their oncogenic potential in patients—proteins from the high-risk HPV types have increased activity in comparison to the low-risk proteins.^{17,353,446,499} The *E6/E7* genes from high-risk HPV can also induce a variety of tumors in transgenic mice, including cervical cancer in mice chronically treated with estrogen.⁷ In addition, the high-risk but not low-risk E7 protein can stimulate DNA synthesis in resting cells and transform rodent cells either on its own or in cooperation with the *v-ras* oncogene.^{244,479} As is the case for LT and E1A/E1B, a major activity of E6 and E7 is to neutralize p53 and Rb.

Human Papillomavirus E7. The E7 protein is not an enzyme, but rather modulates the activity of regulatory proteins encoded by the host cell. Like LT and E1A, the HPV E7 protein contains an LXCXE motif that binds to the Rb family pocket proteins.^{133,326} This results in the displacement of Rb proteins from E2F family members and in degradation of Rb^{45,71} (Fig. 7.12). Although the LXCXE motif is sufficient for Rb binding, sequences outside of this motif are required for degradation and complete inactivation of Rb. The mechanism of Rb degradation is not understood completely but appears to involve the assembly of a cullin 2 ubiquitin ligase complex with Rb, followed by proteosomal degradation.²²⁰

Repression of E7 expression in cervical cancer cells results in the loss of hyperphosphorylated Rb and the induction of the hypophosphorylated, growth inhibitory form of Rb that binds E2F.¹⁸⁸ This in turn results in repression of E2F-responsive genes and growth arrest, demonstrating that continuous association with E7 is required for sustained Rb inhibition.

Several lines of evidence suggest that the transforming activities of E7 depend on its ability to neutralize the Rb family. Both high-risk and low-risk E7 proteins contain the LXCXE motif, but the affinity of HPV E7 proteins for p105^{Rb} correlates with the oncogenic potential of the virus: high-risk E7 binds p105^{Rb} tightly; low-risk E7 binds it poorly.^{166,326} These differences map to a single amino acid immediately adjacent to the LXCXE motif, which determines the ability of E7 to transform rat kidney cells in cooperation with the *ras* oncogene.²¹² In addition, E7 can be replaced in an E6-dependent keratinocyte immortalization assay by down-regulation of the cdk inhibitor p16^{INK4a}, a manipulation that also inactivates p105^{Rb} by allowing its phosphorylation and release from E2F.²⁵⁵ These results suggest that inactivation of the Rb family by E7 is important for cell transformation, keratinocyte immortalization, and cervical carcinogenesis. On the other hand, transformation assays carried out with E7 mutants defective for various activities have not yielded consistent results, although some experiments suggest that Rb binding is essential for transformation.^{237,309} In transgenic mice, the ability of E7 to neutralize Rb function is tightly linked to its ability to support DNA synthesis in differentiating epithelial cells, but not for cervical carcinogenesis.^{13,14}

The E7 proteins can also affect cellular checkpoint control. High-risk E7 interacts with two cdk inhibitors, p21^{CIP1} and p27^{KIP1}, and abrogates their growth inhibitory effects.^{116,165,432,513} These E7 activities may also contribute to the ability of E7 to induce resistance to growth inhibitory cytokines such as transforming growth factor- β , tumor necrosis factor, and interferon.^{18,19,348,358} The signals mobilized by the E7 protein maintain the proliferative status of suprabasal keratinocytes, which otherwise undergo terminal differentiation and become unable to support DNA synthesis. These signals thereby allow viral DNA replication and the production of progeny virus in the stratified epithelium.^{14,76}

The E7 proteins bind to a large number of additional cell proteins.³⁰⁹ Some of these binding partners, such as various cyclins and E2F family members, are likely to reinforce the activities mentioned previously. p600, an Rb-associated protein, associates with both high-risk and low-risk E7 and appears to allow the survival of cells in the absence of anchorage and may thus contribute to malignancy.^{115,221} Other E7 binding partners include histone-modifying enzymes and members of the polycomb group transcriptional repressor complexes,

suggesting that E7 may affect epigenetic status.³⁰⁹ However, the biological relevance of most of the interactions of the E7 protein with cellular proteins is not known.

Cervical cancer cells are often aneuploid and contain other chromosomal abnormalities, which presumably play a role in malignant progression. The high-risk E7 protein induces genomic instability in part by interfering with the synthesis or function of centrosomes, the subcellular structures that form the poles of the mitotic spindle to ensure proper chromosome segregation during mitosis.¹²⁸ Primary human epithelial cells expressing E7 often contain abnormal or supernumerary centrosomes due to the uncoupling of centriole synthesis from cell cycle progression. The molecular mechanism by which E7 interferes with centrosome synthesis is not known in detail, but it is at least in part independent of Rb targeting and may involve interactions with cellular proteins involved in centrosome activity or mitosis.^{129,334}

Human Papillomavirus E6. Like the E7 protein, the HPV E6 protein lacks enzymatic activity and functions by modulating the activity of cellular proteins.²¹⁸ The high-risk E6 protein is able to immortalize human mammary epithelial cells on its own,¹⁵ but most analyses of E6 have been conducted in keratinocytes and focus on its ability to cooperate with E7. A number of the signaling pathways stimulated by the E7 protein, including unscheduled DNA synthesis due to Rb inactivation, tend to drive cells into apoptosis.^{137,445} A major role of E6 is to limit this deleterious cellular response to E7 expression.

The E6 protein from the high-risk HPV types binds to a number of cellular proteins and induces their ubiquitin-mediated degradation.²¹⁸ The proximal target of the E6 protein is the E3 ubiquitin ligase, E6-associated protein (E6-AP).^{222,402} The direct binding of E6 to a leucine-X-X-leucine-leucine motif on E6-AP reprograms it to ubiquitylate a number of cell proteins, which leads to their proteasome-mediated degradation.^{400,402} The most prominent target of E6/E6-AP is p53, which is normally a substrate of a different ubiquitin ligase, mdm2, not E6-AP.^{74,208,489,502} (Fig. 7.13). Although p53 is wild type in most cervical carcinoma cell lines, it has a short half-life and its levels are low in these cells because of E6-mediated degradation.^{312,401} As a consequence, p53-dependent checkpoint controls, for example, in response to Rb inactivation or DNA damage, are blunted although not necessarily eliminated in cells expressing high-risk E6 proteins.^{53,490} The low-risk E6 proteins do not induce p53 degradation. Mutant p53 can replace HPV16 E6 in inducing keratinocyte immortalization, implying that E6-mediated p53 inactivation is important for this activity.⁴¹⁴ In addition to E6-AP-mediated degradation, there appear to be several other mechanisms utilized by E6 to attenuate p53 activity.²¹⁸ Repression of E6 expression in cervical cancer cells results in the elevation of p53 levels and restoration of p53 signaling, indicating that continuous association with E6 is required for sustained p53 inhibition.¹¹³

The E6 proteins also exert p53-independent antiapoptotic effects.²⁷² High-risk E6 blocks several steps in the extrinsic apoptosis signaling cascade by interacting with a death receptor (tumor necrosis factor receptor-1), the FAS-associated via death domain (FADD) adaptor protein, and caspase-8.¹⁵⁵ E6 also inhibits the intrinsic apoptotic pathway by binding to the pro-apoptotic molecule Bak and inducing its proteasome-dependent degradation, thereby blocking release of apoptotic mediators from

mitochondria.⁴⁶⁵ In addition, HPV16 E6 and E7 can up-regulate two cellular inhibitors of apoptosis, c-IAP2 and survivin, which act farther downstream in the apoptosis signaling cascade.^{40,233,512} In concert, this barrage of antiapoptotic action prevents cell death and sets the stage for ongoing proliferation in response to E7 signals. Indeed, the ability of E6 from some HPVs to inhibit ultraviolet light-induced apoptosis has been proposed to be a prime contributor in HPV-associated skin cancer.²³²

In E6-expressing cells, E6-AP (and possibly other ubiquitin ligases) also ubiquitylates and causes the degradation of a series of PDZ domain-containing proteins^{171,303} (Fig. 7.13). These proteins are recruited to the E6/E6-AP ubiquitylation complex by binding to a serine/threonine-X-valine consensus PDZ domain binding motif at the extreme C-terminus of high-risk E6 proteins.^{256,280} These PDZ proteins include a number of proteins with presumed tumor suppressor activity, including Scribble, hDlg, and MAGI-1, which regulate signal transduction, cell-cell contact, and cell polarity. Loss of these proteins disrupts the formation of epithelial tight junctions and may contribute to the invasiveness of cervical cancer cells.^{269,486} E6 also promotes the degradation of several PDZ domain-containing protein tyrosine phosphatases, which regulate growth factor signaling pathways.⁴³⁶ The biological importance of PDZ binding was demonstrated by the finding that deletion of the PDZ binding motif from E6 affected viral genome replication and abrogated its ability to transform keratinocytes and stimulate epithelial hyperplasia in transgenic mice.^{278,335,486} Other viral oncoproteins, such as rhesus papillomavirus E7 (but not E6) and adenovirus E4-orf1, also bind PDZ proteins.⁴⁶⁶

Another consequence of E6 expression is transcriptional induction of the gene encoding hTERT, the catalytic subunit of telomerase, the ribonucleoprotein that maintains the ends of chromosomes.^{259,475} Introduction of hTERT can replace E6 in a keratinocyte immortalization assay,²⁵⁵ suggesting that hTERT induction is a crucial activity of E6. It has been proposed that E6-AP-mediated degradation of NFX1-91, a transcriptional repressor of the *hTERT* gene, is involved in hTERT induction by E6, although E6 probably exerts additional layers of control on telomerase.¹⁷⁷ Indeed, E6 also interacts with the telomerase complex and activates it posttranscriptionally.²⁹⁰ Other targets of E6-AP-mediated degradation include several GAPs, which regulate the activity of ras-like proteins, and proteins involved in DNA replication and repair.²¹⁸ E6 also inhibits O(6)-methylguanine-DNA methyltransferase, a crucial DNA repair enzyme,⁴³⁷ and the DNA repair scaffolding protein, XRCC1.²²⁸

In summary, it appears that the main biochemical activity of E6 is to redirect the E6-AP ubiquitin ligase to a number of cellular proteins with tumor suppressor or related activity, inducing their degradation. Knockout of the *E6-AP* gene in transgenic mice prevents the development of E6-mediated cervical cancer, demonstrating that E6-AP is required for this biological activity of HPV E6.⁴¹⁹

KSHV Oncogenes. Cells latently infected with KSHV express latency-associated nuclear antigen (LANA), a large, multifunctional nuclear protein required for the establishment and maintenance of the extrachromosomal viral genome.^{12,93} LANA can transform cultured fibroblasts in cooperation with activated *ras* and extend the life span of human endothelial

cells. In transgenic mice, LANA causes lymphocyte hyperplasia and, rarely, the development of lymphomas.^{147,368,485} Like the oncoproteins of many other DNA tumor viruses, LANA binds and neutralizes p53 and Rb.^{162,368,379} In addition, by binding to glycogen synthetase kinase 3 β , LANA modulates β -catenin and up-regulates expression of genes involved in cell proliferation such as cyclin D and c-myc.¹⁶³ The KSHV ORF50 and K-bZIP proteins also interfere with p53 activity.^{200,347}

The KSHV K1 protein contains two ITAM motifs and mimics signaling by the B-cell antigen receptor by activating the src family kinase *lyn*.³⁶⁵ The signals constitutively emanating from K1 inhibit apoptosis and can transform cells in a variety of settings.^{279,366} Another KSHV gene with transforming activity is *K12*, which encodes a series of active gene products.³²⁷ These include kaposin B, a mixture of short polypeptides that appear to function by activating the p38-MK2 pathway and stabilizing cellular mRNAs encoding pro-proliferative cytokines.³⁰⁸

In addition to the genes mentioned above, the KSHV genome also contains numerous genes that are clear homologs of cellular genes that stimulate proliferation or block apoptosis.³²⁸ These encode proteins that function as cytokines, a cyclin (v-Cyc), a constitutively active G-protein-coupled receptor (vGPCR), and a variety of other regulatory proteins. v-Cyc activates cdk6 that allow cell cycle progression.^{67,184} v-Cyc appears to be resistant to cdk inhibitors, and a v-Cyc/cdk6 complex can phosphorylate and inactivate p27^{KIP1}, itself a cdk inhibitor, thus reinforcing the pro-proliferative response caused by cdk-mediated phosphorylation of Rb.⁴⁵⁵ vIRF and vIL-6, homologous to interferon regulatory factors and interleukin-6, respectively, can also promote cell growth.^{69,169} vGPCR displays transforming activity in cultured fibroblasts and primary endothelial cells, and it can induce tumors that resemble KS in mice.⁹ Biochemical studies showed that vGPCR activates various mitogenic and pro-survival signaling pathways.^{58,316} KSHV also expresses at least three additional gene products that inhibit apoptosis: v-Bcl, which is homologous to Bcl-2 and acts by maintaining mitochondrial integrity; vIAP (viral inhibitor-of-apoptosis protein), which is homologous to a different cellular antiapoptotic protein, survivin; and vFLIP, which is thought to block apoptosis by inhibiting the Fas death receptor pathway or up-regulating NKkB.^{23,70,75,122,183,199,453,483} These activities contribute to lymphomagenesis in transgenic mice.⁸³ Several KSHV gene products also stimulate angiogenesis⁴³ in this highly vascularized tumor. For example, vGPCR signaling induces the expression of the angiogenic factor vascular endothelial growth factor (VEGF),⁵⁰⁶ and the KSHV chemokine viral macrophage inflammatory protein-1 (v-MIP-1) promotes angiogenesis by inducing VEGF expression and stimulating the chemotaxis of endothelial cells.⁴⁴⁴ Finally, it should be noted that herpesviruses express many gene products that down-regulate the innate and adaptive immune response. These activities are presumably of critical importance for tumor formation in otherwise immunocompetent animals.

Noncoding RNAs and Transformation. Many DNA tumor viruses express microRNAs and other noncoding RNAs (ncRNAs). EBER1 and EBER2, ncRNAs expressed by Epstein-Barr virus, can induce several features of transformation in cultured lymphocytes, including tumorigenicity in immunodeficient mice.^{263,387,504} The biochemical activities of the EBERs responsible for transformation are not known. KSHV encodes miR-K12-11 microRNA, which shows many

similarities to the growth-promoting cellular microRNA, miR-155.¹⁹¹ HVS encodes five small ncRNAs that form ribonucleoprotein complexes and induce the expression of numerous T-cell activation genes, at least in part by causing the degradation of specific cellular microRNAs.⁶³

VIRUSES AND HUMAN CANCER

Because viruses cause cancer in animals, it was natural to ask if they cause cancer in humans. The search for human tumor viruses began in earnest with the discovery of murine leukemia virus in the early 1950s¹⁹⁶ and took on special urgency in the 1960s with the startling discovery that some early stocks of the poliovirus vaccine were contaminated with live SV40, which has potent tumor-inducing activity in newborn hamsters.^{135,176,181} The SV40 contamination resulted from growing poliovirus in primary African green monkey kidney cells, some of which harbored SV40. Despite the suspected association between viruses and cancer, it took many years to assemble convincing evidence that viruses play an etiologic role in human cancer. Epidemiologic and laboratory studies designed to implicate viruses in human cancer were difficult because human tumor viruses do not act rapidly or efficiently. Rather, cancer arises only years or even decades after the initial infection, and most infected people do not get cancer, suggesting that virus infection represents only one step in carcinogenic progression and that additional cellular alterations must occur for cancer formation. In addition, many tumor virus infections (e.g., EBV, HPV, and HBV) are quite common, complicating attempts to forge epidemiologic links to specific cancers. Finally, virally induced tumors usually do not produce virus particles, obscuring the viral cause of some cancers.

Despite these challenges, six viruses are now generally recognized as playing causal roles in cancers that account for approximately 15% of all human cancer deaths worldwide^{310,317} (Table 7.7). These virus-induced cancers tend to be much more common in the developing world, where they can be the most common cause of cancer death. Virus-induced cancers also

occur relatively frequently in immunosuppressed individuals. Because viruses provide well-defined targets for prevention and therapy, virally associated cancers appear to represent a class of tumors that are particularly susceptible to rational prevention and treatment approaches.

Human Papillomaviruses

The HPVs cause papillomas or warts, benign tumors of epithelial cells in the skin and mucous membranes. Although warts are usually self-limited, genital warts (condyloma acuminata) and laryngeal papillomas can be serious medical conditions. The main medical importance of the HPVs derives from their association with human cancer.⁵¹⁸ High-risk strains of HPV such as HPV16 and HPV18, which were first discovered in cervical cancer samples,^{42,132} are responsible for virtually all cancers of the uterine cervix and a substantial fraction of other anogenital and oropharyngeal cancers in both men and women. Additional HPV types are associated with severe warts and skin cancer in immunosuppressed individuals.

As described earlier, the major oncogenes of the high-risk genital HPV are the *E6* and *E7* genes, which are continuously expressed in cancer cells.^{10,412} However, keratinocytes transformed *in vitro* by E6/E7 are not initially tumorigenic in experimental animals, implying that HPV-infected cells must accumulate additional mutations or other events to form cancers.^{131,224} This requirement for additional, presumably rare events may account for the observation that most people infected with high-risk HPV do not develop cancer. It is also likely to account for the long period of time that usually passes between infection and development of cancer. In most cervical cancers, the gene encoding the HPV E2 transcription factor is disrupted or its action is inhibited by mutation or epigenetic modification.⁸¹ This prevents E2-mediated repression of the promoter driving E6 and E7 transcription, and the resulting increased expression of the oncogenes presumably plays a role in carcinogenesis.

The finding that the E6 and E7 proteins from the high-risk HPV types are more effective at inhibiting the p53 and Rb tumor suppressor pathways than are the low-risk types, such as HPV6 and HPV11, implies that these interactions are important in human carcinogenesis.^{166,326} Similarly, the presence of wild-type *p53* and *Rb* genes in most cervical cancers implies that these pathways are neutralized by a nonmutational mechanism in these cells (i.e., by the expression of the viral oncoproteins).^{401,501} Continuous expression of the HPV *E6* and *E7* genes is required to maintain the Rb and p53 pathways in an inactive state in cervical carcinoma cells, and repression of E6/E7 rapidly activates these pathways and inhibits cell survival and proliferation.^{188,225,439,477} The third viral oncogene, the *E5* gene, is expressed in some but not all cervical cancers, and its role in human carcinogenesis is unclear.

Prophylactic vaccines formulated from virus-like particles (VLPs) composed of L1, the major HPV capsid protein, are very effective at raising neutralizing antibodies to the HPV types in the vaccine and preventing infection with these HPV types.^{267,403} These vaccines are likely to prevent many cases of HPV-associated cancers, but because of the long lag between the time of initial infection and cancer development, it will take many years to document this effect. In addition, a therapeutic vaccine composed of HPV16 E7 peptides has shown

TABLE 7.7 Human Tumor Viruses

Epstein-Barr virus	Burkitt lymphoma Nasopharyngeal carcinoma Hodgkin disease Gastric carcinoma
Hepatitis B virus	Hepatocellular carcinoma
Hepatitis C virus	Hepatocellular carcinoma Non-Hodgkin lymphoma
Human papillomaviruses	Cervical carcinoma Other anogenital carcinomas Oropharyngeal carcinoma Nonmelanoma skin cancer
Human T-lymphotropic retrovirus	Adult T-cell leukemia/lymphoma
Kaposi sarcoma-associated herpesvirus	Kaposi sarcoma Multicentric Castleman disease Primary effusion lymphoma

promise in treating women suffering from HPV16-associated vulvar neoplasia.²⁵³

HPV-associated squamous cell carcinoma of the head and neck primarily occurs in the oropharynx and tonsils, and its incidence is rising rapidly. HPV-associated cancer appears to be less aggressive than classic head and neck cancer, which is associated with tobacco and alcohol use, p53 mutations, and low levels of the cdk inhibitor p16.⁴ HPV16 DNA is the predominant HPV type found in these cancers, and repression of HPV16 E6/E7 in head and neck cancer cell lines can inhibit their proliferation.³⁶⁹ Prophylactic VLP vaccines developed to prevent cervical cancer are likely to be effective in preventing head and neck cancer as well.

Epstein-Barr Virus

EBV virus is a ubiquitous human gammaherpesvirus that causes infectious mononucleosis and a variety of cancers including Burkitt lymphoma in Africa and nasopharyngeal cancer (NPC) in certain areas in Southeast Asia.⁴¹⁸ EBV also causes lymphoproliferative disease in individuals with congenital or acquired immunodeficiency, including central nervous system lymphomas in patients with acquired immunodeficiency syndrome (AIDS) and posttransplant lymphoproliferative disease in patients undergoing bone marrow or stem cell transplantation. EBV also appears to play a role in additional cancers including a substantial fraction of Hodgkin disease and some gastric cancers. EBV was the first human virus shown to cause tumors in nonhuman primates.⁴²⁷

A number of viral genes have been implicated in transformation by EBV including LMP1, which mimics an activated TNF receptor, and EBNA2, a transcription factor involved in lymphocyte immortalization. The LMP1 oncoprotein is often expressed in NPC specimens.⁴⁹ Because the EBV genome replicates as a plasmid in latently infected cells and tumors, the viral DNA replication factor EBNA1 is required for persistence of the viral genome. Studies in Burkitt lymphoma cell lines indicate that ongoing expression of EBV genes is required for the survival of these cells.²⁵² Further interesting features of Burkitt lymphoma include its association with malaria and with the consistent presence of chromosomal translocations in the tumor cells, which result in increased expression of the *c-Myc* proto-oncogene by fusing it to regulatory elements of immunoglobulin genes.

Kaposi Sarcoma Herpesvirus

KSHV, also known as human herpesvirus type 8 (HHV-8), is associated with Kaposi Sarcoma (KS), a systemic disease affecting multiple cell types and organs including the skin, with a prominent vascular endothelial component.³ In fact, the KSHV genome was first identified by molecular cloning from KS.⁶⁶ Unlike many other tumors, KS lesions are not monoclonal and display features consistent with paracrine signaling, and cells derived from them often lack properties of transformed cells.

KS, previously a very rare disease, is associated with immunosuppression and appeared in an aggressive form in a relatively high proportion of AIDS patients.¹⁴⁶ In fact, KS was the most common cause of cancer death in this population, but its incidence dropped dramatically with the use of highly active antiretroviral therapy (HAART) for HIV infection. However, because HIV-infected individuals on HAART are now surviving longer, the number of patients with KS and other

HIV-associated cancers may increase in the future. KSHV is also associated with primary effusion lymphoma and some cases of multicentric Castleman disease, a polyclonal lymphoid malignancy. The KSHV genome contains numerous homologs of cellular genes implicated in growth control and evasion of the host cell innate and adaptive immune response, but the pathogenesis of KS and other KSHV-associated cancer is undoubtedly complex and poorly understood.³²⁸

A number of drugs in clinical use inhibit herpesvirus DNA replication. The appearance of new KS lesions in AIDS patients was significantly suppressed by treatment with ganciclovir, a modified nucleoside that is phosphorylated by KSHV thymidine kinase and inhibits the viral DNA polymerase.^{61,301} This finding generates optimism that antiviral drugs have a role in the prevention and treatment of virally induced cancer.

Human T-Cell Leukemia Virus

HTLV-I was the first retrovirus implicated in human disease. Approximately 20 million people are infected with HTLV-I worldwide, with high endemic areas in Latin America, the Caribbean, Africa, and Japan. Infection is associated with adult T-cell leukemia/lymphoma (ATL), a relatively rare tumor, as well as some neurologic diseases.^{178,360,509} ATL typically arises decades after infection and occurs in only a small fraction of infected individuals. As described earlier, Tax protein appears to play a role in latently transforming T lymphocytes. It has multiple effects on transcription of cellular genes, and it affects a variety of other cellular processes as well.^{364,434} Despite the presence of *Tax*, ATL takes many years to develop. An explanation for the long latency of ATL could be that HTLV-I establishes a pool of T lymphocytes with enhanced proliferative potential, but that additional events (likely nonviral), such as accumulation of cellular mutations, are required for development of leukemia. The Tax protein is often not expressed in end-stage ATLs.²⁴⁵ This may reflect immunological selection against continued expression of this viral protein as the tumors progress. ATLs do express another viral protein, HBZ, encoded by an mRNA from the opposite strand of viral DNA.^{173,305} HBZ may contribute to the late stage of tumorigenesis (including down-regulation of Tax expression), while Tax may be involved in early preleukemic steps.²⁴⁵

Hepatitis B Virus

HBV infects more than 1 billion people worldwide and is associated with acute and chronic hepatitis, as well as with the majority of hepatocellular carcinoma (HCC).²¹ Although HBV contains a DNA genome, it undergoes an unusual replication cycle that involves RNA replicative intermediates.⁴⁵² Unlike the other DNA tumor viruses, HBV does not contain well-defined oncogenes, although the X gene has been implicated in cell transformation and tumorigenesis in transgenic mice.^{119,431} X affects cellular gene expression and may interfere with p53 function.¹⁵²

In some HBV-infected individuals (particularly those infected as infants), infection becomes persistent. Over time, persistent infection can lead to chronic liver damage (chronic active hepatitis [CAH]), which has a significant likelihood of progressing to HCC. In individuals with CAH, the liver injury caused by HBV replication and the resulting inflammation and rounds of hepatocyte proliferation and mutagenesis during liver regeneration are thought to cause the accumulation

of cellular mutations that lead to cancer formation. Reactive oxygen species produced by inflammatory cells recruited to sites of liver injury are also likely to cause DNA damage and mutagenesis. Moreover, segments of the HBV genome can show monoclonal integration patterns in HCC, raising the possibility that the viral genome also plays a *cis*-acting role in hepatic carcinogenesis.¹⁵¹ A recombinant HBV subunit vaccine has been deployed for decades and is reducing the incidence of HBV-mediated HCC.⁶⁵

Hepatitis C Virus

HCV is an RNA-containing virus of the *Flavivirus* family. HCV causes viral hepatitis and HCC and is also associated with non-Hodgkin B-cell lymphoma.^{82,332,355,394} HCV lacks a clearly defined viral oncogene, but HCV structural genes have transforming activity in various assays.^{282,321,433} Like HBV, HCV can establish persistent infections and CAH, leading to HCC. The carcinogenic potential of HCV is thought to derive primarily from its cytolytic activity in hepatocytes and the resulting inflammation and regeneration. However, unlike HBV, whose genome is relatively stable, even in a single infected individual the HCV genome is highly variable and constitutes a swarm of closely related viral variants, complicating attempts to develop an effective HCV vaccine. There are reports that successful antiviral treatment of HCV infection can also provide benefit for HCV-associated lymphomas.²¹⁵

Retroviral Oncogenesis in Human Gene Therapy Trials

Because retroviruses can stably deliver genes into host cell DNA, they have been used as gene transfer vectors in clinical trials to correct inherited genetic defects, most notably of the hematopoietic system.³³⁶ In one trial, an MuLV-based vector expressing the cytokine receptor common γ -chain was used to treat patients with X-linked severe combined immunodeficiency (X-SCID).²⁰¹ In this trial, hematopoietic progenitor cells from X-SCID patients were transduced with the vector *ex vivo* and then infused back into the same individuals. This approach corrected the SCID defect, but several of the treated individuals subsequently developed T-cell lymphoma.²⁰¹ Molecular analysis of the lymphomas revealed that the retroviral gene transfer vector caused insertional activation of the LMO2 proto-oncogene, as well as of other proto-oncogenes. Vector-induced malignancies or premalignant conditions have also arisen in similar trials for correction of chronic granulomatous disease and Wiskott-Aldrich syndrome.^{46,270,442} These findings raise cautions about the use of retroviral vectors for gene correction in humans.

In response to these findings, second- and third-generation retroviral vectors have been developed for human gene transfer. The enhancer sequences have been removed from the upstream LTR of the vector to generate a vector that has inactive LTRs after reverse transcription (a self-inactivating or SIN vector).⁵¹¹ The absence of functional viral enhancers in the transduced cells is designed to reduce the likelihood of insertional activation of cellular proto-oncogenes. A second approach has been to use retroviral vectors based on lentiviruses such as HIV-1, because lentiviruses have not been shown to induce cancer. Moreover, while MuLV has a preference for integration near the transcription start sites of genes (and thus may more efficiently activate adjacent genes), HIV-1 tends to integrate within the bodies of genes.^{315,409} A recent human clinical trial

to correct β -thalassemia employed an HIV-1-based SIN vector expressing human β -globin.⁶² In the first treated patient, the clinical symptoms of thalassemia were relieved, but there was an expansion of a clone of cells where the vector was inserted into the gene for HMG2A, which is overexpressed in certain malignancies. Molecular analysis of the integration site in these cells demonstrated that insertion of the provirus truncated the 3' noncoding region of HMG2A mRNA, resulting in removal of binding sequences for a regulatory miRNA and up-regulation of HMG2A mRNA. This is reminiscent of tumors in SL3-3 MuLV-induced T-lymphomas, where *gfi-1* was activated by removal of miRNA binding sequences from the mRNA.¹⁰⁰

Additional Viruses Implicated in Human Cancer

In addition to the six viruses firmly implicated in naturally occurring cancer, it is likely that additional viruses will be shown to be involved in cancer causation in humans, particularly for cancers associated with immunosuppression. Rapid improvements in DNA sequencing technology suggest that sequencing-based efforts are likely to be the prime method of detecting new candidate tumor viruses, as they were in the identification of KSHV and MCV (see next paragraph). Rigorous genetic, cell biological, and epidemiologic studies must be performed to confirm the role of any new candidates in human carcinogenesis.

The next virus likely to be recognized as a human tumor virus is Merkel cell polyomavirus (MCV). Merkel cell carcinoma is a rare but aggressive tumor of neuroendocrine cells in the skin, and it is often associated with sunlight exposure and immunosuppression. Through extensive sequencing of Merkel cell carcinoma RNA, in 2008 Feng et al.¹⁵⁴ identified sequences that were homologous to known polyomaviruses. Further analysis showed that up to 80% of Merkel cell carcinomas worldwide contain an intact, integrated polyomavirus genome, designated MCV. Like all known polyomaviruses, the MCV genome encodes LT that binds Rb family members and *sT* that binds PP2A. Tumors contain monoclonal integrations of MCV DNA in the cellular genome that disrupt the DNA replication activity of LT but leave the Rb binding activity intact,⁴²⁹ providing a plausible mechanism of tumor suppressor inactivation and cell transformation. Recent experiments indicate that Merkel cell carcinoma cell lines require MCV early region expression for continued proliferation²¹⁷ and that *sT* can transform rodent cells in culture. Taken together, these results provide strong evidence that MCV is the seventh human tumor virus. Similar to other oncogenic human DNA viruses, MCV infection appears to be fairly common,²⁵¹ even though Merkel cell cancers are rare.

SV40 itself has been implicated in a number of human cancers, including non-Hodgkin lymphoma, mesothelioma, and childhood brain tumors.¹⁷⁰ However, the evidence in support of these claims, and even of the conclusion that SV40 infection can occur in humans, remains controversial. Two pathogenic human polyomaviruses closely related to SV40, JC virus and BK virus, have also been implicated in human cancer, but the evidence for oncogenic activity in humans is inconclusive.

A retroviral genome closely related to MuLV genomes has been detected in human prostate carcinoma DNA and named xenotropic murine leukemia virus-related virus (XMRV). XMRV DNA was originally found in patients with hereditary prostate cancer, and the viral genome was detected in stromal

cells, not epithelial tumor cells.⁴⁷⁰ Some subsequent reports have detected XMRV DNA and proteins in prostate cancer specimens, but the association of the virus with the hereditary form of prostate cancer and its restriction to stromal cells has not been confirmed.^{157,404} Other groups have failed to detect any association of XMRV with prostate cancer. Recent molecular studies suggest that XMRV was generated by recombination between two endogenous MuLV-related retroviruses during passage of a human prostate cancer in nude mice.³⁴⁴ As a consequence, the prostate cancer cell 22Rv.1 produces high amounts of XMRV, which replicates efficiently in certain human prostate cancer cell lines and appears to have been detected as a contaminant during surveys of prostate cancer tissues by highly sensitive PCR amplification techniques. Viruses related to MMTV have been suggested to play a role in the etiology of human breast cancer, but convincing candidate viruses have not yet emerged.

The foregoing discussion assumes that tumor virus gene products persist in tumor cells. If a virus can cause cancer through a true “hit-and-run” mechanism, there may be many more human tumor viruses than are currently recognized.

Finally, although HIV is not regarded as a tumor virus, the immunosuppression caused by HIV plays an important role in the genesis of many virally induced tumors. AIDS is associated with increased frequencies of many of the virus-associated human cancers (e.g., HPV-associated cancer of the cervix and anus, EBV-associated lymphomas, and Kaposi sarcoma). This suggests that the increased cancer incidence in AIDS patients results from reduced immunological control of the oncogenic virus. However, the incidence of some nonviral cancers is also elevated in individuals with AIDS. Improved prevention and control of HIV infection will reduce worldwide cancer prevalence.

WHY DO VIRUSES TRANSFORM CELLS?

The preceding sections describe how viruses transform cells. But why do so many diverse viruses transform cells and cause tumors? Evolution selects for the ability of viruses to replicate, avoid host cell defenses, and spread to new hosts. Many viruses accomplish these tasks without displaying transforming or tumor-forming activity, so transforming activity *per se* is not essential for virus replication.

Retrovirus transformation is incidental to virus replication. The absence of oncogenes in the replication-competent non-acute retroviruses demonstrates that these genes are not required for virus replication. Rather, the appearance of monoclonal tumors in animals many months after infection indicates that tumorigenic transformation by these viruses is an exceedingly rare event at the cellular level. This conclusion is supported by the inability of these viruses to transform cultured cells. It is only because of the huge number of infected cells in animals and the dramatic consequences of *in vivo* transformation, namely, tumor formation, that these events are even observed by scientists and studied. Presumably, proviruses integrate near many cellular genes, but the vast majority of these integration events do not provide a growth advantage to the cells, and therefore they are not detected. For the acutely transforming retroviruses, oncogenes are acquired from the host cell genome extremely infrequently during virus propagation. But once incorporated in an active form into the viral genome, the transduced gene is introduced into every newly infected cell and is a potent and

rapidly acting carcinogen, allowing scientists to capture these viruses. Tumor-forming activity confers no replication advantage to these viruses, and in fact the acquisition of an oncogene is in almost all cases associated with virus replication defects.

The peculiar lifestyle of retroviruses, involving integrated proviruses that stably persist in infected cells, explains why these viruses are unique among RNA viruses in having transforming potential in cultured cells, because the provirus allows the sustained expression of the viral oncogene or acts *in cis* to regulate the expression of cellular proto-oncogenes or tumor suppressor genes.

DNA tumor virus oncogenes typically are essential, intrinsic viral genes. The biochemical activities of the proteins encoded by these genes were selected during virus evolution for their roles in virus replication and persistence, not for their ability to transform cells. In order for viruses to synthesize the large amounts of viral DNA needed to sustain a productive infection, up to 100,000 or more progeny genomes per infected cell, the cell must be reprogrammed into a DNA replication factory to provide an abundant supply of DNA polymerases and other replication factors. Most cells are resting and not able to support high-level DNA replication when initially exposed to a virus. Therefore, the virus must stimulate the cell to enter S phase to generate a cellular environment conducive to viral DNA replication. What better way to generate this state than to exploit the intrinsic cellular machinery that controls cell cycle progression? Thus, it is no accident that DNA virus oncoproteins stimulate cell proliferation. And when virus replication is aborted, in non-permissive cells for example, and the viral genome undergoes rare recombination events to integrate into cellular DNA, the ongoing proliferative stimulus may result in cell transformation or tumor formation. According to this view, transformation by DNA viruses is an accidental byproduct of their need to drive the cell into S phase for viral DNA replication. Consistent with this hypothesis is the observation that parvoviruses, which do not stimulate S phase and can only replicate in cells induced by other means to enter the cell cycle, are one of the few DNA virus groups devoid of transforming activity.

This aspect of viral oncogene action can be seen clearly with the E7 protein of HPV, because the cells undergoing various responses in stratified squamous epithelia are physically separated. HPV initially infects basal epithelial cells. Normally, when basal cells divide and migrate away from the basement membrane, they undergo a process of terminal differentiation and growth arrest, which is incompatible with vegetative viral DNA replication. However, high-level viral DNA replication must be able to take place in the suprabasal cells because keratinocyte differentiation is required for the virus life cycle to proceed. Therefore, the viral E7 protein inactivates the Rb pathway in suprabasal cells to maintain the DNA replication machinery in an active state without preventing keratinocyte differentiation.⁷⁶ The ability of the E7 protein to inhibit the activity of p21^{WAF1}, which is implicated in keratinocyte differentiation, may assist in this process.^{165,242}

Another aspect of viral tumorigenesis is suggested by the presence of immune evasion genes in the larger DNA viruses such as the herpesviruses. This model posits that transforming activity and tumorigenesis are indirect consequences of viral countermeasures to defense mechanisms erected by the host cell.³¹⁷ Upon initial confrontation with a virus, and even in response to latent infection, cells mobilize innate immune

signaling pathways to block virus replication. In some cases, these pathways activate tumor suppressor pathways or interferon signaling, resulting in growth arrest or apoptosis, which restricts virus replication or spread. The transforming proteins of some viruses may have evolved to neutralize tumor suppressor pathways and thereby circumvent these cell defenses and other innate immune pathways. By removing these blocks to virus propagation, the blocks to cell proliferation are removed as well. Virus genes that inhibit these host defenses thus exert a proliferative and eventually a transforming effect on the cells.

Neither transforming activity nor tumorigenicity is required for replication of RNA and DNA tumor viruses, and these activities have not been directly selected for during evolution. Rather, transforming activity is an accident or at most an unintended consequence of the complex biochemistry of viral genome integration, replication, and persistence. From the scientific and medical point of view, we are the beneficiary of these accidents, because they have generated a set of biological reagents, the viruses themselves, which have provided unprecedented insights into such fundamental cellular and pathologic processes as signal transduction, cell cycle control, and above all carcinogenesis. The dependence of nonviral tumorigenesis on the same pathways mobilized by tumor viruses emphasizes the central role of the regulatory pathways targeted by these viruses and highlights the value of tumor viruses as uniquely powerful probes of cell function.

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Innate Responses to Viral Infections

Sensing Viral Infections

Viral Sensing Through Pattern
Recognition Receptors

Cell-Autonomous Virus Recognition

RIG-I-Like Receptors (RLRs)
NOD-Like Receptors (NLRs)
Cytosolic DNA Sensors

Cell-Extrinsic Virus Recognition

Toll-Like Receptors (TLRs)
Other Strategies of Viral Recognition

Innate Antiviral Cytokines

Type I Interferons
Type I IFN Induction
Amplification of IFN Production by IRF7
IFN- α β R Signaling
Inflammatory Cytokines
Interleukin 6
Interleukin 1- β and Interleukin-18
Tumor Necrosis Factor (TNF)
Interleukin 15
Interferon γ

Cell-Autonomous Antiviral Defense

Mechanisms

Type I IFN-Dependent Antiviral Defense
Retroviral Restriction Factors
Type I IFN-Independent Antiviral Defense
RNA Interference
Xenophagy

Cell Types Involved in Innate Antiviral Responses

NK Cells
Plasmacytoid DCs
Monocytes
Macrophages
Dendritic Cells

Virus Manipulation of Innate Immunity

Virus-TLR Interaction as Viral
Pathogenesis Mechanism
Viral Evasion of the Innate Immune
System

Differences between Evasion Strategy Mechanisms of DNA vs. RNA Viruses

Conclusion

The innate immune system provides a universal form of host protection from infectious diseases. It detects the presence of pathogens using several recognition strategies. The best-characterized innate microbial sensing mechanism is based on pattern recognition receptors (PRRs). These receptors detect conserved microbial structures shared by entire classes of microorganisms. The targets of PRRs are commonly products of metabolic pathways unique to a particular class of microbes, such as lipopolysaccharide in the case of Gram-negative bacteria. In the case of viral pathogens, however, all viral molecular constituents are produced in the host cell. Consequently, the major targets of innate immune recognition are viral nucleic acids. Whenever possible, the innate immune system detects structural features of viral RNA and DNA that are distinct from the host nucleic acid. These include long double-stranded RNA, RNAs containing 5'-triphosphate, and unmethylated CpG motifs in viral DNA genomes. Detection of these structural features, however, is insufficient to reliably distinguish the host and viral nucleic acids. Therefore, additional factors help to determine their origin. For example, viral but not host nucleic acids are normally found in the endolysosomes and viral but not host DNA is present in the cytosol. Innate immune sensors of viral RNA and DNA are present in these locations, where they can be triggered to activate the antiviral responses. In addition to pattern recognition, viral pathogens might be detected indirectly, through alteration in normal cellular processes, such as acute decline in host protein synthesis, altered activity of ion channels, or ER stress. These forms of recognition are still incompletely understood and remain to be fully characterized in future studies.

Detection of viral pathogens by the innate immune system has two major consequences: first, it leads to the induction of the innate antiviral mechanisms, most of which are mediated primarily by type-I interferons (IFNs). Second, it leads to the activation of the adaptive immune response that can provide a more directed, antigen-specific, and long-lasting antiviral immunity. The main host defense strategy against viral pathogens is the elimination of the infected cells. This can be achieved by cell-intrinsic mechanisms that are induced by type-I IFNs and operate in the infected cells, or with the help of cytotoxic lymphocytes: natural killer (NK) cells and CD8 T cells. Another important host defense mechanism is the blockade of viral entry into the host cells. This is primarily a function of neutralizing antibodies. Many additional mechanisms exist that interfere with viral replication, gene expression, virion assembly, and exit from the infected cells. The relative contribution of these defense mechanisms varies depending on the virus and host. Detailed knowledge of the specific antiviral defense mechanisms is difficult to establish due in large part to a high degree of redundancy between different mechanisms.

In this chapter, we will focus on the better-characterized viral sensing mechanisms of the innate immune system and discuss the major defense mechanisms utilized in mammalian hosts.

SENSING VIRAL INFECTIONS

Viral Sensing Through Pattern Recognition Receptors

The mammalian immune system detects the presence of viruses through multiple mechanisms. The best understood mechanisms involve various PRRs. Most common molecular patterns associated with virus infection are the features associated with viral nucleic acids. Innate immune recognition can be cell intrinsic or cell extrinsic, depending on whether it is mediated by infected or non-infected cells. Cell-intrinsic innate immune recognition is mediated by cytosolic sensors, including the NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). Activation of these receptors generally occurs in infected cells. Accordingly, PRRs involved in cell-intrinsic recognition are broadly expressed because viral pathogens target a variety of cell types for replication. In contrast, cell-extrinsic innate immune recognition is mediated by transmembrane receptors (including Toll-like receptors (TLRs) and C-type lectins [CLRs]); their activation does not require the cells expressing these receptors to be infected. Cell-extrinsic recognition is mainly mediated by specialized cells of the immune system, such as the plasmacytoid dendritic cell (pDC), macrophage, and dendritic cell (DC).

PRRs involved in microbial recognition have modular structures and share several structurally related domains for innate recognition, multimerization, interaction with adaptor molecules, and signaling (Fig. 8.1).

CELL-AUTONOMOUS VIRUS RECOGNITION

RIG-I-Like Receptors (RLRs)

RNA viruses are recognized by the cytoplasmic RNA helicases, retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5).¹⁹⁸ The RIG-I-like

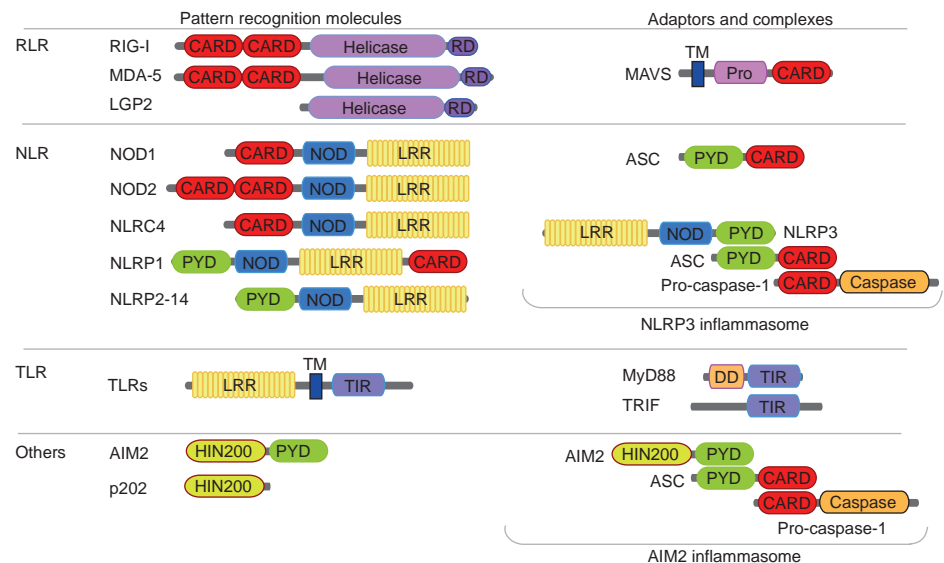
receptors (RLRs) are expressed by most cell types, and both RIG-I and MDA5 recognize viral RNA through their helicase domains. RIG-I also contains the repressor domain (RD) at its C-terminus, which inhibits the activation of RIG-I at steady state. Binding to viral RNA catalyzes a conformational rearrangement, which exposes a caspase activation and recruitment domain (CARD) to initiate antiviral signaling^{152,199} (Fig. 8.1). Both RIG-I and MDA5 utilize a common adaptor molecule called mitochondria antiviral signaling protein (MAVS)¹⁶²—also known as IPS-1,⁹³ Cardif,¹²⁷ or VISA¹⁹⁴—that localizes to the mitochondrial membrane¹⁶² and to the peroxisomes.⁴⁷ MAVS activates two protein kinase complexes, one consisting of FADD and caspases 8/10¹⁷⁵ and the other containing TANK and NAP1¹⁵⁵ (Fig. 8.2). The former activates the IKK α /IKK β /IKK γ complex while the latter activates the TBK1-IKKi complex, leading to the activation and nuclear translocation of transcription factors NF- κ B and IRF3, respectively. In addition, the TRAF6-dependent pathway involving MEKK1 is essential to activate the MAP kinase and NF- κ B pathways.²⁰¹ Despite similarity in overall structure and signaling mechanisms, RIG-I and MDA5 have important differences in their mechanisms of activation. For example, RIG-I but not MDA5 requires ubiquitination by E3 ligase TRIM25 for its activity.⁵⁸ Additional differences in the function of RIG-I and MDA5 likely exist and may reflect distinct features of viral pathogens that they recognize.

Analysis of animals deficient in the RLRs revealed important and distinct roles for these sensors in innate immunity. These molecules cumulatively provide the host with the ability to recognize a large group of viral pathogens in infected cells and mediate a critical aspect of innate antiviral defense.

RIG-I

RIG-I consists of two N-terminal CARDS, a central DExH box RNA helicase/adenosine triphosphatase (ATPase) domain, and a C-terminal regulatory domain (RD) (Fig. 8.1). RIG-I functions as a critical PRR for a number of viruses including Sendai virus (SeV), vesicular stomatitis virus (VSV), influenza, hepatitis C virus, Japanese encephalitis virus, as well as small RNA encoded by the DNA virus, Epstein-Barr virus.⁹² RIG-I

FIGURE 8.1. Domain structure of viral sensors and adaptor molecules. Pattern recognition molecules involved in viral sensing contain distinct domains, some of which are shared by different classes of receptors. These domains include: CARD, caspase activation and recruitment domain; DD, death domain; HIN200, hemopoietic IFN-inducible nuclear proteins is a 200-amino acid motif; LRR, leucine-rich repeat; NOD, nucleotide-binding oligomerization domain; Pro, proline-rich region; PYD, pyrin domain; caspase domain; TIR, toll/interleukin-1 receptor; TM, transmembrane region. Many of these domains can be used for homotypic oligomerization. Examples of molecular complexes are depicted for NLRP3 and AIM2 inflammasomes.



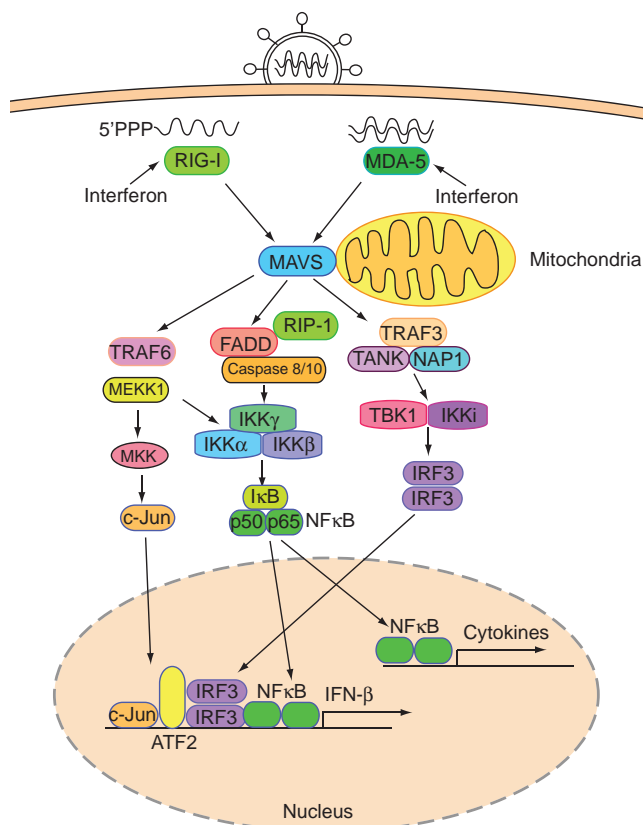


FIGURE 8.2. RIG-I and MDA-5 virus recognition pathways. In a virally infected cell, RNA structures that are unique to viruses are recognized by RIG-I and MDA-5. RIG-I detects ssRNA containing 5' triphosphate, while MDA-5 recognizes a long stretch of dsRNA. RNA binding to RLRs induces conformational changes, enabling their binding to MAVS adaptor protein expressed on the surface of mitochondria and peroxisomes. MAVS activates signaling to activate NF- κ B, MAP kinases, and IRF3, leading to target gene expression including IFN- β and cytokines. Type I IFNs increase the levels of RLRs in bystander cells, enabling robust antiviral signaling upon infection.

recognizes single-stranded (ssRNA) that contains 5' triphosphate end, but not 5' OH or a 5'-methylguanosine cap^{73,144} that is longer than 23 nucleotides¹²³ and contains a uridine- or adenosine-rich ribonucleotide sequence¹⁵³ (Fig. 8.2). RIG-I can also recognize double-stranded (dsRNA) in the absence of 5' triphosphate in some cases with a preference for shorter-length dsRNA compared to those recognized by MDA-5.⁹¹ Analysis of the central DExH box RNA helicase/ATPase domain of RIG-I revealed an ATP-powered dsRNA translocation activity. The CARD domains suppress translocation in the absence of 5'-triphosphate, and the activation by 5'-triphosphate triggers RIG-I to translocate preferentially on dsRNA *in cis*.¹³⁰ This ATPase activity is required for RIG-I signaling, indicating that RIG-I recognizes two distinct features of viral RNA simultaneously: triphosphate at the 5' end and dsRNA. RIG-I indeed selectively detects blunt, short, double-stranded 5'-triphosphate RNA, which is common in the panhandle region of ssRNA viral genomes.¹⁵⁷

MDA5

MDA5 serves as a sensor of picornaviruses and can be activated by cytosolic synthetic dsRNA, such as polyinosinic-polycytidylic acid (poly I:C).^{60,92} There appears to be some level of redundancy in RIG-I and MDA5 in recognition of certain viruses. Dengue virus and West Nile virus are recognized by both MDA5 and RIG-I; either of these sensors is sufficient to induce type I IFN production.¹¹⁵ Comparison of synthetic and viral dsRNA revealed that MDA-5 preferentially recognizes longer dsRNA that is at least 2 k bp in length, while RIG-I is activated by a wider range of dsRNA sizes (400 bp–4 kbp).⁹¹ Many viruses that replicate in the cytoplasm express their own 2'-O-methyltransferases to autonomously modify their messenger RNA (mRNA). Interestingly, infection with viruses deficient in 2'-O-methyltransferase leads to robust activation of MDA5, indicating that lack of ribose 2'-O-methylation is used as a signature of viral RNA for recognition by MDA5.²⁰⁵ Therefore, MDA5 likely recognizes large dsRNA structures lacking 2'-O-methylation generated in the cytosol during virus infection. Of note, 2'-O-methylation is also used by viruses to evade restriction by IFN-induced proteins with tetratricopeptide repeats (IFIT) proteins.^{36,205}

LGP2

The third member of the RLR family, laboratory of genetics and physiology 2 (LGP2), displays sequence homology to the helicase domains of RIG-I and MDA5. It lacks a CARD domain, however, and therefore has been proposed to serve as a negative regulator of both sensors. Overexpression studies indicated that LGP2 does not activate the production of type I IFNs on its own, but inhibits RIG-I- and MDA5-mediated signaling in a dose-dependent manner.^{98,149,200} Interestingly, studies of *lgp2* knockout mice indicated that LGP2 may play both positive and negative regulatory roles in MDA5- and RIG-I-mediated innate immunity.¹⁸⁹ This study found that the loss of *lgp2* augments type I IFN production in response to transfected poly I:C (MDA-5 agonist) or VSV infection (RIG-I agonist). In contrast, LGP2 is required for efficient antiviral responses following EMCV infection (MDA5 agonist). Therefore, LGP2 can serve as a positive or negative regulator of RLR signaling depending on the type of viral infection. The precise mechanism underlying the opposing functions of LGP2 is still unclear.

NOD-Like Receptors (NLRs)

NLRs comprise a large family of intracellular PRRs that regulates innate immunity in response to recognition of various PAMPs and stress signals.¹²⁵ The NLR family consists of multidomain proteins that contain a C-terminal LRR domain, a central NOD domain and an N-terminal effector domain. NLR proteins can be subdivided into three subfamilies depending on the structure of N-terminal domains: CARD-containing subfamily (NOD1, NOD2, NLRCs, CIITA), pyrin domain (PYD)-containing subfamily (NLRPs) (Fig. 8.1), and BIR domain-containing subgroup (NAIPs) (not shown). Both NOD and NLRP members contribute to antiviral defense. The PYD subfamily of NLRPs consists of 14 members. Although the functions of many of the NLRPs are largely unknown, several NLRPs play a key role in the activation of caspase-1 by forming a multiprotein complex known as the

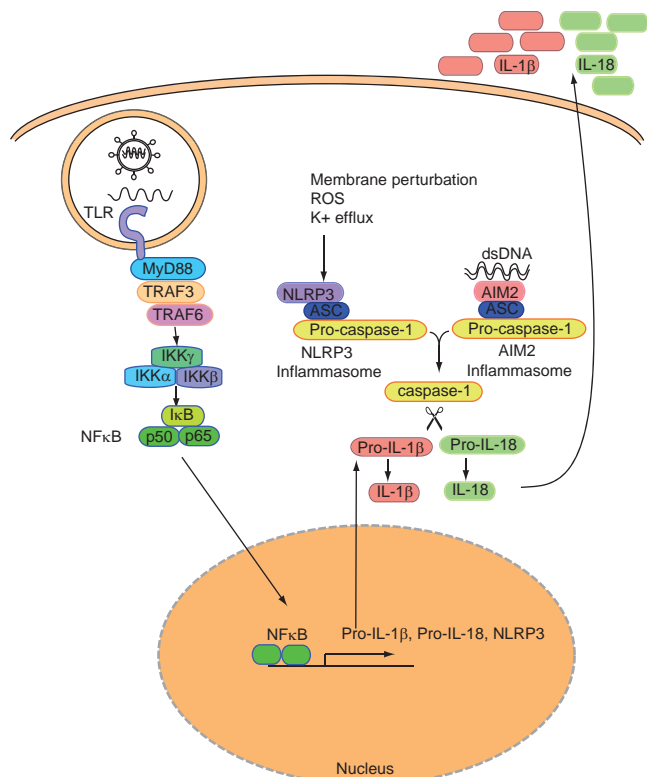


FIGURE 8.3. Inflammasome activation by virus infection. Virus infection can lead to inflammasome activation through two separate pathways. NLRP3 inflammasome is activated upon infection by adenovirus or influenza virus. This pathway is initiated by membrane perturbation, K⁺ efflux, and reactive oxygen species (ROS). The second pathway involves AIM2, which is activated by dsDNA in the cytosol. Both NLRP3 and AIM2 form a complex with ASC and pro-caspase-1 (inflammasome), leading to the self-cleavage and activation of caspase-1. Pro-forms of IL-1 β and IL-18 induced by TLR signals are cleaved by activated caspase-1. Mature forms of these cytokines are then released to the extracellular space.

“inflammasome”¹²⁴ (Fig. 8.3). Caspase-1 is an essential mediator of inflammatory response through its capacity to cleave and generate active forms of IL-1 β and IL-18. IL-1 β and IL-18 are potent proinflammatory cytokines, as described below. Formation and secretion of mature IL-1 β and IL-18 require a two-step activation mechanism: first, transcriptional and translational upregulation of the pro-forms of these cytokines are induced by TLR signaling, and a second signal that leads to the proteolytic activation of caspase-1. The latter process is mediated by the inflammasome. Inflammasome complexes that are important in antiviral defense are described below.

NLRP3-ASC Inflammasome

NLRP3, also known as NALP3/Cryopyrin/CIAS1/PYPAF1,¹⁸¹ forms an ASC-dependent inflammasome (Fig. 8.3). The NLRP3 inflammasome can be activated by a variety of stimuli including endogenous signals from dying cells (uric acid), crystals (asbestos, silica, alum), as well as microbial signals such as whole bacteria, bacterial RNA, extracellular ATP, pore-forming toxins, or viral infections.¹²⁵ It is unclear whether microbial ligands can directly activate the NLRP3

inflammasome. Instead, the NLRP3 inflammasomes likely sense cellular stress such as disruption in membrane integrity and extracellular ATP released from stressed or damaged cells. Virus infection also results in the activation of inflammasomes. Both SeV and influenza virus activated the NLRP3 inflammasome in macrophages pulsed transiently with ATP (signal 2) *in vitro*.⁹⁰ DNA viruses such as adenovirus stimulate the NLRP3-ASC-caspase-1 inflammasomes *in vivo*.¹²⁹ However, inflammasomes are not activated by transfection of RNA, poly I:C, or infection with reovirus (dsRNA virus) or VSV (ssRNA virus),¹²⁹ indicating that viral RNAs are insufficient to trigger inflammasome activation. Influenza virus activates the inflammasome through the activity of the M2 ion channel.⁷⁹ The M2 channel of influenza A virus is a homotetrameric integral membrane protein that associates to form a highly specific proton channel,¹⁴⁵ and is essential for influenza A virus infection and replication.¹⁷⁹ Among other things, the M2 channel exports protons in the acidic trans-Golgi network (TGN) to neutralize the pH of the lumen of the TGN in order to prevent the premature maturation of hemagglutinin to its low-pH fusogenic form.³² Within influenza virus-infected cells, NLRP3 complex detects changes in ionic imbalance in the TGN to activate the inflammasomes. *In vivo*, NLRP3 deficiency resulted in increased susceptibility to high-dose flu challenge.^{4,180} In addition, after a sublethal dose of influenza infection, ASC-dependent inflammasome activation is required to elicit adaptive protective immunity to influenza virus, indicating the importance of NLRs in linking innate viral recognition to adaptive immunity.⁷⁸

AIM2-ASC Inflammasome

Not all inflammasomes are activated by NLRPs. Recent studies showed that AIM2 couples cytosolic dsDNA recognition to ASC-caspase-1 inflammasome.^{25,53,72,148} AIM2 is an HIN200 family of protein that contains a dsDNA binding domain (HIN domain) and the PYD domain, which promotes interaction with the PYD domain of ASC (Fig. 8.1). AIM2 recognizes dsDNA in the cytosol and induces oligomerization of ASC and caspase-1, leading to activation of caspase-1 and cleavage of pro-forms of cytokines including IL-1 β , IL-18, and IL-33 (Fig. 8.3). Vaccinia virus, which contains a dsDNA genome, was shown to require AIM2 for recognition and activation of caspase1 inflammasomes.⁷² It is interesting to note that different classes of dsDNA viruses are recognized by NLRP3 (adenovirus) or AIM2 (vaccinia virus) for inflammasome activation. This likely depends on both the accessibility of the dsDNA genome to intracellular sensors and the viral-induced cellular stress responses. While cytosolic dsDNA can bind to AIM2 directly and activate inflammasomes, how and where exactly NLRP3 becomes activated by dsDNA viruses is yet to be determined.

p202

Mouse HIN200 protein, p202, is a negative regulator of the AIM2 inflammasome.¹⁴⁸ No orthologs of p202 have been found in humans. p202 contains two HIN domains but no PYD domain, and binds specifically to dsDNA. Unlike AIM2, p202 cannot bind ASC and therefore appears to function as an inhibitor of AIM2, presumably by limiting its access to DNA.

Cytosolic DNA Sensors

The existence of a cytoplasmic DNA sensing molecule leading to type I IFN production was suggested from studies of

infection by DNA viruses and bacteria.^{82,170} It is now clear that there are likely multiple sensors that recognize distinct types of DNA. These DNAs include DNA from viruses, bacteria, apoptotic host cells, and synthetic B-form DNAs—particularly poly(dA:dT) and interferon stimulatory DNA (ISD). ISDs are dsDNA containing >25 base-pair oligonucleotides, which in sequence-independent manner, trigger stimulation of type I IFNs through a pathway involving TBK1 and IRF3, but not MAVS.¹⁷⁰ Interestingly, ISD does not engage NK- κ B or MAPK pathways, thus activating only IRF3-induced pathways. ISD recognition pathway exists only in primary cells but is lost from transformed cells.¹⁷⁰ However, the sensor for ISD still remains to be identified. TREX1, an exonuclease, was identified to be a negative regulator of ISD pathway by inhibiting excess accumulation of DNA products from endogenous retroelements.¹⁶⁹ TREX1-deficient humans and mice suffer from autoimmune disease due to hyperstimulation of ISD pathway. TREX1 is also involved in degrading nonproductive RT products generated during HIV-1 infection, enabling HIV-1 to remain undetected by the ISD sensor.¹⁹⁵

In contrast, B-form DNA—or poly(dA-dT)·poly(dT-dA) dsDNA—triggers type I IFNs via IRF3 and NK- κ B, suggesting that this form of DNA triggers a separate sensor from ISD.⁸¹ The sensor(s) for cytosolic DNA remained elusive until recently. First, a molecule called DAI (also known as DLM-1/ZBP1) was identified as a candidate intracellular DNA sensing molecule.¹⁷⁶ IFN induction following poly(dA-dT)·poly(dT-dA) DNA treatment was abrogated by a small interfering RNA (siRNA) directed against DAI, suggesting that DAI is a critical cytoplasmic DNA sensor.¹⁷⁶ However, DAI is not the sole sensor of DNA in the cytosol, as DAI-knockout mice still responded to B-DNA or plasmid DNA.⁸³ More recent studies showed that poly(dA-dT)·poly(dT-dA) DNA is recognized by RIG-I upon transcription by RNA polymerase III (Pol III).^{1,30} Pol III synthesizes 5'-triphosphate RNA using AT-rich dsDNA as a template, generating an RIG-I agonist in the cytosol. Therefore, RIG-I is the sensor for cytosolic poly(dA-dT)·poly(dT-dA) dsDNA upon transcription by Pol III. In addition to the indirect sensing of poly(dA-dT)·poly(dT-dA) by RIG-I, IFI16, a PYHIN protein, can bind to poly(dA-dT)·poly(dT-dA), and knockdown of IFI16 expression led to reduction in IFN- β production.¹⁸⁶

Cyclophilin A

While most pattern recognition of virus infection involves detection of nucleic acids, there are few exceptions in which viral protein serves as a signature for viral infection. Cyclophilin A is a peptidylprolyl isomerase that can catalyze *cis/trans* isomerization of X-Proline epitopes on target proteins. Innate response to HIV-1 in DCs depends on interaction between the newly synthesized HIV-1 capsid and cellular cyclophilin A, leading to activation of type I IFNs through IRF-3-dependent mechanisms.¹²² Human DCs, but not CD4 T cells, have intrinsic machinery for responding to HIV-1 infection through cyclophilin A. However, infected humans are unable to utilize the cyclophilin A-dependent pathway of IFN induction against HIV-1 infection because DCs are resistant to HIV-1 infection. In addition, cyclophilin A is also known to be incorporated into the HIV-1 virions by binding to capsid protein. Virions lacking cyclophilin A possess normal morphology and can penetrate host cells, but are defective in the reverse transcription of viral RNA.²² Therefore, HIV-1 utilizes cyclophilin

A for its infectivity, and renders cyclophilin A useless in antiviral defense by avoiding infection in the relevant cell type.

CELL-EXTRINSIC VIRUS RECOGNITION

Toll-Like Receptors (TLRs)

TLRs are the best characterized members of the PRR family, which recognize evolutionarily conserved molecular patterns associated with a large variety of microorganisms.⁸⁷ There are 11 functional TLRs in mice (TLR1–7, 9, 11–13) and 10 functional TLRs in humans (TLR1–10). All TLRs are type I transmembrane proteins with ectodomains containing leucine rich repeat (LRR) that mediate the recognition of PAMPs. Some TLRs recognize bacterial, fungal, and protozoan pathogens, while others are dedicated for viral recognition.¹⁷⁷ This chapter will focus on the TLRs that recognize viral pathogens.

The transmembrane domains of TLRs dictate the localization of the receptor, and the cytosolic domain contains the toll/interleukin-1 receptor (TIR) domain, which binds to adaptor proteins and ultimately leads to expression of a variety of genes.² TLR activation, following virus detection, catalyzes a complex signaling cascade that bifurcates into two main pathways, culminating in the synthesis of proinflammatory cytokines (NF- κ B-dependent) and antiviral cytokines, type I interferons (IFNs) (IRF-dependent). In addition, TLR signaling results in the activation of the mitogen-activated protein kinases (MAPKs) (see Fig. 8.5). Type I IFNs in turn induce a battery of genes that directly suppress viral replication. Other cytokines and chemokines efficiently recruit immune cells to sites of virus infection. In particular, TLR recognition by DCs, the most potent antigen-presenting cells, enables their activation and antigen-presenting function, effectively linking innate recognition to the induction of adaptive immunity.⁸⁶

TLRs are distributed on specific subcellular locations and are specifically expressed by different cell types. TLRs can be categorized into two major types: those that are expressed on the cell surface (TLR1, 2, 4, 5, 6, 11, and 12) and those that are expressed in the endolysosomes (TLR3, 7, 8, and 9). Although virus–TLR interaction can occur with both types of TLRs, many of the virus interactions with surface TLRs represent viral invasion mechanism (beneficial to the virus) rather than PAMP recognition by TLRs (beneficial to the host) (see discussion under Virus Manipulation of Innate Immunity). In this section, we will discuss the latter type of TLR-virus PAMP recognition leading to antiviral defense.

Viruses are recognized by a group of TLRs that reside in the endosomal membrane—namely, TLR3, 7, 8, and 9—where they can gain access to viral nucleic acids upon endocytosis of virions. A major advantage of this strategy of viral recognition is that the viral sensing and subsequent induction of type I IFN genes are not subjected to viral evasion mechanisms. In addition, since all viruses contain genomes consisting of RNA or DNA, such molecular patterns can be surveyed by a very limited number of receptors. These endosomal TLRs require trafficking by the endoplasmic reticulum (ER) membrane protein, UNC-93B.^{24,174} UNC-93B physically interacts with TLR3, 7, and 9 via the transmembrane domain,²⁴ and transports these TLRs from the ER to the endosome (Fig. 8.4). Mutations in the *Unc93b1* gene were found in two patients with severe HSV encephalitis,²⁶ indicating the importance of transport of endosomal TLRs via

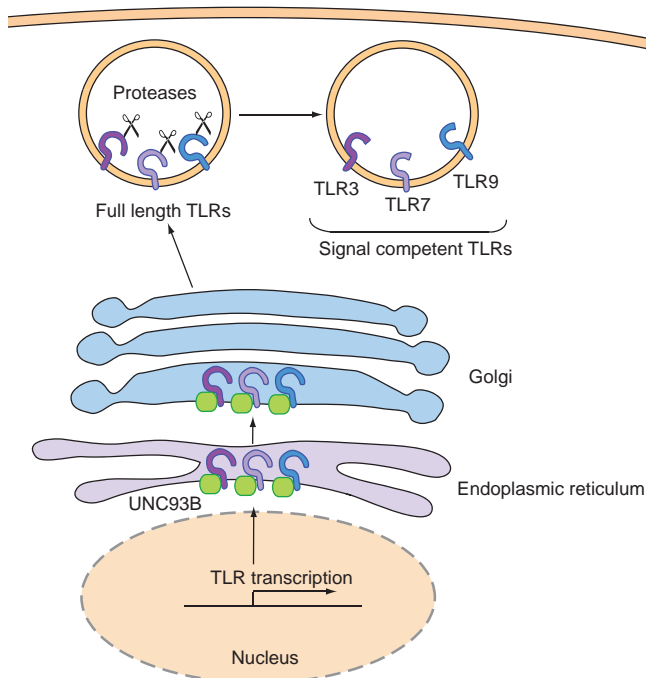


FIGURE 8.4. Endosomal TLR trafficking and processing. Endosomal TLRs, TLR3, TLR7, and TLR9, are synthesized in the ER, transported through Golgi, and are sorted into endosomes. UNC93B is an ER-resident protein, which facilitates TLR transport to the endolysosomes. Once in the endosomes, TLR3, 7, and 9 undergo proteolytic cleavage by proteases, which makes these receptors competent for signaling.

UNC93B in antiviral defense. Upon arrival in the endosomal compartment, these TLRs undergo processing by endosomal proteases. While full-length and cleaved forms of TLR9 are capable of binding ligands, only the processed form recruits MyD88 upon activation.^{52,140} The ectodomain of TLR9, and likely TLR3 and TLR7,⁵² must first be cleaved in the endolysosomes by cathepsins in order to become competent for signaling (Fig. 8.4). Dependence of TLR9 activation on proteolytic processing in the endolysosomes ensures that aberrant activation of TLR9 from cell surface (for example, upon recognition of self DNA) is avoided.

TLR3

TLR3 is expressed by a variety of cells in human and mice, including conventional DCs, B cells, fibroblasts, and epithelial cells but not in plasmacytoid DCs (pDCs). Upon PAMP recognition, TLR3 recruits TRIF via its TIR domain (Fig. 8.5). TRIF is responsible for activation of both NF- κ B and IRF3, leading to proinflammatory cytokines and type I IFN gene expression, respectively. TRIF binds to TRAF6, which is a RING-domain E3 ubiquitin ligase capable of polyubiquitinating TRAF6 and IKK γ . Ubiquitinated TRAF6 and IKK γ recruit TAK1 complex (consisting of TAK1 and TAB proteins), leading to the activation of MAPK. TRIF also binds to receptor-interacting protein (RIP)1 to initiate NF- κ B activation. To activate the type I IFN genes, TRIF also recruits TRAF3, which engages TBK1 and subsequent activation of IRF3. IRF-3 translocates to the nucleus and binds to promoters of type I IFN genes.

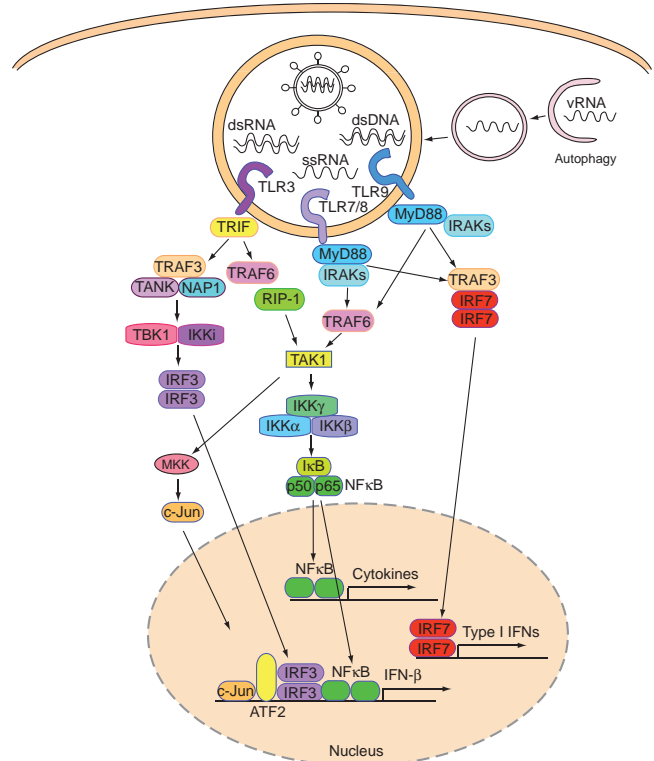


FIGURE 8.5. Endosomal TLR virus recognition and signaling. Viruses or virus-infected cells are endocytosed, and signatures of virus infection become accessible to TLRs upon digestion of membranes and nucleocapsid. TLR3 recognizes dsRNA, TLR7 and TLR8 recognize ssRNA, and TLR9 recognizes DNA. In addition, autophagy delivers TLR7 ligand (cytosolic viral replication intermediate) by fusion with the endosomes containing TLR7. Proteolytically activated TLRs in the endosomes recruit adaptor proteins and induce downstream signaling to activate a variety of transcription factors. IRF3 in combination with NF- κ B, c-Jun, and ATF bind to the promoter and induce IFN- β expression. IRF7 homodimer binds to promoter regions of *IFN- α* genes and induces IFN- α expression. In addition, NF- κ B activates transcription of a large number of genes, including cytokines.

dsRNA intermediates are produced during the replication cycle of most RNA viruses (except for retroviruses). DNA viruses also produce dsRNA by convergent transcription of their genomes.¹⁹³ It has long been known that dsRNA triggers IFN production. The first TLR implicated in viral nucleic acid recognition was TLR3.³ TLR3 responds to the artificial dsRNA mimic, poly I:C, when it is provided extracellularly.^{60,92} However, evidence supporting TLR3-mediated recognition of authentic viral replication intermediates remains elusive, possibly due to overlapping recognition pathways that can compensate for the loss of TLR3. TLR3 may be involved in recognizing virus-produced dsRNA in the context of phagocytized apoptotic cells.¹⁵⁹ Humans with dominant negative mutations in the *tlr3* gene suffer from neonatal herpes infection,²⁰² indicating the importance of this receptor in protection against HSV-1 in the CNS.

TLR7/8

In humans, TLR7 is expressed in plasmacytoid DCs and B cells, while TLR8 is expressed by myeloid DCs and monocytes. In

mice, TLR7 is expressed by pDCs, conventional DCs (except for CD8 α^+ DCs), and B cells. The function of the mouse TLR8 is still poorly understood. However TLR8-deficient mice develop autoimmunity due to hyperstimulation of TLR7.³⁹ TLR7, 8, and 9 are all expressed in the endosomes and share similar signaling pathways. Upon engagement, these receptors recruit MyD88 via the TIR domain. MyD88 subsequently interacts with the death domain of several IRAK proteins to induce both NF- κ B and IRF7 activation. IRAK4 is required for activation of NF- κ B pathway by interacting with TRAF6, which in turn activates the TAK1 complex, leading to activation of NF- κ B and MAPK pathways (Fig. 8.5). NF- κ B activation follows the recruitment of IRF5 to the MyD88 complex. On the other hand, MyD88 also recruits IRF7, which forms a signaling complex with IRAK4 and TRAF6. TRAF3 also binds MyD88 and IRAK1 to induce IRF7 activation. Unlike TLR3, TLR7 and 9 utilize IRF7 and not IRF3 for activation of type I IFN genes in pDCs. Interestingly, in conventional DCs, TLR7 and 9 utilize MyD88 and IRF1 to induce IFN- β but not IFN- α genes.¹⁴³

TLR7 and 8 recognize ssRNA and induce innate immune responses to ssRNA viruses. TLR7 is required for type I IFN and cytokine responses to influenza, SeV, and VSV.^{44,117} Uracil and ribose, the defining signatures of RNA, are both necessary and sufficient for TLR7 stimulation.⁴⁵ Furthermore, viral fusion and/or uncoating and endosomal acidification are required for TLR7-dependent recognition.¹¹⁷ Viral RNA, synthetic poly U RNA, and even nonviral, cellular RNA in the endosome is sufficient to stimulate TLR7-dependent cytokine production,^{44,68} indicating that any RNA localized to the endosome is able to trigger TLR7 activation. While influenza virus is recognized upon endocytosis by TLR7, other viruses such as VSV and SeV require replication in the cytosol prior to recognition by TLR7. The latter type of viruses are recognized by TLR7 in the endosome upon delivery of cytosolic viral replication intermediates through the process of autophagy¹⁰⁶ (see below).

TLR9

TLR9 is expressed in pDCs and B cells in humans. Mice express TLR9 in pDCs, B cells, macrophages, and conventional DCs. TLR9 is located in the endosome and mediates recognition of viral DNA. Originally, bacterial DNA sequences containing hypomethylated CpG motifs were shown to activate TLR9.⁶⁹ More recent studies showed that TLR9 might recognize the sugar-base-backbone, 2-deoxyribose, of phosphodiester DNA irrespective of the CpG content.⁶¹ TLR9 is the principal means by which HSV-1 and HSV-2 stimulate type I IFNs in pDCs *in vivo*.^{100,116} Interestingly, a TLR9 molecule “retargeted” to the plasma membrane, unable to respond to viral nucleic acids, however now responded to self DNA that did not stimulate wild-type TLR9.¹⁵ These results suggest that not only is endosomal localization important to trigger TLR-viral nucleic acid interactions, but that endosomal TLRs may limit access to nonviral nucleic acids via an active sequestering mechanism in the endosome.

C-Type Lectins

In addition to TLRs, C-type lectin receptors (CLRs) expressed on the plasma membrane can bind to certain viruses. Classical CLRs contain carbohydrate recognition domain responsible for the Ca²⁺-dependent binding to their ligands. However,

carbohydrate-recognition domains of many CLRs do not bind to Ca²⁺.⁸ CLRs can be classified into those that are used solely for endocytosis of pathogens and those that are used for inducing signaling. The endocytic CLRs include mannose receptor DEC205 and Langerin, which are expressed on CD8 α^+ DCs and Langerhans cells, respectively. Langerin on Langerhans cells are capable of clearing HIV by receptor-mediated endocytosis and degradation in Birbeck granules.³⁷

The signaling CLRs include Dectin-1 and DC-SIGN.⁴⁰ Dectin-1, which is a receptor for fungal β -glucans, contains an ITAM motif in the cytosolic domain, and engages Syk tyrosine kinase and CARD9, leading to NF- κ B activation.⁹⁵ Dectin-1 plays an important role in antifungal defense, but it is not known to have antiviral functions. DC-SIGN binds to HIV-1 and Ebola virus. Signaling through DC-SIGN alone does not lead to the activation of NF- κ B or expression of cytokines, but can modulate signaling through other PRRs. DC-SIGN engagement by viruses leads to serine/threonine kinase Raf-1 activation. After translocation of NF- κ B by TLR-stimulation, DC-SIGN-activated Raf-1 mediates the phosphorylation of NF- κ B subunit p65, which in turn leads to p65-acetylation. Acetylation of p65 both prolongs and increases IL-10 transcription, resulting in increased IL-10 production. However, the majority of C-type lectin-virus interactions reflect a viral invasion mechanism. A clear example of this is DC-SIGN signaling by HIV-1. This interaction results in impaired DC maturation, enhanced T-cell proliferation and transmission of HIV-1 to T cells, thereby promoting systemic infection of the host. DC-SIGN and a related C-type lectin, DC-SIGNR, also enhance infection by Ebola virus.¹³ Another example is Dengue virus, which binds to CLEC5A and induces signaling through DAP12 to induce proinflammatory cytokines. This leads to lethality, not protection, in mice.²⁹

Other Strategies of Viral Recognition

In addition to PRR-based viral recognition, non-PRR-based, cell-autonomous virus sensing mechanisms exist in mammalian hosts. While molecular mechanisms for such pathways are not yet understood, these pathways likely involve sensing various forms of cellular stresses inflicted by virus infection. One of the hallmarks of virus infection is the production of large amounts of viral proteins. This often leads to ER overload, leading to ER stress and unfolded protein response (UPR) induction. Virus infection also often leads to the generation of reactive oxygen species (ROS) by interfering with proper mitochondrial function. In addition, as discussed below, most viruses express molecules that inhibit vital cellular functions such as transcription, translation and secretion. These types of stress that accompany virus infections might be seen by the mammalian host as a signature of virus infection. In conjunction with pattern recognition, such stress sensing pathways can engage signals leading to type I IFNs synthesis, cell-cycle arrest or cell death to avoid further viral replication and spread.

INNATE ANTIVIRAL CYTOKINES

Type I Interferons

Antiviral mechanisms in vertebrates are highly dependent on the action of type I IFNs. Type I IFNs are a family of cytokines that act early in the innate immune response and are key

cytokines capable of inducing an antiviral state in infected and uninfected neighboring cells.⁸⁰ In addition to this antiviral activity, the interferon cytokines have a role in regulating the ensuing adaptive immune response.¹⁷¹ While type II and type III IFNs also have antiviral activities, in this chapter we will focus on the role of type I IFNs in innate antiviral defense. In humans, the type I IFNs consist of 13 α -genes coding for 12 IFN- α subtypes, one β -gene encoding a single IFN- β subtype and a single gene encoding IFN- ω . Type I IFNs bind to IFN- $\alpha\beta$ R, which is a heterodimer of IFN- α R1 and IFN- α R2 chains. High levels of IFN- α subtypes are rapidly secreted from pDCs upon viral recognition, whereas IFN- β can be induced from most virus-infected cell types.

Type I IFN Induction

As described in detail above, stimulation of various PRRs including TLRs (TLR3, 4, 7, 8, and 9), RLRs (RIG-I, MDA-5), and cytosolic DNA sensor(s) leads to the production of type I IFNs. Most virally infected cells trigger the cell intrinsic pathway of type I IFN production through activation of RLRs or DNA sensors. In contrast, pDCs recognize viral genomes within the endosome via TLR7 or TLR9 and induce robust secretion of IFN- α and IFN- β . pDCs are thought to express high constitutive levels of IRF7 and are the only cells capable of coupling TLRs to IRF7 directly, thereby leading to rapid and robust transcription of type I IFN genes.

Amplification of IFN Production by IRF7

In the cytosol of most cells, IRF3 is constitutively expressed while very low levels of IRF7 are found.⁷¹ While *IFN- α* genes contain ISREs, the *IFN- β* gene also contains binding sites for NF- κ B and activator protein 1 (AP1). IRF3 is a potent activator of the *IFN- β* and *IFN- α 4* gene but not the *IFN- α* genes, whereas IRF7 efficiently activates both *IFN- α* and *IFN- β* genes. Thus, upon activation of RLRs or TLR3, IFN- β and IFN- α 4 production is immediately triggered by the IRF3 homodimer binding to the promoter regions of these genes, while the production of other members of IFN- α occurs at a later time point upon IFN- $\alpha\beta$ R-induced production of IRF7. In addition, IFN- $\alpha\beta$ R signaling increases PRR expression (TLRs and RLRs), leading to further amplification of antiviral signaling pathways.

IFN- $\alpha\beta$ R Signaling

The IFN- $\alpha\beta$ R utilizes the so-called JAK-STAT signaling pathway, which is used by many other cytokine receptors. This pathway consists of a receptor, JAK family tyrosine kinases (JAK1, JAK2, JAK3, and Tyk2), and transcription factors (STAT1–6). The receptor complex typically consists of two or three distinct polypeptides that contain in their cytoplasmic domain binding sites for different combinations of JAK and STAT proteins. Upon receptor engagement by its ligand, the receptor complex is assembled, resulting in activation of JAK kinases with subsequent tyrosine phosphorylation of STAT proteins, leading to their dimerization, nuclear translocation, and activation of target genes (Fig. 8.6).

Thus, upon engagement of Type I IFN receptor complex IFN- α R1 and IFN- α R2 by IFN- α , IFN- β or IFN- ω , these receptor subunits dimerize, resulting in phosphorylation of Tyk2, which is associated with the IFN- α R1, by Janus kinase (JAK) 2. Activated Tyk2 subsequently phosphorylates JAK1, which is

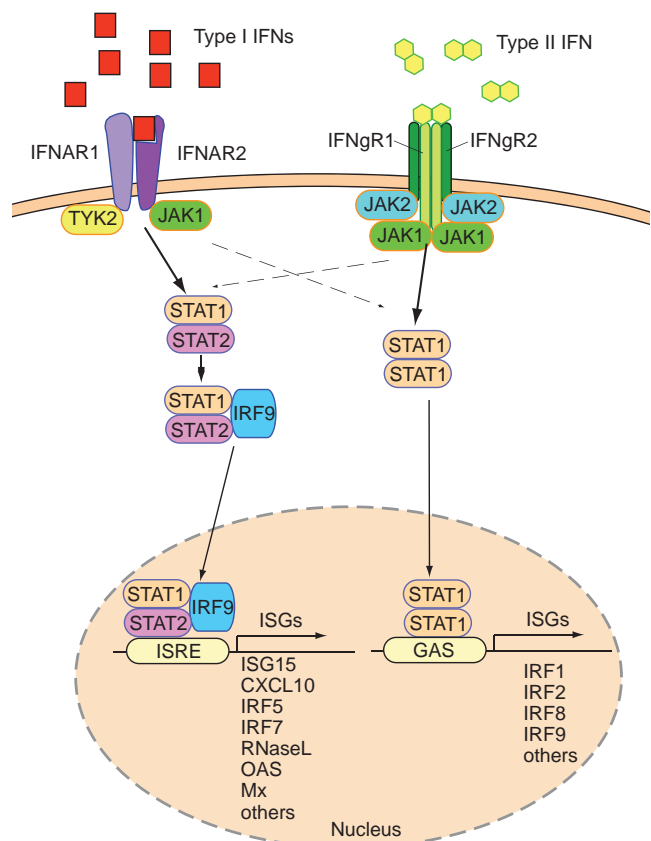


FIGURE 8.6. Type I and type II IFN receptor signaling. Type I and type II IFN receptors consist of IFNAR1 + IFNAR2, and IFNgR1 + IFNgR2, respectively. Engagement of the receptors by cognate cytokines induces activation of prebound kinases (Tyk2 and JAK), leading to phosphorylation of STAT proteins. Phosphorylated STAT molecules form dimers, which translocate into the nucleus to initiate transcription of genes. STAT1/STAT2 dimers recruit IRF9, forming ISGF3, which binds to ISRE sequence found in the promoter of many ISGs. STAT1 homodimer binds to the GAS sequence on different sets of ISGs and induces their transcription.

coupled to the IFN- α R2 chain. Signal transducer and activator of transcription (STAT) 1 and 2 are prebound to the IFN- α R2 chain. Activated JAK1 binds to STAT2 and phosphorylates it, creating a binding site for STAT1, causing their dimerization and transport to the nucleus. The STAT1–2 heterodimer forms a transcription complex, ISGF3, with IRF9 to facilitate transcription of ISGs by binding to the sequence motif called interferon stimulated response element (ISRE). In addition, STAT1 homodimers bind to the IFN- γ -activated site (GAS) sequence motif and induce a different set of ISGs (Fig. 8.6).

Inflammatory Cytokines

In addition to type I IFNs, many inflammatory cytokines play an important role in antiviral defense, by directly inducing antiviral effector molecules or indirectly by stimulating cellular recruitment, phagocytosis of infected cells, and activating adaptive immune responses such as cytotoxic T lymphocytes and neutralizing antibodies. Cytokines can act locally through autocrine and paracrine mechanisms or, if produced at high enough levels, they can gain access to the circulation and

induce systemic effects, such as acute-phase response or fever. The major inflammatory cytokines IL-6, IL-1 β , and TNF are produced by virally infected DCs and macrophages, and act systemically with a wide spectrum of biological activities that help to coordinate the body's response to infections. These cytokines are responsible for causing fever by inducing prostaglandin E2, which acts on the hypothalamus, causing increased heat production from brown fat and by inducing vasoconstriction to prevent heat loss through the skin. Fever is thought to be generally beneficial to host defense, because some pathogens may replicate less efficiently at high temperatures. However, the role of fever in host defense is still incompletely understood. IL-6, IL-1 β , and TNF also stimulate the acute-phase response in the liver. Acute-phase proteins secreted by hepatocytes can have direct antiviral effects, for example, by promoting their killing by complement or phagocytes. Finally, IL-6, IL-1 β , and TNF induce leukocytosis, leading to an increase in circulating neutrophils, which are phagocytes that help to clear virally infected cells.

Interleukin 6

IL-6R belongs to the hemopoietin family of receptors, which are tyrosine kinase-associated receptors that form dimers upon binding their cytokine ligand. IL-6R consists of IL-6R α and gp130 chains. IL-6 bound to the IL-6R α chain causes the association of the complex with gp130, resulting in the activation of Jak1, Jak2 and Tyk2, followed by the phosphorylation of Stat3. Phospho-Stat3 dimerizes and translocates into the nucleus, where it activates transcription of target genes. IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis.⁹⁶ IL-6 is induced by a variety of signals including TLR, NLR, and RLR signaling, and is a key cytokine that triggers the acute-phase response, as well as activation of T cells and antibody-producing plasma cells.

Interleukin 1- β and Interleukin-18

As described above, secretion of IL-1 β and IL-18 requires the activation of inflammasomes. Both IL-1R and IL-18R are heterodimers of a ligand-binding α chain and a coreceptor accessory protein (AcP). Both chains contain a TIR domain in the cytoplasmic tail. As such, these receptors recruit MyD88 and induce NF- κ B signaling (Fig. 8.7). IL-1 β through binding to IL-1R induces the expression of hundreds of genes, including cytokines (IL-6 and TNF- α), chemokines (e.g., IL-8), and adhesion molecules that are important for leukocyte trafficking.⁴⁶ In adaptive antiviral immunity, IL-1 β plays an important role in the antigen-driven expansion and differentiation of CD4 T cells.¹⁶ While IL-1R is widely expressed by many cell types, IL-1 β is mainly produced by the sentinel cells of the innate immune system, including macrophages and DCs, although fibroblasts and keratinocytes can also synthesize this cytokine in response to tissue injury or stress signals.^{46,168} On the other hand, IL-18, which is expressed mainly by macrophages and DCs, work alone or in combination with IL-15 prime NK cells for the production of IFN- γ and enhance their cytolytic activity.^{55,76,178} IL-18 in combination with IL-12 or IL-2 alone can also induce differentiation of either Th1 or Th2 cell types depending on the cytokine milieu.^{75,136} IL-18 is also required for optimal cytokine production by CTLs, including IFN- γ , TNF- α , and IL-2.⁴¹

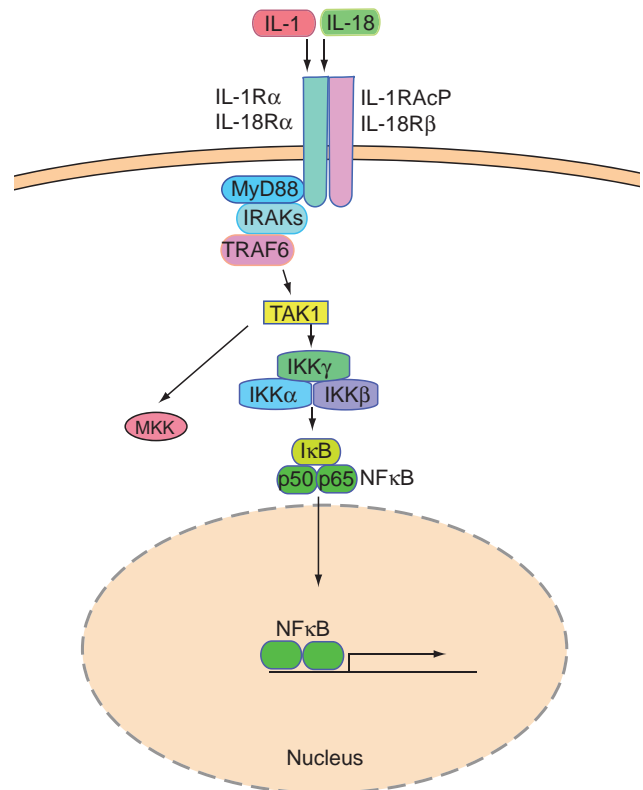


FIGURE 8.7. IL-1R and IL-18R signaling. IL-1R consists of the IL-1R α and IL-1RAcP chains, while IL-18R consists of the IL-18R α and IL-18R β chains. Each chain contains a TIR domain in the cytoplasmic region and, upon receptor engagement by IL-1 or IL-18, recruits MyD88 and induces signaling, resulting in NF- κ B and MAPK activation.

IL-1 β plays an integral role in antiviral immunity against influenza A virus infection. IL-1R^{-/-} mice infected with influenza virus have impaired leukocyte recruitment to the lung, diminished CD4 and CD8 T cell responses, impaired immunoglobulin responses, and succumb to virus-induced fatality.^{78,158} On the other hand, mice deficient in IL-18 were found to have a transient increase in viral titer due to defective NK cytotoxicity after influenza A virus infection but otherwise had normal adaptive immunity to influenza infection.¹¹⁰

Tumor Necrosis Factor (TNF)

TNFR belongs to a group of receptors known as TNFR family, characterized by a cysteine-rich common extracellular binding domain. Upon engagement of the ligand TNF, TNFR forms a trimer (Fig. 8.8). TNFR1 contains a cytoplasmic death domain, which recruits the adaptor TRADD through its death domain. TRADD can assemble two different signaling complexes, involving FADD pro-caspase 8 or RIP1 and TRAF2. RIP1 activates IKK, resulting in the activation of NF- κ B, while TRAF2 stimulates the JNK signaling pathway. Similar to IL-1 and IL-6, TNF is involved in induction of a stereotypic inflammatory response that promotes host defense from a broad range of pathogens, including viruses. Recent studies revealed an additional pathway activated by TNFR—the induction of cell necrosis via the protein kinase RIP3. This TNF-induced necrosis was shown to be important for immune defense against vaccinia virus and CMV.^{31,187}

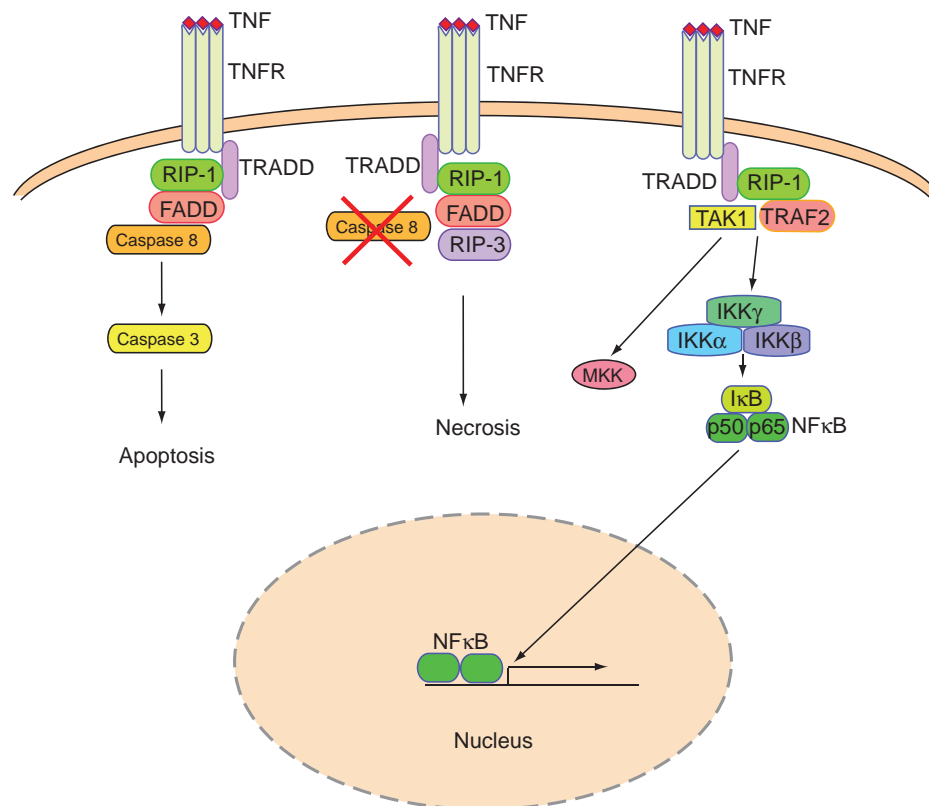


FIGURE 8.8. TNFR signaling leads to three distinct cell fates. TNF binding trimerizes the TNFR. Through the cytoplasmic death domain, TNFR recruits TRADD, which also contains a DD. TRADD can assemble different signaling complexes. TRADD can recruit FADD through DD–DD interaction, which results in caspase-8 activation, leading to apoptosis. In cells in which caspase-8 is rendered nonfunctional, RIP-3 is recruited to the complex to induce necrosis. In addition, TRADD can also recruit RIP-1 and TRAF2, resulting in signal transduction and activation of NF- κ B and MAP kinase pathways.

Interleukin 15

IL-15 is produced by DCs and macrophages upon viral infection. IL-15R is a trimer of the IL-15R α chain, which is specific to IL-15, and β , and the common γ chains that are shared with IL-2 and other cytokines. An unusual feature of IL-15R complex is that the IL-15R α chain is expressed on the cells that produce IL-15, rather than on the target cell that responds to IL-15. IL-15 forms a complex with the IL-15R α chain in the ER and the two proteins are transported to the cell surface, where they remain associated and function as a ligand for the IL-15R β and the common γ chains expressed on target cells. IL-15R induces activation of STAT5, resulting in expression of anti-apoptotic and growth-promoting genes in target cells.¹²⁰ The main target-cell types of IL-15 are cytotoxic lymphocytes: IL-15 is an important growth and survival factor for CD8 T cells, NK cells, and a subset of NKT cells. Importantly, IL-15 can function downstream of type I IFNs in antiviral immune responses.¹³³

Interferon γ

IFN- γ is secreted mainly by three lymphocyte types, NK cells (during early phase of viral infection), CD8 T cells, and Th1 cells (after induction of adaptive immunity). IFN- γ R, like IFN- α β R, belongs to the type II cytokine receptor family (Fig. 8.6). IFN- γ R is expressed by most cells, and consists of two subunits: IFN- γ R1, the ligand-binding chain, and IFN- γ R2, the signal-transducing chain. As the ligand-binding IFN- γ R1 chains interact with IFN- γ , they dimerize and become associated with two signal-transducing IFN- γ R2 chains. Receptor assembly leads to activation of the JAK1 and JAK2 and

phosphorylation of a tyrosine residue on the intracellular domain of IFN- γ R1. This leads to the recruitment and phosphorylation of STAT1, which forms homodimers and translocates to the nucleus to bind to GAS elements, activating a wide range of IFN- γ -responsive genes. IFN- γ has a potent antiviral function, by inducing transcription of genes encoding antiviral effectors such as PKR and viperin. Note that some antiviral effectors are induced only by type I IFNs and not by IFN- γ , including 2'5'OAS, Mx1, Mx2, and RNaseL.⁴² Instead, IFN- γ R signaling induces activation of genes involved in antigen processing and presentation, facilitating the activation of virus-specific T cells.

CELL-AUTONOMOUS ANTIVIRAL DEFENSE MECHANISMS

Type I IFN-Dependent Antiviral Defense

IFN- α β R signaling leads to the expression of over 300 ISGs. Although the functions of majority of these genes are currently unknown, they are all thought to participate in antiviral defense. The effect of IFN- α β R signaling is different in infected and non-infected cells. In infected cells IFN- α β signals in an autocrine fashion to induce ISGs involved in cell autonomous antiviral defenses. These ISG encode proteins that interfere with multiple steps of viral infection cycles. In specialized noninfected cells, such as APCs and cytotoxic lymphocytes, IFN- α β promotes antigen processing and presentation, along with cytotoxic activity of NK cells and CD8 T cells. In addition, IFN- α β signals in a paracrine fashion to induce an antiviral state in neighboring

cells, thereby minimizing the spread of viral infection. Only a handful of ISGs that have direct antiviral effects have been characterized in detail. Their functions are described below.

2'-5' Oligoadenylate Synthetase (OAS) and Ribonuclease L (RNase L)

OAS and RNase L act in concert to degrade viral RNA in the cytosol. While basal levels of OAS and RNase L are found constitutively, stimulation through IFN- $\alpha\beta$ R dramatically increases their expression levels. Activated by dsRNA, (2'-5') OAS converts ATP into 2'-5' oligoadenylate, which functions as a second messenger to activate latent ribonuclease RNase L. Activated RNase L degrades viral and cellular ssRNAs, inhibiting protein synthesis and viral growth (Fig. 8.9). Mice deficient in RNase L suffer from increased susceptibility to RNA viruses including Picornaviridae, Reoviridae, Togaviridae, Paramyxoviridae, Orthomyxoviridae, Flaviviridae, and Retroviridae families.¹⁶⁷ However, the roles of OAS and RNase L pathway in defense against DNA viruses remain less clear. Interestingly, the cleavage products of RNase L can serve as ligands for RIG-I and MDA5, leading to amplification of the RLR pathway.¹²¹

Protein Kinase R (PKR)

PKR is a serine/threonine kinase that phosphorylates the α -subunit of eukaryotic translation initiation factor 2 α (eIF2 α). PKR becomes activated through homodimerization upon binding to viral dsRNA structures via its dsRNA binding domains. This results in inhibition of translation and a decrease in total cellular and viral protein synthesis, effectively reducing viral production. In addition to its translational regulatory function, PKR has a role in signal transduction and transcriptional control through the I κ B/NF- κ B pathway.¹⁰² PKR can also mediate apoptosis, cell-growth arrest, and autophagy, all of which curb viral replication and spread in the host.¹⁵¹ In addition to the virus-restricting function of PKR, recent studies indicate that PKR functions to stabilize IFN- α and IFN- β mRNA, thereby ensuring robust IFN protein production.¹⁶⁰ Mice genetically deficient in PKR are susceptible to infection with viruses including rhabdovirus, orthomyxovirus, and orthobunyavirus.

Orthomyxovirus Resistance Gene (Mx) Proteins

Mx proteins belong to a family of GTPases consisting of MxA and MxB in humans and Mx1 and Mx2 in mice.⁶⁵ The Mx

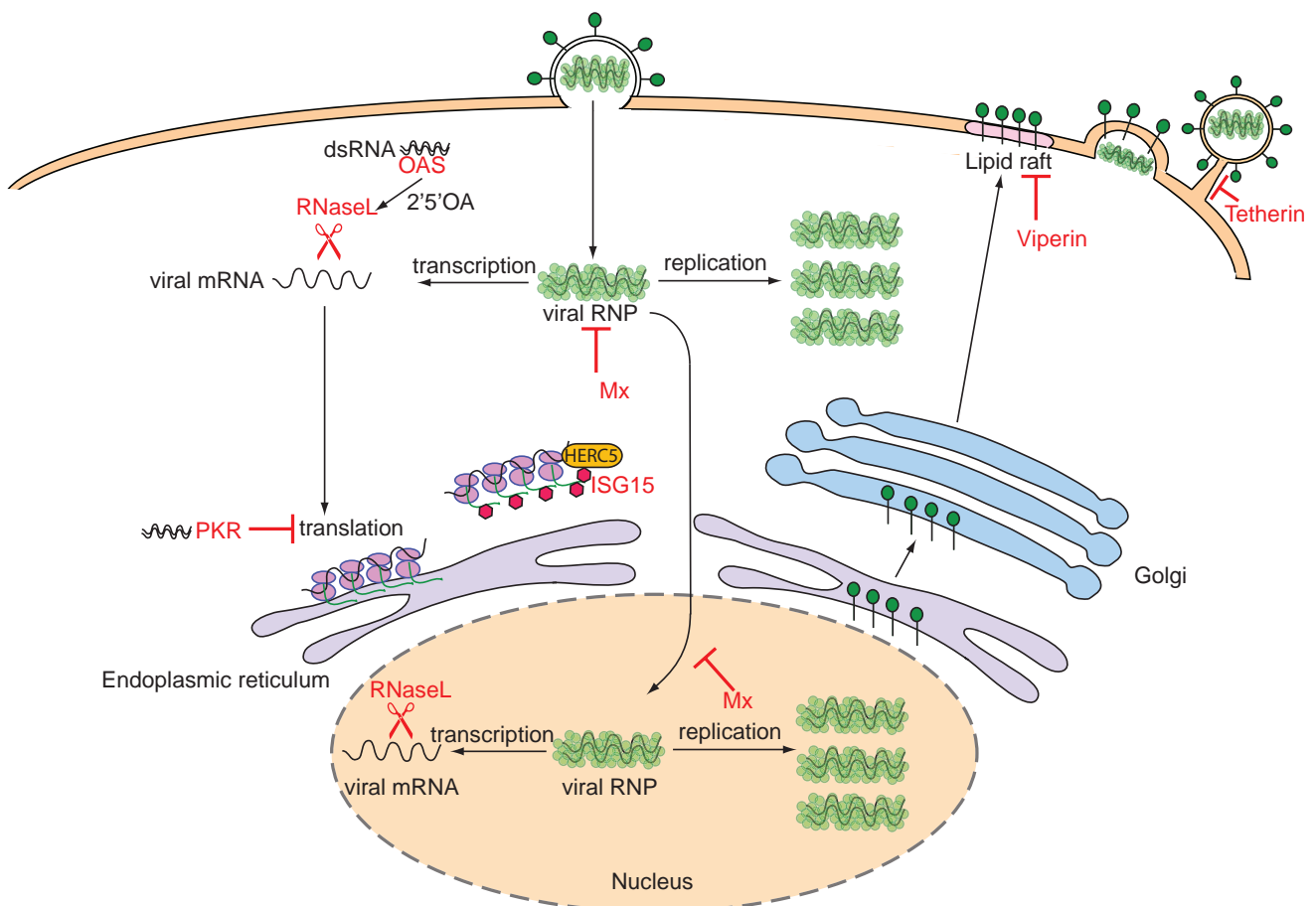


FIGURE 8.9. Type I IFN-induced antiviral mechanisms. Type I IFNs induce over 300 genes, many of which are antiviral effectors. When an IFN-stimulated cell encounters a virus, viral dsRNA activates OAS and PKR. OAS produces 2'-5' oligoadenylate from ATP, which activates a latent ribonuclease RNaseL. RNaseL can cleave viral mRNA (as well as host mRNA). PKR activated by dsRNA inhibits translation, thereby impairing viral protein synthesis. Mx gene products trap viral RNP in the cytosol and prevent its nuclear import. ISG15 modifies newly synthesized proteins (most of these are viral proteins) by forming a complex with HERC5. Viperin impairs viral budding by disrupting lipid rafts. Tetherin inhibits viral particle release.

proteins have a large N-terminal GTPase domain, a central interacting domain (CID) and a C-terminal leucine zipper (LZ) domain. Both the CID and LZ domains are required to recognize target viral structures. Viruses targeted by Mx proteins include orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, and bunyaviruses. Remarkably, transgenic expression of human MxA in IFN- α β R-deficient mice confers full resistance to otherwise fatal infection with Thogoto virus, influenza virus, VSV, LaCrosse virus or, Semliki Forest virus,^{67,142} indicating that this effector molecule is sufficient for protective innate defense against these viruses. The main viral targets seems to be the viral nucleocapsid-like structures.⁹⁷ Mx proteins are thought to survey exocytic events and mediate vesicle trafficking to trap essential viral components. It is interesting to note that most inbred strains of mice harbor defective Mx1 gene.⁶³ Therefore, studies using inbred mice must be interpreted with the caveat that they are Mx-1 deficient.

ISG15

ISG15 is an ubiquitin-like molecule that can be covalently attached to target proteins using E1-, E2-, and E3-like enzymes. UBE1L (E1-like ubiquitin-activating enzyme) was shown to be the specific ISG15-activating enzyme.¹⁴⁷ Two E2 ubiquitin-conjugating enzymes, UBCH6 and UBCH8, serve as ISG15 carriers. Subsequently, two E3 ubiquitin ligases—HERC5 (homologous to the E6-associated protein C terminus (HECT) domain and RCC1-like domain containing protein 5) and TRIM25—conjugate ISG15 to protein substrates. All enzymes identified in the ISGylation pathway are coordinately induced by type I IFNs. There are over 150 targets of ISGylation: RIG-I

and STAT1, as well as several IFN-induced antiviral effector proteins. Interestingly, by physically associating with polyribosomes, HERC5 ensures that ISG15 conjugation is restricted to the newly synthesized pool of proteins, which in infected cells will consist primarily of newly translated viral proteins.⁴⁹ Further, ISGylation of a small percentage of human papillomavirus (HPV) L1 capsid protein has a dominant inhibitory effect on the infectivity of HPV16 pseudoviruses. Given that acute viral replication in general requires a vast amount of *de novo* protein synthesis, ISG15 modification of newly synthesized viral protein might be a powerful virus restriction mechanism that can be broadly applicable to many viruses.

Tetherin and Viperin

Tetherin (also known as BST-2, PDCA-1, and CD317) is a GPI-anchored protein that is highly expressed upon type I IFN stimulation. Tetherin associates with lipid rafts and inhibits retrovirus particle release in the absence of Vpu.¹³² Vpu utilizes the beta-TrCP E3 ubiquitin ligase complex to induce endosomal trafficking events that remove tetherin from the cell surface, rendering it incapable of restricting the release of enveloped viruses.¹²⁸ Thus far, tetherin has been implicated in restricting the release of members of the retrovirus, filovirus, and arenavirus families. Viperin (*virus inhibitory protein, endoplasmic reticulum-associated, interferon inducible*) is another IFN-inducible protein that is known to prevent replication of a variety of viruses including HCMV, influenza virus, hepatitis C virus (HCV), dengue virus, alphaviruses, and HIV-1.⁵⁴ Viperin impairs the release of influenza virus by disrupting lipid rafts via suppression of the activity of farnesyl diphosphate synthase,

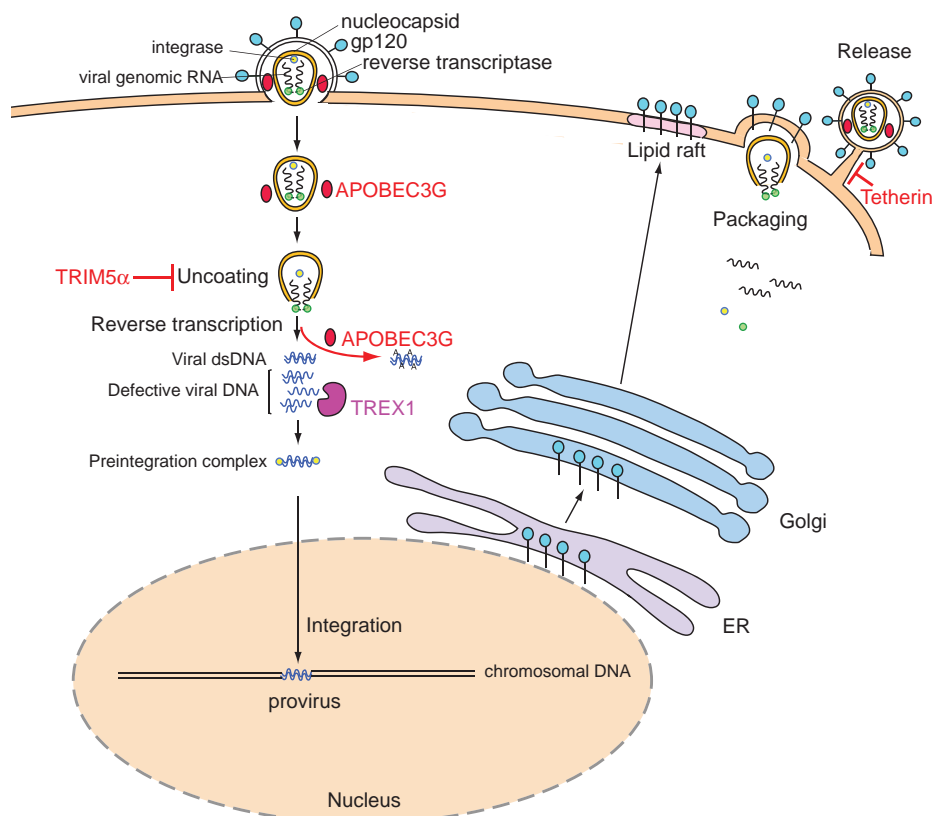


FIGURE 8.10. Innate restriction of retroviruses. TRIM5 α perturbs the controlled uncoating of the subviral particle prior to reverse transcription. APOBEC3G deaminates cytosine residues in nascent retroviral cDNA, causing C/G to T/A hypermutation and degradation of viral cDNA. Tetherin inhibits retroviral particle release. In contrast, TREX1, an exonuclease, inhibits innate immune detection of HIV DNA by degrading nonproductive RT products.

a key enzyme in isoprenoid biosynthesis¹⁹¹ (Fig. 8.9). In addition, viperin localizes to the lipid droplets⁵⁴ and through a yet undefined pathway interferes with replication of other viruses that do not require lipid rafts for synthesis.

Retroviral Restriction Factors

In addition to the IFN-inducible antiviral molecules described above, cells are equipped with “host restriction” factors that are constitutively expressed in some cell types and are induced by IFNs in others.¹⁸ The host restriction factors that specifically counter retrovirus infections are described below.

TRIM5 α

Members of the tripartite motif (TRIM) protein family are involved in various cellular processes, including cell proliferation, differentiation, development, oncogenesis, and apoptosis.¹³⁴ There are 66 known members of TRIM proteins in humans, characterized by the presence of RING, B-box, and coiled-coil domains. Many of the TRIM proteins display antiviral properties, particularly against retrovirus entry and release.¹⁸⁴ TRIM5 α has been extensively studied as a key factor responsible for antiretroviral activities. TRIM5 α is thought to perturb the controlled uncoating of the subviral particle prior to reverse transcription by recognizing and degrading the capsid protein of retroviruses, resulting in a block of viral replication (Fig. 8.10). Interestingly, TRIM5 α from Old World monkeys confers potent resistance to HIV-1, but not SIV, while the human ortholog of TRIM5 α is unable to specifically target HIV. Strikingly, the differential ability of human and monkey TRIM5 α to restrict HIV hinges on a single amino acid, R332 (human) vs. P332 (monkey).¹⁰⁹ It is likely that HIV and SIV have evolved in their natural hosts to evade interaction with TRIM5 α .

APOBEC Family

APOBEC (apolipoprotein BmRNA-editing catalytic polypeptide) proteins are a group of cytidine deaminases. Members of the APOBEC family contain either one or two catalytic deaminase domains. APOBEC is constitutively expressed in various cell types but its expression can be enhanced by type I and type II IFNs. APOBEC3G becomes encapsidated into retroviral virions in infected cells. Upon viral fusion and entry into the new host cell, APOBEC3G deaminates cytosine residues in nascent retroviral cDNA⁶⁵ (Fig. 8.10). The resulting uracil residues function as a template for the incorporation of adenine, which, in turn, can result in strand-specific C/G to T/A transition mutations that affect virus viability. The antiviral activity of APOBEC3G is strongly inhibited by HIV-1 Vif protein, allowing the virus to replicate virtually unimpaired in APOBEC3G-expressing host cells. Vif induces the ubiquitin-dependent degradation of some of the APOBEC proteins. However, Vif is also able to prevent encapsidation of APOBEC3G and APOBEC3F through degradation-independent mechanisms.

Type I IFN-Independent Antiviral Defense

Type I IFN-dependent antiviral mechanisms are only known to exist in vertebrates. Other animals rely on more ancient forms of antiviral defenses, including RNA interference and autophagy.

RNA Interference

RNAi was first identified as a potent antiviral defense mechanism in plants, then subsequently in fungi, nematodes, and insects.¹⁰⁸ The mechanism of RNAi involves two steps (Fig. 8.11). First, viral dsRNA is recognized by members of the Dicer endonucle-

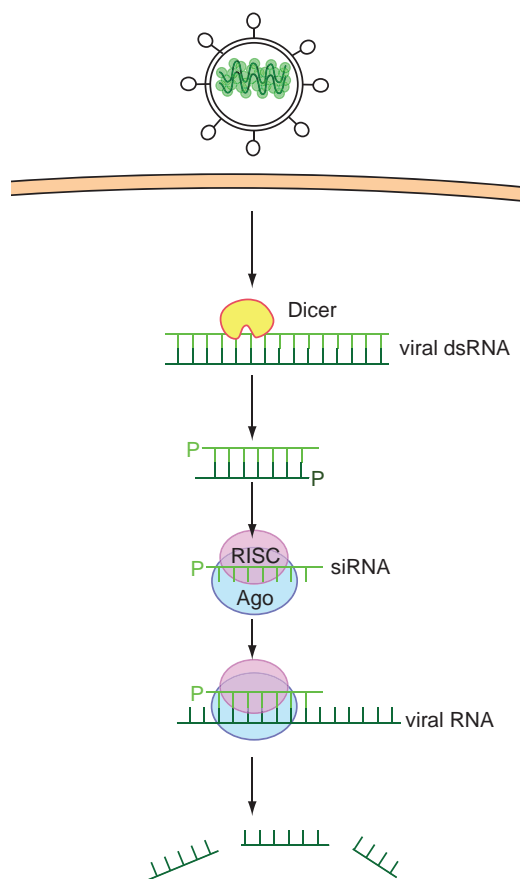


FIGURE 8.11. RNA interference is an ancient antiviral defense mechanism. In plants and invertebrates, the RNAi pathway plays a major role in the recognition and destruction of viral RNA. Viral dsRNA is recognized by Dicer, which processes such RNA into short RNAi (~20 bp). The processed RNAi is incorporated into the RISC complex, which serves as the guide strand. The guide strand base pairs with a complementary sequence of a viral RNA molecule and induces its cleavage by AGO, the catalytic component of the RISC complex.

ase family, which processes it into siRNA. Second, these siRNA are incorporated into RNA-induced silencing complex (RISC), which guides the RNase enzyme AGO to complementary sequences in viral RNA for cleavage and degradation. In plants and nematodes, but not in insects, this antiviral response is further amplified through a secondary wave of siRNAs generated by RNA-dependent RNA polymerases (RdRPs), which greatly increases the pool of siRNAs available to RISC. To combat antiviral RNAi responses, many plant and invertebrate viruses have evolved to encode proteins that act as suppressors of RNA silencing. Interestingly, in addition to generating RNAi, Dicer-2 was shown to trigger a signaling pathway, resulting in the expression of antiviral genes in *Drosophila*. The latter function requires the helicase domain of Dicer-2, and given its phylogenetic relation to RIG-I, suggests a parallel role of Dicer-2 to mammalian RIG-I in the induction of antiviral genes.³⁸ In mammalian cells, RNAi has not been found in virally infected cells. Further, mammalian cells lack the RdRPs to amplify the siRNA and fail to mount a systemic antiviral RNAi response. Therefore, with the evolution of the potent type I IFN system, the RNAi mechanism may have become obsolete for antiviral defense in mammals.

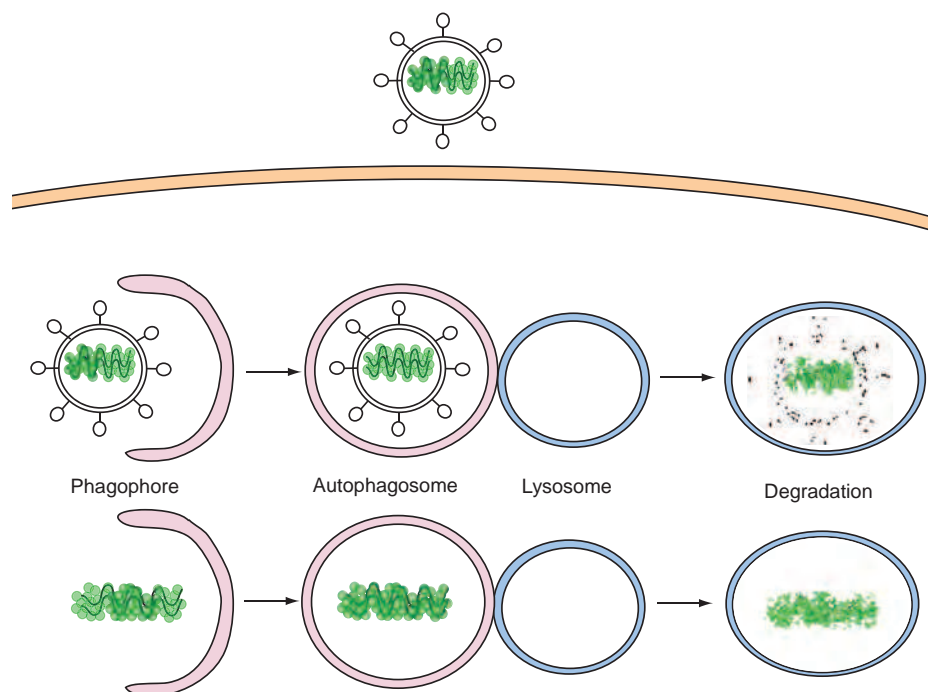


FIGURE 8.12. Autophagy as innate antiviral defense mechanism. From insects to humans, xenophagy is used to limit viral replication in the cytosol. A double membrane structure called a phagophore forms around the invading or replicating virus, engulfing either the virion or replication intermediate into autophagosomes. Contents are degraded when autophagosomes fuse with lysosomes.

Xenophagy

Autophagy is an ancient, highly conserved pathway responsible for the lysosomal degradation of cytosolic constituents and organelles that is critical in maintaining cellular homeostasis. Recent studies have illustrated an important interplay between autophagy and the innate immune system. Signaling through PRRs can lead to the induction of autophagy. Clearance of intracellular pathogens via autophagy followed by degradation in the lysosome is referred to as “xenophagy” and presents an important mechanism of antiviral defense.¹⁰⁷ Upon xenophagocytosis, virions or viral replication products in the cytosol are engulfed into autophagosomes and degraded in the lysosome for clearance (Fig. 8.12). This pathway of viral clearance was shown to be important in protection of *Drosophila* from VSV infection.¹⁶³ Intracranial HSV-1 infection of mice defective in autophagy results in uncontrolled virus replication and neuropathogenesis.¹³⁷ In addition to xenophagic removal of virions within the cytosol, autophagic clearance of viral proteins or other viral products plays a key role in protection of infected cells, particularly terminally differentiated cell types such as neurons.¹³⁸ Given that multiple human viruses—including HSV-1, HCMV, HIV-1, and gamma herpesviruses—have evolved evasion mechanisms to escape destruction by xenophagy,⁴³ this pathway likely plays an important role in viral sequestration and clearance of viruses in humans.

CELL TYPES INVOLVED IN INNATE ANTIVIRAL RESPONSES

NK Cells

NK cells are cytotoxic lymphocytes that play an important role in antiviral immunity. NK cells detect and eliminate virally infected cells and produce IFN- γ to induce other antiviral mechanisms. The importance of NK cells and antiviral immunity, particularly against Herpesviridae, is highlighted by the fact that an individual identified to have isolated NK

deficiency with no other identifiable deficiency suffered from disseminated, life-threatening varicella infection, followed by cytomegalovirus (CMV) pneumonitis and cutaneous HSV infection.¹⁹ A similar observation was reported for a 2 year old who died of recurrent severe varicella infections.⁵⁰

NK cells express two classes of receptors, known as activating and inhibitory receptors. These receptors induce or inhibit the target cell lysis and IFN- γ production upon recognition of their cognate ligands on target cells (Fig. 8.13). The ligands for activating NK receptors are induced in virally infected cells. The ligands for NK inhibitory receptors are MHC class I molecules and other molecules constitutively expressed on most cells and downregulated upon viral infection. The ligands for inhibitory receptors can be downregulated either as a result of sensing the viral infection in the cell or, in the case of MHC-I, as a result of viral downregulation of MHC-I. Many DNA viruses encode molecules dedicated for evasion of MHC I processing and presentation of viral antigens in order to avoid recognition by CD8 T cells.⁶⁴ Through their ability to detect missing self (reduction in MHC class I molecules on the cell surface), NK cells fulfill the gap in immunosurveillance by CD8 T cells. Thus NK cell recognition of infected cells relies on both the sensing of “induced self” (by activating receptors) and the “missing self” (by inhibitory receptors)¹⁴⁶ (Fig. 8.13). The net effect of these changes is detected by NK cells. Only when positive signals dominate, NK cells induce apoptotic death of target cells by unleashing their cytotoxic granules.

NK receptors fall into two structurally distinct families. One group belongs to the killer inhibitory receptor (KIR) family of proteins, which contains immunoglobulin-like domains. Another group of receptors is known as killer lectin-like receptors (KLRs), and contains C-type lectin domains.¹⁹⁷ Both groups of receptors contain activating and inhibitory receptors. The signaling property is defined by the sequence motifs found in cytoplasmic tails of the receptors or their signaling adaptors. Activating receptors typically contain a short (few

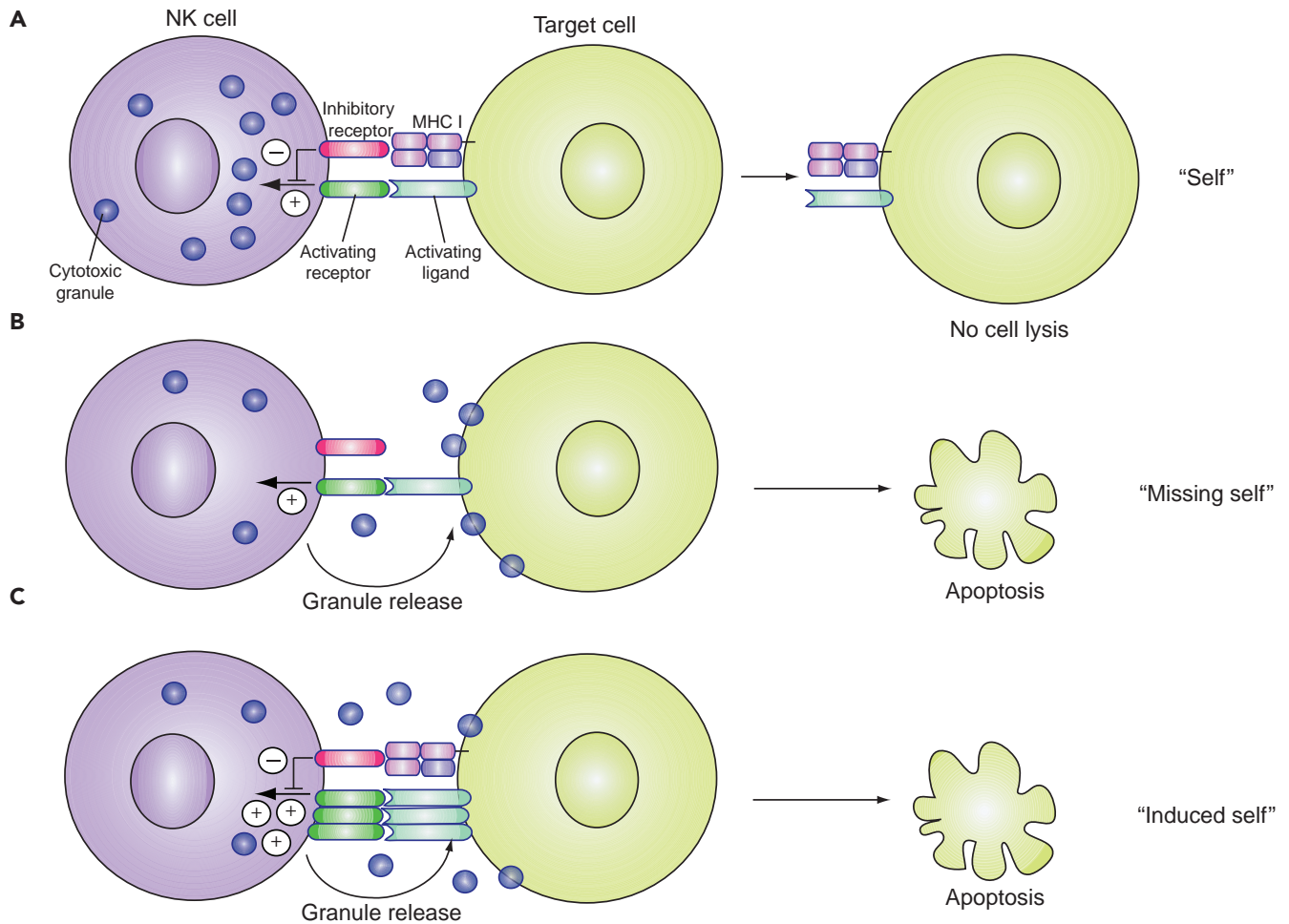


FIGURE 8.13. Natural killer cell activation and inhibition. NK cell recognition of infected cells relies on both the sensing of “induced self” (by activating receptors) and the “missing self” (by inhibitory receptors). NK cells contain cytosolic lytic granules that mediate apoptosis in target cells. **(A)** When an NK cell recognizes a healthy cell, NK cell lysis of the target cell is prevented by engagement of the inhibitory receptor by an normal level of MHC class I on the target cell. **(B)** When an NK cell interacts with virally infected cells that have reduced MHC class I on the cell surface (“missing self”), NK cells induce apoptosis in the target cell. **(C)** When an NK cell recognizes stressed virally infected cell expressing high levels of stimulating ligands (“induced self”), negative signals from inhibitory receptor is overridden by the activating signal, leading to lysis of target cells.

amino acids) cytoplasmic tail and are associated with signaling adaptors via ionic interactions of charged residues within their transmembrane regions. Several adaptor proteins are known to associate with activating receptors: DAP12 (DNAX-activating protein of 12 kDa), which contains the so-called immunoreceptor tyrosine-based activation motif (ITAM), which is a phosphotyrosine signaling module that recruits and activates Syk-72 tyrosine kinase upon recognition of the ligand by the ectodomain of the receptor complex. Another adaptor protein, called DAP10, has a distinct sequence motif that engages the PI3K signaling pathway. This adaptor is used exclusively by the NKG2D receptor, which is discussed in more detail below. In contrast to activating receptors, the inhibitory receptors contain the ITIM motif in their cytoplasmic regions.¹¹⁴ ITIM motifs recruit either tyrosine phosphatase SH2-domain-containing protein tyrosine phosphatase 1 (SHP1) or lipid phosphatase SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP1), which counter the effects of activating receptors. The opposing effects of ITAM and ITIM motifs

explain how the balance of activating and inhibitory receptor ligands determines the outcome of NK cell recognition.

Both humans and mice express the NKG2 family of KLRs, which heterodimerizes with CD94 and interact with nonpolymorphic MHC molecules such as HLA-E (human) and Qa-1 (mouse). In humans, the NKG2 family consists of NKG2A, B, C, D, E, and F, of which NKG2A and B are inhibitory and C and D are activating. In mice, the Ly49 family of KLR proteins is inhibitory except for Ly49H.

NK-activating receptors include the immunoglobulin domain containing proteins NKp30, NKp44, and NKp46, along with the KLR, NKG2D. The NKp proteins associate with CD3 ζ homodimers or the Fc receptor γ chain to induce activating signals. NKG2D is unique among the NKG2 family members in that it recognizes non-MHC ligands and associates with DAP10 and DAP12 signaling molecules, and provides activating signals to NK cells.

The ligands for activating receptors are generally absent or expressed at a low level on normal uninfected cells. Their

expression is upregulated in response to viral infection and cellular stress. The best characterized activating ligands are recognized by NKG2D.¹⁰⁴ Human ligands for NKG2D include MHC class-I-chain-related protein A (MICA) and MICB, which are nonclassical MHC I molecules. In mice, NKG2D binds to several ligands including retinoic acid early transcript 1 (Rae1), a glycosylphosphatidylinositol (GPI) anchored protein, histocompatibility 60 (H60), and mouse UL16-binding protein-like transcript 1 (Mult1). While Rae1, H60, and Mult1 only share 20% amino acid sequence homology, they share a structurally related domain that is distantly related to the MHC I α 1 and α 2 domains. Inducible expression of these ligands can overcome inhibitory signals provided by recognition of normally expressed MHC class I molecules, resulting in target cell lysis (Fig. 8.13C).

NK inhibitory receptors sense the level of MHC class I molecules on target cells. The CD94:NKG2A heterodimers recognize nonpolymorphic MHC class I molecules HLA-E in humans and Qa-1 in mice, which present leader peptides from classical MHC class-I proteins. Thus, when the MHC-I level decreases as a result of viral manipulation, it is reflected in the decreased level of HLA-E/Qa-1 expression, resulting in disengagement of the CD94:NKG2A inhibitory receptor. Not surprisingly, several viruses mimic the activity of HLA-E. UL40 of HCMV confers protection in infected cells by encoding the same leader sequence that can be presented on HLA-E,¹⁸² while HIV-1 selectively avoids downregulation of HLA-E and HLA-C to prevent NK-mediated lysis.³³ The mouse Ly49 family of inhibitory receptors recognizes the polymorphic MHC class I molecules. There are 18 Ly49 proteins in mice that have specificity to different MHC class I haplotypes. This mechanism of NK inhibition has been co-opted by MCMV, which encode m157, which stimulates Ly49I to inhibit NK activation.⁷

Plasmacytoid DCs

Among the leukocytes, pDCs are considered professional viral sensors for several reasons.¹¹¹ First, pDCs are equipped with special cellular machinery to endocytose many types of viruses and transport them to the endolysosomal compartment, whereupon uncoating of viral envelope and capsid, viral genomic DNA or RNA can be detected through TLR9 or TLR7, respectively. Second, pDCs constitutively express molecules involved in signaling to induce type I IFN genes. For instance, these cells express constitutively high levels of IRF7⁹⁴ and utilize IRAK1,¹⁸⁵ TRAF3,^{62,135} IKK α ,⁷⁴ osteopontin,¹⁶⁴ and PDC TREM¹⁹² to induce IRF7-dependent IFN transcription. Third, pDCs form specialized lysosome-related organelles from which TLR9 and TLR7 can engage IRF7 activation to trigger transcription of type I IFN genes. The formation of IFN-inducing organelles depends on the presence of adaptor protein 3 (AP-3).¹⁵⁴

Consequently, pDCs were originally known as IFN-producing cells (IPC), characterized by its potent ability to secrete type I IFNs in response to viruses.^{9,23,59,85} The phenotype of these type I IFN-producing pDCs has been found to differ by species, as they are CD4⁺CD11c⁺BDCA-2⁺CD123⁺ in humans¹⁶⁶ and CD11c^{int}CD11b⁺Gr-1⁺B220⁺SiglecH⁺ in mice.^{9,131} Whereas DCs as a class are thought to be the professional antigen presenting cells specializing in antigen uptake, processing, and presentation to T cells, the predominant role of the pDCs appears to be the secretion of type I IFNs. In particular, pDCs can be induced to secrete large amounts of IFN α when stimulated with viruses including SeV,⁸⁵ influenza,⁹ and HSV-1.^{23,59} However, this

situation may not be universal, as inflammatory monocytes after vaccinia virus infection,¹² and alveolar macrophages after respiratory NDV infection,¹⁰¹ but not pDCs, are the predominant IFN producers, highlighting a differential cell requirement for type I IFN responses dependent on the type of viral stimulus. The pDCs express both L-selectin and CCR7 and enter through the blood vessel into secondary lymphoid tissues.²⁷ At steady state, these cells are not found in peripheral tissues, where they can come in contact with local viral infections. However, they can be induced to migrate into peripheral tissues and secrete antiviral IFNs *in situ*.¹¹⁸ In mice that are depleted of pDCs, reduced early IFN-I production and augmented viral burden was observed after MCMV infection. During VSV infection, pDC depletion enhanced early viral replication and impaired the survival and accumulation of virus-specific cytotoxic T lymphocytes.¹⁷² Thus, pDCs mediate early antiviral IFN-I responses and influence CD8 T cell responses. Given the location of pDCs, these cells likely serve as the initial source for type I IFNs in response to systemic viral infections, and as a secondary source of type I IFNs in response to localized viral infections restricted to the peripheral tissues.

Monocytes

Monocytes are a population of circulating leukocytes that patrol the blood stream and peripheral tissue for infection. Once inside the tissue, monocytes can give rise to macrophages and dendritic cells. Circulating monocytes can be broadly categorized into two types: inflammatory monocytes and patrolling monocytes.¹⁰ Inflammatory monocytes (Ly6C⁺CCR2⁺ in mice and CD14⁺ in humans) are selectively recruited to inflamed tissues and lymph nodes *in vivo*, and produce high levels of TNF- α and IL-1 during infection or tissue damage. Mouse inflammatory monocytes express TLR2 and recognize dsDNA viruses such as vaccinia virus and MCMV, and secrete large amounts of type I IFNs.¹² Interestingly, monocyte recognition of viruses by TLR2 requires endocytosis while recognition of bacterial TLR2 ligands does not. While the exact viral ligands for TLR2-dependent detection by monocytes is unclear, TLR2 is known to be triggered by a variety of viruses including human cytomegalovirus,³⁴ mouse cytomegalovirus (MCMV),¹⁷³ herpes simplex virus types 1 and 2,^{103,156} hepatitis C virus,²⁸ lymphocytic choriomeningitis virus,²⁰³ measles virus,¹⁷ and vaccinia virus.²⁰⁴

The other subset of monocytes (Ly6C⁻ in mice and CD14^{dim} in humans) patrols the blood vasculature, can differentiate into macrophages after extravasation into tissues, and has been suggested to be associated with tissue repair.¹⁰ The human CD14^{dim} monocytes do not express cell surface TLRs but express endosomal TLR7 and TLR8, and after recognition of viral nucleic acids, produce TNF, IL-1 β , and chemokine (C-C motif) ligand 3 (CCL3), but not type I IFNs.³⁵

Macrophages

Macrophages are versatile cells that play central roles in inflammation, wound healing, tissue homeostasis, and tissue remodeling. Macrophages are professional phagocytes, clearing dying cells and pathogens. Macrophage can be found in most organs as specialized cell types, such as Kupffer cells (liver), microglia (neural tissue), and osteoclasts (bone). Macrophages are well known in their defense against bacterial and protozoan pathogens, as they are the chief phagocytes that engulf and degrade these types of pathogens upon activation with IFN- γ . Recent studies highlight the importance of macrophages in

antiviral defense. Macrophages are situated at the forefront of mucosal barriers. In the lung, alveolar macrophages sample and degrade incoming pathogens. In addition, alveolar macrophages become highly infected with respiratory viruses such as Newcastle disease virus (NDV), and produce the highest levels of type I IFNs locally that prevent other cells from becoming infected.¹⁰¹ Not only do macrophages provide the initial type I IFNs in infected tissue, they also serve as “viral sink” to prevent more vulnerable cell types from becoming infected. Subcutaneous injection of VSV results in selective and productive infection of subcapsular sinus macrophages in the draining lymph nodes. These macrophages secrete high levels of type I IFNs that prevent infection of neurons. In mice depleted of such macrophages, local injection of VSV results in infection of innervating neurons in the lymph node and neuropathogenesis and death of mice.⁷⁷ It is interesting to speculate how virus replication niche is supported by the subcapsular macrophages in the face of large amounts of type I IFNs secreted by these cells. Perhaps these subcapsular macrophages have a unique mechanism of resisting viral and IFN-induced cell death.

Dendritic Cells

Dendritic cells are professional antigen-presenting cells capable of stimulating naïve antigen-specific lymphocytes in secondary lymphoid organs. Dendritic cells play an important role in immune responses at multiple levels. First, they are situated at various sites of pathogen entry, and are among the first cells to recognize the incoming pathogens through a set of PRRs. Engagement of PRRs upon viral recognition induces the expression of genes required to both eliminate the pathogens (innate effectors) and to initiate adaptive immune responses.¹²⁶ Second, DCs are the only cell type capable of initiating adaptive immune responses by activating naïve T lymphocytes. Pathogen recognition through PRRs activates DCs to increase their expression of the chemokine receptor, CCR7, which enables them to migrate from the site of infection to the secondary lymphoid tissues, where naïve lymphocytes recirculate (Fig. 8.14). On transit, DCs undergo a maturation program that results in the upregulation of costimulatory molecules and translocation of their MHC class II to the cell surface.¹⁸³ Once in the lymph node, DCs can present antigens derived from the pathogens to naïve T cells and induce their activation (through costimulation) and differentiation (through secretion of appropriate cytokines)¹¹ (Fig. 8.15). A naïve T cell requires three kinds of signals for activation and differentiation. First, a T cell must recognize viral peptides presented by the MHC molecules (signal 1). CD4 T cells bind to peptides presented by MHC class II, while CD8 T cells bind to peptides presented by MHC class I. Second, T cells must receive signals from costimulatory molecules on APCs. PRR stimulation of APCs results in the expression of costimulatory molecules including CD80 and CD86, which bind to CD28 on naïve T cells and provide this second signal that allows optimal clonal expansion. The third signal dictates the differentiation of a naïve T cell into different effector types. CD4 T cells can differentiate into Th1, Th2, and Th17 cells depending on the cytokines provided by the APCs or other accessory cell types. Antiviral responses are mediated by Th1 cells, as they are uniquely capable of secreting IFN- γ .

DCs can be broadly divided into two types, those that reside in the peripheral tissues (mucosa, skin, internal organs; tissue DCs) and those that reside in the blood and lymphoid

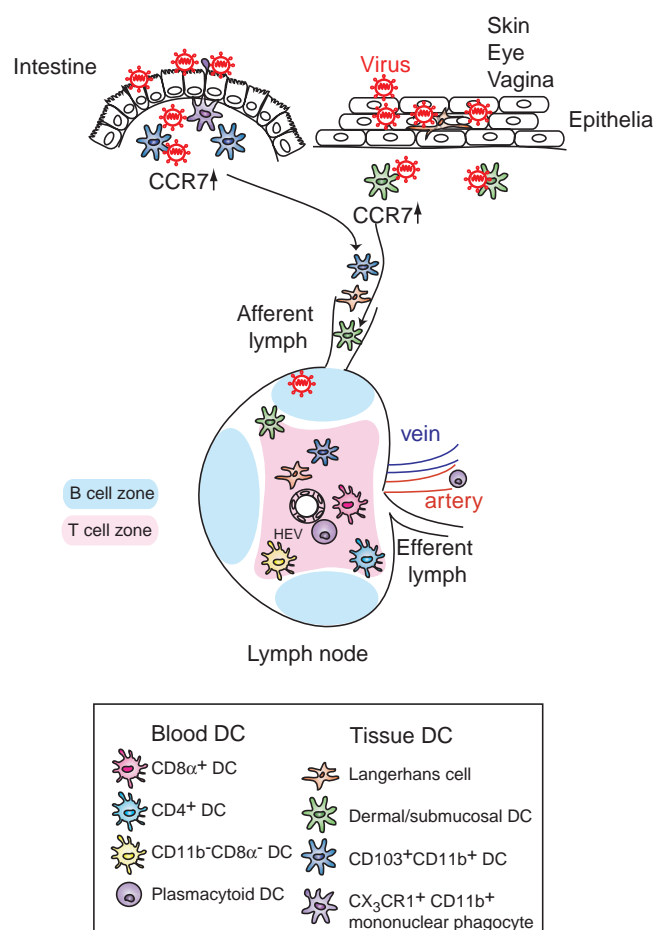


FIGURE 8.14. Dendritic cell populations in the peripheral tissues and in the secondary lymphoid organs.

At the mucosal surfaces and in skin, distinct DC populations are found. In intestinal mucosa, CX3CR1⁺ mononuclear phagocytes extend their dendrites and survey the environment just beneath the epithelial layer, while CD103⁺ CD11b⁺ DCs in the lamina propria can take up antigens and migrate to the mediastinal lymph node to prime T cells. In the epithelial layer of the mucosa of the vagina and eye or in the skin, Langerhans cells are the only DCs present in this layer. In the submucosal or dermis, submucosal/dermal DCs survey the environment for pathogen invasion. Both Langerhans cells and submucosal/dermal DCs are able to migrate to the draining lymph node. In most cases, the latter DC populations are required for T-cell priming. Within the lymph nodes, four different DC subsets appear to perform distinct functions. CD8 α ⁺ DCs are specialized in cross presentation of viral antigens to CD8 T cells, while CD11b⁺ and DN cells are capable of priming CD4 T cells. Plasmacytoid DCs do not participate in induction of effector T cell responses but serve as a key producer of type I IFNs.

tissues (blood DCs).⁸⁴ The blood DCs differentiate from precursors that enter these tissues from peripheral blood. In the mouse lymph nodes and spleen, the blood DCs can be divided into CD8 α ⁺ DC (CD8 α ⁺ CD11b⁻), CD4⁺ DC (CD4⁺ CD11b⁺), DN DC (CD4⁻ CD8⁻), and plasmacytoid DCs (pDCs).^{66,165} In cutaneous lymph nodes or lymph nodes draining vaginal or eye mucosae, two extra DC subsets exist that are derived from the tissue, the Langerhans cells (LCs) and dermal or submucosal DCs^{6,70,150} (Fig. 8.13). In the intestinal lamina propria, a distinct set of tissue DCs surveys the environment.¹⁸⁸ Just beneath

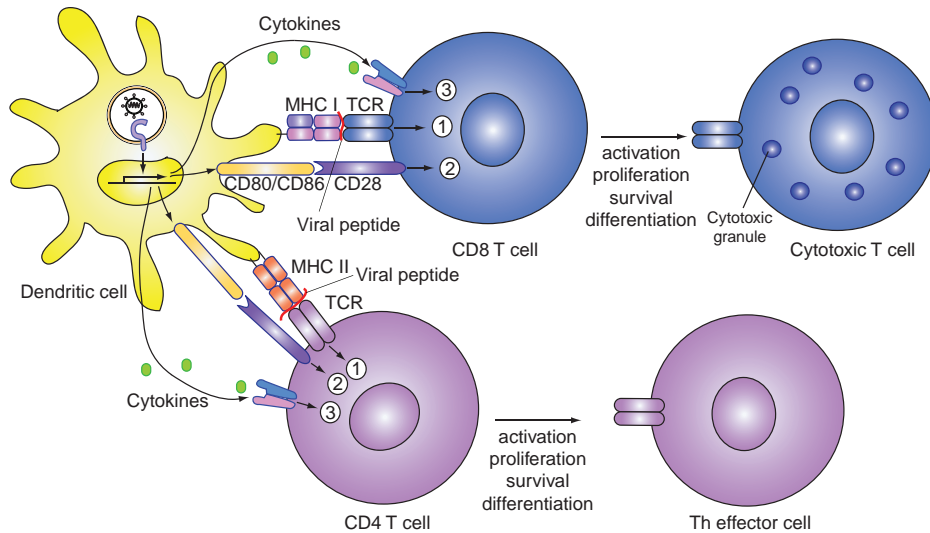


FIGURE 8.15. Innate instruction of adaptive immunity. Within the lymph node, DCs presenting viral antigenic peptides on MHC class I and MHC class II are able to interact with naïve CD8 T and CD4 T cells whose T cell receptors (TCR) are specific to the peptide presented by MHC molecules. PRR engagement (shown here for an endosomal TLR) by viral PAMPs induce expression of costimulatory molecules CD80 and CD86, as well as cytokines that stimulate naïve T cells, providing second and third signals needed for robust T-cell activation and differentiation. T cells receiving all three signals can proliferate and differentiate to become effector T cells. Depending on the cytokines secreted by DCs, CD4 T cells can become various effector Th types including Th1, Th2, or Th17.

the villous epithelial layer, CX₃CR1⁺ mononuclear phagocytes extend their dendrites into the lumen. These cells do not migrate to the lymph node, but play an important role in intestinal homeostasis. In the lamina propria, CD103⁺ CD11b⁺ DCs pick up ingested antigens, and migrate to the mesenteric lymph node to prime immune responses. DC subsets are equipped with specialized cellular machinery to perform distinct functions. For instance, among the blood DC groups, CD8⁺ DCs are equipped with machinery to carry out cross-priming to activate naïve CD8 T cells, while CD8⁻ DCs express proteins involved in the MHC class II presentation pathway leading to the activation of naïve CD4 T cells.⁴⁸ However, migratory DCs from the infected tissues are required to elicit immune responses to localized infections, indicating that both tissue-resident and lymphoid-resident DCs cooperate to prime a robust immune response to pathogens.¹⁰⁵ In contrast, pDCs are specialized in recognizing viruses and secreting high levels of type I IFNs, but are not involved in activation of naïve T cells. Different DC subsets orchestrate innate and adaptive immune responses to viruses, depending on the type of PRR engaged by a given virus infection.

VIRUS MANIPULATION OF INNATE IMMUNITY

Virus–TLR Interaction as Viral Pathogenesis Mechanism

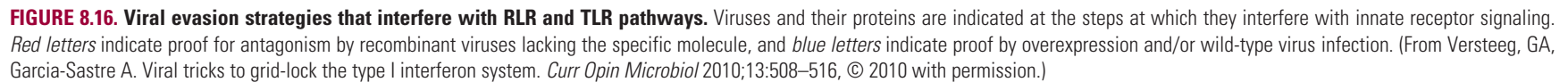
A virus–host PRR interaction reflects either host recognition of viruses to elicit appropriate antiviral defense, or viral strategy to infect the host. As discussed in the previous section, an example of the former is TLR9 recognition of viral DNA within the endosomes. This interaction leads to the activation of type I IFNs and prevention of viral replication in neighboring cells.

However, most TLR–virus interactions that occur at the cell surface belong to the latter category, with the exception of TLR2-mediated recognition of viruses by monocytes (see Cell Types Involved in Innate Antiviral Responses). In fact, certain viruses rely on TLR signaling as a survival mechanism.^{17,89} MMTV persists indefinitely in WT mice, but is rapidly cleared in mice deficient in TLR4 by cytotoxic T-cell response.⁸⁹ MMTV stimulates IL-10 production by B cells through dendritic cell and macrophage activation mediated by TLR4 signaling. IL-10, which is an immune suppressive cytokine, protects the MMTV-infected B cells from removal by cytotoxic T cells. It has been suggested that IL-10 may serve as a master regulator of chronic vs. acute viral infections,²⁰ thus it warrants analyzing whether TLR-dependent IL-10 production plays an important role in the pathogenesis of other chronic viral systems, such as HIV and HCV. Another example in which TLR–virus interaction benefits the virus is the measles virus. The HA protein of measles virus induces cytokine secretion in a TLR2-dependent manner. Interestingly, this interaction also results in upregulated expression of the MV receptor, CD150, suggesting that HA–TLR2 interactions in fact benefit the virus at the expense of the host.¹⁷

Viral Evasion of the Innate Immune System

Virtually all viruses encode factors to counteract the induction, signaling, or antiviral effector functions induced by the type I IFNs.¹⁹⁰ While viruses employ distinct molecules to achieve blockade of the antiviral effects of type I IFNs, these can be categorized into four major strategies: (1) global inhibition of cellular gene expression, (2) evasion from innate recognition, (3) inhibition of molecules involved in the IFN induction and signaling (Fig. 8.16), and (4) inactivation of the IFN-induced effector molecules (Fig. 8.17). A common strategy

Toll-like receptors



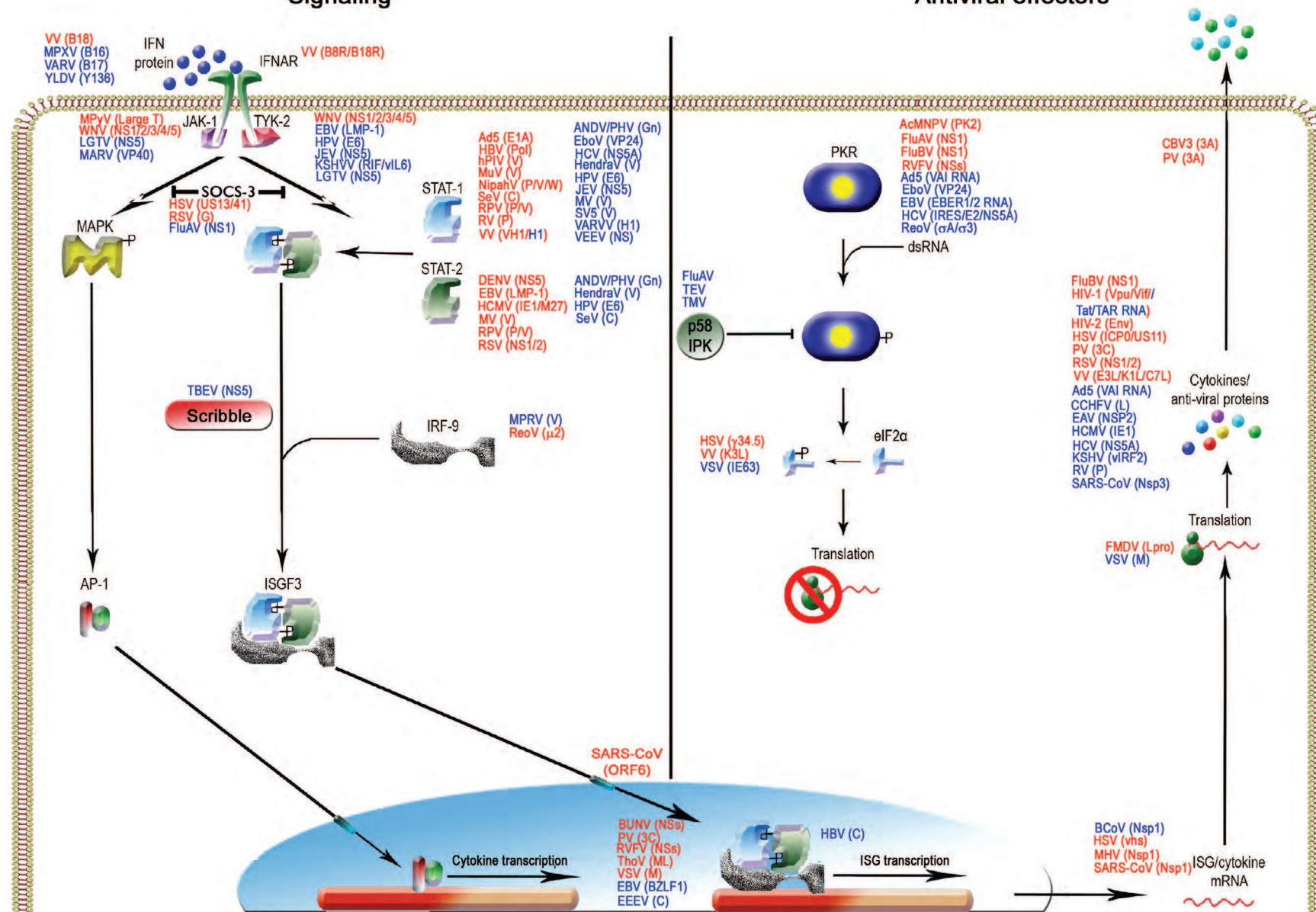


FIGURE 8.17. Viral evasion strategies that interfere with IFN signaling and antiviral effectors. Viruses and their antagonistic proteins are indicated at the steps of the IFN pathway. *Red letters* indicate proof for IFN antagonism by recombinant viruses lacking the specific molecule, and *blue letters* indicate proof by overexpression and/or wild-type virus infection. (From Versteeg, GA, Garcia-Sastre A. Viral tricks to grid-lock the type I interferon system. *Curr Opin Microbiol* 2010;13:508–516, © 2010 with permission.)

employed by many viruses is to inhibit cellular gene expression by interfering with host transcription and/or translation. Viruses that replicate in the cytosol that do not require host transcription for replication often employ strategies to block host transcription altogether. Poliovirus 3C protease and the VSV matrix (M) protein inhibit host gene expression by inactivating transcription initiation factor, TFIID.¹¹⁹ The cleavage of translation initiation factor eIF4G in picornavirus-infected cells is a classic example of virus-induced inhibition of host translation. For this group of viruses, the inhibition of cap-dependent translation has no effect on translation of viral mRNAs because they depend on an internal ribosome entry site (IRES) for translation. Other viruses block the earliest steps in innate recognition by interfering with the function of innate sensors such as MDA-5 and RIG-I. Paramyxovirus V protein prevents MDA-5 dimerization,⁵ and influenza A and B viruses express an NS1 protein preventing the activation of RIG-I by binding and inhibiting the actions of the E3 ligase TRIM25.⁵⁷ A common viral strategy to interfere with IFN gene induction is represented by viral inhibitors of IRF3 and IRF7, master transcription factors of type I IFN genes. Examples include KSHV vIRF3, that block host IRF7 activity,⁸⁸ and rotavirus NSP1, that block IRF3 and IRF7.^{14,161}

Finally, even if the infected cells are able to produce type I IFNs, many viruses encode factors that inhibit signaling through the IFN- $\alpha\beta$ R. SARS-CoV ORF6 protein is involved in preventing nuclear translocation of STAT1 by sequestering nuclear import factors on the ER/Golgi membrane.⁵⁶ Even if the infected cells manage to induce and respond to type I IFNs, influenza virus,¹⁴¹ RSV,¹³⁹ HCV,²¹ and HSV¹⁹⁶ induce SOCS-1 and SOCS-3 expression, which negatively regulates the JAK–STAT pathway.

Differences between Evasion Strategy Mechanisms of DNA vs. RNA Viruses

Even though all viruses encode factors that interfere with host antiviral defense mechanisms, different classes of viruses appear to target different stages in antiviral defense. Nearly all viruses with (–)ssRNA genomes interfere with the innate sensors themselves. By contrast, (+)ssRNA and dsDNA viruses target the downstream IRFs as the means to interfere with IFN induction.¹⁹⁰ Another intriguing feature shared among the dsDNA viruses, particularly poxviruses, is that they encode soluble decoy receptors that bind to secreted type I IFNs and prevent IFN- $\alpha\beta$ R signaling. A key advantage of this strategy, compared to the others, is to be able to prevent neighboring cells from becoming resistant to viral infection.

Another striking difference between the host evasion mechanisms employed by different virus groups is that RNA viruses encode often only one or two molecules dedicated to inhibiting host response, usually through interception at the initiation of innate immune responses. Such a viral factor is usually a nonstructural protein that is expressed in infected cells, and has multiple functions in inhibiting the antiviral mechanisms of the host cells. A good example of this is influenza virus NS1. Despite being only 230aa long, NS1 inhibits almost all stages of antiviral pathways: through sequestration of viral RNA from innate recognition, blocking RIG-I function, inhibition of host mRNA processing and export, inhibition of innate effectors PKR and OAS, and IFN- $\alpha\beta$ R signaling.¹⁹⁰

In contrast, many dsDNA viruses, because of their large genome size, can accommodate multiple genes to specifically inhibit pathways that are useful for their own replicative niche. By expressing soluble IFN-binding proteins that compete with the cellular IFN receptor for its ligand, dsDNA viruses allow for infection and spread to cells that would otherwise be prevented from infection. Another feature associated with dsDNA viruses is their ability to establish latency. To this end, almost all herpesviruses encode molecules that specifically block PKR and its downstream target to block cell-mediated arrest of translation. This strategy is particularly important to ensure survival of the latently infected cells. Other dsDNA viruses have as narrow a target as to inhibit one specific NK receptor ligand. MCMV encodes three proteins—m152, m145, and m155—that reduce surface expression of NKG2D ligands, RAE-1,¹¹² MULT-1,⁹⁹ and H60,¹¹³ respectively, in infected cells. Chronicity of virus infection may result from the ability of dsDNA viruses to manipulate the host immune response by interfering with specific effector mechanisms of various host cell types.

CONCLUSION

In the past 10 years, there has been a tremendous progress in characterization of the innate immune recognition of viral pathogens. The role of type-I IFNs, NK cells, TLRs, and RLRs in antiviral defense is now well appreciated and understood in considerable detail. There are some obvious gaps remaining, such as better characterization of intracellular DNA recognition pathways and their role in immunity to DNA viruses. Most IFN-induced antiviral gene products still have to be functionally characterized. These proteins likely interfere with multiple steps in viral infection cycles. This functional redundancy makes the analysis of their contribution to antiviral defense particularly challenging. Other outstanding questions include the elucidation of the mechanisms that control the expression of ligands for activating and inhibitory NK receptors. These ligands may be regulated by cellular stresses associated with viral infection or by signals from cell-intrinsic pattern recognition receptors. Finally, in the next few years we will likely witness continuous progress in understanding the role of noncoding RNAs, such as micro RNAs and piwi-interacting RNAs in antiviral immunity. Ultimately, the greatest challenge lies with the application of the accumulating knowledge to the management, and potentially even eradication, of major viral infections that continue to threaten our well being.

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Adaptive Immune Response to Viral Infections

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INTRODUCTION

Viruses have been part of human ecology since the human species first evolved on the plains of East Africa. As humans moved out of Africa to populate the continental landmasses,

viruses—already adapted to the human species—were carried along with other microorganisms as part of the human ecological baggage. With the migration of humans around the world, we were exposed to new viruses present in the animal species that humans encountered. Some viruses, such as the herpesviridae family members, Epstein-Barr virus (EBV), and herpes simplex virus (HSV), have co-evolved with humans and have developed complex mechanisms (e.g., latency) to subvert the host defense mechanisms and sustain themselves in the human population. These viruses generally produce mild disease, except where host defenses are absent or weakened (e.g., in immune-deficient or immune-suppressed individuals). Other human viruses (e.g., measles virus) appear to have evolved mechanisms to persist at low levels in some infected individuals and sustain themselves by infecting previously unexposed individuals (typically children) who then go on to develop mild to severe illness and, in turn, serve as reservoirs for further virus propagation. Animal viruses can also *jump* from their host reservoir to infect humans and produce devastating diseases of potentially pandemic proportions. Notable examples of zoonotic viral diseases are the following: (a) the acquired immunodeficiency syndrome (AIDS) pandemic produced by the human immunodeficiency virus (HIV); (b) the outbreak of severe acute respiratory syndrome (SARS) in 2002 produced by the SARS coronavirus; (c) the 2009 H1N1 swine origin influenza A virus pandemic arising from the genetic reassortment in pigs of viruses with donated genes of human, swine, and avian flu origin; and (d) the periodic outbreaks of highly pathogenic avian influenza infection in poultry since 1997 in Asia and most recently in Africa and Europe, which have resulted in sporadic direct infections of humans with mortality rates approaching 50%. Indeed, the recent reconstruction and analysis of the genome of the influenza virus that caused the 1918 *Spanish flu* pandemic suggest that this virus may have been an avian influenza virus that spread directly to humans (without modification of virus in an intermediate host or the acquisition of genetic information from then-circulating human influenza strains).²⁸⁸

Virus infection can result in acute or chronic disease in virtually any organ and tissue of the body. Furthermore, infection with human papillomavirus (HPV), human herpesvirus 8 (HHV-8), and hepatitis B virus (HBV) and hepatitis C virus (HCV) have been directly linked to the development of cervical cancer, Kaposi's sarcoma, and hepatocellular carcinoma, respectively. Given the pandemic outbreak of the swine origin A/California/2009 (H1N1) virus infection, the possibility of another human influenza pandemic from an avian influenza source, as well as the devastating effects of HIV infection all suggest, virus infection represents a continuing threat to human health. We can point to some notable successes in the struggle against viruses (e.g., the eradication of smallpox in the 1970s, and the effectiveness of the live attenuated oral polio vaccine in eliminating paralytic polio in the developing world, raising the prospect of eradicating polio worldwide). Both of these vaccination successes rely on the mobilization of the body's major defense against virus infection—the adaptive immune system. Our enthusiasm about these *successes* must be tempered, however. Because of the success of smallpox eradication, mandatory smallpox vaccination was discontinued in the United States in the early 1970s, making a large fraction of the population (i.e., those younger than age 40) now susceptible to smallpox.

This fact, along with the advances in poxvirus genetics and genetic engineering, now makes smallpox a major bioterrorism threat. Similarly, administration of live oral polio vaccine to children in underdeveloped countries whose immune function is compromised by malnutrition or HIV infection can result in outbreaks of paralytic polio caused by infection with virulent mutants (revertants) of the live attenuated vaccine strain. This realization has prompted a modification of the guidelines for oral polio vaccine administration in the United States and a reevaluation of the prospect of global polio eradication using current vaccination strategies. To counter the continuing threat posed by viruses, it is essential to understand the body's primary defense against virus infection in the immune system.

OVERVIEW OF THE ADAPTIVE IMMUNE RESPONSE TO VIRUS INFECTION

Viruses are obligatory intracellular parasites; that is, they replicate within cells of the infected host and use the cell's biosynthetic machinery for replication and production of progeny virions. Furthermore, most viruses replicate rapidly in infected cells (i.e., within hours) producing progeny virions capable of infecting additional cells, thereby propagating and sustaining infection in the host. These properties of infection by viruses dictate how the immune system must respond to counterinfection. In acute virus infection, the race between the replication of the virus and the host response will result either in the death of the host or in virus clearance and the termination of infection. The tissue injury produced by viruses at the site(s) of infection results both from the cytopathic effects of virus replication and, equally importantly, from the host immune response to infection. In general, therefore, the greater the extent of virus replication in the infected host, the greater the tissue injury and illness severity.

In chronic infection, the time scale of virus replication in the host is not measured in days (as in acute infection) but in weeks, months, or even years, with the host immune response likewise continuing over the same prolonged time scale. Viruses that produce chronic, persistent infection have evolved mechanisms to suppress or alter the immune response (allowing these viruses to persist in the infected host). The tissue injury produced by the immune response to virus infection (termed, *immunopathology*) occurs with both acute and chronic virus infection. In many chronic virus infections (e.g., HCV infection); however, immune-mediated tissue injury predominates (rather than direct virus-mediated injury).³⁸ Furthermore, the clinical manifestations of virus infection (e.g., fever, headache, myalgia, anorexia) are caused primarily by inflammatory mediators (e.g., cytokines) released by cells of the immune system in response to infection. Undoubtedly, chronic (persistent) virus infection also alters or modifies the immune system and its capacity to respond to microbes and environmental antigens.²⁹⁷ Finally, the triggering of the immune system by virus infection can result in the induction of aberrant immune responses directed both to the virus, and to self-cellular constituents, resulting in autoimmune disease.¹⁴³

The primary host defenses against virus infection are physical/chemical barriers to infection and the immune system. The immune system can be divided into two components: the innate immune system (see Chapter 8) and the adaptive immune system. This division is based on the properties of

TABLE 9.1 Cells of the Immune System

Type	Subtype	Onset of effect
Innate immune Cell		
Granulocytic	Neutrophil, basophil, eosinophil	Immediate/inducible (hr)
Monocytic	Macrophage, DCs	Immediate/inducible (hr)
Lymphocytic ^a	NK cell, NK T cell	Immediate/inducible (hr)
Somatic cell (nonhematopoietic)	Epithelial, endothelial cell, etc.	Immediate/inducible (hr)
Adaptive immune Cell		
B Lymphocyte	—	Inducible (d)
T Lymphocyte	CD4 ⁺ , CD8 ⁺ , T _{reg}	Inducible (d)

DC, dendritic cell; NK, natural killer; TCR, T-cell antigen receptor; T_{reg}, regulatory T-cells; hr, hour; d, day.

^aSpecific subtypes of T lymphocytes [e.g., $\gamma\delta$ T cells and intraepithelial (IEL) CD8⁺ $\alpha\alpha$ T lymphocytes] are considered to be part of the innate immune system. These cell types with lymphocytic morphology employ a TCR-type antigen recognition system, but differ in their function and strategy of antigen recognition from conventional T cells.

the immune cell types and molecules involved in the response to infection and the tempo of the response (Table 9.1). The response of the innate immune system to virus infection is either immediate (i.e., constitutively active) or rapidly induced (i.e., typically within hours of infection). The innate immune response is triggered in two ways: first, through recognition of viral constituents (e.g., viral nucleic acids) by a limited set of cellular pattern recognition receptors for foreign molecules displayed on innate immune cells; and, second, through the activation of intracellular signaling mechanisms following virus entry into cells and the initiation of virus replication. Importantly, a defining feature of the innate immune response is that repeat exposure to a particular virus generally evokes an identical response from the innate immune system (although a form of that enhanced efficiency of pathogen recognition has recently been reported for natural killer (NK) cells.²⁹⁸ The genes encoding these receptors and associated signaling mechanisms within innate immune cells are encoded within the germline (i.e., fixed in the genome).

By contrast, the response of the adaptive immune system to a first encounter with a virus takes days to evolve. The response of the cells of the adaptive immune system (i.e., B lymphocytes and T lymphocytes) is triggered by viral constituents, primarily through the engagement of highly specific cell-surface recognition receptors generated by somatic gene rearrangement and displayed by a minuscule fraction of the total repertoire of adaptive immune cells for any given virus. These receptors only recognize the constituents (antigens) of that particular virus. Importantly, this arm of the immune system *adapts* to repeat exposure to a particular virus or viral protein; this results in a more rapid response of higher magnitude by the specific adaptive immune cells on repeat exposure/infection—a phenomenon called *immunological memory*, the basis for vaccination.

The constituents of the innate immune response include proteins of the complement system, and so called *acute phase proteins* (e.g., members of the collectin protein family).²⁵¹ These molecules are present *constitutively* in the blood and tissue fluids or are induced rapidly in the liver following infection and can bind directly to certain viruses, thereby inhibiting virus infection. Leukocytes, in particular, neutrophils, blood monocytes, tissue macrophages, dendritic cells, NK cells and NK T cells are the dominant, innate immune cell types responding to virus infection. They are triggered/activated through

the engagement of pattern recognition receptors (e.g., Toll-like receptors [TLRs], C-type lectins) expressed on their cell surface, which recognize viral constituents (e.g., viral nucleic acids), and in the case of NK and NK T cells, alterations of the cell surface of infected cells. These cells reside in the tissues, and they respond there or are recruited to sites of infection from the bloodstream through the action of cytokines/chemokines released during infection.

Nonhematopoietic body cells (e.g., epithelial cells, endothelial cells, fibroblasts) that are targets of virus infection, must also be considered part of the innate immune response because, on infection, they are triggered to secrete antiviral proinflammatory mediators such as type 1 interferons and certain cytokines/chemokines (the production of which can initiate and amplify the innate immune response to infection). The induction of the innate immune response and the role of innate immune cells and molecules in controlling virus infection are discussed in detail elsewhere (Chapter 8). One of these innate immune cell types, the dendritic cell (DC), however, bridges the innate and adaptive immune response, and its role in the initiation of the adaptive immune response is discussed in a later section of this chapter.

A hallmark of the adaptive immune response is the exquisite specificity of adaptive immune cells for a particular foreign viral antigen, and the capacity of this system to recognize and respond to a myriad of viruses (or other antigens). This remarkable property of the adaptive immune cells is achieved by the generation of a correspondingly diverse array of cell surface recognition receptors, which are randomly generated within the lymphocytes of every individual and clonally distributed among the adaptive immune cells. As a consequence, in a nonimmune individual, only an extremely small fraction of the adaptive immune cell repertoire (e.g., one cell in a million or less) will have an antigen receptor capable of recognizing a specific virus (viral antigen). Understanding how this diverse array of clonally distributed antigen receptors is generated during lymphocyte development and how that rare adaptive immune cell directed to particular virus finds virus or infected cells in a peripheral site of infection (e.g., the skin or lungs) is critical to the understanding of the adaptive immune response to virus infection.

Viruses, because they exist as extracellular virion particles and replicate/assemble within infected cells, pose a unique recognition problem for the adaptive immune system (i.e., how

TABLE 9.2 Properties of T and B Lymphocytes

General properties	B lymphocytes	T lymphocytes
Origin	Bone marrow	Bone marrow
Self/non-self-discrimination	Bone marrow	Thymus
Activation	BCR and other ligand receptors	TCR and other ligand receptors
Antigen recognition	Native (3D) conformation	Nonnative fragments
Antigen receptor	B lymphocytes	T lymphocytes
Somatically generated	+	+
High specificity	+	+
Clonally represented	+	+
Localization	Cell surface/secreted (Ab)	Cell surface only

BCR, B-cell antigen receptor; TCR, T-cell antigen receptor; 3D, three-dimensional; Ab, antibody.

to recognize the virus [viral proteins or genome] both outside of the cell and within the infected cell). The elegant solution to this problem employed by the adaptive immune system is the partitioning of the adaptive immune system into two distinct cell types: B and T lymphocytes (Table 9.2). B lymphocytes displaying a receptor specific for a particular virus will, on encounter with the virus and activation, release their antigen receptor in a soluble form (i.e., as antibody molecules) capable of binding free (extracellular) virions in a highly specific matter. This interaction typically results in virus neutralization and/or elimination. Antiviral B-lymphocyte responses are exquisitely sensitive to the three-dimensional confirmation of the virus (and its constituents), and they play a dominant role in clearing virus during infection, in preventing or limiting reinfection (after previous virus infection), and in vaccination against a specific virus.

Activated antiviral T lymphocytes retain their antigen-specific receptors on the cell surface and are responsible for recognizing and eliminating virus-infected cells. T lymphocytes, however, do not recognize viral proteins in their native conformation. Rather, the antigen receptors on the T lymphocytes recognize *processed* peptide fragments of viral proteins displayed on the infected cell surface bound to cell-surface molecular recognition platforms (i.e., the major histocompatibility complex [MHC] locus gene products). Activated virus-specific T lymphocytes halt the spread of infection by killing virus-infected cells through direct cell-to-cell contact and by the release of soluble mediators (e.g., cytokines, such as IFN- γ and tumor necrosis factor α [TNF- α]). By releasing these and other soluble mediators, the activated T lymphocytes recruit and orchestrate the response of the innate immune cells which, in turn, act to clear infection. Understanding how and where B and T lymphocytes activate, the mechanism of antigen presentation to the cells, and the range of effector activities that these cells use to control and eliminate virus is essential for understanding the adaptive immune response to virus infection. These important topics are reviewed in this chapter.

Finally, the importance of the immune system in general and the adaptive immune response in particular in controlling virus clearance and promoting recovery from infection is exemplified by the fact that viruses have evolved a variety of mechanisms to inhibit or alter the host immune response. Whereas the virus immune evasion strategies are best considered in the context of viral pathogenesis (Chapter 10) and are addressed in chapters dealing with individual viruses, this chapter high-

lights some of the viral evasion strategies directed to the adaptive immune response.

ARCHITECTURE OF THE ADAPTIVE IMMUNE SYSTEM

Generation of T and B Lymphocytes: Primary Lymphoid Organ

Unlike the cells of the innate immune system, T and B lymphocytes possess one unique and profoundly important property. On their surfaces, these cells also display a receptor complex capable of recognizing foreign (nonself) structures—with exquisite specificity. This receptor complex is both randomly generated (by somatic recombination of genetic elements ultimately encoding the mature receptors) and clonally distributed among progeny lymphocytes during their differentiation from progenitor cells. Therefore, of the approximate 10^9 naive T and B lymphocytes in the human adaptive immune system, each cell may, in principle, display a unique receptor directed to a specific foreign antigenic structure. This common property of the antigen receptor on T and B lymphocytes (i.e., random generation by somatic DNA recombination and clonal receptor display) immediately raises two important questions. First, if these antigen receptors are stochastically generated, how is the generation of lymphocytes with receptors to self-structures (e.g., tissue proteins) avoided/prevented? Second, if the frequency of lymphocytes with a receptor directed to a specific foreign antigen (e.g., the coat proteins of an invading virus) is extremely low (1 in 10^7 lymphocytes), how can those rare lymphocytes locate the antigen quickly enough to respond before the infection is beyond control? As is discussed below, it is the unique structure of the primary (central) lymphoid organs where lymphocyte progenitors develop into mature T and B lymphocytes, and of the secondary (peripheral) lymphoid organs where mature T and B lymphocytes respond to invading microorganisms that provides the answers to these questions.

Mature T and B lymphocytes are derived from lymphoid progenitors present in the bone marrow, as well as in sites of hematopoiesis in developing fetus (e.g., the liver) through a process of differentiation and selection linked to, and driven by, the antigen receptors displayed by these cells.^{221,233} As described in Table 9.2, B-lymphocyte lineage development occurs in the bone marrow and follows a well-defined, step-wise program of gene

activation (e.g., expression of the recombination activating gene 1 and 2 [*RAG1* and 2], lymphoid DNA-specific recombinases), and sequential rearrangement of immunoglobulin (Ig) V_H , D_H , J_H , and C_H heavy (H) chain genetic elements (by random somatic recombination), followed by rearrangement of the Ig K/λ V_L - J_L - $C_{LK/\lambda}$ light (L) chain locus elements. Then B-lymphocyte progenitors transit from the pro-B, through the pre-B, and to the surface immunoglobulin-positive immature B-lymphocyte stage. It is during this development/differentiation program that the B-lymphocyte receptor (B-cell receptor [BCR]) diversification occurs through random association of one of the 40 V_H gene segments with one of the 25 D_H and 6 J_H and 70 V_L with 9 J_L to yield (after nucleotide addition and/or excision at the site of gene segment recombination) 10^7 or more different potential BCR H and L chain combinations. During this development process in the bone marrow, T-Lymphocytes expressing a BCR with reactivity to self-molecules (i.e., proteins, lipids, carbohydrates, nucleic acids) will, as a result of BCR engagement, undergo a process of negative selection. These *self-reactive* B cells are largely eliminated/deleted through interaction of the BCR on the developing cells, with self-constituents resulting in apoptosis or in activation ("anergy") of self-reactive B lymphocytes. The immature B lymphocytes that escape negative selection undergo final maturation, migrate from the bone marrow to join the pool of mature B lymphocytes that circulate through the blood, and populate the secondary lymphoid organs. B lymphocytes ultimately express their effector activity by releasing the BCR of the mature B lymphocyte in a secreted form as immunoglobulin (antibody) molecules. This fact has the following two important implications for the recognition of viruses by B lymphocytes: (a) the B-lymphocyte response to viruses will be particularly effective when interacting with free/extracellular virus constituents (e.g., virions); and (b) the BCR and its antibody product will be sensitive to the conformation of viral constituent recognized.

T-Lymphocyte lineage development follows a program similar to that of B lymphocytes. The T-cell antigen receptor (TCR) is also a heterodimer consisting of two polypeptide chains, each of which is formed by somatic recombination of variable (V), diversity (D), and joining (J) segments encoding the variable portion of one chain (or V and J joining for the other chain) to a constant (C) gene segment. As in B lymphocytes, the randomly recombined gene segments encoding the V region gene segment joined to the respective C region gene segment for the two TCR chains encodes the TCR on the developing T lymphocyte. Unlike the BCR, however, the TCR always remains cell associated. Therefore, the TCR is normally directed to foreign antigens (or self-structures) that are cell associated. Consequently, T lymphocytes are uniquely suited to recognize cells that have been invaded by microorganisms (e.g., viruses and intracellular bacteria). Unlike the BCR, the TCR does not recognize intact microbial products, but rather recognizes small fragments of molecules—typically, short peptide fragments of microbial or self-proteins bound to protein products of the mammalian MHC genetic locus (e.g., the classically defined major human transplantation antigens). These MHC gene products expressed on cell surfaces serve as molecular platforms, both selecting and displaying the self-peptides involved in TCR selection during T-lymphocyte precursor development, and presenting microbial peptides to mature T lymphocytes responding to uptake/infection of cells by the microbes. The steps in the processing (fragmenting) and presentation of both self and microbial gene products by the MHC locus protein,

and the structure of the major classes of MHC locus proteins (i.e., MHC class I and II proteins) are discussed below.

The generation of mature T lymphocyte from committed lymphocyte progenitors (arising in the bone marrow) occurs primarily in the thymus (Fig. 9.1). Here, immature thymocytes undergo the developmental program resulting in the formation of mature T lymphocytes, which then leave the thymus, enter the circulation, and ultimately populate secondary lymphoid organs. Two classes of mature T lymphocytes are produced in the thymus. The major population (~80% of the peripheral T lymphocyte pool) are the $\alpha^+\beta^+$ T cells (because their heterodimeric TCR express two chains encoded by TCR α and β loci). The second minor population of mature T cells are the $\gamma^+\delta^+$ T cells (because they express a related two-chain TCR derived from the TCR γ and δ loci). Much of our current information on T-lymphocyte development comes from analysis of $\alpha^+\beta^+$ T-cell development. These T lymphocytes represent the T-cell subset that responds primarily to viral infection, and the selection process of these T cells in the thymus will be emphasized here. The $\gamma^+\delta^+$ T-cell subset likely plays a secondary role in the adaptive response to most virus infections, and the properties/function of this T-cell subset is summarized below.

T-Lymphocyte development in the thymus proceeds in a stepwise fashion with the sequential generation and expression of one (i.e., TCR- β or TCR- δ), and then the other (i.e., TCR- α or TCR- γ) TCR chain by random somatic recombination as thymocytes mature. A complex of four cell-surface molecules called CD3 (CD3 γ , δ , ϵ , and ξ), which are noncovalently associated with the TCR and are critical for signaling through the TCR, are also expressed during T-lymphocyte development. Specific antigen recognition by the TCR- $\alpha:\beta$ (or $\gamma:\delta$) heterodimer is transduced into the cells by the CD3 complex. Concomitant with the expression of the rearranged TCR chains and the signal transducing CD3 complex, the developing thymocytes simultaneously express two co-receptor molecules, CD4 and CD8. Early in the developmental program, thymocytes do not express CD4 or CD8, and they are classified as immature, *double-negative* thymocytes. As thymic development/differentiation proceeds, immature thymocytes that express both CD4 and CD8 co-receptors (as well as CD3 complex and the TCR- α and β chains) and, at this point, are classified as $CD4^+/CD8^+$ *double-positive* thymocytes. CD4 and CD8 molecules recognize structurally conserved domains on the MHC class II and I molecules, respectively. As a result of binding to MHC molecules displaying self-peptides on the surface of thymic stromal cells, CD4 or CD8 also delivers signals to the developing immature thymocytes. The combined signaling by TCR/CD3 complex and the CD4 or CD8 co-receptor directs the development of the immature thymocytes into *single-positive* $CD4^+$ or $CD8^+$ mature thymocytes (expressing TCR, CD3, and either CD4 or CD8).

Because the TCR does not recognize foreign molecules directly (but rather small fragments of molecules bound to MHC molecules), the TCR generated and expressed on developing thymocytes must recognize both MHC molecules and the peptide fragments bound to these molecules. The source of these peptides bound by MHC class I and class II molecules are self-proteins expressed by several different cell types in the thymus. The selection of the thymocytes that ultimately become mature co-receptor *single-positive* T lymphocytes ($CD4^+$ or $CD8^+$) and populate the secondary lymphoid organs is a process in the thymus of positive and negative selection.

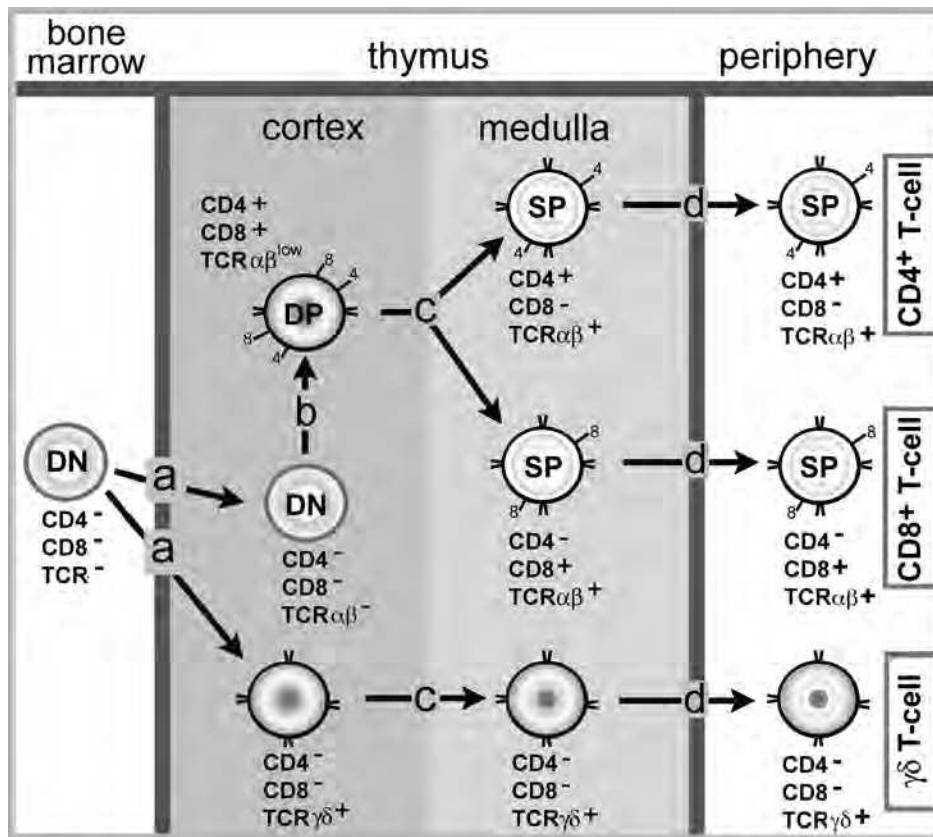


FIGURE 9.1. T-Lymphocyte development and selection in the thymus. The progenitors of mature peripheral T lymphocytes arise in the bone marrow as mononuclear stem cell progenitors committed to T-cell lineage development, but lacking cell surface receptors characteristic of T-lymphocyte lineage (e.g., T-cell receptors [TCRs] or the co-receptors CD4 and CD8). These TCR- and co-receptor-deficient “double negative” (DN) cells enter the thymic cortex (a) from the bloodstream as CD4/CD8-deficient DN lymphocytes. In the case of conventional TCRαβ T cells, the DN cells also lack antigen receptor. The cells then undergo a multistep differentiation process (b), which results in cells simultaneously expressing both CD4 and CD8 as well as a low level expression of the TCRαβ receptor complex—so-called double-positive (DP) thymocytes. Within the thymic cortex and medulla (c), the DP cells first undergo “positive selection”—that is, selection for those cells with TCRαβ and CD4 or CD8 co-receptor complexes capable of interaction with self-peptide/major histocompatibility complex (MHC) ligands. DP thymocytes that interact with self-peptide/MHC ligand with insufficient avidity die (undergo apoptosis). The surviving DP thymocytes then transition into either CD4⁺ or CD8⁺ single-positive (SP) thymocytes, which then undergo a process of “negative selection.” In this case, only those SP thymocytes with an appropriate immediate affinity for self-peptide/MHC complexes survive and go on to traffic through the thymus into the bloodstream to ultimately proceed to the secondary lymphoid organs (d) as mature peripheral CD4⁺ or CD8⁺ T cells. The TCRγδ T cells likewise arrive in the thymus (a) as DN progenitors, which eventually upregulate the γδ TCR (b) and undergo a form of positive and negative selection (c) without upregulation of the canonical co-receptors through a selection process that is not well understood. The TCRγδ T cells will exit the thymus (d) and populate peripheral sites including mucosal surfaces and secondary lymphoid organs. Natural regulatory T-cells (T_{reg}) cells (not shown) undergo selection for recognition of self-peptide/MHC class II ligands late in thymocyte differentiation by a selection process poorly understood at this time.

At a specific development stage, immature double-positive thymocytes (expressing the randomly generated α:β TCR, CD3, and both CD4/CD8) encounter, within the thymic cortex, thymic cortical epithelial cells displaying MHC class I and II molecules complexed with self-peptides. Most of these thymocytes (~80%) have randomly generated TCR that are unable to bind to the self-peptide/MHC molecular complexes. In the absence of a signal delivered through the TCR, these thymocytes will die by apoptosis. Those double-positive thymocytes whose TCR can bind the self-peptide/MHC complexes above a threshold avidity augmented by either a CD4 or a CD8 co-receptor binding to the MHC molecule will undergo

positive selection and survive. If the thymocyte TCR can bind peptides bound to MHC class II molecules in association with the CD4 co-receptor, this interaction will result in the silencing of the CD8 co-receptor gene. This signaling event results in the development of a CD4⁺ single-positive thymocyte whose TCR recognizes (is restricted to) peptides bound to MHC class II molecules. Similarly, the double-positive thymocytes whose TCR and associated CD8 molecule can recognize MHC class I/self-peptide complexes with sufficient avidity to survive (i.e., those that are positively-selected) will give rise to MHC class I-restricted CD8⁺ single-positive thymocytes by a similar silencing mechanism of the CD4 gene.

After undergoing positive selection, the surviving single-positive thymocytes are all auto-reactive (i.e., directed to self-peptide/MHC complexes). Therefore, a process of negative selection must then ensue to eliminate/reduce self-reactive, mature peripheral T cells. Although less well understood, negative selection of the $\alpha:\beta$ TCR-expressing single-positive ($CD4^+$ or $CD8^+$) thymocytes probably occurs in both the thymus cortex and medulla. Negative selection is most efficiently mediated by mononuclear cells of hematopoietic origin (e.g., dendritic cells and macrophages) that simultaneously display MHC class I and II molecules expressing self-peptides. The 10% to 20% of thymocytes that survive positive selection, by definition, are auto-reactive. It is likely (but not certain) that the intracellular signal transduction cascade associated with TCR engagement is altered in the cortical and medullary single-positive thymocytes following positive selection. As a result these cortical and medullary thymocytes acquire the TCR-signaling profile of mature T lymphocytes. Consequently, when the thymocytes encounter the self-peptide/MHC complexes displayed by hematopoietic origin cells like DC in the cortex and medulla, those thymocytes whose TCR/ $CD4$ or $CD8$ co-receptor complex recognizes self-peptide/MHC complexes with a sufficient avidity to activate will be eliminated (i.e., undergo negative selection—death by apoptosis). Thymocytes directed to self-“tissue specific” molecules (e.g., insulin) are deleted through

the action of the autoimmune regulator (*AIRE*) gene. This gene is expressed in thymic medullary stromal cells and triggers the transient low level expression of many extrathymic tissue-specific self-proteins in these cells resulting in negative selection of tissue-reactive single-positive thymocytes. Therefore, only about 2% to 5% of immature thymocytes will survive the positive and negative selection processes. These remaining thymocytes will have the properties of mature $CD4^+$ or $CD8^+$ T lymphocytes (i.e., they have TCR that recognize peptide/MHC complexes, but will only interact with the MHC molecules with sufficient avidity to activate when the appropriate foreign [microbial] peptides are bound to the MHC molecule). Following positive and negative selection, these single-positive $\alpha:\beta$ T lymphocytes ($CD4^+$ or $CD8^+$) will leave the thymus to populate the secondary lymphoid organs as peripheral T cells (Fig. 9.1). The $CD4^+$ or $CD8^+$ $\alpha:\beta$ T cells represent 70% to 80% of the circulating extrathymic T-cell population—the primary T-cell population that responds to virus infection.

The second (minor) population of thymus-derived cells that populates peripheral sites is that of the $\gamma:\delta$ T cells, so named because they employ a heterodimeric TCR whose variable (and constant) region is encoded by a distinct set of genetic loci (the γ locus and the δ locus) homologous to the α and β TCR loci¹⁰³ (Table 9.3). These TCR genes rearrange in the thymus in a manner analogous to that of the

TABLE 9.3 T Lymphocyte Subsets

TCR $\alpha:\beta$	Dominant T-cell Type Responding to Virus Infection
$CD8^+$	Recognize major histocompatibility complex (MHC) class I viral peptide complexes, and are important adaptive immune antiviral effector cells
$CD4^+$	Recognize MHC class II viral peptide complexes, and regulate B-cell differentiation and host inflammatory responses to infection
T_H1	Effector $CD4^+$ T cells that produce proinflammatory antiviral cytokines (e.g., IFN- γ) characterized by mononuclear cell inflammatory infiltrates
T_H2	Effector $CD4^+$ T cells that produce cytokines linked to allergic responses (e.g., IL-4, IL-5)
T_H17	Effector $CD4^+$ T cells that produce pro-inflammatory cytokines (e.g., IL-17A/F, IL-6) associated with antibacterial responses and characterized by neutrophil-rich inflammatory infiltrates
T_{FH}	Activated $CD4^+$ T cells that produce cytokines /chemokines (e.g., IL-21), which facilitate B-cell differentiation in SLO and which are retained in the SLO
TCR $\gamma:\delta$	Play a prominent role as “innate-like” effector cells
	–Express the CD3 complex, but usually not CD4 or CD8
	–The generation process of $\gamma:\delta$ TCR chains in the thymus is similar to that of the dominant TCR $\alpha:\beta$ T-cell TCR
	–Present in the circulation and lymphoid organs and prominently expressed at body surfaces (e.g., gut, skin)
	–Recognize products of stressed cells—no restriction in recognition by MHC class I/II molecules, but can recognize lipid complexed to MHC class Ib molecules
NKT	Act as “innate-like” immune effectors early in immune responses
	–Express conventional TCR $\alpha:\beta$ chains, and can use a restricted number of possible TCR genes (i.e., invariant NK T-cells [iNKT cells])
	–Express CD4 molecules (most NK T cells) and NK cell markers, but not CD8 molecules
	–Recognize products of stressed cells and can recognize lipid moieties displayed by MHC class Ib–related molecules on cell surfaces
	–Activation of TCR triggers release of regulatory cytokines (e.g., IL-4)
T_{reg}	Conventional TCR $\alpha:\beta$ T cells that control the response of T and B lymphocytes to foreign (e.g., viral) antigens and self-proteins
	–Dominant population expresses CD4 and CD25
	–May occur spontaneously during T-cell differentiation (natural Treg cells) or after exposure to foreign (e.g., viral) antigen (inducible Treg cells)
	–Regulates (usually suppresses) the response of $CD4^+$ T cells and $CD8^+$ T cells to infectious agents and self-constituents

dominant $\alpha:\beta$ TCR T-cell homolog. The generation of $\gamma:\delta$ T cells in the thymus precedes $\alpha:\beta$ T-cell development. The $\gamma:\delta$ T cells are generated in two waves during T-cell development in the fetus. The first wave of maturing thymocytes consists of cells with limited TCR V gene diversity (i.e., utilize one or few $V\gamma$ and $V\delta$ rearrangement in their TCR). After selection, these $\gamma:\delta$ T cells leave the thymus, traffick through the circulation, and preferentially localize to epithelial surfaces, particularly the skin, gut, and urogenital tract. The second wave of $\gamma:\delta$ T cells, as with $\alpha:\beta$ T cells, displays a highly diverse TCR array, and these cells localize to the secondary lymphoid tissues. Unlike the $\alpha:\beta$ TCR, however, the $\gamma:\delta$ TCR does not recognize peptides bound to the MHC class I or II locus products. Rather, $\gamma:\delta$ T cells either directly recognize structures such as heat shock proteins displayed on the surface of the infected cells, or recognize lipid or glyco/phospholipids moieties bound to MHC-like molecules (e.g., nonpolymorphic MHC Ib molecules) on the surface of stressed (infected) cells. The subset of intraepithelial $\gamma:\delta$ T cells, in particular, differs from the $\alpha:\beta$ T cells, and appears to function like cells of the innate immune system, recognizing not a specific pathogen, but alterations in epithelial cells induced by infection with microbes such as bacteria. At present, no direct role for $\gamma:\delta$ T cells in the control of virus infection has been firmly established although this T-cell subset has been implicated in the control of HIV and herpesvirus infection in humans.⁵

Among the other T-lymphocyte subsets (Table 9.3) generated during development are NK T cells, so-called because these $\alpha:\beta$ TCR-expressing T cells display cell-surface markers shared with NK cells, most notably the C-type lectin NK 1.1. NK T cells are divided into two subsets $CD4^+ CD8^-$ or $CD4^- CD8^-$ based on co-receptor expression. One subset of NK T cells employs a range of $\alpha:\beta$ V gene combinations in their expressed TCR. The other NK T-cell subset is similar to $CD4^-$ and $CD8^-$ $\gamma:\delta$ T cells, in that the cells utilize a limited number of TCR $\alpha:\beta$ V genes in receptor generation and are classified as invariant NK T cells (iNK T cells). The iNK T cells recognize foreign and self-lipid/glycolipid bound to MHC-like cell-surface CD1 molecules. The NK T cells with diverse TCR usage by contrast, directly recognize self- or microbial gene products rather than peptide/MHC complexes. Although not as yet certain, the CD1/lipid complexes and the self-moieties (e.g., heat shock proteins) recognized by NK T cells are likely produced or upregulated by cells in response to virus infection.

One T-lymphocyte subset has recently been recognized as a critical regulator of the adaptive immune responses to both infectious agents and self-molecules: the regulator T lymphocyte (T_{reg}) subset. The TCR on T_{reg} cells utilize rearranged $\alpha:\beta$ TCR gene products, and appear (at least in experimental animals) to arise late in thymic development (i.e., after the major wave of both $\gamma:\delta$ and $\alpha:\beta$ T-cell generation). As mature thymocytes, T_{reg} cells appear able to recognize self-peptides bound to MHC molecules and directly activate without undergoing negative selection. Therefore, T_{reg} cells that display primarily the $CD4$ co-receptor exist both in the thymus (as mature thymocytes) and in the periphery (i.e., in secondary lymphoid organs) as *activated* $CD4^+$ T cells. The encounter with self and the resulting activation of T_{reg} cells, however, does not result in autoimmunity. Rather, recent evidence suggests that these T cells suppress/downregulate the activity of conventional $CD4^+$

and $CD8^+$ T cells responding to viral infection. Although both the role of T_{reg} cells in adaptive immune responses and their mechanism of action are not yet fully understood, these cells may prevent the development of excessive injury during T-cell responses to infection, and suppress the development of autoimmune responses during viral infection. Recent emerging information on the role of T_{reg} cells in virus infection is discussed in a separate section below.

Secondary Lymphoid Organs: Structure and Function

The mature naïve (resting) B and T lymphocytes that egress from their development site (bone marrow and thymus, respectively) enter the blood, and then migrate to secondary (peripheral) lymphoid organs (SLOs). The SLOs play a critical role in the initiation of the adaptive (T- and B-cell) immune response to viruses and other microbes. The major SLOs are the lymph nodes (LNs), the spleen, and the so-called mucosal-associated lymphoid tissue (MALT). LNs are highly organized structures located at vessel convergence points in the lymphatic system, a system that collects fluids and cells from the tissues of most internal organs (e.g., liver, stomach) and body surfaces (e.g., skin, mucosa of the gastrointestinal [GI], genitourinary [GU], and respiratory tracts). The capacity of lymphatic vessels to collect antigen (e.g., virus, viral constituents) from the body surfaces/tissues, and deliver them to the LNs (where antigen concentrates) is essential for the efficient triggering of the adaptive immune response to microbes. The spleen plays a role similar to that of the LN network; however, the spleen is uniquely suited to collect antigen (virus) directly from the bloodstream, because of its unique architecture and extensive blood circulation. The third class of SLOs, the MALT, is composed of specialized lymphoid aggregates associated with major mucosal surfaces and sites of microbial invasions (e.g., the GI, GU, and respiratory tracts). The gut-associated MALT (also called GALT) includes tonsils, adenoids, appendix, and Peyer's patches in the small intestine. In the respiratory tract are similar, but more diffuse, aggregates of lymphoid tissues associated with the epithelium of small/medium airways (i.e., bronchial-associated lymphoid tissue [BALT]). As with the other SLOs, the MALTs are sites of antigen localization where the initiation/induction of the adaptive immune response can occur.

The common feature of SLOs is the localization of adaptive immune cells (i.e., T and B cells) to distinct regions of the SLOs. For example, most B cells are localized in distinct follicles within the LN cortex. Activation of B cells specific for a pathogen/antigen leads to their proliferation and formation of a germinal center within the LN cortex. T cells within the LN localize to, and are diffusely distributed within, the LN paracortical areas. Other cell types are also distributed within the outer cortex and central medulla of the normal LN. These include stromal cells and cells of macrophage and DC lineage. These cells (particularly DC) play a crucial role in the induction of the adaptive immune response, and serve to direct T-cell/B-cell localization to distinct LN regions through the constitutive release of small (low molecular weight) chemotactic proteins—chemokines. A similar segregation of B cells/T cells to distinct regions occurs within the white pulp of the spleen and the MALT (likewise, under control of chemokines elaborated by SLO-resident cells).

Induction of Adaptive Immune Responses in the Secondary Lymphoid Organs

The body's complement of B and T cells consists of more than 10^9 cells, each of which may display a unique antigen receptor directed to a specific epitope. Therefore, the frequency of B or T cells directed to a specific pathogen is extremely low (e.g., a frequency of 1 of 10^4 to 10^6 naïve B or T cells). The problem faced by the adaptive immune systems is that these rare, antigen-specific cells are distributed randomly throughout the body, and must be rapidly mobilized to respond to invading organism. These rare antigen-specific cells must first contact antigen, then activate and generate effector cells capable of eliminating the pathogen. The solution to this problem is achieved through the process of lymphocyte recirculation through the SLO.^{57,273}

Naïve (primary) T cells and most naïve B cells do not permanently reside in the SLOs. Rather, the T and B cells are constantly migrating from the blood through the SLOs and back to the bloodstream. Lymphocyte recirculation is controlled by the expression of distinct homing receptors (adhesion molecules) whose ligands are expressed on cells within the SLO. Naïve $CD4^+$ and $CD8^+$ T cells within the bloodstream express on their surface the adhesion/homing receptor, CD62L (L-selectin). T cells entering the blood vessels encounter the CD62L ligands—the mucin-like moieties CD34 and GlyCam-1 that are constitutively displayed in the endothelium of specialized high endothelial venules (HEVs) within the LN cortex. The interaction of CD62L (on the T cell) with its endothelial cell ligands arrests the T cell's migration through the blood vessel, which results in its binding to the HEV endothelial cell. Other adhesion receptors displayed on endothelial cells that recognize carbohydrate ligands displayed on the surface of lymphocytes (as well as the $\alpha_1\beta_2$ integrin, lymphocyte function-associated antigen 1 [LFA-1] displayed on lymphocytes) also contribute to the arrest of T-cell migration. The naïve circulating T cells also constitutively express a chemokine receptor (CCR7) that recognizes specific chemokines (CCL19, CCL21) displayed on the surface of the HEV endothelial cells. These chemokines are produced by the HEV cells and by cell types within the LN (e.g., DC and stromal cells). The chemokines produced by the latter cell types diffuse into the HEV and are bound to/displayed on, the surface of the HEV endothelial cell. The interaction between the CCR7 receptor on the arrested T cells and its chemokine ligand triggers the migration of the naïve T cells through the HEV and into the LN cortex (parafollicular T-cell region). If the T cells do not encounter antigen, they traffic through the LN cortex to the LN medulla, and then exit the LN through the efferent lymphatic vessels, and ultimately return to the bloodstream where the process of recirculation through the SLO begins again. The egress of naïve T cells from the LN is as noted above, controlled by a chemotactic receptor S1P1R for the lipid sphingosine-1-P (S1P). S1P is present at higher concentrations in lymph and blood than in the LN. S1P1R engagement attracts naïve T cells away from the LN and back into the circulation. Antigen recognition by T cells transiently downregulates S1P1R retaining the antigen detecting T cells in the LN.

Naïve B cells traffic through the LN by similar mechanisms and transiently localize to the lymphoid follicles along a chemokine gradient mediated by a specific chemokine receptor, CXCR5, displayed on the surface of circulating naïve B

cells. Lymphocyte trafficking through the spleen and MALT are likewise regulated by the interplay of adhesion receptor/ligand interactions and chemokines constitutively expressed in these SLOs. In the case of MALT, a set of unique receptor/ligand interactions (restricted to these sites) guides the trafficking of adaptive immune cells to these specialized mucosal sites.

Afferent lymphatic vessels are localized in all body surfaces/tissues, and they drain into the LN. Virus infection of a body surface/tissue triggers the innate immune system—the initial defense against infection. Innate immune cells located at the body surfaces/tissues (specifically, DC and monocyte/macrophages) encounter pathogen, take up virus/viral antigen, and then activate/migrate through the afferent lymphatics to the draining LN. In the draining LN, these migrating cells (along with DC and, to a lesser extent, monocyte/macrophages present in the LN cortex) serve as antigen-bearing presenting cells (APCs) to the naïve B and T lymphocytes trafficking through the LN. As a result of encounter with antigen displayed on the APCs, the lymphocytes with antigen receptors specific for the antigen or pathogen will no longer traffic through the LN, but rather will be retained in the LN (due to S1P1R downregulation) and initiate the process of activation and differentiation, leading to the generation of specific effector B and T cells directed to the pathogen. Therefore, the SLO concentrate antigen in a specific site where the rare antigen-specific, naïve B and T cells circulating through the body can encounter and respond to the pathogen. Afferent lymphatics can also carry large particulates (e.g., viruses as well as “soluble” viral proteins) directly to the LN where the particulates can be captured by macrophages lining subcapsular lymph node sinuses and subsequently delivered to DC or presented to naïve T cells, as well as directed along with soluble antigens through fibroreticular conduits to B cells and a lymphoid follicles.^{95,125}

If infection by a virus is localized to the body surface (e.g., papillomavirus infection of the skin or influenza virus infection of the respiratory tract), then the adaptive immune response to that pathogen will be induced primarily in the LN draining these sites. Virus infections that result in systemic spread (i.e., viremia) will also induce adaptive immune responses in the spleen. Viruses that infect sites where MALT is prominent (e.g., rotavirus infection in the Peyer's patches of the small intestine) will trigger the local induction of the adaptive immune responses at the site of MALT localization.¹⁹⁶

ANTIGEN PRESENTING CELLS AND THE INDUCTION OF ADAPTIVE IMMUNE RESPONSES

As with most microorganisms, viruses enter the body surfaces where the initial encounter with the immune system occurs. These body surfaces (e.g., skin and mucosa of the GI, GU, and respiratory tracts) contain epithelial lining cells, which can serve as the initial cells supporting virus replication. The epithelial surfaces also contain DC, specialized cells of bone marrow origin, which serve as both infection sentinels and APCs, which migrate to the SLOs and deliver antigen to the adaptive immune cells that are trafficking from the blood to the SLOs. A large body of evidence now suggests that DCs are critical APCs for the induction of the primary adaptive immune (T cell) response.¹⁸⁴

For our purposes, two classes or types of DCs, conventional DCs (cDCs) and plasmacytoid DCs (pDCs) can be distinguished. cDCs are localized to the body surfaces and SLOs where they exist as immature (inactive) DCs prior to infection and carry out the aforementioned sentinel and APC function. As discussed below, a subset of cDCs identified by expression of CD8 α in the SLO and α E integrin CD103 in mucosal tissue has the capacity to efficiently take up and present cellular material—for example, apoptotic-infected cells, particulates like viruses, and soluble proteins—to naïve CD8⁺ T cells without direct infection of the DC. pDCs, so-called because of their plasma cell-like morphology are derived from blood monocyte precursors and recruited to sites of inflammation/infection where, following activation, they are major producers of type 1 α -interferons.⁸⁰ In most circumstances these important innate immune effectors cells have limited capacity to act as APC for naïve T cells.

cDCs localize to the epithelium within the epithelial cell layer (e.g., Langerhans' cells in the skin) at the junction of the epithelial basement membrane in GI and respiratory tracts, as well as in the mucosa and submucosa underlying the epithelial surface. The cDCs at body surfaces are derived from circulating monocytic progenitors originating in the bone marrow, which are present in the blood and migrate to the body surfaces and differentiate into peripheral (tissue) DCs. These tissue DCs exist as immature (inactive) DCs at these sites, turn over at a low variable rate, and are replenished by the circulating blood monocyte progenitors that enter the tissues.

Immature DCs are sessile cells that have a particular set of phenotypic markers or characteristics. They express low to intermediate levels of cell-surface MHC class I and class II molecules; the latter (class II) molecules are found predominately not on the DC surface, but within specialized endocytic vesicles. Immature DCs also express low levels of cell-surface molecules, which serve as co-stimulatory ligands for naïve lymphocytes (e.g., CD80, CD86, CD40, CD83).²⁷² These molecules play a critical accessory role in the triggering of naïve antigen-specific (e.g., virus-specific) T cells, ultimately leading to the generation of effector (i.e., antiviral) T and B cells, and the formation of memory T and B cells directed to the pathogen. In addition, immature DCs have the ability to take up particulate and fluid-phase antigen into pinocytic/phagocytic vesicles with high efficiency. The enhanced capacity of these DCs to take up material (including the remains of infected apoptotic cells) likely accounts for their unique capacity to serve as APCs.¹⁸⁵

The initiation of an inflammatory response at the body surface (as produced by virus infection) triggers the DC activation (maturation) process. Activation of the tissue-resident DC results in the *de novo* expression/upregulation of chemokine receptors (notably, CCR7), which renders the activated DCs capable of migrating from the peripheral tissue, primarily through lymphatic vessels along a gradient of constitutively expressed CCR7 ligands, the CCL19/CCL21 chemokines produced by lymphatic endothelial cells. Activation of the tissue DCs is also accompanied by upregulated expression of MHC class I/II molecules, and co-stimulatory ligands resulting in the maturation of the DCs, which, on activation/maturation, characteristically express high cell-surface levels of MHC class I/II molecules and the co-stimulatory ligands, CD80/86, CD40, and CD83. Mature DCs migrate to the SLOs where they act as the principal APCs for the induction of the adaptive

immune response. Therefore, DCs (and to a lesser extent macrophages) serve the dual role of delivery (concentration) of antigen to the SLOs and acting as APCs to retain/activate the rare antigen-specific adaptive immune cell entering the SLOs from the circulation.

Immature DCs can be triggered to activate by virus infection through at least three mechanisms:

1. *Direct infection* of immature DCs. The stimulus for DC activation/maturation is provided primarily by the interaction of viral nucleic acids with (and signaling through) microbial pattern-recognizing Toll-like receptors (TLRs) displayed by DC,¹²⁹ and/or activation of intracellular antiviral signaling mechanisms mediated by the RNA-activated protein kinase (PKR kinase),²⁴⁰ the retinoic-acid-inducible protein I (RIG-I) helicase systems,¹³⁰ and for some viruses the caspase/IL-1 β -associated inflammasome.²⁸⁹
2. *Uptake of virus* and soluble antigen (or more likely the remnant of infected epithelial cells that have undergone apoptosis in response to infection) with activation of immature DC through binding to one or more scavenger or C-type lectin receptor(s), for example, dendritic and epithelial cells, 205 kDa (DEC-205); dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) displayed on DC with specificity for carbohydrates on viral glycoproteins/glycolipids²⁷⁴ or through the process of phagocytic uptake.
3. Dendritic cell activation/maturation induced by *inflammatory mediators* (i.e., cytokines, such as TNF- α) released by infected epithelial cells and tissue macrophages in response to TLR and/or intracellular antiviral signaling.

The first two types of virus encounters with immature DC will result in the initiation of the adaptive immune response by delivery of virus/viral antigen to the SLO presented by the activated/mature DC. The significance of inflammatory mediator-induced DC maturation in the induction of the antiviral immune response is still not clear, because the activated, mature DC would not directly deliver viral antigen and would not serve as an APC.

Direct infection of immature DCs and the subsequent migration of the activated/mature DCs to the SLOs would appear to be the most likely mechanism to ensure the induction of an effective antiviral adaptive immune response. Whereas this mechanism of viral antigen delivery to T and B lymphocytes in the SLO occurs,³⁶ it raises the important question: *How do viruses with a very narrow cell tropism, which are unable to either infect or express viral genes in hematopoietic cells like DC (e.g., papillomaviruses) initiate an immune response?*

The capacity of certain immature cDCs to take up and internalize cellular debris such as apoptotic remnants of infected epithelial cells provides the most likely mechanism for triggering adaptive immune T-cell responses. This process of the uptake of infected cell material with presentation to T cells by cDCs is called cross-presentation²⁵⁹ and is carried out by CD8 α ⁺ cDCs in SLO and by CD103⁺ cDCs at mucosal surfaces. Indeed, the speculation based on evidence from experimental viral infection is that endocytic uptake of virions or material from virus-infected cells may be the primary/sole mechanism of viral antigen presentation—at least to virus-specific naïve CD8⁺ T cells.²³⁰ The relative contribution of direct presentation (virus infection of DC), and *cross-presentation* of viral antigen remains controversial²⁹⁶ and will differ for different viruses.

Naïve (primary) T lymphocytes have stringent requirements for activation that include both TCR antigen receptor engagement by the appropriate processed antigenic peptide/MHC complexes and engagement of co-stimulatory receptors (e.g., CD28) displayed on naïve T cells by their corresponding ligands (e.g., CD80/86) along with peptide/MHC complexes by APC. Mature, antigen-bearing DCs are uniquely suited to carry out this process. The inflammatory response triggered by virus infection triggers DC maturation/migration. Virus infection also results in the infection of and/or antigen uptake by tissue macrophages. However, the migration of infected DC and macrophages along with infectious virions (either cell-associated or free) from the body surface to the SLO, not only initiates an immune response in the SLO, but also can in many instances spread infection to other sites in the body. One classic example of this is the dissemination of poxvirus from a peripheral infection site (body surface) to the SLOs, where free (infectious) virions and infected cells carried from the inoculation site to the SLOs can result in additional virus replication there, followed by virus entry into the bloodstream.⁷⁸ Migrant macrophages are probably not as efficient as mature DCs in triggering primary immune response, but they may also serve as a potential APC in the induction of recall memory (secondary) immune responses where the requirements for memory T- and B-lymphocyte activation are less stringent.⁷¹ In this regard, it should also be noted that the mature migrant DCs that have entered the SLOs in response to virus infection may not be the primary APCs for T-cell activation. Several lines of evidence suggest the LN-resident DCs (particularly, CD8 α expressing cDCs) can capture viral antigen delivered by migrant DCs (or macrophages), and present that antigen to naïve (primary) T lymphocytes in the SLOs—not by direct infection, but by the transfer of viral antigen from migrant antigen-bearing DCs to SLO-resident DCs through cross-presentation.²⁹⁶ Intact virions and/or soluble (free, intact) viral antigen, however, undoubtedly contribute to the induction of effective antiviral antibody response by B lymphocytes (as discussed in a later section of this chapter).

Studies examining the induction of adaptive immune responses using confocal and intravital multiphoton microscopy in intact viable LN have provided new insight into the early interaction events between DC-APCs and antigen-specific B and T lymphocytes.^{27,255} Some studies indicate that, on entering the draining LN, activated (mature) DCs migrate under a chemokine gradient stimulus to the paracortical T-cell regions in the vicinity of the HEV.³⁵ Therefore, antigen-bearing DCs would be in the precise location to interact with naïve virus-specific B and T lymphocytes that have entered the draining LN from the circulation and, thereby, initiate the adaptive immune response. However, soluble antigens (viral proteins) and particulates such as virions that entered the LN via lymphatics may be presented to T cells, and particularly B cells, by mechanisms not involving DC migration from the site of infection.⁹⁴

Activation of T and B Lymphocytes in the Secondary Lymphoid Organs

The precise localization of T cells, B cells, DCs, and macrophages within the SLOs is controlled by chemokines, which are largely produced by stromal cells of the SLOs, and by bone marrow-derived cells (e.g., SLO-resident DCs). In draining LN, naïve (primary) T cells that are present in the circulation

will enter the LN through the specialized HEV, where they encounter mature DCs (and macrophages) displaying processed antigen bound to MHC molecules, which are localized to sites in the parafollicular cortex of the LN facilitate interaction with T cells. Most naïve CD8⁺ T cell and CD4⁺ T cells entering the LN will not recognize the antigen (i.e., they have TCRs that fail to recognize the processed antigen with sufficient avidity to activate the cells). These T cells will ignore the DCs and migrate from the LN cortex to the medulla, then enter the efferent lymphatics, and ultimately return to the bloodstream to continue their perpetual search for relevant antigen.²⁷³ A similar process of lymphocyte egress through the HEV occurs in the MALT; for example, Peyer's patches of the small intestine where antigen uptake by mucosal DCs and direct viral antigen transfer to the gut MALT through specialized intestinal epithelial cells, called M cells, result in antigen delivery to these mucosal lymphoid aggregates.¹⁵⁰ Viruses that enter the bloodstream (i.e., produce viremia) as well as any antigen-bearing tissue DCs that, on maturation, bypass the LN and gain access to the circulation will localize to the splenic white pulp. Here, circulating T and B lymphocytes enter the splenic white pulp by transiting through splenic arterioles where viral antigen-bearing DCs can encounter lymphocytes. The lymphocytes that do not recognize antigen will exit the splenic white pulp by entering trabecular veins, and re-enter the circulation.

The rare naïve CD8⁺ T cells and CD4⁺ T cells that recognize processed antigenic peptides displayed on the APC surface in the SLO do so through TCR engagement. The activation of naïve T cells leading to the induction of the adaptive immune response is a multistep process (at both the molecular and cellular level).¹¹² For induction of antigen-specific (virus-specific) CD8⁺ T-cell responses, the activation process requires both interaction of the antigen-specific TCR $\alpha:\beta$ chain complex with *processed* antigen fragments bound to MHC class I molecules on the APC surface, with sufficient avidity and interaction of the CD8 molecule on the T cell with a region of the MHC class I molecule distant from the site of peptide binding. To fully activate the naïve CD8⁺ (or CD4⁺) T cells, additional signaling events must occur, including binding of the co-stimulatory receptor, the CD28 molecule, displayed on naïve T lymphocytes with its ligands, CD80/86, which are displayed at high levels on mature (activated), antigen-bearing DCs. The sequence of events resulting in the activation of antigen-specific naïve CD4⁺ T cells parallels the steps in CD8⁺ T-cell activation, except that the antigen-recognizing TCR $\alpha:\beta$ chain complex must bind (with sufficient avidity) the antigenic peptides bound to MHC class II molecules displayed by APC; and the CD4 co-receptor on CD4⁺ T cells, in turn, must interact with a distinct site (outside of the region of peptide binding) on the MHC class II molecule. One important early consequence of CD4⁺ T-cell activation by antigen is the upregulation of the expression of the CD154 molecule on activated CD4⁺ T cells. CD154 (also called CD40 ligand or CD40L) is a member of the *TNF* gene family, and, as its name implies, is a ligand for CD40 (a TNF receptor gene family member). CD40 is expressed on both activated/mature DCs and antigen-activated B cells. Engagement of CD40 on DCs and B cells by CD154 (CD40L) on activated CD4⁺ T cells has important consequences for the function of these cells. Engagement of CD40 on DCs results in increased synthesis of certain cytokines (notably IL-12) by the antigen-bearing DCs in the SLO, which promotes CD4⁺ and CD8⁺

T-cell differentiation into effectors.¹⁴² For B cells, the interaction between CD40-activated B cells and CD40L expressed on activated CD4⁺ T cells is critical for B-cell differentiation into antibody-secreting effector B cells (plasma cells).¹⁸² Strategies to modulate the CD40/CD40L interaction *in vivo* may have important consequences in vaccine development.

The engagement of the TCR/co-receptor complex, and the CD28 co-stimulatory receptor on T cells in the SLO are the first two critical steps in the induction of a T-cell response to a foreign antigen. This initial antigen encounter by the TCR on specific T cells is called *signal 1* in the lexicon of lymphocyte activation/differentiation. The engagement of the CD28 co-stimulatory receptor is designated as *signal 2*. As a consequence of their interaction with antigen and co-stimulatory ligand displaying DC APC, the naïve T cells halt their transit through the SLO (i.e., downregulation of S1P1R, and are retained there). The APC/T cell encounter leading to signal 1 and 2 transmission in the T cells appears to be of short duration (from 6 to 24 hours), after which the initial programming for T-cell activation/differentiation is independent of additional interaction with antigen.^{174,293} Delivery of signals 1 and 2, although necessary, is not sufficient in itself, however, to ensure full T-cell activation/differentiation and the generation of effector and memory T cells to the antigen. A third signal, signal 3, is required to complete the T-cell activation/differentiation program in the SLO. Signal 3 is delivered by soluble mediators (i.e., cytokines and chemokines) produced primarily by cells of the innate immune system (e.g., pDCs mature/activated in cDCs, NK cells, NK T cells), which enter the SLO in response to infection/antigen deposition in the SLO. IL-12 produced by mature (activated) DC in the SLO is a particularly critical type of signal 2 in T-cell responses to virus, because engagement of the IL-12 receptor on activated CD8⁺ T cell and CD4⁺ T cells promotes their differentiation into effector T cells with potent antiviral activity (e.g., the generation of cytolytic T cells).¹⁹⁹

Other cytokines produced during T-cell activation (e.g., type 1 and type II/III IFNs [IFN- α , β , IFN- γ , IFN- λ , respectively], IL-1, IL-4, IL-6, IL-10 and so on) by innate immune cells responding in the SLOs to virus infection will dramatically modulate the magnitude and type of both the effector and memory T-cell responses. These and other soluble mediators modify the course of T-cell activation/differentiation during the T cell's residence in the SLO.¹⁴²

Similarly, in addition to CD28, other co-stimulatory receptors belonging (like CD28) to the Ig receptor gene family (e.g., Cytolytic T-Lymphocyte antigen-4 [CTLA-4]; inducible T-cell co-stimulator [ICOS]; programmed death-1 [PD-1]) and the TNF receptor gene family (e.g., CD27, OX40, 4-1BB, CD40L) whose expression on activated T cells is temporarily regulated following T-cell activation in the SLO, can either positively (ICOS, CD27) or negatively (PD-1, CTLA-4) regulate the tempo and magnitude of T-cell activation and differentiation into effector cells in the SLO or in peripheral sites of virus infection following interaction with their perspective ligands on APC.^{248,252}

The temporal sequence of signal 1, 2, and 3 delivery to T lymphocytes in the SLO is reflected in the morphologic change of the naïve (primary) T cells from small, resting cells to activated lymphoblasts. This is followed by proliferative expansion of the antigen-specific T-cell *clones* in the SLO, and their differentiation into both effector T cells and an expanded population

of memory CD8⁺ T cell and CD4⁺ T cells. T-cell proliferation is extremely rapid (cell cycle time of ≤ 6 hours and in one report ≤ 2 hours,³¹⁷ with estimates of individual naïve T-cell precursors undergoing 8 to 20 divisions during this programmed proliferation.¹³⁷ During their proliferative expansion and differentiation in the SLO, the responding T cells lose cell-surface adhesion molecules (e.g., CD62L) and receptors (e.g., CCR7) necessary for naïve T-cell circulation, and they upregulate the expression or express *de novo* adhesion molecules (e.g., the $\beta 2$ integrin, LFA-1, and $\beta 1$ integrin, very late antigen 4 [VLA-4], respectively), and chemokine receptors (e.g., CCR5, CXCR3) essential for effector T cells to localize to sites of inflammation.¹⁶⁵

At the end of the proliferation/differentiation sequence, most activated antigen-specific effector T cells will upregulate S1P1R and exit the SLO (through the efferent lymphatics in the case of T cells responding in the draining LN), enter the circulation, and traffic to the site of infection/inflammation. The homing of activated T cells is controlled by chemokines released by innate immune cells (as well as by epithelial and endothelial cells and fibroblasts) at the site of inflammation/infection (e.g., CXCL9 [Mig/CXCL9 (Monocline Induced by Gamma interferon)], CXCL10 [IP-10 (Interferon gamma induced Protein-10)], and CXCL11 [I-TAC (Interferon-inducible T cell Alpha Chemoattractant)] for CXCR3; and CCL3 [macrophage inflammatory protein-1 α ; MIP-1 α] and CCL4 [MIP-1 β] for CCR5). This homing is also controlled by the inflammation-induced upregulation of integrin ligands (e.g., intercellular adhesion molecule-1 [ICAM-1] for LFA-1, and vascular adhesion molecule [VCAM] for VLA-4) and adhesion receptors (P-selectin, E-selectin) on endothelial/epithelial cells at these sites.

The generation of effector T cells in the SLOs responding to infection can occur in as few as 5 to 7 days in both experimental models and human viral infection.⁶⁸ Given the rapid replication of most viruses, it is obviously essential to get specific T cells activated and mobilized as soon as possible through the concentration of antigen and the accumulation of the rare antigen-specific T cells in the SLO, followed by rapid proliferative expansion/differentiation of these cells into effectors in the SLO. The rapid induction of an adaptive immune response to virus infection is not a foregone conclusion, however. The process of T- and B-cell activation can be delayed for weeks to months in the case of infection with viruses such as HIV and HCV, which induce chronic infection.^{111,187} In these instances, the virus has the capacity to suppress/delay the induction of the adaptive immune response.

The process of naïve B-cell activation in the SLOs is similar to that of naïve T-cell activation. In the case of B-cell responses to viruses and other T cell-dependent antigens, however, antigen-specific CD4⁺ T cells play a critical role in driving B-cell activation/differentiation. As with naïve T cells, naïve B cells enter the LN through the HEV; unlike T cells, however, the B cells migrate from the paracortical (T-cell rich) area of the LN and into the B cell-enriched LN cortical follicle. This migration is controlled by the chemokine CXCL13, produced by a stromal cell type resident in the follicle (called a *follicular DC* because of its dendritic morphology, but not of hematopoietic origin). CXCL13 is the ligand for CXCR5 chemokine receptor that is constitutively expressed by naïve B cells. If the B cell does not encounter antigen in the follicle, it will ultimately migrate out of the cortical follicle to the medulla, then enter the efferent lymphatics, and rejoin the circulating pool of B cells in the bloodstream.

The B cells that display a BCR antigen receptor (i.e., the cell-surface membrane-bound form of the immunoglobulin molecule that recognizes antigen) for an antigen present in the LN would encounter this antigen immediately after migrating out of the HEV in the parafollicular (T-cell rich area) of the LN. In the case of viral antigen, the relative contribution of free virus, soluble viral proteins, and viral proteins transported by migrant DC/macrophages from the infection site to the reservoir of antigen present in the SLO and available to specific B cells is not certain. However, it has been recently appreciated that structures (i.e., conduits) within the SLO (in the case of LN) direct particulates like viruses and soluble proteins directly to regions of naïve B-cell accumulation in the SLOs.¹²⁵ Binding of antigen to the BCR (i.e., cross-linking cell surface immunoglobulin) provides the initial stimulus (signal 1) for specific B-cell activation. Signal 1 delivery halts the constitutive migration of the B cell from the paracortical T-cell zone to the LN follicle, and activates the chemokine-dependent migration of the B cells to the junction between the T-cell zone and the follicle. The strength of signal 1 can be greatly enhanced if the BCR co-receptor complex²⁰¹ is ligated simultaneously with the BCR by interaction with complement components bound to antigen or antigen-antibody complexes. In addition, cross-linking of the immunoglobulin receptor induces internalization of the antigen-immunoglobulin complex into an endosomal compartment where the antigen is fragmented and associates with MHC class II molecules that are constitutively expressed by B cells. The antigen/MHC class II complexes are then cycled to the B-cell surface, where they are available for recognition by antigen-specific CD4⁺ T cells. Another consequence of signal 1 delivery (by antigen to B cells) is CD40 upregulation. The CD4⁺ T cells, which interact with antigen-stimulated B cells, are themselves activated by prior encounter with antigen/MHC class II complexes on APC (i.e., mature, antigen-bearing DC), and have upregulated CD154 (CD40L) expression. The activated antigen-specific CD4⁺ T cells recognize the antigen/MHC complexes on the antigen-stimulated B cells, and deliver signal 2 to the B cells in two forms: interaction of CD40L (on T cells) with CD40 (on B cells), and the release of T-cell-derived cytokines (notably, IL-4, IL-21, and/or IFN- γ) whose synthesis is triggered in the CD4⁺ T-cell TCR recognition of antigenic peptides displayed by the B cells. Antigen-activated CD4⁺ T cells in LN may support B cell responses by differentiating into so-called T follicular helper (T_{FH}) cells. These activated CD4⁺ T cells appear to represent a distinct CXCR 5⁺/PD-1⁺ CD4⁺ T-cell subset, the generation of which is in part regulated by IL-6 produced within the responding LN. The cells secrete IL-21 in response to TCR engagement and also express CD154 (CD40L). The interaction of the T_{FH} with the naïve parafollicular B cells through CD40 engagement (on B cells) by CD40L on the T cells, and IL-21 signaling through its receptor on naïve T cells helps support the formation of B-cell germinal centers.

The fully activated (signals 1+2⁺) B-cell lymphoblasts undergo programmed proliferation after migration to the follicles to produce germinal center, which are follicular structures consisting primarily of responding/proliferating B-cell lymphoblasts. The proliferation/differentiation of the germinal center B-cell lymphoblasts results in the formation of antibody-secreting plasma cells, which migrate from the LN to the sites of infection/inflammation, and more importantly to the bone marrow where the plasma cells serve as a depot of long-lived antibody-secreting cells.¹⁶⁷ B cells that encounter antigen

without CD4⁺ T-cell *help* (i.e., receive signal 1, but not signal 2) are capable of producing specific secreted antibodies; however, these antibodies will be of the immunoglobulin M (IgM) isotype and of low affinity. The isotype switch of antibody production from IgM to the IgG, IgA, and so on isotypes, as well as, the process of antibody affinity maturation requires T-cell help (signal 2).¹⁶⁷

VIRAL ANTIGEN RECOGNITION BY B CELLS

Viral antigens are found on virions, on virus-infected cells, as soluble molecules produced by virally infected cells, and as breakdown products from virions and infected cells. These antigens can be recognized by antibodies in soluble form in plasma or tissues and by antibodies on the surface of B cells (i.e., BCR). If the individual has had previous exposure to viral antigen(s) through infection or vaccination, then specific soluble high-affinity antibodies will likely be present in the plasma. A minority of these antibodies will be reactive with viral antigens presented on virions and virally infected cells and able to function antivirally. Many will be directed to a range of nonsurface viral antigens; that is, they will be functionally inert, but may be useful in the diagnosis of viral infection. In the case of prior infection or vaccination, memory B cells that express specific antibody will be activated on contact with viral antigen, leading to the production of soluble, specific high-affinity antibody and the increase of specific serum antibody levels. In the absence of a previous encounter with viral antigen, antibodies on the surface of naïve B cells will bind antigen with relatively low affinity, setting in motion antibody affinity maturation and class-switching processes, which are discussed below.

NATURE OF B-CELL EPITOPES

Most of what is known about how antibodies recognize antigens has been determined from studies on the interaction of soluble antibodies or antibody fragments with antigen.^{61,208,308} Given that B-cell surface antibody is essentially identical to soluble monomeric antibody, with an extra segment ensuring membrane localization, the conclusions should apply broadly to cell surface antibody. Antibodies recognize molecular shapes, termed epitopes, on the surface of antigens. The antibody-combining site makes multiple contacts with the surface of the antigen to form complementary surfaces. The more complementary these surfaces are to one another—in terms of geometry and chemical character—the more favorable interactions will be formed between the antibody and antigen and the higher will be the affinity of the antibody for antigen. The affinity of the antibody for the antigen is one of the most important factors in determining the efficacy of the antibody *in vivo*.

The antibody-combining site is made up of residues contributed primarily from six highly variable segments or loops referred to as hypervariable loops or complementary determining regions (CDRs). The site can vary greatly in shape and character depending on the length and characteristics of the CDRs. Generally, most or all of the CDRs contribute to antigen binding, but their relative contributions vary. The heavy chain CDRs (particularly the third heavy chain CDR [CDR H3]) tend to contribute disproportionately to antigen binding. The CDR H3 in human antibodies can be very long, with

distinctive shapes such as fingers and hammerheads that contact viral epitopes. The combining site of antibodies against smaller molecules such as carbohydrates and organic groups (haptens) are often more obviously grooves or pockets, rather than the extended surfaces typically found in antiprotein antibodies.

On the antigen, epitopes come in as many different shapes and sizes as do antibody-combining sites. The area of antigen that contacts antibody, referred to as a footprint, is typically between about 400 and 1,000 Å². All antibodies recognize a topographic surface of a protein antigen. Most usually, key residues in the epitope will arise from widely different positions in the linear amino acid sequence of the protein because of the manner in which proteins are folded. The linear sequence typically traverses from one side of the protein to the other a number of times. Such epitopes are described as discontinuous. Occasionally, key residues arise from a linear amino acid sequence. In such cases, the antibody may bind with relatively high affinity to a peptide incorporating the appropriate linear sequence from the antigen. Furthermore, the peptide may inhibit the antigen binding to the antibody. The epitope in such cases is described as continuous. An example of a continuous epitope would be a loop on the surface of the protein for which an antibody recognized successive residues in the loop. It should be noted, however, that an antibody that recognizes a continuous epitope does not bind a random or disordered structure. Rather, it recognizes a defined structure that is found in the complete protein but can be readily adopted by the shorter peptide.

ANTIGENICITY AND IMMUNOGENICITY

A clear distinction is seen between the ability of an epitope to be recognized by antibody (antigenicity) and its ability to stimulate an antibody response when presented to a host antibody system (immunogenicity). A number of factors appear to be involved in how well a given epitope elicits an antibody response. Perhaps one of the most important factors is the accessibility of the epitope on the protein surface. Loops that protrude from the surface of the folded protein tend to elicit particularly good antibody responses. Figure 9.2 shows epitopes on the hemagglutinin (HA) protein from the surface of the influenza virus, with those at the “top” of the molecule distant from the viral membrane and most accessible to antibody. Natural infection and classical vaccination strategies typically induce antibodies to the top of the HA and mostly to variable regions that are therefore strain specific. Changes within these immunodominant variable regions allow neutralization escape and dictate repeated annual influenza vaccinations to provide robust protection to large populations. More conserved regions in the stem region of the HA appear to induce weaker antibody responses, but if these could be presented in a more immunodominant format they might induce broadly neutralizing antibodies and thereby provide a universal influenza vaccine that would replace the current annual vaccines. HIV is another virus in which highly exposed variable regions on the viral surface glycoprotein are immunogenic.³¹³ Following primary infection, it takes some time (weeks) for neutralizing antibodies to reach a level where they interfere with virus replication.^{229,303} These antibodies are typically elicited to exposed variable loops on the virus. While these antibodies are being elicited, the virus has diversified (i.e., it has become a swarm

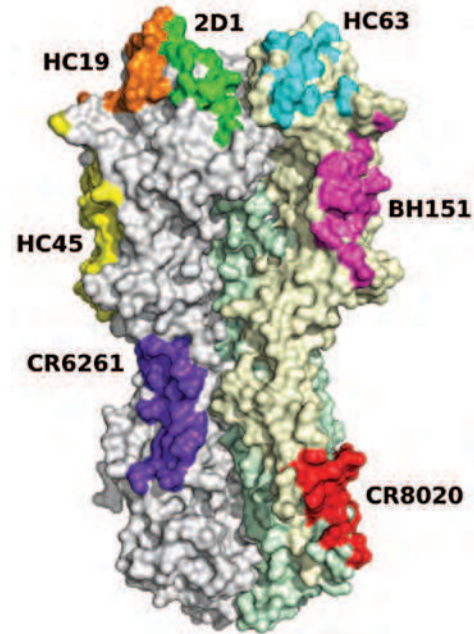


FIGURE 9.2. Neutralizing epitopes on influenza hemagglutinin (HA).

The epitopes were determined from crystal structures of neutralizing Fab fragments in complex with HA. The viral membrane is at the **bottom** of the figure. Most of the epitopes toward the top of the HA molecule are relatively variable in different influenza strains. The epitopes defined by the antibodies CR6261 and CR8020 in the stem of the HA molecule are conserved and might be targeted by a universal influenza vaccine. (Courtesy of Damian Ekiert and Ian Wilson.)

of related viruses through the errors associated with reverse transcription of this RNA retrovirus). Among this swarm is a virus that has sequence changes in the epitopes targeted by the neutralizing antibody response that allows it to escape from the response. This new virus becomes predominant. Eventually, a response is mounted to this virus and a second new virus emerges and so on. The antibody response chases the virus over many years but never appears to gain control.

An important consideration in relation to viral surface antigens and immunogenicity is the spacing of the antigens on the virion surface.¹² It is well established that haptened polymers, in which the haptens are spaced at 5 to 10 nm, can induce antibody responses in the absence of T-cell help. For vesicular stomatitis virus (VSV), in which the G protein is found in a highly organized quasicrystalline state on the virion surface, a strong immunodominant T-cell-independent neutralizing antibody response is induced by infection. A poorly organized recombinant form of VSV-G expressed in micelles induces a weaker B-cell response that is partially T-cell dependent. Soluble VSV-G alone, in the absence of adjuvant, fails to induce a B-cell response.

GENERATION OF THE PRIMARY B-CELL REPERTOIRE

The humoral immune system first senses the presence of novel viral antigens through the interaction of B-cell surface

antibody (monomeric IgM and/or IgD) with antigen. Individual B cells possess multiple BCRs of identical specificity, which when occupied by antigen, are activated to divide and differentiate. The final results include the production of large amounts of specific, soluble high-affinity antibody and the generation of memory B cells that can be readily triggered to produce high-affinity antibody on repeated encounter with viral antigen.²²⁶ The first encounter of viral antigen is with the primary B-cell repertoire of the individual. This repertoire is large, allowing recognition with low affinity of essentially any molecular shape and, therefore, any viral variant. It is produced in B cells by recombination of a limited set of germline segments.^{121,134,169,175} Hence, an individual's primary repertoire is unique to that individual and is in a constant state of flux. Briefly, the variable regions of antibody heavy and light chains are produced by V(D)J recombination in which numerous unique immunoglobulin genes can be made by joining different combinations of the V, D, and J segments at the heavy, and V and J segments at the light, chain loci. In humans, the potential heavy chain repertoire is approximately $50 \text{ VH} \times 27 \text{ DH} \times 6 \text{ JH} = 8 \times 10^3$ different combinations. Similarly, approximately $165 (33 \text{ V}\lambda \times 5 \text{ J}\lambda)$ and $200 (40 \text{ V}\kappa \times 5 \text{ J}\kappa)$ different combinations exist, for a total of 365 light chain (λ and κ) combinations. If we consider that each heavy chain could potentially pair with each light chain, then the diversity of the immunoglobulin repertoire is of the order of 10^6 possible combinations. Further diversity (junctional diversity) is generated because the joining of the V, D, and J gene segments is imprecise.

Although mice and humans use combinatorial and junctional diversity as a mechanism to generate a diverse repertoire, in many species, including birds, cattle, swine, sheep, horses, and rabbits, V(D)J recombination results in assembly and expression of a single functional gene.^{169,179} Repertoire diversification is then achieved by gene conversion, a process in which pseudo-V genes are used as templates to be copied into the assembled variable region exon. For example in the chicken, during B-cell development in the bursa of Fabricius, rapidly proliferating B cells undergo gene conversion to diversify the antibody repertoire. Stretches of sequences from germline variable region pseudogenes, located upstream of the functional V genes, are introduced into the VL and VH regions. This process takes place in the ileal Peyer's patches of cattle, swine, and horses, and in the appendix of rabbits.

B-CELL ACTIVATION BY VIRAL ANTIGENS

B cells can recognize and respond to both soluble and membrane-bound viral antigens, although it is likely that, in vivo, the most relevant interaction is with antigen on the surface of APCs.^{14,15} The encounter of viral antigens with BCR has two consequences. First, a signal is transmitted into the B cell that is amplified via intracellular pathways and results in the activation of transcription factors such as nuclear factor- κ B (NF- κ B) and AP-1. These factors act to induce specific gene transcription promoting B-cell proliferation and differentiation. Second, the BCR delivers viral antigens to intracellular sites where they are proteolytically cleaved and some viral peptides are bound to MHC class II molecules. The peptide/MHC II complexes are returned to the B-cell surface where

they can be recognized by viral-specific TCR on specialized CD4⁺ T cells (T helper cells), known as TFH or T follicular helper cells.^{52,200} These TFH cells are then stimulated to produce cytokines and provide surface costimulatory molecules that promote B-cell proliferation and act on later generations of these B cells to promote differentiation to antibody-producing and memory B cells. Some viral antigens, as described above, can activate B cells directly in the absence of T-cell help (so-called T-independent antigens).

Cell signaling from the BCR occurs when multiple antigen contacts lead to clustering of cell surface IgM molecules.²¹⁹ In the BCRs, IgM monomer is associated with a heterodimer of membrane-bound Ig α and Ig β molecules, which bear immunoreceptor tyrosine activation motifs (ITAM) on their cytoplasmic domains. Following BCR clustering, tyrosines in the ITAM become phosphorylated to trigger a series of events that eventually lead to activation of transcription factors. Signaling by the BCR is modulated by co-receptors, most notably Fc receptor IIB for IgG (FcR γ IIB) and CD19. FcR γ IIB is a potent inhibitor of B-cell signaling¹⁹⁷ by reversing protein kinase activities and by inhibiting the formation of BCR microclusters. CD19 is an activating coreceptor⁶⁴ that functions to recruit activating molecules to the BCR. In addition, CD19 occurs on the surface of B cells alone and is linked to CD21, a receptor for the complement fragment C3d. If, following complement activation, C3d has been linked to viral antigen or to antibody-virus complex, the co-receptor complex can be linked to the BCR and BCR activation enhanced. The involvement of complement can be crucial in the development of normal B-cell memory responses (see below). Mice with deficiency in C3, C4, or CD21/CD35 and infected with HSV show a reduction in specific IgG and germinal centers compared to normal mice.⁵⁹

The interaction of B cells with viral-specific TFH cells in lymphoid tissue leads to B-cell activation and proliferation and differentiation. It is important to note that the B-cell surface antibody and the TCR must recognize epitopes from the same molecular complex for the help signal to be delivered and B-cell activation to occur. Therefore, a B cell that is expressing surface antibody to the surface protein of a virus could be activated by a T helper cell expressing a TCR to an internal viral protein if antigen challenge involved whole virions.²⁴¹ A subunit vaccine consisting only of the envelope protein could not obtain help from such a T helper cell. The process is as follows. The B cell binds a virion through antibody recognizing surface protein. The whole virion is internalized and degraded, and peptides displayed on MHC class II molecules are carried to the B cell surface. Specific TFH cells that have previously been primed by DCs recognize the peptide-MHC class II complex on B cells through specific TCRs.⁴⁴ Antigen recognition results in T-cell adhesion to the B cell and the provision of "help" to the B cell via a range of transmembrane costimulatory receptors and cytokines. TFHs are highly enriched for selective expression of an array of cell surface molecules, reflecting their specialized role for cell-cell interaction with B cells.⁵² The most critical cell-cell interaction is CD40-CD40L. CD40 on B cells engages CD40 ligand (denoted CD40L and also known as CD154) on TFH cells, driving B-cell proliferation. The combination of B- and T-cell interactions and locally released cytokines can further enhance B-cell proliferation or direct differentiation, depending on the signals provided by

the TFH cell and the cell surface molecules expressed by the B cell. Of interest, the process of shared CD4 T-cell help to B cells specific for a different viral protein may occur only for small viruses, as the B cell and antibody responses to infection with vaccinia virus (a large poxvirus used as the smallpox vaccine) are strictly dependent on matched CD4 T-cell help.²⁵⁴ This likely reflects a limit on the pathogen or particle size that a B cell can endocytose whole.

There are two pathways for B cells that have contacted specific T cells. In the first, after several rounds of proliferation, B cells differentiate into plasma cells in extrafollicular foci¹⁶⁸ and secrete relatively large amounts of soluble antibody to the virion surface protein of our example. This antibody is encoded by the unmutated germline repertoire and of the IgM isotype or class-switched to IgG or IgA. The plasma cells are short-lived with a lifespan of a few days. This early antibody is believed to be important as a first-line humoral response against viral infection.

In the second pathway involving germinal centers (GCs) in lymphoid tissues (see Section 2), the quality and versatility of the antibody to the virion surface protein can be improved by affinity maturation and isotype switching.^{40,73,154,166,172,182,188,195} In GCs, B cells divide very rapidly, every 6 to 8 hours. CD4 T cells are required for the formation and maintenance of GCs. GC TFH cells provide signals that regulate maintenance of GC B cells and GC B-cell differentiation into plasma cells and memory B cells.⁵² GC TFH cells critically stimulate somatic hypermutation and selection of high affinity clones. Somatic hypermutation¹⁷⁷ involves the introduction of nontemplated point mutations into V regions of the rapidly proliferating B cells at a rate on the order of 1×10^{-3} mutations per base pair per generation, which is approximately 10^6 times higher than the mutation rate of cellular housekeeping genes. The enzyme activation-induced cytidine deaminase (AID) has been demonstrated to be essential for somatic hypermutation. AID is a cytidine deaminase capable of carrying out targeted deamination of C to U, and shows strong homology with the RNA-editing enzyme APOBEC-1. It seems that AID directly deaminates DNA to produce U:G mismatches that ultimately can result in sequence changes in the V regions. Selection from the array of B cells expressing slightly different mutated forms of cell surface antibody was thought to occur based directly on the ability of higher affinity clones to compete for antigen and receive stronger BCR signals and survive. More recently however,^{56,294} a somewhat different view has emerged in which B cells gather antigen as they move over the network of follicular DCs in GCs. Cells with the highest affinity for antigen acquire the most antigen, thereby leading to greater presentation of peptide/MHC II complexes to T cells, which in turn provide the requisite cytokine signals for B-cell survival and selection.

Isotype switching, in which the constant region of IgM is replaced by that of another isotype, also occurs in germinal centers. This might lead for example to a switch from IgM to IgG or IgA antibodies against a virion surface protein. Switching occurs through deletional DNA recombination events and involves repetitive DNA sequences or switch regions. The enzyme AID, described above, appears to be critically involved in the process. Cytokines such as IFN- γ and IL-17, which are expressed by germinal center TFH cells in a context-dependent manner, control appropriate isotype switching to different pathogens.

The B cells surviving the germinal center differentiate into plasma cells or memory B cells.^{97,171,182,304} Plasma cells accumulate in the spleen or draining lymph nodes during the early stages of acute infection, but as the immune responses subside, the majority of plasma cells are found in the bone marrow.^{11,124,267,318} These cells no longer express surface antibody but are committed to the secretion of soluble antibody—up to 10,000 antibody molecules per second.^{113,118} Because the half-life of IgG is on the order of 1 to 3 weeks, but specific antibody can be present for many years after antigen exposure, it appears that plasma cells maintain serum antibody levels. A favored hypothesis is that plasma cells are very long-lived and continue secreting antibody long after antigen has disappeared (see below). The half-lives of antibody responses in humans to measles and mumps viruses for example are estimated at more than 200 years.⁶ Memory B cells can also last for many decades.^{54,319} Memory B cells maintain surface antibody but secrete little if any antibody, unless activated. They divide slowly if at all. B-cell memory is considered in greater detail below.

VIRAL ANTIGEN RECOGNITION BY T CELLS

The T-cell response to viruses is largely mediated by T cells that utilize the TCR $\alpha:\beta$ chain heterodimer to recognize a foreign antigen. T cells, which employ the $\gamma:\delta$ chain complex (the gamma/delta cells), as well as certain rare TCR $\alpha:\beta$ expressing T cells with restricted variable α (or β) gene segment usage in TCR generation (e.g., NK T cells) have a less clearly defined role in the adaptive immune response to viruses (and will be briefly discussed below). The conventional TCR $\alpha:\beta$ T cells, which have left the thymus and populated the SLOs and bloodstream, express, along with their TCRs, either the CD4 or the CD8 co-receptor molecules. One defining feature of the response of these cells is that $\alpha:\beta$ antigen receptors recognize nonnative peptide fragments of foreign antigen (including viral polypeptides). Therefore, in contrast to the immunoglobulin receptor (BCR), which is sensitive to antigen conformation, T-cell recognition of antigen is insensitive to three-dimensional antigen structure. Furthermore, T-cell recognition of antigens (e.g., viruses) is limited primarily to the polypeptide constituents of the virus or, more precisely, to viral gene products expressed in infected cells (although some rare exceptions exist to this general rule, which are noted later in this chapter).

A second defining feature of the $\alpha:\beta$ TCR is that the antigen receptor recognizes these antigenic peptides bound to MHC molecules displayed on the surface of the antigen-containing (e.g., virus-infected) cells. Therefore, CD4⁺ and CD8⁺ T cells are restricted in their recognition of foreign antigen to cells displaying the foreign peptide complexed to the MHC molecule to which that T cell was selected during development in the thymus. This is the phenomenon of MHC restriction³²⁴ that defines antigen recognition by T cells. CD8⁺ T cells will recognize cells displaying the foreign antigen peptide bound to one of several distinct MHC class I molecules expressed on the cell surface. CD4⁺ T cells, likewise, are restricted in foreign peptide recognition by the cell surface MHC class II molecule to which the peptide fragment is bound.

The implications of MHC-restricted recognition of processed peptide fragments by T cells for virus recognition are immediate and profound. Conventional TCR $\alpha:\beta$ T cells

responding to virus infection will recognize infected cells and not free virions or soluble viral proteins. Therefore, the activation of naïve T cells and the expression of the effector activity of activated T cells responding to virus infection will be primarily mediated through cell-to-cell contact (i.e., contact between naïve T cells and APCs or the infected cell targets of effector T cells, such as epithelial cells). In addition, T cells have the potential to recognize any viral gene product (structural or nonstructural) that can be fragmented into peptides, and bound to/displayed by MHC molecules on the surface of infected cells. Consequently, CD8⁺ T cells will only recognize virus-infected cells that display peptide fragments of viral protein bound to cell surface MHC class I molecules, whereas CD4⁺ T cells will recognize only those cells in which display viral peptide/MHC class II complexes. The differences in the cell types that display MHC class I and II molecules, and the distinct mechanisms by which foreign polypeptides are processed (fragmented) and bound to/presented by MHC class I and II molecules determine how CD8⁺ and CD4⁺ T cells respond to virus infection.

The function of the MHC molecules is to bind peptide fragments from pathogens, and to display them on the cells harboring the pathogen for recognition by T cells expressing the appropriate (i.e., specific) TCR. The consequences of this MHC-TCR-dependent cell-cell interaction are almost always deleterious to the pathogen: virus-infected cells are killed; macrophages are activated to destroy intracellular bacteria living within their intracellular vesicles; and B cells are activated to produce antibodies that eliminate or neutralize extracellular forms of pathogens. The MHC locus is located on chromosome 17 in the human (chromosome 4 in the mouse), and extends over approximately 4 centimorgans of DNA ($\sim 4 \times 10^6$ base pairs). Two properties of the MHC locus product make it difficult for pathogens to evade the adaptive immune response. First, the MHC locus is polygenic.²⁵⁸ It encodes several different MHC class I and II gene products, and these differences in nucleotide/amino acid sequence control the range of peptide fragments that an individual MHC molecule can bind, so that every individual possesses a set of MHC molecules with a different range of peptide-binding specificity. Second, the MHC locus is highly polymorphic. Multiple versions (alleles) exist of each MHC gene product expressed in the population as a whole. In fact, the MHC genes are the most polymorphic genes in the human genome.²⁵⁸

The MHC locus contains three major classes of genes: class I, II, and III. The MHC class I and II genes (and their products) were first identified because of their critical role as targets in graft rejection. Hence, the name for the human MHC locus, HLA, (for human leukocyte antigen locus), and H-2 (histocompatibility-2 locus) for the corresponding genes in the mouse. The MHC class I genes (or more precisely MHC class Ia genes) consist of eight exons and encode a 45-kD protein containing three external domains (α_1 , α_2 , α_3), along with a membrane-spanning segment and a short cytoplasmic tail. The α_1 and α_2 domains fold to form a molecular platform on the upper, outer surface of the molecule, with a *groove* or cleft to accommodate peptide fragments from pathogens.⁸³ Amino acid differences in the α_1 and α_2 domains among several class I molecules encoded within the MHC locus genes of an individual and between the numerous alleles of the gene encoding a given MHC class I gene

product among humans dictate the types of peptides that can be accommodated in the peptide-binding groove. The α_3 domain contains the site for the binding CD8 molecules to the MHC class I. The MHC class I molecule is a heterodimer consisting of the MHC encoded 45-kD heavy (H) chain complexed noncovalently with a non-MHC locus-encoded small molecule, β -2 microglobulin (β -2M), which interacts with the H chain α_3 domain. Stable cell surface expression of the MHC class I molecule usually requires β -2M association. Importantly, MHC class I molecules are expressed at varying levels on most cells of the body, with neurons being a notable exception.²¹⁸ The expression levels of class I molecules on the cell surfaces can be upregulated by exposure of the cell to inflammatory mediators (e.g., type I and II IFN, TLR agonist, and so on).^{85,141} Therefore, the T cells that recognize peptide/MHC class I complexes (i.e., the CD8⁺ T cells) have the capacity to survey most body cells and interact with/destroy cells displaying the appropriate peptide/MHC class I complex. Three class I genes (more precisely, three class Ia genes) exist in the human: HLA-A, HLA-B, and HLA-C (H-2K, H-2D, and H-2L in the mouse), and each gene is polymorphic (i.e., displays multiple different alleles) within the human population.

The MHC class II molecules are also heterodimers, consisting of two chains (α and β), each encoded by a separate gene. These genes have a similar exon-intron structure, with the class II α chain genes encoding two extracellular domains (α_1 and α_2), and the class II β chain gene likewise encoding two extracellular domains (β_1 and β_2). The α_1 and β_1 domains of the class II molecules interact to form a molecular platform with a groove to accommodate the foreign peptide, which is like the α_1 and α_2 domain of the class I molecule on the upper outer surface of the molecule and, therefore, available for recognition by the TCR on CD4⁺ T cells. The CD4 molecule on the T cell interacts with the surface of the β_2 domain, distal to (away from) the TCR binding site. The class II heterodimer is anchored to the cell membrane by transmembrane segments of both chains, and each chain contains a short cytoplasmic tail. In contrast to class I molecules, the expression of class II molecules is largely restricted to immune system cells: B cells, macrophages, DCs, and activated T cells in the human (and to a lesser extent, for activated T cells in the mouse), as well as specialized epithelial cells in the thymus and type II alveolar epithelial cells in the lungs. The expression of MHC class II molecules on these cells can be upregulated by cytokines (in particular, IFN- γ)⁶⁴; evidence indicates that certain somatic (nonimmune) cells can be induced to express MHC class II molecules in response to inflammation.⁸⁵ Three pairs of MHC class II α and β chain genes exist, which are called HLA-DR, HLA-DP, and HLA-DQ (two pairs in the mouse: H-2^{I-A} and H-2^{I-E}). In some instances, the DR cluster contains the genes for two β chains (DR β_1 , DR β_2), each of which can pair with the DR α chain. Again, multiple alleles exist for each gene pair. The amino acid differences among the alleles are localized primarily to the α_1 and β_1 domains, where these residue differences control the range of foreign peptide bound by the various MHC class II allelic gene products.⁶⁶

Although both MHC class I and II molecules are similar in structure and function (i.e., presenting foreign peptides), the difference in the cellular distribution of these molecules points to the role of the T-cell subsets (CD8⁺ and CD4⁺ T cells)

that recognize these molecules in the function of the adaptive immune system. For example, viruses have the potential to infect most nucleated body cells. Because these cells also express (to a varying degree) MHC class I molecules, they would be susceptible to recognition and destruction by activated effector T cells that are restricted by class I molecules in antigen recognition (i.e., CD8⁺ T cells). This simple association suggests an important role of CD8⁺ T cells in eliminating virus-infected cells and recovery from infection.⁷⁰ By contrast, MHC class II molecules are displayed primarily on cells of the immune system. Therefore, the main role of CD4⁺ T cells, which recognize these molecules and the bound foreign peptides, is to activate and regulate the effector response of the other immune cells (e.g., the recognition by activated CD4⁺ T cells of peptide/MHC class II complexes on B cells to stimulate antibody production, the recognition of peptide/MHC class II complexes on macrophages harboring pathogens within intracellular vesicles to stimulate macrophage activation and pathogen destruction, and the recognition of peptide/MHC class II complexes on DCs to stimulate cytokine production during the induction of adaptive immune responses).

Of course, the ability of CD8⁺ and CD4⁺ T cells to interact with cells displaying foreign peptide/MHC complexes also depends on the ability of these foreign antigens to provide peptides to complex with the MHC class I and II molecules. As will be discussed, the pathways by which foreign antigens gain access to and charge MHC class I and II molecules is distinctly different, and dictates the contribution of CD8⁺ T cell and CD4⁺ T cells in the adaptive immune response to invading microorganisms.

ANTIGEN PROCESSING AND PRESENTATION TO T CELLS

The pathways of antigen processing and peptide presentation by MHC class I and II molecules to CD8⁺ and CD4⁺ T cells are historically called the *endogenous* and *exogenous* presentation pathways. In the most basic terms, protein antigens, such as viral polypeptides, which gain access to the cell cytoplasm, have the potential to efficiently enter the MHC class I

processing/presentation pathway—that is, produce peptides that can charge MHC class I molecules in the cells for subsequent recognition by antigen-specific CD8⁺ T cells. During viral infection, access to the cell cytoplasm is perhaps most effectively achieved by infection of the cell that is by *de novo* synthesis of viral gene products.⁹² This endogenous presentation pathway (i.e., infection of the cells) is the typical way that most virus-infected body cells are sensitized for recognition by activated CD8⁺ T cells. Certain bacteria (e.g., *Listeria monocytogenes*, *Salmonella*) and parasites (e.g., malaria) that can gain access to the infected cell cytoplasm can provide protein antigens, which enter the class I presentation pathway, as can strategies that introduce proteins directly into the cytosol of cells (e.g., liposome-cell fusion).^{99,306} Certain cell types that act as professional APCs (most notably, DCs) have the capacity to take up soluble or aggregated antigenic material (i.e., apoptotic, infected cell debris), and process/present antigenic peptide by MHC class I molecules to CD8⁺ T cells by one of several different cross-presentation mechanisms that have been elucidated.²⁵⁹

Protein antigens, both soluble and particulate (e.g., bacteria, virions), which are internalized into the endocytic compartment of cells expressing MHC class II molecules, have the potential to enter the class II processing/presentation pathway and charge MHC class II molecules for recognition by CD4⁺ T cells. This exogenous presentation pathway (i.e., uptake from without) characterizes the handling of soluble antigen (e.g., intact, inactivated virions and subunit vaccines and extracellular bacteria) by the adaptive immune system, leading to the production of activated CD4⁺ T cells and antibodies. Any foreign protein (including a viral membrane glycoprotein) expressed on an infected MHC class II-expressing cell can enter the class II pathway, however, if it can gain access to the endocytic compartment where processing/presentation occurs (Table 9.4).

This somewhat simplified distinction between endogenous (class I) and exogenous (class II) processing/presentation pathways highlights the importance of antigen presentation in dictating the action or effector activity of the T-cell arm of the adaptive immune system. Generally, pathogens that gain access to the cell cytoplasm will generate peptide/MHC class I complexes and trigger a CD8⁺ T-cell response, whereas pathogens/pathogen

TABLE 9.4 Features of Viral Antigen Processing by the Major Histocompatibility Complex Class I and Class II Presentation Pathways

	Location	Proteolysis	MHC loading peptides
MHC Class I Pathway	Cytoplasm ^a	Proteasome ^b neutral pH	Endoplasmic reticulum
MHC Class II Pathway	Endosome ^c	Acid proteases low pH	Endosome ^d

^aViral proteins gain access to the cytoplasm by (a) *de novo* expression of viral proteins in infected cells; (b) direct virus-cell fusion at plasma membrane or in endosomes; (c) in specialized antigen-bearing presenting cells (APCs) (i.e., dendritic cells) by phagocytic uptake of virus or virus-infected cellular material into endosomes followed by endosome–endoplasmic reticulum (ER) fusion with or without retrograde transport of viral proteins into the cytoplasm.

^bOther proteases in the cytoplasm and ER can modify the length of the viral peptide fragment produced by the proteasome before or after transport of the peptide into the ER by the transporter associated with antigen processing (TAP) transporter complex.

^cViral proteins in the form of virions and soluble and membrane-associated viral proteins must gain access to the endosome for fragmentation and binding to major histocompatibility complex (MHC) class II molecules.

^dNewly synthesized MHC class II molecules are blocked from loading peptides in the ER by the class II-associated invariant (Ii) complex which is dissociated from class II molecules in the endosome by step-wise proteolytic cleavage of the Ii chain.

products that remain exclusively in the specialized endocytic compartment of MHC class II-expressing cells will typically trigger a CD4⁺ T-cell response. The steps in the processing (fragmentation) of polypeptides into peptides, as well as the transport and interaction of peptides with MHC molecules, not surprisingly, are different in the class I and class II presentation pathways. Examination of these steps merits review because viruses have developed strategies to suppress the T-cell response by subverting the steps along these pathways.

ANTIGEN PRESENTATION BY THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I PATHWAY

Antigenic peptides usually bind newly synthesized MHC class I molecules in the endoplasmic reticulum (ER) of the antigen-bearing/infected cells. Therefore, in most cell types, the foreign polypeptide must be fragmented in the cytoplasm, transported from the ER, and associate with a newly synthesized class I 45-kD heavy chain/ β_2 M chain complex before transport of the MHC/peptide complex from the ER through the secretory pathway to the cell surface (Fig. 9.3). Antigenic peptide production through fragmentation of cytosolic proteins is primarily carried out by the multisubunit, multicatalytic 20S/26S proteasome complex present in the cell cytoplasm.²³¹ This enzyme complex accounts for most of the cytosolic protein degradation in all cells, and the proteasome is probably responsible for the generation of most self-peptides involved in T-cell selection in the thymus through the turnover (degradation) of cellular proteins in the thymic cortical epithelial cells and medullary DCs. Two important sources of proteasome substrates are newly synthesized self- or foreign (e.g., *de novo*) expressed gene products, such as viral proteins in an infected cell and preformed foreign microbes/microbial proteins that have gained access to the cell cytoplasm. The proteasome recognizes and degrades polyubiquitinated proteins. This protein tagging involves the sequential action of the ubiquitin-activating enzyme (E3), several ubiquitin-conjugating enzymes (E2), and substrate-specific ubiquitin ligases (E3)²³¹ to form the polyubiquitinated substrate of the proteasome. Some evidence suggests that enhancing ubiquitination of a protein may increase the amount of antigenic peptides generated for loading into MHC class I molecules. Furthermore, a significant fraction of newly synthesized proteins are ubiquitinated during the normal turnover of cellular protein, and evidence suggests that defective ribosomal translation products (i.e., products of premature ribosome termination and/or misfolded translation products) are a major source of the protein substrates for MHC class I peptide epitopes.³¹⁶ Therefore, the rate of protein synthesis rather than turnover or expression level would be critical for the loading of MHC I molecules with appropriate antigenic peptides. Such a strategy favors the generation and recognition of newly synthesized viral proteins in infected cells.

The core proteasome cleaves polypeptide substrates after hydrophobic and basic residues.²⁵⁶ Exposure of cells to certain cytokines (particularly, IFN- γ) induces the expression of three new proteasome subunits: b1i, b5i, and b2i (formally called LMP-2, LMP-7, and MECL-7, respectively). These subunits

replace existing subunits in the core proteasome, producing a second proteasome form, the immunoproteasome present in IFN-treated cells.³¹⁴ This replacement of the constitutive proteasome subunits with these inducible subunits changes the proteasome cleavage specificity, thereby increasing the cleavage of polypeptides after hydrophobic and basic residues and decreasing the cleavage after acidic residues (i.e., peptides with acidic C-termini). This is noteworthy because MHC class I molecules preferentially bind peptides (in an MHC class I allele-dependent manner) with hydrophobic or basic *anchor* residues at their C-termini.²⁹

Two of the IFN- γ -inducible proteasome subunits, b1i and b5i, are encoded by genes within the MHC locus,²⁵⁸ suggesting a strong evolutionary selective pressure for the immunoproteasome in MHC function. IFN- γ also increases the production of antigenic peptides by inducing the expression of a multisubunit proteasome adaptor complex PA28 which, on binding to the ends of the core proteasomes/immunoproteasome cylinder, increases the peptide efflux rate from the proteasome. Therefore, a cytokine product, IFN- γ , produced by an innate immune cell type (NK cells) or adaptive immune effector cells (antigen-specific CD4⁺ effector T cells) can enhance the display of antigen peptide fragments recognized by effector CD8⁺ T cells. Proteasomes generate peptides 3 to 20 amino acids (a.a.) in length.⁹³ Studies in which peptide fragments bound to MHC class I molecules isolated from cell surfaces were extracted and analyzed, however, revealed that these class I-bound peptides, in general, are 8 to 10 a.a. in length. Therefore, the proteasome peptide products that have the appropriate C-termini for MHC class I binding must also undergo cleavage at their N-termini. Several cytosolic (and one ER resident) aminopeptidases have been implicated in carrying out this process.^{93,126,159} N-Terminal trimming in the ER of transported peptides having an extended (greater than 8–10 a.a.) length is carried out in this compartment by the ER-associated amino peptidase (ERAAP). One cytosolic peptidase, tripeptidyl peptidase II (TPPII), may be capable of generating a limited range of peptides in the absence of proteasome function.¹⁴⁷ Overall, several studies have suggested that modifications of the proteasome core by inflammatory cytokines, combined with the action of cytosolic proteases, can affect antigenic peptide generation, including the production of antigenic peptides (in cells infected with several different viruses), the recognition of which by CD8⁺ T cells is dependent on these proteasome modifications and/or action of other cytosolic and ER proteases.^{261,290}

Peptides generated in the cytosol are transported to the ER by the heterodimeric transporters associated with antigen presentation (TAP)-1/TAP-2 protein complex. This transporter complex is located within the ER membrane, forming a pore/channel there.⁹⁶ Both proteins are members of the ABC family of ATP-dependent small molecule transporters, and are also encoded by genes mapping to the MHC locus. This transporter complex preferentially translocates peptide of 8 to 16 a.a. in length with hydrophobic or basic C-termini, and the TAP complex exhibits some restriction in transport efficiency of peptides with certain residues at the N-termini. Peptides gaining ER access through the TAP pore encounter the MHC class I peptide loading complex.²⁹¹

Newly synthesized MHC class I molecules must bind to β -2M and have a stably bound peptide (of self or foreign origin) in their peptide binding groove to retain the stable

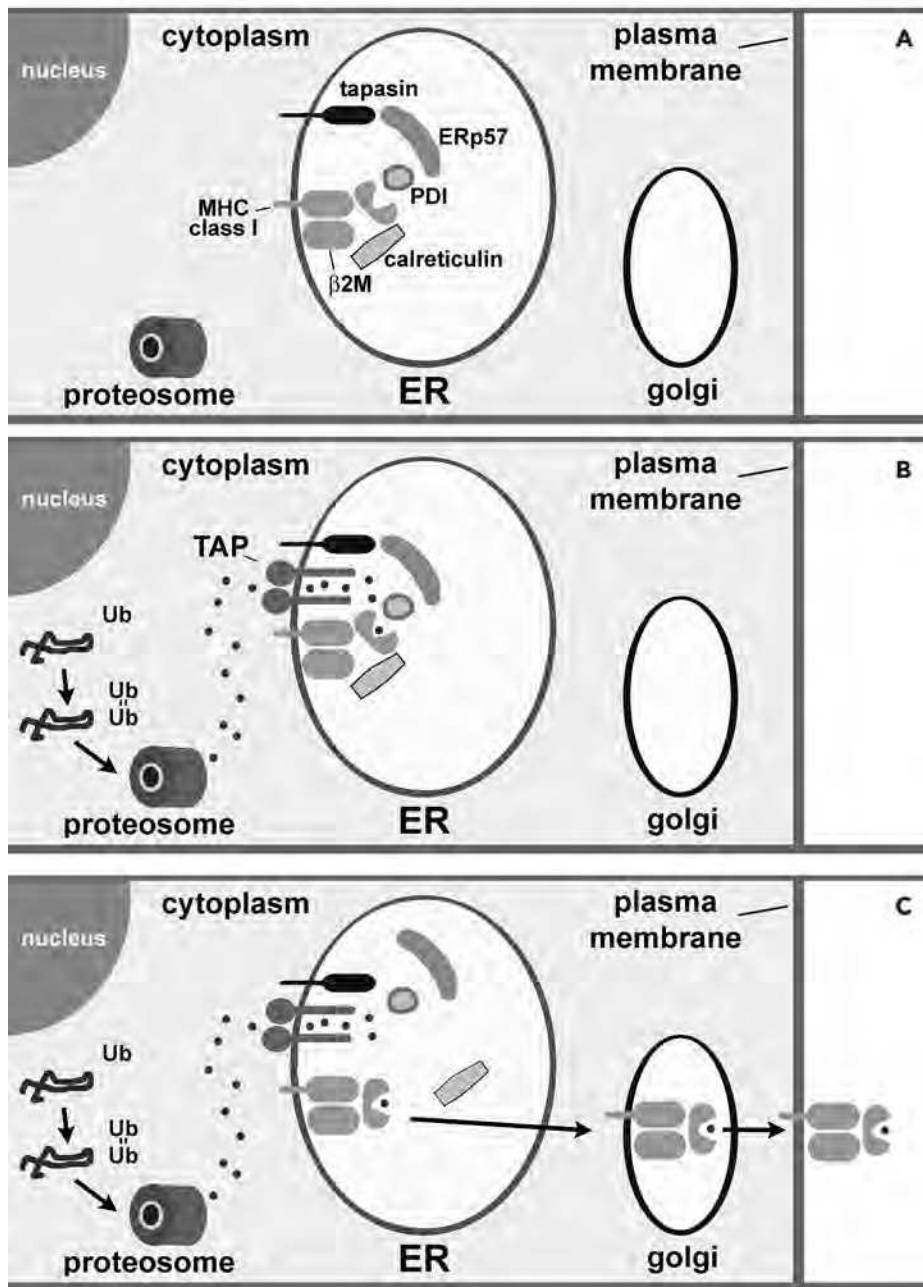


FIGURE 9.3. Major histocompatibility (MHC) class I presentation pathway.

A: Following their co-translational translocation into the endoplasmic reticulum (ER), newly synthesized MHC class I forms a complex consisting of the ER scaffolding protein calreticulin (and calnexin—not shown), peptide and protein modifying enzymes (ERp57, protein disulfide isomerase [PDI]), the antigen presentation transporter associated with antigen presentation (TAP) transporter targeting molecule tapasin and a weak interaction with beta 2 microglobulin ($\beta 2M$). **B:** Processing/presentation through the classical MHC class I pathway requires that a target protein (e.g. viral polypeptide) gain access to the cell cytoplasm where it is ubiquitinated before fragmentation by the cytosolic protein complex, the proteasome. Peptide fragments are then transferred from the cytoplasm to the ER through the TAP transporter complex. The MHC class I complex is targeted to the TAP through the action of tapasin, and here the MHC class I and associated molecules come in contact with transported peptides. Peptides with the appropriate amino acid sequence (binding motif) and length/conformation (following modification by enzymes within the ER) will associate with the nascent MHC class I molecules/ $\beta 2M$ to form a stable complexes. **C:** The new stable MHC class I/peptide/ $\beta 2M$ complexes dissociate from scaffolding proteins and are transported via the secretory pathway to the cell surface where interaction with the CD8⁺ T-lymphocyte antigen receptor occurs.

conformation necessary for it to exit the ER.²⁹¹ Within the ER, nascent class I α chain molecules are bound to the ER chaperone, calnexin, until the 12-kD $\beta 2M$ molecule binds, and calnexin is then replaced by the calreticulin scaffolding protein and the tapasin molecule (an MHC locus-encoded protein that forms a bridge between the class I α chain/ $\beta 2M$ complex and the TAP transporter). The binding of calreticulin and tapasin to the nascent MHC class I molecule stabilizes the complex, allowing the partially folded class I/ $\beta 2M$ complex to maintain a conformation (while it awaits a soluble peptide from the cytosol). The partially folded MHC class I molecule will selectively bind (select) only those peptides containing certain motifs; that is, common or conserved amino acid sequences distributed along the length of the peptide fragment. Each allelic form of the class I molecule has specificity for (binds to)

a peptide containing an MHC class I allele, a specific peptide binding motif. The ability of a specific MHC class I allelic variant to capture peptides displaying this conserved amino acid motif is dictated by the presence of polymorphic amino acid residues in the binding cleft formed by the α_1 and α_2 domains. A third component of the peptide loading complex is the ERp57 thio reductase and protein disulfide isomerase (PDI, which presumably catalyzes disulfide interchange in the class I α chain during peptide loading). In certain cells (e.g., macrophages, DCs), the aforementioned ER amino-peptidase, ERAAP,²⁴⁶ is associated with the loading complex, and acts to trim the N-termini of long peptide (i.e., ≥ 10 a.a.) down to size before final folding of the complex. After proper peptide loading and folding, the stable class I/ $\beta 2M$ /peptide complex is freed from the chaperones, and migrates from the ER to the

cell surface. Note that in the uninfected cells, class I molecules still require a stably bound peptide to exit the ER and traffic to the cell surface. The source of these *antigenic* peptides is degraded cellular (self) proteins.²⁸

The classic MHC class I presentation pathway would appear to be restricted to proteins synthesized/retained in the cytosol. However, this is not the case. Numerous examples are found of secreted and membrane proteins/glycoproteins that contain peptides that are recognized by CD8⁺ T cells and, therefore, bound to MHC class I molecules. The most likely way that these proteins may be degraded is if the polypeptide chains are generated in the cytosol as defective or mistranslated ribosomal products, DRiPs.³¹⁵ Another way is retrograde transport of defective (misfolded) translation products that have been co-translationally translocated into the ER by the Sec61-dependent transport mechanism.¹ Sec61 normally acts in the translocation of the translation products of membrane-bound ribosomes into the ER, but can also shuttle intact (presumably defective) proteins in a retrograde fashion into the cytosol from the ER for degradation by the proteasome.¹ The unique ability of professional APCs (particularly CD8 α^+ and CD103⁺ cDCs) to “cross-present” cellular constituents/fragments of virus-infected cells may reflect the use of such a mechanism (i.e., uptake of virions, infected cellular material, and so on into endosomes followed by retrograde transport of this cargo into the ER) preferentially. Protein processing and peptide presentation to MHC class I molecules may also occur by direct loading peptides into the MHC molecules within endosomes, but the mechanistic basis for this processing pathway is poorly understood, and its *in vivo* significance for virus infection is uncertain.²⁵⁹

ANTIGEN PRESENTATION BY THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II PATHWAY

Antigen processing and presentation along the MHC class II pathway differs from class I presentation in several ways (Table 9.4). First, the class II pathway focuses primarily on antigens (both soluble and particulate) that enter cells by endocytosis/phagocytosis, and are retained in endocytic compartments for subsequent processing (fragmentation) and loading of the peptide fragments into class II molecules. These endocytic compartments include early and late endosomes and, in certain instances, lysosomes.³⁰¹ Second, antigen fragmentation occurs in the low pH environment of the endosome (rather than in the neutral pH of the cytosol where proteasome-dependent MHC class I binding peptides are generated). Third, both newly synthesized and recycled (from the cell surface) MHC class II molecules can capture and present peptide fragments to CD4⁺ T cells. Fourth, unlike the strict length constraints (i.e., 8 to 10 a.a.) for peptides bound to MHC class I molecules, peptides bound to class II molecules are at least 13 amino acids long, and can be longer (i.e., up to 20 a.a.). As with the class I molecules, however, specific class II molecules (alleles) will selectively bind (select) peptides containing particular a.a. motifs (i.e., peptides containing common/conserved a.a. distributed along the length of the peptide). As for class I molecules, these peptide-binding motifs are dictated by the presence

of polymorphic a.a. residues in the class II binding cleft (which differ for each class II locus gene product allele). In summary, class II molecules bind peptides produced from soluble or particulate antigen taken up by endocytosis in the same low pH compartment where protein fragmentation occurs. How then is class II processing achieved?

The MHC class II α and β chains, as with other membrane glycoproteins, are synthesized in the ER. After synthesis, the α : β chain heterodimer engages a type II membrane protein, the MHC class II-associated invariant (Ii) chain—invariant because the Ii gene is nonpolymorphic. The invariant chain forms a trimer complex consisting of three class α : β heterodimers and three Ii subunits, with each Ii subunit binding noncovalently to a class α : β heterodimer. This interaction of the class II α : β heterodimer with Ii serves two important functions. First, a region of Ii binds (with low affinity) to the peptide binding groove/cleft of the nascent class II molecule in the ER, and prevents the loading of peptides transported into the ER from binding to class II in this compartment. Therefore, class II molecules do not bind the same spectrum of peptides as class I molecules, because they do not bind peptides in the ER. Second, Ii targets the class II molecules to the cell's low pH endosomal compartment (rather than the cell surface via the secretory pathway) where protein antigen fragmentation and peptide loading into the class II peptide binding groove occurs.¹⁰⁶

Processing (fragmentation) of protein antigens in the MHC class II pathway is carried out by a variety of acid proteases, and at least one IFN- γ -induced lysosomal thiol reductase (GILT) located in the low pH endosomal compartment. Several related cathepsin cysteine proteases (e.g., cathepsins S, L, B, F), and the unrelated cysteine protease, asparaginendopeptidase (AEP), have been implicated in antigen processing (fragmentation) in endosomes as inhibition of, or deficiency in, one or more of these proteases that can affect the generation of certain antigenic epitopes recognized by CD4⁺ T cells.²⁹⁵

To bind peptides in the endosome, class II molecules must be liberated from Ii. This is achieved by the sequential cleavage of Ii by endosomal proteases (including, AEP, cathepsin S, and cathepsin L), freeing the class II α : β heterodimer from the membrane portion of the Ii, and leaving a short fragment of Ii (called *CLIP* for class II-associated invariant chain peptide), which remains bound to the peptide-binding groove of the class II molecule. CLIP must be removed from the class II molecule to make the class II binding groove accessible to peptide fragmentation in the endosome, which is achieved through the action of a MHC-like molecule, DM (HLA-DM for human, H-2M for mouse) and which is encoded in the MHC locus and resides in the endosome. DM catalyzes the dissociation of CLIP from the class II α : β heterodimer. As with Tapasin for class I molecules, DM stabilizes the class II molecule as the class II molecule interacts with and sorts through the myriad of antigenic peptides in the endosome, selecting those peptides displaying the correct motif to allow these peptides to stably bind to the class II molecule. This peptide selection process by class II molecules typically occurs in 2 to 4 hours (during the time that the class II molecules reside in the endosome). Once a stable peptide/class II complex forms, DM dissociates; and the complex transits to the cell surface of MHC class II-expressing cells. *Empty* class II molecules (i.e., lacking a peptide in the binding groove) are unstable. Consequently, in the absence of infection

or specific exposure to foreign antigen (e.g., vaccination), it is self-peptides derived from the endosomal proteolysis of cellular proteins (e.g., soluble proteins taken up from the extracellular space by pinocytosis or the residues of phagocytosed apoptotic cell) that are bound to the MHC class II molecules on the surface of uninfected, class II-expressing cells.

Although *de novo* synthesized MHC class II molecules likely play a major role in capturing/presenting processed antigen to CD4⁺ T cells, class II molecules can cycle back into the endosomes from the cell surface, and these *recycled* class II molecules may also capture/present antigenic peptide. Class II molecules containing self-peptides (or less frequently, class II molecules that escape the endosome and exit to the cell surface with CLIP peptide in their groove) appear able to re-enter the endocyte compartment, exchange the bound peptide for a new peptide, and cycle back to the cell surface. By employing this recycling mechanism, MHC class II-expressing cells (in particular, professional APC, as with DC) would increase their chances of capturing and displaying foreign antigenic peptides to CD4⁺ T cells. In immature DCs, endosomal/lysosomal proteolytic activity is weak and newly synthesized MHC class II/Ii complexes are shunted to and retained in the endosome where they will be eventually degraded unless the DC are activated.

ANTIGEN PRESENTATION AND PROFESSIONAL ANTIGEN-PRESENTING CELLS

The hallmark of the adaptive immune system is specificity of foreign antigen recognition. For T cells, this means recognition of antigenic peptides bound to MHC class I or II molecules displayed on the cell surface. The T-cell response proceeds, as we have seen, in two phases. First is the inductive phase: the activation of quiescent primary (or memory) T cells in response to recognition of specialized APC displaying the peptide/MHC complex, resulting in the generation of armed effector T cells. Second is the effector phase, where cells displaying the appropriate peptide/MHC complexes are recognized by the activated T cells. The *classic* class I presentation pathway (which utilizes peptides derived from proteins synthesized *de novo* in the cell) is ideally suited to generate the antigenic peptide/MHC class I complexes recognized by CD8⁺ effectors in response to viral infection of any body cell type. Virus infection of a cell leads to the generation of an ample supply of viral protein substrates for processing by the proteasome. The expression of MHC class I molecules, and the molecular machinery of processing (i.e., the TAP transporter complexes and immunoproteasome subunits) are upregulated by type I and, in particular, type II IFN induced during virus infection by infected cells and by responding NK and effector CD4⁺ T cells (increasing the efficiency of infected cell recognition by CD8⁺ T cells). Furthermore, because antigenic peptide/MHC class I complexes are extremely stable,²⁵⁰ viral peptides bound to MHC class I molecules displayed on the surface infected cells are not transferred to surrounding uninfected cells. Consequently, the resulting destruction of bystander cells by CD8⁺ T cells is negligible. Evidence accumulated over several decades suggests that this classic pathway of cytoplasmic generation of viral peptides and

loading of viral peptides into class I molecules in the ER is employed in the recognition of virus-infected cells by CD8⁺ T cells. As discussed below, many viruses have evolved strategies to disrupt the class I presentation pathway to prevent effector CD8⁺ T-cell recognition of infected cells and sustain virus infection.

Due to the more stringent requirements for the activation of resting naïve T cells, there has been considerable attention given over the last decade to the process of antigen processing and/or presentation by professional APCs (in particular DCs) in the generation of peptide/MHC complexes during the inductive phase of the T-cell response. Although a number of DC subsets had been identified and categorized, as noted above, DC can be conveniently divided into two main subsets: cDC (conventional/myeloid DC) and plasmacytoid (lymphoid) DC (based on the bone marrow progenitor cell types that give rise to these two DC subsets and on the expression of certain DC lineage-specific cell surface molecules.²⁶⁰ The cDCs are the primary APCs, which stimulate antiviral T-cell responses.¹⁷ The DC subset derived from lymphoid progenitors, the plasmacytoid DCs (pDCs), has received considerable scrutiny. pDCs, so called because of their plasma cell-like morphology and surface expression of a CD45 isoform (i.e., the B220 molecule, which, characteristically, is found only in B cells) have weak APC activity but have been demonstrated to be the major source of IFN- α produced early (24 to 48 hours) after inflammatory stimuli, including virus infection.⁴⁹ Therefore, in addition to serving as a potential, albeit weak APC for T cells, the pDCs may serve as a major early innate immune defense against virus infection.⁸⁰

In support of their essential role in immune response induction, DCs (when activated/mature) are 10 to 100 times more potent on a per cell basis as activators of naïve T cells when compared with other immune cell APCs (e.g., macrophages, B cells).² Immature DCs can take up virus by the following several mechanisms:

1. Binding of viruses to their cognate receptors displayed on the DC surface with direct fusion or uptake of bound virus into endosomes, and subsequent release of the viral genome to initiate infection.
2. Phagocytosis of virions using a range of lectin-like, innate immune pathogen pattern recognition receptors displayed by DCs (e.g., DEC 205, DC-SIGN). Although triggering of phagocytosis and the likely engagement of TLR capable of recognizing viral nucleic acids/proteins (i.e., TLR-3, 7, 9) should result in DC activation/maturation, engagement of certain lectin receptors by particular viruses (e.g., HIV interaction with DC SIGN) may induce an inhibitory signal suppressing DC activation.⁷⁹
3. DC uptake of virus nonspecifically by fluid phase macropinocytosis. This bulk uptake of fluid phase soluble and particulate material is a characteristic property of immature DCs.
4. For viruses capable of directly activating the complement cascade (i.e., without prior antibody binding) (e.g., HIV), DCs expressing the complement receptor CR3 (i.e., the integrin complex, CD11b/CD18) can take up (by receptor-mediated endocytosis) virions displaying the activated complement C3 product, iC3b.

Any or all of these uptake mechanisms can directly or indirectly deliver viral polypeptides to the MHC class II and classic class I presentation pathways in DC.

Given the critical role of cDCs in the induction of the T-cell response (in particular, the antiviral CD8⁺ T-cell response,¹⁴⁴ viruses might be expected to have evolved strategies to inhibit the induction of CD8⁺ T-cell responses in virus-infected DCs. As discussed elsewhere in this text, for individual virus genera, evidence indicates that infection of DCs by certain viruses may inhibit the APC capacity of DCs.^{242,253} Furthermore, productive virus infection of tissue DCs at body surfaces could also result in virus dissemination when the DCs traffic to the SLOs. These considerations, as well as the fact that certain viruses with narrow cellular tropism (e.g., papillomavirus), therefore, are not capable of infecting DCs and providing polypeptides for processing via the classic class I pathway, led to the concept of cross-presentation of antigen (including, viral polypeptides) by DCs to CD8⁺ T cells. As discussed above, the likely mechanism(s) of antigen cross-presentation by DCs involves phagocytic uptake by DCs of virus or, more likely, of fragments of productively infected apoptotic cells. Recent evidence points to several potential mechanisms of cross-presentation by DCs, including a mechanism where the DC phagosomes containing viral antigen (taken into the DCs by phagocytosis of virus/virus-infected cellular material) fuses with the ER; therefore, viral polypeptides in this ER/phagosome complex would be available for transport through the Sec61 retrograde transport mechanism into the cytosol for proteasome-dependent proteolysis and subsequent transport of viral peptide into the ER for loading onto nascent MHC class I molecules.¹⁸⁰ Cross-presentation of antigens to naïve T cells is believed to be limited to professional APCs, that is, cDCs and possibly certain macrophage subsets that function at the inductive phase of T-cell responses. The concept of cross-presentation as the mechanism of viral antigen presentation to naïve CD8⁺ T cells, is gaining general acceptance, particularly because it solves several of the nagging issues concerning the induction of CD8⁺ T-cell response to viruses such as papilloma and polio viruses, which presumably do not infect DCs (and to tumor cells that cannot serve as professional APCs). It remains uncertain, however, why most pathogens (e.g., extracellular bacteria, fungi) only trigger CD4⁺ T-cell responses (i.e., do not activate the MHC class I presentation pathway) despite the potential for these antigens to enter the phagocytic compartment of DC.

CYTOKINES AND CHEMOKINES

Many cytokines and chemokines act to orchestrate the innate and adaptive immune responses. The cellular and molecular biology of these molecules have been extensively reviewed.^{175,184} Several of the cytokines also play an important role in T-cell activation and are worth consideration. Interleukin-12 (IL-12) is primarily a product of activated DCs and macrophages, and exists in two forms: IL-12p70 (a heterodimer consisting of the p40 and p35 subunits), and a homodimer of IL-12p40. (The p40 subunit is also part of the heterodimer making up the cytokine IL-23—an important regulator of CD4 Th-17 effector T-cell responses as discussed below.) IL12p70 interacts with its receptor on naïve and activated T cells, providing *signal 3* in the naïve T-cell activation sequence through recruitment and

activation of signal transducers and activators of transcription 4 (STAT-4). The outcome of this signaling process is to promote the differentiation of CD8⁺ T cells into activated cytolytic effector cells, and to help drive the differentiation of CD4⁺ T cells along a pathway leading to effector CD4⁺ T cells capable of secreting IFN- γ on TCR engagement (i.e., the generation of T_H1, T helper 1 effector T cells). The effect of IL-12p70 on T cells is to generate activated effector T cells that are efficient at viral clearance.

IL-2 is a cytokine produced by both CD4⁺ T cells and, to a lesser extent, CD8⁺ T cells, during the initial phase of T-cell activation and differentiation.¹³² Engagement of the high affinity IL-2 receptor (expressed only after T-cell activation) by IL-2 drives T-cell proliferative expansion. Although IL-2 was initially believed to be the primary T-cell growth factor (acting in an autocrine fashion to stimulate T-cell proliferation), T-cell activation and proliferative expansion occur normally in the absence of IL-2 (e.g., in IL-2-deficient patients or experimental animals with a targeted disruption of the IL-2 gene). Therefore, it appears that, in the absence of IL-2, other cytokines (e.g., IL-15) can support the normal T-cell expansion in response to antigenic stimulation. As with many cytokines, IL-15 can be produced by a variety of cell types, both constitutively and in response to inflammation. A genetic deficiency in IL-2 results not in a defective T-cell response to antigen, but rather paradoxically, in exaggerated T-cell proliferation (lymphoproliferative disease) and the development of autoimmune responses caused by the defective generation of regulatory T cells.²³⁷ Recently, IL-2 produced by effector CD4⁺ T cells has been shown to regulate the production by CD8⁺ T effector cells of the regulatory cytokine IL-10 during respiratory virus infection.¹²³

Other cytokines, for example, IFN- γ , IL-4, IL-6, and transforming growth factor β (TGF- β) are, like IL-12, sources of *signal 3* and therefore are important regulators of the adaptive immune system and immune cell function during the adaptive immune response to virus infection. IFN- γ is the product of a single gene with minimal allelic variation in the coding sequence of the gene. It is produced during virus infection primarily by activated CD4⁺ T cell and CD8⁺ T cells (and some NK T cells) in response to TCR engagement and by NK cells after engagement of stimulatory NK cell receptors. The role of IFN- γ in augmenting antigen presentation (i.e., upregulation of MHC class I and II expression, immunoproteasome subunit induction) has been discussed earlier. IFN- γ also plays a critical role in activating mononuclear phagocytes (macrophages) to destroy infected intracellular bacteria, and it presumably plays a similar role in the destruction of virus and, possibly, virus-infected cells when armed effector T cells respond to viral antigen at peripheral sites of virus infection. The early production of IFN- γ (likely produced by NK cells entering the SLO in response to infection) during the initiation of T-cell responses to antigen can drive T-cell differentiation to a type 1 (T_H1) pathway (characteristic of antiviral T-cell responses). The IFN- γ gene is rendered transcriptionally active by exposure of responding primary T cells to IL-12p70, resulting in IFN- γ production by activated effector T cells on subsequent TCR engagement by antigen.¹⁹³ IFN- γ signaling through its receptor is mediated primarily by STAT-1.

Interleukin-4 is produced at high levels by activated CD4⁺ T cells that have differentiated along the type 2 (T_H2) pathway.¹⁹³

IL-4 may also be produced at low levels in primary T cells early in the response to antigen and by NK T cells and NK cells. Exposure of responding CD4⁺ T cells to IL-4 early in the activation/differentiation sequence is essential for the generation of T_H2 effector T cells in the SLO. The early low-level production of IL-4 by activated T cells (or by NK T cells and NK cells recruited to the SLO) before their commitment to T_H1 or T_H2 differentiation may be sufficient to render the IL-4 gene locus capable of high-level IL-4 production by CD4⁺ T_H2 effectors on TCR engagement.²⁸³ The NK T-cell subset, which rapidly produces high levels of IL-4 early in the response to pathogens, has been suggested to be a potential source of IL-4, which can regulate conventional $\alpha\beta$:TCR T-cell differentiation in the SLO in response to antigen. IL-4 produced by activated T cells serves both as a B-cell growth-promoting factor and also triggers immunoglobulin type switching during B-cell activation/differentiation. Engagement of the IL-4 receptor activates STAT-6 to mediate the effect of IL-4 on responding B and T cells. As will be discussed, IL-4 and IFN- γ act antagonistically to regulate T-cell differentiation and T-cell effector generation into T_H1 or T_H2 effectors.

The cytokines IL-6 and TGF- β play a critical role in driving naïve CD4 T-cell differentiation into IL-17-producing effector T cells, and in the case of TGF- β , an essential role in the development of T regulatory cells. IL-6 is a proinflammatory cytokine, the expression of which is upregulated following an inflammatory stimulus such as virus infection, and signaling through the IL-6 receptor is mediated by STAT-3. TGF- β exists on cell surfaces in an inactive form and its expression is regulated at the level of transcription, translation, and posttranslation modifications.²⁴⁵ Signaling through of the TGF- β receptor is signal transducing mothers against decapentaplegic protein (SMAD) dependent. Both cytokines can be produced by hematopoietic and nonhematopoietic origin cells. Production of IL-6 and TGF- β in the SLO at the time of infection will trigger naïve CD4⁺ T-cells to differentiate into T_H17 T_E as discussed below (see text under heading “CD4⁺ T_E Effector Mechanisms”).

EFFECTOR ACTIVITIES OF B CELLS

The antiviral activities of B cells are largely mediated by antibodies,^{32,72,91,139,305,325} although B cells may also have modulating effects on innate immunity. Antibodies are distinguished from T-cell effectors in that they have activity against free virus particles as well as against virally infected cells. They are the first line of defense against viral infection. In a very real sense, the adaptive antiviral activities of antibodies and T cells are complementary and should be considered together in attempting to understand immune responses to viruses.⁸ Nevertheless, it is revealing to consider the antiviral activities of antibodies at different layers of complexity³²: *in vitro*, *in vivo* in the absence of other adaptive immune effectors as revealed by passive antibody transfer studies; and *in vivo* in the presence of T-cell responses as in natural infection or following some vaccination strategies.

ANTIVIRAL ACTIVITIES OF ANTIBODY *IN VITRO*

As stated earlier, antibody can act against both free virus and infected cells. This is presented schematically in Figure 9.4 for an enveloped virus. Probably the most studied antiviral activ-

ity of antibody *in vitro* and the one most important for antibody protection *in vivo* is neutralization of free virus particles. Neutralization has been defined as the loss of infectivity that ensues when antibody molecule(s) bind to a virus particle, and usually occurs without the involvement of any other agency. As such, this is an unusual activity of antibody paralleled only by the inhibition of toxins and enzymes.⁶⁷ The mechanism(s) of neutralization have been much debated over the years. The simplest models are based on steric obstruction of virus attachment or virus entry by the antibody molecule.^{116,145,215,269} The relatively large bulk of the antibody molecule, very roughly similar to that of a typical viral spike for an enveloped virus, is suggested to be critical. Such models predict that the neutralizing efficacy of an antibody should be related primarily to its affinity for antigen on the virion surface, and the precise epitope recognized should be of lesser, but potentially significant, importance. Indeed, clear evidence exists that antibodies can neutralize viruses without binding directly to functional sites on the virion surface.^{22,228} The models described can be very loosely termed occupancy or coating models, where the degree of coating of the virus surface by antibody to achieve neutralization differs between models. One model noted a striking linear relationship between the surface area of a set of viruses and the number of antibody molecules bound to virus at neutralization.²¹² This implied that, for these viruses at least, a coating density corresponding to about half the available antibody sites occupied on the virus produced neutralization. This model is likely to be less applicable to complex viruses expressing multiple surface proteins (e.g., the herpes or pox viruses). A related model of neutralization of flaviviruses proposes that highly accessible epitopes may require lower occupancy than poorly accessible epitopes to reach a threshold for neutralization.²²⁰ Further models taking into account kinetic factors in virus neutralization are being developed.

Alternative neutralization models suggest that (a) viruses are neutralized by the binding of one or only a few antibody molecules to critical sites on the virion surface; (b) conformational changes in envelope or capsid molecules are crucial to neutralization; and (c) viral inactivation by antibody can occur following entry to infected cells, for example, by blocking virus uncoating⁶⁷ or by interaction of the Fc of IgG with TRIM21.^{169a} Considerable debate remains in the area of antibody neutralization mechanism and, furthermore, it may be that different mechanisms are operative for different viruses under differing conditions.

Antibody activity against free virus particles can be augmented by Fc-mediated effector systems in several ways. First, complement activation²⁷¹ by virion-bound antibody and deposition of complement components on the virion surface can enhance neutralization.¹⁸³ Occupancy/coating models argue that this reflects an increased coating of molecules on the virion that hinders productive interaction of virion and target cell. Second, complement activation can lead directly to virolysis by deposition of the terminal components of complement in the viral membrane. Third, Fc and complement receptors can bind antibody or complement-coated virions, leading to phagocytosis followed by inactivation in an intracellular compartment within the phagocyte. This process has been described *in vitro* for the picornavirus foot-and-mouth disease virus (FMDV) and is believed to be important *in vivo* in protection against FMDV.¹⁸¹ It is likely that neutralizing antibodies, which tend

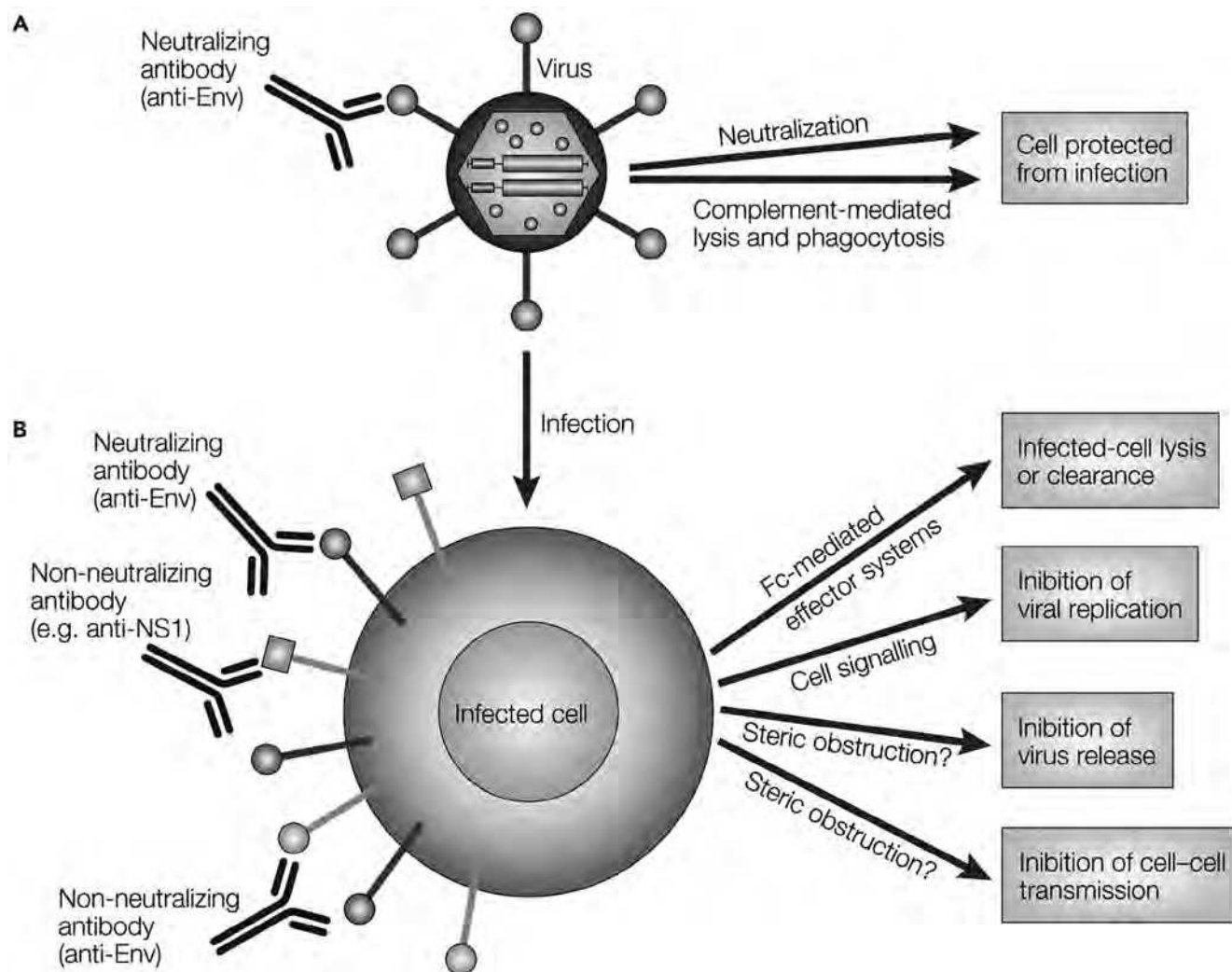


FIGURE 9.4. Antibody activities against free virus particles and virally infected cells. A: Activities against free virus particles. The primary activity is probably neutralization, whereby antibody molecules bind to virion surface proteins and block infection. Virion-bound antibody molecules may also trigger lysis of virions via complement or phagocytosis of virions. An enveloped virus expressing only one surface protein is shown. A virus expressing multiple surface proteins (e.g., a herpes or poxvirus) may not be neutralized by antibody saturation of a single surface protein. Non-functional surface molecules may also be present on infectious virions for some viruses such as HIV.²²⁴ Nonneutralizing antibodies may bind to such molecules and trigger the elimination of infectious virions by effector systems or perhaps trap virus in vivo and prevent contact with target cells. **B:** Activities against virally infected cells. Neutralizing antibodies will bind to the proteins that are also expressed on virions (typically Env proteins). Nonneutralizing antibodies will bind to proteins that are expressed on infected cells but not virions (e.g., NS1 protein of dengue virus) or that are also expressed on virions but whose blockade does not lead to neutralization (e.g., neuraminidase on influenza virions). (Reproduced from Burton DR. Antibodies, viruses and vaccines. *Nat Rev Immunol* 2002;2:706–713, with permission.)

to coat the viral surface, will be most effective in triggering virolysis or phagocytosis. An antigen present at relatively low density on the surface of a virion could, however in principle, bind antibody without mediating neutralization. Such non-neutralizing antibody could nevertheless trigger virolysis or phagocytosis.

Antibodies can act, not only against free virions, but also against infected cells by binding to viral proteins expressed on the surface of virally infected cells (Fig. 9.4). Fc-mediated effector systems can lead to cell lysis or clearance via complement-dependent cytotoxicity (CDC) or antibody-dependent

cellular cytotoxicity (ADCC). In the case of CDC, activation of the classic pathway by antibody that complexes arrayed viral proteins on the infected cell surface can lead to C3b deposition and uptake by effector cells or the deposition of the terminal complement components in the infected cell membrane resulting in lysis.¹⁸ For ADCC, the antibody array on the infected cell surface is recognized by Fc receptors on effector cells, triggering killing of the infected cells. Antibody can also inhibit (a) virus release from infected cells⁹¹ by simply binding to viral proteins at the infected cell surface and (b) cell-to-cell transmission of virus^{30,211} by mechanisms that are thought to be very

similar to those that lead to virus neutralization. Binding of antibody to the infected cell surface, particularly of neurons, has also been suggested to inhibit viral replication inside cells, presumably via signaling mechanisms.^{87,160}

Generally, it appears that antibody is less effective against infected cells than against free virions. For instance, higher concentrations of neutralizing antibody are required to inhibit cell-to-cell transmission than are required to inhibit infection by free virions.^{119,211} Similarly, a higher concentration of antibody has been associated with effective CDC and ADCC than has neutralization.¹¹⁷ It is expected that neutralizing antibodies will be effective against infected cells if they bind to envelope molecule presentations on free infected cells that are also expressed on virions. Antibodies that bind to molecules expressed on infected cells but not virions, for example, the NS1 protein of dengue virus,¹¹⁴ can be effective against infected cells, although they are nonneutralizing.

Polymeric IgA and IgM have been shown to be capable of intracellular neutralization of virus. These antibodies are actively transported over the mucosal epithelium after binding to the polymeric immunoglobulin receptor and may, during transport, contact and neutralize transcytosing viruses.^{25,88,139,178}

Finally, antibody can enhance viral infection *in vitro* under certain circumstances, generally in the presence of subneutralizing concentrations of neutralizing antibodies. Enhancement is mediated by Fc receptors in some cases (e.g., dengue virus),^{16,104,109,191} but not in other cases where enhancement is observed with antibody Fab fragments.²⁷⁷ The occupancy model of neutralization described above explains enhancement of infection as an effect that occurs at low occupancy of virion sites in the presence of permissive cells (e.g., those bearing Fc receptors). As antibody concentrations increase, coating of the virus increases, eventually resulting in neutralization.

ANTIVIRAL ACTIVITIES OF ANTIBODY *IN VIVO*

The classic approach to determine the protective activities of antibody *in vivo* is to passively transfer immune sera or monoclonal antibodies to a naïve animal, challenge with virus, and observe the outcome. This approach has consistently shown—for many different viruses, animal models, and challenge routes—a good correlation between the protection achieved and antibody serum neutralizing activity measured *in vitro*.²¹² It should be noted that this does not necessarily mean that neutralization is the mechanism of protective activity. Neutralizing antibodies are probably those that bind most effectively to free virions and to virus-infected cells (at least for many enveloped viruses) so that, in principle at least, any of the mechanisms of antiviral activity presented in Figure 9.4 could be operative in protection.

Generally speaking, protection in animal models is achieved when neutralizing titers in the serum of the animal at the time of virus challenge are relatively high, often of the order of 1:100 or higher. In other words, the serum of the animal can be diluted 100-fold and 50% to 90% (depending on the study and assay) neutralization achieved *in vitro*. In some instances, protection can be described as sterilizing in that no evidence is seen of viral replication following challenge. Serum neutralizing titers (80%) of 1:380 or greater provide sterilizing immunity in the lungs of cotton rats challenged with respira-

tory syncytial virus (RSV),²²⁵ and titers as high as 1:400 (90%) and 1:38 (~100%) or as low as 1:1 (90%) provide sterilizing immunity against challenge of macaques with chimeric simian-human immunodeficiency virus (SHIV)^{115,198,214} In other instances, such as challenge with lymphocytic choriomeningitis virus (LCMV) in a mouse model³¹² and with Ebola virus in a guinea pig model,²¹³ high titers of neutralizing antibody do not provide sterilizing immunity but do prevent disease. In the latter case, similar titers prevent disease due to Ebola virus in guinea pigs but have no effect on disease course in macaques.²⁰⁵ However, more recently, a cocktail of neutralizing antibodies has been shown to protect macaques against disease due to Ebola virus infection.^{225a} In most instances, however, it has not been established whether sterilizing immunity has been achieved.

A number of possible explanations exist for the neutralizing titers required for protection in many animal studies. If the assumption is that neutralization is the dominant protective mechanism, then for some viruses/animal models/challenge routes it may be necessary for protection for antibody to mop up essentially every last virus particle requiring an excess of antibody. With the same assumption, the protective activity may be achieved at a tissue site with a lower antibody concentration than that of serum, again leading to an apparent overcapacity. Alternatively, protection may require an additional activity of neutralizing antibody distinct from neutralization. For instance, it may be that activity against infected cells, as well as (or in some cases even instead of) activity against free virions, is needed to provide protection. This would be consistent with observations that antibody concentrations required for activities against infected cells (e.g., blocking cell-to-cell transmission) are typically higher than required for neutralization of free virus particles. A further alternative explanation is that the neutralization *in vitro* is not reflective of the process *in vivo*, for example the target cell type used *in vitro* may have important differences from the target cell *in vivo*.

Animal model studies do provide evidence that mechanisms other than neutralization can be important in protection by neutralizing antibodies. In some cases, protection is found to be as effective with F(ab')₂ fragments, which lack the Fc domain and, therefore, are unable to trigger effector functions, as the corresponding whole IgG molecule. In other cases, however, F(ab')₂ fragments that are equally active as whole IgG molecules at neutralization *in vitro* are ineffective at protection. For yellow fever virus,²⁴³ it has been shown that neutralizing mouse IgG1 antibodies (poor activators of effector functions) are ineffective at protection, whereas IgG2a molecules (good activators) of the same specificity are effective. Similarly for vaccinia virus,¹⁸ it has been shown that human IgG1 but not IgG4 isotypes of human mAbs are effective. In many of the examples in mouse models in which the Fc part of IgG is important, protection is independent of complement.²¹² This implies that protection by neutralizing antibodies in these cases may require activity against infected cells and involves ADCC or phagocytosis. Many examples of protective activity due to passively transferred nonneutralizing antibodies have been described. The activity appears to be directed at infected cells (Fig. 9.4) and generally appears to be somewhat less potent than that due to neutralizing antibodies. For instance, a number of cases have been reported where neutralizing antibodies are protective against higher challenge doses or more pathogenic viruses

than nonneutralizing antibodies. In many cases, protection by nonneutralizing antibodies is shown to depend critically on the Fc part of the antibody molecule and to occur in complement-deficient mice, suggesting that ADCC (or phagocytosis) may be crucial in clearing antibody-complexed infected cells. Protection with nonneutralizing antibodies is mostly restricted to those antibodies directed against enveloped viruses.

Passive transfer of antibodies to humans has been shown to provide protection against disease caused by a number of viruses, including hepatitis B, hepatitis A, measles, polio, and RSV.³⁹ Indeed, a humanized, neutralizing anti-RSV monoclonal antibody is in clinical use to protect at-risk infants.¹⁰¹ In some or many of these cases, it is unlikely that the titers of passively transferred neutralizing antibody achieve the levels necessary to offer sterilizing immunity, although this has not been widely studied. Rather, it would seem that neutralizing antibody sufficiently blunts the infection to allow the development of other protective mechanisms, presumably a CD8⁺ T-cell response, active antibody responses, and innate immunity (see below). The protection of young infants by maternal neutralizing antibodies likely falls into this category. Maternal antibodies may “attenuate infection during the initial months of life, thereby creating optimal conditions for the natural immunization of the child as a result of infection”.³²³ In some instances (e.g., rabies virus infection), passive antibody has been shown to protect against disease after exposure, when some measure of infection is clearly established. Once infection is clearly established, however, reports of beneficial effects of passive antibody are limited.

Many important human viral pathogens gain entry to the host via mucosal surfaces. Passive transfer studies show that antibodies present in the mucosal compartments at the time of exposure can protect against viral challenge.^{139,202} Both mucosal secretory IgA (sIgA) and systemic IgG have been shown to be effective. Dramatically, classically nonneutralizing IgA can protect against rotavirus challenge in mice by an intracellular “neutralization” mechanism.³¹ A recent study elegantly shows how different antibody specificities can interfere with HPV infection *in vivo* in a cervicovaginal mouse model at two stages: first to prevent virion binding to the basement membrane and second to prevent virion association with the epithelial cell surface.⁶²

Evidence for the importance of antibody enhancement of viral infection *in vivo* is limited.²⁷⁷ Early observations on dengue virus infection in humans¹⁴⁶ are the most convincing, and the case for antibody enhancement leading to dengue-associated disease has been strengthened by recent observations in animal models.^{13,321}

In terms of human vaccination, it is often stated that neutralizing antibodies are the best correlate of protection.^{7,222} This is not to argue that neutralizing antibodies are the sole or even necessarily the most important mechanism of protection, although the circumstantial evidence in many cases is quite strong. For smallpox, neutralizing titers of 1:20 to 1:32 indicate protective immunity.¹⁰⁵ For measles, low titers of neutralizing antibody (<120 mIU/ml) are strongly associated with clinical disease, intermediate titers (~200 to 900) are associated with a substantial proportion of subclinical infections reflected in boosting of antibody titers following exposure and high titers (>~1,000) with no indication of subclinical infection and possible sterilizing immunity.

EFFECTOR ACTIVITIES OF T CELLS

The categorization of $\alpha:\beta$ TCR T cells into CD8⁺ and CD4⁺ subsets not only demarcates the MHC molecules that restrict recognition (i.e., class I or class II), but also has functional significance at the effector level (Table 9.5). CD8⁺ T-cell effector cells (CD8⁺ T_E) are capable of destroying antigen-displaying cells (e.g., virus-infected cells expressing the appropriate peptide/MHC class I complex) by direct cell-to-cell contact. CD8⁺ T_E also produce/secrete several cytokines with antiviral activity (e.g., IFN- γ , TNF- α) on TCR engagement. CD4⁺ T-cell effectors (CD4⁺ T_E) primarily function by release of cytokines (notably, IFN- γ , and/or IL-4, IL-5) in response to antigenic stimulation. CD4⁺ T_E, however, in rare instances, can also acquire killing capacity by direct contact with cells displaying the appropriate peptide/MHC class II ligand recognized by the TCR/CD4 complex. The differentiation of the naïve T cells into T_E, in particular naïve CD4⁺ T-cell differentiation into T_H1, T_H2, or T_H17 effector cells, is regulated by a variety of factors (as discussed below).

TABLE 9.5 Effector Activation of Activated CD8⁺ and CD4⁺ T Cells

Effector type	Target cell	Effector mechanism
CD8 ⁺ T cell	MHC class I ⁺	–Direct cytotoxicity (cell–cell contact) –Granule exocytosis (perforin/granzyme) –FasL (T cell) – Fas (infected target cell) interaction Cytokine synthesis –IFN- γ , TNF- α , chemokines
CD4 ⁺ T-cell – T _H 1	MHC class II ⁺	Cytokine synthesis –IFN- γ , TNF- α , chemokines
CD4 ⁺ T-cell – T _H 2	MHC class II ⁺	Cytokine synthesis –IL-4, IL-5, IL-13
CD4 ⁺ T-cell – T _H 17	MHC class II ⁺	Cytokine synthesis –IL-17A/F, IL-6, +/-IL-21

MHC, major histocompatibility complex; IFN, interferon; TNF, tumor necrosis factor.

CD8⁺ T_E EFFECTOR MECHANISMS

The CD8⁺ T_E destroy *target* cells (e.g., virus-infected cells) following direct cell-to-cell contact, by two distinct mechanisms. One mechanism is by granule exocytosis.¹⁶¹ Mature (fully differentiated) CD8⁺ T_E contain lytic granules within their cytoplasm, and TCR engagement on the T_E by peptide/MHC class I complexes on the target cell results in the formation of intermolecular signaling clusters (consisting of TCR α : β , CD3, and integrins, e.g., LFA-1). In response to TCR engagement, and signal transduction, the cytoplasmic granules are directed by the cellular microtubule-organizing center along actin filaments to the site of TCR aggregation where the granules are then released onto the surface of the target cell. The adhesive interaction between the T_E and the target cell is stabilized/strengthened through the interaction of LFA-1 (on T_E) with its ligand, ICAM-1 (on the target cell). The lytic granules contain a protein, perforin, which has a sequence homology to the complement component C9. The granules also contain a set of serine proteases, known as *granzymes* (for granule enzymes), which are present in a proenzyme form within the T_E granule as part of a multimeric complex with perforin imbedded within a scaffold consisting of proteoglycan, serglycin, which are activated (by granule-associated cathepsin C) on granule exocytosis. Because of its homology to C9, the perforin monomer released onto the target cell was believed to intercalate and polymerize (in the presence of extracellular Ca²⁺), forming a pore within the target cell plasma membrane that allows the granzymes access to the target cell cytoplasm. More recent evidence suggests that released granzymes bind directly to target cells, possibly through cell surface receptors, and either gain direct access to the cell cytoplasm or are first internalized into endosomes.²⁸ Perforin may act by facilitating the transfer of granzymes from the cell surface or endosome into the cytoplasm. The granzyme family of serine proteases consists of at least five members in the human (granzyme A, B, C).²³⁵ They are believed to function by activating cell death pathways in the target cells, including both direct activation of the caspase-dependent cell death mechanism, and the caspase-independent mitochondrial death pathway associated with granzyme-dependent activation of proapoptotic Bcl-2 family members (e.g., BID, BAX).¹⁰ Although the mechanism of action and function of each granzyme is not completely understood at present, at least one granzyme, granzyme B, is present in both CD8⁺ T_E and NK cell lytic granules, and acts by proteolytic cleavage and activation of procaspases (procaspase 3 caspase 3) and proapoptotic Bcl2 family members (e.g., BID \rightarrow BID*) resulting in the indicative DNA fragmentation and mitochondrial damage characteristic of apoptosis. An additional granule-associated lytic molecule, granulysin (present in human T_E), can form pores in membranes and may play a role in the destruction of certain bacteria by direct lysis.¹⁵¹

The CD8⁺ T_E can also destroy target cells by the upregulation (in response to TCR engagement) of the TNF family protein, CD154 (FasL, CD95L) on the T_E, and engagement of its receptor, the TNF receptor family member, CD95 (Fas), on the infected target cell.⁶⁵ CD95 engagement results in the recruitment/activation of caspases and apoptotic death of target cells. Studies in experimental models of virus infection suggest that both the Fas/FasL and the granule exocytosis mechanisms can contribute to the elimination of virus-infected cells, and virus

clearance by CD8⁺ T_E.^{28,286} The relative contribution of each lytic mechanism to virus clearance differs for different viruses.

The CD8⁺ T_E also produce a number of cytokines in response to TCR engagement by antigen, most notably IFN- γ and TNF- α . The production/secretion of these effector cytokines appears to be tightly regulated and typically requires TCR-dependent signaling in the CD8⁺ T_E.²⁶⁸ IFN- γ exhibits its antiviral effects through upregulation of MHC molecule expression and stimulation of the MHC class I processing/presentation pathway in infected cells. Because IFN- γ is a potent activator of tissue macrophages, activated macrophages would also have the capacity to take up and destroy virus/virus-infected cells. TNF- α , as can the other member of the TNF ligand family, CD154 (FasL), induces apoptosis of infected cells expressing the receptor for TNF α , and in addition can signal the production of proinflammatory/anti-viral cytokines through receptor-dependent NF- κ B activation. In addition, recent evidence has emerged from the analysis of experimental models of acute virus infection that CD8⁺ T_E responding to infection can serve as a major source of the regulatory cytokine IL-10.²⁷⁸ This T-cell derived IL-10 may act to attenuate excess inflammation associated with T cell-dependent virus clearance mechanisms.²⁷⁸

CD4⁺ T_E EFFECTOR MECHANISMS

An essential role of activated CD4⁺ T cells in antiviral immunity is to provide *help* for responding B cells in the SLOs in the production of anti-viral antibody. Emerging evidence suggests that CD4⁺ T cells (presumable CD4⁺ T_E effectors) also play a role, not yet completely defined, in the development and maintenance of the memory CD8⁺ T-cell response during virus infection.^{264,304} CD4⁺ T_E express effector activity through the release of cytokines on TCR engagement (i.e., recognition of specific peptide/MHC class II complexes on target cells). CD4⁺ T_E had originally been categorized as T_H1 (type 1 or T helper 1) or T_H2 (type 2 or T helper 2) T_E, based on the constellation of cytokines produced. More recently a third major CD4⁺ effector T-cell subset or lineage has been identified, the T_H17 CD4⁺ T_E. Among these CD4⁺ T-cell subsets, the T_H1 CD4⁺ T_E, which produce IFN- γ and to a lesser extent TNF, serve the most prominent role as the critical CD4⁺ T_E in virus infection. The hallmark of T_H1-induced inflammation is accumulation of mononuclear cells (inflammatory macrophages, monocytes, DCs, lymphocytes, etc.) at sites of infection. The IL-4, IL-5, IL-13 producing T_H2 CD4⁺ T cells are recognized as a major T_E responding to helminth (worm) and certain parasitic infections, and they play a pivotal role in orchestrating the allergic responses to nonpathogenic environmental antigens. The response of T_H2 T_E is characterized by the development of IgE antibody to antigens and an inflammatory response rich in eosinophils.³⁰⁷

As their name implies, the T_H17 T_E produce IL-17A/F (as well as IL-6 and in some instances IL-21). T_H17 T_E release these cytokines at sites of infection following TCR engagement by antigen. Because of the ability of these cytokines, in particular IL-17, to activate epithelial cells to produce chemokines that are chemotactic for neutrophils, T_H17 T_E are potent inducers of neutrophil recruitment to sites of infection. In keeping with the neutrophil-rich inflammatory response induced by these

T_E , they have been implicated as important adaptive immune effectors in the host response to extracellular bacteria and fungi, as well as in the pathogenesis of certain autoimmune diseases previously believed to be orchestrated by T_H1 T_E . The contribution of T_H17 T_E to the adaptive immune response to the virus infection has yet to be firmly established—although, not surprisingly, T_H17 responses have been reported to be dysregulated in HIV-infected patients,¹⁹² and in one model of experimental virus infection in the CNS, the development of a T_H17 T_E response has been reported to suppress the expression of $CD8^+$ T-cell effector activity.⁹⁸

The strict classification of $CD4^+$ T_E responses into T_H1 , T_H2 , or T_H17 categories is somewhat artificial and based largely on *in vitro* studies where differentiation of naïve $CD4^+$ T cells into one of these $CD4^+$ effector cell subsets can be skewed to produce a single T_E subset exclusively. During the course of antigen exposure/infection *in vivo*, $CD4^+$ T_E capable of producing both the T_H1 and T_H2 cytokines (or T_H1 and T_H17 cytokines) are generated; and it is the relative proportion of the T_H1 (IFN- γ , TNF- α) or T_H2 (IL-4, IL-5) or T_H17 (IL-17, IL-6) T_E subsets in the T cells responding to infection that dictates the overall balance of the $CD4^+$ T_E response. As noted, it is the milieu in which $CD4^+$ T cells activate or differentiate in response to antigen in the SLOs that dictates the balance between T_H1 , T_H2 , or T_H17 $CD4^+$ T_E generation.⁵⁰ Numerous factors control the $CD4^+$ T-cell differentiation program, and the relative proportion of T_H1 or T_H2 T_E generated. Among these factors are (a) the form and dose of antigen/pathogen; (b) the APC response (in particular, DC) that express cell surface molecules after antigen encounter that can influence $CD4^+$ T-cell differentiation; and (c) cytokine production by APCs (e.g., IL-12p70) and by innate immune cells present in the SLO (e.g., IFN- γ from NK cells, IL-4 from NK T cells, and IL-6 and TGF- β from mononuclear phagocytes and stromal cells). Detailed discussion of the contribution of these $T_H1/T_H2/T_H17$ differentiation influences is beyond the scope of this chapter but has been reviewed by others.^{50,270} Whereas virus infection, in general, drives $CD4^+$ T-cell differentiation toward the development of T_H1 $CD4^+$ T_E responses, evidence indicates that, in certain chronic human infections, T_H2 $CD4^+$ T_E responses may predominate.^{34,270} Because the induction of the T_H2 $CD4^+$ T_E response with IL-4 (and IL-10) production can suppress the development of a T_H1 $CD4^+$ T_E response characteristic of the adaptive immune response to virus infection, this form of virus-induced immune deviation could potentially result in the inhibition of virus clearance, leading to the development of persistent infection. Although virus infection typically results in the development of $CD8^+$ T_E , which are *T_H1 -like* (i.e., producing IFN- γ , TNF- α), differentiating $CD8^+$ T cells may also be deviated toward the T_H2 effector response (i.e., weakly cytolytic T_E producing IL-4, IL-5), potentially resulting in decreased virus clearance efficiency by these antiviral T_E .³¹⁰

Whether two other recognized subsets of $CD4^+$ T lymphocytes, the recently appreciated T follicular helper (T_{FH}) T cells and T_{reg} cells can be classified as T_E is up for debate and may simply be a matter of semantics, that is, how one defines an “effector” cell. The factors controlling the differentiation of naïve $CD4^+$ T cells into these various activated T-cell subsets as well as their cytokine profile and role in adaptive immune responses are summarized in Table 9.5. The properties of T_{reg} cells and their role in virus infection are discussed in the next section.

REGULATORY T-CELL FUNCTION IN ANTI-VIRAL ADAPTIVE IMMUNE RESPONSES

T_{reg} cells are a subset of $CD4^+$ T cells that express the FoxP3 lineage commitment transcription factor and are crucial for maintaining self-tolerance in the periphery.^{81,236} Despite the fact that CD25 and FoxP3 expression are reliable markers for T_{reg} cells in mice, both FoxP3 and CD25 are also expressed by activated T cells in humans. Therefore, FoxP3 expression alone does not serve a reliable T_{reg} marker. Recently additional markers, such as CTLA-4 and lymphocyte activation gene-3 associated protein (LAP), have been identified as human T_{reg} markers: CTLA-4 is known to be functionally active for T_{reg} cells⁸⁴ and LAP (a TGF- β binding protein) surface expression is enhanced on functional human T_{reg} .²⁸⁷ In addition, although lower expression of CD127 has been correlated with FoxP3 expression and suppression in humans,¹⁶⁴ activation of T_{reg} is recently shown to result in an increase in CD127 expression.²⁶² Nevertheless, a combination of these markers can allow for improved identification of T_{reg} in humans.

The various regulatory T cells are categorized as (a) naturally occurring, thymus-derived $CD4^+CD25^+$ T cells (named as $CD4^+CD25^+T_{reg}$)^{122,207}; or (b) induced, peripheral T cells, which include $CD4^+CD25^-$ T cells, T_H3 cells, and $CD8^+$ T cells.⁵⁵ T_{reg} subsets are defined by differences in their cell surface marker expression, their cytokine secretion patterns, and their mode of suppression. There is evidence of the generation of FoxP3⁺ suppressive cells from FoxP3⁻ nonsuppressive cells both *in vivo* and *in vitro*. These cells are referred to as inducible T_{reg} , adaptive T_{reg} , or iT_{reg} . The factors involved in *in vivo* conversion are not precisely defined. However, TCR activation in the presence of TGF- β has successfully induced T_{reg} development *in vitro*. Two other types of peripherally induced regulatory cells are named Tr1 and Th3. Tr1 causes immunosuppression by secretion of large amounts of IL-10; whereas Tr1 cells develop in the presence of IL-10, IL-15 promotes the survival of these cells. Tr1 cells have regulatory function, but do not express FoxP3. Th3 cells are developed in the presence of TGF- β and also produce a large quantity of this cytokine. Importantly, T_{reg} activity can be modulated by exposure to costimulatory molecules. For instance, activation of OX40 and 4-1BB on $CD4^+CD25^+$ T_{reg} results in increased proliferation; and stimulation of 4-1BB has also been implicated in the maintenance of suppressive function.

T_{reg} cells play a critical role in the regulation of immune responses to viral infection as well as the prevention of tissue damage caused by viral infection. Particularly, T_{reg} cells are involved in suppressing effector T cells in chronic viral infections such as HSV, Epstein-Barr virus (EBV), and HCV, having the appearance of $CD4^+$ regulatory T cells in chronic viral infection. In case of HCV infection, the frequency of $CD4^+CD25^+$ regulatory T cells in chronic patients is greater than those in recovered or healthy patients,^{24,33,276} suggesting that natural T_{reg} s are associated with the establishment of HCV persistent infection. Apparently, the suppressor function of $CD4^+CD25^+$ T cells derived from chronic HCV patients does not rely on soluble mediator(s) (i.e., cytokines) because neutralizing antibodies to either IL-10 or TGF- β do not reverse suppression.²⁴ In addition, the frequency of $CD4^+CD25^{hi}$ T cells in patients with hepatocellular carcinoma is greater than in individuals who are chronically infected with HCV.²⁰⁴

Besides the role of regulatory T cells in suppression of host immune responses to viral infection, T_{reg} cells are paradoxically

essential to control tissue damage caused by host immune responses to viral infection. Direct evidence of a role for regulatory T cells in preventing immunopathology has come from studies using murine models of viral infection. Theiler's murine encephalomyelitis virus (TMEV) infection induces CD4⁺ T cell-mediated demyelinating disease¹¹⁰ such that TMEV-specific CD4⁺ T cells transferred into susceptible, irradiated C/cByJ mice accelerate clinical disease and enhance TMEV-specific delayed-type hypersensitivity (DTH). Importantly, CD8⁺ T cells from infected C/cByJ mice suppress the *in vivo* disease progression as well as virus-specific T-cell responsiveness in recipients of TMEV-specific CD4⁺ T-cell blasts. So, the transfer of virus-specific CD8⁺ regulatory T cells at the time of Theiler's virus infection reduced tissue damage by preventing the pathogenic role of CD4⁺ T cells. In case of HSV infection, depletion of CD4⁺CD25⁺ T cells before viral infection increases the generation of virus-specific CD8⁺ T-cell responses and viral clearance.^{280,281} In contrast, the severity of T cell-mediated lesions in the cornea of HSV-infected mice was increased upon removal of CD4⁺CD25⁺ T cells. Therefore, CD4⁺CD25⁺ T_{reg} cells are likely involved in reducing the severity of immune-mediated inflammatory lesions by preventing the pathogenic effects of CD4⁺ T_{H1} cells as well as limiting the migration of these cells to inflammatory sites. During chronic viral infections, T_{reg} cells might be beneficial to the host by maintaining a balance between efficient effectors and memory responses, but with a low level of inflammation to limit host tissue damage.

Numerous mechanisms are utilized by eliciting suppressive function of T_{reg} cells. One way is through the production of the suppressive cytokines such as TGF- β and IL-10. Although most studies indicate that the suppressive effect of T_{reg} cells is not mediated by soluble factors,²⁵⁷ that does not exclude the possibility of contribution of cytokines acting in proximity or cell-bound cytokines. In fact, TGF- β blockade appears to reduce the suppressive function of T_{reg} cells following the administration of high amounts of TGF- β antibodies,¹⁹⁴ and T_{reg} production of IL-10 is also necessary for protection in a murine colitis model.²⁷⁹ Another way for suppressive function of T_{reg} is mediated by cell surface molecule such as CTLA-4 to negatively regulate T-cell activity and proliferation. The importance of CTLA-4 to T_{reg} suppressive function is evidenced by the development of fatal autoimmune disease as a result of reduced suppressor function in CTLA-4-deficient mice³⁰⁹ as well as treatment of CTLA-4 blocking antibodies for the action of human T_{reg}.³²² Finally, the induction of effector cell apoptosis as a result of IL-2 consumption is proposed for suppressive function of T_{reg}. T_{reg} cells such as effector T cells require IL-2 to survive and highly express the high-affinity IL-2 receptor containing CD25. However, T_{reg} cells produce no IL-2 and therefore are reliant on other sources of IL-2 for survival. In one study, IL-2 consumption by T_{reg} cells resulted in increased Bim-dependent T-cell apoptosis,²⁰⁹ whereas other modes of T_{reg}-mediated apoptosis include perforin production by natural T_{reg} cells¹⁰⁰ and FasL expression by inducible T_{reg} cells.³⁰²

Furthermore, recent studies indicate that T_{reg} cells exert a suppressive effect through modulation of APCs rather than directly modulating effector T cells; a CTLA-4-dependent reduction in CD80/CD86 expression on DCs by T_{reg} cells,^{309,189} but other studies show that T_{reg} cells exert no change in the expression of costimulatory molecules on APCs and addition of functional APCs did not overcome T_{reg} suppression.²⁸⁵

Direct T-cell to T-cell suppression was demonstrated using peptide-MHC tetramer stimulation in the absence of APCs.²¹⁷ Further research may clarify possible contributions of APCs to act as target for T_{reg} suppression; however, it is evident that T_{reg} cells can suppress T cells directly.

ADAPTIVE IMMUNE MEMORY

Two features that define the adaptive immune system and distinguish it from the innate immune system are (a) the exquisite specificity for antigen and (b) the establishment of immunologic memory. Immunologic memory is perhaps the most important consequence of the induction of an adaptive immune response. As a result of clonal expansion of antigen-specific B or T lymphocytes, and a lower activation threshold for memory cells, the immune system can respond more rapidly and efficiently to previously encountered pathogens. This capacity of B and T cells to adapt to a previously encountered antigen is the basis for vaccination. Memory B and T cells generated after infection or vaccination are generally long-lived. In humans, memory T-cell responses (or circulating antibody titer) can be detected decades after pathogen exposure, under conditions where subsequent reexposure to (subclinical infection with) the organism or persistent infection is unlikely. Memory B and T cells directed to an antigen are usually present at a frequency 100 to 1,000 times higher than the corresponding naïve immune cell precursors of memory T and B cells. The factors controlling the formation/duration of the memory immune responses are not yet well understood, but several cytokines may play an important role in maintaining the viability and basal (homeostatic) proliferation of memory cells.

B-CELL MEMORY

The phrase "B cell memory" is often used as shorthand for the mechanisms that lead to long-term humoral immunity. The following two principal mechanisms exist: (a) specific memory B cells expressing BCR to viral antigens, so that viral infection leads to activation of specific B cells and ultimately the production of high-affinity, class-switched antibody to virus; and (b) preexisting serum and tissue high-affinity, class-switched antibody to virus, probably from long-lived plasma cells in the bone marrow. We consider each in turn.

MEMORY B CELLS

Memory B cells express class-switched, somatically mutated surface antibody and can be detected as antigen-specific cells using labeled antigen and a technique such as flow cytometry.¹⁸² They are in a resting state and do not secrete antibodies unless stimulated by antigen. When this stimulation is carried out *in vitro*, memory B cells can be readily detected by enzyme-linked immunospot (ELISPOT) assays. In mice, multiple subtypes of memory B cells have been defined by a number of cell surface markers and gene activation markers.¹⁸² After clearance of virus on first exposure, memory B cells accumulate in the spleen and other lymphoid tissues. Reexposure to virus leads to rapid secondary antibody responses to viral antigens. This response arises because of rapid proliferation and differentiation

of memory B cells in a CD4⁺ T-cell–dependent manner. The specific B-cell population of spleen and lymph nodes expands massively, and differentiation to plasma cells generates a burst in specific antibody production and in antibody levels. Therefore, repeated exposure to antigen is one way in which memory B cell levels can be maintained.

Although controversies have existed over the years, it appears that memory B cells can be long-lived, even in the absence of antigen. An elegant experiment by Maruyama et al.¹⁷³ is persuasive. Mice were engineered so the memory B cells expressing a BCR against antigen 1 could be switched *in vivo* to express a BCR specific for antigen 2. B cells expressing BCR to antigen 2 were identified as persisting in the mice as well as B cells expressing BCR to antigen 1, although the mice had never encountered antigen 2. It should also be noted that BCR expression is required for B-cell survival,¹⁵³ so the possibility exists that nonspecific BCR stimulation, perhaps via a low affinity antigen interaction, is needed for persistence of B-cell memory. Alternatively, the BCR may receive a signal from another molecule such as a cytokine or a molecule such as a B-cell activating factor (BAFF).

One interesting phenomenon related to memory B cells is that of original antigenic sin, (OAS), which was first proposed to describe a phenomenon whereby an individual, originally infected with a virus and then later with variant of this virus, makes an antibody response during the second viral infection that reacts more strongly to the original virus than the newer variant.⁶⁰ It is suggested that boosting of memory B cells against the first virus may interfere with the activation of naïve B cells to the second virus by an undefined mechanism, resulting in a condition that specific antibody responses against the second virus will not be as great compared with a naïve host that has never encountered the first virus. OAS has been reported in both humans^{76,102} and animals,⁷⁷ although a recent study found no evidence of OAS in healthy adults receiving influenza vaccination.³¹¹

LONG-LIVED PLASMA CELLS

Plasma cells are terminally differentiated, nondividing cells able to secrete relatively large amounts of antibody. Two populations of plasma cells exist. Short-lived plasma cells survive for only a few days, produce antibody in extrafollicular foci, and are probably crucial in the very early response to pathogen.¹⁶⁸ In mice, a second population of plasma cells, secreting high-affinity antibody, is long-lived. Irradiation experiments show that the half-life of plasma cells secreting antibody to LCMV was 94 days in the bone marrow and 172 days in the spleen.²⁶⁶ Pulse chase experiments show that plasma cells secreting specific antibody to ovalbumin survived without cell division for a minimum of 3 months.¹⁷¹ These half-lives constitute a sizable proportion of the total lifespan of the mouse. It may be that plasma cells live for many years in humans and contribute to the maintenance of antibody levels over decades.⁹⁷ The bone marrow niche appears to provide survival signals to plasma cells, because *in vitro* such cells survive for only a few days. Another hypothesis is that the pool of plasma cells is being continually replenished from specific memory B cells that are undergoing antigen-independent or bystander activation.¹⁹ These two hypotheses are not mutually exclusive, and it is possible that multiple mechanisms are involved in maintaining serum antibody levels.

LONG-TERM HUMORAL IMMUNITY

Humoral responses can be measured in the sera of humans many years after the last known contact with the pathogen (Table 9.6). Similarly, many vaccines induce serum antibody responses that are present decades later.²²³ A key question has been: Are these responses maintained by intrinsic mechanisms or is there a periodic boosting of immunity through contact with antigen? This question can be difficult to answer given the

TABLE 9.6 Humoral Response to Acute Viral Infection in Humans

Example	Virus family	Persistence of antibody
Systemic infections		
Chikungunya	<i>Alphaviridae</i>	30 yr
Rift Valley fever	<i>Bunyaviridae</i>	12 yr
Dengue	<i>Flaviviridae</i>	32 yr
Yellow fever	<i>Flaviviridae</i>	75 yr
Measles	<i>Paramyxoviridae</i>	65 yr
Mumps	<i>Paramyxoviridae</i>	12 yr
Polio	<i>Picornaviridae</i>	40 yr
Hepatitis A	<i>Picornaviridae</i>	25 yr
Smallpox	<i>Poxviridae</i>	40 yr
Vaccinia	<i>Poxviridae</i>	75 yr
Rubella	<i>Togaviridae</i>	14 yr
Mucosal infections		
Coronavirus	<i>Coronaviridae</i>	12 mo
Influenza	<i>Orthomyxoviridae</i>	30 mo
RSV	<i>Paramyxoviridae</i>	3 mo
Rotavirus	<i>Reoviridae</i>	12 mo

RSV, respiratory syncytial virus; yr, year; mo, month.

Modified from Slifka MK, Ahmed R. Long-term humoral immunity against viruses: revisiting the issue of plasma cell longevity. Trends Microbiol 1996;4:394–400.

unknowns associated with typical human contacts with pathogens. For at least two vaccines, serum antibody levels appear to be maintained in the absence of antigen boosting.⁵³

A large cross-sectional study of poliovirus immunity in Sweden, a country from which the virus has been eradicated, and which used inactivated poliovirus only as a vaccine, showed substantial anti-poliovirus antibody titers in all age groups.²⁶ Virtually no differences in titers were found in the different age groups, indicating the maintenance of antibody titers over decades in the absence of further vaccination or exposure to live virus. Notably, in the same study, declining antibody titers against tetanus and diphtheria were observed.

Two studies have looked at smallpox vaccination using vaccinia virus.^{54,105} Vaccinia is an excellent model for investigating immune memory, because the virus is typically cleared from the site of infection within a month, does not persist, and does not spread systemically in healthy individuals. Immunization with vaccinia ceased in 1972 and smallpox has been eradicated. Specific memory B cells could be detected more than 60 years after vaccination.^{53,54} Furthermore, memory B cells seemed to show a bimodal kinetics. A drop from peak responses occurred at vaccination to an approximately 10-year time point, but stable levels were seen between 10 and 60 years. Serum antibody responses could be identified at 60 to 75 years after vaccination.^{54,75,105} Because overall antivaccinia antibody levels correlated well with neutralizing antivaccinia antibody levels, it was postulated that different specificities may be equally well preserved. Furthermore, the results suggest, as originally posited by Jenner more than 200 years ago, that protective immunity against lethal smallpox infection may be lifelong.

Finally, it is apparent from Table 9.6, that mucosal antibody responses are much shorter-lived than are serum responses. This may indicate that plasma cells initially produced at mucosal sites migrate to the bone marrow and contribute less to mucosal antibody production.

T-CELL MEMORY

Unlike the immunoglobulin receptors on B cells, the genes encoding the TCR on CD4⁺ T cell and CD8⁺ T cells do not undergo any additional somatic mutations, and memory T cells do not, therefore, exhibit affinity maturation after antigen encounter. Memory T cells distinguished functionally from their naïve precursors by being present at increased frequency (a result of clonal expansion of naïve antigen-specific T cells), and by their lower activation threshold (i.e., decreased requirement for CD28 co-stimulation) to trigger cell proliferation and differentiation.²⁴⁷ Certain memory T cells can also rapidly express effector activity (e.g., proinflammatory/antiviral cytokines, such as IFN- γ and TNF- α) within hours after TCR engagement on these T cells, and without DNA synthesis (additional cell proliferation). Naïve T-cell activation and proliferation and memory T-cell population formation and maintenance are controlled by three cytokines: IL-2, IL-7, and IL-15.²⁴⁴ The receptors on T cells for these three cytokines have a common feature—these heterodimeric and trimeric receptors have a common signaling subunit, the so-called γ chain (γ_c), which participates in both ligand (cytokine) binding and Janus Kinase (JAK)/STAT-dependent signal transduction.¹⁴⁹ IL-2/IL-15 can drive proliferation of activated naïve T cells after

TCR engagement (IL-4, which engages another member of the γ_c family of cytokine receptors, will also support proliferation of differentiating T_H2 CD4⁺ T cells). Current evidence suggests that once memory T cells are formed, they can undergo basal/homeostatic low-level proliferation, which is IL-15-dependent, and long-term memory T-cell viability (i.e., suppression of apoptosis) is supported by IL-7, which upregulates/sustains the expression of anti-apoptotic Bcl-2 gene family members in the developing memory T cells.

Although activated naïve T cells give rise to both effector (T_E) and memory (T_M) T cells, the relationship of the two naïve T-cell products is unclear (i.e., we do not know if T_E give rise to T_M in a linear progression, or if T_E and T_M are separate cellular subsets).²⁴⁷ T_M can be distinguished from naïve T cells and, to a lesser extent, from T_E by the expression of certain cell surface molecules (e.g., isoforms of CD45) differentially expressed on T_M and naïve T cells. Several lines of recent evidence indicate that a further subdivision may exist of CD4⁺ T cell and CD8⁺ T cell T_M into central memory (T_{CM}) and peripheral effector memory (T_{EM}) populations.¹⁵⁵ T_{CM} express cell surface molecules such as CD62L and the chemokine receptor CCR7, which would facilitate the circulation of T_{CM} from the blood into the SLOs (particularly the lymph nodes). So, T_{CM} would mimic the circulation pattern of naïve T cells (from the blood into secondary lymphoid organs (SLO) and then back into the bloodstream), and would respond to antigen delivered to the SLO; and, as with naïve T cells, T_{CM} may require both activation and cell division to express effector activity. By contrast, T_{EM} do not display the homing receptors for the SLOs, and have been suggested to reside primarily in the blood and peripheral tissues, where they can rapidly respond to a pathogen at the initial site of pathogen entry in the periphery. Unlike T_{CM}, the T_{EM} appear to undergo limited proliferation in response to antigen, but rapidly express antimicrobial effector activity in response to infection. Although this classification of memory T cells (T_{CM}/T_{EM}) based on cell surface markers is useful as an experimental framework, evidence from the analysis of the immune response to virus infection in humans suggests that considerable heterogeneity exists in cell surface marker expression on circulating T cells in the peripheral blood.²⁹² Therefore, under conditions of natural infection (and possible vaccination) the memory T-cell response may consist of a continuum of activation and differentiation states—at least, based on activation/differentiation marker expression.

MEMORY CD8⁺ T CELL DIFFERENTIATION

Memory CD8⁺ T cells are characterized by distinct features in terms of quantity and quality of responses from their naïve precursors.^{3,21,136} Substantial progress has been made in defining the phenotypic and functional changes involved in the course of memory CD8⁺ T-cell differentiation. During the acute phase of viral infection, antigen recognition and inflammatory signals such as type I IFN and IL-12 induce a rapid and substantial clonal expansion of naïve antigen-specific T cells, which develop into effector T cells at 1 to 2 weeks after infection.^{133,148} In this expansion phase, there are several changes in phenotype and function of antigen-specific T cells to acquire memory T cells. At the peak of CD8⁺ T cell responses, naïve T-cell expansion leads to two distinct subsets (short-liver effector

cells and memory precursor effector cells). These cells can be defined by expression of the cell surface markers CD127 and KLRG1.^{135,239} CD127 is highly expressed on naïve T cells but appears to be downregulated on all antigen-specific CD8⁺ T cells after activation. These CD127^{lo} T cells consist of KLRG1^{hi} and KLRG1^{lo/int}. Memory CD8⁺ T cells arise from KLRG1^{lo/int} populations by subsequent reexpression of CD127. However, these memory precursor effector cells (CD127^{hi}KLRG1^{lo/int}) efficiently survive the contraction phase, memory T-cell properties, and constitute the majority of the memory T-cell pool. These memory CD8⁺ T cells further differentiate into self-renewing memory T cells, and the extended lifespan depends partly on IL-7/IL-15–dependent homeostatic proliferation having slow cell division and minimal cell number changes.

Recently, factors involved in controlling the differentiation of effector and memory CD8⁺ T cells have been demonstrated. First, T box expressed in T-cells (T bet) has been identified as a transcription factor regulating the differentiation of effector and memory CD8⁺ T-cell responses¹³³; the high levels of inflammation and T-bet promoted effector cell differentiation, whereas mild inflammation and low T-bet generated memory cells. Another T-box transcription factor, Eomesodermin (Eomes), is expressed in a reciprocal manner to T-bet such that it is repressed by inflammatory cytokine, IL-12²⁸⁴ and may play a role in promoting memory responses.¹²⁷ In addition, genetic studies reveal that their function is redundant by promoting both killer T-cell fate and CD8⁺ memory homeostasis.¹²⁶ Lastly, Blimp-1, a regulator of plasma cell differentiation, is required for CD8⁺ T cell differentiation into functional killer cells and is crucial for recall response to reinfection. However, Blimp-1 is not essential for the generation of memory T cells.¹³⁸

VIRAL STRATEGIES TO EVADE THE ADAPTIVE IMMUNE RESPONSE

The immune responses to virus involve complex molecular and cellular interaction between the virus and its host. Therefore, any stage in this interaction could be targeted by a pathogen and used for its own benefit. The systematic study of viral genomes

TABLE 9.7 Viral Strategies to Avoid Host Adaptive Immunity

Escape by mutations
Escape by latency
Escape by destruction of immune cells
Escape by subverting antigen processing and presentation
Inhibition of T cell–mediated target cell lysis
Inhibition of inflammatory responses via modulation of cytokine action
Inhibition of humoral immunity by virally encoded Fc receptor, complement receptor/control protein

reveals that most viruses have evolved means of escaping or subverting immune defenses, and that some of them have many genes devoted to this purpose. Although the large, more complex DNA viruses encode viral proteins to avoid immune recognition, RNA viruses usually generate small numbers of proteins as compared to DNA virus with large genomic materials. Regardless of whether they are DNA and RNA viruses, it seems that many, and possibly all, pathogens that cause chronic infections have evolved strategies to subvert the immune responses of the host as listed in Table 9.7. These strategies include evasion of humoral and cellular immunity by antigen variation, interference with antigen processing and presentation, and modulating the production of cytokines. Viral proteins that interfere with class I MHC antigen presentation and modulate inflammatory cytokines are listed in Tables 9.8 and 9.9. Understanding of the complex interactions between the immune system of the host and the invading viral pathogen will eventually enable researchers to devise better strategies for preventing viral diseases.

As described above, effector function of CD8⁺ T cells and CD4⁺ T_H1 cells plays a pivotal role in controlling viral infection. One critical way to induce virus-specific T-cell responses deals with the generation of antigenic peptides derived from viral products to CD8⁺ T cells and CD4⁺ T_H1 cells. The generation of antigenic peptides also involves the

TABLE 9.8 Inhibition of Antigen Presentation Via the Major Histocompatibility Complex Class I Pathway

Steps to Interfere with MHC class I pathway	Virus-encoded proteins
Inhibit MHC class I synthesis	Lentivirus (Vpu)
Inhibit transporter associated with antigen processing (TAP)	
–Expression	EBV (vIL-10), HCMV (UL111A)
–Function	HCMV (US6), HSV (ICP47)
Inhibit MHC class I transport	
–Retain MHC class I in the ER	HCMV (US3), adenovirus (E3-19K)
–Retain MHC class I in the pre-Golgi compartment	MCMV (m152)
–Dislocate MHC class I to the cytoplasm	HCMV (US11, US2)
–Dislocate MHC class I to lysosomes	MCMV (m6/gp48)
–Bind to cell surface MHC class I molecules	MCMV (gp34)
–Increase endocytosis of MHC class I molecules	HIV (nef), HHV-8 (K3, K4)

EBV, Epstein-Barr virus; ER, endoplasmic reticulum; MHC, major histocompatibility complex; HCMV, human cytomegalovirus; HSV, herpes simplex virus; MCMV, murine cytomegalovirus; HIV, human immunodeficiency virus; HHV, human herpesvirus.

TABLE 9.9 **Viral Modulation of the Cytokine System****Ways to interfere with cytokine function****Interrupt cytokine production**

- Interfere with cytokine and chemokine synthesis
- Inhibit the generation of functional cytokines

Interfere with cytokine action

- Encode homologs of cytokines and cytokine receptors
 - Type I interferon (IFN) homolog: VV (B18R)
 - IFN- γ homolog: VV (B8R)
 - Interleukin (IL)-6: KSHV (K2)
 - IL-8 homolog: HCMV (UL146, 147)
 - IL-10 homolog: EBV (BCRF1), HCMV (UL111A)
- Generate soluble cytokine receptors to neutralize cytokines
 - IFN- γ receptor: myxoma virus (MT-7)
 - IL-1 β R: VV WR (B15R)
 - TNFR homolog: orthopoxvirus (CrmB, CrmD)

Interfere with cytokine effector function

- Alter cytokine signaling pathway

HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; IFN, interferon; IL, interleukin; TNFR, tumor necrosis factor receptor; Crm, cytokine response modifier.

class I II MHC-dependent presentation of antigenic peptides to CD8⁺ and CD4⁺ T cells. The details for virus-mediated class I MHC and class II MHC antigen presentation is well described in.^{131,162} A second critical way for successful induction of antiviral T-cell responses involves the production of cytokines such as IFN- γ , which is pivotal for protective CD8⁺ T cells and CD4⁺ T_H1 cells. It has been well established that IFN- γ production is positively influenced by IL-12, but is negatively regulated by IL-10. It is conceivable that viruses exploit strategies to mimic the immunomodulatory cytokines, IL-12 and IL-10, to modulate virus-specific T-cell responses.⁴ Among the multiple ways to subvert adaptive T-cell responses, we will describe viral immune evasion strategies to evade innate immunity by interfering with the recognition of viral constituents by pattern-recognition receptor (PPR) and the initiation of inflammatory responses.

Innate immunity consists of multiple cellular sensors and signaling pathways, which lead to activation of early host defense mechanisms in response to viral invasion. These early antiviral responses result in the production of type I IFN that activates numerous transcription factors to induce the inflammatory gene expression.^{23,128,140} Thereby the early detection of viruses is the main role of innate immune cells by sensing virus-derived molecules and triggering host defense mechanisms. These sensors include the RIG-I-like helicase family for recognition of viral RNA as well as TLR and NOD-like receptor pathways for sensing various virus-derived molecules.

Activation of the interferon response is triggered by the detection of viral pathogen-associated patterns. All of the PRRs initiate signaling pathways that converge at the activation of transcription factors, IRF-3, IRF-7, and NF- κ B. The activation of these transcription factors leads to the expression of IFN- β . IFN- β initiates an anti-viral effector program in the infected cells and neighboring cells by the expression of numerous IFN-stimulated genes (ISGs). Because the host has evolved strategies for detecting and responding to viral infection, viruses

constantly evade TLR signaling to inhibit antiviral IFN action. One best example is the cleavage of IPS1 by HCV NS3/4a protease to block RIG-I signaling.^{82,90} The influenza A virus NS1 protein inhibits RIG-I-mediated PRR signaling by direct interaction,^{89,206} whereas paramyxovirus V protein binds and inhibits MDA-5 to abrogate PPR signaling.^{42,43}

VIRAL EVASION OF HUMORAL (B CELL) IMMUNITY

Viruses use a number of mechanisms to evade humoral immune responses, and viral neutralizing antibodies in particular (reviewed in¹⁵⁶). Viral strategies to evade neutralizing antibody responses to the viral surface proteins include (a) mutation of surface proteins by antigenic drift and shift, and recombination, most notably for highly variable viruses such as influenza virus, HIV and HCV; (b) masking of conserved epitopes; (c) glycan shielding as seen for HIV and Ebola viruses for example; (d) surface protein decoys that may “mop up” neutralizing antibodies under certain conditions, for example as with HBV; (e) original antigenic sin; (f) irregular surface protein spacing that tends to reduce immunogenicity; and (g) multiple surface proteins and virus forms, for example poxviruses encode multiple proteins for entry. Viruses can also evade humoral responses by spreading directly between cells, upregulating and hijacking host complement regulatory proteins, encoding antagonistic Fc and complement receptors and inducing immunosuppression.

VIRAL EVASION OF CELLULAR (T CELL) IMMUNITY

Escape by Subverting Antigen Processing and Presentation

A number of viruses have devised strategies to impair presentation of their antigens by the MHC class I pathway, thereby reducing activation of antigen-specific CD8⁺ T cells as well as recognition of virus-infected cells by CD8⁺ T cells. This stresses the importance of this cell subset for antiviral defense. Nearly every step of the MHC class I presentation pathway can be interfered with, and some viruses encode multiple proteins that act at different levels of the MHC class I processing pathway (Table 9.8): (a) inhibition of MHC class I synthesis, (b) antigenic peptide generation, (c) transporter associated with antigen processing, and (d) the cell surface expression of MHC class I molecule expression. Although interference of the MHC class II antigen processing pathway by virus is less common, some evidence indicates that viruses are able to inhibit this pathway.

Inhibition of Class I or II Major Histocompatibility Complex Synthesis

Viral proteins, including HIV Tat protein, have been reported to suppress or inhibit MHC gene promoter activity.¹²⁰ It has been shown for HIV Tat protein that the activity of the MHC class I gene promoter was decreased up to 12 times. With respect to the inhibition of MHC class II expression, human cytomegalovirus (HCMV) can impair MHC class II expression through two distinct pathways. MHC class II molecules

are expressed only on a subset of cells, and this expression is regulated at the transcriptional level through control elements that include those that allow both constitutive and cytokine-induced transcription of the MHC class II genes. The MHC class II transactivator (CIITA) is essential for constitutive and induced transcription, and is the rate-limiting factor of MHC class II production. One of the four promoters controlling CIITA production is activated by IFN- γ , which, on binding to its receptors, triggers a signaling cascade through Janus kinases, leading to transcription of IFN-inducible genes. The inhibition of IFN- γ -stimulated MHC class II expression occurs by two different mechanisms: (a) decreased Janus kinases¹⁸⁶ and (b) interference with the CIITA promoter.¹⁵⁸

INHIBITION OF ANTIGENIC PEPTIDE GENERATION

The metalloproteases, CD10 (endopeptidase) and CD13 (aminopeptidase N), are downregulated during HCMV infection.²¹⁶ CD10 expression is apparently blocked at the transcriptional or translational level, whereas CD13 seems to be retained within the ER compartment. These endopeptidases play a role in peptide processing in both MHC class I and II antigen presentation pathways. Both CD10 and CD13 peptidases are expressed on the cell surface and trim antigenic peptides to a size that allows their binding to the groove of class I or II molecules.

Inhibition of Transporter Associated with Antigen Processing

The peptide transporter TAP is needed to shuttle peptides from the cytoplasm to the ER, in which they associate with MHC class I determinants. TAP peptide transport is inhibited by a protein of HCMV, US6, which acts from the luminal site of the ER. HCMV US6 protein specifically binds at the ER-luminal loops of TAP signals across the membrane to the nucleotide-binding domains. This binding prevents ATP hydrolysis of TAP, which is necessary for peptide transport.

The ICP47 polypeptide from HSV inhibits TAP by binding to TAP's peptide binding site, thus preventing its association with other peptides. This results in an insufficient number of peptides in the ER for binding to MHC class I molecules. In contrast, TAP-independent peptide loading of MHC class I molecules is not affected by US6 or ICP47. As described below, EBV encodes a protein that weakly binds the IL-10 receptor. As with cellular IL-10, the EBV protein reduces expression of TAP and of MHC class II.

INHIBITION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I CELL SURFACE EXPRESSION

Modulation of MHC class I expression has been reported in at least three viral systems: adenoviruses, HCMV, and HSV. HCMV US3 protein, a type I membrane protein, prevents maturation of MHC class I molecules by retaining them in the ER compartment. US3 protein binds to MHC class I molecules in a transient fashion, but retains them efficiently in the ER. The US3 luminal domain is responsible for ER retention of

US3 itself, whereas both the US3 luminal and transmembrane domains are necessary for retaining MHC class I in the ER. In contrast to HCMV US3 protein, HCMV US2 and US11 catalyze the dislocation of MHC class I products, resulting in their rapid degradation. US11 protein uses its transmembrane domain to recruit MHC class I products to human homologue of yeast Der1p, a protein essential for the degradation of MHC class I molecules catalyzed by US11, but not by US2. HCMV US2 has been shown to differentially affect surface expression of MHC class I but only targets membrane-bound (not soluble) HLA-G1 antigen for degradation.

Adenovirus expresses a protein E3/19K, which is an ER-retained protein; it has a cytosolic ER-retention signal in the C-terminus of its cytoplasmic tail (-DEKKMP). The 19K binds certain murine MHC class I molecules (e.g., A2, K^d, L^d, but not K^k, or D^d) and holds them in the ER. This results in decreased MHC class I expression and decreased CTL recognition. An immediate early gene product of HSV, the ICP47 gene product, interferes with transport of peptides from the cytoplasm to the ER, thereby depriving MHC class I molecules in the ER of peptides. This results in both the ER peptides and destabilization and retention of MHC class I molecules in the ER.

VIRAL EVASION OF IMMUNE EFFECTOR MECHANISMS

Interference with Cytokine Functions

Cytokines (e.g., IFN and TNF) induce intracellular pathways that activate an antiviral stage or apoptosis, and thereby limit viral replication. A large number of cytokines induce mechanisms that enhance immune recognition and/or immune responses that protect against viral infection. In addition, antiviral cytokines play a pivotal role in removal of infected cells by NK cells or CTL. Therefore, it is not surprising that viruses use immune evasion strategies to control the function of cytokines.

Recently, viral modulation of cytokines by several viruses has been reported. Three different mechanisms have been shown to affect the activity of cytokines (Table 9.9). Viruses produce cytokine homologs, which bind to the same cytokine receptors. Alternatively, viruses generate antigens that mimic cytokine receptors and, thus, neutralize the corresponding factors. Finally, they can directly interfere with the action of cytokines by generating soluble cytokine-binding proteins. The functions of viral homologues of cytokine and their receptors are diverse. The virus-encoded cytokine homologs might be involved in inactivating inflammatory cytokines or redirecting the immune responses.

Inhibition of cytokine production has been shown by poxvirus; cowpox virus (CPV) cytokine response modifier A (CrmA) protein inhibits the production of caspase-1, which prevents the proteolytic cleavage of prointerleukin-1 β (pro-IL-1 β) to mature IL-1 β . In addition, the attachment of measles virus to its cellular receptor, CD46, a complement regulatory protein, inhibits the production of IL-12. Many virus-encoded mechanisms are involved in blocking the effector functions of cytokines, such as the antiviral state induced by IFN or apoptosis triggered by TNF, and intracellular antagonists of TNF or IL-1/Toll-like receptor (TLR) signaling. Interestingly, LMP-1

of EBV recruits components of tumor necrosis factor receptor (TNFR) and CD40 signal-transduction machinery, which induces biological responses to help viral replication.

RECEPTOR BINDING AND MODULATION OF IMMUNE CELL FUNCTION

Measles virus binds to, and enters, monocyte/macrophages using the CD46 molecule (a C3b receptor) as its receptor for entering cells; cross-linking CD46 inhibits the production of IL-12 by macrophages. Therefore, measles-induced immune suppression may be related to the failure of infected APCs to make the proinflammatory cytokine IL-12. Another IL-12 homolog, UL111A, is encoded by HCMV.

CYTOKINE AND CYTOKINE RECEPTOR HOMOLOGS

Alterations in cytokine function represent a newly appreciated mechanism of evasion. One of the most striking examples of this mechanism has been reported for EBV, has apparently cannibalized the gene for the human IL-10 cytokine. The viral genome, gene BCRF1, encodes a protein with 70% amino acid homology to human IL-10, which inhibits cytokine production by T_H1 type T cells and monocytes (macrophages) through its action on macrophage function. IL-10 also activates B lymphocytes and upregulates BCL-2. The viral IL-10 has all of the same activities as human IL-10.

Our understanding of the role of viral immunomodulatory proteins in the context of infection is limited, mainly because of the lack of appropriate experimental models of infection. In many cases, we can only predict the *in vivo* function of a viral protein in view of a known function of cytokines. As expected, inactivation of poxvirus-soluble cytokine receptors (e.g., the myxoma virus vTNFR and vIFN- γ R, or the vaccinia virus vIFN- α/β BP) leads to virus attenuation. The attenuated phenotype of the myxoma virus vIFN- γ R (M-T7) mutant might also be attributed to the ability of M-T7 virus to bind chemokines, which is consistent with increased leukocyte recruitment to sites of infection. The consequences of the inactivation of a viral immunomodulatory protein on the outcome of infection, however, might be unpredictable. Deletion of vaccinia vIL-1 β R exacerbates vaccinia virus infection in a mouse intranasal model because of enhanced systemic activity of the proinflammatory cytokine IL-1 β , which might cause increased fever and weight loss, leading to enhanced mortality of the infected host. This indicates that some viral immunomodulatory proteins might downregulate immune-mediated pathology to favor equilibrium with the host, rather than to increase viral replication.

VIRUS-INDUCED IMMUNE DYSREGULATION AND AUTOIMMUNE DISEASE

Chronic diseases can result when the immune system is overly active, often causing the affected tissues to be inflamed and abnormally infiltrated by lymphocytes and other leukocytes.

However, no active infection is associated with such diseases. Therefore, these diseases are caused by the immune system itself, which attacks cells and tissues of the body. Chronic diseases of this kind are known as autoimmune diseases, as they are caused by immune responses directed toward self-components of the body. Several genetic risk factors and protective elements have shown to influence the susceptibility of autoimmune disease.⁷⁴ However, a considerable discordance in incidence of autoimmune disease comparing identical twins indicates that, in many cases, additional factors such as environment modulators could influence the incidence of autoimmune diseases.⁴⁷

Indeed, viral infections, particularly chronic viruses, are shown to enhance autoimmune disease in susceptible individuals, as infections frequently induce strong inflammatory responses in various organs.^{203,210} There are several major pathways through which viruses can initiate or more likely modulate autoimmunity: (a) direct infection of target cells/organs can cause the release of sequestered autoantigens and enhance antigen presentation; (b) local inflammation might alter the repertoire of self-epitopes presented by APCs by altering Ag degradation properties of the proteasome; (c) Presentation of pathogen epitopes with structural or sequential similarity to self-epitopes might specifically activate autoreactive lymphocytes. However, some viral infections can also act to ameliorate autoimmunity. There are several reports supporting the association of autoimmune diseases with viral infection. Indeed, associations with infectious agents have been suggested for a multitude of autoimmune diseases, including type 1 diabetes (T1D), multiple sclerosis (MS), and ankylosing spondylitis. However, attempts to establish a direct epidemiological, statistically relevant association between microbial infections and various autoimmune disorders have been unsuccessful thus far.

Recently, several animal models for human autoimmune diseases have been established to elucidate the role of molecular mimicry in initiating autoimmune diseases. In transgenic animals that express specific target antigens, challenge with pathogens containing identical or similar antigens act as a triggering factor for the autoimmune disease process.⁴⁶ The induction of autoimmune disease in these models requires sufficient numbers of autoaggressive lymphocytes. Thereby, it is likely that infection with a pathogen having molecular identity to a target self antigen can result in the generation of more lymphocytes with high avidity to the identical transgenic target antigen. In the rat insulin promotor (RIP)-LCMV model for type 1 diabetes, the avidity of self-reactive lymphocytes has been demonstrated to determine the course of disease. RIP-LCMV mice express the glycoprotein or nucleoprotein of LCMV under the control of the RIP, specifically in the β cell of the pancreatic islets of Langerhans.²⁹⁹ However, even in the absence of possible thymic expression of target antigen, mechanism of peripheral tolerance can result in a certain degree of unresponsiveness to the identical antigen present on the triggering virus that might prevent aggressive immune responses. In addition, infection with pathogens with similar but not identical structures might overcome tolerance induced by the host. This has been demonstrated in the development of autoimmune hepatitis in the CYP2D6 mouse model, which is associated with the molecular mimicry. In the model of CYP2D6, adenovirus (Ad-2D6) expressing human cytochrome P450 (CYP) 2D6 (CYP2D6) was used to trigger autoimmune liver damage, and CYP2D6

is a major natural autoantigen in autoimmune hepatitis type 2.^{170,320} As targets, wild-type FVH mice, which express mouse CYP isoenzymes with a structural and sequential similarity to human CYP2D6 (molecular mimicry), or transgenic CYP2D6 mice, which express additional human CYP2D6 (molecular identity). However, it is yet to be known whether autoimmunity initiated by the molecular mimicry generates enough autoaggressive lymphocytes with sufficient avidity to cross the threshold for clinical autoimmune disease.

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Pathogenesis of Viral Infection

Introduction to Viral Pathogenesis

Goals of This Chapter

Definitions and Concepts in Viral Pathogenesis

Productive, Abortive, and Latent Infection
 Acute Versus Chronic or Persistent Infection
 Quasispecies
 Control of Acute Versus Chronic Infection
 Equilibrium and Nonequilibrium States in Pathogenesis
 Disease
 Virulence
 Invasiveness
 Cell-Intrinsic Versus Cell-Extrinsic Mechanisms
 Evasion of Host Molecules and Mechanisms
 Subversion of Host Molecules and Mechanisms
 Tropism
 Essential Genes, Virulence Genes, and Virulence Determinants
 Virulence Genes and Determinants May Not Encode Proteins

Conceptualizing Viral Pathogenesis

Clinical Observations Define Fundamental Pathogenesis Questions
 Conceptualizing Viral Pathogenesis as a Series of Sequential Stages in Infection
 Conceptualizing Viral Pathogenesis as the Interaction Between Stochastic Events and Bottlenecks in Infection
 Conceptualizing Viral Pathogenesis as the Integrated Effects of Host Genetic Variation

The Study of Viral Pathogenesis

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 Animal Models
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 Study of Animal Virus Infections That Resemble Human Infection
 Viral Genetics as a Tool for Analysis of Pathogenesis
 Interactions Between Host and Viral Genes in the Study of Pathogenesis
 Cell Culture

Determinants of Cell, Organ, and Tissue Tropism

Entry into the Host
 Penetration Through Epithelial Barriers

Vertical Spread of Viruses
 Systemic Spread of Virus Infection
 Determinants of Cell, Tissue, and Species Tropism
 The Role of Viral Receptors in Cell, Tissue, and Species Tropism
 Innate Immunity and Intrinsic Cellular Resistance to Infection Determine Tropism
 Immunoprivilege
 Cellular Differentiation as a Determinant of Viral Tropism and Pathogenesis

Fate of the Infected Cell, Tissue, and Host

Age as a Determinant of Susceptibility to Viral Infection
 Fate of Infected and Uninfected Cells in Tissues
 Direct Killing of Cells by Viruses
 Killing of Cells by Cell-Extrinsic Effects of the Immune System
 Clearance of Virus Infection and Chronic Viral Infection
 Tissue Damage During Beneficial Immune Responses Is a Necessary Evil
 Immunopathology Occurs When the Immune System Goes Too Far
 Virus-Induced Autoimmunity

Viral Determinants of Virulence

Niche-Specific Virulence Genes
 Mutation and Selection of Viral Variants
 Virus-Induced Immunosuppression
 Viral Evasion and Subversion of Host Cytokine Responses
 Virus-Host Co-Evolution Drives the Host-Pathogen Interaction
 Virus Genes That Inhibit Pathogenesis of Disease

Future of Viral Pathogenesis Research

Poxvirus Pathogenesis as a Stepwise Process (see eBook)

Rapid Reversion of Venezuelan Equine Encephalitis Virus to Virulence When Confronted with a Bottleneck (see eBook)

FUT2 Gene in Human Norovirus Infection (see eBook)

Vertical Spread of Virus Via the Germ Line (see eBook)

Neural Spread of Viral Pathogens (see eBook)

Receptor Tropism and Viral Pathogenesis in Poliovirus Infection (see eBook)



INTRODUCTION TO VIRAL PATHOGENESIS

Viral pathogenesis is the series of steps that occurs when a virus infects the host. Viruses are obligate parasites of living cells that cannot live independent of an intricate relationship with an infected cell. The cells targeted by viruses during infection survive, differentiate, and function in a tissue that has an intimate relationship with other tissues and physiologic processes in the body. Thus, in the same sense that viruses are obligate parasites of living cells, they are obligate parasites of tissues in living organisms. Living in tissues and spreading from host to host represent processes only approximated in cell culture or biochemical experimentation. The study of viral pathogenesis elucidates this special relationship between the virus and the intact host.

The term *pathogenesis* refers to the processes related to disease induction; therefore, *viral pathogenesis* often refers to disease induction by a virus rather than the process of infection *per se*. However, viral infection does not always result in apparent or immediate disease, and the border between infection and disease becomes less clear as we learn more. It is most useful to consider the pathogenesis of infection independently of whether or not severe or immediate disease is induced. As the pathogenesis of infection is analyzed, the pathogenesis of disease can be considered as a subset of events that occur *in vivo* during infection. Fundamentally important mechanisms are revealed when one considers how the pathogenesis of infection differs between the host with asymptomatic infection or minimal disease and the host doomed to suffer severe consequences of viral infection.

Viral pathogenesis is the integrated result of many complex factors unique to a particular virus, a particular species, and an individual host. The interplay of these factors determines the nature of infection, whether disease occurs, and the severity of disease. In many cases, individual factors involved in pathogenesis can be studied in detail. For example, the crystal structure of a viral immune evasion protein allows predictions as to protein function during infection *in vivo*. However, definitive pathogenesis experiments often falsify such predictions. Thus, all conclusions derived from reductionist experiments utilizing molecular and biochemical approaches need to be validated in living organisms to understand viral pathogenesis.

The complexity of directly testing molecular mechanisms *in vivo* has been a significant stumbling block for pathogenesis research. The difficulty of doing definitive pathogenesis experiments that prove a molecular mechanism *in vivo* has resulted in pathogenesis research being relegated at times to a lesser status as a phenomenological science when compared to studies of molecular and biochemical mechanisms. However, in the end, infection occurs in the intact host; thus, events *in vivo* are highly relevant. With the development of new tools, in particular those that allow genetic analysis of pathogenesis from both the viral and host standpoint, pathogenesis research has rapidly evolved into a mechanistic science. Moreover, classical studies of pathogenesis have often stood the test of time and are useful at a minimum for defining the fundamental questions to be addressed using molecular pathogenesis approaches.

Goals of This Chapter

This chapter will focus on integrating classical concepts of pathogenesis with more current molecular understanding of

viruses. Viral pathogenesis is the subject of many authoritative texts, and the reader is referred to one such text for detailed treatment of many of the important concepts developed here in necessarily shorter form.^{195,196} There is a rich history of how the initial observations that form the basis for our current understanding of viral pathogenesis were made.¹⁹⁶ These founding studies are important to our understanding of the pathogenesis of infection as well as to major advances in human health such as the elimination of smallpox and the development of vaccines against diseases such as polio, measles, mumps, rubella, hepatitis B virus (HBV), and human papillomavirus. Exposure to the historical roots of pathogenesis research is strongly recommended for those who wish to delve into viral pathogenesis as a career or avocation.

Because it is impossible to provide a detailed description of the pathogenesis of all viral infections in a single chapter, the goal of this chapter is to provide a conceptual framework for understanding viral pathogenesis. Principles will be defined and then examples provided from a broad range of infections. For details of specific viral infections, refer to chapters on individual viruses. This chapter will specifically focus on the pathogenesis of infection with nucleic acid-containing viruses in mammals. There is a rich body of knowledge on plant viral diseases and prion diseases that will not be addressed in this chapter.

DEFINITIONS AND CONCEPTS IN VIRAL PATHOGENESIS

The literature on pathogenesis is extensive and more than 100 years old. A search of the recent literature accessed by Entrez using the term *viral pathogenesis* pulls up more than 426,000 references, whereas a search of *viral disease* pulls up more than 695,000 references. Within this complex and voluminous literature, terms are used in multiple ways. Often the utilization of terms is predicated on historical factors rather than more up-to-date considerations. We will therefore begin by defining terms as they will be used in this chapter.

Productive, Abortive, and Latent Infection

Infection is the process by which a virus introduces its genome into a cell. Infection is *productive* if new infectious virus is made and *abortive* if no new infectious virus is produced. Infection is *latent* if the production of infectious virus does not occur immediately but the virus retains the potential to initiate productive infection at a later time. The process of reinitiating a productive infection cycle from the latent state is termed *reactivation*. Latency is not merely a slow productive replication cycle; latency represents a unique transcriptional and translational state where infectious virus is not present, but where a productive replication cycle can be reinitiated when the need arises. A cell is permissive if it can support productive infection and nonpermissive if infection cannot occur at all or is abortive.

Acute Versus Chronic or Persistent Infection

Acute infection occurs when a virus first infects a susceptible host (Fig. 10.1). *Chronic* or *persistent infection* is the continuation of infection beyond the time when the immune system might reasonably be expected to clear acute infection. The

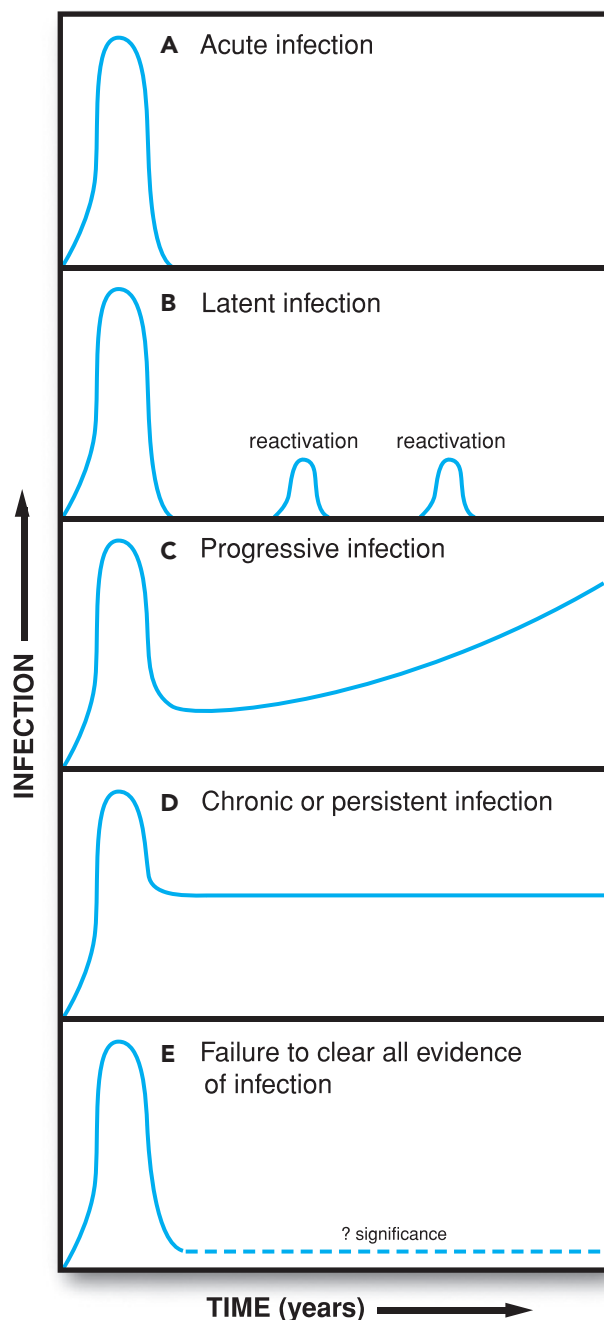


FIGURE 10.1. Schematic of different patterns of viral infection.

terms *chronic* and *persistent* have been used interchangeably for many years; we will continue this convention. It is important to note that these terms denote the presence of viral infection in the host for long periods but do not provide insight as to the mechanism(s) responsible for prolonged survival of the virus in the host. Mechanisms responsible for chronic infection include persistence of nucleic acid, continuous replication, latency, and reactivation. More than one of these processes may occur at the same time. In some cases, viral nucleic acid can be detected in the host for prolonged periods, although the nature of the infectious process has not been defined (see Fig. 10.1). In such

cases, chronic infection may represent continuous replication, latent infection, abortive infection without clearance of residual nucleic acid, or perhaps some as-yet-unidentified form of viral infection.

In some cases, as for HBV or hepatitis C virus (HCV), a proportion of persons become chronically infected while others are cured. In these cases, the transition from acute to chronic is arbitrarily defined as the time when most patients have cleared acute infection. In other cases, essentially all hosts become chronically infected, as is seen with herpesviruses or lentiviruses such as human immunodeficiency virus (HIV). In this case, the transition between acute and chronic infection is defined as the time required for clearance of the initial burst of viral replication and establishment of equilibrium between the host and the virus.

There are two primary mechanisms for establishment of chronic infection: *continuous replication* and *establishment of latency*. During latent viral infection, the virus has a genomic and transcriptional strategy, often involving restricted viral gene expression, which allows the genome to survive even when lytic replication is not occurring. Examples include the proviral form of retroviruses or the circular episomal form with selective expression of viral genes observed for herpesviruses such as Epstein-Barr virus (EBV) and herpes simplex virus (HSV). Often, latently infected cells express no viral proteins, making latency immunologically silent. This is the ultimate form of immune evasion, as the host has no known mechanisms for sensing the presence of the virus. To survive and spread from the latently infected cell, the virus must be able reactivate and reinitiate the lytic cycle of gene expression, potentially generating antigens that the immune system can respond to.

To succeed via continuous replication, a virus must generate new infectious virions despite ongoing innate and adaptive immune responses. Even for viruses that utilize the establishment of latency as a primary strategy, intermittent reactivation and replication may be required to maintain latency and to spread from host to host. Some viruses, such as HIV, persist via both continuous replication and establishment of latency, presenting a particularly difficult challenge for the host immune system.^{107,301}

Quasispecies

The mixture of viruses present in the host at a given time is a *quasispecies*. Although it is convenient to think of a virus as a single homogeneous agent, this is not true because both viral RNA and DNA polymerases make errors that generate mutant viruses during infection. The polymerases of RNA viruses are generally less accurate in copying template molecules than those of DNA viruses; mutation may therefore play a greater role in RNA than DNA virus pathogenesis. However, mutation may play a role in the pathogenesis of any virus. The quasispecies generated by mutation is acted on by complex and powerful selective pressures in the host, with some viruses having a survival or fitness advantage over others. The nature of these selective pressures and the pathogenic potential of the selected viruses are important determinants of viral pathogenesis.

Control of Acute Versus Chronic Infection

The distinctions between acute and chronic or persistent infection are very important. The viral genes and host immune

factors that foster or control acute versus chronic infection are distinct. For example, the cytokine interferon- γ (IFN γ) regulates latency and continuous replication of the murine γ HV68 (also referred to as MHV-68) but has at most a minimal effect during acute infection.¹⁶ This indicates that certain host responses are more relevant to chronic than acute infection. Control of either acute or chronic infection may involve responding to viral quasispecies. This is the case for both HIV and HCV infection, presenting the infected host with the overwhelming problem of dealing with many antigenically distinct viruses with different pathogenic strategies. Many viruses, including herpesviruses, papillomaviruses, and retroviruses, use different gene or gene expression patterns to succeed during acute versus chronic infection. For these reasons, it is fundamentally important not to consider chronic infection as a mere continuation of acute infection.

Equilibrium and Nonequilibrium States in Pathogenesis

A fundamental concept in pathogenesis is that acute infection is a nonequilibrium state, whereas chronic infection is a metastable equilibrium between virus and host. During acute infection, both the host response and virus infection change continuously until infection is resolved or progresses to death of the host or establishment of chronic infection. In contrast, chronic infection, once established, is an equilibrium process with viral and host processes balancing each other. In particular, the immune system of the host brings the acute infection under control and delays or prevents a chronic infection from killing the host. Progression of chronic infection to disease often reflects a change in this equilibrium (see Fig. 10.1). Even when the host and virus are in a stable equilibrium, the infectious process is dynamic with host and viral processes balanced on a knife-edge such that small changes in either virus or host can disrupt the equilibrium with devastating consequences.

An example of nonequilibrium and equilibrium states during acute and chronic infection is provided by EBV, a γ -herpesvirus that causes acute mononucleosis in humans. During the acute stage of infection, EBV causes rapid B-cell expansion (a nonequilibrium process) that is then brought under control by the expansion of virus-specific T cells, which act to maintain the virus in a latent state (an equilibrium process). When levels of latently infected B cells are assessed, significant individual-to-individual variation is observed but levels are remarkably constant within a given individual over time. However, when patients are immune suppressed, the level of latently infected B cells increases,¹² suggesting that interactions between the virus-specific T cells and the virus maintain equilibrium during the chronic stage of infection. This balance between immune and viral processes during chronic infection is incompletely understood in most cases and yet is fundamentally important for understanding many human diseases.

Disease

Disease is a harmful pathologic consequence of infection. In many cases, infection is apparently harmless to the host and does not result in disease. One of the most important goals of pathogenesis research is to define in molecular terms what determines the difference between infection and disease. Even highly virulent viruses often establish infection in a greater

number of hosts than they cause disease. Viruses such as rabies, Ebola, or HIV, which cause significant disease in nearly all infected persons, are the exception.

Disease may be associated with cell and tissue destruction (as in rabies virus killing neurons), induction or secretion of inflammatory cytokines (as in the induction of fever by many viruses), cellular dysfunction induced by viral infection (as in the case with lymphocytic choriomeningitis virus [LCMV] infection of the pituitary), paracrine effects of viral gene products (as in induction of angiogenesis by Kaposi's sarcoma herpesvirus [KSHV]), and the induction of malignant tumors to the effects of the immune system as it responds to infection (as in immunopathology seen with many viruses) or to the presence of a specific virus interacting with allelic polymorphisms in the host to trigger disease.^{40,300}

In many cases, virus-associated disease is defined as a series of nonspecific symptoms or signs such as fever, malaise, or anorexia. The presence of these symptoms and signs is common to infection with many different pathogens and therefore provides little insight into the mechanisms of viral pathogenesis. Recent work with microarray technology suggests that even the nonspecific syndromes associated with acute virus infection with different viruses may be distinguished by the pattern of host gene expression. It is likely that pathogenesis researchers will use such molecular signatures to distinguish between infections with different viruses and to define host genes involved in viral pathogenesis.

In contrast to the nonspecific syndromes commonly associated with virus infection, the presence of specific symptoms or signs of disease such as hepatitis, immunodeficiency, pox on the skin, or paralysis provides important clues as to the nature of the pathogenic process. It is this relationship between the signs and symptoms of disease and the presence of infection with specific viruses that has led to the close link between clinical observations of the natural history of disease and the science of viral pathogenesis.

Virulence

Virulence—the relative capacity of a virus to cause disease—determines the relationship between infection and disease. Virulent viruses cause disease in a greater proportion of infected hosts, and cause more severe disease, than viruses of lower virulence. Virulence comes in many forms, from the induction of rapid death as for variola major (the causative agent in smallpox) to the induction of tumors over prolonged periods, as is the case with certain papillomaviruses or herpesviruses, to the induction of organ failure over many years, as is the case with chronic HBV or HCV infection. Thus, the manifestations of virulence highly depend on the strategies that a given virus uses during infection.

The determination of whether one virus (e.g., Ebola virus) is more virulent than another virus (e.g., papillomavirus) represents a qualitative judgment, because it involves determining whether virus-induced hemorrhagic fever (as is seen with Ebola virus) is worse than metastatic cancer (as is seen with papillomaviruses). To avoid biases inherent in comparing different types of disease, virulence is properly used to compare the disease-inducing capacity of related viruses, such as different strains of the same virus. For example, Ebola Reston, which is not associated with human disease, is less virulent in humans than Ebola Zaire.

It is commonly assumed that viruses that replicate efficiently in the host are more virulent than viruses that replicate less efficiently, and indeed replication is an important determinant of the severity of disease in many cases. However, virulence is much more complex than simply the efficacy of replication. Other aspects of pathogenesis, including tropism, the host response to infection, and interactions between the virus and host tissues, play key roles in viral virulence. Therefore, virulence reflects host resistance to infection functioning in counterpoint to viral virulence genes and determinants with the outcome for the host hanging in the balance.

Invasiveness

Invasiveness is the capacity of a virus to enter into and damage a tissue, a property that distinguishes viruses with high potential virulence but differ in the efficiency with which they enter target tissues. For example, a virus may be highly virulent if directly inoculated into the central nervous system (CNS) but unable to cause disease if inoculated into the periphery, whereas a related virus with a mutation allowing it to cross the blood–brain barrier into the CNS can cause lethal disease following either peripheral or intracranial inoculation. This concept is nicely exemplified by the identification of neuroinvasiveness determinants in Sindbis virus that confer the capacity to cause lethal encephalitis.⁶⁶

Cell-Intrinsic Versus Cell-Extrinsic Mechanisms

Because infected cells live in a tissue in the host, they are affected both by events that occur inside the cell and by events that influence the cell from the outside. Events that occur in a cell independent of events outside of the infected cell are termed *cell intrinsic*. Some cell-intrinsic determinants of infection are owing to intrinsic cellular resistance to infection conferred by the presence of molecules that block viral infection. Events that are dictated by processes that occur outside of the cell are termed *cell extrinsic*. Many cell-extrinsic events are owing to innate and adaptive immunity. It is often the case that processes occurring in infected cells or tissues are affected by both cell-intrinsic and cell-extrinsic mechanisms. An example is the induction of death of infected cells that may be owing to cell-intrinsic induction of programmed cell death or cell-extrinsic immune factors such as cytokines or perforin and granzymes.

Evasion of Host Molecules and Mechanisms

Most viruses have evolved mechanisms to counter host innate and adaptive immunity or to bypass intrinsic cellular resistance molecules so that the virus can complete the infectious process and spread to a new host. These mechanisms constitute viral *evasion* of host responses. Often, evasion strategies involve viral genes with close homology to host genes, as when a virus encodes a host cytokine or cytokine receptor mimic. Other evasion strategies utilize molecules with novel structures to avoid host responses. Because the mechanisms responsible for acute and chronic infection differ, both with regard to viral and host factors, it follows that immune evasion mechanisms are different for acute versus chronic infection. During acute infection, viral immune evasion strategies commonly focus on the host innate immune response, whereas evasion of adaptive immunity is more important for maintaining chronic infection. When analyzing immune evasion mechanisms, it is therefore

important to identify the aspects of the infectious process for which a given molecule is important.

Subversion of Host Molecules and Mechanisms

Viruses must be able to utilize host metabolic and regulatory systems to optimize their growth, survival, and spread. In some cases, the normal functions of the host cell may be sufficient for the virus to replicate. In other cases, host cellular processes may be insufficient or have deleterious effects on the virus. *Subversion* refers to the ability of a virus to redirect or alter normal host processes to the advantage of the virus. For example, some viruses subvert the function of chemokine receptors or other immune-signaling proteins to supply intracellular signals that regulate viral gene expression or encode molecules that subvert the cyclin-dependent progression of cell cycle. There are many different signaling pathways and cellular processes that are subverted by viruses; in fact, it is reasonable to presume that essentially every pathway and process within the cell is subverted by one virus or another. This is the inevitable consequence of the obligate intracellular lifestyle of viruses and their capacity to evolve more rapidly than the mammalian host.

Tropism

Tropism is the capacity of a virus to infect or damage specific cells, tissues, or species. It is a fundamentally important contributor to viral pathogenesis and virulence, as the capacity to induce disease depends on the cell and tissue infected. For example, a neurotropic virus such as West Nile Virus can cause encephalitis or paralysis, whereas a virus with tropism for CD4 T cells such as HIV causes immunodeficiency.

One key determinant of viral tropism is the cognate interaction between the viral cell attachment protein(s) and receptor(s) present on host cells. However, there are many additional factors that determine cell, tissue, and species tropism. The concept of tropism is rapidly evolving with the recognition that essentially every aspect of the viral infectious process within a cell or tissue can be a determinant of tropism. Both cell-intrinsic and cell-extrinsic factors may alter viral tropism.

Essential Genes, Virulence Genes, and Virulence Determinants

Virulence is determined by the capacity of a virus to grow, be invasive, infect vulnerable cells, evade the immune system, subvert cellular processes, and cause tissue damage. These capacities are encoded in the viral genome, by alleles of individual *virulence genes*. That viral genotype confers pathogenic capacity was proven by studies using reassortant viruses, mutant viruses, or molecularly cloned viruses to show that alterations in genotype confer virulence phenotype (e.g., 10,55,56,66,69,121,168,229,265,287,291,308). Subsequent studies have shown that different viral genes can confer capacities for infection in different tissues, indicating that viral virulence genes are tissue specific.^{109,175} Consistent with this, viral variants that arise during chronic infection are often cell and tissue specific.^{2,3,108,143,189}

Any gene essential for replication contributes to virulence, because viruses must replicate to complete their life cycle. In this sense, all viral genes involved in replication are virulence genes. As this is not a very useful concept, viral genes essential for replication in permissive cells are termed *essential genes* rather than virulence genes. Virulence genes are not required for replication *per se* but are important for virulence in the

host. Although the distinction between an essential gene and a virulence gene is useful, it is important to recognize that allelic variations in the function of essential genes can be important determinants of viral virulence. In this case, the specific property associated with virulence is termed a *virulence determinant*. Thus, virulence determinants can be allelic variants of essential genes that confer significant differences in pathogenesis.

The size of the viral genome puts boundaries on the number of genes that a virus can use for pathogenesis and replication. For example, smaller RNA viruses often have genomes of less than 10,000 nucleotides, whereas poxviruses or herpesviruses may have genomes greater than 200,000 nucleotides in length. However, viral proteins often have multiple independent functions—a property particularly well documented for RNA viruses. Thus, the pathogenic capacity of a virus with a small genome should not be underestimated.

Virus particles can contain virulence determinants that are not encoded in their genomes. For example, vaccinia virus incorporates host complement regulatory proteins into its envelope, thereby becoming resistant to inactivation by host complement proteins.²⁹⁴ Another type of epigenetic mechanism is exemplified by herpesviruses such as human cytomegalovirus (HCMV) and HSV that can package host and viral messenger RNAs (mRNAs) into the virion.^{36,93,251,282} Although their role in pathogenesis is not yet clearly defined, carryover of these mRNAs can result in synthesis of viral proteins before the traditional transcription program of the virus is initiated.^{251,282}

It is not necessarily true that viral virulence determinants exert their effects in host cells actually infected by the virus. One example of this is paracrine regulation of tissue pathology by virus-encoded cytokines. Another example is the movement of viral proteins from an infected to an uninfected cell. For example, the HSV protein VP22 can efficiently move from an infected cell to the nucleus of uninfected cells,⁶⁸ can carry other proteins between cells,²²⁴ and can transport mRNAs from one cell to the next.²⁵¹ This is a fascinating example of how a viral virulence determinant can act on uninfected cells to contribute to viral pathogenesis.

Virulence Genes and Determinants May Not Encode Proteins

Virulence can be conferred by viral promoters or other cis elements in the viral genome or noncoding RNAs. For example, EBV infection is associated with the development of B-cell lymphomas. The EBV EBNA1 protein plays an important role in the maintenance of latent EBV infection in B lymphocytes. EBNA1 can be expressed from any of four promoters—Cp, Wp, Fp, and Qp—each of which is regulated in distinct ways by viral and host proteins,²⁶⁴ ensuring that EBNA1 is expressed at the appropriate place and time in B cells. These finely tuned promoters are therefore properly considered virulence determinants for EBV-associated malignancies. Another example of non-protein-coding virulence determinants are virally encoded microRNAs, which are expressed by a number of mammalian viruses, including herpesviruses, adenoviruses, and polyomaviruses.^{228,257} These noncoding RNAs target multiple cellular processes, including cell cycle regulators, apoptotic machinery, or virus-encoded transcript, and play a role in promoting viral persistence. Noncoding elements also play important roles in the pathogenesis of several RNA viruses. For example, attenuating mutations within the internal ribosomal entry site

(IRES) of the Sabin vaccine strain of poliovirus are important for replication in neurons and neurovirulence in mice and primates.^{97,99,141,149} It is becoming increasingly clear that the transcriptional complexity of a broad range of organisms is considerably higher than previously believed,^{23,117} and it is now recognized that this is also the case for viruses such as herpesviruses.^{46,47,133} It is likely that virulence genes that do not encode proteins will increasingly be recognized in many viruses.

CONCEPTUALIZING VIRAL PATHOGENESIS

Every fact or principle presented in this textbook relates in some way to the pathogenesis of infection or disease. Thus, it may seem that pathogenesis is too complex to be studied in any mechanistic detail, or that viruses differ so significantly that common principles do not exist. This problem is further compounded because many events and processes are going on at the same time *in vivo*. Furthermore, processes that occur *in vivo* are nonlinear (e.g., unrestricted viral replication may be exponential) or even stochastic (e.g., whether a mutation occurs when a genome is replicated), or otherwise are the results of as-yet-unidentified allelic variations in host genes that influence pathogenesis.^{1,300}

Given this complexity, it is worth considering how the questions of pathogenesis are formulated and placed in context for interpretation. There are many ways to look at infection—three of which are presented here as a basis for understanding viral pathogenesis. Pathogenesis may be conceptualized as an organized process consisting of sequential stages, as the result of stochastic events under selection by bottlenecks in infection, or as the integration of host allelic variations in genes that determine resistance to infection. Each view of pathogenesis has its limitations and benefits as a basis for understanding the nature and mechanisms of viral infection.

Clinical Observations Define Fundamental Pathogenesis Questions

Viral pathogenesis has been studied for longer than any other aspect of virology; however, in many ways, we know less about pathogenesis than we do about other aspects of virology. The pathogenesis of viral disease was studied before viruses were even identified. Smallpox was known before variola virus was identified, and rabies virus was propagated in animals and an effective vaccine was developed by Pasteur before the virus was characterized.¹³¹ It was known that chickenpox infection conferred lifelong resistance to disease before varicella-zoster virus (VZV) was identified. A study of measles in 1886 in the Faroe Islands identified the incubation period between exposure and disease, resistance of previously exposed individuals to reinfection, and the susceptibility of the very young to disease.^{95,96} These clinical observations often identify the questions that must be answered in pathogenesis studies. For example, mosquito-borne alphaviruses such as chikungunya virus cause severe acute and persistent arthralgia in infected humans, raising the question of how these viruses cause joint pain and whether chronic disease is associated with viral persistence. Through the careful analysis of human clinical samples and the use of appropriate animal models, the viral pathogenesis researcher can begin to define the molecular mechanisms underlying a disease process.

Conceptualizing Viral Pathogenesis as a Series of Sequential Stages in Infection

A very useful way to conceptualize viral pathogenesis is as a series of sequential stages resulting in survival and spread of a virus through the infected host and on to a new host (Figs. 10.2 and 10.3). Different viruses may utilize distinct molecular

mechanisms to accomplish each of these stages. For example, all viruses must infect cells on entry into the host and must spread to new hosts. As the host has mechanisms to inhibit each of these stages of infection, the stages of infection may be considered as hurdles over which the virus must jump to survive.

There may be more than one way for a virus to overcome such a hurdle; however, different viruses may utilize the same strategy to overcome a given hurdle. An example of a common strategy is the relative resistance of some viruses to inactivation in water or sewage, allowing them to spread between hosts by fecal–oral transmission. Another example is infection of the thymus resulting in deletion of virus-specific T cells as *self*-antigens. Yet another common strategy is evading immunity via the establishment of latent infection. Thus, there are many common strategies for infection shared by different viruses.

Dividing viral pathogenesis into stages is a very useful way to conceptualize the infectious process but has significant limitations. First, dividing pathogenesis into stages creates the impression that events are both sequential and depend on each other. This is not always the case; many independent events may be going on at the same time in the host. Second, this conceptual framework suggests that completion of a stage is associated with initiation of the next stage; a significant oversimplification. For example, viremia may continue after a virus has entered the CNS to cause encephalitis. The utility of considering viral pathogenesis as a series of sequential events must be balanced against how well this conceptual framework fits actual events during infection.

Poliovirus pathogenesis provides an excellent example of how pathogenesis can be broken down into a series of steps that culminate in either virus-induced disease or viral control. Clinical questions that drove poliovirus pathogenesis research through the first half of the past century included why poliomyelitis emerged in the 20th century, what mechanisms were responsible for paralysis, and how did protective immunity work.^{183,234,235,274} Solving these questions was central to combating a very important and pressing public health problem. Infection with poliovirus in humans has a wide range of possible outcomes from asymptomatic infection to meningoencephalitis with or without paralysis.^{183,234,235,274}

Studies over many years identified and analyzed a series of stages of poliovirus infection, leading to a relatively simple model for the pathogenesis of disease that elegantly explains paralysis, the low proportion of infected hosts paralyzed, and the lifelong immunity conferred by prior infection. This model, one of the most useful ever constructed, provided a basis for developing the poliovirus vaccines that have largely, although not completely, eliminated paralytic poliomyelitis as a scourge of humanity.

The stages in poliovirus pathogenesis according to this model are outlined in Figure 10.2.^{24,183,234,235} The virus enters the intestine via the fecal–oral route, binds to M cells overlying the Peyer's patch, is transported into the intestinal wall, and then replicates in lymphoid cells, leading to a primary viremia and infection of secondary sites. Replication in secondary sites gives rise to a secondary viremia that reaches a level capable of initiating CNS infection.^{234,235} CNS infection involves passage of the virus across the blood–brain barrier to infect neurons within the CNS. The blood–brain barrier is viewed as an

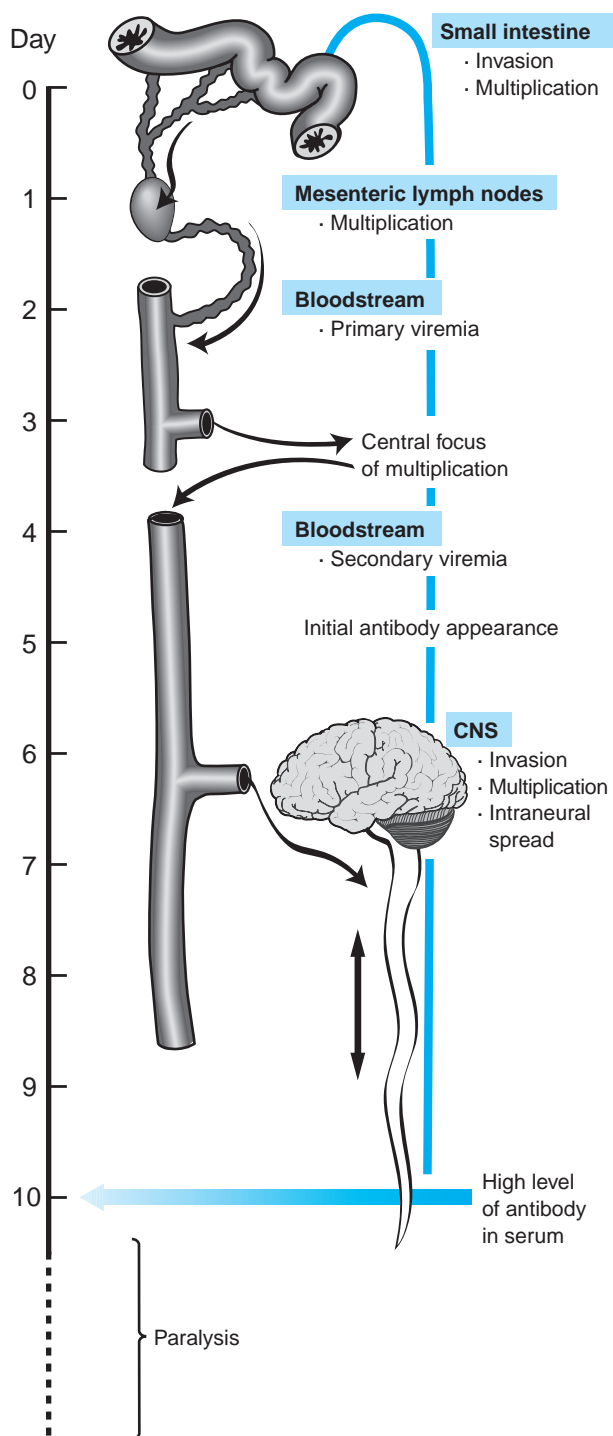


FIGURE 10.2. Model of poliovirus pathogenesis as a series of sequential stages. The steps in poliovirus infection of a human are schematized.

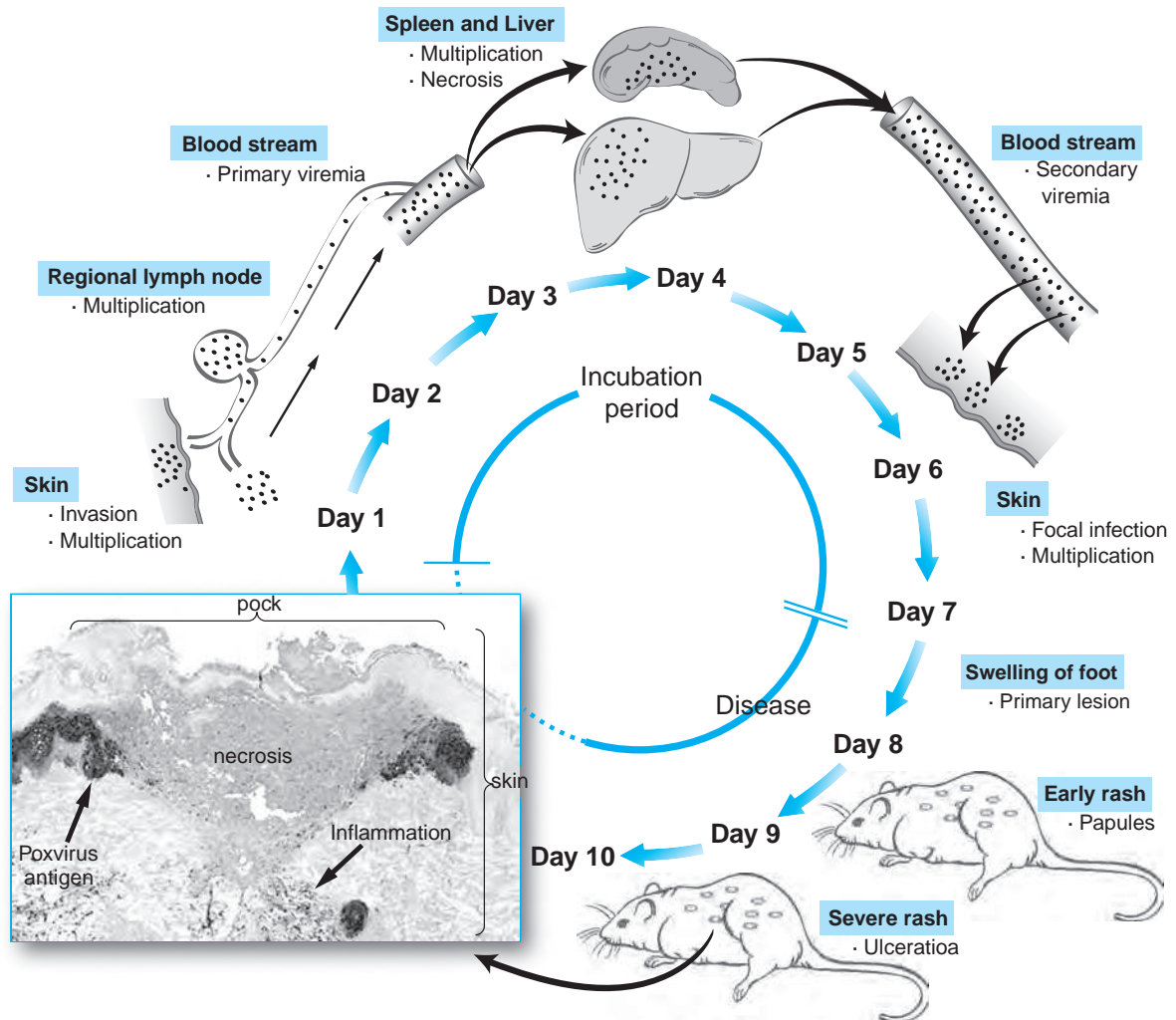


FIGURE 10.3. Model of poxvirus pathogenesis as a series of sequential stages. The steps in ectromelia virus pathogenesis are schematized. The outcome of ectromelia infection varies with mouse and viral strain.^{39,75} The viremia is thought to be cell associated. The histopathologic image of a pock was provided by Drs. Mark Martinez and Peter Jahrling and is skin from a variola-infected monkey stained with specific antisera to identify the infected cells shown as *poxvirus antigen* in the inset image. (Adapted from Fenner F, Buller RM. Mousepox. In: Nathanson N, Ahmed R, Gonzalez-Scarano F, et al, eds. *Viral Pathogenesis*. Philadelphia: Lippincott-Raven, 1997:535–553.)

important anatomic barrier to infection of the CNS, and passage across this barrier is poorly understood. Alternatively, the virus may spread via the blood to peripheral nerves and then spread up the nerves to enter the CNS.^{98,185,205,235,241} Within the CNS, the virus infects motor neurons; destruction of these cells leads to paralysis. Certain motor neurons are hypothesized to be more susceptible to poliovirus infection than others, and some poliovirus strains are either more invasive or more likely to kill neurons than others; these variables contribute to variation in disease penetrance and severity.

Concurrent with entry into the lymphatic system, an immune response is generated (see Fig. 10.2). It is hypothesized in this model that immune antibody limits access to the CNS and prevents paralytic disease.^{183,235} Antibody might act by preventing virus in the circulation from crossing the blood–brain barrier and entering the CNS. However, antibody is capable of inhibiting neural spread of viruses and can

inhibit viral infection by acting directly on or within neurons.^{160,183,290,292,299} Regardless of the mechanisms by which antibody protects, the outcome of infection is a race between the virus and the immune system, presenting another explanation for variations in clinical outcome. The immune system wins if antibody is made early enough to prevent spread to the CNS and neuronal destruction. The virus wins if infection of motor neurons occurs prior to development of protective antibody responses.

This model provides a basis for understanding many aspects of poliovirus infection, disease, immunity, and vaccination; however, many questions are left open. Questions that remain unanswered at the molecular level include how the virus enters the body, the molecular basis of cell and tissue tropism, the mechanism by which antibody acts to prevent CNS disease, the mechanisms of induction of immunity, and the route(s) by which the virus spreads.

Importantly, although the mechanistic details underpinning the pathogenesis of different viruses will differ significantly, the broad stages of the systemic infection process outlined for poliovirus pathogenesis are also relevant for a wide array of other viral pathogens. For example, although poxviruses are very different from small RNA viruses, such as poliovirus, there are remarkable parallels between the models of poliovirus infection (see Fig. 10.2) and those for poxviruses (Fig. 10.3). This includes the concept that both viruses must reach a threshold of viremia to facilitate viral invasion into target organs and that the disease process represents a race between the virus and the host immune response, where the outcome of that race plays a major role in determining whether the virus is controlled or if disease results. Refer to the eBook for a more complete description of poxvirus pathogenesis as a stepwise process. These general principles extend to a wide array of viral pathogens, including arboviruses, herpesviruses, and lentiviruses.



Conceptualizing Viral Pathogenesis as the Interaction Between Stochastic Events and Bottlenecks in Infection

It is important to recognize that the process of infection has, in addition to a series of predictable events that occur in sequence, an element of stochastic variation that contributes to real variability to the outcome of infection. Thus, viral pathogenesis can be conceptualized as a series of stochastic events with the process of infection determined by strong selective pressures in the host referred to as bottlenecks in infection (Fig. 10.4). These bottlenecks can be thought of as analogous to the rate-limiting step in a chemical reaction. Stochastic events such as mutations or the random success of a given virus at a stage in infection provide variation in the substrates for these rate-limiting steps.

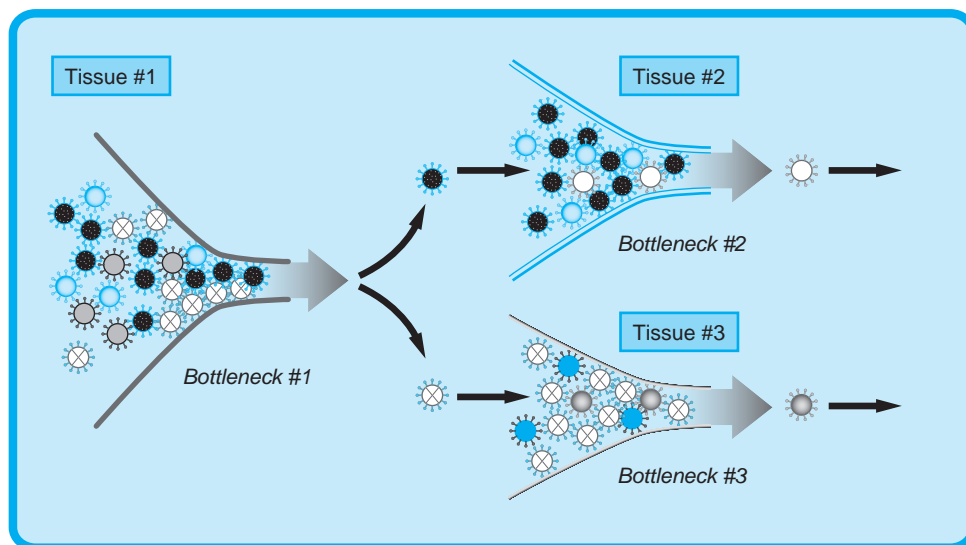
The concept that random events contribute to pathogenesis is not a comfortable one for investigators. However, even using clonal virus stocks for inoculation of genetically identical mice of the same sex and age results in viral titers in tissues that vary considerably (even by orders of magnitude) from one mouse to the next. This variation is not experimental error or noise but is rather a real phenomenon relevant to understanding infection. During natural infection of genetically variable

hosts with viral quasispecies, the variation is likely to be much larger.²⁹⁷

The reasons for the occurrence of significant variations even when conditions of infection are apparently homogeneous are not clear but likely involve several different factors, including epigenetic changes within the host, specific interactions between viruses and certain host genes that confer disease,^{44,300} environmental variables, and viral factors such as viral mutation rates that drive the generation of mutants with selective advantages in the host. This latter process, which involves the random generation of mutant viruses—a subset of which may provide a selective advantage that allow the virus to overcome bottlenecks and efficiently spread throughout the host—represents an important component of viral pathogenesis. For example, polioviruses that have high-fidelity polymerases, and therefore are less able to generate mutant viruses, are less efficient in infecting target tissues and causing disease, suggesting that the generation of viral quasispecies allows the virus to overcome bottlenecks and is essential for disease pathogenesis.²⁹⁶

Selective pressures at bottlenecks in infection can select mutants from within the viral quasispecies that have a fitness advantage (see Fig. 10.4). This is illustrated experimentally by studies with Venezuelan equine encephalitis virus (VEE), an equine pathogen that infects both humans and mice. Following subcutaneous inoculation, VEE initially replicates in the draining lymph node before seeding a serum viremia that leads to viral dissemination and ultimately viral entry into the CNS. Studies that compared molecularly clones of wild-type VEE to a mutant virus with a defined mutation at position 76 in the E2 envelope protein demonstrated that the mutant virus replicated within the draining lymph node but failed to spread systemically. Importantly, infection with the mutant virus led to the rapid generation of viruses that were able to efficiently spread within the host, and characterization of these viruses found that some contained a reversion of the original E2 mutation to wild-type. However, other viruses maintained the original E2 mutation but developed second site mutations that conferred the ability to disseminate systemically.^{10,94} These results demonstrate that there may be many ways to overcome

FIGURE 10.4. Viral pathogenesis as stochastic events followed by selection by bottlenecks in infection. Shown is a representation of a viral quasispecies, only two of which are fit to pass through an initial bottleneck in infection. Subsequent replication of these viruses in different tissues generates further quasispecies that are again acted in by selective forces at further bottlenecks in infection.



a specific bottleneck. Bottlenecks may also be responsible for significant variations in the kinetics of infection between hosts. For example, a variant capable of bypassing a given bottleneck may arise sooner in one host than in another. Refer to the eBook for an expanded discussion of how VEE rapidly mutates to overcome bottlenecks in infection.

An example of a bottleneck in infection exerting a selective pressure comes from HIV infection. When an infected person exposes an uninfected person to the diverse HIV quaspecies that develops during chronic infection, the viruses that emerge in the new host represents only a subset of the viruses in the inoculum.^{106,301} This suggests the existence of a bottleneck early in infection through which some viruses selectively pass. Detailed study shows that HIV viruses that utilize the CCR5 co-receptor are more efficient at spread and initial replication in both men and women than viruses that utilize CXCR4 as a co-receptor.^{167,214,231} This suggests that co-receptor utilization is a critical event for passing through a bottleneck during the initial stages of infection. After passage through this bottleneck, HIV isolates that utilize CCR5 are less fit for later events in infection leading to selection of viruses utilizing CXCR4 as a co-receptor. Thus, a virus that is more effective at passing one bottleneck in infection can be replaced, via mutation and selection, by viruses more fit for bypassing subsequent bottlenecks in infection. For these reasons, one must not assume that the virus initially entering the host is the virus responsible for viral disease but must consider stochastic events and selective pressures in models of pathogenesis.

An important consequence of passage through a bottleneck is contraction of quaspecies heterogeneity. When a mutant virus is selected, allelic variations in regions of the genome that are not under selective pressure are carried along through the bottleneck. Thus, random mutation followed by selection can generate novel viruses with properties that are not selected for at all and may therefore have unique pathogenic properties. For example, a virus may mutate sequences encoding a T-cell epitope to escape control by T lymphocytes, and this new sequence may confer previously unexpected properties to the virus. Therefore, selective pressure may result in the emergence of different viruses in different hosts, providing a

mechanism for the emergence of viruses with new properties in a population of susceptible individuals.

Conceptualizing Viral Pathogenesis as the Integrated Effects of Host Genetic Variation

The previously discussed ways to conceptualize viral pathogenesis are highly focused on the virus, but it has been clear since the earliest studies of viral pathogenesis that hosts differ significantly in genetic susceptibility to infection.^{44,301} The major host determinant of viral virulence and pathogenesis is innate and adaptive immunity, but host genes not involved in immunity also play a role. Allelic variations in these host genes can alter viral pathogenesis (Fig. 10.5).

The extent to which allelic variations in host genes control viral pathogenesis is only now being appreciated. Some examples are well established, whereas others come from single studies and await confirmation. Mutations in CCR5 confer resistance to HIV infection.^{57,164,248} Human noroviruses (type virus Norwalk) are responsible for more than 90% of the epidemic nonbacterial gastroenteritis in the world.^{61,90} Norwalk virus susceptibility is determined by blood group secretor status conferred by the presence of the FUT2 fucosyltransferase.^{127,163} Among human norovirus strains, there are multiple patterns of virus-like particle (VLP) binding to blood group carbohydrates, suggesting that allelic variation in human blood groups contribute to susceptibility to a variety of norovirus strains. Patients with mutations in the IFN γ receptor have been reported to have unusual viral syndromes.²²⁶ Autosomal dominant mutations in the chemokine receptor CXCR4 have been associated with severe warts,¹⁵⁴ and mutations in *EVER1* and *EVER2* have been associated with an unusual clinical presentation of papillomavirus infection called *epidermodysplasia verruciformis*.²³⁷ Allelic variations in mannose-binding lectin and Fc γ RIIA have been linked to the severity of severe acute respiratory syndrome (SARS).^{130,320} A relationship between expression of certain KIR genes, encoding NK cell receptors, and severity and chronicity of infection with HIV, HCV, and EBV have been reported.⁴³ Responsiveness to hepatitis B vaccine may be linked to allelic variations in complement genes.^{25,119} Thus, a significant number of allelic variations in

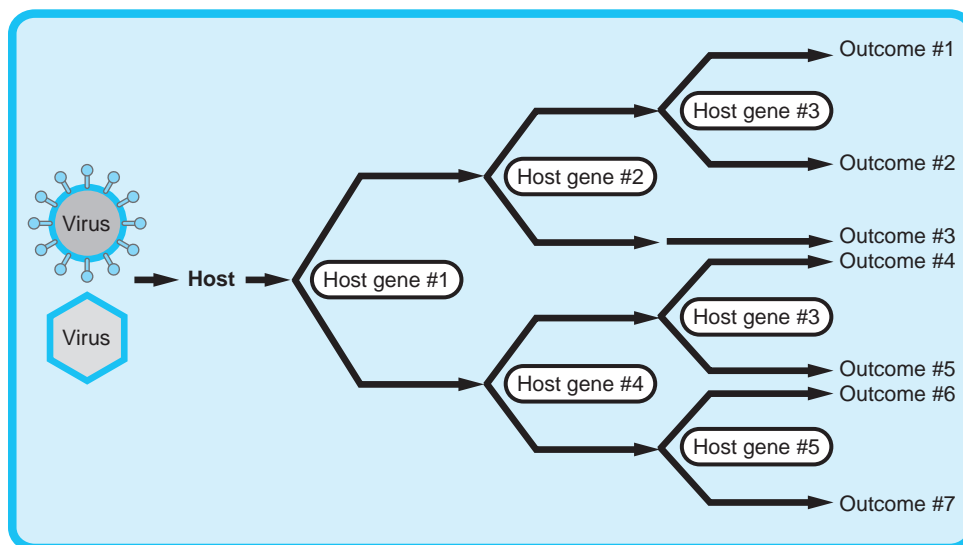


FIGURE 10.5. Viral pathogenesis as the result of the action of a series of host genes. Shown is a representation of infection with different viruses progressing through the host as acted on by host genes that confer either resistance or susceptibility to infection. Depending on the effects of host gene 1, one of two different patterns of infection emerges. In turn, additional host genes interact with the virus, resulting in different outcomes for the host. Note that the more host genes play a determining role, the greater the number of possible outcomes.



humans are important determinants of viral pathogenesis, and additional candidates for such variation are being found at a high rate. Refer to the eBook for further discussion of how variation in blood group secretor status affects susceptibility to human norovirus infection.

Although many studies have focused on the impact of variation in a single gene on viral susceptibility, it is clear that susceptibility or resistance to viral infections is a complex phenotype regulated by multiple interacting genes and gene networks. For example, studies designed to identify genes that regulate resistance to influenza infection between two mouse strains have identified multiple quantitative trait loci (QTL) that contribute to disease susceptibility.²⁸ Although the full number of host genes that vary between susceptible and resistant hosts for a single virus is only beginning to be understood, it is likely that variation in hundreds of different genes may contribute to disease resistance or susceptibility.^{44,302} Recent advances in the area of host genetics, including the application of genome-wide association studies (GWAS), which scan the genome for allelic variants, such as single nucleotide polymorphisms (SNPs), associated with specific phenotypes, as well as whole exome and whole genome sequencing, promise to further enhance our understanding of how different polymorphic genes contribute to disease susceptibility. For example, recent application of GWAS approaches to identify genes that associate with HIV control identified a major association between polymorphisms in the human leukocyte antigen (HLA) locus that affect class I peptide presentation to CD8 T cells and an individual's ability to control HIV.²²¹ These approaches have also been extended to HCV and recently identified a polymorphism within the *IL28B* gene (interferon λ 3) that associates with viral clearance following therapy with interferon.⁸⁴ These types of findings have direct implications for predicting treatment outcomes for infection in patient populations, as has been suggested for inflammatory diseases.³⁰⁰

The examples outlined previously illustrate that allelic variants in host genes can have a significant impact on the pathogenesis of infection (see Fig. 10.5). As multiple independently segregating genes contribute to the susceptibility or resistance to a viral pathogen, many human genotypes exist that may vary significantly in their resistance to even a single viral infection. Therefore, even without considering the impact of viral virulence determinants or other environmental factors, much of the variation in viral pathogenesis within a diverse population could be explained by polymorphisms in host genes. This has implications not only for viral pathogenesis in human populations but also for studying viral pathogenesis in animal models. Although GWAS and candidate gene studies in humans can establish an association between a polymorphic gene and susceptibility to a viral infection, the investigation of the mechanisms by which a polymorphic gene or genes affects pathogenesis requires additional tools, including appropriate animal models. However, pathogenesis studies are often performed in genetically homogeneous experimental hosts (e.g., inbred mouse strains), which results in the loss of any significant effect of host genetic variation on viral pathogenesis and limits investigation of how allelic variants affect pathogenesis outcomes. New model systems that more accurately model the genetic diversity that is present in human populations, such as the Collaborative Cross mouse RI panel,¹¹ as well as classical mouse genetics systems, including BXD recombinant inbred

lines,²⁸ should enhance studies of the role of host variation and gene:gene interactions in viral pathogenesis.

THE STUDY OF VIRAL PATHOGENESIS

Many different tools must be used to understand the complex process of viral pathogenesis. Some of these tools have played essential roles in understanding pathogenesis and will continue to be useful. Examples include assays for infectious virus and histopathologic evaluation of infected tissues. Others are more recently developed and have only been applied in limited circumstances. Understanding the tools of experimental pathogenesis research is essential to understanding the interpretation of experiments that have contributed to our current understanding of virus infection.

Epidemiology

Epidemiology is an essential tool for pathogenesis research for defining patterns of disease and infection and the mode of transmission between hosts. Together with assays for prior infection such as serology or molecular detection of chronic virus infection, epidemiology can define the relationship between infection, immunity, and disease. Epidemiologic studies link a virus to a specific disease and allow formulation of the fundamental questions that must be answered to understand viral pathogenesis. This is nicely illustrated by the identification of Kaposi's sarcoma (KS) herpesvirus, where epidemiology studies suggested that HIV status alone was not an accurate predictor of KS risk, indicating that an additional co-factor was responsible for KS.^{48,250} Following the discovery of KSHV,⁴⁸ additional epidemiologic investigations convincingly linked KSHV infection with KS via demonstration that KSHV sequences were present almost universally in KS lesions and that seroconversion to KSHV preceded the development of KS.¹⁷⁴

In some cases, epidemiologic investigation finds that known viruses explain but a part of the disease burden, leaving open the question of whether unknown agents contribute to disease. The process of associating new viruses with both viral and nonviral diseases continues to this day, with new viruses constantly emerging or being identified as responsible for already described diseases.

Animal Models

Animal models are fundamentally important tools for the study of viral pathogenesis. The need to understand human infection, combined with the complexities of doing human studies, has led to the use of animal models to ask pathogenesis questions that cannot be effectively answered via human studies. There are two types of animal models for human viral disease. In the first, one studies a human virus in infected animals. In the second, one studies an animal virus that is related to a human virus in its animal host. There is an essential tension between these two approaches; in one the "real" pathogen is studied, and in the other a "natural" infection is studied. In truth, each has its advantages and each its limitations.

Study of Human Viruses in Animal Models

Human viruses can be studied in animals that are susceptible to infection either because the virus does not exhibit species tropism or because tropism restrictions are overcome via genetic

manipulation of the host or virus. An important caveat of such studies is that human viruses seldom or never behave in an experimental animal exactly as they do in humans. Nevertheless, this is a very valuable approach. Examples include the use of mice to study HSV, Sindbis virus, yellow fever virus, VEE, LCMV, or chikungunya viruses; the use of humanized mouse models to study HCV and HIV; and the use of primates to study poliovirus, variola, HIV, and Ebola virus.

Excellent examples of this approach are the analysis of infection with filoviruses such as Ebola or Marburg that are very difficult to study in infected patients. However, these viruses cause disease in macaques with significant similarities to human disease, including a striking hemorrhagic diathesis including disseminated intravascular coagulation.^{85–87} These animal models have been used to demonstrate that it is possible to vaccinate against filovirus infection^{136,270,319} and that passive transfer of antibody can be partially protective.^{105,216}

Not all human viruses can replicate in animals. Five approaches have been taken to overcome this hurdle. These approaches are, first, passage-based adaptation of the human virus to growth in an experimental animal; second, engineering of the host to accommodate all or part of the pathogenesis of the human infection; third, expressing the virus as a transgene in an experimental animal; fourth, the creation of humanized mice where immunodeficient mice are reconstituted with aspects of the human immune system and components of the human target organ (e.g., the liver for HCV); and fifth, targeted modification of viruses to allow replication in a model host. Increasing knowledge of the mechanisms of viral pathogenesis and immunity holds out great hope for generations of better animal models.

1. In the first approach, a human virus is adapted to growth in an animal model. Ebola has adapted to infect guinea pigs and mice.^{35,52,89} Ebola infection of small animals is similar to primate and human Ebola infections in some ways. For example, dendritic cells (DCs) and monocytes are early targets of infection in all of the different models. However, mice and guinea pigs do not show the hemorrhagic diathesis seen in humans and macaques, which is a significant limitation for pathogenesis studies.^{35,52,85,89}
2. In the second approach, the host is genetically engineered to allow analysis of a human virus. For example, transgenic expression of the poliovirus or measles virus receptors in mice confers susceptibility to intracerebral infection with poliovirus or measles virus.
3. In the third approach, the virus is expressed as a transgene in a live animal, allowing the replication cycle of the virus to proceed in certain cells even though the host is nonpermissive for infection. This has been accomplished for HBV with mice engineered to generate infectious virus from a transgenic viral genome.^{103,104}
4. In the fourth approach, mice are used as hosts for human tissue allografts that can then be infected with human viruses. This approach is particularly useful for viruses that fail to replicate in nonhuman systems and has been applied to viruses such as HIV¹⁵⁵ and VZV.^{14,249,321}
5. In the fifth approach, the virus is manipulated in a specific way to allow infection of the animal to be used as a model. For example, based on an intimate knowledge of the mechanisms of lentivirus species tropism, it has been possible via

manipulation of the HIV *vif* gene to create an HIV isolate that can replicate in macaques.¹¹³

Study of Animal Virus Infections That Resemble Human Infection

It is often impossible to approximate all aspects of the pathogenesis of a human virus in an animal model. An important alternative approach is to study the pathogenesis of an animal virus that is related to a human virus in an animal host. Examples include the study of murine cytomegalovirus (MCMV) as a model for HCMV, simian immunodeficiency virus (SIV) as a model for HIV, murine γ HV68 as a model for human γ -herpesvirus infection, myxoma virus and ectromelia virus as models for smallpox, and murine norovirus infection as a model for human noroviruses. These models provide, especially in the murine system, tools for doing pathogenesis and host genetics that are unavailable in any other model.

An important caveat of this approach is that animal viruses seldom faithfully reproduce in every detail of a human disease. This is not surprising; after all, animals are not humans and animal viruses are not identical to human viruses. These dissimilarities are often used to argue against the utility of such animal models. However, many principles and mechanisms of viral pathogenesis have first been described in animal models and later proven relevant in humans.

Viral Genetics as a Tool for Analysis of Pathogenesis

Studies taking advantage of genetic differences between viral strains are as old as the study of pathogenesis itself. Comparisons between viral strains can identify correlates of virulence or attenuation but do not necessarily shed light on the mechanisms responsible for the phenomena observed. Reliance on naturally occurring strains of virus has strict limitations for defining pathogenesis mechanisms, because it is only by luck that genetic variation occurs between strains in a manner that allows a specific hypothesis to be tested. Thus, advent of sequencing, structural biology, and directed mutagenesis of viral genomes as tools for pathogenesis research has been a turning point for pathogenesis as an experimental science. Infectious clone technology now exists for most virus families, and by applying directed mutagenesis strategies to these molecularly cloned viruses, mechanistic hypotheses can be tested directly. Often, this is done using a loss-of-function genetic approach in which an entire viral gene is deleted or in which a mutation is used to ablate a specific biochemical function of a viral protein. By evaluating such viruses, one can link structural and biochemical properties of a protein to specific aspects of pathogenesis, thus making loss-of-function genetic approaches a fundamental part of current pathogenesis research.

It is important to understand the limitations of viral mutagenesis as an approach. First, loss-of-function genetic analysis depends on whether a viral property can truly be attributed to a specific mutation. Further, it is necessary to prove that a phenotype is not an artifact of changes that occur during manipulation of the viral genome. This is often accomplished by *marker-rescue*, in which the mutant virus is repaired to wild-type status and the resulting virus characterized. If this marker-rescued virus is different from the wild-type virus, it indicates that other mutations have occurred during the process

of mutant generation, thereby invalidating conclusions drawn using the mutant. This can be particularly problematic for rapidly evolving RNA viruses that may select compensatory mutations during generation of viral stocks. An alternative is to fully sequence the viral mutant—an approach that is now increasingly practical for even the largest viruses.

It is also necessary to prove that a phenotype is owing to a change in a viral protein and not to alterations in cis-elements in the viral genome or in adjacent genes that may confer phenotypes. For example, mutating a residue in a protein might alter a promoter for another viral gene or change the processing of a viral polypeptide. These problems are addressed by studying multiple distinct mutations in a protein, analysis of the expression of other viral proteins, or, when possible, complementing the mutation by expressing the protein in trans from another location in the virus.

A second limitation of loss-of-function genetics is that linking a mutation in a virus to a specific alteration in pathogenesis shows that the gene in question is necessary but does not indicate that the gene is sufficient for the virus to perform a specific task. For example, several genes might be important for an aspect of pathogenesis such that mutation of any one of them would give a phenotype; however, no single gene is independently sufficient for the virus to perform a certain task *in vivo*.

A third limitation of loss-of-function genetics is that the identification of an important role for a gene at a given step in pathogenesis does not provide information on the mechanisms responsible for the phenotype observed. In the absence of additional studies, one can only make correlative statements about the relationship between the behavior of a mutant virus and the biochemical properties of the protein encoded by the altered gene. For example, if a certain amino acid is important for binding of a host protein by a viral protein, and that same mutation causes the virus to be defective in pathogenesis, it is plausible that the phenotype observed *in vivo* relates to the binding between the host and viral protein. Proof of mechanism *in vivo* requires additional studies.

Interactions Between Host and Viral Genes in the Study of Pathogenesis

Defining the mechanism of action of a viral protein or gene *in vivo* is much more complex than showing that the gene is important, requiring that one determine experimentally why pathogenesis is altered by a specific mutation. It is this latter level of analysis that provides the greatest challenges. The attribution of changes in pathogenesis to a specific biochemical property of either the host or the virus is a complex task. The most important question is whether a change in the behavior of a virus *in vivo* is attributable to direct effects of a given mutation or is owing to indirect effects of the mutation. To exemplify this problem, consider the case of a mutant virus that both grows more poorly and induces a lesser inflammatory response than the wild-type virus. One interpretation of such data might be that the mutation influences the function of a gene that evades or subverts the host inflammatory response. A more trivial interpretation is that the host responds to a virus that grows poorly with a lesser inflammatory response. In this situation, one cannot cleanly attribute an immune evasion property to the viral protein in the absence of additional data.

This problem is conceptually similar to the Heisenberg uncertainty principle in physics in which detecting an electron requires the use of a particle that in turn changes the location of the electron. Analogously, one determines the function of the viral gene by mutating the gene; however, mutating the gene can change the process of infection sufficiently to complicate interpretation of any differences observed in pathogenesis.²⁹⁷ A major challenge in defining mechanisms of viral pathogenesis is then to identify genetic, structural, and pathogenesis approaches that allow attribution of events *in vivo* to specific biochemical mechanisms.

One method for doing this involves using a combination of host and viral genetics in a process termed *host complementation*. For example, the *ICP34.5* gene of HSV is essential for viral virulence.^{27,50} One activity of ICP34.5 is to bind protein phosphatase 1A (PP1A) and to redirect PP1A phosphatase activity to dephosphorylation of eIF2 α , thereby reversing the eIF2 α -dependent antiviral effects of PKR.^{51,115,116,245,277} If ICP34.5 is acting to promote virulence through interactions with PKR, the ICP34.5 mutant should exhibit restored virulence specifically in mice lacking PKR, but not mice lacking other antiviral effector molecules. In fact, deletion of PKR, but not RNase L, from mice fully restores the virulence of an ICP34.5 mutant virus,¹⁵⁶ thereby establishing that ICP34.5 interacts with PKR *in vivo*. Other examples of this approach include the role of the influenza virus *NS1* gene in countering STAT-1 dependent innate immunity,⁸³ the role of the murine γ HV68 complement regulatory protein v-RCA in evasion of complement responses,¹³⁸ and the role of the *m157* gene of MCMV as natural killer (NK) cell-activating receptor ligand.^{9,78,260} In all of these studies, deletion of a host gene or cell type has been used to define the mechanisms of action of a viral gene during infection.

It is important to note that this approach is limited to analysis of host genes that do not play an essential role in development or the ontogeny of the animal or the immune system. Moreover, it is essential to use controls including a viral mutant that is unaffected by mutation of a host pathway and a host mutant in which the viral mutant maintains its attenuated phenotype. Simply showing that a mutant virus regains growth or virulence in an immunocompromised host does not constitute genetic proof that a viral gene is countering a specific host pathway.

An important correlate of these experiments is that one must interpret studies of wild-type viruses in animals lacking specific genes with caution. For example, if a mouse lacking a certain gene is capable of resisting infection normally, it is often concluded that the host gene is unimportant. An alternative interpretation is that the virus encodes a molecule that effectively inhibits the host pathway in question, making even a wild-type host effectively deficient in the pathway. The erroneous conclusion that a host pathway cannot play a role in infection has important consequences. For example, inhibiting the viral protein responsible for evading a protective host response might allow the host to effectively control infection. Thus, potential therapeutic approaches for viral infection may be missed by ignoring the possible presence of effective viral strategies for evading host responses.

Cell Culture

Cell culture is an essential tool for the study of viral replication and tropism. However, conditions in cell culture are not

representative of conditions *in vivo*, and thus hypotheses from cell culture experiments must be validated *in vivo*. One obvious limitation to cell culture studies is the absence of a cellular immune response. There are additional important limitations to cell culture studies. Often, a small proportion of cells in a tissue are actually infected at a given time, whereas cell culture is often optimized for synchronized infection of all cells. This obviates effects of infected cells on as-yet-uninfected cells—a fundamentally important part of what happens in tissues. For example, interferon released from one infected cell can protect uninfected cells from viral infection. As interferon effects generally require induction of gene expression, pretreatment of cells in culture is usually required to see full effects of interferon on viral infection, effects that are lost if all cells are simultaneously infected. Furthermore, cultured cells are often transformed or continuous lines whose behavior is at most distantly related to the behavior of primary cells. Even when primary cells are used in tissue culture, it is unlikely that the biology of these cells is the same as the biology of cells residing in a tissue in contact with physiologic extracellular matrix, the circulatory system, the endocrine system, and other primary cells.

DETERMINANTS OF CELL, ORGAN, AND TISSUE TROPISM

One of the most important concepts for understanding pathogenesis is the concept of cell, organ, and species tropism. Tropism is determined by many factors in both the virus and the host, including how the virus enters the host, how the virus spreads within the host (lymphatic, neural, or hematogenous spread), the permissiveness of specific cell types for the virus (as defined by receptors, cellular differentiation, and intrinsic cellular resistance to infection), the nature of innate and adaptive immune responses, and specific properties of tissues such as accessibility and effectiveness of the immune system (immunoprivilege). Each of these factors can play a determining role during viral infection and must be considered when defining mechanisms of viral pathogenesis.

Entry into the Host

A virus must access permissive cells to establish infection and therefore must overcome a series of anatomic and innate immune barriers to enter the host. The route of entry and the mechanisms of spread are therefore important determinants of viral tropism. The route and tissue through which a virus infects the host may be clear from epidemiologic studies. For example, both measles virus and VZV spread by the respiratory route, and polioviruses and noroviruses spread fecal–orally. However, even when the route of infection is known, the precise events involved in entry into the host are mostly unknown. Addressing this apparently simple question is a major challenge for pathogenesis research. The lack of knowledge of this critical step in pathogenesis is owing to the difficulties in studying viral spread under natural infection conditions when only a few infectious virus particles are sufficient to establish infection.

The critical determinants of viral spread include the form of the virus that spreads, the capacity of the virus to survive in the environment, the route of natural exposure to the virus, the mechanisms by which the virus gains entry, and the nature

of host barriers to infection. Host barriers may include non-specific barriers, barriers based on pattern recognition by the immune system, and barriers based on pre-existing adaptive immune responses.

Viruses may enter the body in different forms and via different vehicles.¹⁹⁸ The viral strategy for overcoming host barriers to entry is tightly linked to the form of the virus that spreads and how the virus is shed from the previously infected host. For example, viruses may enter carried in infected cells, may be injected via the mouth parts of arthropods,³⁰⁷ may be contained in droplets or fomites shed from an infected host, or may be ingested as free virus. To spread in a population, viruses may benefit from survival in the environment. For example, HIV is relatively unstable in the environment and does not effectively spread via environmental surfaces. This contrasts dramatically with noroviruses such as Norwalk virus that can spread via contact with contaminated environmental surfaces.¹²⁶

There are six primary portals of entry for viruses, each used by a variety of viruses. Five of these are epithelial surfaces: skin, conjunctiva, respiratory tract, gastrointestinal tract, and genitourinary tract. The sixth is the unique interface between the mother and the germ cell or the developing fetus.

Penetration Through Epithelial Barriers

The body is covered by epithelia, presenting a large surface for viruses to access. However, epithelia share several properties that inhibit viral entry. For example, epithelial cells are constantly turning over and being replenished; thus, cells that are contacted by a virus are shed continuously. The skin has an added protective mechanism, being many cells deep with the surface comprised of metabolically inactive cells that cannot support viral replication. In addition, barriers may be protected by low pH or secretions, including mucus.

Epithelial tissues are highly active immune organs. In all epithelia, DCs (e.g., Langerhans cells in the skin) serve as sentinels for invasion, having the capacity to, when activated, move to lymph nodes to induce immune responses. These sentinel cells play a dual role in infection, both as critical for induction of immunity and as cells targeted by viruses as an initial site of infection. For example, when VEE is inoculated into mice, the first cells infected are DCs; these cells rapidly move to draining lymph nodes, providing the virus access to the lymphatic system but potentially at the cost of the induction of immunity¹⁷¹ (Fig. 10.6). DCs lie beneath the intestinal columnar epithelium, and thus the intestine, as with skin, has a resident DC population plausibly involved in sentinel functions.^{146,202}

Intraepithelial lymphocytes are present in subepithelial and epithelial tissues, providing cells capable of protective immune responses in the most superficial layers of the body. In addition, epithelial cells themselves may be activated to express interferons or other antiviral molecules. In many sites, an invading virus is subject to inactivation by antibodies and complement. Even when a virus passes superficial epithelial barriers, the virus must confront the innate and adaptive immune response as well as an increasingly well understood set of intracellular barriers to infection collectively referred to as intrinsic cellular resistance to infection.

Viruses cross epithelial barriers through mechanical breaches (e.g., vectorborne delivery of arboviruses or bite wound delivery of rabies) or by accessing specialized cells

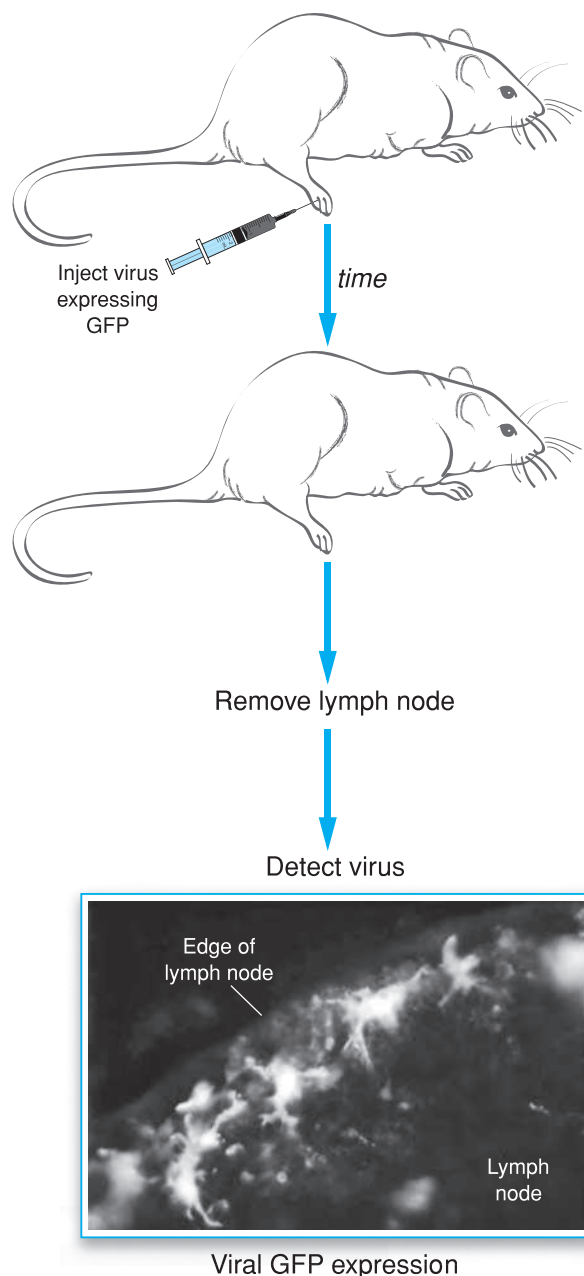


FIGURE 10.6. Infection of dendritic cells early after virus infection. To determine which cells are initially infected with VEE, a mutant virus expressing GFP was constructed and inoculated into the hindlimb (as described in reference 171). This mutant is capable of only a single round of infection; thus, GFP expression is restricted to cells infected by virus in the inoculum. Twelve hours later, the draining lymph node was resected and imaged for GFP expression. In the bottom image, dendritic cells within the lymph node express GFP, demonstrating that dendritic cells are initial targets of VEE infection. VEE, Venezuelan equine encephalitis virus; GFP, green fluorescent protein. (GFP image courtesy of Dr. Robert Johnston.)

within the epithelium. An excellent example of this latter situation is provided by reovirus, which accesses the Peyer's patch through specialized epithelial cells called M cells.^{54,147,200} Upon entry into the intestine, the reovirus virion is proteolyzed, triggering a remarkable structural transition with cleavage and loss

of $\sigma 3$, cleavage of $\mu 1$, and conformational changes in both $\sigma 1$ and $\lambda 2$ that generate the infectious subviral particle (ISVP) (Fig. 10.7). The ISVP binds to M cells, which then transport the virus into the Peyer's patch where productive infection occurs.

Vertical Spread of Viruses

Many viruses infect either the immature fetus or the newborn during the birth—a process referred to as vertical transmission. There are two mechanisms for entry into the developing fetus. The first is via placental penetration, as when a virus enters the fetus after invasion of the fetal circulation or amniotic fluid. Viruses such as HCMV and rubella can spread transplacentally,^{22,201} with devastating consequences for the developing fetus. Viruses such as human endogenous retroviruses (HERVs) can also be vertically transmitted via the germ line and constitute a significant fraction of the human genome.^{132,140} HERVs continue to proliferate within the genome and are likely to exert both beneficial and detrimental effects on their hosts. Refer to the eBook for more discussion of the vertical spread of viral elements encoded in the host genome.



Systemic Spread of Virus Infection

Once a virus has passed through epithelia or penetrated the placenta, the virus may still be far from its target cells and tissue(s). Viruses spread via three host systems that can provide access to a large number of tissues and cells: blood, lymphatics, and nerves. Although the blood is a major highway for spread of viruses through the host, many viruses use nerves or a combination of hematogenous and neural spread to access host tissues.

The level of viremia has been correlated with the severity of acute viral disease, the prognosis of chronic viral disease (as in HIV), the extent of viral dissemination, and the efficiency of viral spread between hosts.^{198,307} The level of viremia is a function of viral access to the blood, viral clearance from the blood, and the vehicle (plasma vs. cell associated) that the virus uses to travel through the blood. Viruses can access the blood either directly via introduction into the circulation, as by a needle or an insect bite, or indirectly after entering into and replicating in tissues. A very common finding is the rapid appearance of a virus in draining lymph nodes or in lymphoid structures such as Peyer's patches or tonsils. The connection between viral spread to lymphoid structures and subsequent spread to the rest of the host was recognized early on.^{73,182}

Entry into lymphoid tissue is a two-edged sword for the virus, as a facile route to access the viscera of the host but one that passes through the very tissues that generate adaptive antiviral immune responses. For viruses that primarily infect mucosal surfaces such as influenza virus, rotavirus, papillomaviruses, rhinoviruses, and noroviruses, it is likely that the primary effect of entry into the lymphoid system is the induction of antiviral immune responses. However, there are clear advantages for lymphoid invasion if the virus has tropism for cells of the immune system that are capable of circulating and entering tissues. For example, HIV and EBV each have tropism for lymphocytes for latent and/or productive infection, and both MCMV and HCMV can spread through the body in infected cells of the monocyte-macrophage lineage.

In many cases, viruses can access the nervous system by infecting neurons in the periphery and then spreading along

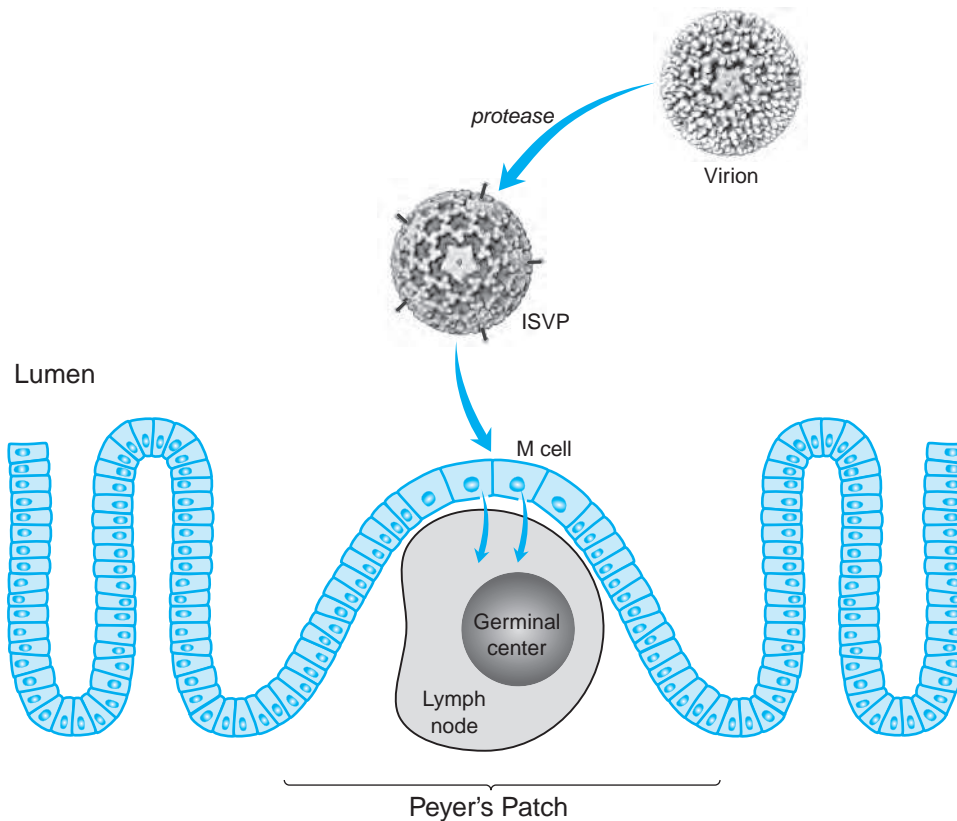


FIGURE 10.7. Reovirus entry into Peyer's patches in the intestine.

Reovirus enters the body as an intact virion. The action of proteases in the intestinal lumen cleaves proteins on the surface of the virion, generating a highly infectious form of the virus—the intermediate subviral particle—which interacts with the microfold or M cell overlying the Peyer's patch and is transported into the Peyer's patch where infection is established. From this point, the virus may enter the bloodstream or enter nerves for spread to organs such as the brain and spinal cord. (Reovirus images courtesy of Dr. Terrence Dermody.)

axons toward the CNS.¹⁹⁸ The classical example of this strategy is rabies virus, which spreads along nerves from the area of inoculation to the CNS.¹³¹ The time between inoculation and development of signs and symptoms of rabies encephalitis depends on the length of the nerves between the site of inoculation and the CNS. The virus travels up nerves toward the CNS at a rate of 50 to 100 mm/day, and the disease can be cured by surgical removal of the infected limb as long as the virus has not entered the CNS.^{13,131} For example, if the initial bite is on a lower extremity, there is a longer time within which vaccination and passive transfer of rabies-immune antibody can be effective than if the bite is on the face.¹³¹ Studies with several viruses, including HSV, pseudorabies virus, and reoviruses, have been used to elucidate the viral and cellular determinants that regulate viral spread through neural tissues, including sophisticated genetic and molecular mechanisms that regulate viral anterograde and retrograde axonal transport. Importantly, the immune system can also modulate neural spread. For example, antibodies can interrupt many of the steps in neural spread of reoviruses to and within the CNS (Fig. 10.8). Refer to the eBook for an expanded discussion of the mechanisms of neural spread of viruses.

Determinants of Cell, Tissue, and Species Tropism

Once a virus has spread via lymphatic, hematogenous, or neural routes, a fundamental determinant of viral pathogenesis becomes the distribution of the virus between and within tissues of the host. Distribution of virus in tissues is a dynamic process determined by competing processes, including the speed of viral replication, the presence of specific viral receptors

or other pro-viral factors that permit viral entry or replication, viral mutation rate, viral virulence genes, host susceptibility and resistance genes, and innate and adaptive immunity. It is useful to think of tissue distribution of a virus as an ongoing battle between the virus and the host being played out in different tissues. This battle has very local aspects, such as the contact between a virus and a specific cell or the contact between an NK cell or cytolytic T cell and a virus-infected cell. However, the outcome of this battle is also determined by effects over short distances in tissues, as, for example, the effects of host cytokines or virus-encoded soluble proteins that evade or subvert host responses. Lastly, there are long-range effects of host responses on infection, including production of antibody, synthesis of stress steroids, activation of the bone marrow to produce inflammatory cells, stimulation of the liver to synthesize and release acute-phase reactants such as complement proteins, and stimulation of autonomic centers in the brain to produce fever.

The Role of Viral Receptors in Cell, Tissue, and Species Tropism

An important step in viral infection, and a primary determinant of the distribution of virus between and within tissues, is the interaction of a virus with specific receptors on permissive cells. Receptor expression plays a major role in determining the tropism of several viruses, including poliovirus and measles virus, and the use of molecular tools, including transgenic mice, expressing the virus-specific receptors provides key insights into the role of receptors in regulating viral tissue tropism and pathogenesis. The importance of viral–receptor interactions is further illustrated by the fact that zoonotic viruses must often

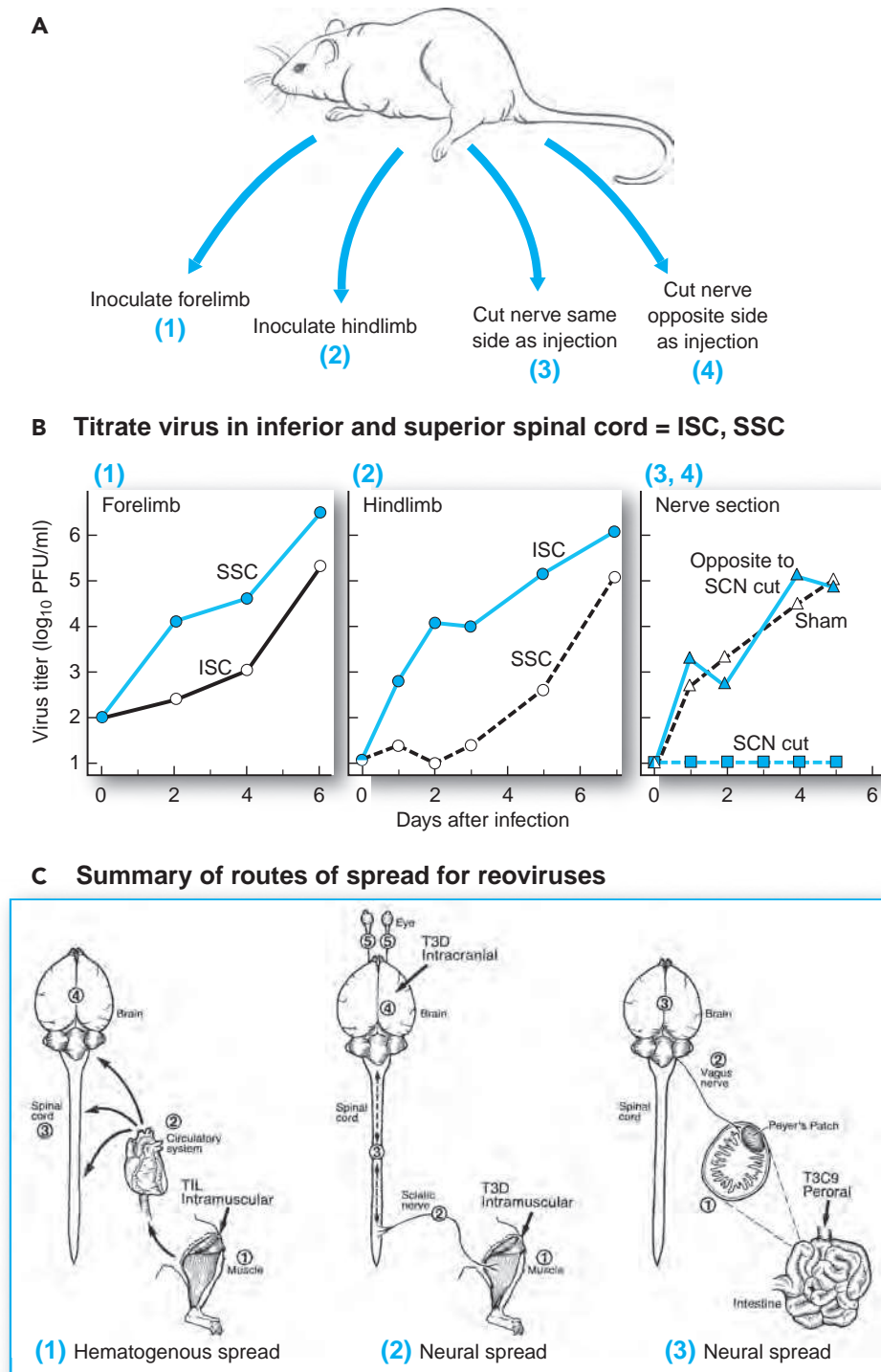


FIGURE 10.8. Neural versus hematogenous spread of reoviruses. **A:** Experimental design. **B:** Viral titrations after various experimental manipulations. **C:** Summary of the routes of spread of reovirus serotype 1 strain Lang (T1L), serotype T3 strain Dearing (T3D), and serotype 3 clone 9 (T3C9). (Adapted from Tyler KL, McPhee DA, Fields BN. Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. *Science* 1986;233:770–774, and from Nathanson N, Murphy FA. Evolution of viral disease. In: Nathanson N, Ahmed R, Gonzalez-Scarano F, et al, eds. *Viral Pathogenesis*. Philadelphia: Lippincott-Raven; 1997:353–369.)

adapt to receptors in their new host species to effectively cause disease and disseminate, as was the case with SARS.¹⁶¹ These binding interactions are necessary for infection but are often not sufficient to explain all aspects of cell, species, and tissue tropism. For example, CD4 and chemokine receptors such as CCR5 or CXCR4 confer susceptibility to HIV infection; however, permissiveness is also controlled by cytoplasmic proteins that restrict HIV infection.

Often, viruses use a binding receptor to increase the concentration of virus at the cell surface and one or more entry receptors. An example of this latter strategy is HSV, which interacts with specific sulfated sugars on heparan sulfate²⁵⁵ in a process that enhances infection but is not required if a cell expresses an entry receptor such as the appropriate nectin.^{88,263,269} Another example of this strategy is utilized by serotype 3 reoviruses, which interact with sialic acid via one portion of their cell attachment protein $\sigma 1$ and the protein JAM1 via another portion of the $\sigma 1$ protein.⁵⁴ Importantly, the interaction with the broadly expressed carbohydrate sialic acid has been shown to be important in the tropism of the reovirus *in vivo*, indicating that even interactions with ubiquitously expressed molecules can play a role in cell and tissue tropism.⁵⁴ Tropism can also be conferred by interactions between viral cell attachment proteins and proteases, as is seen with Newcastle disease and influenza viruses.^{41,191} Refer to the eBook for a more extensive discussion of how receptor tropism affects viral pathogenesis using poliovirus infection as an example.

Innate Immunity and Intrinsic Cellular Resistance to Infection Determine Tropism

Factors in addition to specific receptors play a major role in determining viral tropism. Tropism can be determined by proteins responsible for cell-intrinsic defense against viral infection, transcription factors, cell cycle regulators, and microRNAs. For example, the liver-specific host microRNA miR-122 is essential for robust HCV replication,¹³⁷ and inhibition of miR-122 in nonhuman primates limits HCV replication.¹⁵¹

Once a virus has bound to a cell and delivered its genome or capsid to the cytoplasm, a series of events occur that can have a profound effect on the viral cell tropism. These events likely explain why receptor expression does not always explain the cell and tissue tropism of a virus. The host cell expresses on its cell surface and in its cytoplasm molecules that can either directly inhibit viral replication (intrinsic cellular resistance to infection) or can induce a signaling cascade that in turn generates antiviral molecules (innate immune responses). Together, these molecules and pathways are determinants of both the permissiveness of cells for viral replication and species tropism. It is likely that mechanistic relationships will be discovered between molecules involved in innate immunity and intrinsic cellular resistance to infection as more is learned about each of these two important processes.

Molecules involved in innate immunity can be important components of tissue and species tropism. This is illustrated by myxoma virus, which does not normally infect mice but readily infects murine cell lacking type I interferon responses and causes lethal disease in STAT-1 deficient mice.³⁰⁵ Intrinsic cellular resistance to infection is also conferred by molecules such as the TRIM or APOBEC proteins that restrict retrovirus infection. Importantly, allelic variations in the genes involved in these processes may contribute to regulation of viral pathogenesis.

Immunoprivilege

One important determinant of viral tropism is the fact that the innate and adaptive immune systems are not equally efficacious at clearing virus infection from all tissues. The concept that the immune system is selectively ineffective at clearing virus infection from specific tissues is referred to as immunoprivilege. Immunoprivilege plays a significant part in the pathogenesis of many viruses but has been best studied in experimental murine pathogenesis models. For example, in studies of LCMV infection of mice,^{32,38} many organs including liver, spleen, lung, and pancreas are cleared relatively efficiently within 30 days of transfer of immune cells. However, virus persists in the CNS for up to 90 days and in the kidney and genitourinary system for more than 200 days, indicating that both the CNS and the genitourinary tract are immunoprivileged. These data also show that immunoprivilege is a relative term, with the efficacy of the immune system varying depending on when infection is analyzed. Analysis of the mice at a time point more than 90 days after transfer of immune cells identifies only the genitourinary tract as immunoprivileged (Fig. 10.9).

It is likely that the selective inability of the immune system to clear virus infection from certain tissues is owing to a combination of two interrelated factors. The first is intrinsic limitations of immune system function in certain tissues or to limited capacity to address infection of certain cell types. For example, CD8 T cells may be more effective at eliminating infection from major histocompatibility complex (MHC) class I-expressing hepatocytes than from neurons that do not express MHC class I molecules. The second factor is viral evasion of immunity. An example of this is the capacity of HSV to evade clearance by the host immune system via establishment of latency in neurons. It is plausible that in most cases immunoprivilege is owing to a combination of both viral strategies and cell- or tissue-specific limitations in the efficacy of the immune response. The mechanisms responsible for immunoprivilege are poorly defined at the cellular and molecular level.

Cellular Differentiation as a Determinant of Viral Tropism and Pathogenesis

Another important determinant of viral tissue distribution is cellular differentiation. Thus, cells at different stages of differentiation may have specific properties that favor or disfavor viral infection or replication. This is especially true in a tissue responding to virus-induced damage. For example, when hepatocytes are damaged during chronic HBV or HCV infection, the liver regenerates, providing viruses with access to cells in different differentiation states.

Many viruses take advantage of differentiated functions of the cells that they target. For example, HSV establishes latency in fully differentiated neurons, and EBV latently infects memory B cells; in each case, the reservoir for chronic infection is a particularly long-lived cell type. The lytic cycle of gene expression for both HIV and EBV is triggered by induction of lymphocyte activation and differentiation. In each case, a specific relationship between a virus and a certain differentiation state of an infected cell contributes to viral pathogenesis.

Papillomavirus infection of the skin provides an outstanding example of the impact of cellular differentiation on pathogenesis^{26,190} (Fig. 10.10). In normal skin, basal stem cells give rise to ever more differentiated cells that move toward the skin surface, lose their nucleus, become cornified, and are finally

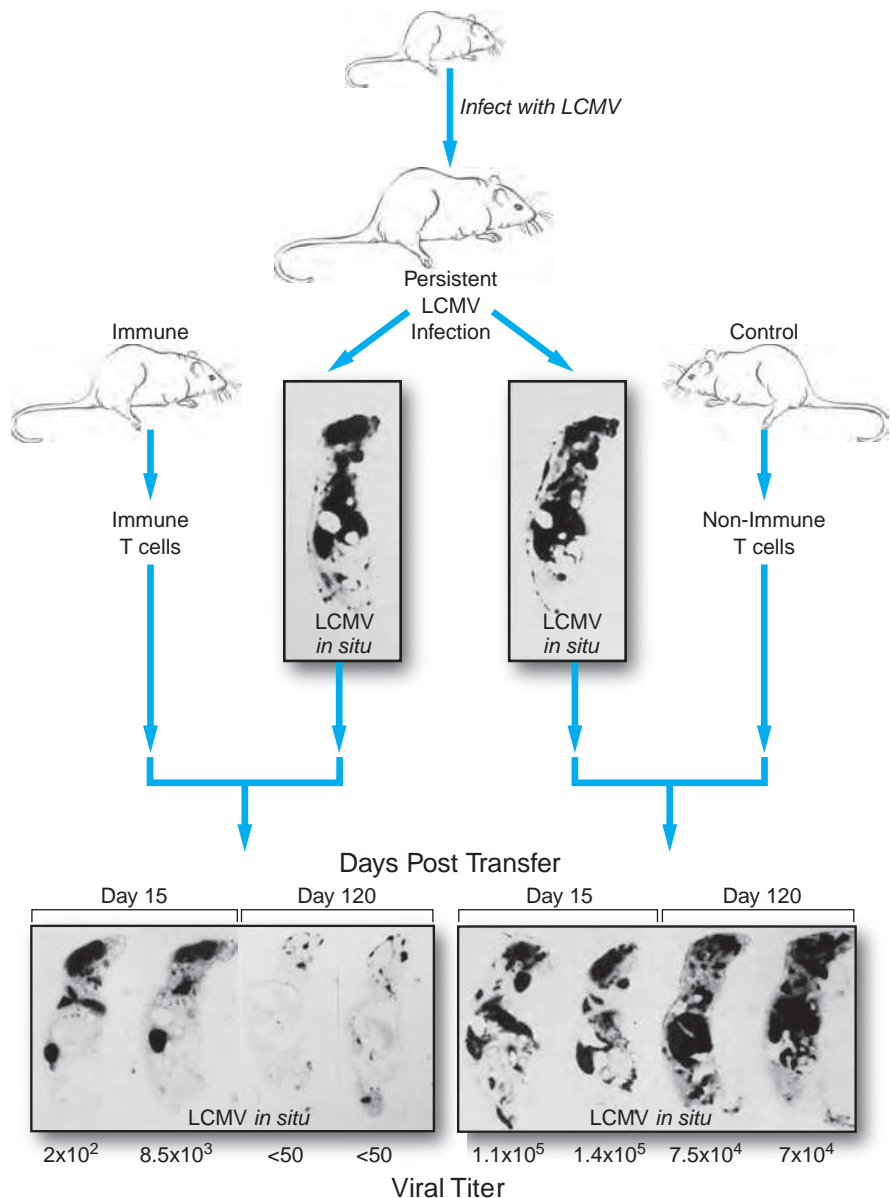


FIGURE 10.9. LCMV clearance by immune T cells reveals immunoprivileged tissues.

Shown is the pattern of clearance of LCMV after transfer of either immune or control T cells into mice with persistent LCMV infection as described in reference 208. The clearance of virus is indicated both by the viral titers shown and by the *in situ* hybridization signal from cross sections of the whole mouse. LCMV, lymphocytic choriomeningitis virus. (Adapted from Oldstone MB, et al. Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature* 1986;321:239–243; courtesy of Dr. Michael Oldstone.)

shed. Papillomaviruses infect basal stem cells of the skin, where they can persist in a latent state for prolonged periods. Activation of a program of gene expression initially involving the expression of E6 and E7 proteins results in cell cycle progression, inhibition of apoptosis, and viral replication. As infected cells differentiate and move toward the skin surface, they become permissive for expression of genes involved in viral replication and assembly (see Fig. 10.10). The normal differentiation process is subverted by the virus, resulting in retention of the nucleus and synthesis of proteins required for viral DNA and protein synthesis. The virus assembles and is released from the skin in shed cells.

The principle that cellular differentiation is a determinant of viral pathogenesis is very important and general. When tissues are damaged, stem cells are activated to generate new cells with consequent proliferation of somatic cells and differentiation into cells such as hepatocytes, endothelial cells,

or epithelial cells. These processes are plausibly subverted by several viruses, many of which grow optimally in replicating cells. One consequence of this principle is that the full potential of a virus's genetic program may not be revealed in a cell line that is transformed or represents a single differentiation state within a particular cell lineage. It is plausible that, as with papillomaviruses, many viruses have genes that play specific roles in the cell at certain stages of differentiation and thereby contribute to pathogenesis.

FATE OF THE INFECTED CELL, TISSUE, AND HOST

The presence of a virus in a tissue may or may not result in damage. The variables that determine the fate of the infected tissue

Virus Infection Changes with Differentiation of a Cell

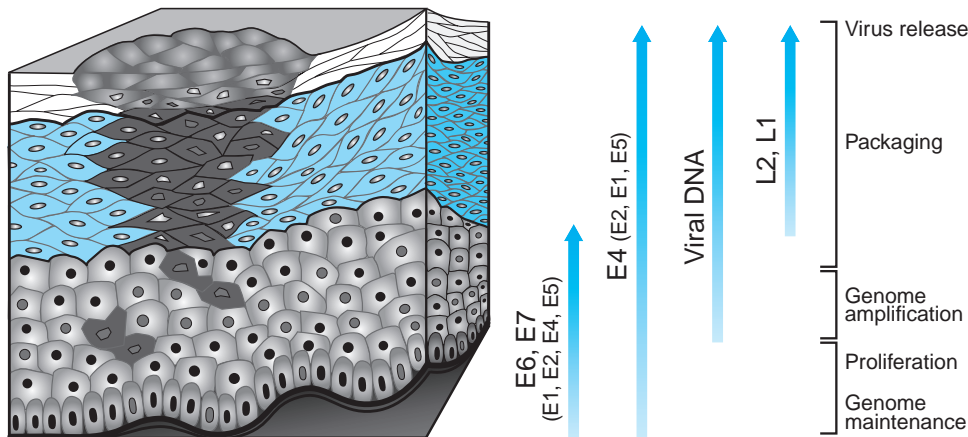


FIGURE 10.10. Critical role of differentiation in papillomavirus pathogenesis. Shown are the relationships between stages in the replication of a papillomavirus on the **far right**, the nature of the progressions of infection through the layers of the skin on the **left**, and the patterns of papillomavirus gene expression as shown by the labeled **blue arrows** in the **center**. Within the skin, virus-infected cells are designated in **dark grey**. (Adapted from Doorbar J. The papillomavirus life cycle. *J Clin Virol* 2005;32:S7–S15.)

and host are complex and involve the interplay between the virus and its cytopathic potential; the age, sex, and nutritional status of the host; the regenerative capacities of different primary cell types; whether the immune system damages the tissue as part of the protective response; whether the virus induces autoimmune responses; and perhaps most importantly, whether the virus is ever cleared from the tissue and, if not, the nature of residual virus infection. Determinants of tissue damage may be local in nature, such as the death of infected cells owing to viral cytopathicity or killing by immune cells, or operative over longer distances, as in the case of vascular damage leading to ischemia, systemic hormonal and cytokine responses, induction of fever or cachexia, cytokine induction of the death of uninfected cells, metastatic tumors, or virus-induced autoimmunity.

Although viruses can directly kill infected cells as they replicate, one is always confronted with the question of whether tissue damage is directly caused by the virus or is indirectly caused by the immune system; what is the balance between the protective and destructive capacities of the immune system? Additionally, viruses can damage the host indirectly by making the host susceptible to secondary infections or by altering fluid and electrolyte balance, as in the case of dehydration induced by gastroenteritis. It has recently been found that virus infection can trigger, in a host with a specific genetic makeup, novel disease phenotypes in uninfected cells,⁴⁰ and therefore that a virus can have a substantial effect on diseases that are highly specific to a given constellation of genes in the host. Thus, in many situations, the pathogenesis of disease may be independent of whether virus replication *per se* induces the death of infected cells.

Age as a Determinant of Susceptibility to Viral Infection

Many viral infections are far more severe in young than older hosts^{159,256}—an increase in severity that often correlates with increased replication and dissemination of virus. For example, suckling mice have long been used to isolate new viruses because they have greater susceptibility to virus infection than mature mice. The susceptibility of young mice to Sindbis virus infection is a striking example.¹⁵⁰ Intracerebral inoculation

of 1-day-old mice with most strains of Sindbis virus results in rapid death, extensive neuronal apoptosis, and high levels of viral replication. In contrast, the same viral inoculum in 4-week-old mice results in a nearly 10-fold lower viral replication, no death, and no neuronal apoptosis. This observation extends to many other viruses, including poliovirus, where 2-week-old poliovirus receptor transgenic mice are 10,000-fold more susceptible to paralytic disease than adult mice.⁵³ All measles virus receptor transgenic mice inoculated at 1 to 6 days of age succumb to lethal infection, whereas mice aged 30 to 60 days at inoculation survive.^{153,236}

The practical importance of this principle is reflected in studies in primates with potential live attenuated SIV vaccines and in the epidemiology of human HBV infection. Vaccination of an adult monkey with live attenuated SIV viruses lacking nef or combinations of nef and other genes significantly protects against SIV challenge.³¹¹ However, inoculation of neonatal animals with the same live attenuated vaccine virus results in progressive immunodeficiency. Similarly, whereas adults infected with HBV often clear the infection, exposure of human neonates to HBV results in a very high level of chronic infection.³¹⁵ Age dependence of virus resistance does not always favor older hosts. For example, West Nile virus encephalitis is more common and more severe in older hosts,¹¹⁴ and lethality after SARS infection is significantly higher in people older than 60 years of age than in younger people.^{62,219} This is also the case in mouse models of SARS infection.^{244,246}

Together, these data indicate that age plays a critical role in determining viral virulence. The mechanism(s) responsible for this are incompletely understood. It is commonly considered that maturation of the immune system explains increased resistance to infections seen in older hosts. However, this has not been rigorously demonstrated as the reason for age-dependent susceptibility to viral infection. Other age-dependent processes, such as cellular differentiation and proliferation, may play a role as large as that of the immune system in age-related susceptibility. For example, the genes expressed in the nervous system during infection of younger versus older hosts differ,^{150,213} indicating that there are fundamental differences in how older and younger tissues respond to viral infection.

Fate of Infected and Uninfected Cells in Tissues

A primary determinant of the tissue damage and pathology observed after viral infection is the fate of the infected cell. It is often unclear why cells in tissues die when infected by viruses, and the extent to which cell death is destructive or protective in tissues is often uncertain. On the one hand, death of infected cells is harmful if those cells are essential to the host; on the other hand, death of an infected cell may inhibit viral replication with the sacrifice of one cell contributing to the protection of other cells. The immune system has many mechanisms for killing infected cells, arguing that the death of infected cells can benefit the host (Chapters 8 and 9). The death of cells in an infected tissue may be indirect, with uninfected cells dying owing to cytokines, bystander killing by leukocytes, or ischemia. The consequences of viral infection range from rapid destruction of tissue cells, as seen with variola or Ebola viruses, to continuous replication in the absence of severe cytopathology, as seen with LCMV, to the establishment of latency, as seen with herpesviruses.

Direct Killing of Cells by Viruses

Cytopathic effect is defined as the destructive consequences of virus infection on cells. Direct killing of cells by viruses may be attributable to viral subversion of cell metabolism for replication or to cell-intrinsic programmed cell death pathways such as necrosis, apoptosis, or nonnecrotic and nonapoptotic cell death. Despite the great importance of understanding the specific mechanisms responsible for virus-induced tissue damage, the balance between virus-induced cell death and cell death owing to host programs induced by the presence of the virus is very poorly understood in most cases.

The term *cytopathic effect* is most commonly used to describe the consequences of infection in cultured cells. Cell death that occurs in a cultured cell may or may not reflect the process of cell death that occurs *in vivo*. An advantage of studying cell death and cytopathicity in cultured cells is that the interaction between the virus and the cell occurs without cell-extrinsic factors, with exception of those owing to autocrine stimulation (e.g., the expression of interferons by infected cells). A disadvantage of this approach to studying cell death is that events in cultured cells may have little relationship to what happens in primary cells in an infected tissue.

It is easiest to ascribe cell killing to direct viral effects, rather than immunopathology or ischemia, when death of virus-infected cells occurs in the absence of inflammation or extensive tissue damage. For example, in both Sindbis and rabies, virus kill neurons in the absence of extensive inflammation. Further proof that viruses can directly damage tissue without invoking indirect effects of immunopathology comes from the susceptibility of SCID mice and *RAG* gene-deficient mice, which cannot mount adaptive T- or B-cell responses, to several different viruses. In these cases, death of host cells cannot be attributed to the immunopathologic consequences of adaptive immunity, although contributions from innate immunity cannot be ruled out. It is important to remember that the presence of inflammation does not necessarily indicate the participation of inflammatory cells in the killing of infected cells. Inflammatory cells may be beneficial or present as a host response to killed cells and tissue damage rather than being participants in the death of infected cells.

Killing of Cells by Cell-Extrinsic Effects of the Immune System

Not all cell death in an infected tissue is a result of direct viral effects or cell-intrinsic mechanisms of programmed cell death triggered by the presence of the virus. The host has many cell-extrinsic mechanisms for initiating apoptosis or lysis of infected cells via cytokines, serum proteins such as complement, and proteins such as the granzymes that are injected via perforin into infected cells by leukocytes (Chapters 8 and 9).

The role of cell killing by the lytic activity of granzymes is important because perforin- and/or granzyme-deficient mice are susceptible to coronaviruses, West Nile virus, LCMV, ectromelia virus, HSV, Theiler's virus, MCMV, and murine γ HV68,^{165,166,188,220,254} and a human with a mutation in perforin has been described with chronic active EBV infection.¹³⁹ As detailed later, these cell killing pathways can contribute to virus-induced immune pathology, as studies in HBV transgenic mice in which cytolytic antiviral CD8 T cells mediate liver disease show that both perforin- and FAS-dependent pathways contribute to liver destruction.¹⁹³

The complement cascade is a two-edged sword important for antiviral immunity but can also contribute to virus-induced tissue damage.^{25,268} Complement proteins are involved in induction of antibody and T-cell responses, trapping of viral antigen on antigen-presenting cells, induction of chemotactic and vascular permeability changes in infected tissues, and activation of leukocytes. In addition to being immunoregulatory, complement can lyse both virus-infected cells and lyse or neutralize virions.^{25,79,178,294}

Clearance of Virus Infection and Chronic Viral Infection

A fundamentally important variable in viral pathogenesis is whether the immune system can clear a virus from the body. Some viruses are readily cleared but can do significant damage to the host during their brief time of residence in the body. An example is variola virus, which causes smallpox. This pattern of acute infection (see Figs. 10.1 and 10.3) is associated with development of sterilizing immunity and effective resistance to reinfection. Many viruses for which effective vaccines are available fall into this category. Other viruses are less amenable to elimination by the immune system, resulting in latent, chronic, or progressive infection (see Fig. 10.1). Examples of chronic viral infections of great medical significance are those caused by HIV, HCV, and HBV. Other chronic infections are less consistently harmful to their hosts but do cause significant disease. Examples include herpesviruses such as EBV, VZV, HCMV, and HSV, each of which permanently infects most human beings. It is possible that such chronic infection can, in addition to causing disease in rare cases, provide a symbiotic benefit to the host via chronic stimulation of the innate immune system, including macrophages and NK cells.^{17,302,310,326,327}

Between the extremes of complete clearance and establishment of chronic infection, there are viruses that persist in a small proportion of hosts. For example, measles virus persists in the CNS of a small proportion of infected persons, causing subacute sclerosing panencephalitis (SSPE). In SSPE, a chronic CNS infection with measles leads to a progressive destruction of neurons and often to death. Importantly, SSPE is associated

with an unusual pattern of mutations in defective measles viruses that persists in the CNS, with mutations in several proteins and a bias toward U to C mutations in the matrix protein.^{45,209,243} In the murine measles virus receptor transgenic system, transient infection with LCMV predisposes to development of an SSPE-like disease.²⁰⁹ This is a paradigmatic example of how a common infection (measles) may cause pathology triggered by a second infection.

Whereas persistence of certain viruses is related to disease in a proportion of infected hosts, the role of persistent infection is more controversial for many other viruses (see Fig. 10.1). For example, coxsackievirus RNA persists in the hearts of some persons with chronic myocarditis, and poliovirus RNA may persist in the CNS of a proportion of persons who develop “postpolio” syndrome.^{142,312} These situations are not as well understood as SSPE or chronic infections with herpesviruses, retroviruses, HCV, or HBV. However, the data suggests the hypothesis that persistence of either virus or viral genomes may contribute to disease in some cases, and it seems highly likely that additional examples of similar phenomena will be detected as unbiased and highly sensitive next-generation sequencing technologies are applied in clinical settings to detect viral genomes.

Tissue Damage During Beneficial Immune Responses Is a Necessary Evil

Certain viruses, such as HCV, HBV, HIV, SIV, and LCMV, can persistently replicate in tissues despite an active adaptive immune response. Although the immune response is ineffective at clearing the virus, activated immune cells and cytokines are still capable of killing host cells. Even when the immune system can clear a virus, these adaptive immune mechanisms may cause significant damage to host tissues. Importantly, the innate immune system can also contribute to host disease in the setting of acute infection, as has been shown, for example, for influenza.²⁸¹

Although there are clear examples in which the immune system is primarily responsible for virus-induced tissue pathology, and thus disease is immunopathologic, the situation is seldom that simple. The antiviral immune response has two faces: a beneficial face as the host system responsible for curing infection and a harmful face via induction of tissue pathology and systemic toxicity. This sets the stage for consideration of one of the most complex topics in viral pathogenesis: the relationship between immune-mediated damage and clearance of infection.

It is not always true that the function of the immune system causes pathology. For example, protective effects of antibody that do not damage tissues are seen in most if not all viral infections. Sindbis virus is cleared from neurons by antibody to the E2 envelope glycoprotein in the absence of neuronal loss,¹⁶⁰ and antibody to measles virus can alter expression of measles proteins in infected cells.^{80,82} Antibody can inhibit the neural spread of viruses, such as reovirus, and can act inside cells to inhibit reovirus and West Nile virus replication.^{283,298,299} Whereas immunity can be beneficial without harming host cells, in other cases, the more harmful face of the immune system is prominent and tissue injury is associated with immune-mediated clearance of infection.

A clear example of the two-faced nature of immunity comes from studies of HBV. HBV DNA replication occurs for weeks before induction of a significant immune and inflammatory response or significant release of serum alanine

aminotransferase (sALT), a marker of hepatocyte lysis, into the circulation^{314,315} (Fig. 10.11). Damage to the liver does occur during the latter stages of clearance of HBV DNA from the liver, as evidenced by increases in both sALT and the number of apoptotic hepatocytes, and the induction of this damage correlates with the appearance of CD8 T cells and IFN γ mRNA in the liver^{314,316} (see Fig. 10.11). Thus, active immune responses and clearance of the last vestiges of infection are associated with significant tissue injury.

The mechanisms underlying these processes have been further elucidated using transgenic mice that express viral antigen and nucleic acids in all hepatocytes and produce infectious HBV in serum. Using these mice, it has been shown that virus replication can be limited and viral protein and nucleic acid can be cleared from hepatocytes without killing the hepatocytes^{100–103,286} via a mechanism that depends on interferons and tumor necrosis factor (TNF). However, these same studies also demonstrated that virus-specific T cells can contribute to the induction of liver injury. These results were further validated by studies in chimpanzees, where depletion of CD8 T cells resulted in delayed viral clearance, and the kinetics of viral clearance and alanine aminotransferase (ALT) induction suggested that virus was cleared in large part by noncytolytic mechanisms but that the CD8 T cells also drove final resolution of infection and subsequent liver damage through cytolytic mechanisms. These studies illustrate the potential dual nature of the immune response in mediating viral clearance while also causing tissue pathology during the clearance process.

Immunopathology Occurs When the Immune System Goes Too Far

In some cases, the balance between the protective effects of immunity and the harmful effects of immunity clearly shifts to immunity being the primary cause of tissue pathology and even death of the host.^{32,323} These processes can be driven by overactive innate immune responses, which result in inflammatory responses that drive virus-induced tissue pathology and disease,³²⁸ nonspecific cell killing, or even induction of autoimmunity by the host adaptive immune response. The balance between acceptable tissue damage associated with viral clearance and immunopathology is determined by the effectiveness of the immune system in clearing the infection. For example, even severe hepatitis would be an acceptable outcome of HCV infection if the virus could be cleared; however, the immune response to HCV infection is often ineffective. Immunopathology can be attributable to either T- or B-cell responses or to innate immunity and may be the consequence of virus-induced immune responses to either viral antigens or, in the case of induced autoimmunity, host antigens.

An excellent example of immunopathology owing to B-cell responses is the kidney damage done by immune complexes generated during the continuous replication of viruses such as LCMV, lactic dehydrogenase virus, Aleutian disease of mink, HBV, HCV, or murine retroviruses.^{110,118,210,211,232,295} In these cases, circulating immune complexes containing viral antigen are deposited in the kidney with consequent activation of complement and cellular inflammation that results in damage to the renal filtration apparatus of the glomerulus.

The existence of T-cell-mediated immune pathology was first demonstrated using the LCMV system.^{32,38} Infection of adult mice by the intracerebral route with LCMV

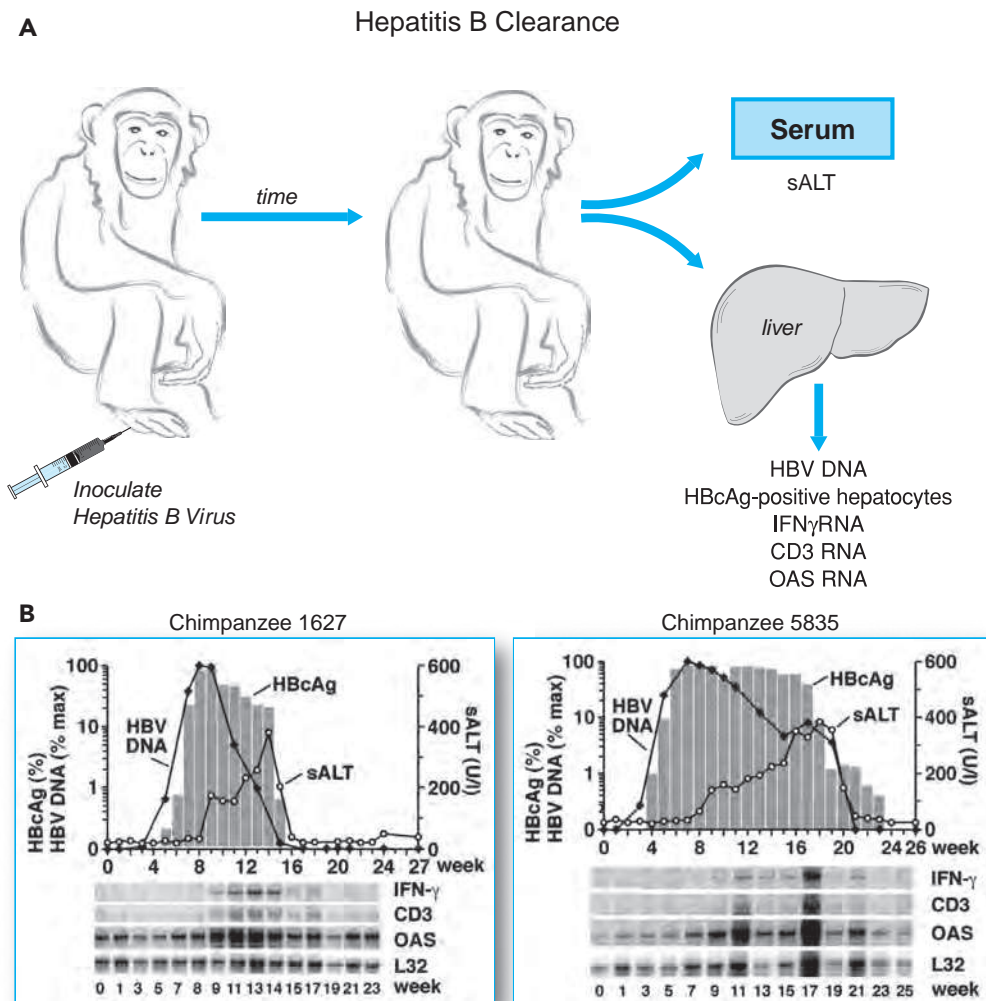


FIGURE 10.11. Clearance of HBV is associated with liver damage. Chimpanzees were infected as shown in **panel A** as described in detail in reference 314; at various times after infection, serum samples or liver biopsies were taken and analyzed for sALT and various parameters of HBV infection. Intrahepatic HBV DNA (*black squares*) is expressed as a percentage (% max) of the peak HBV DNA levels in the liver of each animal. Hepatitis B core antigen–positive hepatocytes (*gray bars*) are expressed as percentage of the total number of hepatocytes. sALT (*open circles*) is expressed in units per liter. Intrahepatic expression profiles for IFN γ , CD3, and 2'5'OAS were determined by RNase protection assay. HBV, hepatitis B virus; sALT, serum alanine aminotransferase; IFN γ , interferon- γ . (Adapted from Wieland S, Thimme R, Purcell RH, et al. Genomic analysis of the host response to hepatitis B virus infection. *Proc Natl Acad Sci U S A* 2004;101:6669–6674.)

results in death attributable to inflammation of the meninges and choroid plexus termed *choriomeningitis*.^{32,38,177} The importance of this model is that, as opposed to most viral infections in which depletion of T cells worsens infection, for LCMV depletion of T cells protects against disease.^{32,38,157,186} Further studies have demonstrated that LCMV-specific CD8 T cells were responsible for virus-induced immune pathology.³²⁵

These experiments in the LCMV system show that CD8 T cells, in addition to their protective capacities, can be responsible for destruction of many different types of virus-infected cells. Why is the balance of protection versus immunopathology tipped toward immunopathology for LCMV? The most obvious reason is that the virus is largely noncytopathic, thus the

presence of the virus in the host even over long periods does limited direct cellular damage. In this view, CD8 T cells kill virus-infected cells that would not otherwise die, resulting in immunopathology. This makes a plausible case for a key role for immunopathology in chronic diseases attributed to largely noncytopathic viruses such as HBV and HCV.

Virus-Induced Autoimmunity

A variant mechanism of immunopathology is the induction of autoimmunity by virus infection.^{21,181,207,304} The contributions of autoimmunity to tissue pathology associated with viral infection, or putatively associated with viral vaccination, remain quite controversial. The role of autoimmunity in virus infection and postinfection syndromes has been studied in

several systems, and viruses have at one time or another been candidate etiologic agents for many autoimmune or inflammatory diseases in humans, including rheumatoid arthritis, multiple sclerosis, Crohn's disease, myocarditis, uveitis, hepatitis, Sjögren's syndrome, and type 1 diabetes.

To date, unequivocal proof of the role of a specific virus in a human autoimmune disease has been lacking, although this is an important area of interest. The presence of immune responses to self-antigens after virus infection is common; however, these responses should not be confused with a pathological role for self-responses in virus-induced pathology. Moreover, when studies in some viral models that have been touted to represent autoimmunity are examined very carefully, the persistence of viral genome, and perhaps protein, provides a plausible alternative to autoimmunity as a mechanism. This is true for coxsackievirus-induced myocarditis,³¹³ underlining the importance of careful virologic assessment of potentially autoimmune phenomena.

A proposed mechanism for the induction of autoimmunity is molecular mimicry between virus and host antigens leading to a breakdown of self-tolerance.²⁰⁷ This might plausibly occur in the setting of virus-induced tissue damage and inflammation. Since the first proof that this can happen, numerous experiments supporting the concept that molecular mimicry can trigger autoimmunity have been published. The opportunity for molecular mimicry for T cells is provided by the degenerate recognition of peptides by even clonally expanded antigen-specific T cells, making it more likely that antiviral T cells could recognize peptides derived from the host proteome.

The potential for molecular mimicry to cause virus-associated pathology has been proven using transgenic mice engineered to express the LCMV nucleoprotein or glycoprotein under the control of the insulin promoter in the insulin-producing islet cells of the pancreas.^{81,204,206,212,303,304} Infection of these mice with LCMV can trigger diabetes. In this case, LCMV infection triggers a T-cell response to the viral protein expressed in the pancreas as a pseudo-self-antigen. Autoimmune T-cell responses can also develop during persistent virus infection when viral and self-proteins do not demonstrably cross-react—a process termed *epitope spreading*.^{181,222} In this process, it may be that the inflammatory response to chronic infection results in the induction of T-cell responses to self-proteins via breakdown in self-tolerance and expansion of rare clones of autoreactive T cells that escape negative selection in the thymus. Although these studies demonstrate that a virus can induce disease by inducing responses to proteins expressed in a host tissue, studies proving that this mechanism is responsible for a specific human disease have not been published.

VIRAL DETERMINANTS OF VIRULENCE

Niche-Specific Virulence Genes

Viral genes and virulence determinants are important indicators of viral pathogenesis and viral virulence. Examples of specific properties of viruses that contribute to virulence abound in the literature, and many—including tropism, the capacity to persist, and the capacity to kill cells—have already been discussed. *Virulence* is a general term representing the severity of viral

infection for the host. However, specific viral genes and properties most often play a role in a very specific tissue, at a certain time, at a very specific bottleneck in infection, or to counter a very specific host response to infection. A useful concept is that such genes are niche specific, having functions in a single aspect of infection defined by time, tissue, and stage of infection or host response. Thus, virulence is the sum of the actions of multiple niche-specific viral genes and virulence determinants that play their specific roles in limited settings *in vivo*.

The concept that viral virulence genes and virulence determinants have niche-specific roles *in vivo* during infection is important for understanding experimental pathogenesis research. *In vivo* readouts of infection often depend on a single route and dose of infection, are often measured in a genetically homogeneous host, and commonly rely on a limited set of assays for viral infection such as death of the infected host, growth of virus in a certain tissue at a certain time, or severity of tissue pathology. The experimentalist is rarely able to mimic the entire process of pathogenesis under physiologic conditions, and so the virus is not asked to perform all of the tasks for which it has evolved in nature. Experiments performed under such conditions may reveal a minor, or even no, contribution of a niche-specific viral virulence gene or allele to infection as measured by a limited set of assays in a given experiment. This type of negative data is fundamentally unrevealing. The gene or allele under study may have a significant role that would be revealed by further experimentation that quantitatively evaluated the niche within which the gene functions. It is plausible that every viral gene has been under considerable evolutionary pressure and that the mere presence of the gene indicates that the gene has an important function for the virus. Exceptions may be found when a virus has recently jumped from one species to another; however, even in that setting, the pressure to utilize the limited viral genome parsimoniously likely results in rapid selection against useless genes.

It is therefore true that niche-specific functions of viral genes may only be identified in specific experimental conditions. Examples of this phenomenon abound and include the role of the *M3* gene of murine γ HV68 that encodes a chemokine scavenger. *M3* mutant viruses are completely normal in a broad range of properties *in vivo* in mice, including replication in many tissues and the establishment of viral latency. However, the gene contributes significantly to virulence after intracranial inoculation.²⁹³ Similarly, a comparison of vaccinia virus mutants inoculated either intranasally or in the pinna of the ear revealed that fully half of a panel of 16 different mutants had a phenotype in only one of the two inoculation models.²⁸⁵ Other examples of niche-specific virulence genes include herpesvirus genes associated with chronic infection or latency. These genes may be dispensable for the lytic phase of the life cycle but then play an essential role in promoting or maintaining the chronic stage of the viral infection. For example, γ HV68 viruses lacking either the *v-cyclin* or *v-Bcl-2* genes have limited or no phenotype during acute infection or in the establishment of latency.¹⁶ However, these genes play an important role in reactivation from latency and in the capacity to continuously replicate in immunocompromised mice. Therefore, when considering the function of a gene or virulence determinant *in vivo*, it is necessary to consider the limitations of the experimental system for revealing the role of a niche-specific viral gene.

Mutation and Selection of Viral Variants

Although viruses have niche-specific genes specialized for specific tasks *in vivo*, it is also possible for mutation and selection to meet specific needs during infection. For this reason, the error-prone RNA polymerases of viruses such as HCV and HIV play a role in viral pathogenesis. Viral RNA polymerases can make a mistake every 10^3 to 10^5 nucleotides while copying an RNA.^{59,60,65,223} In addition, point mutations, duplications, deletions, recombination events, and even acquisition of host mRNAs into RNA and DNA viral genomes have occurred in different viruses. Thus, the total mutational capacity of viruses is very high. It is plausible that a virus with a nearly 9,700-bp genome, such as HCV, will have a large number of mutations generated during replication even in a single cell. However, the variation actually observed *in vivo* or in serial passage in cultured cells is much lower than predicted from this argument. For example, comparison of the sequences of HCV over 13 years of infection of a single host revealed a mutation rate of 1.92×10^{-3} base substitution per genome site per year.²⁰³ Because the observed variation is low compared to the potential variation, it is plausible that the strength of the selective pressure applied to a viral population determines the nature of viruses present in the host at a given time. The capacity of mutation to provide the substrate on which selective pressure operates to generate new viral strains with altered pathogenic capacity has significant implications for understanding and controlling viral infections.

An excellent example of a mutation contributing to the pathogenesis of viral infection is the emergence of immune escape mutants. The principle that viral escape mutants can evade CD8 responses was first identified by study of murine LCMV infection.²²⁷ In this study, investigators created T-cell receptor transgenic (LCMV TCRtg) mice containing a large number of T cells specific for the epitope containing amino acids 32–42 of the LCMV glycoprotein. Within 8 days of infection, many LCMV isolates were no longer recognized by TCRtg T cells and contained mutations in the 32–42 epitope.²²⁷ Importantly, mutations in other T-cell epitopes that were not recognized by TCRtg T cells were not selected, indicating specificity of the selective pressure.

The relevance of this initial observation to events during infection in normal hosts is now well established.^{34,72,225} Both HIV and SIV accumulate mutations in CD8 T-cell epitopes over time, which is associated with progression of disease, escape from vaccine-mediated control of SIV infection, and escape from control of infection by adoptively transferred HIV-specific T cells.^{7,15,31,72,92,144,225} Similar observations have been made during chronic HCV infection, viruses such as MCMV develop mutations that enable escape from NK cell-mediated control, and genetic drift in seasonal influenza virus strains is in large part driven by selective pressure from antiviral antibodies.

Virus-Induced Immunosuppression

Virus infection is often associated with the induction of an immunosuppressed state. Immunosuppression is defined as a virus infection-associated decrease in the capacity of the immune system to respond to antigen. The phenomenon of immunosuppression was first noted as inhibition of tuberculin skin test reactivity during measles virus infection.^{95,96,187,218} That initial observation has been followed by a deluge of reports of altered immune reactivity in virus-infected hosts.

An immunosuppressed state may benefit a virus by inhibiting antiviral responses; however, it is important to distinguish between immunosuppression and viral immune evasion that occurs without inducing a general deficiency in immune function. It is also important not to confuse immunosuppression with the normal down-regulation of virus-specific responses that occurs as viral infection is cleared. There are three categories of immunosuppressive mechanisms.¹⁹⁴ In the first, virus kills significant numbers of critical immune system cells; an example of this is HIV-associated depletion of CD4 T cells. Likewise, LCMV clone 13 causes immunodeficiency owing to destruction of DCs that are critical antigen-presenting cells. In this case, activated CD8 T cells that recognize viral antigens expressed by infected DCs damage the immune system.^{8,30,158,324} A second potential mechanism involves alterations in cytokine secretion by infected cells or the induction of secreted molecules that inhibit immune responses. This mechanism, however, does not provide a clear explanation for a case in which immunosuppression persists long after infection is thought to be cleared. A third but largely speculative potential mechanism only now being explored is viral induction of regulatory T cells that may inhibit the immune response.

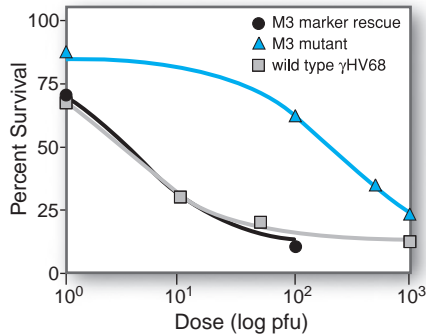
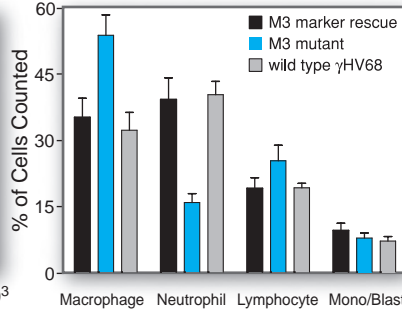
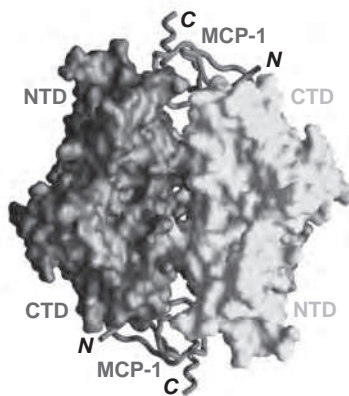
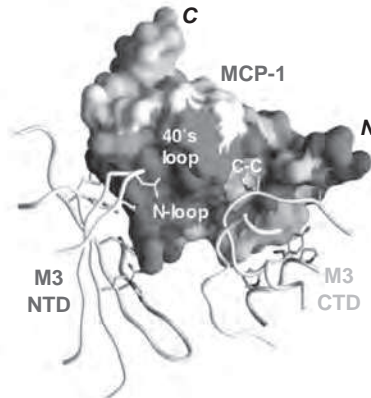
Viral Evasion and Subversion of Host Cytokine Responses

Host molecules present in the extracellular fluid including cytokines, prostaglandins, steroid hormones, peptide hormones, growth factors, and serum components such as complement provide important targets for viral evasion and subversion strategies. Viruses encode molecules that evade or subvert host hormonal and cytokine responses that regulate both innate and adaptive immunity, such as interferons, TNF, IL18, IL6, IL10, complement, and chemokines. These viral evasion and subversion molecules have been studied in detail for herpesviruses and poxviruses, including via the use of viral mutants lacking these proteins to determine their specific role *in vivo*.

The remarkable sophistication of viral cytokine evasion proteins is exemplified by the structure of the γ HV68 chemokine-binding protein M3, which alters the inflammatory response to viral infection^{6,293} (Fig. 10.12). Chemokines are a large group of polypeptides that regulate immune cell trafficking, cell differentiation, and tissue development. The M3 protein has evolved to mimic both the chemokine receptor of the host and the dimer interface between chemokines as a way to bind a broad array of chemokines with a very high affinity. It is likely that similar molecules and mechanisms will be discovered in other viruses and that additional host cytokine systems will be discovered that are targeted by viral immune evasion and/or subversion molecules. In addition, viruses can increase inflammation to foster infection and can induce tissue damage via paracrine effects of virus-infected cells.

Virus–Host Co-Evolution Drives the Host–Pathogen Interaction

The interactions between viral immune evasion proteins in host cytokine networks are highly sophisticated. Given this capacity to manipulate the host, one may question why viruses are not more virulent. A common belief is that the most dangerous and virulent viruses are those that have recently jumped from one species to another—the idea being that virulence *per se* can be the result of incomplete adaptation of the virus to the host. Implicit in this view of viral pathogenesis and evolution is the

A Characterize pathogenesis of M3 mutant**B** Lethality**C** Cellular infiltrate in meninges**D** M3:MCP-1 complex**E** Contact surfaces between M3 and MCP-1**FIGURE 10.12. Role of a viral chemokine-binding protein in herpesvirus pathogenesis.**

A: Experimental protocol for analysis of the pathogenesis of an M3 mutant in murine γ HV68 derived from reference 293. **B:** Lethality observed at different doses of virus administered intracranially. *Marker rescue* refers to a control virus generated by restoring wild-type γ HV68 sequences to the M3 mutant virus. **C:** The nature of the meningeal cellular infiltrate of mice infected with the indicated viruses. Note that inflammation induced by the M3 mutant virus shows increased macrophages and decreased neutrophils compared to wild-type and control viruses. **D:** Three-dimensional structure of M3 homodimer (solid) in complex with the chemokine MCP-1 (tubes) bound at either end of the M3 homodimer in a 2:2 stoichiometry. Note that the M3 dimer is arranged in an antiparallel fashion with the M3 N- and C-terminal domain packed together to form the chemokine-binding niche. **E:** The interfaces between M3 and MCP-1 are shown in more detail than in **D**. M3 chemokine-binding regions are shown as tubes, whereas the sequestered MCP-1 is depicted with its solvent accessible surface. M3 acts as a competitive inhibitor of chemokine function, shown here engaging the same interface of MCP-1 employed to bind the host chemokine receptor CCR2. (**D** and **E** adapted from Alexander JM, Nelson CA, van Berkel V, et al. Structural basis of chemokine sequestration by a herpesvirus decoy receptor. *Cell* 2002;111:343–356; courtesy of Drs. Jennifer Alexander-Brett and Daved Fremont.)

concept that viruses benefit from adapting to lesser virulence. Examples of the virulence of viruses that have recently entered a new host population abound, with the influenza pandemic of 1918, Ebola virus, and HIV being outstanding examples. Each of these virulent viruses has entered humans from another species and is more virulent in the newly invaded species than in the original host species.

What really happens to the virus and the host species as an emergent virus spreads? In a classic series of experiments analyzing the epidemiology, pathogenesis, and virology of myxomatosis in rabbits, Fenner et al. demonstrated virus–host co-evolution. The natural history and evolution of both a virus and its host were followed in real time after the introduction of a virulent virus into a highly susceptible population. Importantly, although both the host and virus evolved in this enormous experiment of nature, this work does not support the concept that viruses necessarily evolve to become completely avirulent in a new species.

The introduction of European rabbits into Australia by European settlers provided the substrate for both an ecological disaster and a series of fundamentally important experiments in viral pathogenesis. Absent natural predators, European rabbits expanded rapidly in Australia with severe ecological

consequences, creating a need for a pest control strategy. Myxomatosis is a relatively benign disease in *Sylvilagus* rabbits from South America but is almost uniformly lethal in the European rabbit *Oryctolagus*.^{74,76} Recognizing that an infection that spread quickly and killed rabbits might be an effective biological weapon, investigators released myxoma virus into the Australian rabbit population in 1950. Initially, the kill rate was calculated as 99.4% to 99.8%, with most surviving rabbits being uninfected.

Over the next several years, a remarkable process of evolution of both the virus and the rabbit host occurred and was experimentally documented.^{76,77} Similar evolution of the rabbit host was observed in laboratory experiments.²⁶² Over the course of a single year, the virus became less virulent, killing only 90% of laboratory European rabbits⁷⁴ (Table 10.1). It is hypothesized that this initial decrease in virulence allowed enough rabbits to survive and breed to allow selection of rabbits capable of resisting virulent myxoma. The virulence of isolates was categorized into five classes, with grade 1 killing more than 99% of rabbits and grade 5 killing less than 50% of rabbits^{74,77} (see Table 10.1). By 1975, less than 2% of isolates were highly virulent class I viruses, whereas more than 60% of isolates were class III viruses. Notably, the distribution of isolates with

TABLE 10.1 Virulence of Field Isolates of Myxoma Virus, 1951–1981

	Virulence grade					Number of samples
	I	II	III	IV	V	
Fatality rate (%)	>99	95–99	70–95	50–70	<50	
Mean survival time (d)	<13	14–16	17–28	29–70	NA	
Years	% of isolates					
1950–1951	100	0.0	0.0	0.0	0.0	1
1952–1955	13	20	53	13	0.0	60
1955–1958	0.7	5	55	24	15	432
1959–1963	1.7	11	61	22	5	449
1964–1966	0.7	0.3	64	34	1.3	306
1967–1969	0.0	0.0	62	36	1.7	229
1970–1974	0.6	5	74	21	0.0	174
1975–1981	1.9	3	67	28	0.0	212

NA, not applicable.

Adapted from Nathanson N, Ahmed R, Gonzalez-Scarano F, et al, eds. *Viral Pathogenesis*. Philadelphia: Lippincott-Raven, 1997, and based on data from Fenner F. Biological control, as exemplified by smallpox eradication and myxomatosis. *Proc R Soc Lond B* 1983;218:259–285.

differing virulence was relatively stable from about 1955 onward, indicating that equilibrium between rabbit and virus was established. The rabbit population similarly evolved. Whereas wild rabbits initially exhibited more than 90% lethality when challenged with a virulent isolate of myxoma virus, after multiple epidemics only 30% of challenged rabbits died and 46% suffered mild to moderate disease (Table 10.2).

Given this evidence for evolution toward resistance in the rabbit population and toward lower virulence in the virus, why didn't myxomatosis become a benign disease in European rabbits? Evolution of the virus to lower virulence, but not avirulence, occurred (see Table 10.1). There were other selective pressures at work in this evolutionary experiment. Field experiments showed that virulent viruses were at a disadvantage compared to less virulent naturally occurring isolates.⁷⁴ Even when virulent isolates were introduced into areas in which attenuated viruses were

endemic, the attenuated viruses were dominant. Very virulent viruses plausibly were at a disadvantage as they killed their hosts rapidly, allowing less time for natural transmission by mosquitoes. Thus, the capacity to persistently infect some hosts or to slow the infectious process might have fostered the capacity to infect new hosts over longer periods, thereby providing a selective advantage. However, the virus had to spread efficiently to survive, and experiments showed that viruses with midrange virulence were present in skin at sufficient levels to spread via mosquitoes. It is believed that selective pressure retained a level of virulence associated with replication to high enough levels to spread,⁷⁴ explaining why the outcome was not evolution to avirulence.

Virus Genes That Inhibit Pathogenesis of Disease

It is a common misconception that viruses primarily encode molecules to cause disease. Although retention of some level

TABLE 10.2 The Susceptibility of Nonimmune Wild Rabbits After Successive Epidemics of Myxomatosis

Number of epidemics	Severity of disease (%) ^a			
	Fatality rate	Severe disease including fatalities	Moderate disease	Mild disease
0	90	93	5	2
2	88	95	5	0
3	80	93	5	2
4	50	61	26	12
5	53	75	14	11
7	30	54	16	30

^aRabbits were challenged with a virus of grade III (intermediate) virulence. (See Table 10.1.)

Adapted from Nathanson N, Ahmed R, Gonzalez-Scarano F, et al, eds. *Viral Pathogenesis*. Philadelphia: Lippincott-Raven, 1997, and based on data from Fenner F. Biological control, as exemplified by smallpox eradication and myxomatosis. *Proc R Soc Lond B* 1983;218:259–285.

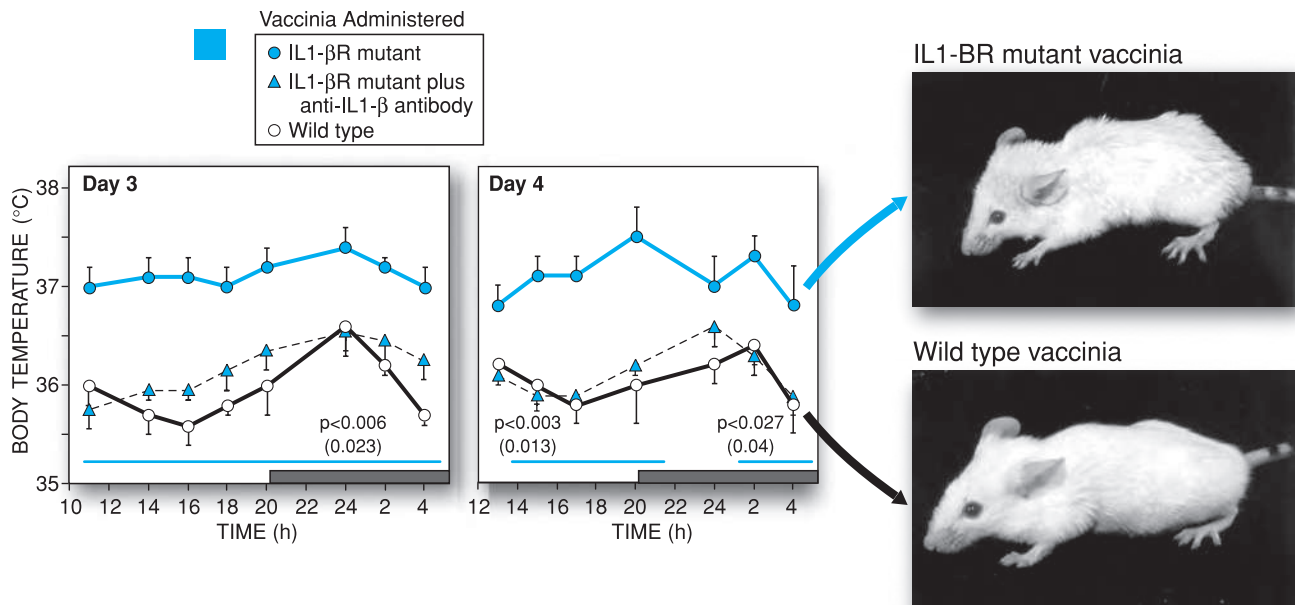


FIGURE 10.13. Protection of the host by the vaccinia virus IL-1 β receptor gene. Shown are body temperatures and clinical appearance of mice infected with the indicated viruses and either left untreated or treated with antibody specific for IL-1 β , IL, interleukin. (Adapted from Alcamì A, Smith GL. A mechanism for the inhibition of fever by a virus. *Proc Natl Acad Sci U S A* 1996;93:11029–11034; images of mice courtesy of Dr. Antonio Alcamì.)

of virulence during adaptation within a host species can be an advantage, evolution to lower virulence did occur in the example presented earlier.^{74,197} One way to lose virulence would be to sequentially lose or inactivate virulence genes or determinants. A second nonexclusive mechanism would be to select for genes that actually protect the host from lethal infection—a prediction borne out by studies of vaccinia virus showing that some viral genes protect their hosts from virus-induced disease.^{4,5} Vaccinia virus expresses a secreted IL-1 β receptor homolog that neutralizes the effects of IL-1 β . Although this gene might plausibly contribute to virulence, instead a mutant virus induced fever and the wild-type virus did not despite similar levels of replication (Fig. 10.13).⁵ The fever induced by the mutant virus was attributed to IL-1 secretion by the host because anti-IL-1 antibody blocked fever induced by the mutant virus (see Fig. 10.13). Expression of the soluble IL-1 β receptor also decreased clinical signs of illness and decreased weight loss. Repair of a nonsense mutation in the soluble IL-1 β receptor in a vaccine strain of vaccinia resulted in a virus that did not induce fever. These data demonstrate that protection of the host can be an active result of viral gene expression rather than a passive process of loss of virulence factors, suggesting that viruses have the capacity to precisely balance virulence and avirulence strategies to foster their survival.

FUTURE OF VIRAL PATHOGENESIS RESEARCH

The study of viral pathogenesis promises to be as important in the future as it has been in the past for understanding fundamental biological and biochemical mechanisms of disease.

Understanding the process of infection is increasingly recognized as the best, and perhaps the only, approach to understanding how to vaccinate against and treat severe unconquered infections such as HIV and HCV and emerging viruses such as Ebola and H5N1 bird influenza. New developments in human genomics combined with ever-improving methods for manipulation of both viral and host genomes will continue to accelerate progress in this critical field of biology. The progress made in other fields as unbiased genetic screens are applied to cell biological problems is breathtaking. In recent years, we have seen the application of these approaches to virology, where whole genome RNA interference (RNAi) screens provide new insights into which host genes are essential for viral replication. Similar approaches are now being applied to identify antiviral and viral restriction factors that regulate the control, tissue tropism, and host range of a wide range of viruses. To date, many of these approaches have been restricted to cell culture-based studies; however, the broadening availability of large panels of genetically modified mice, including knockout and transgenic animals, should continue to facilitate major advances in viral pathogenesis from the standpoint of virus–host interactions.

We have also seen unprecedented progress in our ability to perform studies in genetically complex populations, including the use of GWAS and whole exome and whole genome sequencing to identify polymorphic host genes that are associated with specific phenotypes, including susceptibility to viral infection. The application of these approaches in humans, as well as newly developed mouse models designed to model genetically diverse populations, to the study of viral pathogenesis is likely to revolutionize our understanding of how viral interactions with genetically diverse populations result in different pathogenic outcomes.

Related to these latter approaches, the power of mathematical methods for modeling complex phenomena is improving rapidly and will ultimately be applied to viral pathogenesis. It will be important to apply these tools with a rigorous understanding of the clinical context of disease and an awareness of both the benefits and limitations of studying viral infection in the live host. *In vivo veritas*.

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Virus Evolution

The Origin and Time Scale of Virus Evolution

The Origins of Viruses

The Time Scale of Virus Evolution

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Gene Duplication in Virus Evolution

Lateral Gene Transfer and Modular Evolution

Although Charles Darwin preempted many of the great questions in evolutionary biology, he wrote little about viral infections. Of course, viruses were not formally identified until a full decade after Darwin's death, and, aside from some brief discussion of the origins of yellow fever, his writings make scant reference to what we now know are diseases caused by viruses. This is a great historical shame because it seems certain that Darwin would have held viruses up as some of the best exemplars of evolution by natural selection, and in the case of RNA viruses the evolutionary process is so rapid that it can be effectively followed in "real time".^{123,204}

Although evolutionary analysis arrived relatively late in the science of virology, the study of virus evolution has become one of the most rapidly growing and successful aspects of modern microbiology. The blossoming of evolutionary virology is largely due to two developments. First, viruses, and especially those that possess RNA genomes, have become remarkably powerful research tools for the study of evolutionary processes. The utility of RNA viruses in this respect is a function of the fact that they evolve extremely rapidly, are easy to manipulate *in vitro* and sometimes *in vivo*, often have large and measurable effects on phenotype, and possess such small genomes that the mutations

associated with any phenotype change can be determined relatively easily.⁶⁰ It is therefore no surprise that a growing number of evolutionary researchers are turning to viruses as model systems. For example, studies of viruses represent one of the few cases in which biologists have been able to achieve two of the great aims of modern evolutionary genetics: to measure the fitness effects of individual mutations¹⁹⁵ and to determine the nature of the epistatic interactions between these mutations.¹⁹⁷ Second, the rapidity of RNA virus evolution has acted as a direct stimulus for the development of phylogenetic and coalescent methods that are able to incorporate information on the exact time of sampling of the sequences in question, in turn revolutionizing molecular epidemiology.^{49,132} Indeed, many of the computer programs designed for the evolutionary analysis of gene sequence data were first applied to viruses, such that determining the origin and pathways of spread of specific viruses over epidemiologic time has become a relatively exact science with a myriad of potential applications. The advent of next-generation sequencing promises even more rapid advances in this area, potentially enabling the analysis of many thousands of sequences with detailed associated metadata.⁹⁵ An important spinoff from these studies has been new insights into the patterns and processes of virus evolution.^{79,92} In sum, although their focus is very different, the combination of experimental analyses of model viruses as a means to understand the intricacies of the evolutionary process and studies of molecular epidemiology based on the comparative analysis of virus gene sequence data to document patterns of virus spread has told us a great deal about the nature of virus evolution. Evolutionary virology has blossomed into a well-developed science.

Despite advances on multiple fronts, some fundamental aspects of viral evolution remain unknown, contentious, or both, which will be highlighted in this chapter. For example, there are still major debates over some of the key mechanisms of evolutionary change in RNA viruses, particularly whether their populations routinely form quasispecies, which reflects a wider uncertainty on the roles of mutation, natural selection, and genetic drift as forces of evolutionary change.⁹² For example, it is striking that precise estimates of mutation rates are absent from some important groups of viruses even though they represent a sort of "ground zero" in studies of evolutionary change. Similarly, although we know a great deal more about the origin of viruses than we did 10 years ago, particularly since the discovery and analysis of highly conserved protein structures, exactly when and how viruses first evolved, whether this occurred before or after the appearance of the first cellular organisms, what a precellular world might have looked like, and even if viruses should be classified as living are sources of major debate.^{71,121,138,159} In part, this debate highlights our

profound ignorance of the virosphere. For example, does the apparent absence of RNA viruses in *Archaea* mean that they never existed in these species, that they have been selectively removed, or that we have simply not looked hard enough? Another important issue is that in some respects our knowledge of DNA virus evolution lags behind what we know of the evolution of RNA viruses. We remain ignorant of a number of key aspects of the patterns and processes of DNA virus evolution, particularly whether common principles can be applied to DNA viruses that differ so greatly in size and genome structure. It is hoped that the rise of metagenomics, such that we will sample far more of the virosphere, likely leading to the discovery of a multitude of diverse viruses, will stimulate many advances in this area. Finally, the time scale of evolutionary history in many viruses is unclear, with very different inferences drawn from either the study of endogenous viruses, which are usually indicative of ancient origins, or the molecular clock analysis of recently sampled and often rapidly evolving viral genomes, which usually paints a picture of very recent origins.^{80,94} Advances in this area may require the development of a new class of analytical methods.

Aside from their ability to inform on evolutionary processes, there are a number of practical reasons that the study of virus evolution deserves attention. It is likely that a better understanding of the exact processes of evolutionary change in viruses will assist in the development of improved strategies for their treatment and control and for predicting the spread of newly emerged pathogens. For example, knowing whether natural selection or genetic drift largely controls how mutations spread through a population is essential to understanding the likelihood and rate that a specific drug resistance mutation will become established,¹³¹ while a knowledge of the factors that control how viruses diffuse at the epidemiologic scale represents useful information for any emergent virus.⁹⁵ Similarly, it is likely that a better understanding of the origin of viruses will be essential to obtaining a more precise picture of the earliest events in the early history of life on earth, including the genesis of both RNA and DNA, as it seems reasonable to suppose that some of the earliest replicators resemble what we now know as viruses.

The future of evolutionary virology appears strong. The development and continued refinement of next-generation sequencing methods will doubtless provide unprecedented amounts of data for evolutionary study and stimulate the development of new analytical methods for use in all genetic systems. Innovations in experimental studies of virus evolution will continue, addressing ever more intricate questions, increasingly considering *in vivo* systems, and providing broad-scale evolutionary insights. Metagenomic studies of the virosphere will provide a powerful new perspective on virus biodiversity, in turn bringing important new information on virus ecology, origins, and cross-species transmission and emergence. It therefore seems easy to predict that our understanding of viral evolution 10 years from now will be very different, and more complete, than it is today.

THE ORIGIN AND TIME SCALE OF VIRUS EVOLUTION

The Origins of Viruses

Of all topics in the study of virus evolution, determining exactly how and when viruses first evolved is perhaps the most

difficult. The main hindrance to progress in this area is that viruses likely originated so long ago, and perhaps even before the first cellular species, that the signal of ancient evolutionary history that can be recovered through phylogenetic analysis has largely been eroded. This is particularly true for RNA viruses where rapid rates of evolutionary change ensure that the phylogenetic signal is quickly lost. Accordingly, each individual amino acid and nucleotide site in a viral genome has accumulated so many substitutions since its origin that accurate phylogenetic inference becomes an impossible task. For this reason sequence-based phylogenies have proven to be blunt tools for the study of viral origins, although signs of common ancestry may still reside in aspects of protein structure.

Despite the inherent limitations to understanding viral origins, a number of important theories have been proposed for the genesis of both RNA and DNA viruses, which continue to be debated to this day. Currently two such theories dominate discussions in this area; first, that viruses have a precellular origin, such that they are billions of years old, and may have even contributed to some of the fundamental architecture of the first cells; second, that viruses evolved after the first cellular organisms as “escaped genes” that acquired capsid proteins and the ability to replicate autonomously (Fig. 11.1). Although a third hypothesis—that viruses are regressed copies of cellular species that have shed those genes whose functions are provided by the host—has also been proposed, most notably in the case of the giant mimivirus and megavirus of amoeba,^{9,128} it does appear to be of general applicability. For example, the gene contents of RNA viruses and cellular species have almost no overlap, whereas under the regressive theory virus genes should have their ancestries in cellular genomes. In addition, although often discussed as such, these theories of viral origins are not mutually exclusive, and it is plausible that while some viruses predate the appearance of the first cells, others appeared more recently.

For many years the escaped gene theory dominated discussions on virus origins.¹⁶⁰ Support for this theory was often based on the idea that as viruses are obligate parasites of host cells now, they must have always been so in the past, such that cells must have evolved before viruses. However, this idea is easy to refute. Because it is commonly thought that the first replicating molecules resided in an “RNA world” that existed before the evolution of DNA, it is easy to believe that modern RNA viruses originated from such ancient self-replicating RNA molecules and parasitized cells at a later date. Important recent evidence for the existence of an RNA world was the demonstration that ribonucleotides could be synthesized *de novo* under conditions that might replicate those of early earth.¹⁷⁹ In most cases the escaped gene theory was also taken to mean that viruses could have escaped from host cells on multiple occasions. This is an attractive idea given the huge phenotypic diversity seen in viruses and that there is no one gene that characterizes all viruses. For example, an early idea was that eukaryotic viruses had escaped from eukaryotic cells, while bacteriophages had escaped from bacterial cells.¹⁸⁰ Similarly, it is possible that RNA, DNA, and perhaps retroviruses represent independent episodes of host gene escape, as could the single- (ss) and double-stranded (ds) versions of RNA and DNA viruses, particularly as small ssDNA and large dsDNA viruses clearly have little in common.

However many different origins are postulated, the same general mechanisms are thought to have occurred: that a host

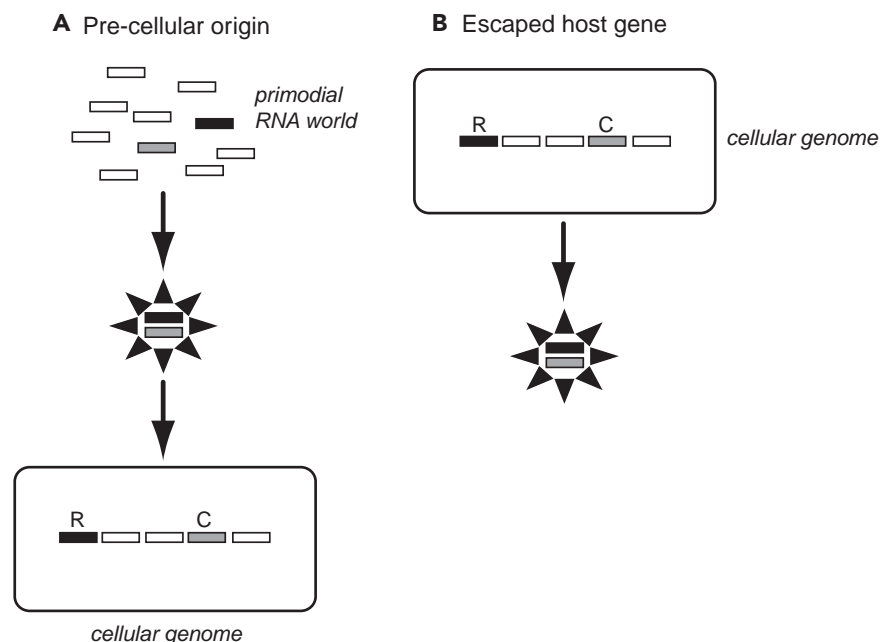


FIGURE 11.1. Schematic representation of two competing models for the origin of viruses. **A:** The precellular origin theory (in this case depicting the origin of RNA viruses). **B:** The escaped gene theory. Cellular genomes are represented by rounded rectangles, and the simplest model virus is shown here to comprise replicase (R) and capsid (C) genes only. (From Holmes EC. *The Evolution and Emergence of RNA Viruses*. Oxford: Oxford University Press, 2009, by permission of Oxford University Press.)

gene that possessed or acquired the ability to self-replicate escaped from the cell, acquiring a protein coat on the way, eventually evolving into an autonomously replicating entity. For example, single-strand positive-sense RNA (ssRNA+) viruses might be descended from escaped cellular messenger RNA (mRNA) molecules that either possessed or evolved RNA polymerase activity, while DNA viruses could be descended from DNA transposable elements or bacterial plasmids. It is also the case that all forms of the escaped gene theory make two important predictions: first, that most virus genes, including the capsid and replicase proteins, ultimately have their ancestries in cellular genomes, and second, because escape events could have occurred multiple times, viruses do not have a single (i.e., monophyletic) origin. In other words, there is no single phylogeny linking all types of virus, as is easily argued from the huge diversity of viruses described today. In theory, both of these predictions are testable, although in practice this is greatly inhibited by the enormous sequence divergence among viruses.

A number of pieces of data have been used to support the idea that viruses had multiple origins after the appearance of cells. At the level of primary amino acid sequence, there is no robust sequence-based phylogeny for either RNA or DNA viruses, nor any gene that contains statistically significant sequence similarity at such vast evolutionary distances. Although there have been attempts to infer the evolutionary history of RNA viruses based on phylogenetic analyses of the RNA-dependent RNA polymerase (RdRp), the phylogenies in question are highly uncertain at the interfamily level where there is often no more sequence similarity than expected by chance alone.^{92,236} However, lack of phylogenetic resolution is not the same thing as an absence of common ancestry, and it is more likely that the inability to accurately infer the evolutionary history of all RNA viruses simply reflects extreme levels of sequence divergence. Indeed, it is striking that the RdRp sequences assigned to different RNA virus families still share a number of short, signature, amino acid motifs (such as a highly

conserved GDD motif), some of which are also found in the reverse transcriptase (RT) protein used by retroviruses.^{81,120} Such conservation, albeit fragmentary, suggests that these replicatory proteins are distantly related. Unfortunately, these motifs are too short to allow the inference of reliable phylogenetic trees. Even more notable is that recent analyses of protein structure have revealed strong similarities between viruses that exhibit no primary sequence similarity, including between RNA and DNA viruses (see later).

In the case of RNA viruses, early phylogenetic analyses of RdRp sequences combined with information on gene order and content were used to construct *supergroup* classification schemes encompassing multiple viral families. For example, one such study suggested that RNA viruses be classified into the alpha-like, carmo-like, corona-like, flavi-like, picorna-like, and sobemo-like supergroups, each of which is characterized by a conserved gene order, distinctive 5' and 3' genome structures, as well as a putative clustering in RdRp phylogenies.⁸¹ However, as noted earlier, extreme sequence divergence means that these RdRp phylogenies are of debatable validity,²³⁶ and it is difficult to construct trees on gene order and content when these differ so dramatically among viral families and in genomes as small as those of RNA viruses. As a consequence, these deep interfamily phylogenies have in reality told us little about virus origins. However, a number of higher-order viral groupings, usually referred to as *orders*, do receive strong phylogenetic support, such that some aspects of the early evolutionary history of RNA viruses can be resolved. These groupings are (a) the *Mononegavirales*, which comprises four families of unsegmented ssRNA- viruses—the *Bornaviridae*, *Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae* (and the *Mononegavirales* clearly cluster together in RdRp trees); (b) the *Nidovirales*, comprising the *Arteriviridae*, *Coronaviridae*, and *Roniviridae* families of ssRNA+ viruses; and (c) the *Picornavirales*, comprising the *Picornaviridae*, *Comoviridae*, *Dicistroviridae*, *Marnaviridae*, and *Sequiviridae* families of ssRNA+ viruses.

Interfamily phylogenetic analyses of DNA viruses have generally proven more successful, in large part because the reliance on high-fidelity DNA polymerases for replication means that dsDNA viruses exhibit lower rates of nucleotide substitution and hence preserve the phylogenetic signal for longer time periods. For example, a number of families of large dsDNA viruses (i.e., those with genomes greater than 100 kb) clearly possess common ancestry such that they can be classified as nucleocytoplasmic large DNA viruses (NCLDVs), comprising ascoviruses, asfarviruses, iridoviruses, phycodnaviruses, and poxviruses.¹⁰⁴ More recent analyses extended the NCLDV group to include the giant amoebal mimivirus, which is most closely related to the phycodnaviruses,¹⁰⁵ as well as the recently described Marcellvirus, which was isolated from the same amoebal host as mimivirus.²³⁵ In other DNA viruses elements of capsid protein structure have been used to link herpesviruses with tailed bacteriophages,¹⁴⁹ while there are clear evolutionary links between the *Papillomaviridae* and *Polyomaviridae* families of small dsDNA viruses.²³² However, there is no phylogeny that encompasses both single- and double-stranded DNA viruses, which again reflects the great divergence between these very different types of virus (which differ massively in genome size) that share no genes in common. More starkly, it is even difficult to infer phylogenetic trees that link all the large dsDNA viruses that infect eukaryotes.¹⁰⁵

The second prediction of the escaped gene theory—that most virus proteins ultimately have a host origin—is equally difficult to resolve. The two most important proteins in this respect, as they essentially define viruses, are the polymerase (a defining feature of all RNA viruses that carry an RdRp or RT) and those that make up the capsid (a defining feature of viruses). The case of the DNA polymerases used by DNA viruses is the easiest to discuss in this context as these enzymes are of the same form, and hence ancestry, as those used by cellular species (i.e., they are classified within the same polymerase families), and small DNA viruses utilize the host DNA polymerases for replication. However, while it is clear that these host and virus DNA polymerases are related,^{65,103} the position of the root, and therefore the direction of evolutionary change, in phylogenetic trees of DNA polymerases is uncertain. Hence, it is difficult to determine whether DNA polymerases are ultimately of host or viral origin,²⁰⁰ particularly as DNA polymerases may also have been involved in ancient lateral gene transfer events.⁶⁵

A similar discussion can be mounted in the case of reverse transcriptase. Proteins that function as reverse transcriptase are a common component of cellular genomes in the form of telomerase, the group II (self-replicating) introns observed in a variety of bacterial species, not to mention the abundant retroelements found in many cellular species, as well as a variety of other genetic elements. Importantly, there are recognizable sequence similarities between the RTs of viruses and those that reside in host genomes such that it is possible to infer phylogenetic trees containing both.^{32,54} These trees have revealed a number of interesting features, including a major division between the long terminal repeat (LTR) and non-LTR retrotransposons, with retroviruses most closely related to the LTR retrotransposons, and that hepadnaviruses and caulimoviruses (small dsDNA viruses that utilize RT) have independent origins and are probably from LTR retrotransposons. However, as with the case of the DNA polymerases, the lack of an outgroup makes the rooting of these phylogenies uncertain, so whether

viral RT genes preceded those present in cells or vice versa is difficult to determine.⁵⁵

The situation is far more complex when it comes to the origin of the RdRp used by RNA viruses. Although the cells of some eukaryotic species contain proteins that function as RdRps, particularly those involved in the production of microRNAs, these exhibit little similarity with the RdRps encoded by viruses, even at the structural level.¹⁰⁶ Similarly, cellular DNA polymerase (Pol) II, which catalyzes the synthesis of RNA from DNA, possesses RdRp activity¹³⁰ yet shares little similarity with the RdRp utilized by RNA viruses, such that their evolutionary origins are currently impossible to resolve. Clearly, determining the evolutionary relationships among these highly diverse polymerase proteins represents a major technical challenge.

While the evidence from phylogenetic trees is ambiguous at best, other pieces of data do provide some support for the escaped gene theory. The most compelling of these is that there is at least one example of a virus whose origins likely lie with a host cellular protein, demonstrating that this mode of viral genesis is possible. The case in point involves hepatitis delta virus (HDV) agent, the ribozyme of which is related to the CPEB3 ribozyme found in a human intron sequence.¹⁹² That HDV is only found in humans and requires hepatitis B virus (HBV) for replication strongly suggests that its origins lie with the human genome.¹⁹²

There has also been considerable debate over the significance of the giant amoebal mimivirus for theories of viral origins and evolution. Although phylogenetic analysis has shown that a small proportion (less than 1%) of mimivirus genes are of host origin, which has been used as support for the idea that viruses are “gene pickpockets” that originated after cellular species,^{158,159} at least 25% of the approximately 1,000 genes in mimivirus clearly link it to the NCLDV group of large DNA viruses,¹⁰⁵ while an even larger set of genes (~70% at the time of writing) have no known homologs, in either viral or cellular genomes, such that they can be regarded as orphans.⁶⁴

Finally, it is striking that, at the time of writing, no RNA viruses have been discovered in *Archaea*. This could mean that either RNA viruses arose as escaped genes after the divergence of *Archaea* from other cellular species or that temperature constraints have led to a major reduction in the frequency of RNA viruses in hyperthermophilic *Archaea*,²³⁷ although this does not explain their absence in nonthermophiles. An alternative, and perhaps more likely, explanation is that RNA viruses do exist in *Archaea* but have simply not been detected as yet.

The competing theory for the origin of viruses, and one that is growing in popularity, is that they originated before the last universal cellular ancestor (LUCA) and represent the modern descendants of the earliest time in earth's history. Hence, modern RNA viruses would be descendants of replicating elements from the RNA world, while DNA viruses would be remnants of the first DNA replicators, and retroviruses perhaps descendants of the first molecules that made the transition from RNA to DNA. For example, because they lack protein-coding regions, possess ribozyme activity, exhibit complex secondary structures, and mutate very rapidly, viroids are potential candidates for extant descendants of the RNA world.⁵⁹ However, although the earliest RNA replicators may share some features with contemporary viroids, because viroids are only seen in plants and likely replicate with the assistance of host cellular

DNA Pol II makes it more likely that they represent escaped host genes or introns that never acquired protein coats.

There are a number of theories for what the pre-LUCA world may have looked like, although all reasonably assume that this precellular stage of evolutionary history contained genetic elements less complex than the viruses we see today. One theory is that there was an *ancient virus world* of primordial replicators that existed before any cellular organisms and that both RNA (first) and DNA (later) viruses originated at this time.¹²¹ A version of this view of virus origins is shown in Figure 11.2. These ancient “viruses” may even have provided some of the features that characterized the first cellular organisms. For example, it has been proposed that the eukaryotic cell nucleus is derived from a virus envelope (the so-called viral eukaryogenesis hypothesis).¹³ An alternative theory for the pre-LUCA world is that RNA cells existed before the LUCA, that RNA viruses parasitized these hypothetical RNA cells, and that DNA evolved later as a way of escaping host cell responses.⁷⁰ Although fascinating, such theories are unfortunately extremely difficult to test.

As sequence-based phylogenetic trees cannot provide insights into the pre-LUCA world, the main evidence for the precellular theory of virus origins is the presence of conserved genes, and more notably protein structures, among divergent viruses. In fact, arguably one of the most important advances in viral evolution in recent years has been the discovery of protein structures that are conserved among diverse viruses that possess little, if any, primary sequence similarity.¹¹ For example, a conserved palm subdomain protein structure, consisting of a four-stranded antiparallel β -sheet and two α -helices, is found in both RNA-dependent and DNA-dependent polymerases.⁸⁴ A more important case in point concerns the jelly-roll capsid, a tightly structured protein barrel that forms the major capsid subunit of virions with an icosahedral structure. Remarkably, the jelly-roll capsid is found in the virions of both RNA and DNA viruses, including such diverse groups as herpesviruses (dsDNA), picornaviruses (ssRNA+), and birnaviruses (dsRNA).^{11,41} Such conservation is strongly suggestive of an ancient common ancestry. Other highly conserved capsid architectures that strongly argue for ancient origins include the PRD1-adenovirus lineage, which is characterized by a double β -barrel fold and found in dsDNA viruses as diverse as bacteriophage PRD1, human adenovirus, and a variety of archaean viruses; the BTV-like lineage, which is found in some dsRNA viruses including members of the *Reoviridae* and *Totiviridae*; and the HK97-like lineage, which encompasses tailed dsDNA viruses that infect archaea, bacteria, and eukaryotes.^{16,17,124} Finally, a common virion architecture has been proposed for some viruses that do not possess an icosahedral capsid, including the archaean virus *Halorubrum* pleomorphic virus type 1 (HRPV-1).¹⁷⁶

Although such structural conservation seems to provide a compelling argument for the antiquity of viruses, it has been proposed that any similarities in protein structure could have arisen more recently due to either strong convergent evolution or lateral gene transfer.¹⁵⁹ While it is theoretically possible that convergent evolution may occur relatively frequently in viral capsid proteins that may be subject to strong selection to be small and perhaps of a specific shape, such large-scale convergence seems highly unlikely given that the similarity in capsid structure covers a huge range of viral taxa. As a consequence, multiple

convergent events need to be invoked from very different starting points, and the more convergent evolution that is required, the less likely it becomes. Frequent lateral gene transfer also seems unlikely. As noted later, current data suggest that lateral gene transfer is relatively rare in RNA viruses (although commonplace in large DNA viruses), in large part because of major selective pressures against the expansion of genome sizes.⁹² As the earliest replicating RNAs likely possessed higher error rates than those of contemporary RNA viruses, the first genomes would have been even more restricted in size, such that lateral gene transfer without exact gene replacement must also have been uncommon at this time. Hence, although DNA viruses may be habitual gene pickpockets, RNA viruses do not seem to be. In conclusion, while not conclusive, the presence of structural similarities among highly divergent viruses currently constitutes the strongest evidence that viruses have a precellular origin.

The Time Scale of Virus Evolution

As our understanding of viral origins is vague, so is our knowledge of the antiquity of those families of viruses that circulate today, in part because viruses lack any sort of fossil record. In general, there are three ways in which the evolutionary history of viruses can be placed on a chronological scale. First, if there is a strong match between the phylogenetic tree of viruses and that of their hosts, such that they have *co-diverged*, then it is possible to use the divergence times of hosts to calibrate the time scale of virus evolution. Second, for viruses that evolve rapidly such that there is *measurable evolution* (i.e., mutations are fixed in viral populations during the time frame of human observation), which has been clearly demonstrated in both RNA viruses and ssDNA viruses, it is possible to determine the number of substitutions that have occurred between viruses sampled at known times (heterochronous samples) and use this information to calibrate the time scale of virus evolution under the assumption of a molecular clock (i.e., that there is an approximately constant rate of nucleotide or amino acid fixation). Third, for viruses where endogenous genome copies are present in the host, it is possible to use the substitution rate of the host to determine when these genome integration events occurred, especially if the endogenous sequences also co-diverge with their host species. All three approaches have limitations and can lead to wildly different interpretations of evolutionary time scales, the resolution of which has yet to be achieved.

Dating the time scale of virus evolution through the use of host divergence times (i.e., co-divergence) is perhaps the simplest and most robust approach to this form of molecular archaeology. This approach has been particularly successful in the study of DNA virus evolution. Good examples of its utility are the dating of herpesvirus evolution through an examination of the phylogenetic relationships of their vertebrate hosts, in which virus–host co-divergence may extend to some 400 million years^{148,149}; of the animal iridoviruses¹⁰⁷; of the baculoviruses of insects⁹⁰; and of the papillomaviruses sampled from a number of vertebrates including humans.¹⁹ Clearly, although each of these virus families can be considered as “ancient,” they are in no way of sufficient age to inform on the question of virus origins. In addition, in some other large DNA viruses, with the poxviruses a good example, frequent host-jumping means that patterns of host–virus co-divergence can be difficult to infer,

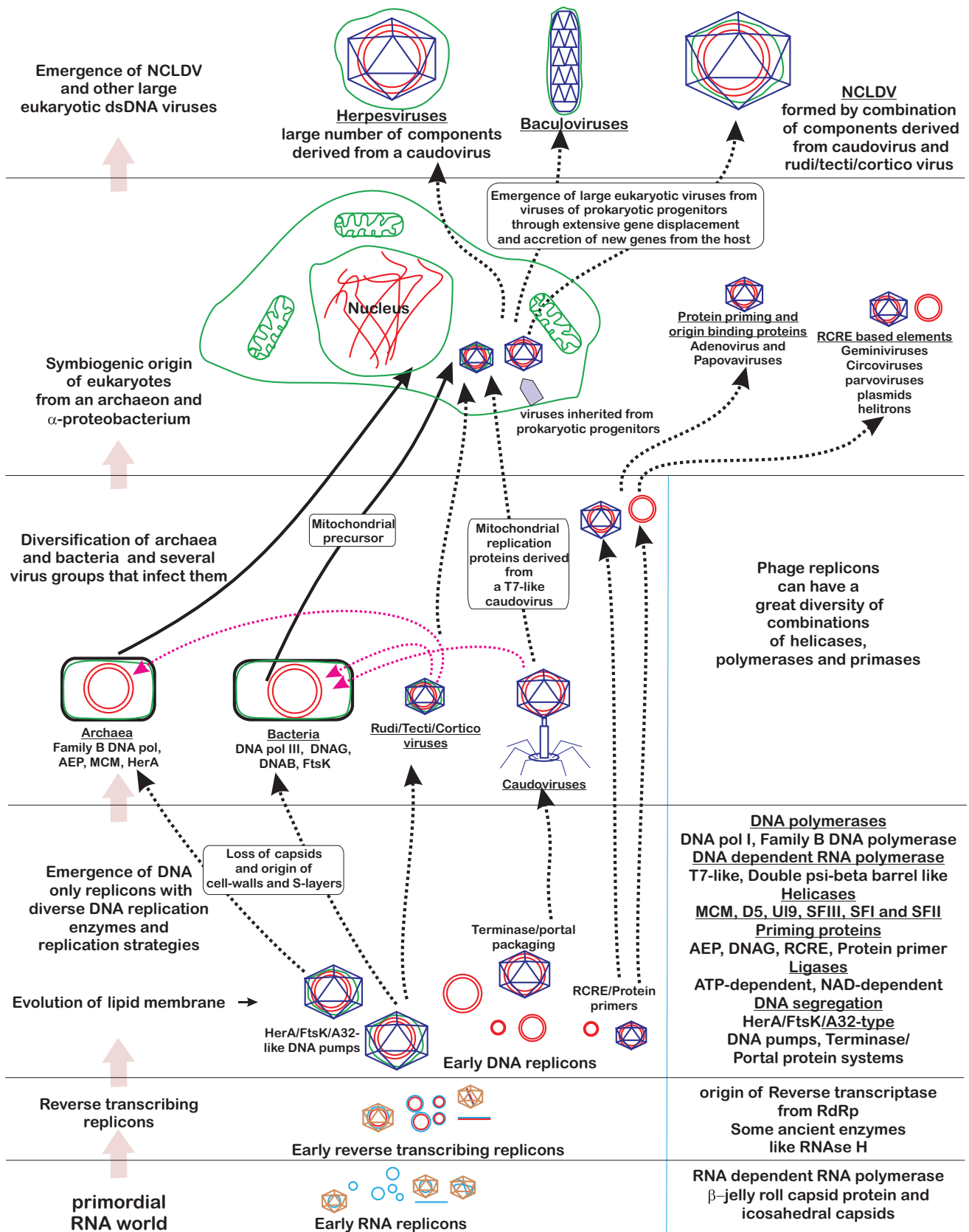


FIGURE 11.2. A plausible scenario for the origin of viruses. Major evolutionary transitions are shown on the **left** of the figure, while the innovations that occurred at each of these transitions are listed on the **right**. Colors are as follows: blue, RNA genomes; red, DNA genomes; green, lipid membranes. (Reprinted from Iyer LM, Balaji S, Koonin EV, et al. Evolutionary genomics of nucleocytoplasmic large DNA viruses. *Virus Res* 2006;117:156–184, with permission from Elsevier. Figure kindly provided by Eugene Koonin.)

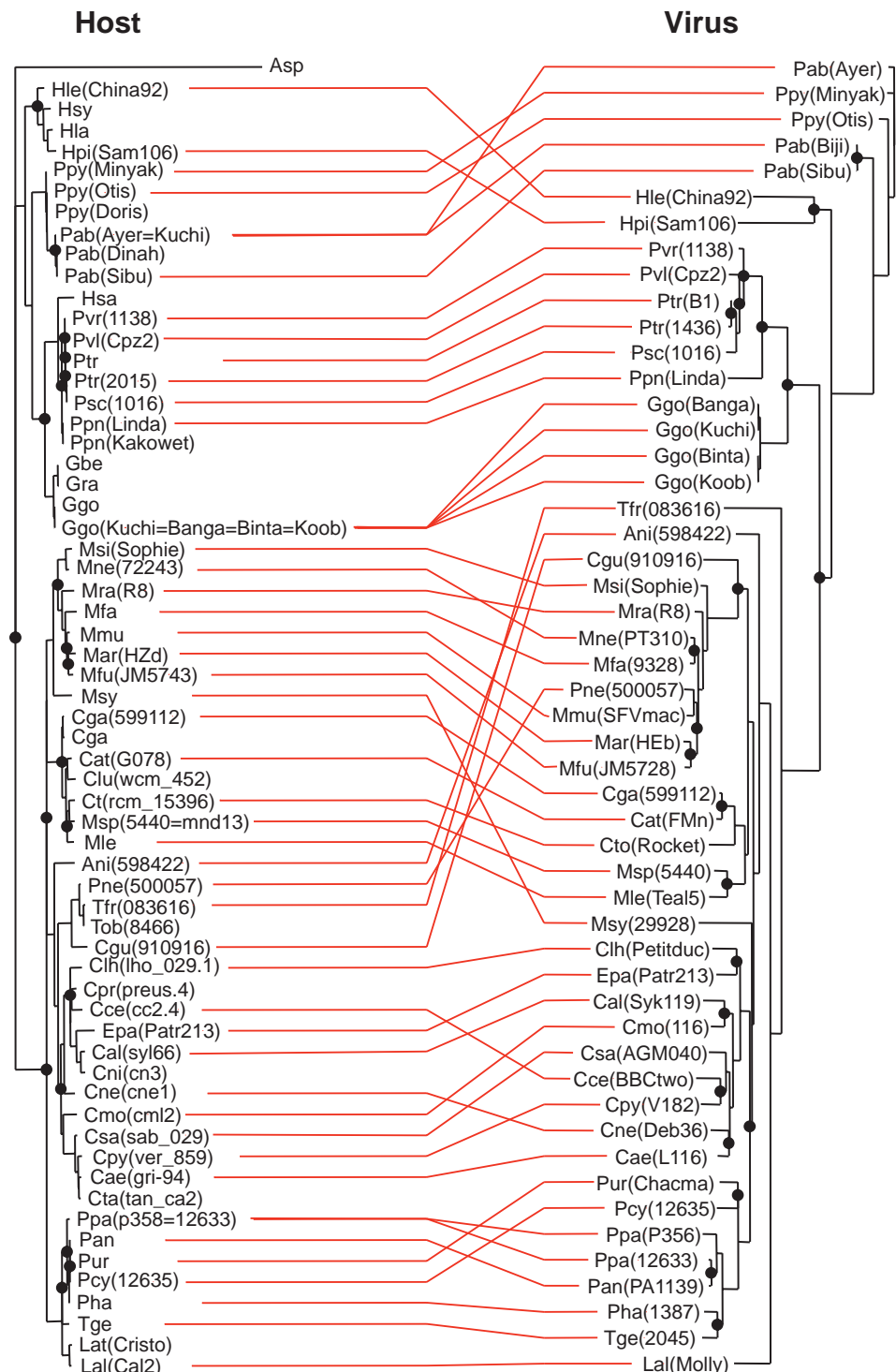
so that the times of origin of key human pathogens like variola virus (VARV; the agent of smallpox) are still the source of considerable debate.^{102,135,206}

Virus–host co-divergence has also been used to date the origin of a number of RNA viruses and retroviruses, although these estimates have sometimes proven controversial. Perhaps the most compelling case to date concerns the retrovirus simian foamy virus (SFV), where a statistically significant match between the phylogenetic trees of host and virus may extend to

at least 30 million years^{137,217} (Fig. 11.3), and where the analysis of endogenous foamy viruses places their evolutionary history in mammals to over 100 million years.¹¹⁴

While *bona fide* examples of virus–host co-divergence constitute a powerful way to date the age of specific viruses, it is also the case that co-divergence is sometimes claimed without any associated statistical test, especially when a small number of taxa are involved such that any resemblance between host and virus phylogeny could occur by chance alone. Given that

FIGURE 11.3. Long-term co-divergence between simian foamy virus (SFV) and its primate hosts. The tree on the left comprises 55 primate species and was inferred using mitochondrial COII sequences. The tree on the right contains 44 SFV sequences and was inferred using the viral *pol* gene. Strong host–virus associations are shown as horizontal lines, while incongruent relationships (i.e., host jumps) are shown as crossed lines (note that some hosts have no SFV associated with them). Host–virus co-divergence events are depicted by solid circles at the nodes. The match between the host and virus trees is far stronger than expected by chance ($p = 0.007$). Branch lengths are drawn to scale of nucleotide substitutions per site. (From Switzer WM, Salemi M, Shanmugam V, et al. Ancient co-speciation of simian foamy viruses and primates. *Nature* 2005;434:376–380; reprinted by permission from Macmillan Publishers Ltd.)



cross-species transmission is a very common mode of virus macroevolution, it can be dangerous to construct a time scale of virus evolution without statistically significant co-divergence. In addition, there are a number of other evolutionary processes that can lead to a match between host and virus phylogenies that do not entail co-divergence. For example, it could be that cross-species transmission occurs more often among closely related host species.²¹⁵ This *preferential host switching*³⁶ may produce phylogenetic patterns that are difficult to distinguish from those of co-divergence. A good example is provided by the primate lentiviruses that infect humans (human immunodeficiency virus [HIV]), chimpanzees (simian immunodeficiency virus [SIV] cpz), and gorillas (SIVgor). That these three viruses are very closely related and infect related hosts might at face value be taken to mean that host and virus have co-diverged for several million years. However, closer inspection of the relevant virus phylogenies revealed that the genetic diversity in each case was in fact due to more recent cross-species transmission. Indeed, cross-species transmission involving very closely related host species appears to be common in viruses.¹¹⁹

Using heterochronous samples to calibrate the virus molecular clock is an extremely powerful and increasingly popular way to study the time scale of virus evolution in the recent past and is the most common method used with RNA viruses where measurable sequence evolution is a routine observation. However, it is also an approach where erroneous conclusions can be drawn if not performed with care. Because large numbers of gene sequences where the precise date of sampling is known are now available, and because virus evolution is often relatively clock-like, it is a straightforward exercise to date the age of samples of genetic diversity.⁴⁹ In some cases divergence times estimated in this manner can be very accurate. For example, an analysis of heterochronous samples of human influenza A virus was able to accurately reconstruct the seasonal peaks and troughs in the population size of this virus.¹⁸⁴ However, while these molecular clock approaches can work well for recent virus evolution—that is, for time scales covering that last few hundred years—they are prone to error at far deeper divergence times, providing a picture of virus evolution that is far too recent. An illustrative example of this effect is provided by the case of the SIVs. While molecular clock studies of SIV evolution using heterochronous samples place this on a time scale of hundreds of years, a calibration based on the biogeographic separation of Bioko Island from the coast of West Africa gave dates of at least 32,000, and perhaps over 100,000 years.²³³ More generally, there are cases in the literature where sensible preconception says that a specific virus should be a certain age, usually because it is thought to have diverged with a particular host species or is associated with a particular event in human history, yet molecular clock studies present a far more recent depiction of its origin.⁹¹

More difficult to explain is precisely why recently calibrated molecular clocks fail so badly in the estimation of ancient divergent times. The most likely explanation is that the statistical models used to estimate the number of nucleotide substitutions separating any two sequences fail to adequately account for all the details of virus evolution, thereby greatly underestimating the true numbers of mutations that have accumulated.^{91,94} In short, there has been excessive site saturation that leads to erroneous estimates of divergence times. At present there is no clear way to resolve this problem, although

a likely path for the future is the development of more sophisticated models of nucleotide and amino acid substitution.

The final, and most recently developed, way to infer the time scale of virus evolution involves the use of endogenous viral sequences that are a common component of eukaryotic genomes. Endogenous genomic copies of exogenous viruses that have entered the germline are particularly commonplace in retroviruses, and it is estimated that approximately 5% to 8% of the human genome is composed of endogenous retrovirus, comprising at least 31 distinct families.¹¹⁵ In addition, there is a growing list of endogenous RNA and small DNA viruses, also referred to as endogenous viral elements (EVEs), usually composed of partial virus genome sequences.^{94,113}

The importance of endogenous viruses is that they represent a sort of “fossil record” of past viral infections; once integrated into host genomes they cease to evolve like viruses and instead assume the low rates of nucleotide substitution that characterize their hosts, replicating using high-fidelity host DNA polymerases and likely experiencing fewer replications per unit time (Fig. 11.4). Consequently, if the mutational differences between endogenous viruses are known to occur postintegration, such as those observed between the LTRs of a single endogenous retrovirus, between duplicated EVEs, or when there is clear evidence for co-divergence, then divergence times can be estimated in a relatively straightforward manner using host substitution rates.

Molecular clock dating in this manner suggests that the exogenous ancestors of some human retroviruses may have diversified relatively early on in mammalian evolution.¹¹⁵ Most dramatic are cases of when both exogenous and endogenous copies of the same virus exist, which have generally resulted in a radically different picture of the time scale of viral evolution than using clock estimates based on heterochronous samples. For example, estimates of the age of primate lentiviruses based on the use of heterochronous sequences generally results in time scales of thousands of years at most,²⁰⁵ while the presence of endogenous lentiviruses in lemurs suggests that these viruses have circulated in primates for at least several million years.¹¹⁶ The same is true of endogenous viruses that are not retroviruses. Perhaps the most compelling of these are the avian hepadnaviruses, in which the observation of EVEs integrated at the same genomic positions in bird species that diverged at least 19 million years ago strongly suggests that hepadnaviruses are at least of the same age.⁸⁰ This antiquity sits in stark contrast to studies of hepadnavirus evolution based on the use of heterochronous sequences, in which divergence times are measured on scales of a few thousand years.²⁴⁰ The same phenomenon has been proposed for a variety of other RNA viruses, including bornaviruses and filoviruses,^{14,97,113,218} as well as ssDNA viruses of the families *Circoviridae* and *Parvoviridae*.^{15,111,113,136} In each case, integrated copies of these viruses are observed in diverse host species, for example, comprising both placental and marsupial mammals in the case of the filoviruses, and sometimes showing virus–host co-divergence such that they are clearly millions of years old.¹¹³ However, there are also many cases where the phylogenies of the endogenous viruses and their hosts do not match, which complicates estimates of virus divergence times. As more host genomes are sequenced, it is certain that more endogenous viral elements will be discovered, which will undoubtedly shed new light on the true time scale of virus evolution.

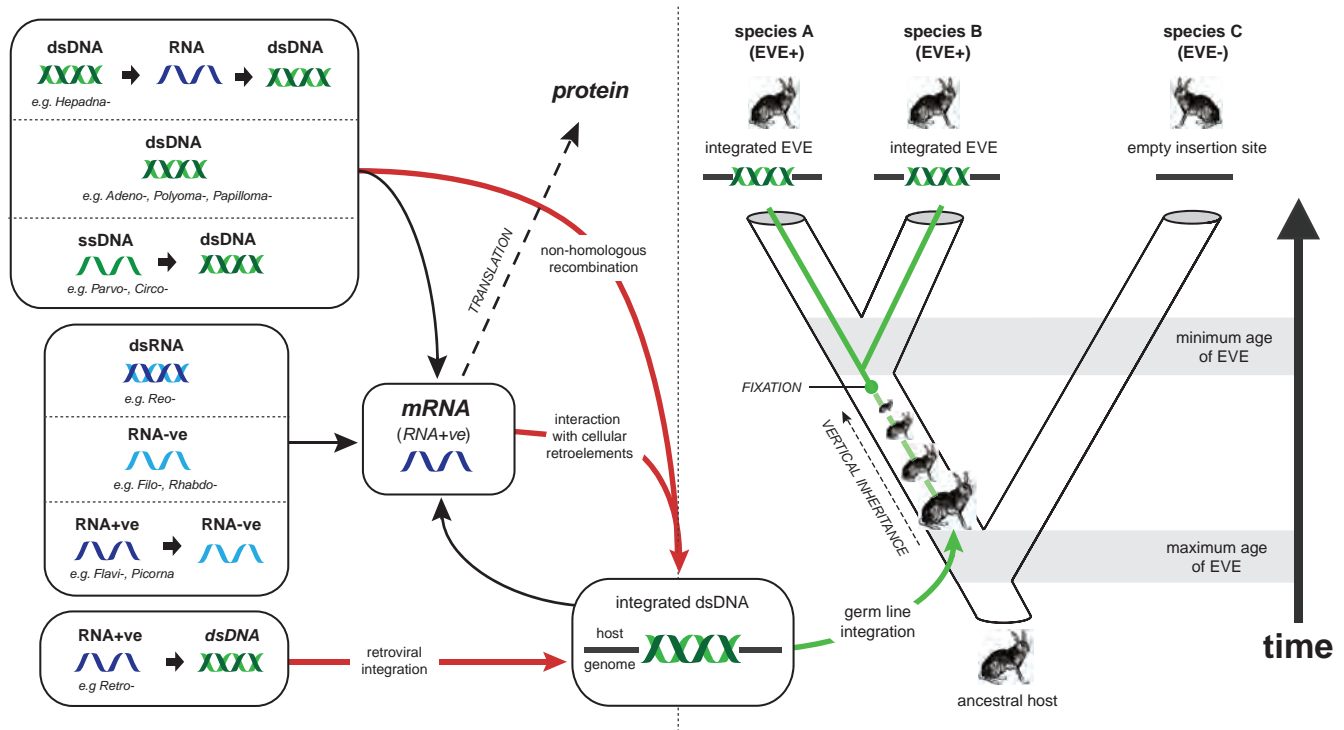


FIGURE 11.4. How endogenous virus elements (EVEs) are generated and can be used to estimate the age of viral families.

Examples of different types of mammalian RNA and DNA viruses are shown. Critically, the presence of EVEs in related host species A and B and integrated into the same genomic position indicates that this integration event occurred prior to the divergence of these two species, such that its minimum age can be estimated if it is known when the host species diverged. (From Katourakis A, Gifford RJ. Endogenous viral elements in animal genomes. *PLoS Genet* 2010;6:e1001191. Original figure kindly provided by Rob Gifford.)

Virus Classification

Finally, and as a brief digression, it is useful to discuss the schemes used for virus classification, ranging from orders to species and genotypes, and what they mean in an evolutionary context, particularly as they indirectly relate to the issue of virus origins. The main points to make here are that although a useful and important aspect of comparative biology, all such classification schemes have a large arbitrary component to them, are not based on a specific evolutionary pattern or process, and do not necessarily mean that the viruses in question exhibit a specific set of biological differences. Indeed, these limitations are likely to apply to most microorganisms. To some extent, this arbitrariness sits in contrast to what is seen in sexually reproducing eukaryotes, where *species* represents a distinct and clearly definable evolutionary group—a population of interbreeding individuals. Hence, although what are termed virus *species* may possess phenotypic characteristics that can be defined for each group and form well-supported clusters on phylogenetic trees, this should not be taken as evidence that they have been formed by a distinct evolutionary process that is analogous to reproductive isolation in eukaryotes. The same is also true of the higher-order classifications proposed for viruses (e.g., supergroups, orders, genera) and for those below the level of species (e.g., genotypes or subtypes). All these groupings can be thought of as points in phylogenetic space rather than describing taxonomic groups that have attained a specific level of phenotypic divergence. In other cases viruses are classified simply through estimates of pairwise genetic diversity, with

different levels of diversity signifying the division into species, genotypes, and so forth. Although simple, classification schemes constructed in this manner similarly have no basis in evolutionary theory and may be biased if different lineages evolve at different rates. In sum, it is overly simplistic to think that nature will generate clear-cut divisions in phenotypic and genotypic space that can be used to construct meaningful classification schemes, and hence that systematics tells us anything about virus origins and evolution.

PROCESSES OF VIRUS EVOLUTION

Irrespective of debates over whether viruses are alive, it is clear that they are subject to the same forces that shape the evolution of cellular species, that is, mutation, natural selection, genetic drift, recombination (and reassortment), and migration. I will discuss the first four of these processes in this chapter. Migration, in the guise of viral epidemiology, is discussed elsewhere in this volume.

Mutation and Nucleotide Substitution in Viruses

The simplest way to examine the role of mutation in virus evolution is to measure the rate of its occurrence. Indeed, understanding the factors that shape the speed at which genetic variation is generated in viruses is central to understanding many aspects of their evolution. For RNA viruses mutation can in some ways be thought of as their defining evolutionary

feature, as it occurs at a pace that greatly exceeds that observed in other organisms.

Although mutation is the ultimate source of genetic variation, the pace of evolutionary change can in fact be measured in two rather different ways. One method is to estimate, experimentally, the rate at which mutations are generated *de novo*. Such rates have usually been presented as the number of mutations per nucleotide, per replication or the number of mutations per genome, per replication. However, because of the inherent complexities and biases in making these estimates, it has been suggested that estimates of mutation rate per nucleotide, per cell infection may be more informative.¹⁹⁶ For example, one important complicating factor is that some viruses employ so-called stamping machine replication, in which a single virus acts as the template for all progeny genomes, so that mutations accumulate linearly, while others utilize “geometric” replication, in which some of the early progeny genomes are used as templates to produce further progeny, in turn increasing the rate of mutation accumulation.⁵²

The power of mutation rate estimates is that they reveal the intrinsic error dynamics of the RNA or DNA polymerases used in viral replication and in theory allow a count of each type of mutation—advantageous, neutral, or deleterious—before they have been shaped by natural selection, although it is always difficult to accurately count the number of lethal mutations that are rapidly removed by purifying selection. A detailed compilation of mutation rate estimates for 23 viruses, and accounting for many of the complexities inherent in analyses of this kind, revealed that these rates varied from 10^{-6} to 10^{-4} mutations/nucleotide/cell infection for RNA viruses to 10^{-8} to 10^{-6} mutations/nucleotide/cell infection for DNA viruses¹⁹⁶ (Fig. 11.5). For RNA viruses that replicate with RdRp, an enzyme that lacks a proofreading or repair function, this equates to mutation rates that are usually a little below approximately one per genome, per replication.^{47,48,52} Similarly, the lower mutation rates observed in large DNA viruses clearly

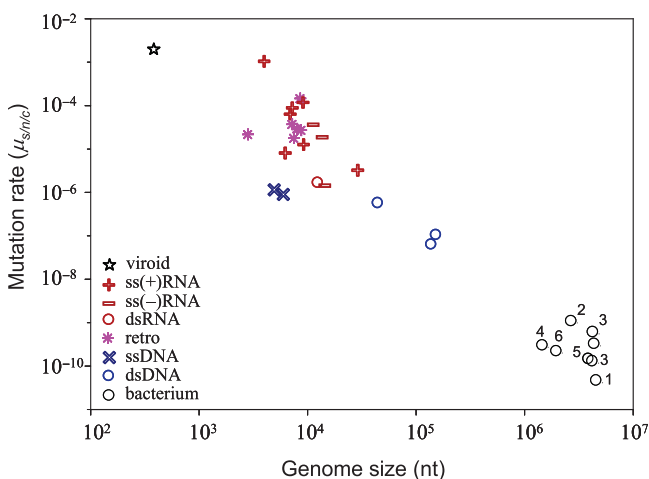


FIGURE 11.5. Comparative rates of mutation in different types of virus and their relationship to genome size. Comparable values from bacteria are also shown. Mutation rates (y-axis) are given per nucleotide per cell infection (s/n/c). See reference 196 for more details including the taxa analyzed. (From Sanjuán R, Nebot MR, Chirico N, et al. Viral mutation rates. *J Virol* 2010;84:9733–9748. Figure kindly provided by Rafa Sanjuán.)

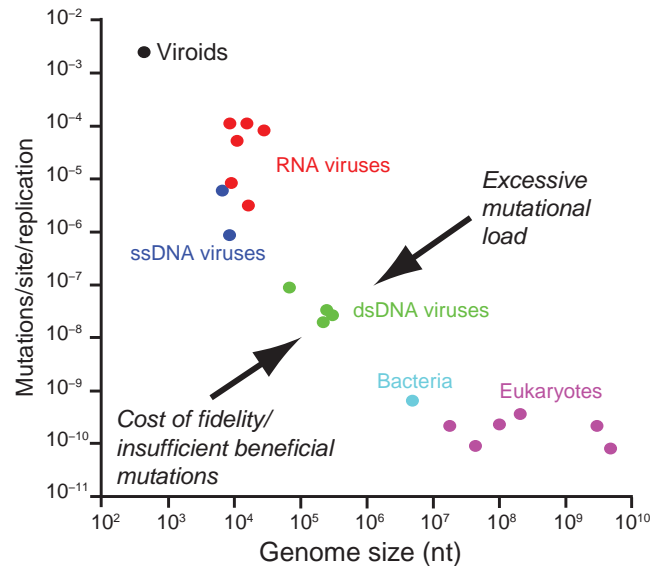


FIGURE 11.6. Relationship between mutation rate and genome size in diverse organisms including RNA and DNA viruses and viroids. The competing evolutionary forces that might be responsible for the limited range of observed error rates and genome sizes are shown. For details see reference 93. Data from reference 75. (From Holmes EC. What does virus evolution tell us about virus origins? *J Virol* 2011;85:5247–5251.)

reflect the higher fidelity of the DNA polymerases employed in their replication cycle. Of particular note is that mutation rate estimates in ssDNA viruses (a maximum of 1.1×10^{-6} , although only two estimates are available) are higher than those of large dsDNA viruses (which range from 5.9×10^{-8} to 5.4×10^{-7}), even though ssDNA viruses have such small genomes that they use host DNA polymerases for replication. It is therefore possible that the relatively high mutation rates in ssDNA viruses reflect less efficient proofreading and excision repair on ssDNA and/or frequent deamination.⁵¹ The study of Sanjuán et al.¹⁹⁶ was also of note in that it revealed that retroviruses such as HIV have error rates that overlapped with those of RdRp-utilizing viruses, even though earlier studies suggested that RT exhibits higher fidelity than RdRp.^{47,48,143}

From a broader perspective these estimates of mutation rate are compatible with the idea that for all living systems there is a strongly inverse relationship between mutation rate and genome size (Fig. 11.6).^{75,93} It also seems likely that the systematic difference in mutation rate and genome size between RNA and DNA viruses is associated with many of the main evolutionary distinctions between these two types of infectious agents that are discussed throughout this chapter, such that they can be thought of as occupying very different regions of evolutionary parameter space (Table 11.1).

Although they utilize related DNA polymerases, mutation rates in large DNA viruses are higher than those of bacteria and eukaryotes, which may reflect an absence of the full set of repair enzymes and pathways in the former. However, there are two possible exceptions to this rule. First, there is currently no estimate of mutation rates in mimivirus or megavirus, although these are expected to fall within the bacterial range (as the genome sizes of these viruses overlap with those of bacteria), and hence are lower than that observed in any virus to date.

TABLE 11.1 The Differing Evolutionary Parameter Spaces Occupied by RNA and DNA Viruses^a

Characteristic	RNA (and ssDNA) viruses	dsDNA viruses
Mutation rate (per nt)	High	Low
Genome size	Small (<32,000 nt)	Can be large (>100,000 nt)
Population sizes	Usually large	Can be small
Recombination	Often low rates	Often high rates
Epistasis	Antagonistic	Synergistic ^b
Gene duplication	Apparently rare	Common
Lateral gene transfer	Apparently rare	Common
Overlapping reading frames	Common	Relatively rare

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

^aNote that the properties shown are “average” ones, and cannot be applied to every taxon in each category (particularly for DNA-based organisms).

^bBy inference only: the extent and sign of epistasis has not been measured in dsDNA viruses.

Second, we similarly lack an estimate of mutation rate in the small dsDNA viruses, such as the papillomaviruses. Although the relationship depicted in Figures 11.5 and 11.6 implies that these viruses will mutate rapidly, most estimates of substitution rate in papillomaviruses are in a similar range to those of large DNA viruses.^{68,187} This implies that papillomaviruses similarly mutate relatively slowly, which would break the simple relationship between mutation rate and genome size.

The relationship between mutation rate and genome size is of twofold importance. First, because it incorporates genetic systems ranging from viroids to eukaryotes, it covers at least eight orders of magnitude of both genome size and mutation rate, and few things in biology encompass such diversity. Second, it implies that mutation rates that are either too high or too low are selected against.⁹³ With respect to the latter, a popular idea is that viral mutation rates (and particularly the high mutation rates of RNA viruses) are the result of an evolutionary trade-off, either between replication rate and replication fidelity⁷⁴ or between the rates of deleterious and advantageous mutation.^{52,213} Data can be cited in support of both relationships.^{92,144,224} The possible trade-off between the rates of deleterious and advantageous mutation seems particularly compelling. On the one hand, as viruses will be commonly exposed to changing environments (i.e., different hosts, a variety of cell types, frequent immune pressure), the generation of some genetic variation via mutation is likely to be selectively advantageous. This idea also has experimental support; RNA polymerases with lower fidelity are sometimes selectively favored over those with higher fidelity.^{92,144,224} On the other hand, there must be an upper limit on the mutation rates experienced by viruses (and all living systems), as excessive error will result in major fitness losses. Powerful evidence for this ceiling on mutation rates is provided by experiments invoking *lethal mutagenesis* (see later), in which artificially increasing error rates with mutagens such as 5-fluorouracil and ribavirin result in an excessive mutational load.^{26,171} It is therefore likely that RNA viruses exist close to their maximum tolerable mutation rates, which in turn imposes an upper limit on genome size. However, it is also likely that RNA viruses are mechanistically unable to reduce their error rates to the levels associated with DNA polymerases. A higher-fidelity RNA polymerase would

need to be more complex, and hence longer, than those that currently exist, yet this cannot evolve because, by increasing genome length, it will result in too many deleterious mutations. This evolutionary conundrum is commonly referred to as *Eigen's paradox* and is a key element in theories for the early evolution of genomic complexity.

The second measure of the pace of virus evolution is the rate of nucleotide substitution per nucleotide site, per year (subs/site/year). As this measure reflects the population success of any mutation, it by necessity incorporates the action of natural selection. Accordingly, deleterious mutations that have been removed by purifying selection will not be counted, while advantageous mutations will be fixed more rapidly than neutral ones. Nucleotide substitution rates are usually far easier to estimate than mutation rates and as such provide a simple and powerful means to compare patterns of virus evolution.

As with rates of mutation, rates of nucleotide substitution vary markedly among viruses. Across RNA and DNA viruses as a whole, nucleotide substitution rates vary by over five orders of magnitude, in large part reflecting the differences in background mutation rate described earlier. Hence, most substitution rates in RNA viruses fall within an order of magnitude of a value of 1×10^{-3} subs/site/year,^{87,110} while the rates in many dsDNA viruses are closer to 1×10^{-8} subs/site/year.^{89,145,148,187} Undoubtedly, our understanding of viral substitution rates would be improved by measures of evolutionary dynamics in the smallest (i.e., viroids) and largest (i.e., mimivirus) viral systems.

Most of the variance in the substitution rates in both RNA and DNA viruses likely reflects virus-specific differences in either mutation rate, replication rate, or both. Mutation rates have been discussed earlier, and some studies have revealed that substitution rates in RNA viruses are negatively associated with genome size as expected if background mutation is the main determinant of substitution rate.¹¹⁰ Although few direct estimates of replication rate are available, they likewise clearly play a major role in shaping substitution rates. For example, although the retrovirus SFV likely has an RT-associated error rate that is similar to those of other retroviruses, its co-divergence with primates for over 30 million years (Fig. 11.3) leads to estimates of the substitution rate of only 1.7×10^{-8} subs/site/year.²¹⁷ This most likely reflects a low rate of replication,

although this merits further investigation. Similarly, replication rates appear to be low in papillomaviruses, in which virus replication occurs simultaneously with the division of host epithelial cells, at approximately 10 to 100 generations per year,¹⁹ which likely contributes to the low substitution rates estimated in this virus. In contrast, that DNA viruses often replicate more rapidly than their hosts may in part explain why virus substitution rates are higher than host substitution rates even though they utilize similar polymerases. For example, the Bo17 protein of bovine herpesvirus 4 represents a viral capture of the mammalian 2 β -1,6-N-acetylglucosaminyltransferase-mucin protein.¹⁴⁵ As a phylogenetic analysis revealed that this gene was captured from the host after the split between cattle and African buffalo approximately 1.5 million years ago, it was possible to estimate that the viral gene had evolved 20 to 30 times faster than its cellular homolog.¹⁴⁵ Finally, for persistently infecting viruses, substitution rates may also differ between periods of intra- and interhost evolution. For example, HIV-1 substitution rates are higher within than among hosts.¹⁴² This may be because intra-host HIV evolution is dominated by the positive selection of immune escape mutations or because some of the mutations that occur within hosts are purged at interhost transmission, thereby reducing the substitution rate.

Although most estimates of substitution rate suggest a fundamental division in virus evolution in which RNA viruses evolve rapidly and DNA viruses evolve slowly, there are a number of important exceptions. First, a number of RNA viruses are reported to evolve anomalously slowly.^{99,216} A much debated example is the rodent hantaviruses (*Bunyaviridae*), which are often claimed to have co-diverged with their murid hosts over many millions of years, which would lead to rates of nucleotide substitution in the range of 10^{-7} subs/site/year.¹⁷⁸ Clearly, these substitution rates are far closer to those of dsDNA viruses than to other RNA viruses. However, there are also important mismatches between the virus and host phylogenies, most notably that hantavirus sequences from various insectivores are mixed with those sampled from rodents,⁶ and hantavirus substitution rates estimated over the short term fall within the usual RNA virus range.¹⁸⁵ As a consequence, the true rate of nucleotide substitution in the rodent hantaviruses is uncertain. Second, ssDNA viruses exhibit substitution rates—often in the realm of 10^{-4} subs/site/year—that are closer to those of RNA viruses than to large DNA viruses. Such high rates have now been described in both carnivore²⁰³ and human^{167,201} parvoviruses, in porcine circovirus,⁶⁷ and in plant geminiviruses.^{51,222} Although the mutation rates of ssDNA viruses are higher than those of large DNA viruses, they do not fully explain the elevated substitution rates.¹⁹⁶ It is therefore possible that rapid nucleotide substitution in ssDNA viruses reflects short generation times and/or strong positive selection, the latter of which is well documented in the carnivore parvoviruses following cross-species transmission.^{172,203} An important caveat is that both endogenous circoviruses and parvoviruses have now been documented, which suggests that these viruses are ancient.^{111,113} Unfortunately, however, the sequences of these endogenous viruses are often so divergent as to challenge any attempt to reliably estimate rates of evolutionary change.

Finally, there are also cases in which large DNA viruses evolve more rapidly than might be expected. A case in point is VARV, in which estimates of the substitution rate fall between 10^{-6} and 10^{-5} subs/site/year, and hence are far higher than those

observed in other large DNA viruses where evolutionary rates have been inferred, particularly the herpesviruses.^{68,102,206} In addition, VARV exhibits a strongly linear relationship between genetic distance and time of sampling (i.e., measurable evolution) that is also indicative of a high substitution rate.⁶⁸ It is therefore possible that VARV has been subject to strong positive selection (at least in the recent past) and that this has elevated the substitution rate to above that expected by neutral mutation pressure alone. Such strong positive selection may be a characteristic common to the orthopoxviruses.¹⁵¹

Natural Selection and Genetic Drift in Virus Evolution

Despite long-standing debates over whether natural selection or genetic drift is the more important process of evolutionary change at the molecular level, there is little doubt that viruses provide some of the very best examples of natural selection in action. The literature contains numerous examples of natural selection for such properties as immune evasion (of antibody, T-cell, or innate responses), antiviral resistance, and the adaptation to new cell types and host species. The strength of these selection pressures is readily apparent in the fact that the genomes of large DNA viruses such as poxviruses and herpesviruses contain many genes dedicated to immune evasion. As there are numerous examples of each of these types of positive (i.e., Darwinian) selection for both RNA and DNA viruses, and they are generally not contentious, I will not discuss them here. A more pressing question is, what proportion of all the mutations that arise and are fixed in viral genomes are entirely free of natural selection and hence evolve in a strictly neutral manner, compared to the proportion that are fixed because they are selectively advantageous? Simple population genetic theory predicts that natural selection should be a potent force in viral evolution. Natural selection dominates molecular evolution when the product $N_e s \gg 1$, where N_e is the effective population size, and s the selection coefficient (i.e., the fitness of the mutation in question). Although easy to state, estimating these parameters is usually thwarted with difficulties, such that the measures obtained are often only approximate. As a consequence, conclusively determining the role of natural selection versus genetic drift in viral evolution is inherently difficult, although a number of important generalities can be made.

In the case of RNA viruses, it is likely that few amino acid mutations are strictly neutral (i.e., $s = 0$), with most clearly deleterious. In particular, their small genomes ensure there is extensive pleiotropy, epistasis, and multifunctionality such that there is little evolutionary elbow room. Indeed, mutagenesis studies of vesicular stomatitis virus (VSV) revealed that nearly 40% of random mutations are lethal, another 29% deleterious, a further 27% neutral, and only 4% beneficial,¹⁹⁵ although these estimates only relate to fitness effects in a single cell and a far larger proportion of mutations are expected to be deleterious (or lethal) when considering the entirety of the virus life cycle. In addition, as recombination is infrequent in many RNA viruses (see later), there will be strong linkage between neutral mutations and those that are either fixed or purged by natural selection, such that their evolutionary fates will be linked. The same is also likely to be true of ssDNA viruses, which are also characterized by very small genome sizes. Accordingly, most estimates of the ratio of nonsynonymous (d_N) to synonymous (d_S) nucleotide substitutions per site (ratio

d_N/d_S , a common measure of selection pressures, with $d_N/d_S = 1$ indicative of selective neutrality) in RNA and ssDNA viruses suggest that purifying selection is the most common evolutionary force (i.e., $d_N/d_S < 1$).^{92,101} However, the case of large DNA viruses is far less clear, in large part because relatively few studies of their molecular evolution have been undertaken. One comparative study of d_N/d_S revealed weaker purifying selection in DNA than RNA viruses,¹⁰¹ suggesting that the former may possess a class of neutrally evolving amino acid sites. Although the explanation for this effect is unclear, it may be a function of the large genome sizes in large dsDNA viruses, which allow more genetic redundancy through the presence of duplicated genes (see later). Alternatively, it may be indicative of greater levels of positive selection in DNA viruses, as also suggested by the high rates of nucleotide substitution documented in some.⁶⁸ Determining the exact nature of the selection pressures acting on large DNA viruses, including the fitness of individual mutations, is clearly an area where a combination of experimental study and evolutionary analysis will be of fundamental importance.

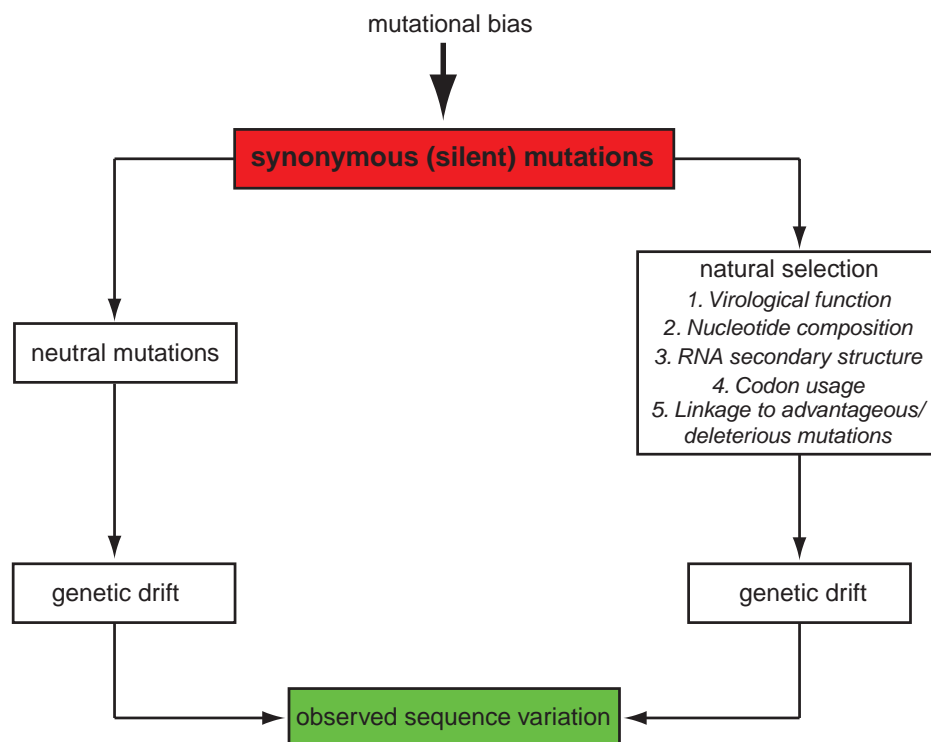
Obviously, the most likely class of neutral sites in viral genomes is those that do not code for protein. For RNA viruses it is debatable how many nucleotide sites fall into this category, particularly as these viruses contain very few, if any, clear-cut examples of “functionless” noncoding RNA, such as pseudogenes and introns. A similar story can be told for small DNA viruses, either single or double stranded, which can be considered as exemplars of genomic efficiency. Indeed, many small DNA viruses, such as circoviruses, parvoviruses, and hepadnaviruses, contain extensive overlapping reading frames, suggesting that they are under strong selective pressure to maximize the phenotypic diversity they can produce from a restricted genomic space. Importantly, the existence of overlapping reading frames changes the selective regime acting on many

nucleotide sites. In particular, synonymous sites in one reading frame are likely to be nonsynonymous in another, such that they must be subject to some sort of purifying selection. Similarly, although some pseudogenes are present in the genomes of large DNA viruses,⁷⁶ there are relatively few gene overlaps, and it is unclear whether they contain stretches of purely nonfunctional DNA. For example, although the genomes of herpesviruses and poxviruses contain many regions that do not code for protein (~5% to 15% of the genome in the case of poxviruses), these may still encode promoters, transcription termination signals, functional RNA molecules, or other functional elements. One candidate for truly nonfunctional sequences is the imperfect tandem repeat seen in the intergenic regions of some poxviruses and that exhibit various deletions and duplications. Understanding the potential functions, if any, of these noncoding regions is an important goal for the future.

Evolution of Synonymous Sites

Perhaps the most intense debates on the nature of selection pressures acting on viral genomes concern synonymous (silent) nucleotide sites. In eukaryotes, and particularly mammals, there is good evidence that many synonymous nucleotides are subject to only weak (if any) selection pressures and therefore evolve in an effectively neutral manner, although selection on silent sites is being increasingly documented³¹ and can take a variety of forms (Fig. 11.7). Under neutral evolution the nucleotide composition of synonymous sites tends to match that of the genome region in which they are located, reflecting the action of background mutation pressure. That in all viruses genome-scale levels of synonymous variation are far higher than those observed at nonsynonymous sites indicates that the fitness effects of synonymous mutations are usually less than those of nonsynonymous mutations (as is true of all living systems). However, this does not necessarily mean that

FIGURE 11.7. Evolutionary processes acting on synonymous (silent) nucleotide sites in viruses. The first bias is generated by the basic process of mutation, which may make some types of mutation (e.g., specific nucleotide transitions) more frequent than others. Those synonymous mutations that do arise may either be neutral, so that they are not subject to natural selection, or exhibit fitness differences (listed), in which case they are put through the sieve of natural selection. Both neutral and advantageous mutations are also subject to random genetic drift.



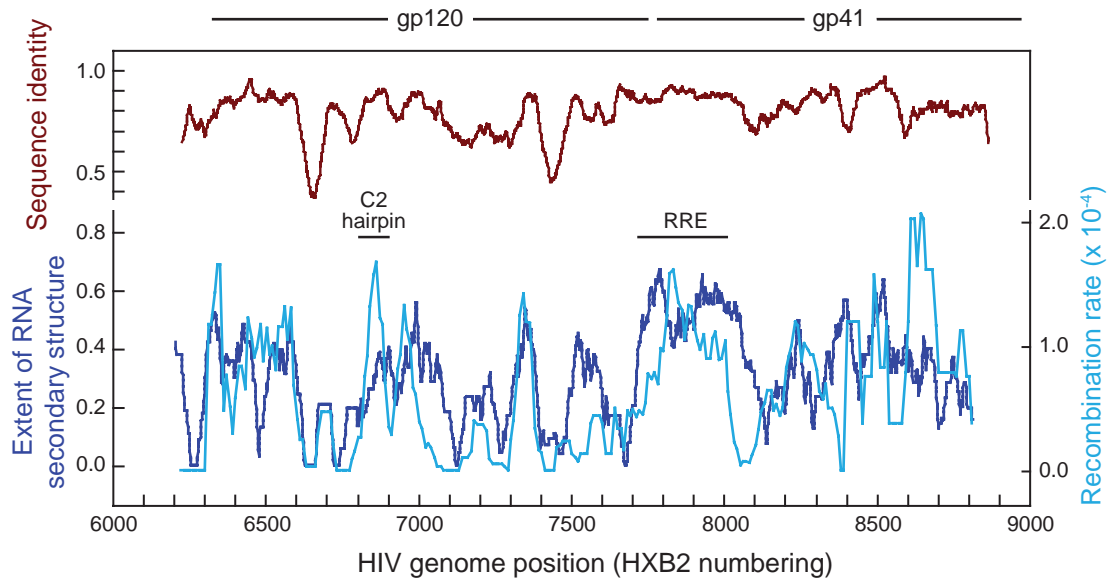


FIGURE 11.8. The relationship between recombination rate, sequence similarity, and RNA secondary structure in the *env* (envelope) gene of human immunodeficiency virus type 1 (HIV-1). Recombination rates are shown in light blue, conserved RNA structures in dark blue, and sequence similarity (identity) in brown. The location of recombination hotspots are marked by light blue bars at the bottom of the figure. (Adapted from Simon-Loriere E, Martin DP, Weeks KM, et al. RNA structures facilitate recombination-mediated gene swapping in HIV-1. *J Virol* 2010;84:12675–12682, which should be consulted for more details. Figure kindly provided by Etienne Simon-Loriere.)

synonymous mutations are strictly neutral, particularly at large effective population sizes where natural selection is especially potent. In fact, there is mounting experimental evidence that synonymous mutations can sometimes have a major effect on viral fitness, as they contain the signals for such processes as promotion, transcription, and encapsidation,^{147,168} which means that all studies of d_N/d_S should be interpreted with care.

The most discussed ways in which natural selection might act on synonymous sites are through RNA secondary structures and codon usage. RNA secondary structures are a common feature of many RNA viruses, can occur in both untranslated regions and coding regions, and can comprise genome-scale interactions.^{210,226} It is well established that these RNA secondary structures can have a major impact on key elements of virus function, such as containing important signals for such processes as replication and translation. RNA secondary structures may impact on other aspects of virus evolution including shaping, to some extent, genome-wide recombination frequencies²¹² (Fig. 11.8). Similarly, functional RNA structures have been noted in a number of DNA viruses.¹⁵⁶ Evidently, the presence of RNA secondary structures strongly argues against the selective neutrality of many synonymous sites,²⁰⁹ although their precise effects on fitness have rarely been documented.

The second form of natural selection that may act on synonymous sites is codon usage. As with RNA secondary structure, changing codon usage may have a profound effect on fitness. As a dramatic case in point, experimental alteration of synonymous codon usage resulted in the attenuation of poliovirus.³⁹ When genetic drift dominates molecular evolution, synonymous codon usage is set by the background (i.e., neutral) mutational bias in the organism in question. In

contrast, natural selection could shape codon choice by optimizing the match between codon and anticodon so as to increase the accuracy and/or efficiency of protein translation (although a variety of other selective explanations exist¹⁷⁷). While viruses often exhibit strong biases in codon usage, the explanations for these biases have usually not been resolved. Many RNA viruses utilize synonymous codons that tend to match the nucleotide composition of the viral genome as a whole, suggesting that selection on codon choice is likely to be relatively weak and mainly set by background mutational pressure¹⁰⁸ or that selection is acting on the overall nucleotide composition (see later). However, direct selection for a specific pattern of codon usage seems to characterize other viruses. For example, codon usage bias in Epstein-Barr virus is associated with levels of viral gene expression, varying between the latent and productive phases,¹¹² while there is a strong match between codon usage and host transfer RNA (tRNA) availability in papillomavirus, which determines the levels of capsid protein expression.²³⁹ Similarly, a study of honeybee viruses revealed the same pattern of codon usage in unrelated RNA viruses, indicative of a bee-specific selection pressure.³³ More interesting is the case of hepatitis A virus in which codon usage seems to be set by the kinetics (rather than the accuracy) of protein translation such that rare codons that utilize nonabundant tRNAs are used, which slows down the translation process to ensure proper protein folding.⁵

As noted earlier, it is also possible that overall nucleotide composition is the selectively determined trait, which has a secondary effect on codon usage bias. Evidence for such a process is the major host-specific differences in nucleotide composition observed in some viruses.^{10,109} For example, nucleotide

composition noticeably changes in influenza A virus following host jumps from birds to mammals, although precisely why is unclear.⁸⁵ Systematic differences in nucleotide composition also characterize DNA viruses, and nucleotide compositions can vary enormously even at the intrafamily level.¹⁴⁹ For example, CpG nucleotide frequency was observed to be the major correlate of codon usage bias in vertebrate DNA viruses, again pointing to the importance of genome-wide mutation pressure.²⁰² However, small DNA viruses were observed to have CpG contents far below that expected given their overall nucleotide composition, in contrast to the situation in large DNA viruses, although the explanations for this difference are unclear.²⁰²

Effective Population Size and Population Bottlenecks

After the selective coefficient of mutations, the second factor that determines the respective roles of natural selection and genetic drift in virus evolution is the effective population size (N_e), which is usually far lower than the census population size, N . Available data indicate that N_e fluctuates dramatically during the life cycle of most viruses. For all exogenous viruses, large values of N_e are most likely observed within an individual host following multiple rounds of replication and at the epidemiologic scale. Natural selection is expected to be an efficient force in these cases. For example, perhaps 10^{10} virions are produced by HIV-1 replication per day in every infected individual,¹⁷⁵ with some 10^7 human hosts HIV-infected on a global scale. In addition, the burst sizes of many acute viruses will be large, such that within-host values of N_e are expected to be high for at least some parts of the virus life cycle. The exception to this rule may be latent viruses, in which effective population sizes are often likely to be relatively small, reflecting a lack of active replication.

Conversely, low values of N_e , reflecting the time when genetic drift is strongest and perhaps overriding the action of natural selection, will generally occur at interhost transmission. At this point in the virus life cycle a major population bottleneck may be a common occurrence, although this is also likely to be at least in part determined by the mode of transmission and hence the infecting dose; for example, vertical transmission (wide bottleneck) might be expected to be associated with the transfer of more viruses between hosts than sexual transmission (narrow bottleneck).¹³⁴ In some cases, including HIV-1, it is possible that the transmission bottleneck is so extensive that new infections are initiated by a single virus particle,⁶⁹ which would obviously result in a major stochastic effect on virus genetic diversity. Similarly, experimental studies of plant viruses have commonly revealed the existence of extensive population bottlenecks, both at interhost transmission and as the virus moves through an individual plant^{3,191} and which may also be true of vector-borne viruses of animals.²¹⁴ In these cases the stochastic effects of genetic drift are expected to be strong, even to the extent of allowing many slightly deleterious mutations to rise to appreciable frequencies, although to date there have been few *in vivo* experiments to address this issue. Interestingly, some comparative sequencing studies have suggested that interhost transmission might be associated with relatively wide population bottlenecks. For example, multiple viral lineages passed among horses during experimental transmissions with equine influenza virus,¹⁶² while the occurrence of

mixed infections in viruses such as influenza^{78,162} and dengue¹ also suggests that transmission bottlenecks might be relatively broad. Overall, it is evident that a great deal more work is needed to fully understand the nature and scale of population bottlenecks in viruses and what this means for the relative frequencies of natural selection versus genetic drift, particularly in natural as opposed to experimental infections.

Evolutionary Interactions: Epistasis, Defecting Interfering Particles, and Complementation

A common assumption in many evolutionary studies is that nucleotide sites evolve independently. However, this assumption is often highly simplistic as epistatic interactions among mutations are a regular occurrence in viral genomes. Not only might such epistasis be of great functional importance, such as that resulting from RNA or protein secondary structure, but it may also tell us a great deal about the basic mechanics of virus evolution.

Epistatic effects can be antagonistic (positive), in which case they reduce the effect of combined mutations on fitness, or synergistic (negative), in which case they increase this effect. Although determining the nature of epistasis represents a major technical challenge because it entails a precise measurement of the combined fitness effects of multiple mutations, there is growing evidence for epistasis in viruses, and particularly in RNA viruses where experimental studies are rather easier to perform. Importantly, most of the epistatic interactions determined to date in RNA viruses are antagonistic rather than synergistic,^{20,125,197} which has major implications for understanding why recombination evolved in viruses (see later). Similar epistatic effects have been observed in small ssDNA viruses.¹⁷⁴

It is useful to discuss the extent of epistasis in the context of mutational robustness, which is a way in which phenotypes can be “protected” against the adverse effects of deleterious mutation pressure. Robustness can be generated in a number of ways, including through genetic redundancies such as duplicated genes, through the creation of neutral spaces as might be contained in RNA or protein secondary structure, or by complementation.^{45,157} There is a strong relationship between the level of genetic redundancy and epistasis, such that antagonistic epistasis is most common in genomes that are characterized by little redundancy and weak robustness, as is the case for RNA and ssDNA viruses (Fig. 11.9).^{58,194} Hence, when mutations occur in viruses with small genomes, which contain many overlapping reading frames and functions, they will tend to repeatedly damage the same functions, leading to antagonistic epistasis.²²⁹ Conversely, synergistic epistasis is more commonplace in larger DNA-based organisms that exhibit a greater level of genetic redundancy and robustness, of which duplicated genes are a good example, which allows them to tolerate a certain number of deleterious mutations (Fig. 11.9). Where large DNA viruses fall on this spectrum is uncertain, as there have been no experimental measures of the rate and sign of epistasis in these organisms to date. However, that multigene families are a common occurrence in large DNA viruses, resulting in a level of genetic redundancy, suggests that most of the epistatic interactions that occur in these viruses will be synergistic.

While individual mutations in viruses can interact through epistasis, whole virus genomes can interact through the presence of defective interfering (DI) particles and complementation.

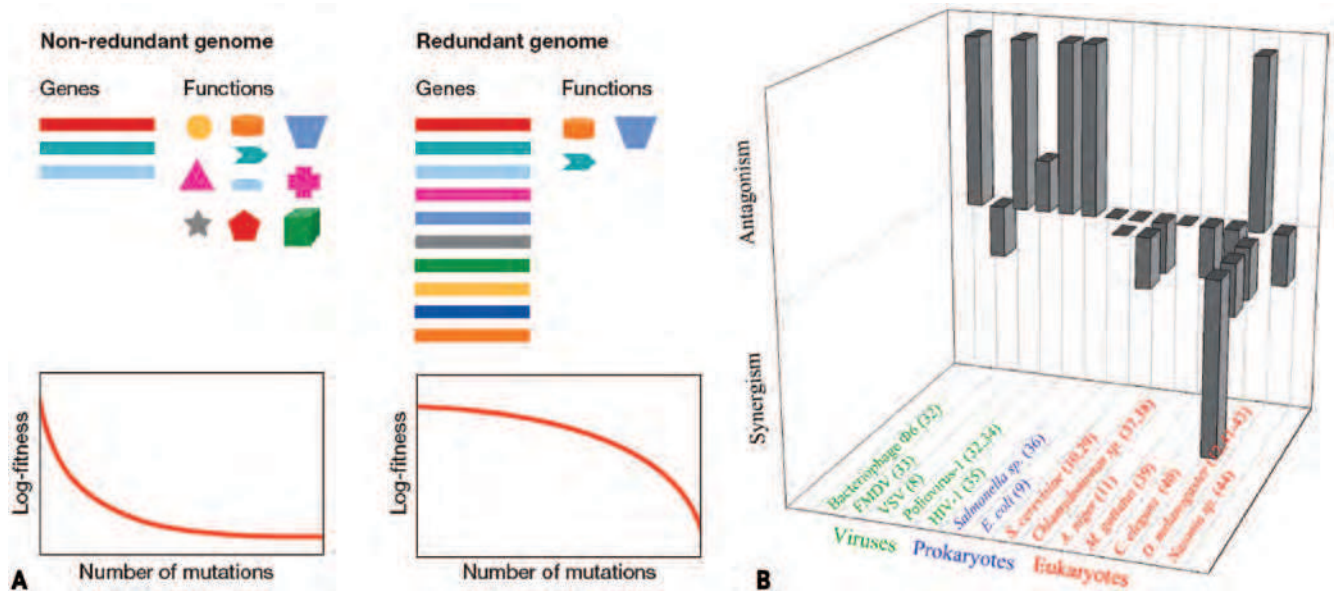


FIGURE 11.9. Redundancy and epistasis in viruses. **A:** Differing properties of robust (redundant) and nonrobust (nonredundant) genetic systems. In robust genetic systems that contain many genes, which may include large DNA viruses, the presence of genetic redundancy means that individual mutations have weak fitness effects and interact through synergistic epistasis. In contrast, in nonrobust systems such as RNA viruses and single-stranded DNA (ssDNA) viruses, there are few genes, and hence little genetic redundancy, so that single genes have multiple functions and single mutations have strong fitness effects. This results in antagonistic epistasis. (From Elena SF, Carrasco P, Daròs JA, et al. Mechanisms of genetic robustness in RNA viruses. *EMBO Rep* 2006;7:168–173.)

B: The type of epistasis seen in a variety of organisms: long bars, significant epistasis; short bars, nonsignificant epistasis; flat bars, no evidence for epistasis. For details see reference 92. (From Sanjuán R, Elena SF. Epistasis correlates to genomic complexity. *Proc Natl Acad Sci U S A* 2006;103:14402–14405; copyright 2006 National Academy of Sciences, U.S.A.)

DI particles are a common observation in many virus families⁹⁸ and have been implicated in a number of virological traits, including persistence.¹⁹⁰ DI particles often harbor large genomic deletions and compete with fully functional viruses during replication because they are able to replicate faster. DI particles have a number of interesting evolutionary consequences, although these will usually be short term. First, the high frequency of DI particles further illustrates the high rate at which deleterious mutations are produced in virus populations, including large-scale deletions. Second, as noted later, the polymerase error process that generates DI particles also has important implications for the evolution of recombination in viruses. Third, because they are able to replicate more rapidly than full-length viral genomes, DI particles may theoretically inhibit the spread of the advantageous mutations that are present on full-length viral genomes, if only transiently.

DI particles are likely to be maintained in virus populations through complementation, which is predicted to be a common occurrence at high multiplicity of infection, and which has been relatively frequently documented in both RNA (e.g., references 77, 221) and DNA viruses (e.g., references 37, 150). Perhaps the most interesting evolutionary consequence of complementation is that it allows deleterious mutations to persist for extended periods.⁷³ Until relatively recently, complementation was a process that had only been observed *in vitro*, such that DI particles were thought to survive just a few generations and therefore had little long-term evolutionary consequence. However, more recent analyses reveal that

complementation may be frequent in nature⁷² and may extend over very long time periods.¹ As a consequence, complementation may play more of a role in virus evolution than is usually envisioned, particularly in the context of how deleterious and advantageous mutations interact with each other, although this will evidently require further study.

The Evolution of Viral Recombination

A combination of comparative genomics and experimental study has provided growing evidence for the action of recombination, and its sister process reassortment, in viral genomes, although rates of virus recombination are often far lower than those documented in many cellular species. At the sequence level recombination is commonly apparent as incongruent phylogenetic trees, in which phylogenies inferred on either side of a recombination breakpoint differ significantly in topology, reflecting the contrasting evolutionary histories of these gene regions. Although such phylogenetic approaches can be biased in two ways—false positives caused by mixed infection or experimental error²¹ and that tree-based methods only detect recombination when there is measurable sequence diversity—they represent a simple means to detect recombination in a wide range of viruses and in doing so allow a direct comparison of recombination frequency.

Mechanisms of recombination differ markedly between viruses, reflecting the very different types of replication strategy employed, although all require co-infection of a single cell by two or more viruses. In the case of dsDNA viruses, recombination

likely occurs in a manner analogous to that observed in other DNA-based organisms, although the precise mechanisms of recombination are not known in all cases. Hence, recombination will involve the occurrence and repair of double-stranded breaks and, in the case of large dsDNA viruses, the presence of multiple recombination enzymes.¹⁰⁵ Recombination appears to be particularly commonplace in poxviruses, an apparent outcome of the form of DNA strain invasion used in replication,^{63,64} as well as in herpesviruses.²³ Recombination in large DNA viruses can be both homologous, in that it occurs at regions exhibiting strong sequence identity, and nonhomologous, involving divergent gene sequences (usually occurring as lateral gene transfer) and can occur at the intra- and interspecific levels.²¹⁹ It therefore plays a major role in shaping genomic architecture.¹⁵¹ For example, in poxviruses recombination may be more commonplace at the extremities of the genome, as these contain most of the species-specific genes, than in the central genomic regions that are conserved among viruses.^{62,151} Rather less is known about the mechanics of recombination in ssDNA viruses, although it is likely to occur as a consequence of the rolling circle replication employed by these viruses,¹⁹⁹ which may both determine the distribution of recombination breakpoints²²³ and result in frequent genome rearrangements, as in the case of Torque teno (TT) virus.¹³³

Evolutionary aspects of recombination are better studied in RNA viruses. Recombination in this case occurs by two rather different mechanisms. The first, sometimes called RNA recombination, occurs when two viruses co-infect a single host cell and a hybrid molecule is produced, most likely through a process of *copy-choice replication*,¹²⁷ although other mechanisms have been proposed. Under the copy-choice model the viral polymerase is thought to jump templates during negative strand synthesis, generating a chimeric RNA molecule.¹²⁷ Although this process is usually homologous, template switching can also occur among genomic regions that do not share sequence similarity. In theory, RNA recombination can occur in any type of RNA virus, irrespective of their genome structure

or orientation, although rates vary hugely among viruses (see later). The second process of recombination in RNA viruses is reassortment, which only occurs in RNA viruses that possess segmented genomes, such that a progeny virus packages segments with different ancestries. Rates of reassortment also vary markedly among RNA viruses, from very frequent in the case of influenza A virus¹⁸² to far less so in the case of hantaviruses.¹⁶⁵

Clearly, recombination can be of great evolutionary importance for viruses²¹¹ and has been associated with such features as the evasion of host immunity,¹⁴¹ the development of antiviral resistance,¹⁶⁶ the ability to infect new hosts,⁹⁶ increases in virulence,¹¹⁸ and even the creation of new viruses.²²⁷ As a consequence, it is important to document cases of viral recombination and reveal the determinants of this process. However, the occasional occurrence of beneficial traits arising from recombination does not necessarily mean that recombination evolved for this purpose, as recombination is as likely to break up beneficial genotypic configurations as create them. Indeed, explaining why recombination has evolved is one of the outstanding questions in evolutionary biology.

For viruses, clues for the reasons underlying the evolution of recombination are provided by the huge variation in recombination rates, from effectively clonal (i.e., asexual) to cases in which the recombination rate per nucleotide exceeds that of mutation. This extensive rate variation is particularly true of RNA viruses. The most common theory for the evolution of recombination is that it functions as a form of sexual reproduction in viruses and as such has been selectively optimized to either create advantageous genetic configurations or remove deleterious mutations (Fig. 11.10). However, this seems unlikely on first principles as high rates of recombination are only seen in a relatively small fraction of virus taxa, such that high recombination rates cannot be universally advantageous.²¹¹ This patchy distribution sits in marked contrast to theories for the evolution of sex in eukaryotes, the aim of which is to explain common recombination and sporadic asexuality.¹⁸⁹ Similarly, the patchy distribution of recombination

Evolutionary Benefit of Recombination

(1) Creation of beneficial genotypes:

Recombination increases the rate of adaptive evolution compared to clonal evolution. It also disassociates advantageous from deleterious mutations, allowing the former to spread.

(2) Purging of deleterious mutations:

Recombination enables deleterious mutations to be placed into a single genome which can be removed by purifying selection. This can be selectively favored if deleterious mutations occur at a high rate (i.e. $U > 1$) and interact through synergistic epistasis.

Schematic Representation

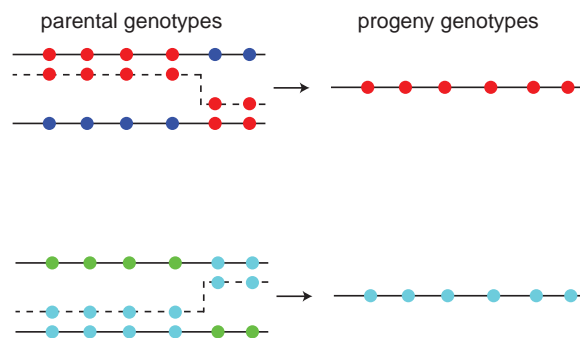


FIGURE 11.10. Potential evolutionary benefits of viral recombination (and reassortment) that might be favored by natural selection and that are commonly invoked in discussions of the evolution of sex. Accordingly, recombination can either (1) create advantageous genetic configurations (red circles), and also disassociate advantageous from deleterious mutations, or (2) purge deleterious mutations (green circles) by placing them in a single individual, which can be removed by purifying selection. In both cases the hatched line denotes the recombination event. (Adapted from Simon-Loriere E, Holmes EC. Why do RNA viruses recombine? *Nat Rev Microbiol* 2011;9:617–626, which should be consulted for more details.)

means that it is unlikely to universally have evolved as a form of repair.¹⁵³

It is obvious that recombination allows viruses to create potentially beneficial genetic configurations more rapidly than asexual populations, and there are many documented cases in viruses in which recombination has been associated with the generation of advantageous characteristics, such as drug-resistant genotypes. However, there are a number of viruses where rates of recombination are very low but where adaptive evolution is extremely common, with hepatitis C virus (HCV) a good case in point.¹⁸⁸ Hence, it may be that RNA viruses create a sufficient number of beneficial genotypes through mutation alone to offset the need for recombination. Recombination has two other important consequences for adaptive evolution. First, it disassociates advantageous from linked deleterious mutations,¹⁸⁹ and second, it prevents *clonal interference*. The latter describes a competition between beneficial mutations as they go to fixation in asexual populations, such that their rate of adaptation is retarded compared to that of sexual populations. Although both these processes are likely to occur in virus populations,¹⁵⁵ their overall importance is unclear.

The second advantage of recombination over purely clonal evolution is that it facilitates the efficient removal of deleterious mutations. This is often called the *mutational deterministic hypothesis* and is a hotly debated theory for the evolution of sex in a wide range of species.^{117,122} The central idea is that rather than many individuals (or genomes) carrying deleterious mutations, which would have a major negative impact on fitness, recombination allows these injurious mutations to be placed in a single individual whose selective removal greatly reduces fitness costs. A related theory is that of Muller's ratchet, which describes a progressive decrease in fitness due to the gradual accumulation of deleterious mutations in asexual populations of finite size and where genetic drift accelerates the loss of mutation-free individuals. Muller's ratchet has been observed in laboratory populations of RNA viruses^{34,35,50} and may therefore play some role when clonal viruses experience small population sizes, such as during transmission bottlenecks, although its effects are likely to be negated when viruses reach large population sizes.

For the mutational deterministic hypothesis to explain the origin of recombination, it is essential that the deleterious mutation per genome replication (U) is greater than 1, and hence very high, and that these deleterious mutations interact through synergistic epistasis.¹²² For RNA viruses $U > 1$ may be attained relatively regularly.^{52,61} This may also be true of ssDNA viruses, which similarly possess small genomes and genes that encode multiple overlapping reading frames and functions. Unfortunately, there are currently no estimates of U in large DNA viruses. However, there is little good evidence for synergistic epistasis in RNA viruses, with most studies undertaken to date suggesting that epistatic interactions are antagonistic rather than synergistic.^{20,28,197} In contrast, the greater genetic redundancy in large DNA viruses makes antagonistic epistasis a more likely occurrence in this instance. In addition, sequence comparisons suggest that the burden of deleterious mutation is high for all RNA viruses studied, irrespective of background recombination rate.¹⁸¹ Hence, it is not recombination that saves RNA viruses from an excessive mutational load, but rather their large population sizes that provide a form of *population robustness* that offsets the effects of deleterious mutation.⁵⁸

An opposing theory for the evolution of viral recombination is that rather than being optimized by natural selection as a form of sexual reproduction, it is simply a mechanistic by-product of differing types of genome architecture, replication strategy, and polymerase processivity.²¹¹ The principal evidence for this theory is that there is an apparent association between recombination frequency and virus genome structure. Hence, recombination is relatively frequent in some retroviruses, and as is reassortment in viruses with segmented genomes. Far more variable recombination rates are observed in ssRNA+ viruses, for example, frequently in coronaviruses and enteroviruses, sporadically in flaviviruses, and currently absent in *Leviviridae* and *Narnaviridae* (although this may be a function of sample size). Finally, recombination is far less common in ssRNA- viruses. A simple example of the disparity of recombination rates is provided by a comparison of HIV-1 and HCV. In the former, recombination rates have been estimated at between 1.38×10^{-4} and 1.4×10^{-5} recombination events per site, per generation,^{164,207} while the equivalent estimates for HCV are only 4×10^{-8} .¹⁸⁸ The low rate in HCV means that recombination is unlikely to be of great evolutionary significance for this virus. Most DNA viruses studied to date seem to experience relatively high levels of recombination (including through lateral gene transfer in the case of large DNA viruses), although there have been few attempts to estimate rates of recombination relative to those of mutation.²³ One DNA virus in which recombination has been particularly well studied is HBV, along with related hepadnaviruses. In this case recombination is commonly depicted using phylogenetic methods and likely represents one of the main reasons that the evolutionary history of this virus has been so hard to determine.²⁰⁸

In each case mentioned previously the frequency of recombination seems to reflect the genome structure of the virus in question.²¹¹ Hence, the high recombination rates of retroviruses are likely a function of the fact that their virions carry two RNA molecules, such that *heterozygous* progeny will be produced when viruses with different ancestries are packaged together. Copy-choice recombination, which is common in this virus, may then produce genetically distinct progeny during reverse transcription. However, not all retroviruses recombine at the same rate as HIV. For example, recombination rates in murine leukemia virus (MLV) are up to 100 times lower than those observed in HIV, despite very similar rates of template switching. The difference between these two viruses is that while HIV genome dimerization occurs randomly in the cytoplasm, genome dimerization in MLV takes place in the nucleus and generally leads to self-associations, rather than to those involving genetically different parental molecules.¹⁷⁰

That heterozygous viral progeny cannot be produced when the genomic material is present as a single molecule obviously acts to reduce recombination rates in most RNA viruses. However, in some cases specific genome organizations can facilitate frequent recombination. Case in point are the coronaviruses, such as murine hepatitis virus (MHV), where up to 25% of the progeny of co-infected cells may be recombinant. Such a high recombination rate reflects the mechanisms controlling gene expression in coronaviruses, in which discontinuous transcription leads to the production of subgenomic RNAs through a copy-choice mechanism.¹⁹⁸ An even more dramatic example of how replication strategy and genome organization may shape recombination rate occurs in the ssRNA- viruses, in which

recombination is relatively infrequent. In these viruses the genomic and antigenomic RNA molecules are quickly bound to multiple nucleoprotein subunits, as well as to other proteins, to form ribonucleoprotein (RNP) complexes from which viral replication and transcription can proceed. However, this tight complex of RNA and protein necessarily limits the probability of hybridization of complementary sequences between the nascent and acceptor nucleic acid molecules, and hence the probability of template switching, and also reduces the potential number of substrates for this process.²¹¹ Accordingly, those phylogenetic studies undertaken to date have revealed relatively few cases of recombination in ssRNA– viruses.^{7,231}

The RNA Virus Quasispecies

One of the most contentious issues in the study of virus evolution is whether RNA viruses evolve according to a particular population genetic model known as the *quasispecies*.⁹² In some aspects, this debate merely reflects a semantic point about how best to describe the intrahost genetic variation commonly seen in RNA viruses. However, a far more important issue, and the true essence of the quasispecies debate, is how natural selection acts on virus genomes.

Quasispecies theory was originally developed by Manfred Eigen and colleagues as a model of the evolution of self-replicating RNA molecules that likely represent the first replicators.^{56,57} The theory was first applied to RNA viruses after genetic variation was observed in a number of experimental systems, beginning with the bacteriophage $\phi\beta$.⁴⁶ Since this time the quasispecies has become a popular descriptor of intrahost RNA virus evolution, particularly because widespread gene sequencing has uncovered abundant genetic diversity. However, because the theory is based on high mutation rates (see later), it should not be applied to large DNA viruses where error frequencies are lower.

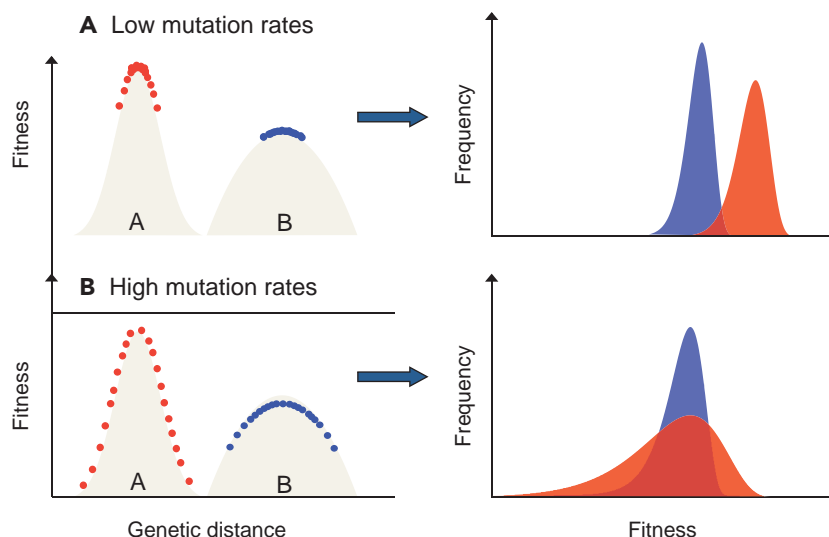
Although commonly used simply as a synonym for genetic variation, quasispecies in fact describes a specific type of mutation-selection balance. Mutation-selection balances are commonly invoked in population genetics and describe an evolutionary equilibrium between the generation of new mutations and their removal by purifying selection. The form of

mutation-selection balance invoked in quasispecies theory is based on the occurrence of an extremely high mutation rate, which ensures that the frequency of any variant in the population is a function of both its individual fitness *plus* the frequency at which it is produced by the erroneous replication of other variants in the population that are linked to it in mutational space. This *mutational coupling* means that viral genomes are not independent entities, such that natural selection favors the entire population as opposed to individual variants. In this manner the group, rather than the individual, becomes the unit of selection.⁵⁷

The central tenet of quasispecies theory is thus that natural selection acts on the viral population as a whole, rather than on individual variants. As a consequence, the entire quasispecies evolves to maximize its average fitness, rather than that of individual variants as is the case in other population genetic models. The most interesting outcome of this particular evolutionary process is that low fitness variants can sometimes outcompete those of higher individual fitness if the former are surrounded by beneficial mutational neighbors. This is sometimes referred to as the *quasispecies effect* or the *survival of the flattest*²³⁰ and describes a situation in which a population whose component mutants have a similar mean fitness can outcompete a population that has a lower average fitness even though it contains variants of higher individual fitness. Under classic *survival of the fittest* population genetic models, these individual high-fitness variants are selectively favored, whereas under *survival of the flattest* (i.e., quasispecies) models, the flatter population is selectively superior as it possesses a higher mean fitness (Fig. 11.11). This can also be thought of as a form of mutational robustness.

A key component of quasispecies theory is that intrahost populations of RNA viruses harbor abundant genetic diversity. There is no doubt that this specific aspect of quasispecies theory is correct, and even so for acute infections, as intrahost genetic variation is commonly observed in RNA viruses.^{44,92} In particular, studies using next-generation sequencing, in which the sequencing coverage of individual nucleotides is very high, have revealed extensive genetic diversity, with HIV-1⁶⁹ and foot-and-mouth disease virus (FMDV)²³⁴ serving as good examples.

FIGURE 11.11. The quasispecies effect (*survival of the flattest*) in experimental populations of RNA viruses. The red population (A) has a high replication rate (i.e., fitness) but low mutational robustness, while the blue population (B) has a lower replication rate but greater mutational robustness. Dots depict mutational variants located on each peak at low and high mutation rates, and the expected distribution of individual fitness values for the two populations is shown on the **right** of the figure. At low mutation rates, population A will always outcompete the flatter population B as it contains the variant of highest individual fitness. However, at very high mutation rates natural selection favors the flatter population (B) as predicted under quasispecies theory. (From Sanjuán R, Cuevas JM, Furió V, et al. Selection for robustness in mutagenized RNA viruses. *PLoS Genet* 2007;3:e93.)



However, a very important caveat is that many estimates of intrahost genetic diversity are likely to have been inflated by erroneous polymerase chain reaction (PCR) and sequencing.¹⁴⁰ Indeed, RT-PCR is a notoriously error-prone process, with artificially induced mutations an inevitable consequence, and different sequencing systems are also characterized by specific error rates. In addition, intrahost genetic diversity in nature is not simply the product of *de novo* mutation because the mixed infection of individual hosts may also play an important role in shaping levels of intrahost genetic variation (see later). More importantly, although the observation of intrahost genetic variation is a necessary criterion for an RNA virus population to be thought of as a quasispecies, it is not sufficient in itself as intrahost genetic diversity is expected under any evolutionary model if the mutation rate is high enough. The quasispecies is therefore not simply another word for intrahost genetic variation.

Both *in silico* and *in vitro* studies have provided some evidence for the existence of quasispecies dynamics defined correctly. As an example of the former, computational studies using *digital organisms* revealed that the survival of the flattest could be induced at very high mutation rates.^{40,230} However, as the mutation rates involved were always higher than one mutation per genome replication, it is uncertain whether such mutation rates could ever be attained for sustained periods in RNA viruses in nature. Similar results have been obtained from some experimental analyses of RNA virus evolution. Studies using both viroids of plants and VSV showed that viral populations with lower replication rates were able to outcompete those with higher replication rates, as expected under the quasispecies model, although this again requires the elevation of mutation rate (by either chemical mutagens or ultraviolet C light) to levels that may not commonly occur in RNA viruses in nature.^{38,193} Additional experimental evidence for the existence of RNA virus quasispecies was the observation that high-fitness clones of bacteriophage $\phi 6$ evolved to a lower mean fitness because their mutational neighbors were of low fitness,²⁷ and that strains of poliovirus that possessed higher fidelity than the wild type, such that the virus population carried less genetic diversity, were unable to infect the full range of tissues that are associated with severe disease.²²⁴ This in turn suggested that quasispecies dynamics might be a central determinant of viral pathogenesis.²²⁴

Another class of experimental studies that have been cited in support of the quasispecies model involves lethal mutagenesis. This entails treating virus populations with mutagens, such as 5-fluorouracil and ribavirin, that increase the error rate to the extent that so many deleterious mutations are produced that fit genotypes are never able to regenerate themselves. Although lethal mutagenesis can clearly result in virus extinction, especially if mutagens are used in combination with more standard antiviral inhibitors,^{4,171} the basis of this effect is more complex. Quasispecies theory predicts that virus extinction in this instance occurs because of an *error catastrophe*, which is the point at which the fittest genotype suffers so many deleterious mutations that it cannot sustain itself in the population (i.e., it has breached an *error threshold*). However, another interpretation is that the virus has instead crossed an *extinction threshold*, the point at which deleterious mutations accumulate faster than they can be eliminated by natural selection, which will also lead to population extinction.²⁶ Importantly, error catastrophe

requires a fundamental shift in viral genotype that is independent of population size, whereas extinction threshold entails a major decline in viral population size.

Studies of RNA virus evolution based on the comparative analysis of gene sequence data have provided less support for the existence of quasispecies. Indeed, there is currently no clear evidence that selection acts on groups rather than individual variants in natural populations of RNA viruses, although this may reflect the fact that even with the most sophisticated tools of gene sequence analysis it is difficult to discern the effects of all but the most strongly favored and disfavored variants. For example, although natural selection is very commonly documented in HIV, the adaptive process always seems to involve the fitness advantage of individual mutants over others in the population, rather than group selection. Similar points can be made for other RNA viruses.⁹² Again, though, it is arguable that natural selection is so strong in these cases as to obscure any quasispecies effect, and that the latter model is a far better description of mutations subject to weaker positive selection, as most natural selection will be in nature. This is clearly a major area for further study.

To conclude, given a sufficiently high mutation rate, quasispecies is a viable and extremely interesting evolutionary model. However, it is less clear whether the quasispecies concept can be successfully applied to RNA viruses in nature, where to date there has been no convincing evidence for its occurrence. This does not necessarily mean that the quasispecies model is wrong, but rather that too few RNA viruses have been studied in sufficient detail through deep amplicon sequencing and precise fitness assays to determine whether they form quasispecies, exemplified by a process of natural selection acting on the virus population as a whole.

PATTERNS AND PROCESSES OF VIRAL GENOME EVOLUTION

The Evolution of Virus Genome Size

Viruses possess a remarkable range of genome sizes (Fig. 11.12). This is especially so for DNA viruses in which genome sizes range from only 1,758 nt in *Porcine circovirus* (ssDNA) to a remarkable 1,259,197 nt in the case of the megavirus *Megavirus chilensis* (dsDNA),⁹ hence covering approximately three orders of magnitude, although all ssDNA viruses have genomes less than 11,000 nt in length. A far narrower range of genome sizes is observed in RNA viruses. The smallest RNA virus currently known is *Ophiostoma novo-ulmi* *mitovirus* 6-Ld, at only 2,343 nt, while the largest RNA viruses are the coronaviruses and roniviruses (order *Nidovirales*), which have genome sizes of approximately 30,000 nt. Mean genome sizes in RNA viruses are around 10,000 nt. Interestingly, there does not appear to be a major difference in genome size between segmented and unsegmented RNA viruses,⁹² with, for example, the unsegmented *Coronaviridae* and *Roniviridae* possessing larger genomes than all segmented RNA viruses. This strongly suggests that genome segmentation has not evolved as a way to increase virus genome sizes.

A variety of theories have been proposed to explain the evolution of genome sizes in viruses. One theory is that virus genome sizes are constrained by the maximum size of the

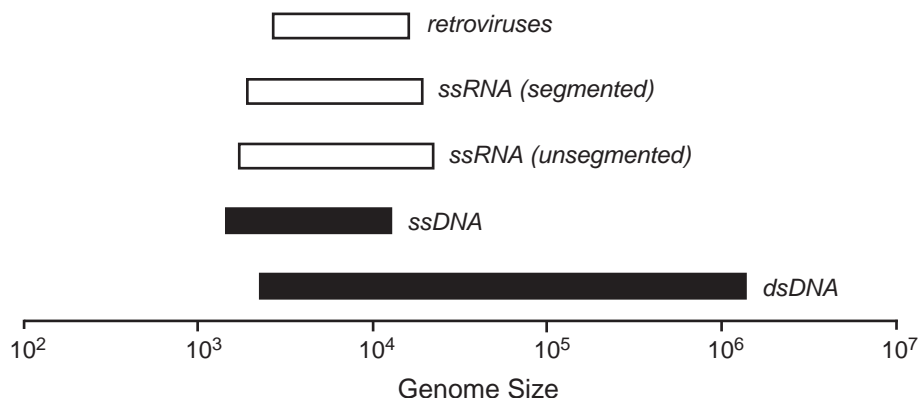


FIGURE 11.12. Distribution of genome sizes in RNA and DNA viruses. Note the similarity in (small) genome sizes between RNA viruses, retroviruses, and single-stranded DNA (ssDNA) viruses.

genetic material that can be contained within a single capsid protein.²³⁸ However, the huge range of genome sizes, especially in DNA viruses, argues against this. The genome content of large DNA viruses in part reflects often frequent lateral gene transfer and gene duplication (see later). In particular, the central part of the genome of many large DNA viruses is composed of a set of core genes that control basic biochemical functions, including replication, while the outer, flanking genes distinguish individual viruses and are often responsible for modulating immunity, host range, and virulence, and it is these that have often been captured from host genomes. This process of gene birth and death has resulted in a great variation in genome sizes, with, for example, a massive increase in genome size from the ancestral NCLDV to those circulating today.¹⁰⁵ This evolutionary process, combined with the discovery of the giant viruses of algae and amoeba, suggests that there are unlikely to be strict constraints against genome sizes in large DNA viruses. Similarly, that bacteriophages are able to transiently carry large parts of bacterial genomes, which evidently plays a key role in lateral gene transfer among bacterial species,¹⁶⁹ also argues against strict constraints on genome size.

However, it is also the case that certain structural features must constrain genome sizes to some extent. First, longer viral genomes are expected to cause an increase in replication times, which may be disadvantageous. Second, in the case of RNA viruses, it is possible that the difficulty in unwinding potentially long regions of dsRNA during replication inhibits the maximum genome size attainable.¹⁸⁶ For example, it has been argued that the unwinding of dsRNA in RNA viruses with genomes greater than 6,000 nt is controlled by the presence of a helicase (HEL) domain,⁸³ the evolution of which allowed RNA viruses to greatly increase their genome size.⁸²

A more plausible explanation for the range of viral genome sizes is that they reflect background mutation rates. As noted previously, there is a fundamental relationship between mutation rate and genome size that seemingly applies to all living systems (Fig. 11.6). Accordingly, dsDNA viruses with relatively low mutation rates will be able to attain relatively large genome sizes, while the small genomes observed in RNA and ssDNA viruses reflect the higher error rates seen in these systems. Perhaps paradoxically, the idea that mutation rates set genome size can be extended to explain the very large (by RNA virus standards) genomes seen in the coronaviruses and roniviruses. The major part of the genomes of these families is composed of a large (greater than 20,000 nt) replicase gene that contains

an ExoN domain encoding a 3' to 5' exoribonuclease. As this ExoN domain is homologous to cellular proteins of the DEDD superfamily of exonucleases that are involved in proofreading and repair,¹⁵⁴ it is possible that coronaviruses and roniviruses reduce their error rate through some sort of proofreading activity of the 3' to 5' exoribonuclease.⁵³ This, in turn, will reduce mutational load and allow larger genome sizes.

A final important difference between RNA and dsDNA viruses with respect to genome size is that the former (as well as ssDNA viruses) frequently utilize overlapping open reading frames, whereas these are less common in the latter (although, for example, the M065R and M066R genes of the poxvirus myxoma virus overlap by ~100 bp). Belshaw et al.¹² noted 819 cases of gene overlap among 701 RNA virus genomes; 56% of the viruses examined possessed some degree of overlap, which nearly always involved a +1 or -1 frame shift. In addition, RNA viruses with longer genomes tend to show less gene overlap than those with shorter genomes.¹² Although the exact evolutionary processes responsible for the evolution of overlapping reading frames are uncertain, they clearly allow an increase in the amount of protein diversity encoded by a single nucleotide sequence.

The Evolution of Genome Organization

It is arguable that viruses contain a greater diversity of genome structures and organizations than any other group of organisms. As well as the obvious division into RNA viruses, DNA viruses, and retroviruses, distinct genome structures include viruses in positive and negative sense orientations, those with single or double strands of the nucleic acid, those with single or multiple segments (which are usually multicomponent in the case of ssRNA+ viruses from plants), those that utilize subgenomic RNAs, and those like the coronaviruses that utilize ribosomal frame shifting. A key challenge for evolutionary virologists is therefore to explain why such a diverse array of structures exists.

One of the most debated issues with respect to the evolution of genome structures in RNA viruses is why some are segmented and others not. As noted previously, one theory for the evolution of segmentation is that it evolved as a way of facilitating reassortment, although this seems unlikely.²¹¹ Similarly, there is no good evidence that segmentation allows the evolution of longer genomes. Another possibility is that genome segmentation, particularly in multicomponent viruses, resulted from the intracellular selection for smaller RNAs that,

because they are shorter, would have had a replication advantage over their full-length counterparts.¹⁶³ However, this theory cannot easily explain why multicomponent viruses are nearly all restricted to plants.

A competing theory for the evolution of genome segmentation is that it allows greater control over gene expression. Clearly, all RNA viruses need to control the levels of each protein they produce. For many ssRNA+ viruses such control occurs at the level of translation as this is necessarily the first step in the virus life cycle. Additional constraints faced by viruses of this type are that eukaryotic ribosomes only recognize the 5' regions of mRNA molecules, so that internal start codons are not utilized, and mRNAs are usually monocistronic.⁹² Many ssRNA+ viruses therefore simply translate a single polyprotein that is proteolytically cleaved into individual protein products, which may represent the ancestral type of genome organization in ssRNA viruses. Although this genomic structure allows efficient replication, similar amounts of each protein product are produced, so that there is relatively little control over gene expression. As a consequence, other ssRNA+ viruses have evolved a variety of more complex ways to control gene expression, all of which can be envisioned as ways of

dividing the viral genome into individual *transcriptional units*, within which transcription (and translation) can occur at different rates. Such a division can involve the creation of multiple genome segments, the utilization of subgenomic RNAs, and the use of a –1 ribosomal frameshift to produce multiple open reading frames as in the case of the coronaviruses and rotaviruses⁹² (Fig. 11.13).

The situation is rather different in the case of ssRNA– viruses. Because ssRNA– viruses by necessity transcribe their genomes before translating them, some control over gene expression can occur at the level of transcription; multiple mRNAs can be produced and there will be a transcriptional gradient from the first (i.e., 3') mRNA, of which most is produced, to the last (5') mRNA, of which least is produced. It is therefore possible that the ability to better control gene expression, itself through the control of transcription, represents the reason that negative-sense genomes evolved in the first instance. In this respect it is significant that the genomes of unsegmented ssRNA– viruses possess a highly conserved gene order, cluster together on polymerase phylogenies, and can easily be classified within the *Mononegavirales*. Moreover, this genome order seems to be a function of the amount of each

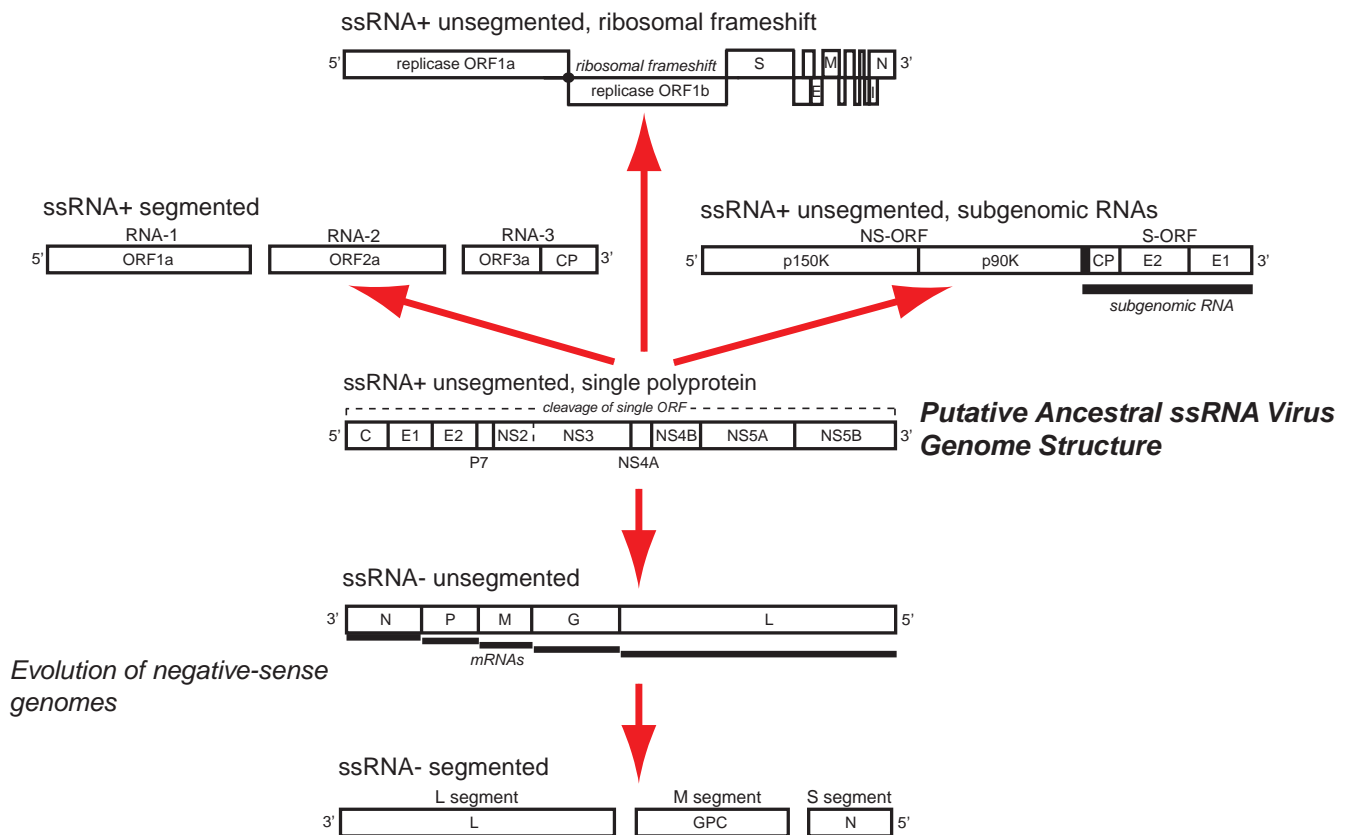


FIGURE 11.13. Schematic representation of the major types of genome organization and replication strategy in RNA viruses and a scenario for their evolutionary origin. Each of these organizations results in a different way to control gene expression, although they should not be considered as mutually exclusive. Unsegmented single-stranded RNA-positive (ssRNA+) viruses that produce a single polyprotein are considered here to be the ancestral type, although this is debatable. Gene and segment sizes are drawn approximately to scale within each of the six organizations, but not among them, and the 5' and 3' terminal sequences have been excluded. (From Holmes EC. *The Evolution and Emergence of RNA Viruses*. Oxford: Oxford University Press, 2009, by permission of Oxford University Press.)

protein product required, such that the 3' gene encodes the nucleocapsid while the 5' gene encodes the RNA polymerase, again suggesting that it is an adaptation to facilitate the control of gene expression.

Gene Duplication in Virus Evolution

One of the most important processes of genome evolution in eukaryotes is gene duplication. This represents an important way in which these organisms create evolutionary novelty; gene duplication is a simple way to create new genes that, following subsequent mutation and adaptation, can exhibit different but related functions, which often appear as multigene families. Because of the evolutionary importance of gene duplication in molecular evolution, it is important to determine its frequency in viruses. Such an analysis again reveals a major division between RNA and ssDNA viruses on the one hand and large dsDNA viruses on the other.

While gene duplication must be responsible for at least some of the genome size variation observed in RNA viruses, particularly during their early evolution, it is striking how rarely this process has been observed in RNA viruses.⁹² An important caveat to make here is that because RNA virus evolution is so rapid and phylogenetic signals lost so quickly, it is possible that gene duplication has occurred more frequently in the past but that the footprints of this process are hidden by frequent multiple substitution. Those gene duplication events documented thus far in RNA viruses tend to occur as short duplications in untranslated or intragenic regions and often result in defective viruses.²⁹ Only occasionally have gene duplication events been described that produce two complete (and sometimes tandemly repeated) genes,^{24,225} although improvements in computational analysis are likely to reveal more. The difficulties in analyzing divergent sequences notwithstanding, there are good reasons for the relative rareness of gene duplication in RNA (and ssDNA) viruses. In particular, given the size limit on virus genome discussed throughout this chapter, increasing genome size through gene duplication is likely to result in major fitness losses through an increase in the load of deleterious mutations.

The situation is very different in large dsDNA viruses, where cases of gene duplication, as well as gene loss, are a common observation and are evidently responsible for some of the size variation among viruses^{100,200} (Fig. 11.14). Gene duplication can occur in both the core and species-specific genes of

large DNA viruses as signified by the presence of related gene pairs and multigene families.¹⁴⁹ For example, gene duplication has been commonplace in the E4 region of some adenoviruses⁴³ and in the terminal inverted repeats of the poxvirus myxoma virus,¹²⁶ although many other examples exist. The process of gene duplication may also be related to that of recombination. For example, in some poxviruses recombination seems to have resulted in the duplication, inversion, and transposition of genes to opposite ends of the genome.¹⁶¹ Finally, the analysis of protein structure suggests that some of these gene duplication events may have occurred in the distant past and assisted in the production of very distinct proteins, such as the head–tail connector protein and tail tube protein of bacteriophage lambda.³⁰

Lateral Gene Transfer and Modular Evolution

A major way for bacteria to create evolutionary novelty is through lateral (or horizontal) gene transfer (LGT), which can sometimes result in genes being transferred for large phylogenetic distances.¹⁶⁹ As LGT will also result in an increase in virus genome size, unless the original gene is directly replaced by the invading gene (which is unlikely unless an exact excision is made, as faulty excision will result in deleterious mutants), it is not surprising that this process has to date only been rarely described in RNA viruses and ssDNA viruses, although it is again important to recall that inferences are compromised by extreme phylogenetic distance. One well-documented case of LGT in an RNA virus involves the acquisition by influenza C virus of the hemagglutinin-esterase (*HE*) gene of coronaviruses.¹³⁹

Lateral gene transfer can occur among viruses and between viruses and hosts. In the case of RNA viruses there are sporadic reports of these infectious agents transiently incorporating host genome sequences. A famous example is provided by the integration of ubiquitin into the genomes of bovine viral diarrhea virus.¹⁵² Similarly, the sequence similarity between the 65-kD protein of closteroviruses (ssRNA+) and the cellular heat shock protein hsp70² may indicate an early LGT event, while the capture of the ExoN domain by coronaviruses has been discussed in more detail earlier. In reality, however, there are few reports of the stable integration of cellular sequences into the genomes of RNA viruses, although more are likely to be documented with the acquisition of increasing numbers of host genome sequences.

In contrast, the capture of host genes is very well documented in the case of large DNA viruses, including those from

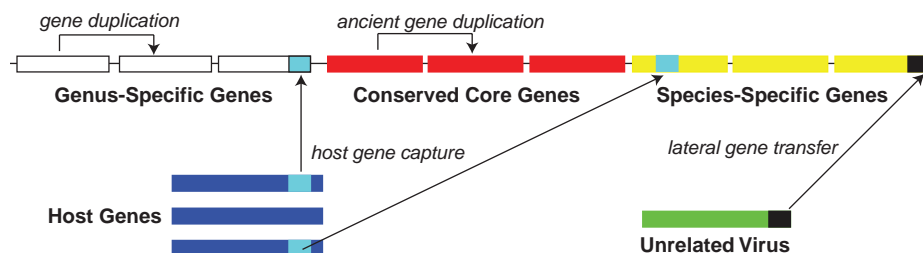


FIGURE 11.14. Processes of lateral gene transfer, from both hosts and other viruses, and gene duplication in large double-stranded DNA (dsDNA) viruses. Core viral genes, which are conserved across divergent taxa, are shown in red and often located in the central part of the genome. Genus and species-specific genes are shown as white and yellow, respectively, and more often located at the terminal regions of the genome. Refer to Figure 11.15 for a real data example. (Adapted from Shackelton LA, Holmes EC. The evolution of large DNA viruses: combining genomic information of viruses and their hosts. *Trends Microbiol* 2004;12:458–465.)

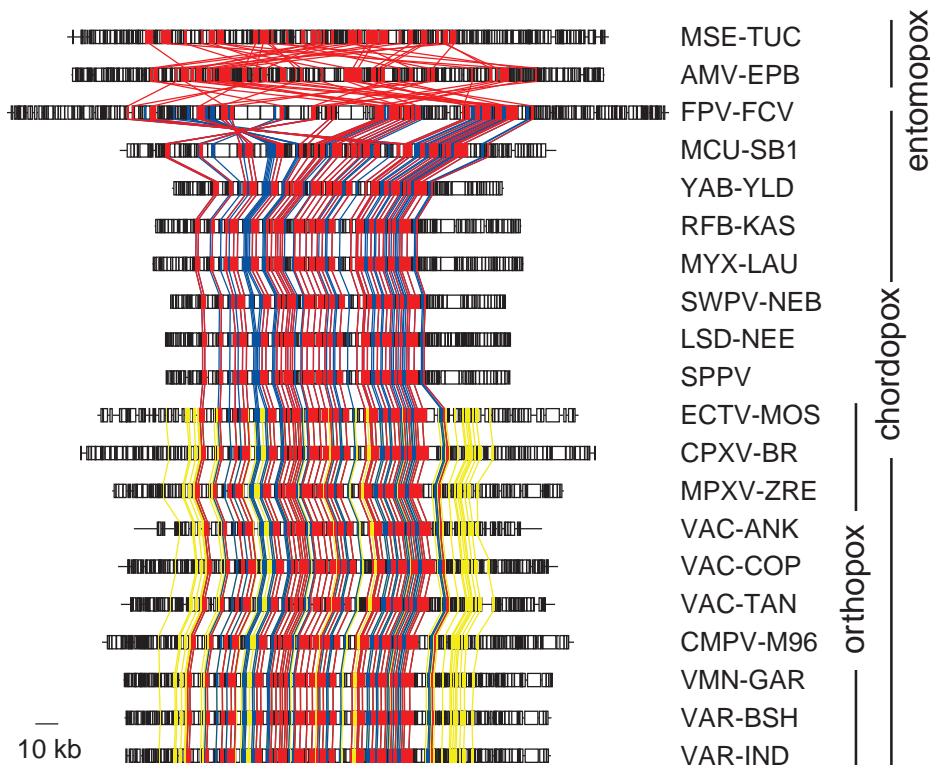


FIGURE 11.15. Comparative genomics of poxviruses. The figure shows a comparison of 92 gene families from 20 different poxviruses. Genes present in all the poxviruses analyzed are shown in red and are located in the central genomic regions. Those genes present in chordopox viruses only are shown in blue, while those present in orthopoxviruses only are shown in yellow. Vertical lines link orthologous genes. Horizontal differences are drawn proportional to genetic distances. (From McLysaght A, Baldi PF, Gaut BS. Extensive gene gain associated with adaptive evolution of poxviruses. *Proc Natl Acad Sci U S A* 2003;100:14960–14965. Figure kindly provided by Aoife McLysaght.)

both bacteria¹⁷³ and eukaryotes,¹⁰⁵ although the complex phylogenetic shadows cast by gene birth and gene loss means that it is sometimes difficult to determine from which host species and when these gene transfer events occurred. The poxviruses have been very well studied in this respect, with the interleukin-10 (*IL-10*) gene family and the vertebrate vascular endothelial growth factor (*VEGFA*) genes, which are distributed among both poxviruses and a range of vertebrates, as well as the DNA ligases, acting as good examples (and the *IL-10* family is also observed in herpesviruses).^{25,102,151} In fact, LGT is a common occurrence in all NCLDV and covers an enormous taxonomic range of hosts.^{66,105} For example, mimivirus may have acquired perhaps 10% of its total gene content from bacterial species, including a number of mobile genetic elements.⁶⁴ In poxviruses gene content seems to change more rapidly than gene order, reflecting the fact that species-specific genes tend to get added to the extremities of genomes (Figs. 11.14 and 11.15). That the flanking genes of large DNA viruses are often the ones captured by the host in turn suggests that internal gene orders are optimized to the extent that disrupting them may result in major fitness losses. Similarly, human herpesviruses contain homologs to such important immune genes as those that encode cytokines, chemokines, and complement system proteins, as well as those of the immunoglobulin superfamily.^{145,183} These genes may modulate the immune response of the host, often by interfering or mimicking their cellular homologs. However, it is not only host immune genes that are acquired by dsDNA viruses. As a case in point, iridoviruses encode a number of cellular proteins that seem essential for virus replication.²²⁰

It is also the case that LGT has occurred among large DNA viruses and is particularly well documented in bacteri-

ophage, where it appears to be a common mode of molecular evolution⁸⁶ (and phage-mediated LGT is of course a key process of bacteria evolution¹⁶⁹), as well as in a number of eukaryotic viruses.⁴³ As another example, the entomopoxvirus *Amsacta moorei* virus (AMV) contains a number of genes that have been acquired from baculoviruses.¹⁰⁵ However, as with the case of LGT among viruses and hosts, it is often hard to pinpoint the exact origin and direction of any gene transfer event.

Less clear is exactly how viruses capture host genes. Although such an event could obviously occur through direct recombination, this would require entry into the nucleus and would result in virus genes containing introns. An alternative possibility is that complementary DNA (cDNA) copies of spliced cellular mRNAs, which do not contain introns, have been inserted into virus genomes, perhaps utilizing the RTs present in cellular retroelements.²⁰⁰ On the other hand, LGT that occurs among viruses, or between viruses and bacteria, most likely occurs during host co-infections.

One of the most interesting developments in studies of virus evolution in recent years is the observation of segments of RNA virus genomes incorporated into host cellular genomes. For retroviruses that make dsDNA and enter the cell nucleus, as well as small ssDNA viruses that are so restricted in size that they do not carry their own polymerase genes and similarly must enter the cell nucleus, the presence of these integrated virus genes is perhaps not a surprise. Indeed, it has been known for many years that much of the eukaryotic genome was composed of endogenous retroviruses.^{18,228} More surprising was the discovery that RNA viruses, which do not make a DNA genomic copy and often replicate in the cytoplasm, could also become integrated into host genomes (in the form of EVEs; see earlier). The first clear-cut case of virus-to-host

LGT to be documented involved sequences closely related to those of insect flaviviruses and found to be integrated into the genomes of *Aedes* mosquitoes.⁴² Since this time a number of other examples have been discovered, including bornavirus,⁹⁷ filoviruses,²¹⁸ and rhabdoviruses,¹¹³ often comprising a very wide range of hosts.⁹⁴ Similarly, EVEs have also been documented in a number of small DNA viruses, including circoviruses, parvoviruses,^{15,111,136} and hepadnaviruses.⁸⁰

As noted at the start of this chapter, the presence of these endogenous virus elements has changed our perspective of the time scale of virus evolution, indicating that some virus families may be far older than previously anticipated. However, these sequences raise other interesting questions, most notably by what mechanisms single-stranded RNA is converted into double-stranded DNA. The most plausible answer involves interaction with the cellular retroelements that are an abundant component of eukaryotic genomes, with the L1 long interspersed nucleotide elements a notable example⁹⁷ (Fig. 11.4). In addition, some EVEs appear to be more conserved than expected if they were evolving in a strictly neutral manner, free of all selective constraints.¹¹³ As such, some of these endogenous virus copies may be of functional importance, perhaps because they confer resistance against related exogenous viruses.⁸

Finally, one process of genome evolution that is mechanistically similar to that of lateral gene transfer, and that has been of great historical importance in studies of virus evolution, is that of *modular evolution*.²² This theory posits that viral genomes can be thought of as comprising a series of functional modules, such as those containing the capsid and the polymerase, that can be exchanged through recombination, and in doing so sometimes create entirely new viruses. For example, it has been suggested that some RNA viruses were created through a process of modular evolution.⁸⁸ In addition, there is evidence that recombination may occasionally reflect aspects of genome modularity in some small DNA viruses,^{129,146} as well as some RNA viruses, which is compatible with a form of modular evolution. However, this pattern more likely reflects essentially random LGT across the viral genome with natural selection then filtering out those transfers that reduce fitness, which tend to occur at intragenic locations, rather than LGT occurring at specific genomic sites as specified in the original modular evolution model.¹⁷³ Similarly, although LGT is a common occurrence in dsDNA viruses, it is another thing to say that these transfer events involve distinct functional *modules*, rather than occasional genes, and there is as yet no good evidence that new DNA viruses have been created by LGT. In sum, the role of modular evolution in the generation of virus diversity remains uncertain.

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Epidemiology

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Acknowledgments

BASIC DEFINITIONS AND METHODS

Epidemiology deals with the occurrence of diseases in populations. Historically, epidemics of viral disease were recognized long before their causal agents were discovered, and viral epidemiology was one of the first aspects of the science of virology to be developed. Evolving insights into the pathogenesis and molecular aspects of virology have provided an increasingly rational basis for understanding the epidemiology of viruses. In this chapter, the biology of viral infections is used to explain the essentials of viral epidemiology.

Incidence and Prevalence

The quantification of disease occurrence is the cardinal feature of epidemiology. To accomplish this, the concept of rates was introduced, and rates have become the basic coinage of epidemiology (Fig. 12.1). Rates are fractions in which the numerator is the number of cases of disease and the denominator is a measure of the population. The *incidence rate* (also called the *attack rate* for acute infectious diseases) is used to quantify the number of new infections. A population and a time frame are defined, and the number of new cases in that population during that interval of time is counted. Note that the denominator includes both the size of population and time frame, and is often expressed as person-years (or any other standard interval of time; e.g., “thousand person-years” or “hundred person-weeks”). The incidence rate is then expressed as “cases per thousand person-years” or a similar term. Also note that the time element may be omitted in expressing incidence; the reader must then determine by context the time frame used.

Prevalence, technically not a rate but a ratio, refers to the total number of cases present within a specified time interval. Thus, the numerator in prevalence includes not just new cases but cases carried forward from the period prior to the specified time interval. When point prevalence is computed, a particular narrow time frame is selected, and the population recorded for that time frame constitutes the denominator. All cases “prevalent” on that date constitute the numerator. Prevalence is expressed as a ratio such as “cases per million”; note that there is no time parameter in this ratio.

Changes in incidence and prevalence may be divergent. A control program for human immunodeficiency virus (HIV) infection, for example, could decrease HIV incidence within a population through effective preventative measures but increase the prevalence of HIV infection through access to antiretroviral therapy and improved survival.

In epidemiology, consistency is a virtue. When cases and populations are counted prior to computing rates, care must be

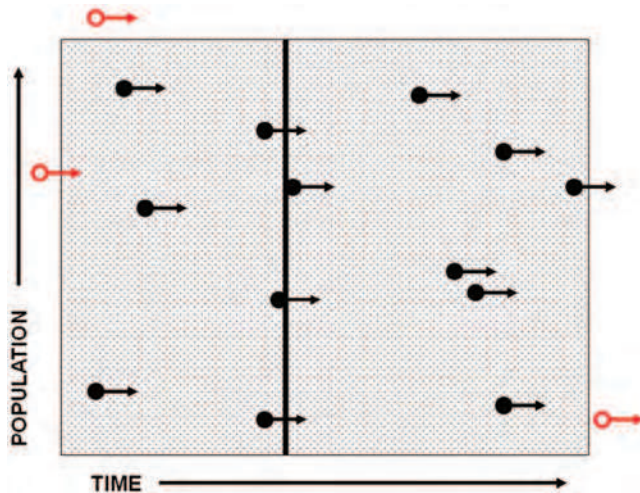


FIGURE 12.1. Computation of incidence rate and prevalence ratio.

The shaded area defines a population and a time frame that can be expressed as person-time units. Cases of disease are indicated by circles (placed according to the date of their onset) and arrows for duration of illness. Solid circles would be counted for computation of incidence, whereas open circles would be excluded because they had onset outside the designated time frame or were resident outside the population boundaries. Incidence equals the number of cases divided by population-time units. Prevalence would be determined by a single vertical line across the hatched area: Cases active at that time point would be divided by the size of the population to compute the prevalence.

taken to use the same definition for numerator and denominator. Persons counted in the denominator should be at risk to be a case in the numerator if infected. For instance, if incidence is computed for the population of Philadelphia for 2011, a case hospitalized in the city but resident outside the city would not be counted. A case with onset in December 2010 but still ill in 2011 would not be counted as an incident case but would be a prevalent case.

What distinguishes infectious disease epidemiology from chronic disease epidemiology is that the former accounts for *dependent happenings*, a term introduced by Ronald Ross,¹⁰² in which the incidence of a disease depends on its prevalence within a population.⁴⁵ Traditional epidemiologic and statistical methods that infer relationships between exposure and disease outcome assume that the outcome in one individual is independent of the outcome in other individuals, an assumption that is invalid for communicable viral infections.^{54,82}

Sources of Data

In practice, the accurate collection of data for the computation of rates is often a major undertaking, whereas the computations are relatively simple. Furthermore, the practicing epidemiologist must frequently work with incomplete and inaccurate information. In most developed countries, denominator (population) information is usually good, and numerator (case) information is the major problem. In resource-limited settings, accurate population data may be unavailable.

For infectious (viral) diseases, there are several sources of case data. Passive surveillance denotes the continuous reporting of disease by healthcare workers. By law, many viral diseases are

designated as “reportable,” and in theory, the reporting of all cases is required. Cases are reported by practicing physicians, hospitals, or laboratories to local health jurisdictions, which in turn relay reports to a central state office whence they are transmitted to a national center (e.g., the U.S. Centers for Disease Control and Prevention). The weakest link is the initial report; in practice, only a small fraction of cases of many common viral diseases (such as 10%–15% of measles or hepatitis B cases) are reported to the national center (called the *reporting efficiency*). However, if the proportion of cases reported is consistent between geographic locales and between years, these reports may be used to monitor trends. Consistency is an important caveat, because the proportion of cases reported may increase if the absolute number of cases decreases. Changes in the case definition also may result in spurious changes in surveillance data. Reporting of certain rare or serious diseases, such as poliomyelitis or acquired immunodeficiency syndrome (AIDS), is often close to 100%; in such instances, reported cases can be used with much greater confidence for calculation of rates. Within increasing interest in the pandemic spread of respiratory viruses and the emergence of new zoonotic viral pathogens, global disease surveillance networks have been established to provide data on disease incidence and transmission in real time,¹⁴ including the Program for Monitoring Emerging Diseases (ProMED) and the World Health Organization’s Global Outbreak Alert and Response Network (GOARN).

Active case detection through epidemic investigation is the traditional approach to collecting information on outbreaks of disease. Such investigations are usually initiated by public health authorities but may be instigated by healthcare workers or patient families. The investigation is tailored to the situation and to the resources at hand, although experience has set certain guidelines, particularly for recurring outbreaks such as food-borne diseases. The purpose of such studies is several: (a) to classify the illness and determine the causative organism, (b) to assess the extent of the outbreak and its economic and health impact, (c) to abort the outbreak or prevent recurrent episodes, and (d) to inform or reassure the public. The first recognition of a new disease or isolation of a previously unknown virus has been accomplished as a result of an epidemic investigation. Examples are the identification of Lassa virus; Marburg virus; Sin Nombre virus (SNV), a hantavirus that is the cause of acute pulmonary syndrome; severe acute respiratory syndrome (SARS)-coronavirus; and the 2009 H1N1 influenza virus.

Serological surveys may be used to detect the footprints that a virus leaves in a population. Serosurveys are particularly useful for viruses because most viral infections leave an imprint on all infected individuals—that is, the presence of immunoglobulin G (IgG) antibody, which often is life long. Because many viruses cause asymptomatic infections or nondescript illnesses in addition to diagnosable diseases, serological surveys identify inapparent as well as apparent infections. Incident viral infection can be identified using assays for immunoglobulin M (IgM) antibody or antibody avidity, whereas IgG antibodies indicate prior infection or vaccination.

Cohort and Case-Control Study Designs

Modern epidemiology has made one outstanding contribution to the discipline—namely, the extension of hypothesis testing from the laboratory to populations. In general, the epidemiologist

TABLE 12.1 Hypothetical Data to Illustrate Computations for a Cohort and a Case-Control Study of Vaccine Efficacy

Group	2012 Cases	Population	Rate per 100,000 person-years	Relative risk
Cohort study^a				
Vaccinated	100	1,000,000	10	0.33
Unvaccinated	900	3,000,000	30	
Group	Cases	Controls	Odds	Odds ratio
Case-control study^b				
Vaccinated	10	25	10/90	0.33
Unvaccinated	90	75	25/75	

Vaccine efficacy = $1 - \text{Relative risk} = 0.67$.

^aCohort study: Two populations, vaccinated and not vaccinated, are followed for 1 year, and cases occurring in each group are recorded. Rates are calculated, and the ratio of rates gives the relative risk for those who were vaccinated. In this instance, relative risk is lower for those with than without the attribute (immunization). The validity of this design depends on the assumption that vaccinated and not vaccinated groups would be at equal risk except for the attribute under study.

^bCase-control study: A group of 100 cases and 100 controls are randomly picked to be representative of the groups from which they are drawn. Subjects in each group are classified as vaccinated or not vaccinated, and two ratios are computed: vaccinated cases/vaccinated controls and unvaccinated cases/unvaccinated controls. The odds of a case being exposed (vaccinated) and the odds of the control being exposed are used to compute the odds ratio, which provides an estimate of the relative risk. The validity of the case-control design depends on two assumptions: (a) the case and control groups are representative of the larger groups from which they are drawn, and (b) the number of cases is very small ($<1/10$) relative to the total population.

attempts to test the hypothesis that one or more population variables influence the occurrence of a specified disease and, if so, to quantify this effect and estimate confidence limits. There are two general designs used for such studies: *cohort* and *case-control* studies. The essence of these designs is summarized next, and the reader is referred to appropriate texts for methodological exposition.³⁵

Cohort Studies

In a cohort study, which may be conducted prospectively or retrospectively, the population is divided into two groups, one with and one without a specified exposure or attribute. Both groups are followed prospectively for the *incidence* of the disease under study, and incidence rates for both groups are then computed. A hypothetical example is shown in Table 12.1. In this example, the population attribute is immunization against a specified viral disease, and the attack rate in the immunized population is one-third the rate in the unimmunized control group. The *relative risk* (RR) associated with immunization is 0.33, and vaccine efficacy is 67% [$(1 - \text{RR}) \times 100$]. There is one important assumption underlying the validity of such a study: The immunized and unimmunized groups are at equal risk of disease. For various reasons, this assumption may not be correct. Differences in the risk of disease between two study groups may be minimized by random allocation of the exposure (in this case, the vaccine) when that is deemed ethical. However, random allocation of exposure is not possible unless the study is of an intervention. Stratifying each study arm according to parameters such as age, race, and socioeconomic status and then comparing rates for each subgroup can be used to identify inequalities between the two groups. Table 12.2 summarizes one such study of polio vaccine and documents the problems of comparing groups (in this instance, vaccinated

and unvaccinated) that are not at equivalent risk of disease (in this instance, poliomyelitis).

More complex study designs are used to measure indirect effects of vaccines or other interventions that affect the transmission of viral pathogens. Such indirect effects include protection of non-vaccinated individuals through decreased transmission from vaccinated individuals, referred to as herd immunity.²⁷ Cluster randomized trials—for example, cohort studies in which communities rather than individuals are randomized to receive the intervention—allow direct measurement of the indirect effects of vaccines by comparing the risk of disease outcomes in nonvaccinated individuals residing in intervention and control communities.^{44,46}

Case-Control Studies

Cohort studies usually require extensive resources and time because they necessitate the enrollment of large numbers of subjects who must be followed for a period of months or years; the less frequent the expectation of disease, the larger the population or longer the follow-up needed. Needless to say, the costs of prospective cohort studies can be great, limiting them to situations such as the introduction of a new drug or vaccine, as illustrated in Table 12.2. Case-control studies can be more cost-effective because they involve smaller numbers of subjects and do not require longitudinal follow-up, particularly if the disease outcome is rare. Table 12.1 shows a simplified version of such a study. In this instance, 100 cases of the study disease and 100 unaffected control subjects are identified and classified according to the exposure or attribute (vaccination) under study. For both vaccinated and unvaccinated individuals, an odds ratio is computed. Because, under certain assumptions, the odds ratios have the same relationship as the rates computed in the prospective study shown in the upper half of Table 12.1,

TABLE 12.2 The 1954 Field Trial of Poliomyelitis Vaccine: Comparison of Attack Rates for Vaccinated and Unvaccinated Children^a

Study area	Vaccination group	Population	Paralytic cases	Rate per 100,000	Estimated efficacy
Placebo areas	Vaccinated	201,000	33	16	72%
	Placebo	201,000	115	57	
	Not inoculated	339,000	121	36	
Observed areas	Vaccinated	222,000	38	17	63%
	Controls	725,000	330	46	
	2nd grade, not inoculated	124,000	43	35	

^aVaccine was administered in the spring of 1954, and children were followed prospectively through the summer poliomyelitis season. Placebo areas were divided into volunteers who were vaccinated, volunteers who received placebo inoculations, and nonvolunteers (not inoculated). Observed areas were divided into second-grade volunteers (vaccinated), second-grade nonvolunteers (not inoculated), and first and third graders (controls).

From Francis T, Jr., Napier JA, Voight RB, et al. *Evaluation of the 1954 Field Trial of Poliomyelitis Vaccine. Final Report.* Ann Arbor: University of Michigan; 1957.

the relative risk can be estimated as the ratio of the two odds ($0.4/1.2 = 0.33$). Two assumptions underlie the validity of the case-control design and analysis, specifically the use of the odds ratio as an estimate of relative risk: The two samples (cases and controls) must be representative of the population from which they are drawn, and the incidence of disease must be low so that cases comprise less than 10% of the total population. Because case-control studies are particularly useful to study risk factors for rare diseases, the second assumption is usually correct. The main challenge in designing case-control studies is the choice of a valid and representative control group, a subject considered in depth in epidemiologic texts.³⁵

BASIC BIOLOGICAL CONCEPTS

Susceptibility and Immunity

Many acute viral infections confer lifelong immunity. Upon re-exposure after initial infection, there is often reinfection but with minimal virus replication and an anamnestic immune response. Such reinfections are usually covert and almost never severe, resulting in minimal or no shedding of infectious virus. For certain viruses, such as poliovirus or rhinovirus, immunity is type specific and confers little protection against exposure to a different serotype. These simple facts have profound implications for viral epidemiology. For epidemiologic purposes, a population may be divided into three groups: susceptible, infected (and infectious), and immune. Persons infected at any time in the past may be considered immune (recovered) and are exempt from disease and unable to act as links in a transmission chain, whereas susceptible individuals can be infected, become infectious, and experience disease. This compartmentalization of the population is the basis for simple SIR (susceptible, infectious, recovered) models of virus transmission⁴ that can explain the periodicity in incidence as the outcome of the buildup and decline of susceptible individuals.

Susceptibility may not be present immediately after birth. IgG antibodies are actively transported across the placenta, conferring protective immunity to neonates and young infants.

For such viral infections, individuals move from a protected state to a susceptible one in the first year of life. Immunity can then be conferred by vaccination or infection.

Persistent viral infections differ from acute infections because the infected individual may be capable of transmitting infection over many years and may develop disease at any time during virus persistence. In the instance of persistent viruses, the population may be divided into uninfected susceptibles and immune-but-infectious persons who carry serological markers of prior infection but who are still capable of acting as links in the chain of infection. Important examples are hepatitis B virus, HIV, and several herpesviruses (herpes simplex virus, varicella-zoster virus, and cytomegalovirus) that establish latent infection capable of reactivation.

Parameters That Determine Incidence

For acute viral infections, the following three parameters determine incidence: the proportion of the population susceptible, the proportion of the population that is infectious, and the rate of contact between susceptible and infectious individuals, with *contact* defined as an encounter sufficient for transmission. The proportion of infected persons who become ill—the case infection ratio—determines the proportion of infected persons detected through case surveillance.

Proportion Susceptible

When a population is exposed to an infectious individual with a specific virus, the susceptible part of the population will determine the spread of the agent and account for all new cases. The proportion susceptible will reflect the past history of infection with the specific virus in the population and the past history of immunization for vaccine-preventable viral infections.

Proportion Infectious

The proportion of susceptibles who become infected, and subsequently infectious, during a fixed period of time (e.g., year or season) can vary widely, depending on the dynamics of transmission, which is influenced by the density of susceptibles and contact patterns between susceptible and infectious individuals.

TABLE 12.3 Estimated Case Infection and Case Fatality Ratios for Selected Viral Infections

Virus	Cases per 100 infections	Fatalities per 100 cases	Comment
Smallpox	>95	<1	Variola minor
	>95	25	Variola major
Measles	>95	0.1	United States, 1970
	>95	2	Africa, 1988
Poliomyelitis	<1	5	United States, 1955
Hepatitis B	50	3	United States, adults
	<1	<0.1	Taiwan, infants

Rate of Contact Between Susceptible and Infectious Individuals

The rate of contact between susceptible and infectious individuals determines the dynamics of viral spread within a population. This rate depends on the mode of transmission (e.g., respiratory, oral–fecal, sexual, or vector-borne), age-specific contact patterns or networks between individuals, and the distribution of susceptible and infectious individuals within a population. Spatial clustering of susceptible individuals can result in outbreaks despite a high proportion of immune individuals within the population.

Case Infection and Fatality Ratios

The proportion of infections resulting in overt disease—the pathogenicity of the organism—is characteristic for each virus and is strikingly different for different agents. One of the most important contributions of modern laboratory methods to viral epidemiology was the identification of inapparent or subclinical infections and the insight that most infections caused by some viruses were asymptomatic. The relative frequency of subclinical infections is expressed as the *case infection ratio*—that is, the number of clinical cases per 100 infections. The lethality of disease is a different parameter that represents the virulence of the organism and is designated as the *case fatality ratio*. The case fatality ratio is the number of deaths attributable to an infection per 100 cases. Table 12.3 lists selected common viral infections and shows estimated case infection ratios and case fatality ratios for each. It is noteworthy that there is no regular relationship between the case infection ratio and severity of illness (i.e., between pathogenicity and virulence). For instance, measles has a very high case infection ratio (>95:100) but a low case fatality ratio in developed countries (<1:1000 in the United States). Conversely, poliomyelitis has a low case infection ratio (<1:100) but a higher case fatality ratio (~5:100). Determining the case infection ratio is an important objective of outbreak investigations of novel pathogens, such as severe acute respiratory syndrome (SARS)-coronavirus and the 2009 pandemic H1N1 influenza virus. Typically, early in an outbreak, only cases that come to clinical attention are counted as incident cases, resulting in an underestimate of the true incidence. Accurate measurement of subclinical infection requires population-based surveys with laboratory confirmation (e.g., serological surveys).

Incubation, Latent, and Infectious Periods

The course of infection in a single individual can be conveniently divided into several periods.²⁹ The interval from acquisition of infection to onset of illness is the *incubation period*. Note that “onset of illness” must be explicitly defined for each disease and is often measured by the first day on which pathognomonic signs or symptoms are reported. The interval from acquisition of infection to onset of infectiousness is the *latent period*. For most viral diseases, the latent period is shorter than the incubation period. Consequently, the infected individual begins to shed virus prior to the onset of illness (smallpox is a notable exception) and continues to shed during and sometimes after recovery from the acute illness. The effectiveness of quarantine measures is reduced when the latent period is significantly shorter than the incubation period as the infectious individual goes unrecognized. The period during which the infected person is potentially infectious for others is the *infectious period*.

Incubation periods for any viral disease vary around the mean, as illustrated in Figure 12.2. When a frequency distribution of individual incubation periods is plotted, it usually has a longer tail at the high end of the distribution (right skewed). If the frequency is plotted against the logarithm of time, the data often approximate a normal distribution.¹⁰⁴ This log-normal distribution is characteristic of the incubation periods of many viral infections.⁶⁰

Generation Time and Serial Interval

The average period between infection of an individual and transmission to others is the *generation time* or transmission interval. From studies of selected outbreaks, the average interval between onsets of successive waves of cases (called the *serial interval*) has been determined, and the generation time is often assumed to be equal to this interval as the best approximation available. This is because it is often impossible to know the exact time of infection. These relationships are shown in Figure 12.3 for a typical acute viral infection.

TRANSMISSION OF VIRUSES

There are two major patterns of transmission into which viruses may be classified: viruses maintained in a single species and viruses that alternately infect different host species. There

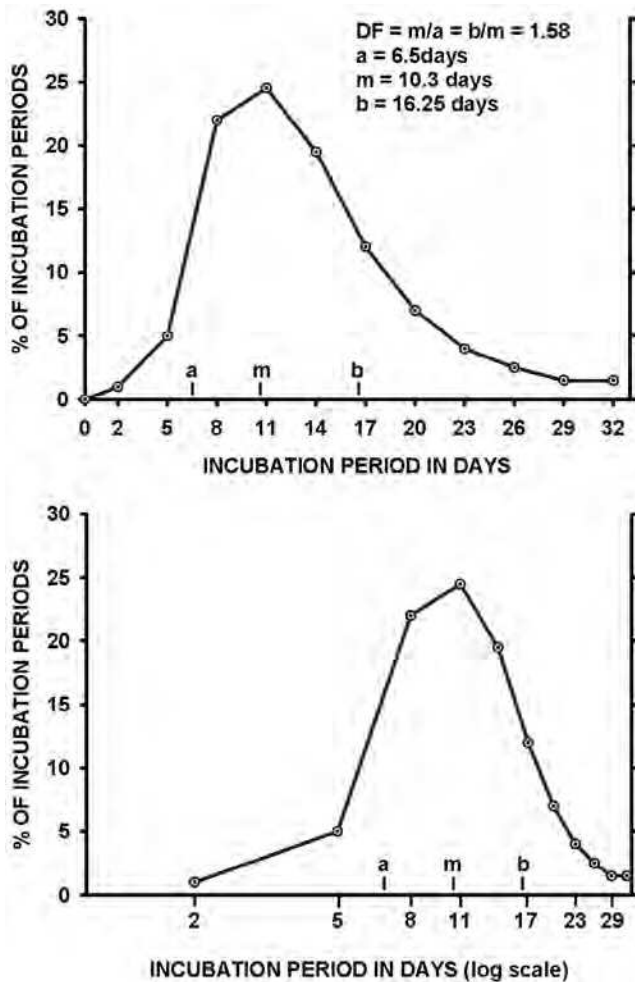


FIGURE 12.2. Log-normal distribution of incubation periods. **Upper panel:** Incubation period distribution is shown for poliomyelitis; there is a right-hand tail. **Lower panel:** The same incubation periods when plotted on a logarithmic scale. Logarithmic incubation period approximates a normal distribution, and the standard deviation may be computed (on the logarithmic scale). The dispersion factor (DF), a measure of the variation of incubation periods, is the antilogarithm of the standard deviation. The mean (m) is shown, together with the standard deviation (points a and b on the graph). The log DF equals the interval ($\log m$ to $\log a$) or the interval ($\log b$ to $\log m$). In this instance, m is about 10.3 days, a is 6.5 days, b is 16.25 days, and DF is 1.58—that is, about two-thirds of the incubation periods fall into the range $(1.58 \times \text{mean})$ to $(\text{mean}/1.58)$. (Data from Aycock WL, Luther EH. The incubation period of poliomyelitis. *J Prev Med* 1929;3:103–120; Casey AE. The incubation period in epidemic poliomyelitis. *JAMA* 1942;120:805–807; Horstmann DM, Paul JR. The incubation period in human poliomyelitis and its implications. *JAMA* 1947;135:11–14; Nathanson N, Langmuir AD. The Cutter incident. *Am J Hyg* 1963;78:16–28; and Sartwell PE. The incubation period of poliomyelitis. *Am J Public Health Nations Health* 1952;42:1403–1408, with permission.)

are a few apparent exceptions, such as rabies and influenza viruses, which spread across species boundaries; however, even in these instances, cycles of transmission between different species may be self-contained. For agents that infect humans, a second distinction may be made between those viruses that rely on human transmission for their perpetuation (the majority)

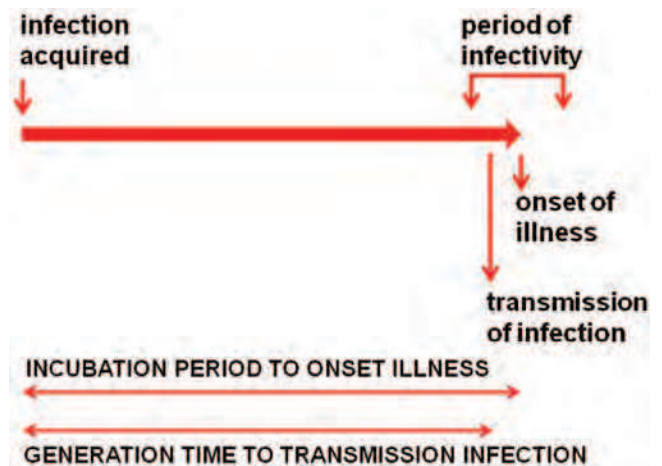


FIGURE 12.3. Incubation period and generation time. Incubation period is the interval between acquisition of infection and onset of illness, whereas generation time is the interval between acquisition of infection and transmission to another person. This diagram shows the mean for each parameter; in practice, there is a spread around this mean.

and those viruses maintained in an extrahuman cycle for which humans are a dead-end host. Examples of these patterns are set forth in Table 12.4.

Viruses Maintained Within a Single Host Population

Each virus has characteristic modes of host-to-host transmission: (a) direct person-to-person transmission through respiratory, fecal–oral, sexual, blood, or from mother to child, or (b) indirect transmission through fomites or vectors. Viruses that cause acute short-term infections require efficient transmission to subsequent hosts. Such infections are characterized by a relatively short infectious period and the excretion of high titers of infectious agent over a limited period of time, as in the instance of influenza, measles, and smallpox viruses. Viruses that cause persistent infections do not require such highly efficient modes of transmission, because they are excreted continuously or intermittently for many years and thus have a long infectious period to maintain virus

TABLE 12.4 Major Transmission Patterns of Viral Infections of Humans

Transmission pattern	Maintenance cycle	Example
Human to human	Human to human	Measles virus Hepatitis A virus Human immunodeficiency virus
Animal to human	Animal to animal	Rabies virus Hantavirus
Vector to human	Vector to human	Dengue virus Urban yellow fever virus
Vector to human	Vector to vertebrate	St. Louis encephalitis virus Western encephalitis virus

TABLE 12.5 Transmission Mechanisms of Human Viruses Maintained by the Person-to-Person Route

General category	Transmission mechanism	Example
Horizontal	Respiratory	Influenza virus
	Fecal–oral	Rotavirus
Vertical	Placental–fetal	Rubella virus
	Maternal parturition	Herpes simplex virus
		Human immunodeficiency virus
	Maternal breast milk	Human immunodeficiency virus
	Germ line	Unknown

transmission. Horizontal transmission is often used to designate these modes of transmission from person to person.

Vertical transmission is used to denote transmission between parent and offspring. Such transmission may occur during gestation, by passage of virus across the placenta (e.g., rubella virus and HIV); perinatally during birth (e.g., herpes simplex or hepatitis B viruses); or from the mother via colostrum or milk (e.g., HIV). Germline transmission, another mode of vertical spread, is seen with certain animal retroviruses such as murine leukemia virus, which are transmitted as an integrated provirus that may be transcribed into infectious virus. Although retroviral sequences are carried in the human genome, there are presently no proven instances where these have been shown to function as infectious virions. Selected examples of horizontal and vertical transmission are listed in Table 12.5.

Viruses That Alternately Infect Different Host Species

Arthropod-borne viruses (arboviruses) are usually maintained by continuously cycling between an insect host and a vertebrate host. In most instances, the virus replicates optimally in a single species or in a few closely related species of insects, which constitute the major, if not exclusive, vectors. Because most blood-feeding insects have a clear feeding preference for a few vertebrate species, this determines and limits the vertebrate species involved in the maintenance cycle. For some arboviruses, alternate maintenance cycles are limited to the insect host, with transovarial or sexual transmission from generation to generation of insects. These insect cycles may be critical to perpetuation of the virus during adverse conditions, such as overwintering, but otherwise play a minor role in viral dissemination.

There are only two arboviruses—dengue and urban yellow fever—of which humans are the major vertebrate host. In other instances, such as St. Louis encephalitis and La Crosse encephalitis, humans are accidentally infected when the vector mosquito happens to feed on humans as an alternate to the preferred vertebrate blood source, such as birds or woodland rodents.

Terminal Hosts

A *dead end* or *terminal host* is one not involved in maintaining viral transmission. Humans are occasional terminal hosts for

several viruses maintained in extrahuman cycles. These include arbovirus infections, with the exceptions of dengue and yellow fever viruses noted earlier. In addition, there are several zoonotic infections, most notably rabies, in which humans are terminal hosts. Transmission of rabies is often by bite, although for most other terminal infections in this group (such as Korean and Bolivian hemorrhagic fever viruses), environmental exposure to fomites is responsible. Table 12.4 lists examples of viral infections in which humans are terminal hosts.

Transmission of Persistent Viral Infections

The transmission of persistent viral infections differs from that of classic acute infections. Those persistent infections that can become latent, such as the herpesviruses, are transmissible intermittently during periods of activation. Thus, one individual may be infected in childhood and transmit infection 50 years later during a recrudescence. The ability for a single link in the infection chain to extend over such an interval has important implications for perpetuation and eradication of infection. Infections such as those caused by hepatitis B virus and HIV, which are transmissible over many years, are often transmitted very inefficiently. Thus, hepatitis B is transmitted to susceptible household contacts at a frequency of less than 1 per 100 person-years exposure, and HIV is transmitted to sexual contacts at a rate of approximately 1 transmission per 100 to 1,000 contact episodes, with the risk of transmission directly correlated with HIV viral load.³⁶ For such infections, transmission requires passage of body fluids or of viable infected cells. Despite inefficient transmission, the number of new infections initiated by each persistent infection may be high because of the long infectious period. An epidemic curve caused by such a virus may stretch over years rather than weeks, and the mathematical modeling of such persistent viral infections is profoundly different from acute infections because infectious individuals are not rapidly converted to noninfectious immunes but continue to accumulate.⁸

Quantitation of Transmission and the Basic Reproductive Rate

The transmissibility of viral infections may be quantified by the basic reproductive rate (R_0), defined as the average number of new infections initiated by a single infectious individual in a completely susceptible population over the course of that individual's infectious period.⁴⁸ The reproductive rate of a pathogen is a function of pathogen characteristics as well as contact patterns within the community. Examples of R_0 are 12 to 18 for measles virus, 5 to 7 for poliovirus and smallpox virus, and 1.5 to 2.0 for influenza viruses.⁴

R_0 is determined by (a) the number of contacts between an infected individual and others, (b) the proportion of these contacts that are susceptible (assumed 100%), and (c) the probability of transmission per susceptible contact. The effective or net reproductive number (R) is the actual average number of secondary cases that occur and equals the product of R_0 and the proportion of susceptible individuals in the population. R is smaller than R_0 because not all individuals in real populations are susceptible, because of either prior infection or immunization. When R is greater than 1, the incidence of infection increases; when R is less than 1, the incidence wanes. When R equals 1, the number of infections is constant. Control programs aim to reduce R to below 1 to achieve disease elimination.

A simple mathematical expression that captures the three parameters for a directly transmitted pathogen is $R = \beta XD$, where β represents the transmission coefficient (a measure of the rate of contact between individuals and the likelihood of transmission during that contact per unit time), X is the proportion of susceptible individuals, and D is the duration of infectiousness. As can be seen from this simple formulation, elimination of a viral infection could be achieved by reducing effective contacts (e.g., quarantine), reducing the proportion of susceptible individuals (e.g., through vaccination), or reducing the infectious period (e.g., treatment to reduce viral load).

Modeling Viral Dynamics

Mathematical models of viral dynamics with varying degrees of sophistication are widely used by infectious disease epidemiologists to understand temporal and spatial patterns of virus transmission within populations and to evaluate the potential impact of control strategies. Mathematical models help to quantify our understanding of infectious disease dynamics and can be used to assess interventions for which epidemiologic studies are not feasible or ethical (e.g., strategies to contain a smallpox outbreak as a result of bioterrorism).⁵⁴ The use of mathematical models in infectious disease epidemiology has a long history, with one of the earliest successful approaches being the model of malaria transmission dynamics developed by Sir Ronald Ross in the early 20th century.¹⁰³ Subsequent models of infectious disease dynamics were developed by Wade Hampton Frost and Lowell Reed (the Reed-Frost model), in which the incidence of newly infected persons was based on the probability of contact between susceptible and infectious hosts.² The models developed by W. O. Kermack and A. G. McKendrick were the first to divide the population into the three classes of individuals discussed earlier (susceptibles, infectious, and recovered; hence, SIR models) and estimated the number of new infections as a function of the number of susceptible and infectious individuals.⁵² These deterministic, compartmental models (based on difference or differential equations depending on whether time is treated as a discrete or continuous variable) were expanded and popularized by Roy Anderson and Robert May⁴ and became the basis for much infectious disease modeling. Variations on the basic SIR model include the addition of other compartments or classes of individuals (e.g., infected but not infectious or a period of maternally acquired protection), age-specific contact patterns, and stochastic processes. Useful models balance the goals of realism and simplicity, as more complex models require a greater number of underlying assumptions and parameter estimates. Useful models clarify our conceptual framework of viral transmission dynamics, suggest which variables are most important for transmission and require careful measurement, and generate new hypotheses. In addition, models can be used to estimate the likely size of an epidemic, its time course and periodicity, the reproductive number R , and the expected impact of various interventions. Finally, model validation against epidemiologic data is a critical component of model building and use.

Measles has long been a favored disease by infectious disease modelers because of the simple transmission dynamics and the long time series available as a result of the readily distinguishable clinical characteristics. Mathematical models of measles virus transmission allow for exploration of the impact of various vaccination strategies, including the optimum age

at measles vaccination and the use of mass vaccination campaigns,^{59,74} that would otherwise be difficult to evaluate in epidemiologic studies. Model results suggest that the best age at which to vaccinate against measles depends critically on the age distribution of cases of infection prior to the introduction of control measures,⁷⁴ the introduction of mass vaccination will induce a temporary phase of low incidence of infection before the system settles to a new pattern of recurrent epidemics,⁷⁴ and that frequent, relatively low coverage mass vaccination campaigns are effective in reducing measles virus transmission.⁵⁹

DESCRIPTIVE EPIDEMIOLOGY

A hallowed maxim in epidemiology is that a complete description of epidemic or endemic disease must include the parameters person, place, and time. The collection and display of this descriptive information is a necessary first step in understanding the epidemiologic mechanisms leading to occurrence, distribution, and course of an epidemic. This simple, systematic approach is a surprisingly powerful tool in analysis. A few selected examples are presented in this section to demonstrate the application of descriptive epidemiology to the understanding of virus transmission.

Person

In tabulating cases of viral infection, the epidemiologist looks for features that distinguish affected persons from the general population to identify risk factors for infection and disease. Clues may exist in demographic features and behavioral characteristics, such as age, sex, race, occupation, residence, or any aspect of personal conduct. Such variables are frequently the most important initial step in understanding an outbreak.

The first recognized outbreak of St. Louis encephalitis in 1933 involved a classical exercise in “shoe leather” epidemiology,⁶⁹ the finding of which led to the proposal that the disease was transmitted by an arthropod vector rather than by person-to-person contact. When this outbreak occurred, neither the disease nor the causal agent was known. Because it was an acute neurological disease, occurring in the summer, it was first thought to be a form of poliomyelitis or to be transmitted in the same manner through the fecal–oral route. The characteristic encephalitic manifestations made it relatively easy to identify many of the cases clinically. When these cases were assembled, and rates estimated, it became apparent that the incidence was greater in the suburbs of St. Louis than in the city proper. Furthermore, there was a striking concentration of cases among the inhabitants of one institution located in the suburbs; however, a comparison of several adjacent institutions revealed even more dramatic discrepancies, illustrated in Table 12.6. First, there were no cases in the personnel in an infectious disease hospital caring for many acute encephalitis cases—strong evidence against person-to-person transmission. Second, there were high rates in an almshouse but very low rates in two insane asylums. Investigation revealed that the almshouse lacked screens, whereas the other three institutions were screened. These observations were strongly reminiscent of the classic findings of the Reed commission investigating yellow fever in Havana⁹⁸ and suggested mosquito transmission. Finally, it was observed that the suburbs of St. Louis, although home to many affluent residents, lacked the system of storm

TABLE 12.6 Attack Rates for St. Louis Encephalitis in Four Institutions in the Suburbs of St. Louis During the Epidemic of 1933^a

Institution	Population	Cases	Rate per 100,000
Mental hospital	400	0	—
Hospital for the insane	4,000	0	—
Isolation hospital	500	0	—
Almshouse (infirmary)	1,200	13	10,800

^aOf these four institutions, three had adequate screening, whereas one (infirmary) did not. The isolation hospital cared for 300 patients with St. Louis encephalitis, but no cases occurred among the staff, estimated at 500.

From Lumsden LL. St. Louis encephalitis in 1933: observations on epidemiological features. *Public Health Rep* 1958;73:340–353.

sewers present in the city. More sites with standing ground-water existed in the suburbs, and in the hot, dry summer of 1933, these sites were prime mosquito breeding sites. These observations suggested the mechanisms of transmission to the investigating epidemiologists.

The “Cutter incident” offers another example where descriptive epidemiology defined an unusual distribution of disease in a population, leading directly to the cause.⁸⁶ In mid-April 1955, newly approved inactivated poliomyelitis vaccine was distributed throughout the United States for immunization of children 5 to 9 years of age—the age group considered the highest risk and therefore given preference in the utilization of limited vaccine supplies. About 2 weeks later, at the end of April, reports were received of cases of acute paralytic poliomyelitis occurring in a small number of recently immunized children. Because this was close to the seasonal trough in poliomyelitis incidence, relatively few cases of poliomyelitis were occurring in the general population, thus a small number of vaccine-associated cases were particularly striking. Furthermore, vaccine-associated cases were confined to a few of the western states. It quickly became apparent that this geographic distribution was related to the manufacturer of vaccine. Vaccine had been produced by five manufacturers, and most cases were

associated with vaccine produced by Cutter Laboratories, which produced the vaccine mainly used in western states. When rates were tabulated for different production pools of Cutter vaccine, the association focused on two high rate pools, as indicated in Table 12.7. Subsequent investigations showed that there were inadequacies in the inactivation and safety testing protocols recommended by the government, permitting the release of vaccine lots containing residual infectious virulent virus.

Age Distribution

The age distribution of viral infection reflects differences in risk. For many endemic human viruses, the cumulative incidence of infection reaches 100% of the population. In such instances, disease is confined to children or to children and young adults, because older individuals are immune as a result of prior subclinical or apparent infection. Poliomyelitis prior to the introduction of vaccine is such an example, and differences in age distribution of disease in different regions or in the same population in different eras reflect differing transmission rates.⁸⁷ Where this enterovirus is transmitted readily, cases are confined to young children; contrariwise, in countries with more rigorous personal hygiene, infection may be delayed so that poliomyelitis occurs up to age 30 or older.⁸⁴ Figure 12.4 compares these age distributions and shows the parallelism with the acquisition of immunity.

For viruses that never infect more than a small proportion of a population, the age distribution of cases reflects differences in exposure or case infection ratio rather than immunity. An example is St. Louis encephalitis, which produces unpredictable outbreaks in different areas of the central United States and is infrequent enough so that most of all age groups are susceptible. Attack rates are typically lower in children and higher in the elderly (10-fold differences). A classic investigation of an epidemic in Houston, Texas, showed that, surprisingly, the frequency of infection was very similar for all ages, indicating that age-specific differences in the case infection ratio accounted for age-specific clinical attack rates.⁹⁵

Age-specific differences in case infection ratios can be a key determinant of epidemiologic patterns of disease. In some instances, infants experience much more severe disease than do adults. A classical example is the outbreak of measles that

TABLE 12.7 The Cutter Incident: Attack Rates Among Children Receiving Cutter Vaccine According to Production Pool, United States, Spring 1955^a

Production pool	Number of inoculations	Paralytic poliomyelitis cases	Rates per 100,000
468	73,700	34	46.1
746	48,000	17	35.4
460	34,100	0	—
463	63,400	1	1.5
467	56,700	1	1.8
762	54,400	1	1.8
766	57,200	0	—
767	3,100	0	—

^aCutter vaccine was administered April 18 through 27, and this table is limited to cases with onset between April 18 and May 14, 1955.

From Nathanson N, Langmuir AD. The Cutter incident. *Am J Hyg* 1963;78:16–28.

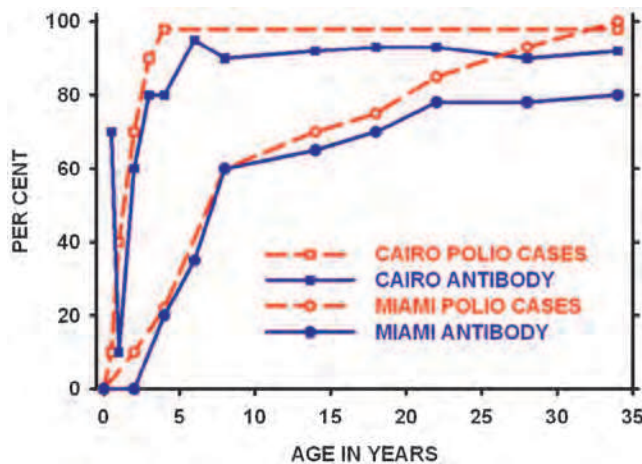


FIGURE 12.4. Age distribution of poliomyelitis cases and immunity.

A comparison of cumulative distribution of cases of poliomyelitis and prevalence of antibodies to type 2 poliovirus in two populations, Cairo and Miami, to show that the age distribution of cases reflects the ages at which infection is acquired. There are few cases above age 3 years in Cairo and above age 30 years in Miami—the ages at which cumulative infection prevalence reaches 100%. (Data from Paul JR. Epidemiology of poliomyelitis. In: *Poliomyelitis*. Geneva: World Health Organization; 1955:9.)

occurred in the Faroe Islands in 1846.⁹³ Because measles had not occurred in this isolated site for more than 50 years, women of childbearing age were seronegative, and their infants lacked maternal antibody. During the epidemic, the case fatality ratio was about 20% in infants and below 1% in children and young adults (Table 12.8). This episode illustrates the biological role of passively acquired maternal antibody. On the other hand, hepatitis B virus causes inapparent but persistent infection in infants but acute liver disease followed by immunity in adults. In developing countries, infections are frequently transmitted from persistently infected mothers to their newborns, whereas

infection in developed countries is mainly transmitted to older children and adults. These differences account for the paradox that the attack rates for hepatitis B are higher in developed countries, whereas cumulative infection rates are much higher and the virus carrier state is more frequent in developing countries, as illustrated in Table 12.9.

Networks

In addition to identifying individual characteristics associated with viral infection or disease, epidemiologists increasingly are interested in transmission networks critical to the dynamics of viral infections within populations.⁶⁸ Network theory and analysis is a complex subject with a long history in mathematics and sociology but has recently been developed by infectious disease epidemiologists, in part because of the ability to characterize complex social networks, such as large-scale social and sexual networks.⁶² The epidemiologic study of social networks is facilitated by unique sampling strategies, including snowball sampling or respondent-driven sampling in which study participants are asked to recruit additional participants among their social contacts. For example, concurrent partnerships (as opposed to serial monogamy) can amplify the spread of HIV and other sexually transmitted infections.⁷⁸ Social networks were shown to affect transmission of 2009 H1N1 influenza virus and were responsible for cyclical patterns of transmission between the school, community, and household.¹⁵

Place

Historically, mapping the distribution of disease preceded the development of epidemiology and became more significant with the introduction of place-specific rates. A famous example is John Snow's mapping of cholera cases during the Broad Street outbreak in London. Mapping spatial patterns of disease distribution remains one of the most powerful and intriguing aspects of descriptive epidemiology and has been enhanced by remote sensing technologies¹⁰¹ and sophisticated spatial analysis tools, including patch and network models.¹⁰⁰

TABLE 12.8 Age-Specific Mortality Rates on the Faroe Islands During the Measles Epidemic of 1846 Compared to Years 1835–1845^a

Age group (Years)	Annual mortality per 100		
	1835–1845	1846	Excess in 1846
<1	10.8	30.0	19.2
1–9	0.5	0.5	—
10–19	0.5	0.4	—
20–29	0.5	0.7	0.2
30–39	0.8	2.1	1.3
40–49	1.1	2.7	1.6
50–59	0.9	4.4	3.5
60–69	2.0	7.7	5.7
70–79	6.5	13.1	6.6
80–100	16.8	26.0	9.2

^aThe mortality for 1846 is for January through August, the period of the epidemic. The excess mortality provides a crude estimate of measles-specific mortality during the epidemic. At least 80% of the population had measles during the epidemic, which was the first to occur since 1781.

From Panum PL. *Observations Made During the Epidemic of Measles on the Faroe Islands in the Year 1846*. New York: Delta Omega Society; 1940.

TABLE 12.9 Prevalence of Hepatitis B in the United States and in Taiwan, Measured by Anti-HBs and HBs Antigenemia^a

Population	Age group (Years)	Anti-HBs (%)	HBs (%)
New York City blood donors (white)	<25	2	<0.1
	25–34	4	0.2
	35–44	3	0.2
	>44	8	<0.1
Taiwan (Chinese)	<10	28	23.0
	10–19	50	20.0
	20–29	48	18.0
	30–39	52	16.0
	40–49	48	15.0
	50–59	48	15.0
	60–69	48	10.0
	>69	48	7.0

Anti-HBs, hepatitis B antibodies; HBs, hepatitis B surface antigen.

^aThe prevalence of antibody and antigen expressed as a percentage of those tested.

From Szmuness W, Harley EJ, Ikram H, et al. Sociodemographic aspects of the epidemiology of hepatitis. In: Vyas GN, ed. *Viral Hepatitis*. Philadelphia: Franklin Institute Press; 1978:297–320.

Two outbreaks of St. Louis encephalitis in two cities of Texas illustrate the complexities of geographic epidemiology. A large outbreak in Houston in 1964 was concentrated in the center of the city with decreasing rates toward the outskirts (Table 12.10). Subsequent serological surveys showed that infection rates paralleled cases and also showed that high rates were associated with the lowest economic strata, open foundations, unscreened windows, and lack of air-conditioning, as well as with areas of standing water close to the banks of the river that flowed through center city. Conversely, in Corpus Christi, attack rates were highest in the suburbs, as shown in Table 12.11. In Corpus Christi, the determining factor was mosquito breeding in pools of standing water associated with lack of a storm sewer system rather than with reduced protection from mosquito attack.

Spatial Patterns and Traveling Waves

Spatial patterns of viral infections are not static but display complex dynamics,¹⁰⁰ one of which is traveling waves of transmission. Measles virus transmission was shown to exhibit spatiotemporal traveling wave patterns in England and Wales,

with regional movement from large cities to small towns.³⁹ The incidence of dengue hemorrhagic fever, a mosquito-borne disease caused by dengue virus, in Thailand was characterized by a spatiotemporal traveling wave emanating from the capital Bangkok.¹⁸ The spatiotemporal dynamics of rotavirus infection in the United States prior to the introduction of rotavirus vaccine, which typically started in the Southwest in late fall and ended in the Northeast 3 months later, was explained by spatiotemporal variation in birth rates and thus the introduction of susceptible hosts.⁹⁶

Time

Seasonality

Many acute viral infections exhibit striking seasonal patterns in incidence.³⁰ In temperate climates, some viral infections peak in the winter, whereas others peak in the summer. In general, respiratory infections spread more readily in the winter, although they may peak at different times. Conversely, the incidence of enteroviral infections peak in the summer, although rotaviruses are a striking exception, as noted earlier. Arbovirus infections are confined to the summer months, when their vectors are active.

TABLE 12.10 Regional and Socioeconomic Differences in Attack Rates (per 100,000) for St. Louis Encephalitis, Houston, Texas, 1964^a

Circle	White upper class	White middle class	White lower class	Nonwhite	Totals
1 (innermost)	—	70	102	77	77
2	34	73	51	50	50
3	14	20	30	20	20
4	2	10	6	7	7
5 (outermost)	3	8	—	6	6

^aThe city was divided into concentric circles and into socioeconomically and racially stratified census tracts within each circle prior to computation of rates.

From Luby JP. St. Louis encephalitis. *Epidemiol Rev* 1979;1:55–73.

TABLE 12.11 Regional and Socioeconomic Differences in Attack Rates for St. Louis Encephalitis, Corpus Christi, Texas, 1966^a

Circle	Socioeconomic stratum	Population	Cases	Rates per 100,000
4 (innermost)	Lower	46,000	5	11
3	Lower middle	49,000	19	39
2	Upper middle	45,000	20	63
1 (outermost)	Upper	46,000	24	52

^aThe city was divided into four zones; zones 4 and 3 were concentric circles, whereas zones 2 and 1 were extensions to the South. Each zone contained a single socioeconomic stratum.

From Williams KH, Hollinger FB, Metzger WR, et al. The epidemiology of St. Louis encephalitis in Corpus Christi, Texas, 1966. *Am J Epidemiol* 1975;102:16–24.

In the tropics, seasonal patterns of viral infections differ from patterns in temperate climates. Influenza virus transmission in the tropics can be either seasonal or relatively constant throughout the year.¹¹⁸ Strong seasonal transmission of measles virus in sub-Saharan Africa generates large, irregular measles outbreaks and is an example of nonlinear, chaotic viral dynamics.²⁶

The underlying explanations for seasonal differences remain elusive. In general, three types of mechanisms have been hypothesized to explain the seasonality of viral infections.⁶⁴ First are seasonal cycles in host resistance to infection, including seasonal differences in vitamin D levels.²² The second involves changes in host behavior and contact patterns, particularly the role of schools as shown for measles virus.²⁸ Third is the role of climatic factors, particularly temperature and humidity. An example is the seasonality of poliomyelitis in the northeastern United States, which contrasts with the

relative absence of seasonality for poliomyelitis in islands of Hawaii, where humidity remains relatively constant throughout the year (Fig. 12.5). This observation led to the hypothesis that viruses differ in their sensitivity to humidity,¹²⁰ with, for example, poliovirus rapidly inactivated during winter months of low humidity,⁸⁷ whereas influenza virus remains viable under similar conditions.¹³² More recently, influenza virus transmission was shown to decrease as vapor pressure (a measure of absolute moisture in air) increases, likely mediated by increased virus survival at low levels of vapor pressure.^{110,111}

The Impact of Viral Pandemics on Secular Trends in Life Expectancy

On rare occasions, the emergence of a new virus may initiate a pandemic so severe that it reduces the life expectancy of the human population. Although exceptional, such events deserve

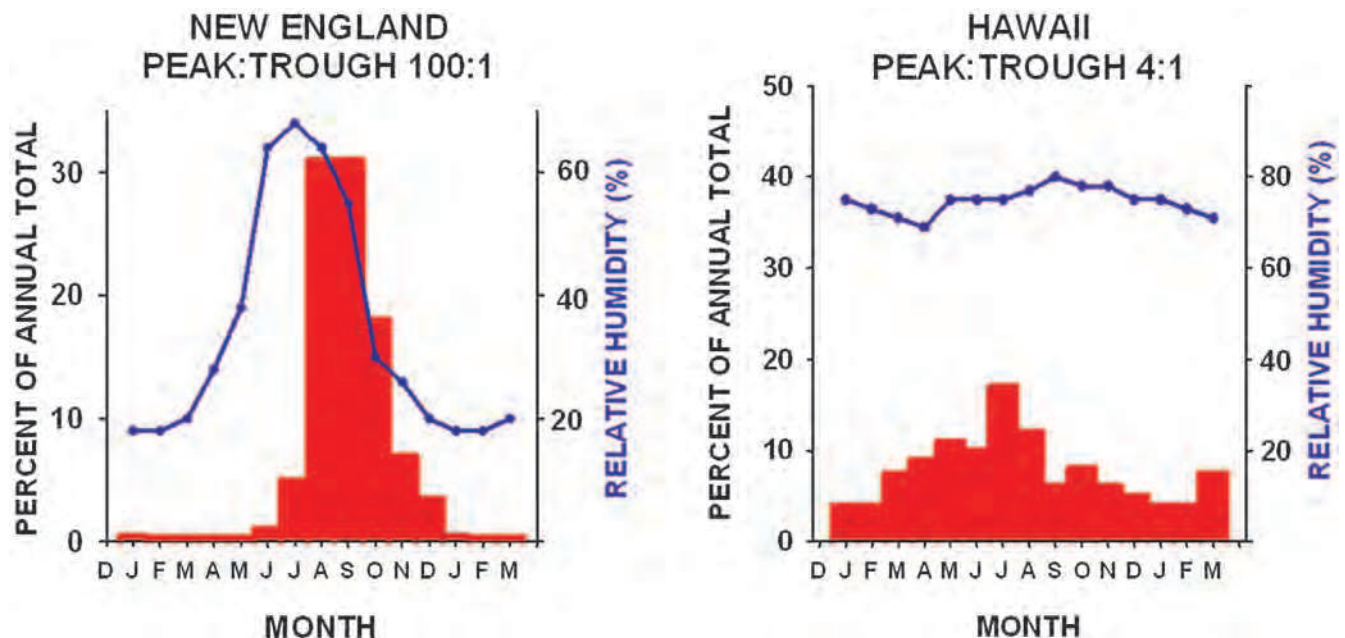


FIGURE 12.5. Seasonal distribution of poliomyelitis in New England and Hawaii, and the seasonal variation in relative humidity. **Upper panel:** Cases for 1942–1951 for New England. **Lower panel:** Cases for 1938–1952 for Hawaii. (Data from Enright JR. Epidemiology of poliomyelitis. *Hawaii Med J* 1954;13:350–354; Nathanson N, Martin JR. The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemicity, and disappearance. *Am J Epidemiol* 1979;110:672–692; and Serfling RE, Sherman IL. Poliomyelitis distribution in the United States. *Public Health Rep* 1953;68:453–466.)

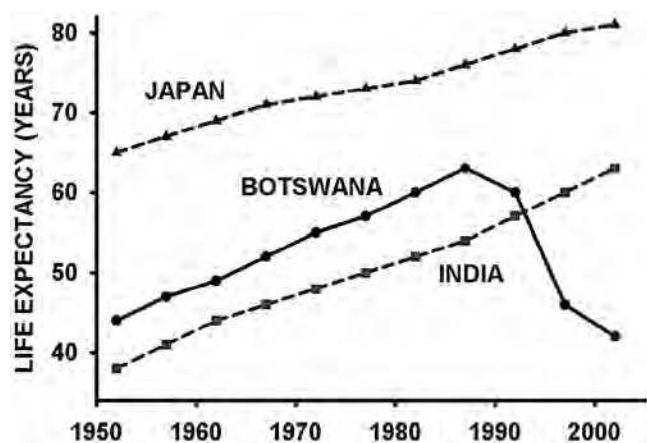


FIGURE 12.6. The drastic effect of the acquired immunodeficiency syndrome pandemic on life expectancy in Botswana, compared with Japan and India. Life expectancy is computed from the age-specific mortality rates for each calendar year, which are averaged to obtain the expectancy for that calendar year. (Data from McMichael AJ, McKee M, Shkolnikov V, et al. Mortality trends and setbacks: global convergence or divergence? *Lancet* 2004;63:1155–1159.)

mention because of their enormous impact. The global influenza pandemic of 1918 was such an event.⁹² A type A influenza virus with an H1N1 genotype emerged, likely a recombinant between an avian and a human influenza virus. Because the human population under age 25 years was negative for H1 antibodies, this virus spread rapidly around the world and is estimated to have caused an excess mortality of about 40 million deaths in less than 1 year, sufficient to cause a transient reduction in life expectancy of about 15 years. Viral archaeology led to reconstruction of the genetic sequence of this virus and the generation of infectious virus through reverse genetics¹²⁵; however, its apparent high virulence has yet to be fully explained in molecular terms.¹²¹ A second striking instance is the impact of HIV/AIDS on life expectancy in some countries of sub-Saharan Africa (Fig. 12.6). In Botswana, for instance, AIDS reduced life expectancy by about 20 years, from age 63 to age 42—an effect whose magnitude has not been seen in recorded medical history.⁷⁵

VIRAL EMERGENCE

One of the most dramatic aspects of historical epidemiology is the appearance of a new viral disease. This may reflect (a) the true appearance of a new virus in the population, a rare but real possibility; (b) an increase in the case infection ratio so that an endemic infection is associated with a marked increase in disease incidence; or lastly, (c) the recognition of an existing but previously unidentified disease that can now be clearly diagnosed owing to new laboratory tests accompanied by heightened practitioner awareness.

Emergence of Novel Viruses

AIDS and other diseases associated with HIV are an only too familiar example of the emergence of a virus new to the population. AIDS first appeared in the United States and Europe

around 1979, and serological studies demonstrated that infection first occurred in the gay population of San Francisco 1 to 2 years prior to the recognition of the disease. The emergence of AIDS was re-enacted in India and Southeast Asia in the mid-1980s, when newly developed serologic and virologic methods permitted the infection to be followed from its earliest entry into the population. Evidence suggests that HIV-2 was derived from viruses indigenous to sooty mangabeys¹¹² and that HIV-1 originated in simian immunodeficiency virus (SIV) strains circulating in chimpanzees.¹¹³

Bovine spongiform encephalopathy (BSE) was first detected in the United Kingdom in the mid-1980s and was quickly identified as a spongiform encephalopathy similar to scrapie, an endemic disease of sheep. Detailed epidemiologic investigation strongly suggested that BSE represented a common source epidemic owing to the contamination of meat and bone meal nutritional supplements routinely fed to dairy cattle.⁹⁹ Scrapie-infected sheep tissues were included in the raw material used to produce the feed supplement, and a change (introduced in the late 1970s) in the production methods permitted scrapie infectivity to survive the production process at a residual level. The rapidly evolving epidemic (Fig. 12.7) led to the exclusion of ruminant tissues from feed supplements, a step that was predicted to terminate the epidemic. However, the long incubation period (averaging about 5 years with a range of 3–9 years) of BSE indicated that the epidemic would continue to evolve. The relatively long tail to the declining epidemic reflected a delay in implementation of the feed ban and a low level of direct cow-to-cow transmission, similar to that observed in scrapie of sheep.

In April 2009, a novel influenza A virus was identified from two children with respiratory symptoms in southern California as a consequence of field epidemiologic investigations.¹¹⁶ Within the first few weeks of identification, field and epidemiologic studies lead to the initial genetic characterization of the virus,³⁴ a description of the clinical and epidemiologic features of infected persons,²⁰ characterization of viral transmission (including the serial interval) within different populations, and assessments of population susceptibility through serological surveys.⁴⁷

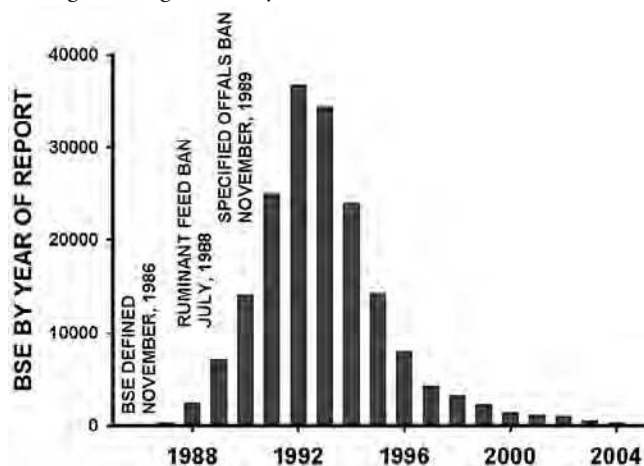


FIGURE 12.7. The course of the epidemic of bovine spongiform encephalopathy (BSE) in Great Britain, with confirmed cases by year of report. (Data from Department of Environment, Food and Rural Affairs. *BSE: Statistics*. 2011, available at <http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/bse/>)

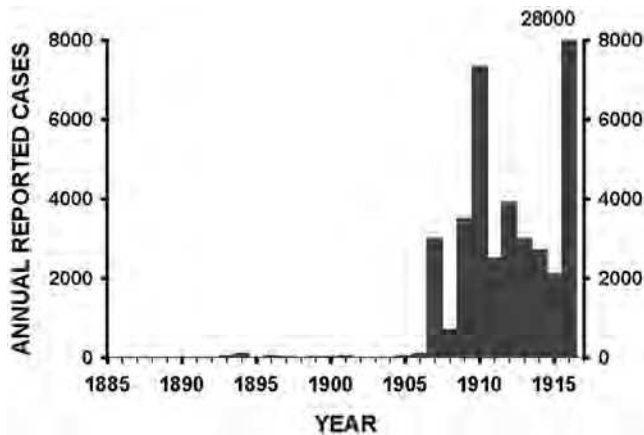


FIGURE 12.8. The appearance of epidemic poliomyelitis in the United States from 1885 to 1916. The graph is based on reported cases (mainly paralytic) during an era when reporting was estimated at about 50%. (Data from Lavinder CH, Freeman SW, Frost WH. Epidemiologic studies of poliomyelitis in New York City and the northeastern United States during the year 1916. *USPHS Hyg Lab Bull* 1918;91:1–150; and Nathanson N, Martin JR. The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemicity, and disappearance. *Am J Epidemiol* 1979;110:672–692.)

Increase in the Case Infection Ratio

The dramatic appearance of epidemic poliomyelitis (then known as *infantile paralysis*) in Europe and the United States in the 19th century is illustrated in Figure 12.8.⁸⁷ In retrospect, it is clear that polioviruses had been endemic for millennia but that cases of paralysis were few and sporadic. The likely explanation is that an improvement in standards of personal hygiene reduced the transmission of this enterovirus, delaying infection of young children beyond the age during which they were protected by maternal antibody (see Fig. 12.4), and resulting in the first epidemics of infantile paralysis.⁹⁰ Support for this view was the re-enactment of the appearance of poliomyelitis in other countries (in the Middle East and Africa) during the 20th century. In such instances, it was possible to conduct serological studies that demonstrated the high prevalence of antibodies prior to the appearance of clinical epidemics. Furthermore, the secular trend continued in the United States,

with a gradual stepwise increase in the age distribution of paralytic poliomyelitis (Table 12.12) so that by the time of vaccine introduction in 1955, the term *infantile paralysis* had become outmoded.

New Recognition of an Existing Virus

La Crosse virus is a mosquito-transmitted bunyavirus that causes La Crosse encephalitis. The causal agent was first isolated from a fatal case in 1964¹²³ and led to the ability to distinguish this disease from the rubric of “arbovirus encephalitis, etiology unknown.” Beginning in 1964, about 100 cases have been reported annually, mainly from the midwestern United States. It is likely that the “emergence” of this disease reflected the ability to make the specific diagnosis, because the incidence did not change and serological studies indicate that infections were occurring at a similar frequency long before isolation of the virus was reported in 1965.⁴¹

A striking acute pulmonary syndrome with high mortality was first reported in the southwestern United States in 1993, and combined epidemiologic and laboratory investigation indicated that the agent, SNV, was a previously unknown bunyavirus belonging to the *Hantavirus* genus.⁸⁸ SNV is an indigenous virus of deer mice (*Peromyscus maniculatus*), in which it causes a persistent infection accompanied by virus excretion leading to production of virus-infected aerosols and exposure of humans. The emergence of SNV was the result of the recognition of a long-existing agent and disease that was brought to attention because of an unusual cluster of cases associated with a transient increase in the deer mouse population.

Possible Increased Frequency of Viral Disease Emergence

New viral diseases may be emerging as zoonoses at an increasing frequency.¹²⁹ Several secular trends have enhanced the probability of emergence of new viral diseases¹²⁷ (Fig. 12.9). First, the population of the world has inexorably continued to increase, and urbanization has concentrated a higher proportion of people in densely populated areas, facilitating the transmission of any new infection. Second, modern transportation permits the carriage of infections around the globe within a single incubation period. Third, man-made perturbations of the environment are occurring at an increasing pace, thus increasing the likelihood of transmission of zoonotic and arboviral infections

TABLE 12.12 Age Distribution of Poliomyelitis in Massachusetts, 1912–1952^a

Years	Age 0–4 years (%)	Age 5–9 years (%)	Age ≥10 years (%)
1912–1916	70	18	12
1920–1924	48	24	28
1925–1929	40	30	30
1930–1934	28	38	34
1935–1939	28	28	44
1940–1944	22	28	50
1948–1952	18	27	55

^aIncludes paralytic and nonparalytic cases.

From Dauer CC. The changing age distribution of paralytic poliomyelitis. *Ann N Y Acad Sci* 1955;61:943–955; and Nathanson N, Martin JR. The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemicity, and disappearance. *Am J Epidemiol* 1979;110:672–692.

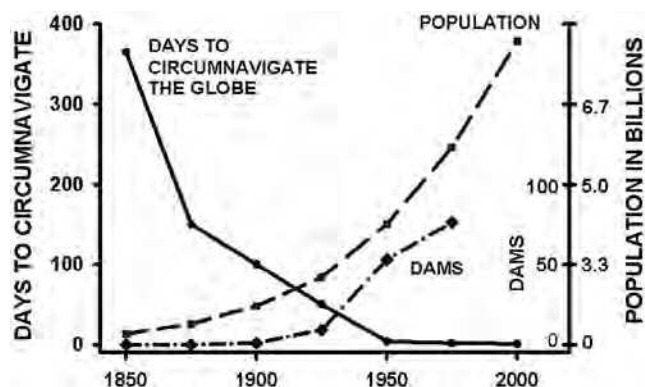


FIGURE 12.9. Since the middle of the 19th century, many global changes have enhanced the probability of the emergence of new viral diseases of humans and animals. This chart depicts three examples of such trends. The rise in the human population has been accompanied by a massive increase in large urban populations, markedly increasing the risk of epidemic spread of infection. Dramatic reductions in the time to travel over long distances have increased the possibility of global transport of infectious agents over short periods of time. The number of large dams (dams >75 meters high built in the United States from 1890 to 1975) exemplifies increases in man-made manipulation of the environment. (Data from Anonymous. *Atlas of World Population History*. New York: Penguin Books and Allen Lane; 1978; Anonymous. *Statistical Yearbook, 38th Issue, 1990/1991*. New York: United Nations Publications; 1993; and Mandzhavidze NF, Mamradze GP. *The High Dams of the World*. Springfield, VA: Academy of the Sciences of the Georgian SSR and Clearinghouse for Federal Scientific and Technical Information; 1994.)

to humans. Finally, advances in fundamental virology have made available a new armamentarium of methods for the detection of previously unknown pathogenic viruses, although numerous challenges are raised by these methods in establishing causal relationships between pathogens and disease.⁶³ The current controversy over the potential association between xenotropic murine leukemia virus-related virus (XMRV) and chronic fatigue syndrome represents an example where the initial report of a causal association between a pathogen and disease based on molecular methods was not confirmed in subsequent studies.^{3,65}

An example that illustrates these forces is the emergence of West Nile virus (WNV) in the United States.⁵⁵ WNV, a mosquito-transmitted flavivirus, was endemic in northern and eastern Africa, the Middle East, and eastern Europe. However, WNV had been limited to these contiguous geographic areas, which is characteristic of the regional distribution of most arboviruses, in contrast to the global distribution of viruses endemic to humans. In 1999, an outbreak of arboviral encephalitis occurred in and around New York City, which was first thought to be caused by St. Louis virus, a flavivirus endemic to the United States. Further study showed that the outbreak was caused by WNV, which had never been found in North America. Although speculative, it appears that WNV was imported from the Middle East, likely by infected mosquitoes that were unpaid passengers on an incoming airliner. Because of the presence of vector mosquitoes permissive for WNV and susceptible avian hosts, WNV spread rapidly and is now endemic across the United States.⁸¹

EPIDEMICS

No medical phenomenon is more dramatic than the occurrence of an epidemic. The immediate cause of any viral epidemic is heightened transmission of the causal agent. Epidemics can be classified according to their principal mode of transmission as either common source or propagated.

Common Source Epidemics

Common source outbreaks are, as the term implies, owing to exposure to the virus from a common source, usually either in food, water, aerosol, or injected product. Common source outbreaks have the potential to be explosive because of the simultaneous exposure of many individuals; however, because the exposure is frequently limited in time, such outbreaks may be of relatively short duration. Finally, common source outbreaks challenge the epidemiologist, because unraveling the source may lead to termination of an ongoing outbreak or prevention of recurrences.

Two examples illustrate common source epidemics. At the beginning of World War II, the military decided to immunize a large number of troops against yellow fever because it was clear that there would be action in several tropical theaters where jungle yellow fever might be encountered. The attenuated 17D strain of yellow fever virus was a newly developed vaccine considered to be a safe and effective immunogen. Because vaccine efficacy depended on the infectivity of 17D virus, its stability was enhanced by including serum in the final formulation. Human serum was used (to avoid serum sickness), and almost 1,000 donors were recruited, most of whom were medical students at Johns Hopkins University. Unfortunately, at least one individual was a carrier of hepatitis B virus. As a result, over 400,000 troops received contaminated vaccine, causing a massive epidemic of hepatitis B infection (about 20,000 cases) in the spring of 1942.¹⁰⁶ Although disease onset was spread over time, when the common source was recognized and cases were plotted from the time of initial immunization with 17D vaccine, they formed a classical log-normal distribution (Fig. 12.10) that provided a definitive estimate of the distribution of incubation period for hepatitis B. Although this epidemic was originally considered to be one of hepatitis A, the great length of the incubation period (mean of 14 weeks) and the absence of secondary spread to contacts provided strong epidemiologic evidence that this disease was a distinct entity.

An outbreak of hepatitis A occurred in 1961 in Pascagoula, Mississippi.⁷³ In some cases, detailed food histories were taken that showed, by comparison with controls, that there was an association with raw oysters. Subsequent investigation disclosed that certain oyster beds in Pascagoula Bay were located near the discharge point for inadequately treated sewage from the city, and cases were associated with oysters supplied from these beds. This was one of the first instances of shellfish-associated hepatitis A, and it was later demonstrated that oysters, through their siphoning system, are capable of concentrating virus up to 1,000-fold. One of the largest outbreaks of hepatitis A virus infection associated with shellfish in the United States within the past two decades occurred in 2005 and involved 39 people.¹⁰ This was the first such outbreak in which identical viral genetic sequences were demonstrated in both cases and food products.

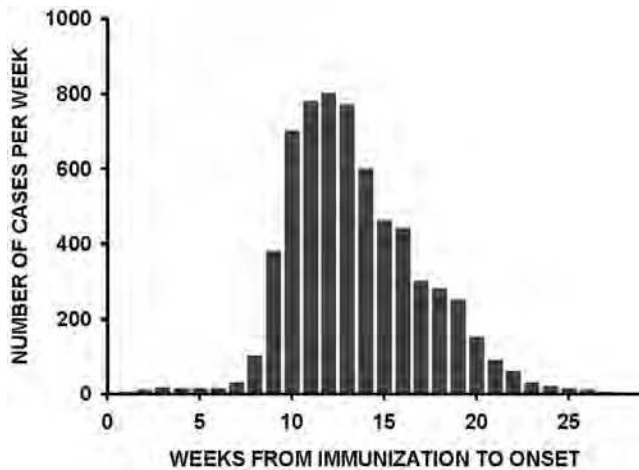


FIGURE 12.10. Distribution of incubation periods for a common source epidemic of vaccine-associated hepatitis B. The graph shows cases of jaundice tabulated by weeks from immunization with 17D yellow fever vaccine to onset for military units in California. (Data from Sawyer WA, Meyer KF, Eaton MD, et al. Jaundice in army personnel in the western region of the United States and its relation to vaccination against yellow fever. *Am J Hyg* 1944;39:337–387.)

Propagated Epidemics

Propagated epidemics, as the term implies, involve host-to-host spread of virus. The occurrence of an epidemic is therefore owing to the action of three parameters that determine clinical disease incidence—namely, the proportion of the population susceptible, the proportion of infected (and infectious) individuals, and the case infection ratio. To produce the unusually high incidence that defines an epidemic, at least one of these parameters must be operating above its usual level. In some but not all outbreaks, it is possible to implicate a specific parameter.

Measles incidence in Iceland over a 50-year period is shown in Figure 12.11, during which epidemics alternated with periods of complete absence of measles. In this extreme situation, the virus invades the population, spreads widely and quickly, and fades out when the concentration of susceptibles drops below the level needed to perpetuate the virus. The virus disappears entirely, allowing susceptibles to accumulate as new

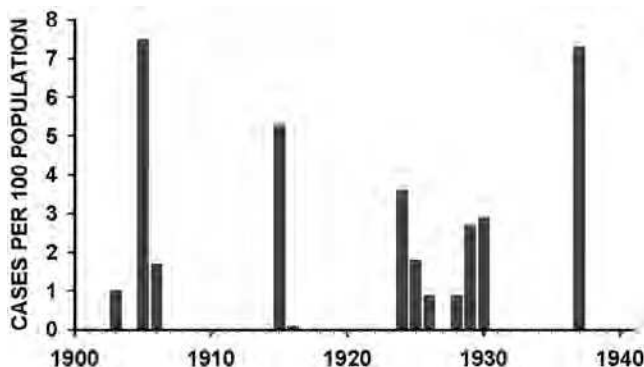


FIGURE 12.11. Measles incidence in Iceland from 1900 to 1940. (Data from Tauxe R. Measles incidence in Iceland, 1900–1940. Unpublished report. 1979.)

cohorts of infants are born. Once enough susceptibles have accumulated, a single importation of measles can initiate a new epidemic. In this situation, the parameter critical for epidemic initiation is the proportion of susceptible individuals in the population.

By contrast, outbreaks of arbovirus disease are unrelated to the proportion of susceptibles, because infection is infrequent and a high proportion of the population is always susceptible (areas with hyperendemic yellow fever or dengue may represent exceptions). Epidemics are a reflection of the proportion of susceptibles who become infected. Because humans are terminal hosts who do not act as links in the infection chain, the key determinants are ecological factors that result in a high concentration of vectors and a high infection rate in the vector population. Epidemics are most commonly preceded by those climatic conditions (rainfall, temperature, and the like) that maximize the vector population.

In some instances, an outbreak is clearly associated with an increase in the ratio of cases to infections, often associated with a virus strain of high pathogenicity. Such occurrences are probably quite frequent but are rarely well documented. One well-studied example is the pandemic of avian influenza that overwhelmed the poultry industry of Pennsylvania in 1983. In this instance, there was a sudden increase in the virulence of an influenza virus, already enzootic in the poultry population, resulting in a pandemic that led to major commercial losses and was only controlled by a widespread slaughter program. Unusually, isolates of the virus from before and after the epidemic were available for analysis, and a classical series of studies showed that a point mutation in the viral hemagglutinin enhanced the replication ability and virulence of the virus.¹²⁶

Epidemics, Viral Pathogenesis, and Molecular Epidemiology

Expanding knowledge of basic virology provides molecular insights into the determinants of viral epidemics. In certain instances, it is possible to identify some of the molecular determinants of each of three parameters (proportion immune, proportion infected, and case infection ratio) that are responsible for propagated epidemics.

The proportion of the population immune is a relatively inflexible parameter for most viruses, with the exception of those against which vaccine exist. Influenza virus is a special instance. This virus is transmitted so effectively that it nearly exhausts the supply of susceptible hosts. The major antigenic protein of influenza virus is the viral hemagglutinin, which serves as the viral attachment protein and the target for neutralizing antibodies. Influenza virus has a genome composed of eight segments, and these segments may reassort when two type A influenza viruses co-infect the same cells, with the creation of viruses containing a mixture of segments from the two parents. On rare occasions, a human strain of virus reassorts with an animal strain and acquires the hemagglutinin of the animal virus. This leads to major shifts in the antigenicity of the virus, such as occurred in 1957, with the appearance of the Asian strain, which had an H2 hemagglutinin whereas previous influenza viruses had an H1 hemagglutinin, and in 2009, resulting in a complex, triple-reassortment H1N1 influenza virus.²⁰ Such a radical shift in antigenicity provides the newly created virus with a global population that is relatively susceptible; as a result, a worldwide influenza pandemic may occur.

TABLE 12.13 Comparison of Case Fatality Rates for Severe (Variola Major) and Mild (Variola Minor) Forms of Smallpox

Disease	Place and years	Cases	Deaths	Case fatality ratio per 100 (%)
Variola major	Minneapolis, 1924–1925	1,430	365	25.5
Variola minor	London, 1928–1934	13,686	34	0.2

From Marsden JP. A critical review of the clinical features of 13,686 cases of smallpox (variola minor). *Bull Hyg* 1948;23:735–746; and Sweitzer SE, Ikeda K. Variola, a clinical study of the Minneapolis epidemic of 1924–1925. *Arch Derm Syph* 1927;15:19–29.

The proportion of susceptibles infected will be determined by two characteristics of a given virus—namely, the generation time and the transmissibility. A virus with a relatively short generation time, such as influenza virus, can spread more rapidly than a virus such as poliovirus despite its lower transmissibility (R_0 for influenza virus is approximately 2 compared to 5–7 for polioviruses). Generation time and transmissibility determine the kinetics of the infection and are related to the titers of virus excreted; these parameters may be influenced by specific viral genes. Analysis of the genetic determinants of reovirus transmission demonstrated that the $\lambda 2$ spike protein encoded by the L2 gene segment is a major determinant of transmissibility.⁵³ Likewise, the transmission of California serogroup bunyaviruses, by their mosquito vector, *Aedes triseriatus*, is determined by the middle RNA segment, which encodes the viral glycoproteins.⁴²

A major determinant of the case infection ratio is viral pathogenicity. Most viruses exhibit great natural variation in pathogenicity. One classical example is variola virus, which caused virulent forms of smallpox (variola major) in India and Africa and much milder disease (alastrim or variola minor) in South America (Table 12.13). It was known for many years that natural isolates of poliovirus varied greatly in their neurovirulence, and attenuated isolates were the starting point for the development of avirulent vaccine strains. Molecular analysis identified the genomic determinants of virulence, which are located both in the structural genes and in the noncoding 5' region of the genome.⁸³ This natural variation might have been a factor in the severity of poliomyelitis epidemics of the past. Another example is the 1983 pandemic of avian influenza cited previously, which was traced to a point mutation that increased the cleavability of the hemagglutinin molecule, thereby enhancing viral maturation and infectiousness with dramatic epidemiologic consequences.¹²⁶

Molecular epidemiology has permitted the unraveling of otherwise enigmatic epidemiologic events. Poliomyelitis was eradicated from many countries, and outbreaks were relatively unusual. However, numerous outbreaks have been traced to revertant strains of oral poliovirus vaccine (OPV), resulting in vaccine-associated poliomyelitis. The genealogy of these revertant viruses was determined by their genomic sequences to be closely aligned with OPV strains but were distinctly different from circulating strains of wild-type poliovirus.⁷⁶

Phylogenetics

Infectious disease epidemiologists are increasingly interested in linking evolutionary and epidemiologic processes, a field

referred to as phylodynamics.⁴⁰ Because of the high mutation rates of viral pathogens, evolutionary and epidemiologic processes take place on a similar time scale.⁹⁷ According to this framework, phylodynamic processes that determine the degree of viral diversity are a function of host immune selective pressures and epidemiologic patterns of transmission.

PERPETUATION AND ERADICATION OF VIRUSES

All viruses, whether they cause acute or chronic infections, are capable of persisting in populations because perpetuation is a requirement for survival. Eradication is the converse of perpetuation and represents the ultimate method for control of an infectious disease. To determine the potential for eradication, it is necessary first to understand the requirements for perpetuation.

The principal ecological patterns of viruses in host populations have been discussed previously, with emphasis on human viruses. In most instances, a virus is perpetuated in a single species, and discussion will be limited to this dominant ecological pattern. Parameters that determine perpetuation include population variables and viral variables.⁴ Population determinants include (a) the size of the population, (b) the turnover rate (rate at which susceptibles are introduced), (c) the density of the population, and (d) the proportion of the population susceptible. Viral determinants include (a) transmissibility, (b) generation time, and (c) duration of infectiousness (acute or persistent). These three viral variables determine the rate of spread of a virus through a population (for a given set of population parameters). Paradoxically, an agent that spreads rapidly may exhaust susceptibles and disappear more quickly from a small population than a virus that moves indolently through the same population. Perpetuation will be discussed separately for large and small populations, because population size is such an important determinant.

Small Populations

For small populations, a crude prediction of perpetuation may be made by determining the number of susceptibles entering the population per generation period. If this number is less than one, persistence will almost certainly not occur.¹³²

Isolated Human Populations

Small primitive human populations that have minimal contact with the outside world are occasionally available for serological

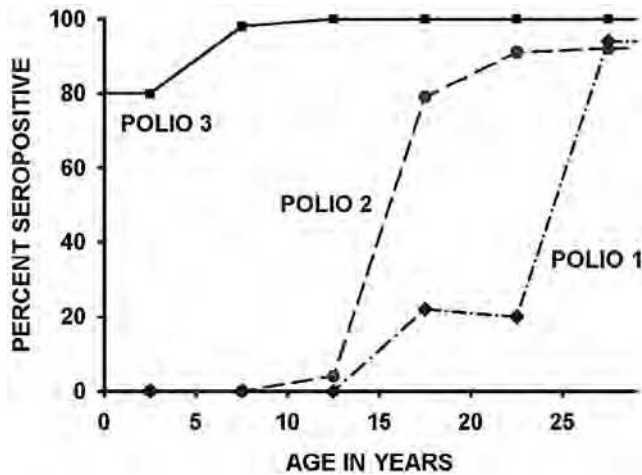


FIGURE 12.12. Age-specific prevalence of antibodies to poliovirus types 1, 2, and 3 in an isolated Eskimo village in Greenland. (Data from Paffenbarger RS, Jr., Bodian D. Poliomyelitis immune status in ecologically diverse populations, in relation to virus spread, clinical incidence, and virus disappearance. *Am J Hyg* 1961;74:311–325.)

study. These populations provide a unique opportunity to determine the ability of viruses to perpetuate in their natural hosts. There are two principal patterns.¹¹ Viruses that cause acute short-term infections produce abrupt outbreaks after introduction into human populations and then die out until reintroduced. Viruses that are capable of persisting in individual hosts are able to perpetuate in small isolated groups.

Figure 12.12 presents the age distribution of antibodies to poliovirus types 1, 2, and 3 in an isolated Eskimo village. The profiles show that type 1 virus had infected essentially the entire population 25 years before the study, type 2 virus had caused a similar wave of infections 15 years before the study, and type 3 virus had been present shortly before the collection of sera. In addition, it is clear that following its intrusion, each virus disappeared from the population, because there were few people younger than 25 years of age with type 1 antibody (the few seropositive individuals probably represent cross-reacting antibody following type 2 infection) and few younger than 15 years of age with type 2 antibody.

In contrast, hepatitis B virus produces a considerable proportion of persistent infections, particularly in primitive populations, where infection occurs early in life. Figure 12.13, from another study of Eskimos, shows that infections occurred at all ages, with a gradual rise in cumulative incidence to 60% by 60 years of age, with no evidence of disappearance of virus from the population or of exhaustion of susceptibles. The curve for hepatitis B surface antigen indicates that there were many persistently infected carriers in the population accounting for perpetuation in this small community.

Small Animal Populations

Animal populations differ radically from human populations in their relatively rapid rate of turnover—a critical variable in virus perpetuation. Mean life expectancy in many animal populations ranges from 6 to 12 months, in contrast to a range of 30 to 70 years for humans. However, the kinetics of infection are similar in an individual human or animal host.

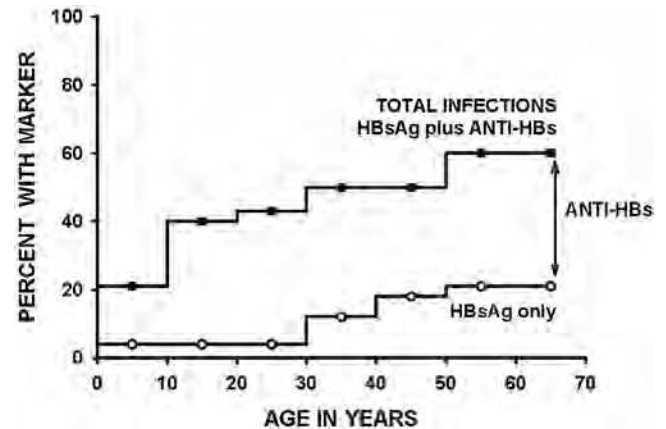


FIGURE 12.13. Age-specific prevalence of hepatitis B antibodies (anti-HBs) and hepatitis B surface antigen (HBsAg) in Eskimos in southwest Greenland. (Data from Skinhoj P. Hepatitis and hepatitis B-antigen in Greenland. II: Occurrence and interrelation of hepatitis B associated surface, core, and “e” antigen-antibody systems in a highly endemic area. *Am J Epidemiol* 1977;105:99–106.)

For instance, the median incubation period (to rash) is about 14 days in smallpox and 10 days in mousepox. The consequence is that perpetuation may occur in animal populations (with rapid turnover) that are much smaller than the minimum human population required to sustain a similar virus.

Laboratory animal colonies have provided an opportunity to document the quantitative impact of population turnover. Perpetuation in such colonies is of more than academic interest, because enzootic infections present an important practical problem that frequently confounds biomedical research. One documented example is ectromelia (mousepox) that closely resembles variola in humans.²⁴ Infection is initiated either by entry through the skin or by inhalation and results in a generalized pox-like rash of the skin from which aerosolized virus is transmitted. As in smallpox, infections can be fatal, although recovered mice are solidly immune. Ectromelia is a relatively infectious agent and a notorious cause of acute and devastating epizootics. When a few infected mice are placed in a larger group of susceptible animals, the virus spreads rapidly, all mice are infected within two incubation periods, and many mice die.

In these circumstances, ectromelia would not be expected to perpetuate in a small mouse population. However, a series of classical studies demonstrated that this virus was readily perpetuated in a population of 100 to 200 animals.³⁷ In one representative experiment, a mouse colony was established with 25 normal and 20 infected mice, and 3 uninfected mice were added daily; animals were removed only if dead. During a 30-month observation period, the total colony size gradually rose to a constant level of about 230 animals. The infection was maintained throughout the observation period; at equilibrium, about one-fifth of the mice were uninfected, one-fifth were actively infected (mortality rate, 50% to 60%), and three-fifths were recovered immune animals. It was possible to maintain this acute highly lethal virus over long periods of time in mouse populations with as few as 70 animals, demonstrating that high turnover markedly reduces the population size required for perpetuation.

TABLE 12.14 Fadeouts of Measles in North American Cities, 1921–1940^a

Size of city (millions)	Number of cities	Average annual measles reports (thousands)	Years of study	Years with fadeout
1.0–7.5	6	3.5–21	120	0
0.5–0.9	9	2.8–4.0	180	1
0.2–0.3	5	0.9–2.1	100	56

^aEstimate of reporting ranged from 14% to 55% of measles incidence. *Large cities*: New York, Chicago, Philadelphia, Detroit, Los Angeles, Montreal; *medium cities*: Cleveland, Baltimore, Boston, Toronto, Washington, Pittsburgh, Milwaukee, Buffalo, Minneapolis; *small cities*: Vancouver, Rochester, Dallas, Akron, Winnipeg.

From Bartlett MS. The critical community size for measles in the United States. *J R Stat Soc A* 1960;123:37–44; and Yorke JA, London WP. Recurrent outbreaks of measles, chickenpox and mumps. II. Systematic differences in contact rates and stochastic effects. *Am J Epidemiol* 1973;98:469–482.

Large Populations

To follow the pattern of viral infection in large populations, it is generally necessary to rely on reportable diseases that can be identified from clinical observations and that exhibit relatively high case infection ratios. Measles is one of the few viral diseases that meets these criteria; however, even measles surveillance has its limitations, considering only a fraction (usually 10%–50%) of cases are reported. Table 12.14 shows that in cities of 200,000 to 300,000, measles faded out for at least 1 month in 56 of the 100 years recorded. Because a population of 300,000 might be expected to experience about 5,000 cases of measles in an average year, it is initially surprising that this would be insufficient to maintain measles virus transmission. The explanation becomes more apparent when the seasonal cycle of measles is examined.^{66,131,132} Data for the city of Baltimore (population 900,000) for 32 years of observation showed that annual reports averaged about 5,000 cases, representing about 35% of cases; incidence rates ranged more than 100-fold from the peak in March to the seasonal low in August, when about 10 cases were reported. Because there were about three generation periods per month, this represents no more than 3 cases per generation period, which is barely sufficient to perpetuate the virus. A similar city of 300,000 would experience only 1 case per generation period at the seasonal trough; in such an instance, it is quite plausible that periodic fadeouts would occur.

This analysis suggests that persistence of a short-cycle infection in a large population depends on the relationship between incidence during the seasonal trough and the generation time. Influenza virus, with a generation time of 1 to 3 days, has difficulty persisting in human populations through the seasonal trough but circumvents the problem by shuttling between Northern and Southern hemispheres each year.

Requirements for Eradication

The history of smallpox, and more recently rinderpest virus, demonstrates that eradication is an attainable objective for selected human and animal viral infections. The salient epidemiologic features that made smallpox eradication possible were its relatively long incubation period (about 14 days) and low infectiousness, the near equivalent durations of the latent and incubation periods, its marked seasonality, a case to infection ratio approaching 1, and the lack of an extrahuman reservoir.⁴⁹ These features made it possible to abandon mass

vaccination in favor of a search-and-containment strategy in which local outbreaks were identified and aborted by intensive immunization around each focus.³¹ The few local outbreaks during the seasonal trough were essential to the success of this strategy.

Eradication of Polioviruses

Two poliovirus vaccines (inactivated poliovirus vaccine [IPV] and OPV) were introduced in the period from 1955 to 1963 in the United States, and their application led to the elimination of circulating wild polioviruses around 1970.^{84,85} Wild polioviruses were eliminated from the Western Hemisphere by 1990 as a result of immunization programs in Latin America, and the World Health Organization set a goal of global eradication in 1988. Worldwide efforts reduced polio cases from an estimated 350,000 in 1988 to fewer than 1,000 in 2000. However, since then, elimination efforts have stalled at a level of 1,000 to 2,000 cases annually, owing to the persistence of wild polioviruses in a few countries: Nigeria and a belt extending from Afghanistan east across Pakistan to northern India. There have been two major impediments in these countries, first the failure to immunize a sufficiently high proportion of infants and young children in settings where transmission of enteric infections is very intense and, second, a low “take” rate of OPV, owing in part to competition between the three serotypes in the vaccine. The latter problem was solved in part by the introduction of monovalent (type 1) and bivalent (types 1 and 3) OPV, whereas concentrated efforts to increase immunization rates have reduced circulating polioviruses in Nigeria and India.¹⁶ At this writing (2011), it is unclear whether these efforts will eliminate residual circulation wild polioviruses, particularly in areas of Pakistan and Afghanistan where there is a breakdown of civil society.

OPV presents another complicating issue, namely the tendency to revert to a more virulent phenotype on human enteric passage. Vaccine-derived polioviruses have caused about 15 documented outbreaks of poliomyelitis in settings where weak immunization programs have left many children susceptible and capable of spreading OPV shed by those who were immunized. Most of these outbreaks have been quite small; however, an outbreak in Nigeria attributable to revertant type 2 vaccine-derived poliovirus has caused nearly 300 paralytic cases over a period of 5 years.¹²⁴ The potential dangers of OPV have led many industrialized countries to shift from OPV to IPV, and

some experts believe that the world will not be freed from paralytic poliomyelitis until immunization with OPV is ended.⁸⁵

Eradication of Measles Virus

Remarkable progress has been made in reducing global measles incidence and mortality as a consequence of measles vaccination. In the Americas, intensive vaccination and surveillance efforts interrupted endemic transmission of measles virus, in part based on the successful Pan American Health Organization strategy of periodic nationwide measles vaccination campaigns and high routine measles vaccine coverage. In the United States, high coverage with two doses of measles vaccine eliminated endemic measles virus transmission in 2000. More recently, progress in reducing measles incidence and mortality has been made in sub-Saharan Africa and Asia as a consequence of increasing routine measles vaccine coverage and provision of a second opportunity for measles vaccination through mass measles vaccination campaigns. The feasibility of measles eradication has been discussed for more than 30 years, beginning in the late 1960s when the long-term protective immunity induced by measles vaccines was becoming evident.¹⁰⁸ Three biological criteria are deemed important for disease eradication, including measles virus: (a) humans are the sole pathogen reservoir; (b) accurate diagnostic tests exist; and (c) an effective, practical intervention is available at reasonable cost.¹ Interruption of transmission in large geographical areas for prolonged periods further supports the feasibility of eradication. Measles is thought by many experts to meet these criteria.⁸⁹

Several potential biological obstacles to measles eradication should be considered.⁷⁹ Persistent infection with transmissible measles virus would pose a biological barrier to eradication. Measles virus is known to establish persistent infection in persons with subacute sclerosing panencephalitis (SSPE); however, virion assembly and budding is defective and multiple mutations occur throughout the measles virus genome.³³ As a consequence, infectious measles virus is not present. Theoretically, selective pressure on measles viruses to mutate neutralizing epitopes and escape protective immune responses induced by vaccines could be a biological obstacle to measles eradication. However, despite the high degree of genetic variation expected of an RNA virus, mutations in the measles virus genome have not reduced the protective immunity induced by measles vaccines.¹¹⁹ Subclinical infection resulting in sustained measles virus transmission also could pose a barrier to eradication,⁸⁰ as it has for polioviruses. However, sustained measles virus transmission among partially immune individuals without clinical disease would be highly unlikely.⁶¹

Eradication of Rinderpest Virus

Rinderpest virus, a pathogen of cattle and many other domestic and wild artiodactyl species, was declared eradicated on May 25, 2011, following widespread vaccination and control efforts coordinated by the Global Rinderpest Eradication Programme.⁷⁷ Rinderpest virus is a *Morbillivirus* closely related to measles virus, transmitted by close contact with an infected animal via inhalation of virus-containing nasal, oral, or fecal secretions. Infection resulted in severe diarrhea leading to dehydration and death. Rinderpest impacted on human health in Europe and Africa by decimating cattle populations, leading to famine, disease, and death.

What is remarkable about rinderpest eradication is that the virus has many potential reservoirs, both domestic and wild, and yet the goal of eradication was achieved through widespread vaccination of cattle and buffalo, establishment of effective surveillance programs, and regional coordination of control efforts.

APPLICATIONS OF EPIDEMIOLOGY

Identification of Etiological Agents

Viruses that cause acute infections, are readily transmitted, and cause intense outbreaks, are often first identified as distinct clinical entities during epidemiologic studies. Examples include HIV (AIDS), hantavirus (pulmonary syndrome), WNV (encephalitis), SARS-coronavirus, and the 2009 H1N1 influenza virus. Epidemiologically, it may be difficult to infer whether the cause is a virus or other infectious agent. Epidemiologic observations, however, may suggest the portal of virus shedding and the period of infectiousness, which is important for the collection of specimens for isolation.

Epidemiologic observations may also contribute insights into more obscure persistent infections associated with atypical chronic diseases. The spongiform encephalopathies or prion diseases are an example. This group of agents includes scrapie of sheep, kuru and Creutzfeldt-Jakob disease of humans, and BSE.¹⁷ The initial definitive evidence that scrapie, a chronic degenerative neurological disease, could be transmitted by an infectious agent and occurred as an unwanted complication of the use of louping ill vaccine in Scotland in 1935.³⁸ Table 12.15 shows that three lots of vaccine against louping ill, a flavivirus, were injected into sheep in Scotland. Unexpectedly, there was a very high incidence of scrapie in recipients of one of the three lots. Subsequent epidemiologic investigation showed that louping ill vaccine was prepared by formalin treatment of virus grown in sheep brain and that several of the sheep used were probably incubating scrapie because their parents subsequently came down with the disease. Prions are extremely resistant to inactivation by formalin; thus, contamination of the vaccine was readily explained. Prior to this episode, it was not generally accepted that scrapie was attributable to a transmissible agent with an incubation period of several years.

TABLE 12.15 Occurrence of Scrapie in Sheep Immunized with Louping Ill Vaccine, Scotland, 1935^a

Vaccine batch	Number of sheep vaccinated	Percentage with scrapie
1	>22,000	<0.1%
2	18,000	5%
3	>4,000	<0.1%

^aBatch 2 was prepared from 122 sheep infected with louping ill; of these, 8 were offspring of scrapie-affected ewes. Interval between subcutaneous injection of vaccine and onset was 20 months or longer.

From Greig JR. Scrapie in sheep. *J Comp Pathol* 1950;60:263–266.

Evaluation of Vaccine Efficacy and Safety

The ultimate test of viral vaccines is their efficacy and safety in humans. Epidemiology provides well-established methods for the design and analysis of prospective studies of vaccine efficacy.⁴⁴ These are illustrated by the 1954 field trial of poliomyelitis vaccine.⁷¹ In this trial, a large number of children in the first three grades of elementary school were enrolled. As shown in Table 12.2, the trial comprised two different study designs—namely, placebo and observed. In the placebo design, children fell into three categories: vaccinated, placebo inoculated, and nonvolunteers (uninoculated). In the observational study, the three groups were vaccinated second graders, nonvolunteer second graders (unvaccinated), and controls (all children in the first and third grades). The most valid comparison was between vaccinated and placebo-inoculated children in the placebo areas: This group yielded an estimated efficacy of 72% for paralytic poliomyelitis, whereas the observed control area yielded an efficacy of 63%. The discrepancy was explained by the fact that the nonvolunteer children in both areas experienced an attack rate that was considerably lower than that of the placebo controls or the observed controls. Apparently, volunteer children were at higher risk (presumably because they included a higher percentage of susceptibles) than were nonvolunteer children. Thus, the strict placebo control was the best comparison group. When the placebo study was further refined by limiting the analysis to laboratory-confirmed cases, the estimated efficacy was even higher (85%), underlining the importance of etiology-proven cases in vaccine evaluation.

Epidemiologic studies can also play a vital role in documenting vaccine complications. The occurrence of Guillain-Barré syndrome associated with the use of swine influenza virus vaccine in the fall of 1976 provides a good illustration.¹⁰⁷ About 40 million people received influenza vaccine in a mass campaign. Cases of a relatively rare neurological disease, Guillain-Barré syndrome, were reported in vaccinees at a frequency considered excessive, and the vaccine was withdrawn. When vaccine-associated cases were compiled by interval after immunization (Fig. 12.14), they exhibited a log-normal distribution typical of many incubation periods. This pathognomonic epidemiologic feature, along with the excessive rates seen during the early weeks after immunization, provided strong evidence of an association and justified termination of the vaccine program. Epidemiologic evidence was the key in this episode because the association (<1 case per 100,000 vaccinees) was only detectable in a massive field study. Because Guillain-Barré syndrome occurs at a baseline rate in the population and those cases associated with the vaccine were not clinically distinguishable from the baseline cases, this episode presented a special epidemiologic challenge. The occurrence of Guillain-Barré syndrome was not predictable by any small-scale human trial or laboratory test, and the biological explanation is still unclear. In fact, epidemiologic studies conducted with influenza vaccines used after 1976 have demonstrated that these vaccines (produced from nonswine strains of virus) apparently carry no risk of Guillain-Barré syndrome.⁵⁸ Nevertheless, the importance of epidemiologic studies to determine background rates of disease remains critical to assessing the risk of adverse events associated with vaccines. In considering mass vaccination against the 2009 H1N1 influenza virus, it was estimated that if a cohort of 10 million individuals were

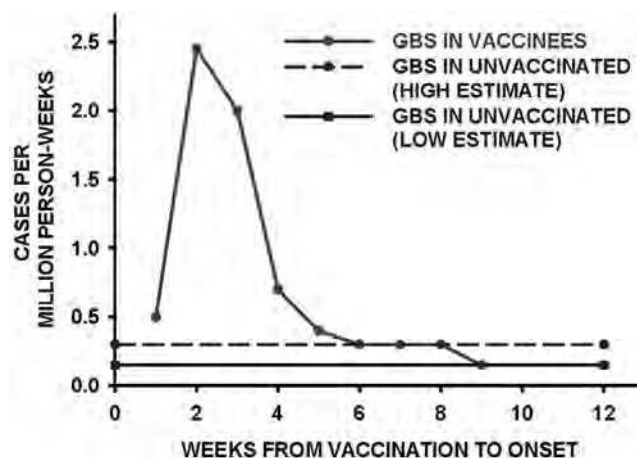


FIGURE 12.14. Guillain-Barré syndrome (limited to cases with extensive involvement) associated with swine influenza vaccine, according to the interval between immunization and onset in the United States in 1976. Two baseline rates are shown to define the expected rate of spontaneous Guillain-Barré syndrome. The period of the epidemic is readily defined (1–6 weeks after immunization) and the intervals during this period of excess incidence form a classical log-normal curve. (Data from Langmuir AD, Bregman DJ, Kurland LT, et al. An epidemiologic and clinical evaluation of Guillain-Barre syndrome reported in association with the administration of swine influenza vaccines. *Am J Epidemiol* 1984;119:841–879.)

vaccinated in the United Kingdom, 21.5 cases of Guillain-Barré syndrome and 5.75 cases of sudden death would be expected to occur within 6 weeks of vaccination as coincident background cases.¹²

Development and Assessment of Control Measures

In addition to vaccines, other control measures are sometimes useful against viral infections. Such approaches seek to reduce the risk of exposure and are usually suggested by epidemiologic studies of transmission mechanisms. Examples include reduction of risk associated with blood or blood products for viruses such as hepatitis B and hepatitis C, HIV, and human T-cell leukemia viruses I and II. Another classical example is the reduction of urban yellow fever and dengue through control of *Aedes aegypti* mosquito populations. The use of epidemiologic management of viral infection is illustrated by a study of Bolivian hemorrhagic fever—an arenavirus that was endemic in certain rural villages.⁵¹ Ecological studies suggested that this virus was enzootic in one species of peridomestic house-dwelling mouse—*Calomys callosus*—and was transmitted by aerosolized fomites. Table 12.16 shows a study in which rodent control was initiated in one-half of a village and then extended to the other half. The dramatic effect of rodenticides confirmed the hypothesis of the virus reservoir and its transmission mechanism and provided a practical approach to control of a serious viral infection. More recently, sophisticated mathematical models have been used to evaluate control measures that cannot be tested in epidemiologic studies, including containment of a potential bioterrorist use of smallpox virus⁴³ and containment of pandemic influenza virus through vaccination¹³⁰ and the use of prophylactic antivirals.²⁵

TABLE 12.16 Control of Bolivian Hemorrhagic Fever by Reduction of the Rodent Reservoir, San Joaquin, Bolivia, 1964^a

Ten-day period beginning	New cases, east sector	New cases, west sector
April 1	12	9
11	8	17
21	12	8
May 1	7	18
11	4	14
21	2	9
31	3	12
June 10	0	10
20	5	7
30	0	0
July 10	0	0

^aRodents were controlled by trapping and poisoning, beginning in the East sector on May 1 and in the West sector on June 15.

From Johnson KM, Halstead SB, Cohen SN. Hemorrhagic fevers of Southeast Asia and South America: a comparative appraisal. *Prog Med Virol* 1967;9:105–158.

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Antiviral Agents

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OVERVIEW

Medical Importance of Antiviral Drugs and Barriers to Their Development

Viruses are a leading cause of disease and death worldwide. Although public health measures and vaccines are the most effective ways to control many viral infections, preventive measures have not succeeded for numerous viral diseases. For some of these diseases, antiviral drugs have been developed. A number of these drugs have been highly successful, saving lives and relieving suffering. This has been most dramatic with

drugs that are active against human immunodeficiency virus (HIV). In the developed world, these drugs have transformed a progressive fatal disease into a manageable condition, in which virus replication can be suppressed presumably for a lifetime as long as antiretroviral therapy is maintained. These dramatic benefits had been extended to more than 7 million people globally by 2011.¹⁸²

Despite these successes, there are still relatively few diseases for which highly effective antiviral drugs have been developed. There are several factors that contribute to this. With very few exceptions, such as the polymerases of related viruses, drug targets are virus specific, and therefore only a few antiviral drugs developed against one virus have been active against other viruses. Some viruses (e.g., herpesviruses, HIV) establish latent infections, so treating active infections does not cure the patient. Different viruses, especially respiratory viruses, can cause similar symptoms, making diagnosis difficult. Often, treatment must begin early to be beneficial, sometimes because the viral diseases are quickly controlled by immune responses.

On top of these factors are economic issues that have often discouraged pharmaceutical companies from developing antiviral drugs. For example, any individual virus disease may represent a relatively small potential market. It is not surprising then that companies have focused mainly on easily diagnosed and chronic viral diseases that require prolonged drug administration and that are relatively widespread in developed countries, because these are diseases for which treatment would earn substantial profits. Perhaps ironically, these diseases are often caused by viruses that establish latent infections and are not curable.

The first highly successful antiviral drug was acyclovir (ACV), initially developed in the late 1970s against herpes simplex virus (HSV) 1 and 2 and varicella zoster virus (VZV).⁹¹ Antiviral drug discovery expanded markedly with the acquired immunodeficiency syndrome (AIDS) epidemic, which led to a variety of drugs against HIV and opportunistic pathogens such as human cytomegalovirus (HCMV). This coincided with expanding knowledge about virus genetics, molecular biology, enzymology, and protein structure, which permitted modern, more rational approaches, to drug discovery. Indeed, antiviral drugs have been a proving ground for these approaches.

The Process of Antiviral Drug Development

Older antiviral drugs such as amantadine and ACV were discovered by the testing of chemicals for their ability to inhibit virus replication in labor- and time-intensive assays of viral replication, such as assays of cytopathic effect or plaque assays.^{75,327} These chemicals had typically been synthesized for some other

purpose, and discovery of their antiviral activities was often serendipitous. When particular chemicals were known to have antiviral activity, a variety of other chemicals with similar structures were tested for activity. Several examples of this somewhat more rational procedure can be found in the sections on specific drugs.

The more modern and rational approach of high-throughput screening of large libraries of chemicals has been used to discover some newer drugs. High throughput screens can be “target-based” or “cell-based.” Target-based screens start with the identification of a viral gene product that would make a good drug target (see below for discussion). It is important that the target protein can be expressed at high levels and purified, and that it can be assayed rapidly with high-throughput robotic technology. Such target-based screens can examine more than one million chemical compounds in no more than a few weeks. Most nonnucleoside reverse transcriptase inhibitors (NNRTIs), which are active against HIV-1, were discovered using this approach (e.g., nevirapine²⁴⁶). Cell-based high-throughput screens are slower, more cumbersome, and more expensive. However, such assays permit interrogation of multiple and unappreciated mechanisms of action, and can be performed relatively rapidly using robots (i.e., within one to a few days per assay). In addition, they automatically identify compounds that can enter cells and block a step of virus replication. Highly potent compounds that target a poorly understood function of hepatitis C virus (HCV) and block HCV replication *in vivo* have been discovered using a cell-based screen.¹¹⁰

A still more rational approach is to exploit detailed knowledge about a viral protein to design drugs that will inhibit its activity. In particular, knowledge of the three-dimensional structure of the protein can enable visualization of its active site and understanding of its mechanism of action, and can also be used to design or refine small molecules that can block the active site and the protein's action. An example of a drug discovered using this approach is the neuraminidase inhibitor, zanamivir, which is active against influenza A and B viruses.³⁷⁰

The approaches outlined in the preceding text result in what is called a “hit”; however, a hit is only an inhibitor of the function of a viral protein or of viral replication in cell culture. The next steps involve using medicinal chemistry to convert the hit into a compound with high potency and desirable pharmacological properties (e.g., bioavailability, long elimination half-life, low toxicity), not to mention patentability. If the structure of the hit compound bound to its target is available, this information can be used to help generate additional compounds to be tested. This process will be illustrated below with the example of the anti-HIV protease inhibitor, ritonavir. Simultaneously, studies to demonstrate the mechanism(s) of action of the compounds are undertaken (see section on antiviral drug mechanisms and drug resistance below). A key, but often overlooked aspect of drug development is developing optimal methods for synthesis of pure compound on a large scale and formulation of the compound for administration to humans.

When a candidate compound with desirable properties has been developed, clinical trials begin. There is no rigid formula by which an antiviral candidate is assessed after it enters the clinic. Typically, the process of clinical development starts with the administration of drug in escalating doses to small

cohorts of volunteers to assess pharmacokinetics and tolerability (phase I trials). With some virus infections, these studies may be performed in patients in whom drug activity can also be assessed, for example by quantifying reductions of chronic HCMV shedding in the genitourinary tract or of HCV in the plasma (proof-of-concept studies). Substantial reductions in viral load encourage progression to more advanced clinical trials. However, with an infection such as HIV, the risk of selecting for drug resistance that might compromise future treatment options often restricts phase I trials to studies of HIV-seronegative subjects. This issue may become important for other viruses (e.g., HCV).

A major advantage of antiviral drug development, compared to drug development for many other diseases, is that quantitative reductions in virus shedding are a precise measure of activity and in general correlate with clinical end points. These quantitative reductions can be measured quickly (days) and permit determination of whether drugs work and of appropriate doses and dosing intervals. Phase II clinical trials assess this antiviral activity along with additional pharmacological and safety information in dozens to several hundred subjects. The data from phase II trials permit the design of larger phase III registrational studies, which provide statistically robust results that fulfill regulatory requirements for drug approval. Trials for drugs for influenza and herpesviruses infections require clinical end points (i.e., amelioration of disease), with virological data supporting those. For HIV, and increasingly for hepatitis B virus (HBV) and HCV, the correlations between levels of viremia and disease, and the correlations between drug-induced reductions in viremia and improvement of disease have resulted in virological end points (quantitative reduction and proportion of patients below the limit of detection) as criteria for drug approval.

Phase III studies of a new antiretroviral drug can pursue two general indications. For patients who failed prior regimens because of drug-resistant virus and who have limited treatment options, an accelerated approval process is available with shorter trials to document efficacy. Historically, patients were randomized to an optimized regimen based on the results of a drug resistance test with or without the addition of the test drug that presumably has activity against drug-resistant virus. Demonstration of superior virological end points after 24 weeks with continued follow-up for at least a year was considered supportive of an unmet medical need to fulfill criteria for registrational approval. Repeated demonstrations that this approach worked have led to the sense that placebo arms were no longer ethically defensible. The proper design of future trials for these patients remains unclear.²⁰⁶

Development of a drug for first-line (initial) therapy faces higher hurdles. Patients starting antiretroviral therapy are now usually asymptomatic and must plan for decades of successful suppression of virus replication. Therefore, a new drug must offer superiority to the current standard of care, and a combination including a new drug “X” (for example, tenofovir and emtricitabine [FTC] plus drug X) might be compared to tenofovir and FTC plus efavirenz. Hundreds of subjects in each arm must be followed for at least 2 years for efficacy and safety to fulfill regulatory criteria for approval of a drug for use in initial treatment. A predefined statistical definition of “non-inferiority” is the criterion for approval of candidate drugs for which there is an approved drug in the same class,

Should the clinical trials yield the desired results, the “hit” will have turned into an approved drug. Even then, its efficacy and safety will be put to the test in much larger populations, and new indications for its use may emerge.

Importance of Antiviral Drugs for Basic Science

Aside from their importance in treating viral diseases, antiviral drugs make excellent laboratory tools. As is illustrated in detail later in this chapter, drugs can block the functions of specific viral proteins and thus specific stages in viral replication. This is crucial for understanding the timing of particular events in the virus replication cycle and which proteins are needed for each step. Studies using selective antiviral drugs, coupled with viral mutations that confer drug resistance, can help to identify the roles of viral proteins and dissect the details of how these proteins function. Drug-resistance mutations can provide selectable markers for engineering interesting mutant viruses and performing viral genetics.

Examples of how studies of antiviral drugs and drug resistance can illuminate virus biology and biochemistry include the role of amantadine in the discovery of an influenza A ion channel and its function in virus uncoating,^{142,289} and the role of ganciclovir in the discovery of an unusual HCMV protein kinase that can phosphorylate nucleoside analogs.³⁵⁰ Crystal structures of drug targets with bound inhibitors have yielded important insights into structure and function of these targets. For example, the first structure of a retroviral (HIV) reverse transcriptase was obtained in complex with nevirapine.¹⁹³ Similarly, clinical investigation has been greatly abetted by antiviral drugs. Intensive monitoring of the dynamics of HIV replication following the administration of drug has permitted important insights about rates of virus production, virus clearance, and cellular sources of virus replication.^{129,282} Studies of patients receiving HIV therapy have elucidated much of our understanding of HIV latency, evolution, and fitness.^{50,98,390} Finally, the remarkable restoration of CD4 cell numbers and function with HIV therapy provides insights regarding both lymphocyte dynamics and immune function.^{7,129}

General Aspects of Antiviral Drug Mechanisms and Drug Resistance

Drug Selectivity, Resistance, and Drug Targets

The value of antiviral drugs to basic science depends on how selective they are and on how well their mechanisms are understood. Selectivity and mechanism are also crucial for the clinical use of antiviral drugs. Biologically, selectivity is the difference between the dose of the drug that exerts its antiviral effect (usually quantified as the dose that reduces viral activity by 50%) and the dose that exerts its cytotoxic effect (or more subtle impairments of the host). Examples of how selectivity is achieved biochemically can be found among the descriptions of the mechanisms of specific antiviral drugs.

It is generally agreed that the best way to understand antiviral selectivity and mechanism is through the study of drug resistance. Because viruses are obligate intracellular parasites, the detection of resistance to an antiviral drug implies that the drug is selective. That is, the drug acts at least in part by interfering directly with a virus-specific process, rather than by incapacitating the host cell.¹⁵⁷ In much the same way, a mutation in a viral gene that confers resistance to an antiviral drug

implies that the gene or its product is necessary for at least part of the selective action of the drug. It is not enough to identify a mutation in a drug-resistant mutant to show that the mutation confers resistance. It is crucial to perform a genetic experiment (e.g., reconstruct a virus with the mutation [and no other] and show that the reconstructed virus is drug-resistant).

In most cases, drug-resistant mutations identify targets of antiviral drugs. However, not all drug-resistant mutations identify drug targets. For example, inhibitors of influenza virus neuraminidase (e.g., zanamivir) can select for mutations in the gene encoding hemagglutinin.^{125,241,341} These mutations would be expected to result in a hemagglutinin that binds less avidly to cell surface glycoproteins. This in turn could result in a reduced requirement for neuraminidase to release the virion from the cell, and thus resistance to the neuraminidase inhibitor. Accordingly, genetics alone is not sufficient to identify a drug target. Ideally, one should also show that the drug interacts with the target, and that the resistance mutation results in a target with an altered interaction with the drug that explains the resistance phenotype. This usually requires biochemical studies, and the analyses are not always straightforward.

Genetics alone is also not sufficient to reveal the detailed mechanism of action of an inhibitor. Mapping a resistance mutation that results in a single amino acid change in the target protein identifies that residue as being necessary for sensitivity to the drug. Other genetic approaches, such as the generation of chimeric genes from closely related viruses that are susceptible or resistant to a drug (e.g., HIV-1 and HIV-2, and NNRTIs⁶⁷), can identify residues that are sufficient to confer susceptibility to the drug to an otherwise resistant protein. However, these results do not identify residues that interact with the drug. Identification of a drug-binding site requires methods such as photoaffinity cross-linking and x-ray crystallography. These two methods have identified the binding site for the NNRTI, nevirapine, on HIV-1 reverse transcriptase (RT).^{62,193,392} Mapping a drug binding site is valuable, but does not tell us how drug binding exerts its physiologic action. In the case of NNRTIs, pre-steady-state kinetic analyses of HIV-1 RT were used to show that the drug slows the enzyme's incorporation of deoxynucleoside triphosphates (dNTPs) into DNA,^{313,338} presumably by altering the orientation of catalytic residues, as suggested by the location of the drug binding site.¹⁹³ Therefore, solving the mechanism of action of an antiviral drug requires multidisciplinary approaches.

For a number of antiviral drugs, we still lack a complete understanding of their mechanisms of action and resistance, and, in some cases, there is considerable controversy regarding these mechanisms. This can even be true for drugs that are designed against a specific target. For example, fomivirsen is an oligonucleotide drug that is designed to inhibit HCMV replication by its complementarity to messenger RNA (mRNA) for an important regulatory protein.^{3,8} However, studies of a drug-resistant mutant did not confirm this mechanism of action.²⁶² Therefore, the mechanism of fomivirsen remains unknown. We will not discuss this and certain other drugs such as the anti-HSV topical treatment, docosanol, whose mechanisms are unknown.

General Aspects of Antiviral Drug Targets

There are two major kinds of virus proteins that serve as antiviral drug targets. In general, it is easier to inhibit a protein's

function (with an antagonist) than to promote its function (with an agonist). Therefore, the most common antiviral drug target is a virus-encoded protein that can be inhibited by a drug. Ideally, this kind of target should be essential for viral replication. Less ideally, it should at least be very important for replication and pathogenesis in the human host. The target should also be sufficiently different from any important host proteins to permit selectivity. It should be *druggable*; that is, a small molecule should be able to inhibit it. Enzymes make especially good targets for inhibition because they are usually at low concentrations inside cells, they are relatively well-understood mechanistically, they often interact with small-molecule substrates, and, as catalysts, their inhibition interrupts multiple reactions. Furthermore, pharmaceutical companies have a great deal of experience in developing enzyme inhibitors as drugs. It is therefore not surprising that most antiviral drug targets are virus-encoded enzymes. A second kind of target is a virus-encoded protein that can activate the drug to make it inhibitory to the virus (*lethal synthesis*), and that is sufficiently different from host counterparts to permit selectivity. An example is HSV thymidine kinase (TK), which activates ACV and its congeners.¹⁰⁸

Host proteins may also serve as antiviral drug targets, if they have an activity that is more important for viral replication than for host functions. A successful example of this approach is targeting the cell surface molecule, C-C chemokine receptor type 5 (CCR5), which led to the development of the anti-HIV drug maraviroc.^{207,297} Targeting of host functions is inherently more likely to result in toxicity than is targeting of viral functions. Conversely, it has been argued that viruses should be less likely to develop resistance against drugs that target host functions. Regardless, such mutants certainly can develop (e.g.,^{328,366}).

Factors Affecting the Development of Drug Resistance

If a drug acts selectively against a virus, the virus will generally be able to develop resistance to it. However, the frequency of drug-resistant mutants in a population, and how rapidly they arise depend on several factors: The first is the mutation rate of the virus. The higher the mutation rate, the more rapidly resistance can develop. Viral mutation rates are largely controlled by the fidelity of the polymerases that replicate the viral genomes. RNA viruses, which replicate using low-fidelity RNA polymerases, usually have the highest mutation rates, averaging one mutation per genome per replication cycle,^{87,232} whereas DNA viruses, whose DNA polymerases include proofreading 3' to 5' exonucleases that contribute to fidelity,¹⁷² have lower mutation rates. However, other factors, including nucleotide pools, which can be modulated by both viral and cellular factors, also contribute to mutation rate.

The second factor is the target size for mutation. The more sites where mutations can confer drug resistance, the more rapidly resistance can arise. For some drugs, such as ACV, many mutations can confer resistance, as any mutation that substantially reduces viral TK activity results in resistance. In addition, some sites within a gene are more or less likely to mutate than others. Homopolymeric runs in the HSV *tk* gene (e.g., a run of seven G's) are hot spots for frameshift mutations that confer ACV resistance.¹⁷⁰

The third factor is magnitude of replication of the virus. The more copies of viral genomes produced, the more opportunities

for resistance to arise. The fourth factor is the preexisting size of the virus population. The more virus present, even in the absence of drug selection, the more likely that drug-resistant mutants will be present among the array of genetic variants. Examples of these two factors that have an impact on HIV drug resistance are provided below in the section on drug resistance in antiviral therapy.

The fifth factor involves fitness (i.e., how well a genetic variant reproduces relative to other genetic variants, which can include “wild-type”). The more fit, the more likely resistance will occur. This is especially important for clinically significant drug resistance. For a virus to cause disease that is resistant to an antiviral drug, it must mutate not only to evade drug action, but also to retain pathogenicity. In vivo fitness and pathogenicity can be inferred from clinical studies, but can be assessed more directly in animal models. Although there is always the question of whether animal models are predictive of human disease, in most cases, there has been an excellent correlation between how drug-resistant mutants behave in clinical and animal studies. Some drug-resistant mutants, such as influenza viruses that are resistant to adamantanes, can be highly fit, both in cell culture and in animal models.^{15,355} Moreover, such mutants appear to be highly fit in human infections as well (reviewed in 144). Most ACV-resistant HSV mutants are highly fit in cell culture, but are much less so in animal models, which correlates with the low frequency that these mutants are recovered in immunocompetent humans (reviewed in 58 and 199). Still other drug-resistant mutants, such as certain HCMV mutants that are resistant to the drug foscarnet, can be less fit in cell culture, but not so impaired that they do not cause disease in patients.¹² Relative fitness is important both in the absence and in the presence of drug. If wild-type virus can replicate relatively well in the presence of drug (e.g., at subtherapeutic concentrations), then resistant mutants will be less likely to predominate. These various factors—mutation rate, target size, replication rate, pre-existing population, and fitness—can be taken into account in mathematical formulas and used to model the emergence of drug resistance (e.g.⁶¹).

In addition, these factors influence how different viral infections are treated. For example, HIV infections are now treated with combinations of antiviral drugs, whereas HSV infections are treated with single agents. This is discussed later in the sections on antiviral therapy.

MECHANISMS OF SPECIFIC ANTIVIRAL DRUGS

This section mainly considers agents that are currently approved for clinical use. No endorsement of the therapeutic value of these drugs is intended. For certain viruses that can be studied usefully in animal models, we discuss the effects of drug-resistant mutations on replication and pathogenesis in vivo.

Targeting Drugs to Specific Stages of Virus Infection

As detailed throughout this book, the infection of cells by viruses can be broken down into a common set of steps or stages, and most of these stages have been targeted by approved antiviral drugs (Fig. 13.1). The order of stages can differ for different viruses, and some viruses have stages that are not

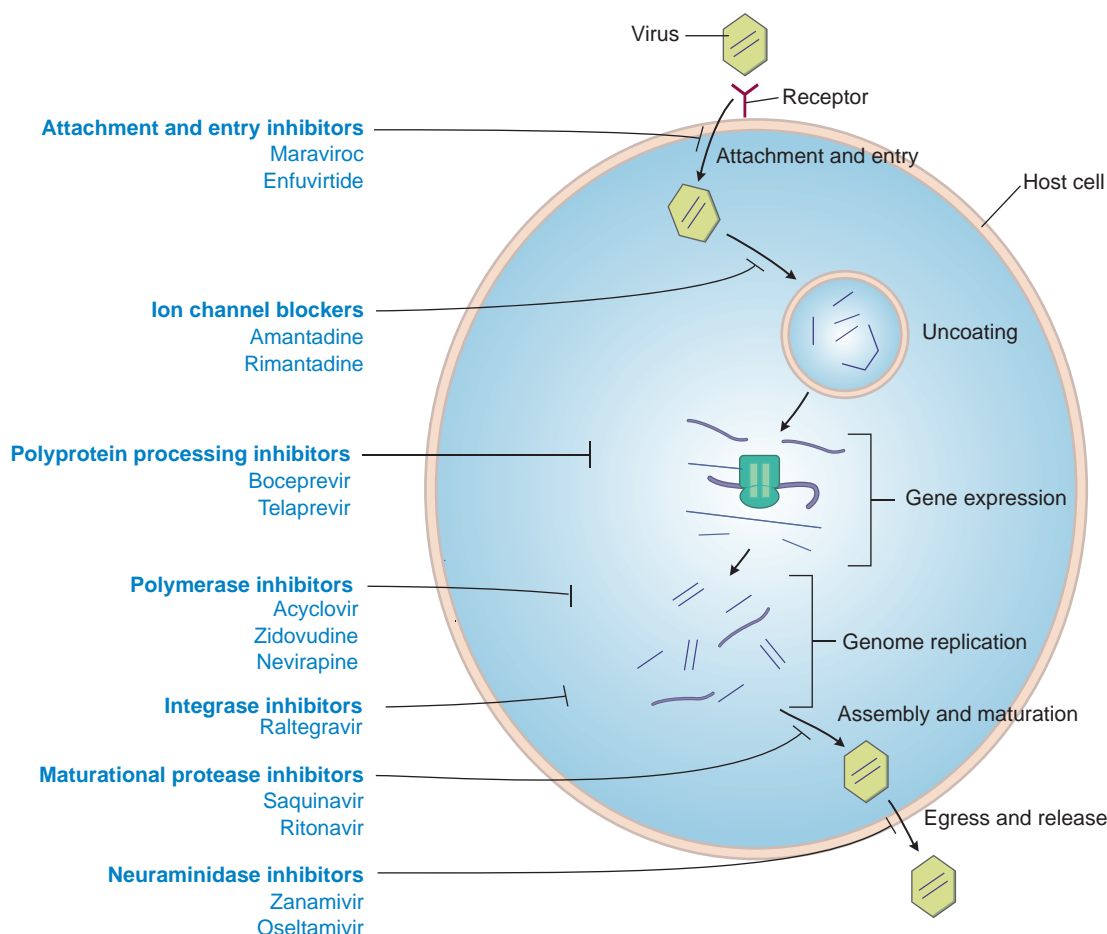


FIGURE 13.1. Antiviral drugs block various stages of viral replication. A generic replication cycle of viruses in cells is cartooned, showing the stages of infection, which different drug classes block. These include attachment and entry, uncoating, gene expression, genome replication, assembly and maturation, and egress and release. Examples of specific drug classes are provided. For some viruses (e.g., human immunodeficiency virus [HIV]), the order of the stages differs from that in this cartoon (see Fig. 13.2). Some viruses lack stages shown here (e.g., release), whereas other viruses have additional stages. (Modified from Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673.)

shared with other viruses. For example, the replication of HIV and the stages that have been targeted by approved antiviral drugs are illustrated in Figure 13.2. In principle, any stage can be targeted for inhibition. There are potential advantages to targeting very early or late stages such as attachment, entry, and release, because inhibitors of these stages do not have to enter cells to exert activity. Stages such as genome replication, assembly, and maturation often require specific viral enzymes, which, as described earlier, are attractive drug targets. Indeed, most antiviral drugs currently available inhibit genome replication. Nevertheless, there are antiviral drugs that act at nearly every stage of viral infection.

Inhibition of Viral Attachment and Entry

Inhibition of attachment and entry prevents all subsequent steps in virus infection and permits the virion to be cleared by immune and other mechanisms. There have been two general approaches for drugs that inhibit attachment and entry, which thus far have resulted in two approved anti-HIV drugs:

maraviroc and enfuvirtide. Both of these drugs have unusual properties; maraviroc targets a host protein, whereas enfuvirtide is a peptide.

CCR5 Blockers

Maraviroc (Fig. 13.3) is a CCR5 blocker; it inhibits binding of HIV to the CCR5 co-receptor. CCR5 is the co-receptor used by the most commonly transmitted HIV-1 strains (R5 tropic), which predominate during the early stages of HIV-1 infection.²⁶ There are several attractive features to CCR5 as a target for anti-HIV drugs: Knockout mice lacking CCR5, and humans with homozygous deletions in the gene encoding CCR5 (CCR5Δ32) lack detectable abnormalities, suggesting that blocking this host function might not have adverse consequences.³⁸ Certain of these CCR5Δ32 homozygotes who have been highly exposed to HIV have remained uninfected, and their cells are resistant to R5-tropic strains of HIV *in vitro*.²²¹ Blockade of CCR5 with a natural ligand (e.g., RANTES) or antibody inhibits R5-tropic HIV replication (e.g.,⁵⁶). These observations suggested that

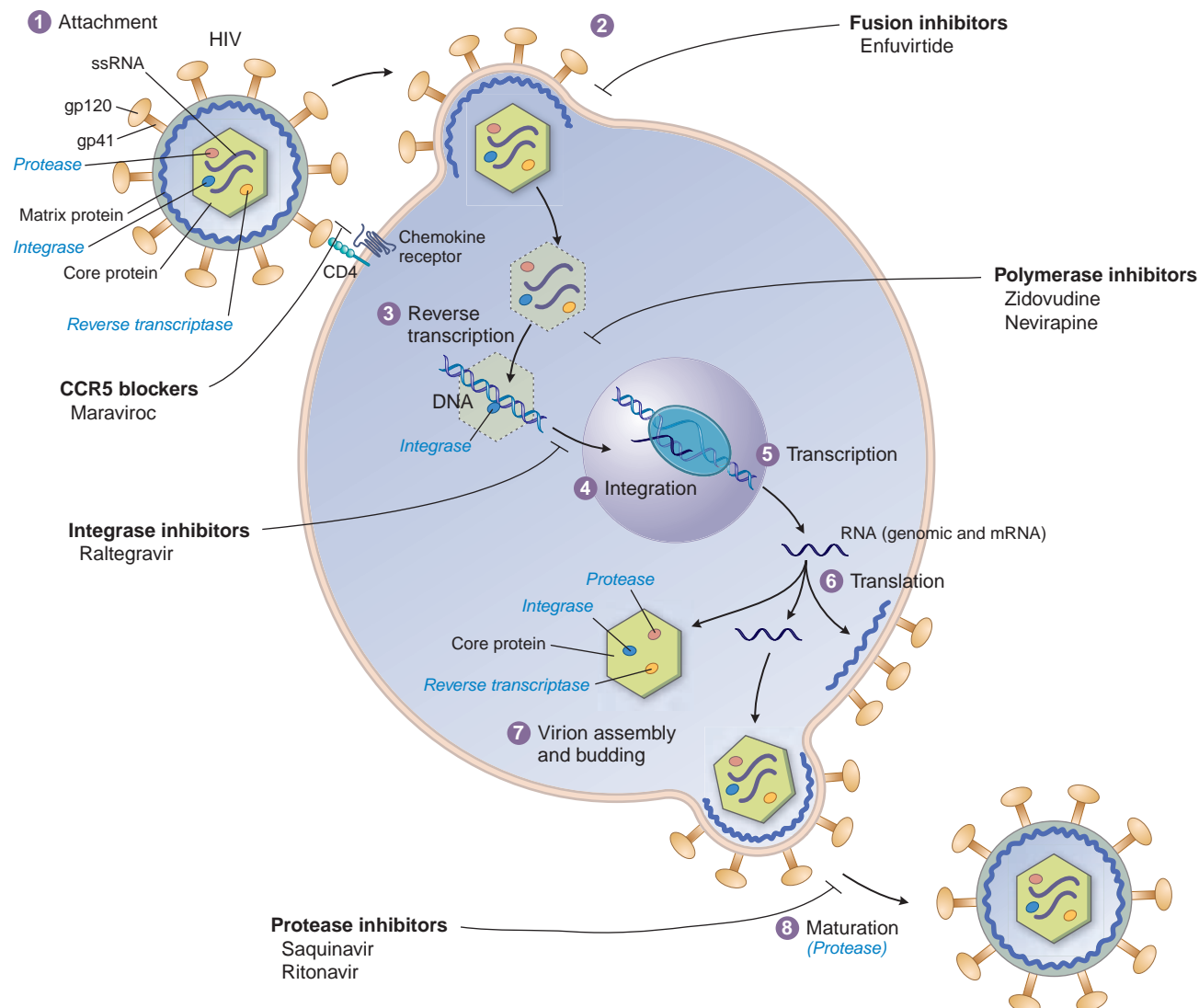


FIGURE 13.2. Stages of HIV infection blocked by different classes of antiviral drugs. The lifecycle of HIV is cartooned, showing the following. **1:** Virus attachment is dependent on binding interactions between viral gp160 (composed of gp41 and gp120 proteins) and host cell CD4 and certain chemokine receptors. **2:** Fusion of the viral membrane (envelope) with the host cell plasma membrane allows the human immunodeficiency (HIV) genome complexed with certain virion proteins to enter the host cell. **3:** Uncoating permits the single-stranded RNA (ssRNA) HIV genome to be copied by reverse transcriptase into double-stranded DNA. **4:** The HIV DNA is integrated into the host cell genome, in a reaction that depends on HIV-encoded integrase. **5:** Gene transcription and posttranscriptional processing by host cell enzymes produce genomic HIV RNA and viral messenger RNA (mRNA). **6:** The viral mRNA is translated into proteins on host cell ribosomes. **7:** The proteins and genomic RNA assemble into immature virions that bud from the host cell membrane. **8:** The virions undergo proteolytic cleavage, maturing into fully infective virions. The steps at which classes of anti-HIV drugs act (attachment, fusion, reverse transcription, integration, and maturation) are indicated, with examples from specific drug classes provided. (Modified from Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673.)

blocking CCR5 could be efficacious against R5-tropic HIV without causing much toxicity.

Maraviroc was discovered using high-throughput screening for small molecules that block binding of CCR5 to a natural ligand, followed by medicinal chemistry efforts to improve upon a hit from the screen.⁸³ Modeling and mutational studies suggest that maraviroc and several other CCR5 blockers bind

to a pocket on CCR5 formed by transmembrane domains,¹⁹⁵ and allosterically inhibit binding to HIV gp120, thereby blocking HIV entry (Fig. 13.3).^{9,83,112,374} R5 tropic HIV mutants resistant to CCR5 blockers including maraviroc contain altered gp120 molecules that are able to bind to the drug-bound form of CCR5.^{366,374} Maraviroc was approved in 2007 for use in combination with other anti-HIV drugs in patients with R5-tropic

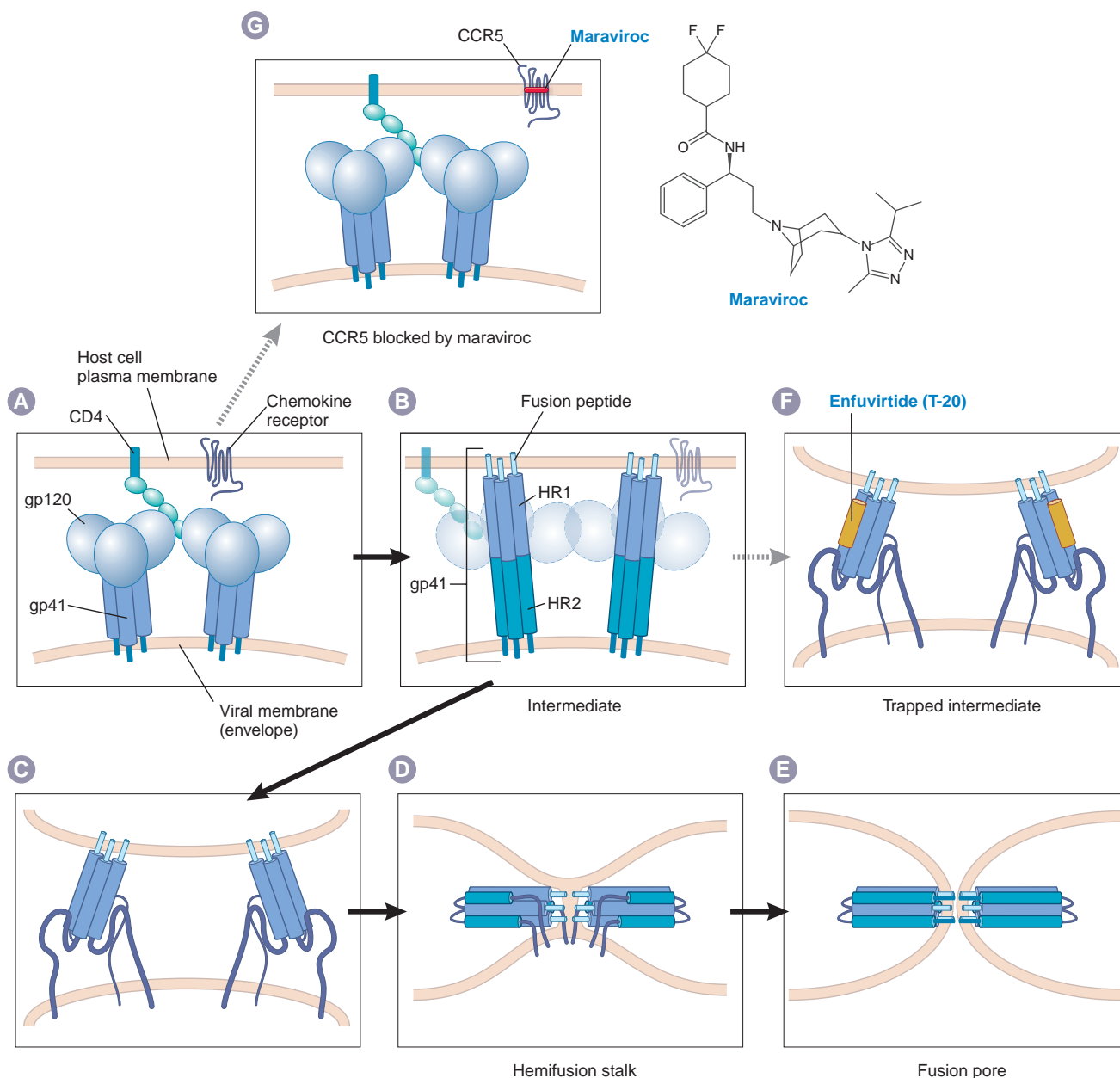


FIGURE 13.3. Model for HIV gp41-mediated fusion and maraviroc and enfuvirtide (T-20) action. **A:** Human immunodeficiency virus (HIV) glycoproteins exist in trimeric form in the viral envelope. Each gp120 molecule is depicted as a ball attached noncovalently to gp41. The CD4 receptor and a chemokine receptor that serves as a co-receptor in the host cell plasma membrane are shown. **B:** The binding of gp120 to CD4 and the co-receptor causes a conformational change in gp41 that exposes the fusion peptide, heptad-repeat region 1 (HR1) and heptad-repeat region 2 (HR2). The fusion peptide inserts into the host cell plasma membrane. **C:** gp41 undergoes further conformational changes, characterized by unfolding and refolding of the HR2 segments. **D:** Completed refolding of the HR regions creates a hemifusion stalk in which the outer leaflets of the viral and host cell membranes are fused. **E:** Formation of a complete fusion pore allows viral entry into the host cell. **F:** Enfuvirtide (T-20) is a synthetic peptide that mimics HR2 and binds to HR1. It prevents the HR1–HR2 interaction (dashed arrow). The drug, therefore, traps the virus–host cell interaction at the attachment stage, preventing membrane fusion and viral entry. **G:** Maraviroc is a small molecule antagonist of the CCR5 chemokine receptor; the drug blocks cellular infection of HIV strains that use CCR5 for attachment and entry (dashed arrow from **A** to **G**). The structure of maraviroc is shown. (From Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673.)

strains. Therefore, patients must be tested to determine if they harbor virus that utilizes a different co-receptor (i.e., CXCR4). Maraviroc treatment failures are more frequently attributable to preexisting minority populations of CXCR4-tropic virus than to selection of R5-tropic virus that is resistant to maraviroc.²⁵⁹ Resistance to CCR5 blockers has not resulted thus far from R5-tropic virus evolving to use a different co-receptor. Nevertheless, this possibility and the effects of antagonizing a host function remain potential issues for the long-term clinical use of maraviroc and other CCR5 blockers, which are in development (e-Table 13.1).

Fusion Inhibitors: Enfuvirtide

Although compounds that bind to virus particles and block attachment and entry have been investigated in the laboratory and clinic for decades (e.g., the anti-picornavirus drug, pleconaril), enfuvirtide was the first drug approved for clinical use that acts this way. This agent was discovered by a rational, directed approach that examined the ability of peptides to inhibit HIV infection in cell culture.^{385,386} The peptide that was most potent (T-20) is similar to a segment of gp41, the HIV protein that mediates membrane fusion. The proposed mechanism for gp41-mediated membrane fusion and T-20 action is illustrated in Figure 13.3: The gp41 protein in the virion is ordinarily trapped in a metastable conformation so that it cannot fuse membranes or bind T-20. Binding of HIV to its cellular receptors triggers a conformational change that exposes the segment of gp41 that is thought to insert into host cell membranes (fusion peptide), a heptad repeat region (HR-1), and the segment that T-20 resembles, which is a second heptad repeat (HR-2). The gp41 then rearranges so that HR-1 and HR-2 bind to each other. If the fusion peptide has inserted into the host cell membrane, this refolding brings the virion envelope and the cell membrane in proximity, thereby allowing membrane fusion to occur (by mechanisms that remain incompletely understood). When T-20 is present, however, it binds to HR-1 and prevents the refolding process, thereby preventing fusion of the HIV envelope with the host cell membrane.

Although this model requires an exogenous peptide to compete with an intramolecular interaction to bind to a target that is available only transiently, it is supported by a number of observations. These include the crystal structure of the rearranged form of gp41, which shows the HR-1–HR-2 interaction^{41,357,373} and evidence that T-20 interacts with HR-1 containing peptides.^{42,205,384} Genetic studies show that HR-1 and HR-2 are crucial for fusion.^{88,383} Perhaps the strongest evidence that T-20 inhibits HIV replication by binding to HR-1 is that HIV mutations that confer resistance to T-20 alter HR-1 residues, and that these alterations weaken binding of T-20 to HR-1.³¹²

Not only is the mechanism of T-20 remarkable, but so is its development into a drug. Because it is a peptide, there were major obstacles to its large-scale synthesis and manufacture, and outpatients must mix the drug and inject it themselves. Regardless, it proved that inhibitors of attachment and entry can be effective antiviral agents.

Inhibition of Viral Uncoating: Adamantane Derivatives

The adamantanes, amantadine and rimantadine (Fig. 13.4), are active exclusively against influenza A virus (and not influenza B or C viruses). In most influenza A virus strains, these drugs

inhibit virus uncoating. It took decades to identify the target of these drugs. A key finding^{142,143} was that amantadine has an unusual dose–response curve, at least for some strains of influenza A virus. It inhibits viral replication at concentrations of approximately 1 μM , which are those attained in patients treated with the drugs; however, at somewhat higher concentrations, it loses antiviral activity. It then regains antiviral activity at concentrations greater than 100 μM . Hay and colleagues¹⁴² then showed that mutants that could replicate in 5 μM amantadine were resistant due to point mutations within the M segment of influenza RNA gene, in an open reading frame that had only recently been recognized. This open reading frame encodes a small membrane protein known as M_2 (the M segment also encodes the long-recognized matrix [M_1] protein.) Subsequent biochemical and electrophysiologic experiments showed that the M_2 protein forms a tetrameric, pH-gated channel for H^+ ions and that amantadine and rimantadine block this channel.^{17,89,163,289,349}

The manner in which amantadine and rimantadine block the M_2 ion channel has been investigated intensively. Viral mutations conferring resistance to these drugs alter any of five residues in the transmembrane segment of the protein that are predicted to be within the pore of the channel, suggesting that the drugs bind to these residues and “plug” the channel.¹⁴¹ Structural studies of drug-bound versions of M_2 followed by electrophysiologic assays using *Xenopus* oocytes and assays of engineered viral mutants support this suggestion.^{36,180,270,343} However, evidence from nuclear magnetic resonance (NMR) studies and liposomal proton flux assays supported the suggestion that these drugs do not act as “plugs,” but instead bind to the outside of the channel and stabilize its closed conformation.^{287,330} This controversy appears to have been resolved in favor of the pore-binding site being the one that mediates drug action, with the NMR structure of rimantadine bound within the pore of a drug-sensitive chimeric M_2 derived from influenza A and B viruses.²⁸⁶

How would blocking an ion channel lead to inhibition of uncoating? A reasonable but not completely verified model is diagrammed in Figure 13.4. Influenza A virus attaches to sialic acid moieties on cell surface glycoproteins, and then, bound to its receptor, is internalized, surrounded by a cell membrane–bounded compartment, the endosome, which normally participates in membrane protein recycling. As part of its normal function, the endosome becomes acidified. During influenza virus entry, this reduction in pH causes a conformational change in the virion hemagglutinin protein, and fusion of the virion envelope and the endosomal membrane via a mechanism similar to that of HIV gp41 (Fig. 13.3). By itself, this action would release viral ribonucleoprotein into the cytoplasm. However, in the presence of amantadine or rimantadine, the matrix protein, M_1 , does not dissociate from the ribonucleoprotein, which remains in the cytoplasm instead of entering the nucleus.^{34,236} Low pH can promote the dissociation of M_1 , and allow nuclear entry of the ribonucleoprotein.³³ Therefore, M_2 in the virion envelope functions to let H^+ ions from the acidified endosome enter the virion, and dissociate M_1 from the ribonucleoprotein. Amantadine and rimantadine would block the entry of H^+ ions, thereby preventing this uncoating event.

With certain influenza A virus strains, amantadine and rimantadine also block late events in virus replication, in particular,

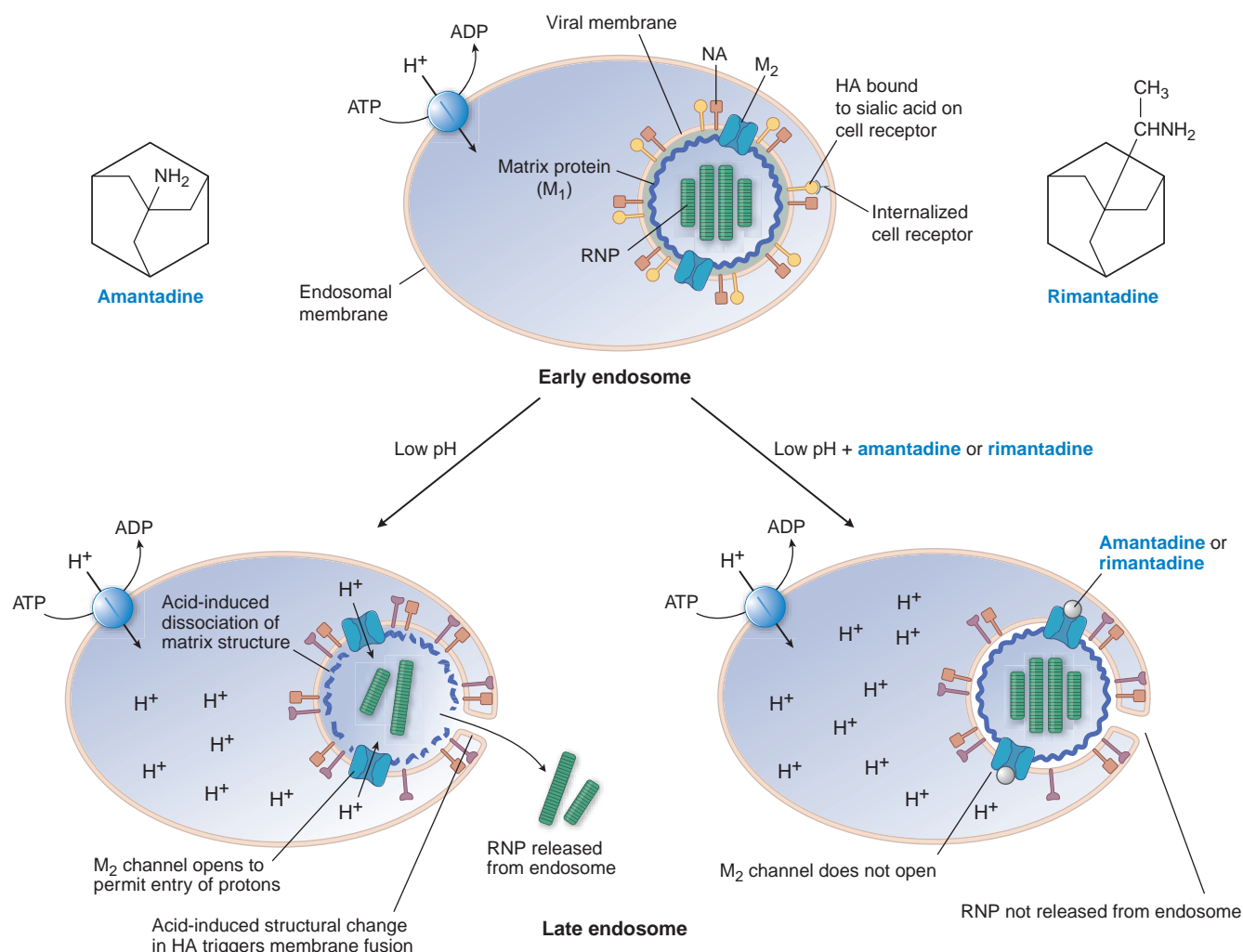


FIGURE 13.4. Model for uncoating of influenza A virus and effect of amantadine or rimantadine. The structures of amantadine and rimantadine are shown. The process of influenza A virus uncoating and drug action is depicted. The virus enters host cells by receptor-mediated endocytosis (not shown) and is contained within an early endosome. The early endosome contains a H⁺-ATPase that acidifies the endosome by pumping protons from the cytosol into the endosome. A low pH-dependent conformational change in the envelope protein, hemagglutinin (HA), triggers fusion of the viral membrane with the endosomal membrane. This fusion event, however, is not sufficient to cause viral uncoating. In addition, protons from the late endosome must enter the virion through the M₂ proton channel, which is a virion envelope protein that opens in response to acidification (pH-gated ion channel). The entry of protons into the virion causes dissociation of the M₁ matrix protein from the influenza A ribonucleoprotein (RNP), releasing it into the cytoplasm. The dissociation of matrix is required for RNP to be transported to the nucleus, where it is transcribed and replicated. Amantadine and rimantadine block M₂ ion channel function and thereby inhibit acidification of the interior of the virion, matrix dissociation, and uncoating. NA, neuraminidase. (Modified from Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673.)

maintenance of hemagglutinin in a proper conformation for infectivity.¹⁴¹

Influenza virus mutants that are resistant to amantadine and rimantadine due to altered M₂ have been assayed in animal models including mice and ferrets, and show little or no diminution in their replication or pathogenesis.^{15,273,355} This correlates well with their behavior in human infections.¹⁴⁴ Since the discoveries of the mechanism of adamantanes and the function of M₂, a number of other viruses have been found to encode proteins that can form ion channels. These also might serve as good drug targets.

Inhibition of Viral Gene Expression: NS3/4A Protease Inhibitors

Viral gene expression entails not only the transcription of the viral genome into mRNA, the translation of mRNA into protein, and the stabilities of RNA and protein gene products, but can also include a variety of processing events including capping, splicing, and polyadenylation of viral mRNAs, and proteolytic cleavage of viral polyproteins into their individual protein units. Many viruses encode functions that execute or abet one or more of these steps in gene expression, and much

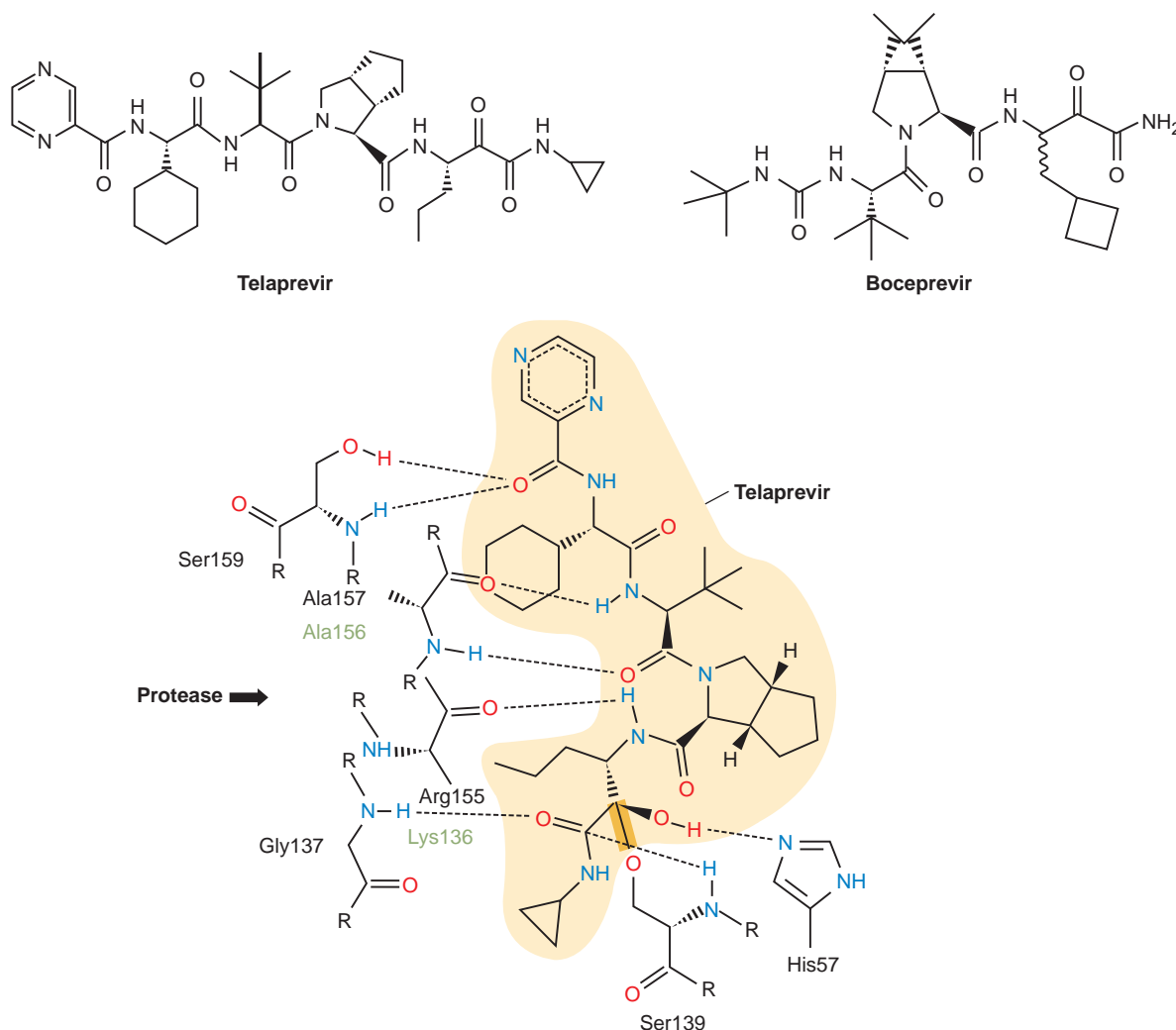


FIGURE 13.5. Anti-HCV (hepatitis C virus) protease inhibitors. The structures of telaprevir (**left**) and boceprevir (**right**) are shown at the **top** of the figure. A schematic of the interaction of telaprevir with the HCV NS3/4A protease is shown at the **bottom** of the figure. Important hydrogen-bonding interactions between protease residues and drug are shown with dashed lines, whereas protease residues that make important hydrophobic interactions are indicated in green. The covalent bond formed between telaprevir and serine 139 of NS3 is highlighted in yellow. (Courtesy of Aysegüi Özen and Celia Schiffer, based on the crystal structure of telaprevir bound to NS3/4A. From Romano KP, Ali A, Soumana D, et al. The molecular basis of drug resistance against hepatitis C virus NS3/4A protease inhibitors. *PLoS Pathog* 2012;8:e1002832.)

effort has gone into targeting these functions for development of antiviral drugs. In particular, viral proteases that generate individual viral proteins have been targeted. The activity of one of these, the HIV protease, is not required until the viral particle is fully assembled, and thus will be discussed under the heading Inhibition of Viral Assembly and Maturation below. The activity of the HCV NS3/4A protease, however, is required for proper expression of viral gene products for essentially all subsequent steps in viral infection. In 2011, the first drugs that target this stage of viral replication were approved by the U.S. Food and Drug Administration (FDA). These two drugs—telaprevir and boceprevir (Fig. 13.5)—are peptidomimetics that inhibit the NS3/4A protease.

Following entry and uncoating of HCV, its genome serves as an mRNA that is translated into a very large (~3,000 residue) polyprotein. This polyprotein is processed into 10

individual proteins by cellular and viral proteases. One viral protease, which is especially important for generating non-structural (NS) proteins, is NS3. NS3 contains an N-terminal protease domain and a C-terminal helicase domain. A second protein, NS4A, forms a complex with NS3 (NS3/NS4A), and enhances its protease activity. During polyprotein processing, NS3 autoproteolytically cleaves between NS3 and NS4A, and then cleaves to generate NS4A (its co-factor), NS4B, NS5A, and NS5B. The NS proteins are essential for HCV genome replication and production of infectious virus. These proteins, especially NS3, are also thought to counteract the innate immune response.¹⁰⁹

Given the success of drugs that inhibit the HIV protease (see below), investigators launched efforts to develop drugs that inhibit the NS3 protease. As described below in more detail for the HIV protease inhibitors, the development

process was iterative involving the synthesis of candidate inhibitors, solving the structures of these inhibitors bound to the protease, and then designing new compounds with higher potency and better pharmacological properties based on those structures.^{213,266} Several features of the HCV NS3/4A protease and its inhibitors differ from those of their HIV counterparts, however. The HIV protease is an aspartyl protease, whereas the NS3/4A protease is a serine protease. The HIV protease's substrate binding pocket is deep, so that there are multiple opportunities for interactions with a small molecule inhibitor, whereas the NS3/4A binding pocket is shallow, with relatively few obvious sites for binding a small molecule. Despite this obstacle, structure-based medicinal chemistry efforts identified sites on NS3 that could mediate tight and selective binding of inhibitors (Fig. 13.5).^{213,266} The anti-HIV protease inhibitors are largely symmetrical, mimicking the protein substrate on both sides of the protease cleavage site, whereas the anti-NS3/4A inhibitors are relatively asymmetric, mainly mimicking the N-terminal product of protease cleavage. The anti-HIV protease inhibitors contain CHOH moieties that mimic the transition state of protease catalysis to achieve tight, but non-covalent binding to the enzyme (transition state analogs). In contrast, the anti-NS3/4A inhibitors contain ketoamide groups that react with the catalytic serine of the enzyme to form a covalent bond with the enzyme (Fig. 13.5), which only very slowly reverses, resulting in time-dependent inhibition of the enzyme (mechanism-based inhibitors) and inhibition constants of ~10 nM.^{213,266}

Both boceprevir and telaprevir exhibit submicromolar potencies (concentrations that inhibit replication by 50%) for inhibition of replication of HCV genomes in a cell-based replicon system.^{214,229} Interestingly, telaprevir was considerably less potent in these assays than a macrocyclic NS3/4A inhibitor, BILN 2061, which binds tightly but noncovalently; yet both drugs eliminated HCV replicons (10⁴-fold reductions) from cells at similar concentrations, which may relate to the very slow reversibility of telaprevir.²¹⁴ This observation helps to illustrate the concept that efficacy (how much viral replication is reduced) is likely to be more important than potency in antiviral development. Both boceprevir and telaprevir inhibit HCV genome replication, at least additively in combination with interferon α (IFN- α).^{214,229} These findings are important due to the use of boceprevir and telaprevir in combination with IFN- α for HCV therapy, and they may relate to a role for NS3 in counteracting IFN action.¹⁰⁹ Once a suitable cell culture system that supports complete replication of HCV was devised, it was shown that NS3/4A inhibitors block production of infectious virus.²¹⁵ Finally, as was first strikingly demonstrated with BILN 2061, NS3/4A inhibitors can drastically reduce levels of HCV in the plasma of patients.¹⁹⁷

HCV replication is rapid and error-prone; therefore, it is not surprising that resistance to NS3/4A protease inhibitors arises rapidly both in cell culture systems and in patients.⁸² Indeed, resistant variants preexist in untreated patients.¹⁴ The different subtypes of HCV complicate HCV drug resistance, with protease inhibitors having different potencies against different subtypes, and with different subtypes requiring different numbers of mutations to achieve resistance.¹³²

Several mutations confer resistance to boceprevir and telaprevir, with some altering NS3 residues that make direct contacts with drug and others altering more distant residues that

contact amino acids close to the drug, such as the catalytic serine.¹³² Certain mutations confer differing degrees of resistance to the two drugs. Notably, some mutations that confer resistance to boceprevir and telaprevir do not confer resistance to newer macrocyclic NS3/4A inhibitors that are in development (e-Table 13.1), and certain mutations that are resistant to the macrocyclic inhibitors do not confer resistance to boceprevir and telaprevir. However, other mutations confer resistance to both classes of NS3/4A drugs.¹³² The effects of the different mutations on fitness vary as measured in cell culture systems for genome replication and production of infectious virus or in human hepatocyte chimeric mice, with some mutations being clearly impaired and others having very little impact.^{158,334,363} Mathematical modeling of HCV infections in humans suggests that at least some protease inhibitor-resistant mutations retain substantial fitness.³¹⁶ These features of resistance to boceprevir and telaprevir are an impetus for continued development of anti-HCV drugs that can be used in combination (e-Table 13.1).

Inhibition of Viral Genome Replication

Most antiviral drugs inhibit viral genome replication, and nearly all of these inhibit a viral polymerase. Those viruses whose genome replication has been successfully targeted to yield FDA-approved drugs include certain human herpesviruses, the retrovirus HIV, and the hepadnavirus HBV. Several inhibitors of the polymerase of the flavivirus HCV are in clinical development. Most of the approved drugs are nucleoside analogs (Figure 13.6). Some of these nucleoside analogs actually mimic nucleoside monophosphates, so they are actually nucleotide analogs. A few approved drugs are nonnucleoside inhibitors of DNA polymerase or reverse transcriptase that act by binding at a site other than the dNTP-binding site, and one approved drug inhibits HIV integrase.

Nucleoside Analogs

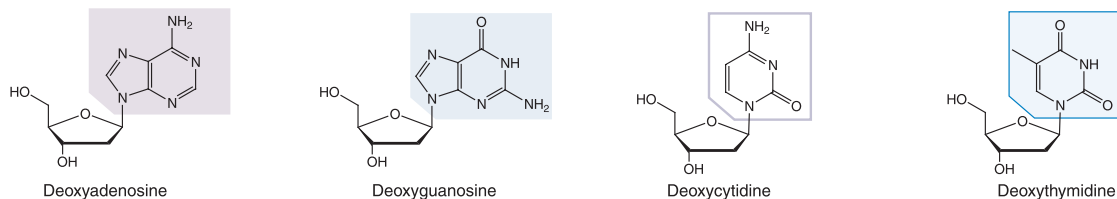
Nucleoside analogs that clearly act by inhibiting viral polymerases are approved for treatment of herpesviruses, HBV, and HIV, and several are in development for treatment of HCV. Another nucleoside analog, ribavirin, is used clinically against HCV and respiratory syncytial virus, but its mechanism of action against those viruses is not well understood (see below). All nucleoside and nucleotide analogs must be activated by phosphorylation, usually to the triphosphate form, to exert their effect. Phosphorylated nucleoside analogs inhibit polymerases by competing with the natural substrate (dNTP for DNA polymerase) and are usually also incorporated into the growing nucleic acid chain, where they often terminate elongation. Either or both of these features—inhibition and incorporation—can be important for antiviral activity.

The more efficiently cellular enzymes phosphorylate the nucleoside analog and the more potently the phosphorylated forms act against cellular enzymes, the more toxic the nucleoside analog will be. Selectivity, therefore, is dependent upon how much more potently and effectively viral genome replication is inhibited than are cellular functions, and for the herpesviruses, how much more efficiently viral enzymes phosphorylate the drug than do cellular enzymes.

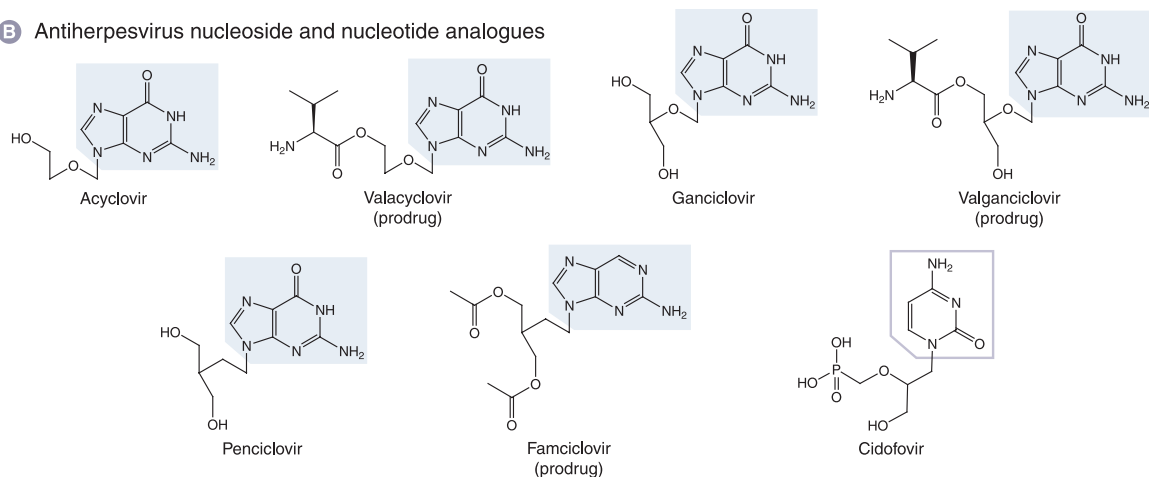
Anti-Herpesvirus DNA Polymerase Inhibitors

The various human herpesviruses encode both kinases and DNA polymerases. The kinases are usually not essential for viral

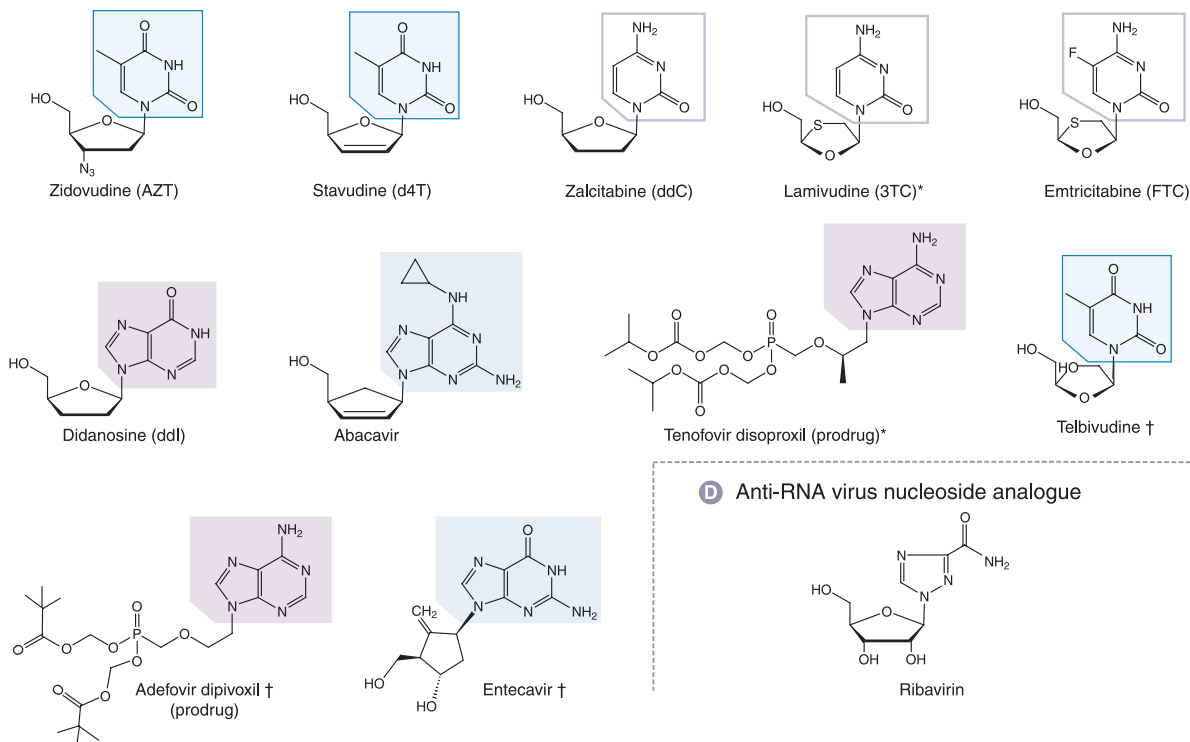
A Native nucleosides



B Antiherpesvirus nucleoside and nucleotide analogues



C Anti-HIV and anti-HBV nucleoside and nucleotide analogues



D Anti-RNA virus nucleoside analogue

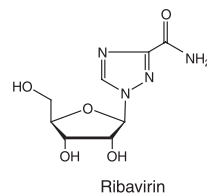


FIGURE 13.6. Structures of antiviral nucleoside analogues. **A:** The deoxynucleosides used as precursors for DNA synthesis are depicted in their *anti* configurations. The base moieties are shaded. **B:** Anti-herpesvirus nucleoside analogs mimic the deoxynucleoside, deoxyguanosine, except for cidofovir, which mimics the deoxynucleotide dCMP. The compounds shown here all contain acyclic moieties that mimic deoxyribose. Valacyclovir and famciclovir are prodrugs of acyclovir (ACV) and penciclovir, respectively. **C:** Anti-HIV (human immunodeficiency virus) and anti-HBV (hepatitis B virus) nucleoside analogs mimic dT, dC, dA, and dG except for tenofovir, and adefovir, which mimic dAMP. All of the analogs contain a base found in **A**, except for didanosine, which mimics deoxyinosine and is converted to dideoxyadenosine, and abacavir, which contains a cyclopropyl-modified guanine. Tenofovir and adefovir are shown as their disoproxil and dipivoxil prodrugs, respectively. Lamivudine, emtricitabine, and telbivudine are L-stereoisomers, whereas the other nucleoside analogs and dT, dC, dA, and dG are D-stereoisomers. All of the compounds shown except entecavir contain deoxyribose mimics that lack the equivalent of a 3'-hydroxyl, and thus are obligate chain terminators. The anti-HIV nucleoside analogs, lamivudine and tenofovir(*), and telbivudine, adefovir, and entecavir (†) are approved for use against HBV. **D:** Ribavirin, which contains a purine mimic attached to ribose, is approved for use against the RNA viruses hepatitis C virus and respiratory syncytial virus. (Modified from Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673.)

replication, but the catalytic subunits of the polymerases (Pols) are. These enzymes differ enough from their cellular counterparts to permit development of selective antiviral nucleoside analog.

A number of antiviral nucleoside analogs including vidarabine, idoxuridine, and trifluridine, were developed and used against HSV infections. However, these drugs have been superseded by more selective compounds, and their mechanisms will not be discussed further.

Acyclovir and Related Drugs

ACV is the paradigmatic antiviral nucleoside analog that illustrates many of the principles of nucleoside analogs. It consists of a guanine base attached to an acyclic sugar-like molecule (Fig. 13.6). ACV and its more orally available valine ester, valacyclovir, and the related drugs, penciclovir and its orally available derivative, famciclovir (structures in Fig. 13.6) have similar mechanisms of action against HSV and VZV. ACV was originally synthesized at Burroughs Wellcome as part of a program to discover adenosine deaminase inhibitors, and only later was it tested for antiviral activity. The mechanism of ACV action⁹¹ is presented in Figure 13.7. The TK encoded by HSV and VZV phosphorylates ACV, despite its differences from the natural substrate of this enzyme, thymidine (also known as deoxythymidine; see Fig. 13.6). Crystal structures of HSV TK in complex with thymidine or ACV^{29,40,387} show that the guanine moiety of ACV interacts with the same residues as does the thymine moiety of thymidine, albeit with differences in water-mediated binding and bond angles. Despite this, ACV is not a particularly good substrate for HSV or VZV TK. Nevertheless, no mammalian enzyme phosphorylates ACV nearly as efficiently as the HSV and VZV TKs. Accordingly, HSV- and VZV-infected cells contain 30- to 100-fold more phosphorylated ACV than do uninfected cells, which accounts for much of ACV's antiviral selectivity.

Phosphorylation of ACV by the viral TK produces the compound ACV-monophosphate, which is converted sequentially to ACV-diphosphate and ACV-triphosphate (ACV-TP) by cellular enzymes.^{252,253} ACV-TP then inhibits HSV and VZV DNA polymerases. Inhibition of HSV DNA polymerase *in vitro* is a three-step process (Fig. 13.7).³⁰⁰ In the first step, ACV-TP competitively inhibits deoxyguanosine triphosphate (dGTP) incorporation (high concentrations of dGTP can reverse inhibition at this early step). Next, ACV-TP acts as a substrate and is incorporated into the growing DNA chain opposite a C residue. Finally, the polymerase translocates to the next position on the template, but cannot add a new deoxynucleoside triphosphate because ACV contains no 3'-hydroxyl; hence ACV-TP is an obligate chain terminator. Provided that the next dNTP is present, the viral polymerase freezes at this final step in a "dead-end complex," leading to apparent inactivation of the enzyme. There is biochemical evidence for selectivity at each of these steps, especially the third.^{174,235,299} The results of enzymological studies of viral polymerases from ACV-resistant mutants¹⁶⁷ argue that in infected cells, as *in vitro*, ACV-TP is not simply a competitive inhibitor of viral DNA polymerase, but rather that its incorporation is crucial, consistent with the three step model (Fig. 13.7).

Stocks of most HSV-1 strains contain ACV-resistant mutants at a level of about 0.01% to 0.1% (reviewed in 57). For at least certain HSV-2 strains, mutation frequencies are an order of magnitude higher.³²³ Compared with the drugs

discussed so far, there is considerable variety in ACV-resistant mutants. ACV selects mainly for *tk* mutants^{60,324,354} that ablate (TK-negative) or reduce (TK-partial) TK activity or alter TK so that it fails to phosphorylate ACV, but continues to phosphorylate dT (TK-altered). ACV-resistance mutations can also alter the viral polymerase to be less inhibited by the drug (reviewed in 199). Crystallographic studies have provided details on how *tk* and *pol* mutations affect binding and catalysis.^{29,40,102,167,222,387}

Nearly all ACV-resistant HSV mutants exhibit some degree of attenuation in assays of pathogenesis in animal models (reviewed in 58 and 199). TK-negative mutants are generally the most attenuated, especially for replication in sensory ganglia and reactivation from latency in those ganglia. However, certain clinical isolates are able to reactivate despite being TK negative.^{123,164} TK-partial mutants are less attenuated. As little as 5% to 10% of wild-type levels of TK activity permit normal ganglionic replication and reactivation from latency,^{44,59} and very low levels of TK produced via these translational mechanisms can suffice to permit some reactivation from latency.^{19,123} Interestingly, certain nonsense and frameshift mutations that might be expected to inactivate TK do not do so. This "leakiness" can result from translational mechanisms, including reinitiation and ribosomal frameshifting, that permit low levels of TK despite these mutations.^{124,165,171,176} Therefore, several mutants that would be expected to be TK negative are actually TK partial. TK-altered and *pol* mutants include the most pathogenic drug-resistant mutants, but some *pol* mutants are highly attenuated.

This picture becomes even more complicated when mixtures of drug-resistant mutants with each other or with drug-sensitive virus are considered, as these can complement each other for both drug resistance and pathogenicity.^{58,199} In addition, *tk* frameshift mutations in homopolymeric sequences, which are the most common drug-resistance mutations, tend to revert, sometimes at remarkably high rates, resulting in mixed populations that reactivate from latency.^{122,124,326} Therefore, it is important to consider heterogeneity as a factor influencing drug resistance and pathogenicity.

Ganciclovir and Valganciclovir

Although ACV has been highly successful in treating HSV and VZV disease, it is much less potent against HCMV,⁷⁴ which causes serious diseases in immunocompromised persons. This is primarily because much less phosphorylated ACV accumulates in HCMV-infected cells than in HSV- or VZV-infected cells.¹⁰⁶ This helped spur the development of ganciclovir. Ganciclovir is much more potent against HCMV than is ACV^{47,336} and was the first drug approved for use against HCMV. Ganciclovir is now also produced as the more orally available agent valganciclovir (see Fig. 13.6).

Ganciclovir, unlike ACV, but like penciclovir, has the equivalent of a 3'-OH moiety (see Fig. 13.6). Much as phosphorylated ACV accumulates in HSV-infected cells, phosphorylated ganciclovir accumulates in HCMV-infected cells.^{21,104} However, as HCMV does not encode a TK, this phosphorylation was initially thought to be due to induction of a host cell kinase. However, studies of a ganciclovir-resistant mutant led to the discovery that an unusual HCMV protein kinase (UL97) phosphorylates ganciclovir in infected cells.^{152,219,248,350,356} Cellular kinases can convert ganciclovir monophosphate to ganciclovir triphosphate. Like ACV triphosphate, ganciclovir

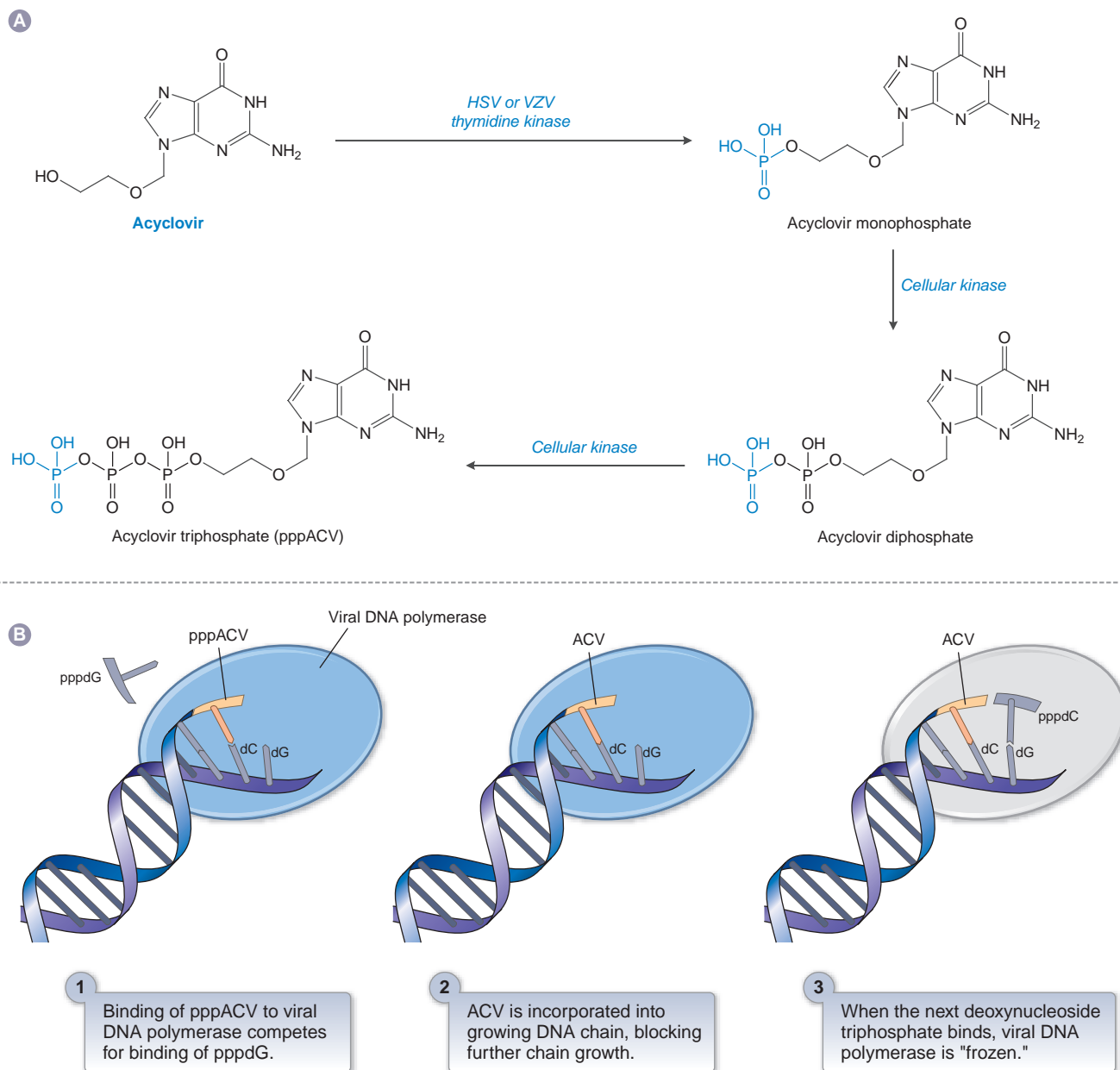


FIGURE 13.7. Mechanism of action of acyclovir (ACV). **A:** ACV is selectively phosphorylated by herpes simplex virus (HSV) or varicella zoster virus (VZV) thymidine kinase to generate ACV monophosphate. Host cellular enzymes then sequentially phosphorylate the drug monophosphate to the diphosphate and triphosphate (pppACV) forms. **B:** ACV triphosphate has a three-step mechanism of inhibition of herpesvirus DNA polymerase *in vitro*: (1) The drug triphosphate acts as a competitive inhibitor of deoxyguanosine triphosphate (dGTP) (pppdG) binding; (2) the drug triphosphate acts as a substrate and is incorporated into the growing DNA chain across from dC in the template, terminating elongation; and (3) the polymerase becomes trapped on the ACV-terminated DNA chain when the deoxynucleotide triphosphate (dNTP) binds (here shown as pppdC, dCTP, which would be templated by dG). (From Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673.)

triphosphate is both a selective competitive inhibitor and substrate for viral DNA polymerase.^{21,115,233,235,303,339} Unlike ACV triphosphate, ganciclovir triphosphate is not an obligate chain terminator. Nevertheless, after incorporating ganciclovir monophosphate, HCMV DNA polymerase can stall after incorporating one additional nucleotide.³⁰³ However, biochemical and genetic studies indicate that selectivity at

the phosphorylation step and the DNA polymerase inhibition step for HCMV are not as great as the selectivity of ACV against HSV. Accordingly, the drug is more toxic than ACV. Toxicity is most commonly manifested in patients as bone marrow suppression, especially neutropenia.²⁶⁷

Most ganciclovir-resistant mutants contain *UL97* mutations, but these have a limited distribution in the gene.^{11,118}

Unlike HSV TK, HCMV UL97 protein kinase is very important for viral replication (i.e., null mutants are much less fit²⁹⁶). It seems likely that clinical *UL97* drug-resistance mutations would affect recognition of ganciclovir without gravely compromising activity on the physiologic protein substrates of *UL97* such as retinoblastoma protein and lamin A.^{135,169,185} However, this has not yet been demonstrated. Numerous different *pol* mutations have been shown to confer ganciclovir resistance (reviewed in 11 and 118). Interestingly, some lie in or near regions not altered in ACV-resistant HSV mutants, but in regions that are thought to be important for 3' to 5' exonuclease activity. How these mutations confer ganciclovir resistance is not yet known.

Cidofovir and Other Nucleotide Analogs

Cidofovir, a phosphonate-containing acyclic cytosine analog, is approved for use against HCMV, and represents a variation on the mechanism of action of nucleoside analogs. Cidofovir, with its phosphonate group, is a nucleotide analog that mimics deoxycytidine monophosphate (dCMP) (see Fig. 13.6). This charged moiety likely accounts for the relatively poor uptake of cidofovir into cells,¹⁶¹ and poor oral bioavailability. Once inside the cell, cidofovir is metabolized via cellular enzymes to its monophosphate (akin to a diphosphate) and diphosphate (akin to a triphosphate), and to a third phosphorylated form that contains a choline adduct.^{52,161} These phosphorylated forms have very long intracellular half-lives, which may be due in part to the choline metabolite serving as a reservoir.^{1,161} This property contributes to the prolonged antiviral activity of cidofovir,²⁶⁰ which provides the therapeutic advantage of infrequent dosing. In addition, because cidofovir uses cellular kinases for its phosphorylation, it is active against *UL97* mutants that are resistant to ganciclovir,²²⁵ although this also removes the selectivity gained by requiring a viral kinase for efficient phosphorylation.

The diphosphorylated form of cidofovir is an analog of dCTP that inhibits HCMV DNA polymerase more potently than it does various cellular DNA polymerases.^{48,161,394} It is also incorporated into DNA, which slows elongation, but does not result in chain termination unless two cidofovir residues are incorporated in a row.^{393,394} Many of the *pol* mutations that confer resistance to ganciclovir confer resistance to cidofovir.

Two related phosphonate-containing drugs are the acyclic deoxyadenosine monophosphate analogues, tenofovir, which is used against HIV and HBV, and adefovir, which has been used against HBV (see Fig. 13.6). The mechanisms of action of these drugs against their respective viruses are similar to those of cidofovir against HCMV, except that these drugs are chain-terminators of DNA synthesis. (See below for discussions of nucleoside analogs active against HIV and HBV.) These two drugs are administered as orally available prodrugs (Fig. 13.6). In contrast, there is currently no such approved prodrug for cidofovir, which must be administered intravenously. Orally available prodrugs of cidovir are being developed with the added potential benefit of activity against poxviruses, adenoviruses, polyomaviruses, and papillomaviruses¹⁶⁶ (e-Table 13.1).

Nonnucleoside Inhibitors of Herpesvirus DNA Polymerase: Foscarnet

As described above, nucleoside analogs can inhibit cellular as well as viral enzymes, and viruses can mutate to resist these

drugs. As a result, efforts have been made to discover compounds that might inhibit viral polymerases by other mechanisms. The first of these to be approved for clinical use was foscarnet (phosphonoformic acid, PFA; structure in Fig. 13.8). Although it is not orally available so that intravenous administration is required, and it is nephrotoxic, it is approved for treatment of severe HSV, VZV, and HCMV infections that are resistant to front-line drugs.

Foscarnet is an analog of pyrophosphate, which is a product of polymerization of nucleic acids. Unlike the nucleoside analogs described above, it does not require activation by either cell or viral enzymes but rather inhibits HCMV DNA polymerase directly and selectively. Inhibition is not competitive with deoxynucleoside triphosphates. Rather, it appears that foscarnet acts as a product analog, preventing normal pyrophosphate release so the polymerase cannot complete the catalytic cycle.^{93,271} The structure of foscarnet bound to a DNA polymerase that is a chimera of a bacteriophage DNA polymerase and segments of HCMV polymerase that are important for foscarnet sensitivity has been solved.³⁹⁸ In the structure, the drug binds to highly conserved basic residues in a closed form of the enzyme that has not translocated to the next base on the template. The drug occupies the position of the beta and gamma phosphates of an incoming dNTP. The structure suggests that foscarnet stabilizes this untranslocated state, thus stalling the polymerase.

Because foscarnet is not a nucleoside analog, HSV *tk* mutants and HCMV *UL97* mutants are not resistant to it. However, although foscarnet inhibits DNA polymerase by a mechanism that differs substantially from the nucleoside analogs, many *pol* mutants that are resistant to nucleoside analogs are resistant to foscarnet. Moreover, most foscarnet-resistant mutants are resistant to one or more nucleoside analogs (reviewed in 118). Therefore, there are patients with serious herpesvirus infections for whom there are no viable treatment options. This should be an impetus to further drug development.

Anti-HIV and HBV Polymerase Inhibitors

The retrovirus HIV and the hepadnavirus hepatitis B virus (HBV) replicate their genomes through DNA and RNA intermediates, respectively, using a DNA polymerase that can copy RNA. The HIV RT and the HBV DNA polymerase are thus essential for viral replication, and differ substantially from cellular polymerases, making them excellent targets for antivirals. The success of anti-herpesvirus nucleoside analogs provided a rationale for the development of nucleoside analogs active against HIV. Within 2 years of the identification of HIV as the etiologic agent of AIDS, a nucleoside analog, zidovudine (AZT), was shown to have antiviral activity in patients.³⁹⁵ Shortly thereafter AZT therapy was shown to have an impact on mortality in the short term.¹⁰⁰ The success of AZT, but also its limitations, led to the development of other anti-HIV nucleoside analogs. These, in turn, provided an impetus for the development of nucleoside analogs against HBV, and, with the RT validated as a target, the nonnucleoside reverse transcriptase inhibitors (NNRTIs) against HIV.

Numerous nucleoside analogs have been approved to treat HIV (see Fig. 13.6). Many of the principles that govern their mechanisms of action and resistance are similar to those of the anti-herpesvirus nucleoside analogs. For the sake of brevity, this chapter focuses on only two anti-HIV nucleoside analogs,

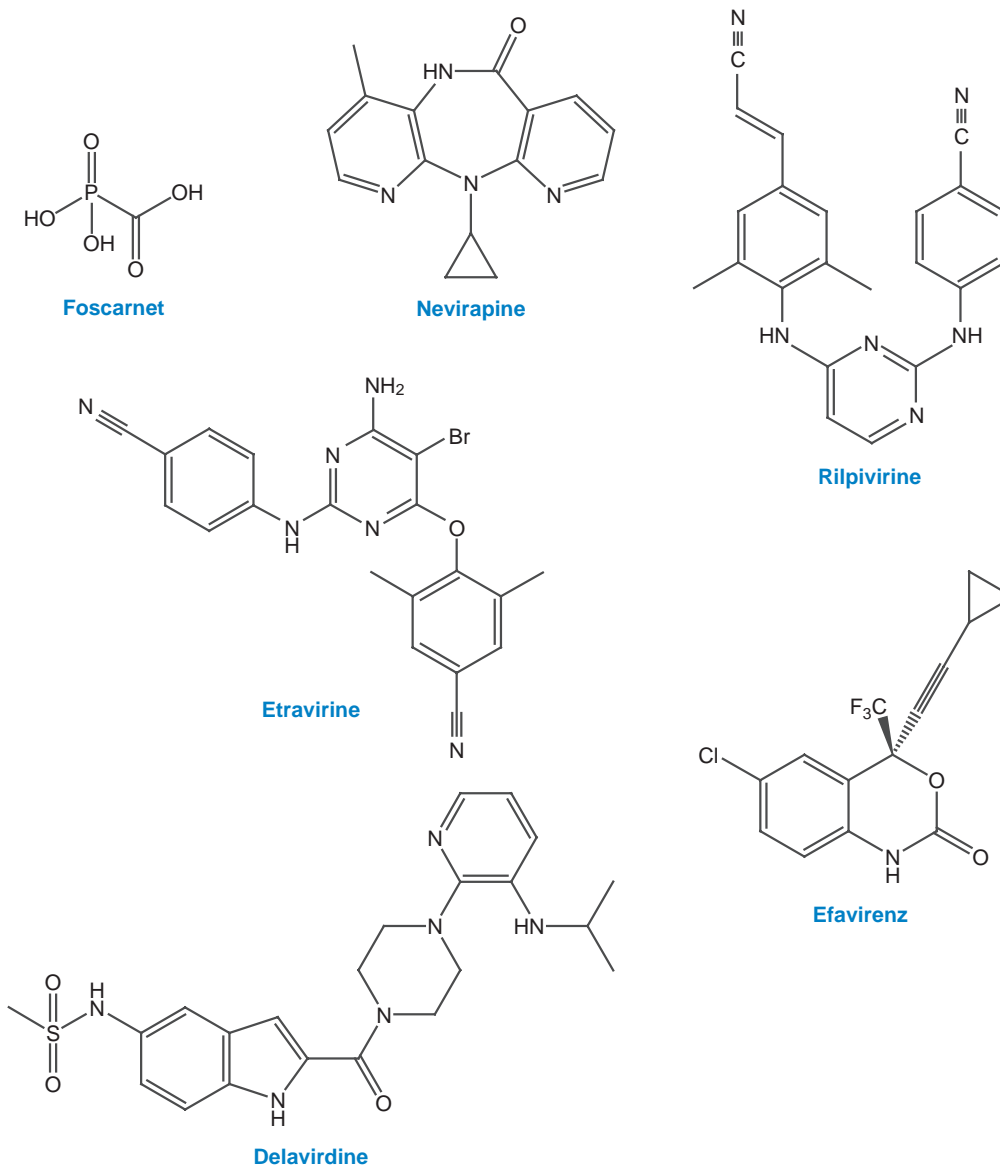


FIGURE 13.8. Nonnucleoside DNA polymerase inhibitors (foscarnet and nonnucleoside reverse transcriptase inhibitors [NNRTIs]). Foscarnet is a pyrophosphate analog that inhibits viral polymerases. It is approved for treatment of herpes simplex virus (HSV) and human cytomegalovirus (HCMV) infections that are resistant to other drugs. Nevirapine, delavirdine, efavirenz, etravirine, and rilpivirine, which do not mimic any known ligands of DNA polymerases, inhibit HIV-1 reverse transcriptase and are approved for treatment of HIV-1 in combination with other drugs. (Modified from Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673.)

zidovudine and lamivudine (which also is an anti-HBV drug), and compares and contrasts their mechanisms with each other and with the anti-herpesvirus drugs.

Zidovudine (AZT)

Zidovudine (azidothymidine, AZT) was synthesized years before the AIDS epidemic as a potential anticancer drug. It was first reported to have anti-HIV activity in 1985.²⁵⁵ Like the anti-herpesvirus drugs described earlier, AZT is a nucleoside analog with an altered sugar moiety. In this case, it is a thymine base attached to a sugar in which the normal 3' hydroxyl has

been replaced by an azido group (see Fig. 13.6). Indeed, all of the currently approved anti-HIV nucleoside analogs lack a 3'-hydroxyl or its equivalent. (This contrasts with penciclovir and ganciclovir.)

Unlike herpesviruses, HIV does not encode kinases that can phosphorylate nucleoside analogs. AZT is an excellent substrate for cellular thymidine kinase, which phosphorylates AZT to AZT-monophosphate.¹⁰⁷ AZT-monophosphate is then converted to the diphosphate form by cellular thymidylate kinase and to the triphosphate form by cellular nucleoside diphosphate kinase.¹⁰⁷ Therefore, unlike ACV and ganciclovir,

there is no selectivity at the activation step, and phosphorylated AZT accumulates in almost all dividing cells in the body, not just infected cells. Moreover, the activity of AZT and other anti-HIV nucleoside analogs can vary depending on the activity of cellular kinases in the infected cell. For example activated lymphocytes upregulate thymidine kinase, which is needed for DNA replication and cellular proliferation; thymidine analogs are thus more potent in activated than in quiescent CD4 lymphocytes.³³⁵

AZT-triphosphate (AZT-TP) is a substantially more potent inhibitor and better substrate of HIV RT than of the human DNA polymerases that have been tested.^{46,107,340} This biochemical selectivity is reflected in the degree of resistance of AZT-resistant mutants, which can be greater than 100-fold in some cell types.²⁰⁰ Like ACV triphosphate, AZT-TP lacks a 3'-hydroxyl, and is an obligatory chain terminator (see Fig. 13.6). The details of the inhibition of RT by AZT-TP are not entirely resolved, but given that AZT resistance acts on incorporated drug (see below), it is clear that the efficient incorporation of AZT-TP is crucial for its selectivity. Therefore, the potency of AZT and other nucleoside analogs depends on the levels of dNTPs that compete with the drug triphosphates for the RT.

Especially given that phosphorylated AZT accumulates in almost all dividing cells in the body, its toxicity is a seri-

ous clinical issue. In particular, AZT causes bone marrow suppression that manifests most commonly as neutropenia and anemia.³⁰⁸ AZT toxicity appears to be due not only to the effects of AZT-TP on cellular polymerases, but also to the effects of AZT-monophosphate, which is both a substrate for cellular thymidylate kinase, and an inhibitor of this essential enzyme.¹⁰⁷ For a number of other anti-HIV nucleoside analogs, a key determinant of toxicity appears to be the mitochondrial DNA polymerase (DNA polymerase γ).^{43,209,235}

The first AZT-resistant mutants of HIV were obtained from patients who had been treated with the drug for months.²⁰⁰ AZT-resistance mutations accumulate in the HIV *pol* gene that encodes RT^{24,53,187,203} (Fig. 13.9). Interestingly, four or more mutations are required to confer high-level resistance, but some of the mutations confer little or no resistance on their own.^{187,202,203} These latter mutations appear to be selected because they boost resistance by the other mutations or compensate for fitness costs of certain mutations. The requirement for multiple mutations for high-level resistance likely explains the relatively slow emergence of AZT resistance in patients and during selection in cell culture.^{111,201} Based on sequencing of virus isolated from patients following cessation of AZT therapy or in individuals newly infected with resistant virus, and of virus from replication competition experiments *in vitro*,

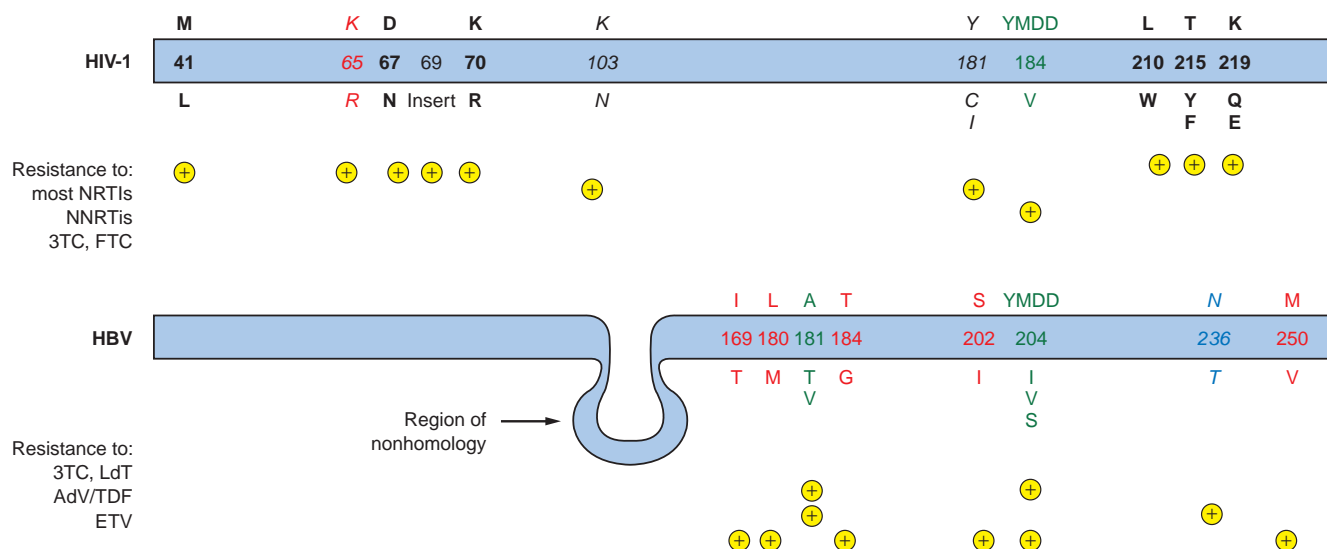


FIGURE 13.9. Locations of drug-resistance alterations in the human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) polymerases. The HIV-1 reverse transcriptase (RT) (**top**) and the HBV DNA polymerase (**bottom**) are related enzymes with sequence and predicted structural homology, and are cartooned as bars. The HBV enzyme has a segment that is unrelated to the HIV enzyme, which is depicted here as looping out. The positions of selected substitutions and insertions that confer drug resistance are indicated in single letter code with the wild-type residue above the bar, the residue number within the bar, and mutant residues or inserts below the bar. Methionine 184 in HIV RT and methionine 204 in HBV polymerase are embedded within a YMDD motif (green) that is important for polymerase catalysis. Alterations in this methionine confer resistance to 3TC and related L-stereoisomer drugs, as does alteration of alanine 181 in HBV polymerase (also green). Boldfaced residues in HIV RT indicate the positions of thymidine analog-associated mutations that affect susceptibility to all anti-HIV nucleoside analogues. An insertion at residue 69 in HIV RT (blue) together with other alterations including M41L, K70R, L210W, T215Y, and K219Q also affect susceptibility to these drugs. Substitution of arginine for lysine 65 (purple) in HIV RT confers resistance to all nucleoside analogs except zidovudine (AZT). Substitutions of lysine 103 and tyrosine 181 (*italics*) confer resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs). Alterations of isoleucine 169, threonine 184, serine 202, or methionine 250 (red), together with a leucine-to-methionine substitution at residue 180 (orange) plus a methionine 204 substitution in HBV polymerase can result in entecavir resistance. A threonine for asparagine 236 substitution in HBV polymerase (blue) can result in resistance to adefovir (high-level) and tenofovir (intermediate).

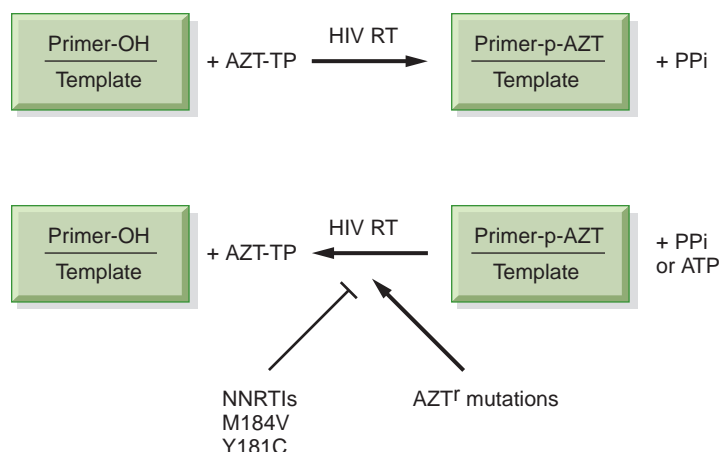


FIGURE 13.10. Biochemical mechanism of resistance to zidovudine (AZT). During synthesis of human immunodeficiency virus (HIV) DNA by HIV reverse transcriptase (RT), AZT-TP (triphosphate) is incorporated into the growing primer strand (Primer-p-AZT), generating pyrophosphate (PPi). This reaction is reversible, so that RT can excise AZT-MP (monophosphate) from the primer terminus and combine it with PPi, regenerating AZT-TP and primer/template. ATP can substitute for PPi in this excision reaction by donating two phosphates to AZT-MP. The excision reaction is enhanced by AZT-resistance mutations, but impaired by NNRTIs and certain HIV RT substitutions that confer resistance to 3TC (e.g., M184V) and NNRTIs (e.g., Y181C).

AZT-resistant mutants appear to be modestly less fit than wild-type virus.^{77,120,121,138,217}

The mechanism of AZT resistance remained elusive for over a decade. In 1998, two sets of investigators explained the mystery.^{5,249} Chain elongation by 3'-5' phosphodiester bond formation with an incoming dNTP is a reversible process. In the presence of pyrophosphate or ATP, RT can catalytically excise the terminal nucleotide by a process termed pyrophosphorolysis (Fig. 13.10). Given the K_m 's of pyrophosphate and ATP for this reaction, and the concentrations of these species in cells, ATP is the likely substrate *in vivo*. The viral DNA chain is now free to resume elongation with the removal of the chain terminating nucleoside analog. AZT-resistance mutations facilitate this reverse reaction of excision repair.^{210,250} Modeling of HIV RT (based on crystal structure) with an AZT-terminated primer/template suggests that the incorporated AZT residue would occupy the site normally used by the next incoming dNTP, and that AZT-resistance mutations, particularly those affecting codon 215, enhance binding of ATP.²⁵

AZT-resistance mutations are also selected by another thymidine analog, stavudine (d4T) (see Fig. 13.6), which has led them to be called *thymidine-associated mutations* (TAMS) (see Fig. 13.9). However, the resistance mechanism of excision repair actually applies to most, if not all, anti-HIV nucleoside analogs, with AZT-terminated primer being the most readily excised by this mechanism.³⁷⁶ Therefore, these mutations have also been termed *nucleoside-associated mutations* (NAMS). The excision repair mechanism accounts for many examples of cross-resistance of HIV toward various nucleoside analogs. Regardless of mechanism, various combinations of mutations affecting HIV RT can confer resistance to every approved nucleoside analog (see Fig. 13.9).¹⁸¹

The limited clinical effectiveness of AZT and problems with toxicity and resistance led to the development of other anti-HIV drugs and the use of combination chemotherapy.

Lamivudine (3TC)

Lamivudine (3TC) was reported to exhibit anti-HIV activity in 1992.^{54,55,329} Lamivudine and its close relative emtricitabine (FTC) appear to exhibit the least toxicity of the anti-HIV nucleoside analogs. This seems to be related to their highly unusual structures: They have an unusual sugar moiety that contains a sulfur atom and is an L-stereoisomer, not the standard D-stereoisomer of normal nucleosides (see Fig. 13.6). Lamivudine is sequentially

phosphorylated by cellular enzymes to its triphosphate.³⁷ Like AZT-triphosphate, 3TC-triphosphate is both a competitive inhibitor and a substrate for RT, and once incorporated, is an obligate chain-terminator of DNA synthesis. It is a more potent inhibitor of HIV RT than cellular polymerases.¹³⁹ The lack of toxicity of 3TC may be due to the negligible inhibition of host polymerases including mitochondrial DNA polymerase by 3TC-triphosphate.¹³⁹

High-level resistance to 3TC develops rapidly both in cell culture and in patients treated with this drug alone (reviewed in 79). A single mutation at codon 184 from Met to Val (M184V) or to Ile (M184I) confers a high degree of resistance to 3TC. M184 is within the conserved YMDD motif, in which the two Asp (D) residues are involved in catalysis of polymerization (see Fig. 13.9). M184I is less fit than M184V, which likely explains the dominance of the M184V mutant in 3TC-treated patients. Nevertheless, the M184V mutant is also less fit than wild-type virus, which may be due to decreased processivity, primer use, or initiation by the mutant enzyme or some combination of these (reviewed in 79). The crystal structure of a 3TC-resistant RT was used to develop a molecular model that posits that codon 184 mutations result in steric hindrance that obstruct incorporation of 3TC-triphosphate, but not normal nucleotides.³²⁰

The M184V mutation also has a number of other interesting effects, including conferring low level resistance to some (e.g. abacavir; see Fig. 13.6) or hypersensitivity to other nucleoside analogs (e.g. AZT and tenofovir; reviewed in 79). This hypersensitivity to AZT and other drugs in the absence of other mutations is also observed in the presence of known drug resistance mutations such that levels of resistance are reduced with the addition of M184V. This phenotypic suppression is best explained by the M184V mutation reducing the excision repair of AZT-terminated primers (Fig. 13.10).⁷⁹ Nevertheless, an accumulation of multiple mutations can result in high level resistance to both AZT and 3TC, and other single mutations such as K65R can confer resistance to 3TC (and most other nucleoside analogs) (see Fig. 13.9).²⁵¹

The mechanisms of action and resistance of FTC are very similar to those of 3TC; however, it may be slightly more potent and have a longer intracellular half-life.³⁰⁷ Its co-formulation with tenofovir has made this combination the nucleoside analog backbone of combination antiretroviral therapy.

Anti-HBV Drugs

Five nucleoside analogs—lamivudine (3TC) and tenofovir (both also approved for use against HIV), adefovir, telbivudine, and entecavir (see Fig. 13.6)—have been approved for the treatment of HBV. The structures of lamivudine, tenofovir, and adefovir were described above. Telbivudine is simply L-thymidine (see Fig. 13.6), thus sharing this isomeric configuration with 3TC and FTC. Entecavir is an unusual deoxyguanosine nucleoside analog in which the ether oxygen of the sugar is replaced with an exo carbon-carbon double bond (see Fig. 13.6). The mechanisms of action of anti-HBV nucleoside analogs are very similar to those of anti-HIV nucleoside analogs: They are converted to triphosphates by cellular enzymes, and the triphosphates inhibit HBV DNA polymerase (which is also a reverse transcriptase). All are incorporated into the growing DNA chain and cause chain termination, even those that are not obligate chain-terminators. Of note, entecavir-triphosphate has a higher affinity for HBV polymerase than does dGTP.³³¹

The similarities in mechanisms of action of these drugs against the two viruses extend to some similarities in mechanisms of resistance, but there are also important differences.²²³ For example, a mutation in the methionine codon in the HBV *pol* gene that corresponds to codon M184 in the HIV *pol* gene can confer high-level resistance to 3TC and telbivudine (see Fig. 13.9); however, in this case, the methionine to isoleucine mutant retains fitness, whereas methionine to valine or serine substitutions are usually only found in the presence of additional mutations. There is little if any evidence for HBV drug-resistance mutations resulting in increased excision of incorporated drug as do AZT-resistance mutations of HIV; rather the mutations appear to affect incorporation of drug-triphosphate by various mechanisms. Interestingly, drug resistance that requires only a single mutation develops relatively quickly, but still more slowly *in vivo* with HBV than with HIV. In at least some cases, this may be because sites of mutation also encode residues in an overlapping reading frame that affect HBV surface antigen.

As is the case with AZT resistance, multiple mutations are required to confer high level resistance to entecavir, and some of these mutations confer little resistance on their own.²²³ This correlates with slow development of resistance *in vivo*. The greater potency and slower rates of resistance to entecavir and tenofovir has led to these drugs becoming preferred for treatment of HBV.²²³ FTC and tenofovir are often used in patients who are infected with both HIV and HBV, because they are active against both viruses (even though FTC is not currently FDA approved for treating HBV infections).

Anti-HIV Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Five NNRTIs—efavirenz, nevirapine, delavirdine, etravirine and, in 2011, rilpivirine (see Fig. 13.8)—have been approved by the FDA for treatment of HIV infections. The first NNRTIs were discovered using high-throughput screening for inhibition of HIV-1 RT (e.g. nevirapine²⁴⁶). Candidate compounds were tested for specificity in a counterscreen by checking their ability to inhibit an unrelated polymerase. The compounds that emerged were then chemically modified to improve their antiviral activities, stabilities, and pharmacokinetics, and to reduce their toxicities. The NNRTIs are highly specific, inhibiting HIV-1 RT at low concentrations while not inhibiting

human DNA polymerases or even the RT of the closely related virus HIV-2.²⁴⁶ This biochemical selectivity is reflected by the high level resistance conferred by HIV-1 *pol* mutations.³⁰⁵ These drugs directly inhibit RT. The NNRTIs do not mimic a natural ligand of a polymerase. Each NNRTI appears to share a polycyclic structure that tends to form a “butterfly wings” conformation upon binding to RT. NNRTIs bind to a pocket near the catalytic Asp residues of RT.¹⁹³ This pocket is not observed in structures of RT in the absence of drug, but rather is stabilized by drug binding.³¹⁴ Therefore, NNRTI binding causes a substantial conformational change in RT, including distortion of the catalytic Asp residues, and slows the enzyme’s incorporation of dNTPs into DNA.^{313,338} NNRTIs inhibit pyrophosphorolysis and the excision of AZT from primer termini by RT (see Fig. 13.10;²⁶⁹), which likely accounts for synergy of these drugs against HIV.³⁰⁴

As with 3TC, resistance to NNRTIs develops quickly both *in vitro* and in patients, with single amino acid substitutions conferring high-level resistance.^{243,305,310} Common resistance mutations arise in codon 181 (see Fig. 13.9), changing Tyr to Cys (Y181C) or to Ile (Y181I), and cause high-level resistance.^{243,305} This change alters a residue known to interact with NNRTIs,^{62,193,392} and thus reduces drug binding. The Y181C mutant is relatively fit in infected cells,^{63,173} and in patients. Some but not all other NNRTI-resistant mutants exhibit reduced fitness in cells and in patients.²¹⁷ The Y181C mutation, like the M184V mutation, sensitizes otherwise AZT-resistant mutants to AZT due to suppression of excision repair (see Fig. 13.10;³³²).

The different mechanisms of action and resistance of the anti-HIV nucleoside analogs and NNRTIs help provide the rationales for various combinations of these drugs in treating HIV (see section on HIV therapies).

Ribavirin, a Nucleoside Analog Active Against RNA Viruses

Ribavirin (see Fig. 13.6) is a nucleoside analog that has been touted as a “broad spectrum antiviral”.³⁴⁸ Indeed it exhibits activity against many viruses *in vitro* and efficacy against certain RNA viruses *in vivo*. In patients, however, it has been approved only as monotherapy for severe respiratory syncytial virus (RSV) infections, and in combination with IFNs for chronic HCV infections (see below for discussion of IFNs). Ribavirin monotherapy results in little reduction in HCV levels in plasma,⁷⁸ but when added to IFNs, substantially increases the success rate of therapy.²⁹³

Structurally, ribavirin differs from the other nucleoside analogs discussed thus far, in that it has a normal sugar moiety (ribose) attached to a base-like moiety, which most resembles a purine (see Fig. 13.6). Its mechanism of action is still not well understood, especially for HCV. Ribavirin is converted to a monophosphate by cellular adenosine kinase and is known to inhibit cellular inosine monophosphate dehydrogenase (IMPDH), thereby lowering GTP pools.³⁴⁸ This could, in principle, confer selective antiviral activity if certain viral functions have higher K_m values for GTP than do most cellular enzymes. Inhibitor and RNA interference experiments suggest that IMPDH inhibition could be important for the anti-HCV activity of ribavirin.^{261,405} A second possible selective mechanism for ribavirin action could be inhibition of viral RNA polymerase. A related possible mechanism, called “error catastrophe,”

is that ribavirin incorporation by viral RNA polymerase increases the already high mutation rate of the virus⁸⁷ “over the edge” of an “error threshold” so that few or no functional viral genomes are produced. This has been investigated most thoroughly with poliovirus,^{72,73,284} but similar studies have been performed with HCV.^{68,198,226,405} This is an appealing concept, but remains unproven, particularly for RSV and HCV. In HCV replicons, ribavirin resistance was found to be due to mutations affecting the NS5A protein rather than polymerase,²⁸⁵ and studies of viral mutagenesis in patients treated with ribavirin have not provided definitive answers.^{227,281} A fourth possible mechanism is that ribavirin induces IFN-stimulated genes, which may be particularly relevant to the use of ribavirin in combination with IFNs.^{96,361} A fifth possible mechanism involves stimulatory effects on T helper cell 1 responses involved in clearing virus from the liver.¹⁶² If anything, there is even less known about the mechanism of ribavirin action against RSV. Further investigations of the mechanisms of ribavirin action and resistance may yield interesting new insights into virus biology and biochemistry.

New nucleoside analog and nonnucleoside inhibitors of viral polymerases are being developed. For example, as of late 2012, several such inhibitors of HCV RNA polymerase were in phase II clinical trials (e-Table 13.1). Therefore, this area of antiviral agents remains highly active.

Anti-Human Immunodeficiency Virus Integrase Inhibitors

A crucial stage in the replication of HIV and other retroviruses is integration of the linear, dsDNA product of reverse transcription into the host genome. The protein that carries out this step, integrase, is an essential enzyme. Integrase assembles onto sequences from the ends of HIV DNA, cleaves dinucleotides from each 3' strand, and transfers these strands to covalently link them with target DNA (Fig. 13.11). The catalytic core of integrase includes three conserved acidic residues called the DDE motif (Fig. 13.11), which binds Mg^{2+} that is essential for integrase activity. Efforts to identify specific inhibitors of HIV integrase that could serve as effective antiviral drugs commenced in the early 1990s. A breakthrough came when Daria Hazuda and her colleagues at Merck developed an assay selective for DNA strand transfer.¹⁵¹ Using this assay, they identified integrase inhibitors that act much more potently at this step than at prior steps (Fig. 13.11). These inhibitors contained a diketoacid moiety, which binds to the metal coordinated by the DDE motif, and additional moieties that bind to the enzyme. Medicinal chemistry studies of various classes of diketoacid scaffolds led to the development of raltegravir (Fig. 13.11).³⁵¹ Raltegravir was tested against a variety of enzymes that are Mg^{2+} -dependent (e.g., HIV RT), and was highly selective for integrase. Its selectivity for the strand-transfer step seems to depend on binding to a conformation of integrase that is stable only after processing of 3' ends. Indeed, viral DNA may form part of the binding site for the drug.^{2,45,238}

Raltegravir inhibits HIV replication at low nanomolar concentrations. As expected, HIV mutants resistant to raltegravir contain mutations in sequences encoding the integrase, with most mutations that confer resistance altering residues close to the inhibitor binding site.²³⁸ Of mechanistic interest, one mutation alters a residue that, in the crystal structure, makes contact with a nucleotide corresponding to the 5' terminus of

viral DNA. There are also mutations that do not themselves confer resistance, but increase the effects of other mutations. Similar mutations are selected by raltegravir in cell culture and in patients. Single mutations seem to be sufficient to confer clinically significant resistance, but most of these mutations decrease HIV fitness at least modestly.²³⁸

In 2012 elvitegravir, another integrase inhibitor, was approved, and dolutegravir, which is active against many raltegravir-resistant mutants, was in late-stage clinical trials. It seems likely that this class of drugs will become increasingly important for HIV therapy.

Inhibition of Viral Assembly and Maturation Protease Inhibitors

Virus assembly and subsequent events to form an infectious virion are attractive targets for drug discovery because they are unique to virus biology. For many viruses, including HIV, mere assembly of proteins and nucleic acid into particles is not sufficient to produce an infectious virion. For such viruses, an additional step—maturation—is required. In most cases, these viruses encode proteases that are essential for maturation. The approved antiviral drugs that target HIV protease are saquinavir, ritonavir (which is now used mainly at low dose to boost plasma drug levels of other protease inhibitors), lopinavir, amprenavir and its prodrug fosamprenavir, indinavir, nelfinavir, atazanavir, darunavir, and tipranavir (Fig. 13.12).

Several features gleaned from a variety of studies established HIV protease as an attractive target for drug discovery and design. Molecular genetic studies showed that the protease is essential for HIV replication, and that a point mutation is sufficient to inactivate the enzyme and viral infectivity, suggesting that a small molecule could be effective.¹⁹² The cleavage sites in the viral polypeptide substrates of HIV protease are conserved and somewhat unusual, suggesting both specificity and a starting point for drug design.^{283,292} Unlike the human proteases most closely related to it, HIV protease is a symmetric homodimer, and each subunit contributes to the active site, again suggesting both specificity and a starting point for drug design.²⁶⁵ The enzyme can be easily overexpressed and assayed, and its crystal structure has been solved,^{263,389} all aiding drug discovery.

The HIV protease inhibitors are paradigms of rational drug design. For simplicity, only the development of ritonavir will be detailed. Ritonavir and the other protease inhibitors are peptidomimetics. Indeed, all but tipranavir retain peptide bonds. Ritonavir's design began with the recognition that a natural substrate of the protease contains a phenylalanine-proline (Phe-Pro) bond; mammalian enzymes rarely if ever cleave at such a site. The transition state of this substrate during cleavage was then modeled (Fig. 13.13, top). Knowing that HIV protease is a symmetric dimer, an analog of the transition state was modeled, using the same residue, phenylalanine, on both sides, and with a CHOH group that mimics the transition state as the center of symmetry. This molecule, A-74702, was a very weak inhibitor of HIV protease, but adding groups symmetrically to both ends to form A-74704, resulted in a greater than 40,000-fold increase in potency ($K_i \sim 5$ nM).⁹²

Attempts to modify A-74704 to improve solubility reduced potency, so a related potent inhibitor, A-75925, in which the center of symmetry was a C-C bond between two CHOH groups, became the scaffold for further additions to both ends.¹⁹⁰ This resulted in a soluble, highly potent inhibitor,

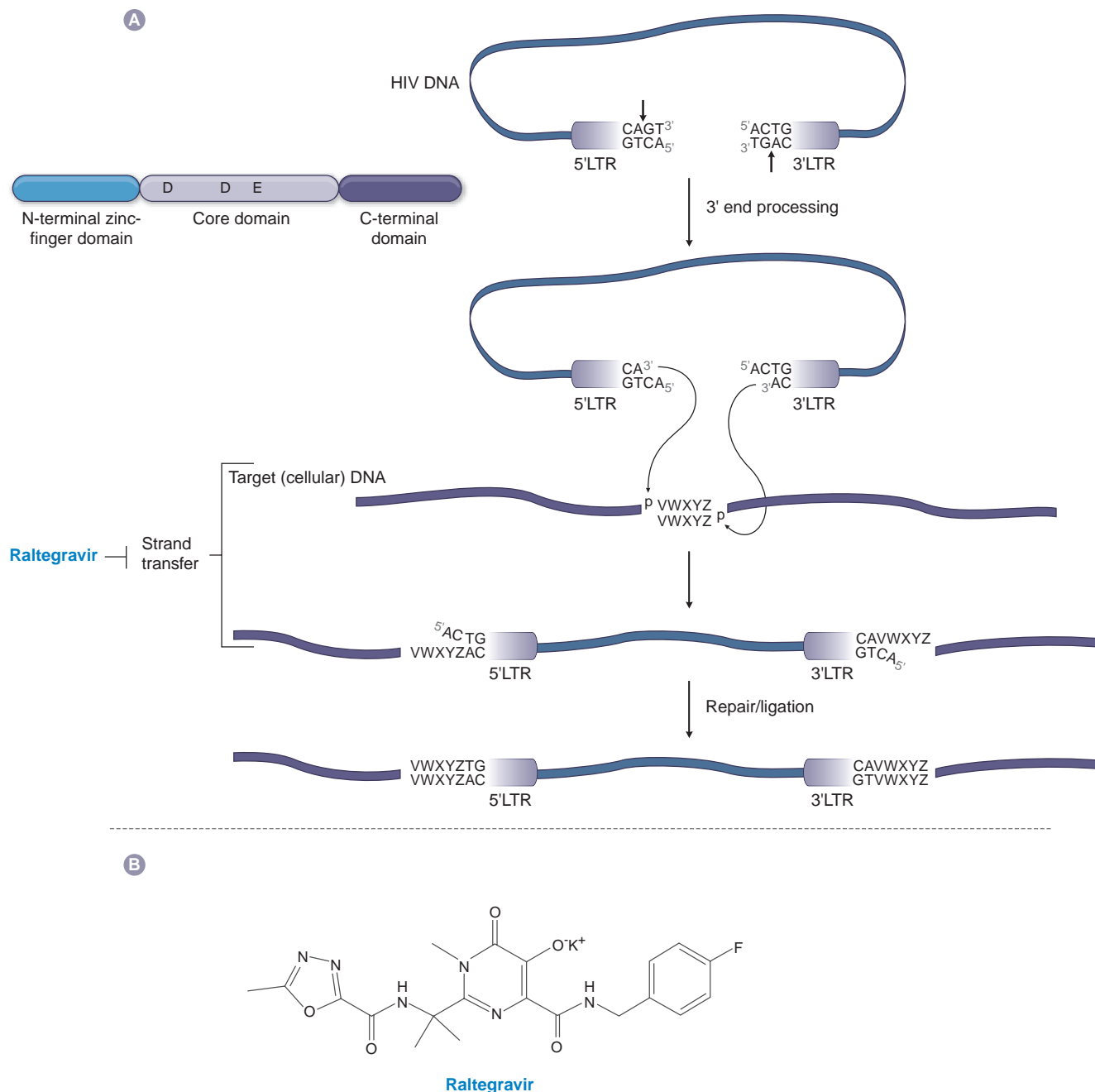
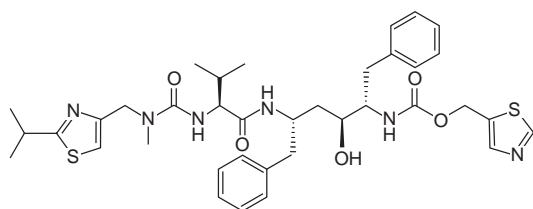
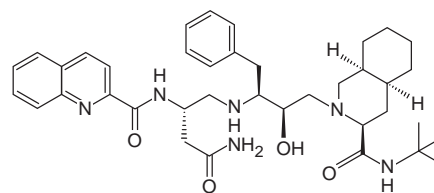


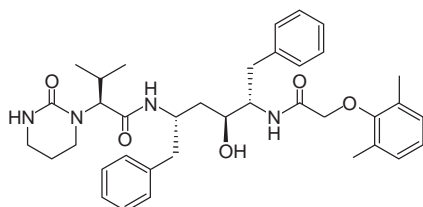
FIGURE 13.11. Human immunodeficiency virus (HIV) integrase and raltegravir action. **A:** Linear, double-stranded HIV DNA, generated by reverse transcription, contains a four-base sequence at each of its ends, as part of its long terminal repeat (LTR). Integrase (IN), which contains an N-terminal zinc-finger domain important for DNA binding, a C-terminal domain, and an internal core domain containing three acidic residues (DDE) that coordinate magnesium ions important for catalysis, binds to HIV DNA (as part of a larger complex) and cleaves a dinucleotide from the 3' strand at each end of viral DNA (3' end processing). The integrase-viral DNA complex then catalyzes the attack of the recessed 3' ends of viral DNA on phosphodiester bonds of target (cellular DNA) (strand transfer). The product results in 5' flaps of viral DNA and gaps in cellular DNA. Integrase releases, and host enzymes remove the flaps and repair the gaps, resulting in integrated viral DNA flanked by duplicated host sequences. Raltegravir inhibits the strand transfer step of this process. (Modified from Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins, 2012:649–673 and Freed EO, Martin MA. HIVs and their replication. In: Knipe DM, Howley PM, Griffin DE, et al., eds. *Fields Virology*. Fifth edition. Philadelphia: Lippincott Williams & Wilkins, 2007.) **B:** Structure of raltegravir.



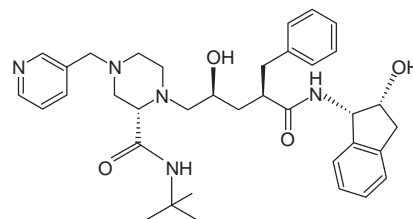
Ritonavir



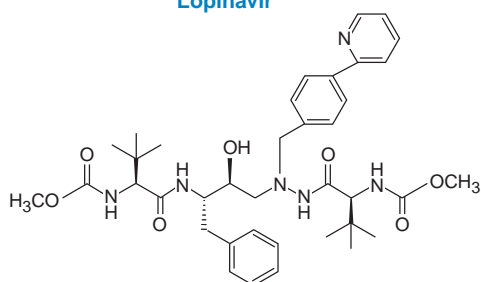
Saquinavir



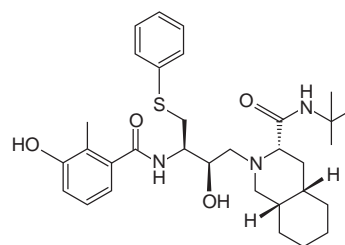
Lopinavir



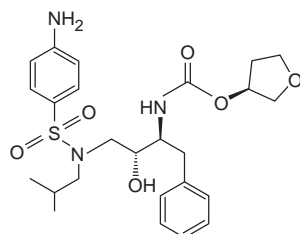
Indinavir



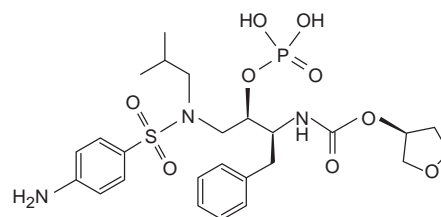
Atazanavir



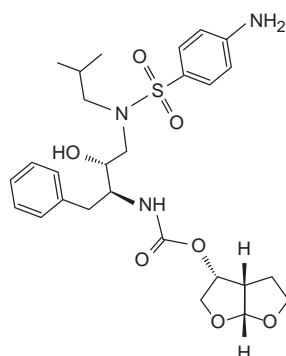
Nelfinavir



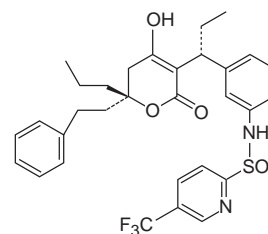
Amprenavir



Fosamprenavir



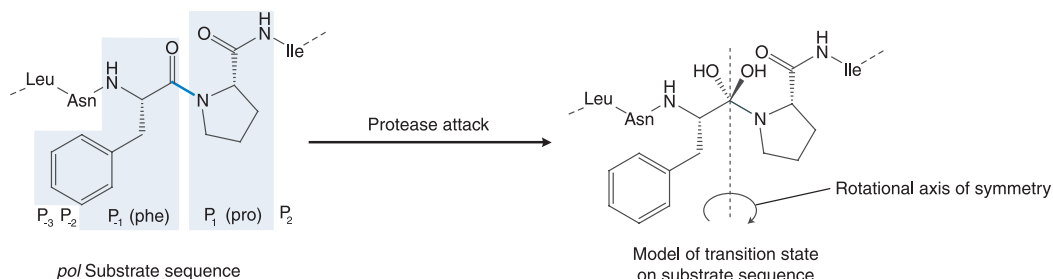
Darunavir



Tipranavir

FIGURE 13.12. Anti-HIV (human immunodeficiency virus) protease inhibitors. The structures of approved anti-HIV protease inhibitors ritonavir, saquinavir, lopinavir, indinavir, atazanavir, nelfinavir, amprenavir, its prodrug, fosamprenavir, darunavir, and tipranavir are shown.

A



B

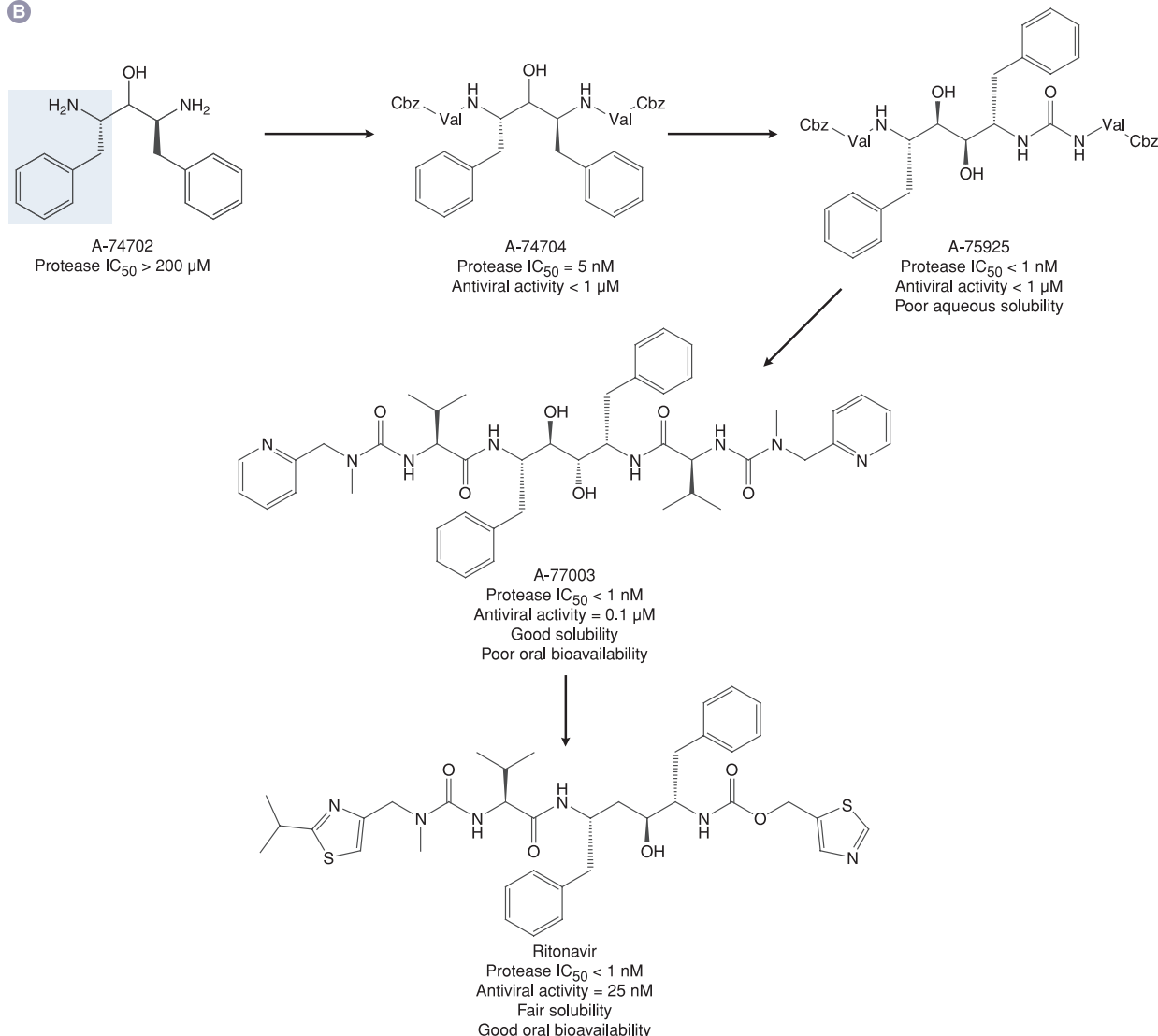


FIGURE 13.13. Steps in the development of ritonavir. **A:** On the left is shown a substrate sequence that is cleaved by human immunodeficiency virus (HIV) protease between a phenylalanine (phe) on the N-terminal side of the cleavage (P_1) and a proline (pro) on the C-terminal side (P_1). On the right, the transition state of this sequence during protease cleavage is modeled. The transition state contains a rotational axis of symmetry **B:** Structure-based development of ritonavir began with A-74072 (**top left**) that mimics the transition state with a single CHOH moiety between two phenylalanines (to maximize symmetry). This compound, which had weak inhibitory activity, was modified by symmetric addition of other groups to yield A-74074, which was more potent (CBZ, carboxybenzyl), but was insufficiently soluble. This was modified to mimic the transition state with CHOH groups on each side of the axis of symmetry (A-75925, **top right**). This was also poorly soluble, but could be improved by adding more groups (A-77003, **middle**). That compound was not very orally available, so additional modifications, including the removal of a central OH group, were made, resulting in ritonavir (**bottom**). The choices of modifications were aided by crystal structures of intermediate compounds bound to HIV-1 protease. For each compound, the inhibitory potencies against HIV-1 protease *in vitro* (IC_{50}) and against HIV in infected cells (antiviral activity) are provided.

A-77003 (Fig. 13.13, bottom). However, this compound was not absorbed orally.¹⁸⁹ It was modified by removing a central OH group and altering other moieties at each end (Fig. 13.13, bottom). This resulted in less solubility, but improved antiviral activity and good oral bioavailability.¹⁸⁸ These improvements took advantage of the x-ray structure of HIV protease complexed with each version of the inhibitors (reviewed in **4 and 388**). By examining these structures, chemists were able to make informed guesses about what groups to add or subtract. The result was ritonavir.

Crystallographic studies reveal that upon binding of protease inhibitors, the enzyme assumes a conformation with closed “flaps” that are unable to undergo conformational changes that help form the active site (reviewed in **4 and 388**). The drugs work in cell culture as expected: HIV-infected cells exposed to protease inhibitors continue to make viral proteins, but these proteins are not processed efficiently.⁷⁰ Viral particles bud from the infected cells, but they are immature and noninfectious.¹¹⁴ Mutations conferring drug-resistance map to HIV sequences encoding the protease, confirming that protease is the target.²⁷²

Resistance to protease inhibitors has taught us a great deal about both the drugs and the virus.^{23,66,181} Mutations in more than 30 of the 99 amino acid residues of protease have been selected with the different protease inhibitors, with as many as 24 conferring resistance to any one drug. Some of the mutations affect the active site or sites known to interact with the inhibitors, and thus the mechanism of resistance can be readily understood. However, other mutations affect more distal sites, and it is not clear how they confer resistance. Resistance is often cumulative. In some patients, after failing multiple regimens containing protease inhibitors, more than 20 of these mutations have accumulated in their virus, demonstrating the remarkable plasticity of this enzyme.^{159,181} There is even an example of a virus whose replication is enhanced by subinhibitory concentrations of a protease inhibitor.²³⁷

Different protease inhibitors tend to select different mutations, at least initially. Therefore, although the drugs all work by similar mechanisms, the first mutation selected for resistance to one protease inhibitor often does not confer resistance to another. This can be understood by considering that although the different drugs tend to share similar functionalities that interact with the protease active site, each drug has a different functionality that confers high affinity binding via interactions with a particular site specific to the drug. Therefore, a single mutation that interferes with binding of one drug will not necessarily affect binding of the other. As mutations accumulate, however, cross-resistance develops among the protease inhibitors. Mutations affecting protease cleavage sites can also confer resistance.⁸⁶ These result in more easily cleaved precursor substrates for the protease. Different protease mutations also have differing effects on viral fitness. Certain mutations can be readily found in untreated patients, suggesting high fitness, whereas others are not detected except following drug treatment, and often decrease fitness. Cleavage site mutations can improve the fitness of protease mutants.^{86,230,399} These different features of resistance to protease inhibitors have important clinical implications (see section on anti-HIV therapy).

Inhibition of Viral Release: Influenza Virus Neuraminidase Inhibitors

Inhibitors of influenza virus neuraminidases block viral release of influenza A and B viruses. The rationale for their action

follows from the mechanism of viral attachment. Influenza attaches to cells via interactions between the virion hemagglutinin and sialic acid, which is present on many cellular membrane glycoproteins. However, upon egress of influenza virus from cells at the end of a round of replication, hemagglutinin on nascent virions again binds to sialic acid and tethers virions, preventing release and initiation of new rounds of infection. To overcome this problem, influenza virus encodes a virion neuraminidase, which cleaves sialic acid from the membrane glycoproteins. Studies using influenza virus mutants or an early neuraminidase inhibitor showed that without neuraminidase, the virus remains tethered and cannot spread to other cells.^{220,275,276} In 1992, structures of the neuraminidase-sialic acid cleavage product were solved.^{35,369} The structure showed that sialic acid occupied two of three well-formed pockets on the enzyme. Based largely on this structure, a new sialic acid transition state analog was designed to make energetically favorable interactions in all three of the pockets, principally by adding a guanidino group³⁷⁰ (Fig. 13.14). This compound, zanamivir, inhibits neuraminidase with a K_i of about 0.1 nM. Zanamivir is active against both influenza A and influenza B viruses with potencies of about 30 nM. However, it is not orally bioavailable, and must be administered as an aerosol.

Efforts to obtain a neuraminidase inhibitor with better pharmacokinetic properties than zanamivir resulted in oseltamivir (Fig. 13.14).^{191,211} Oseltamivir makes use of a carbocyclic ring based on a transition state analog of sialic acid. Rather than use a guanidino group to occupy a third pocket, it makes stronger contacts with one of the two pockets bound by sialic acid by adding a hydrophobic group (Fig. 13.13). Oseltamivir's high oral bioavailability depends in part on its being an ester prodrug, which when cleaved gives rise to the active carboxylate (Fig. 13.14).

Most neuraminidase resistance mutations result in an enzyme that is less inhibited by the drug.²⁴⁵ As would be expected by the differences in drug binding to the enzyme (Fig. 13.14), mutants resistant to one drug are not necessarily resistant to the other. This phenomenon gained worldwide notice when a human who became ill with virulent, avian H5N1 influenza A virus despite prophylaxis with oseltamivir was found to harbor oseltamivir-resistant, but zanamivir-sensitive virus.²²⁸ Still, compared with amantadine, for many years it was relatively difficult to generate mutants resistant to zanamivir or oseltamivir in the laboratory, and they appeared less frequently in the clinic. This was due in part to most such mutants, such as those containing the H275Y substitution that confers oseltamivir-resistance, being less fit than wild-type virus.¹⁷⁷ Indeed, the oseltamivir-resistant H5N1 just mentioned replicated less well in ferrets than did a drug sensitive virus from the same patient.²²⁸

However, during the 2007–2008 influenza season, virulent, readily transmitted H275Y viruses began to appear among seasonal H1N1 isolates, even in the absence of oseltamivir therapy.¹⁴⁶ These viruses containing Y275 appear just as fit *in vitro* as controls containing H275.^{22,298} The fitness of these H1N1 mutants can be attributed to permissive secondary mutations that allow increased expression of the H275Y mutants on the surface of the infected cell.²² As described in the Overview section of this chapter, another class of mutants resistant to the neuraminidase inhibitors contains altered hemagglutinins.

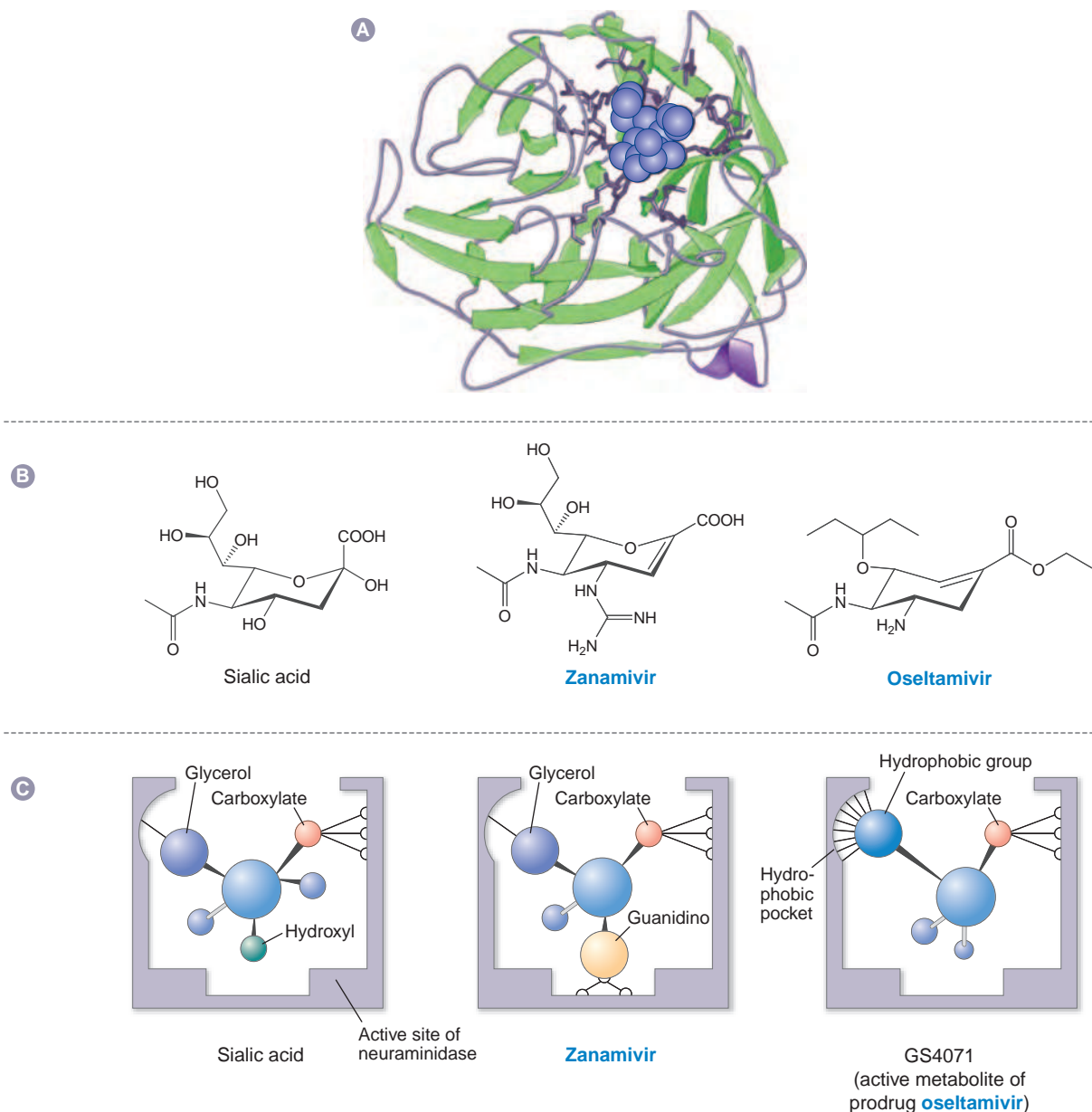


FIGURE 13.14. Structure-based design of inhibitors of influenza A and B virus neuraminidase. **A:** Shown is a model of sialic acid (space-filling structure in dark purple) bound to the influenza neuraminidase (green and light purple), with the amino acids that bind sialic acid shown in stick form. This structure was used to design transition state inhibitors that bind with higher affinity than sialic acid. **B:** Structures of sialic acid and the neuraminidase inhibitors oseltamivir and zanamivir. **C:** Diagram of the active site of influenza virus neuraminidase, showing the binding of sialic acid, zanamivir, and GS 4071 (oseltamivir is the ethyl ester of this compound), showing how the different ligands interact with pockets in the active site. Note that the compounds in **C** are flipped 180 degrees relative to how they are shown in **A** and **B**. (A and B, from Yeh RW, Coen DM. Pharmacology of viral infections. In: Golan DE, Tashjian AH, Jr., Armstrong EJ, et al., eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. Third edition. Philadelphia: Lippincott Williams & Wilkins; 2012:649–673. **C**, modified from Laver WG, Bischofberger N, Webster RG. Disarming flu viruses. *Sci Am* 1999;280:78–87.)

Other neuraminidase inhibitors are in clinical development. One of these, peramivir, is approved in Japan and Korea, but not yet in the United States (e-Table 13.1). Nevertheless, in 2009, it was authorized for emergency use in severe cases of pandemic H1N1 influenza in hospitalized patients, because it

can be administered intravenously. The H275Y mutation confers resistance to peramivir, limiting its use against oseltamivir-resistant virus.

With increasing concerns regarding influenza pandemics, the mechanisms of action and resistance of neuraminidase

inhibitors have become very important, as has the development of new anti-influenza drugs that are active against mutants resistant to neuraminidase inhibitors.

Antiviral Therapies That Target Immune Processes

Two types of drugs approved for treatment of viral infections—imiquimod and IFNs—do not inhibit virus replication directly. Rather, they enhance innate host immune responses to viral infection. Imiquimod (e-Fig. 13.1), which is approved for treating certain diseases caused by human papillomaviruses, interacts with Toll-like receptors TLR7 and TLR8 to boost innate immunity, including secretion of IFNs.^{117,155,183} Interferon alpha is approved for treating HCV; HBV; condyloma acuminata, which is caused by certain human papillomaviruses; and Kaposi's sarcoma, which is caused by Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8). The actions of IFNs are reviewed in Chapter 8. However, which of the many activities induced by IFNs contribute to their therapeutic effects (e.g., against HCV; reviewed in 95) remain poorly understood. Interestingly, genetic polymorphisms in a region encoding certain type III IFNs, which are induced by IFN- α , are associated with response of HCV infections to IFN- α and ribavirin, with lower expression of these interferons associated with lower responses to therapy.^{113,352,358} Follow-up studies of this observation and greater understanding of mechanisms by which viruses inhibit interferon action (e.g., HCV; reviewed in 95) may facilitate the development of more targeted therapies.

PRINCIPLES OF ANTIVIRAL THERAPY

General Concepts

Viral Dynamics and the Role of the Immune System in Antiviral Therapy

Different viruses can have very different dynamics of infection, and these differences have major impacts on the goals and effects of antiviral therapy. Many of these differences relate to the interplay between antiviral therapy and in the immune system. Acute viral infections of immunocompetent patients are usually cleared by the immune system in a matter of days. It is not surprising then that treatment of most acute influenza virus infections in immunocompetent patients with drugs such as oseltamivir reduces the duration of fever and other symptoms by only a day or so. A corollary is that treatment must be started as soon as possible to obtain clinical benefits. Indeed, prophylaxis can be the most effective approach. Many of the same considerations apply to recurrent disease caused by reactivation of latent viral infections in immunocompetent patients. In the case of frequently recurring herpes genitalis, suppressive therapy is much more effective than treating each episode. Another issue in acute infections can be the extent to which symptoms are due to viral replication rather than the immune response to the virus. For example, most of the symptoms of mononucleosis caused by Epstein-Barr virus (EBV) infections occur when viral replication has subsided. As a result, even though several antiviral drugs that are clinically useful against other herpesviruses are potent inhibitors of EBV replication, they are ineffective at treating mononucleosis.

Many of the viruses for which antiviral drugs have been developed cause persistent infections. The primary goal in the

management of persistent viral infections with antiviral therapy is sustained suppression of viral replication. In contrast with acute infections, antiviral therapy can be effective when initiated well after symptoms have appeared. In some cases, such as HCV infections, persistent viral replication occurs in the face of an immune response that usually is incapable of clearing the virus by itself. However, HCV does not form latent infections, and thus can be cured by inhibiting viral replication. In other cases, such as reactivating HCMV in transplant patients, the immune system cannot control virus replication. Antiviral drugs cannot cure HCMV infections, but they can stave off viral disease until an effective immune response is restored. Prophylactic approaches against HCMV are used very selectively in immunosuppressed patients because of the toxicities of anti-HCMV drugs. Physicians often opt for preemptive therapy when a certain level of replicating virus is detected, but before disease symptoms are manifest.

The interplay between antiviral therapy, viral dynamics, and the immune response, and the roles of tissue compartments and cellular reservoirs has been investigated in the most detail with HIV, and is discussed below.

Interplay between Anti-HIV Therapy, Viral Dynamics, and the Immune System

The generation of virions in chronic HIV infection is enormous, and the goal of antiviral therapy is to suppress this. With the initiation of effective treatment, the clearance of virions from the plasma is rapid (minutes to hours). The clearance rate constant varies little among individuals and different stages of disease.²⁸² The steady state levels of viral nucleic acid in the blood are thus determined by the rates of virus production. For HIV these rates of production are a function of the number of infected lymphocytes in the lymphoid tissue.^{129,130} The rate of decline of CD4 lymphocytes is thus directly related to the steady state level of plasma HIV RNA. The higher the RNA levels, the faster the loss of CD4 cells, and the shorter the duration of HIV infection before death.²⁴⁴ Because the CD4 count determines the risk of disease and death, and the level of HIV RNA determines the rate of CD4 cell decline, these values have been routinely used clinically to assess clinical status and urgency to initiate therapy.³⁶²

When potent combination antiretroviral therapy is effectively administered, levels of HIV RNA in both plasma and infected cells in lymphoid tissue rapidly decrease. The rapid, first phase clearance is attributable to the death of infected, activated CD4 lymphocytes and the prevention of infection of new cells.²⁸² The second phase clearance rate is lower and more variable in slope among individuals. It has been attributed to clearance of infected macrophages or to chronically infected CD4 lymphocytes with lower rates of cell death, but may also result from the clearance of the large burden of virions bound to dendritic cells in the germinal centers of lymph nodes.^{39,280,403} Failure to reduce plasma HIV RNA levels to below the limit of detection (20–75 copies/ml with the currently available assays) indicates inadequate suppression and a risk for the outgrowth of resistant virus.³⁶² Even in patients sustaining suppression below this level for 10 years or more, very low levels of viral RNA can often be detected in tissues and blood. This low level viremia is sustained at steady state levels of less than 50 copies of HIV RNA/ml plasma as measured following ultracentrifugation and using very sensitive assays. Although ongoing

replication may be occurring in some patients, several lines of evidence would argue that activation of latently infected CD4⁺ lymphocytes or long-lived persistently infected cells like macrophages or pluripotent multiprogenitor cells are the source of this viremia. Notably, no new drug-resistance mutations emerge, no nucleotide sequence evolution can be discerned, and treatment intensification with additional potent antiretroviral drugs fails to perturb the viremia.^{242,277}

The immunologic consequences of suppressing HIV replication are dramatic. The increase in CD4 lymphocyte numbers has two phases. In the first month or two the increase is often large (20–100 cells per μ l blood).^{7,18,128} The magnitude is proportional to the steady state HIV RNA levels before treatment, which drives the level of generalized activation of the immune system. The normal distribution of lymphocytes is 2% in the circulation and 98% in the lymphoid tissues. With the immune activation of HIV infection, the distribution shifts to 1% and 99%.^{129,404} Therapy largely corrects this shift, and results in redistribution of mostly CD45RO⁺ memory T cells from the lymphoid tissue back to the circulation.^{31,274,404} Production of new cells, mostly of the CD45RA-naïve phenotype, is generated both by restored thymic mass and function, which is age related, and by peripheral proliferation.^{84,239}

The generation of cell numbers is not sufficient to account for the success of antiretroviral therapy. It is the restoration of immune function that has transformed the natural history of AIDS. Both CD4 and CD8 T-cell responses to recall antigens are regenerated.^{7,140,194} Persistent opportunistic infections are often resolved. Occasionally, subclinical chronic infections with *Mycobacterium avium* complex (MAC) or HCMV, for example, are manifested when a restored immune response produces a local inflammatory reaction. Patient care has been transformed with the ability to withdraw prophylactic or suppressive therapy for various opportunistic infections, which had previously been lifelong commitments.

The probability of success of suppressing HIV replication and of preventing the emergence of resistance (see below) is significantly impacted by the level of CD4 cells and of HIV RNA in the blood. This relationship is a practical argument to avoid treating before it is too late, but it also points out that the imperfect efficacy of drug treatments is complemented by having more immunity and less virus. It is noteworthy that the success of treatment of opportunistic infections in AIDS patients, including antiviral therapy for HSV, VZV, and HCMV infections, also is similarly affected by CD4 cell count.

Compartments and Reservoirs

Treatment of HIV is complicated by the existence of tissue compartments and cellular reservoirs. Although there is trafficking between the blood and central nervous system (CNS), much virus in the CNS evolves independently.³⁴⁶ Similar observations have been made with virus in semen.²⁸⁸ Drug penetration into these compartments differs from the circulation and lymphoid tissue, and varies with each drug. Latently infected CD4 lymphocytes represent a small fraction of infected cells during active infection, but they have long half-lives,^{97,311,345} and may be sustained for life by homeostatic proliferation of latently infected memory CD4⁺ lymphocytes.⁴⁹ Consequently, virus archived at any time during infection can reemerge and propagate after the withdrawal of therapy.

Drug Resistance in Antiviral Therapy

The speed, magnitude, and clinical impact of the emergence of resistance differ among antivirals and viruses. As summarized earlier in the chapter, the rate at which mutants that are resistant to a given drug will emerge is a function of mutation rate, target size for mutation, replication rate, preexisting size of the population, and fitness. All of these factors can have clinical impact. For example, at presentation, an HIV-infected individual contains 10^{11} virions,¹³⁰ more than 10 billion (10^{10}) HIV-1 virions are generated daily,²⁸² and on average one mutation is generated for each newly generated genome.²³² Therefore, genomes with each possible mutation, as well as many with double mutations, are likely generated daily. Moreover, during HIV infection, drug-resistant virus is readily archived in latently infected cells to confound treatment modifications for the remainder of the patient's life.^{98,390,311} Therefore, an HIV-infected individual is highly likely to develop an infection that is resistant to any given antiviral drug. In contrast, ACV resistance almost never develops during treatment of HSV or VZV in immunocompetent patients. These patients contain much less virus, and the virus replicates and mutates at a much lower rate than HIV. Nevertheless, ACV resistance does occur more often in immunocompromised patients in whom virus replicates to higher levels and more persistently.

Of particular clinical relevance to antiviral resistance are drug concentrations attained in patients that achieve therapeutic effects. In this regard, fitness is highly important. With increasing drug exposure in a patient, the selective pressure on the replicating virus population increases to promote the more rapid emergence of drug resistant mutants; that is, as drug-sensitive viruses become less fit. For example, higher doses of AZT or zidovudine monotherapy tend to select for drug-resistant HIV more readily than do lower doses.^{256,309} As drug concentrations of monotherapy increase, the likelihood that resistant mutants will arise increases, as long as significant levels of virus replication persist. This can occur through improper dosing, suboptimal adherence, pharmacologic hurdles, and ineffectively treated compartments. Incompletely suppressed viral replication with drug regimens sufficient to exert selective pressure drive the evolution and fixation of drug-resistant virus at a rate Darwin himself never imagined. This scenario may explain the emergence of an oseltamivir-resistant H5N1 influenza virus in a patient who had been given relatively low doses of drug as prophylaxis.²²⁸ With further increases in concentration of antiviral drugs, especially in combination, the amount of virus replication diminishes to the point where the likelihood of emergence of resistance begins to diminish, and becomes nil when virus replication is completely inhibited.³⁰⁶ The ultimate goal of antiviral therapy, especially for viruses like HIV, HBV, and HCV, which have high mutation rates, high replication rates, and high preexisting populations, is to apply drug regimens that completely inhibit virus replication.

Clinical Impact of Drug Resistance

It may seem obvious that drug resistance is important clinically. However, many treatment failures are not due to drug resistance, and many drug-resistant viruses do not cause treatment failures. To demonstrate thoroughly that drug resistance is clinically important, it is necessary to have evidence of treatment failure, to isolate drug-resistant virus, and to successfully

treat the disease with a second drug to which the virus is susceptible.

For acute infections like influenza, which are largely controlled by the immune system, antiviral drug resistance is generally not a major problem in the patient in whom it arises. Therefore, during monotherapy with adamantanes or neuraminidase inhibitors, variants probably emerge in all patients within a week, but in immunocompetent patients, that is when the infection has largely resolved. Transmission of resistant virus to others can occur, however, and the antiviral efficacy will be impaired in these secondary cases. In persistent infections, antiviral drug resistance can be a matter of life and death. As resistance mutations accumulate, drug susceptibility diminishes, progressively reducing the efficacy of antiviral regimens. Continued replication in the presence of drug selects for even greater levels of resistance to each administered drug and progressive cross-resistance to drugs of the same class. This drives a vicious cycle of treatment failure and yet more difficult treatment challenges. Regimens for patients who are failing treatment, while constrained to more limited options by resistance, must still contend with the same obstacles of adherence, pharmacology, and tolerability that challenged the first regimen.

Drug-resistant herpesviruses and HIV can establish latent infections. Drug-resistant HIV, HBV, and influenza A viruses have been transmitted to other individuals. In the case of HIV, resistant virus in blood or genital secretions can be transmitted during sex, needle sharing, or childbirth.²¹⁶ Rates of transmission of drug-resistant HIV appear to have increased, with 5% to 20% of primary infections caused by drug-resistant virus in developed countries.^{216,218} Such patients are more likely to fail their first treatment regimen. Resistance in developing countries has become increasingly appreciated with the rollout of treatment access.²⁹⁵

Strategies to Combat Drug Resistance

The increasing prevalence of antiviral drug resistance raises challenges to the effective treatment of individuals and to the public health similar to those that have resulted from widespread antibiotic resistance. One strategy to combat this problem is to test viruses for drug resistance before choosing antiviral regimens. Resistance testing can help determine which drugs will not work (thereby diminishing cost, toxicity, and inconvenience) and which drugs are most likely to be effective. Such testing is rapidly being incorporated into standard HIV care,¹⁶⁰ and is certain to become part of the management of HBV and HCV. These tests can be performed most rapidly if they are genotypic (i.e., detecting mutations at the level of DNA or RNA) rather than phenotypic (i.e., measuring changes in drug susceptibility). Genotypic assays are much easier to develop if only a few mutations confer clinically relevant resistance. As reviewed in the sections on mechanisms of specific antiviral drugs, this is more likely to be true for some drugs (e.g., lamivudine) than others (e.g., ACV). As numerous mutations in various combinations accumulate, as occurs during treatment of HIV with RT and protease inhibitors, the interpretation of genotypic assays can become very difficult, and phenotypic assays may be required.

A second key strategy is combination chemotherapy. This strategy combats resistance on several levels. First, the probability of a virus being resistant to multiple different drugs (with different mutations conferring resistance) is the product of the

probabilities of resistance to each drug. This makes it much less likely that a preexisting virus in the patient will be resistant to all of the drugs. Second, the combination is likely to suppress replication more completely than would any of the drugs alone. This would provide less chance for resistance to develop. Third, members of the combination might synergize, thereby providing even greater efficacy. It is crucial that drugs in the combination not antagonize each other's activities by mechanism of action or pharmacologic interaction. Fourth, a mutation conferring resistance to one drug in a combination might yield clinical advantages, for example, by making the virus less fit or by sensitizing otherwise resistant viruses to a second drug. Such advantages have been invoked for the M184V mutation in HIV and the M204V/I mutation in HBV that confer resistance to 3TC and FTC (reviewed in 79).

Why not always use combination chemotherapy? Additional drugs add costs, toxicities, and pharmacological interactions, which sometimes exacerbate those of the first drug. Combination regimens can be difficult for the patient, reducing adherence with the regimen. Sometimes too few drugs are available to combine, or if there are multiple drugs, they entail similar mechanisms of action and resistance. Drug resistance is relatively uncommon with certain virus infections, such as herpesvirus infections in the immunocompetent. These points help explain why combination chemotherapy is rarely used for treating herpesvirus or influenza virus infections.

The history and clinical practices as of 2012, and issues particular to therapies of different viruses will now be reviewed.

HIV Therapy

In the 27 years since AZT was first clinically evaluated, 25 drugs have been approved for treating HIV (Fig. 13.15). This remarkable drug development experience helped elucidate many of the principles of antiviral therapy described earlier. A review of the clinical evaluation of each of the anti-HIV drugs and each of the combinations of these drugs for patients in various stages of disease is beyond the scope of this chapter. Guidelines summarizing the practical use of currently available antiretrovirals are frequently updated.^{359,362} With the availability of these treatment alternatives and with an appreciation of the principles of antiviral therapy, HIV has been transformed into a manageable condition for most patients in the developed world and encouraging inroads have been made for millions in resource-limited settings. AIDS mortality and morbidity from opportunistic conditions have been dramatically diminished for those with access to care.

The initial use of AZT monotherapy in patients with late-stage HIV disease had a dramatic effect on mortality over the first several months of follow-up.¹⁰⁰ The benefits dissipated with the emergence of drug-resistant virus.^{99,134,200} The availability of additional nucleoside analog RT inhibitors, as replacement therapy on those failing AZT monotherapy and in combination with AZT in previously untreated patients, provided incremental efficacy.^{64,65,136,184} The use of nucleoside analogs in various combinations and regimens reduced morbidity and prolonged life. However, for most patients, drug resistance and loss of control ultimately ensued.

The approval of the first drugs in additional classes (NNRTIs and protease inhibitors) permitted the trials of two nucleosides and initially either indinavir or nevirapine, which changed the treatment paradigm.^{128,137,257,342} When patients

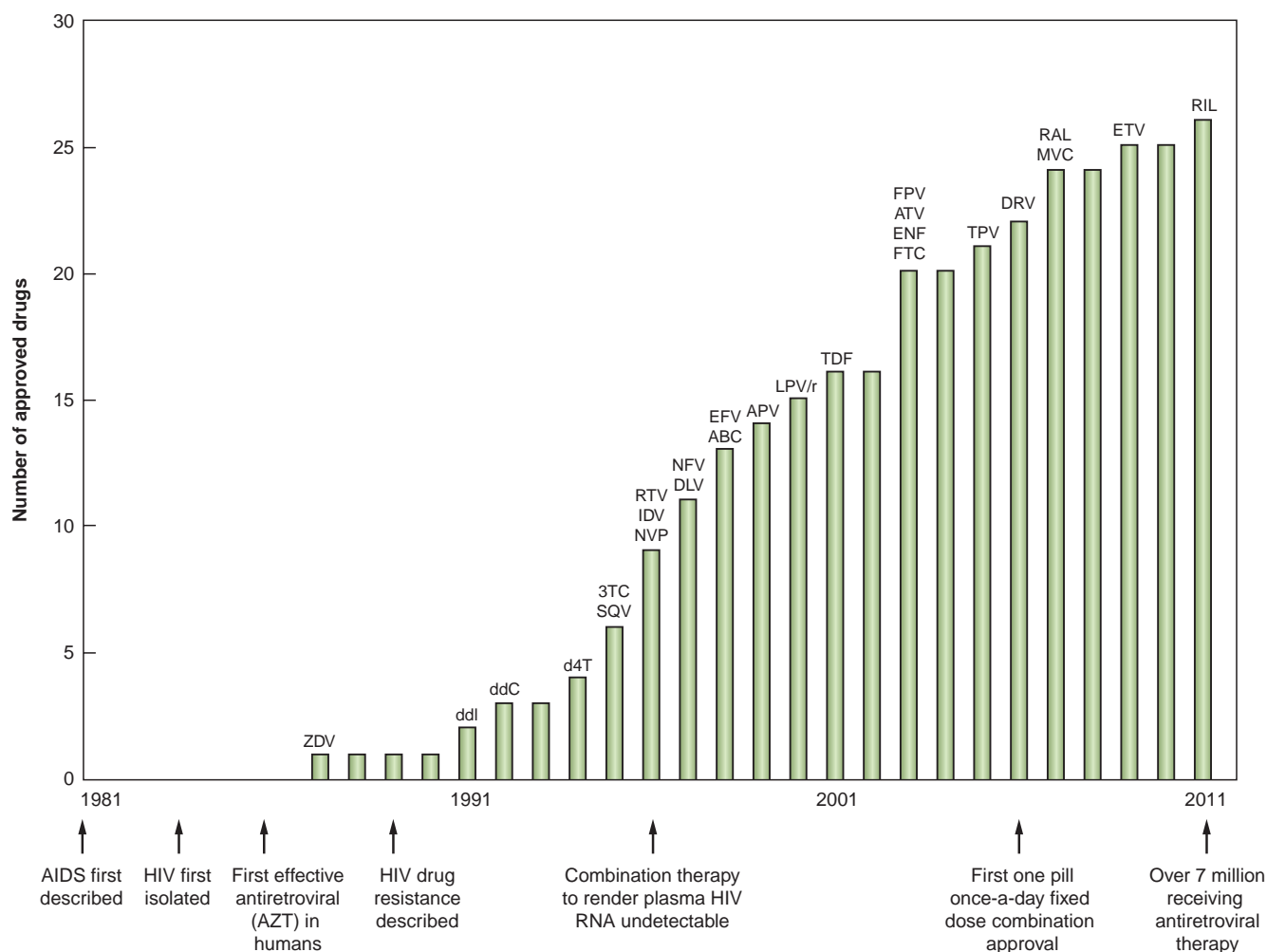


FIGURE 13.15. Time-line of acquired immunodeficiency syndrome (AIDS) therapies. The dates of important events in the understanding and therapy of AIDS are provided together with the dates of U.S. Food and Drug Administration (FDA) approval of antiretroviral drugs. The bars represent the progressive accrual of approved drugs. Abbreviations: ZDV, zidovudine (AZT); ddI, didanosine; ddC, zalcitabine; d4T, stavudine; 3TC, lamivudine; SQV, saquinavir; RTV, ritonavir; IDV, indinavir; NVP, nevirapine; NFV, nelfinavir; DLV, delavirdine; EFV, efavirenz; ABC, abacavir; APV, amprenavir; LPV/r, lopinavir/ritonavir; TDF, tenofovir; FPV, fosamprenavir; ATV, atazanavir; ENF, enfuvirtide; FTC, emtricitabine; TPV, tipranavir; DRV, darunavir; RAL, raltegravir; MVC, maraviroc; ETV, etravirine; RIL, rilpivirine. Elvitegravir was approved in 2012.

who had not yet acquired too many compromising drug-resistance mutations received these triple combinations, they experienced suppression below assay detection for plasma HIV RNA. Many patients are now well into their second decade of HIV RNA suppression and immune restoration. AIDS-related morbidity and mortality are seen primarily in those without access to care. In those who are receiving effective antiretrovirals, the primary complications are related to chronic hepatitis, malignancies, or the accidents and illnesses facing the general population.

The chronic use of antiretrovirals revealed toxicities often not appreciated in the initial preclinical toxicologic assessment of the drugs or clinical trials. Side effects due to nucleoside analogs include bone marrow toxicity (AZT), pancreatitis (ddI), myopathy (AZT), fat wasting in face and limbs (d4T, AZT), lactic acidosis (AZT, d4T, ddI, zalcitabine [ddC]) and peripheral neuropathy (ddC, ddI, d4T, ddC^{28,69}). Most pro-

tease inhibitors were observed to induce hyperlipidemia and insulin resistance.²⁶⁸ The availability of more drug alternatives has led to the gradual selection of regimens that minimize toxicity, maximize potency, and improve convenience in terms of pill burden and dosing frequency to facilitate adherence.^{359,362} As the regimens become more convenient, more efficacious, and less toxic, the proportions of patients whose circulating HIV becomes fully suppressed is approaching 90% and the mortality curves continue to improve each year.^{119,224}

These increasing rates of successful initial treatment diminish the proportion of patients who newly acquire drug resistance. Moreover, the proportion of patients with long-standing resistance to nucleoside analogs acquired before the availability of effective combination therapy is diminishing. The numbers of patients with transmitted drug resistance, after initially rising, now appears to be stable or even diminishing, with improved suppression of ongoing replication in increasing

proportions of the population.¹¹⁹ The availability of multiple alternative drugs is important to address the needs of patients requiring treatment of drug-resistant virus. The phenomenon of cross-resistance within drug classes still leaves many patients with few or no options; however, with newer drugs this number is diminished in developed countries. Therapy for HIV thus requires continuing efforts in drug resistance testing and drug development to contend with a virus so proficient at latency, evolution, and escape.

With the appreciation that treatment is more likely to be effective and less toxic when initiated early, and there is risk with delaying therapy at almost any stage of HIV infection, treatment guidelines have shifted to recommending almost universal treatment if feasible.³⁶² It has been proposed that this practice can also reduce HIV transmission.¹¹⁹

Herpesviruses Therapy

HSV and VZV Therapies

The first effective application of antiviral therapy for a herpesvirus infection was the local application of the nucleoside analog, 5'-iodo-2'-deoxyuridine, for the topical treatment of HSV keratitis.¹⁸⁶ This application has the advantage of permitting high local concentrations of antiviral at the site of infection without the risk of systemic toxicity. Other anti-HSV drugs are now used for this application. The first effective antiviral for systemic use against HSV was vidarabine, a nucleoside analog converted by cellular enzymes to an inhibitor of HSV DNA polymerase. This intravenously administered drug improved biopsy-proven HSV encephalitis in a double-blind, placebo-controlled trial in 1977³⁸² and subsequently was shown to be effective against neonatal herpes and VZV infection in immunocompromised patients.^{379,381}

ACV, the mechanism of which is detailed in this chapter, heralded the second generation of antivirals for herpesviruses and set the standard for the development of antiviral drugs. It became the standard care after randomized, controlled trials of intravenous drug for serious systemic infections such as herpes encephalitis, neonatal herpes, and varicella zoster in immunocompromised hosts.^{13,377, 378–379} In addition, it was shown to be effective for the prophylaxis of HSV and VZV in transplant patients.^{321,322} As an orally available agent with negligible toxicity, it has become widely used for the treatment of primary and recurrent HSV-1 and HSV-2, the prophylaxis of recurrent HSV, and the treatment of uncomplicated VZV.^{85,90,168,247,254,265,302,347,371} The earlier in the presentation of symptoms, the more effective the treatment, and prophylaxis is more dependable than treatment. The appearance of resistant isolates has been remarkably rare in immunocompetent patients and occurs in a minority of immunocompromised patients. ACV-resistant HSV and VZV did become a significant challenge in patients with AIDS preceding the availability of combination antiretroviral therapy.³¹⁷ The expanded number of resistant infections reflected the number of patients with AIDS, the prevalence of recurrent HSV and VZV infection in these patients, and the duration and magnitude of these opportunistic infections, because of the severely reduced antiviral immunity in these hosts. In many, but not all of these patients, cidofovir or foscarnet showed activity against resistant infections,³¹⁸ but resistance to these DNA polymerase inhibitors has developed as well.^{118,319}

Two limitations of oral ACV have been its limited oral bioavailability (~15%) and short half-life. These limitations

require administration of large pills as often as every 4 hours. As described earlier in this chapter, valacyclovir and famciclovir overcome these limitations, and would have completely displaced the use of ACV except for the expiration of the ACV patent resulting in the availability of low-cost generic drug. Both valacyclovir and famciclovir can be used for applications of oral ACV. In addition, because of the more prolonged maintenance of higher levels of antiviral activity, they are also used for the treatment of serious but not immediately life-threatening infections previously treated with intravenous ACV, for example, herpes zoster in the immunocompromised host.^{20,367}

HCMV Therapies

As reviewed earlier in the chapter, the lack of potency of ACV against HCMV led to the development of ganciclovir. Ganciclovir is more toxic than ACV. However, it is the first-line therapy for HCMV disease in the immunocompromised host.

The most common opportunistic complication of HCMV in AIDS is retinitis.¹⁷⁸ Ganciclovir clears viremia and arrests the progression of destructive retinitis.^{32,178,360} HCMV disease develops in patients with AIDS who have fewer than 100 CD4 cells per ml; however, before reconstitution of the immune system with combination antiretroviral therapy, these patients uniformly reactivated disease with withdrawal of treatment, thus requiring chronic suppressive maintenance treatment with the consequences of drug toxicity and emergence of resistant virus.³⁸⁰ Ganciclovir is similarly effective for the treatment of colitis, esophagitis, polyradiculitis, and ventriculitis in patients with AIDS,⁸⁰ and HCMV pneumonia in renal transplant patients.¹⁵⁴

With the difficulties of daily intravenous ganciclovir for maintenance therapy, oral ganciclovir was studied despite its low bioavailability, which resulted in limited efficacy and selection for drug resistance. Some significant benefit for the management of retinitis was the local administration in the eye of ganciclovir (and some other anti-HCMV drugs) by injection or implant.²³⁴ This approach protects the eye with high local concentrations, but fails to address HCMV disease in the rest of the body.³⁸⁰ The eventual development of valganciclovir has now provided an orally bioavailable drug for HCMV that can achieve levels approaching those of intravenous ganciclovir without the cost, inconvenience, and complications. Although a significant advance, the introduction of effective combination antiretroviral therapy was more crucial, as it dramatically diminished the prevalence of HCMV disease and essentially eliminated the need of maintenance therapy for retinitis.

Foscarnet, cidofovir, and fomivirsen are also approved for the treatment of HCMV. Although equally effective for the treatment of HCMV retinitis, foscarnet is used only for ganciclovir-resistant HCMV infections because of inconvenience and toxicity.³⁶⁰ Cidofovir is also used for the treatment of ganciclovir-resistant HCMV, with the advantage of requiring administration only every 1 or 2 weeks due to its very prolonged intracellular half-life, but the disadvantage of renal toxicity.

Antiviral therapy has not yet been shown to significantly impact disease attributable to the other human herpesviruses, EBV, and HHV6, HHV7, and HHV8. Various explanations include the limited activity against these viruses of available drugs, the undesirability of testing a difficult drug like foscarnet that might nevertheless have activity, the difficulty of conducting clinical trials for some of these infections, and the

relatively small size of the perceived market. Aside from therapies against these other herpesviruses, there remains considerable need for new anti-herpesvirus drugs to combat infections resistant to the current drugs, and to overcome the pharmacological and toxicity limitations of the current anti-HCMV drugs. No available anti-herpesvirus drug effectively addresses the problem of viral latency.

Therapy of Viral Hepatitis

HBV Therapy

The complex natural history of HBV infection is described elsewhere in this text. Criteria based on disease stage and level of HBV viremia, and the availability of more effective drugs, have increased the treatment indications and the long-term success of therapy.^{94,391} An important complication is that chronic HBV infection implies that immune responses toward the virus are impaired. In addition, chronic HBV entails the existence of large numbers of cells containing covalently closed circular HBV DNA, a situation akin to herpesvirus latency. For all practical purposes, this may preclude eradication of HBV infection. Therefore, for therapy to be successful, the immune response must be restored, chronic antiviral suppression must be maintained, or both.

A significant proportion, but not all, carriers of HBV progress to cirrhosis, hepatic failure, or hepatocellular carcinoma. Antiviral therapy has been shown to prevent the development of these complications and, in fact, to reverse cirrhotic histopathology.³⁵³ Whom, when, and how to treat are decisions based on risk of progression and therapeutic index of the various treatments.^{94,391} Treatment efficacy is impacted by the regimen used as well as viral genotype, host genetics including ethnic background, disease and virologic status, age, and comorbidities such as HIV co-infection.

The therapy for HBV infections has evolved.^{94,391} The first effective antiviral therapy for HBV infection was recombinant IFN- α , initially injected three times weekly. Daily injections proved more effective. Pegylation of IFN- α to permit effective levels when injected once weekly improved both acceptance and efficacy. Interferon therapy does not suppress plasma HBV DNA in as high a proportion of patients as does therapy with nucleoside analogs when administered for 12 to 24 weeks. Following the cessation of treatment, however, patients receiving nucleoside analogue therapy only rarely sustain responses, whereas a small subset of patients treated with interferon maintain durable responses. Interferon therapy, in contrast to nucleoside analogs, has not been shown to select for resistant mutants. The use of interferon therapy is limited, however, by its cost, inconvenience, and substantial side-effect profile (fatigue, depression, leukopenia).

Five nucleoside analogs have been approved for the treatment of hepatitis B. The experiences with these drugs recapitulate many of the experiences of HIV therapy. 3TC, telbivudine, and adefovir reduce plasma HBV DNA levels an average of 4 logs over a period of 12 weeks, associated with improvements of liver inflammation and progression of disease. Unfortunately, continuing use was associated with increasing proportions of patients with resistant mutants who experience treatment failure.²²³ Entecavir and tenofovir have displaced other drugs for HBV infection because of their high rates of virus suppression with monotherapy and the low rates of emergence of drug-resistant virus during treatment.^{94,337,391} The combination of

tenofovir and emtricitabine, which is active against HBV, has been approved for the treatment of HIV and is used routinely in patients who are co-infected with HIV and HBV.

HCV Therapy

The same issues regarding who and when to treat that apply to HBV apply to HCV. Factors include impact on response of viral genotype, age, sex, disease stage, host genotype, and co-factors like concurrent HIV infection, renal disease, or alcohol use.¹¹⁶ One critical difference is that HCV has no DNA intermediate that precludes eradication. HCV is thus curable.

IFN- α administered three times weekly was the first treatment shown to be effective against HCV; however, only 15% to 20% of patients achieved a sustained response with 48 weeks of therapy.^{293,401} An analysis of viral dynamics concluded that the three-times weekly regimen of IFN- α provided waning activity between doses and that progressively increasing doses provided progressively increasing efficacy,²⁶⁴ leading to incrementally better rates of sustained virological response with daily dosing.^{153,401}

The introduction of pegylated IFN- α , permitting weekly dosing, and provided yet another increment in these rates. The addition of ribavirin added yet another increment to the rates of sustained virology responses,^{105,196,231,240,301} Approximately one-half of patients achieved sustained virological responses with this latter regimen; however, genotypes 2 and 3 respond appreciably better, whereas genotype 1 and patients with HIV co-infection respond less well.^{51,131,364} Other factors including disease stage, viral load, race, age, sex, and weight impact this regimen, as they do all preceding regimens.³⁴⁴ The remarkable impact of the IL-28b genotype on host responsiveness to IFN efficacy provides a substantial explanation for much of the individual variation in treatment responses.¹¹⁶ It is noteworthy that the treatment of HCV, as with HBV, can reverse liver fibrosis and reduce rates of hepatocellular carcinoma.^{175,294}

The resources and commitment to discovery and development of small molecules targeting HCV, called direct acting antivirals (DAAs), has surpassed that directed against HIV. Potent inhibitors of protease, RNA polymerase (both nucleoside analogs and nonnucleosides), NS5A, and other targets have been identified, and entered clinical trials.^{279,290,325} The dynamics of response to treatment are faster with HCV than HIV with as much as 3 to 5 log₁₀ reductions with many drugs in a matter of days. Similarly, with the high levels of HCV replication, drug-resistant mutants emerge with monotherapy in a matter of days. The initial clinical studies thus required examination of the incremental benefit of adding a candidate drug to the standard regimen of pegylated IFN plus ribavirin. The first HCV DAAs to be approved in 2011 were the NS3 protease inhibitors, telaprevir and boceprevir.^{10,179,291,333,400} These drugs have similar efficacies, and increase the sustained virological response (basically a cure) by approximately 25% to 30% over the proportion with such a response with IFN and ribavirin alone. These improved responses are encouraging; however, a substantial proportion of treated subjects fail (and often with protease-resistant virus). Moreover, the regimen still requires the relatively toxic combination of IFN and ribavirin.

The development of drugs with activities against a broad range of genotypes and targeting multiple targets, which include nucleoside NS3 polymerase inhibitors, nonnucleoside NS3 polymerase inhibitors of targeting multiple different sites,

NS5A inhibitors and nonimmunosuppressive cyclophilin-binding compounds among others provide promise for potent combination regimens that could cure HCV infection in 8 to 24 weeks without the need for IFN or ribavirin. The field of HCV antivirals has accelerated remarkably and developments were occurring at an encouraging pace by 2012, such that the state of the field will certainly be transformed. Should this promise be achieved, earlier and more comprehensive treatment would be indicated. One could even imagine efforts to systematically find asymptomatic carriers of HCV and treat them. This might dramatically reduce transmission, chronic hepatic disease, and liver transplantation, approximately one-half of which is attributable to HCV.³⁰

Influenza Therapy

Both the adamantane derivatives and the neuraminidase inhibitors have utility for both prophylaxis and treatment of influenza. The adamantanes, amantadine and rimantadine, reduce rates of illness by 70% to 90% when used prophylactically for influenza A.^{6,71,81,212} They have some quantitative activity in reducing viral shedding and diminishing the duration and severity of symptoms when used early after the onset of illness.^{133,368,397} These treatment effects are limited. Moreover no data have been generated on the treatment of severe disease requiring hospitalization.

Rimantadine has fewer CNS side effects than amantadine, especially in the elderly. In addition, rimantadine is not renally excreted, and thus requires no dosing adjustments in renal impairment. Neither drug is active against influenza B. Moreover, resistance has developed into a major problem. Resistant virus is often shed after several days of treatment and when illness is resolving.¹⁴⁹ This virus can be transmitted, thus eliminating prospects for prophylaxis of contacts of the treated patient. Because of the limited therapeutic efficacy of the adamantanes and their ready selection for resistance, recommendations for the use of the adamantanes have been primarily as prophylaxis for influenza A. Several subtypes of influenza A have become predominantly amantadine resistant, having first been seen in China where amantadine is included in over-the-counter cold remedies,²⁷ and allegations of its use on chicken farms have been made.²⁷⁸ Recent isolates of avian influenza (H5N1) have also been amantadine resistant.¹⁶

The neuraminidase inhibitors, zanamivir and oseltamivir, are effective for prophylaxis^{147,150,258} and treatment.^{145,148,365} of both influenza A and B. Zanamivir requires inhalational administration, risks of uneven distribution in the respiratory tract, and occasionally bronchospasm. Moreover, should systemic activity be needed, especially with H5N1 disease,⁷⁶ oseltamivir is the only option, although parenteral preparations of zanamivir and newer neuraminidase inhibitors are in development.¹⁶ Although the efficacy of neuraminidase inhibitors has not been documented for the treatment of complicated influenza requiring hospitalization, it is recommended as the agent for treatment and prophylaxis of influenza A and B, especially with the expanding prevalence of adamantane resistance. It was stockpiled for a possible epidemic of H5N1 infection for which oseltamivir has been shown to be effective in animal models,¹⁶ and for the pandemic H1N1 epidemic of 2009.

High-level resistance due to His to Tyr mutation in codon 275 of the N1 neuraminidase can emerge during treatment of H1N1 in children and in several patients treated for H5N1 infection.³⁷² This mutation does not confer cross-resistance to

zanamivir.^{127,375} By itself, the H275Y (H274Y in N2) mutation reduces fitness *in vitro* and *in vivo*,¹⁷⁷ although it is transmissible to ferrets.¹⁵⁶ However, as reviewed earlier in the section on neuraminidase inhibitor mechanisms, oseltamivir-resistant H1N1 viruses containing H275Y and secondary mutations that increase fitness have emerged, increasing the need for new therapeutic options and engendering intense interest in the epidemiology of resistance to anti-influenza drugs.¹⁴⁶

Conclusion and Future Directions

Antiviral drugs ameliorate viral disease and save lives. Understanding viral replication and virus–host interactions is crucial for understanding the mechanisms of antiviral agents and for the discovery of new ones. Likewise, antiviral agents provide the virologist with excellent tools with which to investigate viral biology and biochemistry, and the biology and biochemistry of virus hosts. Of the antivirals available today, most inhibit viruses at the genome replication stage. This stage will doubtless continue to be the target for new drugs that become approved for use in patients. However, more and more drugs that target other stages are entering clinical use (e-Table 13.1). There is still a great deal of unmet medical need that can be served by antiviral drugs, and we are still learning how to use them.

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Summary

Acknowledgments

Both innate and adaptive immune responses mediate host resistance to virus infection. Elements of the innate immune system, especially type I interferons (IFNs), strongly influence the clinical outcome of a virus infection. Many viruses have evolved mechanisms to inhibit or evade innate immune responses, indicating the importance of those defenses in limiting virus infection. Innate immune responses also mediate important effects during the induction phase of an immune response and may influence the magnitude and composition of adaptive immune responses. Although memory or “training” of natural killer (NK) cells may occur in response to some antigens (Ags), NK cells do not have mechanisms for somatic diversification of their recognition receptors and are limited to germline sequence.^{315,343} The capacity for somatic diversification of B-cell receptors (BCRs) and T-cell receptors (TCRs), selection, and long-term memory are central properties of adaptive immunity. Therefore, vaccine-induced immunity that is established in advance of virus infection relies primarily on adaptive immune responses for protective efficacy. Vaccination depends critically on the properties of Ag recognition, activation, expansion, memory, trafficking, and effector functions inherent to lymphocytes. Subtle influences of vaccination on the timing, magnitude, specificity, quality, and location of responding lymphocytes and their effector molecules determine whether exposure to a viral pathogen results in infection, protection, subclinical infection, mild disease, or exaggerated disease expression. The extent to which vaccine-induced immunity is successful in an individual can determine the spread of virus within a population (e-Fig. 14.1). Thus, vaccines have a profound effect on public health through the control of epidemics (e-Fig. 14.2). An antigenic stimulus that elicits a virus-specific adaptive immune response that can be recalled during subsequent virus infection defines it as an immunization. A vaccination is an antigenic stimulus delivered by intention.

VIRAL ANTIGENS RECOGNIZED BY THE IMMUNE SYSTEM

Development of an immune response to Ags present on the surface of virions or virus-infected cells is often critical for immunity against virus infection. Antibody (Ab) responses to surface viral protein or glycoprotein Ags often effectively limit infection of new cells and thus spread of the virus. Internal viral Ags can be important targets for T-lymphocyte responses that clear virus-infected cells; however, these responses alone cannot prevent subsequent infections (e-Fig. 14.3). The relative

importance of Abs to surface proteins and of T-cell responses to internal proteins in influenza virus was illustrated by studies of the 1957 Asian influenza pandemic and the 1977 “Russian” influenza epidemic. The 1957 Asian influenza A (H2N2) virus contained surface glycoproteins (the hemagglutinin [HA] and the neuraminidase [NA]) that were novel, such that the human population had not been exposed to strains containing these or related surface Ags. In contrast, the major internal proteins of the new H2N2 virus, the nucleoprotein and the matrix protein, were closely related to corresponding Ags of previous strains of subtype H1N1 to which most individuals had been exposed. Although recent infection with heterotypic viruses may have diminished the level of illness severity in adults,¹²⁶ the population’s prior immunological experience with related internal Ags did not prevent the 1957 Asian H2N2 influenza A virus from causing a major pandemic.³⁰³ In contrast to this experience, observations following the re-emergence of the H1N1 virus in 1977 indicated that homotypic humoral immunity provided a high level of resistance that lasted for decades. Similarly, humans exposed to the 1918 influenza H1N1 virus as infants sustained circulating memory B cells encoding neutralizing Abs to the HA into the 10th decade of life,⁴⁶⁷ and these Abs cross-reacted with the 2009 H1N1 pandemic strain and provided protection.^{233,462} Induction of cross-reactive Abs to influenza may sometimes be harmful. For example, immune complexes with complement deposition have been demonstrated in lung tissues of victims of pandemic influenza.²⁸⁷ Additional evidence for the importance of responses to influenza A surface glycoproteins was provided by passive and active immunization studies in mice. Monoclonal antibodies (mAbs) directed against the HA or NA proteins protected mice against challenge with virulent wild-type virus, whereas mAbs specific for the internal nucleoprotein or matrix protein did not alter the course of disease.³⁰³ Immunization with recombinant vaccinia viruses expressing each of the 10 known influenza A gene products showed that only recombinants expressing the HA or the NA glycoprotein induced resistance to virus challenge.¹²⁴ Whereas the vaccinia vector-induced CD8 T-cell-mediated immunity specific for internal viral proteins provided some measure of heterosubtypic cross-reactivity against influenza challenge,¹²⁵ the aggregate data suggest that a successful strategy of immunoprophylaxis against most virus infections requires the generation of an immune response to the surface Ags displayed on virions and on virus-infected cells. Other viruses (such as poxviruses, herpesviruses, filoviruses, some respiratory viruses, and lentiviruses) that have a more complex pathogenesis may require vaccines that induce more complex immune responses for protection from infection or control of disease. Many of these viruses possess the capacity for persistence, infect immunoprivileged sites, use sophisticated immune evasion strategies, directly infect their target organ without a requirement for viremia, or cause disease by eliciting an aberrant host response. Vaccine development for these viruses probably will require the induction of neutralizing Abs against surface proteins to protect against infection in addition to T-cell responses against structural and regulatory proteins to control virus spread and to modulate the composition of the immune response.¹⁶⁷

The surface glycoproteins of enveloped viruses and the capsid proteins of nonenveloped viruses are the primary target Ags for Ab-mediated protection. The following generalizations can be made about the nature of Abs and their interaction with

virus surface proteins. First, antiviral Abs against the extracellular domain of surface proteins predominantly recognize conformational epitopes. Such conformational epitopes are difficult to mimic with peptides or proteins that have had the structure of antigenic sites modified by vaccine preparation and formulation. Presentation of the native Ag structure in its authentic conformation is critical for induction of neutralizing Abs. Even when the structure is mimicked with atomic-level precision and elicits Abs that bind the epitope, the property of viral neutralization may not be achieved.^{274,326}

Most viral surface proteins assemble into oligomeric states on virions, typically dimers, trimers, or tetramers; however, the monomeric form of the protein is also present in cell debris or shed proteins. The principal neutralizing determinant may be present only in quaternary structures; thus, Abs binding to monomeric protein do not recognize or neutralize virions. In addition, many surface proteins that are targets of neutralizing Abs are fusion proteins present in a metastable state on virions. During the attachment and fusion process, the fusion proteins transition from prefusion to postfusion forms, in some cases drastically altering the structure of neutralizing epitopes. Second, Abs recognize many major antigenic sites on each viral protein, and within each of these antigenic sites, there may be multiple epitopes. For example, the parainfluenza virus attachment glycoprotein (HN) has at least six antigenic sites, three of which are recognized by neutralizing Abs.⁸⁵ After infection, individuals often develop Abs only to a subset of the antigenic sites of the infecting virus.⁸⁶ After one or more reinfections, the Ab response broadens to recognize more antigenic sites. These findings provide a partial explanation for the failure of immunization to induce complete immunity in certain circumstances and also provide a rationale for recommending multiple immunizations to achieve optimal protection. Third, the large size of immunoglobulin (Ig) molecules often precludes direct access to functional sites on viral surface proteins, such as the receptor binding site, sites with enzymatic activity, or fusion peptides. Such domains can be buried within the protein structure of the surface protein or masked by carbohydrate molecules, variable loops, or conformational flexibility.²³⁶ As a consequence, the critical sites vulnerable to Ab-mediated neutralization can remain relatively inaccessible. Therefore, Abs usually exert their antiviral activities by mechanisms other than direct inactivation of functional sites on the surface proteins. Instead, Abs most often act directly or indirectly to impede attachment of virus to the cell or to prevent uncoating of virus that has attached to or entered the cell. Unfortunately, effective Ab-mediated protection against virus infection is not always achievable. For example, the Ab response to the envelope glycoprotein of human immunodeficiency virus (HIV) evolves over time similar to the response elicited by repeated infections with parainfluenza virus; however, the elaboration of the envelope-specific Ab repertoire is not able to keep pace with the rapid genetic evolution of the virus. The Abs present at a given point in time may neutralize virus isolated from the subject 6 months earlier, although they have very low neutralization activity against current strains that have mutated extensively.³⁶⁸ Sufficiently high levels of passively administered broadly neutralizing mAbs can prevent simian-human immunodeficiency virus (SHIV) infection of macaques in some settings.²⁶⁵ SHIV is a chimeric lentivirus with the internal and regulatory proteins of simian immunodeficiency virus (SIV) and the surface

glycoprotein (gp160) of HIV-1. However, immunization with currently available HIV-1 envelope Ags does not elicit Abs with these neutralizing properties. There are also examples from Ebola virus,⁴⁴⁵ vaccinia virus,^{31,271} and lentivirus³⁹³ models that demonstrate settings in which both Ab and T-cell responses contribute to vaccine-induced protection. Ags for eliciting T-cell responses can be surface proteins, internal structural proteins, or regulatory proteins. For example, the major CD8 T-cell responses to HIV-1 are directed toward Gag, Pol, and Nef proteins.³⁶ Often, the nucleocapsid and matrix proteins of viruses are found to encode important cytotoxic T lymphocyte (CTL) epitopes,⁴²⁸ perhaps because they are typically more abundant, more conserved, and produced earlier in the virus life cycle than surface proteins.

VACCINE-INDUCED IMMUNITY

Immune mechanisms have evolved to protect the host against a diverse assortment of viral pathogens. Viruses and their hosts co-evolve; viruses find ways to evade host defenses, and hosts adapt mechanisms to control virus replication. The host is equipped with mechanical barriers and innate immune mechanisms that can limit the initial encounter with a virus and adaptive immune mechanisms that provide immunity against repeated exposures to the same viral pathogen. Although innate responses are important during the induction phase of vaccine-induced immunity, humoral and cellular adaptive responses are the primary effector mechanisms of protection from future infection (e-Fig. 14.4). Humoral and cellular responses are complementary in that Ab is able to prevent infection by free virus; however, when infection occurs despite innate defenses and the presence of neutralizing Abs, T-cell responses are designed to eliminate virus-infected cells and diminish the subsequent release of infectious virus. The repertoires of BCRs and TCRs have also evolved complementary strategies to deal with genetic variation in viruses. For a given antigenic site on a viral protein, most hosts can expand a B-cell population that can recognize the structure. This leads to population-wide protection against reinfection with a particular virus. In turn, this forces antigenic change in the virus, often resulting in a new serotype, which is not recognized by the original Ab. Therefore, the host has to start over in the generation of Ab-mediated type-specific protection. In contrast, T-cell epitopes are not shared between hosts unless they happen to share the same major histocompatibility complex (MHC)-restricting allele. Consequently, each infected host generates T-cell responses against a limited number of viral epitopes that are largely determined by human leukocyte antigen (HLA) haplotype. Unlike Ab epitopes, T-cell epitopes are often cross-reactive between viral serotypes and thereby provide the Ag-experienced host with partial protection against new virus strains until the Ab responses can adapt (e-Fig. 14.5).

When virus breaches the physical and innate barriers and infects a target cell, several rounds of replication may occur in a primary infection before the adaptive immune responses are established (e-Fig. 14.6A). Depending on the virus, this may result in spread to the target organ of disease and/or establishment of a persistent infection. The timing, magnitude, location, and qualitative features of the host response will determine the clinical outcome of infection. In persistent virus infections

(e.g., HIV, hepatitis C virus [HCV], or herpes simplex virus [HSV]), the disease manifestations are often congruent with virus replication. In self-limited infections (e.g., influenza, respiratory syncytial virus [RSV], or dengue), the bulk of disease is often after the peak of viral replication, although in some cases disease can also be temporally associated with viral load (e-Fig. 14.6B). In cases of persistent or latent virus infection, the immune response attempts to limit virus replication or spread. Control of the magnitude and composition of complex immune responses is critical to avoid immunopathology. Fortunately, there is considerable redundancy inherent in immunity to viruses. For example, while CD8 T cells are the primary mediator of cytolytic activity, this function also can be accomplished by complement or by NK cells targeting the Fc portion of Ab labeling virus-infected cells. Another well-studied example of this redundancy involves CD4 and CD8 T cells, either of which are sufficient to achieve clearance of virus, as shown in studies of influenza A virus or poxviruses in mice. Animals lacking CD8 T cells readily clear influenza virus or vaccinia virus from the lungs, indicating that CD4 T cells and Ab are sometimes sufficient for viral clearance.^{271,390} The therapeutic efficacy of Abs acting in the absence of other immune functions has also been demonstrated in experimental influenza A virus infection of mice. Abs to the influenza A virus surface HA have cleared virus from the lungs of persistently infected severe combined immunodeficiency (SCID) mice.³⁹⁰ Whereas CD8 T cells are specialized in recognition and clearance of virus-infected cells, CD4 T cells (plus the Abs secreted by B cells for which they provide help) and CD8 T cells can mediate viral clearance independently. The relative importance of these two arms of the immune response varies in the resolution of particular virus infection. Even in the case of lymphocytic choriomeningitis virus (LCMV), the prototypic arenavirus, which in the murine model is a paradigm for CD8 T-cell-mediated immunity, Abs and CD4 T cells each make a significant contribution to the resolution of primary virus infection.²¹ In general, Abs are the major mediator of resistance to reinfection with virus, and CD8 T cells are the major mediator of clearing virus-infected cells. CD4 T cells are critical for the induction of robust Ab responses and important for “helping” CD8 T-cell responses; they also have potential for direct antiviral effector functions. Abs work best when the target organ for disease pathogenesis is distinct from the initial site of infection, particularly in cases where the virus has to travel through the bloodstream to reach its target organ. For example, in diseases such as measles, polio, hepatitis, or the viral encephalitides, where viremia must be established to cause disease, it often takes very small concentrations of pre-existing Abs to achieve clinical protection. Because it is rarely possible to elicit sufficient Ab through vaccination to prevent virus infection completely, other immune mechanisms must contribute to vaccine-induced immunity.

One reason why live virus vaccines are so effective is that many components of both innate and adaptive immunity are engaged. The protein, nucleic acid, carbohydrate, and lipid components of viruses induce innate responses through a variety of toll-like receptor (TLR) and other pathogen-associated molecular pattern (PAMP) receptor interactions. Virus infection induces all components of the adaptive immune response—Ab responses are induced against viral surface proteins in their native conformation, CD4 T cells are stimulated,

and importantly, viral proteins are produced in the cytoplasm, processed, and presented in MHC class I molecules for induction of CD8 CTL responses. All of these responses are needed to achieve the optimal clinical outcome from a virus infection. Abs and innate defenses provide the initial barrier to infection by neutralizing incoming virus. After the virus has infected a cell, however, it is then incumbent on the innate defenses to control the virus until the adaptive cellular immune response can be activated, expand, and traffic to the affected tissues. In the immune host, the pre-existing Ab will prevent or limit the number of infected cells, and rapid mobilization of memory T-cell responses will rapidly clear virus-infected cells (e-Fig. 14.7A). Although cytolytic T cells are potent in their ability to clear virus-infected cells and reduce the spread of virus, it comes at a cost. The T-cell response can injure the host as it clears the virus and is thus tightly regulated. Turning the immune response off is just as important as turning it on for the well-being of the host. The system is biased toward controlling inflammation, and that may be a reason why the T-cell response is regulated to require several days to activate and expand. Influencing the events in this period between virus exposure and the fully activated primary T-cell response is one of the key factors in determining the efficacy of vaccine-induced immunity, for which the goal is to prevent, abort, or control virus infection to avoid clinical disease (e-Fig. 14.7B).

Vaccine-Induced Cellular Immunity

MHC class I-restricted CD8 cytotoxic CTLs and MHC class II-restricted CD4 helper T cells (Th cells) each function independently as Ag-specific antiviral effector cells (Chapter 9). Intact cellular immunity is critical for protection against some types of viruses. For example, most herpesvirus infections become more severe and destructive when cellular immunity is compromised. Vaccinia can cause progressive tissue invasion in persons with cell-mediated immune deficiencies. RSV and parainfluenza type 3 often become lethal infections in the setting of SCID or in patients who have undergone bone marrow transplantation. In contrast, patients with Ab deficiencies experience more severe disease only from selected viruses, particularly picornaviruses.

CD8 T Lymphocytes

CD8 T cells are the major T-cell effectors of antiviral activity. They are often referred to as CTLs, although they can clear virus-infected cells by both lytic and noncytolytic mechanisms. The CD8 TCR recognizes a short peptide derived from an endogenously produced viral protein in the context of the MHC class I β 2-microglobulin heterodimer expressed on the surface of an infected cell. Ag presentation by the MHC class I β 2-microglobulin heterodimer is restricted to viral peptides that are produced and processed during infection. Therefore, the major CTL functions are to eliminate infected cells¹³⁴ or to inhibit virus replication by the elaboration of soluble mediators such as cytokines^{138,299,425} or chemokines.^{77,464} The net effect of CTL activity is to prevent further spread of virus and to terminate infection in cells that are already infected. The importance of CTLs in recovery from virus infection is indicated not only by the association of cellular immune deficiencies with severe virus infection but also by the diverse strategies that viruses use to escape from CTL immunity.⁴⁰⁸ Viruses such as herpesviruses, poxviruses, and lentiviruses in particular have evolved

strategies for interfering with Ag presentation pathways or CD8 T-cell effector molecules that compromise CTL activity. Lentiviruses are prototypes for escaping CTL killing by genetic variation and selecting mutations of key epitopes. CD8 T-cell activity is critical for controlling SIV replication.³⁹³ When that control is mediated by a dominant CTL response to a single epitope, immune control of viremia is lost when that epitope is altered by mutation.²⁴

Although CTL antiviral activity generally is associated with the clearance of virus and the reduction of virus-associated pathology, disease enhancement can be observed under experimental conditions in which CTLs have been transferred passively to virus-infected recipients.^{58,121,293} Therefore, one can view the immunopathology caused by the T-cell response to virus infection as the cost for virus clearance. A major goal of vaccination is to induce CD8 T cells with properties to clear virus-infected cells efficiently and rapidly to minimize the immunopathology associated with virus infections.

The time course of CTL activation during virus infection in the lungs is consistent with its important role in clearance of primary virus infection. Primary CD8 CTL activity in lung peaks at about day 7 during acute virus infection, such as that produced by influenza A virus, LCMV, or RSV.^{14,134,209} The naive CD8 T cell in the LCMV model undergoes a process of activation and expansion during that time of 10,000- to 50,000-fold.⁴⁰ After about 40 days, the level of detectable virus-specific memory CD8 CTL is about 10% of that during acute infection and is then maintained at constant levels for years even in the absence of Ag.²⁰⁹ Each successive virus infection adds an expanded set of specificities to the pool of memory CD8 T cells over time and shapes the capacity of the host to respond to selected viruses.^{398,399} The memory CD8 T-cell pool not only has an increased precursor frequency against virus-specific epitopes; those cells also have a faster response time. The influence of CD8 CTL memory on the outcome of a subsequent virus exposure can be subtle and may depend on the absence or presence of other components of the immune response. For viruses in which a significant component of the clinical syndrome is related to the immune response clearing the virus, having memory CD8 T-cell responses will result in reduced virus replication a day or two earlier than in primary infection, an earlier peak illness time point, and a lower peak magnitude of illness.^{138,168} In a setting where there is a large viral load (e.g., high inoculum challenge) and an absence of pre-existing neutralizing Abs to diminish the number of virus-infected cells, a large CTL response or infusion of excessive CD8 T cells can result in more severe disease.⁵⁹

To date, T-cell response has not been used successfully as a correlate of immunity that is the basis for licensure of a vaccine. However, the incorporation of CTL epitopes into viral vaccines has been considered valuable because of the important role that CTLs play in clearance of established virus infections. The following characteristics of the CTL response *in vivo* should be kept in mind when designing a vaccine for viruses that may require a CTL component of immunity. First, the ability of a CTL epitope to induce an immune response is MHC dependent. Therefore, the incorporation of large antigenic content is important so that multiple CD8 T-cell specificities can be elicited by the vaccine to allow selection of epitopes by the diverse repertoire of human MHC class I alleles and to reduce the possibility of immune escape through genetic mutation. Second,

when considering vaccine-induced immune protection, CTLs represent a second line of defense against virus infection because they cannot prevent infection and are only effective against infected cells. The localization and phenotype of CD8 T cells can influence the timing and efficiency of viral clearance. For example, immunization of macaques with a recombinant CMV vector that induces a sufficiently high frequency of intraepithelial effector memory CD8 T cells can rapidly clear virus-infected cells following SIV challenge, leading to abortive infection.¹⁸⁵ Technologies such as polychromatic flow cytometry,³⁴⁸ MHC class I tetramer reagents that provide the means to sort epitope-specific T cells,¹⁰⁸ the ability to define functional subsets of T cells with more precision,¹⁰⁵ sequencing technology that allows single-cell clonotyping of epitope-specific cells, and correlation of the TCR-peptide-MHC biophysical interaction and structure with T-cell functions³⁷ may provide the necessary information to harness CD8 T cells more effectively in the future.

CD4 T Cells

CD4 T cells provide a variety of functions, including help to B cells and CD8 T cells, thereby augmenting the two major effector mechanisms of the adaptive immune response. CD4 T cells can also mediate direct antiviral activity *in vivo*^{188,262,296,426}; however, this is not their major function as it is for CD8 T cells. The MHC class II, $\alpha\beta$ heterodimer—the restricting element for CD4 T cells—is present predominantly on antigen-presenting cells (APCs) such as dendritic cells, macrophages, monocytes, and B cells. However, during inflammation, even epithelial cells can express MHC class II molecules, and CD4 T cells have been shown to have compensatory viral clearance capacity in the absence of CD8 T cells. In addition to having antiviral and helper activity, CD4 T cells can mediate immunopathology^{12,279,422} and have been shown to have regulatory and other specialized functions⁴⁷⁶ (e-Fig. 14.8).

The CD4 T-cell response to certain internal viral proteins may play a cooperative role in the development of effective resistance by augmenting the Ab response to a major surface protein.³⁸⁰ For example, immunization with the membrane (M) protein of influenza A virus can prime for a subsequent augmented Ab response to the HA glycoprotein on virus particles.³⁸⁰ In this manner, the entire CD4 T-cell repertoire developed against the viral surface and internal proteins can be called into play to amplify a B-cell response to a surface protein. Because MHC class II–restricted T-cell epitopes tend to be more numerous on viral proteins than MHC class I–restricted epitopes,²³⁴ the repertoire of CD4 T cells capable of augmenting the Ab response is large. Although CD4 T cells are typically thought to provide indirect helper functions to improve CD8 T-cell and Ab responses, there is evidence that they may also contribute direct effector functions to virus clearance. CD4 T cells have been shown to have cytolytic function against HIV.³²⁹ Viral Ags can induce a protective immune response mediated by CD4 T cells in the absence of B cells or CTLs,²⁶² indicating that this arm of the immune response can make an independent contribution to resistance against both acute and chronic virus infections.¹⁸⁸ CD4 T cells also secrete IFN- γ , tumor necrosis factor (TNF)- α , and other soluble factors that may directly inhibit virus replication.^{322,323} For these reasons, it is advisable to include virus Ags capable of inducing CD4 T-cell responses in viral vaccines to achieve maximal

immunogenicity. The processes involved in CD4 T-cell memory induction are distinct from the processes involved in establishing CD8 T-cell memory responses³⁹⁷ and more complex in some ways. Ag processing and presentation in MHC class II molecules can occur through the endocytic pathway; therefore, unlike CD8 T cells, CD4 T cells can be induced by killed virus vaccines or even purified proteins and do not require live virus or gene delivery approaches to allow Ag to reach the cytoplasm. CD4 T cells do not proliferate to the same extent as CD8 T cells during primary infection, and those producing IFN- γ do not survive as long-lived memory cells.⁴⁷²

CD4 T cells have traditionally been considered “helper” T cells (Th), and indeed their activities can improve the potency of B-cell and CD8 T-cell responses. In addition to the original subsets Th1 and Th2, there are now many subpopulations of T cells defined with distinct functions, some of which can alter or diminish effector function. For example, CD4+FoxP3+ T cells have been found to have inhibitory effects on other immune effector functions, hence the name T-regulatory cells, or Tregs.³⁰ The extent to which vaccines induce CD4+FoxP3+ T cells with T-regulatory activity is not well defined. In addition, there are other subsets of CD4 T cells that produce interleukin (IL)-17 and other proinflammatory cytokines that have been associated with immune-mediated pathology but are also important for immunity against some pathogens. More recently, a CD4+CXCR5+ subset of CD4 T cells designated T-follicular helper (Tfh) cells has been identified that resides primarily in lymph node germinal centers and promotes B-cell memory and plasma cell differentiation.⁹⁵ The role of each CD4 T-cell subset in vaccine-induced protection and optimal balance is an area that needs more investigation.

Vaccine-Induced Humoral Immunity

The correlates of immunity that have been established to date for licensed viral vaccines have been associated with Ab responses. In fact, the rationale for development of many of the vaccines in use today was the evidence derived from clinical trials of Ab treatments showing that Abs could mediate protection against disease. Passive transfer studies established the efficacy of Abs in preventing or treating virus infections and disease caused by a wide variety of viruses belonging to diverse RNA or DNA virus families that include the orthomyxoviruses, paramyxoviruses, alphaviruses, flaviviruses, arenaviruses, lentiviruses, picornaviruses, hepadnaviruses, and herpesviruses. Examples of the licensed clinical use of Abs include preparations for hepatitis A and B viruses, measles virus, poliovirus, VZV, rabies virus, RSV, and cytomegalovirus (CMV). A large number of new human mAbs have been discovered in the past decade, and some are under development as prophylactic agents.

A long history of Ab treatments laid the groundwork for current vaccine strategies. Commercial human gamma globulin, which usually contains 16% to 18% immunoglobulin G (IgG) is highly effective in preventing hepatitis A disease and was used widely for that purpose during the past 50 years, even though only a small proportion of the Abs in the preparations are specific for that virus.⁸⁹ Currently, inactivated or recombinant protein vaccines for hepatitis A are licensed and thought to work by inducing serum Abs to the virus. A historic double-blind, prospective clinical trial performed in 1951–1952 showed that human IgG also was effective in preventing

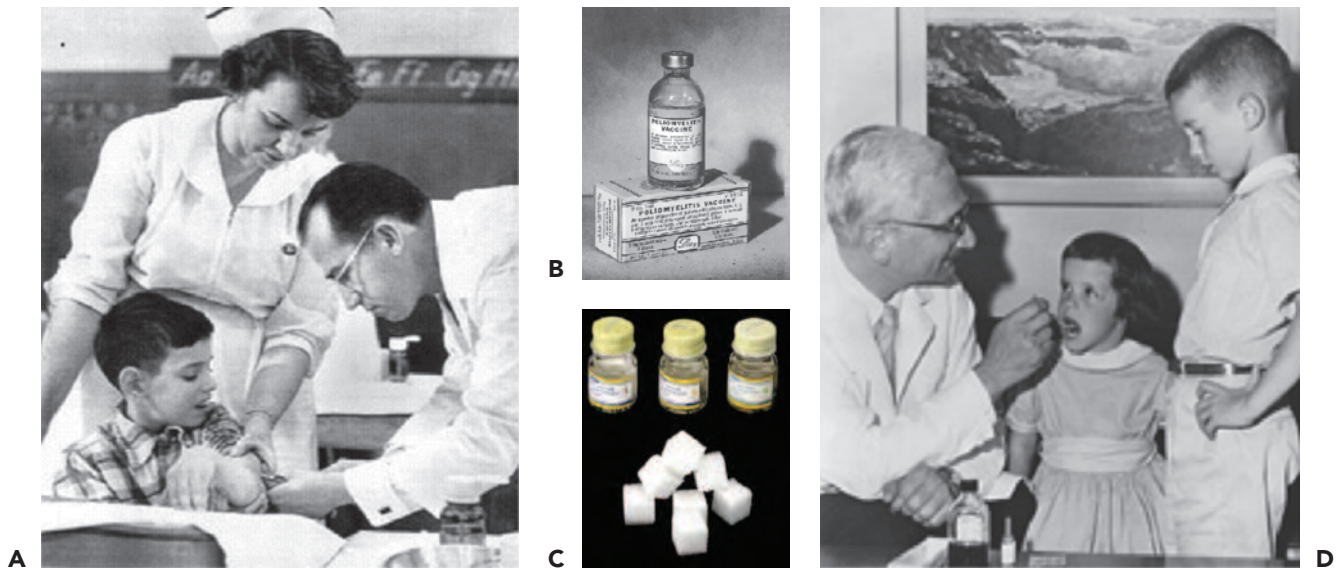


FIGURE 14.1. Polio vaccine development. The development of vaccines for polio brought the process of vaccine development into the public eye and was the first licensed vaccine that relied on mammalian cell culture. Jonas Salk (**A**) spearheaded the development of the inactivated polio vaccine (**B**). The development of the oral polio vaccine (**C**) was headed by Albert Sabin (**D**). These two vaccines are still in use today and over the past 50 years have nearly eliminated poliovirus, making the goal of global eradication feasible. (**A**, © AP Wide World Photos; **C** and **D** courtesy Hauck Center for the Albert B. Sabin Archives, Henry R. Winkler Center for the History of the Health Professions, University of Cincinnati.)

paralytic disease caused by poliovirus.¹⁸¹ Intramuscular inoculation of 0.3 mL of IgG/kg conferred significant resistance that lasted for at least 5 weeks. This seminal observation showed that Abs alone can confer resistance to poliomyelitis and suggested that vaccines inducing such Abs might protect against the disease. Subsequent field trials of inactivated vaccines by Salk and live attenuated vaccines by Sabin validated this view, leading to implementation of universal immunization against poliovirus and ultimately the current worldwide campaign for elimination of polio disease (Fig. 14.1). Before the live measles virus vaccine was licensed for routine use in immunization of young children, human IgG was used for passive prophylaxis and was the mainstay for prevention of disease. IgG given at a dose of 0.5 mL/kg prevented measles, even when administered to exposed individuals as late as 3 days after inoculation with virus.²⁰² Human IgG prepared from pooled plasma selected for a high titer of varicella-zoster virus (VZV) Abs (varicella-zoster immune globulin [VZIG]) is licensed in the United States for use in prevention of severe varicella in immunosuppressed children.⁴⁷¹ Most of the exposed immunosuppressed children receiving treatment still become infected; however, most exhibit attenuated disease or subclinical infection. Live attenuated varicella virus vaccine was licensed and recommended for universal immunization in 1995. Human IgG was effective in preventing chronic hepatitis B virus (HBV) infection in high-risk infants following maternal exposure.²⁸ Hepatitis B immune globulin and subunit vaccine are now used in concert to protect infants in this setting.

Preclinical studies in animals suggested that serum RSV-neutralizing Abs above a threshold titer were protective against lower respiratory tract infection, and this observation was validated during a clinical trial of human IgG containing a high

titer of RSV-neutralizing Abs that led to licensure in 1995.^{1,359} Of interest, the level of serum RSV-neutralizing Abs required for reduction of hospitalization was similar to that needed to prevent infection in cotton rats. A second-generation approach—the use of a humanized murine-neutralizing mAb to RSV—was licensed in 1998.² To date, this is the only mAb licensed for prevention of an infectious disease. These clinical applications strongly suggest that neutralizing Abs are an important correlate of protection for RSV vaccines, which are under development. Immunosuppressed patients who have not been infected previously with CMV, a herpesvirus, are at high risk of developing severe life-threatening disease following exposure to the virus, especially when they receive an organ transplant from a CMV-infected donor. Administration of CMV IgG, prepared from pooled units of plasma selected for a high Ab titer, prevents approximately half of the serious illnesses attributable to CMV but does not prevent infection.⁴⁰⁵ This finding is interesting because the mode of action suggests that Abs suppress disease by their action on CMV-infected cells and that Abs with these properties should be a target for CMV vaccines under development.

Mechanisms of Virus Neutralization

Abs may render free virus noninfectious, principally through neutralization of infectivity. Virus-neutralizing activity typically is measured by *in vitro* assays in which Ab and infectious virus are incubated and then inoculated onto cell culture. Residual infectivity is quantified by plaque count, infectious foci, signal from recombinant reporter virus, percent of infected cells detected by flow cytometry or other methods and compared to untreated suspensions. Viruses are neutralized *in vitro* by Abs by a wide variety of mechanisms. Important Ab

characteristics include affinity/avidity, isotype, concentration, Ab-to-virus ratio, valency, state of polymerization, ability to bind the polyimmunoglobulin receptor (pIgR), ability to fix complement, and specificity for the virus protein and epitope of interest. Virus-related factors important to neutralization include general aspects of the replication cycle of the virus under consideration (receptor use, mode of entry into the cell, pH dependence of fusion, site of replication) and individual characteristics of the specific virus strain (antigenic subgroup and amino acid sequence of viral surface proteins). Host factors influencing the mechanism of neutralization include the type and origin of cells and the level of expression of membrane receptors. The most potent mechanisms of neutralization act prior to infection of cells. First, free infectious virions in solution can be aggregated by multivalent Abs directed against surface glycoproteins.^{17,331} The consolidation of multiple infectious virions into an aggregate reduces the number of available infectious units. Second, Abs can inhibit virus attachment to cells, usually by binding directly to virus attachment proteins. Abs to attachment proteins often are poorly neutralizing *in vitro*, effecting only partial neutralization even at high concentration.²¹¹ This effect may be caused by virus heterogeneity owing to a variable extent of glycosylation that occurs during posttranslational processing at the numerous glycosylation sites on these proteins, or because of multiple routes of entry or mechanisms of attachment. Third, viruses can be neutralized following attachment, at the cell surface prior to cell entry, suggesting that immune serum contains Abs capable of inhibiting entry into the cell. For example, Abs to human papillomavirus (HPV) can interrupt infection at two stages of attachment and entry: the first an initial association with the acellular basement membrane that induces conformational changes in the virion and second the association of virus with the keratinocyte cell surface.¹⁰⁴ Some neutralizing Abs decrease infectivity by inhibition of virus–cell fusion at the plasma membrane. Fusion inhibition (FI) activity for some Abs has been inferred from *in vitro* assays in which Ab is incorporated into overlay medium after virus absorption and penetration, and activity measured by inhibition of postinfection cell-to-cell fusion. Additionally, Abs can inhibit the release of progeny viruses from infected cells. There is even evidence that some influenza virus–specific Abs inhibit virus replication by preventing the primary uncoating of virus that is associated with low-pH membrane fusion in the endosome or at the step of the secondary uncoating of the virion core structure—a step required for primary transcription of the virus genome.^{330,332} Our current understanding of the induction of such Ab functions is not sufficient to allow us to design specific vaccine strategies to induce them.

Structural and Biochemical Features of Antibodies Important to Affinity

Abs induced by primary infection or immunization are generally of low affinity for the target Ag. Affinity maturation of Abs is mediated by somatic mutation of the genes encoding B-cell receptors, which occurs in germinal centers, most prominently following second infections or booster doses of vaccine. Somatic hypermutation (SHM) coupled with B-cell selection results in the promulgation of high-affinity Abs in the antiviral repertoire. Increases in affinity likely increase neutralizing potency for most antiviral Abs directed to surface proteins. Although the molecular basis for affinity maturation during

secondary responses was not understood during the development of early vaccines, the enhanced efficacy of multiple-dose vaccine regimens in clinical trials pointed the way to a practical use of this principle. Most licensed viral vaccines are administered using multiple-dose regimens to enhance the quality, magnitude, and durability of the immune response.

Increased affinity is widely believed to confer enhanced functional competence on Abs. Work using HIV-1–specific Abs derived from combinatorial phage display Ab libraries demonstrated improvements in neutralization associated with affinity maturation caused by SHM.⁴²⁷ The fusion (F) protein—the major protective Ag of RSV—has at least four antigenic sites, each with a differing capacity as a target for virus neutralization.²⁹ Recombinant derivatives of a humanized RSV mAb containing random mutations in the combining site have shown that mutations enhancing the association rate improved neutralization and efficacy, whereas mutations improving the dissociation rate did not, despite their contribution to improved steady-state affinity.⁴⁵⁸ Somatic mutations in human rotavirus Abs have been shown to enhance affinity and inhibitory function, with individual mutations in Ab combining sites conferring several hundred-fold increases in affinity and function.²¹¹ The broadest and most potent human Abs to HIV and influenza virus isolated to date are distinguished by exceptional numbers of somatic mutations in the Ab sequences (on the order of about one-third of nucleotides altered).^{467,474} Hypermutation introduces not only point mutations that enhance function, but in some cases nontemplated insertions of sequences encoding new amino acids that mediate neutralization.²³³ In the vesicular stomatitis virus (VSV) mouse model, investigators have shown only modest increases in the affinities of hypermutated Abs compared with those in germline configuration over the course of infection and after repeated booster injections.²¹⁰ The VSV G glycoprotein is unusual in that it contains only a single immunodominant antigenic site that reliably induces neutralizing Abs. Neutralization activity also is determined by the number of times the neutralizing determinant is displayed on the surface of the virion, and some studies suggest that virion particles may be dynamic in their display of epitopes.¹¹² Understanding the relationship of affinity and neutralization is needed so that we can better appreciate the B-cell response to exposure with foreign Ags in a physiologic context. Furthermore, elucidation of the role of affinity in antiviral Ab function is critical for designing new active and passive immunization strategies against pathogenic human viruses. The absence of somatic mutations in the virus-specific Ab sequences of infants probably accounts for the difficulty in inducing high-affinity, high-potency Abs in this population after a single vaccination.⁴⁴⁸

CDR3 Loops of the Antibody Variable Region in Antigen Binding

A combining site formed by the variable heavy (V_H) and variable light (V_L) domains of the Ab fragment (Fab) mediates Ag recognition. Both the V_H and the V_L chains have three noncontiguous linear sequences of greatest variability—CDRs—that result in flexible loops in the Ig protein and form the classical Ag binding site. HCDR3 shows the greatest variability in terms of length and sequence, particularly in human Abs. Several crystal structures of Abs have demonstrated that human CDRs tend to adopt a limited number of canonical conformations, with the exception of HCDR3.⁷⁵ In each of the crystal

structures of human Abs complexed with Ags, HCDR3 has been shown to make important contacts in every case.^{23,401} Diverse CDR length and gene family usage have been shown to be essential features of immunological health and functional antiviral immune responses.^{153,228,389} Vaccinations that induce a broad Ab repertoire that is rich in CDR diversity are more likely to succeed than highly focused vaccinations inducing limited Ab repertoires. Although not universal, long HCDR3 regions are common in highly active Abs to HIV, and they may facilitate high-affinity binding to complex viral antigenic sites. For example, the crystal structure of the 17b mAb complexed with CD4-triggered HIV gp120 reveals a complex interaction that is accomplished only by the unique long HCDR3 region of this Ab.²³⁵ High-throughput screening for broadly reactive high-potency HIV-neutralizing Abs has isolated two unusual Abs with the longest CDRs ever identified,⁴⁴⁰ and the HCDR3s of these Abs possess fascinating secondary structural elements.³⁴⁶ In contrast, identification of another broadly neutralizing mAb specific for the CD4 binding site has a CDR3 loop of only 13 amino acids, although the variable region of the heavy chains is more than 30% somatically mutated from germ line.⁴⁵⁹

The Role of Isotype in Additional Mechanisms of Antibody-Mediated Virus Neutralization

The complement system is activated by the classical (Ab-dependent), alternative (Ab-independent), and lectin-binding pathways. Viral neutralization by specific Abs is concentration dependent and is often enhanced by complement activation. It has been reported that even apparently “nonneutralizing” Abs can acquire neutralizing activity with the addition of complement—for example, a nonneutralizing RSV murine mAb was shown to exhibit complement-enhanced neutralization *in vivo* in the presence of complement.⁹² Complement fixation is mediated by the C_H2 region of the Ab Fc domain of immunoglobulin M (IgM), IgG1, and IgG3 subclasses in humans and the IgM, IgG2a, and IgG2b subclasses in mice.³²⁸ Understanding the role of complement in enhancing the functional capacity of Abs to neutralize viruses has broad implications for vaccine design, because the cytokine milieu induced by infection or immunization affects the isotype and subclass of the Ab response to the viral Ags by activating selective promoters in the appropriate Ig heavy chain locus. B cells triggered in an environment with high IFN- γ concentrations tend to produce IgG1 and IgG3 Abs. In contrast, in the presence of a dominant IL4 response, B cells produce immunoglobulin E (IgE) and IgG4 Abs. IgE participates in allergic diseases, and it is generally considered desirable to avoid strong IgE responses following vaccination. The Fc region of Abs also mediates some cellular activities, termed *Ab-dependent cell-mediated cytotoxicity* (ADCC), which are discussed below.

Structural Features of Antigens Critical for Inducing Efficient Antibody Interactions

The conformation of epitopes on viral surface proteins often is critical to the ability of vaccine Ags to induce or bind neutralizing Abs. Most neutralizing Abs bind to conformationally dependent epitopes on surface proteins. Linear peptide vaccine candidates derived from the sequence of the natural Ag rarely recapitulate the conformation of the protective Ag, which

is why most peptide-based vaccines are poor immunogens for humoral immunity. Many subunit Ags comprising purified or recombinant viral proteins that have been developed as vaccine candidates contain the full polypeptide sequence of the Ag; however, the protein emerges from the purification process in a conformationally relaxed state that resembles the natural Ag only in part. Such Ags may induce high levels of Abs that bind the vaccine Ag but low levels of Abs that bind or neutralize virus.³⁸⁵ Some viruses appear to present highly antigenic surface loops to the immune system that are structurally non-essential, causing immune focusing on hypervariable domains of viral proteins, such as the influenza HA variable loops surrounding the sialic acid receptor-binding domain or the third variable (V3) region of HIV gp120. Recent studies, however, reveal that some subjects make broadly neutralizing Abs that recognize these hypervariable domains.¹⁵⁶ Many structurally critical domains of viral surface proteins are hidden from B cells because they are buried in the interior of viral molecules or oligomers of molecules, or they are exposed only briefly during rapid conformational changes required for virus attachment or virus–cell membrane fusion. For example, viral hydrophobic fusion peptides, receptor-binding domains, heptad repeats, and other critical regions of fusion proteins usually are not accessible to Abs. Most fusion proteins require cleavage by proteases before they are competent to fuse, and Abs to the precursor protein may not bind the cleaved protein in the mature virus particle.³³⁶ Posttranslational modifications of surface proteins, especially glycosylation, can present a challenge for Ab recognition of protein because of the highly varied nature of the glycoprotein population. Deglycosylated protein Ags may enhance induction of protein-specific Ags; however, these Abs may not efficiently recognize glycosylated proteins *in vivo*. Some virus-neutralizing Abs are directed specifically to carbohydrates on virus glycoproteins.⁵⁶ Some broadly neutralizing Abs to HIV, such as PG9 and PG16, recognize a conformational epitope that depends on glycosylation at specific variable loop N-linked sites.¹¹¹ Posttranslational modification of Abs also can affect the interaction of Ab and virus. For instance, several human Abs to HIV gp120 are sulfated in the combining site, which allows the Ag-combining sites to mimic the structure of the virus coreceptor CCR5, which is sulfated.⁷⁴ These Abs inhibit infectivity by binding to the co-receptor-binding domain on HIV gp120.

Antibody Escape Mechanisms

Viruses, especially RNA viruses with error-prone RNA-dependent RNA polymerases, exhibit facility in generating genetic diversity during multiple replicative cycles. During chronic infections, such as those with HBV, HCV, or retroviruses such as HIV, viruses that accumulate amino acid changes in epitopes that are targets of Abs owing to point missense mutations in the corresponding genes can escape neutralization by reduction or loss of binding. Some viruses easily shed surface glycoproteins (e.g., HIV gp120 or Ebola GP)¹⁸⁷ or encode soluble forms of surface glycoproteins using alternative start codons (e.g., RSV G protein),³⁶⁹ in part as a decoy mechanism, targeting Abs away from infectious virions. The immunity induced by experimental vaccine candidates that are narrowly targeted to single epitopes or single proteins are susceptible to rapid viral escape. To avoid such Ab-resistant mutants, optimal vaccines should induce broad humoral responses to a diversity of antigenic sites.

Antibodies Active Against Virus-Infected Cells

Viruses that mature at the cell surface by budding or that insert viral glycoprotein(s) into the cell surface membrane render the infected cell susceptible to lysis by Ab-dependent complement-mediated lysis or ADCC mechanisms. This lysis is mediated by cytolytic cells, including NK cells, neutrophils, and eosinophils, in an Fc region-dependent manner. Ab-dependent cell-mediated virus inhibition (ADCVI) activity is similar but is typically measured with a readout of reduction of virus replication instead of lysis of target cells. Cytophilic Abs can arm leukocytes in the ADCC reaction, which has been shown to be important in the resistance of infant mice to herpes infection and for clearance of a retrovirus.^{87,226} Fc receptor-mediated mechanisms appear important to neutralization of HIV.¹⁹¹ Some herpesviruses encode an IgG Fc receptor homolog, which enables the virus to evade ADCC by binding Abs, especially virus-specific Abs if they bind virus proteins on infected cells, suggesting the importance of the ADCC effector or other Fc-mediated mechanisms *in vivo*.¹¹⁵ RSV ADCC activity mediated by NK cells has been detected in monkey or human peripheral blood using Abs in serum.^{216,277,395} Because infection of cells must occur for these immune mechanisms to act, these mechanisms represent a second line of defense against infection or disease.

Inadvertent Induction of Antibodies to Host Antigens

Immunization of monkeys with formalin-inactivated SIV produced in human cells induced Abs directed at Ags originating from the cells used to produce the virus.⁴⁰⁷ These anticellular Abs can neutralize the infectivity of SIV produced in human cells, although not virus produced in monkey cells, by interacting with human Ags incorporated into the virion outer membrane. This form of neutralization, which is mediated by complement-mediated virolysis, can yield an overestimate of vaccine immunogenicity. Hence, an effort should be made to avoid immunization with virions into which foreign host cell Ags have become incorporated.⁴⁰⁷ In some cases, even the native virion proteins may mimic structures associated with host cell proteins. For example, many of the original broadly neutralizing mAbs specific for the HIV-1 gp160 envelope glycoprotein have been shown to cross-react with host cell proteins.¹⁹⁰

Inhibitory Antibodies That Lack Classical *In Vitro* Neutralizing Activity

Some Abs that do not exhibit neutralizing activity *in vitro* can mediate resistance to virus infections, although typically they are less effective than neutralizing Abs.²⁹⁷ Such Abs can be directed against Ags on the surface of virus particles or against virion or nonstructural proteins displayed on the surface of infected cells. *In vivo*, these Abs mediate antiviral activities by several different mechanisms. First, virus particles coated with nonneutralizing Abs can undergo accelerated clearance from the bloodstream. Second, Abs directed at surface glycoproteins such as the NA of influenza A virus or the HA-esterase protein of coronavirus (CoV) can decrease the level of virus replication in the target organ, presumably by inhibiting the release of virus from infected cells.^{46,466} Third, Abs can lyse virus-infected cells via IgG Fc-dependent mechanisms.^{92,391}

Antiviral Activity of Antibodies *In Vivo*

Resolution of infection generally depends on CTLs, which reduce infection by lysing virus-infected cells. However, virus-specific Abs also can exert a therapeutic effect. Dramatic clinical therapeutic effect of viral Abs has been observed in patients with Argentine hemorrhagic fever, which is caused by Junin virus (an arenavirus). This disease has a high mortality; however, death can be prevented with a preparation of pooled human serum with a high titer of Junin virus-neutralizing Abs administered within 8 days of onset of symptoms.^{123,261} Mucosal virus infections that are limited to the cells that line the lumen of the respiratory tract also can be cleared by specific Abs that are delivered by parenteral inoculation or by direct instillation into the lungs. RSV immune globulin or RSV-neutralizing Abs protect against severe lower respiratory tract disease.^{1,2} These Abs also reduce established infection in animal models; however, therapeutic trials in humans have not revealed a clinical benefit of treatment of active infection. Influenza A virus HA-specific IgG Abs cleared the lungs of influenza-infected SCID mice in the absence of other recognized immune functions.³³⁴ Abs can exert a therapeutic effect in privileged sites where CD8 CTLs are not active. Central nervous system (CNS) neurons are not good targets for CD8 CTLs, because they have highly regulated expression of class I MHC glycoproteins. Nevertheless, in SCID mice, persistent infection of brain neurons by the alphavirus Sindbis virus can be cleared rapidly by parenteral inoculation of envelope-glycoprotein-specific Abs without causing obvious cell damage.²⁴⁸ Some patients with underlying X-linked agammaglobulinemia complicated by chronic enteroviral meningoencephalitis have been treated successfully with human IgG that contains Abs specific for the infecting virus. A therapeutic effect was achieved with IgG administered by the intravenous or intraventricular routes.^{118,275} Failure of human IgG to alter chronic enteroviral meningoencephalitis in some patients suggests that the concentration of enterovirus-specific Abs in human IgG is often inadequate.

Mucosal Antibodies

Abs have been shown to function within mucosal epithelium.^{268,269} Dimeric immunoglobulin A (IgA) secreted at the basolateral surface of cells or from plasma is transcytosed to the apical surface in association with polymeric Ig receptor to which dimeric IgA with J chain binds. During transcytosis through cells infected with the Sendai virus, virus-specific IgA can reduce intracellular virus and infectivity. The mechanism responsible for this effect is not clear, although it is possible that the endocytic pathway used for IgA transport and the exocytic pathways used for transport of viral glycoproteins intersect at some location within the cell, possibly the apical recycling endosome.¹⁴² Similar studies with IgA Abs to rotavirus and measles virus suggest that IgA molecules may inhibit viruses inside epithelial cells or after transcytosis.^{50,463} Induced IgA molecules to HIV gp41 appear to block transcytosis across genital epithelial surfaces.⁴² The FcRn receptor for IgG also is present in some epithelial cells and could participate in transport or retention of IgG at mucosal surfaces.

Abs present in the systemic circulation efficiently protect internal organs against viruses that are introduced directly into the bloodstream (e.g., HBV and HIV) or that spread via the bloodstream from primary sites of replication such as the respiratory or gastrointestinal mucosa. As mentioned previously,

diseases produced by viruses that fall into the latter category (i.e., measles, polio, hepatitis A, rubella, smallpox, and varicella) can be prevented or modified by Ig prophylaxis, often with small amounts of Ab that are difficult to detect in the blood of recipients. Such Abs are relatively easy to induce by parenteral immunization with live or inactivated virus vaccines. In contrast, Abs present in the systemic circulation do not provide efficient protection against viral diseases that are limited to mucosal surfaces unless these Abs are present in high titer. This is because only a small proportion of such Ig molecules traffic from the plasma to the luminal surface of the mucosal epithelium by transudation. Therefore, to prevent viral diseases that are limited to mucosal tissue, two different strategies can be employed. The first is to induce Abs such as secretory IgA at the mucosal site by virus replication or immunization at that site. The second, and less efficient, strategy is to stimulate a high titer of serum IgG Abs that can protect mucosal surfaces following transudation.

When IgG Abs present in the blood are passively transferred to mucosal surfaces by transudation where they can exert antiviral activity, there is a gradient regarding the ability of serum IgG-derived Abs to restrict virus replication on mucosal surfaces. This gradient is less in lung than it is in the nasopharynx. IgG Abs, if present in the serum in high enough titer, can provide almost complete resistance to pulmonary replication of RSV; however, resistance in the upper respiratory tract is more difficult to achieve. Transudation of IgG also probably occurs in the intestine, especially in inflammatory states, although IgG is more easily subjected to proteolytic degradation in the intestinal lumen than in the respiratory tract. Immunization of the female genital tract with a live virus vaccine can induce the local synthesis of IgG antiviral Abs such that the specific activity of mucosal IgG Abs significantly exceeds that of serum, indicating that IgG Abs present on a mucosal surface can come either from serum via transudation or from IgG Ab-secreting cells present in the submucosal tissues.³⁴⁰ Although passively transferred IgG Abs can provide mucosal immunity in the lower respiratory tract, the major mediators of resistance to virus infection of the upper respiratory tract, the larger airways of the lungs, and the gastrointestinal tract are mucosal Abs, many of which are IgAs selectively transported across mucosal surfaces to exert antiviral effects on the luminal surface. Both nonneutralizing⁵⁰ and neutralizing³⁷⁹ IgA Abs have antiviral effects on mucosal surfaces, with the former active intracellularly during transcytosis and the latter active both intracellularly and on the luminal side of the epithelium. Antiviral IgA Abs function to clear virus infections, to modify the severity of disease on reinfection, and to prevent infection on re-exposure to virus. The level of antiviral IgA Abs, as well as the number of virus-specific IgA-secreting cells, often correlates with resistance to virus infection.^{83,469} The advantages of IgAs in the mucosal fluids are that they are transported more efficiently, they resist proteolytic degradation better than IgG, and the dimeric nature of secretory IgA enhances avidity.

The primary mucosal IgA response peaks within the first 6 weeks after infection and usually decreases to a low, often barely detectable, level by 3 months. The transient nature of the primary mucosal Ab response is a factor in the ability of many viruses to reinfect mucosal surfaces and underlies the need for two or more doses of vaccine to efficiently immunize mucosal surfaces. The virus-specific IgA Ab response is greater

at the site of virus replication or of Ag administration than at distal sites, suggesting that mucosal immunity is best induced by antigenic stimulation of sites directly involved in replication of the wild-type virus.

Adverse Effects of Antibodies

Abs can contribute to the potentiation of disease. For example, heterotypic Abs to the envelope glycoprotein of dengue virus appear to play a role in the severe clinical entity known as dengue hemorrhagic fever/shock syndrome.²³¹ Dengue viruses replicate primarily in cells of the mononuclear phagocyte lineage, and subneutralizing amounts of dengue virus Abs enhance dengue virus infection of these cells *in vitro* by increasing uptake of virus via IgG Fc receptors present on these cells.¹⁸⁰ However, the mechanism by which augmented disease results from enhanced access of dengue virus into mononuclear cells remains unclear.³⁷⁷ Immune complexes were detected in the lung tissues of fatal cases of influenza during the 1957 and 2009 pandemics, suggesting a potential role for enhancing Abs in severe influenza disease.²⁸⁷

OBSTACLES TO IMMUNIZATION IN EARLY LIFE AND IN THE ELDERLY

Immunization of Infants

The developmental immaturity of the immune response to viruses in the very young is seen clearly in the difficulty to immunize neonates and infants.⁴⁰² Young infants are a preferred target population for immunization, because most children present to health systems with the highest frequency at birth or shortly after. A high level of access to infants allows universal immunization, and many viral diseases are most severe early in life. Many obstacles arise, however, in attempts to immunize infants in the first several weeks or months of life against disease caused by viruses.

First, most infants possess maternal Abs that can interfere with response to infection or the infectivity or immunogenicity of primary immunization. Live attenuated measles virus cannot be given effectively prior to 1 year of age, because many infants possess maternal Abs until that time that reduce or eliminate immunogenicity of the vaccine.¹⁰ Ab-mediated suppression can be overcome in some instances by increasing the dose of the vaccine—for example, the inactivated hepatitis A vaccine or a replicating influenza vaccine.^{84,101} The immune suppression by maternal Abs is more potent against B-cell responses than against induction of virus-specific T cells, possibly because the Ab response generally depends more on Ag dose than T-cell responses.⁹⁷

Second, the ability to respond to viral surface proteins with a protective Ab response is acquired gradually over the first year of life, reflecting developmental maturation of the neonatal immune system. Responses of seronegative infants to live measles vaccine remain relatively poor during the second 6 months of life.¹⁴⁴ Responses to live VZV vaccine also are reduced in the first year of life.⁴³⁶ The youngest infants mount Ab responses to experimental mucosal immunization with live influenza, RSV, or parainfluenza virus vaccines that are low in magnitude, quality, and durability.^{84,213,214} The molecular and cellular basis for immunological immaturity is not fully defined. Infant virus-specific Ab sequences are characterized

by marked lack of somatic mutations, resulting in low-affinity Abs of low function.⁴⁴⁸ Infant B cells exhibit limited capability to switch isotype from IgM to IgG2 Abs, and the isotype profile of circulating Abs differs from adults until age 5 to 6 years. Infants possess reduced levels of some components of the complement cascade, especially C8 and C9 that participate in membrane attack complexes, leading to impaired complement-mediated reactions. Infants exhibit a high proportion of transitional immature B cells and poor organization of the splenic marginal zone.

The Ab responses that young infants make to respiratory virus surface glycoproteins are significantly lower in quality than those of adults. Studies have described a dissociation of the level of neutralizing and binding Abs in the very young infant.⁴⁵⁷ This dissociation may be of functional significance, because neutralizing Ab titers correlate well with protective efficacy in studies of protection for viruses. Dissociation between neutralizing and binding Abs also characterized the serum Abs induced by the ill-fated formalin-inactivated RSV experimental vaccine of the 1960s and probably accounts for the lack of protective efficacy of Abs induced by that vaccine candidate.³⁰¹ Ab responses induced by infections of infants are less durable than those of older subjects. Postinfection serum Ab levels to respiratory viruses often wane significantly several months after primary infection, especially if infection occurs at a very young age. Animal models suggest that B-cell priming occurs effectively early in life or in the presence of suppressive Abs^{96,97}; however, the deficient maintenance of long-lived plasma cells in the bone marrow may lead to rapid waning of humoral immunity.³⁴⁹ Reduced humoral immunity to vaccines early in life also may reflect reduced T-helper cell function and altered dendritic cell function. Neonatal CD4 T cells exhibit reduced ability to secrete IFN- γ , reflecting limited secretion of IFN- α and IL-12 by dendritic cells.^{106,155}

Immunization of the Elderly

Obstacles to effective immunization also arise late in life owing to immune senescence. Immune senescence in the elderly is marked by a decrease in the magnitude, quality, and duration of Ab responses and a reduction in functional T cells, especially CD8 T cells. Elderly T cells are distinguished by alterations in cytokine production, co-stimulatory molecule expression, susceptibility to apoptosis, and changes in the level of DNA damage and repair and telomerase length.¹⁴⁵ Chronic virus infection may contribute to immune senescence. For example, CMV-specific T cells progress to a differentiated state in the elderly owing to chronic antigenic stimulation.³⁴⁴ This syndrome has been described as an immune risk phenotype in the elderly, reflecting the accumulation of a high percentage of circulating CMV-specific CD8 T cells at a terminal stage of differentiation that may detract from the flexibility of the T-cell repertoire to respond to other Ags. The CD4 T-cell responses of healthy elderly individuals appear to sustain a good level of function until late in life. Waning immunity to latent viruses may be boosted in the elderly in some cases. Immunization of the elderly with live attenuated varicella virus vaccine reduces the incidence of shingles in that population.³³³

Aging has varying effects on the response to different Ags. For example, the immunogenicity of recombinant hepatitis B vaccine is reduced starting in the fifth decade and is poor in

the elderly.⁴⁵⁵ Ab responses to influenza virus vaccine also are reduced in the elderly. Practically speaking, however, although vaccination in the elderly may be reduced in efficacy and may require frequent boosters or other alterations in strategy, the effectiveness of many vaccines such as influenza virus vaccine are reasonable enough to support vaccination of this group. It also may be possible to improve the immune responses in the elderly by utilizing adjuvants or alternative vaccination strategies. Investigational gene-based vaccines have been shown to be highly immunogenic in older adults compared to the typical age-related decrease in responses seen with many traditional vaccines.²⁴³

GOALS OF IMMUNIZATION AGAINST VIRAL DISEASES

Prevention

The major goals of immunization against viruses are the prevention or modification of disease in an individual and the control of epidemic infection within populations. Most viral vaccines prevent or modify disease without necessarily preventing infection. The ways in which this objective is achieved are diverse. Whereas most viral vaccines are administered before infection occurs, prevention of CNS disease in individuals inoculated with rabies virus by an animal bite can be achieved by administration of a vaccine regimen initiated shortly after infection has occurred. This postexposure prophylaxis is possible because the long incubation period of the infection permits the development of an effective immune response in time to modify infection and prevent disease. Interestingly, even for viruses with a more rapid course, such as variola, individuals can be protected against a fatal outcome if vaccinia virus vaccine is administered up to 4 days after the initial exposure to the virus. This effect may be attributable to augmented innate immune responses or more rapid induction of adaptive immune responses.

The major goal of immunization against rubella virus is the prevention of fetal abnormalities caused by intra-uterine infection. To achieve this goal, both males and females are immunized in the United States. Immunization of males has little direct benefit to the vaccinee other than prevention of the relatively mild illness caused by the rubella virus, and males are not targeted in all national vaccine programs. The fetus of a vaccinated female is protected by immunization of the mother before pregnancy. Herd immunity resulting from extensive immunization of males and females provides some protection to the fetus of an unvaccinated female by decreasing the circulation of virus in the community. An example of herd immunity was seen early after introducing the mumps vaccine. The incidence of mumps declined precipitously before vaccine coverage was significantly expanded. A modest reduction in the number of susceptible persons and transmitters had a significant impact on the epidemic (Fig. 14.2) and e-Fig. 14.2.

In addition to reducing the frequency of susceptible persons and transmitters, some live viral vaccines can improve herd immunity by the spread of vaccine from recipients to contacts. This phenomenon has been observed with oral live poliovirus vaccine³⁸³ resulting in the indirect immunization of a significant proportion of the population and a marked reduction in the incidence of poliomyelitis in both vaccinated and “unvaccinated”

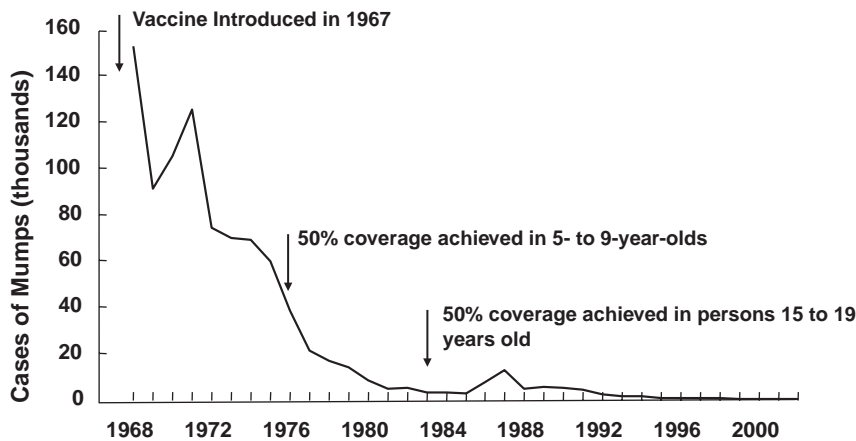


FIGURE 14.2. Incidence of mumps in the United States after introduction of vaccine. The number of mumps cases in the United States began diminishing shortly after the introduction of the vaccine. This effect is considered to be an example of herd immunity, because vaccine distribution and coverage was less than 50% for about 10 years and was not a formal recommendation in the United States until 1976. (Adapted from Cochi SL, Preblud SR, Orenstein WA. Perspectives on the relative resurgence of mumps in the United States. *Am J Dis Child* 1988;142(5):499–507.) Also see e-Fig. 14.2.

individuals. Spread of vaccine virus has been viewed by many as a desirable property because this transmission confers the benefits of immunization on many individuals who were not vaccinated. However, spread of vaccine in the population also raises concern because of the rare occurrence of vaccine-associated paralytic disease among contacts of vaccinees, and immunization of persons without their prior consent presents ethical issues.

Although most vaccines in use today are effective in preventing disease, they usually are inefficient at completely preventing infection. Prevention of infection is a desirable property for specific vaccines such as HBV and HIV. Infection with HBV during the neonatal period is associated with an increased risk of progression to the chronic carrier state and associated with the development of chronic liver disease and hepatocellular carcinoma later in life. Therefore, a major goal of HBV vaccination is to prevent transmission of infection from persistently infected mothers to their infants during the neonatal period. Considerable success in this effort has been achieved through the use of hepatitis B surface antigen (HBsAg) vaccine or hyper-immune HBV antiserum plus vaccine immediately after delivery.¹⁹⁵ HIV is a lentivirus, and the possibility of progression to AIDS can occur many years after initial infection. Therefore, it is desirable to develop a vaccine that induces immunity to prevent infection or result in an abortive or transient HIV infection.

The vaccine for HPV was licensed based on its ability to prevent cervical neoplasia.⁴ In addition to preventing the acute effects of virus infection, the HBV vaccine is also given with the goal of preventing hepatomas.¹⁹⁵

Immunotherapy

Herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) and VZV produce latent infections in sensory ganglia that periodically reactivate, resulting in development of new lesions and increased opportunity for transmission of virus. Therefore, a goal of immunization of persons previously infected with these viruses is to prevent or ameliorate recurrent disease caused by reactivation of their latent infection.³⁵ This form of immunization is referred to as immunotherapy. Other illnesses that are candidates for immunotherapeutic vaccines include warts and cancer caused by HPV and chronic hepatitis caused by HBV or HCV. HIV-1 has also been considered

as a target for immunotherapy to prevent the long-term consequences of chronic infection or to reduce the requirements for ongoing antiretroviral drug therapy. Because of the extent of repeated Ag exposure and damage to many elements of the adaptive immune system, it may be difficult to elicit new immune responses to effectively control virus.⁶² Effective immunotherapeutic vaccines for recurrent HSV face a similar challenge because the vaccinees previously infected by wild-type virus are repeatedly reimmunized during each recurrence. However, for a related herpesvirus, VZV, where recurrence is relatively uncommon, immunization with live attenuated vaccine reduces the incidence of herpes zoster and postherpetic neuralgia.³³³

Eradication or Elimination

Appropriate use of a highly effective vaccine can help to eradicate a major disease when humans are the only natural host for the virus and there is no natural reservoir or intermediate host. During the 1970s, this goal of eradication was achieved for smallpox largely through the use of live vaccinia virus vaccine. This campaign ranks as one of the major public health achievements in all history. Two other human viruses have been targeted similarly for eradication, namely poliovirus and measles virus, and significant progress toward these goals has been made for both viral pathogens. The United Nations officially declared eradication of the morbillivirus animal pathogen rinderpest in 2011, after a concerted worldwide eradication program. Unfortunately, eradication of viral pathogens usually is an elusive goal because of setbacks from social instability, anti-vaccine sentiments, the threat of bioterrorism, and the potential for intentional reintroduction or resurgence of previously eradicated viral pathogens. Therefore, it will be prudent to find ways of maintaining immunity to serious virus pathogens even after eradication of virus in the natural reservoir has been achieved. The new technologies that have improved our ability to identify emerging pathogens and to develop biologics for vaccines and therapeutics have also created an intellectual reservoir that is a formidable barrier to true eradication efforts. Elimination of a virus pathogen suggests that epidemic and endemic disease is controlled and no active cases are present. However, setting the goal of elimination acknowledges the possibility of re-emergence and would include maintenance of active vaccine-induced immunity.

HISTORY OF VIRAL VACCINE DEVELOPMENT AND FUTURE PROSPECTS

The history of discovery and development for each viral vaccine is unique and instructional. Although space limitations prevent the full description of each vaccine, some of the major events are outlined. Although each vaccine development story is different, there are common events and themes that have been catalogued in a supplementary table (e-Table 14.1) that includes (a) disease description and virus discovery, (b) vaccine

discovery and/or proof of concept, (c) vaccine licensure or first human use, and (d) unique or unanticipated events.

Origins of Vaccinology

Origin of Live Virus Vaccination

Most viral vaccines were developed as a result of both fortuitous events and empirical findings. The origin of modern vaccinology, and particularly live virus vaccination, is based on the practice of variolization (mechanical attenuation and



FIGURE 14.3. Origins of live attenuated viral vaccines. **A:** Edward Jenner is credited for performing the first vaccine experiment in which he demonstrated that inoculation of a boy with purulent material obtained from a cowpox lesion on the hand of a milkmaid protected him against subsequent challenge with variola. This event launched the formal concept of vaccination and established the prototypical approach for developing live attenuated viral vaccines. **B:** The implementation of smallpox vaccination faced many of the same issues of fear and misunderstanding that are prevalent today and impede the deployment of effective vaccines, as illustrated by a political cartoon published in 1804. **C:** The successful eradication of smallpox yielded many lessons about the value of simple, low-cost, local production of vaccine, as illustrated by the collection of vaccine material from the hide of a cow during the initial phases of the World Health Organization–sponsored eradication campaign. There were also lessons on the importance of product stability and simple delivery approaches. **D:** Once vaccinia was lyophilized, it could be reconstituted on site and was not as dependent on maintenance of the cold chain. The bifurcated needle (**D**) was an innovation that provided a simple standardized approach to inoculation without the need for needles or mechanical devices and allowed vaccination to penetrate remote locations. (**A** and **B**, courtesy of the National Library of Medicine; **C**, courtesy of J. Mohr, World Health Organization; **D**, courtesy of James Gathany/Centers for Disease Control and Prevention.)

intentional low-dose infection) to reduce the virulence of subsequent smallpox infection, which was initiated more than 1,000 years ago in India and China. From this arose the concept of vaccination (using an alternative agent to provide immunity against an infectious disease) and the first vaccine used in humans—live cowpox virus. In 1796, Edward Jenner inoculated 8-year old James Phipps with purulent material taken from a cowpox pustule on the hand of milkmaid Sarah Nelmes and introduced it into an incision on the boy's arm. Jenner subsequently proved that the boy was protected from an inoculation of material from a smallpox lesion. He published the work in 1798 and coined the word *vaccine* from the Latin *vacca* for cow, designating the process vaccination. For 80 years, live virus was transferred almost exclusively from human arm to arm by collection of purulent material. Subsequently, vaccine was propagated on the skin of calves using serial inoculation, resulting in vaccinia virus as the distinct vaccine strain.²⁶⁰ This vaccine was used successfully to eradicate smallpox from the population, and smallpox remains the only human virus infection to be eradicated through a vaccination campaign (Fig. 14.3).

Origin of Inactivated Virus Vaccination

In the 1880s, Louis Pasteur and his colleagues attributed rabies to virus replication to the CNS. Next, they discovered that drying rabies-infected tissue at room temperature attenuated the infectivity of the tissue. The dried rabies-infected tissue was determined to be noninfectious, although serial inoculation of dogs with the tissues provided protection against an otherwise lethal rabies virus challenge. Inoculation of dried rabies-infected CNS tissue was tested in a single human postexposure case in 1885 when Pasteur serially inoculated Joseph Meister, a 9-year-old boy bitten by a rabid dog more than 2 days earlier. The initial inoculations contained highly attenuated virus from rabbit spinal cord dried for 15 days, followed by inoculations with less attenuated virus-infected tissue. In total, the regimen included 13 immunizations over 10 days. The boy was fully protected from disease, representing the beginning of an era in which inactivated vaccines were used for other viral diseases following Pasteur's paradigm of identification, inactivation, and inoculation (Fig. 14.4) and e-Table 14.1.

Critical Events in the History of Viral Vaccines

Since the introduction of vaccinia virus (Chapters 66 and 67), licensed vaccines have been developed for 15 other viral pathogens (Table 14.1). Notable advances in viral vaccine development over the past century include semieradication of many virus infections; processes for optimization of manufacturing cell lines for next-generation vaccines; utilization of human immune correlates to establish efficacy for licensure; and improvements in vaccine safety monitoring, which is important for public safety but also serves to keep safe and efficacious vaccines from unwarranted negative assessment.

Semieradication Secondary to Effective Vaccine Campaigns

Several vaccines have resulted in regional semieradication of virus disease or dramatic reduction in the incidence or morbidity from infection (polio, measles, mumps, rubella, rabies, and VZV). Other vaccines are highly effective and have been reduced to use only for travelers to endemic areas of the world

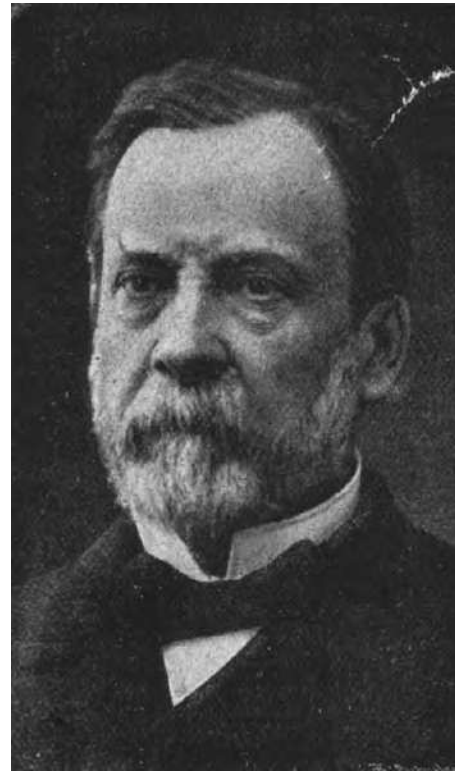


FIGURE 14.4. Origins of inactivated viral vaccines. Louis Pasteur is pictured here as the person responsible for the inactivated viral vaccine concept. His work on the rabies vaccine produced by desiccation of spinal cords from rabid dogs also anticipated the public debates that still occur involving the balance of risk and benefit to the individual and the public from the implementation of vaccines. (Courtesy of the National Library of Medicine.)

(yellow fever, Japanese encephalitis). Licensed vaccines for influenza and hepatitis A and B are highly effective, and their impact on frequency of infection has been improved by new strategies for distribution (Table 14.2).

Modernization of Vaccine Manufacturing

The process of manufacturing a vaccine is complex, and the primary cornerstone in overall production is the substrate used for vaccine growth or antigen production. Early-generation vaccines were often grown in animal organ cells, including adenovirus vaccine in monkey kidney cells and Japanese encephalitis virus (JEV) vaccine in mouse brains. During the second half of the 20th century, modernization of vaccine production included the progression to use of cell lines as substrates, including current JEV vaccine in Vero cells and hepatitis B vaccine in yeast. Influenza vaccine researchers continue to develop U.S. marketed vaccines using chicken eggs as the substrate but have developed cell culture–based substrates that are used effectively in some European countries. The transition to cell-based substrates offers a more efficient, more reliable, and more stable manufacturing process.

Surrogate End Points for Vaccine Efficacy

Traditionally, the assessment of vaccine efficacy has been made by determining whether infection or disease has been

TABLE 14.1 Viral Vaccines Licensed in the United States^a

Virus vaccine	Number of serotypes covered by vaccine	Type of vaccine			Target population	Comments
		Live	Inactivated	VLP		
Adenovirus ^b	2 (types 4 & 7)	+			Military recruits	WT virus in enteric-coated capsules orally to selectively infect the gut; lapse in manufacturing
Hepatitis A	1		+		Travelers, healthcare workers	IM; 2 doses; combined hepatitis A & B vaccine also available
Hepatitis B	1			+	All children	IM or SQ; 3 doses
Human papillomavirus	4 (types 6, 11, 16, & 18)			+	Preteen girls	IM to prevent cervical cancer; 3 doses
Influenza A & B	3 (H1N1, H3N2, & type B)	+	+		Elderly, patients with cardiopulmonary disease, school-age children, others	IM or ID; repeated annually with disrupted virus vaccine, or intranasal delivery of live attenuated vaccine
Japanese encephalitis	1		+		Travelers to endemic region	IM; single dose
Measles	1	+			All children	IM or SQ; single dose; booster recommended at 4–6 y of age
Mumps	1	+			All children	IM or SQ; single dose; booster recommended at 4–6 y of age
Poliovirus	3	+	+		All children	IM or SQ; 3 doses of inactivated vaccine recommended in United States; booster recommended at 4–6 y of age
Rabies	1		+		High-risk or exposed persons	IM; prophylactic 3 doses; therapeutic 4 doses
Rotavirus ^b	4 (G1, G2, G3, & G4)	+			All children	Oral vaccine based on rhesus rotavirus; 3 doses; removed from market because of concerns related to intussusception
	5 (G1, G2, G3, G4, & P[8])	+			All children	Oral vaccine; 3 doses; must start prior to 3 mo of age
	1 (G1P[8])	+			All children	Oral vaccine; 2 doses given at 2 & 4 mo of age
Rubella	1	+			All children	IM or SQ; single dose; booster recommended at 4–6 y of age
Smallpox	1	+			Military & first responder healthcare workers; recommended for laboratory workers using recombinant vaccinia virus	Intradermal vaccine used to eradicate smallpox
Varicella	1	+			All children; the elderly to diminish herpes zoster	IM or SQ; single dose; booster recommended at 4–6 y of age
Yellow fever	1	+	Travelers to endemic region	IM, single dose		
Total number of viruses covered	27	19	9	5		

VLP, virus-like particle; WT, wild-type; IM, intramuscular; SQ, subcutaneous; ID, intradermal.

^aSee individual chapters for specifics on target populations and immunization schedules for individual agents, which may vary or change from this overview. In addition, see the following website for a list of licensed vaccine products, including combination products: <http://www.fda.gov/cber/vaccine/licvacc.htm>.

^bRotaShield tetravalent vaccine is no longer available. RotaTeg pentavalent and Rotarix monovalent vaccines are available.

TABLE 14.2 Impact of Licensed Vaccines on Viral Diseases With Well-Defined Surveillance and Estimated Impact on Disease Burden for Other Viral Vaccines

Viral disease	Year of peak U.S. prevalence	Peak number of cases per year in U.S.	Number of annual U.S. cases in modern vaccine era
Impact of licensed vaccines on viral diseases with well-defined surveillance			
Hepatitis A	1971	59,606	1,670 ^a
Hepatitis B	1985	26,654	3,374 ^a
Measles	1958–1962	503,282	63 ^a
Mumps	1967	185,691	2,612 ^a
Polio	1951–1954	16,316	0 ^a
Rubella	1966–1968	47,745	5 ^a
Congenital rubella	1966–1968	823	0 ^a
Smallpox	1900–1904	48,164	0 ^a
Estimated impact on disease burden for other viral vaccines			
Influenza A & B	Annual epidemics	~50,000 excess deaths per year ^b	Reduces morbidity & mortality; however, effectiveness varies from year to year because of variations in circulating virus strains
Japanese encephalitis	—	—	Only necessary for travel to endemic regions, highly effective
Rabies	<1940	>100 deaths/y	2–3/y ^c
Varicella	<1994	>4 million/y	Vaccine offers 83%–96% efficacy against primary infection ^d
Yellow fever	1905 ^e	8,399	Only necessary for travel to endemic regions, highly effective

^a<http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5953a1.htm>.^b<http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5933a1.htm>.^c<http://www.cdc.gov/rabies/location/usa/surveillance/index.html>.^dhttp://www.merck.com/product/usa/pi_circulars/v/varivax/varivax_pi.pdf.^eLast U.S. epidemic in New Orleans.

prevented. In the process of establishing vaccine efficacy, the use of a human immune correlate of protection is potentially more efficient and has been implemented successfully in the yearly approval process for influenza vaccines. Influenza vaccines typically are approved based on the accepted surrogate of protection, a hemagglutination inhibition (HAI) titer of 1:40 or higher or a fourfold increase over the prevaccination HAI titer. In this case, the immune response serves as a surrogate marker that is predetermined to predict clinical benefit; therefore, the more lengthy process of assessing the presence or absence of influenza infection in a clinical trial is not always needed (<http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074794.htm>). Similarly, assessing the end result of infection (or absence of) rather than documenting an effect on the virus itself can also be used to assess protective efficacy. HPV vaccines were licensed based on efficacy derived from the prevention of cervical and vulvar cancer, precancerous lesions, and dysplastic lesions (<http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm111283.htm>).

Maintaining Vaccine Availability

Vaccine efficacy, need, utilization, and safety (or perceived safety) all have a significant impact on the success of licensure and sustained availability of a vaccine. Two licensed vaccines that are no longer available are the adenovirus serotypes 4 and 7 and the tetravalent rhesus rotavirus vaccine. Production of the

adenovirus vaccine, selectively given to new military recruits, was stopped by the sole manufacturer in 1995 for economic reasons. Since then, morbidity from adenovirus infections has increased among military recruits, and epidemics have occurred in training centers.¹⁶⁹ In addition to economic concerns, safety concerns have also prompted an abrupt end to the use of vaccines. Specifically, RotaShield, a live tetravalent rhesus rotavirus reassortant vaccine against the four major prevalent serotypes (based on VP7, a major outer capsid glycoprotein), was effective but was later withdrawn from market. A postmarketing analysis revealed that the vaccine was associated with an excess rate of intussusception of up to 1 per 2,500 vaccinees that occurred in the immediate postvaccination period, predominantly following the first dose of vaccine.³⁰⁶ Following withdrawal of RotaShield, subsequent studies led to a significant downward revision of these estimates and have suggested that the early period of increase in intussusception seen after the first dose was followed by a period of compensatory decrease such that there was no net overall increase in the number of cases in vaccinees less than 1 year of age.³⁰⁶ Furthermore, the excess cases of intussusception occurred mostly when the vaccine was used in children 3 months of age or older to “catch up” rather than in vaccinees who received the ideally recommended schedule of 2, 4, and 6 months of age.^{212,403} This case highlights the importance of the analysis of postmarketing data, which needs to be especially thorough and comprehensive because it can dramatically impact the availability of a vaccine that may otherwise have a

favorable risk/benefit ratio. Despite the initial conclusions and withdrawal of RotaShield from the market, two newer versions of a rotavirus vaccine have been developed. Pentavalent RotaTeq has been licensed in the United States for infants less than 3 months of age. It consists of a mixture of live attenuated, bovine/human reassortant viruses that express a single VP4 (P1A) as well as VP7 (G) Ag from G1 through G4 strains of rotavirus and VP4 (P1A[8]). P1A is the most common VP4 in G1, G3, and G4 strains and is present in a major portion of G9 viruses that are becoming more prevalent in some regions. The second new vaccine is a monovalent VP7 G1:P1A[8] attenuated human rotavirus strain that was first licensed in Mexico and the Dominican Republic¹⁵¹ and later in 2008 in the United States. These two vaccines protect against severe gastroenteritis attributable to rotavirus. Large-scale postlicensure evaluations will be essential to more clearly elucidate the overall risks associated with rotavirus vaccines in the general population.

Future Directions in Vaccinology

In recent decades, major advances in viral vaccine development have coincided with the emergence of new technologies (Fig. 14.5). Therefore, selected technologies that are likely to influence future vaccine design are discussed briefly.

Impact of Advances in High-Throughput Sequencing Technology

The Human Genome Project stimulated major investments in sequencing technology, resulting in breakthroughs that have increased throughput by orders of magnitude. As a result, it is now feasible to sequence all nucleic acids in samples of interest, such as vaccine preparations. Not surprisingly, such experiments have identified previously unidentified agents or nucleic acids in licensed vaccines, such as porcine circovirus type 1 (PCV1) in the commercial rotavirus vaccine Rotarix.⁴³⁸ The U.S. Food and Drug Administration (FDA) temporarily suspended use of the vaccine in 2010; however, experts agreed

that this highly prevalent nonpathogenic pig virus does not infect humans, and the use of vaccine was reinstituted. Some vaccines are produced in avian or primate cell lines, and thus it was not surprising that RNAs from avian leukosis virus, which is noninfectious for humans, or genetically defective DNAs from simian retrovirus (SRV) were present in products of such cell lines. Another interesting finding of such sequencing efforts is that the vaccine virus populations contain minority variants with polymorphisms or mutations (compared to the known sequence of the vaccine strains). Such variants have been observed for oral polio vaccine (OPV), mumps virus, and VZVs but are not known to affect attenuation.

Advances in Monoclonal Antibody Isolation

The neutralizing determinants on vaccine Ags are *de facto* the epitopes recognized by human Abs that mediate antiviral function. The study of the molecular and structural basis for neutralization has been limited by the difficulty in isolating human mAbs. Recent breakthroughs in this field using enhanced EBV transformation with CpG,³⁴ human hybridoma formation,⁴⁶⁷ plasmablast sorting and cloning,⁴⁵⁶ single B-cell sorting and expansion,⁴⁴⁰ and other techniques have increased the numbers of human Abs for study by several orders of magnitude. Structural studies of complexes of human Abs with viral Ags have begun to yield fascinating insights into the features of protective Ab epitopes, which often are conformational, quaternary, and otherwise complex.²³⁷

Advances in Structural Biology

The ability to design optimal antigenic surfaces for induction of neutralizing Abs has long been a dream of vaccine scientists. The goal is to develop synthetic constructs that elicit Abs with predetermined structural specificity. In particular, induction of Abs against epitopes that are hidden in the native viral protein has been a challenge for vaccine design. Investigators working in the areas of structural biology and computational modeling

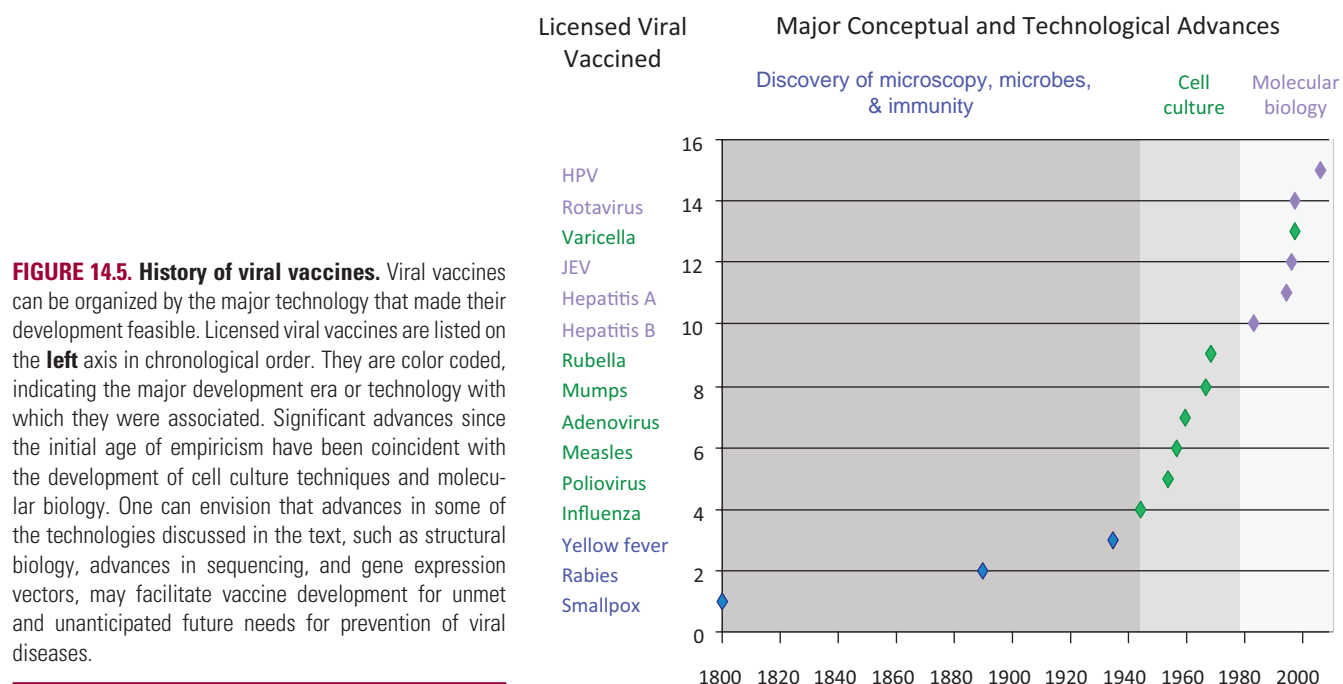


FIGURE 14.5. History of viral vaccines. Viral vaccines can be organized by the major technology that made their development feasible. Licensed viral vaccines are listed on the left axis in chronological order. They are color coded, indicating the major development era or technology with which they were associated. Significant advances since the initial age of empiricism have been coincident with the development of cell culture techniques and molecular biology. One can envision that advances in some of the technologies discussed in the text, such as structural biology, advances in sequencing, and gene expression vectors, may facilitate vaccine development for unmet and unanticipated future needs for prevention of viral diseases.

recently have made significant progress in this regard. Computationally designed epitope-scaffolds that are immunogenic have been developed for the HIV-1 gp41 epitopes of the broadly neutralizing mAbs 2F5³²⁶ and 4E10⁹³ and the RSV F motavizumab epitope.²⁷⁴ Structural information from HIV gp120 CD4 binding site epitope recognized by the neutralizing Ab b12 has been used to design gp120 reagents to select additional CD4 binding site Abs that have broad neutralizing activity against HIV.⁴⁷⁴ Fragments of influenza HA have been expressed to focus the immune response to conserved epitopes in the HA stem region.⁴¹ Computational and structural tools also can be used for design of viral inhibitors—for example, the design of proteins targeting the influenza HA stem that mimic the action of neutralizing Abs. It seems likely that computationally designed Ags will increasingly influence viral vaccine design.

LIVE VIRUS VACCINES

History of Methods for Isolating Attenuated Strains

Varying methods have been used to achieve attenuation for live virus vaccines. Type 2 poliovirus, strain 712, one of the three poliovirus vaccine strains in the live vaccine preparation, is a naturally occurring attenuated human poliovirus that was identified by its lack of virulence for the brain and spinal cord of monkeys.³⁸² Adenovirus vaccine strains (types 4 and 7) are wild-type human respiratory viruses used in the U.S. military that produced an asymptomatic infection following oral administration that was restricted in replication to the intestinal tract.⁶⁸ The remaining attenuated vaccine viruses were derived from wild-type human viruses by serial passage in cell cultures prepared from an unnatural host, leading to the emergence of mutants that were partially restricted in humans at the portal of entry and/or the target organ(s). In this manner, attenuated mutants of rubella virus and types 1 and 3 poliovirus were selected after passage in monkey kidney tissue culture. Vaccine strains of yellow fever virus (17D strain) and measles virus were generated by multiple passage in chick embryo cell culture, whereas mumps virus was attenuated by multiple passages in embryonated eggs.

A critical feature of most successful live vaccine development programs is the identification of preclinical laboratory predictors of attenuation. For the yellow fever virus and poliovirus vaccines, promising vaccine strains were identified by their attenuation for experimental animals. In monkeys, the 17D strain of yellow fever virus exhibited decreased tropism for the liver, suggesting that this mutant might be suitably attenuated for humans⁴²³—a hypothesis that was confirmed during subsequent clinical trials. Naturally occurring or tissue culture-passaged strains of poliovirus were evaluated for neurovirulence in monkeys by intraspinal inoculation, which was a central innovation that allowed Sabin to identify attenuated strains.^{381,382} This testing system was selected because several observations suggested that the spinal cord of monkeys was more permissive than that of humans to the neuronolytic effect of poliovirus.³⁸² Viruses that caused the least neurovirulence in monkeys were identified, and these strains then were cloned and recloned biologically by the plaque technique to yield progeny with the lowest possible level of monkey neurovirulence. These

candidate vaccine strains then were subjected to additional cycles of selection, which led to the identification of mutants that replicated with high efficiency in the intestinal tract without significant increase in neurovirulence for nonhuman primates. These viruses were evaluated in clinical trials and were shown to be satisfactorily attenuated and immunogenic.

Experimental animal systems were not available for evaluating virulence of the other vaccine viruses. Therefore, these viruses were tested for attenuation directly in humans. Initial candidate measles and rubella virus vaccines were insufficiently attenuated, although further passage of rubella virus in monkey kidney cell culture and selection of a cold-adapted, temperature-sensitive mutant of measles virus yielded satisfactory vaccine strains. Attenuation of mumps virus passaged in eggs also was determined in humans. Interestingly, the Jeryl Lynn strain of mumps virus vaccine developed by Maurice Hilleman as a clinical isolate from his daughter was found to be a mixture of two closely related strains of mumps virus.⁷ Several other vaccines have been found to be mixtures of virus strains, including the yellow fever virus vaccine and some vaccinia stocks used for smallpox vaccination.

Poliovirus and adenovirus vaccines are administered orally, whereas yellow fever, rubella, measles, and mumps virus vaccines are given parenterally. Each of the currently licensed live virus vaccines is directed against a virus that has a complex pathogenesis of infection in which virus is introduced by inoculation (yellow fever) or by implantation on a mucosal surface (measles, mumps, rubella, and poliovirus) with subsequent spread systemically to the target organ(s). The requirement of such viruses to pass through blood to reach the target organ of disease makes them generally susceptible to serum Abs. It is more difficult to protect against infection by viruses that cause disease at a mucosal portal of entry without the need for viremia.

Advantages of Live Virus Vaccines

The major advantage of live virus vaccines is the activation of all components of the immune system yielding both a balanced systemic and local immune response and a broad humoral and cell-mediated response. This type of comprehensive induction of immune effectors is particularly important for infections in which cell-mediated immunity plays an important role and for mucosal infections in which both local and systemic immunity are required for optimal resistance. As noted, mucosal infection with a live virus vaccine is much more effective in stimulating a local response in the unprimed host than inactivated virus vaccines administered parenterally.³⁰⁵ Live virus vaccines also can stimulate an immune response to each of the protective Ags of a virus, and this obviates the difficulties that arise from selective destruction of one of the protective Ags that may occur during the inactivation process and can lead to disease potentiation. This advantage is especially important for complex viruses encoding many protective Ags, because it is prohibitively difficult and expensive to immunize with multiple purified proteins or to construct multivalent vectors. Furthermore, immunity induced by live virus vaccines is generally more durable and more effective.²⁰⁴ There are practical advantages to many live virus vaccines, such as relatively low cost of production and ease of administration. Live viruses contribute to protection in populations because of the induction of herd immunity, sometimes facilitated by transmission of vaccine viruses from a

recipient to a contact. The effectiveness of live vaccines in settings of high transmission is illustrated by the use of adenovirus vaccines. Adenovirus vaccines have reduced military respiratory disease morbidity significantly over the past three decades. In 1995, however, the manufacturer of these vaccines ceased production owing to budgetary constraints. Major outbreaks of serious adenovirus infections occurred in military facilities as a consequence.

Disadvantages of Live Virus Vaccines

The major concerns with live virus vaccines are that they (a) can contain adventitious agents; (b) can cause illness directly; (c) can lose their attenuation during manufacture or during replication in vaccinees by reversion or by second-site compensatory mutations; (d) can spread to contacts; and (e) can lose infectivity during storage, transport, or use. The potential for contamination with live adventitious agents always exists as discussed previously. Sources include the original clinical sample from which the virus was isolated, cell culture substrates, laboratory reagents of animal origin (such as serum supplements and trypsin), and contamination from laboratory workers or environments. Cross-contamination of vaccine lots with other viruses in a laboratory facility is possible. Fortunately, contamination of clinical lots of vaccine with major human pathogenic adventitious agents has rarely been a problem in the modern era. Some of the early lots of live poliovirus vaccine were contaminated with live SV40, and the live yellow fever virus vaccine was initially contaminated with an avian leukosis virus. Follow-up of individuals who were given live vaccine contaminated with SV40 or avian leukosis virus has failed to identify any long-term adverse effect, including cancer, associated with exposure to these adventitious viruses.^{291,412,446}

Some live virus vaccines, such as the measles virus, rubella virus, and yellow fever virus vaccines, retain a low level of residual virulence. The reactions produced by these vaccines generally are minor and have not interfered with acceptance and widespread use of these products. A more serious problem is that of restoration of a varying degree of virulence during infection by vaccine virus. This occurs with the poliovirus vaccine at an extremely low frequency—that is, about one in 10^6 to 10^7 immunizations.³¹⁹ Most vaccine-associated cases of paralysis in vaccinees or their close contacts occur after the first dose and involve the type 3 vaccine strain.³¹⁹ A significant proportion of the paralytic illnesses associated with poliovirus vaccine occurs in individuals who are immunocompromised; however, this may not represent a manifestation of genetic alteration of vaccine virus.³¹⁹

The genetic basis for the very rare increase in virulence of the poliovirus vaccine strains during replication *in vivo* is relatively well understood. However, a paradox exists in that mutation toward a higher level of virulence for the CNS occurs rapidly and frequently during replication of poliovirus in the intestinal tract of vaccinees, although vaccine-associated paralytic disease is extremely rare. During manufacture of vaccine, the mutations that are associated with restoration of virulence can be detected at variable low frequency by sequence analysis, and neurovirulence of the final product can be held to a low level by rejecting vaccine lots that exceed the accepted standard for frequency of these mutations.⁷⁶ Genetic instability was also a problem with the 17D yellow fever virus vaccine during early field trials. Encephalitis was observed in 1% to 2% of young

vaccinees administered virus that had been passaged 20 times beyond the original seed lot.¹³⁷

The problem of genetic instability of live attenuated virus vaccines following replication *in vivo* is an even larger concern for viruses that can cause latent or persistent infection *in vivo* such as herpes viruses or lentiviruses (e.g., HIV). Despite this concern, successful vaccines have been developed for VZV—a herpesvirus that causes chickenpox and zoster—indicating that a successful live attenuated vaccine can be developed for a virus that causes a lifelong infection in humans. This vaccine was licensed first for prevention of childhood chickenpox and has recently been shown to significantly reduce the risk of VZV reactivation or shingles.³³³

Rubella virus can be recovered from the lymphocytes of immunologically normal individuals with arthritis up to 6 years after immunization or natural infection⁶⁹; however, the rubella virus vaccine does not appear to be associated with chronic arthritis.³⁶⁴ Interestingly, persistence of measles vaccine virus has not been detected, nor has the vaccine virus been implicated in subacute sclerosing panencephalitis.²⁸¹ Instead, widespread use of live measles vaccine has almost eradicated this rare but serious sequelae of persistent wild-type measles virus infection.

Several other concerns have been identified for live attenuated virus vaccines. First, there is a theoretical risk that infection of the fetus could occur following vaccination during the first trimester of pregnancy. For rubella vaccine, there is concern that vaccination might lead to development of the congenital rubella syndrome; however, the actual risk appears to be negligible.³³⁹ Second, naturally occurring wild-type viruses may interfere with infection by a live virus vaccine, resulting in a decrease in vaccine efficacy. Such interference has been observed primarily with live poliovirus vaccine strains and is caused by a wide variety of enteric viruses. This phenomenon has also been seen with experimental live attenuated alphavirus vaccines.²⁷⁰ To overcome this viral–viral interference, multiple doses of polyvalent live virus vaccines are required to assure a protective immune response to each component. Third, defective interfering particles have been identified in preparations of live virus vaccines,⁵⁵ and it is likely that they are present in all live virus vaccines to some degree. Fourth, live attenuated measles virus vaccine has been associated with a generalized immunosuppression that was observed to be directly correlated with the level of immune response to the vaccine.¹⁹⁹

Finally, it should be noted that stability is a serious practical problem with thermolabile vaccine viruses such as measles virus. The need for storage and transport of measles vaccine at low temperature (4°C) has limited its usefulness in some tropical areas where maintenance of a “cold chain” for transport and storage is difficult. Some simple solutions for stabilizing other live virus vaccines have been identified, such as lyophilization of vaccinia virus or using deuterium oxide for water in live poliovirus or influenza vaccine formulations,^{200,283} although additional work is needed.

Genetic Basis for Attenuation of Live Viruses

Mutants selected by passage in an unnatural host accumulate many mutations, often making it difficult to define in a precise manner the genetic basis for their attenuation. In some cases, the molecular basis of attenuation has been defined. The genetic basis of this neuroattenuation of each of the three poliovirus

vaccine strains has been partially defined.¹⁷⁰ Surprisingly, few mutations are involved. Analyses have identified attenuating nucleotide substitutions in 5' noncoding sequences and others attenuating in the 3' noncoding region or in the structural or nonstructural coding regions of type 1, type 2, or type 3 polioviruses.^{45,147,367} These mutations can now be monitored during manufacture and following replication *in vivo*. Vaccine lots with an increased frequency of reversion at some residues have been shown to exhibit increased neurovirulence, and such lots can be discarded as unacceptable for human use.⁷⁶ Thus, it is now possible to use molecular virological techniques to control the manufacture of vaccines by identifying the presence of the desired attenuating mutations as well as the absence, or low frequency, of unwanted reversions.⁷⁶

The Jennerian approach to the development of live attenuated viruses involves the use of a virus strain of mammalian or avian origin to immunize humans against a human virus that is related antigenically to the animal or avian strain. Mammalian and avian viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. At present, we lack a complete understanding of the genetic basis for this form of host-range restriction. However, those mammalian or avian viral genes that have been identified as being responsible for host-range restriction in humans exhibit significant divergence of nucleotide sequence from that of the corresponding human viral genes. Quadrivalent and pentavalent rotavirus vaccine are examples of the Jennerian approach to vaccine development in which a nonhuman rotavirus strain, the rhesus rotavirus or bovine rotavirus, was found to be attenuated in humans. Because there was a need for a multivalent vaccine that would induce resistance to each of the major human rotavirus serotypes, the Jennerian approach was modified by constructing three reassortant viruses, each of which contained 10 rhesus rotavirus or bovine rotavirus genes plus a single human rotavirus gene encoding one of the major neutralization Ags of the targeted serotypes.

Conventional techniques for attenuation of viruses such as passage of virus at low temperature, mutagenesis followed by selection of mutants with the desired phenotype(s), or passage of virus in heterologous tissues continue to play a significant role in the development of live virus vaccines. These techniques have been used to generate candidate vaccine strains for CMV, ortho- and paramyxoviruses, alphaviruses, flaviviruses, hepatitis A virus, and arenaviruses. Selection for a combination of phenotypes (such as cold adapted and *ts*) is thought to be desirable because the different mutations can have synergistic effects on the stability of the attenuation phenotype *in vivo*.³⁰⁴ Viruses bearing stable, molecularly defined, identifiable attenuating mutations or attenuating gene constellations represent the vaccine strains of the future, because the genetic basis for attenuation will be known and can be monitored directly during all phases of vaccine development, manufacture, and utilization in humans.

The function of viral proteins can be altered by mis-sense mutations, insertions, or deletions. Classically, attenuated viruses were identified using biologic phenotypes: (a) *ts* mutants; (b) cold-passaged (*cp*) or cold-adapted (*ca*) mutants; (c) small plaque mutants; (d) protease activation (*pa*) mutants (also designated as cleavage mutants); and (e) mutants with altered interaction with host cell receptors. With modern molecular techniques, virulent viruses also can be attenuated

by a wide variety of creative approaches including gene deletion, shuffling of gene order, alteration of noncoding regulatory regions contain cis-acting signals required for efficient replication, the use of gene incompatibility in chimeric genomes, and the insertion of genes that encode proteins with known antiviral activities or with known immunoregulatory functions.

INACTIVATED VIRUS VACCINES

Inactivated virus vaccines are available in the United States for the prevention of disease caused by nine separate viral agents (see Table 14.1). The available vaccines are based on either whole inactivated virus or virus-like particles (VLPs) produced from purified protein. The viruses are grown in a variety of cell substrates, including eggs (influenza types A and B), a continuous monkey kidney cell line (poliovirus types 1, 2, and 3), human diploid fibroblasts (rabies, hepatitis A), or mouse brain (JEV vaccine). Virus is then inactivated with a chemical such as formalin or disrupted with a detergent (influenza). The level of efficacy of inactivated viral vaccines differs: Inactivated poliovirus vaccine is highly effective in preventing disease, whereas influenza virus vaccine is only partially protective.

Features of Inactivated Virus Vaccines

Inactivated virus vaccines offer the advantage of immunization with the entire antigenic content of the virus with little or no risk of infection. Only rarely do such vaccines contain a contaminating adventitious agent or residual infectious virus that has resisted inactivation. For example, paralytic disease was produced by some of the early lots of inactivated poliovirus vaccine that contained residual infectious virus.^{313,327} Contamination by an infectious adventitious agent was also detected retrospectively in some early lots of inactivated poliovirus. This was of some concern because the contaminating simian virus, SV40, was oncogenic in hamsters.¹⁹⁴ Fortunately, long-term follow-up of individuals who were inoculated with SV40-contaminated vaccine during early infancy failed to show evidence of a vaccine-induced oncogenic effect.⁴¹² Another incident occurred in a research setting where live vaccinia virus was found to be present in a preparation of inactivated vaccinia–HIV recombinant virus being evaluated as a therapeutic vaccine in HIV-infected subjects. Unfortunately, an immunodeficient recipient of this vaccine developed progressive vaccinia caused by residual live virus in the inoculum.⁴⁷⁰ Several inactivated whole virus vaccines have potentiated disease rather than prevented it.^{143,219} This finding was first observed with formalin-inactivated measles virus vaccine.¹⁴³ Initially, this vaccine prevented measles; however, after several years, vaccinees lost their resistance to infection. When subsequently infected with naturally circulating measles virus, the vaccinees developed an atypical illness with accentuated systemic symptoms and pneumonia.^{143,310} Retrospective analysis showed that formalin inactivation destroyed the ability of the measles fusion (F) protein to induce hemolysis-inhibiting Abs, although it did not destroy the ability of the H (HA or attachment) protein to induce neutralizing Abs.^{320,321} When the H-specific Ab had waned sufficiently to permit extensive infection with measles virus, an altered and sometimes more severe disease was seen at the sites of measles virus replication.⁵³ This atypical disease is believed to be mediated in part by an altered cell-mediated immune response in which Th2 cells³⁵⁶ or

a delayed-type hypersensitivity reaction²⁴⁶ were induced preferentially, leading to heightened disease manifestations at the sites of viral replication. Immune complex deposition also plays a role in the pathogenesis of atypical measles.³⁵⁷

Potential of disease also was observed after parenteral administration of an experimental formalin-inactivated respiratory syncytial virus (FI-RSV) vaccine.²¹⁹ In clinical trials conducted in the mid-1960s, an FI-RSV vaccine induced a measurable serum-neutralizing Ab response but did not protect the youngest vaccinees. Unexpectedly, on subsequent natural infection with RSV, the FI-RSV vaccinees developed severe RSV lower respiratory tract disease significantly more often than did infants and young children who had received an inactivated parainfluenza virus vaccine.²¹⁹ Speculation on the mechanism of disease potentiation by the inactivated RSV vaccine has centered on several possible aberrations of the immune response to vaccine that involve an imbalance between or within various compartments of the immune system. Most likely, the protective antigenic sites on the F protein were altered by fixation, and the altered Ag induced an aberrant T-cell and cytokine response leading to enhanced disease as recently reviewed.¹⁶⁵ The major lesson learned from the FI-RSV vaccine-enhanced illness is that the chemical treatment and physical purification procedures used for the preparation of each new inactivated vaccine should be evaluated not only for their effect on the magnitude of immune responses but also on the functional characteristics of the response. Typically, the Ab induced should have neutralizing activity, and the T-cell responses should mediate efficient virus clearance.

Potential of disease by an inactivated virus vaccine is not limited to vaccines containing measles virus or RSV and has occurred with both human and veterinary vaccines. For example, administration of an inactivated caprine lentivirus vaccine has also been associated with an accelerated, more severe disease in animals subsequently challenged with virus.²⁷³ Vaccine-enhanced pathology is also not limited to inactivated virus vaccines. Immunization with a subunit vaccine of another lentivirus—equine infectious anemia virus (EIAV)—enhanced disease caused by subsequent challenge with a heterologous EIAV strain.²⁰¹ A similar phenomenon occurred during the development of a vaccine for feline infectious peritonitis virus (FIPV). FIPV is a CoV that developed more notoriety after the emergence of the severe acute respiratory syndrome (SARS) CoV in 2003. Attempts to develop a vaccine against FIPV have been largely unsuccessful and associated with a vaccine-enhanced illness. A major problem is that Abs are not protective even when given passively for prophylaxis, and they can facilitate virus entry through Fc receptors. Whole virus,³⁴⁵ recombinant vaccinia expressing the major surface S (spike) glycoprotein,⁴³⁷ or DNA immunization with IL-12¹⁵⁰ all have led to enhanced disease syndromes. Immunization of ferrets with a recombinant modified vaccinia virus Ankara (MVA) expressing the S glycoprotein has also caused an enhanced disease with hepatitis after SARS CoV immunization. Although the immunological mechanisms responsible for these examples of enhanced disease have not been identified, there are two common features present in these settings to consider. First, there was induction of a memory T-cell response in the absence of prechallenge Ab that could effectively neutralize virus. This is analogous to the conditions associated with measles and RSV vaccine-enhanced disease. Second, these are viruses that have

tropism for Fc receptor bearing cells. The presence of binding Ab that does not effectively neutralize virus could thereby facilitate entry and amplify viral replication. Vaccine studies with DNA or recombinant vectors, subunit proteins, or inactivated virus alone or in combination that have induced effective neutralizing Ab responses to the SARS CoV in animal models have not been associated with enhanced illness postchallenge.

Another disadvantage of inactivated virus particle vaccines is that they typically have to be given parenterally because without replication or gene expression, it is difficult for mucosally delivered Ag to elicit a significant inductive event. Parenteral induction of systemic immunity may not fully protect against viruses that infect and cause disease primarily at mucosal surfaces if the gradient of Ab between serum and mucosa is too large or the kinetics of viral entry are too rapid. Therefore, resistance to viral challenge in the upper respiratory tract or in the intestines may be less robust than that conferred by local immunization or infection. This may be the reason inactivated influenza vaccines are only partially protective, and why the inactivated polio vaccine (IPV) can prevent paralytic polio but cannot prevent poliovirus infection and intestinal carriage. There are clear exceptions to this principle, which are best exemplified by the success of parenterally administered HPV vaccine. Although HPV is a purely mucosal pathogen, it has recently been shown that entry into basal epithelial cells is a two-step process that may take several hours.²²² Virus neutralization can be accomplished with much less Ab when given longer access to the viral antigenic sites.

Inactivated virus vaccines are clearly at a disadvantage compared to either live attenuated virus vaccines or vector-based gene delivery with respect to induction of a CTL response. CD8 CTL responses require Ags to be processed through the proteasome so that peptides can be transported into the endoplasmic reticulum and associate with MHC class I molecules. This is best achieved when proteins are synthesized in the host cell cytoplasm and is rarely achieved in primates when Ags enter the APC through the endocytic pathway. For example, inactivated influenza virus is considerably less effective than virus infection in stimulating a primary CTL response in mice. However, inactivated influenza A virus or purified surface glycoproteins derived from it can sometimes stimulate a secondary CTL response in sensitized mice or humans.^{5,122} There are new adjuvants and delivery vehicles that have the capacity for moving purified proteins into the MHC class I presentation pathway, and these may be considerations for future vaccine development.

The Guillain-Barré syndrome was associated with widespread use of inactivated influenza A H1N1 (swine) virus vaccine during 1976 and 1977,²⁴⁰ indicating that unanticipated, delayed, or untoward side effects can be induced by inactivated virus vaccines. This syndrome has been associated only rarely with subsequent influenza virus vaccines and was not reported at all following the rapid introduction of vaccines against the 2009 H1N1 pandemic strain, even though these products were prepared in the same manner as the 1976 vaccine.²⁴⁰ The basis for this idiosyncratic syndrome associated with the 1976 influenza vaccine is still not defined.

Future Considerations for Inactivated Virus Vaccine Development

Formaldehyde, heat, and oxidation have been shown to produce reactive carbonyl groups on protein Ags that promote

CD4 T-cell responses with Th2 differentiation.²⁸⁵ Chemical inactivation of poliovirus during vaccine manufacturing can result in modification of a major neutralization epitope. Therefore, inactivating infectivity of nonenveloped and enveloped viruses can adversely affect the antigenicity of the vaccine.¹³⁰ It has also been recognized that antigenic changes can occur during selection and amplification of influenza viruses in the allantoic cavity of embryonated eggs—the substrate used to produce virus for the inactivated vaccine.³⁷⁰ The genetic basis for these mutations involves host cell selection of mutants that replicate more efficiently in eggs. These variants acquire a mutation in or near the receptor binding pocket of the HA that increases their efficiency of replication in eggs but also alters antigenicity and immunogenicity. Nevertheless, influenza viruses that are used to prepare inactivated vaccine for humans are grown in eggs, and such vaccines have been repeatedly shown to be protective. Although this would not be a problem for influenza vaccines produced in mammalian cell culture, vaccine Ags may be modified or processed in ways that compromise antigenicity even in mammalian cell substrates. For example, poliovirus grown in tissue culture contains an uncleaved outer capsid protein, VP1, which is usually cleaved in a natural infection by intestinal proteases. Although tissue culture–grown poliovirus has limited ability to induce Abs that neutralize viruses containing a cleaved VP1,³⁷¹ both cleaved and uncleaved VP1 can induce protection from replication of subsequently administered live attenuated poliovirus vaccine.³⁵⁰ Nevertheless, careful assessment of the substrate-dependent antigenicity and consequences of inactivation is warranted in the development of inactivated virus vaccines.

VIRUS-LIKE PARTICLE VACCINES

There are licensed VLP vaccines for two viral pathogens (HBV and HPV). The VLPs are produced as self-assembling capsid proteins in yeast or insect cells. For the purposes of this section, we will define VLP as self-assembling viral proteins that can mimic the particle structure of virions and present the quaternary structures of oligomeric viral surface proteins but do not package the virus genome. There are other self-assembling nonviral proteins that can be used to display vaccine Ags that are mentioned with nanoparticles in the Delivery Vehicles section.

The initial version of the HBV vaccine was based on surface Ag purified from the blood of persons persistently infected with HBV. The Australia Ag was associated with the disease caused by HBV, and later the Dane particle was found to represent the Australia Ag. Interestingly, electron microscopy revealed not only the Dane particle, which is a 42-nm structure representing the complete infectious virion, but also smaller particles and filaments. The 22-nm particle was found to be comprised of just HBsAg and is noninfectious because it does not contain nucleic acid. This small particle, purified from human plasma, was the first licensed HBV vaccine. An effective second-generation HBsAg vaccine was developed using a recombinant plasmid to express the gene for HBsAg in yeast. Yeast-produced HBsAg self-assembles into uniform 22-nm particles. Although the 22-nm noninfectious particle is smaller in size and technically a defective genomeless subvirion particle, it is considered a VLP because it is made of

the same capsid protein (HBsAg) as the full particle. Licensed HBV VLPs are formulated with alum and are now part of the routine pediatric vaccine schedule. This vaccine often is protective against HBV infection and hepatitis even in the absence of detectable Ab response. Immunization against HBV also significantly reduces the frequency of hepatoma and is regarded as the first anticancer vaccine.⁶⁷ The immunogenicity of HBV VLP in patients with renal failure, HIV-1 infection, or other immunocompromising conditions is diminished. New adjuvant approaches such as formulation with CpG (TLR-9 agonist), monophosphoryl lipid A (MPL)+alum, or MPL+QS21 are being explored to improve vaccine-induced immunity in those settings.^{32,33,91} HBV VLPs are now produced for human use in nine countries using yeast, bacteria, or mammalian cells for manufacturing, and other substrates including transgenic plants are being explored.³⁷⁵

HPV particles were first identified by electron microscopy in 1949. In 1983, the association between HPV infection and cervical dysplasia and malignancy was proven.¹¹⁷ In 1991, it was shown that VLPs lacking genomes could be produced by expression of the L1 and L2⁴⁷³ or just the L1²²⁴ capsid proteins of papillomaviruses. L1 self-assembles into pentamers, and each VLP is comprised of 72 pentameric capsomeres. If present, one L2 protein is associated with the center of each L1 pentamer on the inner surface of the capsid. These key discoveries led to the development of candidate VLP vaccines. Importantly, the end points for the efficacy trial were chosen carefully. Although prevention of cervical carcinoma was the goal, this target was not an acceptable end point because ablative treatment would be performed if premalignant lesions were detected. Therefore, cervical intraepithelial neoplasia grade 2 or higher was used as the end point for vaccine efficacy trials as a surrogate for cervical carcinoma. Prevention of persistent serotype-specific HPV infection was also a primary end point. Yeast-produced HPV 16 VLP formulated in alum and delivered parenterally was 100% effective in preventing cervical intraepithelial neoplasia.²³² A three-dose regimen of a tetravalent L1-based VLP vaccine based on HPV 16 and 18 (most common causes of cervical intraepithelial neoplasia) and HPV 6 and 11 (most common causes of anogenital warts) is also highly effective.⁴³⁹ Subsequent studies have shown that HPV VLP immunization can prevent HPV-induced cervical lesions in women⁴ and anogenital lesions in both women and men.^{146,149} Three doses containing 30 µg of each of the four VLPs formulated with aluminum hydroxyphosphate sulfate (Gardasil) or 20 µg of each of two VLPs formulated with a mixture of aluminum hydroxide and 3-deacylated MPL (AS04) (Cervarix) are sufficient to achieve protective immunity that is durable for more than 5 years.³⁷⁴ The protection has been correlated with type-specific neutralizing Ab. A nonavalent VLP vaccine including serotypes 6, 11, 16, 18, 31, 33, 45, 52, and 58 is being evaluated in efficacy trials in an attempt to extend the coverage against cervical neoplasia from 70% to more than 90%.

Advantages of Virus-Like Particle Vaccines

Capsid proteins from many nonenveloped viruses and the nucleocapsid or matrix proteins together with surface proteins from some enveloped viruses can self-assemble into VLPs and provide a platform technology that may be applicable as vaccines for multiple virus families. VLPs offer several advantages as immunogens. First, the viral nucleic acid is not present in

VLPs, thus there is no concern that viral oncogenes or other pathogenic features of the live virus are present. Second, VLPs present conformational epitopes to the immune system in the same way as native infectious particles so that neutralizing Abs and other protective immune responses are induced efficiently. Some of these epitopes are formed by the juxtaposition of parts of two different capsid proteins. Third, many of the non-enveloped viruses from which immunogenic VLPs have been developed replicate poorly or not at all in tissue culture (e.g., B19 parvovirus, papillomaviruses, and hepatitis E virus), thus precluding the use of purified virus as immunogen. Fourth, because VLPs are noninfectious, chemical inactivation is not required. For this reason, VLPs might prove to be better immunogens than formalin-inactivated whole virus vaccines because the deleterious effects of formalin on structure and immunogenicity can be avoided. Fifth, the immune system responds better to a viral Ag presented as an assembled multimeric particle rather than as an individual monomeric protein. Sixth, the stability of VLPs from nonenveloped viruses may simplify vaccine storage and distribution and also make successful oral delivery feasible in some cases.³¹⁸

Disadvantages of Virus-Like Particle Vaccines

The major disadvantage of VLP vaccines is related to the cost and complexity of manufacture, although costs should become lower with advances and expansion of the options for cell substrates. The biological disadvantage of VLPs compared to replication-competent viruses or vectors is that there is not ongoing production of Ag following vaccination. However, these particles tend to be highly antigenic and stable, and immunogenicity can be enhanced with adjuvants.

Future Considerations for Virus-Like Particle Vaccine Development

VLPs have been engineered successfully for viruses belonging to a wide range of virus families, including both nonenveloped and enveloped viruses. Because of the advantages listed previously and track record of success, they should be considered as a vaccine platform technology for other virus families when possible.

Nonenveloped Virus-Like Particles

The first characterization of synthetic VLPs followed the observation that picornavirus capsid proteins could self-assemble during the *in vitro* translation of viral RNA to yield 75S to 85S particles.³³⁵ Later studies indicated that VLPs could form *in vitro* following translation of picornavirus viral RNA lacking most of the 5' noncoding region.⁸⁰ Next, it was observed that expression of the open reading frames of capsid proteins of non-enveloped viruses by a baculovirus or vaccinia vector in appropriate eukaryotic cells resulted in generation of VLPs.^{81,224,473} For picornaviruses, particle assembly requires expression of the entire open reading frame to permit the proteolytic processing of the structural proteins by nonstructural viral-encoded proteases. There are current vaccine development approaches being used for enterovirus 71 that involve VLP technology.⁷⁸

Human parvovirus B19 causes erythema infectiosum, acute and chronic red cell aplasia, and other illnesses. A vaccine candidate consisting of the human parvovirus B19 VP1 and VP2 capsid proteins that form VLPs is being developed.²² Immunization of humans with this vaccine induces neutralizing

Abs that achieve levels equivalent in magnitude and neutralizing activity to those seen following natural infection.⁴⁴ Antigenic VLPs can be readily produced for other human (bocavirus)⁶⁶ and veterinary parvoviruses,³⁷³ suggesting that VLP-based vaccines may be an appropriate approach for pathogens for this viral family.

Norovirus, the prototypic member of the calicivirus family, also may be a good candidate target for a VLP vaccine. The virus is a 28- to 37-nm nonenveloped particle that is formed by a single major capsid protein (VP1). VP1 is a protein with two domains connected by a short linker. The P domain forms the outer surface of the particle, and the S domain forms an inner shell. The P portion of VP1 can self-assemble 24 protomers to form a 20-nm particle that is immunogenic.⁴¹⁷

Enveloped Virus-Like Particles

Expression of the nucleoprotein or matrix proteins of some enveloped viruses has also resulted in the production of VLP or virus pseudoparticles. This finding was noted first for the Gag protein of HIV when it was expressed in recombinant vaccinia-infected cells.^{178,196} Production of purified HIV pseudovirions is being developed as a vaccine strategy,¹²⁷ and the construction of vectors that are competent to produce pseudovirions *in vivo* may improve immunogenicity beyond that of the simple expression of the isolated gene products.^{72,182} VLPs have now been produced for several respiratory viral pathogens. Expression of M1, M2, NA, and HA can form influenza VLPs²⁴¹; however, HA is sufficient in the presence of NA.⁷¹ These are potent immunogens that can be constructed to express Ags that can confer broad cross-reactivity⁴⁰⁶ or TLR agonists that can provide adjuvant properties.⁴⁴¹ SARS CoV pseudoparticles are formed by transfection of 293 cells with the M (membrane) and N (nucleocapsid) genes.¹⁹⁷ The M protein of Newcastle disease virus (NDV) is sufficient to produce VLPs³³⁷ that can support the incorporation and display of RSV F and G and is immunogenic and protective in animal models.²⁷² The NDV VLPs appear to be a stable platform for presentation of viral glycoproteins from other viruses as well.²⁹⁰ Alphaviruses and flaviviruses are also potential viral targets for VLP vaccines. For example, West Nile virus pseudoparticles are formed by co-expression of prM and E.¹⁸⁴ Recently, chikungunya virus VLPs were produced that protected rhesus macaques from lethal challenge.⁸

Novel chimeric particles in which viral Ags are built onto the hepatitis B core VLP⁴³ are immunogenic in mice. Relatively large structures can be mounted on the recombinant VLPs if inserted covalently into the outer loops or attached through chemical conjugation in the right place and orientation to allow access to the vaccine Ag being displayed. Other chimeric VLPs can be constructed by inserting candidate vaccine Ags into the variable loops that face outward, including norovirus P particles,⁴¹⁸ Chikungunya,⁸ or others, and could be selected based on the desired particle size and Ag valency (Fig. 14.6).

OTHER VACCINE APPROACHES

Molecular biology has been an important stimulus for new approaches to make live attenuated virus vaccines. As well, it has provided avenues for production of subunit vaccines and gene delivery vehicles that express viral Ags (e-Fig. 14.9). MAB

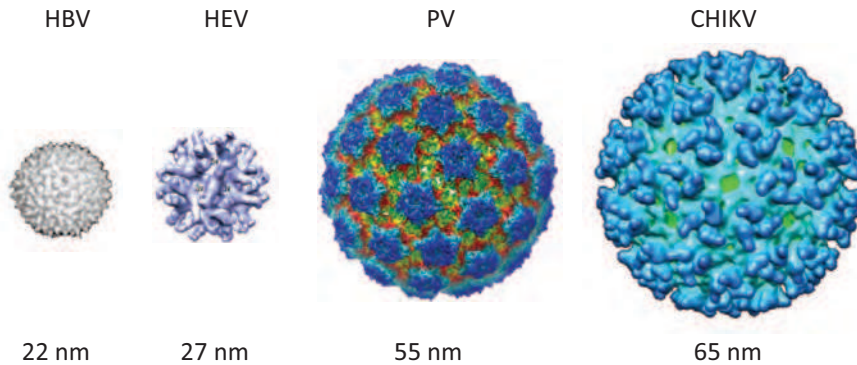


FIGURE 14.6. Variations on VLP size and structure. VLPs assume a wide range of sizes and are shown here as images derived from cryo-electron microscopy. Hepatitis B (HBV),¹¹³ hepatitis E (HEV),⁴⁶³ bovine papillomavirus (PV) representative of other papillomaviruses,⁴⁵⁵ and chikungunya (CHIKV)⁹ are shown by order of increasing size. VLP, virus-like particle.

and recombinant DNA technology are now used routinely to produce large quantities of purified viral Ags for use in immunoprophylaxis. MAb immunoaffinity chromatography or lectin chromatography facilitates purification of viral Ags from infected cells, whereas recombinant DNA technology makes it possible to express viral proteins in eukaryotic cells of yeast, insect, or mammalian origin. The use of these techniques has yielded candidate vaccines encompassing a large array of viral proteins that are capable of inducing protective Abs in experimental animals. Immunization by gene delivery is emerging as an important way to mimic certain aspects of live virus infection and can be accomplished by a growing list of delivery methods.

Vaccination With Proteins or Peptides

There are several theoretical advantages to immunizing with selected viral Ags instead of using whole virus particles. Production of recombinant viral proteins or synthetic peptides diminishes the risk of contamination by unrecognized pathogens. In addition, the preparation of vaccines free of nucleic acid eliminates theoretical concerns about recombination or integration of viral nucleic acid. Immunizing with individual Ags or epitopes from the virus might also reduce antigenic competition and focus the response on the most relevant protective antigenic sites.

Production of Viral Proteins in Eukaryotic Cells

Before expression of viral proteins in eukaryotic cells can be used as a method for vaccine production, several obstacles must be overcome. First, it is essential to identify the correct immunogen. Second, it is necessary to produce protein in a highly efficient manner to make this approach economically feasible. Mechanisms to enhance expression of viral proteins that have been pursued focus primarily on identification of strong promoters of gene expression and development of techniques for efficient gene amplification. Third, viral proteins must be separated effectively from host cell protein, DNA, and endogenous adventitious agents. Lastly, the proteins must be produced with the desired structure and activity. This latter requirement is of special concern, as immortalized mammalian cell lines are ideal for producing viral proteins because posttranslational modifications are more likely to be authentic than when these proteins are produced from prokaryotic hosts. Host cell DNA must be greatly reduced in the final vaccine (FDA guidance is <10 ng/dose) to reduce the theoretical potential for oncogenic events. Additionally, removal and inactivation of endogenous

viruses must be performed for products made from continuous rodent and other mammalian cell lines. Recombinant DNA techniques to promote secretion of viral glycoproteins into the medium have been developed to facilitate effective separation of viral proteins from host cell proteins. Fourth, the procedures used to produce and purify viral protein must be gentle enough to maintain the protein in its native state, thereby preserving conformationally dependent neutralization epitopes needed to induce protective Abs. Finally, because purified monomeric proteins are typically weak immunogens, they need to be formulated with adjuvants or produced as oligomers or particles to enhance their immunogenicity. The following discussion of these approaches is not intended to be exhaustive in scope but illustrative of current strategies in use or in development.

Recombinant DNA techniques are commonly used to express viral proteins in yeast, insect, or mammalian cells. The usefulness of yeast expression has been described previously for the production of the HBV vaccine and is also used to produce HPV VLPs.²⁵⁴ Some viral proteins such as the rabies G glycoprotein are more difficult to produce in yeast.³⁸⁴

A major advance in viral Ag production has been the development of baculovirus vectors to express foreign genes to high levels in insect cells under control of a strong baculovirus promoter, such as the polyhedrin promoter.²⁵⁷ Protein can be produced in large quantity (1 mg/10⁶ cells) using this system. Viral glycoproteins also can be produced in this system. For example, baculovirus-produced influenza HAs can be produced efficiently and have equivalent or superior immunogenicity to the traditional trivalent split product in adults²⁷ even though immunogenicity is significantly reduced in more immunologically naive children.²²³ The baculovirus system also can be used to express more complicated viral immunogens that require polyprotein processing to generate native immunogenic surface proteins. An example of this is expression of the entire structural protein region (C-E3-E2-6K-E1) of an alphavirus to generate authentic, processed viral proteins capable of inducing an efficient neutralizing Ab response in experimental animals.¹⁹³

Mammalian cells also have been used for the production of viral proteins that can be incorporated in a subunit vaccine. Mammalian cell cultures represent an optimal system for the production of viral proteins because protein folding, transport, and processing closely approximate those that occur in the infected host. Initial approaches were to purify viral proteins from infected cells or from cells transduced with a recombinant virus expression system engineered for high level of expression

of the foreign viral Ag^{140,141} or by transient transfection with DNA. More recently, cell lines have been developed that can stably produce recombinant proteins or Abs, can be modified for large-scale production, and have achieved regulatory approval for clinical products.

Chinese hamster ovary (CHO) cells have long been a preferred cell line for the production of protein-based therapeutics.⁴³¹ These cells have been used to produce viral glycoproteins from HIV and HSV that have been advanced to efficacy trials^{135,366,409} and can be modified to improve the manufacturing efficiency and capacity even further. Immortalized human cell lines derived from healthy human embryonic material have also emerged as producer cell lines for protein production such as 293 and PER.C6. Both lines have been used extensively to produce gene delivery vectors, have fully documented passage histories, and have had no adventitious agents or oncogenic or teratogenic potential identified to date. PER.C6 was derived from embryonic retina cells and immortalized with the adenovirus *E1* gene. The 293-ORF6 cell line has a similar development history and contains the adenovirus open reading frame 6 from *E4* as well as the *E1* gene.⁵⁴ These fully human cell lines may provide advantages for producing virus glycoproteins that authentically replicate the structures present in human infection, can be adapted to different growth conditions, and can stably produce high levels of recombinant protein without gene amplification.²⁰⁸ In general, identifying immortalized human cell line platforms for the production of proteins, VLPs, whole virus, or vaccine vectors will be preferable to using primary cell lines that have to be revalidated repeatedly or using less efficient production methods that require transient infection or transfection.

Production of Viral Proteins in Prokaryotic Cells

Production of immunogenic viral proteins in prokaryotic cells is difficult and requires extensive re-engineering of the gene to achieve immunogenic structures because of differences in protein processing and microenvironment. However, there have been some notable successes, especially in the production of VLPs. In particular, a portion of the structural protein from hepatitis E ORF2 produced in *Escherichia coli* self-assembles into particles and was shown to be efficacious in a 100,000-person study.⁴⁷⁵ HPV VLPs have also been produced in bacteria, although clinical trials have not yet been reported.^{249,311}

Production of Viral Proteins in Plant Cells

Plants have been modified genetically to express virus proteins^{63,415} and VLPs,⁹⁹ some of which have advanced to clinical trials. Plants can be engineered with selected mammalian glycosidases to provide more authentic posttranslational modifications.⁴¹¹ The major advantage of this approach is the potential for inexpensive and widely distributed manufacturing capacity.

Protein Purification

Immunoaffinity chromatography, lectin chromatography, and physical separation techniques have been used to purify a variety of viral glycoproteins that have induced partial to complete resistance in animals. When directly compared in animals, resistance induced by immunization is greatest when the purified viral glycoprotein is in its native conformation.⁴³³ In the case of RSV and EBV, purified glycoprotein vaccines induced Abs that were able to bind to glycoprotein in enzyme-

linked immunosorbent assays (ELISAs); however, these Abs had a very low level of neutralizing activity.^{302,452} In contrast to purified protein immunogens, RSV glycoproteins expressed by a vaccinia virus RSV F or G recombinant induced a titer of neutralizing Abs comparable to that developed by animals infected with the virus.⁸⁸ Observations of this type underscore the potential importance of quaternary structures and presentation of proteins in the context of membranes and organized on particles to achieve functional Ab responses.

Synthetic Peptides as Immunogens

During the early 1970s, the use of synthetic peptides as immunogens was studied using a synthetic icosapeptide representing the immunodominant domain of the outer coat protein of bacteriophage MS-2.²³⁹ This peptide, conjugated to a carrier and emulsified in adjuvant, induced Abs in rabbits that neutralized the infectivity of the bacteriophage when goat antirabbit Ig Abs were added to the mixture. Although synthetic peptides were shown subsequently to induce neutralizing Abs that did not depend on the aggregating effect of anti-Ig Abs and to stimulate immunity to certain animal viruses *in vivo*,³⁸ the immunity induced was less than that achieved when full-length protein, inactivated whole virus, or live virus was used as immunogen. The decreased immunogenicity of synthetic peptides is a consequence of their failure to mimic the conformational epitopes of viral surface proteins. Strategies to augment the immunogenicity of synthetic peptides, such as (a) inclusion of both B-cell and CD4 T-cell epitopes in the peptide, (b) synthesis of oligomeric peptide structures by using polylysine scaffolding,³¹² (c) presentation of peptide in a noninfectious viral particle, (d) expression of peptide by infectious virus, or (e) scaffolding of peptide epitopes with constrained conformational structure have not overcome the inherent deficiencies of this approach. Neutralizing epitopes from the membrane proximal region of HIV gp41 and the Synagis epitope from RSV were designed on protein scaffolds to mimic the structure and display the identical contact residues defined by the original peptide-Ab crystal structures. Although these novel Ags were immunogenic and induced Abs that bound HIV gp41 and RSV F, vaccinee sera did not have neutralizing activity.^{274,326} Using a much longer peptide may sometimes retain sufficient structural integrity to induce protective Ab responses. For example, a 100-aa peptide from the central region of RSV G can elicit neutralizing Ab to a central conserved region of the protein.³⁵⁸ Also, a 56-aa peptide from the stem region of the influenza HA can induce Abs to a conserved epitope that has some cross-neutralizing activity.⁴⁴⁴

The fact that CD8 T-cell epitopes are short peptides of 8 to 10 amino acids in length presented in the context of the MHC class I heterodimer suggested that it might be possible to produce peptide vaccines to induce CTL-mediated immunity.¹²⁰ CTL induction by peptide vaccines has been achieved in several experimental systems. For example, a peptide in adjuvant induced CTLs and protected mice against Sendai virus infection.²¹⁵ Thus far, peptide vaccines have not achieved significant immunogenicity when used in immunologically naive humans unless formulated in preparations with strong adjuvants that are associated with unacceptable reactogenicity. Also, the reductionist approach to vaccine Ags has not been successful when tested in humans. In general, it is better to use a larger antigenic content in vaccines for viruses so that the individual hosts can

select the most relevant and effective epitopes for themselves. This principle is also consistent with the concept that vaccination has its largest impact on public health at the population level. Developing tailored, personalized vaccination approaches may be in the distant future for preventive antiviral vaccines, and this type of approach is being explored for therapeutic vaccines against neoplasms and some chronic virus infections. Indeed, immunization with mixtures of long peptides derived from HPV E6 and E7 oncoproteins formulated in incomplete Freund's adjuvant (IFA) has led to remission of HPV16-induced vulvar intraepithelial neoplasia.²¹⁷

Vaccination by Gene Delivery

The age of molecular biology has made it possible to build expression vectors that can serve as a vehicle for gene delivery of vaccine Ags. The development of this technology is attractive because it combines the immunogenicity advantages of live vaccines and the safety advantages of nonliving vaccines. Genes can be modified to be nonfunctional and harmless to the host, improve expression, and yet retain the antigenicity of the expressed protein. Using the host cell for transient protein production provides the opportunity for expression of more native structures than are possible with *in vitro* protein production including glycosylation patterns that may influence antigenicity. Genes can be produced synthetically so that no component of the original virus pathogen is needed in the manufacturing process to avoid the potential complications of infection with live viruses or adventitious agents. Selected immunogenic components of the pathogens can be expressed and combined with molecular adjuvants that enhance and guide the composition of immune responses. In addition, the mechanisms that viruses use to evade or alter immune responses can be removed, or genes can be modified (e.g., by adding ubiquitination sites) to improve processing and Ag presentation.⁴⁰⁴ Therefore, it may be possible to improve on the immunity afforded by natural infection by using vector-based expression of viral Ags. New delivery approaches, including vector technology, provide the opportunity to combine Ags from multiple strains or different pathogens to potentially allow broadening of vaccine-induced immunity while simplifying vaccination regimens. They can be categorized by their functional properties (e-Fig. 14.10) and other features that may influence vaccine strategies for a particular virus (e-Table 14.2).

DNA Vaccines

The concept of using bacteria-derived plasmid DNA to deliver vaccine Ags has many attractive features, including ease and flexibility of construction, scalable manufacturing capacity, stability, production of vaccine Ag in host cells, transient expression, no induction of antivector immunity, induction of a balanced CD4 and CD8 T-cell response in addition to an Ab response, and minimal local or systemic reactogenicity.²⁴²

It was originally found to be possible to express transgenes by direct intramuscular or intradermal inoculation of plasmid DNA in the early 1990s.^{419,454} The feasibility of immunization with DNA was demonstrated by the induction of Abs against human growth hormone in mice immunized with a plasmid encoding this foreign protein.⁴¹⁹ The first demonstration of DNA vaccine protection against a virus was in mice challenged with influenza.²⁸⁸ Subsequently, immunization with DNA that encoded viral Ags, including Ags that can form VLPs, from

a wide range of viruses has induced functional (i.e., neutralizing or syncytium-inhibiting) Abs and/or resistance to virus infection.¹¹⁰ Gene-based immunization promotes host cell synthesis and expression of the vaccine Ag and physiological posttranslational processing and folding in the cell cytoplasm. For these reasons, DNA vaccination can elicit both CD4 and CD8 T-lymphocyte responses with a variety of immunogens in animal models.

Immunization with DNA has several advantages over immunization with purified viral Ags. The most important advantage is that vaccine Ags such as viral glycoproteins can be expressed on the surface of transfected cells and are presented to the immune system in a native state. During the processes of purification of viral proteins, purification and assembly of VLPs, or inactivation of viruses with chemicals, epitopes on viral proteins can be altered that compromise immunogenicity. DNA immunization, which circumvents these problems, more closely resembles immunization with a live virus vaccine than with an inactivated viral Ag. Viral Ags encoded by DNA, whether entire proteins or minimal CTL epitopes, are presented efficiently in the context of MHC class I molecules and are able to induce CD4 T cells and CTLs.^{65,243,264,468} In this way, it is possible to induce a balanced immune response more similar to immunity induced by natural infection than to immunity induced by administration of preformed viral Ags. A second advantage of immunization with DNA is its ability to transfect cells without interference by antiviral or antivector Abs. Live attenuated vaccine virus, such as that present in the measles virus vaccine, is effectively rendered noninfectious by Abs. Likewise, Abs to gene-delivery vectors, such as adenovirus serotype 5 (Ad5), can diminish the immunogenicity of this approach, whereas DNA immunization is not affected by pre-existing Abs. Finally, it has been observed that the foreign Ag can be expressed *in vivo* for several months following DNA immunization.⁴⁶⁵ Protein expression can also be optimized by modifying the promoter elements (e.g., adding the translation enhancer element from the human T-lymphotropic virus type 1 [HTLV-1] LTR).^{26,243} It is thought that the major factor limiting the immunogenicity of DNA vaccines is inefficient transfection of cells. This is partially improved by using delivery devices such as Biojector, and new approaches utilizing electroporation offer the potential for improved potency of DNA vaccines.³⁸⁸

The safety profile of DNA vaccines in clinical trials has been excellent to date, without evidence of integration of the vaccine DNA into the human genome or induction of autoimmune reactions, which were some of the early theoretical concerns. Initially, DNA immunization showed limited immunogenicity in humans, despite many examples of vaccine-induced protection in mice and nonhuman primates.¹¹⁰ The first DNA vaccine demonstrated to be immunogenic in Ag-naïve humans expressed the circumsporozoite Ag from *Plasmodium falciparum* and was shown to induce CD8 CTL responses detected by *in vitro* expansion of effectors.⁴⁴³ Another report described a DNA plasmid expressing the HBsAg that induced Ab as well as vaccine-specific T-cell responses in Ag-naïve humans.³⁷⁸ Through improvements in promoter design, manufacturing process, and delivery, DNA immunization in humans is now showing promise as a platform for inducing significant neutralizing Ab^{243,264} and T-cell responses.¹⁶³

Replication-Competent Vectors

The molecular technologies that have made gene delivery possible in general have been applied to the development of stable attenuated virus vaccines, some of which have been used to construct viable recombinants that express the surface proteins of other viruses, thus blurring the distinction between live attenuated viruses and vaccine vectors. Chimeric or live recombinant virus vectors can be designed to deliver the foreign gene from a virulent pathogen in the context of a nonpathogenic expression system, thereby improving safety. The vector design can also take into account manufacturing issues to improve overall production efficiency. The use of a viral vector for immunization has the advantage that the foreign viral Ags are expressed naturally in the context of an infected cell, thereby inducing cellular and humoral immune responses. Vector selection and design can focus vaccine Ag expression to a particular target cell or immunological compartment to tailor the organization of an immune response to a particular pathogen. Delivering the vaccine Ag in a complex structure has the benefit of broadly engaging both the innate and adaptive immune responses to better simulate the immunity to natural infection. Replication-competent vectors have additional immunogenicity advantages over replication-defective vectors, because more prolonged Ag expression and more diverse stimulation of TLR pathways often translate into better Ab production and more durable immune responses.

CHIMERIC LIVE VIRUS REASSORTANT AND RECOMBINANT VACCINES

Two major platforms have emerged to create chimeric viruses. One is based on reassortment of genes that occurs in segmented RNA viruses and is used in two currently licensed vaccines. The other utilizes reverse genetic systems developed to make molecular clones of viruses with nonsegmented RNA genomes. Live attenuated chimeric virus vaccines are distinguished from live viral vectors because the viral vaccine Ag being expressed is derived from the same virus family and replaces the homologous protein from the virus that supplies the coding and noncoding regions constituting the replication-competent particle.

The rhesus and bovine rotavirus vaccines are examples of live attenuated chimeric viruses produced by reassortment. The chimeric viruses contain the attenuating background of the rhesus or bovine rotavirus plus the gene for a human rotavirus VP7 surface protein. Similarly, the live attenuated influenza virus vaccines are chimeric viruses that can be antigenically updated by replacement of the HA and NA genes of the attenuated donor virus with those of a new epidemic or pandemic virus. This process was utilized in the response to the 2009 H1N1 pandemic. While the replication of A/California/7/2009 H1N1-like isolates in eggs was relatively low, the chimeric live virus produced as FluMist had outstanding growth properties that facilitated production.

Construction of recombinant live attenuated chimeric virus vaccines by gene replacement in molecular clones has most commonly utilized flaviviruses and paramyxovirus backgrounds. For flaviviruses, a live attenuated virus vaccine candidate for JEV has been made by the replacement of genes encoding the membrane precursor (prM) and envelope (E) proteins of the attenuated yellow fever virus (YFV) vaccine with those from an attenuated strain of JEV. The resulting

JEV-YFV antigenic chimeric recombinant vaccine candidate was attenuated and immunogenic *in vivo*.¹⁷³ Both components of this chimeric virus came from attenuated viruses. It has now been evaluated in advanced clinical trials²⁸⁶ and is being submitted for registration.¹⁷⁵ Chimeric recombinant candidate vaccines have been made between a naturally attenuated tick-borne flavivirus (Langat virus) and a wild-type mosquito-borne dengue type 4 virus (DENV-4), and the resulting recombinant was found to be significantly more attenuated than its tick-borne parent virus for mice.³⁵⁵ This is an example of an attenuating effect that stems from partial incompatibility between the two components of the chimeric virus. Another strategy is being pursued for the production of a tetravalent dengue virus vaccine in which a DENV-4 background containing an attenuating deletion mutation in the 3' noncoding region is used to construct antigenic chimeric viruses containing the surface proteins of other dengue serotypes.²⁷⁸ Alternatively, the 3' attenuating mutation is reproduced in the other serotypes. Chimeric flavivirus constructs appear to be a stable and effective vaccine platform for these types of viruses. Tetravalent, live attenuated dengue vaccines based on either the attenuated DENV-4 background or the chimeric YFV expressing prM and E from each serotype are immunogenic in humans and are being evaluated in advanced clinical trials.^{175,307}

A chimeric virus containing the HN and F surface proteins of parainfluenza virus type 1 (PIV1) substituted for those of an attenuated parainfluenza virus type 3 (PIV3) virus has been constructed and shown to be attenuated and protective in experimental animals.⁴²¹ Both bovine and human PIV3 chimeras are being pursued to make vaccines against several other paramyxoviruses including PIV1, -2, and -3, RSV, metapneumovirus, and measles virus. Both human and bovine PIV3 are very amenable to use as a background for chimeric paramyxovirus vaccines; however, other paramyxoviruses are also being developed as vaccine vectors including Sendai virus, NDV, canine distemper virus, and measles virus.¹⁹⁸ Paramyxoviruses have also been constructed as vectors to deliver vaccine Ags from unrelated viruses such as HIV or Ebola.¹⁰⁷

POXVIRUS VECTORS

Vaccinia virus is the most commonly used recombinant vector to date.²⁹⁵ Poxviruses have large genomes into which a variety of foreign viral genes can be inserted and expressed without seriously compromising the capacity of vaccinia virus to replicate. Recombinant vaccinia viruses expressing the surface protein(s) of a large number of viruses have been constructed and are shown to be protective in experimental animals. Polyvalent live vaccinia virus recombinants have been used to successfully immunize experimental animals, even in the presence of immunity to one of the foreign viral Ags,¹³² and vaccinia-rabies virus recombinants are being used to control rabies in foxes³⁴² (e-Table 14.3). Recombinant vaccinia has been evaluated in clinical trials,^{158,160,161} although they are not sufficiently attenuated for use in immunocompromised hosts^{129,172,365} and are complicated by other idiosyncratic side effects such as myopericarditis.¹⁸ However, new versions of attenuated replication-competent poxvirus vectors such as the Tianan strain of vaccinia have been constructed as candidate HIV vaccines and are currently being evaluated in clinical trials in China.¹⁰³

HERPESVIRUS VECTORS

Herpesvirus vectors have been developed using HSV-1, EBV, CMV, and some animal alphaherpesviruses. The primary advantages of this vector system are the capacity to package up to 100 kb of foreign DNA and the potential for long-term gene expression from latent episomal DNA that can be present in both neuronal and nonneuronal cells.⁵² Herpesviruses have tropism for a wide variety of cells and are efficient at cell binding and attachment even at low particle-to-cell ratios. Vectors can also be pseudotyped to help target delivery into selected host cells. They can be given mucosally as well as parenterally and can be engineered to eliminate immune evasion genes such as the HSV-1 ICP47 that interferes with the transporter associated with antigen processing (TAP) to diminish MHC class I presentation. Live herpesvirus vectors can be attenuated to avoid vector-mediated disease and can also be made replication defective (see later discussion). Because HSV is ubiquitous, a potential disadvantage for this approach is that pre-existing Ab to the vector may diminish immune responses to the transgene product. In a murine model, it was shown that pre-existing HSV immunity did not alter the Ab or proliferative responses to recombinant protein encoded by the HSV vector.⁴⁷ Using animal herpesvirus vectors may be an alternative approach to circumvent the potential problem of pre-existing immunity to the vector, although replication is severely restricted when species barriers are crossed.⁴²⁹ There is a licensed veterinary vaccine for Marek's disease in chickens based on this technology (see e-Table 14.3), and this technology has also been applied in HIV vaccine development. Initially, it was shown that replication-competent HSV vectors expressing Env and Nef showed partial protection against challenge with SIVmac239.³⁰⁸ Subsequently, rhesus CMV vectors were constructed expressing Env, Gag, Pol, and Rev/Nef/Tat. Animals immunized with either recombinant CMV vectors alone or primed with CMV and boosted with recombinant adenovirus (rAd) vectors had significant protection from a SIVmac239 mucosal challenge. The correlate of protection is thought to be persistence of tissue-resident effector memory CD8 T cells, sustained by the persistence of the vector.¹⁸⁵

RHABDOVIRUS VECTORS

VSV has been engineered to serve as a vector for recombinant gene delivery. Recombinant VSV can be produced at high titer, is relatively stable, has broad tropism, and can be given mucosally.²²⁰ VSV is a rhabdovirus and is known to have neurovirulence. Therefore, extensive work has been done to attenuate the parent vector by altering the gene order and by truncating the cytoplasmic tail of the major surface glycoprotein.³⁶⁰ Gene delivery and subsequent immune responses to the recombinant gene product are potent,¹⁷⁹ and rhesus macaques immunized by a VSV vector expressing Env and Gag have been protected from SHIV challenge.³⁷⁶ Replication-defective VSV vectors have also been developed (see later discussion).

LIVE ADENOVIRUS VECTORS

The live adenovirus vaccine for serotypes 4 and 7 is a licensed and highly effective vaccine that is administered mucosally. Therefore, it has ideal properties for a gene-delivery vehicle and has been developed as a candidate HIV-1 vaccine.^{256,347} The advantages are the track record of the vector for both safety and efficacy, as well as the potential for delivery in capsules to the lower intestinal tract.^{174,416}

PICORNAVIRUS VECTORS

Poliovirus vectors have been developed that can express recombinant foreign proteins that are excised by the virus-encoded protease after translation of the recombinant polypeptide.¹⁵ The major advantages of this approach are the long history of successful vaccination with the vector and the capacity for oral delivery and induction of mucosal immunity. The major disadvantage of this system is the relatively small packaging capacity (~200 aa) that requires a library of recombinant viruses in the vaccine to deliver large Ags. There is also the concern of reversion to neurovirulent forms of poliovirus, although there are new design approaches that can probably overcome this problem by manipulating the fidelity of the poliovirus polymerase¹⁹ or substituting the internal ribosomal entry site (IRES) in the 5' untranslated region with a similar stem-loop structure from rhinovirus.⁵⁷ Other issues complicating the use of live poliovirus vectors include pre-existing immunity against the vector in vaccinated persons and the recommendation to only use inactivated poliovirus vaccine in the United States. Although the global eradication campaign seeks to eliminate the need for polio vaccination, as with variola, as long as the virus remains in freezers or the sequence is available in databases, the idea of maintaining immunity to polio in the general population by using poliovirus as a vector for other pathogens has merit. Enteroviruses and rhinovirus vectors have been developed using similar technology and to target different mucosal inductive sites, and new innovations such as removing part of the capsid gene can provide a larger coding region for the vaccine Ag.^{108,298}

BACTERIAL VECTORS

Bacterial vectors have also been used to deliver viral Ags. Recombinant bacilli Calmette-Guérin (BCG) has received the most attention. Some advantages of BCG as a vector include (a) its safety record after administration to millions of persons, particularly in developing countries; (b) its persistence and therefore prolonged Ag production that may provide a more durable immune response; (c) its potential for mucosal administration; (d) its innate chemical composition that provides an adjuvant effect through TLR-1, TLR-3, TLR-4, and possibly other pattern recognition receptors; and (e) that immunity against tuberculosis could be a by-product of immune responses to the vector. Advances in mycobacterial genetics have made construction of recombinant vectors feasible,³⁸⁶ and because of the large capacity for accepting foreign genes, co-expression of cytokines and other immunomodulators is possible.^{11,309} In addition, discoveries about how mycobacteria inhibit apoptosis of macrophages to delay immune-mediated clearance have led to the development of attenuated mycobacteria for vaccination against tuberculosis. New promoters, other vector design modifications, and combining with other modalities may further enhance immune responses to the foreign gene products.³⁷⁵ Other bacterial vectors that have been considered for delivering viral Ags include lactobacilli, salmonella, shigella, and listeria. The salmonella and shigella vectors have also been designed to deliver the gene-based vaccine Ag by plasmid and thereby combine mucosal delivery and adjuvanticity with authentic glycosylation of the gene product.¹³⁶ This concept has been tested in early-phase clinical trials to express an HIV-1 envelope protein, although there was poor immunogenicity.

Replication-Defective Vectors

Replication-defective virus vectors have had genes deleted that are essential for replication *in vitro* or *in vivo*. Replication-defective viruses can replicate to high titer in cell lines engineered to express the missing protein that would be encoded by the deleted gene. The viruses produced in such complementing cells can attach and deliver their genetic payload to cells *in vivo* but are unable to replicate. Such vectors undergo an abortive infection *in vivo* but express the foreign protein during this abortive infection and can thereby induce both the humoral and cellular arms of the immune response. Non-replicating vectors do not retain the virulence of the original agent from which they were derived and would be expected to be safe even in hosts with compromised immune systems, should they encounter the vector. Many vectors retain the antigenicity of structural proteins from the original agent. This raises two issues. The first is reactogenicity. Many vectors can trigger innate immune responses that cause local and systemic reactions in the vaccinee. This is an advantage from the perspective of adjuvant activity but can be limiting at higher doses of the vector. The second is pre-existing immunity to the vector. If the vector is derived from an agent to which the host has had prior exposure, the pre-existing Ab can dampen the immunogenicity of the delivered vaccine Ag. Vector-based vaccines are of particular value when the vaccine target is a virus for which live attenuated or whole inactivated virus vaccines are not considered for safety or other reasons. Vectors have added value when the vaccine strategy is focused on inducing CD8 T-cell responses because they deliver the vaccine Ag to the cytoplasm, allowing access to the MHC class I processing pathway for Ag presentation. Because this characterizes the current strategies for HIV vaccine development, most vector-based vaccine strategies have been evaluated for the induction of protective immunity against lentivirus infection. Therefore, antiviral vaccine vectors will be discussed primarily in the context of HIV vaccine development even though they are also being developed for many other antiviral vaccine programs.

The regulatory approval pathway for vector-based gene delivery will involve scrutiny around issues such as integration, recombination, reversion to replication competence, and reactogenicity. The origins of many vaccine vectors are from the field of gene therapy, and some of the clinical events associated with gene therapy trials still affect the evaluation of vaccine vectors. For example, the 1999 death of a teenager in a gene therapy trial at the University of Pennsylvania prompted extensive reviews of safety data from both human and animal studies of vector-based gene delivery. The 18-year-old patient, who had a rare liver disorder called *ornithine transcarbamylase deficiency*, died after receiving a large dose (3.8×10^{13} particle units [PU]) of E1/E4-deleted serotype 5 adenoviral vector directly into the liver through the right hepatic artery. The death was most likely attributable to an adenovirus-induced cytokine storm with subsequent disseminated intravascular coagulation, acute respiratory distress, and multiorgan failure.³ This incident compels a cautious approach to dose and route evaluation of any antigenic substance but particularly vector-based products. Another gene therapy incident involved a retrovirus vector used to successfully treat children with X-linked SCID. After 3 years, some of the children developed leukemia associated with the integration of vector DNA into the LMO2 proto-oncogene locus.^{176,177}

ADENOVIRUS VECTORS

Replication-defective rAds exhibit characteristics that make them ideal vaccine vector candidates, and substantial resources are being invested to develop safe and immunogenic rAd vaccines for infectious agents including HIV, Ebola, plague, anthrax, influenza, tuberculosis, and malaria.²⁵ These vectors possess desirable qualities for use in vaccination strategies, including efficient transduction of host cells, limited duration of gene expression, vector nonpersistence, the ability to rapidly induce both humoral and cell-mediated immunity, induction of innate immune responses that may provide an adjuvant effect, relatively high stability, and ability to scale up production for manufacturing purposes.²⁵ Most rAd vaccine candidates are replication-defective owing to a deletion of the E1 region. Some vectors have additional deletions of E3 and E4 to increase capacity for gene insertion and to further reduce the chance of recombination in the packaging cell line to form replication-competent adenovirus. Deletion of E4 also prevents transcription of adenoviral genes encoding structural proteins to reduce antigenic competition with the vaccine Ag. Complementary human embryonic cell lines such as PER.C6²⁵⁸ or 293-ORF6⁴⁸ are used to package the deficient rAd *in vitro* and allow large-scale production. Pre-existing immunity to a given adenovirus serotype has been shown in animal models and in human clinic trials to diminish the immunogenicity of the vaccine Ag. Adenovirus serotype 5 (Ad5) immunity has a high prevalence, particularly in developing countries.²²⁹ Although Ad5 has been commonly used in the initial studies, there are 51 human adenovirus serotypes, and many have now been engineered to express foreign genes. In addition, new vectors have been constructed from adenoviruses derived from chimpanzees and macaques that have favorable properties and will provide options to avoid pre-existing vector-specific immunity. An alternative to using a rare serotype rAd vector is to construct chimeric vectors. For example, adenovirus vectors have been developed in which the shaft and knob portions of the fiber proteins are exchanged.⁴⁶⁰ Another novel approach involved making hexon loop chimeras in which the neutralizing targets in hexon are exchanged for those of an uncommon adenovirus serotype.⁴¹⁴ A mucosal route of administration is another potential option for overcoming pre-existing vector immunity, with the added advantage of better induction of immunity in the mucosal compartment. This may be particularly important for inducing immunity against viruses that primarily enter the host through a mucosal surface such as HIV and influenza. Replication-defective rAd has been successfully given mucosally without adverse reactions,⁴³⁴ even though one study in mice showed that intranasal administration could result in Ag expression in the olfactory bulb.²⁴⁵ The effects of pre-existing immunity to the vector can also be partially overcome by increasing the dose or by priming with a heterologous vector expressing the vaccine Ag of interest. Finally, one can avoid pre-existing immunity by carefully selecting the target population. For example, children between 6 months and 2 years of age are Ad5 seronegative, which makes recombinant Ad5 vectors a viable choice for immunization of that age group.

The Step study was a phase IIb trial that evaluated E1-deleted recombinant Ad5 vectors expressing HIV-1 Gag, Pol, and Nef in individuals at high-risk of HIV infection. It was stopped prematurely in 2007 because there was a higher frequency of HIV-1 infections among vaccinees than placebo

recipients.⁴⁹ The increased rate of infection was associated with being uncircumcised and with pre-existing Ad5 Ab. The biological basis for this outcome is not known. HIV-1 vaccine clinical trials utilizing rare serotype rAd vectors and recombinant Ad5 vectors (E1, E3, E4-deleted expressing HIV-1 Env, Gag, and Pol) in heterologous prime-boost combinations with DNA vaccines are still in progress.

ADENO-ASSOCIATED VIRUS VECTORS

Adeno-associated virus (AAV) vectors are unique because the wild-type virus itself is replication-defective and requires the presence of wild-type adenovirus as a helper virus to propagate. The vector is very simple and contains the promoter, foreign gene, and polyadenylation sequence surrounded by two short noncoding inverted terminal repeats from AAV. No genes from the wild-type AAV are included in the vector. Particles are produced in a packaging cell line that provides the AAV structural proteins. The AAV capsid induces an immune response, and as with other vectors, anti-AAV Ab can diminish immune responses to the foreign gene product expressed by recombinant AAV vectors. Using alternative capsid serotypes and incorporating immune modulators may overcome any limitations posed by pre-existing AAV immunity or allow heterologous boosting and further amplification of immunogenicity induced by primary vaccination. Transduction of muscle cells and APCs is efficient, and expression can be prolonged. A single dose of recombinant AAV has been shown to induce both humoral and cellular immunity sufficient to control SIV infection in macaques.²⁰⁵ AAV-transduced cells achieve persistent gene expression by delivering concatameric circular episomes that remain transcriptionally active, and there is no evidence for integration.³⁹⁴ Long-term episomal gene expression has been uniquely demonstrated by delivering neutralizing Ab genes to muscle cells in rhesus macaques and achieving sufficient titers in serum to protect against SIV challenge.²⁰⁶

ALPHAVIRUS VECTORS

Alphaviruses such as Venezuelan equine encephalitis (VEE) virus, Sindbis virus, and Semliki Forest virus (SFV) have been modified to produce three distinct types of immunogens. All take advantage of the alphavirus replicon that is a minimal genome with replication origins and packaging signals. The vectors include the alphavirus nonstructural genes including the replicase and the subgenomic promoter upstream of a foreign gene that replaces the genes encoding alphavirus structural proteins. The replicon itself can be injected as either plasmid DNA or as naked RNA. Alternatively, helper cell lines can be constructed that provide the structural gene products needed for packaging of replicons and particle assembly of nonpropagating vectors.¹⁴⁰ These replication-defective viral particles are extremely efficient in targeting APCs, particularly dendritic cells, and because of the amplification of the RNA, they achieve high-level expression of the foreign protein. They have induced significant humoral and cellular immune responses to a variety of viral proteins in animal models,^{192,207,361} and a VEE vector expressing HIV Ags has advanced into clinical trials.⁴⁵¹ The immunogenicity of the VEE vector is in part owing to its tropism for dendritic cells. When minor changes are made in the envelope protein to diminish dendritic cell entry, the induction of immune response is compromised.²⁵⁹ One unique property of VEE vector-induced immunity is that parenteral immunization has

been shown to induce mucosal IgA responses.¹⁸⁶ The mechanism underlying this phenomenon relates to the rapid and dramatic effect that the VEE particles and replicons have on the systemic innate immune response²²⁷—an effect that occurs in the first 24 hours after inoculation.⁴²⁴ Because the stimulation of the innate immune system by VEE replicons also appears to enhance adaptive immune responses, replicons that do not express a vaccine Ag can be used as an adjuvant to enhance the protective response to subunit vaccines.⁶¹

HERPESVIRUS VECTORS

Replication-defective HSVs have been produced in a manner analogous to that of the E1A-deficient adenoviruses—that is, they have had one or more genes deleted that are essential for replication; however, replication *in vitro* is supported in a cell line that constitutively expresses the essential protein needed for replication.^{100,289} In contrast to the E1-deficient adenoviruses, the replication-defective HSVs are being developed as vaccines to protect against disease caused by HSV itself, as well as a vector to deliver foreign viral proteins. One potential advantage of herpesvirus vectors is persistent Ag production that may be required for the optimal induction of cellular immune responses. Another approach to vector construction is to package replication-defective HSV amplicons in HSV particles using bacterial artificial chromosome (BAC)-encoded helper viruses with deleted packaging sequences resulting in foreign gene expression without the influence of any residual HSV genes.¹⁵⁴

POXVIRUS VECTORS

Attenuated replication-defective poxvirus vectors have been developed to address the known complications of vaccinia virus in immunocompromised persons. The most developed vectors include MVA, avipox (canarypox and fowlpox), and NYVAC (deliberate deletion of 18 genes associated with virulence).³³⁸ MVA was derived by more than 500 passages in chick embryo cells^{266,280} and has been evaluated as a vaccine vector for a variety of viral Ags. MVA is a highly attenuated host-range mutant that replicates well in avian cells and has limited replication in BSC-40 and baby hamster kidney (BHK) cells but does not propagate in most mammalian cells.^{39,60,113} MVA is nonpathogenic in immunocompromised hosts and has been administered to large numbers of humans without incident.^{267,410,449} Attenuation of the MVA recombinant resulted from the loss of nearly 30 kb of genome including genes for immune evasion and host range during its many passages in embryonated eggs.¹⁶ The use of avian poxviruses, referred to as avipox viruses, as vectors represents a second type of host-range restricted poxvirus vector. Avipox viruses are naturally occurring host-range restricted viruses that are replication-deficient in mammalian cells and mammal hosts.³³⁸ Each of these poxvirus vectors has the property of efficient replication *in vitro* in avian cell culture but restricted replication in primates. Avipox vectors have an added advantage because pre-existing immunity to vaccinia does not impact vector delivery. Poxvirus vectors have the following advantages: (a) thermostability, (b) the history of being associated with the extraordinary success of the smallpox eradication campaign, (c) large packaging capacity for foreign genes, and (d) lack of persistence or genomic integration. The major disadvantages are that (a) attenuated vectors are grown in primary cell lines (chicken embryo fibroblasts),

and production is difficult to scale up; (b) the foreign gene insert is sometimes unstable; and (c) poxviruses are antigenically complex, and there may be competition between the vector and the inserted gene products for Ag processing and presentation.

After extensive phase I and phase II evaluation,^{152,352} a recombinant canarypox vaccine was evaluated in RV144—a controversial phase III efficacy study.⁵¹ This landmark study provides a benchmark as the first demonstration of efficacy for an HIV vaccine.³⁶⁶ A general population cohort of 16,402 people in Thailand was enrolled in the study and randomized 1:1 vaccine to placebo recipient. Vaccinees received intramuscular injections of a recombinant canarypox vector expressing Env, Gag, and parts of Pol and Nef given at 0, 1, 3, and 6 months and rgp120 formulated in alum given at months 3 and 6. At 18 months, 74 infections had occurred in placebo recipients, and 51 infections had occurred in vaccinees, which was statistically significant. A similar rgp120 did not protect men-who-have-sex-with-men (MSM)¹⁴⁸ or intravenous drug users (IVDU)³⁵³—populations with much higher incidence. The immune correlate of protection is not yet defined; however, protection appears to be temporally associated with serum Ab titers, even though vaccinee sera does not neutralize commonly transmitted strains using traditional assays.

Limitations of Gene-Based Vectors

It is beyond the scope of this chapter to cover the growing list of potential vectors to deliver genes encoding viral Ags that may be considered for future vaccine development. For example, arenavirus¹³¹ and lentivirus¹⁰² vectors have not been discussed here, and reverse genetic systems have been developed for other virus families²²⁵ that will make recombinant vector development feasible. Each system has a unique set of properties that should be considered when devising a strategy for a particular pathogen. One of the primary considerations is seroprevalence for the vector in the target population. Pre-existing vector-specific immunity can affect both safety and potency of the vaccine. This may be a problem if there is natural immunity to the vector or if competing vaccine programs for other important pathogens converge on the same vector platforms. There are several factors involving safety, Ag selection, immunogenicity, manufacturing, and stability that are important to consider when developing a vector-based vaccine strategy that are pathogen and target population specific (see e-Table 14.2).

Viral Vectors as Vaccines Against the Virus from Which the Vector Was Derived

A practical by-product of the development of gene-based replication-defective viral vaccine vectors is that the vector itself may serve as a novel vaccine platform for the virus from which the vector was derived. The most advanced examples of this are the poxvirus vectors such as MVA or NYVAC. These have been evaluated as candidate smallpox vaccines in clinical trials using replication-competent vaccinia (Dryvax) as a surrogate challenge.^{139,341} This type of approach has also been proposed for herpesviruses¹¹⁶ and adenoviruses. Using the viral vector creates the opportunity to express additional viral Ags⁹ or immunomodulatory proteins such as cytokines⁷⁰ that could provide adjuvant effects and increase immunogenicity compared to the live attenuated virus platform. Another advantage of this approach is that the vector delivers the antigenic

complexity of a live attenuated virus vaccine with a more favorable risk profile because of lack of replication. In addition, the vector provides more options for achieving robust manufacturing capacity, although this is often a challenge for live attenuated viral vaccines.

VACCINE FORMULATION AND DELIVERY

Adjuvants

Adaptive immune responses are controlled by the amount, duration, and formulation of the Ag in conjunction with innate immunity. The Ag sequence and structure determines specificity; however, the adjuvant and context of delivery and Ag presentation determine the composition, location, kinetics, and magnitude of vaccine-induced immune responses (e-Fig. 14.11). Although specificity is a property of the adaptive immune response, many adjuvant effects are mediated by the innate immune system. The most potent and broad-based adaptive immune responses are induced by replication-competent virus vaccines and gene-based viral vectors. These vectors provide efficient Ag presentation and inherently stimulate innate immunity through a variety of signaling pathways, thus limiting a requirement for exogenous adjuvants. By contrast, inactivated virus vaccines, purified viral proteins, or peptide Ags by themselves have relatively weak immunogenicity in a naive host. There are several reasons why replication-competent viral vaccines and gene-based vectors are inherently more immunogenic than inactivated virus or subunit vaccines. First, gene expression provides an ongoing source of the relevant protective Ag that is amplified through replication beyond the dose contained in the original inoculum. Second, Ags produced during transcription in the host cells have native quaternary and multimeric particulate structures and authentic posttranslational modifications. Third, replication-competent viruses and gene-based vectors can broadly engage multiple distinct innate immune and Ag presentation pathways. Indeed, a major advantage of live or attenuated viral vaccines is that expressed antigenic epitopes are processed efficiently through both MHC class I and II pathways. This mechanism contrasts with that of inactivated vaccines where Ag processing is mostly limited to MHC class II presentation, which elicits CD4 T-cell responses but is limited for generating CD8 immunity. Finally, PAMP recognition receptors are engaged on the cell surface, in endocytic vesicles, and in the cytoplasm with live vaccines (e-Fig. 14.12). Therefore, adjuvants are needed to compensate for the diminished immunogenicity of vaccines that are not transcriptionally active in host cells by recruiting additional signaling pathways to activate innate immunity, promoting APC maturation and co-stimulation, and prolonging Ag persistence.

Empirically Derived Adjuvants

Traditionally, adjuvants have been derived from natural products empirically found to have immunostimulating properties. Adjuvants can improve immunogenicity by optimizing the duration of Ag presentation through a “depot” effect and/or through enhancing certain innate stimulatory pathways. It was first discovered that bacterial toxins were more protective when mixed with other substances.³⁶² A prototypic adjuvant used exclusively in laboratory research is Freund’s adjuvant prepared from mycobacterial cell walls. A modern version of IFA

(Montanide ISA-51) is a water-in-oil emulsion composed of mineral oil mixed with the surfactant mannose monooleate in a 1:1 ratio with the aqueous phase. Although vaccines formulated with mineral oil have been administered to more than one million people since 1945, with the emergence of aluminum-based adjuvants, they fell out of favor because of the reactogenicity profile and potential for causing sterile abscesses.¹⁶⁴ They are still frequently used in the context of therapeutic vaccines.

There are two currently licensed adjuvants in the United States for human use in preventive viral vaccines: alum and a combination of alum+MPL. Alum is derived from aluminum hydroxide or aluminum phosphate and is formulated as a gel or used to precipitate protein Ags to create aggregates. It functions as a short-term Ag depot in which Ag that is electrostatically bound to alum is slowly released at the site of inoculation. More recent studies have shown that some of the adjuvant activity conferred by alum is through activation of the “inflammasome” innate signaling pathway.¹¹⁹ Alum has been the standard adjuvant used in licensed vaccines for several decades. All currently licensed inactivated viral vaccines and hepatitis B VLPs are formulated with alum. Gardasil, the Merck HPV VLP vaccine, is also formulated with alum. More recently, Ab responses can be improved by combining additional immune stimulatory molecules to alum. Indeed, AS04 (Adjuvant System 04, GlaxoSmith-Kline, London, UK) is a combination of alum and a modified synthetic form of MPL, which is a key component of lipopolysaccharide (LPS) that is the ligand for TLR-4. Cervarix, the HPV VLP from GlaxoSmithKline, is formulated with AS04 and is the first adjuvant containing a TLR ligand to be approved for use in humans. The MPL-based adjuvants when combined either with alum (AS04) or with QS21 (AS02) have been shown to have a significant dose-sparing effect and may have additional advantages in terms of the speed and durability of the humoral responses. Formulations that include MPL also stimulate IFN- γ , producing CD4 T-cell responses that may have additional benefit.³¹⁶

After alum, the other most widely used adjuvant in humans is MF59. MF59 is a squalene oil-in-water squalene emulsion that is licensed for human use in Europe. MF59 was designed originally to provide an Ag depot from which Ag would be slowly released as the biodegradable oil was metabolized or removed by macrophages, and as a vehicle for the immunostimulant muramyl tripeptide-phosphatidyl ethanolamine (MTP-PE), a derivative of muramyl dipeptide, the active component of the mycobacterium cell wall fraction in Freund's complete adjuvant. However, clinical studies in humans indicated that MF59 itself was as immunostimulatory as MF59/MTP-PE with less toxicity.¹⁶² Its adjuvant effect is not TLR dependent, but there is evidence that MF59 has some innate immune stimulatory capacity. More than 45 million doses have now been given to humans formulated with seasonal influenza vaccines. MF59 is dose sparing, and clinical studies with H5N1, H1N1, or seasonal trivalent influenza vaccines show that protective levels of immunity can be achieved with as little as 3.75 μ g of vaccine Ag.⁷⁹ A similar squalene oil-in-water emulsion (AS03) has achieved similar dose-sparing effects when formulated with influenza Ags.³¹⁷ MF59-formulated influenza vaccines also result in Abs with greater cross-reactivity and greater avidity to conformational epitopes than

alum-formulated vaccines in head-to-head comparisons.²¹⁸ As with alum, MF59 tends to promote Th2 responses.⁴³²

QS21, the active ingredient of immune stimulating complexes (ISCOMs), was discovered in the bark of *Quilaja saponaria*, Molina (soap bark tree). It was named for the tree's scientific name and for being the 21st peak on the high-performance liquid chromatography analysis of the bark extract. The basis for its adjuvant properties is not known.

Rationally Designed Adjuvants

As discoveries were made in the 1980s and early 1990s about the regulation of immune responses by cytokines,²⁹⁴ chemokines,⁷ and co-stimulatory molecules,³⁹² a more targeted molecular approach was taken to vaccine adjuvants. The trend during the 1990s was to attempt to build the optimal adjuvant effect one cytokine at a time and try to precisely control the processes of cell activation and differentiation.^{251,420} This approach may still be possible but has underestimated the complexity of the molecular milieu required to produce a specific effect, and also has underestimated the complexity of effector phenotypes and the importance of timing involved in the successive waves of regulatory events participating in nascent immune responses. For example, in a study evaluating HIV Ags delivering by DNA together with an IL-2-Ig fusion protein also expressed as a plasmid, it was found that the cytokine only provided an adjuvant effect if delivered 48 hours after the vaccine Ag.²⁰

More recently, the understanding of how PAMPS²⁰³ are recognized has helped to explain the molecular basis for some traditional adjuvants and has resulted in better characterized products that are now being developed as vaccine adjuvants. PAMPS are recognized by TLRs—a family of type 1 integral membrane proteins—present on plasma membranes or within endosomes.²⁷⁶ Ligands for any of the 11 currently known human TLRs can produce an integrated set of responses by APCs and other components of the innate immune system to stimulate adaptive immune responses (Chapter 8). Using TLR ligands instead of individual cytokines to adjuvant vaccine-induced immune responses helps to avoid the unanticipated problems that arise when using individual molecular adjuvants in isolation, providing a more authentic stimulus for the host to decide what pattern of cytokines, chemokines, and co-stimulation will be needed for a particular immune response. Empirically developed adjuvant products such as muramyl dipeptide derivatives or MPL are known to stimulate TLR-2 and TLR-4, respectively. Components of live and inactivated virus vaccines such as double-stranded RNA and single-stranded RNA are known to stimulate TLR-3 and TLR-7/8, respectively. Experimental adjuvants such as PolyI:C (TLR-3 agonist) and imidazoquinoline compounds (imiquimod or resiquimod TLR-7/8 agonists) are being explored to mimic these features of live virus infection. Palindromic CpG motifs present in bacterial DNA that are ligands of TLR-9 are also used as experimental adjuvants.

It is beyond the scope of this chapter to review all of the ways that cytokines, chemokines, and activation of TLR signaling pathways are being explored in antiviral vaccine formulations. As described previously, the discovery of PAMP recognition receptors provides an explanation for the activity of some empirically developed adjuvants (e.g., TLR-2 and TLR-4 agonists) and a platform for the rational design of future vaccine adjuvants.

Delivery Vehicles

Delivery of vaccine Ag can determine the immunological compartment that is stimulated and influence the types of APCs that process the vaccine Ag. Many delivery vehicles are formulated with adjuvants or have innate adjuvant properties; however, in this section the term will refer to approaches for transporting Ag to a particular location. A delivery vehicle may allow the mucosal delivery of an Ag that could otherwise only be given parenterally. Alternatively, a delivery vehicle could function as a way of co-delivering a mixture of Ags and adjuvant, produce a depot effect, or otherwise control the timing of release of Ag. More recently, vaccine delivery vehicles have been designed to carry Ag into particular subcellular compartments with the intent of stimulating specific immune responses by accessing distinct Ag processing pathways.

Lipid-Based Carriers

Many virus surface proteins are spatially oriented in lipid envelopes, and that is one rationale to use lipids in the formulation of virus vaccines. Another is that the Ag may be protected by the lipid and allow delivery through harsh environments such as the gastric mucosa or blood, or promote a depot effect when injected. Liposomal vaccine formulations may facilitate DNA transport into target cells and have been used extensively for transfection of cells *in vitro*. Liposomes are vesicles with a roughly spherical lipid bilayer surrounding an aqueous center.¹²⁸ If virus-derived proteins are inserted into the membranes (proteoliposomes), they are often referred to as virosomes. Liposomes have been used to deliver peptides, proteins, whole virus, and DNA with and without adjuvants and can be designed to target or co-stimulate selected APCs. The intent is to produce an inert particle that concentrates in the target organ of interest and then releases its payload. Typically, when used for purposes of DNA delivery, cationic lipids are used in the liposome formulation to improve the interaction with the negatively charged nucleic acid. The lipid composition of the liposome can be modified to affect charge, pH sensitivity, thermal stability, and fusogenicity to control release of the products. Virosomes are used in licensed products (non U.S. products) to deliver vaccine Ags for influenza and hepatitis A.²⁹² More elaborate virosomes in which the proteoliposome is built around a polystyrene bead are being developed as a candidate HIV-1 vaccine, using the scaffolding of the particle to produce more stable conformations of the envelope glycoprotein.¹⁷¹ As a natural source of liposomes, exosomes are 50- to 90-nm vesicles produced by an endosomal process in which vesicles bud into endosomes (multivesicular bodies) and are then secreted from APCs. Interestingly, these exocytic vesicles contain many of the proteins involved in Ag presentation, including MHC and co-stimulatory molecules.²³⁸ In addition, they have lipid content similar to many enveloped viruses and lipid rafts with high cholesterol content.¹⁵⁷ ISCOMs are another lipid-based vehicle for delivering vaccine Ag. They are 40-nm cage-like structures formed by a complex mixture of lipids and saponins that can be formulated with viral Ags.⁶ ISCOMs are able to deliver proteins or peptides to the cytoplasmic compartment, where they can be processed to activate CD8 T cells, and this has also been demonstrated in clinical trials of HPV and HCV vaccine candidates.³⁸⁷ ISCOMs also have adjuvant properties derived primarily from the saponin component QS21, as described previously.

Synthetic Particles

Synthetic polymers have been used in creative ways to contain or hold vaccine Ags for presentation to the immune system. Microparticles are made from biodegradable polymers complexed with viral Ags.⁹⁴ The primary polymer used is poly lactic-co-glycolic acid (PLGA), which can encapsulate DNA by itself or with multiple proteins, adjuvants, or other immunomodulators. PLGA has an extensive history of safety in humans because it is the polymer used in absorbable sutures. Some other advantages of microparticles (1 to 10 μm) include (a) preferential uptake through the phagocytic pathway of dendritic cells; (b) protection of the vaccine product from the environment, resulting in a slow-release depot effect³²⁴; and (c) a potential payload capacity that is higher than for liposomes or viral vectors. PLGA microparticles coated with cationic surfactants have also been used as a vehicle for DNA delivery by absorption of plasmid DNA to its charged surface.³²⁴ In general, these approaches have not produced the robust gene expression and immunogenicity needed for gene-based vaccination, and the formulations are being empirically modified. One approach has been to produce a hybrid polymer by combining PLGA with other substances such as poly- β amino ester to improve the sensitivity to pH change and more rapidly liberate the DNA.²⁵² Another approach for DNA delivery has been to use alternative polymers such as poly (ortho esters) (POEs), which had been developed for drug and peptide delivery.⁴⁴²

Nanoparticles also have been explored as vehicles to deliver DNA vaccines. Generally sizes of less than 200 nm (close to the size of many viruses) have been used, which are less likely to be forced into the phagolysosome pathway than microparticles. One approach has involved polylysine cationic polymers with imidazole side chains to facilitate escape from endosomes.²⁵³ Another utilizes an oil-in-water microemulsion to produce wax cationic nanoparticles⁹⁸ that can be modified to encapsulate DNA.³⁰⁰ Finally, nonionic block co-polymers have been used to deliver DNA vaccines with promising results in animal models but not in human trials.⁴⁰⁰

Synthetic self-assembling polypeptides can be used to display vaccine Ags on particles to achieve multivalency similar to the use of VLP as Ag scaffolds.³⁵¹ Another approach is to use self-assembling proteins from other (nonviral) biological sources such as the yeast protein Ty,²⁴⁴ although evaluation of these structures in human trials did not induce significant immune responses.⁴⁴⁷

Cell-based Carriers

Dendritic cells are important for Ag presentation, for initiating the innate responses, and for bridging the innate and adaptive immune systems to coordinate a comprehensive response to a virus infection. Therefore, they have been central to several vaccine development efforts. Not surprisingly, dendritic cells themselves have been used as the vehicle for Ag delivery. Autologous dendritic cells have been sorted, expanded *in vitro* with selected cytokines, pulsed with peptide, inactivated whole virus, or vectors and then reinfused into subjects.^{255,263,325} Another cell-based vaccine approach utilizes transgenic plants. Potatoes, lettuce, tomatoes, carrots, and corn have all been engineered to produce vaccine Ags that have been evaluated in clinical trials.⁴¹⁵ This technology is also in early stages but has partially overcome some of the initial theoretical concerns that delivery of edible vaccines would result in oral tolerance or

possibly result in food allergies to the carrier. A major advantage of transgenic plants is that production does not require an extensive physical infrastructure, and therefore manufacturing is inexpensive and easily distributed.

Conjugates

Vaccines derived by conjugating protein to carbohydrate have been extraordinarily successful for the control of encapsulated bacteria. The protein in this setting helps to redirect the Ag presentation so that alternative Ab isotypes are produced to control the bacteria. Because the carbohydrate structures of viruses are made by the host cell, they are not antigenic targets and often protect the virus from host immune defenses, as exemplified by the “glycan shield” of the HIV-1 gp120. Conjugates for viral Ags have typically been designed to carry the viral Ag into the cytoplasmic compartment of the APC so that processing and MHC class I presentation and CD8 T-cell activation can occur. This objective has been accomplished by using heat shock proteins (HSP70),⁶⁴ alpha-2 macroglobulin,²⁵⁰ and leader sequences that promote membrane translocation to help the protein directly enter the cytoplasm.^{189,372} Protein conjugate vaccines with Ab Fc receptors, CpG motifs, and TLR-7/8 agonists^{73,450} also have been used to improve the efficiency of uptake of viral Ags to dendritic cells. An additional approach is to target Ag directly to dendritic cells through incorporating the viral Ag into an Ab such as the DEC205 Ab conjugate that delivers Ag directly to dendritic cells.^{133,314} This provides an efficient *in vivo* approach for targeting dendritic cells instead of the *ex vivo* approach mentioned earlier, which may be limited to therapeutic vaccines for chronic virus infection and cancer.

Mechanical Devices

Currently licensed vaccines are given orally or by needle and syringe, aerosol, or bifurcated needle in the case of vaccinia. Future vaccines and adjuvants or immunomodulators may utilize more technically sophisticated devices such as microneedles²²¹ or micromechanical abrasion.²⁸² Other delivery devices have been adapted or developed in large part to address the need for improving the efficacy of DNA vaccines in humans. Needle-free injection systems such as Biojector or PowderJet have been used to deliver DNA vaccines in clinical trials,^{163,378,443} and in both animal models and humans, electroporation has been shown to improve Ag expression and immunogenicity of DNA vaccination.^{388,435} In addition, there continue to be new designs for delivery by traditional aerosol or needle and syringe devices that have been designed to improve effectiveness or safety. In particular, there are rapid advances in microneedle (<1 μm) technology in which arrays of needles made from a wide variety of materials (ranging from stainless steel to dissolving crystalline sugars) can effectively deliver vaccines.

Combination Approaches

Co-Delivery or Multivalent Vaccines

Co-delivery of vaccines has become a matter of practicality with more than 13 pathogens being targeted during the childhood immunization series alone. Therefore, combined vaccine formulations such as MMR (measles, mumps, rubella), the tetravalent product that includes varicella vaccine with the MMR combination, IPV (polio serotypes 1, 2, and 3), and influenza (B and A H3N2 and H1N1) are commonly used. Factors such as antigenic competition, interference, and

unanticipated adverse reactions need to be considered during the development of combination products.

Co-delivery of different vaccines against the same pathogen can sometimes augment immunogenicity. Simultaneous administration of two different influenza virus vaccines to elderly subjects—namely, an intranasally administered live attenuated⁴¹⁵ influenza A virus candidate vaccine and a parenterally administered licensed inactivated virus vaccine—induced greater resistance to illness than did administration of the inactivated virus vaccine alone.⁴³⁰ In this situation, the greater immunogenicity of the live virus vaccine in the respiratory tract coupled with the greater efficiency of the inactivated virus vaccine in stimulating systemic immunity appear to act in concert to optimize efficacy. A replication-defective HSV vaccine was found to be most immunogenic when administered by both parenteral and mucosal routes, indicating that the same vaccine preparation can also be given by a combination of routes to augment protection.²⁸⁹

Sequential Combination of Vaccines

Sequential combination of different vaccine modalities that focus on inducing immune responses to the same pathogen have recently been referred to as prime-boost strategies.³⁶³ This concept takes advantage of the memory inherent in the adaptive immune response by sequentially amplifying the response to selected Ags. This can be done with combinations of replication-competent or gene-based vectors with inactivated or subunit vaccine approaches or by heterologous vector combinations. Combining vaccine approaches has the potential for eliciting new components of the immune response and for broadening the response to new specificities and immunological compartments. Combining heterologous vectors has the added value of the boost, avoiding pre-existing antivektor immunity while amplifying the response to the expressed vaccine Ag.

Combination approaches can be used both for improving the safety of the booster vaccine and for improving immunogenicity beyond either approach alone. Polio vaccination is an example where using combined modalities improved both safety and immunogenicity. It is now recommended that only IPV be used for polio vaccination in the United States. Prior to this, sequential immunization with inactivated poliovirus vaccine followed by live attenuated virus vaccine was used as a safer alternative to OPV, the three-dose live virus vaccine. The combination approach provided immunity against the rare cases of vaccine-associated polio caused by the live virus vaccine and also primed for a booster effect from OPV, which expanded the immune response to the mucosal compartment. Thus, immunogenicity was optimized in the individual and herd immunity was provided to the population by inducing sufficient mucosal immunity in the gastrointestinal tract to prevent the spread of imported strains of poliovirus.^{284,350} Sequential immunization with a live attenuated alphavirus vaccine candidate followed with a boost with an inactivated virus vaccine induced higher levels of Ab than live virus alone.³⁵⁴ In the field of HIV vaccine development, a variety of prime-boost strategies have been evaluated. In some cases, priming has been done with a DNA or gene-based vector followed by subunit protein boost.^{82,90,160} This approach led to the first demonstration of efficacy against HIV acquisition using a recombinant canarypox vector expressing Env, Gag, and Pol boosted with recombinant gp120

protein.³⁶⁶ Other approaches have utilized heterologous vector combinations or DNA priming followed by gene-based vector boosting.^{13,183,230,396} These studies have shown that DNA priming followed by recombinant Ad5 boosting can protect against SIV infection in macaques and reduce viral load in vaccinated animals that experience breakthrough infections.²⁴⁷ This concept is being evaluated in HVTN 505, a phase IIb efficacy trial.

SUMMARY

Vaccine development has occurred primarily through observation and empiricism. In some cases, the emergence of new technologies allowed advances in vaccine development, and occasionally basic research discoveries have led to successful new vaccine approaches. The earliest observations that smallpox did not occur twice in the same individual led to the development of insuflation and variolization. Observing that a relatively innocuous cowpox infection seemed to protect against smallpox led Jenner to vaccinate. When Pasteur observed that an aged bacterial culture did not cause disease in chickens, and subsequently found that those chickens were protected from challenge with fresh cultures, an era of intentional modification and attenuation of microbes began that led to the introduction of many new vaccines. New technologies, such as cell culture, led to a series of new vaccines because viruses such as measles, mumps, rubella, and polio could be cultivated and produced in large quantities. The advent of molecular biology has led to the production of subunit proteins for successful hepatitis B and HPV vaccines and the technical ability to produce reassortant and recombinant viruses. Modern immunology, driven largely by the response to the HIV/acquired immunodeficiency syndrome (AIDS) crisis, has introduced new methods for measuring T-cell function that will help define the role of T cells in vaccine-induced immunity and guide the use of molecular adjuvants. Structural biology is providing an increasingly rapid way to view the atomic structures of viruses and their antigenic determinants bound to Abs and suggesting new approaches for vaccine design. The expanded capacity for nucleic acid sequencing has facilitated the discovery and surveillance of viral threats and has provided new insights to the biology of viruses and immune effectors. Technological advances in engineering and manufacturing have made vaccines safer and have created many new approaches for delivery that will have additional benefits on safety, stability, and ease of administration.

Vaccine development typically has required decades to accomplish and has never been a process that could occur in response to a crisis. However, a better awareness of the inevitability of new emerging infectious diseases and heightened concerns about bioterrorism have challenged traditional processes and compelled the consideration of new approaches. For example, the FDA has created the Two Animal Rule, which is a provision for vaccine licensure based on efficacy data in animal models combined with safety data in humans for diseases that are not conducive to efficacy trials.⁴¹⁵ A test case for the ability to develop vaccines rapidly for emerging infectious diseases occurred during the outbreak of the SARS CoV. This disease was recognized in the winter of 2003; the virus was identified in March 2003; a full-length sequence was obtained in April 2003; and vaccine development of whole inactivated, vector-based, DNA, and subunit vaccines began immediately.

Phase I clinical trials of the whole inactivated virus vaccine began in the summer of 2004, and DNA trials started in December 2004. Fortunately, by that time, the epidemic had been contained through classical public health approaches of surveillance and quarantine. In 2009, when the pandemic H1N1/A/California/2009 virus appeared, both commercially licensed approaches and some experimental vaccines were in clinical use or testing within 4 months. It is possible that in the future, platform technologies for classes of viruses will allow even more rapid development time lines. Just as toxoids and polysaccharide conjugates have been successful platform technologies for toxin-producing and encapsulated bacteria, respectively, patterns are emerging for vaccine approaches that have a high likelihood of success against some types of virus. For example, using reassortment to produce live attenuated vaccines for viruses with segmented genomes, or producing VLPs with the capsid proteins of nonenveloped and some enveloped viruses, appear to be viable platforms for those types of viruses. Although this chapter has focused on antiviral vaccines for human pathogens, much can be learned from the development of veterinary vaccines. The list of licensed veterinary antiviral vaccines that are shown in [e-Table 14.3](#) is provided to demonstrate the enormous variety of viruses to which vaccines have been developed, to demonstrate the types of approaches that have had some measure of success against various virus families, and to illustrate that the introduction of gene-based vaccine approaches into veterinary practice is anticipating the evolution of new human vaccines (see [e-Table 14.3](#)).

The goal for future vaccine development is to identify preventive strategies for selected pathogens that cause a large disease burden (e.g., HIV-1, RSV, dengue, and HSV) and have the potential for sporadic epidemics or intentional release and high disease severity (e.g., orthopoxviruses, filoviruses, flaviviruses), or emerging pathogens with the potential for causing widespread epidemics (e.g., chikungunya or H5N1 influenza), and at the same time develop platform technologies that can be deployed more rapidly when new virus diseases emerge (e.g., SARS CoV and West Nile virus). The 21st century promises higher capacity for protein production, structure-based Ag design, molecular adjuvants, and delivery devices to direct immune response patterns, a better understanding of the genetics of human immune response polymorphisms and repertoire, and more complete knowledge of immune effector mechanisms. In addition, the safety and efficacy of several classes of gene-based delivery vectors will be determined. These advances will hopefully provide the knowledge and infrastructure needed to develop vaccines for new emerging viruses and for difficult viruses that have eluded vaccine solutions and continue to impact human health.

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Diagnostic Virology

History of Diagnostic Virology**Specimens for Viral Diagnosis****Significance of Viral Detection****Methods Used in Diagnostic Virology**

Viral Culture

Electron Microscopy

Light Microscopy

Cytology and Histology

Antigen Detection

Nucleic Acid Detection

Serology

Antiviral Susceptibility Testing

Selected Clinical Problems**Virus Discovery**Novel Virus Discovery Strategies
(2000–2010)Summary of Pathogen Discovery
MethodsRepresentative Novel Human Viruses
Discovered (2000–2010)Prospects and Challenges for the
Future**Perspective****Disclosures**

Diagnostic virology continues to evolve rapidly. Viral testing is now essential for the care of a number of patient groups, including hospitalized patients with acute respiratory infections; transplant recipients and other immunocompromised patients; patients infected with human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV); and infants with possible congenital infection. Multiple test methods continue to be used, but molecular tests are emerging as the dominant technology. A variety of commercial molecular assays have been or are in the process of being approved or cleared as *in vitro* diagnostic tests by the Food and Drug Administration (FDA). This is an important development because it makes viral diagnostic testing available to more laboratories and it improves the standardization of diagnostic testing. The scope of diagnostic virology has broadened. General categories of viral diagnostic testing and the viruses included in those categories are shown in Table 15.1.

HISTORY OF DIAGNOSTIC VIROLOGY

Modern diagnostic virology dates to the first growth of human viruses in tissue culture reported by Weller and Enders in 1948.¹⁴⁹ This and other landmarks in the history of diagnostic virology are shown in Table 15.2.

SPECIMENS FOR VIRAL DIAGNOSIS

The likelihood of making a specific viral diagnosis depends largely on the quality of the specimen that is received in the laboratory. Important variables include the timing of the specimen in relation to the patient's illness, the type of specimen, the quality and amount of specimen material obtained, and the time and conditions of transport to the laboratory. Although this concept is so basic as to seem trivial, optimizing the variables listed requires knowledge on the part of the physician and attention to logistic considerations that can present substantial barriers to diagnosis.

For the diagnosis of acute viral infections, the best specimens are usually obtained from the site of disease. For example, in the patient with suspected viral meningitis, cerebrospinal fluid (CSF) is the best specimen. In infections involving skin or mucosal surfaces, specimens obtained from those surfaces are usually the only ones required. Viral titers are highest early in the course of an acute illness, so that specimens obtained within the first few days after onset are most likely to be positive.

Viral culture requires more attention to conditions of transport than specimens submitted for detection of viral antigens or nucleic acids, because the viability of the virus must be preserved. A number of clinically important viruses are labile and will not survive prolonged transport. General instructions for transporting specimens for viral culture are shown in Table 15.3. These conditions are usually also suitable for detection of viral antigens and nucleic acids, but consultation with the laboratory performing testing is advised.

For serologic diagnosis, an acute phase serum specimen should be obtained within the first few days of illness and a convalescent phase specimen 2 to 4 weeks later. If a virus-specific immunoglobulin M (IgM) assay is available, the acute phase specimen may be sufficient by itself. Immunoglobulins are stable in serum or plasma. Proper handling of specimens for serologic diagnosis begins with separation of serum or plasma. If testing is performed within several days, the serum or plasma can be stored at 4°C. If a longer delay is involved, the specimen should be frozen at –20°C or –70°C. For certain viral infections, serologic tests can also be performed on saliva or urine.

TABLE 15.1 Categories of Testing Performed in Diagnostic Virology

Category of testing	Specific viruses	Methodology
Respiratory viruses	Influenza A and B, RSV, PIV 1–4, hMPV, rhinoviruses, enteroviruses, coronaviruses, adenoviruses	Rapid antigen tests (influenza A and B, RSV), fluorescent antibody staining (influenza A and B, RSV, PIV 1–3, adenoviruses, hMPV), culture, multiplex NAAT
Gastrointestinal viruses	Rotavirus, norovirus, adenovirus, astrovirus	Rapid antigen tests (rotavirus, norovirus, adenovirus), NAAT
Mucocutaneous viruses	HSV, VZV, HPV	Fluorescent antibody staining (HSV and VZV), culture (HSV and VZV), NAAT
Central nervous system viruses	HSV, VZV, CMV, EBV, HHV-6, JCV, enteroviruses, parechoviruses, West Nile virus, other arboviruses	NAAT, serology (West Nile and other arboviruses)
Opportunistic agents	CMV, EBV, BKV, HHV-6, adenoviruses	NAAT, antigen detection (CMV pp65 assay), cytology (BKV)
Mononucleosis syndrome in nonimmunocompromised individuals	EBV, CMV, HIV	Serology, NAAT
HIV, HCV, HBV viral loads	HIV, HCV, HBV	NAAT
Viral genotyping	HCV, HBV, HPV	Nucleotide sequencing, reverse hybridization, NAAT (Cleavase reaction for HPV)
HIV, HCV, HBV diagnosis	HIV, HCV, HBV	Serology, NAAT
Systemic infections of childhood	Parvovirus B19, measles virus, rubella virus, mumps virus	NAAT, serology
Tropical and emerging infections	Dengue and other flaviviruses; chikungunya and other alphaviruses; hemorrhagic fever viruses including arenaviruses, bunyaviruses, and filoviruses; Hendra and Nipah viruses	Serology, culture, NAAT (hemorrhagic fever testing is done in BSL-4 laboratories)
Unknown virus	Any	Culture, microarray, nucleotide sequencing

BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV-6, human herpesvirus 6; HIV, human immunodeficiency virus; hMPV, human metapneumovirus; HPV, human papillomavirus; HSV, herpes simplex virus; JCV, JC virus; NAAT, nucleic acid amplification testing; PIV, parainfluenza virus; RSV, respiratory syncytial virus; VZV, varicella-zoster virus.

TABLE 15.2 Landmarks in the History of Diagnostic Virology

Year	Landmark
1892	Intranuclear and intracytoplasmic inclusions noted at the base of smallpox lesions ⁴⁷
1898	Discovery by Loeffler and Frosch that foot-and-mouth disease of cattle is caused by a filterable agent, referred to as a virus
1929	Complement fixation method described for detection of antibodies to smallpox, vaccinia, and varicella-zoster viruses ⁶
1948	First growth of pathogenic human viruses in tissue culture ¹⁴⁹
1956	Detection of influenza virus in respiratory secretions using fluorescent antibody staining ^{36,80}
1975	Development of monoclonal antibodies as diagnostic reagents ⁶⁸
1985	Discovery of PCR ¹¹⁸
1992	Development of real-time PCR ⁵³
2002	Beginning of systematic approaches to virus discovery ^{45,91,148}
2007	Description of multiplex PCR for respiratory viruses ⁸⁵

PCR, polymerase chain reaction.

SIGNIFICANCE OF VIRAL DETECTION

As in other areas of microbiology, the detection of a virus in a clinical sample is not proof in and of itself that the detected virus is the cause of the patient's illness. This problem regarding viral causality is exacerbated by polymerase chain reaction (PCR) and other very sensitive detection methods that may reveal very low-level persistent infection or even latent infection, unrelated to the patient's current illness. A determination of whether a causal relationship exists requires consideration of several factors. Two of the most important are (a) the nature of the virus–host interaction and (b) whether the virus is known to cause the disease manifestations that the patient is experiencing. If the virus detected is one known to be associated with persistent or latent infection, its detection may require other supportive evidence before it can be accepted as causal of the patient's current illness. For example, detection of the presence of virus-specific IgM antibodies or of seroconversion, in addition to the presence of the virus, favors acute infection, which, in turn, favors a causal relationship. An alternative approach using molecular methods may be to detect a specific viral RNA that encodes a structural or other protein that is expressed only in active infection. Obviously, detection of a virus known to cause the disease that the patient has is much more likely to be causal than detection of a virus not

TABLE 15.3 Instructions For Obtaining and Transporting Specimens for Viral Testing^a

Specimen	Instructions ^b
Blood	Volume requirement varies according to the specific test required. Molecular methods generally require lower blood volumes than does culture. The appropriate tube is determined by the test to be performed, the blood component on which the test is performed (whole blood, leukocytes, plasma, or serum), and the preference of the laboratory. Whole blood, leukocytes, and plasma require collection into a tube containing an anticoagulant. The most commonly used anticoagulants are ethylenediaminetetraacetic acid (EDTA) (purple top tube), heparin (green top tube), and acid-citrate-dextrose (yellow top tube). Note: Heparin is inhibitory for some polymerase chain reaction (PCR) procedures, especially those performed on plasma. Some nonculture tests performed on blood may have specific collection tube requirements that can be determined through communication with the laboratory. Most serologic procedures are performed on serum, although some tests can also be performed on plasma. Serum specimens require collection into a tube that does not contain an anticoagulant (red top tube or gold top serum separator tubes). Blood specimens do not have to be placed on ice if transport requires less than 1 day. Note: Prolonged transport can severely compromise the ability to recover cytomegalovirus (CMV) in culture from blood specimens. ¹¹⁴
Swab	Swabs with Dacron or rayon tips are preferred. If available, swabs designed specifically for collection of viral specimens should be used. Recently, flocked swabs have been shown to be superior to standard swabs. ^{1,90} Swabs can also be placed in viral transport media. In this case, the shaft of the swab may have to be cut to allow the top of the transport vial to be securely closed. The swab or tube of transport media should be placed on ice if transport will require more than 1 hour.
Fluid	Place in a sterile container.
Tissue	Place in a sterile container with a small amount of viral transport media or sterile saline or phosphate-buffered saline to maintain moisture.
Stool	Obtain at least 4 g of stool. Place in a clean or sterile container.

^aSpecimens other than blood should be transported on ice if more than 1 hour is required for arrival in the laboratory. If a delay of more than 24 hours is anticipated, specimens should be frozen at -70°C and transported on dry ice. Note: Some viruses such as respiratory syncytial virus (RSV) and varicella-zoster virus (VZV) are unlikely to retain viability with freezing. Specimens should not be frozen at -20°C .

^bCommercial tests may have specific recommendations for collection and transport. These should always be followed to ensure proper assay performance.

known to be associated with that disease. This applies particularly to viruses such as human herpesvirus type 6 (HHV-6) and type 7 (HHV-7), human polyomaviruses such as JC virus, non–high-risk types of human papillomavirus (HPV), and even respiratory or gastrointestinal viruses such as rhinovirus or adenoviruses that may be present in patients who are not ill.

METHODS USED IN DIAGNOSTIC VIROLOGY

Viral Culture

For approximately 50 years, viral culture was the signature method of diagnostic virology laboratories, and it was the method that led to the establishment of virology laboratories as distinct entities apart from other areas of the clinical laboratory. With the advent of immunological and especially of molecular methods, which are more rapid than viral culture and potentially detect a broader range of viruses, cell culture is playing a diminishing role. For this reason, the treatment of cell culture in this chapter will be brief, and the interested reader is referred to other sources.^{77,78,125,144}

By their very nature, viruses require living systems for propagation. In the past, inoculation of animals such as suckling mice or of embryonated eggs was used to grow viruses. These methods were largely supplanted by cell culture in the diagnostic laboratory. To grow viruses in cell culture, a clinical sample is prepared and then inoculated onto one or more cell culture types. Typically several cell culture types are inoculated, because no one type supports the growth of all clinically

relevant viruses. Cell culture types that are commonly used are shown in Table 15.4. After inoculation, viral growth is detected in one of several ways. Traditionally, the principal method was the appearance of cytopathic effect, which refers to morphologic changes observable by microscopy that occur in virally infected cells (Fig. 15.1). Alternatively, hemadsorption, which refers to the surface binding of erythrocytes by virally infected cells, was used for the detection of influenza, parainfluenza, and mumps viruses. Interference, used to detect rubella virus, refers to the phenomenon that cells infected with certain viruses such as rubella virus become resistant to infection with other viruses that would readily infect the cells if they were uninfected by the first virus. Confirmation of virus growth, suspected based on one of the indicators described previously, can be achieved by immunofluorescence using specific antiviral antibodies. When cytopathic effect is present but immunological stains are negative, electron microscopy of cell culture material can be used to examine the culture. More recently, molecular methods such as VIDISCA (virus discovery based on complementary DNA [cDNA]-amplified fragment length polymorphism) have been used in this situation to detect new viruses growing in cell culture.^{107,132}

Presently, most viral culture done in diagnostic virology laboratories is performed using centrifugation cultures, also often referred to as shell-vial cultures (Fig. 15.2). The shell-vial culture method was originally developed for cytomegalovirus (CMV)^{42,46} but has subsequently been applied to many other viruses. In this method, viral entry is enhanced by a low-speed centrifugation after inoculation of the clinical sample onto the cell culture, which may be grown on a cover

TABLE 15.4 Cell Culture Types Used to Detect Medically Important Viruses

Primary	Monkey kidney Rabbit kidney Human embryonic kidney	Influenza viruses, parainfluenza viruses, enteroviruses Herpes simplex virus Adenovirus, enteroviruses
Diploid	Fibroblasts (e.g., MRC-5, WI-38)	CMV, VZV, HSV, rhinovirus, enteroviruses (some), adenovirus, RSV
Continuous	HEp-2 A549 MDCK LLC-MK2 Rhabdosarcoma (RD) Buffalo green monkey	HSV, adenovirus, HSV, parainfluenza viruses (some), enteroviruses (some) HSV, adenovirus, enteroviruses Influenza viruses Parainfluenza viruses, hMPV Echoviruses Coxsackieviruses

CMV, cytomegalovirus; hMPV, human metapneumovirus; HSV, herpes simplex virus; RSV, respiratory syncytial virus; VZV, varicella-zoster virus.

slip within a 1-dram vial referred to as a shell vial, but can also be grown within other devices such as 24-well plates that can be conveniently centrifuged. Viral growth is detected by immunofluorescence, typically at 16 and 40 hours after inoculation, allowing detection of viruses much sooner than could be accomplished using conventional tube cultures. It is now possible to purchase cell cultures for centrifugation cultures in which more than one type of cell is present. For example, a commercial system called R-Mix (Diagnostic Hybrids, Inc., Athens, Ohio) combines human adenocarcinoma (A549) and mink lung cell lines for growth of respiratory viruses. After incubation, the R-Mix cells are stained with a mixture of monoclonal antibodies specific for seven respiratory viruses. Similar mixed cell culture systems also exist to detect herpes simplex

virus (HSV) and varicella-zoster virus (VZV) and to detect enteroviruses.

Genetically Engineered Cell Lines

Techniques of genetic engineering have been used to modify cell lines either to make them susceptible to viruses to which they are not otherwise susceptible or to create novel means of detecting virus growth. An example is a cell line developed by Stabell and Olivo¹²⁶ for the detection of HSV. This cell line, shown in Figure 15.3, consists of baby hamster kidney (BHK) cells that have been transfected with the β -galactosidase gene from *Escherichia coli* under the control of a promoter from the herpes simplex gene *UL39*. The promoter is activated by exposure to HSV proteins ICP0 and VP16. Exposure of the cells to

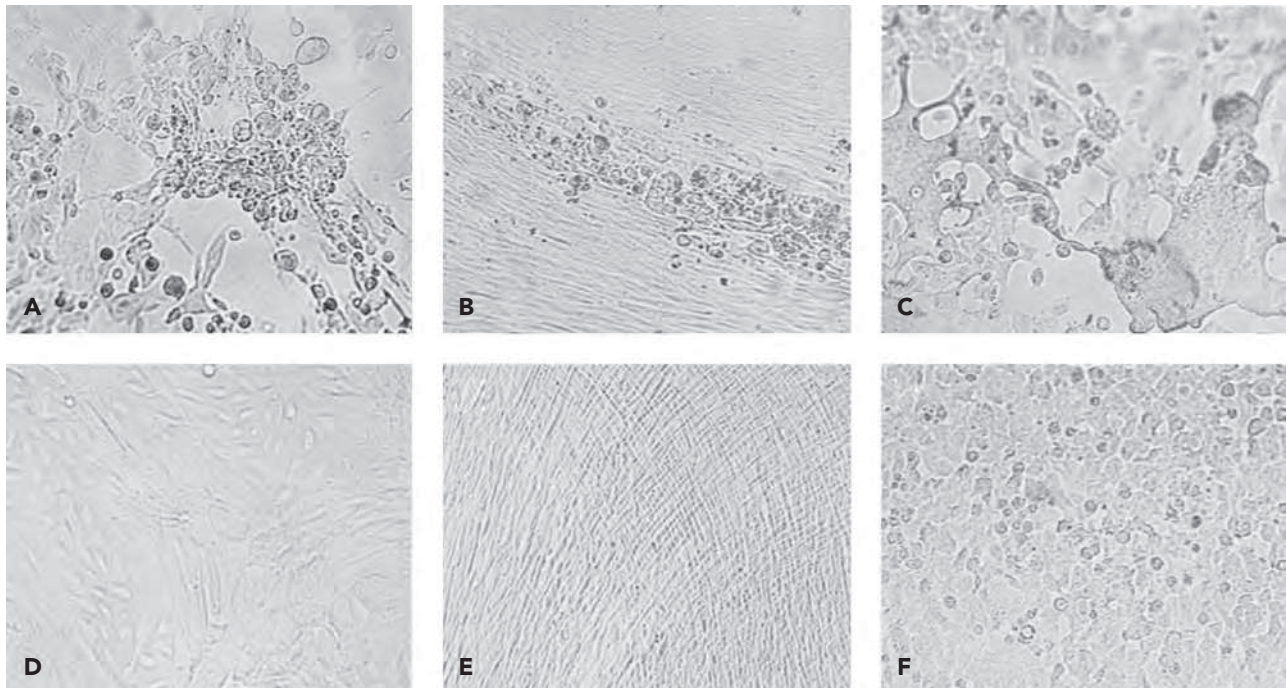


FIGURE 15.1. Cytopathic effect caused by viruses growing in cell culture. Herpes simplex virus growing in primary rabbit kidney cells (A); uninfected primary rabbit kidney cells (D). Cytomegalovirus growing in human embryonic lung fibroblast cells (B); uninfected human embryonic lung fibroblast cells (E). Respiratory syncytial virus growing in HEp-2 cells (C); uninfected HEp-2 cells (F).

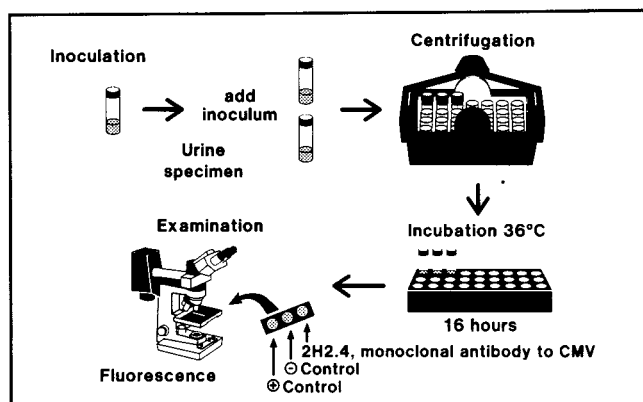


FIGURE 15.2. Shell-vial assay for cytomegalovirus. (From Shuster EA, Beneke JS, Tegtmeier GE, et al. Monoclonal antibody for rapid laboratory detection of cytomegalovirus infections: characterization and diagnostic application. *Mayo Clin Proc* 1985;60:577–585, with permission.)

a specimen containing HSV specifically activates the promoter, causing production of β -galactosidase, which can be detected by a simple histochemical stain of the culture, performed 16 to 24 hours after inoculation. A commercial version of this system, called ELVIS HSV (Diagnostic Hybrids, San Diego, CA), has been shown to have sensitivity comparable to conventional viral culture for detection of HSV in clinical specimens.¹²⁷ The system has also been adapted to the performance of HSV

antiviral drug susceptibility assays.¹³³ Another example is the creation of a line of buffalo green monkey (BGM) cells that have been modified to express human decay-accelerating factor, which is a receptor for some enterovirus serotypes.⁵⁶ Addition of this molecule increases the ability of unaltered BGM cells to grow enteroviruses. These cells are combined with A549 cells in a commercial product called Super E-Mix (Diagnostic Hybrids).

Electron Microscopy

Viral infections are rarely diagnosed using electron microscopy for the direct visualization of viral particles in specimens. Advantages include speed, lack of requirement for viral viability, and the potential to visualize many different kinds of viral particles. Disadvantages include the cost and complexity of maintaining an electron microscopy, the need for a skilled operator, and limited sensitivity related to the relatively high concentration of viral particles (10^5 to 10^6 /L) that is required for visualization.⁸⁹ Historically, electron microscopy was used for the evaluation of stool specimens from patients with suspected viral gastroenteritis. Viruses such as rotavirus, astrovirus, and adenovirus could be identified based on their characteristic morphology, but this has been replaced by rapid immunoassays and PCR assays. Electron microscopy can also be used to detect and classify viral particles in fixed tissue obtained by biopsy or at autopsy. Screening of tissue samples by electron microscopy is not useful because of the small area of tissue that can be examined. Thus, electron microscopy is best employed when it is directed by evidence of viral infection detected by routine histology.

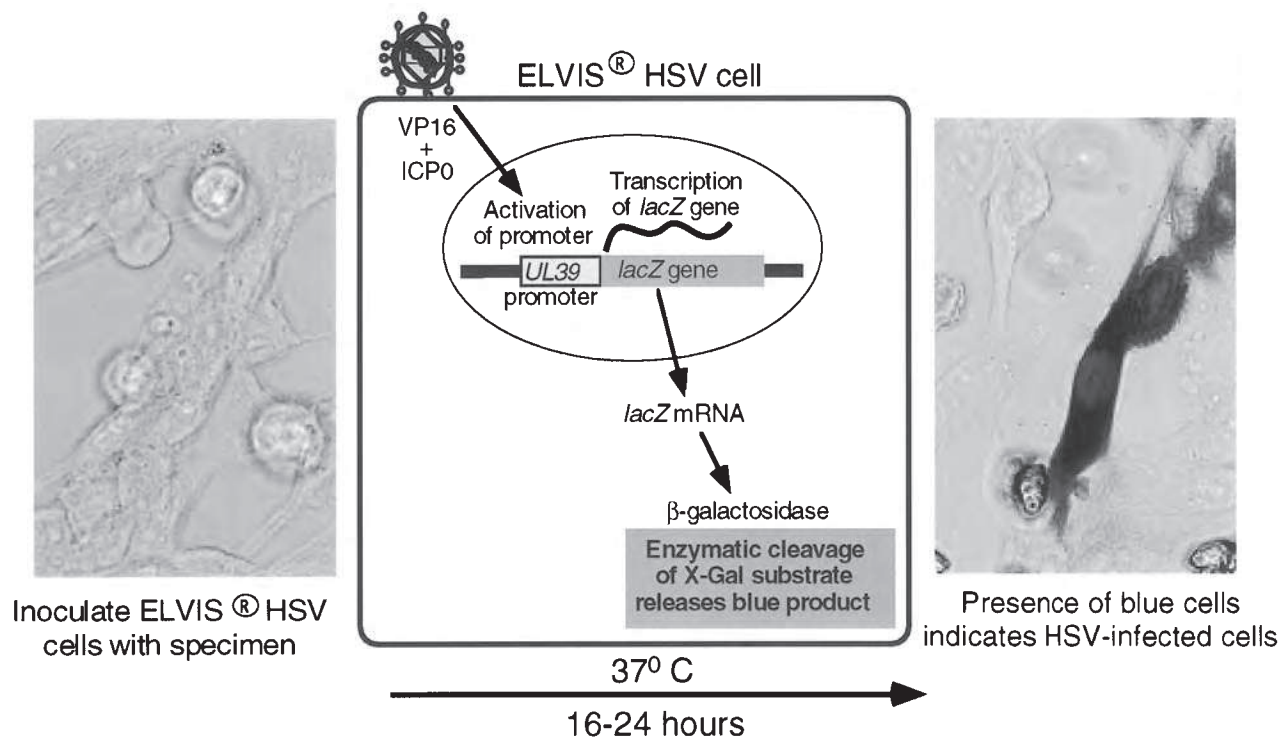


FIGURE 15.3. ELVIS herpes simplex virus (HSV), a genetically engineered cell line for the detection of HSV clinical samples. ELVIS cells are baby hamster kidney (BHK) cells that contain a β -galactosidase gene under the control of the HSV promoter UL39. If HSV is present in the specimen, viral particles enter the cells and the HSV proteins ICP0 and VP16 activate the UL39 promoter, leading to synthesis of β -galactosidase. The presence of β -galactosidase is detected by adding X-gal, a substrate for the enzyme. The activity of β -galactosidase on X-gal results in blue staining of ELVIS cells, visible microscopically.

TABLE 15.5 Viral Inclusions

Virus	Nuclear	Cytoplasmic
Herpes simplex, varicella-zoster	×	
Cytomegalovirus	×	×
Adenovirus	×	
Polyomaviruses (JC and BK)	×	
Parvovirus B19	×	
Poxviruses		×
Measles	×	×
Parainfluenza		×
Rabies		×

Light Microscopy

Although viral particles, with the exception of poxviruses, cannot be directly visualized by light microscopy, indirect evidence of viral infection can be detected. The most characteristic signs of viral infection are inclusion bodies (composed of masses of virions), multinucleated cells, and syncytial cells. These findings may be present in cytologic specimens or in tissue examined after histologic staining. Viral inclusions can be found in the nucleus or the cytoplasm of infected cells. The location is characteristic of the responsible virus. The location of viral inclusions is summarized in Table 15.5. Microscopy for detection of the effects of viral infections in clinical specimens is generally carried out in surgical pathology rather than diagnostic virology laboratories.

Cytology and Histology

Cytologic examination for evidence of viral infection can be performed on smears prepared by applying a specimen directly to a microscope slide, on slides prepared by cytocentrifugation of fluids (e.g., bronchoalveolar lavage fluid), and on touch preparations prepared from pieces of unfixed tissue. Viruses that can produce cytologic evidence of infection in respiratory specimens include HSV, CMV, adenoviruses, polyomaviruses, and measles virus (Warthin-Finkeldey cells). Cytologic examination is usually not a sensitive method for detection but provides strong evidence for tissue involvement. Examples of cytologic findings suggestive of viral infection are shown in Figure 15.4. The Tzanck smear is a method sometimes used to detect cytologic evidence of HSV or VZV infection. It is rarely used in diagnostic virology laboratories because techniques such as fluorescent antibody staining and PCR are more sensitive and specific. The Papanicolaou stain (Pap smear) is used to detect evidence of HPV infection in cells obtained from the uterine cervix. HPV infection produces characteristic changes in keratinocytes, including a condensed nucleus with a prominent perinuclear clear zone referred to as *koilocytosis*. More specific evidence of the presence of HPV can be provided by histochemical stains or molecular techniques. Urine cytology may reveal intranuclear inclusions indicative of infection with either CMV or the polyomaviruses, JC and BK. Culture or PCR for CMV and PCR for polyomaviruses, however, are more sensitive and specific techniques. Cytology does not distinguish between JC and BK viruses.

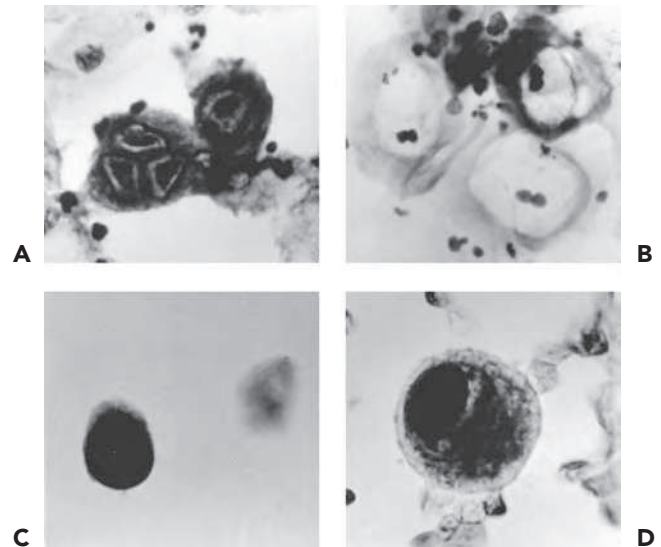


FIGURE 15.4. Cytologic findings suggestive of viral infection.

A: Cervical smear showing multinucleated cells and the Cowdry type A intranuclear inclusions of herpes simplex infection. **B:** Papanicolaou smear showing binucleate squamous epithelial cells with distinct perinuclear halos. These characteristics, described as *koilocytosis*, are the cellular features associated with human papillomavirus infection. **C:** Urinary epithelial cell containing an enlarged nucleus with smudgy chromatin and a small pale *glassy* intranuclear inclusion indicative of polyomavirus infection. **D:** Cell from bronchoalveolar lavage with a large intranuclear inclusion with a perinuclear clear space (owl's eye cell) indicative of cytomegalovirus infection. (Photographs provided by Dr. Leslie Boucher, Department of Pathology, Washington University, St. Louis, Missouri.)

Histologic examination of stained tissue can provide unique information about the role of viral infection in producing tissue inflammation and injury and can be very useful in distinguishing between asymptomatic viral shedding and clinically significant infection. This is particularly useful for CMV infections in which prolonged viral shedding can occur without producing disease. For example, the presence of cytomegalic inclusion cells in tissue obtained by biopsy or at autopsy is often considered to be the gold standard for diagnosing clinically significant CMV infection localized in a specific organ.

Antigen Detection

The detection of viral antigens directly in clinical specimens is widely used to obtain rapid evidence of viral infection. The lack of requirement for virus viability is an important advantage. For some viruses, especially respiratory syncytial virus (RSV) and CMV, the sensitivity of antigen detection may exceed that of viral culture.^{111,135} Antigen detection methods can be applied when the following conditions are met: (a) viral antigen is expressed and is present in an accessible specimen, (b) an appropriate antibody (usually but not necessarily monoclonal) is available, (c) antigenic variability does not preclude recognition by immunologic reagents of different strains of the target virus, and (d) the antigen being detected is sufficiently stable that it does not degrade during transport and processing of the specimen. Methods used for viral antigen detection include fluorescent antibody (FA) staining, immunoperoxidase staining, and enzyme immunoassay. The latter can be subdivided

TABLE 15.6 Viral Antigen Detection: Specimens Used and Viruses Detected

Specimen	Viruses detected
Respiratory (nasopharyngeal swab or aspirate, nasopharyngeal wash, tracheal aspirate, bronchoalveolar lavage)	Respiratory syncytial Influenza A, B Parainfluenza 1–3 Adenovirus Human metapneumovirus Measles
Skin or mucous membrane scraping	Herpes simplex Varicella-zoster
Conjunctival or corneal scraping	Herpes simplex Adenovirus
Stool	Rotavirus Adenovirus (enteric serotypes) Norovirus
Blood	Cytomegalovirus (pp65) Hepatitis B (surface antigen) Human immunodeficiency (p24)

into solid phase and lateral flow immunochromatographic assays. The viruses for which antigen detection assays are in widespread use are shown in Table 15.6.

Antigen detection can also be used to detect viral antigens in fixed tissue (immunohistochemistry [IHC]). This method can document the specific viral etiology of a nonspecific finding such as an inclusion body that could be produced by several different viruses. In some cases, IHC also enhances the sensitivity of detection of viruses in tissue compared to standard light microscopy without immunostaining. Immunoperoxidase staining is the most common technique used for viral antigen detection in fixed tissue. IHC can often be performed on formalin-fixed tissue, but for some antigen–antibody combinations, sensitivity is better on fresh frozen tissue.

Fluorescent Antibody Staining

Fluorescent antibody staining is widely used to detect cell-associated viral antigens. In the direct format, a fluorescent label, usually fluorescein isothiocyanate (FITC), is conjugated directly to the antibody that recognizes the viral antigen. In the indirect format, the antiviral antibody is unlabeled and is detected by a second antibody that recognizes immunoglobulins from the animal species of origin of the antiviral antibody. The second antibody carries the fluorescent label. After staining, the specimen is viewed with epi-illumination using ultraviolet (UV) light of the wavelength needed to excite the fluorescent label. The direct method is simpler to use but requires conjugation of each antiviral antibody with the fluorescent label. The indirect method is slightly more sensitive and more versatile, because only the anti-immunoglobulin antibody has to be conjugated with the fluorescent label.

The main applications of FA staining have been to detect respiratory, ocular, cutaneous, and bloodstream pathogens. Many different respiratory specimens can be used, including nasopharyngeal swabs or aspirates, nasal washes, tracheal aspirates, and bronchoalveolar lavage fluid. In each case, the specimen is processed in the laboratory to prepare a pellet of cells, which is spotted onto one or more microscope slides. The cells

are air dried, fixed in acetone, and stained with monoclonal antibodies to one or more viruses. For ocular infections, material is scraped from the conjunctiva or cornea, placed on microscope slides, and processed as described for respiratory specimens. Staining is usually directed at detection of HSV and adenovirus antigens. For cutaneous infections, the lesion is scraped with a scalpel blade or swab, and cellular material obtained is placed directly on one or more microscope slides. Alternatively, the base of the lesion can be rubbed vigorously with a swab and the swab submitted to the laboratory, where cellular material is washed from the swab and spotted onto a microscope slide. The use of cyto centrifugation to prepare the slides has been shown to improve the results.⁷⁵ Staining is directed at detection of HSV and VZV. In the CMV pp65 antigenemia assay, peripheral blood leukocytes are separated from anticoagulated blood; spotted onto a microscope slide, usually by cyto centrifugation; and stained using a monoclonal antibody specific for the CMV pp65 antigen. The major limitation of FA staining is having an adequate number of cells in the specimen. Immunoperoxidase (IP) staining is similar to FA staining, except that horseradish peroxidase is used in place of a fluorescent label, making it possible to view the stain using light microscopy rather than requiring fluorescence microscopy. It is more often used to detect viral antigens in fixed tissue rather than directly in patient samples.

Enzyme Immunoassay

Enzyme immunoassay (EIA) is a versatile and widely used method that can be applied to the detection of antigens, regardless of whether they are cell associated or in fluid phase. Because intact cells in the specimen are not required, specimen integrity is less important than for FA or IP staining. This may be advantageous when specimen transport time is prolonged. A common assay format for antigen detection is the double antibody sandwich technique, shown in Figure 15.5. In this assay, a *capture* antibody specific for the viral antigen being sought is bound to a reaction surface, for example, the wells of a plastic microtiter tray or the surface of a plastic bead. When the specimen is added, viral antigen present in the specimen binds to the capture antibody. Bound antigen is detected using a different antiviral antibody, the *detector* antibody. The detector antibody can be labeled with an enzyme or can be detected by

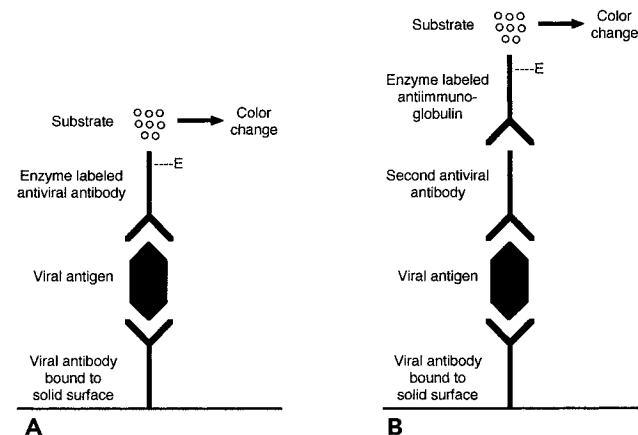


FIGURE 15.5. Enzyme immunoassay (EIA) for antigen detection. **A:** Direct. **B:** Indirect.

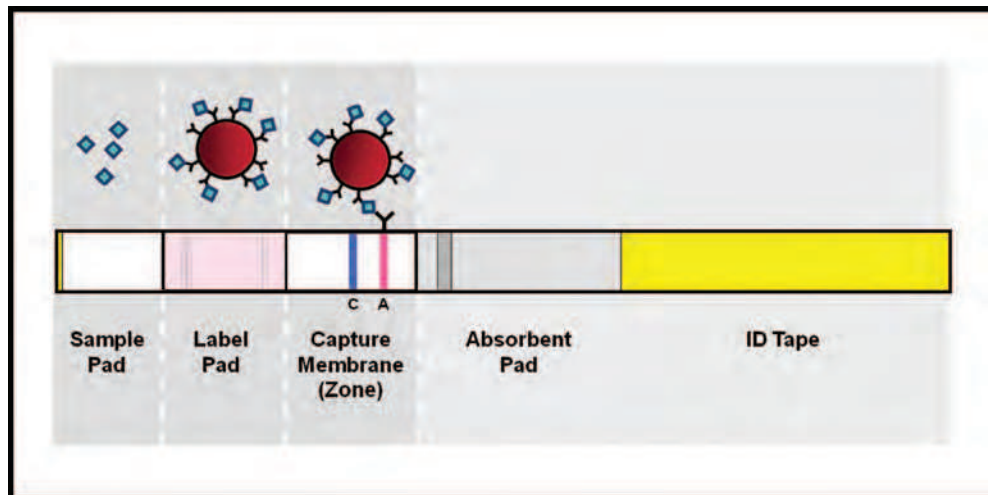


FIGURE 15.6. Lateral flow immunochromatographic assay. The sample is applied to the sample pad. The sample flows across the unit from left to right, drawn by the absorbent pad. Located in the label pad are one or more antibodies, each of which is conjugated to a label such as gold particles. These antibodies bind to antigen present in the sample as it moves across, forming complexes of antigen with conjugated antibody. If present, these complexes are then captured by second antibodies, located in lines in the capture membrane zone. When complexes are captured, the presence of the conjugated label causes the line to become visible. In the figure, antibodies against the target antigen are indicated by “A,” and antibodies against a control antigen are indicated by “C.” Presence of a colored line corresponding to A is a positive test. Presence of a line corresponding to C in the absence of a line corresponding to A indicates a negative test. Absence of both lines indicates that the test is not valid. (Figure provided by John Tamerius, Quidel Inc.)

the addition of an enzyme-labeled third antibody with specificity for immunoglobulin of the species from which the second antibody was derived. The addition of an enzyme substrate produces a color change or light emission if the enzyme is present. Thus, color change or light emission indicates that viral antigen was present in the specimen being tested. Advantages of EIA include applicability to diverse specimens and potential for automation. Laboratory instruments are now available that can perform EIA to detect either antigens or antibodies. Viruses for which antigen EIA have been widely used are RSV, influenza, rotavirus, enteric adenoviruses, HSV, HBV, and HIV.

Membrane Immunoassay

The lateral flow immunochromatographic assay is a variant of EIA that was first used in home pregnancy tests. A schematic diagram of a lateral flow unit is shown in Figure 15.6. In these tests, a sample is applied directly to a membrane and is drawn across the membrane by capillary action. Antigens in the sample react first with an antibody with specificity for the antigen being detected. This antibody is conjugated to a detector label such as gold particles or fluorescein. If binding occurs, the antigen–antibody complexes sweep across the membrane until they are captured by a second antibody that is bound to the membrane. When the labeled antigen–antibody complexes are captured, a line becomes visible because of the concentration of the label into a limited physical space. Most assays also include a positive control. These assays can be configured as dipsticks or as self-contained cassettes. Standard lateral flow immunochromatographic assays do not require an instrument and are convenient for testing single samples, with results available in 5 to 20 minutes. Multiple commercial versions are now available, mainly for

detection of influenza, RSV, and rotavirus. Some of the tests are sufficiently simple to perform that they have been assigned *waived* status under the Clinical Laboratory Improvement Amendments (CLIA), meaning that they can be performed by individuals without specific training who can be working within or outside of a certified clinical laboratory. Unfortunately, sensitivity of these tests has generally been less than that for fluorescent antibody staining, culture, or PCR. This problem was especially true for commercial influenza lateral flow tests in detecting the 2009 H1N1 pandemic.¹⁰⁶ A recent test developed by 3M (3M Rapid Detection RSV or Influenza tests, 3M, St. Paul, MN) achieves increased sensitivity by using fluorescent beads conjugated to a detector antibody and reading the captured antigen–antibody complexes in a fluorescence reader.⁴¹

Nucleic Acid Detection

Diagnostic virology has been revolutionized by the application of nucleic acid detection techniques, which detect specific DNA or RNA sequences, and can be applied to the detection of virtually any virus. Depending on the target sequence, the assays can be specific for a single virus species or for a group of related viruses. The latter characteristic is particularly advantageous, because it allows nucleic acid detection techniques to be applied to groups of viruses (e.g., the enteroviruses) for which antigenic diversity has precluded successful application of antigen detection techniques. For example, enteroviruses, for which a rapid nonmolecular detection method had not been available, can be detected using reverse-transcription PCR assays that amplify a highly conserved sequence in the 5′ nontranslated region of the genome.^{14,115} An overview of applications of nucleic acid testing in diagnostic virology is shown in Table 15.7.

TABLE 15.7 Nucleic Acid Detection for Viral Diagnosis

Target	Specimen(s)	Application(s)
HIV (DNA)	Leukocytes from infant born to HIV-infected mother	Diagnosis of perinatal infection
HIV (quantitative RNA)	Plasma	Viral load (prognosis, response to treatment)
HIV (qualitative RNA)	Plasma	Confirmation of diagnosis
HSV	CSF, ocular fluid, swabs from mucocutaneous lesions	Diagnosis of encephalitis, meningitis, retinitis, mucocutaneous lesions
VZV	CSF, ocular fluid, swabs from mucocutaneous lesions	Diagnosis of encephalitis, meningitis, retinitis, mucocutaneous lesions
CMV	Blood (whole blood, leukocytes, or plasma), CSF, ocular fluid, amniotic fluid	Diagnosis of systemic infection after organ transplantation, encephalitis, radiculomyelitis, retinitis, congenital infection. Quantitative assay performed on blood can be used to assess severity of infection and monitor response to therapy.
EBV	CSF, blood (whole blood, leukocytes, or plasma)	Primary central nervous system lymphoma in AIDS, other EBV infection of the central nervous system, posttransplant lymphoproliferative disorder. Quantitative assay performed on blood can be used to assess the risk of PTLD and the response to therapeutic interventions.
HHV-6, -7	Blood, CSF	Diagnosis of systemic infection and of encephalitis
HHV-8 (Kaposi sarcoma virus)	Blood, pleural or pericardial fluid	Diagnosis of body cavity lymphoma
Adenovirus	Blood, respiratory secretions	Diagnosis and monitoring of disseminated infection in immunocompromised hosts, diagnosis of respiratory tract infection
Parvovirus B19	Serum, amniotic fluid	Diagnosis of acute or chronic parvovirus B19 infection, aplastic crisis, congenital infection
BK virus	Urine, plasma	Assessment of risk of transplant nephropathy in renal transplant recipients and of hemorrhagic cystitis in hematopoietic stem cell transplant recipients
Human papillomavirus	Genital secretions	Detection of viral types associated with cervical carcinoma
Enteroviruses	CSF, blood (whole blood, serum, or plasma)	Diagnosis of meningitis, encephalitis, or systemic infection
Hepatitis C	Plasma	Diagnosis of infection. Quantitative assay is used to assess response to therapy.
Hepatitis B	Serum	Diagnosis of infection when serologic studies are ambiguous. Recognition of virologically active infection. Quantitative assay is used to assess response to therapy and recognize emergence of drug resistance.
Rabies	Saliva, CSF	Diagnosis of rabies
Respiratory viruses	Respiratory samples	Enhanced sensitivity, including detection of noncultivable agents
Gastrointestinal viruses	Feces	Enhanced sensitivity, including detection of noncultivable agents
West Nile and other flavivirus infections	CSF, blood	Rapid diagnosis of CNS infection, especially in immunocompromised hosts, screening of blood units for presymptomatic infection
Dengue	Plasma	Diagnosis of early infection, genotyping
Arenaviruses	Serum or whole blood	Diagnosis of acute infection
Filoviruses	Serum or whole blood	Diagnosis of acute infection
Nipah and Hendra viruses	CSF, throat swabs, urine	Diagnosis of acute infection
Vaccinia and other pox virus infections	Lesion	Diagnosis of acute infection

CMV, cytomegalovirus; CNS, central nervous system; CSF, cerebrospinal fluid; EBV, Epstein-Barr virus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; PTLD, posttransplant lymphoproliferative disorder.

The earliest attempts at nucleic acid–based diagnosis involved direct hybridization of nucleic acid probes to viral nucleic acids present in clinical specimens. Direct hybridization was never widely adopted because it lacked adequate sensitivity, requiring the presence of 10^4 to 10^5 copies of the target nucleic acid. The development of PCR¹¹⁸ and other nucleic

acid amplification techniques overcame that sensitivity barrier and has led to the development of nucleic acid–based diagnostic tests for many viruses. Nucleic acid amplification tests (NAATs) were originally directed at viruses that were difficult or impossible to cultivate, viruses that grew very slowly in culture, and viruses for which antigen detection could not be applied

because of antigenic diversity or because the level of viral antigen in clinical specimens was too low to permit successful detection. NAATs were also very advantageous for specimens such as CSF or ocular fluid for which sample volume could be limiting. Still another advantage of nucleic acid detection as a diagnostic method is the stability of DNA as an analyte, so that detection of viral nucleic acids can be done even when conditions of transport lead to loss of virus viability. Currently NAATs are being applied to all viruses that are of interest in diagnostic virology.

Nucleic Acid Amplification Assays

TARGET AMPLIFICATION

Polymerase Chain Reaction. PCR, which is the prototype of target amplification assays, employs short oligonucleotide primers and a thermostable DNA polymerase such as *Taq* polymerase to amplify a segment of target DNA that is typically 100 to 1,000 base pairs (bp) in length. Classic PCR includes repetitive cycles, each consisting of denaturation, primer annealing, and extension steps that take place at different temperatures. Progression through the steps of the cycle is controlled by a thermal cycler that controls the temperature of the reaction. After PCR amplification, the PCR product (also known as the *amplicon*) is detected by gel electrophoresis or by one of several probe-hybridization techniques (e.g., Southern blotting). The analytic sensitivity of PCR can be as low as 1 to 10 copies of target DNA. Because of its simplicity and broad applicability, PCR remains the most widely used NAAT.

Currently, most PCR in diagnostic virology laboratories is carried out using real-time PCR in which reaction products are detected as they are synthesized.^{40,52} Numerous specialized instruments for running real-time PCR are now commercially available, including the LightCycler (Roche Diagnostics, Indianapolis, IN), the SmartCycler (Cepheid, Sunnyvale, CA), the ABI TaqMan 7000 series (Applied Biosystems, Foster City, CA), the Rotor Gene (Qiagen, San Diego CA), and many others. Compared with conventional PCR, real-time PCR has several important advantages. Because the accumulation of PCR product is monitored in the reaction tube, no separate detection method (e.g., gel electrophoresis) is required, thus shortening the effective assay time markedly and decreasing the risk of contamination of the laboratory environment by the amplified PCR product. The time from setting up the assay to completion can be less than 1 hour. The use of multiple fluorescent dyes with different emission wavelengths makes it possible to perform multiplex reactions with simultaneous amplification of more than one product. Of great importance, quantification of PCR targets is readily achieved because the generated fluorescence is proportional to the amount of PCR product.

Detection of PCR products in real-time PCR has generally used one of three methods, although other detection methods have also been introduced more recently. The simplest system uses the DNA binding dye SYBR Green, which emits fluorescence when it is bound to double-stranded DNA (dsDNA). When SYBR Green is included in a PCR reaction, the intensity of fluorescence is proportional to the amount of PCR product. Because SYBR Green binds nonspecifically to any dsDNA, signal is generated by undesired amplification products such as primer dimers, as well as by intended amplicons. Discrimination between different amplification products can be achieved through the use of dissociation curves, referred to as *melting*

point analysis. Melting point analysis is performed after completion of the amplification reaction by recording fluorescence as the temperature of the reaction mix is gradually increased. When the dissociation temperature (melting point) of the dsDNA reaction product is reached, SYBR Green is released and fluorescence decreases. The melting point is affected by both length and sequence of the PCR product, and thus is precisely defined for a specific PCR product. Unintended amplification products will usually have different melting points, allowing easy discrimination from the intended PCR product.

The other two detection systems are based on the use of oligonucleotide probes that are labeled with fluorescent dyes that interact with one another according to principles of fluorescence resonance energy transfer (FRET). The hybridization probe assay format requires two oligonucleotide probes that are homologous to adjacent portions of one of the strands of the amplified DNA. The probes are chosen so that the 5' end of one probe is within a few nucleotides of the 3' end of the other probe. These adjacent ends are each labeled with a fluorescent dye. The dye on the 3' end is termed the *donor dye* and the dye on the 5' end is termed the *acceptor dye*. The required property of these dyes is that when they are in close proximity (within a distance of several nucleotides), excitation of the donor dye leads to emission of light by the acceptor dye. When both probes bind to their target sequences, the two dyes are within the proximity required for excitation of the acceptor dye and fluorescence occurs. The fluorescence intensity is proportional to the amount of PCR product.

The other detection system based on interacting fluorescent dyes is the fluorogenic 5' exonuclease assay (also referred to as the *Taqman assay*). This assay uses a probe that is complementary to a segment of the intended PCR product located between the PCR primers. This probe is labeled with two fluorescent dyes, one called the *reporter* that is linked to the 5' end of the probe, and the other called the *quencher* that is linked to the 3' end. When they are in close proximity (i.e., bound to opposite ends of an oligonucleotide probe), the quencher prevents fluorescence by the reporter. During the extension step of PCR, the probe labeled with both dyes binds to the PCR product as it is being synthesized. During extension, the 5' exonuclease activity of *Taq* polymerase cleaves nucleotides from the 5' end of the bound probe, releasing the reporter dye away from the quencher, thus allowing it to emit light on excitation. As in the hybridization probe assay, the intensity of fluorescence is proportional to the amount of PCR product.

Because *Taq* polymerase uses only DNA as a template, the use of PCR to detect viral RNA sequences requires the inclusion of a reverse-transcription (RT) step before PCR (RT-PCR). The RT reaction can be performed using a devoted enzyme such as Moloney murine leukemia virus RT or avian myeloblastosis virus RT. Alternatively, heat-stable, multifunctional enzymes such as recombinant *Thermus thermophilus* DNA polymerase are now available that can carry out RT as well as DNA polymerase reactions. Special care must be used in specimen processing because of the susceptibility of RNA to digestion by ribonucleases that may be present in clinical samples. In addition to detecting virion RNA, RT-PCR can also be applied to the detection of viral messenger RNA. This may be particularly useful in the diagnosis of infection caused by viruses that have a latent phase in their life cycle. For these viruses, detection of viral DNA might not distinguish between

latent and productive infection, whereas detection of a messenger RNA (mRNA) expressed only in productive infection would be evidence of active viral infection.

RNA Amplification Assays. Several reactions have been developed that are directed at amplification of RNA. *Transcription-based amplification* (Fig. 15.7)^{48,73} uses three enzymes, RT, ribonuclease (RNase) H, and T7 RNA polymerase, to amplify a target RNA sequence, employing a series of reactions that mimic the retrovirus replication scheme. An advantage of transcription-based amplification assays is that they are isothermal and do not require complicated instrumentation. The assays begin with synthesis of a DNA strand that is complementary to the RNA target, using a primer that contains a T7 polymerase binding site at its 5' end. The resulting DNA–RNA hybrid is converted to dsDNA by the action of RNase H and a second primer that also contains a 5' T7 polymerase binding site. The dsDNA product then serves as a template for transcription driven by T7 RNA polymerase. The newly synthesized RNA transcripts serve as templates for additional cycles of the

reaction. Variants of transcription-based amplification include transcription-mediated amplification (TMA),³⁹ self-sustained sequence replication (3SR),⁴⁸ and nucleic acid sequence-based amplification (NASBA).²¹ Currently, NASBA assays for HIV, enteroviruses, and CMV pp67 have been approved by the FDA and are marketed by bioMérieux Inc. (Durham, NC) under the trade name NucliSENS. TMA is the basis for the Procleix assays for HIV, HCV, HBV, and West Nile virus marketed by Gen-Probe (San Diego, CA).

SIGNAL AMPLIFICATION

Examples of signal amplification assays include the branched-chain DNA (bDNA) assay,²² the hybrid capture assay,⁵¹ and the cleavase reaction.⁴⁹ The bDNA assay (Fig. 15.8) uses short, branched-chain oligonucleotides to capture the target nucleic acid sequence. Other branched-chain oligonucleotides link multiple molecules of detector enzyme to the captured target. A chemiluminescent substrate allows detection of the target, and measurement of the intensity of emitted light makes it possible to quantify the input target accurately. Because the target itself is not amplified, this reaction is less susceptible than PCR to carryover contamination. Currently, FDA-approved quantitative bDNA assays for HIV and HCV are marketed by Siemens USA (Deerfield, IL) under the trade name Versant.

The hybrid capture assay (Fig. 15.9) is a signal amplification assay that involves a liquid hybridization reaction between the denatured DNA target and RNA probes specific for the viral DNA sequence of interest. If the viral DNA is present, DNA–RNA hybrid molecules are formed and are captured and detected using an antibody specific for DNA–RNA hybrids. This assay can be used as either a qualitative or quantitative assay. Currently, FDA-approved or cleared hybrid capture assays for HPV and CMV are marketed by Qiagen (Valencia, CA).^{83,88}

The Invader assay (Fig. 15.10) is an isothermal reaction that makes use of (a) an enzyme referred to as Cleavase that functions as an FEN-1–encoded flap endonuclease,¹³⁶ plus (b) two short sequence-specific oligonucleotides termed the probe oligo and the Invader oligo. To initiate the reaction, the two oligos bind to the target DNA with a short region of overlap that is designed to occur at a targeted single nucleotide polymorphism (SNP). The overlap structure occurs only when exact base pairing occurs with the targeted SNP. The resulting structure forms a substrate for the Cleavase enzyme, which cleaves the primary probe oligo at the position of overlap, releasing the 5' portion, which is termed the *flap oligonucleotide*. The primary probe and the Invader oligos are present in large molar excess, resulting in generation of many cleaved flap oligos if the targeted SNP is present in the template. The flap oligo itself functions as an Invader oligo, binding to a hairpin region of a third oligo, termed a fluorescence resonance energy transfer (FRET) cassette because it contains a fluorophore and a quencher adjacent to one another at one end of the cassette. The sequence of the FRET oligo causes it to assume a hairpin configuration in which the quencher damps fluorescence from the fluorophore. Binding of the flap Invader generates another tertiary DNA structure that also functions as a substrate for the Cleavase enzyme. The resulting cleavage releases the fluorophore away from the quencher, which leads to the generation of a fluorescent signal. Reaction conditions that allow cycling of the probe oligo and the hairpin oligos cycle on and off their

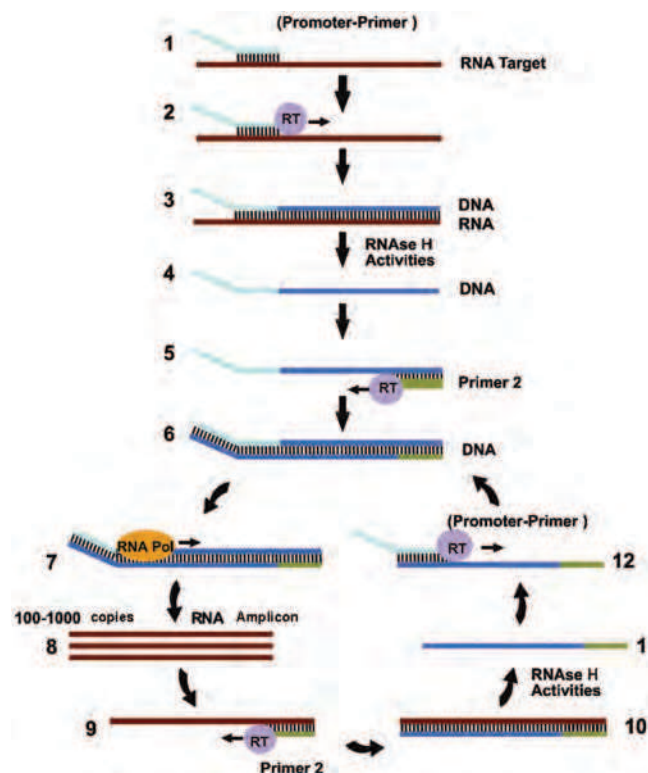


FIGURE 15.7. Transcription-mediated assay, also called 3SR. The 3SR reaction depends on a continuous cycle of reverse transcription and transcription reactions to replicate an RNA target by means of complementary DNA (cDNA) intermediates. Steps 1–6 depict the synthesis of a double-stranded cDNA, which is a transcription template for T7 RNA polymerase. Complete cDNA synthesis is dependent on the digestion of the RNA in the intermediate RNA–DNA hybrid (step 4) by ribonuclease (RNase) H. Transcription-competent cDNAs yield antisense RNA copies of the original target (step 7, right). These transcripts are converted to cDNA containing double-stranded promoters on both ends in an inverted repeat orientation (steps 7–12). These cDNAs can yield either sense or antisense RNA, which can re-enter the cycle.

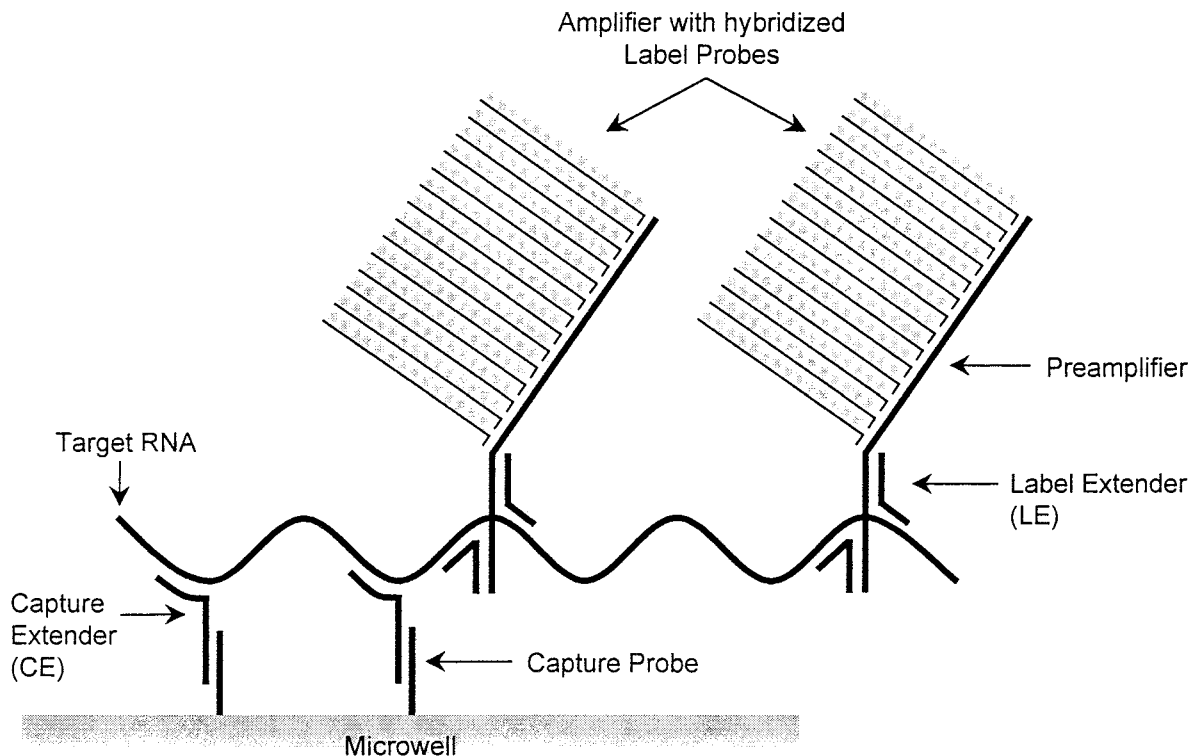


FIGURE 15.8. Branched-chain DNA assay as used for ultrasensitive detection of human immunodeficiency virus (HIV).

After liberation of viral nucleic acid from the clinical specimen by a lysis buffer, viral nucleic acid is hybridized with two sets of bifunctional oligonucleotide probes, each of which contains sequences complementary to the target. One set of probes also contains a generic sequence complementary to a capture probe that is bound to the surface of a microtiter tray and serves to bind the target to the solid surface. The other set of probes contains a sequence complementary to *preamplifier* molecules. Additional specificity is achieved because two of the target-specific probes must be juxtaposed in the correct orientation to stabilize the binding of the preamplifier molecule. Each preamplifier molecule binds numerous amplifier molecules, each of which binds many subsequently added alkaline phosphatase-labeled probes. A chemiluminescent substrate is added and generated light is read by a luminometer. The cascade effect results in amplification of the signal generated from initial binding of probes to the target and allows for detection and quantification of nucleic acid present in the specimen. (Courtesy of Bayer Diagnostics, Emeryville, California.)

respective targets result in the generation of 10^6 - to 10^7 -fold signal amplification per hour. Currently, FDA-approved tests using the Cleavase reaction to detect high-risk HPV types and to genotype HPV types 16 and 18 are marketed by Hologic (Bedford, MA) (formerly Third Wave Technologies) under the trade name Cervista.

The loop-mediated isothermal amplification (LAMP) (Fig. 15.11) is an amplification reaction that employs four primers and a strand-displacing DNA polymerase.⁹⁶ It was developed originally by Eiken Chemical Co. (Tokyo, Japan), and additional detailed diagrams of the assay can be viewed on the Eiken web page: <http://loopamp.eiken.co.jp/e/lamp/index.html>. The inner primers have sense and antisense sequences. When they initiate DNA synthesis, the result is a stem-loop structure, which itself serves as a target for additional amplification. The resulting reaction can produce 10^9 copies of target DNA or RNA in less than 1 hour under isothermal conditions. The reaction can be monitored by measuring turbidity, which results from the accumulation of magnesium pyrophosphate, a by-product of the reaction. Alternatively, the synthesis of dsDNA can be measured using an agent such as SYBR Green, which generates fluorescence when it intercalates between the strands of dsDNA. The reaction has high specificity by virtue

of the inclusion of multiple primer sets. The isothermal nature of the reaction, which obviates the requirement for a thermal cycler, makes LAMP attractive for use as a point-of-care test or for use in the developing world.

CONTAMINATION

A general concern regarding highly sensitive nucleic acid amplification assays is the occurrence of false-positive findings resulting from contamination of the reaction with exogenous nucleic acids. The source of contaminating nucleic acids can be either amplified products from previous reactions or viral nucleic acids present in a different specimen, especially one containing a high level of the intended target. Prevention of contamination requires fastidious attention to procedural detail within the laboratory. A number of specific preventive techniques are widely used to prevent carryover contamination after PCR, including the use of positive displacement pipettes or plugged pipette tips to block aerosol production, UV irradiation of reaction components (not including *Taq* polymerase and PCR primers that can be inactivated by UV irradiation), use of small working aliquots, and frequent cleaning of equipment and laboratory surfaces with 10% bleach. An important precaution is the physical separation of the laboratory into

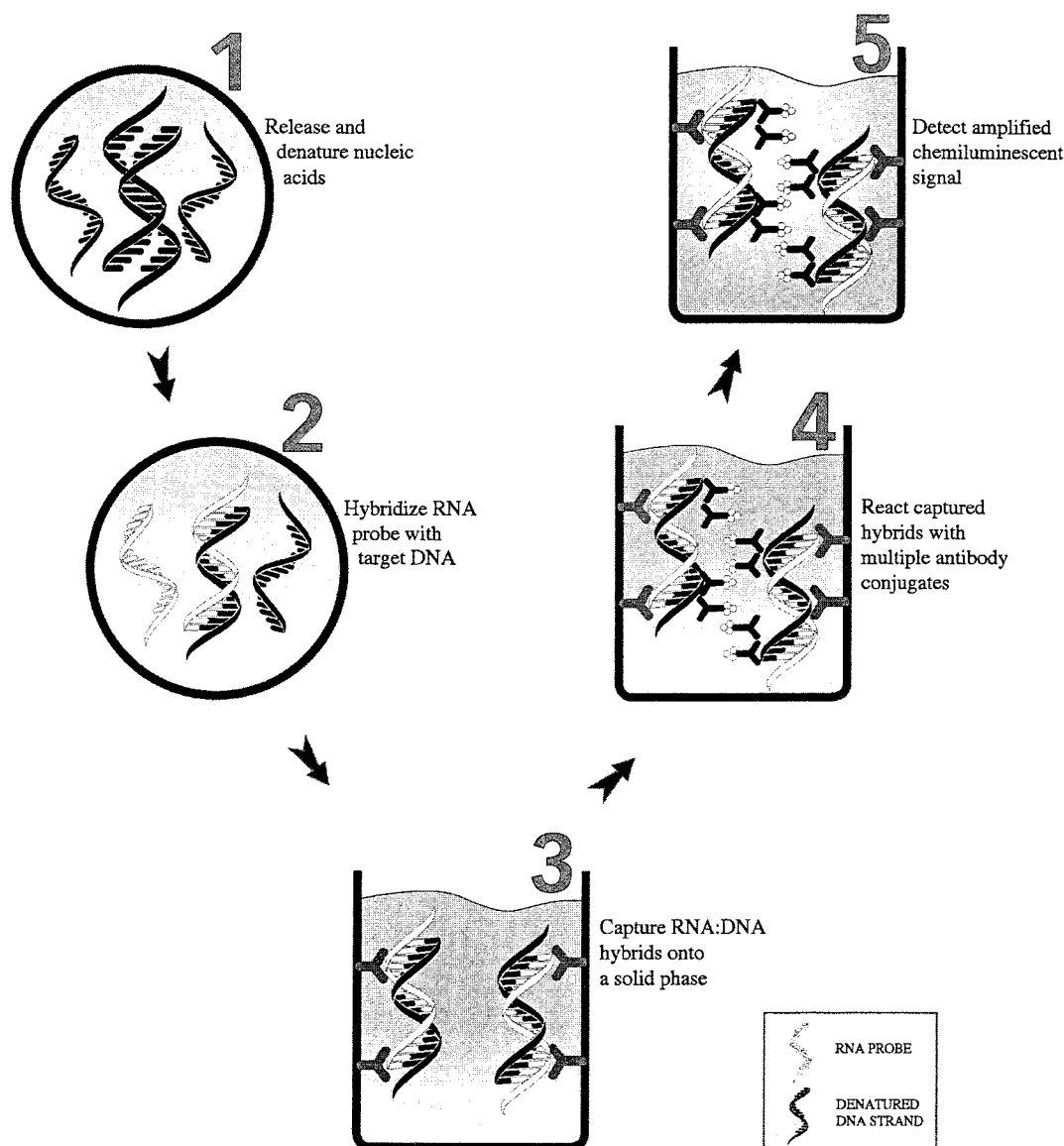


FIGURE 15.9. Hybrid capture assay. In step 1, target DNA is released and denatured. In step 2, RNA probe hybridizes with target DNA. In step 3, RNA–DNA hybrids are captured by an antibody to RNA–DNA hybrids that is bound to the sides of the reaction vessel. In step 4, an enzyme-conjugated antibody that recognizes RNA–DNA hybrids binds to the hybrids. In step 5, a chemiluminescent substrate is added, and light is emitted if hybrids have been formed. (Courtesy of Digene Corporation, Beltsville, Maryland.)

sections where (a) the specimen is prepared, (b) the PCR reaction is set up, (c) the PCR amplification reaction takes place, and (d) the detection of amplification products is carried out. After PCR has taken place, the reaction tube is opened only in the section of the laboratory devoted to product detection. Techniques such as the inclusion of uracil-N-glycosylase or psoralen and isopsoralen derivatives in the reaction mixture were developed to minimize the danger of PCR contamination.¹⁰⁴ Real-time PCR is less susceptible to contamination because product detection does not require manipulating the amplified product. For any form of nucleic acid testing, an additional concern is contamination from one specimen to another, which may occur during specimen handling in the

laboratory. Inclusion of negative controls in the reaction setup is important in order to detect this form of contamination.

Nucleotide Sequencing

Sequencing of PCR amplification products can be carried out using the cycle-sequencing reaction⁵⁷ or pyrosequencing. Sequence information can be used for several purposes including precise identification of a virus, genotyping, and the presence of mutations associated with antiviral drug resistance or unusual clinical manifestations. Genotypic resistance assays are performed most commonly for HIV, CMV, HBV, and influenza A virus (see section on antiviral susceptibility testing). For HBV, nucleotide sequencing of the nucleocapsid gene

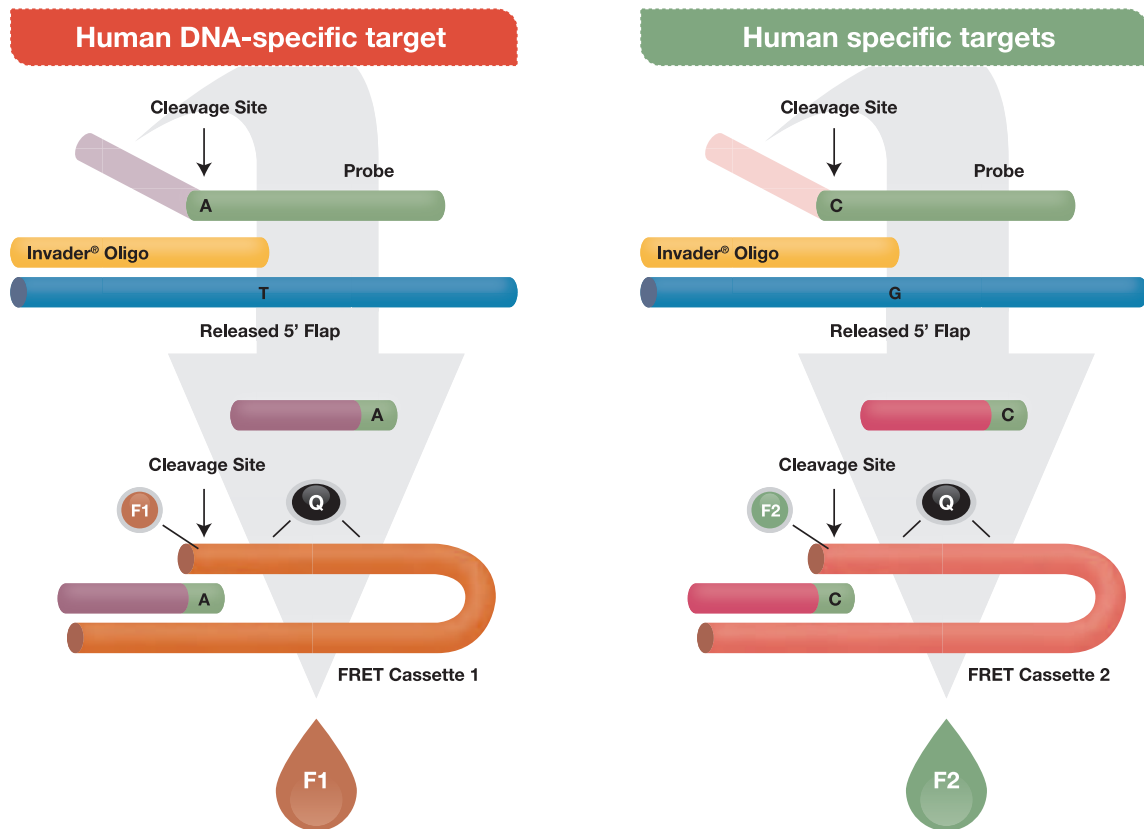


FIGURE 15.10. Invader assay. The **left side** of the figure shows a positive control reaction for human histone 2DNA and the **right side** shows the reaction to detect human papillomavirus (HPV). For each reaction, the probe oligo and the Invader oligo bind to the target, with a short region of overlap occurring at a targeted single nucleotide polymorphism (SNP). This results in a structure that is cleaved by the activity of an enzyme called “Cleavase,” releasing the 5’ end of the probe oligo at the position of overlap. The sequence of the released portion is homologous to the 3’ end of a third oligo, which includes a fluorophore (F1 for the human DNA reaction and F2 for the HPV reaction) and a quencher (Q) and is referred to in the figure as the FRET cassette. The hairpin configuration assumed by the FRET cassette brings the quencher sufficiently close to the fluorophore to allow its fluorescence to be quenched. Binding of the released portion of the probe oligo to the FRET cassette results in a region that is another substrate for Cleavase. The activity of Cleavase releases the fluorophore, resulting in generation of a fluorescent signal. (Courtesy of Hologic.)

and its associated promoter is also used for detection of core promoter and precore mutations, which have been associated with unusually severe disease and progression to chronic infection.⁹⁷ High-throughput (“next-generation”) sequencing will probably have important applications in diagnostic virology, including for antiviral susceptibility testing, for identification of viruses growing in cell culture that are not identifiable by conventional means,¹³⁰ and for discovery of known^{15,154} and novel viruses.^{20,99}

Microarray Technology

High-density microarrays consist of hundreds or thousands of oligonucleotide probes bound to a solid phase, usually a small silicon chip. Amplified nucleic acid can be hybridized to the array, and binding to specific probes can be identified. Use of probes representing all possible nucleotide sequence variations within a target sequence allows very rapid determination of nucleotide sequence.⁴⁵ Multiple sequence variants present within the specimen can also be detected. Microarray technology has the potential to allow simultaneous detection of multiple infectious disease pathogens, viral and nonviral. An early

application in diagnostic virology has been for rapid sequencing to detect HIV mutations associated with resistance to antiretroviral drugs.⁷⁰ In another application, sequences representing all sequenced viruses have been selected to create a microarray that can be used to discover unknown viruses. This microarray was successful in categorizing the agent of severe acute respiratory syndrome (SARS) as a new coronavirus.¹⁴⁵ The major application of microarray technology in diagnostic virology to date has been the use of liquid arrays, which are used for the simultaneous detection of multiple respiratory viruses, as exemplified by the xTAG RVP described later. Future uses of liquid arrays and other microarray technology under development include assays to detect multiple viruses (as well as other classes of pathogens) associated with gastroenteritis, sexually transmitted diseases, sepsis, and bioterrorism.

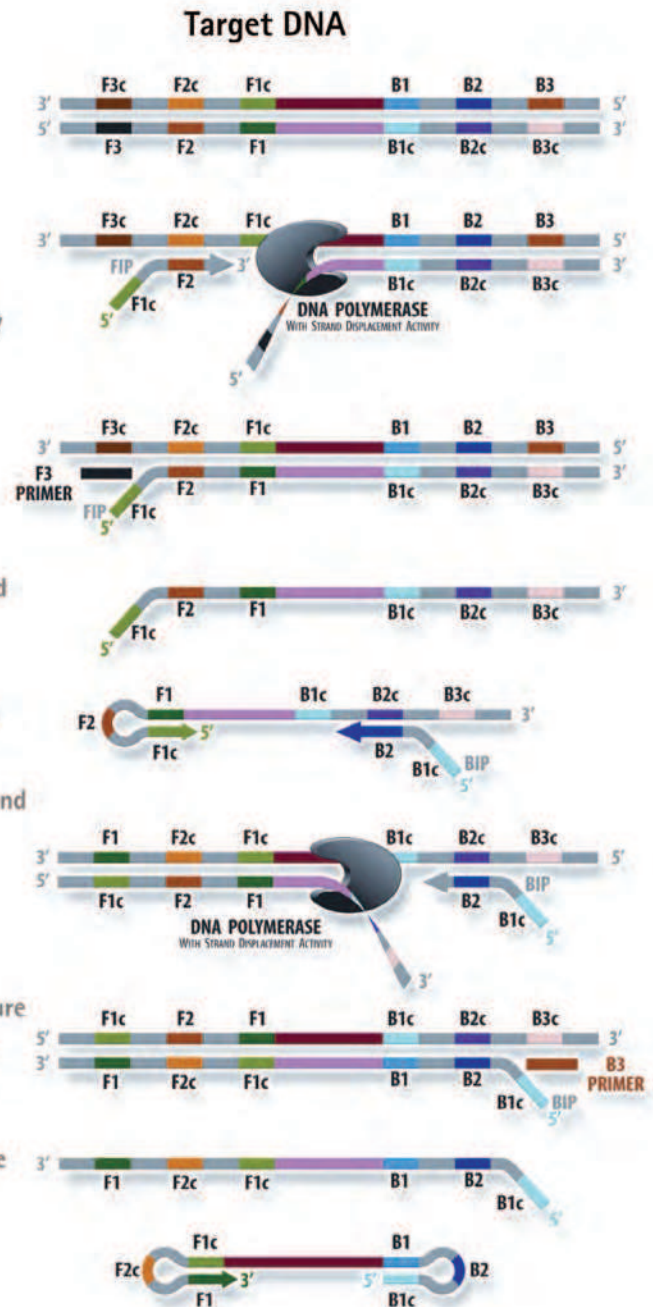
Commercial Nucleic Acid Amplification Platforms and Tests

GOVERNMENT REGULATION

In the United States, commercially marketed diagnostic tests and test platforms are regulated by the FDA. Before marketing

LAMP Process

1. Solution temperature at 60°–65° C
2. Forward Initiating Primer (FIP) anneals to Target Sequence
3. DNA Polymerase initiates synthesis that displaces single strand template DNA
4. Through polymerase activity, a strand complementary to the DNA template is formed
5. The F3 Primer anneals to the F3c Region
6. DNA Polymerase initiates synthesis and the FIP-linked complementary strand is replaced
7. This strand forms a "stem loop" at the 5' end due to complementary F1 and F1c regions
8. The BIP anneals to the 3' end of the "stem loop" strand
9. The B3 Primer anneals to the B3c target
10. From the 3' end, polymerase synthesizes a complementary DNA strand
11. DNA reverts from a Loop structure to a linear structure
12. The BIP linked complementary strand is displaced as a single strand
13. This strand forms stem-loops at either end due to the activity of the dual complementary primers
14. This is the starting structure for LAMP Cycling



A

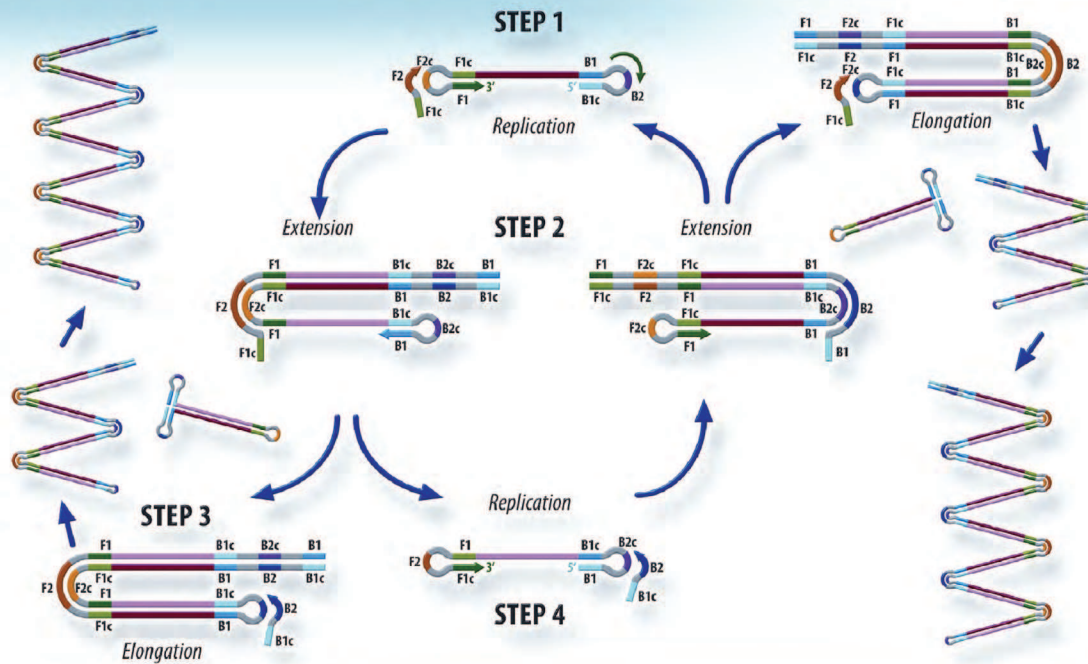
FIGURE 15.11. Loop-mediated amplification reaction (LAMP assay). A: Steps leading up to the formation of the "dumbbell" structure. (continued)

Cycling Amplification

A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions.

Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (STEP2).

The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (STEP4).



This structure is the 'turn over' structure of the structure formed in STEP1. Similar to the STEPS 1 THROUGH 4, structure in STEP4 leads to self-primed DNA synthesis starting from the 3' end of the B1 region.

Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to STEPS 2 AND 3 as well as the same structure as STEP 1 are produced. With the structure produced in STEP 3, the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.

B

FIGURE 15.11. (Continued) B: The cycling steps of the reaction, which lead to extremely rapid amplification. (Courtesy of Meridian Biosciences.)

a test for diagnostic use, a manufacturer must receive authorization from the FDA. This authorization can take one of several forms. If there is no similar test on the market, the manufacturer must apply for premarket approval (PMA). This is a rigorous and expensive process that requires the manufacturer to provide evidence for the accuracy and utility of the proposed test. A test that is “substantially equivalent” to a test that has been previously approved or cleared by the FDA is cleared for use through a mechanism referred to as 510K, after the section of the Federal Food, Drug, and Cosmetic Act that describes the process. Some diagnostic test materials are marketed under the classification “analyte-specific reagent (ASR).” This term is used by the FDA to refer to reagents or materials, including nucleic acid primers and probes, that must meet certain specifications set by the FDA, including being produced utilizing current “good manufacturing practices.” In addition, clinical laboratory tests carried out using ASRs must have appropriate labeling appended to the reported results stating that the test was developed and validated by the laboratory and has not undergone FDA clearance or approval. ASRs are intended to be used as components of laboratory-developed tests and can be sold only to laboratories that are qualified under CLIA to perform highly complex testing. ASRs are excluded from the need for premarket approval by the FDA. They cannot be marketed as complete test kits and must be sold without instructions for use or claims regarding performance characteristics.

Individual laboratories may also develop their own assays that are intended for use only in that laboratory. These tests, referred to as “laboratory-developed tests” (sometimes also referred to as “home-brew assays”), are not actively regulated by the FDA, but instead are under the jurisdiction of CLIA passed by the U.S. Congress in 1988. Laboratory-developed tests may use ASRs as components of the test and may also use reagents obtained from diverse sources not specifically labeled as ASRs. The performance of laboratory-developed tests must be verified according to specific criteria established by CLIA.

OVERVIEW OF COMMERCIAL ASSAYS AND PLATFORMS

Since the last edition of this book, there has been a dramatic increase in molecular diagnostic tests and test platforms that have been approved or cleared by the FDA for the detection of pathogenic viruses. Tests and platforms approved or cleared by the FDA at the time this chapter was written are shown in Tables 15.8 and 15.9. These tables are adapted from tables that are maintained by the Association for Molecular Pathology (AMP) and are accessible on the AMP website (<http://www.amp.org>). No single platform permits the detection of all medically important viruses, so laboratories whose mission is broad-based viral diagnosis will need to employ multiple platforms. Currently, laboratories vary widely in the relative proportion of testing that is carried out using commercial platforms versus laboratory-developed tests. Likewise, laboratories that provide different types of service will employ different platforms. For example, hospital-based laboratories may emphasize platforms that provide rapid turnaround time, commercial laboratories may employ platforms that allow high specimen throughput, and government laboratories may employ platforms that allow for testing of viruses of public health significance. The following paragraphs describe several categories of commercial molecular diagnostic tests.

Multiplex Assays

Multiplex PCR refers to PCR reactions in which more than one primer set is incorporated into the reaction mix, allowing the detection of multiple targets. Multiplex assays may be developed either as commercial tests or as laboratory-developed tests. Currently, multiplex assays are being developed for important specimen types to amplify various viral and other microbial agents that are pathogenic at the specific body site. This process is most highly developed for respiratory samples, for which five different multiplex assays are currently cleared by the FDA.

The Proflu+ assay marketed by Prodesse (now Gen-Probe, San Diego, CA), the Simplexa Flu A/B & RSV Test assay marketed by Focus (Cypress, CA), and the Verigene RV+ (Nanosphere, Northbrook, IL) are multiplex real-time assays that use separate primer pairs to simultaneously amplify influenza A and B viruses and RSV plus an internal control. In the Proflu+ and Simplexa assays, unique probes for each virus and the internal control are labeled with separate fluorophores, allowing determination of which virus or viruses have been amplified. The Simplexa assay is run on the 3M Integrated Cycler, an innovative instrument produced by 3M (St. Paul, MN) that has the capability to run 96 assays in 1 hour. Multiple different specimens can be loaded on the instrument and multiple assays can be run simultaneously. The Verigene RV assay uses a very innovative system to detect amplified products in which the amplicons are captured by capture probes that are bound to specific sites on a microarray. Captured amplicons are then detected by binding of silver-coated gold nanospheres that have been tagged with oligonucleotides that are specific for the amplicons and are visualized by light scatter.

The xTAG Respiratory Virus Panel (RVP) marketed by Luminex (Toronto, Ontario, Canada) was the first large multiplex assay to be cleared by the FDA. This assay detects the following viruses in nasopharyngeal swabs: influenza A and B, influenza A subtypes H1 and H3, RSV A and B, parainfluenza 1 through 3, human metapneumovirus (hMPV), enterovirus/rhinovirus, and adenovirus.^{85,98} Primers for influenza A hemagglutinin subtype H5, parainfluenza 4, and coronaviruses OC43, 229E, NL63, HKU1, and SARS are also included in the reaction but are not currently cleared for use in the United States. The first step in the reaction is reverse transcription followed by a multiplex PCR reaction that includes primer sets for each target. The resulting amplicons are prepared for detection through a reaction called target-specific primer extension (TSPE). The 3' end of each of the primers used in TSPE is homologous to one of the amplicons from the multiplex PCR reaction. The 5' end of the primer consists of a short “tag” sequence that will be used to capture the TSPE product. During the extension reaction that follows annealing of the TSPE primers, biotin-labeled deoxycytidine triphosphate (dCTP) is incorporated into the primer extension product. Detection of the TSPE products uses a set of polystyrene microspheres composed of 100 members. Each member microsphere incorporates two fluorescent dyes in a unique ratio that allows identification by flow cytometry using two lasers, each of which emits light of the appropriate wavelength to excite one of the two fluorescent dyes. Bound to each member of the microsphere set is a short oligonucleotide (“antitag”) with sequence homology to one of the tags introduced in the TSPE reaction. These tags are specific for each amplicon amplified by the

TABLE 15.8 Viral Molecular Tests Approved or Cleared by the Food and Drug Administration^a

Virus	Test Name	Method	Manufacturer
Adenovirus	ProAdeno™+ Assay	Multiplex real-time PCR	Gen-Probe (Prodesse), San Diego, CA
Cytomegalovirus	CMV pp67 mRNA	NASBA	bioMérieux, Durham, NC
Cytomegalovirus	HC1® CMV DNA Test	Hybrid capture	Qiagen, Germantown, MD
Cytomegalovirus (quantification)	COBAS AmpliPrep/COBAS Taqman CMV Test	Real-time PCR	Roche Molecular Diagnostics, Pleasanton, CA
Dengue	CDC DENV 1-4 Real Time RT-PCR Assay	Real-time PCR	Centers for Disease Control and Prevention, Atlanta, GA
Enteroviruses	NucliSENS EasyQ® Enterovirus	NASBA	bioMérieux, Durham, NC
Enteroviruses	Xpert™ EV	Real-time PCR	Cepheid, Sunnyvale, CA
Hepatitis B virus (quantification)	Abbott Real-time HBV	Real-time PCR	Abbott Molecular, Des Plaines, IL
Hepatitis B virus (quantification)	COBAS® TaqMan® HBV Test and COBAS® AmpliPrep/COBAS® TaqMan® HBV Test v 2.0 and COBAS TaqmanHBV Test for use with the HighPure System	Real-time PCR	Roche Molecular Diagnostics, Pleasanton, CA
Hepatitis C virus (quantification)	Abbott Real-Time HCV	Real-time RT-PCR	Abbott Molecular, Des Plaines, IL
Hepatitis C virus (quantification)	VERSANT® HCV RNA 3.0 Assay	bdNA	Siemens Healthcare Diagnostics, Deerfield, IL
Hepatitis C virus (quantification)	COBAS® AmpliPrep/COBAS® TaqMan® HCV Test v 2.0, COBAS® TaqMan® HCV v2.0 for use with the HighPure System, AMPLICOR HCV Test v 2.0, COBAS AMPLICOR HCV Test v 2.0	Real-time PCR	Roche Molecular Diagnostics, Pleasanton, CA
HIV (drug resistance)	ViroSeq™ HIV-1 Genotyping System	Sequencing	Celera Diagnostics, Alameda, CA
HIV (drug resistance)	TruGene™ HIV-1 Genotyping and Open Gene DNA Sequencing System	Sequencing	Siemens Healthcare Diagnostics, Deerfield, IL
HIV (quantification)	Abbott Real-Time HIV-1	Real-time RT-PCR	Abbott Molecular, Des Plaines, IL
HIV (quantification)	NucliSENS® HIV-1 QT	NASBA	bioMérieux, Durham, NC
HIV (quantification)	AMPLICOR HIV-1 MONITOR™ Test v1.5, COBAS® AMPLICOR HIV-1 MONITOR™ Test v1.5, COBAS AmpliPrep/COBAS Taqman HIV-1 Test v 2.0, COBAS Taqman HIV-1 Test v 2.0, COBAS® AmpliScreen™ HIV-1 Test, v1.5, COBAS® TaqScreen MPX®	RT-PCR	Roche Molecular Diagnostics, Pleasanton, CA
HIV (quantification)	VERSANT HIV-1 RNA 3.0 Assay (bdNA)	bdNA	Siemens Healthcare Diagnostics, Deerfield, IL
Herpes simplex virus	BD ProbeTec Herpes Simplex Viruses Qx Amplified DNA Test	SDA	BD Diagnostic Systems, Sparks, MD
Herpes simplex virus	Multicode-RTX Herpes Simplex 1 and 2 kit	Multicode chemistry real-time PCR	Eragen Biosciences, Madison, WI
Herpes simplex virus	IsoAmp HSV Assay	HAD	BioHelix, Beverly, MA
Human metapneumovirus	Pro hMPV+™ Assay	Real time RT-PCR	Gen-Probe (Prodesse), San Diego, CA
Human papilloma- virus	Cervista™ HPV HR and HPV 16/18	Invader® chemistry	Hologic (Third Wave Technologies), Bedford, MA
Human papilloma-virus	HC2® HR and LR, HR, DNA with Pap	Hybrid capture	Qiagen, Germantown, MD
Human papilloma-virus	COBAS® HPV Test	Multiplex real-time PCR and RT-PCR	Roche Molecular Diagnostics, Pleasanton, CA
Human papilloma-virus	Aptima HPV	TMA, HPA	Gen-Probe, San Diego, CA
Influenza virus	Human Influenza Virus Real-Time RT-PCR Detection and Characterization Panel	Real-time RT-PCR	Centers for Disease Control and Prevention, Atlanta, GA
Influenza virus	Xpert™ flu	Real-time PCR	Cepheid, Sunnyvale, CA
Influenza virus	Liat Influenza A/B Assay	Real-Time PCR	IQumm, Marlborough, MA
Influenza virus	Artus Infl A/B RG RT-PCR Jut	Real-Time RT-PCR	Qiagen, Germantown, MD
Influenza virus	Influenza A + B Assay	Real-Time RT-PCR	Quidel, San Diego, CA
Influenza virus	ProFlu Assay	Real-time RT-PCR	Gen-Probe (Prodesse), San Diego, CA
Influenza virus H1N1	Simplexa™ Influenza Test	Real-time RT-PCR	Focus Diagnostics, Cypress, CA

(continued)

TABLE 15.8 Viral Molecular Tests Approved or Cleared by the Food and Drug Administration^a (*continued*)

Virus	Test Name	Method	Manufacturer
Influenza virus (avian)	Influenza A/H5	Real-time PCR	Centers for Disease Control and Prevention, Atlanta, GA
Parainfluenza viruses 1-3	ProParaFlu+™ Assay	Real-time PCR	Gen-Probe (Prodesse), San Diego, CA
Respiratory viruses (influenza A and B, RSV, parainfluenza 1-3, human metapneumovirus, enterovirus, rhinovirus, adenovirus)	xTAG Respiratory Virus Panel	PCR, RT-PCR, TSPE, Tag sorting	Luminex Molecular Diagnostics, Toronto, CA
Respiratory viruses (influenza A and B, RSV, parainfluenza 1-4, human metapneumovirus, enterovirus, rhinovirus, adenovirus, coronaviruses NL63, HKU1, OC43, 229E)	FilmArray Respiratory Panel	Multiplex, nested PCR and RT-PCR	Biofire Diagnostics, Salt Lake City, UT
Respiratory viruses (influenza A and B, RSV, parainfluenza 1-3, human metapneumovirus, rhinovirus, adenovirus)	eSensor Respiratory Virus Panel	PCR, RT-PCR, Probe Hybridization	Gen-Mark Diagnostics, Pasadena, CA
Respiratory viruses (influenza A and B, RSV)	Verigene® Respiratory Virus Plus Nucleic Acid Test	Multiplex gold particle nanoparticle probes	Nanosphere, Northbrook, IL
Respiratory viruses (influenza A and B, RSV)	ProFlu+™ Flu A/B & RSV Test	Multiplex real-time RT-PCR	Focus Diagnostics, Cypress, CA

bDNA, branched chain DNA assay; HAD, helix displacement assay; HPA, hybrid protection assay; SDA, strand displacement assay; TMA, transcription-mediated amplification; TSPE, target-specific primer extension.

^aAdapted from the web site of the Association for Molecular Pathology (AMP), www.amp.org, accessed November 26, 2012.

TABLE 15.9 Molecular Diagnostic Systems Approved/Cleared by the FDA^a

System	Viruses detected	Manufacturer
NucISENS EasyQ® System	Enterovirus	bioMérieux, Durham, NC
Procleix® Semi-Automated and TIGRIS Systems	HIV-1, HCV, HBV, WNV	Gen-Probe, San Diego, CA
VERSANT™ 440 Molecular System	HCV	Siemens, Deerfield, IL
Luminex LX 100/200-xTAG Respiratory virus Panel	Respiratory viruses	Luminex, Toronto, Canada
Verigene® System	Influenza A and B, RSV	Nanosphere, Northbrook, IL
Abbott m2000™	HIV-1, HCV	Abbott, Des Plaines, IL
COBAS AmpliPrep/COBAS Taqman, COBAS 4800, COBAS Taqman 48 Analyzer	HIV-1, HBV, HCV, HPV	Roche Diagnostics, Pleasanton, CA
7500 Fast Dx Real-time PCR Instrument	Influenza	Applied Biosystems, Foster City, CA
FilmArray System	Respiratory viruses	Biofire Diagnostics, Salt Lake City, UT
eSensor XT-8 System	Respiratory viruses	GenMark Diagnostics, Pasadena, CA
GeneXpert™ Real-time PCR System	Enteroviruses, Influenza A and B	Cepheid, Sunnyvale, CA
3M Integrated	Influenza A and B	Focus Diagnostics, Cypress CA
Cycler/Simplexa™ reagent kits and assay protocols		

^aAdapted from the web site of the Association for Molecular Pathology (AMP), www.amp.org, accessed August 6, 2011.

multiplex reaction. Following the TSPE reaction, the reaction mix is incubated with the microspheres, allowing the microspheres to specifically capture any TSPE products that have been produced. A fluorescent detection signal is generated through the inclusion of streptavidin-phycoerythrin, which binds to the biotin molecules incorporated into the amplicons. As the microspheres pass through the flow cell, they are interrogated by the lasers, allowing identification of the specific microsphere set member and determination of whether or not a TSPE product has been bound. Computer software analyzes the fluorescent events and determines for each virus and internal control whether a threshold of detection has been surpassed. Recently a version that requires less time to complete, called xTAG RVP Fast, was cleared by the FDA for detection of influenza A, influenza A subtype H1, influenza A subtype H3, influenza B, RSV, hMPV, rhinovirus, and adenovirus.

The other large multiplex assay that has been approved by the FDA is the FilmArray Respiratory Panel, which was cleared by the FDA in 2011 for detection in nasopharyngeal swabs of the following viruses: influenza A and B; influenza A subtypes H1, H1 2009, and H3; RSV; hMPV; adenovirus; parainfluenza viruses 1 through 4; rhinovirus/enterovirus; and coronaviruses NL63 and HKU1.¹¹⁰ Extraction, amplification, and detection are carried out in a self-contained unit referred to as a “pouch.” Nucleic acid extraction is accomplished using magnetic beads. The nucleic acid amplification that follows is a multiplex nested PCR assay. The first amplification reaction is a multiplex reaction containing outer primers for each target virus. The reaction mix is then diluted and combined with a new primer-free master mix that contains the fluorescent dye LC Green Plus, which binds to dsDNA. This mix is then moved by microfluidic techniques into an array of individual wells, each of which contains inner primers specific for one target virus. Each target is represented in at least three wells. Production of viral amplicons is confirmed by analyzing melting curves. The entire process from inoculation of sample to availability of results takes approximately 1 hour, with only 2 minutes of hands-on time. The manufacturer also plans to develop a test for gastroenteritis that will include but not be limited to viruses.

Integrated Platforms. The term *integrated platform* refers to test platforms in which nucleic acid extraction, amplification, and product detection are carried out within the same device or instrument. These platforms represent an important advance in diagnostic microbiology because their simplicity allows them to be performed in many laboratories that would not otherwise be able to provide viral diagnostic testing. In fact, they are so simple that they could be performed in a physician's office, although they do not currently have CLIA-waived status, which would permit testing outside of laboratories approved for “high-complexity” testing. To date, two integrated platforms have been approved or cleared by the FDA. The first is the GeneXpert Real-time PCR system, marketed by Cepheid (Sunnyvale, CA). This platform employs test cartridges into which a specimen is inoculated. The cartridge is inserted into the GeneXpert instrument, which performs nucleic acid extraction, amplification, and product detection without any intervention by the user. Tests for enteroviruses⁶⁹ and for influenza A and B viruses¹⁰⁵ have currently been cleared by the FDA. The enterovirus assay requires a total of approximately 2½ hours to perform, while the influenza assay requires less

than 1 hour. Several GeneXpert bacterial assays have also been cleared by the FDA. The second integrated platform cleared by the FDA is the FilmArray, described previously.

HIGH-THROUGHPUT QUANTITATIVE SYSTEMS

Commercial systems that provide standardized quantitative testing for HIV and HCV RNA and HBV DNA have been approved by the FDA. These systems are the Roche COBAS AmpliPrep/COBAS TaqMan and the Abbott m2000. Both systems include separate automated instruments for nucleic acid extraction and real-time PCR. While these systems are highly automated, they require more hands-on time from laboratory personnel than the truly integrated platforms. Both systems have the capability for moderate to high throughput. Test results are intended for diagnosis, to determine the need for treatment, and to monitor the response to therapy. Tests for quantitative detection of HIV, HCV, and HBV have been approved on both systems. The m2000 system is also FDA approved for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoea*. Outside of the United States, additional viral tests are available for the m2000, including HCV genotyping and detection of CMV, Epstein-Barr virus (EBV), HPV, *C. trachomatis*, and *N. gonorrhoea*.

Specialized highly automated platforms have also been approved by the FDA for screening donated blood. The Procleix Tigris system has been developed by Gen-Probe in collaboration with Novartis Diagnostics (Emeryville, CA). This is a highly automated instrument that uses FDA-cleared assays (Procleix Ultrio and Ultrio Plus) to test for HIV, HCV, HBV, and West Nile virus (Procleix West Nile Virus Assay). The tests for RNA viruses use the TMA reaction for amplifying RNA. Roche has developed the COBAS s201 system for screening donated blood. This system includes the COBAS AmpliPrep instrument for nucleic acid extraction and the COBAS TaqMan 96 analyzer for performing real-time PCR. The system provides testing for HIV, HCV, HBV, and West Nile virus (TaqScreen MPX Test and TaqScreen West Nile Virus test). Non-real-time PCR assays (COBAS AmpliScreen) have also been approved by the FDA for screening donated blood for HIV, HCV, and HBV.

Serology

Measurement of antiviral antibodies was one of the first methods used for the specific diagnosis of viral infections and it remains an important tool in the diagnostic virology laboratory. The role of serology can be for the diagnosis of acute or current infection or for the determination of immune status to specific viruses. Serologic diagnosis is important for viruses that cannot be cultured readily or for which culture is slow or otherwise impractical. Viruses for which serology is useful in diagnosing acute infection are shown in Table 15.10. Serology is uniquely useful for defining immunity with respect to specific viruses. It is important to use sensitive assays such as EIA for this purpose, because antibody levels indicative of past infection can decline to very low levels years after the infection. The specific assay used must also be selected with care to ensure that it measures antibodies that correlate with immunity. Viruses for which serologic testing is used to determine immune status are shown in Table 15.11.

Kinetics of the Antibody Response

Serologic diagnosis is based on the kinetics of the antibody response to viral infection. Virus-specific antibodies are absent

TABLE 15.10 Serology for Diagnosis of Acute Infection

Epstein-Barr ^a
Cytomegalovirus (mononucleosis syndrome) ^a
Hepatitis viruses (A–E) ^b
Measles ^a
Rubella ^a
Mumps ^a
Parvovirus B19 ^a
Encephalitis viruses (e.g., West Nile virus) ^a
Rabies
Hemorrhagic fever viruses ^a
Dengue fever ^a
Human immunodeficiency virus (HIV)
Human T-cell lymphoma or leukemia virus types 1/2 (HTLV-1/2)

^aVirus-specific immunoglobulin M (IgM) assays are used.

^bVirus-specific IgM assays are used for hepatitis A, B, and E.

in a susceptible individual but typically become detectable several weeks after the onset of infection. Virus-specific IgM antibodies are often detectable for a short period before virus-specific IgG antibodies. The IgM response tends to decline within approximately 1 to 2 months, although low levels can persist for 1 year or more in some viral infections. IgG antibodies are much more long-lasting and can persist for the life span of the individual. Thus, the absence of virus-specific IgG and IgM antibodies signifies susceptibility to infection; the presence of virus-specific IgM antibodies, with or without virus-specific IgG antibodies, signifies current or very recent infection; and the presence of virus-specific IgG but not IgM antibodies signifies past infection. The latter pattern often, but not always, indicates immunity to subsequent infection.

An alternative serologic approach to the diagnosis of recent infection is based on the concept that the avidity of antigen-specific antibodies increases as the immune response matures.²⁸ Thus, antibodies produced shortly after infection have low affinity for the causative agent, and antibodies produced late after infection have high affinity. Detection of low-affinity antibodies has been used to diagnose recent infection with CMV,²⁷ rubella,¹¹⁶ and HIV.¹²⁹

TABLE 15.11 Serology to Determine Immune Status

Varicella-zoster virus
Herpes simplex virus
Cytomegalovirus
Epstein-Barr virus (immunoglobulin G antibodies to the viral capsid antigen)
Human herpesvirus type 6
Rubella
Measles
Mumps
Parvovirus B19
Hepatitis A virus (total antibodies)
Hepatitis B virus (anti-HBs)
Hepatitis E virus

The relationship between the onset of clinical manifestations of infection and the presence of virus-specific antibodies depends on the incubation period of the infection. In infections with short incubation periods (e.g., acute respiratory infections), antibodies are absent at the onset of symptoms. In viruses with incubation periods of 1 month or longer, however, virus-specific IgG and IgM antibodies are typically present at the onset of symptoms.

Serology in Reinfection and Reactivation

The serologic diagnoses of reinfection and reactivated infection are more complicated than that of initial infection. In both of these cases, the infection occurs in an individual with preexisting, virus-specific IgG antibodies. In reinfection an anamnestic response may occur that results in an increase in the level of virus-specific IgG antibodies with continued absence of virus-specific IgM antibodies. Reactivation of latent infection, for example, in the case of some herpesvirus infections, may result in the appearance of virus-specific IgM as well as IgG antibodies.

Serology in Chronic Infections

The interpretation of serology is unique for viruses that typically cause chronic infection (e.g., HIV) and the related retroviruses human T-cell lymphoma or leukemia virus (HTLV) types 1 and 2. For these agents, the detection of antibodies of any isotype to these viruses (in the absence of artificial immunization) virtually always signifies current infection. In the case of HCV, the confirmed presence of antibodies to the virus corresponds to active infection in approximately 85% of individuals. A recent innovation is the development of tests that simultaneously measure HIV p24 antigen in addition to HIV antibodies. Because p24 antigen may appear some days before the appearance of HIV antibodies, the combined test decreases the “window” during which a recently infected individual is serologically negative but capable of transmitting infection.¹⁰⁰ The Abbott Architect HIV Ag/Ab Combo assay was recently approved by the FDA as an aid in the diagnosis of HIV-1 or -2 infection.

Serologic Assays

BINDING ASSAYS

The most widely used assays are those that directly measure binding of antibodies to viral antigens. Examples of binding assays include EIA, radioimmunoassay (RIA), and the indirect immunofluorescent antibody assay (IFA). Binding assays can be used in many different formats. In one common format, a viral antigen, which can consist of virally infected cells, a purified viral preparation, or a recombinant viral protein, is attached to a solid surface such as the inner surface of the well of a microtiter tray, a plastic bead, or a microscope slide in the case of IFA. Serum is added, allowing the binding of antiviral antibodies that might be present in the serum. After an appropriate incubation period, the serum is removed and the well is extensively washed. The next step is the addition of a second antibody (also referred to as a detector antibody) with specificity for human immunoglobulins. The second antibody is typically a mouse monoclonal or a polyclonal antibody from a nonhuman species. It is linked to an enzyme such as horseradish peroxidase. The second antibody binds to any human antibody present (ideally, because of specific binding to the viral antigen). The well is again extensively washed, and the presence of the second antibody is detected based on a colorimetric, radiometric, or fluorescent signal. An

important advantage of binding assays is that they can be modified to detect IgM- or IgA-specific antiviral antibodies through the use of isotype-specific detector antibodies.

IMMUNOBINDING ASSAYS

The western blot and related recombinant immunobinding assays are binding assays that allow the identification of antibodies to specific viral proteins. In the western blot assay, viral antigens present in infected cells are denatured, separated by gel electrophoresis, transferred to a membrane, and allowed to react with the serum specimen. Binding of antiviral antibodies is detected through the use of labeled detector antibodies. The western blot is cumbersome but provides a very high level of specificity because of the ability to identify binding to individual viral proteins and the production of a characteristic blot fingerprint. Thus, its main application in diagnostic laboratories is the confirmation of screening assays (e.g., EIA) that are less specific. For example, until recently, the western blot was widely used to confirm a positive HIV EIA. Western blot is also considered the gold standard in determining the type specificity of HSV antibodies. The recombinant immunobinding assay differs from the western blot in that specific viral antigens are produced in *in vitro* expression systems and artificially attached to membranes in a predetermined configuration, often in dots or in bands. The membranes are then incubated with serum, and bound antibodies are detected, as in the western blot. Because the antigens used in the immunobinding assay are not denatured as they are in routine western blots, these assays may have advantages in detection of antibodies to conformational epitopes. A recombinant immunobinding assay is used to confirm positive hepatitis C EIA results.

FUNCTIONAL ASSAYS

Functional assays are based on detection of specific activities resulting from the binding of specific antibodies to viral antigens. An important example is the neutralization assay, which measures the ability of antibodies to block viral infectivity. In neutralization assays, serial dilutions of the serum to be tested are incubated with a standardized quantity of infectious virus. After a short incubation period, the serum-virus mixtures are added to cells that support the growth of the virus, in parallel with a similar inoculum of virus that has not been incubated with serum. The neutralizing antibody titer is the highest serum dilution that prevents infection of the cells. The neutralization assay correlates well with protection from infection and is sometimes considered the standard against which other serologic assays should be measured. Because it is cumbersome and expensive, the neutralization assay is now rarely used in routine diagnostic laboratories. Other examples of functional assays are the hemagglutination-inhibition assay, in which the presence of antiviral antibodies is detected by their ability to block virus-induced hemagglutination, and the complement fixation assay, which measures the ability of antiviral antibodies to fix complement, preventing the complement from lysing indicator erythrocytes. Although hemagglutination-inhibition and complement fixation assays have been widely used in the past, they are increasingly being replaced by binding assays.

AGGLUTINATION ASSAYS

Viral antigens can be bound to a variety of particles, including fixed erythrocytes and latex particles. Agglutination assays are performed by mixing dilutions of serum with a suspension of antigen-coated particles. The agglutination titer is the

highest serum dilution that results in visible agglutination of particles. The advantage of agglutination assays is simplicity; they are rapid and do not require sophisticated equipment. Therefore, they are well suited for *stat* testing and for use under field conditions. For example, a rapid latex agglutination assay for varicella antibodies can be used for rapid determination of the varicella immune status of health care personnel who are exposed to a patient with varicella.

Virus-Specific IgM Assays

Assays for virus-specific IgM antibodies are important because they often allow a specific diagnosis of an acute infection to be made based on analysis of a single serum specimen obtained during the acute phase of illness. These assays are most likely to be useful in diseases with incubation periods that are sufficiently long so that virus-specific IgM levels are present at detectable levels at the onset of illness. Hepatitis A provides an excellent example; because of its incubation period of 2 to 6 weeks, hepatitis A IgM antibodies are virtually always detectable at the time patients seek medical care.

A number of methods are used to measure virus-specific IgM antibodies. Two older methods include (a) separating IgG and IgM fractions before performing the virus-specific antibody assay and (b) performing the virus-specific assay before and after procedures, such as treatment with β -mercaptoethanol, that selectively destroy IgM antibodies. Many modern virus-specific IgM antibody assays use IFA or EIA with a detector antibody that binds only to human IgM. The IgM capture assay is an alternative approach in which IgM antibodies present in the serum sample being tested are first *captured* using an antibody specific for human IgM. A preparation of viral antigen is added, followed by an enzyme-labeled antibody specific for the viral antigen. Binding is detected by the addition of the appropriate enzyme substrate.

Virus-specific IgM assays have several potential pitfalls and must be interpreted in conjunction with clinical findings. False-positive results can occur if the serum being tested contains rheumatoid factor (IgM antibodies to human IgG) plus virus-specific IgG antibodies that form complexes that may be falsely detected by the assay as virus-specific IgM antibodies. This problem can be avoided by pretreatment of the serum to remove either rheumatoid factor or IgG antibodies. One or the other of these pretreatments is included routinely in many commercial virus-specific IgM antibody assays. False-negative results can occur if the serum being tested contains high levels of virus-specific IgG antibodies that may compete with the virus-specific IgM antibodies. The IgM-capture assay configuration avoids this problem, as does serum pretreatment to remove IgG.

Complexities in the nature of the IgM antibody response must also be considered in interpreting virus-specific IgM antibody tests. In some patients, the IgM antibody response is transient or low level, leading to failure to detect virus-specific IgM antibodies, whereas in others, low levels of IgM antibodies may persist for 1 year or longer, decreasing the specificity of the assay as an indicator of very recent infection. In herpesvirus infections, IgM antibodies can sometimes be detected in reactivations as well as in primary infection.

Saliva and Urine Assays

Virus-specific antibodies can often be detected in saliva and urine. These body substances are attractive for use in serologic

assays because they avoid the need for phlebotomy. Assays for HIV antibodies in these fluids are commercially available and have performance characteristics similar to assays carried out on serum.^{35,138}

Cerebrospinal Fluid Serology

Serologic testing can be applied to CSF for the diagnosis of central nervous system (CNS) infection. For unusual viruses (e.g., rabies), the presence of any virus-specific antibodies within the CSF is diagnostic of active infection. For the diagnosis of encephalitis caused by the alphaviruses, bunyaviruses, or flaviviruses, the presence of virus-specific antibodies in CSF is highly suspicious and the presence of virus-specific IgM antibodies in CSF is diagnostic. For more common viruses (e.g., herpesviruses) or the common respiratory viruses, the mere presence of virus-specific antibodies in CSF is not diagnostic of CNS infection because antibodies produced in the blood are present in the CSF even in the absence of CNS infection. The problem is further complicated by the fact that defects in the blood–brain barrier that can occur during many neurologic diseases can increase the passage of antibodies from blood to CSF. Therefore, for the common viruses, intrathecal synthesis of specific antiviral antibodies must be demonstrated to provide evidence of CNS infection.

Intrathecal synthesis of a specific antiviral antibody is evaluated by determining the quotient of two ratios: (a) the ratio of specific antiviral antibody level in CSF to the level in serum, and (b) the ratio of total IgG in CSF to total IgG in serum ($\text{CSF}_{\text{specific antibody}} \cdot \text{serum}_{\text{specific antibody}}$ divided by $\text{CSF}_{\text{IgG}} \cdot \text{serum}_{\text{IgG}}$). A quotient greater than 1.5 is evidence of intrathecal antibody synthesis of the specific antibody. The antibody assay used to measure the specific antiviral antibody must have certain special characteristics. First, it must be very sensitive, because CSF antibody levels are usually low (~1,000 times less than serum levels). Second, it must be capable of providing a quantitative result that allows calculation of the CSF-to-serum ratio of specific antibody level. EIA is usually used, but testing of a series of dilutions of CSF and serum is required to obtain linear quantitative estimates of antibody level suitable for calculating the necessary ratio. Because of the complexities involved, accurate determination of intrathecal antibody synthesis of specific antiviral antibodies is performed in only a limited number of reference laboratories.

Measurement of intrathecal antibody synthesis has been used in a variety of CNS infections. The applications for which experience is most extensive are encephalitis caused by HSV and VZV. In HSV encephalitis, the utility of determinations of intrathecal antibody synthesis is limited because intrathecal antibody synthesis may not be detectable until 7 to 10 days after the onset of illness. Interestingly, intrathecal antibody synthesis can persist for years after the acute infection. Therefore, the main application is in cases in which specimens were not available early in the illness to allow a definitive diagnosis based on PCR. A caveat concerning diagnosis by demonstration of intrathecal antibody synthesis is that nonspecific polyclonal intrathecal antibody synthesis can occur in some diseases (e.g., multiple sclerosis).¹¹³ This pitfall can be avoided by evaluating intrathecal synthesis of antibodies to at least one other virus other than the one of primary interest. Serologic diagnosis has been shown to be more sensitive than PCR for diagnosis of VZV vasculopathy.⁹¹ This is explained partly by the fact that

CSF samples from some patients in this study were obtained months after the onset of neurologic manifestations.

Antiviral Susceptibility Testing

In recent years, antiviral drugs have been licensed for the treatment of infections caused by HSV, VZV, CMV, influenza A and B, RSV, HIV, HCV, and HBV. Not surprisingly, increasing use of these drugs has been accompanied by the appearance of antiviral drug resistance. Testing for resistance may be required for optimal patient management. Antiviral drug susceptibility testing is performed in some diagnostic virology laboratories and is also available through reference laboratories.

Phenotypic Assays

Antiviral susceptibility testing is divided into phenotypic and genotypic methods. Phenotypic methods analyze the effect of antiviral drugs on the replication of virus, which can be measured by infectivity, viral antigen or nucleic acid production, or effect on the activity of a viral enzyme such as influenza neuraminidase. Phenotypic assays are important because they directly measure the effects of antiviral drugs on viral replication. In addition, they are more universal than genotypic assays because they can be used when the genetic basis for resistance is unknown. In the case of HIV, phenotypic assays are useful in analyzing viruses in which the presence of multiple mutations makes it difficult to predict phenotype from genotypic data. Finally, phenotypic assays must be used to determine the significance of candidate mutations found in drug-resistant isolates. However, from a practical standpoint, phenotypic assays tend to be labor-intensive, slow, and poorly standardized. In addition, there is a theoretical concern that the growth of the virus in cell culture may lead to selection of nonrepresentative viral variants, especially because antiviral resistance may in some instances be associated with decreased viral fitness. For these reasons, phenotypic assays are not performed in most diagnostic virology laboratories. Table 15.12 shows recommended *cut-offs* that define resistance to several drugs used for HSV and CMV infections.

The plaque assay was the traditional method for performing phenotypic susceptibility testing. However, this method is rarely used in diagnostic virology laboratories for the reasons mentioned previously and will not be discussed further here. Assays measuring viral antigen or nucleic acid production are alternatives to infectivity-based assays. A variety of assays have been used in individual laboratories, but they are not commercially available. The ELVIS assay illustrated in Figure 15.3 has also been adapted for the performance of antiviral susceptibility testing.^{128,134} For influenza, resistance to neuraminidase inhibitors has been measured using neuraminidase inhibition assays based on either fluorescent or chemiluminescent signal generation.^{12,92,93}

Recombinant phenotypic assays have been developed and widely used for HIV. These assays involve insertion of HIV genes that are targets for antiretroviral drugs (polymerase, protease, integrase) from a patient being evaluated into a vector consisting of a rapidly replicating laboratory strain of HIV that also contains a reporter gene (e.g., luciferase) that is used to measure viral growth.¹⁰² These assays allow relatively rapid testing of the susceptibility of HIV to multiple drugs as well as to drug combinations. Commercial tests have been developed by Monogram Biosciences (San Francisco, CA) and Virco

TABLE 15.12 Cut-Offs for Antiviral Susceptibility Testing for Herpes Simplex Virus (HSV) and Cytomegalovirus (CMV) Using the Plaque Reduction Assay

Virus (reference)	Drug	Cut-off (μM)		
		Sensitive	Intermediate	Resistant
HSV (117)	Acyclovir	<8		≥ 8
	Foscarnet	<330		≥ 330
CMV (18,84)	Ganciclovir	≤ 6	6–12	>12
	Foscarnet	≤ 400		>400
	Cidofovir	≤ 2		>4

(Antwerp, Belgium) and are available in the United States through national reference laboratories. A special case is the HIV drug maraviroc, which blocks HIV entry by binding to the CCR5 receptor. Viruses that use the CXCR4 receptor rather than the CCR5 receptor are resistant to maraviroc. A commercial assay called Trofile (Monogram Biosciences)¹⁵⁰ is used to predict response to maraviroc by determining the receptor tropism of a patient's isolate.

Genotypic Assays

Genotypic assays test for the genetic basis for resistant phenotypes. A variety of methods are used, including detection of restriction fragment length polymorphisms, PCR assays that detect mutations by virtue of differential primer or probe binding, and nucleotide sequencing. With rapid improvements in sequencing technology including the development of pyrosequencing, sequencing-based methods are becoming increasingly used. Genotypic assays are informative only when the genetic basis for resistance is known and are most useful when the resistance being evaluated is accounted for by a limited number of genetic changes.

Genotypic assays have been most widely used for CMV, HIV, HBV, and influenza A, because phenotypic assays for these viruses are slow and expensive or not widely available. Application of genotypic assays requires an extensive body of knowledge concerning the effect of specific mutations on antiviral resistance. Some single mutations may confer high-level resistance, while others may confer only partial resistance, and others are not associated with resistance at all.

For CMV, resistance to the currently FDA-approved drugs ganciclovir, foscarnet, and cidofovir has been related to mutations in the phosphotransferase (*UL97*) and polymerase (*UL54*) genes.^{18,84} Most laboratories now use sequencing to detect these mutations. Because ganciclovir-resistance mutations generally arise first in the *UL97* gene, some laboratories sequence this gene first, proceeding to sequencing *UL54* only if no significant mutations are detected in *UL97*, or if the patient is being treated with foscarnet or cidofovir, which exert their antiviral effect by inhibiting this gene. Different mutations within *UL97* or *UL54* are associated with different levels of antiviral resistance.⁸⁴

For HIV, genotypic testing for resistance is commonly used by physicians in the developed world in the management of antiretroviral drug therapy. This testing involves RT-PCR amplification of regions of the polymerase and protease genes and sometimes the integrase gene, followed by nucleotide sequencing of the amplicons. Certain mutations are highly

associated with phenotypic resistance and drug failure, whereas the significance of other mutations found in drug-resistant strains is less clear. Interpretation of HIV genotypic testing is most straightforward in patients who are not highly drug experienced and have relatively small numbers of mutations. Patients who have been treated more extensively may have multiple interacting mutations and may also have mutations that lie outside of the segments that are sequenced but nevertheless affect susceptibility to antiretroviral drugs.⁴³ Several FDA-approved sequencing systems are commercially available to detect HIV drug resistance mutations, including the ViroSeq system marketed by Celera Diagnostics (Alameda, CA) and the TruGene system marketed by Siemens (Deerfield, IL). High-throughput sequencing can be used to detect rare drug-resistant mutants that make up a small percentage of viral genomes.¹²² The clinical significance of small minority populations of drug-resistant mutants is still under investigation.¹¹

Resistance testing for influenza is related to the two existing classes of influenza antiviral drugs, the adamantanes and the neuroaminidase inhibitors. Resistance to the adamantanes occurs when there are mutations at positions 26, 27, 30, 31, or 34 in the gene encoding the M2 matrix protein, which is the target of the drugs.¹¹ Resistance to the neuraminidase inhibitors is related to specific mutations in the gene encoding neuraminidase, especially a histidine to tyrosine change at position 274 (N2 numbering), designated H274Y. This mutation became widespread during the 2007–2008 influenza season.^{24,50} Pyrosequencing has been effectively used to detect these mutations,²³ as have a variety of PCR-based assays.^{19,151}

The presence of resistance of HBV to antiviral agents is detected by sequencing of the DNA polymerase gene. Genotypic testing for HSV is less useful than phenotypic testing because of the diverse genetic loci within the HSV thymidine kinase and polymerase genes that can account for resistance.³⁷

SELECTED CLINICAL PROBLEMS

Tables 15.13 through 15.18 provide information related to specific areas of diagnostic virology not covered elsewhere in the chapter.

VIRUS DISCOVERY

Experimental methodologies used for viral discovery have traditionally relied on classic approaches such as electron

TABLE 15.13 Laboratory Diagnosis of Human Herpesvirus Infections

Virus	Clinical problem	Test(s)	Specimen
Herpes simplex	Mucocutaneous lesions	Culture, NAAT, FA stain	Swab of lesion
	Encephalitis or meningitis	NAAT	CSF
Varicella-zoster	Mucocutaneous lesions	Culture, NAAT, FA stain	Swab of lesion
	Encephalitis or meningitis	NAAT	CSF
	CNS vasculopathy	Intrathecal antibody synthesis, NAAT ^a	Serum or plasma plus CSF
Cytomegalovirus	Systemic infection in immunocompromised individual	Quantitative NAAT, pp65 antigenemia assay	Plasma or whole blood
	Mononucleosis syndrome in nonimmunocompromised individual	CMV IgM antibody assay, NAAT	Serum or plasma for antibody assay, plasma or whole blood for NAAT
	Tissue-invasive disease (e.g., involving the gastrointestinal or respiratory tract)	Histology, immunohistochemistry <i>in situ</i> hybridization, culture, NAAT	Tissue from involved organ
	Encephalitis	NAAT	CSF
	Retinitis	NAAT	Vitreous or aqueous fluid
Epstein-Barr virus (EBV)	Infectious mononucleosis	Heterophile antibody assay, ^b IgM antibodies to the viral capsid antigen	Serum or plasma
	Posttransplant lymphoproliferative disorder	Quantitative NAAT	Whole blood or plasma
	Primary central nervous system lymphoma	NAAT	CSF
	Encephalitis or meningitis	NAAT ^c	CSF ^c
Human herpesviruses types 6 (HHV-6) and 7 (HHV-7)	Acute infection ^d	NAAT, ^e IgM antibody assay, IgG avidity assay ¹⁴⁷	Plasma (NAAT), plasma or serum for IgM antibody assay or IgG avidity assay ¹⁴⁷
	Systemic infection in immunocompromised individual	NAAT ^e	Whole blood or plasma ^e
	Encephalitis	NAAT ^e	CSF
Human herpesvirus type 8 (HHV-8) (Kaposi sarcoma virus)	Systemic infection ^f	NAAT	Blood or body fluid

CSF, cerebrospinal fluid; FA, fluorescent antibody; Ig, immunoglobulin; NAAT, nucleic acid amplification test.

^aIntrathecal antibody synthesis has been shown to be more sensitive than NAAT for diagnosis of varicella-zoster virus central nervous system vasculopathy.⁹¹

^bSlide tests or other modified heterophile assays are usually used.

^cShould be performed only in the setting of primary EBV infection. In other clinical settings, specificity and positive predictive values are low.

^dSome but not all cases have a rash and are diagnosed as roseola.

^eApproximately 1% of the population has chromosomal integration of HHV-6 and has very high levels of HHV-6 DNA in blood and tissue, which is not indicative of disease attributable to HHV-6. Chromosomal integration in immunocompetent patients can be excluded by testing a follow-up sample (those with chromosomal integration will have persistently high levels of HHV-6 DNA) or a pretransplant sample from the transplant recipient (those with chromosomal integration will have high levels of HHV-6 DNA in the pretransplant sample).

^fSystemic HHV-8 infection may be associated with body cavity lymphoma or multicentric Castleman disease.

microscopy, cell culture, and antibody-based detection. Despite the success of these approaches, which have resulted in the discovery of many viruses throughout the years, each of these methods suffers from significant limitations. For example, electron microscopy requires high viral titers and may not provide the necessary resolution to unambiguously identify the type of virus even when viral particles are observed. Many viruses cannot be cultured, and those that can often require very specific cell types; crucially, there is no universal cell line capable of broadly supporting growth of all, or even a majority of, known viruses. Antibody-based methods are limited

in breadth, require the development of specific reagents for a given viral group, and, most critically, require selection of specific candidates based on prior knowledge in order to be useful in identifying a virus. Due to these limitations, there have been ongoing efforts to develop additional complementary strategies for detecting and discovering novel viruses. In the last decade of the 20th century, various molecular methods were developed in efforts to circumvent limitations of these classic approaches. These molecular methods, including library immunoscreening, degenerate PCR, and representational difference analysis, led to the discovery of many clinically important viruses including

TABLE 15.14 Laboratory Diagnosis of Viral Respiratory and Gastrointestinal Tract Infections

Virus	Test method	Comments
Respiratory tract infections		
Influenza A, B	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
	Culture	Less sensitive than NAAT, requires viable virus
	Antigen detection	Low sensitivity, especially for pandemic 2009 H1N1 virus
Respiratory syncytial	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
	Culture	Requires viable virus
	Rapid tests	Less sensitive than other methods
Parainfluenza	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
	Culture	Requires viable virus
Human metapneumovirus	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
Rhinovirus	NAAT	Most sensitive test; primers and probes must be selected carefully to detect all serotypes/genotypes. May be difficult or impossible to distinguish from enteroviruses
	Culture	Requires viable virus; is less sensitive than NAAT. Clade C rhinoviruses have not been successfully cultured as of time of writing
Coronaviruses (OC43, 229E, NL63, HKU1)	NAAT	Most sensitive test
Adenoviruses	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT or culture
	FA	Less sensitive than culture
Gastrointestinal tract infections		
Rotavirus	Antigen detection	Very sensitive
Norovirus	NAAT	Most sensitive test
	ELISA	Less sensitive than NAAT
Adenoviruses (group F, serotypes 40 and 41)	ELISA	Specific assay for serotypes 40 and 41
	NAAT	Most sensitive test
Astroviruses	NAAT	Most sensitive test

ELISA, enzyme-linked immunosorbent assay; FA, fluorescent antibody; NAAT, nucleic acid amplification test.

HCV, Kaposi sarcoma herpesvirus (KSHV), and others. As these methods have been the subject of other reviews,⁶⁵ they will not be discussed in detail here.

The 21st century has witnessed a revolution in the field of virus discovery as evidenced by the dramatic increase in the rate of identification of novel viruses. This increase has been driven largely by the advent of the genomic era, which has created novel sequencing technologies that in turn have geometrically increased the volume of sequencing data that can be generated. The combination of increased sequences in public databases and increased sequencing capacity has enabled the development of new, sophisticated sequence-based approaches for viral detection and discovery. Even existing methods, such as consensus PCR approaches, have benefited from the geometric growth in sequence databases. With the availability of many more viral genome sequences, improved consensus PCR primers can be designed for many viral taxa.

All of the recently developed viral discovery methods are based on detection of viral nucleic acids. Fundamentally, the major challenge in sequence-based viral discovery is identifying the relatively few copies of viral DNA or RNA in specimens that typically contain a vast excess of host genomic DNA and/

or RNA. In theory, given unlimited sequencing capacity, there would be no need to be concerned with the host; exhaustive direct sequencing of a given sample should reveal the presence of any viral sequences in the sample no matter how low the abundance of the virus. However, practical limitations on sequencing, either in terms of sheer capacity or cost, have until recently required that additional methods be employed to effectively increase the relative percentage of viral sequence to host sequence. For example, propagation of a virus in cell culture is one means of increasing the relative abundance of viral sequences. Physical purification of virions, by ultracentrifugation or nuclease treatment to eliminate nonencapsidated nucleic acids, is another means of enriching for viral sequences. Subtractive hybridization strategies achieve a similar end point by physically reducing the amount of host sequences. However, we are rapidly approaching a point where sequencing capacity and costs are no longer limiting.

One potential limitation in sequence-based virus discovery is the quantity of nucleic acids available for sequencing. To circumvent this challenge, multiple experimental approaches to increase the total abundance of nucleic acids to levels amenable to further analysis have also been developed. An essential feature

TABLE 15.15 Laboratory Diagnosis of Central Nervous System Infections

Syndrome	Virus	Method ^a
Meningitis	Enterovirus	NAAT
	Herpes simplex virus	NAAT
	Varicella-zoster virus	NAAT
	Human immunodeficiency virus	NAAT on plasma
Encephalitis in nonimmunosuppressed individuals	Herpes simplex	NAAT
	West Nile virus and other arboviruses	IgM antibody assay on serum and CSF ^b
	Varicella-zoster virus	NAAT, intrathecal antibody synthesis ^c
	Epstein-Barr virus	NAAT, IgM antibody assay on serum and plasma
	HHV-6	NAAT
	Rabies	NAAT on CSF and saliva, rabies antibody assay on CSF and serum, ^d FA stain of skin biopsy from nape of neck
	Mumps	NAAT on CSF and saliva, IgM antibody assay on serum
	Lymphocytic choriomeningitis virus	IgG and IgM antibody assays on CSF and serum (acute and convalescent samples), NAAT, culture
Encephalitis in immunosuppressed individuals	Cytomegalovirus	NAAT
	Epstein-Barr virus	NAAT
	Varicella-zoster virus	NAAT, intrathecal antibody synthesis ^c
	JC virus	NAAT

CSF, cerebrospinal fluid; HHV-6, human herpesvirus type 6; Ig, immunoglobulin; NAAT, nucleic acid amplification test.

^aIndicated test is performed on CSF unless otherwise indicated.

^bTesting should be performed on CSF and serum. Testing of CSF is more specific for current infection.

^cDetection of intrathecal antibody synthesis has been shown to be more sensitive than NAAT for the diagnosis of varicella-zoster virus central nervous system vasculopathy.⁹¹

^dCSF is preferred if the individual being evaluated has previously received rabies immunization.

TABLE 15.16 Laboratory Diagnosis of Congenital and Neonatal Viral Infections

Virus	Recommended testing
Cytomegalovirus	NAAT or culture of urine and/or saliva during first 2 weeks of life
Herpes simplex	NAAT or culture of a vesicle, CSF, blood (plasma, serum, or whole blood). Conjunctiva, nasopharynx, oropharynx, urine, and rectum/stool may also be tested
Human immunodeficiency virus (HIV)	NAAT for HIV RNA on plasma or DNA on whole blood
Rubella	IgM antibody assay of serum or plasma, NAAT or culture of urine, oropharynx, blood, stool, CSF. Amniotic fluid and products of conception can also be tested
Parvovirus B19	IgM antibody assay of serum or plasma, NAAT of plasma or serum
Hepatitis B	Hepatitis B surface antigen test on serum or plasma
Varicella-zoster virus	NAAT, culture, or FA stain on vesicle, CSF, or blood. NAAT on amniotic fluid
Enteroviruses	NAAT on plasma or serum, CSF, nasopharyngeal or oropharyngeal swab, stool, urine
Hepatitis C	NAAT on plasma or serum on two occasions between 2 and 6 months of age or HCV antibody assay at age ≥15 months of age

CSF, cerebrospinal fluid; FA, fluorescent antibody; Ig, immunoglobulin; NAAT, nucleic acid amplification assay.

TABLE 15.17 Laboratory Diagnosis of Human Immunodeficiency and Other Retrovirus Infections

Indication	Test
Routine diagnosis	ELISA for HIV antibodies ^a
Confirmation of a positive ELISA	Samples that are reactive on the screening HIV antibody or combined HIV antigen–antibody assay should be tested with a confirmatory antibody test that differentiates HIV-1 and HIV-2 antibodies. ^b Patients who are positive with this assay should have a second sample tested for HIV RNA. Rare patients who are reactive on the screening test and the confirmatory HIV-1/HIV-2 antibody assay but negative for HIV RNA should have additional testing performed under the direction of an HIV specialist. This testing might include a repeat of the screening assay, an HIV western blot, or an HIV DNA PCR assay. Patients who are negative with the confirmatory HIV-1/HIV-2 antibody assay should have an HIV RNA assay performed. Patients who are reactive on the screening assay, negative on the confirmatory HIV-1/HIV-2 antibody assay, and positive for HIV RNA are presumed to have recent infection and should be referred immediately to an HIV specialist. ⁸
Blood donor screening	ELISA for antibodies to HIV-1 and -2, HIV RNA assay
Infant born to infected mother	HIV RNA or DNA assay at 14–21 days of age, 1–2 months, and 4–6 months ^c
Acute antiretroviral syndrome	HIV RNA assay
Prognosis	Quantitative HIV RNA assay
Response to therapy	Quantitative HIV RNA assay
Resistance to antiretroviral drugs	Genotypic assay based on nucleotide sequencing of the relevant genes, or phenotypic assay based on inserting relevant genes from the patient's HIV strain into a laboratory vector
Diagnosis of HIV-2 infection	ELISA for HIV-2 antibodies ^d
Monitoring of HIV-2 infection	HIV-2 RNA assay ^e
Diagnosis of HTLV-1 or -2 infection	HTLV-1 and 2 antibody ELISA assays
Confirmation of HTLV-1 or -2 infection	HTLV-1 and -2 western blot, line immunoassay or immunofluorescent antibody assay ^e

ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; HTLV, human T-cell lymphoma or leukemia virus; PCR, polymerase chain reaction.

^aMost FDA licensed tests detect antibodies to both HIV-1 and -2. Third-generation antibody assays are preferred because they detect immunoglobulin M (IgM) as well as IgG antibodies, and thus become positive earlier after infection than assays that detect only IgG antibodies. An alternative approach is to use a fourth-generation test that measures HIV p24 antigen in addition to HIV antibodies.

^bA licensed rapid HIV antibody test approved by the Food and Drug Administration (FDA) is now available that differentiates HIV-1 and HIV-2 antibodies.

^cDNA PCR may be preferred while the infant is on prophylactic antiretroviral treatment because of the possibility that antiretroviral therapy may make HIV RNA undetectable.

^dMost HIV antibody assays currently in use detect antibodies to both HIV-1 and HIV-2, but only one FDA-licensed test differentiates between HIV-1 and HIV-2 antibodies.

^eAvailable only in selected reference laboratories.

TABLE 15.18 Laboratory Diagnosis of Tropical and Other Geographically Localized Viral Infections

Virus or syndrome	Location	Preferred tests
Sin nombre virus	Western United States	Virus-specific IgM antibody assay, NAAT
Other hantaviruses	Asia, Europe	Virus-specific IgM antibody assay, NAAT
Colorado tick fever	Western United States	Virus-specific IgM antibody assay, NAAT
Dengue fever	Asia, Central and South America	Virus-specific IgM antibody assay, NAAT
Filoviruses (Ebola and Marburg viruses)	Central Africa	Virus-specific IgM antibody assay, NAAT
Arenaviruses (e.g., Lassa, Junin, many others)	West Africa (Lassa fever), South American (Junin)	Virus-specific IgM antibody assay, NAAT, culture
Hendra and Nipah viruses	Australia, Malaysia, Bangladesh, India	Virus-specific IgM antibody assay, NAAT
Yellow fever	Central Africa, Amazon region	Virus-specific IgM antibody assay, NAAT
Crimean-Congo hemorrhagic fever and other bunyavirus infections	Asia, Africa, Europe	Virus-specific IgM antibody assay, antigen-capture ELISA, culture, NAAT
Rift Valley fever	East Africa	Virus-specific IgM antibody assay, antigen-capture ELISA, culture, NAAT, culture
Chikungunya and other alphavirus infections (O'nyong nyong, sindbis, Mayaro, Ross River)	Africa, India, Central and South America, Europe, Australia (Ross River)	Virus-specific IgM antibody assay, culture, NAAT

ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M; NAAT, nucleic acid amplification test.

of all of these amplification strategies is that no prior knowledge regarding the nature of the sequences to be amplified is required because it is unknown what type of virus may be present.

For sequence-based detection technologies, a key step is the computational analysis required to identify a sequence as viral in nature. In early efforts, only a few sequences were generated and thus analysis could easily be done via commonly used web-interface software. With advances in sequencing capacity, the number of sequences generated from a sample increased initially to hundreds or thousands and now can easily surpass a million sequences or more. In order to efficiently analyze this volume of sequence data, automated high-throughput bioinformatics pipelines had to be developed. Although many variations on this theme exist, most computational pipelines utilize very similar overall strategies. Typically, sequence alignment software, such as BLAST, is used to compare the sequences generated from a sample to established public databases. Sequences with perfect or near-perfect alignments to known sequences (host, viral, bacterial, etc.) can then be categorized based on the identity of the reference sequence to which a given read aligned. Sequences derived from novel viruses are those that have only limited similarity to known viruses in the available databases. For more definitive characterization of the relatedness of these sequences to known viruses, phylogenetic methods are subsequently used.

As mentioned previously, a key driving factor in the advances in virus discovery has been the evolution of sequencing technology. For over 30 years, from its inception in the late 1970s until the late 2000s, Sanger dideoxy sequencing was the dominant sequencing technology in use, and all efforts to identify viruses using sequencing-based strategies relied on this technology. At its highest throughput, automated 96-capillary Sanger sequencing could generate up to approximately 100,000 bp of sequence in a single run. Starting in 2005, the first of the so-called Next Generation (NextGen) sequencing platforms was introduced.⁸⁷ These platforms dramatically increased the quantity of sequence that could be produced. For example, the Roche/454 platform in its initial format was capable of generating 100,000 reads of 100 bp length in a single run, or approximately 100-fold greater sequence than a run on the previous instrument standard, the ABI 3730. Over the next few years, this platform rapidly evolved such that by 2009, the Roche/454 platform could readily generate 1,000,000 reads that averaged 400 bp, or 400 megabases (MB) of sequence. Two other major NextGen sequencing platforms, Solexa (Illumina) and SOLiD (ABI), also emerged during this time (reviewed in reference 86). Both of these platforms generate more reads than Roche/454, but of shorter length (up to 125 bp for Illumina, up to 75 for SOLiD). At the current time, these platforms are capable of generating on the order of 100 gigabases (GB) of sequence in a single run. It is the unprecedented growth in sequencing capacity offered by these new platforms that has transformed the process of virus discovery.

Novel Virus Discovery Strategies (2000–2010)

Random Arbitrarily Primed PCR

The 2001 discovery of human metapneumovirus relied on differential display of amplicons generated by a sequence-independent PCR amplification strategy.¹³⁹ An unknown virus was passaged in tertiary monkey kidney cells from respiratory

secretions of multiple children with respiratory tract infections. From nucleic acids purified from one such culture, a defined set of random, arbitrary primers was hybridized under low stringency to amplify nucleic acids from the culture and an uninfected control. Comparison of the resulting gel electrophoresis patterns yielded unique bands present only in the infected sample. These bands were selectively purified from the gel, sequenced, and found to share only limited sequence identity to avian pneumoviruses. Cultured virus was used to infect cynomolgus macaques, several of which developed mild respiratory symptoms and replicated virus as measured by RT-PCR, thus fulfilling Koch's postulates. Serologic analysis demonstrated that the virus is commonly acquired in childhood, with the majority of adults being seropositive. The human metapneumovirus is now established as one of the leading causes of viral respiratory infection.

DNase-SISPA

In 2001, Allander et al.² described DNase-SISPA (sequence-independent single primer amplification), a key virus discovery methodology that would be used to identify numerous novel viruses in the following years. Importantly, specific enrichment for capsid-protected viral nucleic acids in the specimen was carried out prior to nucleic acid extraction, using a combination of ultracentrifugation to pellet virions followed by treatment with DNase to degrade any free nucleic acids, presumably derived primarily from the host. RNA and DNA were independently extracted and analyzed in parallel. RNA was converted to double-stranded cDNA and then treated exactly as the extracted DNA. DNA was digested by restriction enzymes, a common linker was ligated to both ends of the double-stranded DNA fragments, and then the DNA was PCR amplified using a single common linker primer. Amplification products were visualized by gel electrophoresis, and individual bands were recovered from the gel, cloned, and sequenced using standard Sanger sequencing technology. In this proof of principle study, two novel bovine parvoviruses were identified in commercial bovine serum.

Random PCR Amplification-DNA Microarray Hybridization or "Virus Chip"

With the advent of DNA microarrays in the early 1990s, it rapidly became clear that the inherently parallel nature of microarrays could be utilized for massively parallel viral detection. Conceptually, the presence of a virus in a sample could be deduced from the pattern of hybridization to virus-derived oligonucleotide sequences on the microarray or so-called virus chip. The spectrum of viruses that could be detected in a single microarray hybridization was limited in theory only by the availability of viral sequences that could be included in a given microarray. The first broad-range viral detection microarray, which contained ~1,600 oligonucleotides capable of detecting ~140 viruses from all virus families with members known to cause respiratory disease, was described in 2002.¹⁴⁵ Critically, the most highly conserved 70-mer regions from these viruses were incorporated into the microarray to maximize the probability of detecting not only known viruses but also unsequenced or novel viruses by cross-hybridization to these highly conserved elements. The resulting pan-viral microarray is shown in Figure 15.12. The ability of the microarray

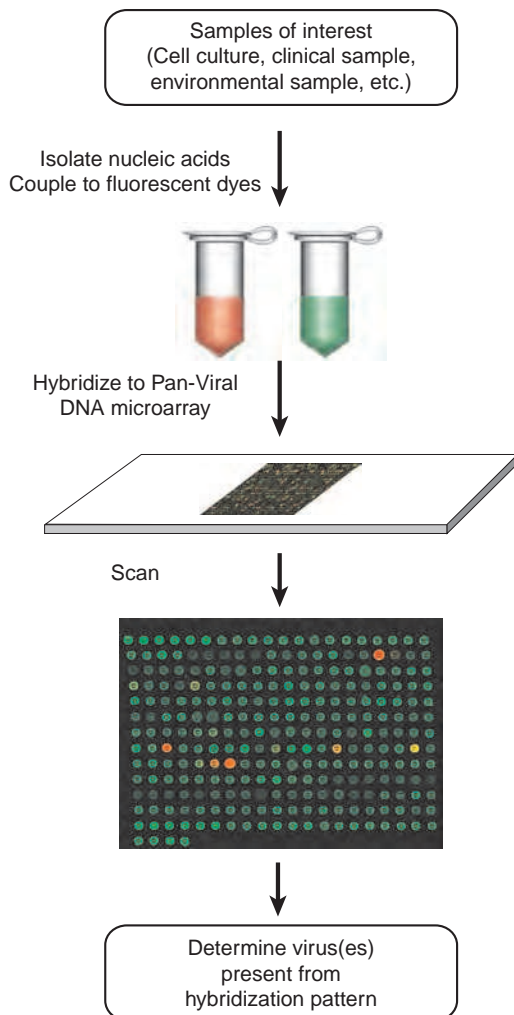


FIGURE 15.12. Pan-viral microarray used for virus discovery. (Wang D, Coscoy L, Zylberberg M, et al. Microarray-based detection and genotyping of viral pathogens. *Proc Natl Acad Sci U S A* 2002;99:15687–15692.)

to detect known viruses was validated using a series of known respiratory viruses grown in cell culture. To demonstrate the feasibility of detecting unknown viruses, multiple unsequenced strains of rhinoviruses grown in culture were hybridized to the microarray. Based on hybridization to many rhinovirus-derived sequences, these viruses could clearly be classified as rhinoviruses. Furthermore, to demonstrate that the method could be applied to human clinical samples and not just to cultured viruses, random PCR amplification of nucleic acids extracted directly from human respiratory specimens was employed. In this strategy, a single primer composed of a fixed linker region at the 5' end and five or more totally degenerate positions at the 3' end is the critical reagent.^{7,34} The degenerate 3' end can theoretically hybridize at any position on any DNA template, and when two primers hybridize to complementary strands in close proximity, the subsequent thermocycling generates an amplicon. In this fashion, all nucleic acids in a sample can be amplified at random. Microarray hybridization of randomly amplified nucleic acids extracted from the clinical specimens enabled detection of diverse known human respiratory viruses.

The first application of this approach to identify a novel virus occurred during the SARS outbreak of 2003.^{72,146} A second-generation pan-viral DNA microarray that contained ~11,000 70-mers derived from every fully sequenced viral genome in GenBank at the time was used to analyze an unknown virus cultured in Vero cells from the respiratory swab of a SARS patient. Random PCR followed by hybridization to the microarray yielded a hybridization pattern consistent with the presence of a divergent member of the family *Coronaviridae*; only a limited number of coronavirus-derived probes had high signal intensity. In order to rapidly sequence portions of the viral genome, fragments of the unknown virus that were bound to various 70-mers on the microarray were physically recovered, cloned, and sequenced.¹⁴⁶ These sequences shared only 33% identity to murine hepatitis virus, a murine coronavirus, definitively demonstrating that SARS was highly divergent from all previously described viruses.

VIDISCA

The discovery of human coronavirus NL63 in 2004 relied on VIDISCA (*Virus Discovery cDNA-AFLP* [amplified fragment length polymorphism]), a variant of the DNase-SISPA strategy¹⁴⁰ in combination with classic viral culture. From a 7-month-old child with bronchiolitis, cytopathic effect was detected following culture in tertiary monkey kidney cells. Material from those cultures was further passaged into LLC-MK2 cells. To identify the virus, supernatants from infected and uninfected cells were generated and subjected to VIDISCA. In this protocol, the initial steps were essentially identical to DNase-SISPA: ultracentrifugation, DNase treatment, nucleic acid purification, restriction endonuclease digestion, linker ligation, and PCR amplification. At this stage, a second set of PCR reactions was performed using all pairwise combinations of four PCR primers; each primer used contained one additional nucleotide (A, T, G, or C) appended to the 3' end of the linker primer used in the first round of PCR. Thus, 16 PCR reactions were performed with the goal of identifying specific bands present only in the infected cell culture and absent from the uninfected control. This process reduces the complexity of the banding pattern during gel electrophoresis compared to standard DNase-SISPA as each reaction should contain only one-sixteenth of the amplicons. In this instance, 16 bands unique to the infected sample were cloned, and 13 were determined to have only limited sequence similarity to known coronaviruses. Analysis of the complete genome of coronavirus NL63 determined that it shares 65% nucleotide identity to its closest relative, coronavirus 229E. Follow-up epidemiologic studies have defined an association of coronavirus-NL63 with croup.¹⁴¹

Rolling Circle Amplification/Restriction Digest/Gel Electrophoresis

The methods described previously have all utilized sequence-independent PCR amplification. An alternative non-PCR-based, sequence-independent amplification strategy relies on isothermal amplification using phi29 polymerase with random primers. This amplification strategy, also referred to variously as *rolling circle amplification*, *multiple displacement amplification*, and *whole genome amplification*, takes advantage of the highly processive nature of phi29 polymerase (greater than 70,000 average nucleotide extension) and its strand displacement

activity, which can lead to branching and thereby exponential amplification. When incubated with phi29 polymerase and random primers, small circular DNA viruses can be amplified by a factor of 10^4 . The products of this amplification, long double-stranded linear concatemers of viral genome monomers, can then be digested by restriction enzymes and visualized by gel electrophoresis. Bands can then be cloned and sequenced. In demonstrating proof of principle, Rector et al.¹¹² described the detection of a bovine papillomavirus from tissue. Subsequently, this approach would be used to identify the novel human polyomavirus 6.¹²⁰

Random PCR-Sanger High-Throughput Sequencing

The discovery of human bocavirus was the first example where PCR products from randomly amplified clinical material were sequenced en masse³; prior examples of sequencing-based discovery described previously relied on gel purification of specific individual target bands. In this study, respiratory secretions from multiple patients with unexplained respiratory illness were pooled, ultracentrifuged to concentrate viral particles, and DNase-treated to enrich for capsid-protected sequences. Following nucleic acid extraction, random PCR amplification, and size selection of 600- to 1,500-bp amplicons for cloning, 384 clones were sequenced using standard Sanger technology. Sequences with limited amino acid similarity to known parvoviruses were detected. Phylogenetic analysis demonstrated that this novel genome was a previously uncharacterized species of the genus *Bocavirus*. This experimental strategy would be used in many subsequent instances to identify novel viruses. With the evolution of NextGen sequencing platforms, many laboratories subsequently adapted their protocols to utilize NextGen sequencing technologies in place of the traditional Sanger sequencing. Multiple examples of viruses discovered by random PCR-NextGen sequencing will be described in the following sections.

NextGen High-Throughput Sequencing of a cDNA Library

The identification of Merkel cell polyomavirus in Merkel carcinomas was the first instance of the use of a NextGen sequencing platform (Roche/454 FLX platform) to discover a novel virus.²⁹ The experimental strategy, termed *digital transcriptome subtraction*, entailed sequencing of mRNA isolated from tumor tissue and subsequent computational subtraction of host sequences followed by alignment of the remaining sequences. Proof of concept for this type of strategy was first demonstrated in 2002, wherein an expressed sequence tag (EST) library derived from HeLa cells was subjected to computational subtraction of human sequences.¹⁴⁸ Among the remaining ESTs that did not match to any human sequences were human papillomavirus-18 sequences, which were known to be present in HeLa cells. In the Merkel carcinoma study, a total of four tumor samples were pooled and ~382,000 high-quality sequence reads were generated; from the data, one sequence possessed detectable sequence similarity to the T antigen of known polyomaviruses. The complete genome sequence of the novel virus, designated Merkel polyomavirus, was obtained, and phylogenetic analysis demonstrated that it was a highly divergent polyomavirus. Southern blotting determined that Merkel polyomavirus DNA was clonally integrated in a number of Merkel tumors. Multiple subsequent prevalence studies have determined that ~80%

of Merkel carcinoma tissues contain Merkel polyomavirus. This landmark study demonstrated the increased sensitivity achievable by deep sequencing with NextGen platforms as compared to previous efforts, wherein a single sequence of a novel virus (out of >380,000 total sequences sampled) could be detected. Moreover, no steps were taken to enrich the ratio of virus to host.

NextGen High-Throughput Sequencing of Small RNAs

Two studies demonstrated the feasibility of identifying novel viruses by sequencing and assembly of small RNAs.^{71,153} Following the discovery of RNA interference (RNAi), it became clear that RNAi has an antiviral function in plants and lower eukaryotic organisms; as a consequence, viral RNA sequences are cleaved to small RNAs ranging in size from ~21 to 25 nt. The presence of these small RNAs could readily be detected by gel-purifying small RNAs, using NextGen platforms to sequence the small RNAs. From these sequences, the application of assembly algorithms to reconstitute larger RNA fragments yielded contigs that could be clearly recognized as viral in nature. In plant experiments designed to analyze the effect of known sweet potato viruses on RNAi, previously undescribed badnaviruses and mastreviruses were detected by assembly of small RNAs.⁷¹ Similarly, analysis of small RNA sequencing data from *Drosophila* cell lines demonstrated that multiple novel viruses, including members of *Nodaviridae*, *Titiviridae*, *Birnaviridae*, and *Tetraviridae*, could be identified in this fashion.¹⁵³

Summary of Pathogen Discovery Methods

The methods described for virus discovery in the preceding paragraphs share a common end goal, albeit with multiple variations, namely, to identify nucleic acid sequences derived from viruses present in a sample of interest. The overall process can be dissected into distinct component modules, which in general can be mixed and matched, as illustrated in Figure 15.13. For a given specimen, different methods of processing the initial sample can be applied, different types of nucleic acid can be isolated, different sequence-independent amplification strategies can be used, and, ultimately, a range of technologies can be used as the readout. New combinations of these components continue to evolve.

Representative Novel Human Viruses Discovered (2000–2010)

Parvovirus 4

Screening of human plasma from patients with febrile illness by DNase-SISPA led to the discovery of sequences with only limited identity to known members of the family *Parvoviridae* in 2005.⁵⁸ The genome of a novel parvovirus, parvovirus 4, was sequenced. It has been detected to date in blood, bone marrow, and lymphoid tissue from patients with either HCV or HIV/acquired immunodeficiency syndrome (AIDS), in plasma of kidney transplant patients, and in CSF of encephalitis patients. The virus is believed to be parenterally transmitted based on the observation of high detection rates in hemophiliacs and injection drug users, although additional routes of transmission may also exist.

Anelloviruses

TT virus, the prototype anellovirus, was discovered in 1997 using representational difference analysis on human serum⁹⁵

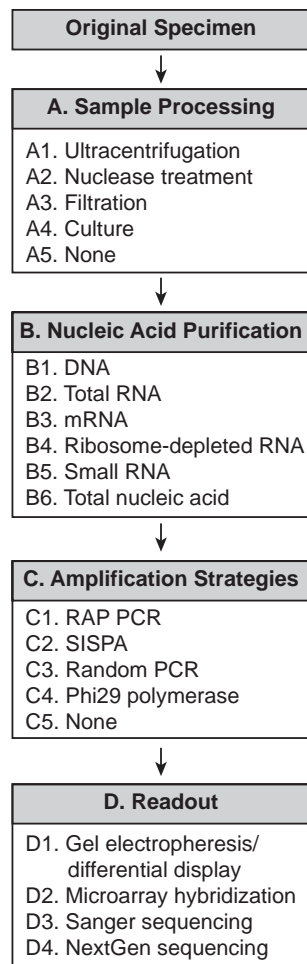


FIGURE 15.13. Pathogen discovery methods. Recently developed sequence-based pathogen discovery methods all fundamentally follow a similar overall scheme. A clinical specimen of interest is collected. **A:** If desired, samples can be manipulated to increase the ratio of virus to host by various methods. **B:** Nucleic acid species of interest is isolated. **C:** In cases where the quantity of purified nucleic acid is limiting, random sequencing-independent amplification is applied. **D:** A readout method is selected. For example, the identification of human bocavirus used the sequence of steps A1-A2-B1-C3-D3. The identification of Merkel polyomavirus used the sequence A5-B3-C5-D4.

from a transfusion-associated hepatitis patient. Since its discovery, TT virus and TT-related viruses have been found at high frequencies in a variety of human specimens, including respiratory secretions, stool, and serum. There is currently no known disease association for any of the anelloviruses. Two studies in 2005 described the presence of novel anelloviruses in human blood.^{9,58} In one study using DNase-SISPA to analyze serum from patients with febrile illness, two novel anelloviruses, SA1 and SA2, were identified that shared only 32% to 35% similarity to TT virus.⁵⁸ In the other study, plasma from healthy donors was enriched for virions by centrifugation through a cesium chloride (CsCl) step gradient. The fractions expected to contain known DNA viruses (1.2 to 1.5 g/mL density) were collected, DNase treated, and then extracted. Two amplification strategies were then used: (a) shearing followed by linker

ligation and sequence-independent PCR (as in DNase-SISPA) and (b) sequence-independent amplification using phi29 polymerase. In both instances, shotgun libraries were constructed and then sequenced. In this study, seven sequences with limited similarity to anelloviruses were detected. Since these studies, novel anelloviruses have been detected in numerous studies.^{30,143}

Novel Bocaviruses

Following the discovery of human bocavirus in respiratory secretions, a second novel bocavirus, named human bocavirus 2, was discovered by two independent groups using similar Sanger sequencing-based screening of stool samples of patients with acute flaccid paralysis⁶² and acute gastroenteritis.⁵ In the latter study, human bocavirus 2 prevalence of 17% in diarrhea cases was described, as was a statistically significant association between infection with human bocavirus 2 and acute gastroenteritis; however, in another study, no association was detected.⁶³ During efforts to describe the prevalence of human bocavirus and bocavirus 2 in human stools using consensus PCR, two additional human bocaviruses, human bocavirus 3⁵ and human bocavirus 4,⁶³ were discovered.

KI Polyomavirus

Using Sanger sequencing of pooled respiratory secretions physically enriched for virions, KI polyomavirus (KIV), a novel member of the family *Polyomaviridae*, was discovered in 2007.⁴ From the sequences generated, one 363-bp read possessed limited sequence similarity to the simian virus 40 (SV40) VP1 protein. From this one fragment, the complete 5,040-bp circular genome was sequenced. KIV has been detected in respiratory secretions in populations around the world as well as in feces. Seroepidemiologic studies have determined that a high percentage of the general population has been previously infected by KIV.^{13,64,94}

WU Polyomavirus

Nearly simultaneously with the discovery of KIV, WU polyomavirus (WUV) was discovered by a similar strategy of high-throughput Sanger sequencing analysis of individual respiratory secretions.³⁸ From 384 clones that were sequenced from the nasopharyngeal aspirate of a child with pneumonia, six sequence reads that shared 35% to 50% amino acid identity to JC virus and SV40 were detected. The complete genome of 5,229 bp was sequenced, and based on phylogenetic analysis, WUV was found to be most closely related to KIV. To date, WUV has been detected primarily in respiratory secretions, but it has also been detected in a few instances in feces, blood (whole blood, plasma, and serum), CSF, and lymphoid tissue. As with KIV, infection by WUV appears to occur primarily in childhood, with ~90% of adult populations harboring antibodies to WUV.^{13,64,94}

Human Polyomaviruses 6 and 7

During efforts to clone genomes of Merkel polyomavirus using rolling circle amplification of DNA collected from skin swab samples, a 1.6-kb BamHI fragment of a novel polyomavirus was detected. Subsequently, the complete genome of a novel virus designated human polyomavirus 6 was sequenced.¹²⁰ Based on this sequence, screening of skin swabs using degenerate PCR primers designed from human polyomavirus 6 and WU polyomavirus

yielded an additional virus, human polyomavirus 7. Serologic analysis of 95 blood donors using recombinant VP1 capsid protein demonstrated 69% and 35% seropositivity for human polyomavirus 6 and human polyomavirus 7, respectively.¹²⁰

TSV Polyomavirus

Trichodysplasia spinulosa (TSV) is a rare skin disease that occurs exclusively in immunocompromised patients. It is characterized by the development of spicules and follicular papules primarily on the face. Multiple case reports included electron micrographs demonstrating the presence of clusters of ~40-nm viral particles consistent with viruses in the polyomavirus or papillomavirus families. In 2010, DNA from the spicules collected from a heart transplant recipient suffering symptoms of TSV was subjected to rolling circle amplification (RCA). Restriction analysis of the RCA product yielded specific bands, which upon cloning and sequencing yielded a novel polyomavirus genome of 5,232 bp.¹⁴² Quantitative PCR demonstrated that the virus was present at 2×10^5 copies per cell. Prevalence studies performed on plucked eyebrows from 69 immunocompromised renal transplant patients yielded 3 (4%) that were TSV positive. TSV polyomavirus has subsequently been detected in two urine samples from two additional patients.¹²¹

Human Polyomavirus 9

Human polyomavirus 9 was discovered using a degenerate PCR amplification strategy from the serum of a kidney transplant patient.¹²¹ Sequencing of the complete genome demonstrated that human polyomavirus 9 was most closely related to the African green monkey lymphotropic virus. PCR-based screening of a cohort primarily composed of immunocompromised individuals yielded human polyomavirus 9 in three additional blood samples and one urine sample.

Asfarivirus-Like Virus

Random PCR-Roche/454 high-throughput sequencing was applied to human serum samples collected from patients suffering acute febrile illness and healthy controls in the Middle East. Multiple sequence fragments were detected in a total of four distinct serum samples that shared limited sequence identity (36% to 64% amino acid identity) to African swine fever virus proteins.⁸¹ African swine fever virus is the only known member of the family *Asfarviridae*, and the divergence between the detected sequences and known African swine fever viruses clearly demonstrate that the sequences represent one or more novel members of this family. No human infection with African swine fever virus has been reported. In addition, sequencing of raw sewage collected in Spain also yielded multiple sequence fragments that were most closely related to African swine fever virus. In total, 36 unique fragments were identified, including multiple fragments that shared sequence identity to genes specifically found only in African swine fever virus.

Novel Astroviruses

Astrovirus MLB1 was identified in the first metagenomic analysis of stools from patients with diarrhea.³⁰ Following filtration of diluted stool supernatant, RNA was extracted and randomly amplified. The amplicons were cloned and 384 clones were sequenced using standard Sanger sequencing. From the stool sample of a 3-year-old child from Australia with acute diarrhea, seven sequences with less than 68% amino acid identity to

known astroviruses were detected. The complete genome was sequenced, and phylogenetic analysis demonstrated it was a highly divergent astrovirus. The virus has been detected in stool samples of patients with and without diarrhea from the United States, Mexico, Hong Kong, India, and Nigeria.

Another novel astrovirus, astrovirus VA1, was discovered in stool samples collected from an unexplained outbreak of gastroenteritis in the United States by random PCR high-throughput sequencing. Parallel sequencing efforts using Sanger technology and the Roche/454 platform were used, in combination with conventional rapid amplification of cDNA ends (RACE) protocols, to sequence the entire genome.³² The robustness of NextGen platforms such as Roche/454 was demonstrated by the fact that the majority of the genome (a contig of 6,581 nt out of the complete genome of 6,586 nt) could be assembled from the initial Roche/454 data. Astrovirus VA1 shared only 61% amino acid identity to its closest relative, mink astrovirus. Real-time RT-PCR screening for astrovirus VA1 demonstrated that astrovirus VA1 was present at high copy number in three out of the six available stool samples from the outbreak. These results raise the possibility that astrovirus VA1 may play a role in causing diarrhea. Viruses very closely related to astrovirus VA1 were subsequently detected in three stool samples from children in Nepal⁶¹ and a patient in the Netherlands with celiac disease.¹²⁴ Furthermore, Roche/454 sequencing of encephalitic brain tissue from a child suffering from X-linked agammaglobulinemia yielded a virus closely related to astrovirus VA1.¹⁰⁹ Additional consensus astrovirus RT-PCR screening of stool samples led to the discovery of three additional astroviruses in human stool specimens in two independent studies: astrovirus MLB2, astrovirus VA2/HMO-A, and astrovirus VA3/HMO-B.^{31,61}

Saffold Cardiovirus

Saffold virus, a novel member of the *Cardiovirus* genus, was discovered by DNase-SISPA analysis of an unknown virus cultured from a patient stool sample.⁵⁹ Saffold-like viruses were subsequently detected directly in a sample of human respiratory secretions using a pan-viral microarray approach¹⁶ as well as in human stool from patients with gastroenteritis by PCR screening.^{16,26} Serologic studies using neutralization assays have yielded seropositivity rates of 80% to 90% by adulthood, demonstrating that Saffold infection is a common occurrence.^{17,155}

Rhinovirus C

By a consensus PCR strategy, a novel clade of rhinoviruses was identified in patients with influenza-like illness.⁷⁴ The many known serotypes of rhinoviruses have traditionally been classified into two clades, rhinovirus A and rhinovirus B. With the discovery in numerous studies of a novel set of related rhinoviruses, a new clade, designated rhinovirus C, has been established.⁶⁶⁻⁷⁶ These viruses appear to be globally widespread and may contribute to 50% or more of rhinovirus infections. No member of the rhinovirus C clade has been successfully cultivated in the cell lines traditionally used for detection of rhinoviruses.

Cosavirus

Random PCR followed by Sanger sequencing led to the discovery of a novel picornavirus, cosavirus A1, from a stool sample of a child with nonpolio acute flaccid paralysis from Pakistan.⁶⁰ Phylogenetic analysis of the complete genome of 7,634 bp determined that it is most closely related to Seneca Valley virus

in the genus *Cardiovirus*. In this study, PCR screening identified an additional 34 cosavirus-positive samples in cases and in healthy controls that, based on phylogenetic analysis, were further classified into four distinct genetic groups (A through D). An independent study also using random PCR and Sanger sequencing led to the identification of a proposed genetic group E of cosavirus.⁵⁴

Klassevirus/Salivirus

The identification of the novel picornavirus human klassevirus,^{55,144} also known as salivirus,⁷⁹ by three independent groups in 2009 relied primarily on Roche/454 sequencing. This virus is most similar to members of the genus *Kobuvirus* and has been detected both in human stool specimens from the United States,^{44,55,79} Australia,⁵⁵ Nigeria, Tunisia, and Nepal⁷⁹ and in raw sewage from Spain.⁵⁵ These results demonstrate that klassevirus/salivirus is globally widespread. In one study, 2 of 751 stool samples were positive for klassevirus, with the two positive patients twin siblings.⁴⁴ One case-control study in a Nepalese cohort demonstrated a positive association with gastroenteritis ($p = 0.0056$).⁷⁹

Coronavirus HKU1

Degenerate PCR screening of respiratory secretions resulted in the discovery of human coronavirus HKU1.¹⁵² This virus has been detected in numerous studies from respiratory samples from patients around the world.

Lymphocytic Choriomeningitis Virus–Like Arenavirus

In one of the earliest applications of Roche/454 high-throughput sequencing to viral detection, an arenavirus was identified in multiple recipients of organs from a common donor.⁹⁹ In this case cluster, three organ recipients from the same donor died 4 to 6 weeks after transplantation. RNA was extracted from the CSF, serum, brain, kidney, and liver from two patients, pooled, and sequenced. From a total of 94,043 high-quality sequence reads, 14 had detectable sequence similarity to lymphocytic choriomeningitis virus (LCMV). Following PCR to sequence the complete genome of the virus, RT-PCR testing of multiple specimens from each of the three individuals revealed identical viral sequences in all three patients. Virus was isolated by culture in Vero E6 cells. Using antibodies against LCMV, viral antigen was detected in liver and kidney sections, and seroconversion was observed in one patient.

Lujo Virus

In 2008, a cluster of five cases of undiagnosed hemorrhagic fever was reported in South Africa with four fatalities. Random PCR-Roche/454 high-throughput sequencing was performed on RNA extracted from two postmortem liver biopsies and one serum sample. Analysis of the resulting ~300,000 sequences yielded nine fragments with limited sequence similarity to known arenaviruses.¹⁰ Upon sequencing of the complete genome, phylogenetic analysis demonstrated that the virus, named Lujo virus, was highly divergent from known arenaviruses. Specimens collected from all five patients were positive for Lujo virus by virus isolation using cell culture and by RT-PCR.¹⁰³

Xenotropic Murine Leukemia Virus–Related Virus

In 2006, a novel retrovirus closely related to xenotropic murine leukemia viruses, named xenotropic murine leukemia virus–

related virus (XMRV), was first identified by random PCR pan-viral DNA microarray analysis.¹³⁷ The samples analyzed were prostate tumor specimens, and presence of the virus was associated with a specific polymorphism of the *RNaseL* gene, a key component of the interferon-induced antiviral defense system. In further studies of prostate cancer specimens, XMRV was also detected in one instance¹¹⁹ but not in another.¹³¹ Subsequently, one study suggested an association between chronic fatigue syndrome and XMRV.⁸² This finding, however, has been contradicted by numerous studies that have failed to detect XMRV in chronic fatigue patients, including a large multicenter blinded study¹²³ and a study that retested patients previously identified to have the virus in the original study.⁶⁷ Furthermore, multiple studies have demonstrated that reagents often used in RT-PCR and other manipulations can be contaminated with XMRV-like sequences, raising the possibility that amplification artifacts and contamination contribute to some of the observations.¹³¹ Finally, one study demonstrated that XMRV likely arose via recombination between two murine retroviruses in the course of prostate tumor xenograft experiments, suggesting that this virus is an artifact of laboratory experimentation.¹⁰¹ These studies illustrate some of the potential challenges of defining the biological relevance of newly identified agents using primarily molecular approaches.

Prospects and Challenges for the Future

Advances in molecular methods have dramatically increased the rate at which viral genomes can be detected, resulting in our awareness of the existence of many more viral genomes than just a few years ago. For example, following the initial discoveries of BK and JC viruses by culture in 1971, there were no additional polyomaviruses of humans identified until 2007. Since then, a total of seven new polyomaviruses have been identified in humans, all by molecular methods. Parallel situations exist in many viral taxa, wherein geometric increases in the number of known members have been observed. With the continued growth in sequencing capacity, there is little doubt that many more viruses will be identified in the upcoming years. Recently, specific efforts to comprehensively catalog the spectrum of viruses present in humans have begun. One objective of the international Human Microbiome Project is to define the set of viruses present in different anatomic sites in a set of “healthy” adults. These and other similar efforts to define the “viromes” of humans, animals, plants, or environmental niches will undoubtedly uncover a tremendous diversity of new viral genomes. While our ability to identify viral nucleic acids and genomes has grown at an astounding rate, our ability to define the molecular properties and the biological relevance of these new agents has unfortunately lagged behind. In order to effectively study these new viruses, commensurate new developments in traditional virology must occur. For example, novel methods of virus culture must be developed in order to keep pace with the rate of discovery of new viral sequences, as the ability to propagate a virus in culture is, in many instances, the rate-limiting step in our ability to further characterize a virus and to define its pathogenicity. Many of the viruses being discovered today by sequencing-based molecular methods are unlikely to grow in the traditional cell lines utilized by virologists. As an example, the advent of differentiated primary respiratory epithelial cultures has opened new frontiers by enabling the culture of several newly identified respiratory viruses, such

as human bocavirus²⁵ and coronavirus HKU,¹⁰⁸ that have failed to grow previously in traditional respiratory tract–derived cell lines. Another area of great importance is the development of robust animal models for these new agents, which will be a critical part of efforts to define pathogenicity and disease causality. Finally, the criteria used for defining “pathogenicity” must also be revisited. While Koch’s postulates, the 100-plus-year-old gold standard for proof of disease causation, have served us well in the past, this era of molecular virus discovery necessitates an enhanced emphasis on the role of epidemiologic and seroepidemiologic approaches in defining disease associations.³³ Furthermore, more nuanced conceptions of microbially induced disease, such as disease as a consequence of imbalance or perturbation of host microbial populations, must also be considered. In summary, technology-driven advances in sequencing have led to a golden era of discovery of new viruses in the first decade of the 21st century. Hopefully, parallel advances in understanding the biology of these new agents will come to fruition in the next decades of the 21st century.

PERSPECTIVE

Diagnostic virology is at a critical point in its history. The field has matured, with detection of a selected group of viral pathogens emerging as an important component of clinical laboratory services at major medical centers. For example, the detection of respiratory viruses; diagnosis and monitoring of viral infections, especially CMV, EBV, and BK virus in immunocompromised patients; viral load and antiviral susceptibility testing for HIV, HCV, and HBV; and testing for HPV have all become mainstream diagnostic procedures. The methodology of diagnostic virology is evolving rapidly, with increasing conversion to molecular methods. The development of standardized commercial molecular tests that have passed through the review processes of the FDA has been relatively slow but is now beginning to gain momentum. At the same time, the process of virus discovery, described earlier, is dramatically expanding the vistas of medical virology. In the coming years, it may become necessary to detect many viruses other than those for which FDA-approved assays are under development. Relatively unbiased methods such as NextGen sequencing, currently being used mainly for virus discovery, will undoubtedly be evaluated in the clinical laboratory setting. In addition, new paradigms, including recognition and diagnosis of diseases that result from multiple microbial interactions as well as diseases that occur uniquely in an individual based on that individual’s genetic makeup, may require profound changes in the way in which diagnostic testing is performed. Assays to detect the presence of viruses may need to be combined with assays to evaluate biomarkers that provide information on how the host is reacting to the presence of the virus. Genetic markers may need to be evaluated to help define how an individual patient is likely to respond to viral infection. In addition, there is little doubt that unforeseen technological advances will drive the evolution of the field in directions that are currently impossible to predict.

DISCLOSURES

Dr. Storch serves on the medical advisory board of Roche Molecular Systems and has served as a consultant to Idaho Technology,

PrimeraDx, Abbott, and Diagnostic Hybrids. He has received an honorarium from GenProbe and Meridian. He is currently performing research funded by Luminex and Meridian. Dr. Wang has no disclosures.

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Picornaviridae: The Viruses and Their Replication

Classification

Virion Structure

- Physical Properties
- Ratio of Particles to Infectious Viruses
- High-Resolution Structure of the Virion
- Surface of the Virion
- Interior of the Virion
- Hydrophobic Pocket
- Myristate
- Neutralizing Antigenic Sites

Genome Structure and Organization

- General Features

Infectious DNA Clones of Picornavirus Genomes

Stages of Replication

Attachment

- Cellular Receptors and Coreceptors
- One Type of Receptor Molecule for Virus
 - Binding and Entry
- Receptors and Coreceptors Required for Infection
- Alternative Receptors
- How Picornaviruses Attach to Cell Receptors
- Kinetics and Affinity of the Virus–Receptor Interaction

Entry into Cells

- Entry by Clathrin-Mediated Endocytosis
- Entry by Caveolin-Mediated Endocytosis
- Caveolin- and Clathrin-Independent Endocytosis
- Uncoating
- Regulation of Uncoating by Cellular Molecules

Translation of the Viral RNA

- Internal Ribosome Binding: The Internal Ribosome Entry Site
- Mechanism of Internal Ribosome Binding
- Processing of the Viral Polyprotein

Viral RNA Synthesis

- Viral RNA-Dependent RNA Polymerase, 3D^{pol}

- Viral Accessory Proteins

- 2A Protein

- 2B Protein

- 2C Protein

- 2BC Protein

- 3AB Protein

- Cellular Accessory Proteins

- Protein-Primed RNA Synthesis

- Cellular Site of RNA Synthesis

- Translation and Replication of the Same RNA Molecule

- Discrimination of Viral and Cellular RNA

Origins of Diversity

- Misincorporation of Nucleotides

- Recombination

Assembly of Virus Particles

Effects of Viral Multiplication on the Host Cell

- Inhibition of 5' End-Dependent mRNA Translation

- Modulation of eIF4F Activity

- Stress-Associated RNA Granules

- Inhibition of Cellular RNA Synthesis

- Inhibition of Nucleocytoplasmic Trafficking

- Inhibition of Protein Secretion

- Cell Death and Virus Release

Perspectives

Viruses in the family *Picornaviridae* have nonenveloped particles with a single-stranded RNA (ssRNA) genome of positive polarity. Among its many members are numerous important human and animal pathogens, such as poliovirus, hepatitis A virus, foot-and-mouth disease virus (FMDV), enterovirus 71,

and rhinovirus. The name of the virus family was intended to convey the small size of the viruses (pico, a small unit of measurement [10^{-12}]) and the type of nucleic acid that constitutes the viral genome (RNA).

Picornaviruses have played important roles in the development of modern virology. Foot-and-mouth disease virus was the first animal virus to be discovered, by Loeffler and Frosch in 1898.³⁰⁵ Poliovirus was isolated 10 years later,²⁸⁹ a discovery spurred by the emergence of epidemic poliomyelitis at the turn of the 20th century. The discovery in 1949 that poliovirus could be propagated in cultured cells led to studies of viral replication.¹³⁵ The plaque assay, an essential method for quantification of viral infectivity, was developed with poliovirus.¹³⁰ The first RNA-dependent RNA polymerase identified was that of mengovirus,²⁹ a picornavirus, and the synthesis of a precursor polyprotein from which viral proteins are derived by proteolytic processing was first identified in poliovirus-infected cells.⁴⁷⁷ The first infectious DNA clone of an animal RNA virus was that of poliovirus,⁴¹⁶ and the first three-dimensional structures of animal viruses determined by x-ray crystallography were those of poliovirus²²³ and rhinovirus.⁴³⁵ Poliovirus RNA was the first messenger RNA (mRNA) shown to lack a 5' cap structure.^{215,361} This observation was subsequently explained by the finding that the genome RNA of poliovirus and other picornaviruses is translated by internal ribosome binding,^{243,385} a process now known to occur on cellular mRNA.^{248,313}

Because they cause serious diseases, poliovirus and FMDV have been the best-studied picornaviruses. Research on poliovirus has produced two effective vaccines; it is likely that poliomyelitis will be eradicated from the globe in the near future. The World Health Organization (WHO) has established a goal of cessation of vaccination, at which time all poliovirus stocks must be destroyed. When this historic event takes place, all research on this virus will cease. Poliovirus is truly a virus with a brilliant past, but with no future.

CLASSIFICATION

The family *Picornaviridae* belongs to the order *Picornavirales* and comprises 12 genera (Table 16.1), which all contain viruses that infect vertebrates.²⁸⁰ Information on selected viruses is summarized below.

The genus *Aphthovirus* consists of four species: *Foot-and-mouth disease virus* (FMDV), *Bovine rhinitis A virus*, *Bovine rhinitis B virus*, and *Equine rhinitis A virus*. FMDV infects cloven-footed animals (e.g., cattle, goats, pigs, and sheep), primarily via the upper respiratory tract, and has been isolated from at least 70 species of mammals. Seven serotypes of FMDV have been identified; within each serotype are many subtypes. These viruses are highly labile and rapidly lose infectivity at pH values of less than 6.8.

There are two species within the genus *Cardiovirus*: *Encephalomyocarditis virus* and *Theilovirus*. The encephalomyocarditis viruses (which include strains known as encephalomyocarditis virus, Columbia SK virus, Maus Elberfeld virus, and Mengovirus) are murine viruses, which can also infect many other hosts, including humans, monkeys, pigs, elephants, and squirrels. The second species includes the Theiler's murine encephalomyelitis viruses and human Saffold viruses.

TABLE 16.1 Members of the Family *Picornaviridae*

Genus	Species
<i>Aphthovirus</i>	<i>Foot-and-mouth disease virus</i> <i>Equine rhinitis A virus</i> <i>Equine rhinitis B virus</i>
<i>Avihepatovirus</i>	<i>Duck hepatitis A virus</i>
<i>Cardiovirus</i>	<i>Encephalomyocarditis virus</i> <i>Theilovirus</i>
<i>Enterovirus</i>	<i>Bovine enterovirus</i> <i>Human enterovirus A (Coxsackievirus, enterovirus)</i> <i>Human enterovirus B (Coxsackievirus, echovirus, enterovirus)</i> <i>Human enterovirus C (poliovirus, Coxsackievirus, enterovirus)</i> <i>Human enterovirus D (enterovirus)</i> <i>Porcine enterovirus B</i> <i>Simian enterovirus A</i> <i>Human rhinovirus A</i> <i>Human rhinovirus B</i> <i>Human rhinovirus C</i>
<i>Erbovirus</i>	<i>Equine rhinitis B virus</i>
<i>Hepatovirus</i>	<i>Hepatitis A virus</i>
<i>Kobuvirus</i>	<i>Aichi virus</i> <i>Bovine kobuvirus</i>
<i>Parechovirus</i>	<i>Human parechovirus</i> <i>Ljungan virus</i>
<i>Sapelovirus</i>	<i>Porcine sapelovirus</i> <i>Simian sapelovirus</i> <i>Avian sapelovirus</i>
<i>Senecavirus</i>	<i>Seneca Valley virus</i>
<i>Tremovirus</i>	<i>Avian encephalomyelitis virus</i>
<i>Teschovirus</i>	<i>Porcine teschovirus</i>

There are 10 species in the *Enterovirus* genus: *Human enterovirus A, B, C, D*; *Simian enterovirus A*, *Bovine enterovirus*, *Porcine enterovirus B*, and *Human rhinovirus A, B, C*. Members of this genus include poliovirus (3 serotypes), Coxsackievirus (25 serotypes), echovirus (28 serotypes), human enterovirus (43 serotypes), and many nonhuman enteric viruses. Enteroviruses such as poliovirus (3 serotypes) and Coxsackievirus (25 serotypes) replicate in the alimentary tract and are resistant to low pH. The acid-labile rhinoviruses (so named because they replicate in the nasopharynx) are important agents of the common cold. There are 100 serotypes of *Human rhinovirus A and B*. Rhinoviruses may also replicate in the lower respiratory tract; the newly discovered *Human rhinovirus C* (49 types) have been associated with severe lower respiratory tract disease.³²⁷

The genus *Hepatovirus* contains a single species, the human pathogen *Hepatitis A virus* (one serotype). Virions are highly stable and resistant to acid pH and high temperatures (60°C for 10 min). The virus infects epithelial cells of the small intestine and hepatocytes.

The *Parechovirus* genus contains two species, *Human parechovirus* and *Ljungan virus*, a virus of rodents. Parechoviruses are etiologic agents of respiratory and gastrointestinal disease.

Proteins of parechoviruses are substantially diverged from those of other picornaviruses, with no greater than 30% amino acid identity.

The *Erbovirus* genus contains three types of *Equine rhinitis B virus*. These viruses cause upper respiratory tract disease in horses that is associated with viremia and fecal shedding of virus particles. The *Kobuvirus* genus contains two species: *Aichi virus*, which causes gastroenteritis in humans, and *Bovine kobuvirus*. The *Teschovirus* genus consists of 12 serotypes of *Porcine teschovirus*. Some strains can cause polioencephalitis in pigs, also called Teschen/Talfan disease or teschovirus encephalomyelitis. There are three species in the *Sapelovirus* genus: *Porcine sapelovirus*, *Simian sapelovirus*, and *Avian sapelovirus*. The sole species in the genus *Senecavirus* is *Seneca Valley virus*, found in pigs throughout the United States, but there is no known associated disease. It is currently in clinical trials to assess its value for treating human tumors.⁴⁴⁰ *Avian encephalomyelitis virus*, which causes the eponymous disease in young chickens, pheasants, quail, and turkeys, is the sole species in the genus *Tremovirus*.

A number of other related viruses may be members of the *Picornaviridae* but have not been approved as species by the International Committee on Taxonomy of Viruses (ICTV). These include human cosaviruses, identified in the stools of south Asian children,^{224,262} human klassevirus/salivirus,^{183,225,297} and many others.²⁸⁰

VIRION STRUCTURE

Physical Properties

Picornavirus virions are spherical, with a diameter of about 30 nm (Fig. 16.1). The particles are simple, consisting of a protein shell surrounding the naked RNA genome. The virus particles lack a lipid envelope, and their infectivity is insensitive to organic solvents. Cardioviruses, enteroviruses (except rhinoviruses), hepatoviruses, and parechoviruses are acid stable and retain infectivity at pH values of 3.0 and lower. In contrast, rhinoviruses and aphthoviruses are labile at pH values of less than 6.0. Differences in pH stability influence the sites of replication of the virus. For example, rhinoviruses and aphthoviruses replicate in the respiratory tract and need not be acid stable. Because they are acid labile, they cannot replicate in the alimentary tract. Cardioviruses, enteroviruses, hepatoviruses, and parechoviruses pass through the stomach to gain access to the intestine and, therefore, must be resistant to low pH. The structural basis for the acid lability of FMDV is partly understood (see *Entry into Cells*).

The buoyant densities of picornaviruses are quite different (Table 16.2). Cardioviruses and enteroviruses have a buoyant density of 1.34 g/mL, that of FMDV is 1.45 g/mL, and rhinoviruses have an intermediate value (1.40 g/mL). The reason for the difference lies in the permeability of the viral capsid to cesium. The capsid of poliovirus does not allow cesium to reach the RNA interior; thus, the virus bands at an abnormally light buoyant density.¹⁴⁶ In contrast, aphthovirus capsids contain pores that allow cesium to enter.¹ The rhinovirus capsid is permeable to cesium, but the presence of polyamines in the capsid interior limits the amount of cesium that can enter, which provides an explanation for the intermediate buoyant density value of these viruses.¹⁴⁶

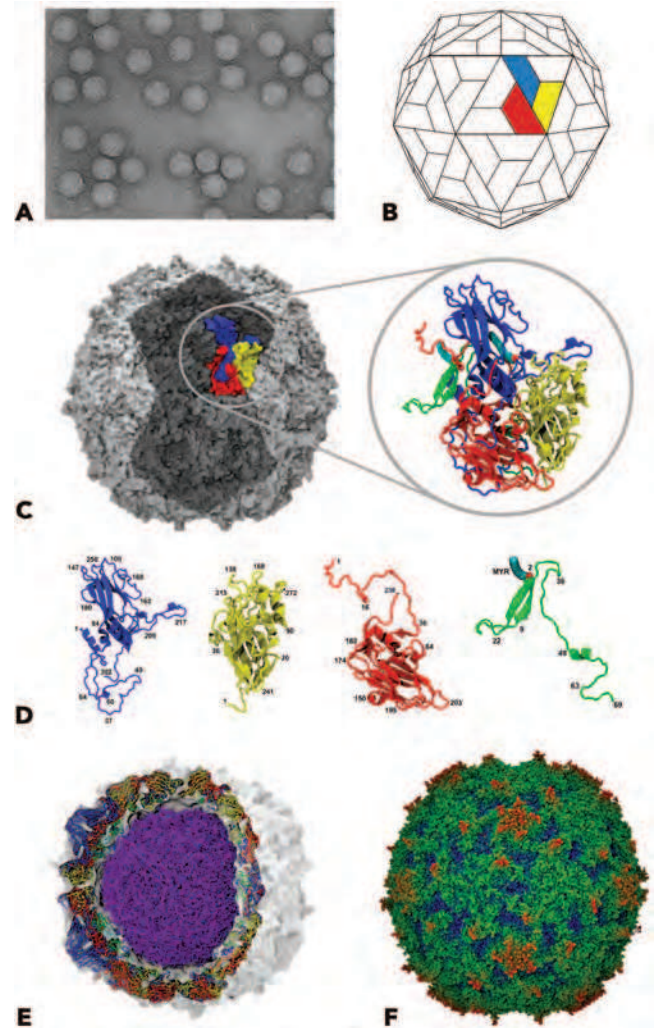


FIGURE 16.1. Structural features of picornaviruses. **A:** Electron micrograph of negatively stained poliovirus ($\times 270,000$ magnification). **B:** Schematic of the picornavirus capsid, showing the pseudoequivalent packing arrangement of VP1 (blue), VP2 (yellow), and VP3 (red). VP4 (green) is on the interior of the capsid. A single biologic protomer is colored. **C:** Model of poliovirus type 1, Mahoney, based on x-ray crystallographic structure determined at 2.9 Å. Two adjacent pentamers aligned at the twofold axis of symmetry are shaded dark gray. A single protomer is colored and also expanded at right as a cartoon of the alpha carbon backbone; capsid proteins are color coded according to **B**. **D:** Individual capsid proteins VP1, VP2, VP3, and VP4 are shown as cartoon representations of the alpha carbon backbone. **E:** Virion after 10 nanoseconds of atomistic molecular dynamics simulation. Volumetric (density) map representation is white with protein backbone shown as tubes and colored as in **B**. Lipids are represented as Van der Waals models with coloring by element. RNA is colored purple and associated magnesium ions are colored blue. **F:** All-atom Van der Waals representation of the poliovirus capsid with radial coloring depicting the relative distance from the center of the particle; blue = 133 Å to red = 166 Å. (**A** courtesy of N. Cheng and D. M. Belnap; **C** to **F** courtesy of Jason A. Roberts, WHO Poliomyelitis Regional Reference Laboratory, Victorian Infectious Diseases Reference Laboratory, North Melbourne, Australia.)

TABLE 16.2 Physical Properties of Some Picornaviruses

Genus	pH stability	Virion buoyant density	Sedimentation coefficient
<i>Aphthovirus</i>	Labile, <6.8	1.43–1.45	142–146S
<i>Cardiovirus</i>	Stable, 3–9	1.33–1.34	160S
<i>Enterovirus</i>			
Enteroviruses	Stable, 3–9	1.34	160S
Rhinoviruses	Labile, <6	1.40	160S
<i>Hepatovirus</i>	Stable	1.32–1.34	160S
<i>Parechovirus</i>	Stable	1.36	160S

Ratio of Particles to Infectious Viruses

The ratio of particles to infectious virus is determined by dividing the number of virus particles in a sample (determined by electron microscopy [EM] or spectrophotometric measurements) by the plaque titer, yielding the particle-to-plaque forming unit (pfu) ratio. This ratio is a measurement of the fraction of virus particles that can complete an infectious cycle. The particle-to-pfu ratio of poliovirus ranges from 30 to 1,000, and that of other picornaviruses is in the same range. The high particle-to-pfu ratio may be caused by the presence of lethal mutations in the viral genome. This explanation, however, probably does not apply to all picornaviruses; it has been shown that the infectivity of aphthovirus RNA approaches one infectious unit per molecule. An alternative explanation is that all viruses do not successfully complete an infectious cycle because they fail at one of several steps that must be completed, including attachment, entry, replication, and assembly.

High-Resolution Structure of the Virion

The capsids of picornaviruses are composed of four structural proteins: VP1, VP2, VP3, and VP4. The results of x-ray diffraction studies, EM observations, and biochemical studies of virus particles and their dissociation products led to the hypothesis that the picornavirus capsid contains 60 structural proteins arranged into an icosahedral lattice.⁴⁴¹ Our understanding of the structure of picornaviruses was substantially advanced in 1985 when the atomic structures of poliovirus type 1²²³ and human rhinovirus type 14⁴³⁵ were determined by x-ray crystallography. Since then, the high-resolution structures of many other picornaviruses have been determined.

The basic building block of the picornavirus capsid is the protomer, which contains one copy each of VP1, VP2, VP3, and VP4. The shell is formed by VP1 to VP3, and VP4 lies on its inner surface (Fig. 16.1). VP1, VP2, and VP3 have no sequence homology, yet all three proteins have the same topology: they form an eight-stranded, antiparallel β -barrel (also called a β -barrel jelly roll or a Swiss-roll β -barrel). This domain is a wedge-shaped structure made up of two antiparallel β -sheets. One β -sheet forms the wall of the wedge, and the second, which has a bend in the center, forms both a wall and the floor. The wedge shape facilitates the packing of structural units to form a dense, rigid protein shell. Packing of the β -barrel domains is strengthened by a network of protein–protein contacts on the interior of the capsid, particularly around the fivefold axes. This network, which is formed by the N-terminal extensions of VP1 to VP3 as well as VP4, is essential for the stability of the virion.

VP4 differs significantly from the other three proteins in that it has an extended conformation. This protein is similar in position and conformation to the NH₂-terminal sequences of VP1 and VP3 and functions as a detached NH₂-terminal extension of VP2 rather than an independent capsid protein.

The main structural differences among VP1, VP2, and VP3 lie in the loops that connect the β -strands and the N- and C-terminal sequences that extend from the β -barrel domain. These amino acid sequences give each picornavirus its distinct morphology and antigenicity. The C-termini are located on the surface of the virion, and the N-termini are on the interior, indicating that significant rearrangement of the P1 precursor occurs on proteolytic cleavage.

Resolution of the structures of poliovirus and rhinovirus revealed that the β -barrel domains are strikingly similar in structure to those of plant viruses such as southern bean mosaic virus and tomato bushy stunt virus. The capsid proteins of these viruses bear no sequence homology with those of the picornaviruses. It has since become apparent that similar protein topology is found in the capsid proteins of many plant, insect, and vertebrate positive-stranded RNA viruses as well as in the DNA-containing papovaviruses and adenoviruses. These findings suggest that either the polypeptides evolved from a common ancestor or that the β -barrel domain is one of the few ways to allow proteins to pack to form a sphere.

In some viruses, such as the parechoviruses, VP0, the precursor to VP2 + VP4, remains uncleaved, while in hepatitis A virus, VP4 is very small.

Surface of the Virion

Resolution of the structures of poliovirus and human rhinovirus revealed that the surfaces of these viruses have a corrugated topography; a prominent, star-shaped plateau (mesa) is found at the fivefold axis of symmetry, surrounded by a deep depression (canyon) and another protrusion at the threefold axis (Fig. 16.1). It was originally proposed that the canyon is the receptor-binding site—this hypothesis has been proved for a number of enteroviruses. Not all picornaviruses have canyons. The surface of cardioviruses bears a series of depressions, or “pits,” which are involved in receptor binding, while that of FMDV is much smoother. As will be discussed later, a flexible loop that projects from the capsid surface binds to the cellular receptor for FMDV.

Interior of the Virion

A network formed by the N-termini on the interior of the capsid contributes significantly to the stability of the virion.

At the fivefold axis of symmetry, the N-termini of five VP3 molecules form a cylindrically parallel β -sheet. This structure is surrounded by five three-stranded β -sheets formed by the N-termini of VP4 and VP1. The myristate group attached to the N-terminus of VP4 mediates the interaction between these two structures.⁹³ Interactions among pentamers are stabilized by a seven-stranded β -sheet, composed of four β -strands of the VP3 β -barrel and one strand from the N-terminus of VP1 that surround a two-stranded β -sheet made from the N-terminus of VP2 from a neighboring pentamer.¹³⁸

Poliovirus genomes containing an extra 1,500 nucleotides can be successfully packaged, indicating that the interior of the capsid is not fully occupied.⁹ It has been suggested that picornaviral capsids are stabilized by interactions with the RNA genome, based on findings with bean pod mottle virus, which is related to picornaviruses. In this virus, ordered RNA can be observed at the threefold axis, and packaging of viral RNA stabilizes subunit interactions.^{91,299} Several nucleotides have been tentatively identified in a similar location in the structures of P3/Sabin and rhinovirus type 14.^{25,138} In the atomic structure of poliovirus P2/Lansing, RNA bases have been observed stacking with conserved aromatic residues of VP4.²⁹⁴ The structure of *Seneca Valley virus* revealed the arrangement of the RNA within the capsid.⁵¹¹ Much of the nucleic acid contacts the inner surface of the capsid, particularly near the twofold axes under VP2. The RNA forms a shell that contacts both VP2 and VP4 and which could serve as a scaffold for capsid assembly or might contribute to virion stability.

Hydrophobic Pocket

Within the core of VP1, just beneath the canyon floor of many picornaviruses, is a hydrophobic tunnel or pocket (Fig. 16.2). Electron density observed in this area has been interpreted to be cell-derived lipids called “pocket factors.” In poliovirus types 1 and 3, the pocket is believed to contain sphingosine.¹³⁸ An unidentified lipid has been found in human rhinoviruses types 1A and 16, a C16 fatty acid has been modeled in the pocket of coxsackievirus B3,^{194,271,348} coxsackievirus A21 is believed to carry myristic acid,⁵³⁵ and bovine enterovirus contains a mixture of palmitic and myristic acid.⁴⁶⁴ In the enterovirus 71 virion, the pocket factor is partly exposed on the floor of the canyon.⁴⁰³ In contrast, the pocket of human rhinovirus type 14 is empty.²⁵ The results of introducing amino acid changes in the pocket of rhinovirus 16 suggest that the hydrophobic pocket, and not the pocket factor, is important for maintaining capsid dynamics.²⁶⁵

The hydrophobic pocket is also the binding site for anti-picornavirus drugs such as the WIN compounds produced by Sterling-Winthrop⁴⁶² as well as similar molecules produced by Janssen Pharmaceuticals (Titusville, NJ)^{19,104} (Fig. 16.2). Some of these drugs have been evaluated in clinical trials, such as pleconaril for treatment of common colds caused by rhinoviruses³⁹² and for enteroviral sepsis syndrome.³⁵⁶ These hydrophobic, sausage-shaped compounds bind tightly in the hydrophobic tunnel, displacing any lipid that is present, inhibiting either binding or uncoating. Drug-dependent mutants of poliovirus spontaneously lose infectivity at 37°C, probably because they do not contain lipid in the pocket.³⁴⁷

Myristate

Myristic acid (*n*-tetradecanoic acid) is covalently linked to glycine at the amino terminus of VP4 of most picornaviruses.⁹³

This fatty acid is an integral part of the viral capsid. The N-termini of VP3 intertwine around the fivefold axis to form a twisted tube of parallel β -structure.²²³ The five myristyl groups extend from the N-termini of VP4 and cradle the twisted tube formed by VP3.⁹³ The myristyl groups interact with amino acid side chains of VP4 and VP3. Mutagenesis of VP4 has revealed a role for myristic acid modification in virus assembly and in the stability of the capsid.^{20,314–316,344}

Neutralizing Antigenic Sites

Viral serotype is determined by the connecting loops and C-termini of the capsid proteins that decorate the outer surface of the virion. These contain the major neutralization antigenic sites of the virus, the amino acid sequences that are recognized by antibodies that block viral infectivity. Such sites are defined by mutations that confer resistance to neutralization with monoclonal antibodies directed against the viral capsid.^{334,335,458} Human sera contain antibodies directed against poliovirus antigenic sites identified in mice.⁴²³

The large area of contact of the poliovirus receptor (PVR) CD155 on the virion surface, compared with other picornavirus–receptor interactions, might provide a clue to why there are many rhinovirus serotypes but only three serotypes of poliovirus.²⁰⁹ This large interaction area could limit the viability of antibody-escape mutants because they would also compromise their ability to bind CD155.

The capsids of rhinovirus, poliovirus, and other picornaviruses are dynamic, leading to transient display of the buried N-termini of VP1.^{246,266,298,411,432,446} Antibodies to this sequence of VP1 neutralize viral infectivity.

GENOME STRUCTURE AND ORGANIZATION

General Features

The genome of picornaviruses is a single positive-stranded RNA molecule (Fig. 16.3). The viral RNA is infectious because it is translated on entry into the cell to produce all the viral proteins required for viral replication. Picornavirus genomic RNA is unique because it is covalently linked at the 5′ end to a protein called VPg (virion protein, genome linked).^{142,293} VPg is covalently joined to the 5′-uridylylate moiety of the viral RNA by an O4-(5′-uridylyl)-tyrosine linkage. The tyrosine that is linked to the viral RNA is always the third amino acid from the N-terminus. VPg of different picornaviruses varies in length from 22 to 24 amino acid residues and is encoded by a single viral gene, except in the genome of FMDV, which encodes three VPg genes.¹⁴⁵ VPg is not required for infectivity of poliovirus RNA; if it is removed from viral RNA by treatment with proteinase, the specific infectivity of the viral RNA is not reduced. VPg is not found on viral mRNA that is associated with cellular ribosomes and undergoing translation; these mRNA contain only uridine 5′-phosphate (pU) at their 5′ ends. Poliovirus mRNA differs from virion RNA only by the lack of VPg.^{363,389} VPg is removed from virion RNA by a host protein called *unlinking enzyme*.¹² It is not known whether removal of VPg is a prerequisite for association with ribosomes or is a result of that association. Although VPg-linked RNA can be translated in cell-free extracts, it is possible that VPg is rapidly cleaved from the RNA such that only RNA lacking VPg are translated.^{169,338,514} VPg is present on nascent RNA

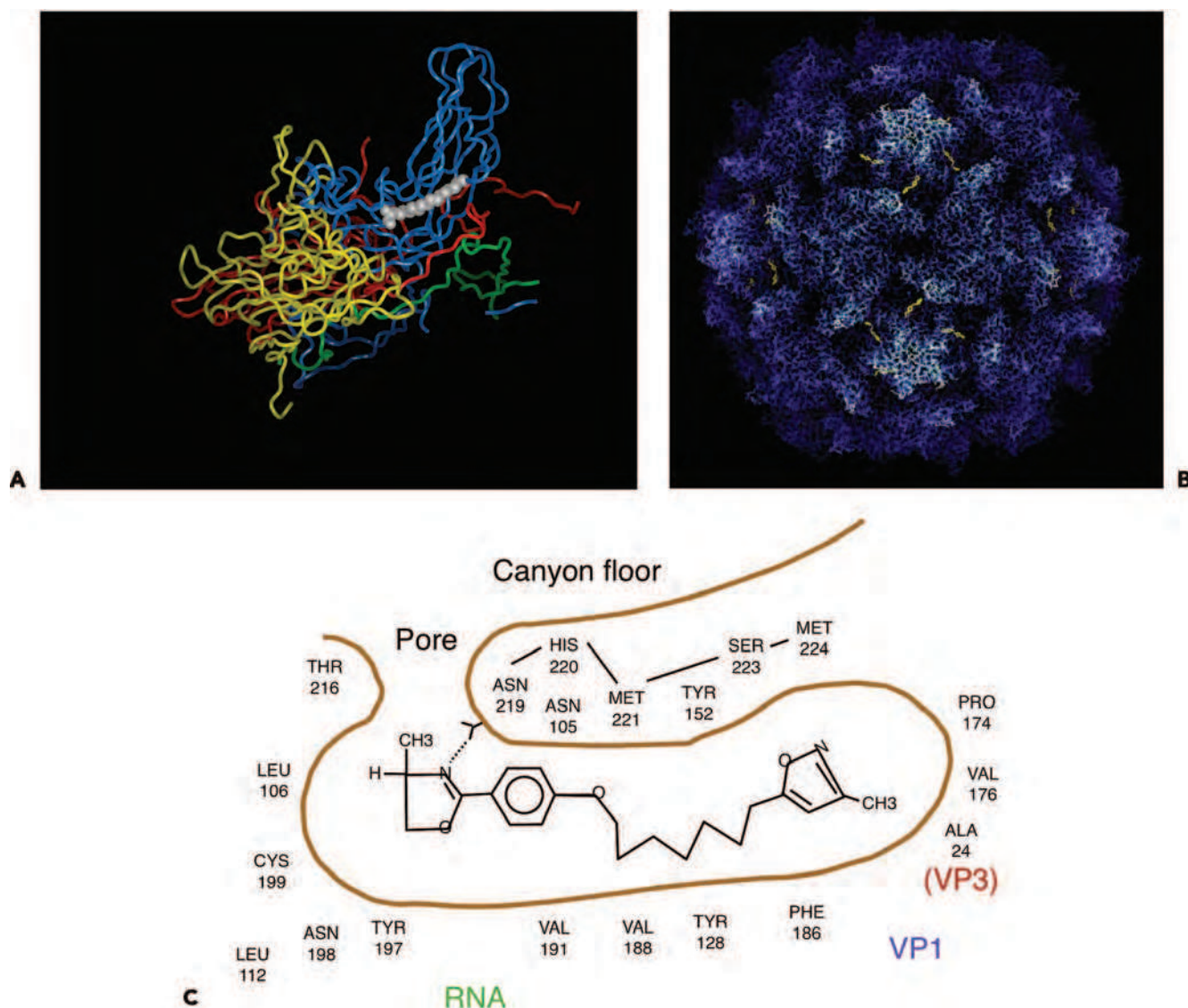


FIGURE 16.2. The hydrophobic pocket in the picornavirus capsid. **A:** Cellular lipid bound in the hydrophobic pocket of poliovirus type 1. Side view of a protomer, consisting of one copy each of VP1, VP2, VP3, and VP4. RNA is below, and the fivefold axis of symmetry is at the upper right. Gray spheres represent what is believed to be a molecule of sphingosine bound in the hydrophobic pocket. The lipid is just below the canyon floor. **B:** Drug bound in the poliovirus pocket. R78206, a WIN-like compound, bound in the poliovirus capsid. The bound drug is the small yellow molecule at the base of the canyon. The drug appears to be exposed on the surface but is actually in the hydrophobic pocket. The capsid model is shown in a radial depth-cued representation, in which atoms are colored according to whether they are near the center of the virus (blue) or far from the center (white). Residues at the bottom of the canyon are dark because they are at a position of low radius. The bound drug is not depth cued. **C:** WIN52084 bound in the hydrophobic pocket. These drugs displace the lipid from the pocket, thereby blocking infectivity.

chains of the replicative intermediate RNA and on negative-stranded RNA, which has led to the suggestion that VPg is a primer for poliovirus RNA synthesis.^{362,389} The role of VPg in viral RNA synthesis is discussed in subsequent sections.

Nucleotide sequence analysis of many picornavirus RNAs has revealed a common organizational pattern (Fig. 16.3). The genomes vary in length from 7 to 8.8 kb. The 5′-noncoding regions of picornaviruses are long (0.5 to 1.5 kb) and highly structured. This region of the genome contains sequences that

control genome replication and translation. The 5′-noncoding region contains the internal ribosome entry site (IRES) that directs translation of the mRNA by internal ribosome binding. The 5′-untranslated regions of aphthoviruses, cardioviruses, and erboviruses contain a poly(C) tract that varies in length among different virus strains (80 to 250 nucleotides in cardioviruses, 40 to 400 nucleotides in aphthoviruses). Among cardioviruses, longer poly(C) length is associated with higher virulence in animals.^{129,198}

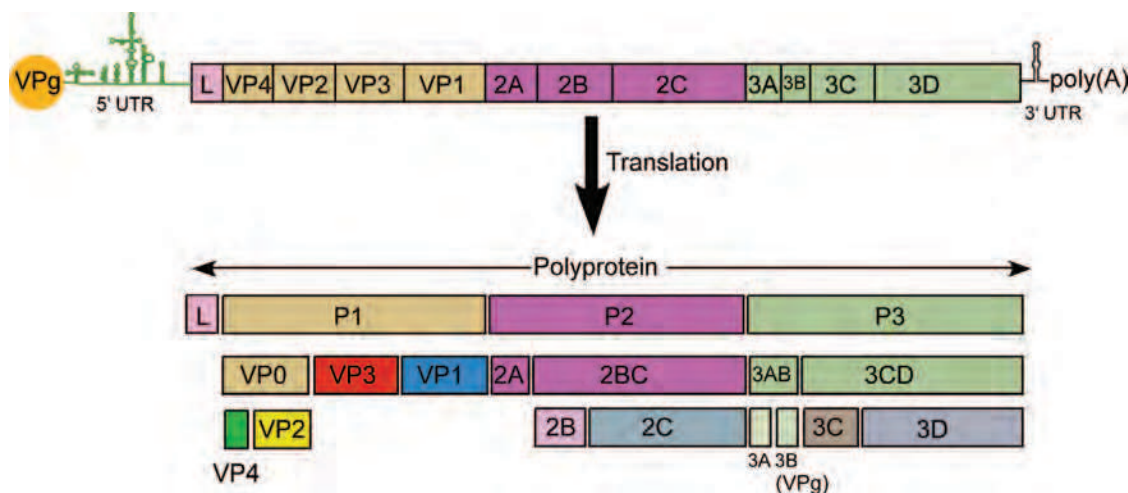


FIGURE 16.3. Organization of a picornavirus genome. **Top:** Schematic of the viral RNA genome, with the genome-linked protein VPg at the 5' end, the 5' untranslated region containing the IRES, the protein coding region, the 3' untranslated region containing a pseudoknot, and the poly(A) tail. L is a leader protein encoded in the genomes of erboviruses, cardioviruses, and aphthoviruses but not other picornaviruses. Coding regions for the viral proteins are indicated. **Bottom:** Processing pattern of picornavirus polyprotein. Some genomes encode multiple copies of protein coding regions, e.g., there are three VPgs in the FMDV genome, two 2A motifs in Ljungan virus, and three 2A motifs in the duck hepatitis A virus genome.

The 3'-noncoding region of picornaviruses is short, ranging in length from 40 to 330 nucleotides. This region may also contain a secondary structure, a pseudoknot, that has been implicated in controlling viral RNA synthesis.²⁴¹ The entire 3'-noncoding region of poliovirus and rhinovirus is not required for infectivity, however.^{77,489} Both virion RNA and mRNA contain a 3' stretch of poly(A).⁵⁴¹ Negative-stranded RNA contains a 5' stretch of poly(U), which is copied to form poly(A) of the positive strand.⁵⁴² The average length of the poly(A) tail varies from 35 nucleotides in encephalomyocarditis virus to 100 nucleotides in aphthoviruses.⁵ Viral RNA from which the poly(A) tract is removed is noninfectious.⁴⁶⁹

The results of biochemical studies of poliovirus-infected cells had predicted the presence of a single, long, open reading frame (ORF) on the viral RNA that is processed to form individual viral proteins.⁴⁷⁷ This hypothesis was proved when the nucleotide sequence of the poliovirus genome was determined, which revealed that the viral RNA encodes a single ORF.^{276,415} A similar strategy for viral gene expression occurs during the replication of all picornaviruses. The polyprotein is cleaved during translation, so that the full-length product is not observed. Cleavage is carried out by virus-encoded proteinases to yield 11 to 15 final cleavage products. Some of the uncleaved precursors also have functions during replication.

To unify the nomenclature of picornavirus proteins, the polyprotein has been divided into three regions: P1, P2, and P3 (Fig. 16.3). The genomes of some picornaviruses encode a leader (L) protein before the P1 region. The P1 region encodes the viral capsid proteins, whereas the P2 and P3 regions encode proteins involved in protein processing (2A^{pro}, 3C^{pro}, 3CD^{pro}) and genome replication (2B, 2C, 3AB, 3B^{VPg}, 3CD^{pro}, 3D^{pro}). The genome of FMDV encodes three VPg proteins. The genome of Ljungan virus, a parechovirus, may encode two unrelated 2A proteins²⁴⁹ and that of duck hepatitis A virus in the genus *Avihepatovirus* encodes three 2A motifs.

INFECTIOUS DNA CLONES OF PICORNAVIRUS GENOMES

Recombinant DNA techniques allow the introduction of mutations anywhere in the genome of most animal viruses. An infectious DNA clone—a double-stranded copy of the viral genome carried on a bacterial plasmid—or RNA transcripts derived by *in vitro* transcription, can be introduced into cultured cells by transfection to recover infectious virus. The first infectious DNA clone of an animal RNA virus was that of poliovirus.⁴¹⁶ The infectivity of cloned poliovirus DNA (10³ pfu/μg) is much lower than that of genomic RNA (10⁶ pfu/μg). The development of plasmid vectors incorporating promoters for bacteriophage SP6, T7, or T3 RNA polymerase for the production of RNA transcripts *in vitro* enabled the production of infectious picornavirus RNA from cloned DNA.⁵⁰³ Such RNA transcripts have an infectivity approaching that of genomic RNA. A similar approach has been adopted for the recovery of many other picornaviruses from cloned DNA copies of the viral genome.

STAGES OF REPLICATION

Replication of picornaviruses occurs in the cell cytoplasm. The first step is attachment to a cell receptor (Fig. 16.4). The RNA genome is then uncoated, a process that involves structural changes in the capsid. Once the positive-stranded viral RNA enters the cytoplasm, it is translated to provide viral proteins essential for genome replication and the production of new virus particles. The viral proteins are synthesized from a polyprotein precursor, which is cleaved nascently. Cleavages are carried out mainly by two viral proteinases, 2A^{pro} and 3C^{pro} or 3CD^{pro}. Among the proteins synthesized are the viral

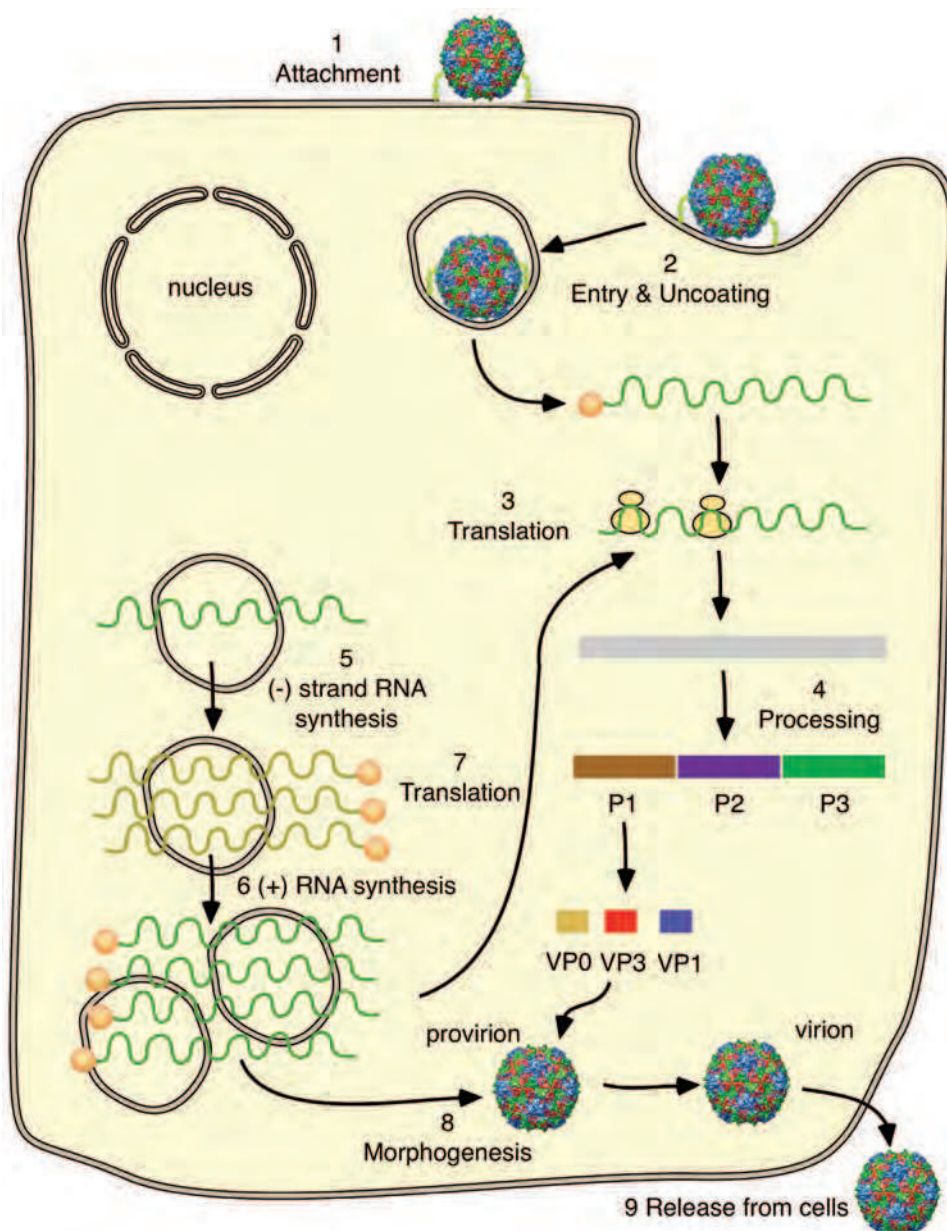


FIGURE 16.4. Overview of the picornavirus replication cycle. Virus binds to a cellular receptor (1) and the genome is uncoated (2). VPg (viral protein, genome linked) is removed from the viral RNA, which is then translated (3). The polyprotein is cleaved nascently to produce individual viral proteins (4). RNA synthesis occurs on membrane vesicles induced by viral proteins (not drawn to scale). Viral (–) strand RNA is copied by the viral RNA polymerase to form full-length (–) strand RNAs (5), which are then copied to produce additional (+) strand RNA (6). Early in infection, newly synthesized (+) strand RNA is translated to produce additional viral proteins (7). Later in infection, the (+) strands enter the morphogenetic pathway (8). Newly synthesized virus particles are released from the cell by lysis (9).

RNA-dependent RNA polymerase and accessory proteins required for genome replication and mRNA synthesis. The first step in genome replication is copying of the positive-stranded RNA to form a negative-stranded intermediate; this step is followed by the production of additional positive strands. These events occur on small membranous vesicles that are induced by several virus proteins. Once the pool of capsid proteins is sufficiently large, encapsidation begins. Coat protein precursor P1 is cleaved to produce an immature protomer, which then assembles into pentamers. Newly synthesized, positive-stranded RNA associates with pentamers to form the infectious virus. Empty capsids that are found in infected cells are likely to be a storage form of pentamers.

The entire time required for a single replication cycle ranges from 5 to 10 hours, depending on many variables, including the particular virus, temperature, pH, host cell, and

multiplicity of infection. Many picornaviruses are released as the cell loses its integrity and lyses. Other picornaviruses (e.g., hepatitis A virus) are released from cells in the absence of cytopathic effect.

ATTACHMENT

Cellular Receptors and Coreceptors

Like most viruses, picornaviruses initiate infection of cells by first attaching to a receptor on the host cell plasma membrane. The nature of picornavirus receptors remained obscure until 1989, when the receptors for poliovirus and the major group rhinoviruses were identified.^{184,332,471} Receptors for many other members of this virus family have since been identified (Table 16.3). Different types of cell surface molecules serve as

TABLE 16.3 Some Cell Receptors for *Picornaviruses*

Virus	Virus receptor	Type of receptor	Coreceptor	References
Aphthovirus				
Foot-and-mouth disease virus (cell culture adapted)	Heparan sulfate	Glycosaminoglycan		(236)
Foot-and-mouth disease virus	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_8$	Integrin		(237,238,351)
Equine rhinitis A virus	Sialic acid	Carbohydrate		(473)
Cardiovirus				
Encephalomyocarditis virus	Vcam-1	Ig-like		(227)
	Sialylated glycophorin A (for hemagglutination only)	Carbohydrate		
Theiler's murine encephalomyelitis virus	Sialic acid	Carbohydrate		(193)
Low neurovirulence strains	Sialic acid	Carbohydrate		(302)
High neurovirulence strains	Heparin sulfate	Proteoglycan		
Enterovirus				
Bovine enterovirus	Sialic acid	Carbohydrate		(547)
Coxsackievirus A9	$\alpha_v\beta_3$, $\alpha_v\beta_6$	Integrin	β_2 -Microglobulin, GRP78, MHC-1	(431,498,499, 531)
Coxsackieviruses A13, A18, A21	Icam-1 (CD)	Ig-like		(99)
Coxsackievirus A16	P-selectin glycoprotein ligand-1 (PSGL-1), Scavenger receptor class B (SCARB2)	Mucin-like		(360,539)
Coxsackievirus A21	Decay-accelerating factor (CD55)	SCR-like (complement cascade)	Icam-1	(453)
Coxsackievirus A24	Sialic-acid containing, O-linked glycoconjugates	Carbohydrate		(336,359)
Coxsackieviruses B1–B6	Car (coxsackievirus-adenovirus receptor)	Ig-like		(58)
Coxsackievirus B1, B3, B5	Decay-accelerating factor (CD55)	scr-like (complement cascade)	$\alpha_v\beta_6$ -Integrin (CVB1) CAR (CVB3)	(4,452,454)
Echovirus 1, 8	$\alpha_2\beta_1$ -Integrin (Vla-2)	Integrin	β_2 -Microglobulin	(56,521)
Echovirus 3, 6, 7, 11–13, 20, 21, 24, 29, 30, 33	Decay-accelerating factor (CD55)	SCR-like (complement cascade)	β_2 -Microglobulin, CD59 (E7)	(57,172,409, 437,520,521)
Echovirus 5	Heparan sulfate	Proteoglycan		(234)
Echovirus 9	$\alpha_v\beta_3$ -Integrin	Integrin		(352)
Enterovirus 70	Decay-accelerating factor (CD55) sialic acid	SCR-like (complement cascade) carbohydrate		(8,264)
Enterovirus 71	P-selectin glycoprotein ligand-1 (PSGL-1), Scavenger receptor class B (SCARB2)	Mucin-like		(360,539)
Parechovirus 1	$\alpha_v\beta_3$, $\alpha_v\beta_6$			(497)
Parechovirus 1	$\alpha_v\beta_1$, $\alpha_v\beta_3$ (Vitronectin receptor)	Integrin		(497)
Hepatitis A virus	HAVcr-1	T-cell Ig-like, mucin-like (TIM)		(261)
Polioviruses 1–3	Pvr (CD155)	Ig-like		(332)
Rhinoviruses (major group, 91 serotypes)	Icam-1	Ig-like		(184,471,492)
Rhinoviruses (minor group, 10 serotypes)	Low-density lipoprotein receptor protein family	Signaling receptor		(222)

Ig, Immunoglobulin; SCR, short consensus repeat.

cellular receptors for picornaviruses; some are shared among picornaviruses and members of other virus families. For example, the cell surface protein CD55 is a receptor for certain Coxsackieviruses, echoviruses, and enterovirus 70, and the PVR CD155 is a receptor for alphaherpesviruses. For some picornaviruses (e.g., poliovirus and rhinovirus), a single type of receptor is sufficient for entry of viruses into cells. For other viruses, a second molecule, or coreceptor, is needed for virus entry into cells. For example, Coxsackievirus A21, which attaches to CD55, requires intercellular adhesion molecule 1 (ICAM-1) for entry into cells.⁴⁵³

One Type of Receptor Molecule for Virus Binding and Entry

For certain picornaviruses, a single type of receptor molecule is sufficient for virus binding and entry. These include the cell receptors for poliovirus (PVR/CD155), rhinoviruses (ICAM-1, low-density lipoprotein receptor LDLR, LDLR-related protein, very-low-density lipoprotein receptor), EMCV (VCAM-1), hepatitis A virus (TIM-1), and enterovirus 71 (SCBR2, PSGL-1).

The PVR is a type I transmembrane protein and a member of the immunoglobulin (Ig) superfamily of proteins, with three extracellular Ig-like domains: a membrane-distal V-type domain followed by two C2-type domains.³³² Production of PVR in mice is sufficient to overcome the lack of susceptibility of this species to poliovirus infection.^{282,421} Because PVR transgenic mice develop poliomyelitis after inoculation with poliovirus by different routes, they have proved to be a valuable model for studying the pathogenesis of poliomyelitis.⁴¹⁷ PVR transgenic mice are not susceptible to infection by the oral route, the natural route of infection in humans, unless the gene encoding type I interferon receptors has been deleted.^{370,549} PVR is synthesized in many tissues in transgenic mice, yet the main sites of poliovirus replication are the brain and spinal cord.^{282,422} This restricted tropism is regulated by the interferon (IFN)- α/β response, which limits viral replication in extraneural organs.²³²

Expression of PVR on cultured cells derived from different animal species leads to susceptibility to poliovirus infection. Therefore it is likely that PVR is the only molecule required for poliovirus binding and entry. The observation that a monoclonal antibody directed against the lymphocyte homing receptor CD44 blocks poliovirus binding to cells suggests that this protein might be a coreceptor for poliovirus entry.^{456,457} It was subsequently shown that CD44 is not a receptor for poliovirus and is not required for poliovirus infection of cells that produce PVR.^{72,149} It seems likely that PVR and CD44 are associated in the cell membrane¹⁵⁰ and that anti-CD44 antibodies block poliovirus attachment by blocking the poliovirus-binding sites on PVR.

Orthologs of the *pvr* gene are present in the genomes of a number of mammals, including those not susceptible to poliovirus infection.²³¹ The amino acid sequence of domain 1 of PVR varies extensively among the nonsusceptible mammals, especially in the regions known to contact poliovirus. The absence of a poliovirus-binding site on these PVR molecules, therefore, explains why poliovirus infection is restricted to simians.

The PVR is an adhesion molecule that participates in the formation of adherens junctions through interaction with nectin-3, a related immunoglobulin-like protein.³⁴⁹ The PVR is

also a recognition molecule for natural killer (NK) cells, and interacts with CD226 and CD96 on NK cells to stimulate their cytotoxic activity.^{71,153} It also interacts with T-cell Ig and ITIM domain (TIGIT), regulating T-cell function.³⁰⁸ The UL141 protein of cytomegalovirus (CMV) blocks surface expression of PVR, leading to evasion of NK cell-mediated killing.⁴⁹¹

The cell surface receptor for the major group of human rhinoviruses (90 serotypes) was identified by using monoclonal antibodies directed against the cellular binding site to isolate the receptor protein from susceptible cells. Amino acid sequence analysis of the purified protein revealed that it is ICAM-1, a type I transmembrane protein with five immunoglobulin-like domains.^{184,471,492} The normal cellular functions of ICAM-1 are to bind its ligand, lymphocyte function-associated antigen 1 (LFA-1) on the surface of lymphocytes and to promote a wide range of immunologic functions.⁵⁰² ICAM-1 is expressed on the surfaces of many tissues, including the nasal epithelium, which is the entry site for rhinoviruses.

Three members of the low-density lipoprotein receptor family are receptors for minor group rhinoviruses (10 serotypes). These proteins consist of 7 (LDLR), 8 (VLDLR), or 31 (LRP) ligand-binding repeats, transmembrane and cytoplasmic domains.

Many picornaviruses bind integrins, which are dimeric cell adhesion receptors with α and β subunits. Many integrin receptors recognize the tripeptide Arg-Gly-Asp (RGD) whose presence in the viral capsid suggests interaction with this type of receptor. A number of integrins can serve as entry receptors for FMDV (Table 16.3), although all may not be utilized during infection of animals. Although integrins are sufficient for FMDV infection, their interaction with the viral capsid does not lead to uncoating (see *Entry Into Cells*).

Receptors and Coreceptors Required for Infection

Many enteroviruses bind to decay-accelerating factor (DAF, or CD55), a member of the complement cascade (Table 16.3); it is composed of four extracellular short consensus repeat modules and is attached to the plasma membrane by a glycosylphosphatidyl inositol (GPI) anchor. For most of these viruses, however, interaction with DAF is not sufficient for infection; this molecule is an attachment receptor but does not lead to virion uncoating. For example, Coxsackievirus A21 binds to DAF, but infection does not occur unless ICAM-1 is also bound (Fig. 16.5).⁴⁵³ In this case, ICAM-1 inserts into the canyon where it triggers capsid uncoating.⁵³⁴ Coxsackievirus B3 binds DAF but virion uncoating does not occur unless Coxsackievirus-adenovirus receptor (CAR; see below) binds in the canyon.²⁰⁸

Echovirus 7, which normally binds DAF, can infect some DAF-negative cells; in these cases CD59 or β_2 -microglobulin can serve as coreceptors for entry.^{172,521} Some Coxsackie B viruses that bind CD55 may require $\alpha_5\beta_1$ -integrin as a coreceptor.⁴

A specific role for coreceptors in virus entry is illustrated by Coxsackievirus B3 entry into polarized epithelial cells.¹⁰⁵ The Coxsackievirus and adenovirus receptor, CAR, mediates cell entry of all Coxsackie B viruses.⁵⁸ CAR is not present on the apical surface of epithelial cells that line the intestinal and respiratory tracts, but is a component of the tight junction and is inaccessible to virus entry. Coxsackie B viruses first bind an attachment receptor, DAF, which is present on the apical

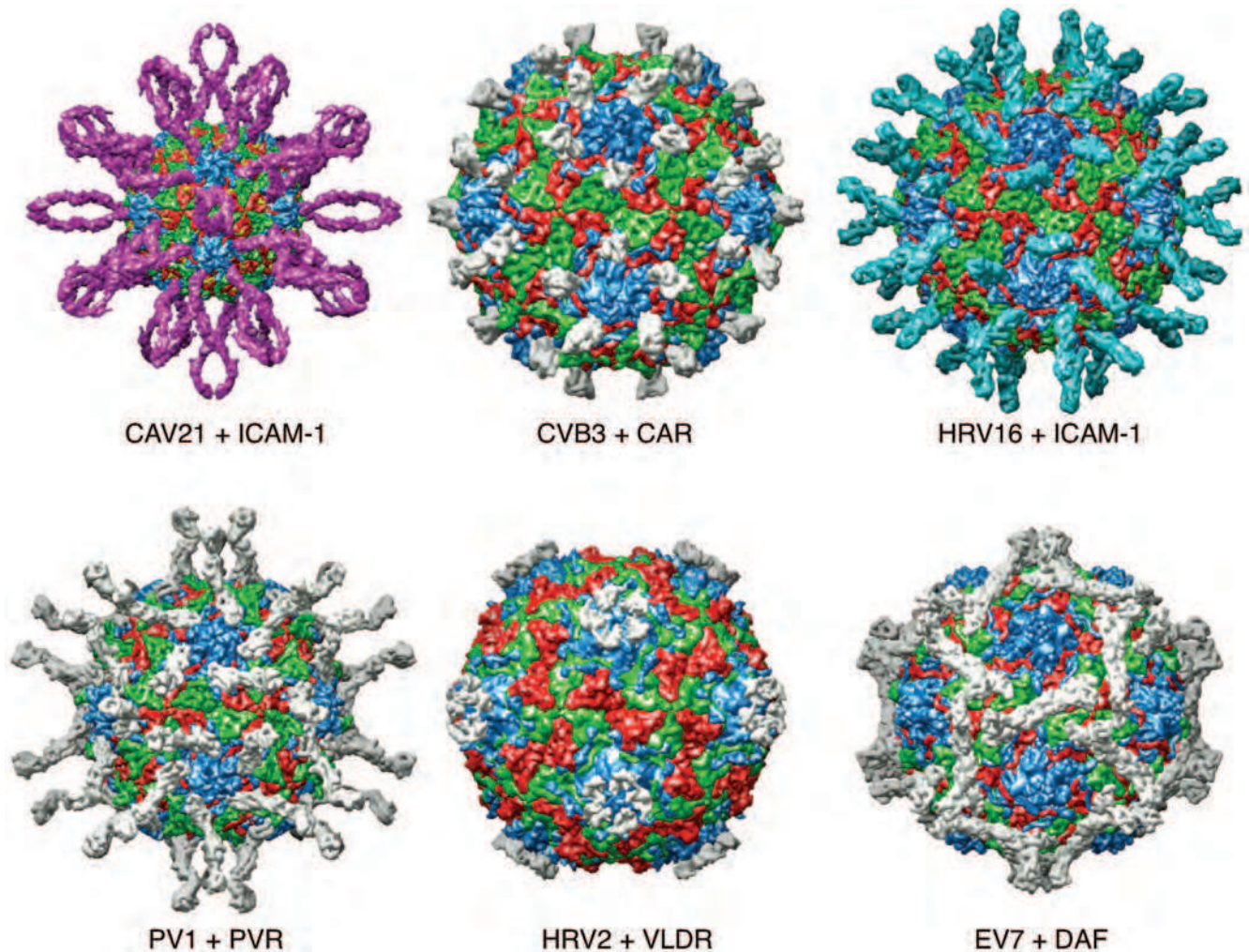


FIGURE 16.5. Interactions of six different picornaviruses with cellular receptors. Immunoglobulin-like cell receptors bind in the canyon, as illustrated for coxsackievirus 21 and ICAM-1, coxsackievirus B3 and CAR, rhinovirus 16 and ICAM-1, and poliovirus type 1 with PVR. A receptor for rhinovirus type 2, very-low-density lipoprotein receptor, binds on the plateau at the fivefold axis, and DAF binds echovirus 7 near the twofold icosahedral axes. Images produced with the Virus Particle Explorer at viberdb.scripps.edu.⁸³

surface of epithelial cells. Coxsackievirus B3 binding to DAF activates Abl kinase, which in turn triggers Rac-dependent actin rearrangements, leading to virus movement to the tight junction where it can bind CAR and enter cells.¹⁰⁵

Enteroviruses that bind integrins typically require a coreceptor for cell entry. For CAV9, it has been suggested that β_2 -microglobulin,⁴⁹⁹ GRP78, and MHC-1⁴⁹⁸ might fulfill this role.

Alternative Receptors

Some viruses bind to different cell surface receptors, depending on the virus isolate or the cell line (Table 16.3). Clinical isolates of FMDV bind to integrin receptors, but passage in cell culture can select for viruses that bind to heparin sulfate, a sulfated glycan.^{236,320,443} Cell culture passage may also produce a virus that infects cells independent of heparin sulfate and integrins.³³

How Picornaviruses Attach to Cell Receptors

Among the picornavirus members, the four capsid proteins are arranged similarly, but the surface architecture varies. These dif-

ferences account for both the diverse serotypes and the varied modes of interaction with cell receptors. For example, the capsids of enteroviruses have a groove, or canyon, surrounding each fivefold axis of symmetry. In contrast, coronaviruses and aphthoviruses do not have canyons.

The canyons of enteroviruses are the sites of interaction with Ig-like cell receptors. The results of genetic and structural experiments demonstrate that the first Ig-like domain contains the site that binds poliovirus. Cells expressing the first Ig-like domain of PVR, either alone or as a hybrid with other Ig-like proteins, are susceptible to infection with poliovirus.^{281,342,450,451} Mutations in the first Ig-like domain of PVR interfere with poliovirus binding.^{21,60,343} Mutagenesis of ICAM-1 DNA has revealed that the binding site for rhinovirus is located in the first Ig-like domain.^{325,420,472} Models of the interaction of poliovirus, rhinovirus, and Coxsackievirus with their cellular receptors have been produced from cryo-EM and x-ray crystallographic data (Fig. 16.5).^{51,53,84,85,207,283,372,536} These models reveal that only domain 1 of PVR or ICAM-1 penetrates the

canyon of the respective virus. Mutations that affect receptor binding map to the virion–receptor interface as determined by these structural studies. Mutation of amino acids that line the canyons of poliovirus and rhinovirus can alter the affinity of binding to receptors.^{100–102,204,300}

Although the capsids of at least two minor group rhinoviruses possess a canyon around the fivefold axes, it is not the binding site for the minor group receptor, members of the low-density lipoprotein receptor family (Fig. 16.5). Rather, the minor group receptor binds close to the fivefold axis, on the star-shaped plateau that is surrounded by the canyon.^{213,413,418,512} In this way multiple low-affinity interactions are combined to yield a high-avidity virus–receptor complex.

Sequence and structural comparisons have revealed why major and minor group rhinoviruses recognize different receptors. A lysine at position 224 of VP1, which is conserved in all minor group rhinoviruses, is the key amino acid that interacts with a negatively charged cluster of LDLR.⁵¹² The electrostatic attraction between Lys1224 and the acidic cluster in LDLR might initiate contact between virus and receptor. Neighboring hydrophobic and basic residues in VP1 could then lead to tight binding between virus and receptor. The conserved lysine is not present in VP1 of major group rhinoviruses, providing an explanation for failure of these viruses to bind LDLR. An exception is rhinovirus 85, a major group serotype that has the conserved lysine; presumably, it does not bind LDLR because of other amino acid differences in neighboring hydrophobic and basic VP1 residues.

It was originally believed that the picornavirus canyons were too deep and narrow to allow penetration by antibody molecules, which contain adjacent immunoglobulin domains.⁴³⁶ This physical barrier was believed to hide amino acids crucial for receptor binding from the immune system. Structural studies of a rhinovirus–antibody complex, however, revealed that antibody does penetrate deep into the canyon, as does ICAM-1.⁴⁶³ The shape of the picornavirus canyon, therefore, is not likely to play a role in concealing virus from the immune system.

In contrast to the Ig-like receptors that bind the canyons of enteroviruses, the binding sites for DAF on the virion are diverse. For example, DAF bridges the canyon of Coxsackievirus B3.¹⁹⁶ In contrast, the binding site of DAF on echovirus 7 (Fig. 16.5) and 12 is near the twofold axis.^{390,402} These interactions are not sufficient for virion uncoating. Coxsackievirus A21 must also bind ICAM-1, which inserts into the canyon, triggering capsid uncoating.^{453,534} Similarly, Coxsackievirus B3 binds DAF but virion uncoating does not occur unless CAR binds in the canyon.²⁰⁸

Integrin-binding picornaviruses attach to cell receptors through surface loops. In FMDV, an Arg-Gly-Asp sequence in the flexible, exposed β G- β H loop of the capsid protein VP1 is recognized by integrin receptors on cells.^{121,147,306} Arg-Gly-Asp-containing peptides block attachment of FMDV,⁴⁴ and alteration of this sequence interferes with virus binding.³²² In Coxsackievirus A9, the Arg-Gly-Asp sequence is present in a 17–amino acid extension of the C-terminus of VP1 and is also the site of attachment to cell receptors.^{87,431} Alteration of this sequence does not abolish binding to cells, suggesting that the virus can bind to another cell surface receptor.²²⁸ Echovirus 1 is unusual in that it binds the RGD-independent integrin $\alpha_v\beta_1$ in the canyon.⁵³⁷

As discussed, FMDV binds alternative receptors, either integrin or heparan sulfate, depending on the virus isolate. The binding site for heparan sulfate on cell culture–adapted FMDV

is a shallow depression on the virion surface, where the three major capsid proteins, VP1, VP2, and VP3, are located.¹⁵² Binding specificity is controlled by two preformed sulfate-binding sites on the capsid. Residue 56 of VP3 is a critical regulator of receptor recognition. In field isolates of the virus, this amino acid is histidine. Adaptation to cell culture selects for viruses with an arginine at this position, which forms the high-affinity, heparan sulfate-binding site.

The interaction of EMCV with its cellular receptor, VCAM-1, has not been studied in detail. The EMCV capsid does not have a canyon, therefore VCAM-1, an Ig-like protein, must interact with the virion in a manner different from the Ig-like receptors of poliovirus and rhinovirus.

Kinetics and Affinity of the Virus–Receptor Interaction

The affinity and kinetics of picornaviruses binding to soluble forms of their receptors have been studied by surface plasmon resonance. Two classes of receptor-binding sites, with distinct binding affinities, exist on the capsids of poliovirus and rhinovirus.^{84,326,536} The association rates for the two binding classes are 25 and 13 times higher for the poliovirus–sPVR interaction than for the rhinovirus–sICAM interaction at 20°C. The greater association rate of poliovirus and PVR may be caused, in part, by differences in the extent of contact between virus and receptor. In contrast, whereas two dissociation rate constants exist for the poliovirus–PVR interaction, only one has been reported for the rhinovirus 3–ICAM interaction. The dissociation rates for the poliovirus–sPVR interaction are 1.5 times and 2.0 times faster than for the rhinovirus–sICAM interaction, indicating greater instability of the former complex. The affinity constants for the poliovirus–sPVR interaction are 19 times and 6 times greater than those reported for the rhinovirus–sICAM-1 complex.

In contrast to the observations with poliovirus and rhinovirus, a single class of binding site was found on echovirus 11 for a soluble form of its receptor, CD55.²⁹² The affinity of this interaction is at least fourfold lower than either of the binding sites on poliovirus for sPVR. The association rate for the interaction between echovirus 11 and CD55 is faster than that of poliovirus–sPVR and rhinovirus–sICAM-1. One explanation for these findings is that the contact between echovirus 11 and CD55 is more extensive than that of the other two virus–receptor complexes. The binding site for CD55 on echovirus 11 may also be more accessible than those of PVR and ICAM-1. The dissociation rate for the echovirus–CD55 interaction is at least 97 times faster than that of either the poliovirus–sPVR or the rhinovirus–sICAM-1 interaction. These findings are consistent with a more accessible binding site for CD55 on echovirus 11, compared with the receptor-binding sites on poliovirus and rhinovirus. Atomic interactions between CD55 and echovirus 11 may be weaker than between the other two viruses and their receptors. The faster dissociation rate of the echovirus 11–CD55 complex may be related to the finding that the interaction does not lead to structural changes of the virus particle,⁴⁰⁹ as occurs with poliovirus and rhinovirus. In general, there is a higher affinity for virions of receptors that can uncoat particles (“unzippers”—poliovirus/PVR, HRV/ICAM-1, CVB3/CAR) compared with attachment receptors (E6, 7, 11, 12, CVB3 with DAF). This may reflect the requirement for higher affinity to release the viral RNA.

Why do poliovirus and rhinovirus have two classes of receptor-binding sites? The receptors for both viruses make contacts at two major sites on the virus surface, one in a cleft on the south rim of the canyon, and a second on the side of the mesa on the north rim. These two contact sites may correspond to the two classes of binding sites. Two classes of binding sites may also be a consequence of the structural flexibility exhibited by both viruses, which may cause exposure of different binding sites. Normally internal parts of the poliovirus and rhinovirus capsid proteins have been shown to be transiently displayed on the virion surface, a process called *breathing*.^{296,298} As to be discussed later, the interaction of poliovirus and rhinovirus with their cellular receptors leads to irreversible and more extensive structural changes. In contrast to the findings with poliovirus and rhinovirus, binding of echovirus 11 with CD55 can be described by a simple 1:1 binding model. Such behavior, which would be expected for the interaction of two preformed binding sites, is consistent with the fact that the echovirus–CD55 interaction does not result in detectable structural changes in the capsid.⁴⁰⁹

ENTRY INTO CELLS

Once picornaviruses have attached to their cellular receptor, the viral capsid is brought into the cell by the endocytic pathway, followed by genome release into the cytoplasm, the site of picornavirus replication. For some picornaviruses, interaction with a cell receptor serves only to concentrate virus on the cell surface; release of the genome is a consequence of low pH or perhaps the activity of a coreceptor. For other picornaviruses, the cell receptor is also an unzipper and initiates conformational changes in the virus that lead to release of the genome.

Entry by Clathrin-Mediated Endocytosis

Several lines of evidence indicate that FMDVs enter cells by clathrin-mediated endocytosis. Infection is inhibited by sucrose, which eliminates clathrin-coated pits and induces clathrin to polymerize into empty cages, and by expression of a dominant negative form of the clathrin coat assembly protein AP180 that is needed for assembly of clathrin cages.⁶¹ Confocal microscopy also revealed that FMDV enters cells via a clathrin-dependent mechanism.³⁶⁶ There is also some evidence that entry is dependent on cholesterol in the plasma membrane, a requirement generally observed for lipid rafts³¹⁹; however, cholesterol might be required for clathrin-induced membrane curving.⁴²⁸ Another aphthovirus, equine rhinitis A virus, binds sialic acid-containing receptors but also enters into cells via clathrin-mediated endocytosis.¹⁸⁵

LDLR family members that are receptors for minor group HRVs possess C-terminal cytoplasmic domains with tyrosine- and di-leucine-based internalization signals that lead to clustering of the receptors in clathrin-coated pits.³⁸⁴ Evidence for HRV entry via this pathway includes the inhibition of infection in cells producing dominant negative inhibitors of the clathrin pathway.^{47,465} There is some evidence that ICAM-1 binding major group HRVs also enter cells via clathrin-mediated endocytosis, including transmission EM, which shows virions in clathrin-coated pits and vesicles 5 minutes after infection¹⁸⁷ and the fact that dominant negative dynamin inhibits infection.¹¹⁸ HRV infection also activates signaling pathways with

links to the endocytic machinery. The cytoplasmic domain of ICAM-1 binds the adaptor protein ezrin, which links the receptor to Syk, a tyrosine kinase.^{519a} When HRV binds ICAM-1, Syk is recruited from the cytoplasm to the plasma membrane together with clathrin. Functional Syk is required for HRV entry via ICAM-1.²⁹¹

When virions enter cells by clathrin-dependent endocytosis they encounter low pH, which triggers release of the viral RNA from the capsid. A role for low pH in infection can be demonstrated by determining the effect on entry of compounds that block acidification, such as weak bases (ammonium chloride, chloroquine, methylamine) ionophores (monensin, nigericin, X537A) or inhibitors of the vacuolar proton ATPase (concanamycin A, bafilomycin A). The pH of early endosomes is 6.5; as these vesicles mature to late endosomes the pH drops to 5.5. Endosomal maturation is dependent not only on vacuolar ATPases but also on microtubules and membrane GTPases of the Rab family. Early to late endosome maturation can be inhibited by drugs that depolymerize microtubules (nocodazole), dominant negative Rabs, or inhibitors of PI3K signaling (wortmannin). In this way it is possible to determine if viral entry occurs from early or late endosomes.

Uncoating by FMDV clearly requires low pH because concanamycin A, monensin, and ammonium chloride all inhibit infection.^{43,61,366,517} Entry occurs from the early endosome as determined by experiments with dominant negative Rab proteins.²⁵⁰ FMDV that bind heparan sulfate enter cells via a caveolae-dependent route, but low pH is still required for infection.³⁶⁷ Consistent with this mechanism of uncoating, FMDV that has been coated with antibody can bind to, and infect, cells that express Fc receptors, in contrast to poliovirus, which cannot productively infect cells via this pathway.³²¹ Cell receptors for FMDV are, therefore, *hooks*: they do not induce uncoating-related changes in the virus particle, but rather serve only to tether the virus to the cell and bring it into the endocytic pathway.

Entry of minor group HRVs requires low pH of the late endosome, as infection is inhibited by monensin, bafilomycin A1, nocodazole, and wortmannin.^{46,59,74,353,410} ICAM-binding HRVs are also sensitive to bafilomycin and monensin, suggesting that endosome acidification is required for entry.^{187,387,483}

Echovirus 7, which binds DAF, enters cells by clathrin-mediated endocytosis, and trafficking into late endosomes is required.²⁷⁰ However, infection does not appear to require low pH, and therefore the trigger for uncoating and its intracellular location remains in question.

Entry by Caveolin-Mediated Endocytosis

As discussed above, CVB3 binds DAF, a GPI-linked protein localized within lipid rafts on the apical surface of polarized cells. The virus/DAF complex then moves to tight junctions (TJ) where it engages CAR. The virus is then internalized along with the TJ protein occludin by caveolin-1-dependent endocytosis.¹⁰⁷ The role of occludin is not known but it could provide a scaffold for recruiting other molecules. Within 60 minutes, the virus is within caveolin-1-containing vesicles (caveolae and caveosomes). Phosphorylation of caveolin-1 by tyrosine kinases is required for CVB3 entry, but the role of this modification is not known. Dynamin is not required for uptake of this virus, suggesting that other routes of entry are involved. Inhibitors of micropinocytosis (rotterin and dominant negative Rab34) block infection, indicating a role for this type of uptake.

Echovirus 1, which binds the integrin $\alpha 2\beta 1$, is taken into the cell by the caveolin-mediated endocytic pathway. The receptor is present in raft-like membrane domains that do not contain caveolin. Internalization of the virus does not depend on dynamin, but components of the macropinocytosis pathway, such as PKC, Pak1, and Rac1, are involved. By 30 minutes after infection, the virus appears in vesicular structures that appear to be caveolae.³¹⁸ These fuse with caveosomes, delivering the virus and its receptor to the perinuclear region.³⁹⁷ These may be novel multivesicular bodies; virus transport to them appears to depend on ESCRT proteins.²⁶³

Caveolin- and Clathrin-Independent Endocytosis

In HeLa cells, poliovirus is taken up into cells by an endocytic pathway that is dependent on actin, ATP, and a tyrosine kinase, but independent of clathrin, caveolin, flotillin, microtubules, and pinocytosis.⁷⁶ RNA release from the particle occurs rapidly and within 100 to 200 nm of the cell surface. Entry was different in a highly polarized human brain microvascular endothelial cell line.¹⁰⁶ Poliovirus enters these cells very slowly via dynamin-dependent caveolar endocytosis. Virus binding to PVR induces tyrosine phosphorylation of the receptor cytoplasmic domain, which in turn recruits and activates SHP-2, which is required for infection. These observations emphasize that virus entry pathways are likely to differ substantially according to cell type.

Uncoating

The interaction of enteroviruses and major group rhinoviruses with susceptible cells leads to the conversion of virions to a

more slowly sedimenting form (135S versus 160S for native particles).^{112,252} The resulting particles, called *altered* (or *A*) *particles*, contain the viral RNA but have lost the internal capsid protein VP4. In addition, the N-terminus of VP1, which is normally on the interior of the capsid, is on the surface of the A particle.¹⁵¹ This sequence of VP1 is hydrophobic and, as a result, the A particles have an increased affinity for membranes compared with the native virus particle. It is believed that A particles represent a stable intermediate structure in the entry process that terminates with exit of RNA and the production of empty (80S) capsids. In one hypothesis for poliovirus entry, receptor binding leads to these conformational changes; the exposed lipophilic N-terminus of VP1 then inserts into the cell membrane, tethering the A particle to the membrane. A membrane pore is then formed, possibly by both VP4 and VP1, through which the viral RNA can travel to the cytoplasm (Fig. 16.6). The finding that A particles, when added to lipid bilayers, induce the formation of ion channels supports this hypothesis.⁴⁹³ The trigger for conversion of minor group HRVs is not receptor binding but low pH.⁴¹⁰

Native poliovirus and rhinovirus particles have been shown to transiently and reversibly expose VP4 and the N-terminus of VP1, a process called “breathing”.^{296,298} Receptor binding (poliovirus) or low pH (rhinoviruses, cardioviruses, aphthoviruses) lowers the energy barrier for conversion to the A particle, a process that enables genome delivery to the cell. The structure of poliovirus bound to a monoclonal antibody that recognizes the N-terminus of VP1 reveals that this viral protein exits the capsid near the twofold axes, instead of near the propeller tip in 135S particles (see below).

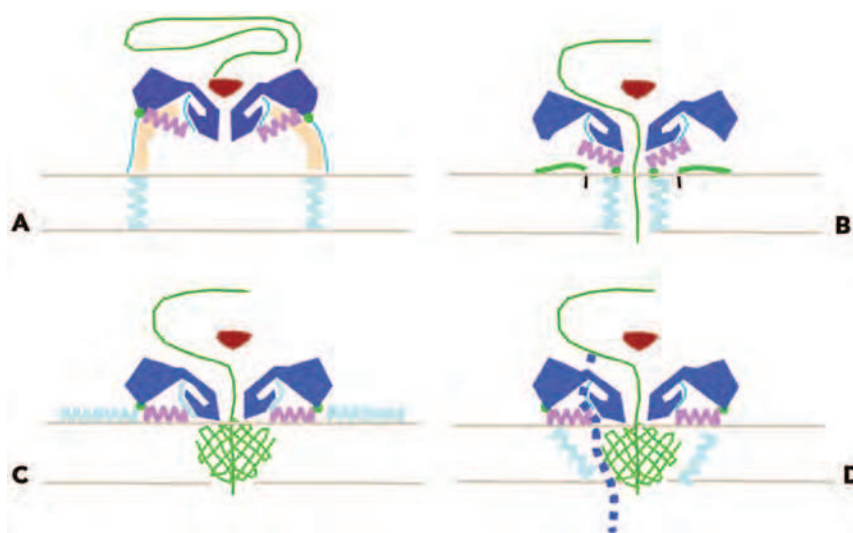


FIGURE 16.6. Hypothetical mechanism for translocation of poliovirus RNA across the cell membrane. **A:** Cross-section of a virus particle that has just bound poliovirus receptor (PVR) at the cell surface. PVR docks on the capsid in the canyon, above the hydrophobic pocket. The path of VP1 egress would not preclude continued binding to PVR. The viral RNA is in the capsid, and lipid has exited from the hydrophobic pocket. The capsid is colored blue, VP4 is green, and the N-terminus of VP1 is cyan and magenta, PVR is tan, RNA is a green line. At this stage the VP3 β -cylinder (red) blocks a channel at the fivefold axis. **B–D** illustrate alternative models for anchoring of virus particle to the cell membrane by the N-terminus of VP1 and formation of a pore for passage of viral RNA. Upon binding, PVR structural changes occur that move the VP3 β -cylinder out of the way like a float valve and open a channel at the fivefold axis that is contiguous with a pore in the membrane. **(B)** Amphipathic helices at the N-terminus of VP1 form a pore through the membrane. In **C** and **D**, VP4 (green mesh) is playing a central role in pore formation. In this case, VP1 may anchor the particle to the membrane. An alternative pathway for release of the genome from the base of the canyon is shown.

Low-resolution, cryo-EM reconstructions of the poliovirus 135S and 80S particles did not reveal openings that could allow for release of the viral genome.⁵² Subsequently, higher-resolution structures of the poliovirus A particle and a derivative in which the N-terminal 31 amino acids of VP1 were removed revealed that this sequence of VP1 is likely to exit the virion at the base of the canyon, between the tips of the “stars” at the fivefold axis and the propeller-shaped feature around the threefold axis.⁷⁸ It has also been suggested that VP4 exits near the twofold axis, based on the observation that insertion of a cysteine in the N-terminus of VP4 leads to disulfide linking upon breathing.²⁶⁶

Cryo-EM reconstructions of 80S particles of HRV2,²¹³ HRV14, and poliovirus²⁹⁵ reveal two populations of particles, one with more internal density (presumably RNA) than the other. The poliovirus structures reveal different particle states: some in the process of releasing RNA, others with nucleic acid in the particle, crossing the particle walls, and outside the particle. Analysis of these particles indicates that the viral RNA exits the capsid from openings at the base of the canyon, near where the release of the VP1 N-terminus occurs.⁷⁰ The trigger for release of RNA from the capsid is unknown, but may require unfolding of secondary structure.⁷⁶

VP4 is released from the virion during conversion to A particles, but a small amount of this protein might remain and participate with VP1 in membrane channel formation. A virus containing an amino acid change at position 28 of VP4 can bind to cells and be converted to A particles, but these are blocked at a subsequent step in virus entry.³⁴⁵ Amino acid changes at this position of VP4 reduce the conductance of ion channels and the translocation of viral RNA.¹¹⁵ These findings suggest that VP4 might play a central role in pore formation. VP4 and VP2 are produced, during virus assembly, from the precursor VP0, which remains uncleaved until RNA encapsidation. Cleavage of VP0, therefore, can be viewed as a way of priming the capsid for uncoating because cleavage separates VP4 from VP2.

While the enterovirus capsid maintains its icosahedral form during uncoating, the acid-labile aphthoviruses dissociate into pentamers at low pH, a process that releases the viral RNA.⁵¹⁰ The mechanism by which low pH causes disassembly of the FMDV capsid has been illuminated by structural and genetic studies. Examination of the atomic structure of the virus revealed a high density of histidine residues lining the pentamer interfaces, which are stabilized by β -sheet interactions.¹ These residues confer stability to the capsid; because the pKa of histidine is 6.8, close to the pH at which the virus dissociates, protonation of the side chains of the histidines might cause electrostatic repulsion, leading to disassembly.¹¹³ To test this hypothesis, a histidine residue at position 142 of VP3 was changed to arginine by mutagenesis. The resulting capsids were more stable at low pH than wild-type capsids,¹³⁴ supporting the proposed role of the histidine residue in acid-catalyzed disassembly. Enteroviruses are stable at low pH in part because there are extra β -sheet interactions in VP1.¹³⁸ Equine rhinitis A virus is also acid labile; it dissociates into pentamers due to rearrangement of loops in VP2 that disrupt the pentamer interfaces.⁵⁰¹ How aphthoviruses breach the endosome membrane is not known; these viruses do not have a hydrophobic VP1 N-terminus, and A particles are not produced.

If aphthoviruses dissociate into pentamers in the endosome, how is the integrity of viral RNA preserved before it exits to the cytoplasm? A clue is provided by the finding that equine rhinitis A virus capsid dissociation is preceded by the formation of a transient empty particle.⁵⁰¹ It is possible that this protects the viral RNA until it leaves the endosome.

Low pH causes particle expansion of minor group rhinoviruses and the opening of a 10 Å diameter pore at the fivefold axis through which the RNA is presumed to exit.²¹⁴

Regulation of Uncoating by Cellular Molecules

Beneath the canyon floor is a hydrophobic pocket that opens at the base of the canyon and extends toward the fivefold axis of symmetry. The pocket appears to be occupied in many picornaviruses with a fatty acid or related compound (Fig. 16.2). In some picornaviruses (e.g., rhinovirus types 3 and 14), the pockets are apparently empty.^{25,550} The hydrophobic pocket appears to be a critical regulator of the receptor-induced structural transitions of enteroviruses. The icosahedral symmetry of the capsid would allow each virion to contain up to 60 lipid molecules. Certain antiviral drugs (e.g., the WIN compounds first identified by Sterling-Winthrop, Inc.) displace the lipid and bind tightly within the pocket.⁴⁶² Binding of such drugs to rhinovirus 14 causes conformational changes in the canyon that prevent attachment to cells.³⁹¹ In contrast, drug binding to rhinoviruses 1A, 3, and 16 and to poliovirus causes smaller structural changes in the capsid.^{182,195,218} Inhibition of rhinovirus 16 binding by these compounds is probably not a consequence of altering the receptor-binding site, but rather the result of preventing conformational changes required for receptor binding. Such compounds do not inhibit binding of poliovirus, but rather uncoating.³³⁰

The lipids that occupy the hydrophobic pocket were originally believed to contribute to the stability of the native virus particle by locking the capsid in a stable configuration and preventing conformational changes. Removal of the lipid was therefore necessary to provide the capsid with sufficient flexibility to undergo the changes that permit the RNA to leave the shell. This hypothesis comes from the study of antiviral drugs, such as the WIN compounds, that displace the lipid and bind tightly in the hydrophobic pocket. These antiviral compounds block breathing of the rhinovirus capsid, the process by which normally internal parts of the capsid proteins are transiently displayed on the virion surface.²⁹⁶ Polioviruses containing bound WIN compounds can bind to cells, but the interaction with PVR does not result in the production of A particles.^{148,548} WIN compounds appear to inhibit poliovirus infectivity by preventing PVR-mediated conformational alterations that are required for uncoating. Additional support for the role of lipids in uncoating comes from the analysis of poliovirus mutants that cannot replicate unless WIN compounds are present.³⁴⁷ Such WIN-dependent mutants spontaneously convert to altered particles at 37°C, in the absence of the cell receptor, probably because of the absence of lipid in the hydrophobic pocket. The lipids can be considered to be switches that determine whether the virus is stable (lipid present) or will uncoat (lipid absent). It is not known what causes lipid release from the capsid. PVR docks onto the poliovirus capsid just above the hydrophobic pocket (Fig. 16.6), which suggests that the interaction of the virus with receptor may initiate structural changes in the virion that lead to the release of the lipid.

Incubation of poliovirus with PVR for short periods at low temperatures appears to result in loss of the lipid.⁵³ The results of computational modeling and kinetic studies suggest that stabilization of virus particles by drugs that replace the lipid is a consequence of increased compressibility rather than increased rigidity.^{395,470,500}

TRANSLATION OF THE VIRAL RNA

Internal Ribosome Binding: The Internal Ribosome Entry Site

Once the picornavirus positive-stranded genomic RNA is released into the cell cytoplasm, it must be translated because it cannot be copied by any cellular RNA polymerase and no viral enzymes are brought into the cell within the viral capsid. Several experimental findings led to the belief that translation of the picornavirus genome was accomplished by an unusual mechanism. The positive-stranded RNA genomes lack 5'-terminal cap

structures; although virion RNA is linked to the viral protein VPg, this protein is removed by a cellular unlinking enzyme on entry of the RNA into the cell.¹³ Furthermore, picornavirus genomes are efficiently translated in infected cells despite inhibition of cellular mRNA translation. Determination of the nucleotide sequence of poliovirus positive-stranded RNA revealed a 741-nucleotide 5'-untranslated region that contains seven AUG codons.^{276,415} Similar 5'-noncoding regions were subsequently found in other picornaviruses and shown to contain highly ordered RNA structures.^{426,461} These findings led to the suggestion that ribosomes do not scan through picornaviral 5'-untranslated regions, but rather bind to an internal sequence. The 5'-untranslated region of poliovirus positive-stranded RNA was subsequently shown to contain a sequence that promotes internal binding of the 40S ribosomal subunit; it was called the *internal ribosome entry site* (IRES) (Fig. 16.7).

All picornavirus RNA contain an IRES, as do other viral and some cellular mRNAs.³¹³ Viral IRES have been placed in four groups based on a variety of criteria, including primary

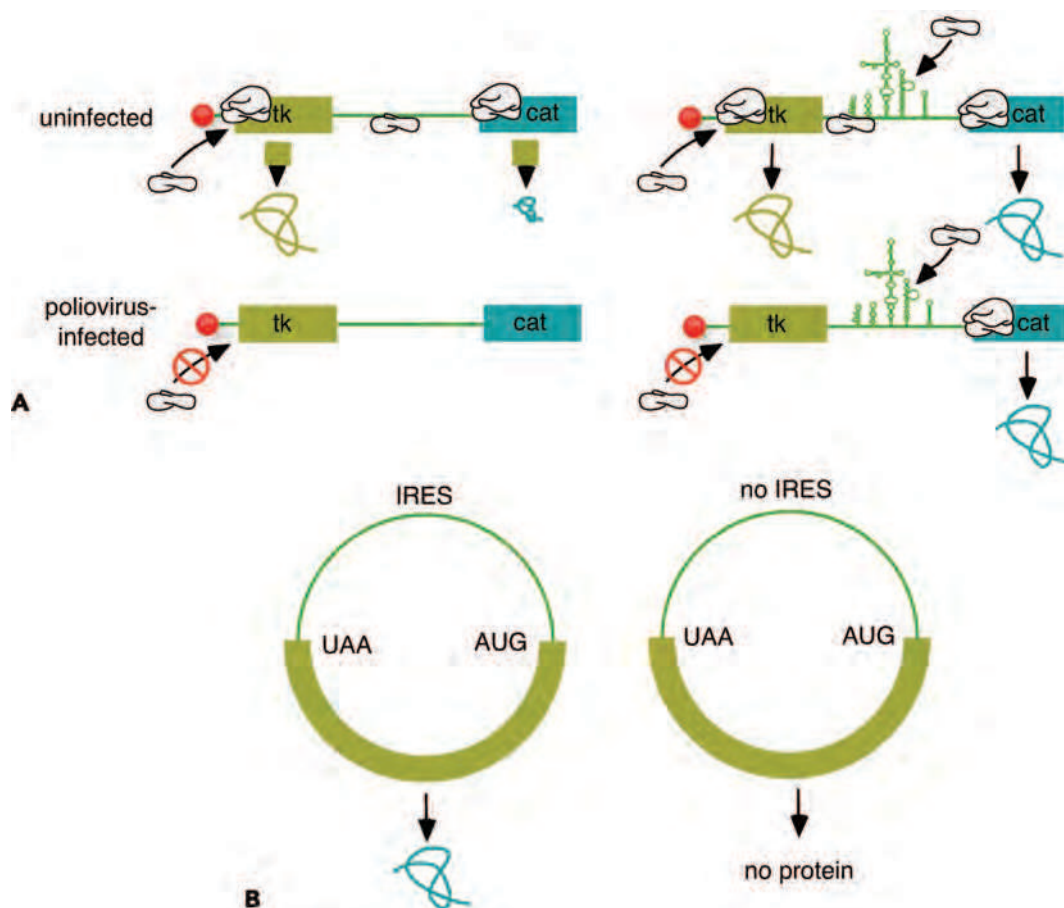


FIGURE 16.7. Discovery of the internal ribosome entry site (IRES). **A:** Bicistronic messenger RNA (mRNA) assay used to discover the IRES. Plasmids were constructed that encode two reporter molecules, thymidine kinase (tk) and chloramphenicol acetyl transferase (cat), separated by an IRES or a spacer. After introduction into mammalian cells, the plasmids give rise to mRNA of the structure shown in the figure. In uninfected cells (*top line*), both reporter molecules can be detected, although cat synthesis is inefficient without an IRES and is probably caused by reinitiation. In poliovirus-infected cells, 5' end-dependent initiation is inhibited, and no proteins are detected without an IRES, demonstrating internal ribosome binding. **B:** Circular mRNA assay for an IRES. Circular mRNA were constructed and translated *in vitro* in cell extracts. In the absence of an IRES, no protein is observed because 5'-end initiation requires a free 5' end. Inclusion of an IRES allows protein translation from the circular mRNA, demonstrating internal ribosome binding.

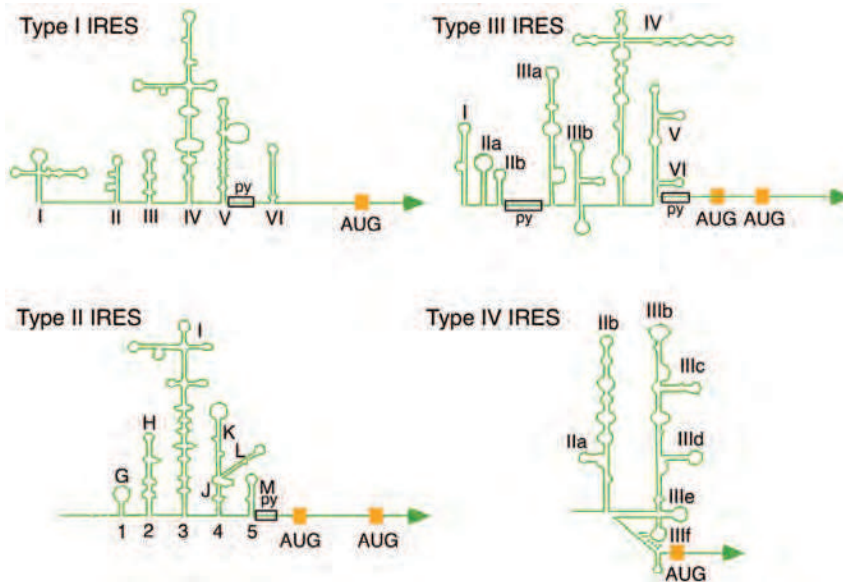


FIGURE 16.8. Four major types of picornaviral internal ribosome entry sites (IRES). The type I IRES is found in the genomes of enteroviruses. The genomes of cardioviruses and aphthoviruses contain a type II IRES. The IRES of hepatitis A virus is type III, and the type IV IRES is represented by porcine teschovirus 1; other picornaviruses with this type of IRES include simian virus 2, porcine enterovirus 8, simian picornavirus type 9, avian encephalomyelitis virus, and Seneca Valley virus.

sequence, secondary structure, location of the initiation codon, and activity in different cell types (Fig. 16.8). In the type I IRES (found in the genomes of enteroviruses and rhinoviruses), and the type III IRES (hepatitis A virus), the initiation codon is located 50 to 100 nucleotides beyond the 3'-end of the IRES, whereas it is located at the 3'-end of a type II IRES (cardioviruses and aphthoviruses). The IRES of porcine teschovirus, avian encephalomyelitis virus, and hepatitis C virus (a member of the *Flaviviridae*) are classified as a type IV IRES.

There is little nucleotide sequence conservation among different IRES elements. The picornavirus IRES contains extensive regions of RNA secondary structure (Fig. 16.7) that is not strictly conserved but is crucial for ribosome binding. One sequence motif that is conserved among picornavirus IRES is a GNRA sequence (G, guanine; N, any nucleotide; R, purine; A, adenine) in stem-loop IV of the type I IRES and in stem-loop I of the type II IRES. Another conserved element is an Yn-Xm-AUG motif, in which Yn is a pyrimidine-rich region and Xm is a 15- to 25-nucleotide spacer followed by an AUG codon. Translation initiation mediated by a type I IRES involves binding of the 40S ribosomal subunit to the IRES and scanning of the subunit to the AUG initiation codon. The 40S subunit probably binds at the AUG initiation codon of a type II IRES.

The type III IRES has little homology with type I and type II IRES except for the Yn-Xm-AUG motif. It is structurally distinct, consisting of two major domains, and in addition requires intact eIF4F complex.¹¹ The type IV IRES functions in a prokaryotic-like manner. The secondary structures of these RNA elements facilitate direct interaction with the small 40S ribosome.^{143,144,242,468} These interactions place a portion of the RNA within the P site of the 40S ribosomal subunit as observed by *in vitro* toeprinting and structural analyses of RNA-protein complexes. Initiation of translation mediated by the type IV IRES is therefore independent of all canonical translation proteins including the ternary complex of eIF2 α -GTP-met-tRNAi. The only initiation protein required is DHX29, necessary to unwind secondary structure surrounding the

initiation codon.^{401,546} No met-tRNAi is required, as the first amino acid of this polypeptide is glycine.²⁴²

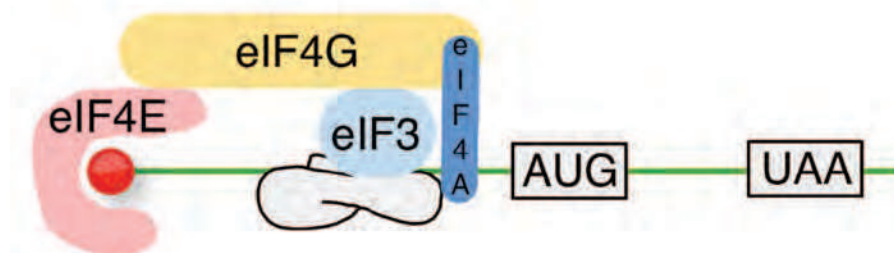
The IRES of Aichi virus is distinct from types I, II, and III IRES and may represent a distinct class.⁵⁴⁶ Domain I is not related to elements found in any other IRES. Domain J consists of a long, interrupted basal helix and an apical four-way helical junction, similar to but smaller than domain IV in type I IRES. Its apical subdomain (Jb) also includes a GNRA tetraloop, which is essential for the function of type I and type II IRES. The apex of AV domain K contains an element identical to an apical motif in domain J of type II IRES that is essential for specific interaction with eIF4G.^{42,95} These domains are otherwise unrelated. An equivalent of domain K of type 2 IRES is also absent. Finally, the initiation codon is preceded by an Yn motif as in the type I/II IRES, but in contrast, it is sequestered in a long, stable hairpin, explaining why this IRES requires the DExH-box protein DHX29.

Mechanism of Internal Ribosome Binding

Different sets of translation initiation proteins are needed for internal initiation mediated by various IRES. Internal ribosome binding via the type III IRES requires all of the initiation proteins, including eIF4E. A subset of translation initiation proteins is required for the activity of most picornavirus IRES.

Translation initiation via a type I IRES involves binding of the 40S ribosomal subunit to the IRES, followed by scanning of the subunit to the initiation codon. The 40S subunit may bind directly to the RNA, or might be recruited to the IRES by interacting with translation initiation proteins. In cells infected with some picornaviruses, eIF4G is cleaved, inactivating the translation of most cellular mRNA. This observation led to the belief that eIF4G is not required for function of the IRES of poliovirus and other picornaviruses. The C-terminal fragment of eIF4G, which contains binding sites for eIF3 and eIF4A (Fig. 16.9), however, stimulates IRES-mediated translation.^{79,371} These findings have led to a model in which the 40S ribosomal subunit is recruited to the IRES through interaction with eIF3 bound to the C-terminal domain of eIF4G, which

5'-end dependent



IRES-dependent

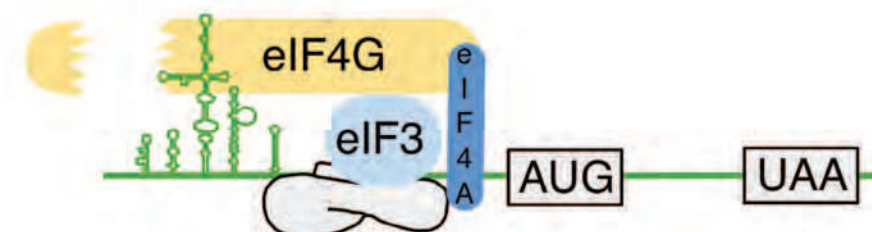


FIGURE 16.9. Models for translation initiation complex formation. In 5′ end–dependent initiation, the 40S subunit is recruited to the messenger RNA (mRNA) through its interaction with eIF3, which binds eIF4G. The latter initiation factor is part of eIF4F, which also contains eIF4A, a helicase to unwind RNA secondary structure, and eIF4E, the cap-binding protein. Binding of eIF4E to the cap thus positions eIF4E at the 5′ end and positions the 40S subunit on the mRNA. In IRES-dependent translation, a 5′ end is not required. The eIF3–40S complex is believed to be recruited to the RNA by the interaction of eIF4G with the IRES.

binds directly to the IRES. Stimulation of IRES function by the C-terminal proteolytic fragment of eIF4G may explain the observation that IRES activity is enhanced in cells in which the poliovirus protease 2A^{pro} is expressed.²⁰⁰ Protease 2A^{pro} is one of the picornaviral proteinases responsible for cleavage of eIF4G. Although the IRES of most picornaviruses function with cleaved eIF4G, that of hepatitis A virus requires intact eIF4G.⁶⁹ The 40S ribosomal subunit binds at or near the AUG initiation codon of the type II IRES, and no scanning occurs.

The poliovirus IRES functions poorly in reticulocyte lysates, in which capped mRNA can be translated efficiently. Addition of a cytoplasmic extract to reticulocyte lysates restores efficient translation from this IRES. These observations led to the suggestion that ribosome binding to the IRES requires cell proteins other than the canonical translation proteins. Such proteins have been identified by their ability to bind the IRES and restore internal initiation in reticulocyte lysates (reviewed in¹³⁹). One host protein identified by this approach is the La protein, which binds to the 3′-end of the poliovirus IRES.³³¹ This protein is associated with the 3′-termini of newly synthesized small RNA, including transcripts of cellular RNA polymerase III. La protein is present in low amounts in reticulocyte lysates; addition of the protein to such lysates stimulates the activity of the poliovirus IRES.³³¹ La is a nuclear protein that is relocalized to the cytoplasm in poliovirus-infected cells.⁴⁵⁹ La protein is also required for efficient function of the encephalomyocarditis virus IRES.²⁷²

Polypyrimidine tract-binding protein (PTB) is composed of four RNA binding domains and functions as a regulator of pre-mRNA splicing. It binds the poliovirus IRES²¹¹ and is required by all type I IRES.²³⁰ Removal of this protein from a cell extract with an RNA affinity column inhibits the func-

tion of the FMDV IRES^{358,398} and that of encephalomyocarditis virus, but does not affect translation by 5′ end–dependent initiation.²⁵⁸ The deficiency in the function of these IRES is restored by adding the purified protein back to the lysate. The depleted lysate, however, still supports the function of the IRES from Theiler’s murine encephalomyocarditis virus, another picornavirus. It was subsequently shown that the requirement for polypyrimidine tract-binding protein by the encephalomyocarditis virus IRES depends on the nature of the reporter and the size of an A-rich bulge in the IRES.²⁵⁹ It is thought that PTB protein facilitates initiation via the type I IRES by modulating binding of eIF4G to the viral RNA through the interaction between the RNA-binding motifs 1 and 2 of PTB protein, the bottom of domain V of the type I IRES, and the RNA-binding motifs 3 and 4 within the single-stranded region of the viral RNA surrounding domain V of the IRES.²⁵⁷ Unlike the type I IRES, multiple copies of PTB protein are required to bind the FMDV, EMCV, and other type II IRES. RNA-binding domains 1 and 2 of one PTB molecule binds domain F of the IRES, and RNA-binding domains 3 and 4 bind domains D and E of the IRES. RNA-binding domains 1 and 2 of a second molecule of PTB bind domain K of the IRES, and RNA-binding domains 3 and 4 interact with IRES domains H, I, and L, all with lower affinity.²⁵⁶

HeLa cell extracts also contain unr, an RNA-binding protein with five cold-shock domains that is required for IRES function.²²⁹ Recombinant unr stimulates the function of the rhinovirus IRES in the reticulocyte lysate and acts synergistically with recombinant PTB protein to stimulate translation mediated by the rhinovirus IRES *in vitro*. However, the poliovirus IRES inefficiently mediates translation initiation in unr^{−/−} cells even though PTB is present.⁷³

Ribosome-associated poly r(C)-binding proteins bind at multiple sites within the poliovirus IRES.^{65,155} One binding site for these proteins has been identified within stem loop IV of the poliovirus IRES.⁶⁵ Mutations in this region that abolish binding of poly r(C)-binding proteins cause decreased translation *in vitro*. Furthermore, depletion of poly r(C)-binding proteins from HeLa cell translation extracts results in inhibition of poliovirus IRES function.⁶⁶ When this assay was used to survey a wide range of picornaviral IRES elements, it was found that poly r(C)-binding proteins are required for function of the type I, but not the type II, IRES.⁵¹⁸ A second binding site for poly r(C)-binding proteins has been identified within a cloverleaf RNA structure that forms within the first 108 nucleotides of the positive-stranded poliovirus RNA genome.^{155,377} The interaction of poly r(C)-binding proteins with this part of the RNA has been proposed to regulate whether a positive-stranded RNA molecule is translated or replicated.

The nuclear-cytoplasmic protein SRp20 functions with poly r(C)-binding protein 2 to promote initiation on poliovirus mRNA.⁵⁰ SRp20 binds to the KH3 domain of poly r(C)-binding protein 2. Depletion of SRp20 from cellular lysates by monoclonal antibodies or from cells by short interfering RNAs reduced IRES-mediated translation by 50%. Polysome analysis of infected cells by sucrose gradient fractionation demonstrated that both SRp20 and poly r(C)-binding protein 2 are at least partly associated with translation initiation complexes bound to stem loop V of the poliovirus 5'-UTR.¹⁴⁰

Murine proliferation-associated protein 1 (Mpp-1) is required for the function of the foot-and-mouth disease virus IRES. This protein binds to a central domain of the viral IRES and acts synergistically with polypyrimidine tract-binding protein to increase the binding of eIF4E. It has been suggested that Mpp-1 may determine the tissues in which the IRES functions. To test this hypothesis, a recombinant virus was constructed by replacing the IRES of Theiler's virus with that of FMDV. Theiler's virus replicates in the mouse brain, but the recombinant virus cannot, possibly because of the absence of Mpp-1 in this organ.³⁹⁸

The DEXH-box helicase DHX29 is necessary for the activity of the Aichi virus IRES.⁵⁴⁶ Initially this protein was identified because it enabled efficient 48S complex formation on cellular mRNAs possessing highly structured 5'UTRs.⁴⁰¹ It is believed to be required for proper placement of the ribosome on RNAs possessing prokaryotic-like IRES elements, such as those of classic swine fever virus and the intergenic IRES of CrPV.⁴⁰¹

No single cellular protein has been identified that is essential for the function of all viral IRES. A common property of cellular proteins needed for IRES activity is that they are RNA-binding proteins that can form multimers with the potential to contact the IRES at multiple points. This observation has led to the hypothesis that such proteins may act as RNA chaperones, maintaining the IRES in a structure that permits it to bind directly to the translational machinery.²³⁵ IRES that do not require such chaperones may fold properly without the need for cellular proteins.

Processing of the Viral Polyprotein

Picornavirus proteins are synthesized by the translation of a single, long, ORF encoded by the viral positive-stranded RNA genome, followed by cleavage of the polyprotein by virus-

encoded proteinases (Figs. 16.3 and 16.10). This strategy allows the synthesis of multiple protein products from a single RNA genome. The polyprotein is not observed in infected cells because it is processed as soon as the protease coding sequences have been translated. The polyprotein precursor is processed cotranslationally by intramolecular reactions (in *cis*) in what are called *primary cleavages*, followed by secondary processing in *cis* or in *trans* (intermolecular). All picornavirus genomes encode at least one proteinase, 3C^{pro}/3CD^{pro}, and some encode L^{pro} or 2A^{pro}.

The first protein encoded in the genome of aphthoviruses, cardioviruses, and erboviruses is the L protein (Figs. 16.3 and 16.10). The L protein of aphthoviruses and erboviruses is a proteinase that releases itself from the polyprotein by cleaving between its C-terminus and the N-terminus of VP4.^{217,475} Based on sequence analysis, it was suggested that FMDV L^{pro} is related to thiol proteases.¹⁷⁸ This prediction was supported by the results of site-directed mutagenesis, which showed that Cys-51 and His-148 are the active-site amino acids.^{396,427} The atomic structure of L^{pro} reveals that it consists of two domains, with a topology related to that of papain, a thiol proteinase¹⁸⁹ (Fig. 16.11). The active-site His is located at the top of the central α -helix, and substrate binds in the groove between the two domains. Besides releasing itself from the polyprotein, L^{pro} also cleaves the translation initiation factor eIF4G, causing inhibition of cellular translation.¹¹⁹ The cardiovirus L protein, which does not have proteolytic activity, is released from the P1 precursor by 3C^{pro}.³⁷⁴

In cells infected with enteroviruses (and possibly sapeloviruses), the primary cleavage between P1 and P2 is mediated by 2A^{pro}. Cellular proteins are also cleaved by 2A^{pro}, including eIF4GI, eIF4GII, Pabp, heart muscle dystrophin, and nucleoporins.^{27,86,180,181,191,192,247,268,286,375} In the protein precursor of rhinovirus, poliovirus, and some other enteroviruses, the cleavage site for 2A^{pro} is between tyrosine and glycine. Other sites cleaved by 2A^{pro} include threonine-glycine and phenylalanine-glycine in certain Cocksackieviruses and echoviruses. Based on sequence alignments, it was suggested that the structure of 2A^{pro} would resemble that of small bacterial chymotrypsin-like proteinases (e.g., *Streptomyces griseus* proteinase A) and would possess a catalytic triad consisting of His-20, Asp-38, and an active-site nucleophile of Cys-109 rather than serine.⁴⁸ The results of site-directed mutagenesis and the resolution of the atomic structure of rhinovirus and Cocksackievirus B4 2A^{pro} confirm that these residues comprise the active site and that the fold of 2A^{pro} is very similar to that of *Streptomyces griseus* proteinase A.^{45,388,467,545} (Fig. 16.11). However, 2A^{pro} differs from all known chymotrypsin-like proteinases in that the N-terminal domain is not a β -barrel, but rather a four-stranded antiparallel β -sheet. The larger C-terminal domain contains a six-stranded antiparallel β -barrel. The active-site catalytic triad is located in a cleft between the two domains. Another unusual feature of 2A^{pro} is a tightly bound zinc ion located at the beginning of the C-terminal domain. Biochemical and structural studies indicate that zinc is essential for the structure of the enzyme.^{388,466,516}

The 2A/2B junction of aphthoviruses, avihepatoviruses, cardioviruses, erboviruses, senecaviruses, teschoviruses, Ljungan virus and duck hepatitis virus is cleaved not by proteolysis but by an unusual mechanism called ribosomal skipping.¹²⁶ Changes within the conserved amino acid sequence Asn-Pro-Gly-Pro,

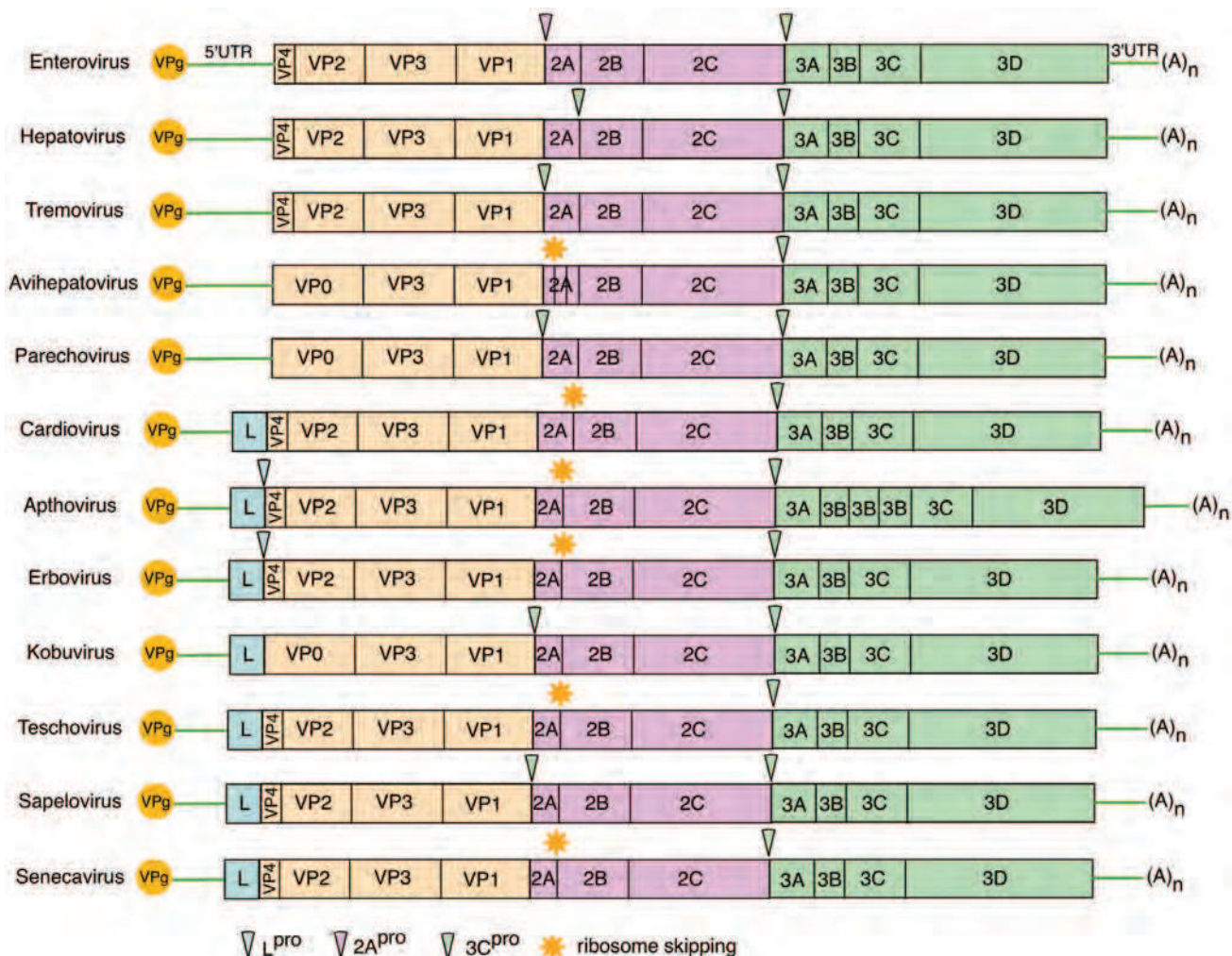


FIGURE 16.10. Primary cleavages of picornavirus polyprotein. In all viruses shown, the P2–P3 cleavage is carried out by 3C^{pro} (green triangle). In some picornaviruses, the P1–P2 cleavage is carried out by 2A^{pro} (magenta triangle) or 3C^{pro}; in others, the 2A/2B bond is separated by ribosome skipping (orange asterisk). The L^{pro} proteinase (blue triangle) of aphthoviruses and erboviruses catalyzes its release from VP4.

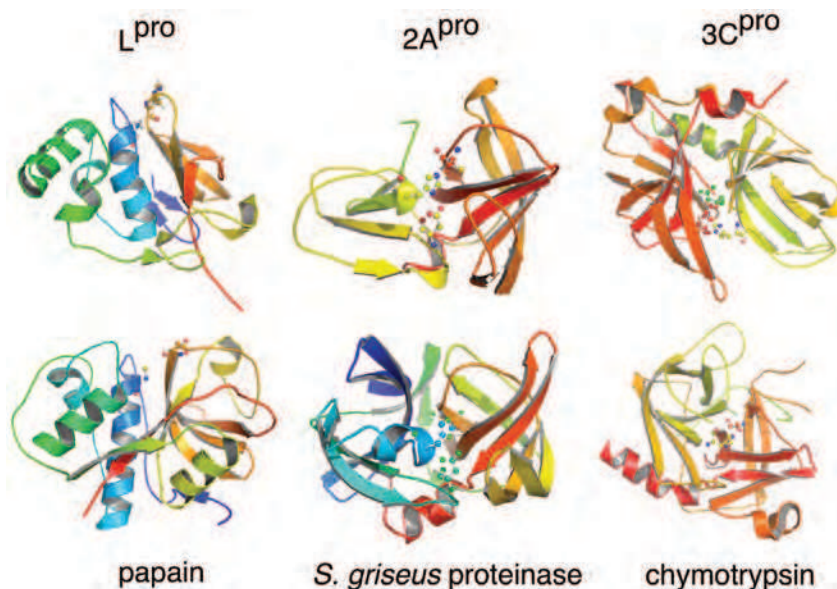


FIGURE 16.11. Three-dimensional structures of L^{pro} of foot-and-mouth disease virus, rhinovirus 2A^{pro}, and hepatitis A virus 3C^{pro}. Below each is the cellular proteinase that is structurally similar to each viral enzyme. Catalytic residues are drawn as balls and sticks. Images drawn with MacPyMol using the following pdb files: 2sga, 2hrv, 2jqg, 1pip, 1hav, 5cha.

which contains the cleavage site Gly-Pro, disrupt cleavage.³⁷⁴ The mechanism of NPG/P-mediated cleavage is not understood. It has been suggested that aphthovirus and cardiovirus 2A might modify the ribosome, causing the translational machinery to skip the synthesis of a glycyl-prolyl peptide bond at the C-terminus of 2A.¹²⁷ If this model is correct, it would represent a novel mechanism for the release of proteins from a polyprotein precursor without enzymatic cleavage. Similar 2A-like sequences have been found in other viral genomes and trypanosome non-LTR retrotransposons.³¹⁰ The ability of the 2A sequence to yield two proteins from a single open reading frame, without proteinase activity, has led to its use in many research and biomedical applications.⁴³⁸

Because only enteroviruses and possibly sapeloviruses have proteolytically active 2A^{pro}, in cells infected with other picornaviruses, the VP1–2A cleavage is either carried out by 3C^{pro}^{245,374,448,449} or in Ljungan virus by an NPG/P sequence following the VP1 protein²⁴⁹ (Fig. 16.10).

The polyprotein of Aichi virus, a member of the *Kobuvirus* genus, is unusual because the L and 2A proteins are not proteinases and there is no NPG/P motif at the 2A/2B junction. The only active proteinase encoded in the genome of this virus is 3C^{pro} and 3CD^{pro}, which can process all cleavage sites in the polyprotein, including the VP1/2A site.⁴⁴⁵ Efficient cleavage of the VP1/2A site requires tight binding of 3CD to the 2A region of the substrate.

All picornaviruses encode 3C^{pro}, which carries out a primary cleavage between 2C and 3A (Fig. 16.10). Unlike the other picornavirus proteinases, 3C^{pro} also carries out secondary cleavages of the P1 and P2 precursors. Poliovirus 3C^{pro} cleaves only at the Gln-Gly dipeptide; however, 3C^{pro} of other picornaviruses has less strict cleavage specificities and cleaves at other sites, including Gln-Ser, Gln-Ile, Gln-Asn, Gln-Ala, Gln-Thr, and Gln-Val. Clearly, other determinants of cleavage exist because not all such dipeptides in picornavirus polyproteins are cleaved by 3C^{pro}. Additional determinants include accessibility of the cleavage site to the enzyme, recognition of secondary and tertiary structures in the substrate by the enzyme, and amino acid sequences surrounding the cleavage site. For example, efficient cleavage of poliovirus Gln-Gly pairs requires an Ala at the P4 position (Gln is residue P1, numbering is toward the N-terminus).⁶⁴

Sequence comparisons with cellular proteinases led to the prediction that 3C^{pro} folds similarly to the chymotrypsin-like serine proteinases, in particular *Staphylococcus aureus* proteinase.^{48,174–177} The putative catalytic triad was believed to consist of His-40, Asp-71 (aphthoviruses and cardioviruses) or Glu-71 (enteroviruses and rhinoviruses), and Cys-147 as the nucleophilic residue, in contrast to serine in cellular serine proteinases. These predictions have been confirmed by site-directed mutagenesis and by resolution of the atomic structures of rhinovirus, hepatitis A virus, poliovirus, FMDV, and enterovirus 71 3C^{pro}.^{89,186,201,267,323,346,388,519} The viral enzyme folds into two equivalent β -barrels like chymotrypsin (Fig. 16.11), but differs in some of the connecting loops, the orientation of the catalytic residues, and in areas needed for transition-state stabilization. The acidic member of the catalytic triad, Glu or Asp, points away from the active-site His and, therefore, is not believed to assist in catalysis. However, 3C^{pro} also binds viral RNA (see discussion of genome replication and mRNA synthesis), and this binding site is distal from the active site of the enzyme.

The presence of this RNA-binding site imposes evolutionary constraints on 3C^{pro} that are not found in other proteinases.

Both 3C^{pro} and 2A^{pro} are active in the nascent polypeptide and release themselves from the polyprotein by self-cleavage. After the proteinases have been released, they cleave the polyprotein in *trans*. The cascade of processing events varies for different picornaviruses. In cells infected with rhinovirus and enteroviruses, the initial event in the processing cascade is the release of the P1 precursor from the nascent P2–P3 protein by 2A^{pro}. The activity of 2A^{pro} does not depend on whether it is cleaved from the precursor,²¹⁰ but further processing of P1 by 3CD^{pro} does not occur unless 2A^{pro} is released from P1.^{357,543} Next, 3CD^{pro} is released from the P3 precursor by autocatalytic cleavage. This proteinase, which contains the entire sequence of the viral RNA polymerase, carries out secondary cleavage of glutamine-glycine dipeptides in poliovirus P1 far more efficiently than 3C^{pro}.^{253,544} Both 3C^{pro} and 3CD^{pro} process proteins of the P2 and P3 regions with similar efficiency. The 3D^{pol} sequence within 3CD^{pro} may be required to recognize structural motifs in properly folded P1, allowing efficient processing by the 3C^{pro} part of the enzyme. The presence of multiple activities in a single protein is not found among eukaryotic proteinases and is an example of how the coding capacity of small viral genomes can be maximized. Not all picornaviruses require 3CD^{pro} to process P1; aphthoviruses, cardioviruses, and hepatoviruses produce 3C^{pro} that can cleave P1 without additional viral protein sequence.

An advantage of the polyprotein strategy is that expression can be controlled by the rate and extent of proteolytic processing. Alternative use of cleavage sites can also produce proteins with different activities. For example, because 3CD^{pro} is required for processing of the poliovirus capsid protein precursor P1, the extent of capsid protein processing can be controlled by regulating the amount of 3CD^{pro} that is produced. Because 3CD^{pro} does not possess RNA polymerase activity, some of it must be cleaved to allow RNA replication.

A final processing step occurs during maturation, when VP0 is cleaved to form VP4 and VP2. This event is discussed later.

VIRAL RNA SYNTHESIS

In the 1950s, it was believed that the genome of RNA viruses was replicated by the cellular DNA-dependent RNA polymerase, through an intermediate DNA strand. The replication of RNA viruses, therefore, was thought to occur entirely in the cell nucleus. In the early 1960s, studies of mengovirus showed that virus infection results in the induction of a cytoplasmic enzyme that can synthesize viral RNA in the presence of actinomycin D.³⁰ This observation suggested that viral genome replication occurred through a virus-specific, RNA-dependent RNA polymerase because cellular RNA synthesis is DNA dependent; it occurs in the nucleus and is sensitive to actinomycin D. A similar cytoplasmic, actinomycin D-resistant genome replication system was discovered in poliovirus-infected cells.²⁸

In poliovirus-infected cells, the positive-stranded genome is amplified to about 50,000 copies per cell through a negative-stranded intermediate. Three forms of viral RNA have been identified in the cell; single-stranded RNA, replicative intermediate (RI), and replicative form (RF). Single-stranded RNA,

the most abundant form, is exclusively positive stranded; free negative strands have never been detected in infected cells. RI is full-length RNA from which six to eight nascent strands are attached. RI is largely of positive polarity with nascent negative strands, although the opposite configuration has been detected. RF is a double-stranded structure, consisting of one full-length copy of the positive and negative strands. Viral RNA synthesis is asymmetric; the synthesis of positive strands is 30 to 70 times greater than the synthesis of negative strands.^{162,364}

Viral RNA-Dependent RNA Polymerase, 3D^{pol}

The first evidence for a viral RNA-dependent RNA polymerase activity came from experiments in which lysates from cells infected with mengovirus or poliovirus were assayed for viral RNA polymerase activity by the incorporation of a radioactive nucleotide into viral RNA.²⁹ Initial experiments demonstrated that the viral RNA polymerase is associated with a cellular membrane fraction, subsequently shown to be comprised of smooth membranes, which was called the *RNA replication complex*.¹⁶⁶ A major component of the replication complex was a viral protein that migrated at 63,000 d on polyacrylamide gels (therefore, called p63), which was suggested to be the viral RNA-dependent RNA polymerase. Other viral and host proteins, including 2BC, 2C, 3AB, and 3C^{pro}, however, were detected in the RNA replication complexes.

A limitation of this early work was that replication complexes only copied viral RNA already present in the complex, and did not respond to added RNA. Attempts were made, therefore, to purify a template-dependent enzyme from membrane fractions of infected cells, using a poly(A) template and an oligo(U) primer. A poly(U) polymerase activity was purified from poliovirus-infected cells, which could also copy poliovirus RNA in the presence of an oligo(U) primer. Highly purified preparations contained only p63, the major viral protein found in membranous replication complexes.^{141,504} This protein is the poliovirus RNA polymerase, now known as 3D^{pol}. In the absence of an oligo(U) primer, 3D^{pol} cannot copy poliovirus RNA. Recombinant 3D^{pol} purified from bacteria or insect cells also requires the presence of an oligo(U) primer to copy poliovirus RNA.^{354,404} 3D^{pol}, therefore, is a template- and primer-dependent enzyme that can copy poliovirus RNA. Its molecular weight predicted from the amino acid sequence is 53 kd.

The structures of three of the four types of polymerases—DNA-dependent DNA polymerase, DNA-dependent RNA polymerase, and RNA-dependent DNA polymerase (reverse transcriptase)—are characterized by analogy with a right hand, consisting of a palm, fingers, and thumb. The palm domain contains the active site of the enzyme. The first structure of an RNA-dependent RNA polymerase is that of poliovirus 3D^{pol}, first determined at 2.6 Å resolution by x-ray crystallography.²⁰² Additional structures of 3D^{pol} from poliovirus, three rhinovirus serotypes, and coxsackievirus B3 provide resolution of virtually all amino acids in the protein, offering a complete picture of the enzyme.^{22,81,307,488} The enzyme has the same overall shape as other polymerases, although the fingers and thumb differ (Fig. 16.12). The fingers and thumb domains interact, resulting in an encircled active site. The palm domain contains four conserved amino acid motifs (B, C, D, E) that are found in other RNA-dependent polymerases. Amino acids 1 to 68 (forming the ring finger) cross the active site under the index finger and

extend to the enzyme surface. The ring finger, which is the top of the channel through which nucleoside triphosphates enter, includes conserved motif F that is important for interacting with the triphosphates. Template and primer molecules that contact the front face of 3D^{pol} probably use the opening for nucleotide exchange, as do other polymerases. A template RNA channel in the fingers domain of all the picornavirus polymerases is lined with basic amino acids that are predicted to interact with viral template as it enters the active site. Alteration of amino acids in this channel identifies key residues for template binding or elongation.²⁸⁴

The RNA polymerase 3D^{pol} is produced by cleavage of a precursor protein, 3CD^{pro}, which is highly active as a proteinase, binds *cre* (see below), but has no polymerase activity. It was suggested that processing of 3CD affected the location of the N-terminus of 3D^{pol}, which is buried in a surface pocket in 3D^{pol}. There it makes hydrogen bonds that position Asp238, an essential residue that selects for the 2' OH group of substrate rNTP (ribonucleoside triphosphate), in the active site. On proteolysis, the N-terminal Gly was believed to push Asp-238 a distance of 1.4 Å into the catalytic site. However, a comparison of structures of 3D^{pol} and 3CD^{pro} did not show a significant difference in the position of Asp238.^{317,488} Resolution of the structure of the coxsackievirus B3 3D^{pol} reveals an unusual conformation for residue 5, which is located at a distortion within a β-strand that is conserved in all known 3D^{pol} structures.⁸¹ Substitution of more hydrophobic residues at this position results in higher enzymatic activity. It has been proposed that this residue becomes buried during the repositioning of the nucleotide that takes place before phosphoryl transfer, which cannot occur if the N-terminus has not been properly generated by proteolytic processing.

The first poliovirus 3D^{pol} structure revealed that the polymerase molecules interacted in a head-to-tail manner and formed fibers; subsequently, the protein was shown to form a lattice.³¹¹ The implication was that RNA would be replicated as it moved along the lattice, rather than the polymerase moving on the template. The head-to-tail fibers were formed by interface I, which involves more than 23 amino acid side chains between the thumb of one polymerase and the back of the palm of another (Fig. 16.13). Amino acid changes in the back of the thumb that disrupt this interface impaired replication.^{120,221} Repetition of this interaction in a head-to-tail fashion results in long fibers of polymerase molecules 50 Å in diameter. A similar interface was observed in crystals of 3D^{pol} of rhinovirus 14 but not rhinoviruses 16 or 1b.³⁰⁷ Interface II is formed by N-terminal polypeptide segments, which may lead to a network of polymerase fibers in combination with interactions at interface I. These N-terminal polypeptide segments may originate from different polymerase molecules. The N-terminus of 3D^{pol} is required for enzyme activity, which would support the idea that interface II interactions are of functional consequence. Furthermore, intermolecular cross-linking has been observed between cysteines engineered at Ala-29 and Ile-441 of poliovirus 3D^{pol}²²¹ and disruption of these interactions led to lethality.⁴⁸⁶ Polymerase-containing oligomeric structures resembling those seen with purified 3D^{pol} were observed on the surface of vesicles isolated from poliovirus-infected cells.³¹¹ Because picornavirus RNA synthesis occurs on membranous vesicles, the concept of a flat, catalytic lattice is mechanistically attractive.

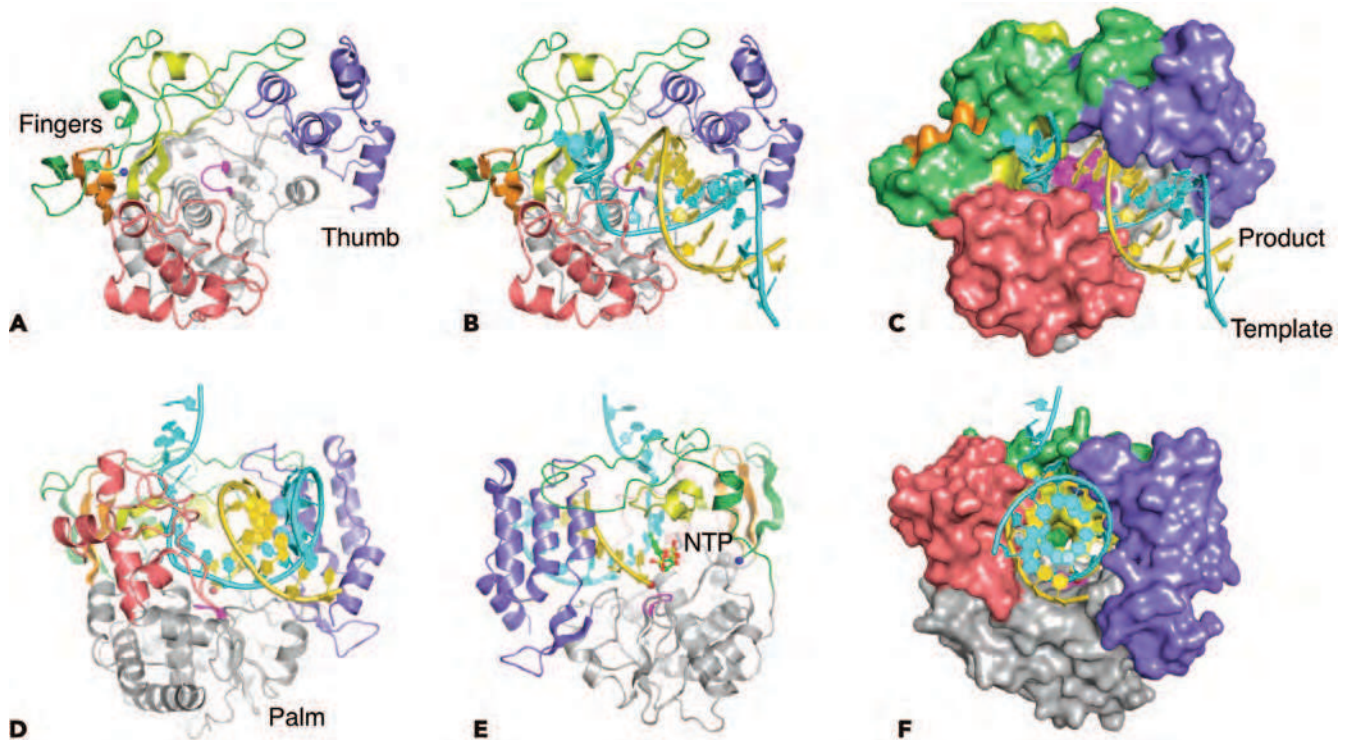


FIGURE 16.12. Three-dimensional structure of poliovirus 3D^{pol}. Structure of poliovirus 3D^{pol} polymerase and its elongation complex with primer-template RNA. The polymerase structure can be described by analogy to a cupped right hand, consisting of palm (gray) and thumb (blue) domains and a fingers domains with four discrete structural elements known as the index (green), middle (orange), ring (yellow), and pinky (red) fingers. The structure of the elongation complex shows the path of the RNA as the template strand (cyan) enters the polymerase from the top and of the template-product (gold) duplex as it exits the polymerase between the pinky finger and thumb structures.²²⁰ **A-C:** Views from the top of the polymerase looking down into the active site (magenta) of 3D^{pol} in the absence of RNA (**A**), the 3D^{pol} elongation complex with bound RNA (**B**), and the elongation complex with a surface representation of the polymerase to show how the product RNA duplex is clamped in place between the pinky finger and thumb structures (**C**). **D:** Front view of the elongation complex showing the path template and product RNA strands. **E:** Back view of the elongation complex showing the NTP entry channel with the priming 3' OH (red sphere) positioned above the active site and a bound di-deoxy-CTP (green ball-and-stick with phosphate groups in red and yellow). **F:** Surface representation of the elongation complex looking down the axis of the exiting RNA duplex. 3D^{pol} from poliovirus and closely related picornaviruses is activated upon cleavage from the viral polyprotein, resulting in a newly created N-terminus that becomes buried in a pocket at the base of the finger domain that is ≈15 Å away from the active site itself (blue sphere in **A** and **E**). (Courtesy of Olve Peersen, Colorado State University.)

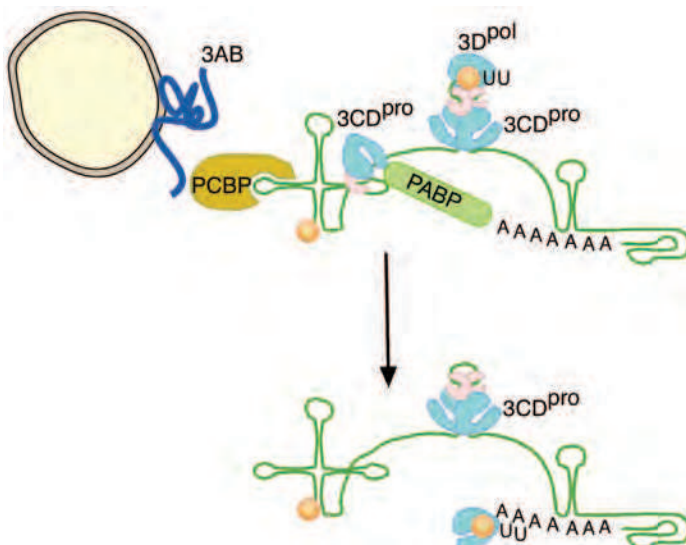


FIGURE 16.13. Model for the synthesis of poliovirus (–) strand RNA. The (+) strand template is shown in green with the 5'-cloverleaf structure, the internal *cre* sequence, and the 3' pseudoknot. A ribonucleoprotein complex is formed when poly r(C)-binding protein (PCBP) and 3CD^{pro} bind the cloverleaf structure. The ribonucleoprotein complex interacts with PABP1, which is bound to the 3'-poly(A) sequence, producing a circular template. Protease 3CD^{pro} cleaves membrane-bound 3AB to produce VPg and 3A. The *cre* sequence binds 3D^{pol}, 3CD^{pro}, and VPg. VPg-pUpU is synthesized by 3D^{pol} using the sequence AAACA of *cre* as template. The complex is transferred to the 3' end of the genome, and 3D^{pol} uses VPg-pUpU to prime RNA synthesis.

Viral Accessory Proteins

The poliovirus capsid proteins are not required for viral RNA synthesis; the region of the viral genome that encodes these proteins can be deleted without affecting the ability of viral RNA to replicate in cells.²⁶⁰ The capsid coding regions of rhinovirus, Theiler's virus, and Mengovirus, however, contain a *cis*-acting RNA sequence required for genome replication.^{304,328,329} A *cis*-acting RNA sequence required for RNA replication has also been identified in the poliovirus protein 2C coding region.¹⁷¹ Genetic and biochemical studies implicate most proteins of the P2 and P3 regions of the genome in RNA synthesis.

2A Protein

As discussed above, 2A^{pro} protein is necessary for proteolytic cleavage of the polyprotein. The protein also appears to have a role in RNA replication. A deletion within the 2A^{pro} coding sequence severely inhibits replication of subgenomic replicons, which lack the capsid region and are not dependent on proteolytic activity of 2A^{pro} to release the P1 region.⁹⁸ In another approach, a second IRES element was placed in the poliovirus genome before the 2A^{pro} coding region, effectively alleviating the need for the processing activity of the enzyme. Such viruses are viable.³³⁹ Deletion of part of the coding region for 2A^{pro} is lethal, however. These results suggest that 2A^{pro} also plays a role in viral RNA replication. By using a cell-free replication system, it was possible to determine the effect of 2A^{pro} on each step of the replication cycle.²⁵⁵ The results show that 2A^{pro} stimulates the initiation of negative-strand synthesis, but has no effect on positive-strand synthesis. How 2A^{pro} achieves this affect is not known, but it has been suggested that the proteinase might modify a cellular protein required for negative-strand RNA synthesis.²⁵⁵

2B Protein

The 2B protein is a small, hydrophobic, membrane-associated protein that is involved at an early step of viral RNA synthesis. Alterations of the 2B protein of poliovirus and coxsackievirus lead to viruses with defects in RNA synthesis.^{251,505} Adaptation of rhinoviruses to mouse cells is mediated in part by amino acid changes in 2B, which allow viral RNA synthesis in this host cell.²⁰⁶ The C-terminus of 2B contains a hydrophobic region and a conserved putative amphipathic α -helix that appear to be crucial to the function of the protein and its association with membranes.⁵⁰⁶ Protein 2B has been called a *viroporin*, a protein that oligomerizes and inserts into membranes to create channels.^{2,170} The exact role of 2B in RNA synthesis, however, is not known. Synthesis of protein 2B leads to an inhibition of protein secretion from the Golgi apparatus^{35,123} and permeabilization of membranes,^{2,7,444,508,509} which may play a role in release of virus from cells. Protein 2B is also partly responsible for the proliferation of membranous vesicles in infected cells, which are the sites of viral RNA replication. Because protein 3A has the same effect, it is not clear whether altered membrane proliferation is responsible for the RNA-defective phenotype in 2B mutants.

2C Protein

Protein 2C is a highly conserved protein with membrane-, RNA-, and NTP-binding regions.^{131,429,430,508,509} The structure of 2C suggests that it contains three domains, with amphipathic α -helices at both the N- and C-termini that mediate peripheral association with membranes^{285,487} and a central

region with NTP-binding domains. Mutations responsible for resistance of poliovirus and echovirus to guanidine hydrochloride, which blocks viral RNA replication, are located in the 2C protein.^{278,399} This compound has been shown to inhibit specifically the initiation of negative-stranded RNA synthesis and has no effect on initiation of positive-stranded RNA synthesis or on elongation on either strand.⁴⁰ The nucleoside triphosphatase (NTPase) activity of protein 2C is inhibited by guanidine.³⁹⁴ Protein 2C shares amino acid homology with known RNA helicases, proteins encoded by most positive-stranded RNA viruses with large genomes. These enzymes are believed to be necessary to unwind double-stranded RNA structures that form during RNA replication. Purified protein 2C, however, does not have RNA helicase activity.⁴²⁹ Alteration of conserved amino acids within the NTPase domain of 2C results in loss of viral infectivity; 2C, therefore, may have two functions during viral RNA synthesis: as an NTPase and directing replication complexes to cell membranes. Synthesis of 2C causes disassembly of the Golgi apparatus and endoplasmic reticulum (ER), and formation of vesicular structures similar, but not identical to, those that constitute the replication complex.^{6,92}

2BC Protein

Much of protein 2BC, the precursor to 2B and 2C, remains uncleaved during infection, and its presence is critical to viral replication.⁹² Synthesis of 2BC causes membrane permeabilization to a greater degree than 2B synthesis alone,⁷ and leads to the formation of vesicles that are more similar to those formed during viral infection than does protein 2C.^{6,92} The C-terminus of 2B and the N-terminus of 2C may interact intramolecularly in 2BC, and protein cleavage may cause a conformational change that alters the properties of 2B and 2C individually.^{35,507} Larger 2BC-containing precursors are also required for certain steps of replication,²⁵⁴ although an intact 2C–3A junction is not strictly required.³⁸⁰

3AB Protein

A strongly hydrophobic region in the C-terminus of 3A mediates the association of 3A and its precursor, 3AB, with membranes.^{124,474,495} Amino acid changes in the hydrophobic region of 3A yield replication-defective viruses.^{163,287} Changes in viral host range have been mapped to changes in 3A protein.^{49,206,287,365} The solution structure of 3A demonstrates that it is a homodimer, with the dimer interface located in the central region of the protein.⁴⁷⁴ The requirement for 3A dimerization during infection has not been explored. Protein 3B, also known as VPg, plays an indispensable role in viral replication by acting as a protein primer for viral RNA synthesis (see below). Protein 3AB is believed to anchor VPg in membranes for the priming step of RNA synthesis. The purified protein greatly stimulates 3D^{pol} activity *in vitro*^{379,405,424} as well as the proteolytic activity of 3CD^{pro}.³⁴⁰ 3AB interacts with 3D^{pol} and 3CD^{pro} in infected cells and with 3D^{pol} in the yeast two-hybrid system.²²⁶ Amino acid changes in 3D^{pol} that disrupt its interaction with 3AB result in viruses with defects in protein processing and viral RNA synthesis.^{161,163} A complex of 3AB and 3CD^{pro} also binds the 3'-terminal sequence of poliovirus RNA.

Cellular Accessory Proteins

Early studies of poliovirus replication using purified components suggested that a host cell protein is required for copying

the viral RNA by 3D^{pol} in the absence of an oligo(U) primer.¹¹⁶ Although this protein was never identified, the concept of a host factor required for poliovirus replication endured, largely because an ample precedent was seen for the participation of host cell components in a viral RNA replicase. The best-studied example is the RNA replicase of the bacteriophage Q β , which is a multisubunit enzyme consisting of a 65-kd virus-encoded protein and three host proteins: ribosomal protein S1 and translation factors EF-Tu and EF-Ts.²⁰³ The 65-kd viral protein has no RNA polymerase activity in the absence of the host factors but has sequence similarity with known RNA-dependent RNA polymerases. A subunit of the translation initiation factor eIF-3 is part of the polymerase from brome mosaic virus.⁴¹² Two different experimental systems have been used to provide additional evidence that poliovirus RNA synthesis requires host cell components.

When purified poliovirus RNA is incubated *in vitro* with a cytoplasmic extract prepared from cultured, permissive cells, the viral RNA is translated, the resulting protein is proteolytically processed, and the genome is replicated and assembled into new infectious virus particles.³³⁸ When guanidine, an inhibitor of poliovirus RNA synthesis, is included in the reaction, complexes are formed, but elongation cannot occur.³⁸ The preinitiation complexes can be isolated free of guanidine and, when added to new cytoplasmic extracts, RNA synthesis occurs. In the absence of cytoplasmic extract, preinitiation complexes do not synthesize viral RNA.³⁷ These results indicate that one or more soluble cellular components are required for the initiation of viral RNA replication. A similar conclusion comes from studies in which poliovirus RNA is injected into oocytes derived from the African clawed toad, *Xenopus laevis*. Poliovirus RNA cannot replicate in *Xenopus* oocytes unless it is coinjected with a cytoplasmic extract from human cells.¹⁵⁴

Cellular poly r(C)-binding proteins are required for poliovirus RNA synthesis. These proteins bind to a cloverleaf-like secondary structure, also called a stem-loop I, that forms in the first 108 nucleotides of positive-stranded RNA (Fig. 16.13). Binding of poly r(C)-binding protein to the cloverleaf is necessary for the binding of viral protein 3CD^{pro} to the opposite side of the same cloverleaf.^{16,18,155,377} Formation of a ribonucleoprotein complex composed of the 5' cloverleaf, 3CD^{pro}, and a cellular protein is essential for the initiation of viral RNA synthesis.¹⁸ The human cell protein required for replication of poliovirus RNA in *Xenopus* oocytes has also been identified as poly r(C)-binding protein.¹⁵⁵ A model of how these interactions lead to viral RNA synthesis is shown in Figure 16.14. The ternary complex with stem-loop I may also function during positive-strand RNA synthesis.⁵¹⁵

Another candidate for a host protein that is essential for poliovirus RNA synthesis is poly(A)-binding protein 1. This protein interacts with poly r(C)-binding protein, 3CD^{pro}, and the 3'-poly(A) tail of poliovirus RNA, circularizing the genome^{40,212} (Fig. 16.13). Formation of this circular ribonucleoprotein complex is required for negative-strand RNA synthesis.

Protein-Primed RNA Synthesis

As discussed, poliovirus 3D^{pol} is a primer-dependent enzyme that will not copy poliovirus RNA *in vitro* without an oligo(U) primer. The discovery of VPg linked to poliovirus genome RNA, as well as to the 5' end of newly synthesized positive-

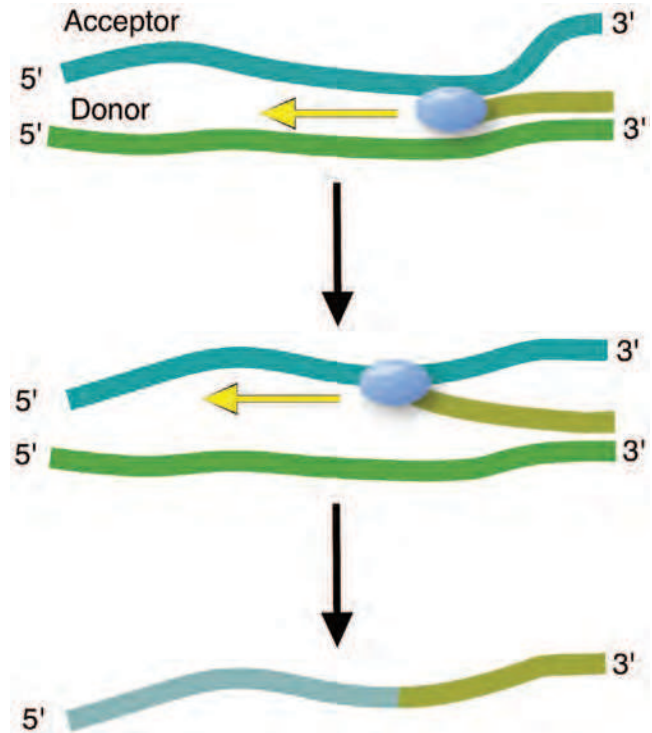


FIGURE 16.14. Schematic diagram of RNA recombination in picornavirus-infected cells by template switching, or copy choice.

Two parental genomes, the acceptor and donor, are shown. The RNA polymerase (blue) is shown copying the 3' end of donor RNA and switching to the acceptor genome (middle). As a result of this template switch, the recombinant RNA shown is formed (bottom).

and negative-stranded RNA, suggested that VPg might be involved in the initiation of RNA synthesis. This hypothesis was supported by the finding that both VPg and a uridylylated form of the protein, VPg-pUpU, can be found in infected cells.¹¹⁰ Furthermore, VPg-pUpU can be synthesized *in vitro* in a membrane fraction from poliovirus-infected cells.⁴⁸¹ It was also argued that a precursor of VPg, known as 3AB, is likely to participate in the initiation reaction. 3AB is a membrane-bound polypeptide and, therefore, an ideal candidate to act as a VPg donor in membranous replication complexes. Viruses containing a mutation in 3AB that decreases its hydrophobicity are defective in initiation of RNA synthesis, *in vitro* uridylylation of VPg, and *in vivo* synthesis of positive-stranded viral RNA.^{161,163} Because no protein larger than VPg has been detected linked to nascent RNA strands, it is likely that 3AB is rapidly cleaved by the proteinase 3C^{pro} to form VPg-linked RNA. Additional evidence that VPg can serve as a primer for poliovirus RNA synthesis comes from experiments in which synthetic VPg is first uridylylated *in vitro*, then added to an *in vitro* polymerase reaction containing a poly(A) template and 3D^{pol}.³⁸¹ The labeled, uridylylated VPg is extended to form poly(U). Poliovirus RNA replication is primed by a genome-linked protein, a mechanism also involved in adenovirus DNA replication. A model for VPg priming of poliovirus RNA synthesis is shown in Figure 16.13.

The template for uridylylation of VPg is an RNA hairpin, the *cis*-acting replication element, *cre*, located in the coding

region of picornaviruses.^{382,425,540} The cre functions independent of position in the genome, and its location differs among viruses. Protein 3C is a major determinant of RNA binding activity, but 3AB may also be involved in cre recognition.^{378,533} Whether cre-dependent VPg uridylation is involved only in (+) strand RNA synthesis, or also during synthesis of the (–) strand, is controversial.^{173,341,350,425}

The results of genetic analyses indicate that the binding site for VPg (as part of 3AB) on 3D^{pol} is on the surface of the enzyme near conserved motif E, on the back side of the palm near the base of the thumb, which is distant from the catalytic center.³¹² Because uridylation of VPg takes place at the same polymerase active site used for chain elongation, it must occur through the opposite side of the nucleotide channel used for chain elongation. How the uridylated VPg is transferred to the front side of the polymerase for use in priming RNA synthesis is not known. The primer might be transferred through the nucleotide channel, or, as suggested by experimental results, to another polymerase molecule.⁴⁸⁵ Resolution of the structure of rhinovirus 3D^{pol} suggested that VPg could bind via the front face of the polymerase.²² However, structure of the FMDV 3D^{pol} bound to VPg revealed that the protein does not bind to the active site, consistent with the results of genetic analyses. Whether there are virus-specific mechanisms of VPg priming remains to be determined.

Cellular Site of RNA Synthesis

Picornaviral RNA synthesis, like that of most RNA viruses, occurs in the cytoplasm of the cell. Picornavirus infection leads to the proliferation and rearrangement of intracellular membranes in infected cells. The ER and Golgi apparatus are destroyed in this process, and the cytoplasm fills with double-membraned vesicles.^{115,447} Viral RNA replication occurs on the cytoplasmic surfaces of these vesicles.^{63,92,133} Membrane localization of viral RNA replication proteins may ensure high local concentrations of replication components, increasing the rates or efficiencies of replication reactions. It has also been suggested that membrane localization of viral RNA replication proteins could promote their oligomerization.³¹¹

Poliovirus proteins 3A and 2BC are sufficient to induce the formation of vesicles that are biochemically and ultrastructurally similar to those observed in virus-infected cells.⁴⁷⁶ Because viral proteins 2A and 2BC are known to localize to the ER, a plausible hypothesis is that they promote vesicle formation from this site.¹²⁴ One source of virus-induced vesicles may be those that participate in ER-to-Golgi trafficking. Budding of vesicles from the ER is initiated by COPII coats; these fuse to create the ER-Golgi intermediate compartment (ERGIC). COPI coats initiate budding of vesicles from the ERGIC that move to the Golgi. It has been suggested that poliovirus-induced vesicles are morphologically similar to COPII vesicles, which are involved in anterograde transport.⁴⁴² The COPII proteins Sec13/31 were shown to colocalize with the viral 2B protein on the surface of the vesicles. The inhibition of poliovirus and rhinovirus RNA replication by brefeldin A, however, apparently contradicts this hypothesis.^{158,233,324} Brefeldin A interferes with formation of COPI, but not COPII or autophagic vesicles, by inhibiting the exchange of guanosine triphosphate (GDP) for guanosine triphosphate (GTP) by the adenosine diphosphate (ADP) ribosylation factor, Arf. Brefeldin prevents COPI coat assembly by targeting the Arf1 guanine exchange factors

GBF1, BIG1 and BIG2. A brefeldin-resistant form of GBF1 was found to allow poliovirus replication in the presence of the drug.⁵⁴ Viral protein 3A binds GBF1, recruiting it to replication complexes, and this interaction, which BFA functionally interferes with, is required for replication.^{54,290} When poliovirus proteins are synthesized in cells in the absence of viral RNA replication, membrane remodeling is insensitive to brefeldin A, indicating that the drug inhibits viral replication (by interfering with GBF1), not remodeling of cellular membranes.⁵⁴

The binding of 3A to GBF1 diverts the protein from its normal function in the secretory pathway, and explains inhibition of protein secretion by viral infection.¹²³ The result is an inhibition of COPI vesicle formation; these may be diverted to form viral replication complexes. In support of this hypothesis, components of COPI coats have been found in association with echovirus replication complexes.¹⁵⁸

The vesicles induced during poliovirus and rhinovirus infection may also be derived from the cellular autophagosomal pathway. In response to a variety of stimuli, including cellular starvation, cells break down cytoplasmic proteins and organelles within autophagosomes, double-membraned structures that mature and become degradative. The poliovirus-induced vesicles bear several hallmarks of autophagosomes, including their double-membraned structure, the presence of cytoplasmic content within the vesicles,⁴⁴⁷ and colocalization with autophagosomal markers latency-associated membrane protein 1 (LAMP1) and LC3.²³⁹ In support of the autophagosomal origin of virus-induced vesicles, stimulation of autophagy increased virus yield, whereas inhibition of the autophagosomal pathway by drugs or small interfering RNA (siRNA) reduced virus yield.²³⁹ These findings indicate that components of the cellular autophagosomal pathway are subverted to provide membranous supports for viral RNA replication complexes. Autophagosomes are induced during infection with other picornaviruses, including FMDV, enterovirus 71, EMCV, rhinoviruses, and coxsackieviruses (reviewed in²⁷⁷). They are not present in rhinovirus 1A-infected cells,⁴¹⁴ however, and it is not clear if they are needed for replication of rhinovirus 2.⁷⁵ Replication membranes in rhinovirus 1A-infected cells appear to be derived from the Golgi apparatus, whose fragmentation correlates with the presence of the viral 3A protein.⁴¹⁴

A recent study on the three-dimensional architecture of the poliovirus-induced replication complexes revealed that membrane remodeling begins with the formation, often in association with a Golgi antigen, of a network of irregularly shaped, single-membrane, branching tubular structures.⁵⁵ Later in infection these become double-membraned structures, but the highest rates of viral RNA synthesis occur when the mainly single-membraned convoluted tubules are present.

At least two viral proteins, 2C and 3AB, bring the replication complex to membranous vesicles. As discussed, 3AB is a hydrophobic protein that anchors the protein primer VPg in the membrane for RNA synthesis. Protein 3AB binds 3D^{pol} and 3CD^{pro}, thereby recruiting the replication complex to membranes. Protein 2C has an RNA-binding domain, which could also anchor viral RNA to membranes in the replication complexes.¹³²

Translation and Replication of the Same RNA Molecule

The genomic RNA of picornaviruses is not only mRNA but also the template for synthesis of negative-stranded RNA. How

does the viral polymerase, traveling in a 3' to 5' direction on the positive strand, avoid collisions with ribosomes translating in the opposite direction? It is believed that a mechanism exists to avoid the two processes occurring simultaneously. *In vitro* experiments using inhibitors of protein synthesis demonstrate that, when ribosomes are frozen on the viral RNA, replication of the RNA is inhibited. In contrast, when ribosomes are released from the viral RNA, its replication is increased.³⁹ These results suggest that replication and translation cannot occur on the same template simultaneously.

A mechanism for regulating viral RNA translation and replication involves cleavage of poly r(C)-binding protein. This protein functions in IRES-dependent translation by binding stem-loop IV^{65,155} (Fig. 16.15), and in viral RNA synthesis by binding stem-loop I.^{157,377} Poly r(C)-binding protein is cleaved by viral 3C^{pro}; the cleaved protein can no longer stimulate IRES-dependent protein synthesis but is competent to participate in the initiation of viral RNA synthesis.³⁸⁶ Another mechanism involves binding of 3CD to stem-loop I, which increases the binding affinity of poly r(C)-binding protein for stem-loop I and decreases it for stem-loop IV.¹⁵⁶ Cleavage of PTB by 3C^{pro} also leads to reduced viral translation.²⁶ The consequence of these modifications is that viral IRES-dependent translation is down-regulated, and ribosomes are cleared from the viral mRNA, allowing unimpeded transit of RNA polymerase.

Whether there exist mechanisms to regulate translation and replication of RNA, experimental evidence indicates that some ribosome and RNA polymerase collisions do occur. This conclusion is based on the isolation of a poliovirus variant whose genome contains an insertion of a 15 nucleotide sequence from 28S ribosomal RNA (rRNA).⁸⁸ Apparently, the RNA polymerase collided with a ribosome, copied 15 nucleotides of rRNA, and then returned to the viral RNA template.

Discrimination of Viral and Cellular RNA

The RNA-dependent RNA polymerases of picornaviruses are template-specific enzymes. Poliovirus 3D^{pol} copies only viral RNA, not cellular mRNA, in infected cells. The purified enzyme, however, will copy any polyadenylated RNA if provided with an oligo(U) primer. This observation has led to the suggestion that template specificity probably resides in the interaction of replication proteins with sequence elements in the viral RNA. The *cis*-acting RNA elements located within the coding region of picornaviruses, which direct the uridylylation of VPg, are binding sites for 3CD^{pro} (Fig. 16.13). The 3'-noncoding region of the viral positive-stranded RNA contains an RNA pseudoknot conserved among picornaviruses that is believed to play a role in the specificity of copying by 3D^{pol}.²⁴¹ Disruption of the pseudoknot by mutagenesis produces viruses that have impaired RNA synthesis, indicating the importance of the structure in the synthesis of negative-stranded RNA. 3D^{pol} or 3CD^{pro} cannot bind to the 3' end of poliovirus RNA unless 3AB is present. The interaction of 3AB-3D^{pol} may determine the specificity of binding to the 3' pseudoknot.

Despite experimental results that underscore the importance of the pseudoknot in RNA synthesis, polioviruses from which the entire 3'-noncoding region of the viral RNA has been removed are able to replicate.^{77,489} This finding has led to the suggestion that template specificity imparted by termi-

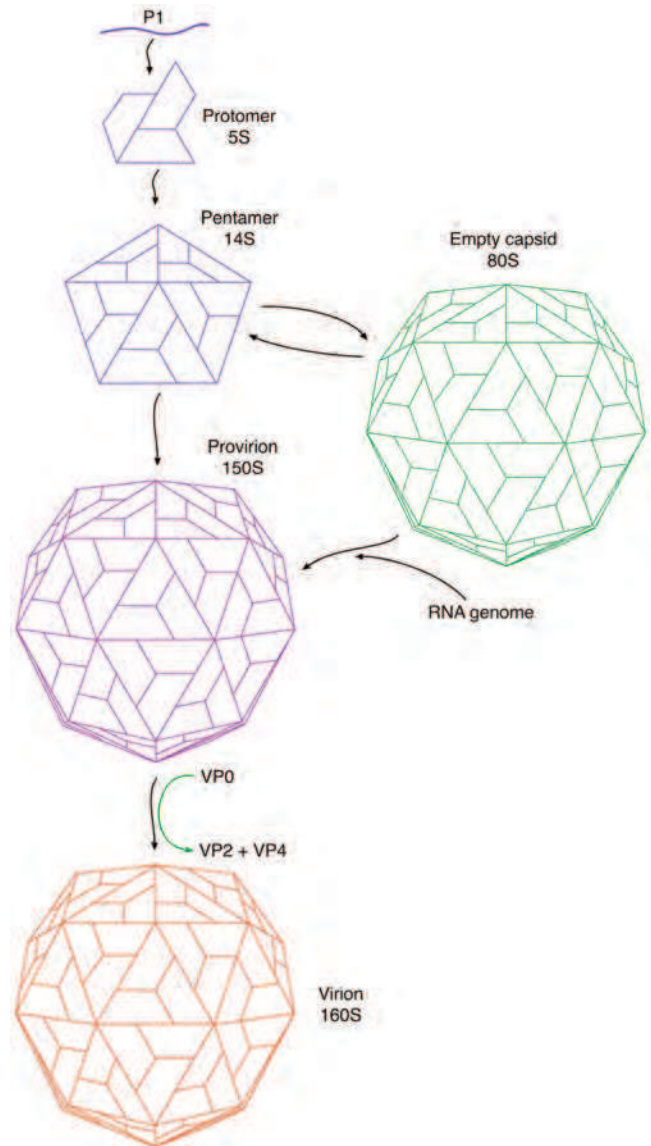


FIGURE 16.15. Morphogenesis of picornaviruses. The capsid protein precursor, P1, folds nascently, is cleaved from P2, and then is further cleaved to VP0 + VP3 + VP1 by 3CD^{pro}. Protomers (5S) self-assemble into 14S pentamers, and pentamers assemble into 80S empty capsids. In one model of encapsidation, RNA is threaded into the empty capsid, producing a 150S provirion in which VP0 is uncleaved. Another possibility is that pentamers assemble around the RNA genome and that empty capsids are storage depots for pentamers. Cleavage of VP0 is the final morphogenetic step that produces the infectious 160S virion. The proteinase responsible for the cleavage of VP0 is unknown.

nal structures of RNA might be of greater importance early in infection. During the initiation phases of replication, the 3' pseudoknot structure might facilitate template selection when few viral polymerase molecules are available and membrane association has not yet provided high concentrations of replication components. Later, determinants of template selection by the polymerase might include the membrane association of the RNA polymerase. Template specificity may also be conferred

by the position of the 3D^{pol} gene at the very 3' end of the viral RNA; translation places the polymerase at the 3' end of the genome, ready for initiation.

A cloverleaf-like structure that forms in the 5'-noncoding region also plays an important role in template specificity. The finding that a mutation in a cloverleaf-like structure in the 5'-noncoding region that affects RNA synthesis could be complemented by a suppressor mutation in 3C^{pro} led to the suggestion that 3C^{pro} might bind the cloverleaf and play a role in viral RNA replication.¹⁷ It was subsequently found that 3CD^{pro} binds the cloverleaf structure in the positive strand, together with a cellular protein, now known to be poly r(C)-binding protein, that is required for complex formation.^{16,18,377} The RNA-binding domain of 3CD^{pro} is contained within the 3C^{pro} portion of the protein, on the opposite face of the molecule from the site involved in proteolysis. Mutations within this domain abolish complex formation and RNA replication without affecting viral protein processing. 3CD^{pro}, therefore, plays an important role in viral RNA synthesis by participating in formation of a ribonucleoprotein complex at the 5' end of the positive-stranded RNA. A structural model of rhinovirus 3C^{pro} bound to stem-loop I shows that RNA binding induces changes in the proteinase active site, although their effect on catalytic activity is not known.⁹⁴ A role for these interactions in viral RNA replication is suggested in the model in Figure 16.13.

ORIGINS OF DIVERSITY

Misincorporation of Nucleotides

As with all other RNA viruses, the picornaviruses have very high error rates, because of misincorporation during chain elongation and the lack of proofreading ability in RNA polymerases. With error frequencies as high as one misincorporation per 10³ to 10⁴ nucleotides, RNA virus populations exist as *quasispecies*, or mixtures of many different genome sequences.¹²⁵

It has been suggested that RNA viruses exist on the threshold of error catastrophe, to maximize diversity and adaptability.¹²⁵ A moderate increase in error frequency would be expected to destroy the virus population. In one study, it was estimated that each poliovirus genome synthesized after multiple rounds of replication in an infected cell contains two point mutations.¹¹¹ In the presence of the antiviral drug ribavirin, each poliovirus genome contained 15 point mutations, and yields of poliovirus in infected cells were 0.00001% of untreated cells. Similar observations have been made with FMDV¹⁸⁸ and Coxsackievirus B3.¹⁷⁹ These findings demonstrate that RNA viruses do exist at the error threshold, and that ribavirin is an RNA virus mutagen that inhibits virus replication by increasing the RNA polymerase error rate beyond the threshold.

High RNA virus error rates are believed to be necessary to enable survival of the virus population under selective pressure. Consequently, viruses with less error-prone RNA polymerases should be at a competitive disadvantage in complex environments such as an infected animal. To test this hypothesis, a poliovirus mutant resistant to the antiviral effects of ribavirin was isolated.^{393,513} Resistance to ribavirin was conferred by a single amino acid change in 3D^{pol} that reduces errors during replication. The high-fidelity mutant virus replicated and spread

poorly in mice, and was unable to compete with a low-fidelity virus. The results indicate that mutations, and the formation of a diverse quasispecies, benefits viral populations, particularly in an infected animal. Analysis of a ribavirin-resistant mutant of FMDV reveals no restriction of the viral quasispecies.²³ This apparent paradox is explained by the observation that the RNA polymerase mutation increased the frequency of misincorporation of natural nucleotides while decreasing the frequency of the incorporation of ribavirin nucleotide.

Recombination

Recombination, the exchange of nucleotide sequences among different genome RNA molecules, was first discovered in cells infected with poliovirus, and was subsequently found to occur during infection with other positive- and negative-stranded RNA viruses. The frequency of recombination, which is calculated by dividing the yield of recombinant virus by the sum of the yields of parental viruses, can be relatively high. In one study of poliovirus and FMDV, the recombination frequency was 0.9%, leading to the estimation that 10% to 20% of the viral genomes recombine in one growth cycle. When poliovirus recombination is studied by quantitative polymerase chain reaction, obviating the necessity to select for viable viruses, the recombination frequency for marker loci 600 nucleotides apart is 2×10^{-3} , similar to estimates obtained using selectable markers.²⁴⁴ RNA recombination also occurs in natural infections. For example, intertypic recombinants among the three serotypes of Sabin poliovirus vaccine strains are readily isolated from the intestines of vaccinees; some recombinants contain sequences from all three serotypes.⁸⁰ The significance of these recombinants is unknown, but it has been suggested that such viruses are selected for their improved ability to replicate in the human alimentary tract compared with the parental viruses. Recombination in nature has also been demonstrated among nonpolio enteroviruses⁴⁶⁰ (reviewed in³⁰⁹).

Poliovirus recombination, which mainly occurs between nucleotide sequences of the two parental genome RNA strands that have a high percentage of nucleotide identity, is called *base pairing dependent* (Fig. 16.14). RNA recombination is believed to be coupled with the process of genome RNA replication: it occurs by template switching during negative-strand synthesis, as first demonstrated in poliovirus-infected cells²⁷⁵ and subsequently in cell-free extracts.⁴⁸² The RNA polymerase first copies the 3' end of one parental positive strand, then switches templates and continues synthesis at the corresponding position on a second parental positive strand. Template switching in poliovirus-infected cells occurs predominantly during negative-strand synthesis because the concentration of positive-strand acceptors for template switching is 30 to 70 times higher than that of negative-strand acceptors. This template-switching mechanism of recombination is also known as *copy-choice*. A prediction of the copy-choice mechanism is that recombination frequencies should be lower between different poliovirus serotypes, a prediction that has been verified experimentally. For example, recombination between poliovirus types 1 and 2 occurs about 100 times less frequently than among type 1 polioviruses (the different poliovirus serotypes differ by about 15% in their nucleotide sequences).²⁷³ The cause of template switching is not known, but it might be triggered by pausing of the polymerase during chain elongation.

ASSEMBLY OF VIRUS PARTICLES

Morphogenesis of picornaviruses has been studied extensively because the 60-subunit capsid is relatively simple and the assembly intermediates can be readily detected in infected cells (Fig. 16.15). During the synthesis of the P1 protein, the capsid protein precursor, the central β -barrel domains form, and intramolecular interactions among the surfaces of these domains lead to formation of the structural units. Once P1 is released from the 2A protein, the VP0–VP3 and VP3–VP1 bonds are cleaved by proteinase 3CD^{pro}. These cleavage sites are located in flexible regions between the β -barrels; considerable movement of the aminotermini and carboxyltermini occurs after cleavage, but the contacts between β -barrels are not disturbed.²²³ In the mature capsid, the carboxyltermini of VP1, VP2, and VP3 are on the outer surface of the capsid, whereas the aminotermini are on the interior, where they participate in an extensive network of interactions among protomers. This process produces the first assembly intermediate in the poliovirus pathway, the 5S protomer, the immature structural unit consisting of one copy each of VP0, VP3, and VP1. Five protomers then assemble to form a pentamer, which sediments at 14S. Cleavage of P1 is probably required for assembly of the pentamer.³⁷³ This conclusion is supported by examination of the virion structure. The β -cylinder at the fivefold axis of symmetry is formed from the N-termini of neighboring VP3 molecules; the cylinder is surrounded by a bundle composed of the aminotermini of VP0 and VP1. For these interactions to occur, proteolytic cleavage of the capsid proteins must occur to allow movement of the aminotermini.

Pentamers are important intermediates in the assembly of all picornaviruses.^{67,433} They can self-assemble *in vitro* or *in vivo* into 80S empty capsids; in one model of assembly, newly synthesized viral RNA is inserted into these particles to form the provirion,²⁴⁰ in which the capsid protein VP0 remains uncleaved. This assembly model would seemingly require an opening in the empty capsid through which the RNA can enter. Examination of the high-resolution x-ray crystallographic structure of these particles does not provide evidence for such an opening.⁴¹ This finding does not exclude this morphogenetic pathway because the pore might be dynamic and not observed in the crystals. In an alternative morphogenesis pathway, 4S pentamers assemble with virion RNA to form provirions. In this model, for which there is some experimental support,³⁶⁵ the empty capsids found in infected cells serve as storage depots for 14S pentamers.

The final morphogenetic step involves cleavage of most of the VP0 molecules to VP4 + VP2. The proteinase that carries out this final maturation cleavage has not been identified. The VP0 scissile bond is located on the interior of empty capsids and mature virions and is inaccessible to viral or cellular proteinases. The presence of a conserved serine in VP2 near one of the cleaved termini of VP2 led to a model that cleavage occurs by a novel autocatalytic serine protease-like mechanism in which basic viral RNA groups serve as proton abstracters during the cleavage reaction.²⁴ Replacement of ser-10, however, does not impair VP0 cleavage.²⁰⁵ In another hypothesis, a conserved histidine in VP2 is involved in catalysis, together with the viral RNA.^{41,114} Replacement of this histidine with different amino acids leads to lack of infectivity or highly unstable

particles, supporting the involvement of VP2 histidine 195 in mediating VP0 cleavage during assembly.²¹⁶

The structure of the 80S particle reveals differences in the network formed by the N-terminal extensions of the capsid proteins on the inner surface of the shell, compared with the native virion.⁴¹ In empty capsids, VP4 and the entire N-terminal extensions of VP1 and VP2 are disordered, and many stabilizing interactions that are present in the mature virion are not present. Thus, cleavage of VP0 establishes the ordered N-terminal network, an interlocking seven-stranded β -sheet formed by residues from adjacent pentamers. This network results in an increase in particle stability and the acquisition of infectivity.

The picornavirus encapsidation process is highly specific, resulting in packaging of only positive-stranded RNA, and not viral mRNA, negative-stranded viral RNA, or any cellular RNA.^{363,364} VPg is probably not an encapsidation signal because VPg-containing negative-stranded RNA is not packaged. The coupling of encapsidation to viral RNA synthesis may explain the selective packaging of viral positive-stranded RNA. In infected cells, newly synthesized RNA is packaged into virions within 5 minutes, whereas incorporation of capsid proteins in virions requires at least 20 minutes.³¹ The pool of viral RNA available for packaging, therefore, is small, and the pool of capsid proteins is large. If capsid formation is inhibited with *p*-fluorophenylalanine, the accumulated RNA cannot be packaged after removal of the inhibitor.¹⁹⁹ These results suggest that packaging of the viral genome is linked to RNA synthesis, and would explain why only RNA containing VPg are encapsidated. This conclusion is questioned by more recent findings showing that inhibition of virion assembly by hydantoin, which targets the 2C protein, does not effect genome replication. Furthermore, when hydantoin from infected cells after RNA synthesis is complete, and in the presence of an inhibitor of replication, normal levels of virions are produced.³⁶⁹

During its synthesis, the P1 capsid protein precursor is linked to myristic acid at the aminoterminal glycine residue of VP4 that is exposed after removal of the initiation Met residue.⁹³ The myristate groups, which form part of a network of interactions between subunits that form when protomers assemble into pentamers, cluster around the fivefold axis of symmetry and stabilize the β -cylinder that is made by the aminotermini of five copies of VP3. Mutagenesis indicates that the myristate group plays a role in stabilizing pentamers and, therefore, virions.^{20,314–316,344}

EFFECTS OF VIRAL MULTIPLICATION ON THE HOST CELL

Inhibition of 5' End–Dependent mRNA Translation Cleavage of eIF4G

In cultured mammalian cells, poliovirus infection results in inhibition of cellular protein synthesis. By 2 hours after infection, polyribosomes are disrupted and translation of nearly all cellular mRNA stops, replaced by viral mRNA translation (Fig. 16.16). Poliovirus mRNA, but not capped mRNA, can be translated in extracts from infected cells. In such extracts, the eIF4GI/II component of the translation initiation factor eIF4F has been cleaved.^{136,434,496} Cleavage of eIF4G separates the N-terminal eIF4E-binding domain of eIF4G from the C-terminal fragment

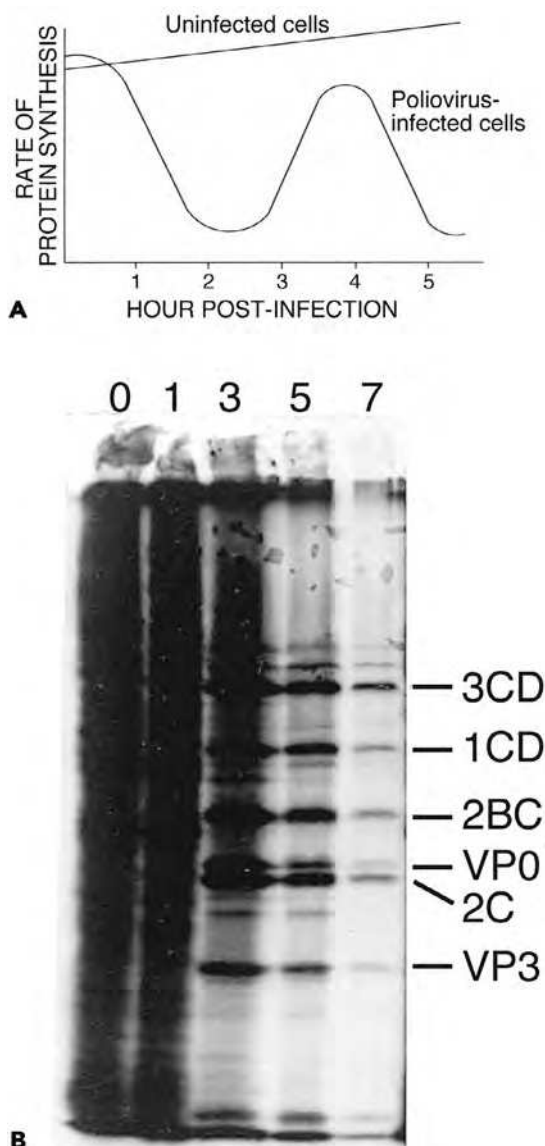


FIGURE 16.16. Inhibition of cellular translation in cells infected with poliovirus. **A:** Protein synthesis in poliovirus-infected and uninfected cells at different times after infection. Poliovirus infection results in inhibition of host cell translation beginning about 1 hour after infection. The increase in translation beginning 3 hours after infection is caused by the synthesis of viral proteins. **B:** Polyacrylamide gel showing inhibition of cellular translation. At different times after infection (top of each lane), cells were incubated with ^{35}S -methionine for 15 minutes; the cell extracts were then fractionated on an SDS-polyacrylamide gel. By 5 hours after infection, host translation is markedly inhibited and replaced by the synthesis of viral proteins, identified at the right.

(Fig. 16.17). The assumption that the C-terminal fragment of eIF4G cannot support the translation of capped mRNA has been proved incorrect.¹⁰ It is now believed that picornavirus-induced translational inhibition is not caused by inability of p100 to support capped mRNA translation, but to the viral RNA outcompeting host cell mRNA for the limiting concentration of p100. Optimal function of the IRES does require the C-terminal frag-

ment of eIF4G, which, as discussed previously, is necessary to anchor 40S ribosomal subunits to the IRES. Although both eIF4GI and eIF4GII are cleaved in poliovirus- and rhinovirus-infected cells, the kinetics of shutoff of host translation correlates with cleavage of eIF4GII and not eIF4GI.^{180,478}

Both forms of eIF4G are cleaved by protease 2A^{pro} of poliovirus and rhinovirus.^{180,478} *In vitro* cleavage of eIF4G by purified 2A^{pro} of rhinovirus is inefficient unless eIF4G is bound to eIF4E.¹⁹⁷ This finding indicates that eIF4G is not cleaved as an individual polypeptide, but rather as part of the eIF4F complex. Binding of eIF4E to eIF4G may induce conformational changes in eIF4G that make it a more efficient substrate for the protease. Poliovirus 2A^{pro} efficiently cleaves eIF4GI, but not eIF4GII, consistent with the differential cleavage of these proteins during virus infection.¹⁸⁰ The L^{pro} protein cleaves eIF4GI in cells infected with FMDV. The cleavage sites in eIF4GI for the two proteinases are different: L^{pro} cleaves between Gly-479 and Arg-480, whereas 2A^{pro} cleaves between Arg-486, and Gly-487.^{274,288}

Modulation of eIF4F Activity

Two related low-molecular-weight cell proteins, 4E-BP1 and 4E-BP2, bind to eIF4E and inhibit translation by 5' end-dependent scanning, but not by internal ribosome entry (Fig. 16.17).³⁸³ 4E-BP1 is identical to a protein called PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin), which was previously known to be an important phosphorylation substrate in cells treated with insulin and growth factors.³⁰¹ Phosphorylation of 4E-BP1 *in vitro* blocks its association with eIF4E. Binding of either 4E-BP1 or 4E-BP2 to eIF4E does not prevent it from interacting with the 5' cap but does inhibit binding to eIF4G. Consequently, active eIF4F is not formed. eIF4G and the 4E-BP have a common sequence motif that binds eIF4E. Treatment of cells with hormones and growth factors leads to the phosphorylation of 4E-BP1 and its release from eIF4E. Those mRNAs with extensive secondary structure in the 5'-untranslated region, which are translated poorly, are preferentially sensitive to the phosphorylation state of 4E-BP1. As expected, translation by internal ribosome binding is not affected when 4E-BP1 is dephosphorylated. Binding of extracellular ligands leads to phosphorylation of 4E-BP through a signaling pathway that includes the target of rapamycin.⁵³²

Infection with several picornaviruses causes alteration of the phosphorylation state of 4E-BP1 and 4E-BP2 (Fig. 16.17). Infection of cells with encephalomyocarditis virus causes inhibition of cellular translation, but in contrast to the shutoff that occurs in poliovirus-infected cells, shutoff of cellular protein synthesis occurs late in infection and is not mediated by cleavage of eIF4G. Infection with encephalomyocarditis virus induces dephosphorylation of 4E-BP1, which then binds eIF4E to prevent it from forming eIF4F.¹³² Translation of cellular mRNA is inhibited, but that of the viral RNA is not inhibited because it contains an IRES. Dephosphorylation of 4E-BP1 also occurs late in cells infected with poliovirus, but this event does not coincide with inhibition of cellular translation, which occurs earlier in infection.¹⁶⁵ Inhibition of translation by dephosphorylation of 4E-BP also influences the ability of the cell to productively combat viral infection by reducing production of type I interferons.⁹⁷

Efficient translation initiation requires the formation of a closed loop on mRNA. Circularization of the RNA is mediated

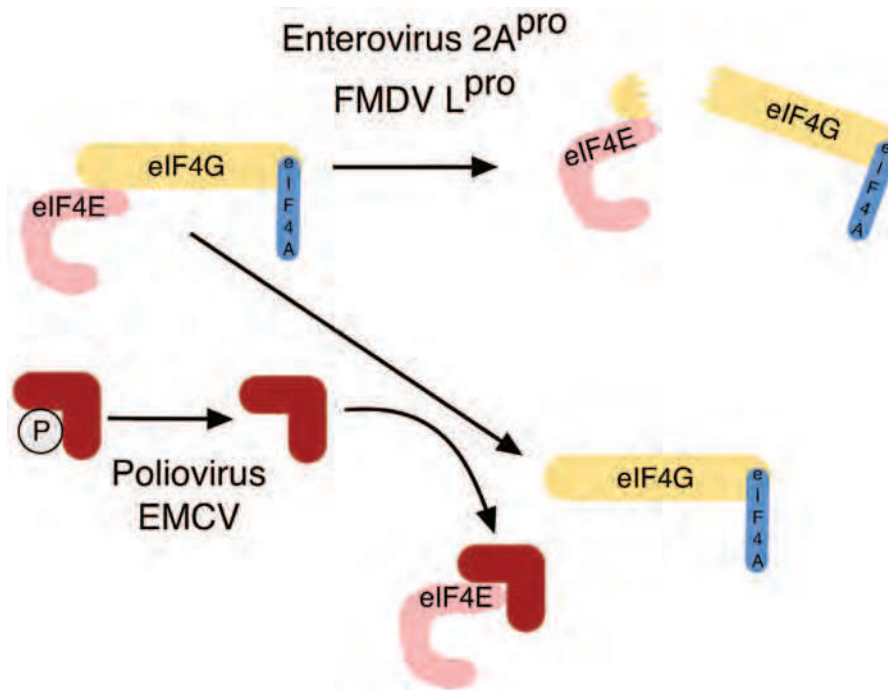


FIGURE 16.17. Two mechanisms for regulating eIF4F activity in picornavirus-infected cells. The proteins 4E-BP1 and 4E-BP1 bind eIF4E and prevent it from interacting with eIF4G. 4E-BP1 binds eIF4E when it is dephosphorylated, an event that occurs in cells infected with poliovirus and encephalomyocarditis virus. Cleavage of eIF4G takes place in cells infected with poliovirus, rhinovirus, and foot-and-mouth disease virus, among others. Cleavage reduces the efficiency of translation of capped messenger RNA (mRNA). 5' end-dependent initiation is inhibited because capped mRNA cannot compete with viral mRNA for the translation machinery.

by the interaction of eIF4G bound at the 5' end of the RNA and poly(A) binding protein bound to the poly(A) tail at the 3' end. The initial synthesis of picornavirus specific proteins is stimulated by the interaction of eIF4G with poly(A) binding protein.^{333,479} Like eIF4G, poly(A) binding protein is cleaved during enterovirus infection by viral proteinases 2A^{pro} and 3C^{pro}; however, the kinetics of poly(A) binding protein cleavage are slower than those of eIF4G.²⁴⁷ Cleavage of poly(A) binding protein is necessary but not sufficient for efficient inhibition of cellular translation; instead, cleavage of this protein is believed to participate in the switch from RNA translation to replication (see “Translation and Replication of the Same RNA molecule”).⁶⁸ The effect of poly(A) binding protein cleavage during this switch is controversial, as depletion of poly(A) binding protein from cell lysates did not reduce virus production.⁴⁸⁰ The cellular polyadenylation factor CstF-64 is cleaved by enterovirus 71 3C^{pro} proteinase, impairing the addition of poly(A) to host cell mRNAs.⁵²⁴

Formation of the eIF4F complex can also be regulated by miRNAs. During enterovirus infection, transcription of miR-141 is enhanced. Because multiple binding sites for this miRNA are found within the 3'-UTR of the eIF4E mRNA, levels of eIF4E—and consequently the eIF4F complex—are reduced, and cellular protein synthesis is impaired.²¹⁹ Viral translation is unaffected as it does not require eIF4E.

The production of double-stranded RNA during infection with many viruses leads to activation of Pkr, phosphorylation of eIF2 α , and inhibition of translation. It occurs very late in the replication cycle of poliovirus.³⁶⁸ Like most cellular mRNAs, the polyprotein encoded by picornaviral mRNA begins with an AUG, and early in infection initiation of protein synthesis requires the ternary complex of eIF2-GTP-met-tRNA_i. Late in infection, phosphorylation of serine 52 of the alpha subunit of eIF2 prevents the recycling of the ternary complex after initiation. However, poliovirus specific protein synthesis continues

throughout infection. It is believed that initiation of translation late during infection occurs independently of eIF2.^{523,530} The exact mechanism for this mode of initiation is not well understood but is thought to be dependent on the generation of a fragment of eIF5 that functions in a similar manner as eIF2 in recruiting the initiating tRNA to the small 40S ribosomal subunit while bound to the mRNA.⁵³⁰ The proteolytic activity of 2A^{pro} is believed to be required to enable poliovirus mRNA translation in the absence of eIF2.⁴¹⁹

Stress-Associated RNA Granules

Sequestering of mRNA away from the translation apparatus in processing (P) bodies and stress granules is another mechanism by which cellular mRNA translation can be impaired.¹⁵ P bodies and stress granules are two nonmembranous cytoplasmic aggregates composed of RNA and protein, including many proteins involved in mRNA translation. These granules are believed to form when translation is inhibited in the presence of intracellular and extracellular stresses, including viral infection. When stress conditions are alleviated, the mRNAs found in these aggregates can either be deadenylated and degraded or returned to the pool of actively translated RNAs.

Stress granules are thought to be nucleated on two cellular proteins, T-cell-restricted intracellular antigen-1 (TIA-1) and the RasGAP SH3-domain binding protein 1 (G3BP). Reduction of either protein impairs formation of stress granules, and overproduction of either component stimulates formation of these aggregates.^{164,494} Stalled translation complexes consisting of mRNA, eIF4E, eIF4G, eIF4A, eIF3, poly(A) binding protein, and phosphorylated eIF2 α are found within these granules. The presence of phosphorylated eIF2 α is a hallmark of stress granules. Formation of stress granules is enhanced during early picornavirus infection, and correlates with the inhibition of cellular translation but is independent of eIF2 α phosphorylation. Late in viral infection, the viral proteinase 3C^{pro} cleaves

RasGAP SH3-domain binding protein 1 α , disassembling stress granules, an event required for efficient viral replication. The presence of an altered form of cleaved RasGAP SH3-domain binding protein 1^{Q326E} prevents the disassembly of stress granules and impairs viral replication.⁵²⁸ Instead, a noncanonical form of stress granules remains, an aggregate containing only TIA-1.^{400,529} The opposite is observed during infection with members of the *Discitroviridae*: cricket paralysis virus infection prohibits stress granule formation as defined by foci of the *Drosophila* homologs of TIA-1 and RasGAP SH3-domain binding protein 1, Rcx8 and Rin. Addition of potent inducers of stress granules such as arsenite and patemine A to the culture medium is unable to overcome viral inhibition.²⁶⁹

Processing (P-) bodies are a second type of nonmembrane bound aggregate found in the cytoplasm. These aggregates, which are the sites of RNA deadenylation and mRNA repression, are composed of proteins such as the decapping enzymes Dcp1a, Dcp2, and proteins that mediate mRNA deadenylation including Pan3, the CCR4/Not 1 complex, Xrn-1, a 5'-3' RNA exonuclease, and the DEAD-box helicase p54/Rck. In uninfected cells, P-bodies are the consequence of micro RNA (miRNA)-mediated repression.¹³⁷ Ultrastructural analysis of these granules suggests that there is an anchoring core composed of proteins required for repression with proteins mediating decay on the periphery. The protein that bridges between the two regions of the aggregate is p54/Rck.¹⁰³ P-bodies are found in proximity to both the ribosome and mitochondria. Although cellular mRNA translation is inhibited during picornavirus infection, the RNA is not degraded, possibly due to inhibition of P-body formation. The 3C^{pro} proteinase of poliovirus cleaves several P-body components including Xrn1, Dcp1a, and Pan 3, disrupting P-body formation.¹²⁸

Inhibition of Cellular RNA Synthesis

Infection of cells with picornaviruses leads to a rapid inhibition of host cell RNA synthesis catalyzed by all three classes of DNA-dependent RNA polymerase. RNA polymerases I, II, and III from poliovirus-infected cells are enzymatically active, suggesting that accessory proteins may be the target of transcriptional inhibition. Studies of *in vitro* systems have demonstrated the inhibition of specific transcription factors required by each of the three RNA polymerases. The RNA polymerase factor TFIID, which is a multiprotein complex, is inactivated in poliovirus-infected cells.²⁷⁹ This inactivation appears to be caused, at least in part, by cleavage of a subunit of TFIID, the TATA-binding protein, by protease 3C^{pro}.⁷⁹ A pol III DNA-binding transcription factor, TFIIC, is also cleaved and inactivated by 3C^{pro}.⁹⁶ The target of 3C^{pro} is a subunit of TFIIC, which contacts the pol III promoter. The pol I transcription factors SL-1 (selectivity factor) and UBF (upstream binding factor) are inactivated in poliovirus-infected cells by 3C^{pro}, resulting in inhibition of pol I transcription.^{32,439}

Because poliovirus replication occurs in the cytoplasm, cleavage of RNA polymerase transcription factors requires that the viral proteinase 3C^{pro} enter the nucleus. 3C^{pro} lacks a nuclear localization signal (NLS), but the precursor 3CD^{pro} enters the nucleus by virtue of an NLS in protein 3D^{pol}. Transcription factors in the nucleus are then cleaved by either 3CD^{pro} or 3C^{pro} that is released by autocatalysis.⁴⁵⁵ Rhinovirus 16 3CD^{pro} has also been shown to enter the nucleus.¹⁴

Inhibition of Nucleocytoplasmic Trafficking

Infection of cells with picornaviruses leads to the disruption of nucleocytoplasmic trafficking. One mechanism by which this is achieved is the proteolysis of nucleoporins, proteins that constitute the nuclear pore complex. In cells infected with poliovirus, two protein components of the nuclear pore complex are cleaved, Nup153 and Nup62.^{191,192} The proteinase responsible for cleavage of Nup62 in rhinovirus-infected cells is 2A^{pro}.³⁷⁶ This cleavage results in the cytoplasmic accumulation of nuclear proteins, some of which, such as La protein⁴⁵⁹ and SRp20,¹⁴⁰ are required for viral replication. The rates and processing profiles of Nup by 2A^{pro} proteinases of different rhinoviruses vary widely.⁵²² The rhinovirus 3C^{pro} and its precursor 3CD^{pro} are imported into the nucleus, leading to the degradation of nuclear pore components.¹⁶⁰

Another mechanism of disruption of nucleocytoplasmic trafficking involves the L protein of cardioviruses (the 2A protein of these viruses is not a proteinase). The L protein binds directly to the Ran-GTPase, a key regulator of nucleocytoplasmic transport.⁴⁰⁶ Infection also leads to phosphorylation of nucleoporins Nup62, Nup153, and Nup214.⁴⁰⁷ Staurosporine, a broad-spectrum protein kinase inhibitor, and ERK and p38 MAP kinase inhibitors block Nup phosphorylation and restore normal nuclear trafficking.⁴⁰⁸ Inhibition of Nup activity is therefore achieved by phosphorylation in cardiovirus-infected cells and by proteolysis in cells infected with enteroviruses.

The disruption of nucleocytoplasmic trafficking by picornaviruses not only provides cytoplasmic access to nuclear proteins needed for viral replication, but blocks the export of cell mRNAs with antiviral activity produced as part of the innate immune response.

Inhibition of Protein Secretion

Transport of both secretory and plasma membrane proteins is blocked in picornavirus-infected cells.¹²³ The 2B and 2BC proteins block protein secretion from the Golgi apparatus, and the 3A protein blocks vesicular traffic from the ERGIC to the Golgi complex.^{34,62,124,444} Inhibition of protein secretion by poliovirus protein 3A is not required for viral growth in cell culture.¹²² Protein 3A inhibits protein transport by binding to and inhibiting GBF1, a guanine exchange factor that is needed for activity of Arf1 and is required for the transport through the secretory pathway.⁵⁴ Cells infected with the poliovirus mutant 3A-2, which has a single amino acid change in the protein, have reduced inhibition of protein transport.¹²⁴ This amino acid change abrogates binding of 3A to GBF1 and therefore does not inhibit activity of the protein.^{525,527} Because the 3A protein prevents secretion of cytokines¹²² and major histocompatibility complex I (MHC-I)-dependent antigen presentation,¹¹⁷ it is likely to modulate the innate and adaptive immune responses of the host and, therefore, the outcome of infection. Consistent with this hypothesis, when the 3A mutation is introduced into the genome of Coxsackievirus B3, the mutant is less pathogenic in mice, although the mechanism of attenuation is not known.⁵²⁶ Inhibition of protein secretion in cells infected by FMDV is caused not by protein 3A but by protein 2BC.³³⁷

Cell Death and Virus Release

When cells are productively infected with poliovirus, they develop the characteristic morphologic changes known as

cytopathic effects. These include condensation of chromatin, nuclear blebbing, proliferation of membranous vesicles, changes in membrane permeability, leakage of intracellular components, and shriveling of the entire cell. The cause of cytopathic effects is unknown. One hypothesis is that leakage of lysosomal contents is partly responsible.¹⁹⁰ Although cellular RNA, protein, and DNA synthesis are inhibited during the first few hours of infection, they cannot account for cytopathic effects.

When poliovirus reproduction is hindered by certain drugs or other restrictive conditions, cell death occurs through induction of apoptosis.⁴⁹⁰ Although certain manifestations of cytopathic effects and apoptosis are similar (e.g., chromatin condensation and nuclear deformation), the pathways leading to their induction differ.³ During productive infections of cultured cells with poliovirus, apoptosis is blocked by a virus-encoded inhibitor.⁴⁹⁰ Viral replication and central nervous system injury in mice infected with poliovirus, however, are associated with apoptosis.¹⁶⁷ Viral inducers of apoptosis include proteins 2C, 2A^{pro}, and 3C^{pro},^{36,168,303} and inhibitors of apoptosis include L^{pro}, 2B, and 3A.^{82,122,159,355,538} The ability of different strains of Theiler's murine encephalomyelitis virus to induce apoptosis may be a determinant of disease. The TO strain of Theiler's virus, which causes a persistent demyelinating disease in mice, encodes an additional protein, L*.⁹⁰ This protein is produced by initiation from an AUG that is 13 nucleotides downstream from the initiator AUG of the polyprotein, in a different reading frame. In contrast, nondemyelinating strains of the virus (e.g., GDVII) do not encode L*. It was subsequently found that L* has antiapoptotic properties in macrophages, is critical for virus persistence,¹⁵⁹ and prevents antiviral cytotoxic T-cell activation.⁵³⁸ The ability of L* to inhibit apoptosis may be a key factor in determining whether infection of mice results in acute disease or persistence and demyelination.

The autophagosomes that form in cells infected with some picornaviruses (see "Cellular Site of Viral RNA Synthesis") may also play a role in virus release from the cell. Suppressing proteins in the autophagic pathway reduces viral release, suggesting a mechanism for nonlytic exit of newly synthesized virus from the infected cell.⁴⁸⁴

PERSPECTIVES

Since the identification in 1908 of poliovirus as the etiologic agent of poliomyelitis, research on the virus has waxed and waned. With each lull in activity, questions were raised about whether it was productive to continue research on the virus. Each time, new technologies emerged that allowed the field to advance and become active once again. Today, research on poliovirus is as vibrant as ever. The difficulties encountered in the effort to globally eradicate this virus have allowed research to continue. Nevertheless, work on other picornaviruses has become highly productive, and research on a wide range of cardioviruses, aphthoviruses, enteroviruses, and members of many other genera flourishes. Many questions remain about nearly every stage of the replicative cycle, and an unprecedented array of experimental techniques and reagents are available to address them. Genome-wide RNA interference screens are being applied to picornaviruses, and have already

identified hundreds of cell genes that not only are required for picornavirus infection but regulate replication.^{108,109} It can be anticipated that cellular genes will be involved in all aspects of the picornaviral replication cycle, from receptor binding and entry to macromolecular synthesis, assembly of new virus particles, and release. Studies of these processes will contribute to understanding questions about how the viral RNA is released from the capsid, whether RNA synthesis proceeds in infected cells as has been learned from *in vitro* systems, how replication complexes are produced in infected cells, and how the viral RNA enters the virion and becomes folded to fit in a very small space. Because poliovirus is a model system, its study provides a unique opportunity to address fundamental questions in virology. The polio eradication program has made impressive gains—as of this writing in April 2012 there have been fewer than 47 cases of paralytic disease, and remarkably, India has been polio free for over 1 year. When the time comes for research on poliovirus to cease, the picornavirus field will be ready to move on to other fascinating subjects. As viral discovery in general has greatly accelerated in the past 5 years, the size of the *Picornaviridae* has grown remarkably. With this growth come new opportunities to study fascinating new viral systems, one of which might some day become the next model system for this virus family.

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Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses

History

Infectious Agents

- Physical and Chemical Properties
- Antigenic Characteristics and Taxonomy
- Propagation and Assay in Cell Culture
- Infection in Experimental Animals:
 - Host Range
 - Other Human Picornaviruses

Pathogenesis and Pathology

- Entry into the Host
- Site of Primary Replication
- Spread in the Host
- Cell and Tissue Tropism
- Immune Response
- Release from Host
- Virulence
- Persistence

Epidemiology

- Demographics
- Transmission
- Prevalence and Disease Incidence
- Molecular Epidemiology

Clinical Features

- Poliomyelitis
- Meningitis and Encephalitis
- Cardiac Disease
- Muscle Disease Including Pleurodynia
- Diabetes
- Eye Infections
- Respiratory Infections, Herpangina, and Hand-Foot-and-Mouth Disease
- Neonate and Infant Disease

Diagnosis

- Differential and Presumptive Diagnosis
- Laboratory Diagnosis

Prevention and Control

- Treatment

Vaccines

Poliovirus Vaccine and Eradication

Perspectives

Acknowledgments

HISTORY

The history of enteroviruses (EVs) is very much the history of poliovirus (PV). In fact, many of the PV milestones are landmarks in the study of EV and, in fact, all of virology.

Poliomyelitis is believed to be an ancient disease. It has been suggested that the depiction of a young man with an atrophic limb on an Egyptian stele from the second millennium bc represents a sequela of poliomyelitis.¹⁹⁷ The first clinical descriptions of poliomyelitis were made in the 1800s, with reports of cases of paralysis with fever. In 1840, von Heine¹⁵³ published a monograph more specifically describing the affliction. His contributions and those published later by Medin²⁷¹ from Sweden led to paralytic poliomyelitis being referred to as *Heine-Medin disease*. Another early report, by Charcot and Joffroy,⁶⁵ described the pathologic changes in the anterior horn motor neurons of the spinal cord in poliomyelitis.

The 1900s began a new era in poliomyelitis investigations and the beginning of an understanding of the infectious nature of this disease. Wickman⁴¹⁸ and others recognized the communicable nature of poliomyelitis, the importance of asymptomatic infected individuals in transmission of PV, and the role of enteric infection in disease pathogenesis. The role of the gastrointestinal tract in the initiation and spread of PV infection was later confirmed by Trask et al.³⁹⁶ In a classic study, Viennese investigators Landsteiner and Popper²⁴¹ proved the infectious nature of poliomyelitis by successfully transmitting the clinical disease and its pathology to monkeys following inoculation of central nervous system (CNS) tissue homogenates from human cases.

Despite this progress, a number of unfortunate misconceptions emerged about poliomyelitis that initially confused scientists and misdirected efforts for control. These misconceptions included a belief that the virus was exclusively neurotropic, that

the nasopharynx was a major site for virus entry into the CNS, and that the virus spread to the nervous system before viremia and by way of the olfactory nerve. As a result of these misconceptions and the failure of several poorly conceived immunization attempts, some with rather disastrous results,³²⁸ an atmosphere of pessimism existed by the middle of the 20th century concerning the eventual control of poliomyelitis, even among scientists working in the field. In 1945, Burnet⁴⁹ wrote, "The practical problem of preventing infantile paralysis has not been solved. It is even doubtful whether it ever will be solved." The eventual realization that virus entered via the oral–gastrointestinal route and that CNS disease followed a viremia did much to boost hopes for effective immunization.³⁶

Building on studies of others, Enders et al.¹⁰⁵ performed a landmark study showing that PV could be propagated in non-neural tissue culture. These investigations had implications for all of virology because they indicated, first, that PV grew in various tissue culture cells that did not correspond to the tissues infected during the human disease, and second, that PV destroyed cells with a specific cytopathic effect. Neutralization tests showed that PV has three serotypes,³⁹ and serologic tests²⁵ confirmed that most infected individuals do not manifest clinical disease. These investigations laid a critical framework for the development of a vaccine, and they clarified a host of confusing data, such as the apparent presence of second attacks of poliomyelitis.

A variety of vaccines were subsequently produced, with the most well known being the Salk inactivated polio vaccine (IPV) delivered via the intramuscular route (licensed in 1955 in the United States) and the Sabin live, attenuated vaccine (oral polio vaccine [OPV]) delivered via the oral route (licensed in 1961–1962). The importance of these vaccines and the individuals who produced them can begin to be realized by noting that more Americans knew the name of Jonas Salk than the president of the United States. The real impact of these vaccines will ultimately be felt with the complete global eradication of poliomyelitis. The eradication will undoubtedly provide a fitting dramatic finale to the compelling story of poliomyelitis.

Poliovirus work has had a continuing significant impact on the field of molecular virology. PV was the first animal virus completely cloned and sequenced,^{227,337} the first RNA animal virus for which an infectious clone was constructed,³³⁶ and the first human virus that had its three-dimensional structure solved by x-ray crystallography.¹⁶⁴ In 1989, Mendelsohn et al.²⁷⁹ identified the PV receptor, CD155, a finding that was followed by the generation of mice carrying CD155 as a transgene.^{234,344}

Coxsackieviruses (group A) were first isolated during poliomyelitis outbreaks in 1947 from the feces of paralyzed children in Coxsackie, New York.⁸⁹ These isolates were obtained by inoculation of suckling mice, the pathogenicity in mice clearly differentiating these viruses from PV. In the following year, the first coxsackievirus (CV) group B was isolated from cases of aseptic meningitis.²⁷⁶ The original CV group A (CVA) isolates produced myositis with flaccid hind limb paralysis in newborn mice, whereas the coxsackieviruses group B (CVB) produced a spastic paralysis and generalized infection in newborn mice, with myositis and involvement of the brain, pancreas, heart, and brown fat.

In 1951, echoviruses were first isolated from the stool of asymptomatic individuals.³⁴⁹ Echoviruses received their name because they were enteric isolates, cytopathogenic in tissue culture, isolated from humans, and orphans (i.e., unassociated with a known clinical disease). Subsequent studies have shown

that echoviruses, in fact, do cause a variety of human diseases. After this period of rapid growth in the number of enteroviruses, there were several decades where new enteroviruses were uncommonly identified. This changed with the introduction of molecular detection methods, and the last 15 years have seen a rapid expansion in the number of recognized enteroviruses. This period of discovery is still in progress.

INFECTIOUS AGENTS

Physical and Chemical Properties

Enteroviruses are distinguished from other picornaviruses on the basis of physical properties, such as buoyant density in cesium chloride and stability in weak acid. Many aspects of enteroviral pathology, transmission, and general epidemiology are directly related to the biophysical properties and their cytolytic life cycle. The infectious virus is relatively resistant to many common laboratory disinfectants, including 70% ethanol, isopropanol, dilute Lysol, and quaternary ammonium compounds. The virus is insensitive to lipid solvents, including ether and chloroform, and it is stable in many detergents at ambient temperature. Formaldehyde, glutaraldehyde, strong acid, sodium hypochlorite, and free residual chlorine inactivate enteroviruses. Concentration, pH, extraneous organic materials, and contact time affect the degree of inactivation by these compounds. Similar inactivation is achieved when virus is present on fomites, although conditions may not be exactly comparable.¹ In general, most reagents that inactivate EV depend on active chemical modification of the virion, whereas most extractive solvents have no effect.

Enteroviruses are relatively thermostable, but less so than hepatitis A virus. Most enteroviruses are readily inactivated at 42°C, although some sulfhydryl reducing agents and magnesium cations can stabilize viruses so that they are relatively stable at 50°C.^{8,99} The relative sensitivity to modest elevations in temperature makes it possible to use pasteurization to inactivate EV in many biologically active preparations.¹⁶⁰

As with other infectious agents, ultraviolet light can be used to inactivate EV, particularly on surfaces. In addition, the process of drying on surfaces significantly reduces virus titers. The degree of virus loss by drying is related to porosity of the surfaces and the presence of organic material.² Many studies of EV inactivation have been conducted using PV as a model enterovirus. A report describing strain-specific differences for glutaraldehyde inactivation among echovirus 25 isolates implies, however, that the assumption that PV is representative of all EV may not be valid.⁶² The inactivation of infectivity may not be directly related to the destruction of the viral genome, because the polymerase chain reaction (PCR) can be used to amplify viral RNA, even after inactivation of virus has occurred.²⁵⁹ This would suggest that reactivation of infectivity may be possible in some circumstances. In fact, some examples of recovered infectivity have been reported through increased multiplicity of infection in cell culture,^{372,443} but the practical significance of these observations is not clear.

Antigenic Characteristics and Taxonomy

As described in Chapter 16, the picornaviruses are among the simplest RNA viruses, having a highly structured capsid with little place for elaboration. Yet, despite the limited

genetic material and structural constraints, evolution within the picornaviruses has resulted in a large number of readily distinguishable members. This variability has been categorized antigenically as serotype.

Each of the serotypes correlates with the immunologic response of the human host, protection from disease, receptor usage, and, to a lesser extent, the spectrum of clinical disease. These correlations, however, have only a partial relationship with the original classification of enteroviruses into polioviruses, coxsackie A or B viruses, and echoviruses, based on biological activity and disease: human CNS disease with flaccid paralysis (poliomyelitis); flaccid paralysis in newborn mice, human CNS disease, and herpangina (coxsackie A viruses); spastic paralysis in newborn mice and human CNS and cardiac disease (coxsackie B viruses); and no disease in mice and (originally) no human disease (echoviruses). Within each of these groups, isolates can be readily distinguished on the basis of antigenicity as measured with antisera raised in animals. The original classification scheme broke down with the identification of viruses serologically identical to known echoviruses that were found to cause disease in mice and humans. This and other inconsistencies led to a numbering of new EV serotypes starting with EV68. These antigenic groupings, which define the serotypes, became increasingly more complicated as the number of different viruses grew. Despite these limitations, the serotype remains the single most important physical and immunologic property that distinguishes the different EVs. Most of the prototype EV strains are maintained in the American Type Culture Collection, Manassas, Virginia, and in many of the World Health Organization (WHO) collaborating reference laboratories.

Despite the importance of the antigenic properties, the introduction of molecular typing methods and a reassessment of the limitations of the old classification scheme led to the development of the current classification system that divides the members of the EV genus into species on the basis of genome organization and sequence similarity as well as biological properties (Table 17.1).

The human enteroviruses are now classified into four species: *Enterovirus A* (EV-A), EV-B, EV-C, and EV-D. In this system, members within an EV species:

share greater than 70% aa [amino acid] identity in the polyprotein, share greater than 60% aa identity in P1, share greater than 70% aa identity in the nonstructural proteins 2C + 3CD, share a limited range of host cell receptors, share a limited natural host range, have a genome base composition (G + C) which varies by no more than 2.5%, share a significant degree of compatibility in proteolytic processing, replication, encapsidation, and genetic recombination.²³⁰

Coding for the capsid proteins, the P1 region provides a reliable correlation between sequence relatedness and the traditional definition of serotype. This also appears to be true for the various individual capsid protein regions, with the exception of VP4; the VP4 sequence does not always correlate with serotype and, therefore, is not reliable for serotype identification.

The molecular studies have also provided a framework in which the EV antigenic relationships can be better understood. These studies suggest that the nucleotide sequence of VP1 can function as an excellent surrogate for the reference antigenic typing methods that use neutralization tests in order to distinguish EV serotypes. VP1 nucleotide sequence identity of at

least 75% (85% aa identity) between an isolate and a serotype prototype strain suggests that the isolate is serotypically identical to the prototype (assuming that the next highest identity with other prototype strains is less than 70%). For example, a capsid sequence identity of 85.4% aa between CVA3 and A8 compared with a mean sequence identity among prototype strains of 71.5% confirmed the antigenic relationships that had previously been described and suggested that these two viruses probably derived relatively recently from a common ancestor.³¹⁸ Similarly, capsid sequences with more than 96% identity confirmed the antigenic relationships between CVA11 and A15 and between A13 and A18;⁴⁶ as a result, CVA15 and A18 have been reclassified as A11 and A15, respectively. In this way, the use of VP1 sequencing studies has supported and clarified early serologic data and also led to proposals regarding the classification of isolates into new EV serotypes.^{312,316}

No general correlation is found, however, between sequence similarities with serotype in genome segments outside of the capsid region because of frequent recombination in the noncapsid regions. For example, sequencing studies have shown that phylogenetic trees constructed from sequences from varying genome regions of members of EV-C and *Poliovirus* have incongruities between the capsid region and noncapsid regions,⁴⁶ suggesting that viruses with a PV capsid may recombine with these CV nonstructural protein coding regions to acquire different nonstructural protein sequences and, similarly, viruses with a CV capsid protein sequence may recombine to acquire different nonstructural protein sequences.²⁵⁵ These findings imply that recombination occurs between PV and other EV-C viruses within the nonstructural protein coding regions, and this shuffling of different nonstructural protein coding regions may lead to serotypes with selective advantages that become dominant. The frequency of recombination in the noncapsid region supports the idea that serotype is defined by the capsid region and that limited correlations likely exist between the serotype of isolates and other phenotypic characteristics not associated with the capsid proteins. The findings also demonstrated that the phylogenetic clustering of prototype strains changes, depending on the nonstructural region that is analyzed.

Recombination within the nonstructural proteins was also found among EV-A and EV-B prototype members with other members of the same species, consistent with their classification into two separate species.^{314,318} An analysis of multiple isolates within several EV-B serotypes³¹⁹ found relatively frequent interserotypic recombination of the noncoding regions, which appeared to occur at least once every 6 years for the isolates that were analyzed. Although no evidence was found of interserotypic recombination within the capsid (perhaps because of structural constraints specific for a particular serotype), intraserotypic recombination within this region appeared to occur.

Additional sequencing studies showed that the 5' untranslated region (UTR) of enteroviruses forms two clusters: the viruses of EV-C and EV-D compose cluster I, whereas EV-A and EV-B compose cluster II.^{46,188}

Sequencing studies have also demonstrated significant similarities between human rhinoviruses and the human enteroviruses, resulting in a reclassification of the human rhinoviruses as members of three different species within the genus *Enterovirus*.²³⁰ Despite the reclassification, the rhinoviruses have several unique properties and are covered separately in this volume (see Chapter 18).

TABLE 17.1 Picornavirus Genera, Species,^a and (Sero)Types

Genera and species	No. of types	Comments
Genus <i>Enterovirus</i>	269	
Human enterovirus A ^b	22	Five have been found only in nonhuman primates
Human enterovirus B ^b	60	Two have been found only in nonhuman primates
Human enterovirus C ^b	21	
Human enterovirus D ^b	4	
Human rhinovirus A ^b	77	
Human rhinovirus B ^b	25	
Human rhinovirus C ^b	49	
Simian enterovirus A	1	
Bovine enterovirus	2	Possible third type detected in sheep
Porcine enterovirus B	3	
Unclassified	5	Detected in nonhuman primates
Genus <i>Hepatovirus</i> ^b	1	
Genus <i>Cardiovirus</i>	12	
Encephalomyocarditis virus ^b	1	
Theilovirus ^b	11	
Genus <i>Kobuvirus</i>	3	
Aichi virus ^b	1	
Bovine kobuvirus	1	Also found in sheep and pigs
Porcine kobuvirus	1	
Unclassified	2	One virus detected in rodents and one in dogs
Genus <i>Teschovirus</i>	11	
Genus <i>Erbovirus</i>	3	
Genus <i>Aphthovirus</i>	11	
Foot-and-mouth disease virus	7	
Bovine rhinitis A virus	1	
Bovine rhinitis B virus	2	
Equine rhinitis A virus	1	
Genus <i>Parechovirus</i>	20	
Human parechovirus ^b	16	Types 1 and 2 formerly classified in genus <i>Enterovirus</i>
Ljungan virus	4	
Genus <i>Sapelovirus</i>	8	
Simian sapelovirus	3	
Porcine sapelovirus	1	Formerly porcine enterovirus A in genus <i>Enterovirus</i>
Avian sapelovirus	1	
Unclassified	3	One virus detected in rodents and two in sea lions
Genus <i>Senecavirus</i>	1	
Genus <i>Tremovirus</i>	1	
Genus <i>Avihepatovirus</i>	3	
Proposed genus Aquamavirus	1	
Proposed genus Cosavirus ^b	4	
Proposed genus Megrivirus	1	
Proposed genus Salivirus ^b	1	
Unclassified picornaviruses	18	Viruses detected in bats, cats, rodents, sheep, birds, fish, and reptiles

^aThe classification scheme shown is from the Picornavirus Study Group of the International Committee on the Taxonomy of Viruses (Knowles NJ, Hovi T, Hyypiä T, et al. Picornaviridae. In: King AMQ, Adams MJ, Carstens EB, et al., eds. *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. San Diego: Elsevier, 2011:855–880; and <http://www.picornaviridae.com>). The types that compose the human enterovirus species are listed in Tables 17.4 through 17.7.

^bAt least one virus in the genus or species has been detected in humans.

In addition to the genetic relatedness, many different EV serotypes share some antigenicity. For example, PV1 and 2 share a common antigen, and antigenic relationships also exist between coxsackieviruses A3 and A8, A16 and EV71, and A24 and EV70, and between echoviruses 6 and 30, and 12 and 29. When virions are disrupted by heating, particularly in the presence of detergent, nonsurface antigens are exposed that are shared broadly among many EVs.²⁸⁰

Despite this lack of understanding of molecular variation in virus structure as measured by polyclonal antibodies, high-resolution studies of the virion surface have been particularly useful in identifying the targets of neutralization of EV by monoclonal antibodies.^{165,327} Other less investigated antigenic sites elicit immune responses that are not neutralizing but nevertheless contribute to serotype identity. The observed structure of some antigenic sites has been shown to span non-continuous polypeptide chains, providing an explanation for why antigenicity of the virus is destroyed by disruption of the virion structure.

Antisera raised in animals to each of the enteroviruses are largely type specific and are used to determine serotype in a neutralization assay. The PV neutralizing antibody response is serotype specific, with the exception of some minor cross-reaction between PV1 and 2. A monoclonal antibody has been described that reacts with this shared site that is not found on PV3.⁴⁰³ As noted, heat-disrupted virions, particularly those heated in the presence of detergent, induce antibodies that react with many EVs.²⁸⁰ These broadly reacting antibodies are generally not neutralizing, and at least one of these epitopes has been mapped to the amino-terminal region of capsid protein VP1.³⁶² Although measurable *in vitro* differences are found in antigenic properties among strains within a serotype, the significance of these differences during natural infection has not been determined. Several PVs isolated during outbreaks have demonstrated different antigenic properties when compared with the reference vaccine strains.^{140,186} In all cases, immunity derived from vaccination has been sufficient to provide protection and control of these strains.⁴³ In addition, even in the face of massive immunization campaigns, no antigenic *escape mutants* resistant to neutralization have ever been observed, and successive genotypes of PV have been eliminated. Natural antigenic variants have also been identified with panels of monoclonal antibodies for several nonpolio EVs.^{148,329}

Propagation and Assay in Cell Culture

One of the prominent characteristics of enteroviruses is the cytolytic nature of growth in cell culture. For many years, PV was the prototype of a lytic viral infection. At the microscopic level, infection is usually manifest within 1 to 7 days by the appearance of a characteristic cytopathic effect, which features visible rounding, shrinking, nuclear pyknosis, refractility, and cell degeneration (Fig. 17.1). The earliest effects can be seen in less than 24 hours if the inoculum contains many infectious particles. With fewer virions, however, visible changes are not recognizable for several days, although a sufficient number of cells are infected. In addition, some EVs either do not cause cytopathic effect at all or do so only after several passages. In general, once focal cytopathic effect is detected, infection spreads rapidly throughout the cell sheet with total destruction of the monolayer, sometimes in a matter of hours.

All known EVs can be propagated in either cell culture or in suckling mice. Most of the serotypes can be grown in at least one human or primate continuous cell culture. No cell line, however, can support the growth of all cultivable EVs. Even after many years of experimentation, a few serotypes (e.g., CVA19) can be propagated only in suckling mice. The typical host range of human EV in cell cultures or animals is shown in a broad, generalized way in Table 17.2 and is not clearly associated specifically with a given virus species.²⁷⁵ Infection of target cells depends on viruses binding to specific receptors on the cell surface. Collectively, the EVs use many different receptors. A practical adaptation resulting from the identification and genetic cloning of EV receptors is the introduction of the receptor into animals and cells that do not normally permit virus infection. This approach has resulted in advances in understanding both the pathogenesis of PV infection in a nonprimate animal model system and its practical application in the diagnostic laboratory. The L20B cells, which are murine cells that express CD155, are now used routinely to selectively isolate PV (see Diagnosis) as part of the global PV laboratory network supporting the poliomyelitis eradication initiative.³³³

Infection in Experimental Animals: Host Range

The natural host for all human enteroviruses is the human. Although serologically distinct picornaviruses with the same physical properties as human EV have been found in many animals, human beings do not usually have recognizable infections with these animal EV. On the other hand, some animals are susceptible to experimental infection with human EV. These include nonhuman primates and CD155 transgenic mice for polioviruses, mice and some monkeys for coxsackieviruses A and B, and monkeys for echoviruses. Human EV can infect nonhuman primates, perhaps related to the homology that several simian EVs share with human viruses, but the infections appear to be largely subclinical.^{313,334} Among higher primates, chimpanzees and gorillas appear to be able to acquire PV infection and develop disease from humans through natural exposure.¹⁰⁰ CVB5 is closely related antigenically to the porcine EV causing swine vesicular disease, with about 50% genetic homology over the entire genome. Genetic studies of a number of strains of swine vesicular disease virus, as well as epidemiologic information gleaned from outbreaks, strongly suggest that a human CVB5 was specifically introduced into swine decades ago and led to establishment in this new host.⁴⁴⁸

Although most coxsackie A viruses have been successfully grown in various cell culture systems, isolation from clinical specimens is sometimes unsuccessful, necessitating the inoculation of suckling mice. Inoculation of suckling mice and subsequent virus identification is a process analogous to that of cell culture inoculation. A blind passage in mice may be necessary if the inoculum is of very low titer or, possibly, because passage of the virus is needed for it to adapt to growth in mice. The two groups of coxsackieviruses can be distinguished by the distinct pathology that they cause in mice (Figs. 17.2 and 17.3). With CVA infection, newborn mice develop flaccid paralysis and severe, extensive degeneration of skeletal muscle (sparing the tongue, heart, and CNS), and they may have renal lesions. Death usually occurs within a week. CVB infection proceeds more slowly and is characterized by spastic paralysis and tremors associated with encephalomyelitis, focal myositis, necrosis of brown fat pads, myocarditis, hepatitis, and acinar cell

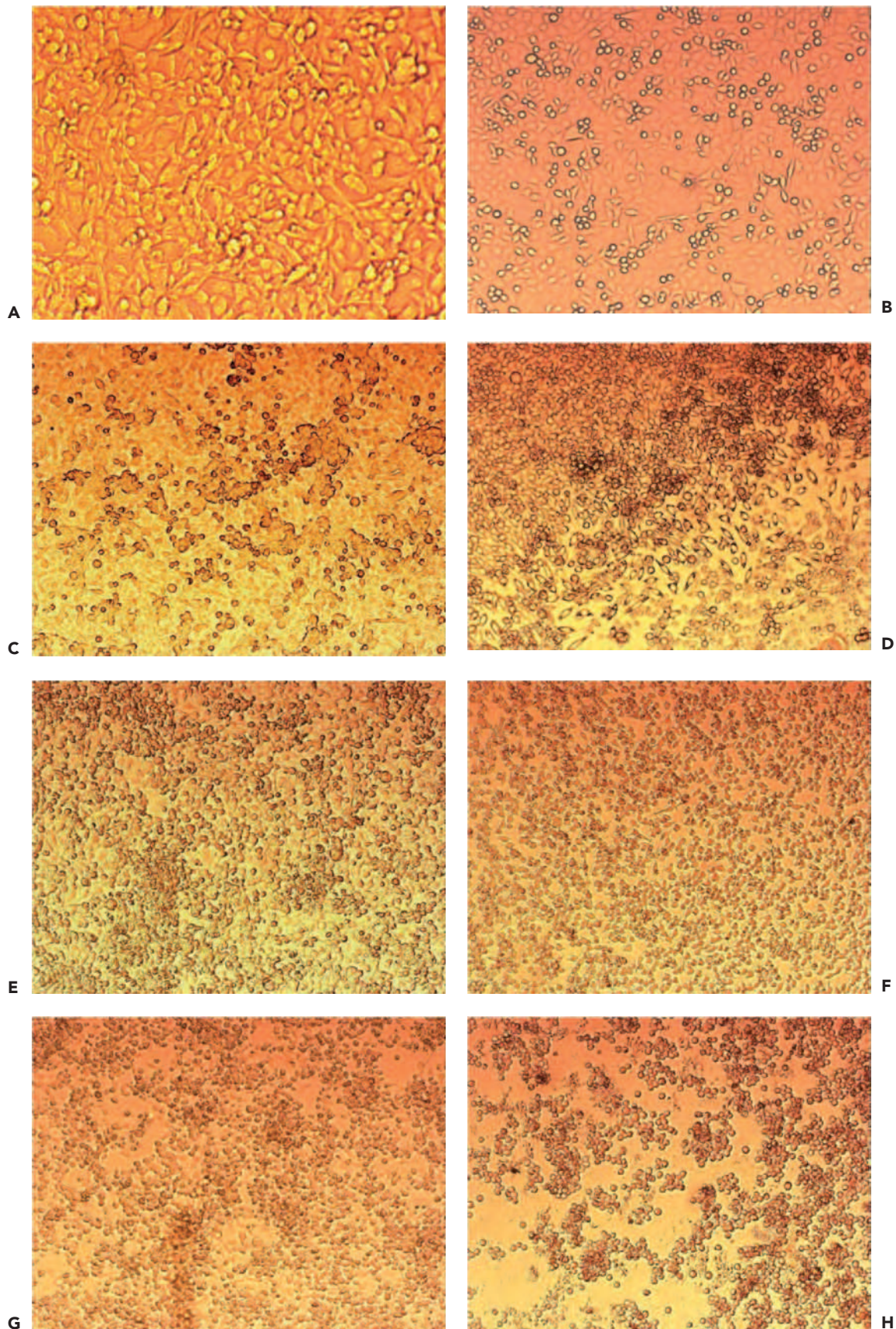


FIGURE 17.1. Normal and poliovirus (PV)-infected rhabdomyosarcoma (RD) cells and CD155 transgenic mouse L cells (L20B) in culture. **A:** Monolayer of normal RD cells in culture. **B:** RD cell culture showing early stage of cytopathic effect typical of PV infection. Approximately 25% of the cells in the culture show cytopathic effect (especially rounding) indicative of virus multiplication (1+ cytopathic effect score). **C:** RD cell culture illustrating more advanced cytopathic effect (3+ to 4+ cytopathic effect). **D:** Almost 100% of the cells are affected, and most of the cell sheet has come loose from the wall of the culture tube. **E–H:** Similar stages of cytopathic effect are shown as in **A–D**, but in this case L20B cells are infected with PV.

TABLE 17.2 Usual Host Range of Human Enteroviruses: Animal and Tissue Culture Spectrum^a

Virus	Antigenic types ^b	Cytopathic effect		Illness and pathology	
		Monkey kidney tissue culture	Human tissue culture	Suckling mouse	Monkey
Polioviruses	1–3	+	+	–	+
Coxsackieviruses, group A	1–22, 24	±	±	+	–
Coxsackieviruses, group B	1–6	+	+	+	–
Echoviruses	1–33	+	±	–	–
Enteroviruses	68–116	+	+	–	–

^aMany enteroviral strains have been isolated that do not conform to these categories.

^bNew types, beginning with type 68, are now assigned enterovirus type numbers instead of coxsackievirus or echovirus numbers. Types 68–116 have been identified.

Adapted from Melnick JL. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM, eds. *Fields Virology*. 3rd ed. Philadelphia: Lippincott-Raven, 1996:655–712.

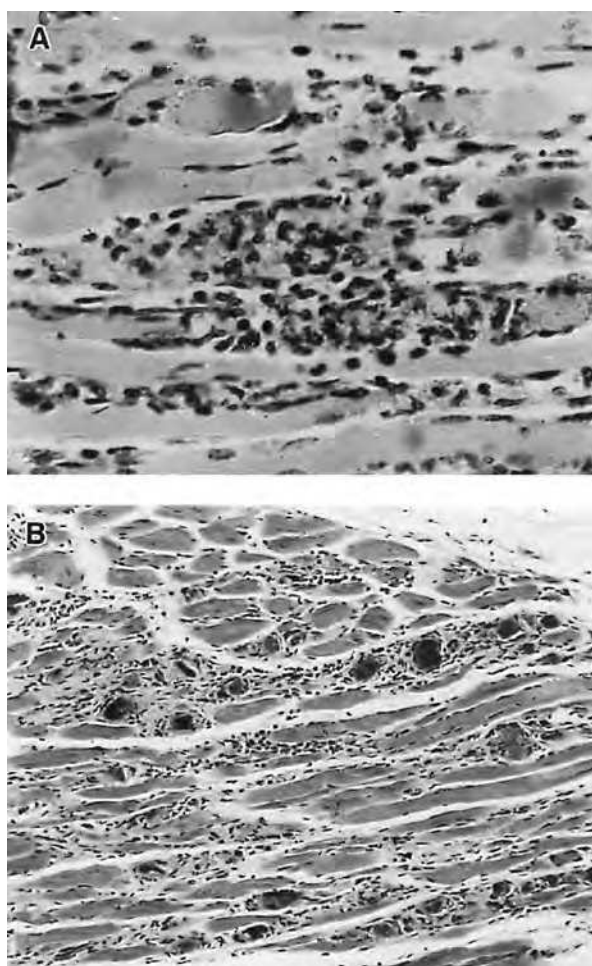


FIGURE 17.2. Photomicrographs of lesions of striated muscle in suckling mice infected with coxsackieviruses. A: Two sarcolemmic tubes with numerous mononuclear phagocytes and remnants of hyaline material within the sarcolemmic sheaths. Acute stage of infection with Conn-5 strain, prototype of coxsackievirus B1 (CVB1) (×450). **B:** Partly mineralized residual masses surrounded by fibrous capsules, and sometimes by giant cells. The remainder of the muscle is completely restored. Texas-1 strain of CVA4, 11 days after onset of paralysis (×180). (From Melnick JL. Current status of poliovirus infections. *Clin Microbiol Rev* 1996;9[3]:293–300, with permission.)

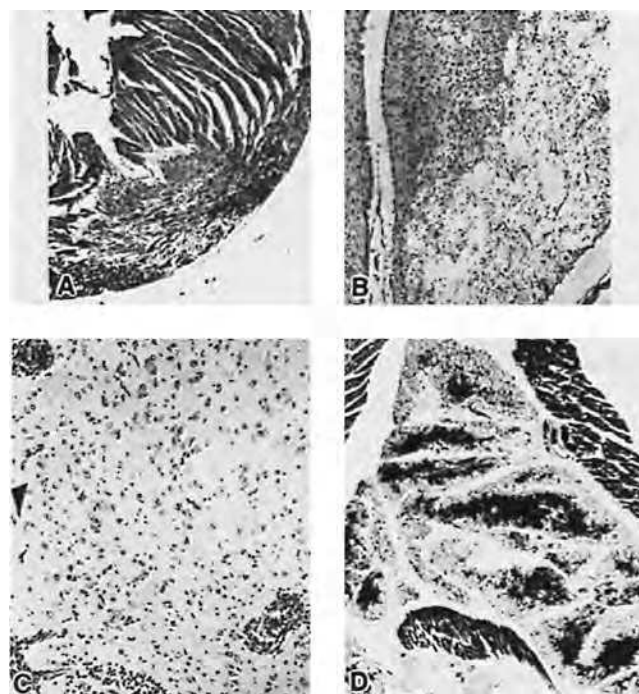


FIGURE 17.3. Photomicrographs of lesions in heart, brain, and fat lobules of suckling mice in acute stage of infection with Conn-5 strain of coxsackievirus B1 (CVB1) (90). A: Heart. Large zone of myocardial necrosis at apex of left ventricle (×170). **B:** Brain. Rarefaction after necrosis in cerebrum (×140). **C:** Brain. Encephalitis showing marked perivascular cuffing and leptomeningitis (×190). **D:** Fat lobules. Interlobular edema and acute necrotizing steatitis, illustrating the selective destruction of the peripheral parts of the lobules, which are shown as pale margins, with preservation of the central parts, which are shown as dark fuchsinophilic areas (×72). (From Melnick JL. Current status of poliovirus infections. *Clin Microbiol Rev* 1996;9[3]:293–300, with permission.)

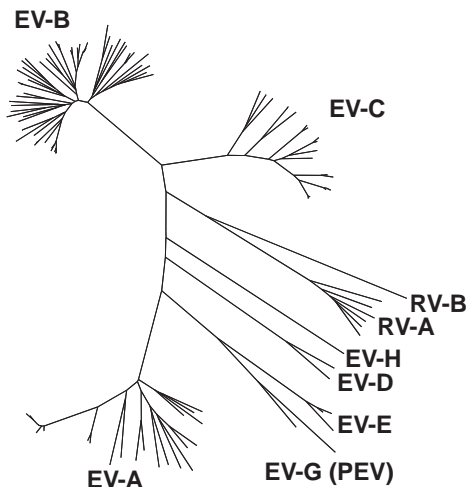


FIGURE 17.4. Dendrogram of genus *Enterovirus*. The figure illustrates the phylogenetic relationship among the prototype strains (see Tables 17.4 through 17.7) within the genus *Enterovirus* and the distinct grouping of isolates into each of the species of viruses that affects humans and other related animal enteroviruses based on the amino acid sequence of the P1 (capsid) coding region of the genomes. Species: EV-A (formerly *Human enterovirus A*); EV-B (formerly *Human enterovirus B*); EV-C (formerly *Human enterovirus C*); EV-D (formerly *Human enterovirus D*); RV-A (formerly *Human rhinovirus A*); RV-B (formerly *Human rhinovirus B*); EV-E (formerly *Bovine enterovirus*); EV-G (formerly *Porcine enterovirus B*); EV-H (formerly *Simian enterovirus B*). Not shown: EV-F (*Bovine enterovirus 2*), EV-J (*Simian virus 6* and related viruses), and RV-C. (Data from M. S. Oberste.)

pancreatitis. Echoviruses, except for some isolates of echovirus type 9, do not generally cause disease in mice.

Other Human Picornaviruses

In addition to the enteroviruses, rhinoviruses (Chapter 18), and hepatitis A (Chapter 19), other picornaviruses that infect humans have been recently discovered or previously considered to be enteroviruses and reclassified as a separate genus (Table 17.1). These genera are genetically distinct from the EV genus (Fig. 17.4) but share some physical and structural similarity with EV. On the basis of a very low genetic relationship, differences in viral proteins and processing, and a novel 2A protease, echoviruses 22 and 23 were reclassified as a new genus, *Parechovirus*. Additional members of this genus exist, including additional serotypes of human parechovirus,¹⁹² as well as a separate species first isolated in Swedish bank voles, Ljungan virus; Ljungan virus has been associated with diabetes in its natural host and may have a possible role in human disease.³⁰² The human parechoviruses (HPEVs) cause a similar spectrum of illnesses as the EVs and can often be detected in cerebrospinal fluid (CSF) from meningitis cases at a frequency similar to that of the EVs.^{409,422} Serologic studies suggest that HPEV infection occurs at an early age, as most children were seropositive by the age of 2 years.^{3,198,385} HPEV3 has been associated with sepsis-like illness and CNS disease in infants.^{32,41}

Another distinct picornavirus genus associated with human infection is *Kobuvirus*.⁴³⁴ Although little information is currently available about this virus, it appears that it is often associated with gastroenteritis in young children and infection

is common. Members of the genus *Cardiovirus* have also been associated with disease in humans, but they do not appear to be a major cause of human illness.^{301,309} Viruses in the proposed genus *Salivirus* are related to kobuviruses and have also been associated with gastroenteritis in humans.^{167,250} Another proposed genus, *Cosavirus*, has been detected at a relatively high frequency in stool, but there is no known association with disease. What is notable about all of these newer genera is that the currently available molecular reagents for the detection of EV do not detect these viruses (see Diagnosis).

PATHOGENESIS AND PATHOLOGY

Entry into the Host

Virus infection normally requires that the virion can attach to the cell surface, and it was long imagined that each virus would have a single receptor. For poliovirus, at least, this may be the case: the virus binds to the poliovirus receptor (PVR,²⁷⁹ now named CD155), a transmembrane glycoprotein in the immunoglobulin superfamily that mediates adhesion of natural killer (NK) cells, and triggers their effector functions. PVR (human CD155) appears to be the major factor regulating the virus's natural host range, which is limited to humans and Old World primates. CD155 homologs/orthologs have been identified and characterized in mice²⁸⁹ and in New World monkeys; the extracellular domains share ~70% amino acid homology with hCD155, and these homologs do not support efficient PV binding or infection. Several laboratories have generated transgenic mice that express PVR, and in many of the resulting models, PV was shown to induce neurologic disease and paralysis following parenteral administration.^{82,117,189,233,279,295,342–344,449} However, oral administration did not cause disease even when the PVR transgene was regulated by a promoter that drove protein expression in enterocytes and microfold (M) cells; PV appeared to bind to the intestinal cells, but productive infection was not observed following oral inoculation of greater than 10⁸ plaque-forming units/mL (pfu) of virus.⁴⁴⁹ These findings are consistent with studies of humans and susceptible primates, in which hCD155 expression has been identified in many tissues, but productive infection is limited largely to the CNS. Thus, factors other than PVR expression play a key role in determining *in vivo* tropism.

It is now generally accepted that some viruses, or viral strains, may have more than one receptor, perhaps expanding their potential host range. EV71, most frequently associated with hand-foot-and-mouth disease in children but capable of causing devastating neurologic pathology,³⁸¹ has at least two receptors: scavenger receptor B2⁴³⁵ and P-selectin glycoprotein ligand-1.³⁰³ Indeed, some viruses appear to interact with two different surface molecules on a single cell, perhaps in series, with one protein acting as the binding moiety, before “handing over” the virus to a second protein that facilitates its entry into the cell. This is thought to occur for some CVBs that bind to decay accelerating factor (DAF, CD55) but then must interact with another protein, the coxsackievirus and adenovirus receptor (CAR), in order to enter the cell.

Site of Primary Replication

Human enteroviruses are spread by the fecal–oral route and respiratory droplets, so systemic infection requires the virus to

cross the gastrointestinal wall, most of which is lined with epithelial cells that form a barrier to invasion. Perhaps surprisingly, given the many years of study, the primary site of PV infection and replication in the intestine remain unknown. PV has been identified in lymphoid tissues, such as the tonsils,³⁶ and in lymphoid aggregates, commonly termed Peyer's patches (PPs), that are present in the ileum of the small intestine. PPs are overlaid with a specialized follicle-associated epithelium (FAE) that contains M cells, which can transport certain molecules from the gastrointestinal lumen, across the epithelial layer, and into cells in the PP. Some studies suggest that PV may replicate within these epithelial cells and lymphoid cells,²⁰⁴ while others suggest that the virus may be transcytosed through the M cell, subsequently establishing infection in an unidentified cell in the PP.³⁷⁵ The cells in which CVB initially replicates are also uncertain; this issue is further clouded by the predominant use, in mouse models, of the intraperitoneal route of infection. Human rhinovirus (HRV) infects epithelial cells of the airways. Infection of the nasal epithelium causes few detectable pathologic changes, even if rhinitis is quite severe, and—as is true for many virus-induced diseases—many of the symptoms appear to be caused by the host response rather than by direct virus-mediated tissue damage.^{420,421}

Spread in the Host

Following replication in the alimentary tract, PV enters the blood, thereby potentially gaining access to all tissues; however, in normal hosts, viral replication is highly restricted, being readily detected mainly in the CNS.³⁶ PV can enter the CNS in two ways: first, from the blood—the virus is thought to be able to cross the blood brain barrier (BBB), perhaps independently of the PVR,⁴³⁷ thereby accessing the CNS parenchyma; and second, by retrograde axonal transport, in which the virus (apparently in the form of an intact virion) ascends the neuronal axon, perhaps in endosomes, and uncoating begins when PV reaches the cell body.^{320–322} This may underpin provocation poliomyelitis, a phenomenon in which a traumatized limb is more susceptible to paralytic polio. The trauma may be directly associated with the virus, as reported in 1935, when it was noted that paralysis first appeared in (or was most severe in) the limb that had received an intramuscular inoculation of “pre-Salk” polio vaccine.²⁴⁴ Such inoculation poliomyelitis also was observed in the “Cutter incident,” when an incompletely inactivated Salk vaccine was administered.²⁹⁷ However, provocation poliomyelitis does not require that trauma and virus be administered to the same limb; when PV was administered intravascularly to monkeys that had received innocuous injections into one limb, paralysis was more likely to develop in the injected muscles.³⁸ Provocation poliomyelitis also has been reproduced in a mouse model.¹³⁹ Mechanisms other than retrograde axonal transport may contribute. For example, the peripheral trauma may increase the permeability of the BBB locally, in the region that innervates the injured muscle; this could explain why provocation poliomyelitis still occurs in traumatized limbs despite scission of the ipsilateral peroneal muscle.²⁹⁶ Dissemination of CVB and other enteroviruses appears to occur largely by the hematogenous route. Viremia is frequently found, when sought; this is true even in rhinovirus infection of normal or asthmatic children,⁴³² in which spread to the lower respiratory tract was thought to occur by more direct means.

Cell and Tissue Tropism

It has been proposed that the observed *in vivo* tropism of PV for the CNS may result from differing efficiencies of viral internal ribosome entry site (IRES) utilization by the various cell types. However, this explanation appears to be incorrect; the PV IRES is equally functional in many cell types *in vivo*, including those that do not support virus replication in the living animal.²¹¹ Rather, the explanation may lie in the ability of an infected cell (or tissue) to respond to type I interferons (T1IFNs). Following PV infection, PVR transgenic mice lacking the receptor for these key antiviral cytokines developed severe lesions not only in the CNS but also in the liver, spleen, and pancreas.¹⁹⁰ Thus, in immunocompetent PVR transgenic mice (and, by extension, in the natural hosts), the absence of apparent PV infection in most hCD155+ peripheral tissues may result from these cells' being able to mount rapid and strong T1IFN responses; and the apparent neurotropism of the virus in immunocompetent hosts may reflect a reduced or delayed T1IFN response within the CNS.¹⁹⁰

CVB3 can cause pancreatitis, myocarditis, and meningoencephalitis. Enteroviruses, and especially CVB, have been implicated in up to one-third of human pancreatitis cases.^{22,326,404} In the mouse model, the pancreas appears to be the first major site of abundant CVB3 replication, with virus titers reaching $\sim 10^4$ pfu/gram after only 12 hours, and $\sim 10^{10}$ pfu/gram at 24 hours postinfection.^{277,278} As shown in Figure 17.5A, there is a large inflammatory infiltrate and widespread destruction of the exocrine pancreas, but the islets of Langerhans remain apparently unaffected. Transmission electron microscopy (Fig. 17.5B and C) reveals, in infected acinar cells, nuclear pyknosis and the accumulation of small double-membraned autophagy-like vesicles, also termed compound membrane vesicles, which are characteristic of enterovirus-infected cells. CVB4 also causes pancreatitis,^{339,340,406} the severity of which depends on the virus isolate that is used; a single amino acid change in VP1 appears to be largely responsible for switching the phenotype from mild pancreatitis to a more severe form.^{51,142} CVB has been found in acinar cells but not in islets of Langerhans^{356,407,410} by immunohistochemistry^{23,406} and by *in situ* hybridization.^{23,407,410} CAR expression correlates with the observed pathology: the receptor messenger RNA (mRNA) is expressed at very high levels in acinar cells but not in the pancreatic ducts or the islets of Langerhans (204), consistent with the observation that CVB-infected mice do not develop hyperglycemia.^{277,338} Cre recombinase-mediated deletion of CAR from the pancreas confers substantial, albeit incomplete, protection against organ damage during CVB infection.²⁰³

A role for EV-B, and in particular CVB, also has been suggested in type 1 diabetes (T1D), and evidence is available to support this notion.^{40,75,341} Several studies have implicated antibody or T-cell cross-reactivity between CVB and host proteins to explain the observed correlations.^{107,179,180,299,300,353} The innate immune system, too, may be involved; a human genome-wide scan for nonsynonymous single nucleotide polymorphisms, comparing healthy individuals to diabetic patients, identified MDA5 as a T1D susceptibility locus.³⁷⁹ However, despite many years of study, a causal role for CVB (or any enterovirus) in T1D has not been convincingly demonstrated in humans, and the issue remains controversial. It is possible that, as has been proposed for pancreatitis, enteroviruses are a co-factor in T1D, very rarely initiating disease in healthy islets but tipping

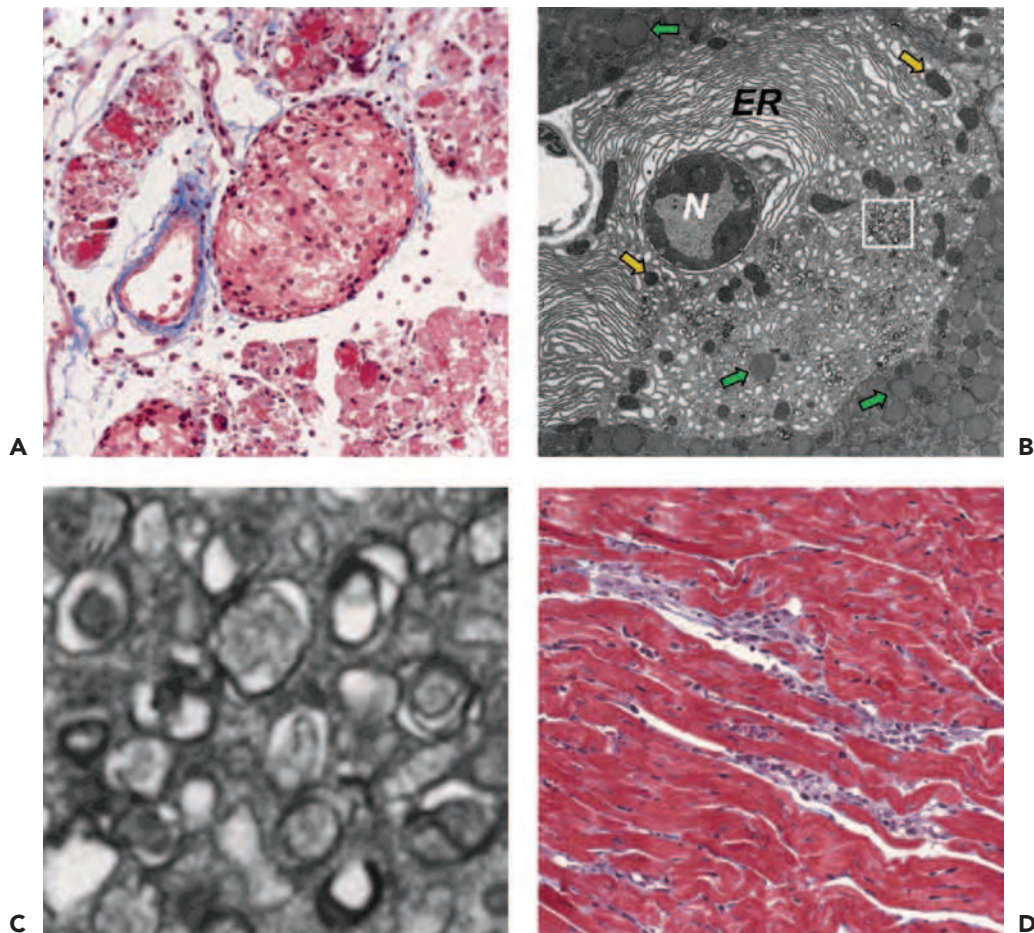


FIGURE 17.5. Histopathology and electron microscopy of coxsackievirus B3 (CVB3)-infected mice. **A:** Inflammatory infiltrate of pancreas with destruction of the exocrine pancreas but sparing of the islets of Langerhans. **B:** Transmission electron microscopy of infected acinar cells showing nuclear pyknosis. **C:** Further magnification of **B** showing accumulation of small double-membraned autophagy-like vesicles. **D:** Infected heart myocardium showing inflammatory infiltrate that contains predominantly macrophages, T cells, and natural killer cells.

the balance in individuals who—unbeknownst to them—have ongoing islet inflammation and are in prediabetic status.

CVB has long been considered one of the principal causes of viral myocarditis,⁸¹ and this view has been confirmed in several recent reports.^{16,106,262,264,266} The inflammatory infiltrate (Fig. 17.5D) contains predominantly macrophages, T cells, and natural killer cells. Several mechanisms have been proposed to explain CVB-mediated cardiomyocyte destruction. The first is direct, virus-mediated damage. Cardiomyocytes can be infected *in vitro*,^{152,206} and infected cells are rapidly lysed.¹⁵⁸ These *in vitro* findings are corroborated by *in vivo* ultrastructural studies of myocardial tissue, which show clear evidence of virus infection of cardiac muscle cells and cell death.^{131,163,228} The second proposed mechanism is immunopathologic damage. The inflammatory infiltrate contains CD8+ T cells, natural killer cells, and macrophages,^{67,129,156,370} and other studies have implicated $\gamma\delta$ T cells in CVB pathogenesis.^{174–176,178} Finally, studies have implicated autoimmunity in CVB-triggered myocarditis.^{176,299,353–355} One potential means by which this could occur is via molecular mimicry (i.e., immunologic cross-reactivity between viral and heart proteins), and there is evidence

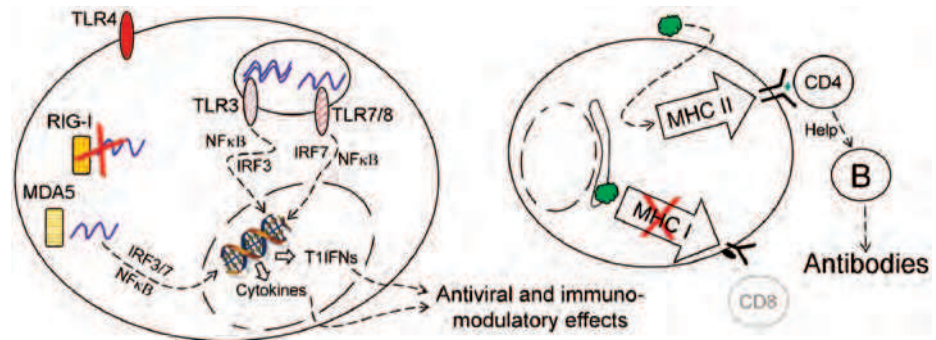
that this occurs at both the antibody^{121,122} and T-cell^{176,177,181} levels. However, recent studies in mice that lack CAR expression only on cardiomyocytes casts doubt on the importance of molecular mimicry in CVB-induced myocarditis. Thus, virus replication in the heart is a prerequisite for myocardial destruction, and this is difficult to reconcile with molecular mimicry; these data do not, of course, exclude a role for autoimmunity induced by other means, for example, by virus-driven exposure of sequestered cardiac antigens.

Immune Response

Innate Immunity

The innate immune response plays a central role in regulating virus infection, as illustrated earlier by the important role played by T1IFNs in regulating PV tissue tropism and pathogenesis. RNA viruses may trigger one (or more) of at least three sensor groups:²⁰⁸ Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and NOD-like receptors (NLRs, some of which assemble into larger structures termed inflammasomes). The interactions between enteroviruses and NLRs have not been reported, so

FIGURE 17.6. Signaling through Toll-like receptors (TLRs) during enterovirus infections. TLR4 on human pancreatic cells is triggered by infection. TLR3 senses double-stranded RNA (dsRNA) molecules and a strong TLR3-triggered response may protect against enteroviral myocarditis. Other internal TLRs also can contribute to the control of coxsackievirus B (CVB) infections.



herein we focus on the first two sensor groups. Triggering of TLRs and RLRs alters the expression of hundreds of genes including a variety of cytokines, chemokines, and other proteins, some of which can directly counter virus infection (e.g., protein kinase regulated by RNA [PKR, discussed later] and type I interferons), while others may regulate the development of the adaptive antiviral immune response. The roles of cell surface and internal TLRs during enterovirus infections have been evaluated in the CVB model (Fig. 17.6). TLR4 on human pancreatic cells is triggered by CVB4,³⁹⁸ and TLR4 knockout (KO) mice infected with CVB3 show reduced virus titers and myocarditis.¹⁰⁹ A comparison of male and female mice confirmed that TLR4 signaling was correlated with the severity of myocarditis.¹¹⁸ However, the administration of TLR4 stimulants such as lipopolysaccharides (LPSs) greatly increased the severity of CVB-induced myocarditis, suggesting that CVB-mediated triggering of TLR4 *in vivo* is likely to be submaximal.^{242,346} TLR3 senses double-stranded RNA (dsRNA) molecules, which are commonly produced during the replication of RNA viruses.¹³ One study of CVB4 infection of TLR3-deficient mice suggested that TLR3 was almost indispensable for the innate response to this virus³⁴⁷ and, when compared to wild-type (wt) mice, TLR3KO mice showed increased mortality and developed more severe myocarditis following CVB3 infection.^{298,416} Genomic screening of patients diagnosed with enteroviral myocarditis or dilated cardiomyopathy (DCM) revealed two TLR3 sequence variants, both of which showed reduced responsiveness to ligand;¹²⁰ this suggests that a strong TLR3-triggered response may protect against enteroviral myocarditis. Other internal TLRs also can contribute to the control of CVB infections. For example, human cardiac inflammatory responses to CVB are reported to be dependent largely on TLR7 and TLR8,³⁹⁷ both of which recognize single-stranded RNA (ssRNA) and other small molecules.¹⁵¹ Contrary to the reported beneficial effects of a strong TLR3 response to enteroviruses, a strong TLR8 response may be associated with adverse outcomes in patients with enterovirus-associated DCM.³⁶⁵ Autophagy is up-regulated by TLRs, and the most potent pro-autophagic effects are mediated by ssRNA/TLR7 signaling.⁹¹ Electron microscopic (EM) studies of poliovirus-infected cells revealed an association between PV and double-membraned intracellular vesicles,⁸⁸ subsequently shown to be autophagy related.³⁶⁸ Extensive membrane remodeling occurs in a poliovirus-infected cell, mediated by the viral 2BC and 3A proteins,³⁸³ and the resulting vesicles carry several autophagosome-related proteins.^{391,392} A similar relationship between CVB and autophagic vesicles has been reported,¹³⁴ and there is a marked increase in double-mem-

bran structures within CVB-infected cells, both in tissue culture^{424,441} and *in vivo*.²¹⁴ Although autophagy is generally thought to support the replication of these viruses, to date, the effects have been determined only in tissue culture cells and are extremely modest; inhibiting autophagy in cells infected with PV,¹⁹³ CVB3,⁴²⁴ CVB4,⁴⁴¹ or EV71¹⁷³ reduces production of each of these viruses by only 1.5- to 4-fold. To date, few studies have evaluated autophagy during enteroviral replication *in vivo*.

Three RLRs have been identified: RIG-I, MDA5 (melanoma differentiation-associated gene 5), and LGP2 (laboratory of genetics and physiology 2). Unlike TLRs, these proteins are expressed in most cell types. All are activated by nucleic acids, and all are cytosolic, although it has been reported that RIG-I co-localizes with F-actin, and thus is associated with the cytoskeleton.²⁹³ Many RNA viruses produce abundant dsRNA and ssRNA with 5' triphosphate and, therefore, strongly activate RIG-I. In the absence of RIG-I, the innate response to several RNA virus families is abrogated.²⁵⁷ The 5' terminus of an enteroviral RNA lacks the 5' triphosphate moiety, instead bearing a modified protein (VPg), and, for this reason, these viruses do not stimulate RIG-I. Infected cells appear to rely largely on MDA5 to alert them to the presence of picornaviral RNA. This sensor is tripped by the cardiovirus encephalomyocarditis virus (EMCV),^{127,210} and two recent publications using MDA5 knockout mice suggest that enteroviruses, too, trigger MDA5 signaling.^{183,414} MDA5, like RIG-I, is degraded in poliovirus-infected HeLa cells, providing one possible mechanism by which enteroviruses might paralyze the innate immune response.²⁸ However, the observation that MDA5 deficiency has a marked effect during CVB3 infection suggests that, if MDA5 degradation occurs in CVB-infected cells, the process does not prevent MDA5-mediated triggering of the innate response.

At least two observations have been made that may be relevant to both TLR- and RLR-mediated responses to CVB (and, possibly, other enteroviruses). First, many cells—exemplified by macrophages and dendritic cells—are phagocytic and engulf dead or dying cells. Thus, although human dendritic cells (DCs) cannot be productively infected by CVB,²³⁹ these cells can consume debris from CVB-infected cells, thereby potentially introducing viral materials to the cytosolic and intravesicular sensors, potentially inducing an antiviral state in the DCs.²³⁸ Second, as well as degrading the sensors themselves, some enteroviruses can interrupt the downstream signaling upon which the innate response depends. Signaling via TLR3 and the RLRs rely, respectively, on proteins named TRIF and MAVS, both of which are degraded in CVB-infected cells, apparently by the viral 3C protease.²⁹²

NK cells, which are part of the innate response to many infections, are important in protecting against CVB-induced pancreatitis in the mouse model.⁴⁰⁸ The importance of NK cells in combating human enteroviral infections is unknown, but human NK cells can produce interferon- γ (IFN- γ) in response to CVB-infected cells.¹⁸²

Adaptive Immunity

Antibodies and CD8+ T cells together provide a strong antigen-specific barrier against virus infections. Under normal circumstances, these two arms of the adaptive response complement each other. However, members of the *Enterovirus* species appear to be an exception to this general rule. Patients with X-linked agammaglobulinemia are highly susceptible to enteroviral infections,²⁸² and after receiving live poliovirus vaccine, such individuals may continue to shed virulent poliovirus for up to ~20 years.^{219,267} The near-absolute requirement for antibodies in protecting against enteroviruses has been confirmed in an animal model of CVB3 infection, using B-cell knockout (BcKO) mice; these mice cannot eradicate the virus, and high titers are present in many organs.²⁷⁸ These observations suggest that, for agammaglobulinemic hosts infected with an enterovirus, there may be some deficit in the backup system that, for most viruses, is provided by CD8+ T-cell responses. This may, in part, explain why most studies of enteroviral infections, in mouse and man, have identified strong antibody responses, while CD8+ T-cell responses—so easily detected in most virus infections—are weak (if detected at all). The enterovirus genus is large, and the adaptive responses to only a select few enteroviral species will be discussed later.

HUMAN ENTEROVIRUS A

The best-studied pathogen in this species is EV71. The virus triggers an immunoglobulin M (IgM) response that is detectable as early as 2 days postinfection,³⁹⁹ as well as a strong neutralizing IgG response that recognizes epitopes in the N-terminal segment of VP1.³⁸⁷ Neutralizing antibodies, when transferred to uninfected neonatal recipient mice, are able to protect against a lethal challenge infection.⁴⁴⁵ Studies of knockout mice showed that B cells, in particular, were important for survival following EV71 infection, and B-cell-deficient mice treated with virus-specific antibody either before or during EV71 infection had lower virus titers, less severe disease, and lower mortality.²⁵³ Memory T-helper 1 (Th1) CD4+ T-cell responses specific for three epitopes in VP1 have been identified in EV71-positive individuals,¹¹⁶ but no CD8+ T-cell epitopes have been reported.

HUMAN ENTEROVIRUS B

CVBs are the best-studied pathogens in this species and, as described earlier, can trigger severe acute and chronic diseases including myocarditis, DCM, pancreatitis, and aseptic meningitis. Infection by these viruses triggers a rapid and strong neutralizing antibody response. Virus-specific IgM appears during the first week of infection, followed by a strong neutralizing IgG response. The CVB3-specific IgM titer wanes over time, but IgG antibodies persist.²⁵⁶ Work in T-cell-deficient (nude) mice indicates that at least some of the CVB-specific antibody response is T-cell independent,^{150,364,426} although some studies suggest that CD4+ T-cell help may be important for the induction of strong neutralizing antibody responses.²⁴⁶ B cells appear to be targeted by CVB²⁷⁸ and may provide a reservoir for the virus during persistent CVB infection. Viral RNA-positive cells, most probably B cells, can be found in the splenic follicles and

germinal centers;^{14,195,207,229,278} approximately 1% of B cells are infected with CVB3 *in vivo*, and these cells may accelerate the systemic distribution of virus.²⁷⁸ T cells can help control CVB infection, although much less effectively than antibodies. *In vivo* analyses of CVB-specific T cells has been challenging because these viruses, despite replicating to high titers in mice, induce remarkably weak CD8+ T-cell responses.^{378,213a} Nevertheless, some responses can be identified, exemplified by CD4+ T-cell responses against epitopes expressed by CVB4.^{143,144} CD8+ T-cell responses are particularly meager. Epitopes in several viral proteins have been identified in human studies, but their detection required ~2 weeks of *in vitro* peptide antigen restimulation.⁴¹⁶

HUMAN ENTEROVIRUS C

This species contains a number of coxsackie A viruses, but the most important pathogen is PV, which induces a strong neutralizing antibody response that is necessary to control the infection. Virus infection and vaccination induce strong and long-lasting humoral responses,⁷⁹ but the immunity is not sterilizing and secondary infections of the gut can occur. Susceptibility to such reinfections, and subsequent shedding of PV, may be controlled by IgA.⁴⁸ PV-driven T-cell proliferation has been observed in infant vaccinees, and the responses appear to be cross-reactive across different enteroviruses.²⁰² However, PV-specific T-cell responses in OPV-vaccinated infants may be weaker than those in adults.⁴⁰⁵ OPV induces major histocompatibility complex (MHC) class II-restricted memory CD4+ T-cell responses targeted to epitopes in all four capsid proteins.³⁷⁷ CD8+ T-cell responses to PV vaccination are long-lived but, as is true for most CVB-specific responses, they became detectable only after several rounds of *in vitro* restimulation, suggesting that T-cell numbers *in vivo* were low.⁴¹³ Mice are not naturally infected by PV, but PVR-transgenic (PVR-Tg) animals have allowed the analysis of T- and B-cell responses and their roles in antiviral protection. Adoptive transfer of PV-primed B cells together with a VP4-specific CD4+ T-cell clone protected PVR-Tg mice against a lethal challenge of PV, but neither cell population alone was protective, indicating that the virus-specific B cells required T-cell help.²⁶⁵

Release from Host

Virion assembly and RNA packaging of enteroviruses remain very poorly understood. Although enteroviruses are generally considered to be highly lytic—and, therefore, released from cells upon their lysis—there is some evidence from cell culture studies consistent with PV release by nonlytic means.^{330,401} It has been proposed that autophagy-mediated release of PV may occur, permitting the virus to exit a cell in a noncytolytic manner;¹⁹³ this proposed mechanism has, memorably, been termed AWOL (*autophagy-mediated exit without lysis*).³⁹⁰

Virulence

Viral virulence is a complex interplay between virus and host, and some of the contributing elements, such as receptor distribution and host responses, have been discussed earlier. Here, we will focus on selected enteroviral sequences and proteins and their contributions to a virulent phenotype. Wild-type PV is more neurovirulent than the attenuated viruses that constitute the oral vaccine, and studies have identified, in all three PV serotypes, changes in the 5' noncoding region that can alter neurovirulence. For example, sequence comparison between the attenuated Sabin 3 virus and revertants from cases of

vaccine-associated poliomyelitis showed that a single U-C change in the viral IRES, at position 472, conferred a growth advantage in the human intestine and resulted in increased neurovirulence, although, on its own, the change was insufficient to confer full neurovirulence;¹⁰⁸ a second change, leading to an amino acid substitution in VP3, almost completely restored virulence.⁴¹⁷ Mutations in the IRES of PV types 1 and 2 also modulate the neurovirulent phenotype.^{212,260,261} The *in vivo* neuroattenuating phenotype imposed by changes in the IRES, together with tissue culture studies showing apparent cell-specific effects of the IRES mutations,¹⁴⁶ led to the proposal that neuroattenuation might be explained by a neuron-specific reduction in usage of the mutated IRES. However, *in vivo* analyses have demonstrated that, while the Sabin 3 IRES sequence is indeed less effectively utilized in neurons, this defect also is observed in other cells and tissues.²¹² Thus, another explanation was sought. The importance of T1IFNs in modulating PV neurovirulence *in vivo* has been described earlier. Cardiovirulence in CVB3 also has been mapped to various locations in the 5' nontranslated region (NTR), ~80 to 240 bases from the 5' end of the genome,^{64,104,400} although the capsid region, too, plays a part.³⁹⁵ In contrast to the extensive mapping of the limits of, and functional domains within, the PV IRES, analysis of the CVB IRES has been limited.

Persistence

Although both PV and CVB are rapidly cytolytic in many of the cell types that they infect, both viruses can establish persistent or chronic infection in tissue culture.^{78,119,269,331} In neuroblastoma cells, PV persistence is associated with accrual of mutations in the capsid region,³³⁰ and for both PV and CVB, moving from a cytolytic to persistent phenotype in cell culture may be inversely related to the capacity of the virus to adsorb to the receptor and/or to the level of receptor expression.^{53,240} It recently has been reported that, during persistent infection of cultured cells that express low levels of CAR, CVB accumulated changes in the capsid that allow the variant virus to bind to novel (non-DAF, non-CAR) molecules, and this more promiscuous activity conferred a replicative advantage upon the variant.⁵² Cellular factors contribute to the establishment of PV persistence.¹²⁶ Cell cycle status may play a role in the case of CVB, which does not replicate efficiently in tissue culture cells rendered quiescent by drugs or by serum starvation but undergoes productive and cytolytic replication when the cell cycle is triggered.^{112,113}

Enteroviral persistent infections can take place under two general scenarios. First, a chronic productive enterovirus infection can occur in immunocompromised hosts. As noted earlier, agammaglobulinemic individuals who receive oral polio vaccine may retain, and excrete, the virus for many years. One study of individuals with primary immunodeficiency who had developed vaccine-associated paralytic poliomyelitis found that approximately one in five secreted vaccine-derived PV at 6 months after their last OPV dose, but this frequency declined to 0% when the interval was 10 years.²²² The low prevalence of the underlying condition means that such persons are rare; only ~40 such individuals have been identified.⁴²⁹ Fortunately, a more common potential cause of immunosuppression, human immunodeficiency virus (HIV) infection, seems not to correlate with PV persistence/shedding.^{24,157} Second, immunocompetent hosts may carry virus

(or at least viral RNA) for many years. Given the frequency of enteroviral infections, it is reasonable to suppose that this is the more common of the two types of enterovirus persistence. In the vast majority of cases in which enteroviral persistence *in vivo* has been reported in immunocompetent hosts, infectious virus was not identified; rather, viral materials (most commonly RNA) were reported and infectious particles, if sought, were not found. CVB RNA has frequently been detected by PCR in many analyses of cardiac biopsies from individuals with DCM^{20,21,45,232} or inflammatory peripheral myopathy.⁴⁴ From results obtained in a murine model of polymyositis, the authors concluded that the RNA was maintained in double-stranded form, with little indication of virus mutation/evolution.³⁸⁶ However, recent analyses of CVB3 genomes isolated from the hearts of persistently infected mice suggest that CVB persistence *in vivo* may be dependent upon the deletion of nucleotides at the 5' end of the genome.²²⁶ Several deletions were reported, some extending to nucleotide 49, and all affecting the 5' cloverleaf structure that is considered vital for RNA replication. Importantly, the materials were infectious; although replicating very slowly, they could be maintained in culture and did not require a helper virus. The VPg protein was present on several of the deletion mutant genomes and, notably, ~25% of RNA encapsidated into virions was negative sense. The authors proposed that the encapsidation of negative strands might occur because the terminal deletions, by altering RNA replication, markedly reduced the ratio of positive to negative strands. Similar 5' terminal deletion variants subsequently were identified in CVB3 that had been passed in primary tissue culture cells²²⁵ and, critically, in a CVB2 genome isolated from the heart of a human who had succumbed to enteroviral myocarditis.⁶³ The poor infectivity of these mutated viruses may explain why infectious virus was not identified in the vast majority of previous studies in which enterovirus RNA was found. To date, there is no evidence to suggest that these terminally deleted variants can be transmitted under normal circumstances.

EPIDEMIOLOGY

Demographics

Despite the nearly ubiquitous nature of EV infections and the wide variety of clinical presentations, the demographics of the various infections and diseases have some consistent characteristics. In particular, several factors, including age, sex, and socioeconomic status, have largely predictable effects.

One of the most important determinants of EV infection outcome is age. Different age groups have different susceptibilities to infection, severity of illness, clinical manifestations, and prognoses following EV infection. Understanding these age effects on outcome of infection is complicated by the widely divergent prior history of infection and resulting immunity. Nevertheless, it is possible to make certain generalizations.

The largest amount and duration of virus shedding occurs on primary infection with a given EV serotype. Because infection is so common, most primary infections occur during childhood. For these reasons, young children are probably the most important transmitters of EV, particularly within households. The greater exposure of children to virus during infection may make them more likely to have significant clinical

symptoms. For example, in outbreaks of meningitis, children typically have higher rates of disease than adults.^{134,191} Most studies, however, do not separately determine age-specific infection rates and disease rates, and the relative rates at which adults are infected are not generally known.

The incidence of poliomyelitis is relatively low for the first 4 to 6 months of life in countries in which control through vaccination has not yet been achieved, because of the frequent presence of protective maternal antibody. In these countries, an increased incidence is seen of paralytic disease in children older than 6 months compared with children in wealthier developed countries, presumably related to an earlier exposure to virus as a result of poor sanitary conditions. Ironically, areas with improved hygiene may have a decrease in infant exposure, leaving an older (unexposed) population susceptible to epidemic disease, with high rates of paralytic disease during an outbreak.³³⁵ Adults are more likely to be severely affected in both developing and developed countries, tending to acquire paralytic poliomyelitis rather than nonparalytic CNS disease (i.e., aseptic meningitis), abortive illness, or asymptomatic infection.^{56,57,170} The reason for the increase in severity later in life is unknown. A possible reason relates to the finding that fast axonal flow, which appears important in the spread of PV within the CNS,²⁰⁰ increases with age. In addition, it may be that CD155 expression or host factors important in replication change with age.

Severity of a number of enteroviral diseases besides poliomyelitis may be strikingly age related. An indirect indication is that a delay in first infection with a number of EVs increases risk of more severe disease. For example, exanthema associated with CVA and echoviruses are for the most part milder in children than in adults. On the other hand, some EVs cause more severe disease in newborns than in older children and adults, possibly inducing a fulminant *viral sepsis* with myocarditis, encephalitis, and sometimes death (see Clinical Features: Neonate and Infant Disease).^{68,194} In addition, recent outbreaks of hand-foot-and-mouth disease caused by EV71 have been associated with a significant CNS complication, fatal brainstem encephalitis, that was restricted largely to young children (Table 17.3) (see Clinical Features: Meningitis and Encephalitis).^{235,258,428}

In general, encephalitis and aseptic meningitis caused by EV appear to be most frequent among those 5 to 14 years of age rather than those older or younger. In a 10-year surveillance summary from the United States,²⁸⁶ adults tended to be overrepresented among cases of severe disease (paralysis, encephalitis, meningitis, carditis) when compared with the age distribution of the EV-infected population as a whole. In another study, the mean age among patients with CVB meningitis (7.7 years) or pericarditis (9.9 years) was greater than the mean age of patients with CVB gastroenteritis (1.3 years).⁹⁶

Enterovirus infections are more prevalent among persons of lower socioeconomic status and those living in urban areas.^{145,196} In a study utilizing active surveillance of healthy children for EV infections in West Virginia during 1951–1953, the rate of isolations among children in a lower socioeconomic setting was two- to sevenfold higher than among children in a higher socioeconomic setting.¹⁶⁸ A similar study in Ghana during 1971–1973 further indicated that EV isolations were significantly more frequent among children in areas with poorer sanitation and in urban areas during both rainy and dry seasons.³²⁵

TABLE 17.3 Some Enterovirus 71 Outbreaks, 1969–2009

Year	Location	Clinical findings
1969–1973	California	Aseptic meningitis, encephalitis
1972	New York	Aseptic meningitis, encephalitis, hand-foot-and-mouth disease
1972	Australia	Aseptic meningitis, rash, polyneuritis, acute respiratory infection
1973	Sweden	Aseptic meningitis, hand-foot-and-mouth disease
1973	Japan	Hand-foot-and-mouth disease, aseptic meningitis
1975	Bulgaria	Aseptic meningitis, encephalitis, acute myocarditis, acute flaccid paralysis
1978 ^a	Hungary	Aseptic meningitis, encephalitis, acute flaccid paralysis
1985	Hong Kong	Monoplegia
1986	Australia	Central nervous system involvement
1987	United States	Acute flaccid paralysis, meningitis, encephalitis
1989	China	Hand-foot-and-mouth disease
1997	Malaysia	Fatal encephalitis, acute flaccid paralysis, hand-foot-and-mouth disease
1998	Taiwan	Fatal encephalitis, hand-foot-and-mouth disease
2000–2001	Australia	Severe neurologic disease, pulmonary edema
2005–2009	Viet Nam	Severe neurologic disease, hand-foot-and-mouth disease
2006	Brunei Darussalam	Severe neurologic disease, hand-foot-and-mouth disease, herpangina
2007–2009	China	Fatal encephalitis, hand-foot-and-mouth disease

^aA mixed epidemic of tick-borne encephalitis (chiefly in adults) and enterovirus 71 disease (chiefly in children).

Adapted from Melnick JL. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM, eds. *Fields Virology*. 3rd ed. Philadelphia: Lippincott-Raven, 1996:655–712; with additional data from World Health Organization. *A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD)*. Geneva: World Health Organization, 2011.

Paradoxically, poliomyelitis and possibly other EV diseases tend to be *diseases of development*. In the case of poliomyelitis, improvement in a country's hygienic and socioeconomic conditions (before vaccination programs) successfully reduces the incidence of paralysis caused by PV and leads to a transitional period in which there is a delay in age of first infection with a subsequent temporary increase in the paralysis-to-infection ratio. Before the introduction of the PV vaccine in the United States and other developed countries, paralytic poliomyelitis was disproportionately a disease of the middle and upper socioeconomic classes; this disease distribution was a result of infection at an older age, when paralysis was a more frequent

complication. Ironically, the delay was a result of improved hygiene. The infant mortality rate, a general indicator of a country's level of health development, may be inversely correlated with the age-specific incidence of poliomyelitis.²⁷⁴

Enterovirus diseases, and possibly also EV infections, occur more frequently in males than in females,^{132,287} although some exceptions have been described.⁹⁴ In numerous reports, the male-to-female ratio appears generally to range between about 1.2 and 2.5:1; that is, approximately 55% to 70% of such diseases occur in males. Male predominance tends to be greater for the more severe diseases (e.g., CNS disease or carditis) than for less-severe disease (e.g., pleurodynia, hand-foot-and-mouth disease, respiratory disease, acute hemorrhagic conjunctivitis, rash, or undifferentiated febrile illness).

The apparent predominance of enteroviral infections among males may have both sociologic and biological explanations. Population-based measurements of infection (e.g., serosurveys), which should be gender neutral, have not consistently demonstrated a higher infection rate for males. Several additional explanations for the male predominance have been proposed on the basis of studies of infections in healthy children:¹²³ a longer duration of virus excretion occurs in males than in females (leading to increased chance of identifying infected males); and a higher virus titer occurs in the feces of males (leading to a similar increase in diagnosis). Another possibility is that, indeed, more frequent infections occur in males because of a greater exposure to the pathogen, perhaps because of differences in the parental treatment and play habits of younger boys and greater activity among older boys. An additional possibility is that males are more likely to develop a serious illness from a given EV infection than females. For example, the reason that human myopericarditis is more common in adolescent and adult males than in females (except in pregnant and postpartum women)⁴²³ could be caused by sex-related endocrine effects leading to differences in disease susceptibility.

Transmission

Enteroviruses can be isolated from both the lower and upper alimentary tract and can be transmitted by both fecal–oral and respiratory routes. Fecal–oral transmission may predominate in areas with poor sanitary conditions, whereas respiratory transmission may be important in more developed areas.¹⁶⁹ The relative importance of the different modes of transmission probably varies with the particular EV and environmental setting. It is believed that almost all EVs, except possibly EV70, can be transmitted by the fecal–oral route; however, it is not known whether most can also be transmitted by the respiratory route. EV70 and CVA24 variant, the agents that cause acute hemorrhagic conjunctivitis, are seldom isolated from the respiratory tract or stool specimens and are probably primarily spread by direct or indirect contact with eye secretions.²³⁶ Enteroviruses that cause a vesicular exanthem presumably can be spread by direct or indirect contact with vesicular fluid, which contains infectious virus.

It is likely that EVs are transmitted in the same manner as are other viruses causing the common cold—that is, by hand contact with secretions (e.g., on the hand of another person) and autoinoculation to the mouth, nose, or eyes. Direct bloodstream inoculation, usually by laboratory accidents (e.g., needlesticks) can result in EV infection; however, neither blood transfusion nor mosquito or other insect bite appears to be a

significant route of transmission. The isolation of EV from flies has led to a suspicion that houseflies (*Musca domestica*) and various filth flies may be vehicles of mechanical transmission. No evidence indicates that venereal transmission is important.

Transmission within households has been well studied for both PV and nonpolio EV. Small children generally introduce EV into the family, although young adults make up the majority of index cases in some outbreaks of acute hemorrhagic conjunctivitis.³⁶⁶ Intrafamily transmission can be rapid and relatively complete, depending on duration of virus excretion, household size, number of siblings, socioeconomic status, immune status of household members, and other risk factors.¹⁴⁵ Transmission has been generally found to be greatest in large families of lower socioeconomic status with a greater number of children 5 to 9 years of age and with no evidence of serologic immunity to the virus type. Not surprisingly, infections in different family members can result in different clinical manifestations.

Observations of household transmission of various EVs have documented that many infected contacts do not become ill and that the extent of secondary transmission varies with different EVs. Household secondary attack rates in susceptible members may be greatest for the agents of acute hemorrhagic conjunctivitis (EV70 and CVA24 variant) and for PV, and of lesser magnitude for the coxsackieviruses and echoviruses. In some studies, secondary attack rates may be 90% or greater, although they are typically lower. New York Virus Watch data indicate that EV infections were more frequent among children 2 to 9 years of age and that secondary CV infections were more frequent in mothers (78%) than in fathers (47%).²³¹ In the same study, coxsackieviruses spread to 76% of exposed susceptible persons versus 25% of exposed persons who had detectable antibody to the infecting type; echoviruses infected 43% of those who were susceptible and only one person with antibody. The greater spread of polioviruses and coxsackieviruses may derive from longer periods of virus excretion.

Transmission occurs within the neighborhood and community, particularly where people congregate. In addition, as with many other viruses, EV can be rapidly transmitted within institutions when circumstances permit (e.g., crowding, poor hygiene, or contaminated water). School teams or activity groups and institutionalized ambulatory retarded children or adults may be at special risk.¹¹ Despite crowding, EV transmission is not usually accelerated to a noticeable degree in institutions where good sanitation is found.

As a result of widespread but incomplete PV immunization, rare PV-susceptible enclaves have arisen. These usually consist of unvaccinated religious groups in countries with an otherwise high prevalence of PV immunity. Despite the barrier of millions of immune persons, PV outbreaks have occurred in some of these enclaves.³²⁴ The frequency and ease of international travel may result in the continuous introduction of wild-type PV in all regions of the world, indicating that a large proportion of the population must be vaccinated if poliomyelitis epidemics are to be prevented. This suggests that herd immunity may be of only limited value in protecting groups of susceptible persons who have regular contact with outside populations, and it raises questions about the risks that such groups may pose to the community at large.

Nosocomial transmission of various CVA and B and the echoviruses has also been well documented, typically in newborn

nurseries. Hospital staff may have been involved in mediating transmission in some of these outbreaks. EV70, as well as CVA24 variant, is highly transmissible and can cause outbreaks in ophthalmology clinics when instruments are inadequately cleaned between patients. An apparent outbreak of CVA1, which included some fatal cases, has been reported in bone marrow transplant recipients.³⁹⁴

Although human EVs have been isolated from various environmental sources, humans are thought to make up the only important natural reservoir.^{101,110} Survival beyond a few weeks does not generally occur, although EV can survive for months in favorable environmental conditions; these favorable conditions include neutral pH, moisture, and low temperatures, especially in the presence of organic matter, which protects against inactivation. Simian enteroviruses have been identified that are closely related to a number of human viruses,^{149,305,307,315,334} and human enteroviruses have been detected in free-living nonhuman primates,³⁰⁸ but it is not known whether primates can serve as a reservoir for human infection.

Although little evidence suggests that EVs found in the environment are of public health importance, concern has been expressed about possible dangers of contaminated sources of water (Fig. 17.7). Recreational swimming water has been investigated in several studies, and EVs have been isolated from swimming and wading pools in the absence of fecal coliforms and in the presence of *recommended* levels of free residual chlo-

rine. CVB5 was isolated from an unchlorinated lake swimming area during an outbreak at a boys' camp in Vermont, although the outbreak itself was explained by person-to-person transmission. In one study, the relative risk of EV infection among children was found to be significantly higher for beach swimmers, especially for those younger than 4 years of age.⁸⁴ These reports suggest that swallowing of contaminated pool or lake water may theoretically account for transmission, but no proof exists that this type of transmission is significant in recreational settings.

Enteroviruses have been found in surface and ground waters throughout the world. In the tropics, virus survival is more prolonged in ground water because it is cooler than surface water. As in the case with swimming pools, EV can be found in these waters even after chlorination and even in the absence of fecal coliforms. In industrialized countries, EV transmission from potable water is apparently uncommon but is a constant source of concern for public health investigators, because the usual conditions under which city drinking water is chlorinated may be insufficient to completely inactivate enteroviruses.

Enteroviruses have been isolated from raw or partly cooked mollusks and crustacea and their overlying waters.¹³⁴ Shellfish rapidly concentrate many viruses, including EV. These viruses can survive in oysters for 3 weeks at temperatures of 1°C to 21°C. To date, no outbreak of EV disease has been attributed to consumption of shellfish. Other food-borne transmission

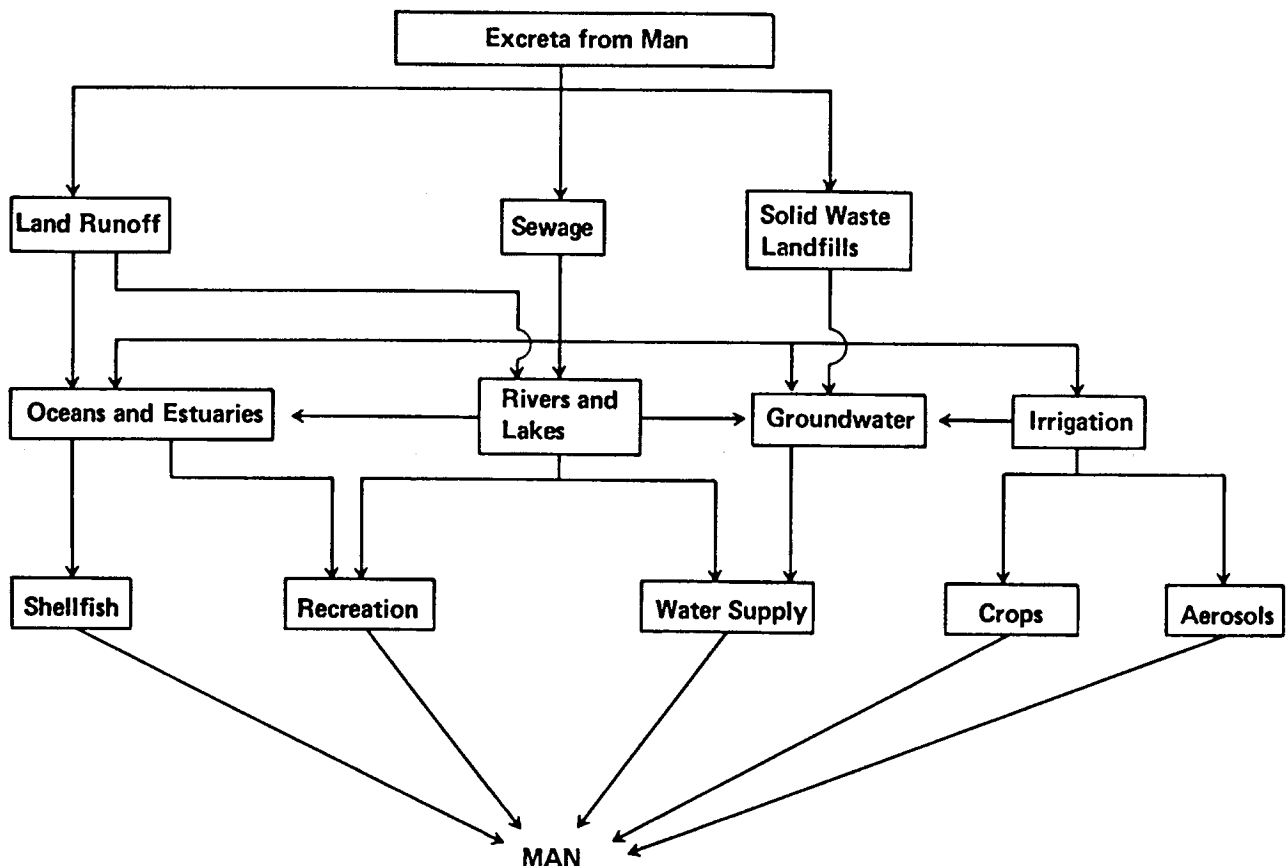


FIGURE 17.7. Routes of potential enteric virus transmission in the environment. (From Melnick JL. Current status of poliovirus infections. *Clin Microbiol Rev* 1996;9[3]:293–300, with permission.)

has been documented but is thought to be uncommon. A 1976 outbreak of aseptic meningitis attributed to echovirus type 4 was apparently caused by consumption of contaminated coleslaw at a large picnic (Centers for Disease Control and Prevention, unpublished data, 1976).

Enteroviruses, especially polioviruses, are regularly found in sewage. Enteroviruses are more prevalent in sewage from areas with low socioeconomic conditions or with large proportions of young children. In addition, sewage workers have been shown to have a higher prevalence of serum antibodies to EV than highway maintenance workers, which is consistent with an occupational risk.⁷⁴

Soil and crops also provide conditions favorable to EV. Enteroviruses survive well in sludge and remain on the surface of sludge-treated soil and even on crops. Air samples from aerosolized spray irrigants using contaminated effluents have also been found to contain EV.²⁸⁵ Survival of EV on vegetable food crops exposed to contaminated water or fertilizer has not been proved to be associated with virus transmission.

Prevalence and Disease Incidence

Incidence data about diseases caused by particular EV types can be derived from prospective longitudinal surveillance of a defined population or from a sample of the population in which the occurrence of disease or infection can be reliably determined. The Virus Watch program in the 1960s in U.S. cities exemplifies this type of surveillance study, in which specimens from subject children were obtained every 2 weeks for virologic evaluation.^{80,231,382} Although difficult and extremely expensive, such prospective cohort studies avoid many of the pitfalls of passive surveillance, and they allow interpretations about both infection and disease incidence.

Less-useful information is based on passive case finding. Ascertainment may be incomplete because the surveillance system is likely to identify a case only if it is easily recognizable and diagnosed by someone who decides to report it. Because such data indicate neither how many ill persons were not reported nor how many ill persons had negative laboratory tests, the information is mostly of qualitative value; however, it may be useful in indicating trends. Despite these limitations, occasional reports do appear.^{29,221}

In the United States, EV surveillance data are collected and analyzed by the Centers for Disease Control and Prevention (CDC). The data have been reported irregularly since the beginning of the program in 1961.^{221,286,288} In the United States, the only notifiable enteroviral diseases are poliomyelitis and encephalitis. These are reportable by diagnosis only (e.g., encephalitis) and not etiology (e.g., echovirus encephalitis). Such disease-based surveillance is the most accessible but least representative of all surveillance data.

Enterovirus excretion does not necessarily imply association with disease, because most such excretion is asymptomatic. This applies particularly to developing countries where EVs are ubiquitous and childhood infections commonplace and characteristically silent.

Enterovirus activity in populations can be either sporadic or epidemic, and certain EV types are associated with both sporadic and epidemic disease occurrences. The reported incidence or prevalence of a given EV disease may actually or artifactually be increased in an outbreak situation when sudden focus of attention improves diagnosis and reporting of cases,

but this may also increase reporting of *noncases*. In addition, there may be a tendency for other strains to be excluded when a particular strain is predominant in a community; however, large communities with summer enteroviral disease typically support co-circulation of several different types simultaneously and in no particular pattern.

An important concept in understanding the epidemiology of the EVs is variation: by serotype, by time, by geographic location, and by disease. This concept is illustrated in surveillance studies of nonpolio EV infections. For example, Figure 17.8 summarizes the data for the years 1970–1998 for CVB3, echovirus 11, and echovirus 30 isolates in the United States collected and analyzed by the CDC. These data illustrate endemic and epidemic patterns of EV prevalence. The epidemic pattern, as typified by E11 and E30, is characterized by peaks in numbers of isolations followed by periods with few

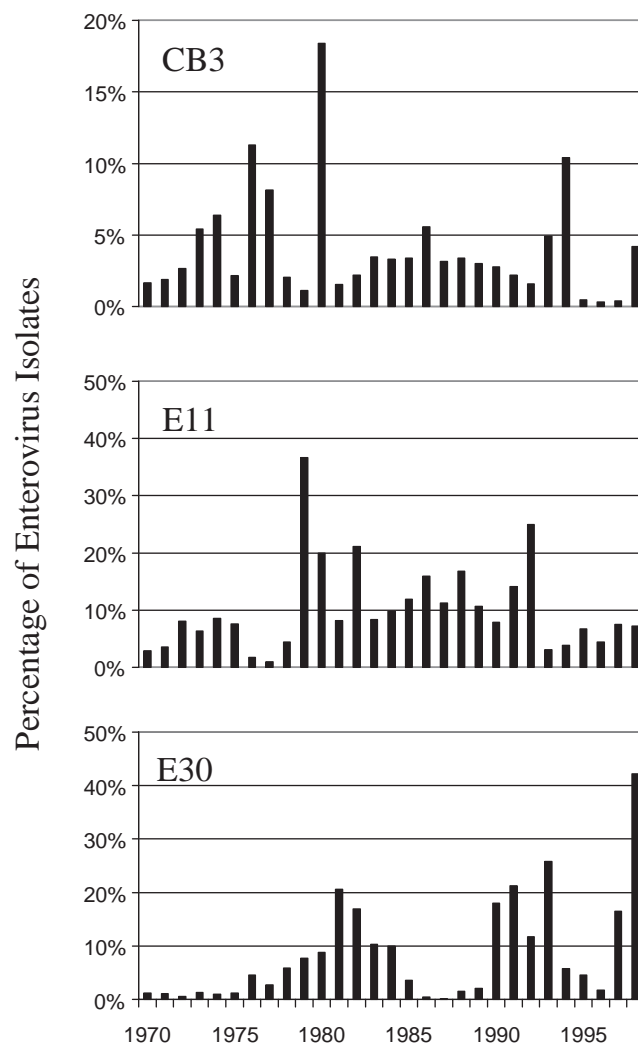


FIGURE 17.8. Reported enterovirus isolations in the United States, 1970–1998. The graphs represent the percentage of total enterovirus isolations in a given year for each of three common serotypes, coxsackievirus B3 (CB3), echovirus 11 (E11), and echovirus 30 (E30). Note that full scale for the coxsackievirus B3 panel is 20%, whereas it is 50% for both of the echoviruses.

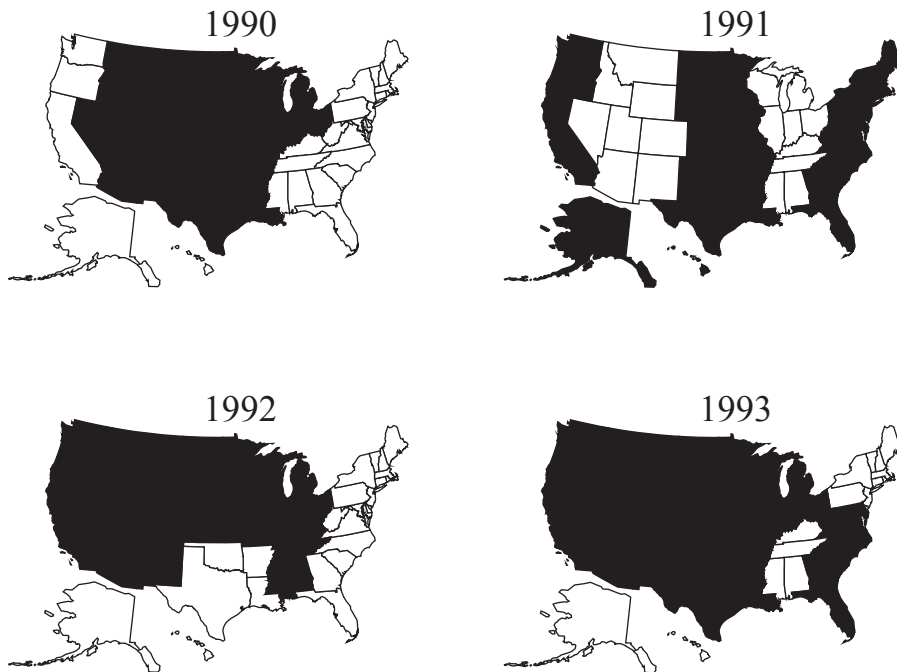


FIGURE 17.9. Geographic distribution of echovirus 30 isolates in the United States, 1990–1993. Maps represent regions of the United States where echovirus 30 was one of the three most common enterovirus isolates for the given year. States are shaded on a regional basis, because not all states report enterovirus isolation data.

isolations.²²¹ These peaks may be sharp (1- or 2-year) or broad (multiyear) periods of increased virus isolations. For example, during the study period, several major epidemics occurred of echovirus 30 in the United States: outbreaks from 1981 to 1982, 1990 to 1994, and 1997 to 1998. By contrast, endemic viruses (e.g., CVB3) are isolated nearly every year and in similar numbers each year. Even with endemic viruses, larger outbreaks do occasionally occur, as with CVB3 in 1980. Similar endemic and epidemic patterns are seen for the other echoviruses and coxsackie A viruses.

Variation by location is also a major characteristic of EV. Outbreaks can be restricted to small groups (e.g., schools and day care centers) or to select communities, or they may become widespread at the regional, national, or even international level. Outbreaks in small groups can sometimes be linked epidemiologically to a breakdown in hygiene practices. Even during national outbreaks of a specific serotype, the location of virus activity may not be uniform. During the period 1990–1993, echovirus 30 was the most commonly isolated EV in the United States (Fig. 17.9). As can be seen in the figure, not all parts of the country had echovirus 30 isolates during the entire period. Some areas, such as the New England states, had extensive circulation in only 1 year, whereas other areas, such as the entire western United States, had extensive virus circulation for 3 or more of the 4 years. It is important to note, therefore, that aggregate national data can obscure significant regional and local variation in viral prevalence.

In temperate climates, EVs are characteristically found during the summer and early autumn, although outbreaks can continue into the winter. In fact, naturally occurring EVs have a distinct seasonal pattern of circulation that varies by geographic area; in contrast, live attenuated PV (mostly vaccine strains) are isolated year round, reflecting the routine administration of poliomyelitis vaccine to children. In tropical and semitropical areas, circulation tends to be year-round or associated with

the rainy season. In the United States, 23 years of surveillance indicated that 78% of EV isolations were made during the five summer or fall months of June to October.²²¹ In a 6-year study of viral CNS disease, 85% of enteroviral disease, compared with 12% to 26% of diseases caused by other viral agents, occurred between June and November.²⁸⁶

Many studies have examined the prevalence of antibodies to the EVs in specific populations, as has been reviewed elsewhere,^{273,284} with several important conclusions. First, the number of persons who have neutralizing antibody to any given EV is large, indicating a high incidence of past infection. A high incidence of recent infection is also suggested by surveys of IgM antibodies to EV, which typically show 4% to 6% positivity. Second, infections with one serotype of EV can boost antibody titers to other EV serotypes as measured by either IgM or neutralization. The pattern of the heterotypic response varies by serotype and among individuals. The nature of this heterotypic response has been explored through the identification of specific epitopes using monoclonal antibodies and peptide antisera.^{141,362,433} Third, the pattern of antibody prevalence by serotype varies by geographic location, time, and age. Thus, prevalence data from different years and locations are not directly comparable. These three points must be considered when interpreting the findings of serologic studies of associations between EV infection and disease.

Molecular Epidemiology

Study of the molecular variation of viral proteins or nucleic acid may contribute significant epidemiologic information on viral diseases (see Diagnosis). Molecular epidemiologic studies have helped in our understanding of EVs including the following: providing the opportunity for unequivocal strain identification, providing insights into EV classification and taxonomy, clarifying the origins of outbreaks, and allowing identification of strains transmitted between outbreaks. For the EVs, and in

particular PV, the primary method used to generate epidemiologic information is direct analysis of genomic variation using nucleic acid sequencing. Previously, both monoclonal antibodies and oligonucleotide fingerprinting were also used to study variation in PV and EV; however, these approaches are limited by their ability to show similarities and small differences only among relatively closely related viruses. Neither technique, however, is able to readily detect any patterns among seemingly unrelated virus isolates. The introduction of the technique of genomic nucleic acid sequencing and its application to the study of wild PV isolates from different parts of the world has significantly extended the epidemiologic power of molecular studies.³⁴⁸ By analyzing the random mutations that occur in the genome of different PVs, closely related viruses were easily detected, and, in addition, more distantly related viruses were clustered into distinct geographic groupings of endemic circulation. This approach allowed epidemiologic links to be extended beyond those identified with other techniques.

Nucleic acid sequencing technology has been most comprehensively applied to studies of PV, where the information has proved valuable for supporting the global PV eradication program.^{215–217} From these studies, it is possible to determine (a) if an isolated PV is related to vaccine virus, (b) similarities among isolates in an epidemic, and (c) differences among isolates from different geographic areas. By comparing the changes that are observed between virus strains, the geographic and temporal origin of a virus can be determined. Building on a nucleic acid sequence database of PV strains worldwide, it has been possible to develop rapid approaches to tracking wild PV strains.^{9,254,294,348,450}

Studies on the molecular epidemiology of nonpolio EV have focused on the evolutionary inference derived from the comparison of virus isolates within a serotype over time, as well as the comparison of isolates from different serotypes and even between different genera within the *Picornaviridae*. Molecular

epidemiologic studies using sequencing have been reported for CVB1, CVB5, echovirus 30, and EV71.^{47,102,237,310,452} One of the studies of CVB5 isolates examined the pattern of genetic changes over three separate outbreaks in the United States. The nucleotide sequence from multiple isolates from the epidemics showed that each of the epidemics was caused by a single genotype. The genotype of CVB5 observed in the 1967 epidemic showed more similarity to the virus observed in the 1983 epidemic than to viruses isolated during the intervening years,²³⁷ suggesting discontinuous transmission of epidemic CVB5 in the United States during this time. In an analogous manner, echovirus 30 genotypes have demonstrated an overlapping succession among the isolates characterized in the United States.³¹⁰ More than one genotype may be found in certain periods, and the displaced genotype can be found in other parts of the world after isolations have ceased in the United States for many years. In studies of EV71 isolates, three distinct genotypes have been characterized.⁴⁷ Unlike the situation with echovirus 30 and more similar to the CVB5 example, the transition from one genotype to another occurred during a single year, 1987, and the older genotype has not been isolated since in the United States despite isolation in other parts of the world.

CLINICAL FEATURES

Most EV infections are asymptomatic. On the other hand, these viruses can cause a spectrum of clinically distinct syndromes when they lead to disease. Tables 17.4 through 17.7 list the prototype EV strains and the illness, if any, in the person from whom the prototype virus was isolated. Individual serotypes generally lead to varied symptomatology and disease processes. Similarly, individual clinical disorders can generally be caused by a number of different EVs. On occasion, however, particular syndromes are associated with specific EVs

TABLE 17.4 Enterovirus Species A (EV-A)

Type	Prototype strain	Geographic origin	Illness or source prototype virus	Accession number
CVA2	Fleetwood	Delaware	Poliomyelitis	AY421760
CVA3	Olson	New York	Meningitis	AY421761
CVA4	High Point	North Carolina	Sewage of community with polio	AY421762
CVA5	Swartz	New York	Poliomyelitis	AY421763
CVA6	Gdula	New York	Meningitis	AY421764
CVA7	Parker	New York	Meningitis	AY421765
CVA8	Donovan	New York	Poliomyelitis	AY421766
CVA10	Kowalik	New York	Meningitis	AY421767
CVA12	Texas-12	Texas	Flies in community with polio	AY421768
CVA14	G-14	South Africa	None	AY421769
CVA16	G-10	South Africa	None	U05876
EV71	BrCr	California	Meningitis ^a	U22521
EV76	10226	France	Gastroenteritis	AY697458
EV89	10359	Bangladesh	Acute flaccid paralysis	AY697459
EV90	10399	Bangladesh	Acute flaccid paralysis	AY697460
EV91	10406	Bangladesh	Acute flaccid paralysis	AY697461
EV114	11610	Bangladesh	Acute flaccid paralysis	NA

NA, information not available.

^aAn identical strain was isolated from the brain of a fatal encephalitis case in the same local outbreak of central nervous system disease.

TABLE 17.5 Enterovirus Species B (EV-B)^a

Type	Prototype strain	Geographic origin	Illness yielding prototype virus	Accession number
CVA9	Bozek	New York	Meningitis	D00627
CVB1	Conn-5	Connecticut	Meningitis	M16560
CVB2	Ohio-1	Ohio	Summer grippé	AF085363
CVB3	Nancy	Connecticut	Minor febrile illness	M16572
CVB4	JVB	New York	Chest and abdominal pain	X05690
CVB5	Faulkner	Kentucky	Mild paralytic disease with atrophy	AF114383
CVB6	Schmidt	Philippine Islands	None	AF105342
E1	Farouk	Egypt	None	AF029859
E2	Cornelis	Connecticut	Meningitis	AY302545
E3	Morrisey	Connecticut	Meningitis	AY302553
E4	Pesascsek	Connecticut	Meningitis	AY302557
E5	Noyce	Maine	Meningitis	AF083069
E6	D'Amori	Rhode Island	Meningitis	AY302558
E7	Wallace	Ohio	None	AY302559
E9	Hill	Ohio	None	X84981
E11	Gregory	Ohio	None	X80059
E12	Travis	Philippine Islands	None	X79047
E13	Del Carmen	Philippine Islands	None	AY302539
E14	Tow	Rhode Island	Meningitis	AY302540
E15	CH 96-51	West Virginia	None	AY302541
E16	Harrington	Massachusetts	Meningitis	AY302542
E17	CHHE-29	Mexico City	None	AY302543
E18	Metcalf	Ohio	Diarrhea	AF317694
E19	Burke	Ohio	Diarrhea	AY302544
E20	JV-1	Washington, DC	Fever	AY302546
E21	Farina	Massachusetts	Meningitis	AY302547
E24	DeCamp	Ohio	Diarrhea	AY302548
E25	JV-4	Washington, DC	Diarrhea	AY302549
E26	Coronel	Philippine Islands	None	AY302550
E27	Bacon	Philippine Islands	None	AY302551
E29	JV-10	Washington, DC	None	AY302552
E30	Bastianni	New York	Meningitis	AF162711
E31	Caldwell	Kansas	Meningitis	AY302554
E32	PR-10	Puerto Rico	Meningitis	AY302555
E33	Toluca-3	Mexico	None	AY302556
EV69	Toluca-1	Mexico	None	AY302560
EV73	CA55-1988	California	Unknown	AF241359
EV74	10213	California	Unknown	AY556057
EV75	10219	Oklahoma	Unknown	AY556070
EV77	CF496-99	France	Unknown	AJ493062
EV78	W137-126/99	France	Unknown	AY208120
EV79	10384	California	Unknown	AY843297
EV80	10387	California	Unknown	AY843298
EV81	10389	California	Unknown	AY843299
EV82	10390	California	Unknown	AY843300
EV83	10392	California	Unknown	AY843301
EV84	10603	Côte d'Ivoire	None	DQ902712
EV85	10353	Bangladesh	Acute flaccid paralysis	AY843303
EV86	10354	Bangladesh	Acute flaccid paralysis	AY843304
EV87	10396	Bangladesh	Acute flaccid paralysis	AY843305
EV88	10398	Bangladesh	Acute flaccid paralysis	AY843306
EV97	10355	Bangladesh	Acute flaccid paralysis	AY843307
EV100	10500	Bangladesh	Acute flaccid paralysis	DQ902713
EV101	10361	Côte d'Ivoire	None	AY843308
EV106	10634	Bangladesh	Acute flaccid paralysis	NA
EV107	TN94-0349	Thailand	None	AB266609

NA, information not available.

^aEchovirus types 1 and 8 share antigens, type 1 having the broader spectrum. Type 10 was soon excluded from this group: it turned out to be a larger RNA virus and was reclassified as a prototypic reovirus. Type 28 was reclassified as rhinovirus type 1. Types 22 and 23 have been reclassified as members of the genus *Parechovirus* and are named parechovirus 1 and 2; these viruses along with Ljungan virus represent the only members of this new genus. Type 34, DN-19, is now considered a prime strain of CV A24, rather than a distinct echovirus. Additional newer serotypes (EV73 and higher) are proposed new types defined on the basis of genetic sequence information.

TABLE 17.6 Enterovirus Species C (EV-C)

Type	Prototype strain	Geographic origin	Illness in person with prototype	Accession number
CVA1	Tompkins	Coxsackie, NY	Poliomyelitis	AF499635
CVA11	Belgium-1	Belgium	Epidemic myalgia	AF499636
CVA13	Flores	Mexico	None	AF499637
CVA17	G-12	South Africa	None	AF499639
CVA19	NIH-8663	Japan	Guillain-Barré syndrome	AF499641
CVA20	IH-35	New York	Infectious hepatitis	AF499642
CVA21	Kuykendall; Coe	California	Poliomyelitis, mild respiratory disease	AF546702
CVA22	Chulman	New York	Vomiting and diarrhea	AF499643
CVA24	Joseph	South Africa	None	D90457
PV1	Brunhilde	Maryland	Paralytic poliomyelitis	AY560657
PV2	Lansing	Michigan	Fatal paralytic poliomyelitis	AY082680
PV3	Leon	California	Fatal paralytic poliomyelitis	K01392
EV96	10358	Bangladesh	Acute flaccid paralysis	EF015886
EV99	10461	Bangladesh	Acute flaccid paralysis	EF555644
EV102	10424	Bangladesh	Acute flaccid paralysis	EF555645
EV104	CL-1231094	Switzerland	Acute respiratory illness	EU840733
EV105	TW/NTU07	NA	NA	NA
EV109	NICA08-4327	Nicaragua	Acute respiratory illness	GQ865517
EV116	126	Russia	NA	JX514942
EV117	LIT22	NA	Pneumonia	JQ446368
EV118	ISR10	NA	Pneumonia	JQ768163

NA, information not available.

(Table 17.8). For example, acute hemorrhagic conjunctivitis is usually caused by the CVA24 variant or EV70. Acute flaccid paralysis is usually caused by PV or EV71. The occasional isolates from cases of diabetes are usually CVB serotypes.

Poliomyelitis

As is true for nonpolio EV, infection of most patients with PV does not result in disease or lead to any symptomatology. The most common symptomatic disease caused by PV, known as *abortive poliomyelitis*, is a mild febrile illness with or without gastrointestinal signs that occurs in 4% to 8% of individuals. The incubation period from infection to the onset of abortive poliomyelitis is usually 1 to 3 days, although symptoms can be seen as late as 5 days after infection. Less frequently, PV infection results in aseptic meningitis. This nonparalytic illness has the typical features of viral meningitis, with fever, headache, and meningeal signs but an absence of signs of CNS parenchymal involvement. The meningitis has a self-limited course and lasts for a few days to 2 weeks.

On average, only about 1 in 200 PV infections in a fully susceptible population results in the paralytic disease known as *poliomyelitis*. The incubation period from infection to the onset of paralysis is usually 4 to 10 days, although it can be as short as 3 days or longer than a month. The paralysis generally occurs 2 to 5 days after headaches occur and peaks within a few days. Usually, a prodrome occurs with sensory complaints and shooting or aching pains in muscle. The muscle pains may reflect growth of the virus in this tissue, which is known to occur.²⁷⁰ In children can be seen a biphasic or *dromedary* course of neurologic involvement, and paralysis can occur as the initial symptom.

Paralysis is classified as either spinal or bulbar, depending on whether the spinal cord or brainstem, respectively, is involved. Not infrequently, the spinal form becomes associated with the bulbar form during the course of the disease, resulting in so-called *bulbospinal polio*. Spinal polio is usually asymmetric, flaccid, and limited to the extremities and trunk and varies from mild weakness to quadriplegia. Only about 10% to 15%

TABLE 17.7 Enterovirus Species D (EV-D)

Type	Prototype strain	Geographic origin	Illness in person with prototype	Accession number
EV68	Fermon	California	Lower respiratory illness	AY426531
EV70	J670/71	Japan and Singapore	Acute hemorrhagic conjunctivitis	D00820
EV94	E210	Egypt	Detected in sewage	DQ916376
EV111	KK2640	Cameroon	None ^a	JF416935

^aThe prototype strain was detected in a chimpanzee, but another strain of EV111 was detected in a human with acute flaccid paralysis in the Democratic Republic of the Congo.

TABLE 17.8 Clinical Syndromes Associated with Infections by Enteroviruses

Polioviruses, types 1–3
Paralysis (complete to slight muscle weakness)
Aseptic meningitis
Undifferentiated febrile illness, particularly during the summer
Coxsackieviruses, group A, types 1–24
Herpangina
Acute lymphatic or nodular pharyngitis
Aseptic meningitis
Paralysis
Exanthema
Hand-foot-and-mouth disease (A10, A16)
Pneumonitis of infants
“Common cold”
Hepatitis
Infantile diarrhea
Acute hemorrhagic conjunctivitis (type A24 variant)
Coxsackieviruses, group B, types 1–6
Pleurodynia
Aseptic meningitis
Paralysis (infrequently)
Severe systemic infection in infants, meningoencephalitis, and myocarditis
Pericarditis, myocarditis
Upper respiratory illness and pneumonia
Rash
Hepatitis
Undifferentiated febrile illness
Echoviruses, types 1–33
Aseptic meningitis
Paralysis
Encephalitis, ataxia, or Guillain-Barré syndrome
Exanthema
Respiratory disease
Others: Diarrhea
Pericarditis and myocarditis
Hepatic disturbance
Enterovirus, types 68–116 ^a
Pneumonia and bronchiolitis
Acute hemorrhagic conjunctivitis (type 70)
Paralysis (types 70, 71) ^b
Meningoencephalitis (types 70, 71)
Hand-foot-and-mouth disease (type 71)

^aSince 1969, new enterovirus types have been assigned enterovirus type numbers rather than being subclassified as coxsackieviruses or echoviruses. The vernacular names of the previously identified enteroviruses have been retained.

^bNumerous additional types have been identified in stool from acute flaccid paralysis cases, but an etiologic link has not been confirmed.

Adapted from Melnick JL. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM, eds. *Fields Virology*. 3rd ed. Philadelphia: Lippincott-Raven, 1996:655–712.

of poliomyelitis cases are bulbar, a term indicating involvement of the motor cranial nerves or medullary centers controlling respiration and the vasomotor system. Cranial nerves IX and X, those most affected, lead to paralysis of the pharyngeal and laryngeal muscles with resultant difficulty swallowing and talking. Involvement of other cranial nerves can lead to

weakness of the face (VII) and tongue (XII). Most dreaded is involvement of the brainstem reticular formation, resulting in respiratory compromise, potentially requiring ventilatory support. Also seen is autonomic involvement, which manifests as abnormalities of sweating, urination, defecation, and blood pressure control. Recovery can be delayed significantly. Not infrequently, one extremity is left severely weak and atrophic, with a relatively normal contralateral limb.

The pathology of poliomyelitis is one of inflammation and destruction of the gray matter of the CNS, especially of the spinal cord. Motor neurons up and down the neuraxis can be infected, including upper motor neurons located rostrally in the brainstem and cerebral hemispheres in addition to the anterior horn lower motor neurons in the spinal cord. The widespread nature of the gray matter infection demonstrates that the disease is frequently a polioencephalomyelitis (i.e., inflammation of the gray matter of the brain and spinal cord) rather than merely a poliomyelitis (inflammation of the gray matter of the spinal cord). Interestingly, perivascular mononuclear inflammatory cells can persist for months, although virus is difficult to culture from the spinal cord after a week.³⁷ Although the focus of pathology in the spinal cord is in the anterior horn, abnormalities also occur outside the motor system in the posterior horn and intermediolateral column. Similarly, the brainstem shows involvement of a number of sensory cranial nerve nuclei and of the reticular formation in addition to the motor cranial nerve nuclei. Neurons die with evidence of chromatolysis followed by neuronophagia. Recent investigations of CD155 transgenic mice have reported that spinal cord neurons die by apoptosis.¹²⁵

Poliomyelitis or acute flaccid paralysis can occur as a result of infection with other EVs besides PV. EV71 is emerging as the most important virulent neurotropic EV during the poliomyelitis eradication period. This virus causes epidemics of poliomyelitis-type disease, including bulbar disease.⁷² In an EV71 epidemic in Bulgaria in 1975, a paralytic disease occurred in as many as 21% of approximately 700 patients, with a case fatality rate approaching 30%.³⁷³ EV71 is also associated with epidemic encephalitis and meningitis (see Clinical Features: Meningitis and Encephalitis). EV70, a cause of epidemics of acute hemorrhagic conjunctivitis, can lead to a severe and acute paralytic disease.⁴¹¹ The incidence of paralysis is probably 1 of 10,000 infections.¹⁹⁷ These patients can also have cranial nerve palsies, autonomic abnormalities, and sensory signs. Sometimes, EV70 infections cause isolated cranial nerve palsies, most commonly involving the facial nerve. The eye disease is usually spread by direct or indirect contamination of the eye rather than the fecal–oral route. Some echoviruses and coxsackieviruses have also been associated with acute flaccid paralysis (e.g., CVA7 virus).¹³⁷

Postpolio Syndrome and Amyotrophic Lateral Sclerosis

Patients with postpolio syndrome complain of new weakness, fatigue, and pain decades after paralytic poliomyelitis. One subgroup of this syndrome, called *postpoliomyelitis progressive muscular atrophy*, is an uncommon primary neurologic disorder manifested by slowly progressive atrophy of muscles with evidence of ongoing motor nerve damage.⁵⁴ Some investigators have reported a persistent PV infection in the spinal fluid or

CNS tissue from patients with postpoliomyelitis progressive muscular atrophy,^{248,249,290,333,371} but others have failed to confirm these findings.^{201,272,361} One of the groups that originally reported EV genome in patients with postpolio syndrome later claimed that both patients and controls had evidence of enteroviral genome in the CNS.²⁹¹ Although the PV genome of a mouse-adapted mutant PV has been reported to persist at low levels following experimental infection of mice,⁹⁷ the only consistent evidence for persistent PV infection in humans has been found in individuals who are immunocompromised. We await more convincing data demonstrating that a persistent infection underlies the postpolio syndrome.

The issue of whether PV can persist in postpoliomyelitis progressive muscular atrophy has raised questions about whether PV or another EV is involved in amyotrophic lateral sclerosis, a chronic progressive weakening disease of unknown cause associated with death of motor neurons. A possible role for EV in amyotrophic lateral sclerosis seems unlikely given the immunocompetence of these patients and the noninflammatory pathology of the disease. Although some studies have reported enteroviral genomic sequences in tissues of patients with amyotrophic lateral sclerosis and motor neuron diseases, a recent investigation failed to find evidence to support this.³⁰⁴

Meningitis and Encephalitis

Aseptic meningitis is a nonbacterial inflammation of the meninges associated with fever, headache, photophobia, and meningeal signs in the absence of signs of brain parenchymal involvement.³⁵⁸ The syndrome is the most common CNS infection, with 7,000 cases of aseptic meningitis reported per year in the United States and an actual incidence believed to be 10-fold higher.³⁵⁷

Enteroviruses are the main recognized cause of aseptic meningitis in both children and adults in developed countries. Enteroviruses were identified in 85% to 95% of cases in which a specific pathogen was cultured.³⁵⁸ In one study, 62% of infants younger than 3 months of age with aseptic meningitis had CVB as the etiologic agent.²⁰⁹ Of note, aseptic meningitis is the most common clinical syndrome caused by EV that results in medical attention.

Enteroviruses should be suspected as the causative agent for aseptic meningitis occurring in the summer and fall in temperate zones. The seasonal increase in incidence of enteroviral aseptic meningitis may result because the fecal–oral route of transmission is facilitated in warm periods when less clothing is worn.¹⁹⁷ Fever is common in patients with EV-induced aseptic meningitis, as with most cases of aseptic meningitis. At times, the fever has a biphasic pattern, initially associated with constitutional symptoms and then returning with meningeal signs. One may see nonneurologic abnormalities associated with enteroviral meningitis (e.g., rash), which may be helpful in the diagnosis and identification of the particular enteroviral serotype.¹⁹⁷ For example, rashes have been associated with CNS disease caused by CVA5, A9, and A16 and echoviruses 4, 6, 9, and 16. The rash associated with echovirus 9 meningitis can be petechial, resembling that seen with meningococcemia.

Encephalitis signifies that the brain parenchyma is infected and is not infrequently associated with a disturbed state of consciousness, focal neurologic signs, and seizures. The distinction between aseptic meningitis and encephalitis is important because the absence of parenchymal involvement in aseptic

meningitis suggests a more benign condition and more favorable prognosis. Encephalitis is usually associated with aseptic meningitis, usually resulting in a meningoencephalitis.

Although a specific virus is not usually identified in most cases of encephalitis, and although the number of cases in which an EV has been identified as the cause of encephalitis is low, EVs nevertheless rank second to herpes simplex virus and comparable to arboviruses as a recognized cause of encephalitis in the United States. The New York State Department of Health found evidence of EV genome by PCR in the spinal fluid of 3 of 41 cases of encephalitis over the period from July 1997 to November 1998;¹⁷² herpes simplex virus genome and arbovirus genome were each found in 5 cases.

Patients with EV encephalitis usually have a global neurologic depression in function, although occasionally seen are focal neurologic signs, resembling herpes simplex virus encephalitis.²⁸³ Cases associated with an acute cerebellar ataxia have been reported in children infected with various EVs, including PV, echoviruses 6 and 9, and coxsackieviruses A2 and A9.¹⁹⁷ Other uncommon clinical manifestations reported include acute hemiplegia, opsoclonus-myoclonus, movement disorders, and Guillain-Barré syndrome.¹⁹⁷

The meningeal syndrome is usually self-limited and benign, with evidence of improvement in days to a week. Deaths have been reported, however, following infection with a number of enteroviruses, including CVB1 and echoviruses 9, 17, and 21.¹⁹⁷ Morbidity and mortality are also increased in the neonate. The neonate's disease tends to be especially severe when the disease appears soon after birth, perhaps reflecting perinatal spread of virus to the fetus from the mother. Mortality is increased in the neonate partly because the aseptic meningitis can be associated with systemic disease (e.g., hepatitis and myocarditis) as well as encephalitis. The largest study of recovered patients failed to find any neurodevelopmental abnormalities above levels seen in controls.³⁵²

Isolation of EV may be unsuccessful because a particular EV may grow poorly in cell culture or because inhibitory factors (e.g., neutralizing antibody) may be present in the spinal fluid. With the use of reverse transcriptase (RT)-PCR, EV genome may be found in a significant number of culture-negative spinal fluid samples from cases of aseptic meningitis. The potential usefulness of RT-PCR can be seen in a report of a Swiss epidemic of aseptic meningitis caused by echovirus 30, when the EV genome was identified using this technique in 42 of 50 spinal fluid cultures (84%) that were negative for virus isolation.¹³³ On the other hand, the use of RT-PCR may be unsuccessful because some so-called generic RT-PCR primers may fail to hybridize well to particular EVs because of a difference in target sequence.

In some areas of the world, epidemics of EV71^{72,373} have been associated with a high incidence of aseptic meningitis as well as CNS parenchymal involvement; the CNS infection has caused acute flaccid paralysis as well as a more varied clinical symptomatology.²⁷⁴ As noted, EV71 is emerging as the most significant neurotropic EV in some areas of the world. This virus circulates in the United States, and 26% of adults tested in a seroepidemiologic study in New York in 1972 had antibody.⁹⁵ An epidemic of EV71 infection in 1998 in Taiwan caused frequent CNS disease and more than 100,000 reports of hand-foot-and-mouth disease or herpangina (see Respiratory Infections, Herpangina, and Hand-Foot-and-Mouth

Disease), which may correspond to 1 million actual cases of EV infection.¹⁶¹ The patients with hand-foot-and-mouth disease had vesicular lesions on their hands, feet, mouth, and, at times, buttocks, whereas the patients with herpangina had vesicular lesions of the palate and pharynx. Of special concern was the finding of 405 severe cases with associated complications, 78 of which were fatal. Among the more severe cases, the case fatality rate in different areas ranged from 7.7% to 31.0%. Most of the hospitalized cases (80%) and the deaths (91%) were in children younger than 5 years of age. The most frequent complication was encephalitis. Other serious complications, which were associated at times with encephalitis, included aseptic meningitis, myocarditis, and pulmonary edema and hemorrhage. It is unclear whether the pulmonary signs were related to viral invasion of the lungs or to brain injury from the viral encephalitis.

A review¹⁷² of 41 hospitalized children with neurologic complications and culture-confirmed EV71 infection during the Taiwan epidemic in 1998 demonstrated an unusual and rather distinctive neurologic manifestation, brainstem encephalitis or rhombencephalitis. The mean age of the 41 children was 2.5 years (with a range from 3 months to 8.2 years). There was frequently an associated condition: hand-foot-and-mouth disease in 68%, or herpangina in 15%. The neurologic disease usually followed the initial illness of fever and skin or mucosal lesions, and it was manifested as rhombencephalitis (37 patients), aseptic meningitis (3 patients), and acute flaccid paralysis (associated with the disease in 4 patients, and following rhombencephalitis in 3 of the 4). At times, breathing difficulties and coma were seen in the patients with rhombencephalitis, progressing to death in five. The brainstem and spinal cord pathology was presumably related to direct viral injury, because virus was cultured from the spinal cord and brainstem of the one patient who underwent autopsy. Five of the patients who survived had neurologic sequelae. EV71 brainstem involvement was also seen during an epidemic in Malaysia in 1997, but not in earlier epidemics. In contrast, hand-foot-and-mouth disease and herpangina did not occur during the previous epidemics in Bulgaria and Hungary. It is not clear whether the changes in EV71 disease phenotype represent the emergence (or re-emergence) of a more virulent strain or whether it is related to the serologic status of the at-risk populations.

The presence of immunodeficiency predisposes to a syndrome of EV-induced aseptic meningitis or meningoencephalitis, at times producing a persistent CNS infection. McKinney et al.²⁷⁰ reviewed more than 40 cases of chronic enteroviral meningoencephalitis in patients with congenital immunodeficiencies, most commonly X-linked agammaglobulinemia. Clinical features were remarkably varied and included headaches, seizures, ataxia, a disturbed state of consciousness, motor deficits, personality changes with cognitive decline, and sensory disturbances. At times, patients had other associated abnormalities, such as dermatomyositis syndrome (in 21 of 41 patients; see Clinical Features: Muscle Disease), rashes (16 of 35 cases), edema (20 of 40 cases), and hepatitis (15 of 32 patients). Although some cases started abruptly, most tended to be slowly progressive, at times lasting years, and with a frequent fatal outcome. Most patients had a spinal fluid mononuclear cell pleocytosis with an increase in protein. Some cases at autopsy had evidence of inflammatory infiltrates in the heart, lungs, kidneys, adrenal glands, thyroid, and pancreas, in addition to the CNS.

Cardiac Disease

Myocarditis is an inflammation of the myocardium associated with damage that is unrelated to an ischemic injury. Myocarditis is frequently self-limited and subclinical, with few if any sequelae. On the other hand, the acute disease can lead to significant morbidity and even death. On the basis of well-established criteria, one study found evidence of myocarditis in approximately 1% of autopsies.¹³⁶

In some cases, myocardial inflammation may persist, producing a chronic myocarditis that can progress to dilated cardiomyopathy.^{61,205,354} In dilated cardiomyopathy, the heart is large, with impaired function and evidence of heart failure but with little or no inflammation. The incidence of dilated cardiomyopathy in the United States has been reported to be 6 per 100,000 cases,⁷⁷ with approximately 100,000 new cases each year. Because of the high mortality of dilated cardiomyopathy, patients are frequent recipients of heart transplants, making up perhaps 50% of all cardiac transplant patients. Of note is the observation that nonpolio EVs, especially CVB3, cause acute and chronic myocarditis in experimental animals, and that a contribution from the immune system to the chronic disease has been proposed (see Pathogenesis earlier).

Acute Cardiac Disease

CVB has long been considered one of the principal causes of viral myocarditis,⁸¹ and this view has been confirmed in several recent reports.^{16,106,262,264,266} Epidemiologic and other studies suggest that 70% of the general public will be exposed to cardiotropic viruses, and half of these individuals will develop acute viral myocarditis.³⁰⁶ It is believed that 1.5% of enteroviral infections, including 3.2% of CVB infections, result in overt cardiac signs or symptoms.¹³⁷ Myocarditis is a frequent autopsy finding in children who die of overwhelming CV infection.²⁰⁹ The peak age group in which myocarditis caused by CVB occurs is young adults, primarily between the ages of 20 and 39 years, with a higher prevalence among men.⁴²⁵

However, even the larger group of symptom-free individuals is at risk; collapse and death of young and vigorous individuals, especially during exertion, can result from catastrophic dysfunction of the electrical pathways in the heart, as a consequence of unsuspected acute viral myocarditis.^{31,415} A remarkably high prevalence of asymptomatic myocarditis has been shown by necropsy studies of victims of violent or accidental deaths; in this relatively random human population, approximately 1% had active myocarditis.¹³⁶

A summary of data from varied sources shows that the prevalence of enteroviral infection in acute myocarditis on the basis of serologic studies is 34% (214 positives in 636 patients) compared with 4% (44 of 1,139) of controls (199). However, these serologic studies demonstrating seroconversion with an EV do not prove that EV caused the cardiomyopathy, as they may result merely from an unrelated EV infection. In addition, evidence suggests that multiple EVs can circulate at the time of an epidemic, so that isolation of an EV does not prove its role in a disease; for example, more than 10 EVs were recovered from the community during an echovirus 9 epidemic.¹⁹⁷

A meta-analysis of data obtained from molecular studies (slot blot hybridization, *in situ* hybridization, and RT-PCR) published in 12 reports found 23% of cases (68 of 289) and 6% of controls (14 of 216) had evidence of EV genome in heart tissue, giving an odds ratio of 4.4 with a 95% confidence

interval of 2.4 to 8.2.²⁷ These data, coupled with reports of positive virus isolations from the heart, indicate that EV may represent a common cause of acute myocarditis. The surprisingly large number of positive findings in controls is presumed to result from difficulties in obtaining appropriately controlled heart tissue.

Chronic Cardiac Disease

Human DCM has multiple causes, both inherited and sporadic. A prior history of enteroviral infection of the heart, especially with CVB, has been implicated in sporadic human dilated cardiomyopathy.^{26,382} Although the majority of patients with symptoms recover well from acute myocarditis, the disease can have serious long-term sequelae; some 10% to 20% of people with symptoms (i.e., ~20,000 to 40,000 patients per year in the United States) will develop chronic disease, progressing over time to DCM.^{306,380} A summary of serologic data from multiple sources found that the prevalence of EV in dilated cardiomyopathy was 25% (64 of 260) compared to 10% of controls (26 of 255).²⁶⁸ These serologic data suffer from the same drawbacks that are noted earlier.

In contrast to acute myocarditis, no reports are found of isolation of EV from chronic dilated cardiomyopathy, suggesting that the virus may have a restricted expression or disappear following the acute infection; however, some reports exist of EV VP1 antigen in the heart of patients with dilated cardiomyopathy and chronic coronary disease.¹⁵

The difficulty in isolating infectious virus led to studies probing affected tissues for persistent enteroviral genome. A meta-analysis of data from molecular studies described in 17 published reports found 23% of the patients and 7% of controls had evidence of enteroviral genome, giving an odds ratio of 3.8 with a 95% confidence interval of 2.1 to 4.6.²⁷ The meta-analysis data are supportive of an association between EV infection and chronic cardiac disease; however, it remains a possibility that RNA from other laboratory-based enteroviral studies contaminated test samples in the highly sensitive PCR studies. Most of the hybridization and PCR studies did not include sequencing of the viral genome and, therefore, failed to prove more directly that an EV was involved. In a few cases, however, partial sequencing was done of the RNA found in clinical samples; for example, Archard et al.²⁰ identified the amplified sequence as CVB.

Not all the viral genomic studies have been positive. One investigation involving a nested PCR failed to find evidence of EV RNA in 287 heart biopsy specimens from 38 patients with dilated cardiomyopathy and 39 patients with heart failure of unknown cause.⁹² At least two other studies have also resulted in negative findings.^{251,369} The lack of consistent reproducible results regarding the presence of enteroviral genome in tissue from patients with cardiomyopathy indicates that further studies are needed under careful, blinded conditions.

Muscle Disease Including Pleurodynia

The relationship of EV to inflammatory muscle diseases was initially recognized because of the myotropism of coxsackieviruses in suckling mice. This observation was fueled by the association of these viruses with epidemic pleurodynia on the Danish island of Bornholm. The latter disease, called *Bornholm disease*, is an acute febrile illness with myalgia, especially involving the chest and abdomen, but without muscle weakness.

It has occurred as an epidemic and also sporadically in various locales. Relapses can occur. CVB3 and B5 are the most frequently recognized causative agents, although other EVs have also been isolated.⁴⁴⁶ The limited information from muscle biopsy findings suggests that the inflammation in this disease may be confined to the endomysial part of the muscle and, therefore, is not a true myositis.⁸⁷

Enteroviruses have been implicated in acute and chronic inflammatory muscle disease. The acute diseases, which are usually called *acute polymyositis* or *myositis*, are characterized by fever with myalgia, elevated muscle enzymes, and, at times, myoglobinuria. Chronic inflammatory muscle diseases, which are generally classified as polymyositis, dermatomyositis, or inclusion body myositis, have a subacute to chronic progressive weakness with a distinctive pathology on muscle biopsy. Dermatomyositis is distinguished from the other two inflammatory myopathies because it is associated with a characteristic rash.

The causes of chronic inflammatory myopathy are generally unknown. Hypotheses concerning the etiology of polymyositis and dermatomyositis include a direct virus infection, especially an EV infection, or an autoimmune process in which the virus infection triggers a reaction against muscle (see Pathogenesis earlier). That coxsackieviruses cause acute and chronic inflammatory myopathy in experimental animals provides additional support for their involvement in human inflammatory muscle diseases. Investigations of these model systems may help clarify the pathogenesis of these diseases in humans.

Enteroviruses have been isolated from cases of inflammatory myopathies, but these isolations have generally been rare and from single cases with acute³³ or atypical clinical pictures.³⁸⁹ The inability to isolate virus from cases of chronic inflammatory myopathy has raised the possibility of a restricted expression of the virus with little infectious virus present. For this reason, investigators have probed muscle tissue from patients with inflammatory myopathy for picornaviral genome. Some reports involving slot blot or *in situ* hybridization studies of muscle tissues from patients with inflammatory myopathies have shown positive results using EV-specific probes.⁴⁴⁴ In some cases, the product amplified in an RT-PCR has been identified as CVB sequence.²¹ In contrast, other studies using RT-PCR and nucleic acid hybridization have found negative results.^{245,247,328} It remains a possibility that an EV could trigger an autoimmune inflammatory muscle disease and then disappear. Studies regarding the possible role of EV in chronic fatigue syndrome have similarly failed to find reproducible evidence of EV involvement.⁸⁷

Of special interest with respect to the issue of EV involvement in inflammatory muscle disease is the observation that patients with immunodeficient states can manifest a disease similar to dermatomyositis with an accompanying persistent echovirus infection.^{412,419} It should be noted, however, that questions have been raised whether these patients had a true myositis (i.e., inflammation of the muscle) or a fasciitis with interstitial inflammation in the endomysium.⁸⁷ The inflammatory muscle disease is associated with a chronic encephalomyelitis as well as a more disseminated disease in which EV, especially echoviruses, can be cultured from the spinal fluid (see Clinical Features: Meningitis and Encephalitis earlier).⁴⁵¹ Although virus has occasionally been isolated from affected muscle, it remains unclear whether the dermatomyositis syndrome in patients who are immunodeficient is a result of

direct virus invasion of the muscle or an immune-mediated disease associated with virus persistence. The existence of this syndrome similar to dermatomyositis demonstrates that echoviruses, and perhaps other EVs, can produce a chronic myositis in humans that is associated with persistent infection and chronic inflammation.

Diabetes

Both genetic and environmental factors, including EV infection, have been implicated in the cause of insulin-dependent diabetes. A number of epidemiologic and serologic studies have demonstrated a relationship between EV infection and the development of diabetes. D'Alessio⁸⁴ found that 15 of 84 cases (17.8%) of newly diagnosed insulin-dependent diabetes had evidence of IgM antibody against CVB, compared with 5 of 71 controls (7.0%). The individuals with IgM antibody were especially common in association with human leukocyte antigen (HLA)-DR3 positivity. A prospective study in Finland found a greater incidence of serologic conversion of EV antibodies among children who developed insulin-dependent diabetes than among those who did not.¹⁸⁷ The antibody present in the prediabetic period was directed against a number of different EV serotypes, including CVA9, B1, B2, B3, and B5.³⁵¹ At times, the EV antibodies are associated with autoantibodies to GAD₆₅ and other important targets.

Rare, but well-documented, isolations of CVB4 have come from the pancreases of patients with acute-onset as well as fatal cases of insulin-dependent diabetes.^{341,345} Some of the isolates have been demonstrated to be diabetogenic when inoculated into certain mouse strains⁴⁴¹ and nonhuman primates.⁴⁴² Patients dying from coxsackieviral myocarditis can have an associated pancreatitis, including isletitis. More recent investigations have involved probing tissues from patients for evidence of EV genome. An *in situ* hybridization study showed evidence of EV genome in the islets of autopsy pancreases from 7 of 12 newborn infants who died of fulminant CV infection (and 6 of the 7 had isletitis), in the islets of autopsy pancreases of 4 of 65 adults with type 1 diabetes, and in one of the pancreatic control tissues from 40 nondiabetic patients.⁴⁴⁰

Although it is likely that EVs are capable of causing diabetes mellitus in animals and humans, it remains unclear how often this occurs in humans and what if any immune mechanisms are involved.

Eye Infections

Acute hemorrhagic conjunctivitis is characterized by a short incubation period of 24 to 48 hours preceding a rapid onset of unilateral or binocular symptoms and signs. Patients manifest excessive lacrimation, pain, periorbital swelling, and redness of the conjunctiva (from subconjunctival petechiae to frank hemorrhages).⁴³⁸ Keratitis with accompanying pain and possible visual impairment as well as anterior uveitis may be seen. The epidemic disease can also be associated with nonophthalmic symptoms and signs, such as neurologic dysfunction (see Clinical Features earlier) and respiratory and gastrointestinal disturbances. The disease usually resolves without sequelae in 1 to 2 weeks.

The first pandemic of acute hemorrhagic conjunctivitis was recognized in 1969 in Africa. Within 2 years, two EVs, a new antigenic variant of CVA24 and a previously unknown EV serotype designated EV70, were implicated as causative

agents responsible for the epidemics of this disease.²⁵² During the first pandemic from 1969 to 1971, hundreds of millions of people were likely to have been infected. Subsequent epidemics of acute hemorrhagic conjunctivitis have continued in various locations throughout the world. Other EVs have also been recognized as causes of acute hemorrhagic conjunctivitis (e.g., echovirus type 7)³⁶³ and as causes of sporadic conjunctivitis and keratoconjunctivitis.²¹³ The ocular disease caused by EV is often indistinguishable from acute hemorrhagic conjunctivitis caused by various adenovirus serotypes.

Respiratory Infections, Herpangina, and Hand-Foot-and-Mouth Disease

Enteroviruses are a common cause of respiratory illnesses. Enteroviruses were isolated in 1.7% of 3,119 respiratory specimens submitted for viral culture from 1983 to 1994⁷⁰ and in 6.4% of all the viruses that were isolated from these specimens (54 of 838). Respiratory illness occurred in 15% of EV infections collected by the WHO from 1967 to 1974¹³⁷ and 21% of cases from the CDC obtained from 1970 to 1979.¹⁸ The EVs implicated in these respiratory infections included CVA (12.4%), CVB (20.3%), and echoviruses (12.6%).¹³⁷

Enteroviral respiratory infections are more frequently associated with upper respiratory infections (e.g., the common cold, croup, and epiglottitis) than with lower respiratory infections (e.g., pneumonia). The infections are frequently subclinical, but if they cause clinical disease it tends to be self-limited and mild, with a short incubation period of 1 to 3 days. More severe lower respiratory infections may be related to a higher dose of virus in the inoculum. In a series of infants with CVB infections, 30 of 77 patients had marked abnormalities (e.g., interstitial inflammation and hemorrhage), and 12 had virus isolated from the lung.²⁰⁹

Herpangina is a febrile illness of relatively sudden onset with complaints of fever and sore throat. Characteristic lesions are found on the anterior tonsillar pillars, soft palate, uvula, and tonsils, and on the posterior pharynx. The illness, which has a predilection for the young, is usually self-limited and disappears within a few days. At times, the disease is associated with more significant clinical abnormalities (e.g., meningitis). Hand-foot-and-mouth disease is an illness associated with vesicular lesions of the hands, feet, mouth, and, at times, buttocks.

A number of EVs have been identified as causes for herpangina, including CVA and B serotypes; echovirus types 6, 9, 11, 16, 17, 22, and 25;⁷⁰ and EV71. The main causes of hand-foot-and-mouth disease are CVA10 and A16 and EV71.¹³⁷ The pathogenesis of the lesions seen in herpangina is not clear; however, experimental infection of rhesus monkeys with CVA4 may provide a model system for the study of the pathogenesis of herpangina. In this system, ingestion of the virus by the oral route leads to multiplication in the lower gastrointestinal tract, followed by viremia, and then multiplication in the oropharynx.³⁷⁶

Neonate and Infant Disease

Neonates are at increased risk from enteroviral infections. This increased susceptibility is present in humans as well as experimental animals infected with varied EVs.⁷¹

The increased risk of neonates to EV infection was apparent in the prevaccine era with respect to PV. Although the incidence of paralytic disease among neonates approached 40%

born to mothers with poliomyelitis at the time of delivery, the overall incidence of neonatal poliomyelitis was an infrequent occurrence, perhaps related to the protective effect of maternal antibody.⁴³¹ Neonatal poliomyelitis generally had a shorter incubation period and a higher case fatality rate than found with disease later in life, demonstrating the increased susceptibility of immature hosts to EV infection. Infection of the mother early in gestation was associated with an increased risk of abortion, stillbirth, and prematurity.⁷¹

Nonpolio EVs are a not infrequent cause of infection in neonates and infants. In a series from the CDC, EV isolates from patients younger than 2 months of age included echoviruses (51%), CVB (45%), and CVA (4%).²⁸⁸ Echovirus serotypes included 4, 9, 11, 17 to 20, 22 (now classified as human parechovirus 1), and 31.

The most frequent presentation in the neonate with nonpolio EV is asymptomatic infection.¹⁹⁶ Symptomatic cases most commonly manifest a self-contained febrile illness, at times associated with irritability and nonspecific signs of infection, making EV infection a leading cause of fever in the infant. A rash of variable character is seen in more than 30% of cases.⁴ More serious infections can be seen, with the peak of symptoms correlating with viremia.⁸⁶ Nonpolio EVs are the most frequently identified cause of aseptic meningitis in infants younger than 1 month of age and are believed responsible for more than one-third of these cases.³⁰¹ An increased susceptibility of the neonatal CNS to CV infection may be related to the virus's predilection for neonatal stem cells.¹¹¹ In a small series of patients with pneumonia in the first month of life, EVs were implicated in 15% of cases (6 of 40).⁵ Usually, the mother has a history of fever or respiratory symptoms a week before the delivery, although mothers can be asymptomatic or have a more severe disease.

One of the serious infections caused by EVs is a sepsis-like disease. In one series, EVs were implicated in 65% of infants younger than 3 months of age admitted to the hospital for suspected sepsis.⁸⁵ In another series, evidence was seen of enteroviral genome in 80 of 345 infants younger than 90 days of age admitted to a medical center with suspected sepsis.⁵⁰ A number of other studies have yielded similar results, emphasizing the importance of EV as a cause for a sepsis-like syndrome among neonates and infants.⁸⁶ In fact, EVs tend to be a more common cause of this syndrome than bacteria in the summer and fall.⁵⁰ There may be extensive multiorgan involvement in severely affected cases, especially ones that go on to a fatal outcome; the organs affected include liver, lung, heart, pancreas, and brain.

The incidence and severity of EV infections among infants can be better appreciated by reviewing a study conducted in Nassau County, New York, from 1970 to 1979.²⁰⁹ Of 153,250 live births, 77 infants younger than 3 months required hospitalization for CVB infection. These 77 cases were from a pool of 602 infants who tested positive for CVB, demonstrating the high frequency of CVB infection in infants. The attack rate might have been even higher than was found, because the positive samples from this study were from only a limited number of infants hospitalized in the community. A total of 24 mothers had evidence of a viral-like infection occurring within a period from 10 days before delivery to 5 days after delivery. The most common syndrome that was seen was aseptic meningitis. Some of the infections in the infants were very serious, as evidenced by the finding that eight children died from

overwhelming CVB infection. All but one of these eight had evidence of myocarditis.

Severe EV disease in the young is associated with an early age of illness, prematurity, a more severe illness in the mother, multiorgan disease, low socioeconomic status, bottle-feeding, specific EV types, and an absence of neutralizing antibody to the pathogen.⁴ The level of maternal neutralizing antibody appears to be important both for determining the risk of developing infection and for modulating disease severity.⁷ Long-term sequelae following neonatal myocarditis or CNS infection appear infrequent.^{4,357}

Nonpolio enteroviral infection can affect the fetus, inducing clinical abnormalities and causing overt disease in the neonate. Reports exist of abortion and stillbirth associated with maternal enteroviral infections, although these are infrequent.⁴ The relationship between maternal EV infection and congenital anomalies in the newborn remains unclear.

A number of routes exist by which EV can cause neonatal disease. Studies have found evidence of placentitis and viral infection in fetal tissues.⁴ Virus can enter the placenta or fetus from contaminated maternal blood, feces, or vaginal secretions, which can occur during pregnancy, with virus penetrating the fetal membranes, or at the time of the delivery. In addition, infection of the neonate can result from a viremia, because EVs have been cultured from cord blood,¹⁹⁹ but it is unclear how often this route of transmission occurs. Infection can also occur from virus shed by other neonates or hospital staff in the nursery. In cases involving nursery outbreaks, implementation of infection controls (e.g., strict hand washing and the isolation of affected patients) has a role in prevention of new cases.

DIAGNOSIS

Differential and Presumptive Diagnosis

The process of diagnosing an EV infection or establishing that an EV infection produced a particular clinical syndrome can be complicated and challenging. This problem results from the biology and epidemiology of EV infections, as well as from limitations in current diagnostic methodologies. Although it is possible to demonstrate that a person is infected with an enterovirus, this association does not necessarily prove likely disease causation. On the other hand, a presumptive diagnosis in certain epidemiologic and clinical situations can be possible with a high degree of certainty on clinical grounds alone.

Several related biological properties complicate the diagnosis of EV-induced disease. The first is that most virus replication and infection typically occur in the respiratory and gastrointestinal tract. These infections are often asymptomatic, with few if any systemic clinical signs. Because these infections are extremely common, even random sampling of healthy individuals can demonstrate EV infections at substantial rates. For this reason, the simple recovery or detection of virus from certain nonsterile sites does not establish a firm linkage to disease, and it may be merely coincidental.

A second difficulty is that even when illness results from the EV infection, most of the signs and symptoms are relatively generic and usually lack specificity. The collection of appropriate clinical specimens for virus isolation or detection is critical to laboratory confirmation of EV infection. For example, the exclusive use of CNS or cardiac specimens to diagnose

meningitis and encephalitis limits the sensitivity for detection of infection. For example, although it is possible to detect EV in the CSF from meningitis cases, it is uncommon to find virus in the CSF from cases of encephalitis wherein is seen a reasonable suspicion of EV as the cause; in other cases, the virus can be readily recovered from CSF, such as during the recent outbreaks of fatal EV71 encephalitis.¹⁷ In general, however, the specimen with the highest sensitivity for establishing an acute infection is a stool specimen, regardless of clinical presentation.²⁸¹ The difficulty with virus detection in some clinical conditions may be further compounded because virus shedding from the gastrointestinal tract may stop before the onset of symptoms and, therefore, sensitivity for virus detection may be lower.

Sometimes a presumptive diagnosis can be made on the basis of limited information because of the pronounced seasonality of EV infection in temperate latitudes and the tendency for EV to cause community outbreaks. For example, cases of aseptic meningitis occurring during the late summer and early autumn have a high probability of EV etiology. In this situation, a presumptive diagnosis of an EV infection could be made on the basis of signs and symptoms, exclusion of other nonviral pathogens, and evidence of characteristic cytopathology of inoculated cell cultures or detection from some nonsterile site. Another example pertains to epidemic acute hemorrhagic conjunctivitis; in this case, the identification of an EV (either CVA24 variant or EV70) precludes the need for further testing of specimens from other patients in that community with similar symptoms.

Laboratory Diagnosis

Molecular Detection

The application of molecular biology techniques to clinical virology has significantly changed approaches to EV diagnostics (see Epidemiology earlier). Because of distinct advantages in speed, many of these procedures have already supplanted traditional methods of detection and characterization. Continued development and refinement of these procedures will undoubtedly lead to even further application of the methodologies to a broader spectrum of EV diagnostic problems and, ultimately, into routine primary usage.

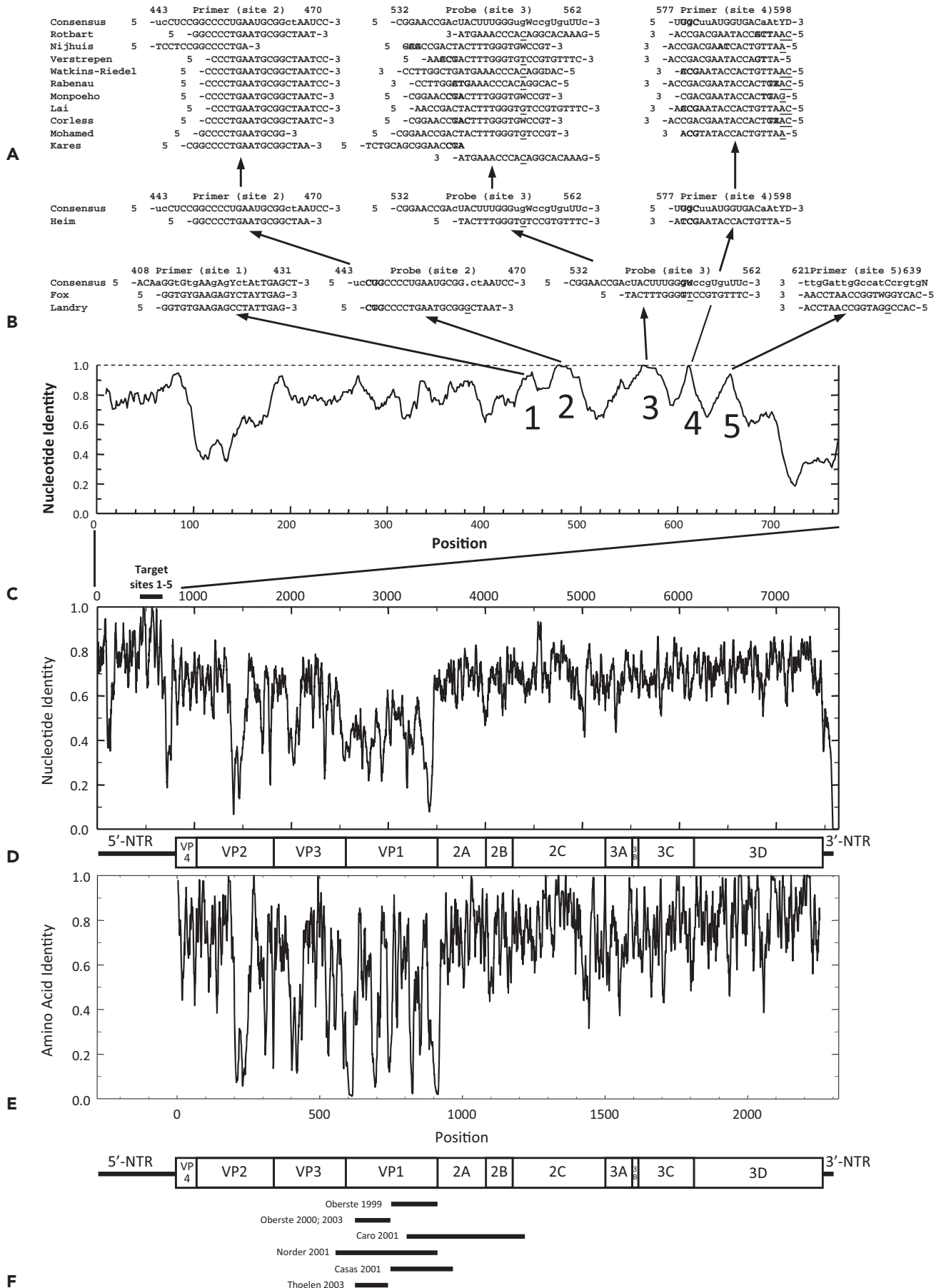
As with virus isolation and serotyping, the molecular methods attempt to detect the presence of EVs in a specimen and, in some procedures, to further characterize the detected virus. The techniques can be grouped on the basis of their infrastructure and technical requirements and the types of specimens to which they are applicable. The most commonly used tests are based on the PCR, which is used primarily to detect EV genome in cell cultures, clinical specimens, and biopsy or autopsy tissues.³¹⁷ The newest procedures utilize genomic sequencing for the characterization of EV at the highest levels of specificity.

By far the most common use of PCR for EV diagnosis is the direct detection of virus in clinical specimens.^{214,317,323,360} Numerous variations on the details of the procedures are found, but all methods that can generically detect EV have several common features. The most important property of these tests is that the primers are targeted to amplify the 5' UTR of the virus genome (Fig. 17.10). Many different primers targeted to this region have been published, targeting different sequences and providing different product sizes. Many of these primers, however, have not been completely evaluated on a

large number of clinical samples to confirm reactivity with all EV serotypes and strains within serotypes, and therefore, they have not been validated sufficiently for diagnostic use.²⁹¹ The major advantage of the pan-EV PCR is that rapid detection of an EV is possible, even with very small amounts of clinical specimens such as spinal fluid. It is also possible to detect EVs that do not readily grow in cell culture. As with all PCRs, the sensitivity of amplification of RNA from biological specimens is extremely variable, depending on the nature of the specimen. Although the PCR procedure can be shown to give a positive result even from only one or a few copies of viral RNA, it is not unusual for the sensitivity to be many orders of magnitude lower in certain specimens (e.g., stool). For this reason, despite the theoretic advantage of the PCR to greatly improve EV detection from clinical specimens, the gains in practice are much more modest (e.g., with spinal fluid) or nonexistent (e.g., with stool).³⁸⁸

By changing the target for amplification, it is possible to characterize a particular EV using the PCR. Because the antigenic property of viruses that defines serotype is a property of the viral capsid proteins, certain sequences correspond to this conserved antigenic property within the capsid-coding region of the virus. Extensive studies of the capsid region of PV isolates have led to the design of primers that can selectively amplify isolates from a single serotype but not from other isolates of heterologous serotypes.²²³ This is accomplished, despite the high rate of synonymous nucleotide substitutions, through the use of inosine in the primer synthesis. In an analogous manner, the ability of PV to uniquely bind to the CD155 is also an intrinsic property of the capsid proteins. The amino acids that are involved in receptor binding have been well characterized from studies of the three-dimensional structure of the virion. When primers are designed that correspond to these presumably PV-specific sequences, amplification is observed with all PV isolates but not with other EVs.²²⁴ It should be possible to extend these principles to other EV receptor groups and serotypes and to provide tools for rapid serotype characterization of EVs without the requirement for cell culture procedures. Nucleic acid probes, including the use of RNA probes to improve sensitivity, the introduction of nonradiochemical labeling of probes, and the application of these techniques to *in situ* hybridization in myocardial tissue,¹⁶⁶ have been particularly useful for understanding the cytopathology of EV infection, especially in studies of myocarditis. The technique, however, is relatively insensitive and cannot be used to identify the EV serotype in a specimen because of probe binding to related serotypes.

A goal in virus identification is knowledge of the sequence of the viral genome. Encoded within this sequence are determinants for all the biological properties that are attributable to a given virus. Therefore, the nucleic acid sequence information of a virus represents its ultimate characterization. All important information about a virus could potentially be obtained directly by PCR in conjunction with nucleic acid sequencing if all the molecular correlates of viral phenotypic determinants were understood. At present, however, the genetic location for many properties of the virus remains uncertain. Nevertheless, it is possible at present to use sequence information to assign an EV isolate to a particular serotype.³¹¹ The molecular typing system is based on RT-PCR and nucleotide sequencing of all or a portion of the genomic region encoding VP1. The serotype of an unknown isolate is inferred by comparison of the



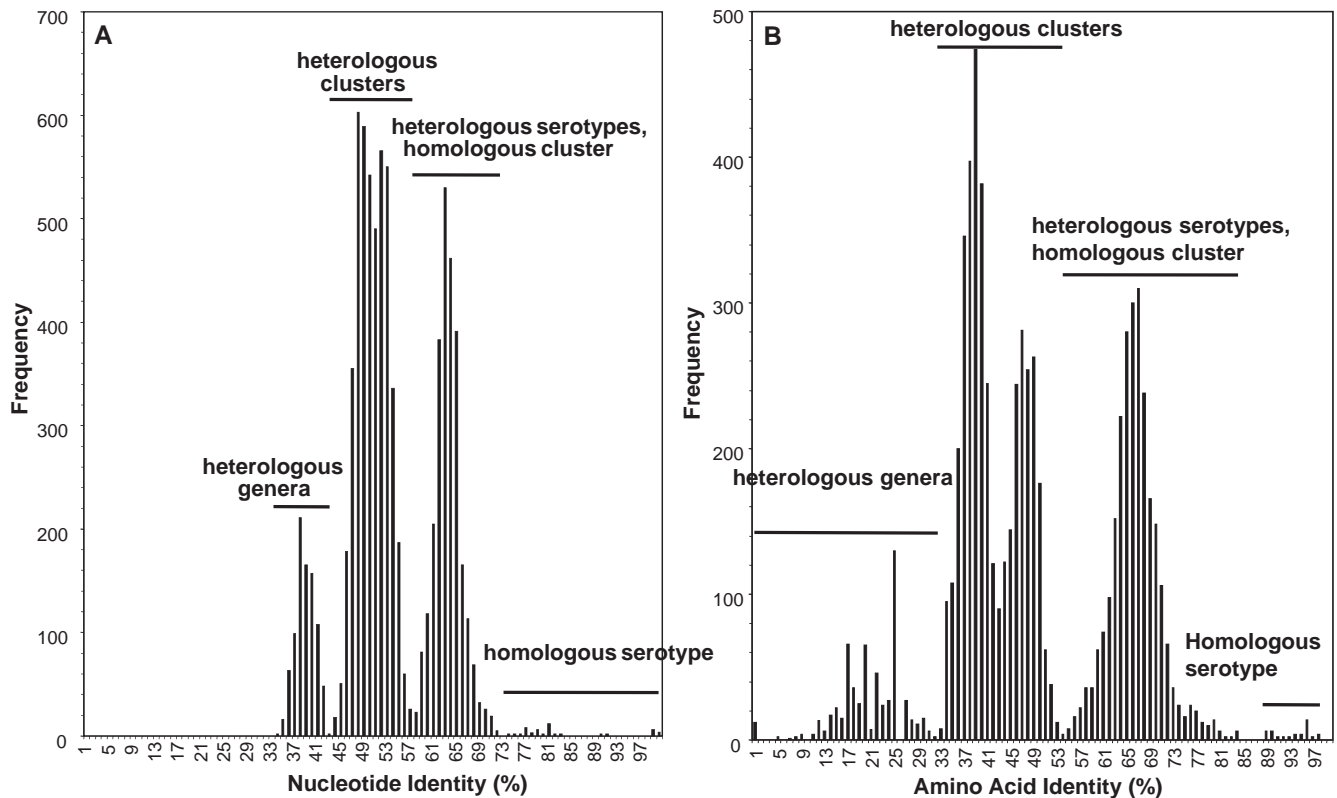


FIGURE 17.11. Serotype identification using sequence data. Frequency distribution of pairwise identity scores for comparison of VP1 nucleotide and deduced amino acid sequences. Serotype identification of an isolate can be achieved by comparing the sequence of the isolate with known sequences in VP1 for all serotypes and looking for identities of greater than 75% in nucleotide sequence or greater than 88% in amino acid. **A:** Nucleotide sequence distribution. **B:** Amino acid sequence distribution. (From: Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enteroviruses by partial sequencing of VP1. *J Clin Microbiol.* 1999;37:1288–1293.)

VP1 sequence with a database containing VP1 sequences for the prototype and variant strains of all human EV serotypes. The following guidelines have been suggested:

- (i) a partial or complete VP1 nucleotide sequence identity of $\geq 75\%$ ($>85\%$ amino acid identity) between a clinical EV isolate and serotype prototype strain may be used to establish the serotype of the isolate, on the provision that the second highest score is $<70\%$; (ii) a best-match nucleotide sequence identity of $<70\%$ may indicate that the isolate represents an unknown (that is, new) serotype; and (iii) a sequence identity between 70% and

75% indicates that further characterization is required before the isolate can be identified firmly.³¹⁶

Using these guidelines, strains of homologous serotypes can be easily discriminated from heterologous serotypes and new serotypes can be identified (Fig. 17.11). This method can greatly reduce the time required to type an EV isolate and can be used to type isolates that are difficult or impossible to type using standard immunologic reagents. The technique is also useful to rapidly determine whether viruses isolated during an outbreak are epidemiologically related.

FIGURE 17.10. Enterovirus nucleotide and amino acid sequence variation, and location of primers used for virus detection and identification.

A: Sequence conservation at sites in the enterovirus (EV) 5' nontranslated region (NTR) that are targeted by published real-time reverse transcriptase-polymerase chain reaction (RT-PCR) primers. The consensus sequence at each site and the sequences of the widely used Rotbart primers and probe are indicated for comparison. Mismatches between a primer and the human enterovirus consensus are underlined. Numbers indicate nucleotide positions relative to the genome of poliovirus 1, Mahoney strain (GenBank J02281). **B:** Sequence conservation at sites in the EV 5' NTR that are targeted by the published nucleic acid sequence-based amplification (NASBA) methods, as in panel **A**. **C:** Nucleotide sequence variation across the enterovirus genome. Complete genome sequences for reference strains of all human enterovirus serotypes were aligned, the sequence identity within each window of 18 residues was plotted versus the nucleotide position, and the window was advanced in one-residue increments across the genome. An expanded view of the 5' NTR analysis is shown in the upper plot. Peaks labeled 1–5 are sites commonly targeted by enterovirus molecular detection assays. **D:** Enterovirus genome map. Boundaries of mature protein products are approximate. **E:** Amino acid sequence variation across the enterovirus genome. Deduced polypeptide amino acid sequences for reference strains of all human enterovirus serotypes were aligned; the sequence identity within each window of six residues was plotted versus the amino acid position and the window was advanced in one-residue increments across the genome. **F:** Locations of RT-PCR products used for molecular serotyping.

Virus Isolation

Many of the detailed procedures for the established laboratory diagnosis of EV infections using virus isolation have been described.¹³⁵ The traditional techniques for detecting and characterizing EV rely on the time-consuming and labor-intensive procedures of viral isolation in cell culture and neutralization by reference antisera. Isolation of EV from specimens using appropriate cultured cell lines is often possible within 2 or 3 days and remains a very sensitive method for detecting these viruses. The best specimens for isolation of virus are, in order of preference, stool specimens or rectal swabs, throat swabs or washings, and CSF. Throat swabs or washings and CSF are most likely to yield virus isolates if they are obtained early in the acute phase of the illness. For cases of acute hemorrhagic conjunctivitis, the best specimens are conjunctival swabs,⁴³⁸ although occasionally virus can be isolated from tears.⁴³⁹ Since the major pandemic in 1981, however, isolation of EV70 from patients with acute hemorrhagic conjunctivitis has been very difficult, and molecular methods provide the only sensitive method to detect this agent.³⁷⁴

The procedure for virus isolation involves inoculation of appropriate specimens onto susceptible cultured cells. No single cell line exists that is capable of growing all human enteroviruses. It is common practice to use several types of human and primate cells to increase the spectrum of viruses that can be detected.⁶⁹ Even with a variety of cells, however, several CVA serotypes fail to propagate in culture. The coxsackieviruses, including those that do not grow in cell culture, can be isolated and propagated in suckling mice.

As a consequence of current PV eradication activities and the importance of PV as a public health problem, specific diagnostic procedures have been developed to detect this virus. In general, PV grows well on a variety of primate and human cell culture lines, but it cannot be distinguished from other EVs solely on the basis of cytopathic effect. Polioviruses are unique in the use of CD155, which is distinct from receptors used by all other EVs to infect cells. This receptor has been transfected and expressed in a murine cell line that normally cannot be infected by most EVs but is permissive to viral replication when the viral genome is present within the cell. One of these transfected murine cells, L20B, can grow PV and has been exploited selectively to isolate PV, even in the presence of other EVs.¹⁷¹ When a specimen is inoculated onto these cells and a characteristic EV cytopathic effect is seen, the virus can be presumptively identified as a PV. A few strains of certain nonpolio EV serotypes are able to grow on the parent murine cells, however, and therefore growth on L20B cells is not a definitive identification of PV, and confirmatory testing is required.

In routine diagnostic testing, all EV growth in cell culture is detected by its cytopathic effect, and the isolate is typically confirmed as a specific EV by neutralization with type-specific antisera, by immunofluorescence with type-specific monoclonal antibodies, or by PCR coupled with sequencing. For clinical management of routine cases, it is seldom critical to identify the specific nonpolio EV type. A high index of suspicion for an EV infection can be developed by reflecting on the clinical picture, the virus isolate's cytopathic effect and cell culture systems utilized, and knowledge of basic EV epidemiology.

Antibody Tests

Serologic diagnosis of EV infection can be made by comparing titers in acute and convalescent phase (*paired*) serum specimens. In general, however, EV serodiagnosis is more relevant to epidemiologic studies than to clinical diagnosis (see Epidemiology earlier). The most basic serologic test is that of neutralization in cell culture. Many serologic studies rely on the detection of IgM antibody as evidence for recent EV infection, and this is now widely used as an alternative to the neutralization and complement fixation test. Several groups have developed an enzyme-linked immunosorbent assay (ELISA) for EV-specific IgM.^{30,263} These tests have been found positive for nearly 90% of culture-confirmed CVB infections and can be performed rapidly. The ELISA has been successfully applied for epidemiologic investigations of outbreaks,¹³⁰ as well as for specific diagnostic use.⁹⁰

In most cases, the IgM ELISA test is not serotype specific. Depending on the configuration and sensitivity of the test, from 10% to nearly 70% of serum samples show a heterotypic response caused by other EV infections. This heterotypic response has been exploited to measure broadly reactive antibody, and the assay used to detect EV infection generically.^{42,384} In attempting to characterize the exact nature of the response using different antigens, it is clear that the human immune response to EV infection includes antibodies that react with both serotype-specific epitopes and shared epitopes.¹²⁰ Despite this problem, which is inherently biological, a fairly high concordance of results remains between assays of different configurations.¹⁶² In summary, the IgM assays that are generally used in epidemiologic studies have very good sensitivity and appear to be very specific for EV infection; however, these assays detect heterotypic antibodies resulting from other EV infections and, therefore, cannot be considered strictly serotype specific.

PREVENTION AND CONTROL

Treatment

Although no currently available drug treatment for enteroviral infections is in clinical use, the effectiveness of a variety of drugs *in vitro*, as well as in animal models, has been documented. In addition, varied new directions for future therapeutic intervention are being pursued.

Several potential therapies (e.g., IFN and antiviral antibody) target early stages in the virus life cycle, such as spread. IFN- α and IFN- β have been found to be effective in CVA24 *in vitro* infections.²⁴³ Pilot studies have been conducted administering intravenous immunoglobulin in neonates suspected of having enteroviral infection.⁷ Pooled immunoglobulin delivered intravenously or via a shunt into the spinal fluid has also been used in patients who are agammaglobulinemic with chronic encephalitis and meningitis associated with nonpolio enteroviruses. Patients who are immunodeficient with persistent EV infections, including PV infections, represent a particular challenge to effective treatment. Although intravenous immunoglobulin may protect these patients from poliomyelitis and may appear to stabilize and improve some of the infections, the disease may progress; most of these infections, however, spontaneously cease. In some cases, efficacy may be limited by inadequate amounts of the relevant antibody in the immunoglobulin pool (e.g., if the infection involves an

unusual and rare EV serotype), as well as problems in the delivery of adequate levels of antibody to the infected cells. One report documented the failure to clear persistent PV excretion despite treatment with intravenous immunoglobulin, breast milk, and ribavirin.^{261a} Interestingly, a successful clearance of PV may apparently follow intercurrent diarrheal infections caused by other pathogens, perhaps because of damage to gut lymphoid tissue, which acts as a main site of PV replication.²²²

A number of specific antiviral compounds have been developed to target enteroviral proteins and steps in the virus's life cycle. The *WIN compounds* and related derivatives, which were originally shown to be effective against rhinovirus, have shown the most consistent results and have been those most studied mechanistically. These drugs bind a hydrophobic site near the surface of the virion called the *pocket*,⁴⁴⁷ which lies in the floor of the *canyon* where the virion binds to the cellular receptor. By binding to the pocket, these compounds are believed to interfere with viral attachment and uncoating. Variations in activity of WIN compounds against different picornaviruses are presumably related to the particular fit of the drug into the pocket of a specific EV strain. Oral administration of WIN 54954 significantly decreased the number of upper respiratory infections following challenge with CVA21 (i.e., 3 of 27 patients in the treated group had an upper respiratory infection versus 15 of 23 in the placebo group) with decreased associated symptoms and viral titers.³⁶⁷ Adverse reactions, however, curtailed further investigations with this drug. Pleconaril or VP 63843 is a more recently developed pocket-binding compound with a broad *in vitro* inhibitory activity against 95% of the 215 nonpolio EVs that were tested.³³² Significant activity was noted against some serotypes, such as echovirus 11. A randomized, double-blind study involving the administration of pleconaril following a challenge with CVA21 showed statistically significant decreases in viral shedding in nasal secretions, nasal mucous production, and total respiratory illness symptom scores in patients treated with pleconaril compared with subjects treated with placebo.³⁶⁷ Another phase II trial of the same drug against enteroviral meningitis showed a statistically significant decrease in disease duration (9.5 days in the placebo group versus 4.0 days in the controls).³⁵⁹ A subsequent study, however, failed to have the statistical power to show efficacy in infants with enteroviral meningitis.⁶ A problem with all of these antiviral compounds is that mutant viruses resistant to the drug can arise. In the case of resistance to rhinovirus, the mutant viruses tend to have bulky amino acid substitutions that sterically block entry of the drug into the pocket.¹⁵⁴ These mutant viruses may not be as significant a problem as expected, because drug-resistant CVB3 mutants that appeared in tissue culture following exposure to the WIN compounds tended to be attenuated when inoculated into mice.¹³⁸

Enviroxime is an antiviral drug that targets nonstructural protein 3A, leading to a block in the synthesis of plus-strand viral RNA. Although this compound inhibits EV and rhinovirus *in vitro* infections, it is toxic and not effective in humans.⁹⁸ Resistance to enviroxime is determined by changes in the amino acid at position 30 in protein 3A.¹⁵⁵

An antiviral strategy promoted recently involves the use of drug-sensitive dominantly inhibitory viruses, generated as a result of a targeted drug treatment, which interfere with growth of drug-resistant viruses.⁸³ Regions of the virus that can serve as targets for drugs that lead to the generation of these dominant

defective viruses include (a) the capsid and polymerase coding region, because of the proteins' oligomeric properties (i.e., there is interference during interactions with the respective wild-type protein); (b) cre and VPg (genome-linked virus protein), perhaps because their malfunction leads to inhibitory intermediates; and (c) 2A, perhaps because the uncleaved intramolecular cleavage of VP1–2A is inhibitory during assembly of the virus capsids. The 2A proteinase seemed to be an especially attractive target because of its inability to be rescued in *trans* and the dominant inhibition of the uncleaved product on virus growth.

The administration of small interfering RNA (siRNA) has also been examined as a possible strategy for control of EV infections. This approach has been used to inhibit *in vitro* infections of PV as well as CVB3.^{10,128} The use of a pool of siRNA to target multiple sites throughout the virus genome may be able to limit the emergence of resistant viruses arising from mutations. The possibility of designing an siRNA that is effective against many closely related EV makes this an especially attractive approach for human infections. As with all siRNA approaches, further testing in varied EV infections of animals and then humans is necessary, including the development of an efficient and appropriate delivery system.

Vaccines

Efforts have been initiated to develop a vaccine against EV71, and several approaches have been evaluated in animal models. Mice immunized with a DNA vaccine encoding VP1,^{402,430} or with synthetic peptides encoding B-cell epitopes from VP1,¹¹⁵ mount a neutralizing VP1-specific IgG response; passive transfer of vaccine-induced antibodies to neonatal mice conferred substantial protection against a normally lethal EV71 challenge.¹¹⁴ A virus-like particle (VLP) vaccine induced a strong and sustained neutralizing IgG response.⁷³ Finally, transgenic mice have been developed in which VP1 is expressed in the milk of nursing mothers; EV71-specific antibodies were induced in the suckling pups.⁷³ In both of the latter studies, the antibody responses protected neonatal mice against a normally lethal EV71 challenge.^{66,73}

Poliovirus Vaccine and Eradication

The PV field was fortunate to have more than one excellent vaccine, because both the killed intramuscular vaccine of Salk and the oral attenuated vaccine of Sabin were available. Both of these vaccines result in production of anti-PV antibody (Fig. 17.12) with subsequent protection from disease.³⁰⁰ Although there has been a continuing advocacy for one or the other vaccine over the years, it is clear that each has advantages and disadvantages (Tables 17.9 and 17.10) and that appropriate circumstances exist for the use of each. With the incidence of PV declining dramatically, the United States has switched from OPV to the exclusive use of IPV in 2000 (at 2 months, 4 months, 6 to 18 months, and 4 to 6 years of age). The main benefit for this change was the elimination of vaccine-associated paralytic poliomyelitis (VAPP), and subsequent studies have shown that this was accomplished.¹²

In 1988, in part based on the rapid progress of eradication activities in the Americas, the World Health Assembly unanimously adopted a resolution calling for the global eradication of PV before the end of the 20th century. This resolution was reaffirmed in May of 1999, 2004, and 2011, and an acceleration of activities was urged with particular focus on the

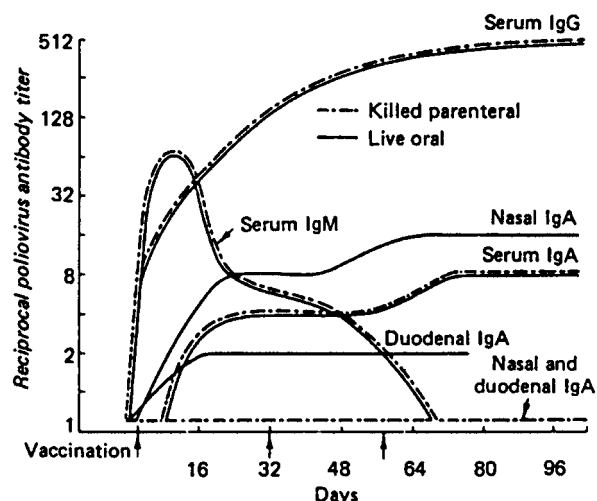


FIGURE 17.12. Serum and secretory antibody responses to oral administration of live attenuated polio vaccine and to intramuscular inoculation of killed poliovirus (PV) vaccine. (From: Ogra PL, Karzon DT. Formation and function of poliovirus antibody on different tissues. *Prog Med Virol.* 1971;13:156–193.)

remaining endemic areas. These accelerated activities include additional rounds of National Immunization Days, intensified surveillance in high-risk communities, and mopping up in focal reservoirs.

A progress report on the global efforts to eradicate PV is provided on a website of the WHO.⁴²⁷ Four fundamental components compose the strategy to eradicate PV.¹⁸⁵ First is the achievement and maintenance of high levels of routine immunization. With the accelerated activities resulting from the Expanded Program on Immunization of the WHO, routine coverage with three doses of OPV in children younger than 1 year of age reached nearly 90% of the world's children by 1990; however, maintenance of these levels has not been completely successful, and some erosion of routine immunization has occurred. Because of the inability of routine immunization to control PV circulation in many developing countries, the second element of the strategy is the use of National Immunization Days for the delivery of vaccine to all children younger than 5 years of age in a very short period of time, usually from 1 to a few days.³⁴ The mass immunization interferes with the spread of wild PV through a rapid increase in population

TABLE 17.9 Killed Poliovirus Vaccine: Advantages and Disadvantages

Advantages

- Safe because inactivation ensures no infectious virus exists
- Can be used in immunodeficient and immunosuppressed individuals
- Provides excellent systemic immunity

Disadvantages

- Requires intramuscular injection with repeated doses
- More expensive than live vaccine
- Potential hazards because of the use of wild seed virus in production
- Reduced intestinal immunity compared with natural infection

TABLE 17.10 Live Poliovirus Vaccine: Advantages and Disadvantages

Advantages

- Confers strong systemic and intestinal immunity
- Relatively inexpensive
- Provides herd immunity because the virus is excreted into the environment, expanding immunity
- Oral delivery
- Relatively safe

Disadvantages

- Can mutate to neurovirulent form, causing vaccine-associated paralytic poliomyelitis
- Contraindicated in immunodeficient and immunosuppressed individuals
- Requires monkeys for safety testing
- Can lead to circulating vaccine-derived polioviruses causing outbreaks of poliomyelitis

immunity and abruptly decreases the *chains of transmission* in a country. This strategy was used in many early immunization efforts in the 1960s and was applied successfully in Cuba to achieve and maintain the elimination of PV from that country following 3 successive years of annual campaigns.³⁵⁰ Multiple rounds in a given year are now routinely carried out for all endemic countries, and the National Immunization Days have grown in an extraordinary way as more countries have adopted this strategy. Some campaigns now represent the largest public health activities on record and often represent the largest multinational health events as well.

The third basic element is the use of surveillance based on cases of acute flaccid paralysis.³⁵ One of the major differences between the smallpox eradication program and the efforts to eradicate PV is the low rate of clinical disease following PV infection. Because less than 1% of infected susceptible individuals will develop paralytic illness, most infections are not clinically recognized. Therefore, unlike smallpox, where almost all infections were symptomatic, PV is difficult to detect; this is certainly a challenge to eradication efforts. To improve the sensitivity of detecting PV infection and yet achieve a practical system, surveillance was developed around the unique clinical presentation of paralytic poliomyelitis. To avoid the requirement for extensive neurologic examinations, which are not feasible in many developing countries, the surveillance was simplified to include any case of acute flaccid paralysis. This system reports many other diseases in addition to poliomyelitis, such as Guillain-Barré syndrome, transverse myelitis, and traumatic neuropathy. This loss of specificity, however, is compensated for by a gain in sensitivity, because most cases of true poliomyelitis are reported. The incidence of non-PV-induced flaccid paralysis is also used as an indicator of surveillance sensitivity (>2 cases per 100,000 population younger than 15 years of age is considered an operational indicator of adequate quality), although it is not clear if the expected rate is the same in different countries or would be expected to be constant over time. Regardless, acute flaccid paralysis surveillance has proved to be remarkably efficient for detection of wild PV circulation.

The remaining part of surveillance is focused on detection of the virus. Two stool specimens are collected from all cases of

acute flaccid paralysis and tested in a global network of laboratories to attempt isolation of PV.¹⁸⁴ The major advantages of this surveillance system are simplicity, practicality, and reasonable sensitivity for detection of PV. The major disadvantages are the requirements to (a) rapidly collect, transport, and test a large number of specimens from all areas of the world and (b) have high-quality laboratory testing. Despite these challenges, the global network of 146 laboratories was able to process more than 200,000 specimens in 2011 and provide this information in a timely manner to the Eradication Program.⁵⁹

The last element of the eradication strategy is the use of *mopping-up* activities.⁹³ This strategy focuses on an area or country where the previous three parts of the program have successfully reduced the number of PV cases to a small number, and where surveillance has localized the remaining reservoirs of transmission. It is then possible to intensify immunization activities in those targeted communities that contain the remaining circulating virus, or the last *chains of transmission*. These intensified activities usually involve active searches in communities for children, including house-to-house or boat-to-boat immunization.¹⁹ Teams visit all residences in the area and ensure that children are not missed. With further reduc-

tion of poliovirus circulation to only parts of a limited number of countries, a further increase in vaccine coverage was achieved by more focused Supplemental Immunization Activities. These immunization campaigns were focused on the remaining reservoirs of polio circulation within the endemic countries and conducted many times nearly year round. As a result of this intensified effort, by the end of 2011, only four countries continued to maintain polio circulation.

These elements of PV eradication strategy have proved successful in large parts of the world. Since the program began, almost all countries are now free of indigenous PV circulation. This can be seen in Figure 17.13, where in 1988 PV was found on all the continents (except Australia), with estimates of more than 350,000 cases of PV each year. In 2011, the number of countries with PV was only 16, and all of the Americas, Europe, and the Far East have been certified to be free of PV.⁵⁸ The only countries with endemic PV cases were in south Asia (Pakistan and Afghanistan) and Sub-Saharan Africa (Nigeria).

Numerous unknowns and potential obstacles remain to be faced in the eradication efforts. Because of the inherent insensitivity of detecting PV infection, it is important that surveillance quality be achieved and maintained for a period of time.

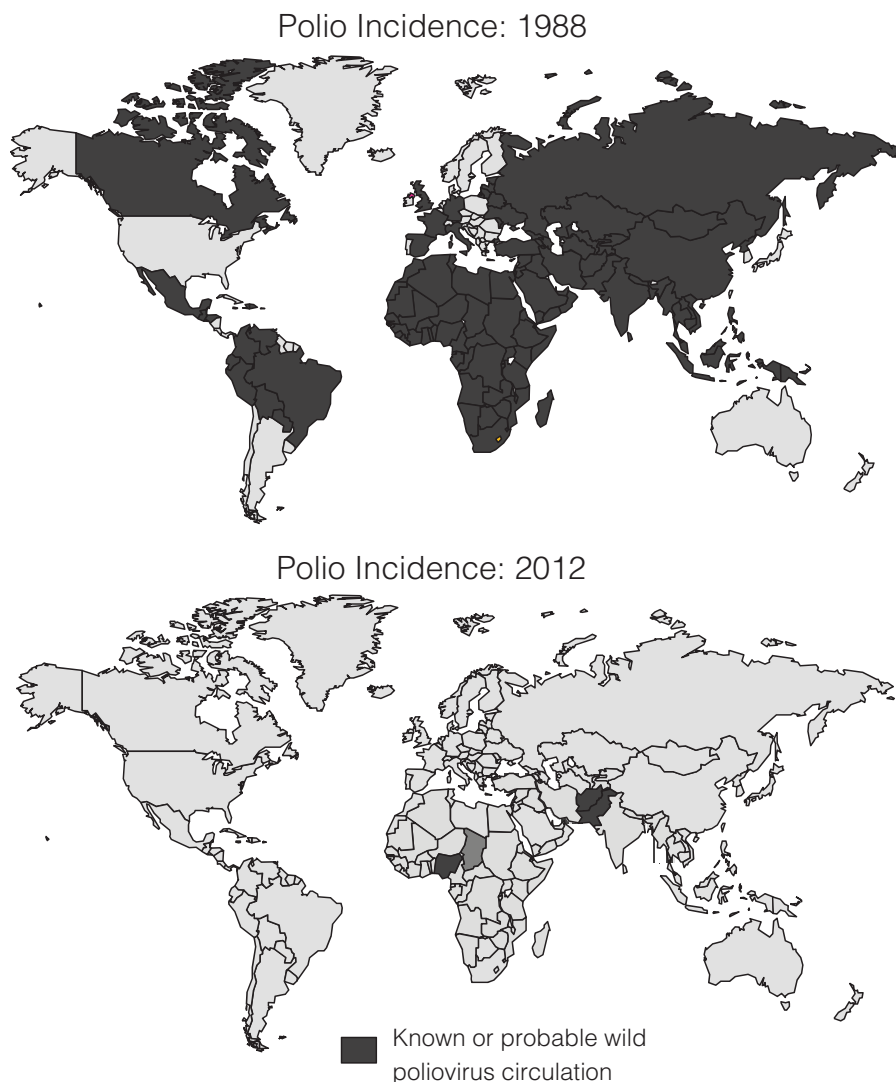


FIGURE 17.13. World map depicting the circulation of wild poliovirus (PV) for 1988 and 2012 as reported to the World Health Organization. In 2012, intermediate shaded country had eliminated indigenous transmission but following importation re-established transmission in the country for a period exceeding 12 months. (Adapted from Centers for Disease Control and Prevention, unpublished data.)

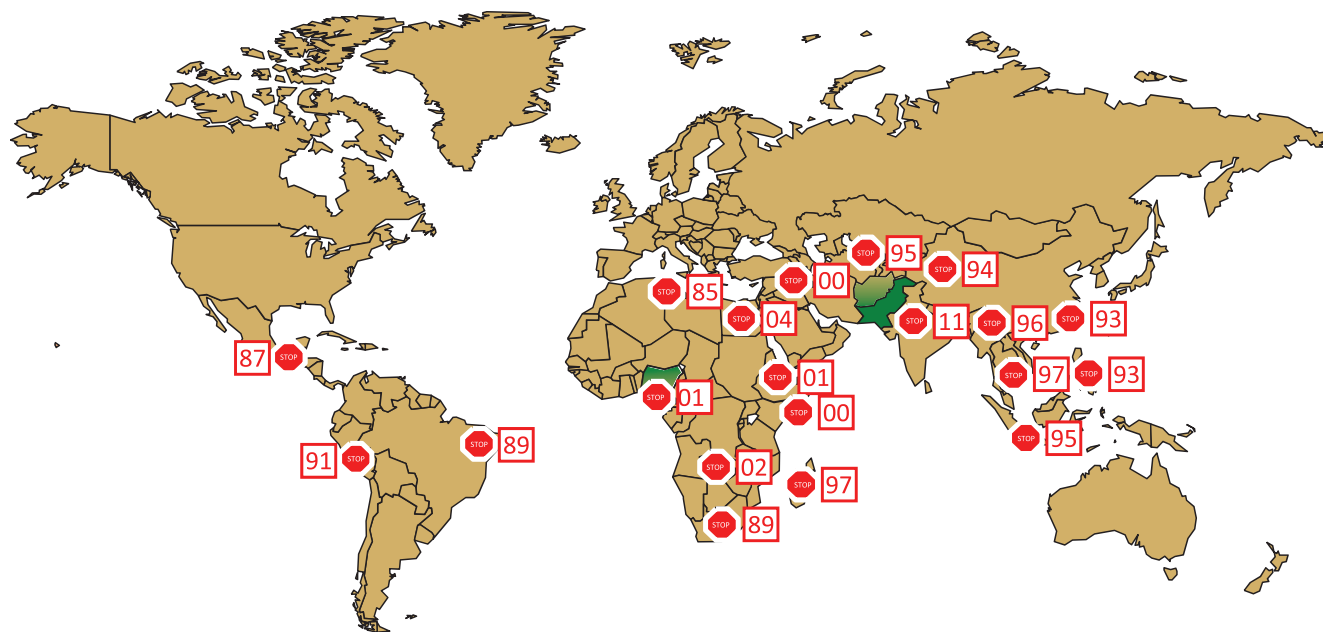


FIGURE 17.14. World map depicting type 1 poliovirus (PV) genotypes in different regions from 1985/2011 and the years in which the eradication occurred. Shaded countries represent remaining endemic circulation for the two remaining type 1 genotypes. Each symbol represents a distinct genotype that has been eradicated and the year when that occurred. (Adapted from Centers for Disease Control and Prevention, unpublished data.)

Although the exact length of time required to be assured of success is not known in all circumstances, the minimal requirement for certification is 3 years. Previous experience indicates that if excellent surveillance is achieved and maintained, PV genotypes that have not been detected for a period of 1 year will not be detected ever again. This has been true for more than three dozen genotypes that have been eliminated from circulation (Fig. 17.14).

After wild virus circulation is interrupted, the accidental release of viruses from laboratories or vaccine-manufacturing facilities will represent a significant risk to the PV-free world. Therefore, it will be necessary to institute proper containment of the virus. A plan for these steps of containment has been drafted and will be implemented in phases as eradication proceeds. In the first pre-eradication phase, preparation for later phases is to be undertaken by conducting an inventory of all institutions and laboratories that have wild PV or potentially infectious materials. This survey for wild PV and potentially infectious materials has been completed in the polio-free regions of the world and has been completed in more than 100 countries, including the United States.⁵⁵ Wherever possible, use of vaccine strains of PV in the laboratory should be substituted for wild strains. In the second phase, after the last wild PV has been isolated anywhere in the world, all work with wild PV will be done at a higher biosafety level of containment (BSL-3/polio). Once all countries and regions have been certified as being free of circulating PV, and once all laboratories have properly contained, referred, or destroyed their PV stocks and potentially infectious materials, the Global Certification Commission will declare wild PV eradicated. The last phase of containment will occur sometime in the future when the decision is made to stop vaccination with OPV. At that time, all infectious PV will need

to be in containment. The containment requirements for this last phase are being developed. In addition, the special containment needs associated with IPV production are being assessed. Of particular concern are the current use of wild strains and the associated risk of an accidental release of virus during vaccine production. One way to limit this danger that is under consideration and active development is to prepare the IPV from the live Sabin attenuated vaccine strains.⁷⁶

Once eradication of wild PV is achieved, the only disease caused by PV in the world will be as a result of the use of live OPV. In most parts of the world, the disease burden caused by VAPP already exceeds that of wild PV. In addition, the continued use of OPV poses a risk for outbreaks from vaccine-derived polioviruses (VDPVs). VDPVs are derived from OPV but differ from the OPV strains and from the frequent PV isolates seen soon after vaccination with OPV by having more than 1% nucleotide changes in the coding region for VP1. The demarcation of more than 1% is somewhat arbitrary but is consistent with the approximate 1 year of circulation of the virus. More recently, this definition has been modified for PV type 2 to be greater than 0.6% difference from the parent vaccine strain. Most OPV-like isolates and most PV excreted from patients with VAPP have less than 1% nucleotide change in the VP1 coding region and, therefore, are not classified as VDPVs because the duration of infection is short and because random mutations do not accumulate as observed with VDPVs. VDPVs fall into three groups: (a) circulating VDPVs (cVDPVs), (b) VDPVs isolated from individuals with immune deficiencies (iVDPVs), and (c) ambiguous VDPVs (aVDPVs) in which the source of infection is unknown.

Although recognized as a potential problem associated with OPV for more than 40 years, the occurrence of multiple

episodes involving cVDPVs within the last 12 years has explicitly demonstrated this risk. The first documented cVDPV outbreak of poliomyelitis occurred on the island of Hispaniola in 2000–2001, with 21 virologically confirmed cases in Haiti and the Dominican Republic, and with many more apparent cases from which no specimens were collected.^{218,220} Since this outbreak in Hispaniola, cVDPV outbreaks have occurred in numerous countries, including the large multiyear outbreak of type 2 cVDPV in Nigeria (<http://www.polioeradication.org/Dataandmonitoring/Poliothisweek/Circulatingvaccine-derivedpoliovirus.aspx>). In addition, cVDPVs have been recognized retrospectively in other countries, particularly Egypt, where the type 2 cVDPV was endemic for possibly 10 years.⁴³⁶ cVDPVs circulate in regions that have inadequate vaccine coverage along with an absence of natural infection from circulating wild PV of the same serotype. The strains are of particular concern because they have caused paralytic disease and have a capacity for sustained person-to-person spread similar to wild-type PV.^{106a} The presence of cVDPVs underscores the importance of maintaining excellent surveillance, the need to maintain high vaccine coverage, and the continued risk from suboptimal use of OPV, even in polio-free areas of the world.

Although not as great a concern as cVDPVs, an issue that complicates PV eradication is the demonstrated excretion of iVDPVs, at times for periods of more than a decade. The occurrence of iVDPVs is rare, however, even in immunosuppressed patients. Although both iVDPVs and cVDPVs can occur in the absence of any recombination, recombination has only been observed in the case of iVDPVs with other vaccine PV strains and not with other members of EV-C. This is presumably because of the absence of EV-C co-infections in the tissue supporting the chronic PV infection, in contrast to the frequent recombination observed between vaccine strains that occurs in normal vaccinees. Interestingly, prolonged excretion with PV occurs with viruses derived from OPV, and not with wild PV,²²² perhaps because the greater neurovirulence of wild PV would be expected to lead to a uniformly fatal outcome in immunosuppressed individuals or because individuals with severe immune deficiencies are likely to live in PV-free countries and, therefore, are only exposed to vaccine strains. Currently no evidence indicates that patients with cell-mediated deficiencies are at increased risk for chronic PV infections. Prolonged excretion of PV has not been observed in studies undertaken specifically to look at HIV-infected children and adults.^{147,157}

Included in the VDPV group are ambiguous VDPVs (aVDPVs), in which the source of infection is unknown (e.g., VDPV isolates from environmental sources), no known immunodeficiency is found in the patient, or the isolate is not associated with a PV outbreak. For example, in more than 10,000 vaccine-related isolates studied in WHO Network laboratories in 2011, there were 67 cVDPVs (from 6 recent outbreaks).^{60,427}

Because of the risks evident from VAPP and VDPVs, the WHO has concluded that continued use of OPV following wild PV eradication is incompatible with the ultimate goal of polio eradication. Therefore, sometime after the eradication of wild PV, all use of OPV for routine immunization will stop. This has significant implications for immunization policy decisions and raises two very important basic questions regarding the management of risks: What vaccination policies will be recommended for PV? How will future PV outbreaks or cases be controlled after OPV use has stopped? With regard to the

first question, one option that countries could elect would be to completely stop vaccination for PV. In the absence of wild PV and competing health priorities, this option may be very attractive to many countries, particularly those with limited access to resources. In contrast, concerns about the potential use of PV as a bioterrorism agent means that some countries will continue to vaccinate indefinitely with IPV, as they are doing now. For some countries, particularly middle-income countries, however, the decision about which option to choose may not be simple.

Ideally, it should be possible to describe accurately all of the advantages and disadvantages of choices related to future vaccination policy. It is difficult, however, to estimate and quantitate risks for all populations associated with the cessation of OPV use because of inadequate knowledge about reversion of vaccine strains and viral genetic determinants for neurovirulence and transmissibility. It is also not clear what protective benefits are possible in the absence of high levels of routine IPV use. An attempt to use elements of decision analysis and disease modeling has been made to try to provide better information regarding risk assessments.^{103,393}

Regardless of decisions made about immunization policies, future generations will have an increasing susceptibility to PV as immunization becomes the only source of immunity. For this reason, another key component of activities related to posteradication risk management is the establishment of a global stockpile of vaccine to respond to any outbreak in the future. In most developed countries in temperate climates, IPV has proved very effective in preventing outbreaks in the general population and may remain the vaccine of choice for small outbreaks in the countries. Much remains unknown about the potential for IPV to control PV circulation in developing countries because IPV induces a lower level of mucosal immunity than OPV.¹²⁴ Because OPV is the only vaccine that has ever been demonstrated to stop circulation of PV in developing countries, it will likely remain the vaccine of choice for the global stockpile. If an outbreak does occur, it is reasonable to expect that it will be caused by a single serotype in a specific place or time, prompting the maintenance of monovalent OPV strains in the stockpile. Monovalent OPV strains of all three types are already licensed, and types 1 and 3 are being used for the last stages of polio eradication.⁷⁶ The use of monovalent OPV strains will eliminate the need to introduce undesirable additional strains into the population. Details remain, however, about the response plans and the use of the stockpile that need to be completed. In addition, issues remain about what should be done at the boundaries of the response population. It may be that the process of eliminating OPV may be as complicated and difficult as the process of eradicating the wild virus.

When the eradication program began, it was assumed that the vaccines available at the time were sufficient for the successful achievement of the goal. With the growing knowledge of the risks associated with OPV and the possible difficulties associated with either continuing or stopping its use in the future, hindsight indicates that the availability of additional options would be highly desirable. In the intervening years many new approaches to vaccinology have been developed, raising the possibility of developing new PV vaccines (e.g., genetically engineered PV, noninfectious DNA, or immunogenic peptides). Although some or all of these vaccines might allow protection with greater safety than the present vaccines, no

easy way exists to assess the effectiveness of an untested vaccine against poliomyelitis, given the licensing requirements and demonstration of efficacy. The testing would be expensive and extensive, and passing the test may not ensure universal acceptance of a new intervention. Despite the significant difficulties involving testing these new interventions, it seems appropriate to continue active investigations in this area.¹⁵⁹

The world has endured the crippling effects of PV for millennia, yet the end of these particular viruses is approaching. It is possible that the next edition of this chapter will treat PV quite differently, possibly even as a historic footnote rather than a major focus of public health activities.

PERSPECTIVES

For 25 to 50 years before the identification of acquired immunodeficiency syndrome, much of human virology was focused on PV. With the eradication of poliomyelitis imminent, the picornavirology field is changing and will never be the same again. It is somewhat ironic to realize that, despite the incredible advances in our understanding of the molecular aspects of PV and the availability of extraordinarily powerful molecular tools for studying PV, the eradication of poliomyelitis will become a reality as a result of the use of the Salk and Sabin vaccines, vaccines that were generated empirically in the middle of the last century before the breakthroughs in our understanding of the molecular biology of PV.

But perhaps it is unfair to belittle the achievements of modern techniques in the eradication of PV. Certainly, the tools of molecular biology were and will continue to be important in monitoring and tracking PV and identifying strains; it also may be that new technology and knowledge will make a better PV vaccine. Novel diagnostic methods developed to assist in surveillance of poliomyelitis are likely to aid in the identification of new enteroviruses, provide better and more accurate descriptions of the epidemiology of EV infections, and lead to new anti-EV drugs, nonpolio EV vaccines, and a better understanding of the relationship of EV to acute and chronic human disease.

What new directions will drive the field of picornavirology? Pressure exists to develop new antiviral treatments and vaccines. New antiviral approaches will take advantage of knowledge of virus receptors, structures of capsid and nonstructural proteins, and immunologic features of the disease. New vaccines will be pursued and developed. The ability of EV71 to mount large epidemics and to be a major cause of neurologic disease, especially acute flaccid paralysis, is likely to have already generated special interest with respect to vaccine development. The many diseases that the coxsackieviruses cause also makes this group of viruses of great interest from the point of view of vaccine development. In addition, investigations of coxsackieviral diseases may clarify the pathogenesis of virus-induced autoimmune diseases.

Despite the development of new treatments and vaccines, enteroviruses will still be very much with us, even after the eradication of PV.

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Rhinoviruses

History

- Infectious Agent
- Pathogenesis and Pathology
- Epidemiology
- Clinical Features
- Diagnosis
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- Perspective

HISTORY

The common cold has been recognized for millennia, and both the illness and prescribed remedies have been influenced by and engendered a broad body of folklore. In fact, the name of the illness stems from the belief that being chilled causes the illness, a concept that studies using experimental inoculation techniques have been unable to verify.⁵³ In Chinese traditional medicine, colds were considered an illness of wind and cold, and the Roman physician Galen wrote “The white-colored substance (the phlegma) collects mostly... in those who have been chilled in some way”.¹⁴ Through the ages, ideas about pathogenesis have varied widely, and suggestions for common cold cures were creative and occasionally bizarre, but seldom helpful. Enthusiasm for a cure for the common cold remains quite high today; a web-based search yielded 4,120,000 hits in response to the terms “common cold cure.”

In modern times, Kruse in 1914¹³⁹ demonstrated that cell-free filtrates of nasal secretions from affected individuals could transmit colds. In 1930, Dochez et al.⁵⁰ confirmed these findings by transmitting colds to volunteers and apes using filtered nasal secretions that were free of bacteria, indicating a viral etiology. Progress in finding the cause for common colds was accelerated by the establishment of the Common Cold Unit (CCU) by the UK Medical Research Council in 1946.²³⁷ The building was originally a hospital established by Harvard University and the American Red Cross to support Great Britain in World War II, and after the war the building was donated to the British government. At the time one virus was presumed to cause colds, and another to cause influenza.²³⁷ The goals of the CCU, led by Christopher Andrewes and later David Tyrrell, were to identify the common cold virus, its means of transmission, and host characteristics that promoted more severe illness. From 1946 until the unit closed in 1989, more than 20,000 volunteers participated in these studies.

Rhinoviruses were discovered beginning in 1956 by two groups working independently.^{190,198} It was not long before

researchers realized that several families of viruses caused common cold illnesses, and that human rhinovirus (HRV) serotypes were numerous. In 1967, a collaborative program classified the known rhinoviruses into 55 different serotypes,¹ serotypes 56 through 89 were added in 1971,² and the remainder of the classical serotypes were added in 1987.⁸⁸

The development of molecular techniques for detecting HRV in a variety of clinical specimens led to a renewed period of discovery related to HRV classification and epidemiology. Recent findings include the discovery of the genetically distinct HRV-C species viruses that do not grow in standard tissue culture, and additional insights into the role of HRV not only in common colds, but also otitis, sinusitis, lower respiratory infections, asthma, and acute exacerbations of chronic respiratory diseases such as asthma.

Infectious Agent Classification

The human rhinoviruses comprise the HRV-A, HRV-B, and HRV-C species of the *Enterovirus* genus in the *Picornaviridae* family. Classification is based on overt similarities in genome organization, capsid properties, and primary sequence conservation.¹⁸⁵ Viruses are assigned to these species classifications if they share greater than 70% amino acid identity in the P1, 2C, and 3CD regions with other members. Within species, isolates are subdivided into numeric genotypes (Fig. 18.1). For the HRV-A and HRV-B, several historic clinical panels archived by the American Type Culture Collection, were combined and indexed into 101 original HRV types after assessment of antigenic crossreactivity or serotyping in rabbits. HRV-A87 was subsequently reassigned to the *Enterovirus D* (HEV-D68) after reevaluation of genetic, immunogenic, and receptor use (decay-accelerating factor as a receptor) properties.²¹⁰ New HRV isolates, especially for the HRV-C, are now rarely tested for immunogenicity. Type assignments still respect the historic naming system, but rely more heavily on sequence comparisons, primarily of the VP1 protein or VP4/VP2. Strains within a common genotype generally share greater than 12% to 15% aligned amino acid identity in either or both of these regions. Full genome sequencing indicated that some historic types are more closely related than this (e.g., A09 and A95, or A29 and A44), and others such as A45 and A95 defining “clade D” may be sufficiently different as to warrant eventual designation as a fourth species (Fig. 18.1). Because many HRV-Cs are not yet fully sequenced, genotype assignments in this species still rely on comparative VP1 data. Nomenclature conventions for all HRVs cite both the species letter and genotype assignment (e.g., A16, B14, C01) to prevent ambiguity among assigned HRV-A (77 types), HRV-B (25 types), and HRV-C (49 types).

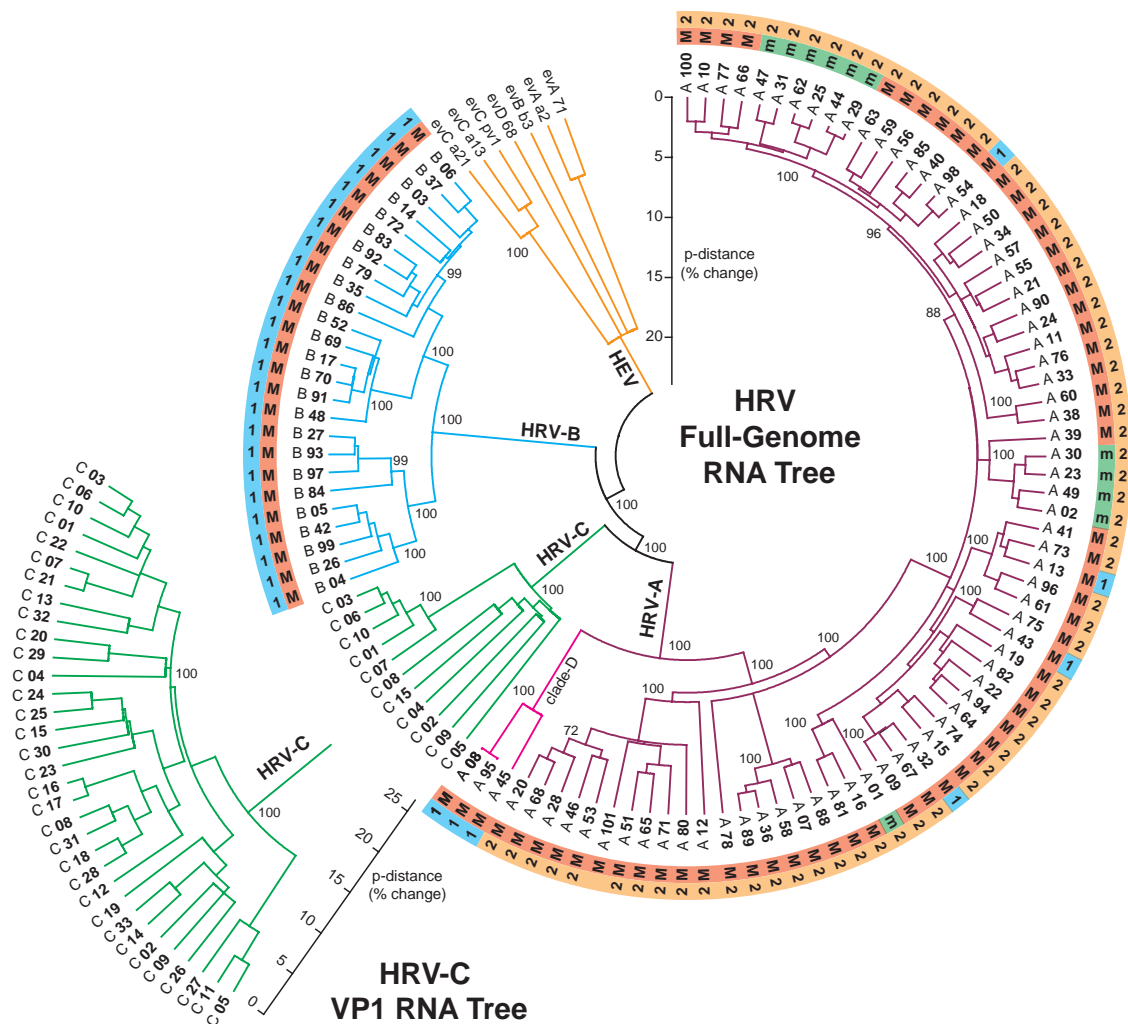


FIGURE 18.1. Circle phylogram relationships for known genotypes of human rhinovirus (HRV)-A, HRV-B, and HRV-C. The tree was calculated with neighbor-joining methods from aligned, full-genome RNA sequences, and rooted with data from human enterovirus (HEV)-A, HEV-B, and HEV-C species (similar to 186). The outer ring ("1" or "2") indicates anticapsid drug group types, if known.⁷ The inner ring shows members of the Major ("M," intercellular adhesion molecule 1 [ICAM-1]) and minor ("m," low-density lipoprotein receptor [LDL-R]) receptor groups. The HRV-C receptor is unknown. Because few HRV-C are fully sequenced, relationships among these genotypes rely on partial VP1 RNA data (lower left). Bootstrap values (percent of 2,000 replicates) are indicated at key nodes.

Physical Characteristics

As enteroviruses, the HRV have genome organizations (Fig. 18.2) and capsid structures (Fig. 18.3) similar to those of polioviruses, coxsackie viruses, and enteric cytopathic human orphan (ECHO) viruses. But unlike other enteroviruses that remain viable at pH 3.0, HRV particles are unstable at pH below 5 to 6. The icosahedral capsid (~30 nm diameter) has 60 copies each, of proteins VP1, VP2, VP3, and VP4, named in order of descending electrophoretic mobility. The protein shell surrounds a densely packed, single-stranded, positive-sense RNA genome of 7079 (C01) to 7233 (B92) bases, a count that does not include the variable length 3' poly(A) tail. Several HRV capsids have been resolved at the atomic level resolution, including A01 (*1r1a*), A02 (*1fpn*), A16 (*1ayn*), B03 (*1rhi*), and B14 (*4hrv*), with multiple structure variants showing

receptor interactions (e.g., A16 with intercellular adhesion molecule 1 [ICAM-1], *1d3e*) or antiviral drug interactions (e.g., A16 with pleconaril, *1c8m*). Like poliovirus, the surfaces of HRV-A&B capsids are dominated by the three largest proteins (Fig. 18.3). VP4 is internal to the structure, centered near the fivefold axis. Around the exterior fivefold plateau, a symmetrical "canyon" provides receptor binding sites and immunogenic surfaces. There are no current structures for an HRV-C, but available sequences indicate significant deletions in the VP1 regions contributing to the fivefold plateau, so it is likely the HRV-C have unique topologies.¹⁶¹ Common to all HRV, the VP1 cores surround a hydrophobic "pocket" or cavity, which can uptake and bind antiviral drugs like pleconaril or other capsid-binding agents. Type-1 long (e.g., B14) or type-2 short (e.g., A16) pocket shapes are defined for most

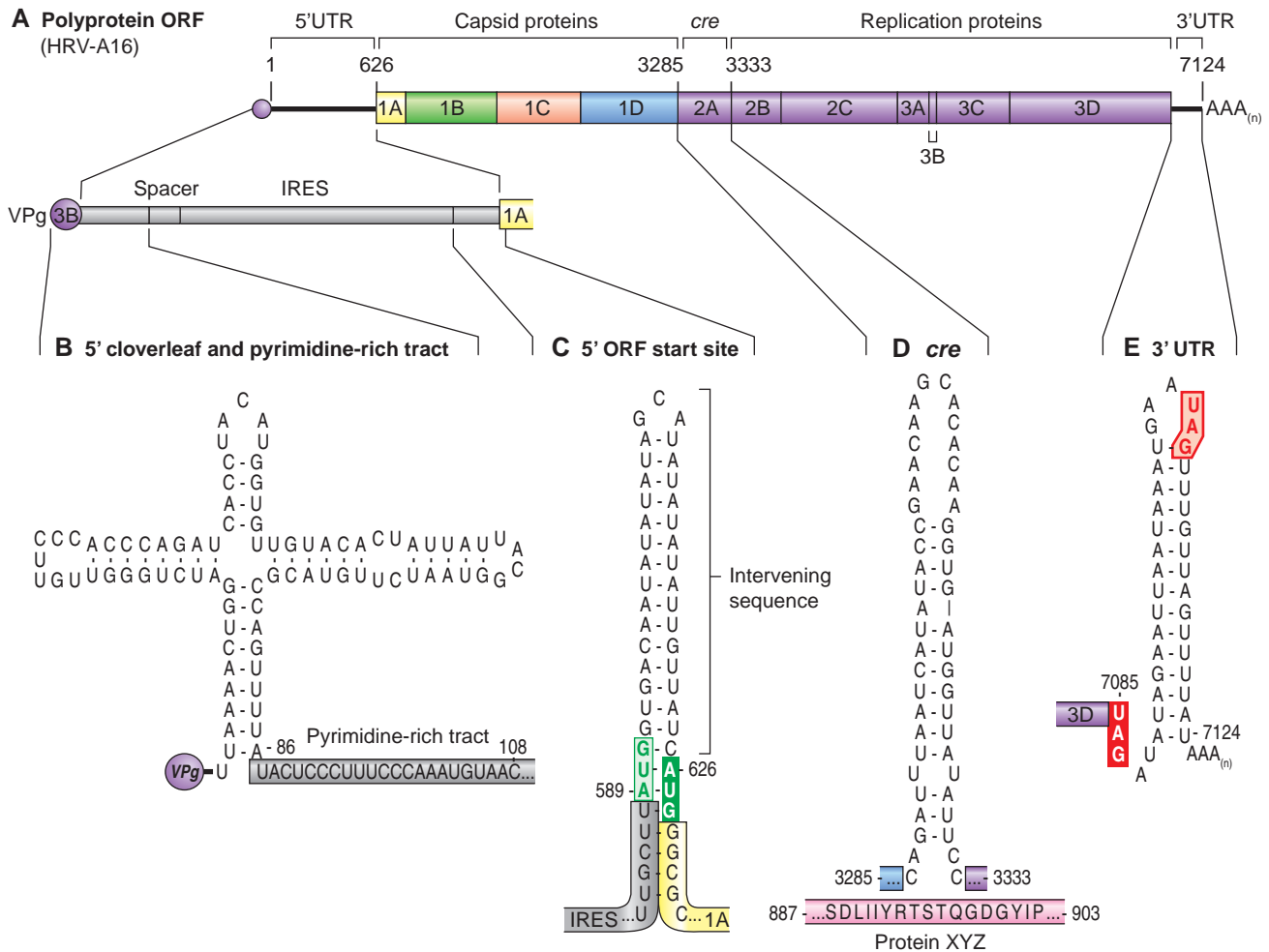


FIGURE 18.2. The genome of an HRV encodes a single polyprotein open reading frame (ORF) (A). Important RNA structural motifs include a 5' cloverleaf (B), ORF start-site stem (C), a *cre* element (D), and 3' stem motif (E).

HRV-A&B and determine whether a virus is susceptible to particular drugs aimed at inhibiting the uncoating process.⁷

The HRV genome is messenger-sense, encoding the polyprotein open reading frame (ORF) and multiple important RNA structural motifs (Fig. 18.2). Adjacent to the 5' cloverleaf, a regulatory feature for translation and replication, each HRV encodes a strain-specific pyrimidine-rich tract that may be involved in suppressing innate immunity triggers.¹⁸⁶ The type-1 internal ribosome entry site (IRES) 3' to this tract, includes a variable-length stem structure pairing the ORF start site (AUG) with an upstream AUG. Unlike poliovirus, intervening sequences between these AUGs are probably not scanned by initiating ribosomes.¹¹⁴ The picornavirus VPg uridylation reaction, required for RNA synthesis, is templated by a special structure called the *cre* (*cis*-acting replication element) whose location varies in every species of picornavirus. For the HRV-A, the *cre* is in the 2A gene.²²⁵ For the HRV-B, the *cre* is in the 2C gene.²²⁵ The HRV-C *cre* has been proposed as one of two sites in the 1B gene.^{37,186,225} Neither has been confirmed experimentally. The short, 3' untranslated sequences (untranslated regions, UTRs) are of highly variable sequence. Invariably, they configure as an inclusive stem motif display-

ing at least one bogus termination codon in the terminal loop. This codon may be in-frame or out-of-frame with the authentic ORF stop site, and has been proposed to play a role in the recruitment of translation termination factors.¹⁸⁶

Pathogenesis and Pathology

Entry into the Host

The primary portal of entry for HRV infections is through inoculation of either the eyes or nose. Studies of seronegative infected volunteers have shown that very low doses of HRV, substantially less than the amount needed to infect cells *in vitro* (one tissue culture infectious dose₅₀ [TCID₅₀]), can cause infection when introduced via the conjunctiva or nasal mucosa.⁴¹ In contrast, approximately 10,000 times as much virus is needed to cause productive infection when the inoculation site is the tongue or external nares.⁴¹

Site of Primary Replication

The primary site of infection is the airway epithelium. In studies of airway tissues from either natural or experimentally induced colds, detection of viral protein or RNA is largely confined to the epithelial layer, along with an occasional cell in

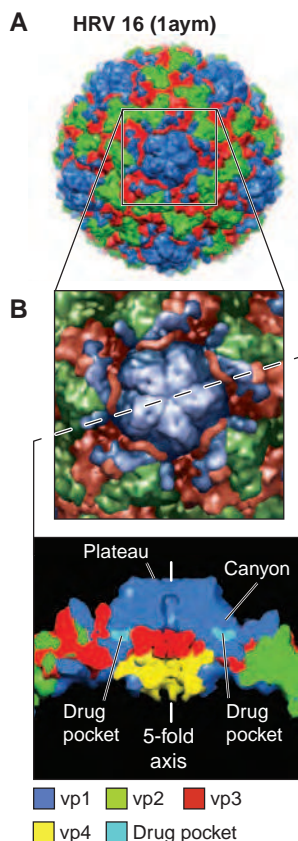


FIGURE 18.3. Capsid structure of a human rhinovirus (HRV) (**A**), illustrates the icosahedral surface topography of VP1, VP2 and VP3 proteins. The VP4 is internal to the capsid (**B**). Type 1 and type 2 antiviral capsid drugs bind in a pocket, internal to VP1.

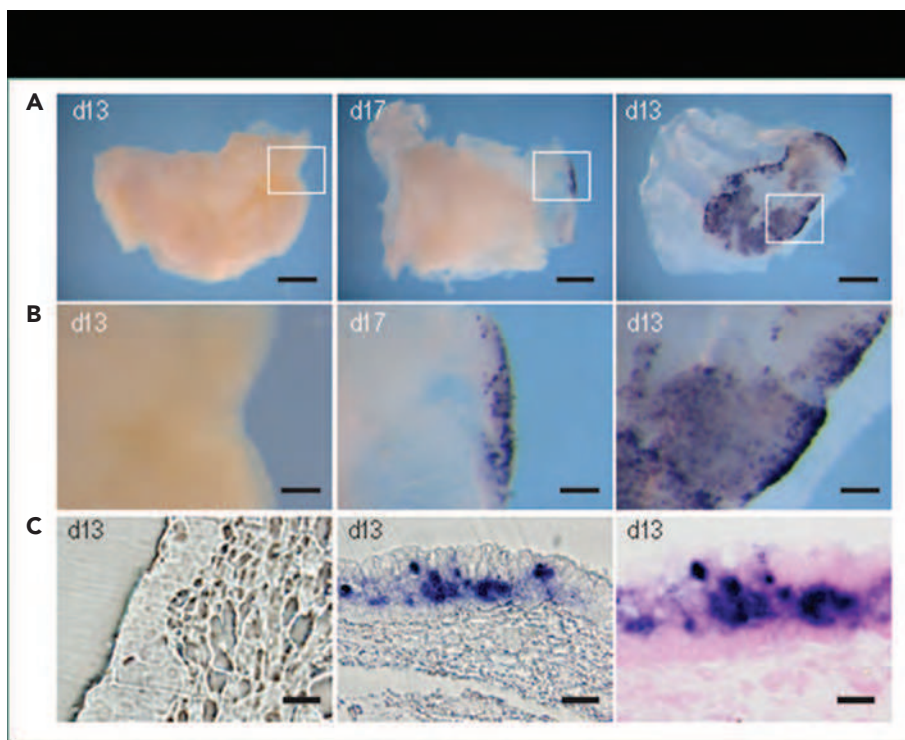
the subepithelial layer (Fig. 18.4).^{11,23,175} Highly differentiated epithelial cells grown at air–liquid interface are more resistant to HRV infection compared to undifferentiated monolayers of cells.¹⁵⁴ In addition, mechanical damage to well-differentiated cells significantly enhances viral replication *in vitro*.¹¹⁶ These findings suggest that the epithelial barrier function is an important contributor to the resistance against HRV infections. In addition, differentiation of epithelial cells in tissue culture in the presence of interleukin-13 (IL-13) *in vitro* causes increased numbers of goblet cells and greater HRV replication *in vitro*, suggesting that HRV replication is increased in goblet cells.¹⁴³

Spread

For years it was assumed that HRV infection was confined to the upper airway and did not affect the chest except under unusual circumstances. In fact, one of the breakthroughs that led to successful propagation of HRV in cell culture was the discovery that this virus replicated best at 33°C to 35°C. It was assumed that the lower airways were at core temperature (37°C), and this characteristic was thought to limit HRV replication to the cooler upper airways. Contrary to these initial assumptions, direct measurements in the lower airways have shown that large- and medium-sized airways are at the ideal temperature for HRV replication (Fig. 18.5).¹⁶³ In addition, cultured lower airway epithelial cells support HRV replication *in vitro* at least as well, and perhaps even better, than cells derived from the upper airways.^{155,174}

Following experimental inoculation of the upper airway, HRV has been detected in the lower airways of individuals with a variety of techniques. Secretions from the lower airways sampled by bronchoscopy and bronchial lavage were analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR), and more than half of the lower airway specimens tested positive for HRV.^{73,214} These findings were extended by subsequent

FIGURE 18.4. Human rhinovirus (HRV)-C15 replication within epithelial cells in sinus organ culture. **A:** Sinus tissue in organ culture was inoculated with HRV-C15 (center and right) or medium alone (left), and whole mounts of the tissue were analyzed for HRV-C15 RNA by *in situ* hybridization (purple stain). Scale bars, 1 mm. **B:** Higher magnification view of the areas boxed in panel **a**, showing uninfected cells (left) or cells containing viral RNA (center and right). Scale bars, 0.15 mm. **C:** Sections of mock (left) or HRV-C infected (center and right) sinus tissue. Right panel is counterstained with eosin (pink). Scale bars, 15 μ m. (From Bochkov YA, Palmenberg AC, Lee WM, et al. Molecular modeling, organ culture and reverse genetics for the emerging respiratory pathogen human rhinovirus C. *Nat Med* 2011;17: 627–632; with permission.)



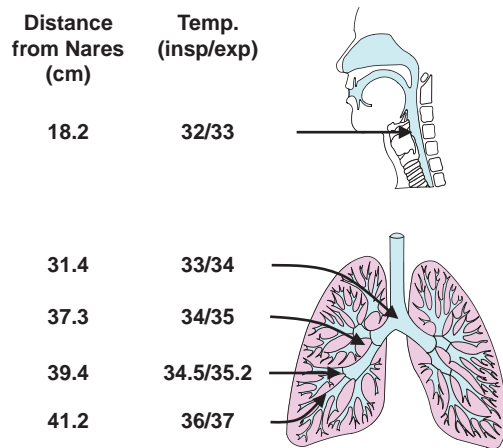


FIGURE 18.5. Temperatures in the lower airways are ideal for human rhinovirus (HRV) replication. Direct measurements of temperature in lower airways have been recorded at measured distances from the nares using a bronchoscope equipped with a small thermistor.¹⁶³ Even when the inspired air is at room temperature (26.7°C), airway temperatures in the medium and large airways are in the range of 33°C to 35°C. In contrast, small airway temperatures approach core temperature (37°C). insp, inspiration; exp, expiration.

studies demonstrating the presence of intracellular HRV RNA and protein using *in situ* hybridization and immunohistochemistry, respectively.^{175,187} Analysis of sputum has been used to provide an estimate of the quantity and kinetics of viral shedding from the large lower airways. After experimental inoculation of seronegative volunteers, viral shedding from the upper airway peaks 2 to 4 days later. Analysis of sputum specimens 3, 7, and 14 days after inoculation revealed that peak levels of virus in the sputum occurred 3 to 7 days after inoculation.¹⁷⁵ In addition, about half of the volunteers had viral shedding in the sputum that was equal to or exceeded that found in the nasal secretions. Notably, only small amounts of virus were detected in bronchial lavage specimens, which originate from distal airways and alveoli.¹⁷⁵ These findings suggest that HRV replication is greatest in the upper airway in most individuals, and that high-level replication can also occur in the large- and medium-sized airways.

Data from natural cold studies also provide evidence of lower airway infections with HRV. Viral recovery from sputum during colds can exceed that obtained from upper airway samples.¹⁰⁶ In epidemiologic studies, HRV is frequently detected in children who are hospitalized with wheezing illnesses and in infants with pneumonia.^{166,167,193} In wheezing infants, HRV has been detected in lower airway biopsies, and HRV detection was associated with reduced lung function in these infants.¹⁵⁸ In children with tracheostomies, samples of nasal mucus can be obtained directly from the lower airway without contamination from nasal secretions, and HRV detection rates from upper versus lower airway specimens are similar.²¹⁷

In addition to infecting the nasopharynx, conjunctiva, and lower airways, HRV has also been recovered in specimens obtained from the middle ear and sinuses.^{29,117,194,196} The respiratory epithelium in each of these two locations is contiguous with that of the nasopharynx, and the virus presumably spreads

via local extension. Rhinovirus viremia has been detected in infected children by PCR but not by recovery of infectious virus,²⁶⁰ and a study of experimentally infected adults showed no evidence of circulating HRV RNA.⁴⁵ Therefore, systemic infections do not occur in immune competent individuals. HRV are inactivated at pH less than 6, thus preventing swallowed virus from replicating in the gastrointestinal tract.

Cell and Tissue Tropism

Biopsies of the upper airway from infected volunteers show a patchy pattern of infection with small foci of infected cells.^{10,195,256} Point cultures of the airway have demonstrated high levels of HRV shedding in the nasopharynx and especially in the adenoidal region. Examination of biopsies obtained during experimentally induced colds suggest that a specific type of nonciliated adenoidal epithelial cell, resembling the intestinal M cell, expresses high levels of ICAM-1 and supports high-level viral replication.²⁵⁵ It is possible that these cells play a sentinel role in the detection of viral respiratory infections.

The receptors for HRV are also expressed by airway cells other than epithelial cells. Besides epithelial cells, HRV can bind to macrophages, monocytes, eosinophils, and fibroblasts.^{72,75,90} Macrophages and monocytes are good sources of type I and type III interferons,^{131,136} which may explain why there is little or no HRV replication in these cells. Airway fibroblasts⁷⁵ and possibly smooth muscle cells¹⁴⁰ support HRV replication in tissue culture, but it has not been established whether these cells, which are located several cell layers under the epithelial surface, are infected *in vivo*.

RECEPTORS

All HRV-A&B use ICAM-1 (88 “major” types) or alternatively, low-density lipoprotein receptor (LDL-R, 11 “minor” types) for recognition and attachment to cells.^{78,243} In addition, the major group virus HRV-A89 binds to heparan sulfate in the absence of ICAM-1.²⁴² The external cellular receptor(s) used by the HRV-C are most certainly different but yet to be identified.^{23,161} Studies utilizing atomic force spectroscopy indicate that after initial HRV binding, multiple receptors are rapidly recruited (within 200 ms).²⁰⁵

SPECIES TROPISM

Rhinoviruses primarily infect humans. Chimpanzees can be infected, but although viral shedding can be detected, there are few or no signs or symptoms of illness.¹⁰⁸ HRV replication in rabbit smooth muscle cells has been reported by a single group.⁸⁶ HRV do not bind to murine ICAM-1, but can bind to murine LDL-R.²⁶¹ Accordingly, minor group viruses can replicate in murine epithelial cells,^{24,236} and strains have been selected for this property by serial passage in murine cell lines.^{17,91} In addition, epithelial cells from transgenic mice engineered to express human ICAM-1 support replication of major group viruses.¹⁷

The demonstration that HRV can grow in murine epithelial cells has led to the development of murine models of HRV infection.^{17,24,178,180,236} Several features of these models resemble infection of the human, including replication in the respiratory epithelium, induction of type I interferons and neutrophil chemokines, and neutrophilic airway inflammation.^{17,180} In addition, HRV infection of mice increases inflammation in response to allergen exposure, suggesting that these models could be informative for asthma.¹⁷ Limitations of the models include the requirement of a large inoculating dose,

and short duration (<24 hours) of significant viral replication and shedding. A second rodent model of picornavirus respiratory infection was developed using a genetically altered strain of Mengovirus, a natural mouse pathogen that causes a polio-like syndrome.²⁰⁶ Intranasal inoculation of rats with attenuated Mengovirus causes infection of the airway epithelium with similarities to HRV infections in humans. The ability to investigate HRV host–cell interactions *in vivo* using the genetic tools available in rodent models should yield important new insights into the pathogenesis of infection in humans.

Immune Response

IMMUNOHISTOCHEMISTRY

HRV infections produce relatively little cytopathology. Findings on biopsy during the peak of the illness include tissue edema and a sparse infiltration of neutrophils and mononuclear cells.^{253,254} The relative lack of pathology together with the small number of infected cells suggests that the immune response to HRV rather than direct virus-induced injury contributes most to respiratory symptoms.⁹⁷

MEDIATORS OF INFLAMMATION

HRV infection induced a variety of proinflammatory cytokines and mediators (Table 18.1). Most of these factors are present in peak concentrations during the acute cold, and correlate with illness severity and viral shedding.¹⁶ There is only circumstantial evidence that these immune mediators contribute to the pathogenesis of cold signs and symptoms, and the specific roles for most of these factors have yet to be defined. Kinins, leukotrienes, and histamine are of special interest due to their effects on vasodilation and edema, which are prominent findings during acute colds.^{110,262} HRV infection of volunteers with asthma induced increased mRNA and activity of human

kallikrein, a critical enzyme for the release of kinins, in the lower airways.³³ These mediators can also cause smooth muscle contraction as well as edema, and thus could promote airway obstruction in patients with asthma. Notably, common colds have little effect on concentrations of histamine in the nose,⁶¹ and second-generation antihistamines (which are specific H1-receptor antagonists), have no effect on cold symptoms.¹⁷⁶ Leukotrienes (LTs) are overproduced during viral respiratory infections,⁷⁰ but the cystinyl leukotriene receptor antagonist montelukast did not affect cold symptoms in experimentally infected volunteers.¹³⁴ In addition, administration of LTB4 to the nose before experimental inoculation had no significant effects on either viral replication or symptoms.²⁵¹ Kinins are increased early in the course of a cold,²⁰⁰ but testing of their role in pathogenesis awaits the development of safe and effective kinin inhibitors. HRV-induced cytokines may also promote vascular permeability; experiments conducted *in vitro* demonstrate that tumor necrosis factor α (TNF- α), IL-1 β , and IFN- γ can each increase vascular permeability.²¹² Analysis of nasal secretions after experimental inoculation demonstrates that the thin rhinorrhea during the early stages of a cold contains serum proteins (e.g., immunoglobulin [IgG], albumin), and as the cold progresses the composition shifts to mucin proteins.^{110,262} This progression suggests that the pathophysiology of rhinorrhea is time-dependent, and shifts from vascular leakage initially to local secretion of mucus during the mid to latter stages of a cold.

EPITHELIUM

The airway epithelium is the first line of defense against HRV infections. Mucins, antimicrobial peptides, and surfactant proteins in the mucous layer nonspecifically deter infection.¹⁰³ In addition, well-differentiated epithelial cells are relatively

TABLE 18.1 HRV-Induced Mediators and Cytokines

Category	Examples	Comments	References
Mediators	Kinins	Vasodilation and edema	(110,200,262)
	Leukotrienes	↑Vascular permeability and	(70)
	Nitric oxide	bronchoconstriction	(43,64,208)
Antivirals		Vasodilation, bronchoconstriction	
	IFN- α	Low concentrations in nasal secretions ^a	(131,137)
	IFN- β	Possibly deficient in asthma	(131,247)
	IFN- λ (IL-28, IL-29)	Possibly deficient in asthma	(36,131)
	β -Defensins	Activity against bacteria and enveloped viruses	(58,201)
Chemokines	CXCL10	Attracts Th1 cells	(137,223,246)
	CXCL8	Attracts neutrophils	(74,230,235)
	CCL2	Attracts monocytes, T cells, and dendritic cells	(87)
Cytokines	IL-1	Proinflammatory	(199)
	IL-6	Proinflammatory	(154)
Growth factors and tissue repair	G-CSF	↑Production of neutrophils in bone marrow	(118)
	TGF- β	↑Collagen synthesis, immunoregulation	(52)
	VEGF	Angiogenesis and airway remodeling	(44)

^aTrue for all interferons.

IFN, interferon; IL, interleukin; G-CSF, granulocyte-colony stimulating factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

resistant to HRV infection.¹⁵⁴ The epithelium may serve as a barrier against HRV infection; HRV replication is enhanced when apical cells of well-differentiated epithelial cell cultures are either damaged or stripped away.¹¹⁶ This property may help to explain how exposure to factors such as pollutants and allergic inflammation that can damage epithelium also increase the risk and/or severity of HRV illnesses. HRV infection itself can disrupt the barrier function of the epithelium,²⁰⁷ which may contribute to synergistic effects with bacteria, allergens, or irritants.

In addition to being the principal host cells for HRV infections, airway epithelial cells initiate antiviral and proinflammatory immune responses (Fig. 18.6). Attachment of HRV to ICAM-1 activates signaling cascades that lead to the

activation of chemokine genes such as *CXCL10*.^{69,136} Once HRV particles uncoat in the endosome, the release of single-stranded RNA activates additional innate immune sensors. These include toll-like receptors (TLRs) (e.g., TLR-3, TLR-7) that are expressed on endosomal membranes, and the double-stranded RNA-dependent protein kinase (PKR) and RNA helicases (retinoic acid-induced gene I [RIG-I] and melanoma-differentiation-associated gene-5 [MDA-5]). MDA-5 binds to double-stranded RNA that is formed during the replication process, and appears to be an important contributor to anti-HRV IFN responses.^{220,244} The role of RIG-I, which binds to the 5' triphosphate motif and longer stretches of double-stranded RNA,¹⁹ is more controversial. One recent *in vitro* study found that TLR-3 was constitutively expressed on airway

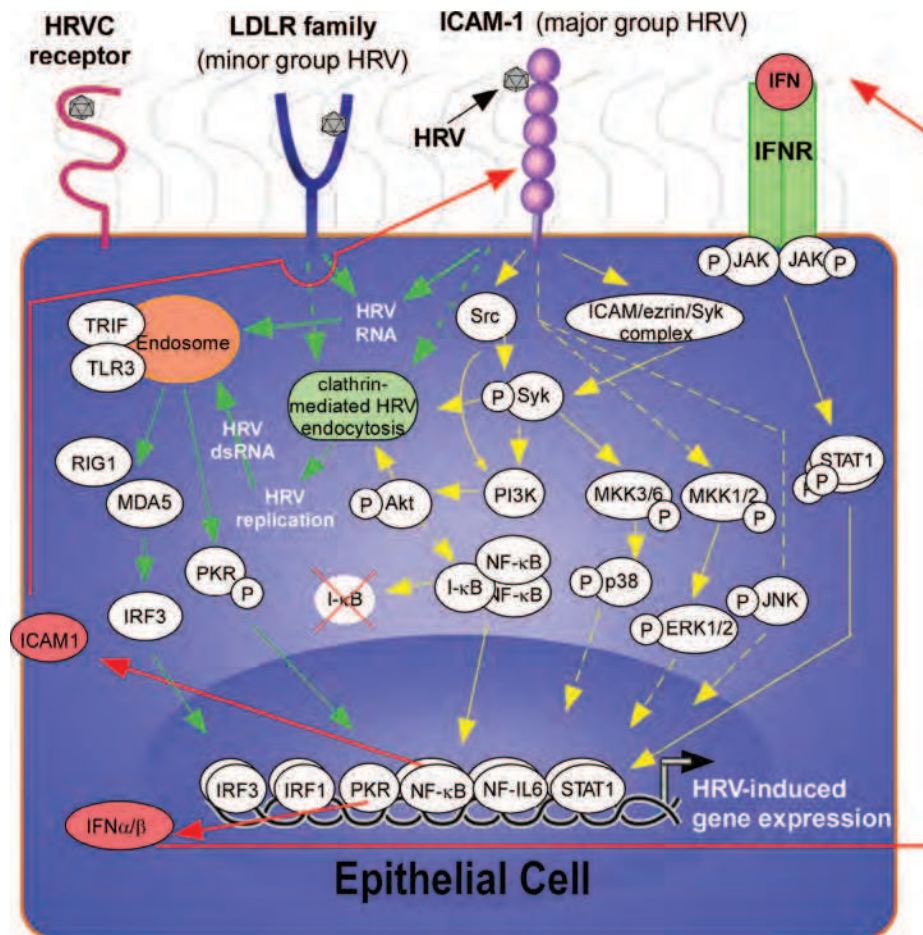


FIGURE 18.6. Airway epithelial cells initiate the immune response to human rhinovirus (HRV). Major group HRV binds to intercellular adhesion molecule 1 (ICAM-1) and minor group HRV binds to low-density lipoprotein receptors (LDL-Rs) on the surface of cells, such as epithelial cells and leukocytes, that would be abundant in the airway, to induce downstream signaling. The receptor and signaling pathways induced by HRV-C types has yet to be elucidated. Upon ligation of HRV to surface receptors on epithelial cells, direct uncoating of viral RNA or clathrin-mediated endocytosis leads to the release/replication of HRV RNA into the cytoplasm. This viral RNA is detected by endosomal receptors, such as toll-like receptor 3 (TLR-3), to propagate downstream signaling and induction of gene expression, including the production of interferons (IFNs) that can exert an autocrine/paracrine effect by binding to IFN receptors and triggering a janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling cascade. Replication-independent signaling is also induced upon HRV infection, and includes the activation of Src, Syk, and mitogen-activated protein kinase (MAPK) signaling cascades. Yellow solid lines, data to support link; dashed lines, indirectly connected (may have signaling molecules in between); red lines, signaling induced by product of HRV-induced gene transcription; green lines, replication-dependent signaling. (From Gavala ML, Bertics PJ, Gern JE. Rhinoviruses, allergic inflammation, and asthma. *Immunol Rev* 2011;242:69–90, with permission.)

epithelial cells, and activation of TLR-3 led to induction of RIG-I and MDA-5.²²⁰ Subsequently, all three of these molecules contributed to upregulation of innate IFN responses to HRV infection.

Engagement of cell surface receptors and RNA-sensing molecules leads to activation of several signaling cascades, including nuclear factor kappa B (NF- κ B), janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathways, and mitogen-activated protein kinases (MAPKs).^{60,87,99,132,136,144,146,179,180,245,263} Viral proteases can also contribute to cell activation; the 3C protease cleaves the transcription factor organic cation transporter-1 (OCT-1),⁶ which negatively regulates CXCL8 (interleukin-8) transcription. The net result of these actions is activation of viral sensing and antiviral effector pathways, IFNs, and a variety of proinflammatory cytokines and chemokines.^{22,31,202} Type I IFNs inhibit HRV replication by inducing multiple antiviral pathways, and also prime epithelial cells to synthesize chemokines such as CXCL10.^{6,137} IFN- γ has several effects on viral replication and inflammatory responses. It is a potent inducer of ICAM-1¹³⁵ and soluble ICAM-1,²⁵⁰ and effects on viral replication in cultured epithelial cells depend on experimental conditions. In addition to affecting viral receptor expression, IFN- γ also enhances the HRV-induced secretion of chemokines such as CCL5.¹³⁵ HRV-induced chemokines from epithelial cells and other sources attract a variety of cells into the airway, including dendritic cells, monocytes, macrophages, epithelial cells, and lymphocytes (Table 18.1). The ensuing cellular response promotes antiviral effects and killing of infected cells, but also increases inflammation that contributes to the pathophysiology of illness.

CELLULAR IMMUNE RESPONSES

Macrophages are the principal cells in lower airway secretions, and it is likely that these cells are important contributors to the innate antiviral response. Considering that little to no replication of HRV has been observed in airway macrophages or blood monocytes,^{72,146} the signaling pathways could differ from those in epithelial cells, in which viral replication processes provide potent innate immune stimulation. As in epithelial cells, HRV activate NF- κ B and STAT-1-dependent pathways. For example, HRV-induced secretion of CCL2 involves activation of NF- κ B and phosphorylation of activating transcription factor-2 (ATF-2).⁸⁷ Furthermore, HRV-A16 induces STAT-1 tyrosine phosphorylation and IFN- α release in monocytes.¹³⁶ NF- κ B activation, but not the p38 MAPK, has been reported to be involved in HRV-mediated TNF- α release by monocytes.¹⁴⁶

Macrophages are important sources of cytokines (e.g., TNF- α), chemokines (e.g., CXCL8 [IL-8], CXCL10 [IP-10], CCL2 [MCP-1]), and type I and type III IFNs during HRV infections.^{87,127,131,146} Although epithelial cells and macrophages in isolation can both respond to HRV infection, co-culture of these cells leads to synergistic enhancement of selected chemokines such as CXCL10, and this synergy has been linked to macrophage secretion of IFN- α .¹³⁷ Airway dendritic cells are interdigitated into the epithelium, and as major producers of type I IFNs²¹⁵ they are likely to be important in orchestrating the early innate response to HRV infection.

Neutrophils are the most numerous cells in airway secretions during an HRV cold.^{118,231,254} Experiments conducted after experimental HRV inoculation demonstrate that granulocyte-colony stimulating factor (G-CSF) is secreted into the

nasal secretions within 24 hours, followed closely by increased in circulating G-CSF that can boost the production of neutrophil precursors in the bone marrow.¹¹⁸ Neutrophils increase in the blood 1 to 2 days after inoculation, and are then recruited to upper and lower airway secretions. Neutrophilic inflammation correlates with respiratory symptoms, and it is suspected that these cells have both antiviral and proinflammatory effects during the cold.^{16,79,118,230,231} The P2 \times ₇ receptor is a cation channel expressed on epithelial cells and leukocytes that is activated after binding ATP, and can therefore initiate inflammatory responses secondary to virus-induced cellular damage and other stimuli that release ATP. In volunteers who were experimentally inoculated with HRV, P2 \times ₇ pore activity measured in blood cells correlated with the influx of neutrophils into upper airway secretions during the acute cold.⁴⁶

Lymphocyte numbers dip briefly in the blood during an acute cold, and rise in nasal secretions, but to a lesser extent than neutrophils.^{151,152} T-cell responses are critical for resolution of HRV infections, as indicated by reports of prolonged and sometimes severe symptoms in immunosuppressed individuals or those with primary immune deficiencies.^{68,112} T-cell responses can be directed at either serotype-specific or shared antigens.⁷¹ Vigorous HRV-specific proliferative responses in seronegative volunteers at baseline were associated with milder clinical symptoms, suggesting a potential role for lymphocytes in the initial antiviral response.¹⁸⁸

ROLE OF ANTIBODY

Antibody responses can be detected as early as 7 to 14 days following experimental inoculation, and it is likely that both IgG and IgA responses to HRV contribute to protection from reinfection.^{41,66,192} Preexisting antibody reduces the rates of HRV infection and illness. Studies of natural infections among families found that the infection rate for individuals with serum antibody was half that of antibody-negative subjects,^{41,66} and similar findings were reported in trials of experimental inoculation.^{4,28} In addition to protection associated with antibody responses to a homotypic virus, serial challenges with heterotypic virus also produced a reduced frequency of infection, suggesting either the presence of cross-reacting antibodies or protective mechanisms not involving antibody.⁶⁵

Release from Host and Mode of Transmission

The transmission of HRV infections has been the subject of a number of creative and innovative studies. Dick and colleagues⁴⁹ compared the HRV transmission rates from experimentally infected donors to seronegative recipients under controlled conditions. Some volunteers wore arm restraints or plastic collars designed to prevent hand to face contact; rates of transmission were similar for these volunteers compared to those with no restraints. These results are in agreement with observations that a related virus (coxsackievirus A21) could be spread by aerosol droplets.⁴⁰ On the other hand, Dr. Jack Gwaltney and colleagues⁸¹ found that contact with coffee cups or plastic tiles contaminated with wet secretions could transmit HRV infections to volunteers who handled the fomites and then rubbed their eyes and nares with their fingertips. In addition, transmission of HRV can be inhibited by cleaning the hands with virucidal solutions, or by the use of virucidal tissues when blowing the nose.⁴⁸ Notably, items with dried secretions contain little virus and were not able to transmit colds. Collectively, these

findings indicate that HRV can be transmitted by either aerosol or fomites, and that the predominant mode of transmission may depend on age, personal hygiene, and degree of viral shedding.

Virulence

With the very large number of HRV serotypes and genotypes, it is likely that some are more or less virulent. Early studies relying on tissue culture documented that at least 20 different HRV serotypes could cause wheezing illnesses. Larger studies using molecular diagnostics indicate that HRV-C infections appear to be overrepresented in more severe illnesses, including wheezing in infants and young children,⁸ and acute exacerbations of childhood asthma.²⁰ There is also a single report of HRV-C recovery from pericardial fluid in a child with signs and symptoms of systemic infection.²²⁸ Given that prevailing HRV strains change almost completely, additional studies include community surveillance over multiple seasons to definitively establish whether HRV-C are more virulent, and whether there are selected types of HRV-A and HRV-B with the same properties.

Persistence

Several studies have observed that following acute HRV infections, virus can sometimes be detected for weeks,^{120,259} and this has led to speculation that HRV might cause chronic infection. In fact, studies that have performed HRV typing following either natural or experimentally induced infections provide definitive evidence that most HRV infections are cleared after 1 to 2 weeks.^{45,184} Prolonged detection of HRV is usually explained by serial infections with different HRV types, and shedding of a single virus for four weeks or longer is a rare event in immunocompetent individuals.¹¹⁹ In contrast, immunodeficient or immunocompromised patients can shed the same HRV type for prolonged periods, and can have more severe illnesses.^{68,112,130}

Detection of HRV in the absence of respiratory symptoms is common, and rates of asymptomatic infection in infants are especially high (25% to 44%).^{141,150,240} This could be a reflection of the age-related increased susceptibility to all HRV infections (symptomatic or not), but it is also true that mild symptoms (e.g., sore throat) may not be recognized in infants. In surveillance studies of infants, 10% to 20% of these “asymptomatic infections” reflect viral shedding follow-

ing recovery from a cold, or a prodromal period just before the onset of respiratory illness.^{109,120,240,257} Studies that have performed serial sampling of respiratory secretions have found no evidence that HRV establishes a “carrier state” characterized by persistent low-level viral shedding.^{119,184,191} The quantity of viral shedding is not a good differentiator of symptomatic versus asymptomatic infection.¹⁹¹ Notably, both symptomatic and asymptomatic infections can elicit immune responses.¹²³ When considered together, these findings indicate that detection of HRV shedding by culture or PCR is indicative of infection, and that asymptomatic infections are common and need to be considered in epidemiologic studies linking infection to illness outcomes.

Epidemiology

Age

HRV infections are most common in infants and young children. There is evidence that infection rates are somewhat lower in the first 6 months of life, and then rates are relatively high throughout infancy and early childhood.^{18,47,66,105,113,238} Among young adults, infection rates are greater in mothers and women of similar age compared to men. In older adults, this relationship is reversed and illness rates are higher in men.⁸²

Morbidity

HRV cause a broad spectrum of illness including asymptomatic infections, common colds, and other upper respiratory illnesses, bronchitis and wheezing illnesses, and lower respiratory tract infections including bronchiolitis, bronchitis, wheezing, and pneumonia (Fig. 18.7). The wide range of illness suggests that there are a number of factors related to the virus, the host, and the environment that contribute to the severity of illness. With 100 traditional serotypes and more than 40 HRV-C types, it is likely that there are species or strain-specific patterns of virulence. The C viruses appear to be overrepresented in studies of hospitalized children^{51,89,145,148,153,156,162,166} and acute exacerbations of asthma,²⁰ but longitudinal data including large numbers of HRV illnesses of varying severity are needed to confirm these findings.

Most of the morbidity associated with HRV infections is in high-risk groups: infants, the elderly, immunocompromised individuals, and patients with chronic respiratory diseases such as asthma,^{39,129,182} chronic obstructive lung disease,²⁵⁸ and cystic

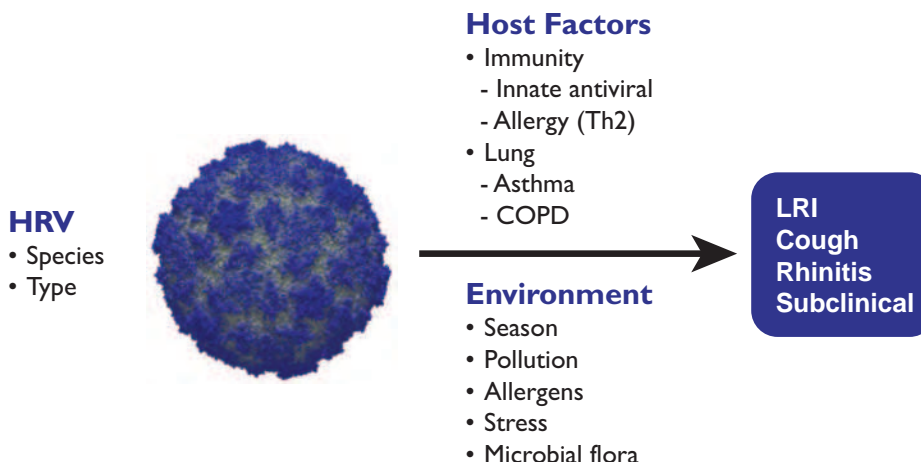


FIGURE 18.7. The spectrum of human rhinovirus (HRV) illness (see text).

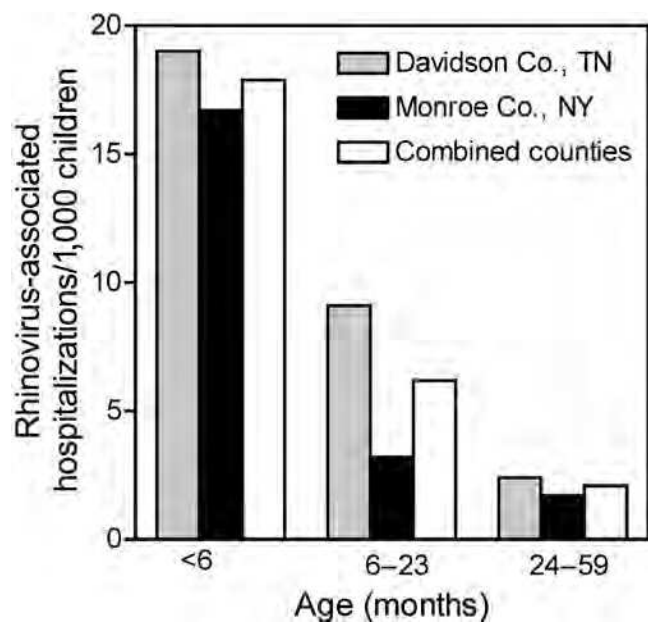


FIGURE 18.8. Age-related hospitalization rates for rhinovirus lower respiratory infection. (From Miller EK, Lu X, Erdman DD, et al. Rhinovirus-associated hospitalizations in young children. *J Infect Dis* 2007;195:773–781, with permission.)

fibrosis.^{35,222,249} Risk factors for HRV wheezing illnesses in early life include maternal atopy, a history of previous wheezing episodes, and increased airway resistance on infant lung function tests.^{122,168,224,239} Population-based hospitalization rates were calculated for children hospitalized in Rochester, New York, and Nashville, Tennessee (Fig. 18.8); one major children's hospital serves the population in each of these communities.¹⁶⁷ Young age and a history of previous asthma or wheezing were two risk factors for HRV lower respiratory illness. Finally, several studies indicate that allergic children are especially likely to develop acute wheezing illnesses with HRV infections.^{100,184,204}

Environmental factors that contribute to the severity of HRV illness include tobacco smoke and other pollutants.^{57,181,241} Several studies have provided evidence that stress increases the severity of natural and experimentally induced colds.^{34,226} Seasonal influences have been linked to cold prevalence, and likely also affect severity of illness. Notably, vitamin D status has been shown to be associated with the prevalence of upper respiratory illnesses, although specific links to HRV infections have not been established.⁷⁶

HRV infections have been closely linked to the inception and exacerbation of asthma (Fig. 18.9). Many respiratory viruses can cause wheezing episodes in infancy, and low lung function and environmental exposures such as tobacco smoke increase the risk for wheeze. Most children recover fully, but a subset subsequently develop recurrent wheezing illnesses and asthma. Wheezing with HRV infections during infancy and early childhood is one of the strongest indicators of risk for subsequent childhood asthma, especially in those individuals who develop allergen-specific IgE (allergic sensitization) at an early age.^{113,138,142} The causality of this relationship is uncertain. Once asthma has become established, viral respiratory illnesses (most commonly caused by HRV) are the most com-

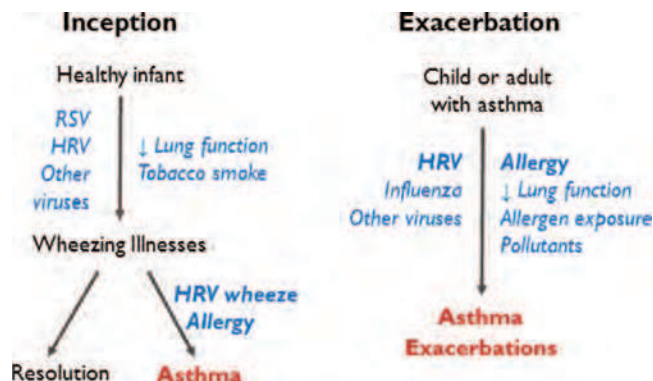


FIGURE 18.9. Human rhinovirus (HRV) infections and asthma (see text).

mon trigger for acute exacerbations of asthma. The relationship between HRV infections and acute asthma is especially close in childhood; HRV infection is detected in up to 85% of asthma exacerbations in children compared to about half of exacerbations in adults.^{13,100,101,129,182,184} There is a remarkable similarity in the seasonal patterns of HRV infections and exacerbations of asthma, especially in school-aged children in whom hospitalizations due to asthma have a strong fall and spring preponderance year after year.^{125,126,128} Even so, the use of routine monitoring of respiratory secretions for HRV in children with asthma demonstrates that only a subset of HRV infections cause exacerbations.¹⁸⁴ Children with respiratory allergies are at greatest risk for virus-induced wheezing, and other risk factors include exposure to allergens or pollutants, obstructive changes on lung function testing, and failure to use asthma-control medications.^{30,57,77,125,177,241}

Origin and Spread of HRV Infections

In general, transmission of HRV infections is relatively inefficient. Children have the highest rates of HRV illnesses, and also appear to transmit infections most effectively. In family surveillance studies, transmission from children to other children and adults was much more common than transmission from adult to children or other adults.^{98,191} Epidemics with HRV are uncommon, although they have been reported to occur associated with “flu-like” illnesses in nursing homes.^{62,102} Secondary attack rates within families vary between 30% and 70%.

Conditions that affect transmission of HRV infections were identified in studies of experimentally inoculated HRV “donors” and seronegative adult “recipients”. Short-term (15 minute to 3 hours) contact led to transmission rates of less than 10%, even in the presence of loud vocalization, card playing, and kissing.^{41,83} Higher rates of transmission were observed among donor and recipient married couples (38% transmission rate),⁴² and in a crowded research hut in Antarctica (88% to 100% transmission rate).¹⁰⁴ Transmission of natural colds in a more spacious Antarctic hut occurred at a relatively low rate (~1% per day among 200 inhabitants), and it was notable that transmission rates were similar among long-term residents and recent arrivals to the research station.²⁴⁸ In clinical studies that reproduced the close contact of the studies in Antarctica, the best predictor of transmission of a cold from a donor to a seronegative recipient, was the duration of exposure

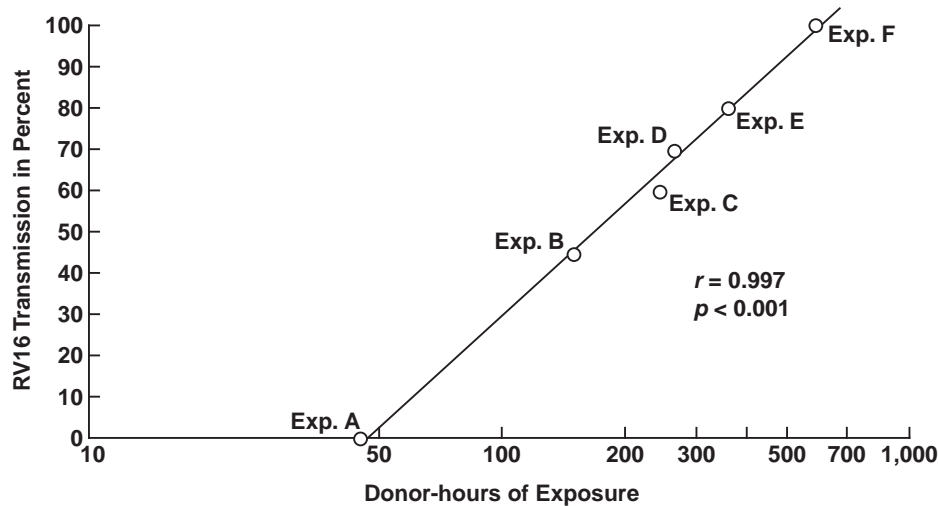


FIGURE 18.10. Relationship between the exposure and transmission of human rhinovirus (HRV)-A16. A series of experiments (A–F) was conducted in which donors with severe experimentally induced colds (HRV-A16) were housed with seronegative recipients for varying lengths of time. The groups were housed in a clinical research unit from 5 to 79 hours, and samples of nasal lavage fluid were obtained for 10 days and analyzed for viral shedding. One donor-hour of exposure (DHE) was defined as exposure to one infected donor for 1 hour. There was a straight-line relationship between the logarithm of DHE and the risk of HRV transmission. (From Meschievitz CK, Schultz SB, Dick EC. A model for obtaining predictable natural transmission of rhinoviruses in human volunteers. *J Infect Dis* 1984;150:195–201, with permission.)

(Fig. 18.10).¹⁶⁵ In general, factors that promote transmission include young age, symptomatic illness, crowding, high-level viral shedding from the index case, and seronegativity of the recipient.^{124,172}

Prevalence and Seroepidemiology

HRV infections occur year round, with increased prevalence in the fall and spring (Fig. 18.11).^{66,157,171,257} The increased prevalence in the fall has led to speculation that school-based

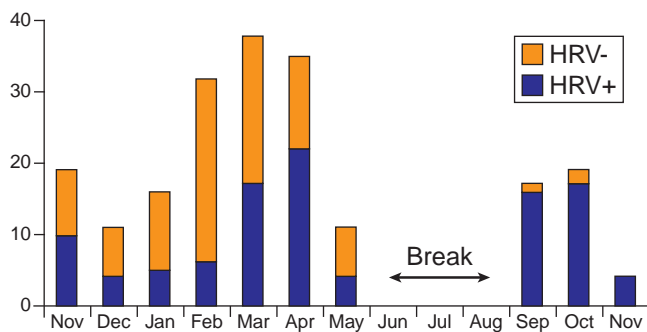


FIGURE 18.11. Seasonality of human rhinovirus (HRV) infections. Between October 1994 and November 1995, 200 young adults (mean age 24 years, mostly university students) were followed for the occurrence of common cold symptoms. Nasopharyngeal aspirates obtained during periods of illness were analyzed by polymerase chain reaction (PCR) for rhinoviruses and other respiratory viruses. The graph depicts the monthly occurrence of upper respiratory illnesses, and illnesses that were positive for HRV by PCR-based diagnostics are indicated by the blue bars. Data from.¹⁵⁷

transmission among children may drive this trend.¹²⁶ HRV infections continue during the wintertime, but account for less than 50% of infections during this period of time due to the plethora of other respiratory viruses found at this time of year. The prevalence of HRV infections increases again in the spring, and is lowest in the summertime. Overall, HRV infections account for about half of common cold infections over the entire year, and more than 80% of infections during peak seasons in the spring and fall HRV.

More than 20 types of HRV can circulate within a community at any given period of time, and the composition of HRV types in a single community changes almost completely from season to season and year to year.^{170,184} Some studies have reported species-specific patterns of HRV prevalence,^{166,184} but because the range of viruses differs markedly with season and location, several years of data will be necessary to confirm these patterns.

Serologic responses to the various rhinovirus types can be detected in infants, and the number of serotypes to which antibodies are present increased throughout childhood and adolescence.^{80,172} The prevalence of serotype-specific antibodies peaks in young adults (mean percent positive = 60%), and remains at 40% to 50% throughout adulthood. Type-specific antibody in the same individual can persist at relatively stable levels for years.²²⁹

Epidemiologic studies of HRV prevalence are best conducted with molecular diagnostics because of difficulties in culturing these viruses (especially the C species viruses). Serologic tests can be performed for culturable viruses, but due to the large number of serotypes, antibody responses are measured mainly in experimental inoculation studies performed with a single serotype.^{84,234}

FIGURE 18.12. Recombinant origins for many human rhinovirus (HRV). A&B were uncovered by genome sequencing.¹⁸⁶ Parents (solid boxes) or progeny (two-color boxes) are founders of many clades.

that the HRV-A and HRV-C frequently recombine, and when they do, they exchange not the expected capsid Nims, but 5' UTR regions, including the pyrimidine-rich tracts, and their respective 2A protease genes.^{107,164} Comparative 2A work is underway to document why evolution apparently favors these particular recombinants. Possibly, divergent protease specificities may help these viruses regulate the overall cell response to infection.

Picornavirus polymerases have a high error rate, estimated at 10^{-3} to 10^{-4} errors/nucleotide/cycle of replication. As a result, HRV types exist as quasispecies populations. Analysis of 34 full HRV sequences of prototype strains demonstrated that the HRV genome as a whole is under purifying selective pressure, with focal areas of diversifying pressure at antigenic sites in the structural genes, and in the 3C protease and 3D polymerase.¹³³ Mutation rates have also been determined for HRV-A39 after experimental inoculation.³⁸ The calculated mutation rate after 5 days of replication *in vivo* was 3.4×10^{-4} mutations/nucleotides over the whole ORF, and specific hyper-variable mutation sites were located within VP1, VP2, VP3, 2C, and 3C sequences.³⁸

Clinical Features

Experimental inoculation studies have enabled detailed observations of the time course of clinical manifestations of HRV infection.^{21,42,49,54,56,104,165} Following inoculation with virus, the first signs of illness are usually sore or scratchy throat with an onset as soon as 16 hours postinoculation, followed by malaise and rhinitis. The first nasal symptoms are rhinorrhea, which usually begins with a watery discharge. During the peak of the cold, the discharge becomes mucoid or purulent, and is accompanied by nasal congestion, cough, and headache. Low grade fevers can occur in infected children, and are uncommon among adults with HRV infections. The latent period after infection is generally 1 to 2 days, and peak symptoms generally occur between 2 and 4 days following inoculation. Most colds are either over or are clearly subsiding within one week; cough and nasal symptoms that persist for two weeks or longer suggest either secondary bacterial sinusitis,²²¹ or else a second infection with a different virus.¹¹⁹ There are few symptoms to differentiate HRV illnesses from those caused from other viruses. Studies of adults indicate that HRV may be more likely to present with

sore throat, have prominent nasal symptoms in most individuals, and are less likely to be associated with fever.¹²

HRV can also cause infections of the middle ear, sinuses, and lower airways. Eustation tube dysfunction occurs in about half of experimentally induced or naturally occurring HRV infections.^{25,160,173} Studies of natural colds also link detection of HRVs in the nasal secretions or middle ear fluid to otitis media (OM).^{9,29,32,196,211,227,252} A prospective one-year study of the role of viral URI in OM enrolled 294 children between the ages of 6 months and 3 years, and the viruses most often detected were HRV and adenoviruses (27% of OM for each virus).³² Approximately one third of HRV infections were complicated by acute OM, and another 20% of cases had asymptomatic middle ear effusions. Another recent longitudinal study prospectively sampled nasal secretions of 1- to 5-year-old children and siblings at the onset of either cold or otologic symptoms.⁵ HRV infection was detected at least once in 70% of the children, and 44% of these infections were associated with either OM with effusion or acute OM.

To investigate effects of HRV on the paranasal sinuses, Gwaltney and colleagues⁸⁵ performed sinus computerized tomography (CT) scans during the peak symptom period following experimental HRV inoculation of 31 adults, and during the acute cold 27 subjects (87%) had swelling and or mucosal thickening and fluid collection in one or both of the maxillary sinuses (Fig. 18.13). A subset of the study subjects had repeat scans two weeks later, and these abnormalities had spontaneously subsided in 79%. Likewise, Puhakka et al.²⁰³ tracked findings on serial plain radiographs in individuals with naturally acquired common colds, and found that 38% developed mucosal thickening and either air fluid levels or opacification of the sinuses on day 7 of the cold. These abnormalities resolved without treatment on follow-up films obtained on day 21. Together, these findings provide evidence that HRV infections can cause acute sinusitis. Whether the illness is caused by HRV or a combination of HRV plus bacteria has not been resolved, but an important clinical point is that the illness subsided without antibiotic treatment.

In infants, HRV can cause bronchiolitis, which is a syndrome consisting of upper airway symptoms that progress to severe cough, wheezing, and tachypnea. In severe cases, significant air trapping and hypoventilation can lead to hypoxia

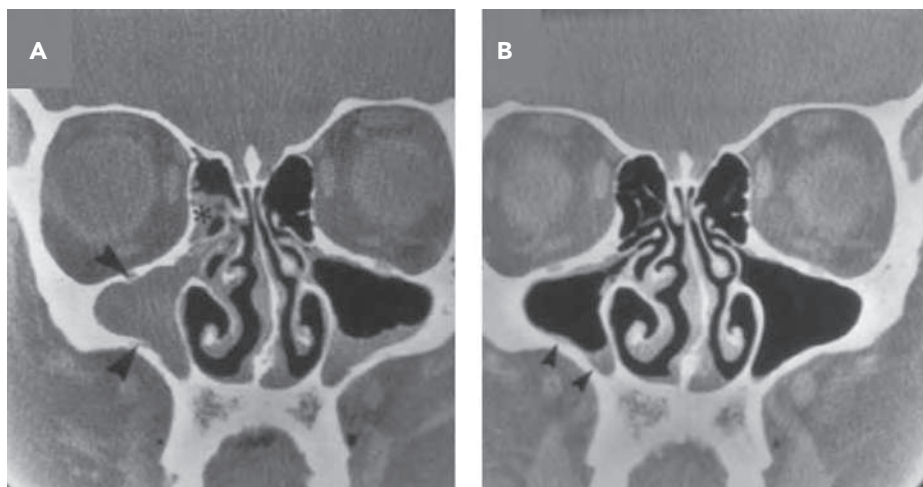


FIGURE 18.13. Computerized tomography (CT) scans during an experimentally induced human rhinovirus (HRV) infection. The initial scan obtained on day 4 postinoculation (**A**) shows bilateral effusions in the maxillary sinuses (arrows) and abnormalities in the right ethmoid sinuses (asterisk). The follow-up scan on day 13 of the illness (**B**) shows some residual abnormality of the right maxillary sinus (arrows). (From Gwaltney JM, Jr, Phillips CD, Miller RD, et al. Computed tomographic study of the common cold. *N Engl J Med* 1994;330:25–30, with permission © Massachusetts Medical Society.)

and need for hospitalization for supportive care. HRV is second only to respiratory syncytial virus as a cause for bronchiolitis in infants.^{26,115,121,122,149,169,218} In patients with chronic asthma, HRV infections often begin with typical cold symptoms and then progress to asthma exacerbations that include severe cough, wheezing, shortness of breath, and in severe cases, hypoxia. Similar exacerbations of chronic respiratory symptoms also occur in patients with cystic fibrosis or chronic obstructive pulmonary disease (COPD).^{35,222,249,258}

Diagnosis

Differential

HRV infections cause respiratory symptoms that are quite similar to those caused by other respiratory viruses. Compared to influenza, HRV is less likely to cause fever, myalgia, and headache. In general, signs and symptoms of rhinitis are prominent, but there are few true differentiating features. The spring and fall peaks in HRV epidemiology follow the same general pattern as hay fever in many geographic locations. Differentiation of common cold and allergy symptoms can be difficult, but there are some differences in symptom profiles. Compared to respiratory allergies, HRV infections are of shorter duration, are more likely to cause sore throat, and are less likely to cause nasal and ocular pruritus.

Laboratory

VIRUS ISOLATION AND IDENTIFICATION

Tissue culture is a relatively insensitive method for HRV detection. Fetal lung fibroblast cells (WI-38, MRC-5, and Wis.L cells) or HRV-sensitive lines of HeLa cells are commonly used for HRV detection. Notably, HRV-C viruses cannot be cultured in standard cell lines, which lack the receptor to bind C species viruses, but have been propagated in organ cultures of sinus epithelium.²³ Typing of HRV-A and HRV-B species isolates is possible using specific antisera.

HRV detection is greatly improved through the use of RT-PCR^{67,111,147} or other molecular techniques,^{51,133} which are available mainly through research laboratories. The 5' untranslated region contains several highly conserved regions that are usually targeted for primer design (Fig. 18.14). Because HRV-C's do not grow in standard tissue culture conditions, partial genomic sequencing has been used to classify these viruses

into "types".²¹⁶ The regions usually targeted for genotyping are the 5' UTR, VP2-4, or VP1. Sequences in the capsid coding region are more variable, which helps to distinguish genotypes but also complicates the design of broadly applicable primers. Higher rates of success (close to 100%) have been achieved by targeting the 5' UTR.¹⁴⁸ Classification based on partial sequences must be considered as tentative, since recombination among HRV can occur.¹⁸⁶

Nasopharyngeal lavage specimens or swabs are the best specimens for HRV detection, and yields are lower from throat swabs.^{165,224} A "nose-blow" technique has been used to obtain nasal secretions for clinical research protocols using PCR-based diagnostics with good recovery rates.^{95,184,197} Viral transport medium containing a protein source such as gelatin or albumin helps to stabilize the viral capsid for best results with tissue culture or molecular techniques. Nasal swabs obtained at home and mailed to the laboratory also provide specimens suitable for analysis by molecular techniques.¹⁹¹

Prevention and Control

The cure for the common cold remains enigmatic. Many approaches have been explored, including nutritional supplements, immune modulators, antiviral agents, and mediator antagonists to block specific common cold symptoms.²³² Vitamin C has long been touted for common cold treatment; however, a meta-analysis of common cold treatment studies found no significant effects on either prevention or treatment.⁵⁵ A Cochrane review of clinical studies found evidence that zinc lozenges reduce common cold duration, but have significant side effects including bad taste and nausea.²¹⁹ Topical zinc was marketed as a homeopathic treatment for colds, but was taken off the market due to association with anosmia.³ Large-scale trials of Echinacea have provided no evidence of efficacy.^{15,233} There is evidence that warm drinks, as recommended for generations, can provide symptomatic relief from malaise and nasal symptoms without troublesome side effects.²⁰⁹

Improved knowledge of HRV molecular virology has led to several attempts to develop antivirals. IFN- α has antiviral effects *in vitro* and shortens the duration and severity of colds, but topical application led to nasal irritation and bleeding.^{63,92,94} Anti-ICAM-1 and soluble ICAM-1 were developed to prevent binding of major group viruses to their receptor.^{27,159,183} Capsid

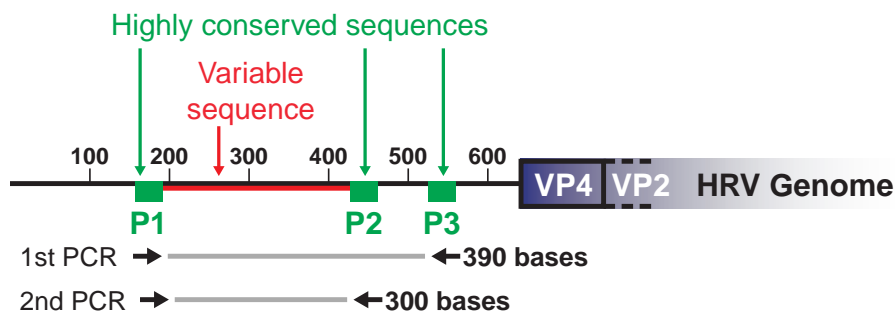


FIGURE 18.14. Selection of primers for polymerase chain reaction (PCR)-based diagnostic tests for human rhinovirus (HRV) infection. The most conserved sites in the HRV genome are in the 5' untranslated region in the area of stem loop structures that bind ribosomal proteins. These sites are targeted for primer design (P1, P2, P3) that can be used for single or nested reverse-transcriptase PCR. (Figure courtesy of Wai Ming Lee, PhD, University of Wisconsin-Madison.)

binding agents that bind to the VP1 pocket and inhibit viral binding and/or uncoating^{7,213,234} have shown modest antiviral effects and efficacy in clinical trials.^{93,95} An inhibitor to the 3C protease (rupintrivir) also showed broad anti-HRV activity *in vitro* and efficacy in clinical trials.⁹⁶ Unfortunately, these antiviral approaches have not so far led to development of a clinically useful medication. The molecules tested to date have been limited by combinations of modest efficacy, side effects, and/or drug interactions.²³²

Medications targeting specific symptoms can be helpful in relieving some aspects of the common cold. Examples include use of decongestants and topical ipratropium for relief of congestion and rhinorrhea, respectively.⁵⁹ Cough syrups are ineffective. Notably, marketing of combination cough and cold medications to children has been stopped by the U.S. Food and Drug Administration (FDA) due to reports of toxicity with overdoses, and even a small number of children receiving standard doses of these medications. A recent study suggests that vapor rub (petroleum jelly with aromatic oils) may relieve common cold symptoms, perhaps by stimulation of ion channels.¹⁸⁹

Perspective

The development of molecular diagnostic tests for HRV has led to a better appreciation of their role in respiratory illnesses, especially with respect to lower respiratory illness in young children and patients with chronic lung diseases. Findings from recent studies underscore the ubiquitous nature of HRV infections, and at the same time have led to some caution in interpretation of viral detection due to high rates of asymptomatic illness. That HRV is linked to a full spectrum of respiratory illness also raises questions about the nature of factors that modify illness severity. Identification of environmental and personal determinants of illness severity could provide new preventive strategies, and this is especially important for high risk populations.

Another revelation for common cold researchers was the discovery of the HRV-C species in 2006. After a hiatus of almost 20 years following the cataloguing of what most virologists considered to be the final HRV serotypes, clinical studies utilizing molecular diagnostics led to the discovery of another estimated 50 to 60 HRV types. There is growing evidence that HRV-C viruses may be more likely than other species to cause lower respiratory illness in infants and children, and acute exacerbations of asthma.

Finally, the cure for the common cold remains elusive. After some initial failures took the wind out of the sails of these efforts, the recognition that HRV infections are an important cause of lower respiratory illnesses has generated renewed interest in this effort. Recent advances in understanding the molecular pathogenesis of HRV illnesses, including HRV-C, provide reason for renewed optimism that this goal will be achieved.

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Hepatitis A Virus

History

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Virion Structure and Physicochemical Properties

Hepatitis A Virus Resistance to Physical and Chemical Agents

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HISTORY

Reports of icteric disease in ancient Chinese literature, as reviewed by Zuckerman,⁴¹⁹ and a letter from Pope Zacharias to Archbishop St. Boniface of Mainz during the 8th century AD describing an outbreak of jaundice may refer to hepatitis A; however, it cannot be distinguished from jaundice attributable to other causes. It was not until the 17th, 18th, and 19th centuries that scattered epidemics of jaundice affecting diverse populations were recorded more frequently. The disease, which was common in military troops, was called *campaign jaundice*. In time, an infectious cause was suspected by several clinicians, especially for the milder, more contagious forms called *epidemic catarrhal jaundice*.¹⁴³ In 1912, Cockayne⁵⁴ used the term *infectious hepatitis* to describe this epidemic form of the disease, and support for a viral cause gained favor from that point forward.²⁶⁰

Epidemiologic studies and human volunteer experiments performed during and after World War II confirmed the viral cause of hepatitis A and defined its relatively short incubation period and its fecal–oral mode of transmission. These studies also demonstrated the uniqueness of this disease from *homologous serum jaundice*, another form of hepatitis having a longer incubation period.^{30,153,154,205} In 1947, MacCallum²³⁵ introduced the terms *hepatitis A* and *hepatitis B* to categorize these diseases, terms that were adopted in 1973 by the World Health Organization (WHO) Expert Committee on Viral Hepatitis.⁴⁰¹ Between the mid-1950s and early 1970s, the studies of Murray²⁷⁰ and of Krugman et al^{205,207} at the Willowbrook State School for the mentally handicapped were instrumental in further defining the seroepidemiologic relationships between hepatitis A and B that eventually led to the evaluation of new methods for the immunoprophylaxis of these diseases. One of the hepatitis viruses, designated MS-1, was transmitted primarily by the fecal–oral route. The disease was highly infectious, had a relatively short incubation period of 4 ± 2 weeks, and defined classic viral hepatitis type A. The MS-1 strain of hepatitis A virus (HAV) was later used to infect adult volunteers,³⁰ leading to the identification of the virus in their feces by immune electron microscopy, as reported by Feinstone et al¹⁰¹ in 1973. Highly sensitive immunoassays were subsequently developed for the detection of HAV antigens and specific antibodies,¹⁶² culminating in the development of immunoglobulin M (IgM)-specific anti-HAV tests to distinguish recent HAV infection from a previous infection.²²³ In 1967, Deinhardt et al⁷⁹ provided the first evidence for propagation of HAV in small primates, leading to the subsequent recovery of the CR326 strain of human HAV from an experimentally infected *Saguinus mystax*.²⁵² In 1979, Provost and Hilleman³⁰⁴ were successful in cultivating and serially

passaging this HAV strain in cell culture—an important step that ultimately contributed to the development of a vaccine.

VIRUS CLASSIFICATION

HAV is a nonenveloped, positive-sense, single-stranded RNA virus classified within the family *Picornaviridae* that otherwise include many medically and veterinary important pathogens subclassified into 12 genera based on genotypic and serologic characterization (Fig. 19.1). HAV differs from other picornaviruses by several attributes, as reviewed by Martin and Lemon²⁴⁹: (a) HAV is resistant to high temperatures, low pH, and drugs that inactivate many picornaviruses; (b) HAV replicates very slowly and generally without cytopathic effect in cell culture; (c) nucleotide and amino acid sequences, as well as predicted sizes of several HAV proteins are dissimilar; and (d) only one serotype of HAV has been identified, and one antigenic neutralization site is immunodominant. Therefore, HAV has been classified as the type species of a separate genus—*Hepatovirus*—and is still the only identified member of this genus. One other distinct species of picornavirus found in chickens and other birds, avian encephalomyelitis virus,²⁵¹ was tentatively classified within the *Hepatovirus* genus on the

basis of a close phylogenetic relationship to HAV.³⁵¹ However, based on substantial molecular divergence in several proteins and its polyprotein translation mechanism, this virus has been assigned recently to a separate new genus within the *Picornaviridae* family—the *Tremovirus* genus.

VIRUS HOST RANGE AND EXPERIMENTAL MODEL SYSTEMS

HAV infects humans and nonhuman primates. Serologic and experimental infectivity studies in nonhuman primates using acute-phase sera from HAV-infected patients have revealed that HAV can be transmitted to chimpanzees (*Pan troglodytes*)^{78,82,256} and other Old World primates, such as vervet (*Chlorocebus* sp.), African green monkeys (*Cercopithecus aethiops*), rhesus and cynomolgus macaques (*Macaca mulatta* and *M. fascicularis*), as well as several species of New World primates, such as tamarins (*Saguinus* sp.), marmosets (*Callithrix* sp.), squirrel (*Saimiri* sp.), and owl monkeys (*Aotus* sp.).^{18,79,165,307} Disease in nonhuman primates resembles that in humans but is usually milder.³⁰⁷ There are several reports of isolation of HAV-related viruses from Old World monkeys in the wild, several of which have significant sequence variation and minor

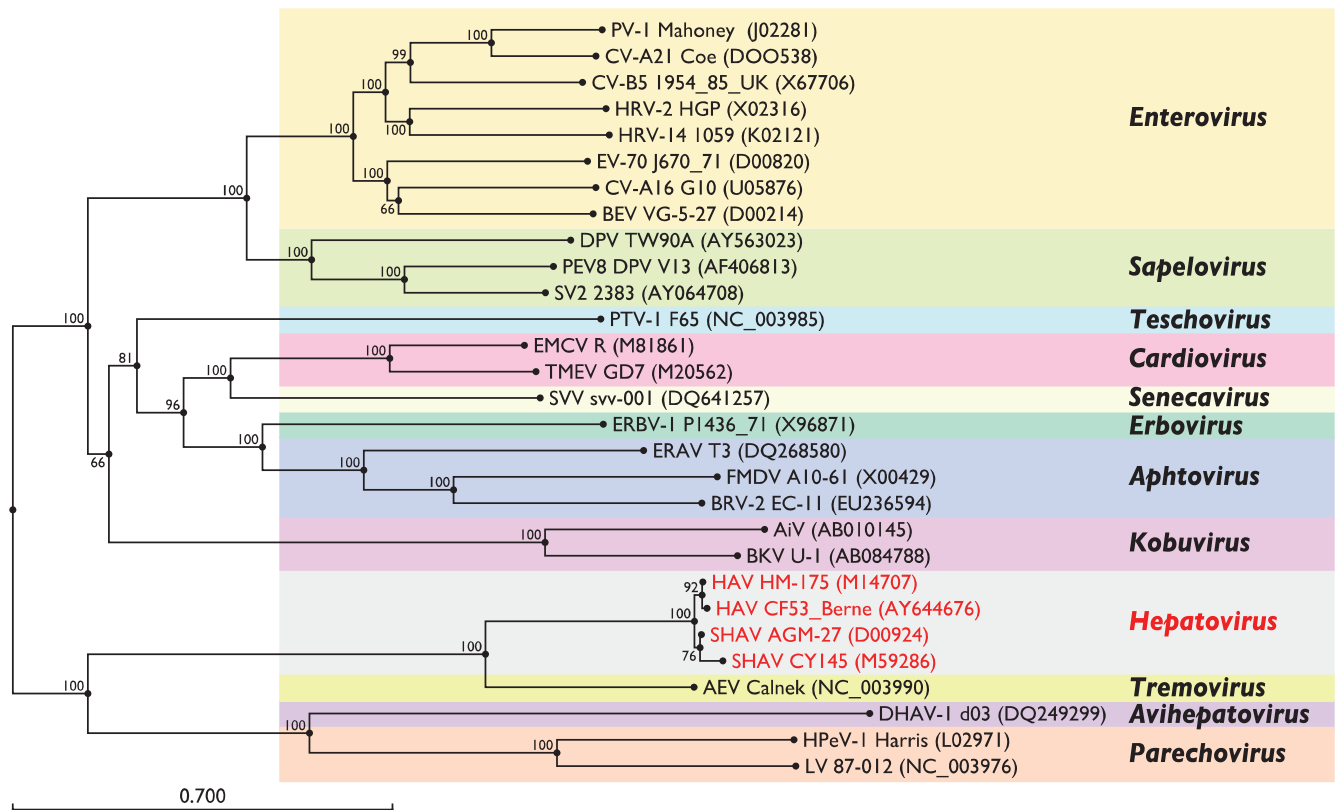


FIGURE 19.1. Phylogenetic relationship of hepatitis A virus (HAV) and simian HAV (SHAV) to representative species of other *Picornaviridae* genera. The relationship was examined by mid-pointed rooted neighbor-joining tree based on P1 (capsid) amino acid similarities using Clustal alignments (accession numbers for sequences of selected picornavirus strains are indicated in parentheses). Bootstrap values (%) are illustrated at nodes, and the genetic distance per amino acid per site is shown by the horizontal bar. The grouping in 12 genera is marked by colored boxes. HAV appears most closely related to tremoviruses (avian encephalomyelitis virus, AEV).

antigenic differences with human HAV^{45,94,274,376} (see the Diversity of the Infectious Agent section). The simian virus isolated from an African green monkey caused severe hepatitis in African green monkeys, rhesus macaques, and tamarins but not in chimpanzees (although the virus replicated and the animals seroconverted).⁹⁴ Correspondingly, after 20 passages in marmosets, human HAV MS-1 was more virulent for marmosets but was attenuated for chimpanzees.⁴⁰ This suggests that host, species-specific variants may have been selected. To date, no rodent animal model has been shown to be susceptible to HAV, although experimental infection in guinea pigs resulted in virus replication in the absence of clinical disease.¹⁶⁶ Chimpanzees, tamarins, and marmosets have been most extensively used as animal models of HAV infection.

HAV is usually difficult to adapt and grow *in vitro*. The virus was first isolated *ex vivo* in marmoset liver explant cultures and was subsequently propagated in continuous fetal rhesus monkey kidney cells³⁰⁴ and in a variety of different types of mammalian cells. Primary African green monkey kidney (AGMK) cells, primary human fibroblast cells, and continuous human diploid lung (MRC5) cells often have been used because of their suitability as potential vaccine substrates. Cell lines derived from AGMK (e.g., BS-C-1, Vero), fetal rhesus kidney (FRhK4), or human hepatoma (PLC/PRF/5) tissue also have been useful for studying virus replication and for propagating large amounts of virus.^{71,106,308} Surprisingly, HAV also replicates in cells of guinea pig, porcine, or dolphin origin.⁸⁷

Wild-type HAV strains collected from infected patients usually replicate very slowly and to relatively low titers in cultured cells, often requiring weeks or even months and blind passages to reach maximal titers. With continued *in vitro* passage, however, the virus becomes progressively adapted to growth in cell culture, replicating more rapidly and achieving higher titers.^{57,59,71} During this process, the viral genome accumulates several mutations (see the Molecular Determinants of Hepatitis A Virus Adaptation to Cell Culture and Attenuation *In Vivo* section). Even when well adapted, HAV usually takes several days to weeks to reach maximal concentrations of propagated virus, achieving typical yields of 10^6 to 10^8 50% tissue culture infectious doses (TCID₅₀)/mL of virus. Recently, a subpopulation of human hepatoma cells (Huh7) has been selected that support efficient growth of wild-type HAV strains without requiring further genetic adaptation.²⁰² These cells may prove useful to characterize wild-type HAV strains isolated from patients and environmental samples.

In contrast to many other picornaviruses, HAV usually has few or no visible effects on the host cell. Thus, detection of HAV multiplication has usually required indirect methods: immunologic assays for viral antigens and hybridization or real-time polymerase chain reaction (RT-PCR) assays for HAV RNA. Radioimmunofocus or infrared fluorescent immunofocus assays can be used to quantify infectious HAV, select viral clones, and measure neutralizing anti-HAV antibodies.^{66,222}

As with other positive-strand viruses, purified genomic RNA, whether extracted from virions or produced synthetically from cloned complementary DNA (cDNA) of cell culture-adapted HAV, is replication competent when transfected into permissive cultured cells.⁵⁸ Accordingly, using synthetic RNA in cell culture and also applying findings from other picornaviruses, it has been possible to elucidate several features of the HAV replication strategy.

MORPHOLOGY AND PROPERTIES OF HEPATITIS A VIRIONS

Virion Structure and Physicochemical Properties

HAV is a nonenveloped, approximately 27-nm spherical virus particle as first determined by Feinstone et al.,¹⁰¹ who used immune-electron microscopy and negative staining techniques on stool specimens collected from acute-phase hepatitis A patients (Fig. 19.2). Mature HAV virions purified from feces collected from infected humans or chimpanzees band at 1.32 to 1.34 g/cm³ in cesium chloride (CsCl) and sediment at approximately 156S to 160S in neutral sucrose solutions.³⁴² Additional populations of viral particles with different sedimentation characteristics have been isolated from human feces and cell culture. Particles with lower density that band at about 1.27 g/cm³ in CsCl and sediment at 70S to 80S are abundant in feces collected during early infection and probably represent empty capsids devoid of genomic RNA (also called *procapsids*) (see Fig. 19.2). Other premature or defective particles, including particles harboring capsid protein precursors, also can be observed.^{26,224,342} These alternative structures are

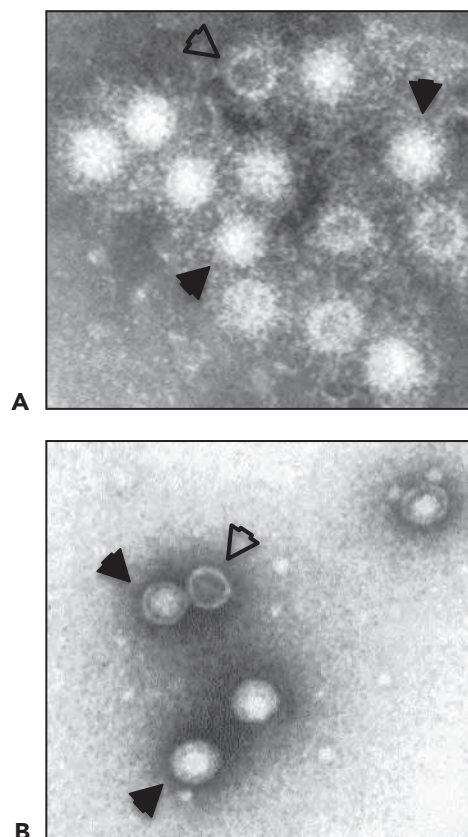


FIGURE 19.2. Hepatitis A virus (HAV) particles observed by electron microscopy. A: Immune electron micrograph of HAV particles from human stool heavily coated with and aggregated by human convalescent antibody. **B:** Negative staining showing 27- to 28-nm HAV particles with cubical symmetry purified from human stool. Both virions (*closed arrowheads*) and procapsids devoid of genomic material (*open arrowheads*) can be found. (Courtesy of Dr. Stephen M. Feinstone, Food and Drug Administration, Bethesda, MD.)

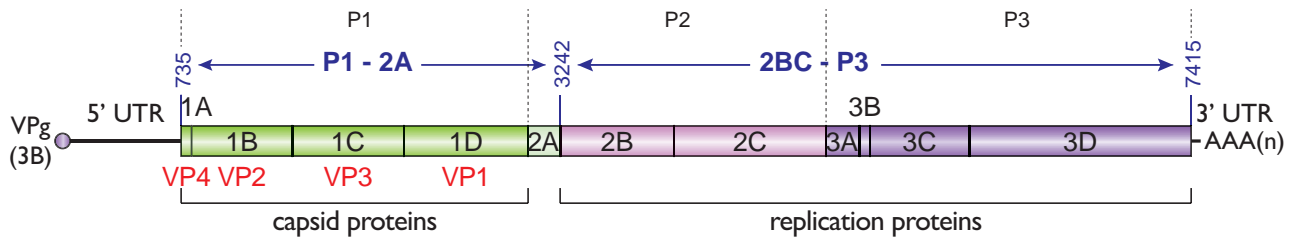


FIGURE 19.3. Hepatitis A virus (HAV) genome organization. The positive-strand RNA genome contains a single open reading frame (ORF), 5' and 3' untranslated regions (UTRs), is linked at its 5' terminus to the virus-encoded protein 3B (also referred to as VPg), and is polyadenylated [AAA(n)] at its 3' terminus. The ORF encodes capsid proteins 1A-D (also referred to as VP1-4 in the order indicated below the genome scheme; 1A or VP4-VP2 precursor also is known as VP0), protein 2A that is involved in particle assembly as 1D-2A (VP1-2A) precursor, and proteins 2B-C and 3A-D involved in genome replication. *Dotted lines* delineate commonly termed P1, P2, and P3 coding segments in picornaviruses, whereas primary cleavage of HAV polyprotein generates P1-2A and 2B-C-P3 polypeptides (which sequences are indicated by *double-headed arrows*). Numbering above the genome indicates nucleotide positions within the wild-type HM175 strain of HAV (GenBank accession number M14707).

morphologically indistinguishable from typical HAV and have the same major surface antigens.

The HAV capsid encloses the viral genome, which is a linear, single-stranded, 33S RNA molecule of positive polarity approximately 7.5 kilobases (kb) in length.^{59,65,342} The viral genome encodes a single polyprotein that is subsequently cleaved primarily by the unique virus-encoded protease 3C to generate four capsid proteins (VP1-VP4) at its N-terminus and the nonstructural proteins involved in genome replication at its C-terminus (Fig. 19.3).

The HAV capsid contains 60 copies of the three major capsid proteins VP1, VP2, and VP3.^{120,342} Attempts to determine the atomic structure of HAV by X-ray crystallography have not been successful, although such studies have provided high-resolution images of virus particles from several other picornaviral genera. Medium-resolution images of the HAV particle, obtained by cryo-electron microscopy (Fig. 19.4) and generated by R. H. Cheng and collaborators (personal communication, 2011), suggest that there are prominent features at the icosahedral surface of the particle around fivefold and threefold axes of symmetry but no marked depression around the fivefold axes in contrast to the canyon found in enteroviruses.²⁵⁰

Hepatitis A Virus Resistance to Physical and Chemical Agents

In common with the enteroviruses, HAV is stable at low pH.³²⁷ It retains most of its infectivity when subjected to pH 1.0 for 2 hours at room temperature and is still infectious after 5 hours. The thermal stability of HAV, however, is considerably greater than that of other picornaviruses.³⁴⁴ Incubation of the virus for at least 4 weeks at room temperature (25°C) results in only a 100-fold decrease in infectivity.^{259,350} Significant loss of infectivity starts to occur with exposure at 60°C for short periods, and infectivity is destroyed almost instantaneously by heating above 85°C at neutral pH.^{285,305,344}

HAV has been found to survive for days to months in experimentally contaminated fresh water, seawater, wastewater, soils, marine sediment, live oysters, and cream-filled cookies.³⁵⁰ Outbreaks of hepatitis A have been reported following ingestion of partially cooked bivalve mollusks, suggesting that even steaming may be insufficient to destroy the virus in this

food source. In addition, HAV infectivity is highly resistant to drying, and infectious virus has been recovered from acetone-fixed cell sheets. The virus is also highly resistant to detergents, surviving a 1% concentration of sodium dodecyl sulfate, as well as to organic solvents such as diethyl ether, chloroform, or trichlorotrifluoroethane.^{294,344} Thus, solvent-detergent inactivation procedures do not reduce the infectivity of HAV, explaining why hepatitis A transmission has occasionally been associated with the administration of high purity, solvent-

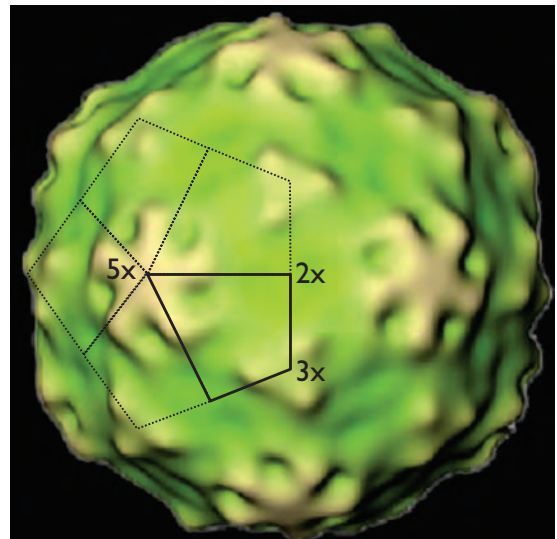


FIGURE 19.4. Medium-resolution image of the structure of the hepatitis A virus (HAV) particle as revealed by cryo-electron microscopy. The solid kite defines a protomer that is likely occupied by single copies of each capsid protein VP1, VP2, and VP3. Five copies of such a protomer are linked at the fivefold (5 \times) axis of symmetry to form a pentamer assembly subunit. The mature particle contains 12 pentamers arranged on an icosahedral surface or 60 copies of each individual capsid protein. Threefold (3 \times) and twofold (2 \times) axes also are indicated. Coloring is based on the distance from the center of the particle, with *yellow* depicting exposed regions at the surface of the particle. (Courtesy of Profs. R. Holland Cheng, University of California at Davis, Davis, CA, and Tatsuo Miyamura, National Institute of Infectious Diseases, Tokyo, Japan.)

detergent-treated clotting factor concentrates.²²⁷ These properties of the virus are likely to contribute significantly to its ability to spread through the environment and to cause common-source outbreaks of hepatitis.

Inactivation of HAV was reviewed by Coulepis et al.⁶³ Appropriate measures rely on autoclaving (121°C for 20 minutes), exposure to chlorine-containing compounds (sodium hypochlorite, 3–10 mg/L at 20°C for 5–15 minutes or free residual chlorine concentrations of 2.0–2.5 mg/L for 15 minutes),²⁹⁴ or to a quaternary ammonium formulation containing 23% hydrogen chloride (HCl). HAV also is inactivated by incubation in 3% formaldehyde for 5 minutes, or in 70% ethanol for 60 minutes, and by β -propiolactone or ultraviolet irradiation.²⁵⁸

GENOME STRUCTURE

Genomic Organization

Similar to all picornaviral genomes, the HAV genome is a single-stranded RNA molecule of positive polarity that can be divided into three parts (see Fig. 19.3): (a) a relatively lengthy 5′ untranslated region (UTR) that does not have a cap structure at its 5′ end but instead has a 2.5 kDa, covalently bound, virus-encoded protein—VPg (also known as 3B)³⁸⁹; (b) a single, large open reading frame (ORF) encoding a polyprotein of approximately 2,227 amino acids in length that is proteolytically processed into structural (P1-2A) and nonstructural (2B-C and P3 or 3A-D) viral polypeptides; and (c) a short 3′ UTR of 63 nucleotides that is followed by a poly(A) tail of varying lengths (40–80 nucleotides) typical of picornavirus genomes. Complete sequence lengths range from 7,470 to 7,487 nucleotides excluding the 3′ poly(A) tail.

The HAV genome is very rich in A+U bases (62%) as compared to the median A+U base composition of 54% found among other picornaviruses—a feature that is likely to affect base-pair composition and biologically relevant folds of the genome. As in other picornaviruses, however, the HAV genome also is predicted to exist as a circular entity with both 5′ and 3′ ends in close proximity to facilitate RNA translation to replication switches.²⁸⁴

Untranslated Regions

The nucleotide sequence of the HAV genome begins at the 5′ end with two uridyl residues typical of picornaviruses. The 5′ UTR is approximately 729 to 749 nucleotides long and has a high level of secondary RNA structure comprising several complex stem-loop structures (Fig. 19.5). RNA secondary structure and tertiary interactions (pseudoknots) within this segment of the genome have been established by a combination of (a) phylogenetic comparative sequence analyses to predict thermodynamically stable base pairing, (b) functional genetic studies, and (c) direct biophysical and nuclease mapping techniques with single- or double-strand-specific ribonucleases (RNases).^{41,215,336} Among HAV strains, the 5′ UTR appears to be the most conserved region of the genome ($\geq 89\%$ nucleotide identity among strains representing genotypes I, II, and III).⁴¹ All picornavirus genomes have secondary structural elements at their extreme 5′ end that differ in length and folding.²⁸⁴ In the case of HAV, the 5′ UTR has an unbranched terminal stem of very low free energy comprising the 41 5′ terminal nucleotides.

This element (stem-loop I; see Fig. 19.5) is likely to be important for RNA replication.

As in all picornaviruses, the most dominant structural unit in the HAV 5′ UTR is the internal ribosome entry site (IRES), which determines the initiation of viral translation in a 5′ cap-independent fashion (see Fig. 19.5). Depending on structural similarities at the secondary and tertiary levels, and the nature of cellular translation factors required to initiate translation, picornaviral IRESs are divided into four groups.²⁸⁴ The HAV IRES makes up a group on its own, designated type III IRES, which is distinct from all other known IRESs.^{20,284} The boundaries of the HAV IRES were mapped by using bicistronic messenger RNAs (mRNAs) comprised of a first reporter gene followed by various segments of the HAV 5′ UTR that controlled translation of a second, downstream reporter gene. Characterization of their translation activities in reticulocyte lysates or in cell culture revealed that the IRES is located between nucleotides 154 and 735.^{43,125}

Between the 5′ terminal stem-loop I and the IRES lies a sequence of approximately 110 nucleotides (nucleotides 42–154) that includes (a) two pseudoknots (IIa and IIb, nucleotides 42–94),³³⁶ the sequence of which is highly conserved among HAV strains, and (b) a partially ordered polypyrimidine tract and a single-stranded region (nucleotides 95–154),¹⁴⁹ which are susceptible to naturally occurring mutations, particularly deletions.⁴¹ This 5′ spacer domain corresponds to the most variable segment in length and sequence among all picornaviruses.²⁸⁴ These sequences may function as replication elements and/or contribute essential virus–host interactions.

The 3′ UTR of HAV RNA shows a high proportion of differences between HAV strains (up to 20%). It has been less extensively studied than the 5′ UTR but also has a high propensity to form higher-order structures. Hypothetical structural models of the HAV 3′ UTR have been proposed based on probing with specific RNases and computer-aided predictions, suggesting that a pseudoknot structure is favored⁸⁵ (e-Fig. 19.1).

Structural Proteins and Replication Proteins

Uniquely among *Picornaviridae* genera, the primary cleavage of the HAV polyprotein occurs between the P1-2A and 2BC-P3 segments of the polyprotein under the direction of the only protease encoded by the virus—the 3C protein^{184,248} (see Fig. 19.3). The P1-2A segment comprises four structural polypeptides (in order): VP4 (1A), VP2 (1B), VP3 (1C), and VP1 (1D), named according to their homologs in the poliovirus capsid based on relative molecular masses, with VP1 being the largest. These proteins are approximately 23, 222, 246, and 274 amino acids in length, respectively (Table 19.1).^{59,138,247} VP1, however, has a heterogeneous carboxy terminus,¹³⁸ reflecting a unique maturation mechanism. Whereas VP2, VP3, and VP1 comprise the viral capsid, VP4 is substantially smaller than its homologs in other picornaviruses and has never been experimentally determined to be part of the HAV capsid. The 2A protein of HAV does not encode a primary cleavage function as found in the 2A proteins of most other picornaviruses, neither is it involved in genome replication.²⁵⁰ HAV 2A functions in capsid assembly as a fusion precursor with VP1⁶⁰ and remains attached to some otherwise fully formed virions.⁹ In-frame insertions within the HAV genome of exogenous protein coding sequences, flanked by 3C cleavage sites at the 2A/2B junction, has resulted in replication-competent virus, further

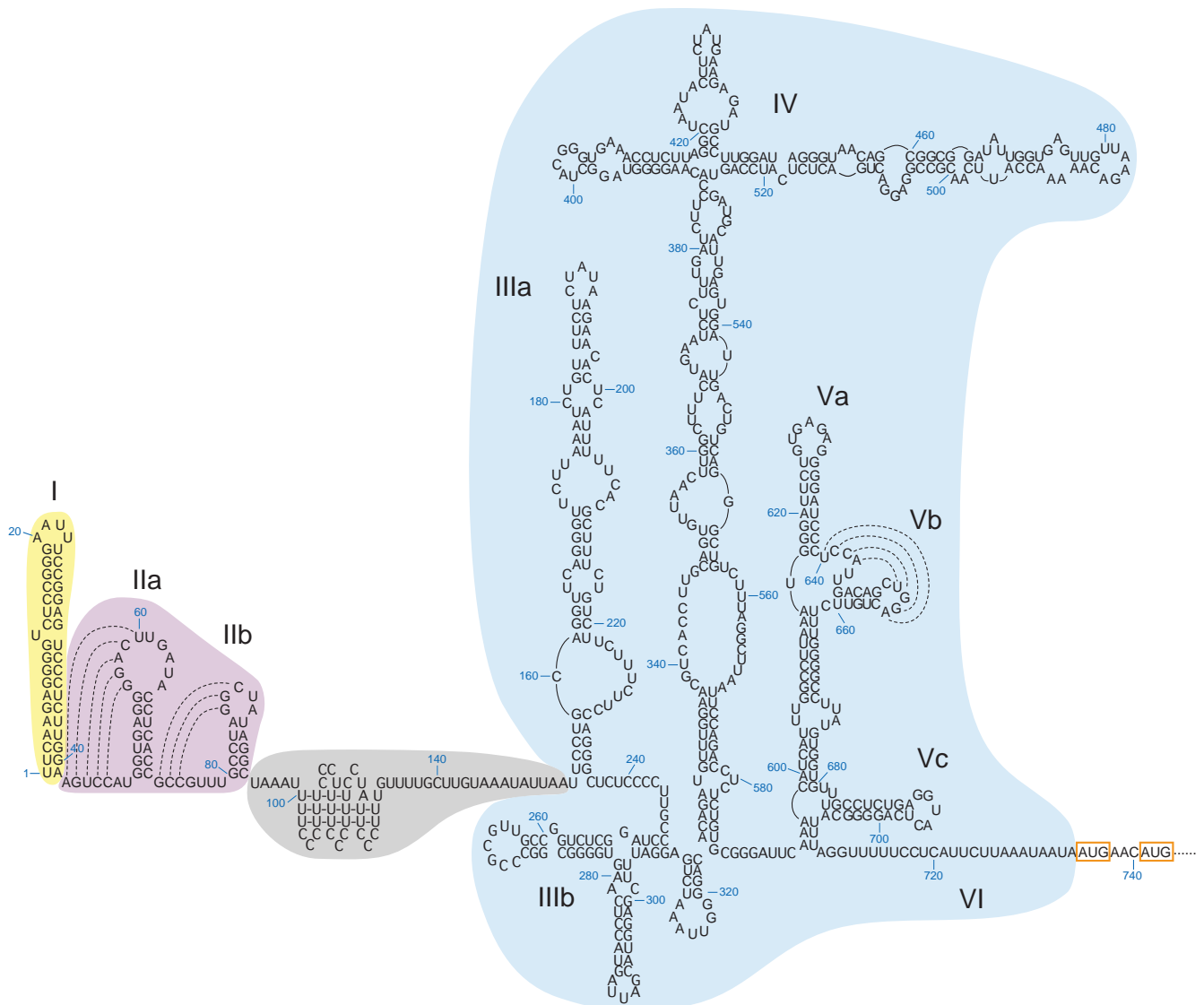


FIGURE 19.5. Proposed RNA structure of the 5' untranslated region (5' UTR) of hepatitis A virus (HAV; wild-type HM175 strain, accession number M14707). Nucleotides (in blue) are numbered according to their positions within the HAV genome. Subdomains comprised of stem-loop structures are numbered sequentially (Roman numerals). Putative tertiary pseudoknots are represented by *dotted lines* to indicate possible interactions between nucleotides. The 5' terminal stem-loop structure (I), pseudoknot elements (IIa-b), and polypyrimidine tract/single-stranded region (see text) are represented by *yellow*, *pink*, and *gray-shaded areas*, respectively. The internal ribosome entry site (IRES) is delineated by the *blue-shaded area* and comprises RNA structures IIIa to VI (nucleotides 154–735). The two in-frame AUG initiator codons of the viral polyprotein are boxed. (Modified from Martin A, Lemon S. The molecular biology of hepatitis A virus. In: Ou J-H, ed. *Hepatitis Viruses*. Norwell, MA: Kluwer Academic Publishers; 2002:23–50, with kind permission from Springer Science+Business Media B.V.)

confirming that 2A has no function *in cis* with the remainder of the nonstructural proteins.²¹

Nonstructural proteins derived from the 2BC-P3 segment of the polyprotein probably all contribute directly to the assembly of the membrane-bound viral replicase complex (see Table 19.1). Proteins 2B and 2C, and probably also the precursor 2BC, are involved in directing the rearrangements of cellular membranes required for replicase assembly.^{130,369} It has been suggested that 2B, containing hydrophobic sequences, anchors the replicase complexes to altered intracellular membranes.¹³⁰ 2C has NTPase activity¹²⁶ and contains an RNA helicase sequence motif.⁸⁴

Among the P3 nonstructural proteins, 3D is assumed to be the viral RNA-dependent RNA polymerase, although no direct data exist. 3C has been crystallized and is a chymotrypsin-like cysteine protease^{3,23,409} (Fig. 19.6). It appears to be the only virus-encoded protease of HAV, which is responsible for all co- or posttranslational cleavage events within the HAV polyprotein except for those at VP4/VP2 and VP1/2A junctions.^{151,242} In addition, HAV 3C protease and/or its precursors 3ABC and 3CD also appear to cleave cellular IRES-transacting factors and adaptor molecules acting in interferon signaling pathways (see the Infectious Viral Life Cycle and the Pathogenesis and

TABLE 19.1 Hepatitis A Virus Structural and Nonstructural Proteins

Viral Protein	Number of amino acids	Function	Properties
1A = VP4	21–23	Capsid protein?	Found as 1AB precursor in immature particles Presence not demonstrated in virions
1B = VP2	222	Capsid protein	Contributes to the major neutralization antigenic site Heterogeneous C-terminus Also found as 1D-2A precursor in immature particles Contributes to the major neutralization antigenic site Only characterized as P1-2A and 1D-2A precursors Also found as 2BC precursor
1C = VP3	246	Capsid protein	
1D = VP1	272–274	Capsid protein	
2A	71	Essential for capsid assembly	Also found as 2BC precursor 3ABC precursor associates with mitochondrial membranes Covalently linked to the 5' end of the genome Cleaves all protein junctions except for 1D/2A and 1A/1B 3ABC and 3CD precursors cleave adaptor proteins of the host interferon signaling pathways
2B	251	Probable membrane anchoring for the virus replication complex	
2C	335	NTPase and helicase activities	
3A	74	Probable membrane anchoring for 3B	
3B = VPg	23	Initiation of RNA replication?	
3C	219	Chymotrypsin-like cysteine protease	
3D	489	RNA-dependent RNA polymerase	

Host Immune Responses sections). P3 also generates protein 3B (also referred to as VPg), which is attached to the 5' end of both positive- and negative-strand RNAs,³⁸⁹ and protein 3A, which comprises a hydrophobic 21 amino acid stretch that is believed to anchor the 3AB precursor of VPg (3B) to cellular membranes and to target the 3ABC precursor to mitochondrial membranes.⁴⁰⁴

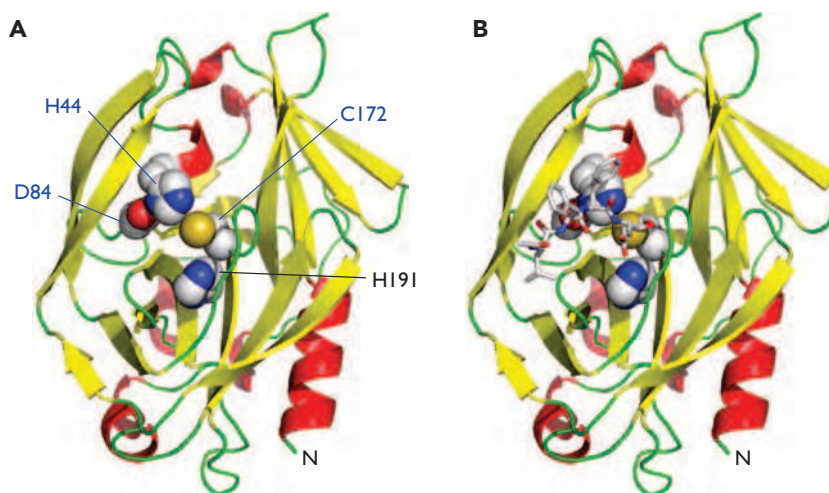
DIVERSITY OF THE INFECTIOUS AGENT

Genetic Diversity

HAV identification and initial characterization were done with strain MS-1 isolated in 1964 from the blood and stools of a patient in New York.^{101,204} In the mid-1980s, the first comparative study of the nucleotide sequences of HAV performed by

RNase T1 oligonucleotide mapping of eight strains originating from diverse geographic sources revealed a variation in the order of magnitude reported for other picornaviruses.³⁹⁰ Several other laboratories reported nucleotide sequencing of full-length genomes from several HAV strains isolated directly from the stools of patients (e.g., HM175; Australia, 1976),^{59,71} or after a few passages in small primates or cell culture (e.g., LA; USA, 1976),²⁷⁵ MBB (North Africa, 1978),²⁸⁶ and GBM (Germany, 1976).¹³⁶ Wild-type strain HM175 has been adapted to yield the widest range of mutants in cell culture, including attenuated, persistently infecting, cytopathic and neutralization-resistant variants (see the Molecular Determinants of Hepatitis A Virus Adaptation to Cell Culture section). Attenuated mutants of HM175,¹⁹³ CR326 (Costa Rica, 1960),²³¹ H2 (China, 1982),²⁴⁴ and variants of strains GBM³⁸⁴ and MBB¹⁶⁷ are the best characterized candidates for live attenuated vaccines,

FIGURE 19.6. Ribbon representation of the crystal structure of hepatitis A virus (HAV) 3C protease (PDB accession 2HAL).⁴⁰⁹ **A:** Ribbon is colored by secondary structure with red, helix; yellow, sheet; green, loop. Side chains of the catalytic triad (D84, H44, C172) are shown as spheres across the top of the active site. The side chain of H191, the primary residue responsible for the glutamine specificity in P1, is also shown as spheres. **B:** A stick representation of the LFFE-FMK inhibitor, which mimics substrate residues P1-P4, is shown bound in the active site. Spheres and sticks are colored by atom type. (Courtesy of Dr. Bruce A. Malcolm, Johnson and Johnson Infectious Diseases and Vaccines, Beerse, Belgium.)



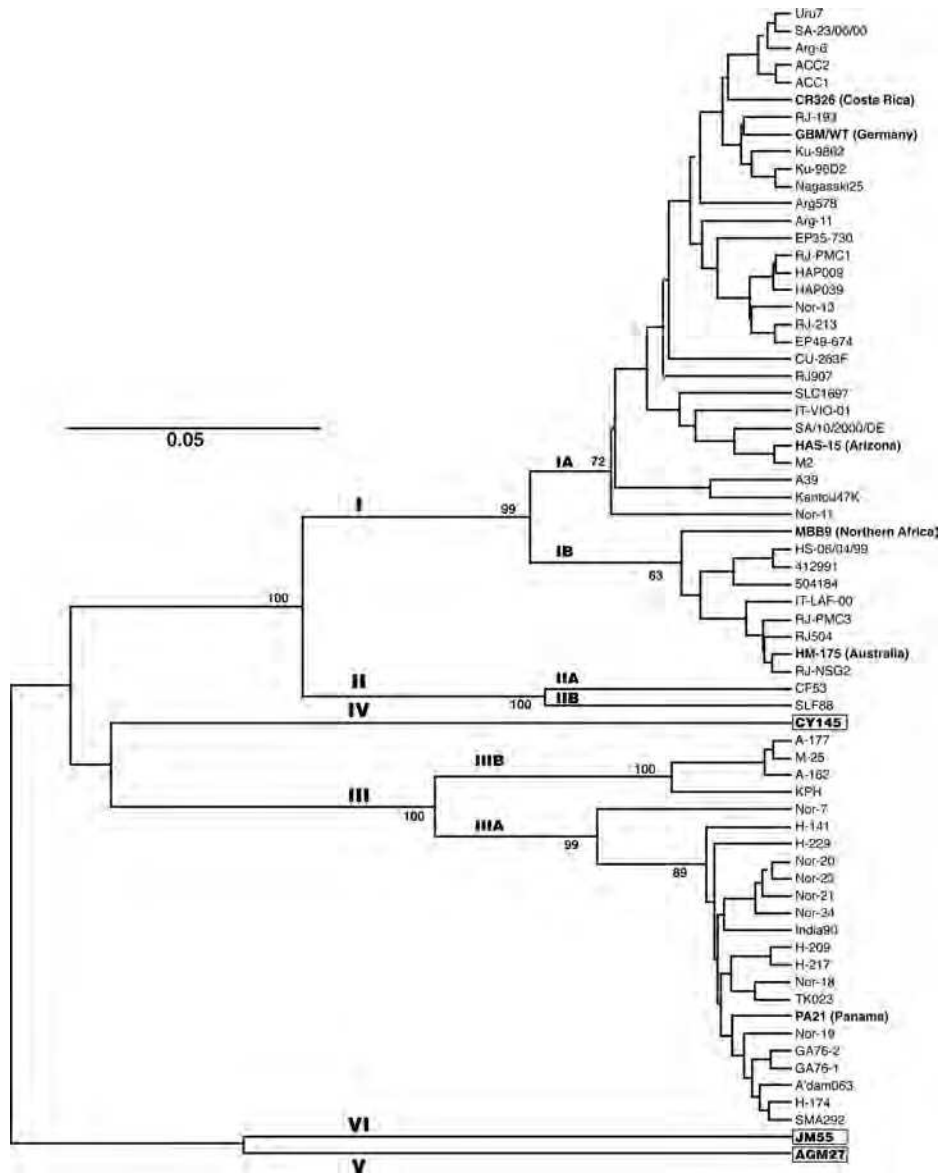


FIGURE 19.7. Phylogenetic tree based on hepatitis A virus (HAV) sequences (168 nucleotides) in the 2A region. Statistically significant bootstrap values (%) are given at the nodes, and genetic distance per nucleotide per site is shown by the horizontal bar. Genotypes are indicated by Roman numerals, whereas subgenotypes are indicated by A and B. The country of origin is indicated for best characterized strains of genotypes I, II, and III. The prototype MS-1 strain of HAV (not shown) belongs to subgenotype IA. The only known representatives of genotypes IV, V, and VI were collected from Old World monkeys. (Modified from Lu L, Ching KZ, de Paula VS, et al. Characterization of the complete genomic sequence of genotype II hepatitis A virus (CF53/Berne isolate). *J Gen Virol* 2004;85:2943–2952, with permission.)

as reviewed by Flehmig et al,¹⁰⁷ Hu et al,¹⁶⁸ and Provost et al¹³⁰³ (see the Hepatitis A Vaccines section).

The genetic diversity of HAV has further been investigated by determining the partial genomic nucleotide sequences of 152 HAV strains recovered from various human or simian sources and geographical areas²³⁴ (Fig. 19.7). Following virus purification by capture with an HAV monoclonal antibody, reverse transcription of viral RNA, and amplification by RT-PCR,¹⁸¹ genotypic analysis was based on the nucleotide sequence of a short genomic segment (168 nucleotides) that encompassed the relatively variable 2A coding sequence (originally thought to span the VP1/2A junction).

Additional phylogenetic studies were more recently carried out using a wider range of HAV isolates that were under-represented in the initial genotyping studies. This included HAV strains from South America, North and Central Africa, and India. In addition, full-length VP1 sequences (900 nucleotides) were chosen to establish nucleotide comparisons, con-

sidering that several residues of VP1 contribute to the major immunodominant site of HAV. Overall strain variation in the complete VP1 gene was found to be higher than 20% at the nucleotide level and around 10% at the amino acid level.⁶²

The current view is that HAV circulates worldwide as a total of six genotypes ($\leq 85\%$ identity) with three genotypes (I, II and III) subdivided into subtypes A and B (86%–92.5% identity within subtypes)²³⁴ (see Fig. 19.7). Genotypes I, II, and III comprise all human strains, among which subgenotype IA and genotype III are the most prevalent worldwide (e-Fig. 19.2). Each of the three simian HAV genotypes are defined by a single strain isolated from naturally infected Old World monkeys, cynomolgus macaques (genotypes IV and VI),^{274,318} and African green monkeys (genotype V).³⁷⁶ All simian HAV strains have a typical signature sequence at the VP3/VP1 junction that distinguishes them from human strains.^{42,318,376}

Clusters within genotypes predominate in certain geographic regions (see e-Fig. 19.2), such as a group of subgenotype

IA strains with more than 97% identity that represent nearly all of the viruses from the United States, suggesting endemic spread. Other regions, such as Europe and Japan, have a greater genetic mixture of viruses, suggesting regular introduction of strains by travelers returning from endemic regions. A higher degree of heterogeneity than reported previously has been found in strains isolated in South America.⁶⁷

To better define the HAV mode of evolution in populations, full-length VP1 sequences of a collection of genotype IA HAV strains isolated in France from 1984 to 2001 were analyzed. This revealed a mean rate of 9.76×10^{-4} nucleotide substitutions per site per year, and an estimation of a synonymous substitution rate around 5×10^{-3} substitutions per site per year. The latter is clearly lower than that observed for other representative members of the family *Picornaviridae* and may be related to the slower replication cycle of HAV, its particular tropism, or its transmission mode.²⁶⁵

Virion Antigenic Structure and Diversity

Comparisons of the nucleotide sequences of various human HAV strains from widely separated geographic regions have demonstrated high amino acid conservation in the sequences of the viral capsid proteins (>70%). Likewise, viruses belonging to distinct genotypes elicit antibodies with substantial cross-neutralizing activity, suggesting that there is only one HAV serotype among human strains.³⁵³ A primate HAV strain recovered from a captive New World monkey (owl monkey) was considered to be a human (genotype IIIA) rather than a simian strain following biological characterization and sequence analysis^{42,318} (see e-Fig. 19.2). Conversely, in strains of HAV isolated from naturally infected Old World monkeys (cynomolgus and African green monkeys), some monoclonal antibodies are capable of distinguishing unique epitopes, indicating some degree of antigenic singularity in simian strains.^{274,376} However, whereas African green monkey virus from genotype V caused no disease in chimpanzees, it elicited an anti-HAV response that protected the chimpanzees against disease after challenge with the virulent human-derived HM175 strain.⁹⁴ These observations suggest that even simian and human strains of HAV demonstrate substantial antigenic cross-reactivity.

Neutralization epitopes of HAV are conformational and generally are not displayed on isolated HAV proteins. Correspondingly, neutralizing murine monoclonal antibodies do not recognize denatured capsid proteins, and antisera raised against proteins expressed by recombinant DNA techniques show only weak reactivity with native capsids and have very limited virus neutralization activity.¹⁷⁰ Most murine monoclonal antibodies to HAV compete with each other and are able to substantially block the binding of polyclonal human antibodies to virus in solid-phase competition immunoassays.^{171,354} Similarly, human and chimpanzee neutralizing monoclonal anti-HAV antibodies recovered by phage display have been shown to compete with murine monoclonal antibodies.^{199,326} This suggests that there may be only a limited number of antigenic epitopes that are closely clustered on the surface of the virus.

Antigenic variants of HAV that resist neutralization by murine monoclonal antibodies have been selected in cell culture by continued passage of HM175 virus in the presence of these antibodies.³⁵⁴ Sequencing of the genomic regions encoding their capsid proteins revealed single amino acid changes in each mutant with a limited number of changes confined to

VP3 (two mutations) and VP1 (four mutations).^{296,297} Similarly, in strain HAS-15 (Arizona, 1979), a small number of mutations that confer resistance to neutralizing antibodies also were selected in VP3 and VP1.²⁷³ Competition studies with these mutants confirmed the existence of an immunodominant site composed of residues of VP1 (at positions 102, 171, and 176) and VP3 (at positions 70 and 74) that are likely to be closely positioned on the surface of the HAV particle and reside on exposed loops that connect β strands of the capsid proteins. This feature is likely to distinguish HAV from enteroviruses, for which equivalent residues do not lie in close vicinity at the surface of the capsid.¹⁶⁰ A crystallographic structure of the HAV capsid would shed light on the conformational nature of the immunodominant antigenic site. An apparently distinct site involving C-terminal residues of VP1 has been identified from HM175 neutralization escape mutants.²⁹⁷ In addition, two continuous epitopes near the N-terminus of VP1 (residues 11–25) and within VP3 (residues 110–121), which elicit weak neutralizing activity as peptides also may represent secondary neutralization sites.^{34,95} In the case of poliovirus, the VP1 N-terminus is internally located in the virion capsid but is exposed during virus attachment to cells.⁴⁴

The finding of limited amino acid changes among multiple neutralization escape mutants suggests that there are important constraints to the antigenic or structural variations of HAV. Recently, however, several HAV variants bearing substitutions or deletions at or around the immunodominant neutralization site or secondary neutralization sites were isolated in sporadic cases or during outbreaks of hepatitis A. These strains showed at least partial resistance to antiserum generated against HAV vaccine or to murine monoclonal anti-HAV antibodies, suggesting a potential for the emergence of HAV antigenic variants or new serotypes in the population.^{62,119,123,292,323}

INFECTIOUS VIRAL LIFE CYCLE

Attachment and Entry

HAV has been shown to bind to a wide range of cultured animal cells, including nonpermissive cells. Although nothing is known about viral determinants involved in HAV cell attachment, it has been observed that mature virions attach relatively rapidly and in a calcium-dependent way^{25,352,394,412} but undergo comparatively slow uncoating with release of viral RNA into the cytoplasm about 4 hours postinfection.²⁷

A candidate cellular receptor (HAVcr1), a mucin-like class 1 integral membrane glycoprotein of 451 amino acids³⁷⁰ (Fig. 19.8A), was first identified in HAV-susceptible AGMK cells using an expression cloning strategy and a monoclonal antibody that blocked binding of HAV to the cell surface.¹⁹¹ A molecule with 95% amino acid similarity was concomitantly identified in an HAV-susceptible hybrid marmoset-Vero cell line.¹⁴ A human homolog of HAVcr1 (with 79% identity) was later shown to interact with cell culture–adapted HAV.¹⁰⁰ The N-terminal cysteine-rich immunoglobulin-like region of the HAVcr1 ectodomain is sufficient for binding HAV, but both this region and the mucin-like region are required for viral particle conformational changes leading to HAV uncoating³⁴⁶ (Fig. 19.8A,B). A soluble form of HAVcr1 also is able to neutralize wild-type HAV,³⁴⁵ providing additional evidence for the role of this molecule as the HAV receptor *in vivo*. Recent

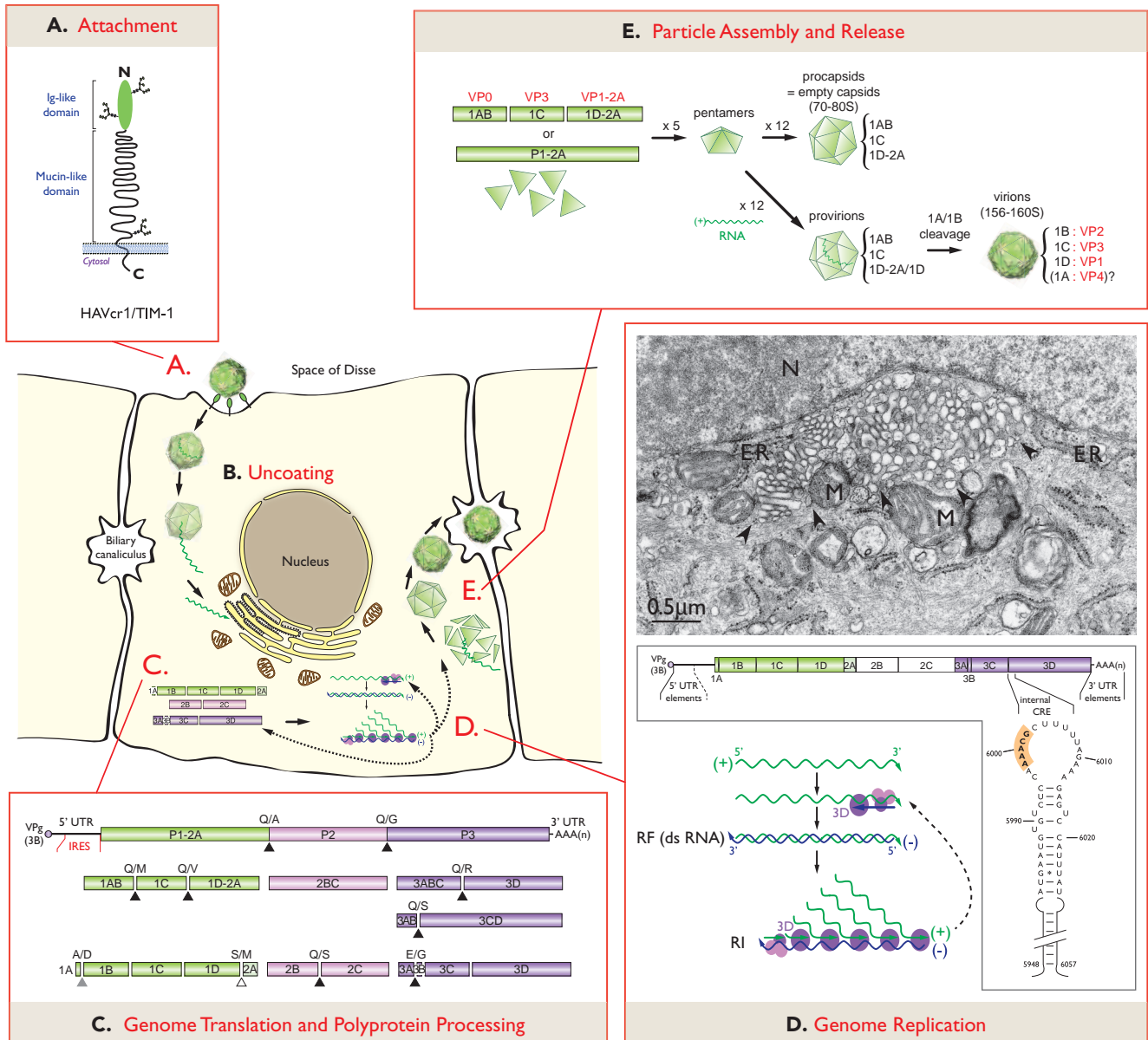


FIGURE 19.8. Hepatitis A virus (HAV) infectious life cycle. **A:** HAV enters hepatocytes by attachment to a cellular receptor, likely HAVcr1/TIM-1. This receptor, as schematically represented, comprises an N-terminal immunoglobulin-like domain and a mucin-like domain with three N-glycosylated sites (branched elements).³⁷⁰ **B:** Details of HAV uncoating remain largely unknown. **C:** Once released within the cell cytoplasm, virion RNA serves as a template for translation of the viral polyprotein, which is subsequently cleaved as depicted. Cleavages are carried out by HAV protease 3C at most junctions (black triangles), likely by a cellular protease at the 1D/2A junction (open triangle), or upon RNA encapsidation at the 1A/1B junction (gray triangle). Dipeptides representing cleavage sites between stable polypeptide precursors or mature proteins are indicated above corresponding boxes. **D:** Genome replication subsequently takes place within the virus-induced tubular vesicular network. The electron micrograph of FRhK-4 cells infected with cell culture-adapted HAV demonstrates rearrangement of cytosolic membranes into a network of tubular-vesicular structures (delineated by arrowheads), which appear closely associated with membranes of the endoplasmic reticulum (ER) and mitochondria (M) in the vicinity of the nucleus (N).¹³⁰ The positive-strand RNA is used as a template by the viral replicase complex comprised of polymerase 3D and other nonstructural proteins for minus-strand RNA synthesis, thereby generating replicating forms (RF). The minus-strand RNA serves in turn as a template for the synthesis of multiple positive-strand RNAs (replication intermediates, RI). *Cis*-active RNA elements (CRE) located at the 5' and 3' terminal regions of the genome, as well as within the 3D coding sequence are required for RNA replication. The stem-loop structure of the internal CRE, as depicted below the genome, includes a relatively lengthy stem (nucleotide numbering from HAV full-length genome) and a large loop that contains a motif boxed in orange, typical of all picornaviral CREs and necessary for their function.⁴⁰⁵ Newly synthesized positive-strand RNAs can be either engaged in translation, replication, or encapsidation, as represented by dashed arrows within the cell. (Electron micrograph courtesy of Dr. Rainer Gosert, Institute for Medical Microbiology, Basel, Switzerland.) **E:** Virion assembly is likely to proceed through indicated assembly intermediates, ultimately leading to the production of infectious virions, which are subsequently released into biliary canaliculi.

studies have identified *TIM-1*, an atopy susceptibility gene, as the gene that encodes HAVcr1.²⁶¹ The T-cell immunoglobulin mucin (TIM) gene family, particularly TIM-1, are cell surface receptors that are important in T-cell regulation and Th-cell differentiation (see the Clinical Features section). HAVcr1 is widely distributed in different tissues¹⁰⁰; thus, the tropism of HAV for the liver remains an enigma waiting to be resolved.

A surrogate entry mechanism of HAV has been proposed via immunocomplexes with HAV-specific immunoglobulin A (IgA), which are present in significant amounts during acute infection. This was based on the observation that HAV/IgA complexes can be endocytosed in hepatocyte cultures via the hepatocellular asialoglycoprotein receptor (ASGPR), which mediates uptake of IgA.⁸⁸ IgA-mediated transcytosis of HAV via polymeric immunoglobulin receptor also was observed *in vitro* and proposed as a mechanism for HAV to overcome the epithelial barrier of the intestinal tract.⁸⁶

Genome Translation, Polyprotein Processing, and Replication

The entire HAV life cycle occurs in the cytoplasm of the cell (Fig. 19.8). HAV genome translation and replication appear to take place in association with a tubular-vesicular membranous network specifically rearranged from cytosolic membranes by proteins 2B, 2C, or 2BC in HAV-infected cells,^{130,369} as is the case for other picornaviruses (Fig. 19.8D). The HAV 2B protein is localized predominantly in the endoplasmic reticulum (ER),⁷⁶ suggesting that the tubular vesicular structures may be derived from the ER.¹⁹⁷ Unlike enterovirus homologous proteins, HAV 2B has little, if any, effect on ER and Golgi calcium homeostasis, or on protein trafficking through the secretory pathway,⁷⁶ which is consistent with the hypothesis that hepatitis A virions may use the secretory pathway to exit the cell.²⁸

Translation

Uncoated RNA is translated via an IRES-dependent mechanism that involves cellular factors so that 40S ribosomal subunits bind and begin translation at the correct initiation codon and not at multiple AUG codons scattered throughout the 5' UTR. This mechanism contrasts with typical eukaryotic translation, in which 40S subunits recognize an m7G-cap at the 5' mRNA terminus (not present in the HAV genome) and then scan for the nearest AUG within an appropriate sequence motif.²⁰³ Initiation of HAV translation may start at either of two in-frame AUG codons at positions 735 through 737 and 741 through 743³⁶⁶ (see Fig. 19.5). Although either codon could function independently, selective deletion experiments have demonstrated that the second of these codons is preferentially used *in vitro* and in cells.³⁶⁶

The HAV IRES is far less efficient for directing translation in acellular systems and in various cell lines than are encephalomyocarditis virus (EMCV) and foot-and-mouth disease virus (FMDV) IRES, for example.^{32,43,125,396} Inefficient translation is likely owing to the fact that the HAV IRES uniquely requires intact cellular initiation factor eIF-4G to function, unlike other picornavirus IRESs that utilize a cleaved fragment of this factor,³¹ and that it probably competes poorly for this and other necessary cellular factors. HAV translation and replication are thus likely to depend on continued synthesis of host factors. Consistent with this hypothesis is the fact that HAV generally does not shut off host protein synthesis, unlike other picorna-

viruses. In the context of virus/cell competition, HAV appears to have adopted a naturally biased, deoptimized codon usage with respect to that of its cellular host.^{12,322} It has been hypothesized that this finely tuned strategy, observed specifically in the capsid coding region, might avoid competition between HAV and the host cell for cellular transfer RNAs (tRNAs), modulate translation kinetics, and allow proper protein folding to reach the highest viral fitness.¹²

HAV IRES activity appears to be modulated by several cellular proteins, including polypyrimidine tract-binding protein (PTB),^{51,129} glyceraldehyde 3-phosphate dehydrogenase (GAPDH),^{51,331,408} poly(rC) binding protein 2 (PCBP2),¹³³ and lupus autoantigen (La).⁶¹ GAPDH and PTB compete for binding to overlapping sites in the IRES (stem-loop III at the 5' end of the IRES)³³¹ (see Fig. 19.5). Although the mechanisms by which these cellular proteins modulate the translational activities of HAV IRES are unknown, it has been speculated that GAPDH may have a destabilization effect on RNA secondary structure, leading to translation suppression,⁴⁰⁸ whereas PTB may have a stabilizing action on higher-ordered RNA structures, thereby reversing GAPDH action and stimulating translation.^{129,408} In support of this hypothesis, a naturally occurring mutation that enhances translation in AGMK cells³³² decreases the affinity of GAPDH for stem-loop III in the IRES.⁴⁰⁸

Polyprotein Processing

Hepatoviruses follow a unique cleavage cascade distinct from all other picornavirus genera, as reviewed in Martin and Lemon.²⁴⁹ The primary, co-translational cleavage during HAV polyprotein translation is mediated by the unique virus-encoded protease (3C) at the 2A/2B junction, thus releasing a P1-2A precursor of 836 amino acids from the downstream nonstructural proteins^{128,248} (Fig. 19.8C). In most picornavirus genera, the primary polyprotein cleavage also occurs at the 2A/2B junction, although by a different mechanism; a unique sequence of amino acids (Asn-Pro-Gly-Pro) located at the 2A/2B junction possesses an inherent propensity to induce ribosome skipping, resulting in "cleavage" of the nascent polyprotein between Gly and Pro residues. Enteroviruses encode a second protease (2A) that autocatalytically cleaves its own N-terminus at the P1/2A junction.²⁸⁴

The key protease 3C carries out most other cleavages in the HAV polyprotein,^{151,182,242,330,368} as in other picornaviruses. The 3C-specific cleavage sites are formed by at least six residues surrounding the scissile bonds (amino acids at positions P4-P1 upstream, and P'1-P'2 downstream, of the cleavage site). HAV 3C is in general less discriminating than other picornaviral 3C proteases and recognizes Gln or Glu residues at position P1, any small residue or even charged residues with large side chains at position P'1 (e.g., Gln/Arg at the 3C/3D site), virtually any amino acid at position P'2, and residues with large side chains at position P4²⁴¹ (see Figs. 19.6 and 19.8C). The 3C-mediated cleavages occur according to preferential site order and also release stable intermediate precursors, such as 2BC, 3ABC, 3CD that appear to contribute specific functions in the HAV life cycle (see Table 19.1).^{210,301,310,404}

Cleavage at two junctions, VP1(1D)/2A and VP4(1A)/VP2(1B), is not carried out by the 3C protease. Uniquely among picornaviruses, VP1 is apparently released from 2A by cellular proteases.^{138,247} The C-terminus of VP1 is heterogeneous,¹³⁸ with a predominant form releasing a 2A protein of

71 amino acids. It has been proposed that, as for Mengovirus, cellular proteases may trim 2A sequences from VP1-2A to generate VP1 intermediate forms, as well as mature VP1 protein.^{138,247} The maturation cleavage at the VP4 (1A)/VP2 (1B) junction, similar to other picornaviruses, takes place in late stages of virion morphogenesis (see Fig. 19.8E) and occurs by an unknown, perhaps autocatalytic, mechanism to yield capsid proteins VP4 and VP2.

RNA Replication

Within the membrane-bound replicase complex, the virion RNA serves as a template for negative-strand RNA synthesis by the RNA-dependent, RNA polymerase 3D, generating double-stranded replicative forms (dsRNA) (Fig. 19.8D). This negative-strand intermediate then acts in turn as a template for the synthesis of multiple positive-strand RNA molecules, yielding replication intermediates (RIs). This asymmetric process suggests that mechanisms of replication of positive and negative strands may show some differences. Newly synthesized positive-strand RNAs can be translated into proteins, replicated, or packaged into new virions.

There are several genomic *cis*-active RNA elements (CREs) required for the initiation of viral RNA replication. Terminal sequences and/or structures present at the 5′ and 3′ ends of the genome, as well as the poly(A) 3′ terminal tail are likely to contain important replication elements. Consistent with this hypothesis, HAV genome-length RNAs that lacked the two 5′ terminal nucleotides (UU) or nucleotides 2 and 3 (UC), which are thought to be involved in a stem-loop structure (see Fig. 19.5), were not infectious.¹⁵⁰ One study suggested that elements required for RNA replication may extend downstream of nucleotide 151 of the 5′ UTR (i.e., beyond the polypyrimidine tract), because a genome containing only the 5′ terminal 151 nucleotides of the 5′ UTR followed by the EMCV IRES was noninfectious, in contrast to chimeric genomes containing the first 237 HAV nucleotides fused to EMCV IRES.¹⁸⁵ Likewise, whereas deletion of the pyrimidine-rich tract (nucleotides 96–139) did not alter viral replication in cell culture, deletions that also included nucleotides 140 through 144 immediately downstream of it (in a predicted single-stranded region (see Fig. 19.5) yielded a temperature-sensitive virus³³⁶ with defective RNA synthesis.³³⁸ The importance of the poly(A) 3′ terminal tail has been corroborated by observations that viral replication is delayed in cells transfected with genomes lacking part or all of the poly(A) sequence, and the poly(A) tail is concomitantly regenerated in progeny virus.²¹²

In addition, other internal CREs first identified in a rhinovirus genome²⁶² have been subsequently identified in several other picornavirus genera and are often located within genomic coding sequences. These elements comprise stem-loop structures of variable lengths with a common apical loop containing an “AAAC” motif.³⁵⁶ In poliovirus, the CRE was shown to template the uridylation of 3B (VPg)—the small genome-linked protein—generating VPg-pUpU that functions as a protein primer for viral RNA synthesis.²⁸⁷ It is still debated whether both negative- and positive-strand RNA synthesis require CRE-dependent VPg uridylation or whether negative-strand synthesis can be primed by VPg.³⁵⁶ For HAV, a CRE has been identified in the 3D coding sequence by combined phylogenetic and thermodynamic predictive strategies, as well as mutational analyses.⁴⁰⁵ HAV CRE comprises

a larger stem loop with lower free-folding energy than CREs of other picornaviruses (see Fig. 19.8D), suggesting that there are likely to be important differences in the details of the VPg uridylation templated process for HAV. This is further supported by the apparent targeting of HAV 3A (and likely also the 3AB precursor of VPg) to mitochondrial rather than ER membranes.^{389,404} Evidence suggests that internal CREs function coordinately with the other *cis*-active RNA sequences and structures at the 5′ and 3′ termini of picornavirus RNAs to mediate RNA replication, presumably via ribonucleoprotein interactions that might be facilitated by the NTPase activity of 2C.³⁵⁶ Along these lines, interactions between HAV proteins 3C and 3AB with structures formed at both termini of the HAV genome have been documented *in vitro*.^{22,211,213,293}

Translation to Replication Switches

Interestingly, several cellular proteins may be involved in the finely tuned regulation of HAV RNA translation to replication switches, given that the same genomic molecule may be engaged in either process and that viral polysomes must be cleared of ribosomes before RNA replication can occur. Several sites for binding of GAPDH in the HAV 3′ UTR have been described.⁸⁵ Therefore, binding of GAPDH to both 5′^{51,331,408} (see earlier discussion) and 3′ termini of the HAV genome might promote the establishment of their close spatial proximity and have a role in RNA translation-replication switches. Viral protein and RNA syntheses also might be coordinated by the binding of full-length PCBP2 to the polypyrimidine tract of the 5′ UTR, whereas a truncated form of this cellular protein, which is cleaved by HAV 3C protease, has lesser affinity for HAV RNA, thus possibly facilitating the switch toward replication.⁴¹⁴ Similarly, another IRES *trans*-acting factor—PTB¹²⁹—is cleaved upon overexpression of HAV 3C protease, resulting in translation inhibition and hypothetically replication turn on.¹⁸⁹ However, the biological relevance of these observations is uncertain, because cleavage of these cellular factors has not been confirmed in HAV-infected cells, maybe as the result of low 3C quantities being present owing to protracted virus growth. HAV IRES was shown to be stimulated by polyadenylation in an acellular system, in contrast to FMDV IRES, suggesting a possible role for the 3′ terminal poly(A) residues of the genome in regulating HAV translation.²⁹⁰ More recently, poly(A)-binding protein (PABP) was shown to interact with polyadenylated HAV 3′ UTR.⁴¹³ This could hypothetically allow the bridging of HAV IRES via PABP-eIF4G interaction, resulting in the circularization of the RNA, such as in poliovirus and cellular mRNAs.¹⁵⁸ PABP also is targeted by HAV 3C protease in HAV-infected cells yielding a C-terminally truncated polypeptide that is able to bind to the polypyrimidine tract of HAV 5′ UTR. This might result in the curtailment of translation and the promotion of RNA replication.⁴¹³

Particle Assembly and Release

The mechanisms of HAV particle assembly are likely to differ significantly from those of other picornaviruses, consistent with detection of immature particle intermediates in HAV-infected cells.^{26,33} Recombinant vaccinia virus-mediated expression of HAV polyprotein or of capsid protein precursor P1-2A, on the one hand, and nonstructural protein precursor 2BC-P3, on the other hand, results in the assembly of

capsid-like structures.^{60,400} Using this system and C-terminally truncated P1-2A polypeptides, the presence of nonstructural protein 2A as a C-terminal extension of P1 was shown to be essential for both efficient 3C-mediated internal processing of P1-2A and the first step in virion morphogenesis.⁶⁰ The N-terminal domain of 2A is actually instrumental for assembling five copies of 1AB (VP4-VP2 or VP0), 1C (VP3), and 1D-2A (VP1-2A) polypeptides into pentamers, as a direct primary signal and/or as a consequence of its critical role in 3C-mediated processing of P1-2A, notably at the VP0/VP3 junction. However, whether pentamers are first assembled with five copies of uncleaved P1-2A precursors or cleaved VP0, VP3, VP1-2A polypeptides remains uncertain.^{33,60,300} (Fig. 19.8E). In other picornaviruses, the assembly of pentamers is initiated by N-terminally myristoylated VP4 protein.²⁸⁴ VP4 of HAV, however, is considerably smaller than VP4 proteins of other picornaviruses (at most 23 amino acids) and is not myristoylated, despite the presence of an internal consensus myristoylation signal.³⁶⁷ Twelve pentamers subsequently assemble into complete procapsids (empty capsids) or incorporate virion RNA to form provirions. N-terminal fusion of heterologous sequences to VP0 did not prevent the assembly of procapsids,⁴¹⁶ further suggesting that VP4 is not a critical determinant of HAV capsid assembly. The presence of the 3ABC precursor of 3C appears to improve P1-2A cleavage and particle assembly.²¹⁰ Cleavage at the VP1/2A junction begins after assembly of complete capsids and releases the mature VP1 capsid protein as well as larger VP1 polypeptides of intermediate lengths that, along with uncleaved VP1-2A, can still be detected in low amounts in procapsids and provirions.^{33,60,300} C-terminal residues of 2A are important for efficient cleavage at the VP1/2A junction,⁶⁰ which is likely carried out by a cellular protease that remains to be identified. Maturation cleavage of VP0 to release VP2 depends on RNA encapsidation, occurs by an as yet undetermined mechanism, and is associated with a conformational change to generate mature virions.²⁶ Cleavage of VP1-2A and VP0 into corresponding mature capsid proteins is accompanied by an increase in the specific infectivity of viral particles.^{26,60} The fate of 2A and VP4 in this process is unknown, and efforts to detect them in infected cells or virions have been unsuccessful.

Although a large proportion of the newly synthesized particles remains cell associated, there is extensive release of progeny virus into cell culture supernatant fluids by an as yet unknown mechanism. In polarized, human colonic epithelial cell cultures (Caco-2), release of virus occurs almost exclusively into apical supernatant fluids, mimicking the secretion of HAV across the apical canalicular membrane of the hepatocyte into the biliary system.²⁸ Interestingly, this process is largely blocked by an inhibitor of the cellular secretory pathway (Brefeldin A), suggesting that virus release may involve vectorial vesicular transport mechanisms.²⁸

MOLECULAR DETERMINANTS OF HEPATITIS A VIRUS ADAPTATION TO CELL CULTURE AND ATTENUATION *IN VIVO*

In contrast to the invariably transient nature of HAV infections in humans, infection of cultured cells, such as FRhK4,

BS-C-1, or MRC5, is typically not associated with any dramatic cellular injury and commonly leads to long-term persistence of the virus in cells. In addition, the HAV replication cycle in cell culture is typically slow. Most cell culture-adapted HAV variants attain maximal accumulation within 3 to 13 days but are not cytolytic. However, highly adapted variants of HAV that replicate rapidly (2–3 days) and cause cytopathic effects in cultured cells have been described.^{38,68,226,264} The development of a cytopathic effect depends on the interaction between particular HAV strains and cells at certain passage levels. In most cases, rapid cytolytic growth was selected by passaging HAV HM175 from persistently infected cells at intervals of 3 to 7 days.³⁸ Some strains of HAV, however, were cytopathic within several passages after direct isolation from human feces.²⁶⁴ Cellular injury appears to arise from the induction of apoptotic pathways leading to programmed cell death.^{38,130,131} Continuous passage of the virus in cell culture frequently results in a reduction in the ability of the virus to replicate in primates, resulting in attenuation of HAV virulence *in vivo*.³⁰²

Infectious cloned cDNAs of both wild-type and cell culture-adapted HAV strains are available and have made it possible to study the effects of mutations on the growth of HAV in cell culture and on its virulence for primates, as reviewed by Purcell and Emerson.³⁰⁸ The development of reporter subgenomic replicons lacking structural protein sequences that are capable of autonomous replication also have contributed to a better understanding of whether cell culture-adaptive mutations acted at the level of genome replication.⁴⁰⁷ Several concepts have emerged about mutations selected during adaptation or passage in cultured cells (Fig. 19.9): (a) mutations in gene 2B or 2C appear to be essential for the adaptation and efficient genome replication of HM175^{56,91,93,407} and probably other strains¹³⁵; (b) mutations in the 5' UTR (especially in the variable region around nucleotide 200) are not essential but enhance growth in a cell-type-restricted fashion^{75,115,116,117,137}—a cluster of mutations in the IRES appear to act by modulating translation efficiency in certain cells, perhaps through cell-type-specific interactions with accessory cellular proteins^{75,117,332}; and (c) other mutations or deletions throughout the genome, notably in 3A and 3D coding sequences, also contribute to enhanced growth of various HAV strains.^{135,136,201,264,308,415}

The effect of several cell culture-adapting mutations on HAV virulence has been evaluated following inoculation of tamarins (or occasionally chimpanzees) with virus or genomic RNA corresponding to wild-type HAV carrying cell culture-adapting mutations, or chimeras comprised of an attenuated HM175 variant and a virulent African green monkey strain. These experiments revealed that neither the 2B gene nor the 5' UTR (including the polypyrimidine tract and IRES) are implicated in HAV virulence,^{56,92,116,134,337} whereas the 2C gene (in particular, nucleotide 4563) plays a major role in virulence of HM175 in tamarins^{92,312} (see Fig. 19.9). The VP1-2A region also is an important determinant for HAV virulence, although not for adaptation to cultured cells.⁹² Genetic analysis of clinical viral isolates collected in patients experiencing self-limited acute, severe acute, or fulminant hepatitis failed to demonstrate clear associations of particular viral genome polymorphisms in the 5' UTR/IRES, 2B, or 2C sequences with disease phenotypes.^{111,113,237}

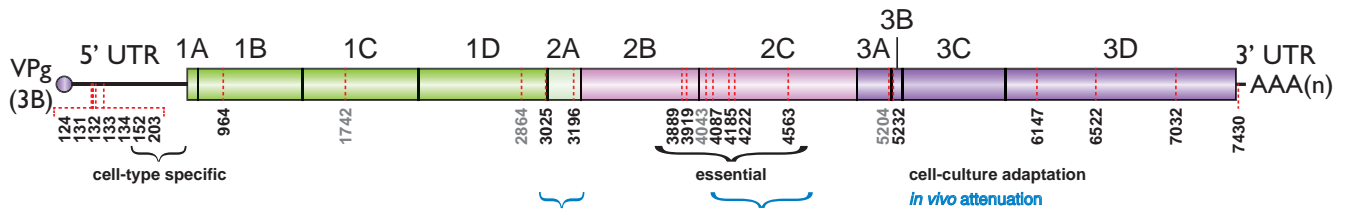


FIGURE 19.9. Substitutions existing between the cell culture–adapted HM175/p35 strain, used to produce inactivated hepatitis A virus (HAV) vaccines (GenBank accession number M16632), and its wild-type HM175 counterpart (GenBank accession number M14707) are represented by *dotted red lines* with their nucleotide position within the HAV full-length genome indicated below. Substitutions in the untranslated regions and those that result in amino acid changes are shown in *black font*, whereas silent changes are shown in *gray font*. Substitutions that are essential to cell culture adaptation and/or *in vivo* attenuation, or involved in cell-type modulation of HAV translation or replication (see text), are indicated by *curly braces*.

PATHOGENESIS AND HOST IMMUNE RESPONSES

Virus Replication *In Vivo*

Natural infection with HAV usually follows ingestion of virus from material contaminated with feces containing HAV. The sequence of events that begins with entry via the gastrointestinal tract and ultimately results in hepatitis has not been completely resolved. Consistent with the hypothesis that some cells of the gastrointestinal tract may be susceptible to HAV are studies in orally infected owl monkeys in which virus has been isolated from various levels of the gastrointestinal tract 3 to 35 days postinfection, and viral antigen has been identified by immunofluorescence in the epithelial cells of the intestinal

crypts and in cells of the lamina propria from the duodenum, jejunum, and ileum.¹³ Recognition that a transient rebound in fecal HAV RNA shedding has been observed in HAV-infected chimpanzees in the absence of a concomitant relapsing rise of virus-specific RNA in the serum or liver also suggests the possibility of an independent gastrointestinal replication compartment.²¹⁴ Whether this represents a *bona fide* site of replication of HAV remains to be clarified, however, because previous studies could not identify HAV antigen or genomic material in the intestinal mucosa of experimentally infected tamarins.^{204,253,254}

The primary site of replication for HAV is the liver, as demonstrated by virus detection in hepatocytes within days and even hours after infection.^{204,219,254,298} HAV is then released from

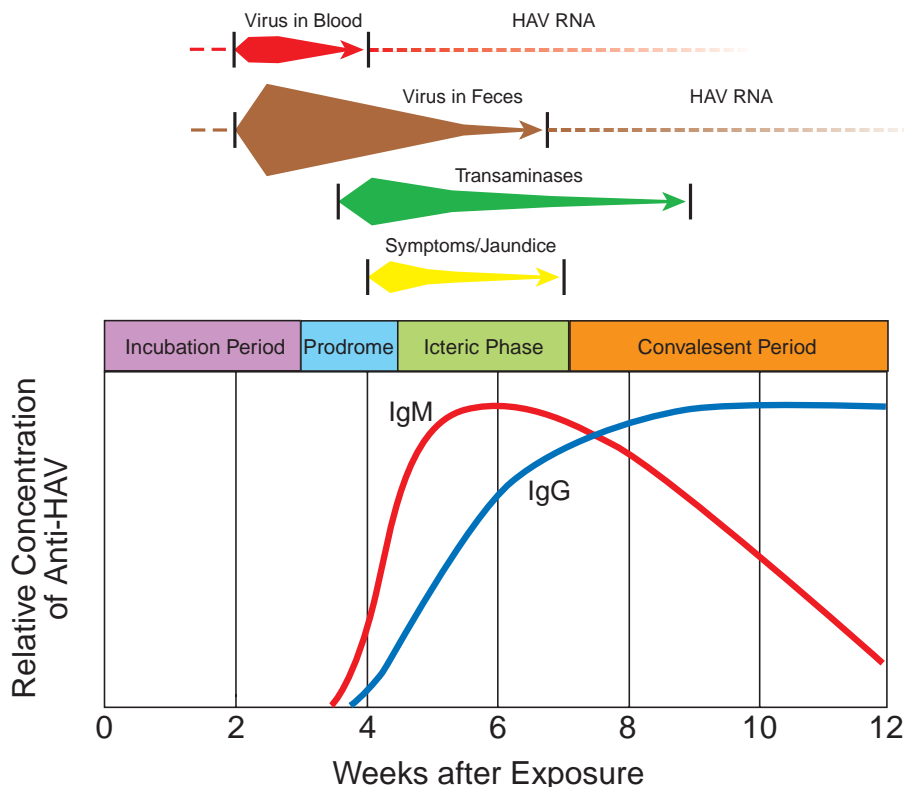
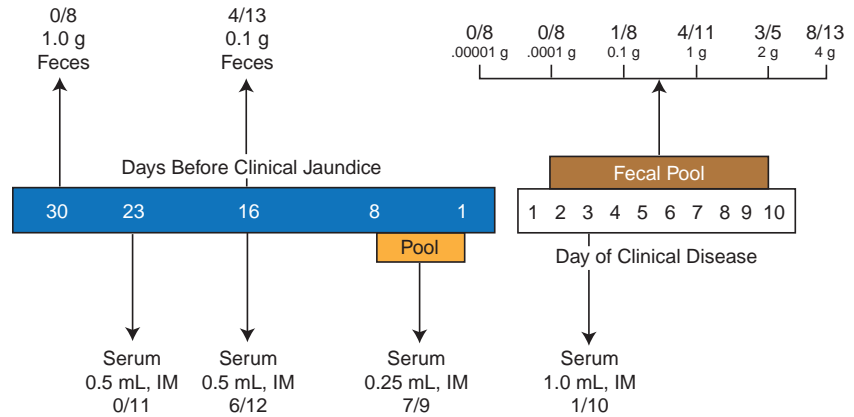


FIGURE 19.10. Clinically relevant immunologic and biologic events associated with hepatitis A virus (HAV) infection in humans divided into the four clinical stages. Following a period when virus in blood and feces has been shown to be transmissible, HAV RNA genomic material continues to be detected over a finite period of time (*dotted lines*) in some samples in the absence of documented infectivity (see text).

FIGURE 19.11. Relative levels of hepatitis A virus (HAV) infectivity observed for fecal and serum samples collected during the incubation period before clinical jaundice and the early stages of acute icteric hepatitis A. The number of subjects tested preceded by the number developing clinical hepatitis A is provided for each quantity of sample evaluated. In the absence of a diagnostic HAV assay in the 1960s to determine susceptibility, the number tested is not necessarily equivalent to the number of susceptible subjects; thus, the infectivity rate may be underestimated.²⁰⁵ IM, intramuscular.



infected hepatocytes into the bile canaliculi and passes into the intestine, after which it is detected in stools.^{308,328} According to this virus replication sequence, relatively high concentrations of HAV (up to 10^9 infectious virions per gram of stool)^{309,371} are shed in the feces before the alanine aminotransferase (ALT) level initially becomes elevated and before the onset of clinical symptoms or jaundice^{64,109,145,162,311} (Fig. 19.10). Communicability is highest during this interval.^{169,186,206,311} Early human studies have documented excretion of infectious HAV in stool specimens collected as early as 2 to 3 weeks before and up to 8 days after the onset of jaundice^{152,206} (Fig. 19.11). By contrast, transmission is severely curtailed during the acute and early convalescent stages of the disease, requiring increasingly larger quantities of fecal material to elicit an infection.^{206,207,233,386} In adults, viral particles are more often detected in the stools of jaundiced patients in contrast to patients who develop clinical symptoms without jaundice or who display no clinical disease.^{83,109} It is noteworthy that fecal shedding of presumably encapsidated HAV RNA can occur for several weeks after hepatitis develops, notably in infants,^{316,319,364} whereas recurrent shedding of HAV RNA has been observed in patients who have relapsing illness.³⁴⁷ In one study,³¹⁹ HAV RNA was detected in fecal samples from three preterm infants up to 4 to 5 months after they had developed acute hepatitis A. In addition to these observations, viral RNA and HAV antigen persist in the liver of infected chimpanzees more than 30 weeks after ALT levels have returned to normal and hepatic inflammation has resolved.²¹⁴

Virus is observed in blood (viremia) at about the same time extensive shedding of the virus in feces is occurring (see Fig. 19.10), although at levels generally 2 to 4 \log_{10} units lower than those in stool.^{35,55,219} Viremia precedes the appearance of clinical and laboratory evidence of hepatitis by at least 2 weeks, and its level tends to diminish dramatically during the period of liver enzyme elevation. In some situations, HAV appears to circulate in the blood enclosed in lipid-associated membrane fragments that may transiently protect the virus from neutralizing antibody.²²¹ In parallel with what has been observed in stool samples, HAV genomic material has been detected in the blood of infected patients and chimpanzees for much longer periods after onset of the disease than originally assumed (>10 weeks), although at concentrations several orders of magnitude lower than in acute-phase samples and always accompanied by

virus-specific antibody.^{35,112,219,280,410} The clinical significance of these observations has not been determined, because transmission or infectivity of blood components or fecal material containing HAV genomic material at these later time intervals has never been documented.

Evidence has been obtained for the replication of HAV in the oropharynx and salivary glands in orally inoculated chimpanzees and marmosets shortly after the appearance of virus in the blood; this could represent an early replicative event for the virus.^{55,298} Correspondingly, the presence of low titers of infectious virus in saliva collected from patients during the acute phase of the disease^{5,238,309} and in macaques infected by the intravenous route⁴ supports this conclusion. It is also possible, however, that the saliva may be contaminated by HAV entering the mouth during mastication through crevicular fluid that originates at the root–capillary interface, is released at the gingival surface, and contains serous material.¹⁶³ Finally, HAV antigen and/or genomic material has been observed in the kidney and spleen of infected owl monkeys, the biological relevance of which remains unknown.¹³

Immune Responses and Liver Disease

Early clinical studies in patients with acute hepatitis A reported either the absence of type I interferon³⁹⁵ or only transient increases in serum levels of interferons and type I interferon-induced human MxA protein^{180,228,411}—key components of the host innate immune responses that exert an antiviral effect on several viruses. Recently, a transcriptome analysis in HAV-infected chimpanzees revealed that the first evidence of an interferon response occurred at week 1 postinfection, as indicated by low-level increases of intrahepatic interferon-stimulated gene (ISG)-15 transcripts.²¹⁴ However, unlike many other genes, most ISG transcripts were up-regulated to a minimal degree and for only a few weeks in these HAV-infected animals. This paucity of type I interferon-induced ISG expression contrasts sharply with early and high-level induction of such genes in acute-resolving hepatitis C virus (HCV) infection. This is despite similar levels of viremia and 100-fold higher levels and much longer persistence of HAV RNA in the liver, and despite the presence of dsRNA in up to 10% of hepatocytes. The dsRNA corresponds to HAV replicative intermediates and forms (see Fig. 19.8D) and represents a potent pathogen-associated molecular pattern (PAMP) recognized by innate immune sensors.²¹⁴

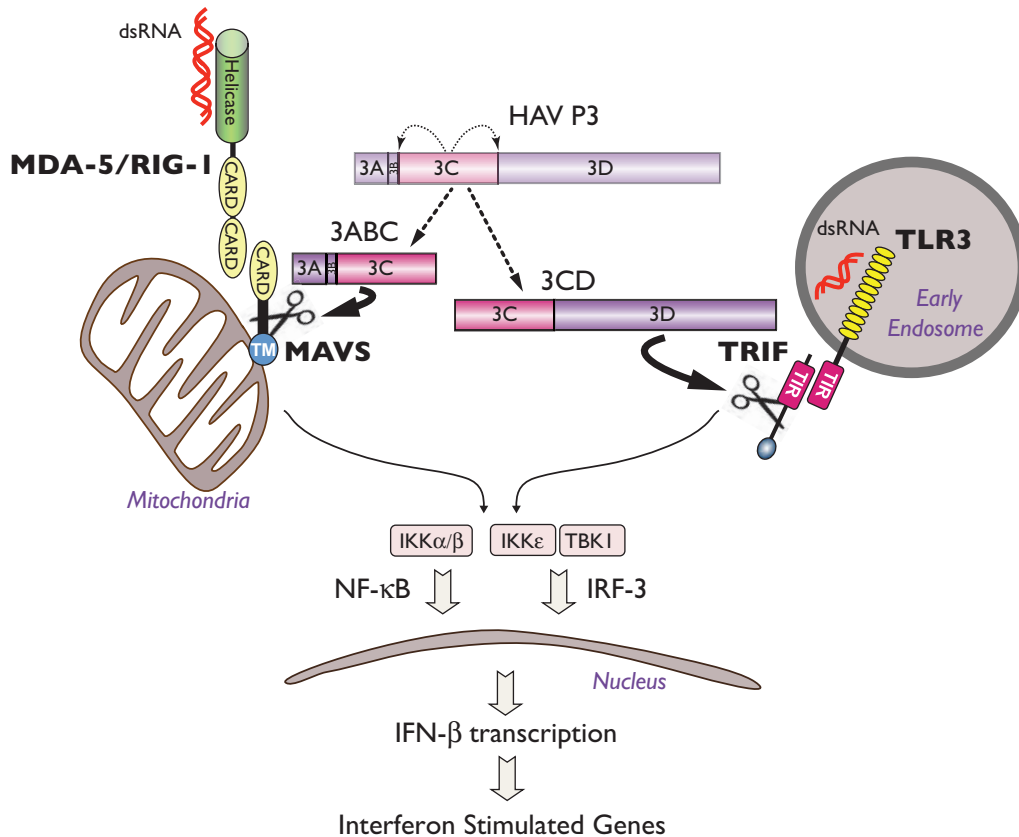


FIGURE 19.12. Interferon-activating pathways disrupted by hepatitis A virus (HAV) 3C protease precursors. Cytosolic HAV RNA (double-stranded RNA [dsRNA]) is most likely sensed by the cellular RNA helicase MDA-5, which interacts through caspase-recruitment domains (CARD) with the adaptor protein MAVS localized on the mitochondrial outer membrane. This induces the activation of kinases (IKK α/β , TBK-1, IKK ϵ) of the I κ B complex and subsequent activation of latent cytoplasmic transcription factors IRF-3 and NF- κ B that results in the synthesis of interferon (IFN)- β as well as interferon-stimulated genes (ISG). Within an endosomal compartment, sensing of dsRNA by toll-like receptor 3 (TLR3) induces the dimerization of TLR3 and subsequent recruitment of the adaptor protein TRIF through shared toll/interleukin-1 receptor (TIR) domains. This also results in the signalization of IFN- β and ISG synthesis. Two different 3C protease processing intermediates derived from HAV P3 polypeptide block these signaling pathways by directing cleavage of the adaptor proteins: polypeptide 3ABC cleaves MAVS⁴⁰⁴ and polypeptide 3CD possesses altered substrate specificity compared to mature 3C that allows cleavage of TRIF.³¹⁰ (Modified from a scheme courtesy of Prof. Stanley M. Lemon, University of North Carolina, Chapel Hill, NC, USA)

Altogether, these observations suggest that HAV has evolved strategies to evade protective host innate immune responses induced by dsRNA that can be summarized as follows (Fig. 19.12). Initial studies revealed that HAV infection blocked interferon- β expression in cell culture by inhibiting interferon regulatory factor 3 (IRF3) activation mediated by retinoic acid-inducible gene I (RIG-I) or melanoma differentiation-associated gene 5 (MDA5); signal transduction appeared disrupted between RIG-I/MDA5 and downstream kinases.^{37,102} It was further shown that this disruption operated through cleavage of the adaptor molecule (MAVS) of the MDA-5/RIG-I-mediated signaling pathway by HAV 3ABC polypeptide. A stable and proteolytically active 3C protease precursor, 3ABC is directed to the mitochondrial membrane where MAVS localizes, owing to the presence of a mitochondrial targeting transmembrane domain in 3A.⁴⁰⁴ In addition, evidence indicates that the 3CD protease-polymerase precursor processing intermediate disrupts toll-like receptor 3 (TLR3) interferon signaling by directing the cleavage of

the corresponding adaptor molecule TRIF at two noncanonical 3C cleavage sites.³¹⁰ Thus, HAV proteins interfere with the two major cellular antiviral response pathways (RIG-I/MDA5/MAVS and TLR3/TRIF), possibly facilitating HAV replication during the clinically silent early weeks of infection.

The mechanisms responsible for hepatocellular injury and virus clearance in hepatitis A are incompletely characterized. The presence of large quantities of virus in the liver and stools prior to the onset of hepatic inflammation argues against a major direct cytopathic effect of HAV. Furthermore, in the absence of extensive adaptation of HAV to cell culture, HAV infections *in vitro* are noncytopathic and cell metabolism is relatively unaffected. Clinical hepatitis coincides with the appearance of robust humoral¹⁸³ and cellular (see below) immune responses. Because of this, the possibility of humoral immunopathogenesis has been examined, although no complement-dependent, antibody-mediated cytolytic activity has been demonstrated against HAV-infected cells.¹¹⁸ Circulating

immune complexes containing HAV and HAV-specific antibodies (primarily immunoglobulin M [IgM]) have been found during infection.^{17,39,246} However, although immune complexes may be present in the kidney,¹⁶⁴ immunoglobulin and complement deposits were not found at the sites of liver cell damage, and resolution of disease occurred at a time when antibody levels were rising and hepatitis A antigen could still be easily detected in the liver.²⁴⁵

Natural killer cells capable of lysing HAV-infected cultured cells have been isolated from patients with acute hepatitis A,¹⁶ suggesting that nonspecific immune mechanisms involving natural killer cells may play a role in hepatocellular damage. Clinical studies have revealed that lymphocytes recovered from the liver and blood of patients with acute hepatitis A contain human leukocyte antigen (HLA)-restricted, HAV-specific, CD8+ cytotoxic T cells that produce interferon- γ in response to HAV-infected cultured cells.^{108,209,239,379,380} This is consistent with the pathology of hepatitis A being largely the result of cell-mediated immune responses to the infection, as reviewed by Vallbracht and Fleischer³⁷⁸ and Siegl and Weitz.³⁴³ Highly conserved HAV-specific epitopes restricted by HLA-A2 and other alleles were recognized by CD8+ T cells collected from patients with acute, postacute, and resolved HAV infections.³²⁹ Most patients showed a multispecific T-cell response against at least two epitopes located within HAV VP1, VP2, VP3, 2B, 2C, and 3D regions of the HAV polyprotein, with the response directed against the 3D epitope being reproducibly immunodominant in HLA-A2 patients. These results and the existence of a single serotype suggest that immune escape in HAV infection does not appear to play a major role in the pathogenesis of this infection.

Although a dominant role for CD8+ T cells in both liver injury and control of HAV infections in humans has been postulated, a recent study in HAV-infected chimpanzees pointed to a potentially significant role for CD4+ T cells in HAV clear-

ance. CD4+ T cells produced multiple cytokines when viremia first declined, rebounded with a transient relapse of HAV fecal shedding, and remained detectable for many weeks until the HAV genome was cleared from the liver, whereas CD8+ T-cell frequency and effector functions did not appear to be as closely associated with virus control, at least in chimpanzees.⁴¹⁷ It is noteworthy that viremia declines rapidly following the appearance of the humoral response, suggesting that neutralizing antibodies also play a major role in clearing viremia.²²⁵ Correspondingly, a transcriptome analysis in HAV-infected chimpanzees revealed a remarkable activation of genes involved in B-cell development at 3 to 4 weeks postinfection.²¹⁴

EPIDEMIOLOGY

Hepatitis A is one of the most common causes of infectious jaundice in the world today and is frequently associated with recurrent epidemics. Humans and other primates are the only natural reservoirs for HAV. Based on incidence rates from confirmed acute HAV cases, the geographic distribution of HAV infection appears to be changing as countries improve their level of sanitation and personal hygiene (D. Shouval, personal communication, 2011)¹⁷⁸ (Fig. 19.13). In populations in which living conditions are crowded and sanitation is nonexistent or inadequate, prevalence studies reveal that infections occur at an early age and that close to 100% of children acquire immunity during the first decade of life^{110,141,179,257,361} (Fig. 19.14). In contrast, in modern urban societies or in developing nations where improvements in sanitation and personal hygiene have delayed infection, a reduction in anti-HAV prevalence among younger persons has been found.^{142,177,179,257,324,360} Thus, large segments of the population remain susceptible, and epidemics can occur after chance introduction of the virus to such an area. This has resulted in a paradoxical increase in the number



FIGURE 19.13. Estimated global hepatitis A virus incidence levels based on age-seroprevalence rates by country or region from samples that are representative of the general population, 2005. (Courtesy of Prof. Daniel Shouval, Hadassah-Hebrew University Hospital, Jerusalem, Israel.)

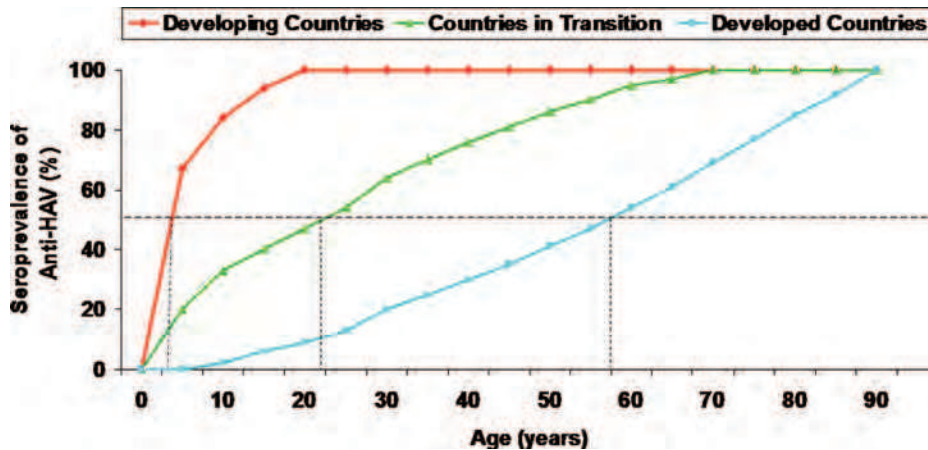


FIGURE 19.14. Age-specific hepatitis A virus seroprevalence curves in developed and developing countries and those in transition demonstrating age at which 50% of the people have immunity.¹⁷⁹

of cases observed in adults as they escape early childhood infection where disease manifestations are mild, and transition to adulthood where clinical disease is more overt and severe. An example of this changing epidemiology was the large epidemic that occurred in Shanghai in 1988 with more than 300,000 cases recorded.⁴⁰⁶

In the United States, the incidence of acute, symptomatic cases of HAV declined from 12.8 cases/100,000 population in 1980 to 0.9 cases/100,000 in 2008⁴⁹ (Fig. 19.15). At least a part of this decline is attributable to implementation of vaccine programs in high-risk populations (see the Prevention and Control section). The actual incidence of disease throughout the United States is estimated to be much higher because (a) many persons contract such a mild form of hepatitis that they do not seek treatment, and (b) physicians underreport hospitalized cases, although deaths are usually reported.

The incubation period for hepatitis A ranges from 10 to 50 days, with a mode of approximately 1 month, regardless of the route of infection.^{276,288,289} Higher doses of virus, however,

lead to a shorter incubation period.^{289,309} When two cases occur less than 14 days apart during a point source epidemic, they are more likely to be coprimary, rather than secondary, cases. Correspondingly, cases occurring more than 60 days apart presumably result from secondary spread of the infection.^{207,386} Although the median incubation periods for viral hepatitis A through E are distinctly different, there is considerable overlap; thus, knowledge of the incubation period is rarely useful in determining etiology. In addition, the patient may not know the time of exposure.

Of those with overt hepatitis A, from 21% to 53% are hospitalized, with the lowest being among children and the highest among persons 60 years of age or older. More than 70% of the adult clinical cases are jaundiced. In the United States, an estimated 100 persons die of fulminant hepatitis annually. Table 19.2 provides mortality data by age group for hospitalized patients with icteric hepatitis A increasing from 0.23% for those younger than 30 years to 1.8% to 2.1% for those older than 49 years. An overall case fatality rate of less than 0.015%

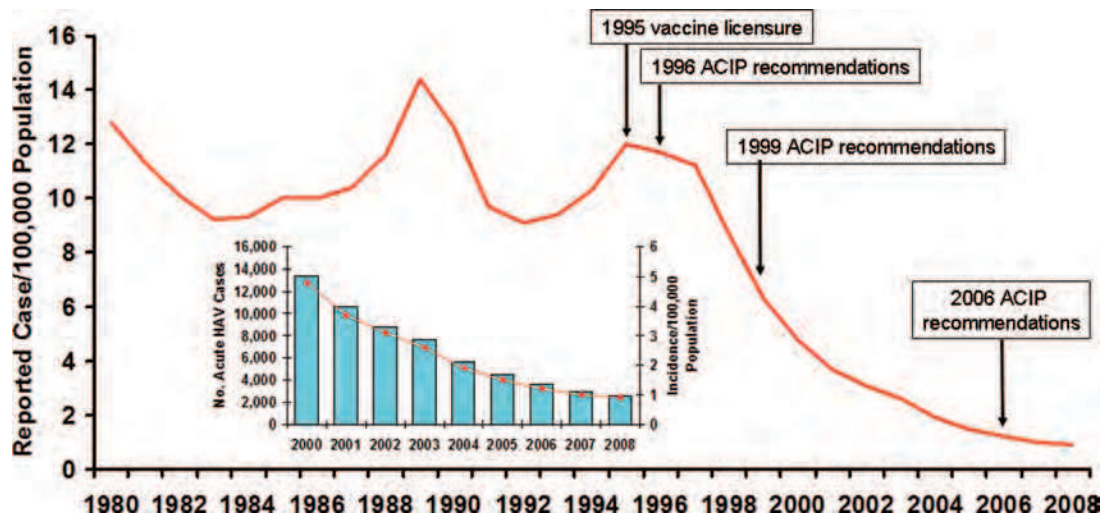


FIGURE 19.15. Incidence of acute hepatitis A per 100,000 population, United States, by year and Advisory Committee on Immunization Practices (ACIP) recommendations.⁴⁹ The inset covers the years 2000 to 2008 and shows the number of acute hepatitis A virus cases and the incidence/100,000 population in more detail.

TABLE 19.2 Predicted Outcome After an Infection With Hepatitis A Virus^a

Parameter	Predicted outcome	
	Children (<5 y) (%)	Adults (%)
Inapparent infection	80–95	10–25
Anicteric or icteric disease	5–20	75–90
Complete recovery	≥99	≥98
Chronic disease	None	
Mortality rate (hospitalized cases)		
<30 y	0.23	
30–49 y	0.3–0.6	
>49 y	1.8–2.1	

^aRefer to the text regarding atypical manifestations of hepatitis A.

was recorded among 310,746 cases of hepatitis A that occurred in Shanghai in 1988.⁴⁰⁶ This translated to a mortality rate of 0.3% to 0.6% for the 8,647 patients who were hospitalized, of whom 90.8% were between the ages of 20 and 40 years. Some of these patients had an underlying hepatitis B infection.

As expected, the presence of HAV in feces facilitates virus dissemination and accounts for the person-to-person mode of spread of hepatitis A. In the United States, the epidemiologic characteristics of acute hepatitis A cases reported to the Centers for Disease Control and Prevention (CDC) from 2001 to 2007 changed annually⁴⁷ to include household or sexual contacts with a person who had hepatitis A, international travel, men who had sex with other men, illicit drug users, and children and employees associated with daycare centers or with contacts of these individuals (e-Fig. 19.3). No risk factor was disclosed in more than 50% of the cases, although HAV-contaminated food was often suspected. Percentages are found to vary each year; however, a trend exists toward proportionally more cases being attributed to international travel, especially to Mexico and Central/South America, although sexual or household contact also is frequently implicated.

In contrast to this person-to-person mode of spread, sudden, explosive epidemics of hepatitis A within communities or confined populations usually result from fecal contamination from a single source,⁶³ such as drinking water,²⁶⁶ milk, or food.¹⁰⁴ Contamination of food can occur during cultivation, harvesting, processing, distribution, or preparation. Most food handlers with hepatitis A do not transmit HAV to patrons; when this occurs, however, the incriminated food is usually an item that is served uncooked or without further cooking (e.g., sandwiches, salads, pastries). Recent sources of large outbreaks have implicated frozen raspberries and strawberries, as well as iceberg lettuce.¹⁰⁴ An example of foodborne outbreaks in three states of the United States following the consumption of contaminated green onions³⁹³ is illustrated by an increase in the number of cases reported in 2003 (see e-Fig. 19.3). Several other foodborne outbreaks of hepatitis A have resulted from the consumption of raw or inadequately cooked oysters and clams obtained from water polluted with sewage.^{147,321,406} The role of mollusks has been that of virus concentrators of water polluted by sewage and not as a source for virus replication. During feeding, physiologically active bivalves (e.g., oysters or

mussels) can filter up to 10 gallons of water per hour over a short period during which HAV can be concentrated at least 100 times and persist for about 7 days.⁹⁶ As expected, non-bivalve shellfish (e.g., lobsters, shrimp) do not impart the same risk.

Hepatitis A molecular epidemiology is being widely and successfully used to characterize sources of infection and transmission patterns.²⁷² These molecular studies can provide strong epidemiologic links of disease transmission between persons who travel to an endemic area^{316,318,372} or during investigations of foodborne outbreaks.⁸ Such analyses are facilitated by the use of nucleic acid sequencing and phylogenetic evaluations to identify unique strains and determine genetic relatedness. Current phylogenetic analyses generally use a 315-bp nucleotide fragment from the 1D-2AB region of the genome because of its high degree of variability.²⁷² Population-based studies provide evidence that HAV is often transmitted by networks of persons who display similar risk factors for infection.³⁷²

As discussed previously, HAV communicability is apparently highest during the clinically silent incubation period when virus replication reaches a peak^{169,186,206,311} (see Fig. 19.11). By the time the patient seeks medical attention or the transaminases reach a peak and the patient becomes jaundiced, clinically relevant viremia also has significantly diminished. In one study, an acute-phase serum sample from a human with clinical disease was a million-fold less infectious when inoculated into chimpanzees than a stool sample collected on the same day,³⁰⁹ and serum samples from three other strains of HAV were at least 1,000 times less infectious. Despite these observations, HAV transmission through blood remains a rare but potential cause of posttransfusion hepatitis.^{19,148,164,219} In the past, the problem was amplified in neonatal intensive care units where infants developed a covert infection after receiving blood components from an infected donor. They subsequently transmitted the virus to healthcare workers and family members, presumably by the fecal–oral route.^{15,200,279,319} The rarity of transfusion-associated hepatitis A in adults probably can be attributed to several closely associated factors. First, the viremic stage is short, and the concentration of HAV in the blood is relatively low, as previously reviewed. The absence of an HAV carrier state²⁰⁷ also contributes to the rarity of HAV transmission through blood. Thus, for transmission to occur, an infected donor would have to donate blood to a susceptible recipient during a very restricted interval. In addition, because most recipients are given more than 1 unit of blood, an anti-HAV-positive unit may be transfused concurrently with the HAV-positive unit, thereby neutralizing the virus. Several of these scenarios often do not apply to neonates, which is why the risk is greater in this group. Current blood bank practices do not include specific screening of prospective donors for evidence of active HAV infection. However, nucleic acid testing for HAV is being done by the plasma industry, and whereas there is a wide variation in pool sizes and sensitivities, the general conclusion is that the HAV nucleic acid–reactive yield is relatively low with a range of 1 positive donation in 120,000 to 1,805,500 donations (S. Stramer, personal communication, 2011).

From 1989 to 1995, several outbreaks of hepatitis A occurred among hemophiliacs in Europe, South Africa, and the United States who received solvent- and detergent-treated factor VIII.^{243,315} Implicated lots were devoid of appreciable amounts of neutralizing anti-HAV and contained HAV

genomic material whose genetic sequence was identical to that obtained from infected patients. The solvent or detergent treatment had no effect on infectivity of this nonenveloped virus (see the Morphology and Properties of Hepatitis A Virions section) but could have been responsible for dispersing HAV in the product by releasing the virus from a nonneutralizable, lipid-associated environment. Effective heat-inactivation procedures, a preference for recombinant factor VIII, and vaccination have significantly reduced this risk.

Little evidence is found for transmission of HAV by exposure to urine or the nasopharyngeal secretions of infected patients.^{1,124,154,187,236,277} Other identified potential sources of exposure to HAV are nonhuman primates, usually chimpanzees, that have infected caretakers and other zoo personnel in close contact with them.^{74,81,159} In these situations, vaccination has been highly effective.

CLINICAL FEATURES

The outcome of hepatitis A can be extremely variable depending on the age when acquired (see Table 19.2). Patients with inapparent or subclinical hepatitis have neither symptoms nor jaundice. These asymptomatic cases previously went unrecognized but now can be identified by detecting biochemical or serologic alterations in the blood. Other patients can develop anicteric hepatitis or icteric hepatitis. Symptoms ranging from mild and transient to severe and prolonged can accompany anicteric or icteric hepatitis. Most patients recover completely; however, some develop fulminant hepatitis and die. As noted in Table 19.2, the disease is milder in children than in adults, complete recovery is the rule, and chronic disease has not been observed.²¹⁶ Hospitalization for and death caused by fulminant hepatic failure is age dependent. Whereas two-thirds of the clinical cases occur in children and young adults, more than 70% of the deaths are observed in patients older than 50 years. The mortality rate is considerably higher among patients with chronic hepatitis B or C who are superinfected with HAV,^{144,196,382,406} primarily when the underlying liver disease is advanced.

Symptoms and Signs

A typical course of acute hepatitis A can be divided into four clinical phases: (a) incubation or preclinical period (time between exposure and the first day of symptoms), (b) prodromal or preicteric stage, (c) icteric phase, and (d) convalescent period (see Fig. 19.10).

During the incubation phase, the patient remains asymptomatic despite active replication of the virus. A short prodromal or preicteric phase, varying from a few days to more than a week, precedes the onset of jaundice. In more than half of patients, the prodromal state typically is characterized by anorexia, fever, fatigue, malaise, myalgia, nausea, and vomiting. In hepatitis A, the transition from well-being to acutely ill occurs abruptly (within a period of 24 hours) in more than 60% of the cases, whereas the onset is more insidious in hepatitis B. Fever higher than 100.5°F is more common in acute hepatitis A than in acute hepatitis B.³⁸⁶ Diarrhea, nausea, and vomiting are more frequent in children than in adults.²²⁰ Weight loss of 2 to 10 pounds is common as a result of the anorexia that accompanies disorders of taste and smell.^{157,349} Older children

and adults often complain of right upper quadrant pain or discomfort as a consequence of hepatomegaly.³⁸⁶

The icteric phase of acute viral hepatitis is ushered in by the appearance of golden-brown urine caused by bilirubinuria, followed one to several days later by pale stools and yellowish discoloration of the mucous membranes, conjunctivae, sclerae, and skin. This icteric phase begins within 10 days of the initial symptoms in more than 85% of HAV cases,⁴²⁰ prompting patients to seek medical attention. Fever, if present, usually subsides after the first few days of jaundice.

Physical examination of the patient with a typical case of acute disease reveals the presence of jaundice accompanied by tenderness to palpation or percussion of the liver, which may be enlarged. Measurement by percussion is essential, because a reduction in size in a patient whose condition is deteriorating often heralds massive necrosis. Approximately 5% to 15% of patients have splenomegaly; however, generalized adenopathy is not a component of the disease and should trigger further investigation. Palmar erythema and spider angiomas may be observed.

Several atypical manifestations of hepatitis A have been reported, including the development of cholestasis, the possible induction of type 1 autoimmune hepatitis, extrahepatic manifestations, and relapsing hepatitis.^{188,325} HAV-infected patients who present with cholestatic hepatitis A initially have pruritus, fever, diarrhea, and weight loss. The serum bilirubin usually remains above 10 mg/dL for more than 12 weeks, whereas the ALT level is often less than 500 IU/L. Resolution of the disease is slow; however, recovery is complete within 2 to 8 months.^{127,334} HAV also has been associated with acute acalculous cholecystitis with jaundice on rare occasions^{268,283} and to trigger the onset of autoimmune hepatitis. Extrahepatic manifestations of hepatitis A are unusual,⁶⁹ although transient rashes are observed infrequently, as are arthralgia, arthritis, hemolysis, leukocytoclastic vasculitis, membranoproliferative glomerulonephritis, and pancreatitis.^{164,174,399} Finally, relapsing hepatitis occurs in 3% to 20% of the cases of acute hepatitis A. Recrudescence of the disease, usually less severe than the original episode, occurs 4 to 15 weeks after the initial symptoms have resolved. In some patients, only biochemical changes are observed. IgM anti-HAV either reappears or increases in titer, and HAV genomic material is detected in feces and serum. More than one relapse can occur, and enzyme elevations can persist for 5 to 12 months, although chronic sequelae are not observed.

Occasionally, more extensive necrosis of the liver occurs during acute viral hepatitis A, leading to severe impairment of hepatic synthetic processes, excretory functions, and detoxifying mechanisms. This entity, designated *fulminant hepatitis* if hepatic encephalopathy occurs during the first 6 to 8 weeks of illness or within 1 to 4 weeks after jaundice, is characterized by the sudden onset of high fever, marked abdominal pain, vomiting, and jaundice followed by the development of encephalopathy associated with deep coma and seizures.²⁸² A jaundice-to-encephalopathy time >7 days is an important prognostic indicator when determining the need for a liver transplant. This, however, is only one of several factors that are employed to determine survival in cases of fulminant hepatitis A. Other variables include coagulopathy (international normalized ratio [INR] >3.85), age (<10 or >40 years), elevated serum bilirubin and creatinine levels, intubation, and the use of

pressors.^{281,365} Although ascites, a bleeding diathesis, and decerebrate rigidity can lead to death in 70% to 90% of patients, in at least one study,³⁶⁵ the overall survival rate in fulminant hepatitis A among those spontaneously recovering and not requiring a liver transplant was 55%. Fortunately, fulminant liver failure is rare, occurring in less than 1.0% of the icteric patients hospitalized for acute viral hepatitis A.

Host factors that might influence the severity of hepatitis A infection are not well known. Viremia in patients with fulminant hepatitis A was found to be significantly lower than in classical forms of the disease, raising the hypothesis that fulminant hepatitis A might be associated with a strong immunological response.³¹⁴ A recent study found a relationship between HAV-induced severe liver disease and a six amino acid insertion in HAVcr1/TIM-1, a potential functional receptor for HAV^{52,198} (see the Infectious Viral Life Cycle section). This gene polymorphism appeared to offer protection against the development of atopic diseases while predisposing the individual to severe HAV-induced disease. A possible explanation for this may stem from observations that the long form of TIM-1 binds HAV more efficiently and that natural killer T cells expressing the long form of TIM-1 are more cytotoxic for HAV-infected hepatocytes.¹⁹⁸ In addition, it was found that the association of IgA with HAVcr1/TIM-1 enhances the interaction of HAVcr1/TIM-1 with HAV, suggesting a potential role for IgA/HAVcr1 interaction in the pathogenesis of HAV by enhancing viral entry in cells expressing low levels of HAVcr1/TIM-1.³⁶²

Laboratory Features

The initial laboratory evaluation of the patient with acute hepatitis should include a biochemical liver panel, complete blood count (CBC), and INR. Serum ALT and aspartate aminotransferase (AST) concentrations are highly sensitive indicators of hepatocellular injury. They provide a quantitative assessment of acute damage sustained by the liver, although the eventual outcome cannot be predicted from the level attained. Within hepatocytes, AST is found in mitochondria (80%) and the cytosol (20%), whereas ALT is limited to the cytosol, which may partially explain the aminotransferase patterns seen in acute hepatitis (i.e., ALT > AST). Damage to the plasma membrane leading to ionic and water shifts³⁷⁵ is followed by ballooning degeneration of hepatocytes (Fig. 19.16) and leakage of ALT from the cytosol. Consequently, high ALT:AST ratios (>1.4) are observed in uncomplicated acute viral hepatitis cases. Exceptions include those situations in which severe tissue necrosis develops, resulting in the release of mitochondrial AST into the blood. In these situations, the lactic dehydrogenase (LDH) enzyme also is significantly elevated. For the jaundiced patient, total serum bilirubin usually remains below 10 mg/dL, although levels of 20 mg/dL are occasionally observed. After reaching a peak in 1 to 2 weeks, the rate of decline is more gradual, with resolution occurring within 6 weeks of onset in most patients.³²⁰ As liver injury improves, discordant findings between serum and urine bilirubin can occur when elevated levels of serum bilirubin may not be accompanied by bilirubinuria owing to the *de novo* synthesis of a conjugated form of bilirubin (delta bilirubin) that is irreversibly (covalently) bound to albumin,³⁸⁸ thereby impairing its excretion in the urine.

Certain serum proteins (e.g., albumin, prothrombin, and fibrinogen) are synthesized exclusively or predominantly by the liver and can be affected by HAV injury. However, because

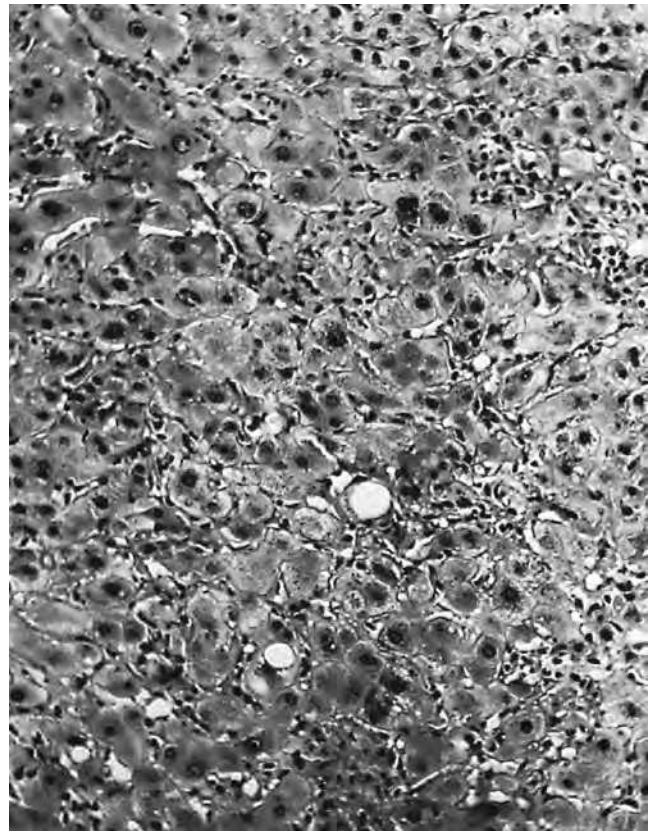


FIGURE 19.16. Classic acute viral hepatitis. Lobular disarray characterized by anisocytosis, anisonucleosis, ballooning, and Kupffer cell hypertrophy (H&E, $\times 190$). (From Ishak KG. Light microscopic morphology of viral hepatitis. *Am J Clin Pathol* 1976;65(Suppl 5):787–827, with permission.)

the intravascular half-lives of the various plasma-clotting factors are relatively short (hours to days), measurement of both the INR and partial thromboplastin time may more accurately reflect early and potentially serious derangements in liver function than does measurement of serum albumin, which has a half-life of 3 weeks.

Generally, the patient with typical acute viral hepatitis has a normal or slightly reduced number of neutrophils and a relative lymphocytosis.¹⁵⁵ When the white blood count is more than 12,000/mm³, a more serious form of the disease should be anticipated. Although mild to moderate reduction in red blood cell survival is frequent in acute viral hepatitis,¹⁹⁵ the hematocrit and hemoglobin remain within normal limits. Occasionally, however, hemolytic anemia occurs, often in association with glucose-6-phosphate dehydrogenase deficiency.^{132,173,240} Rarely does agranulocytosis, thrombocytopenia, red cell aplasia, or aplastic anemia accompany acute viral hepatitis.^{69,97,271}

Histologic Findings

In acute hepatitis A, both degenerative and regenerative parenchymal changes coexist with a diffusely distributed accumulation of mononuclear inflammatory cells.¹⁷⁶ Lobular disarray (see Fig. 19.16) is accompanied by swollen hepatocytes (ballooning degeneration) with indistinct plasma membranes, enlarged nuclei, and a featureless cytoplasm except for some cytoplasmic remnants condensed around the nuclei (e-Fig. 19.4). Acidophilic

degeneration (apoptosis) also occurs with cells becoming shriveled and angulated while displaying increased eosinophilia. Both types of degeneration result in the migration of affected cells or cellular debris into the spaces of Disse and eventually into the sinusoids, where they are surrounded by Kupffer cells and undergo phagocytosis and digestion. Activation of Kupffer cells in the sinusoids and macrophages in the portal tracts results in marked hypertrophy and hyperplasia of these cells. The portal tracts are enlarged as a result of edema accompanied by a moderate to heavy infiltration of lymphocytes, although neutrophils and eosinophils can be present. Plasma cells are uncommon. Inflammatory cells can spill over into the adjacent parenchyma, a process called *interface hepatitis*; however, less than 25% of the limiting plate is involved. During the recovery stage, regeneration is prominent, as manifested by anisonucleosis, the presence of numerous bi- and trinucleated cells, and mitosis. The inflammatory response diminishes, although Kupffer cell hypertrophy persists for a few weeks. The damaged hepatic tissue is usually restored within 8 to 12 weeks.

In 5% to 10% of patients, focal necrosis gives way to zones of necrosis that bridge portal tracts or join portal areas to terminal hepatic veins.^{36,103} When this process involves entire lobules or adjacent lobules, the terms *submassive* (involving the central and middle zones of lobules) or *massive* (involving

entire lobules) are applied. The latter is characterized further by the proliferation of cholangioles in the periportal areas infiltrated by neutrophils (Fig. 19.17). Confluent hepatic necrosis is potentially a progressive lesion that can lead to fulminant hepatitis and death, which appears to be inevitable when necrosis involves more than 65% to 80% of the total hepatocyte fraction.^{121,335} Conversely, complete regeneration of the liver is observed in survivors.^{53,194}

In rare cases of acute viral hepatitis A accompanied by jaundice, severe centrilobular cholestasis associated with disruption of bile canaliculi and periportal inflammation is observed that sometimes resembles chronic hepatitis, which never occurs in hepatitis A. This appears to be more common in hepatitis A than in hepatitis B or C but is less common than in acute hepatitis E.^{139,334} Plasma cells are often prominent in this condition.

Just as it is difficult to determine the cause of acute hepatitis without serologic data, the pathologic changes in acute-phase liver tissue obtained from humans with different forms of viral hepatitis are qualitatively similar.^{103,176} In general, liver tissue from patients with acute hepatitis A has less conspicuous parenchymal changes (including focal necrosis, Kupffer cell proliferation, acidophilic bodies, and ballooning degeneration) and less occurrence of steatosis than does tissue from patients with acute hepatitis C; however, necrosis and mononuclear cell inflammation of the periportal region are more evident than in biopsies from patients with acute hepatitis B.^{2,103,208} In addition, the limiting plate is more likely to be disrupted in hepatitis A.

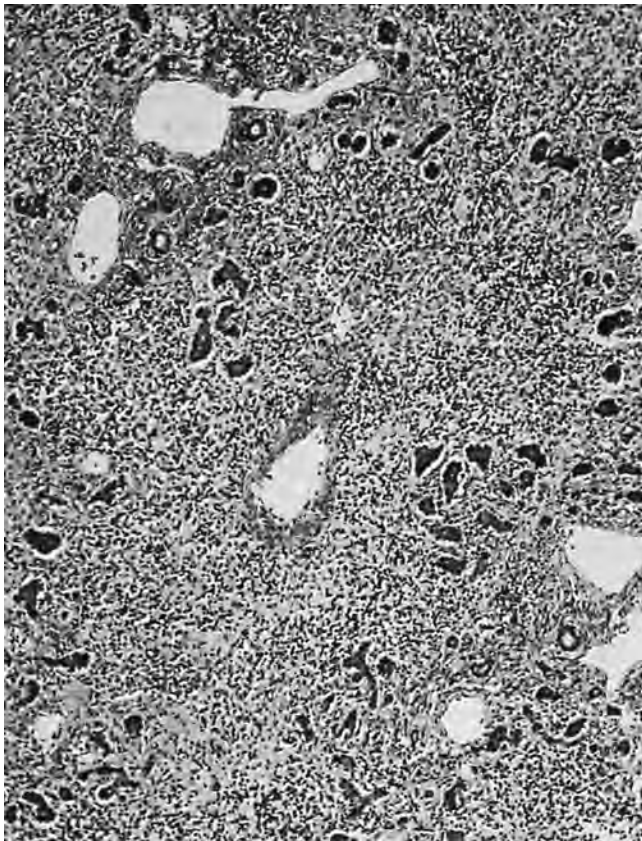


FIGURE 19.17. Acute viral hepatitis with massive necrosis. All liver cells have “dropped out.” Note proliferating periportal cholangioles and infiltration of stroma and portal areas with inflammatory cells (H&E, $\times 73$). (From Ishak KG. Light microscopic morphology of viral hepatitis. *Am J Clin Pathol* 1976;65 (Suppl 5):787–827, with permission.)

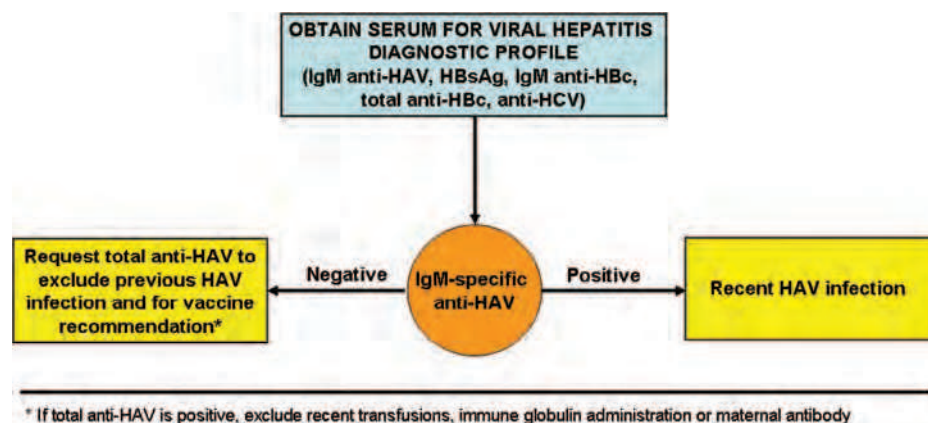
DIAGNOSIS

Because the agents of viral hepatitis often cannot be distinguished clinically and HAV superinfection of hepatitis B and C carriers can occur, serologic means are required for identification. The most favored acute viral hepatitis profile includes assays for IgM anti-HAV, hepatitis B surface antigen (HBsAg), IgM antibodies to the hepatitis B core antigen (IgM anti-HBc), and antibodies to HCV (anti-HCV). Idiosyncrasies of the assays, however, often make it desirable to perform ancillary tests that will confirm a positive response. Figure 19.18 is the approach used to diagnose acute hepatitis A, determine immunity, and assess the need for vaccination.

Amplification and sequencing of HAV RNA is being used to determine the genetic relatedness of isolates to identify infection sources and transmission patterns during epidemiologic case investigations^{80,172,315,316,318,372} (see the Epidemiology section). Immunocytochemical staining and physicochemical studies have been used to identify HAV and viral antigen in liver tissue from infected primates obtained early in the course of the disease.^{169,197,214,254,269,339} None of these methods, however, is practical in a clinical setting; they are not necessary unless incontrovertible proof for the presence of infectious virus or its source is needed.

To establish the diagnosis of acute or recent hepatitis A, blood samples are examined for the presence of acute-phase IgM-specific anti-HAV^{39,77,232} (see Figs. 19.10 and 19.18). Rarely, this test may be nonreactive when the patient first seeks medical assistance but will invariably become positive within the next 7 to 10 days.²³⁰ Conceptually, the *total* anti-HAV test also will be positive when the IgM anti-HAV assay is positive,

FIGURE 19.18. Serologic diagnosis of acute hepatitis A and immunity. IgM anti-HAV (immunoglobulin M antibodies to the hepatitis A virus); HBsAg (hepatitis B surface antigen); IgM anti-HBc (immunoglobulin M antibodies to the hepatitis B core antigen); total anti-HBc (immunoglobulin G [IgG] and IgM antibodies to the hepatitis B core antigen); anti-HCV (antibodies to the hepatitis C virus); total anti-HAV (IgG and IgM antibodies to the hepatitis A virus).



because it detects both immunoglobulin G (IgG) and IgM antibodies. Should it be negative, a false-positive IgM anti-HAV result should be entertained. The IgM anti-HAV antibody rapidly increases in titer over a period of 4 to 6 weeks and then declines to nondetectable levels within 3 to 6 months in most patients (range of <30 to >420 days), with persistence for more than 200 days in 13.5% of cases.¹⁹⁰ In more than 85% of patients, the liver enzymes will be normal before or at the time of disappearance of IgM anti-HAV. IgG anti-HAV eventually replaces the IgM antibody, persists for years after infection, and appears to confer lifelong immunity³⁴⁸ (see Fig. 19.10).

The total anti-HAV assay is used primarily to determine the immune status of an individual after vaccination or to assess risk in a person traveling to an endemic region or working in a high-risk area. In the absence of either IgM anti-HAV or an abnormal ALT level, clinicians can assume that the antibody being detected by this assay is of the IgG type, indicating previous infection with HAV—or successful vaccination—and protection against future infection. These conclusions are valid only if passively acquired antibody from blood components, maternal antibodies crossing the placenta, or immune globulin immunoprophylaxis can be excluded.

MANAGEMENT OF ACUTE VIRAL HEPATITIS

No specific treatment for acute viral hepatitis exists, and hospitalization is not ordinarily indicated. Therapy should be supportive and aimed at maintaining comfort and adequate nutritional balance. For most jaundiced patients, strict bed rest and prolonged confinement are probably not indicated.⁵⁰ In one study, for example, strenuous activity did not appear to have any adverse effect on the clinical course of acute viral hepatitis.³¹³

The diet should conform to the patient's appetite and wishes; it must, however, supply adequate protein and calories. As a general rule, modest consumption of alcohol (<30 g/day for men and <15 g/day for women) during convalescence (6–24 weeks after symptoms develop) does not seem to be harmful when the ALT level has decreased to less than 1.5 times the upper limit of normal.³⁷³ Nevertheless, because of the direct hepatotoxic effect of ethanol, abstinence from alcoholic beverages during the acute phase of hepatitis seems prudent. Finally, women who are taking oral contraceptives need not discontinue their use during the course of hepatitis.³³³

No indication exists for corticosteroids in the treatment of acute, uncomplicated hepatitis A. They have no effect on the rate of resolution of the underlying disease process and may increase the rate of relapse or result in death from fulminant hepatitis, especially if administered during the incubation or prodromal stage of infection.^{29,98,99} Conversely, a 4-week course of prednisone with tapering doses can hasten resolution in some patients who have developed prolonged, moderate to severe cholestasis, although complete recovery without therapy will eventually occur in all.³²⁵ Abruptly stopping prednisone or shortening the interval of therapy can result in a relapse.

Specific antiviral therapy is not yet available for HAV, partly because chronicity is not an issue and outcome of the disease is generally favorable. However, amantadine and interferon-alpha have been reported to interfere with HAV replication in cell culture.^{397,403} Amantadine was shown to inhibit HAV IRES-mediated translation in hepatoma cells. However, whether effective treatment doses could be transposed to the clinical setting remains to be demonstrated.

Treatment of fulminant hepatitis A is based on a thorough understanding of the pathophysiology of this disorder, which has been reviewed.^{299,359} Although mortality rates are relatively high, the immediate transfer of the patient to a liver transplant facility and the aggressive management of complications have been responsible for much of the improvement in survival rates. Orthotopic liver transplantation (OLT) or auxiliary partial OLT remain the only therapeutic modalities available for achieving a nonfatal outcome in the most severely affected patients with fulminant hepatitis A, leading to 1-year survival rates of 80% or more. The use of artificial or bioartificial liver support systems to perform all of the functions of the liver remains elusive and cannot currently be recommended outside of clinical trials.

PREVENTION AND CONTROL

Decisions concerning the prevention of hepatitis A must consider the immunobiology of the disease and the principal routes of transmission.¹⁶¹ The most effective control measures must prevent fecal contamination by infected individuals before their clinical disease becomes apparent. Thus, the most critical control measure is proper hand washing and the avoidance of work practices that facilitate contamination of hands when

taking care of children younger than 2 years, especially infected neonates where nosocomial transmission has occurred in relatives, family, staff, and physicians having direct contact with these individuals.^{15,90,200,279,319} Because the personal hygiene of young children is often difficult to control, they should be restrained from close contact with their susceptible playmates during the first 2 weeks of illness or for at least 2 weeks after the onset of jaundice.

Considering the low order of infectivity that is present during the icteric stage of disease in adults (see Fig. 19.11), many authorities have suggested that strict enteric precautions within the hospital are not necessary.^{255,267} In general, universal precautions should be adequate. Thus, isolation of patients in private rooms and the use of gowns or masks are unnecessary unless direct and excessive exposure to feces or fecally contaminated items is anticipated.

Immune Globulin Immunoprophylaxis

Prevention of clinical manifestations of hepatitis A through the administration of conventional intramuscular immune globulin (IGIM) was documented initially by Stokes and Neefe³⁵⁸ in 1944 during a common-source outbreak at a summer camp in which they observed an 87% reduction in the attack rate among those receiving IGIM. Similar immunoprophylaxis studies were carried out simultaneously in military personnel and institutionalized children.^{122,156} In each instance, IGIM was capable of preventing anicteric or icteric hepatitis A in 80% to 90% of the participants when given up to 6 days before the expected onset of illness, and doses as low as 0.01 to 0.02 mL/kg were found to be protective.

IGIM collected from any geographic region should be protective against HAV infection because only a single human HAV serotype exists. Current lots of IGIM are sterile preparations of concentrated antibodies that have tested negative for HBsAg, human immunodeficiency virus (HIV), and HCV. Although the U.S. Food and Drug Administration (FDA) has not yet set minimal concentration requirements for IGIM, the level of anti-HAV in the 16.5% formulation has ranged from about 33 IU/mL to 63 IU/mL in lots assayed between 2005 and 2007 (M-Y Yu, CBER, FDA, personal communication, 2011). Peak levels of antibody are achieved approximately 2 days after intramuscular administration, and the half-life of IgG is 23 days.

Studies performed in institutions and those conducted during community outbreaks indicate that IGIM can either completely suppress or modify the infection in HAV-susceptible persons, depending on the concentration of anti-HAV administered and the interval from exposure to treatment.^{89,146,357} (e-Fig. 19.5). Larger doses of IGIM may provide protection lasting a few more weeks; however, more importantly, passive-active responses are curtailed, leaving a larger percentage of susceptibles available for infection following another exposure. IGIM is unlikely to be effective if given more than 3 weeks after exposure. The theoretical concern—that individuals might acquire HAV from persons who develop subclinical hepatitis after IGIM treatment—has not been borne out by epidemiologic investigations.³⁵⁷ Once the occupants of a household have received adequate prophylactic treatment, rigid isolation or compulsive cleanliness is not necessary. Commercial enzyme-linked immunosorbent (EIA) assays for anti-HAV following IGIM administration are usually unable to detect anti-HAV in the blood unless the procedure is optimized.⁷ When sera

were evaluated with a sensitive neutralization assay, however, low-level neutralizing antibody was found to persist for at least 14 weeks, depending on dose and concentration of antibody.^{114,353} Based on these observations, it can be assumed that very low levels of anti-HAV are sufficient to prevent infection.

Although IGIM may not interfere with the immune response to inactivated vaccines or to oral poliovirus or yellow fever vaccine,¹⁹² it may interfere with the response to other live attenuated vaccines such as measles, mumps, rubella, and varicella. Therefore, IGIM should be delayed at least 2 weeks after such immunizations, or, if IGIM is administered initially for hepatitis A prophylaxis, at least 3 months should pass before these other vaccines are administered.⁴⁸

Hepatitis A Vaccines

As nations improve their level of sanitation and hygiene, the age at which individuals will become infected is being delayed until adulthood, at which time the likelihood of developing symptomatic or serious illness is considerably higher (see Fig. 19.14 and Table 19.2). With the development, evaluation, and subsequent licensure of cell culture–derived hepatitis A vaccines in Europe in 1991 and the United States in 1995,^{11,218} the potential for long-term immunity has become a reality.

Two types of hepatitis A vaccines have been developed: either alum- or influenza virosome-adjuvanted, formaldehyde-inactivated vaccines that are available worldwide (Havrix, Vaqta, Avaxim, and Epaxal) (e-Table 19.1), or freeze-dried, live attenuated vaccines that have been used primarily in China and recently introduced into India.³⁴⁰ In general, these vaccines were initially evaluated in nonhuman primates, then their safety and efficacy were confirmed in clinical trials in humans. Both types use cell culture–derived HAV grown in human diploid cell cultures. In addition to these monovalent vaccines, the HAV-specific antigen has been combined with other vaccines such as the hepatitis B vaccine (Twinrix, Twinrix Junior, and Ambirix from GlaxoSmithKline [GSK]) or typhoid vaccines (ViATIM or Vivaxim from Sanofi Pasteur and Hepatyrrix from GSK). None of the HAV vaccines induce antigenic competition with other vaccines or increase the frequency of adverse events.^{6,24} Except for Epaxal, all other inactivated vaccines are free of thimerosal as a preservative.

All inactivated vaccines have similar immunogenicity and require two injections given 6 to 12 months apart, although this interval can be extended to 36 months. The vaccines should be administered by the intramuscular route, because suboptimal responses can result if the vaccine is administered in the gluteal region, especially in obese adults. Accelerated stability tests at 37°C for up to 3 weeks and after 15 months at 2°C to 8°C have revealed no loss of immunogenicity. In contrast, freezing may be deleterious to the product.

To prepare the alum-adsorbed Havrix hepatitis A vaccine, SmithKline Beecham (now GSK) extracted the cell culture–adapted HM175 strain of HAV from MRC5 cells¹⁴⁰ by freezing and thawing. This crude harvest is tested for the absence of extraneous agents, microbiologic sterility, and antigen identity²⁹¹ and then treated with formalin to ensure viral inactivation. Residual MRC5 cellular protein and traces of formalin and neomycin remain in the vaccine. No serious adverse events caused by this inactivated vaccine have been recorded in either outbreak settings or in field trials involving approximately 24,000 individuals.^{10,278} Postmarketing experience of unsolicited

events from a population of uncertain size precludes establishing reliable estimates of frequency of adverse events or their causal relationship to the vaccine.

The formalin-inactivated, alum-adsorbed, hepatitis A vaccine Vaqta, distributed by Merck & Co. Inc. is prepared from MRC5 cell monolayers infected with the attenuated strain of HAV CR326F.^{229,303} Antigen is extracted by lysis of infected cells and then concentrated, purified, and treated with formalin. The purified preparation is free from detectable MRC5 protein and contains only trace amounts of nonviral protein, DNA, bovine albumin, formaldehyde, and neomycin. The antigen is composed of monodispersed, full and empty, HAV particles. As with Havrix, undesirable side effects (e.g., injection-site pain/tenderness, fever, and headache) were mild in nature and of short duration. In a postmarketing surveillance study, no adverse events were identified among more than 42,000 individuals.²⁷⁸

Immunogenicity in adults, adolescents, and children 1 year of age or older is excellent, with protective levels of antibody (≥ 20 mIU/mL) being achieved in virtually all subjects after the primary injection. Two large efficacy trials involving more than 19,000 children have been conducted with the Havrix and Vaqta vaccines.^{175,391} The only cases that were observed in the Vaqta group occurred during the first 16 days, presumably among persons who had been infected before or shortly after the initial injection. The two cases that occurred in the Havrix group were relatively mild when compared with the cases in the control group.¹⁷⁵ Adverse events in these trials were mild and self-limited, and vaccine efficacy ranged from 95% to 100% (Fig. 19.19). During a 9-year follow-up period

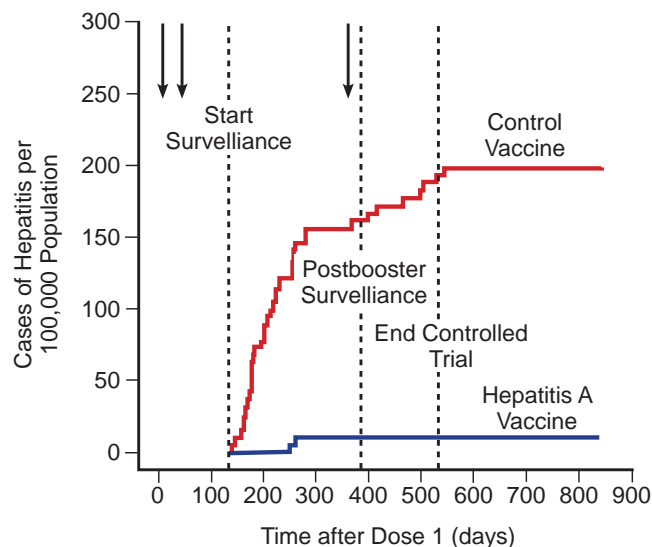


FIGURE 19.19. Cumulative rates of symptomatic infection with hepatitis A virus in healthy children receiving hepatitis B surface antigen vaccine (control group) and hepatitis A vaccine (Havrix, GlaxoSmithKline, Research Triangle Park, NC). Timing of vaccine administrations is represented (arrows) with doses being given at 0, 1, and 12 months. Dashed lines indicate when surveillance was started, when postbooster surveillance was begun, and when the controlled trial was terminated at crossover. (Modified from Innis BL, Snitbhan R, Kunasol P, et al. Protection against hepatitis A by an inactivated vaccine. *JAMA* 1994;271:1328–1334, with permission.)

in a community-wide Vaqta study, no vaccine recipient developed hepatitis A despite continuing community exposure.³⁹²

Estimates using kinetic models suggest that protective levels of neutralizing antibody could persist in most vaccinees for 20 years or more.^{381,398} Development of IgM anti-HAV after vaccination is rare,³⁴¹ and the ALT level remains normal, so confusion with recent viral hepatitis should not be an issue. Immunogenicity data for infants are limited; however, initial studies have indicated excellent responses in seronegative infants. In contrast, responses in infants with pre-existing maternal antibody have resulted in a suboptimal response, especially in those under 6 months of age.^{72,105,295,374,377} A subsequent booster dose of vaccine, however, results in an anamnestic response in most, indicating that priming of the immune response had occurred despite the lower anti-HAV concentrations observed initially.^{72,105} Currently, the vaccine is only recommended for children 1 year of age and older. Other factors associated with reduced immunogenicity include immunosuppression from disease or drugs and advanced age.

The bivalent Twinrix vaccine contains a pooled formulation of the inactivated HAV and a purified recombinant HBsAg hepatitis B vaccine. It is indicated for susceptible individuals 18 years of age and older who are, or will be, at risk for exposure to both viruses. This risk includes (a) travelers to endemic areas of high or intermediate endemicity for both HAV and hepatitis B virus (HBV) who are at increased risk because of behavioral or occupational factors; (b) patients with chronic liver disease; (c) persons at risk through their work, such as emergency medical assistance teams; (d) men who have sex with men; (e) military recruits; (f) illicit drug users, injecting or noninjecting; and (g) persons with clotting factor disorders. In patients with chronic liver disease, baseline serology suggests that fewer than 40% may qualify for the bivalent vaccine because of natural immunity to one or the other virus. Primary immunization with the Twinrix vaccine includes three doses given on a 0-, 1-, and 6-month schedule, although a four-dose regimen (0, 7, and 21–30 days with a booster at 12 months) may be used if time is of the essence. Seroconversion and protective levels of anti-HAV were observed after one dose in 93.8% of healthy adult volunteers, although the anti-HBs response was unexceptional until after the third dose, as it is with the conventional hepatitis B vaccine. In contrast, responsiveness is reduced for both vaccines, but especially HBV, in patients with chronic liver disease with bridging fibrosis or cirrhosis. For the HBsAg nonresponders, booster injections with high concentrations of HBsAg (e.g., the Merck dialysis formulation) are sometimes required to achieve immunity. Adverse reactions have been rare and include hypersensitivity reactions and anaphylaxis.

At least two live attenuated hepatitis A vaccines used in China over the past decade have been reported to be safe and immunogenic and to achieve comparable seroprotection levels for more than 10 years following a single-dose immunization schedule.^{244,402,418} These vaccines contain the H2 and LA-1 strains of HAV that were adapted for growth in human diploid cell cultures at lower temperature (32°C). In a recent clinical trial comparing these two vaccines, a 95% protective efficacy to prevent clinical infection was observed in more than 450,000 children.⁴⁰² Herd immunity in the nonvaccinated population also was observed. Although subclinical infection may not be averted in all recipients, there is no evidence to indicate that the vaccine virus is shed in the feces or that it

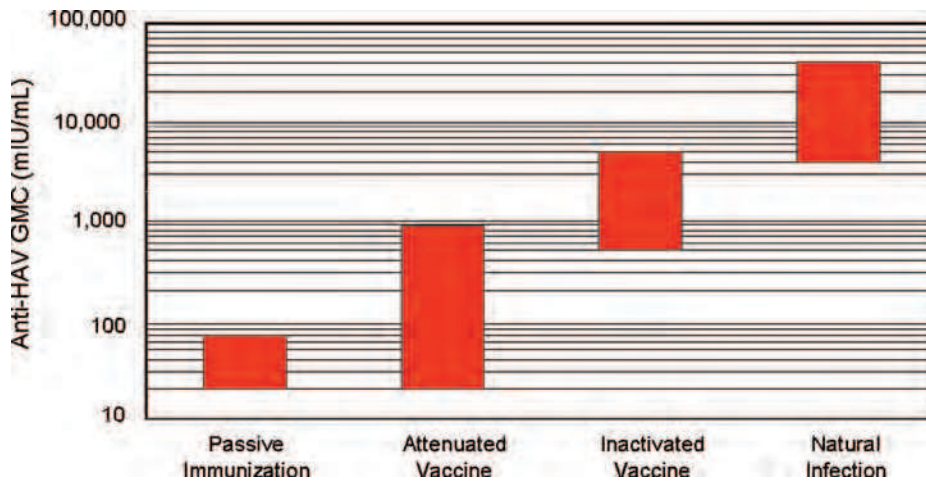


FIGURE 19.20. Anti-hepatitis A virus (HAV) geometric mean concentration (GMC; mIU/mL) observed in persons after passive and active immunization and after natural HAV infection. (Modified from Lemon S. Hepatitis A virus: current concepts of the molecular virology, immunobiology and approaches to vaccine development. *Rev Med Virol* 1992;73–87, with permission.)

causes vaccine-associated infections. Accordingly, reversion to a more virulent strain has not occurred. Oral inoculation has been unsuccessful, however, even when higher doses of vaccine were administered.

Strategies for the Use of Hepatitis A Virus Vaccine and Immune Globulin

Anti-HAV Responses After Passive and Active Immunization

Qualitative differences in protective antibody responses are observed following active and passive immunization probably because of differences in the affinity of antibodies.²²⁵ Figure 19.20 compares anti-HAV responses in the following situations²¹⁷: (a) at 3 to 5 days after receiving 5 mL of IGIM (passive immunization), (b) at 6 to 12 months after one injection of live attenuated HAV vaccine, (c) at 6 to 12 months after two injections of inactivated hepatitis A vaccine, and (d) at 10 to 20 years after a natural infection. Despite these disparities, protection against disease is comparable, implying that very low and even nondetectable levels of anti-HAV may confer protection.

Pre-Exposure Prophylaxis

Although universal childhood immunization for hepatitis A is a worthy goal, it is unlikely that such a recommendation will be issued by policy makers in developing countries or regions with transitional economies, given the economic and political realities of global immunization programs. Even in developed countries, the integration of hepatitis A vaccination into routine childhood immunization schedules has been slow to materialize. Within the United States, however, several recommendations were formulated by the Advisory Committee on Immunization Practices (ACIP) beginning shortly after the vaccine was introduced in 1995, even though the annual number of prevaccination deaths from hepatitis A placed it at the bottom of the list of other infectious diseases for which vaccination was subsequently recommended. At first the focus was on vaccinating high- and intermediate-risk groups and children living in communities with the highest disease rates. In 1999, this was expanded to include children residing in states, counties, or communities that had consistently elevated rates of hepatitis A (≥ 10 cases/100,000 population or twice the national average).⁴⁸ The final step in this strategy came in

2006, when routine hepatitis A vaccination of children was recommended nationwide. Administration of IGIM was relegated to more specific indications (see later discussion).

A recent assessment of the impact of this novel immunization strategy revealed that the incidence of hepatitis A in 2003, when compared with a prevaccination baseline period (1990–1997), declined 76% to 2.6 cases/100,000 population³⁸⁷ (see Fig. 19.15). Although this decline was seen throughout the United States regardless of vaccination policy, it was more pronounced in those states where vaccination was implemented (88% vs. 53% decline in rate). A decline in the incidence rates also was seen in all age groups. A similar reduction in disease incidence in the vaccinated and older nonvaccinated population also was observed following universal immunization of toddlers 18 to 24 months of age in Israel, suggesting marked herd protection and the possibility that catch-up vaccination programs may not be necessary⁷³ (e-Fig. 19.6).

As shown in Table 19.3, immunization should be offered to persons traveling from industrialized countries to regions of the world where HAV is endemic and where the risk of acquiring hepatitis A is enhanced because of living conditions and the length of stay (e.g., Asia [except Japan]; Africa, Latin America and the Caribbean, eastern Europe, the Middle East, and the Commonwealth of Independent States). Routine immunization for U.S. military personnel could be reserved for those who regularly travel to endemic areas or those in rapid-deployment

TABLE 19.3 Risk Groups Potentially Targeted for Pre-Exposure Prophylaxis

- Travelers to or workers in foreign countries (including the military) where the risk of acquiring hepatitis A is enhanced
- American Indians/Alaskan natives
- Persons who work with HAV or with nonhuman primates
- Persons with chronic liver disease
- Men who have sex with men
- Persons who have clotting factor disorders
- Persons residing in areas where extended community outbreaks exist
- Refugees residing in temporary camps after catastrophes

units. For other soldiers, typical reassignment or deployment times of several weeks would be sufficient for generating a protective response to vaccination.

It has been reported that the incidence of symptomatic hepatitis A for a 1-month trip to a developing country is 3 to 6 cases/1,000 susceptible travelers.³⁵⁵ In contrast, the incidence rate is sixfold higher (20 cases/1,000) in persons whose travel arrangements or personal hygiene place them in jeopardy of acquiring an infection. In making a recommendation for vaccination, it is important to recognize that hepatitis A is contracted at least 100 times more frequently than is typhoid fever or cholera.³⁵⁵ For most travelers, vaccine alone will suffice if the first dose is administered at least 2 weeks prior to expected exposure to HAV. For both immediate and long-term protection of older adults (>40 years), immunocompromised persons and persons with chronic liver disease or other chronic medical conditions where the immune response may be muted, and who are planning to depart for an endemic area in 2 weeks or less, the simultaneous administration of inactivated hepatitis A vaccine with IGIM (0.02–0.06 mL/kg) should provide optimal protection with the stipulation that separate syringes and injection sites are used.^{46,385} The anti-HAV titers, however, are about twofold lower when compared with those observed when vaccine alone is administered but more than 100 times higher than levels considered to be protective.

At-risk individuals who are allergic to components in the vaccine or who do not elect to receive the vaccine can be given small doses of IGIM (0.02–0.04 mL/kg or 2.0 mL for adults) if the anticipated period of exposure is 2 to 3 months. Larger doses (0.05–0.06 mL/kg or 5.0 mL for adults) should be administered every 4 to 6 months for more prolonged exposure.

Vaccine also should be offered to staff and residents of institutions for the mentally disabled or to members of other relatively closed communities who are at high risk for acquiring hepatitis A. It also should be offered to preschool children attending daycare centers, including the staff, parents and siblings, and to any other group whose occupation, lifestyle, or behavior places them in the high-risk category (e.g., illicit drug users, injecting or noninjecting, and men who have sex with other men). Other groups that have been targeted for possible immunization for hepatitis A are listed in Table 19.3.

One of the more contentious recommendations concerns the administration of hepatitis A vaccine to patients with chronic liver disease, especially those with hepatitis B or C. Several reports have suggested that the occurrence of hepatitis A in these patients is associated with a higher frequency of severe illness or fulminant hepatic failure, leading to substantially higher case fatality rates.^{196,382} Other investigators, however, have reported conflicting data on the clinical course and outcome of hepatitis A in chronic liver disease.^{263,363} In the interim, it seems prudent to offer vaccination to all patients with chronic liver disease as recommended by the ACIP. Physicians should recognize, however, that no data indicate that a concurrent hepatitis A infection will place patients with chronic liver disease at increased risk for a life-threatening outcome unless they have advanced liver disease (e.g., cirrhosis).

Postexposure Prophylaxis

There has been considerable movement to revamp the strategy on postexposure prophylaxis favoring the use of hepatitis A vac-

TABLE 19.4 Advisory Committee on Immunization Practices Recommendations for Postexposure Prophylaxis Using Vaccine or Immunoglobulin M Alone

- Close personal contact (household and sexual contacts)^a
- Outbreak in childcare centers (staff and attendees; consider household members of diapered attendees)
- Common-source exposure (food handlers of index worker and patrons if identified and treated within 2 weeks of exposure)
- Outbreak confirmed in schools, hospitals, and work settings

^aConsider vaccine alone or both vaccine and immunoglobulin M if the contact shared illicit drugs with case.

cine over IGIM following exposure to HAV. Advantages include long-term protection, ease of administration, higher acceptability and availability, and comparable cost. Initial data was limited to experiments in marmosets and chimpanzees.^{70,306,317} Primates were vaccinated 1 to 3 days after being challenged orally or parenterally and were protected against hepatitis A disease or had an attenuated infection without virus shedding. A randomized, double-blind, active-control, noninferiority trial conducted in Kazakhstan and supported by the CDC, was designed to compare the efficacy of hepatitis A vaccine (n = 568) to IGIM (n = 522) in preventing laboratory-confirmed, symptomatic HAV infection when given to at-risk subjects within 14 days after exposure.³⁸³ This study found that HAV occurred infrequently in either treatment group, meeting a prespecified criterion for noninferiority for these prophylactic measures. Rates of infection, however, were higher in the vaccine group (8.6%) than in the IGIM group (6.7% overall) and for all HAV study points examined (clinical and subclinical), suggesting that the IGIM performed modestly better than the vaccine in these per-protocol populations (e-Fig. 19.7). Regardless, the ACIP in 2007 updated their recommendations for postexposure prophylaxis to include either hepatitis A vaccine alone or IGIM alone in most exposure circumstances (Table 19.4). The only situation where both vaccine and IGIM were recommended was in a person who shared illicit drugs with a case, and even in this situation, vaccine alone also was an option.

PERSPECTIVES

Detailed studies of early innate and adaptive immune responses to HAV that have been undertaken recently are likely to provide a better understanding of critical responses that lead to the elimination of virus from the host. In addition, through comparative analyses with host responses to another hepatotropic virus—HCV—such studies may help understand why HCV may establish persistent infections in humans whereas HAV never does. A better understanding of HAV virulence mechanisms could lead to the development of novel therapies designed to subvert severe outcomes. As developing countries transition from high endemicity to low endemicity, the need for universal vaccination of the population will become more compelling. The development of an oral vaccine would be of great benefit in this situation.

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Caliciviridae: The Noroviruses

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Perspective

The family *Caliciviridae* is composed of small (27 to 40 nm), nonenveloped, icosahedral viruses that possess a linear, positive-sense, single-stranded RNA (ssRNA) genome. The five genera of the family are *Norovirus*, *Sapovirus*, *Nebovirus*, *Lagovirus*, and *Vesivirus*. The major human pathogens in the family are the noroviruses and sapoviruses, which cause acute gastroenteritis. Important veterinary pathogens include vesiviruses such as feline calicivirus (FCV), which causes a respiratory disease in cats, and lagoviruses such as rabbit hemorrhagic disease virus (RHDV), which causes an often fatal hemorrhagic disease in rabbits. This chapter provides a description of the family *Caliciviridae*, with major emphasis on the noroviruses because of their prominent role in sporadic and epidemic gastroenteritis.¹⁴⁷

HISTORY

The establishment of a viral etiology for gastroenteritis in humans was a decades-long process that was hampered by the fastidious nature of many of these viruses for growth in cell culture.²¹³ Volunteer studies carried out in the 1940s and 1950s in the United States and Japan played a major role in establishing that filterable, nonbacterial infectious agents can cause enteric disease.²¹³ An important advance occurred in 1972 with the discovery of Norwalk virus (NV) by Kapikian et al.²¹⁴ Stool material from a rectal swab obtained from an ill individual involved in a gastroenteritis outbreak that had occurred at an elementary school in Norwalk, Ohio, in October 1968 was administered to adult volunteers as a bacteria-free filtrate and serially passed to other volunteers, inducing acute gastroenteritis in certain individuals.^{112,113} Stool material from these volunteers was then examined for the presence of viruses by the technique of immune electron microscopy (IEM), which involves the direct observation of antigen–antibody complexes by EM.^{11,13,18,214} The fecal filtrate was incubated with prechallenge or convalescent-phase serum from a volunteer who had become ill following ingestion of the filtrate. Figure 20.1 shows the striking difference between the appearance of the small, round virus particles (described as 27 to 30 nm in diameter with a hint of surface structure) after incubation with the prechallenge serum (part A and B) and after incubation with convalescent-phase serum (parts C and D). The increase in visible virus-specific antibodies in the convalescent serum from individuals involved in the original Norwalk outbreak, as well as in volunteers challenged with the virus derived from the outbreak, led to the conclusion that NV was the etiologic agent.²¹⁴ Norwalk virus would become the prototype strain for a large group of related *Norwalk-like viruses* or *small round structured viruses*, known now as the noroviruses.

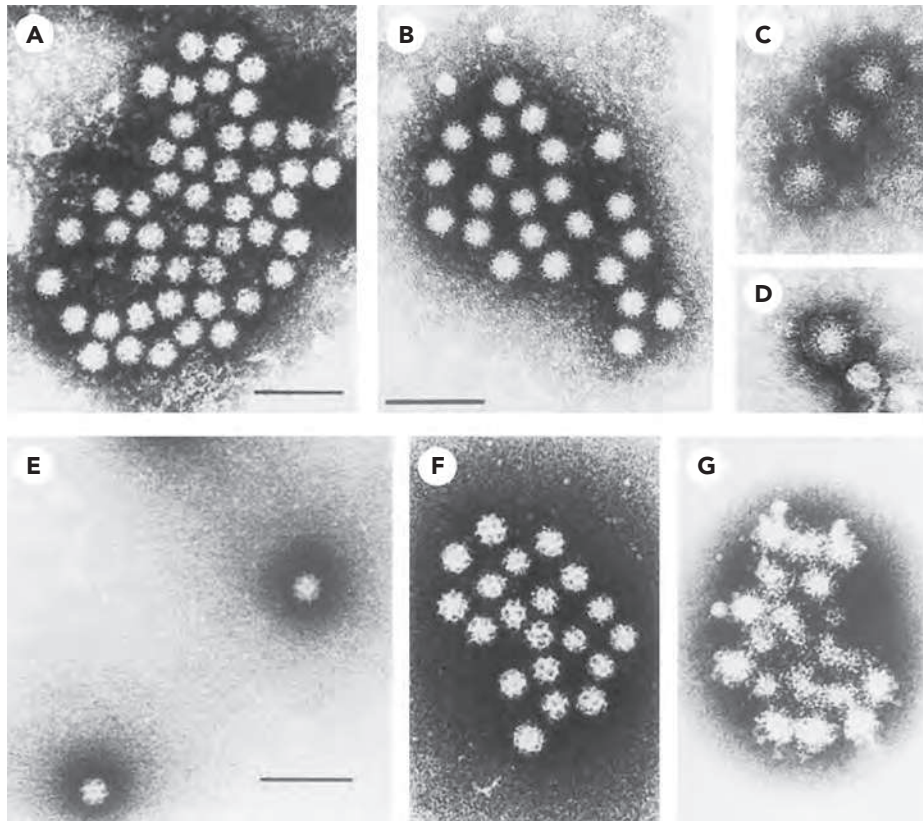


FIGURE 20.1. Norwalk virus (NV) (genus *Norovirus*) and Sapporo virus (genus *Sapovirus*) in stool material. **A: NV particles in a stool filtrate visualized by immune electron microscopy (IEM). **B:** An aggregate observed after incubation of NV stool filtrate with a 1:5 dilution of volunteer's prechallenge serum. Particles have a light coating of antibody molecules. The amount of antibody present on this aggregate was given a rating of 1 to 2 to 2+ and the serum was given an overall rating of 1 to 2+ on a scale of 1 to 4. **C, D:** Three single particles (**C**) and one individual particle (**D**) observed after incubation of the NV stool filtrate with a 1:5 dilution of the same volunteer's postchallenge convalescent serum. Particles are heavily coated with antibody molecules. At high antibody levels (antibody excess), large aggregates may not be seen. The amount of antibody was given a rating of 4+ and the serum was given an overall rating of 4+ on a scale of 1 to 4. **E:** Sapporo virus particles in stool material visualized by direct electron microscopy (EM). The distinct, hollow cup-like structures are apparent. **F:** Sapporo virus particles after incubation with guinea pig hyperimmune serum. The amount of antibody was given a 1+ rating. **G:** An aggregate observed after incubation of Sapporo virus stool filtrate with guinea pig hyperimmune serum. The amount of antibody was given a rating of 4+ on a scale of 1 to 4. (**A–D** from Kapikian AZ, Wyatt RG, Dolin R, et al. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol* 1972;10:1075–1081, with permission. **E–G** from Nakata S, Kogawa K, Numata K, et al. The epidemiology of human calicivirus/Sapporo/82/Japan. *Arch Virol Suppl* 1996;12:263–270, with permission.)**

The techniques used in the discovery of the NV proved instrumental in the subsequent characterization of other fastidious enteric viruses (including human rotaviruses, astroviruses, parvoviruses, enteroviruses, and hepatitis A virus) by an approach that became known as *particle* or *direct virology*.²¹³ Norovirus reference strains such as Hawaii virus (from a family outbreak of gastroenteritis that occurred in Honolulu in 1971) and Snow Mountain virus (from an outbreak in a Colorado resort camp in 1976) were discovered in 1977 and 1982, respectively.^{116,468} In 1976, Madeley and Cosgrove²⁹⁶ reported the presence of “typical” caliciviruses in the stools of infants and young children (2 to 18 months of age), and these viruses showed a striking morphologic similarity to previously characterized animal caliciviruses that were known to exhibit “classical” distinct cup-like depressions on the surface of the virion. That same year, Flewett and Davies¹³⁰ observed similar calicivi-

rus particles in a sample from the intestinal lumen of a child at autopsy and in the feces of a child with gastroenteritis. Chiba et al.⁹⁰ described a “classical” calicivirus, associated with gastroenteritis, in infants and young children living in an infant home in Sapporo, Japan, in 1977. Another virus (later designated Sapporo/82/Japan) that exhibited classical calicivirus morphology was subsequently detected from this same infant home (Fig. 20.1E).³⁴⁰ Hyperimmune serum was raised in guinea pigs against Sapporo/82/Japan virus particles purified from stool material, and IEM was used to establish its antigenic characteristics (Fig. 20.1F and 20.1G, respectively). The Sapporo virus would become the prototype strain for the *Sapporo-like viruses* or *classical caliciviruses*, known now as the sapoviruses.

In the late 1980s, the stool filtrate derived from the Norwalk, Ohio, outbreak was again fed to adults in volunteer studies to obtain adequate quantities of virus particles to

TABLE 20.1 Taxonomic Structure of the *Caliciviridae*

Genus	Species	Representative strain
<i>Norovirus</i> (NoV)	<i>Norwalk virus</i> (NV)	Hu/NoV/GI.1/Norwalk/1968/US
<i>Sapovirus</i> (SaV)	<i>Sapporo virus</i> (SV)	Hu/SaV/GI.1/Sapporo/1982/JP
<i>Lagovirus</i> (LaV)	<i>Rabbit hemorrhagic disease virus</i> (RHDV)	Ra/LaV/RHDV/GH/1988/DE
	<i>European brown hare syndrome virus</i> (EBHSV)	Ha/LaV/EBHSV/GD/1989/FR
<i>Nebovirus</i> (NeV)	<i>Newbury-1 virus</i> (NBV)	Bo/NeV/NBV/Newbury-1/1976/UK
<i>Vesivirus</i> (VeV)	<i>Vesicular exanthema of swine virus</i> (VESV)	Po/VeV/VESV/VESV-A48/1948/US
	<i>Feline calicivirus</i> (FCV)	Fe/VeV/FCV/F9/1958/US

The cryptograms are organized as follows: host species from which the virus was obtained/genus/species (or genogroup)/strain name/year of occurrence/country of origin. Abbreviations for the host species are Fe, feline; Ha, Hare; Hu, Human; Po, Porcine; Ra, Rabbit. Country abbreviations are DE, Germany; FR, France; JP, Japan; US, United States.

GenBank Accession numbers and references for description of representative viruses: Norwalk virus, M87661^{202,214}; Sapporo virus, U65427^{89,352}; RHDV, M67473³²⁵; EBHSV, Z69620²⁵⁸; VESV, AF181082^{345,498}; FCV, M86379⁶⁴; Newbury-1 virus, DQ013304.^{53,361}

Adapted from Virus taxonomy: 2011 release. International Committee on the Taxonomy of Viruses web page. <http://ictvonline.org/virusTaxonomy.asp?version=2011>

characterize the viral genome, a major advance by Jiang et al¹⁹⁹ that established the classification of Norwalk virus as a member of the *Caliciviridae*. The complete RNA genome sequences of Norwalk virus and the closely related Southampton virus were determined and found to be organized into three major open reading frames (ORF1, -2, and -3) with a polyadenylated 3' end^{176,202,253,254} (see Genome Structure and Organization). The ORF1 was shown to encode a large polypeptide that was proteolytically processed into the mature nonstructural proteins.^{273,276} The ORF2 encoded the major capsid protein, VP1, and ORF3 encoded a minor structural protein, VP2. The human noroviruses initially segregated into two major phylogenetic groups within what would become the genus *Norovirus* of the *Caliciviridae* that were designated as genogroups I (GI) and II (GII), with NV belonging to GI and the Hawaii and Snow Mountain viruses belonging to GII.^{266,487} In addition, sequence analysis of the “classical” caliciviruses confirmed that they were distinct from the noroviruses, ultimately forming a separate genus, *Sapovirus*, within the *Caliciviridae*.^{252,274,309}

CLASSIFICATION

Members of the virus family *Caliciviridae* have a virion protein, genome (VPg)-linked, positive-sense RNA genome that is polyadenylated and surrounded by a nonenveloped, icosahedral capsid of 27 to 40 nm in diameter. The capsid is constructed predominantly from a major structural protein, VP1, of approximately 60,000 D. The five genera of the family *Caliciviridae*—*Norovirus*, *Sapovirus*, *Lagovirus*, *Nebovirus*, and *Vesivirus*—each represent a distinct phylogenetic clade in the family^{92,152} (e-Fig. 20.1). Within each genus, one or more species has been defined based primarily on genetic relatedness, and the current taxonomic structure of the *Caliciviridae* is shown in Table 20.1. It is possible that additional genera will be established, following characterization of the

unique genomes of Tulane virus (simian),¹²⁸ St. Valérian virus (porcine),²⁵¹ and Bayern virus (avian)⁵⁰⁹ (e-Fig. 20.1). Below the level of species in certain genera (*Norovirus* and *Sapovirus*), provisional genetic typing systems (consisting of genogroups subdivided into genetic clusters or genotypes) have proven useful in epidemiologic studies (see Molecular Epidemiology).

VIRION STRUCTURE

Calicivirus virions exhibit $T = 3$ icosahedral symmetry. The capsid contains 90 dimers of the VP1 capsid protein that form a shell from which 90 arch-like capsomeres protrude at the local and strict twofold axes.^{86,381,382,384} These arches are arranged in such a way that 32 large hollows are seen at the icosahedral five- and threefold positions, and these hollows are seen as cup-like structures on the surface of caliciviruses (*calici* is derived from the Latin word *calyx*, or “cup”). Electron cryomicroscopy and computer image processing studies of representative caliciviruses show subtle variation in the capsid structures that are consistent with their differences in appearance by negative-stain EM, which can range from a feathery appearance (many noroviruses, such as Norwalk virus in Fig. 20.1A) to the presence of sharply defined “cups” (many sapoviruses, such as Sapporo virus in Fig. 20.1E and the vesiviruses).^{86,383}

Self-assembly of the norovirus VP1 into virus-like particles (VLPs) is an efficient process and does not require RNA²⁰¹ or the minor capsid protein, VP2.^{256,263} This feature has been especially useful in the study of the fastidious caliciviruses, because recombinant (r) VLPs expressed in the baculovirus system have served as a surrogate for native virions.^{154,201,203} The NV rVP1 (180 copies) characteristically self-assembles into 38-nm particles with $T = 3$ symmetry, but it can form smaller VLPs (23 nm) with $T = 1$ symmetry composed of 60 copies of VP1.⁴⁹⁸

The atomic structure of the Norwalk rVLP has been determined by x-ray crystallography.³⁸² These structural studies

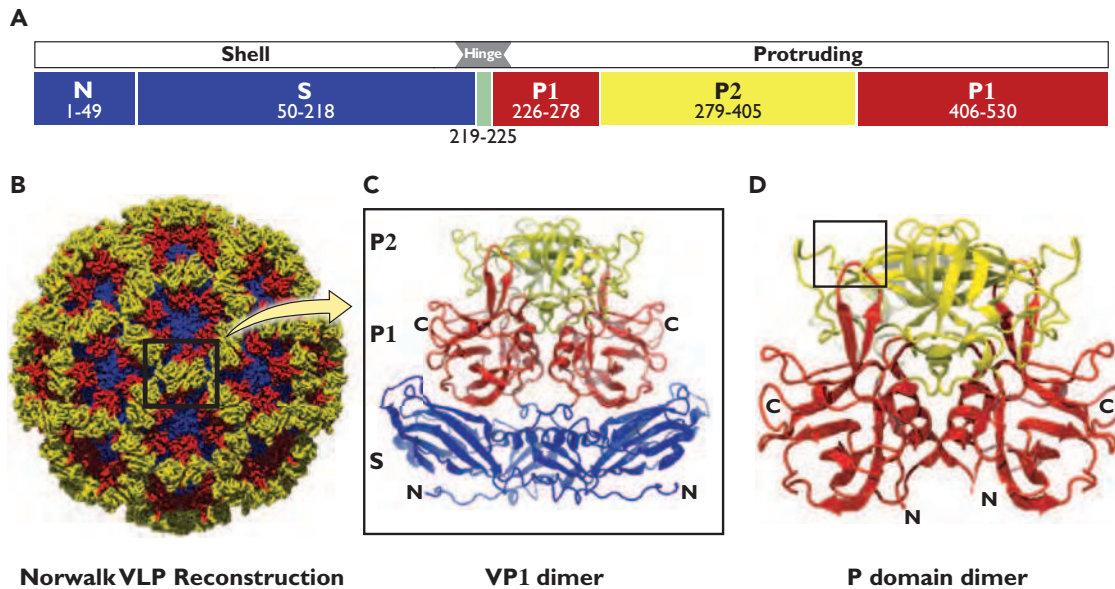


FIGURE 20.2. Organization of the norovirus major capsid protein, VP1. **A:** The Norwalk virus major capsid protein VP1 is 539 amino acids (aa) in length and is organized into two major parts, shell (S) and protruding (P), connected by a hinge (H) region. The aa borders of the defined domains are N-terminal (N) arm, 1 to 49; S, 50 to 218; hinge, 219 to 225; P1, 226 to 278 and 406 to 530; and P2, 279 to 405. (Adapted from Prasad BV, Hardy ME, Dokland T, et al. X-ray crystallographic structure of the Norwalk virus capsid. *Science* 1999;286:287–290.) **B:** Model of the T = 3 Norwalk virus whole capsid, determined at 3.4-Å resolution.³⁸² The capsid is composed of 90 dimers of VP1, with the S, P1, and P2 domains shown in blue, red, and yellow, respectively. The box highlights the arrangement of a VP1 dimer as it is displayed on the surface of the virion. **C:** Three-dimensional ribbon representation of a VP1 dimer derived from x-ray crystallography studies of Norwalk virus recombinant virus-like particles (rVLPs) at 3.4 Å, showing the presentation of the P2 domain at the top of an arch supported by two P1 domain “arms”.³⁸² The S domain forms the internal scaffold of the virion that surrounds the RNA genome and positions the arch to the virion surface. The N-terminus (N) and C-terminus (C) of the VP1 are indicated. **D:** Three-dimensional ribbon representation of a P domain-only dimer, determined at 1.4-Å resolution.⁹¹ The box highlights the location of a histo-blood group antigen (HBGA) carbohydrate binding site mapped within the P2 domain (see Fig. 20.7). (Images provided by B. V. V. Prasad.)

have defined two major domains in the VP1—the shell (S) and the protruding (P) arm (Fig. 20.2). The S domain forms the inner part of the capsid that surrounds the RNA genome and maintains the icosahedral contacts of the T = 3 structure, and the P domain forms the arch-like protrusions that emanate from the shell and contain the dimeric contacts.³⁸² The amino acid residues that correspond to the S and P subdomains in the NV VP1 (530 amino acids in length) are diagrammed in Figure 20.2A. A ribbon model of the VP1 dimeric subunit derived from the crystallographic structure of the capsid shows a more detailed view of these domains and their interactions (Fig. 20.2B, C). The NH₂-terminal (N) arm, located within the S domain, consists of residues 1 to 49 and faces the interior of the capsid. The part of the S domain that forms a classic eight-stranded antiparallel β -sandwich fold consists of amino acids (aa) 50 to 225 (which includes a flexible hinge). The entire S domain (aa 1 to 225) corresponds to the N-terminal region of the capsid protein that is relatively conserved among noroviruses in sequence comparisons. The P domain, which is linked to the S domain through a flexible hinge (aa 219 to 225), corresponds to the C-terminal half of the VP1, which is more variable in amino acid sequence. The P domain is divided into the P1 subdomain, encompassing aa 226 to 278 and 406 to 520, and the P2 subdomain, encompassing aa 279 to 405. The P1 subdomains form the sides of

the arch of the capsomeres and position the highly variable P2 subdomain at the top of the arch. In the dimeric form of the capsid protein, two P2 subdomains form what appears to be a bilobed structure at the surface of the virion. The exposure of the variable P2 region on the surface is consistent with its role in the formation of a major antigenic site and in receptor binding.^{10,47,85,86,107,110,172,178,185,221,271,281,348,382,398,459,497} It has been proposed that the highly conserved S domain may function as an icosahedral scaffold with the N-terminal arm providing a switch to facilitate the appropriate curvature, and that the P domain may be a replaceable module for conferring strain differences and antigenic specificity.^{85,86,382} Structural studies of other caliciviruses have shown a similar organization, with an internal shell domain serving as the scaffold for the more variable protruding domain.^{85,220}

Knowledge of the capsid structure has informed study of capsid assembly and facilitated the expression of subviral forms of the capsid that have proven useful in several areas of research (e-Fig. 20.2).⁴⁶⁰ The extreme N-terminus of the NV VP1 protein (first 20 aa) was not required for the assembly of native-sized 38-nm VLPs with T = 3 symmetry.³⁶ Expression of the NV VP1 S region alone (beginning with the extreme N-terminus of the VP1 and including amino acid residues 1 to 227) resulted in the formation of smooth particles (designated “S” particles in e-Fig. 20.2) of approximately 30 nm in diameter.³⁶

Although initial expression of the NV P region alone did not yield VLPs in baculovirus or bacterial systems,^{36,458} the native P domain from NV and other noroviruses formed *P dimers* that were recognized by antibodies and carbohydrates similarly to intact VLPs.⁴⁵⁸ The ability to produce rapidly *P dimers* (and mutagenized forms) accelerated structure and function studies of the norovirus capsid protein.^{61,463,464,465} Further genetic engineering to include a four to seven arginine-rich sequence at the C-terminus of the P domain resulted in the generation of small, subviral particles termed *P particles* that were assembled from 12 dimers of the P domain and that showed T = 1 symmetry.^{460,463} (e-Fig. 20.2). Recently, expression of the P domain with a further modified terminus allowed the production of *small P particles*.⁴⁵⁷

GENOME STRUCTURE AND ORGANIZATION

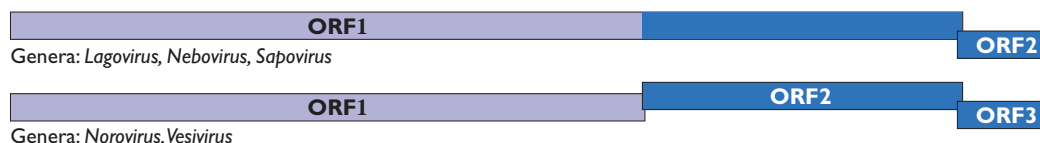
Caliciviruses have a linear, single-stranded, positive-sense RNA genome (ranging from approximately 7.3 to 8.5 kb [kilobases]

in length) (Fig. 20.3A). Genomes characteristically begin with a 5' end terminal pGpU sequence that is covalently linked to a small protein, VPg. A short conserved region (CR) at the 5' end is repeated internally in the genome near the beginning of a subgenomic-sized RNA transcript that is co-terminal with the 3' end of the genome.^{179,254,324,331,344} The nonstructural proteins are encoded beginning near the 5' end of the genome, and the structural proteins (VP1 and VP2) are encoded toward the 3' end of the genome in the region corresponding to a subgenomic RNA. Calicivirus genomes are organized into two or more major ORFs, depending on the genus. The noroviruses and vesiviruses encode the VP1 structural protein in a separate ORF (ORF2), whereas sapoviruses, lagoviruses, and neboviruses encode a VP1 that is contiguous with the large nonstructural polyprotein in ORF1 (Fig. 20.3B). All caliciviruses have a relatively small ORF near the 3' end that encodes the minor structural protein, VP2. The VP2 is variable in size (12,000 to 29,000 D) and sequence identity among the caliciviruses.^{65,418} Murine norovirus genomes analyzed thus far contain a unique conserved ORF (ORF4).⁴⁶⁷ with an encoded protein of 23,800 D

A Genome Organization



B Reading Frame Usage



C Gene Order and Cleavage Sites

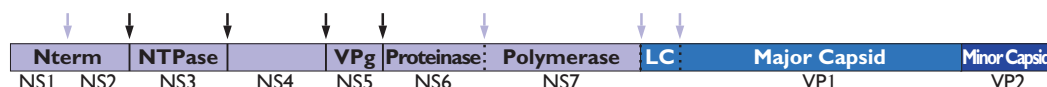


FIGURE 20.3. Comparative features of calicivirus genomes. **A:** The positive-sense RNA genome is covalently linked to a virion protein genome (VPg) at the 5' end and polyadenylated at the 3' end. Nonstructural proteins are encoded beginning from the 5' end of the genome and the structural proteins are encoded toward the 3' end. A conserved region (CR) of nucleotide sequence is shared between the 5' end of the viral genome and the 5' end of an abundant subgenomic RNA species produced during replication that serves as a template for translation of the viral structural proteins. **B:** Calicivirus genome organization. Calicivirus genomes are organized so that the major capsid protein coding sequence is either in frame or not with the upstream nonstructural protein sequence. The genomic organization and reading frame usage are shown for representative strains in the genera *Lagovirus* (Ra/LaV/RHDV/GH/1988/DE, GenBank Accession Number M67473), *Nebovirus* (Bo/NeV/NBV/Newbury-1/1976/UK, GenBank Accession Number DQ013304), *Sapovirus* (Hu/SaV/Manchester/1993/UK, GenBank Accession Number X86560), *Norovirus* (Hu/NoV/Norwalk/1968/US, GenBank Accession Number M87661), and *Vesivirus* (Fe/VeV/FCV/F9/1958/US, GenBank Accession Number M86379). **C:** A large polyprotein (encoded in open reading frame 1 [ORF1]) is translated from the viral RNA genome, and it is processed into precursors and final products by a virus-encoded protease. The proteolytic processing strategy varies among the caliciviruses, but all viruses encode domains for at least seven protein functions indicated here as nonstructural (NS) proteins NS1 through NS7.⁴³⁴ An extra cleavage site is present in the ORF1 of caliciviruses in which the capsid protein sequence is in frame with the nonstructural polyprotein encoded in ORF1. Cleavage at this site is thought to release the VP1 from the polyprotein so that the RNA-dependent RNA polymerase (NS7) can adopt an active conformation early in the replicative cycle. A cleavage site unique to the vesiviruses is present to release the leader of the capsid protein (LC) from a capsid precursor encoded in ORF2.⁴³⁸ Mapped cleavage sites conserved and utilized among all calicivirus genera are indicated with a dark arrow; cleavage sites that vary in utilization among the family are indicated with a light arrow.^{238,273,276,358,359,434,435,438,506}

(designated VF-1) that has been implicated in modulation of the host innate immune response *in vivo*.³¹⁸

Viral Proteins

Structural Proteins

Three proteins are found in mature calicivirus virions: VP1, VP2, and VPg.⁴³⁶ The VP1 (~60,000 D), which is the major structural protein of the virus, is present in 180 copies (90 dimers) per virion.³⁸³ The predominance of VP1 in the formation of the viral capsid structure (see Virion Structure) is consistent with its critical role in determining the antigenic phenotype of the virus and its interactions with host cells.

The VP2 (12,000 to 29,000 D) is considered a minor structural protein because it is present in only one to two copies per virion, and its function is unknown.^{145,146,436} The ablation of VP2 expression in an infectious FCV complementary DNA (cDNA) clone by the introduction of a stop codon in its reading frame did not abolish RNA replication; however, infectious virions could not be recovered without an intact VP2.⁴³³ Although VP1 can self-assemble into rVLPs independently of the presence of VP2,^{256,263} the presence of VP2 may increase the efficiency of VP1 expression and enhance the stability of rVLPs generated in the baculovirus expression system.³⁵ Evidence for a direct interaction between the VP1 and VP2 capsid proteins has been reported for both NV^{74,145,146} and FCV,^{108,211} further suggesting a role in particle maturation or stability.

The VPg is covalently linked to the genomic and sub-genomic RNA in infected cells,^{120,180} and is a minor component in virions at an estimated one or two copies per particle.^{410,436} Although the VPg is present in virions, it likely functions primarily as a nonstructural protein during replication (see Nonstructural Proteins).

Nonstructural Proteins

Caliciviruses derive their mature nonstructural proteins (designated here as NS1 through NS7) by proteolytic cleavage of a large polyprotein encoded in ORF1 (Fig. 20.3C). The length of the noncleaved polyprotein precursor is approximately 200,000 D (excluding the in-frame VP1 capsid protein sequences of the lagoviruses, neboviruses, and sapoviruses). This large precursor has never been observed, most likely because proteolytic processing is rapid and co-translational.^{273,276,439} The proteolytic cleavages are mediated by a virus-encoded cysteine proteinase (NS6^{Pro}).⁵⁰ The location of the cleavage sites in the ORF1 polyprotein that define the borders of the final nonstructural protein cleavage products has been determined for calicivirus strains representing the genera *Norovirus*,^{34,273,276,434} *Vesivirus*,⁴³⁵ *Lagovirus*,^{238,506} and *Sapovirus*.³⁵⁹ Some variation is seen among the proteolytic processing strategies, but the overall gene order of the calicivirus nonstructural proteins is conserved. The lagoviruses, represented by RHDV, have the highest number (six) of mapped cleavage sites. The polyprotein of RHDV is cleaved at these six sites to release seven final products (designated as NS1 through NS7 in Fig. 20.3C and Table 20.2). Five cleavage sites for the norovirus ORF1 polyprotein (represented by Southampton virus) have been mapped that would release six mature products.^{273,276} The noroviruses differ from the lagoviruses in that a protease cleavage site has not been directly mapped in the extreme N-terminal protein, although evidence suggests that additional processing (or modification) of the protein can occur in cells.^{419,434} The vesivirus cleavage

map (represented by FCV) contains five mapped cleavage sites to release six mature nonstructural proteins, and each cleavage event is essential in the virus replication cycle.⁴³⁵ The vesiviruses show yet another variation in processing, in that no evidence exists for efficient viral protease-mediated cleavage between the Pro and polymerase (Pol) proteins, even in virus-infected cells.^{306,357,435,439} In addition, vesiviruses bear a unique cleavage site in a capsid protein precursor protein that is processed by NS6^{Pro} to release the leader of the capsid (LC) and the major capsid protein, VP1^{438,470} (Fig. 20.3C). The predicted nonstructural protein cleavage map of human sapovirus strain, Mc10, shows an overall similarity with that of the vesiviruses.³⁵⁹ Sapoviruses (and other caliciviruses in which the capsid region is in frame with the nonstructural polyprotein) bear an additional protease cleavage site in the ORF1 polyprotein between the polymerase and capsid coding regions. A number of stable precursor proteins have been described also for the caliciviruses, and it is likely that these proteins have defined functions in replication.^{34,39,93,238,325,326,434,435}

The calicivirus dipeptide cleavage recognition sites are consistent with those described for the picornavirus 3C cysteine proteinase.¹⁷⁴ The calicivirus cleavage sites identified thus far have either a negatively charged glutamic acid (E) or polar glutamine (Q) in the first position (designated P1). More variation exists within the second position of the dipeptide cleavage site (designated P1'). Studies of the calicivirus proteinase substrate specificity have shown some tolerance in the P1' position at certain cleavage sites.^{174,438,505} The conformation of the protein surrounding the dipeptide recognition site is also important for efficient cleavage by the proteinase.^{174,439}

The availability of proteolytic cleavage maps for the calicivirus nonstructural polyproteins has enabled studies that elucidate the functions and structures of individual proteins.¹⁷⁵ Biochemical studies first confirmed enzymatic activities in calicivirus proteins corresponding to an NTPase (NTPase or NS3^{NTPase}),³⁰⁰ a chymotrypsin-like cysteine proteinase (Pro or NS6^{Pro}),⁵⁰ and an RNA-dependent RNA polymerase (Pol or NS7^{Pol}).⁴⁷⁹ The three-dimensional structures for the latter two enzymes have been reported. The norovirus Pro shares structural similarities with classical chymotrypsin-like serine proteases^{188,189,339,518} (Fig. 20.4A). A cleft containing the active site catalytic residues (His 30, Glu 54, and Cys 139 for Norwalk virus) involved in substrate cleavage is located between two domains. The N-terminal domain (blue) starts with an α -helix followed by a five-stranded twisted antiparallel β -sheet. The structure of this domain contains features of both the simpler four-stranded β -sheet found in the N-terminal domain of picornaviral 2A proteinases and the more commonly found complete β -barrels observed in the N-terminal domains of most chymotrypsin-like proteinases. The C-terminal domain (red) adopts the structure of a classical six-stranded β -barrel found in a wide range of viral and nonviral chymotrypsin-like proteinases. Co-crystallization of the protease with an active site-directed peptide inhibitor (acetyl-Glu-Phe-Gln-Leu-Gln-propenyl ethyl ester) has given detailed insight into the interaction of substrate with the catalytic residues such as Cys 139 (the active site nucleophile) within the cleft¹⁸⁹ (Fig. 20.4B). The norovirus RNA-dependent RNA polymerase has a classical "right hand" (finger, thumb, and palm) organization,^{346,347,517} with the C-terminus of the protein positioned in the active site cleft (Fig. 20.5A). Modeling of the

TABLE 20.2 Calicivirus Nonstructural Proteins

Calicivirus nonstructural protein (NS)	Picornavirus counterpart	Function	Reported properties (genus)	References
NS1	2A	Unknown	Cleavage and release essential for replication (VeV)	(435)
NS2	2B	Unknown	Binding partner with host cell VAP-A (NoV)	(125)
			Golgi co-localization and disassembly (NoV)	(129)
			C-terminal hydrophobic region (NoV)	(125,129)
			Present in membranous replication complexes (VeV)	(155)
			Interacts with NS3, NS4, NS6–7 (VeV)	(211)
NS3	2C	NTPase	NTP binding, hydrolysis of NTP (LaV, NoV)	(300,375)
			Present in membranous replication complexes (VeV)	(155)
NS4	3A	Unknown	Forms stable precursor with VPg: proposed VPg anchor (VeV)	(435)
			Present in membranous replication complexes (VeV)	(155)
			Inhibits actin cytoskeleton remodeling (NoV)	(182)
			ER/Golgi trafficking antagonist (NoV)	(422)
NS5	3B	VPg	Present in virions, covalently linked to RNA (VeV)	(58,180)
			Binding partner with eIF3 (NoV)	(103)
			Binding partner with eIF4E (VeV)	(148)
			Nucleotidylated by RdRP at conserved tyrosine (LaV)	(295)
			Functions in protein-primed RNA synthesis (NoV)	(399)
			Conserved tyrosine essential for replication of virus (VeV, NoV)	(327,446)
			Sequence homology with eIF1A (NoV, VeV)	(439)
			Present in membranous replication complexes (VeV)	(155)
			Linked to cellular trans-Golgi network protein 2 RNA in cells (NoV)	(446)
NS6	3C	Proteinase (Pro)	Proteinase activity, cysteine in active site (NoV, VeV, LaV, SaV)	(50,276,359,439)
			Mediates cleavage and release of ORF1-encoded proteins (LaV, NoV, VeV, SaV)	(276,359,439,506)
			Mediates cleavage of capsid precursor protein, PreVP1 (VeV)	(438)
			Cleaves cellular proteins (VeV, NoV)	(250,503)
NS7	3D	RNA-dependent RNA polymerase (Pol)	Structural analysis shows chymotrypsin-like folding (NoV)	(339)
			Contains Pol motif hYGDDhhY/V (VeV, NoV, LaV, SaV)	(199,309,325,342)
			<i>In vitro</i> activity is primer independent (LaV, NoV, VeV)	(134,479,493)
			Transcription activity on negative-strand template (LaV)	(331)
			Enzymatically active as ProPol (NS6–7) precursor form (VeV, NoV)	(33,493)
			Interacts with VPg, PreVP1, VP2 (VeV)	(211)
			Present in membranous replication complexes (VeV)	(155)
			Structural analysis reveals carboxyl-terminus in active site cleft (NoV)	(347)

ER, endoplasmic reticulum; LaV, Lagovirus; NoV, Norovirus; NTP, nucleotide triphosphate; NTPase, nucleotide triphosphatase; ORF1, open reading frame 1; RdRP, RNA-dependent RNA polymerase; SaV, Sapovirus; VeV, Vesivirus; VPg, virion protein, genome.

interaction of Pol with an RNA template in the presence of manganese and cytidine triphosphate (CTP) shows that the initiation of RNA synthesis occurs within the active site cleft (Fig. 20.5B–D). Binding of the primer/template RNA duplex displaces the C-terminal tail away from the active site, allowing the central helix of the thumb domain to position itself for interaction with the primer strand and minor groove of the primer–template duplex.⁵¹⁷ Two divalent metal ions (likely

Mg²⁺ in cells) help mediate catalysis by forming coordination bonds with three highly conserved aspartic acid residues and the nucleoside triphosphate (NTP). After nucleotidyl transfer has occurred, the pyrophosphate is released from the enzyme. The primer–template duplex is predicted to translocate in a manner that places the newly incorporated nucleotide into the same position as that of the 3' end of the primer strand immediately prior to nucleotidyl transfer. This translocation process

Norovirus Proteinase

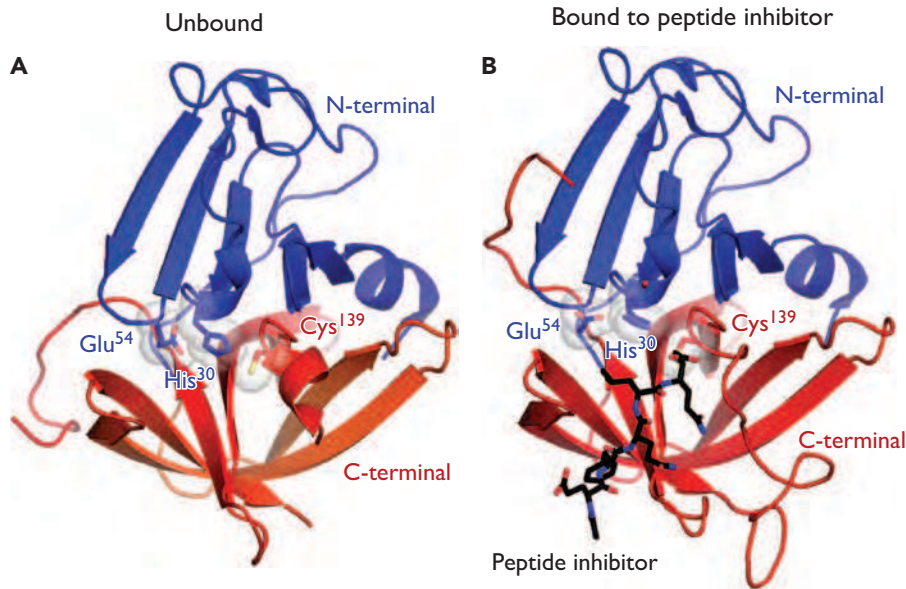


FIGURE 20.4. Structure of the norovirus proteinase. **A:** Three-dimensional ribbon representation of the Norwalk virus protease structure resolved at 1.5 Å shows that it adopts the conformation of a classical serine protease, in which a cleft containing the enzymatic active site is located between two β -barrel domains. The active site is positioned at the opening of the cleft and composed of residues His 30, Glu 54, and Cys 139 (the *catalytic triad*). The conformation of the loops and β -strands are thought to play a role in positioning the active site residues for proteolysis. **B:** Modeled interaction of Norwalk virus protease with an inhibitor that blocks proteolysis.^{188,189,339,518} (Images provided by K. Ng.)

provides the space needed to form the binding site for the next incoming nucleoside triphosphate.

Although the gene order of the nonstructural proteins and strong structural and functional homology in the Pro and Pol enzymes suggest a common ancestor for the caliciviruses and picornaviruses,³⁴² it is striking that several proteins encoded in the calicivirus ORF1 share little or no detectable sequence

relatedness with the picornaviruses. These include the extreme N-terminal proteins NS1 and NS2 (corresponding in gene order to the picornavirus 2A and 2B proteins), the NS4 (corresponding to the picornavirus 3A protein), and the NS5 (corresponding to the VPg). Although the roles of these proteins in replication require further investigation, evidence for some functional homology with the picornaviruses has been

Norovirus RNA-dependent RNA Polymerase

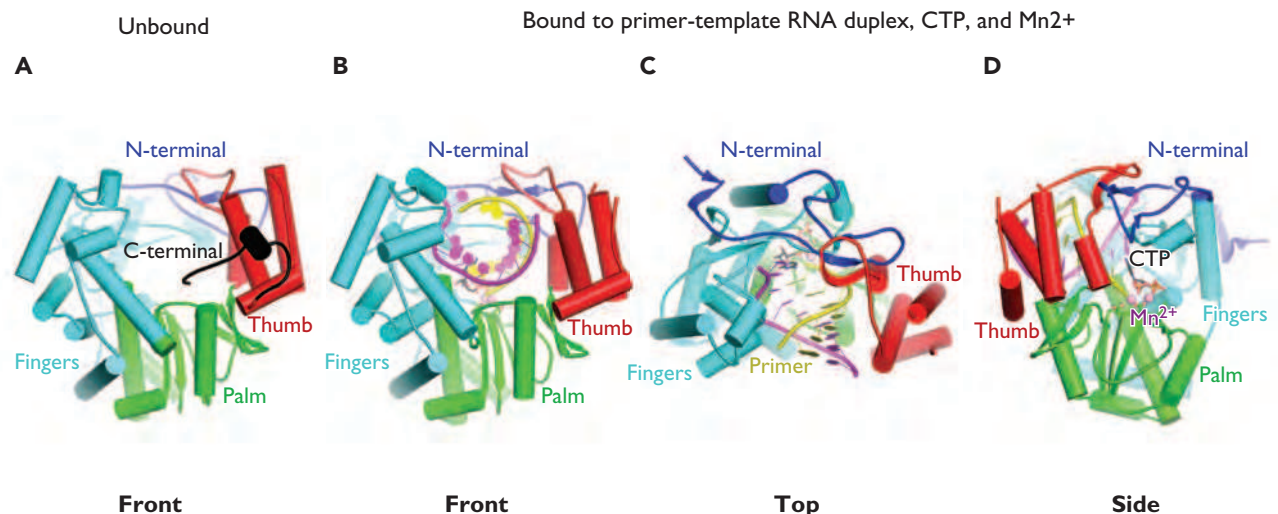


FIGURE 20.5. Structure of the norovirus RNA-dependent RNA polymerase. **A:** The norovirus RNA-dependent RNA polymerase adopts the classic “right hand” structure characteristic of polynucleotide polymerases as shown in this stick model. The fingers (blue) and palm (green) domains form a rigid unit, while the thumb (red) domain is flexible and can assume either a “closed” or “open” conformation. An N-terminal domain bridges the fingers and thumb domains. When the polymerase is unbound to template, the C-terminal end of the protein appears to lie within the active site cleft.³⁴⁷ **B–D:** The front, top, and side views, respectively, of the Norwalk RNA polymerase bound to a primer–RNA duplex, cytidine triphosphate (CTP), and metal divalent cation, Mn²⁺.^{24, 346,347,517} (Images provided by K. Ng.)

proposed.^{25,129,422} A summary of the calicivirus nonstructural proteins and their known properties is shown in Table 20.2.

STAGES OF REPLICATION

Replication Strategy

The replication strategy elucidated thus far for the caliciviruses shares many features with those of other positive-strand RNA viruses (Fig. 20.6). Caliciviruses attach and enter the cell, the

RNA genome is released, and translation of the genome occurs via the host cell machinery. Certain newly translated viral proteins interact with the host cell to establish defined sites of virus replication (characteristically involving reorganized intracellular membranes), while other proteins function as replicative enzymes. Newly synthesized positive-strand RNA genomes are covalently linked to VPg and packaged into virions that are released from lysed cells. The replication cycle of a calicivirus is rapid: new viral progeny can be detected within hours after infection.

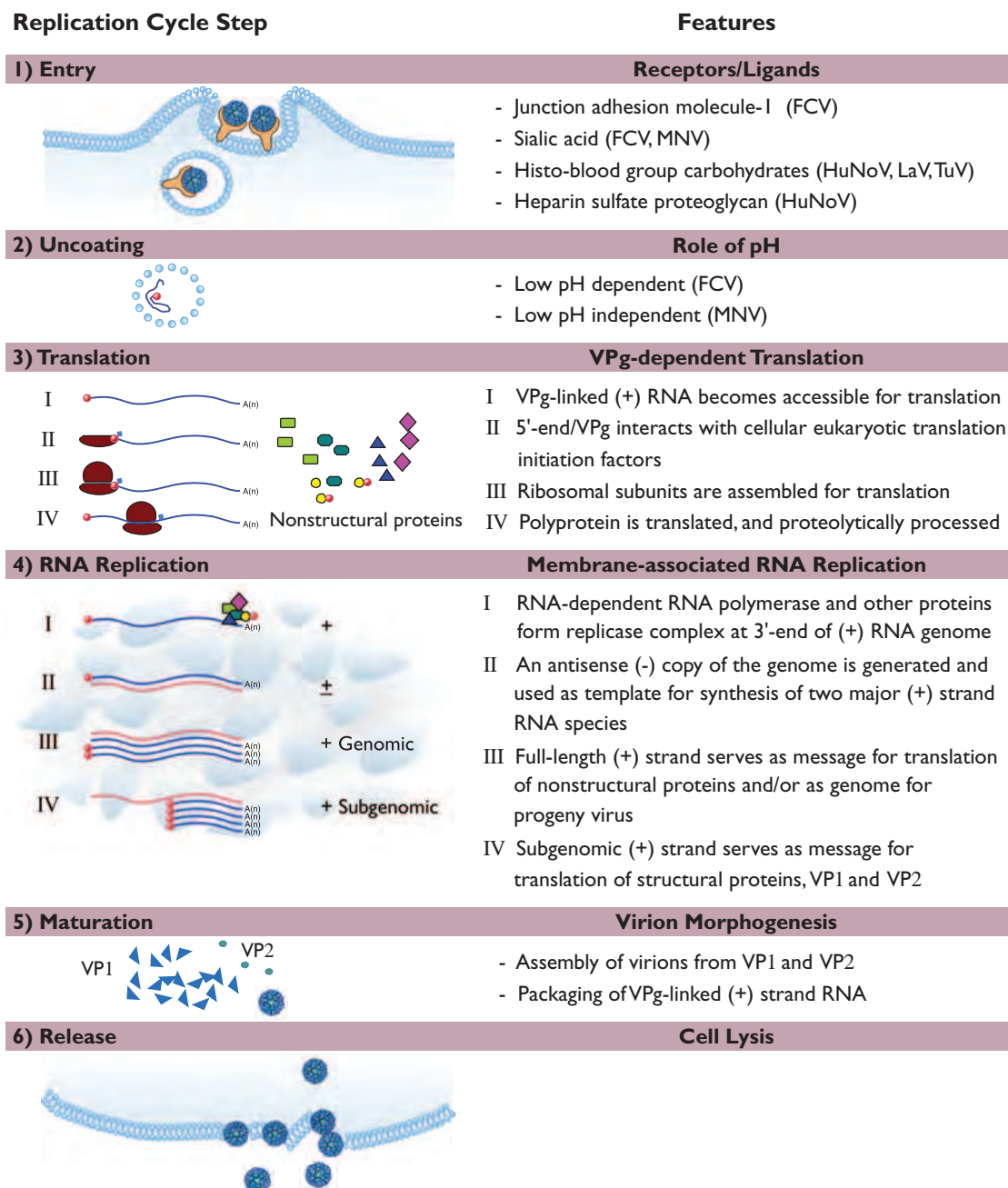


FIGURE 20.6. Schematic diagram of the proposed replication strategy of the caliciviruses. Consistent with other positive-strand RNA viruses, the replication cycle of a calicivirus involves the following stages: **(1)** entry, **(2)** uncoating, **(3)** translation, **(4)** RNA replication, **(5)** maturation, and **(6)** release, as reviewed in the text. FCV, feline calicivirus; LaV, lagovirus; MNV, murine norovirus; HuNoV, human norovirus; TuV, Tulane virus.

Reverse genetics systems have been developed for several caliciviruses, including FCV,⁴³² porcine enteric calicivirus,⁷⁷ Tulane virus,⁴⁹² RHDV,²⁷⁷ and murine norovirus,^{83,489,515} based on the construction of infectious full-length cDNA clones of the viral genome. Murine norovirus is presently the only norovirus that grows efficiently in cell culture,⁵⁰⁷ making this virus an important model.⁵⁰⁸ Human norovirus infectious cDNA clones have remained elusive in the absence of a fully permissive cell culture system to verify recovery of virus, but replication in cells can be studied following expression of proteins from full-length cDNA clones^{21,219} or in stable RNA replicon-bearing cell lines.⁷⁹

Mechanism of Attachment

Calicivirus virions must first interact with the host cell. Carbohydrates, including those present on various histo-blood group antigens (HBGAs), have been implicated in the binding of a number of calicivirus strains to cells^{245,301,403,443,455,462,516} (Fig. 20.6). Structural studies have verified that the norovirus VP1 interacts with HBGA carbohydrates^{61,91} (Fig. 20.7). Receptor recognition is essential for the caliciviruses, as transfection of infectious calicivirus RNA into nonpermissive cells (i.e., those that cannot be infected with virions) allows replication and recovery of infectious progeny virus.^{162,245,298} The junction adhesion molecule-1 (JAM-1), an immunoglobulin-like cellular membrane protein, has been identified as a functional receptor for FCV,²⁹⁹ making this the first experimentally verified cellular receptor in the family. Structural and modeling studies have shown that FCV interacts with feline (f) JAM-1 through binding of the P2 domain of the capsid to the distal membrane domain (D1) of fJAM-1^{37,38,363} (e-Fig. 20.3).

Nearly all norovirus VLPs bind to one or more HBGA carbohydrates, and as noted earlier, structural studies have defined interactions with certain HBGA saccharides at the amino acid level⁶¹ (Fig. 20.7). A correlation was found between susceptibility to Norwalk virus infection in adult volunteers and HBGA secretor status, which is linked to the *FUT2* gene²⁶⁹ (see Cell and Tissue Tropism). A similar pattern of genetic susceptibility to RHDV (lagovirus) infection in rabbits was associated with genes involved in the modification of HBGAs, indicating a role for blood group carbohydrates in host cell recognition by lagoviruses.¹⁶¹ Experiments to verify that HBGAs can serve as a functional receptor for the noroviruses have been unsuccessful, hampered in part by the unavailability of a permissive cell culture system. Expression of the human *FUT2* gene product (fucosyltransferase-2) in nonpermissive cells enhanced binding of Norwalk virus VLPs to cells *in vitro* but did not render them permissive for the virus.¹⁶²

Mechanism of Entry and Intracellular Trafficking

Variation has been noted among caliciviruses in their requirements for entry into cells and subsequent infection. Feline calicivirus replication was inhibited by chloroquine, a reagent that raises lysosomal pH, indicating dependence on a low pH step during entry.^{244,444} In contrast, murine norovirus (strain MNV-1) was not dependent on the acidification of endosomes for infectivity.^{143,373} Feline caliciviruses use clathrin-mediated endocytosis for entry into mammalian cells,⁴⁴⁴ whereas MNV-1 apparently does not enter via clathrin- or caveolin-mediated pathways.^{143,373} An endocytic pathway was proposed for MNV-1 entry that likely involves cholesterol-sensitive lipid rafts and dynamin II (at least in RAW264.7 cells).^{143,373,374}

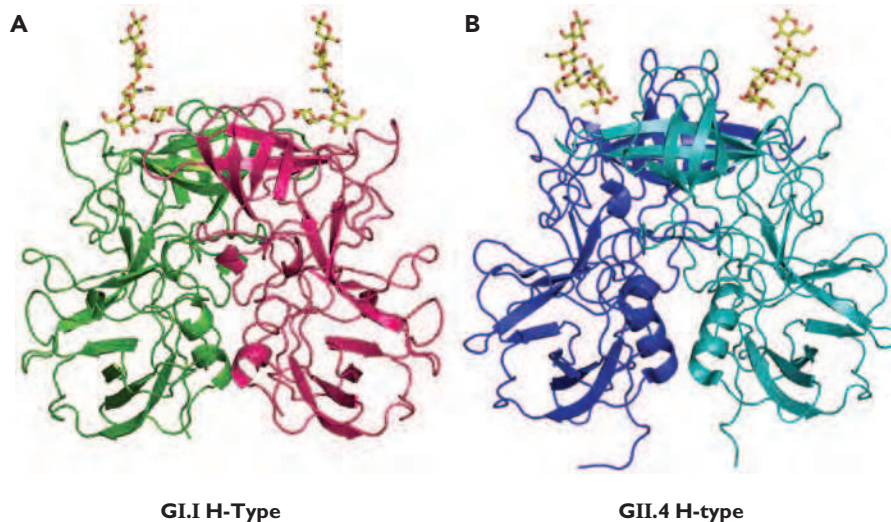


FIGURE 20.7. Interaction of representative norovirus VP1 dimers with H carbohydrates from histo-blood group antigens (HBGAs). For both GI.1 and GII.4 noroviruses, HBGA carbohydrates interact with the distal surface of the capsid, but differences in these interactions have been noted. The Norwalk virus (GI.1) carbohydrate binding site contains residues that project from a well-structured antiparallel β -sheet near the P domain dimeric interface and favors a precise and limited recognition of a terminal Gal-Fuc or Gal-acetamido combination. In contrast, the VA387 (GII.4) binding site is formed by residues located in two surface-exposed loops near the P domain dimeric interface that broadly recognize a terminal fucosyl moiety. (Courtesy of B. V. V. Prasad with data adapted from Cao S, Lou Z, Tan M, et al. Structural basis for the recognition of blood group trisaccharides by norovirus. *J Virol* 2007;81:5949–5957; and Choi JM, Hutson AM, Estes MK, et al. Atomic resolution structural characterization of recognition of histo-blood group antigens by Norwalk virus. *Proc Natl Acad Sci U S A* 2008;105:9175–9180.)

Uncoating

The uncoating events that allow the calicivirus positive-sense RNA genome to become accessible to the cellular translational machinery have not been defined. Uncoating is a rapid process: MNV genome was released within 1 hour after infection.³⁷⁴ Binding to the cellular receptor induces a conformational change in the capsid of FCV, suggesting that uncoating may, in part, be receptor binding mediated.³⁷ FCV infection is associated with an increase in permeability of the host cell membrane soon after virus entry via clathrin-coated endosomes and exposure to low pH.⁴⁴⁴ It was proposed that this acidification process might change the conformation of the FCV virion structure to facilitate release of the viral RNA.⁴⁴⁴ Analysis of norovirus (Norwalk) VLPs by mass spectrometry has shown that they are stable under acidic conditions; a disassembly model has been proposed in which T = 3 particles (containing 180 copies of VP1) disassociate into predominantly dimers in the presence of alkaline conditions.⁴²⁶

Translation

Calicivirus genomic RNA in virions requires the presence of the covalently linked VPg protein in order to establish an infection after release (or transfection) into cells.⁵⁸ The initiation of translation of the incoming positive-strand genome is likely mediated through interactions of the VPg protein with the cellular translation machinery.^{82,103,148,165} Calicivirus replication is associated with an inhibition of host cell translation, and the viral proteinase (NS6^{Pro}) has been shown to cleave certain cellular proteins involved in translation, which may, in part, give the viral RNA a competitive advantage.²⁵⁰ The ORF1 of the virus is translated first to produce a large polyprotein, which is processed rapidly into precursors and products by NS6^{Pro} at several essential cleavage sites.⁴³⁵ Some of these nonstructural proteins and their precursors function to set up replication sites within the host cell,^{25,194} while others (such as the RNA-dependent RNA polymerase, NS7^{Pol}) play a role in replication of the viral RNA (Table 20.2).

An abundant VPg-linked subgenomic positive-strand RNA serves as a bicistronic message for translation of the structural proteins VP1 and VP2,^{179,344} and the regulation of translation of VP2 from the subgenomic RNA (translated at approximately 20% of the levels of VP1) was mapped to an upstream RNA sequence element in the VP1 coding region of approximately 70 nucleotides designated as the termination upstream ribosomal binding site (TURBS).^{322,323} The TURBS site contains two motifs, one of which mediates base pairing between the viral subgenomic messenger RNA and the cellular 18S ribosomal subunit.³²² This interaction may function as a “tether,” positioning the ribosome for immediate reinitiation of translation following termination of translation of the VP1 gene.^{291,292}

Replication of Genomic Nucleic Acid

As with all other positive-strand RNA viruses, the replication of calicivirus RNA is associated with host cell membranes.¹⁵⁵ A marked rearrangement of intracellular membranes occurs, and evidence exists for the initiation of RNA replication in a perinuclear site that contains membranes associated with endoplasmic reticulum, trans-Golgi network, and endosomal membrane markers.^{25,194} The initiation of synthesis of an anti-sense (negative)-strand RNA from the genomic RNA template occurs beginning at the 3′ end of the genomic positive-strand

RNA and likely involves interactions with cellular proteins.¹⁶⁶ The negative-strand RNA, in turn, serves as a template for transcription of two major positive-strand RNA species corresponding to the full-length genome (genomic RNA) and the approximately terminal one-third of the genome toward the 3′ end (subgenomic RNA).³⁴⁴

The calicivirus RNA genome bears conserved regions of secondary structure,^{292,378,429} and functional RNA regulatory elements have been mapped both internally and near the ends of the genome.²⁶ Transcription from the start site for the subgenomic RNA species on the negative-strand template (nt 5,296 of RHDV) was found to require an upstream sequence of 50 nt for full polymerase activity in *in vitro* studies,³³¹ consistent with its role in the formation of a subgenomic promoter. The corresponding region of MNV was mapped also as bearing a promoter for subgenomic RNA synthesis from the negative-strand template.⁴²⁹

Assembly

A two-stage process has been proposed for the maturation of calicivirus (FCV) particles.²³⁷ The first stage involves the rapid aggregation and assembly of capsid precursor proteins into 5S subunits, which then pass through several intermediate forms (with varying stability) to form stable 15S subunits. The second stage involves the association of 15S subunits with newly synthesized RNA genomes to form infectious particles (that sediment at 170S). Protein–protein interactions have been detected between the FCV VPg (covalently linked to the RNA genome) and the capsid precursor as well as between the RNA-dependent RNA polymerase NS7^{Pol} and the capsid precursor, suggesting that these interactions may be related to packaging of the newly synthesized RNA into the viral capsid.²¹¹ In addition, successful assembly of the infectious FCV virions was linked to an efficient expression of the virus minor capsid protein, VP2.⁴³³

The VPg-linked genomic and subgenomic positive-strand RNA species are found in FCV and RHDV particles of distinct densities, indicating that they are not packaged together in the same virion.^{324,343} The incorporation of subgenomic RNA into the lower-density (LD) particles suggests that a packaging signal is located within the 3′ terminal 2,400 nucleotides of the genome. It has been suggested that LD particles (which would not be infectious) may be associated with FCV strains of higher virulence, but their function, if any, in the virus life cycle is unknown.³⁴³

Release

Calicivirus-infected cells undergo lysis, and it is presumed that the majority of progeny viruses are released during this process. The triggering of apoptosis has been associated with calicivirus infection both *in vitro*^{7,49,135,341,437} and *in vivo*,^{12,84,208,223,334,337,386,472} and apoptotic changes in cellular membranes may be one of the mechanisms by which cells lyse and release viral particles.

PATHOGENESIS AND PATHOLOGY

Caliciviruses cause a broad range of diseases in many different animal hosts (Table 20.3). This section will focus on the noroviruses, but there are important common themes in pathogenesis shared by all caliciviruses. Illnesses range from

TABLE 20.3 Pathogenesis and Disease Manifestations of Representative Calicivirus Strains

Calicivirus strain	Genus	Host	Site of replication ^a	Clinical disease ^b
Norwalk virus	Norovirus	Human	Enteric	Gastroenteritis
Jena virus	Norovirus	Cattle	Enteric	Gastroenteritis
Murine norovirus-1 (MNV-1)	Norovirus	Mouse	Enteric, lymphoid cells	Fulminant organ dysfunction ^c
Pistoia virus	Norovirus	Lion	Enteric	Hemorrhagic enteritis ^d
Canine norovirus	Norovirus	Dog	Enteric	Gastroenteritis
Swine 43	Norovirus	Pig	Enteric	Not established
Sapporo virus	Sapovirus	Human	Enteric	Gastroenteritis
Porcine enteric calicivirus (Cowden)	Sapovirus	Pig	Enteric	Gastroenteritis
Mink enteric calicivirus	Sapovirus	Mink	Enteric	Gastroenteritis
Rabbit hemorrhagic disease virus	Lagovirus	Rabbit	Liver, systemic	Organ dysfunction, pulmonary hemorrhage ^e
European brown hare syndrome virus	Lagovirus	Hare	Liver, systemic	Organ dysfunction, pulmonary hemorrhage
Bovine enteric calicivirus (Newbury-1 virus)	Nebovirus	Cattle	Enteric	Gastroenteritis
Bovine enteric calicivirus (Nebraska virus)	Nebovirus	Cattle	Enteric	Gastroenteritis
Feline calicivirus	Vesivirus	Cat	Mouth, upper respiratory	Stomatitis, pneumonia
Feline calicivirus-VS	Vesivirus	Cat	Systemic	Fulminant organ dysfunction ^f
San Miguel sea lion virus	Vesivirus	Sea lion	Mucosal, systemic	Skin (flipper) lesions, pneumonia
Canine calicivirus (No. 48)	Vesivirus	Dog	Enteric	Gastroenteritis
Vesivirus isolate 2117	Vesivirus	Unknown	Unknown	Unknown ^g
Tulane virus	Unclassified	Monkey	Enteric	Gastroenteritis
St. Valérian virus (AB90)	Unclassified	Pig	Enteric	Not established
Bayern virus	Unclassified	Chicken	Enteric	Not established

^aSite of replication is based on observed pathologic effects in tissue and in some cases, confirmed by immunohistochemistry. For many caliciviruses, the primary site of replication in the host has not been fully established.

^bThe characteristic clinical disease associated with each representative calicivirus strain is shown. Swine norovirus,⁴⁴⁸ St. Valérian virus,²⁵¹ and Bayern virus⁵⁰⁹ were detected in screening surveys of nonselected stool specimens: the clinical disease outcome has not been established. Norwalk virus, representative of the human noroviruses associated with acute gastroenteritis, is generally associated with self-limiting vomiting and diarrhea of 24 to 48 hours' duration. However, the illness can be severe and life-threatening in certain individuals (see text). Sapporo virus, representative of the human sapoviruses associated with acute gastroenteritis, has most often been associated with self-limiting gastroenteritis in younger age groups.⁸⁹ In general, infection outcome in the family *Caliciviridae* can range from asymptomatic (subclinical) to lethal.

^cMost variants of the MNV-1 strain of murine norovirus are lethal only in certain mice that lack an innate immune system, but evidence exists for varying pathogenicity among MNV strains and variants.^{26,217,467,507}

^dFirst case report described illness in one lion cub,³⁰⁴ natural history not established.

^eRabbit hemorrhagic disease virus emerged as a highly lethal disease in rabbits in China in 1984,²⁹⁰ but was likely endemic in rabbits worldwide prior to 1984.²²⁴

^fFeline calicivirus strains have emerged in recent years designated as "virulent systemic" (VS) that are associated with high morbidity and mortality in cats. In addition, the virus can cause a persistent, asymptomatic infection in cats.³⁸⁵

^gVesivirus 2117 was discovered as a contaminant in cultured Chinese hamster ovary cells.³⁵⁷

mild to life-threatening, and evidence exists for the emergence of calicivirus strains with increased virulence. Asymptomatic infection occurs in susceptible populations,^{106,377} and shedding of virus can extend days to weeks after resolution of acute symptoms.^{24,140}

Adult volunteer studies have been successful in defining important features of norovirus gastroenteritis, pathogenesis, and immunity.²¹³ Presently, there is no animal model that directly recapitulates the full range of disease symptoms observed in humans (see Clinical Features), but animals in which evidence for infection has been reported following challenge with human noroviruses include gnotobiotic piglets⁸⁴ and calves,⁴⁴⁰ monkeys,^{394,447} and chimpanzees.^{48,158,512}

Noroviruses have been detected in the feces of mice (e.g., strain MNV-1),^{210,217,337} pigs (strain SW418),⁴⁴⁸ cattle (strain Jena virus),²⁷⁵ and dogs (strain 170/07),³⁰⁵ leading to the investigation of these viruses as model systems for the human

noroviruses. Bovine norovirus (strain Jena) caused diarrhea and severe blunting of the small intestinal villi in newborn calves similar to that observed in human norovirus infection, and virus antigen-positive cells were observed in epithelial cells of the villi and the lamina propria.³⁶⁴

Entry into the Host

Noroviruses enter the body predominantly via the oral route. Virions are acid stable, consistent with an ability to survive passage through the stomach. Indirect evidence from epidemiologic studies suggests that viruses can enter also via aerosols, such as in those generated from the explosive vomiting that often occurs during illness.^{66,70,302,303,372}

Based on volunteer studies with NV, the incubation period is short, ranging from 10 to 51 hours, with a mean of 24 hours.^{24,43,112,113,442,511} Acute illness usually lasts about 24 to 48 hours. The incubation period recorded in 22 naturally

occurring outbreaks of norovirus gastroenteritis was between 24 and 48 hours in 20 of the outbreaks, and the range was from 4 to 77 hours.²¹⁶ The incubation period of experimentally induced Snow Mountain virus illness ranged from 19 to 41 hours, with a mean of 27 hours.¹¹⁶

Studies in volunteers demonstrated that the Norwalk, Hawaii, Montgomery County, and Snow Mountain viruses induced gastroenteritis when administered by the oral route.^{112,113,206,265,332,442,511} Transmission via the respiratory route has not been established for this group of agents, although it has been suggested from epidemiologic observations in selected settings.^{66,71,302,408} The virus was detected in vomitus obtained from infected volunteers¹⁵⁷; vomitus has been considered to be a source of transmission in certain settings.⁷⁰ Oral-to-oral transmission has been suggested: norovirus RNA was detected in washings from the mouths of individuals with norovirus infection (in one case, up to 2 weeks postillness) and in 10/17 (59%) of patients hospitalized with norovirus gastroenteritis who had vomited within 24 hours prior to sample collection.²³¹ Nasopharyngeal washings from a volunteer with experimentally induced Norwalk gastroenteritis did not induce illness in three volunteers.¹¹²

Site of Primary Replication

The site of primary replication for the noroviruses has not been established, but it is assumed that they replicate in the upper intestinal tract. Biopsies of the jejunum of volunteers who develop gastrointestinal illness following oral administra-

tion of the Norwalk or Hawaii virus exhibit histopathologic lesions.^{3,114,413,414} A broadening and blunting of the villi of the proximal small intestine occurs, although the mucosa itself remains histologically intact (Fig. 20.8). Infiltration with mononuclear cells and cytoplasmic vacuolization is also observed. Biopsies obtained during the convalescent phase of illness are normal. Blunting and atrophy of the villi was observed also in the small intestine of pigs infected with porcine enteric calicivirus (PEC), a sapovirus,¹³² and in newborn calves infected with Jena virus, a bovine norovirus.³⁶⁴ When viewed by transmission EM, the epithelial cells are intact, but there is shortening of the microvilli. Virus has not been observed in epithelial cells of the mucosa by EM. Of interest is that the characteristic jejunal lesion has also been observed in volunteers who were fed NV or Hawaii virus but who did not become ill.^{320,413,414} Histologic lesions are not observed in the gastric fundus, antrum, or rectal mucosa of volunteers with NV-induced illness.⁵⁰² The examination of intestinal biopsies from pediatric patients infected with norovirus who had undergone small intestinal transplantation showed increased mononuclear infiltrates in the lamina propria and villous blunting when compared with uninfected controls.³³⁴ Duodenal biopsies from immunocompetent individuals with acute norovirus gastroenteritis showed evidence of shortened villus height (which decreased the overall villus surface area by approximately 47%) and noted also the infiltration of a dense population of intraepithelial CD8 lymphocytes.⁴⁷²

A transient malabsorption of fat, d-xylose, and lactose is observed during experimentally induced NV illness.^{43,413} Levels

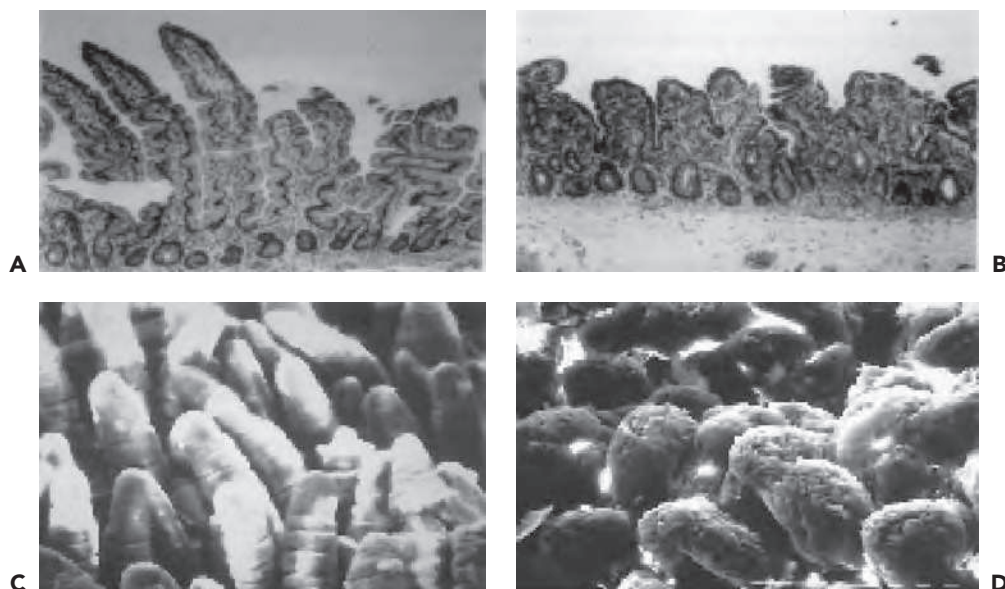


FIGURE 20.8. Pathology of norovirus and sapovirus infection in the jejunum. **A:** Normal-appearing jejunal tissue from biopsy of a volunteer prior to challenge with Norwalk virus (NV). Hematoxylin and eosin (H&E), 390. **B:** Broadened and flattened villi in jejunal biopsy tissue from same volunteer during illness with Norwalk-induced gastroenteritis. H&E, 390. **C:** Scanning electron microscopy (EM) showing normal-appearing jejunal tissue from biopsy of pig prior to challenge with porcine enteric calicivirus. **D:** Scanning EM showing shortening, blunting, fusion, or absence of villi in jejunal biopsy from same pig following challenge with porcine enteric calicivirus. (**A,B** from Agus SG, Dolin R, Wyatt RG, et al. Acute infectious nonbacterial gastroenteritis: intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man. *Ann Intern Med* 1973;79:18–25, with permission. **C,D** from Flynn WT, Saif LJ, Moorhead PD. Pathogenesis of porcine enteric calicivirus-like virus in four-day-old gnotobiotic pigs. *Am J Vet Res* 1988;49:819–825, with permission.)

of small intestinal brush-border enzymes (trehalase and alkaline phosphatase) were significantly decreased when compared with baseline and convalescent-phase values, whereas adenylate cyclase activity in the jejunum was not elevated following NV- or Hawaii virus—induced illness.^{3,265} Gastric secretion of hydrochloric acid, pepsin, and intrinsic factor did not appear to be altered during NV illness.³²⁰ Elevation of serum transaminase levels was reported in four pediatric patients at approximately two weeks following acute illness.⁴⁷⁴

A marked delay in gastric emptying was observed in infected volunteers who became ill and developed the typical jejunal mucosal lesion.³²⁰ It has been proposed that abnormal gastric motor function is responsible for the nausea and vomiting associated with these viral agents.³²⁰ An analysis of epithelial barrier and secretion function in norovirus-positive human duodenal biopsy tissue showed that the tight junction proteins occludin, claudin-4, and claudin-5 were reduced, and anion secretion was stimulated.⁴⁷² It was proposed that norovirus diarrhea could result from both epithelial barrier and secretory pathway dysfunction.

Spread in the Host

The major site of norovirus replication is presumably the upper small intestinal tract (duodenum and upper jejunum), but there is evidence that extraintestinal spread of the virus can occur. Viral RNA has been detected in serum and cerebrospinal fluid of a 23-month-old patient with encephalopathy.¹⁹⁶ In an analysis of 39 patients with acute norovirus gastroenteritis (confirmed by the detection of viral RNA in stool), norovirus RNA was detected in the serum of 6 (15%) patients by real-time PCR.⁴⁵³ Human noroviruses can establish an enteric infection in gnotobiotic piglets⁸⁴ and chimpanzees⁴⁸ when administered by the intravenous route.

Cell and Tissue Tropism

Human noroviruses are presumed to target cells in the small intestine due to the observed pathologic lesion (blunted villi), but the specificity of this interaction has been difficult to establish. Radiolabeled Norwalk rVLPs bind to a variety of cultured cell types, and in certain cells, they are apparently internalized with a low efficiency.⁴⁹⁷ Norwalk VLPs bind to the surface of human intestinal epithelial cells bearing H1 or H2/3 HBGAs present in fixed tissue sections from secretor-positive (but not secretor-negative) individuals,³⁰¹ but the expression of these antigens on the surface of Huh-7 cells (a human liver cell line) does not confer full susceptibility to a productive Norwalk virus infection.¹⁶² It was speculated that human noroviruses might bind nonepithelial cells in the gut: Sakai virus (a GII norovirus) was shown to bind to cells within the lamina propria and Brunner gland of human duodenum,⁷³ and chimpanzees infected with Norwalk virus displayed Norwalk virus antigen-positive cells within the lamina propria of the upper small intestine.⁴⁸ The murine norovirus infects macrophage and dendritic cells of the mouse immune system,⁵⁰⁷ but this cell tropism has not been confirmed for human noroviruses in *in vitro* studies.²⁵⁷ Gnotobiotic piglets challenged with a GII.4 norovirus strain displayed virus-positive enterocytes in the duodenum and jejunum.⁸⁴

For years speculation was that a genetically determined variation in virus receptors in the intestinal tract might be responsible for long-term resistance to NV.^{30,41,98,239,369} Recent studies

have found evidence for such a mechanism that involves the ABH and Lewis carbohydrate antigens present on gut epithelial cells,³⁰¹ which is reviewed elsewhere.^{117,191,261,262,461} Expression of these carbohydrate antigens on cells is controlled by enzymes that are products of alleles at the ABO, FUT2, and FUT3 loci. Individuals with two mutated FUT2 alleles, and therefore devoid of H-type antigens on their gut (and other mucosal) epithelial cells, are called *nonsecretors*. It was first observed that RHDV (lagovirus) virions and RHDV rVLPs were able to bind to ABH histo-blood group antigens (present also on human blood cells) that were expressed on epithelial cells of the respiratory and digestive tracts of rabbits.⁴⁰³ Furthermore, the binding of RHDV to human blood cells was blocked by saliva from human individuals who were secretor positive for H carbohydrates, but not from individuals who were nonsecretors. The subsequent discovery that Norwalk VLPs could bind to HBGAs expressed on human intestinal epithelial cells³⁰¹ suggested that genetically determined host factors relating to histo-blood group antigens might play a role in mediating susceptibility to infection. This hypothesis was confirmed in human volunteers, in which the secretor status of the individual was a major correlate in susceptibility to infection with NV.²⁶⁹ On oral challenge with NV, only secretor individuals with H type 1 antigens were susceptible to infection, whereas nonsecretors were resistant. In a retrospective analysis of volunteers in earlier NV challenge studies, the secretor status was again identified as a major correlate of susceptibility to infection.¹⁹⁰ Furthermore, the ABO blood group antigens have also been linked to susceptibility to Norwalk infection, with B blood group antigen individuals rarely showing evidence of infection and illness with NV.^{192,269} Variation likely exists among the noroviruses in their recognition of host cell carbohydrates.^{61,91,186,461} Infection in volunteers challenged with GII Snow Mountain virus (unlike those challenged with NV) showed no correlation between ABH secretor status and susceptibility,²⁶⁸ suggesting that the link to protection in type B individuals might be limited to GI noroviruses.³⁹⁵ Attempts to correlate susceptibility with ABH secretor status or blood type in naturally occurring disease have yielded variable results.^{170,255,351,441}

The role of HBGA recognition by noroviruses has been the subject of intense study from the perspective of structure,²⁴² evolution,^{47,105,272} and host restriction.^{63,262} It is not yet clear whether these molecules serve only as attachment ligands on host cells or are functional receptors that enable the entry of virus (see Mechanism of Attachment).

Immune Response

Immunity to noroviruses in humans is poorly understood.³¹⁰ Adults consistently demonstrate a high degree of susceptibility to both naturally occurring and experimentally induced NV illness. In some norovirus outbreaks, more than 80% of adults became ill.²¹⁶ In addition, approximately 50% of unselected adult volunteers consistently developed illness following challenge with NV.^{43,112,442,511} Resistance to norovirus illness likely involves a complex interplay between the genetic and immunological susceptibility of the host and exposure to evolving norovirus strains.

Adaptive Immunity

Because serum or intestinal secretory neutralizing antibodies to the noroviruses cannot yet be measured in tissue culture, most

of the information about immunity comes from early volunteer studies. Although the use of high doses of challenge virus in these early studies has been noted,¹⁴⁷ they established that there are two forms of immunity to NV: one is short term and the other is long term.^{112,369,511} Short-term immunity, which follows the traditional pattern, is apparently virus (possibly serotype) specific. Thus, volunteers who become ill following NV (GI.1) challenge are usually resistant to rechallenge with this virus 6 to 14 weeks later. Challenge of such volunteers with the heterotypic Hawaii virus (GII.1), however, induces illness. Similarly, volunteers who recently became ill following infection with Hawaii virus are susceptible to challenge with NV.⁵¹¹ Long-term immunity, however, deviates from the traditional pattern. Twelve volunteers who were challenged with the NV on two occasions, 27 to 42 months apart, exhibited two different patterns of resistance to sequential challenge.³⁶⁹ Six volunteers developed gastrointestinal illness following the initial challenge, and they developed it again following rechallenge 27 to 42 months later. In contrast, the other six individuals failed to become ill following the initial challenge and were also resistant after rechallenge 31 to 34 months later. Serologic studies in which prechallenge serum antibodies to NV were measured by IEM and radioimmunoassay (RIA) failed to provide an explanation for the difference in susceptibility.^{99,156,369} Paradoxically, volunteers who did not become ill had little, if any, antibody to NV measurable by IEM in either prechallenge serum specimen.³⁶⁹ Also, they failed to develop a significant serologic response following each challenge. Volunteers who became ill following each challenge, and who were evaluated serologically, developed a serologic response after each challenge, however. The observation that serum antibody to NV does not correlate with resistance to illness has been reproduced in more recent volunteer studies.^{149,206,269} It was found also that the levels of local jejunal antibodies did not correlate with resistance to illness in volunteer studies and during natural outbreaks of disease.^{30,42,156,205,206,297} Naturally acquired immunity, however, may play some role in protection at the mucosal level. A recent volunteer study showed that the development of a rapid mucosal IgA response (indicating prior exposure to NV or a related norovirus) was associated with resistance to illness following challenge with NV.²⁶⁹ Furthermore, antibody was shown to be critical for the clearance of MNV in the mouse model.⁶⁸

The discovery that NV and other human noroviruses recognize HBGAs has led to the development of assays that measured HBGA carbohydrate-blocking activity (or "blockade") of serum antibodies.²⁷⁹ The presence of antibodies that blocked binding of Norwalk VLPs to H type 1 or H type 3 glycans at the time of Norwalk virus challenge was shown to correlate with protection from illness in adult volunteers, leading to the proposal that HBGA blocking assays might serve as a surrogate test for neutralization in the absence of traditional cell culture-based virus neutralization assays.³⁸⁷ Consistent with this, chimpanzees vaccinated with a Norwalk VLP immunogen and resistant to Norwalk virus infection when subsequently challenged were shown also to develop serum antibodies that block binding of Norwalk VLPs to synthetic HBGA carbohydrates.⁴⁸ Application of this technique in the first efficacy study of norovirus vaccines in adult volunteers showed a correlation between levels of prechallenge HBGA-blocking titers and protection from illness.²² A hemagglutination inhibition assay (HAI) to

measure serum antibodies was shown also to correlate with protection from illness in volunteers challenged with Norwalk virus.^{101,193}

Cell-Mediated Immunity

Cell-mediated immune responses were studied in volunteers following oral immunization with Norwalk rVLPs.⁴⁵² The VLPs elicited a cell-mediated immune response that included virus-specific proliferative responses. There was an increase in γ -interferon (IFN) in the absence of IL-4 production, suggesting a dominant T-helper type 1 (Th-1) pattern of cytokine production. In a study of 15 volunteers infected with Snow Mountain virus, significant increases in serum γ -IFN and IL-2, but not IL-6 or IL-10, were detected on day 2 after challenge, again showing a dominant Th-1 response.²⁶⁸ Depletion of CD4+ cells prior to stimulation of peripheral blood mononuclear cells with norovirus antigen led to a decrease in γ -IFN, again consistent with a Th-1 dominant response that is characteristic of cell-mediated immunity.²⁶⁸

Both CD4 and CD8 T cells are required for virus clearance from the intestine following challenge with MNV.⁶⁷ Evidence for cross-reactive T-cell epitopes shared among different norovirus genogroups and genotypes was found in mice immunized with VLPs representing human and murine noroviruses.²⁸⁰

Innate Immunity

The innate immune response was found to be important in the control of murine norovirus (strain MNV-1) in mice: certain mouse strains deficient in components of the innate immune system (such as STAT1 or receptors for interferon) developed a disseminated, lethal infection when challenged with MNV-1.²¹⁷ It has been reported that MDA-5, an intracellular sensor of double-stranded RNA, may be involved in the recognition and control of MNV.³¹⁵ Caliciviruses have been shown to be sensitive to various types of interferon *in vitro*.^{76,78,79,80,229,328}

Release from Host and Transmission

Noroviruses are released from the enteric tract of the host in feces and have been detected also in vomitus.^{151,157,227,317} Viral RNA can be detected in feces before the onset of symptoms and shedding in stool can last several days to weeks in immunocompetent individuals^{24,141,149,360} and even longer in immunocompromised patients.^{136,222,223,289,406,450} In a study of 13 elderly patients (60 to 98 years of age), the average duration of norovirus shedding was 14.3 days, with some individuals shedding up to 32 days.¹⁶ In pediatric patients (3 months to 7 years of age), the average length of shedding was 16 days, with three patients younger than 6 months of age shedding for more than 40 days.³³⁸ Studies of the natural history of norovirus in a community found that 26% of 99 patients examined shed virus (detected by reverse transcriptase [RT]-PCR) up to 3 weeks after the onset of illness, with the highest rate (38%) of prolonged shedding in children younger than 1 year of age.³⁹³ This observation indicates that infected individuals recovering from norovirus illness can continue shedding beyond the symptomatic period, a finding that has implications in the management of outbreaks.^{100,329,367,388,477,496} Norovirus RNA has been detected in washings from the mouths of individuals who experienced norovirus infection for several days after symptoms subsided, suggesting that oral-to-oral transmission of norovirus might occur.²³¹ The detection of norovirus RNA

in the sera of children with gastroenteritis has been reported, but it is not known whether this RNA corresponds to that of circulating infectious virus (viremia) or noninfectious (inactivated) virus present in circulating immune cells.⁴⁵³

Noroviruses are spread by several modes of transmission (see Fig. 20.9B for a summary of the reported modes of transmission in 5,036 norovirus outbreaks that occurred in Europe

between 2000 and 2011). The predominant modes of transmission for the noroviruses are person-to-person contact and food- or water-borne spread.²⁴⁸ Contamination of surfaces and objects by infected individuals can lead to inadvertent exposure.^{184,283,477} Several epidemiologic investigations have linked exposure to noroviruses in air or in aerosolized vomitus with infection.^{31,66,142,302,303,408}

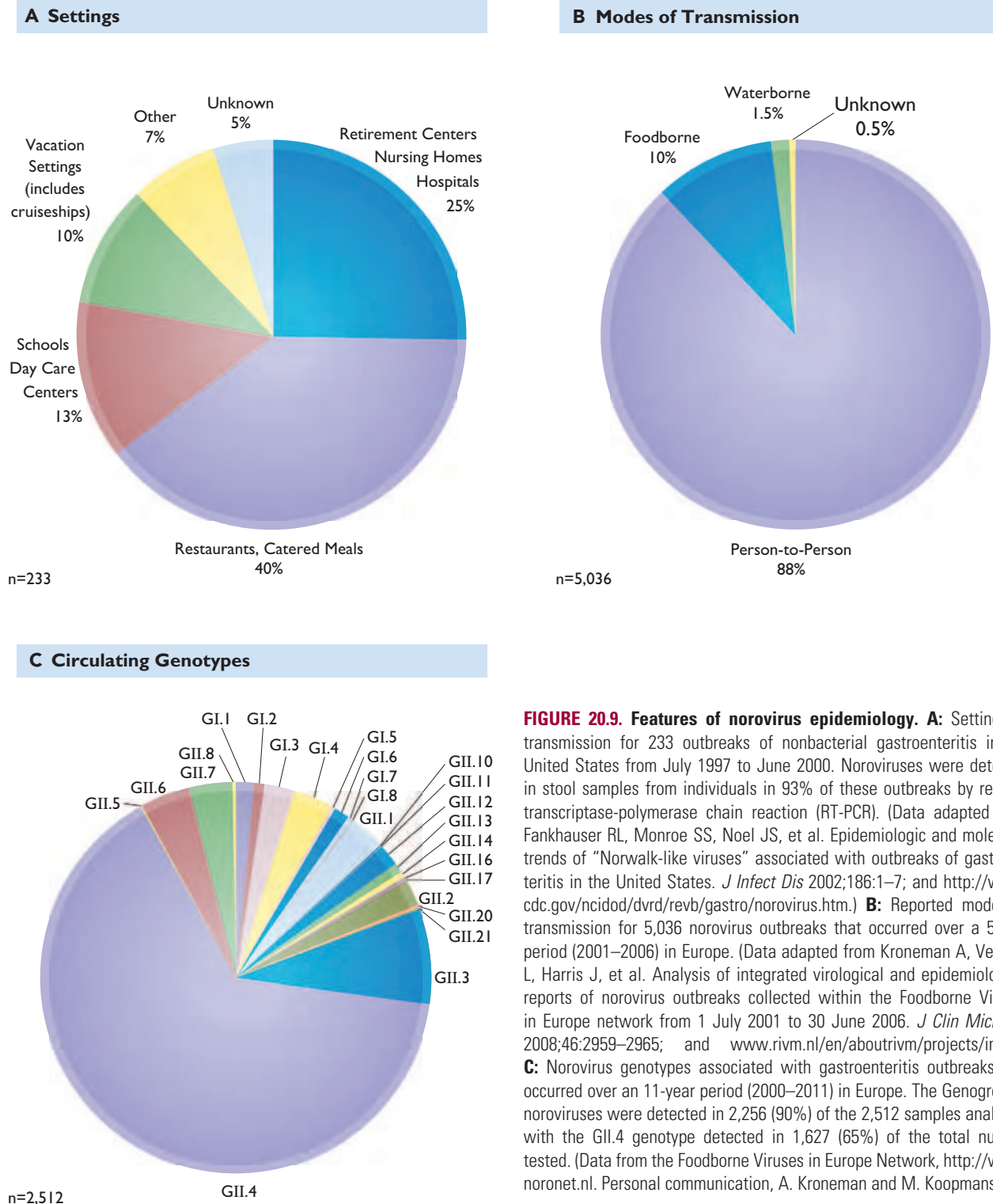


FIGURE 20.9. Features of norovirus epidemiology. **A:** Settings of transmission for 233 outbreaks of nonbacterial gastroenteritis in the United States from July 1997 to June 2000. Noroviruses were detected in stool samples from individuals in 93% of these outbreaks by reverse transcriptase-polymerase chain reaction (RT-PCR). (Data adapted from Fankhauser RL, Monroe SS, Noel JS, et al. Epidemiologic and molecular trends of “Norwalk-like viruses” associated with outbreaks of gastroenteritis in the United States. *J Infect Dis* 2002;186:1–7; and <http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus.htm>.) **B:** Reported modes of transmission for 5,036 norovirus outbreaks that occurred over a 5-year period (2001–2006) in Europe. (Data adapted from Kroneman A, Verhoef L, Harris J, et al. Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the Foodborne Viruses in Europe network from 1 July 2001 to 30 June 2006. *J Clin Microbiol* 2008;46:2959–2965; and www.rivm.nl/en/aboutrivm/projects/index.) **C:** Norovirus genotypes associated with gastroenteritis outbreaks that occurred over an 11-year period (2000–2011) in Europe. The Genogroup II noroviruses were detected in 2,256 (90%) of the 2,512 samples analyzed, with the GII.4 genotype detected in 1,627 (65%) of the total number tested. (Data from the Foodborne Viruses in Europe Network, <http://www.noronet.nl>. Personal communication, A. Kroneman and M. Koopmans.)

The explosive nature of some NV outbreaks, in which a large number of persons become ill within 24 to 48 hours, indicates that infection is often acquired from a common source. This was suggested in the original NV outbreak, but a common-source exposure could not be identified.² Later, a review of 38 NV-associated outbreaks suggested that a common source of infection was likely in 31 (82%).²¹⁵ The vehicle of transmission could be identified in 17 of the 31 outbreaks, including water in 13 instances and food in four others. Water-borne infection was attributed to a municipal water system (twice), semipublic water supply (seven times), stored water on a cruise ship (twice), and recreational swimming (twice). The food-borne outbreaks were associated with the ingestion of oysters or salad. Primary person-to-person transmission occurred in 7 of the 38 outbreaks.²¹⁶ Sufficient data were available from 26 of the outbreaks to permit estimation of secondary attack rates.²¹⁶ In 20 of 23 common-source outbreaks and in each of 3 person-to-person outbreaks, secondary transmission was observed, with attack rates ranging from 4% to 32%. In a large outbreak for which age data were available, the secondary attack rate was highest in children younger than 10 years of age. The median duration of the 38 outbreaks was 7 days (ranging from 1 day to 3 months). The number of individuals who became ill ranged from 2 to 2,000, with the attack rate being higher in common-source outbreaks (median 60%, range 23% to 93%) than in primary person-to-person outbreaks (median 39%, range 31% to 42%).²¹⁶ In the Colorado outbreak caused by the Snow Mountain virus, 61% of the 418 illnesses began on a single day.^{116,332} A water-borne source was suggested as the etiologic agent because the attack rate was directly related to the amount of water- or ice-containing beverages consumed. In addition, the water supply was contaminated by a leaking septic tank and was inadequately chlorinated. The efficient transmission of human caliciviruses by contaminated food and water vehicles has raised concerns about public safety in a global market economy.^{147,379,411,482,491}

The infectious dose for noroviruses is low, with an estimated median infectious dose of 18 viruses.⁴⁶⁶ Exposure to higher levels of virus likely increases the risk of illness.²⁸⁸ In a study that examined norovirus fecal load, higher numbers (greater than 100-fold) of norovirus genome copies were detected in individuals shedding GII (median, 3.0×10^8 genome copies per gram feces) strains compared to GI (median, 8.4×10^5 genome copies per gram feces).⁷⁵ It was proposed that the higher levels of GII shedding might account for a higher transmissibility of these viruses through the fecal–oral route.⁷⁵ However, high levels of GI shedding have been reported: the median peak of GI Norwalk virus shed in the stool of 16 volunteers was 9.5×10^{10} genome copies per gram feces.²⁴

Noroviruses are remarkably stable. The NV retains infectivity for volunteers following (a) exposure of the stool filtrate to pH 2.7 for 3 hours at room temperature, (b) treatment with 20% ether at 4°C for 18 hours, or (c) incubation at 60°C for 30 minutes.¹¹² Norwalk virions remain infectious when stored in ground water at room temperature in the dark for as long as 61 days, and RNA could be detected in such samples after storage at room temperature for 3 years.⁴²⁰ NV is resistant to inactivation following treatment with 3.75 to 6.25 mg/L of chlorine (free residual chlorine of 0.5 to 1.0 mg/L), a chlorine concentration consistent with that found in a drinking water distribution system.²²⁵ NV, however, is inactivated following

treatment with 10 mg/L of chlorine, a concentration that is used to treat water supply systems after contamination has been detected. NV is more resistant to inactivation by chlorine than poliovirus type 1, human rotavirus (Wa), simian rotavirus (SA11), or f2 bacteriophage.²²⁵

Virulence

Virulence determinants have not been defined for the human noroviruses, but differences in clinical outcome, ranging from asymptomatic infection to life-threatening diarrhea, suggest that strain differences exist,¹⁸⁷ as shown for other caliciviruses (Table 20.3). A compelling feature of norovirus epidemiology that supports strain differences (especially in the GII.4 genotype) is the striking variation in disease prevalence in certain years, with sharp increases in global outbreaks and disease burden.^{123,147}

Persistence

Noroviruses have been associated with prolonged infection in immunocompromised patients,^{139,223,348,396} and, therefore, nosocomial transmission of noroviruses to such patients should be prevented by precautionary measures.⁴³⁰ Nine adult patients receiving kidney allografts and undergoing immunosuppression were shown to shed norovirus for periods ranging from 97 to 898 days.⁴¹² One study documented chronic diarrhea in a heart transplant patient undergoing immunosuppressive therapy who shed a GII norovirus strain over 2 years.³⁴⁸ A 36-yr year-old patient with human immunodeficiency virus (HIV) infection and admitted to the hospital for chronic diarrhea was reported to shed norovirus for 15 months (with stools collected 5 months prior to hospitalization identified as norovirus-positive).⁵⁰⁴ Deaths in two chronic lymphocytic leukemia (CLL) patients were associated with prolonged norovirus diarrhea.⁶² Immunocompromised patients with norovirus infection were reported to be at increased risk for the development of pneumonia intestinalis.²²⁸

EPIDEMIOLOGY

Age

Noroviruses have been associated with infection and disease in all age groups. The estimated overall prevalence of norovirus in infants and young children hospitalized for the treatment of gastroenteritis is approximately 12% according to a recent review of published studies.³⁷¹ making the noroviruses second only to the rotaviruses as etiologic agents of severe gastroenteritis in this young age group. In the surveillance period following efficacious rotavirus vaccination in Finnish infants, noroviruses became the single most important cause of acute gastroenteritis.⁵¹⁹

The noroviruses have frequently been associated with gastroenteritis in the elderly, especially those living in institutional settings such as nursing homes.^{126,153} An estimated 25% of 233 nonbacterial gastroenteritis outbreaks reported to the Centers for Disease Control and Prevention (CDC) from July 1997 to June 2000 occurred in the hospital or nursing home setting.¹²⁶

Morbidity/Mortality

Although precise data on morbidity and mortality are not available, several studies estimate a significant burden from norovirus-associated disease.^{169,409} In the United States, an estimated 71,000 hospitalizations are associated with norovirus

gastroenteritis each year, at a cost of \$493 million per year.²⁸⁶ In a review of published studies, noroviruses were consistently reported as the second most important cause (after rotavirus) of severe gastroenteritis in infants and young children worldwide, with an estimated 200,000 deaths per year in children younger than 5 years of age in developing countries.³⁷¹ Noroviruses have been linked to gastrointestinal illness in neonates.³²¹ Norovirus infection and disease in preterm infants receiving intensive care in a hospital nursery unit have been reported,¹⁹ and in one such setting necrotizing enterocolitis occurred more frequently in preterm infants with norovirus infection.⁴⁴⁵ In an analysis of medical registrations (such as doctor visits, hospitalizations, and deaths) associated with gastroenteritis of unspecified cause during periods of high norovirus outbreak activity in the Netherlands, it was estimated that elderly individuals (65 years of age and older) were at increased risk for serious illness requiring medical intervention and death.⁴⁷⁸ In Sweden, an overall excess mortality rate was found in elderly patients hospitalized for the treatment of severe norovirus gastroenteritis that was acquired in the community,¹⁶⁴ and a case-fatality rate of 2% was reported in a large norovirus outbreak that occurred in six nursing homes in Israel.⁶⁰ An analysis of the role of various enteric pathogens in hospitalized patients in Canada (from 2001 to 2004) estimated that noroviruses were responsible for a mean hospitalization incidence rate of 1.6 cases per 100,000 and that the average age of hospitalized patients was 59 years old.⁴⁰⁴ Over the 4-year period, 43 deaths were attributed to noroviruses, making it a leading cause of mortality among the enteric pathogens studied.⁴⁰⁴ Noroviruses have been associated with increased morbidity and mortality in patients with an underlying illness such as cardiovascular disease or who are immunosuppressed or receiving chemotherapy.^{62,216,223,232,312,400,417,430}

Origin and Spread of Epidemics

Noroviruses are the single most important cause of nonbacterial gastroenteritis outbreaks. In an analysis of 233 nonbacterial gastroenteritis outbreaks reported to the CDC between July 1997 and June 2000, 217 (93%) were associated with noroviruses.¹²⁶ In a larger survey of 3,714 nonbacterial gastroenteritis outbreaks that occurred in Europe between 1995 and 2000, 85% were associated with noroviruses.²⁸⁷ Outbreaks often occur in hospitals, long-term care facilities, camps, recreational areas, elementary schools, daycare centers, cruise ships, retirement centers, colleges, restaurants, social events with catered meals, families, the military, prisons, and community settings (see Fig. 20.9A for predominant settings of outbreaks in the United States between 1997 and 2000). Outbreaks can vary in size, involving small family groups to hundreds of individuals.^{195,311,513}

Especially noteworthy is the predominant role of the noroviruses in food-related outbreaks of gastroenteritis. Noroviruses are the leading cause of food-borne illness in the United States (followed by *Salmonella* [nontyphoidal], *Clostridium perfringens*, *Campylobacter* spp., and *Staphylococcus aureus*), accounting for approximately 26% of all reported outbreaks.⁴⁰⁹ An analysis of 8,271 food-borne outbreaks reported to the CDC (1991–2000) showed that norovirus outbreaks were often larger than bacterial outbreaks (median persons affected: 25 vs. 15), with 10% of the affected individuals seeking medical care and 1% being hospitalized.⁵⁰¹ In a study of water-borne outbreaks in Finland, 18 (64%) of 28 outbreaks evaluated were

associated with norovirus.³¹³ Bivalve mollusks (such as oysters and mussels) are an important cause of food-borne norovirus outbreaks.^{9,234,285,316,335,495}

Noroviruses have also been documented as important agents of gastroenteritis in military populations in several different areas of the world.^{4,20,104,314} Among U.S. military personnel deployed to South America or West Africa, NV infection was detected in 10% of ill personnel, second in importance to enterotoxigenic *Escherichia coli*, which was the most frequently encountered pathogen (17%).⁵² In addition, large-scale outbreaks of gastroenteritis have been attributed to the noroviruses on ships such as aircraft carriers on which hundreds of crew members became ill.^{45,94,365,423}

Although the majority of traveler's diarrhea has been attributed to enterotoxigenic *E. coli*,⁴⁰ an increasing number of reports have linked noroviruses to such illness.^{5,6,17,81,121,405} Norovirus transmission during airplane travel, in addition to that on recreational cruise ships, has been reported, illustrating the potential ease with which strains can be spread globally.^{233,469,481,500}

Prevalence and Seroepidemiology

Noroviruses have a worldwide distribution. Most individuals in both developed and developing countries show evidence of infection with norovirus before adulthood, reflecting the global distribution and endemic nature of these viruses.²⁰³ A study of Finnish infants and children, aged 0 to 14 years, found that antibody prevalence against GII.4 noroviruses reached 91.2% in children older than 5 years of age.³⁵³ The prevalence of antibody to the GII viruses (Mexico, Hawaii, or Lordsdale) appears to be greater than that of the GI viruses in most^{97,204,355,431} but not all^{102,109} studies, which likely reflects the predominance of circulating GII strains. The prevalence of norovirus antibodies characteristically increased more gradually by age in developed than in developing countries.²⁰³

The incidence of norovirus gastroenteritis has been estimated in several community-based studies. In the United States, an enhanced surveillance study of acute gastroenteritis (that included pathogen identification with diagnostic assays) in a single state (Georgia) estimated that norovirus was the predominant cause of acute gastroenteritis, accounting for 6,500 (16%) and 640 (12%) per 100,000 person-years of community and outpatient acute gastroenteritis episodes, respectively.¹⁶⁹ In the Netherlands, 18% of the community cases of gastroenteritis over a single winter season and at least 5% of gastroenteritis cases that resulted in a visit to a physician were associated with norovirus infection.²⁴¹ Furthermore, a 1-year prospective population-based cohort study showed that noroviruses were the single most important cause of gastroenteritis overall in nearly all age groups in the Netherlands.¹⁰⁶ In Germany, the incidence of norovirus gastroenteritis in the community requiring medical attention was reported as 626 cases/per 100,000 person-years, making it the predominant known cause of acute gastroenteritis in that country.²¹⁸ In England, the community incidence of norovirus gastroenteritis was 4.5 cases/per 100 person-years, corresponding to approximately 2 million episodes per/year.³⁷⁶

Genetic Diversity of Virus

Molecular epidemiologic studies have demonstrated a marked genetic diversity among circulating noroviruses. Genetic typing

of circulating strains has proven to be a useful tool in elucidating the source and spread of outbreaks,^{247,482,483} and regional data-sharing networks have been established in several areas of the world.^{133,246,336,480} Genetic typing systems for the noroviruses have been proposed based on relatedness in the complete VP1 capsid protein, which presumably would correlate with the antigenic specificity.^{247,520} One such system (Table 20.4) shows the division of the genus *Norovirus* into

six major phylogenetic clades, or genogroups, designated GI through GV. Genogroups I, II, and III are further subdivided into 9, 21, and 3 genotypes, respectively.

Large-scale molecular epidemiologic studies have given insight into important genetic features of the noroviruses. An analysis of the genetic typing data from 2,512 noroviruses associated with outbreaks in Europe from 2000 to 2011 shows the marked genetic variation in the genotypes of circulating

TABLE 20.4 Norovirus Genogroups and Genotypes as Determined by VP1 Relatedness

Reference virus	Genogroup	Genotype	GenBank accession number
Hu/NoV/GI.1/Norwalk/1968/US	I	1	M87661
Hu/NoV/GI.2/Southampton/1991/UK	I	2	L07418
Hu/NoV/GI.3/Desert Shield 395/1990/SA	I	3	U04469
Hu/NoV/GI.4/Chiba 407/1987/JP	I	4	AB022679
Hu/NoV/GI.5/Musgrove/1989/UK	I	5	AJ277614
Hu/NoV/GI.6/BS5(Hesse3)/1997/DE	I	6	AF093797
Hu/NoV/GI.7/Winchester/1994/UK	I	7	AJ277609
Hu/NoV/GI.8/Boxer/2001/US	I	8	AF538679
Hu/NoV/GI.9/Vancouver730/2004/CA	I	9	HQ637267
Hu/NoV/GII.1/Hawaii/1971/US	II	1	U07611
Hu/NoV/GII.2/Melksham/1994/UK	II	2	X81879
Hu/NoV/GII.3/Toronto 24/1991/CA	II	3	U02030
Hu/NoV/GII.4/Bristol/1993/UK	II	4	X76716
Hu/NoV/GII.5/Hillingdon/1990/UK	II	5	AJ277607
Hu/NoV/GII.6/Seacroft/1990/UK	II	6	AJ277620
Hu/NoV/GII.7/Leeds/1990/UK	II	7	AJ277608
Hu/NoV/GII.8/Amsterdam/1998/NL	II	8	AF195848
Hu/NoV/GII.9/VA97207/1996/US	II	9	AY038599
Hu/NoV/GII.10/Erfurt546/2000/DE	II	10	AF427118
Po/NoV/GII.11/Sw918/1997/JP	II	11	AB074893
Hu/NoV/GII.12/Wortley/1990/UK	II	12	AJ277618
Hu/NoV/GII.13/Fayetteville/1998/US	II	13	AY113106
Hu/NoV/GII.14/M7/1999/US	II	14	AY130761
Hu/NoV/GII.15/J23/1999/US	II	15	AY130762
Hu/NoV/GII.16/Tiffin/1999/US	II	16	AY502010
Hu/NoV/GII.17/CS-E1/2002/US	II	17	AY502009
Po/NoV/GII.18/OH-QW101/2003/US	II	18	AY823304
Po/NoV/GII.19/OH-QW170/2003/US	II	19	AY823306
Hu/NoV/GII.20/Luckenwalde591/2002/DE	II	20	EU373815
Hu/NoV/GII.21/IF1998/2003/IR	II	21	AY675554
Hu/NoV/GII.22/Yuri/2002/JP	II	22	AB083780
Bo/NoV/GIII.1/Jena/1980/DE	III	1	AJ011099
Bo/NoV/GIII.2/Newbury-2/1976/UK	III	2	AF097917
Ov/NoV/GIII.3/Norsewood30/2007/NZ	III	3	EU193658
Hu/NoV/GIV.1/Alphatron 98–2/1998/NL	IV	1	AF195847
Lion/NoV/GIV.2/Pistoia/387/2006/IT	IV	2	EF450827
Mu/NoV/GV.1/MNV-1/2002/US	V	1	AY228235
Ca/NoV/GVI/Bari/91/2007/IT	VI	1	FJ875027
Ca/NoV/GVI/Visau/2007/PT	VI	2	GQ443611

Country abbreviations are CA, Canada; DE, Germany; IR, Iraq; IT, Italy; JP, Japan; NL, Netherlands; NZ, New Zealand; PT, Portugal; SA, Saudi Arabia; UK, United Kingdom; US, United States. Species abbreviations are Bo, bovine; Ca, canine; Hu, human; Mu, murine; Po, porcine; Ov, ovine

Note: According to classification system of the online norovirus typing tool at <http://www.rivm.nl/mpf/norovirus/typingtool>. Krone-man A, Vennema H, Deforche K, et al. An automated genotyping tool for enteroviruses and noroviruses. *J Clin Virol* 2011;51:121–125.

strains (Fig. 20.9C). The GII noroviruses, particularly those of the GII.4 cluster, were the predominant viruses detected, and this distribution reflects the epidemiologic pattern observed in most other parts of the world. In an analysis of 773 norovirus outbreaks reported to the CDC from 1994 to 2006, 629 (81.4%) were caused by GII viruses and 342 (44.2%) of these were caused by GII.4 strains.⁵²¹ Major shifts in the predominant circulating strain can occur,^{56,127,138,284,350,389,485,499,521} and the factors (host or viral) responsible for the emergence of an epidemic norovirus strain are under investigation. Sequence analysis of the GII.4 noroviruses that emerged on a global level in the early 2000s identified the presence of an amino acid insertion in the VP1 P2 domain, suggesting a possible change in the antigenic or receptor recognition phenotype.¹¹⁰ Further evolution in the GII.4 cluster was described,⁵⁶ and several studies have examined the possibly unique propensity of this genotype to undergo genetic (and antigenic) drift.^{10,47,51,57,118,271,272,336,421,428} Genetic variation has been detected in other regions of the genome such as the emergence of the “GGIIB-pol” polymerase type that was found in norovirus strains in combination with several different VP1 genotypes.^{389,390} Additional reports of unique polymerase sequences have led to the conclusion that recombination is a driving mechanism of norovirus evolution.^{55,144}

Evidence for mixed norovirus infections within the same individual or within the same outbreak has been reported in many epidemiologic studies.^{14,54,150,167,282,449} In addition, a marked genetic diversity among norovirus strains has been documented in pediatric patients.^{137,173,235,307,522} Such diverse and mixed infections may allow recombination between RNA genomes, a possibility suggested from sequence analyses of naturally occurring noroviruses in several species.^{55,124,171,177,198,362,398,488} The presence of diverse norovirus sequences in shellfish samples is common,^{87,95,260} suggesting another potential source for mixed infection. Infection of feline kidney cells with two distinct recombinant FCV strains bearing different fluorescent markers has demonstrated that co-infection of the same cell can readily occur, further supporting the potential for recombination of RNA genomes during replication.¹

CLINICAL FEATURES

Viral gastroenteritis is generally considered to be mild and self-limiting, although the illness can be incapacitating during the symptomatic phase that usually lasts 24 to 48 hours (Fig. 20.10). Illness induced by the noroviruses can be sufficiently severe to require medical intervention, with an increased risk for life-threatening dehydration at both ends of the age spectrum.^{20,104,216,232,366,400,423} Immunocompromised patients with norovirus infection are at increased risk for morbidity and mortality, and an increasing number of reports have linked norovirus to chronic gastroenteritis in patients undergoing transplantation or chemotherapy.^{46,131,312,401,406,417,430,450,494} (see Treatment).

Clinical manifestations observed in 31 volunteers experimentally infected with noroviruses who became ill included the following: fever above 99.4°F (45%), diarrhea (81%), vomiting (65%), abdominal discomfort (68%), anorexia (90%), headache (81%), and myalgias (58%).⁵¹¹ The illnesses were characteristically mild and usually lasted 24 to 48 hours; however, one volunteer was given parenteral fluid because he

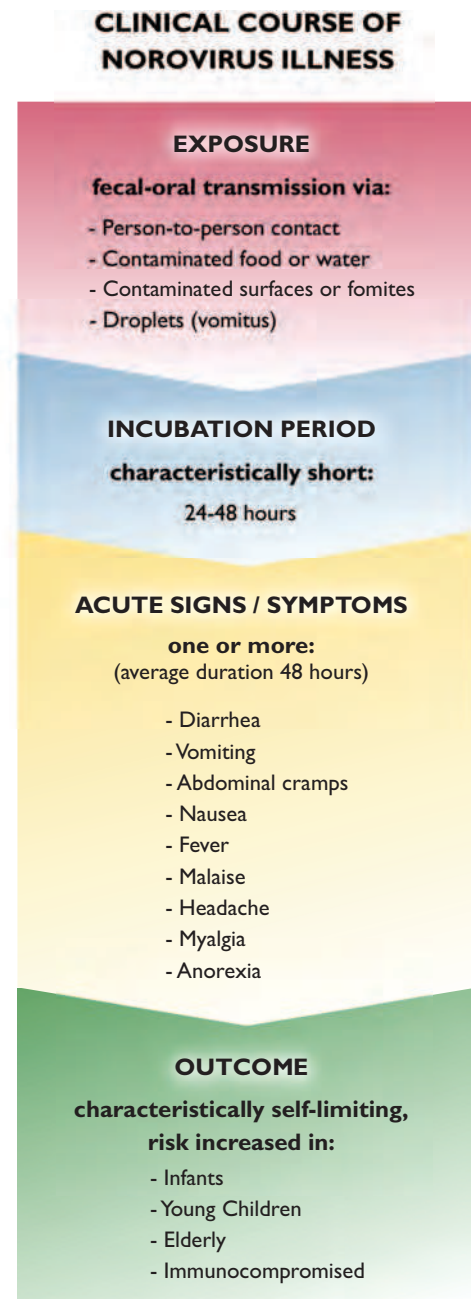


FIGURE 20.10. Clinical course of norovirus disease.

vomited 20 times within a 24-hour period. The number of clinical signs and symptoms can vary among volunteers receiving the same inoculum.¹¹³ Illnesses induced by the Hawaii, Montgomery County, and Snow Mountain viruses in volunteers cannot be distinguished clinically from those caused by Norwalk virus.^{116,511} Subclinical infections with Norwalk or Hawaii virus have been observed under experimental as well as natural conditions.

Of 16 volunteers who developed illness following Norwalk or Hawaii virus infection, 14 developed transient lymphopenia.¹¹⁵ This was attributed to a redistribution of circulating lymphocytes to the site of viral infection in the small intestine.

The lymphocytes remaining in the circulation responded normally or exhibited an exaggerated response to mitogenic stimuli.

Clinical manifestations observed in 38 outbreaks associated with NV included the following (expressed as the median percentage of patients): nausea (79%), vomiting (69%), diarrhea (66%), abdominal cramps (30%), headache (22%), fever (subjective) (37%), chills (32%), myalgias (26%), and sore throat (18%).²¹⁶ Bloody stools were not reported. Vomiting occurred more frequently than diarrhea in children, whereas in adults the reverse was observed. The duration of illness in 28 outbreaks ranged from 2 hours to several days, with a mean or median of between 12 and 60 hours in 26 of the 28 outbreaks. In six outbreaks, illness lasted more than 3 days in up to 15% of the affected individuals. The attack rates did not differ significantly with age or sex in six outbreaks in which this was analyzed.²¹⁶

A 2-year study in children (5 years of age or younger) compared the severity of acute diarrheal episodes caused by noroviruses with rotaviruses in hospital emergency and outpatient settings; most cases for both viruses ranged from moderate to severe, although illness was overall less severe for norovirus-infected individuals.³⁵⁴ The clinical course of norovirus illness in preterm infants has been reported to include a distended abdomen and symptoms such as apnea or a sepsis-like appearance: vomiting was not a predominant symptom in these patients.¹⁹ Noroviruses have been reported as associated with the following rare conditions or sequelae: convulsions,^{72,319} encephalopathy,^{196,356} and necrotizing enterocolitis.⁴⁴⁵

DIAGNOSIS

Differential

An analysis of the common features of 38 NV outbreaks indicates that a provisional diagnosis of illness by the noroviruses can be made during an outbreak if the following criteria are met: (a) bacterial or parasitic pathogens are not detected, (b) vomiting occurs in more than 50% of cases, (c) the mean or median duration of illness ranges from 12 to 60 hours, and (d) the incubation period is 24 to 48 hours.²¹⁵ These so-called Kaplan criteria were found to be 99% specific and 68% specific for the provisional diagnosis of a norovirus outbreak when re-evaluated with samples confirmed as norovirus positive.⁴⁷⁶

Differential diagnosis of sporadic norovirus illness in individual patients is difficult, due to shared clinical features (see Clinical Features) with a wide range of enteric pathogens and disease syndromes.

Laboratory

Reverse Transcriptase-Polymerase Chain Reaction

Currently, RT-PCR is the most widely used technique for detection of noroviruses.²³ With this method, noroviruses can be detected in clinical specimens (feces or vomitus) and contaminated food, water, or fomites. The application of real-time quantitative (q) RT-PCR has gained widespread use because it allows rapid detection as well as comparison of viral RNA levels.^{209,473} There are several considerations for optimal performance of RT-PCR techniques.²³ First, the viral RNA extraction procedure should allow the purification of an RNA template that is nondegraded and free of inhibitors of

the RT-PCR reaction. Internal RNA controls can be used in the assay of clinical and environmental samples to avoid false-negative results.^{370,415} Second, the choice of primers is important, because considerable genetic diversity exists among circulating strains. Several primer pairs have been described that were deduced from highly conserved regions of the RNA genome (usually the polymerase region).⁴⁸⁴ RT-PCR, coupled with sequence analysis of the amplicons, has been used extensively to detect and characterize noroviruses in various outbreaks (see Molecular Epidemiology). Norovirus identification is increasingly included in multiplex assays that detect a wide array of enteric pathogens, facilitated by an accumulating norovirus database that has allowed the design of broadly reactive primers and probes.^{160,197,278,424,510}

Immunoassays

Immunoassays for the detection of noroviruses have been developed that employ hyperimmune antisera prepared against rVLPs.^{201,203} Although sensitive, these assays are often highly specific for the immunizing VLP.^{149,200,203,212} Progress has been made in the development of norovirus-specific and cross-reactive monoclonal antibodies^{168,178,181,236,267,270,368,425,514} for use in enzyme-linked immunosorbent assays (ELISAs) that can detect viral antigen in clinical specimens; commercial diagnostic ELISA kits are now available.

The ELISAs that use rVLPs as antigen are specific, sensitive, and efficient for detecting infection with the human caliciviruses and have been used in several large-scale sero-epidemiologic studies (see Prevalence and Seroepidemiology). The Norwalk rVLP ELISA has been shown to detect broadly reactive antibody responses in volunteers given NV, Hawaii virus, or Snow Mountain virus, although the maximal response was observed in volunteers challenged with NV.^{149,154,330,471} Thus, it is impossible to identify the antigenic type of an infecting norovirus strain by serologic analysis because of the cross-reactive antibodies detected by ELISA.^{32,349} The demonstration of an antibody response in 50% or more of the individuals involved (or examined) in an outbreak to a calicivirus antigen is strong evidence linking the virus with the outbreak.²¹⁶

PREVENTION AND CONTROL

Treatment

The noroviruses characteristically induce a mild, self-limited gastroenteritis that normally resolves without complications.^{43,111,112,113,206,442,511} As noted, hospitalization for severe dehydration, although rare, can occur with norovirus gastroenteritis. Oral fluid and electrolyte replacement therapy is usually sufficient to replace fluid loss.^{96,380,477} Oral rehydration therapy should not be administered to patients with depressed consciousness because of the possibility of fluid aspiration. Parenteral administration of fluids may be necessary, however, if severe vomiting or diarrhea occurs.

Oral administration of bismuth subsalicylate after onset of symptoms significantly reduced the severity and duration of abdominal cramps during experimentally induced norovirus illness in adults.⁴⁴² In addition, the median duration of gastrointestinal symptoms was reduced from 20 to 14 hours. The number, weight, and water content of stools, as well as the extent of virus excretion, were not significantly affected by

TABLE 20.5 Various Treatment Protocols Tested in Normal and Immunocompromised Patients with Norovirus Gastroenteritis

Patient status at time of norovirus illness	Age	Norovirus treatment	Dose or modification of immunosuppressive therapy (IST)	Reported outcome ^b	Reference
Normal	Adult	Bismuth subsalicylate	420 mg	Reduced abdominal cramping	(442)
Normal	12–60 years	Nitazoxanide	Oral, 500 mg, 2× x daily	Shorter duration of illness	(402)
AML	43 years	Nitazoxanide	Oral, 500 mg, 2× daily	Improvement	(427)
Immunocompromised	All ^a	Immunoglobulin	Oral, 25 mg/kg, 4× x daily	Decreased stool output	(131)
HIV	36 years	Immunoglobulin	IV, 400 mg/kg, 1× daily	Minimal 2-day improvement	(504)
Stem cell/lung transplant	56 years	Modify IST	Drug switch	Improvement	(46)
Intestinal transplant	<2 years	Modify IST	Dose reduction	Improvement	(223)
Renal transplant	Adult	Modify IST	Dose reduction	Improvement	(401)
Renal transplant	Adult	Modify IST	Dose reduction	Improvement	(412)

AML, acute myelogenous leukemia; HIV, human immunodeficiency virus; IST, immunosuppressive therapy.

^aTwo study groups: one with average age of 2 years old, the other with male adults.

^bLong term follow-up information unavailable for most patients.

treatment. The use of various medications for symptomatic treatment of acute diarrhea in infants and young children (aged 1 month to 5 years) was reviewed: bismuth subsalicylate, loperamide, anticholinergic agents, adsorbents, or Lactobacillus-containing compounds were not recommended by the American Academy of Pediatrics and, in addition, the use of opiates as well as opiate and atropine combination drugs was contraindicated.³⁸⁰

Although direct-acting antivirals for the treatment of norovirus gastroenteritis are not yet available, treatment protocols implemented for other pathogens have been evaluated in a small number of individuals with norovirus disease, with varying success (Table 20.5). Improvement in chronic norovirus diarrhea has been reported in immunocompromised patients following reduction in immunosuppressive therapy (IST) drugs; however, careful monitoring of these patients is required (Table 20.5). Efforts are in progress to develop antiviral drugs to target specific stages of the norovirus life cycle, but their safety and clinical efficacy have not been established.^{188,229,392,397}

Vaccines

A vaccine for the control of norovirus gastroenteritis is not yet available, but a promising efficacy study in adult volunteers has shown recently that administration of Norwalk virus rVLPs as vaccine provided protection against illness when vaccinees were challenged with Norwalk virus.²² Additional work will be needed to determine the number of antigenic components required to provide protection against a broad range of norovirus antigenic types and variants. Various routes of administration, formulations, and expression systems for rVLPs (or subunit forms) are under investigation.^{27,28,48,122,163,451,452,454,456,460} A safe and effective vaccine could reduce the incidence of epidemic viral gastroenteritis. A vaccine would be of special importance to college students, military personnel, nursing home residents, and individuals in various institutional settings. In addition, it might reduce the number of episodes of severe gastroenteritis in infants and children. Although norovirus gastroenteritis tends to be a mild illness, a reduction in diarrheal episodes may be especially important in the debilitated, mal-

nourished infant, because it has been suggested that repeated diarrheal episodes may be a precipitating factor in the development of malnutrition through sequential damage to the intestinal mucosa.³⁰⁸ The impact of norovirus infection on normal gastrointestinal microflora or underlying disease conditions,⁵⁹ when elucidated, may provide additional data to support norovirus immunoprophylaxis.

Infection Control

Specific methods are not available for the prevention of human calicivirus infection or illness. Outbreak management generally focuses on containment by the prevention of spread to other areas by ill or exposed individuals, frequent handwashing, and effective environmental decontamination.^{8,69,88,226,249,293,469,477}

The noroviruses are generally resistant to detergent or ethanol-based cleaning of environmental surfaces and fomites and require additional chemical disinfection.^{29,119,477} Reliance on the use of alcohol-based hand sanitizers over handwashing has been reported to actually increase the risk of norovirus transmission and infection in patient care settings.^{44,486} Effective disinfectants have been reported to include hypochlorite at 5,000 ppm (domestic bleach is approximately 5% sodium hypochlorite and can be used as a 10% solution), hydrogen peroxide-based cleaners, and phenolic-based cleaners.^{29,477}

Special care must be given to the hygienic processing of food in view of the frequent occurrence of food-borne outbreaks.^{240,475} Depuration of oysters does not adequately clear tissues of NV,^{15,159,416} and recent studies have shown that NV binds to oyster tissues via carbohydrate structures similar to those of human histo-blood group antigens.^{259,294} Precaution must be taken to prevent contamination of oyster beds with feces, vomitus, or sewage treatment plant effluent. Measures that increase the purity of drinking water or swimming pool water should also decrease the frequency of outbreaks.²⁸² Various technological processes are under development to inactivate noroviruses in agricultural food products and shellfish prior to market^{183,230,243,264,333}; human volunteer studies remain important in establishing their efficacy in the absence of an *in vitro* infectivity assay for the human strains.³⁹¹

PERSPECTIVE

Caliciviruses are a highly diverse and evolving family of single-stranded RNA viruses. Because these viruses have a proven ability to persist silently in nature, next-generation pathogen discovery techniques, such as deep sequencing, are sure to uncover many new host species for these viruses. Rabbit hemorrhagic disease virus remained undetected as a pathogen until its sudden emergence as the etiologic agent of a deadly rabbit hemorrhagic disease three decades ago. Murine norovirus, likely present in laboratory mice for decades, was discovered only when genetically engineered laboratory mice lacking a functional innate immune system developed unexplained illness and died, thus prompting investigation. Even recognized calicivirus pathogens may shift suddenly in virulence, as shown by the recent emergence of lethal “virulent systemic” FCV strains. Human GII.4 noroviruses, currently the predominant genotype, periodically undergo genetic drift to cause sharp increases in the number of global gastroenteritis outbreaks, affecting millions.

The diversity and rapid evolution of viruses in the *Caliciviridae* present a constant challenge in the management of disease and in the development of vaccines, and noroviruses are no exception. Human norovirus research is complicated even further by the continued absence of a fully permissive cell culture system, thus hindering the ability to determine serotypic diversity. Despite this stubborn, lingering technical obstacle, important strides have been made in the last few years, and more are on the horizon. Norovirus vaccines containing antigens from the major capsid protein, VP1, have advanced in clinical trials, and the first demonstration of vaccine efficacy has been achieved. The serious nature of prolonged norovirus shedding and illness in immunocompromised patients has become apparent, and intense efforts are in progress to identify an effective antiviral drug. Drug development for human noroviruses is facilitated by the availability of molecular biology-driven tools such as recombinant enzymes expressed from cDNA clones and a human norovirus replicon-bearing cell line. These tools, coupled with the investigation of caliciviruses that grow in cell culture such as feline calicivirus and murine norovirus, each with powerful reverse genetics systems, are bringing the field closer to the realization of effective approaches for norovirus control. The next few years should be remarkable.

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Astroviruses

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Acknowledgments

The family *Astroviridae* includes human and animal astroviruses that show icosahedral morphology; they are nonenveloped and their genome is composed of plus-sense, single-stranded RNA (ssRNA), with three open-reading frames, whose organization distinguishes them from other virus families.

Astroviruses (AstV) have been isolated from a variety of animal species. In most mammals, astrovirus infections are associated with gastroenteritis. In particular, human astroviruses (HAstV) have been found to be the second or third most

common cause of viral diarrhea in young children and cause of sporadic gastroenteritis outbreaks. Avian AstV, on the other hand, have been linked with more severe intestinal and extraintestinal manifestations of disease.

HISTORY

The term *astrovirus* was coined by Madeley and Cosgrove in 1975 to describe small, round viruses with a distinctive five- or six-pointed, star-like appearance (astron, *star* in Greek) of about 28 to 30 nm in diameter.⁸⁶ They were observed by direct electron microscopy (EM) in the stools of infants hospitalized with diarrhea and in outbreaks of gastroenteritis in newborn nurseries (Fig. 21.1). Subsequently, viral particles of similar size and morphology were identified by EM in association with gastroenteritis in a wide variety of young mammals and birds.

An important milestone was achieved in 1981 when Lee and Kurtz reported the isolation and passage of HAstV in primary cell cultures.⁸² This achievement led to the recognition of five HAstV serotypes in 1984,⁷⁹ development of an enzyme immunoassay (EIA) to detect viral antigen in the late 1980s,⁶⁰ and confirmation of its medical importance.⁶¹ The molecular characterization of astrovirus isolates subsequently permitted the recognition of 8 serotypes of HAstV and the design of molecular probes for use as diagnostic tools. Metagenomic approaches have allowed the identification of novel AstV from humans and animal species in recent years.^{16,33,35,115} The efficient propagation of HAstV in cell lines¹⁴⁷ and of turkey isolates in animal models.⁷² has further advanced our knowledge of the molecular and structural biology, as well as of pathogenesis of these viruses.

CLASSIFICATION

The general organization of the astrovirus genome places the open reading frames (ORF) encoding the nonstructural proteins at the 5' end, and the ORF encoding the structural proteins at the 3' end. Distinctive features of this family include the morphology of viruses,¹¹⁶ the lack of an RNA-helicase domain encoded in the genome, and the usage of a ribosomal frameshifting mechanism to translate the RNA-dependent RNA polymerase (RdRp).⁶⁵

Astroviruses were originally classified into genera and species based only on the host of origin; however, recent characterization of novel AstV has shown that isolates from different animal species can be genetically similar, while genetically

*Deceased.

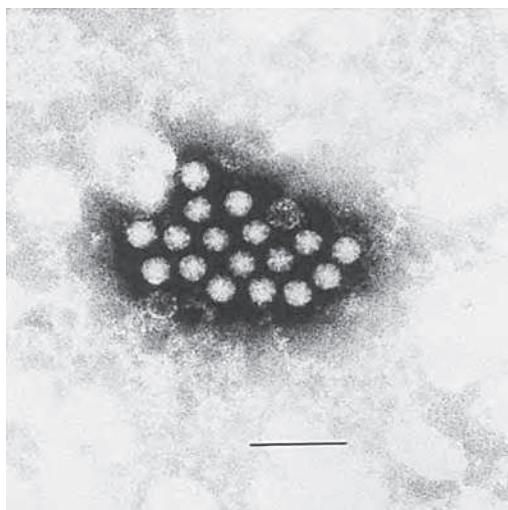


FIGURE 21.1. Electron micrograph of human astrovirus in a fecal specimen. Bar = 100 nm. (Courtesy of T. W. Lee and J. B. Kurtz.)

diverse viruses can be isolated from the same animal species.^{69,85,152} These findings have led to a proposed new classification scheme based on the amino acid sequence of ORF2 (Astroviruses Study Group, 9th Report ICTV, 2010),¹⁷ which encodes the capsid polyprotein and represents the most variable region of the genome (see below). Two genera are distinguished within the *Astroviridae* family: *Mamastrovirus* and *Avastrovirus* (Fig. 21.2). Viruses belonging to the genus *Mamastrovirus* include isolates from a number of mammals, including humans, pigs (PAstV), cats (FeAstV), minks (MAstV), sheep (OAsV), calves (BoAstV), dogs (CaAstV), bats (BAstV), rats

(RAstV), deer (CcAstV), and marine mammals, such as sea lions (CSIAstV) and bottlenose dolphins (BdAstV), among others. This genus includes two genogroups, GI and GII, with 10 and 9 genotype species, respectively. Both genogroups comprise viruses from human and animal origin. Of note, recently identified human viruses are very similar to animal isolates, such as mink and sheep,⁶⁹ among others. HAstV previously classified within one species that comprised serotypes 1 to 8 (based on their reactivity to hyperimmune sera; HAstV-1 to -8) are now included in the proposed genotype G1 of genogroup I. The genetic diversity found among pig,⁸⁵ bat,¹⁵² and human⁶⁹ isolates places them into highly divergent groups, suggesting that they have different ancestors that probably emerged during interspecies transmission.⁸⁵

Viruses from the genus *Avastrovirus* include isolates from turkeys (TAstV), ducks (DAstV), chicken (CAstV), and guinea fowl. This genus includes two proposed species in genogroup GI (GIA, GIB) and one in genogroup II (GIIA). Similarly to canonical HAstV, members of some of these species can be distinguished by serology, indicating the existence of viral serotypes in some, such as in TAstV-2 and ANV.^{132,134} In general, avastroviruses show higher diversity than mamastroviruses.

VIRION STRUCTURE AND COMPOSITION

Virion Structure

Ultrastructural analysis by EM of human viruses propagated in cell culture in the presence of trypsin revealed icosahedral particles of 41 nm, with spikes protruding from the surface.¹¹⁶ The star-like form of the particles was observed only after alkaline treatment. Recent studies by cryo-EM and image processing of trypsin-treated and untreated HAstV particles confirmed the spiked icosahedral structure of virions (Fig. 21.3) (K. Dryden et al.,

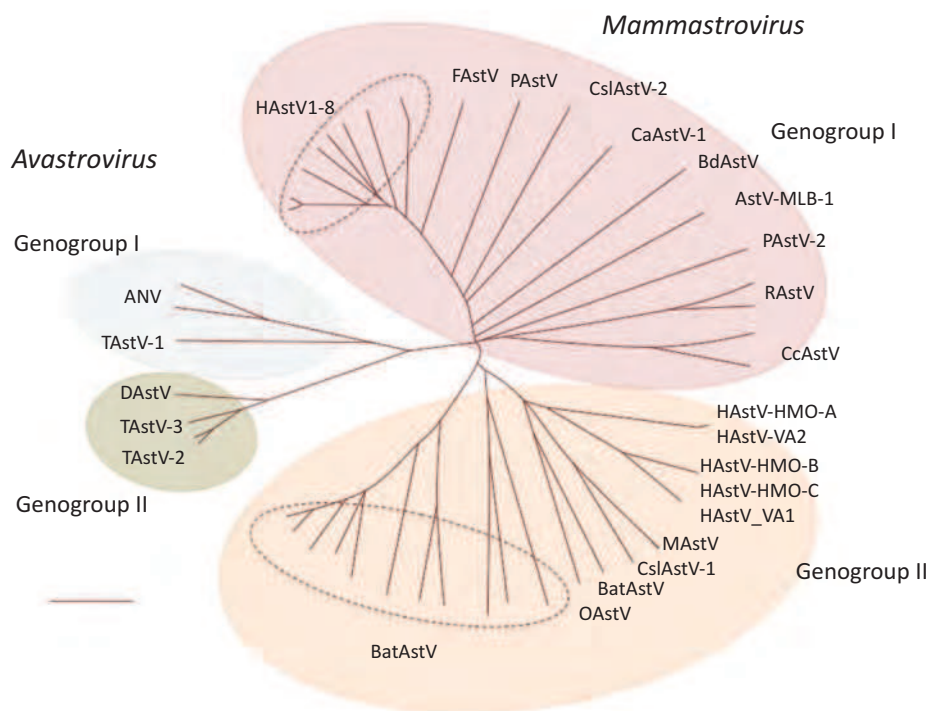


FIGURE 21.2. The *Astroviridae* family includes two genera with two genogroups each. Virus species are classified based on the ORF2 amino acid sequence distances. (Classification proposed by the Astroviruses Study Group, 9th Report ICTV, 2010.¹⁷)

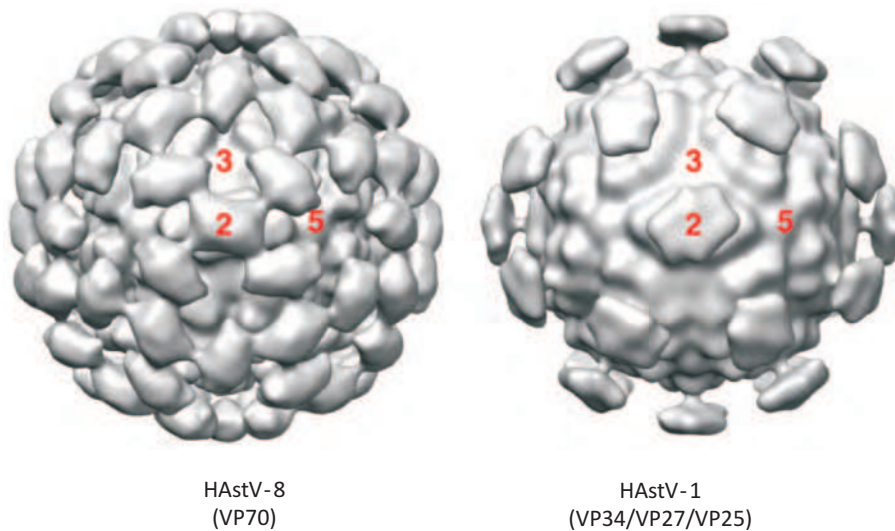


FIGURE 21.3. Three-dimensional reconstruction of HAdV obtained by cryo-electron microscopy. Purified particles of HAdV-8 and HAdV-1 by cesium chloride gradients, untreated or treated with trypsin, respectively. Protein composition is indicated. Images were obtained at 23–25 Å of resolution. (Courtesy of K. Dryden, University of Virginia.)

unpublished results). Remarkable differences are observed between the two types of particles. The untreated virus, 46 nm in diameter, contains 180 copies of a single protein of 70 kd arranged in a $T=3$ icosahedral symmetry. Two kinds of spikes, localized at two- and fivefold vertices, can be observed in these particles. Two protein layers can be distinguished. The internal layer forms the capsid core and is almost identical to that of treated particles, at the highest resolution reached (23–25 Å); however, the distal layer that forms the spikes shows dramatic changes after trypsin treatment. This treatment results in the cleavage of the 70-kd protein into three polypeptides that is required for virus infectivity (see below). The main difference between the two types of particles is the number of spikes observed, more likely due to disordering of the projections located around the fivefold vertices in the trypsin-treated virions (K. Dryden et al., personal communication).

Virion Composition

Astrovirus particles are formed by the viral genome surrounded by an icosahedral capsid formed by a single protein of 70 to 90 kd, or by at least three proteins in the range of 25 to 34 kd, depending on the extent of proteolytic processing of the virion.^{12,93,119} Extracellular particles, released from HAdV-8 infected cells, are formed by protein VP70, which results from the intracellular processing of VP90, the full-length primary protein product encoded in ORF2⁹⁴ (see below). Fully

infectious particles obtained by exhaustive treatment of the virus with trypsin are constituted by three proteins, in the range of 32–34, 27–29, and 25–26 kd, depending on the virus strain, with the last two proteins overlapping in sequence.^{93,119} Thus, proteins of different size (between 24 and 90 kd), probably representing final and intermediate cleavage products, can be found in particles of HAdV.¹² The size of some of the proteins identified in animal viruses is similar to that of proteins found in human strains; thus, PAstV contains proteins of 31, 30, and 36 kd,¹²⁵ and OAstV contains two proteins of approximately 33 kd⁵⁸; however, PAstV also contains proteins of 39 and 13 kd,¹²⁵ not usually detected in mature HAdV particles. An isolated report indicates that HAdV particles may contain a small protein of 5.2 kd, but its nature was not established.⁷⁸

In addition to proteins derived from ORF2, proteins coded by ORF1a have been suggested to be present in viral particles, since antibodies to purified HAdV-1 virions recognize a recombinant protein containing amino acids 757 to 899 of nspl a.⁸⁹

GENOME STRUCTURE AND ORGANIZATION

Astroviruses have an ssRNA genome of positive polarity [ssRNA(+)] (Fig. 21.4) that varies in length from 6.17 kb for the human strain MLB-1³³ to 7.72 kb for DAdV-2,³⁷

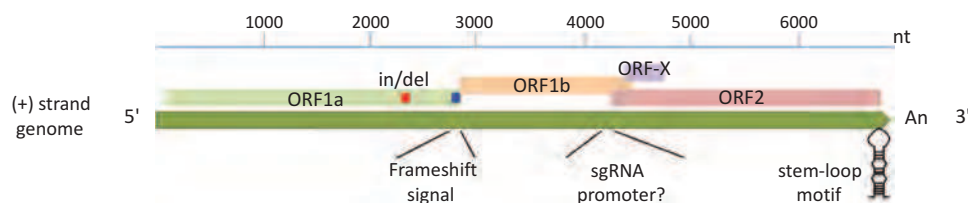


FIGURE 21.4. Genome organization of human astrovirus. The genomic RNA, approximately 6.8 kb, contains three recognized open reading frames (ORF1a, ORF1b, and ORF2). ORF-X has been proposed as functional, given its conservation among AstVs. The genome contains three elements conserved among all members of the family: the frameshift signal (blue square), the sequence upstream of ORF2 that putatively acts as promoter for synthesis of the subgenomic (sg) RNA, and the stem-loop at the ORF2 3' end. Also shown are the insertion/deletion (in/del) region (red square) and the presence of a poly(A) tail at the 3' end.

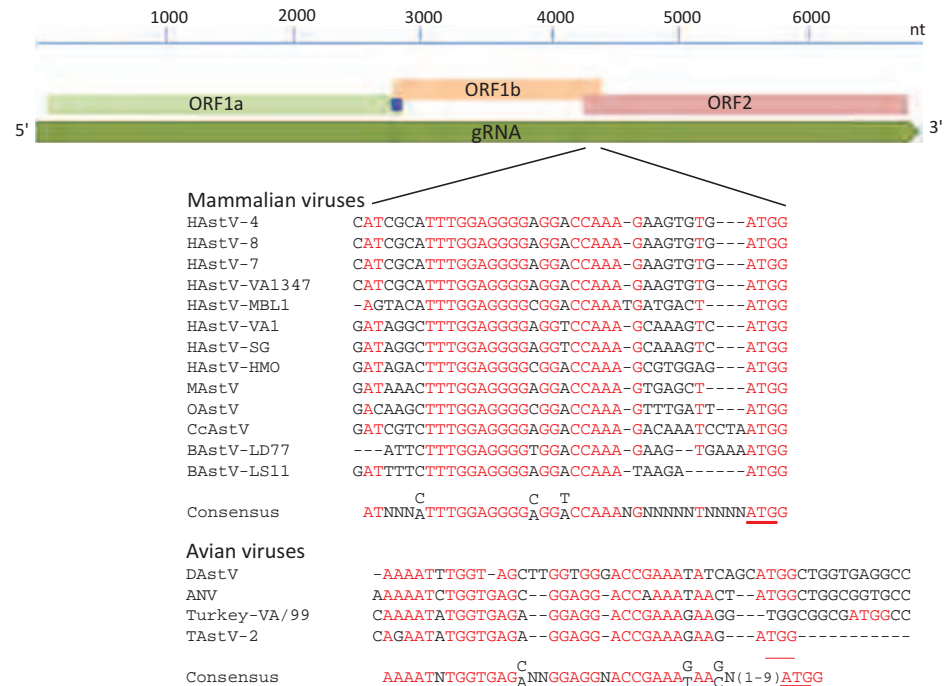


FIGURE 21.5. Conserved sequences upstream of the ORF2 initiation codon that might represent the promoter for sgRNA synthesis. Red letters indicate those completely conserved among viruses of the same genus; the ORF2 initiation codon is underlined. Viruses within each genus have a different consensus sequence.

excluding the poly(A) tail at the 3' end (e-Table 21.1 and e-Fig. 21.1). The RNA extracted from AstV particles, as well as RNA transcribed from a full-length genomic copy of complementary DNA (cDNA) clone, are able to initiate a productive infection in cultured cells,⁴² although with different efficiencies. The viral genome includes 5' and 3' untranslated regions (UTRs), and three ORFs of variable length in different isolates (e-Table 21.1). The two ORFs located towards the 5' end of the genome, designated ORF1a and ORF1b, encode nonstructural proteins that are presumed to be involved in transcription and replication of the virus genome, based on the sequence motifs they contain (see below). Variation of the ORF1a length in HastV is mainly due to insertions or deletions (in/del regions) present near the 3' end of ORF1a.^{55,144} The third ORF, found at the 3' end of the genome and designated ORF2, encodes the capsid polypeptide. ORF1a and ORF1b overlap in 10 to 148 nucleotides (nt) in the genome of mammalian viruses, and between 10 and 45 nt in avian viruses. The overlapping region contains an essential signal for translation of the viral RNA polymerase (encoded in ORF1b) through a frameshift mechanism⁶⁵ (see below).

Two positive-sense RNA species have been identified in astrovirus-infected cells: the full-length genomic RNA (gRNA), and a subgenomic RNA (sgRNA) of ~2.4 kb¹⁰⁴ (see below, Transcription/Replication section). Based on the transcription initiation site determined for the sgRNA in HastV-1 and HastV-2,^{103,146} ORF1b and ORF2 overlap in 8 nt; however, the length of this overlapping may vary, and it is not present in DastV; rather, it is 24 nt apart in this virus.³⁷ The highly conserved sequence around the ORF2 start codon has been suggested to be part of the promoter for synthesis of the sgRNA,⁶⁸ as described for alphaviruses; however, this region differs in length and sequence between mammalian and avian viruses (Fig. 21.5). This conserved sequence shows partial identity with the 5' end of the gRNA,⁶⁸ suggesting that it has an important role for the synthesis of both gRNA

and sgRNA. Analysis of recombinant AstV isolated from different species has identified this region as a hot spot for recombination.^{132,140,148} Of note, the terminal 19 nt of ORF2 and the adjacent 3' -UTR are highly conserved among most

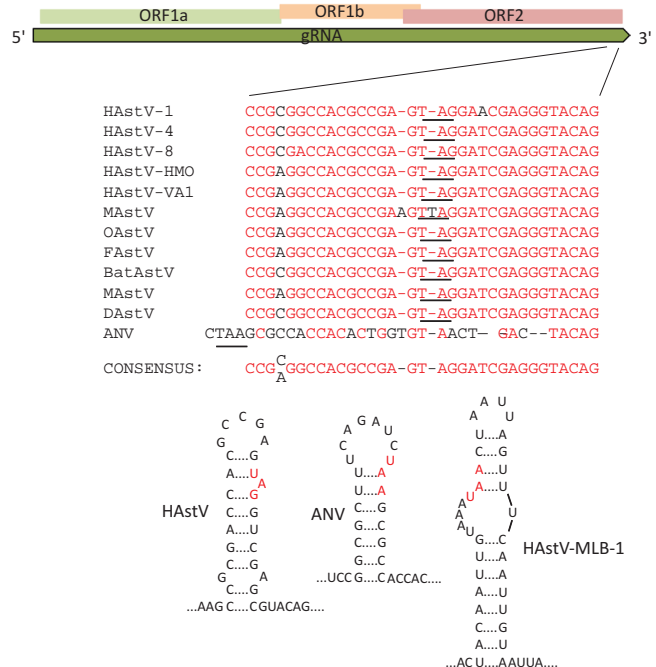


FIGURE 21.6. Conserved sequence and secondary structure at the 3' end of the ORF2 sequence. The relative position of these sequences is indicated in the upper diagram. Red letters on the sequence comparison indicate conserved nucleotides. The stop codon is underlined in the sequence and in red letters in the stem-loop structures. Although some strains lack this sequence conservation, such as ANV and HastV-MLB1, a stem-loop structure in this region is also predicted.

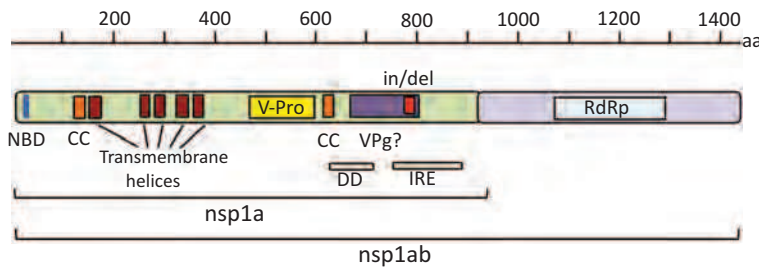


FIGURE 21.7. Nonstructural proteins predicted from the nucleotide sequence of ORF1a and ORF1b. NBM, nucleotide binding motif; CC, coiled-coil; v-Pro, viral protease; VPg, viral protein attached to the genome; in/del, insertion/deletion; DD, putative death domain; IRE, immune reactive epitope; RdRp, RNA-dependent RNA polymerase.

AstV, and similarities have been observed in the sequence and folding of the 3' -UTR of viruses from other families, such as avian infectious bronchitis virus (a coronavirus), a dog norovirus, and equine rhinovirus serotype 2 (a picornavirus),^{85,101} suggesting that it is relevant for astrovirus genome replication (Fig. 21.6).

ORF1a and ORF1b

The polypeptide encoded by ORF1a (nsp1a) is 874 to 936 amino acids (aa) in length in most mammalian viruses, although it can be as short as 787 in a human isolate and can reach 1,240 in avian viruses (e-Table 21.1). Five to six helical transmembrane motifs followed by a viral serine protease motif (v-Pro) are predicted in nsp1a⁶⁸ (Fig. 21.7). The v-Pro has features consistent with trypsin-like proteases, with a serine at the third catalytic amino acid residue.¹³¹ A viral protein genome-linked (VPg) encoded downstream of the protease motif has also been predicted based on its similarity with the VPg of calicivirus³; however, its synthesis in astrovirus-infected cells has not been investigated. Two predicted coiled-coil structures are present in nsp1a, one just upstream of the first helical transmembrane motif, and the second one downstream of the protease motif, suggesting that some protein products of nsp1a might form oligomers.⁶⁸ One region containing insertion/deletions (in/del), located downstream of the VPg motif, was related to the efficiency of HAstV to synthesize viral RNA species and to adaptation to cultured cells^{55,144}; however, it is not known whether the RNA sequence or the encoded protein is involved in modulating those events. ORF1b encodes a polypeptide of 515 to 539 aa that contains motifs of an RdRp, which are similar to RdRp of picornaviruses, caliciviruses, and certain plant viruses.^{3,65}

Regions that could potentially encode an RNA helicase and a methyltransferase have not been identified in the astrovirus genome.⁶⁵ Given the putative existence of a VPg, the absence of a methyltransferase could be understandable; however, the absence of an RNA helicase domain is unusual for a plus-strand RNA virus with a genome length of more than 6,000 nt, like astrovirus.⁶⁵ The amino terminus of nsp1a shows similarity with an NTP binding motif of some helicases,³ but lacks other motifs, such as the substrate binding and NTP hydrolysis domains present in these enzymes.¹³⁵

ORF2

The largest sequence variability in the astrovirus genome is found in ORF2, which codes for the virus structural polyprotein. This polypeptide varies from 672 to 851 aa in length in TastV-1 and a porcine strain, respectively. In general, the ORF2 of avian viruses codes for shorter polyproteins (e-Table 21.1). The N-terminal half of the polyprotein is more conserved than the C-terminal domain, encompasses a region of basic character that is conserved among astroviruses,^{67,142} and is thought to interact with the genomic RNA in the virion.⁴³ On the other hand, the C-terminal half of the protein shows considerable sequence variability among astroviruses isolated from different species, even among different isolates from the same animal species; the sequence of this region defines the serotypes in HAstV (Fig. 21.8). A delimited region with abundant insertion or deletions among different astroviruses is also present in the carboxy half of the capsid protein.¹⁴² The acidic character of a small portion of the protein, located close to the carboxy terminus (residues 649 to 702 for HAstV-8), is highly conserved among all members of the *Astroviridae* family.⁹² The last five amino acid residues at the C-terminal end

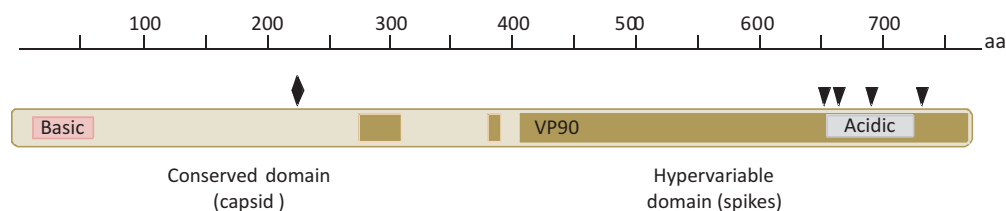


FIGURE 21.8. Features of the structural protein. The primary ORF2 product (named VP90 in HAstV-8), contains two domains that can be distinguished by their degree of conservation: the N-terminal domain is highly conserved and forms the core of the capsid, while the hypervariable C-terminal domain forms the spikes of the virus particle. VP90 contains basic and acidic regions that are highly conserved among all AstVs characterized. The diamond in the conserved domain indicates a lethal mutation identified in HAstV-1 and the black arrows indicate motifs recognized by caspases, important for virus maturation and cell egress.

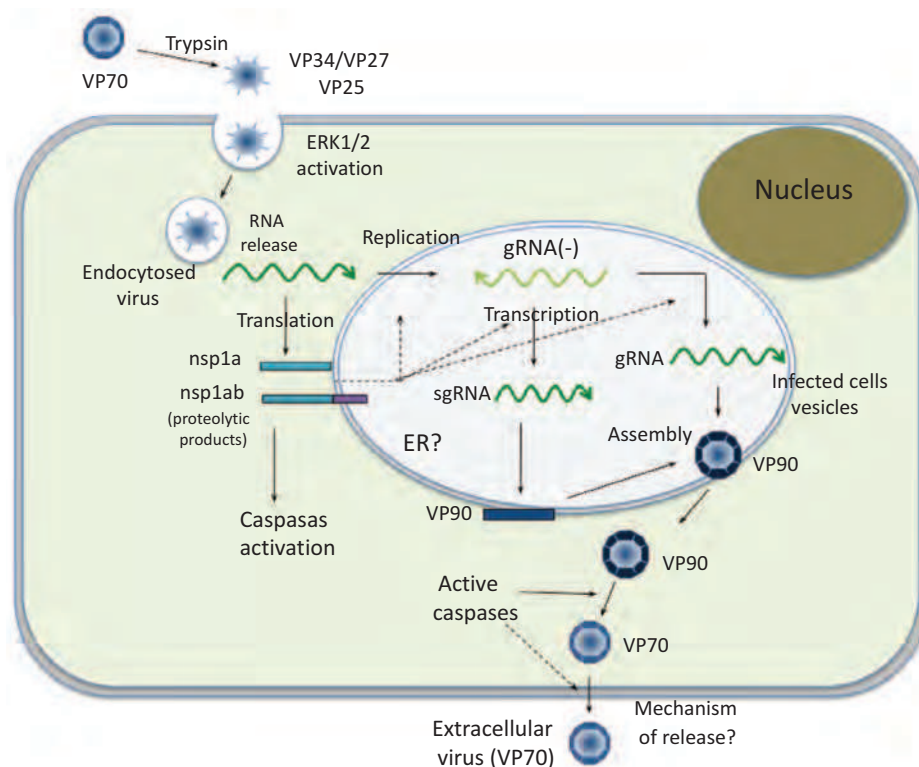


FIGURE 21.9. Replication cycle of HAdV. See details in the text.

are conserved, particularly among human and some other mammalian strains.

The conserved domain of the structural protein forms the capsid core of the particle, whereas the hypervariable C-terminal domain forms the spikes of the virion; thus, this last domain is predicted to participate in the early interactions of the virus with the host cell.⁷⁶

An alternative ORF (named ORF-X) of 91 to 122 codons overlapping ORF2 in a +1 reading frame has been described in all HAdV and some other mammalian viruses³⁶ (Fig. 21.4). Its initiation codon, usually located 41–50 nt downstream of the ORF2 AUG, is placed in a better Kozak's consensus sequence than that of ORF2, and might be translated through a leaky scanning mechanism. It remains to be determined whether ORF-X is actually translated and, if it is, its significance for virus replication.

STAGES OF REPLICATION

Attachment and Entry

Studies directed toward understanding the early interactions of astroviruses with their host cells have been limited; however, a general view of the replication cycle can be depicted (Fig. 21.9). Cell receptor molecules for these viruses have not been identified. Different HAdV strains show different tropism in cultured cells¹⁹; thus, it is likely that their initial interactions with the host cells might be different.

The infectivity of human astroviruses is greatly enhanced (3 to 5 logs) by, and probably requires, the treatment of the virus particles with trypsin.^{8,93,119} Although the proteolytic pathway of VP70 processing present in the virion has been elu-

cidated for HAdV-8,⁹³ the mechanism by which this treatment enhances virus infectivity is still unknown. Trypsin cleavage of the precursor polypeptide induces drastic structural changes in the particles⁸ (Fig. 21.3) and the generation in HAdV-8 of three final products: VP34, VP25, and VP27 (see details in the Protein Synthesis and Processing section). VP34 represents the conserved domain and forms the capsid core, while both VP25 and VP27 form the spikes on the virion surface. In spite of its high variability, VP25 contains two conserved structural motifs that have been suggested to be involved in the interaction of the virus with the host cell, one of which includes residues Lys455, Ser554, Thr575, and Glu610, predicted to interact with oligosaccharide moieties that could act as cell receptors or co-receptors (Fig. 21.10) (Dr. Y.J. Tao, unpublished data). Antibodies that recognize the spike proteins VP25 and VP27 neutralize virus infectivity,^{9,119} probably by blocking virus binding.

Early studies on HAdV entry in HEK-293 Graham cells using endocytosis-blocking agents (ammonium chloride, methylamine, and dansylcadaverine), as well as ultrastructural analysis, indicated that an endocytic, coated pit-dependent pathway is used by the virus to enter cells.³⁰ Recently, a clathrin-dependent endocytosis was confirmed to be a functional pathway for the entry of HAdV-8 into Caco-2 cells (unpublished results).

The interaction of HAdV with the host cell provokes activation of the ERK1/2 signaling pathway within the first 15 minutes after virus attachment.¹⁰⁷ Although the mechanism for this activation is unknown, it was independent of virus replication, suggesting that this pathway is triggered during virus binding or entry into the cell. Accordingly, ERK1/2 seems to be required at early times to establish a productive infection,

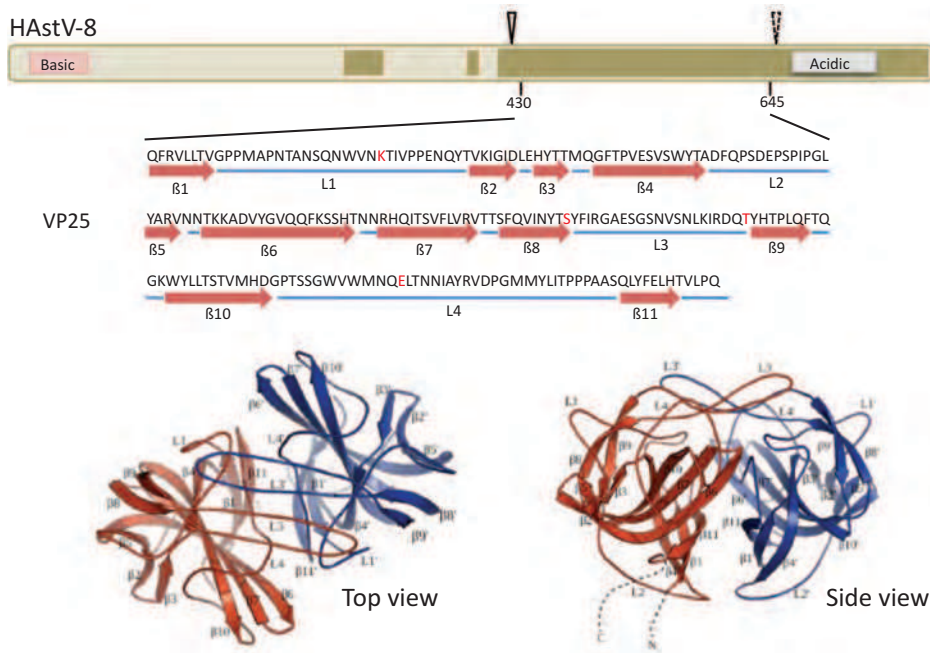


FIGURE 21.10. Three-dimensional structure of the spike protein VP25.

The hypervariable region of HAstV-8 VP90, including residues 430 to 645, crystallized to 1.8 Å resolution as a dimer. The upper diagram shows the relative position of the crystallized protein; the cleavage carried out by trypsin is indicated (arrowheads, as in Fig. 21.15). Amino acid residues Lys455, Ser554, Thr575, and Glu610 (red in the sequence) are strictly conserved in all eight HAstV serotypes, and have been proposed as potentially interacting with cell surface carbohydrate motifs during the early virus–cell interactions. Top and side views of the spike are shown. (Courtesy of Dr. Y. J. Tao, Rice University.)

since inhibitors of this kinase blocked synthesis of viral proteins and RNA and, consequently, reduced virus yield. HAstV also induce an increase in the epithelial barrier permeability that is independent of virus replication, apparently due to an interaction between the capsid protein and the cell surface.¹⁰⁵ It has been suggested that HAstV could trigger tight junction instability to reach putative receptors present in the basolateral membrane; however, the fact that the increase in permeability is observed after at least 16 hours of addition of the virus suggests that more than a requirement for infection, it is a consequence of the cellular transduction signal pathways induced by the virus.

Uncoating

The mechanism through which the viral genomic RNA is released from the infecting virus particle into the cytoplasm for translation, the cell site where it occurs, and the cellular and viral factors involved in this event are unknown.

Translation

No detectable changes in cellular protein synthesis are observed upon HAstV infection. As do most cellular mRNAs, astroviral RNA contains a polyA tail at the 3' end, but the presence of a cap structure at its 5' end has not been described. Since a VPg has been suggested to be encoded in astrovirus ORF1a,³ this protein could modulate the translation of viral mRNAs by interacting with translation initiation factors, as has been described for other viruses, such as calicivirus.^{23,29}

Nonstructural Polyproteins Synthesis and Processing

After uncoating, the gRNA is translated into nonstructural proteins that are produced as polyprotein precursors and subsequently proteolytically processed into smaller proteins. ORF1a directs the synthesis of protein nsp1a (of about 100 kd), whereas protein nsp1ab (160 kd) is derived from both ORF1a and ORF1b, through a frameshift mechanism of translation

(see below). Proteins nsp1a and nsp1ab are apparently processed co-translationally at their amino terminus, so that the expected full-length proteins are not, or very rarely, observed in HAstV-infected cells (Fig. 21.11). Information regarding processing of the nonstructural polyproteins has been obtained by *in vitro* translation, transient expression of cDNA clones, and analysis of HAstV-infected cells, using antibodies to different regions of nsp1a and nsp1ab.^{41,45,95,145} No specific processing of nsp1a and nsp1ab were observed by *in vitro* translation of cDNA-derived transcripts,⁴⁵ suggesting a requirement for cellular factors.

In HAstV-1-infected Caco-2 cells, Willcocks et al.¹⁴⁵ detected proteins of 74, 34, 20, 6.5, and 5.5 kd with antibodies raised to the predicted C-terminal 298 aa of nsp1a. Proteins of 20 and 74 kd—in addition to products of 88, 27, and 19 kd—were also detected in Caco-2 cells infected with an HAstV-8 strain using antibodies to several regions of nsp1a.⁹⁵ The 19- and 20-kd proteins likely represent the most N-terminal products of nsp1a, apparently cleaved out by a cellular protease, probably a signalase, because it is produced by *in vitro* translation experiments only in the presence of microsomes,⁹¹ it occurs co-translationally,^{41,95} and it is independent of the v-Pro activity.⁴¹ The 27-kd protein represents the v-Pro and probably spans from around amino acid residue 410 to 654 of nsp1a.⁴¹ Proteins of 160, 75, and 38 to 40 kd, and phosphorylated forms of 21 to 27 kd were also detected with antibodies to a synthetic peptide that comprises aa residues 778 to 792 of nsp1a.⁵⁴

Translation of the RdRp occurs through a ribosomal-1 frameshift mechanism in the overlapping region between ORF1a and ORF1b. The signal that modulates this event has two key features, conserved among all astroviruses: a heptameric sequence (AAAAAAC) and the potential to form a downstream stem-loop structure¹⁸ (Fig. 21.12). Translation of heterologous proteins, with the HAstV frameshift signal included, revealed that its efficiency varies between

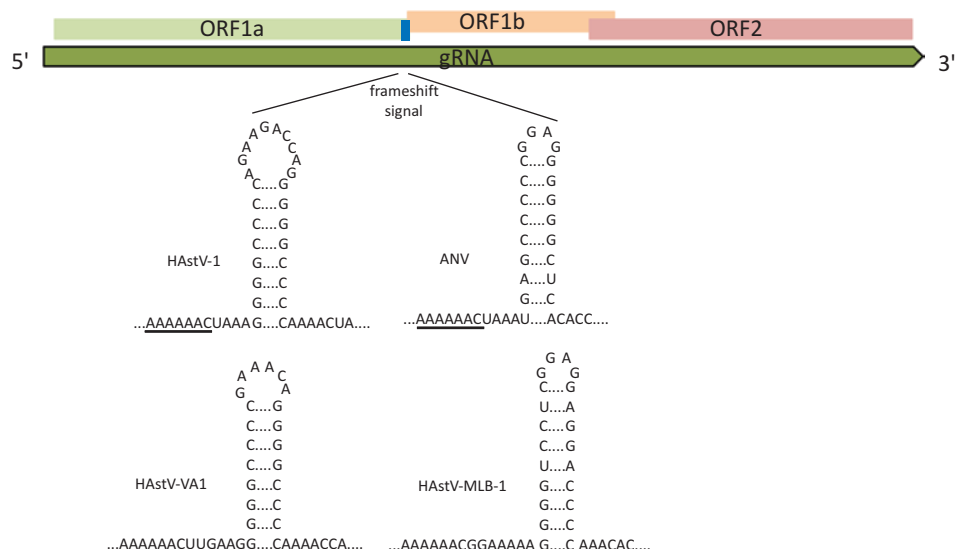


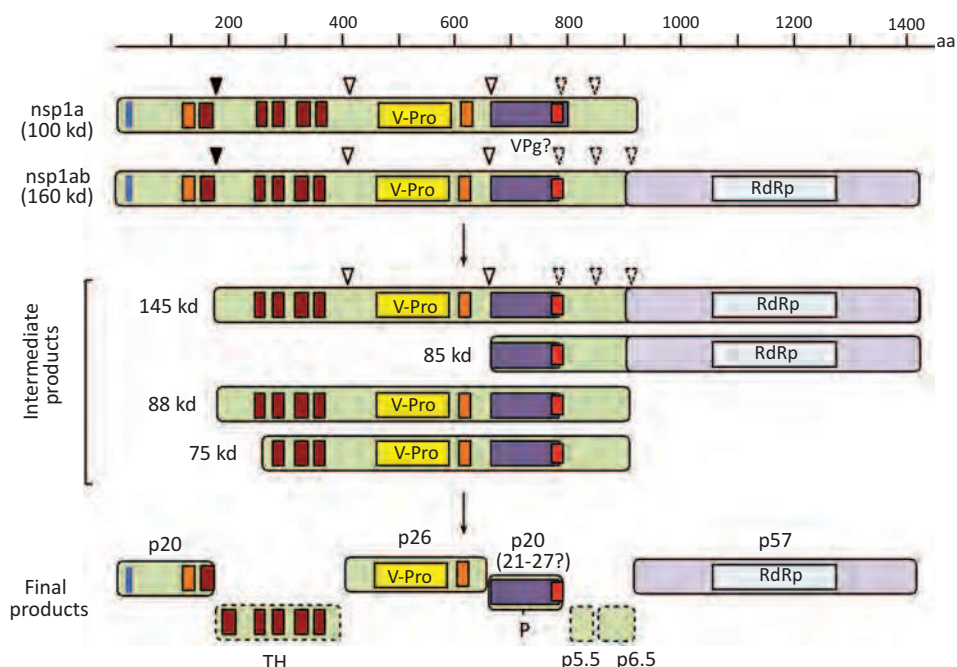
FIGURE 21.11. The frameshift signal (fss) of astroviruses is composed by a slippery sequence (underlined) and a stem-loop structure. The length and sequence of the loop and of the region between the slippery motif and stem-loop structure are not important for its function,¹⁸ as seen for the viruses shown.

7% and 28%, depending on the system in which it was evaluated⁸⁴; however, the efficiency of the frameshift has not been determined in HAsV-infected cells. Antibodies raised to recombinant proteins containing the RdRp motif identified a protein of 57 kD as the final product in Caco-2 cells infected with an HAsV-8 strain.⁹⁵

In summary, proteins of 27, 20, 19, 6.5, and 5.5 kD, as well as the phosphorylated 21 to 27 kD polypeptides, seem to represent the final processing products of polyprotein nsp1a, whereas a 57 to 59 kD protein represents the mature protein derived from ORF1b. The proteins observed of 145, 88, 85, 75, and 34 kD most likely represent intermediate products of the nonstructural protein processing (Fig. 21.11). With excep-

tion of the cleavage that releases the 20 kD most amino-terminal polypeptide, all other cleavages at nsp1a and nsp1ab polypeptides are believed to depend on the v-Pro activity, although only those identified at around amino acid residues 410 and 654 have been confirmed.⁴¹ Crystal analysis of the v-Pro domain (Fig. 21.13) showed that the enzyme contains a basic S1 pocket that recognizes and cleaves acidic residues (Glu and Asp) at position P1.¹³¹ Thus, Asp-413, but not Ala-409, and Glu-654 seem to be the actual residues cleaved by v-Pro on nsp1a. The C-terminal region of nsp1a contains many acidic residues that prevent the prediction of cleavage sites downstream of Glu-654 recognized by the viral enzyme to generate the 6.5 and 5.5 kD products observed.

FIGURE 21.12. Processing of the nonstructural proteins. Nsp1a and nsp1ab are processed by cellular (*closed triangle*) and viral (*open triangles*) proteases. Although only the cleavages around amino acid residues 410 and 654 have been confirmed to be due to v-Pro, indirect evidence that would explain the protein processing products observed suggests that the downstream cleavages (*dashed triangles*) are also due to viral protease. No products from the hydrophobic region have been identified and the smaller products of 5.5 and 6.5 kD have not been mapped into nsp1a (*dotted boxes*).



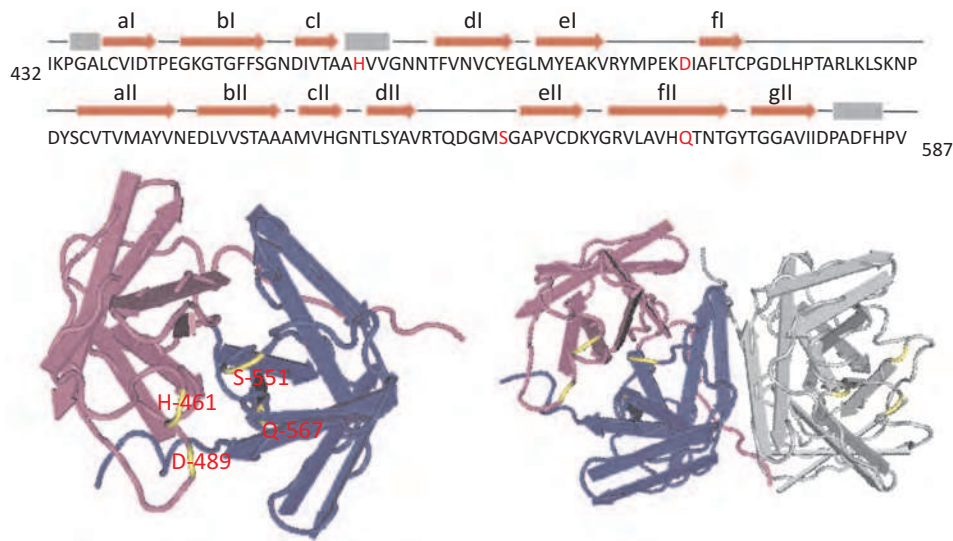


FIGURE 21.13. Diagram representing the structure of the HAstV-1 viral protease. Residues 432 to 587 of HAstV-1 nsP1a (upper sequence) were crystallized to 2.0 Å resolution. Monomer (left) with the two domains in blue and pink, and the catalytic residues in yellow and red letters is shown on the left. In the dimer (right), two domains of a monomer are colored and the other monomer is in grey. Structure was obtained from Protein Data Bank (ID code 2w5e).

Structural Polyprotein Synthesis and Processing

The structural proteins of HAstV, encoded in ORF2, are synthesized from the sgRNA as a polyprotein precursor of 87 to 90 kd. Studies with HAstV-8 have revealed that the 90-kd primary translation product is intracellularly cleaved at Asp-657 to yield a product of 70 kd (named VP70)⁷ through intermediates of 75 to 85 kd,⁹⁴ whose biological relevance, if any, is unknown (Fig. 21.14). Processing of VP90 to VP70 is carried out by cellular enzymes (caspases) that are involved in apoptotic processes,⁷ and whose activity is triggered during viral infection by an unknown mechanism.^{7,53} The pan-caspases inhibitor Z-VAD-fmk strongly inhibits this processing, whereas specific inhibitors of caspase activity partially block it. On the contrary, some proapoptotic factors—such as TNF-related apoptosis-inducing ligand (TRAIL) and staurosporine, which triggers different apoptotic pathways—enhance the cleavage of VP90 to VP70.⁹⁴ It is believed that caspases are important for processing the structural polyprotein precursor in most, if not all, astroviruses since the caspase-recognition motifs present

at the carboxy-terminal region of VP90 are highly conserved among different strains. For HAstV-8, caspase-3 and caspase-9 seem particularly important for processing VP90; however, other caspases might also be involved, since VP90 is substrate of caspase-8 and caspase-4 *in vitro*.⁷ Also, it cannot be excluded that different caspases are responsible for cleavage of VP90 from different astrovirus strains, since the motifs recognized by caspases in this precursor protein vary among viruses. Of interest, the VP90 to VP70 processing is not required for VP90 to assemble as viral particles, but it is important for the egress of the virus from infected cells^{7,91} (see below).

Extracellular particles of HAstV-8 formed by VP70 are weakly infectious, but its infectivity is strongly enhanced by treatment of the viral particles with trypsin,^{8,93} which is present in the intestinal lumen. Protein VP70 present in the extracellular virion is initially cleaved to yield 41 kd (VP41, the N-terminal product) and 28 kd (VP28, the C-terminal product) polypeptides. VP41 is subsequently cleaved at its carboxy terminus to yield a mature protein of 34 kd (VP34), whereas

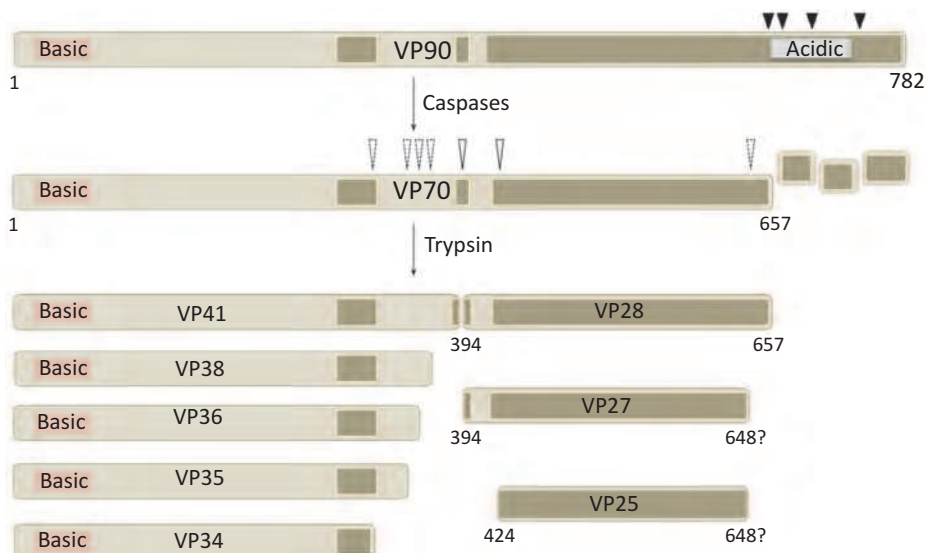


FIGURE 21.14. Processing of the structural protein VP90. The primary product of ORF2 is sequentially processed at its carboxy terminus by caspases to generate VP70, the protein present in the extracellular particles. These particles are processed by trypsin to generate protein intermediates of variable size, and the final products VP34, VP27, and VP25. Closed arrowheads represent cleavages carried out by caspases; open, long arrowheads represent cleavages by trypsin. The cleavage sites indicated by dashed arrowheads have not been precisely determined, therefore the carboxy-terminus of the intermediate and final products is unknown.

VP28 is cleaved to final products of 27 and 25 kd (VP27 and VP25, respectively) that share their carboxy terminus.⁹³ Thus, the full infectious virus is constituted by VP34, VP27, and VP25.

The C-terminal products VP25 and VP27 (or VP26 and VP29 in HAsV-2) are recognized by neutralizing antibodies,^{9,119} whereas VP34 (VP32 in HAsV-2) contain cross-reaction epitopes that have been used for diagnosis.⁵⁹ Although not tested, the infectivity of other astroviruses may also depend on trypsin treatment, since sites susceptible to trypsin cleavage are conserved in the capsid protein from avian and mammalian viruses.

Transcription/Replication

Astrovirus RNA synthesis has been poorly studied. Given the structure and organization of the astrovirus genome, it is believed that these viruses follow a strategy similar to that of alphaviruses to replicate and transcribe their genome.⁴⁴ Based on this assumption, the gRNA would be used as a template to synthesize a full-length negative-sense RNA, gRNA(-), which in turn would be used as a template to produce both the full-length gRNA and the sgRNA. These positive-strand RNA molecules are initially observed in HAsV-8-infected Caco-2 cells at 8 hours postinfection (hpi) (Fig. 21.15), indicating that at this time gRNA(-) is already synthesized, although at undetectable levels. In a different study gRNA(-) was detected starting at 9 hpi and it accumulated to 0.7% to 4% of gRNA.⁶⁴ Similarly to alphavirus, it is believed that the viral transcriptase recognizes a *cis*-element that acts as a promoter on gRNA(-) to synthesize the sgRNA, which in the case of HAsV can reach five- to tenfold higher molar abundance than the full length gRNA.¹⁰⁴ A sequence located around the ORF2 initiation codon that is highly conserved among most known astroviruses might represent that promoter (see above, Fig. 21.5).

Synthesis of gRNA(-) and accumulation of gRNA require cell protein synthesis, but not cellular DNA transcription.⁶⁴ v-Pro, RdRp, VP90, gRNA(-), and viral particles have all been found to be associated with internal cell membranes.⁹¹ This suggests that RNA replication and the first steps of morphogenesis are carried out associated with the observed membranous structures that probably derive from the ER, since viral phosphoproteins of 21 to 27 kd, which interact with the

RdRp,³⁸ and are likely involved in RNA replication, localize to this organelle.⁵⁴

Besides v-Pro and RdRp, no specific function has been assigned to the rest of the ORF1a and ORF1b products. Since the 20-kd N-terminal product of nsp1a is cleaved co-translationally, it is likely that the cleavage is carried out by a signalase.⁹⁵ This protein thus might target the replication complexes to membranes, although the predicted region containing highly hydrophobic transmembrane helices (amino acid residues 178 to 409) could also contribute to association of the replication complexes to membranes. A potential VPg has been suggested as possible product of nsp1a,³ just downstream of the protease, opening the possibility that AstV RNA synthesis would be primer dependent. Further studies are required to confirm this hypothesis.

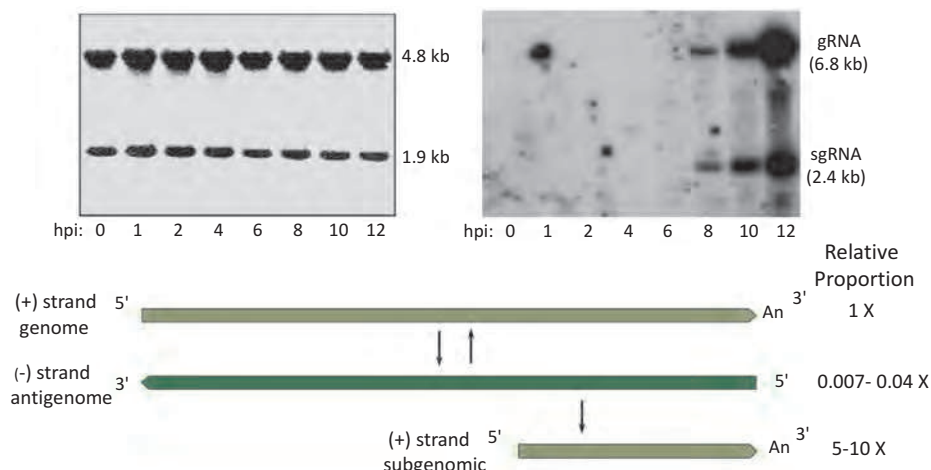
Assembly and Release

The expression of ORF2 in cultured cells using recombinant vaccinia virus²⁸ or baculovirus²² as vectors leads to the assembly of virus-like particles, indicating that the encoded protein is able to assemble in the absence of viral RNA; however, these particles were unstable and showed atypical morphology when purified, indicating a defective assembly.^{22,105}

Virus assembly tolerates some deletion or changes in the 70 N-terminal basic amino acids of VP90/VP70, which are thought to interact with the gRNA in the particles.⁴³ The infectivity of a recombinant virus in which aa residues 11 to 30 were replaced by 8 or 9 amino acids of a foreign sequence reduced to about 50%. In contrast, viruses carrying the same foreign sequence in place of aa residues 31 to 50 of the capsid protein showed a drastic reduction to 0.1% of infectivity. Substituting the 5' C-terminal aa of ORF2 was also very deleterious to virus infectivity.⁴³ A single-point lethal mutation was described in HAsV-1 VP34 (Thr-227 to Ala or Ser),⁸⁸ however, whether the defect on infectivity was due to a defective assembly was not studied.

The ORF2 primary product of HAsV-8, VP90, forms intracellular particles that can be found associated with membranes or in the cytosol.⁹¹ Apparently, VP90 assembles into particles associated with membranous structures, where viral nonstructural proteins and the sense and antisense genomic RNA are also present⁹¹; therefore RNA replication is also thought to occur associated with these structures (Fig. 21.16).

FIGURE 21.15. Astrovirus RNA species and their relative proportion produced during infection of HAsV. Total RNA from HAsV-8-infected cells at a multiplicity of infection of 2 were separated by gel electrophoresis. Ribosomal RNA was detected by staining with ethidium bromide (**upper left**), and viral RNAs by Northern blot with a negative-polarity RNA probe that is able to detect both gRNA and sgRNA (**upper right**). The hours postinfection (hpi) at which each sample was taken is shown. The lower scheme depicts the astrovirus RNA species and their relative amounts in infected cells.^{64,104}



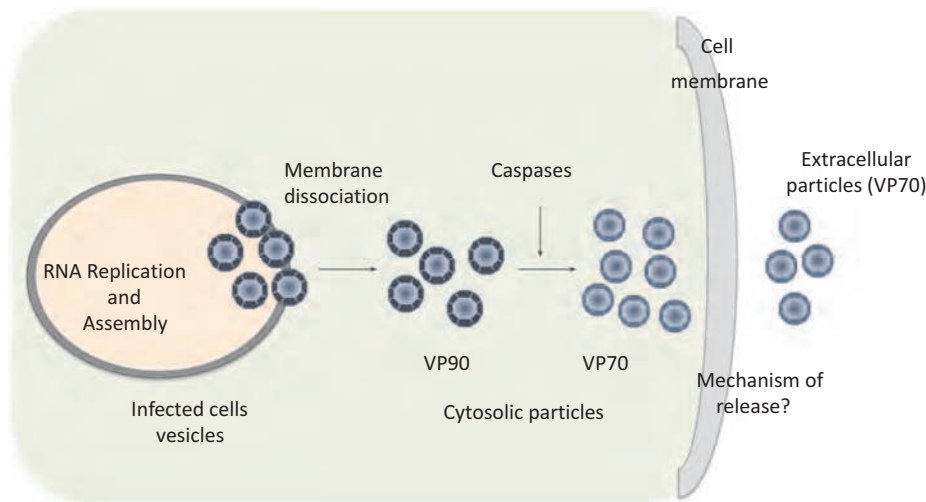


FIGURE 21.16. Model of VP90 processing and virus maturation. See details in the text.

It is believed that rearrangement of internal membranes is induced by AstV infection, since it is observed in intestinal epithelial cells of lambs infected with OAstV but not in uninfected cells.⁴⁸

HAsV infection induces the activation of caspases in Caco-2 cells through an unknown mechanism. Activity of initiator (caspase-8, -9, and -4), and executioner (caspase-3 and -7) caspases is clearly detected at 12 hpi. Apoptotic markers, such as lamin A and poly (ADP-ribose)-polymerase (PARP) cleavage, are also detected at 12 hpi, although cell death is not observed up to 48 hpi, even at high moi.⁷ A putative death domain (pDD), localized downstream of the v-Pro domain (aa residues 620 to 714 of nspl1a), was suggested as responsible for activation of caspases⁵³; however, given that cleavage at Glu-654 by v-Pro would split this putative domain, its role in caspase activation requires further investigation. Recent findings that v-Pro may cleave Asp residues¹³¹ open the possibility that this enzyme might cleave and activate pro-caspases.

Proteolytic processing by caspases of the VP90 assembled into particles, to yield VP70, is required for cell egress of the virus, since inhibitors of these proteases prevents it, while it is promoted by pro-apoptotic factors, such as TRAIL.⁹⁴ It is believed that after their initial assembly associated with membranes, the viral particles formed by VP90 separate from these structures, exposing its carboxy terminus, such that it is now available to caspase cleavage.⁹¹ In addition to its role in cleaving VP90 to VP70, the activity of Cap-3 seems to be required by the virus to exit the cell.⁷ Although the cellular process mediated by this caspase to allow virus egress is not known, the cell death induced by this protease seems not to be necessary; thus, a nonlytic mechanism seems to be involved in the release of HAsV. On the other hand, since a cytopathic effect was observed upon infection with porcine,¹²⁵ and chicken¹⁰ astrovirus strains, these viruses could use more than one mechanism to exit the cell.

PATHOGENESIS AND PATHOLOGY

As a gastrointestinal pathogen, HAsV can be transmitted to a host through the fecal–oral route, as shown by human vol-

unteer studies, but also person to person, as it was found in an outbreak of gastroenteritis in California.⁹⁷ Illness in adults can also be due to exposure to a larger dose of astrovirus or through fomites, and contaminated food or water.^{2,13} In the case of avian viruses transmission is also through a fecal–oral route, facilitated by the large amount of viruses secreted in feces and the usual contact of animals with them, although it has also been proposed to occur by vertical transmission from parents to their offspring.¹³⁷

Mammalian astroviruses affect mainly epithelial cells of the intestinal tract. Histopathologic studies of an immunocompromised child persistently infected, having a pronounced diarrhea, showed that astrovirus infection was limited to the small intestine. Infection involves the mature epithelial cells near the microvilli tips; it was more extensive in the jejunum than in the duodenum, but not in the stomach (Fig. 21.17).¹²² Morphologic abnormalities in the intestine suggested that, despite severe diarrhea, an inflammatory response does not occur. Studies with other mamastroviruses indicate that AstV can infect epithelial cells (OAstV and BoAstV) as well as subepithelial macrophages (OAstV) and M cells (BoAstV) of the small intestine.^{130,150} OAstV particles were observed in vacuoles of the enterocytes, very similar to those found during infection of HAsV in cultured cells. OAstV infection resulted in diarrhea on days 2 to 4 postinfection and a transient villus atrophy and crypt hypertrophy.⁴⁸ Although BoAstV was considered as nonpathogenic because it was unable to induce diarrhea in gnotobiotic animals, inflammatory mononuclear cells above the dome villi were observed on infection with this virus. In addition, the lamina propria was infiltrated with neutrophils and contained cells with degenerate nuclei. Lymphoid-cell depletion was noted in the central region of germinal centers beneath infected dome villi.¹⁵⁰

Astrovirus was recently found as the only pathogen in the central nervous system (CNS) of an immunodeficient patient who died of encephalitis.¹¹⁵ Expression of structural proteins was specifically localized in astrocytes, but not in macrophages or neurons. Infiltration of macrophages and inflammation were found in the CNS of the patient, although evidence of astrovirus infection was not found in gastrointestinal postmortem samples. Of note, the human strain associated with encephalitis

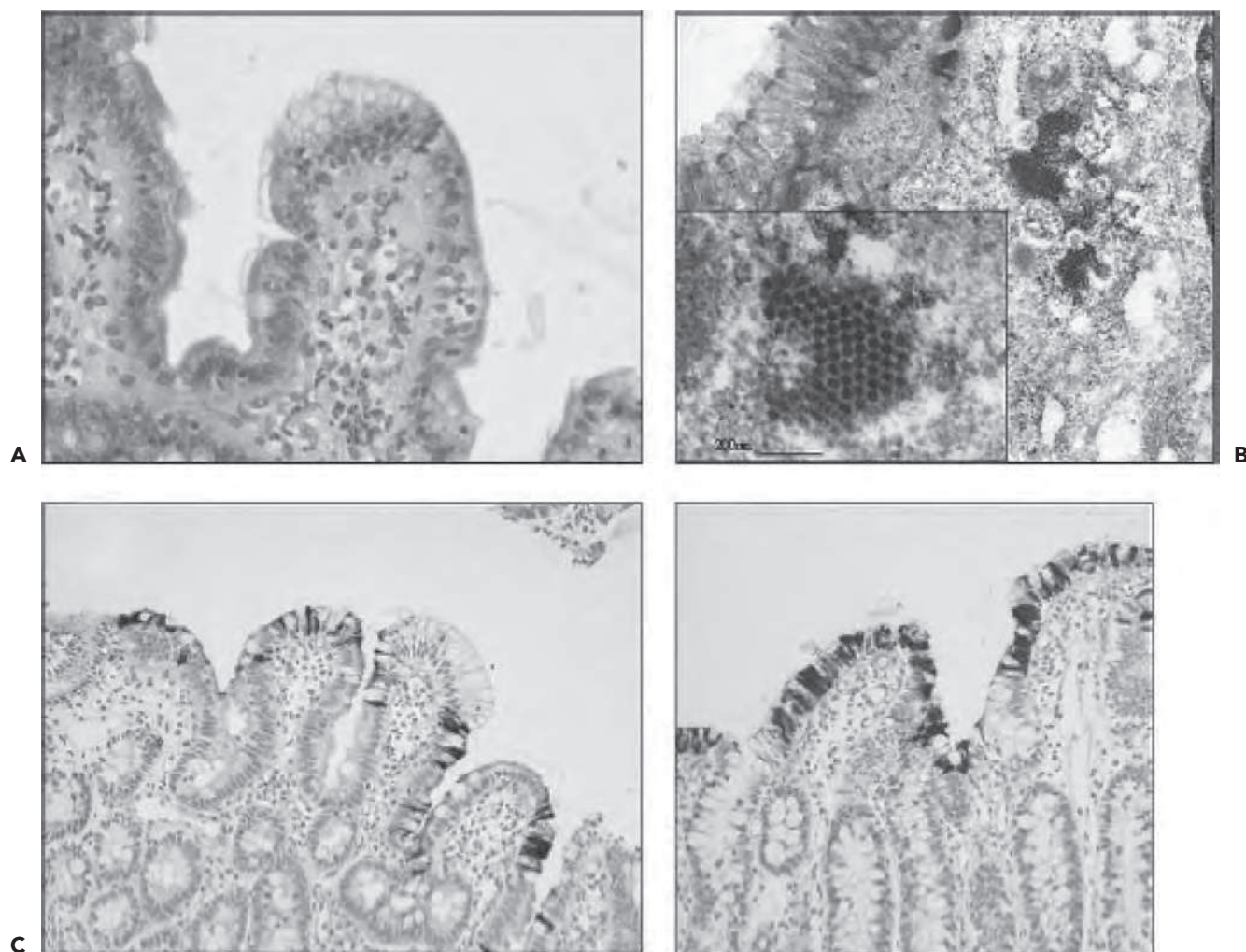


FIGURE 21.17. **A:** Photomicrograph of a jejunal biopsy specimen from a bone marrow transplant recipient with astrovirus infection demonstrating villus blunting, nonspecific alterations in surface epithelial cells and a mixed lamina propria inflammatory infiltrate, but without the presence of viral inclusion bodies (original magnification, $\times 100$). Photomicrographs of **(B)** duodenal and **(C)** jejunal biopsies from a bone marrow transplant recipient with astrovirus infection immunostained with anti-astrovirus antibody and demonstrating progressively more extensive staining of surface epithelial cells, most commonly near the villus tips (original magnifications, $\times 40$ and $\times 100$, respectively). **D:** Electron micrographs of a jejunal enterocyte demonstrating cytoplasmic paracrystalline viral arrays of astrovirus [original magnifications, $\times 32,000$ and $\times 100,000$ (inset)]. (From Sebire NJ, Malone M, Shah N, et al. Pathology of astrovirus associated diarrhoea in a paediatric bone marrow transplant recipient. *J Clin Pathol* 2004;57:1001–1003, with permission.)

was genetically related to a novel human astrovirus associated with an outbreak of gastroenteritis (strain VA1)³⁵ and with celiac disease.¹²⁷ It was also found in feces of five children with nonpoliovirus acute flaccid paralysis,⁶⁹ although it was also present in one healthy child. In addition, an astrovirus strain was found in the CNS of minks suffering from a neurologic disease (shaking mink syndrome) by metagenomics.¹⁶ In this last case, the syndrome was transmitted from infected to healthy animals by inoculation of brain homogenates. These findings suggest that AstV might cause neurologic diseases in mammals; however, the mechanism by which astrovirus could reach the CNS after infecting the gastrointestinal tract is unknown.

Lack of inflammation after intestinal astrovirus infection in humans¹²² and turkeys⁷² and the ability of HAstV to induce apoptosis in cultured cells^{53,94} suggest that this form of pro-

grammed cell death, in which inflammation is not frequently observed, could contribute to gastrointestinal disease. However, more than one mechanism could participate in the disease produced by these viruses since, for instance, inflammation was observed in the CNS upon AstV infection.¹¹⁵ Inflammation was also observed in premature children with necrotizing enterocolitis associated with HAstV, although in this case it was unclear whether inflammation was due to bacteria or HAstV spread.^{5,6}

Structural proteins may also be involved in pathogenesis, since the capsid protein is able to increase the permeability of monolayers of epithelium cells in culture.¹⁰⁵ Apparently, no virus replication is needed for the capsid protein to have this effect, but transduction signals are likely involved since barrier permeability was clearly increased at 20 hours after treatment with inactivated virus or the purified protein, but not earlier.

The HAsV-1 capsid protein binds C1q and mannose-binding lectin, blocking the complement activation through the classical and lectin pathways, respectively. Interaction of the virion with C1q is likely conserved among astroviruses, given the high conservation of the complement system in animals as well as of the region through which the capsid protein interacts with C1q (aa residues 79 to 108).

TAsV-2, isolated from birds with poult enteritis mortality syndrome (characterized by enteritis, growth depression, lymphoid atrophy, immunosuppression, and high mortality rates), has been used as a model to study astrovirus pathogenesis in young turkeys.⁷² Experimental infection of turkeys with TAsV-2 has shown that infectious virus can be recovered from many tissues, including blood, indicating the occurrence of viremia. The intestine, however, appears to be the only organ in which astrovirus replicates in this disease model.¹¹ Turkeys developed diarrhea on days 1 through 3 that persisted through 4 more days. In the intestines, no drastic morphologic changes or apoptosis were observed by light microscopy in infected animals; however, the F-actin distribution pattern changed at the apical region of jejunum tight junctions, which correlated with zones of infected cells. It has been suggested that these changes, together with defects in Na⁺ absorption probably due to modifications of expression and cell distribution of sodium/hydrogen exchangers, might provoke osmotic diarrhea.¹¹⁰ Intracytoplasmic aggregates of astrovirus were found in enterocytes on the sides and base of villi in the ileum and distal jejunum on day 3.

Avian astroviruses may spread to other tissues besides intestine, such as kidney, pancreas, lymphoid organs and liver, causing more severe diseases; however, this effect might depend on many factors, such as animal age and route of infection (vertical or horizontal), maternal antibody levels, size of the infecting dose, virus strain, and the presence of co-infecting pathogens.^{66,128,133,136}

Immunity

Determinants of immunity to astrovirus are not well understood. Symptomatic astrovirus infection is found primarily in two age groups: young children and elderly, institutionalized patients. Indirect evidence suggests that astrovirus-specific antibodies could play a role in limiting infection in the host. The existence of astrovirus serotypes in different animal species, classified mainly on the basis of neutralizing antibody reactivity against the carboxy region of the capsid protein, suggests that antibodies exert immune pressure on the virus.⁷⁰ The biphasic age distribution of symptomatic infection suggests that antibody acquired early in life provides some kind of protection from illness through most of adult life and that immunity to astrovirus wanes late in life. Immunoglobulin therapy of immunocompromised patients with persistent astrovirus infection resulted in virus clearance and diarrhea elimination.¹⁵ However, the role of astrovirus-specific antibodies to clear the virus was not defined because no neutralizing antibodies were measured in the immunoglobulin preparation utilized. On the other hand, bone marrow transplant patients with chronic diarrhea did not respond to the immunoglobulin treatment, although this preparation was demonstrated to have antibodies to the homologous infecting astrovirus serotype.²⁷ Thus, the response of patients to immunoglobulin treatment for virus clearance could be caused by additional unspecific factors more than to astrovirus-specific antibodies.

The normal mucosal immune system could be important in protecting individuals from repeated infections with human astrovirus.¹⁰⁰ T cells that recognize astrovirus antigens in a human leukocyte antigen (HLA)-restricted manner were found to reside in the intestinal lamina propria of healthy adults. These human astrovirus-specific CD4⁺ T cells produced helper T-cell subtype 1 (T_H1)-type cytokines, interferon gamma, and tumor necrosis factor, when activated.

The complement system is one of the initial host responses to pathogens, important in both innate and adaptive immunity. By blocking the complement system,^{51,57} as mentioned earlier, HAsV could alter an effective immune response of the host. However, on the other hand, binding of C1q to the capsid protein already assembled in the virion, if it occurs, could facilitate phagocytosis and elimination of the virus by C1q acting as opsonin.

In animals, the role of the humoral immune response to limit astrovirus infection is also not clear. Koci et al.⁷¹ found that virus replication in small turkeys infected with TAsV was limited, even though the infection did not induce a significant adaptive immune response, evaluated through measurement of a specific increase in the population of CD4⁺ and CD8⁺ T cells. Because there was no protection against TAsV upon secondary challenge, the limitation of virus replication was attributed to an innate response, mediated by production of nitric oxide. In chickens, protective immunity can be conferred by the capsid protein, since inoculation of this protein partially protected animals from disease,¹²³ although the role of humoral and cellular immunity in this partial protection is still unknown.

EPIDEMIOLOGY

HAsV infections have been found worldwide primarily, but not exclusively, in young children with diarrhea. Several studies associate astroviruses with diarrhea in immunocompromised patients, including adults,^{15,25,27,40,50,149} and with necrotizing enterocolitis (NEC) in premature children.^{5,6} Sporadic outbreaks of gastroenteritis caused by astrovirus have been reported among elderly patients⁸³ and military recruits.¹³ Large, food-borne outbreaks, affecting thousands of individuals in Japan, have occurred among school-aged children and adults as well.¹¹² In children, studies in different populations^{26,113} have revealed HAsV as the second most important cause of gastroenteritis, after rotavirus, with incidences varying between 4.2% and 7.3%, although incidences lower than 1% have also been reported. Higher incidences of HAsV have been rarely reported in children with diarrhea, such as in native populations of Brazil and southeast Mexico, with incidences of 56%³⁹ and 28%,⁸⁷ respectively. The age distribution of HAsV can vary depending on several factors; however, in a study carried out in Spain, approximately 80% of the astrovirus infections occurred in children under 3 years of age.⁵⁶ The age-specific HAsV incidences (episodes per year) found in an Egyptian study (0.38 for infants <6 months, 0.40 for infants 6 to 11 months, and 0.16 for children between 12 and 23 months) were similar to those found for rotavirus.¹⁰⁹

Most astrovirus infections in humans are detected in the winter months in temperate regions and in the rainy season in more tropical climates.^{26,141} Both community-acquired and

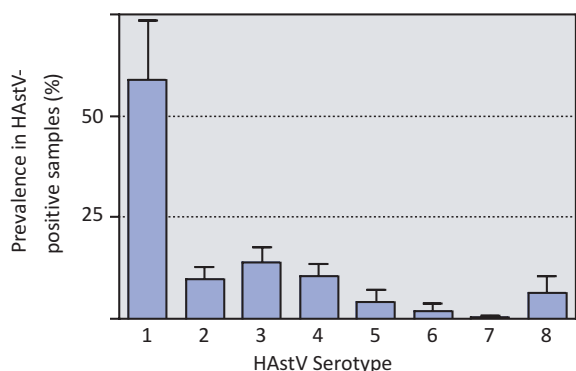


FIGURE 21.18. Prevalence of HAstV serotypes in children with diarrhea. This graph compiles data from eight studies in different countries with 461 astrovirus-positive samples. (Adapted from Monroe, SS. Molecular epidemiology of human astroviruses, In: Desselberger U, Gray JJ, eds. *Perspectives in Medical Virology. Viral Gastroenteritis*. Vol. 9. Amsterdam: Elsevier, 2003:607–616.)

nosocomial infections, particularly among immunocompromised patients^{25,40} and premature children, have been observed.

Eight serotypes have been mainly described among viruses that circulate in the human population. In general, serotype 1 of HAstV is the most common type found in children¹⁰² (Fig. 21.18), but the predominant serotype can vary with time and location. Thus, in a study in the United Kingdom, 72% of the community-acquired astroviruses encountered between 1975 and 1987 were serotype 1⁷⁸; while in Australia serotypes 1, 3, and 4 were regularly found in a period of 18 years.¹²⁰ In contrast, a longitudinal study of diarrhea in a cohort of Mexican children found that astrovirus serotype 2 was the most common (35%).⁵² In a different study with samples from different geographic regions in Mexico, all eight serotypes, except serotype 5, were found, with serotype 8 being as frequent as serotype 1 in one of the studied regions.⁹⁶ Analyzing AstV from different regions of India, putative recombinant viruses were found to be the most prevalent in children under 24 months of age.¹³⁸ Recombinant viruses containing RdRp and the capsid protein from different canonical HAstV have also been identified in different populations of Kenya.¹⁴⁸

Epidemiologic studies of AstV in the human population have been limited so far to the canonical HAstV serotypes 1 to 8. However, the recent identification of novel AstV in humans^{33–35,69} by highly sensitive methods highlights the necessity to analyze the prevalence of these novel viruses to determine their actual impact in public health, especially because they were found to be genetically related to animal viruses and some were isolated from patients with more severe diseases, such as encephalitis. Limited studies suggest that these viruses, similarly to the canonical HAstV, may be distributed worldwide in the human population.^{32,69} Given their genetic relationship with animal viruses and the potential for interspecies transmission, it would be possible that wild animals may become part of the epidemiologic equation.

Morbidity and Mortality

Although the gastroenteritis caused by HAstV is usually a mild, self-limiting illness that does not require hospitalization, more

severe gastroenterologic disease that may result in death has also been reported.¹¹² Astrovirus infection has been associated with intestinal diseases other than gastroenteritis, such as celiac disease¹²⁷ and intussusception,^{4,63} and it was found as the only pathogen in the CNS of a child with encephalitis.¹¹⁵ In a population of Mayan infants, astrovirus was the most common enteric pathogen identified in stool samples collected during a prospective study of oral poliovirus immunogenicity.⁸⁷ In 61% of the infants, astrovirus was detected by EIA in at least one stool sample over the 18-week period each infant was followed. Of 305 diarrheal episodes reported at study visits, 26% were associated with astrovirus. The enzyme immuno assay (EIA) results were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) in a subset of EIA-positive and -negative samples. The reason for the high prevalence of astrovirus infection and the low rate of rotavirus infection (4%) in this semiclosed community is not known, but it may be related to temperature, rainfall, humidity, or other environmental factors.

HAstV were shown to be an important cause of outbreaks of diarrhea in the childcare setting. Children up to 36 months of age attending childcare centers were at greatest risk of developing diarrhea.⁹⁹ An investigation of eight outbreaks of astrovirus diarrhea in six childcare centers showed that 20% of the children with diarrhea shed astrovirus.⁹⁸

Origin and Transmission

Epidemiologic data indicate that contaminated food is the main source for HAstV infection. HAstV has been found in bivalve mollusks, indicating that seafood may contribute to gastroenteritis caused by HAstV.¹³⁹ HAstV has also been detected in water from different origins, including drinking water, rivers, dams, wastewater, and effluents from water treatment plants.^{81,90,108,114} Sequence analysis of HAstV strains recovered from water and clinical samples from hospitalized patients showed that virus strains from both origins were identical,¹⁰⁸ confirming that water supplies are an important source of HAstV contamination. In ground water, the infectivity of astrovirus can be maintained for months; besides, it is partially resistant to chlorination,³¹ which is widely used for wastewater treatment in many countries.

The identification of astroviruses in the human population—different from canonical HAstV but genetically related to AstV isolated from rats,²⁴ minks,⁶⁹ marine mammals,¹¹⁷ and pigs⁸⁵—suggested that wild mammals might act as reservoirs for human AstV, and that interspecies transmission occurs. The identification of a recombinant virus containing sequences derived from HAstV-4 and CslAstV in sea lions supports this hypothesis.¹¹⁷ A high diversity of AstV has been found in mammals, such as bats¹⁵² and pigs,⁸⁵ suggesting that the emergence of novel viruses in humans is possible.

Seroprevalence

Antibodies to canonical HAstV tend to be acquired in early childhood, which is consistent with the age at which children are primarily infected. An early survey of 87 children less than 10 years of age in the Oxford region of the United Kingdom revealed that antibody prevalence rises rapidly from 7% in 6- to 12-month-old infants to 70% by school age.⁷⁷ This study was focused on detecting HAstV antibodies by immunofluorescence of astrovirus-infected cells; therefore, the prevalence of antibodies to astrovirus in this population could be underestimated.

The presence of high titers of antibodies to HAsV, particularly to HAsV-1 capsid protein,²⁷ in gammaglobulin pools from the United States indicates that HAsV infection with this serotype is very common. As expected, based on the most frequent HAsV serotype found to cause infection in different studies, one study of seroprevalence among children treated at a hospital in the United Kingdom found rates of 86% for serotype 1, 1% for serotype 2, 8% for serotype 3, and 6% for serotype 4.¹¹¹ In an age-stratified sample from The Netherlands, seroprevalence of neutralizing antibodies was again higher for serotype 1 (91%), but it was different for the other serotypes.⁷⁵ Studies to determine the seroprevalence of antibodies to the recently identified human isolates—such as HAsV-HMO, -MLB, and -VA1—are still pending, although limited studies with the HAsV-MLB-like virus strain indicate that they are also widely distributed in the human population.^{34,69}

CLINICAL FEATURES

Characteristics of Human Illness

Gastroenteritis caused by astrovirus infection primarily affects young children throughout the world. Table 21.1 summarizes the clinical features associated with this disease; however, these features can vary depending on the population studied. For instance, 12.4% of outpatients under 36 months of age infected with HAsV in Argentina got diarrhea, 41.6% had fever, and 16.6% of the patients required hospitalization⁴⁶; in Egypt, diarrhea caused by HAsV in children under the age of 3 years was similar to that caused by rotavirus, and severe dehydration was present in 17% of these patients.¹⁰⁹ Complications such as dehydration can develop in patients with underlying

gastrointestinal disease, poor nutritional status, and mixed infection.²⁶

Adult human volunteer studies established the incubation period to be 3 to 4 days and the highest viral shedding at day 6, which appeared to be proportional to the severity of the illness; seroconversion was confirmed at day 28.⁹⁷ Development of disease in these patients was limited and dependent on the size of the inoculum. A shorter incubation period of 24 to 36 hours was calculated from the secondary spread characteristics during an outbreak of gastroenteritis in a Japanese kindergarten.⁷⁴ In general, astrovirus diarrhea is milder than that caused by rotavirus and does not lead to significant dehydration or hospitalization.¹⁴¹ Persistent gastroenteritis caused by astrovirus has been associated with strains belonging to serotype 3.²¹ Deaths associated with gastrointestinal astrovirus infection are extremely rare but have been reported.¹²⁶

Additional gastrointestinal diseases associated with canonical HAsV include intussusception^{4,63} and necrotizing enterocolitis,^{5,6} while the recently described HAsV-VA1 was found in celiac disease.¹²⁷ HAsV was found in 3 out of 6 pediatric patients (between 4 and 7 months) with ileocolic intussusception; in two, HAsV was the only pathogen found and one died.⁴ In premature children, HAsV was the most frequently associated viral pathogen with necrotizing enterocolitis.⁶

HAsV has been found to be the cause of chronic diarrhea among immunocompromised patients, children, and adults, and of outbreaks in pediatric patients undergoing bone marrow transplantation²⁷ and pediatric primary immunodeficiency units.⁴⁰ Among gastrointestinal viruses, HAsV have been found more frequently in patients with a number of immune diseases, such as human immunodeficiency (HIV positives), combined immunodeficiency, congenital T-cell immunodeficiency, chronic lymphocytic leukemia, Waldenström's macroglobulinemia, and immunodeficiency polyendocrinopathy.^{15,40,50,149} Patients treated with fludarabine, which is known to deplete CD4+ T cells, developed persistent diarrhea caused by HAsV.¹⁵

Astroviruses have been identified in feces of children with nonpoliovirus flaccid paralysis⁶⁹ and in frontal cortex biopsy of a patient with X-linked agammaglobulinemia who developed encephalitis.¹¹⁵

Clinical Features in Avian Species

Intestinal and extraintestinal illness has been noted in avian species infected with astroviruses. Avian nephritis virus (ANV) infects chickens, and the viral antigen can be found in different organs (e.g., liver, spleen, pancreas, kidney, jejunum, and ileum) of this animal. This virus has been associated with growth retardation and acute interstitial nephritis.⁶² Antigenically ANV-unrelated astrovirus (confirmed to be different by sequence analysis of a specific region of the nonstructural polyprotein) isolated from chickens were found in the small intestine, but rarely in other organs; these viruses were associated with mild diarrhea.¹⁰ In ducks, astrovirus infection is associated with fatal hepatitis, with mortality rates of up to 50%.⁴⁷ Subcutaneous injection of a 20% (w/v) suspension of astrovirus-infected livers into 2- to 3-day-old ducklings recapitulated the hepatitis with all of the histologic features of the original animals and caused death within 2 to 4 days in 25% of the ducklings. Serial passage of this agent in ducks resulted in mortality rates of 25% to 55%. However, asymptomatic DAsV infections seem to be present at high frequency since

TABLE 21.1 Clinical Symptoms of Human Astrovirus Infection

Diarrhea	72%–100%
Duration of diarrhea (days, average)	2–3
Maximum number of stools/24 h	4
Bloody diarrhea	0%
Abdominal pain	50%
Vomiting	20%–70%
Duration of vomiting (days, average)	1
Maximum number of vomiting episodes/24 h	1
Fever	20%–25%
Maximum fever (Celsius)	37.9
Dehydration to some degree	24%–30%
Severe dehydration	0%–5%
Hospitalization	6%
Duration of hospitalization (days, average)	6
Severity score ^a (1–20) (average)	5
Otitis	13%
Bronchiolitis	33%
Admission diagnosis of gastroenteritis	18.7%–48%

^aTwenty point scoring system (Ruuska T, Vesikari T. Rotavirus disease in Finnish children: use of numerical scores for clinical severity of diarrhoeal episodes. *Scand J Infect Dis*. 1990;22:259–267.).

Adapted from Walter JE, Mitchell DK. Astrovirus infection in children. *Curr Opin Infect Dis* 2003;16:247–253.

a significant seroprevalence on healthy animals can be found; a high seroprevalence to DAsV was also found in flocks that present a very low mortality rate.¹³⁷ Turkey astrovirus has been associated with diarrhea and the high mortality disease poult enteritis mortality syndrome (PEMS). TAsV was isolated from the thymus of an infected animal with PEMS,⁷³ but it could be detected in different tissues (e.g., bursa, thymus, spleen, kidney, pancreas, and skeletal muscle).¹⁰⁶ The small intestine, however, seems to be the main tissue where virus replication occurs.¹¹ Asymptomatic infections have been also found in turkeys as well as in chickens infected with AsV.¹³⁷

DIAGNOSIS

Electron Microscopy

Astroviruses have been detected by direct EM examination of negative-stained fecal specimens. The sensitivity of this methodology has been estimated to be 10^6 to 10^7 virus particles per gram of stool. Usually, patients with diarrhea caused by astrovirus shed large numbers of viral particles (equivalent to approximately 10^{10} or 10^{11} of virus genomes per gram of feces).⁵⁵ However, in those patients who may be excreting fewer viral particles, usage of antiviral antibodies for immunoelectron microscopy facilitates identification of the virus.

Enzyme Immunoassays

Herrmann et al. developed an EIA that relies on a group-reactive monoclonal antibody (8E7) that maps to the conserved domain of the capsid protein of HAsV serotypes 1 to 8 (amino acid residues 71 to 260) to capture viral antigen, and a polyclonal antiserum as the detector antibody.⁶⁰ This EIA had a comparable sensitivity (91%) and specificity (98%) to immune EM. EIA has been useful for rapid detection of astrovirus antigen in studies in which a large number of human samples have been assayed. However, antibodies developed by Herrmann do not recognize the recently described novel HAsV strains.

The high antigenic divergence of avian viruses has not allowed the obtention of antibodies to common epitopes to be used in immunoassays to detect all AsV that infect flocks; the DAsV capsid carboxy-terminal region has been successfully used to detect prevalence of antibodies to this virus in ducks.¹⁴³

Molecular Techniques

As more information regarding the sequence of astrovirus genomes became available, detection techniques that employ molecular probes and RT-PCR were developed. At present, the complete genome sequence of several human and animal AsV strains, as well as the ORF2 sequences from a high number of isolates, have been determined. The high sequence variability that exists among these viruses does not allow the use of universal primers for all members of the *Astroviridae* family. However, based on these sequences, oligonucleotides from selected regions of the genome have been used as primers to diagnose closely related viruses by RT-PCR and sequencing of the amplicon. Particularly, primers derived from different regions along the genome have been successfully used for RT-PCR to detect typical HAsV and animal viruses. The sensitivity of this methodology increased when it was coupled to a cell-culture system in clinical and environmental water samples.⁴⁹ Given

that different pathogens are associated with enteric infections in humans and animals, methods based in RT-PCR have been developed recently for diagnosis of multiple human or animal viruses, including astroviruses, in a single tube.^{14,118,124,151}

Diagnosis by RT-PCR has also been used to detect the presence of astrovirus in animals; however, in the case of avian astroviruses, as mentioned, common oligonucleotides cannot be used due to the high genome diversity, which is even higher than among mammastroviruses. In spite of this, quantitative RT-PCR assays for ANV and CAsV, able to detect 180 and 105 RNA copies, respectively, have been used to specifically demonstrate the presence of these viruses in the gut and kidney of field and experimental infected animals.¹²⁹ Sensitivity for ANV detection increased (detecting up to 18 RNA copies) when oligonucleotides that amplify the 3'-UTR conserved region were used.¹³⁶

Metagenomic analysis has revealed the existence of novel astroviruses in samples from patients with diseases that had not been previously associated with these viruses,^{69,115} contributing to changing the virologist community's view of their behavior and relevance. This powerful technology can help to identify emerging astroviruses, as well as many other novel pathogens, and will provide the basis to design biological reagents for specific diagnosis.

Oligonucleotide microarrays have also been used as a powerful technology to identify previously unknown viruses belonging to different families. This methodology has been evaluated to detect and type HAsV using viral cDNA or RNA from reference strains adapted to cultured cells or wild-type strains from feces.²⁰ More studies are needed to determine the usefulness and sensitivity of this methodology with clinical samples.

PREVENTION AND CONTROL

Gastroenteritis caused by astrovirus is generally a mild, self-limiting illness that can disrupt an individual's activities for a few days but does not require specific therapy. In the young child or adult patient who becomes dehydrated, oral or intravenous fluid resuscitation may be necessary. Intravenous immunoglobulin may be a useful adjunct in severely immunodeficient patients who fail to respond to conservative measures,¹⁵ but larger studies are needed to determine efficacy and establish indications. Of note, immunoglobulin treatment has been suggested as a possible cause of encephalitis in an immunodeficient patient,¹¹⁵ although this was not confirmed.

Interruption of transmission is the key factor in preventing astrovirus infection. This is especially important in hospitals and other institutions, daycare centers, and families, where person-to-person transmission is likely to occur. Astrovirus is resistant to a number of treatments, including normal disinfectants,^{80,121} and it can survive in water after chlorination.^{1,81} Therefore, universal hygienic procedures must be enforced in these settings. No vaccines have been developed for astrovirus infections in any host animal; however, inoculation of the recombinant, baculovirus-expressed capsid protein to hens partially protected the offspring for the presence of gut lesions and weight depression after virus challenge.¹²³ Thus, this kind of antigen eventually could be useful in evaluating vaccine candidates in other animal species, including humans.

PERSPECTIVES

The perception of the relevance of AstV in public health is changing given the discovery in recent years of novel viruses isolated from humans that show genetic similarities with animal strains, some of which have been associated with diseases other than gastroenteritis. Further characterization of HAsTV genetically related to animal strains will help to better understand the public health importance of astroviruses. The available evidence indicates that interspecies transmission might be common in astroviruses. Epidemiologic studies designed to search for AstV in nongastrointestinal diseases, such as those related to the CNS, will be useful in understanding the spectrum of pathology these viruses may be associated with, and could change the current knowledge we have about the epidemiology and pathology of astrovirus-associated disease. Although animal models have been established for avian viruses, models for mammalian viruses are required to study the determinants of protective immunity in these viruses and the pathogenesis of the diseases they cause. The structural analysis of AstV proteins that has already started with viral protease, and spike protein should continue to help in the understanding of the molecular determinants of infection as well as the role specific viral proteins play in the virus replication cycle. So far, neither cellular receptors nor the virus capsid protein domains involved in virus entry and cell egress have been identified for AstV. It is expected that further structural characterization of viral capsid proteins will allow us to undertake new approaches targeted to understand these processes, and also probably to control infection. Significant gaps exist in our understanding of astrovirus genome replication, especially since it occurs in the absence of a viral encoded RNA-helicase, a peculiar feature of AstV; therefore, the identification of viral and cellular proteins that participate in this process will be useful in elucidating its mechanism. The cell response to HAsTV infection has proven to be partially beneficial for virus cell entry and egress; however, the cell factors and mechanisms involved in these processes are only beginning to be characterized. Further studies are required to advance our understanding of these stages of the virus lifecycle. Although infectious clones for HAsTV and ANV have been available for some time, reverse genetics in AstV has been poorly exploited. The systems for reverse genetics should be improved and used to identify the virus molecular determinants involved in the virus replication cycle.

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Togaviridae

Classification of Viruses within the Togaviridae Family

Virion Structure

- Structure of Mature Virion
- Structure of Immature Virion
- Structural Proteins of the Virion

Genome Structure and Organization

Alphavirus Replication

- Mechanism of Attachment and Receptors
- Mechanisms of Entry, Membrane Fusion, and Uncoating
- Translation and the Role of Viral-Encoded Replication Proteins
- Transcription and Replication of Genomic Nucleic Acid
- Assembly of Nucleocapsid Core, Glycoprotein Synthesis, and Processing
- Virion Budding
- Effects on the Host Cell
- Defective Interfering Genomes and Replicon Systems

Rubivirus Replication

- Virion Structure and Entry
- Transcription, Translation, and Genome Replication
- Virus Assembly

Perspectives

The togaviruses are simple enveloped plus-strand RNA viruses that are spherical in appearance and contribute significantly to human disease. Although they were originally classified together with several groups of viruses that are transmitted predominantly by insects, more recent analyses have categorized them into a distinct family with two genera: *Alphavirus* and the *Rubivirus*.^{167,208} The *Alphavirus* genus is by far the larger of the two, with about 30 recognized members, whereas the *Rubivirus* genus is composed of a single member, rubella virus. Virus classification into each group is determined by genome organization and nucleotide homologies. The alphaviruses are responsible for a variety of human and animal diseases, involving encephalitis, arthritis, fever, and rash, and are transmitted primarily by arthropod vectors. Viruses such as Chikungunya (CHIKV) and Venezuelan equine encephalitis virus (VEEV) have been responsible for recent human outbreaks and have raised awareness of the

significance and potential threat of alphaviruses to human health. Rubella virus is a common childhood illness for which an effective vaccine is available. However, in the absence of immunity, the virus can induce severe congenital defects in the fetuses of infected women.

Sindbis virus (SINV), the type-member of the alphavirus genus, has been studied extensively, in large part due to its facile growth in cell culture and its ability to cause mild or inapparent illness in humans. The virus has an 11.7-kb RNA genome that is capped at its 5' end and contains a poly A tract at its 3' terminus.²⁶⁵ Virions have a spherical icosahedral arrangement of proteins that has facilitated their structural analysis. The detailed knowledge of the viral life cycle, which is the focus of this chapter, has been exploited for the development of alphavirus gene expression vectors. Many members of this virus group have been studied for their role in pathogenesis. Rubella virus, as expected from its classification, shares a number of properties with the alphaviruses, yet has several important distinctions that are highlighted throughout the chapter.

CLASSIFICATION OF VIRUSES WITHIN THE TOGAVIRIDAE FAMILY

Viruses transmitted by arthropods have been referred to as arboviruses.²⁰⁸ It was originally noted that many arboviruses had a similar morphologic structure, as observed by electron microscopy, that resembled a Roman cloak (in Latin, *toga*), hence the name togaviruses. Originally, the family *Togaviridae* consisted of group A (alphaviruses) and group B (flaviviruses); however, the genera *Rubivirus* and *Pestivirus* were later added based on their similar physical properties but despite their lack of arthropod transmission. With the development of sequencing, it became apparent that the original joint classification for these viruses was in error. The *togaviruses* have nonstructural or replication proteins encoded at the 5' end of their genome RNA, whereas the 3' end encodes the proteins that comprise the virus particle, or virion. In the togaviruses, these structural proteins are translated from a subgenomic messenger RNA (mRNA) that derives from, and is co-terminal with, the 3' end of the genome.^{219,265}

The larger of the two genera within togaviruses, the *Alphaviruses*, has been classified into seven antigenically related complexes.²⁰⁹ Most phylogenetic analyses support this classification, but several recent additions add complexity to the organization. The alphaviruses have a worldwide geographic distribution, including the continent of Antarctica. The alphaviruses have classically been described as either Old World or New World viruses, depending on their distribution, and it is

likely that several transoceanic exchanges have occurred.²⁰⁹ Most alphaviruses are transmitted by arthropod vectors that probably control their geographic dispersal. However, the recent identification of the salmonid viruses, salmon pancreas disease virus, and sleeping disease virus (infecting rainbow trout), present examples of alphaviruses for which arthropod transmission is unlikely.^{293,309} These salmonid viruses appear to have diverged from the Old World–New World lineages early in alphavirus evolution, with no present-day close relatives. Another identified alphavirus, southern elephant seal virus, has been isolated from the louse *Lepidophthirus macrorhini*.¹⁵¹ This isolation demonstrates not only that alphaviruses are transmitted by lice, but that they can also infect marine mammals, and a recent report suggests a marine origin for the alphaviruses.⁵¹ Given this wide host range, it seems probable that amphibian- and reptile-specific alphaviruses also await future identification.

Although the alphaviruses and rubella virus have been classified within the same family, the evolutionary relationship between them is obscure.⁵⁴ They have a similar genome organization, and their virions share physical similarities, yet their replication and assembly strategies are sufficiently diverse to question whether they arose from a direct ancestor.

VIRION STRUCTURE

Structure of Mature Virion

The structure of the alphavirus virion has been extensively studied, and numerous high-resolution structural studies now provide a near-atomic view of the virion. Although a variety of biophysical methods were used to elaborate the alphavirus structure, work that has advanced the field the most has come from cryo-electron microscopy (cryo-EM) and image reconstruction techniques.^{93,94,287,288} The virion is 70 nm in diameter, with a molecular mass of 52×10^6 D and a density of 1.22 g per cc. It is composed of repeating units of the E1 and E2 transmembrane

glycoproteins, the capsid or nucleocapsid protein (C), a host-derived lipid bilayer, and a single molecule of genome RNA. The protein components of the virion are arranged as a T = 4 icosahedral lattice, with 240 copies of each subunit.^{27,198,201} These subunits interact with one another to form a rigid structure across the membrane in a one-to-one relationship between glycoprotein E2 and the capsid protein. Smaller amounts of another membrane-associated protein, 6K, are also found in the virus particle.^{64,162} More recently it has been discovered that another small protein, the TransFrame (TF) protein, is found in substoichiometric amounts in the virion.⁴⁷ The lipid bilayer is derived from the host plasma membrane and is enriched in cholesterol and sphingolipid, molecules that are required for entry and budding.¹¹⁴ Inside the bilayer, the C surrounds the genome RNA and forms an icosahedral shell. Thus, the alphaviruses are composed of multiple organized shells of molecules that effectively protect and deliver the viral RNA to susceptible host cells.

Cryo-EM has been used extensively to study the structure of alphaviruses, including SINV,¹⁹⁸ Semliki Forest virus (SFV),²⁸³ Ross River virus (RRV),²⁷ VEEV,^{197,315} Western equine encephalitis virus (WEEV),²⁴⁷ Aura virus,³¹⁶ CHIKV,^{298a} and Barmah Forest virus (BFV).¹²² The most recent studies with VEEV, BFV, and SINV are the most advanced, with a resolution between 4.4 and 7.0 Å reported for these viruses.^{122,315} The surface view of the virion, seen in Figure 22.1 for SINV, reveals a spherical particle with spike protrusions rising 100 Å from the surface. The icosahedral nature of the particle results in an ordered distribution of the petal-like spikes. The asymmetric unit, which is shaded in green in Figure 22.1A, contains four E1–E2 heterodimers. Each spike consists of three heterodimers of E1 and E2 glycoproteins. A total of 80 spikes reside on icosahedral threefold (solid black triangle in Fig. 22.1A) and quasi-threefold axes (open white triangles in Fig. 22.1A). Earlier biochemical studies established the nature of the heterodimer, and this relationship has been confirmed

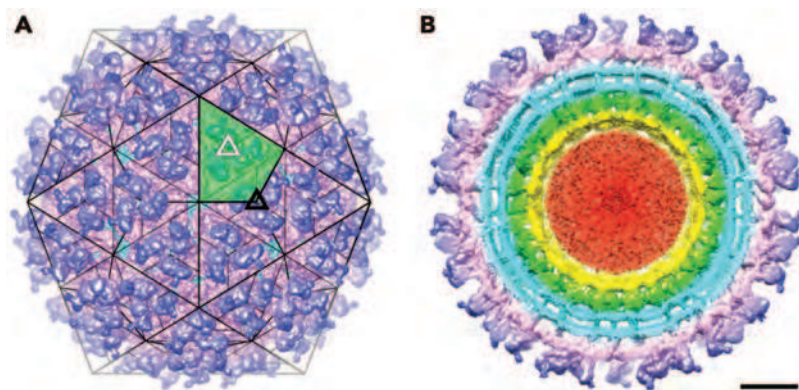


FIGURE 22.1. Structure of alphaviruses determined using cryo-EM. **A:** Surface-shaded view of Sindbis virus as determined by cryo-electron microscopy (cryo-EM) at 7.0 Å. The trimeric petal-shaped spikes are visible, with solid triangles representing the threefold axes, and white triangles representing quasi-threefold axes. One of the asymmetric units is highlighted by green shading. **B:** The same view as shown in A but with the front half of the reconstructed structure removed. The outer layer containing spikes is shown in blue, whereas the underlying skirt density is in magenta. Crossing the lipid bilayer (cyan) reveals the ordered capsid protein (green; residues 114–264), a disordered region containing a mix of protein and RNA (yellow), and a region containing the remainder of the RNA genome (red). The transmembrane densities of E1 and E2 are seen spanning the outer and inner leaflets of the lipid bilayer (cyan). (Reproduced from Tang J, Jose J, Chipman P, et al. Molecular links between the E2 envelope glycoprotein and nucleocapsid core in Sindbis virus. *J Mol Biol* 2011;414:442–459; copyright 2011, with permission from Elsevier.)

by cryo-EM reconstructions^{218,321} and by x-ray crystallography.^{145,289} Although the protein lattice occupies a substantial surface area, small openings are present in the virion that reveal the underlying lipid bilayer. These openings are most pronounced at the twofold axes, but can also be found at the fivefold axes and around the base of each spike.

The transmembrane components of the two glycoproteins are clearly seen in the cryo-EM reconstructions (Fig. 22.1B). The shape of density suggests that each transmembrane segment traverses the bilayer as a helix, although the E1 transmembrane domain is better represented by two alpha helices separated by a two amino acid kink.³¹⁵ For SINV the E1 glycoprotein has five amino acid residues that penetrate across to the inner side of the membrane (cdE1), whereas E2 has 33 amino acids (cdE2) that interact with the nucleocapsid core.^{14,148,153,271} This interaction is observed in cryo-EM reconstructions and demonstrates that each E2 molecule makes specific contacts with each capsid protein. The nucleocapsid core has a $T = 4$ arrangement of capsid protein, with the C-terminal protease domain forming pentameric and hexameric projections that appear as capsomeres on the surface of nucleocapsid cores (shown in green in Fig. 22.1B). The genome RNA does not appear to assume regular symmetry within the nucleocapsid core and is not ordered in the reconstructions (red in Fig. 22.1B).

Structure of Immature Virion

The structure of the immature virus containing an uncleaved precursor to E2, called PE2 or p62, has also been solved using cryo-EM.^{46,200} Mutant versions of both SINV and SFV were used for independent structure determinations resulting in similar structures. The extra density corresponding to the small protein E3 was found predominantly between the petals of the spike resulting in a dual-lobed petal. At a resolution of 25 Å, no apparent differences were found in the skirt or other regions of the spike and suggest that following cleavage of PE2 and release of E3, no significant conformational changes occur in the virus structure. The immature form of the virus that contains PE2 has been proposed to stabilize the fusion protein as it transits the mildly acidic environment of the Golgi.^{155,157,291}

Structural Proteins of the Virion

The proteins that comprise the alphavirus virion are synthesized as a polyprotein from a subgenomic RNA and are shown in Figure 22.2. The structures of the three major virion proteins have been determined by x-ray crystallography to high resolution. The capsid protein functions to encapsidate the genome RNA and forms a $T = 4$ icosahedron prior to release from the cell. The 264 amino acid capsid protein of SINV was crystallized, although the N-terminal 105 residues have been absent in all of the final structures.²⁹ It was suggested that the highly basic N-terminal domain was susceptible to proteases and degraded during crystal growth. A similar observation was made for the SFV capsid protein.²⁸ The C-terminal residues 114 to 264 of the SINV capsid protein form a chymotrypsin-like fold with the ultimate residue tryptophan 264 bound in the active-site protease pocket. The fold contains two Greek key β -barrel domains that bring the catalytic triad of Ser215, His141, and Asp163 into juxtaposition for activity.⁸⁶ This structure substantiated biochemical and genetic data that proposed that the capsid protein had an autocatalytic serine protease activity.^{10,85,168} The atomic structure of the capsid protein was used to generate a “pseudoatomic” resolution structure of the alphavirus nucleocapsid core (Figure 22.3) by fitting its coordinates into the cryo-EM density.^{27,179,228} This was accomplished and it was suggested that the missing residues of the N-terminus would point inward from the core surface and interact with the negatively charged viral RNA. The recent 4.4 Å cryo-EM structure of VEEV provides support for the earlier fitted structures, but the N-terminal basic domain residues (1–110). Remains obscure as ordered density within the nucleocapsid core has not been observed.³¹⁵

The E1 protein functions as a class II fusion machine to promote the joining of viral and cellular membranes under conditions of low pH. The structure of the E1 molecule from SFV was solved using x-ray crystallography.¹³⁸ The starting material for crystallization was the SFV spike cleaved with subtilisin, which released the ectodomains of the E1 and E2 glycoproteins.³⁰⁷ Unfortunately, the crystals contained only the E1 polypeptide, and it was assumed that E2 was proteolyzed.

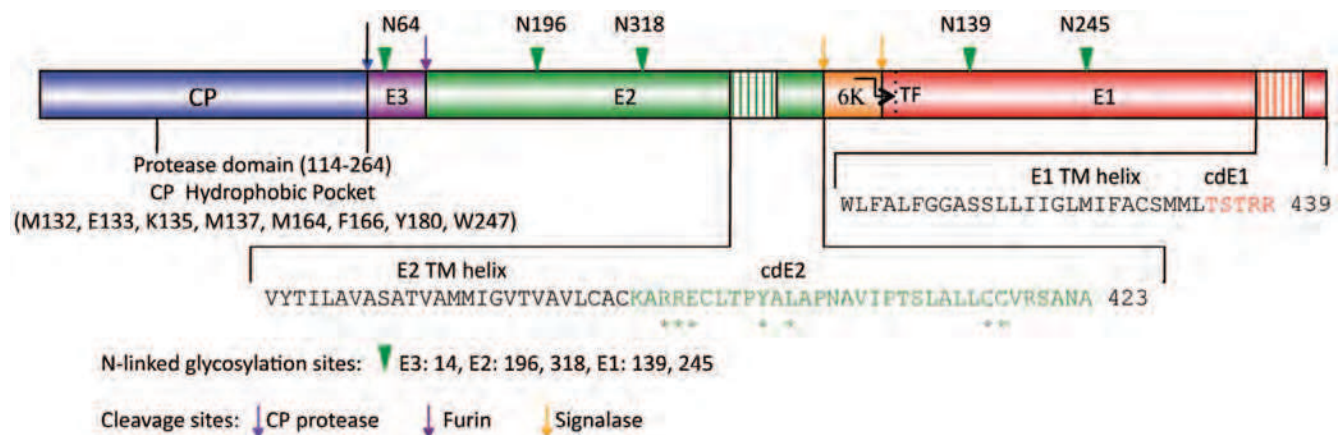


FIGURE 22.2. A schematic of the Sindbis virus structural polyprotein. The individual proteins are color coded, with protease cleavage sites indicated by an arrow. N-linked glycosylation sites are shown by green arrowheads. The amino acids that comprise the transmembrane and cytoplasmic domains of both E1 and E2 are shown. Asterisks indicate residues of cdE2 that interact with the capsid protein. The TransFrame (TF) protein is identified by the bent arrow followed by the dotted line crossing E1.

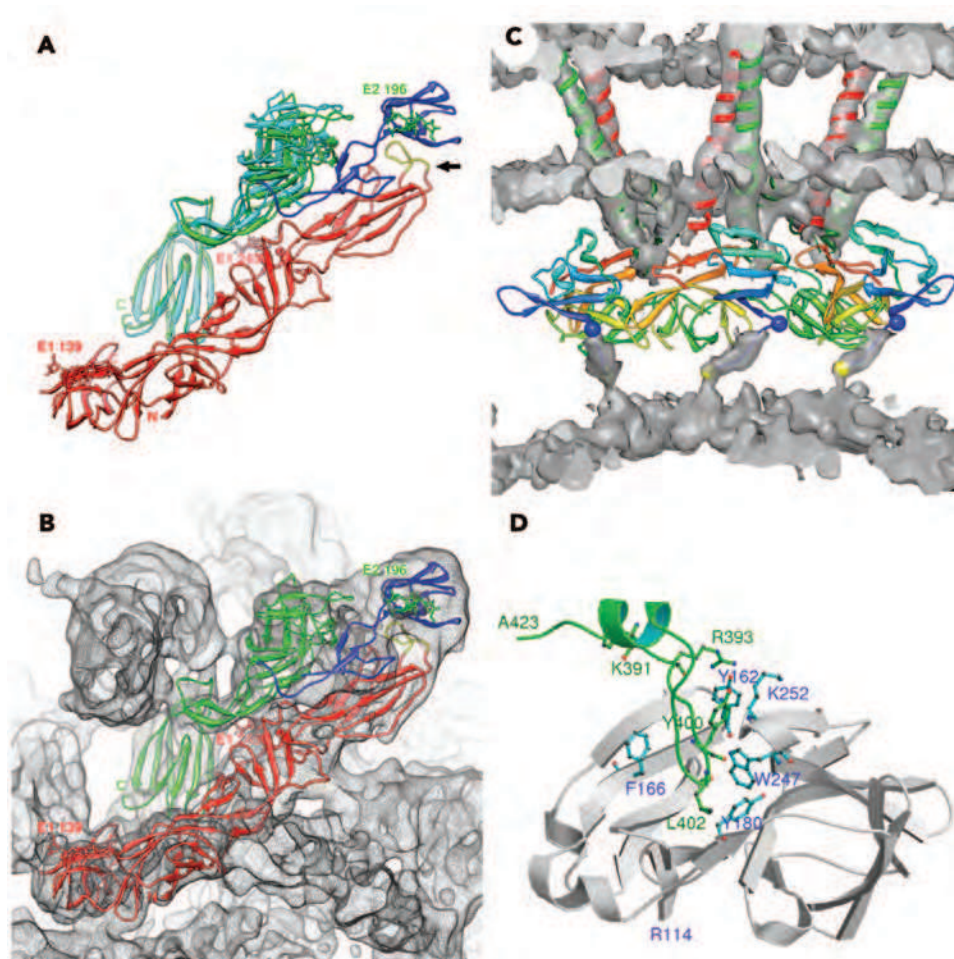


FIGURE 22.3. Pseudoatomic representation of the Sindbis virus structural proteins based on the 7.0 Å cryo-electron microscopy (cryo-EM) reconstruction. **A:** The structure of E1 and E2 of Sindbis virus. E1 is shown in red. The A and C domains of E2 are shown in two colors, with one representing the structure from the crystal structure (cyan) and the other obtained by fitting the crystal structure into the cryo-EM map (green). The B domain of E2 (blue) is a homology-modeled structure derived from the Chikungunya crystal structure. The arrow points to the E1 fusion peptide (yellow). **B:** Same as in **A** but fitted into the cryo-EM density (grey mesh). **C:** Cross-section of the fitted cryo-EM structure showing the transmembrane helices of E1 (red) and E2 (green) and the underlying N-terminal protease domain of the capsid protein (residues 114–264). Below capsid residue 114 (blue ball), the remaining N-terminal residues of the capsid are not identified but must interact with the underlying genome RNA. Additional EM density is shown in gray. **D:** Interactions between the capsid protein and the cdE2. The residues that comprise cdE2 (amino acids 391–423) were modeled according to the cryo-EM density and are shown as they interact with the hydrophobic pocket of the capsid protein. Residues of the capsid that have been shown as important in this interaction—F166, Y180, and W247—are highlighted. (Reproduced from Tang J, Jose J, Chipman P, et al. Molecular links between the E2 envelope glycoprotein and nucleocapsid core in Sindbis virus. *J Mol Biol* 2011;414:442–459; copyright 2011, with permission from Elsevier.)

The structure of the E1 ectodomain (residues 1–383) is shown in Figure 22.3 and contains three β -barrel domains. Domain I, the so-called central domain, links domains II and III. The extended domain II contains at its distal end the fusion peptide, a short loop of hydrophobic amino acids that promotes insertion of the protein into the target membrane. Domain III has an immunoglobulin (Ig)-like fold and connects at its C-terminus with the transmembrane domain of the protein.

The structure of E1 is remarkably similar to that of the flavivirus E protein.^{217,269} Unlike the alphaviruses, the flavivirus E protein functions both in receptor attachment and in membrane fusion. In the alphaviruses, these two activities are carried

out by proteins E2 and E1, respectively. In the flaviviruses, the E protein is oriented roughly parallel to the lipid membrane, and it was anticipated that E1 might assume a similar orientation in the alphavirus virion.¹²⁴ This was essentially verified once the E1 atomic structure was fitted into the cryo-EM density. This fitting was accomplished for both SFV and SINV, and most recently for CHIKV.^{138,145,207,289} Furthermore, for SINV, the two sites of E1 glycosylation were mapped onto the cryo-EM structure, and these sites at Asn139 and Asn245 were used as positional markers to fix the position of E1 in the virion.²⁰⁷ E1 lies at an angle of ~50 degrees relative to the surface of the membrane (Figure 22.3B), and it forms an icosahedral lattice

comprising the region that has been referred to as the skirt.^{138,207} The crystallographic E1 dimer, which is a back-to-back dimer as opposed to a face-to-face dimer seen in the flaviviruses, is essentially preserved in the arrangement of E1 in the virion. The interface residues that make contact in the crystallographic dimer are presumably those that are important for forming the icosahedral lattice in the virion.

Although E1 comprises the skirt region of the alphavirus surface, the majority of the protruding spike is comprised of the E2 molecule. E2 constitutes the petals that make up the spike, and covers the distal end of the E1 molecule that points outward. E2 serves to engage cell surface receptor molecules required for entry of the virion into the cell. The crystal structure of E2 together with both E1 and E3 for CHIKV was solved and reveals a three-immunoglobulin domain protein.²⁸⁹ Domain B is located at the outermost point of the spike and contains the residues that have been implicated in receptor binding and as well as those involved in binding neutralizing antibodies. Domain B exhibits mobility in the context of the spike and was absent from the intact SINV E1–E2 trimer structure done at low pH.¹⁴⁵ At the other end of the protein, located closest to the viral membrane, is the C-terminal domain C. The N-terminal domain A (residues 1–132 in SINV) is the central bridge of E2, and connections are made to the B domain by β -ribbon connectors. The β -ribbon connector is flanked by a pair of well-conserved histidine residues that might function as an acid switch to release the connector and B domain under acidic conditions. Although E3 does not directly contact E1, its contact with the β -ribbon connector holds it in place to prevent movement of the B domain while the virus is in the process of egress from the cell in an immature state. The furin cleavage of the p62 to release E3, therefore, releases the clamp

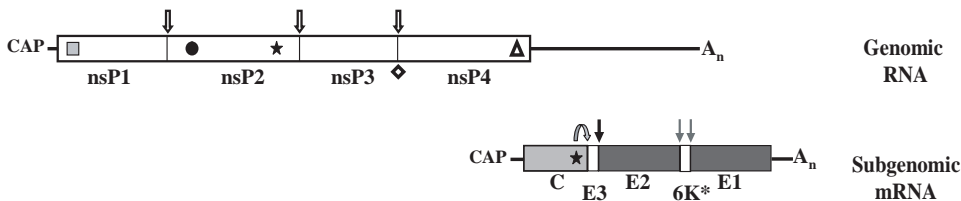
holding the B domain and activating the complex for pH-triggered fusion. E1 and E2 have extensive contacts with each other, promoting the spike architecture (Fig. 22.3).

In addition to the three major structural proteins identified by cryo-EM in the virion, two small transmembrane proteins are present in sub-stoichiometric amounts and can be identified using purified virus and mass spectrometry. The existence of the 6K protein in the virion has been well established, but more recently the TF protein (~8 kD) has also been found within SFV particles.⁴⁷ Given the low abundance and presumably random distribution of these small transmembrane proteins, they have not been detected using high-resolution cryo-EM, and their membrane architecture has to date prevented x-ray crystal structures. Whether the TF and 6K proteins exist as oligomers is also unknown.

GENOME STRUCTURE AND ORGANIZATION

The togavirus genome resides on a positive-strand RNA that contains a 5' terminal 7-methylguanosine and a 3' terminus that is polyadenylated. The alphavirus genome, represented in Figure 22.4 by the type virus SINV, is approximately 11.7 kb in length, whereas rubella virus is nearly 2 kb shorter, at 9.8 kb.^{40,265} The genomes segregate their replication and virion protein coding regions into two segments, with the replication region mapping to the 5' two-thirds and the structural region mapping to the 3' one-third. Limited nucleotide homology exists between genomes in the two genera, although there are several sequences in both translated and nontranslated regions that do have homology; however, most evidence suggests that their replication and assembly strategies are quite different.⁴⁰

Alphavirus (Sindbis virus)



Rubivirus (rubella virus)

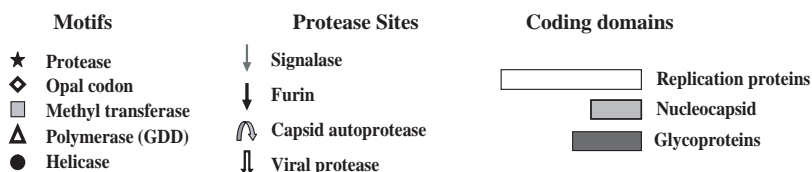
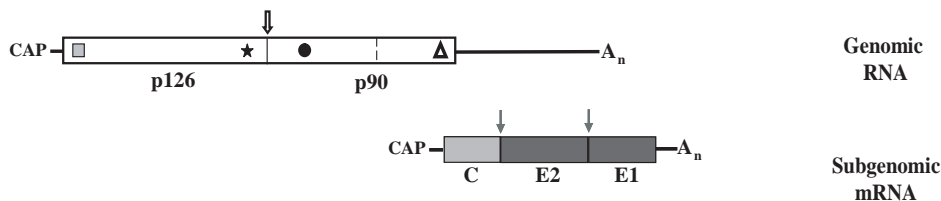


FIGURE 22.4. The genomes of Sindbis virus and rubella virus. Nontranslated regions are shown by the solid line and translated regions are shown in boxes. Open boxes indicate replication proteins and shaded boxes represent structural or virion proteins. Motifs and cleavage sites are indicated according to the scheme. The subgenomic mRNAs are not shown to scale with the genomic RNAs.

The nonstructural or replication proteins are translated from the genome RNA, whereas the structural or virion proteins are translated from a subgenomic mRNA.^{191,251} In SINV, the 5′ nontranslated region (NTR) is 59 nucleotides, about average for the alphaviruses, whereas the 3′ NTR is also close to the average in length at 322 nucleotides.

Using comparative genome analyses and functional genetic studies of defective interfering particles and viruses, four conserved regions (conserved sequence element [CSE]) of the alphavirus genome were identified as *cis*-acting elements important for replication.^{184,185–186} Two conserved regions are found near the 5′ end of the genome, one is found in the junction region between nonstructural and structural genes, and one is found at the 3′ end immediately preceding the poly (A). Three presumably similar functioning CSEs can also be found in the rubella genome.⁴⁰ In the alphaviruses, each CSE has been shown to interact in a host-dependent manner, suggesting that host factors may play a role in their function.^{44,97,98,123,187,188} It has been shown that a U-rich region in the 3′ NTR of SINV contains elements responsible for viral RNA stability and that the cellular HuR protein binds this region, thus decreasing the rate of cell-mediated decay of the genome RNA.²⁶⁰ In addition, studies have shown that host proteins bind to the 3′ end of the minus-strand RNA of SINV, and in one case the protein was identified as the mosquito homolog to the La protein.^{193,194–195}

ALPHAVIRUS REPLICATION

Mechanism of Attachment and Receptors

Alphaviruses display an extremely broad host range, both in terms of susceptible animal species and in terms of cells in culture. This broad host range has prompted speculation as to the nature of the receptor, with two hypotheses proposed to explain this phenomenon.²⁶⁶ In the first, the virus E2 glycoprotein contains multiple receptor-binding sites so that distinct cellular receptors can bind the viral surface protein. The second hypothesis proposes that the virus uses a ubiquitous receptor that is highly conserved across species, including both mammals and mosquitoes. Data exist to support each model, and it is likely that a combination of the two is the true strategy for alphavirus attachment to the cell.

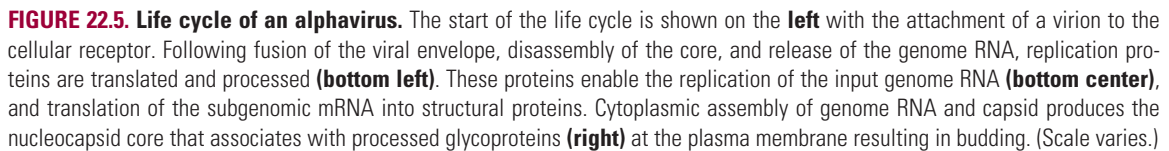
The variety of molecules that have been implicated in SINV argues for multiple distinct receptor-binding sites on E2.²⁶⁶ The use of the laminin receptor with its high conservation across species suggests that it might serve as a receptor in multiple cell types and multiple host species.²⁹⁵ However, the picture remains obscure for SINV because laminin receptor functions in baby hamster kidney cells but not in chicken embryo fibroblasts, where a 63-kD protein has been implicated.²⁹⁶ In mouse neuroblastoma cells, proteins of 74 and 110 kD have been reported as possible SINV cellular receptors.²⁷⁸ DC-SIGN and L-SIGN, C-type lectins that bind mannose-enriched carbohydrates, have also been implicated as receptors of alphaviruses that have been produced in mosquito cells.¹¹⁸ Very recent studies using a genome-wide RNA interference (RNAi) screen in *Drosophila* cells identified the natural resistance-associated macrophage protein (NRAMP) as a cellular receptor functioning to permit SINV infection.²²⁶ Likewise, NRAMP2, the vertebrate homolog, allowed for SINV, but not RRV, entry into mammalian cells. This new approach and

finding raises the possibility that a family of related conserved multipass membrane proteins may serve as receptors for other alphaviruses.

Natural isolates of EEEV utilize cell surface heparan sulfate as an attachment receptor.⁶⁵ This use of heparan sulfate may direct tropism of EEEV and promote enhanced neurovirulence. In contrast, passage of several other alphaviruses in culture leads to the accumulation of adaptive mutations, some of which introduce basic amino acids in their E2 glycoprotein.^{18,20,21,119} This increase in positively charged amino acids in E2 leads to high efficiency attachment to cells through heparan sulfate molecules. The importance of this interaction was demonstrated genetically by the generation of a Chinese hamster ovary cell line using retroviral insertional mutagenesis that was deficient in the expression of heparan sulfate and chondroitin sulfate.¹⁰⁸ These cells were resistant to SINV infection and defective in binding virus. The substitution of a single residue on the E2 glycoprotein of RRV was sufficient to permit heparan sulfate binding, and this attachment was mapped using cryo-EM to the distal tip of the spike.^{97,317} The binding of heparan sulfate does not result in conformational changes in the virion, nor does it enhance the fusion process.^{257,317} Therefore, it likely serves simply as a mechanism to attach the particle to the cell surface with high affinity so an efficient interaction with the entry receptor can occur. In contrast to what is observed in cell culture with SINV, infection of mice results in the development of large-plaque viral mutants with a reduced affinity for heparan sulfate and a greater viremia.²¹ However, given these diverse observations, the utilization of heparan sulfate and its role in pathogenesis among the different alphaviruses requires further investigation.

Mechanisms of Entry, Membrane Fusion, and Uncoating

Following attachment to cells and engagement with an entry receptor, alphaviruses proceed via the endocytic pathway to gain access to the cell interior (Fig. 22.5).^{38,98,166} Structural and biochemical experiments demonstrate that binding of RRV to heparan sulfate does not induce conformational changes in the virion.^{257,317} However, other receptors may induce conformational changes in the particle that might mediate entry such as the reduction of disulfide bonds to disrupt protein–protein interactions.^{1,11,199,237} Despite these suggestions that disulfide exchange may play a role in alphavirus entry, the use of thiol-blocking reagents failed to show a significant inhibition of infection.⁷⁷ However, it is clear that the attachment of SINV to cells results in the exposure of new epitopes defined by monoclonal antibodies and suggests that protein rearrangements occur following receptor engagement.^{48,172} Similar observations have been made using purified virus and treatment with heat, pH, and reducing agents.¹⁷¹ In addition, the recent observations on the role of virion and protein dynamics in the flavivirus life cycle, suggest that other structurally related viruses might employ particle dynamics to sense their environment as they search for an entry receptor.^{40a} In addition to the role of thiol exchange reactions that have been proposed to play a role in alphavirus entry, there has even been a suggestion that cell penetration may occur at the cell surface in the absence of fusion by conformational rearrangements in the envelope glycoproteins.^{1,199} However, it has been fairly well established that membrane fusion triggered by an exposure to acidic conditions

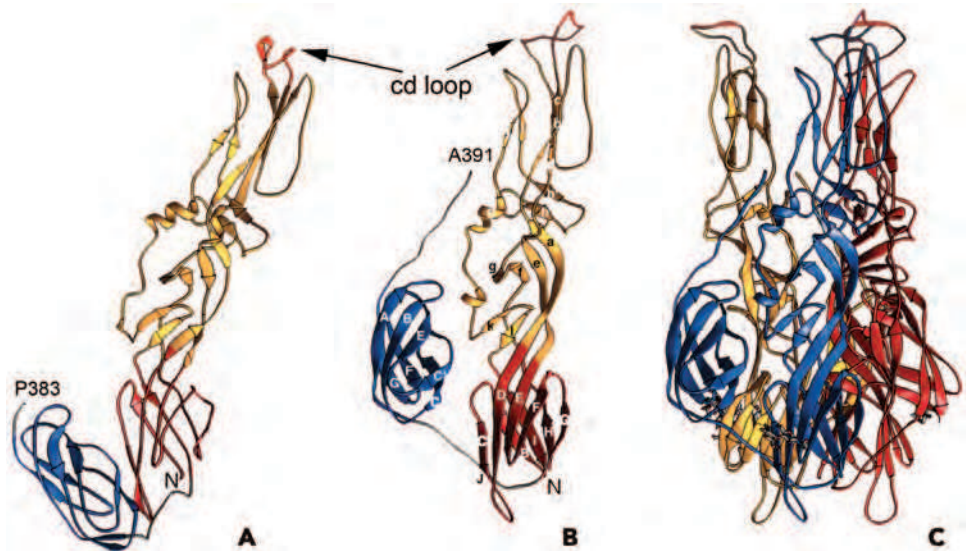


Receptor-bound viruses undergo endocytosis into coated vesicles using a clathrin-dependent pathway. This pathway was demonstrated by DeTulleo and Kirchhausen³⁸ using dominant-negative mutants of dynamin to block the formation of clathrin-coated pits and prevent entry of SFV and SINV. The vesicles are subsequently acidified, providing the trigger for fusion between viral and cellular membranes. Acid-induced fusion is supported by numerous studies, but most convincingly by the use of lysosomotropic weak bases that raise the pH of endocytic vesicles and prevent entry of alphaviruses.^{77,99,310} Although it has been argued that viral RNA replication might also be affected by the acidification, pseudotyped viruses con-

The role of the alphavirus E1 and E2 glycoproteins in the entry process has been firmly established (Figs. 22.6 and 22.7). In the presence of acidic pH, the E1–E2 heterodimer is destabilized and the two proteins dissociate.^{291,293} The dissociation of the proteins results in the exposure of the fusion peptide that is found on the distal tip of E1.^{4,74,88,138} The fusion peptide of E1 inserts into the target membrane in a cholesterol-dependent manner followed by the trimerization of E1.^{3,73,116,189,206,292} A large conformational change in E1 results in domain III and the stem-anchor region of the protein packing against domain II, resulting in the viral and target membranes being brought into close opposition (Fig. 22.6).⁷⁶ A set of E1 trimers, possibly

FIGURE 22.6. Postfusion structure of the Semliki Forest virus E1 protein.

A and **B**: Neutral and low pH forms of the soluble E1 ectodomain lacking amino acid residues 392–438, respectively. The orientation of the protein presents the fusion loop (cd loop) toward the target membrane (**top**). **C**: This rearrangement would occur after E1 has undergone trimerization as shown here. (Modified from an image provided by Dr. Felix Rey; 76.)



resembling what has been seen in liposomes, results in membrane deformation and promotes membrane mixing.⁷⁴ Finally, a fusion pore will form as the two membranes complete the process, and the nucleocapsid core will be released into the cytoplasm (Fig. 22.7).

Rey et al.¹³⁸ recognized that the structural features of the alphavirus E1 and flavivirus E proteins were distinct from the structures of other previously identified fusion proteins such as hemagglutinin (HA) from influenza, and proposed that they represented a novel class of fusion machines. They termed these class II membrane fusion proteins and elaborated several distinguishing features that separated the two classes. Class II fusion proteins are composed predominantly of β -strands, contain an internal fusion peptide, and have a companion protein that stabilizes the structure; this companion protein forms an activated metastable structure following proteolytic processing of a precursor protein. In the alphaviruses, PE2 is proteolytically activated by cleavage to generate E3 and E2, with E2 and E1 forming a stable heterodimer.^{155,156} Mutagenesis experiments have provided insight into the fusion process and the residues that play regulatory and supporting roles in this process. As predicted, a pH-sensitive histidine residue in E1 (SFV H3) appears to regulate the low pH-dependent folding of E1 that is required for fusion.²¹⁵ Several other well-conserved histidines in E1 were evaluated and found unable to influence the activity. In addition, Kielian and colleagues¹⁵² found that a salt bridge is formed within domain II of the E1 trimer core, which appears critical for stabilizing the homotrimer fusion intermediate.

Biochemical experiments with SFV have defined the requirements and steps in the alphavirus fusion process. Kielian et al.^{116,159,206} demonstrated a strict dependence on cholesterol for fusion. This requirement has been narrowed down to the sterol β hydroxyl group in cholesterol.^{31,176,290,312} A mutant was isolated (named *srf-3* for sterol requirement in function) that was cholesterol independent and had an amino acid substitution of a proline to serine at E1 residue 226. This mutation did not affect fusion with normal membranes but significantly enhanced fusion to cholesterol-free membranes.^{24,280} In mosquito cells, the *srf-3* mutant grew better than wild-type virus.⁵ The importance of this region of E1, which lies in the ij loop,

for alphavirus fusion was further demonstrated by mutation of a conserved histidine at position 230 to alanine with the resulting virus particles being noninfectious, but capable of proceeding through E1 homotrimer formation under low pH conditions and suggesting a late step in fusion.²³ However, the exact role of cholesterol to promote fusion is not known. The structure of the postfusion form of E1 has been determined using x-ray crystallography.^{74,75,76} This was accomplished using a soluble form of the E1 ectodomain known as E1* following exposure to low pH in the presence of liposomes, and solubilization with detergents. The structure of this homotrimer reveals the movement of domain III by 37 Å toward the fusion peptide and target membrane (Fig. 22.6)⁷⁶ and is similar to changes that are induced by class I fusion protein activation.¹¹⁵ It is important to note that the addition of exogenous domain III can inhibit fusion by binding to E1 and preventing fold-back of the endogenous domain III.¹⁴⁶ This fold-back process appears to be reversible and is mediated by a series of interactions between the domain I and domain III linker region.³²⁰ A complete picture of the virion during the fusion process is not available, despite several attempts using cryo-EM to examine these steps.^{46,62,84,199}

Following the fusion of the viral and cellular membranes, the nucleocapsid core is released into the cytoplasm. The stability of that core is not known, but uncoating has been suggested to require the interaction of the core with ribosomes. This is based on several reports that suggest that ribosomal RNA competes for a site on the capsid protein and that displacement of this site results in disassembly.^{253,254,305} This site has been identified in SINV capsid protein between residues 94 and 105 and coincides with a predicted site for genome RNA binding.³⁰⁸ It has also been suggested that the core might be primed for uncoating by exposure to low pH, which causes the core to become unstable.^{303,306} This exposure to low pH could occur in the endosome because both E1 and 6K have been proposed to have ion channel properties.^{158,169,304} However, studies using pre-formed cores microinjected into naïve cells demonstrated that these cores do uncoat presumably devoid of conditions of low pH.²⁵⁸ Whether these cores interact with ribosomes to promote disassembly and release of genome RNA remains to be shown.

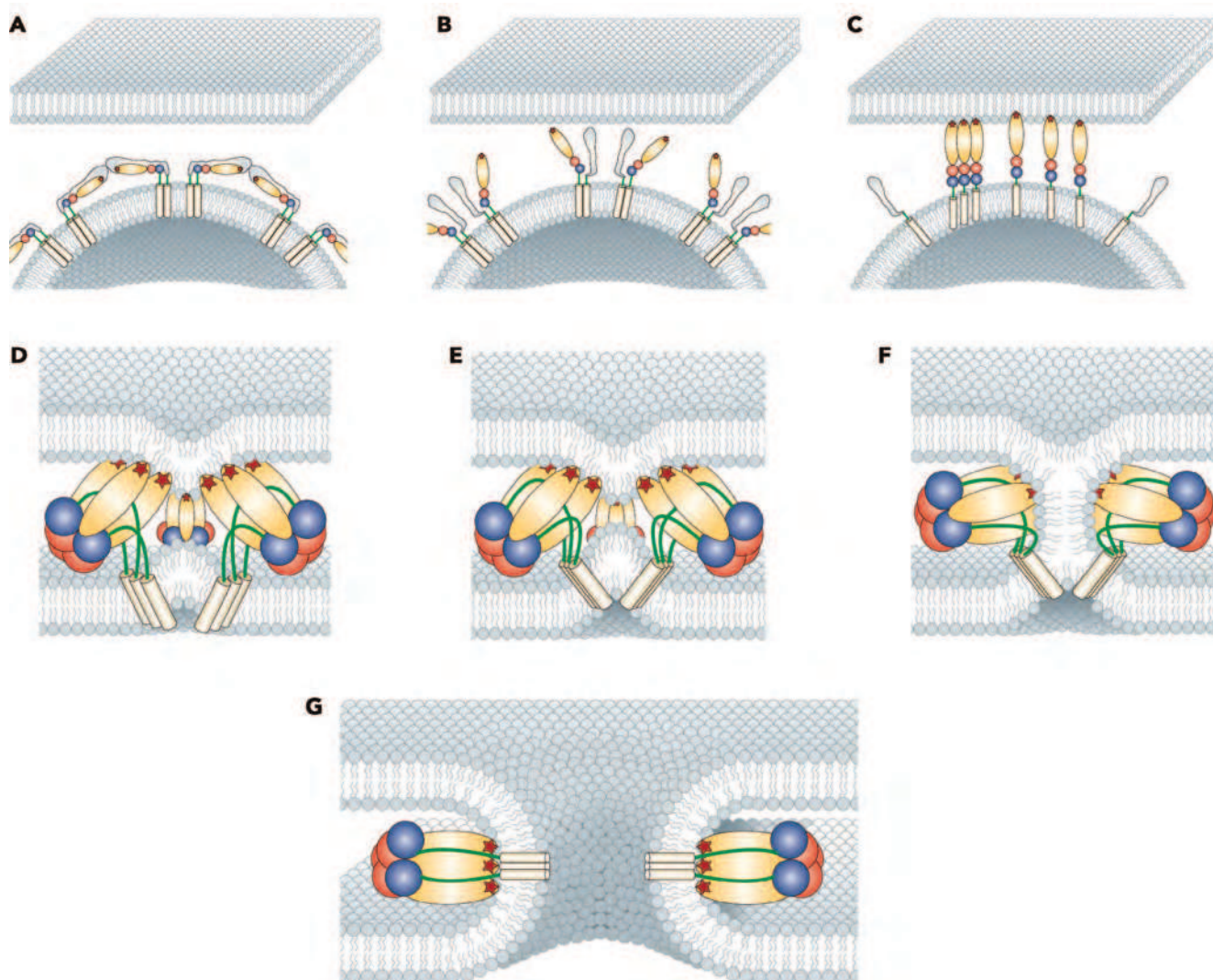


FIGURE 22.7. Model of alphavirus fusion. **A:** Native virions with E1 represented by two circles (domains I and III) and an extended oval (domain II), and E2 in the background and covering the E1 fusion peptide (star). **B:** Low pH triggers dissociation of E1 and E2 and fusion peptide exposure. **C:** Low pH and cholesterol-dependent insertion of the fusion loop into the target membrane, aligning E1 and promoting trimerization. **D:** Fold-back process by which domain III and the stem region move toward the fusion loop. **E:** Folding of domain III and the stem region against domain II of the trimer pulls the transmembrane domains toward one another distorting the viral membrane. **F:** Opposing dome-like deformations in the two membranes leads to mixing of the outer leaflets (termed hemifusion). **G:** Close opposition of the fusion peptides and transmembrane domains resolve into the fusion pore. (Courtesy of Dr. Margaret Kielian. Reprinted from Kielian M, Rey FA. Virus membrane-fusion proteins: more than one way to make a hairpin. *Nat Rev Microbiol* 2006;4:67–76, with permission from Macmillan Publishers Ltd: Nature Review Microbiology, © 2006.)

Translation and the Role of Viral-Encoded Replication Proteins

The genome RNA serves as an mRNA for the synthesis of the nonstructural or replication proteins (Fig. 22.4). These are produced by two polyproteins that originate translation at nucleotide 60 in SINV.²⁶⁸ The smaller but more abundant polyprotein P123 terminates translation at an opal codon following 1,897 amino acids. Readthrough of the opal codon occurs with a low frequency (~10% to 20%) and results in the production of the larger P1234 polyprotein. Not all alphaviruses have a termination codon to control the production of the two polyproteins, but mutagenesis of the opal codon in SINV

adversely affects replication.¹⁴⁴ Processing of the polyproteins occurs through the action of a virus-encoded protease located within the nonstructural protein 2 (nsP2).^{39,91,92} The processing of the polyprotein to generate precursor and end-product nsP's is believed to regulate the synthesis of viral RNAs.^{35,137,250} Translation of the structural polyprotein proceeds through a subgenomic mRNA that is initiated near the coding region for the C-terminus of P1234. The subgenomic mRNA is 3' co-terminal with the genome RNA and is produced later in the infection.²⁶⁸

Initial studies of the replication proteins used temperature-sensitive mutants that were conditional lethal for viral

TABLE 22.1 Translation Products of Alphaviruses (Sindbis Virus)

Protein	Size (aa)	Function
Nonstructural Proteins		
nsP1	540	Methyltransferase and guanylyltransferase; anchors replicase complex to membranes
nsP2	807	NTPase, helicase, RNA triphosphatase, protease responsible for processing of non-structural polyprotein
nsP3	556	Phosphoprotein with unknown function(s) but important for minus-strand synthesis; contains macro domain and SH3-binding regions
nsP4	610	RNA-dependent RNA polymerase (RdRp), terminal transferase
Structural Proteins		
Capsid	264	Encapsidates genomic RNA to form nucleocapsid core; carboxyl domain is an autocatalytic serine protease
E3	64	N-terminal domain is uncleaved leader peptide for E2; E3 + E2 = pE2.
E2	423	Presents the major neutralizing epitopes and is responsible for receptor binding
6K	55	Leader peptide for E1, enhances particle release, putative ion channel
TF	70	TransFrame (TF) protein, putative ion channel, enhances particle infectivity, expression prevents synthesis of E1
E1	439	Responsible for membrane fusion activity

replication. These studies established complementation groups and identified specific functions of the replication proteins.²⁶⁴ Four complementation groups were identified, and these correlated with the four nsP's. Once sequence information was available, motifs were identified that verified function and permitted phylogenetic relationships between other virus families to be established. This resulted in the suggestion of an alphavirus-like superfamily that contained RNA virus members from several plant families and argued for an evolutionary relationship between them.^{2,267}

The nsP's are multifunctional proteins with their known activities shown in Table 22.1. However, it is likely that additional unknown activities exist. Guanine-7-methyltransferase and guanylyltransferase activities necessary for mRNA and genome RNA capping have been shown to reside within nsP1.^{127,173,174} This capping activity is distinct from cellular capping enzymes in substrate preference.⁶ Several genetic studies have confirmed the identity of amino acids critical for the methyltransferase function, but the domain required for guanylyltransferase activity has not been identified and may flank the conserved methyltransferase domain.^{8,227} A *ts* mutant that mapped to Ala348 of nsP1 in SINV demonstrated a role of the protein in minus-strand RNA synthesis.⁸⁷ A defect in minus-strand synthesis has also been seen with mutations in nsP4, and some of these have been complemented with changes in

nsP1 at residues 349 and 374, suggesting sites for nsP1 and nsP4 interaction.⁴³ nsP1 is the only alphavirus nonstructural protein that has been shown to be membrane associated.^{128,203} The membrane association has been suggested to occur by a palmitoylated cysteine at residue 420 (in SINV).^{7,127} Mutations that disrupt palmitoylation did not alter the distribution of replication complexes and show only modest reductions in growth. However, nsP1 can still bind to membranes through a patch of positively charged and hydrophobic amino acids between residues 245 and 264.⁹ Nuclear magnetic resonance spectroscopy of a corresponding peptide suggests that this sequence can form an amphipathic α -helix that can interact with liposomes.¹²⁹ This membrane anchoring of the replication complex associated with nsP1 is probably required for efficient replicase activity.

The largest of the replication proteins is nsP2, with a length of about 800 amino acids. The N-terminal half of the protein has helicase, nucleoside triphosphatase, and RNA triphosphatase activities,^{78,79,221,281} whereas the C-terminal half contains a novel cysteine protease domain and a nonfunctional methyltransferase domain.^{263,282} The structure for the C-terminal two domains of the VEEV nsP2 was solved by Watowich and colleagues using x-ray crystallography (Fig. 22.8).²³⁴ The structure shows that the active site of the protease is positioned close to the interface between the two domains. Although related to proteases papain and cathepsin X, the fold of the nsP2 protease domain appears to be unique and a new



FIGURE 22.8. Structure of the Venezuelan equine encephalitis virus nsP2 protease. A ribbon diagram showing the protease colored from blue (N-terminus) through red (C-terminus) representing residues N468 to S787. The catalytic dyad residues, C477 and H546, are found in the N-terminal protease domain (**top**), whereas the methyltransferase-like domain (**bottom**) comprises the C-terminal domain. (Courtesy of Dr. Joyce Jose; generated in PyMol using the PDB coordinates 2HWK from Russo AT, White MA, Watowich SJ. The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. *Structure* 2006;14:1449–1458.)

form of cysteine protease structure. Although having a similar tertiary structure with known methyltransferases such as FtsJ, the S-adenosylmethionine (SAM) substrate binding site is very different in backbone alignment and sequence, arguing against any methyltransferase activity. However, by mapping several previously identified *ts* mutants affected in RNA synthesis onto the C-terminal domain, it was suggested that the domain functions as an RNA-binding scaffold that regulates protease activity and RNA synthesis.^{233,234}

The nsP2 protein has a nuclear localization sequence that results in 50% of the protein reaching the nucleus,^{222,223} and it has been reported that nsP2 of VEEV undergoes both nuclear import as well as export.¹⁷⁷ Abrogation of the signal results in a slightly defective virus, but at least in SFV the mutant has lost neuropathogenesis, arguing for a role of nsP2 in host interactions.²²⁰ Studies have identified a role for nsP2 from the Old World alphaviruses in the induction of cytopathic effects and the establishment of persistent infections, and is discussed later in the chapter.^{41,55} In addition, experiments have provided a link between nsP2 and the host response, resulting in shutoff of minus-strand RNA synthesis.²⁴⁰ Genetic studies identified conditional lethal mutations demonstrating RNA-defective phenotypes that implicated nsP2 in the regulation of minus-strand synthesis and in the initiation of subgenomic RNA synthesis.^{37,87,241} Furthermore, the role of the protease activity to regulate the temporal control of RNA synthesis has been well established and is described later.^{35,137}

The function of the nsP3 protein remains obscure, although genetic analyses indicate that it plays a role in RNA synthesis and neurovirulence.^{131,276,297} The protein is highly conserved among alphaviruses at its N-terminus, whereas the C-terminal 200 amino acids are rich in serine and threonine residues. The protein is phosphorylated on serines and threonine; however, this modification is not required for replication, and its function in the virus life cycle is not known.^{130,143,204,284,285} The protein has a weak affinity for membranes and will associate with them when expressed in the absence of the other nsPs.^{202,284} A crystal structure of both the CHIKV and VEEV N-terminal 160 residues of nsP3 confirmed previous suggestions that this region contains a macro domain (the VEEV structure is shown in Fig. 22.9).^{80,163} These domains function as ADP-ribose binding modules and have also been shown capable of single-strand RNA binding. The exact function(s) of the macro domain awaits additional studies. Although the C-terminal end of nsP3 is not well conserved, a proline-rich sequence found in most alphaviruses was identified as a target site for Src-homology 3 domain containing proteins amphiphysin-1 and amphiphysin-2.¹⁸⁵ Disruption of the binding sequence by mutation or reduction of amphiphysin-2 by RNAi reduced replication in both SINV and SFV. It is unclear how this interaction influences RNA replication; however, the amphiphysins have been implicated as membrane-binding proteins, involved in endocytosis, and membrane trafficking, and the virus may usurp these functions to facilitate RNA synthesis.

The core of the virus replication complex is the RNA-dependent RNA polymerase (RdRp) that maps to nsP4.^{87,121} Interestingly, because of the opal codon in SINV, synthesis of P123 is significantly greater than the level of P1234 and thus nsP4 levels are lower than that of the other nsPs. Furthermore, modifications that increase the synthesis of nsP4, such as the removal of the opal termination codon, result in reduced virus replication.¹⁴⁴ The majority of the protein from the C-terminus constitutes the RdRp domain based



FIGURE 22.9. Structure of the Venezuelan equine encephalitis virus nsP3 macro domain. A ribbon diagram showing the nsP3 macro domain colored from blue (N-terminus) through red (C-terminus) representing residues A1 to E160. The structure consists of a six-stranded β -sheet ringed by three α -helices. (Courtesy of Dr. Joyce Jose; generated in PyMol using the PDB coordinates 3GQE from Malet H, Coutard B, Jamel S, et al. The crystal structures of Chikungunya and Venezuelan equine encephalitis virus nsP3 macro domains define a conserved adenosine binding pocket. *J Virol* 2009;83:6534–6545.)

on homology with other polymerases and predicted secondary structures.¹¹¹ A short region exists at the N-terminus that lacks a counterpart in other viral polymerases, and it has been suggested that it might be a binding domain for the other nsPs.^{87,249} The N-terminus of nsP4 also contains a conserved tyrosine residue, and this serves to make the protein unstable in infected cells.^{36,248,252} deGroot et al.³⁶ examined the degradation of nsP4 and showed that it was degraded by the N-end rule pathway. It has been suggested that free nsP4 is rapidly degraded, whereas nsP4 that is a component of replicase complexes is protected and relatively stable. Therefore, it was not a surprise that attempts to express the full-length nsP4 in heterologous systems were initially unsuccessful. However, expression of nsP4 lacking the first 97 amino acids was successful, and this truncated nsP4 displayed a terminal adenylyltransferase activity.²⁷⁵ This terminal transferase activity was suggested to play a role in the maintenance of the poly (A) tract at the 3' end of positive-strand RNAs. The nsP4 protein was subsequently expressed as an N-terminal small ubiquitin-like modifier (SUMO) fusion protein that was cleaved after purification, and nsP4 was shown to possess de novo minus-strand synthesis activity that was dependent on the correct 3' end of positive-strand template.²²⁹ Proteomic studies have been used to identify host proteins that might interact with nsP4 during virus infection.³³ In this study the authors demonstrated that a total of 29 host proteins were associated with nsP4 in a temporally regulated pattern. Among the proteins identified, two proteins known as GTPase-activating protein SH3-domain binding protein 1 and 2 (G3BP1 and G3BP2) were

also shown to interact with nsP2 and nsP3.³² However, the role of these proteins is unclear; they may function to reduce the pool of RNAs available for translation by recruiting the viral RNA to the stress granule pathway.

Transcription and Replication of Genomic Nucleic Acid

Alphavirus-infected cells produce three species of RNAs: genome plus-strand RNA, complementary minus-strand RNA, and sub-genomic mRNA. The synthesis of these three species is tightly regulated by the availability of specific nsP's (Fig. 22.10).^{35,136,137} Replication is initiated on the cytoplasmic surface of endosomes and lysosomes on structures termed cytopathic vacuoles.⁶¹ All four nsP's can be found associated with each other and within these vacuoles.¹²⁶ In elegant studies by Strauss et al.³⁵ it was shown that proteolytic site selection controlled the processing of the nsP's and determined the components of the replicase complex. This was accomplished by assessing cleavage-site preferences and determining which enzymes (nsP2 or its precursors) could affect processing. Additional studies by the Sawicki laboratory using temperature-sensitive mutants established the biochemical nature of the replicase complex.^{15,37,239,242} In complementary work, the Rice laboratory carried out *in vivo* replica-

tion studies using nsP's expressed in a vaccinia vector to discern the functional complexes.^{135–137} Despite earlier problems, they were successful in developing a system for template-dependent initiation of SINV.¹³⁴

RNA replication begins with the initiation of minus-strand synthesis. This event requires the 3' CSE and host proteins/factors.^{56,72,90,123} *In vitro* studies using polymerase extracts suggest the poly (A) tract may not serve as template for the initiation of minus-strand synthesis, although the details of this initiation event are not known.⁸⁹ Minus-strand synthesis requires P123 or P23 and nsP4, but a cleavage-defective P1234 is not functional.^{135–137,250} Similarly, in the vaccinia system, expression of the individual nsP's was not sufficient for complex formation and minus-strand synthesis.^{135,136} As minus-strand synthesis continues, nsP's continue to be translated and concentrations of the protease precursors increase. Cleavage at the nsP1–nsP2 and nsP2–nsP3 junctions results in the switch over to plus-strand synthesis, presumably by a change in the conformation and composition of the replicase complex (Fig. 22.10). Synthesis of minus strands by the replicase requires continuous protein synthesis, and it has been suggested that nsP2 engages the host response by using the RNase L-dependent pathway to inhibit host cell translation.^{82,240} As

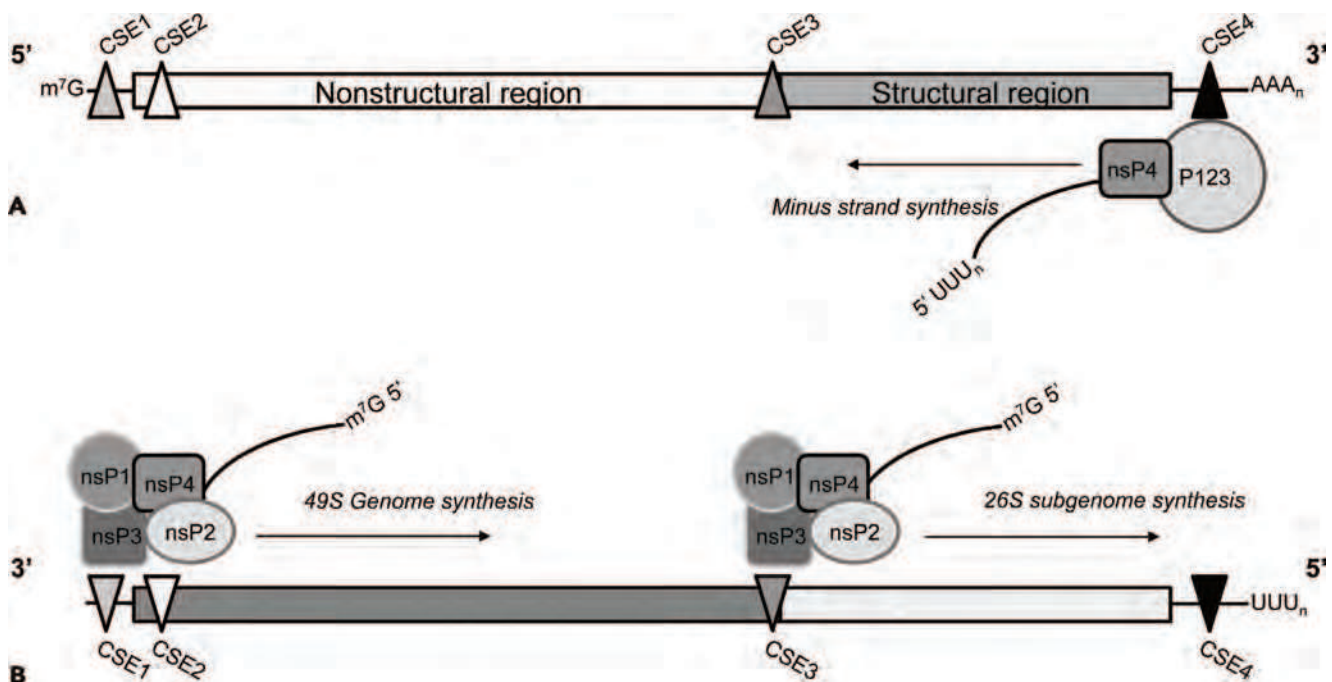


FIGURE 22.10. The conserved sequence elements (CSEs) and nonstructural proteins involved in alphavirus genome replication. A: A schematic of minus-strand synthesis from a plus-strand template. A protein complex composed of P123 and nsP4, and presumably host proteins (not shown), initiate synthesis of the minus strand from the 3' end of the genome. CSE4, a 19nt element, is found just upstream of the polyA tract. CSE4 is thought to act as a promoter for minus-strand synthesis, perhaps via a cyclization event with CSE2, a 51nt element located within the nsP1 coding region (not shown). **B:** A schematic of full-length 49S genomic and 26S subgenomic RNA syntheses from a minus-strand template. An accumulation of P123 allows processing of P123 polyproteins *in trans* into the individual nonstructural proteins. Presumably, the altered conformation of the replicase complex shifts template preference to the minus strand. CSE1, comprising the first 44nt of the 5' genome, acts as a promoter for synthesis of full-length 49S genomic RNA from a minus-strand template, perhaps in conjunction with CSE2. Fully processed replicase complexes also associate on the CSE3 element, which span the 3' end of the nsP4 coding sequence and the junction region between the nonstructural and structural genes. These CSE3-associated replicase complexes efficiently transcribe the 26S subgenomic message. Note that a replicase complex composed of nsP1, P23, and nsP4 may also be capable of plus-strand synthesis (not shown). (Courtesy of Jonathan Snyder.)

with most plus-strand RNA viruses, synthesis is asymmetric with minus-strand synthesis about 2% to 5% the level of plus-strand genome RNA.²⁹⁸

Although the viral protein composition of the minus- and plus-strand replicases is known, the role of host proteins in the complex is not. From studies with the CSEs, host cell-dependent effects were observed and several host proteins were shown to bind to the conserved RNA elements.^{56,193,194,195} Furthermore, Fayzulín and Frolov⁴⁴ showed that although the 51 nt CSE is dispensable in mammalian cells, in mosquito cells mutations have a deleterious effect. Interestingly, adaptive mutations occur in the 5' NTR, as well as in nsP2 and nsP3, suggesting their involvement in CSE function. Frolov et al.,⁵⁶ using chimeric templates and *trans*-competition experiments, showed that the 5' NTR is a component of the promoter for not only plus-strand synthesis, but also for minus-strand synthesis. From these data, they proposed a model for the initiation of minus-strand RNA synthesis that requires the 5' and 3' ends of the genome RNA to be brought together. This would be accomplished using components of the host translational machinery, which is involved in cap and poly (A) binding. Despite the attractiveness of the model, the lack of a purified

reconstituted system for RNA synthesis has hampered progress in understanding alphavirus RNA replication.

The synthesis of the subgenomic mRNA is controlled by a minimal promoter element that spans from -19 to +5 relative to the start of mRNA synthesis. A larger fragment from -98 to +14 provides three- to sixfold more activity and constitutes the fully active promoter.²¹⁴ As with the 5' and 3' CSEs, the subgenomic promoter appears to interact with host factors, as mutations in the promoter have differential effects, depending on replication in vertebrate versus invertebrate hosts.^{101,102,311} This promoter has been extensively employed in gene expression and replicon studies using alphaviruses, including the use of multiple tandem promoters.^{19,57,59}

Perhaps the most informative studies on alphavirus replication in recent years have emerged using advanced imaging techniques. These approaches have shed light on the spatial and temporal assembly of replication complexes. Most intriguing is the observation that membrane-derived structures competent for RNA synthesis appear to form initially at the plasma membrane (Fig. 22.11). Studies from both SFV and SINV demonstrate that the membrane invaginations referred to as spherules first accumulate on the plasma membrane and are

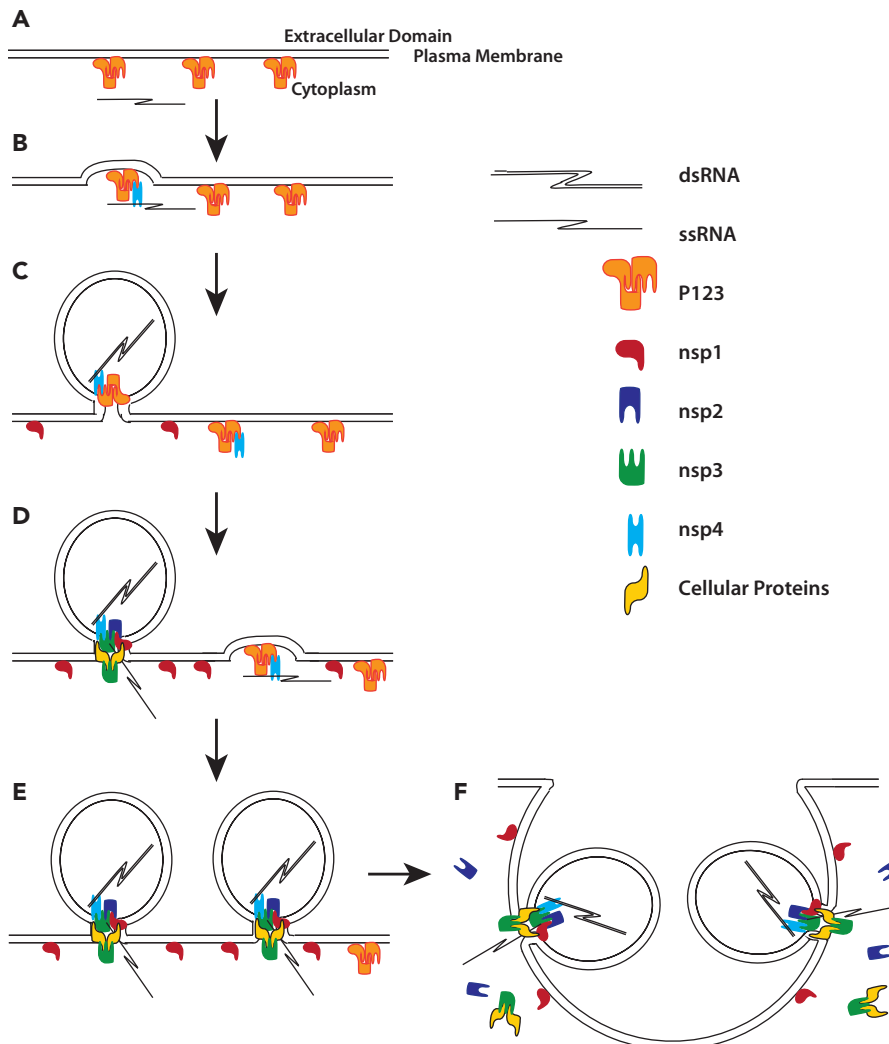


FIGURE 22.11. Assembly of alphavirus replication complexes. **A:** Replication complexes consisting of P123 and genome RNA are found at the plasma membrane. With the addition of nsP4 (**B**), the RNA-dependent RNA synthesis (RdRp), synthesis of double-stranded DNA (dsRNA) occurs (**C**) along with the formation of membrane spherules. Upon processing of P123 (**D**), replication complexes switch to the synthesis of genomic and subgenomic RNA. As replication continues (**E** and **F**) free nsP1, nsP2, and nsP3 are found, with the latter protein associating with specific host proteins and nsP1 remaining membrane associated. At later stages, multiple spherules coalesce into cytopathic vacuoles type 1 (CPV-1) within the cytoplasm. (Adapted by Thomas Edwards and R.J.K. with permission from Drs. Elena Frolova and Ilya Frolov.⁶⁰)

later internalized using the actin–myosin network.^{60,262} These structures appear to contain all of the nonstructural proteins as well as double-stranded RNA but are devoid of any structural proteins. Structures known as cytopathic vacuoles type 1 and type 2 (CPVI and CPVII) were described previously as containing the replication proteins and viral glycoproteins, respectively.^{61,202} The role of CPV2 appears to guide the glycoproteins to the site of budding; however, the role of CPV1 for RNA synthesis is not as clear. CPV1 structures form later in RNA replication, probably as a result of spherule recruitment from the plasma membrane, but it has been suggested that they may not be the major site for viral RNA synthesis at least in mammalian cells.⁶⁰

Assembly of Nucleocapsid Core, Glycoprotein Synthesis, and Processing

The subgenomic RNA, which is made at approximately three times the level of the genomic RNA, is translated to produce the structural or virion proteins.²¹⁶ The order of translation is capsid-PE2(E3+E2)-6K-E1 (Fig. 22.2). Translation of the structural polyprotein is enhanced due to the presence of a hairpin secondary structure in the subgenomic mRNA between residues 77 and 139.⁵⁹ The polyprotein is processed by host and viral proteases to generate the authentic structural proteins that will end up in the virion, and the membrane topology of the glycoproteins is shown in Figure 22.12. The capsid protein is translated first and is released by proteolysis immediately after the ribosome clears the junction between it and PE2. The capsid functions as an autoprotease, and sequence and mutational analyses suggested that the C-terminal domain of the capsid contained a serine-like protease.^{10,86} This hypothesis was confirmed by the x-ray crystal structure of the C-terminal domain from SINV.²⁹ The protein has a chymotrypsin-like fold, with His141, Asp163, and Ser215 forming the catalytic triad. Interestingly, the C-terminal resi-

due, Trp264, remains in the active site pocket and presumably prevents transcleavage by the protease. With the self-cleavage of the capsid protein, the new N-terminus of the polyprotein now contains a signal sequence for translocation of the PE2 sequence across the endoplasmic reticulum (ER) membrane.⁶⁹ Additional signal sequences are present at the C-terminus of E2, permitting translocation of 6K, and at the C-terminus of 6K, permitting translocation of E1 (Fig. 22.12). The expression of TF protein, which contains the first 43 amino acids of 6K, and then shifts to the -1 reading frame that prevents the synthesis of E1.⁴⁷ Proteins E1, E2, 6K, and TF are transmembrane proteins, whereas E3 is released from most alphavirus particles following cleavage of its PE2 precursor.^{95,210,211,232,236} SFV retains the cleaved E3 with the virion; however, it is unclear whether it has a postcleavage function.³²¹

Following autoproteolysis, the capsid protein transiently associates with the ribosome, and assembly into a core particle appears to be both rapid and efficient, with no observed intermediates.^{259,279} A specific “packaging sequence” has been identified in the genome RNA in SINV that promotes encapsidation of RNA into the assembling core.³⁰⁰ In SINV, this sequence has been identified as nucleotides 945 to 1076, and attempts to identify the nature of the recognition element have met with limited success.¹⁵⁰ However, there is conservation of structural and functional components of packaging signal across diverse alphaviruses.¹¹⁷ The packaging sequence on the genome RNA is recognized by residues 81 to 113 in the capsid protein.^{71,299} A deletion of residues 97 to 106 results in a failure to efficiently package genome RNA, although cores still form with heterologous RNA incorporated.¹⁹² Although most alphaviruses package their genomes with high efficiency, Aura virus has been described as an alphavirus that packages its subgenomic mRNA as well.^{230,231} The development of alphaviruses as gene expression vectors has prompted much investigation into the packaging requirements, and in VEEV, packaging has been shown to

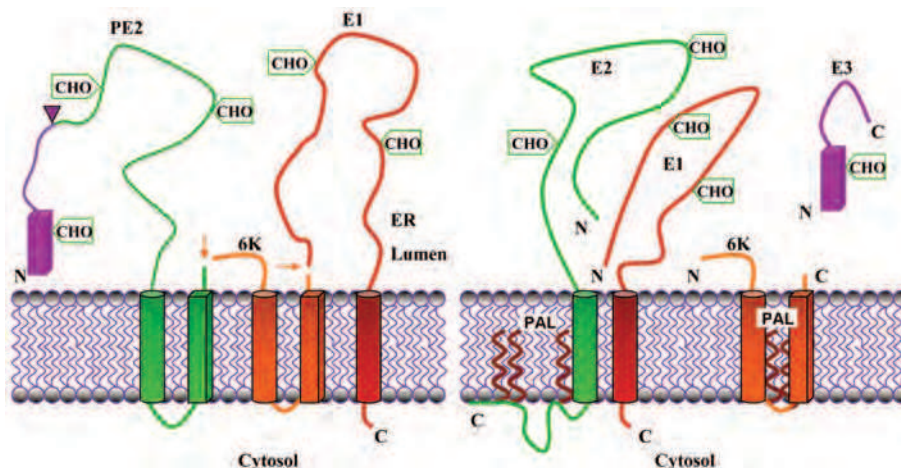


FIGURE 22.12. Schematic model for the configuration of the E1 and E2 glycoproteins in the membrane. **Left:** Configuration of the glycoproteins after signalase cleavage of 6K and E1, but before cleavage of PE2. **Right:** Configuration of glycoproteins after the maturation cleavage of PE2 into E3 and E2. Note the *dark red jagged lines* on the cytoplasmic side of E2 and 6K that represent palmitoylation sites (PALs). Glycosylation sites are indicated by CHO. Signal sequences are indicated as *colored rectangular blocks*. Stop-transfer sequences are indicated as *colored cylinders*. The shapes of the polypeptides do not imply their native configuration. (Courtesy of Dr. Joyce Jose.)

require expression of nsP123, although the mechanism and the generality of this requirement have not been shown.²⁸⁶

Although *in vitro* systems for core assembly have been described, the stepwise assembly process has not been clearly elaborated.^{272–274,301,302} The capsid protein requires the addition of nucleic acid to initiate the assembly process, and in the presence of full-length wild-type protein, assembly proceeds rapidly. Using a truncated capsid protein, the initial step appears to involve a protein dimer complexed with RNA.^{273,274} Cores are found in the cytoplasm and attached to membranes, probably through their interaction with E2 (reviewed in^{68,110}). Cytoplasmic cores have a well-defined size and $T = 4$ icosahedral symmetry, similar to *in vitro* assembled cores, but not identical to the well-ordered $T = 4$ symmetry in cores found within virus particles.^{178,196} However, the symmetry of the core does not necessarily dictate the symmetry of the virion, and it is likely that the icosahedral architecture of the glycoprotein scaffold is the driving force behind the strict $T = 4$ organization of alphavirus.^{52,138,207} This is supported by the observation from Forsell et al.,⁵³ who produced a SFV capsid protein with a deletion in residues 40 to 118. This mutant was unable to assemble cores, but virus particles were produced that had the expected $T = 4$ symmetry.

In parallel with the formation of the nucleocapsid in the cytoplasm, the envelope proteins that were translocated into the ER are processed and undergo posttranslational modifications (Fig. 22.5). High mannose chains are added to all potential N-linked glycosylation sites, and the oligosaccharide chains are trimmed depending on the availability of the site.^{243,245} Palmitoylation occurs at several sites in E2 and 6K/TF.^{17,64} In a set of elegant studies, Brown et al. showed that E1 and PE2 undergo a complex series of folding intermediates.^{22,120,180,181} These intermediates require chaperones and disulfide bond formation and exchange. The E1 and E2 glycoproteins form a heterodimer in the ER, but it is not known whether higher-order oligomerization takes place here.^{218,321} Oligomerization of PE2 and E1 is also a requirement for the transport of the glycoproteins, but the presence of the capsid (CP) is not required.^{70,105,157} It has been shown that PE2 oligomerizes with a partially folded intermediate of E1 and that this oligomerization is sufficient for the proteins to exit the ER. After the heterodimer reaches the trans-Golgi network, but prior to arrival at the plasma membrane, PE2 is cleaved by furin.³⁴ This cleavage is required for virion entry and fusion activation in new cells, although revertants can be readily isolated that suppress the requirement for cleavage.^{96,211}

Virion Budding

The final stage of the virus life cycle is the effective interaction between the capsid protein and the glycoproteins to promote virus budding. Thin-section electron microscopy of infected cells has shown a clustering of nucleocapsid cores at the plasma membrane at sites of budding but it is likely that the interaction occurs earlier, perhaps in the vicinity of CPVII.²⁶¹ All evidence suggests that a proper interaction between the cores and glycoprotein spikes is required for budding.¹⁰⁹ When the glycoproteins have been expressed in the absence of the capsid protein, virus-like particles have not been observed. In SFV, virions have been shown to bud from specific sites in polarized cells,²²⁵ and virus has been reported to bud intracellularly in insect cells.¹⁷⁵ There also appears to be a requirement for cholesterol in the membrane to support budding,^{159,164} and host

cell lipid metabolism has also been implicated.¹⁸⁶ Several systems have been reported that show the exogenously produced capsid cores can be introduced into naïve cells expressing the viral glycoproteins and particles can be released, suggesting that RNA synthesis is not required for budding.^{26,258}

The interaction between the capsid protein and the envelope glycoproteins has been investigated extensively, with most of the available data coming from molecular genetic and structural studies.^{109,271} X-ray crystallography of the capsid protein identified a hydrophobic pocket that was occupied by the amino-terminal arm of a neighboring capsid protein. The nature of the arm residues bound in the hydrophobic pocket suggested that a tyr-ala-leu motif found in the cytoplasmic domain of the E2 glycoprotein might function in a similar manner and bind into the capsid protein pocket.^{133,256} An extensive number of mutagenesis experiments involving SFV and SINV have established residues of the E2 cytoplasmic domain that were important for the interaction with the tyrosine, which is conserved in the alphaviruses, as an important one.^{64,109,270,319} E2 peptides have also been used to inhibit budding, suggesting residues involved in process and similar peptides were shown to bind to capsids.^{30,170} In addition, cryo-EM studies have shown that the cytoplasmic domain of E2 clearly extends down into the core to the site of the hydrophobic pocket.^{271,179,271,318} As was mentioned previously, deletions that disrupt the accumulation of nucleocapsid cores do not prevent budding because lateral interaction between the glycoproteins appear to be the driving force as long as capsid interactions do occur.²⁸⁷

The 6K protein, which has been estimated at 5 to 10 molecules per virion, has been implicated in the budding process and in the formation of virions.^{63,64} Removal of 6K from the genome of SFV did not influence the formation of the E1–E2 heterodimer or its transport to the cell surface, but it did reduce budding.¹⁴⁹ Other studies have shown that mutations in 6K can influence glycoprotein trafficking and virion assembly.²³⁸ E2 and 6K appear to interact, as mutations in 6K can be suppressed by mutations in E2,¹⁰⁷ and chimeric viruses containing an SINV glycoprotein and an RRV 6K are highly defective for virus formation.³¹⁴ Recently, it has been shown that a frameshift occurs at a low frequency during translation of the region encoding 6K resulting in the production of the TF protein shown in Figure 22.13.⁴⁷ The TF protein shares 47 amino acids with 6K and contains the transmembrane domain that has been implicated in channel formation. The remaining 23 residues are unique to TF and since there is a –1 frameshift followed by a termination codon, no E1 is produced from this polyprotein. Preliminary data suggest that TF also has a role in virus replication and is incorporated into the virion but whether its function(s) overlaps with 6K is not yet known.

Effects on the Host Cell

Alphaviruses have a wide host range and must interact with a variety of cellular receptors, either ubiquitous or unrelated molecules. Because the nature of these receptors is largely unknown, equally unknown is the signaling that such molecules might engage in following virion attachment and early steps in entry. Clearly, the response of most vertebrate cells to viral infection is distinct from the response of invertebrate cells. However, in both cases there appears to be a balance between the needs of the virus to effectively propagate and the needs of the host to control virus infection and dissemination. Host

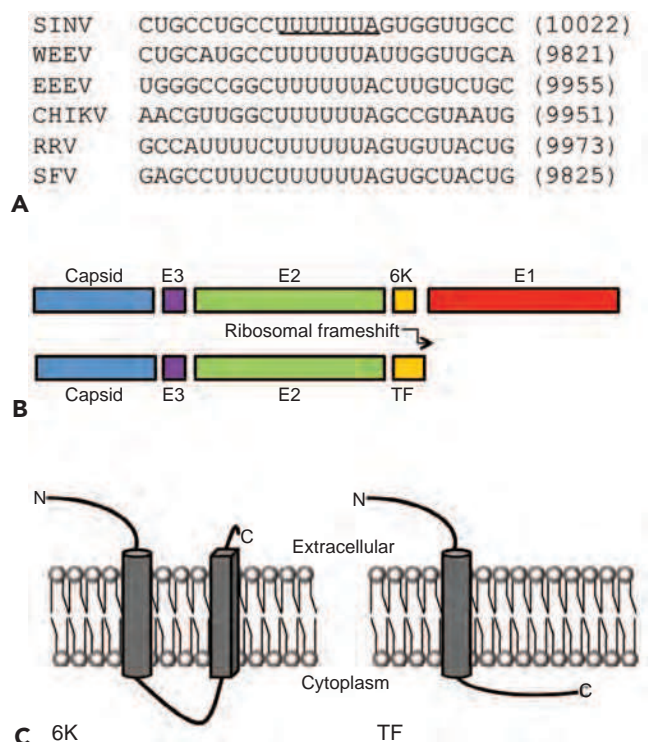


FIGURE 22.13. Ribosomal frameshifting during translation of the 26S subgenomic RNA yields a newly described protein, TransFrame (TF). **A:** Sequence alignment of various alphaviruses demonstrates the conservation of a putative slippery site motif within the 6K gene (underlined for Sindbus virus). The coordinate of the first nucleotide of the slippery site is indicated in parentheses for each virus. **B:** The typical protein products obtained from normal translation of the 26S subgenomic RNA (**top**), and the protein products obtained in the case of a frameshift event (**bottom**). Note that the amino terminal regions of the 6K and TF proteins share the same sequence. **C:** A model of the putative membrane topology of the mature 6K and TF proteins. The N- and C-termini are denoted. 6K (**left**) contains both a transmembrane anchor (cylindrical), and a membrane spanning region that acts as the signal sequence for E1 (rectangular). TF (**right**) contains only the transmembrane anchor (cylindrical); the frameshift event prevents production of the E1 signal sequence. (Courtesy of Jonathan Snyder.)

macromolecular synthesis is inhibited in vertebrate cells shortly after infection (reviewed in 268). Host protein synthesis is shut off at 3 hours after infection, although virus protein translation continues unabated. This has been an intensive area of investigation, and four mechanisms for shutoff have been proposed: (a) an altered intracellular environment such as K^+ concentration that would favor viral translation, (b) direct competition for translational machinery, (c) inhibition of cellular translation by the capsid protein, and (d) inhibition of translation by one of the nonstructural proteins. The development and use of replicon systems that contain only the *cis*-acting replication signals and the coding region for replication proteins suggested that the structural proteins were not responsible for translational shutoff.⁵⁸ Furthermore, studies investigating the establishment of persistence identified changes in SINV nsP2, at Pro726 to serine, which reduced cytopathic effects of the virus.⁵⁵ A variety of studies suggest that alphavirus infection

promotes the double-stranded RNA-activated protein kinase (PKR)-dependent and PKR-independent pathways to reduce host cell translation.^{66,235} Mutations in nsP2 suggest that the shutoff of host cell transcription and translation are distinct events and strongly influence the decreased production of a α/β interferon.⁸¹ However, the role of nsP2 in host cell shutoff appears to function only in the Old World alphaviruses. It has been shown that in VEEV and other New World alphaviruses that a region of the capsid protein encompassing residues 33-68 and not nsP2 is responsible for transcriptional shutoff and cytopathogenicity.⁶⁷ This region of the VEEV capsid protein has also been shown to be responsible for nuclear trafficking of the protein to the nucleus.¹²

Most infections by alphaviruses of vertebrate cells in culture lead to the induction of apoptosis.¹³⁹ Apoptosis of infected neurons is a major determinant of neurovirulence, as demonstrated for SINV.¹⁴² In contrast, mosquito cells can undergo a variety of effects from persistent infections to cell death caused by necrosis.¹¹² It has also been possible to establish persistent infection of vertebrate cells using defective interfering particles, followed by genetic changes to the helper virus in nsP2.⁴¹ An alternative method was used by selecting for replicons that were noncytopathic to BHK cells, and again, amino acids substitutions were detected in the coding region for nsP2,⁵⁵ suggesting a major role for this protein in modulating the virus–host interaction.

A number of antiviral proteins produced in infected cells have been described for the alphaviruses. A small hydrophobic peptide of 3,200 D was shown to be produced in persistently infected mosquito cells, and this peptide could activate an antiviral state.¹⁶¹ This peptide induced the synthesis of a 55-kD protein and inhibited the replication of alphavirus RNA.¹⁶⁰ The rat zinc-finger antiviral protein (ZAP), originally identified as a retrovirus resistance protein, was shown to inhibit multiple alphaviruses.¹⁶ This protein inhibited viral translation by binding to viral mRNA, although its exact mechanism of action is not known.⁸³ It is likely that many additional proteins, such as interferon-stimulated genes, will demonstrate direct antiviral activity and might be exploited to control alphavirus infection.

Defective Interfering Genomes and Replicon Systems

Defective interfering (DI) genomes replicate and are packaged in the presence of helper virus, and retain all *cis*-acting sequences necessary for RNA replication. Several alphavirus DI genomes have been molecularly characterized, and all retain the 3' CSE that was previously described. The 5' end of the DI genomes was found to be more heterogeneous, with the 5' CSE, cellular transfer RNA (tRNA) sequences, or the 5' 142 nucleotides from the subgenomic mRNA located at the 5' end of the DI RNA. The study of DI genomes provided a powerful genetic tool to identify the location and function of required *cis*-acting sequence elements.¹⁴¹ The development of DI genomes for genetic purposes gave way to the construction of replicons, which supported RNA replication but were incapable of infection of new cells because they lacked the structural proteins.^{140,244,313} The structural proteins could be supplied by additional helper RNAs so that the replicons could be packaged and used to efficiently infect target cells.¹⁹

The alphavirus replicon has become a standard gene expression system. The system has proven useful for examining

protein expression in heterogenous systems and the development of vaccines.²⁴⁴ SINV, SFV, and VEEV replicons have been widely used and can allow for targeting to specific cells.^{148,214,244} By introducing a mutation in nsP2 that renders the replicon noncytopathic, continuous replication in the absence of cell death can occur for SINV.⁵⁵ For VEEV, mutations in the 5' nontranslated region and nsP3 were also required for persistent infection by the replicon.²⁰⁵ Multiple subgenomic promoters can be employed for the expression of several proteins of interest in a regulated fashion. To further reduce the chance of recombination between replicon and helper RNAs to generate an infectious genome, tricomponent replicon systems have been developed that can produce at least 1,000 packaged replicons per cell.⁴⁵

RUBIVIRUS REPLICATION

Virion Structure and Entry

Although once considered a close cousin of the alphaviruses, molecular analyses of rubella virus have revealed significant differences.⁵⁴ Whereas rubella virions are similar to alphaviruses in protein composition and morphology, the particles have yet to yield to high-resolution structural analysis, and it is unlikely that the particles share the property of icosahedral symmetry with the alphaviruses. The rubella virion is composed of three structural proteins that share the same name as alphaviruses, yet differ in amino acid sequence.⁴⁰ The virions are pleomorphic in shape and are around 60 to 70 nm in diameter.¹⁸² The two type I envelope glycoproteins, E1 and E2, form heterodimers, and have 13 and 7 amino acids on their inner cytoplasmic face. All three structural proteins are membrane associated with the C-terminus of the capsid containing the signal sequence for E2.¹⁹⁰ The structural proteins are cleaved by signal peptidase, with the signal sequence for E1 present at the C-terminus of E2.¹⁰³ The capsid protein is a phosphoprotein of 293 or 300 amino acids, depending on which AUG codon is used to initiate the polyprotein.¹⁶⁵

Rubella virus is restricted to growth in humans and is not transmitted by insects, as are the alphaviruses. However, the virus can replicate in a wide range of mammalian cell types and can infect experimental animals producing subclinical results. Thus, like alphaviruses, a ubiquitous cellular receptor may function in entry, although none has yet been identified. The virus also appears to enter through a receptor-mediated endocytosis pathway with membrane fusion promoted by an acidified endosome,¹¹³ and a class II fusion mechanism is expected.

Transcription, Translation, and Genome Replication

The placement of rubella virus in the family *Togaviridae* implies a common genome structure and replication strategy. Complete nucleotide sequences are available for several strains of rubella virus.^{40,212} The 9,762 nucleotide genome RNA contains a 5' terminal 7-methylguanosine and a 3' terminus that is polyadenylated.^{191,294} The genomes of rubiviruses and alphaviruses are compared in Figure 22.4, and a more complete discussion of rubella replication is provided in Chapter 24. The replication proteins P150 and P90 are encoded by the genome RNA, whereas the structural proteins are derived from the subgenomic mRNA. Although there is no amino acid sequence

homology among the structural proteins, limited homologies do exist within the replication proteins.⁴⁰ The construction of a rubella virus complementary DNA (cDNA) clone from which infectious RNA could be generated permitted molecular genetic studies on the virus that were, up until then, quite limited.^{213,294}

Three species of RNA are synthesized in infected cells: complementary minus-strand RNA, genome RNA, and subgenomic mRNA. On infection, the genome RNA is translated into a 200-kD polyprotein that is cleaved by a virus-encoded protease.⁵⁰ Unlike its alphavirus counterpart, the rubella enzyme is a metalloprotease that contains zinc-binding domains, and it cleaves P200 in trans.²⁹⁵ Virus replication complexes can be found in association with cellular membranes. Virus-specific vacuoles have been identified and colocalize with lysosomal markers, similar to those found for alphaviruses.¹²⁵ There is a close association between these replication complexes, identified with antibodies to P150, and ER and Golgi membranes, presumably to facilitate translation and packaging of genome RNAs.²²⁴

Attempts to identify cellular proteins that might participate in RNA replication have focused on proteins that bind to the 5' and 3' NTRs. A 5' stem-loop structure predicted to form on the plus-strand RNA was shown to bind to the La autoantigen. Several cellular proteins were shown to bind to the 3' NTR.^{183,184} One of these proteins was identified as calreticulin, although decreased binding of calreticulin did not correlate with reduced virus replication.²⁵⁵ The nonstructural protein P90 has been shown to bind to the retinoblastoma protein Rb through an Rb-binding motif.¹³ Mutation of this motif reduces virus replication, but it is unclear whether the defect is related to a reduction in binding.⁴⁹

The capsid protein has also been implicated in RNA replication. It has been shown to complement a replication defect resulting from a deletion of 169 amino acids from P150.²⁷⁷ Exactly how this might function is not known, but the amino terminal 88 residues of the capsid were sufficient for complementation, and these might function by binding to RNA. In addition, the capsid protein has been shown to influence the replication of rubella replicons,²⁵ although once again the mechanism is not known. The capsid protein has also been shown to bind to mitochondrial matrix protein p32 and the proapoptotic protein Bax.¹⁰⁶ The function of capsid in binding these proteins is to prevent apoptosis and enhance RNA replication.

Virus Assembly

A packaging signal has been located between nucleotides 347 and 375 of the genome RNA, and it interacts with capsid residues 28 to 56.¹⁵⁴ Phosphorylation of the capsid protein occurs and has been suggested to act as a regulatory mechanism to prevent binding of nonviral RNAs to the capsid.¹³² With the retention of the capsid protein signal sequence at its C-terminus, the capsid remains associated with the ER membrane. This association may be important for ensuring close connectivity with the envelope glycoproteins through their transmembrane domains. Glycoproteins E1 and E2 are believed to have functions similar to their alphavirus counterparts, although rubella virions bud into the Golgi.¹⁰⁴ Virions undergo a maturation step after Golgi budding that may release the capsid signal sequence because morphologic changes occur in the core of the virion.²²⁴ The structures of the mature and immature rubella virions await structural techniques but will probably require

electron tomography, as they lack the icosahedral symmetry present in the alphaviruses.

PERSPECTIVES

Our knowledge of togaviruses has grown dramatically thanks in large part to the ability to genetically manipulate these relatively “simple” plus-strand RNA viruses. Insights into alphaviruses have been obtained more quickly than for rubella virus due to their greater replication efficiency in cultured cells and their well-organized virions. Structural studies of the alphaviruses have progressed rapidly, leveraging the icosahedral nature of the virus particles and the ability to obtain large quantities of homogeneous preparations. The ability to express capsid proteins in heterologous systems has facilitated both structural and biochemical studies of capsid structure and its assembly pathway. The structure determinations of the native and postfusion forms of the E1 protein have provided exceptional insights into the entry process and the structure of the virion. The atomic structures of the E1 and E2 heterodimer, in both low pH and neutral forms, have provided insight into the sequential entry process employed by these viruses.

Significant gaps still persist in understanding the replication process of the togaviruses. Although the protease domain of nsP2 and the macro domain of nsP3 have been determined, the full-length proteins have proven difficult to express in large quantities, to purify to homogeneity, and to crystallize. Furthermore, it is expected that the functional entities will be complexes of viral and probably cellular proteins that will further complicate structural analyses. The ability to reconstitute purified and functional replication complexes will be an important milestone to decipher RNA replication. In contrast, significant progress has been made in understanding the cellular response to viral infection and the recruitment of cellular proteins to promote or inhibit the virus replication complex. Systems-level studies to evaluate the total cellular environment altered in virus infection are beginning to yield a comprehensive picture of how the virus perturbs and subjugates the cell. The use of advanced and real-time optical imaging as well as electron tomography promises to provide a temporal and spatial view of virus infection. However, most studies continue to rely on standard cell culture systems and it will be important to verify what occurs in more natural target cells such as neurons.

With the growing knowledge of togaviruses, their utility in gene expression and gene therapy continues to be exploited and expanded. Future directions that will further benefit this system will be in understanding the nature of the cellular receptors and the structure of the E2 protein that is involved in binding these receptors. With this knowledge, newly designed alphavirus vectors will be engineered to specifically and efficiently target the replicon to cells and tissues of interest.

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Alphaviruses

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Perspective

The genus *Alphavirus* includes 29 species that can be classified antigenically into at least eight complexes (Table 23.1). Alphaviruses are geographically restricted in their distributions and have been found on all continents and on many islands. In nature, alphaviruses cycle between invertebrate insect vectors and vertebrate hosts. For most alphaviruses, the insect vectors are mosquitoes; however, other hematophagous arthropods, such as lice or mites, are vectors for a few. The vertebrate hosts are generally mammals or birds, although fish are hosts for the aquatic alphaviruses. In general, the pathogenic alphaviruses are divided into the viruses that cause human disease characterized by rash and arthritis, primarily found in the Old World, and viruses that cause encephalitis, primarily found in the New World. For many alphaviruses, no human or veterinary disease has been recognized. Larger mammals, such as humans and horses, that tend to develop severe or fatal disease are often dead-end hosts unimportant to the endemic virus transmission cycles but can be important for sustaining epidemics.

HISTORY

Records of diseases almost certainly attributable to alphaviruses date to the 18th and 19th centuries, when epidemics of fatal encephalitis in horses in the northeastern United States and outbreaks of arthritis in Southeast Asia were recognized and recorded.^{111,300,788} The first clear report of epidemic encephalitis comes from the summer of 1831, when 75 horses died in Massachusetts.³⁰⁰ Over the next 100 years, several local outbreaks of encephalitis in horses were noted along the Atlantic seaboard of the United States and in the Pampas region of South America.⁶⁸³ However, the first alphavirus to be cultured was western equine encephalitis virus (WEEV). This virus was isolated in 1930 from the central nervous system (CNS) tissues of 2 horses involved in an epidemic of equine encephalitis in the San Joaquin Valley of California.⁵²² Eastern equine encephalitis virus (EEEV) was isolated from the brains of affected horses in New Jersey and Virginia in 1933.⁷⁸⁶ Both diseases occurred in summertime epidemics, suggesting an arthropod vector, and

TABLE 23.1 Alphaviruses, Abbreviations, Biological Features, and Association with Disease

Virus (abbreviation)	Antigenic complex	Principal vertebrate reservoir host	Geographic distribution	Human disease	Animal disease
Aura (AURA)	WEE	?	South America		
Barmah Forest (BF)	BF	Birds	Australia	Fever, arthritis, rash	
Bebaru (BEB)	SF	?	Asia		
Cabassou (CAB)	VEE	?	French Guiana		
Chikungunya (CHIK)	SF	Primates	Africa, Southeast Asia, Philippines, Indonesia	Fever, arthritis, rash	
Eastern equine encephalitis (EEE)	EEE	Birds	North America, South America, Caribbean	Fever, encephalitis	Horse, pheasant, emu, pigeon, turkey
Everglades (EVE)	VEE	Mammals	Florida	Fever, encephalitis	
Fort Morgan (FM)	WEE	Birds	Colorado		
Getah (GET)	SF	Mammals	Asia	Fever	Horse
Highlands J (HJ)	WEE	Birds	North America		Horse, turkey, emu, pheasant, duck, crane
Mayaro (MAY)	SF	Mammals	South America	Fever, arthritis, rash	
Middelburg (MID)	MID	?	Africa		
Mosso das Pedras/78V3531	VEE	?	South America		
Mucambo (MUC)	VEE	?	South America, Caribbean		
Ndumu (NDU)	NDU	?	Africa		
O'nyong-nyong (ONN)	SF	?	East Africa	Fever, arthritis, rash	
Pixuna (PIX)	VEE	Mammals	Brazil		
Rio Negro/AG80 (RN)	VEE	Mammals	Argentina		
Ross River (RR)	SF	Mammals	Australia, South Pacific	Fever, arthritis, rash	
Salmonid alphavirus (SAV)	?	Fish	North Atlantic		Trout, salmon
Semliki Forest (SF)	SF	?	Africa	Fever, encephalitis	Horse
Sindbis (SIN)	WEE	Birds	Australia, Africa, Northern Europe, Middle East	Fever, arthritis, rash	
Southern elephant seal (SES)	?	Seals	Antarctica		
Tonate (TON)	VEE	?	South America	Fever, encephalitis	
Trocará (TRO)	WEE	?	South America		
Una (UNA)	SF	?	South America, Trinidad		Horse
Venezuelan equine encephalitis (VEE)	VEE	Mammals	South America, North America	Fever, encephalitis	Horse
Western equine encephalitis (WEE)	WEE	Birds, mammals	North America, South America	Fever, encephalitis	Horse, emu
Whataroa (WHA)	WEE	Birds	New Zealand, Australia		

in 1933, Kelser showed WEEV transmission by mosquitoes.³⁸⁸ In 1936, an epizootic of equine encephalitis occurred in the Guajira region of Venezuela; the virus isolated was not neutralized by antisera against EEEV or WEEV and was designated Venezuelan equine encephalitis virus (VEEV).⁴²⁰

Summertime epidemics of polyarthritis were recognized in Australia and New Guinea in 1928,^{183,572} and subsequent outbreaks were reported in Northern Europe, Africa, and Southeast Asia.⁷²⁷ It is likely, of course, that the alphavirus-induced

arthritic diseases are much older than these dates but were not clearly described or differentiated from more prevalent infections in these regions, such as dengue. This is considered to be particularly true of outbreaks of chikungunya virus (CHIKV) that have occurred in India and Southeast Asia over the past 200 years.^{111,788} Viruses associated with epidemic polyarthritis were eventually isolated, both from mosquitoes collected in the areas of human disease and later from humans. The first of these viruses was isolated in 1952 from a pool of *Culex* spp.

mosquitoes collected near Sindbis, Egypt.⁷⁸² However, it was many years before Sindbis virus (SINV) was linked to human disease.⁴⁹³

The first clear association of an alphavirus with arthritic disease came in 1953, when CHIKV was isolated in Tanzania from the blood of people with severe arthritis.⁶⁶⁸ During the next several years, several viruses that cause arthritis, often accompanied by a rash, were isolated in Africa, Australia, and South America.^{122,537,884} These viruses were added to the growing list of arthropod-borne (arbo) viruses, defined by the World Health Organization in 1967 as “viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation”.⁶²¹

In 1954, arboviruses were divided by Casals and Brown into three serologic groups—A, B, and C—based on cross-reactivity in hemagglutination inhibition (HI) tests.^{556,621} EEEV, WEEV, and VEEV constituted the group A arboviruses. A second cross-reacting set, including dengue, St. Louis encephalitis, and yellow fever viruses, constituted the group B arboviruses, and the nonreactive viruses were designated group C. As viruses became classified on the fundamental properties of the virion and the genome, the group A viruses became the *Alphavirus* genus within the *Togaviridae* family of enveloped RNA viruses.

INFECTIOUS AGENTS

Alphaviruses are enveloped plus-strand RNA viruses with icosahedral symmetry (Fig. 23.1). The virions are 60 to 70 nm

in diameter and sensitive to ether and detergent. Cryo-electron microscopy structures are available for many.^{18,418,496,549,618,722,901–903} The RNA is contained within a capsid formed by a single protein arranged as an icosahedron with $T = 4$ symmetry. The nucleocapsid is enclosed in a lipid envelope derived from the host cell plasma membrane that contains the viral-encoded glycoproteins E1 and E2. These proteins form heterodimers that are grouped as trimers to form 80 knobs on the virion surface. Glycoproteins are arranged such that 240 copies of each interact with 240 copies of capsid protein.

The 49S genome is composed of a single-strand, non-segmented, capped, and polyadenylated message sense RNA that is infectious. Complete sequence information is available for representatives of all currently known *Alphavirus* species.^{129,161,206,219,297,402,431,434,437,518,764} The genomes are 11 to 12 kb in size and are organized with the nonstructural proteins (nsPs) at the 5′ end and the structural proteins at the 3′ end. The nsPs are translated from genomic RNA and the structural proteins from a subgenomic RNA.⁷⁶⁵ Four nsPs function to replicate the viral RNA and produce the subgenomic RNA (see Chapter 22).

Five potential structural proteins (C, E3, E2, 6K, and E1) are encoded in the subgenomic RNA as a polyprotein, and an additional transframe protein (TF) is produced by −1 ribosomal frameshifting within the 6K coding region.^{141,215} The N-terminal portion of C is basic and is presumed to bind the viral genomic RNA, whereas the more conserved C-terminal portion interacts with other copies of the C protein to form the nucleocapsid and also interacts with the cytoplasmic tail of E2.^{765,901}

E3 is a small, cysteine-rich glycoprotein that serves as a signal sequence for pE2 (the precursor containing E3 and E2), mediates proper folding of E2, and is necessary for pE2 to

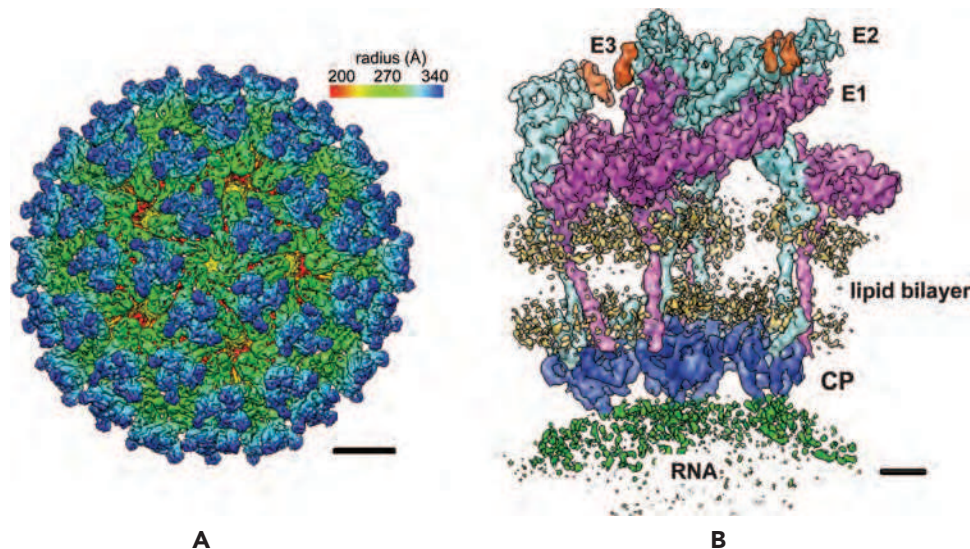


FIGURE 23.1. Three-dimensional (3D) reconstruction of the Venezuelan equine encephalitis virus (VEEV) virion. A: Radially colored 3D reconstruction of VEEV showing the E1 basal triangle (green) and E2 central protrusion (blue) for each spike. Scale bar: 10 nm. **B:** One asymmetric unit of the virus containing four unique copies of E1 (magenta), E2 (cyan), E3 (orange), and capsid (CP, blue). The cryo-electron microscopy densities for the viral membrane (yellow) and genomic RNA (green) are also displayed at slightly lower isosurface threshold. (Courtesy of Wah Chiu; reproduced with permission from Zhang R, Hryck CF, Cong Y, et al. 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. *EMBO J* 2011;30:3854–3863.)

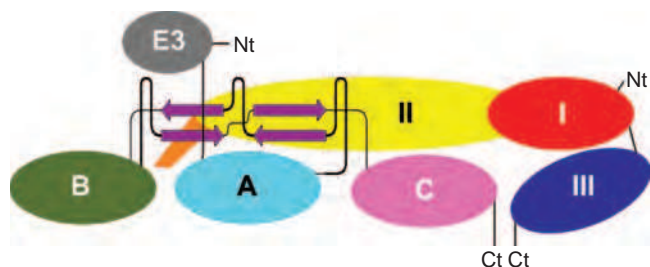


FIGURE 23.2. Domain structure of the glycoprotein spike. Schematic diagram of the E1pE2 heterodimer drawn “untwisted” to show the domains positioned with respect to one another and their connectivity. Domains of E1 (I, red; II, yellow; III, blue; fusion loop, orange) and pE2 (A, cyan; B, dark green; C, pink; E3, gray) are shown. Ct, C-terminus; Nt, N-terminus. (Courtesy of Felix Rey; reproduced with permission from Voss JE, Vaney MC, Duquerroy S, et al. Glycoprotein organization of chikungunya virus particles revealed by x-ray crystallography. *Nature* 2011;468:709–712.)

heterodimerize with E1 for transport to the cell surface.^{468,599} As part of the pE2-E1 heterodimer, E3 prevents premature activation of E1.^{362,840,844} E3 is cleaved from E2 by furin in the trans-Golgi, remains associated with the spike under acidic conditions, and is usually shed when virions bud from the cell surface.^{737,904}

The E2 glycoprotein is a transmembrane protein that has two or three N-linked carbohydrates and contains the most important epitopes for neutralizing antibody. E2 is organized into three immunoglobulin domains (A, B, and C) (Fig. 23.2). Domain A (residues 1–132) is at the center and top of the heterotrimer and has receptor and neutralizing antibody-binding sites. Domain B is at the spike tip, and C is toward the viral membrane.^{455,840} The intracytoplasmic portion interacts with the capsid and has a second stretch of hydrophobic amino acids and myristoylation sites that tether it to the inner surface of the membrane.

The 6K protein serves as a signal peptide for E1, is cleaved from E1 and E2 by signal peptidase, and is important for budding, and small amounts are incorporated into the virion.^{238,461} The E1 protein has one or two N-linked carbohydrates, a short (one or two residue) intracytoplasmic tail, and a positionally conserved internal hydrophobic stretch of amino acids in the N-terminal portion that serves as the fusion peptide for virion entry into the cell. E1 is organized into three β -sheet-rich domains (I, II, and III) similar to the flavivirus E protein with the internal fusion loop at the tip of domain II^{253,443} (see Fig. 23.2). The function of TF has not yet been defined but can be incorporated into virions.²¹⁵

Propagation and Assay in Tissue Culture

Initial isolations of alphaviruses were accomplished by intracerebral inoculation into suckling mice—a host very susceptible to infection with most alphaviruses.^{335,668,689,781,782,867,884} Many alphaviruses can also be isolated and propagated efficiently in primary chick embryo fibroblasts (CEFs) and in various continuous mammalian cell lines such as human epithelial (HeLa, MRC5) cells, baby hamster kidney (BHK) cells, monkey kidney (Vero) cells, and mouse fibroblast and neuroblastoma cells^{108,581} (Fig. 23.3).

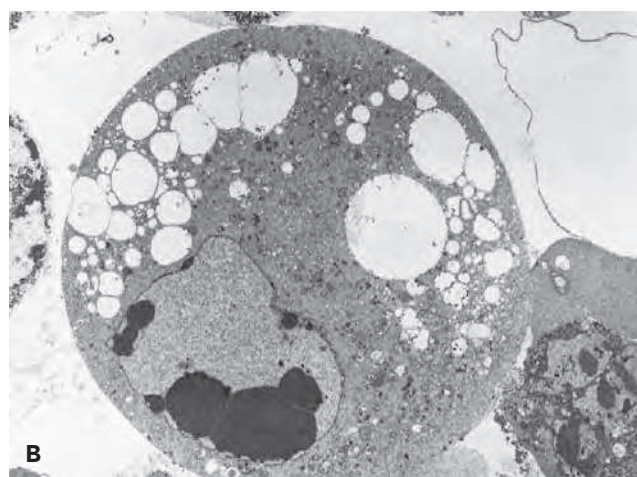
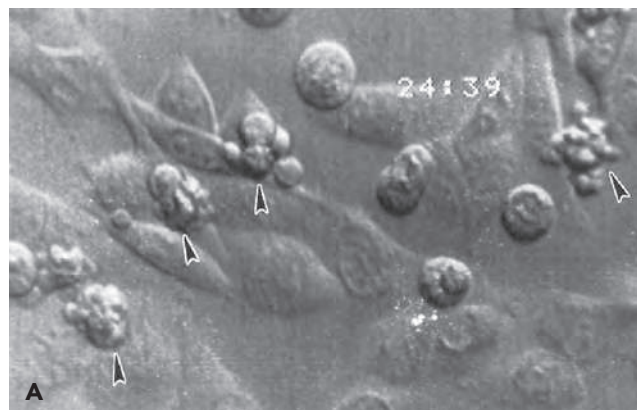


FIGURE 23.3. Effects of Sindbis virus infection on vertebrate cells.

A: Cytopathic effects (CPE) of rounding, shrinkage, and cytoplasmic blebbing in baby hamster kidney (BHK) cells infected at a low multiplicity. **B:** Electron micrograph of an infected BHK cell showing chromosomal condensation and cytopathic vacuoles.

Most alphaviruses will form plaques on susceptible mammalian or avian cells under an agar overlay. Mosquito cell lines also support replication, although often without cytopathic effect (CPE).^{635,761,763,803} The first plaque assay of an animal virus was performed using WEEV on CEF cells,¹⁹⁰ and plaque assay remains a convenient and sensitive way to quantify infectious virus. Plaque size has been used to differentiate strains and is determined by the type of overlay used, by relative virus binding to negatively charged sulfated polysaccharides present in the overlay, and by replication efficiency.^{87,96,775}

Biological Characteristics

Hemagglutination

Alphaviruses can hemagglutinate avian (e.g., goose, chicken) erythrocytes,^{581,684} and hemagglutination has been used as a method for quantifying virus and HI for measuring antiviral antibody.¹⁴⁴ Hemagglutination requires prior exposure of the virus to acidic pH, depends primarily on the E1 glycoprotein, and reflects binding of the fusion domain of the E1 glycoprotein to lipids in the erythrocyte membrane.^{130,144,829,873} E2 also participates in hemagglutination because some monoclonal

antibodies (MAbs) specific for E2 also have HI activity.^{67,640} The HI test has been useful for determining antigenic relationships among alphaviruses.¹⁰⁷

Cellular Receptors

Binding of virus to the cell surface and entry into the cell is a multistep process that depends on virus glycoproteins E1 and E2, cell surface molecules, low pH in the endosome, and fusion of membrane lipids. Variations in any of these components will affect the efficiency of infection and the likelihood that any particular cell will become infected *in vivo*. Virus-specific attachment to cells is primarily a function of the E2 glycoprotein. The important role for E2 in initiating virus–cell interaction is evidenced by the ability of anti-E2 MAbs to inhibit binding to cells,^{96,474} of anti-idiotypic antibodies to E2-specific MAbs to recognize putative virus receptors on cells,^{823,852} and of amino acid changes in E2 to alter virus binding to cells of different types.^{189,438}

Identification of specific alphavirus receptors has been difficult and may be complicated by experimental use of virus strains that are adapted to replicate in tissue culture.⁴¹¹ Because each alphavirus infects a wide range of hosts, often including birds, mammals, and mosquitoes, they must either use an evolutionarily well-conserved cell surface molecule or multiple molecules as receptors. None of the alphavirus receptors identified to date appears to be used exclusively, suggesting the possibility of several receptors. Alternatively, alphaviruses may use receptor–coreceptor combinations to achieve wide host range and the specific tropisms observed *in vivo*.

The first alphavirus receptor to be identified was the major histocompatibility complex (MHC) class I molecule receptor for Semliki Forest virus (SFV) on mouse and human cells.³¹⁵ This molecule is not absolutely required because cells lacking MHC molecules can still be infected with SFV.⁵⁸³ The high-affinity laminin receptor is a receptor for SINV on BHK cells and a potential receptor for SINV and VEEV on C6/36 mosquito cells.^{474,850} Again, this molecule appears to account for only a portion of the total virus interaction with the cells studied and does not contribute to SINV binding to avian cells.⁸⁵⁰ Ross River virus (RRV) uses the $\alpha_1\beta_1$ integrin on HeLa cells for binding.⁴²⁶ SINV and VEEV can use natural resistance-associated macrophage protein (NRAMP) for binding to insect and mammalian cells.⁶⁶⁴ The type of cell in which the virus is grown can also influence initial virus–receptor interactions. For instance, SINV grown in mosquito cells is enriched in high-mannose carbohydrates that bind the C-type lectins DC-SIGN and L-SIGN on the surface of dendritic cells (DCs).⁴¹⁰

Heparan sulfate (HS), a ubiquitously expressed glycosaminoglycan is an important initial binding molecule for certain strains of many alphaviruses. The use of HS is selected for by virus passage in vertebrate cells. However, wild-type strains of EEEV bind HS, so this property is not exclusively determined by passage in tissue culture.²⁴² For these viruses, addition of heparin or lactoferrin, treatment of cells with heparinase, or the use of cells deficient in HS decreases binding to cells and plaque formation.^{59,96,242,312,409,411,426,740,843} Interaction with HS—a highly sulfated, negatively charged molecule—probably explains the effects of ionic strength and charge on the attachment of virus to cells.^{499,501,611} The heparin-binding domain of the E2 glycoprotein is on domain A and overlaps a neutralizing epitope (see Fig. 23.2). Changes toward more positively charged amino acids

in the region around E2–70 increase the efficiency of attachment to cells in tissue culture.^{59,97,242,411}

Entry requires endocytosis followed by a conformational change in the trimer of E1–E2 heterodimers induced by exposure to low pH.^{313,729,739,879} The E1 fusion peptide is protected in the virion heterotrimer by the B domain of E2, and this association is stabilized by E3.^{455,840} When exposed to acidic pH, E1 dissociates from E2 and forms stable E1 homotrimers in the presence of cholesterol-containing membranes. During this conformational change, the fusion peptide is exposed and inserted into the outer leaflet of the lipid bilayer.^{83,252,253} Fusion with the cell membrane to initiate infection depends on the presence of a membrane potential and sphingolipid.^{314,570,899}

Effects on Vertebrate Cells

Alphaviruses replicate rapidly in most vertebrate cell lines with the release of progeny virus typically within 4 to 6 hours after infection. At the time of virus entry, there is an increase in permeability perhaps owing to pore formation by the E1, 6K, and/or TF proteins.^{492,871,872} Insertion of newly synthesized glycoproteins into the plasma membrane renders infected cells capable of adenosine triphosphatase (ATP)-dependent polykaryocyte formation on exposure to acid pH.³⁸⁹ Infection causes extensive CPE characterized by cell rounding, shrinkage, and cytoplasmic blebbing (see Fig. 23.3A), with the death of infected cells within 24 to 48 hours.^{449,635} Alphavirus-induced CPE has been linked to shut off of host cell transcription and translation,²⁴⁶ endoplasmic reticulum (ER) stress,⁵⁷⁴ and induction of apoptosis.^{20,26,257,270,351,449,656,699} The ability of alphaviruses to induce cell death, combined with the ability to express heterologous genes and activate natural killer (NK) cells, has led to their development as potential oncolytic agents.^{344,454,552,633,634,808,809,830}

Shut off of host cell functions in Old World viruses (e.g., SINV, SFV) has been linked to the effects of nuclear nsP2 and in New World viruses (e.g., VEEV, EEEV) to the effects of C.^{14,32,247} Activation of protein kinase R (PKR) results in phosphorylation of eIF2 α , which shuts down host protein synthesis without affecting translation of viral 26S subgenomic RNA.⁸³⁴ Alphavirus escape from eIF2 α phosphorylation is owing to the presence of a hairpin loop structure in the subgenomic messenger RNA (mRNA) downstream of the initiation codon that allows the 40S ribosome to initiate translation in the absence of eIF2.^{185,804,834} This initiation process utilizes host cell factors ligatin and MCT-1/DFNR⁷³⁸ and does not depend on mTOR signaling.⁵³⁰ Alphavirus-induced inhibition of host protein synthesis can also be PKR-independent perhaps through late suppression of mTOR.^{265,530}

The apoptotic process is associated with blebbing of the plasma membrane, condensation of nuclear chromatin, and formation of apoptotic bodies (see Fig. 23.3B). Viral proteins are concentrated in the surface blebs from which budding continues to occur.⁶⁶⁵ This process does not hamper, and may enhance, virus replication because inhibition of apoptosis usually decreases virus yield.^{445,458,699}

The mechanism(s) by which alphaviruses induce apoptosis is not completely understood and likely differs with virus and type of target cell. Apoptosis of cultured cells can be initiated at the endosomal membrane during SINV fusion.³⁶⁴ Membrane-bound sphingomyelinases are activated releasing ceramide, an efficient inducer of cellular apoptosis.^{363,366} SFV-induced apoptosis requires RNA synthesis and accumulation^{777,828} and is

independent of p53.^{258,828} Other events in alphavirus-induced cell death often include early activation of poly(adenosine diphosphate [ADP] ribose) polymerase,^{564,596} activation of pro-apoptotic Bcl-2 family member proteins Bad or Bak,⁵³⁹ loss of mitochondrial membrane integrity, and release of cytochrome c.^{50,828} Cellular caspases are activated with cleavage of caspase-3 substrates and fragmentation of chromosomal DNA.⁸²⁵

Apoptotic death is accelerated by glycoprotein-induced ER stress,^{50,234,574} sphingomyelinase deficiency,⁵⁶⁷ low levels of extracellular Ca^{++} ,⁸²⁵ expression of Bax,⁵⁷⁴ and activation of Bid.⁸²⁸ Alphavirus-induced apoptosis can be slowed or prevented by expression of ceramidase,³⁶³ altered Ras signaling,^{365,413} expression of p21^{WAF1/CIP1},³³⁸ expression of Bcl-2 family member and interacting proteins,^{270,449,451,458,539,552,574,699,826,828} expression of the ER stress protective protein Parkin,⁵⁷⁴ mutation of nsP2,^{246,574} phosphorylation of PKC δ ,⁹¹⁰ inhibition of constitutive expression of NF κ B,⁴⁶² and caspase inhibition.^{565,695,828}

Alphavirus-induced vertebrate cell death can also occur by caspase-independent, nonapoptotic mechanisms. Alphaviruses efficiently shut down protein, ribosomal RNA (rRNA) and mRNA synthesis in infected cells,^{246,264,510,556} deplete nicotinamide adenine dinucleotide (NAD) and energy stores,^{199,825} and induce dysfunction of Na^+K^+ adenosine triphosphatase (ATPase) causing loss of membrane potential and changes in intracellular cation concentrations.^{51,827} Genome replication without structural protein synthesis can induce cell death.⁸²⁸ For Old World viruses, expression of nsP2 alone is cytotoxic, and this property is independent of its protease activity and can be separated from the effects of nsP2 on host transcription.^{187,233,246,247,264,507,605,777} Although immature neurons die by apoptosis, mature neurons are more resistant to apoptotic cell death.^{257,307} This resistance is attributable to an intrinsic ability to suppress virus replication.^{120,835} Mature motor neurons, infected by virulent strains of virus, die by a necrotic process and are not protected from death by Bcl-2 family member proteins.^{307,393} Autophagic clearance of viral proteins may promote neuronal survival.⁵⁸⁸

Persistent infection can occasionally be established in mammalian cell cultures *in vitro*. Mouse fibroblasts producing interferon (IFN), or BHK cells with a high concentration of defective interfering particles, can establish SINV persistent infection.^{345,869} Infection with SINV or SINV replicons that have mutations in the C-terminal methyl transferase-like domain of nsP2 results in reduced viral RNA synthesis, decreased CPE, and persistent infection in some vertebrate cell lines.^{7,187,233,235,507,605} Persistent infection can also be established if the cell infected is resistant to virus-induced apoptosis.^{90,449,824,835}

Effects on Invertebrate Cells

Studies of alphavirus infection of cell lines derived from *Aedes albopictus* (e.g., C6/36, U4.4) and *Aedes aegypti* (e.g., Aag2) larvae demonstrate differences in alphavirus replication between vertebrate and invertebrate cells. The time course of virus replication is similar; however, frequently, virus matures within vesicular structures and virions are released by exocytosis rather than at the plasma membrane.^{523,763} Virions are relatively deficient in cholesterol and have detectable differences in structure.^{296,310,754} There is only a modest effect on host gene expression,^{225,696} and persistent noncytopathic infection, or death by a nonapoptotic process,³⁸³ is more likely than in vertebrate cells.

The uncloned lines derived from *Ae. albopictus* larvae contain many types of cells with properties representative of different mosquito tissues. Lytic infection occurs in clones that support high levels of virus replication,^{523,803} whereas persistent infection is associated with a short period of relatively high virus replication followed by a decrease in virus production and often in the numbers of cells in the culture that are producing virus.^{164,547,548} The decrease in virus production is not associated with activation of the signal transducer and activator of transcription (STAT), immune deficiency (IMD), or toll insect innate response pathways but is associated with decreased processing of the nonstructural polypeptide and expression of the protease inhibitor TEPII.^{225,547} Lytic infection can also be induced by viruses engineered to express death-inducing insect proteins such as reaper or michelob_x.⁸⁴⁹ Transcriptional analysis of persistently infected cells shows an increase in mRNAs associated with vesicle formation and the Notch signaling pathway.⁵⁴⁷

RNA interference (RNAi) is an important insect defense mechanism, and SFV infection of U4.4 or Aag2 cells leads to production of virus-derived small interfering RNAs (viRNAs) derived from replicative double-stranded (dsRNA) that are unevenly distributed across the genome and variable in efficiency for mediating antiviral RNAi.⁷³⁶ This RNAi signal can spread from cell to cell and inhibit replication.³⁷ The RNAi pathway is defective in C6/36 cells⁷⁴; however, the IMD, and not the toll innate response pathway, can suppress SINV replication in these cells.⁴¹

Superinfection Exclusion

Vertebrate and invertebrate cells infected with one alphavirus cannot be productively infected with the same, or a closely related, alphavirus at a later time. Exclusion is established after translation of the nsP genes of the first virus to enter. The superinfecting genome can be translated but not replicated,⁷⁶² probably owing to the presence of the transacting nsP2 protease that prematurely cleaves the replicase polyprotein required for minus-strand synthesis.³⁸⁴

Antigenic Composition

All alphaviruses are related and share common antigenic sites, as revealed by HI and complement fixation (CF) tests with polyclonal immune sera¹⁰⁷ and by cytotoxic T-cell lysis of infected cells.^{465,550} Antigenic cross-reactivities may confer some cross protection and interfere with sequential alphavirus immunizations.^{106,181,277,465,508} These cross-reactivities formed the basis for the original classification into the group A arboviruses and continue to be a valuable means for initial identification and classification of alphaviruses.¹⁰⁷ Closely related viruses within a serogroup form a complex. Seven broad antigenic complexes have been identified within the alphavirus serogroup: Barmah Forest, eastern equine encephalitis (EEE), Middelburg (MID), Ndumu (NDU), Semliki Forest, Venezuelan equine encephalitis (VEE), and western equine encephalitis (WEE).^{427,626,806} A few viruses remain unclassified and will probably form one or more additional complexes (see Table 23.1). The Barmah Forest, EEE, MID, and NDU complexes each contain only a single virus, whereas the Semliki Forest, VEE, and WEE complexes include several viruses. Viruses within each complex can be subtyped using reactivity with MAbs, kinetic HI, or neutralization assays.⁹⁸

Antibodies to E1 are more likely to cross-react with other alphaviruses than are antibodies to E2.^{67,343,892} This is

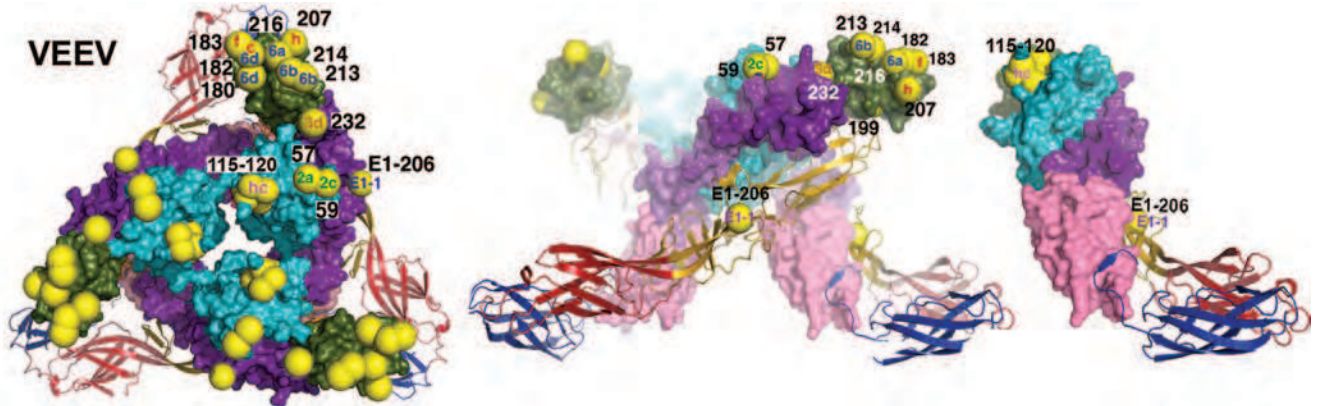


FIGURE 23.4. Neutralization escape mutations and positions affecting host range and tissue tropism mapped on the Venezuelan equine encephalitis virus spike. Neutralizing antibody escape mutations are displayed as yellow spheres on the spike with epitope written in the spheres. E1 is shown in ribbons and E2 in surface rendering using the colors defined in Figure 23.2. The **left panel** shows a top-down view of the spike, the **middle panel** shows the spike from the side, and the **right panel** shows the far right dimer from the middle panel. (Courtesy of Felix Rey; reproduced with permission from Voss JE, Vaney MC, Duquerroy S, et al. Glycoprotein organization of chikungunya virus particles revealed by x-ray crystallography. *Nature* 2011;468:709–712.)

consistent with the documented sequence conservation in the E1 protein. Competitive binding assays using MAbs have identified approximately seven epitopes on the E1 glycoproteins of SINV, SFV, WEEV, and VEEV.^{67,343,519,662,703} Most E1 epitopes are not exposed on the virion surface but are present on the surface of infected cells or on acid-exposed virions.^{15,282,320,519,703} These transitional epitopes map to domain III in a region buried at the spike interfaces⁸⁴⁰ (Fig. 23.4). The *in vitro* biological activities of antibodies to E1 include HI, neutralization of virus infectivity, and inhibition of fusion.^{15,67,282,343,703} Neutralizing epitopes map to domains I, II, and III.^{282,455}

Antibodies to E2 are usually alphavirus specific. Competitive binding assays using MAbs identify four to five epitopes on the E2 glycoproteins of SINV, SFV, RRV, and VEEV.^{67,394,519,586,662} *In vitro* biological activities of antibodies to E2 include HI, neutralization of virus infectivity, and blocking of virus binding to the cell surface.⁶⁶² Many anti-E2 MAbs have both neutralizing and HI activity, suggesting that these functions overlap.²⁸² Neutralization escape mutants, naturally occurring variants, λ gt11 expression libraries, site-directed mutagenesis, and recombinant viruses have been used to identify amino acids contributing to the various epitopes on E2 and have identified two major neutralizing sites^{704,758,851} that have been mapped onto the crystal structure of the E1-E2 heterodimer and trimerized spike and are in domain B.^{8,282,367,455,840} (see Fig. 23.4). This is an exposed hydrophilic region that often includes an N-linked carbohydrate. There are linear, as well as conformational, determinants in this region because these MAbs frequently react in Western blots and recognize λ fusion proteins, and antibodies to peptides from this region are protective against challenge.⁸⁵¹

The second neutralizing epitope on E2 appears to be primarily conformational and is in domain A. This region is responsible for binding to HS and is obscured if pE2 is not cleaved.^{96,675,840} Visualization by cryo-electron microscopy of the binding of HS and MAbs to this epitope on SINV and RRV identifies the domain A knob on the glycoprotein spike.^{742,902}

Evolution and Phylogeny

Alphaviruses, which replicate in arthropods, birds, reptiles, fish, and mammals, derive from a single unknown protoalphavirus and are part of the alphavirus superfamily of viruses. Viruses in this superfamily, including many RNA plant viruses, have a similar genetic organization and replicase proteins but diverse coat proteins.^{417,766} Among alphaviruses, amino acids important in secondary structure (e.g., cysteines and those close to one another in adjacent β sheets and α helices) have been conserved for the glycoproteins E1, E2, and E3, consistent with a similar three-dimensional structure of the virion for all.²¹⁹ The most variable regions of the genome are in the C-terminus of nsP3 and the N-terminus of C.²¹⁹ Highly conserved regions in the nsP1 and nsP4 genes have allowed development of primers to detect a broad range of alphaviruses by reverse transcription polymerase chain reaction (RT-PCR).^{201,290} Sequence information from the entire genome generally groups the viruses similarly to that derived by antigenic analysis (Fig. 23.5) and has detected at least one recombination event.²⁹⁷ Criteria for species demarcation of alphaviruses combine genetic, ecological, and antigenic information. Species generally have distinct transmission cycles and differ by more than 23% at the nucleotide level and 10% in amino acid sequence when E1 genes are compared.

The origin of the alphaviruses is unclear. Partial genome sequencing has suggested origins both in the Americas and in the Old World.^{107,267,434,626,859} Recently, comparison of whole genome sequences from all known alphaviruses has suggested an origin from the louse-borne aquatic alphaviruses⁵¹⁹ (see Fig. 23.5). All of these scenarios require repeated movement across the globe to explain the current virus distributions.

Like other RNA viruses, alphaviruses undergo genetic change primarily by accumulation of point mutations in the genomic RNA; however, deletions and duplications also occur.^{1,217} Mutation occurs at a rate that is slower ($1\text{--}7 \times 10^{-4}$ substitutions/nucleotide/year) than is estimated for other RNA viruses,^{93,146} presumably because fitness must be maintained in both insect vectors and vertebrate hosts.¹⁴⁷ Recombination

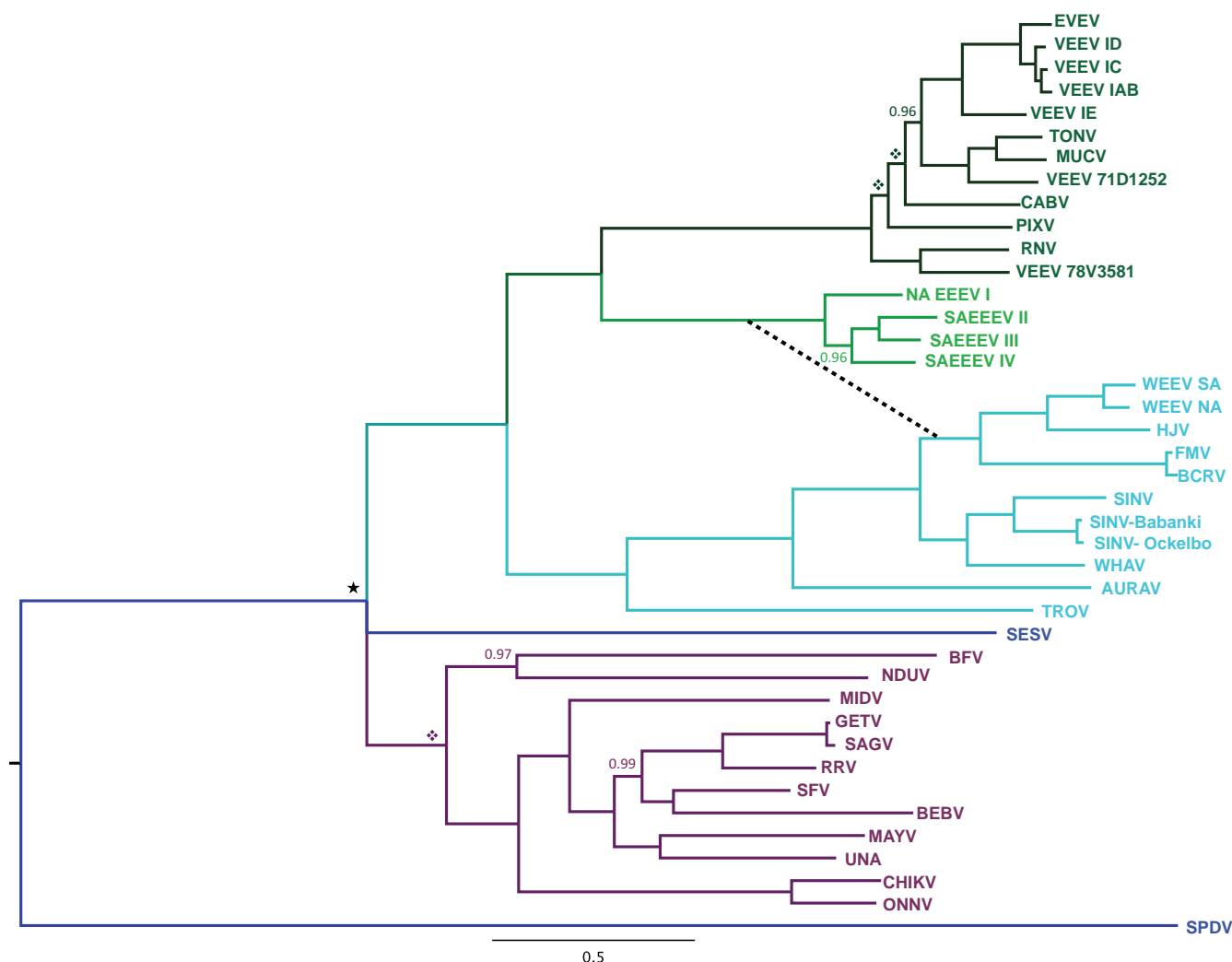


FIGURE 23.5. Alphavirus phylogenetic tree produced using the E2, 6K, and E1 structural protein genes and Bayesian methods with midpoint rooting. The tree includes representatives from all alphavirus species, and the *dashed line* indicates the recombination between ancestors of Sindbis and eastern equine encephalitis viruses that led to the western equine encephalitis virus (WEEV) group. Roman numerals indicate major subtypes of some species, and scale indicates 50% nucleotide sequence divergence. All posterior probabilities were 1 except as indicated; nodes with a diamond had posterior probabilities less than 0.9, and nodes with a star had no posterior support. A similar tree utilizing full-length sequences without the WEEV recombinant group showed a similar topology except Middelburg virus was basal to chikungunya and o'nyong-nyong viruses, and Una, Mayaro, Semliki Forest, and Bebaru viruses were grouped separately together with Getah, Sagiya, and Ross River viruses.²¹⁹ (Courtesy of Scott Weaver; from Forrester NL, Palacios G, Tesh RB, et al. 2012. Genome-scale phylogeny of the alphavirus genus suggests a marine origin. *J Virol* 2012;86(5):2729–2738.)

between alphaviruses can be demonstrated *in vitro* but is infrequent and usually puts the chimeric virus at a replicative disadvantage.^{93,146} However, successful recombination has occurred at least occasionally in nature because WEEV resulted from recombination between EEE- and Sindbis-like viruses (see Fig. 23.5) at the junction of E3 and E2, an event that is estimated to have occurred thousands of years ago.^{219,421,766}

Alphaviruses replicate in and are transmitted horizontally by a wide range of invertebrate, primarily mosquito, species. However, each virus usually has a principal or preferred vector for the enzootic cycle. Most alphaviruses can infect various vertebrates but have birds, mammals, or fish as their primary amplifying and reservoir hosts (see Table 23.1). The specific

invertebrate vector and vertebrate host used by an alphavirus will contribute significantly to determining the geographic distribution of that virus. Experiments modeling evolution *in vitro* show that fewer mutations accumulate if replication alternates between vertebrate and invertebrate cells, although diversity, fitness, and adaptability are greater with serial passage.^{149,150,153,276,856} Experiments employing *in vivo* passage show that serial passage in mosquitoes increases mosquito infection and that passage in vertebrates produces higher viremias; however, alternately passaged viruses do not change fitness.¹⁴⁹ It is hypothesized that short transmission seasons and host mobility influence alphavirus genetic diversity and evolution in a geographic region.^{146,858} Viruses using avian enzootic hosts

(e.g., EEEV, WEEV, SINV) extend over wide geographic regions and evolve as a few highly conserved genotypes, whereas viruses using mammalian enzootic hosts with a more limited range of dispersal (e.g., RRV, VEEV) evolve within multiple geographically restricted genotypes.^{490,688}

Some strains of alphaviruses associated with epidemics or epizootics are antigenically and biologically distinguishable from enzootic strains. Phylogenetic evidence indicates that, at least for VEEV and WEEV, the virulent epizootic strains evolve by mutation from avirulent viruses being maintained in the enzootic cycle.^{23,61,629,657}

PATHOGENESIS AND PATHOLOGY IN VERTEBRATES

Excellent and well-studied model systems exist for several alphaviruses, and much of our detailed knowledge about alphavirus pathogenesis comes from investigations in mice. Information from these models will be combined where appropriate with information from studies of humans with alphavirus-induced disease to deduce the pathogenesis of infection. Specifics will be covered in sections on the individual viruses.

Entry

The primary mode of alphavirus transmission to vertebrates is through the bite of an infected insect, most often a mosquito. Mosquitoes salivate during feeding and deposit virus-infected saliva extravascularly.⁸¹⁹ Saliva virus titers are highest early after the mosquito is infected and decline, along with transmission rates, after 1 to 2 weeks; however, mosquitoes remain infected for life.^{525,833} The high-mannose glycans on virus from mosquitoes inhibits induction of IFN by myeloid DCs,⁷¹⁸ and proteins in saliva further facilitate transmission by skewing the host cellular immune response toward Th2 cytokines.⁷⁹⁴

Sites of Primary Replication

The initial sites of virus replication vary with the virus and host. Mice have received the most extensive study. After subcutaneous inoculation, viruses may infect skeletal muscle or fibroblasts at the local site (e.g., EEEV, WEEV, SFV, RRV, SINV, and Getah virus) or be taken up by and infect Langerhans cells in the skin (e.g., VEEV)^{287,325,466,553} (Fig. 23.6). Langerhans cells and DCs transport virus to lymph nodes draining the site of inoculation that also may become infected.^{242,487} *In vitro*, human DCs are susceptible to infection with VEEV but not to infection with CHIKV or EEEV^{241,573,702,707,753}; thus, the importance of DC infection after mosquito inoculation is likely to differ with the infecting virus.

Spread

Alphaviruses induce a substantial plasma viremia in their amplifying hosts and in hosts susceptible to disease (Fig. 23.7). The ability to mount and sustain a viremia depends on the continued efficient production of virus, delivery of virus into the vascular system, and slow clearance from the blood. Animal studies have shown that small plaque viruses are generally less virulent because they are cleared more rapidly from the circulation than are large plaque viruses.^{354,360,623} This phenomenon is related to the ability of small plaque viruses to bind HS and

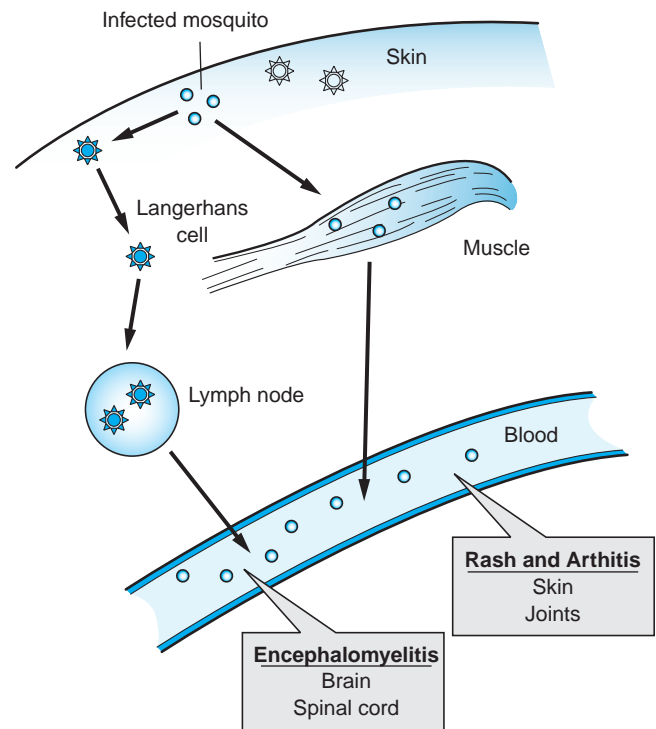


FIGURE 23.6. Basic steps in alphavirus infection of vertebrates. Virus is delivered extravascularly by an infected mosquito and infects local muscle cells or Langerhans cells in the skin. Langerhans cells can carry virus to local lymph nodes, where further replication may occur. Virus is delivered to the blood and spreads to target tissues such as skin, joints, and the central nervous system, in addition to distant muscle and lymphatic tissue.

thus to be rapidly removed from the circulation by the highly sulfated glycosaminoglycans in the liver.⁹⁷ Ability to invade target organs depends in part on the duration and height of the viremia but also on other characteristics of the virus important for tissue invasion.⁴⁸⁴

Cell and Tissue Tropism

Viruses that replicate initially in skeletal muscle and lymph nodes near the site of inoculation often spread through the bloodstream to more distant skeletal muscles and other lymphatic tissues. In addition, cardiac myocytes, osteoblasts, brain and spinal cord neurons, and brown fat cells are secondary sites of replication for many alphaviruses in mice^{9,466,535,554} (see Fig. 23.6). Getah virus causes polymyositis.^{325,554,713} EEEV, WEEV, SFV, and SINV cause encephalitis^{44,242,350,466,838}; RRV and CHIKV cause myositis and arthritis^{244,543,907}; and VEEV causes lymphoid depletion and encephalitis.^{260,359,845}

In humans, the skin is a target for alphaviruses that cause a rash,^{230,493} the joints for alphaviruses that cause arthritis,^{229,328,543,772} muscle for alphaviruses that cause myalgia,^{543,590,907} and the nervous system for alphaviruses that cause encephalitis.^{245,760} RRV and SINV have been recovered from skin biopsies.^{423,424,493} RRV replicates in skin basal epidermal and eccrine duct epithelial cells.²³⁰ CHIKV is found in fibroblasts and macrophages.^{328,428} Human synovial cells support RRV infection *in vitro*,³⁷³ and RRV RNA is detected in synovial biopsy specimens.⁷⁴⁷ Joint fluid taken from humans with acute arthritis has

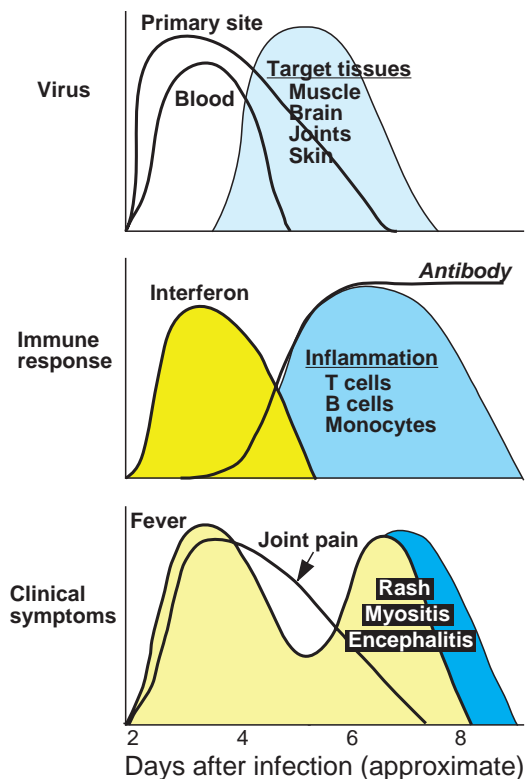


FIGURE 23.7. Schematic diagram of the pathogenesis of alphavirus-induced disease. Viremia may be accompanied by production of interferon, other proinflammatory cytokines, and fever. Virus then spreads through the blood to other target tissues. As the immune response is induced, the viremia is terminated; however, fever is renewed with appearance of a mononuclear inflammatory response in the infected tissue. In infections that lead to rash and arthritis, joint pain usually appears early after infection and prior to the appearance of the rash.

not yielded infectious virus; however, viral antigen and RNA can be detected in macrophages, and these cells support RRV and CHIKV replication *in vitro*.^{328,373,854}

The mechanism by which encephalitic alphaviruses enter the CNS is not entirely clear. Neuroinvasiveness is a component of virulence that varies between viruses and virus strains.¹⁸⁸ Murine studies have shown infection or transport by cerebrovascular endothelial cells,^{396,602,748} infection of choroid plexus epithelial cells,⁴⁶⁶ infection of olfactory neurons,^{133,670,792,837} and transport by peripheral nerves.¹⁵² Once within the CNS, virus can spread cell to cell or through the cerebrospinal fluid (CSF).^{349,584,792} For most encephalitic alphaviruses, the targeted cell within the CNS is the neuron,^{350,466,584} where cellular protein synthesis is suppressed⁸⁰⁴ and damage can be severe and irreversible. In mice that recover from neuronal infection, infectious virus is cleared but viral RNA persists.^{223,446,521,821} SFV, RRV, and VEEV can cause persistent infection of microglial and oligodendroglial cells leading to demyelination.^{94,160,226,715}

Immune Responses

Innate Responses

Early responses to alphavirus infection include production of cytokines and chemokines and activation of NK cells.²⁷¹

Type I (α/β) IFN is induced *in vivo* after many, but not all, alphavirus infections of experimental animals^{242,255,358,721,807} and humans^{328,677,702,853} (see Fig. 23.7). The amount of IFN produced by infected tissues is usually linked to the level of virus replication, and IFN production continues if virus is not cleared.^{91,224,294,328,358,702} IFN rapidly appears in serum, and levels are diminished by splenectomy, suggesting that lymphoid tissue is one important source.^{358,415,416}

Several different cell systems have been used to determine the mechanisms by which alphaviruses induce and control synthesis of IFN in response to infection. Viruses vary in their ability to induce IFN production by different types of cells,^{623,702,721} and the cellular sources of IFN probably differ with time after infection. In tissue culture cell lines, IFN production, shut-off of host protein synthesis, and CPE are controlled either by nsP2 (SINV, SFV) or C (VEEV, EEEV).^{14,81,235,246,247} In these cells, shutoff of host gene expression suppresses antiviral responses.^{14,81,247,894}

The primary source of early IFN *in vivo* may be plasmacytoid DCs for some alphaviruses⁷³⁰ but not for others.⁷⁰² SFV induction of IFN by myeloid DCs requires fusion but not replication, and it is independent of MyD88.³²² For SINV and RRV, N-linked glycans on E2 are important determinants of IFN production by DCs.⁷¹⁹

Induction of IFN requires viral entry and RNA synthesis.^{57,322,881} Data from *ts* mutants suggest that formation of dsRNA is the necessary step in replication for IFN induction. Viruses with mutations in the protease domain of nsP2, which cannot process the nonstructural polyprotein and thus cannot initiate plus-strand RNA synthesis, do not induce IFN.^{298,497} In infected fibroblasts and epithelial cells, dsRNA and higher-ordered RNA structures activate MDA5 and PKR, which are important intracellular sensors of alphavirus infection,⁴⁹ and stimulate phosphorylation of interferon regulatory factor (IRF) 3^{610,706} and formation of the IRF3/CBP/p300 transcriptional activation complex for immediate early IFNs.⁵⁷ This process is independent of mTOR pathway activation.¹⁷⁵ NsP1/nsP2 cleavage affects IRF3 activation and IFN induction by SINV without affecting shutoff of host transcription or translation.¹⁵⁸ Virulent strains of EEEV do not induce IFN after fibroblast infection, whereas attenuated strains do, which is potentially associated with decreased binding to HS and increased infection of lymphoid tissue.^{242,243}

Production of IFN follows the initial release of virus from infected cells by 2 to 3 hours³²⁷ and for some, but not all, alphavirus strains is regulated by nuclear importation of nsP2 and by whether host protein synthesis is shut off before IFN can be synthesized.^{81,92,881} The ability of CHIKV to induce IFN mRNA and protein is cell type dependent.⁷⁰² Primary human monocytes infected by CHIKV produce IFN- α , interleukin (IL)-6, and IL-12³¹⁹; however, similarly infected primary human fibroblasts produce IFN- β mRNA but no protein, because the mRNA is not translated.⁸⁸¹

IFN is an important part of the host response to alphavirus infection, and virus replication is sensitive to its effects.^{12,155,172,441,680,718,753,787,905} Animals can be protected from lethal infection if treated with IFN or IFN inducers before or soon after infection.^{12,358,375,477,714} Animals unable to respond to IFN owing to deletions of the IFN receptor or crucial IFN signaling molecules develop more severe disease than wild-type mice.^{12,95,155,157,210,224,279,679,702,756,880} Furthermore, absence

of an IFN response allows virus replication in cells previously resistant to infection.^{224,678,679,810} IFN appears to act primarily to limit virus replication early, during the time the specific immune response is being induced (see Fig. 23.7). Treatment of cells with IFN inhibits alphavirus replication^{90,172,358,540,551,681}; however, the mechanism by which this occurs, and therefore the IFN-induced host responses important for control of replication, are not known. Attachment and entry are not affected,^{172,641} although later replication steps, including formation of replication complexes, structural protein synthesis, and morphogenesis, are inhibited.^{551,641,787}

In addition to inhibiting host cell protein synthesis, alphaviruses can also interfere with IFN signaling by decreasing Janus kinase (JAK) activation and STAT phosphorylation and nuclear translocation.^{236,733,894} This is a property of either nsP1 or nsP2.^{236,734}

Antiviral proteins PKR and RNase L have a limited role in the IFN-induced antiviral response *in vitro* and *in vivo*.⁶⁸¹ However, activation of PKR improves the stability of IFN mRNA.^{49,706} Interestingly, infected ribonuclease (RNase) L-deficient fibroblasts fail to shut off minus-strand RNA synthesis or form stable replication complexes and establish persistent infection, suggesting a role for RNase L in virus replication.⁶⁹⁸

IFN-induced proteins that can inhibit alphavirus replication include human MxA,⁴³² zinc-finger antiviral protein (ZAP),^{63,292,392,435,488,905} viperin,⁹⁰⁵ the large form of 2',5'-oligoadenylate synthetase,⁸² interferon-stimulated gene (ISG) 20,⁹⁰⁵ and ISG15.^{440,441} Transgenic expression of MxA, a large cytoplasmic guanosine triphosphatase (GTPase), in IFN- α/β receptor-deficient mice results in decreased SFV replication by preventing accumulation of genomic and subgenomic RNA and provides some protection against fatal disease.³¹¹ ZAP is an RNA-binding protein that prevents accumulation of viral RNA by blocking translation of incoming viral genomic RNA.^{63,291} This is accomplished by binding to specific viral mRNA sequences and interaction with the host DEAD box helicase p72 and RNA-processing exosome.^{135,292} ISG15 is an ubiquitin-like molecule that exerts its antiviral effect by conjugating proteins, although the mechanism for suppression of virus replication remains unknown.²⁵¹

Viruses and virus strains vary in their sensitivity to the antiviral activities of IFN, and this may or may not correlate with virulence.^{24,68,174,756,894} Mutations associated with altered sensitivity to IFN have been mapped to the 5' nontranslated region (NTR), nsP1, and nsP2.^{235,667,756,880}

IFN may also contribute to alphavirus-induced disease. Fever during the viremic phase of infection, as is seen with CHIKV and RRV, is probably a response to the IFN induced early after infection (see Fig. 23.7). It has been postulated that the rapidly fatal disease induced by alphaviruses in newborn mice may be owing to the production of large amounts of IFN and pro-inflammatory cytokines.⁸⁰⁷ Acute-phase responses induced by alphaviruses prior to the virus-specific immune response include up-regulation of toll-like receptor expression and increases in tumor necrosis factor (TNF), IL-1, and IL-6, and levels generally correlate with the extent of virus replication.^{278,512,702,807,874} Adult mice deficient in IL-1 β have reduced mortality after CNS infection with a neurovirulent strain of SINRV, again suggesting that cytokine effects may contribute to mortality.⁴⁵⁷

Virus-Specific Adaptive Responses

Both cellular and humoral immune responses are induced by infection (see Fig. 23.7). In experimentally infected adult mice, antiviral antibody is usually detected in serum within 3 to 4 days after infection.^{280,592,713} The cellular immune response, manifested by the presence of virus-reactive lymphocytes in draining lymph nodes and blood and the infiltration of mononuclear cells into infected tissues, also appears within 3 to 4 days after infection.^{283,509} These responses appear later (7–10 days after infection) in neonatal mice.⁷²¹ Both appear to play a role in recovery from infection and protection against reinfection.

HUMORAL IMMUNITY

Virus-specific immunoglobulin M (IgM) antibody is detectable very early in human disease, often provides a means for rapid diagnosis of infection, and can persist for many months after recovery.^{69,99,103,112,115,288,424,495,571} Virus-specific immunoglobulin A (IgA) also appears early in infection but declines rapidly.¹¹⁴ Immunoglobulin G (IgG) antibody is present in serum after 7 to 14 days and is maintained at relatively high levels for years.^{101,183,396} Many lines of evidence support the hypothesis that recovery from alphavirus infection depends in large part on the antibody response.^{91,284,906} Rapidity of antibody synthesis is predictive of outcome from encephalitis because patients without evidence of antibody at the time of illness onset are most likely to die.⁹⁹ Antibody can neutralize virus infectivity and promote virus clearance by the reticuloendothelial system in conjunction with complement.³⁵⁶ Appearance of antibody correlates with cessation of viremia (see Fig. 23.7).

The most extensive experimental studies to define the antibody specificity and the mechanisms of antibody-mediated recovery and protection have been done using VEEV, SFV, and SINRV infection of mice. Passive transfer of antibody before or after infection is protective. Both neutralizing and nonneutralizing MABs against multiple epitopes on the E1, E2, and E3 glycoproteins can protect against alphavirus challenge and promote recovery.^{65,66,289,343,502,519,597,757,892} There is a correlation of protection with the ability of the MAB to bind to the surface of infected cells, although this is not absolute.⁷⁵⁷ Protection requires intact bivalent antibody but does not require complement.^{323,503} However, virus clearance from blood is delayed in complement-deficient mice.³²⁴

Treatment of immune deficient mice persistently infected with SINRV or SFV with antiviral antibody clears infectious virus from the CNS without causing neurologic damage.^{21,448} Clearance of infectious virus is rapid, whereas the decline in viral RNA occurs more slowly.^{448,521} E2-specific MABs can down-regulate intracellular virus replication *in vivo* and *in vitro* by a nonlytic mechanism.⁴⁴⁸ Antibody against an N-terminal peptide of VEEV E2 that is not neutralizing can limit virus replication *in vivo*,³⁴² and a nonneutralizing MAB to SFV E2 can limit virus replication *in vitro*.⁶⁶ Anti-E3 MABs inhibit production of VEEV.⁵⁹⁷ Anti-E1 MABs may also be able to alter intracellular virus replication.¹²⁸

In vitro studies show that the process by which antibody alters intracellular virus replication requires bivalent antibody but does not require the Fc portion of the MAB, complement, or other cells^{448,824}; however, the effects of antibody are amplified by treatment of infected cells with IFN- α .¹⁷² Soon after antibody binding, virion budding from the plasma membrane is inhibited.¹⁷³ *In vivo* studies also show that IFN and antibody act

synergistically to promote recovery, although the mechanisms by which these systems interact have not been identified.^{95,154}

After recovery from encephalitis, viral RNA remains detectable in the CNS for life. Therefore, one consequence of a nonlytic mechanism for clearance of virus from tissue is that the virus genome is not completely eliminated if the originally infected cells survive.^{446,447} This leads to a need for long-term control of virus replication that is accomplished in part by infiltration of antibody-producing B cells into the CNS.^{281,286,521,821}

Antibody also is important for protection from infection.⁵³⁸ Inactivated vaccines protect against EEE, VEE, and WEE.^{636,638} Delivered before or shortly after infection, passive transfer of antibody can protect from acute fatal disease but may predispose to late disease.^{284,399,712}

CELLULAR IMMUNITY

Alphavirus infection induces virus-specific lymphoproliferative, cytokine, and cytotoxic T-cell responses.^{4,283,385,498,531} Cytokines increased in plasma during acute disease include IL-4, IL-6, IL-10, IL-12, IL-13, and IFN- γ .^{328,853} After epidermal virus inoculation, Langerhans cells increase expression of MHC class II antigens and accessory and costimulatory molecules that enhance activation of naive T cells.³⁷⁰ The mononuclear inflammatory process in alphavirus encephalitis is immunologically specific⁵⁰⁹ and includes infiltration of NK cells, CD4⁺ and CD8⁺ T lymphocytes, B cells, and macrophages.^{346,521,529,541} Relative proportions of these mononuclear cells vary with the time after infection.^{346,521,529} T cells play a role in virus clearance and in protection from challenge.^{200,897} Viral RNA levels in the CNS of SINV-infected mice decrease more rapidly when CD8⁺ T cells are present.⁴⁰⁰ Mice lacking the ability to produce antibody can clear infectious virus from some populations of neurons through production of IFN- γ ,^{64,84} and IFN- γ down-regulates SINV replication in mature neurons *in vitro* through a JAK/STAT-dependent mechanism.^{89,90} In animals infected with virulent strains of virus, cellular immune responses contribute to tissue damage and fatal disease.^{273,399,563,669}

Pathologic Changes

Encephalomyelitis

Pathologic changes in the CNS of humans with fatal neurologic disease and mice with experimentally induced encephalomyelitis begin with perivascular infiltration of mononuclear and polymorphonuclear inflammatory cells.^{509,529,577} Adhesion molecules (e.g., ICAM-1, VCAM-1) are up-regulated on endothelial cells and integrins LFA-1 and VLA-4 are important mediators of mononuclear cell entry.^{347,741,750} This phase of infection may include extravasation of red blood cells and endothelial cell swelling and hyperplasia.⁵⁷⁷ Lymphocytes and monocytes move from the perivascular regions to areas of the parenchyma with virus-infected neurons. This inflammatory process is accompanied by gliosis and evidence of inflammatory and glial cell apoptosis.²⁴⁵

Neonatal mice and human infants may die with widespread virus-induced neuronal cell death before the inflammatory process—a manifestation of the cellular immune response—can be initiated.⁵⁵⁵ Immature neurons die by an apoptotic process,⁴⁵² whereas death of mature neurons may be characterized by cytoplasmic swelling, vacuolation, membrane breakdown, and cellular degeneration suggesting necrosis.^{245,307,555} Demyelination has been described as a consequence of EEEV

and WEEV infection in humans^{52,576,577} and of WEEV, RRV, and SFV infection of mice, probably as a result of infection of oligodendrocytes.^{94,532,715}

Reticuloendothelial Infection

The pathology of VEE in horses includes cellular depletion of bone marrow, spleen, and lymph node tissue, and pancreatic necrosis.⁴⁰⁵ Small mammals also develop widespread infection of reticuloendothelial system tissues and may develop ileal necrosis.^{40,266,845} Leukopenia is commonly observed during human infection.⁵³⁴

Arthritis

In CHIKV- and RRV-induced arthritis, there is hyperplasia of the synovial lining, vascular proliferation, and mononuclear cell infiltration.^{328,747} Synovial fluid contains increased protein, CD4⁺ T lymphocytes, activated NK cells and macrophages, and increased levels of monocyte chemotactic protein (MCP)-1/CCL2, IL-6, and IL-8.^{145,229,328} Persistent infection is suggested by the presence of RRV RNA 5 weeks after onset of symptoms⁷⁴⁷ and CHIKV antigen and RNA in synovial macrophages 18 months after acute disease in a patient with chronic arthritis.³²⁸

Rash

Skin biopsies taken from patients with RRV-induced rash show perivascular infiltration of lymphocytes (primarily CD8⁺ T cells) and monocytes without evidence of immune complex deposition.²³⁰

Release and Transmission

A common feature of alphaviruses is their transmission by insects and maintenance in a natural cycle of replication in vertebrate and invertebrate hosts. Arthropod vectors become infected by feeding on a viremic host, are able to transmit the virus 4 to 10 days later (external incubation), and remain persistently infected. Maintenance of this cycle requires an amplifying host that develops a viremia of sufficient magnitude to infect feeding mosquitoes. For many alphaviruses, humans are dead-end hosts unable to infect mosquitoes efficiently. However, human-mosquito-human transmission has been important in epidemics of RRV, o'nyong nyong virus (ONNV), and CHIKV-induced polyarthritis,^{478,689,790} and horse-mosquito-horse transmission is important in epizootics of VEE.^{659,771}

Other modes of transmission are occasionally important. Horses infected with VEEV may shed virus in nasal, eye, and mouth secretions, as well as in urine and milk, resulting in the potential for transmission by the respiratory route.^{118,405} Aerosol transmission of VEEV, CHIKV, and Mayaro virus has occurred in laboratory settings,^{118,378,439,788,799} and aerosolized VEEV has been developed as an agent of biological warfare.⁷²⁸ EEEV persists in the feather follicles of infected pheasants, and transmission among penned pheasants can occur through feather picking and cannibalism.⁶⁹⁷ Person-to-person transmission has not been documented.^{439,659}

Veterinary Correlates and Animal Models

WEEV, EEEV, and VEEV—the first alphaviruses to be cultured—came to the attention of virologists because they caused fatal disease in horses; these viruses remain important equine pathogens.^{182,522,786} EEEV and WEEV cause encephalitis

in horses, whereas VEEV causes severe respiratory disease associated with leukopenia; encephalitis may or may not be present. Getah virus causes an urticarial rash and hind leg edema in horses.⁷¹⁷ WEEV, EEEV, and Highlands J virus (HJV) cause disease in domesticated birds such as chickens, pigeons, pheasants, turkeys, and emus.^{42,167,213,221,476,822,868} The alphaviruses associated with arthritis in humans have not been recognized as important causes of disease in domestic animals.

Good small animal models exist for the encephalitogenic alphaviruses but are less satisfactory for study of the arthritogenic alphaviruses. In mice, alphaviruses generally infect lymphatic tissue, muscle, brown fat, brain, and spinal cord; however, the extent and relative importance of infection at these sites differs among these viruses. For instance, RRV and Getah virus cause primarily myositis, VEEV causes reticuloendothelial infection, and WEEV and EEEV cause encephalitis with neurons as the main target cells.^{9,526,554} In mice infected with relatively avirulent strains of SFV, RRV, and VEEV, the acute encephalitic phase is accompanied by infection of oligodendroglial cells and demyelination.^{94,715} For all alphaviruses, fatal disease in mice is usually associated with CNS infection even if encephalitis is not a manifestation of the human infection. For instance, SINV infection of mice is studied as a model for acute viral encephalitis, although SINV causes arthritis and rash, not encephalitis, in humans.^{349,489,820} Specifics of these animal model systems are discussed with the individual viruses.

Virulence

Virulence is a measure of the ability of the virus to cause fatal disease; for alphaviruses, this usually reflects the severity of neurologic disease. Outcome is influenced by characteristics of both the host and the virus. An early virus determinant of virulence is induction of IFN and susceptibility to IFN-mediated inhibition of replication. Viruses that induce IFN and are susceptible to IFN are generally attenuated.^{12,92,158,235,243,734,756,880} Most alphaviruses show an age-dependent susceptibility to disease.^{9,75,294,554,584,713} Resistance increases with maturation and is associated with decreased virus replication in tissues at the site of virus inoculation and in target tissues (e.g., brain), not with changes in induction of IFN or the ability of infected mice to mount a virus-specific immune response.^{280,287,554} The ability of a virus strain to cause fatal disease or a particular complication of infection also often depends on the genetic background of the host.^{186,759,792,814}; however, the specific genetic determinants of susceptibility are just beginning to be identified.⁷⁹³ Avirulent alphavirus strains may replicate poorly even in newborn animals, whereas virulent strains can usually replicate well and cause disease in adult and newborn animals. The role of the response to IFN is unclear, although older mice increase ISG12 whereas young mice do not.⁴²⁹

For encephalitic alphaviruses, another viral determinant of virulence is their ability to enter the CNS efficiently (neuroinvasiveness). Many alphavirus strains can cause fatal disease after intracerebral or intranasal inoculation but not after subcutaneous or intraperitoneal inoculation. The duration of viremia often correlates with virulence with virulent strains sustaining longer viremias than avirulent strains.^{355,357,360} Peripheral replication, viremia, neuroinvasiveness, and neurotropism (ability to replicate in CNS cells) all contribute to virulence.

The viral determinants of virulence have been most extensively studied in murine models for SINV, SFV, RRV, and

VEEV infections. Viruses with altered virulence have been selected after chemical mutagenesis,^{48,85} by passage in tissue culture,^{58,166,395,783} by passage in mice,^{284,517,783} by isolation of MAb escape mutants⁸⁴² or plaque variants,³⁵⁴ and by manipulation of complementary DNA (cDNA) virus clones.^{166,485,779,815} Nucleotide and amino acid changes affecting virulence have been mapped to the 5' NTR, nsP1, nsP2, nsP3, E1, and E2. Virulence determinants in the glycoproteins map to receptor-binding regions of the E2 A and B domains⁸⁴⁰ (see Fig. 23.4). Specifics are covered in the sections dealing with each of these viruses.

Persistence

There is substantial evidence that alphaviruses can persist after appearance of an immune response and clearance of infectious virus from the circulation and from tissue.⁴⁴⁷ Pathologic examination of CNS tissue from human cases of progressive WEEV months to years after resolution of acute encephalitis has shown an active inflammatory process.^{575,577} Viral RNA and proteins can be detected in the nervous system long after recovery of mice from SINV or SFV-induced encephalitis and for several weeks in the joints of humans with RRV-induced arthritis.^{186,285,396,447,521,747,821} It is postulated that this persistence of RNA is attributable to failure of the virus or the immune system to eliminate the infected cells. Interestingly, passive antibody protection predisposes to persistent infection and the late onset of progressive disease.^{399,712}

Congenital Infection

Alphaviruses can be transmitted transplacentally. This has been documented in mice for RRV, SFV, VEEV, and Getah virus.^{3,30,35,524,755} and in humans for RRV, WEEV, VEEV, and CHIKV.^{6,231,380,723} In mice, the virus infects the placenta, where it is able to persist and spread to the fetus despite the development of maternal antibody. The outcome of fetal infection depends on the timing of infection relative to transfer of maternal antiviral IgG to the fetus. Fetuses are protected if transfer occurs prior to infection; however, transfer of antibody after fetal infection does not mediate recovery.⁵²⁴ In monkeys, congenital infection with VEEV induces malformations of the brain and eye.⁴⁷¹ In humans, no abnormalities were observed in infants infected with RRV at 11 to 19 weeks gestation; however, earlier infection may lead to fetal death.⁶ Epidemics of VEE are associated with increases in spontaneous abortion.^{659,862} No effect on pregnancy outcome was identified during the CHIKV outbreak on Reunion Island.²³¹

PATHOGENESIS AND PATHOLOGY IN MOSQUITOES

The ability of alphaviruses to infect mosquitoes efficiently with spread to and replication in the salivary glands is essential for maintaining the natural cycle of transmission. Not all mosquitoes taking a blood meal from a viremic host will become infected, and not all infected mosquitoes develop the ability to transmit virus. Many alphaviruses preferentially infect a narrow range of mosquito species, and this host specificity plays an important role in determining the geographic distribution of the virus. Even within a species, strains of mosquitoes may vary in

susceptibility to infection. *Ae. albopictus* collected from different geographic regions show differences in susceptibility to infection with CHIKV and in the amount of virus produced after infection.⁷⁸⁹ Field and laboratory populations of *Culex tarsalis* differ in susceptibility to WEEV.³³⁴ Strains of virus also differ in their abilities to infect mosquitoes, and laboratory-adapted strains may establish infection relatively inefficiently.^{557,710,711,831,833}

The extrinsic incubation period—or the time between taking an infected blood meal and ability to transmit infection—depends on the rapidity of virus replication and dissemination to the salivary glands. This period is relatively short (2–7 days) for alphaviruses compared to other arboviruses.⁷¹⁰

Entry and Sites of Primary Replication

Posterior midgut epithelial cells are the initial sites of infection,^{77,558,612,708,864} and infection is facilitated when virus in the serum is concentrated next to these cells as the blood meal clots⁸⁶⁶ (Fig. 23.8). Susceptibility of mosquitoes to alphavirus infection is determined in large part by the ability of the virus to infect midgut epithelial cells.^{334,397,710} Changes both in the virus and vector can affect this interaction.⁸⁸⁹ WEEV rapidly fuses with microvillar membrane preparations from *Cx. tarsalis* mosquitoes, and both WEEV and CHIKV bind better to membranes from susceptible mosquitoes than to membranes from refractory mosquitoes,^{334,546} which is consistent with a role for viral structural proteins as determinants of vector specificity, midgut infection, and dissemination.^{612,813,832,833} The high-affinity laminin receptor A is a receptor for VEEV and SINV on the surface of C6/36 larval *Ae. albopictus* cells,^{474,850} and proteins of 60 and 38 kDa have been identified as putative receptors for CHIKV on brush-border membranes from *Ae. aegypti*.⁵⁴⁶

Host cell membrane cholesterol levels affect the efficiency of alphavirus entry.⁴⁷³ Mosquitoes, like other insects, do not synthesize cholesterol and obtain sterols needed for reproduction and development from dietary blood. Cholesterol-independent

mutants of SFV replicate better than parental SFV in cholesterol-depleted C6/36 cells and in adult *Ae. albopictus* mosquitoes.¹⁶

Replication in midgut epithelial cells is regulated by the RNAi innate antiviral response that is triggered by virus-derived dsRNA and small interfering RNAs.^{109,387,397,559} RNAi affects infection rate of the midgut, intensity of infection, and virus dissemination to secondary tissues and is probably an important determinant of vector competence.^{387,397}

Spread

For the mosquito to become capable of transmission, virus must reach the salivary glands and replicate there (see Fig. 23.8). For most alphaviruses, spread beyond the midgut to other tissues is through the hemolymph.⁷¹⁰ Virus buds primarily from the basolateral surface of the infected midgut epithelial cells and accumulates next to the basal lamina,⁷¹⁰ which is a layered structure composed of mucopolysaccharide that acts as a barrier to hemocoel entry. Dissemination of SINV from the midgut of *Ae. aegypti* mosquitoes depends on expression of endosomal proteins UNC93A and synaptic vesicle-2.¹¹⁰ Replication of ONNV in *Anopheles gambiae* is controlled by expression of heat shock protein cognate 70B.^{731,732} Infected midgut epithelial cells often degenerate and slough 36 to 48 hours after infection; this process may facilitate penetration of the virus into the hemocoel.^{710,865}

Infection of salivary gland acinar cells from the hemolymph requires that the virus again traverse a basal lamina—a process that may depend primarily on hemolymph titer. The fat body is an important site for virus amplification.^{855,864} EEEV infects the midgut, fat body, muscle, and salivary glands without involving the nervous system or the ovarioles,^{708,864} whereas VEEV infects the nervous system⁸⁵⁵ and SINV infects respiratory tissue.⁷³

Once salivary gland infection is established, virus matures by budding into apical cavities or randomly into vesicles basolaterally and apically. This process may be associated with cytopathic changes in the salivary glands.⁵²⁵ The rapidity of virus growth and dissemination to the salivary glands depend on the ambient temperature. Higher temperatures accelerate the transmission cycle in warm months.^{650,816} This can be as short as 2 to 3 days.⁷¹⁰ In general, virus content of an individual mosquito reaches a peak within 4 to 7 days after infection.^{525,710} Some tissues (e.g., fat bodies) produce large amounts of virus, and some (e.g., head ganglia) produce small amounts; others (e.g., ovarioles and Malpighian tubules) remain virus negative.⁵²³

For transovarial transmission to occur, virus must be able to infect oocytes early in development. Failure to infect ovarioles precludes efficient transovarial transmission of most alphaviruses.^{73,176,708,855} However, the presence of RRV and CHIKV in field-caught male mosquitoes suggests that vertical transmission can occur.^{463,639,795} Eggs can also be infected after they have been fertilized. Low levels of vertical transmission have been documented in the laboratory for RRV in *Aedes vigilax*, for SINV in *Aedes australis*, and for CHIKV in *Ae. aegypti*.^{506,589} Vertical transmission in nature has been reported for RRV, SINV, and WEEV.^{176,237,463}

Pathology, Persistence, and Host Response

Although generally considered to be benign for the invertebrate vector, infection may reduce survival and reproductive capacity.^{709,710} The IMD pathway of innate immunity can

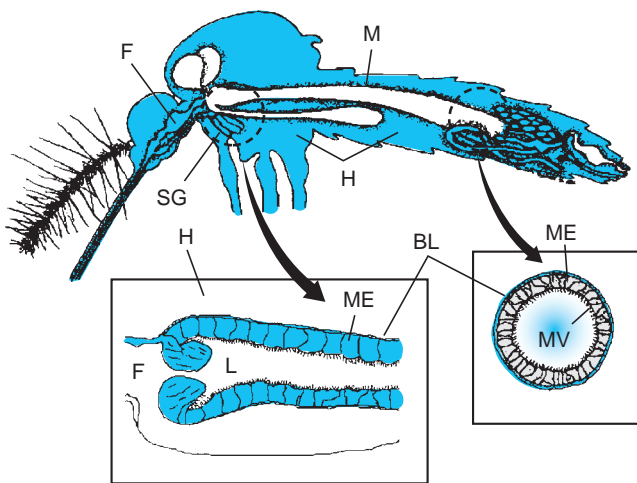


FIGURE 23.8. Diagram of mosquito internal anatomy showing the essential sites for alphavirus infection. BL, basal lamina; L, lumen; F, foregut; M, midgut; ME, midgut epithelium; MV, microvilli or brush border; SG, salivary gland. (Adapted from Weaver SC, Scott TW, Lorenz LH, et al. Togavirus-associated pathologic changes in the midgut of a natural mosquito vector. *J Virol* 1988;62:2083–2090.)

be activated by infection to decrease virus replication.⁴¹ Suppression of the RNAi response increases virus replication and mosquito mortality after infection.^{143,387} Mosquitoes remain infected and infectious for life.^{525,710} In the fat body and salivary glands, infection is persistent, although titers decline after the acute phase of infection, decreasing efficiency of transmission.⁷⁰⁸

ALPHAVIRUSES ASSOCIATED PRIMARILY WITH ENCEPHALITIS

Eastern Equine Encephalitis

EEE was first recognized as a disease of horses in the north-eastern United States. In the summer of 1831, more than 75 horses died in three coastal counties of Massachusetts.³⁰⁰ Between 1845 and 1912, epizootics were recorded on Long Island and in North Carolina, New Jersey, Florida, Maryland, and Virginia.^{300,710} The virus responsible for EEE was first isolated in 1933 from the brains of affected horses in New Jersey and Virginia during a widespread outbreak that also involved coastal areas of Delaware and Maryland.⁷⁸⁶ South American eastern equine encephalitis virus (SA-EEEV) was first isolated in 1936 from a horse in Argentina.⁶⁸³ Although human cases were suspected earlier, it was not demonstrated until 1938 when an outbreak in the northeastern United States resulted in 30 cases of fatal encephalitis in children living in the same areas as equine cases. At that time, EEEV was isolated from the CNS of humans,⁸⁶⁷ as well as from pigeons²²¹ and pheasants.⁸⁶⁸

Based on seasonality, location of cases near salt marsh areas, lack of evidence of transmission from horse to horse through contact, short period of equine viremia, and geographic distribution of cases, Ten Broeck et al.⁷⁸⁵ postulated that insects transmitted infection and that birds were likely to be the reservoir host. The first arthropod isolates were actually from chicken mites and lice, which can transmit infection only inefficiently.^{193,710} Transmission of EEEV by *Aedes sollicitans* in the laboratory was accomplished in 1934⁵²⁰; subsequently, multiple *Aedes* species were shown to be competent vectors, although recovery of EEEV from naturally infected mosquitoes did not occur until 1949 with isolates from *Mansonia perturbans* in Georgia³³⁷ and *Culiseta melanura* in Louisiana.¹²⁷ Subsequent work showing the competence of *Cs. melanura* and a consistent association between infected birds and the isolation of virus from this vector has led to the current understanding that *Cs. melanura* is the primary enzootic vector for North American eastern equine encephalitis virus (NA-EEEV) strains.^{27,162,436,710}

Epidemiology

NA-EEEV causes localized outbreaks of equine, pheasant, and human encephalitis in the summer. The virus is enzootic in North America from Maine southward along the Atlantic seaboard and Gulf Coast to Texas, in the Caribbean, and in Central America²⁵⁴ (Fig. 23.9). Inland foci exist in the Great Lakes Region and have extended to South Dakota and Quebec.¹³⁶ The primary enzootic cycle is maintained in shaded swamps where the vector is the ornithophilic mosquito *Cs. melanura*^{27,159,710} (Fig. 23.10). Birds are the primary reservoir host, and many species are susceptible to infection.⁴⁰⁶ The amplifying species for NA-EEEV are wading birds, migratory passerine songbirds, and starlings.^{162,204,414,514} Young birds are probably important for

virus amplification because they are more susceptible to infection, have a prolonged viremia, and are less defensive toward mosquitoes.¹⁶² Multiple mosquito species, such as *Coquillettidia perturbans*, *Ochlerotatus canadensis*, and various *Aedes* species, may serve as bridge vectors for transmission to susceptible mammals.^{27,159,528,710} In temperate areas, the virus is most likely periodically reintroduced by migratory viremic birds or wind-borne infected mosquitoes from subtropical areas of year-round transmission.^{716,861,878,895} Reptiles with viremias that are prolonged and persist through hibernation may maintain the virus focally.⁸⁷⁷ There is no convincing evidence for overwintering in mosquitoes, and ovarioles are not involved after experimental infection of *Cs. melanura* with NA-EEEV.⁷⁰⁸

SA-EEEV is enzootic along the northern and eastern coasts of South America and in the Amazon Basin¹²³ (see Fig. 23.9). In South and Central America, the enzootic cycle is maintained in moist forests where *Culex (Melanoconion)* spp. appear to be the primary vectors.^{710,818} Forest-dwelling rodents, bats, and marsupials are frequently infected and may provide a reservoir; however, these transmission cycles are not well characterized.^{28,710} Infection of reptiles and amphibians is also likely.¹⁵⁹

MORBIDITY AND MORTALITY

NA-EEEV is the most virulent of the encephalitic alphaviruses, with a high mortality owing to encephalitis. Most cases are associated with exposure to wooded areas adjacent to swamps and marshes,⁹⁸ and approximately equal numbers occur in females and males.²¹¹ Children under 10 years of age are most susceptible,²¹¹ with 1 in 8 infections resulting in encephalitis; in adults, 1 in 23 infections result in encephalitis.²⁶¹ The case fatality rate was 60% to 70% in earlier studies and 30% to 50% in more recent studies, with the highest rates in children and the elderly.^{98,125,171,207,211,632} SA-EEEV is much less virulent and causes little human disease despite evidence of human infection in areas of endemic transmission.^{13,123,180,682}

ORIGIN AND SPREAD OF EPIDEMICS

In the northern part of the North American region, human and equine cases of EEE occur between July and October; in the southern region, cases can occur throughout the year.^{125,632} In the Caribbean, outbreaks appear to be linked to virus introduction by southbound migratory songbirds.⁷¹⁰ In North America, human and equine cases usually occur near swamps maintaining enzootic transmission. Outbreaks are initiated when the virus spreads from the enzootic cycle involving ornithophilic mosquitoes into mosquito populations that feed on various hosts (see Fig. 23.10). Multiple species of mosquitoes with catholic feeding habits have been implicated as potential bridge vectors in different regions.^{27,159,528,694,710}

Cases of equine encephalitis are usually the first indication of an outbreak. In the absence of immunization, epizootics appear approximately every 5 to 10 years and are associated with heavy rainfall and warmer water temperatures that increase the populations of enzootic and epizootic mosquito vectors.⁴⁴⁴ Most of the epidemics have occurred in Massachusetts and surrounding states²¹¹; however, the largest recorded outbreak occurred in 1947 in Louisiana and Texas, with 14,344 cases of equine encephalitis and 11,722 horse deaths.¹²⁹ Since 1955, an average of eight cases of EEE have been diagnosed in humans in the United States each year.^{125,126} (Fig. 23.11).

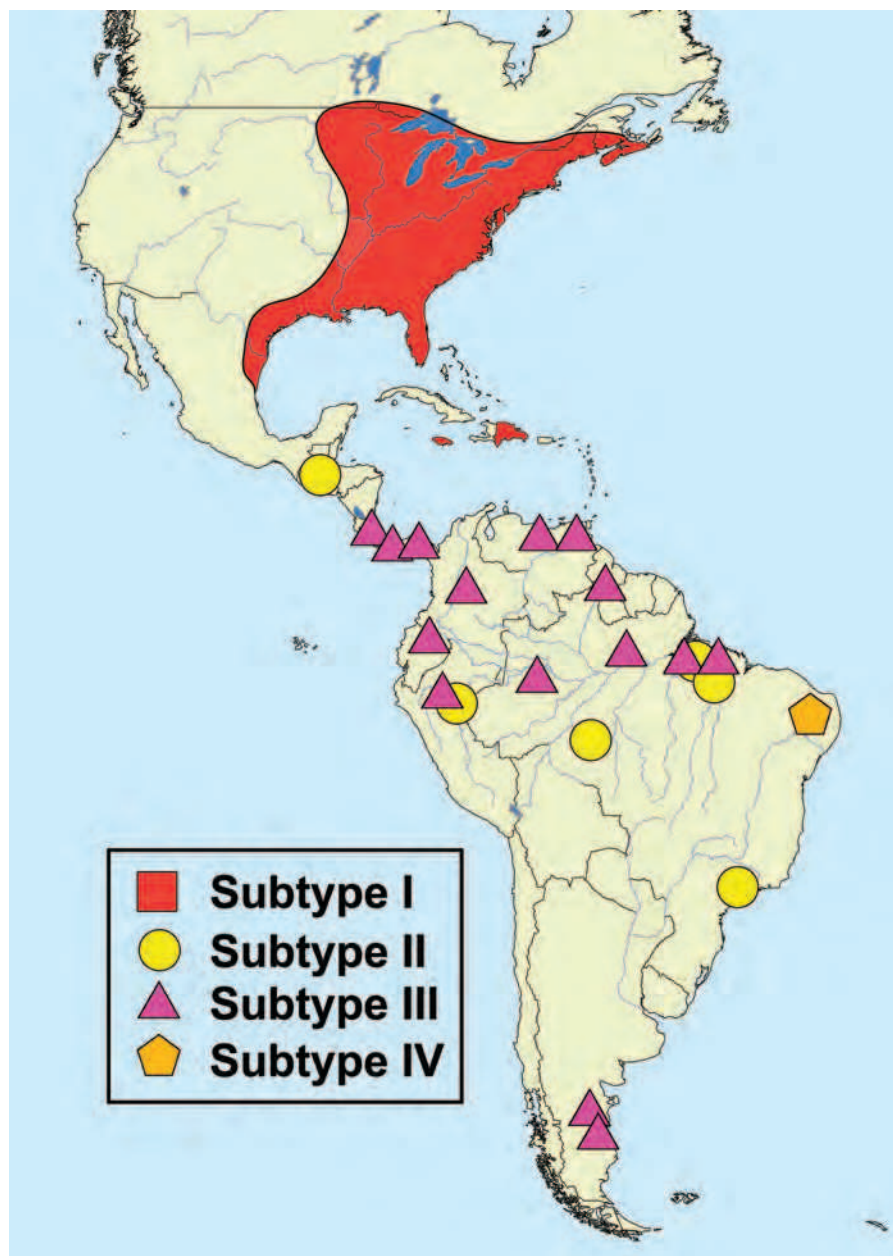


FIGURE 23.9. Geographic distribution of eastern equine encephalitis virus. The map shows the locations for isolation of North American eastern equine encephalitis virus (subtype I) and the three South American eastern equine encephalitis virus lineages (subtypes I, II, and III).⁸⁶¹ (Courtesy of Scott Weaver; from Weaver SC, Powers AM, Brault AC, et al. Molecular epidemiologic studies of veterinary arboviral encephalitides. *Vet J* 1999; 157:123–138.)

MOLECULAR EPIDEMIOLOGY

Antigenic differences between North and South American strains of EEEV have long been recognized,¹¹⁷ and the strains can be distinguished by reactivity with MAbs to the E1 glycoprotein.⁶⁶³ Sequence comparisons indicate that EEEV has evolved independently in North and South America over the past 1,000 years, with 23% to 24% nucleotide and 9% to 11% amino acid sequence divergence.²⁹ There is one lineage of NA-EEEV (lineage I) and three lineages of SA-EEEV: on the coasts of South and Central America (lineage II), in the Amazon basin (lineage III), and in Brazil (lineage IV)^{78,861} (see Fig. 23.9). Isolates of NA-EEEV are highly conserved, differing over a large geographic range by less than 3% in nucleotide sequence consistent with birds as the vertebrate reservoir hosts. The calculated yearly nucleotide substitution rate is 2.7×10^{-4} .^{78,861} The two main lineages of SA-EEEV (II and III)

exhibit 17% to 21% nucleotide divergence and 3% to 5% amino acid divergence and are evolving at a slower rate (1.2×10^{-4} substitutions/nucleotide/year).²⁹ The South American groups are estimated to have diverged 1,000 to 2,000 years ago and appear to be evolving locally, which is consistent with small mammals as the reservoir hosts.^{29,858,861}

Clinical Features and Pathology

Illness caused by infection with NA-EEEV may consist of 1 to 2 weeks of fever, chills, malaise, and myalgias followed by recovery. In cases of encephalitis, these prodromal symptoms are followed by the fulminant onset of increased headache, vomiting, restlessness, irritability, seizures, obtundation, and coma.^{171,207,211} Meningismus is frequent, and focal signs including cranial nerve palsies and hemipareses are not uncommon.^{171,632} Hyponatremia owing to inappropriate secretion of

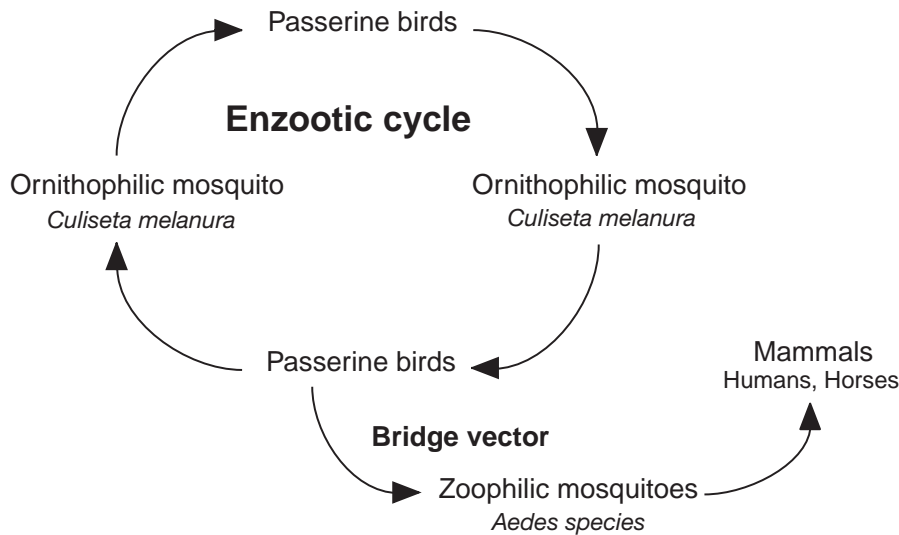


FIGURE 23.10. Enzootic and epidemic/epizootic cycles of eastern equine encephalitis virus as an example of an alphavirus that uses a species of mosquito with different host feeding preferences to bridge from the enzootic avian-ornithophilic mosquito cycle to infect mammals such as humans and horses. Similar mechanisms are operative in transmission of Sindbis virus to humans.

antidiuretic hormone is a common complication, and edema of the face and extremities has been noted.^{171,207} Death typically occurs within 2 to 10 days after onset of encephalitis.

CSF examination shows increased pressure and white cell counts ranging from 10 to 2,000/ μ L. Polymorphonuclear leukocytes are often abundant early in disease, with a shift to mononuclear cells over the first few days of illness.¹⁷¹ The presence of red blood cells or xanthochromia is not uncommon.⁶³² CSF protein levels are increased, whereas glucose is low to normal.^{207,632} Electroencephalographic (EEG) findings are relatively nonspecific, usually showing slowing.¹⁷¹ Computed tomography (CT) scans may be normal or show only edema.^{171,632} Magnetic resonance imaging (MRI) scans are more often abnormal, with focal lesions most commonly observed in the thalamus, basal ganglia, and brain stem.¹⁷¹

Poor outcome is predicted by high CSF white cell count or severe hyponatremia but not by the size of the radiographic lesions.¹⁷¹ Recovery is more likely in individuals who have a long (5–7 day) prodrome and do not develop coma.⁶³² Sequelae, including paralysis, seizures, and mental retardation, are common, with 35% to 80% of survivors, particularly children, having significant long-term neurological impairment.^{171,207,211}

Histopathology on fatal cases demonstrates a diffuse meningoencephalitis with widespread neuronal destruction; perivascular cuffing with polymorphonuclear, as well as mononuclear, leukocytes; and vasculitis with vessel occlusion in the cortex, basal ganglia, and brain stem. The spinal cord is frequently spared.^{207,222,760} Virus antigen is localized to neurons, and neuronal death is marked by cytoplasmic swelling and

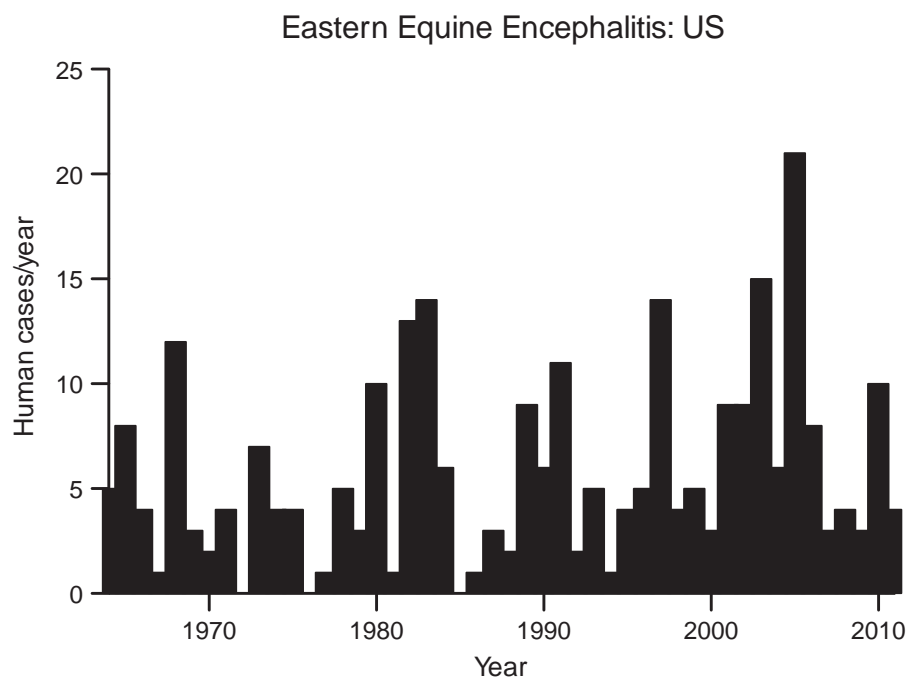


FIGURE 23.11. Numbers of human cases of eastern equine encephalitis reported annually in the United States since 1964. (Based on data from the Centers for Disease Control and Prevention.)

nuclear pyknosis.²⁴⁵ Apoptotic glial and inflammatory cells are frequently found in the regions of affected neurons.^{207,245}

Veterinary Correlates, Host Range, and Animal Models

NA-EEEV is an important cause of disease in horses, pheasants, emus, and turkeys and can also cause encephalitis in penguins, sheep, deer, dogs, and pigs.^{53,167,221,293,476,697,780} Horses develop signs of depression, progressive incoordination, seizures, and prostration. The case fatality rate is 80% to 90%. Most survivors are left with neurologic sequelae.⁷¹⁰ Birds vary in their susceptibility—some species develop disease, whereas many others show no morbidity or mortality despite a prolonged viremia.^{406,476} Chickens, turkeys, emus, and whooping cranes often develop fatal viscerotropic disease with multifocal necrosis in the heart, kidney, and pancreas and lymphoid depletion in thymus, spleen, and bursa.^{167,293,822} Pheasants develop encephalitis with 50% to 70% mortality, whereas penguins develop nonfatal encephalitis.^{476,697}

Laboratory studies of macaques, marmosets, mice, guinea pigs, and hamsters generally confirm the neurovirulence of NA-EEEV for mammals.^{222,466,592,670,760,786,838,893} NA-EEEV can initiate CNS infection in experimental animals by infecting choroid plexus epithelial cells or olfactory neurons.^{466,670} Peripheral replication is in fibroblasts, skeletal muscle, and osteoblasts.⁸³⁸ Macaques and marmosets develop encephalitis, whereas New World *Aotus* monkeys develop viremia without evidence of disease.²⁰² Young mice show extensive neuronal damage and rapid death.⁸³⁸ Older mice are relatively resistant to peripheral inoculation; however, after intracerebral inoculation, they develop seizures and die rapidly.^{242,466} Guinea pigs develop encephalitis after aerosol exposure to NA-EEEV with neuronal death, inflammation, and vasculitis.⁶⁷⁰ Hamsters develop a biphasic fatal illness with hepatitis and lymphatic organ infection followed by encephalitis characterized by extensive vasculitis and microhemorrhages.⁵⁹²

Virulence

NA-EEEV is more virulent than SA-EEEV in humans and experimental animals.^{12,123,710} Construction and testing of chimeric viruses in mice indicate that both structural and nsPs contribute to virulence.¹⁰ Viruses with a temperature-sensitive, small plaque phenotype, and decreased virulence for mice and hamsters have been selected after chemical mutagenesis.⁸⁵ One determinant of the neurovirulence of NA-EEEV is the ability to bind HS. Increased HS binding is associated with decreased tropism for lymphoid tissue, decreased production of IFN, and increased replication in the CNS.²⁴²

Diagnosis, Treatment, and Prevention

Diagnosis is based on virus isolation or detection of RNA or antibody. Virus can be isolated from CSF, blood, or CNS tissue by inoculation into newborn mice or onto various tissue culture cells. Detection and identification of virus in field and clinical samples can also be accomplished through various nucleic acid amplification assays.⁴³⁰ Antibody is usually measured by enzyme immunoassay (EIA), with detection of IgM in serum and CSF particularly useful.^{99,100} No specific therapies have been developed for treatment of EEE; thus, treatment is supportive.⁶⁴⁵

Infection in mosquito populations can be monitored by virus isolation, by nucleic acid amplification, or by seroconver-

sion of sentinel pheasants or chickens. This information can be used to guide insecticide spraying to reduce adult and larval mosquito populations and prevent human cases. A formalin-inactivated vaccine based on an NA-EEEV strain (PE-6) is available for horses and emus and for investigational use in humans to protect laboratory workers. This vaccine does not induce significant neutralizing or anti-E2 antibody to SA-EEEV.⁷⁶⁷

Western Equine Encephalitis

Epizootics of viral encephalitis in horses were described in 1908 in Argentina. In 1912, 25,000 horses were estimated to have died in the central plains of the United States.⁶⁸³ In the summer of 1930, a similar epizootic occurred in the San Joaquin Valley of California, causing an estimated 6,000 cases of equine encephalitis. During this outbreak, WEEV was isolated from the brains of two affected horses by intraocular inoculation of another horse, and this virus was subsequently used to infect other animals.⁵²² WEEV was suspected at that time to be a cause of human encephalitis. In 1938, WEEV was recovered from the brain of a child with fatal encephalitis.³³⁵

Mosquitoes were implicated in disease transmission when it was demonstrated that horses developed a viremia³³⁶ and that experimentally infected *Ae. aegypti* mosquitoes were capable of transmitting the virus to horses.³⁸⁸ However, it was not until the summer of 1941 during a widespread epidemic in the northern plains of the United States and Canada that the virus was isolated from naturally infected *Cx. tarsalis* mosquitoes.²⁹⁹ Subsequently, WEEV was isolated from *Cx. tarsalis* in other epizootic sites and recognized to be the principal vector.

Other New World Western Equine Encephalitis Complex Viruses

The WEE complex in the New World includes three viruses in addition to WEEV: Highlands J (HJ), Buggy Creek, and Aura viruses.¹⁰² These viruses have different ecological niches and vary in virulence. Only WEEV is recognized to cause human disease.⁹⁸ Viruses in the WEE complex found in the Old World (e.g., SINV and Whataroa virus) are discussed in the later Sindbis section. WEEV, HJV, and Fort Morgan virus all belong to the lineage that diverged since recombination between a Sindbis-like virus and an EEE-like virus.²⁹⁷ Aura virus is a “pre-recombinant” virus (see Fig. 23.5).

HIGHLANDS J

WEEV-like viruses were first isolated in the eastern part of the United States in 1952. In 1960, the prototype HJV was isolated from a blue jay in Florida.³¹⁷ HJV is enzootic on the U.S. East Coast and is maintained in a cycle similar to that of EEEV, with *Cs. melanura* the primary vector and migrating birds the primary reservoir. All alphaviruses in the WEEV complex isolated in the eastern United States are strains of HJV.³⁰⁹ Rates of divergence of WEEV and HJV of 0.1% to 0.2% per year have been estimated.¹⁴⁶ HJV can occasionally cause encephalitis in horses³⁸¹ and is a recognized pathogen for various avian species, including turkeys, pheasants, partridges, ducks, emus, hawks, and whooping cranes.^{19,213,293,861}

FORT MORGAN, BUGGY CREEK, AND STONE LAKES

Fort Morgan virus and its close relative Buggy Creek virus were originally isolated from swallows and sparrows in eastern Colorado¹⁰⁵ and Oklahoma.³³² These viruses exist as two lineages

found primarily in the western plains of North America^{591,607} and are transmitted to cliff swallows and sparrows by cimicid swallow nest bugs (*Oeciacus vicarius*).⁸⁶ Neither virus is recognized as a pathogen for humans; however, the viruses cause encephalitis in nestling house sparrows.⁵⁷⁸ Recently, a third related virus, Stone Lakes virus, was isolated from swallow bugs in California.⁷⁶

AURA

Aura virus was isolated in 1959 in Brazil from *Culex (Melanoconion)* spp. collected near the Aura River and later from *Aedes serratus* in Brazil and Argentina.¹²¹ This virus is likely to be related to the “Sindbis-like” virus that recombined with EEEV to produce WEEV.⁶⁷⁴ It has not been linked to human disease and is relatively nonpathogenic for mice.

Epidemiology

WEEV is widely distributed in the western plains and valleys of the United States and Canada and in South America. In North America, WEEV is maintained in an endemic cycle involving domestic and passerine birds (particularly finches and sparrows) and *Cx. tarsalis*, a mosquito well adapted to irrigated agricultural areas.⁹⁸ Occasional isolations have been made from *Aedes melanimon* and *Aedes dorsalis*, which also are competent vectors. There is evidence for a transmission cycle involving *Ae. melanimon* and the black-tailed jackrabbit^{98,302} in addition to the *Cx. tarsalis*–avian cycle. Increased transmission is associated with greater abundance of *Cx. tarsalis*.⁴⁶ Serosurveys and virus isolations provide evidence of natural infection in chickens, pheasants, rodents, rabbits, ungulates, tortoises, and snakes. In some areas of South America, most mosquitoes from which WEEV has been isolated feed primarily on mammals and antibodies are common in small mammals including rats and rabbits, whereas in other areas, antibodies are found primarily in birds.^{726,861} A recent survey of horses in the Pantanal region of Brazil found 36% seropositivity for WEEV.⁶⁰³ Interseasonal persistence can occur in saltwater marshes, perhaps by overwintering in adults.^{648,649}

MORBIDITY AND MORTALITY

WEEV in the western United States has caused epidemics of encephalitis in humans, horses, and emus; however, the case fatality rate of 10% for humans, 20% to 40% for horses, and 10% for emus is lower than for NA-EEEV. In older children and adults, males are two to three times more likely to develop disease than females.⁴⁷² The estimated case to infection ratio is 1:58 in children younger than 5 years and 1:1150 in adults.⁹⁸ Clinically apparent disease is most common in the very young and those older than 50 years.⁴⁷² Severe disease, seizures, fatal encephalitis, and significant sequelae are more likely to occur in infants and in young children.^{194,412} The overall case fatality rate is 3% but rises to 8% in those older than 50 years.⁴⁷² Accidental infections involving aerosolized virus in the laboratory have been reported²²² and have led WEEV to be regarded as a potential bioterrorist threat.⁷²⁸

The rare occurrence of significant human disease during equine outbreaks of WEE in South America may be related to the feeding habits of the vector or to a lower virulence of South American strains for humans and horses.^{169,683}

ORIGIN AND SPREAD OF EPIDEMICS

There have been large, often widespread epidemics of equine encephalitis occurring from mid June to late September in North America with significant spillover into the human population. These outbreaks correlated with regional increases in the population densities of *Cx. tarsalis*.⁵⁸⁷ Major epizootics occurred every 2 to 3 years in the United States from 1931 to 1952. In 1941, more than 3,400 human WEE cases were reported in the plains of the western United States and Canada, with attack rates of up to 167 cases/100,000 population.⁹⁸ During the 1952 epidemic in California's Central Valley, attack rates were 36 cases/100,000 humans and 1120/100,000 equids.⁹⁸ Seroprevalence in humans was 34% in rural areas of California endemic for WEEV in 1960²³² but only 1.3% to 2.6% in similar areas from 1993 to 1995.⁶⁴⁶ Human cases of WEE have steadily declined to fewer than one to two human cases/year over the past 20 years (Fig. 23.12). This decline is

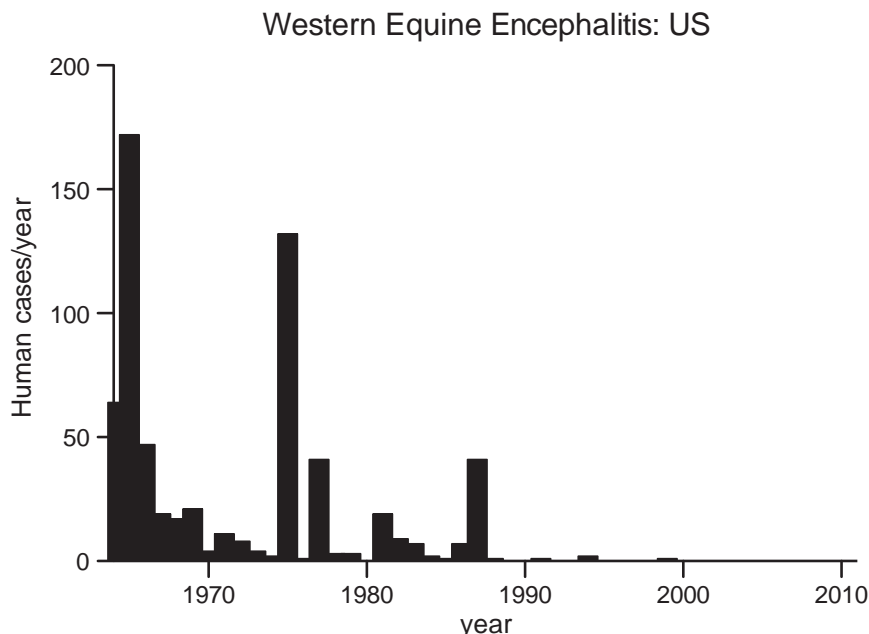


FIGURE 23.12. Numbers of human cases of western equine encephalitis reported annually in the United States since 1964. (Based on data from the Centers for Disease Control and Prevention.)

not understood but does not appear to be owing to a decrease in virus virulence or change in vector competence.^{218,647,900}

Studies of differential virulence of epizootic and enzootic strains in South America first suggested that epizootic strains arise from nonpathogenic enzootic strains.⁶¹ Sequence analysis of the viruses found at the initial focus of the 1982 WEE epizootic in Argentina indicated that the enzootic virus was the source of a virulent variant, which emerged by mutation or selection to cause the epizootic. The epizootic vector in South America is *Aedes albifasciatus*, and rabbits may serve as an amplifying host.⁸⁶¹

MOLECULAR EPIDEMIOLOGY

WEEV has four major lineages—two in South America and two widely distributed in North and South America and the Caribbean.^{859,861} One was isolated during the 1982–1983 epizootic in Argentina, a second is related to the McMillan strain found in North and South America and Cuba from 1930 to 1972, a third was found in the same regions from 1964 to 1993, and a fourth was found in Brazil and Argentina. Within California, separately evolving lineages have been identified in the Central Valley and the southern part of the state.⁴¹⁹

Clinical Features and Pathology

WEEV causes encephalitis with signs and symptoms similar to those of EEE. Transmission can occur by aerosols as well as by mosquitoes.⁶⁴² There is a 3- to 5-day prodrome of fever and headache that may progress to restlessness, tremor, irritability, nuchal rigidity, photophobia, altered mental status, and paralysis.^{214,412,516} Infants often present with rigidity, seizures, and a bulging fontanel.^{214,516} Transplacental transmission results in perinatal infection manifesting within the first week of life as fever, failure to feed, and seizures.^{516,723} CSF pleocytosis is typical with 100 to 1,500 cells/ μ L. Neutrophils are present early in disease and mononuclear cells later.⁵¹⁶ In infants less than 1 year of age, approximately 60% of survivors have brain damage, and in some the disease is progressive.^{516,576} Common problems are cognitive disabilities associated with quadriplegia, spasticity, recurring seizures, cortical atrophy, ventricular dilation, and intracranial calcification.^{194,214,576,751} In older individuals, recovery is typically rapid, with remission of signs and symptoms within 5 to 10 days, and sequelae are less common (5%).²¹⁴ Age-dependent susceptibility is likely related to the maturation-dependent ability of neurons to respond to IFN and limit virus replication.¹²⁰

Pathology of acute cases of WEE shows leptomeningitis and perivascular cuffing with neutrophils in the earliest cases and lymphocytes, plasma cells, and macrophages at later times. In areas of neuronal degeneration, there is inflammation accompanied by endothelial hyperplasia, petechial hemorrhages, and glial nodules. Lesions are found primarily in the basal ganglia, brain stem, cerebellum, cerebral cortex, and spinal cord, with areas of focal necrosis and demyelination in the subcortical white matter and basal ganglia.^{214,576,577}

Occasionally in infants and children, there is pathologic evidence of progressive disease consistent with persistent infection.^{516,576,577} Individuals surviving months to years after onset of encephalitis (often with progressive disease) may have cystic lesions, gliosis, and demyelination with areas of active mononuclear inflammation.^{576,577}

Veterinary Correlates, Host Range, and Animal Models

WEE was first recognized as a neurologic disease of equids characterized by fever, incoordination, drowsiness, and anorexia leading to prostration, coma, and death in approximately 40% of affected animals.¹⁸² Emus also develop symptomatic, often fatal disease characterized by ataxia, paralysis, and tremors.⁴² As a part of its enzootic cycle, WEEV infects sparrows, finches, blackbirds, mourning doves, pheasants, cowbirds, swallows, and chickens. The virus can cause fatal disease in sparrows, whereas chickens, which are often used as sentinels, develop asymptomatic infection.⁶⁵¹

Experimentally infected newborn mice die within 48 hours after infection with involvement of skeletal muscle, cartilage, and bone marrow. In weanling mice, the brain, heart, lung, and brown fat appear to be the primary target tissues.⁹ After intracerebral inoculation, there is infection of the choroid plexus and ependyma with spread to neurons and glial cells in the brain, cerebellum, and brain stem and to motor neurons in the spinal cord.⁴⁶⁶ After peripheral inoculation, WEEV replicates in skeletal and cardiac muscle cells, causes a necrotizing myocarditis, and occasionally spreads to the CNS.^{466,535} Hamsters are more susceptible to WEEV-induced disease than mice, with high mortality owing to encephalitis after intranasal or intraperitoneal inoculation.³⁷⁵ Neurons are infected, and neuropathologic changes include perivascular inflammation, microcavitation, and astrocytic hypertrophy.⁹⁰⁹ Cynomolgus and rhesus macaques are susceptible to intranasal, aerosol, and intracerebral infection and develop dose-dependent signs of encephalitis with fever, tremors, and altered consciousness. Pathology shows encephalitis with infection of neurons accompanied by mononuclear inflammation.^{642,893}

Virulence

Variants of WEEV with decreased pathogenicity for mice and hamsters (e.g., B628 clone 15) have been selected by passage on CEF cells.⁹⁰⁹ Large plaque strains tend to be more virulent than small plaque strains.³⁵⁴ Epizootic strains appear to be optimized for viremia and neuroinvasiveness and are generally more virulent for mice and guinea pigs than are enzootic strains.^{61,62,303} North American isolates vary substantially in their virulence for mice but are generally more virulent than South American strains.^{218,469,561} The molecular bases for any of these differences have not been defined; however, preliminary sequencing suggests that structural region genes are important determinants of virulence.⁵⁶¹

Diagnosis, Treatment, and Prevention

Diagnosis can be made by detection of WEEV-specific IgM in serum, by isolation of virus in mice or cultured cells, or by nucleic acid amplification.^{99,430} Small molecule screening has identified potential lead compounds that inhibit WEEV replication and are partially protective in mice.^{377,604} An inactivated vaccine is available for horses and as an experimental preparation for laboratory workers.⁶³⁸ Preclinical studies of approaches to protection include activation of innate immunity,⁴⁷⁰ DNA virus vectors expressing IFN or viral proteins, and chimeric alphaviruses.^{33,45,248,560,774,890,891}

Venezuelan Equine Encephalitis

Equine disease was recognized in South America in the 1920s. In 1936, a virus (VEEV) was isolated from the brains of encephalitic horses during an outbreak of equine encephalitis that spread from the central river valleys of Colombia into the Guajira region of Venezuela. This virus was antigenically distinct from the viruses causing equine encephalitis in the eastern and western portions of North America (EEEV and WEEV) and became the third encephalitic alphavirus identified in the Americas.^{56,420} Between 1943 and 1963, many VEEV-related viruses, including the prototypic Trinidad donkey (TrD) strain,⁶³⁷ were isolated in South America, Central America, and southern regions of the United States.¹¹ The first reported human cases of VEE were in laboratory workers^{118,439}; subsequently, human disease was documented in the general population during equine outbreaks, and virus was isolated from ill humans.^{637,692,857}

Studies in Central America during this period indicated an enzootic cycle involving *Culex (Melanoconion)* spp. mosquitoes and rodents.²³⁹ In 1969, an epizootic/epidemic of VEE appeared and spread from Peru through Central America and into Texas, causing human disease and a high mortality in horses.¹¹ Virus isolations during the epizootic were primarily from *Psorophora confinnis* and *Ae. sollicitans* mosquitoes and from horses, suggesting that the epizootic and enzootic transmission cycles differed.^{404,771}

Venezuelan Equine Encephalitis Complex Viruses

Using a short incubation HI test, isolates of viruses related to VEEV were originally classified into subtypes I through IV:

VEE (I), Everglades (EVE, II), Mucambo (MUC, III), and Pixuna (PIX, IV).⁸⁹⁶ When Cabassou (CAB) and AG80–663 (Rio Negro [RN]) viruses were isolated and shown to be within the VEEV antigenic complex, they became subtypes V and VI.^{104,181} The VEE subtype I viruses have been further subdivided serologically into IAB, IC, ID, IE, and IF (Mosso das Pedras)^{104,857,896} and the MUC subtype III viruses into IIIA, IIIB (Tonate [TON]/Bijou Bridge), IIIC, and IIID.^{403,857} Analysis of the phylogenetic relationships of the VEE complex viruses gained from sequencing has led to a recognition of eight species within the VEEV complex^{402,518,629} (Fig. 23.13; see Table 23.1).

EVERGLADES

EVE virus (serotype II) was first recognized in southern Florida in the 1960s in persons living near Everglades National Park. Transmission is widespread in Florida with *Culex (Melanoconion) cedecei* as the primary vector and cotton rats (*Sigmodon hispidus*) the main vertebrate reservoir.^{148,863} Disease in humans is usually mild.¹⁹⁸

MUCAMBO

MUC virus (serotype IIIA) was first isolated (BeAn 8) from a Brazilian monkey in 1954.¹²³ Three clades have been identified, with temporally defined clades 1 and 2 from Trinidad and clade 3 from Brazil.^{38,39} The virus causes fatal disease in newborn mice and in adult mice after intracerebral, but not peripheral, inoculation. Experimentally inoculated guinea pigs and horses survive infection.⁷²⁵

TONATE

TON virus (serotype IIIB) was first isolated in 1973 from a bird (*Psarocolius decumanus*) captured in French Guiana¹⁸¹ and

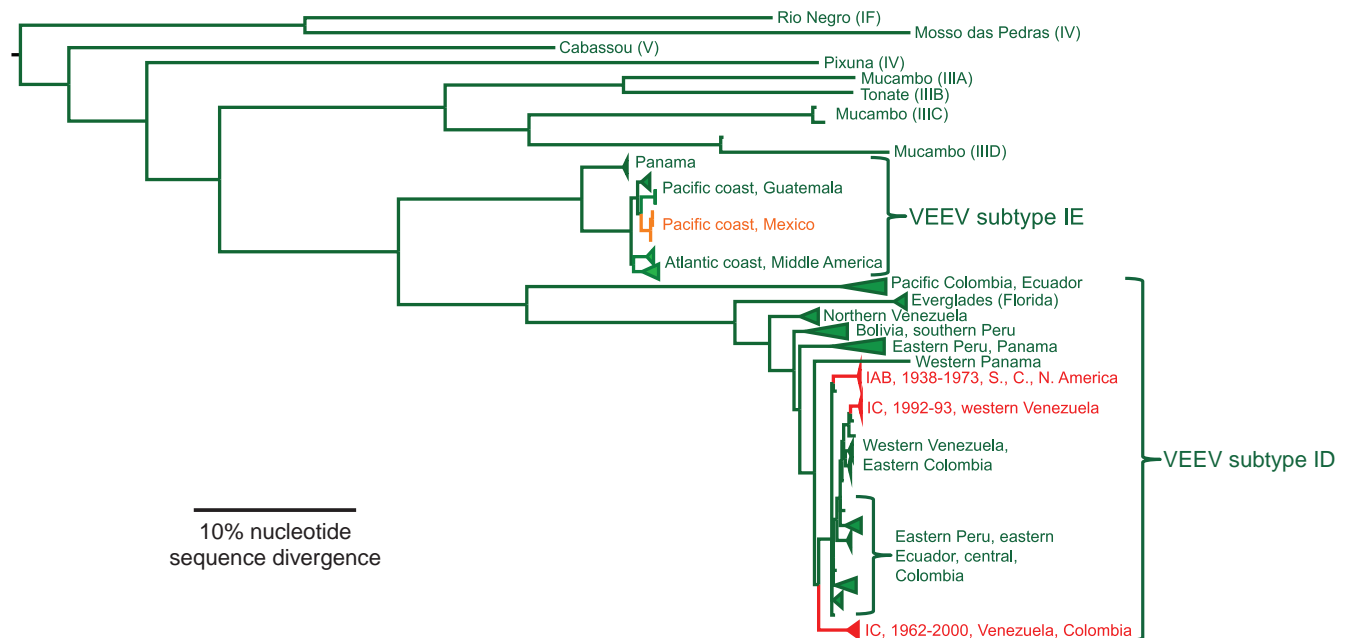


FIGURE 23.13. Venezuelan equine encephalitis virus phylogenetic tree showing relationships of epizootic (colored red and orange) and enzootic/endemic strains (colored green). Strains are abbreviated with country followed by year of collection. The tree was generated using 817 nucleotide sequences from the PE2 envelope glycoprotein gene using maximum likelihood methods. (Courtesy of Scott Weaver.)

subsequently from *Culex portesi*.⁵⁹⁵ TON virus (Bijou Bridge) has also been recovered from cliff swallow bugs and birds in North America.⁵³⁶ Human seropositivity is 11.9% in French Guiana, with the highest rates in savannah areas.⁷⁷⁶ Infection is most often associated with a mild dengue-like illness but has caused fatal encephalitis in a young child.³³¹

PIXUNA

PIX virus (serotype IV) was first isolated (BeAr 35645) from *Anopheles (Stethomyia) nimbus* mosquitoes in Belem, Brazil, in 1961⁷²⁵ and has also been identified in Argentina.^{613,614} There is no evidence that it causes disease in humans or horses.

CABASSOU

CAB virus (serotype V) was first isolated (CaAr 508) from mosquitoes in French Guiana in 1974.¹⁸¹ This virus is not neurovirulent for adult mice or guinea pigs.

RIO NEGRO

RN virus (serotype VI) was first isolated (AG80–663) from *Culex (Melanoconion) delpontei* near the Rio Negro in Argentina in 1980.¹⁰⁴ The virus circulates in neotropical regions of Argentina,^{527,613,614} where it has caused outbreaks of acute febrile illness.¹⁵¹ Suckling mice die within 2 to 3 days; however, adult mice and guinea pigs survive infection.¹⁰⁴

Epidemiology

Enzootic VEE complex viruses are involved in perennially active transmission cycles in subtropical and tropical areas of the Americas (e.g., EVE in Florida; VEE ID and IE in Central America; Mosso das Pedras, MUC, PIX, CAB, and RN in South America). In enzootic areas, mosquito isolates are primarily from *Culex (Melanoconion)* spp. mosquitoes that live in tropical and subtropical swamps throughout the Americas, breed near aquatic plants, and feed at dawn and dusk on various rodents, birds, and other vertebrates.^{11,212,239,272} Wild birds are susceptible to infection; however, mammals, such as cotton rats, spiny rats, bats, and opossums, are the most likely reservoir hosts, as determined by virus isolation, levels of viremia, serology, and resistance to disease.^{47,113,205,272,857}

MORBIDITY AND MORTALITY

Clinically evident human infection can occur with enzootic, as well as epizootic, VEE complex viruses.^{11,198,331,776,857} Humans living in areas of enzootic transmission have a high prevalence of antibody owing to infection associated mostly with undiagnosed mild febrile illnesses, often assumed to be dengue.^{11,205,272,701,776} During epizootics, human attack rates vary widely.⁶⁵⁹ The apparent to inapparent infection ratio is estimated at 1:11.⁷² All ages and both sexes are equally susceptible to infection; however, disease manifestations vary with age.⁶⁵⁹ Individuals younger than 15 years are more likely to develop fulminant disease with reticuloendothelial infection, lymphoid depletion, and encephalitis. In older children and young adults, a relatively benign influenza-like illness is most common.¹⁹⁸ Individuals older than 50 years are prone to develop encephalitis, although most recover.¹⁹⁸ The incidence of encephalitis in clinically ill humans is generally less than 5% and the mortality less than 1%.⁶⁵⁹ Essentially all deaths occur in children.

ORIGIN AND SPREAD OF EPIDEMICS

VEE epizootics/epidemics have occurred at approximately 10- to 20-year intervals in cattle ranching areas of Venezuela,

Colombia, Peru, and Ecuador when heavy rainfall leads to increased populations of epizootic mosquito vectors and herd immunity decreases.⁶⁵⁹ Formalinized vaccines containing residual live virus were probably responsible for initiating the IAB outbreaks in South America that spread to Central America and Texas between 1969 and 1972⁴⁰⁴ and in Peru in 1973.^{629,860} Viruses causing epizootics are subtype IAB, IC, or IE, whereas enzootic viruses are ID, IE, or IF. During 1995, a major VEE IC outbreak occurred in coastal areas of Venezuela and Colombia that caused disease in 75,000 to 100,000 people. This region had experienced a similar outbreak in 1962–1964. A ID virus obtained from a mosquito pool collected in Venezuela in 1983, when there was no epidemic activity, was similar in sequence and indicated that the epizootic IC virus was not maintained in a separate cycle but rather evolved by mutation from an enzootic ID virus.^{629,657,862} (Figs. 23.13 and 23.14). A similar process accounted for the emergence of a IC virus in 1992–1993 from a ID virus.²³ Epizootic potential is correlated with positive-charge mutations in E2 that increase the level of viremia in equines and may also increase infectivity for the mosquito vector *Ochlerotatus taeniorhynchus*.^{23,80,275} During epizootics, horses are an important amplifying species, and susceptible equines provide a means for virus spread.⁷⁷¹

Detailed phylogenetic studies have delineated six major lineages of enzootic VEEV, including five subtype ID strains and the subtype IE lineage (see Fig. 23.13). All epizootic strains from major outbreaks fall into a clade nested within one of these lineages.⁸⁵⁷ Enzootic IE viruses were the source of epizootic IE strains in two recent equine epizootics in Mexico.⁵⁷⁹ Interestingly, these epizootic IE strains cause encephalitis in horses with little viremia.^{262,685}

During outbreaks, virus has been isolated from several species of mosquitoes. Those most incriminated as VEEV vectors include *Ochlerotatus sollicitans*, *Oc. taeniorhynchus*, *Psorophora columbiae*, and *P. confinnis*.^{771,857} After an epizootic, IC viruses may persist in a natural cycle and serve as a source for subsequent smaller outbreaks.⁵⁶⁶

MOLECULAR EPIDEMIOLOGY

Sequence analysis of the VEE complex viruses shows the greatest divergence for the E2 glycoprotein and the C-terminal region of nsP3.^{518,629} Five enzootic subtype ID lineages have been identified: Colombia and coastal Ecuador, Panama, Colombia, western Venezuela, and northern Peru (see Fig. 23.13). All epizootic IAB and IC strains are related to ID strains from Colombia, Venezuela, and Peru.^{629,857} Three distinct geographically separate IE lineages have also been recognized: northwestern Panama, Pacific coast (Mexico and Guatemala), and Gulf/Caribbean coast (Mexico and Belize).⁵⁸⁰

Clinical Features and Pathology

Infection with enzootic, as well as epizootic, strains of VEEV can cause significant human disease, with the most common signs and symptoms being fever, headache, tremors, prostration, nausea, and vomiting that last from 3 to 4 days.¹¹ Accidental laboratory aerosol infections of young adults with epizootic strains of VEEV caused a febrile illness with the abrupt onset of chills, headache, myalgia, somnolence, vomiting, diarrhea, and pharyngitis 2 to 5 days after exposure without evidence of encephalitis.^{118,198,439} Natural epidemic human infection was first described in 1952 in Colombia and subsequently

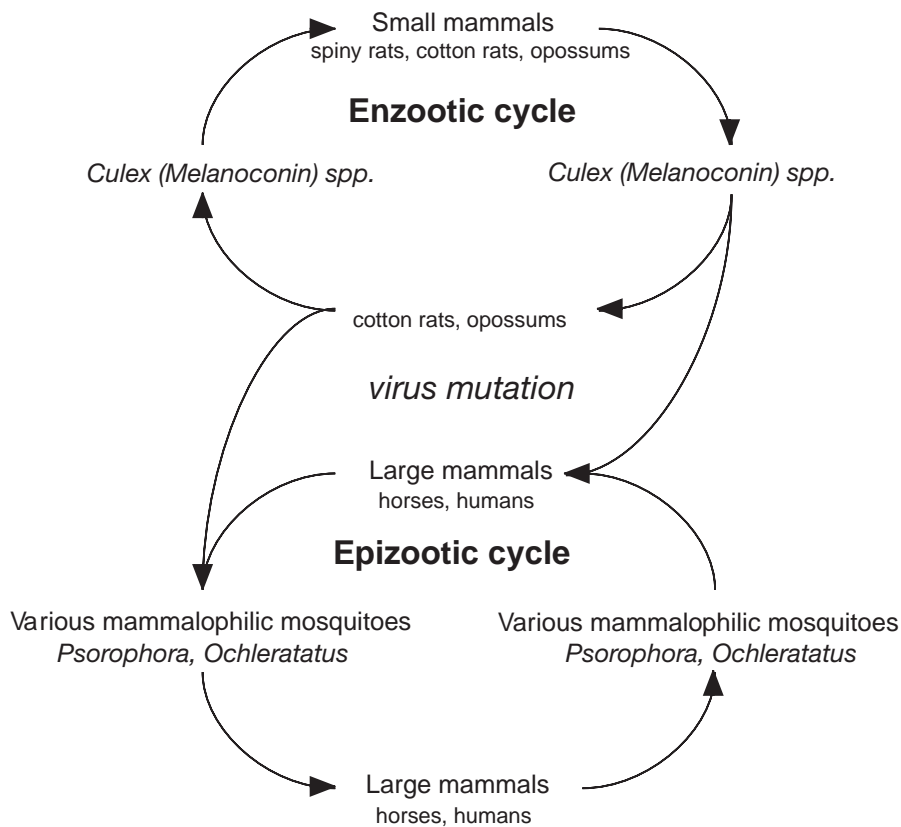


FIGURE 23.14. Enzootic and epidemic/epizootic cycles of Venezuelan equine encephalitis virus as an example of an alphavirus for which the epizootic strain arises by mutation from the enzootic virus. Similar mechanisms may be operative in transmission of epidemic western equine encephalitis virus.

in Venezuela and Panama during the 1962–1964 outbreak that resulted in 32,000 human cases and 190 deaths.^{198,368} The 1995 outbreak in Colombia caused an estimated 75,000 human cases—3,000 with neurologic complications and 300 deaths.^{659,862} The case fatality rate is 0.7% to 1%. Neurologic disease tends to appear 4 to 10 days after onset of illness, with headache and vomiting the most common initial symptoms. Specific neurologic signs include focal or generalized seizures, paresis, behavioral changes, and stupor or coma.^{659,862} Children recovering from encephalitis may be left with neurological deficits, particularly seizure disorders.⁴⁴² Fetal abnormalities, spontaneous abortions, and stillbirths may occur with infection during pregnancy.^{659,862}

Laboratory studies often show lymphopenia. Pathology on fatal cases has shown myocarditis, focal centrilobular hepatic necrosis and inflammation, generalized lymphoid depletion, cerebral edema, and vasculitis.³⁶⁸ Congenitally infected infants show severe neurologic damage with widespread necrosis, hemorrhage, and hypoplasia resulting—in the most severe cases—in hydranencephaly.⁸⁷⁰

Veterinary Correlates, Host Range, and Animal Models

Enzootic strains of the VEE complex can infect horses, although these infections are asymptomatic or cause short-term fever, low-level viremia, and little clinical illness and may immunize horses against infection with epizootic strains (IAB, IC, and IE).^{316,857} Equine disease induced by epizootic strains is characterized by fever, depression, and diarrhea leading to death 6 to 8 days after infection. Viremia persists until death, with titers up to 10^8 infectious units/mL blood. Virus

can also be recovered from eye washings, nasal washings, and urine. Leukopenia coincides with the viremia and is progressive in fatal cases. In animals that recover, antibody appears approximately 7 days after infection.⁴⁰⁵ Pathology on fatal cases shows pancreatic necrosis and cellular depletion in bone marrow, lymph nodes, and spleen. The brains of horses with signs of neurologic disease show swollen cerebrovascular endothelial cells, edema, extravasation of blood, and leukocytic infiltration into the perivenular spaces.^{260,405}

Infection of macaques by aerosol or subcutaneous inoculation with enzootic and epizootic strains of virus elicits a biphasic febrile response—the first phase is coincident with the viremia and the second phase with termination of viremia—that is, the appearance of the immune response.^{260,534,644} (see Fig. 23.7). Leukopenia is common. Symptoms are usually mild, consisting of anorexia, loose stools, and irritability, and occasionally loss of balance, tremor, or myoclonus.^{534,760} Examination of tissues shows lymphocyte depletion early, mild hepatitis, myocarditis, and encephalitis. Lesions in the brain are found primarily in the olfactory cortex and basal ganglia and consist of lymphocytic perivascular cuffing and glial nodules.²⁶⁰

Experimental infection of small laboratory animals with VEEV produces various disease patterns. After subcutaneous inoculation of guinea pigs, rabbits, or hamsters with virulent strains of VEEV, there is a viremia. Virus replicates in bone marrow, lymph nodes, spleen, and brain, with rapid destruction of myeloid and lymphoid cells in lymph nodes, spleen, thymus, intestinal and conjunctival lymphoid tissue, liver, and bone marrow; damage to the intestinal wall and pancreas; cerebral hemorrhage; and neuronal cell death.^{260,359,845} Death occurs 2 to 4 days after infection and may be associated with

ileal necrosis, bacteremia, and endotoxemia.²⁶⁶ EVE, MUC, and PIX viruses are progressively less virulent.³⁵⁹

In addition to myeloid and lymphoid necrosis, susceptible strains of mice develop encephalomyelitis leading to death in 6 to 9 days after infection with the TC-83 vaccine strain, as well as wild-type strains of VEEV.^{260,376,475} After subcutaneous inoculation, virus replicates first in DCs or Langerhans cells, which migrate to the draining lymph node where virus is amplified⁴⁸⁷ (see Fig. 23.6). Virus enters the CNS by the olfactory route after respiratory or peripheral inoculation. Initial infection is of olfactory epithelium, with spread to olfactory neurons and then caudally to all regions of the brain, causing encephalitis and neuronal apoptosis.^{133,351,759,760,837} Virus also infects the pancreas, liver, and teeth. Fatal disease has an immunopathologic component that depends on the strain of mouse infected.^{132,475,760} There can also be transplacental transmission of infection.⁷⁵⁵

Virulence

Comparative studies of the virulent TrD and avirulent TC-83 strains of the IAB serotype and construction of recombinant viruses have led to identification of the 5' NTR (nt 3) and the E2 glycoprotein (residue 120) as important determinants of VEEV virulence for mice.^{166,401} Attenuated viruses infect DCs less efficiently and replicate less well in lymphoid tissue and in the CNS than virulent viruses.^{487,759} Virulence for guinea pigs is determined by both envelope and nonenvelope genes.^{274,625} Determinants of equine virulence are different from determinants of murine virulence but also lie largely within the E2 glycoprotein.^{79,275,629} Changes most frequently associated with acquisition of equine virulence for ID strains are a Thr to Met change at position 360 of nsP3 and replacement of uncharged residues with Arg at positions 193 and 213 of the E2 glycoprotein.^{23,846} Acquisition of surface charge changes in E2 are also associated with emergence of epizootic IE strains.⁷⁹

Diagnosis, Treatment, and Prevention

Diagnosis can be made by virus isolation from blood or pharynx^{118,439,862} or by documenting the presence of anti-VEEV IgM or a rise in IgG antibody. HI, CF, neutralization, or EIA tests can be used for serologic diagnosis and nucleic acid amplification for detection of viral RNA. Antibody responses to enzootic versus epizootic strains can be differentiated with an epitope-blocking EIA.⁸⁴⁸

Treatment is generally supportive; however, passive transfer of antibody can protect mice before, or up to 24 hours after, challenge.^{263,340,341} The D-(-) enantiomer of carbodine, an inhibitor of cellular cytidine triphosphate synthetase, and antisense morpholino oligomers suppress VEEV replication *in vitro* and improve the outcome of infected mice.^{374,593}

The earliest vaccines developed for horses and laboratory workers were formalin-inactivated preparations.^{636,638} These vaccines had repeated problems with residual live virus producing disease and with poor immunogenicity and are no longer in use.^{404,629,860} A live attenuated vaccine (TC-83), developed by serial passage of the virulent TrD strain in guinea pig heart tissue culture cells,⁵⁸ is protective for horses and laboratory workers; however, 15% to 30% of recipients develop fever and pharyngeal viral shedding.⁶¹⁶ Therefore, a formalin-inactivated TC-83 vaccine (C-84) was produced.¹⁹⁶ Both the live and inactivated vaccines are immunogenic, although live TC-83 provides better protection against aerosol challenge in hamsters

than C-84 and is therefore preferred despite the reactogenicity.³⁶¹ To produce a more optimal vaccine, several experimental DNA, inactivated, and attenuated live virus vaccines are under development.^{131,191,192,500,594,643}

Several measures are effective in controlling outbreaks. These include immunizing equines, limiting equine movements from regions of infection, applying larvicides to mosquito breeding sites, and spraying insecticides to control adult mosquitoes.^{659,771,857} Protection of human populations relies primarily on personal protection from mosquito bites.

ALPHAVIRUSES ASSOCIATED PRIMARILY WITH POLYARTHRITIS AND RASH

Chikungunya

An outbreak of a crippling arthritic disease of sudden onset was first recorded in the Newala District of Tanzania in 1952.⁶⁶¹ Retrospective case reviews have suggested that CHIKV epidemics in Africa occurred as early as 1779 but were confused with dengue.¹¹¹ Because of the severe arthritic symptoms, the disease was given the name *chikungunya*, meaning “to walk bent over” in the Kimakonde language of Mozambique.^{328,661} The virus, isolated in 1953 from serum and from *Aedes* spp. and *Culex* spp. mosquitoes, was related to the group A arboviruses⁶⁶⁸ (see Fig. 23.5). Subsequent epidemics were recognized in the Transvaal of South Africa, Zambia, India, Southeast Asia, and the Philippines. From 2004 to 2007, a large epidemic affected islands in the Indian Ocean and India, with spread to Southeast Asia and Europe.^{628,654}

Epidemiology

CHIKV causes epidemics of rash and arthritis in India, Southeast Asia, Indonesia, the Philippines, most of sub-Saharan Africa, and Indian Ocean islands with recent extension into southern Europe.^{269,390,433,655,788} (Fig. 23.15). In Africa, the virus

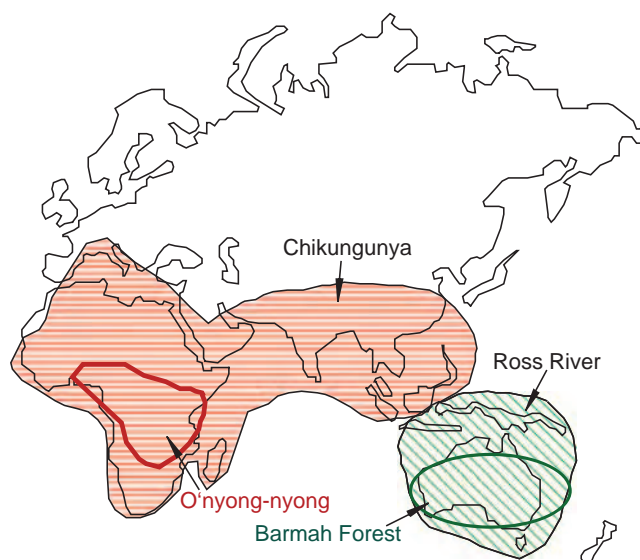


FIGURE 23.15. Geographic distributions of several alphaviruses that cause epidemic polyarthritis and rash: chikungunya, o'nyong-nyong, Barmah Forest, and Ross River viruses.

is maintained in cycles similar to that of yellow fever virus. There is a rural cycle involving *Aedes africanus*, *Aedes furcifer*, nonhuman primates, and other mammals and an urban cycle involving *Ae. aegypti* or *Ae. albopictus* and humans.^{137,177,812} In rural areas, the disease is endemic with small numbers of cases occurring most years.¹⁷⁷ In urban areas, outbreaks are sporadic and explosive, with infection of a large proportion of the susceptible population within a few weeks.^{250,601,654,661} In Asia, there is no evidence for a sylvatic cycle; rather, urban transmission is by *Ae. aegypti* and rural transmission is by *Ae. albopictus* in a human-mosquito-human cycle.^{326,568,627} Laboratory-acquired infections have also been reported.⁷⁹⁹

MORBIDITY AND MORTALITY

All ages and both sexes are susceptible to chikungunya, and disease is usually self-limiting and rarely life threatening. However, approximately 0.3% of cases are atypical or severe with nephritis, hepatitis, meningoencephalitis, thrombocytopenia, or encephalopathy. The case fatality rate has been estimated at 1 in 1,000, with most of the deaths either in neonates infected peripartum, adults with underlying conditions, or the elderly.^{119,195,654,660,707} The epidemic on Reunion Island affected almost 40% of the population (estimated 300,000 cases) and led to an excess of

254 deaths.^{250,318,653} Infants infected at birth are susceptible to CNS complications including cerebral edema and hemorrhage resulting in long-term disabilities.²⁴⁹ Musculoskeletal symptoms are recurrent or persistent in approximately 40% of adults and are more likely to affect females than males.^{752,887}

ORIGIN AND SPREAD OF EPIDEMICS

Outbreaks typically occur during the rainy season and are associated with increased population densities of mosquitoes.^{112,390,433,478} After an epidemic, the disease usually vanishes from an affected region for years, possibly because large portions of the population have become immune. The recent epidemics that began in Kenya in 2004 and spread to Comoros, Reunion Island, the Seychelles, Mauritius, and Mayotte in 2005 were associated with the emergence of a new strain that arose from the East African lineage^{382,705} (Fig. 23.16). Related outbreaks have been reported in India, Sri Lanka, Thailand, Malaysia, Singapore, France, and Italy, with imported cases recognized in many countries.^{318,598} Many of the recent outbreaks have been associated with a strain of virus that can be efficiently transmitted by *Ae. albopictus*.^{326,833} Because susceptible mosquitoes are widely distributed, travelers provide a source of CHIKV introduction into many other geographic regions.^{318,598,652,796}

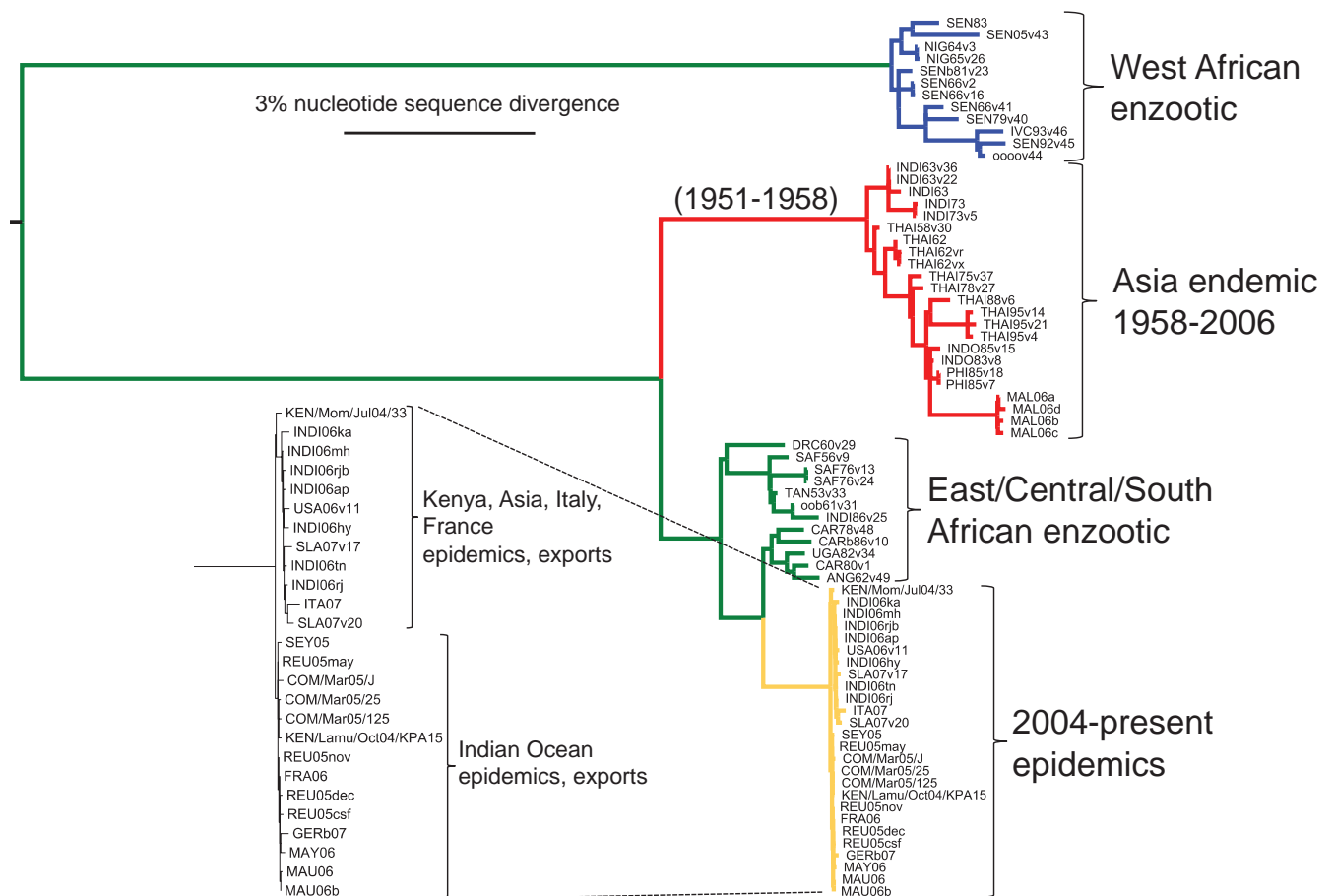


FIGURE 23.16. Chikungunya virus phylogenetic tree derived from genomic nucleotide sequences and Bayesian methods.

Strains are abbreviated with country followed by year of collection. All posterior probability values were 1.0 except for some within the 2004 to present epidemic clade shown in yellow. Estimated time frame for emergence of the 1958–2006 Asian endemic clade is indicated in parentheses. (Courtesy of Scott Weaver.)

There is some evidence that virus can be maintained within mosquito populations by low rates of transovarial or venereal transmission.^{506,639,795}

MOLECULAR EPIDEMIOLOGY

CHIKV is a member of the SFV complex, evolved from an ancestor in Africa, and was probably introduced into Asia 70 to 90 years ago.⁸³⁹ Two distinct lineages exist—one in western Africa and another in southern and eastern Africa and Asia^{627,628,705} (see Fig. 23.16). In the latter lineage, the Asian strains group as a genotype distinct from the African strains, and analysis of isolates from recent outbreaks suggest that the Indian Ocean strains originated in Kenya and evolved separately from the viruses that spread to Sri Lanka, Singapore, and the Maldives.^{301,382,705,839} Analysis of genetic changes over time suggests positive selection driven by cellular immune responses to the virus and enhanced transmissibility by *Ae. albopictus*, an important vector for outbreaks, particularly in rural areas.^{301,568,705,800,813,833} Adaptation of endemic Asian strains to *Ae. albopictus* was restricted by interaction between threonine at E1-98 and the adaptive E1-226 alanine to valine substitution. This allowed African strains that had adapted to this vector to replace the endemic strains in many countries^{326,568,811,812-813} and possibly increase virulence.⁶²⁸

Clinical Features and Pathology

Chikungunya is of sudden onset with an incubation period estimated at 3 to 12 days.⁶⁶¹ No prodrome is recognized.¹¹² Fever rises rapidly to 103°F to 104°F and may be accompanied by a rigor. The onset of fever corresponds to the period of viremia and may be related to the ability of this virus to induce large amounts of type I IFN^{140,255,328,853} (see Fig. 23.7). Monocytes are infected and are a source of IFN during infection.³¹⁹ Virus titers in blood can reach greater than 10⁶ plaque-forming units (pfu)/mL, and viral load correlates with disease severity and IFN production.^{112,140,328,702} Joint pain appears suddenly and can be incapacitating. Essentially any joint can be involved, and pain may be accompanied by swelling and paresthesias.^{69,391,569} Headache, conjunctivitis, and gastrointestinal symptoms are common; in 80%, a maculopapular rash appears 4 to 8 days after the initial illness. The rash may be associated with a second rise in fever (see Fig. 23.7), lasts approximately 2 days, and is described as “irritating” or “itchy.”^{70,661} Atypical cases with severe disease and increased mortality can develop bullous dermatosis, encephalitis, myelopathy, pneumonia, and diabetes.^{142,195,778} Fibroblasts in skin and joints and satellite cells in muscle are targets for infection.^{155,590} Leukopenia is frequent.^{70,140,328,887} Plasma levels of TNF, IFN- γ , and MCP-1 are increased.⁶⁷²

Joint pains and myalgias may continue in a milder form for many months after the original illness.^{168,668,752} Chronic pain is more likely in females and those older than 60 years with a high viral load during the acute illness.^{328,887} Synovial tissue harbors persistently infected perivascular macrophages, fibroblast hyperplasia, activated NK cells, and CD4⁺ T cells but few CD8⁺ T cells.³²⁸ Cytokines are increased during the response to infection, and persistent arthralgia is associated with elevated plasma levels of IL-6 and granulocyte macrophage colony-stimulating factor.^{139,140,569} Joint x-rays are usually normal or show soft tissue swelling without evidence of bone or joint damage, although erosive arthritis has been reported.^{391,495} In

India, but not Africa, inguinal lymphadenopathy and red swollen ears are also observed as part of the clinical picture.¹¹²

Animal Models and Host Range

Mice and nonhuman primates can be infected with CHIKV. In wild-type mice, disease severity is age dependent.¹⁵⁵ Neonatal mice develop fatal disease.⁷⁸⁴ Two-week-old mice survive infection but develop weight loss, difficulty walking, myositis, foot swelling, tenosynovitis, and vasculitis.^{543,907} Local inoculation of adult mice causes foot swelling associated with macrophage infiltration and production of inflammatory cytokines, particularly TNF, IFN- γ , and MCP-1.^{244,672}

Fibroblasts are target cells for CHIKV infection and a major source of IFN- α/β .⁷⁰² Adult interferon α/β receptor (IFNAR)- and STAT1-deficient mice develop fatal disease with replication in the liver, muscle, joint, and skin fibroblasts and occasional dissemination to the choroid plexus, leptomeninges, and ependyma of the CNS.^{155,157,702} Infection of pregnant mice does not result in fetal infection.¹⁵⁷

Adult cynomolgus macaques infected intravenously or intradermally develop a dose-dependent viremia, fever, rash, liver enzyme elevation, arthritis, and meningoencephalitis.⁴²⁸ Peak production of IFN- α/β coincides with peak viremia. Pathology shows persistent mononuclear cell infiltration into lymphatic tissue, joints, muscle, and liver associated with prolonged presence of CHIKV RNA in macrophages at these sites.⁴²⁸ Pregnant rhesus macaques infected with enzootic West African and epidemic Indian strains of CHIKV develop viremia, rash, joint swelling, leucopenia, and cytokine increases after infection; however, fetuses did not become infected, providing further evidence that transplacental transmission is infrequent.¹³⁴

Diagnosis, Treatment, and Prevention

The primary differential diagnoses for chikungunya fever are dengue and o'nyong-nyong (ONN) fevers. Dengue overlaps the chikungunya geographic distribution extensively but is characterized more by myalgia than arthralgia.³⁹¹ ONN is clinically similar but has geographic overlap only in Africa¹¹² (see Fig. 23.15). Laboratory parameters are variable and not particularly helpful in the diagnosis.⁶²⁸ CHIKV can be isolated from plasma or detected by nucleic acid amplification during the initial fever.^{288,600,668,784} Detection of IgM antibody provides a means of early diagnosis and can persist for months, particularly in those with persistent symptoms.^{69,100,288}

Treatment is generally symptomatic with anti-inflammatory agents. Drugs that inhibit MCP-1 synthesis decrease muscle and joint inflammation in mice.⁶⁷² Passive transfer of immunoglobulin containing antibody to CHIKV can protect neonatal wild-type mice and IFNAR-/- mice from fatal infection.¹⁵⁶ IFN- α is protective in mice, although only if given prior to infection.²⁴⁴

A live attenuated vaccine (TSI-GSD-218) has been developed by passage of a CHIKV isolate from Thailand in MRC-5 cells.¹⁹⁷ This vaccine induces long-term production of neutralizing antibody, can be used to protect laboratory workers from infection,⁵⁰⁸ and is undergoing further development.⁶²⁸ A formalin-inactivated vaccine can elicit protective immune responses in mice.^{244,797} Additional approaches to vaccination include DNA, chimeric engineered viruses, and virus-like particles.^{18,398,494,617,796,847}

O'nyong-nyong

In 1959, an outbreak of a new disease, originally mistaken for dengue, was reported from northwestern Uganda.⁷²⁷ It is likely that a similar epidemic occurred in the same region in 1904–1906.⁷²⁷ The name *o'nyong-nyong* originated from one of the first tribes to be affected—the Acholi—and refers to the painful joints that are characteristic of the disease.²⁹⁵ During the 1959 outbreak, ONNV was isolated from the serum of a patient with acute arthritis⁸⁸⁴ and from anopheline mosquitoes.⁸⁸⁶ ONNV is a member of the SFV complex and antigenically is most closely related to CHIKV, from which it is estimated to have diverged thousands of years ago.^{627,884} The virus re-emerged in southern Uganda in 1996–1997, suggesting a 30- to 50-year epidemic cycle.⁴³¹ In 1967, Igbo Ora, now recognized to be a strain of ONNV,^{431,627} was isolated from humans in western Nigeria.⁵³⁷

Epidemiology

ONNV causes sporadic, widespread outbreaks of fever, rash, and arthritis with high attack rates. The first epidemic recognized originated in northwestern Uganda in 1959 and spread south and east to Kenya, Tanzania, Zaire, Malawi, Mozambique, and Zambia to affect more than two million people.⁸⁸⁵ More recent outbreaks of ONNV/Igbo Ora infection have been documented in West and Central Africa.^{60,422,453,622}

The enzootic vector and vertebrate reservoir host for ONNV are unknown. Interepidemic seroconversions and mosquito isolations indicate continuous sporadic transmission in East Africa, and it is possible that humans or nonhuman primates are the primary reservoir.⁴³¹ ONNV is transmitted principally by *Anopheles funestus* and *An. gambiae* and is the only alphavirus known to be transmitted by anopheline mosquitoes.^{486,886} Human-mosquito-human transmission occurs during epidemics, and spread from one region to another occurs through the movement of viremic humans.⁶⁸⁹ The most recent outbreak began near swamps and lakes in the rural Rakai District of south-central Uganda. Serosurveys showed infection rates of 45% to 96% in areas of epidemic transmission, with the ratio of apparent to inapparent infection ranging from 1:4 to 1:24.^{453,537,689} All ages and both sexes are equally susceptible.⁴⁰⁷ Among domestic animals in the same region, cattle have the highest seroprevalence (40%),⁵⁸² although the enzootic reservoir for this virus is not known.

Clinical Features and Pathology

The onset of fever associated with ONNV is sudden and often accompanied by a rigor. The characteristic syndrome includes joint pains, rash, lymphadenitis, and conjunctivitis. Fever is typically moderate (100°F–101°F) and lasts approximately 5 days. Joint pain most often occurs in the knees, although ankles, elbows, wrists, and fingers can also be affected. The pain usually lasts 6 to 7 days and is severe enough to be immobilizing in 80% to 90% of patients but can persist for up to 3 months. The generalized morbilliform maculopapular rash erupts 4 to 7 days after the onset of symptoms and is similar to that of chikungunya. It begins on the face and extends to the trunk and extremities, occasionally affecting the palms. Cervical lymphadenopathy distinguishes the disease from chikungunya, occurs in approximately half of patients, and most frequently involves posterior cervical lymph nodes. Leukopenia is common. Fatalities have not been described; however, morbidity is substantial.^{407,453,537,727}

Diagnosis

ONNV is often clinically confused with malaria, measles, dengue, and rubella^{622,727} but also resembles chikungunya. Virus can be isolated, or nucleic acid amplified, from blood early in the illness.^{60,622,885} Diagnostic serology includes a positive IgM EIA or neutralizing antibody to ONNV that is more than two-fold greater than that to CHIKV.⁶⁸⁹ IgM persists for about 60 days after infection.⁴⁰⁷

Ross River

Epidemics of polyarthritis in Australia probably date from at least 1886 but were first clearly described in towns on the Murrumbidgee River in New South Wales in 1928 and then in troops stationed in the Northern Territory and Queensland during World War II.^{306,353,489} Serologic studies suggested an alphavirus as the causative agent, and RRV (strain T48) was first isolated from *Ae. vigilax* mosquitoes trapped near the Ross River in Queensland in 1959.^{306,489} The first human isolate was made in 1971 from the blood of a child with fever.¹⁸⁴ In 1979, RRV spread from Australia to Fiji, Samoa, and the Cook Islands, causing an explosive outbreak of tens of thousands of cases of polyarthritis on these Pacific islands.³⁰⁶ Virus was first isolated from the serum of patients with polyarthritis during this epidemic.

Epidemiology

RRV is endemic throughout the coastal regions of much of northern and central Australia and epidemic in the rest of Australia, Papua New Guinea, and the Solomon Islands.^{666,790} In general, the Wallace and Weber hypothetical lines in the Indo-Australian archipelago that separate the fauna of the Oriental and Australian regions appear also to separate the geographic distributions of CHIKV and RRV⁴⁸⁹ (see Fig. 23.15). RRV is the most common mosquito-borne pathogen in Australia, with approximately 5,000 cases reported each year, an annual incidence of 14 to 50/100,000 population, and 10-year seroconversion rates of 24% in some areas.⁶⁷⁶ Cases are most common in the north and occur primarily in late summer and early autumn with some evidence of an increasing incidence.^{306,489,676} Two salt marsh–breeding mosquitoes—*Aedes camptorhynchus* and *Ae. vigilax*—are probably the major vectors in coastal regions of Australia.⁶⁷⁶ In inland areas, the freshwater-breeding mosquito *Culex annulirostris* is the major vector; in urban areas, *Aedes notoscriptus* appears to be involved in transmission.⁶⁷⁶ Various mammals, primarily macropods such as kangaroos and wallabies, and potentially pteropid bats, appear to serve as vertebrate hosts for the enzootic cycle.^{116,306,676} In urban areas, horses and brushtail possums are likely involved in transmission.³⁸⁶

MORBIDITY AND MORTALITY

Infection rates can be as high as 1:30 during outbreaks.⁴⁸⁹ Estimates of apparent to inapparent infections range from 1:3 to 1:1.^{306,772} Seroprevalence and disease incidence are similar in males and females. Clinically apparent infections are rare in children, and the highest incidence of disease is in the 25- to 40-year age group.^{306,353}

ORIGIN AND SPREAD OF EPIDEMICS

In Australia, outbreaks are seasonal and have been associated with prolonged inundation of salt marshes owing to increased tidal heights or heavy rains during the summer.^{339,353,489,801,883}

and with a loss of herd immunity in reservoir populations.¹¹⁶ In arid regions, outbreaks often occur within 2 or 3 weeks of heavy rains, suggesting that RRV survives in desiccation-resistant mosquito eggs with vertical transmission.⁴⁶³ This is further suggested by isolation of RRV from field-caught immature and male *Ae. vigilax* and *Aedes tremulus* mosquitoes.⁴⁶³ Both urban and rural cycles of transmission occur. Epidemic polyarthritis, initiated by a viremic traveler from Australia to Fiji, spread to several South Pacific Islands between 1979 and 1980 in explosive outbreaks similar to urban chikungunya, with a man-mosquito-man transmission cycle and *Aedes polynesiensis* as the vector.^{666,790} Human viremias of more than 10⁶ mosquito infectious dose 50 (ID₅₀)/mL have been documented.⁷⁹⁰ Cases in travelers suggest that RRV has been reintroduced to Fiji.⁴⁰⁸

MOLECULAR EPIDEMIOLOGY

Strains of RRV originally isolated from the northeastern (Queensland), southeastern (New South Wales), and western regions of Australia are antigenically and genetically distinguishable.^{371,687,888} However, the northeastern lineage has not been recovered since 1977 and, along with the western lineage, has been replaced by the southeastern lineage.^{1,371} This replacement accompanied the appearance of duplication in the C-terminal domain of nsP3 first identified in a virus recovered in 1979 from a patient in Fiji.^{1,5} The data suggest genetic divergence and independent evolution of RRV within geographically isolated enzootic foci consistent with mammals as a vertebrate reservoir.⁶⁸⁷ However, overall diversity is low, perhaps owing to purifying selection.³⁷¹ Sequencing of intrahost populations of RRV reveal substantial diversity and evidence of mixed infections.⁴⁶⁷

Clinical Features and Pathology

RRV infection most often induces fever, arthralgia, and rash (see Fig. 23.6), although not all patients develop all three symptoms.^{308,788} Usually the illness has a sudden onset. Fever is the initial symptom, which is low grade, and is typically followed by the onset of pain, swelling, and tenderness in multiple joints. Joint involvement is usually symmetrical, and both small and large joints can be affected. Characteristically, the rash appears on the third or fourth day of illness; is maculopapular, nonpruritic, and present on the face, trunk, and extremities with occasional involvement of the palms and soles; and generally lasts 3 to 4 days.²²⁸ Frequent accompanying signs and symptoms include lymphadenopathy, lethargy, headache, myalgias, and photophobia. The average period of incapacity is 6 weeks, and symptoms gradually resolve in most affected individuals over 3 to 6 months.^{304,308}

Examination of synovial fluid shows increased protein; the presence of CD4⁺ T lymphocytes, monocytes, and activated macrophages; and increased levels of TNF, IFN- γ , and MCP-1.^{145,229,460} Cell counts are moderately increased, ranging from 1,500 to 15,000 cells/mm³,²²⁹ and RRV RNA is present.⁷⁴⁷ RRV replicates in, and can cause persistent infection of, synovial macrophages *in vitro*.⁸⁵⁴ It is postulated that infection of these cells leads to cytokine production and inflammatory arthritis *in vivo*.⁷⁷²

The skin lesions show mononuclear perivascular inflammation without evidence of immune complex deposition. Most of the infiltrating cells are CD8⁺ T lymphocytes. RRV antigen is present in the basal epidermal and eccrine duct epithelial cells.²³⁰

Animal Models and Host Range

Despite its extensive host range, RRV induces clinically evident disease only in humans, mice, and horses.^{43,783} The prototype T48 strain has been extensively passaged in mice, and virulence is age dependent.^{526,554} In newborn mice, T48 infects cardiac muscle, causing myocardial necrosis and death 3 to 4 days after infection.^{554,715} One-week-old mice survive, and infection of the CNS leads to demyelination and destruction of the internal granule cell layer of the cerebellum.⁷¹⁵ In 2- to 3-week-old mice, RRV causes myositis, arthritis, and weakness, with virus replication in muscle, perichondrium, periosteum, and skin.^{545,554} Macrophages are prominent components of the disease-inducing inflammatory process.^{460,673} Soluble mediators that contribute to disease severity include complement, TNF, IFN- α/β , IFN- γ , MCP-1, and macrophage inhibitory factor (MIF).^{321,459,460,542,544} Adult mice develop an asymptomatic viremia that persists for 5 to 9 days.⁷²⁰ In pregnant mice, virus can replicate in the placenta and spread to the fetus.⁵²⁴

The less virulent NB5092 strain induces severe myositis and muscle cell death in newborn mice after subcutaneous inoculation.⁵⁵⁴ Mice become stiff and unable to move and then gradually regenerate muscle and recover function after virus is cleared.⁵⁵⁴ CNS disease is minimal, with patchy infection of ependymal cells and ependymitis leading to hydrocephalus. Neurons are only occasionally infected.⁵²⁶ Brown fat is another important site of virus replication.⁵⁵⁴ By 1 week of age, mice no longer develop signs of disease.⁵⁵⁴

Virulence

To identify determinants of virulence for mice, virulent T48 has been compared to the less virulent NB5092 and DC5692 strains with different passage histories.^{206,240} Virulence has been associated primarily with amino acid differences in nsP1 and E2.^{379,395,517,783,841,842} Changes in nsP1 affect disease without affecting virus replication, whereas changes in E2 usually affect replication. Induction and susceptibility to IFN also influence disease development.⁴⁵⁹ Of unproven but possible significance is the fact that the epidemic strain of RRV in the Pacific differed from the parent Australian strain by a change from Thr to Ala at E2-219 and duplication in nsP3.^{1,93}

Diagnosis, Treatment, and Prevention

Diagnosis is aided by geographic location and travel history. The differential diagnosis includes rubella, other alphavirus-induced arthritides, and Henoch-Schönlein syndrome.²²⁸ Virus can be recovered from blood early in disease^{666,790}; however, most diagnoses are made using serology. The most sensitive and commonly used serologic test is an IgM capture EIA that can remain positive for 1 to 2 years after the onset of disease.¹¹⁵

In mice, treatment with sulfasalazine to block NF κ B or with bindarit to inhibit MCP-1 synthesis decreases inflammation and tissue damage.^{460,671} In contrast, treatment with etanercept to block TNF enhances virus replication, inflammation, and tissue damage.⁸⁹⁸

Vector control and personal protection against mosquito bites remain the primary means of prevention.^{305,798} An aluminum hydroxide adjuvanted inactivated whole virus vaccine that protects mice from infection and disease induces neutralizing antibody in humans at levels predicted to be protective and is in clinical development.^{2,17,330}

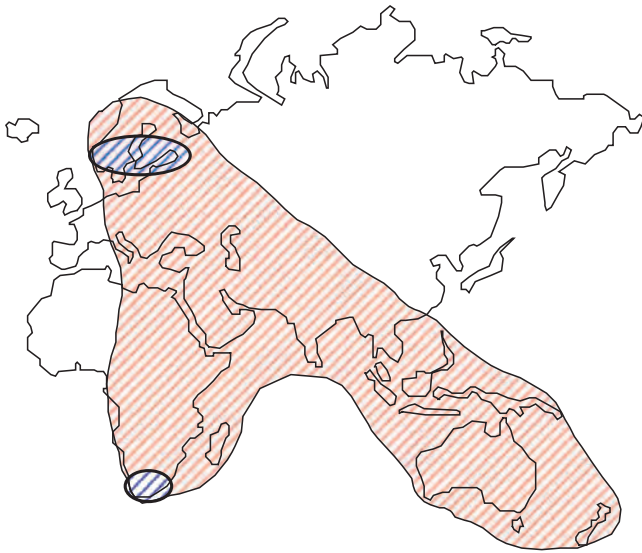


FIGURE 23.17. Geographic distribution of Sindbis virus. Northern Europe and South Africa, regions with significant outbreaks of arthritis, and rash disease in humans are designated in blue.

Sindbis

The prototype strain of SINV—AR339—was initially isolated from *Culex univittatus* mosquitoes collected near Sindbis, Egypt, in 1952.⁷⁸² Humans living in the Nile Delta at that time had a seroprevalence of 27%, although no disease was associated with infection.⁷⁸² Over the next 25 years, SINV was isolated in Europe, the Middle East, Africa, India, Asia, Australia, and the Philippines from various mosquito and vertebrate species. SINV was first isolated from the blood of febrile humans in Uganda in 1961 and recognized in South Africa as a cause of rash and arthritis in 1963.⁴⁹³

Epidemiology

SINV is the most widely distributed of the alphaviruses causing arthritis in man (Fig. 23.17). The primary sites of recognized SINV-mediated human disease are in northern Europe (Ockelbo in Sweden, Pogosta fever in Finland, and Karelian fever in Russia)²⁰³ and South Africa.³⁸⁰ In the many other regions where the virus exists, human infection occurs but results only in subclinical disease, fever, or mild arthralgias. The basic maintenance cycle of SINV is between *Culex* spp. or *Culiseta* spp. mosquitoes and wild birds.⁴⁸⁹ In Sweden, the enzootic cycle involves *Culex torrentium*, *Culiseta morsitans*, and passerine birds.^{227,480,482} In Finland, grouse are an important vertebrate host.⁴²⁵ Vertical transmission in mosquitoes can occur.¹⁷⁶

MORBIDITY AND MORTALITY

The age-adjusted prevalence for SINV is 2% to 8% in endemic regions of northern Europe and 0.1% to 0.2% in neighboring nonendemic areas. Risk is highest between the 60th and 64th parallels and is associated with spending time in the woods or marshland and exposure to mosquito bites.^{88,203,425,479,691} The ratio of symptomatic to asymptomatic cases is 1:17, with 45- to 65-year-old women most likely to develop disease.⁸⁸

ORIGIN AND SPREAD OF EPIDEMICS

Spread of SINV from its enzootic cycle between birds and ornithophilic mosquitoes to humans involves bridge vectors with less specialized feeding habits⁸¹⁷ (see Fig. 23.10). In northern Europe, the primary bridge vector is *Aedes cinereus*, and availability of this species may determine the frequency of human infection. Cases begin to appear in late July and continue into the fall. Large outbreaks occur approximately every 7 years, perhaps in association with fluctuating grouse populations.^{479,690}

MOLECULAR EPIDEMIOLOGY

Antigenic and genetic analyses of strains from different locations indicate the presence of five geographically distributed distinct genotypes: Africa and Europe (I), Australia (II), East Asia (III), Azerbaijan and China (IV), and New Zealand (V, Whataroa).^{481,482,686,688} Strains isolated in northern Europe and South Africa, where most SINV-induced disease occurs, are more closely related to each other than to strains isolated in south and central Europe and the Middle East, where disease is rare.^{372,724} The phylogeographic distribution is consistent with migratory birds as the major vertebrate host.^{481,688}

Clinical Features

The primary clinical manifestations are itching rash, arthritis, fever, and muscle pain.^{203,424,820} The rash is distributed diffusely over the trunk and limbs and can affect the palms and soles. Skin lesions have a macular base with central vesicle formation and are occasionally hemorrhagic.^{203,493} Joint pain preferentially affects large joints and may be severe enough to be immobilizing. Macrophages infected with SINV become activated; release MIF, TNE, IL-1 β , and IL-6; and express matrix metalloproteinases.³¹ Most patients recover within 14 days; however, joint pain and stiffness may persist for months to years.^{203,424,820} In Australia, SINV-induced arthritis and rash is milder and less frequent than RRV-induced disease or SINV-induced disease in northern Europe and Africa.⁴⁸⁹

Animal Models and Host Range

SINV can infect various vertebrates and has been extensively studied in mice as a model for acute encephalitis. In mice, there is an age-dependent susceptibility to fatal encephalitis.³⁶⁹ In young mice, virus replicates to high titer and spreads rapidly, causing death in 3 to 5 days. In older mice, virus replication is more restricted, and they often recover. After peripheral inoculation, virus replicates in muscle, produces a viremia, and then spreads to the brain and spinal cord, where the primary target cells are neurons.³⁴⁹ Ability of SINV to spread to the CNS and cause fatal disease depends on the strain of virus and the genetic background of the mouse.^{350,483,484,721,792,814} C57BL/6 mice are most susceptible to fatal encephalomyelitis after infection with neurovirulent strains, and this is determined in part by a gene on chromosome 2.^{792,793,814} Neuronal death requires contributions of the host, such as glutamate excitotoxicity and immunopathology.^{163,273,563,669} Deficiencies of IFN signaling or acid sphingomyelinase increase susceptibility to fatal encephalitis.^{95,567}

In nonfatal encephalitis, neutralizing antibody and an SINV-specific perivascular mononuclear inflammatory response consisting of T cells and monocytes appears within 3 to 4 days after infection.⁵²⁹ Spread of infection is limited initially by IFN- α/β ,⁹⁵ and infectious virus is cleared within 7 to 8 days after

infection, primarily through the effects of antiviral antibody and IFN- γ .^{64,89–91,286,448} Viral RNA persists in the CNS after clearance of infectious virus, and reactivation of infection appears to be prevented by continued presence of T lymphocytes and antibody-secreting B cells within the CNS.^{285,447,521,821}

Virulence

Strains of SINV differing in virulence have been derived from independent isolates from Egypt (AR339), South Africa (SR86), and Israel (SV-Peleg). Variants of AR339 and SV-Peleg differing in virulence have been derived by passage in mice and in tissue culture.^{284,483} Virulence is determined primarily by the 5' NTR and the E2 glycoprotein but can be influenced by changes in E1 and the nsPs.^{165,485,513,773} Changes in nucleotide 5 or 8 from A to G increase neurovirulence by unknown mechanisms.^{188,513} Several amino acid changes in the E2 glycoprotein affect virulence by altering efficiency of virus entry into the CNS or by enhancing neuronal infection.^{54,165,438,815} Neuroinvasion is affected by changes at residues 55 and 190 of E2.¹⁸⁸ A Gln to His change at E2-55 increases efficiency of infection of neurons and is a major determinant of increased virulence in older mice.^{438,815}

Diagnosis, Treatment, and Prevention

The major differential in SINV is with other causes of acute rash and arthritis, such as parvovirus B19 and rubella. The virus can be recovered from skin lesions and from blood.^{423,424,493}; however, the diagnosis is most often made by serology. SINV-specific IgM increases during the acute phase of the disease and then tends to decrease slowly for 3 to 4 years, independent of persistent symptoms.⁵⁷¹

Mice can be protected from fatal encephalitis by treatment with drugs that inhibit inflammation and glutamate excitotoxicity.^{273,348,631} Mice can also be passively protected with antibody but develop progressive destruction of infected regions of the brain.³⁹⁹

Barmah Forest

Barmah Forest virus (BFV) was first isolated in 1974 from *Cx. annulirostris* mosquitoes collected in the Barmah Forest in the Murray River Valley region of southeastern Australia and soon thereafter in southwest Queensland.²⁵ It is the only recognized member of the BFV complex and has a unique E2 protein without N-linked glycosylation.^{418,437} Human disease was first reported in 1986 with an epidemic of polyarthritis in New South Wales,⁷¹ and BFV was first isolated from the blood of a patient in 1988.⁶⁰⁹ The geographic range of BFV is expanding.⁸⁰² In 1989, BFV—previously restricted to eastern portions of Australia—was isolated in western Australia with subsequent outbreaks of human disease.⁴⁶⁴

The vertebrate reservoir host is unknown; however, serosurveys suggest that horses and marsupials, particularly brushtail possums, may play a role.^{386,464} On the other hand, sequence analysis shows a high degree of homology over the geographic distribution consistent with an avian vertebrate host.⁶²⁰ The main mosquito vectors are not well established but appear to include *Cx. annulirostris*, *Ae. vigilax*, *Ae. notoscriptus*, and *Ae. camptorhynchus*.^{352,464} Transmission in coastal areas is influenced by temperature, tides, and socioeconomic factors.⁵⁶²

Disease is most common in the 30- to 50-year age group, with men and women equally affected.⁵⁵ The most common clinical features are fever, lethargy, polyarthritis, and myalgia accompanied by a vesicular rash.^{25,55,609} Diagnosis may not be made because the illness is frequently mild and overlaps the clinical spectrum and geographic distribution of RRV and SINV infections (see Figs. 23.15 and 23.17). Rash is more prominent in BFV, whereas arthritis is more prominent in RRV²¹⁶ and the serology is distinct.⁶⁰⁹ In over half of infected individuals, recovery takes several weeks, with lethargy the most prominent persisting symptom.⁵⁵

Mayaro and Una

Mayaro virus was first isolated in 1954 from febrile forest workers in Trinidad and then from several individuals with fever and frontal headache in the Guama River area of Brazil.^{22,122} The virus is widely distributed in South America.^{220,505,791} Human cases are sporadic and occur primarily in persons with recent contact with humid tropical forests.⁸⁰⁵ There are two distinct genotypes. Genotype D contains isolates from Trinidad and north central South America, and genotype L contains isolates from Brazil.⁶²⁴ The principal mosquito vectors are in the forest-dwelling genus *Haemagogus*, and the vertebrate hosts are mammals, mainly nonhuman primates. Airborne transmission to laboratory personnel has also occurred.³⁷⁸ Symptoms include fever, headache, arthralgia, myalgia, vomiting, diarrhea, and rash that last from 3 to 5 days.⁵⁰⁵ The diagnosis can be made by isolation of virus from blood or by serology using an IgM capture EIA.¹⁰⁰ Una virus is closely related to Mayaro virus and was first isolated from *Psoraphora ferox* mosquitoes in the Amazonian region of Brazil in 1959.¹²¹ It is widely distributed in Central and South America.^{121,178,179} Vertebrate hosts include nonhuman primates.¹⁷⁸ This virus is pathogenic for mice but is not recognized to cause human disease.

OTHER ALPHAVIRUSES

Semliki Forest

SFV was first isolated in 1942 from *Aedes abnormalis* collected in the Semliki Forest of western Uganda.⁷⁴³ It is widely distributed in Africa with mosquito (e.g., *Ae. africanus*, *Aedes argenteopunctatus*) isolates documented from Mozambique, Nigeria, and the Central African Republic.^{491,504,511} Although SFV is one of the most extensively studied of the alphaviruses and serosurveys indicate that human infection is relatively common,^{333,504,743} SFV has been linked to human disease on only two occasions. In the first case, reported in 1979, a 26-year-old laboratory worker in Germany with a 1-year history of “purulent bronchitis” working with the Osterrieth strain of SFV developed fever and headache followed by seizures, coma, and death from encephalitis. SFV was isolated from CSF and from brain. No antiviral antibody was detectable at the time the CNS symptoms began, although it was detected at the time of death 1 week later.⁸⁸² The history of chronic pulmonary infections and failure to produce antiviral antibody rapidly suggest that this individual had an immunodeficiency disorder involving antibody production. In 1987, SFV was isolated from serum samples of individuals in the Central African Republic with fever, persistent headache, myalgias, and arthralgias.⁵⁰⁴

Animal Models

SFV can cause encephalitis in horses, mice, rats, hamsters, rabbits, and guinea pigs.^{36,75,743,908} Severity and type of disease depend on the age of the animal at the time of infection, the route of inoculation, and the virulence of the strain of SFV used for infection.^{75,287} Mice have been most extensively studied, and the strain of inbred mouse infected can also influence outcome.⁷⁶⁹

In newborn and suckling mice inoculated peripherally, virulent and avirulent strains of SFV replicate rapidly and extensively in muscle, elicit a high-titered viremia, spread to the CNS, and cause death within 2 to 4 days.^{208,287,553} Evidence suggests that SFV enters the brain across cerebrovascular endothelial cells.^{208,396,748} Once within the CNS, the virus replicates primarily in neurons and spreads rapidly along neural pathways, producing neuronal cell death.⁵⁸⁴

In weanling (3- to 5-week-old) mice, SFV replicates rapidly but reaches lower peak titers in muscle, blood, and brain than in younger animals.²⁸⁷ Virus enters perivascular regions of the brain and initiates foci of infection within the CNS. After intranasal inoculation, virus infects olfactory neurons first and then spreads within the CNS.⁵⁸⁴ The primary target cells in the brain are neurons, although oligodendrocytes are also infected.^{36,208,396} Neurons control virus replication more effectively than oligodendrocytes.²²⁶ A mononuclear inflammatory response consisting of T lymphocytes, B lymphocytes, and monocytes is apparent 3 to 4 days after infection, peaks at 2 to 3 weeks, and is mostly resolved by 6 weeks.⁵⁴¹

Mice infected with virulent strains can be passively protected from fatal encephalitis with immune serum but then develop a delayed disease associated with persistent infection, inflammation, and neuronal degeneration.⁷¹² Mice that survive infection develop demyelination, which is accompanied by mild paralysis, 2 to 4 weeks after infection.^{138,770} Clearance of infectious virus is complete 7 to 10 days after infection, and this clearance is mediated by antibody.²⁰⁸ Viral RNA and protein persist for months.^{186,396} Focal areas of demyelination are found 14 to 21 days after SFV infection and are characterized initially by swelling and vacuolation of oligodendrocytes and loss of myelin sheaths followed by remyelination.⁹⁴ Demyelination is macrophage mediated and appears to be the result of oligodendrocyte infection, the immune response to infection, and induction of an autoimmune response to myelin.^{209,532,768} SJL mice have prolonged inflammatory responses and demyelination after infection compared with other strains of mice.^{186,744}

SFV infection of the CNS can also increase the susceptibility of mice to induction of experimental autoimmune encephalomyelitis,⁵³³ apparently by damaging the blood-brain barrier, increasing adhesion molecule expression on endothelial cells, and facilitating entry of autoimmune T lymphocytes into the CNS.^{748,749}

Virulence

Isolates from mosquitoes collected in 1942 in Bwamba, Uganda,⁷⁴³ in 1948 in Kumba, Nigeria,⁴⁹¹ and in 1959 in Namacurra, Mozambique⁵¹¹ have given rise to various laboratory strains of SFV with differing levels of virulence. The most commonly studied are virulent strains V12, V13, and L10 (Uganda strain independently passaged in mice in Bethesda [V] and London [L]) and avirulent strain A7 (Mozambique strain AR2066 passaged in mice) and its less virulent derivative A7-74.⁷⁵ In

addition, avirulent strains of L10 have been derived by chemical mutagenesis (e.g., m9).⁴⁸ Virulent and avirulent strains differ in their ability to invade and replicate in the CNS of weanling mice and rats after peripheral inoculation, although all strains cause fatal disease in newborn or suckling mice.^{36,208,287,553} In 3- to 4-week-old mice, avirulent SFV is restricted in replication and spread in the CNS compared to virulent strains of virus and compared to avirulent strains in younger mice.⁵⁸⁵ This difference in replication is associated with decreased budding of infectious virus and is independent of the host immune response.²⁰⁸ Mature neurons and pancreatic and myocardial cells can be made more susceptible to avirulent virus by treatment with aurothiomalate compounds that induce intracellular membrane proliferation.^{208,700} In general, reduced virulence correlates with reduced replication in neurons.⁴⁴

In vitro studies of the differences between virulent and avirulent strains of SFV have shown differential replication in neuronal cells³⁴ and differences in susceptibility to IFN.¹⁷⁴ Efforts to identify specific nucleotide and amino acid changes important for virulence have utilized comparative sequence analysis and an infectious SFV cDNA clone pSP6-SFV4 derived from the prototype virulent L10 strain. Construction of SFV4/A7 chimeric viruses has shown that determinants of virulence reside in both the structural and nonstructural regions of the genome.⁷⁷⁹ E2, nsP2, and nsP3 are important determinants of virulence.^{208,256,259,658,693}

Other Semliki Forest-Related Viruses

The SFV complex includes eight viruses and has representatives in both the Old World (Bebaru virus, chikungunya, Getah, ONN, Ross River, and Simliki Forest) and the New World (Mayaro and Una) (see Table 23.1 and Fig. 23.5). Recently identified southern elephant seal virus is phylogenetically, but not antigenically, related to SFV. Human disease, when present, is generally characterized by fever that may be accompanied by arthritis and rash. CHIKV, ONNV, RRV, and SFV have been discussed previously.

Getah

Getah virus was first isolated from *Culex* spp. mosquitoes collected in Malaysia in 1955 and causes myositis when inoculated into mice.³²⁵ It is maintained in a cycle similar to Japanese encephalitis virus with transmission by *Culex tritaeniorhynchus* and amplification in domestic pigs.⁷³⁵ Getah virus is widespread and ranges from Eurasia to Southeast and Far East Asia, the Pacific Islands, and Australasia. Disease in humans is limited to fever,⁴⁵⁶ although it causes abortion in pigs and is an important pathogen of horses.⁷¹⁷ The equine disease is characterized by fever, an urticarial rash, and hind leg edema but is not life threatening.⁷¹⁷

Southern Elephant Seal

Southern elephant seal virus was isolated from lice residing on southern elephant seals on Macquarie Island, Australia. It is phylogenetically related to SFV but does not cross-react serologically. No disease has been recognized in infected seals.⁴²⁷

Salmonid Alphavirus

Salmonid alphavirus (SAV) causes sleeping disease in rainbow trout and pancreas disease in farmed Atlantic salmon.^{329,836,875,876} SAV has unusually large E1 and E2 structural glycoproteins

and relatively low (30%–34%) homology to other alphaviruses.^{836,876} Six subtypes have been identified.⁷⁴⁵ Subtypes 1, 4, and 5 are closely related and have been isolated from farms in Britain with pancreas disease. Subtype 2 causes sleeping disease in Europe, and subtype 3 causes disease on salmon farms in Norway. Subtype 6 is represented by a single isolate from Ireland. SAV causes significant disease on fish farms, which is characterized by abnormal swimming behavior and lack of appetite. Histopathology shows degeneration of the pancreas and of cardiac and skeletal muscles.⁵¹⁵ SAV is shed in feces and mucus and can be horizontally transmitted.²⁶⁸ Sea lice can be infected; however, their role in transmission is unclear.⁶⁰⁶ Subtype 5 SAV RNA sequences have been detected in wild marine fish, suggesting that marine reservoirs exist.⁷⁴⁶

DIAGNOSIS

The differential diagnosis of alphavirus-induced diseases often includes more than one alphavirus in addition to other mosquito-borne viral diseases such as dengue and West Nile fevers, other rash diseases such as rubella and parvovirus B19, and other causes of encephalitis. IgM capture EIAs can be used for diagnosis early in disease.^{99,100,103} The IgM response is relatively specific for each antigenic complex and is useful even at later times, because IgM persists for at least 2 to 3 weeks after onset of disease.¹⁰⁰ Virus isolation and identification remain useful; however, nucleic acid amplification tests have simplified virus identification in clinical samples.⁶³⁰ RT-PCR primers have been designed that can amplify the conserved region of all alphaviruses,^{201,290,608} as well as alphavirus-specific regions,⁴³⁰ and should be useful for rapid diagnosis.

PREVENTION AND CONTROL

Treatment

At this time, there is no available specific antiviral treatment for any alphavirus-induced disease, although compound screening has identified potential antiviral therapies for evaluation in animals.^{170,374,377,593,604,619,645} Therapeutic MAbs and immune system activators are being evaluated in animal models.^{341,470} Several neuroprotective drugs protect mice from fatal encephalitis.^{163,273,348,563,631} Supportive treatment for those with encephalitis can be lifesaving because individuals may make remarkable recoveries from coma. Symptomatic treatment for arthritis with anti-inflammatory drugs and immune modulators can be beneficial.

Vaccines

Formalin-inactivated vaccines against EEE, WEE, and VEE are available for horses and against EEE for birds. Experimental inactivated vaccines against EEE, WEE, and VEE are also available for laboratory workers exposed to these agents, with yearly booster doses required for EEE and WEE.¹⁹⁶ PE-6—the investigational inactivated EEE vaccine for humans—induces good immunoreactivity against the NA-EEEV but not SA-EEEV.⁷⁶⁷ An inactivated VEE vaccine that uses the V3526 strain is under evaluation,⁵⁰⁰ and an inactivated RRV vaccine is in human trials.¹⁷

Live attenuated vaccines have been developed for VEEV (TC-83) and CHIKV.⁴⁵⁰ Substantial side effects are common after immunization with TC-83,^{196,616} and new attenuated strains are under investigation.^{594,643} Alternative approaches in preclinical development include MAbs, DNA vaccines, chimeric alphaviruses, alphavirus replicons, and adenovirus-vectored vaccines.^{191,192,263,340,594,774} In both horses and humans, prior vaccination against one alphavirus can interfere with development of neutralizing antibody to subsequent alphavirus vaccines.^{106,181,508,615}

Other

Prevention of infection with most alphaviruses relies primarily on efforts to control mosquito populations by spraying and reducing breeding places. Various means can be used for assessing the need for mosquito abatement. These include monitoring mosquito population densities, seroconversion of sentinel animals, and presence of virus in populations of mosquitoes capable of transmitting virus to humans or domestic animals. Individual use of protective measures, such as mosquito repellents and protective clothing, are important.

PERSPECTIVE

The ability to construct full-length cDNA alphavirus clones that can be transcribed into infectious RNA has advanced understanding of the functions of various genes and their importance for replication and virulence in the multiple hosts necessary for maintenance of these viruses in their natural cycles. An understanding of the three-dimensional structures of proteins in the virion has greatly aided interpretation of much of the sequence and virulence data previously acquired. Further sequence information on virulent and avirulent strains, functional and structural analysis of the nsPs, and assessment of host–cell interactions is likely to provide the next level of understanding of virus–host relationships.

In addition, there is a need for improved approaches to prevention and treatment. There is a particular need for understanding the components of the immune response necessary for noncytolytic virus clearance, protection from reinfection, and immune modulation of disease. New and improved vaccines are needed for protection during outbreaks and for laboratory workers. In addition, there is a need for effective anti-alphaviral drugs, and an understanding of the structure of viral proteins may offer new approaches to therapeutics. Both of these areas have implications for biological defense purposes, because many alphaviruses can be transmitted by aerosol and VEEV, EEEV, and WEEV are on the U.S. Centers for Disease Control and Prevention category B list of critical biological agents.¹²⁴

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Rubella Virus

Infectious Agent

- Morphology and Physicochemical Properties of the Virion
- Biological Characteristics
- Animal Models of Rubella Virus Infection
- Antigenic Composition and Determinants

Epidemiology

- Incidence
- Age
- Origin and Spread of Epidemics
- Molecular Epidemiology

Clinical Features

- Acute Rubella
- Congenital Rubella Syndrome

Diagnosis

- Differential
- Laboratory

Prevention and Control

- Vaccines

Perspective

Rubella virus (RV) is the etiologic agent of *rubella*, a mild exanthematous disease associated with low-grade fever, lymphadenopathy, and a short-lived morbilliform rash. Rubella was first described as a distinct disease in the early 1800s.¹⁴³ Prior to acquiring its more common name, the disease was named *Rōtheln* by the German physician de Bergen,⁶⁵ leading to the common name of German measles, a milder form of the much more serious exanthem caused by the paramyxovirus, measles virus. Subsequently, in 1866 the disease name was anglicized to *rubella* by the British physician Veale.²⁴³

Predominantly a childhood disease, RV is endemic in many parts of the world. The introduction of comprehensive vaccination programs in most industrialized regions including the Americas, Western Europe, Japan, and Australia has drastically reduced the incidence of disease in these areas. Indeed, the indigenous circulation of rubella was officially declared eliminated in the United States in 2005. However, developed nations are still very much at risk for rubella outbreaks due to immigration from areas where RV vaccination is lacking. For example, in Africa and Southeast Asia, scheduled infant immunization against rubella is 0.1% and 4%, respectively.²⁵⁸ Because a very large proportion of the world's population is still

susceptible to RV (Fig. 24.1), we can expect that epidemics of rubella will still occur on a regular basis.

Until the keen observations of the ophthalmologist Norman Gregg, rubella was considered a relatively benign infection, associated with considerably less morbidity than measles. In 1941, Gregg encountered a large number of children with cataracts, many of whom had additional serious congenital defects. He noted that an apparent epidemic of congenital cataracts was directly preceded by a large rubella outbreak. Gregg proposed that the cataracts and the often associated congenital cardiac abnormalities were the consequence of maternal infection during pregnancy.⁸³ Further studies by other investigators confirmed that the virus could have devastating effects on a developing fetus when acquired by the mother in early pregnancy.²⁵⁴ The realization that viruses can act as teratogenic agents spurred the efforts to develop an attenuated vaccine.

A key step in vaccine development was isolation and growth of RV in cultured cells.^{182,255} In 1969, the first rubella vaccine was licensed in the United States, 5 years after the last major epidemic in that country and shortly before the next significant outbreak was predicted to occur. Fortunately, no other major rubella epidemics occurred, largely in part because of the adoption of universal vaccination in infancy, which has been remarkably successful in controlling natural rubella and its devastating teratogenic effects.²¹³

INFECTIOUS AGENT

RV is an enveloped positive-strand RNA virus in the family *Togaviridae*. There are two genera that compose the *Togaviridae*: *Alphavirus*, which includes Sindbis and Semliki Forest viruses, and *Rubivirus*, whose sole member is RV. Togaviruses share a common genome organization and replication strategy (Fig. 24.2). Whereas alphaviruses employ animal reservoirs and arthropod vectors for transmission, humans appear to be the only natural host and reservoir for RV. Although only one serotype exists, there are at least 10 genotypes of RV (Fig. 24.3), which can be grouped into two clades.²⁶⁴ Circulation of clade 2 viruses, which contain three genotypes, is limited to Eurasia, while clade 1 viruses are more widely distributed. The genome of RV is a 5' capped positive-sense RNA molecule of approximately 10 kilobases (kb) with a poly (A) tail. There is no serologic cross-reactivity between the alphaviruses and RV, and only limited genome sequence similarity, predominantly within the nonstructural genes in regions that encode functional domains such as the polymerase and protease activities.

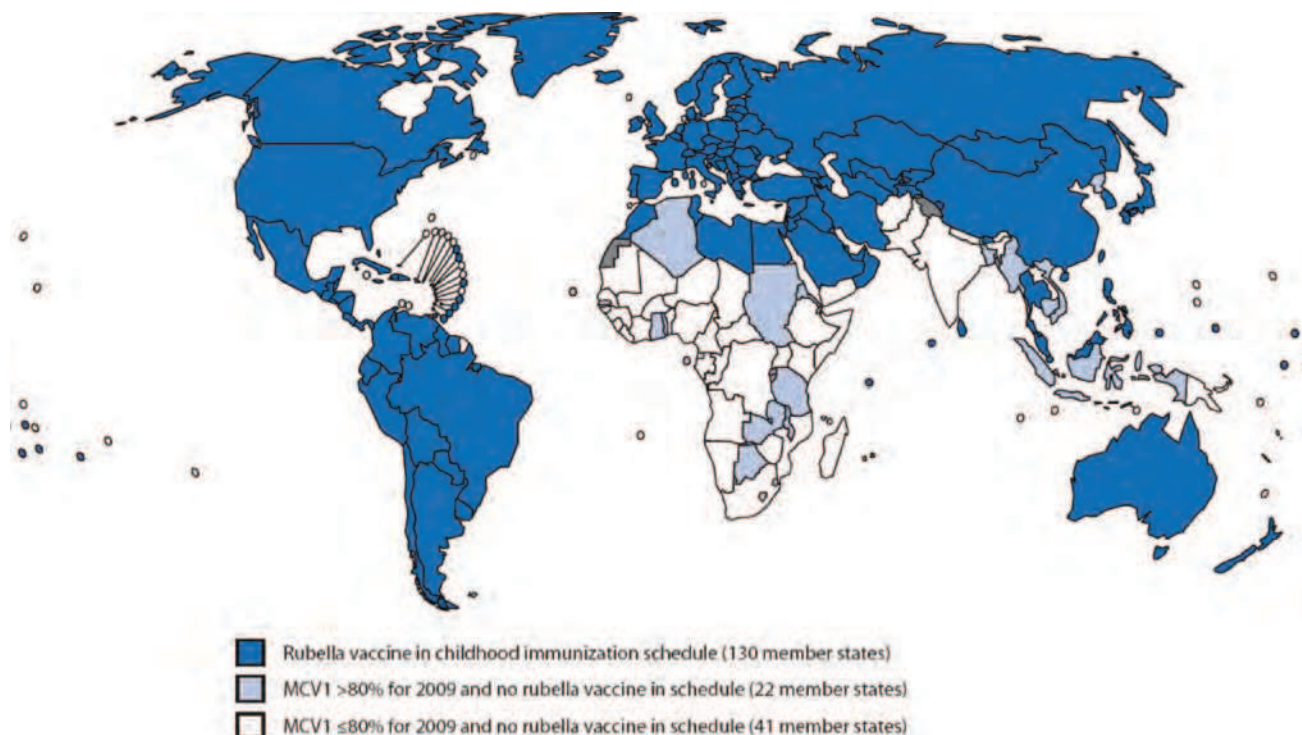


FIGURE 24.1. World map showing countries that have comprehensive rubella vaccination strategies. (From Progress toward control of rubella and prevention of congenital rubella syndrome—worldwide, 2009. *MMWR Morb Mortal Wkly Rep* 2010;59[40]: 1307–1310.)

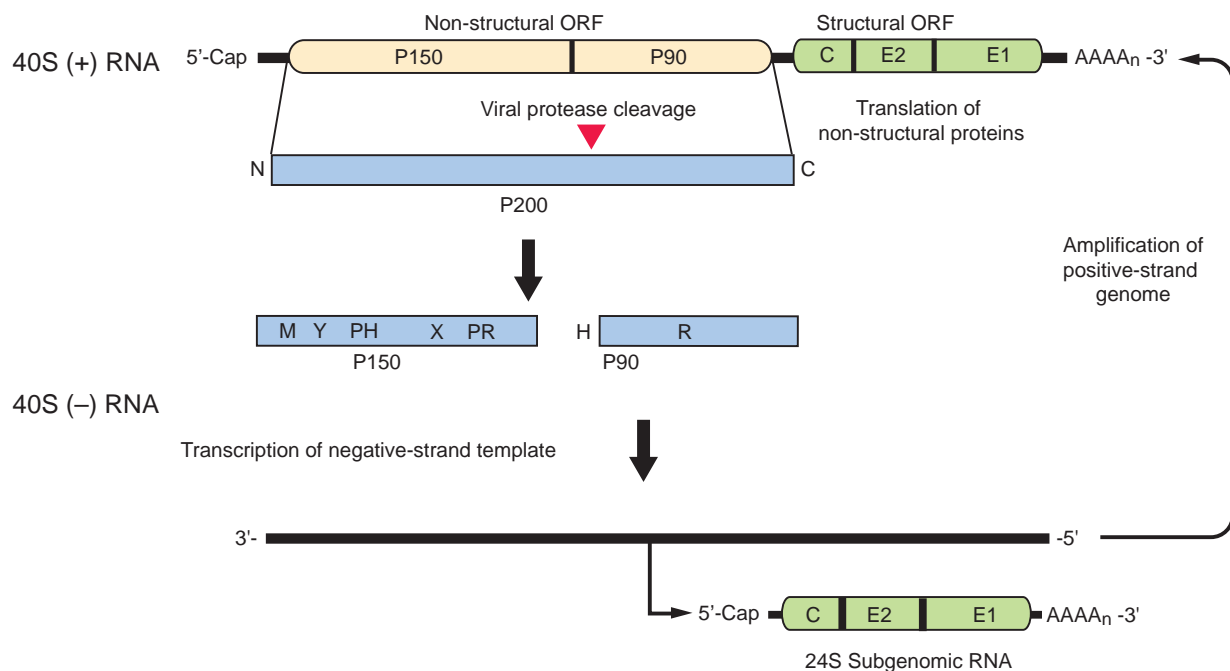
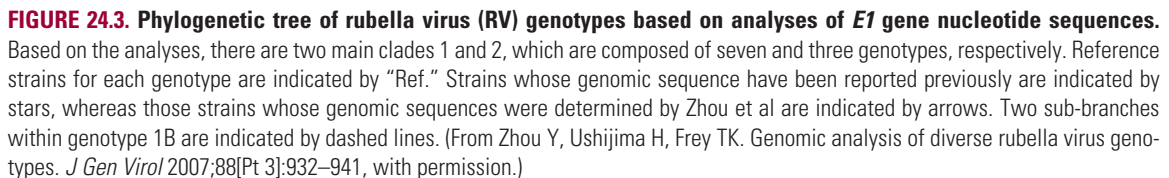


FIGURE 24.2. Organization and expression of the rubella virus (RV) genome. The genome is a single-stranded 40S RNA containing two open reading frames (ORFs). The 5' proximal ORF encodes the nonstructural proteins p150 and p90. The 40S genome serves as a messenger RNA (mRNA) for translation of the nonstructural protein precursor p200, which is cleaved by a virus-encoded protease to produce p150 and p90. The relative positions of methyltransferase (M), Y, proline hinge (PH), X, protease (PR), helicase (H), and RNA-dependent polymerase (R) domains within p150 and p90 proteins are shown. These two proteins form the viral replicase that synthesizes a negative-sense 40S RNA, which then serves as a template for synthesis of more genomic RNA and the 24S subgenomic RNA. Both the 40S and 24S positive-sense RNAs are capped and polyadenylated.



By transmission electron microscopy, RV virions appear as spherical particles with a mean diameter of 61 nm.¹⁶⁰ Virus particles can often be observed budding into the Golgi complex of infected cells (Fig. 24.4). They have an electron-dense nucleocapsid core surrounded by a host-derived envelope.¹⁵⁹ The nucleocapsid (30 to 40 nm in diameter) is composed of a single molecule of genomic RNA and multiple copies of capsid protein. Between the core and the envelope is an electron-lucent region giving the virions the appearance of having a ring or “toga” surrounding the nucleocapsid, from which the name is derived. Virus-encoded glycoprotein spikes project 6 to 8 nm outward from the lipid envelope²³⁶ and denote the virion surface. Early electron microscopic studies suggest that the RV

The buoyant density of rubella virions in sucrose gradients is 1.18 to 1.19 g/mL.¹⁰⁴ In comparison, the buoyant density of alphavirus virions is 1.20 g/mL, a difference that may be attributable to a wider electron-lucent zone between the core and envelope in rubella virions. The reported sedimentation coefficient of RV virions ranges from 240S and 350S.⁹ The reason for this heterogeneity is not known but may be due in part to contamination of the virus preparations by host cell-derived membranous material. The difficulty in obtaining highly purified homogenous preparations of RV virions is one of the numerous technical reasons that has impeded the progress of structural analysis.

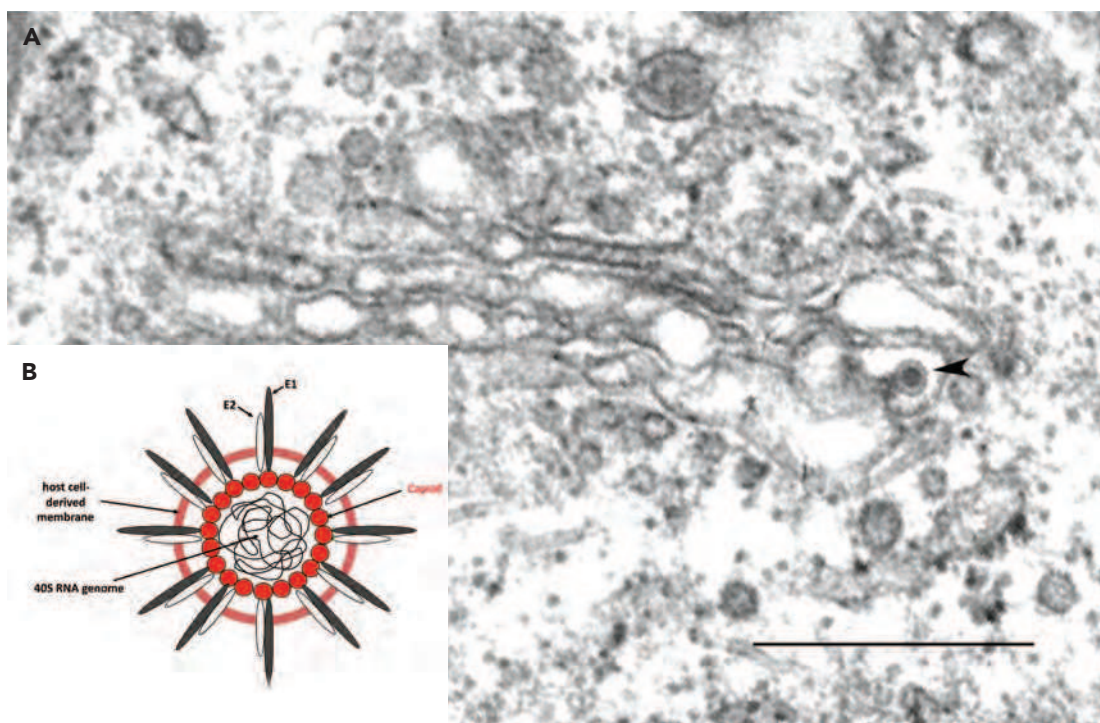


FIGURE 24.4. A: Electron micrograph of a rubella virion budding into the lumen of a Golgi cisterna in an infected Vero cell. Note the electron-lucent halo that surrounds the electron-dense nucleocapsid. Bar = 0.5 μm . (Courtesy of Dr. John Law and Ms. Honey Chan). **B: Schematic of a rubella virus (RV) virion.**

The infectivity of RV virions is rapidly lost following exposure to protein denaturing agents (formaldehyde, ethylene oxide, and β -propiolactone) or treatments/reagents that cause lesions in nucleic acids (ultraviolet light or photodynamic dyes). Moreover, because it is an enveloped virus, exposure of RV preparations to nonionic or ionic detergents and lipid solvents abrogates the infectivity of virions.¹⁸¹ Virions are rapidly inactivated by incubation at 56°C for less than 20 minutes, and even at much lower temperatures such as 37°C, RV loses activity slowly ($t_{1/2}$ = 48 hours).¹⁵² When stored at temperatures less than -60°C in the presence of protein stabilizers, RV preparations maintain their infectivity for many years. Fortunately, with respect to vaccine distribution, lyophilized RV preparations are stable for years at 4°C and for months at ambient temperatures.¹⁸⁴

Biological Characteristics

RV has a restricted host range (only humans) *in vivo* but replicates in a wide variety of cultured mammalian cell lines, including primary African green monkey kidney cells, BHK21, RK13, and Vero cell lines. All strains of the virus (including wt+ strains) are slightly temperature sensitive, with higher yields of virus obtained when infected cells are cultured at 35°C rather than at 37°C. The vaccine strains RA27/3 and Cendehill are completely growth restricted at 39°C, while some wt+ isolates are still able to replicate at 41°C, albeit at lower levels.³⁶

In comparison to the rapid lytic infection of mammalian cells by the alphaviruses, RV replicates more slowly, with an eclipse phase of 10 to 12 hours (c.f. 4 hours for Sindbis or

Semliki Forest viruses), and peak titers of secreted virus are not observed until 36 to 48 hours.^{87,242} By comparison, peak viral titers for alphaviruses are observed within 8 hours. As well as exhibiting relatively slow replication kinetics, the amount of infectious virus progeny released per cell is estimated to be 1,000-fold less than for alphaviruses. Under optimal conditions, RV-infected BHK-21 and Vero cells can produce titers of 10^7 and 10^8 plaque-forming units (pfu) per mL, respectively.^{10,241} The relatively feeble replication of RV may be due to a number of factors including low uptake of virus and/or inefficiencies associated with the replicase/transcriptase complex or limitations of the assembly process. For example, RV buds into the Golgi complex, which is a much smaller membrane compartment than the endoplasmic reticulum or the plasma membrane. Another factor that may play a role is the high G + C content of the viral genome (69.5%), which is predicted to form stable secondary and tertiary structures that may impede replication/transcription or translation.⁶⁸ Also related to the G + C content, the codon usage by RV is different from that of mammalian cells, requiring utilization of transfer RNAs (tRNAs) that may be of low abundance, a factor that has been suggested to limit the rate of protein synthesis.²²⁵

Effects on Host Cell

Although RV can have devastating effects on the developing human fetus, infection of most cultured primate or rodent cell lines does not result in major cytopathology. However, in some cell lines (Vero, BHK-21, and RK13), when high multiplicities of infection (MOIs) are employed, the virus causes rounding and detachment of cells commencing at 24 hours postinfection

and increasing over the next 72 hours. Much of the RV-induced cytopathic effect is likely the result of apoptosis.^{61,149,195} Virions that are first inactivated by ultraviolet light do not induce apoptosis, indicating that viral replication is required to cause cell death.^{61,100} The cytopathic determinants of RV have been mapped to the nonstructural genes,¹⁹³ and in agreement with this, expression of RV structural proteins alone in Vero cells does not induce apoptosis.¹⁰⁰ Paradoxically, primary human embryonic fibroblasts seem to be particularly immune to RV-induced apoptosis.³

At low (less than 1) or moderate (1 to 3) MOIs, little virus cytopathic effect is observed in most cultured cells^{61,116} and chronic infections can readily be established (see Replication Strategy section). Induction of apoptosis in response to RV infection is undoubtedly an important antiviral defense mechanism that is employed by cells against many RNA viruses. Because viruses are obligate parasites, they require a living cell for replication. Depending on the apoptotic stimuli, cells can be killed in a matter of hours, a situation that would be seemingly problematic for RV, which has a long eclipse period and whose replication peak occurs between 2 and 4 days postinfection.⁸⁷ However, the maximum RV-induced apoptosis does not occur until after 5 days postinfection,¹⁴⁹ which is consistent with a scenario where the virus actively blocks apoptosis early in infection. Indeed, a recent study revealed that RV-infected cells are highly resistant to apoptotic stimuli.¹⁰⁷ Mapping studies showed that the capsid protein is responsible for this process, which presumably provides a window of opportunity for the virus to replicate before apoptosis is initiated.

In most cell types, cellular macromolecular synthesis on a global level does not appear to be significantly affected, even following high-multiplicity infections (5 to 10 pfu per cell).

For example, host cell RNA synthesis continues normally and protein synthesis is only slightly reduced at 72 hours postinfection in several highly permissive cell lines.⁸⁷ However, in mitogen-stimulated peripheral blood mononuclear cells (PBMCs), host cell protein synthesis was reportedly inhibited by more than 90% at 48 hours postinfection.³⁸ The capsid protein has been shown to block translation *in vitro*,¹⁰⁸ but it has yet to be determined whether this viral protein is responsible for inhibition of protein synthesis in PBMCs or other cell types.

A number of characteristic morphologic changes occur in RV-infected cells. Similar to other togaviruses, RV infection is accompanied by drastic rearrangement of cellular membranes. For example, the endoplasmic reticulum (ER), Golgi complex, and mitochondria are often closely arranged around the virus replication complexes (Fig. 24.5), which are derived from endosomes and/or lysosomes.^{125,127} This arrangement of organelles could in theory facilitate the efficient transfer of virus genome from the site of RNA replication (endosomes) to the area of virus assembly (Golgi complex). Whereas organelle rearrangement is common in togavirus-infected cells, the formation of electron-dense plaques (22 to 25 nm in thickness) between organelles is unique to RV infection.¹²³ The plaques (Fig. 24.6) and associated organelles have been termed confronting membranes or confronting cisternae and commonly involve outer membranes of mitochondria and rough ER, adjacent mitochondria, and adjacent ER membranes, respectively. Expression of capsid protein in the absence of other viral proteins induces mitochondrial clustering and formation of plaques,¹⁵ and immunoelectron microscopy revealed that capsid protein is a major component of the plaques.¹⁰⁹ It has been known for years that a large pool of capsid protein is targeted to the surface

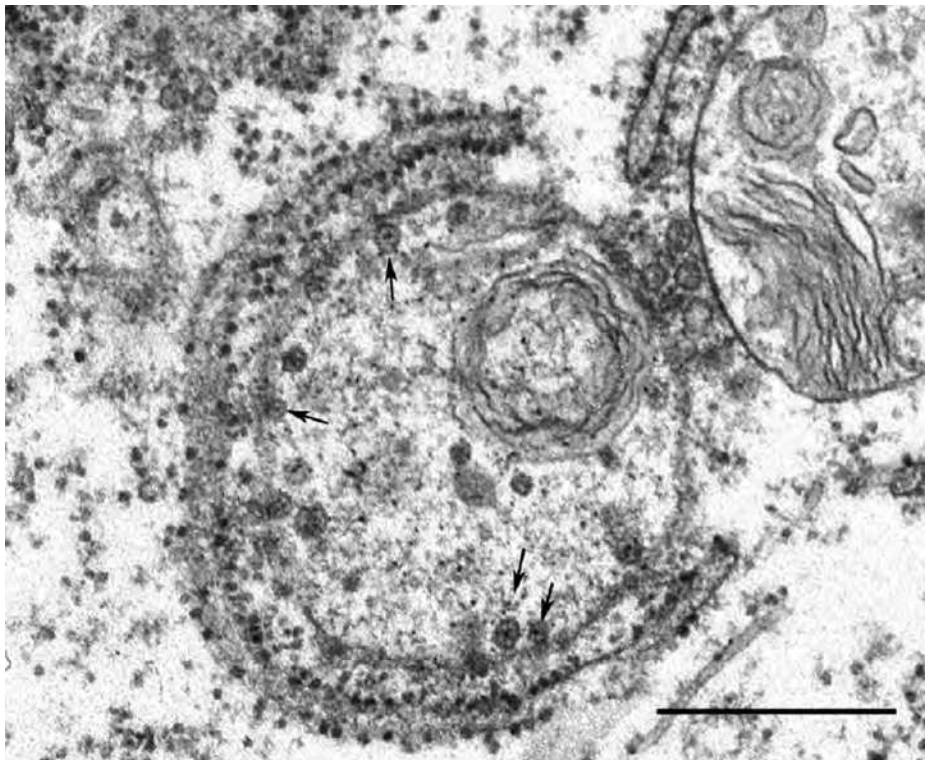


FIGURE 24.5. Electron micrograph of viral replication complex is associated with rough endoplasmic reticulum. Spherules are indicated by arrows. Bar = 0.5 μm . (Courtesy of Dr. John Law and Ms. Honey Chan.)

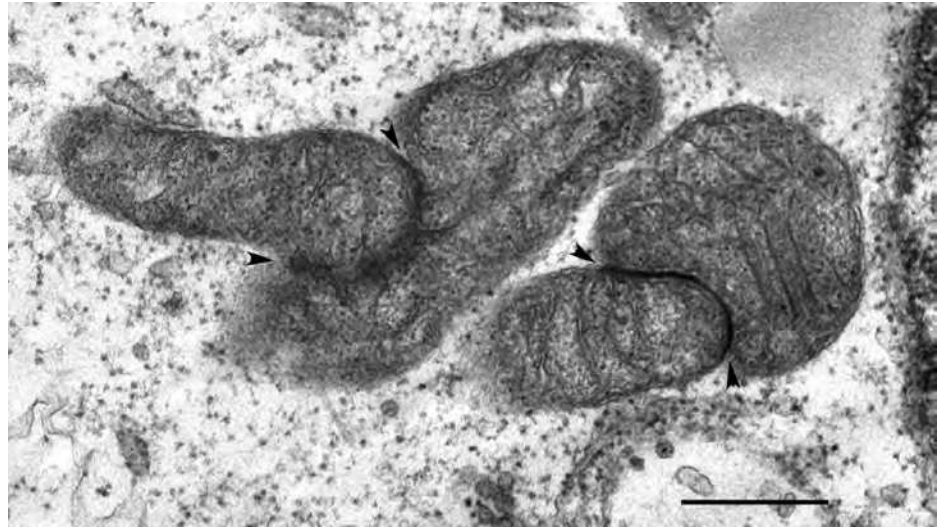


FIGURE 24.6. Aggregation of mitochondria and formation of electron-dense intermitochondrial plaques in rubella virus (RV)-infected cells. Bar = 0.5 μm . (Courtesy of Dr. John Law and Ms. Honey Chan.)

of mitochondria,^{16,126} and recently it was discovered that capsid affects mitochondrial import.¹⁰⁹ The latter may explain the loss of cristae and dysmorphic (club-shaped) mitochondria in RV-infected cells.¹²² Together, these observations reflect the close link between RV replication and mitochondrial physiology.

Phenotypic Variation

Although there are now recognized to be at least 10 genotypes of RV (Fig. 24.3), there is only one serotype, and infection with one strain provides protection against all others recognized to date. However, the limited genetic differences seen (0.8% to 2.1% at the amino acid level^{221,263}) are associated with differences in hemagglutination,¹³³ plaque morphology,¹¹⁶ temperature sensitivity, virus yield, and cell tropism.³⁶ Compared to natural RV infection, administration of vaccine strains is associated with milder acute symptoms and a lower incidence of complications such as joint and neurological symptoms,^{21,66,229} as well as teratogenic effects.¹³

The major neutralization epitopes appear to be highly conserved among RV strains,^{22,69} although differences have been noted in the kinetics of neutralization⁸¹ and in reactivity to certain monoclonal antibodies.³⁶ However, both infection and immunization are believed to provide protection against all other strains for the duration of the immune response elicited, except in cases where incomplete immunity is induced.²³⁰

The vaccine strains RA27/3 and Cendehill display different tissue tropisms from wt+ strains, including growth restriction in both PBMCs and the B-cell lines, Raji and Cess cells.³⁶ These two strains also show limited replication in cells derived from human joint tissue,¹⁵² with the Cendehill strain completely inhibited in synovial organ cultures and chondrocytes. In comparison, wt+ strains replicate to high titer (10^6 to 10^7 pfu/mL) in joint cells. The determinants that govern growth in joint tissue have been mapped to the 5' end of the genome, which encodes the nonstructural proteins, and as such, it is likely that replication rather than binding and entry events determine tropism.¹³⁵

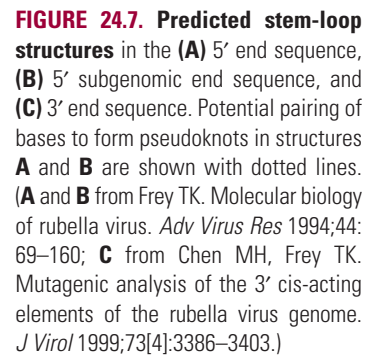
Structure and Organization of the Genome

The genome of RV is a single molecule of positive-strand RNA of approximately 10 kb with a GC content of 69.5%,

by far the highest of any RNA virus sequenced to date.⁶ The 5' end of the RNA has a 7-methyl-guanosine cap,¹⁶⁶ while at the 3' end there is a poly (A) tract with a mean length of 53 nucleotides.²⁴⁶ The genome consists of two nonoverlapping polycistronic open reading frames (ORFs) separated by a nontranslated region of 123 nucleotides (Fig. 24.2).¹⁹² The 5' proximal ORF (approximately 6,385 nucleotides) encodes the nonstructural proteins, while the 3' proximal ORF (3,189 nucleotides) encodes the three structural proteins, capsid, E2, and E1. The structural proteins are translated from a 5' capped and polyadenylated subgenomic RNA that is collinear with the 3' one-third of the 40S genome.¹⁶⁶ The complete nucleotide sequences have been determined for the genomes of several wild-type and vaccine strains of RV⁶⁸ and are available in GenBank. In addition, infectious complementary DNA (cDNA) clones of several strains have been produced and used to map genetic elements involved in viral replication and attenuation.^{135,196,246,259}

Cis-Acting Elements

The availability of infectious RV cDNA clones^{135,196,259} and replicons²³⁷ has enabled the application of reverse genetics to study the roles of *cis*-acting elements in genome replication. Both the 5' and 3' untranslated regions (UTRs) of the RV genome are believed to form secondary structures that influence transcription and translation events. For example, the predicted 5' stem-loop (5'SL), which encompasses nucleotides 15 to 65, has a terminal loop as well as a bulge in the stem and a hinge region (Fig. 24.7). It is believed to form a pseudoknot,¹⁹⁴ a structure that has been shown in a number of plant and animal viruses to enhance binding of proteins that function in RNA replication.^{57,110} Within the single-stranded leader sequence (nucleotides 1 to 14) and the stem-loop, there are three AUG codons starting at nucleotides 3, 41, and 57. Translation of the long ORF encoding the nonstructural genes is initiated at AUG₄₁, while the first and third AUGs are in a different reading frame and are terminated at nucleotides 54 and 90, respectively. Indeed, site-specific mutagenesis studies suggest that AUG₃ is not essential for viral replication,¹⁹⁴ nor is it known whether short peptides that originate from either AUG₃ or AUG₅₇ play any role in virus biology. However, it is notable that the



Between the nonstructural and structural genes is a 123-bp region that is predicted to form a series of stem-loops

(Fig. 24.7). This region shares 58% identity with the equivalent region in the Sindbis virus genome and may be important for regulating synthesis of the subgenomic RNA. In addition, translation of the structural genes is affected by mutations in this region, which forms the 5' end of the subgenomic RNA.¹⁸⁰ In the 3' UTR, there is a stretch of 59 nucleotides following the stop codon at nucleotide 9701. A complex secondary structure involving three major stem-loop structures⁴³ has been predicted for the 3' terminal 240 nucleotides (Fig. 24.7).

These 3' UTR sequences function in *cis* to regulate transcription, specifically, synthesis of plus-strand viral RNAs but not negative-sense RNA.⁴⁴

Encapsidation Signal

Mapping studies revealed that capsid protein binds with relatively high affinity to a 29-nucleotide segment located in the 5' nonstructural gene region between nucleotides 347 and 375.¹³²

Nonstructural ORF and Protein Products

The nonstructural (NS) genes are located in the 5' end of the viral genome within a long ORF commencing at AUG.⁴¹ The NS ORF is translated as a single polypeptide greater than 200 kD that is cleaved into two products, p150 and p90,¹⁴⁰ that function in replication and transcription of viral RNAs (Fig. 24.2). The gene order of the 5' ORF is NH₂-p150-p90-COOH. The two nonstructural proteins have several enzymatic activities including RNA polymerase, protease, and helicase and together form the viral replicase. Proteolytic processing of the p200 polypeptide marks the switch from synthesis of negative-strand genomic RNA to synthesis of plus-strand RNA.¹²⁸

p150 contains several domains that are conserved among other RNA virus-encoded proteins. A protease domain located in the carboxyl portion of p150 (Fig. 24.2) is responsible for cleavage of the nonstructural precursor protein p200 and is critical for virus replication.^{42,260} Mutagenesis studies have shown that the RV protease is a metalloprotease that requires divalent cations for activity^{130,131} and functions in both *cis* and *trans* modes.¹²⁹ A putative methyltransferase domain in the amino terminus is believed to have a role in capping the viral RNA.²¹¹ p150 also contains an X domain that has homology to the nonstructural proteins of alphaviruses, coronaviruses, and hepatitis E virus.⁸⁰ The function of the X domain is not well characterized, but it is required for *trans* cleavage by the RV protease and in alphaviruses at least is important for replication.⁸⁶ Interestingly, the X domain is the region that is most highly conserved between RV and alphaviruses.⁶⁰ Two other domains of unknown function, Y and a proline hinge, are also present in p150. The order of these domains in p150 is NH₂-methyltransferase-Y domain-proline hinge domain-X domain-protease-COOH. The second nonstructural protein, p90, comprises 905 amino acid residues and contains the replicase and helicase motifs. Based on comparison with global RNA-dependent RNA polymerase consensus sequences,¹¹¹ the GDD tripeptide at amino acids 1965 to 1967 of p90 is the active site of the RV replicase. An RNA-stimulated nucleoside triphosphatase (NTPase) that promotes unwinding of the template during RNA replication is associated with the helicase motif.⁸⁵

The localization of RV nonstructural proteins, p150 and p90, is less well studied than that of the structural proteins. This is because the nonstructural proteins are present at relatively low concentrations in infected cells, and furthermore, attempts to produce useful antibodies against these antigens has met with limited success. However, it has been reported that p150 associates with long tubular structures that correspond to sites of viral RNA synthesis.¹¹⁸ p90 has been detected in discrete cytoplasmic puncta that form linear chains.⁵ The identity of the p90-associated structures was not determined, but it is likely that they are replication complexes derived from endocytic vacuoles. Presumably, a large pool of p150 is also

localized to the p90-positive foci. No information regarding sequences that target the nonstructural proteins to endocytic membranes has been reported.

Structural ORF and Protein Products

The structural genes are contained within the 3' ORF of the 40S genomic RNA; however, they are actually translated from a subgenomic 24S RNA that is produced in infected cells. There are two in-frame AUG initiation codons in the subgenomic RNA separated by 21 nucleotides. The downstream AUG is in the more favorable context for translation initiation, but *in vitro* mutagenesis experiments indicate that both codons can be used.⁴⁷ The 24S subgenomic RNA encodes three structural proteins, which are translated in the order NH₂-C-E2-E1-COOH.¹⁶⁵ Unlike alphavirus structural proteins, which require both host cell signal peptidase and a capsid-associated protease for complete processing,¹⁵⁰ processing of the RV structural polypeptide (Fig. 24.8) into C, E2, and E1 only requires signal peptidase, which is encoded by the host cell.^{139,224}

In the absence of genomic RNA, coordinated expression of the RV structural proteins in mammalian cells results in the assembly and secretion of rubella virus-like particles (VLPs).^{94,199} The VLPs, which resemble native virions in terms of morphology and antigenicity, have served as a useful tool for studying virus assembly, and as a consequence, the roles of RV structural proteins and their domains in the assembly process are relatively well understood. The fact that rubella VLPs are efficiently assembled and secreted in the absence of genomic RNA indicates that virus budding is not tightly linked to nucleocapsid assembly.

Capsid

The capsid protein is a phosphoprotein with an apparent molecular mass of 33 to 35 kD.¹³⁹ The protein contains 300 amino acid residues and is rich in arginine and proline residues, particularly at its amino terminus, which gives it a net positive charge⁴⁶ and facilitates its interaction with genomic RNA during nucleocapsid formation. Capsid protein often migrates as a doublet on SDS-PAGE gels. The reason for this remains unknown, but it is not due to the use of an alternate translation start site.^{47,139} Virion-associated capsid protein can be isolated as disulfide-linked dimers, but intermolecular disulfide bonding is not necessary for formation of virus particles.^{12,124}

Cleavage of the RV capsid protein from the polypeptide precursor is distinct from that of alphavirus capsid proteins, which contains an autoprotease that separates the capsid protein from the polypeptide.¹⁵⁰ As a consequence, alphavirus capsids are free in the cytoplasm of infected cells. RV capsid protein, in contrast, lacks autoprotease activity, and separation from E2 is carried out by host cell signal peptidase.⁴⁶ The E2 signal peptide is therefore retained as the carboxyl terminus of capsid protein (Fig. 24.8), where it serves to confer membrane association of the protein.^{91,139,224} The membrane association of RV capsid protein is unique among togaviruses and may account for some of the unusual morphogenetic features of the virus. For example, retention of the E2 signal peptide on capsid protein is necessary for assembly of VLPs and presumably infectious virions.¹²⁰

Role of the Capsid Protein in Virus Assembly. The primary function of capsid protein during virus assembly is to homo-oligomerize and bind viral genomic RNA to form

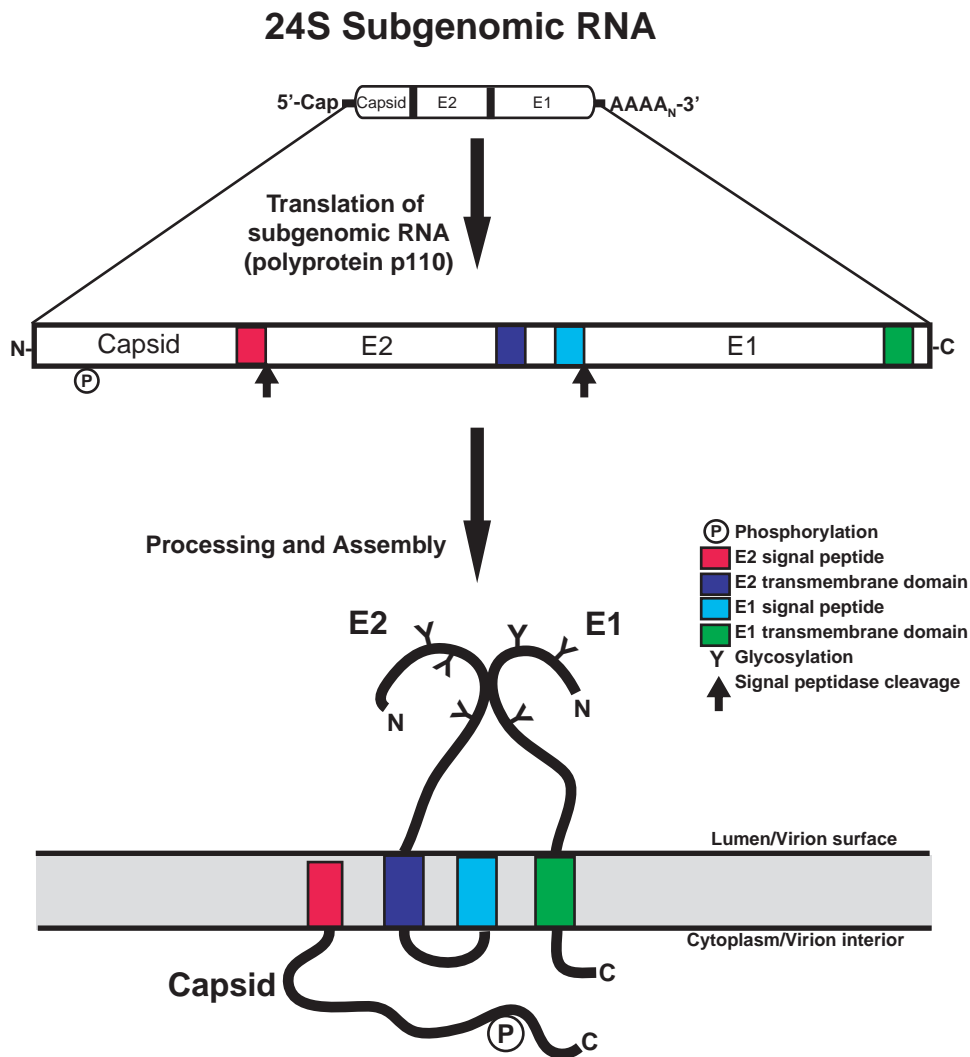


FIGURE 24.8. Processing of rubella virus (RV) structural proteins. The 24S RNA serves as the messenger RNA (mRNA) for translation of a structural polyprotein precursor, which is then cleaved by host signal peptidase to produce capsid, E2 and E1. The relative membrane orientations of the structural proteins immediately following translocation into the endoplasmic reticulum (ER) are shown. The amino (N) and carboxyl (C) termini of the proteins are indicated.

the nucleocapsid. The RNA-binding domain of capsid protein resides within amino acid residues 28 to 56 and binds to a packaging signal (nucleotides 347 to 375) in the genomic RNA.¹³² Posttranslational modification of capsid protein may play an important role in regulating the formation of nucleocapsids because phosphorylation of serine 46 within the RNA-binding domain negatively regulates RNA binding.^{119,121} This is believed to prevent nonspecific binding of cellular RNAs to capsid protein and to delay binding of genomic RNA until the virion components are targeted to the budding site. *In vitro*, capsid protein is a substrate for protein phosphatase 1A, an enzyme that has been implicated in Golgi-associated functions.¹²¹ Accordingly, targeting of capsid protein to the Golgi complex, followed by dephosphorylation at this site, could explain how nucleocapsid assembly is synchronized with virus assembly (Fig. 24.9). Supporting this scenario is the observation that virion-associated capsid protein has a higher affinity for viral RNA and contains significantly less phosphate than cell-associated capsid protein.^{119,121} Similar to alphaviruses,²²³ it is thought that capsid protein drives budding of RV through interactions with the envelope glycoproteins; however, direct binding between RV capsid protein and E2 and/or E1 has yet to be demonstrated.

Nonstructural Roles of the Capsid Protein. In addition to nucleocapsid assembly, the RV capsid protein plays a role in regulating viral transcription and replication. The first indication of this nonstructural function came from the observation that expression of capsid protein rescues the replication of an RV replicon containing an in-frame deletion of p150.²³⁸ Further analyses indicated that the capsid protein is involved in modulating viral genomic and subgenomic RNA synthesis.²³⁹ In the absence of capsid protein expression, the ratio of genomic RNA to subgenomic RNA is substantially lower. It is believed that a pool of capsid protein that associates with the replication complexes is responsible for regulating synthesis of viral RNAs. Interestingly, the effects of capsid protein on transcription depend on the levels of replicon RNA.⁴⁵ At low levels of RNA, capsid protein expression enhances replication of viral RNA, but with higher levels of RNA, capsid protein is inhibitory for this process.

Given its multiple roles, it is not surprising that the RV capsid protein is associated with multiple organelles. Consistent with its function in virus assembly, pools of capsid protein are localized to the Golgi.^{11,94} Transport of capsid to the Golgi region from the site of its synthesis, the ER, depends on E1 and E2.^{74,120} Capsid also localizes to mitochondria and to virus

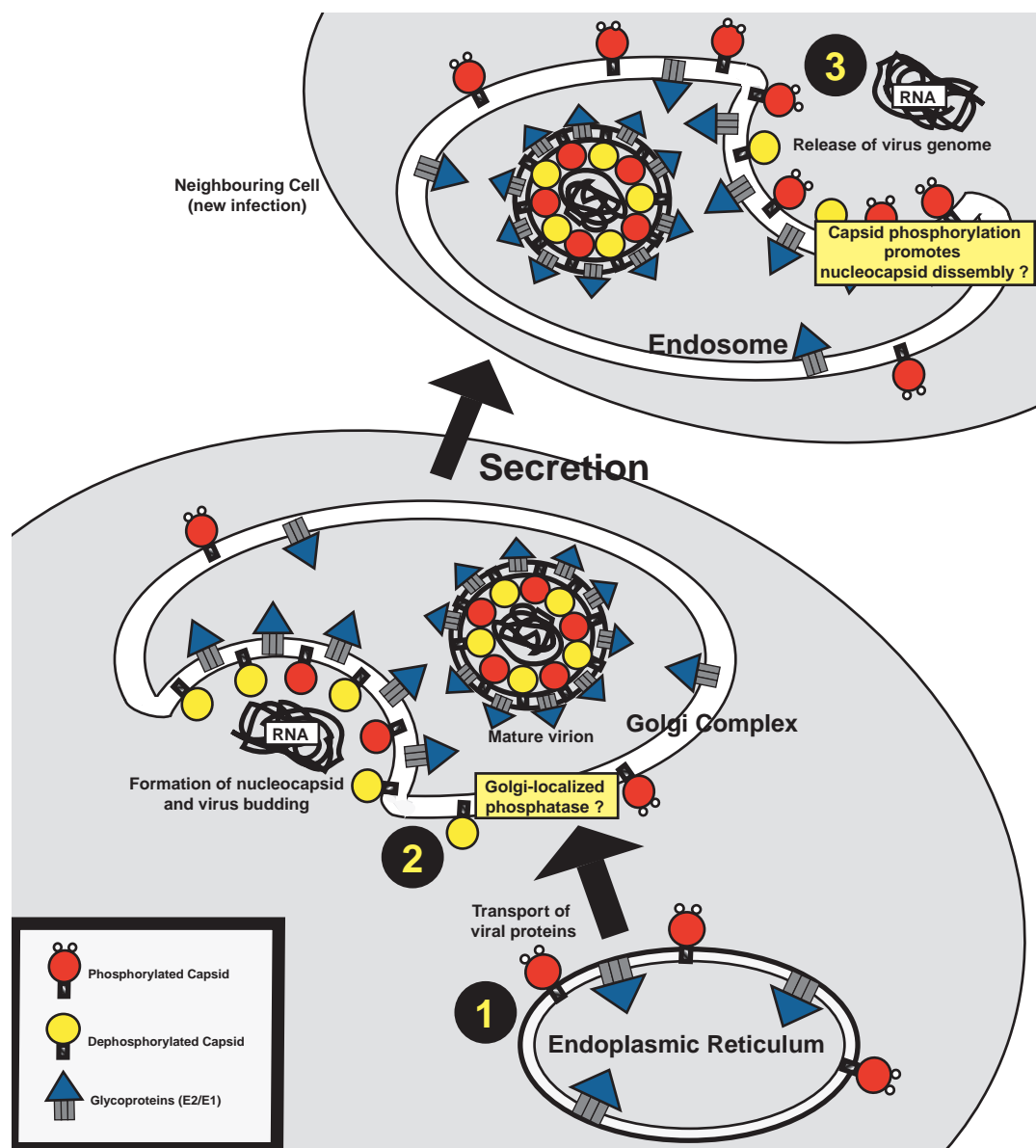


FIGURE 24.9. Model to illustrate the putative roles of dynamic phosphorylation of capsid protein in virus replication. **1:** Phosphorylation of newly synthesized capsid protein prevents nonspecific binding of RNA and premature formation of nucleocapsid at the early stages of virus assembly. **2:** Capsid protein is subsequently targeted to the Golgi complex and dephosphorylation of the protein at this stage allows interaction with the genomic RNA, formation of the nucleocapsid, and subsequent virus budding. **3:** Timely rephosphorylation of capsid protein before or during virus entry promotes the disassembly of nucleocapsid.

replication complexes.^{16,123} In contrast, the glycoproteins E1 and E2 are targeted only to the virus budding site (Golgi), suggesting that they do not have major nonstructural roles. Together with p150, replication complex–localized capsid protein¹¹⁸ is thought to modulate replication of viral RNA, whereas the mitochondrial pool of capsid plays one or more critical roles in virus–host interactions (Fig. 24.10). For example, it potently blocks apoptosis by sequestering the proapoptotic host protein Bax into nonfunctional complexes.¹⁰⁷ RV capsid also interferes with mitochondrial import,¹⁰⁹ a critical process that is linked to apoptosis in mammalian cells.¹⁸³ Finally, it has been known for

more than a decade that capsid interacts with the mitochondrial matrix protein p32.¹⁶ The precise function of this interaction is not clear, but several lines of evidence suggest that it is important for virus replication. First, ablation of the p32-binding site in capsid protein reduces the replication efficiency of RV by 1,000-fold.¹⁵ Second, depletion of cellular p32 pools by RNA interference results in a 10-fold reduction in virus replication.⁴⁹ Capsid protein–p32 interactions may also be important for synthesis of subgenomic RNA and/or translation of structural proteins. Finally, loss of stable binding between capsid protein and p32 is correlated with reduced mitochondrial clustering in

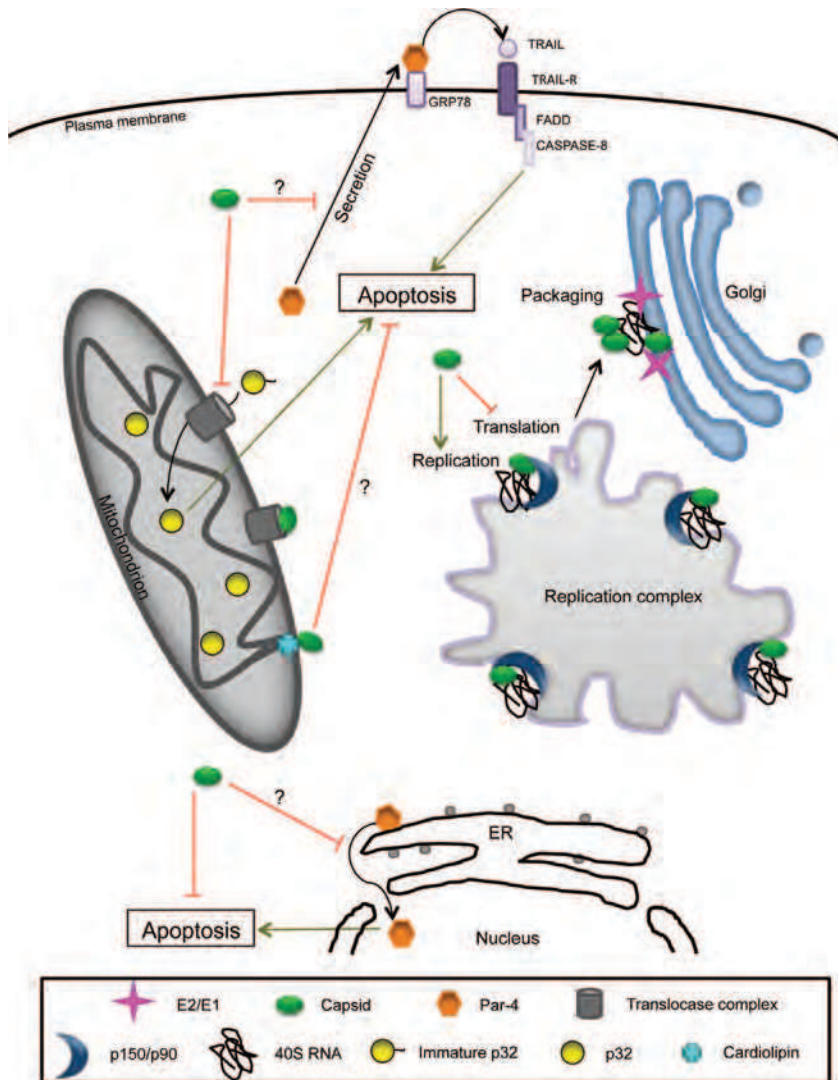


FIGURE 24.10. Integrated model of rubella virus (RV) capsid nonstructural functions. At the replication complex, capsid associates with nonstructural proteins (p150 and p90) and regulates transcription and replication of viral RNA. Late in the infection cycle, local sequestration of translation initiation factors such as poly(A) binding protein (PABP) may modulate the switch from translation to packaging of the genomic RNA, a mechanism that would leave the viral RNA available for packaging into nucleocapsids at the Golgi complex. Capsid prevents apoptosis by binding to Bax and inducing the formation of heterooligomers that are incompetent for pore formation at the mitochondria and potentially at the endoplasmic reticulum (ER) membranes. Capsid may also inhibit apoptosis by preventing translocation of pro-apoptotic proteins such as p32 into the mitochondria or by engaging in complexes with the mitochondrial lipid, cardiolipin. (Modified from Ilkow CS, Willows SD, Hobman TC. Rubella virus capsid protein: a small protein with big functions. *Future Microbiol* 2010;5[4]:571–584.)

the perinuclear region. Together, these results suggest that interactions between capsid protein and p32 are important for RV-induced rearrangement of mitochondria, a phenomenon that is associated with optimal replication.

ENVELOPE PROTEINS

E1 and E2 are type I membrane proteins that dimerize to form the spike complexes on the surface of the virion (Fig. 24.8). The major functions of these spikes are to bind receptors on the host cell and to mediate fusion with cell membranes.¹¹⁴ The envelope proteins, E1 in particular, are the major antigenic determinants against which neutralizing antibodies are directed.²⁴⁹

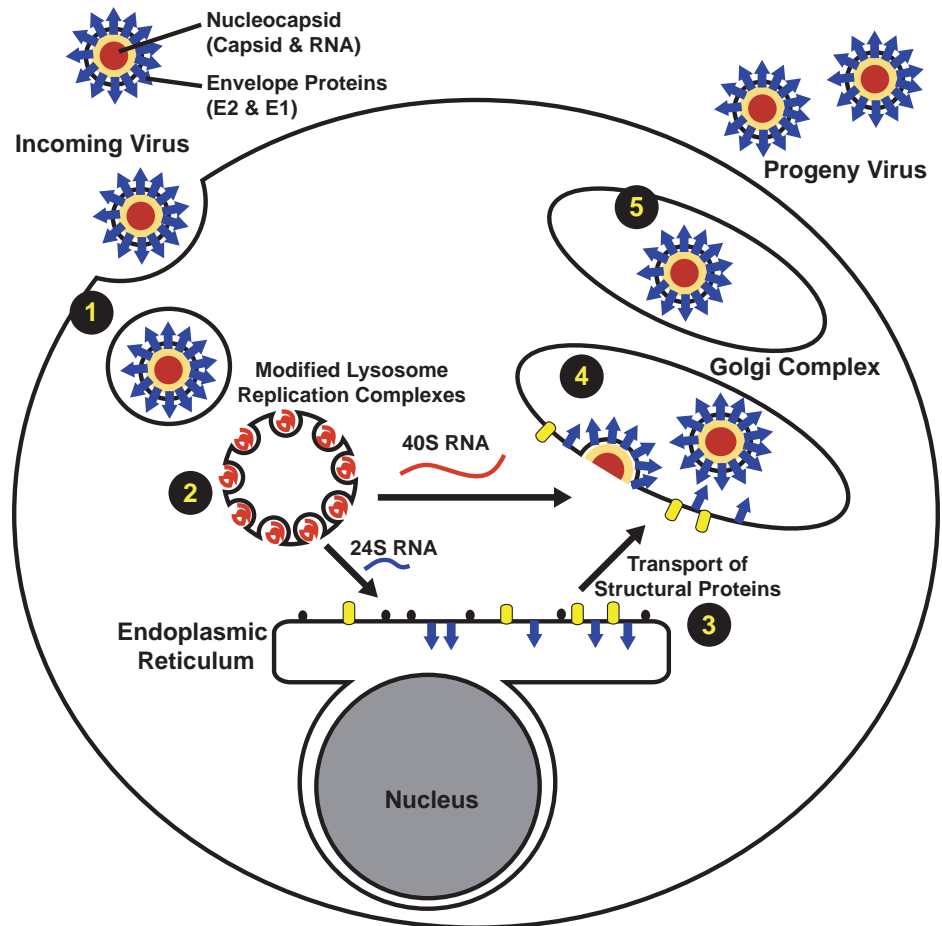
E1 is 481 amino acid residues in length and exhibits an apparent molecular mass of 58 kD when analyzed by SDS-PAGE. The mature protein contains three asparagine-linked oligosaccharide moieties.⁹⁵ An amino terminal signal peptide facilitates translocation of E1 into the ER and a 22-amino acid hydrophobic sequence at its carboxyl terminus mediates membrane association.⁹⁶ E1 also contains a carboxyl terminal tail of 13 amino acid residues that is exposed to the cytoplasm. E2 (282 amino acids residues) contains two stretches of hydrophobic amino acid residues near its carboxy terminus,

an 18-amino acid residue transmembrane domain, and the E1 signal peptide.^{11,46} These two hydrophobic domains are separated by a 7-amino acid residue loop that is rich in basic amino acid residues. Similar to E1, translocation of E2 into ER membranes is mediated by an amino terminal signal peptide.⁹¹ E2 is heavily glycosylated and contains both asparagine-linked and O-linked carbohydrates.^{96,136} Finally, both glycoproteins are modified by the addition of palmitate prior to virus assembly.^{93,249} The role of this modification in spike protein function has not been investigated, but it is possible that acylation may serve to further stabilize and/or orientate E2 and E1 in the membrane. The membrane orientations of the RV structural proteins are shown in Figure 28.8.

Replication Strategy

The replication strategy of RV is similar to that of alphaviruses in many respects, but there are several quantitative and qualitative differences. As discussed previously, RV replication is less robust and much slower than that of alphaviruses.⁸⁷ In addition, it is not possible to obtain a uniformly infected population of cells within 24 hours, even at high multiplicities of infection.²¹⁴ The reason for this phenomenon is not clear, but

FIGURE 24.11. Entry and exit strategy of rubella virus (RV). 1: Uptake of RV is dependent upon receptor-mediated endocytosis. The low pH of the endosome/lysosome induces virus uncoating. 2: RNA replication occurs at the cytopathic vacuoles that originate from endosomes/lysosomes. Genome amplification and synthesis of the 24S subgenomic RNA also occurs at these sites. 3: Synthesis of structural proteins takes place at the endoplasmic reticulum and processing by the host cell signal peptidase separates the polyprotein into individual structural proteins. Subsequently, the structural proteins assemble and are transported to the Golgi complex. 4: Nucleocapsid assembly and virus budding occur at the Golgi complex. 5: Rubella virions undergo a series of maturation events before exocytosis.



given the fact that eventually all cells within a permissive culture become infected, it is possible that a cell cycle-dependent factor is required for RV entry or early viral replication events. Another major difference from the alphaviruses is that a large pool of the nascent RV capsid protein remains membrane associated.^{120,224} This may be one of the reasons that RV nucleocapsid assembly differs from that of alphaviruses, which are not membrane associated. Free nucleocapsids are rarely observed in RV-infected cells but rather assembly is coincident with virus budding at the Golgi complex, a process that is hypothesized to be regulated by reversible phosphorylation of the capsid protein.¹²¹ A schematic diagram of how RV enters, replicates, and exits from host cells is shown in Figure 24.11.

Virus Entry

Host cell–encoded proteins and lipids are important for virion binding,^{50,142} after which the virus is internalized by receptor-mediated endocytosis.¹⁸⁶ At low pH, the envelope glycoproteins become fusogenic, and capsid undergoes a conformational change and becomes hydrophobic,^{114,145} indicating that the acidic environment within endosomes induces fusion of the viral envelope with cellular membranes followed by release of genomic RNA from the nucleocapsid. The type I membrane protein myelin oligodendrocyte glycoprotein binds to the E1 glycoprotein and was recently identified as a host receptor for RV.⁵⁰ This protein is most highly expressed in the central nervous system, but in epithelial and lymphoid cells, which serve as the portals

of entry and initial sites of replication, myelin oligodendrocyte glycoprotein levels are much lower. As such, it cannot be ruled out that the virus uses different receptors in different cell types.

Replication Complexes

Similar to all other plus-strand RNA viruses that infect mammalian cells, replication of RV RNA occurs in association with cellular membranes. Cytoplasmic vacuoles (Fig. 24.5) with regularly shaped invaginations or spherules (60 nm in diameter) are observed in RV-infected cells.¹²⁷ The spherules are connected to the vacuolar membranes by thin membranous necks and can be stained with antibodies against p150 and double-stranded RNA, suggesting that they are the sites of viral RNA synthesis.^{118,125} These vacuoles also co-localize with lysosomal markers, indicating that they are derived from endosomes and/or possibly lysosomes.¹³⁸

Recently, replication complexes in RV-infected cells have been analyzed using electron microscopy and freeze fracture techniques.⁶⁴ Replication of viral RNA occurs within protected membranous pockets of the cytopathic vacuoles. The vacuoles are surrounded by rough ER, an arrangement that may facilitate binding between RNA and the capsid protein (which is synthesized on the surface of the ER) to promote nucleocapsid formation. In a number of fundamental aspects, RV replication complexes are similar to those found in alphavirus-infected cells.⁷¹ However, there are several important distinctions between them. First, assembled nucleocapsids are rarely

observed near replication complexes in RV-infected cells. In contrast, nucleocapsids are regularly observed surrounding the replication complexes of alphavirus-infected cells. In RV-infected cells, formation of the replication complexes coincides with the rearrangement of other cellular structures. Specifically, rough ER and Golgi are in close proximity to the virus-modified endocytic organelles, and mitochondria cluster to regions of the cell containing replication complexes.^{123,127} Although mitochondrial aggregation in the vicinity of replication complexes also occurs in alphavirus-infected cells, the close association of the rough ER and Golgi with replication complexes is unique to RV-infected cells. Recruitment of ER and Golgi membranes to the replication complexes may be necessary to coordinate translation of the structural proteins and packaging of the genomic RNA. Mitochondria are also recruited to the replication complexes, but the reason for this is not clear. It has been suggested that this arrangement of organelles would increase the availability of adenosine triphosphate (ATP) for virus replication, but solid evidence to support this theory is lacking.

Targeting of Structural Proteins to the Budding Site

Following synthesis of the subgenomic RNA, the RV structural proteins are translated in association with ER membranes. The structural polyprotein precursor is cleaved by signal peptidase at two sites (Fig. 24.8) to generate the three structural proteins.^{91,96} The structural proteins are then posttranslationally modified and transported to the Golgi complex, which is the primary site of virus budding. Processing and transport of the RV structural proteins have been extensively studied using transfection experiments. When co-expressed, E2 and E1 heterodimerize, form intramolecular disulfide bonds in the ER, and are transported to and retained in the Golgi.^{11,99} Targeting of the virus glycoproteins to the Golgi complex is independent of capsid, indicating that accumulation of E2 and E1 in this organelle is the major factor in determining the budding site. In the absence of E2, E1 does not target to the Golgi but rather is retained in a smooth ER compartment.⁹⁸ The membrane proximal regions of the structural proteins have important roles in regulating the localization and transport of these proteins. For example, within the transmembrane and the cytoplasmic domains of E1 resides an ER retention signal that prevents or delays transport of nascent viral glycoproteins from the ER.⁹² Co-expression of E2 appears to mask the ER retention signal in E1, thereby allowing transport of the E2-E1 heterocomplex from the ER to the Golgi after a maturation period. The transmembrane domain of E2, in turn, contains a retention signal that mediates retention of the E2-E1 heterodimer in the Golgi.⁹⁷

The highly regulated transport of RV glycoproteins may serve as a quality control mechanism to ensure only properly assembled subunits reach the virus budding site. The half-life for transport of the RV glycoproteins to the Golgi is 60 to 90 minutes (whereas for alphavirus glycoproteins it is approximately 25 minutes).⁹⁰ Dimerization of E1 and E2 in the ER is not rate limiting as evidenced by the fact that these two proteins associate rapidly following synthesis. E2 achieves its mature conformation shortly after synthesis and has been proposed to function as a scaffold for E1. Accordingly, the rate-limiting step for transport of the glycoproteins is the relatively slow maturation and folding of E1 in the ER.⁹⁹ The mature ectodomain of E1 contains 20 cysteine residues that participate in the formation of intramolecular disulfide bonds.⁸⁴ It is hypothesized

that the ER retention signal in E1 is required to retain nascent E1 in the ER until it has achieved the proper conformation, which includes the formation of intramolecular disulfide bonds. Indeed, replacing the E1 transmembrane domain with analogous domains from other membrane glycoproteins actually increases the rate of ER-to-Golgi transport for E1 and E2.⁷⁴ The E2 transmembrane and cytoplasmic domains are required for assembly of E2-E1 heterodimers and therefore have an indirect role in regulating transport of the RV glycoproteins from the ER to the Golgi. Finally, mutagenesis studies have shown that N-linked glycosylation is important for maturation and intracellular transport of both E1 and E2 glycoproteins.^{95,198}

As well as regulating targeting of E1 to the Golgi complex, E2 is required for transport of the capsid protein to the intracellular budding site.^{11,94} The carboxyl terminus of E2, which includes the transmembrane and cytoplasmic domains, is essential for this process⁷⁴ and formation of VLPs¹²⁰ and likely infectious virions. Lateral interactions between the E2 signal peptide and the transmembrane regions of E2 and/or E1 may facilitate transport of capsid from the ER to the Golgi by incorporation into ER-derived transport vesicles. In contrast, the analogous regions of E1 are not required for localization of capsid to the juxtannuclear region. Together, these results indicate that the E2 carboxyl terminus governs the targeting of all three RV structural proteins to the virus budding site.

Virus Assembly and Secretion

RV budding has been reported to occur at both the Golgi complex and the plasma membrane, depending on cell type and the time postinfection.^{10,244} However, several lines of evidence suggest that the primary site for virus budding is the Golgi complex. First, the presence of a signal in E2 mediates retention of the structural proteins in the Golgi complex, and consequently, only a small fraction of the RV structural proteins reaches the plasma membrane.^{97,99} Second, budding at the plasma membrane reportedly occurs late in infection, suggesting that this is not the principal budding site. Furthermore, virion membrane composition is more similar to that of intracellular membranes rather than the plasma membrane.⁸

Unlike alphavirus nucleocapsids, which are formed in the cytoplasm of infected cells prior to budding, RV nucleocapsids form in association with cellular membranes coincident with budding and are rarely observed in the cytoplasm of infected cells.¹²⁶ It has also been hypothesized that RV employs a mechanism to prevent premature assembly of nucleocapsids (Fig. 24.9). Specifically, reversible phosphorylation of the RNA-binding region of capsid may regulate assembly and disassembly of RV nucleocapsids.^{119,121} Whether phosphorylation of capsid proteins has any role in assembly of alphavirus nucleocapsids has not been investigated.

The mechanisms that regulate interactions between the nucleocapsid and spike glycoproteins during virus budding are largely unknown. With respect to the budding reaction, it is tempting to speculate that electrostatic interactions between the cytoplasmic domain of E2, which is rich in basic amino acid residues, and clusters of acidic amino acid residues in the capsid are involved. Indeed, nonconservative mutations in this E2 domain drastically affect the assembly of VLPs.⁷⁴ It is also well established that the transmembrane domains of the structural proteins are important for targeting to the budding site. In this respect, lateral interactions between the transmembrane

domains of E1, E2, and capsid protein may be important for the budding reaction. Finally, it is important to consider that interactions between RV structural proteins and host cell proteins contribute to efficient budding of virus particles.

A morphologic study that employed freeze-substitution electron microscopy revealed that rubella virions undergo a maturation process following budding into the Golgi.²⁰⁶ Specifically, at early time points, intra-Golgi virions exhibited homogenous interiors with fine contacts between the core and the particle membrane. At later time points the virion cores appeared denser and were smaller in diameter such that they were clearly delineated from the virion membrane. These results indicate that the virion maturation process involves compaction of the nucleocapsid. Several lines of evidence suggest that the E1 transmembrane and cytoplasmic domains also function in virion maturation and/or secretion. Replacement of the E1 transmembrane domain does not affect budding of VLPs in the Golgi, but secretion of the particles into the extracellular space is blocked.⁷³ Similarly, introduction of point mutations into the E1 transmembrane and/or cytoplasmic domains drastically reduces virus secretion without affecting the assembly process at the Golgi.^{200,259} These rather surprising data indicate that virus assembly at the Golgi complex is not coupled to secretion and that E1 is involved in a late-stage maturation event that is necessary for virus secretion.

There is limited information regarding the interactions between RV virions and polarized cells. Because it causes a systemic infection, the virus must cross one or more epithelial layers, including the upper respiratory epithelium. Cultured epithelial cells can be infected from the apical and basal membranes, indicating that RV receptors are not confined to one surface.⁷³ The secretion of virus particles varies according to cell type. In two of the three polarized cell lines examined, virions were released primarily from the apical surface, but significant quantities were also secreted from the basolateral membrane. Presumably, secretion of rubella virions from the apical surface facilitates virus spread from person to person, whereas basolateral secretion could be important for establishing a systemic infection and/or crossing the placenta prior to fetal infection.

RV Persistence

Although cell death by apoptosis occurs in highly susceptible cell lines infected at high MOIs, infection of a wide variety of cells *in vitro* with low MOIs results in little cytopathology and viral persistence.^{1,3,70,255} In fact, a recent study has shown that RV-infected cells are quite resistant to a variety of apoptotic stimuli as a result of interaction between the capsid protein and the host protein Bax.¹⁰⁷ These findings are consistent with earlier observations that while temperature-sensitive mutants and defective interfering (DI) particles develop in and may play a role in controlling replication in persistently infected cells, neither is required to establish persistent infection.^{70,255} During long-term persistence in RV-infected Vero cells, DI RNAs become the dominant species of viral RNA, with genomic RNA decreasing to low levels. Persistence in cultured cells is thus a chronic infection, with the majority of cells expressing viral antigen and RNA, much of which is DI RNA. These cultures release low levels of temperature-sensitive progeny virus and DI particles. Finally, while it cannot be ruled out that interferons play a role in viral persistence, they are clearly not essential because persistent RV infections can be established

in interferon-deficient cell lines such as Vero and BHK-21 cells.²²² Moreover, exogenous interferon was not found to have an effect on RV persistence in these cell lines.

The virus can persist for many months in congenitally infected human fetuses, and multiple organ systems are affected (see Mechanisms of Teratogenesis section). Following postnatal infection, PBMCs are an established site of RV persistence *in vivo*, particularly in adults who develop rubella-associated arthritis following natural infection or immunization.³⁵ Viral persistence *in vivo* occurs in the presence of high levels of neutralizing antibody, which not only may limit viral spread but also has been suggested to actually promote viral persistence. To date, however, this theory has yet to be substantiated. Another site of persistence *in vivo* is joint tissue,^{39,67} which has led to speculation that RV plays a role in development of degenerative joint disease (see Complications section).

In vitro, both wt+ and vaccine strains of RV can infect and persist in chondrocyte-derived cell lines³⁶ and in primary cultures of human joint tissue.¹⁵² Except for the Cendehill vaccine strain, all other RV strains can replicate and persist in joint tissue for more than 3 months. Interestingly, virus derived from these chronically infected cultures was temperature sensitive, but no DI particles were detected.

Animal Models of Rubella Virus Infection

There are no reliable animal models for the study of clinically symptomatic rubella infection. However, a variety of laboratory animals (including nonhuman primates) can be asymptomatically infected with RV. For example, rhesus and African green monkeys develop viremia, shed virus in respiratory secretions, and produce humoral immune responses in a manner that is similar to acute rubella in humans.¹⁰¹ However, attempts to model the teratogenic effects of rubella in various animals, including baboons, cynomolgus monkeys, marmosets, and rats, have produced inconsistent results with, at best, low incidence of defects such as cataracts or stillbirths.^{248,251} Infection of the central nervous system (CNS) has been shown to occur in immunosuppressed BALB/c mice, and as such, it is possible that this system could work as a small animal model to understand the effects of RV on the brain.¹⁴¹ Unfortunately, none of the animal systems has proven sufficiently reliable to study details of the pathogenesis of either acquired or congenital rubella.

Antigenic Composition and Determinants

Complement-Fixing and Hemagglutinating Antigens

Early serologic tests for rubella used crude complement fixation antigens extracted from virus-infected cells and purified on sucrose gradients. A more slowly sedimenting fraction from the gradients with a density of 1.08 to 1.10 in sucrose (as compared to a density of 1.18 to 1.20 for intact virions) was shown to have hemagglutinating (HA) activity.²³⁶ HA activity is also associated with cell-free virus preparations¹⁰⁵ and can be extracted from infected cells or supernatant virus using Tween 80 and ether, resulting in a 26S particle that retains biological activity. By electron microscopy, it appears as a 15-nm rosette with a hollow core.²³⁵

B-Cell Epitopes

The E1 glycoprotein is the immunodominant surface molecule of the virus particle as evidenced by the fact that it is the major target for the host's humoral immune system.²⁵⁶ Antibodies to

both E2 and capsid are also found in humans, although at lower levels and of lower avidity. Mapping of antigenic domains on the viral proteins was first carried out using monoclonal antibodies.²⁵⁶ Later, detailed studies on the fine antigenic structure of each viral protein were carried out using recombinant proteins, proteolytic peptide fragments, and synthetic peptides to deduce antigenic sites.^{40,147,154} At least five distinct nonoverlapping immunoreactive regions were identified in the E1 protein (E1_{11–39}, E1_{154–179}, E1_{199–239}, E1_{226–277}, and E1_{389–412}). Cellular proliferative responses to these peptides were found in 29% to 83% of the subjects tested,¹⁵⁴ including one peptide (E1_{208–239}) that contained a previously identified neutralization domain.²⁵⁷ Reactivity with this peptide in an enzyme-linked immunoassay (ELISA) correlates well with assays that measure hemagglutination inhibition (HAI) and virus neutralization.^{54,157} To date, two B-cell epitopes have been identified on the capsid protein, C_{1–30} and C_{96–123}, and one (E2_{31–105}) on the E2 glycoprotein.^{40,147,173}

T-Cell Epitopes

There are at least 17 RV-specific T-cell epitopes, but the precise limit of each epitope is not known.^{147,154,172} A minimum of four immunodominant sites are located on E1, and these display reactivity to T cells from several donors with different HLA haplotype backgrounds.¹⁷⁸ Subsequently, minimal T-helper-cell epitopes were identified at E1_{280–287}, E1_{385–393}, and E1_{410–420} using T-cell lines from seropositive healthy donors, with variation in responsiveness found between individuals.¹⁴¹ Interestingly, binding of the peptide E1_{272–285} to HLA-DR and its recognition by RV-specific clones is influenced by DR β polymorphisms.¹⁷⁷

At least three T-cell epitopes have been found on the E2 protein.^{154,172} One of these peptides (E2_{54–65}) is present in approximately 50% of T-cell lines derived from donors with the HLA-DR7 phenotype.¹⁷⁰ With respect to the capsid protein, a peptide (C_{265–273}) is recognized by T-cell clones derived from donors expressing the DRB1*0403 and DRB1*0901 alleles.¹⁷¹ However, this peptide is recognized promiscuously by HLA-DR molecules that have common residues in pocket 4 of the peptide-binding groove and therefore likely defines a DR supertype.¹⁷⁶

EPIDEMIOLOGY

Incidence

Prior to the introduction of immunization programs, rubella was endemic worldwide, with regular seasonal peaks occurring in the spring months in temperate climates. In addition, epidemics of rubella occurred at intervals of 6 to 9 years as the pool of susceptible individuals reached a threshold. Epidemics still occur in developing and tropical countries, but the lack of effective monitoring programs, together with the absence of serious clinical symptoms in affected children, has made them difficult to assess.²⁰⁹ Regional variations in the age of onset of rubella, the incidence of the disease, and the appearance and spread of epidemics are determined by population densities, socioeconomic factors, and levels of medical sophistication in a given community. When these rubella outbreaks occur, they are accompanied by birth defects associated with congenital rubella syndrome (CRS). The teratogenic effects of rubella were a significant factor that spurred vaccine development following the rubella pandemic between 1962 and 1965. In this epidemic, an estimated 12.5 million cases of rubella

occurred in the United States alone, resulting in up to 20,000 children born with congenital abnormalities.²⁸ Today, CRS has been reduced to a handful of cases in both Europe and North America, with a declaration by the Centers for Disease Control and Prevention (CDC) in 2005 that endemic CRS had been eliminated from the United States.² However, residual cases are seen in immigrants who may not have been immunized in their country of origin.¹⁸ Despite an increase in rubella vaccination worldwide, less than 40% of the global birth cohort was covered as of 2009.²⁵⁸ Indeed, most of Africa and India and parts of Asia are not vaccinated against rubella (Fig. 24.1). It has been estimated that even in nonepidemic years, there are more than 100,000 infants born with CRS annually.

Age

Prior to the introduction of universal rubella immunization, peak infection occurred in the 5- to 9-year-old age group.¹⁰² After vaccine implementation, the disease shifted from children to young adults until its virtual elimination from Europe and North America in recent years.³⁰ In countries that have not implemented vaccination programs, infection at an early age is still the norm, with high seroconversion rates found in both preschool populations and in the 5- to 9-year-old age group.⁷⁹ In many developing countries, women of childbearing age are susceptible to rubella and the incidence of CRS during rubella outbreaks is 1 to 2 per 1,000 live births.⁵⁸

Origin and Spread of Epidemics

There are no animal reservoirs for RV, and therefore, continued cycling of the virus within the human population is required for perpetuation of the disease between seasons of maximal endemic occurrence. In countries where rubella immunization is not carried out and congenital infection is still common, the surviving infants shed high levels of virus for many months, forming a potential source for maintenance of the virus for relatively long periods. Enhanced vaccination programs have been very effective in disrupting virus circulation in much of the developed world. However, localized outbreaks of rubella still occur with regular frequency in countries where vaccination is practiced. Often, vaccine refusal on the basis of religious grounds is the major factor in such outbreaks. For example, in 2005, 214 cases of rubella occurred in an Amish community in Canada.¹⁴ Similar outbreaks have been reported in the United States among religious communities and Hispanic populations.²⁸ More recently, between 2009 and 2010, an outbreak of 1,900 cases of rubella was reported in Bosnia and Herzegovina, a situation that resulted from decreased vaccine uptake during the 1992–1995 war.¹⁰⁶ Together, these studies demonstrate the importance of maintaining high levels of herd immunity in developed countries, because rubella can be reintroduced into the population by foreign travel or from recent immigrants travelling from endemic areas. To prevent outbreaks, it has been estimated that at least 85% of the population must be immune to RV,⁴ whereas to maintain elimination, a 90% level of immunity among children is required.⁸⁸

Molecular Epidemiology

Based on sequence comparison of the E1 gene, at least 10 genotypes of RV representing two clades (RG1 and 2) have been identified²⁶⁴ (Fig. 24.3). The RG1 clade comprises viral isolates from North America, Europe, and Japan, whereas RG2 was



FIGURE 24.12. Characteristic skin rash on patient with acute rubella.

(Reproduced with permission from Logical Images, Inc.)

identified using isolates from China and India. RG1 and RG2 differ by 8% to 10% at the nucleotide level, as opposed to less than 5% among genotypes within each clade. RG2 has more genetic diversity than RG1 and may in fact consist of multiple clades. These genetic differences have been useful in tracking RV spread around the world and changes in endemic strains over time. Nevertheless, the differences in *E1* genes result in only 1% to 3% substitutions at the amino acid level, attesting to a high degree of genetic conservation between rubella strains. Finally, only one strain from clade 2 contained a mutation in one of the known epitopes defined by monoclonal antibodies, the first indication of a serotype variant.

CLINICAL FEATURES

Acute Rubella

Postnatal infection with RV is usually mild and frequently subclinical.^{7,62} Symptoms, when present, typically include sore throat and low-grade fever, a maculopapular rash, lymphadenopathy, and, in some cases, conjunctivitis and/or arthralgia. The rash (Fig. 24.12) is first seen on the face and spreads in centripetal fashion. The lesions appear as distinct pink maculopapules that fade rapidly over several days. A pronounced posterior cervical and suboccipital adenopathy is often present. In the majority of cases, the clinical syndrome clears in days and is seldom attended by more significant symptoms, but arthropathy, thrombocytopenia, and encephalopathy can occur.¹⁹ However, these serious sequelae and death as a result of RV infection are rare. The course of the acquired infection and the accompanying immune response are shown in Figure 24.13.

The virus is spread through respiratory secretions, and the mucosa of the upper respiratory tract and the nasopharyngeal

lymphoid tissue serve as portals of virus entry as well as the initial sites for viral replication. Spread of virus via lymphatics or a transient viremia then seeds regional lymph nodes. Local replication of virus in these nodes accounts for the posterior cervical and occipital nodal enlargement that typically appears 5 to 9 days before the onset of the rash. The incubation period (approximately 14 days) is followed by the appearance of virus in serum and the onset of viral shedding into the nasopharynx and stool, providing a source of spread to susceptible individuals. High levels of virus can be found in nasopharyngeal

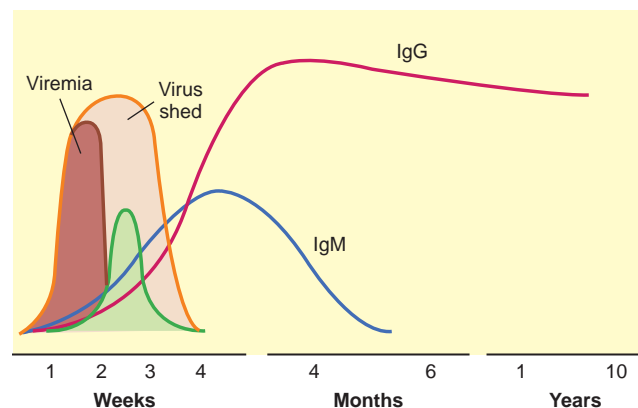


FIGURE 24.13. Time course showing viremic phase, rash, and immunoglobulin M (IgM) and IgG development during acute rubella infection from the time of infection initiation by droplet spray. Patients are infectious and shed virus from the time of infection until 1 to 2 weeks after the appearance of rash. IgM is present by day 10 and peaks around 4 weeks postinfection, by which time low-avidity IgG is also present.

secretions, exceeding 10^5 tissue culture infectious dose 50 (TCID₅₀) per 0.1 mL even in vaccinated individuals.⁷ The viremic phase may be marked by mild prodromal symptoms and malaise. The maculopapular rash appears at 14 to 21 days after natural exposure but somewhat earlier following experimental infection or vaccination. The presence of virus in serum ceases shortly after the rash appears, coincident with the onset of detectable circulating antibodies.

Immune Response

A serologic response can be detected at the onset of rash and continues to evolve over the next few weeks (Fig. 24.13). Generally, immunoglobulin M (IgM) antibody is first detected at 10 days postinfection, after which peak levels occur at 4 weeks postinfection but can persist for more than 7 months after acute infection.^{212,228} By 3 weeks, RV-specific antibodies are present in all immunoglobulin classes, including IgG, IgA, IgD, and IgE. At early stages of primary infection, IgG is of low avidity, maturing gradually during the next 3 months.²⁵

When measured by immunoprecipitation or immunoblot techniques, the majority of the immunoglobulin response appears to be directed at the E1 glycoprotein, with proportionally lesser amounts of the response against E2 or capsid.⁴¹ Interestingly, males have a more rapid and robust antibody response than females, although females have a higher anti-E2 response.¹⁵⁸ Whether there are pathologic consequences of these differences is not known.

Transient depression of lymphocyte responsiveness to mitogenic stimulation follows natural or vaccine infections in children and adults.²⁶ Despite this generalized immune suppression, RV-specific cell-mediated immune responses develop and can be measured *in vitro* within 1 or 2 weeks of onset of clinical illness. The cellular responses wane over the next few years but persist at low levels indefinitely after natural rubella. In contrast, they are difficult to detect following vaccination. More detailed studies have defined the epitope specificity of defined CD4+ and CD8+ T-cell clones^{134,172,175} (see Antigenic Composition and Determinants section).

Complications

JOINT SYMPTOMS

The most common complications of natural rubella are acute arthralgia and/or arthritis, particularly in adolescent and adult women.²¹⁹ Incidence rates for joint involvement range between 30% and 60% during outbreaks. For example, in one report, 52% of adult females and 9% of adult males developed arthropathy, with symptoms being more severe among women.²²⁹ The joint symptoms usually begin within a week of the appearance of the rash and may involve any joint, with the fingers and knees being most commonly affected. Although these symptoms usually resolve within several weeks, they can persist for months or even years, in which case they are episodic. Rarely, chronic severe arthropathy that is significantly disabling has been reported.³⁵ A similar range of less severe symptoms occurs 9 to 27 days following vaccination but is less common than after natural rubella.²²⁹ A report on rubella vaccine-associated arthritis, based on analysis of the Vaccine Adverse Events Reporting System (VAERS) database, has confirmed that rubella vaccine in adult women is associated with chronic arthritis that can persist for at least 1 year.⁷⁶

Factors that affect the incidence of joint symptoms include age and gender,²²⁹ as well as major histocompatibility complex (MHC) type of the infected individual.¹⁵⁵ Hormonal influences also affect the incidence of joint symptoms, with adult females being most susceptible. The underlying pathogenesis of rubella arthritis is unknown, but the mechanism may involve local viral replication. Several groups have reported the isolation of RV from synovial fluids of symptomatic joints for up to a month following acute infection.^{67,162} Virus has also been isolated from PBMCs of symptomatic individuals³⁵ and can be detected in women with chronic arthropathy postvaccination by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of PBMC RNA using RV-specific primers.¹⁵⁶ Replication and persistence of virus in extra-articular sites and deposition of immune complexes on articular surfaces may therefore play a role in the acute stage.

RV can also infect and persist in joint tissue for prolonged periods *in vitro*, forming foci of infection that are positive for viral antigen.¹⁵² Overall, the body of evidence indicates that RV is a highly arthrotropic virus and a plausible candidate as one of several viral triggers (including human parvovirus, hepatitis C, and human T-cell lymphotropic virus type 1 [HTLV-1]) of chronic degenerative forms of arthritis.

THROMBOCYTOPENIA

A number of different viral infections result in reduced platelets either by inhibiting their production or by causing their lysis.²⁰² For example, binding of viral immune complexes to Fc receptors on the platelet surface triggers immune-mediated clearing of both the complex and its associated platelet.¹¹⁵ This causes a transient asymptomatic depression of thrombocyte counts and is quite common with rubella.⁵² Thrombocytopenic purpura is seen following rubella infection; however, this condition is relatively rare (1 in 1,500 cases)²⁴⁰ and is usually self-limiting. However, it may even occur in the absence of rash. Accordingly, undiagnosed RV infections are likely the cause of some cases of idiopathic thrombocytopenic purpura. Very rarely is epidemic rubella associated with hemolytic anemia.

ENCEPHALOPATHY

The most serious complication of postnatal rubella is postinfectious encephalopathy or encephalomyelitis.¹⁷ Estimated to occur in 1 in 6,000 cases of natural infection, rubella encephalitis is rarely reported in countries with comprehensive vaccination policies. However, occasionally, case reports indicate that this disorder can appear during rubella outbreaks. The symptoms of rubella encephalopathy appear abruptly 1 to 6 days following the onset of rash in an otherwise typical case of rubella. The most frequently encountered symptoms include headache, vomiting, stiff neck, lethargy, and generalized convulsions.²⁵⁰ In rare cases, RV antigens have been detected within brain tissue,⁵¹ and virus has been isolated from cerebrospinal fluid (CSF), indicating that it has the capacity to invade the mature CNS.²²⁰

Postnatal rubella encephalopathy usually requires only supportive treatment, and the disease course is generally concluded within a few days; the survival rate approximates 80%. Among the 20% of patients who do not survive, the disease course typically includes coma, respiratory distress, apnea, and then death, usually within a few days of onset of symptoms.

Congenital Rubella Syndrome

Although postnatal rubella is rarely associated with severe complications, infection *in utero* following transplacental transmission of virus from the mother has dire consequences for the developing fetus. These are reflected in a constellation of symptoms collectively called congenital rubella syndrome.^{52,251}

Pathogenesis of Congenital Rubella Syndrome

In general, maternal infection shortly before conception does not lead to intrauterine infection.⁶³ However, when infection occurs after conception, the virus is present in placental villi approximately 10 days after the onset of rash in the mother and can be detected in the fetus after 20 to 30 days.¹¹³ Transplacental transmission occurs in up to 90% of cases during the first 8 weeks of gestation, falling to a low of 25% to 35% during the second trimester and rising again near term.^{7,75,251} This fluctuating incidence of fetal infection is likely related to changes in the placenta during pregnancy. In early gestation, infection of the placenta causes scattered foci of necrotic syncytiotrophoblast and cytotrophoblast cells, as well as damage to the vascular endothelium, resulting in placental hypoplasia.⁷⁵ Infection at later stages is associated with multifocal mononuclear cell infiltrates in the placental membranes, cord, and decidua, along with vasculitis.²⁵¹ In cases where fetal infection occurs, the virus can spread widely and almost any organ may be infected. A chronic and generally nonlytic infection is then established in the fetus.¹⁸⁷

Clinical Consequences

The effects of RV invasion of fetal tissue are quite varied, and early infection may result in resorption of the embryo. Whether placental infection alone can lead to spontaneous abortion or abnormalities of fetal development is not established. In the majority of cases, the infected fetus will survive and the pregnancy continues to term, with premature delivery or stillbirths being rare outcomes.¹⁵³ In many countries, clinically recognized maternal rubella during the first 8 weeks of gestation is an indication for therapeutic abortion due to the high incidence of congenital defects. Although these are not universal, some degree of neurologic deficit including sensorineural deafness is found in most cases. The current rubella vaccine RA27/3 has extremely low teratogenicity, and inadvertent vaccination in early pregnancy is not considered an indication for therapeutic abortion, although immunization at this time should be avoided.^{31,191}

The most common clinical manifestations of congenital rubella are listed in Table 24.1. In addition to the high incidence of sensorineural deafness (~80%), cataracts are detected in 50% to 60% of neonates infected in the first 8 weeks of pregnancy.⁷ Congenital heart disease is also found in more than half of CRS babies, usually manifested as patent ductus arteriosus or pulmonary artery or valvular stenosis. Other common defects include glaucoma, retinopathy, psychomotor retardation, neonatal thrombocytopenia purpura, hepatomegaly and/or splenomegaly, and intrauterine growth retardation.⁵² Less frequent features (present in 5% to 10%) include adenopathy, bony radiolucencies, hepatitis, and hemolytic anemia. Many of the clinical manifestations of congenital rubella are evident at birth or shortly thereafter. This includes a reddish-blue (purpuric) maculopapular rash termed the *blueberry muffin* rash (Fig. 24.14). Other clinical

TABLE 24.1 Manifestations of Congenital Rubella Syndrome

Group A	Group B
Eye manifestations	Purpura
• Cataracts	Hepatosplenomegaly
• Congenital glaucoma	Jaundice
• Retinitis	Microcephaly
Congenital heart defects	Meningoencephalitis
• Patent ductus arteriosus	Radiolucent bone disease
• Pulmonary artery stenosis	Progressive or late-onset manifestations
Sensorineural hearing loss	• Mental retardation
Pigmentary neuropathy	• Diabetes mellitus
	• Progressive panencephalitis

cal signs, including hepatosplenomegaly and jaundice, usually resolve within weeks. In addition, there are defects that are not recognized at birth but may be manifest in childhood (e.g., mental or physical retardation) or in adolescence (type 1 diabetes).

At birth, CRS is accompanied by clinical signs that include bulging anterior fontanel, microcephaly, lethargy, irritability, and motor tone abnormalities.²⁵¹ RV can be isolated from almost any organ at birth and from selected tissues for up to 1 year or more in surviving infants.¹⁵¹ More than 80% of congenitally infected newborns contain substantial amounts of RV in their nasopharyngeal secretions and urine, and 3% will continue to shed virus for as long as 20 months. This chronic shedding of RV by neonates is an indicator of early gestational infection⁵⁶ and a major source of virus for dissemination to others.

Mechanisms of Teratogenesis

Most of our information on rubella embryopathy comes from careful pathologic analyses conducted following the major



FIGURE 24.14. Infant with congenital rubella showing characteristic “blueberry muffin” maculopapular rash. (From Dermatology Online Journal, dermatology.cdlib.org.)

rubella pandemic of the 1960s. Data from these studies led to the idea that direct cytolytic effects of RV to cells in the retinal epithelium, myocardium, skeletal muscle, and neural tissue underlie much of the observed damage in CRS.^{117,210,233} The widespread nature of the tissue damage indicates that RV infects most fetal organ systems. Infection of focal clones of cells and their progeny during critical stages of the ontogeny of fetal organs was believed to give rise to the wide range of abnormalities that together compose CRS.

Because there are no animal models that recapitulate the teratogenic effects of RV infection, direct effects on human fetal cells *in situ* can only be extrapolated from studies in cell culture. Multiple studies reporting that cultured cells infected with RV exhibited signs of apoptosis^{61,100,122,149,195} and that human embryonic cells persistently infected with RV produce lower levels of collagen and are less responsive to epidermal growth factor²⁶¹ led to the hypothesis that altered cell physiology and induction of programmed cell death were integral to the teratogenic activity of the virus. However, a later study reported that RV infection does not induce apoptosis in human embryonic fibroblasts, but rather induces expression of multiple of antiapoptotic genes.³ These findings are consistent with a more recent report showing that the capsid protein blocks apoptosis in human embryonic and adult cultured cell lines.¹⁰⁷ Accordingly, it is quite possible that a predominantly noncytopathic RV infection of selected embryonic cell types *in utero* upsets the normal delicate balance of cellular growth and differentiation and has profound effects on organogenesis. These effects, alone or in concert, could explain the observation that the small but otherwise apparently normal organs of congenital rubella infants contain reduced numbers of cells.¹⁶¹ In addition to these direct effects of RV replication in host tissue, it is possible that immune-mediated damage occurs in CRS.

Fetal Immune Response in CRS

The persistence of RV in fetal tissue throughout gestation, and in infants with CRS for prolonged periods after birth, raises the question of how the virus avoids immune elimination. Clearly, neither transferred maternal IgG nor fetal IgM (detected at around 15 weeks' gestation²⁵¹) can eliminate virus *in utero*, although both have neutralizing capacity *in vitro*.⁵³ It has been suggested that antibody may in fact promote persistence; however, this phenomenon has only been observed *in vitro*.^{1,34} and it is not at all clear whether it can occur in the absence of the complement system and phagocytic cells. Two recent studies^{3,107} suggest that the ability of RV to block apoptosis may also play a role in establishment and/or maintenance of persistence by thwarting innate antiviral defenses. Moreover, because the thymus does not mature until 15 weeks, the fetus is highly vulnerable to viral infection prior to this time. Interferon- α is present early in gestation and therefore may limit viral spread, a scenario that could explain why only 0.1% to 0.001% of cells from fetal organs contain detectable virus.²⁰³

Postnatal infection induces IgG class antibodies against each of the three structural proteins of the virus; however, CRS infants often lack antibodies to the capsid protein and demonstrate weak humoral reactivity to the E2 protein.^{59,197} Perhaps more telling, these children show selective tolerance to the E1 protein¹⁴⁶ and, more specifically, possess little or no antibody to the putative E1 neutralization domain.^{157,197}

Infants with CRS also demonstrate prolonged impairment of RV-specific cell-mediated immune responses, including cytotoxicity, and lymphokine secretion *in vitro*.^{26,59} T-cell lines derived from several congenital rubella children and adults failed to respond to RV peptides that stimulate lymphocyte proliferative responses in normal immune adult donor cells.¹⁷² These data suggest that early fetal infection results in selective immune tolerance to a limited but critical number of RV epitopes that must be recognized to clear the virus.

Late-Onset Sequelae

Although many of the effects of fetal rubella that manifest at birth are transient, some defects such as retinopathy, mental retardation, hearing loss, and endocrine abnormalities may not become clinically apparent for several years.^{148,215} Psychomotor retardation (62%), cardiac abnormalities (58%), and mental retardation (42%) are also associated with CRS, and multi-organ disease is found in 88% of patients.⁷⁸

DELAYED ENDOCRINE DISEASE

Congenital RV infection is associated with a variety of delayed endocrine abnormalities, including type 1 diabetes, thyroid disease, and polyglandular autoimmunity.^{201,215} Early reports suggested that type 1 diabetes occurs in as many as 10% to 40% of CRS patients. However, while more recent analyses from a diabetes-focused perspective confirmed that *in utero* RV infection undoubtedly predisposes to diabetes, the proportion is probably less than 10%.⁷² Conversely, postnatal rubella does not have a clear association with type 1 diabetes.²³

The mechanism by which RV causes diabetes is not understood, and certainly the lack of a suitable animal model has limited progress in this area. However, a number of older studies in hamsters have provided some intriguing results. For example, Syrian hamster pups infected with an adapted rubella RA 27/3 vaccine virus exhibited hypoinsulinemia and hyperglycemia.²⁰⁴ In these animals, cell-free RV was recoverable from pancreas, viral antigens were present in islet cells, and a mononuclear infiltration of the islets occurred over the first 3 weeks of the infection. Moreover, islet cell antibodies developed in 40% of the infected pups.

RV-induced damage to the pancreas may therefore be a combination of viral replication in islets, compounded by the triggering of an autoimmune reaction that perpetuates beta-cell damage. Evidence in favor of this theory includes the identification of a monoclonal antibody to RV capsid protein that reacts with pancreatic islet cell Ia2 protein.¹¹² In addition, CD4 and CD8 T-cell clones isolated from CRS patients recognize peptides of the diabetes-associated autoantigen GAD65 in an HLA-restricted manner.^{174,179} Also, 20% to 40% of sera from CRS patients in a selected series were found to contain antibodies that react with thyroid tissue.^{48,77,216} Finally, CRS is also associated with growth hormone deficiency, and therefore, pituitary involvement is another complication. Relatedly, hamsters immunized with recombinant RV E1 or E2 develop pituitary autoantibodies.²⁶² In summary, these observations suggest that viral-induced autoimmunity may partially account for the late appearance of polyendocrine diseases that frequently complicate congenital rubella.

DELAYED NEUROLOGIC DISEASE

A rare late-onset encephalitis following rubella has been described, referred to as progressive rubella panencephalitis

(PRP).^{234,252} Like other slow viral diseases of the CNS, PRP is characterized by a prolonged asymptomatic period, followed by the onset of symptoms of neural deterioration during the second decade of life. The pathogenesis of PRP is unclear; however, immune complexes are consistently identified, suggesting ongoing viral replication.⁵⁵ The near-complete destruction of Purkinje cells indicates either some degree of selective tropism of RV for these cells within the CNS or a negative effect on their metabolism. This could also account for the selective loss of myelin and oligodendroglia, although immunopathologic mechanisms could also be active.

DIAGNOSIS

Differential

The most common symptoms of rubella (lymphadenopathy, erythematous rash, and low-grade fever) can be readily confused with similar illnesses associated with maculopapular rash caused by other common viral and nonviral pathogens or even some drug treatments. The differential diagnosis includes parvovirus, measles, human herpesvirus 6 (roseola), and rash-associated enteroviruses, such as echovirus 9 and coxsackievirus A9.⁷ In particular, parvovirus infections can be confused with rubella because their clinical presentations are so similar and both can be associated with arthritis or arthralgia.²¹⁷ Moreover, in endemic areas, dengue, West Nile, Sindbis, chikungunya, and Ross River virus infections should be considered.⁷ Therefore, a definitive diagnosis of rubella can only be made using specific laboratory tests.

Similarly, confirmation of a diagnosis of CRS cannot be established solely on the basis of clinical findings. It requires either the direct isolation of RV or serologic evidence of acute infection in the infant. In the absence of confirmatory laboratory data, a clinical diagnosis compatible with CRS requires the presence of any two of the following: cataracts and/or congenital glaucoma, congenital heart disease, hearing loss, or pigmentary retinopathy. In the presence of only one of the preceding manifestations, the additional finding of purpura, hepatosplenomegaly, jaundice, microcephaly, mental retardation, meningoencephalitis, or radiolucent bone disease is indicative of CRS (Table 24.1).³³ In countries in which rubella vaccination is not carried out (or is in the process of being implemented), surveillance for CRS is important to monitor for prevalence or effectiveness of the vaccination policy. A combination of eye and congenital heart anomalies has been proposed as a sensitive and specific sentinel for CRS to identify infants for further laboratory investigation.²⁰⁷

Laboratory

Diagnosis of postnatal rubella and congenital infection (following birth) is normally carried out by detection of rubella-specific IgM by enzyme immunoassay (EIA) using commercial assays.²³² The capture assay is preferred over indirect assays, which have been shown to give false-positive results with other acute viral infections such as parvovirus, Epstein-Barr virus, or cytomegalovirus,²²⁶ or when rheumatoid factor is present.⁸² IgM is usually detectable for 6 to 8 weeks after acute rubella⁷ but can be detected for longer in some patients and is present for up to a year in congenitally infected infants.²²⁷

Virus isolation may occasionally be warranted, particularly to confirm infection during pregnancy. In acute infection, RV is readily isolated from throat swabs or nasopharyngeal secretions for approximately 1 week before and up to 2 weeks after the appearance of rash.²⁶⁵ Virus can also be isolated from circulating lymphocytes for up to 1 month postinfection,³⁷ but this is too expensive for routine clinical diagnostic purposes. Cord blood or placental tissue may be used to confirm congenital infection at the time of birth. In addition, virus can readily be isolated from throat swabs or urine of the neonate.⁵⁶

Over the last 10 to 15 years, a variety of nucleic acid amplification-based techniques have been developed to detect RV genomic RNA in clinical samples. Reverse transcription of RNA isolated from patient tissue/cells or fluid is required prior to amplification of the cDNA by PCR using primers specific for a selected region of the RV genome (usually the *E1* gene). PCR-based detection of RV RNA has been used to detect virus in cases of suspected congenital infection using samples obtained by amniocentesis, cordocentesis, and chorionic villus sampling for *in utero* diagnosis.^{24,205} A comparison of nested RT-PCR on amniotic fluid with measurement of rubella IgM in fetal blood has confirmed both the specificity and the sensitivity of the genome amplification technique.¹³⁷ Finally, adaption of multiplexed real time-based PCR assays allows molecular genotyping as well as exquisitely accurate and sensitive detection of as little as one plaque-forming unit of RV in clinical samples.^{163,164}

PREVENTION AND CONTROL

Vaccines

Development

Following isolation of RV in cell culture in 1962,¹⁸² attenuation of the virus was carried out by serial passage in a variety of cell lines, giving rise to several vaccine strains that came into use around 1970. These included the original vaccine used widely in North America, HPV77/DE5²⁷; the Cendehill strain, used more extensively in Europe¹⁸⁵; and several Japanese strains.²¹⁸ The HPV77/DE5 strain was the predominant strain used in North America until 1979, when it was replaced by the RA27/3 vaccine due to concerns of waning immunity in HPV77/DE5 vaccine recipients.¹⁰³ The RA27/3 strain was derived from an isolate from the kidney of a RV-infected fetus and was attenuated by passaging 4 times in human embryonic kidney cells followed by passage 17 to 25 times in WI-38 fibroblasts.¹⁸⁸ This strain of virus induces a more vigorous immune response than HPV77/DE5 and has the added advantage that it was attenuated in human cells and is therefore not subject to possible side effects associated with vaccines grown in nonhuman cells. RA27/3 was licensed in 1979 and is now the only rubella vaccine available in North America. It is also widely used in other countries, including the majority of those in Europe, in Australia and New Zealand, and in South America. However, in parts of Asia, locally produced rubella vaccine strains are used. In Japan, five vaccine strains (KRT, TO-336, Matsuura, TCRB19, and Matsuba) have been developed,¹⁶⁹ whereas in China, BRD-2 has been compared in clinical studies to RA27/3.²⁴⁷ The most recent reporting indicates that 130 of the 193 World Health Organization (WHO) member states (Fig. 24.1) now employ RV vaccine in their national immunization programs.²⁹ Yet, this

does not include most of Africa and India and large areas of Asia, and as such, it represents less than 50% of the global birth cohort. The WHO initiated a campaign to eliminate rubella and CRS from the Americas by 2010 and the European region by 2015.

Vaccine Administration

Rubella vaccine is usually given in combination with measles and mumps as the measles-mumps-rubella (MMR) vaccine between 12 and 15 months of age, with a subsequent booster dose prior to school entry or in adolescence. Subcutaneous administration induces an antibody response in approximately 95% of recipients older than 12 months of age, detectable at 4 weeks postimmunization. The majority of vaccinated children report only mild symptoms, and transmission to susceptible bystanders seems not to occur. Although the antibody titers are lower than those following natural infection, protection is believed to endure for more than 21 years in the majority of those immunized.¹⁶⁸ However, in 10% of women tested in the United States, titers had dropped to low or undetectable levels in 5 years, although they responded rapidly to challenge with high-titer RA27/3.¹⁶⁷ In view of this, rubella vaccination should be given to all women of childbearing age found to have low or undetectable antibody titers, preferably 1 month before conception or postpartum. Immunization during pregnancy should be avoided (see Risk in Pregnancy section).

Adverse Reactions

The current RA27/3 vaccine is well tolerated but not problem free. Certainly, the incidence of acute adverse reactions is lower than after natural rubella²²⁹ but still higher than that reported for some other vaccine strains.²¹ Symptoms include fever, lymphadenopathy, arthralgia/arthritis, paresthesia, and carpal tunnel syndrome. Between 10% and 30% of adult rubella seronegative women vaccinated with the RA27/3 vaccine develop acute, usually transient, arthritis or arthralgia.^{189,229,231} The joint reactions can be recurrent or persistent, similar to symptoms following natural rubella, and viral persistence may be associated with ongoing symptoms.³⁵ Subsequent studies have questioned the link between RV vaccination and joint manifestations, and it is probably prudent to conclude that the incidence of vaccine-induced chronic arthritis is lower than previously reported.²²⁹ However, the finding of an association of certain HLA-DR haplotypes with a higher incidence of joint symptoms following RA27/3 rubella vaccination¹⁵⁵ suggests that the virus may cause joint symptoms in small numbers of genetically predisposed individuals. This factor should be taken into account in any future studies based on analysis of populations.

Vaccine side effects and complications are a serious challenge to maintaining herd immunity, and the higher incidence of arthralgia and arthritis particularly in female vaccines has most certainly had negative consequences. However, by far the most damage to effective uptake of MMR vaccine stems from a report by Wakefield et al²⁴⁵ that ultimately led to the belief among a significant number of people that this vaccine was linked to autism. Numerous larger and much better controlled studies failed to confirm a causal relationship between the MMR vaccine and autism, but unfortunately the damage was done and the rate of vaccine uptake dropped dramatically. Predictably, this led to an increase in the number of cases of measles and, to a lesser extent, rubella. Fortunately, the journal that

published the Wakefield et al paper, the *Lancet*, retracted the paper from the published record in 2010. However, it will still be some time before we have fully recovered from the effects of this fraudulent report.

Risk in Pregnancy

A serious concern of rubella vaccination programs has been the potential risk to the developing fetus in mothers who are immunized during early pregnancy. In such instances, the placenta and fetus may become infected, although the isolation rate is only 3%.²⁰⁸ Moreover, one survey of 515 children born of mothers inadvertently immunized within 3 months of conception showed that none had malformations compatible with CRS.²⁰ This included cases of vaccination with the earlier Cendehill and HPV-77 vaccines, as well as with the current RA27/3 vaccine. Vaccine strains of RV therefore appear to be far less teratogenic than wt+ RV, and even in one case where the fetus was infected no signs of CRS were detected at birth. Because a potential risk to the fetus still exists (estimated at 1.3% when the mother is vaccinated 1 to 2 weeks before to 4 to 6 weeks after conception³²), pregnancy remains a contraindication to rubella vaccination. However, inadvertent vaccination of a seronegative pregnant woman is not sufficient reason for termination of pregnancy.

New Vaccine Strategies

Because the current rubella vaccines are considered highly effective, there is limited interest in developing a subunit vaccine. Nevertheless, live attenuated vaccines are not recommended for children with immunodeficiency diseases or for women in the early stages of pregnancy who might be living or working in a situation where they risk exposure. For these situations, a subunit vaccine that provides some protection, even if only for a limited time, may be appropriate. Use of the E1 protein, which contains the major neutralizing epitopes, has been proposed,²⁵⁷ but such vaccine platforms have not been developed further. In addition, DNA vaccines incorporating genes for all three structural proteins, or just E1 and E2, have been constructed and have shown some promise in mice.¹⁹⁰ However, for general use, a live attenuated vaccine that promotes the strongest and most enduring immune response is still advisable. With current knowledge on the genetic determinants of virulence and joint tropism, the potential exists for development of a recombinant vaccine designed to be highly immunogenic but not associated with unwanted side effects. Nevertheless, the overall success of current vaccines likely precludes further development of next-generation recombinant vaccines.

PERSPECTIVE

With the availability of relatively inexpensive and safe vaccines, rubella is most certainly a preventable disease. Indeed, much of the industrialized world has adopted comprehensive immunization policies that have curtailed virus circulation and CRS; however, more than 50% of the global birth cohort is still susceptible to rubella.²⁹ The cost of medical care for children with CRS easily justifies implementation of comprehensive immunization programs on economic grounds alone.⁸⁹ Yet RV is still endemic in many developing countries, and sadly, these are the least well-equipped nations to deal with the very high costs of caring for CRS children.

Compounding matters is the fact that to prevent RV transmission, herd immunity needs to exceed 85%⁴ and the cost of proper surveillance for RV can be prohibitive. As a result of inadequate surveillance, the actual numbers of rubella and CRS are vastly underreported. For example, based on seroprevalence data and statistical modeling for the Southeast Asia region, it is estimated that more than 46,000 infants are born with CRS each year, yet only 13 cases of CRS were reported between 2000 and 2009.²⁹ Accordingly, RV is still a significant human pathogen that takes an enormous toll on the health of the world's population through the widespread manifestations of CRS, most notably in relation to blindness and hearing defects.

On the positive side, significant progress has been made with respect to understanding virus–host interactions at the molecular level, and the complete sequences of multiple wild-type and vaccine strains of RV are known. By pooling the knowledge from these studies through further investigation, we will be in a better position to understand the underlying mechanisms of teratogenicity and virus-induced autoimmune diseases such as diabetes and arthritis.

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Flaviviridae

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INTRODUCTION

The first human virus was discovered over one century ago when Walter Reed demonstrated that yellow fever could be experimentally transferred via the filtered serum of an infected individual, and that this infectious agent was transmitted to humans by mosquitoes.⁸¹⁷ It is now appreciated that yellow fever virus (YFV) is but one representative of a large family of related positive-strand RNA viruses, the *Flaviviridae* (from the Latin *flavus*, “yellow”). This family currently consists of three genera: *Flavivirus*, *Pestivirus* (from the Latin *pestis*, “plague”), and *Hepacivirus* (from the Greek *hepar*, *hepatos*, “liver”).⁸⁵¹ (Table 25.1). A fourth genus, *Pegivirus* (persistent GB virus), has recently been proposed to encompass the previously unclassified GB virus A (GBV-A), GBV-C, and GBV-D.⁸⁰⁵ As detailed later, the *Flaviviridae* share similarities in virion morphology, genome organization, and replication strategy but exhibit diverse biological properties and lack serologic cross-reactivity. The phylogenetic relationships of the *Flaviviridae* are shown in Figure 25.1. The increasing significance of *Flaviviridae* as human and animal pathogens emphasizes that their study remains no less pertinent than in Reed’s time.

Family Classification

Positive-stranded RNA viruses are classified into three superfamilies based on the evolutionary relatedness of their RNA-dependent RNA polymerases (RdRPs). The *Flaviviridae* are members of superfamily 2, bearing distant similarity to coliphages and the plant-infecting carmo-, tombus-, diantho-, and subgroup I luteoviruses.⁴¹⁸ Before the era of molecular biology, some members of the family *Flaviviridae* were classified as *Togaviridae*.

Family Characteristics and Replication Cycle

This chapter is organized around common features of the family *Flaviviridae* life cycle (Fig. 25.2). The enveloped virions are composed of a lipid bilayer with two or more species of envelope (E) glycoprotein surrounding a nucleocapsid, which consists of a single-stranded, positive-sense RNA genome complexed with multiple copies of a small, basic capsid (C) protein. Binding and uptake are believed to involve receptor-mediated endocytosis. The low pH of the endosome induces fusion of the virion envelope with cellular membranes. Following uncoating of the nucleocapsid, the RNA genome is released into the cytoplasm. The genome serves three discrete roles within the life cycle: as the messenger RNA (mRNA) for translation of all viral proteins, a template during RNA replication, and the genetic material packaged within new virus particles. The organization

TABLE 25.1 Members of the *Flaviviridae*

Taxonomic unit	Representative examples
Genus <i>Flavivirus</i> ⁵⁴	
Mosquito-borne viruses	<i>Yellow fever virus</i> (YFV) <i>Dengue virus</i> , types 1 to 4 (DENV-1 to DENV-4) <i>Japanese encephalitis virus</i> (JEV) <i>Kokobera virus</i> (KOKV) <i>West Nile virus</i> (WNV)
Tick-borne viruses	<i>Tick-borne encephalitis virus</i> , European subtype (TBEV-Eu) <i>Tick-borne encephalitis virus</i> , Far Eastern subtype (TBEV-FE) <i>Omsk hemorrhagic fever virus</i> (OHFV) <i>Tyuleniy virus</i> (TYEV)
Viruses with no known vector	<i>Modoc virus</i> (MODV) <i>Rio Bravo virus</i> (RBV)
Unclassified	<i>Cell fusing agent virus</i> (CFAV)
Genus <i>Hepacivirus</i> ¹	<i>Hepatitis C virus</i> (HCV), seven genotypes <i>GB virus B</i> (GBV-B; unclassified) <i>Canine hepacivirus</i> (CHV; unclassified)
Genus <i>Pestivirus</i> ⁴	<i>Bovine viral diarrhea virus 1</i> (BVDV-1) <i>Bovine viral diarrhea virus 2</i> (BVDV-2) <i>Border disease virus</i> (BDV) <i>Classical swine fever virus</i> (CSFV) ^a <i>Giraffe-1 pestivirus</i> (unclassified)
Genus <i>Pegivirus</i> (proposed)	<i>GB virus A</i> (GBV-A) <i>GB virus C</i> (GBV-C), “ <i>Hepatitis G virus</i> (HGV)” <i>GB virus D</i> (GBV-D)

Numbers in superscript indicate the current number of virus species recognized within each group.

^aCSFV was formerly called *hog cholera virus* (HCV). The name was changed to avoid confusion with *hepatitis C virus*.

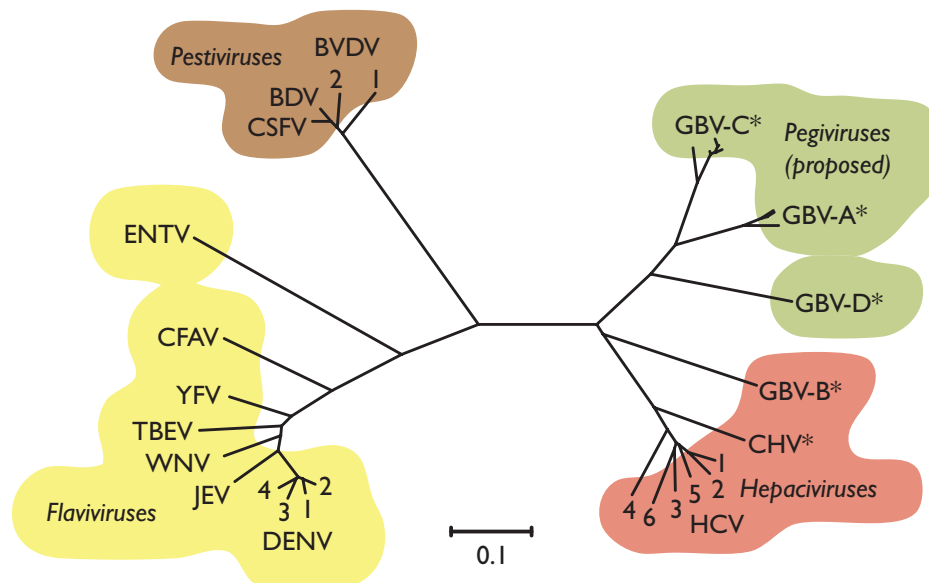


FIGURE 25.1. The family *Flaviviridae*. Phylogenetic tree based on neighbor-joining analysis of the viral RNA-dependent RNA polymerases (RdRPs). Shown are members of the *Flavivirus* genus: yellow fever virus (YFV), dengue virus (DENV) serotypes 1 through 4, West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), cell-fusing agent virus (CFAV), and Entebbe bat virus (ENTV); the *Pestivirus* genus: bovine viral diarrhea virus (BVDV) types 1 and 2, classical swine fever virus (CSFV), and border disease virus (BDV); the *Hepacivirus* genus: hepatitis C virus (HCV) genotypes 1 through 6, GB virus B (GBV-B, proposed assignment), and canine hepacivirus (CHV, proposed assignment); and the proposed *Pegivirus* genus: GB virus A (GBV-A), GB virus C (GBV-C), and GB virus D (GBV-D). The scale bar indicates amino acid substitutions per position.

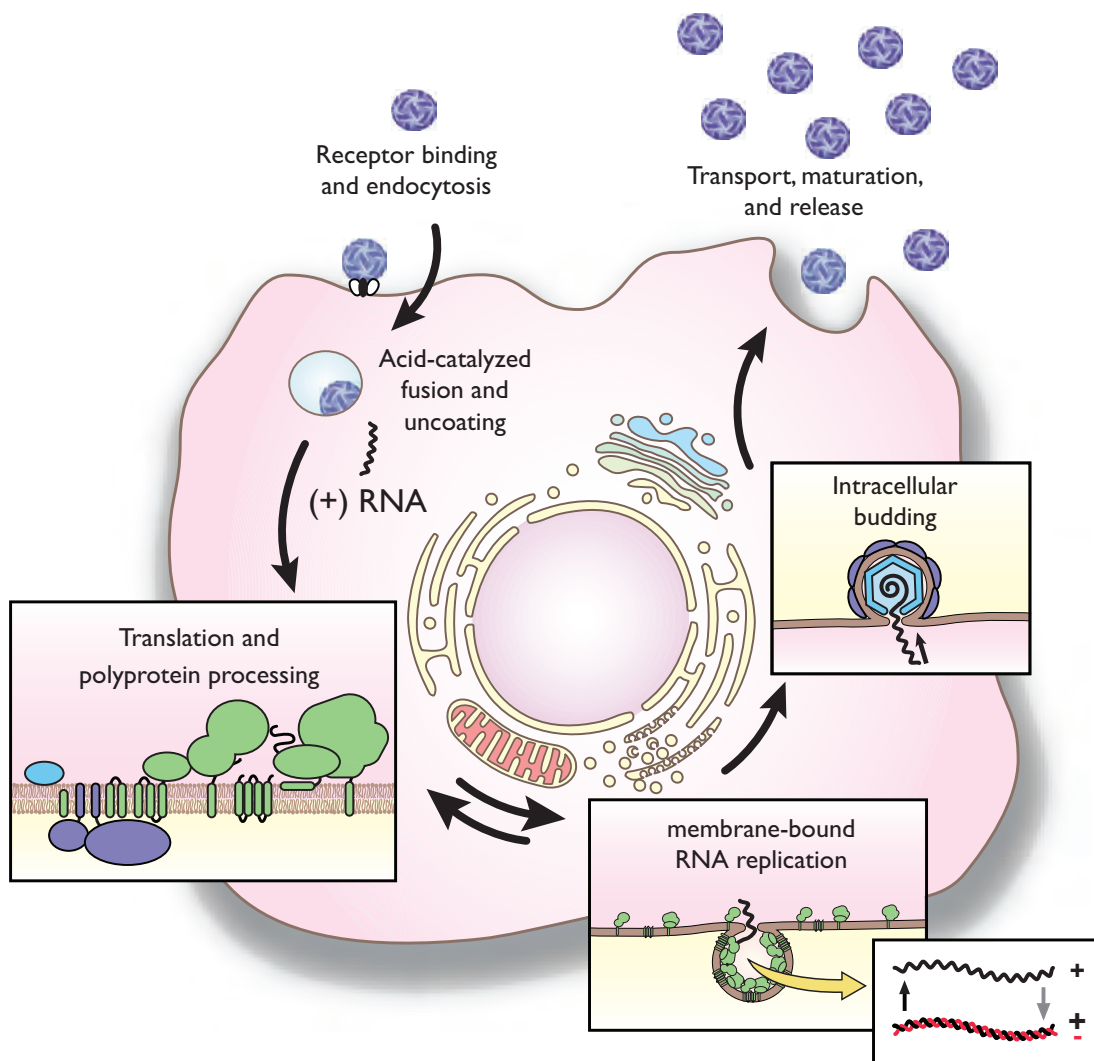


FIGURE 25.2. The life cycle of the *Flaviviridae*. See text for further details.

of the genome is similar for all genera. Viral proteins are produced as part of a single polypeptide that is cleaved by a combination of host and viral proteases. The structural proteins are located in the N-terminal portion of the polypeptide with the nonstructural (NS) proteins in the remainder. Sequence motifs characteristic of a serine protease, RNA helicase, and an RdRP are found in similar locations in the polypeptides of all three genera.⁵⁸⁵ RNA replication occurs entirely in the cytoplasm in close association with intracellular membranes; the synthesis of a genome-length minus-strand RNA provides the intermediate. Progeny virions assemble by budding into an intracellular membrane compartment, most likely the endoplasmic reticulum (ER), then transit through the host secretory pathway and are released at the cell surface.

FLAVIVIRUSES

Background and Classification

The *Flavivirus* genus consists of more than 50 species, many of which are arthropod-borne human pathogens. Flaviviruses cause

a variety of diseases, including fever, encephalitis, and hemorrhagic fevers. Entities of major global concern include dengue virus (DENV)—with its associated dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)—Japanese encephalitis virus (JEV), West Nile virus (WNV), and YFV (reviewed in⁵³⁷). Other flaviviruses with regional or endemic distribution include Murray Valley encephalitis virus (MVEV) and St. Louis encephalitis virus (SLEV). *Tick-borne encephalitis virus* (TBEV) is a name commonly applied to either central European encephalitis virus or Far Eastern encephalitis virus, although these are clearly distinct species.²⁰⁸ Decreases in mosquito control efforts during the latter part of the 20th century, coupled with societal factors (e.g., increased transportation and dense urbanization), have contributed to the re-emergence of flaviviruses such as DENV in South and Central America. Following an outbreak in New York City in 1999, WNV has spread throughout much of North America and Central America.

Flavivirus species are further categorized into antigenic complexes and subcomplexes based on serologic criteria or into clusters, clades, and species, according to molecular phylogenetics.¹¹⁷ Mosquito-borne and tick-borne flaviviruses, although

distinct, appear to have evolved via a common ancestral line that diverged from viruses with no known arthropod vector. DENV circulates as four distinct serotypes, which show significant sequence diversity (reviewed in³³⁷). Some reports have documented intertypic recombination among DENV isolates, although the taxonomic status of these isolates is currently unclear.

The development of the first live-attenuated flavivirus vaccine, YFV strain 17D,⁸⁴⁹ led to Max Theiler's recognition by the Nobel Prize committee in 1951. Only a limited number of flavivirus vaccines are available, including inactivated TBEV and JEV for use in humans and inactivated WNV for use in animals.⁶⁹⁴ Development of effective DENV vaccines that exhibit cross-protection between serotypes is proving to be particularly challenging. The ability to genetically manipulate flaviviruses has led to novel approaches, including live attenuated chimeric vaccines based on the YFV-17D backbone.

Structure and Physical Properties of the Virion

Infectious flavivirus particles are roughly spherical, approximately 50 nm in diameter, and surrounded by a lipid envelope⁶¹⁵ (Fig. 25.3). Viruses sediment between 170 and 210S and have buoyant densities of 1.19 to 1.23 g/cm³ depending on the lipid composition, which can vary by host.⁷⁴⁵ The outer shell of the particle is made up of two viral proteins, envelope (E) and membrane (M). The E glycoprotein is the major antigenic determinant of the virion and mediates binding and fusion during virus entry. The M protein is a small proteolytic fragment of the precursor (pr)M protein and is produced during viral maturation within the secretory pathway. Removal of the lipid envelope with nonionic detergents reveals discrete nucleocapsids (120 to 140S; 1.30 to 1.31 g/cm³), which consist of capsid (C) protein and genomic RNA (reviewed in⁷⁴⁵). Isolated nucleocapsids become unstable under high salt conditions, disassembling into C protein dimers.³⁹⁹

Cryo-electron microscopy and image reconstruction have provided a wealth of information on flavivirus structure. Mature infectious particles of DENV⁴³⁰ and WNV⁶¹⁰ display a relatively

smooth outer surface. Fitting the E protein crystal structure⁷²² into the electron density maps showed that glycoprotein dimers lie flat across the surface of the virion. Interestingly, the 180 copies of E are tightly packed in an unusual herringbone array that completely covers the lipid bilayer. Beneath the protein shell, the M protein associates closely with the membrane. Notably, the nucleocapsid lacks discernible symmetry, and neither E nor M sequences extend through the membrane to make contacts with the nucleocapsid.⁹⁵⁶

Immature flavivirus particles adopt various appearances as they egress through the secretory pathway.⁶⁶⁷ Soon after they are formed, immature virions are larger (60 nm in diameter) than mature virions and display 60 prominent spikes on their surface.⁹⁵⁷ Each protrusion is composed of three E-prM heterodimers, with the prM molecule capping the E fusion peptide. As immature particles pass through the low pH environment of the *trans*-Golgi network, a dramatic rearrangement of the glycoproteins occurs. The immature virions now adopt a smooth appearance almost indistinguishable from mature particles, with the exception that prM remains attached.⁹⁴⁶ This conformational change is followed by cleavage of prM by the host cell enzyme furin⁸⁰² and the release of the protective fragment upon exit from the cell, revealing the mature virions.⁹⁴⁶ This process is not always efficient, and immature or partially mature particles can be released in significant quantities. Although immature particles are deemed noninfectious because they cannot undergo fusion,³⁰³ they have recently been shown to initiate infection when internalized in complex with anti-prM antibodies by cells bearing the Fc receptor.⁷³⁵ The mechanism of this is not well understood, but it may invoke prM cleavage in the endosome and could be especially relevant during secondary infection when antibody levels are high. In addition, partially matured particles that retain only some prM have been visualized in flavivirus populations and can undergo attachment and fusion to initiate infection similar to fully mature particles.¹³⁷

Small, noninfectious subviral particles (SVPs) are the final class of particles released from flavivirus-infected cells. SVPs

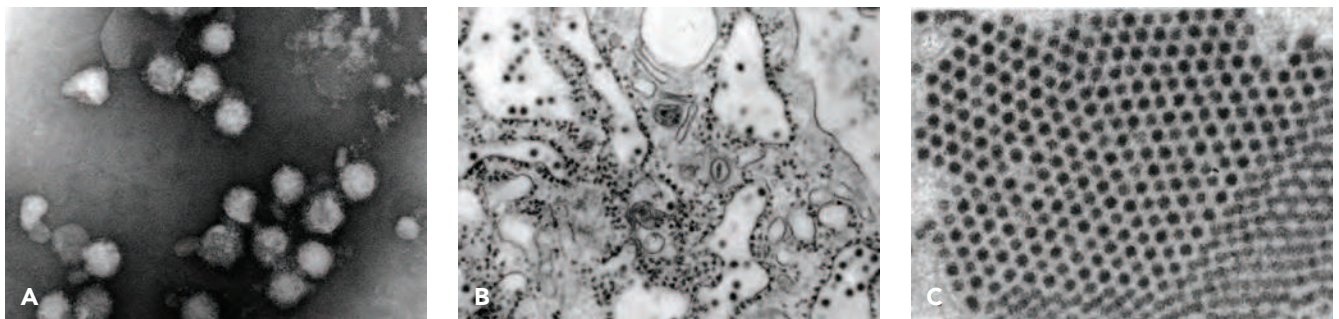


FIGURE 25.3. Electron micrographs of flavivirus particles and virus-infected cells. **A:** Purified St. Louis encephalitis virus (SLEV) negatively stained with ammonium molybdate (Murphy FA. *Togavirus morphology and morphogenesis*. In: Schlesinger RW. *The Togaviruses: Biology, Structure, Replication*. Academic Press: New York; 1980:241–316). Surface projections appear as a very thin, indistinct layer. (Courtesy of Dr. Frederick A. Murphy.) **B:** Thin section of a baby hamster kidney (BHK)-21 cell at 48 hours after infection showing SLEV particles in the cisternae of the endoplasmic reticulum (Whitfield SG, Murphy FA, Sudia WD. *St. Louis encephalitis virus: an ultrastructural study of infection in a mosquito vector*. *Virology* 1973;56:70–87). (Courtesy of Drs. Frederick A. Murphy, Sylvia G. Whitfield, and A. K. Harrison.) **C:** Paracrystalline array of SLEV particles in the salivary gland of a *Culex pipiens* mosquito 25 days after blood meal feeding on an infected suckling mouse. (Courtesy of Sylvia G. Whitfield, Frederick A. Murphy, and W. Daniel Sudia.)

contain E and M proteins but lack C and RNA.⁷⁹⁷ They complete the same maturation process as whole virions and can undergo fusion with a target cell⁷⁶⁰; due to lack of a genome, however, they are not infectious. Recombinant subviral particles (RSPs) form in cells experimentally transfected with only prM and E, indicating that interactions between these envelope proteins are sufficient to drive budding.^{14,512,760} RSPs are generally about 30 nm in diameter and slightly less dense than infectious virus (1.14 g/cm³),⁷⁶⁰ although virion-sized particles have also been observed in these expression systems.^{16,512} Cryo-electron microscopy and image reconstruction of TBEV RSPs suggests a markedly different arrangement of the E proteins compared to infectious virions. Thirty E dimers lie flat against the surface in a $T = 1$ icosahedral shell²³² rather than a herringbone array.⁴³⁰ It is hypothesized that this arrangement may resemble a fusion intermediate that is adopted as E dimers rearrange to trimers upon virus entry.⁶¹¹



Binding and Entry (see Video in e-Book)

Flaviviruses infect a variety of target cells through receptor-mediated endocytosis, followed by intracellular membrane fusion. Flavivirus receptors are not well characterized, perhaps because these viruses use a range of entry factors for different cell types and employ more than one host molecule to enter a target cell. Highly sulphated glycosaminoglycans, such as heparin sulphate, are ubiquitously expressed molecules used as initial attachment factors by many viruses. These factors have also been shown to play a role in binding and entry of flaviviruses, such as DENV,¹³² YFV,²⁶⁹ TBE,⁴²⁶ and JEV.¹³⁰ However, high affinity for glycosaminoglycans in tissue culture-adapted strains is associated with *in vivo* attenuation.⁴⁵⁸ C-type lectins are cellular proteins that bind mannose-rich glycans and are involved in flavivirus infection of dendritic cells (DCs). Intradermal DCs are often the primary targets encountered by the arthropod-delivered pathogens and can transport the virus to draining lymph nodes where a second round of replication initiates viremia. C-type lectin Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) is thought to function as an attachment receptor for DENV infection of DCs.^{516,624,833} WNV preferentially uses DC-SIGN-related (DC-SIGNR),¹⁸² while YFV-17D, which lacks glycan modifications on E, can infect DC cells in a lectin-independent manner.³⁹ The mannose receptor is a C-type lectin that is constitutively internalized by clathrin-mediated endocytosis and has been suggested to play a role in endocytosis of DENV, JEV, and TBEV.⁵⁸⁴ Interestingly, C-type lectin domain family 5, member A (CLEC5) interacts with DENV but does not mediate its entry; instead, CLEC5 binding triggers the release of inflammatory cytokines, leading to DHF/DSS-like symptoms in mice.¹³¹

Additional receptors that have been implicated in flavivirus entry include the glycosphingolipid neolactotetraosylceramide,²⁶ low-density lipoprotein receptor (LDL-R),¹³⁸ laminin receptor 1,^{850,857} $\alpha\beta 3$ integrins,¹⁴⁴ and a CD14-containing complex.¹³⁴ Heat shock proteins have also been suggested as entry factors. GRP78 (BIP) plays a role in liver cell uptake of DENV,^{112,368} and Hsp90/Hsp70 acts in DENV and JEV entry into human monocytes/macrophages, neuroblastoma cell lines, and mosquito cells.^{180,720,723} Finally, virus particles opsonized with subneutralizing concentrations of immunoglobulins show enhanced binding and infection of cells expressing Fc receptors.^{661,764} It is widely speculated that antibody-enhanced

infection is relevant to the pathogenesis of DSS and DHF, which occur more frequently in people previously exposed to other DENV serotypes.

After capture by the appropriate receptor(s), flaviviruses are internalized by endocytosis. Single particle tracking of fluorescently labeled DENV particles has shown that virions diffuse across the surface of the cell until they encounter a preformed clathrin-coated pit.⁸⁷¹ Following internalization, DENV particles are delivered to early or intermediate endosomes, which then mature into late endosomes.⁸⁷¹ Fusion of the viral and host membranes occurs during endosomal trafficking, although the exact compartment that triggers this event seems to differ between strains and flavivirus species, perhaps indicating an optimal pH.⁸⁷¹ In the acidic environment, E protein dimers dissociate and undergo an irreversible conformational change to become fusogenic trimers.^{13,810} The fusion peptide, previously buried at the E homodimer interface, is exposed and inserts into the endosomal membrane (Video 25.1). The efficiency of fusion is influenced by the lipid composition of target membranes: cholesterol, oleic acid, and anionic lipids such as bis(monoacylglycerol)phosphate and phosphatidylserine enhance fusion, whereas lyophosphatidylcholine inhibits the process.^{812,813,950} Lipid composition can also influence the pH threshold of fusion.⁴²⁰ Following fusion, viral genomes are immediately accessible for translation.⁴²⁰

Genome Structure

As for other positive-strand RNA viruses, the genomes of flaviviruses are infectious.⁶⁶² Full-length infectious complementary DNA (cDNA) clones have been constructed for several species, allowing flavivirus biology to be dissected by reverse genetics.^{491,725,742} Flavivirus genomes consist of a single, positive-strand RNA of approximately 11 kilobases (kb) in length (sedimentation, 42S) with a 5' type 1 cap, m⁷GpppAmN^{151,901} (Fig. 25.4). The cap structure serves to stabilize the viral RNA, initiate translation, and subvert innate antiviral defenses.^{178,253} Unlike cellular mRNAs, flavivirus genomes lack a 3' polyadenylate tail.⁹⁰¹ Genomes encode a single long open reading frame (ORF, ~3,400 codons) flanked by 5' and 3' noncoding regions (NCRs) of ~100 nucleotides (nt) and 400 to 700 nt, respectively.⁵⁴⁴ (Fig. 25.4).

The sequence of the 5' NCR is not well conserved between flaviviruses, although common secondary structural elements have been identified, including a bifurcating 5' stem-loop (5' SL). These structures influence viral genome translation, as antisense oligonucleotides complementary to the 5' SL abolish DENV translation and replication, and second-site mutations in this region compensate for replication defects caused by reduced viral cap methylation. In addition, 5' SL likely acts as a promoter to initiate RNA replication by binding the viral NS5 polymerase/methyltransferase protein.^{196,236,237,506} Consistent with this, deletions within the 5' NCR cause severe defects in DENV-4 replication, but not viral translation.¹¹³ Interestingly, one of the viable mutants exhibited a limited host-range growth phenotype, suggesting that host-specific factors interact with either the 5' NCR or the complementary 3' end of the negative strand. Indeed, several human proteins, including La and TIAR, can bind to the 3' end of negative-strand RNA.^{472,778,938} WNV replication is inhibited in a TIAR-knockout cell line,⁴⁷² and mutagenesis of the TIA-1/TIAR binding sites suggests a role in initiating positive-strand RNA synthesis.²¹⁷

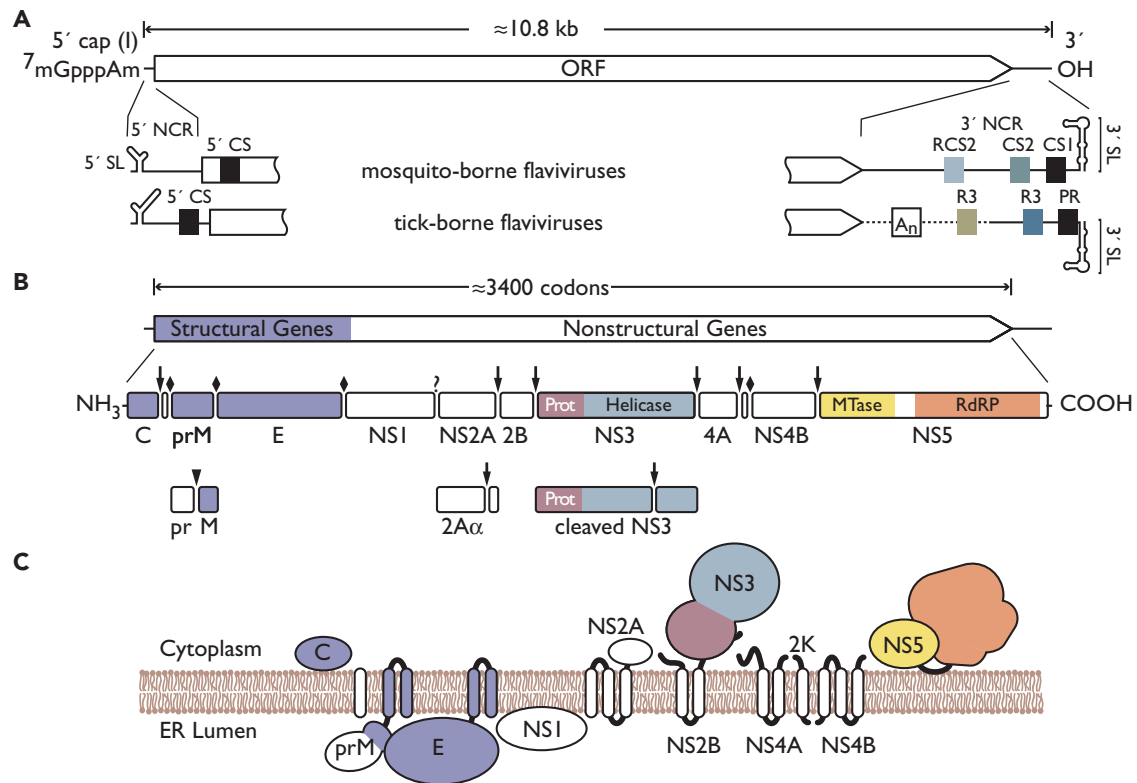


FIGURE 25.4. Flavivirus genome structure and protein expression. **A:** Genome structure and RNA elements. The viral genome is depicted with the open reading frame (ORF), the 5' cap, and the 5' and 3' noncoding regions (NCR) indicated. Functionally significant RNA structures within the viral genome are indicated. (See the text for further details.) **B:** Polyprotein processing and cleavage products. Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. Structural proteins are colored purple, while nonstructural (NS) proteins are white or shaded according to their enzymatic subunits, as indicated. Cleavage sites for host signalase (♦), the viral serine protease (downward arrow), furin or related protease (triangle), or unknown proteases (?) are indicated. **C:** Polyprotein membrane topology. The proposed membrane orientation of the flavivirus proteins is shown. The proteins are approximately to scale (areas are proportional to the number of amino acids) and arranged in order (left to right) of their appearance in the polyprotein.

The organization of the 3' NCR differs between mosquito-borne viruses, tick-borne viruses, and viruses with no known vector. Nevertheless, conserved regions, sequence duplications, and predicted RNA secondary structures are shared among the groups (Fig. 25.4). The greatest structural similarity is a long (90 to 120 nt) 3' stem-loop (3' SL) that differs in primary sequence between mosquito-borne and tick-borne flaviviruses.²⁹⁶ Mutational analysis of DENV-2 and WNV revealed essential virus-specific and host-specific functional regions within the 3' SL.^{216,856,947,953} The 3' SL enhances translation of reporter mRNAs containing the DENV 3' NCR,^{140,335} while DENV-2 translation and replication were inhibited by a corresponding antisense oligonucleotide.³³⁶ The 3' SL also interacts with several important proteins, including the viral NS2A, NS3, and NS5 proteins^{129,175,535} and translation elongation factor 1A (EF1A).^{75,183,185} These results are intriguing, because EF1A, and its prokaryotic homolog EF-Tu, contribute to the replication of other positive-strand RNA viruses.^{81,314,379,951} In addition, the human La autoantigen,^{185,262,875} polypyrimidine tract binding (PTB) protein,¹⁸⁵ and murine Mov34 protein⁸²² were found to bind 3' SL of DENV-4 and JEV, although the functional relevance of these interactions is presently unknown.

Upstream of the 3' SL lie conserved sequence repeats (CS1, CS2, CS3, RCS2, and RCS3), secondary structures, and putative pseudoknots.²⁹⁶ Some of these structures confer resistance to the cellular 5'-3' exoribonuclease Xrn-1,^{252,785} suggesting that this region of the genome forms a compact structure.

Flavivirus genomes can be circularized through long-distance base pairing between elements located near the 5' and 3' ends. In mosquito-borne flaviviruses, these interactions are mediated by the 5' UAR (upstream of AUG region), 5' DAR (downstream of AUG region), and 5' CS (conserved sequence), which base-pair with the 3' UAR, 3' DAR, and 3' CS1 regions, respectively, located more than 10 kb downstream at the base of the 3' SL.³⁵⁴ A distinct set of long-distance interactions circularizes the genomes of tick-borne flaviviruses.^{394,410} These long-range base pairs are important for RNA replication, presumably by bringing the 5' SL-bound NS5 protein in proximity to the 3' site of minus-strand initiation.^{18,96,167,196,236,237,248,354,394,410,940} It should be noted that genome circularization requires the melting of local secondary structures within the 5' and 3' ends and leads to the occlusion of the translation start site. Thus, large-scale conformation changes within the flavivirus genome may regulate the switch from translation to RNA replication.

Translation and Proteolytic Processing

The efficiency of genome translation is a primary determinant of flavivirus infectivity.²⁰⁹ The viruses therefore use several mechanisms to ensure translational competence, including specialized structures within the 5′ and 3′ NCRs. Translation is cap dependent, and 2′-O methylation of the 5′ cap helps to overcome innate antiviral defenses that down-regulate translation in infected cells.¹⁷⁸ While translation initiates via ribosomal scanning, many mosquito-borne flaviviruses lack a canonical Kozak initiation motif and contain several AUG codons near the correct start site. To help ensure proper AUG selection, DENV uses a small RNA stem-loop embedded within the C gene to induce ribosomal pausing over the authentic initiation codon.¹⁵⁵

Translation of the single, long ORF produces a large polyprotein that is co- and posttranslationally cleaved into at least 10 proteins (Fig. 25.4B). The N-terminal region of the polyprotein encodes the structural proteins (C-prM-E), which are followed by the NS proteins (NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5).^{119,120,726} Host signal peptidase is responsible for cleavages between C/prM, prM/E, E/NS1, and 2K-NS4B. A virus-encoded serine protease, NS2B-3, processes at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/2K, and NS4B/NS5 junctions. The enzyme responsible for NS1-2A cleavage is presently unknown. The expected topology of the flavivirus polyprotein is depicted in Figure 25.4C.

Features of the Structural Proteins

C Protein

Capsid (C) protein is a highly basic protein of ~11 kD. The nascent protein contains a C-terminal hydrophobic tail that serves as a signal peptide for ER translocation of prM. This anchor is cleaved in two steps, first by the viral NS2B-3 protease and then by signal peptidase.⁵⁰³ Mature C protein folds into a compact dimer with each monomer containing four α -helices^{193,372,529} (Fig. 25.5). The N-terminal region of the

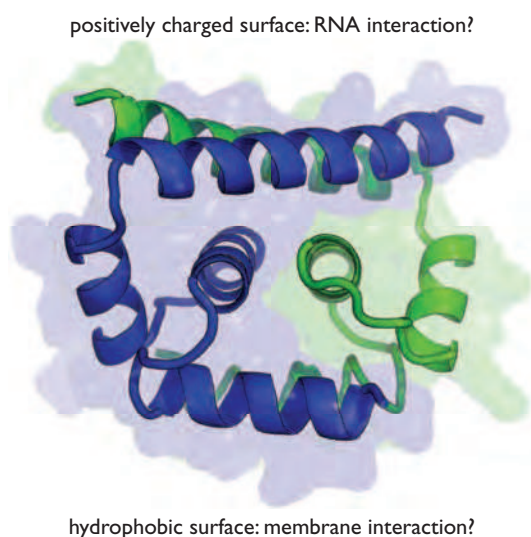


FIGURE 25.5. Flavivirus C protein structure. The WNV-KUN C protein is shown as a ribbon diagram with the protein surface rendered transparent, from PDB accession number 1SFK.¹⁹³ One monomer of the dimer is colored blue, the other green.

protein remains unstructured and, along with charged residues at the C-terminus, is thought to be involved in RNA binding.³⁹⁸ An internal hydrophobic region mediates membrane association of C.⁵²⁹

Overall, flavivirus C proteins demonstrate remarkable functional flexibility, with tolerance for large deletions. YFV C retains its ability to package RNA even after deletion of nearly 40 residues from the N-terminus or 27 residues of the C-terminus; internal deletions of the hydrophobic sequence are less tolerated.⁶⁵⁴ The TBEV C protein can accept deletions of up to 16 amino acids from the central hydrophobic helix, albeit with increased production of empty particles.⁴⁰⁹ Mutants containing larger deletions are not viable but can be rescued by second-site changes that increase the hydrophobicity of downstream sequences.⁴¹¹ WNV tolerates small deletions in hydrophobic helix $\alpha 2$ to various degrees. Remarkably, infectivity of the deleted genomes was improved by even larger deletions—up to one-third of the C protein sequence—encompassing all of helix $\alpha 3$.⁷⁶⁵ This results in the loss of a hydrophilic stretch, again suggesting the importance of hydrophobicity. It is not yet clear how C protein dimers are organized within the apparently disordered nucleocapsids, but interaction with RNA or DNA can induce isolated C protein dimers to assemble into nucleocapsid-like particles *in vitro*.³⁹⁹

Membrane Glycoprotein prM

The glycoprotein precursor of M, prM (~26 kD), is translocated into the ER via a signal sequence provided by the hydrophobic tail of C. Signal peptidase cleavage is delayed, however, until the viral serine protease cleaves on the cytosolic side of the membrane to generate the mature form of C.^{19,503,924} This delay seems to result from the combination of a fairly short (14 to 22 amino acids) signal sequence, suboptimal residues at the signalase cleavage site, and residues in downstream regions of prM^{504,815} and E protein.⁵¹¹ Interestingly, uncoupling signal peptidase cleavage from NS2B-3 processing leads to increased production of empty virus particles.^{459,504,505} Coordinated cleavage therefore serves to delay structural protein processing until the viral serine protease has accumulated and replication is under way, which may limit the release of immunogenic but noninfectious SVPs early in infection.

The N-terminal region of prM contains one to three N-linked glycosylation sites¹²² and six conserved cysteine residues, all of which are disulfide linked.⁶³³ The prM protein folds rapidly and assists in the proper folding of E.^{415,511} The C-terminal transmembrane (TM) domains of prM and E act as ER retention signals and may assist in their heterodimerization.^{486,641,643} A major function of prM is to prevent E from undergoing acid-catalyzed rearrangement and fusion during transit of the virions through the secretory pathway.^{302,322} The crystal structure of DENV prM in complex with E has recently been solved and demonstrates how this function is performed.⁴⁷¹ The pr domain is a unique fold consisting of seven β strands, with the previously identified disulfide bonds stabilizing the structure. In immature particles, the pr region sits at the tip of the E protein, forming the pr-E spike and shielding the fusion peptide from the cellular environment (Fig. 25.6A). prM is not accessible to furin cleavage in these particles due to steric hindrance.⁴⁷¹ The acidity of the *trans*-Golgi compartment induces a global rearrangement that exposes the furin cleavage site.⁹⁴⁶ After cleavage, the pr peptide does not immediately

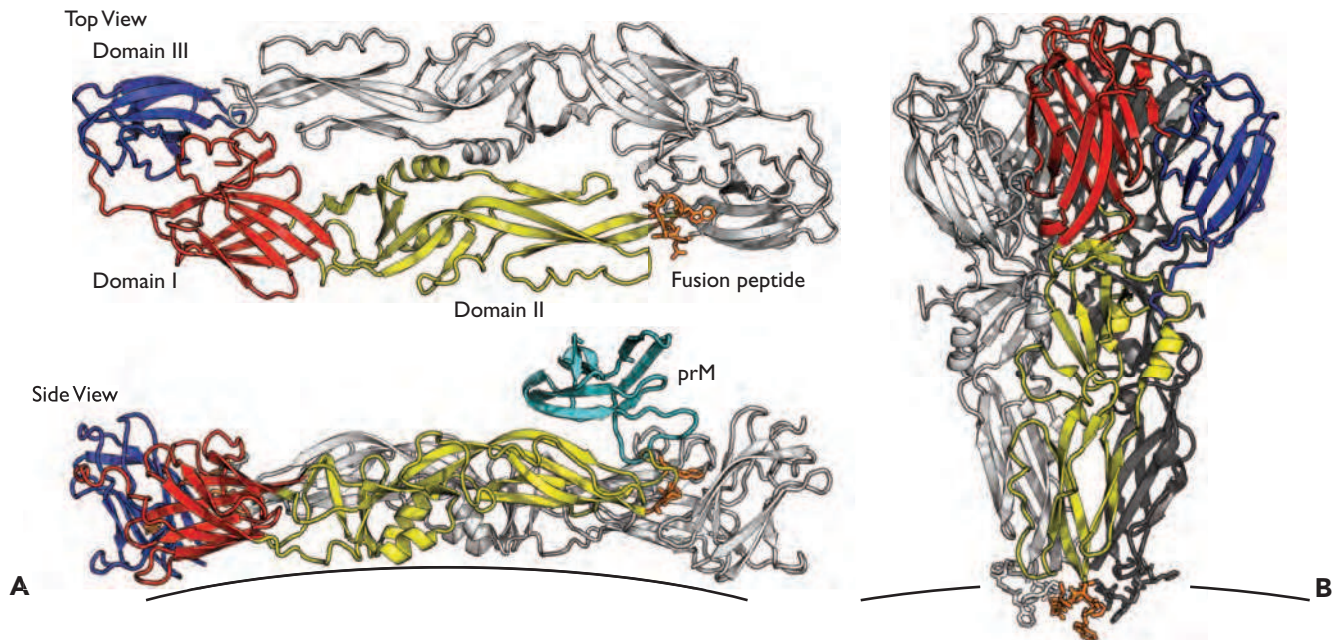


FIGURE 25.6. Flavivirus glycoprotein structures. **A:** The structure of a dengue virus 2 (DENV-2) E glycoprotein dimer is represented in this ribbon diagram, as viewed perpendicular (**top**) or laterally (**bottom**) with respect to the lipid bilayer. One E monomer is colored red (domain I), yellow (domain II), and blue gray (domain III). The amino acid side chains of the fusion peptide are shown (orange). Rendered from PDB 10AN.⁵⁹¹ In the **bottom panel**, the low pH conformation of the DENV-2 pr protein from PDB 3CSX⁴⁷¹ was modeled onto the structure of 10AN. **B:** tick-borne encephalitis virus (TBEV) E protein trimers in their postfusion form, colored as in **A**. Rendered from PDB 1URZ.⁹⁸

disassociate from the virus particle.⁸⁰² Exposure to the neutral pH of the extracellular space is required to release pr and reveal the fusion-competent mature virion. This delay prevents the cleaved particles from undergoing premature membrane fusion within the Golgi.

Envelope Glycoprotein

E protein (~53 kD) is the major protein on the surface of flavivirus virions. E is synthesized as a type I membrane protein containing 12 conserved cysteines that form disulfide bonds⁶³⁴; in some viral species E is N-glycosylated.^{123,909} Proper folding, stabilization in low pH, and secretion of E depends on co-expression with prM.^{415,511} E is a class II fusion protein that mediates both receptor binding and membrane fusion.

Atomic resolution structures of E proteins from several flaviviruses have been solved in pre- and postfusion conformations.^{98,382,591–592,593,625,635,722,958} In its prefusion form, E folds into an elongated structure rich in β -sheets and forming head-to-tail homodimers that lie parallel to the virus envelope.^{591,722} Each E protein is composed of three domains: DI, which forms an eight-stranded β -barrel; DII, a long, finger-like domain that projects along the virus surface; and DIII, which maintains an immunoglobulin-like fold (Fig. 25.6A). The fusion peptide¹² is located at the tip of DII and remains covered by the pr peptide or buried in a hydrophobic pocket formed by DI and DIII of the partner monomer until triggered to insert into the target cell membrane.⁷²² DIII projects slightly from the virion surface and is thought to be involved in receptor binding; it is a major target of neutralizing antibodies.¹⁴⁵ Between the ectodomain of E and the membrane is a short but functionally important

stem region composed of two α -helices that lie parallel to the plane of the membrane.^{15,956}

On exposure to low pH, E protein dimers dissociate into monomeric subunits, which then form fusogenic trimers.^{13,810,811} Interestingly, the WNV E protein crystal structure shows an array of perpendicular monomers, suggesting a mechanism for E protein rotation without exposing the fusion loop.^{635,814} Crystal structures of postfusion E show the protein folded back onto itself, bringing the N-terminal fusion peptide, with its associated cellular membrane, into proximity with C-terminal TM domain, which is still integrated in the viral membrane^{98,592} (Fig. 25.6B). To accomplish this, DIII must rotate and fold back more than 30 Å in relation to DI. Indeed, neutralizing antibodies against DIII can inhibit a postattachment step of viral entry,⁶³⁶ and a soluble, recombinant form of DIII is a potent dominant-negative inhibitor of fusion.⁴⁷⁸ In addition, DII rotates relative to DI,^{98,592} with similar displacement of DII seen in crystals of native E protein grown in the presence of the detergent β -octylglucoside.^{591,958} Residues that influence the pH threshold for membrane fusion surround the DI/DII pocket.⁵⁹¹ Protonation of conserved histidines at the interface of DI and DIII also contribute to E domain rearrangements in TBEV RSPs.²⁴⁹ Mutagenesis studies of WNV, however, failed to identify histidine residues that entirely control the switch.⁶²⁹

Features of the Nonstructural Proteins

NS1 Glycoprotein

The NS1 glycoprotein (~46 kD) is translocated into the ER during synthesis and processed at its N-terminus by host signal

peptidase. The C-terminus of NS1 arises through NS1–2A cleavage by an unknown ER-resident host enzyme, which requires the eight C-terminal residues of NS1 and greater than 140 amino acids of NS2A.^{227,228,340} In addition, JEV expresses an elongated form of NS1, termed NS1', which arises through a ribosomal –1 frameshifting event.^{80,238,547,565}

NS1 contains two or three N-linked glycosylation sites and 12 conserved cysteines that form disulfide bonds.^{79,461,547,797,884} Around 30 minutes after synthesis, NS1 simultaneously forms highly stable homodimers and acquires an affinity for membranes.^{910,911} As NS1 lacks a known membrane interaction domain, the nature of its membrane association remains unclear. One possibility is that dimerization creates a hydrophobic surface for peripheral membrane binding. Alternatively, it has been reported that DENV-2 NS1 exhibits properties of a glycosylphosphatidylinositol (GPI)-anchored protein, although this mechanism seems inconsistent with the C-terminal peptide sequence of this protein.⁹⁰²

NS1 is retained within a secretory-derived compartment, expressed on the surface of infected cells, and efficiently secreted from mammalian, but not insect, cells.^{491,816} The relative distribution of NS1 within these compartments is regulated through an unknown mechanism involving a short, N-terminal region of the protein.⁹⁴² The secreted form of NS1 accumulates to high levels in human sera and tissues and can be used to diagnose flavivirus infections at an early stage.^{8,147,531} Secreted NS1 forms soluble, hexameric lipoprotein particles of ~10 nm that appear as three dimers held together in a barrel configuration.^{172,239,304} The secreted form of NS1 can bind to uninfected cells by interaction with sulfated glycosaminoglycans³³ and can be internalized and trafficked to late endosomes, where it accumulates.⁹ The function of endocytosed NS1 is not yet clear, but it may enhance subsequent infection with the homologous virus.⁹

The intracellular form of NS1 localizes to sites of viral RNA synthesis and plays an essential role in genome replication.^{533,904} Mutations in NS1 can lead to dramatic defects in RNA replication and infectious virus production.^{170,619,620} *trans*-Complementation studies revealed that NS1 functions at an early stage in RNA replication through a genetic interaction with NS4A.^{395,396,490,492}

The extracellular forms of NS1 are highly antigenic and induce a strong humoral response. Secreted NS1 was originally characterized as a soluble, complement-fixing antigen present in the serum and tissues of DENV-infected animals. Antibodies that recognize cell surface-bound NS1 can direct complement-mediated lysis of infected cells and protect animals from lethal disease; other NS1-specific antibodies are protective in a complement-independent manner.^{148,149} Antibody-mediated cross-linking of cell surface NS1 can also induce signaling cascades in DENV-2-infected cells, and it has been proposed that NS1 may contribute to pathogenesis by inducing antibodies that cross-react with human proteins. Despite the strong link between NS1-specific humoral responses and complement fixation, recent evidence indicates that WNV NS1 can inhibit the alternative pathway of complement activation by binding to and inhibiting the serum protein factor H. Furthermore, DENV, WNV, and YFV NS1 inhibit the classical pathway of complement fixation by binding to and increasing the turnover of complement factor C4. NS1 clearly plays an important role in flavivirus-specific humoral responses.

NS2A and NS2B Proteins

NS2A is a relatively small (~22 kD) hydrophobic protein. Its N-terminus is generated by an unidentified ER-resident host enzyme,²²⁸ whereas the C-terminus is generated by NS2B-3 cleavage in the cytoplasm. Thus, NS2A is membrane spanning, although the precise topology of the protein is unknown. In addition, the YFV serine protease can cleave at an internal site in NS2A to generate a C-terminally truncated form, NS2A α .^{123,630} Interestingly, mutations at the YFV NS2A α cleavage site block virus particle production and can be suppressed by a second mutation on the surface of the NS3 helicase domain.⁴³⁴ Mutations in KUNV NS2A similarly block virus assembly, while the protein also localizes to subcellular sites of RNA replication and interacts with replicase components NS3, NS5, and the 3' NCR of genome RNA. The involvement of NS proteins, in particular the NS2–3 region, in replication and infectious virus assembly appears to be an emerging theme for all three genera of the family *Flaviviridae*.

DENV-2 and WNV NS2A can also inhibit interferon (IFN) signaling, as evidenced by specific mutations in the protein that diminish this inhibitory activity and attenuate virulence in mice.^{498,500,613} Interestingly, these mutations are cell culture adaptive and enhance the ability of KUNV replicons to establish persistence in IFN-competent cell lines. Remarkably, NS2A of the tick-borne flavivirus Langat does not share this property, which appears to be carried out instead by NS5.⁶⁶

NS2B is also a small (~14 kD) membrane-associated protein.¹⁵⁴ NS2B forms a stable complex with NS3, serves to anchor this complex to cellular membranes, and acts as an essential co-factor for the NS2B-3 serine protease.²²⁹ The co-factor activity lies in a central peptide that intercalates within the fold of the serine protease domain.^{31,125,221}

NS3 Protein

NS3 is a large (~70 kD) multifunctional protein, encoding enzymatic activities required for polypeptide processing and RNA replication. The N-terminal third of the protein is the catalytic domain of the NS2B-3 serine protease,^{50,124,279} which has specificity for substrates containing adjacent basic residues at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions.¹²² In addition, this protease generates the C-termini of mature capsid protein^{19,924} and NS4A⁴⁸¹ and can cleave at internal sites within NS2A and NS3.

Soluble, recombinant forms of the NS2B-3 serine protease domain have been purified and crystallized for x-ray diffraction.^{125,221,733} These structures show that the co-factor region of NS2B contributes a β -strand to complete the chymotrypsin-like fold of the protease (Fig. 25.7A), similar to hepatitis C virus (HCV) NS3-4A. The C-terminal region of the NS2B co-factor can adopt multiple conformations that may alternately help to form the substrate-binding pocket or project outward from the protease fold; it is not yet clear whether these structural rearrangements are biologically significant.

As for other members of the *Flaviviridae*, the C-terminal region of NS3 encodes a supergroup 2 RNA helicase–nucleoside triphosphatase (NTPase).²⁸⁰ NS3 demonstrates RNA-stimulated NTPase and RNA unwinding activities^{890,899} and mutagenesis of the active site residues confirmed that these activities are essential for viral replication.⁵⁴⁹ This region of NS3 also exhibits RNA triphosphatase (RTPase) activity, proposed to dephosphorylate the 5' end of the genome before cap addition.⁹⁰⁰ Recent

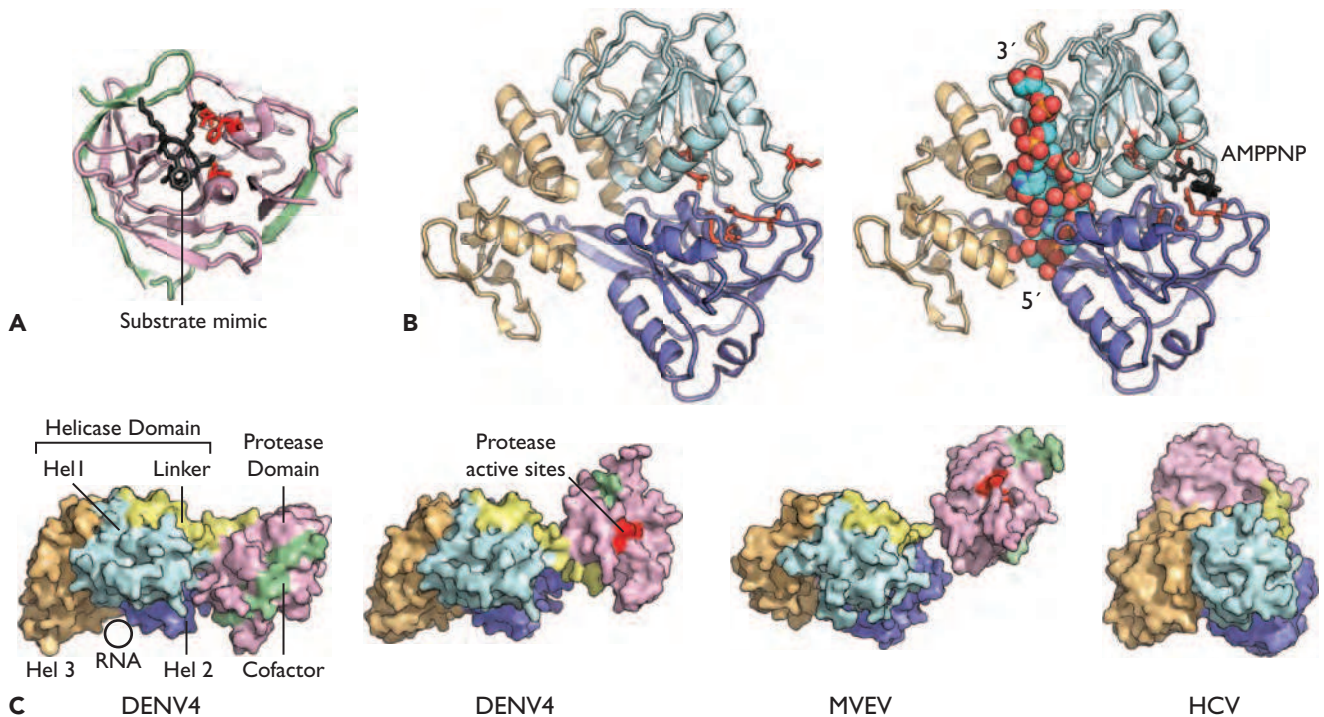


FIGURE 25.7. Viral NS3 proteins. **A:** The structure of the West Nile virus (WNV) NS2B-3 serine protease domain, with NS2B co-factor peptide (green), NS3 protease domain (pink), and a substrate-based inhibitor (black). Active-site residues are shown in red. Rendered from PDB number 2FP7.²²¹ **B:** Structure of the dengue fever 4 (DENV-4) NS3 helicase domain with (left) or without (right) bound RNA substrate (colored spheres) and adenosine triphosphate (ATP) analog (black). Note the adenosine triphosphatase (ATPase) active site (red) becomes structured at the interface of domain I (cyan) and domain II (purple) upon RNA binding. The RNA is bound within a cleft formed by the first two domains and domain III (gold). Rendered from PDB numbers 2JLQ and 2JLV.⁵²³ **C:** Structural interdomain flexibility within full-length NS3. Shown are two conformations of full-length DENV-4 NS3 (PDBs 2VBC⁵²² and 2WHX,⁵²¹ respectively), Murray Valley encephalitis virus (MVEV) (PDB 2WV9⁹¹), and hepatitis C virus (HCV) (PDB 1CU1⁹³⁰). Shown is a structural alignment of the helicases, with molecular surfaces colored as in **A** and **B** and the protease-helicase linker in yellow.

studies show that RTPase is dependent on the Walker B motif in the helicase–NTPase catalytic core for phosphodiester bond hydrolysis.^{41,58} Thus, all three nucleic acid–modifying activities of NS3 rely on a common active center. In addition to its roles in RNA replication, the helicase domain of NS3 has been implicated in virus assembly,^{434,655} a role that is separable from the known enzymatic activities and can function *in trans*.⁶⁵⁵

Crystal structures of isolated DENV and YFV NS3 helicase domains show three subdomains, two structurally conserved RecA-like domains that are involved in NTP hydrolysis, and a unique C-terminal domain that may be involved in virus-specific RNA and protein recognition.^{523,916,921} Cocrystallization of the DENV helicase with or without RNA substrates and nucleoside analogs revealed the structural basis for RNA-stimulated adenosine triphosphatase (ATPase) activity and substrate unwinding⁵²³ (Fig. 25.7B). The structures of full-length DENV and MVEV NS3 have recently been solved in complex with their corresponding NS2B co-factors.^{31,521,522} In these structures, the serine protease and helicase regions largely retain their domain folds, forming an elongated binary complex. However, the relative orientation of the domains differ, and greatly differ from the orientation of the related domains in HCV NS3, implying that the flexible linker region may play an important role in coordinating enzyme activities

(Fig. 25.7C). The DENV structure also revealed that the serine protease domain can contribute to RNA helicase activity.⁵²²

Truncated forms of NS3, which result from alternative serine protease cleavage events in the helicase domain, have been observed *in vitro* and *in vivo*. The role of these cleavages is unclear, although it is possible that the products could have a distinct function. In this regard, replication defects caused by deletions in the KUNV helicase domain can be complemented *in trans*, while deletions in the serine protease domain cannot be complemented.^{374,396,499}

Finally, the NS3 proteins of Langat, DENV-2, and WNV have been shown to induce apoptosis, in some cases through activation of caspase-8.^{691,704,776} Flaviviruses are often cytopathic in mammalian cells, although whether this is the normal pathway for cell killing requires further study. The DENV NS2B-3 serine protease can also down-regulate the activation of type I IFN in human dendritic cells, although the relevant protease substrate(s) have not been identified.⁷³⁶

The NS4A and NS4B Proteins

NS4A and NS4B are small (16 kD and 27 kD, respectively) hydrophobic proteins. NS4A has been implicated in RNA replication through a genetic interaction with NS1⁴⁹⁰ and co-localization with replication complexes.⁵³⁵ Similar to the

coordinated processing of C protein, signal peptidase cleavage at the 2K/NS4B junction requires prior cleavage by the NS2B-3 serine protease at a site just upstream of the 2K internal signal peptide.^{481,690} Overexpression studies showed that NS4A can induce membrane rearrangements and/or formation of autophagosomes, and that regulated NS4A/2K/4B cleavage is necessary for this activity.^{560,586,738} Mutations in NS4A and 2K have been found to confer resistance to a potent inhibitor of flavivirus RNA replication and to overcome superinfection exclusion, further implicating this region in RNA replication.^{966,967}

NS4B is a polytopic membrane protein that co-localizes with NS3 at the presumed sites of RNA replication.^{481,587} NS4B is posttranslationally modified to a form that migrates faster on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),^{123,690} although the identity and function of this modification remain to be determined. Similar to NS2A, DENV NS4A and NS4B can block type I IFN signaling.⁶¹³ NS4B has the strongest antagonistic effect, which requires either proper processing of the NS4A–NS4B polypeptide or expression of NS4B with an N-terminal signal peptide.⁶¹² Similarly, WNV NS4A and NS4B block IFN signaling by inducing an unfolded protein response in the ER, which can down-regulate Jak-STAT signaling.²⁰

NS5 Protein

NS5 is a large (103 kD), highly conserved, multifunctional phosphoprotein with RNA capping and RdRP activities encoded within its N- and C-terminal regions, respectively.¹⁸¹ Formation of a type 1 RNA cap involves multiple steps, including (a) removal of one phosphate from a 5' triphosphorylated RNA substrate by an RTPase, (b) addition of a 5'-5' guanosine cap (from guanosine triphosphate [GTP]) by a guanylyltransferase, (c) N7-methylation of the guanylyl cap by a methyltransferase (MTase), and (d) 2'-O methylation of the second residue by the same or another MTase. As mentioned earlier, the NS3 helicase–NTPase exhibits RTPase activity, which suggests that NS3

and NS5 function together during RNA capping.^{41,900} The flavivirus guanylyltransferase proved to be elusive for many years, although recent evidence indicates that the N-terminal domain of NS5 is capable of performing this reaction.³⁶¹ Finally, the N-terminal region of NS5 encodes conserved MTase motifs and is capable of performing both N7 and 2'-O methylation in a coordinated fashion.^{211,417,713} The structure of the NS5 capping domain has been solved by x-ray crystallography under a variety of conditions, revealing high-resolution structures of these reaction pathways^{181,211,266,931} (Fig. 25.8A). Mutagenesis of the NS5 capping domain showed that the methylation events are separable and that N7 methylation is required for viral translation and replication, while 2'-O methylation allows the virus to avoid innate antiviral defenses.^{178,195,427,964} Interestingly, cellular casein kinase 1 can phosphorylate YFV NS5 *in vitro* at a serine residue near the methyltransferase active site, which inhibits 2'-O methylation.^{67,68} While inhibitors of this kinase affect YFV replication, it is not known if this site is phosphorylated *in vivo*.

The C-terminal domain of NS5 contains conserved RdRP motifs^{417,726} and structurally resembles other RNA polymerases, forming a “right hand” structure with palm, fingers, and thumb subdomains^{181,543,932} (Fig. 25.8B). NS5 RNA polymerase activity has been confirmed with purified, recombinant protein^{1,181,305,826}; mutagenesis of the polymerase active site; and supplying the activity *in trans* from a KUNV replicon. The major product of *in vitro* RdRP reactions is often a self-primed copy-back RNA. However, NS5 is capable of initiating RNA synthesis *de novo*,^{1,632,948} which likely reflects the authentic mechanism in infected cells. NS5 forms a complex with NS3^{371,389} and stimulates NS3 NTPase and RTPase activities.^{175,939} Cross-linking studies have shown that both proteins bind to the 3' SL of the viral genome.¹²⁹ Along with genome circularization, this may serve to initiate minus-strand synthesis by 5' SL-bound NS5.^{196,237,354,940}

WNV and DENV-2 NS5 have been shown to localize at sites of viral RNA synthesis,^{534,898} although this has been

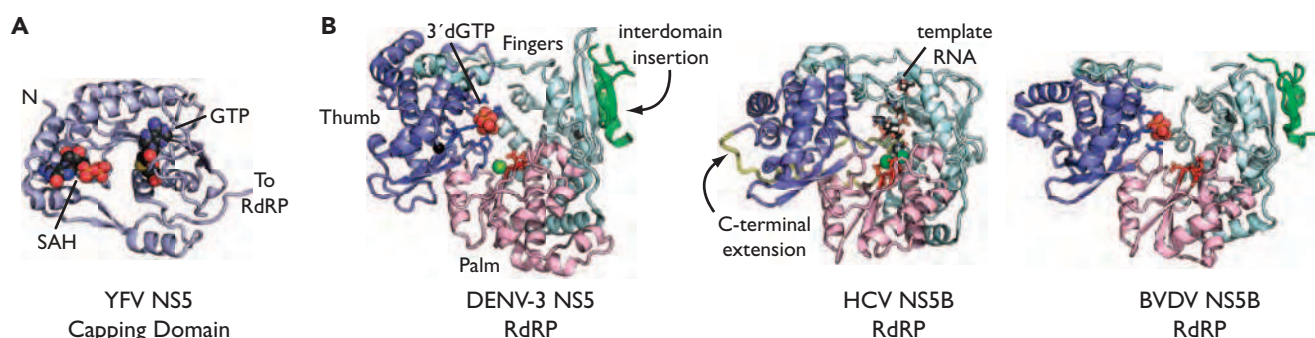


FIGURE 25.8. Viral NS5 and NS5B proteins. **A:** The yellow fever virus (YFV) capping domain is shown, with bound methyl donor S-adenosyl-L-homocysteine (SAH) and guanosine triphosphate (GTP). Rendered from PDB number 3EVC.²⁶⁶ **B:** A structural comparison of RNA-dependent RNA polymerases (RdRP) domains across the *Flaviviridae*. The dengue virus 3 (DENV-3) RdRP domain is shown, with the canonical finger (cyan), palm (pink), and thumb (purple) domains indicated. A flavivirus-specific interdomain insertion is shown in green. This structure shows the binding site of a GTP analog, which is important for activating *de novo* RNA synthesis. GTP-binding residues are blue, RdRP active-site residues are red, and a catalytic Mg²⁺ ion is shown as a green sphere. Structural Zn²⁺ ions are shown in black. This model is a composite structure, rendered from PDB numbers 2J7U and 2J7W.⁹³² The hepatitis C virus (HCV) NS5B RdRP is shown with bound template RNA; the HCV-specific C-terminal extension is shown in yellow. Rendered from PDB number 1NB7.⁶³⁷ The bovine viral diarrhea virus 1 (BVDV-1) NS5B RdRP is shown modeled in the GTP-bound state by rendering PDB number 2JCQ and the GTP analog from 2J7W. The pestivirus-specific N-terminal extension is shown in green.

difficult to show for other viruses. Biochemical studies indicate that only a small fraction of NS5 co-fractionates with replicase activity,^{298,868} and the protein is frequently localized to the nucleus of flavivirus-infected cells.^{181,389} These results suggest that NS5 may play additional roles, other than in RNA replication, in the virus life cycle. In this regard, DENV-2 NS5 induces interleukin-8 (IL-8) transcription and secretion, which may enhance viral spread or disease by recruiting inflammatory cells to the site of infection.⁵⁶² In addition, NS5 has been shown to block the Jak-STAT pathway of IFN signaling.^{66,181}

RNA Replication

The flavivirus NS proteins presumably recruit the viral genome out of translation and into a replication complex. Replication begins with the synthesis of a genome-length minus-strand RNA, which then serves as a template for new plus-strand genomes. Minus-strand RNA has been detected as early as 3 hours after infection.⁴⁹² Viral RNA synthesis is asymmetric, with approximately 10-fold more positive strands accumulating compared to minus strands.^{152,620} Flavivirus replication can be followed by metabolic labeling of virus-specific RNA in the presence of actinomycin D, an inhibitor of DNA-dependent RNA polymerases. Three major species of labeled flavivirus RNA have been described, including the plus-strand genome, a double-stranded replicative form (RF), and a heterogeneous population of replicative intermediates (RIs) that most likely represent duplex regions and recently synthesized RNAs displaced by nascent strands undergoing elongation.^{146,152} Pulse-chase analyses indicate that RF and RI are precursors to genome RNA,^{146,152} indicating semiconservative and asymmetric replication.¹⁴⁶

In addition to genome-length plus- and minus-strand products of RNA replication, 0.2- to 0.6-kb subgenomic flavivirus RNAs (sfRNAs) are produced in infected cells.^{485,762,869}

sfRNAs are co-linear with the 3' end of the genome and are produced through incomplete degradation of the genome by the cellular 5'-3' exoribonuclease Xrn1.^{678,785} The Xrn1 resistance of this region is due to conserved secondary structures and pseudoknots located within the 3' NCR.^{252,785} While controversy exists over whether sfRNAs are needed for efficient RNA replication, a WNV mutant that does not produce sfRNAs is less cytopathic in cell culture and less pathogenic in mice.^{252,678,785} Interestingly, supplying sfRNA *in trans* restored WNV cytopathic effect in cell culture, suggesting that these small RNAs may have a specific but as yet unknown target.⁶⁷⁸

Membrane Reorganization and the Compartmentalization of Flavivirus Replication

Biochemical studies of flavivirus-infected cells show that replicase activity is concentrated in dense membrane fractions that are enriched for most viral NS proteins. Treatment with non-ionic detergents increases sensitivity to nucleases and proteases, indicating that the active replicase resides within a membrane-bound compartment. Consistent with this, ultrastructural changes in perinuclear membranes can be detected in flavivirus-infected cells.⁸⁹⁸ In general, the earliest event is the proliferation of ER membranes, followed by the appearance of smooth vesicular structures around the time of early logarithmic virus production. These structures, sometimes referred to as double-membrane vesicles or vesicle packets, are small clusters of ~90-nm vesicles within the lumen of the ER.^{898,904} They are frequently adjacent to ER-derived convoluted membranes, which can appear as randomly folded membranes or highly ordered "paracrystalline arrays".^{454,616,898} Electron tomography revealed that these vesicles are invaginations of the ER and retain connectivity to the cytosol through a neck-like pore, sometimes apposed to sites of virus assembly⁸⁹⁸ (Fig. 25.9).

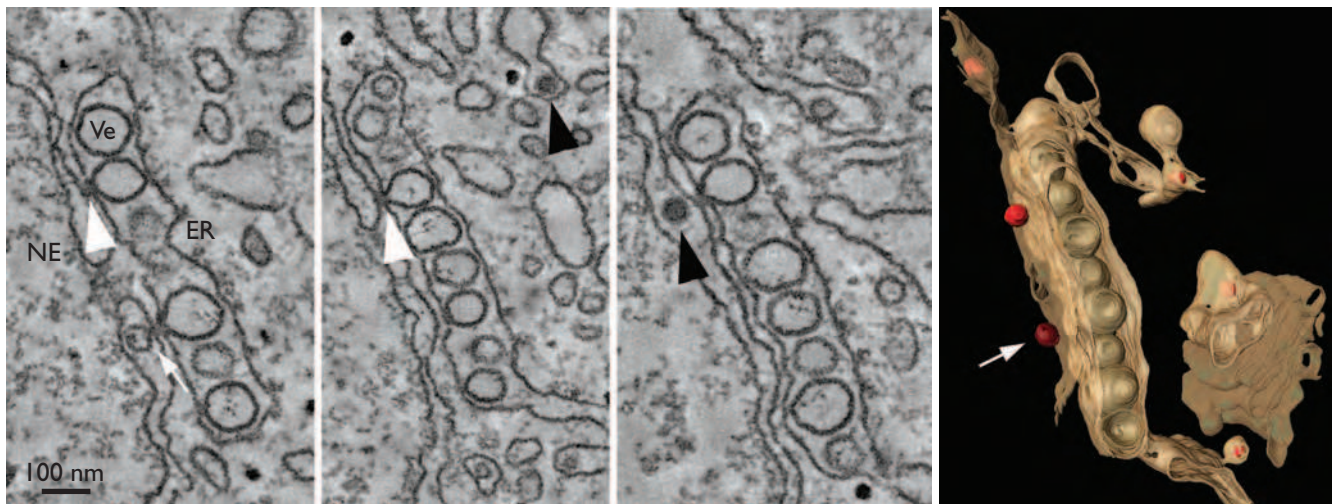


FIGURE 25.9. Sites of dengue virus (DENV) RNA replication and virus particle assembly. The three tomographic slices, each ~2 nm thick, show DENV-induced vesicles (Ve) associated with the endoplasmic reticulum (ER) and nuclear envelope (NE). White arrowheads indicate necked connections between the Ve and ER membranes; black arrowheads indicate virus particles. To the right is a three-dimensional reconstruction of the membrane surfaces (tan) and virus particles (red). The white arrow indicates a putative site of virus budding. (Courtesy of Drs. Sonja Welsch and Ralf Bartenschlager. Adapted from Welsch S, Miller S, Romero-Brey I, et al. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host Microbe* 2009;5:365–375, with permission from Elsevier.)

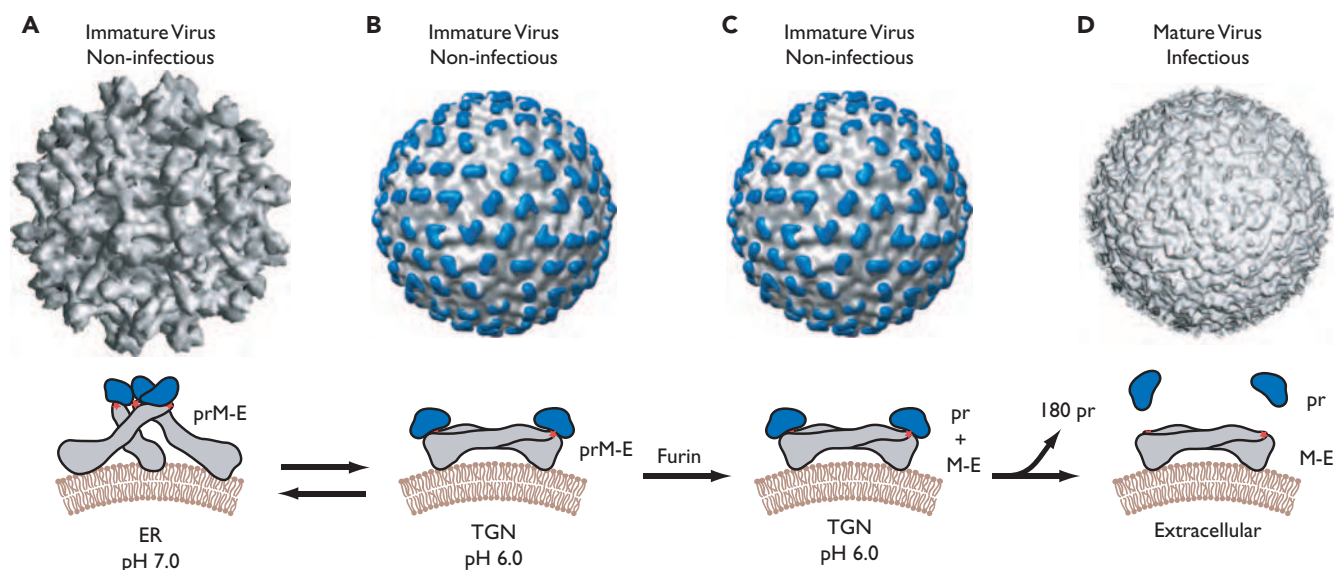


FIGURE 25.10. Flavivirus particle maturation. Nascent, noninfectious, immature particles undergo a pH-dependent conformational rearrangement of the viral glycoproteins E (gray) and precursor M (prM) (blue). Mature, infectious particles form upon furin cleavage of prM and release of the pr fragment. (Adapted from Perera R, Kuhn RJ. Structural proteomics of dengue virus. *Curr Opin Microbiol* 2008;11:369–377, with permission from Elsevier.)

Perinuclear vesicle packets have been confirmed as the sites of viral RNA synthesis by metabolic labeling of nascent RNA, *in situ* hybridization, and immunodetection of double-stranded RNA (dsRNA) (presumably RF and RI).

The pathways of flavivirus-induced membrane reorganization are currently being worked out. One important clue comes from the ability of DENV-2 NS3 to recruit the cellular fatty acid synthase to sites of replication, at least by late times of infection.³¹⁹ In addition, DENV induces autophagosome formation in infected cells,^{393,463,560,651} leading to turnover of triglycerides into free fatty acids, β -oxidation of lipids, and increased adenosine triphosphate (ATP) levels that promote virus replication.³²⁰

Assembly and Release of Particles from Flavivirus-Infected Cells

Similar to replication, early ultrastructural studies indicated that flavivirus morphogenesis occurs in close association with intracellular membranes.⁶¹⁵ The assembly process is thought to commence by association of C protein dimers with genomic RNA, followed by budding into ER membranes containing the E–prM glycoprotein complex. Recent electron tomography studies of DENV-infected cells have shown that replicase-containing vesicle packets and ER-associated sites of virus budding are part of one continuous network. Pores within the replication vesicles appear to release newly synthesized viral RNA directly adjacent to budding DENV particles.⁸⁹⁸ These findings suggest a potential mechanism for coupling of flavivirus replication and assembly.³⁹⁷ Interdependence of these processes is a common theme among RNA viruses and might serve to reduce the propagation of defective genomes. Coupling flavivirus replication and assembly may also explain the roles of NS2A and NS3 proteins in infectious virus assembly.^{434,465,497,655}

Following assembly, nascent virions are transported through the secretory pathway and released at the cell surface.⁵³⁶

It is during secretion that immature particles undergo acid-induced E–prM rearrangement and prM cleavage (Fig. 25.10). Additional virion maturation steps occur during egress, including glycan modification of prM and E (for some viruses) by trimming and terminal addition.^{123,169,309,547} This implies that virions move through an exocytosis pathway similar to that used for host cell surface glycoproteins. In addition to the secretory pathway machinery, other host factors have been implicated in flavivirus morphogenesis. Inhibitor and RNA interference studies implicate the Src family kinase c-Yes in WNV egress from the ER.³³³ A serine/threonine protein phosphatase inhibitor, I(2)(PP2A), has also been found to bind the WNV C protein, although its importance for infectivity was minimal.³⁵⁰

HEPATITIS C VIRUSES

Background and Classification

HCV was identified in 1989 through expression cloning of immunoreactive cDNA from the plasma of a chimpanzee infected with an etiologic agent known to cause non-A non-B viral hepatitis.¹⁴³ The virus currently infects more than 130 million people worldwide and remains a significant public health concern. HCV typically causes chronic hepatotropic infections, which can progress over decades to fibrosis, cirrhosis, and liver cancer. Based on difficulties in detecting HCV *in vivo*, the virus was originally thought to replicate poorly. Yet mathematical models of HCV dynamics indicate that a chronically infected patient produces approximately 10^{12} virions per day, with a virion half-life of only a few hours.⁶⁶⁶

Based on its evolutionary history, HCV is classified into seven genotypes, which differ from each other by more than 30% at the nucleotide level.^{614,788} Each genotype is further divided into numerous subtypes. HCV genotypes show differences in worldwide distribution, disease progression, and susceptibility to

treatment. In addition, intergenotypic and intersubtype recombinant HCV genomes have been described.⁷⁸⁸

While no HCV vaccine exists, advances in treatment have been made. Pegylated IFN- α and ribavirin—the standard of care for over a decade—have burdensome side effects and do not cure many of the patients treated. The most common HCV genotype, genotype 1, is also the most difficult to treat with this therapy.³³⁴ Recently, however, HCV-specific antiviral drugs—such as protease inhibitors—have begun to reach the clinic. While the new drugs must still be used in combination with IFN and ribavirin, first-generation protease inhibitors have almost doubled the chances of treatment success.

In addition to HCV, the genus *Hepacivirus* may contain a few related viruses that are awaiting taxonomic classification. An HCV-like virus was recently identified in domestic dogs with respiratory illness.³⁸⁸ This virus appears to be closely related to HCV and has been tentatively designated canine hepacivirus (CHV), although it has not been formally classified within this genus. In addition, GB virus B (see later) appears to be closely related to the hepaciviruses.

Experimental Systems

HCV research was limited for years by the lack of convenient laboratory culture systems and small animal models. However, many of these technical hurdles have been overcome in the past decade.

Shortly after the sequence of the HCV genome was fully elucidated, the first consensus cDNA clones were constructed. RNA transcripts from these cDNAs were shown to be infectious by direct intrahepatic inoculation into chimpanzees.^{412,925} Chimpanzee infectious clones have been used to show that all viral enzymes, the *p7* gene, and 3' NCR are essential for HCV replication *in vivo*.^{414,750,926} Furthermore, the ability to use genetically defined inocula to initiate clonal infections provided a useful tool to study virus evolution and immune responses.¹⁰⁶ Despite their demonstrated utility *in vivo*, however, these infectious transcripts failed to replicate after transfection into cultured cells.

Subgenomic HCV replicons were developed in 1999 and proved to be the first broadly useful system for studying HCV RNA replication in cell culture.⁵⁰⁹ Subgenomic replicons are bicistronic constructs in which the HCV internal ribosome entry site (IRES) drives expression of the neomycin resistance gene and the encephalomyocarditis virus (EMCV) IRES drives expression of the HCV NS proteins. Following RNA transfection into a human hepatoma line, Huh-7, the original genotype 1b (Con1 strain) replicon replicated to low levels.⁵⁰⁹ Cell culture-adaptive mutations were later found to increase RNA replication efficiency up to 10,000 times.⁷⁷ These mutations, many of which cluster in NS5A, are thought to increase RNA accumulation at the expense of infectious virus production, although their mechanisms of action are not well understood (reviewed in⁴³). In addition to Con1, subgenomic replicons have now been constructed for additional genotype 1b strains, as well as genotype 1a and 2a isolates.

Full-length genotype 1a and 1b genomes bearing adaptive mutations were shown to replicate in cell culture but did not produce infectious virus particles.^{78,355,676} The HCV pseudoparticle (HCVpp) system was therefore developed to examine the role of the viral glycoproteins in entry. HCVpp are defective retrovirus particles expressing a reporter gene and

displaying the HCV envelope proteins on their surface.^{46,201,343} Pseudoparticles undergo low-pH-mediated entry by using the known HCV entry factors and continue to be a valuable system to study entry in isolation from RNA replication. HCVpp differ from authentic particles, however, in their acid susceptibility³⁴³ and their assembly in a post-Golgi compartment rather than in the ER.⁷⁵¹

In 2005, production of authentic HCV in cell culture (HCVcc) was achieved for the first time. An HCV isolate from an unusual case of acute fulminant hepatitis in Japan (JFH-1) was found to replicate in culture without the need for adaptive mutations.³⁹² Remarkably, full-length JFH-1 also produced low levels of infectious virus.^{883,959} Infectious titers could be increased with passage or by engineering chimeric genomes based on a related genotype 2a strain, J6.^{487,675} A cell culture-adapted genotype 1a genome that produces infectious HCVcc has also been developed, albeit with lower infectivity.⁹³⁷

The chimpanzee remains the only animal model that reproduces clinical aspects of HCV infection, including a high rate of viral persistence and the development of HCV-specific innate and adaptive immune responses. Advances have been made, however, in generating small animal models for HCV infection. Immunodeficient mouse strains with liver injury can be repopulated with human hepatocytes and infected with HCV. Two commonly used liver injury models are the toxic urokinase plasminogen activator transgene (Alb-uPA mice)^{568,570} and a conditionally lethal fumaryl acetoacrylate hydrolase deficiency (FAH^{-/-} mice).⁷³ Recently, immunocompetent mice that express human HCV entry factors have been developed as a model for the early steps of infection.¹⁹⁸

Structure and Physical Properties of the Virion

HCV particles are enveloped and contain the viral proteins core (C), E1, and E2, as well as the genomic RNA. Virions are between 30 and 80 nm in diameter.^{88,317,949} For a virus of this size, HCV exhibits an unusually low and heterogeneous buoyant density. In highly infectious acute-phase chimpanzee serum, HCV-specific RNA is detected in fractions with densities ranging from 1.03 to 1.10 g/mL.^{87,331} The particle density is inversely correlated with infectivity, with low-density virions being more infectious than high-density particles. Similar to serum-derived virus, the peak of HCVcc RNA has a buoyant density of approximately 1.15 g/mL,^{116,487,883,937,959} whereas the peak infectivity is near 1.10 g/mL.^{116,487,959} Passage of cell culture-grown virus in animals⁴⁸⁸ or primary hepatocyte cultures⁶⁸² increases the proportion of low-density, high-infectivity virus, highlighting the impact of host cell environment on virion composition.

The high density observed for noninfectious particles may come from immunoglobulin binding to the virus and/or represent nonenveloped nucleocapsids.²³ The low buoyant density of infectious HCV appears to reflect the ability of the virus to interact with serum lipoproteins.^{22,631,692,855} The lipidome of HCV particles is very similar to low-density lipoproteins (LDLs) and very-low-density lipoproteins (VLDLs), including an enrichment of cholesteryl esters.⁵⁶⁹ Immunoprecipitation and immunogold-electron microscopy (EM) studies have detected apolipoprotein (Apo) AI,⁴¹⁶ ApoB,^{416,631,855} ApoC1,⁵⁷² and ApoE⁶³¹ in association with serum-derived HCV. Apolipoprotein E and ApoC1 are also associated with HCVcc,^{126,569,572} but ApoB is generally lacking, leading to controversy about

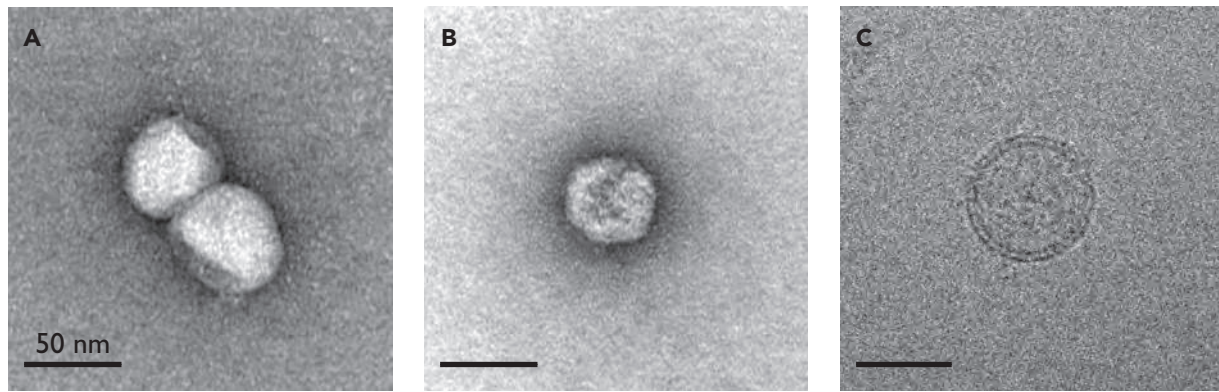


FIGURE 25.11. Hepatitis C virus (HCV) particles produced in cell culture. **A:** Virus particles were purified and negative stained with uranyl acetate. **B:** A representative virus particle after extraction with 0.2% NP-40 and negative staining. **C:** A representative enveloped virus particle as imaged through cryoelectron microscopy. (Adapted from Gastaminza P, Dryden KA, Boyd B, et al. Ultrastructural and biophysical characterization of hepatitis C virus particles produced in cell culture. *J Virol* 2010;84:10999–11009, with permission of the American Society for Microbiology Press.)

its functional importance (reviewed in⁴⁴). The propensity of HCV to interact closely with lipids has led to the hypothesis that at least a portion of circulating virus is internalized in host lipoproteins to form a “lipoviral” particle,²² which may provide camouflage for the virus to escape neutralizing antibodies.

Although unambiguous visualization of HCV from patient samples remains a challenge, negative stain and cryoelectron microscopy studies have started to reveal structural features of HCV grown in cell culture^{264,569,883} (Fig. 25.11). One recent study detected two predominant classes of HCV particles, each making up approximately half of the preparation. The first were spherical, pleomorphic particles, ~55 nm in diameter, containing a lipid bilayer and an electron-dense core (Fig. 25.11C). These particles, which are presumably infectious HCV, were enriched in low-density fractions and had a relatively smooth, featureless surface that reacted with E2- and ApoE-specific antibodies. The second class of particles, electron-dense structures ~45 nm in diameter, is presumed to be naked nucleocapsids. This class was enriched in high-density, low-infectivity fractions and lacked a membrane or visible surface projections. Similar particles were seen after detergent treatment of infectious virus, which is expected to remove the viral envelope.²⁶⁴ Unfortunately, the reconstruction of high-resolution models of the virus particle is currently limited by particle heterogeneity, relatively low yields, and lack of symmetry in both classes of HCV particles.

Binding and Entry

HCV infects target hepatocytes through a multistep process that involves several cell surface molecules. Virus particles initially attach to glycosaminoglycans^{45,268} and possibly the LDL receptor (LDL-R),^{4,598} which may interact with virion-associated ApoB and ApoE. Following attachment, virus particles employ four specific entry factors to mediate uptake: scavenger receptor class B type I (SR-BI, aka SCARBI),⁷⁵⁸ CD81,⁶⁷⁹ claudin 1 (CLDN1),²²⁴ and occludin (OCLN).⁶⁸¹

SR-BI, which is highly expressed on hepatocytes, plays important physiologic roles in the selective uptake of cholesterol and cholesteryl esters from lipoproteins. SR-BI can interact with the E2 hypervariable region 1 (HVR1),⁷⁵⁸ and

its overexpression enhances virion binding to CHO cells,²²⁴ suggesting a role for SR-BI in primary attachment. Blocking antibody studies have both supported a role for SR-BI in binding,¹²¹ as well as shown the importance of SR-BI at a post-binding step that is closely linked to CD81.^{386,952} Antibodies directed at E2 HVR1 similarly block a postattachment step of HCV entry.⁸⁷⁸ The physiologic SR-BI binding partner, PDZK1, is also important for entry.²²⁵ Perhaps because the lipoviral particle shares commonalities with the natural ligands of SR-BI, various lipoproteins modulate HCV infectivity. Specifically, high-density lipoproteins (HDLs) can enhance HCV fusion in a manner dependent on SR-BI lipid transfer, ApoCI, and the E2 HVR1.^{47,199,571,880} In contrast, oxidized LDL,⁸⁸² VLDL,⁵⁴² and serum amyloid A^{114,453} inhibit HCV entry.

The tetraspanin CD81 binds tightly to the glycoprotein E2 via residues in the CD81 large extracellular loop.^{328,670,679} Antibodies targeting CD81 do not affect HCVpp binding to hepatocytes¹⁶⁶ but inhibit HCV infection after virus attachment.⁴²¹ Because HVR1 masks the CD81 binding site, a conformational change may be required to prime E2 for binding to this co-receptor.³⁷ CD81 interacts with another HCV entry factor, CLDN1,^{313,929} forming a complex that is essential for HCV infection.³¹² Receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), appear to regulate CD81–CLDN1 interactions and to promote HCV membrane fusion.⁵²⁷ Interaction of CD81 with another host factor, EWI-2wint, inhibits virus uptake.⁷³⁴ In addition to its role in entry, interaction of HCV with lymphocyte-expressed CD81 may contribute to the immune dysregulation seen in some patients with chronic infection.^{173,739,865}

Two tight junction proteins, CLDN1²²⁴ and OCLN,^{59,496,681} are essential for postbinding steps of HCV uptake. In addition, CLDN family members-6 and -9 can also mediate entry.⁵⁶³ The involvement of tight junction proteins suggests that HCV could traffic across the surface of the hepatocyte in order to engage all of its entry factors. Indeed, binding to CD81 activates a Rho guanosine triphosphatase (GTPase), which may mediate cytoskeleton rearrangements. Furthermore, recombinant E2 promotes relocation of CD81 to tight junctions.⁹⁴ In conflict with this hypothesis, however, is the finding that CLDN1

can form complexes with CD81 on the basolateral surface of hepatocytes.³¹² In addition, single particle tracking has visualized HCV internalization outside tight junctions—although this may represent the nonpolarized nature of the cultured hepatoma cells.¹⁵⁹

Following attachment, HCV is taken up via clathrin-mediated endocytosis.^{76,564} Internalized virions co-localize with clathrin light chain and the E3 ubiquitin ligase c-Cbl prior to uncoating.¹⁵⁹ Intracellular HCV is delivered to Rab5a-positive early endosomes,¹⁵⁹ which likely provide the acidic environment necessary to induce rearrangement of the HCV glycoproteins into their fusogenic form.^{343,864} Surprisingly, extracellular HCVcc particles are resistant to inactivation by low pH, suggesting that interactions at or near the cell surface prime the virus for fusion.⁸⁶⁴ This is functionally similar to pestiviruses⁴²⁵ but in contrast to flavivirus particles, which are primed during maturation,⁹⁴⁶ and to HCVpp,^{343,644} which have structural and compositional differences from authentic HCV particles.

Genome Structure

The HCV genome is an uncapped, 9.6-kb RNA containing highly structured 5' and 3' ends (Fig. 25.12A). The 5' NCR is a well-conserved, 341-nt sequence element that folds into a complex structure consisting of four major domains and a pseudoknot.³²⁴ The first 120 nt serves as a minimal replication element, although compelling genetic evidence shows that

the entire 5' NCR (or its complement in the negative strand) plays an important role in efficient positive-strand RNA synthesis.^{70,247,282,290,404,525,721} The 5' NCR also contains an IRES, which directs the cap-independent translation of the single large ORF.

Interestingly, the liver-specific micro-RNA (miR)-122 binds to two sites within the HCV 5' NCR, and these interactions are required for efficient viral replication.^{377,469} While miR-122 can stimulate IRES-mediated translation in an Argonaute 2-dependent manner,^{325,366,732,907} elegant genetic experiments revealed that a major function of miR-122 is to sequester the 5' end of the genome, perhaps protecting it from RNA degradation or from activation of innate antiviral responses.⁵³² Nuclease-resistant oligonucleotides that antagonize miR-122 function *in vivo* profoundly decrease HCV replication in experimentally infected chimpanzees and show promise for future therapeutics,⁴⁴⁷ although viral escape can occur in cell culture.⁴⁷⁴

A number of functionally important RNA structures have been identified within the HCV ORF.⁸⁶⁷ Genetic analysis indicated that an RNA stem-loop within the C gene is important for viral fitness.^{561,876} An RNA structure in NS5B, termed 5BSL3.2, forms a long-distance base-pairing interaction with upstream RNA sequences with the 3' NCR.²⁴⁶ This “kissing” interaction is essential for replication, although its function remains to be determined. In addition, the HCV ORF contains fewer UA and UU dinucleotides than expected

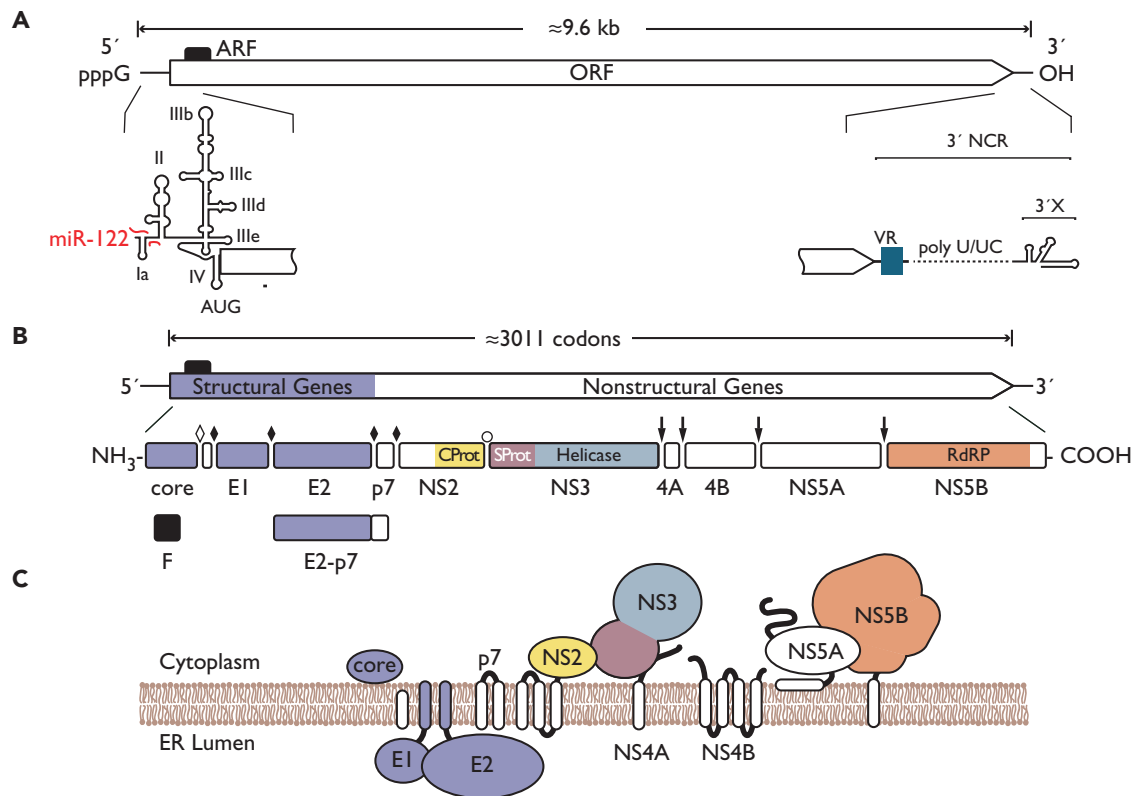


FIGURE 25.12. Hepacivirus genome structure and protein expression. **A:** Hepatitis C virus (HCV) genome structure and RNA elements. Important RNA elements are indicated as in Figure 25.4. The binding sites of micro-RNA (miR)-122 are indicated in red; the alternative reading frame (ARF) is indicated by a black box. **B:** Polyprotein processing and cleavage products. Cleavage sites are indicated as in Figure 25.4 except the NS2/3 cleavage, which is mediated by the NS2 cysteine autoprotease (open bullet). **C:** Polyprotein membrane topology. See the legend of Figure 25.4 for symbol definitions and the text for further details.

by chance.³⁰⁸ This is significant because these dinucleotides are the preferred cleavage sites of the IFN-stimulated RNase L. Based on the large-scale computerized folding of numerous positive-strand RNA viral genomes, the HCV ORF is also predicted to contain an unusually high rate of internal base pairing.⁷⁸⁹ Intriguingly, this feature is common among viruses that cause persistent infections. Thus, the observed bias in nucleotide composition and propensity for internal base pairing within the HCV ORF may reflect adaptation to evolutionary pressure exerted by innate antiviral pathways.

The 3' NCR was originally thought to terminate in polyadenosine or polyuridine. Improved methods for producing 3' terminal cDNAs later revealed, however, that the HCV 3' NCR actually consists of a short (~40 nt) variable domain and a polyuridine/polypyrimidine (polyU/UC) tract, followed by a highly conserved 98-nt 3' X domain.^{413,827} Mutagenesis studies revealed that the 3' X domain is essential for RNA replication and that the polyU/UC tract must be at least 26 nt in length.^{245,926,935,941} A number of cellular factors have been found to bind to the HCV 3' NCR, including RNA-binding proteins polypyrimidine-tract binding (PTB) protein,^{278,363,866} heterogeneous ribonuclear protein C,^{150,277} glyceraldehyde-3-phosphate dehydrogenase,⁶⁷¹ HuR,⁸⁰¹ and La autoantigen.⁷⁹⁹

Translation and Proteolytic Processing

In addition to its roles in replication, the HCV 5' NCR acts as an IRES to direct cap-independent translation of the viral genome. The IRES is encoded by 5' NCR domains II through IV (Fig. 25.12A), although flanking sequences can influence activity. In the current model of HCV IRES function, free 40S ribosomal subunits directly bind to the 5' NCR domains IIIId through f and adopt an mRNA-bound conformation.^{645,669,798} The IRES-40S complex then binds eIF3 and the ternary complex eIF2-GTP-Met-tRNA_i to form a 48S intermediate complex, in which the initiating AUG codon at nt 342 is positioned within the ribosomal P site.^{367,645} The HCV IRES directly interacts with eIF3 via determinants in domain IIIb¹⁶⁴ and thereby functionally mimics the 5' cap-binding complex eIF4E.⁷⁹³ Following GTP hydrolysis and release of eIF2, the 60S ribosomal subunit is recruited to form a translationally active 80S complex.^{367,645,669} Under some conditions the HCV IRES does not require eIF3 or ternary complex and is also capable of directing translation from non-AUG initiation sites.^{444,846} This mechanism, which utilizes eIF5B to recruit Met-tRNA_i, may allow the virus to initiate translation despite cellular eIF2 phosphorylation by the viral RNA sensor protein kinase R (PKR).

A number of cellular factors have been reported to contribute to HCV IRES activity. The human La protein stimulates translation by binding near the initiator AUG and recruiting the 40S ribosomal subunit^{168,693}; inhibiting La strongly decreases HCV gene expression and replication.^{168,194} Other host factors that stimulate HCV IRES activity include proteasome α -subunit PSMA7⁴²⁸ and nucleolin.³⁶⁵ In addition, polycytidine binding proteins 1 and 2 and PTB were shown to bind the 5' NCR, although their functions in the virus life cycle remain to be defined.⁴³ IRES-mediated translation can be inhibited by long-distance base pairing of 5' NCR nt 24 to 38 to a stem-loop region (nt 428 to 442) within the C gene region.⁴⁰⁵ Interestingly, this part of the 5' NCR overlaps with one of the miR-122 binding sites.³⁷⁷

Translation of the HCV genome produces a large polyprotein (~3,000 amino acids) that is proteolytically cleaved to produce 10 viral proteins (Fig. 25.12B): the virion structural components core (C) and glycoproteins E1 and E2, and the presumed NS proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Mature forms of the HCV proteins arise via co- and posttranslational cleavage mediated by cellular and host-encoded proteases. During structural region processing, cleavages between C/E1, E1/E2, and E2/p7 are mediated by signal peptidase; mature C protein is released from the E1 signal peptide via signal peptide peptidase (SPP). Within the NS region, signal peptidase mediates cleavage of p7/NS2. The remainder of the NS region is processed by two virus-encoded proteases: the NS2 cysteine autoprotease mediates cleavage at the NS2/3 junction, whereas the NS3-4A serine protease cleaves at all downstream junctions (Fig. 25.12C).

In addition to proteins encoded by the large ORF, it appears that small protein products can be produced from the +1 reading frame of the C gene. At least three different forms of alternative reading frame protein (ARFP) have been described: ARFP/F (frameshift), ARFP/DF (double frameshift), or ARFP/S (short form). Although it is thought that these products are produced via ribosomal frameshifting, alternate translational initiation sites may also be involved.⁸⁹ It is equally unclear what role, if any, ARFP expression plays in the virus life cycle. Mutant genomes with a disrupted +1 reading frame are highly attenuated *in vivo*,^{561,876} although this may be due to disruption of conserved RNA secondary structures within the C gene.

Features of the Structural Proteins

C Protein

Core (C) is the first protein encoded by the HCV genome. It is among the most conserved of the viral proteins and is thought to multimerize and bind RNA to form the viral nucleocapsid. Proteolytic processing of the C protein occurs in two steps. First, the C/E1 junction is cleaved by host signal peptidase to yield a 191-amino acid, membrane-anchored form of C and the N-terminus of E1.³²⁹ Subsequent cleavage within the C-terminal membrane anchor by SPP liberates the mature form of C^{559,640,752,933} and is essential for infectivity.⁸³² The authentic SPP cleavage site has not yet been determined but likely occurs somewhere between residues 173 and 182³⁵¹; recent genetic evidence suggests that the C-terminus is at position 177.⁴¹⁹ In addition, N-terminally truncated forms of C have been described, but their mechanism of production is unknown.²¹⁸

Following removal of the membrane anchor, the mature C protein consists of two domains.⁸⁶ The N-terminal domain I (DI, ~120 amino acids) is hydrophilic and can bind RNA nonspecifically,⁷⁵² associate with the 5' NCR,^{783,828} and mediate dimerization of the RNA 3'X region.¹⁷¹ *In vitro*, interaction of DI with structured RNA can induce the assembly of nucleocapsid-like particles.⁴³¹ C protein likely dimerizes through homotypic binding elements in domains I and II,^{86,406,518,548} as well as a potential disulfide bond at Cys 128.⁴³² C protein has also been shown to associate with E1 and E2.^{49,502,623} Domain I may also be the target of phosphorylation by protein kinases A and C.⁵¹⁷

Domain II (~50 amino acids) contains primarily hydrophobic residues. *In vitro*, this domain is important for folding of truncated recombinant protein (residues 1 to 169) into an

α -helix-rich dimer.⁸⁶ DII is also important for localizing C to ER-associated lipid droplets,^{38,85,604} which play an important but unclear role in virus particle assembly.^{588,777} Lipid droplet association requires prior cleavage with SPP⁵⁵⁹ and is enhanced by the actions of diacylglycerol acyltransferase-1 (DGAT-1), an ER-resident enzyme that catalyzes a final step in triglyceride synthesis.³²⁶ It has been proposed that trafficking of C to lipid droplets increases the risk of liver steatosis in patients with chronic HCV, as well as promotes the development of steatosis in certain transgenic mice that overexpress the protein. Indeed, C binding to lipid droplets displaces adipocyte differentiation-related protein (ADRP) and leads to redistribution, clustering, and increased synthesis of these organelles in cell culture.^{84,190}

The HCV C protein has been implicated in numerous cellular pathways, including altered signaling, transcriptional control, apoptosis, and cellular transformation.^{558,845}

Envelope Glycoproteins

The HCV glycoproteins E1 (~30 kD) and E2 (~70 kD) form a noncovalent heterodimer that mediates viral attachment and membrane fusion. Both E1 and E2 are type I TM glycoproteins that contain a large extracellular domain and a single C-terminal TM domain. To generate this topology, the E1 and E2 TM segments, which each contain two short (<20 amino acids) hydrophobic stretches separated by charged residues, adopt an intramembrane hairpin structure during translocation. Following signal peptidase cleavage, the luminal C-termini reorient to face the cytoplasm, yielding tandem proteins each with a single membrane anchor.¹⁵⁶ Following biogenesis, the glycoproteins are retained in the ER.^{203,207,740}

Structural information regarding E1 is not available. Recently, however, biophysical characterization of the E2 ectodomain has produced a structural model that can be compared to flavivirus protein E.^{422,905} E2 is a three-domain protein with a high proportion of β -sheet, as well as random coil, β -turns and other natively unfolded regions; the overall structure of E2 is the same at both acidic and neutral pH.^{422,905} Three variable regions, including HVR1, can be deleted without affecting CD81 binding.⁵⁵⁴ HVR1 is followed by domain I (DI), which likely contains eight β -strands and includes the determinants of CD81 interaction. Several glycosylation sites cluster on DI and may shield the CD81 binding site from antibody neutralization. The highly conserved domain II (DII) is largely unstructured and includes the candidate fusion loop at residues 502 to 520; it also encompasses HVR2. The fusion peptide may be buried by contacts with E1 prior to fusion. Domain III (DIII) is connected to DII by an extendable linker encompassing the intergenotypic variable region (IgVR) and contains residues that may assist DI in CD81 binding. DIII is followed by a flexible stem that connects it to the TM domain.⁴²²

E1 and E2 likely associate as a class II fusion protein complex.⁹²² Formation of E1-E2 heterodimers is a slow process, with folding of each glycoprotein dependent on the other,^{95,582,653} and on the chaperone calnexin.²⁰⁴ Residues within the E1 and E2 TM domains are important for heterodimerization,⁶⁴² as are interactions of the ectodomains.⁹³⁶ The membrane proximal linker in E2 is also important for heterodimerization and HCVpp entry.²⁰² Construction of HCVpp bearing E1 and E2 from different genotypes indicates regions of intergenotypic

incompatibility in DI and HVRII and additional sequences in DII, IgVR, and the stem region of E2, confirming functionally important determinants.⁷ Although E1 and E2 interactions in the ER membrane are noncovalent, recent studies of infectious virions suggest that the assembled glycoproteins form large covalent complexes stabilized by disulfide bonds.⁸⁷⁹ These bridges may block acid-induced conformational changes until a trigger initiates entry, explaining the pH resistance of HCV particles.⁸⁶⁴

The HCV glycoproteins are important humoral antigens that can lead to virus neutralization and therefore employ strategies to evade the immune response. HVR1 is a major target of neutralizing antibodies.^{391,895} HVR1-specific antibodies can protect chimpanzees from infection with homologous HCV strains,²³¹ and early induction of HVR1-specific antibodies correlates with viral clearance in humans.^{11,965} To avoid neutralization, HVR1 exhibits a high level of sequence variability even within a single patient, largely driven by immunogenic selection.^{230,553,599,896} In addition, protein glycosylation likely also contributes to immune evasion by the envelope proteins. The E1 and E2 ectodomains are heavily N-glycosylated (generally 4 and 11 sites, respectively). Certain glycosylation site mutants are highly sensitive to neutralizing antibodies, suggesting the glycans mask conserved epitopes from the immune response.³²³ Glycosylation is also important for the proper folding and secretion of E2,⁷⁹⁵ as well as for the assembly and infectivity of HCVpp²⁷⁴ and HCVcc.³²³

Features of the Nonstructural Proteins

p7 Protein

p7 is a small (~7 kD) hydrophobic protein that spans the membrane twice, with ER-luminal N- and C-termini and a short, basic, cytoplasmic loop (Fig. 25.13A). Incomplete or delayed processing by host signal peptidase between E2, p7, and NS2 can lead to the production of E2-p7 and E2-p7-NS2.^{482,590,676,773} In model membrane systems, p7 multimerizes to form hexameric, cation-conductive membrane channels that are inhibited by amantadine and hexamethylene amiloride.^{294,519,600,659,689} (Fig. 25.13B).

HCV p7 is essential for virus assembly and infectivity *in vitro* and *in vivo*.^{373,750,809} In virus-producing cells, p7 was found to equilibrate pH gradients, reducing the number of acidic vesicles and raising the pH of lysosomes.⁹¹⁵ Interestingly, a p7 mutant that lacks channel activity could be complemented by the influenza M2 protein or by treatment with bafilomycin A1, demonstrating that p7 acts as a viroporin to modulate intracellular pH during virus production.⁹¹⁵ However, a p7 deletion mutant was not complemented by these treatments, suggesting that the protein plays an additional role during virus assembly. In this regard, p7 appears to interact with NS2 at a very early stage of virus morphogenesis.^{83,370,530}

NS2 Protein

NS2 (~23 kD) is a membrane-spanning protein with cysteine protease activity.^{287,330,513,770} The membrane topology of NS2 has not been fully elucidated but likely incorporates three N-terminal TM segments^{369,370} (Fig. 25.13C). The C-terminal region of NS2 contains the protease active site residues, His-952, Glu-972, and Cys-993.^{287,330,513} The only known function of this enzyme is to cleave the NS2/3 junction. Optimal autoprotease activity requires co-expression of NS2

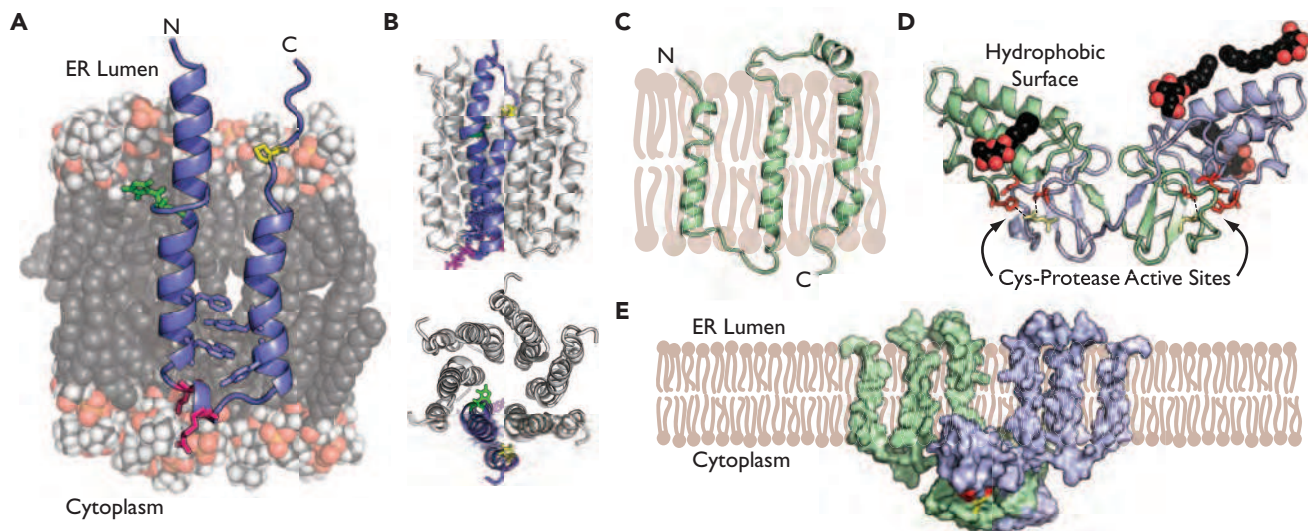


FIGURE 25.13. Structure of the hepatitis C virus (HCV) p7 and NS2 proteins. **A:** Model of the p7 monomer in a lipid bilayer.⁶⁰⁰ Functionally important, conserved residues His-17 (green); Lys-33 and Arg-35 (magenta); Pro-58 (yellow); and Phe-26, Trp-30, Tyr-42, and Tyr-45 (slate) are shown. (Nuclear magnetic resonance [NMR] coordinates and membrane modeling kindly provided by C. Chipot and F. Penin.) **B:** Model of a p7 hexamer as viewed from the side or from the endoplasmic reticulum (ER) lumen. (Modeling coordinates kindly provided by C. Chipot and F. Penin.) **C:** Model of NS2 transmembrane (TM) domains, based on NMR data.³⁷⁰ **D:** The dimeric NS2 cysteine protease domain. Monomeric subunits are colored green and blue; active-site residues, red; C-terminal residues, yellow; co-crystallized detergent molecules are shown as spheres. Rendered from PDB number 2HD0.⁵¹³ **E:** A model of the full-length NS2, based on the models presented in **C** and **D**.

with the N-terminal zinc-binding domain of NS3, although the mechanism of enhancement is not clear.^{287,330,770} Notably, NS2/3 cleavage is inhibited by NS4A, the co-factor that promotes NS3 folding into a compact serine protease structure.¹⁷⁹

The x-ray crystal structure of the NS2 C-terminal domain demonstrated a cysteine protease with the active-site geometry of a serine protease.⁵¹³ The C-terminus of NS2 is bound in the substrate-binding pocket, suggesting that further protease activity is blocked following NS2/3 cleavage (Fig. 25.13D). Interestingly, the protease domain homodimerizes, leading to the formation of two composite active sites at the dimer interface. This architecture reveals a mechanism that can control polypeptide processing and explains the ability of some NS2-3 mutants bearing distinct defects to reform an active autoprotease *in trans*.^{287,716}

NS2 has an important albeit unclear role in virus particle assembly. Genetic experiments suggested that the N-terminal region of NS2 interacts with the structural proteins and/or p7, whereas downstream regions of NS2 interact with other NS proteins.⁶⁷⁵ Indeed, further genetic and biochemical studies confirm NS2 interactions with E1, E2, p7, NS3, and NS4A, indicating that NS2 contributes to virus particle assembly by bringing together viral structural and NS proteins.^{83,370,400,530,672,688,803}

NS3 Protein

HCV NS3 (~70 kD) is a multifunctional protein, containing an N-terminal serine protease domain and a C-terminal RNA helicase/NTPase domain.⁶⁰⁵ Both enzyme activities are critical for viral replication and have been characterized biochemically and structurally.

The serine protease domain of NS3 requires interaction with NS4A, as well as coordination of a structural Zn²⁺ ion,

for complete folding and enzyme activity.^{42,226,483} The NS3-4A protease is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites.⁶⁰⁶ The protease first cleaves the NS3/4A site *in cis*, whereas subsequent cleavages occur *in trans*, with the preferred order NS5A/B > NS4A/B > NS4B/5A. Cleavage sites are well conserved and conform to the sequence (Asp/Glu)XXXX(Cys/Thr)/(Ser/Ala). In addition, NS3 has been shown to mediate internal cleavages within NS3, NS4B, and NS5A, although the functional relevance of these events is not yet known.⁴³

NS3-4A is structurally similar to other chymotrypsin-like proteases, with active site residues His-57, Asp-81, and Ser-139, and a substrate-binding surface located in a cleft between two β -barrel subdomains.⁶⁰⁶ The first subdomain includes an intercalated β -strand from the central region of NS4A. In the absence of the NS4A co-factor, the N-terminal 28 residues of NS3 remain flexible⁵⁵⁷ and the protein is rapidly degraded.⁹¹⁴ NS3 interacts with cellular membranes through two N-terminal amphipathic helices and through its interaction with NS4A.^{90,914} Interestingly, the structure of a single-chain, full-length NS3 shows the C-terminus coordinated by the serine protease, as would be expected from the *cis*-cleavage reaction⁹³⁰ (Fig. 25.7C).

The mechanism of protease activity initiates through deprotonation of Ser-139 by His-57; Ser-139 in turn acts as a nucleophile to attack the carbonyl carbon of the scissile peptide bond, forming a transient covalent link between the catalytic serine and C-terminal substrate. Dissociation of the N-terminal product allows hydrolysis of the tetrahedral intermediate to occur, regenerating the active site and C-terminal product. NS3-4A has an unusually shallow and hydrophobic substrate-binding surface that accommodates six amino acids.

However, the recognition of *trans*-cleaved substrates (NS4A/B, NS4B/5A, and NS5A/B) involves a relatively stable interaction between Cys residues at the substrate P1 position and a conserved Phe residue within NS3.⁶⁸⁰ This interaction slows the release of N-terminal products, which can inhibit subsequent catalysis.^{501,808} The discovery of this product-based inhibition led to the development of peptidomimetic compounds that potently inhibit NS3-4A serine protease activity and HCV replication *in vivo*.^{442,606} Interestingly, the preference for threonine at the P1 position of the NS3/4A cleavage site appears to reflect the selection of a substrate that is easily released following autocleavage.^{887,930}

In addition to its essential role in HCV polyprotein processing, NS3-4A serine protease manipulates the host cellular environment by cleaving the human mitochondrial antiviral-signaling (MAVS), TIR-domain-containing adapter-inducing interferon- β (TRIF), and tyrosine-protein phosphatase non-receptor type 2 (PTPN2) proteins (reviewed in⁶⁰⁶). MAVS is a key molecule involved in transducing signals from retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated 5 (MDA5) proteins, which are cytoplasmic sensors of viral RNA that activate interferon regulatory factor-3 (IRF-3). TRIF is involved in the Toll-like receptor 3 pathway, which senses dsRNA in the extracellular and endolysosomal compartments. Thus, cleavage of MAVS and TRIF serves to subvert innate antiviral defenses.^{470,473,581} PTPN2 is a protein tyrosine phosphatase that resets the epithelial growth factor (EGF) receptor and other growth factor signals. Cleavage of PTPN2 may therefore contribute to altered cell growth and the development of liver cancer.⁹⁷

Similar to other *Flaviviridae*, the C-terminal domain of HCV NS3 encodes a superfamily 2 RNA helicase/NTPase. These enzymes utilize the energy derived from NTP hydrolysis to translocate along and unwind double-stranded nucleic acids.⁶⁹⁷ NS3 has been shown to unwind RNA and DNA homo- and heteroduplexes by binding to an unpaired region of a template strand and translocating in a 3' to 5' direction.^{43,306,339}

Like other RNA helicases, HCV NS3 contains two RecA-like subdomains with ATPase active site residues at their interface. *Flaviviridae* helicases also contain an α -helix-rich, third subdomain of unclear function. Co-crystallization studies revealed that nucleic acids are bound with a cleft formed between the first two subdomains and subdomain 3.^{402,538} The mechanism of NS3-4A helicase activity has recently been revealed through elegant structural biology, traditional ensemble enzymology, and single-molecule biophysical studies.^{29,205,301,467,468,774,775} NS3 sequentially translocates in the 3' to 5' direction along the tracking strand's phosphodiester backbone, unwinding one base pair for each ATP hydrolyzed.³⁰¹ Other studies have suggested a spring-loaded mechanism can lead to larger apparent step sizes of up to 3 nt.²⁹ Dimerization of NS3 helicase domains appears to facilitate cooperative unwinding of long templates. Helicase activity and substrate recognition are also stimulated by the NS3 serine protease domain and NS4A, and the helicase in turn stimulates NS3-4A serine protease activity.^{62,63,300,342,429,648} The helicase function of full-length NS3 can also be up-regulated by NS5B, presumably through interactions with the serine protease domain.⁹⁵⁴ Likewise, the NS3 helicase activity facilitates NS5B-mediated RNA synthesis.⁶⁷⁴ Helicase activity can be down-regulated by the cellular enzyme protein arginine N-methyltransferase 1 (PRMT1), which methylates one or

more arginines within the NTP-coordinating motif VI.^{206,724} In addition, many cell culture-adaptive mutations map to the surface of the helicase and to the region linking the protease and helicase,⁴³ suggesting that these are critical sites of protein interaction and/or conformational changes.

Although the precise roles of the NS3 helicase are not fully understood, the NTPase and helicase activities are essential for HCV replication and viral infectivity,^{414,441} and the helicase domain has been implicated in virus particle assembly.⁶¹⁷

NS4A and NS4B Proteins

NS4A is the smallest (~8 kD) NS protein, yet it has multiple functions in the virus life cycle. The central region of the protein acts as a co-factor for the serine protease and facilitates recognition of RNA substrates by the full-length NS3 protease/helicase.⁶¹ The hydrophobic N-terminal TM region of NS4A anchors NS3-4A to cellular membranes. NS4A also physically interacts with NS4B, NS5A, and uncleaved NS4B-5A.^{30,484} The acidic C-terminal region of NS4A contributes to HCV helicase activity,⁶¹ NS5A phosphorylation,^{383,407} RNA replication,⁴⁸⁹ and virus particle assembly.⁶⁷³

NS4B (~27 kD) is an integral membrane protein containing four central TM domains separating cytoplasmic N- and C-terminal regions.²⁸⁴ The N-terminal region of NS4B encodes two amphipathic helices, the second of which can alter its topology to insert in the membrane, and thereby invert the orientation of the first helix.^{284,520} The C-terminus of NS4B also encodes two α -helices, the second of which is membrane associated²⁸³ and can be palmitoylated.⁹⁴³ In addition, the central region of NS4B encodes a partially conserved nucleotide-binding motif and exhibits NTPase and adenylate kinase activities.^{212,854} NS4B can also bind RNA, with an affinity for the 3' terminus of the minus strand, and a small-molecule inhibitor of this activity decreases HCV replication.²¹³

NS4B forms oligomers and plays a critical role in organizing the membrane-bound replication complex.^{285,657,943} Expression of NS4B is sufficient to induce membrane alterations resembling the *membranous web*, where HCV RNA replication occurs.^{210,281} Consistent with an important role in RNA replication, a number of cell culture-adaptive mutations have been mapped to NS4B (reviewed in⁴³). NS4B has also been genetically linked to virus particle assembly, although its function in this process is not yet clear.³⁷⁵

NS5A Protein

NS5A (~56 to 58 kD) is a proline-rich, homodimeric, RNA-binding phosphoprotein that plays multiple roles in the viral life cycle. NS5A is a multidomain protein that contains an N-terminal Zn²⁺-binding domain I (DI), a central conserved domain II (DII), and a C-terminal variable domain III (DIII); these domains are separated by two short linkers of low complexity sequence (LCS).⁸⁴² DI includes an N-terminal amphipathic helix that mediates membrane association.^{91,214,665} X-ray crystallography revealed that DI adopts a novel protein fold and contains a unique Zn²⁺-binding motif⁸⁴³ (Fig. 25.14A, B). Interestingly, the same DI structure has been crystallized in two distinct homodimeric conformations: in one case DI formed a homodimeric claw with a basic groove postulated to bind RNA⁸⁴³; in the other case DI dimers formed a barrel-shaped structure⁵¹⁴ (Fig. 25.14B, C). Both dimer forms contain well-conserved residues at their interface, suggesting that these

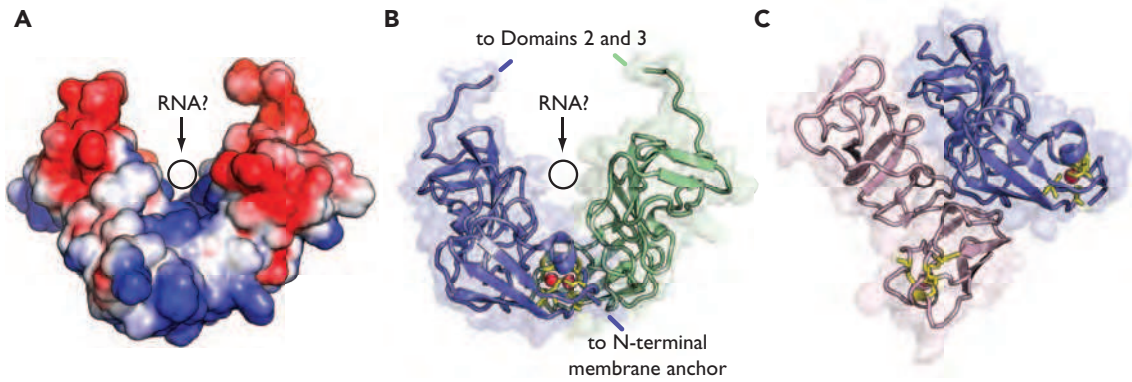


FIGURE 25.14. Structures of hepatitis C virus (HCV) NS5A protein. **A:** Surface electrostatic potential of the dimeric NS5A zinc-binding domain, as rendered from PDB 1ZH1.⁸⁴³ Negative charges in red and positive charges in blue. **B:** The NS5A zinc-binding domain (PDB 1ZH1), with individual monomeric subunits colored purple and green. Zn^{2+} ions are shown in red, and coordinating residues are shown in yellow. **C:** An alternative structure of the NS5A zinc-binding domain, as rendered from PDB 3FQM.⁵¹⁴ The purple subunit is structurally aligned the same as the purple subunit in **B**.

alternate conformations may be functionally important—perhaps representing a molecular switch or a mechanism of higher-order oligomerization. NS5A homodimers have been observed in cell culture, although the dimer form was not resolved.³⁵² DII and DIII appear to contain natively unfolded regions, although NMR spectroscopy supports α -helical content within DIII.^{477,877} In line with this flexibility, DIII is tolerant of large deletions and insertions.^{27,345,495,556,605,841} One unusual feature of NS5A is a central region (amino acids 227 to 277), which overlaps the first LCS and part of DII, termed the IFN sensitivity-determining region (ISDR). Sequence variation within the ISDR was originally thought to correlate with IFN responsiveness in chronically infected patients, although the predictive value of this variation was later discredited.^{219,660} Nevertheless, the ISDR likely has other important functions, described later.

NS5A is phosphorylated by multiple cellular serine kinases and can be found in basally phosphorylated (56 kD) and hyperphosphorylated (58 kD) forms. The relevant cellular kinases and phosphoacceptor sites in NS5A are not fully characterized. Basal phosphorylation involves two regions of NS5A: a central region that encompasses the ISDR and a large C-terminal region of DIII.⁸³⁰ Basal phosphorylation likely involves casein kinase II or a related member of the CMCG kinase family, although other kinases have been implicated.^{158,346,347,353,401,717} Hyperphosphorylation involves residues in the first LCS, Ser-224, Ser-228, and Ser-231,⁸³⁰ although it is not clear whether these are the sites of hypophosphorylation or whether they are required for downstream phosphorylation events. NS5A hyperphosphorylation appears to be mediated by casein kinase I α ,^{702,703} although Polo-like kinase 1 has also been implicated in this process.¹³³ Furthermore, NS5A hyperphosphorylation requires the co-expression of NS3–5A *in cis*^{407,626} and interaction between NS5A and NS4A.^{30,383}

NS5A plays multiple key roles in RNA replication. The first line of evidence came from the identification of cell culture–adaptive mutations that enhance the replication efficiency of genotype 1a and 1b replicons, many of which mapped to hyperphosphorylation determinants within NS5A.^{43,77} Several adaptive mutations (including those in other NS proteins)

are associated with decreased NS5A hyperphosphorylation, suggesting that hyperphosphorylation negatively regulates replication.^{43,77} However small molecules that block NS5A hyperphosphorylation can either inhibit or enhance HCV replication depending on the viral genetic background,⁶²⁷ indicating that replication in cell culture may require a balance between different NS5A phosphoforms. Furthermore, cell culture–adaptive mutations associated with low hyperphosphorylation strongly inhibit HCV replication in chimpanzees.¹¹⁰ While the consequences of NS5A hyperphosphorylation are not fully understood, adaptive mutations associated with decreased hyperphosphorylation correlate with increased interaction between NS5A and hVAP-A,²²³ a cellular vesicle trafficking protein and putative HCV replication factor.²⁶⁰ Furthermore, an important function of NS5A is to bind to and activate PI4KIII α , a cellular lipid kinase that is essential for HCV replication complex formation.^{719,823} Consistent with this, NS5A localizes to sites of viral RNA synthesis.²⁸¹ NS5A also interacts with NS5B and can inhibit its RdRP activity *in vitro*,⁷⁸⁴ and NS5A mutations that block these interactions are detrimental for replication in cell culture.⁷⁸¹ Furthermore, NS5A binds G/U-rich RNA and exhibits a high affinity for the polypyrimidine tract of the HCV 3' NCR.³⁴⁵ The minimal RNA-binding domain of NS5A consists of DI and the first LCS,³⁵² although DII and DIII may contribute to RNA binding as well.²⁴² Finally, the RNA-binding ability of NS5A can be regulated by cyclophilin A (CypA), a cellular proline *cis-trans* isomerase that is essential for HCV replication.^{128,157,233,234,243,622,646,696,877,891,927}

NS5A also plays an important role in virus particle assembly, primarily through determinants in DIII.^{28,840} Specifically, virus assembly requires phosphorylation of NS5A residue Ser-457 by casein kinase II α .⁸⁴⁰ Interestingly, mutation of this residue resulted in decreased hyperphosphorylation, suggesting a possible switch between genome replication and virion assembly. Furthermore, virus morphogenesis is dependent on recruitment of NS5A to lipid droplets through its interaction with the C protein.^{28,546,588} NS5A also interacts with ApoE, an essential host factor for HCV particle assembly, and recruits annexin A2, a cellular phospholipid-binding protein that enhances virus production.³⁴

In addition to replication and assembly factors, NS5A has been reported to interact with numerous cellular partners in pathways such as signal transduction, transcriptional control, cell death regulation, and cell cycle control.³¹⁸ While the molecular details for many of these observations have not yet been elucidated, NS5A has been found to interact with p53, a tumor suppressor protein; Grb-2, an adaptor protein involved in mitogen signaling; phosphatidylinositol 3 kinase (PI3K), a lipid kinase involved in cell survival through the AKT pathway; FBL-2, a geranylgeranylated F-box protein that is required for the replication of genotype 1b replicons; SRCAP, an ATPase that activates cellular transcription; karyopherin β 3, a protein involved in nuclear trafficking; Cdk1/2, cyclin-dependent kinases that regulate cell cycle control; and Fyn, Hck, Lck, and Lyn, Src-family kinases.^{43,318} In addition, although the molecular consequences of the ISDR have not been explained, NS5A does appear to manipulate innate antiviral defenses. NS5A can bind to and antagonize PKR, a cytoplasmic sensor of dsRNA, as well as induce IL-8 expression, which can antagonize type I IFN responses.^{254–256,684–686}

Because of its important roles in the virus life cycle, NS5A is a promising target for antiviral drug design. Based on the requirement for interaction with CypA, HCV replication is potentially inhibited by cyclosporin A and derivatives.^{622,891} One promising candidate is DEBIO-025, which inhibits HCV replication but lacks cyclosporin's immunosuppressive effects.^{157,646} A new class of NS5A inhibitors was recently identified in a replicon-based screen, leading to compounds that appear to target DI and inhibit HCV replication at picomolar concentrations.^{261,464} Interestingly, the most potent compounds are symmetric, suggesting that they may bind to NS5A dimers.

NS5B Protein

NS5B (~68 kD) is the major enzyme of HCV RNA replication, the RdRP. Similar to other polymerases, NS5B has a right-hand structure, with distinct finger, palm, and thumb domains^{5,99,100} (Fig. 25.8B). HCV NS5B was the first RdRP to be structurally solved in a closed, active conformation, demonstrating extensive contacts between the finger and thumb domains surrounding a preformed active site. Subsequent studies showed that the active structure opens into an inactive form via movement of the thumb domain.⁷⁴ Conserved RdRP motifs and catalytic residues are primarily located in the palm domain and serve to properly align the RNA template, NTP substrates, and two divalent cations that catalyze nucleotide transfer (reviewed in⁸⁷²). Structures of NS5B in complex with divalent cations and NTP revealed an active-site geometry remarkably similar to human immunodeficiency virus (HIV) reverse transcriptase (an RNA-dependent DNA polymerase [RdDP]) and the RdRP of the dsRNA bacteriophage ϕ 6.^{99,637} In addition, a low-affinity GTP-binding pocket was identified at the interface of the thumb and finger domains.⁹⁹ Mutation of this GTP binding site has shown that it is dispensable for RdRP activity *in vitro* but critical for RNA replication in cell culture.^{115,707} The finger domain also contains a polar groove, which holds the template RNA; correct positioning of the template 3' end is ensured by a β -hairpin that protrudes from the thumb into the active-site cavity.^{338,637,707} This structure may act as a flap that is displaced during RNA synthesis to allow the dsRNA product to exit the polymerase core. The

polymerase also contains a C-terminal regulatory loop that wraps around the thumb and inserts into the active site, decreasing RNA binding and RdRP activity.^{466,707} In addition to the core RdRP structure, NS5B contains a 21-amino acid C-terminal hydrophobic tail that posttranslationally inserts into the ER membrane.^{364,766} Mutations that interfere with membrane association destroy RNA replication.^{462,603} Although the tail anchor can be functionally replaced with a heterologous membrane anchor,⁴⁶⁰ other evidence suggests that it contains important determinants for intramembrane protein–protein interaction.⁹² Nevertheless, tail anchor deletion mutants retain RdRP activity and permit the efficient expression and purification of soluble, active, recombinant NS5B for biochemical and structural studies.⁹²³

In vitro, NS5B has been shown to elongate annealed primers or self-priming copy-back templates.⁵⁶ RNA synthesis utilizes the divalent cations Mg^{2+} or Mn^{2+} to catalyze nucleotide incorporation at a rate of 150 to 200 nt/minute.^{508,638,708} NTP analogs containing 2'C-methyl groups are potent chain-terminating RdRP inhibitors of HCV and other *Flaviviridae*.¹⁸⁴ As the purine 2' position is not involved in catalysis, these compounds may impose steric constraints within the catalytic core. Resistance to these inhibitors is easily acquired in HCV by mutation of Ser-282-Thr in the NTP binding pocket, although this mutation has adverse effects on RNA replication.⁵⁸³

During authentic genome replication, NS5B is thought to initiate RNA synthesis *de novo* (i.e., without a primer).^{524,708,710,963} Comparative enzymatic studies showed that the efficiency of *de novo* initiation correlates with the efficiency of replication in cell culture, and that residue 405 on the thumb domain is an important determinant of initiation.^{767,786} As for other RNA polymerases, NS5B initiates with a purine nucleotide, which can be mono-, di-, or triphosphorylated.^{706,707,780} *De novo* initiation is destroyed by NS5B mutations that affect GTP binding but do not disrupt NTP incorporation.⁷¹² The β -hairpin and C-terminal regulatory loop of NS5B, which limit dsRNA binding, also control *de novo* initiation versus primed synthesis.^{136,466,707} High GTP concentrations may serve to structurally rearrange these elements, thereby favoring *de novo* initiation.³¹⁵ RNA templates that are efficiently used for *de novo* initiation, at least *in vitro*, contain limited secondary structure and an unpaired 3' end.³⁸⁵ NS5B can also utilize circular RNA templates, suggesting that a free 3' end is not absolutely required and that RNA is loaded when NS5B is in the open conformation.⁷⁰⁹ Nevertheless, the aforementioned template requirements are notably different from the natural site of HCV minus-strand initiation, the 3' NCR, which terminates with a uridylyl base paired in a stable stem-loop. When the HCV 3' NCR is used as a template in *de novo* initiation reactions, only internally initiated minus strands are produced.^{385,403,639,820} Addition of a few unpaired 3' nt, however, leads to the production of template-length minus-strand products.⁶³⁹ Thus, authentic initiation of HCV minus-strand synthesis may depend on the local unwinding of 3' secondary structures, perhaps by the NS3-4A helicase.

NS5B RdRP activity depends on higher-order interactions. Important contacts between NS5B domains were revealed through structural studies with nonnucleoside RdRP inhibitors (NNIs), which can allosterically block polymerase activity.^{74,139,153,191,299,515,859,860,885} In addition to intramolecular

interactions, oligomerization of NS5B leads to cooperative stimulation of polymerase activity,^{699,886} and the NS3-4A helicase can enhance primed RNA synthesis.⁶⁷⁴ Although both NS4B and NS5A mutations inhibit RNA synthesis,^{674,784} mutations in NS5A that inhibit interaction with NS5B are detrimental for RNA replication.⁷⁸¹ Genetic studies indicate that NS5B binds NS5A primarily via residues on the back of the thumb and inner surface of the fingers.⁶⁹⁹

In addition to template-directed RNA synthesis, NS5B may have terminal nucleotide transferase (TnTase) activity, adding one or a few untemplated nt to the 3' end of an RNA substrate.^{56,705,780} It should be noted that several reports did not detect this TnTase activity or showed that a cellular TnTase copurified with NS5B.^{390,508,638,923} Nevertheless, the TnTase activity of a highly purified NS5B preparation was shown to depend on RdRP active-site residues.^{705,712} Moreover, NS5B TnTase activity can convert an RNA lacking a 3' initiation site into a useful template for *de novo* initiation,⁷⁰⁵ suggesting that TnTase activity may contribute to maintaining genome integrity.

RNA Replication

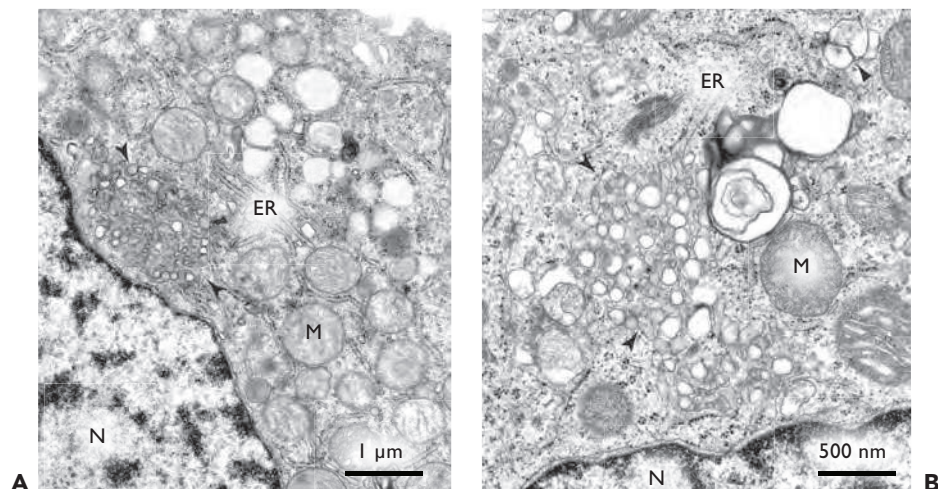
Because many copies of the structural proteins are needed to package each nascent genome, *Flaviviridae* genomes must be translated more frequently than they are replicated. Indeed, HCV subgenomic replicon-bearing cells produce a ~1,000-to-1 molar ratio of viral proteins to viral RNA.⁷⁰¹ One way to achieve this is by cross-talk between the determinants that control translation and genome replication. For instance, the cellular PTB protein binds to the HCV 5' NCR and C coding region where it may modulate IRES activity,^{25,362,858} and to the 3' NCR where it may repress replication.^{362,866} Similarly, La protein was shown to bind to both HCV NCRs. For the related pestiviruses, several NF/NFAR proteins bind the 5' and 3' NCRs and regulate genome circularization, and might also be involved in regulating HCV translation versus replication.^{359,360} HCV translation may also be autoregulated through product inhibition: low levels of C protein can enhance IRES-mediated translation, whereas high concentrations inhibit it.^{82,955} Finally, it is interesting to note that polycytidine-binding protein 2 (PCBP-2) binds to the HCV 5' NCR.^{251,800} PCBP-2 also interacts with the 5' NCR and RdRP of another positive-strand RNA virus, poliovirus, to control the switch between translation and replication.²⁵⁹

HCV RNA replication takes place in a dense perinuclear matrix of ~85-nm vesicles termed the membranous web^{210,281} (Fig. 25.15). Several studies have shown that the membranous web is likely to be derived from the rough ER,⁴³ although these structures can be insoluble in nonionic detergents, suggesting that they are sphingomyelin rich.^{6,779} Formation of the membranous web is mediated by both NS4B²¹⁰ and NS5A⁷¹⁹ and may utilize autophagic pathways²⁰⁰ and activate ER stress.⁸³¹ The process of HCV replication also induces the expression of genes involved in lipid metabolism.^{192,387,818} Furthermore, replication is stimulated by increased availability of saturated and monounsaturated fatty acids and inhibited by polyunsaturated fatty acids or inhibitors of fatty acid synthesis,³⁸⁷ suggesting that specific lipids and/or membrane fluidity are important for the function of the membranous web. In addition, altering cholesterol metabolism pharmacologically can lead to the disassembly of the replicase and inhibit RNA replication due to reduced geranylgeranylation.^{387,934}

The HCV replicase can be accessed biochemically by using permeabilized cells, cell lysates, or membrane preparations isolated from HCV replicon-bearing cells.^{6,10,311,439,589,701,928} These *in vitro* systems allow the elongation of endogenous RNA templates to be studied, but they do not accept exogenous RNA. Nevertheless, the sensitivity of RNA synthesis to heparin³¹¹ and the pulse-chase metabolic labeling of single-stranded RNA (ssRNA) into dsRNA⁴³⁹ suggest that at least a limited amount of *de novo* synthesis occurs *in vitro*. Furthermore, *in vitro* RdRP activity is protected from nuclease and protease degradation by a detergent-sensitive membrane,^{589,701} suggesting that RNA synthesis is enclosed within the membranous web. These data support the hypothesis that active replicase is bound by a limiting membrane and demonstrate that a vast excess of NS proteins are produced. This enclosed replicase presumably includes a channel for the exchange of NTPs with nascent RNA and pyrophosphate, similar to the spherule structures proposed for other positive-strand RNA viruses.

Similar to other *Flaviviridae*, HCV RNA replication initiates with the synthesis of genome-length, negative-strand RNAs, which exist as partially double-stranded replicative intermediates or fully double-stranded replicative forms.¹⁰ Negative-strand RNA then serves as a template for multiple rounds of positive-strand synthesis, leading to the asymmetric

FIGURE 25.15. Membranous webs, the site of hepatitis C virus (HCV) RNA replication. A: Membrane alterations in Huh-7 cells harboring an HCV subgenomic replicon. **B:** Higher magnification of the membranous web (arrows). M, mitochondria; N, nucleus. (Courtesy of R. Gosert and D. Moradpour. Adapted from Gosert R, Egger D, Lohmann V, et al. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J Virol* 2003;77[9]:5487–5492. Used with permission of the American Society for Microbiology Press.)



accumulation of nearly 10 positive strands for every negative strand.^{6,445,509,589,701} At least for cell culture–adapted genotype 1b replicons that do not make infectious virus, each cell contains approximately 100 negative strands, 1,000 positive-strands, and 1,000,000 copies of each viral protein.⁷⁰¹ A number of factors, however, influence the rate of HCV RNA replication. In Huh-7 cells, genotype 1b replication is robust in exponentially growing cells and repressed in growth-arrested cells.^{628,677,769} Interestingly, this block may be caused by a reduced pool of pyrimidine nucleotides, because replication can be restored in confluent cells by supplementing media with uridine and cytosine.⁶²⁸ In addition, co-transfected replicons interfere with each other, suggesting that they compete for limiting cellular factors.^{222,507} Given that replication-defective genomes also compete but that translation-defective genomes do not, one of these limiting cellular factors may interact with an NS protein.⁵⁰⁷

Virus Assembly

Similar to the related *Flaviviridae*, HCV particles bud directly into the ER, transit the secretory pathway, and are released through exocytosis. A model of HCV particle assembly has recently emerged, tying this process to the unique lipid metabolism of hepatocytes. Infectious virus production begins when SPP-processed core protein migrates to ER-associated cytoplasmic lipid droplets.⁵⁵⁹ Core is thought to recruit the membrane-associated replication complex through its interaction with NS5A,^{546,588,28} while the p7–NS2 complex recruits viral structural and NS proteins to the sites of virus assembly.^{83,370,530,672,688,803}

HCV particle formation is associated with VLDL assembly, which also takes place in the secretory pathway of hepatocytes. Consistent with this, inhibitors of serum lipoprotein assembly strongly inhibit HCV particle production,^{263,344} and NS5A has been shown to interact with apolipoproteins.^{60,176,223} During budding, nascent HCV particles may become associated with ER-luminal lipid droplets displaying ApoE. Lipid droplets are the triglyceride source for maturing VLDL particles and, *in vivo*, a large proportion may fuse with nascent ApoB-positive lipoproteins (reviewed in⁴⁴). HCV egresses through the secretory pathway, where the ion channel activity of p7 may protect nascent virions from premature fusion induced by the low pH of the secretory compartment.⁹¹⁵ While intracellular HCV particles are infectious, their buoyant density becomes lower as they egress in a maturation process that parallels VLDL.²⁶⁵ Like VLDL, blocking secretion of HCV leads to degradation of the high-density particles in a post-ER compartment.²⁶³

In addition to the VLDL secretory pathway, a number of other host factors have been implicated in HCV assembly, including the NS5A-associated factor annexin A2,³⁴ autophagy proteins Atg7 and beclin-1,⁸²⁹ and heat shock protein HSC70.⁶⁵²

PESTIVIRUSES

Background and Classification

Pestiviruses are animal pathogens of major economic importance for the livestock industry. They include the type member, bovine viral diarrhea virus (BVDV), as well as classical swine fever virus (CSFV) and border disease virus (BDV) of sheep.⁸⁵¹ New pestiviruses are frequently being isolated from

animals, bovine serum, or cell cultures. The International Committee on the Taxonomy of Viruses currently recognizes four pestivirus species (BVDV-1, BVDV-2, CSFV, and BDV) and four tentative species (atypical pestivirus, Bungereh virus, giraffe-1 pestivirus, and pronghorn antelope pestivirus).^{271,494} Within the family *Flaviviridae*, pestiviruses show greater similarity in genome structure and mechanism of initiating translation to the hepaciviruses than to the flaviviruses.

Pestivirus infections can be subclinical or produce a range of symptoms, including acute diarrhea, hemorrhagic syndrome, acute fatal disease, and wasting disease (reviewed in⁸⁵²). CSFV, typically transmitted oronasally, leads to acute or chronic hemorrhagic syndromes with significant mortality. In contrast, ruminant pestiviruses usually result in subclinical infection or cause mild symptoms in adult animals. A notable exception is BVDV-2, which has been associated with a severe, acute hemorrhagic condition in cattle.^{165,664,714} In addition, diaplacental transmission of pestiviruses can cause fetal death, malformation, or persistent infection of the fetus; for cattle this can lead to the development of mucosal disease. Two biotypes of pestiviruses, cytopathic (cp) and noncytopathic (ncp) viruses, are distinguished by their ability to cause cytopathic effects in cell culture.

Live attenuated strains, inactivated virus preparations, and subunit vaccines are available for immunization against pestivirus-induced diseases.⁵⁹⁴ Such vaccines should prevent diaplacental infection. Early attempts at vaccination with an attenuated cpBVDV strain resulted in genome recombination and the emergence of fatal mucosal disease in persistently infected cattle.⁵³ While improved vaccines have been developed by combining multiple attenuating mutations,⁵⁷⁴ genetic recombination remains a concern.

Structure and Physical Properties of the Virion

Pestiviruses have been difficult to purify because of modest growth in cell culture, inefficient release from infected cells, and association with serum components and cellular debris.⁴⁵² Virus particles visualized by electron microscopy^{594,894} are spherical and 40 to 60 nm in diameter³⁴¹ (Fig. 25.16). Structure and symmetry of the core have not been characterized. In addition to the genome RNA and lipid envelope, the particles are composed of four structural proteins: the core protein (C) and three envelope glycoproteins, E^{ms} (for ribonuclease, secreted), E1, and E2.^{749,853} E^{ms} and E2 have been detected on the surface of CSFV and BVDV particles by immunogold labeling,⁸⁹⁴ and disulfide bonds connect the envelope proteins on the virion surface.⁸⁵³ Pestivirus virions have a buoyant density of 1.134 g/mL and are inactivated by heat, organic solvents, and detergents.⁷⁴⁶ Similar to HCV, virion infectivity is stable over a relatively broad range of pH.⁴⁸⁰

Binding and Entry

Binding and entry of pestiviruses involves initial attachment, interaction with specific receptor(s), internalization, and membrane fusion. Pestiviruses can be detected in a variety of tissue types *in vivo*, including epithelial cells, endothelial cells, PBMCs, the gastrointestinal tract, and neurons. Highly permissive cell lines for the propagation of pestiviruses and infectious cDNA clones have allowed the study of viral entry in culture. Bovine CD46 has been identified as a cellular receptor for

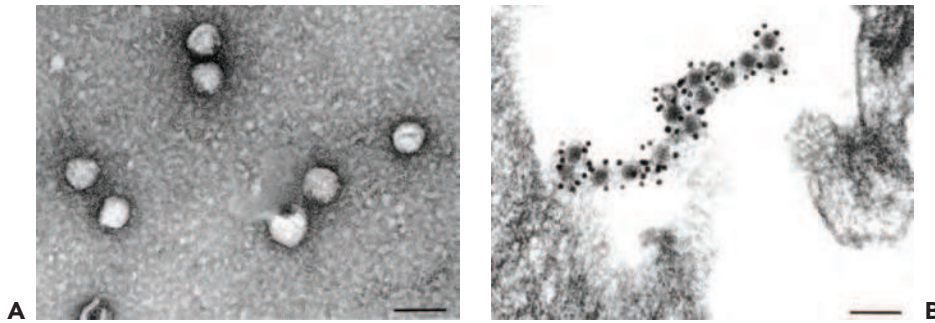


FIGURE 25.16. Pestivirus particles. **A:** Classical swine fever virus (CSFV) virions negatively stained with uranyl acetate **B:** Ultrathin section of swine testicular epitheloid cells infected with CSFV and immunostained with E^{ms}-specific monoclonal antibody (mAb) 24/16 and colloidal gold. Bar = 100 nm. (Courtesy of F. Weiland. Adapted from Weiland F, Weiland E, Unger G et al. Localization of pestiviral envelope proteins E^{ms} and E2 at the cell surface and on isolated particles. *J Gen Virol* 1999; 80[5]:1157–1165. Used with permission of Society for General Microbiology).

BVDV-1 and BVDV-2, including primary clinical isolates.⁵⁵¹ Experiments using chimeric CD46 molecules identified a discrete subregion within complement control protein repeat 1 as essential for BVDV binding and uptake.⁴²³ The viral ligand for CD46 is probably E2, which is also the major determinant of cell culture tropism, at least for ruminant pestiviruses.^{476,718} The LDL receptor was also suggested to assist in BVDV entry,⁴ although later evidence found no role for this molecule in bovine cell infection.⁴²⁴ In addition, E^{ms} of cell culture–adapted viruses binds cell surface glycosaminoglycans, which may act as an initial attachment factor.^{356,357} Finally, ectopically expressed E2 ectodomain inhibits BVDV entry at a step downstream of viral interaction with CD46, suggesting the involvement of an as-yet-unidentified entry factor.⁸⁶²

After binding, BVDV enters target cells via clathrin-dependent endocytosis followed by acid-dependent fusion in the endosome.^{297,425,457} Similar to HCV, BVDV must be primed to respond to low pH. Breakage of disulfide bonds between the glycoproteins during endocytosis may contribute to destabilizing the virion.⁴²⁵

Interestingly, E1 and E2 are sufficient for entry of CSFV or BVDV glycoprotein-pseudotyped particles; E^{ms} is nonessential in this system.^{737,888} Charged residues in the TM domains of E1 and E2 play a critical role in protein heterodimer formation and pseudoparticle entry.⁷³⁷

Genome Structure

Pestivirus genomes are approximately 12.3 kb in length.^{162,601} Similar to HCV, pestivirus genomes lack a 5′ cap and 3′ poly(A) tract^{102,601} (Fig. 25.17A). A long ORF of approximately 4,000 codons is flanked by a 5′ NCR of 372 to 385 nt and a 3′ NCR of 185 to 273 nt.^{32,102,160} Two 5′ terminal stem-loop structures in the BVDV genome (domains Ia and Ib in Fig. 25.17A) are important for efficient RNA replication.^{250,945} A 5′ terminal GUAU sequence is essential for BVDV replication, as its complement may be a promoter for plus-strand synthesis.²⁵⁰ Provided that this tetranucleotide sequence is retained, substitutions and deletions of hairpin Ia and part of Ib do not abolish replication.⁵² Thus, the 5′ signals essential for pestivirus genome replication are significantly shorter than for the hepaciviruses. Following the ORF, the 3′ NCR consists of a variable region,

a single-stranded region, and a conserved 3′ terminal stem-loop.^{189,944} Mutational analyses indicate that the terminal stem-loop and the upstream single-stranded region harbor important primary and secondary structural elements that probably function *in cis* to direct minus-strand initiation. In contrast, deletions in the variable region are well tolerated.^{360,650,944}

Translation and Polyprotein Processing

Cap-independent translation initiation of the pestivirus genome is mediated by an IRES that bears structural and functional similarity to that of HCV (compare Figs. 25.12A and 25.17A).^{103,189,687,729} The minimal pestivirus IRES includes 5′ NCR domains II and III and can be influenced by structured sequences downstream from the initiator AUG.^{142,621,687,729} As for HCV, the pestivirus IRES binds ribosomal 40S subunits without the need for translation initiation factors eIF4A, eIF4B, and eIF4F.^{668,669,794} The pestivirus genome encodes a large polyprotein that is processed into individual viral proteins: N^{pro}-C-E^{ms}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Fig. 25.17B).^{161,163,578,602,695}

Unlike other *Flaviviridae*, the first pestivirus protein is an NS protein, N^{pro}. This is an autoprotease responsible for cleavage at the N^{pro}/C site.^{806,912} Processing of the pestivirus structural region appears to be mediated by at least three additional proteases. Host signal peptidase is believed to cleave at the C/E^{ms}, E1/E2, E2/p7, and p7/NS2 sites, with incomplete cleavage at the E2/p7 site leading to accumulation of an uncleaved product.^{215,310,749} SPP mediates further processing of the pestivirus C protein in the TM region.³²¹ The E^{ms}-E1 polyprotein (gp62) is processed slowly at a novel type of signal peptidase cleavage site.^{71,749} The NS region is processed by the NS2 autoprotease,⁴³⁷ which performs an incomplete cleavage at the NS2/3 junction,³ and the NS3-4A serine protease, which cleaves the remainder of the polyprotein.^{834,913,920} As detailed later, certain cytopathic pestiviruses generate the authentic N-terminus of NS3 via several different mechanisms.

N^{pro} Autoprotease

N^{pro} is an NS autoprotease that cleaves at a conserved site between Cys-168 and Ser-169 of the polyprotein.^{806,912} The active-site residues involved in this activity include Glu-22,

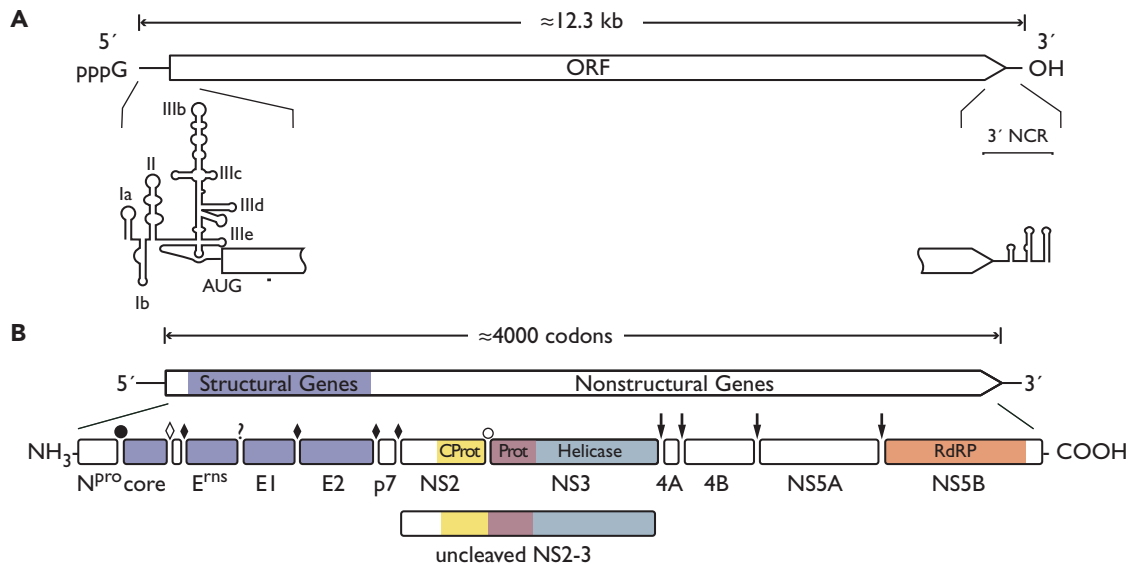


FIGURE 25.17. Pestivirus genome structure and protein expression. **A:** Genome structure and RNA elements. Important RNA elements are indicated as in Figure 25.4. **B:** Polyprotein processing and cleavage products. Symbols identifying proteolytic cleavages for the cpBVDV NADL strain are the same as those described in Figure 25.4 except for the proposed autocatalytic cleavage releasing the N-terminal nonstructural protein Npro from the pestivirus polyprotein, which is indicated by a closed bullet. See text for details.

His-49, and Cys-69, leading to the suggestion that N^{pro} may be an unusual subtilisin-like cysteine protease.⁷⁴⁷ N^{pro} is dispensable for pestivirus replication in cell culture but is required for virulence in animals.^{55,552,607,835,861} N^{pro} inhibits IFN production^{135,272,435,741,744} by targeting the cellular transcription factor IRF-3 for degradation.^{48,135,332,771} However, CSFV mutants that lack this activity remain virulent in animals.⁷⁴³

Pestivirus Structural Proteins

The capsid (C) protein is a 14-kD, highly basic, RNA-binding protein.⁶¹⁸ The N-terminus of C is generated by the autocleavage of N^{pro}.⁹¹² The nascent C protein encodes a C-terminal signal peptide that leads to translocation of E^{trns} into the ER. Similar to HCV, pestivirus C protein undergoes sequential maturational cleavages by signal peptidase and SPI.³²¹ Mature C is a natively unfolded protein that nonspecifically binds RNA with low affinity.⁶¹⁸ Remarkably, functional C protein can tolerate sizeable deletions, duplications, and insertions; for one of these mutants, severe defects in virus assembly were suppressed by a second site mutation in the NS3 helicase domain.⁷²⁷ These data suggest that C protein does not form an icosahedral nucleocapsid but may function like a histone protein to condense nucleic acids.

The E^{trns} glycoprotein (44 to 48 kD, formerly known as E0 or gp44/48) is heavily glycosylated on seven to nine asparagine residues and forms disulfide-linked homodimers.^{449,853} E^{trns} associates with membranes and virus particles via a C-terminal amphipathic helix^{235,847} but is also secreted from infected cells in soluble form.^{749,892,894} The most unusual feature of E^{trns} is that it encodes a ribonuclease (RNase) activity with specificity for uridine residues.^{316,768} This RNase activity contributes to the ability of pestiviruses to inhibit the induction of type I IFN by exogenous dsRNA.^{358,526,540,550,574} Antibodies that inhibit RNase activity tend to neutralize virus infectivity,⁹⁰⁸ and mutations in E^{trns} that destroy enzymatic activity give rise to viruses that are

attenuated *in vivo*.^{573,574,881} While homodimerization is dispensable for RNase activity,⁹⁰⁸ viral mutants that are unable to form E^{trns} complexes are less virulent,⁸⁴⁸ suggesting that the glycoprotein may have additional functions *in vivo*. Recombinant E^{trns} is toxic to lymphocytes *in vitro*,¹⁰⁴ which may contribute to the leukopenia seen in natural infections.⁸²¹ Although cytotoxicity is a feature of other soluble RNases,⁷⁶¹ it is not yet clear whether the enzymatic activity of E^{trns} is related to its toxicity. The C-terminal domain of E^{trns} can promote its translocation across cellular membranes, suggesting that it may have an intracellular target or function.⁴⁴⁸ Recombinant E^{trns}, however, can also bind strongly to the surface of cells, probably via interaction with glycosaminoglycans, and inhibit viral infection.^{356,894}

E1 and E2 are integral membrane glycoproteins that contain two to three and four to six N-linked glycosylation motifs, respectively.⁸⁹³ E2 forms homodimers,^{853,893} as well as disulfide-linked heterodimers with E1.⁷⁴⁹ Heterodimer formation is essential for viral entry and involves the interaction of charged residues within the TM domains of E1 and E2. Recombinant CSFV E2 can bind to cells and block infection of CSFV and BVDV, suggesting a common receptor or co-receptor for binding and entry of these pestiviruses.³⁴⁸ In addition, E2 expression inhibits BVDV superinfection at the level of viral entry.^{862,863} Infectious pestivirus particles are neutralized by monoclonal antibodies that recognize E^{trns} or E2,^{197,656,873,892,893,903} and these antigens can elicit protective immunity.^{349,748,874}

p7 Protein

The p7 protein consists of a central charged region separating hydrophobic termini. Similar to the HCV protein, pestivirus p7 is dispensable for RNA replication⁵⁵ but required for the production of infectious virus particles.^{310,475} Another similarity is that E2–p7 cleavage, most likely by host signal peptidase, is inefficient.²¹⁵ Uncleaved E2–p7 is not required for replication

in cell culture and both E2-p7 and p7 appear to remain cell associated.³¹⁰ Furthermore, pestivirus p7 protein may form ion channels *in vitro* and *in vivo*.^{295,528}

Pestivirus Nonstructural Proteins

The NS2 protein (~54 kD) is a cysteine autoprotease, distantly related to the NS2-3 autoprotease of HCV and GB viruses, which is responsible for processing NS2-3 (~125 kD).^{436,438} Remarkably, a cellular chaperone protein, DNAJC14 (originally identified as Jiv for J-domain protein interacting with viral protein), is an essential co-factor for NS2-mediated proteolysis. DNAJC14, a member of the heat shock protein (HSP) 40 family of chaperones, interacts irreversibly with NS2 and facilitates interaction of the catalytic and substrate residues.⁴³⁶ As discussed later, NS2-3 cleavage is essential for pestivirus RNA replication, and the efficiency of processing is a key regulator of RNA accumulation and cytopathogenicity. Interestingly, pestivirus NS2-3 cleavage is incomplete, and the unprocessed form of the protein is essential for infectious virus production.^{3,608}

As for other *Flaviviridae*, the pestivirus NS3 protein (~80 kD) contains an N-terminal serine protease domain^{50,279,913} and a C-terminal RNA helicase.²⁸⁰ The NS3 serine protease, along with its NS4A co-factor,^{836,920} cleaves between leucine and small uncharged amino acids: L↓(S/A/N).^{834,920} Substitutions that eliminate serine protease activity abolish viral RNA replication, confirming its essential role in virus viability.^{288,920} Interestingly, protease activity is retained when threonine is substituted for the serine nucleophile.⁸³⁶ The NS3 protein of BVDV has been purified and shown to possess RNA helicase⁸⁸⁹ and RNA-stimulated NTPase⁸²⁵ activities. Site-directed mutagenesis of the conserved helicase and NTPase motifs abolished viral replication.

The pestivirus NS4A (~10 kD) and NS4B (~38 kD) proteins share similar size, organization, and function with their HCV analogs, although sequence homology between the genera is negligible. NS4A is an essential co-factor for the NS3 serine protease activity.^{836,920} NS4B is a multispanning membrane protein that associates with rearranged cellular membranes involved in RNA replication.⁸⁹⁷ Similar to HCV, the pestivirus NS4B protein encodes an NTPase activity of unclear function.²⁷³ While a ~45 kD NS4A-4B precursor is transiently produced in pestivirus-infected cells, genetic analysis revealed that it is not essential for BVDV replication in cell culture.²⁵⁷

The remaining two proteins, NS5A (~58 kD) and NS5B (~75 kD), are present as mature cleavage products, as well as an uncleaved NS5A-5B precursor.^{161,163,443} NS5A is essential for RNA replication, although its precise functions have not been fully elucidated.^{289,844} Similar to HCV, pestivirus NS5A proteins contain an N-terminal amphipathic helix and a zinc-coordinating motif.^{93,753,844} Furthermore, NS5A is phosphorylated by a cellular serine or threonine kinase with properties similar to enzyme(s) that modify flavivirus NS5 and hepatitis virus NS5A.⁷¹⁵ Genetic analysis revealed that defects in the *NS5A* gene can be efficiently complemented *in trans*, whereas mutations in other pestivirus NS genes cannot.²⁸⁹

NS5B contains motifs characteristic of RdRP.¹⁶⁰ The RNA polymerase activity of recombinant NS5B has been characterized *in vitro* and found to extend template-primed RNA into double-stranded copy-back products^{440,510,807,960} or to catalyze *de novo* initiation from short, synthetic RNA or DNA templates.^{384,440} The structure of BVDV NS5B is similar to the

HCV polymerase and to other RdRPs, containing a palm subdomain surrounded by finger and thumb subdomains.¹⁴¹ The pestivirus NS5B structure reveals a unique N-terminal region, which suggests a role for GTP in *de novo* initiation, and provides a framework for understanding the molecular mechanisms of small-molecule inhibitors of BVDV replication.^{35,177,647,819}

RNA Replication

The basic mechanisms of pestivirus RNA replication appear to be similar to those described for HCV. RNA accumulation is associated with cytoplasmic membranes and requires NS3 through NS5B. Cellular components are also involved; for example, NFAR proteins associate specifically with the 5' and 3' termini of the BVDV genome.³⁵⁹ The 3' NCR also contains determinants that ensure efficient termination of translation, which is essential for efficient pestivirus RNA replication.³⁶⁰ Negative- and positive-strand RNAs have been detected from 4 to 6 hours after pestivirus infection, followed by the asymmetric synthesis of additional minus- and excess plus-strand RNA.²⁷⁵ Double-stranded RF RNA and partial duplex RI RNA have been tentatively identified.^{275,276,695}

Insights into the regulation of BVDV RNA replication and virus assembly have emerged from the study of ncp and cpBVDV. In addition to displaying differences in cytopathogenicity, both biotypes show altered NS2-3 processing. cpBVDV produces both NS3 and uncleaved NS2-3 in large amounts, whereas ncpBVDV was thought to express only the uncleaved protein. This suggested that uncleaved NS2-3 could serve as a functional RNA replicase component. It has been shown, however, that NS2/3 cleavage is absolutely required for RNA replication and that the efficiency of this process is regulated by the NS2 co-factor, DNAJC14.⁴³⁶ Early after ncpBVDV infection, NS2/3 cleavage is nearly complete, allowing efficient NS3 production and the initiation of RNA replication. At later time points, when uncomplexed DNAJC14 levels are limiting, autoprocessing becomes inefficient and viral RNA synthesis rates decline.⁴³⁷ cpBVDV viruses overcome the decline of endogenous DNAJC14 and promote continuous NS2-3 cleavage through a variety of genetic variations, as described later.

During replication, nonhomologous recombination can occur within pestivirus genomes and between pestivirus RNA and host cellular mRNA (reviewed in⁵⁸⁰). One likely mechanism is copy-choice template recruitment during minus-strand synthesis, which is consistent with the coding orientation of cellular inserts. An alternative mechanism of RNA recombination has been demonstrated by using a cell culture-based system in which homologous and nonhomologous recombination occurred between two overlapping transcripts that each lacked different essential parts of the viral RdRP.²⁵⁸ Statistical analysis of recombination sites also supports the hypothesis that homologous recombination contributes to pestivirus diversity in nature.³⁷⁶

Assembly and Release of Virus Particles

Other than the features of the virion structural proteins described previously, little information is available on the assembly and release of pestiviruses. Electron microscopic examination of infected cells suggests that pestiviruses mature in intracellular vesicles and are released by exocytosis.^{69,291} Consistent with intracellular budding, pestivirus envelope proteins are retained within the secretory pathway^{292,894} and

brefeldin A, a potent inhibitor of ER–Golgi transport, inhibits the secretion of viral particles but does not block their assembly.⁵³⁹ Interestingly, E^{rns} and E2 have been immunolocalized on isolated virus particles by electron microscopy, but E2 was not detected in particles undergoing secretion (or perhaps reattachment) at the cell surface.⁸⁹⁴ This suggests that E2 may be conformationally inaccessible to antibodies before maturation. As with other members of the *Flaviviridae*, NS proteins play an important role in pestivirus virion assembly or release, including p7, NS2-3, NS3-4A, and NS5B.^{3,24,475,608,617}

Pathogenesis of Mucosal Disease and the Generation of Cytopathic Pestiviruses

Mucosal disease, the most severe outcome of BVDV infection, is usually fatal.⁵⁸⁰ This disease occurs only after *in utero* infection with an ncpBVDV strain between 40 and 125 days of gestation, leading to the birth of immunotolerant animals that remain persistently infected for life. In the case of an animal exhibiting mucosal disease, both cp and ncp biotypes of BVDV can be found.⁵⁵⁵ The close serologic relatedness of isolated ncp–cp pairs led to the suggestion that cpBVDV might arise from ncpBVDV by a rare mutational event. Genetic characterization of a number of these ncp–cp pairs has verified this hypothesis and led to the remarkable discovery that most cpBVDV strains are generated via RNA recombination,⁵⁴ although a few cpBVDV strains lack obvious genome rearrangements.^{580,663} The presence of these genome rearrangements strongly cor-

relates with increased NS3 expression, enhanced RNA replication, and cytopathic effects in cell culture. Figure 25.18 illustrates a few of the remarkable cpBVDV genome alterations that have been discovered.

Common features of some cpBVDV variants include genome rearrangements or mutations that activate NS2-3 autoprotease activity, leading to increased production of NS3 and augmented RNA replication. For instance, strain NADL (Fig. 25.18A) contains a fragment of the cellular *DNAJC14* gene (also known as Jiv) inserted within NS2.⁷³¹ As described earlier, DNAJC14 is an essential co-factor of the NS2 protease, and overexpression of a critical 90-amino acid DNAJC14 subdomain enhances NS2-3 cleavage, regardless of whether the fragment is provided *in cis* or *in trans*.⁴³⁷ Interestingly, a much smaller insertion is found in the NS2 gene of cpBVDV strain CP7, which contains a 27-nt duplication from an upstream region of the NS2 gene in an alternate reading frame.⁸³⁷ As with the DNAJC14 fragment, this insertion leads to increased NS2-3 processing and a virus that is cytopathic in culture.^{567,576} Other viral insertions at or very close to the same site have been described.³⁶ For other cpBVDV isolates, increased NS2-3 autoprotease activity appears to result from point mutations that have accumulated within the NS2 gene.⁴³³

Another common rearrangement in cpBVDV isolates is the insertion of ubiquitin or ubiquitin-like genes immediately upstream of NS3,^{40,577,698,838} leading to NS2/3 processing by ubiquitin C-terminal hydrolase or related enzymes (e.g., strain

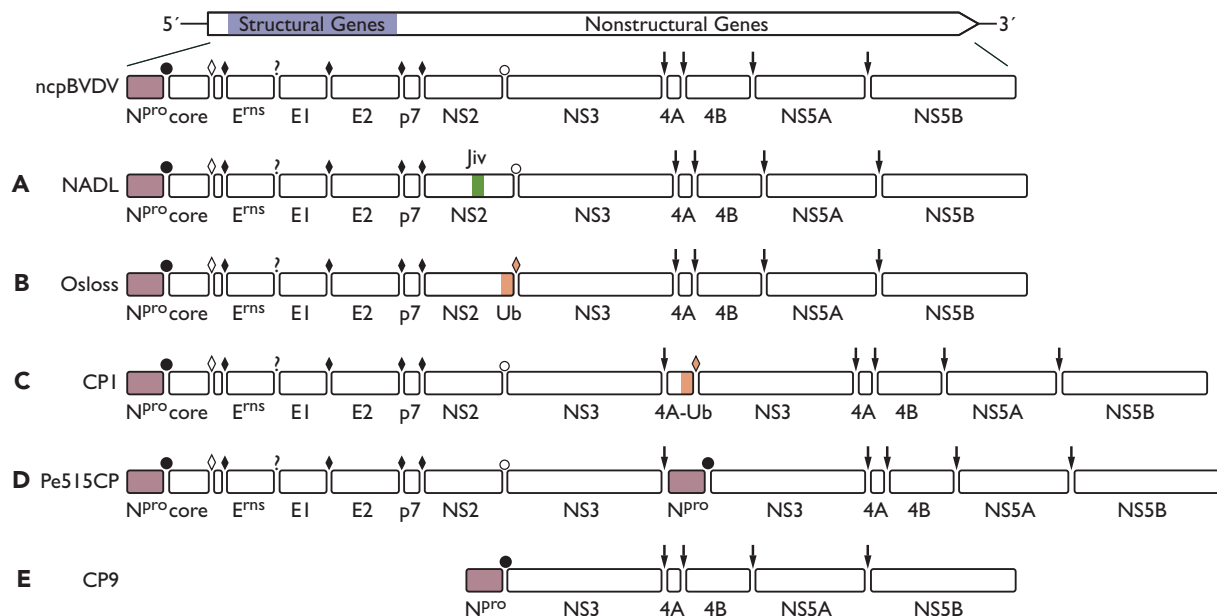


FIGURE 25.18. Genome rearrangements associated with cytopathic bovine viral diarrhea virus (cpBVDV). The **top diagram** indicates the polyprotein of a typical noncytopathic BVDV (ncpBVDV) isolate. Below, the polyproteins encoded by five different cpBVDV isolates generated by RNA recombination are shown. **A:** The genome of BVDV1 strain NADL. **B:** The genome of BVDV1 strain Osloss. **C:** The genome of BVDV1 strain CPI. **D:** The genome of BVDV1 strain Pe515CP. **E:** The genome of BVDV1 strain CP9. As discussed in the text, these cpBVDV polyprotein structures allow the production of both NS2-3 and NS3. In-frame insertions of host sequences (colored boxes) are present in NADL, Osloss, and CPI. The NS2 autoprotease is responsible for NS3 production in the NADL strain, but the inserted ubiquitin sequences in Osloss and CPI provide sites for processing by host ubiquitin C-terminal hydrolase (orange diamond). For Pe515CP and the CP9 DI RNA, the N^{pro} autoprotease (maroon box) mediates the cleavage producing the NS3 N terminus. The nomenclature and organization of the cleavage products and the symbols for the normal processing enzymes are defined in Figure 25.17.

Osloss) (Fig. 25.18B). In strains such as CP1, this may be accompanied by additional genome rearrangements, such as duplication of the *NS3* and *NS4A* genes⁵⁷⁸ (Fig. 25.18C). All the strains described previously also express uncleaved NS2-3, which is important for infectious virion production.³ Another type of insertion includes the light chain 3 gene of cellular microtubule-associated proteins, which is targeted for cleavage by a cellular protease.^{244,575}

A third type of cpBVDV genome configuration repositions the N^{pro} autoprotease immediately upstream of NS3. In strain Pe515CP, N^{pro} is duplicated together with the *NS3* and *NS4A* genes⁵⁷⁸ (Fig. 25.18D). Other cpBVDV isolates, such as CP9, contain a precise deletion of the C-E^{ms}-E1-E2-p7-NS2 coding region, resulting in an in-frame fusion of N^{pro} and NS3⁸³⁹ (Fig. 25.18E). Such subgenomic RNAs replicate autonomously but require ncpBVDV helper viruses to provide packaging functions *in trans*.⁵⁵ For CSFV, cp subgenomic RNAs have been isolated in which the entire coding sequence upstream of NS3 has been deleted.⁵⁷⁹

cpBVDV genome rearrangements strongly correlate with increased NS3 expression and enhanced RNA replication. These phenotypes, however, can be uncoupled from cytopathogenicity in cell culture. For instance, a temperature-sensitive mutant of CP7 containing a point mutation in NS2 is ncp at 39.5°C but retains high NS3 expression, although RNA replication is reduced.⁶⁴⁹ Selection for variants of cpBVDV in cell culture resulted in an ncpBVDV strain that still produces NS3 and viral RNA at levels comparable to the cp parent but encodes a point mutation in NS4B that attenuates the cytopathic effect.⁷⁰⁰ Other ncpBVDV strains also show changes in NS4B.²⁵⁷ Given that cpBVDV can cause ER stress³⁷⁸ and NS4B is involved in membrane reorganization,⁸⁹⁷ it seems plausible that cytopathic effects may result from overcommitment of cellular membranes to viral replication. Proteome analysis of infected cell cultures showed that cp or ncp viruses differentially regulate host signal transduction pathways, although these studies were not conducted with an isotype-matched cp–ncp pair of viruses.²¹ In infected animals, increased cell death may be sufficient to induce widespread tissue injury and inflammation. Animals with mucosal disease also show increased numbers of infected cells, suggesting that differences in cpBVDV tropism may also contribute to the disease.⁴⁷⁹

GB VIRUSES

Discovery and Classification

In the early 1990s, a residual number of hepatitis cases were still not attributable to hepatitis A through E viruses. Efforts aimed at identifying additional hepatitis agents revealed three novel viruses that have been tentatively assigned to the family *Flaviviridae*. Two of these viruses, GBV-A and GBV-B, were cloned via representational difference analysis from the sera of tamarins experimentally infected with the GB hepatitis agent.⁷⁹² The GB agent was originally derived from the serum of a 34-year-old surgeon, “GB,” who had acute hepatitis, by serial passage in tamarins.¹⁸⁸ Both viruses are similar to HCV yet genetically quite distinct.⁶⁰⁹ Although originally derived from a human hepatitis case, subsequent work showed that GBV-A is an indigenous monkey virus that was likely acquired during passage in tamarins.^{107,456} Some human cases that are not A through E

hepatitis show serologic reactivity to both GBV-A and GBV-B, but reverse transcription-polymerase chain reaction (RT-PCR) has failed to detect either virus in human samples. Rather, a third related virus, GBV-C, was subsequently identified in humans.⁷⁹¹ Working independently, another group that was immunoscreening cDNA libraries from non-A, non-B hepatitis cases identified an agent, initially termed hepatitis G virus (HGV), which later turned out to be an independent isolate of GBV-C.⁴⁹³ Because this virus has not been convincingly shown to cause human disease, including hepatitis, we will refer to it by its original designation, GBV-C.

A virus distantly related to GBV-A and GBV-C was recently discovered in Old World frugivorous bats in Bangladesh.²²⁰ This virus has been designated GBV-D.

Based on sequence relatedness and overall genome structure, GBVs have been classified as members of the family *Flaviviridae*. GBV-B is considered to be a member of the genus *Hepacivirus*.⁸⁵¹ It has recently been proposed that GBV-A, GBV-C, and GBV-D be classified as a separate genus, *Pegivirus*, although this awaits formal ratification by taxonomists.⁸⁰⁵ According to the proposed classification strategy, GBV-B would be renamed GBV and other GBVs would be renamed as pegiviruses according to their host of origin.

The inability to detect GBV-A or GBV-B in human samples led to investigation into their origins. Interestingly, GBV-A has been detected in several species of New World monkeys in the absence of experimental infection or overt disease.^{107,455} Viral sequences isolated from a single primate species are highly related, whereas sequences derived from separate species show greater divergence, indicating that GBV-A has adapted to its primate hosts over extended periods of time.^{107,127,455} The distribution of GBV-B in nature is unknown because the only source of this virus is the original tamarin-passaged GB serum. Despite intensive efforts, it has not been reisolated from natural sources.

Since its initial discovery, GBV-C infection has been found to be surprisingly common in the human population. Approximately 15% of healthy volunteer blood donors have markers of previous or ongoing infection with this virus,⁸⁰⁴ and GBV-C has also been found in chimpanzees.^{2,72,595} Phylogenetic analysis of GBV-C sequences has been complicated by an apparent bias against synonymous substitutions in some parts of the genome, leading to differences in inferred evolutionary relationships.⁷⁸⁷ The molecular basis for this bias is unclear but may involve evolutionary constraints imposed by RNA structures⁷⁸⁹ or cryptic ORFs.^{658,796} Nevertheless, GBV-C has been classified into four or five genotypes.⁷⁸⁷ Remarkably, variation among GBV-C isolates reflects the geographic distribution of human migration, suggesting the long-term co-evolution of this virus and its host. Given the rate at which RNA viruses typically evolve, this finding suggests that GBV-C is subject to unusual evolutionary constraints.

Clinical Perspective

Although GBV-A and GBV-B were originally derived from a case of human hepatitis, it is unclear whether either virus was the cause of the disease. It is now clear that GBV-A is not associated with any known disease and is likely to have been acquired during tamarin passage. GBV-B can infect and cause hepatitis in New World monkeys such as tamarins, marmosets, and owl monkeys, but it does not infect chimpanzees.^{101,108,446} Because

of this preference for lower primates, GBV-B is unlikely to be a human virus. Attempts to identify GBVs in the original GB clinical sample have failed, possibly because of degradation over prolonged storage.^{2,763}

Human infection with GBV-C is well documented, although direct association of this virus with any human disease has proved to be elusive.⁸⁰⁴ While the virus is usually cleared within 2 years,⁶⁴ persistent infections can last for years without clinical effects.¹⁷ Clearance usually correlates with the appearance of antibodies against the viral E2 glycoprotein.⁸⁰⁴ GBV-C appears to be primarily lymphotropic *in vivo*,^{450,451} although evidence also exists for hepatotropic isolates.²⁴⁰ GBV-C is transmitted parenterally or sexually, and a vertical transmission route is also likely.^{65,804} Because these routes also transmit many other human viruses, GBV-C co-infections with HBV, HCV, or HIV are not uncommon. Needless to say, co-infection with human hepatitis viruses has likely contributed to the confusing association of GBV-C with disease.

Intriguingly, there is a possible interaction between GBV-C and HIV. It has been noted that patients co-infected with the two viruses tend to have higher CD4+ T-cell counts, lower HIV titers, and slower HIV disease progression.⁸⁰⁴ Based on these observations, as well as *in vitro* experiments, it has been proposed that GBV-C may interfere with HIV replication by altering expression of cytokines, chemokines, and chemokine receptors,^{683,918} decreasing T-cell activation,⁵⁴¹ directly inhibiting HIV-1 entry,^{327,381,408,596} or eliciting cross-reactive antibodies that neutralize HIV particles.⁵⁹⁷ It has also been argued, however, that as a lymphotropic virus, the presence of GBV-C viremia may simply reflect the higher CD4+ counts in HIV nonprogressors.⁸⁷⁰ Thus, the underlying reasons for the correlation between GBV-C infection and slower HIV progression are not yet clear.

Experimental Systems

Little work has been done on GBV-A because it is an indigenous monkey virus that is not associated with disease. On the other hand, GBV-B is the closest relative of HCV and has been extensively studied as a surrogate model system. GBV-B can be readily cultured in primary tamarin or marmoset hepatocytes^{51,101,446} but replicates poorly (if at all) in many immortalized cell lines.¹⁰⁵ Full-length GBV-B cDNAs have been assembled and shown to be infectious and cause hepatitis in tamarins.^{109,545,757} Based on these functional clones, subgenomic GBV-B replicons have been constructed and can replicate in the human hepatoma lines Huh-7 and Hep3B, albeit with low efficiency.^{186,187}

GBV-C has been reportedly cultured in human hepatoma lines,⁷⁷² primary human lymphocytes,²⁶⁷ peripheral blood mononuclear cells,²⁴¹ and a derivative of the Daudi Burkitt lymphoma line.⁷⁸² Reminiscent of HCV, replication levels are low in these cell culture systems. Nevertheless, a GBV-C cDNA clone was constructed and shown to be infectious in primary human CD4+ T-cells.⁹¹⁹ GBV-C subgenomic replicons can persistently replicate in Huh-7 cells.¹¹⁸

Virion Structure and Entry

Particles of GBV-A and GBV-B have not been characterized. Similar to HCV, GBV-C particles exhibit unusually low and heterogeneous buoyant density, with peaks near 1.07 to 1.09 g/mL and 1.17 g/mL^{566,754,917} due to interaction with lipoproteins.

Treatment with detergents or organic solvents removes the viral envelope and shifts the peak of viral RNA to a higher-density form that may represent nucleocapsids,^{566,754,917} although, paradoxically, GBV-A and GBV-C do not encode an obvious capsid gene. Little is known about the entry mechanism of GBV, although it has been proposed that GBV-C utilizes the LDL receptor.⁴

Genome Structure and Expression

As with other *Flaviviridae*, the GBVs encode a single long ORF containing structural genes followed by NS genes, flanked by 5' and 3' NCRs.^{456,609} As for HCV and the pestiviruses, GBVs utilize an IRES to direct cap-independent translation.^{286,790} Compared to other *Flaviviridae*, however, the GBV-A and GBV-C 5' NCRs are much longer (>500 nt) and appear to fold into a similar structure that differs from other family members. The GBV-B 5' NCR is also much longer than the corresponding HCV sequence, but the two regions share significant similarities in primary, secondary, and tertiary structure. In fact, critical regions of the GBV-B and HCV IRESs can be functionally exchanged.^{380,728,730}

The GBV-A and GBV-C 3' NCRs lack a poly(U/UC) tract and are highly conserved only within these virus groups, with the exception of more broadly conserved terminal stem-loop structures.^{174,919} The GBV-B 3' NCR is 361 nt long, containing a short poly(U) stretch 30 nt downstream of the stop codon, followed by a unique 309-nt sequence.^{109,756} Although this region of the GBV-B genome does not display homology to HCV, the terminal 82 nt of the sequence can fold into a structure reminiscent of the HCV 3' X region.

As with HCV, GBV-B contains a basic capsid protein followed by two envelope glycoproteins, E1 and E2. The genomes for GBV-A and GBV-C also contain E1 and E2 glycoproteins, but they lack any obvious capsid-like sequence.^{493,609} The initiation codons used by these viruses have not been firmly mapped but appear to be conserved AUG codons immediately upstream of the E1 gene.⁷⁹⁰ It has been observed, however, that individuals infected with GBV-C generate antibodies against a small basic peptide that can be translated from an in-frame upstream AUG, suggesting that such a protein is expressed *in vivo*.⁹¹⁷ Alternative explanations for the lack of a capsid-like protein include the possibilities that GBV-A and GBV-C might usurp a capsid-like protein from the host cell or a co-infecting virus, or that additional GBV proteins may be involved. In this regard, a region of the GBV-C NS5A gene exhibiting a bias against synonymous mutation has been noted to potentially encode a small basic protein (10 kD, pI 11.5) in an alternate reading frame.⁶⁵⁸ Further characterization of GBV-A and GBV-C particles will be needed to demonstrate the nature of their nucleocapsid.

GBV-B encodes a 13-kD protein that shows partial homology to HCV p7.²⁷⁰ This protein is predicted to span the membrane four times and can be processed by signal peptidase into two tandem p7-like proteins.^{270,824} Remarkably, only the second half of p13, which has greater similarity to the HCV p7 gene, is needed for infectivity in tamarins.⁸²⁴ Furthermore, the GBV-B p13 gene can be functionally replaced by HCV p7.²⁹³

The NS proteins of GBVs show the greatest similarity to HCV, and the boundaries of cognate NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins have been proposed.^{456,609} Catalytic residues of the HCV NS2/3 autoprotease are conserved

among GBV NS2 proteins, and this enzymatic activity has been demonstrated for GBV-C.⁵⁷ Similarly, the GBV NS3 proteins encode an N-terminal serine protease and C-terminal RNA helicases.^{456,609} The GBV-B serine protease activity shares substrate specificity with the HCV enzyme and requires the virus-specific NS4A co-factor.^{111,755,759} Consistent with this high degree of similarity, inhibitors of the HCV serine protease also inhibit the GBV-B protease.¹⁰¹ NTP-dependent RNA helicase activity has also been demonstrated for NS3 proteins of GBV-B and GBV-C.^{307,961} The GBV-B NS5B has been shown to possess primer-dependent and *de novo* initiation RdRP and terminal transferase activities, albeit with different cation selectivity.^{711,962} The tail anchor of GBV-B NS5B can functionally substitute for the tail anchor of HCV NS5B.⁹²

PERSPECTIVES

Our understanding of the *Flaviviridae* has increased tremendously in the recent years, although significant gaps remain. The recent identification of new members of the *Flaviviridae* such as the GBVs and CHV has deep implications for understanding the origins and diversity of these viruses. For a few viruses in the family, viral and host proteins required for genome replication have been identified and some of them have been characterized in molecular detail. A major remaining task is to understand how these components come together to form a functional replicase. Similarly, the processes of virus entry and particle assembly are only incompletely understood. It is very curious that flaviviruses and pestiviruses can tolerate large deletions and insertions within their C genes, and that some GBVs lack recognizable nucleocapsid genes altogether. These observations suggest that alternative mechanisms must allow viral genomes to condense into small virus particles. It is also curious that HCV assembly has been tied to VLDL assembly, although the molecular basis for this association remains unclear. Finally, a major overarching goal is to translate our knowledge of these viruses into improvements in human and animal health. The recent development of HCV-specific antivirals demonstrates the feasibility of this approach, although major challenges remain to develop broadly acting antiviral strategies. Similarly, vaccine development remains an important priority, particularly for dengue and HCV, with no clear solutions in sight. Clearly, we have only just begun to understand the *Flaviviridae*.

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Flaviviruses

Flavivirus Diversity, Evolution, and Distribution

- Molecular Phylogeny
- Evolution
- Global Distribution

Flavivirus Composition and Antigenic Structure

- Structure
- The Antigenic Surface

Clinical and Pathologic Syndromes of the Flaviviruses

Dengue Virus

- History, Global Distribution, and Epidemic Cycle
- DENV Diversity
- Clinical Features of Acute DF: Primary DENV Infection
- Clinical Features of DHF/DSS: Secondary and Infant DENV Infection
- Pathologic Features of DHF/DSS

Yellow Fever Virus

- History, Global Distribution, and Epidemic Cycle
- YFV Diversity
- Clinical Features of YFV Infection
- Pathologic Features of YFV Infection

West Nile Virus

- History, Global Distribution, and Epidemic Cycle
- WNV Diversity
- Clinical Features of WNV Infection
- Pathologic Features of WNV Infection

Japanese Encephalitis Virus

- History, Global Distribution, and Epidemic Cycle
- JEV Diversity
- Clinical Features of JEV Infection
- Pathologic Features of JEV Infection

St. Louis Encephalitis Virus

- History, Global Distribution, and Epidemic Cycle
- SLEV Diversity
- Clinical and Pathologic Features of SLEV Infection

Tick-Borne Encephalitis Viruses

- History, Global Distribution, and Epidemic Cycle
- TBEV Diversity
- Clinical Features of TBEV Infection
- Pathologic Features of TBEV Infection

Pathogenesis and Immunity

- Virus Entry and Tropism
- Mechanisms of Dissemination
- Mechanisms of Immune Control: Innate Immunity

Animal Models of Flavivirus Pathogenesis and Disease

- Dengue Virus
- Yellow Fever Virus
- West Nile Virus

Prevention and Control

- Flavivirus Vaccines
- Therapeutics
- Passive Antibody Therapy
- Nucleic Acid Inhibitors
- Flavivirus Antiviral Peptides
- Iminosugars
- High-Throughput Screens with Small Molecule Inhibitors

Acknowledgments

Flaviviruses acquired their name from the jaundice associated with the liver dysfunction caused by yellow fever virus (YFV) infections. YFV played an important historical role in defining the nature of viruses in general. Seminal studies by Walter Reed and colleagues⁷⁸⁰ demonstrated that the etiology of yellow fever was a filterable agent that could be transmitted through the bite of a mosquito, confirming the postulates of Carlos Finlay. YFV was the first flavivirus isolated (in 1927) and the first to be propagated *in vitro*.^{790,820} These advances led remarkably rapidly to the development of an effective YFV vaccine that remains in use today.⁵⁶⁹ Experiments with the louping ill virus (LIV) in 1931 established that ticks also were capable of transmitting viruses associated with human disease.¹¹⁹ The discovery that antisera raised against some, but not all, viruses that caused similar diseases (e.g., encephalitis) cross-reacted with heterologous viruses provided a method to investigate the relatedness of flaviviruses.^{126–128,876} This was refined further with the development of a standardized hemagglutination inhibition test that allowed classification of 10 different flaviviruses and distinguished them from alphaviruses.¹²⁷ These two groups of viruses were referred to thereafter as group A and B arboviruses, respectively. The first full-length flavivirus genome (YFV) were sequenced in 1985 by Charles Rice and colleagues.⁶⁸⁰ Subsequent advances in the molecular genetics of flaviviruses have increased our understanding of the relationships between viruses that was originally revealed by serology (described below). Seventy-three viruses of the *Flavivirus* genus (classified as 53 distinct species) have since been defined (<http://www.ICTVonline.org/index.asp>).

FLAVIVIRUS DIVERSITY, EVOLUTION, AND DISTRIBUTION

Molecular Phylogeny

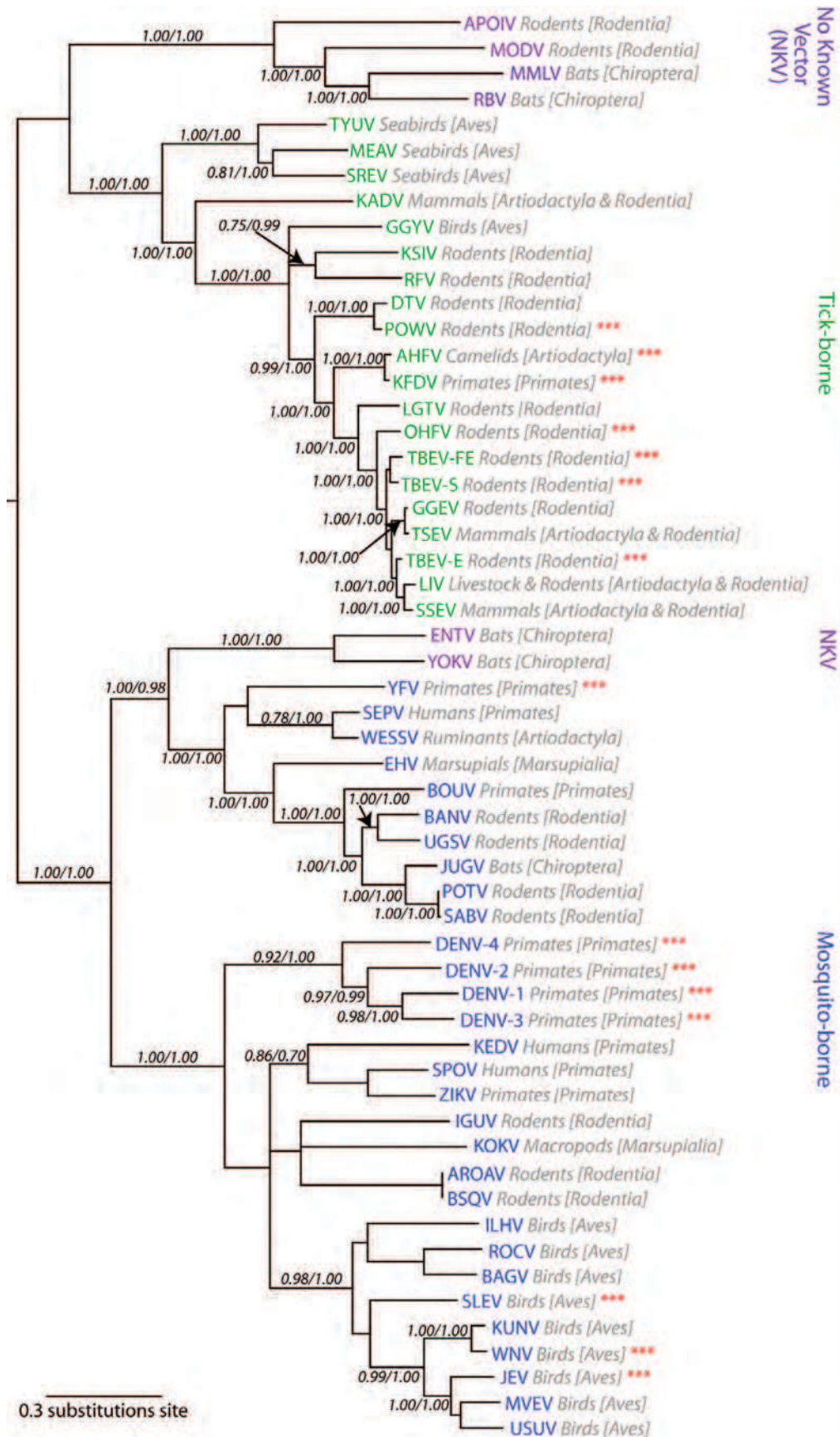
Phylogenetic relationships among members of the flavivirus genus have been established through the analysis of individual genes, and, more recently, the entire open reading frame of the genome.^{73,160,248,274,275,402,435,910} Analysis of the phylogenetic tree of flaviviruses with respect to key features of the biology and ecology of these viruses has proven insightful.^{248,271} Three groups of viruses are defined based on their mode of transmission: tick-borne flaviviruses (TBFVs), mosquito-borne flaviviruses (MBFVs), and those flaviviruses with no known vector (NKV). The earliest divergence from a monophyletic origin separates flaviviruses based on their mode of transmission.^{435,528} One lineage arising from the earliest branch of the phylogenetic tree contains viruses transmitted by ticks and two groups of NKV viruses (Fig. 26.1). The second includes the mosquito-borne viruses and the Entebbe bat virus (ENTV) group of NKV viruses.⁴⁰² The MBFVs are grouped further as a function of their association with mosquitoes of the *Aedes* and *Culex* genera. Viruses in these two clades cause hemorrhagic disease and encephalitis in humans and livestock, respectively. Although many of the viruses in the *Culex* clade infect avian hosts, *Aedes* viruses generally do not. Conversely, *Culex* viruses are not maintained in nature in infection cycles involving primates.

The tick-borne flaviviruses include 12 species divided into three groups^{274,402} (Fig. 26.1). The largest group of TBFVs is associated with mammalian hosts (typically rodents), and includes viruses that cause encephalitis (e.g., tick-borne encephalitis viruses [TBEVs]) and hemorrhagic fever (Omsk hemorrhagic fever virus [OHFV] and Kyasanur Forest disease virus [KFDV]) in humans. In addition, three species of mammalian TBFVs have not been associated with disease (Royal Farm virus [RFV], Karshi virus [KSIV], and Gadgets Gully virus [GGYV]). Viruses of the TBEV serocomplex are thought to represent a continuous evolutionary cline (a genetic gradient) that originated in Africa and moved from east to west across the Northern Hemisphere; the genetic distance between viruses in the mammalian tick-borne group correlates with increases in geographic distance.^{274,910} This is reflected by the asymmetric branching pattern of the phylogenetic tree of the

mammalian viruses⁹¹¹ (Fig. 26.1). The evolution of RFV, KSIV, and GGYV is not associated with this TBEV serocomplex cline, nor understood. This second group of TBFVs replicates within seabirds and ornithophilic ticks but does not cause disease in humans. These viruses have a broad geographic range that presumably reflects the migratory patterns of their avian hosts.^{271,274} The sole member of the final group of TBFVs is the Kadam virus (KADV). KADV is found in Africa and is typically associated with livestock.²⁷¹ An understanding of the relationship between KADV and other members of the TBFVs has evolved. Although these viruses have been assigned to both the mammalian or sea bird groups of flaviviruses, a more recent analysis of the complete coding sequence of TBFVs places this virus in its own group, which is supported by unique features of its envelope protein and the fact that it encodes a polyprotein that is smaller than the rest of the TBFVs.²⁷⁴

MBFVs diverged early into two lineages; viruses within each of these lineages are subdivided based on their association with mosquitoes of the *Aedes* genus or *Culex* genus^{275,402} (Fig. 26.1). Viruses of the *Aedes* clade are a paraphyletic group thought to predate and give rise to the *Culex* viruses.²⁷¹ One branch of the MBFV portion of the phylogenetic tree includes viruses of the YFV group, the recently proposed Edge Hill virus (EHV) group, and two NKV viruses, discussed below. The YFV group includes Wesselsbron virus (WESSV), Sepik virus (SEPV), and YFV. WESSV is a veterinary pathogen transmitted by *Aedes* mosquitoes that causes a nonfatal febrile illness in humans. Very little is known about the clinical significance and vector biology of SEPV infection. Both SEPV and WESSV are found in Africa and Asia.²⁷⁵ The seven viruses of the EHV group are transmitted predominantly by *Aedes* mosquitoes, are found primarily in Africa (except for EHV, which is present in Australia), and share the unique property of encoding five (instead of six) disulfide bridges in the envelope glycoprotein. Human cases have been associated only with Banzi virus infection (BANV).²⁷⁵ The second branch of the MBFV phylogenetic tree contains dengue viruses (DENVs), which are transmitted by *Aedes* mosquitoes, a large group of viruses vectored by *Culex* mosquitoes (e.g., Japanese encephalitis virus [JEV], West Nile virus [WNV]), and a group of *Aedes*-vectored viruses closely related to the *Culex* flaviviruses (e.g., Spondweni virus, SPOV).⁴⁰² The diversity of viruses in the JEV serocomplex and DENV is described in detail below.

FIGURE 26.1. Phylogenetic tree of viruses in the genus *Flavivirus* reveals the evolutionary relationships among viruses transmitted by different vectors. A maximum-likelihood tree was generated using the complete polyprotein sequence of the indicated flaviviruses as detailed by Kitchen and colleagues.⁴⁰² The viral taxa are abbreviated and colored according to their mode of transmission. The host reservoir for each virus is indicated in gray. Viruses that frequently cause disease in humans are indicated with red asterisks. APOIV, Apoi virus; MODV, Modoc virus; MMLV, Montana myotis leukoencephalitis virus; RBV, Rio Bravo virus; TYUV, Tyulenyi virus; MEAV, Meaban virus; SREV, Saumaurez Reef virus; KADV, Kadam virus; GGYV, Gadgets Gully virus; KSIV, Karshi virus; RFV, Royal Farm virus; DTV, Deer tick virus; POWV, Powassan virus; AHFV, Alkhurma hemorrhagic fever virus; KFDV, Kyasanur Forest disease virus; LGTV, Langat virus (LGTV); OHFV, Omsk hemorrhagic fever virus; TBEV-FE, Tick-borne encephalitis virus-far eastern subtype; TBEV-S, Tick-borne encephalitis virus-Siberian subtype; GGEV, Greek goat encephalomyelitis virus; TSEV, Turkish sheep encephalitis virus; TBEV-E, Tick-borne encephalitis virus-European subtype; LIV, Louping ill virus; SSEV, Spanish sheep encephalomyelitis virus; ENTV, Entebbe bat virus; YOKV, Yokose virus; YFV, Yellow fever virus; SEPV, Sepik virus; WESSV, Wesselsbron virus; EHV, Edge Hill virus; BOUV, Bouboui virus; BANV, Banzi virus; UGSV, Uganda S virus; JUGV, Jugra virus; POTV, Potiskum virus; SABV, Saboya virus; DENV-4, Dengue virus serotype 4; DENV-2, Dengue virus serotype 2; DENV-1, Dengue virus serotype 1; DENV-3, Dengue virus serotype 3; KEDV, Kedougou virus; SPOV, Spondweni virus; ZIKV, Zika virus; IGUV, Iguape virus; KOKV, Kokobera virus; AROAV, Aroa virus; BSQV, Bussuquara virus; ILHV, Ilheus virus; ROCV, Rocio virus; BAGV, Bagaza virus; SLEV, St. Louis encephalitis virus; KUNV, Kunjin virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; and USUV, Usutu virus. The tree was kindly provided by Dr. Edward Holmes and modified with permission.



By comparison to the vector-borne flaviviruses, relatively little is known about the NKV viruses. These viruses are most commonly found in rodents or bats, in which they do not appear to cause disease or a high viremia. NKV viruses of rodents are found only in the New World, whereas those capable of infecting bats are found in both the Old and New Worlds.²⁷⁰ Although the majority of NKV viruses (12 species) diverged with the TBFVs, two species of bat-associated viruses appear to have evolved from a lineage transmitted by mosquitoes and then lost this trait secondarily (Yokose virus [YOKV] and ENTV)^{270,434,806} (Fig. 26.1). These viruses are most similar to the *Aedes* species–vectored viruses of the YFV group. Of interest, studies with chimeric YFV and DENV engineered to express the envelope genes of Modoc virus (MODV) suggest that the host-restriction of NKV viruses may be at a postentry step of the virus life cycle.¹³⁸

Evolution

Many flaviviruses are transmitted by insect vectors, which is a unique feature of the genus not shared by the pestiviruses and hepaciviruses of the *Flaviviridae* family. One hypothesis is that flaviviruses evolved from an ancestral virus that was not vectored. Phylogenetic analysis of NS5 sequences supports this notion^{271,435}; these studies indicate that the majority of NKV viruses diverged from a lineage that gave rise to vector-borne viruses early in the evolution of flaviviruses. In this context,

MBFVs arose from the TBFV lineage. An alternative hypothesis, supported by the analysis of NS3 and complete genome sequences, is that MBFVs diverged first and then gave rise to TBFVs and the majority of NVK viruses^{73,275,274,402} (Fig. 26.1). Flaviviruses likely originated in the Old World during the last 10,000 years since the last ice age.^{160,270} All of the TBFVs, with the exception of Powassan virus (POWV), are found in the Old World.²⁷¹ POWV is found in far eastern Russia and Canada. Deer tick virus (DTV) is a subtype of POWV isolated in New England.⁸¹³ That significant speciation of POWV in the New World has not yet occurred suggests a relatively recent introduction. The most divergent MBFVs are found in the Old World.²⁷¹ The earliest lineages of the *Aedes* clade viruses are thought to originate in Africa; only DENV and YFV are now found in the New World.²⁷¹ Finally, the distribution of the NKV viruses also appears consistent with an Old World origin. Viruses that infect bats are found in either the Old or New World, whereas those associated with rodents occupy restricted niches in the New World. It is possible that bats played an important role in the introduction of these viruses into the New World.²⁴⁸

Inspection of the phylogenetic trees for MBFV and TBFVs revealed striking differences that may reflect distinct biology of the vectors that transmit them. The portion of the tree that includes the TBEV group is highly asymmetric, with a step-wise branching pattern associated with the evolutionary cline of these

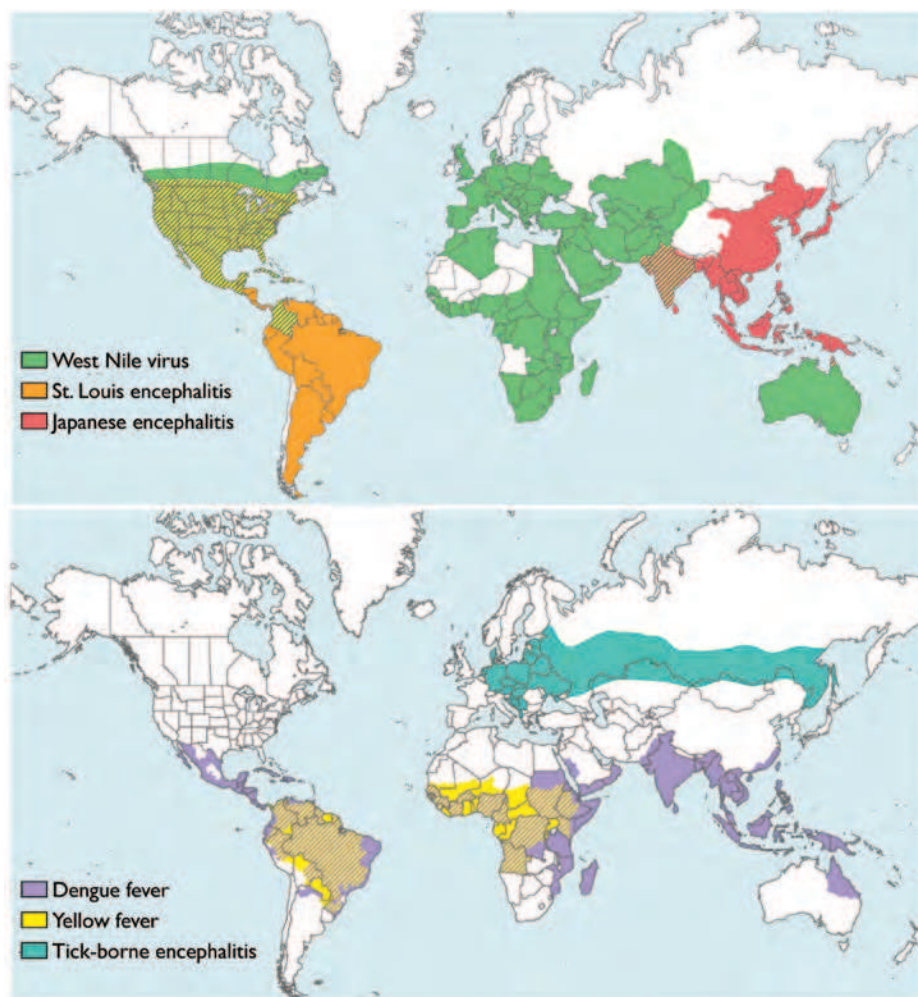


FIGURE 26.2. Global distribution of flaviviruses. The global distribution of flaviviruses with significant impact on global health.

viruses.^{910,911} In contrast, the phylogenetic tree for MBFVs is more balanced and does not result in a greater number of branches than predicted by chance. MBFVs evolution appears to involve slow phases punctuated by periods of rapid change and diversification. The last two centuries have been characterized by extensive cladogenesis (change that results in new branches on the phylogenetic tree) for the DENV and JEV complexes.

Overall, TBFVs appear to have evolved more slowly than the MBFVs (0.56 times the rate of mosquito-borne viruses).⁹¹¹ Several aspects of tick biology may limit the number of replication cycles and dispersal of TBFVs in nature that contribute to clinal pattern and modest rate of evolution: (a) ticks live for relatively long periods (2 to 7 years), (b) ticks feed only three times during their lifespan, (c) ticks may transmit viruses to other ticks during co-feeding, minimizing the importance of the vertebrate host for increasing replication cycles, and (d) ticks are relatively immobile unless carried by a vertebrate host. By comparison, MBFVs are transmitted by vectors with the capacity for wider distributions and are quickly replicated through many cycles in the mosquito vector and vertebrate hosts. MBFVs are found in overlapping distributions (e.g., the four serotypes of DENV), whereas TBFVs characteristically occupy defined and nonoverlapping niches.²⁷⁰

Global Distribution

Flaviviruses are found on six different continents where they are responsible for endemic and epidemic disease each year (Fig. 26.2). The geographic distribution of flaviviruses has proven quite dynamic, enabling emergence in new geographic areas and increased disease incidence.⁵¹⁴ For example, since its introduction into the Western Hemisphere in 1999, it took only 4 years for the WNV to spread across the United States, where it is now an endemic pathogen. The contribution of human activity toward the spread of flaviviruses is significant.²⁷¹ Prior to the development of rapid intercontinental transportation, the movement of flaviviruses between the Old World and New World was uncommon. YFV (and potentially the *Aedes aegypti* mosquito) were introduced into the Americas during the slave trade 300–400 years ago. Importation of YFV by travelers into nonendemic areas, and DENV and WNV into the New World, has been described.¹¹⁰

FLAVIVIRUS COMPOSITION AND ANTIGENIC STRUCTURE

Structure

Flaviviruses are small spherical particles composed of three structural proteins, an ~11 kb positive-sense genomic RNA, and a lipid envelope. The envelope (E) protein is a ~53 kD structural protein that functions in multiple steps of the virus life cycle including assembly, budding, attachment to target cells, and viral membrane fusion (reviewed by⁵⁹¹). The E protein is also the major target of neutralizing antibodies (reviewed by^{647,688}). The structure of the ectodomain of the E protein has been determined at the atomic level for several flaviviruses.^{557,559,591,623,647,679,918} Flavivirus E protein is an elongated, type II viral fusion protein composed of three distinct domains connected by short flexible hinges (Fig. 26.3AB). Domain I (E-DI) is an eight-stranded β -barrel located in the center of the E protein molecule. This central domain contains two of the six disulfide bonds present

in the E protein structure, as well as a site for the addition of an asparagine-linked (N-linked) carbohydrate. Domain II (E-DII) is an elongated structure that mediates dimerization of E proteins on the mature virion. A highly conserved glycine-rich loop composed of 13 amino acids located at the tip of E-DII is thought to insert into the membranes of target cells.^{10,98,558} In the context of the dimer, the E-DII fusion loop (E-DII-FL) sits in a hydrophobic pocket formed at the interface of E-DI and domain III (E-DIII). The introduction of mutations into the fusion loop blocks fusion between virions and the membranes of synthetic liposomes.¹⁶² For some flaviviruses, E-DII contains a second N-linked glycosylation site. E-DIII adopts an immunoglobulin-like fold at the carboxy-terminus (C-terminus) of the E protein ectodomain and is stabilized by a single disulfide bridge. E-DIII is the portion of the E protein that projects farthest from the surface of the mature virion and is speculated to contain binding sites for cellular factors involved in virus attachment and entry.^{71,153,455,520,679} Many of the most potent neutralizing antibodies characterized to date recognize epitopes on E-DIII (discussed below). The E protein is tethered to the viral membrane by a helical stem (the stem anchor) and two transmembrane domains.^{11,591,915}

The precursor to membrane protein (prM) is a ~20 kD protein that facilitates E protein folding and trafficking.⁵⁰¹ In addition, interactions with the E protein prevent the adventitious fusion of the virus during egress.³²¹ Virion maturation is regulated by the proteolytic cleavage of prM, which results in the formation of a “pr” protein that is ultimately released from the virion and an ~8 kD membrane-associated M peptide. The structure of the “pr” peptide has been determined at the atomic level and is composed of seven β strands held together by three disulfide bonds (Fig. 26.4A).⁴⁶⁶ prM interacts with the E protein near at the tip of E-DII adjacent to the fusion loop.^{916,917} prM is anchored into the viral membrane via two transmembrane domains.^{591,915} Recent studies suggest that antibodies specific for prM are commonly produced *in vivo*.^{61,191}

Flaviviruses assemble on virus-induced membranes derived from the endoplasmic reticulum (ER).^{353,502,513,861} Virus particles bud into the lumen of these membrane structures as immature virions on which E and prM proteins form heterotrimeric spikes that project from the surface of the virion. Within each spike, the prM protein is located at the tip of the trimer. Immature virions incorporate 60 trimers arranged with icosahedral symmetry (Fig. 26.4B).^{907,916,917} Transit of the immature virion through the mildly acidic compartments of the trans-Golgi network (TGN) triggers an extensive rearrangement of E proteins on the immature virion; the lower pH induces a structural transition such that E proteins lie flat as antiparallel dimers on the surface of the virion, analogous to the structure of the mature virion discussed below.⁹⁰⁷ Under acidic conditions, prM remains associated with the fusion loop on this structure and protrudes from the surface of an otherwise smooth virus particle. This pH-dependent conformational change increases the susceptibility of prM for a furin-like serine protease.⁷⁷⁷ Cleavage of prM is the hallmark of the virion maturation process, and is a required step in the virus life cycle.²²³ Release of the virion into the neutral conditions of the extracellular milieu results in the dissociation of the pr peptide.^{466,907} Mature virions are relatively smooth virus particles that incorporate 180 copies of the E protein arranged with an unusual herringbone pseudo-T = 3 icosahedral symmetry^{425,590,591} (Fig. 26.5A).

FIGURE 26.3. Structure of the flavivirus E protein.

The envelope (E) proteins of flaviviruses are elongated class II viral fusion proteins composed of three structurally distinct domains. **A:** Ribbon diagram of the Dengue virus (DENV) E protein dimer as seen from the top; individual domains of each E protein monomer are indicated (domain I, E-DI, red; domain II, E-DII, yellow; and domain III, E-DIII, blue). The fusion loop at the tip of E-DII is shown in green. **B:** DENV E protein as viewed from the side. The stem anchor connecting the E protein to the viral membrane is not shown. The two N-linked carbohydrate modifications at positions Asn67 and Asn154 are shown. **C:** Structure of the West Nile virus (WNV) E-DIII highlighting amino acids that form the epitope recognized by the type-specific neutralizing mAb E16. Residues identified in structural studies as antibody contacts are shown in brown; the side chains of residues demonstrated to be critical for antibody binding are shown and labeled. **D:** Structure of the DENV E-DIII highlighting the epitope recognized by the group-reactive mAb 1A1D-2. Residues identified in structural studies as antibody contacts are shown in brown; the side chains of residues demonstrated to be critical for antibody binding are shown and labeled. We thank Mr. Phong Lee (National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]) for preparation of the figure.

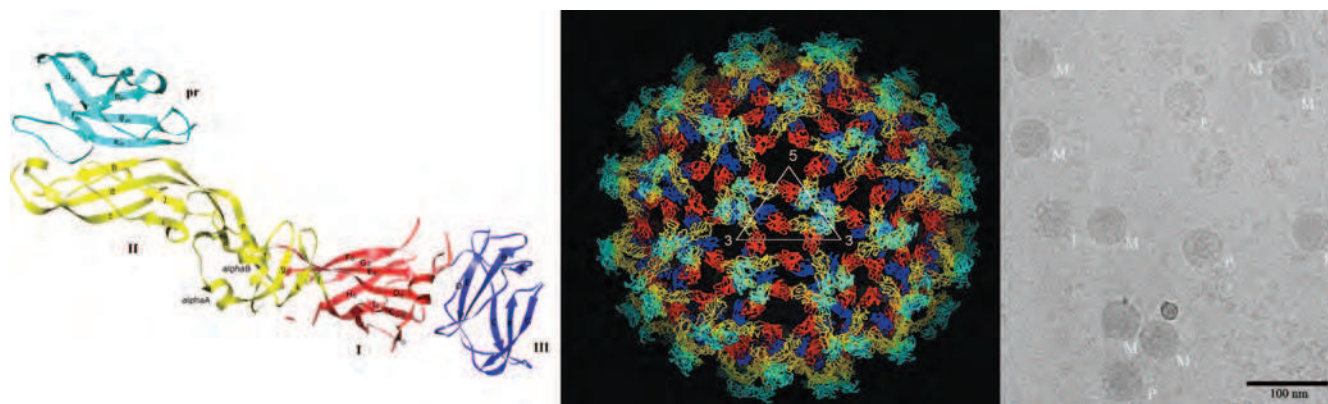
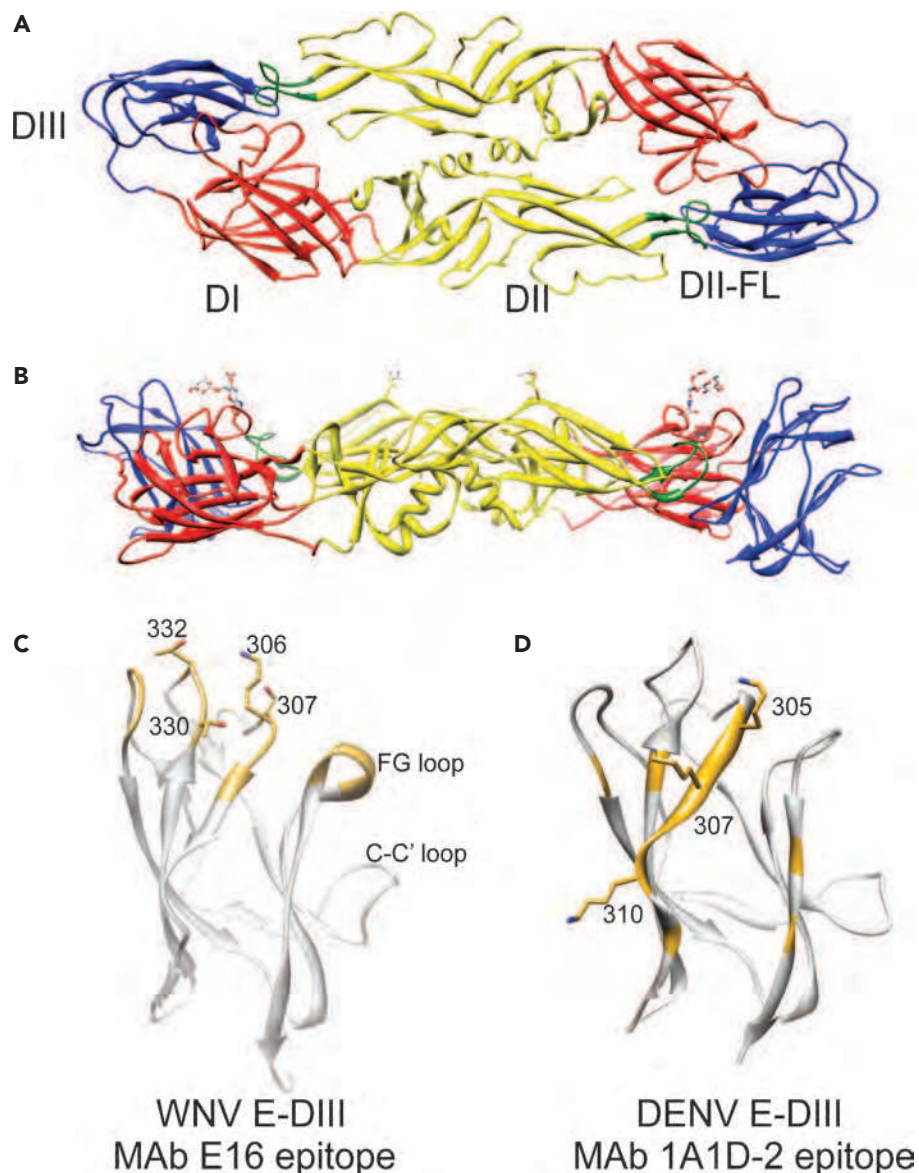


FIGURE 26.4. The immature flavivirus virion. The structure of prM and the immature flavivirus virion. **A:** Ribbon representation of the Dengue virus (DENV) pr peptide complexed with the DENV E protein. The pr peptide is shown in cyan. Domains I, II, and III of the E protein are shown in red, yellow, and blue, respectively. **B:** Surface-shaded representation of the immature DENV virion at neutral pH. **C:** Cryo-electron microscopy visualization of extracellular DENV reveals the heterogeneity of virions released from mosquito cell cultures. Immature (I) and mature (M) virions are indicated. Partially mature virions (P) characterized by the appearance of smooth and spiky features on the same virion comprise a significant fraction of the virions released from cells.

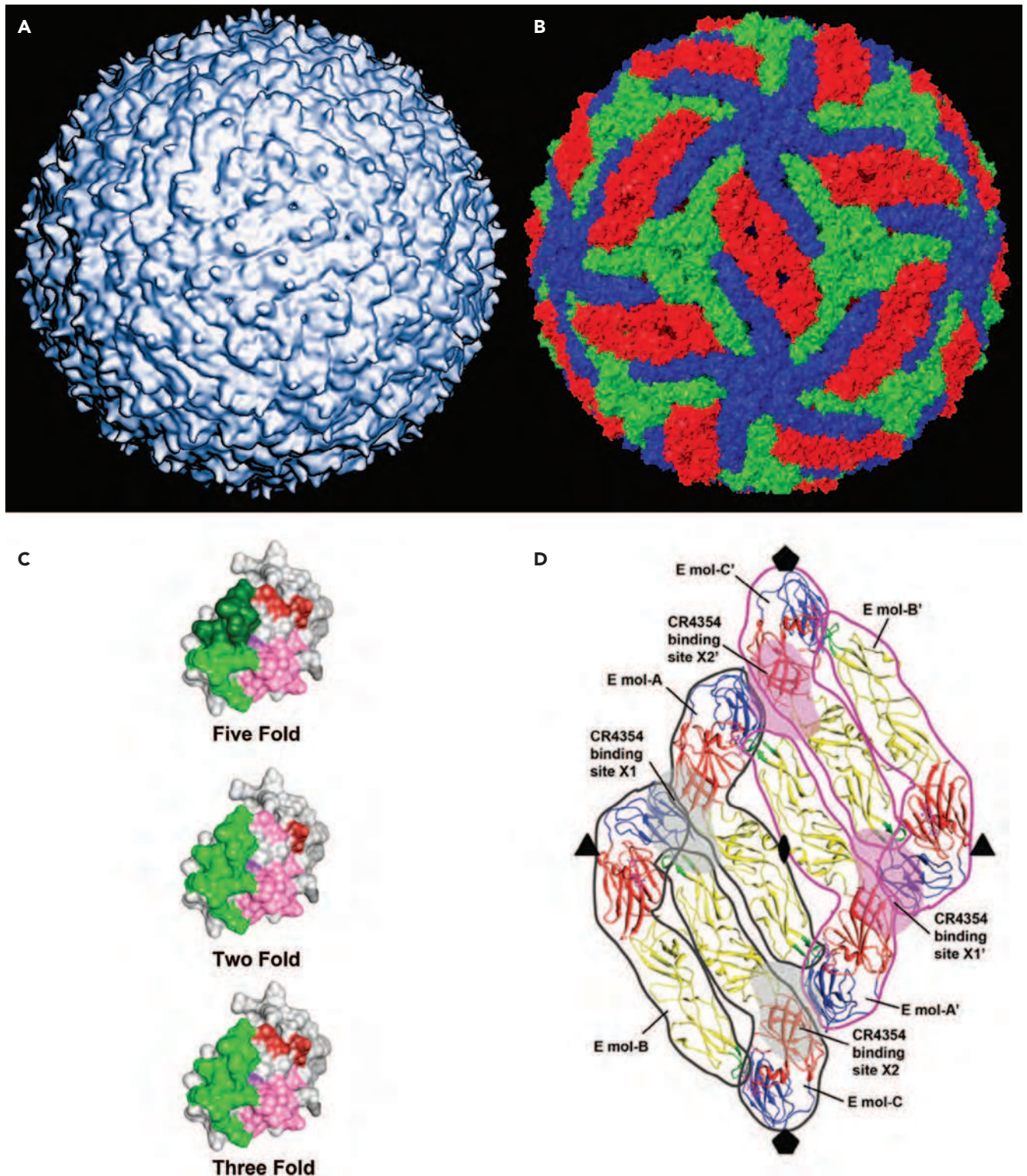


FIGURE 26.5. The arrangement of E proteins on the mature flavivirus increases the complexity of the antigenic surface of the virus particle. **A:** Structure of the mature DENV virion. **B:** Structure of the mature virion highlighting the association of E proteins proximal to the two-, three-, and five-fold symmetry axis of the virion, shown in *red*, *green*, and *blue*, respectively. **C:** Epitope accessibility varies as a function of the location of a particular E protein on the surface of the pseudo-icosahedral mature virion. Residues important for the binding of mAb E16 are highlighted in *green* for E proteins of each symmetry environment. The steric conflicts that prevent binding of E16 to E proteins proximal to the fivefold symmetry axis are shown in *dark green*. Accessibility of amino acids involved in 1A1D-2 binding are shown in *pink*; steric conflicts that prevent binding to E proteins on the mature virion are shown in *red*. **D:** The complex epitope of mAb 4354 composed of multiple E proteins is shown. Individual domains of each E protein monomer are indicated (domain I, E-DI, *red*; domain II, E-DII, *yellow*; and domain III, E-DIII, *blue*).

Each virion is composed of 30 sets of three antiparallel dimers. In this configuration, E proteins exist in one of three chemically distinct dimer environments defined by their proximity to the two-, three-, or fivefold symmetry axis (Fig. 26.5B).

The Antigenic Surface

Flaviviruses were first classified according to serologic reactivity.^{127,188} These early studies generally agreed with approaches that group viruses as a function of genetic relatedness (discussed above, Fig. 26.1).⁵²² Flavivirus-reactive antibodies are classified as a function of their capacity to discriminate between the antigens of viruses within and between related serologic groups of viruses.⁸²⁹ For example, monoclonal antibodies that react with DENV may be type-specific (a single DENV serotype), subcomplex-specific (more than one DENV serotype), complex reactive (all DENV viruses), or flavivirus-group reactive (multiple flaviviruses).³²² Type-specific mAbs provided a rapid and specific method for distinguishing between antigenically related viruses.^{324,572} Furthermore, recent studies indicate that antibodies differentially recognize different genotypes within a given serotype.^{99,751,852}

E Protein Epitopes Recognized by Neutralizing Antibodies

The majority of neutralizing antibodies bind epitopes on the E protein (reviewed in⁶⁸⁸). Early studies distinguished epitopes on the E protein based on the biochemical and functional properties of mAbs including a capacity to bind and compete for viral antigens, neutralize virus, and inhibit hemagglutination of red blood cells.^{251,319,322,394,395,411,642,690} An early model of the antigenic structure of the E protein was proposed by Heinz and colleagues that featured three nonoverlapping antigenic domains (A, B, and C); more refined clusters of epitopes within each domain were based on functional differences.^{286,318} These studies also identified a small number of antibodies that bound the E protein outside of these domains. This advance not only provided a framework to classify antibodies based on their epitope, but also provided context to consider relationships between epitope location and the functional properties of mAbs.⁶⁸⁹ Antigenic domain A epitopes were recognized by cross- and group-reactive antibodies, domain B epitopes were typically type specific, and domain C epitopes were recognized by subtype-specific mAbs.^{286,521} These epitopes were subsequently shown to correspond to E-DII, E-DIII, and E-DI, respectively.⁶⁷⁹

All three domains of the E protein are recognized by neutralizing antibodies, albeit with widely varying potency. Epitopes of some of the most well-characterized antibodies to date are described below, although it should be anticipated that this list will expand as greater insight to the complexity of the antigenic surface of the virion is obtained:

E-DIII-LR

Many of the most potent neutralizing antibodies characterized to date bind epitopes on E-DIII. Potently neutralizing antibodies that bind an epitope on the domain III lateral ridge (E-DIII-LR) of several flaviviruses have been identified.^{52,150,170,279,627,647,689,708,751} mAb E16 is a type-specific WNV-reactive mAb that neutralizes at picomolar concentrations *in vitro* and protects mice from lethal challenge when administered 5 days after infection.^{581,582,627,648} The structure of E16

bound to E-DIII has been determined and revealed a binding footprint composed of four discontinuous loops centered on amino acids of the BC loop and amino-terminal region (positions 306, 307, 330, and 332)⁶²⁴ (see Fig. 26.3C). Type-specific neutralizing antibodies against DENV-2 have been mapped to an epitope composed of multiple regions of the upper lateral surface of E-DIII, including the FG loop.^{279,360,380,794,795} The binding of many of these antibodies to DIII is also sensitive to amino acid substitutions on the BC loop, C-C' loop, the amino-terminal region (residue 301), or the A-strand (e.g., residue 304); the latter structure is also recognized by antibodies that neutralize DENV with subcomplex specificity, as discussed below. For example, the highly characterized mAb 3H5 recognizes an epitope composed of residues on both the FG loops and the A strand.⁷⁹⁵ Mapping experiments with type-specific DENV-1,⁷⁵¹ DENV-3,^{99,852} JEV,⁸⁹¹ and TBEV⁷⁸⁶ antibodies have identified similar epitopes.

E-DIII A-STRAND

Antibodies with a pattern of complex and subcomplex reactivity have been mapped to the A-strand of E-DIII.^{497,671,795,824} mAb 1A1D-2 potently neutralizes DENV-1, DENV-2, and DENV-3, but fails to bind DENV-4 viruses. The structure of mAb 1A1D-2 bound to E-DIII was solved and revealed that this antibody binds an epitope on the A strand and is sensitive to mutation of DENV residues 305, 307, and 310 (see Fig. 26.3D).⁴⁹⁷ In addition, these studies identified residues in the G strand that play a role in antibody binding, consistent with mapping studies of other complex- and group-reactive antibodies.^{280,489,751,824}

E-DII-FL

Antibodies that bind the fusion loop of E-DII are highly cross-reactive.^{168,171,629,787} Although mutation of conserved fusion loop residues reduces antibody binding, other adjacent structures also may contribute to the fine specificity of antibody binding and the functional properties of these antibodies.^{266,629} The structure of the WNV fusion loop-reactive antibody mAb E53 bound to soluble E proteins has been determined.¹⁴⁹ Residues shown to be important antibody contacts include those of the fusion loop (residues 104–107, and 109–110) as well as residues of the BC-loop of E-DII.⁶²⁹ The fusion loop epitope is also poorly accessible on the mature virion, as discussed in detail below.^{629,787}

E-DI

Antibodies that bind E-DI have also been characterized. mAb 5H2 is a type-specific antibody that neutralizes DENV-4. Neutralization escape studies mapped 5H2 binding to an epitope that includes residue 174 of E-DI.⁴⁴⁵ DI-reactive antibodies that bind WNV (E121; residues 175, 191, 193, and 194), DENV2 (mAb DV2–48, residue 177), and TBEV (IC3 and i2, residues D181 and K171, respectively) also have been characterized.^{338,521,629,794} The recently described WNV mAb CR4354 that binds a complex epitope that includes the linker between E-DI and E-DII will be described in detail below.³⁸⁴

Antibodies That Bind the prM Protein

Antibodies that react with the prM protein have been described.^{61,121,191,229,844} Generally, these antibodies are characterized by low neutralizing activity *in vitro*. Recent studies suggest that anti-prM antibodies are elicited frequently *in vivo*,

and may contribute to the pathogenesis of DENV infection as discussed below.^{191,685} Human monoclonal antibodies to WNV prM protein have been isolated and mapped to residues V19, T20, T24, and L33.¹²¹

Complexities of Antibody Recognition of the Virion

The complex and dynamic arrangement of the E proteins on the surface of flaviviruses complicates an understanding of how antibodies interact with flaviviruses. E proteins exist on the mature virus particle in different chemical environments (Fig. 26.5B; defined in relation to the two-, three-, and five-fold symmetry axes of the pseudo-icosahedral particle), which impacts antibody recognition. Amino acids involved in antibody recognition may be differentially accessible for antibody binding depending on their location on the virus particle.^{624,629,787} As mentioned above, the WNV mAb E16 binds a cluster of residues on the upper lateral surface of E-DIII.⁶²⁴ However, this epitope is not uniformly accessible for antibody binding on all E proteins on the mature virus; steric constraints imposed by the tight packing of E-DIII on E proteins at the fivefold axis of symmetry prevent antibody binding to these molecules (Fig. 26.5C).^{383,624} Therefore, although the mature virion incorporates 180 copies of the E protein, a maximum of 120 antibodies physically can bind the virus particle. This is not an unusual feature of this antibody as none of the antibodies studied to date using structural methods appear capable of binding all E proteins on the intact mature virus particle. In fact, the molecular basis for recognition by many antibodies cannot be explained using static models of virion structure.

EPITOPES CAN BE COMPOSED OF MORE THAN ONE PROTEIN ON THE SURFACE OF THE VIRION

Virions contain 180 individual E proteins. To date, most of the well-characterized antibodies are capable of binding monomeric E proteins, indicating their epitopes are composed of residues contained within a single E protein molecule. However, complex epitopes composed of contact residues from adjacent E proteins have been described.^{168,384,521} mAb CR4354 is a human antibody that binds the hinge between E-DI and E-DII. Mapping studies using recombinant forms of the E protein failed to identify the CR4354 epitope because this antibody was unable to bind soluble forms of the E protein. A loss-of-function substitution at position K136 was defined by neutralization escape studies.⁸⁵⁰ Cryoelectron microscopic reconstructions of CR4354 Fab fragments bound to the mature virion revealed a complex epitope composed of residues on neighboring E proteins (Fig. 26.5D).³⁸⁴

INCOMPLETE VIRION MATURATION IMPACTS ANTIBODY-MEDIATED NEUTRALIZATION

Cleavage of prM is a required step in the flavivirus life cycle; mutation of the RRXR/S motif in prM recognized by furin-like proteases renders TBEV noninfectious.²²³ However, biochemical analysis of preparations of flaviviruses released from cells indicated that a substantial amount of prM may remain uncleaved. Recent studies demonstrate that more than 90% of DENV virions could be precipitated with anti-prM antibodies.³⁶⁸ Electron microscopy studies identified virus particles with structural features of both mature and immature virions (hereafter referred to as “partially mature virions”) (Fig. 26.4C).⁶⁵³

Several lines of evidence suggest that partially mature virions are infectious. Virions produced in the presence of ammonium chloride display a reduced sensitivity to inactivation when exposed to acid, presumably because pH-mediated changes in the conformation of E protein are reversible when complexed with prM.²⁸⁵ In addition, the carbohydrate on prM can mediate attachment of the lineage II 956 strain of WNV (which lacks an N-linked carbohydrate on the E protein) onto cells expressing the c-type lectin CD209L.¹⁸³ Although these studies demonstrate that virions containing prM may be infectious, the stoichiometric requirements of prM cleavage have not yet been determined.

The presence of partially mature secreted virions impacts antibody recognition in at least two ways. Increasing the efficiency of virion maturation resulted in a marked reduction in the sensitivity of WNV to neutralization by antibodies that bind several structurally distinct epitopes, including the DI-LR and DII-FL epitopes recognized by mAbs E121 and E53, respectively.⁶⁰⁷ Conversely, decreasing the extent of virion maturation enhanced neutralization by these mAbs. An analysis of the sensitivity of polyclonal antibody elicited by vaccination with two distinct candidate WNV vaccines revealed maturation state-dependent changes in neutralization potency in roughly half the volunteers.⁶⁰⁷ The structural basis for this pattern of recognition has been investigated.¹⁴⁹ mAb E53 does not efficiently bind the E protein on mature virions due to poor accessibility of the fusion loop epitope on the mature virus particle. In addition to modulating the potency of neutralizing antibodies, uncleaved prM on infectious virus particles may interact directly with antibodies, resulting in enhanced infection of Fcγ-receptor bearing cells *in vitro* and *in vivo* as discussed below.^{61,191,347,685,912}

IMPACT OF STRUCTURAL DYNAMICS OF THE VIRUS PARTICLE

Flaviviruses present a complex and dynamic antigenic surface to the immune system that is not fully captured by the static models of virion structure. It has long been appreciated that proteins are in constant motion and sample an ensemble of conformations at equilibrium.⁸⁴ Proteins incorporated into virus particles also are structurally dynamic.^{361,888} Virus “breathing” has been demonstrated for several unrelated classes of viruses,^{88,459} and may affect antibody recognition.^{467,905} As an example, the accessibility of the A-strand epitope on the mature virion is limited by steric constraints arising from the arrangement of E proteins on the virus particle. The binding of Fab fragments of the subcomplex-reactive A-strand-specific mAb 1A1D-2 to mature DENV virions was shown to be temperature dependent; significant Fab binding was observed only after incubation at 37°C.⁴⁹⁷ Cryoelectron microscopic reconstruction of mature DENV bound by the 1A1D-2 Fab revealed significant changes in the arrangement and orientation of E proteins on the surface of the virus particle. These results suggested that the binding of 1A1D-2 stabilized the E proteins in a state distinct from the herringbone arrangement found on mature virions. More recent studies suggest the impact of viral dynamics on antibody-mediated neutralization is widespread among antibodies of differing specificity. Analysis of the neutralizing activity of a panel of mAbs specific for structurally distinct epitopes revealed a time- and temperature-dependent aspect of neutralization of WNV and DENV attributed to changes in epitope accessibility arising from the dynamic

motion of E proteins on the virion.²¹⁰ Given sufficient time, even epitopes that are poorly accessible in all three symmetry axes of the mature virion may support some level of neutralization. The scope of the structural conformations sampled by flaviviruses at equilibrium is not yet understood. Changes in the configuration of E proteins on dynamic virions have the potential to affect several aspects of antibody binding (functional affinity, bivalency, antibody binding orientation), and thus, complicates our understanding of the antigenic surface of the flavivirus virion.

Antibodies to NS1 Can Protect *In vivo*

NS1, a protein that is absent from the virion, is secreted at high levels into the extracellular environment during flavivirus infection, predominantly as a hexamer,²³⁸ with significant accumulation (up to 50 µg/ml) in the sera of DENV-infected patients.^{6,28,470,906} In addition, soluble NS1 can bind back to the plasma membrane of cells through an interaction with specific sulfated glycosaminoglycans.²⁹ Furthermore, NS1 is expressed directly on the surface of infected cells, possibly via glycosyl phosphatidyl inositol (GPI) linkage,³⁵⁴ lipid raft association,⁶²¹ or through an as-yet undefined mechanism. Several groups also have generated nonneutralizing, yet protective mAbs against NS1.^{155,156,194,230,323,665,720,721,723-725} Therefore, protection against flavivirus infections *in vivo* does not always correlate with neutralizing activity *in vitro*.^{93,690,722} Beyond direct virus neutralization, antibody binding to virions or virus-infected cells can trigger protective Fc-dependent antiviral activities through complement activation or Fc-γ receptor-mediated immune complex clearance mechanisms. Fc-γ receptors can activate or inhibit immune responses depending on their cytoplasmic domain and association with specific signaling molecules.⁶¹⁷ A requirement for Fc effector function has been established for protective anti-NS1 mAbs. NS1 is expressed on the cell surface or secreted into the extracellular space and antagonizes complement control of flavivirus infection by binding the negative regulator factor H or by promoting C4 degradation.^{24,154} Passive transfer of mAbs against NS1 can protect mice against lethal infection by WNV and YFV,^{155,722} and this requires an intact Fc moiety.⁷²⁵ Mechanistic studies using immunodeficient mice demonstrate that protective anti-NS1 mAbs recognize cell surface-associated NS1 and trigger Fc-γ receptor-dependent phagocytosis and clearance of WNV-infected cells.¹⁵⁶

CLINICAL AND PATHOLOGIC SYNDROMES OF THE FLAVIVIRUSES

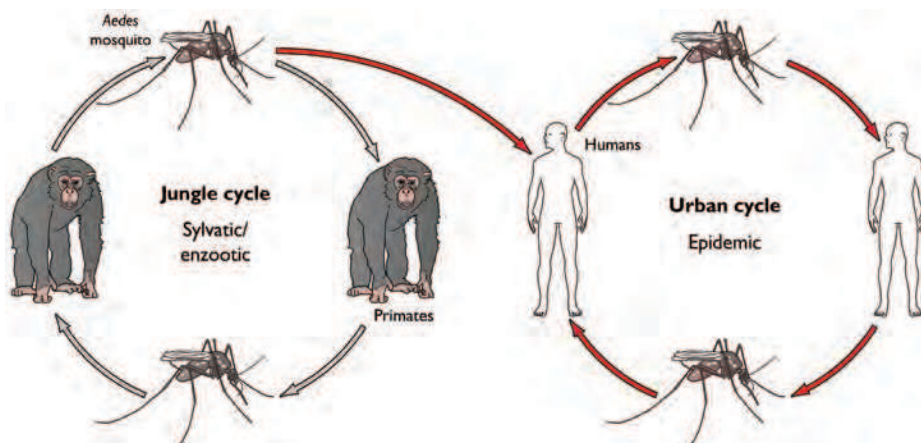
DENGUE VIRUS

History, Global Distribution, and Epidemic Cycle

The natural cycle of epidemic Dengue virus (DENV) infection is between the mosquito vector (*Aedes albopictus* or *Aedes aegypti*) and humans (Fig. 26.6). After mosquito inoculation, DENV infection causes a spectrum of clinical disease ranging from self-limited Dengue fever (DF) to a life-threatening hemorrhagic and capillary leak syndrome (Dengue hemorrhagic fever [DHF]/Dengue shock syndrome [DSS]). Globally, there is significant diversity among DENV strains, including four distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) that differ at the amino acid level in the viral envelope proteins by 25% to 40%. DENV causes an estimated 25 to 100 million infections and 250,000 cases of DHF/DSS per year worldwide, with 2.5 billion people at risk.^{298,562}

Although a dengue-like syndrome may have occurred in China several times during the first millennium AD, the initial description of a DENV epidemic is attributed to Benjamin Rush, a physician in Philadelphia, in his article reporting a febrile outbreak in 1780.⁶⁹⁴ Primary DENV infection and epidemics were common in North America, the Caribbean, Asia, and Australia during the 18th and 19th centuries, presumably due to the widespread ecology of the mosquito vectors. During World War II, DENV spread to and through Southeast Asia. Troop movement and the destruction of the environment and human settlements are believed to have promoted the spread of DENV and their mosquito vectors throughout Southeast Asia and the Western Pacific.⁴³³ Since 1950, the number of people infected has risen steadily, such that today DENV is the leading arthropod-borne viral disease in the world. With the spread and co-circulation of multiple DENV serotypes, secondary infection with heterologous serotypes and epidemic DHF/DSS emerged 50 years ago in Southeast Asia,³¹⁰ and more recently in the Americas in 1981⁴²⁰ and South Asia in 1989.⁵⁵¹ Since the 1950s, epidemics involving thousands of people with multiple DENV serotypes and strains occur annually in multiple parts of the world, including the Americas, Asia, Africa, and Australia, in essence wherever the primary mosquito vector *Aedes aegypti* is present. Indeed, after a recent outbreak of DENV

FIGURE 26.6. Life cycle of Dengue virus (DENV). DENV circulates in nature in two relatively distinct transmission cycles vectored by *Aedes* sp. mosquitoes. DENV infection of humans results in a sufficiently high viremia to support infection of feeding mosquitoes; transmission cycles of DENV do not require an enzootic amplifying host. DENV may also replicate in a sylvatic cycle. Although incompletely understood, the contribution of sylvatic strains of DENV to human infections appears minimal.



in Key West Florida in 2009, a serosurvey conducted by the Centers for Disease Control and Prevention (CDC) reported that 5.4% of households had evidence of recent DENV infection.¹ As a reflection of this, the global incidence of DHF/DSS has increased more than 500-fold, with more than 100 countries affected by outbreaks of dengue.⁴⁴²

DENV Diversity

Globally, there is significant diversity among DENV strains. The four serotypes of DENV (DENV-1, DENV-2, DENV-3, and DENV-4) are genetically distinct but cause similar diseases and share epidemiologic features. All DENV strains are members of the Dengue antigenic complex; inclusion of a strain as DENV is based on antigen cross-reactivity, sequence homology, and genome organization.¹²⁰ The four serotypes of DENV were historically distinguished by limited cross-neutralization or hemagglutination inhibition using serum from infected individuals. Subsequent sequencing analysis revealed that individual serotypes of DENV can differ from one another at the amino acid level significantly, with 30% to 40% variation in the viral envelope proteins. However, within a given serotype, amino acid homology is much greater, with conservation levels at approximately 90% or higher. Therefore, individual DENV serotypes (e.g., DENV-1 versus DENV-4) vary far more than distinct viruses in Japanese encephalitis serocomplex (e.g., WNVs and JEVs vary by 10% to 15% at the amino acid level), which has led some to consider DENV as a group of four different viruses that are linked by serology, epidemiology, and disease pathogenesis. Differences in severity associated with individual serotypes or particular sequences of serotypes in sequential infection have been observed, and it still is unclear whether some serotypes are inherently more pathogenic than others. DENV-2 viruses have been commonly associated with DHF/DSS,^{36,112,821} as are DENV-1 and DENV-3 viruses.^{273,314,551} In comparison, DENV-4 appears more commonly to be clinically mild, although it can cause severe disease.⁶¹⁹

Genetic variation of DENV, however, is not limited to serotype. Geographic variants within a serotype were initially identified by RNase fingerprint assays.^{678,847} Subsequently, nucleic acid sequencing confirmed differences within each serotype, allowing for classification of genotypes that vary further by up to approximately 6% and 3% at the nucleotide and amino acid levels, respectively.^{337,681} DENV genotype classification was originally defined by sequence variation within a given genomic region (e.g., *E* and *NS1* genes). More recent analysis has used high-throughput full genome sequencing technologies to assign phylogenetic classification. Although there remains some dissonance among investigators, most classification schemes include five DENV-1 genotypes, four or five DENV-2 genotypes, four DENV-3 genotypes, and two or three DENV-4 genotypes.^{682,874} Beyond serotype and genotype, two further types of DENV complexity should be mentioned: strain variation and quasispecies. DENV strain variation refers to the limited change that occurs among individual isolates; this was classically described as within a serotype, although as DENV continues to emerge and evolve, variation now occurs within a genotype. Strain variation within a genotype may be functionally important, as it can affect antibody neutralization, presumably due to changes at key sites within exposed epitopes.^{99,794,852,925}

In addition to serotype, genotype, and strain variation, DENV has the capacity to accumulate variation rapidly within

an individual host. Viral quasispecies comprises a cloud of variants that are genetically linked through mutation. It is observed during infection by many RNA viruses (e.g., hepatitis C virus [HCV], human immunodeficiency virus [HIV], and influenza) and creates diversity that allows a viral population to adapt rapidly to dynamic environments and evolve resistance to immune responses, vaccines, and antiviral drugs.^{450,848} DENV exists as a collection of highly similar variants forming a quasispecies⁸⁶⁹ by virtue of its error-prone NS5 polymerase, which has an estimated mutation rate of 10^3 to 10^5 substitutions per nucleotide copied per round of replication.^{137,836} Preliminary studies suggest that genetic diversity is greater in the structural proteins, which may have less constraint to maintain integral functions. The study of genetic and intrahost diversity for DENV is still in its relative infancy, and thus more analysis is warranted to define how mutation and variation impact fitness, tropism, and resistance.

Clinical Features of Acute DF: Primary DENV Infection

DENV infection of humans after mosquito inoculation causes a spectrum of clinical disease ranging from inapparent disease (~50% of infections^{37,112,224}), self-limited dengue fever (DF) to severe DHF and DSS. A classical presentation of DF is an abrupt onset of a debilitating febrile illness characterized by headache, retroorbital pain, myalgias, arthralgias, and a maculopapular rash that occurs after a 2- to 7-day incubation period after mosquito inoculation.⁷⁰⁰ Some individuals experience severe bone and joint pain (“break-bone fever”) and develop petechial hemorrhages that are associated with mild to severe thrombocytopenia. There is no specific constellation of signs or symptoms to differentiate DF from other acute flu-like viral syndromes, so a health care provider must have a high index of suspicion for diagnosis in the setting of the appropriate epidemiology. DF also may present in a less classical form as an undifferentiated febrile illness with rash along with mild upper respiratory symptoms (cough, pharyngitis, rhinitis), particularly in children. DF is usually self-limited, lasting 1 to 2 weeks, although some (up to 25% of hospitalized patients) experience a prolonged postinfectious fatigue and depression syndrome that can persist for weeks, akin to that seen after Epstein-Barr virus (EBV) infection and mononucleosis.⁷³⁹ Because of the debilitating fever and musculoskeletal symptoms, the morbidity toll is high in clinically apparent DF, whereas the mortality rate is exceedingly low. Primary DF usually occurs during the initial DENV infection of an individual, with the exception of infants from immune mothers that have acquired antibodies transplacentally.

Clinical Features of DHF/DSS: Secondary and Infant DENV Infection

The incidence of the most severe form of DENV disease, DHF/DSS, varies considerably between primary and secondary infections. A secondary DENV infection results when a person previously infected with one serotype is exposed to a different serotype, and is the single most important risk factor for severe dengue disease.^{112,224,294,305} Epidemiologic data in Thailand has shown greater than 10-fold higher rates of DHF/DSS during secondary compared to primary infection of children.⁸⁴³ It should be pointed out that even during secondary infection, DHF/DSS is quite rare, with only 0.5% of

secondary infections progressing to the most severe forms of dengue disease. DHF/DSS is characterized by rapid onset of capillary leakage accompanied by thrombocytopenia and mild to moderate liver damage, reflected by increases in serum levels of hepatic enzyme (e.g., aspartate aminotransferase [AST] and alanine aminotransferase [ALT]).³⁰¹ DHF/DSS usually occurs as a second phase of the illness, after a short period of deferescence from the initial fever. Hemorrhagic manifestations are observed in a subset of DHF/DSS cases and include petechiae, epistaxis, gastrointestinal bleeding (hematemesis or melena), menorrhagia, and a positive tourniquet test. Use of the term hemorrhagic fever instead of dengue capillary-leak syndrome has led many to anticipate that bleeding is the greatest threat. Rather, fluid loss into tissue spaces with hemoconcentration and hypotension can result in shock, which carries the highest risk of mortality.⁶¹² From a diagnostic standpoint, an elevated hematocrit and upper abdominal ultrasonogram showing a thickened gall bladder wall, hepatomegaly, ascites, or pleural effusions are evidence of fluid shifts associated with a capillary leak syndrome.

Whereas DHF/DSS occur largely after secondary infection by a different DENV serotype in children and adults,²⁷³ in infants younger than age one born to dengue-immune mothers, primary infection can cause severe DHF/DSS.^{305,356,764} In clinical studies, maternal anti-DENV neutralization antibody titers and age of the infant correlated with disease. The actual age at which DHF/DSS occur in infants (peak at 7 months) corresponds to the age at which maximum enhancing activity for DENV infection in primary monocytes is observed *in vitro*.⁴⁰⁵ Severe clinical manifestations of DHF/DSS are more prevalent in infants³¹¹ and there is an approximately fourfold higher mortality rate compared to other age groups.³⁷³ Infants represent approximately 5% of children hospitalized with DHF/DSS in many parts of Southeast Asia.^{140,273,303} The more prevalent or severe clinical manifestations associated with infant DHF/DSS include seizures, hepatic dysfunction, thrombocytopenia, high-grade fever, diffuse rash, peripheral edema, ascites, and frank shock.³⁵⁶

Although it is not fully accepted by the field, some clinical studies have suggested that severe DENV infection also can have neurologic manifestations including transverse myelitis, Guillain-Barre syndrome, encephalitis, and encephalopathy,^{769,841} occurring in as many as 1% to 6% of DHF/DSS cases.^{122,325} In contrast to other encephalitic flaviviruses (e.g., JEV, WNV, or tick-borne encephalitis viruses), DENV historically has not been considered as neurotropic. However, the discovery of DENV and anti-DENV immunoglobulin M (IgM) in the cerebrospinal fluid of patients with encephalopathy suggests that it may be capable of causing central nervous system (CNS) infection as part of a severe DHF/DSS syndrome, at least in a subset of individuals.^{507,769} In support of this, focal imaging abnormalities have been detected in brain MRI scans of DENV-infected patients.^{122,872} Although these results are suggestive, bona fide DENV encephalitis and CNS disease may not be fully accepted until its antigens are reliably detected in the brains of encephalopathic patients and a more complete understanding of the molecular determinants for neurotropism is acquired.

Pathologic Features of DHF/DSS

Although DENV is the leading mosquito-borne transmitted viral infection in the world, there are few detailed autopsies

series of patients who succumbed to DHF/DSS, and fewer performed with newer molecular techniques and markers. Detailed histopathologic studies that might inform a basic understanding of DENV pathogenesis are rare because much of the lethal disease occurs in regions lacking sophisticated laboratory infrastructure, highly trained personnel, and repositories for long-term tissue storage. Forensic studies also are complicated by lack of standardization of histologic procedures and variation in the quality of specimen preparation and storage.

A recent summary of the autopsy literature from a total of 160 fatal DHF/DSS cases occurring primarily in children and adolescents was published.⁵³¹ Pathologic findings in the liver of DHF/DSS cases include centrilobular necrosis, changes in fatty tissue, inflammatory leukocyte infiltration, and Kupffer cell hyperplasia.^{70,113} Gross macroscopic examination revealed multiple hemorrhagic foci. Microscopic analysis has shown increased inflammatory infiltrates around the portal vessels, sinusoidal congestion, small hemorrhages, midzonal hepatocyte necrosis, and microvesicular steatosis.^{228,293} In other tissues (spleen or lung) hemorrhage, tissue edema, and plasma leakage have been observed.⁵⁰

A key to understanding the pathogenesis of severe DENV infection is defining cellular tropism of infection, which could influence the host inflammatory response that results in the capillary leakage syndrome. Autopsy series have shown the presence of DENV antigen or nucleic acid in cells of the skin, liver, spleen, lymph nodes, kidney, lung, thymus, or brain.^{38,50,165,293,377,392,553,692} However, several of these studies used *in situ* hybridization or reverse transcriptase polymerase chain reaction (RT-PCR) based assays, and thus have not definitively shown that infectious virus is produced in a given cell of a target tissue. In severe DENV cases, infectious virus can be reliably isolated from blood, lymphoid tissues, and the liver, although the cellular source of the virus remains controversial. Studies in humans, nonhuman primates, and small animal models support a role for infection of myeloid cells (blood monocytes, tissue macrophages, Kupffer cells), and possibly other cells including hepatocytes²⁹⁶ and endothelial cells.^{50,912}

YELLOW FEVER VIRUS

History, Global Distribution, and Epidemic Cycle

Several recent excellent reviews have described the epidemiology and historical details of yellow fever virus (YFV) infection.^{41,222,246} YFV, the causative agent of yellow fever virus (YFV), was first isolated (strain Asibi) in 1927 after inoculation of a rhesus monkey with the blood of a patient from Ghana.⁷⁸⁹ YFV originated in Africa, was imported into the Americas during the slave trade, and had the first reported epidemic in the Yucatan in 1648.⁴⁵ Historically, large epidemics of YF disease occurred beyond these regions and were described in the 17th through 20th centuries as far north in the Americas as Canada, as well as in parts of Europe including Spain, Italy, France, and England.⁵⁶⁶ Despite the presence of an effective vaccine (17D strain) that was developed in 1937 by Max Theiler and colleagues,⁸²⁰ with more than 500 million doses administered to humans,²⁴⁶ YFV infection has remained a public health threat in restricted parts of the world. Currently, YFV is endemic in the tropical regions of Africa and the Americas, infects humans and

nonhuman primates, and is transmitted by mosquitoes including *Aedes aegypti*. The World Health Organization (WHO) estimates an incidence of 200,000 cases per year, leading to about 30,000 deaths, with the majority occurring in sub-Saharan Africa.⁸⁹⁰ Overall, 44 countries in Africa and the Americas are considered within the modern YFV endemic zone, with almost 900 million people at risk of infection.^{222,246}

The sylvatic or jungle cycle of YFV in which transmission occurs between mosquitoes and wild monkeys explains why extensive vaccination campaigns have reduced but not eradicated infection. In East Africa, YFV infection is maintained enzootically in monkey transmission cycles in the jungle with the *Aedes africanus* mosquito vector. Periodically, infection may cross into humans during an intermediate savannah cycle, with transmission by several different *Aedes* mosquito species (e.g., *Aedes bromeliae*). Indeed, in the Americas, most cases appear to be a result of humans intruding on the jungle cycle of YFV.⁴⁷ Epidemic YFV infection (human–mosquito–human cycle) ensues in urban or domestic areas with *Aedes aegypti* as the principal mosquito vector. Rapid urbanization in Africa and the Americas with population shifts from rural to urban settings combined with the collapse of mosquito eradication programs has allowed the *A. aegypti* vector to repopulate many parts of the world, and caused YFV to be classified as a reemerging threat for humans.²⁴⁶

YFV Diversity

YFV does not belong to an antigenic subgroup based on plaque reduction neutralization assays,¹²⁰ but shows greater genetic relationship to other African flaviviruses including Banzai, Zika, Wesselsbron, and Bouboui viruses. Indeed, cross-protection against YFV infection in monkeys has been shown after immunization with some of these related viruses.³²⁶ Although there is only one serotype of YFV, there is significant diversity within the genus. Seven genotypes have been proposed including two West African genotypes, a single Central/South African genotype, two East African genotypes, and two South American genotypes.²²² These genotypes of YFV were originally defined based on nucleotide variation of greater than 9% in the prM, E, and 3' UTR gene regions^{601,858} and have been confirmed with full-genome sequencing of YFV isolates.⁸⁵¹ Phylogenetic studies suggest that YFV originated in East or Central Africa and was introduced subsequently into West Africa and South America.^{600,601} Beyond genotypes, sequence analysis of 79 YFV strains isolated from 1935 to 2001 in Brazil revealed further strain divergence into clades that differ at the nucleotide and amino acid level by up to 7% and 5%, respectively.⁸⁴² The physiologic basis for genotype-specific amino acid variation between YFV isolates remains uncertain, although it is likely that selection confers a phenotypic advantage in a given host.

Clinical Features of YFV Infection

In humans, YFV infection causes a variable clinical syndrome ranging from no symptoms, to mild febrile flu-like illness, to fulminate and possibly fatal disease. Approximately 15% of people who become infected develop severe visceral disease, and in this group there is a 20% to 50% case fatality rate.⁵⁶⁴ Symptoms occur within 3 to 6 days of mosquito inoculation and include an abrupt onset of fever, chills, myalgia, back pain, and headache or the first “period of infection,” which usually lasts 3 days and corresponds to peak viremia. During

this phase, individuals are infectious to mosquitoes. In some, this stage may be followed by a short “period of remission,” with defervescence and improvement of clinical signs and symptoms. Shortly after, in a subset (20%) of patients, fever and symptoms worsen (“period of intoxication”) with vomiting, epigastric pain, and jaundice (which gives yellow fever its name); this is associated with YFV replication in the liver, an absence of viremia, and measurable anti-YFV antibodies in serum. As time progresses, severe YFV infection evolves into a hemorrhagic fever characterized by severe hepatitis, renal failure, hemorrhage, shock, and multiorgan failure. A bleeding diathesis manifests with melena, hematemesis, epistaxis, ecchymosis, menorrhagia, petechial hemorrhages, and blood oozing from mucous membranes. Renal failure is associated with an abrupt decrease in urine output and with albuminuria. Laboratory tests show leukopenia, thrombocytopenia, and a coagulopathy. Death occurs on the 7th to 10th day of illness and is preceded by hemodynamic and cardiovascular instability, acute liver failure, hypothermia, hypoglycemia, and coma. For those individuals surviving severe YFV infection, convalescence is prolonged with hepatitis and associated constitutional symptoms persisting sometimes for months.

Pathologic Features of YFV Infection

Macroscopic gross pathology of tissues from YFV infection autopsy studies show an enlarged and icteric liver and edematous and enlarged kidneys and heart. Microscopic pathologic analyses of the liver reveal six major features,^{246,408,567} which occur primarily during the last “period of intoxication”: (a) eosinophilic degeneration of hepatocytes and Kupffer cells; (b) midzonal hepatocellular swelling and necrosis, with sparing of the cells in the portal area; (c) the presence of Councilman bodies coincident with hepatocyte cell death; (d) absence of leukocyte inflammatory infiltrates; (e) microvesicular fatty changes and lipid accumulation, likely secondary to decreased apolipoprotein synthesis by hepatocytes; and (f) retention of the reticulin structure. YFV antigen and RNA are demonstrable in hepatocytes by immunohistochemistry or in situ hybridization,¹⁸⁷ and this coupled with the absence of inflammation, suggests that the cell death is mediated directly by virus infection, likely via apoptotic mechanisms.^{567,666}

In the kidney, severe eosinophilic degeneration and a microvesicular fatty change of renal tubular epithelium are observed, analogous to that seen in the liver. Viral antigen can be detected by immunohistochemistry in renal tubular cells.¹⁸⁷ Glomerular damage and albuminuria with changes in the basement membrane and degeneration of cells lining Bowman's capsule may be due to direct viral injury⁵⁶⁷ or secondary to decreased blood flow during the sepsis syndrome.²⁴⁶ The spleen shows an overall loss of lymphocytes, hyperplasia of the follicle, appearance of large mononuclear tissue histiocytes, and significant degeneration of cells with accumulation of fragmented nuclei.⁴⁰⁹ In monkeys, necrosis of B-cell follicular areas of the spleen is more apparent.⁵⁶⁸ In the heart, myocardial cells also undergo apoptotic changes as in other organs, in the absence of a significant cellular inflammatory response. Patchy lesions have been described in sinoatrial (SA) node and bundle of His,⁴⁹⁵ which could explain the paradoxical bradycardia (Faget's sign) and late cardiac death, observed in some severe YFV cases.

Hemorrhagic manifestations and damage to and plasma leakage from capillaries are characteristic findings of severe

YFV infection.⁵⁶⁷ The bleeding manifestations are attributed to decreased synthesis of vitamin K–dependent coagulation factors by the injured liver, disseminated intravascular coagulation, and reduced platelet numbers and function. Beyond direct bleeding, there is additional vascular dysfunction, with pleural and peritoneal effusions, and edema of several other organs, including the brain. At present, the precise pathogenesis of the vascular leakage syndrome associated with YFV remains unknown, although highly elevated levels of proinflammatory and vasoactive cytokines are observed.⁸¹⁴

WEST NILE VIRUS

History, Global Distribution, and Epidemic Cycle

West Nile virus (WNV) was first isolated in 1937 in the West Nile district of Uganda from a woman with an undiagnosed febrile illness.⁷⁶⁶ Historically, WNV caused sporadic outbreaks of a mild febrile illness in regions of Africa, the Middle East, Asia, and Australia. Indeed, in the 1950s, detailed studies of WNV showed recurrent outbreaks in Israel^{66,260} and high levels of seroconversion in adults from Egypt^{352,549}; these outbreaks and others in Africa generally were not associated with severe human disease. However, in the 1990s, the epidemiology of infection changed. New outbreaks in Eastern Europe were associated with higher rates of neurologic disease.³⁴⁸ In 1999, WNV entered North America, and caused seven human fatalities in the New York area as well a large number of avian and equine deaths. Over the last decade, WNV has spread to all 48 of the lower United States as well as to parts of Canada, Mexico, the Caribbean, and South America. Because of the increased range, the number of human cases has continued to rise: in the United States between 1999 and 2012, 36,500 cases were confirmed and associated with 1,500 deaths (<http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm>).

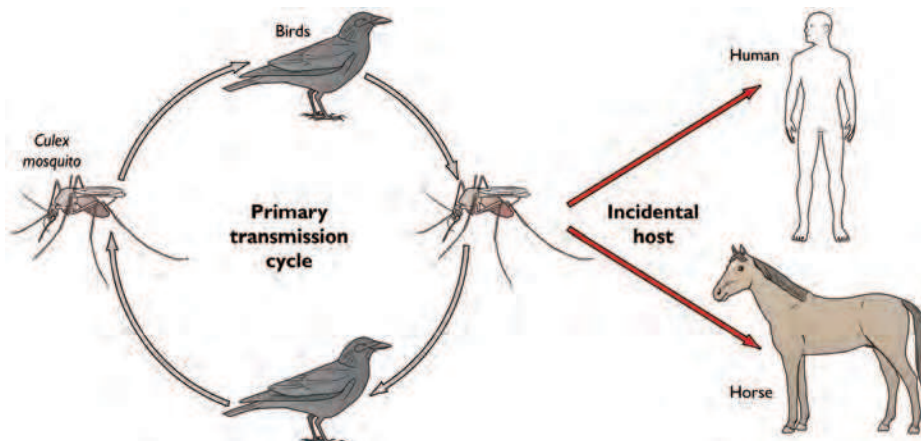
WNV cycles in nature between *Culex* mosquitoes and birds, but also infects and causes disease in humans, horses, and other vertebrate species. (Fig. 26.7) Ticks also have been implicated as having a minor role in transmission in some parts of the world,⁵¹⁰ although few isolates have been obtained. Although its enzootic cycle is overwhelmingly between mosquitoes and birds, with vertebrate species serving as “dead-end” hosts because of low-level and transient viremia, nonviremic transmission of WNV between co-feeding mosquitoes³³⁰

suggests that vertebrates could act as reservoirs for mosquito infection. Most (~85%) human infections in the Northern Hemisphere occur in the late summer, with a peak number of cases in August and September. This reflects the seasonal activity of *Culex* mosquito vectors and a requirement for virus amplification in the late spring and early summer in avian hosts. In warmer parts of the world, virtually year-round transmission has been observed. Although more than 100 avian species are susceptible to WNV infection, in the United States, some are particularly vulnerable, with a large number of deaths in crows, blue jays, and hawks. The magnitude of dying birds in a community in the early summer often predicts the severity of human or equine disease weeks later.⁴¹⁵ Ecologic studies suggest that *Culex pipiens*, the dominant enzootic (bird-to-bird) and bridge (bird-to-human) vector of WNV in urbanized areas in the northeast and north-central United States, shifts its feeding preferences from birds to humans during the late summer and early fall, coincident with the dispersal of its preferred host, the American robin (*Turdus migratorius*).³⁹³

WNV Diversity

Sequencing and phylogenetic analysis of full-length genomes has resulted in a division of WNV strains into four distinct lineages,^{67,358,447,510} with lineage 1 strains further separated into three clades (1a, 1b, and 1c). This topic has been analyzed in great detail in a recent study.⁵³⁷ Clade 1a comprises isolates from Europe, the Middle East, Russia, and the Americas, and includes all strains from the recent epoch in the United States and Canada. Clade 1b contains the naturally attenuated Australian variant, Kunjin virus, which forms a tight cluster with approximately 2% to 3% difference at the amino acid level from North American WNV strains.⁷¹⁹ Clade 1c comprises isolates from India only. Historically, lineage 2 isolates were isolated from sub-Saharan Africa and Madagascar, and generally showed less ability to cause disease in humans and animals^{54,348}; a more recent study suggests that lineage 2 isolates now circulate in parts of Eastern Europe, some of which cause severe disease.²²⁶ There are fewer sequenced strains from lineage 3 and 4 WNV, with only one lineage 3 isolate from Austria in 1997³³ and several lineage 4 isolates⁵¹⁰ from Russia between 2002 and 2006. Within a given ecological niche, possibly because of the enzootic cycle, WNV has remarkable genetic stability despite its error-prone RNA-dependent RNA polymerase; full-length sequencing analysis of North American isolates over the past decade

FIGURE 26.7. Transmission cycle of West Nile virus (WNV). WNV is maintained in nature in an enzootic transmission cycle between mosquitoes and birds. Many vertebrate species, including humans, may also be infected as “dead-end” hosts for WNV. The resulting transient low-level viremia in mammalian hosts does not support sufficient infection of the mosquito vector to continue the transmission cycle.



has revealed a rate of approximately five nucleotide and fewer than one amino acid mutation per genome per year, with little geographic subdivision.^{181,810}

Clinical Features of WNV Infection

Seroprevalence studies suggest that most (~80%) cases are subclinical, without significant symptoms. Among clinical cases, many develop a self-limiting illness that is termed WNV fever. This syndrome begins after a 2- to 14-day incubation period and is characterized by fever accompanied by myalgias, arthralgias, headache, fatigue, gastrointestinal complaints, maculopapular rash, or lymphadenopathy. This nonneuroinvasive form of WNV infection can be severe, as 38% of patients with WNV fever were hospitalized with a mean length of stay of 5.4 days.³⁵⁰ A subset of the symptomatic cases progress to the neuroinvasive forms of WNV infection, including acute flaccid paralysis, meningitis, encephalitis, and ocular manifestations^{34,742}; in many instances, a combination of these syndromes is present. Overall, about 1 in 150 WNV infections result in the most severe and potentially lethal form of the disease. During an epidemic, on a human population scale, the seroconversion rate is approximately 3%^{588,832} and the attack rate for severe disease during an epidemic is about 7 per 100,000.³⁵⁰ The risk of severe WNV infection is greatest in the elderly.^{151,603,832} At least two studies have estimated a 20-fold increased risk of neuroinvasive disease and death in those older than 50 years of age.^{350,603} Persistent movement disorders, cognitive complaints, and functional disability may occur after West Nile neuroinvasive disease. West Nile poliomyelitis-like disease may result in limb weakness and long-term morbidity. Moreover, even patients with apparently mild cases of acute disease have sustained subjective and somatic sequelae following WNV infection. Therefore, the neurologic and functional disability associated with WNV infection represents a considerable source of morbidity in patients long after their recovery from acute illness.^{740,741,742,743}

Although most human WNV infections occur after the bite of an infected *Culex* mosquito, other routes including transfusion, organ transplantation, and placental and breast milk transmission. In 2002, 23 cases of WNV infection were identified after transfusion of blood products.⁶³⁹ These cases led to the development and implementation of nucleic acid amplification tests, which have been used to identify infected pools or individual blood product samples^{115,644} and largely prevent transmission by transfusion. Nucleic acid screening of blood donors have not completely eliminated transfusion-transmitted WNV infections, as “breakthrough” infections have occurred, and were attributed to units that had levels of viremia below the sensitivity of the screening assay.¹¹⁶ In addition to transfusion-associated WNV infection, several cases by organ transplantation have been reported.^{427,428} Because of the relatively low incidence of WNV infection in organ transplantation and risk of false-positives that can occur with wide scale testing, screening is not mandated.

Pathologic Features of WNV Infection

WNV causes encephalitis in several vertebrate species including humans, horses, and birds, by virtue of its ability to infect and cause injury to neurons through direct (viral-induced) and indirect (immune response induced) mechanisms.¹²⁹ Pathologic observations in humans, however, is limited by the

small number of autopsy studies on individuals succumbing to WNV infection. Gross macroscopic examination of organs (brain, lung, kidney, and spleen) tends to be unremarkable.⁶³⁰ Microscopic examination of the brain in humans and other animals reveals histologic changes that are consistent with the clinical disease.^{221,630} This includes neuronal cell death, activation of resident microglia and infiltrating macrophages, perivascular and parenchymal accumulation of CD4⁺ and CD8⁺ lymphocytes and CD138⁺ plasma cells, and formation of microglial nodules. These lesions, which tend to be patchy in distribution, occur in the brainstem, cerebral cortex, hippocampus, thalamus, and cerebellum.⁶³⁰ In addition, overt meningitis with cellular infiltrates in the meninges can be readily apparent. In some cases, destruction of vascular structures with focal hemorrhage is present, suggestive of a vasculitis; this may be associated with local compromise of the blood-brain barrier.^{200,868} Immunohistochemical analysis confirms that WNV antigen is present in neurons from multiple regions of the brain, although other cells (e.g., astrocytes or CD11b⁺ myeloid cells) may be infected to lesser degrees.^{176,208}

In addition, WNV infection can cause a poliomyelitis-like syndrome of acute flaccid paralysis.^{257,458} Patients show markedly decreased motor responses in the paretic limbs, preserved sensory responses, and widespread asymmetric muscle denervation without evidence of demyelination or myopathy.⁴⁵⁸ Microscopically, in the spinal cord, an intense inflammatory infiltrate around large and small blood vessels is observed with large numbers of microglia in the ventral horn. Anterior horn motor neurons are targeted by WNV,^{458,754} and studies suggest that axonal transport from peripheral neurons can mediate WNV entry into the spinal cord and induce acute flaccid paralysis.⁷⁰⁵

Although most mammalian WNV infections are cleared by the adaptive immune response, persistence in the kidney has been described, albeit infrequently. Hamsters experimentally infected with WNV developed chronic renal infection and viremia for up to 8 months, despite clearance from blood and the appearance of neutralizing antibodies. Although minimal histopathology was reported, WNV antigen staining was detected in the renal epithelium, interstitial cells, and tubules.⁸¹⁶ Of interest, these persistent viruses evolved genetically and no longer caused neuroinvasive disease on challenge of naïve animals.⁸⁹⁵ Analogous to the studies in hamsters, WNV RNA was demonstrated in 5 of 25 urine samples from convalescent humans 1.6 to 6.7 years after the initial infection, although infectious virus was not successfully isolated.⁵⁹⁹ However, a separate larger study did not detect viral RNA in urine, and thus analysis of additional patient cohorts may be required to better define renal persistence and its significance in humans.²⁵⁴

Persistent WNV infection in the CNS also has been suggested by experimental infection studies in monkeys, hamsters, and mice. In monkeys, WNV persisted at least 5.5 months after initial infection and was isolated in the cerebellum and cerebral subcortical ganglia but had lost its neurovirulence and cytopathic properties.⁶⁵⁵ In hamsters, persistent WNV RNA and foci of WNV antigen-positive cells were identified in the CNS of hamsters between 28 to 86 days after infection,⁷⁵⁸ and this was associated with long-term neurologic sequelae. In mice, infectious WNV persisted in the brains of wild-type animals up to 4 months, and viral RNA could be detected at 6 months in up 12% of mice, even in animals with subclinical infection.¹⁶ Consistent with this, virus-specific B- and

T-cell immune responses persisted in the CNS of mice up to 4 months after infection.⁷⁸⁵

JAPANESE ENCEPHALITIS VIRUS

History, Global Distribution, and Epidemic Cycle

Japanese encephalitis virus (JEV) is a mosquito-transmitted flavivirus and the prototype virus of the JEV antigenic serocomplex. JEV causes severe neurologic disease, primarily in Asia, where it accounts for about 35,000 to 50,000 cases and 10,000 to 15,000 deaths annually.⁸³¹ JEV epidemics were originally described in Japan in the 1870s, and the virus was initially recovered in 1935 from the brain of an infected human in Tokyo; this isolate was established as the prototype Nakayama JEV strain.⁴⁶⁰ Although most human infections are asymptomatic or result in mild symptoms, greater than 50% of the severe clinical cases are fatal or result in devastating long-term neurologic sequelae.⁷³⁶ Moreover, as JEV-induced disease largely occurs in children living in rural areas, it is likely vastly underreported in most regions of Asia.^{736,768}

The enzootic cycle of JEV is between waterbirds (e.g., egrets and herons) and mosquitoes, with pigs also serving as an amplifying host. JEV is transmitted primarily by *Culex* mosquitoes (principally *Culex tritaeniorhynchus*) that breed in rice fields and stagnant water. Humans and other vertebrate animals are considered incidental targets and dead-end hosts, as they do not produce a viremia sufficient to infect mosquitoes. Two epidemiologic patterns are observed: in northern temperate areas JEV infections occur during the summer months, whereas as in tropical climates, year-round transmission of JEV has been described.²⁶⁷

Globally, despite the introduction of several inactivated and live-attenuated vaccines (see Vaccine section below), JEV remains the most important cause of arthropod-transmitted viral encephalitis. Disease caused by JEV is widely distributed in Asia, with outbreaks historically occurring in Japan, China, Taiwan, Korea, the Philippines, India, parts of Southeast Asia, and the far-eastern region of Russia. Although cases in China appear to be declining, possibly due to large-scale vaccination campaigns, epidemic activity in India, Nepal, and other parts of Southeast Asia appears to be escalating. More recently, JEV has been described in Pakistan, Papua New Guinea, and Australia, suggesting that its geographic range may be expanding.^{312,313}

JEV Diversity

Phylogenetic analysis suggests that JEV evolved from an ancestral flavivirus in Africa within the last few centuries.²⁶⁸ Based on sequence analysis primarily of the viral structural genes, JEV was initially classified into one single serotype with four distinct genotypes (I–IV),^{145,146,835} with as much as 12% variation at the nucleotide level. These divisions have been confirmed by full-length genome sequencing on a subset of isolates. Genotype I includes isolates from Thailand, Cambodia, Korea, China, Japan, Vietnam, Taiwan, and Australia from 1967 to the present. Genotype II includes strains from Thailand, Malaysia, Indonesia, Papua New Guinea, and Australia from 1951 to 1999. Genotype III includes isolates recovered from mostly temperate areas of Asia including Japan, China, Taiwan, the Philippines, and the Asian subcontinent between 1935 and the present. Finally, genotype IV includes strains

from Indonesia that were isolated only in 1980 and 1981. More recently, a fifth, more divergent genotype (V) has been proposed based on full-genome sequencing of a 1952 isolate from a patient in the Muar region Malaysia.⁵⁶⁰ This strain has approximately 20% and 9% nucleotide and amino acid divergence, respectively, and shows significant variation with respect to neutralization by JEV-specific monoclonal antibodies.³¹⁵

Because genotypes I and III largely occurred in epidemic regions and genotypes II and IV were associated with endemic transmission, differences in strain virulence were hypothesized to explain the epidemiologic patterns of JEV.¹⁴⁶ However, as the geographic range of JEV has expanded, there are now several examples in which strains of individual genotypes cause either epidemic or endemic disease depending on the region or country.⁷⁷²

Clinical Features of JEV Infection

In humans, the JEV infection can be asymptomatic or produce a range of clinical syndromes including a mild nonspecific febrile illness, aseptic meningitis, seizures, encephalitis, and poliomyelitis-like flaccid paralysis. Disease onset usually begins with a 1- to 2-week period of flu-like symptoms including headache, fever, cough, and upper respiratory symptoms, as well as gastrointestinal complaints such as nausea, vomiting, and diarrhea. In infants and young children the disease can progress rapidly as the virus invades the CNS and infects and injures neurons. CNS invasion is heralded by nuchal rigidity, photophobia, and altered mental status. JEV infection in the CNS can share features with Parkinson's disease including mask-like facies, hypertonia, tremor, and cogwheel rigidity. Other CNS symptoms include seizures (more common in children than adults), ataxia, involuntary movements (e.g., choreoathetosis, facial grimacing, and lip-smacking), and cranial nerve palsies. Associated with this are elevated white blood cell counts and pressure in the cerebrospinal fluid (CSF) and abnormal electroencephalography (EEG) examinations. Imaging studies in the brain have revealed thalamic and basal ganglia abnormalities during the acute phase of disease.⁷⁶⁸ Upper rather than lower extremity paralysis is more common, and lower motor neuron disease of the spinal cord can develop. Death can occur, especially in children, within 3 to 5 days of CNS symptoms, or much later due to complications associated with hospitalization or cardiopulmonary status. A recent prospective study evaluated the clinical features and long-term prognosis of 118 children with encephalitis due to JEV in Malaysia.⁶³³ Only 44% of patients had full recovery, with 8% dying during the acute phase of the illness and 31% having persistent and severe neurologic sequelae. These included chronic seizures, motor dysfunction, and neuropsychiatric symptoms such as mental retardation and psychiatric disorders.

Pathologic Features of JEV Infection

JEV infection in the brain results in neuronal degeneration, necrosis, microglial nodule formation, and perivascular and parenchymal leukocyte infiltrates as well as focal hemorrhage. Parenchymal damage in the CNS is attributed to both direct cytopathic effect of the virus in nonrenewing populations of neurons and the resultant inflammatory state induced by activated microglia and infiltrating leukocytes. Although these histologic findings can occur throughout the brain, they usually are more restricted to the gray matter in the cortex, midbrain,

and brainstem, providing anatomic correlates for the tremor and dystonias associated with CNS infection. Focal lesions are seen predominantly in the thalamus and cerebral peduncles but also are commonly observed in the substantia nigra, cerebral and cerebellar cortices, and the anterior horn of the spinal cord,⁷⁶⁸ the latter of which is associated with a poliomyelitis-like acute flaccid paralysis.⁷⁷¹ In patients who die rapidly, there may be little histologic evidence of inflammation, but instead, high levels of JEV antigen can be detected in morphologically intact neurons.³⁶²

ST. LOUIS ENCEPHALITIS VIRUS

History, Global Distribution, and Epidemic Cycle

St. Louis encephalitis virus (SLEV) is a mosquito-borne member of the JEV serocomplex capable of causing severe neurologic disease in humans. SLEV was first discovered in 1933 following a large epidemic of encephalitis in St. Louis, Missouri (1,095 cases and 225 deaths).^{185,508,675} More than 10,000 cases of severe illness and 1,000 deaths have since been attributed to SLEV infection, reflecting annual endemic transmission (~50 cases/year) punctuated by epidemic periods that occur every 5 to 15 years.⁵⁶³ At least 41 epidemics of SLEV have occurred in the United States since 1933,¹⁸⁵ the largest of these in 1975.¹⁶⁷ During this epidemic, SLEV cases were reported in 29 states and the District of Columbia; the greatest number of illnesses occurred in Ohio, Mississippi, Indiana, and Illinois. Roughly 1,500 confirmed cases were reported, resulting in 171 fatalities. The most recent large outbreak of SLEV occurred in central Florida during 1990, resulting in 222 laboratory-confirmed cases and 14 deaths.⁵⁴³

SLEV is found in much of the New World; distribution ranges from Canada to Argentina, and across North America.⁶⁷⁵ SLEV is maintained in nature in enzootic cycles between *Culex* mosquitoes and passeriform and columbiform birds. Of interest, the transmission cycle of this virus varies by region due to differences in the biology of the primary vector mosquitoes.⁶⁷⁵ In the eastern and central United States, the principal vectors of SLEV are *Culex pipiens* and *Culex quinquefasciatus* mosquitoes. *Culex tarsalis* is the primary vector for SLEV in Western states, whereas *Culex nigripalpus* transmits SLEV in Florida. The avian hosts of SLEV in these transmission cycles include house finches, house sparrows, and mourning doves. The mechanism of virus transmission and amplification in South and Central America is less clear. SLEV has been isolated from 11 different mosquito genera, many of which feed primarily on mammals.

Both WNV and SLEV are antigenically related members of the JEV serogroup that share a similar transmission cycle between *Culex* mosquitoes and birds. How the introduction of WNV in North America has impacted the epidemiology of SLEV is of significant interest. Analysis of the number of neuroinvasive cases attributed to SLEV reported to the CDC between 1999 and 2007 revealed a threefold reduction by comparison with data in the pre-WNV era.⁶⁷⁴ Interpretation of this finding is complicated by changes in the intensity of surveillance and local testing for arboviral diseases in the years after the introduction of WNV. Because major epidemics of SLEV have occurred infrequently in the past, the modest number of clinical cases may simply reflect a nadir in the natural cycle of

this virus. Alternatively, the existence of cross-reactive antibodies in WNV-immune avian reservoirs may disrupt the transmission cycle of SLEV via competition for avian hosts. Although the infection of house finches with WNV has been shown to confer protection from subsequent infection by SLEV, the reciprocal is not true. Prior exposure of finches to SLEV prevents mortality following WNV infection but not the low-level of viremia that is sufficient for transmission of WNV.²³² Similar findings were reported in a golden hamster model of infection.⁸¹⁷ The disappearance of SLEV from regions of California following introduction of WNV is consistent with the notion that competition may allow for the local displacement of the virus from historically endemic areas.⁶⁷⁶ Additional study and surveillance are required to clarify the dynamics and interactions between these two related pathogens in North America.

SLEV Diversity

Phylogenetic studies grouped SLEV isolates into seven genetic lineages (I–VII), many of which were divided further into clades of related genotypes.^{421,538} These groups correspond roughly to the geographic distribution of each lineage of SLEV.⁸³⁰ For example, lineage I include viruses isolated in the western United States, whereas lineage V contains South American strains and an isolate from Trinidad. However, the relationship between phylogenetic relatedness and geographic region is imperfect. SLEV strains vary considerably with respect to virulence in avian and mammalian hosts; these differences correlate roughly with geographic distribution.^{90,571} In addition to regional persistence, sequence analysis reveals that SLEV may be transported between regions.⁴²¹

Clinical and Pathologic Features of SLEV Infection

As is the case for both WNV and JEV, the majority of SLEV infections of humans are clinically asymptomatic. The ratio of apparent to inapparent infections has been reported to range from 1:16 to 1:425.⁵⁶³ Increasing age is a significant factor influencing susceptibility to severe illness. Symptomatic illness is noted after an incubation period of 5 to 15 days and is characterized by mild malaise, fever, headache, nausea, myalgia, sore throat, and cough.¹⁰³ Severe neurologic manifestations including encephalitis and aseptic meningitis may occur and can be fatal. Case fatality rates for SLEV range from 5% to 20%, with fatalities increasing in the elderly.⁶⁷⁵ Although most SLEV cases resolve spontaneously and without sequelae, many patients (30% to 50%) experience an extended convalescence lasting up to 3 years. This phase is characterized by headache, depression, memory loss, and weakness.^{103,675}

TICK-BORNE ENCEPHALITIS VIRUSES

History, Global Distribution, and Epidemic Cycle

Tick-borne encephalitis virus (TBEV) causes a fatal neurologic syndrome that primarily affects individuals ranging from northern China and Japan, through Russia, to parts of Northern Europe.⁵²⁵ TBEV infection was first described in 1931 after a pattern of seasonal meningoencephalitis cases in Austria was observed.⁷³² In 1939, experiments confirmed that this seasonal encephalitis in humans was caused by a virus transmission by the tick, *Ixodes persulcatus*.⁹²³ Although a highly effective

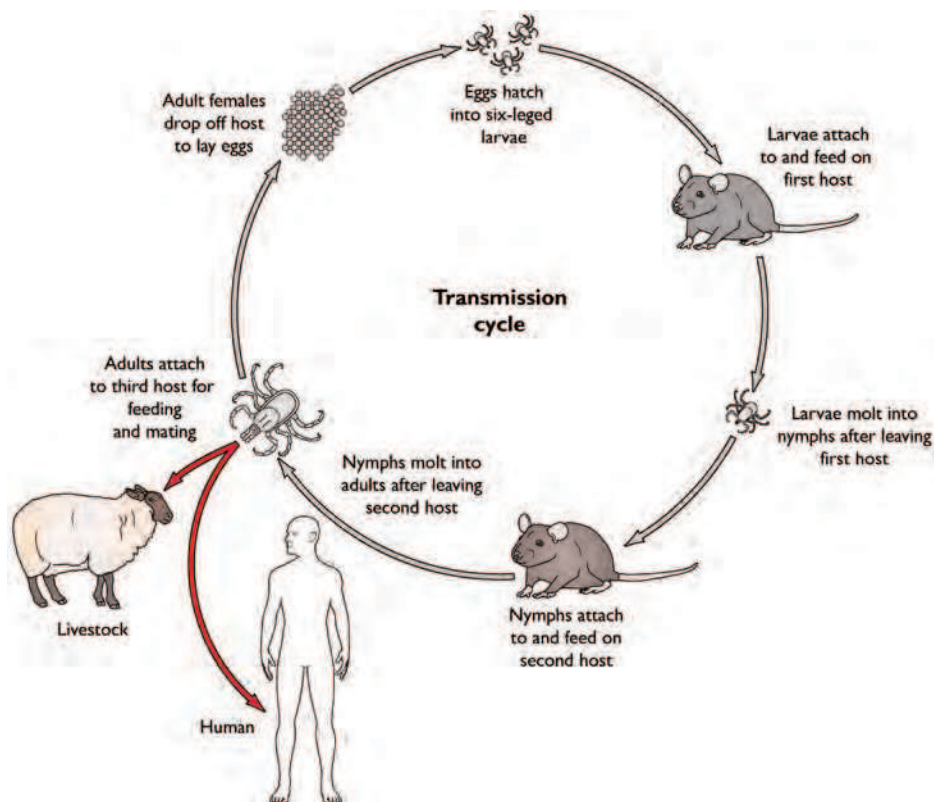


FIGURE 26.8. Life cycle of tick-borne encephalitis virus (TBEV). The transmission of tick-borne flaviviruses is connected to the life cycle of the vector due to a requirement for the tick to feed prior to transition through each of its developmental stage. Ticks are infected during this blood meal, molt, and then may infect a mammalian host. Nonviremic transmission between co-feeding ticks has also been shown to be an important mechanism of transmission and is not pictured.

formalin-inactivated vaccine has been implemented in some European countries (e.g., Austria) with marked reductions in case numbers,³²⁰ TBEV-induced morbidity and mortality continue to rise.⁷⁹⁹ Between 1990 and 2007, about 9,000 cases per year were reported in Europe and Russia⁷⁹⁹; currently, TBEV is believed to cause approximately 14,000 human cases per year, the majority of which occur in parts of Russia.²⁷⁷ This increase is thought to be due to changes in climate, population dynamics and range of permissive ticks, and shifts in land usage. Within Russia, Siberia has the highest number of TBEV cases, whereas the Czech Republic has the greatest incidence.⁵²⁵ The relative virulence of TBEV decreases with its westward spread, with the far-eastern subtype having a case-fatality rate of almost 40%.

In the enzootic cycle, TBEV is maintained between ticks and different vertebrate hosts, with humans as incidental hosts (Fig. 26.8). TBEV is transmitted primarily by the hard tick *Ixodes ricinus*, although in Eastern Europe and Russia the principal vector is *Ixodes persulcatus*. Infection is seasonal, usually occurring between March and November,³⁷¹ and coincides with seasonal peaks of feeding activity of the particular tick involved in transmission. TBEV is endemic from central Europe to Far East Asia, with cases reported in 34 countries.⁷⁹⁸ Ticks can become chronically infected after sampling viremic blood, or by transstadial or transovarial transmission. In addition, infected ticks can transmit virus to uninfected ticks during co-feeding on rodents.^{277,444} This is because the local skin environment supports TBEV replication, and migratory infected cells transport virus within the skin allowing for transmission in the absence of viremia.⁴⁴³

One exception to TBEV transmission by tick inoculation is the syndrome of biphasic milk fever, which results from oral

infection and was first identified in Russia between 1947 and 1951. During milk fever epidemics, whole families contracted TBEV infection, and this was associated with the consumption of goat milk. Goats develop subclinical TBEV infection after tick bite and become the source of infectious virus after secretion into milk. Analogously, TBEV transmission to humans has been reported after consumption of unpasteurized cow or sheep milk or dairy products.²⁷⁷ These findings are supported by experiments in mice in which TBEV infection was established after oral feeding.⁶⁵⁴

TBEV Diversity

Based on sequence similarity, three main subtypes of TBEV exist: the Far Eastern genotype 1 (previously Russian Spring and Summer encephalitis), European genotype 2 (previously Central European encephalitis), and Siberian genotype 3 (previously west-Siberian). These TBEV genotypes are closely related^{218,339} and transmitted by the ticks *Ixodes ricinus* (European subtypes) and *Ixodes persulcatus* (Asian subtypes).²⁷⁷ Within these three genotypes, there is an approximately 1.2% to 1.7% difference at the amino acid level. The Far Eastern, European, and Siberian genotypes 1, 2, and 3 differ from each other by approximately 5% to 7% at the amino acid level. In addition to these three TBEV genotypes, two additional genotypes (4 and 5) have been described based on nucleotide and amino acid differences.¹⁹³ Other viruses that are antigenically related across Europe, Asia, and North America are classified as part of the TBEV serocomplex,¹¹⁸ also termed the mammalian group of tick-borne flaviviruses. In addition to TBEV, this group includes Omsk hemorrhagic fever virus (OHFV), Louping ill virus (LIV), Langat virus (LGTV), Powassan virus (POWV), Kyasanur Forest disease virus (KFDV), Kadam

virus (KADV), Royal Farm virus (RFV), Gadgets Gully virus (GGYV), Alkhurma hemorrhagic fever virus (AHFV), and Karshi virus (KSIV). Of these viruses, TBEV, LIV, and POWV cause encephalitis in humans and animals, whereas OHFV, KFDV, and AHFV cause hemorrhagic fever.^{278,695} LGTV is a naturally occurring avirulent virus (analogous to Kunjin virus among WNV strains), and no clinical disease has been reported for KSIV, RFV, or GGYV.

Clinical Features of TBEV Infection

About one-third of patients after inoculation with an infected tick will become symptomatic,³⁷¹ with men affected twice as frequently as women, although this could reflect exposure bias. The incubation period for TBEV infection in humans varies, but for most individuals is approximately 1 to 2 weeks. A prodrome of fatigue, musculoskeletal pain, and headache lasts a few days, and is followed by an abrupt onset of fever, nausea, vomiting, and myalgias; this phase is associated with thrombocytopenia, leukopenia, and mildly elevated levels of liver enzymes in the serum. Subsequent to this, several clinical syndromes of TBEV infection develop, as reviewed previously^{277,371}:

1. *Febrile syndrome.* This illness is characterized by high fever (39°C) with no evidence of neuroinvasion. It lasts from 1 to 5 days, and upon defervescence, patients recover completely.
2. *Meningitis.* This is the most common form of clinically apparent TBEV infection occurring in approximately 50% of individuals. After the onset of fever, symptoms worsen with progressive headache, nausea, vomiting, and photophobia. All patients exhibit a CSF leukocyte pleocytosis after lumbar puncture. Fever lasts 1 to 2 weeks, with gradual recovery.
3. *Meningoencephalitis.* This form occurs in approximately 10% of cases, is more severe, and is associated with damage to the CNS. Individuals become weak, lethargic, and develop focal signs of disease including hemiparesis, hemiplegia, seizures, and autonomic instability. Up to 30% of these cases are fatal, and survivors have long-term neurologic sequelae with slow convalescence.
4. *Poliomyelitis-like disease.* This is characterized by a prodrome of limb weakness or numbness that progresses to paralysis. Paralysis occurs more frequently in the upper limbs, with the proximal segments affected more often. Recovery is slow, partial, and occurs in only one-half of patients, with the remainder showing progressive deterioration.
5. *Polyradiculitis.* This syndrome has a biphasic course with fever, headache, and myalgia followed by defervescence. Approximately one week later the second phase starts and is characterized by pain and damage in peripheral nerves, sometimes coupled with meningitis. Recovery from this form of TBEV infection is usually complete.
6. *Chronic or persistent infection.* This form has been described in Siberia and Far East Russia, although not in Europe, and is believed to associate uniquely with the Siberian subtype of TBEV. Chronic or persistent infection is characterized by a late phase (months or even years later) deterioration of the neurologic sequelae that developed during the acute illness. Alternatively, chronic TBEV infection can begin with the acute phase of disease, such that neurologic symptoms occur years after a tick bite. Clinical symptoms can include epilepsy, Parkinsonian movement and cognitive disorders,

and progressive muscle atrophy, ultimately with dementia and death ensuing. Although infectious virus has not been routinely recovered in autopsy studies, a TBEV strain was isolated from a patient who died of a progressive (2-year) form of tick-borne encephalitis 10 years after experiencing a tick bite.²⁷⁶

7. *Postencephalitic syndrome.* Both retrospective and prospective clinical trials have shown that TBEV infection is associated with a slow recovery period that has considerable long-term morbidity.^{289,370,554} This postencephalitic syndrome occurs in approximately 40% to 60% of patients, and includes memory disturbances, headache, and affective and gait disorders. The frequency of these symptoms was proportionately higher in more severe cases.

Pathologic Features of TBEV Infection

Gross pathologic analysis of the brain of humans who succumb to lethal TBEV infection shows edema and hyperemia. Microscopic lesions occur in a patchy distribution throughout the CNS but are most prominent in the brainstem, basal ganglia, thalamus, cerebellum, and spinal cord. The cerebral and spinal meninges show a diffuse leukocyte infiltration, predominantly with lymphocytes. In the parenchyma of the brain and spinal cord, perivascular infiltrates, microglial nodules, and necrosis of neurons is observed. Notably, Purkinje cell neurons in the cerebellum and anterior horn motor neurons in the spinal cord are preferentially targeted and injured by TBEV.³⁷¹ Immunohistochemical analysis of brains from 28 autopsy cases²⁵⁰ showed prominent TBEV antigen staining in Purkinje cells, neurons of the dentate gyrus, the brainstem, and basal ganglia, with T lymphocytes detected in direct apposition to TBEV-infected neurons.

PATHOGENESIS AND IMMUNITY

Virus Entry and Tropism

Flavivirus entry into cells is mediated by the envelope proteins and can be considered in three relatively discrete steps (Fig. 26.9). The first step involves the attachment of the virus particle to the cell. Collisions between virions and target cells are not always productive. “Attachment factors” promote infection by increasing the duration of contact between the virion and cell surface, and thereby increase the likelihood that subsequent steps in the virus entry pathway will occur. Attachment factors are not strictly required for infection. In contrast, interactions with viral “receptors” promote required events during virus entry. Although the distinction between these two types of cellular factors is clear for some viruses (e.g., HIV), the cell biology of flavivirus entry remains poorly understood.

Several cellular factors have been suggested to function as attachment factors or receptors during the flavivirus entry (reviewed by Lindenbach, Murray, Thiel, and Rice in Chapter 25 of this volume). The interaction between flaviviruses and glycosaminoglycans (GAGs) have been documented.^{147,332,455,523} The binding site for these sulfated polysaccharides on the virion has been mapped to positively charged surfaces of the E protein.^{147,523} Passage of virus in cell culture selects for variants that bind more efficiently to GAGs, although this adaptation appears to be associated with reduced fitness *in vivo*.^{454,456,523} Treatment

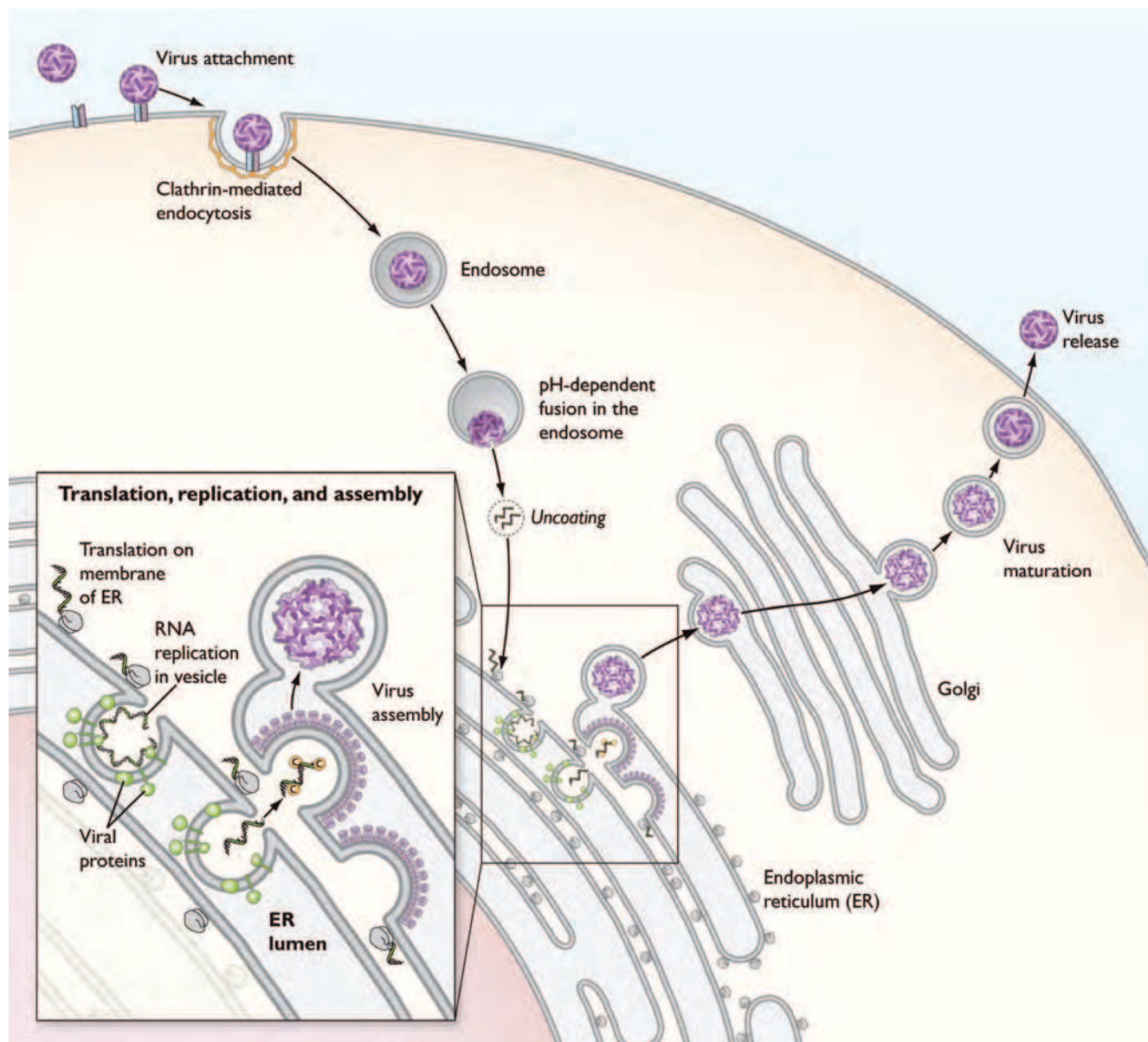


FIGURE 26.9. The replication cycle of flaviviruses. Flaviviruses bind cells of the host through poorly defined interactions with one or more molecules on target cells. Viruses are internalized via clathrin-mediated endocytosis and fuse with membranes of the late endosome in a pH-dependent manner. Viral RNA replication begins shortly thereafter in association with membranes of the host cell. Cells actively replicating flaviviruses reveal striking host membrane rearrangements thought to coordinate the processes of genomic RNA replication and virus assembly. Virus particles assemble at and bud into the endoplasmic reticulum and are secreted from the cell. During egress, virion maturation occurs in the acidic compartments of the Golgi and is characterized by cleavage of the prM protein by a furin-like protease.

of cells with heparan sulfate can inhibit infection.^{147,457,485} GAGs are thought to promote more efficient attachment to cells via electrostatic interactions with the virus particle.

Cellular lectins also increase the efficiency of flavivirus attachment. CD209 Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin (DC-SIGN) is a calcium-dependent c-type lectin that serves as an attachment factor for several classes of viruses (reviewed in⁸³⁹), including some flaviviruses.^{183,605,811} These interactions are mediated by N-linked sugars on the prM and E proteins of the virion.^{182,406,480,504} CD209 is expressed *in vivo* on a subset of

dendritic cells (DCs) and macrophages.⁸³⁹ The infectivity of DCs by DENV correlates with CD209 expression; immature DCs express CD209 and are more permissive to infection than mature DCs expressing lower levels of CD209.^{811,893} Antibodies against CD209 or soluble forms of this lectin are capable of blocking DENV infection of DCs.^{605,811} Experiments with truncated forms of CD209 suggest that internalization of CD209 is not required to increase the efficiency of virus attachment to selected cell types.⁵⁰³ CD209L Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin-Related protein (DC-SIGNR)^{183,811} and the mannose

receptor (MR)⁵⁵⁶ have also been identified as attachment factors for flaviviruses.

Recent studies have identified members of the TIM and TAM families of phosphatidylserine receptors that function as attachment and potentially signaling factors for flaviviruses. These cellular proteins directly (in the case of TIM1) or indirectly (in the case of TAM proteins) bind lipids incorporated into the membrane of the virus particle. The expression pattern of this family of molecules may explain, in part, the broad cellular tropism of these viruses *in vitro*. Furthermore, the interaction of cellular proteins with the lipid envelope of the virion, thought to be buried by the dense icosahedral array of E proteins described above), raises questions about the structure(s) of infectious flaviviruses.^{543a}

Flavivirus enter cells via clathrin-mediated endocytosis.^{2,152,262,424,838} Elegant single-particle tracking studies of DENV suggest that virions move across the surface of cells until they encounter preformed clathrin-coated pits. Virus particles are then internalized and traffic into late Rab7-positive endosomal compartments where viral fusion occurs.⁸³⁸ Fusion between viral and cellular membranes is triggered by the acidic environment of endosomes. How viruses sense the low-pH environment is not completely understood, but may involve the protonation of key histidine residues on the E protein.^{241,375,608} Fusion may also be governed by the lipid composition of the endosome.⁹⁰⁹ That flaviviruses have the ability to fuse with synthetic liposomes devoid of proteins indicates that this process does not require interactions with a cellular receptor.^{162,264}

The molecular basis for the tropism of flaviviruses is unknown. A wide variety of cell lines representing different lineages and species may be infected *in vitro*. This suggests cellular factors involved in virus entry are either highly conserved (from mosquitoes to man) or redundant. Targets for flavivirus infection *in vivo* appear more restricted and include monocytes, macrophages, hepatocytes, neurons, endothelial cells, and DCs.^{38,704} Tropism may be regulated at a postentry level through the activities of interferon and interferon-stimulated genes.^{704,859}

Mechanisms of Dissemination

Blood-Borne Viruses

For both viscerotropic (e.g., DENV and YFV) and encephalitic (e.g., WNV, JEV, and TBEV) flaviviruses, the skin is the likely initial infection site after insect inoculation, with resident dendritic cells^{117,529} or epidermal keratinocytes⁴⁷⁸ believed to be the primary target cells. The dose of virus inoculated by the mosquito under conditions of natural infection is not known precisely, but likely ranges from 10^3 to 10^5 plaque-forming units (PFU),^{731,791} depending on the flavivirus and insect vector. Active WNV replication can be detected at the subcutaneous site of infection within one day of infection,¹⁰⁷ and virus spread to the lymph node occurs in animals infected by mosquitoes or with mosquito salivary extracts.^{731,792} Proteins in mosquito saliva alter cytokine levels and other components of innate immunity, leading to local immunosuppression or dysregulation,⁷²⁹ and enhanced spread and replication.

Flaviviruses disseminate to local lymph nodes either associated with migratory infected dendritic cells⁶⁶³ or as free virus that transudates directly into lymphatic fluid.³⁶⁹ Macrophages on the floor of the subcapsular sinus and in the medulla of lymph nodes capture viral particles efficiently, serving as possible targets of virus amplification infection and initiators of innate and adaptive immune responses.³⁶⁹ Virus produced in the draining

lymph nodes likely spreads to intravascular venous compartments via efferent lymphatic drainage. Virus in the bloodstream can directly infect blood cells or visceral tissues, which can result in further dissemination and secondary viremia.

The infectivity of flaviviruses in plasma, the fluid component of blood, appears remarkably short, with a half-life in mice ranging from 2 to 10 minutes for DENV and WNV, respectively.²⁴² The loss of infectivity is due in part, to complement (C3 and C4 components) opsonization via mannose-binding lectin recognition of N-linked glycans on the surface of virions.²⁴² The short half-life of infectious flavivirus in plasma may also reflect sequestration and removal by different visceral organs.³⁵⁵ Alternatively, flaviviruses may transit rapidly into the cellular compartment of blood. One study of patients with DENV infections of different disease severity showed DENV antigen (prM and NS3) predominantly in cells of monocyte (CD14⁺, CD32⁺) lineage, with up to 80% to 90% of cells of expressing viral antigen.²¹⁶ This finding of DENV in blood monocytes is consistent with prior literature⁵²⁶ but contrasts with newer studies in rhesus macaques, suggesting that platelets become positive for dengue antigen during the course of infection.⁶³² Finally, another explanation for the rapid drop of plasma infectivity is that flaviviruses adhere readily to erythrocytes in whole blood.⁶⁸³

Neurotropic Viruses

Flavivirus neuropathogenesis requires neuroinvasiveness, the capacity to enter the CNS, and neurovirulence, the ability to propagate efficiently within cells of the CNS. In classical studies, phenotypic distinctions were made among different arthropod-borne viruses on the basis of replication efficiency and pathogenic potential in peripheral versus CNS tissues.⁵ A main principle was the relationship between peripheral virus burden and the propensity to cause neuroinvasion. Viruses with a low capacity to replicate in the periphery generally had less neuroinvasive potential, regardless of their intrinsic neurovirulence. Aerosol-acquired and, perhaps, mucosal infections are possible exceptions, as these may use alternate routes of CNS entry.

Data from several studies indicate that the time of onset, magnitude, and duration of viremia, as well as the integrity of the host immune system influences the risk of entry into the CNS. Therefore, the neuropathogenic potential of most flaviviruses is a balance between the replication efficiency and the effectiveness of early host defenses in clearing viremia. Neuroinvasiveness is affected by both viral and host factors. Based on genetic analysis of virulent and attenuated strains of JEV, TBEV, YFV, and WNV, viral determinants of neuroinvasiveness map primarily to the E protein.^{54,56,130,541,615,616} The mechanisms associated with these genetic determinants have not been determined, but are believed to relate to increased viral infectivity of key target cells through enhanced binding and penetration.

Animal models of infection of encephalitic flaviviruses have begun to define factors that govern virus entry into the brain and spinal cord. Crossing of the blood-brain barrier (BBB) likely occurs through a hematogenous route, as increased viral burden in the serum correlates with earlier and enhanced viral entry into the brain.²⁰⁴ Accordingly, changes in endothelial cell permeability may facilitate CNS entry; these may be triggered by vasoactive cytokines^{509,868} or activation of matrix metalloproteinases that degrade the BBB extracellular matrix.^{845,864} Additional possible mechanisms may contribute to CNS infection of flaviviruses, including the following: (a)

direct infection or passive transport through the endothelium,^{212,488,846} and (b) infection of olfactory neurons and rostral spread from the olfactory bulb.¹⁰⁷ Access through the olfactory bulb is believed to occur either after infection by the aerosol or intranasal route^{602,618,669} or in the context of hematogenous dissemination of virus.⁵⁴² The olfactory bulb is vulnerable to direct infection because of the exposure of its nerve terminals within the olfactory mucosa; (c) a “Trojan horse” mechanism in which virus is transported by infected immune cells that traffic to the CNS⁸⁶⁵; (d) access to the CNS after breakdown of BBB integrity^{139,412}; and (e) direct axonal retrograde transport from infected peripheral neurons.^{351,584,705,860} Although much has been learned from infection studies in mice and hamsters, the precise mechanisms of CNS entry of encephalitic flaviviruses in humans and other animals requires additional study.

Mechanisms of Immune Control: Innate Immunity

Cellular Innate Immunity

MACROPHAGES

Although only limited studies have directly addressed the function of cellular innate immunity in flavivirus infection, emerging data suggest that macrophages play key roles in orchestrating control of infection. Macrophages can limit infection through direct viral clearance, enhanced antigen presentation to B and T cells, and production of proinflammatory or antiviral cytokines and chemokines.^{426,527} The protective role of macrophages is highlighted by studies in mice, which demonstrated exacerbated WNV, TBEV, DENV, or YFV disease after selective macrophage depletion.^{63,234,390,663,924} Macrophages may control flaviviruses through the production of nitric oxide (NO) and other reactive oxygen intermediates after stimulation of inducible nitric oxide synthetase (NOS-2).^{422,484,712,713} Activation of macrophages in response to flavivirus infection also promotes release of type I interferon (IFN), tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-8, and other cytokines, some of which have antiviral activity and reduce viral replication, at least in culture.⁷⁵⁷ Despite their protective role in innate defense, macrophages also are targets of infection by some flaviviruses,^{416,441,684} and have the potential to contribute to pathogenesis through antibody-dependent enhancement of infection mediated by Fc- γ and complement receptors.^{123,261,641} The macrophage cell surface receptor CLEC5a independently has been reported to interact with DENV directly, resulting in DAP12 phosphorylation and the release of proinflammatory cytokines.¹⁴⁴ Therefore, in some circumstances macrophages can contribute to flavivirus-induced disease, although the contribution to clearance versus pathogenesis may vary depending on the specific virus, the presence of preexisting nonneutralizing antibodies, and the specific proinflammatory molecules that are produced.

NEUTROPHILS

Although polymorphonuclear leukocytes (neutrophils) are among the first circulating leukocytes to respond to infection or inflammatory stimuli, their function in flavivirus infection remains uncertain. Some studies suggest a protective function; however, others indicate that neutrophils can contribute to flavivirus pathogenesis. A protective role was reported in the context of WNV infection as macrophages produced neutrophil chemoattractive chemokines (CXCL1 and CXCL2),

neutrophils rapidly migrated to the site of infection, and mice depleted of neutrophils 1 or 2 days after virus infection developed higher viremia and experienced earlier death.³⁰ Paradoxically, if neutrophils were depleted prior to infection, viremia was reduced and survival was enhanced.³⁰ Analogously, depletion of neutrophils resulted in prolonged survival and decreased mortality in Murray Valley encephalitis virus-infected mice, and neutrophil infiltration and disease correlated with NOS-2 expression within the CNS.¹⁴ Finally, transcriptional gene signatures from whole blood showed a greater abundance of neutrophil transcripts in patients who progressed to DSS, a finding supported by higher plasma levels of proteins associated with neutrophil degranulation.³³⁴ Although further studies are warranted, neutrophils may prevent or promote flavivirus disease, depending on the specific virus and immunologic context.

DENDRITIC CELLS

Human peripheral blood contains two types of dendritic cells (DCs), plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), which can be distinguished based on function and distinctive surface markers. pDCs lack phagocytic capacity and are less efficient in capturing and presenting antigens to T cells, but they produce extraordinarily high levels of type I IFN in the presence of viruses or bacteria,⁷⁵⁹ and are thus considered to play a crucial role in antiviral immunity.^{802,803} Low levels of DENV replication were observed in pDCs, but proinflammatory cytokines were produced rapidly and could accumulate to high levels. This cytokine response was not dependent on viral replication, but dependent on endosomal toll-like receptor 7 (TLR7), and could be induced by purified DENV RNA.^{797,862} In prospective clinical studies, the absolute number of circulating pDCs remained stable early in moderately ill children with dengue fever or other nondengue, febrile illnesses. However, there was an early decrease in circulating pDCs in children who subsequently developed DHF, as a blunted blood pDC response was associated with an altered innate immune response, higher viremia levels, and severe disease.⁶⁴⁶ Of interest, the host origin of the flavivirus influences the response that is generated by pDCs, as WNV grown in mammalian cells was a potent inducer of IFN- α secretion in pDCs, whereas pDCs failed to produce IFN- α when exposed to WNV grown in mosquito cells.⁷⁶²

mDCs reside and circulate throughout the body, enabling them to transport antigens from the periphery to lymphoid tissues. They are professional antigen-presenting cells that transmit incoming infectious signals to B and T cells to orchestrate rapid and efficient adaptive immune responses.⁷⁸³ mDCs are more readily infected by flaviviruses *ex vivo*, and are thought to contribute to viral spread and early immune system priming depending on the particular virus. For example, WNV efficiently infects mouse mDCs and induces a type I IFN and proinflammatory cytokine response through RIG-I-like pattern recognition receptors and IPS-1-dependent signaling cascade.^{178,800} In comparison, also in mice, JEV induced impaired responses through MyD88-dependent and -independent pathways, with blunted co-stimulatory molecule expression and production of the antiinflammatory cytokine IL-10, which resulted in poor T-cell priming.⁷ DENV productively infects human mDCs and induces release of high levels of chemokines and proinflammatory cytokines, with the notable exception of type I IFN,^{686,687} although this latter finding has not been observed with all strains of DENV.^{35,469} Moreover, mature mDCs were capable of

supporting antibody-dependent enhancement (ADE) of DENV, whereas immature DCs, due to expression of higher levels of DC-SIGN, did not promote ADE.⁸⁷

Despite an accumulating wealth of data on purified mDC *ex vivo*, few studies have assessed their direct function *in vivo* in the context of flavivirus infection. A recent report showed that selective genetic deletion of CD8 α ⁺ mDCs resulted in defective cross-presentation and virus-specific CD8⁺ T-cell responses to WNV.³³¹ The generation of diphtheria toxin receptor transgenic mice that selectively deplete DC subsets may allow further dissection of the net function of mDCs in flavivirus infection.

NATURAL KILLER CELLS

Natural killer (NK) cells are innate immune lymphocytes that serve as a first line of defense against a variety of infections.⁷⁵ NK cells mediate protection through the recognition and killing of target cells and the production of immunomodulatory cytokines, particularly IFN- γ , which enhances innate immunity and shapes the subsequent adaptive immune response.¹⁶¹ Unlike adaptive T and B lymphocytes, NK cells do not rearrange their receptor genes somatically, but rely on a fixed number of inhibitory and activating cell receptors that recognize major histocompatibility complex (MHC) class I and class I-like molecules, as well as other ligands.¹¹¹ The function of NK cells in flavivirus infection remains uncertain. Some *in vitro* studies suggest that human NK cells can expand and inhibit WNV infection of cells through both cytolytic (antibody-dependent cell-mediated cytotoxicity [ADCC]) and non-cytolytic (IFN- γ) activities.⁹¹⁴ The activating human NK-cell receptor, NKp44 has been reported to directly bind to domain III of DENV and WNV E proteins. This interaction induced IFN- γ secretion and lysis of WNV-infected targets by NK cells.³²⁸ However, flavivirus infection may inhibit NK-cell killing by increasing the cell surface expression of class I MHC molecules,^{209,329,400} which sends a negative signal to NK cells.³⁶³ *In vivo*, the function of NK cells in flavivirus infection also remains unclear. Although NK cells expand and become activated in YFV- and DENV-infected humans and mice,^{140,610,747} antibody depletion of NK cells in mice did not alter morbidity or mortality after WNV infection.⁷⁵⁵

$\gamma\delta$ T CELLS

$\gamma\delta$ T cells contribute to the innate defense against several viruses by virtue of their relative frequency in blood and epithelial sites and ability to respond rapidly to nonpeptide antigens and secrete proinflammatory cytokines and chemokines. Because they lack classical MHC restriction, $\gamma\delta$ T cells can react with viral antigens in the absence of conventional antigen processing.⁷⁸¹ $\gamma\delta$ T cells are divided into functionally distinct subsets, which have disparate effects on host immunity to pathogen infection. Splenic V γ 1⁺ $\gamma\delta$ T cells contribute to eliminating *Listeria* infection by virtue of their IFN- γ activity.⁵³⁶ In comparison, V γ 4⁺ $\gamma\delta$ T cells enhance Th1-cell activation through IFN- γ - and CD1-dependent mechanisms.³⁴⁹ To date, much of the initial analysis of $\gamma\delta$ T cell function during flavivirus infection has focused on studies with WNV in mice, although recent studies confirm that human and monkey $\gamma\delta$ T cells also are activated rapidly after YFV infection.^{196,611} Mice deficient in $\gamma\delta$ T cells were more susceptible to WNV infection,⁸⁶⁷ and this was in part due to their ability to produce IFN- γ , which has direct antiviral effects.⁷⁵⁶ Moreover, mice depleted of V γ 1⁺

$\gamma\delta$ T cells have enhanced viremia and higher WNV mortality, whereas the opposite is observed with depletion of V γ 4⁺ $\gamma\delta$ T cells.⁸⁷⁷ Subsequent work showed that $\gamma\delta$ T cells also contribute to the development of a protective CD8⁺ T-cell response against WNV, as TCR δ ^{-/-} mice were more susceptible than wild type mice to secondary WNV challenge.⁸⁶⁶ This priming effect may reflect DC maturation (increased expression of surface co-stimulatory and class II MHC molecules and secretion of IL-12) that is promoted by $\gamma\delta$ T cells after activation by WNV.²³¹

MAST CELLS

Mast cells contribute to a variety of inflammatory reactions and host defense against pathogens⁵¹⁹ by secreting chemokines, cytokines, and inflammatory lipid mediators and granule-associated products. Mast cells express several Fc receptors, reside primarily in tissues, and associate closely with vascular beds.⁷⁴⁴ Recent studies suggest that infection or activation of mast cells in tissues by DENV can promote viral clearance⁷⁷⁶ or have immunopathologic consequences that contribute to the vasculopathy associated with secondary infection. DENV infection of mast cells *ex vivo* resulted in increased secretion of chemokines, including CCL5 without inducing degranulation,³⁹⁸ and production of vasoactive cytokines was enhanced in the presence of subneutralizing concentrations of antibody that promotes ADE.³⁹⁹ Antibody-enhanced DENV infection of mast cells in culture also resulted in significant production of TNF- α , which can stimulate endothelial cells,¹⁰⁸ as well as massive mast cell apoptosis that occurs via global caspase activation.¹⁰⁹ Although more investigation is needed, DENV-infected mast cells may contribute to endothelial cell activation and permeability via local production of cytokines.

Cell-Intrinsic Immunity

RECOGNITION AND CONTROL OF FLAVIVIRUSES BY HOST SENSORS

Interferon (IFN) responses are an essential host defense program against many viruses, including flaviviruses. IFNs are produced during the earliest stages of viral infection after recognition of pathogen-associated molecular patterns (PAMPs) by specific pathogen recognition receptors (PRRs). In mammalian cells, the host detects and responds to infection by flaviviruses by primarily recognizing viral RNA through several distinct PRRs including the cell surface and endosomal RNA sensors TLR3 and TLR7, and the cytoplasmic RNA sensors retinoic acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) (Fig. 26.10). Binding of single- and/or double-stranded (ds) viral RNA to these PRR results in downstream activation of transcription factors, such as IFN regulatory factors 3 and 7 (IRF-3 and IRF-7) and NF- κ B, and induction of IFN- α and IFN- β . Secretion of IFNs followed by engagement of the IFN- $\alpha\beta$ receptor (IFNAR) in an autocrine and paracrine fashion activates janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway-dependent and independent signal transduction cascades⁴⁶⁵ that induce the expression of hundreds of IFN-stimulated genes (ISGs), a subset of which likely have antiviral activity against flaviviruses (Fig. 26.11).⁷³³

Recent studies suggest that RIG-I and MDA5 contribute to the induction of host IFN and antiviral response to flaviviruses (Fig. 26.10). Murine embryonic fibroblasts (MEFs) deficient in RIG-I and MDA5 showed decreased IRF-3 activation, delayed induction of host interferon and ISG responses, and augmented WNV and DENV replication.^{239,240,500,604} RIG-I

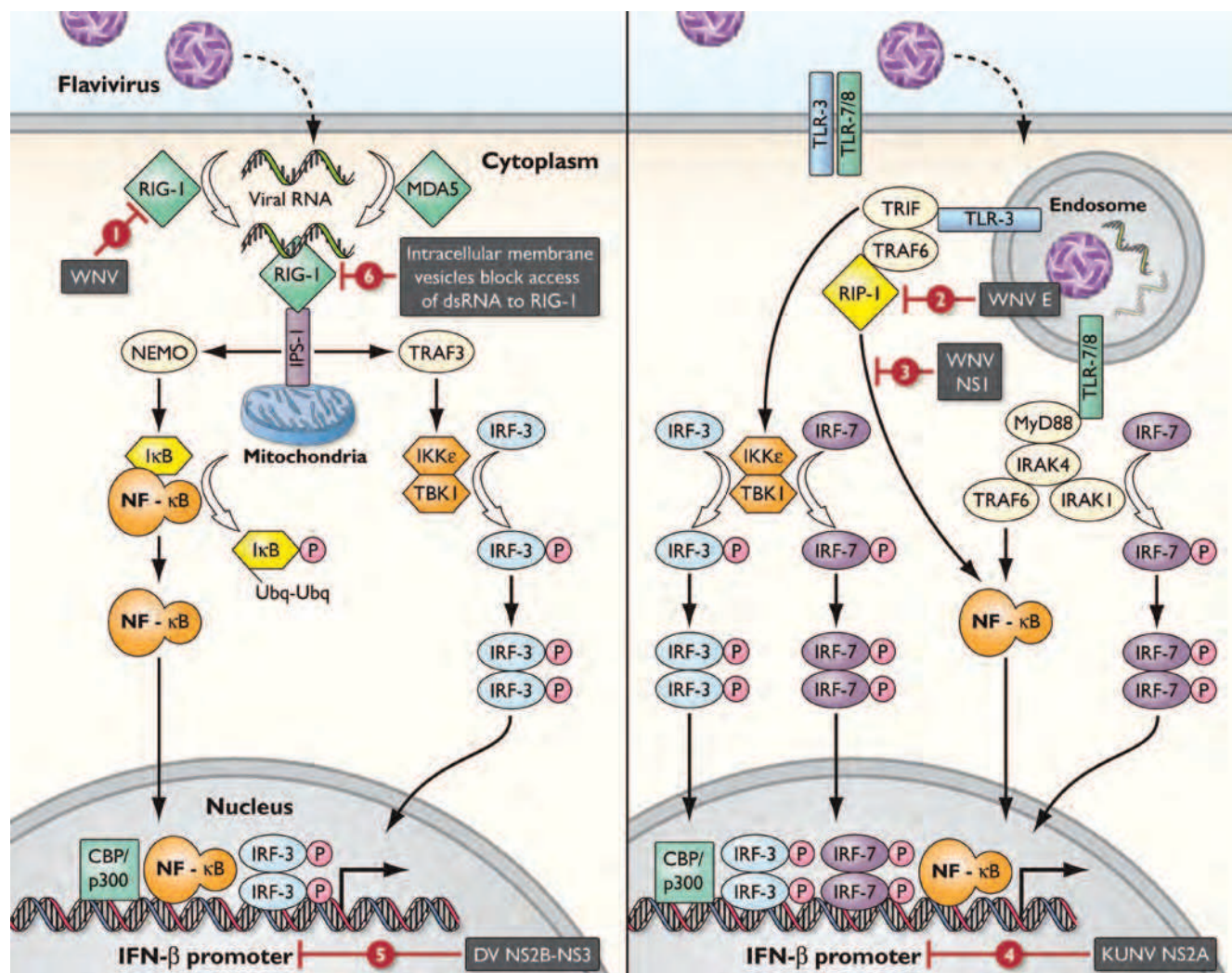


FIGURE 26.10. Detection of flavivirus RNA by pathogen recognition receptors (PRRs) and mechanisms of viral evasion.

(Left) Cytoplasmic PRR and signaling cascade. Infection by flaviviruses produces double-stranded RNA (dsRNA) replication intermediates that display motifs recognized by retinoic acid-inducible protein I (RIG-I) and possibly, the melanoma disassociation-associated 5 (MDA5) helicase. Binding of viral RNA promotes an interaction with IPS-1 that results in recruitment of signaling proteins (NEMO and TRAF3) that activate interferon regulatory factor 3 (IRF-3) and nuclear factor kappa B (NF- κ B). These transcription factors translocate to the nucleus and bind to the promoter region of the IFN- β gene leading to transcription and translation. **(Right)** Toll-like receptor (TLR) signaling cascade. In some cells, the transmembrane PRRs TLR3 and TLR7/8 in endosomes recognize double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) motifs leading to recruitment of cytoplasmic adaptor molecules (TIR-domain-containing adapter-inducing interferon- β [TRIF] and Mmeloid differentiation primary response gene (88) [MYD88]), respectively), which initiate signaling cascades (via I kappaB kinase [IKK] TANK-binding kinase 1 [TBK1] Receptor-Interacting Protein 1 [RIP-1] and interleukin-1 receptor-associated kinase 4 [IRAK-4]) that activate IRF-3, IRF-7, and NF- κ B, resulting in IFN- β gene transcription. Mechanisms of evasion by flaviviruses are believed to include the following: (1) a delay in recognition of West Nile virus (WNV) RNA by RIG-I; (2) impairment of RIP-1 signaling by high mannose carbohydrates on the structural E protein; (3) attenuation of TLR3 signaling by the NS1 protein; (4) reduction in IFN- β gene transcription by the Kunjin virus (KUNV) NS2A protein; (5) reduction of type I IFN production by catalytically active dengue virus (DENV) NS2B-NS3 protein; and (6) viral dsRNA intermediates localized to specialized membrane vesicles, which prevent rapid detection by intracellular sensors such as RIG-I.

appears to prime the early type I IFN response, whereas MDA5 has a more apparent role in a second phase of IFN-dependent gene expression that occurs later in the course of infection. A genetic deficiency of IPS-1 (also known as Cardif, mitochondrial antiviral signaling protein [MAVS], or virus-induced signaling adaptor [VISA]), an essential RIG-I and MDA5 adaptor molecule that is anchored to the outer leaflet of mitochondria, completely disabled the innate IFN response^{178,240} and was asso-

ciated with enhanced WNV lethality in mice with dysregulated immune responses.⁸⁰⁰ RIG-I-dependent signaling appears dominant in mice, as animals deficient in RIG-I were more vulnerable to JEV infection,³⁷⁹ and a deficiency of proteins that regulate the Tripartite motif-containing protein 25 (TRIM25)-mediated ubiquitination and activation of RIG-I resulted in enhanced WNV replication and mortality.⁸⁶³ Consistent with this, JEV and DENV induce the host type I IFN response

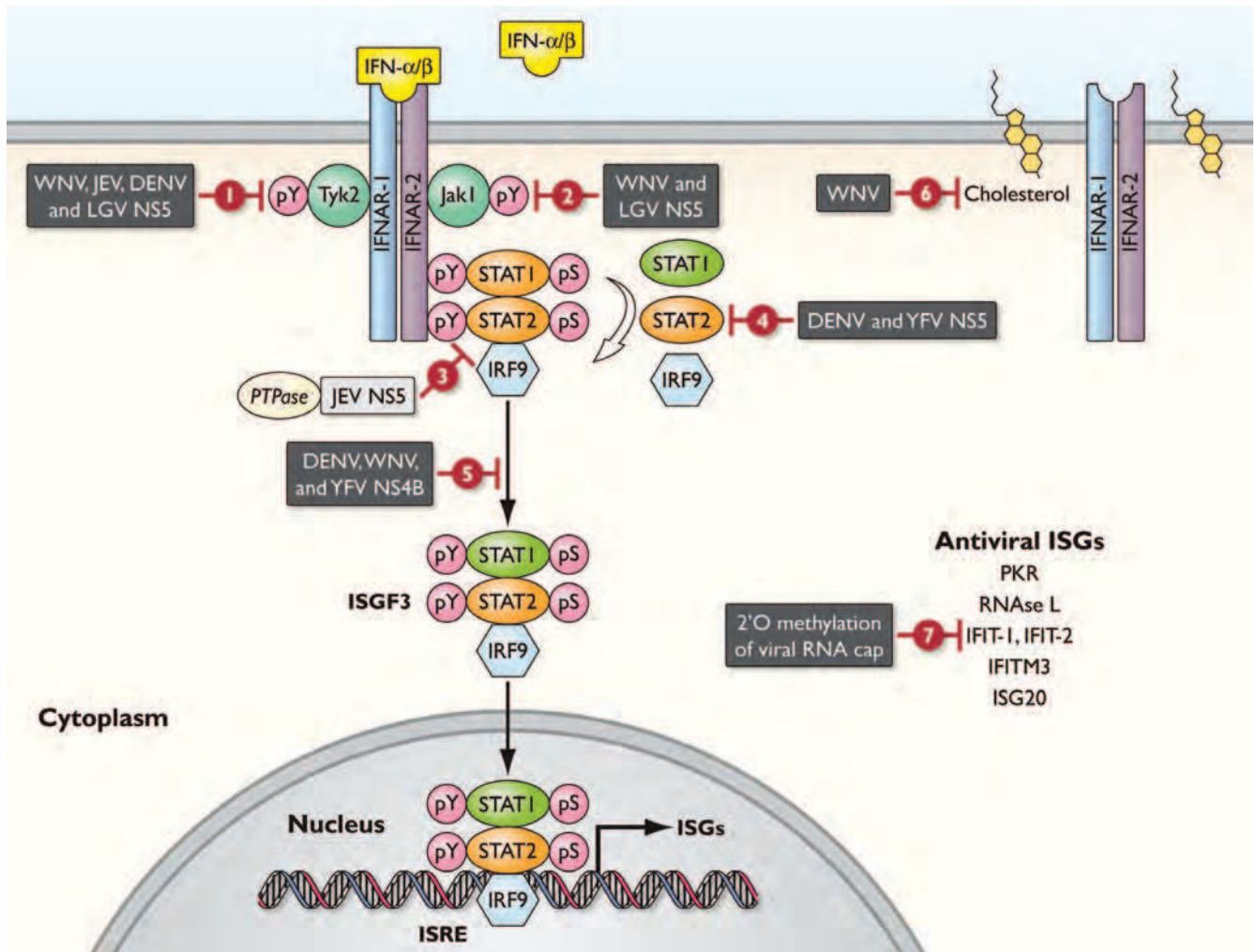


FIGURE 26.11. Type I interferon (IFN) signaling and mechanisms of disruption by flaviviruses. Secretion of IFN by a flavivirus-infected cell results in autocrine and paracrine signaling through the heterodimeric IFN- $\alpha\beta$ receptor (IFNAR). Binding by IFN results in activation and tyrosine phosphorylation of Janus kinase (JAK) family members (JAK1 and Tyk2) and the cytoplasmic tail of the IFNAR. This promotes recruitment of the signal transducers and activators of transcription (STAT1) and STAT2, which themselves become phosphorylated by the JAKs. Phosphorylated STAT1 and STAT2 proteins heterodimerize, associate with IRF-9, and translocate to the nucleus, where they bind interferon-sensitive response element (ISRE) sequences to induce expression of hundreds of interferon-stimulated genes (ISGs). Mechanisms of evasion by flaviviruses are believed to include the following: blockade of phosphorylation of (1) Tyk2 and (2) JAK1 by flavivirus NS5; (3) activation of a phosphotyrosine phosphatase by JEV NS5; (4) reduction in STAT2 gene and protein expression by Dengue virus (DENV) and Yellow fever virus (YFV) NS5; (5) attenuation of STAT signaling by flavivirus NS4B; (6) downregulation of the IFNAR through virus-induced redistribution of cellular cholesterol; and (7) antagonism of interferon-induced protein with tetratricopeptide repeats (IFIT) family genes effector functions by 2' O methylation of flavivirus RNA.

through a mechanism involving RIG-I/IRF-3 and NF- κ B.¹³⁶ MDA5 may be less important for flavivirus recognition, as IFN production by MDA5^{-/-} myeloid dendritic cells remains largely intact after WNV infection,²⁵⁶ and a deficiency of MDA5 in mice did not affect survival after JEV,³⁷⁹ although higher mortality rates are observed after WNV infection (H. Lazear and M. Diamond, unpublished results). Despite data from murine embryonic fibroblasts (MEF) suggesting that RIG-I and likely MDA5 recognize WNV RNA and induce type I IFN responses, IFN- α and β production in mice appears independent of IPS-1⁸⁰⁰ or the downstream transcription factor IRF-3.^{89,174} Therefore, individual cell types (myeloid, fibroblast, and neuronal) use distinct PRR responses to protect

against flavivirus infection through both IFN-dependent and IFN-independent pathways.¹⁷⁷

TLR3, which is expressed on the surface of fibroblasts and in the endosomes of myeloid cells, promotes IRF-3 phosphorylation after binding ds viral RNA through a complex signaling cascade that includes recruitment of TIR-domain-containing adapter-inducing interferon- β (TRIF) and activation of the kinases TANK-binding kinase 1 (TBK1) and I kappaB kinase (IKK ϵ).^{535,735} Initial studies with TRIF-deficient MEF suggested that TLR3 may be dispensable for recognition of flaviviruses in cells,²³⁹ although subsequent cell culture studies showed a proinflammatory and protective effect of TLR3 after DENV infection.^{604,834} Experiments in TLR3^{-/-} mice have had

conflicting results. TLR3^{-/-} mice injected by an intraperitoneal route paradoxically showed decreased WNV lethality despite higher peripheral viral titers, presumably because of blunted cytokine responses (e.g., TNF- α) that normally facilitate virus entry into the CNS.⁸⁶⁸ Consistent with a possible pathologic role, preliminary studies suggest a functional TLR3 allele is a risk factor for severe human TBEV infection.³⁹⁷ In comparison, other studies with TLR3^{-/-} mice and a different North American WNV strain showed increased viral burden in the brain and enhanced lethality,¹⁷⁶ as might be anticipated for a PRR that triggers a protective host immune response. *Ex vivo* and *in vivo* experiments suggest a cell-specific role for TLR3, as it protected against WNV largely by restricting replication in neurons.

TLR7 is an endosomal PRR that detects guanosine and uridine-rich single-stranded RNA²⁰⁷ and activates IRF-7 via the Myeloid differentiation primary response gene (88) (MYD88) adaptor molecule. IRF-7 was identified as a primary regulator of antiviral gene induction after YFV infection,²⁴⁷ with some of this activation occurring through TLR7 recognition of viral RNA.^{667,668,797,862} Similarly, DENV stimulates IFN production in pDCs in a TLR7-dependent manner after virus uncoating.⁸⁶² The antiviral IFN- α response against WNV is mediated primarily by IRF-7,¹⁷⁵ and at least some of this signal is attributed to recognition of viral RNA by TLR7. Indeed, both TLR7^{-/-} and MyD88^{-/-} mice show increased susceptibility to WNV infection, and this was associated with increased local infection and decreased production of IL-1 β , IL-6, IL-12, IL-23, and several chemokines, which altered leukocyte trafficking and virus control in several tissues.^{572a,805,828,878} In addition to its possible antiviral effects as an IFN effector molecule (see below), dsRNA-dependent protein kinase R (PKR) also may serve as a PRR for inducing interferon responses. In three different human cell lines, small interfering RNA (siRNA) knockdown and chemical inhibition of PKR blocked WNV-induced IFN synthesis.²⁵⁵

TYPE I IFN SIGNALING PATHWAY

Type I IFNs induce an antiviral state by upregulating genes with both direct and indirect inhibitory functions.⁶⁵¹ In mice, for example, there are at least 14 IFN- α and one IFN- β isoforms, in addition to multiple other subtypes.⁸⁴⁰ IFN- α and IFN- β are considered the dominant functional type I IFN in humans and are secreted by many cell types following virus infection. Type I IFN primes adaptive immune responses through stimulation of DCs, activation of B and T cells, and by preventing death of recently activated T cells.^{451,452,784} Pretreatment of cells with IFN- α or IFN- β inhibits flavivirus replication *in vitro*,^{68,203,482,505,849} but treatment after infection is less effective.^{13,166,203,704,778} Although flaviviruses can antagonize IFN-induced responses after infection, IFN still restricts replication and spread *in vivo*. Mice lacking the type I IFN receptor (IFNAR^{-/-}) or downstream signaling components (e.g., STAT1) show enhanced lethality and replication after infection with WNV,^{385,704} DENV,⁷⁴⁸ YFV,⁵⁴⁸ or MVEV.⁴⁹⁶ Increased infection occurred in normally resistant cell populations and tissues after flavivirus infection of IFNAR^{-/-} mice, suggesting that IFN acts, in part, to restrict viral tropism. The importance of type I IFN in controlling flavivirus infection has been confirmed in therapeutic disease models. Pretreatment of animals with IFN- α or inducers of IFN- α attenuates infection by SLEV, WNV, YFV, and Modoc viruses.^{106,366,463,464,650,812} The relevance of these pathways has been confirmed *in vivo* as

several recent microarray analyses have shown that animals or primary cells infected with flaviviruses produce a potent innate antiviral transcriptional gene signature characterized by genes downstream of type I IFN signaling.^{35,334,668,710}

TYPE I IFN-INDUCED GENES THAT CONTROL FLAVIVIRUS INFECTION

Progress has been made in defining the specific IFN-induced antiviral genes that limit flavivirus infection (Fig. 26.11). Initial studies showed that dsRNA-dependent PKR and 2'-5'-oligoadenylate synthase (Oas) proteins mediated intrinsic cell resistance to WNV.⁷⁰⁶ PKR is activated by binding dsRNA and phosphorylates the eukaryotic translation initiation factor 2 (eIF2- α) resulting in attenuation of protein synthesis.⁵⁵² PKR also may have independent antiviral effects by activating signaling pathways that augment type I IFN production^{48,255} and directly regulating IFN- β mRNA stability.⁷³⁸ RNase L is activated by 2'-5'-linked oligoadenylates that are synthesized by Oas enzymes. RNase L inhibits viral infections by functioning as an endoribonuclease that cleaves viral RNA^{920,921} and by generating small self-RNA PAMPs that amplify antiviral immunity through a RIG-I and MDA5-dependent pathway.^{517,518} RNase L^{-/-} MEF and macrophages supported increased WNV replication *in vitro*,^{706,717} and knockdown of RNase L enhanced infectivity in human cells.⁴⁸³ Moreover, mice deficient in RNase L showed increased lethality following WNV infection, with higher viral loads in peripheral tissues at early time points after infection.⁷⁰⁶

Although susceptibility to flaviviruses in mice has been mapped to a mutation in the *Oas* gene 1b, resulting in the expression of a truncated Oas isoform,^{532,643} the mechanism of control by this gene appears independent of RNase L⁷¹⁷ and the type I IFN signaling pathway.¹⁰⁴ Knock-in of the wild-type Oas1b allele into a flavivirus-induced disease susceptible mouse generated a resistant phenotype,⁷¹⁶ and murine cells that ectopically expressed Oas1b resisted WNV infection by preventing viral RNA accumulation inside infected cells.³⁷² Although biochemical studies have shown that Oas1b itself is an inactive 2'-5' Oas, recent experiments suggest that Oas1b inhibits Oas1a activity, resulting in reduced 2'-5' oligoA production in response to poly(I:C).²²⁰ Negative regulation of 2'-5' Oas by inactive Oas1b proteins may tune the RNase L response that could cause significant damage in cells, if it were not tightly controlled.

More recent studies have used ectopic expression and siRNA or short hairpin RNA (shRNA) knockdown strategies to identify key and novel ISGs that restrict infection of different flaviviruses. A large-scale ectopic lentivirus screen identified several regulatory and effector ISGs that inhibit infection of WNV and YFV in human cells.⁷³³ Ectopic expression of ISG15, a ubiquitin-like protein that conjugates to key proteins of the cellular innate immune response,²⁵³ inhibited replication of the JEV in human medulloblastoma cells,³⁴³ although ISG15^{-/-} mice or neurons are not more susceptible to WNV infection (M. Samuel, D. Lenschow, and M. Diamond, unpublished observations). Members of the Interferon-inducible transmembrane (IFITM) proteins were recently shown to inhibit an early entry step in infection of DENV and WNV in cells.⁹⁴ This observation was confirmed by ectopic expression studies in HEK293 cells.³⁵⁹ Other studies have suggested that viperin^{359,804} and ISG20^{359,922} also may inhibit infection by flaviviruses. Although the field is rapidly advancing with respect to identifying antiviral ISG against

flaviviruses, definitive studies in genetically deficient animals may be required to establish the cell- and tissue-specific nonredundant effects of individual ISG in controlling flavivirus infection in the context of a robust type I IFN response.

Chemokines

Depending on the specific flavivirus infection, individual chemokines and cytokines can either protect or contribute to pathogenesis. For encephalitic flaviviruses, production of inflammatory chemokines in the brain by neuronal and non-neuronal cells coordinates recruitment of lymphocytes for clearance of viral infection. Chemokines that have been detected in the brain or CSF after WNV, JEV, or TBEV infection of mice include CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CCL2 (MCP-1), CCL3 (MIP-1a), CCL4 (MIP-1b), and CCL5 (RANTES).^{245,291,404,746,825} WNV infection in the brain is associated with the early expression of the T-cell chemoattractant CXCL10 by virally infected neurons⁴⁰⁴; this expression proceeds in a caudal to rostral direction with higher levels detected in the cerebellum. This regional heterogeneity in CXCL10 expression is due to differential regulation by WNV-infected cortical versus cerebellar granule cell neurons and leads to enhanced trafficking of WNV-specific T cells that express the CXCL10 receptor CXCR3 into the cerebellum.⁹¹³ Loss of CXCL10 or CXCR3 via targeted deletion or neutralizing antibody administration leads to decreased recruitment of WNV-specific CD8⁺ T cells into the CNS, especially within the cerebellum, increased viral loads, and enhanced mortality.^{404,913} In contrast, antagonism of polarized CXCR4–CXCL12 interactions along the BBB improved survival from lethal WNV infection through enhanced intraparenchymal migration of WNV-specific CD8⁺ T cells within the brain, leading to reduced viral loads and decreased immunopathology.⁵⁴⁰

A genetic deficiency in CCR2, a chemokine receptor on inflammatory monocytes and other leukocyte subtypes, resulted in markedly increased WNV-induced mortality in C57BL/6 mice,⁴⁷⁷ and was associated with a selective reduction of monocyte accumulation in the brain. Subsequent experiments showed that CCR2 mediates selective peripheral blood monocytoysis in the context of WNV infection, and this is critical for accumulation of protective monocytes in the brain. Although a protective role for CCL2–CCR2 interactions was observed with a virulent WNV isolate, an opposing phenotype was seen after infection with an attenuated strain; neutralization of CCL2 reduced the number of microglia in the brain during WNV infection but prolonged the life of infected animals.²⁵² Therefore, depending on the virulence of the strain, CCL2–CCR2-dependent monocyte accumulation and migration may differentially affect disease outcome after WNV infection.

Additional studies have established that the chemokines CCL3, CCL4, and CCL5, all of which bind to the chemokine receptor CCR5, are strongly induced within the brain after WNV infection.^{258,404,746} Moreover, targeted deletion of CCR5 is associated with depressed leukocyte trafficking, increased viral burden, and enhanced mortality.²⁵⁸ An analysis of WNV infection in humans with CCR5Δ32, a defective CCR5 allele, showed that homozygosity for the allele correlated with an increased risk of symptomatic disease.^{259,475,476} Because the mouse studies examined the entire brain with regard to expression and leukocyte trafficking, and the human studies did not report on specific neurologic symptoms, it remains

unclear whether CCR5-expressing leukocytes also exhibit regional specificity during CNS recruitment. Finally, a case of YFV vaccine-associated viscerotropic disease was associated with genetic polymorphisms in both *CCR5* and *CCL5* genes.⁶⁶²

For viscerotropic flaviviruses such as DENV, the function of chemokine interaction with their receptors remains less certain. Although DENV-infected wild-type mice produce high levels of chemokines CCL2, CCL3, and CCL5 in their spleen and liver, CCR1^{−/−} mice had a phenotype similar to wild-type mice, whereas infection of CCR2^{−/−} or CCR4^{−/−} mice showed attenuated lethality, liver damage, leukocyte activation, and levels of IL-6 and IFN-γ without significant differences in viral load.²⁸³ Therefore, chemokine–chemokine receptor interactions in the context of DENV infection appear to contribute to the development of disease. Nevertheless, in an encephalitic mouse model of DENV infection, CXCL10 interaction with CXCR3 was required for clearance and resistance to infection.³⁴⁴

Complement Activation and Flaviviruses

The complement system is a family of serum and cell surface proteins that recognize PAMPs, altered-self ligands, and immune complexes. Although complement activation inhibits infection of many viruses (reviewed in^{27,788}), it has both protective and pathogenic roles in flavivirus infection depending on the specific virus, phase of the infection, and immune status of the host. Activation of the complement cascade triggers several antiviral functions, including pathogen opsonization and/or lysis, and priming of adaptive immune responses. Complement is activated through the classical, lectin, and alternative pathways depending on specific recognition molecules.^{855,856} Classical pathway activity is triggered by C1q binding to antigen–antibody complexes on the surface of pathogens. The lectin pathway is initiated by mannose binding lectin (MBL) or ficolin recognition of carbohydrate structures on the surface of microbes or apoptotic cells. The alternative pathway is constitutively active at low levels through the spontaneous hydrolysis of C3 and also amplifies activation of the classical and lectin pathways. The classical, lectin, and alternative pathways generate convertase enzymes (C4bC2a for classical and lectin, and C3bBb for the alternative), which cleave C3, the central component of the complement system, and expose a reactive internal thioester bond on C3b necessary for covalent attachment to target surfaces. The binding of C3b back to C4b2a and C3bBb C3 convertases forms the classical and alternative pathway C5 convertases, respectively. These enzymes cleave C5 and promote assembly of C5b-9 membrane attack complex (MAC), which lyses pathogens or infected cells. Sublytic amounts of C5b-9 on a cell surface can activate granulocytes and endothelial cells, whereas soluble C5b-9 independently induces inflammation through cytokine induction. The release of anaphylatoxins (C3a and C5a) by the C3 and C5 convertases also promotes chemotaxis of immune cells via the interaction with specific G-protein coupled transmembrane receptors (C3aR and C5aR). Deposition of C3 and C4 fragments (C3b and C4b) on a pathogen facilitates binding and phagocytosis by complement receptors (CR1, CR3, CR4, and CRIg), a process called opsonization, which helps to clear microbial infections.¹²⁵

PROTECTIVE ACTIVITY OF COMPLEMENT ON FLAVIVIRUS INFECTION

Complement can limit flavivirus infection by stimulating adaptive immune responses or by directly neutralizing infection. In support of an immune priming role for complement, C3^{−/−} mice are more

susceptible to lethal WNV infection and show greater viral burden and reduced antiviral antibody titers.⁵⁴⁷ Infection studies with mice lacking C1q, MBL, C4, or factor B establish that all complement activation pathways protect against WNV infection.^{243,545} However, each activation pathway appears to exert distinct effects in response to WNV infection. Humoral IgM responses to WNV depend upon activation of C3 by the lectin recognition pathway. In contrast, both the lectin and alternative pathways appear necessary for efficient T-cell priming, as C4^{-/-}, factor B^{-/-}, and factor D^{-/-} mice exhibited reduced WNV-specific CD8⁺ T-cell responses.⁵⁴⁵ The T-cell defects in C4^{-/-} mice may be indirect as depressed IgM responses could affect viral opsonization and antigen presentation. The terminal lytic complement components (C5–C9) do not appear to serve a major function in protection, as C5 neither contributed to protection against WNV pathogenesis nor augmented the neutralizing efficacy of complement-fixing anti-WNV neutralizing antibodies in mice.⁵⁴⁶

Flaviviruses directly trigger complement activation, which can inhibit infectivity. Increasing concentrations of serum complement neutralize WNV or DENV in cell culture and *in vivo* in the absence of antibody, and this depends on recognition of N-linked glycans on the surface of the virion by MBL.²⁴² Complement activation by flaviviruses occurs *in vivo*, as C3 and C4 consumption occur prior to the induction of a specific antibody response.⁵⁴⁵

Complement augments antibody-mediated neutralization of flaviviruses, including YFV, DENV, and WNV.^{192,547,775} The C1q component of complement is sufficient to enhance the potency of antibody neutralization as it reduces the number of antibodies that must bind the virion to neutralize infectivity.⁵⁴⁶ The protective efficacy of flavivirus neutralizing antibodies *in vivo* correlates with IgG subclasses that efficiently fix complement.^{546,724}

PATHOLOGIC EFFECTS OF COMPLEMENT ON FLAVIVIRUS INFECTION

In myeloid cells that express complement receptors, antibody-dependent complement activation paradoxically may enhance viral infection.^{123,124} Blockade of complement receptor-3 (CD11b/CD18) abrogated the complement-dependent enhancement of WNV infection in this model system. Therefore, under certain circumstances, antibody and complement-dependent opsonization of flaviviruses may increase infection in myeloid cells.

During severe secondary DENV infection, a vascular leakage syndrome occurs with fluid transudation into serosal spaces. Although the pathogenesis of DENV infection remains incompletely understood, a pathologic role for complement activation has been proposed.^{28,85} In early clinical studies, reduced levels of C3 and C4 and factor B and increased catabolic rates of C3 and C1q were observed, particularly in patients with severe DENV disease.⁸⁵ In addition, C3 breakdown products and anaphylatoxins accumulated in the circulation of severely ill patients and peaked at the day of maximum vascular leakage.^{157,516} Circulating immune complexes formed by virions and DENV-specific antibodies have been hypothesized to cause the pathologic complement activation.⁸⁵ One alternative hypothesis is that infected cells express sufficient amounts of DENV antigens (E or NS1 proteins) on their surface, thereby facilitating immune complex formation and complement deposition.⁶⁹ Indeed, DENV-infected endothelial cells activate human complement in the presence of antibodies resulting in C5b-9 deposition.^{26,28}

Humoral Immunity

Humoral immunity contributes significantly to the host response to flavivirus infection. Virus challenge experiments using inbred strains of mice identified the importance of B cells during a protective response.^{204,205} The importance of antiviral antibody has been established directly. Passive administration of virus-reactive mAbs, purified polyclonal γ -globulin, and immune sera confers significant protection in small animal models of flavivirus infection.²⁰² For example, transfer of heat-inactivated WNV-immune serum into wild-type mice completely protects from lethal infection with WNV; administration of antibody into *uMT* or *RAG1* KO mouse strains prior to virus challenge delays mortality but does not protect from death.²⁰⁴ Antibodies may also protect from disease when administered therapeutically after infection.²⁰² Although neutralizing antibody titers correlate with protection by several flavivirus vaccines,^{60,320,533,575} the relationship may be imperfect.⁸¹ Antibodies also may exert protective effects via effector functions mediated by the Fc portion of the antibody molecule, including complement fixation, antibody-mediated cellular cytotoxicity, and facilitating virus and clearance.^{546,724,725} Protective antibodies that bind epitopes on the prM and E structural proteins incorporated into virions, as well as the nonstructural protein NS1, have been characterized.^{211,381}

STOICHIOMETRIC REQUIREMENTS FOR NEUTRALIZATION

Antibody-mediated neutralization of flaviviruses requires engagement by antibodies with a stoichiometry that exceeds a particular threshold (reviewed by²¹¹). From this perspective, the number of antibodies bound to the virion is controlled by the functional avidity of the antibody for viral antigens on the virion, and the number of times an epitope is displayed on the virion in a context in which it is accessible for antibody binding. Complexities that modulate the accessibility of viral epitopes include the dense arrangement of E proteins on the surface of the mature virion,^{497,624,629,787} the extent of virion maturation,⁶⁰⁷ and the structural dynamics of the virus particle.^{210,497} Antibody avidity determines the fraction of accessible antibody epitopes bound by antibody molecules at any concentration of antibody.^{211,403} The mAb E16 binds a relatively accessible epitope on the E-DIII-LR and supports neutralization at a low occupancy.⁶⁴⁸ Similar findings were reported for neutralizing anti-DENV antibodies that bind a similar epitope.²⁷⁹ By comparison, antibodies that bind poorly accessible “cryptic” epitopes may neutralize only at saturation.^{280,607,648,787} It is notable that some epitopes are not displayed with a frequency that allows engagement of the virion with a stoichiometry that allows for neutralization, even when fully occupied. Therefore, antibodies that bind cryptic epitopes may be incapable of neutralization regardless of their functional affinity for the virion.^{607,648} The limited neutralizing activity of prM-specific antibodies may simply reflect an inability to bind the virion enough times (Fig. 26.12).

The stoichiometric requirements for neutralization of WNV have been estimated.⁶⁴⁸ Experiments with E-DIII-LR-specific mAbs demonstrate that neutralization requires occupancy of 20% to 25% of accessible epitopes on the mature virion. Because only 120 epitopes on the mature virion are accessible for antibody binding, this translates into a requirement for engagement of the virion by roughly 30 antibody molecules. The functional significance of this number is not yet clear. Of potential interest, it does agree with predictions of the “coating

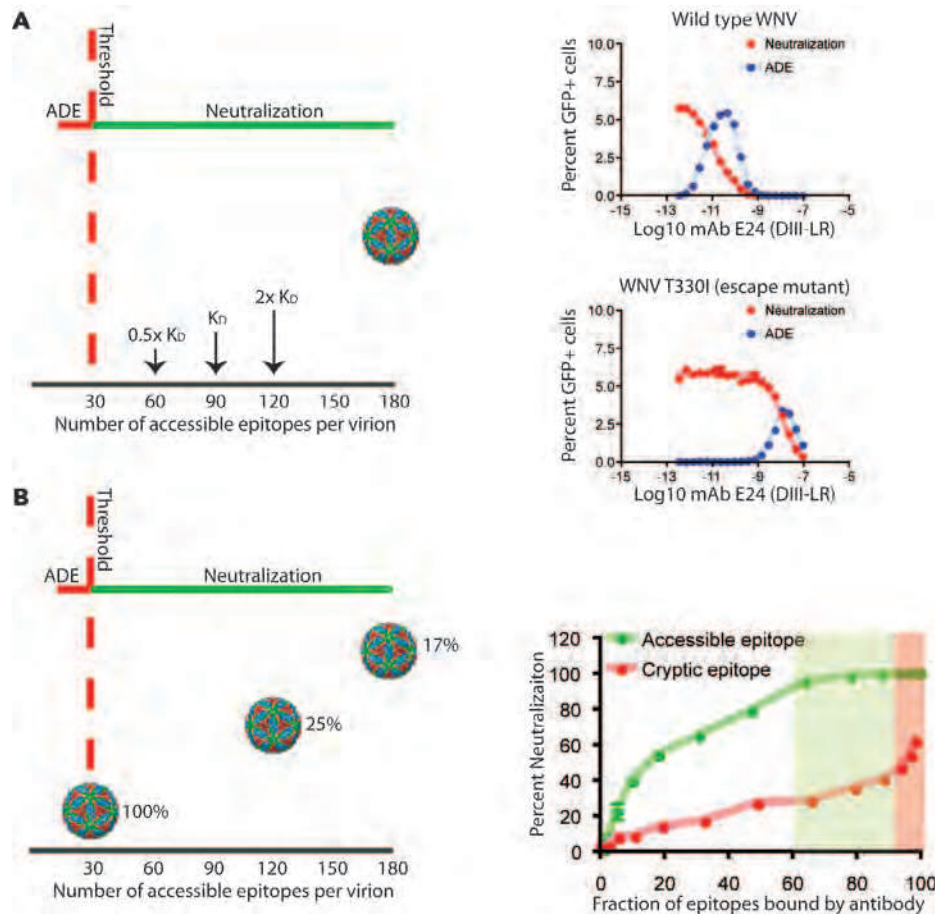


FIGURE 26.12. Antibody affinity and epitope accessibility govern the neutralization potency of anti-flavivirus antibodies.

Neutralization of flaviviruses is a multiple-hit phenomenon that requires engagement of the virion by antibody with a stoichiometry that exceeds a threshold estimated at approximately 30 antibody molecules. The number of antibody molecules bound to the virion at any given antibody concentration is determined in part by the strength of the antibody–virion interaction and epitope accessibility. **A:** The affinity of antibody–virion interactions determines the fraction of epitopes displayed on the virion bound by antibody at given concentration. **(Right)** Therefore, changes in antibody affinity (conferred in this example via mutation of the antibody epitope) results in a change in the number of antibodies bound to the virus particle at each antibody dilution and a shift in the neutralization profile toward higher concentrations of antibody. **B:** Epitope accessibility, which may vary considerably depending on its location on the virion and the maturation state of the virion, governs the occupancy requirements for neutralization. For a theoretical flavivirus displaying 180 epitopes, an epitope occupancy of 17% is required to exceed a stoichiometric threshold of 30 antibody molecules. A reduction in epitope accessibility translates into increases in the fraction of epitopes that must be engaged to support virus neutralization. **(Right)** Antibodies that bind highly accessible determinants may completely neutralize infection at relatively modest occupancy (60%, *green shading*), whereas antibodies that bind poorly accessible structures neutralize infection only at full occupancy (*red shading*). (Reproduced from Dowd KA, Pierson TC. Antibody-mediated neutralization of flaviviruses: a reductionist view. *Virology*. 2011;411(2):306–315, with permission.)

theory” model that suggest the number of antibodies required to neutralize a virion is determined by surface area of the virus particle.¹¹⁴ It will be important to determine the stoichiometric requirements for neutralization by antibodies that bind other epitopes; this is complicated experimentally as antibody-mediated neutralization of many other epitopes is modulated by the extent of maturation and structural dynamics of the virion.

MECHANISMS OF NEUTRALIZATION

Antibodies have the capacity to neutralize directly the infectivity of viruses via several mechanisms that act at distinct steps in the virus life cycle. Anti-flavivirus antibodies can block virus attachment to host cells.^{170,317,624} At present, it is unknown whether

antibodies that block attachment to cells do so by interfering with specific interactions with receptor on the target cells or via a general steric hindrance mechanism. Flavivirus-reactive antibodies also may block infection to virions after the attachment step. Studies by Gollins and Porterfield demonstrated that antibodies could block the uncoating and infectivity of WNV, even when added after virions attached to cells. Furthermore, they demonstrated that antibodies could directly block fusion of virions to synthetic liposomes.²⁶³ These observations have been expanded to include other flaviviruses and antibodies.^{689,822} Analysis of the ability of a large panel of TBEV-reactive antibodies to block liposomal fusion reveal mAbs capable of blocking fusion completely (25% of antibodies tested), partially (58% of

antibodies tested), or not at all (17% of antibodies tested).⁷⁸⁶ These results suggest that an ability to block fusion is a relatively common functional property of neutralizing antibodies. Two recent atomic structures illustrate different ways antibodies that decorate the virus particle may block fusion. Experiments with Fab fragments of mAb E16 suggest this antibody blocks the radial expansion of the virus particle and traps it in an intermediate step of the fusion process following exposure of the virus to acidic conditions.³⁸² In contrast, the complex epitope recognized by the mAb CR4354, which was composed of multiple E proteins, suggests a mechanism of inhibition by which E proteins on the surface are cross-linked together, preventing the rearrangements that propel fusion.³⁸⁴

How an antibody neutralizes flavivirus infection may depend on context, as an individual antibody may block virus infection at more than one step of the virus entry pathway. For example, mAb E16 partially blocks attachment at relatively high antibody concentration. Virus binding is not significantly inhibited at lower concentrations of antibody at which significant virus neutralization is observed.^{210,624} This observation suggests that the stoichiometric requirements for neutralization may differ depending on the mechanism of inhibition.

ANTIBODY-DEPENDENT ENHANCEMENT OF INFECTION

Antibody-dependent enhancement (ADE) of infection describes a phenomenon in which a significant increase in the efficiency of virus infection is observed in the presence of virus-reactive antibody.^{306,307} Although ADE has been demonstrated for several families of viruses *in vitro*, a role for enhancing antibodies *in vivo* has been suggested in only a few contexts, including secondary DENV infection.³⁰⁰ Passive transfer of DENV-reactive antibodies increases viral burden and exacerbates disease in an IFN- $\alpha\beta\gamma$ receptor-deficient murine model (AG129 mice) of infection and pathogenesis^{39,912} and increases viremia in primates.^{265,297} The mechanism of ADE has been studied extensively, but remains incompletely understood. ADE is most commonly, although not exclusively, observed on cells that express Fc- γ or complement receptors.³⁰⁰ Antibodies enhance infection by increasing the efficiency of virus attachment to target cells and thus, is of significantly reduced magnitude on cells with the capacity to bind viruses via other attachment factors.⁶⁴⁸ For example, Fc- γ -receptor expressing immature dendritic cells do not support ADE due to the expression of CD209. By comparison, ADE occurs with mature dendritic cells that lack expression of CD209.⁸¹¹ ADE can be inhibited by antibodies that block Fc- γ -receptor interactions,⁶⁴⁰ enzymatic removal of the heavy chain of the antibody molecule,⁹¹² and removal of the N-linked sugar on IgG molecules.^{39,265,648}

What are the properties of antibodies that enhance infection? Virus neutralization and the phenomenon of ADE are related by the number of antibody molecules bound to the virion; antibodies that neutralize flaviviruses also have the potential to enhance infection at subneutralizing concentrations.²¹¹ Antibodies that bind the virion with low affinity will enhance infection at higher concentrations relative to molecules that engage the virion via high affinity interactions. Furthermore, antibodies that recognize poorly accessible epitopes support ADE at higher concentrations than antibodies that bind readily accessible determinants. Estimates of the stoichiometric requirements for ADE identified a requirement for more than a single antibody molecule; enhancement of Fc- γ -RII-expressing cells

required engagement of at least 15 DIII-LR-specific mAbs.⁶⁴⁸ Presumably this is the minimal number of antibodies required for stable attachment of the virion-antibody complex to cells. Whether the requirements for ADE differ on cells expressing other Fc- γ -receptor molecules has not been investigated.

Recent studies indicate that complement can restrict ADE. Complement minimized ADE of WNV and DENV infection in Fc- γ R-expressing cells.^{544,899} Experiments with mouse sera deficient in individual complement components indicate that C1q is sufficient to restrict ADE of WNV infection *in vitro*. This effect was IgG subclass-dependent, as C1q restricted ADE by a human IgG₃ isotype-switch variant, but had little effect on IgG₂ and IgG₄ subclass variants; these results correlate with the known affinity of human IgG subclasses for C1q.⁷⁴ The addition of complement reduces the stoichiometry of neutralization by antibodies such that for IgG subclasses that bind C1q avidly, the reduced threshold of neutralization falls below the minimal number of antibodies required for ADE of infection.^{647,648}

THE REPERTOIRE OF ANTIBODIES ELICITED *IN VIVO*

The composition of the polyclonal antibody response elicited by infection is incompletely understood. Recent studies suggest that the humoral immune response of humans is directed against the highly conserved fusion loop of E-DII.^{61,191,823} In agreement, biochemical studies with recombinant proteins and virus particles incorporating mutations in the fusion loop suggest a significant portion of the reactivity maps to this conserved structure.^{168,169,628} Functional approaches to measure the relative contribution of epitopes on the E protein toward the neutralizing and protective activity of sera are being developed.^{186,607,628,853}

T-Cell-Mediated Control

CD8⁺ T CELLS

CD8⁺ T cells, by virtue of their ability to lyse infected target cells and produce inflammatory cytokines (e.g., IFN- γ and TNF- α), can have either protective or pathologic effects depending on the context. Indeed, depending on the flavivirus strain and experimental system, beneficial or adverse functions of CD8⁺ T cells have been reported. Experiments in small animal models and *in vitro* demonstrate that T lymphocytes can be an essential component of protection against infection by several different flaviviruses, including WNV, DENV, YFV, and JEV.^{77,100,101-102,490,595,596,664,752,765,901,903} Consistent with this, individuals with hematologic malignancies and impaired T-cell function have an increased risk of neuroinvasive WNV infection.^{598,658} Upon recognition of a flavivirus-infected cell that expresses class I MHC molecules, antigen-restricted cytotoxic T lymphocytes (CTLs) proliferate, release proinflammatory cytokines,^{101,209,388,426,664} and lyse cells directly through the delivery of perforin and granzymes A and B, or via Fas-Fas ligand interactions. After WNV infection, mice deficient in CD8⁺ T cells had higher and sustained WNV burdens in the spleen and CNS and increased mortality.^{752,870} CD8⁺ T cells require perforin and Fas ligand interactions to control infection of virulent North American WNV strains, as mice deficient in these molecules had increased CNS viral burdens and lethality.^{753,755} Moreover, adoptive transfer of wild-type but not perforin or Fas-ligand-deficient CD8⁺ T cells decreased CNS viral burden and enhanced survival. In comparison, granzymes appear important for the control of the lineage II isolate Sarafend, with perforin, Fas, and Fas ligand having a

more limited role in modulating infection.⁸⁷¹ The net function of CD8⁺ T cells in infection by other encephalitic flaviviruses (e.g., JEV or MVEV) also varies. Initial reports showed that JEV-specific cytotoxic CD8⁺ T cells could reduce production of infectious virus from infected macrophage and neuronal-like cells *in vitro*.⁵⁹⁵ Moreover, adoptive transfer of anti-JEV CD8⁺ T cells by an intracerebral route protected adult but not newborn or suckling BALB/c mice against lethal JEV challenge.⁵⁹⁶ However, in vaccine immunization studies, challenge experiments in CD8 T cell^{-/-} mice, indicate that CD8⁺ T cells are dispensable and that antibody was the most critical component of protection.⁶³⁷ CD8⁺ T cells may have a lesser role *in vivo* in JEV infection because of active subversion of the antigen-presentation pathway by the virus; recent reports suggest that JEV infection leads to active depletion and impairment of CD8 α ⁺CD11c⁺ dendritic cells,^{7,8} which are the cells that predominantly mediate cross-presentation of antigen and priming of CD8⁺ T-cell responses *in vivo*.³³¹ With MVEV infection, effector CD8⁺ T cells in the brain appear pathologic, as mice deficient in granule exocytosis (perforin or granzyme B) or Fas-mediated cytotoxicity showed delayed and reduced mortality.⁴⁷¹

For DENV, which generally does not cause encephalitis, the protective or pathologic function of CD8⁺ T cells depends on whether the response is primary or memory. During primary infection of mice, depletion of CD8⁺ T cells before infection resulted in significantly higher viral loads. DENV-specific CD8⁺ T cells produced IFN- γ and TNF- α , and exhibited cytotoxic activity *in vivo*.⁹⁰³ In comparison, a pathogenic role of CD8⁺ T cells has been described during secondary DENV infection. Due to the significant amino acid sequence homology among the four serotypes, there is a high potential for T-cell cross-reactivity during secondary heterologous DENV infection. Serotype cross-reactive CD8⁺ T cells are preferentially activated during secondary infection in humans in a phenomenon termed “original antigenic sin”.⁵⁷⁷ These cross-reactive CD8⁺ T cells exhibit altered cytokine production and reduced cytolytic activity.^{49,57,524,578} Aberrant cytokine production by T cells could contribute to severe DENV disease, as higher levels of proinflammatory mediators may contribute to endothelial cell dysfunction or damage, leading to plasma leakage.⁵³⁴

CD4⁺ T CELLS

CD4⁺ T cells can restrict or contribute to pathogenesis depending on the flavivirus, and whether the response is primary or anamnestic. Studies in mice have shown that CD4⁺ T cells restrict pathogenesis of primary WNV infection. A genetic or acquired deficiency of CD4⁺ T-cell function resulted in protracted WNV infection in the CNS that culminated in uniform lethality by 50 days after infection. CD4⁺ T cells protect against primary WNV infection by providing help for antibody responses, sustaining WNV-specific CD8⁺ T-cell responses in the CNS that enable viral clearance, producing antiviral cytokines, and killing cells.^{102,765} A protective role for CD4⁺ T cells against lethal JEV infection in mice was observed as depletion reduced and adoptive transfer promoted survival.⁷⁷ Moreover, in humans, impaired JEV-specific CD4⁺ T-cell function (e.g., IFN- γ secretion) was seen preferentially in patients with encephalitis and neurologic sequelae.⁴³⁰ Consistent with this, CD4^{-/-} mice also showed greater susceptibility to CNS infection by a neuroadapted strain of YFV.⁴⁹⁰ In comparison,

depletion of CD4⁺ T cells prior to DENV infection in mice had no effect on tissue viral burden, DENV-specific antibody titers or neutralizing activity, or CD8⁺ T-cell responses.⁹⁰¹

Memory CD4⁺ T cells can have protective or pathologic consequences, depending on the context. For DENV, immunization schemes that elicit antigen-specific CD4⁺ T cells prior to infection of mice resulted in significantly lower viral burden after challenge with homologous DENV.⁹⁰¹ Therefore, induction of CD4⁺ T cells by immunization can contribute to viral clearance. However, during heterologous secondary DENV infection, cross-reactive CD4⁺ memory T cells may be stimulated by antigen from the secondary infection. These CD4⁺ T cells then augment the response of memory CD8⁺ T cells, which can result in an overexuberant production of inflammatory cytokines and an increased risk for severe DENV disease.⁵⁷

CD4⁺CD25⁺FoxP3⁺ REGULATORY T CELLS

Regulatory CD4⁺ T cells (Tregs) are a subset of CD4⁺ T cells that can suppress effector T cells to control reactivity to self-antigens and pathogens.^{702,801} These cells function to blunt inflammation and to maintain antigen-specific T-cell homeostasis.^{432,625} A recent study showed that Tregs control the development of symptomatic WNV infection in humans and mice.⁴⁴⁹ Symptomatic WNV-infected mice and humans had lower Treg frequencies compared with asymptomatic cohorts, and Treg-deficient mice developed lethal WNV infection at a higher rate than controls. Of interest, in severe DENV infection in humans, although Tregs expand and function normally, their relative frequencies are insufficient to control the immunopathology of severe disease.⁵⁰⁶ Given their relatively recent identification, future studies will undoubtedly clarify the role of Tregs in preventing or promoting flavivirus pathogenesis.

FLAVIVIRUS IMMUNE EVASION

Evasion of the Type I IFN Pathway. Flaviviruses have evolved several strategies to avoid and/or attenuate induction of type I IFN and its effector responses. In cell culture, flaviviruses are largely resistant to the antiviral effects of IFN once infection is established.²⁰³ This may explain in part, the relatively modest therapeutic window for IFN- α administration that has been observed clinically in animal models or humans infected with JEV, SLEV, and WNV.^{132,374,670,770} Experiments by several groups have demonstrated that individual flaviviruses attenuate IFN signaling at distinct steps in the cascade.

Inhibition of IFN- β Gene Induction. Three mechanisms have been described by which flaviviruses minimize the induction of IFN- β (Fig. 12.10).

1. IFN- β gene transcription. Studies with KUNV have identified the nonstructural protein NS2A as an inhibitor of IFN- β gene transcription.^{491,492} Incorporation of an A30P mutation of NS2A into a KUNV genome resulted in a virus that elicits more rapid and sustained synthesis of type I IFN; infection of this mutant virus *in vitro* and *in vivo* was highly attenuated. The exact cellular target of NS2A and its mechanism of inhibition remain unknown.
2. PRR detection. Highly pathogenic WNV strains evade IRF-3-dependent recognition pathways without actively antagonizing the host defense signaling pathways.²³⁹ Virulent WNV strains delay activation of PRR, such as RIG-I,

through mechanisms that are not clear to provide the virus with a kinetic advantage in the infected cell to elude host detection during replication at early times after infection.³⁸⁶ In contrast, less pathogenic strains of WNV induce greater levels of IFN at early time points.³⁸⁵

3. PRR signaling. Studies in human mDCs suggest that DENV infection interferes with the type I IFN production⁷⁹⁷ prior to *IFN-β* gene induction as IRF-3 phosphorylation is not induced.^{686,687} Although the precise mechanism remains uncertain, ectopic expression studies show that a catalytically active NS2B–NS3 complex is sufficient for IFN antagonism. Activation of IRF-3 in response to dsRNA (poly (I:C)) also was inhibited in HeLa cells infected with WNV or stably propagating a subgenomic replicon.⁷³⁴ Although initial experiments suggested that NS1 might mediate this inhibitory effect,⁸⁸⁶ more recent work has questioned these results.⁴² Alternatively, the high mannose carbohydrates on the E protein may independently block the production of IFN-β, IL-6, and TNF-α that is induced by dsRNA in macrophages. This effect was not directly dependent on TLR3 but instead occurred downstream at the level of the signaling intermediate and NF-κB activator, receptor-interacting protein (RIP)-1.¹⁷ Based on studies with macrophages from different age cohorts, this E protein-dependent inhibitory pathway may be dysregulated in elderly humans, leading to a pathogenic cytokine response.⁴¹⁶ Although the mechanistic basis for how specific forms of the E protein alter antiviral signaling programs remains uncertain, glycosylated E proteins can potentially signal through multiple cell surface lectins including the mannose receptor⁵⁵⁶ and CLEC5a.¹⁴⁴

Impaired IFNAR Pathway Signaling. In addition to antagonizing induction of *IFN-β* gene responses, several flaviviruses target the JAK-STAT signaling pathway for evasion to prevent the induction of antiviral ISG with possible antiviral activity (Fig 26.11). Therefore, even when type I IFN is produced, it may not achieve its inhibitory effect because of attenuated signaling capacity. Because the nonstructural proteins NS2A, NS3, NS4A, NS4B, and NS5 mediate many of the viral evasion mechanisms described below, these countermeasures are largely intrinsic to infected cells.

1. Phosphorylation of JAKs. Studies with LGV and WNV have shown interference with phosphorylation of both JAK1 and Tyk2.^{68,290} A variation on this was observed with JEV, which showed complete inhibition of phosphorylation of Tyk2 with little effect on JAK1 phosphorylation.⁴⁸² Expression of a subgenomic replicon or infection of cells with DENV also inhibited Tyk2 phosphorylation and had no effect on IFNAR expression.³³³ However, there may be cell- or virus-specific effects as JEV also inhibits STAT1 and STAT2 activation in the setting of normal levels of Tyk2 phosphorylation.⁴⁷⁹
2. STAT2 gene expression. DENV antagonizes IFN function by reducing STAT2 expression.³⁶⁴ Cell lines that stably propagated subgenomic DENV replicons were resistant to the antiviral effects of IFN-α, had reduced levels of STAT2, and blunted ISG responses. DENV NS5 protein mediates binding and degradation of human but not mouse STAT2 via a ubiquitin and proteasome-dependent process,²⁰ and this species-specific effect in part explains the restriction of DENV infection in wild-type mice.²¹

3. Cholesterol redistribution. Flavivirus infection can actively promote re-localization of cholesterol to intracellular membranous sites of replication. This redistribution diminishes the formation of cholesterol-rich lipid rafts in the plasma membrane and attenuates the IFN antiviral signaling response.⁵¹²
4. NS proteins as specific IFN antagonists. Several groups have begun to define the viral determinants and mechanisms that mediate IFN attenuation. Ectopic expression studies in A549 cells with DENV showed that NS2A, NS4A, or NS4B enhanced replication of an IFN-sensitive virus by blocking nuclear localization of STAT1.⁵⁹⁴ Subsequent experiments showed that NS4B of DENV, WNV, and YFV partially block STAT1 activation and ISG induction.⁵⁹³ Mutagenesis studies have identified a sequence determinant on WNV NS4B (E22/K24) that controls IFN resistance in cells that express subgenomic replicons.²²⁷

Although NS5 attenuates JAK-STAT signaling after LGV, JEV, and TBEV infection, the mechanism of NS5 inhibition appears to have virus-specific characteristics. For TBEV, a sequence in the methyltransferase domain of NS5 binds the PDZ protein scribble to inhibit JAK-STAT signaling.⁸⁷⁹ For JEV, the N-terminal 83 residues of NS5 inhibit JAK-STAT signaling through a protein-tyrosine phosphatase-dependent mechanism.⁴⁸¹ Finally, for LGV, the JAK-STAT inhibitory domain was mapped to sites within the RNA-dependent RNA polymerase domain.⁶³⁸

Impaired IFN Effector Functions. Although flaviviruses devote a significant segment of their genome to inhibiting JAK-STAT signaling, they also target individual downstream antiviral effector molecules. Viperin is a candidate antiviral ISG with inhibitory activity against HCV, influenza virus, HIV, and Sindbis virus, possibly because of its ability to alter lipid raft formation. JEV, however, counteracts viperin by promoting rapid proteasome-dependent degradation.¹³¹ The mechanism of this inhibition remains unclear, as transfection of individual JEV proteins failed to explain the phenotype, suggesting that a combined effect of viral proteins or replication is required.

More recent studies have shown that 2′O methylation modification of flavivirus RNA encoded by the methyltransferase activity of NS5 can antagonize the antiviral effects of the IFN-induced genes, IFIT-1 and IFIT-2.¹⁷⁹ A WNV mutant in NS5 (E218A) that specifically lost 2′O methylation activity replicated poorly in primary macrophages and mice, but showed restored virulence in cells and animals lacking IFNαβR or IFIT-1.

Evasion of the Complement Pathway by NS1. To minimize recognition and/or destruction by complement, viruses have evolved strategies to evade or exploit complement to establish infection.^{27,788} Flavivirus NS1 is expressed on cell surfaces, secreted from infected cells, and accumulates in the serum of infected individuals, with high circulating levels correlating with severe DENV disease.^{28,468} WNV NS1 attenuates complement activation of the alternative pathway by enhancing the cofactor activity of factor H for factor I-mediated cleavage of C3b to iC3b, which decreases deposition of C3b and the C5b–C9 membrane attack complex on cell surfaces.¹⁵⁴ As an additional mechanism by which flaviviruses can evade complement, NS1 also binds to C4 and C1s, which enhanced the cleavage of C4 to C4b and reduced C4b and C3b deposition on cell surfaces.²⁴ Soluble NS1 has also been reported to bind the complement regulatory factors

C4bp²⁵ and clusterin, the latter of which normally inhibits the formation of the C5b–C9 membrane attack complex.⁴³⁹

Class I MHC and NK Cell Evasion. Because of their capacity to directly kill virally infected cells or produce inflammatory cytokines that control early stages of infection, NK cells are an important initial defense against many viruses. NK cells lyse infected cells by releasing cytotoxic granules that contain perforin and granzymes, or by binding to apoptosis-inducing receptors on target cells. NK cell activation is finely regulated through a balance of activating (Ly49D, Ly49H, and NKG2D) and inhibitory cell surface receptors (killer-cell immunoglobulin-like receptors (KIRs), immunoglobulin-like inhibitory receptors (ILTR), and CD94–NKG2A). To control the consequences of untoward activation of NK cells, inhibitory receptors are expressed constitutively, some of which bind to host MHC class I molecules on opposing cells and transmit inhibitory signals through intracellular tyrosine-based inhibitory motifs in their cytoplasmic domains. A decrease in expression of class I MHC molecules on a cell may prompt NK cell activation by attenuating the inhibitory signals. Therefore, NK-cell target recognition occurs after ligation of activating receptors and repression of inhibitory receptors on the cell surface.

Although many viruses attempt to avoid NK responses by expressing MHC class I homologs, flaviviruses may evade NK cell cytotoxicity by increasing surface expression of class I MHC molecules.^{400,493,494} Expression of class I MHC molecules is stimulated by increasing the transport activity of transporter associated with antigen processing (TAP)^{561,592} and by NF- κ B-dependent transcriptional activation of MHC class I genes.³⁸⁹ The rapid increase in expression of MHC class I suggests that early in the course of infection, flaviviruses may overcome susceptibility to NK cell-mediated lysis, even if it is at the expense of later recognition by an adaptive CD8⁺ T-cell response. Consistent with this, splenocytes from WNV-immunized mice had poor NK-cell lytic activity⁵⁶¹ and mice with acquired deficiencies in NK cells demonstrated no increased morbidity or mortality compared to wild type controls.⁷⁵⁵

Intrinsic ADE. The ligation of monocyte or macrophage Fc- γ receptors by IgG immune complexes, rather than aiding host defenses, have been hypothesized to suppress innate immunity, increase production of IL-10, and bias T-helper cell responses, leading to increased infectious output by infected cells.^{304,587} Initial studies with the unrelated Ross River alphavirus in RAW 264.7 macrophage-like cells showed that infection by ADE suppressed expression of CXCL10, NOS-2, IRF-1, TNF- α , and IFN- γ .^{472,515} Subsequent experiments with DENV in the THP-1 monocytic cell line confirmed that ADE attenuated innate immune responses by downregulating the RIG-I/MDA5 signaling pathway and decreasing production of type I IFN and ISGs.⁸³⁷ However, more recent experiments with DENV infection of primary human monocytes^{86,419} did not demonstrate suppressed production of inhibitory or immunomodulatory cytokines in the context of ADE. One caveat to the concept of intrinsic ADE is that enhanced viral entry and infectivity (via DENV immune complex interaction with Fc- γ receptor) yields higher levels of viral nonstructural proteins in a cell, which themselves independently suppress innate immunity^{197,198} irrespective of Fc- γ receptor signaling. Although the idea that intrinsic ADE of infection suppresses

innate immunity and modulates disease severity of DENV infection is appealing,³⁰⁴ it remains to be distinguished from the enhanced infectivity *per se* and confirmed in a physiologically relevant setting.

ANIMAL MODELS OF FLAVIVIRUS PATHOGENESIS AND DISEASE

Animal models of viral infections are used to address fundamental questions that are difficult to answer in human studies. These investigations are often directed toward defining basic mechanisms of viral pathogenesis (tropism, dissemination, and virulence) and host immune responses (protective and pathologic), but also are important for determining relative efficacy of candidate vaccines and antiviral agents. Although what constitutes a good animal model varies among investigators, in general, the most useful surrogate models mimic features of human disease, are reproducible, and have the capacity for high-throughput experimentation. The weakness of many animal models is they often do not fully recapitulate human disease with respect to kinetics, viral replication and spread, or disease phenotype, and thus restraint is required in applying these results to the human condition. Animal models of flavivirus infection are varied in their fidelity to human disease, and thus in their utility in providing basic insight into pathogenesis, immune control, and likely efficacy of vaccines or antiviral agents. This section reviews the strengths and weaknesses of key animal models, and what investigators in the field have learned by using them.

Dengue Virus

One of the major limitations in identifying and working with animal models of DENV infection is that humans are the only known host to develop disease after infection. A second consideration is that severe DENV infection and its plasma leakage syndrome is associated with preexisting maternal antibody in infants and secondary infection in children and adults, suggesting an immunopathogenesis mechanism, which has been difficult to recapitulate in animals. Although each of the animal models described below has been informative for understanding DENV infection, their inability to mimic human disease has limited the insight on human DENV infection.

Non-Human Primate Model of DENV Infection

Although humans are the natural host for DENV, serologic data support the existence of a sylvatic cycle between mosquitoes and nonhuman primates (NHPs).⁸⁵⁷ Several species of monkeys (e.g., chimpanzees and rhesus macaques) have been infected experimentally with DENV and develop viremia and adaptive immune responses,^{308,309,718} although in most cases, there is limited evidence of the severe disease seen in humans. One study in macaques showed thrombocytopenia, transiently reduced complement levels, and enhanced peak viremia after secondary infection with heterologous DENV serotype, although only 1 of 44 animals developed a syndrome that shared features of severe human disease.³⁰⁹ A more recent study observed features of DHF in six rhesus macaques after high-dose (10^7 PFU per animal) intravenous infection with a DENV-2 strain,⁶³² including neutropenia, thrombocytopenia, clotting abnormalities, and petechial hemorrhage.

In addition, NHPs have been used as a model to study ADE and its consequences *in vivo*. *In vivo* enhancement of viremia was observed in juvenile rhesus monkeys after passive transfer of antibody and heterologous DENV challenge.²⁹⁷ Analogously, an approximately 100-fold increase of DENV-4 viremia was demonstrated in juvenile rhesus monkeys that received a cross-reactive mAb recognizing the fusion loop in DII.²⁶⁵ In neither model, however, was evidence of severe vascular leak observed despite the increase in DENV replication. NHPs also have been used to evaluate adaptive immune response and protection of live-attenuated or subunit-based DENV vaccine candidates.^{158,213,418}

Mouse Models

A recent review describes the utility and clinical features of individual mouse models of DENV infection in great detail (see Table 1 in Yauch and Shresta⁹⁰²). Below, we describe some features of the more commonly used models in the field. In general, there have been several hurdles to establishing mouse models of DENV disease pathogenesis: (a) the majority of models are not ideal because most mice do not develop the same clinical disease as humans; (b) it has been difficult to infect mice reliably and reproducibly with low passage clinical and mosquito isolates (hence, many studies are performed with laboratory- or mouse-adapted strains that have uncertain relevance to the strains that cause human disease); and (c) DENV is virulent in humans because it has evolved specific countermeasures to evade the human immune response.¹⁹⁸ In mice, these evasion mechanisms may not function, resulting in rapid control. One example is the recent finding that DENV NS5 promotes degradation of human but not mouse STAT2, a key protein in the type I IFN signaling cascade.²¹

IFN-SIGNALING DEFICIENT MICE

Because of the importance of STAT2 and the IFN response in restricting DENV infection, mice (AG129) lacking receptors for both type I (IFN- α/β) and type II (IFN- γ) were tested and shown vulnerable to intraperitoneal (i.p.) infection with a mouse-adapted (New Guinea C) DENV-2 strain³⁶⁰ or intravenous (i.v.) infection with a laboratory-adapted (PL046) DENV-2 strain.⁷⁴⁸ In these studies, however, mice succumbed to DENV infection because of rapid spread to the CNS, resulting in encephalitis and paralysis, which are not common features of human disease. Similar results were observed in STAT1^{-/-} mice,⁷⁴⁹ although in some cases hemorrhage was observed after inoculation at multiple sites.¹⁴⁴ Subsequent investigation identified mouse adapted (DENV-2 D2S10) and nonadapted strains (DENV-2 Y98P) that cause rapid death of AG129 mice associated with some characteristics of human disease, including cytokine storm, vascular leakage, and high TNF- α levels^{750,808} after i.v. or i.p. infection.

AG129 mice have been used as a model to test antiviral candidates^{148,737,782} or to explore the role of ADE in disease severity. Two groups showed that preexisting cross-reactive monoclonal or polyclonal antibody facilitate ADE *in vivo* and promote more severe DENV disease including vascular leakage.^{39,912} Importantly, when the Fc fragment of the antibody was eliminated by proteolysis or modified genetically, enhanced replication and disease were no longer observed, thus confirming the longstanding hypothesis that ADE can cause severe disease in an animal.²⁹⁹ Cellular and tissue tropism have been

examined in the ADE model in AG129 mice^{39,912}; the virus targets appear similar to that described in human autopsy studies, with antigen present in the lymph node, spleen, and bone marrow, with significant infection in myeloid cells, and possibly sinusoidal endothelial cells in the liver. Although the comparative data are intriguing, the absence of IFN in mice independently broadens cellular and tissue tropism of flaviviruses,⁷⁰⁴ and thus some caution in interpretation is warranted.

IMMUNOCOMPETENT MICE

The successful infection of immunocompetent mice with DENV strains would allow more detailed analysis of the kinetics and function of protective immune responses. Although most DENV strains replicate poorly in wild-type laboratory strains of mice, recent reports suggest that infection may be possible, with the development of a spectrum of disease. Subcutaneous and systemic hemorrhage was induced in wild-type C57BL/6 mice after intradermal (i.d.) infection with a laboratory passaged DENV-2 16681 strain.¹⁴¹ With this strain, C57BL/6 and BALB/c mice also developed thrombocytopenia, elevated levels of systemic TNF- α , and liver damage.^{142,635} However, none of these experiments showed evidence of vascular leakage, the hallmark of severe DENV disease in humans. Studies with a mouse-adapted DENV-2 strain (P23085) that was injected i.p. in 4 week-old BALB/c or C57BL/6 mice showed thrombocytopenia, liver injury, and the development of a vascular permeability and shock-like syndrome.^{23,774} This promising mouse model, which has not yet been validated extensively, recently was used to assess the function of platelet-activating factor, macrophage migration inhibitory factor, and chemokine receptors in the pathogenesis of DENV.^{22,283,774}

MOUSE-HUMAN CHIMERAS

Because most mouse strains do not sustain DENV replication after infection, mouse-human chimeras have been developed. Early studies using severe combined immunodeficient (SCID) mice engrafted with human peripheral blood lymphocytes showed marginal infection with a DENV-1 strain.⁸⁹⁴ Subsequent studies engrafted human tumor cells (K562, HepG2, Huh-7),^{12,80,486} which supported DENV replication but caused CNS disease and not a vascular leakage syndrome. Nonobese diabetic (NOD)/SCID or NOD/SCID IL2R γ ^{-/-} have been engrafted with CD34⁺ human cord blood hematopoietic progenitor cells. After infection with DENV-2, these chimeric mice developed some of the signs of severe human disease including fever, rash, and thrombocytopenia.^{65,357,589} In an analogous model, RAG2^{-/-} \times γ chain^{-/-} mice engrafted with CD34⁺ human fetal liver stem cells and infected with DENV-2, developed viremia and fever and produced human-specific anti-DENV antibody responses.⁴⁴⁰ Although engraftment of human cells is advantageous as the response of human cells, pathogenesis, and possibly tropism can be analyzed, the chimeric models have limitations: (a) the disease phenotype generated to date recapitulates only some of the features of severe DENV; (b) technically, the mouse-to-mouse level of chimerism is variable, making phenotypic analysis challenging; (c) the throughput of experiments is low, making these models less practical for vaccine or antiviral testing; and (d) the immune cross-talk between human and mouse cells within an animal may be altered, limiting interpretation of effects on immunity.

Yellow Fever Virus

Despite the fact that YFV was isolated in 1927 and that a vaccine was developed 10 years later, our understanding of the mechanisms underlying the virulence and pathogenesis of virulent YFV remains surprisingly limited. Analogous to DENV, part of this stems from the lack of a small animal model that recapitulates the viscerotropism of human infection. Given the reemergence of YFV, an improved understanding of its pathogenesis and a vehicle for testing novel vaccines and antiviral agents through the use of existing and new animal models of disease is now a research priority.

Human Vaccine Model

Vaccination with the attenuated 17D strain of YFV has conferred protection to hundreds of millions of humans worldwide. Recent prospective analyses have examined the interaction of 17D YFV with the innate immune system and how this might be important for triggering long-term protective adaptive immunity.⁶⁶¹ A systems biology approach defined early gene signatures that predicted immune responses in humans vaccinated with yellow fever vaccine YF-17D. Computational analyses identified induction of genes (e.g., complement protein C1qB, TNFRS17, and eukaryotic translation initiation factor 2 alpha kinase 4) that correlated with and predicted protective B- and T-cell responses with high accuracy in an independent, blinded trial.⁶⁶⁸

Nonhuman Primate Model of Severe YFV Infection

YFV cycles in nature as part of a sylvatic cycle between *Aedes* mosquitoes and wild monkeys. Rhesus and cynomolgus monkeys develop viscerotropic disease, analogous to humans, ranging from mild to fulminate hepatitis, whereas African and New World NHPs have milder or silent infections.⁵⁶⁷ The pathogenesis of YFV infection in rhesus monkeys resembles severe human disease with the development of jaundice, acute renal failure, coagulopathy, and shock,⁵⁶⁸ although the course is more severe, not biphasic, produces markedly higher viral burden, and is also associated with severe necrosis of lymphoid tissue.^{409,568} The coagulopathy in monkeys is associated with a global decrease in synthesis of clotting factors secondary to direct hepatic damage and impaired hemostasis associated with abnormalities of platelet function.⁵⁶⁷

In contrast to that described for DENV, preexisting immunity to heterologous flaviviruses results in protection rather than enhanced pathogenesis in NHPs. Rhesus monkeys that were infected previously with DENV were protected against YFV challenge, and recipients of anti-DENV antibodies by passive transfer showed no evidence of enhanced disease.^{567,818} Monkeys immunized with other flaviviruses,³²⁶ similar to humans with prior exposure to flaviviruses,⁵⁷⁰ manifest a lower incidence of severe YFV disease.

Rodent Models of YFV Infection

Historical infection studies in mice and hamsters with non-adapted YFV did not cause viscerotropic disease. Syrian golden hamsters, however, did develop disease that more closely resembled human YFV infection (hepatitis, hepatic necrosis, splenic necrosis), but this phenotype requires serial passage of YFV *in vivo*, and renal disease was not observed.^{539,897} In comparison, peripheral infection of wild-type mice does not cause viscerotropic disease. However, YFV-induced encephalitis can be

induced in suckling mice after i.p. or intracranial (i.c.) inoculation, in adult mice if the blood-brain barrier is disturbed, or if mouse-adapted strains are used.^{44,236,237,567,711} Because these models do not cause viscerotropism, they are of limited relevance to understanding the pathophysiology of human YFV infection, and have been largely restricted to vaccine and antiviral testing. More recent subcutaneous infection studies of mice that are deficient in IFN-signaling revealed viscerotropic YFV infection and disease (liver and spleen necrosis) without a requirement for virus adaptation.⁵⁴⁸ This study suggests that nonadapted YFV has little ability to evade the antiviral activity of IFN- α/β in mice, whereas species-specific antagonism of IFN- α/β antiviral activity in primate hosts may contribute to infection outcome.

West Nile Virus

WNV and other encephalitic flaviviruses are generally more promiscuous in their ability to infect and cause disease in different species of animals. Beyond its endemic cycle in multiple species of birds, WNV causes severe disease in horses, and can periodically infect other mammals sometimes with severe consequences.⁹¹ Although the molecular basis for its broad animal tropism remains uncharacterized, as a result of this, it has been easier to develop animal models of infection that recapitulate features of human disease using low-passage field isolates. However, the frequency of neuroinvasive disease may vary significantly among animal strains, making some models preferred for studying pathogenesis and disease outcome.

Non-Human Primate Model of WNV Infection

NHP models of WNV infection are important because of their potential for use in evaluating vaccine and therapeutic candidates. In one study of five intradermally infected rhesus macaques, the clinical course, level and duration of viremia, and antibody response were similar to that occurring in uncomplicated human WNV infection, although it was unclear whether virus entered the brain in these animals.⁶⁷² This model of sustained viremia and measurable immune responses has been used to evaluate the efficacy of WNV vaccine candidates.⁸⁸⁴ Analogously, in baboons, after intradermal infection, WNV accumulated to high levels in blood and was associated with a transient macular rash, but failed to cause encephalitis or other severe clinical signs.⁸⁸⁹ Although these NHP models do not develop WNV encephalitis, it remains possible that the frequency of neuroinvasive disease parallels human infection (1:150), and thus would require much larger studies to identify severe cases. In contrast to infection via a peripheral route, i.c. inoculation of rhesus monkeys with different African and Asian WNV strains results in persistent viral infection in the CNS and other organs.⁶⁵⁵ These animals sustained a prolonged infection course and showed evidence of fatal encephalitis with diffuse neuronal degeneration and necrosis and inflammation. Similar severe clinical manifestations (fever, tremors, and spasticity) were observed in rhesus macaques challenged via a frontal lobe injection with the New York 1999 strain of WNV.¹⁹

Hamster Model

Syrian golden hamsters are an excellent small animal model for studying WNV pathogenesis, vaccine efficacy, and antiviral screening. Intraperitoneal or even oral infection of a New York isolate of WNV results in viremia of 5 to 6 days in duration,

followed by the development of virus-specific antibodies.^{714,896} Clinical signs of encephalitis (weakness, tremor, ataxia, and paralysis) were apparent within 6 to 7 days of infection with an approximately 50% mortality rate. WNV disease correlated with the detection of viral antigen and neuronal degeneration in several regions of the brain including the cerebral cortex, basal ganglia, hippocampus, cerebellum, and brainstem. Because of their larger size relative to mice, hamsters have been used to elucidate particular aspects of neuropathogenesis. WNV spread to the CNS can occur through a retrograde axonal transport mechanism as the virus moves from peripheral motor neurons into the spinal cord.^{705,860} Electrophysiology studies have shown that respiratory distress associated with WNV infection is caused by diaphragmatic suppression through lesions in the brainstem and cervical spinal cord, or altered vagal afferent function.⁵⁸³

In the hamster model, infectious WNV can be cultured from the brains of hamsters up to 53 days after initial infection,⁸⁹⁶ suggesting that persistent replication occurs. Persistent WNV infection or protein production in the spinal cord causes continued neuronal dysfunction, chronic neuropathologic lesions, and poliomyelitis-like disease, and can be measured using electrophysiologic approaches.⁷⁵⁸ Consistent with persistent infection in the brain, hamsters also develop persistent viremia, as infectious WNV can be cultured from urine for several weeks.⁸²⁶

The hamster model has been used to evaluate candidate therapeutics or vaccines against WNV disease. Studies with small molecule inhibitors,⁵⁸⁶ antiviral cytokines,⁵⁸⁰ synthetic oligonucleotides,⁸²⁷ and humanized monoclonal antibodies⁵⁸¹ have been performed with varying efficacy, especially when administered as postexposure therapy.¹⁹⁹ Analogously, immunization with single-cycle,⁸⁸⁵ recombinant subunit,⁷⁶¹ or live-attenuated⁸¹⁵ vaccines have elicited durable protective immunity, and thus has provided a robust preclinical small animal model for assessment and comparison of the surrogate markers of protection.

Mouse Models

Infection studies in several inbred laboratory strains of mice have provided insight into the fundamental mechanisms of WNV dissemination, pathogenesis, and immune system control. Over the last decade, most studies have been performed with North American WNV strains and wild-type and immunodeficient strains of C57BL/6 mice. The strengths of this particular model include the following: (a) depending on the dose of virus and age of mice, a subset of wild-type mice develop neuroinvasive disease, whereas the remainder are infected with minimal or limited spread to the CNS. Therefore, the mechanisms by which the immune system restricts viral entry or facilitates viral clearance can be studied; (b) many features of pathogenesis and neuropathology appear remarkably similar to that observed in humans; (c) nonadapted low-passage WNV isolates cause disease in wild-type mice. Therefore, this model can be used to define the genetics of virus attenuation; (d) there are a large number of transgenic, knockout, and conditional knock-out mice available from academic laboratories and public consortia to study the role of specific genes or cells in pathogenesis; and (e) genes (e.g., CCR5 and OAS-1b) that predict susceptibility in mice have been corroborated as risk factors for human WNV disease.²⁰¹ Nonetheless, there are limitations to

the model including the compressed disease time course, the difficulty in obtaining CSF samples in live animals because of size, and a rather flat virus dose–response curve after peripheral infection. Moreover, the mouse anti-WNV antibody response appears directed at a distinct dominant neutralizing epitope than the human response.⁶²⁸

Following peripheral inoculation of mice, initial WNV replication is thought to occur in keratinocytes and skin Langerhans dendritic cells,¹¹⁷ with mosquito saliva modulating the local proinflammatory cytokine response.⁷³⁰ Dendritic cells migrate to and seed draining lymph nodes, resulting in a primary viremia and subsequent infection of peripheral tissues such as the spleen and occasionally, the kidney. By the end of the first week, WNV is largely cleared from the serum and peripheral organs, and infection in the CNS is observed in a subset of immunocompetent animals. Mice that succumb to infection develop CNS pathology similar to that observed in human WNV cases, including infection and injury of brainstem, hippocampal, and spinal cord neurons.⁷⁵⁴ WNV infection is detected at much lower levels in nonneuronal CNS cell populations, such as CD11b⁺ cells^{176,828} or astrocytes.²⁰⁸ In most surviving wild-type mice, WNV is cleared from all tissue compartments within 2 to 3 weeks after infection. However, persistent WNV infection in the brains of class II MHC,⁷⁶⁵ CD8⁺ T-cell⁷⁵² or perforin deficient mice⁷⁵⁵ was routinely observed. Analogously, a small subset of wild-type mice sustained WNV persistence in the CNS, even in the setting of a robust antibody response and inflammation.¹⁶ Remarkably, WNV persistence in the CNS was observed even in mice with subclinical infections, as treatment with the immunosuppressive drug cyclophosphamide resulted in active viral replication.

PREVENTION AND CONTROL

Flavivirus Vaccines

Successful vaccination programs have dramatically reduced the global health burden of flavivirus infections. More than 500 million doses of vaccine to prevent YFV infection have been administered since its development in 1937, and effective vaccines have blunted the impact of JEV and TBEV as discussed below. Nonetheless, safe and effective vaccines for several clinically significant flaviviruses still remain elusive. As an example, up to 100 million DENV infections occur each year, and severe disease manifestations are occurring with an increased frequency.⁸⁸² New flavivirus vaccines and improvement on safety of existing vaccines is urgently needed. The development of molecular clone technology, more sophisticated animal models of infection, and insights from structural biology have aided recent efforts in these areas.

Yellow Fever Virus

The live-attenuated YFV 17D vaccine is considered among the most safe and effective ever developed, an achievement for which Max Theiler was awarded the Nobel Prize in 1951. An excellent historical account of the development of this vaccine has been written by Monath.⁵⁶⁹ The current YFV vaccine was derived from a virus (the Asibi strain) isolated in 1927 from a West African man with a mild febrile illness.⁷⁸⁹ The Asibi strain was passaged 176 times in the embryonic tissue of mice and chickens to yield the YF-17D virus with considerably reduced

neurotropic and viscerotropic properties.^{565,819} Vaccines currently in use are substrains of YF-17D; strain YF-17DD is used in vaccines produced for South America (passage 287–289), whereas the YF-17D-204 strain (passages 235–240) is distributed elsewhere, including the United States.^{46,779} The consensus sequence of the vaccine strains differs from the parent Asibi strain by approximately 20 amino acids as well as by 4 nucleotide changes in the 3' UTR.⁵⁶⁵ Vaccine is produced in chicken embryos, lyophilized, and administered by subcutaneous injection following reconstitution in saline. A single dose of YFV vaccine contains roughly 10^4 to 10^6 PFU.⁴⁶

THE IMMUNE RESPONSE TO YF-17 INFECTION

The host response to YF-17D infection involves both the adaptive and innate arms of the immune system.⁵⁶⁹ Recent studies highlight the significance of the innate response to YF-17D in shaping the adaptive immune response to vaccination.^{247,661,667} YF-17D activates mDCs and pDCs through multiple TLR proteins, including TLR2, TLR7, TLR8, and TLR9, resulting in the induction of proinflammatory cytokines and IFN- α .^{667,668} Of interest, a capacity to interact with multiple TLR pathways does not appear functionally redundant; these interactions tune the adaptive response by influencing the balance of Th1 and Th2 cytokines and the quality of the anti-YFV T cell response.⁶⁶⁷ Indeed, YF-17D infection induces a mixture of Th1 and Th2 cytokines *in vivo*,^{247,709} and YF-17D infected DCs present viral antigen to T cells despite inefficient replication in these cells.^{40,636}

YF-17D vaccination induces a low-grade and transient viremia that peaks on day 5.⁵⁶⁵ Defervescence is coincident with a reduction in viremia and the detection of cellular and humoral responses. YF-17D infection induces a polyfunctional CD8⁺ T-cell response of considerable magnitude (2% to 13% of CD8⁺ T cells) that peaks roughly 2 weeks postimmunization.³ Analysis of the breadth of this response demonstrates that all 10 viral proteins contain epitopes recognized by CD8⁺ T cells; reactivity with epitopes in E, NS3, and NS5 proteins were most common.^{4,247} The virus-specific CD8⁺ T-cell response contracts at approximately day 30 postinfection to a size that corresponds to approximately 5% to 10% of the magnitude of the original response, with memory CD8⁺ T cells persisting for years.^{4,555}

Vaccination with YF-17D also elicits a rapid neutralizing antibody response in virtually all recipients.^{60,645} Kinetic analysis of vaccinated adults revealed that approximately 87% have neutralizing antibodies at 2 weeks postvaccination, with virtually 100% of subjects developing neutralizing antibody by day 28.⁴⁴⁸ YFV-reactive IgM can be detected by day 9, peaks between days 14 and 17, and persists for more than one year. YFV-specific IgG is detected between days 10 and 17 and peaks approximately 1 month postvaccination.⁵⁶⁹ Neutralizing antibodies persist for decades. More than 90% of vaccinated subjects had neutralizing antibody when examined 16 to 19 years postimmunization.⁶⁹³ Indeed, neutralizing antibody was detected in 80% of vaccinated U.S. military personnel when assayed 30 to 35 years after receiving YF-17D.⁶⁵⁶ Despite the impressive longevity of the antibody response, booster immunizations are still recommended every 10 years.⁷⁷⁹

The neutralizing antibody response correlates with protection from infection.^{60,533,569,575} Roughly 94% of primates with a neutralizing antibody titer greater or equal to 0.7 logs (1/5

dilution of serum) were protected from a lethal challenge with YF-Asibi.⁵³³ As a comparison, the mean neutralizing antibody titer of recipients of the YF-17D vaccine at 28 days postimmunization is 2.2 logs (1/160 serum dilution).⁵⁷⁵

ADVERSE EVENTS ARISING FROM YF-17D VACCINATION

More than 500 million doses of YF-17D have been administered to humans with a high track record of safety.²⁴⁶ The most common side effects from YF-17D vaccination are transient headache, myalgia, and low-grade fever.⁷⁷⁹ Severe adverse events (SAEs) following vaccination have been reported, albeit at a very low frequency. The risk of SAEs following vaccination increases with age; the incidence of SAEs in vaccine recipients greater than 70 years of age is roughly 10-fold higher than that for individuals aged 19 to 29.³⁹¹ Three main classes of SAEs have been reported:

1. Anaphylactic reactions are infrequent (1 in 135,000) and likely a result of allergic responses to components of the vaccine including egg and chicken proteins, gelatin, and latex.^{387,779} Hypersensitivity to eggs is a contraindication for vaccination.⁵⁶⁹
2. Vaccine-associated neurologic disease (YEL-AND) is associated with invasion of the CNS by the vaccine strain. This SAE was most commonly reported prior to the establishment of the vaccine seed system (in 1945) and in infants prior to changes in the recommendations for vaccination of children less than 6 months old (in 1960).⁵⁶⁵ The mechanism underlying the increased risk of YEL-AND in infants remains uncertain, but may reflect differences in the level or duration of viremia, the integrity of the BBB, or a failure to mount an effective immune response.⁵⁶⁹ Twenty-nine cases of YEL-AND have been reported since 1990 with a case fatality ratio of 6.9%⁵⁶⁹ with an incidence of 0.4 to 0.8 per 100,000 doses.^{487,779}
3. Vaccine-associated viscerotropic disease (YEL-AVD) is a recently reported SAE that mimics many aspects of naturally acquired YFV infection. YEL-AVD is characterized by the rapid onset of high fever (within 2 to 5 days of vaccination), malaise, and myalgia that is followed by jaundice, oliguria, cardiovascular instability, and hemorrhage. Analysis of the sequence of viruses recovered from vaccine recipients with YEL-AVD failed to identify mutations associated with this SAE.⁴⁶ Risk factors for YEL-AVD include advanced age and a history of thymus disease or thymectomy. As of 2010, 57 cases of YEL-AVD have been reported with a case fatality rate of 64%. In the United States, the incidence is estimated as 0.3 to 0.5 per 100,000 doses.⁷⁷⁹

NEW VACCINE APPROACHES

Despite the demonstrable success of the live-attenuated YF-17D vaccine, the potential for SAE has prompted efforts to develop new vaccines with improved safety. Perspectives supporting a need for new vaccine approaches have been given.³¹⁶ A new inactivated whole virus vaccine candidate, XRX-001, is being developed to complement the existing YF-17D vaccine, particularly for contraindicated populations. XRX-001 is a β -propiolactone inactivated YF-17D virus that is produced in Vero cells and adsorbed to aluminum hydroxide.⁵⁷³ Two doses of vaccine in the presence or absence of adjuvant was sufficient to elicit a neutralizing antibody response in mice. The neutralizing

antibody titers achieved following XRX-001 vaccination were equivalent or better than those with live-attenuated YF-17D vaccination experiments performed in parallel. Experiments in hamsters and NHPs confirmed that vaccination protected against lethal challenge with YFV.⁵⁷³ Evaluation of this candidate vaccine in human clinical trials is underway (clinical trials.gov identified NCT00995865).

Dengue Virus

Four antigenically related serotypes of DENV circulate in nature. Although natural infection by DENV is thought to confer protection from re-infection by a homologous DENV serotype,⁷⁰⁰ an increased risk of severe clinical manifestations following secondary infection by a heterologous DENV has been demonstrated.^{112,224,294,305} Therefore, the potential for an exacerbated clinical outcome in a DENV sensitized-individual complicates the development of a safe and effective vaccine. A perceived requirement of candidate DENV vaccines is that administration confers simultaneous, durable protection against all four different DENV serotypes. Given the considerable effort and resources required to bring a safe and effective vaccine against a single pathogen to market, a tetravalent DENV vaccine is among the most ambitious vaccine development efforts undertaken. DENV vaccine research traces its roots to the 1940s⁷⁰¹ and has advanced from empirical administration of passaged strains to rational design that exploits the advances of molecular and structural virology.²¹⁷

LIVE ATTENUATED DENGUE VACCINES DERIVED FROM EXTENSIVE PASSAGING

The earliest efforts to produce a vaccine against DENV were undertaken by Sabin and colleagues.⁷⁰¹ DENV-1 (Hawaii strain) was passaged in mice via intracranial inoculation, isolated as a brain homogenate, and used to challenge human volunteers. Although the first six passages of DENV in mice did not sufficiently attenuate the virus and caused fever in human subjects, experiments with virus passaged seven or more times conferred protection following challenge with DENV-infected mosquitoes.^{700,701} Similar experiments were performed using the DENV-1 Mochizuki strain.^{340,341} A more extensively passaged mouse brain-derived DENV-1 isolate was the first DENV vaccine candidate evaluated in the field during an outbreak of DENV-3 in Puerto Rico; this heterologous protection experiment suggested an efficacy of about 40%.^{59,703}

Extensive passage in tissue culture also has been used to attenuate DENV for use in vaccines. Investigators in Thailand developed vaccine candidates for all four serotypes of DENV via serial passages in primary dog kidney (PDK) or primary green monkey kidney (PGMK) cells.⁸⁸² Aventis Pasteur/Sanofi Pasteur licensed these strains for clinical vaccine development. The safety and immunogenicity of tetravalent formulations was investigated in clinical trials in adults^{376,696} and in children.⁶⁹⁷ Although these vaccines were generally well tolerated, reactogenicity was noted, particularly after the first dose of vaccine. Furthermore, the DENV-3 component of the vaccine replicated more robustly in vaccinated subjects and was immunodominant. Although changes in the relative doses of each component strain of the vaccine were tested to reduce the dominance of DENV-3 and reactogenicity, development of this tetravalent candidate was halted. These studies highlight the complexity and challenge of eliciting a balanced immune

response against four different viruses representing all DENV serotypes.

Investigators at the Walter Reed Army Institute of Research (WRAIR) developed a tetravalent vaccine composed of four highly passaged DENV. These viruses were produced by serial passage of clinical isolates of DENV in PDK cells, and the formulation of candidate tetravalent vaccines were studied in several monovalent and tetravalent clinical trials.²¹⁷ A phase I clinical evaluation of two doses of vaccine administered 6 months apart demonstrated that the vaccine was well-tolerated and elicited a tetravalent neutralizing antibody response in all subjects.⁷⁶³ Concerns as to the durability of the tetravalent humoral response in humans has prompted the commercial partner (GlaxoSmith-Kline) to suspend clinical trials (J Toussaint, personal communication) with this vaccine candidate.

LIVE-ATTENUATED DENGUE VACCINES: RATIONAL DESIGN USING MOLECULAR BIOLOGY

The development of molecular infectious clones of flaviviruses has enabled the construction and characterization of variants with attenuating mutations. The 3' UTR of flaviviruses folds into RNA structures that function to regulate genomic RNA replication, translation, and cytopathicity.^{159,649} The introduction of a 30 nucleotide deletion in the 3' UTR of DENV-4 (strain 814669) yielded a markedly attenuated virus *in vivo*, which still elicited a robust humoral response in monkeys and humans.^{213,550} Vaccination with DENV-4Δ30 resulted in low-level viremia (~1.6 logs) in 70% of recipients,²¹³ which is not sufficient for blood-meal transmission of the vaccine to mosquito vectors. Similar results were reported with a DENV-1Δ30 virus constructed from the Western Pacific 1974 strain.²¹⁴ In contrast, DENV-2 and DENV-3 viruses incorporating the Δ30 deletion were not attenuated sufficiently to warrant further study as vaccine candidates.^{78,79} A second DENV-3 virus, however, encoding two deletions in the 3' UTR (DENV-3Δ30/31) appears more promising; monkeys immunized with this variant were not viremic, mounted a robust neutralizing antibody response, and were protected from challenge by wild-type virus.⁸²

The development of chimeric flaviviruses encoding the structural genes of heterologous viruses is a second attenuation approach that has yielded several promising vaccine candidates. The first chimeric viruses were constructed by introducing the C, prM, and E genes of DENV-1 or DENV-2 into the genetic background of DENV-4.⁹⁵ These viruses were immunogenic and attenuated as immunization of monkeys elicited neutralizing antibodies and reduced viremia following challenge with a homologous wild-type strain.⁹⁶ These early studies established that the structural proteins conferred the serologic specificity of chimeric DENV. Chimeric vaccine candidates encoding the heterologous C-prM-E and prM-E cassettes have been characterized; viruses constructed using the latter replicated more efficiently than those encoding all three structural genes, perhaps due to a requirement for interaction between RNA elements in the capsid gene and the 3' UTR.⁹⁶ Several tetravalent chimeric flavivirus vaccine candidates using molecular backbones of DENV4Δ30, YF-17D, and DENV-2 viruses are in advanced stages of clinical development.^{446,597}

A tetravalent DENV vaccine using chimeras constructed with the DENV-4Δ30 backbone is being developed by the National Institute of Allergy and Infectious Disease (NIAID),

National Institutes of Health (NIH). Because the DENV-4 backbone of these viruses is already attenuated by the $\Delta 30$ deletion, viruses were constructed using the structural genes from wild-type strains. A chimeric DENV-2/DENV-4 $\Delta 30$ virus encoding the prM-E of the New Guinea C DENV-2 strain has been evaluated in phase I clinical studies.^{215,883} Although chimeric DENV4 $\Delta 30$ encoding the structural proteins of DENV-3 could be recovered, these viruses were over-attenuated *in vivo*.⁷⁸ A second chimeric virus strategy involved replacing the 3' UTR of a DENV-3 virus with the DENV-4 $\Delta 30$ UTR described above is being evaluated.⁸² Tetravalent formulations of the NIAID vaccine candidates are in phase I clinical trials and include both chimeric viruses and those encoding a $\Delta 30$ deletion.

Because of the extensive safety profile of the YF-17D vaccine, several chimeric viruses also have been constructed by replacing genes encoding the structural proteins of YF-17D with those of heterologous flaviviruses.²⁹² This platform, called ChimeriVax™ (licensed by Sanofi Pasteur), has been used to create chimeric viruses expressing the prM-E proteins of all four serotypes of DENV.²⁸⁴ The structural genes of these chimeric viruses were obtained from low-passage primary isolates of human dengue cases. Preclinical and clinical studies demonstrate that ChimeriVax-DENV is safe, with minimal and nonsevere adverse reactions (reviewed by²⁹²). Studies in suckling mice infected via the intracranial route demonstrated that chimeric DENV-YF-17D has lost the neurovirulent phenotype associated with the YF-17D backbone. Vaccination of monkeys and humans results in a low and transient viremia of reduced magnitude compared to the parental viruses. Preclinical studies in monkeys immunized with a single dose of a tetravalent formulation showed excellent immunogenicity, and 92% percent of immunized animals remained aviremic after challenge with wild-type DENV.²⁸⁷ A randomized phase IIb study of this vaccine candidate in dengue-experienced subjects (clinicaltrials.gov identifier NCT00842530) revealed only modest efficacy (30.2%) that was not uniform among serotypes; no protection against infection by DENV2 viruses.^{697a}

A third chimeric tetravalent DENV vaccine formulation has been generated using the Thai DENV-2 PDK-53 vaccine strain as the backbone.^{345,346} Although these vectors are immunogenic and protective in mouse models, they have only recently entered phase I clinical trials in humans (clinical trials.gov identifier NCT01110551).

ALTERNATIVE VACCINE STRATEGIES

DNA Vaccines. DNA-based DENV vaccines offer several advantages including ease of production, transport, and storage. Furthermore, administration of multiple DNA constructs encoding different flavivirus antigens avoids viral interference.⁷²⁶ The first DNA vaccine for DENV encoded the prM and a secreted form of the E protein lacking the transmembrane domains of the DENV-2 New Guinea C strain.⁴¹³ Plasmids that express prM and E proteins may be particularly immunogenic, as they can produce secreted subviral particles *in vivo*, which display the E protein in a highly ordered array analogous to that present on infectious virions.²³³ Two vaccinations with four plasmids encoding the envelope proteins of each DENV serotype were sufficiently immunogenic in mice to confer protection from heterologous challenge with any of the four dengue viruses.⁴¹⁷ However, a phase I clinical evaluation of three

doses of a monovalent DENV-1 DNA vaccine revealed only modest immunogenicity.⁵⁸

Recombinant Subunit Vaccines. DENV vaccines composed of recombinant E proteins also have been studied. Analogous to DNA vaccines, this approach may simplify the task of eliciting a balance tetravalent response.²¹⁷ High-level expression of soluble forms of the E protein has been achieved using insect cell gene expression technologies. Whether the antigens in subunit vaccines are capable of eliciting the full spectrum of antibody specificities required for a maximally protective response, particularly those spanning multiple E protein oligomers on the intact virion, remains uncertain.

Other/Modified Viral Vectors. Several viral expression vectors have been studied in mice and primates as possible DENV vaccine platforms including vaccinia virus,^{97,195} alphavirus,^{143,881} adenovirus,⁶⁷³ and measles virus.⁹²

Japanese Encephalitis Virus

JEV is a principal cause of pediatric encephalitis in Asia and has been a focus of vaccine development efforts since before World War II.³⁰² Inactivated suspensions of JEV-infected mouse brains were administered to military personnel in response to an outbreak of Japanese B virus (now recognized as JEV) on Okinawa in 1945.⁶⁹⁹ Although circumstances did not permit a complete evaluation of efficacy, this vaccine elicited neutralizing antibodies in a subset of vaccinated subjects at titers that protected mice from lethal infection.⁶⁹⁸ Since that time, considerable progress has been made toward developing a safe and effective JEV vaccine. Three vaccination approaches have reduced the incidence of JEV in countries with the means to utilize them. However, ~35,000 to 50,000 cases of JEV disease annually are still seen; as such, efforts to develop effective and economical vaccines with improved safety profiles continue.⁵³

MOUSE-BRAIN DERIVED JEV VACCINES

Vaccines produced from JEV-infected mouse brains have been effective at controlling JEV in many parts of Asia, including Japan, South Korea, Taiwan, and Thailand.²³⁵ The Research Foundation for Microbial Diseases of Osaka University (BIKEN) produced the majority of mouse-brain derived JEV vaccine licensed for international use between 1954 and 2005. This vaccine platform uses genotype III Nakayama or Beijing-1 strains for different markets.⁵³ Brain tissue from intracranially infected mice is homogenized, and virus is purified by ultracentrifugation and filtration steps. The materials are inactivated by formalin during this process.³³⁵ These vaccines were used primarily in Japan, Korea, Thailand, Malaysia, Sri Lanka, and Vietnam to protect against endemic JEV.⁵³ The BIKEN vaccine was licensed for travelers in the United States and elsewhere and marketed as JE-VAX™.²³⁵

Efficacy studies of mouse brain-derived JEV vaccines suggest that they are modestly immunogenic, and thus require multiple boosts. A placebo-controlled double-blind evaluation of monovalent (Nakayama strain) or bivalent (Nakayama and Beijing strains) formulations of JE-VAX conducted in Thailand revealed an efficacy of 91% in children receiving two doses of vaccine 7 days apart.³³⁵ However, interpretation of these studies is complicated by the prevalence of individuals with prior flavivirus experience in JEV-endemic regions. Studies of the

immunogenicity of two doses of JE-VAX in flavivirus-naïve subjects revealed only 33% seroconversion at 26 weeks postvaccination; in subsequent studies, near complete seroconversion was achieved using a third dose.^{657,707} Attempts to measure the durability of antibody responses elicited by mouse brain-derived vaccine in endemic regions have been complicated by the potential boosting by naturally acquired flavivirus infection. Studies of military vaccine recipients suggest that neutralizing antibodies may persist for at least 3 years.²⁴⁴ Vaccine is typically administered in two doses separated by 1 to 4 weeks, followed by a booster 1 to 2 years later. Travelers require a rapid three-dose vaccination regimen (days 0, 7, and 30).²³⁵

Although mouse brain-derived JEV vaccines have shown efficacy in humans, they are reactogenic and raise concerns about vaccine safety. Roughly 20% of JE-VAX recipients experience local adverse events including swelling, redness, and tenderness at the vaccination site, and mild systemic symptoms are also relatively common. Severe allergic and neurologic (acute disseminated encephalomyelitis [ADEM]) complications of vaccination have been observed (10 to 260 and 0.1 to 2 per 100,000 vaccinees, respectively).^{53,235} Fatalities from ADEM resulted in cessation of production of JE-VAX in 2005 in favor of newer vaccines with more favorable safety profiles.

LIVE ATTENUATED JEV VACCINES

SA14-14-2 is a live-attenuated JEV vaccine that has been administered to more than 300 million individuals.³⁰² The parental SA14 strain was isolated from the larvae of *Culex pipiens* mosquitoes collected in China in 1954, and causes lethal neurologic disease when inoculated into weanling mice via the intracranial route. The attenuated strain was developed after extensive passaging of the SA14 strain in cell culture, and in hamsters and suckling mice.^{613,898} The SA12-1-7 strain was isolated by passage of SA14 in newborn mice 10 times and 100 passages in primary hamster kidney cells.⁶¹⁴ Although this virus was considerably less virulent than SA14, it was not genetically stable and reverted to a neurovirulent phenotype following a single passage in mice, or several passages in primary cell cultures.⁹⁰⁸ Derivatives of this virus with greater genetic stability were subsequently developed and evaluated. SA14-5-3 was derived from SA12-1-7 by additional passages in cell culture and plaque purification steps. Clinical studies demonstrated vaccine safety in humans but only modest immunogenicity, with seroconversion rates of 85% and 61% in endemic and nonendemic regions, respectively.³⁰² SA14-5-3 was licensed for use in China; altogether about 5 million children were vaccinated.⁹⁰⁸ To improve immunogenicity, additional passages of SA14-5-2 in suckling mice yielded the SA14-14-2 strain. A single dose of SA14-14-2 induced a neutralizing antibody response in 85 to 100% of non-immune recipients.^{767,833,898} Case-controlled studies demonstrated that a single dose of SA14-14-2 vaccine provided considerable protection (80% to 99%),^{76,327,431,626} which was durable even after 5 years.⁸⁰⁹ SA-14-14-2 was licensed for use in China in 1988, and subsequently distributed in Nepal, South Korea, Sri Lanka, Thailand, and India.

INACTIVATED JEV VACCINES PRODUCED IN CELL CULTURE

The IC51 (or IXIARO) vaccine is a formalin-inactivated vaccine produced in certified Vero cells under serum free conditions and adjuvanted with aluminum hydroxide.²³⁵ IC51 uses the SA14-14-2 JEV strain,⁴¹⁴ and is administered in two doses

28 days apart, each containing ~6 µg of purified virus. Licensure was granted based on a noninferiority immunogenicity study comparing the response of recipients receiving IC51 to those vaccinated with a three-dose regimen of JE-VAX. Although a single dose of IC51 was poorly immunogenic (41% seroconversion), 4 weeks after the last of three doses, 96% of recipients had detectable neutralizing antibodies, and these persisted in most subjects 6 months after vaccination.⁴¹⁴ No significant local or systemic adverse events were associated with vaccination; rare events await a more detailed analysis of larger populations of vaccine recipients. IC51 was licensed for use in the United States as a traveler's vaccine in 2009 for individuals older than 17 years of age. An inactivated JEV vaccine derived from the genotype III P3 strain and produced in hamster cell cultures also has been used extensively in China, with as many as 70 million doses of this vaccine administered each year.³⁰²

NEW VACCINATION APPROACHES

ChimeriVax-JE, or IMOJEV, is a promising live attenuated vaccine constructed using the attenuated YF17D backbone described above (reviewed by⁵⁵). ChimeriVax-JE was constructed by inserting the prM-E genes of YF-17D, with genes encoding the envelope proteins of SA-14-14-2. Vaccination with ChimeriVax-JE elicits a protective response in mice and primate studies.^{55,288,576} Clinical studies in humans reveal the vaccine is well tolerated and immunogenic (reviewed by²⁹²).

Tick-Borne Encephalitis Virus

Two inactivated TBEV vaccines are used to prevent infection in Europe, but are not licensed in the United States.⁶⁷⁷ Kunz and colleagues^{436,437} developed the first licensed vaccine in 1973 using the Austrian Neudörfl strain of TBEV grown in chick embryo fibroblasts, after inactivation with formalin and adjuvanting with aluminum hydroxide. Today, this vaccine is distributed as FSME-IMMUN by Baxter Biosciences. A second vaccine, produced by Novartis and marketed as Encepur,^{83,407} was licensed in 1991. It is produced using similar methods, except the German K23 strain of TBEV is substituted. Both inactivated TBEV vaccines are administered in three doses and require boosting. The conventional schedule requires three vaccinations at 0, 1–3 months, and 9–12 months, and a booster at 3 years, followed by additional booster vaccinations every 5 years. These vaccines are highly immunogenic; virtually 100% of vaccinated subjects develop significant neutralizing antibody titers following their third dose,⁴³⁶ and antibodies persist for at least 5 years.^{652,887} The effectiveness of current TBEV vaccines in the field has been estimated at about 99%.³²⁰ Despite the availability of effective vaccine, TBEV incidence in parts of Europe recently has increased, coincident with poor vaccine coverage.⁴³⁸

West Nile Virus

Several strategies for vaccination against WNV have been developed and evaluated in clinical studies (reviewed in⁵¹). Live-attenuated flavivirus chimeras have been developed for WNV using the strategies described above for DENV and JEV. ChimeriVax WNV was constructed by replacing the genes encoding the prM-E proteins of YF-17D with those of WNV.²⁹² Two vaccines were developed using this approach. ChimeriVax-WN01 encodes the unmodified sequence of the NY99 strain, and was developed as a veterinary vaccine that has been in use in horses since roughly 2006.^{498,499} ChimeriVax-

WN02 differs from the veterinary vaccine by three amino acid substitutions in the E protein introduced to reduce neurovirulence, and a fourth adventitious substitution that arose during adaptation of the vaccine lot to growth on Vero cells.²⁹² ChimeriVax-WN02 has been shown to be safe and immunogenic in phase I and phase II clinical trials in humans.^{72,574} Chimeric flavivirus vaccine candidates have also been constructed using the DENV4Δ30 backbone described earlier (see DENV section) and have proven to be safe and immunogenic in preclinical⁶⁰⁶ and clinical studies (A. Durbin, S. Whitehead, and colleagues, unpublished data; ClinicalTrials.gov identifier: NCT00094718).

Despite their effectiveness, concerns about the potential hazards associated with the use of live-attenuated viral vaccines provide a strong rationale for the development of other approaches. DNA vaccination has also shown promise for WNV vaccination. DNA vaccine constructs typically express genes encoding the prM and E structural proteins. Expression of prM-E *in vitro* is sufficient for the production of small virus-like subviral particles on which the E proteins are arrayed with icosahedral symmetry²³³; DNA vaccines are thought to produce subviral particles upon administration *in vivo*. Two phase I clinical studies of a WNV nucleic acid vaccine have been performed.^{180,453,530} These trials demonstrated that three doses of vaccine were well-tolerated and capable of eliciting both a T-cell and neutralizing antibody response. A similar construct has proven to be efficacious at reducing WNV incidence in horses and a variety of birds.⁵¹

An adjuvanted subunit vaccine containing a soluble fragment of the WNV E protein has been developed^{473,474,873} and studied in humans (ClinicalTrials.gov identifier: NCT00707642). A truncated form of the E protein lacking the transmembrane domains (referred to as 80% E) was produced in *Drosophila* S2 cells and purified using immunoaffinity chromatography. Preclinical studies demonstrate that administration of a single dose of adjuvanted protein was immunogenic and capable of eliciting a neutralizing antibody response.^{473,474,873} To date, the results of a phase I clinical study of this vaccine have not been published.

Replivax-WN is a truncated form of the WNV genome encoding a large deletion in the gene encoding the capsid protein. This construct can be complemented *in vitro* using cell lines that express the capsid protein to yield pseudoinfectious virus particles. Infection of cells with these virus particles is not productive, yet it results in the production of subviral particles composed of prM and E. Immunization with Replivax particles has been shown to be safe in preclinical studies and stimulates a robust adaptive response.^{606,884,885} A similar modification of the Kunjin virus genome has been evaluated as a candidate WNV vaccine.¹³³

Altogether four veterinary vaccines have been licensed for use including a formalin-inactivated adjuvanted whole virus vaccine (WN-Innovator, Fort Dodge Animal Health), a canarypox vector expressing prM and E (Recombitek equine WNV vaccine),^{219,378,760} ChimeriVax-WN01 (PreveNile),²⁹² and a DNA vaccine construct that served as the basis for the human vaccine described above (WN-Innovator DNA, Fort Dodge Animal Health).¹⁸⁰

Therapeutics

At present, no specific therapy has been approved for use in humans with any flavivirus infection, as all current treatments

are supportive. For example, treatment of severe DHF/DSS currently consists of careful patient monitoring and aggressive fluid management. Although tissue culture and animal model studies have applied multiple screening strategies to generate novel therapies against flaviviruses, development has remained challenging. Among the impediments are the rapid development of resistance for monotherapy, a need to efficiently cross the BBB for inhibitors of the encephalitic flaviviruses, and regulatory hurdles for the design and implementation of multicenter trials, given the sporadic temporal and spatial occurrence of many flavivirus infections.

Existing Antiviral Agents: Ribavirin, Mycophenolic Acid and Human IFN- α

Ribavirin is a broad-spectrum antiviral agent and has been used clinically to treat respiratory syncytial, hepatitis C, Lassa fever, and Crimean-Congo hemorrhagic fever viruses. It acts as a guanosine analog and competitively inhibits inosine monophosphate dehydrogenase (IMP), resulting in depleted intracellular guanosine pools.⁴⁶² This activity has been proposed to interfere with the guanylation step of RNA capping, inhibit viral polymerases, or compromise the integrity of the viral genome by being incorporated directly into the nascent RNA strand and serving as a template for both cytidine and uridine.^{172,173,184} Ribavirin has inhibitory activity against flaviviruses infection in cell culture,^{166,365,807} albeit at relatively high micromolar concentrations. Limited animal studies have been performed with varying results. Although a beneficial therapeutic effect of ribavirin was observed in YFV-infected hamsters,^{366,715} treatment of YFV-infected NHP had minimal positive effect,⁵⁶⁴ and increased mortality was observed in WNV-infected hamsters.⁵⁸⁰ A combination of ribavirin with IFN- α_{2b} also failed to improve outcome of flavivirus-induced encephalitis in mice.⁴⁶³ Finally, during a WNV outbreak in Israel in 2000, in an uncontrolled study, 37 patients received ribavirin and had a higher mortality rate.¹⁵¹

Mycophenolic acid (MPA) is a nonnucleoside inhibitor of IMP dehydrogenase that is used clinically to prevent rejection of transplanted organs. The immunosuppressive properties of MPA are attributed to its antiproliferative effect on lymphocytes *in vitro*. MPA inhibits to varying degrees the replication of a number of DNA, RNA, and retroviruses. Several studies have demonstrated that MPA inhibits flavivirus infection in cells by limiting viral RNA replication.^{206,585,807} Although MPA blocks flavivirus infection in cell culture, its immunosuppressive properties *in vivo* likely overshadow its direct antiviral effects, as no study has reported therapeutic benefit in animals.

Type I IFN induces an antiviral state within cells through the induction of antiviral proteins and by modulating adaptive immune responses. Despite the ability of flaviviruses to antagonize its signaling pathways, pretreatment of cells with type I IFN potently inhibits infection by many flaviviruses. Nonetheless, IFN may still have therapeutic potential. Pretreatment of rodents with IFN- α inhibited SLEV infection and resulted in decreased WNV viral loads and mortality.^{106,580} Analogously, treatment of before or after YFV infection also improved survival rates.³⁶⁶ Administration of IFN- α reduced complications in human SLEV cases and has been used in an uncontrolled manner to treat small numbers of human cases of WNV encephalitis.^{374,461,670} However, in Vietnam, a double-blinded, randomized placebo-controlled clinical trial was performed in

1,112 children with suspected or documented encephalitis virus infection; treatment with IFN- α_{2a} failed to improve outcome.⁷⁷⁰

Passive Antibody Therapy

Through experiments in a variety of experimental systems, it is well established that antibodies can neutralize flavivirus infection *in vitro* and *in vivo*, and prophylaxis or immunization can provide sterilizing immunity and prevent infection. The ability to cure animals with established flavivirus infection by passive transfer of antibodies, however, is more challenging and depends on the dosage, time of administration, and individual flavivirus.⁶⁹¹ For some flaviviruses, there are concerns that treatment could promote ADE and paradoxically exacerbate disease. In both monkeys and mice, subneutralizing concentrations of antibody enhanced DENV infectivity^{39,265,309,912} and thus could complicate the antibody therapy. Apart from or perhaps related to ADE, an “early-death” phenomenon⁵⁷⁹ has been reported that could also limit the utility of antibody therapy. According to this model, animals that have preexisting humoral immunity but do not respond well to viral challenge may succumb to infection more rapidly than animals without preexisting immunity. Although it has been described after passive acquisition of antibodies against YFV and Langat encephalitis viruses,^{43,269,875} this phenomenon was not observed after transfer of monoclonal or polyclonal antibodies against WNV,^{64,225,627} JEV,³⁹⁶ or TBEV.⁴²³

Although preexposure passive transfer of neutralizing antibodies protects successfully against infection by many flaviviruses,^{199,691} postexposure therapeutic studies have been performed in a more limited fashion, primarily with antibodies against WNV, TBEV, and DENV. In therapeutic trials, immune human γ -globulin protected mice and hamsters against WNV-induced mortality.^{62,64,225,367} Therapeutic intervention even 5 days after WNV infection reduced mortality; this time point is significant because in rodents WNV spreads to the brain and spinal cord by day 4. Therefore, passive transfer of immune antibody improved clinical outcome even after WNV had disseminated into the CNS. Analogously, postexposure treatment with polyclonal immune immunoglobulin decreased TBEV lethality in mice, with the degree of protection correlating with the amount of antibody administered and the time interval between infection and treatment.⁴²³

Humans have received passive therapy with immune γ -globulin against flavivirus infection. The largest experience is with commercial anti-TBEV antibody preparations (Encegam® and FSME-Bulin®). These products were available beginning in the 1970s, and recommended for treatment within a few days of a tick bite at risk for TBEV infection,¹⁰⁵ with between 70,000 and 200,000 doses administered.¹⁸ However, worsened illness after treatment was reported in three children,^{410,854} although no definitive clinical trial was ever conducted. Subsequently, immune γ -globulin production was suspended in the European Union, and Latvia remains the only European country where TBEV-specific immunoglobulin is still given.¹⁰⁵ In comparison, case reports have described improvement in humans with neuroinvasive WNV infection after receiving immune γ -globulin.^{295,745} Although promising, γ -globulin immunotherapy against WNV infection in humans has limitations: (a) batch variability may affect the quantitative titer and functional activity; (b) it is purified from human plasma and has a theoretical risk of transmitting infectious agents; and (c) it requires a large

volume of administration, which can increase adverse events in patients with cardiac or renal comorbidities.

More recently, humanized or human monoclonal antibodies or antibody derivatives with therapeutic activity against WNV infection^{272,627,823,850} have been developed. These human or humanized antibody fragments have high neutralizing activity *in vitro* and provide excellent protection in rodents. When some humanized mAbs were given as a single dose 5 or 6 days after infection, 90% of mice or hamsters were protected.^{581,627} Acute flaccid paralysis in hamsters also was blocked by treatment several days after infection using one anti-WNV neutralizing antibody.⁷⁰⁵ A phase II randomized, double-blinded clinical trial to evaluate safety and efficacy of this humanized antibody (E16, also termed MGAWN1) against severe WNV infection was recently completed (ClinicalTrials.gov identifier: NCT00515385). Neutralizing antibody therapeutics show promise, as they directly inhibit transneuronal spread of WNV infection and prevent the development of paralysis *in vivo*. Future use of a combination of monoclonal antibodies that bind distinct epitopes and neutralize by independent mechanisms could diminish the potential risk of selecting escape variants *in vivo*, especially in immunocompromised individuals who generate high-grade viremia and tissue viral burden.

Antibody-based therapeutics more recently have been proposed as a possible treatment for DENV infection. Although somewhat counterintuitive, because of the theoretical risk of ADE and immune-enhanced disease *in vivo*, genetically engineered antibody variants (E60-N297Q, 82.11-LALA, and 87.11-LALA) that recognize conserved epitopes in domain II or domain III and cannot bind Fc γ R exhibited prophylactic and therapeutic efficacy against ADE-induced lethal challenge of DENV-2 in mice.^{39,61} These observations suggest a novel strategy for the design of antibody-based therapeutics against DENV.

Nucleic Acid Inhibitors

RNA Interference

RNA interference (RNAi) is a cellular process that specifically degrades RNA within the cytoplasm of cells in a sequence-specific manner. RNAi occurs in plants, nematodes, parasites, insects, and mammalian cells and functions as a regulator of cellular gene expression and an innate defense against RNA viruses. RNAi uses dsRNA to target and degrade sequence-specific single-stranded RNA (ssRNA). The cytoplasmic ribonuclease Dicer recognizes and cleaves long dsRNA molecules into approximately 21 to 25 base pair small interfering RNA (siRNA) molecules; these associate with the RNA-induced silencing complex (RISC) to target and degrade complementary ssRNA molecules.⁷⁷³ The viral targets of RNAi have included double-stranded replicative-intermediate RNA or highly structured hairpin regions in single-stranded viral genomic RNA. In addition, any single-stranded viral RNA may be targeted and converted first to dsRNA by a cellular RNA-dependent RNA polymerase before recognition by Dicer.⁹

Many mammalian viruses appear susceptible to treatment with exogenous siRNA. Cells that express virus-specific siRNA or shRNA are resistant to infection by WNV,^{15,249,631,900} DENV,⁷⁹³ YFV,⁶³⁴ SLEV,⁹⁰⁴ and JEV.⁴²⁹ The sequence-specific activity of siRNA against viruses has led to great interest in its potential as antiviral therapy. Administration of siRNA to mice reduces flavivirus infection and affords partial protection against lethal challenge.^{32,429,634,904} WNV-specific siRNA could

act efficiently as a therapeutic after viral challenge, although administration within 6 hours of infection was required.⁴²⁹ No significant protection was observed when siRNA was delivered 24 hours after infection.³² Although promising, RNAi-based therapeutics against viruses may await the development of delivery systems that allow more effective activity against actively replicating viruses.

Antisense Technology

Antisense oligomers have been used to modulate gene expression of pathogenic viruses, and several are in various stages of clinical development.⁵¹¹ These compounds inhibit flaviviruses by binding RNA in a sequence-specific manner, effectively blocking access to a particular region of the viral genome. The development of phosphorodiamidate morpholino oligomers (PMOs) has enhanced water solubility and nuclease resistance,⁷⁹⁶ and the conjugation of arginine-rich peptides to PMOs has facilitated cellular uptake and inhibitory activity.⁶⁰⁹ Sequence-specific antisense oligomers have inhibitory activity in cell culture against several flaviviruses, including WNV^{189,190} and DENV.^{336,401} Low micromolar concentrations of arginine-rich peptide-conjugated PMOs that target the 5' untranslated or 3' cyclization sequences inhibited flavivirus infection by 5 to 6 log₁₀ PFU/ml^{190,401} when administered as pretreatment. However, when given either 2 or 4 days after infection, peptide-conjugated PMOs had little or no antiviral effect. PMO directed against the 5' and 3' conserved sequences partially protected mice from WNV or DENV infection and disease without causing appreciable toxicity,^{189,782} although selection of resistant mutants was observed.¹⁸⁹ Some clinical improvement was observed when PPO was administered to mice at day 5 after infection, although statistically significant differences were not achieved. AVI Biopharma initiated a phase I human clinical trial for treatment of WNV infection (ClinicalTrials.gov identifier: NCT00091845) with AVI-4020, but the study was terminated prematurely due to a limited pool of eligible subjects.

Flavivirus Antiviral Peptides

FuzeonTM is a peptide-based fusion inhibitor approved for clinical use in HIV-infected patients. Although structurally distinct, the flavivirus E proteins undergo an analogous series of pH-dependent conformational changes that permit entry, fusion, and nucleocapsid escape into the cytoplasm (see section on Virus Entry and Tropism). Exogenous administration of peptides corresponding to prM protein⁹¹⁹ or the stem anchor domains^{342,727,728} of WNV and DENV E proteins inhibit infectivity in cell culture, likely during a late stage of the fusion process. Peptides corresponding to sites in domain II and the domain I–II hinge interface also inhibited DENV infection in cell culture at the level of virus–cell attachment.¹⁶³ Finally, another group identified two E protein peptides that could inhibit WNV infection with half maximal effective concentration (EC₅₀) values as low as about 3 mM. Mice challenged with WNV that had been administered these inhibitory peptides showed reduced viremia and lethality.³¹ Although this is an emerging area of therapeutic development for flaviviruses with multiple possible targets, clinical studies have not yet been initiated.

Iminosugars

In flavivirus-infected mammalian cells, a 14-residue oligosaccharide (Glc)₃(Man)₉(GlcNAc)₂ is added in the ER to specific

asparagine residues on the prM and E virion proteins. This high-mannose carbohydrate is sequentially modified in the ER and Golgi by resident glucosidases to generate N-linked glycans that lack the terminal $\alpha(1,2)$ and $\alpha(1,3)$ glucose residues. Trimming of N-linked glycans in the ER is required for efficient assembly and secretion of flaviviruses in mammalian cells.^{164,892} Iminosugar derivatives, such as deoxynojirimycin or castanospermine, inhibit endoplasmic reticulum α -glucosidases I and II. This prevents processing of high-mannose N-linked glycans from nascent glycoproteins, a step that is required for interaction with the ER chaperones, calnexin, and calreticulin. Several flaviviruses are strongly inhibited by α -glucosidase inhibitors *in vitro*^{135,164,281,737,880,892} and *in vivo*.^{134,880} One possible advantage of α -glucosidase inhibitors is that they target a host enzyme that is an essential step in virus secretion rather than the virus directly, and are thus less likely to select for resistant variants.

High-Throughput Screens with Small Molecule Inhibitors

Over the last decade, high-throughput screens (HTS) with small molecule libraries have identified classes of “druggable” compounds that inhibit flavivirus infection. Several strategies have been utilized in high-throughput platforms including approaches that target viral enzymes, host proteins, key viral protein structures, and viral replication.⁶²⁰ HTS screens that directly or indirectly assess flavivirus replication have measured inhibition of cytopathic effect of viral infection, reporter gene expression in the context of a flavivirus replicon, or viral antigen expression by immunofluorescence and automated microscopy.^{282,622,659,660} Among viral targets, inhibitors that attenuate NS2B-NS3 protease activity, NS5 methyltransferase activity, and NS5 RNA-dependent RNA polymerase activity of different flaviviruses have been identified. Possible host targets for HTS include furin-like enzymes or signal peptidases that promote virion maturation, c-Src and c-Yes kinases that are required for assembly and maturation, cholesterol and lipid biosynthesis, and immune response genes. High-resolution x-ray crystal structures of key viral proteins (e.g., C, prM, E, NS3, and NS5) have informed structure-based design and *in silico* screening of inhibitors to augment the potency of lead antiviral compounds. Co-crystallization of lead candidate inhibitors with protein targets can determine the topology and consequences of binding, so that structure-activity related variants with augmented efficacy can be designed.

More limited studies have been performed with small molecule inhibitors in animals to assess therapeutic potential. One oral pyrazine derivative with broad-spectrum antiviral activity, T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) was protective in rodents when administered twice daily beginning 2 days after WNV infection.⁵⁸⁶ However, administration of T-705 at days 3 or 4 after infection showed little apparent efficacy. A small molecule nucleoside analog (e.g., NITD203) that targets flavivirus RNA synthesis showed efficacy against DENV in mouse models,¹⁴⁸ although its significance as a possible therapeutic agent appears limited by *in vivo* toxicity.

Given their continued global emergence and expansion, the development of antiviral agents against flaviviruses as a complement to intensive vaccine design strategies is essential. At present, several candidate therapies that act through distinct mechanisms are moving through various stages of preclinical and clinical development. Based on the pathogenesis of infection by different

flaviviruses, therapeutic agents may pair potent and direct antivirals with drugs that mitigate immune system-mediated inflammation and damage. For the encephalitic flaviviruses, inhibitors may need to cross the BBB efficiently to allow for control of local replication within neurons. For some flaviviruses where disease is sporadic and less predictable on an annual basis, regulatory hurdles will be encountered in implementing multicenter trials. Because of this, extensive preclinical experiments in small animals and NHPs may be useful to define whether a candidate therapeutic should reach human clinical trials. Ongoing pathogenesis and infection studies will inform novel drug design strategies that target individual viral proteins. Experiments in animals should continue to define the essential components of the protective immune response, and the immunologic risk factors that predispose to severe neurologic disease. Ultimately, a combination drug strategy that blocks viral infection, minimizes tissue injury, and limits the development of resistant variants will likely be more effective than single agents.

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Hepatitis C Virus

History

Pathogenesis and Pathology

- Entry into the Host
- Cell and Tissue Tropism
- The First Infectious Clone
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- HCV Cell Culture
- HCV Pseudoparticles
- Virion Production without HCV Replication
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Prevention and Control

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- Rapid Virologic Response (Week 4 Response)
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- Drug–Drug Interactions
- Vaccines
- Prevention of Transmission of HCV

Perspective

Acknowledgments

HISTORY

By the mid-1970s, it was apparent that at least one viral hepatitis agent other than hepatitis A virus (HAV) or hepatitis B virus (HBV) was the primary agent of posttransfusion hepatitis, a syndrome termed “non-A, non-B” hepatitis (NANBH).^{197,335} Studies of transfusion recipients revealed that NANBH tended to be milder in its acute form than HBV but could cause severe complications including cirrhosis and liver failure.¹⁶ Inoculation of chimpanzees with blood components from humans having both acute and chronic NANBH resulted in characteristic elevations of hepatic transaminases, providing a valuable animal model for NANBH and establishing the chronic nature of NANBH.¹⁷ By the mid-1980s, physicochemical studies of infectious inocula had revealed that the NANBH agent was a small (less than 80 nm), enveloped virus; however, the agent defied efforts directed at conventional viral cultivation and immunological detection.^{76,159}

Serial passage of NANBH in chimpanzees provided key pathologic, physiologic, and biochemical insights, as well as a well-characterized pool of specimens in which the agent was known to be present. A team led by Michael Houghton assembled a lambda phage library of complementary DNA (cDNA) derived from one such high-titer chimpanzee plasma specimen and then screened more than 1 million expression clones using serum from a chronic NANBH patient to find a single positive cDNA clone called 5-1-1.¹¹⁶ This discovery led to initial assays for detection of antibodies to the newly named hepatitis C virus (HCV),^{18,116,352} and the 5-1-1 antigen continues to be a component of anti-HCV serologic tests.

The first cDNA clone enabled further characterization of the genome as a positive-strand RNA molecule of almost 10,000 nucleotides containing a single open reading frame with an organization consistent with the *Flaviviridae*.¹¹⁷ Discovery of the authentic 5′ and 3′ untranslated regions (UTRs) led to a full-length cDNA clone of the HCV genome that, when transcribed, was infectious by direct intrahepatic injection in chimpanzees.³³⁷ The development of *in vitro* model systems was relatively intractable until the development of subgenomic RNA replicons³⁸⁷ and then successful passage in cell culture of a clone from one strain.⁶⁷⁰

HCV continues to present unresolved scientific and clinical challenges. Questions persist regarding fundamental aspects of the HCV life cycle, replication dynamics *in vivo*, mechanisms of persistence, and pathogenesis. Screening of blood products using antibody- and then nucleic acid–based testing, combined with other blood banking practices, provides a sound basis for the virtual elimination of transfusion-transmitted HCV infection; nonetheless, new infections continue to occur via other routes. Nearly 3% of humans remain chronically infected with

HCV, and although treatment continues to improve in efficacy and availability, HCV infection remains a major cause of death and disability worldwide.

PATHOGENESIS AND PATHOLOGY

Entry into the Host

As discussed in the Transmission section later (under Epidemiology), the primary route of HCV entry is percutaneous, although permucosal infection has also been described. Experimentally, HCV infection can be achieved by intravenous injection of HCV virions or intrahepatic injection of HCV genomic RNA.^{337,694}

Cell and Tissue Tropism

As depicted in Figure 27.1, HCV replication *in vivo* occurs primarily or exclusively in hepatocytes, the major parenchymal cell of the liver.⁵⁰⁵ The basis for this tropism is likely to be multifactorial, including entry facilitated by proteins expressed at particularly high levels on hepatocytes (e.g., low-density lipoprotein receptor [LDL-R]⁴⁴⁶ and scavenger receptor class B type I [SR-BI]^{185,248,557}), dependence on liver-specific miR-122 for efficient replication,²⁹⁹ and utilization of the liver's lipoprotein assembly pathway for virion production.²⁸⁰ Tissue-specific subcellular localization and dynamic interaction between viral components and more broadly expressed proteins on which HCV replication depends (such as CD81,^{195,248,270,320,499} claudins [CLDNs],^{183,267,270,422} occludin [OCLN],⁵⁰⁰ epidermal growth factor receptor,³⁹² and cyclophilins²⁵⁷) may also contrib-

ute to liver-specific tropism. HCV entry is discussed in detail in Chapter 25.

Extrahepatic Replication

Productive infection of other cell types is controversial,^{70,362} but there is evidence for extrahepatic detection of HCV negative-strand replicative intermediates,^{106,375,701} sequence variant compartmentalization,^{459,562} and *in vitro* replication in a variety of cell types.^{602,608} Viral dynamic modeling of data from the anhepatic phase of liver transplantation suggested that in a subset of patients with end-stage liver disease, an extrahepatic compartment exists that contributes no more than 3% to 4% of plasma viremia.^{142,505}

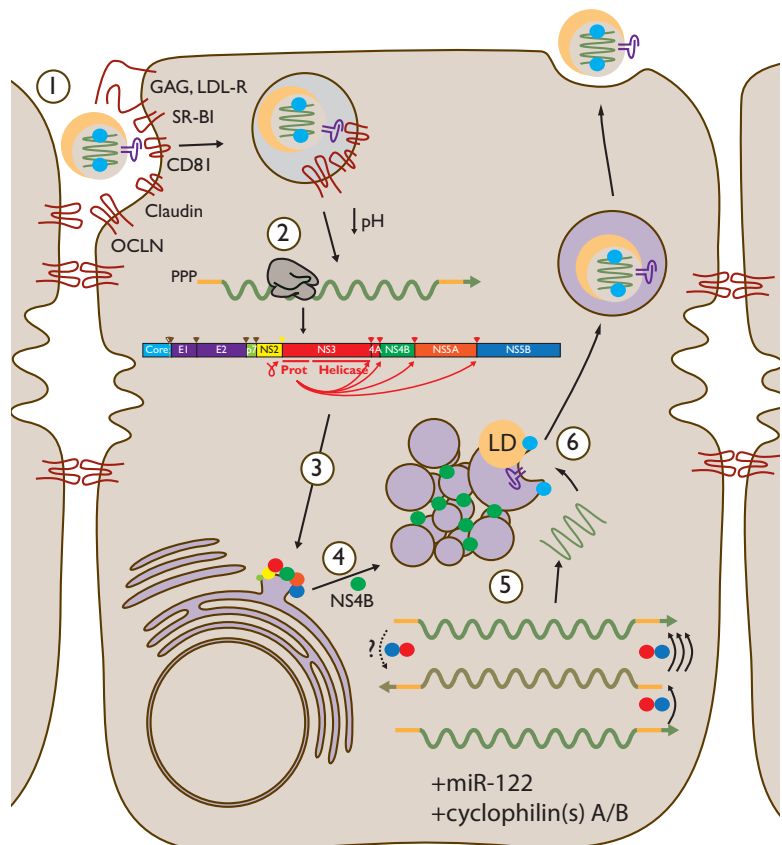
Model Systems *In Vitro*

During the first decade after HCV was discovered, the only robust model was the chimpanzee infected with primary or chimpanzee-passaged HCV isolates. Efforts to culture HCV from human and chimpanzee serum using primary hepatocytes or hepatoma cell lines were limited by relatively insensitive tools for measuring and visualizing infection, inconsistent cell lines, and the inherent variability of HCV isolates (see Genetic Diversity, later).^{284,290,313,444,445,454,570,697} Without an efficient culture system, screening of candidate antivirals was hampered.

The First Infectious Clone

In 1997, two groups separately reported chimpanzee infection with infectious clones of HCV,^{337,694} generated from acute phase isolate H77¹⁷ by using overlapping sequences spanning the genome (Fig. 27.2) to identify low-frequency polymorphisms

FIGURE 27.1. Life cycle of hepatitis C virus (HCV). Initial binding and internalization (1) probably involve glycosaminoglycans (GAGs) and low-density lipoprotein receptor (LDL-R), which may interact with viral envelope proteins or with virion-associated lipoproteins. Entry depends directly on binding of E2 with the tetraspanin CD81, as well as interactions with scavenger receptor BI (SR-BI) and tight junction proteins claudin-1 and occludin (OCLN). The viral genome is released from late endosomes (2) in a pH-dependent manner, followed by internal ribosome entry site (IRES)-dependent polyprotein synthesis (3) with initial cleavages among the structural proteins mediated by signalase and signal peptide peptidase followed by cleavage of the NS2–NS3 junction by NS2–NS3 cysteine protease; the remaining junctions are cleaved by the NS3–NS4A serine protease. NS4B recruits and rearranges endoplasmic reticulum (ER) membranes (4) to form a *membranous web*, the principal site of viral replication. Minus-strand and subsequent plus-strand RNA syntheses are affected by the NS5B RNA-dependent RNA polymerase (RdRp) (5) and depend on miR-122 and cyclophilin B, as well as conserved structural elements at the 5' and 3' ends of the genome. Core protein associates with lipid droplets (LDs) in the lipoprotein assembly pathway (6), linked to NS5A and other members of the replication complex by interaction with NS2. Viroporin p7 is necessary for production of stable viral particles coated with E1 and E2, which fold in a cooperative manner and are glycosylated in a manner consistent with ER but not Golgi processing.



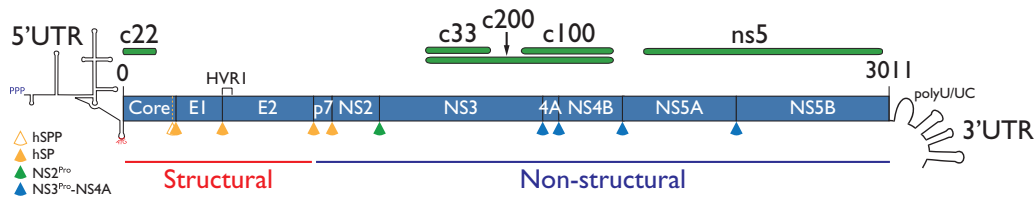


FIGURE 27.2. Map of the hepatitis C virus (HCV) genome, depicting the 5' untranslated region (5'UTR), capsid core, envelope genes *E1* and *E2*, viroporin *p7*, membrane-anchored cysteine protease *NS2*, serine protease-helicase *NS3*, *NS3* protease co-factor *NS4A*, membrane remodeling protein *NS4B*, phosphoprotein *NS5A*, RNA-dependent RNA polymerase *NS5B*, and the 3'UTR. Depicted as green bars are protein segments used as antigens in HCV enzyme immune assay (EIA) and recombinant immunoblot assay (RIBA) (c22, ^{268,609} c100-3³⁵²). Cleavages of the polyprotein are due to the action of signal peptidase (solid orange), signal peptide peptidase (open orange), *NS2* cysteine autoprotease (green), and *NS3-NS4A* serine protease (blue).

that were likely to be either artifactual (generated during cDNA cloning) or biologically genuine but less infectious. By removing such minor sequence variations, they constructed consensus sequences that were infectious, paving the way for the generation of consensus sequences from other isolates in search of clones that would replicate efficiently *in vitro*.⁵¹

HCV Replicons

In 1999, the efficient replication of subgenomic replicons in hepatoma cell line Huh-7 was reported, representing the first culture system that depended on HCV enzymes for propagation of a selectable marker (driven in a 2-internal ribosome entry site [IRES] bicistronic construct) and permitting more direct study of the viral life cycle.³⁸⁷ Highly replicative subtype subgenomic replicons were developed and were shown to depend on intact viral enzymatic sequences and negative-strand subgenome intermediates, inhibited by interferon- α .^{64,386} This latter characteristic opened the door to “curing” of cultures with interferon, resulting in Huh-7–derived cell lines like Huh-7.5⁶⁵ that were more permissive for HCV replication, an important tool for subsequent achievements including authentic culture of HCV *in vitro*.

Although cell culture adaptation of replicons permitted greater dynamic range of replication sufficient for screening inhibitors and studying viral protein interactions, the adaptive changes^{64,343,386} were often found to impair infectivity *in vivo*,⁸⁵ and when they included the structural and *p7* genes, these replicons did not produce structural proteins or viral RNA in the supernatant. Strains that were assembled from human isolates as consensus clones but required no adaptive changes as replicons were identified, including subtype 1b strain HCV-N, which was also infectious in chimpanzees,²⁸⁵ and subtype 2a strain JFH-1.^{315,316} When these were assembled as full-genome replicons, JFH-1 was found to produce infectious viral particles.

HCV Cell Culture

In 2005, the complete replication cycle of HCV in culture was described, using the subtype 2a strain JFH-1 in Huh-7–derived cells that had been made more permissive by eradication of HCV subgenomic replicon infection with interferon.^{670,706} Subsequently, the HCV cell culture (HCVcc) platform was broadened to include subtype 1a strain H77-S.⁶⁹⁶ As discussed in Chapter 25, this system and derivatives such as chimeric structural and non-structural regions²⁴⁸ and reporter constructs³²⁸ provide new avenues for investigation of HCV biology and immunity.

HCV Pseudoparticles

Prior to the advent of HCVcc, HCV pseudoparticles (HCVpp) were developed to study HCV entry, which was found to be similar to other members of the *Flaviviridae*, and CD81 was found to be necessary but not sufficient for *E1E2*-mediated entry.^{46,279} HCVpp expressing a variety of *E1E2* genes have facilitated study of cross-subtype and cross-genotype neutralization^{436,478} and have been used to demonstrate that neutralizing antibodies drive the rapid evolution of *E2* during acute HCV infection.^{170,382}

Virion Production without HCV Replication

There are many uses for high-titer stocks of HCV, but these can be difficult to generate and achievable titers are dependent on genomic characteristics (e.g., culture adaptive mutations) that may interfere with intended uses. A novel system for generating virions was recently developed to address these challenges, using cells conditioned by replication of a West Nile virus replicon.⁶³⁸ Because this system does not depend on HCV replication, it is potentially HCV sequence (i.e., genotype) independent.

Model Systems In Vivo

The initial model for HCV infection was the chimpanzee, essential to the discovery of this virus and to key experiments described herein (e.g., Transmission, Immune Response, and Genetic Diversity sections). The chimpanzee remains the only model for studying the full range of host–HCV interactions, from acute to chronic infection.⁸⁴ The availability and use of chimpanzees is very limited,²² and other models are available that are relevant to specific areas.

Mice with Implanted Ectopic Human Liver Grafts

The Trimera severe combined immunodeficiency (SCID) mouse model, in which liver fragments remain viable for weeks after ectopic implantation (e.g., under the kidney capsule), can be used for studying HCV infection. The level of replication is modest ($10^{4.8}$ IU/mL) but high enough for testing antiviral regimens.²⁸⁶ The liver tissue in this model does not maintain normal architecture.

Mice with Liver Injury and Human Hepatocyte Xenografts

SCID mice transgenic for urokinase plasminogen activator (uPA) driven by the albumin promoter develop severe neonatal liver injury that is rescued by infused hepatocytes that engraft and

occupy the space of the involuting liver.⁴³¹ Similarly, RAG^{-/-}/interleukin-2 receptor- γ -deficient (IL2R γ ^{-/-}) mice bred for fumaryl acetoacetate hydrolase deficiency (FAH^{-/-}) develop hepatic toxicity but can be rescued pharmacologically with NTBC (2-[2-nitro-4-trifluoromethylbenzoyl]-1,3-cyclohexanedione) or by transfer of the FAH gene. These FRG mice (FAH^{-/-}, RAG^{-/-}, IL2R γ ^{-/-}) will accept infusions of human hepatocytes after infection with a uPA-expressing adenovirus (presumably to proteolytically damage the liver stroma).³⁵ These mice can achieve physiologic levels of human albumin and lipoprotein levels, and after infection with HCV they can develop high levels of HCV RNA (10⁶ IU/mL) and maintain these levels for months. The mice are difficult to breed and remain immunodeficient, and hepatocyte engraftment is highly variable; however, they are a useful model for HCV replication and have enabled mechanistic study of phenomena such as HCV neutralization *in vivo*.⁴³⁴

Humanized Mouse

A different approach from those described earlier was the recent development of a genetically humanized mouse model of HCV infection that partially addresses host restriction factors that block HCV infection of mouse hepatocytes.¹⁶⁸ Using adenovirus gene delivery to induce expression of potential restriction factors for entry, CD81,⁴⁹⁹ SR-BI,⁵⁵⁷ CLDN-1,¹⁸³ and OCLN,⁵⁰⁰ they found that human CD81 and OCLN were required for entry in the mouse. Mouse SR-BI knockout and human SR-BI complementation confirmed the necessity of SR-BI for HCV entry and that mouse SR-BI could substitute for human SR-BI in HCV entry. The remaining host restrictions for HCV infection of mice are unknown, but the use of adenovirus gene delivery may have enhanced innate antiviral responses, and the stable expression of CD81 and OCLN in the mouse will facilitate further study. At present, the genetically humanized mouse model supports entry but not replication of HCV.

Spread

The mode of spread of HCV throughout the liver is poorly understood. High-level viremia achieved by HCV provides ample opportunity for virions to interact with hepatocytes, yet it appears that only about 10% to 20% of hepatocytes are infected during chronic infection.³⁷⁸ Lack of uniform infection may be explained by innate responses that could render cells refractory and adaptive immunity that could interfere with entry of free virions.⁵⁸⁸ Cell-to-cell spread⁴⁴⁹ within the liver could circumvent antibody responses, and data from *in vitro* culture on human hepatoma cell lines support this mode of spread,⁶³⁰ suggesting that virus spread *in vivo* is relatively resistant to neutralizing antibody compared with infection with free virions, yet is dependent on the same key entry factors (HCV envelope, CD81, SR-BI, OCLN, and CLDN family members).⁷⁹ These data are supported by the observation of foci of infection during *in vitro* culture, suggesting that this is a potential mode of local spread; however, it is clear that humoral immune pressure drives HCV evolution during chronic infection,³⁸² suggesting that a major component of spread during chronic HCV infection remains subject to antibody-mediated neutralization.

Immune Response

Each component of the host immune response to HCV is balanced in some way by viral components. As a result, multiple viral proteins (depicted in Figs. 27.1 and 27.2, with a detailed

functional discussion in Chapter 25) have immune-evasive roles in addition to more direct functions in the viral life cycle. These include Core (capsid), E1 and E2 (envelope), NS3-NS4A (serine protease), NS5A (polyfunctional phosphoprotein), and NS5B (RNA-dependent RNA polymerase).

Innate Immune Response

The innate immune response is of great importance in control of HCV infection,²¹⁴ and the virus has evolved a variety of mechanisms to evade this response (Fig. 27.3). Interferon signaling is a key component of the innate responses against HCV. Type I, II, and III interferons (IFN- α and IFN- β ; IFN- γ ; and IFN- λ , respectively) have all been shown to be important, early intrahepatic responses in HCV infection.^{60,363,374,626} Type I and II interferons are induced by overlapping signaling pathways. IFN-regulatory factor 3 (IRF3), a latent cytoplasmic transcription factor, can be activated by viral infection and translocated to the nucleus where it induces the transcription of IFN- β . In autocrine and paracrine fashion, IFN- β stimulates activation of the Janus-activated kinase and signal transducer and activators of transcription (JAK-STAT) signaling pathway and synthesis of IFN- α , as well as multiple other antiviral cytokines and chemokines, inhibiting viral replication and orchestrating the subsequent adaptive immune response.²²¹ HCV NS3-NS4A blocks IRF3 activation by proteolytically cleaving TIR domain-containing adapter-inducing interferon- β (TRIF) and mitochondrial antiviral signaling protein (MAVS). TRIF is an adapter protein for the double-stranded RNA (dsRNA) sensing molecule Toll-like receptor 3,³⁷⁷ and MAVS is an adapter protein in the retinoic-acid-inducible gene I (RIG-I) signaling cascade.⁴³⁹ These cleavages underscore the importance of these pathways for antiviral immunity, and impairment of interferon stimulated gene (ISG) expression was reversed by treatment with small-molecule inhibitors of the NS3-NS4A protease.^{377,389,439}

Defects in JAK-STAT signaling have also been described in HCV transgenic mice.⁶⁶ In HCV transgenic mouse and human liver biopsies, impairment of JAK-STAT signaling is linked to hypomethylation of STAT1 and increased expression of protein phosphatase 2A (PP2A).¹⁷⁵ There is also evidence that NS5A protein can stimulate IL-8, inhibit dsRNA-activated protein kinase (PKR), and interfere with 2',5'-oligoadenylate synthetase (2,5-OAS), antagonizing type 1 interferon signaling.^{223,305,344,501} Overexpression of HCV core protein also interferes with IFN signaling, likely through direct interaction with STAT1.^{67,381} Other studies suggest that ubiquitin-specific peptidase 18 (USP18) may be up-regulated by long-term interferon stimulation, blocking activation of ISG15 and suppressing JAK-STAT signaling, and leading to refractoriness to type 1 IFN stimulation.^{401,553} The inhibitory effect of HCV proteins on the interferon activation cascade is incomplete, as gene expression microarray studies have shown type 1 interferon responses in the livers of acutely and chronically HCV-infected chimpanzees.^{60,592} Hepatic levels of ISG expression in chronically infected humans vary significantly for unclear reasons.^{109,552}

Natural killer (NK) cells are also likely to play a very important role in control of HCV infection. NK and natural killer T-lymphocyte (NKT) cells are abundant in the liver and prime cellular immune responses through production of IFN- γ and other cytokines.^{138,639} Binding of the E2 protein to CD81 has been associated with inhibition of NK cell activity.^{138,639} HLA Cw*04 and related haplotypes, which bind inhibitory

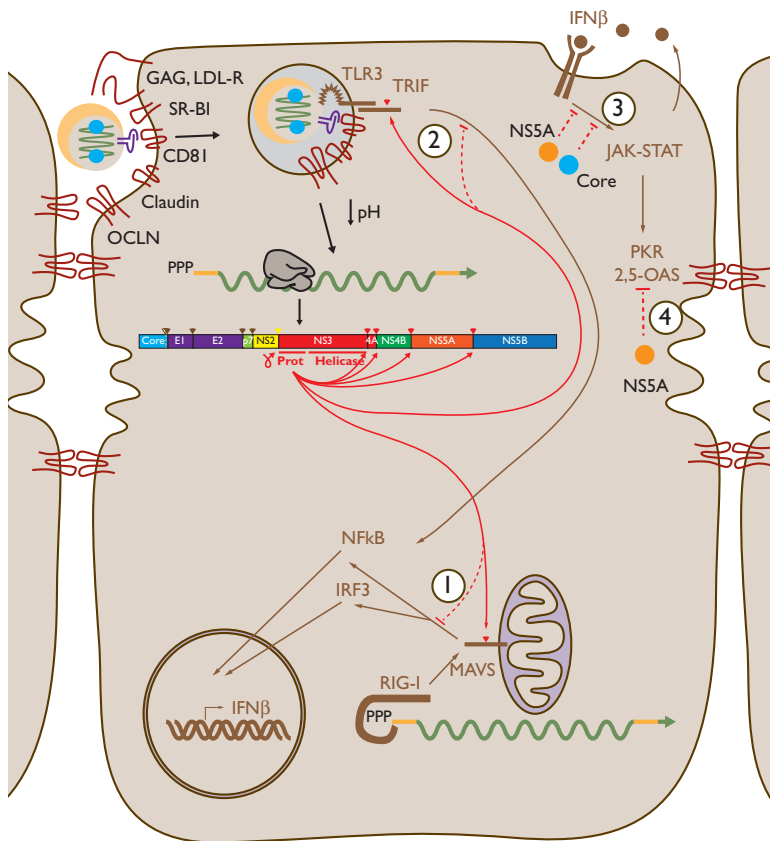


FIGURE 27.3. Innate responses to hepatitis C virus (HCV) and their evasion by the virus. Cytoplasmic HCV double-stranded RNA (dsRNA) can be sensed by RIG-I (1), resulting in signaling through mitochondrial antiviral signaling protein (MAVS) and subsequent nuclear translocation of nuclear factor (NF)- κ B and phosphorylated IRF3 that activate an antiviral program including secretion of interferon- β (IFN- β), which has autocrine and paracrine activity; HCV NS3-NS4A protease cleaves MAVS, blocking this signaling.^{213,389,439} Viral dsRNA may be sensed in endosomes by Toll-like receptor 3 (TLR3) (2), which signals via the adapter molecule TRIF domain-containing adapter-inducing interferon- β (TRIF), resulting in nuclear translocation of NF- κ B and expression of IFN- β ; NS3-NS4A protease cleaves TRIF, interfering with this response.³⁷⁷ IFN- β effects depend on the JAK-STAT signaling pathway, which is inhibited (3) by HCV core and NS5A proteins. Inflammatory responses including type I interferons activate host antiviral molecules including protein kinase (PKR) and 2',5'-oligoadenylate synthetase (2,5-OAS), which are antagonized (4) by NS5A. Further details and additional innate responses and evasive mechanisms are described in the text and Table 27.1.

killer immunoglobulin-like receptors (KIR) on NK cells, have been associated with viral persistence.³²⁵ The least inhibitory human leukocyte antigen (HLA)-C-KIR haplotypes are most strongly associated with recovery.

Dendritic cells are critical for orchestration of both innate and adaptive immune responses. Toll-like receptor 7 (TLR7) is expressed by plasmacytoid dendritic cells (pDCs), and pDCs produce type I interferon when co-cultured with Huh-7 cells containing replicating HCV RNA⁶⁰⁶; however, data regarding the effect of HCV infection on pDCs *in vivo* are less clear.^{153,306} HCV may interfere with NK cell activation of dendritic cells,²⁹⁷ and there is some evidence that HCV infection may be associated with impaired peripheral dendritic cell function.^{40,307} This impairment may explain the collapse of the cellular immune response during the transition from acute to chronic infection.^{133,564}

Cellular Immune Response

There is strong evidence that both CD4 and CD8 T-cell responses are critical for control of HCV infection, but there is limited understanding of failure of these responses leading to chronicity. HCV-specific T cells develop rapidly during acute infection and are then detectable for years in blood and liver in individuals after clearance of infection. During chronic infection, stronger polyclonal CD8 T-cell responses in the liver and circulation have been associated with lower circulating HCV loads,^{465,525} though CD8 T-cell responses to HCV epitopes are only detectable *ex vivo* in half of human immunodeficiency virus (HIV)-negative individuals chronically infected with HCV, whether obtained from peripheral blood or liver.^{327,340}

INDUCTION OF T-CELL RESPONSES

HCV-specific T cells typically become detectable in the blood 5 to 10 weeks after infection.^{127,371,573,615,643} In experimentally infected chimpanzees, intrahepatic T-cell responses appear another 4 to 8 weeks later.^{127,615} In intravenous drug users, there is significant overlap in the number of T-cell epitopes targeted during acute infection in individuals with subsequent resolving versus persistent infection outcomes.^{133,564} Even at the height of the response, HCV-specific CD8 T cells rarely target more than 10 epitopes, regardless of outcome, with little evidence of immunodominance.^{133,340} While acute phase CD4 and CD8 T-cell responses are usually detectable regardless of outcome, in individuals who progress to chronic infection, they disappear rapidly and may be less vigorous.^{133,160,259,308,441,564,616,644} HCV-specific T cells produce IL-2 and IFN- γ in individuals who go on to clear infection, and acquisition of full effector function may be a key factor leading to viral control in individuals with spontaneous clearance. In individuals who clear infection, functional effector CD8 T cells peak in the blood just after the initial drop in viremia, usually about 8 to 12 weeks after infection.^{127,251,368,371,615,616}

Anti-HCV T-cell responses are not focused on one viral protein or genomic region, and there is little evidence of immunodominance in general^{133,340,564} when compared with responses to HIV and influenza.^{10,379} Comprehensive analyses of CD4 and CD8 T-cell responses in persons with acute infection, using overlapping peptides composing the HCV polyprotein, have revealed widely dispersed epitopes (Fig. 27.4).^{133,368,564} In persons with certain uncommon alleles, such as HLA-B*27 and HLA-B*57, immunodominant responses to functionally

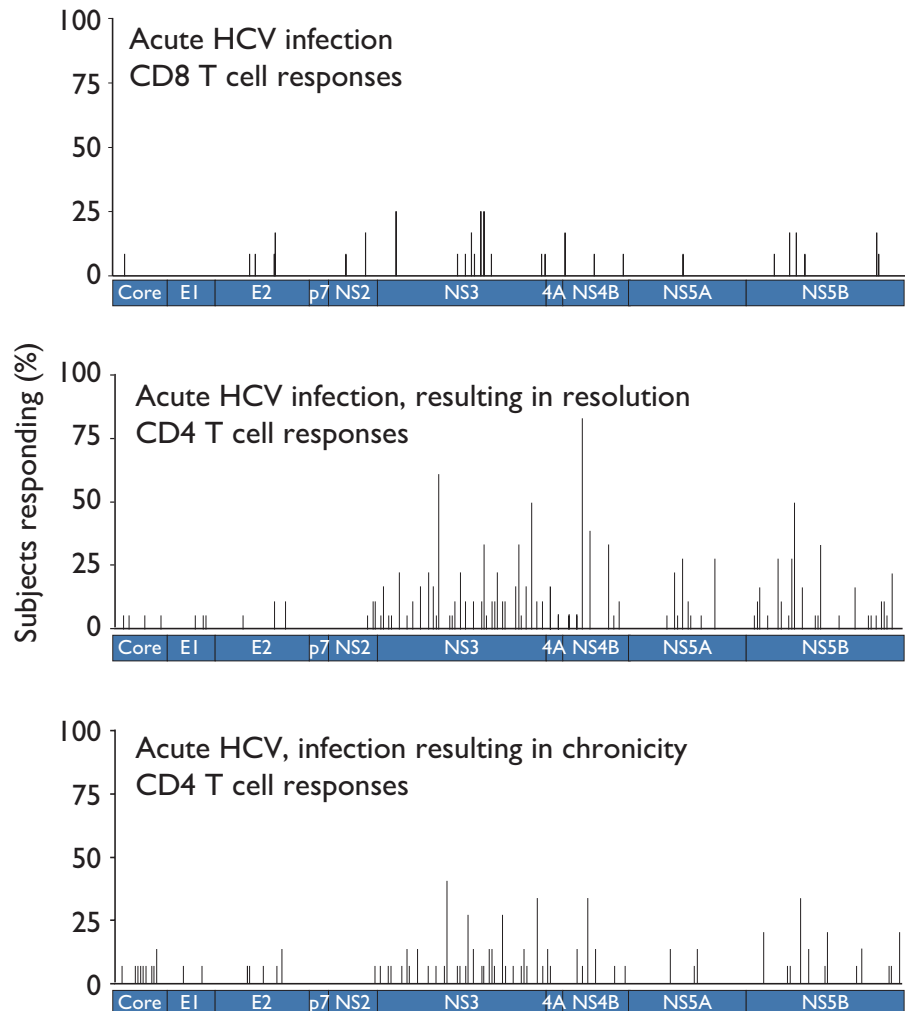


FIGURE 27.4. Anti-hepatitis C virus (HCV) T-cell epitopes are widely dispersed across the polyprotein during acute infection. The per-person frequency at which responses were detected using ELISpot is indicated for epitopes centered at positions indicated along the polyprotein. CD8 T-cell responses in 12 persons with acute HCV infection are indicated in the upper panel (adapted from two reports, with many outcomes unknown due to early interferon treatment^{133,368}), while the middle and lower panels depict CD4 T-cell responses from 18 subjects with resolving acute HCV and 13 subjects who progressed to chronicity, respectively.⁵⁶⁴ (Middle and lower panels © 2012, Schulze zur Wiesch et al. Originally published in *The Journal of Experimental Medicine*. 209:61–75.)

constrained epitopes have been described,^{469,470,477,549} whereas each person with the more common allele HLA-A*02 targets a few of the dozens of epitopes restricted by that allele.⁶⁷³

ROLE OF T CELLS IN CLEARANCE OF HCV

A central role for T cells in clearance of HCV was illustrated by studies in which T cells (CD4 or CD8) were depleted in chimpanzees in the context of acute HCV infection.^{251,573} Depletion of CD8 T cells, from an animal that had rapidly and spontaneously cleared HCV infection twice, led to prolonged viremia after reinfection, with control occurring when CD8 T cells returned.⁵⁷³ Depletion of CD4 T cells had a somewhat different effect, with widely fluctuating levels of viremia associated with progressive escape mutations at epitopes targeted by previously primed CD8 T-cell epitope responses.^{251,573}

In humans, spontaneous clearance of HCV has been associated with expression of certain major histocompatibility complex (MHC) class I molecules, with various studies showing association with the presence of HLA-B*57, HLA-B*27, HLA-A*11, HLA-A*03, or HLA-Cw*01, and the absence of HLA-Cw*04.^{326,349,420,617} Some of the differences in observed associations may be due to variation in predominant circulating HCV genotypes. The mechanism by which these particular alleles favor clearance of viremia is generally unknown, though there is evidence that some protective alleles bind and present

to T cells epitopes that are particularly immunogenic and/or functionally conserved,⁴⁷⁰ whereas risk alleles may be ligands for inhibitory receptors of NK cells.⁶¹⁷ The latter mechanism is supported by evidence that polymorphisms in NK receptors may also play an important role in HCV clearance.³²⁵

HCV clearance has also been associated with MHC class II genes, particularly DQB1*0301 and HLA-DRB1*1101.^{269,617} In a study using peptides spanning the HCV polyprotein, individuals with resolved infections targeted an average of 10 MHC class II epitopes (range 3 to 28), whereas individuals with chronic infection targeted an average of 1 epitope (range 0 to 8). Epitopes most frequently recognized were in core and nonstructural proteins, which may reflect differences in protein processing or mismatch in other regions between the circulating virus and library peptides.^{150,235,489,560,565} In chimpanzees, a subset of dominant CD4 T-cell epitopes were targeted prior to clearance of infection, and subdominant populations were detected only after clearance.²⁶⁹

MEMORY RESPONSES

Anti-HCV CD8 T-cell responses have been detected in individuals who have been exposed to HCV but have not seroconverted.^{81,340} Studies in chimpanzees have also shown that CD4 and CD8 memory T-cell responses are important for protection against reinfection with HCV.^{48,189,251,400,458,507,573,681} Control of

a second infection is associated with rapid expansion of memory CD4 and CD8 cells.³⁶³ Expansion of HCV-specific T cells was observed 2 to 3 weeks after reinfection compared to 10 to 12 weeks after initial exposure to virus. Depletion of CD8 T cells led to prolonged viremia after reinfection, and depletion of CD4 T cells also led to impaired control, despite the presence of previously primed HCV-specific CD8 T cells.^{251,573} Even more importantly, a recent study demonstrated that reinfecting humans tend to develop broader T-cell responses and lower peak viremia and are more likely to spontaneously clear their second HCV infection.⁴⁷⁸

T-CELL RESPONSES DURING CHRONIC VIREMIA

Despite T-cell responses, most HCV-infected individuals remain persistently infected. In individuals who progress to chronic infection, HCV-specific CD8 T cells become dysfunctional, possibly due to CD4 T-cell dysfunction.^{29,258,390,564,675} HCV-specific CD8 T cells obtained from peripheral blood during chronic infection show poor *ex vivo* proliferation and IFN- γ production, low intracellular stores of perforin, and decreased ability to lyse target cells.^{29,258,390,675} These functions are not consistently restored after successful treatment.⁴⁴²

Anti-HCV T-cell responses have been studied primarily in peripheral blood; due to compartmentalization of the T-cell response, such studies are likely to underestimate the breadth and magnitude of intrahepatic responses. Approximately a third of chronically infected individuals have intrahepatic anti-HCV T-cell responses that can be expanded *ex vivo*.^{339,687} Intrahepatic anti-HCV T-cell responses may be associated with lower serum HCV RNA levels, higher degrees of hepatic inflammation, and higher rates of response to interferon-based treatment;^{465,466} thus, responses that contribute to clearance may, if unsuccessful, contribute to injury.¹⁰² In spite of quantitative differences in number and breadth and some differences in phenotype⁵³ of T-cell responses, there are many similarities.⁵⁸⁵ Such inferences from PBMCs have been supported by indirect correlations such as viral escape substitutions in epitopes that were detected in assays of PBMCs.^{134,629}

Many HCV-specific CD8 T cells express the counterregulatory molecule PD-1.^{104,258,541,675} Studies in murine models have shown that PD-1 binds to programmed death ligands 1 and 2 (PD-L1 and PD-L2), and ligation leads to dephosphorylation of signaling molecules downstream of the T-cell receptor (TCR), decreasing T-cell sensitivity to stimulation.^{108,366,476} Blockade by anti-PD-L1 antibodies leads to increased proliferation of both CD8 and CD4 T cells directed against HCV.^{312,455,495,511,524,637} Interestingly, levels of PD-1 expression in acute infection do not appear to correlate with outcome of infection. In model systems, direct activation of CD8 T cells by hepatic parenchymal cells, without help from CD4 T cells, may result in impaired CD8 T cells that express high levels of PD-1.⁶⁹² Some recent studies have suggested that high levels of PD-1 may indicate a high level of immune activation, but not necessarily T-cell exhaustion.¹⁷⁶

T-cell immunoglobulin and mucin domain-containing molecule-3 (TIM-3) may also play a role in modulation of HCV-specific T-cell responses. Expression of both TIM-3 and PD-1 on CD8 cells during acute infection was associated with persistence, and like PD-1, blockade of TIM-3 increased proliferation of HCV-specific CD8 T cells.⁴²¹ Recent studies have suggested that 2B4 (CD244), another inhibitory molecule on

exhausted T cells in the lymphocytic choriomeningitis virus model of chronic infection, may also play a role in modulating HCV-specific CD8 T cells. HCV-specific CD8 T cells show increased expression of 2B4, and 2B4 stimulation reduced the increase in proliferation of HCV-specific T cells usually seen after PD-1 blockade.⁵⁶¹

Interaction with other immune cells likely also modulates antiviral T-cell activity. Liver-infiltrating CD8 T cells may have decreased expression of the co-stimulatory molecule CD86.^{301,355} Regulatory T cells (T_{reg})^{298,376} may modulate HCV-specific T-cell activity, and increased early IL-10 production during chronic HCV infection may drive CD4 T cells to become T_{reg}.²⁰⁹ These CD4CD25^{high} T cells are enriched in peripheral blood during chronic HCV infection and may infiltrate the chronically infected liver, potentially protecting it from injury.^{69,71,92,590,678}

HCV EVASION OF T-CELL RESPONSE

In addition to innate and adaptive host responses that are functionally inadequate for clearing infection, there appears to be selection for HCV mutations that enable response evasion while maintaining adequate replicative fitness to sustain infection. T-cell recognition of HCV is reduced by amino acid replacements that occur *in vivo*,^{103,134,628} and such changes have been correlated with persistence.^{134,181} Multiple cross-sectional studies have shown enrichment of amino acid changes in predicted or confirmed cytotoxic T-lymphocyte (CTL) epitopes among chronically infected individuals with corresponding HLA types.^{207,232,365,521,540,629} Observed mechanisms of reduced recognition during HCV infection (Fig. 27.5) include changes adjacent to epitopes that result in impaired processing for MHC class I presentation,^{329,566} changes in anchor residues that reduce binding affinity for MHC class I,^{134,628} and mutations that affect TCR contact residues.⁶⁸⁶

Amino acid replacements have potential fitness costs that may balance the fitness gain associated with escaping a T-cell response. These substitutions could disrupt functions of HCV proteins or RNA genomic elements^{299,642} or create neoantigen. Loss of protein function in this context has been observed, as have compensatory changes that appear to restore function.^{470,477,539,549}; such compensatory changes must be considered when analyzing HCV evolution in the context of an immune response and may be detected in searches for long-range interactions across the genome.¹⁶⁵ Neoantigen could be recognized by other T cells, as has been observed for HIV¹⁵; however, this does not appear to be common in HCV. Lack of recognition of the neoantigen produced by escape substitutions could be due to repertoire fixation,⁶⁸⁶ analogous to the phenomenon of original antigenic sin observed in repeated infections.³³⁴ A novel additional mechanism, the exploitation by HCV of a hole in the human T-cell repertoire⁶⁸⁶ such that the mutant form is not recognized at all, may not be surprising for a virus that has been adapting to humans for a very long time.⁵⁷⁶ Consistent with that mechanism, CD8 T cells specific for epitopes that have escape substitutions, though low in frequency, may express high levels of the memory marker CD127 similar to those found in persons with spontaneous clearance.^{53,311} Escape mutations are not generally observed in epitopes targeted by CD4 T cells^{220,564}; this is not surprising given the indirect role of CD4 T cells in antiviral responses, because a viral variant with an escape substitution in a CD4

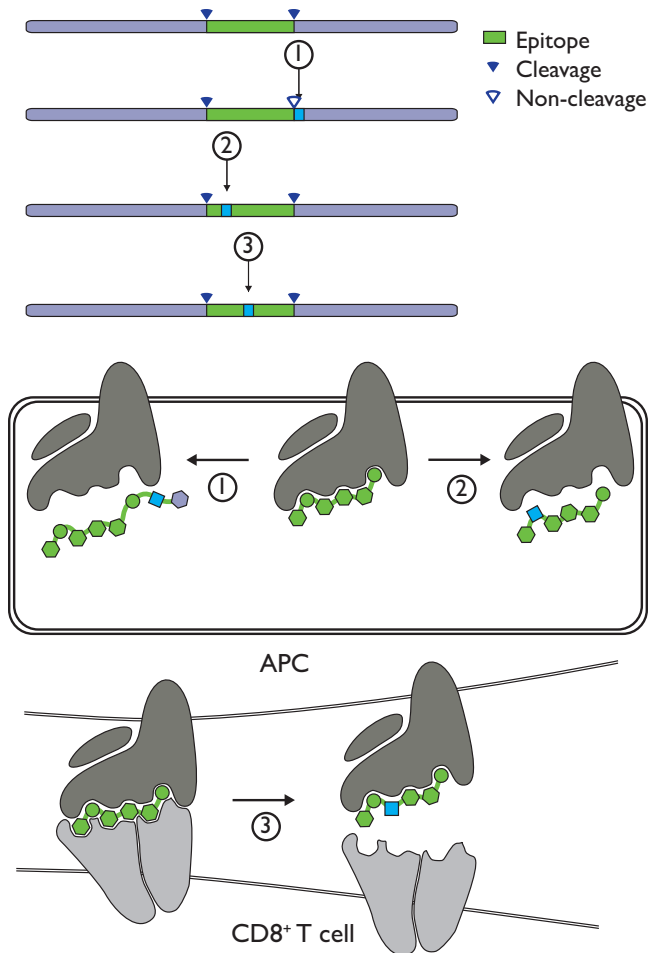


FIGURE 27.5. Evolutionary escape from CD8 T-cell response. Mechanisms for escape from recognition from CD8 T cells that have been demonstrated for hepatitis C virus (HCV) include (1) change in a residue affecting proteasomal processing, resulting in C-terminal extensions that cannot be trimmed in the endoplasmic reticulum (ER); (2) change in an anchor residue, resulting in loss of affinity for major histocompatibility complex (MHC) class I; and (3) change in a T-cell receptor (TCR) contact residue.

T-cell epitope might not have a survival advantage relative to nearby variants lacking such a substitution.

Humoral Immune Response

There is increasing evidence for the importance of the humoral immune response in control and clearance of HCV infection. Antibodies against HCV are not absolutely required for clearance of infection, as demonstrated in individuals with congenital agammaglobulinemia.⁴ In individuals with normal humoral immunity, however, binding antibody responses against structural and nonstructural HCV proteins are detectable within weeks to months of infection.^{110,467} Using autologous virus, neutralizing antibodies can sometimes be detected within this same time period.^{170,494}

Envelope proteins E1 and E2 are type I transmembrane proteins that exist on infectious virions as a cross-linked heterodimer.⁶⁶⁰ The structure of E1 is unknown, but recent mutational and computational analysis has produced a draft

structure of E2³⁴² that is supported by functional and antibody mapping studies.^{11,321} HCV envelope binds directly to CD81, and mutational analysis suggests that this binding involves E2 residues in domain I (Fig. 27.6). Although anti-E1 and anti-E2 antibodies can be detected in persons with acute and chronic HCV infection,⁴⁶⁷ almost all neutralizing antibodies target E2 and inhibit entry at a postattachment step.^{272,544,659}

There are numerous direct and indirect lines of evidence to suggest that antibodies against HCV decrease the risk of infection after exposure (see also Passive Immunization section, later). First, there were fewer HCV infections in liver transplant recipients who received immune globulin prior to 1990, the first year that immune globulin preparations were screened for HCV seroreactivity.¹⁹⁹ Second, immune globulin in a randomized controlled study reduced the incidence of sexual HCV transmission.⁴⁹⁷ In addition, inoculum-specific neutralizing antibodies directed at the hypervariable region 1 (HVR-1) reduced infection in chimpanzees; however, HVR-1 variability resulted in breakthrough infection.^{192,194,680} More recently, prophylactic treatment with a broadly neutralizing monoclonal antibody protected against HCV challenge in a human liver chimeric mouse model.³⁶⁹

Neutralizing antibodies may also play a role in modulating ongoing HCV infection. Individuals with primary hypogammaglobulinemia had more rapid progression of disease and poorer response to interferon treatment,⁶³ and individuals with humoral immune defects have fewer amino acid changes in E2.^{73,231}

EVASION OF THE NEUTRALIZING ANTIBODY RESPONSE

The development of pseudotyped lentiviruses for measuring neutralizing antibodies to HCV^{46,385,532} has revealed that chronic infection is associated with significant titers of neutralizing antibodies,³⁸⁵ and a case report of a chronically infected individual showed continuous rounds of escape from neutralizing antibody.⁶⁶⁶ During acute infection, neutralizing antibodies drive sequence evolution, suggesting that they have an impact on fitness *in vivo*, and early appearance of HVR-1-specific and/or neutralizing antibodies is associated with an increased likelihood of spontaneous viral clearance.^{13,170,494,708,709} Individuals reinfected after clearance of infection have lower second peak viremia, increased likelihood of clearance of the second infection, and a more broadly neutralizing antibody response.^{424,478}

The enormous diversity of the virus and tolerance of amino acid changes in E1E2 contribute to escape from this host response (Table 27.1). As with HIV-1, heavy glycosylation of E1 and E2 may provide a “glycan shield” that obscures conserved, functionally important domains (Fig. 27.6).²⁷² During the transition from acute to chronic infection, acceleration of evolution in HCV envelope genes is likely to be due to the appearance of neutralizing antibodies.³⁸² Because they are immunodominant targets of humoral immunity while also tolerating extensive non-synonymous variation, HVR-1 and, to a lesser degree, HVR-2 and the intergenotypic variable region (igVR) contribute to neutralizing antibody escape (Fig. 27.6, marker 2).^{42,47,193,523,659} Antibodies targeting the HVR-1 are common *in vivo*, but, given the variability of the region, they tend to be strain specific. In a study of neutralizing antibody development in acute HCV infection, neutralizing antibody escape mutations were mapped to the HVR-1.¹⁷⁰ Most broadly neutralizing antibodies bind to the E2-CD81-binding site.^{80,320,369,479,544,659} Some of these epitopes are linear, while others are conformational in nature. While the

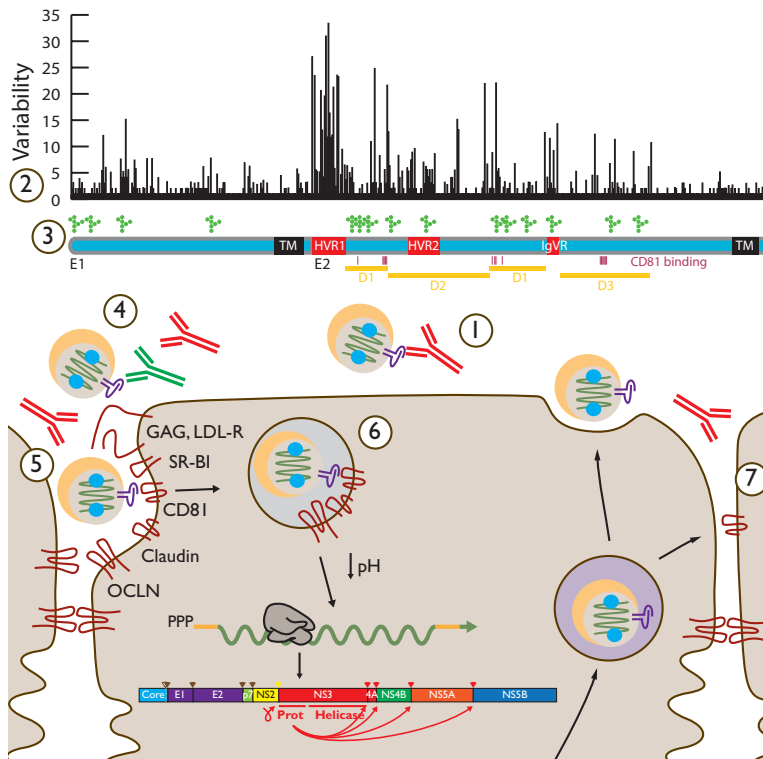


FIGURE 27.6. Evasion of anti-hepatitis C virus (HCV) antibody-mediated responses. Neutralization of HCV by antibodies can block infection of the cell (1). Binding of neutralizing antibodies (red) can be evaded by variability in the envelope proteins (2) illustrated here in a plot of Wu-Kabat amino acid variability⁶⁹¹ and by dense glycosylation at approximately 15 positions (3). Nonneutralizing antibodies (4, green) and lipoproteins (5) may hinder neutralizing antibody binding to HCV envelope glycoproteins, and delayed exposure of conserved domains until late in the entry process may prevent their recognition on free virions (6). Cell-to-cell transfer of virions is resistant to neutralizing antibodies *in vitro*, suggesting an additional mode of escape for local spread of infection (7). Along the envelope gene map are indicated transmembrane regions (TMs), hypervariable regions (HVRs), intergenotypic variable regions (IgVRs), putative tertiary domains (D1–D3), and CD81-binding residues (vertical lines).

CD81-binding site is highly conserved, many of these broadly neutralizing antibodies can induce escape mutations in HCV cell culture, suggesting that replication-competent escape variants may also exist *in vivo*.³²⁰

Several other mechanisms in addition to antigenic variability may contribute to HCV resistance to antibody-mediated neutralization. It appears that the CD81-binding site may be partially shielded from neutralizing antibodies by the HVR-1 and by N-linked glycosylation (Fig. 27.6, marker 3).^{42,187,272} Lipid shielding of the virion may also play a role, because studies have suggested that some neutralization epitopes are less accessible in particles associated with very low-density lipoproteins (VLDLs) or high-density lipoproteins (HDLs) (Fig. 27.6, marker 5).^{90,665} Additional mechanisms of evasion of neutralizing antibodies may include nonneutralizing antibodies that

bind E1E2 in a manner that interferes with binding of neutralizing antibodies, and postendocytic conformational changes in E1E2 revealing conserved determinants of entry (Figure 27.6, markers 4 and 6, respectively).^{545,705} *In vitro* demonstration of direct cell-to-cell spread of HCV, resistant to most neutralizing antibodies, suggests an additional potential mechanism for immune evasion (Fig. 27.6, marker 7)^{79,630,685}; however, strong evidence for antibody-driven HCV evolution^{170,382} suggests that neutralizing antibodies apply significant selection pressure *in vivo*.

Release from Host and Transmission

HCV RNA has been detected in small amounts in a variety of secreted body fluids including saliva, tears, and urine,^{111,204,430} but transmission primarily results from percutaneous exposure

TABLE 27.1 HCV Proteins Contributing to Persistence

Protein	Immune function	Evasion mechanism	References
Core	TNF- α and lymphotoxin signaling	Interference with intracellular signaling	(107,414,707)
E1, E2	Antibody binding, neutralization	Glycan shield	(272)
		Cell-to-cell spread	(79,630,685)
		Evolution/escape	(170,187,318–320,382,545,568,666,680)
NS3-NS4A	TLR3 signaling	TRIF cleavage	(377)
	RIG-I signaling	MAVS cleavage	(389,439)
NS5A	PKR, 2,5-OAS responses	Direct interaction	(222,223)
		IL-8 stimulation	(501)
Any	MHC epitope recognition by T cells	Evolution/escape (see Fig. 27.5)	(134,351,521,614,628)

MAVS, mitochondrial antiviral signaling protein; MHC, major histocompatibility complex; 2,5-OAS, 2',5'-oligoadenylate synthetase; PKR, protein kinase RNA-activated; RIG-I, Toll-like receptor; TNF, tumor necrosis factor; TRIF, TIR domain-containing adapter-inducing interferon- β .

to blood or rarely from mucosal exposure to genital secretions, as discussed in the section on Transmission later.

Virulence

In spite of their extreme heterogeneity, genetic variants of HCV (genotypes and subtypes) have remarkably similar clinical manifestations; for example, there have been no reported outbreaks of acute fulminant hepatitis, and persons infected in common-source outbreaks have displayed a wide range of outcomes.^{151,322} Moreover, efforts to identify viral determinants of fibrosis progression have not revealed consistent associations. Response to treatment is strongly affected by viral genotype, with genotype 1 being relatively refractory to interferon-based therapy as discussed in the Treatment section.

HCV subverts hepatic lipoprotein metabolism (see Virus Assembly section for HCV in Chapter 25), so it is not surprising that steatosis and insulin resistance are common features in HCV infection (see Clinical Features, later).²⁸¹ Multiple studies have found a significantly stronger association between genotype 3 HCV infection and steatosis¹⁵¹ than for other HCV genotypes; this association may be related to genotype-specific disruption of lipid biosynthesis pathways.¹¹⁸ Steatosis is also strongly associated with visceral obesity.⁵ Geographic variation in host factors as well as viral genetic types (see Genetic Diversity, later) could confound association of viral genotype with some manifestations.

During initial HCV infection, the peak of hepatic injury (illustrated by the peak in alanine aminotransferase [ALT] in Fig. 27.7) follows, rather than coinciding with, the peak of viremia.¹⁰² This consistent observation, combined with observations of liver pathology and cell culture, suggests that lysis of HCV-infected cells results primarily from the host antiviral immune response.^{102,115} The association of chronic infection with progressive liver disease and hepatocellular carcinoma is discussed in the Clinical Features section later.

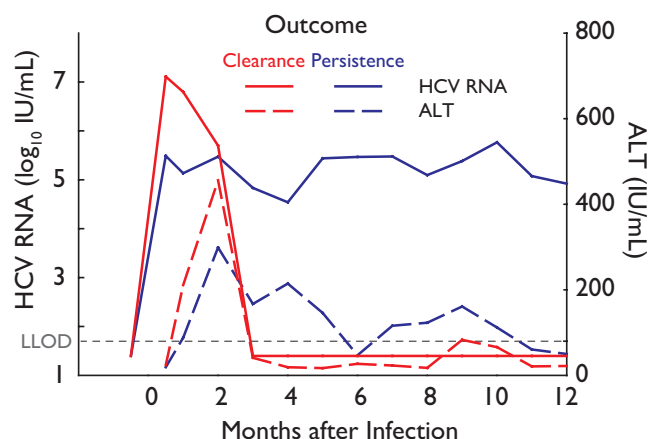


FIGURE 27.7. Patterns of acute hepatitis C virus (HCV) infection, resulting in spontaneous resolution or chronicity. The initial peak of viremia is followed by a peak in alanine aminotransferase (ALT) indicating cytotoxicity, temporally associated with the detection of cell-mediated responses to HCV that do not differ qualitatively by outcome when measured *ex vivo*.¹³³ Initial level of viremia is higher in those who clear compared with those who later progress to chronic infection.³⁸³

Persistence

When untreated, acute infection with HCV may spontaneously resolve or persist as chronic HCV infection (Fig. 27.7, discussed further in Clinical Features, later). Spontaneous clearance of HCV occurs in approximately one-third of untreated infections. This resolution occurs in the first 2 years and is generally complete, with no residual viral RNA in serum or liver.^{135,424}

Persistent HCV infection occurs in two-thirds of infected persons, is attributable to the evasion mechanisms discussed earlier, and is associated with persistent viremia at a level of 5 to 7 log₁₀ IU/mL in 90% of individuals.⁶²⁷ Spontaneous resolution during chronicity is rare.⁹ Because persistence is marked by high-level viremia and constant evolution in immunocompetent hosts, HCV appears to persist dynamically and there is no evidence of a stable, latent reservoir or archive of previously dominant variants. See Clinical Features of chronic infection, later.

EPIDEMIOLOGY

Morbidity/Mortality

The morbidity and mortality that is most clearly caused by HCV is liver failure and/or liver cancer as a result of chronic infection. In the United States, the Centers for Disease Control and Prevention estimates that chronic HCV infection contributes to 15,000 deaths per year, is the leading cause of liver failure leading to transplantation, and in 2007 superseded HIV as a cause of death (Fig. 27.8).^{184,394,684} HCV-related liver morbidity and mortality increase with older age and greater duration of HCV infection and are expected to rise in the coming decades. Using multistate disease models, one group recently estimated that HCV-related liver failure and cancer will continue to increase until 2020–2023 without widespread treatment.¹⁴⁷ Liver-related mortality is predicted to rise from 146,667 cases in 2000–2009 to 254,550 cases in 2010–2019 and 283,378 in 2020–2029. Reliable worldwide estimates of HCV-related mortality are not available.

HCV-infected persons are at increased risk of more than liver failure. In one study, 10,259 HCV antibody–positive blood donors were compared to donors matched by year of donation, age, gender, and zip code and followed for a mean of 7.7 years.²⁶¹ Compared to the HCV-uninfected donors, the risk of death was 3.13-fold higher in HCV-infected donors, who were more likely to die of not just liver-related but also drug/alcohol-related events, trauma/suicide, and cardiovascular causes. Persons with HCV infection are also at much higher risk of some medical conditions such as mixed cryoglobulinemic vasculitis and porphyria cutanea tarda (see Clinical Features later).^{6,154} The degree to which HCV infection contributes to less specific medical syndromes such as chronic fatigue/arthritis or mental illness is more difficult to establish.

Origin and Spread of Epidemics

HCV infection spread during the 20th century, strongly correlated with expanded production of syringes and their worldwide use for both conventional medicine and illicit drugs.^{172,215,397} Drucker and co-workers¹⁷² estimated that global syringe production rose from 100,000 per year in 1920 to 7.5 million per year by 1952. Widespread use of percutaneous injections for medicinal (and then illicit) drug use antedated appreciation of blood-borne transmission of infection

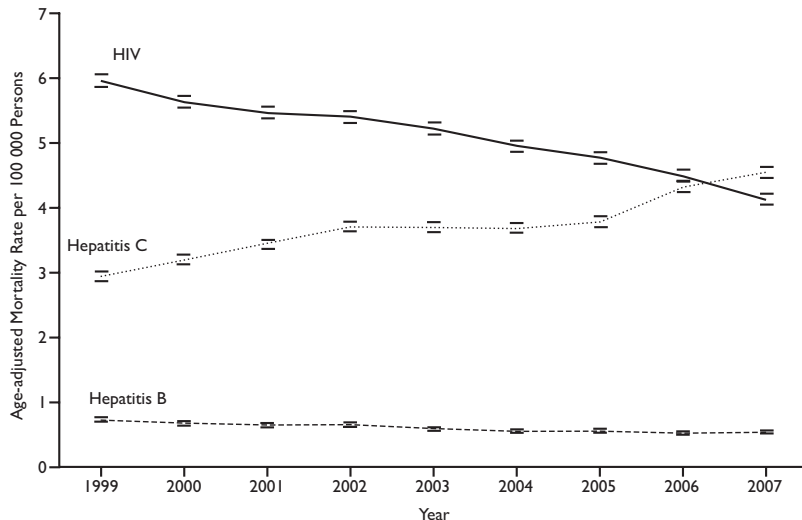


FIGURE 27.8. Annual age-adjusted mortality rates from hepatitis B and hepatitis C virus and human immunodeficiency virus (HIV) infections listed as causes of death in the United States between 1999 and 2007. Because a decedent can have multiple causes of death, a record listing more than one type of infection was counted for each type of infection. (From Ly KN, Xing J, Kleven RM, et al. The increasing burden of mortality from viral hepatitis in the United States between 1999 and 2007. *Ann Intern Med* 2012;156:271–278.)

and spread HCV throughout the world. This trend explains the 5- to 20-fold increased HCV prevalence rates in certain regions where unsafe injections were widespread and among injection drug users (IDUs) (see Global Burden, Incidence, and Prevalence).²¹⁵ Transfusions of blood products also contributed to HCV infection, especially when donors were paid and no measures were in place to screen blood for infection.³⁹⁹

Prior to the 20th century, HCV infection was probably sustained by percutaneous practices such as scarification rituals and circumcision. This conjecture is supported by evidence of transmission by such practices where they still occur and by molecular clock estimates derived from analyses of worldwide HCV RNA sequences (see Genetic Diversity).^{332,509}

Prevalence and Seroepidemiology

Transmission

HCV can be transmitted by percutaneous exposure to contaminated blood, from a mother to her infant, and by sexual intercourse. There is no evidence HCV can penetrate intact skin, but permucosal transmission has occurred when blood was splashed into eyes.

The likelihood that HCV transmission will occur is directly related to the inoculum and the exposure type. Blood is the usual inoculum, typically contains 5 to 7 log₁₀ copies of HCV RNA per mL, and rarely transmits HCV when viremia is not detected.^{166,627} Although HCV RNA has been amplified from most other body fluids, it is not clear to what extent other body fluids harbor infectious virions.^{204,430,671}

Percutaneous exposures such as unsafe medical procedures and injection drug use are the usual routes of HCV transmission worldwide. HCV transmission almost always occurs following very large percutaneous inocula, such as transfusion of a contaminated unit of blood.^{182,669} However, even very small (less than 10 µL) blood inocula may contain infectious virions to establish infection in a recipient if injected percutaneously, and nosocomial exposure may occur if strict universal precautions are not observed.^{14,293} Blood spiked with an HCV reporter virus was loaded into syringes and viability was recovered from 71% of tuberculin syringes kept at 22°C for 7 days.⁴⁸⁰ This finding correlates with studies of health care personnel with accidental needlestick exposures in whom transmission occurs

in 1% to 2% overall and more often from hollow-bore needles, which contain a larger inoculum than a solid-bore needle.^{330,528} Repeated small-volume exposures to HCV explain the high rates of HCV among injection drug users (see later).

Nonmedical percutaneous exposures such as body piercing and tattooing are plausible risks and epidemiologically linked to HCV prevalence in many countries, though they are likely to be confounded by other risky behaviors in some populations.^{276,336,395,411,426,438,462,506,601}

The frequency by which HCV is transmitted sexually is controversial. On the one hand, long-term monogamous partners of individuals with HCV infection almost never acquire HCV.³⁰ In one study, 895 monogamous sexual partners of persons with chronic HCV infection were followed for over 8,000 person-years, and there were no instances of sexual HCV transmission, despite unprotected intercourse occurring an average of 1.8 times per week.⁶⁵⁴ On the other hand, HCV infection occurs in persons acknowledging high-risk sexual practices (and no other exposure),⁶⁵⁰ and there are multiple outbreaks among HIV-infected men who have high-risk sexual exposures with other men.^{145,648} One speculation is that, as with HIV, the risk of sexual HCV transmission is greater during the acute phase of infection when viremia peaks and prior to formation of neutralizing antibodies. In addition, anal intercourse may cause mucosal tears that promote HCV transmission. Permucosal spread of HCV may also explain the association of HCV infection with intranasal use of cocaine.¹²⁵

HCV transmission from a mother to her infant occurs infrequently (2% to 10%).^{413,527,700} How and when infection occurs in this setting is not known, but risk is increased by maternal HIV infection and/or high HCV RNA levels, prolonged rupture of membranes, and internal fetal monitoring.^{413,624}

Global Burden, Incidence, and Prevalence

There are an estimated 185 million HCV-infected persons in the world, or 2.2% of the human population.^{688,689} There are marked differences in HCV prevalence between regions (Fig. 27.9) and, even within countries, between age and risk groups. Egypt appears to have the highest HCV prevalence, which is as high as 50% in persons born before 1960.²¹⁵ The history of HCV infection in Egypt is exemplary of global

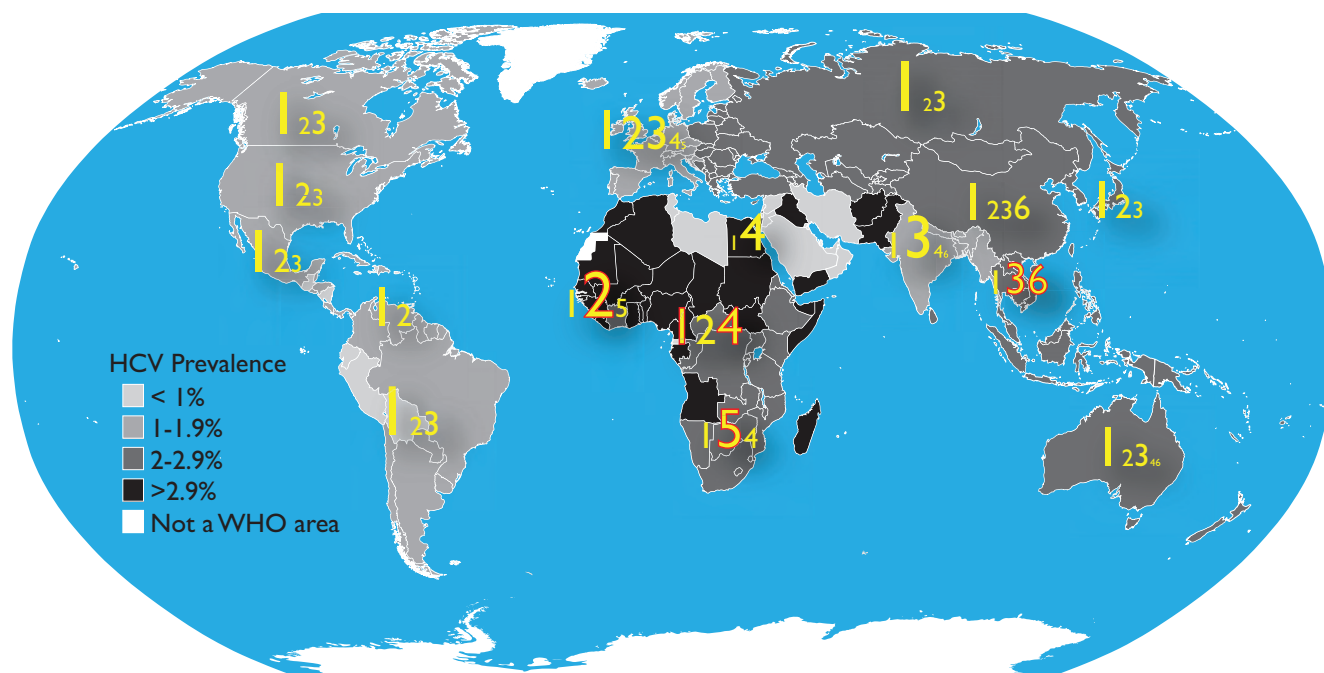


FIGURE 27.9. Map depicting geographic variation in the relative prevalence of hepatitis C virus (HCV) infection and genotypes. Shading of country indicates prevalence. Yellow numerals indicate prevalent HCV genotypes in different regions, with font size corresponding to relative genotype prevalence within each region; red outlining indicates genotypes with greatest intraregional diversity. HCV prevalence is highest, and genetically most diverse, in Africa. Genotype 1 is prevalent worldwide, whereas genotypes 4 and 5 are almost exclusively found in north-central and southern Africa, respectively. Genotype 7 (provisionally assigned and not depicted) has been reported very rarely in persons with epidemiologic links to central Africa. (HCV prevalence estimates adapted from World Health Organization. Global burden of disease [GBD] for hepatitis C. *J Clin Pharmacol* 2004;44:20–29, with permission. Relative genotype prevalence within each region based on sources cited in the text.)

transmission patterns. From the 1950s to the 1980s, the Egyptian Ministry of Health embarked on a campaign to eradicate schistosomiasis infection by intravenously administering tartar emetic to millions of citizens.⁵⁸⁹ The effort, commended at the time as a public health model, occurred before there was widespread appreciation for blood-borne transmission of infectious agents. HCV was transmitted extensively because of the widespread reuse of insufficiently cleaned injection equipment.²¹⁵ Consequently, the prevalence of HCV infection can exceed 50% in persons alive during that campaign while being 1% to 2% in those born after. In addition, more than 90% of HCV infections in Egypt are genotype 4, which make up less than 10% of genotypes in most other regions of the world.⁵²⁰

There is molecular and epidemiologic evidence of similar transmission patterns elsewhere. In studies modeling HCV sequences, Tanaka and co-workers⁶¹⁰ estimated rapid expansion of HCV-1b in Japan in the 1920s, in Europe in the 1940s, and in the United States (HCV-1a) in the 1960s.⁶¹⁰ Population data from southern Italy show that the HCV prevalence is 1.3% in subjects younger than 30 years and 33% in those older than 60 years of age; the odds of infection were doubled in those who recalled reusable glass syringe use.²⁶⁰ Thus, as mentioned earlier (in Origin and Spread), it appears HCV was widely transmitted worldwide during the 1900s due to stepped-up production of syringes and their worldwide use both for conventional and illicit drugs.^{172,397,509}

The overall prevalence of HCV infection in Europe is 1% to 2%. However, country-specific rates vary considerably, with

the lowest HCV prevalence (less than 0.5%) reported from Sweden, Germany, and the Netherlands while prevalence rates of 2% to 3% have been reported in some Mediterranean countries.²⁷¹ There is less information on incident HCV infection. Although HCV surveillance is required in European countries, new infection information is restricted to symptomatic events (which are the minority of HCV infections) and thus data on trends and comparisons across regions are crude. Overall, because procedures to screen blood donations were implemented, most new infections in Europe are linked to injection drug use or recent health care exposure.⁴⁰⁸

United States Prevalence and Incidence

In the United States, an estimated 3 million persons have chronic HCV infection. Several key epidemiologic trends explain the incidence and prevalence of HCV infection. As in other parts of the world, unsafe medical injections probably contributed to an early expansion of HCV prevalence following World War II. Transfusion of blood and blood products caused new HCV infections until 1992, when the most effective screening measures were adopted. However, it was the epidemic of injection drug use from the 1950s to the 1980s that caused most HCV infections in the United States. Whereas there were probably fewer than 500,000 persons with chronic HCV infection in the early 1950s, by the mid-1990s there were an estimated 3.5 million persons with chronic HCV infection and another 1 million to 1.5 million who had recovered.³¹ Much of that epidemic spread was due to injection drug use.

Not only does injection drug use cause most HCV infections in the United States, but also most injection drug users have been HCV infected. HCV infection generally occurs within the first years of initiating the illicit use of injected drugs with annual incidence rates of 10% to 30%.^{135,228,266} In one cohort, 80% of subjects acknowledging 2 or more years of injection use were infected with the virus, a prevalence that was higher than that of HIV or HBV infection.^{229,625} Early acquisition of HCV is probably related to the practice of older (infected) IDUs teaching new (uninfected) initiates by demonstrating first on themselves and then on the new initiate.²²⁸ Although sharing of needles and syringes causes some HCV transmission, Hagan and co-workers²⁶⁵ estimated that 37% of new cases were due to sharing of other equipment.

After peaking in the 1980s, HCV incidence has dropped markedly in the United States.⁹⁸ Elimination of transfusion-related transmission contributed to the reduction in incidence. However, most of the decline is attributed to a reduction of HCV due to injection drug use that is not fully explained.¹⁹ However, because HCV serology remains positive in most instances even when viremia is cleared, the 20-year surge in HCV incidence among persons born between 1945 and 1964 remains serologically evident.

The best data on HCV prevalence in the United States come from the serial National Health and Nutrition Examination Surveys (NHANES).³² By testing blood collected from a subset of persons representing households in the United States around 1990, it was estimated that 4.1 million individuals had been infected with HCV, or 1.6% of the general population. Approximately two-thirds of those infected were born between

1945 and 1965. The survey was repeated 10 years later and showed the same number of HCV-infected individuals in the same age cohort that was 10 years older. Omitted from this survey were nearly 2 million incarcerated persons in the United States, who probably represent another 250,000 to 500,000 HCV-infected persons.³⁹ The prevalence of HCV infection in the United States is also higher among racial minorities than in Caucasian Americans, and greater in African Americans than in Mexican Americans. In non-Hispanic Blacks 40 to 49 years of age, the HCV prevalence was 14% compared to a general population prevalence of 1.6%. HCV infection was detected in only 1% of those 20 to 29 years of age.

Genetic Diversity

Genetic variability is one of the most remarkable features of HCV, contributing to evasion of host immune responses and complicating development of diagnostics, therapeutics, and effective vaccines. HCV genomic sequences can be clustered phylogenetically into related groups (genotypes and subtypes), are distinct between individuals, and are highly variable within each infected individual at any given point in time (i.e., quasispecies diversity^{314,406}) and over time (i.e., quasispecies divergence).

Global Diversity of HCV

Soon after HCV was discovered, it was apparent that genetically distinct strains were prevalent in different geographic areas. International standards for nomenclature established six major genotypes that are phylogenetically distinct, and subsequent reports have resulted in the proposal of a seventh genotype (Fig. 27.10).^{248,346,451,530,577} Within genotypes, phylogenetically

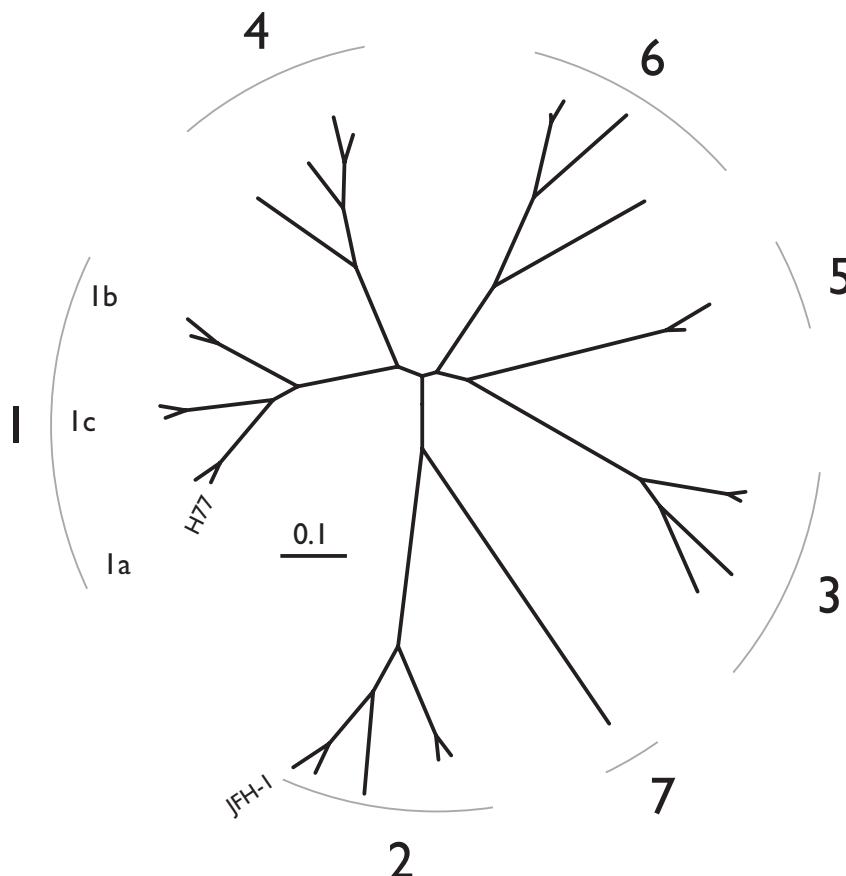


FIGURE 27.10. Phylogenetic tree of representative sequence from the seven proposed genotypes of hepatitis C virus (HCV). Full-genome nucleotide sequences were aligned and analyzed using a maximum likelihood model with estimation of invariant sites and modeling of variable rates using the gamma distribution, with bootstrap re-sampling to confirm support for each genotype cluster. Reference isolates H77 (AF009606) and JFH-1 (AB047639) are indicated. Subtypes of genotype 1 (1a, 1b, 1c) are indicated for illustration.

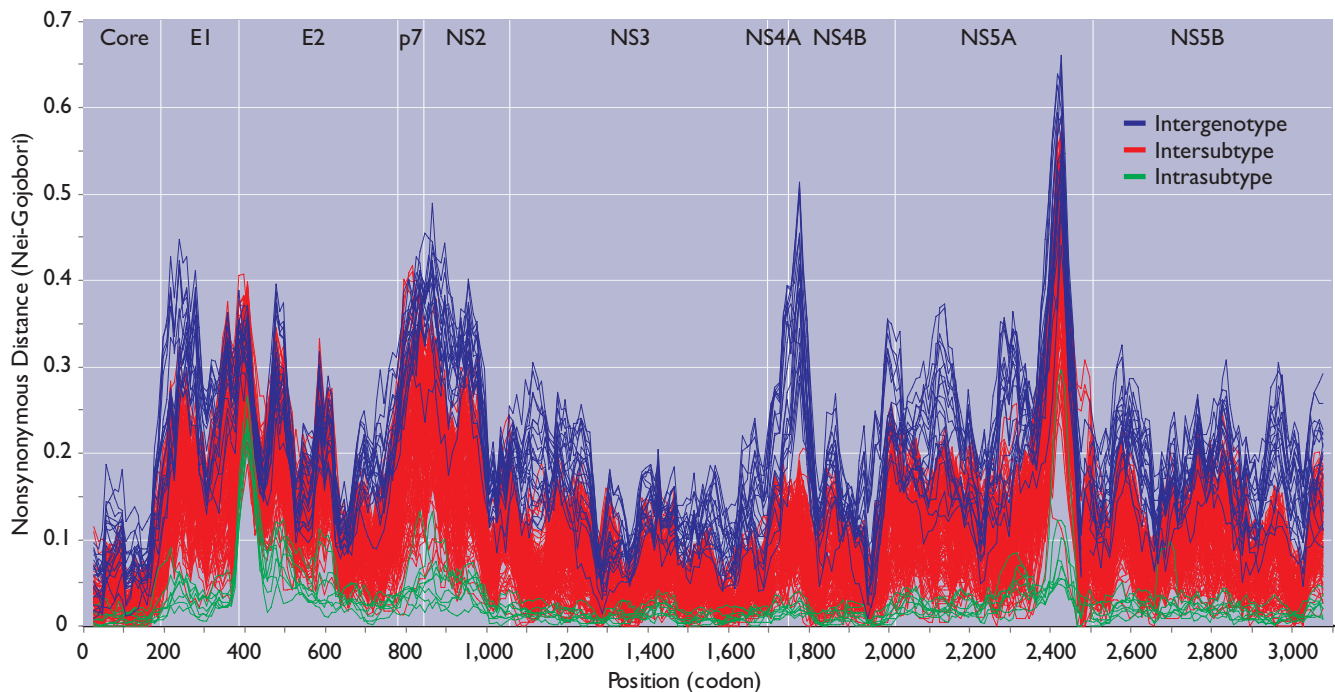


FIGURE 27.11. Genetic variability of hepatitis C virus (HCV) by genomic region. Using an alignment of full-genome sequences from all seven proposed genotypes, the polyprotein open reading frame (ORF) was analyzed using VarPlot (described in⁵²³) to calculate mean pairwise nonsynonymous distance in a sliding window of 50 codons at intervals of 10 codons. For intergenotype comparisons (blue), there are 21 curves representing each pairwise comparison among the seven genotypes. For genotypes having at least two subtype representatives, there is a curve for each pair of subtypes (red). There is one curve representing mean intrasubtype distance for each subtype having at least four available sequences (green), including subtypes of genotypes 1, 2, 3, 4, and 6; for subtypes having many full-genome sequences, 10 were identified randomly.

distinct clusters may be found that are called subtypes. Clinically, HCV genotypes and subtypes are very similar (see Virulence), though they vary in responsiveness to interferons (genotypes 1 and 4 are less responsive) and, in more complex ways, in susceptibility to direct-acting antiviral agents (see Treatment).

The pairwise distance between genotypes ranges from 29% to 34% for genomic nucleotide sequences and 24% to 33% for amino acid sequences spanning the polyprotein. The protein-coding differences between genotypes are not uniformly distributed across the genome; rather, they are greatest in the E1, E2, p7, NS2, N-terminus of NS4B, and V3 region²⁸⁷ of NS5A, and most conserved in core (blue curves in Fig. 27.11). As that figure illustrates, the contours of pairwise distance are remarkably consistent between genotypes, reflecting neutral evolution as a dominant force in the divergence of genotypes.⁵⁷⁶ Even in the most variable regions (e.g., HVR-1, positions 384 to 410 in the polyprotein using standard genome numbering³⁴⁵), there are severe constraints on amino acids with regard to specific amino acids⁴¹⁵ and biophysical properties.⁴⁸⁸

Variation within genotype (i.e., genetic distance between pairs of subtypes) is about half of the intergenotype diversity, with similar regions of divergence and conservation (red curves in Fig. 27.11); a notable exception is the N-terminus of NS4B, the significance of which is unclear. Variation within subtype (green curves in Fig. 27.11) is more restricted, yet remarkably consistent considering that each tracing represents one subtype

from widely divergent genotypes (1, 2, 3, 4, and 6, which sufficient representatives). Hypervariable region locations that differ among genotypes suggest different selection pressures.²⁸²

Potentially confounding genotype assignment and diagnostics is recombination, which is a dominant mode of evolution in HBV and HIV.^{531,579} Intergenotype recombination has been reported in HCV sequences,^{59,300,302,373,474} and standards for confirmation and nomenclature of recombinant forms are available.³⁴⁶ While recombination among HCV subtypes has clearly occurred *in vivo* and hybrid genomes can be generated *in vitro*,^{248,498,559} this appears to be a rare occurrence given how often distinct HCV genomes simultaneously infect the same host.^{56,580,658} HCV recombination may be limited by superinfection exclusion, which has been demonstrated for HCV *in vitro*.⁵⁵⁸

Because intergenotypic recombination is rare, phylogenetic trees obtained from any segment of the genome will reflect genotypic clustering seen on the full-genome tree, on a scale determined by the variability in that segment of the genome.⁵³⁰ By international consensus,³⁴⁶ the reference genomic regions for HCV genotype/subtype assignment are core/E1^{86,128} and NS5B⁵⁹¹ (see Diagnosis section).

Global Molecular Epidemiology of HCV

Accurate estimation of the relative prevalence of HCV genotypes in a region depends on population-based sampling,^{33,452,520} which is rarely performed. Nonetheless, it is clear that HCV genotypes

are nonuniformly distributed among geographic regions, with dominant features depicted in Figure 27.9.^{131,323,347,464,575}

Genotype 1 is the most widely dispersed worldwide, and phylodynamic analyses are consistent with global expansion in the population and dispersion from 1940 to 1980 and suggest that subtype 1b may have disseminated, possibly in blood products, earlier than subtype 1a.^{397,509} These trends are corroborated by other studies,^{354,536} and subtype 1a is often associated with recent or ongoing drug use in North America and northern Europe.^{55,135,241,452}

Independent analyses of sequence data from other countries in the western hemisphere are consistent with expansion of epidemics during the second half of the 20th century but involving a wider variety of subtypes.^{25,360} Taken together, these studies do not suggest that HCV subtypes differ significantly in terms of transmissibility or association with specific routes of transmission; rather, specific HCV subtypes appear to dominate epidemics as a result of founder effects, and the presence of highly diverse variants of one genotype in a region suggests a local origin.⁵⁷⁶

The epidemic in Egypt²¹⁵ is dominated by subtype 4a but includes diverse subtypes of genotypes 1 and 4,^{514,520} perhaps reflecting its proximity to Central Africa where genotypes 1 and 4 appear to be endemic.^{216,338,427,460,473,482,672} and may share a common origin.^{461,547} Genotype 2 is highly diverse in West Africa, where subtype boundaries can be indistinct, suggesting a local origin of this subtype.^{96,295,538} In Southeast Asia, genotypes 3 and 6 appear to be epidemic, including among IDUs, with subtypes 1b and 2a associated with older infections.^{428,506,578,631–634} (note that in 2005, sequences that had been assigned to subtypes 7a, 8a, 9a, 10a, and 11a were reclassified as subtypes 6d, 6k, 6h, 3k, and 6g, respectively.⁵⁷⁷) Genotype 5 is found in southern Africa, with limited dispersal.²⁶

Origins of HCV

Available evidence summarized in the previous section suggests that HCV is endemic in widely separated regions of the globe. While nonprimate hepaciviruses have been discovered recently in dogs and horses,^{89,309} those isolates are very distinct from HCV, and their origin, prevalence, and tropism are not known.

HCV genotypes appear to have arisen hundreds or perhaps thousands of years ago.^{397,509,583} Without sequence data older than 50 years to “calibrate” such molecular clock analyses, phylogenetic saturation at the HCV genotype level may preclude accurate estimation of the age of HCV.⁵⁷⁶

Quasispecies Variability: Mechanisms

HCV exists in each infected host as a swarm of genetically related but distinct variants, collectively called a quasispecies.^{162,163,177,314,406} This characteristically diverse set of viruses in an individual arises from one or more “founder” sequences from the predictably diverse quasispecies present in the donor(s), selected randomly or based on phenotypic characteristics by a transmission bottleneck that is poorly understood.⁸⁸

Diversity is generated by mutations introduced by the NS5B RNA-dependent RNA polymerase, which lacks a proof-reading function and has an estimated error rate of 10^{-3} to 10^{-5} per nucleotide per replication cycle.^{45,174} Enhancing this diversity is the high rate of viral replication, with 10^{10} to 10^{12} virions produced per day.⁴⁶⁸ This dynamic, error-prone replication is likely to generate a vast array of mutants every day.⁵³⁵ Because

each HCV genome is produced by the error-prone NS5B polymerase and the number of intracellular replication events (from positive to negative, and negative to positive strand) is at least two but could be larger, each infected cell can generate a diverse population of viral genomes. In contrast, HIV undergoes a single round of error-prone cDNA synthesis per infected cell, with subsequent proviral replication (during cell division) and RNA genome synthesis by host DNA and RNA polymerases, respectively.³⁸

Due to neutral drift and sequential selection events (addressed in the next section), HCV quasispecies sequences have motifs that gradually change over time and during passage among individuals, making sequence analysis suitable for forensic and epidemiologic linkage studies.^{243,273,322,484}

Assessment of a complex quasispecies can be confounded by methodologic artifacts. Nucleic acid contamination of specimens and analytical intermediates is a common problem, which can be reduced (if not completely eliminated) by taking appropriate precautions.³⁵⁷ Amplification in multiple cycles, as with the polymerase chain reaction, can introduce sequence artifacts and distort the frequency distribution of sequence variants. These phenomena can inflate or suppress estimates of diversity and evolutionary change and must be considered at all stages from specimen selection and processing through analysis and interpretation.^{384,582,664} The biological site of specimen selection may also affect the results due to compartmentalization,^{91,396,459,562,569} though the limitations of quasispecies sampling may confound such analyses, and the biological implications of compartmentalization remain controversial.

Quasispecies Evolution During Acute HCV Infection

A diverse viral population, under influence from a variety of selection pressures in a complex host environment, is an ideal situation for viral adaptation in a Darwinian manner.⁶⁸⁰ The quasispecies is shaped by positive selection pressure from the host (see Immune Response) and negative selection pressure due to functional constraints imposed by requirements of the viral life cycle (see Chapter 25 for discussion of essential protein motifs and constrained RNA structures at both ends of the genome); therefore, each host's HCV quasispecies directly reflects dynamic aspects of both the host and pathogen.

During acute HCV infection, the diverse quasispecies may be targeted by cellular and humoral immune responses (see Immune Response), which have the potential to reduce the fitness of variants carrying epitopes they recognize and therefore apply positive selection pressure. In people studied during the first months of acute HCV viremia, this selection pressure has been observed in individual epitopes,⁶²⁸ and more broadly as an excess of amino acid replacements in CD8 T-cell epitopes (studied in nonenvelope genes, to avoid confounding by antibody responses).^{134,614} Detailed analysis spanning the HCV polyprotein at multiple time points in four subjects displayed the same phenomena and also demonstrated that nontargeted substitutions represented reversions to the subtype consensus sequence at a rate that was directly related to the conservation of that site in reference sequences.³⁵¹ In that study, the rate of mutation in nonenvelope genes declined during the transition to chronicity, consistent with progressive T-cell dysfunction.^{133,541,564} Some escape mutations require compensatory changes to restore fitness,^{470,539} possibly accounting for additional changes observed during acute HCV infection that

do not fall within targeted epitopes.^{134,351} In comparison with HIV, HCV evolution during acute infection is relatively limited, reflecting CD8 T-cell dysfunction, HCV genomic inflexibility, or both.⁴⁹⁶

The envelope (E1E2) region of the HCV genome is highly variable (as noted earlier) and has a much higher rate of evolution within hosts than other regions of the genome.²⁵² Humoral immune responses directed against envelope genes *E1* and *E2* have the potential to neutralize HCV, and the HCVpp and HCVcc systems provide the means to correlate E1E2 evolution with neutralizing antibody responses (see section on the Humoral Immune Response). HCV escape from neutralizing antibodies⁶⁶⁶ drives the evolution of envelope sequences during acute infection.¹⁷⁰ This is contrasted with relative stasis of HCV

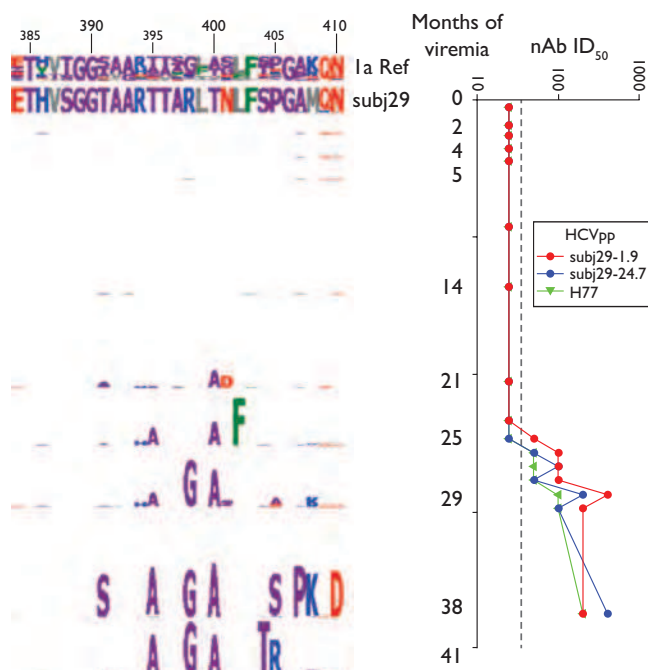


FIGURE 27.12. Evolution of hypervariable region 1 (HVR-1) during the transition from acute to chronic infection. HVR-1 evolution (left panel) correlated with neutralizing antibody (nAb) responses (right panel) in subject 29 (subj29) from the BBAASH cohort¹³⁶ studied from initial viremia (month 0) to chronicity. Type 1 sequence logos²⁴⁷ were used to demonstrate the variability among 388 reference sequences (1aRef) as well as the initial viral quasispecies (subj29, month 0). Amino acid sequence positions are indicated according to H77.³⁴⁵ For months 2 through 41, type 2 logos²⁴⁷ were used to compare amino acid sequences to month 0 sequence, with the height of each amino acid determined by the \log_2 unlikelihood of an amino acid at a given position relative to the initial sequence. To determine nAb infectious dose 50 (ID₅₀) titers, autologous HCV pseudoparticles (HCVpp) expressing E1E2 from month 2 and month 25 visits, as well as HCVpp-H77, were incubated with serial twofold dilutions of autologous plasma. When 50% neutralization was not detected at the starting plasma dilution of 1:50 (dashed line), the result was recorded as one-half this value, a titer of 1:25. (From Liu L, Fisher BE, Dowd KA, et al. Acceleration of hepatitis C virus envelope evolution in humans is consistent with progressive humoral immune selection during the transition from acute to chronic infection. *J Virol* 2010;84:5067–5077. Copyright © 2010, American Society for Microbiology.)

envelope sequences in persons with severely impaired humoral immunity^{73,231,348} and in chimpanzees with poor anti-E2 responses⁴⁹ even during 8 to 10 years of chronic viremia.⁵²² Both stasis and driven evolution are illustrated by HVR-1 evolution in a subject with acute HCV infection progressing to chronicity (Fig. 27.12) in whom neutralizing antibody responses were not detected in the first 2 years of high-level viremia, during which there was no evolution of HVR-1, whereas there was rapid evolution following the detection of neutralizing antibodies.³⁸² Delayed onset of neutralizing antibody responses is typical in those developing persistent HCV infection^{13,170,494,708,709} and appears to explain the acceleration of envelope evolution during the transition from acute to chronic infection.³⁸²

Common-source outbreaks (like the one illustrated in Fig. 27.13) facilitate examination of HCV evolution with respect to host–pathogen interactions and provide strong evidence for nonrandom evolution.^{521,614} HLA allele-specific adaptations of HCV subtype 1b in the Irish outbreak (due to HCV contamination of anti-D immunoglobulin, which occurred in 1977–1978) were distinct from previously identified adaptations in other populations with HCV subtype 1a and 3a infections, suggesting HCV subtype-specific pathways for evolution.⁴²⁰ Study of this cohort revealed a protective effect (associated with spontaneous clearance) of HLA-A*03, and the “footprints” of this response have been observed in HLA-A*03⁺ members of the anti-D immunoglobulin HCV infection cohort; additional study demonstrated that escape mutation was associated with impaired viral replication *in vitro*.²⁰⁷

Implications for Virologic Inference *In Vitro*

In vitro HCV replication systems have provided important insights regarding the viral life cycle, but these observations depend primarily on a very limited set of isolates growing in cell lines with impaired innate immunity³²⁴; most human isolates of HCV do not propagate in cell culture, for reasons that are not clear. *In vivo* models that support replication of more diverse isolates in hepatocytes (rather than hepatoma cell lines) are described in Chapter 25 (HCV Experimental Systems section) and may enable investigation of a more representative variety of HCV strains.

CLINICAL FEATURES

Acute HCV

Acute HCV infection is usually asymptomatic,^{135,448} though a minority of persons will present with more typical symptoms of acute viral hepatitis (malaise, fatigue, anorexia, nausea, abdominal pain, jaundice, dark urine, and sometimes pale stool). While HCV can cause fulminant hepatitis, this presentation is rare.^{190,246,304,398,690,693,698} In general, the latent period (from exposure to symptoms or laboratory abnormalities) is approximately 7 weeks (range 1 to 16 weeks).⁴⁴⁸ In many cases, the only sign of acute infection may be elevation of “hepatic transaminases”^{1,135,661} (ALT and aspartate aminotransferase [AST] from damaged or dead hepatocytes; these enzymes may also be released from nonhepatic cells, particularly AST from cardiac or skeletal myocytes, in association with other conditions).

Most exposures that result in HCV infection are percutaneous (see Transmission), after which hematogenous infection of the liver is presumed to occur. Viremia is detectable within days⁴⁴⁸

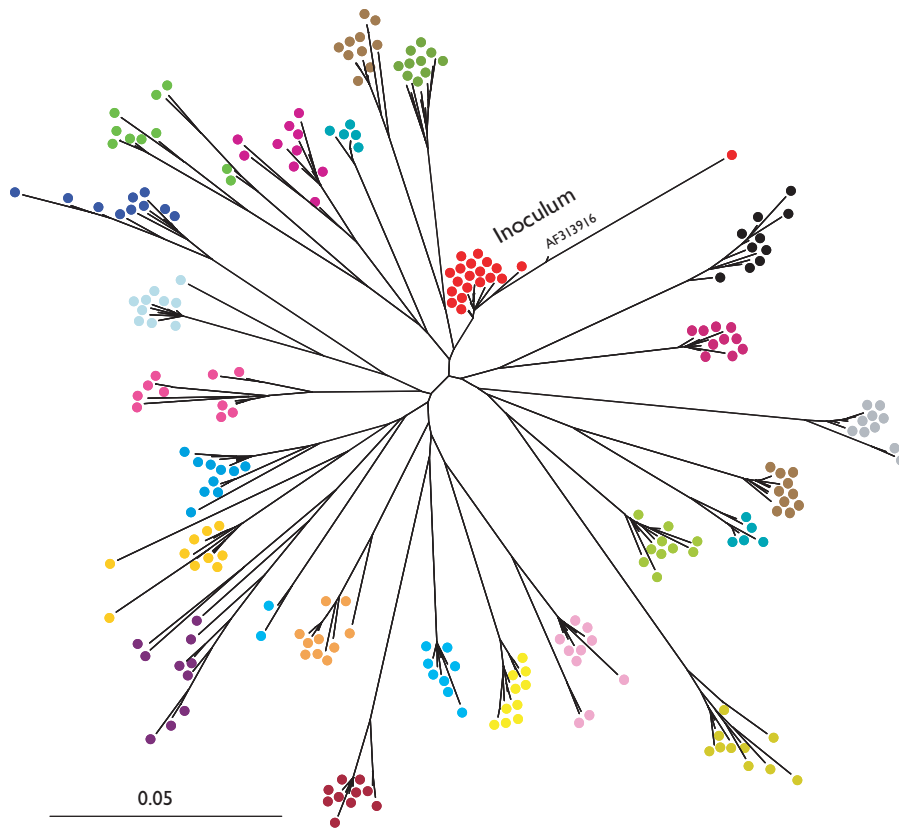


FIGURE 27.13. Phylogenetic analysis of hepatitis C virus (HCV) 18 to 22 years after common-source outbreak, using 10 complementary DNA (cDNA) clones from each study subject to obtain the sequence of a 698-nt region spanning the E1/E2 junction. “Inoculum” indicates 20 clones from inoculum source plasma (10 each from 2 specimens) and a full-length clone (AF313916) obtained in an independent study of this material using smaller amplicons. (From Ray SC, Fanning L, Wang XH, et al. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. *J Exp Med* 2005;201:1753–1759. Copyright © Ray SC et al, 2005.)

and reaches levels of 10^5 to 10^7 IU/mL within weeks. A decrease in viremia, 1 to 2 weeks later, is associated with a sharp rise in hepatic transaminase levels in blood. This sharp rise is thought to result from immune-mediated cytolysis as adaptive responses to HCV develop (Fig. 27.7),^{2,48,135,191,448,571} with more severe hepatitis and higher initial viremia being associated with higher rates of spontaneous clearance of viremia.^{234,383,661} When treatment is not instituted and spontaneous clearance does not occur within 12 months, late spontaneous resolution is rare.

Predictors of Spontaneous Clearance

Spontaneous clearance of HCV RNA usually occurs within 6 months of infection and is associated with having overt symptoms of hepatitis, non-African descent, and lack of HIV infection.^{234,661} The recognition that a linked set of alleles surrounding the IL28B gene (encoding IFN- λ 3) was strongly associated with success of interferon-based therapy²³³ (see Treatment, later) was echoed by the discovery that the same protective genotype was associated with spontaneous recovery from acute HCV,⁶²³ apparently independent of viral genotype.^{353,515,623} The protective IL28B genotype is more frequently found in Asians and least frequently among persons with African ancestry, in keeping with the clinically observed effect of race.⁶²³ The mechanistic basis of these observations remains unknown.⁴¹

Chronic HCV

For the 60% to 85% in whom spontaneous resolution does not occur, chronic HCV infection is a heterogeneous condition, with highly individual manifestations and rates of progression (Fig. 27.14).²⁴⁵ Associated morbidity and mortality

occur almost exclusively when the disease progresses to cirrhosis and end-stage liver disease that may manifest as hepatocellular carcinoma (see subsection later). Chronic HCV infection is characterized by high-level viremia and fluctuating hepatic inflammation and transaminase levels,^{331,492,683} yet chronically infected people typically have few symptoms that are directly attributable to HCV infection. It is difficult to establish a causal

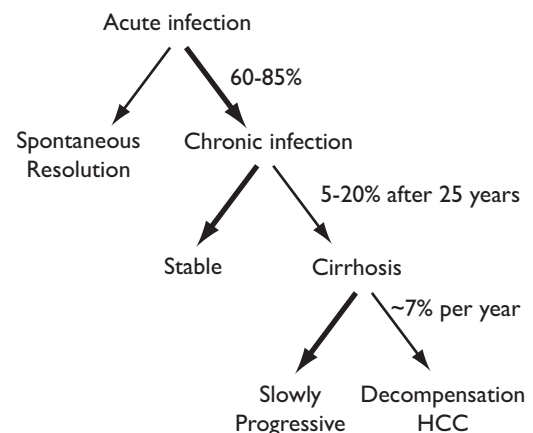


FIGURE 27.14. Progression of hepatitis C virus (HCV) infection. Most HCV infections are persistent and stable, but a minority will progress to cirrhosis within 20 to 30 years. Of those with cirrhosis, most are slowly progressive, but 7% per year will develop either hepatocellular carcinoma (HCC) or decompensated liver disease.^{291,550}

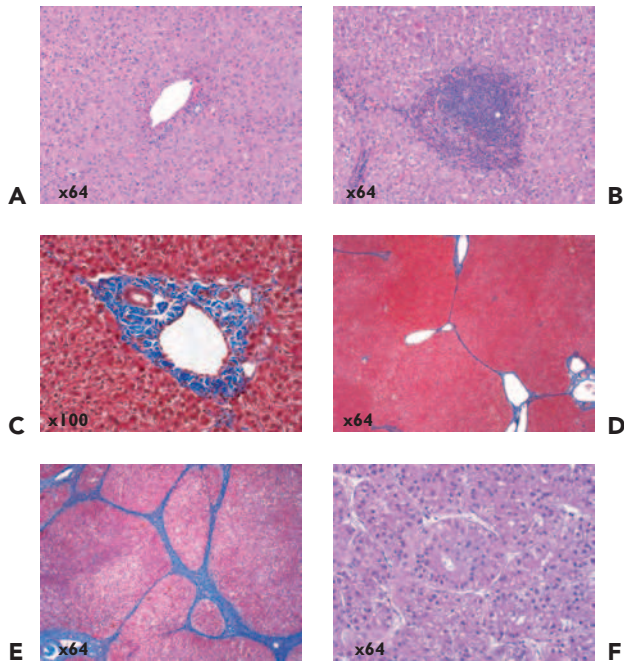


FIGURE 27.15. Histology illustrating progression of hepatitis C virus (HCV)-related liver disease. **A:** Normal portal tract (hematoxylin and eosin [H&E] stain). **B:** Portal inflammation (H&E stain). **C:** No fibrosis (trichrome stain). **D:** Bridging fibrosis (trichrome stain). **E:** Cirrhosis (trichrome stain). **F:** Hepatocellular carcinoma (HCC) (H&E stain). (Courtesy of Michael Torbenson, MD.)

relation between HCV and nonspecific symptoms, though they do tend to improve after successful therapy.^{58,212,586}

The level of HCV RNA in the blood tends to be stable over long periods of time within 1 log₁₀ of 10⁶ IU/mL in 90% of individuals. Conditions associated with modest increases include HIV infection, male gender, and increasing age and body mass index, whereas lower levels may be found in those with ongoing HBV infection and more advanced stage of liver disease.^{21,125,288,621,627,635} Although the HCV RNA level in the blood is correlated with the level in the liver,⁶⁶⁸ there is not a strong correlation between HCV RNA level and fibrosis progression.

Chronic HCV infection is associated with varying degrees of chronic inflammation and steatosis. Lymphocytic infiltrates are typically found in periportal regions of the liver (Fig. 27.15), though these do not correlate strongly with liver disease progression.⁵⁶⁷ For reasons that are not clear, many individuals will not develop significant fibrosis despite decades of high-level viral infection, while others have more production than resorption of collagen, also initially in the periportal region. This process may be stable or progress to formation of septae that expand to form bridges between lobules, and further expansion may result in the severe scarring and regeneration that characterize cirrhosis. Increasing portal venous pressure may lead to portal hypertension and neoplastic transformation may lead to hepatocellular carcinoma, hallmarks of end-stage liver disease.

Although people of African descent are more likely to develop chronic HCV than Caucasians, they appear to have

a milder course, with less inflammation and progression to fibrosis.^{137,613} One potential explanation for this is differences in frequencies of alleles near certain genes such as *IL28B* that are known to affect clearance and may modulate inflammation, though the mechanisms remain poorly understood.

Alcohol consumption is frequent in persons with chronic HCV and is strongly associated with liver disease progression.^{12,493} Along with its well-known hepatotoxicity, alcohol use is associated with reduced access to and early discontinuation of HCV treatment.²³ Elimination of alcohol consumption should be attempted to reduce complications in all persons with chronic HCV.^{24,237,443,584,695}

Chronic HCV infection is also associated with metabolic dysfunction including insulin resistance, type 2 diabetes, lipid derangement, and steatosis that may be more common in (but not exclusive to) persons infected with HCV genotype 3 (see Virulence, earlier).^{129,281,412,423,463} This association is stronger for HCV than for HBV, suggesting that the mechanism may be specific to HCV.^{572,682} Potential contributing mechanisms to metabolic dysfunction in HCV include down-regulation of hepatocyte insulin receptor substrate 1⁷⁵ and glucose transporter 2³¹⁰ and up-regulation of PP2A (see Innate Immune Response, earlier).^{57,175}

Flares of hepatitis (with elevated serum transaminases and/or bilirubin), common in HBV infection,⁴⁹¹ are rare in chronic HCV and should prompt a search for other causes. For example, acute HAV infection during chronic HCV infection may be associated with severe acute hepatitis, including liver failure⁶⁵⁶; for this reason, persons with chronic HCV infection should be vaccinated for HAV and HBV if susceptible.²⁴

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a growing problem in countries like the United States with relatively recent HCV epidemics and is a major established problem in countries like Japan and Egypt where the epidemic of HCV infection occurred 10 to 20 years earlier.⁶¹⁰ Because cirrhosis is often unrecognized, and HCC causes few symptoms until advanced, screening for cirrhosis and HCC is an important aspect of HCV management. Unfortunately, there are few effective, inexpensive screening tools. Serum testing for α -fetoprotein (AFP) has limited diagnostic utility.⁸²

Unlike HBV, which is associated with a substantially elevated risk of HCC at all stages of infection, the association of HCC with HCV primarily arises after a person develops cirrhosis.^{83,146,550,581} While a direct pathogenetic role of HCV in HCC cannot be dismissed,^{101,196,225} and studies in which the core protein was overexpressed under control of a strong promoter have suggested potential for induction of proto-oncogenes and suppression of apoptosis,^{105,107,112,414,517–519,551,574,604,707} the data are conflicting,⁴⁸³ and chronic inflammation may be sufficient to trigger HCC in those cases that precede cirrhosis.⁴⁵⁶

Extrahepatic Manifestations

Although the liver is the principal site of HCV replication, chronic infection is associated with a wide variety of extrahepatic manifestations.²⁶² A common feature of these conditions is chronic inflammation.

Essential mixed cryoglobulinemia, a condition in which cold-precipitating immune complexes are deposited in multiple organ systems, is strongly associated with HCV infection,

though other inflammatory conditions may be implicated. Manifestations often include purpuric rash, weakness, and joint pain but may also include Raynaud syndrome and vasculitis complicated by membranoproliferative glomerulonephritis and neuropathy.^{94,404,440} HCV tests are positive for anti-HCV antibodies and HCV RNA in a large proportion of affected individuals,⁶ HCV treatment can result in remission,^{93,201} and rituximab-mediated B-cell depletion may augment therapeutic response.⁵⁴² The chronic stimulation of B cells implicated in HCV-related cryoglobulinemia⁵⁴³ may also explain the elevated risk (approximately 2.5-fold) of non-Hodgkin lymphoma¹⁴⁴; mechanistically, the potential for HCV E2 to cross-link the B-cell receptor with the co-stimulatory CD19/CD21/CD81 receptor complex may play a role in development of lymphoma (reviewed in⁶⁶²).

Porphyria cutanea tarda (PCT), characterized primarily by disorders of the skin (blistering, hyperpigmentation) and nails (onycholysis) worsened by sun exposure and complicated by scarring, is associated with liver disease and is caused by reduced activity of uroporphyrinogen decarboxylase. It is a multifactorial disease, potentiated by mutations in the *HFE* gene (associated with hereditary hemochromatosis and found in 15% of persons with PCT), as well as HCV infection, alcohol, and estrogen use—all of which should be evaluated in persons presenting with PCT.⁷²

Other conditions that may be observed at increased frequency include detection of antithyroid antibodies and, in some studies, increased prevalence of thyroiditis even before interferon therapy (see also Adverse Effects of Peginterferon and Ribavirin Therapy, later).^{121,140,157,173,636} The association between chronic HCV infection and thyroiditis has been supported by a meta-analysis, though the mechanism remains unclear.^{27,28}

DIAGNOSIS

Clinically, HCV is suspected in persons with otherwise unexplained liver disease. In asymptomatic persons, this infection should be suspected in any person reporting risk factors for infection (see Epidemiology, earlier) or having elevated hepatic transaminases even in the absence of known risk factors, because the infection is highly prevalent. Because of shared risk factors and increased severity of disease, the U.S. Public Health Service recommends HCV testing for all HIV-infected persons upon entry into health care. Because more than two-thirds of persons with HCV infection in the United States were born between 1945 and 1965, the U.S. Public Health Service has recommended that everyone in that “birth cohort” be tested once for HCV infection. The cost-effectiveness of birth cohort testing has already been demonstrated.⁵²⁶

Differential Diagnosis

Other viral causes of acute hepatitis (described in Clinical Features, earlier) include the named hepatitis viruses (HAV, HBV with or without the delta hepatitis agent, or hepatitis E virus [HEV]), yellow fever virus, and a wide range of viruses with broader tropism including Epstein-Barr virus (EBV) and cytomegalovirus (CMV). In immunocompromised hosts, adenovirus and other agents with broad tropism can also cause

hepatitis. Nonviral causes of acute hepatitis include leptospirosis, tuberculosis, rickettsia and rickettsia-like organisms, numerous toxins (alcohol, acetaminophen, isoniazid, and *Amanita phalloides* toxin being prominent), anoxia/hypoperfusion, and autoimmune disease.

Chronic hepatitis may be caused by HBV and occasionally HEV (in immunocompromised hosts), as well as toxoplasmosis, autoimmune hepatitis, and nonalcoholic steatohepatitis.

Laboratory

The recommended approach for diagnosis of HCV infection is testing for HCV antibodies by enzyme immune assay (EIA). When the screening EIA is positive, the next step is usually to test for HCV RNA to establish that there is ongoing infection.²³⁷

Serologic Testing

The standard screen for HCV infection is anti-HCV EIA,²⁴ which detects antibodies to recombinant HCV proteins core, NS3, NS4, and NS5 (see Fig. 27.2).^{113,417,457} Additional antibody testing can also be performed using recombinant immunoblot assay (RIBA),⁶⁵³ which indicates separately the reactivity to antigens that are tested together in the EIA. RIBA is not an independent confirmatory test. Instead, the results indicate whether the antibodies detected on EIA are to HCV or due to nonspecific cross-reactivity.²⁵⁵ In the situation with a positive HCV EIA and negative HCV RNA result, a positive RIBA indicates that there was indeed prior HCV infection, which may have resolved spontaneously (or from treatment). In that setting a negative RIBA would imply the EIA was falsely positive. The signal strength of EIA may also provide an indication of the specificity of the result,^{20,486} though there is no clear cut-off value⁵⁶³ and current versions of the EIA interpreted according to manufacturer specifications have a specificity of greater than 99%.¹²²

Current versions of HCV EIA have increased sensitivity to about 97%^{132,647} and become positive within 4 to 8 weeks of infection.^{44,135,255} Because specificity is not 100%, positive tests in low-risk individuals may be false-positives, and additional testing such as HCV RNA may be needed.^{237,255} Clearance of HCV viremia (spontaneously or after successful treatment) is associated with decreasing antibody levels, sometimes below the level of what would be considered positive on EIA (seroreversion),^{372,607} which can complicate inference of past events.

Early reports of false-negative EIA tests in immunocompromised individuals¹⁰⁰ have been addressed in third-generation immunoassays, resulting in a very low rate of false-negatives even in HIV infection and dialysis settings^{512,618}; nevertheless, RNA testing should be used if suspected on the basis of elevated risk or hepatic transaminases.^{512,598}

Viral Nucleic Acid Detection and Quantitation

Direct viral testing currently depends on detection of the viral genome in plasma or serum, with concentrations expressed in terms of international units (IU).⁵⁴⁶ HCV RNA detection methods use reverse transcriptase-polymerase chain reaction (RT-PCR) (qualitative and quantitative), transcription-mediated amplification (TMA), and branched DNA (bDNA) for signal amplification. Approved RT-PCR assays have linear ranges from 1.7 to 7 log₁₀ IU/mL, while bDNA has somewhat lower sensitivity but greater reproducibility.⁹⁵ Although TMA

has been shown to detect low-level viremia in some specimens that are negative by RT-PCR, TMA and RT-PCR assays appear to have equivalent utility in detecting HCV RNA during or at the end of treatment to predict sustained virologic response (SVR).^{74,447} Though most assays have targeted the 5' UTR due to its relatively high conservation, it is feasible to detect or quantitate HCV RNA by targeting the extremely conserved 3' terminus of the genome.¹⁷¹

HCV Genotype Determination

HCV genotype is the strongest single biological predictor of HCV treatment success.²⁴ Commercially available assays depend on amplification of targets near the 5' end of the genome and use sequencing or reverse hybridization to determine genotype (and, in some cases, subtype). Mixed infections occur but are uncommon. The gold standard for genotype determination is phylogenetic analysis of nucleotide sequences obtained from a phylogenetically informative genomic region; by international consensus, either the E1 or NS5B region may be used for definitive assignment.^{128,577,591}

Liver Disease Staging

Because the progression of HCV-related liver disease is highly variable, staging is important for informing treatment decisions, lifestyle modification, and prognosis. Although the liver biopsy remains the reference standard for staging and provides a wealth of information beyond fibrosis stage, it is invasive and expensive, and other modalities are gaining prominence as they are validated.

Liver biopsy is the standard reference tool for assessing liver fibrosis grade (inflammation) and stage (fibrosis). Tissue examination may detect other causes of liver disease, as well as conditions such as iron overload and steatosis that are important contributors to HCV-related liver disease and may help guide therapy. Histologic stage is assigned on a standard scale, with widely used examples including Batts-Ludwig,⁵⁰ International Association for the Study of the Liver (IASL),¹⁵⁶ Metavir, and Ishak.^{51a,289} Scores range from no fibrosis to cirrhosis, staged as 0 to 4 (for Batts-Ludwig, IASL, and Metavir) or 0 to 6 (for Ishak). In addition to cost and discomfort, the value of a liver biopsy is limited by sampling error and imprecision¹²⁰; its value is being reassessed and noninvasive alternatives are being evaluated.

Noninvasive markers of fibrosis offer the potential for more frequent assessment than the standard liver biopsy interval of 4 to 5 years. Serum markers (only some of which are approved by the Food and Drug Administration [FDA]) and sonographic elastography (a measure of liver stiffness, not yet evaluated by the FDA, but widely available in Europe) are most informative at the extremes, whereas intermediate values may not discriminate well between mild and severe fibrosis.^{237,533}

PREVENTION AND CONTROL

Treatment

All currently approved regimens for treating HCV infection include type I interferon, with the highest response rates associated with pegylated interferon (peginterferon) alfa plus ribavirin, with or without a protease inhibitor (reviewed in⁵⁵⁴). While effective in most participants in clinical trials, HCV treatment

TABLE 27.2 Hepatitis C Virus Direct-Acting Antivirals in Current Phase III Studies

Agent	Drug Class
Asunaprevir (BMS-650032)	NS3-NS4A protease inhibitor
Faldaprevir (BI201335)	NS3-NS4A protease inhibitor
Simeprevir (TMC435)	NS3-NS4A protease inhibitor
Vaniprevir (MK-7009)	NS3-NS4A protease inhibitor
Daclatasvir (BMS-790052)	NS5A inhibitor
Sofosbuvir (GS-7977)	NS5B polymerase inhibitor
Silymarin	NS5B Polymerase inhibitor

remains expensive and is associated with significant side effects. When liver transplantation is necessary, HCV infection of the graft is inevitable without treatment.^{188,505,513}

The only direct-acting antivirals (DAAs) currently approved in the United States or Europe for HCV treatment are the protease inhibitors boceprevir³⁵⁶ and telaprevir,^{275,418} which are reversibly covalent inhibitors of the NS3-NS4A serine protease. Additional DAAs are in advanced (phase III) development (Table 27.2), including noncovalent NS3-NS4A protease inhibitors (asunaprevir/BMS-650032, faldaprevir/BI201335, simeprevir/TMC435, and vaniprevir/MK-7009), an NS5A inhibitor (daclatasvir), and NS5B polymerase inhibitors (sofosbuvir/GS-7977 and silymarin).

Goals of Treatment

The principal goal of antiviral therapy is the amelioration or prevention of disease. Because not all patients are affected by HCV in the same way and because there can be serious adverse events, treatment is individualized. In 2009, guidelines for use of peginterferon and ribavirin suggested the benefits of treatment would outweigh the risks for persons most likely to respond (e.g., genotype 2 infection) and those most in need (e.g., significant fibrosis) and those with the fewest complicating medical problems (e.g., no depression). However, because there are so many factors to consider, treatment decisions were often individualized.²³⁷ Recent therapeutic advances have added even more variables, and selection of the patient for treatment remains a highly individualized practice.

The primary goal of therapy is SVR, defined as undetectable HCV RNA 24 weeks after the end of a treatment regimen (Fig. 27.16); SVR appears to represent a cure of infection. This outcome is associated with loss of intrahepatic RNA and histologic improvement.^{62,367,403} The durability of SVR was assessed in a study of 1,343 people from nine randomized multicenter trials who had SVR after interferon-based treatment,⁶⁰⁵ demonstrating that 99.1% remained HCV RNA negative after an average follow-up of 4 years. Whether the 0.9% of persons with recurrent viremia had relapse or were reinfected was not known.

In contrast to SVR, other outcomes are often described as follows (Fig. 27.16): null nonresponse, consistently detectable HCV RNA with less than 2 log₁₀ reduction by week 24; partial response, consistently detectable HCV RNA with reduction by more than 2 log₁₀ at week 24; breakthrough, suppression to undetectable HCV RNA level that becomes detectable again during therapy; and relapse, suppression of HCV RNA to undetectable levels through the completion of treatment

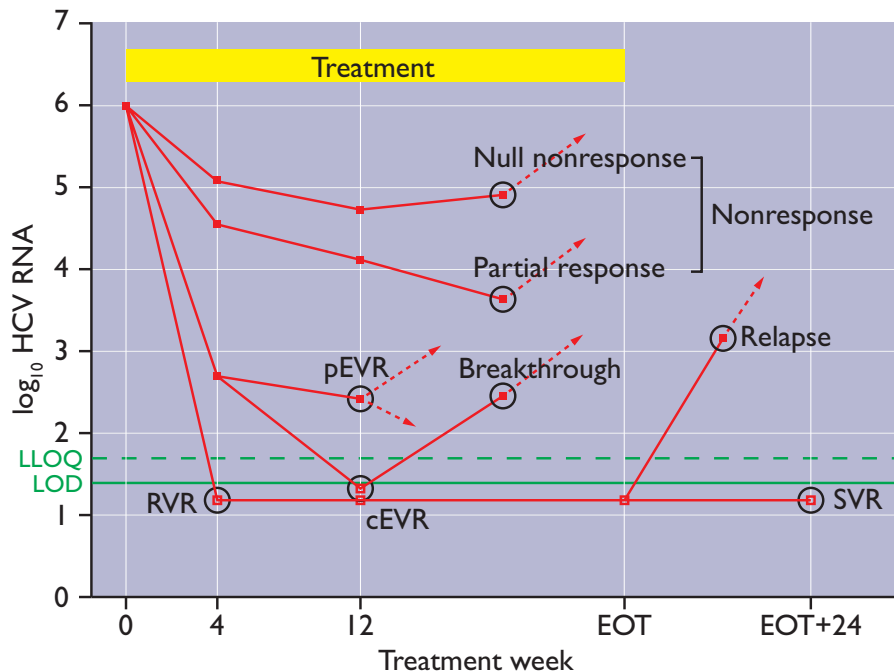


FIGURE 27.16. Outcomes and patterns of treatment response at times during treatment and at end of treatment (EOT). Outcomes (sustained virologic response [SVR], relapse, breakthrough, and nonresponse) are predicted by early patterns of viral response: rapid, complete early, and partial early virologic response (RVR, cEVR, and pEVR, respectively). Dashed arrows indicate subsequent trajectory of viremia. LLOQ, lower limit of quantitation; LOD, limit of detection.

followed by viremia during subsequent follow-up. Nonresponse can be used to describe collectively the null response and incomplete response patterns (i.e., to distinguish from those who achieve viral suppression), though these terms are not used uniformly (nonresponse is sometimes used to refer specifically to null response).

Mechanisms of Action

INTERFERON- α

Type I interferons are antiviral and regulate proliferation and immunity. Encoded by more than 10 IFN- α genes and one IFN- β gene, these proteins bind the type I interferon receptor, a heterodimer of IFNAR1 and IFNAR2¹¹⁴ that signals through the JAK-STAT pathway to induce hundreds of interferon-stimulated genes (ISGs).³¹⁷ Among these ISGs are PKR, 2,5-OAS, and MxA. These ISGs and others are typically up-regulated during acute HCV infection.⁶⁰ Antiviral effects of activated PKR include phosphorylation and inactivation of eukaryotic translation initiation factor 2A (EIF2A) and nuclear localization of nuclear factor (NF)- κ B; in addition, HCV NS5A can bind and interfere with PKR function.²²⁴ Type I interferons also up-regulate MHC class I expression and NK cell killing functions.⁶¹ Currently recommended therapy is based on peginterferon α due to greater efficacy than other interferon preparations currently available.²³⁷ These are derivatives of IFN- α (2a or 2b) to which polyethylene glycol has been added for improved pharmacokinetics and efficacy.^{219,264,402}

Ribavirin

Like type I interferons, ribavirin is broadly antiviral and is effective as a component of HCV therapy; however, its principal mode of anti-HCV action is unclear.⁴⁸⁵ As an inhibitor of inosine monophosphate dehydrogenase and via direct interaction with RNA polymerases, ribavirin may increase the error rate of the HCV RNA-directed RNA polymerase to a level that

induces lethal mutagenesis,¹³⁹ an effect that may be inapparent at high replication levels but may be unmasked in combination therapy.^{161,361} Alternatively, ribavirin may have immunomodulatory effects.⁵²⁹ In clinical trials, the most pronounced effect of ribavirin is reduction of viremia relapse after completion of therapy.²¹⁹

Efficacy of Combination Therapy with Pegylated Interferon and Ribavirin

The standard regimen for treatment of chronic HCV infection is subcutaneous injection of peginterferon α (180 μ g of 2a, or 1.5 μ g/kg of 2b) weekly. Patients take ribavirin orally with half the daily dose each morning and night; those with genotypes 2 and 3 receive 800 mg daily and those with genotype 1 receive 800–1,400 mg based on body weight.²³⁷

Outcomes with the two peginterferon α formulations (2a or 2b) in combination with ribavirin are comparable, with approximately 40% and 80% of previously untreated patients with chronic HCV genotype 1 versus genotype 2 or 3 achieving a sustained virologic response, respectively.^{219,402} The optimal duration of therapy varies by genotype, with genotype 1 requiring 48 weeks of therapy and persons without HIV infection who have HCV genotype 2 or 3 achieving high rates of SVR with 24 weeks of therapy.²⁶⁴

Predictors of Treatment Success During Interferon-Based Therapy

Well-established determinants of clinical response to these drugs include HCV genotype (genotypes 2, 3, 5, and 6 most responsive, genotype 1 least responsive), HIV infection (coinfection less responsive), stage of liver disease (early stage more responsive than cirrhosis), race (Asians more responsive than Caucasians, who are more responsive than persons with African ancestry), age (<40 years more responsive), sex (females more responsive than males), baseline HCV RNA

level (lower levels more responsive), and body weight (lower weight more responsive).^{26,36,52,148,158,167,202,303,409,410,487,640,641,667,702,703,710} Though not uniformly measured, insulin resistance (see Clinical Features, earlier) is strongly associated with failure of interferon-based therapy.^{124,155,646} Within genotype, there appear to be viral determinants of treatment responsiveness, particularly in the NS5A (specifically, the interferon sensitivity determining region, or ISDR¹⁸⁰) and core proteins, summarized in Tavis et al⁶¹¹; inconsistent detection of ISDRs, with differences among distinct populations, may be related to other differences among those populations,⁴⁸¹ with subtler virologic determinants unmasked only when the impact of host genetics is minimized. Long-range correlations spanning the HCV genome^{34,165} might affect treatment response and constrain evolution of resistance,¹⁶⁴ but their biological significance remains to be demonstrated.

It was recently discovered through a genome-wide association study that a series of polymorphisms clustered around the *IL28B* gene (encoding IFN- λ 3) has a major impact on the outcome of HCV treatment²³³ (as well as spontaneous recovery⁶²³), summarized in.⁴¹ The protective haplotype is enriched in Asian populations, uncommon in African populations, and intermediate in Caucasians; this genotype correlates well with overall success rates of interferon-based therapy, explains about half of the effect of race on treatment success, and may explain divergent findings from different races regarding the importance of HCV polymorphisms in ISDR and core noted earlier. Clinical testing for *IL28B* polymorphisms became available within a year of that discovery, ostensibly to assist in prediction of treatment response, yet the mechanism of this effect remains poorly understood. The presence of the protective *IL28B* haplotype is also associated prior to the start of therapy with lower intrahepatic ISG expression,²⁷⁷ which in turn was previously shown to predict treatment success.^{109,552} Taken together, these results suggest that *IL28B* genotype is one of multiple factors contributing to the phenotype associated with treatment response.⁴¹

Uncommonly, HCV infection is detected during the acute phase.³⁹⁸ Compared to chronic infection, acute HCV infection is more responsive to interferon-based treatment with overall SVR rates greater than 80%, and the response is significantly less genotype dependent than during chronic infection.²⁹⁴ A recent meta-analysis supports monitoring for up to 12 weeks after presentation with acute HCV and in those with persistent viremia using interferon-based therapy¹³⁰ such as peginterferon with ribavirin.⁶⁸ Rates of SVR in HIV-infected individuals with acute HCV may be lower.³⁵⁸

Adverse Effects of Pegylated Interferon and Ribavirin Therapy

Most recipients of the combination of peginterferon α and ribavirin experience adverse medication effects. Patients receiving standard doses of peginterferon α -2a or -2b in a large trial (more than 1,000 participants receiving each drug),⁴¹⁹ the following adverse effects were reported: fatigue (63% to 66%), headache (42% to 50%), nausea (36% to 42%), insomnia (39% to 41%), pyrexia (23% to 35%), anemia (34%), myalgia (22% to 27%), neutropenia (26% to 31%), depression (21% to 25%), irritability (25%), or rash (22% to 28%).

Adverse events resulted in early discontinuation of treatment in 13% of those participants and dose reduction in

43%. Serious adverse events (resulting in new or prolonged hospitalization, disability/incapacity, life-threatening complication, or death) attributed to the medications occurred in 4% of participants.

In addition to those mentioned previously, major adverse effects²⁷⁸ reported commonly (greater than 5%) by patients include arthralgia, anorexia, diarrhea, anxiety, poor concentration, memory loss, hair loss, photosensitivity, itching, nasal congestion, and injection site reactions (erythema, pain, or abscess). Hematologic effects are also common, including reduced neutrophil count, hemoglobin (see later regarding anemia), and platelet count. Uncommon (1% to 5%) but significant adverse effects include marked depression and anxiety, relapse of substance or alcohol abuse, severe bacterial infection, and induction of autoantibodies. Similarly, rare (less than 1%) but significant adverse effects include major psychiatric events, neurologic complications, autoimmune disease, cardiac complications, worsening of hepatitis, and renal, cardiac, or pulmonary failure.

The principal adverse effect of ribavirin is hemolytic anemia. The degree of anemia is highly variable, in part due to polymorphisms in the gene for inosine triphosphatase (ITPA); the protective alleles are associated with reduced ITPA activity and intraerythrocyte accumulation of inosine triphosphate, providing potential for pharmacologic intervention that would make ribavirin less toxic.¹⁹⁸ In combination with peginterferon (which contributes to anemia), ribavirin dose reduction is required in 25% of patients treated for genotype 1 infection and must be discontinued in less than 5%.⁴¹⁹

Viral Dynamics

As increasingly potent therapy approaches an abrupt halt in virus production, mathematical modeling has provided increasingly precise estimates of viral dynamics because at steady state (just prior to initiation of therapy) the rate of virion clearance must equal the rate of virion production.^{405,468,490} The implications of viral dynamic modeling include providing testable hypotheses regarding therapeutic mechanisms, evaluating the contributions of immunologic control to therapeutic efficacy, and predicting clinical outcomes including drug resistance.⁵³⁵

Initiation of antiviral treatment is followed by a delay that varies among drug regimens and then a decline in viremia that fits a biphasic decay model.⁴⁶⁸ Current models provide estimates of an HCV half-life as short as 45 minutes (reviewed in¹⁴³). The 7- to 10-hour delay observed following treatment initiation of interferon-based therapy may be due to interferon signaling¹⁴³ and is shortened to 2 hours with DAA-based therapy.²²⁷ The subsequent first phase of decline, lasting 1 to 2 days, is rapid and attributed primarily to viral clearance unmasked by reduced production. A second, slower phase of decline may be attributed to clearance of infected cells (either through cell death or termination of productive HCV infection). Because early viral dynamics are linked to drug efficacy, it is reasonable to expect that they would be predictive of outcome.

In clinical trials, early viral dynamics have been found to predict outcome with sufficient accuracy so that they are now used to guide early therapeutic decisions from stopping rules to adjustments in treatment duration. Increasingly potent regimens have required enhanced precision of these definitions, and such changes are likely to continue.

Early Virologic Response (Week 12 Response)

Lack of HCV RNA suppression at week 12 (lack of early virologic response [EVR]) consistently predicts failure of treatment. When defined as reduction of HCV by at least 2 log₁₀, the proportion of treatment-naïve individuals who went on to achieve SVR in the context of treatment with peginterferon α -2a or -2b was 0% to 3%,^{149,219} and EVR was generally adopted as a “stopping rule” due to futility. The positive predictive value of EVR in those studies was low, with 65% to 72% achieving SVR. When EVR was subdivided into complete EVR (cEVR, lack of detectable HCV RNA at week 12, Fig. 27.16) versus partial EVR (pEVR, EVR with detectable HCV RNA at week 12), the proportion going on to SVR was 74% to 83% versus 21% to 38%, but cEVR was achieved in only 64% to 65% of those treated with combination therapy^{149,219} for mixed genotype cohorts, and in only 40% to 45% of persons with genotype 1.⁴¹⁹

Rapid Virologic Response (Week 4 Response)

Just as slow response is highly predictive of treatment failure, rapid response is highly predictive of treatment success. In treatment-naïve persons with chronic HCV genotype 1, 80% to 92% of patients treated with peginterferon α -2a or -2b and ribavirin who achieved a rapid virologic response (RVR, undetectable viremia at week 4) went on to achieve SVR,^{200,419} though only 11% to 12% of treatment-naïve patients with HCV genotype 1 achieved RVR when treated with peginterferon α -2a or -2b.

RVR is also associated with comparable success rates with shorter durations of therapy. For genotype 1–infected individuals, shortening the duration of peginterferon α -2a therapy from 48 to 24 weeks is associated with higher failure rates overall, but in the subset with RVR the outcomes are comparable.^{264,296} For genotype 2– and 3–infected individuals with early-stage fibrosis and low baseline HCV RNA levels, shortening the treatment duration from 24 to 12 or 16 weeks in those with RVR may be appropriate.²³⁷

Less than 1 log₁₀ reduction in HCV RNA at week 4 (which occurred in 10% to 11% of patients) also had strong negative predictive value in genotype 1–infected patients treated with peginterferon and ribavirin, with less than 5% of these individuals achieving SVR.⁴¹⁹ In contrast, lack of RVR in this setting has a poor negative predictive value.

Boceprevir and Telaprevir

In May 2011, the NS3-NS4A serine protease inhibitors boceprevir (formerly SCH503034) and telaprevir (formerly VX-950) were approved by the FDA for use in combination with peginterferon and ribavirin for treatment of chronic HCV genotype 1 infection.⁵³⁷ These linear tetrapeptide analogs bind to the NS3 protease active site with a ketoamide center that functions as a covalent serine trap.^{380,472}

Short-term monotherapy with these agents demonstrated significant but incomplete reductions in HCV RNA level, and more detailed study of specimens from the telaprevir trial showed rapid emergence of resistance.^{555,556} Clinical trials of boceprevir (recommended dose 800 mg with food every 7 to 9 hours) or telaprevir (recommended dose 750 mg with fat-containing food every 7 to 9 hours), in combination with peginterferon and ribavirin at standard doses, resulted in approximately 70% SVR, compared with 40% SVR in those treated with the prior standard of care.^{275,356,418}

Boceprevir therapy includes a 4-week lead-in phase of peginterferon and ribavirin, followed by addition of boceprevir. The total duration can be fixed at 48 weeks (4-week lead-in plus 44 weeks of triple therapy). Alternatively, treatment-naïve patients without cirrhosis may be treated using response-guided therapy (RGT) during which early HCV RNA dynamics determine treatment duration: if HCV RNA is undetectable at week 8 (week 4 of boceprevir), the duration of triple therapy is 28 weeks (24 weeks of boceprevir); if HCV RNA is detectable at any visit after week 8 but negative at week 24 and subsequent visits, boceprevir is discontinued at week 36 and peginterferon and ribavirin are continued for an additional 12 weeks. RGT is recommended only for treatment-naïve patients without cirrhosis, and all treatment should be discontinued if the HCV RNA level is greater than 100 IU/mL at treatment week 12, or detectable at week 24.²³⁶

Telaprevir is initiated as part of triple therapy and continued for 12 weeks, after which peginterferon and ribavirin are continued to the end of treatment. For treatment-naïve patients without cirrhosis that have an *extended RVR*, defined as HCV RNA undetected at weeks 4 and 12, duration of therapy can be abbreviated at 24 weeks; for others (with cirrhosis and/or slower response dynamics), peginterferon and ribavirin are continued to complete an overall treatment duration of 48 weeks. All treatment should be discontinued if the HCV RNA level is greater than 1,000 IU/mL at week 4 or week 12 or detectable at week 24.²³⁶

In treatment-experienced patients with HCV genotype 1, boceprevir- or telaprevir-containing triple therapy resulted in overall rates of SVR that were much higher than for peginterferon plus ribavirin, 64% to 66% versus 17% to 21%.^{37,704} Not surprisingly, these rates varied depending on the dynamics of the prior treatment response, with 75% to 83% of prior relapsed patients achieving SVR and only 52% to 59% of prior partial responders achieving SVR after triple therapy. Prior null responders (Fig. 27.16) were excluded from the boceprevir trial,³⁷ but in the telaprevir trial 29% of prior null responders achieved an SVR; most prior null responders who did not achieve SVR had detectable treatment-emergent resistance mutations.⁷⁰⁴

Resistance to Boceprevir and Telaprevir

Boceprevir and telaprevir select for characteristic changes at NS3 codons 36 (V to M, A, or L), 54 (T to A or S), 155 (R to K or T), 156 (A to S, T, or V), and 168 (D to N) both *in vitro* and *in vivo*.^{432,612} Based on *in vitro* models of single substitutions, V36A/M is expected to have a modest impact both on resistance (4-fold increased IC₅₀) and replication capacity (less than 10% reduction), A156V/T the greatest impact on resistance (greater than 50-fold increased IC₅₀) and replication capacity (>50% reduction), and the other substitutions are expected to be intermediate between those extremes.⁵⁵⁵ Combined substitutions at codons 36+155 or codons 36+156 have enhanced resistance and fitness relative to the 155 and 156 single substitutions. Changes at other NS3 codons, including 43 and 55, are less consistently observed and may have less-pronounced impact on resistance.

In the pivotal phase 3 trials in which boceprevir was combined with peginterferon and ribavirin, baseline viral sequence data were available for 980 patients treated with boceprevir. Analysis of these NS3 sequences revealed 43 patients (4%)

with pretreatment resistance-associated variants V36M, T54A/S, V55A, and/or R155K.⁴³² The SVR rate in this population was 65% (28/43). Among the 36 patients in this subgroup who were interferon responsive (greater than or equal to 1 log₁₀ reduction in HCV RNA during 4-week peginterferon/ribavirin lead-in therapy), the SVR rate was 78% (28/36), comparable to the 73% to 81% SVR rate observed in the overall population of interferon-responsive patients. Therefore, in this small subpopulation, the response did not appear to be hampered by the presence of high-level resistance mutations at baseline as long as patients were responsive to lead-in peginterferon/ribavirin. In the seven patients with high-level resistance mutations at baseline who were nonresponsive to interferon as defined by a less than 1 log₁₀ reduction in HCV RNA during the 4-week peginterferon/ribavirin lead-in therapy, SVR was not observed. By contrast, in the overall population, 28% to 38% of patients who were nonresponsive to interferon during the 4-week lead-in period achieved SVR. Currently, there is no clinical indication for baseline resistance testing.²³⁶

In agreement with the aforementioned baseline data, the R155K substitution has been observed in untreated individuals with chronic HCV³⁵⁰ and may represent an escape mutant for an HLA-A*68-restricted T-cell epitope.⁵⁴⁸ At baseline and after unsuccessful treatment, R155K resistance substitutions are observed more frequently in persons with HCV subtype 1a infection than those with subtype 1b infection, reflecting a lower genetic barrier for subtype 1a.⁵⁵⁵ Specifically, for subtype 1a codon R155 is AGG, requiring only one nucleotide substitution to become AAG (K), whereas for subtype 1b the same R155 is encoded by CGG and requires two changes to become AAG.

Following termination of unsuccessful treatment with boceprevir or telaprevir, the frequency of resistant variants tends to decay and wild-type variants predominate in peripheral blood.⁶⁰³ Whether the frequency of resistant variants returns to pretreatment baseline or will have an impact on future treatment success is not known. Current understanding of viral dynamics and resistance supports discontinuation of failing DAA-based therapy as early as possible to avoid an increase in the frequency and fitness (through compensatory mutation) of resistant variants, to preserve future treatment options.²³⁶

Adverse Effects of Boceprevir and Telaprevir

Both boceprevir and telaprevir are associated with increased frequency and severity of anemia relative to peginterferon and ribavirin.^{292,504} Management of anemia by reducing ribavirin dose was not associated with a reduced rate of SVR in these trials.²³⁶

Rash is the most prominent adverse effect associated with telaprevir, with rash noted in 56% versus 32% of those receiving telaprevir, peginterferon, and ribavirin versus those receiving only peginterferon and ribavirin, respectively.²⁹² In 4% of cases the rash was severe (involving at least 50% of the body surface area), in 6% of cases telaprevir was discontinued due to rash, and in 1% of cases the entire regimen was discontinued due to rash.

Drug–Drug Interactions

In a manner analogous to (but distinct from) HIV protease inhibitors, preliminary studies reveal that boceprevir and telaprevir have significant interactions with other drugs as a result of metabolism by and inhibition of hepatic cytochromes. The

range of drugs potentially affected is large and likely to change frequently; clinicians must consult authoritative references for specific information about concomitant medications.

Investigational Agents

Discoveries revealing key steps in the life cycle of HCV have provided promising targets for antiviral development, including HCV p7,³⁹³ NS3-NS4A protease, NS3 helicase, NS4B,^{178,179} NS5A,³⁷⁰ NS5B polymerase,⁴²⁹ NS5A–cyclophilin interaction,^{119,208,257,453} and miR122.^{364,657} Drugs discussed here have completed or are currently in phase 3 trials (Table 27.2). A notable challenge for drug design is the NS3 helicase, which has been difficult to target selectively.²¹⁸

NS3 protease inhibitor classes include linear covalent (FDA-approved agents boceprevir and telaprevir), linear non-covalent, and macrocyclic compounds. The pharmacokinetics, potency, and resistance profiles of NS3 protease inhibitors vary. NS3 protease inhibitors currently in FDA phase 3 development (Table 27.2) include two noncovalent linear inhibitors (asunaprevir/BMS-650032 and BI 201335) and two macrocyclic inhibitors (TMC435350 and vaniprevir/MK-7009).

Daclatasvir/BMC-790052 is a potent inhibitor of NS5A that was used, in combination with asunaprevir, in the first interferon-sparing regimen with a significant rate of SVR (36% in prior nonresponders to peginterferon and ribavirin).³⁸⁸ In this small phase 2a study of patients with chronic HCV genotype 1, 4 of 11 patients who received the interferon-sparing regimen had SVR, and 9 of 10 patients who received peginterferon alfa-2a and ribavirin in addition to asunaprevir and daclatasvir had an SVR. Mild to moderate diarrhea affected most patients in both groups but did not require dose modification.

Small-molecule NS5B polymerase inhibitors include active site nucleoside/nucleotide analogs (competitors and chain terminators) and drugs that bind outside the active site. The latter have multiple potential binding sites in the thumb and palm domains of the NS5B crystal structure. GS-7977 (previously PSI-7977), a chain terminator nucleotide analog prodrug,⁴⁵⁰ which has *in vitro* activity against genotypes 1, 2, and 3,³⁵⁹ was recently reported to achieve SVR in some subjects with HCV genotypes 2 and 3 who received an interferon-free regimen.²²⁶

Silymarin, an extract of milk thistle, has been shown to have anti-inflammatory properties,^{502,503} but it has been difficult to assess such studies due to the fact that silymarin is a mixture of many compounds and standardization has been difficult. Silibinin, which is a component of silymarin, directly inhibits NS5B polymerase.⁷

Vaccines

There is no licensed vaccine for HCV, in spite of substantial interest given the high burden of disease worldwide,^{688,689} ongoing community and nosocomial transmission,^{97,358,484} limited access to treatment in populations at highest risk,^{211,425} evidence for protection from passive immunization (see later), and evidence for protective immunity against chronic infection after primary infection in humans^{424,478} and chimpanzees.³⁶³ The two major vaccine categories, prophylactic (preventive) and therapeutic, may share mechanistic features but have different goals and rationales. It is important to note that unlike most viral infections, a prophylactic vaccine for HCV might include a vaccine that allows (typically mild) acute infection but prevents chronic infection.

Passive Immunization

Passive immunization can be highly protective for some viral infections (e.g., hepatitis B virus, varicella-zoster virus), though its role in HCV infection has not been established and no FDA-licensed anti-HCV immune globulin preparation is available. Unlike HBV, HCV viremia was delayed but not prevented in chimpanzees treated with anti-HCV immune globulin postexposure.³⁴¹ Indirect evidence for protection by passive immunization was gleaned from HCV infections that occurred after anti-HCV–positive donations were excluded from commercial immunoglobulin preparations prior to the implementation of nucleic acid testing for HCV.⁷⁸ More recent studies, augmented by pseudoparticle-based neutralization assays (see Humoral Immune Response, earlier)⁴⁶ and use of the chimpanzee model, revealed evidence for passive protection by neutralizing antibodies *in vitro* and *in vivo*.⁶⁹⁹

Investigations of passive immunization have been facilitated by the development of humanized mouse models of HCV entry and infection (see Model Systems In Vivo, earlier). A genetically humanized mouse model demonstrated protection by passive immunization, using entry (without replication) as a surrogate for infection.¹⁶⁸ Similarly, use of the Alb-uPA SCID mouse model with engrafted human liver has demonstrated neutralization *in vivo* by polyclonal serum,⁶⁵⁵ though neutralization may be less efficient *in vivo* than *in vitro*.⁴³⁴ These systems also support identification and evaluation of broadly neutralizing monoclonal antibodies.^{80,238,369} Studies of maternal–infant transmission of HCV, a setting in which passive immunization might be anticipated, has not revealed correlation between protection and the presence of neutralizing antibodies in the serum of mother or child.^{169,435}

Prophylactic Vaccine Development

Natural infection with HCV generates an immune response that is initially robust and is largely maintained in those who spontaneously clear viremia (see Immune Response, earlier). Prior infection does not prevent reinfection in humans^{8,253,478} or chimpanzees,^{87,189} but chronicity is greatly reduced in secondary infections^{254,424,478} even with heterologous challenge.³⁶³ Although a portion of this apparent protection may be due to host differences,^{41,325,617,623} known genetic markers explain only a minority of the clearance phenotype.

A meta-analysis of vaccine studies in chimpanzees (including 63 naïve, 53 vaccinated, and 36 rechallenged animals),¹⁴¹ most of which were challenged with homologous virus, revealed that HCV-specific immune responses were generated and reduced the rate of chronic infection ($p < .001$) in vaccinated animals (28%) relative to naïve animals (62%) and similarly to rechallenged animals (17%). Peak RNA levels and duration of viremia were also reduced by vaccination. Assays for T-cell responses by IFN- γ ELISpot, performed in only a small number of animals, did not predict efficacy; however, vaccine antigens based on structural proteins were significantly ($p = .01$) more protective (14% chronicity) than those containing nonstructural proteins (46% chronicity). This latter finding is at odds with the highly protective effect of prior cleared HCV infection (i.e., rechallenge after exposure to the full genome) and the consistent importance of HLA class I alleles⁴⁶⁹ and CD8 T cells⁵⁷³ in spontaneous resolution. Therefore, past vaccines have not achieved the protection afforded by natural infection, and

further optimization of antigen, adjuvant, vector, route, and/or schedule is needed.

The protective role of antibody responses in HCV infection remains controversial, in part due to data demonstrating an essential role for CD8 T cells in spontaneous clearance⁵⁷³ in association with a strong CD4 T-cell response²⁵¹ and evidence that individuals with congenital agammaglobulinemia can clear infection without treatment.⁴ Additionally, hyperimmune serum appeared to be only partially protective in the chimpanzee model¹⁹⁴ due to breakthrough of minor sequence variants, and the extreme variability of the HCV envelope gene (see Genetic Diversity) may limit the breadth of protection provided by an antibody-based vaccine or passive immunization. Nonetheless, antibodies generated by chimpanzees after two vaccinations with recombinant E1 + E2 from the HCV-1 strain (subtype 1a) neutralized HCVpp and HCVcc constructs with envelope proteins derived from genotypes 1, 4, 5, and 6.⁴³⁷ Similarly, vaccination of mice and macaques with HCVpp displaying E1 and/or E2 from subtype 1a stimulated production of antibodies that neutralized HCV genotypes 1, 2, 4, and 5.²³⁰ E1E2 subunit vaccination of a small number of healthy human volunteers elicited antibody responses that neutralized heterologous isolates.^{217,516,587}

Vaccines that elicit robust T-cell responses without neutralizing antibodies may not prevent new HCV infections, but they may prevent chronic infection²¹⁰ and associated serious complications. As noted previously (see Immune Response), spontaneous resolution is associated with broadly targeted, polyfunctional CD4 and CD8 T-cell responses. Adenovirus serotypes rarely or never found in humans (addressing the problem of pre-existing immunity¹²³) and expressing HCV NS3–NS5B proteins were recently used to vaccinate healthy volunteers, resulting in sustained T-cell responses targeting multiple viral proteins.⁴³ It is not known whether such a vaccine will prevent chronic HCV infection in humans.

Therapeutic Vaccine Development

A therapeutic vaccine, to be used during chronic infection to augment pharmacologic therapy, modulate chronic disease outcome, or clear chronic infection, is an attractive goal for improving the health of many millions of people with limited treatment options. As an adjunct to conventional antiviral therapy, a therapeutic vaccine would have the potential to reduce duration and/or dose of antivirals, thereby reducing toxicity, cost, and failure rate. Evidence of low-level viremia at end of treatment in persons who subsequently experience relapse underscores the potential value of this approach; in addition, suppression of viremia during therapy may optimize the immune system's ability to augment responses that were previously suppressed by the tolerizing effects of chronic viremia.

Therapeutic vaccines have demonstrated modest efficacy in pilot studies, illustrating the challenge of stimulating immune responses to a chronic infection. Humans injected with E1 protein subunit developed anti-E1 responses but no significant change in viremia or inflammation.^{471,676} IC41, a multiepitope peptide-based vaccine, stimulated peptide-specific CD4 and CD8 responses in healthy volunteers,^{205,206} had inconsistent effects on the level of viremia in chronic infection,³³³ and induced HCV-specific T-cell responses near the end of interferon-based treatment without preventing relapse.⁶⁷⁷ TG4040, a modified vaccinia Ankara (MVA) vaccine using an attenuated

poxvirus to express NS3-NS5B proteins, stimulated measurable T-cell responses in a minority of recipients, some of whom had transient reductions in viremia.²⁶³

Prevention of Transmission of HCV

Because the transmission routes of HCV are well defined (see Transmission, earlier), behavioral measures for prevention can be effective if applied consistently. HCV transmission by medical procedures can be stopped by strict observance of infection control protocols.^{239,293,512} Although blood transfusions were once an important route of HCV transmission, transfusion transmission has virtually been eliminated by testing donors for HCV antibodies and RNA.³⁹⁹ Nonetheless, even in some economically developed nations, HCV transmission still occurs in hospitals and traditional medical centers. In one report from Spain, 67% of acute HCV infections were linked to receipt of medical care.⁴⁰⁸ The World Health Organization estimates that unsafe injections continue to cause 2.3 million to 4.7 million new HCV infections each year.²⁸³

HIV Co-Infection with HCV

The complications of HCV are more frequent and severe in persons with HIV, leading to HCV's designation in 1999 as an opportunistic infection,^{99,595} and liver disease related to viral hepatitis continues to be a major cause of death among persons with HIV worldwide.⁶⁷⁴ For these reasons, people with HIV should be screened for HCV upon entry into care, for any unexplained elevation of hepatic transaminases, and periodically in the setting of risky behavior.

Due primarily to shared routes of transmission, approximately one-quarter of persons with HIV infection also have HCV infection.⁶¹⁹ HIV infection increases the likelihood of transmission of HCV from mother to child^{274,624,700} and among men who have sex with men.^{240,249,645,648,649,651,652} After exposure to HCV infection, the likelihood of becoming chronically infected is higher in persons with HIV infection,^{391,424,433,620} and in those with chronic infection, progression to end-stage liver disease is more frequent and rapid in those with HIV.^{242,250,596} HCV RNA levels in persons with HIV are higher than in those without HIV.^{186,621,622,627} There has been concern about evidence of fibrosis progression in the first 1 to 2 years of HCV infection in some cohorts,²⁰³ though pre-existing liver disease could not be excluded, fibrosis has not previously been assessed during acute infection, and longer follow-up is needed to clarify this issue.⁶⁶³

Whether chronic HCV infection alters the course of HIV or the response to antiretroviral therapy is not clear. Greub and co-workers²⁵⁶ suggested that even among the subset of HIV/HCV co-infected persons in whom HIV was fully suppressed by antiretroviral therapy, CD4 lymphocyte responses were reduced compared to HIV-infected controls without HCV. Sulkowski and co-workers⁵⁹⁷ failed to detect an association once the strong interaction of HCV and injection drug use was considered. What is clear is that adherence to antiretroviral therapy is the dominant determinant of clinical outcomes in HIV/HCV co-infected persons.

Even though treatment of HIV in persons with HIV may be complicated by hepatotoxicity,^{3,126,152,244,256,416,475,508,534,593,598,599-600,619} significant complications are rare, therapy can be monitored and adjusted, and antiretroviral therapy appears to reduce liver disease progression.^{54,77,510} Treatment of HCV in

those co-infected with HIV is more complicated due to cytopenias and other toxicities, and concomitant therapies increase the potential for drug-drug interactions, particularly with direct-acting antiviral agents for HCV.⁵⁹⁴ These challenges, and the lower likelihood of SVR in persons with HIV infection (discussed in the Treatment section earlier), underscore the need for safer and more effective antiviral agents for HCV.

PERSPECTIVE

A more detailed understanding of the HCV life cycle remains a high priority; we have limited understanding of the trade-offs between replication and virion production, their basis in the viral sequence, and how these are modulated. Current model systems are incompatible with “wild” isolates, and a robust immunocompetent small-animal model is sorely needed. Our nascent understanding of the systems biology of antiviral immunity is underscored by the puzzle of *IL28B* polymorphisms and HCV: intense study has failed to identify the key genetic determinant and its mechanism, with potentially broad implications for innate and adaptive antiviral immunity; this is made more interesting by the relative lack of effect of *IL28B* polymorphisms on HBV or HIV pathogenesis.⁴⁰⁷ Epidemiology of HCV, including genotype distribution, is rarely obtained on a population basis; more accurate assessment would have a broad impact on treatment and prevention efforts. Although the safety and efficacy of treatment are likely to improve, worldwide eradication of HCV will depend on a multifaceted approach; therefore, development of a vaccine that prevents chronic infection continues to be an important goal.

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Coronaviridae

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and severe hepatitis and neurologic diseases in mice.^{75,186} It was not until the 1960s, however, that these viruses,^{27,32} as well as certain human respiratory viruses,^{8,391} were recognized to share characteristics that merited their being grouped together. Their most notable common feature, revealed by electron microscopy, was a fringe of widely spaced, club-shaped spikes that projected from the virion surface; these spikes were morphologically distinct from the surface projections of ortho- and paramyxoviruses. The halo of spikes was described as giving the viral particle the appearance of the solar corona, which prompted the name that was adopted for this new virus group.⁷

Over the next 40 years, coronaviruses were studied mainly because they cause economically significant respiratory and gastrointestinal diseases in domestic animals and because they provide unique models for viral pathogenesis. In humans, two coronaviruses were known to be responsible for a substantial fraction of common colds, particularly those that circulate in winter months. This situation changed dramatically with the emergence in 2002 of a devastating new human disease, severe acute respiratory syndrome (SARS), which was caused by a previously unknown coronavirus.^{143,288,440} Research stimulated by the SARS outbreak has led to great strides in our understanding of coronaviruses; by 2005, two additional, widespread human respiratory coronaviruses had been discovered.^{573,615} Moreover, the search for animal virus reservoirs has nearly tripled the total number of identified coronaviruses,^{255,394,616} although most of the recently discovered species are known only as genomic sequences and have yet to be isolated or propagated experimentally.

CLASSIFICATION

The coronaviruses are the largest group within the *Nidovirales* (Fig. 28.1), an order that comprises the families *Coronaviridae*, *Arteriviridae*,⁵²⁴ and *Roniviridae*.¹⁰² The arteriviruses, a small group of mammalian pathogens, are discussed in Chapter 29. The roniviruses, which infect shrimp, and a very recently isolated mosquito-borne virus,^{416,663} which is not yet classified, are currently the only members of the order having invertebrate hosts. Nidoviruses are membrane-enveloped, nonsegmented positive-strand RNA viruses that are set apart from other RNA viruses by certain distinctive characteristics.¹⁹⁴ Their most significant common features are (a) an invariant general genomic organization, with a very large replicase gene upstream of the structural protein genes; (b) the expression of the replicase-transcriptase polypeptide by means of ribosomal frame shifting; (c) a collection of unique enzymatic activities contained within the replicase-transcriptase protein products; and (d) the expression of downstream genes via transcription of multiple

HISTORY

Coronaviruses are enveloped RNA viruses that are broadly distributed among humans, other mammals, and birds, causing acute and persistent infections. Members of this family were isolated as early as the 1930s as the causative agents of infectious bronchitis in chickens,²⁵ transmissible gastroenteritis in pigs,¹⁴²

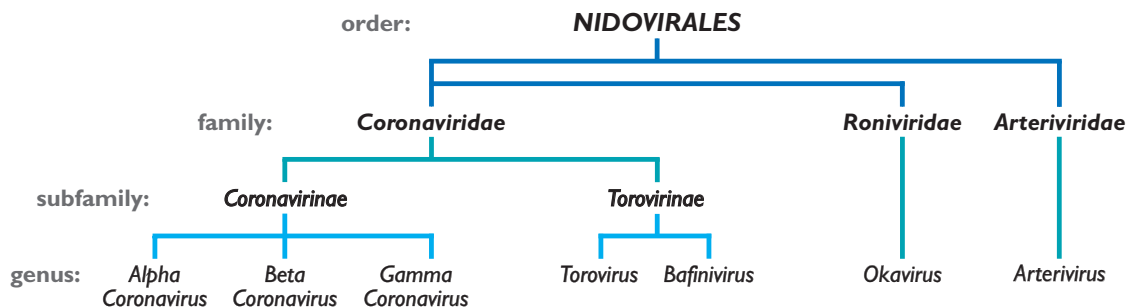


FIGURE 28.1. Taxonomy of the order *Nidovirales*.

3'-nested subgenomic messenger RNAs (mRNAs). This last property has provided the name for the order, which comes from the Latin *nido*, for "nest".¹⁵⁷ It should be noted that the replicative similarities among the three nidovirus families are offset by marked differences in the numbers, types, and sizes of their structural proteins and great variation among the morphologies of their virions and nucleocapsids.

Coronaviruses are now classified as one of two subfamilies (*Coronavirinae*) in the family *Coronaviridae* (see Fig. 28.1). The other subfamily, *Torovirinae*, includes the toroviruses, which are pathogens of cattle, horses, and swine,⁵²³ and the bafiniviruses, whose sole member is the only nidovirus currently known to infect fish.⁵⁰⁵ This chapter will concentrate almost exclusively on the *Coronavirinae*.

Coronaviruses have long been sorted into three groups, originally on the basis of serologic relationships and, subsequently, on the basis of phylogenetic clustering.^{193,195} Following proposals that were recently ratified by the International Committee on Taxonomy of Viruses (ICTV),⁵⁷ these groups—the alpha-, beta-, and gammacoronaviruses—have now been accorded the taxonomic status of genera (see Fig. 28.1). The ICTV classifications have also established rigorous criteria for coronavirus species definitions, in a manner consistent with those used for other viral families. As a consequence, some viruses previously considered to be separate species are currently recognized as a single species—for example, the viruses now grouped within alphacoronavirus 1 or betacoronavirus 1 (Table 28.1). Additionally, the new classification criteria resolve any previous uncertainty about the taxonomic assignment of the virus that caused SARS (severe acute respiratory syndrome coronavirus [SARS-CoV]) as a betacoronavirus.^{153,197,374,473,483,521,534,535}

Almost all alpha- and betacoronaviruses have mammalian hosts. In contrast, the gammacoronaviruses, with a single exception, have been isolated from avian hosts. Several of the viruses listed in Table 28.1 have been studied for decades, specifically those included in the species alphacoronavirus 1, betacoronavirus 1, murine coronavirus, and avian coronavirus. The focus on these viruses came about largely because they were amenable to isolation and growth in tissue culture. However, since 2004, molecular surveillance and genomics efforts initiated in the wake of the SARS epidemic have led to the discovery of a multitude of previously unknown coronaviruses that now constitute most members of this subfamily.⁶¹⁶ Notably, most of the newly recognized species were identified in bats, which constitute one of the largest orders within the mammals. Diverse coronaviruses have been described from bats, principally in Asia but also in Africa, Europe, and North and South

America. These viruses include likely predecessors of SARS-CoV^{308,332} but also four unique species of alphacoronaviruses and three species of betacoronaviruses. Birds have also proven to be a rich source of new viruses. Novel avian coronaviruses have been found to infect geese, pigeons, and ducks,²⁵⁵ and highly divergent coronaviruses recently identified in bulbuls, thrushes, and munias⁶¹⁷ have the potential to define a fourth genus in the *Coronavirinae*. It has been proposed that bats and birds are ideally suited as reservoirs for the incubation and evolution of coronaviruses, owing to their common ability to fly and their propensity to roost and flock.⁶¹⁶

Five of the viruses in Table 28.1 are associated with human disease. The most categorically harmful of these, SARS-CoV, which is discussed at length later in this chapter, does not currently infect the human population. The remaining four human coronaviruses (HCoVs), the alphacoronaviruses HCoV-229E and HCoV-NL63, and the betacoronaviruses HCoV-OC43 and HCoV-HKU1, typically cause common colds. Remarkably, HCoV-NL63 and HCoV-HKU1 were only discovered recently, in the post-SARS era,^{573,615} despite the fact that each has a worldwide prevalence and has been in circulation for a long time.^{461,618} Although generally associated with upper respiratory tract infections, the extant HCoVs can also cause lower respiratory tract infections and have more serious consequences in the young, the elderly, and immunocompromised individuals. In particular, HCoV-NL63 is strongly associated with childhood croup,⁵⁷⁴ and the most severe HCoV-HKU1, -OC43, and -229E infections are manifest in patients with other underlying illnesses.⁴⁶⁰

VIRION STRUCTURE

Virus and Nucleocapsid

Virions of coronaviruses are roughly spherical and exhibit a moderate degree of pleomorphism. In the earlier literature, viral particles were reported to have average diameters of 80 to 120 nm but were far from uniform, with extreme sizes from 50 to 200 nm.³⁸⁹ The spikes of coronaviruses, typically described as club-like or petal-shaped, emerge from the virion surface as stalks with bulb-like distal termini. Some of the variation in particle size and shape was likely attributable to stresses exerted by virion purification or distortions introduced by negative staining of samples for electron microscopy. More recent studies, employing cryo-electron microscopy and cryo-electron tomography,^{21,30,413,415} have produced images (e.g., Fig. 28.2A) in which virion size and shape are far more regular, although still

TABLE 28.1 Classification of Coronaviruses

Species ^a	GenBank accession ^b	Previous names for viruses included in newly defined species
Genus <i>Alphacoronavirus</i>		
Alphacoronavirus 1	EU186072 AY994055 GQ477367 AJ271965 AF304460 AY567487 AF353511 EF203067 DQ648858 EU420138 EU420139	Feline coronavirus type I (FeCoV I) Feline coronavirus type II (FeCoV II), Feline infectious peritonitis virus (FIPV) Canine coronavirus (CCoV) Transmissible gastroenteritis virus (TGEV)
Human coronavirus 229E (HCoV-229E)		
Human coronavirus NL63 (HCoV-NL63)		
Porcine epidemic diarrhea virus (PEDV)		
<i>Rhinolophus</i> bat coronavirus HKU2 (<i>Rh</i> -BatCoV HKU2)		
<i>Scotophilus</i> bat coronavirus 512 (<i>Sc</i> -BatCoV 512)		
<i>Miniopterus</i> bat coronavirus 1 (<i>Mi</i> -BatCoV 1)		
<i>Miniopterus</i> bat coronavirus HKU8 (<i>Mi</i> -BatCoV HKU8)		
Genus <i>Betacoronavirus</i>		
Betacoronavirus 1 ^c	U00735 EF446615 AY903460 DQ011855	Bovine coronavirus (BCoV) Equine coronavirus (EqCoV) Human coronavirus OC43 (HCoV-OC43) Porcine hemagglutinating encephalomyelitis virus (PHEV)
Murine coronavirus ^d	AY700211 FJ938068 AY597011 AY278741 DQ022305 DQ071615	Mouse hepatitis virus (MHV) Rat coronavirus (RCoV) Human severe acute respiratory syndrome coronavirus (SARS-CoV) Severe acute respiratory syndrome–related <i>Rhinolophus</i> bat coronavirus HKU3 (SARSr- <i>Rh</i> -BatCoV HKU3) Severe acute respiratory syndrome–related <i>Rhinolophus</i> bat coronavirus Rp3 (SARSr- <i>Rh</i> -BatCoV Rp3)
<i>Tylonycteris</i> bat coronavirus HKU4 (<i>Ty</i> -BatCoV HKU4)	EF065505	
<i>Pipistrellus</i> bat coronavirus HKU5 (<i>Pi</i> -BatCoV HKU5)	EF065509	
<i>Rousettus</i> bat coronavirus HKU9 (<i>Ro</i> -BatCoV HKU9)	EF065513	
Genus <i>Gammacoronavirus</i>		
Avian coronavirus ^e	AJ311317 EU022526 EU111742	Infectious bronchitis virus (IBV) Turkey coronavirus (TuCoV)
Beluga whale coronavirus SW1		

^aListed viruses are those for which complete genome sequences are available. Novel viruses that have not yet been formally classified include Bulbul coronavirus HKU11,⁶¹⁷ Thrush coronavirus HKU12,⁶¹⁷ Munia coronavirus HKU13,⁶¹⁷ Asian leopard cat coronavirus,¹³⁹ and Mink coronavirus.⁵⁹²

^bRepresentative GenBank accession numbers are given for viruses in each species; in many cases, multiple genomic sequences for a given virus are available.

^cOther viruses included in the species Betacoronavirus 1 are Human enteric coronavirus (HECoV) and Canine respiratory coronavirus (CRCoV), for which only partial genomic sequences are available.

^dOther viruses included in the species Murine coronavirus are Puffinosis virus (PCoV) and Sialodacryoadenitis virus (SDAV), for which only partial genomic sequences are available.

^eOther viruses included in the species Avian coronavirus are Pheasant coronavirus (PhCoV), Goose coronavirus (GCoV), Pigeon coronavirus (PCoV), and Duck coronavirus (DCoV), for which only partial genomic sequences are available.²⁵⁵

pleomorphic. These studies, which examined a number of alpha- and betacoronaviruses, converge on mean particle diameters of 118 to 136 nm, including the contributions of the spikes, which project some 16 to 21 nm from the virion envelope.

Enclosed within the virion envelope is the nucleocapsid—a ribonucleoprotein that contains the viral genome. The struc-

ture of this component is relatively obscure in images of whole virions; however, its makeup has been partially displayed by electron micrographs of spontaneously disrupted virions or of virions solubilized with nonionic detergents.^{59,109,183,269,366} Such studies revealed another distinguishing characteristic of coronaviruses: They have helically symmetric nucleocapsids.

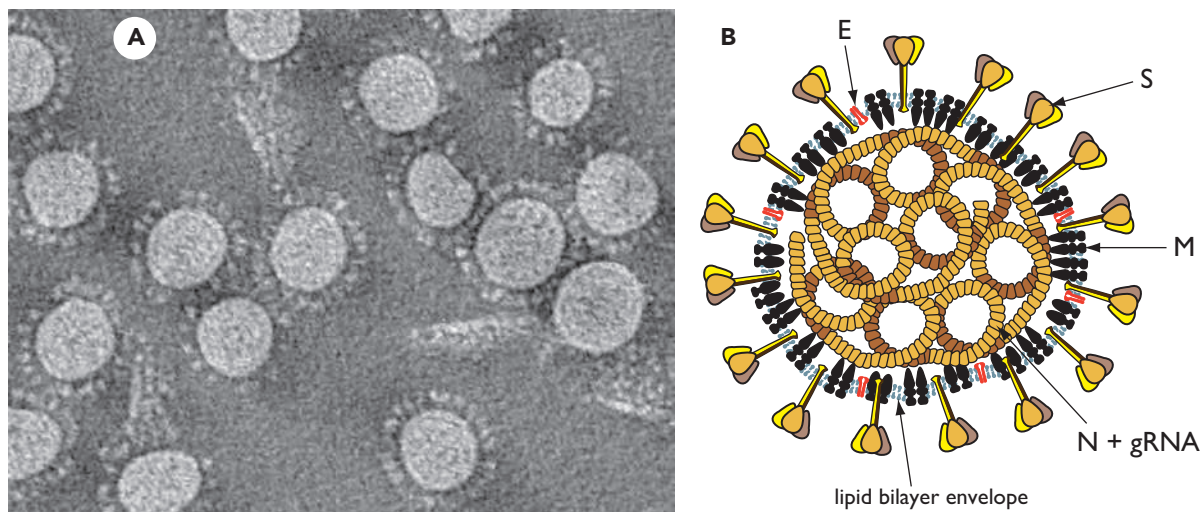


FIGURE 28.2. Coronavirus structure. **A:** Cryo-electron tomographic image of purified virions of mouse hepatitis virus (MHV), reconstructed as described in reference 415. (Courtesy of Benjamin Neuman, David Bhella, and Stanley Sawicki.) **B:** Schematic showing the major structural proteins of the coronavirus virion: S, spike protein; M, membrane protein; E, envelope protein; and N, nucleocapsid protein.

Helical symmetry is common for negative-strand RNA virus nucleocapsids, although it is highly unusual for positive-strand RNA animal viruses, almost all of which have icosahedral capsids. The best-resolved images of the coronavirus nucleocapsid, which were obtained with HCoV-229E, showed filamentous structures 9 to 13 nm in diameter, with 3- to 4-nm-wide central canals⁵⁹; these filaments were thinner and less sharply segmented than paramyxovirus nucleocapsids. However, widely ranging and sometimes discrepant parameters have been reported for the nucleocapsids of other coronaviruses,³⁷⁸ varying with both the viral species and the method of preparation.^{109,183,269,366,476} Thus, further work is needed to clearly define the diameter, symmetry, length, and protein:RNA stoichiometry of this virion component in isolation. More recent coronavirus ultrastructural studies suggest that when packaged within the virion envelope, the helical nucleocapsid is quite flexible, forming coils and other structures that fold back on themselves.^{21,413}

Virion Structural Proteins

Coronaviruses contain a canonical set of four major structural proteins: the spike (S), membrane (M), and envelope (E) proteins, all of which are located in the membrane envelope, and the nucleocapsid (N) protein, which is found in the ribonucleoprotein core (see Fig. 28.2B).

The distinctive surface spikes of coronaviruses are composed of trimers of S molecules.^{30,129,529} S is a class I viral fusion protein⁴¹ that binds to host cell receptors and mediates the earliest steps of infection.⁹⁵ In some cases, S protein can also induce cell–cell fusion late in infection. The S monomer is a transmembrane protein of 128 to 160 kDa, composed of a very large N-terminal ectodomain and a tiny C-terminal endodomain (Fig. 28.3). This protein is inserted, via a cleaved signal peptide,⁶² into the endoplasmic reticulum (ER), where it obtains N-linked glycosylation increasing its mass by some 40 kDa.^{224,487} Comprehensive mapping of glycosylation sites has not been carried out for any S protein; however, an analysis

of the SARS-CoV S protein showed that at least half of its 23 candidate sites are glycosylated.²⁸⁷ The early steps of glycosylation occur co-translationally, and this modification assists monomer folding and proper oligomerization; terminal glycosylation is then completed subsequent to trimerization.¹²⁹ S protein monomer folding is also accompanied by the formation of intramolecular disulfide bonds among a subset of the numerous cysteine residues of the ectodomain.⁴²⁵ The positions of S protein cysteines are well conserved in each coronavirus genus^{2,153}; disulfide linkages have yet to be mapped.

In many beta- and gammacoronaviruses (e.g., mouse hepatitis virus [MHV], bovine coronavirus [BCoV], and infectious bronchitis virus [IBV]), the S protein is partially or completely cleaved by a furin-like host cell protease into two polypeptides, denoted S1 and S2, which are roughly equal in size. Correspondingly, in coronaviruses that do not have detectably cleaved mature S proteins, the N-terminal and C-terminal halves of the molecule are also designated S1 and S2, respectively. S protein cleavage occurs immediately downstream of a highly basic pentapeptide motif,^{2,62,361} and the extent of proteolysis correlates with the number of positively charged residues in the motif.³⁶ The S1 domain is extremely variable, exhibiting very low homology across the three genera and often diverging extensively among different isolates of a single coronavirus.^{181,430,597} By contrast, the S2 domain is highly conserved.¹¹¹ For those coronaviruses in which it occurs, S1–S2 cleavage is a late event in virion assembly and release from infected cells. For many other coronaviruses, an alternative type of S protein cleavage (S2′) takes place during the initiation of infection, activating the molecule for fusion.²⁸ The differing functions of S1 and S2 and the role of proteolysis are discussed later (see the Viral Entry and Uncoating section).

A complete high-resolution structure has not yet been determined for any coronavirus S protein, although a cryo-electron microscopic reconstruction of the SARS-CoV S protein is available,³⁰ and partial crystal structures have been solved for particular S protein domains.^{144,208,323,325,624,630,655}

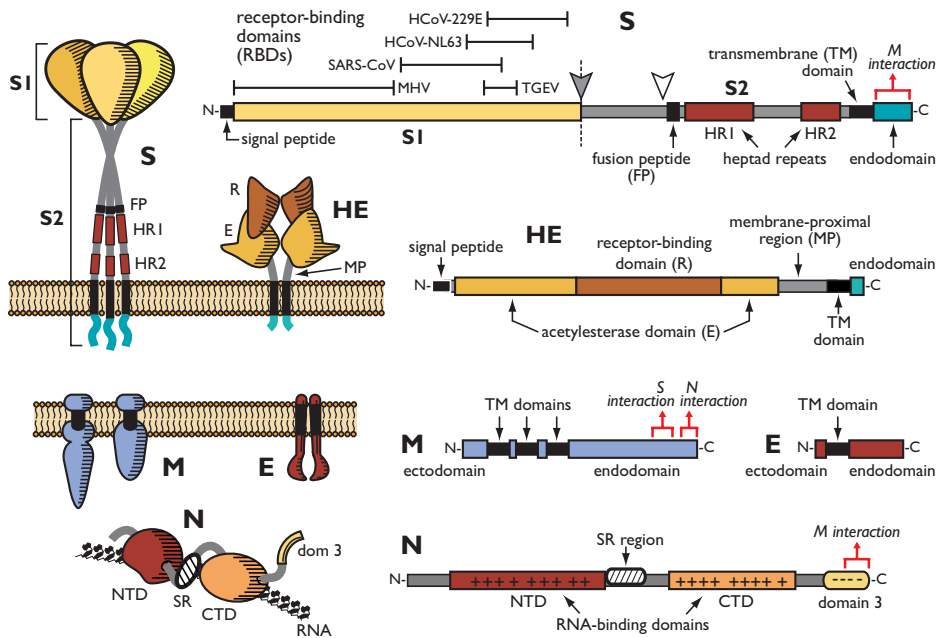


FIGURE 28.3. Virion structural proteins.

Folded and linear representations of the spike (S), hemagglutinin-esterase (HE), membrane (M), envelope (E), and nucleocapsid (N) proteins. The size scale for the linear diagram of S is half of that for the other proteins. In the linear diagram of S, *solid* and *open arrowheads* indicate the S1-S2 and alternative (S2') cleavage sites, respectively. In the linear diagrams of S, M, and N, *red brackets* indicate mapped regions involved in assembly interactions (see the Assembly and Release of Virions section).

Nevertheless, all currently available structural and biochemical evidence accords well with an early proposal that S is functionally analogous to the influenza HA protein.¹¹¹ In this model, the S1 domains of the S protein oligomer make up the bulbous, receptor-binding portion of the spike. The narrow stalk of the spike, distancing the bulb from the membrane, is a coiled-coil structure formed by association of heptad repeat regions (HR1 and HR2) of the S2 domains of monomers (see Fig. 28.3).

The most abundant structural protein in coronaviruses—the M protein^{544,546}—gives the virion envelope its shape. The M monomer, which ranges from 25 to 30 kDa, is a polytopic membrane protein that is embedded in the envelope by three transmembrane domains.^{14,486} At its amino terminus is a very small ectodomain; the C-terminal endodomain of M accounts for the major part of the molecule and is situated in the interior of the virion or on the cytoplasmic face of intracellular membranes (see Fig. 28.3). Although it is inserted co-translationally into the ER membrane, the M protein generally does not bear an amino-terminal signal peptide.^{62,486} For IBV and MHV, either the first or the third transmembrane domain of M alone suffices as a signal for insertion and anchoring of the protein in its native membrane orientation.^{350,363,384} Anomalous, M proteins of the alphacoronavirus 1 species do contain cleavable N-terminal signal peptides, although it is not clear whether these are necessary for membrane insertion.^{263,584} The ectodomain of M is modified by glycosylation, which is usually N linked.^{60,251,402,536,632} However, a subset of betacoronavirus M proteins exhibit O-linked glycosylation, and the MHV M protein has served as a model for study of this type of post-translational modification.^{116,349,419} Glycosylation of M influences both organ tropism and the interferon (IFN)-inducing capacity of some coronaviruses.^{72,113,311}

M proteins are moderately well conserved within each coronavirus genus but diverge considerably across genera. The most variable part of the molecule is the ectodomain. By contrast, a short segment, overlapping the third transmembrane domain and the start of the endodomain, exhibits a high degree

of sequence conservation that is seen even in torovirus M proteins.¹³² Like most multispanning membrane proteins, the M protein has been refractory to crystallization; however, recent cryo-electron microscopic and tomographic reconstructions have provided a glimpse of the structure of this protein within the virion envelope.^{21,413,415} These studies reveal that the large carboxy terminus of M extends some 6 to 8 nm into the viral particle and is compressed into a globular domain, consistent with early work showing that the endodomain is very resistant to proteases.^{61,384,486,490} The observed M structures are likely to be dimers, the monomers of which are associated through multiple interacting regions. M dimers appear to adopt two different conformations: a compact form that promotes greater membrane curvature and a more elongated form that contacts the nucleocapsid.⁴¹⁵

The E protein is a small polypeptide of 8 to 12 kDa that is found in limited amounts in the virion envelope.^{189,344,647} Despite its minor presence, no wild-type coronavirus has been discovered to lack this protein. Engineered knockout or deletion of the *E* gene has effects ranging from moderate¹²⁴ to severe^{293,296} to lethal.^{105,428} Thus, although E is not always essential, it is critical for coronavirus infectivity (see the Assembly and Release of Virions section). E protein sequences are widely divergent, even among closely related coronaviruses.²⁹³ However, all E proteins share a common architecture: a short hydrophilic amino terminus, followed by a large hydrophobic region, and, lastly, a large hydrophilic C-terminal tail (see Fig. 28.3). E is an integral membrane protein,^{100,335,582} but it does not have a cleavable signal peptide⁴⁶⁵ and is not glycosylated. Beta- and gammacoronavirus E proteins are palmitoylated on cysteine residues downstream and adjacent to the hydrophobic region^{38,101,335,354,647}; this modification remains to be found in an alphacoronavirus E protein.¹⁸⁹ The membrane topology of E is not completely resolved. Most evidence indicates that this polypeptide transits the membrane once, with an N-terminal exodomain and a C-terminal endodomain.^{101,420,465,564,582} Contrary to this are reports that E has a

hairpin conformation, placing both of its termini on the cytoplasmic face of membranes,^{12,368} or that E can have multiple membrane topologies.⁶⁴⁸ Also unresolved is the oligomeric state of E protein. The hydrophobic region of the SARS-CoV E protein forms multimers, from dimers through pentamers.^{564,610} A pentameric alpha-helical bundle structure has been solved for this domain,⁴⁴⁹ although it is not yet clear whether this reflects the organization of the native protein.

Residing in the interior of the virion, the N protein is the sole protein constituent of the helical nucleocapsid.²²² Monomers of this 43- to 50-kDa protein bind along the RNA genome in a beads-on-a-string configuration common to other helical viral nucleocapsids (see Fig. 28.2B). However, unlike the nucleoproteins of rhabdo- and paramyxoviruses, the coronavirus N protein provides little or no protection for its genome against the action of ribonucleases.^{366,408} The bulk of the N protein monomer is made up of two independently folding domains—designated the N-terminal domain (NTD) and the C-terminal domain (CTD)—although neither includes its respective terminus of the N molecule (see Fig. 28.3). Crystal or solution structures have been determined for NTDs and CTDs of SARS-CoV, IBV, and MHV.^{76,164,200,234,253,493,555,646} Flanking the NTD and CTD are three spacer segments, the central one of which contains a serine- and arginine-rich tract (the SR region), which was noted to resemble the SR domains of RNA-splicing factors.⁴⁴² Another functionally distinct region of N, the carboxy-terminal domain 3, has been defined genetically.^{236,279,441,442} The spacer segments and domain 3 are each likely to be intrinsically disordered polypeptides.^{66,67} Most of the N molecule, including the NTD and CTD, is highly basic; by contrast, domain 3 is acidic. There is only a moderate degree of sequence homology among N proteins across the three genera, with the exception of a stretch of 30 amino acids within the NTD that is highly conserved among all coronaviruses.³⁸⁰

The N protein is a phosphoprotein,^{272,352,515,542} modified at a limited number of serine and threonine residues. Phosphorylation sites have been mapped for a representative coronavirus from each genus, and targeted sites, collectively, fall in every domain and spacer region of the N molecule.^{55,77,604,619} Thus, a general pattern for N protein phosphorylation cannot yet be discerned, nor have all responsible kinases been identified, although there is evidence linking glycogen synthase kinase-3 to phosphorylation of the SR region.⁶¹⁹ The role of phosphorylation is also not known but is thought to have regulatory impact. Phosphorylation has been suggested to trigger a conformational change in N protein,⁵⁴¹ and it may enhance the affinity of N for viral versus nonviral RNA.⁷⁷

The most conspicuous function of the N protein is to bind to viral RNA. Nucleocapsid formation must involve both sequence-specific and nonspecific modes of RNA binding. Specific RNA substrates that have been identified for N protein include the transcription-regulating sequence (TRS)^{200,412,539} (see the Viral RNA Synthesis section) and the genomic RNA packaging signal^{96,396} (see the Assembly and Release of Virions section). The NTD and the CTD are each separately capable of binding to RNA ligands *in vitro*, and the structures of these domains offer some clues as to how this is accomplished. The NTD consists of a U-shaped β -platform with an extruding β -hairpin, which presents a putative RNA-binding groove rich in basic and aromatic amino acid residues.^{164,200,493} The CTD forms a tightly interconnected dimer, which exhibits a potential

RNA-binding groove lined by basic α -helices.^{253,555} Some work suggests that in the intact N protein, optimal RNA binding requires concerted contributions from both the NTD and the CTD.^{67,235} A significant fraction of nucleocapsid stability also results from interactions among N monomers.⁴⁰⁸ This level of association is generally attributed to the CTD^{67,164,253,646}; however, additional regions of N–N interaction have been mapped to the NTD and to domain 3.^{164,235,253} Another crucial function of N protein is to bind to M protein.^{162,546} This capability is provided by domain 3 of N.^{236,295,585}

A fifth prominent structural protein—the hemagglutinin-esterase (HE) protein—is found in only a subset of the beta-coronaviruses, including murine coronavirus, betacoronavirus 1, and HCoV-HKU1. In virions of these species, HE forms a secondary set of short projections of 5 to 10 nm arrayed beneath the canopy of S protein spikes.^{204,435,550} The 48-kDa HE monomer is composed almost entirely of an N-terminal ectodomain; this is followed by a transmembrane anchor and a very short C-terminal endodomain (see Fig. 28.3). HE is inserted into the ER by means of a cleaved signal peptide and acquires an additional 17 kDa of N-linked glycosylation at multiple sites.^{221,271,640} The assembled protein is a homodimer, the subunits of which are connected by disulfide bonds.²²¹ As its name indicates, the HE protein contains a pair of associated activities. First, it is a hemagglutinin—that is, it has the capability to bind to sialic acid moieties found on cell surface glycoproteins and glycolipids.^{54,272} Second, HE exhibits acylesterase activity with specificity for either 9-*O*- or 4-*O*-acetylated sialic acids.^{274,472,520,590,591} These characteristics are thought to allow HE to act as a cofactor for S protein, assisting attachment of virus to host cells, as well as expediting the travel of virus through the extracellular mucosa.⁹⁹ Consistent with this notion, the presence of HE in MHV dramatically enhances neurovirulence in the mouse host.²⁶⁵ Conversely, the HE protein is a burden to the virus in tissue culture, where its expression is rapidly counterselected.³⁴³ The two activities of the HE protein are strikingly similar to the receptor-binding and receptor-destroying activities found in influenza C virus,^{590,591} and, remarkably, the coronavirus HE gene is clearly related to the influenza C virus HEF gene.³⁵⁹ Moreover, toroviruses also possess a homolog of the HE gene,^{99,305} raising the possibility that all three of these virus groups evolved from a common ancestor.^{359,522} This kinship is further corroborated by the crystal structure of the BCoV HE protein, which reveals separate receptor-binding and acylesterase domains perched atop a truncated membrane-proximal region.⁶⁵⁰ The HE protein thus resembles a squat version of its influenza virus counterpart, shortened because it lacks the fusion domain stalk of the HEF protein.

GENOME STRUCTURE AND ORGANIZATION

Basic and Accessory Genes

The coronavirus genome, which ranges from 26 to 32 kb, is the largest among all RNA viruses, including RNA viruses that have segmented genomes. This exceptional RNA molecule acts in at least three capacities^{50,194}: as the initial mRNA of the infectious cycle (see the Expression of the Replicase-Transcriptase Complex section), as the template for RNA replication and transcription (see the Viral RNA Synthesis section), and as the substrate for packaging into progeny viruses (see

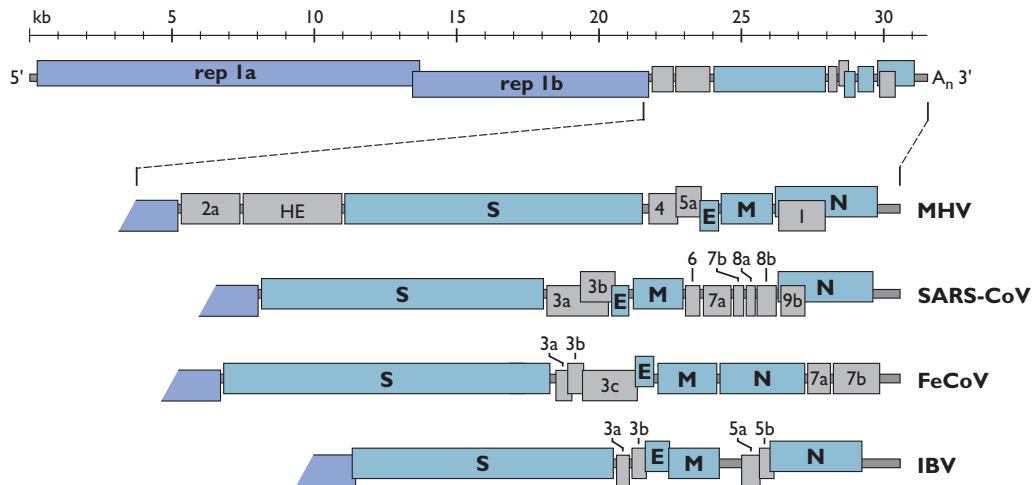


FIGURE 28.4. Coronavirus genome organization. A schematic of the complete genome of MHV is shown at the top. The replicase gene constitutes two ORFs, rep 1a and rep 1b, which are expressed by a ribosomal frameshifting mechanism (see the Expression of the Replicase-Transcriptase Complex section). The expanded region shows the downstream portion of the genomes of two betacoronaviruses (MHV and SARS-CoV), an alphacoronavirus (FeCoV), and a gammacoronavirus (IBV). The sizes and positions of accessory genes are indicated, relative to the basic genes *S*, *E*, *M*, and *N*. MHV, mouse hepatitis virus; ORFs, open reading frames; SARS-CoV, severe acute respiratory syndrome coronavirus; FeCoV, feline coronavirus; IBV, infectious bronchitis virus.

the Assembly and Release of Virions section). Consistent with its role as an mRNA, the coronavirus genome has a standard eukaryotic 5'-terminal cap structure³⁰¹ and a 3' polyadenylate tail.^{302,351,503,599} The genome comprises a basic set of genes in the invariant order 5'-*replicase*-*S*-*E*-*M*-*N*-3', with the huge *replicase* gene occupying two-thirds of the available coding capacity (Fig. 28.4). The replicase-transcriptase is the only protein translated from the genome; the products of all downstream open reading frames (ORFs) are derived from subgenomic mRNAs. The 5'-most position of the *replicase* gene is dictated by the requirement for expression of the replicase to set in motion all subsequent events of infection. The organization of the other basic genes, however, does not seem to reflect any underlying principle, because engineered rearrangement of the downstream gene order is completely tolerated.¹²¹

Dispersed among the basic genes in the 3'-most third of the genome, there are from one to as many as eight additional ORFs, which are designated accessory genes^{378,407} (see Fig. 28.4). These can fall in any of the intergenic intervals downstream of the *replicase* gene,⁶¹⁶ except, curiously, never between the *E* and *M* genes. In some cases, an accessory gene can be partially or entirely embedded as an alternate reading frame within another gene—for example, the internal (*I*) gene of MHV or the *3b* gene of SARS-CoV. Accessory genes are generally numbered according to the smallest transcript in which they fall. Consequently, there is usually no relatedness among identically named accessory genes in coronaviruses of different genera, such as the *3a* genes of SARS-CoV, feline coronavirus (FeCoV), and IBV (see Fig. 28.4). Some of these extra ORFs are thought to have been acquired through ancestral recombination with RNA from cellular or heterologous viral sources. The *HE* gene is the best-supported example of this type of horizontal genetic transfer.³⁵⁹ Two other such candidates are the *2a* gene found in murine coronavirus and betacoronavirus 1, which encodes a putative 2',3'-cyclic phosphodiesterase,^{385,485}

and gene 10 of beluga whale coronavirus, which encodes a putative uridine-cytidine kinase.³⁹⁴ Notably, the *2a* gene has a homolog embedded as a module within the *replicase* gene of the toroviruses,⁵²² which is a situation also consistent with horizontal transfer. The origin of most accessory genes, however, remains an open question. It is plausible that some of them evolved through intragenomic recombination, resulting in gene duplication and subsequent divergence, as suggested for several of the accessory genes of SARS-CoV.²⁴¹

Almost all accessory genes that have been examined are expressed during infection, although their functions are incompletely understood. The protein products of most accessory genes are nonstructural; however, this rule is not without exception. The *HE* protein, the MHV *I* protein,¹⁶⁵ and the products of SARS-CoV ORFs 3a, 6, 7a, 7b, and 9b^{231,407,502,627} are all components of virions. Mutational knockout or deletion of accessory genes has revealed that none are essential for viral replication in tissue culture. Conversely, accessory gene ablation,^{103,115,206} or transfer to another virus,^{452,559} can have profound effects on viral pathogenesis. In some cases, the basis for this is understood to result from interactions with host innate immunity (see the Immune Response and Viral Evasion of the Immune Response section). For other accessory genes, though, potential *in vivo* functions have not yet been elucidated.^{125,165,645}

Coronavirus Genetics

Classical coronavirus genetics focused principally on two types of mutants.²⁹⁹ The first were naturally arising viral variants, particularly deletion mutants, which offered clues to genetic changes responsible for different pathogenic traits.^{430,583,603} The second were temperature-sensitive (*ts*) mutants isolated from MHV following chemical mutagenesis.^{282,477,501,545} Some of these proved to be valuable in analyses of the functions of structural proteins.^{279,360,380,474} However, owing to the large target size of the *replicase* gene, most of such randomly generated mutants

had conditional-lethal, RNA-negative phenotypes. Complementation analyses of these latter mutants yielded early insights into the multiplicity of functions entailed by coronavirus RNA synthesis.^{22,176,177,501} There has been a recent resurgence of interest in classical replicase *ts* mutants, which are currently sorted into five complementation groups, because they can now be fully examined by the tools of reverse genetics.^{138,499,543}

The development of coronavirus reverse genetics proceeded in two phases.¹³⁰ Initially, a method called *targeted RNA recombination* was devised at a time when it was uncertain whether the construction of full-length infectious complementary DNA (cDNA) clones of coronavirus genomes would ever become technically feasible. With this method, a synthetic donor RNA bearing mutations of interest is transfected into cells that have been infected with a recipient parent virus possessing some characteristic that can be selected against.^{279,377,380} In its current form, for manipulation of MHV, the technique uses a chimeric recipient parent virus designated fMHV (Fig. 28.5A). The fMHV chimera is a mutant of MHV that contains the S protein ectodomain from the FeCoV feline infectious peritonitis virus (FIPV) and can therefore only grow in feline cells (see the Virion Attachment to Host Cells section). The restoration of its ability to grow in murine cells, via recombination with donor RNA containing the MHV S gene, enables a strong selection for viruses bearing site-specific mutations^{292,381}; unwanted secondary crossover events distal to the S gene are eliminated owing to the rearrangement of downstream genes in fMHV.¹⁹⁰ Targeted RNA recombination remains a powerful method to recover structural or accessory protein or 3' untranslated region (UTR) mutants.

To obtain access to the major part of the coronavirus genome, however, it was necessary to create full-length cDNAs, despite the barriers presented by the huge size of the *replicase* gene and the high instability of various regions when propagated in bacterial clones. Three innovative strategies were developed to overcome these inherent difficulties.¹³⁰ In the first (see Fig. 28.5B), a full-length cDNA copy of a coronavirus genome is assembled downstream of a cytomegalovirus (CMV) promoter in a bacterial artificial chromosome (BAC) vector, which is stable by virtue of its low copy number.^{5,6} The infection is then launched from transfected BAC DNA through transcription of infectious coronavirus RNA by host RNA polymerase II. This method of initiating infection obviates potential limitations of *in vitro* capping and synthesis of genomic RNA. In the second strategy (see Fig. 28.5C), a full-length genomic cDNA is assembled by *in vitro* ligation of smaller cloned cDNA fragments, some of the boundaries of which have been chosen so as to interrupt regions of instability.^{642,643} The ligation occurs in a directed order that is dictated by the use of asymmetric restriction sites. Infectious genomic RNA is then transcribed *in vitro* and used to transfect susceptible host cells. An extension of this method has demonstrated the construction of a coronavirus genome entirely from synthetic cDNAs.²⁶ In the third strategy (see Fig. 28.5D), the genome of vaccinia virus is used as the cloning vector for a full-length coronavirus cDNA that is generated by long-range reverse transcription polymerase chain reaction (RT-PCR).^{94,561} The cDNA is then amenable to manipulation by the repertoire of techniques available for poxvirus reverse genetics.^{51,94} Infections are launched from *in vitro*-synthesized RNA or else from transfected cDNA

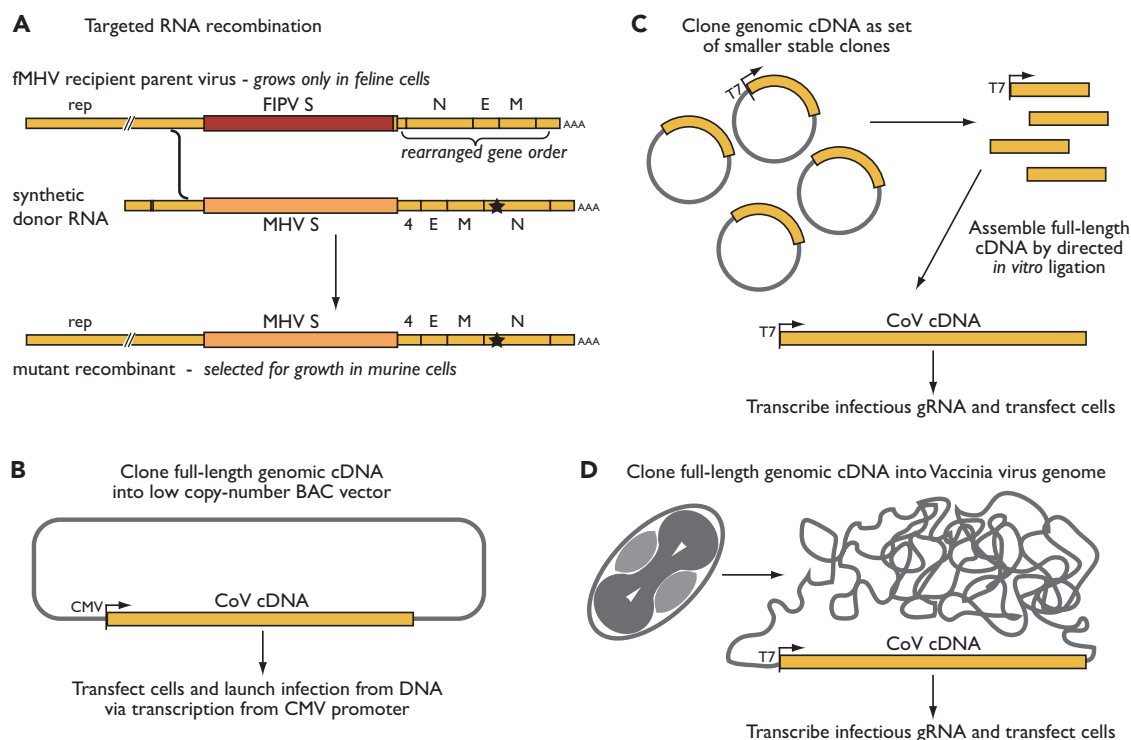


FIGURE 28.5. Methods for coronavirus reverse genetics. **A:** Targeted RNA recombination, which is applicable to the downstream third of the genome, shown here for transduction of a mutation (*star*) into the mouse hepatitis virus *N* gene. **B–D:** Three schemes developed for complete reverse genetics, based on stable production of full-length genomic complementary DNAs.

transcribed *in vivo* by fowlpox-encoded T7 RNA polymerase.⁵⁸ Collectively, these systems developed for complete reverse genetics provide an important pathway toward unraveling the complexities of the coronavirus replicase.

CORONAVIRUS REPLICATION

Virion Attachment to Host Cells

Coronavirus infections are initiated by the binding of virions to cellular receptors (Fig. 28.6). There then follows a series of events culminating in the delivery of the nucleocapsid to the cytoplasm, where the viral genome becomes available for translation. Individual coronaviruses usually infect only one or a few closely related hosts. The interaction between the viral S protein and its cognate receptor constitutes the principal determinant governing coronavirus host species range and tissue tropism. This has been most convincingly shown in two ways. First, the expression of a particular receptor in nonpermissive cells of a heterologous species renders those cells permissive for the corresponding coronavirus.^{127,146,330,331,399,567,639} Second, the engineered replacement of the S protein ectodomain changes the host cell species specificity or tissue tropism of a coronavirus in a predictable fashion.^{207,292,410,453,495} The amino-terminal, more variable half of the spike protein, S1, is the part that binds to receptor. Binding leads to conformational changes that result in fusion between virion and cell membranes, medi-

ated by the more conserved half of the spike protein, S2. The region of S1 that contacts the receptor—the receptor-binding domain (RBD)—varies among different coronaviruses (see Fig. 28.3). For MHV, the RBD maps to the N-terminal section of S1.^{290,554} By contrast, RBDs for SARS-CoV,^{614,625} HCoV-NL63,³³⁷ transmissible gastroenteritis virus (TGEV),¹⁸⁸ and HCoV-229E³⁴ fall in the middle or C-terminal sections of S1.

The known cellular receptors for alpha- and betacoronaviruses are listed in Table 28.2; to date, no receptors have been identified for gammacoronaviruses. The MHV receptor mCEACAM1 was the first discovered coronavirus receptor (as well as one of the first receptors defined for *any* virus).^{606,607} That this molecule is the only biologically relevant receptor for MHV was made clear by the demonstration that homozygous *Ceacam1*^{-/-} knockout mice are totally resistant to infection by high doses of MHV.²¹⁵ CEACAM1 is a member of the carcinoembryonic antigen (CEA) family within the immunoglobulin (Ig) superfamily and, in its full-length form, contains four Ig-like domains.¹⁴⁶ A diversity of two- and four-Ig domain isoforms is generated by multiple alleles and alternative splicing variants of *Ceacam1*.^{97,145,147,422,423,641} The wide range of pathogenicity of MHV in mice is thought to be strongly affected by the interactions of S proteins of different virus strains with the array of receptor isoforms that are expressed in mice of different genetic backgrounds. Although their S proteins are phylogenetically very close to that of MHV, the betacoronaviruses BCoV and HCoV-OC43 do not use CEACAMs to infect their

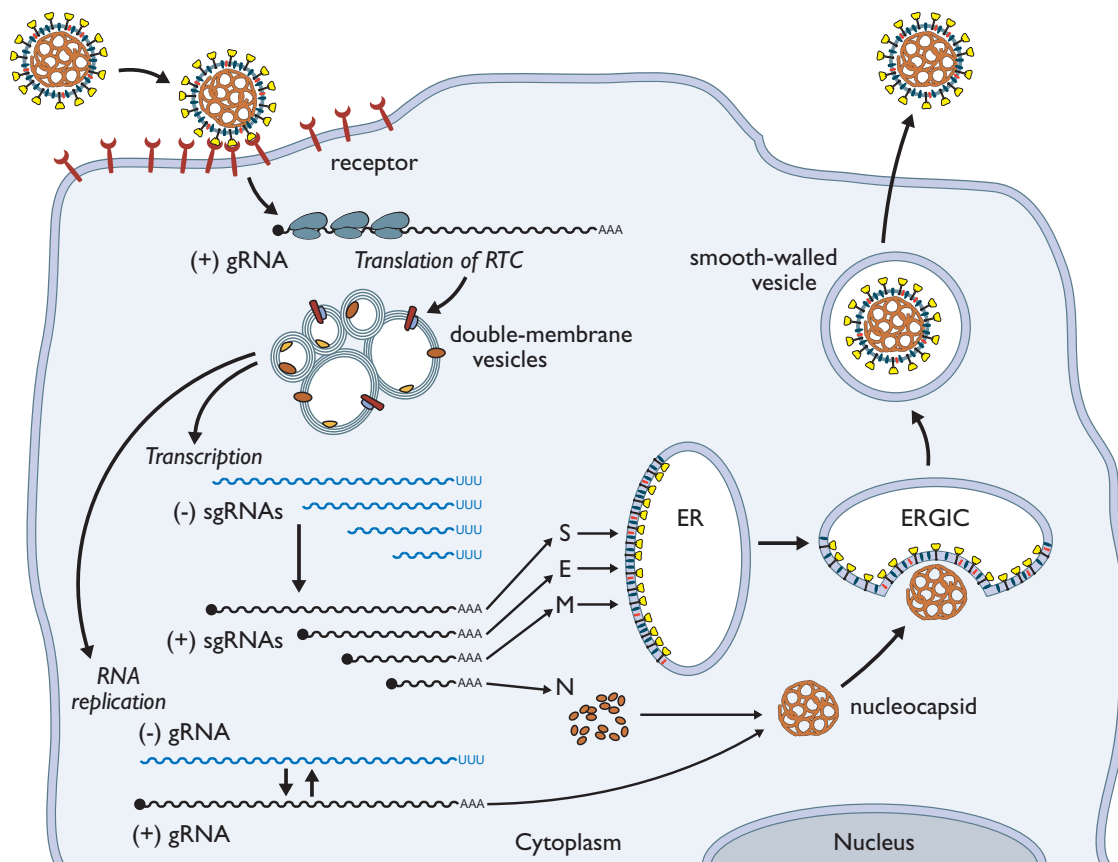


FIGURE 28.6. Overview of coronavirus replication (see text for details).

TABLE 28.2 Coronavirus Receptors

Virus	Receptor	References
Alphacoronaviruses		
TGEV	pAPN ^a	127
PRCoV	pAPN	128
PEDV	pAPN	322
FeCoV II, FIPV	fAPN ^b	567
FeCoV I	Unknown, but <i>not</i> fAPN ^b	148,223
CCoV	cAPN	29
HCoV-229E	hAPN	639
HCoV-NL63	ACE2	219
Betacoronaviruses		
MHV	mCEACAM1 ^c	411,606
BCoV	N-acetyl-9- <i>O</i> -acetylneuraminic acid	504
HCoV-OC43	N-acetyl-9- <i>O</i> -acetylneuraminic acid	291
SARS-CoV	ACE2 ^d	331

TGEV, transmissible gastroenteritis virus; pAPN, porcine aminopeptidase N; PRCoV, porcine respiratory coronavirus; PEDV, porcine epidemic diarrhea virus; FeCoV, feline coronavirus; fAPN, feline aminopeptidase N; FIPV, feline infectious peritonitis virus; CCoV, canine coronavirus; cAPN, canine aminopeptidase N; HCoV, human coronavirus; hAPN, human aminopeptidase N; ACE2, angiotensin-converting enzyme 2; MHV, mouse hepatitis virus; mCEACAM1, murine carcinoembryonic antigen–related adhesion molecule 1; BCoV, bovine coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus.

^aMammalian aminopeptidase N is also known as CD13.

^bAlthough the receptor for FeCoV I remains to be identified, the lectin fDC-SIGN serves as a coreceptor for both FeCoV I and FeCoV II.⁴⁷¹

^cThe related molecule mCEACAM2 functions weakly as an MHV receptor in tissue culture; however, it is not an alternate receptor in the mouse host *in vivo*.²¹⁵

^dHuman CD209L (L-SIGN), a lectin family member, can also act as a receptor for SARS-CoV but with much lower efficiency than ACE2²⁵⁴; a related lectin, DC-SIGN, can serve as a coreceptor.^{376,635}

hosts; rather, the only currently known attachment factor for these viruses is N-acetyl-9-*O*-acetylneuraminic acid.^{291,504} The recently solved structure of the MHV RBD complexed with mCEACAM1 has allowed the identification of key residues at the S protein–receptor interface.⁴⁴³ Coupled with mutational analysis, this structure reveals why the S proteins of BCoV and HCoV-OC43 cannot bind the MHV receptor and, conversely, why MHV does not bind to bovine or human CEACAMs.

Many alphacoronaviruses use aminopeptidase N (APN) of their respective host species as a receptor (see Table 28.2).^{127,567,639} APN (also called CD13) is a cell-surface, zinc-binding protease that is resident in respiratory and enteric epithelia and in neural tissue. The APN molecule is a heavily glycosylated homodimer. Mutational and inhibitor studies have shown that its enzymatic activity is not required for viral attachment and entry.¹²⁶ In general, the receptor activities of APN homologs are not interchangeable among species^{126,281}; however, feline aminopeptidase N (fAPN) can serve as a receptor not only for FIPV but also for canine coronavirus (CCoV), TGEV, and HCoV-229E.⁵⁶⁷ This circumstance has been exploited for the construction of chimeric APN molecules to map the basis for receptor recognition. Such studies have found

three small, linearly discontinuous determinants in APN that govern the species specificity of this subgroup of alphacoronaviruses.^{29,214,280,569}

The receptor for SARS-CoV—angiotensin-converting enzyme 2 (ACE2)—was discovered with notable rapidity following the isolation of the virus.³³¹ ACE2 is a cell-surface, zinc-binding carboxypeptidase involved in regulation of cardiac function and blood pressure. It is expressed in epithelial cells of the lung and the small intestine, which are the primary targets of SARS-CoV, as well as in heart, kidney, and other tissues.²⁰⁹ As with APN, the receptor role of ACE2 appears to be independent of its enzymatic activity. Although the SARS-CoV S protein binds to the catalytic domain of ACE2, active-site mutation or chemical inhibition does not detectably affect the ability of ACE2 to associate with S protein or to promote syncytia formation.^{331,333,398} The crystal structure of the SARS-CoV S protein RBD in complex with ACE2 shows the RBD cradling one lobe of the claw-like catalytic domain of its receptor.³²⁵ Remarkably, ACE2 also serves as the receptor for the alphacoronavirus HCoV-NL63,²¹⁹ and the corresponding structural complex for that virus reveals that the HCoV-NL63 RBD and the SARS-CoV RBD bind to the same motifs.⁶²⁴ Because the SARS-CoV and HCoV-NL63 RBDs have neither sequence nor structural homology, this finding strongly supports the notion that they have independently evolved to bind to the same hotspot on the ACE2 surface.^{623,624} Analyses of the SARS-CoV RBD–ACE2 interface have additionally demonstrated the structural basis for the final jump of SARS-CoV from palm civets to human hosts (see the Epidemiology section). These studies found that merely four critical residues constitute the major species barrier between the civet and human ACE2 molecules, and that mutation of only two key RBD residues was sufficient for civet SARS-CoV S protein to gain the ability to productively bind human ACE2.^{323,333}

Viral Entry and Uncoating

The entry of virions into cells results from large-scale rearrangements of the S protein that lead to the fusion of viral and cellular membranes.⁴¹ These rearrangements are triggered by some combination of receptor binding, proteolytic cleavage of S, and exposure to acidic pH. The S proteins of many coronaviruses are uncleaved in mature virions and require an encounter with a protease at the entry step of infection to separate the receptor-binding and fusion components of the spike. The details of proteolytic activation are still incompletely understood but have been best studied for SARS-CoV. In the cell types in which this virus is most commonly grown in tissue culture, viral entry depends on cathepsins, which are acid-activated endosomal proteases. The infectivity of SARS-CoV is thus suppressed by cathepsin inhibitors or by lysosomotropic agents.⁵¹⁷ However, cell-bound SARS-CoV can alternatively be activated by treatment with extracellular proteases, such as trypsin or elastase. This route of activation greatly enhances the infectivity of SARS-CoV and allows the virus to enter from the cell surface, thereby rendering the infection insensitive to lysosomotropic agents.³⁸³ The same pattern of proteolytic activation—cathepsin-dependence and its circumvention by exogenously added protease—is observed with a particular strain of MHV (MHV-2) that is unique in having an uncleaved S protein.⁴⁶⁴

The site of cleavage of receptor-bound SARS-CoV S protein by cathepsin or by exogenous trypsin differs from that of the S1-S2 cleavage, which occurs in other coronaviruses upon exit from cells. Cleavage at entry takes place at a locus (S2') within the S2 half of the molecule, immediately upstream of the putative fusion peptide²⁸ (see Fig. 28.3). It is not yet clear if cleavage at analogous S2' sites is the pattern for all coronavirus S proteins; however, the emerging pattern is that proteolytic activation of S protein is required for infectivity and that coronaviruses have evolved in different ways to ensure that this occurs.⁴¹ Recent studies provide evidence that for the SARS-CoV S protein, the most biologically relevant protease may be TMPRSS2.^{187,382,514} This transmembrane serine protease, which is expressed in pneumocytes, co-localizes with and binds to ACE2. In cells expressing TMPRSS2, SARS-CoV enters at the cell surface and is insensitive to cathepsin inhibitors and lysosomotropic agents.

Just as the mechanism of S protein proteolytic activation is variable, so too is its location. Some coronaviruses, such as most strains of MHV, fuse with the plasma membrane,^{547,601} whereas others, such as TGEV,²¹² HCoV-229E,⁴²¹ and SARS-CoV,⁵¹⁷ can enter cells through receptor-mediated endocytosis and then fuse with the membranes of acidified endosomes. The boundary between these two modes of entry may easily shift. For one strain of MHV (MHV-4), as few as three amino acid changes in the heptad repeat region of S2 switches the virus from plasma membrane fusion to acid pH-dependent fusion.¹⁸⁰ It remains unresolved whether acidic pH, *per se*, is required for S protein conformational changes^{90,154,324} or whether this reflects the requirements for activation of endosomal proteases during infection of some types of cells.⁵¹⁷

The coronavirus S protein is a class I viral fusion protein with domains functionally similar to those of the fusion proteins of phylogenetically distant RNA viruses, such as influenza virus, human immunodeficiency virus (HIV), and Ebola virus, but on a much larger scale.^{41,42} As in those other viral fusion proteins, the coronavirus S2 moiety contains two separated heptad repeats—HR1 and HR2—with a fusion peptide upstream of HR1 and the transmembrane domain immediately downstream of HR2 (see Fig. 28.3). The exact assignment of the fusion peptide is not agreed upon, however.^{41,367,450} Receptor-mediated conformational changes in S1, and the dissociation of S1 from S2, are thought to initiate major rearrangements in the remaining S2 trimer that proceed through multiple intermediate states.^{133,324} These rearrangements ultimately expose the fusion peptide, which interacts with the host cellular membrane, and the two heptad repeats in each monomer are brought together to form an antiparallel, six-helix bundle. The six-helix bundle is an extremely stable, rod-like complex, the biophysical properties of which have been extensively studied.^{40,42,242,348,568} Highly similar crystallographic structures have been solved for the six-helix complexes from both the MHV S protein⁶²⁹ and the SARS-CoV S protein.^{144,552,630} These show the three HR1 helices forming a central, coiled-coil core some two to three times larger than its counterparts in other viruses. Arrayed around this, the three shorter HR2 helices, in an antiparallel orientation, pack into the grooves between the HR1 monomers via hydrophobic interactions. The outcome of the formation of the six-helix bundle is the juxtaposition of the viral and cellular membranes in sufficient proximity to allow mixing of their lipid bilayers and the deposition of the contents of the virion into the cytoplasm.

Expression of the Replicase-Transcriptase Complex

Following delivery of the viral nucleocapsid to the cytoplasm, the next event is the translation of the *replicase* gene from the genomic RNA. This gene consists of two large ORFs—rep 1a and rep 1b—that share a small region of overlap (see Fig. 28.4). Translation of the entire replicase depends on a mechanism called *ribosomal frameshifting*, whereby, with a fixed probability, a translating ribosome shifts one nucleotide in the –1 direction, from the rep 1a reading frame into the rep 1b reading frame.³⁷⁸ This repositioning is programmed by two RNA elements (Fig. 28.7A), embedded near the region of overlap, that were discovered in studies of IBV.^{46,47} The first element is the 5'-UUUAAAC-3' heptanucleotide slippery sequence, which is identical for all known coronaviruses and has apparently been selected as optimal for its role.^{48,457} The second element, located a short distance downstream of the slippery sequence, is an extensively characterized RNA pseudoknot structure.^{49,405} This latter component was initially thought to be a classic two-stem (H-type) pseudoknot; however, recent analyses of SARS-CoV frameshifting support a more elaborate structure that includes a third stem loop within pseudoknot loop 2.^{20,141,456}

The two elements act together to produce the coterminal polypeptide products pp1a and pp1ab. During most rounds of translation, the elongating ribosome unwinds the pseudoknot and translation terminates at the rep 1a stop codon, yielding the smaller product, pp1a. Some fraction of the time, however, the pseudoknot blocks the mRNA entrance channel of the ribosome.^{213,403,528} The consequent pause required for the ribosome to melt out the mRNA structure allows the simultaneous slippage of the P and A site transfer RNAs (tRNAs) into the rep 1b reading frame. This results in the synthesis of pp1ab when elongation resumes.^{20,47} Studies of reporter gene expression suggest that the incidence of coronavirus ribosomal frameshifting is as high as 25% to 30%; however, the *in vivo* frequency in infected cells remains to be quantitated. It is thought that the role of programmed frameshifting is to provide a fixed ratio of translation products for assembly into a macromolecular complex.⁴⁵⁷ It is also possible that frameshifting forestalls expression of the enzymatic products of rep 1b until the products of rep 1a have prepared a suitable environment for RNA synthesis.

Polypeptides pp1a (440–500 kDa) and pp1ab (740–810 kDa) are autoproteolytically processed into mature products that are designated nsp1 to nsp16 (except for the gammacoronaviruses, which do not have a counterpart of nsp1). From work begun with early studies of MHV,^{134,135,525} complete processing schemes have now been solved for replicases of multiple coronaviruses representing all three genera^{659,661} (see Fig. 28.7B). Processing also generates many long-lived partial proteolytic products, which may have functional importance. There are two types of polypeptide cleavage activity.^{17,358} One or two papain-like proteases (PL^{pro}), which are situated in nsp3, carry out the relatively specialized separation of nsp1, nsp2, and nsp3. The main protease (M^{pro})—nsp5—performs the remaining 11 cleavage events. M^{pro} is often designated the 3C-like protease (3CL^{pro}) to point out its distant relationship to the 3C proteins of picornaviruses. Several crystal structures have been determined for PL^{pro} and M^{pro} of SARS-CoV and other

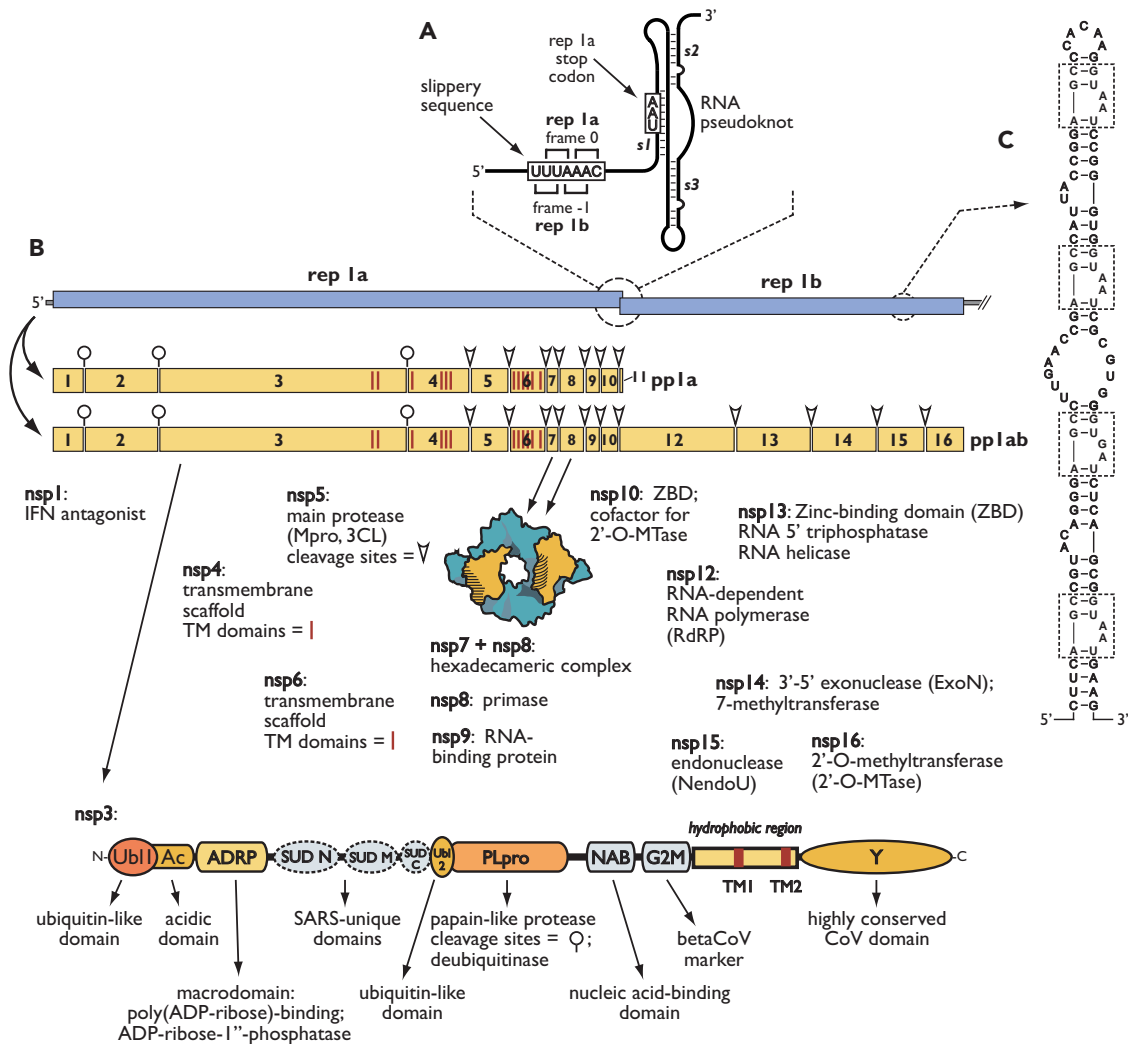


FIGURE 28.7. Coronavirus replicase gene and protein products. **A:** Ribosomal frameshifting elements of the SARS-CoV *replicase* gene. Pseudoknot stems are indicated as s1, s2, and s3. **B:** Polyprotein pp1a and pp1ab processing scheme for alpha- and betacoronaviruses. The gammacoronavirus processing scheme is identical, except for the absence of nsp1. Known functions and properties of nsp1 through nsp16 are listed; nsp11 is an oligopeptide generated when ribosomal frameshifting does not occur. Transmembrane domains in nsp3, nsp4, and nsp6 are indicated by red vertical lines. The nsp3 schematic shown is for SARS-CoV⁴¹⁴; some modules differ in other coronaviruses. **C:** The RNA packaging signal located in the nsp15-encoding region of the MHV genome.⁸¹ This element is found only in a subset of the betacoronaviruses (MHV, betacoronavirus 1, and HCoV-HKU1); repeat units are boxed. SARS-CoV, severe acute respiratory syndrome coronavirus; MHV, mouse hepatitis virus; HCoV, human coronavirus.

coronaviruses,^{9,469,612,631} and these enzymes present attractive targets for antiviral drug design.^{468,633,634}

The processed nsps assemble to form the coronavirus replicase, which is also referred to as the replicase-transcriptase complex (RTC).⁶⁶⁰ The challenge of defining the roles of the many nsp components of the RTC was initially addressed by foundational studies in bioinformatics,^{196,317} which is a discipline that continues to inform the analysis of this intricate molecular machinery.^{414,521} Besides PL^{pro} and M^{pro}, the products of rep 1a contain several activities that establish cellular conditions favorable for infection. Some of these are directly linked to RNA synthesis. Others are nonessential for viral replication in tissue culture; however, they can have major effects on virus–host interactions (see the Immune Response and Viral Evasion

of the Immune Response section). The very first polyprotein product—nsp1—exhibits a broad repertoire of antagonistic activities that selectively inhibit host protein synthesis and IFN signaling.^{230,258,259} By contrast, nsp2 is completely expendable and, as yet, has no demonstrated function.¹⁹⁹

Nsp3 is by far the largest of the RTC proteins. It consists of a concatenation of individual structural modules that are arranged as globular domains separated by flexibly disordered linkers⁴¹⁴ (see Fig. 28.7B). At the amino terminus of nsp3 are ubiquitin-like (Ubl1) and acidic (Ac) domains⁵⁰⁶ that interact with the SR region of the N protein.²³⁷ It is proposed that this interaction tethers the genome to the assembling RTC to allow formation of the initiation complex for RNA synthesis. As mentioned earlier, located within nsp3 are one (in SARS-CoV and

gammacoronaviruses) or two PL^{pro} modules (in most other coronaviruses). In addition to protease activity, PL^{pro} domains possess deubiquitinase activity,^{341,469,612} which forms another part of the viral arsenal that counters host innate immunity.^{136,174} A highly conserved domain of nsp3 has adenosine diphosphate-ribose-1"-phosphatase (ADRP) and poly(adenosine diphosphate [ADP]-ribose)-binding activities,^{152,494} which, although nonessential for replication, help confer resistance to host defenses.^{158,297} At the C-terminus of nsp3 is a conserved region, designated the Y domain, containing three metal-binding clusters of cysteine and histidine residues.^{414,662} The potential functions of other domains of nsp3 (NAB, G2M, SUD),^{73,414,507,521} which appear only in various subsets of coronaviruses, remain to be elucidated.

Notably, the rep 1a products nsp3, nsp4, and nsp6 each contain multiple transmembrane helices that anchor the RTC to intracellular membranes.^{262,424} These proteins also appear to be responsible for remodeling cellular membranes to form structures that are dedicated to viral RNA synthesis.^{92,178} Recent cryo-electron tomographic imaging has revealed an extensive network of convoluted membranes, double-membrane vesicles (DMVs), and vesicle packets, all continuous with the ER, induced by coronavirus infection²⁷⁷ (Fig. 28.8). Anchorage and compartmentalization of the RTC are thought to provide a scaffold for recruitment of soluble nsps, to offer protection from ribonucleases, and to sequester double-stranded viral

RNA intermediates that might activate host innate immunity (see the Immune Response and Viral Evasion of the Immune Response section).

The most C-terminal rep 1a products are nsp7 through nsp10, a cluster of essential small proteins.¹³¹ Structural studies have revealed that two of these—nsp7 and nsp8—form a hexadecameric supercomplex with a central channel large enough to accommodate double-stranded RNA.⁶⁵¹ This formidable assembly has thus been proposed to act as a processivity clamp for the RNA polymerase. Nsp9 is a single-stranded RNA-binding protein,^{151,553} and nsp10 defines a novel structural class of zinc finger proteins.^{257,548}

The processed products encoded by rep 1b contain several well-studied enzymatic activities, including many that are common to all positive-strand RNA viruses. Most prominent in this latter class is the coronavirus RNA-dependent RNA polymerase (RdRp), which is contained in nsp12. Sequence alignment and homology modeling indicate that nsp12 has the fingers, palm, and thumb domains characteristic of several viral RdRps and reverse transcriptases⁶²⁸; however, to date, this protein has proven refractory to structural determination. Additionally, nsp12 has an unusually large NTD, at least part of which mediates targeting to the RTC.⁵² Coronavirus RdRp activity, *in vitro*, is primer dependent.^{83,560} Remarkably, a second RdRp activity resides in nsp8 and is capable of synthesizing short RNA oligomers.²⁴⁰ Nsp8 is thus the optimal candidate for the requisite primase. Another enzyme crucial to RNA synthesis is the helicase of nsp13. This activity unwinds RNA duplexes with a 5' to 3' polarity, suggesting that its role is to prepare the template ahead of the RdRp.^{247,248} The nsp13 helicase has an amino-terminal zinc finger domain that is found only in nidoviruses.⁵¹⁰

Like many RNA viruses, coronaviruses contain machinery capable of catalyzing multiple steps of the pathway for synthesis of the 5'-terminal cap structure of mRNA. An RNA 5'-triphosphatase, which would be required for the first step, is yet another property of nsp13.^{247,248} Intriguingly, a guanylyltransferase has thus far not been identified among the nsps. The nsp14 C-terminus and nsp16, respectively, harbor N7-methyltransferase and 2'-O-methyltransferase activities.^{82,123} These enzymes operate in an obligatory sequential manner, with guanosine-N7 methylation preceding ribose-2'-O methylation. Activation of the nsp16 methyltransferase requires nsp10 as a cofactor, and the crystal structure of a heterodimer of these two proteins suggests that nsp10 serves as a platform to stabilize nsp16.^{43,122} Genetic evidence also implicates nsp10 as a regulator of polyprotein processing by the nsp5 M^{pro}.¹³⁸

Finally, there are two rep 1b-encoded activities that are not found outside the order *Nidovirales*^{194,521}; surprisingly, both are ribonucleases. The first is an endonuclease, designated NendoU, which resides in nsp15. NendoU hydrolyzes both single- and double-stranded RNA and specifically cleaves downstream of uridylate residues, producing 2'-3' cyclic phosphates.^{33,246} Although it bears homology to XendoU, an enzyme involved in small nucleolar RNA (snoRNA) processing, the potential role of NendoU in coronavirus RNA synthesis is not clear. It is also unresolved whether NendoU is essential or if lethal mutations constructed in nsp15 affect some other function of that protein.^{246,260} The second activity is ExoN, a 3'-5' exonuclease that is associated with the amino-terminal portion

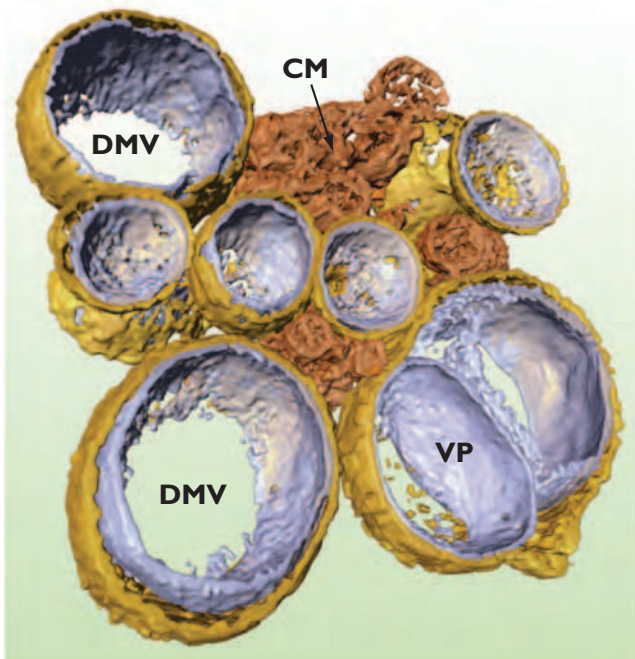


FIGURE 28.8. Membranous compartments for RNA replication and transcription induced by coronavirus infection. Shown is a cryo-electron tomographic reconstruction of the network of intracellular membrane rearrangements found in SARS-CoV-infected Vero cells. There are three types of structures: convoluted membranes (CM), which are the major sites of nsp accumulation; double-membrane vesicles (DMV), which appear to be the sites of active RNA synthesis; and vesicle packets (VP), which are formed by the merger of DMV. (From Knoops K, Kikkert M, Worm SH, et al. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biol* 2008;6:e226.)

of nsp14.³⁹⁵ This enzyme is not essential for viral replication; however, nsp14 mutants have a greatly enhanced mutation rate, supporting the notion that ExoN provides a proofreading function for the coronavirus RdRp.^{149,150} Such a corrective activity may be critical for maintenance of the stability of the exceptionally large coronavirus genome.

Viral RNA Synthesis

Expression and assembly of the RTC sets the stage for viral RNA synthesis (see Fig. 28.6), a process resulting in the replication of genomic RNA and the transcription of multiple subgenomic RNAs (sgRNAs).^{299,433,577} The latter species serve as mRNAs for the genes downstream of the *replicase* gene. Each sgRNA consists of a leader RNA of 70 to 100 nucleotides, which is identical to the 5' end of the genome, joined to a body RNA, which is identical to a segment of the 3' end of the genome. The fusion of the leader RNA to body RNAs occurs at short motifs—TRSs—examples of which are listed in

Figure 28.9. Like the genome, the sgRNAs have 5' caps and 3' polyadenylate tails. Together, these transcripts form a 3'-nested set—the single most distinctive feature of the order *Nidovirales*.^{157,194} Synthesis of both genomic RNA and sgRNAs proceeds through negative-strand intermediates.^{24,509} The negative sense RNAs, which possess 5' oligouridylylate tracts²²⁰ and 3' antileaders,⁵⁰⁸ are roughly a tenth to a hundredth as abundant as their positive sense counterparts.

At their 5' and 3' termini, coronavirus genomes contain *cis*-acting RNA elements that allow their selective recognition as templates for the RTC and play essential roles in RNA synthesis (see Fig. 28.9). The initial localization of these elements was carried out in studies of defective interfering (DI) RNAs, which are extensively deleted genomic variants that propagate by competing for the viral RNA synthesis machinery.^{69,371,393,445,575} Manipulations of natural and artificially constructed DI RNAs, evaluated by transfection into helper-virus-infected cells, made possible the mapping of sequences

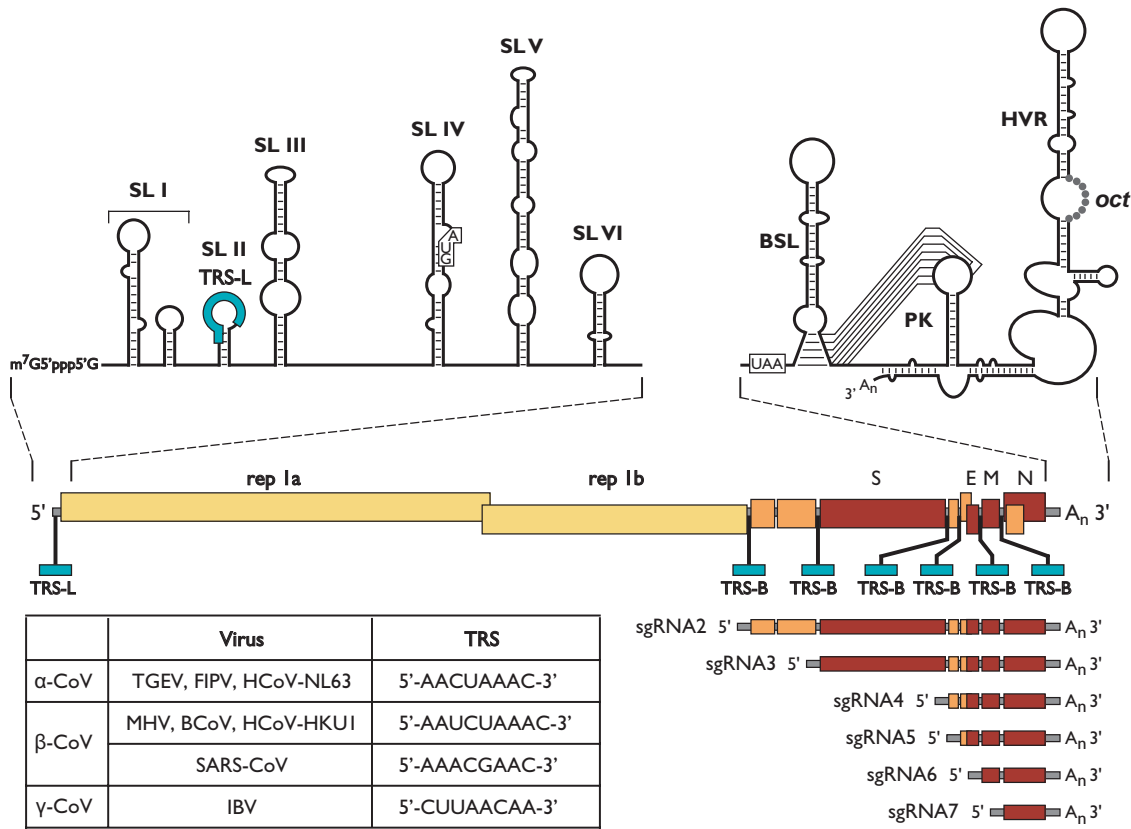


FIGURE 28.9. Coronavirus RNA synthesis. Shown are a schematic of MHV genomic RNA and the nested set of transcribed subgenomic RNA species that are a defining feature of the order *Nidovirales*. The leader and body copies of the TRS (TRS-L and TRS-B, respectively) are denoted by green boxes. At the left are listed examples of consensus TRSs that have been experimentally confirmed^{462,463,531,562}; the inferred TRSs of other coronaviruses are identical or highly similar to these. Expanded regions above the genome depict *cis*-acting RNA structures at the genome termini. The structures shown are those characterized for MHV.^{190,202,346,667} Homologous structures exist in the BCoV^{53,622} and SARS-CoV genomes,^{192,261} and counterparts of some of these elements appear in other coronaviruses.^{80,107,605} The 5' expanded region represents the 210-nt 5' UTR and the first 140 nt of the *rep 1a* gene; the elements shown are SLs I through VI, numbered as originally described for BCoV.^{53,202} TRS-L is denoted in green in SL II; the start codon of *rep 1a* is boxed in SL IV. The 3' expanded region represents the 301-nt 3' UTR. The elements shown are the bulged stem loop (BSL), the pseudoknot (PK), the hypervariable region (HVR), and the conserved coronavirus octanucleotide motif (*oct*); the stop codon for the upstream *N* gene is boxed. MHV, mouse hepatitis virus; TRS, transcription-regulating sequence; BCoV, bovine coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; UTR, untranslated region; nt, nucleotide; SL, stem loop.

that are critical for the replication and transcription of DI RNA and, presumably, also for genomic RNA.^{45,379} More recently, *cis*-acting RNA elements have been dissected through reverse genetics of the intact viral genome, complemented by *in vitro* biochemical and structural analyses. The most completely characterized structures and sequences are those of the betacoronaviruses MHV, BCoV, and SARS-CoV (see Fig. 28.9).

At the 5' end of the genome, the elements that participate in viral RNA synthesis extend well beyond the 5' UTR into the replicase coding region, making up a set of seven stem loops.^{53,202,346,467} One of these displays the leader copy of the TRS (TRS-L) in its loop, and another sequesters the start codon of the *rep 1a* gene within its stem. Many, but not all, of these defined structures can be exchanged among the genomes of different betacoronaviruses.^{202,261} Significantly, functional analyses have shown that either the stability^{202,346} or the instability³²⁹ of a given RNA stem can be critical for viral fitness, suggesting that these structures operate in a dynamic manner during RNA synthesis.

At the 3' end of the genome, *cis*-acting RNA elements are confined entirely to the 3' UTR¹⁹⁰ and are functionally interchangeable among the betacoronaviruses.^{192,228,622} These elements consist of a bulged stem loop²²⁸ and an adjacent pseudoknot⁶⁰⁵ that have each been demonstrated to be essential for viral replication. Further downstream is a hypervariable region, which is completely dispensable for viral replication but yet harbors 5'-GGAAGAGC-3', an octanucleotide motif that is universally conserved in the coronaviruses.^{191,347} Notably, the bulged stem loop and the pseudoknot partially overlap, and they therefore can not fold up simultaneously. The two structures are thus thought to constitute a molecular switch between different steps of RNA synthesis.^{190,227} In addition, the first loop of the pseudoknot forms a duplex with the extreme 3' end of the genome and genetically interacts with the RTC subunits nsp8 and nsp9.⁶⁶⁷ On this basis, a mechanism has been proposed in which alternate RNA conformations of the 3' UTR facilitate the transition between initiation of negative-strand RNA synthesis by the nsp8 primase and elongation by the nsp12 RdRp. However, this scheme does not yet incorporate potential cross talk between the 5' and 3' ends of the genome,³²⁹ and much remains to be learned about how *cis*-acting RNA elements are recognized by, and cooperate with, the RTC.

A central issue in coronavirus RNA synthesis is how the leader RNA becomes attached to the body segments of the sgRNAs. It became clear from early work that transcription involves a discontinuous process. Ultraviolet (UV) transcriptional mapping demonstrated that sgRNAs are not processed from a genome-length precursor,^{250,537} and mixed infections with two different strains of MHV showed that leader RNAs could reassort between separate sgRNA body segments.³⁷² It was also clearly established by DI RNA studies, and later confirmed by genomic reverse genetics,^{527,664} that the TRSs play key roles in sgRNA formation. The efficiency of fusion at an individual body TRS (TRS-B) is, in part, governed by how closely it conforms to the leader TRS (TRS-L).^{217,369,576} Nonetheless, factors such as the local sequence context of the TRS and the position of the TRS relative to the 3' end of the genome also profoundly influence transcription levels.^{286,429,580}

Originally, the leader-to-body fusion event was envisioned to occur by a leader-primed mechanism during positive-strand RNA synthesis.^{298,300,652} However, there is now broad,

although not universal, agreement that fusion takes place through discontinuous extension of negative-strand RNA synthesis.^{433,496,498,664} In this model, both genomic and subgenomic negative-strand RNAs are initiated by the RTC at the 3' end of the (positive-strand) genome template (Fig. 28.10). A pause in RNA synthesis occurs when the RdRp crosses a TRS-B. At this point, the RdRp may continue to elongate the growing negative strand. Alternatively, it may switch to the leader at the 5' end of the genome template, guided by the complementarity between the 3' end of the nascent negative strand and the TRS-L of the genome. The resulting negative-strand sgRNA, in partial duplex with positive-strand gRNA, then serves as the template for synthesis of multiple copies of the corresponding positive-strand sgRNA.

Leader-to-body fusion during negative-strand synthesis is amply supported by accumulated experimental results with coronaviruses and the closely related arteriviruses. First, as necessitated by the model, negative-strand sgRNAs contain antileaders at their 3' ends.⁵⁰⁸ Second, in infected cells, there exist transcription intermediates containing negative-strand sgRNAs in association with the genome. These complexes actively participate in transcription^{24,497} and can be biochemically separated from replication intermediates containing genome-length negative-strand RNAs.⁵⁰⁰ Finally, as would be predicted for discontinuous negative-strand synthesis, engineered (or naturally occurring) variant nucleotides incorporated into the TRS-B, rather than the TRS-L, end up in the leader-body junction of the resulting sgRNA.^{238,434,579,664} There remains, however, considerable further work to be done to elucidate the details of the model.^{433,498} It is not clear how the transcribing RdRp might continuously monitor the ability of its nascent product to base pair to the TRS-L. Additionally, the synthesis of genome-length negative strands would require the RdRp to bypass all of the TRS-B sites in the genome template. This may come about through a stochastic process, or it may be actively promoted by some RTC component under certain conditions. These and other questions will need to be addressed, possibly with the aid of a robust *in vitro* viral RNA synthesizing system.⁵⁷⁸ Such a system may also be decisive in assessing the potential roles of host factors in transcription and replication. Several cellular proteins, including hnRNP A1,^{327,512,513} polypyrimidine tract-binding protein,^{326,526} mitochondrial aconitase,⁴⁰⁴ and polyadenylate-binding protein,⁵³² have been proposed to take part in coronavirus RNA synthesis, mainly based on their ability to bind *in vitro* to genomic RNA segments. Because many putative host factors also play critical or essential roles in normal cellular functions, it has been difficult to convincingly demonstrate their specific involvement in viral processes. As yet, only a single candidate host factor has been shown to be required for *in vitro* viral RNA synthesis.⁵⁷⁸

In addition to its central role in sgRNA formation, template switching is also at the heart of RNA recombination—another prominent feature of coronavirus RNA synthesis. Significant rates of both homologous and nonhomologous RNA recombination have been found among selected and unselected markers during the course of infection.^{266,267,268,370} It is presumed, but remains to be formally demonstrated, that coronavirus RNA recombination results from a copy-choice mechanism, as originally established for poliovirus.²⁷³ In MHV, recombination takes place at an estimated frequency of 1% per 1.3 kb (almost 25% over the entire genome)—the

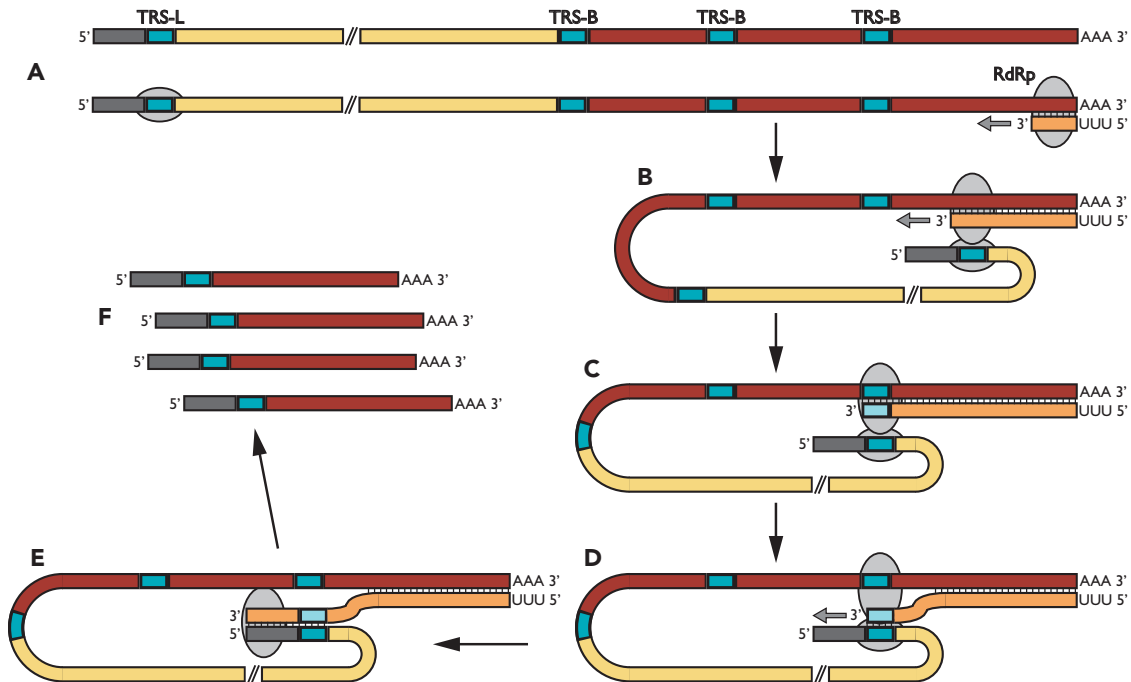


FIGURE 28.10. Coronavirus transcription through discontinuous extension of negative-strand RNA synthesis.^{496,498,664} **A, B:** Negative-strand sgRNA synthesis initiates at the 3' end of the positive-strand genomic RNA template. In the version of the model shown here, the genomic template loops out in such a way as to allow a component of the RTC to constantly monitor the potential complementarity of the 3' end of the nascent negative-strand RNA with the TRS-L. **C:** Transcription pauses at a TRS-B. At this point, elongation may resume, thereby bypassing the TRS-B. **D:** Alternatively, the nascent negative strand may switch templates, binding to the TRS-L. **E:** Resumption of elongation results in completion of synthesis of an antileader-containing negative-strand sgRNA. **F:** The resulting complex of genome and negative-strand sgRNA acts as template for the synthesis of multiple copies of the corresponding positive-strand sgRNA. sgRNA, subgenomic RNA; RTC, replicase-transcriptase complex; TRS, transcription-regulating sequence. (Adapted from Zúñiga S, Sola I, Alonso S, et al. Sequence motifs involved in the regulation of discontinuous coronavirus subgenomic RNA synthesis. *J Virol* 2004;78:980–994.)

highest rate observed for any RNA virus.²² On a fine scale, the sites of crossover are random,¹⁹ although selective pressures can generate the appearance of local clustering of recombinational hot spots.¹⁸ This facility for RdRp strand switching may make a major contribution to the ability of the huge coronavirus genome to evolve and to circumvent the accumulation of deleterious mutations. It also serves as the basis for targeted RNA recombination (see the Coronavirus Genetics section).

Assembly and Release of Virions

The immediate outcome of transcription is to enable translation of the proteins that build progeny viruses. The membrane-bound proteins M, S, and E are initially inserted into the ER; from there, they transit to the site of virion assembly, the endoplasmic reticulum–Golgi intermediate compartment (ERGIC).^{275,285,563} Here, nucleocapsids composed of progeny genomes encapsidated by N protein coalesce with the envelope components to form virions, which bud into the ERGIC^{117,222,378} (see Fig. 28.6).

Coronavirus assembly occurs through a network of cooperative interactions, most of which involve M protein. However, despite its central role, M is not assembly competent by itself. Expression of M protein alone does not result in virion-like structures, and M traverses the secretory pathway beyond the budding site, as far as the *trans*-Golgi.^{275,362,363,489} The first

virus-like particle (VLP) systems developed for coronaviruses led to the key finding that co-expression of E protein with M protein is sufficient to yield the formation of particles that are released from cells and appear morphologically identical to coronavirus envelopes.^{37,582} More recently, it has been shown that the additional co-expression of N protein substantially increases the efficiency of VLP formation^{38,519} and can even compensate for mutational defects in M.¹⁵ Other viral structural proteins, in particular S protein, are gathered into virions but are not specifically required for the assembly process. Because virions and VLPs contain very little E protein, this indicates that lateral interactions between M molecules provide the driving force for envelope morphogenesis. Investigations of the ability of M protein mutants to support VLP assembly concluded that M–M interactions occur via multiple contacts throughout the molecule, especially between the transmembrane domains.^{114,120} Recent cryo-electron tomographic reconstructions of whole virions suggest that the M protein forms dimers that are maintained through multiple monomer–monomer contacts, while dimer–dimer interactions occur among the globular endodomains.⁴¹⁵

It remains enigmatic how E protein critically assists M in envelope formation. Like M, E protein by itself moves to a compartment past the ERGIC^{93,100}; however, co-expression or infection somehow secures localization of M and E at the

budding site. Some evidence suggests that E protein promotes assembly by inducing membrane curvature.^{100,166,465} Other work indicates a role for E in maintaining M protein in an assembly-competent state by preventing its nonproductive aggregation—a function that crucially depends on palmitoylation of E.³⁸ Such a chaperone-like role would be consistent with demonstrations that diverse heterologous E proteins, and even truncated versions of M protein, can functionally replace E protein in MHV.^{293,294} Finally, there are reports that point to a need for E protein to facilitate the release of assembled virions from infected cells.^{364,427} These roles are not mutually exclusive, and some recent studies have begun to assign individual functions to various regions of the E molecule. The C-terminal endodomain of the IBV E protein governs Golgi localization^{100,101} and when linked to a heterologous transmembrane domain can support VLP and virion assembly.^{364,491} Conversely, the transmembrane domain of E alters the host secretory pathway in a way that promotes virus release.⁴⁹¹ This latter effect is potentially a consequence of the putative ion channel properties of the E transmembrane domain^{449,609,610,638}; however, it is unresolved whether native E protein acts as an ion channel at intracellular membranes *in vivo*.⁴²⁰

The dispensability of S protein for VLP formation is consistent with earlier observations that spikeless (noninfectious) virions were formed by infected cells treated with the glycosylation inhibitor tunicamycin^{224,487} or by cells infected with particular S mutants.^{360,474} S protein thus appears to play a passive role in assembly; however, during its passage through the secretory pathway, it is captured by M protein for virion incorporation.^{387,426} For some S proteins, localization at or near the budding compartment is abetted by targeting signals contained in the endodomain.^{353,386,611} The S endodomain is also the region of the protein that interacts with M during assembly.^{39,636} Conversely, the ability of M protein to interact with S maps to a locus close to the C-terminus of the M endodomain¹¹⁸ (see Fig. 28.3).

Virion assembly is completed by condensation of the nucleocapsid with the envelope components. This is brought about principally by N and M protein interactions, which have been mapped to domain 3 of N^{236,585} and the extreme C-terminus of the M endodomain^{162,295} (see Fig. 28.3). These interacting regions likely account for the thread-like connections that have been visualized between the M protein endodomain and the nucleocapsid in virion reconstructions.^{21,415} Nucleocapsid formation is presumed to be concomitant with genome replication; however, the details of how the nucleocapsid traffics to the budding compartment are not known. It is also not well understood how coronaviruses selectively package genomic RNA from among the many positive- and negative-strand viral RNA species that are synthesized during infection. DI RNA analyses have mapped the genomic packaging signal of MHV to a small span of RNA sequence embedded in the region of the *replicase* gene that encodes nsp15^{169,373,575} (see Fig. 28.7C). Highly homologous structures exist in the genomes of BCoV and HCoV-HKU1.^{81,96} However, for most coronaviruses, including SARS-CoV,²⁵⁶ packaging signals are clearly not found at the same locus, and the relevant structures for these viruses may occur at a large distance, near the 5' ends of their respective genomes.^{80,161} The mechanism by which the MHV packaging signal operates is undetermined. Some studies have shown that it is specifically bound by N protein,^{96,396} although

other work demonstrates that M protein, in the absence of N, acts as the discriminatory factor for packaging signal recognition.^{406,409}

Following assembly and budding, progeny virions are exported from infected cells by transport to the plasma membrane in smooth-walled vesicles and are released by exocytosis. It remains to be more clearly defined whether coronaviruses follow the constitutive pathway for post-Golgi transport of large cargo or, alternatively, if specialized cellular machinery must be diverted for their exit.²²² For some coronaviruses, but not others, a fraction of S protein that has not been assembled into virions transits to the plasma membrane, where it can mediate fusion between infected cells and adjacent, uninfected cells. This leads to the formation of large, multinucleate syncytia, enabling the spread of infection by a means not subject to neutralization by antibody. For MHV, cell–cell fusion depends on S1–S2 cleavage carried out by a furin-like protease late in infection.¹¹⁹ However, this form of proteolytic activation of S does not appear to affect virus–cell fusion that occurs at the initiation of infection. Similarly, the SARS-CoV S protein has different proteolytic requirements for cell–cell and virus–cell fusion.^{168,516} On the opposite side of the membrane from the cleaved ectodomain, the cysteine-rich region of the S protein endodomain also plays a critical role in cell–cell fusion^{36,68,636}; specifically, this has been shown to depend on the palmitoylation of a subset of endodomain cysteine residues.³⁸⁸

PATHOGENESIS AND PATHOLOGY OF CORONAVIRUS INFECTIONS

General Principles

Most coronaviruses spread to susceptible hosts by respiratory or fecal–oral routes of infection, with replication first occurring in epithelial cells (Table 28.3). Some, including HCoV-OC43, HCoV-229E, and porcine respiratory coronavirus (PRCoV), replicate principally in respiratory epithelial cells, where they produce virus and cause local respiratory symptoms. Other coronaviruses, including TGEV, BCoV, porcine hemagglutinating encephalomyelitis virus (PHEV), CCoV, FeCoV, and enteric strains of MHV, infect epithelial cells of the enteric tract. Some of these viruses, such as TGEV, cause diarrhea that is particularly severe, and sometimes fatal, in young animals.⁴⁹² Inapparent enteric infection of adult animals maintains the virus in the population.⁹⁸ In addition to local infection of the respiratory or enteric tracts, several coronaviruses cause severe disease. For example, SARS-CoV spreads from the upper airway to cause a severe lower respiratory tract infection, whereas FIPV spreads systemically to cause a generalized wasting disease in felines.^{439,448} Rat coronavirus strains cause respiratory infection or sialodacryoadenitis owing to infection of the salivary and lacrimal glands⁴⁴⁶ but can also interfere with reproduction by infecting the female urogenital tract.⁵⁷¹ PHEV of swine predominantly causes enteric infection but is also neurotropic.³⁸⁹ Infection spreads to nerves that innervate the stomach of infected piglets and prevents gastric emptying, resulting in vomiting and wasting disease. The ability to cause localized versus systemic disease is mirrored in polarized tissue culture cells. Thus, coronaviruses such as MHV, which can cause systemic disease, enter the apical side of cells and exit the basolateral side, whereas others, such as HCoV-229E, which causes only a localized infection, enter and

TABLE 28.3 Representative Coronaviruses and Associated Diseases

Virus	Host species	Sites of infection	Clinical disease
Alphacoronaviruses			
CCoV	Canine	GI tract	Gastroenteritis
FeCoV	Felidae	GI tract, respiratory	Gastroenteritis
FIPV	Felidae	Systemic disease	Peritonitis, wasting disease
HCoV-229E	Human	Respiratory	Upper respiratory tract infection
HCoV-NL63	Human	Respiratory	Upper respiratory tract infection, croup
PEDV	Pig	GI tract	Gastroenteritis
TGEV	Pig	GI tract, respiratory	Gastroenteritis
BatCoV	Bat	GI tract, respiratory	Unknown
Rabbit CoV	Rabbit	Heart, GI tract, respiratory	Enteritis, myocarditis
Betacoronaviruses			
BCoV	Bovine, ruminants	GI tract, respiratory	Enteritis, upper and lower respiratory tract infection
HCoV-OC43	Human	Respiratory	Upper respiratory tract infection
HCoV-HKU1	Human	Respiratory	Upper and lower respiratory tract infection
MHV	Mouse, rat	GI tract, liver, brain, lungs	Gastroenteritis, hepatitis, encephalitis, chronic demyelination
PHEV	Pig	Respiratory, brain	Vomiting, wasting, encephalomyelitis
RCoV	Rat	Respiratory, salivary and lachrymal glands, urogenital tract	Respiratory tract infection, metritis, sialodacryoadenitis
SARS-CoV	Human	Respiratory, GI tract	Pneumonia (SARS)
BatCoV	Bat	GI tract, respiratory	Unknown
Gammacoronaviruses			
IBV	Chicken	Respiratory, kidney	Bronchitis, nephritis
TuCoV	Turkey	GI tract	Gastroenteritis

CCoV, canine coronavirus; GI, gastrointestinal; FeCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; HCoV, human coronavirus; PEDV, porcine epidemic diarrhea virus; TGEV, transmissible gastroenteritis virus; BatCoV, bat coronavirus; CoV, coronavirus; BCoV, bovine coronavirus; MHV, mouse hepatitis virus; PHEV, porcine hemagglutinating encephalomyelitis virus; RCoV, rat coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS, severe acute respiratory syndrome; IBV, infectious bronchitis virus; TuCoV, turkey coronavirus.

exit the cell apically.^{481,482,595} Specific examples are described in more detail later.

Animal Coronavirus Infections

Several coronavirus infections have been extensively studied in their natural hosts. Here, we will focus on murine and feline coronavirus infections.

Mouse Hepatitis Virus

MHV, which until the advent of SARS was the most widely studied coronavirus, causes enteric, hepatic, and neurologic infections of susceptible strains of rodents. Remarkably, closely related strains of MHV, all of which use the same host cell receptor for entry,⁶⁰⁶ infect different organs. Enteric strains, such as MHV-Y and MHV-RI, are a major problem in animal research facilities.⁹⁸ These viruses spread within infected colonies to young, uninfected animals. They do not generally cause symptomatic disease but may subtly impair the host immune response to other pathogens and immunological stimuli.^{98,540} Studies of MHV pathogenesis predominantly use the neurotropic JHM and A59 strains of virus (JHM virus [JHMOV] and MHV-A59), in part because they cause a demyelinating encephalomyelitis with similarities to the human disease multiple sclerosis (MS). Originally isolated from a mouse with hind-limb paralysis, JHMOV became progressively more virulent on

passage in mice.^{16,75} The most virulent strains of JHMOV cause rapidly fatal acute encephalitis with widespread neuronal infection.⁶⁰⁰ Subsequently, most studies have used either attenuated JHMOV variants or the mildly neurovirulent MHV-A59 strain for studies of demyelination. Infection with these viruses results in minimal infection of neurons, with oligodendrocytes, microglia, and astrocytes commonly infected.^{167,276,313} Myelin destruction occurs during the process of virus clearance from infected glia.⁵⁹⁴ Initial studies suggested that demyelination resulted from virus-mediated lysis of oligodendrocytes.^{304,600} However, more recent studies show that demyelination is largely immune mediated. In support of this, irradiated mice or congenitally immunodeficient mice (mice with severe combined immunodeficiency [SCID]) or with a disrupted recombination activation gene [*RAG*^{-/-}] do not develop demyelination after infection with JHMOV. When these mice, which lack T and B cells, are reconstituted with virus-specific T cells, demyelination rapidly develops^{593,620} (Fig. 28.11). Demyelination is accompanied by infiltration of macrophages and activated microglia into the white matter of the spinal cord.⁶²¹ Little is known, however, about how macrophages and microglia are actually attracted to the spinal cord or about the nature of the signals that cause these cells to phagocytose infected myelin. Both CD4 and CD8 T cells are required for virus clearance from the central nervous system (CNS), with CD8 T cells considered most important in

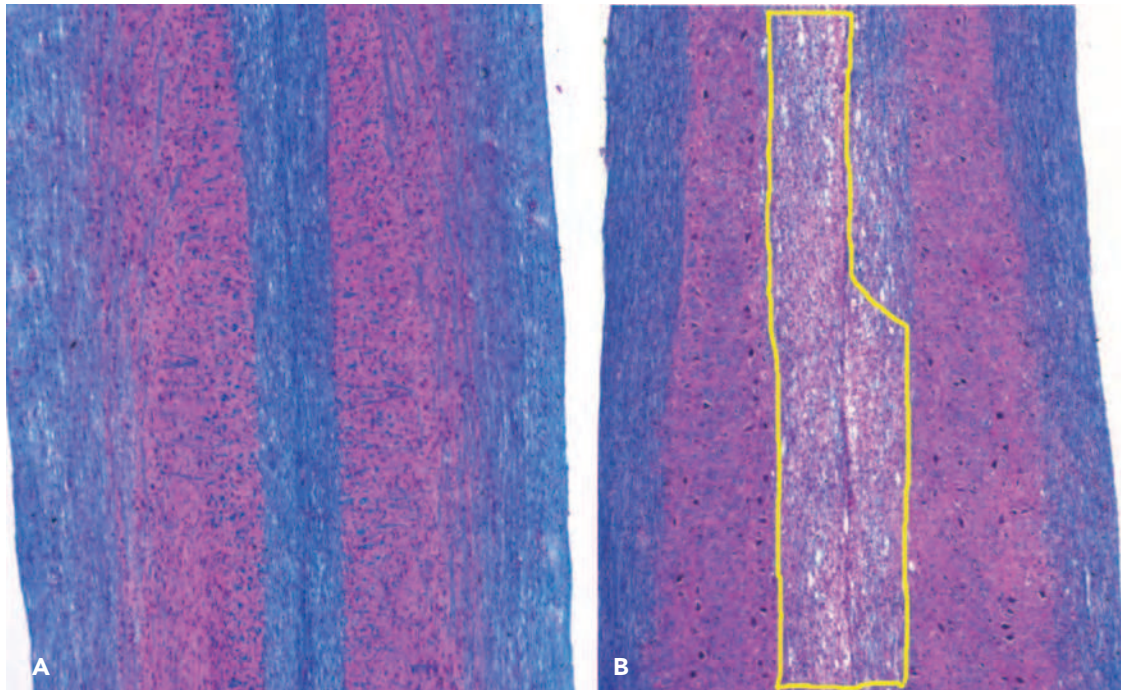


FIGURE 28.11. Immune-mediated demyelination in mice infected with a neurotropic MHV. RAG1^{-/-} mice, lacking T and B cells, were infected with a neurotropic coronavirus as described.⁶²⁰ Four days later, some mice received adoptively transferred spleen cells from a wild-type C57Bl/6 mouse that was previously immunized intraperitoneally with MHV (**B**). All mice were sacrificed 8 days later and analyzed for demyelination (marked with a yellow line in **B**). Demyelination was observed only in mice that received adoptively transferred MHV-immune cells (**B**) and not in those that did not (**A**), showing that myelin destruction is largely mediated by T cells during the process of virus clearance. MHV, mouse hepatitis virus.

this process.⁶⁰⁸ CD8 T cells eliminate virus from infected astrocytes and microglia by perforin-dependent pathways, whereas clearance from oligodendrocytes is IFN- γ dependent.^{340,432} However, T-cell-mediated virus clearance is not complete, and antiviral antibody is required to prevent virus recrudescence.³³⁸ Virus persistence in neonatal mice occurs, in part, because virus variants mutated in an immunodominant CD8 T-cell epitope are selected in specific strains of mice, with subsequent evasion of the cytotoxic T-cell immune response.⁴⁵¹ However, this mechanism of immune evasion has not been detected in older mice that are persistently infected with JHMV. The anti-virus CD4 T-cell response, while critical for virus clearance, is also pathogenic. Partial diminution of this response decreases morbidity and mortality, whereas enhancement of the antiviral CD4 T-cell response increases disease severity.¹¹

Other strains of MHV, including MHV-A59, MHV-2, and MHV-3, infect both the liver and the CNS. Most notably, MHV-3 causes a fulminant hepatitis in susceptible strains of mice and chronic neurologic infections in semisusceptible strains.⁶⁴⁹ In susceptible strains, MHV-3 infects macrophages, resulting in up-regulation of several proinflammatory cytokines, including fibrinogen-like protein 2 (FGL2), a transmembrane procoagulant molecule.⁴³¹ FGL2 is also expressed by Foxp3⁺ regulatory T cells.⁵¹¹ Expression of this molecule results in prothrombin cleavage, with consequent disseminated intravascular coagulation (DIC), hepatic hypoperfusion, and necrosis.³⁷⁵ Levels of FGL2 are better predictors of a fatal outcome than virus titers. It is known that the propensity to develop severe disease occurs at a postentry stage because the MHV-3 receptor,

CEACAM1, is expressed in both resistant and susceptible strains of mice. Like JHMV, MHV-3 also infects the CNS; however, infection of this organ occurs only in strains that do not develop a fulminant hepatitis. MHV-3 does not cause a demyelinating disease but rather ependymitis, hydrocephalus, encephalitis, and thrombotic vasculitis.^{315,589} The pathogenesis of these entities is not well studied but appears to be immune mediated. Unlike most other strains of MHV, MHV-3 directly infects T and B cells, resulting in lymphocyte apoptosis and lymphopenia.³⁰³ Lymphopenia, with consequent immunosuppression, facilitates virus persistence and its immunopathologic consequences.

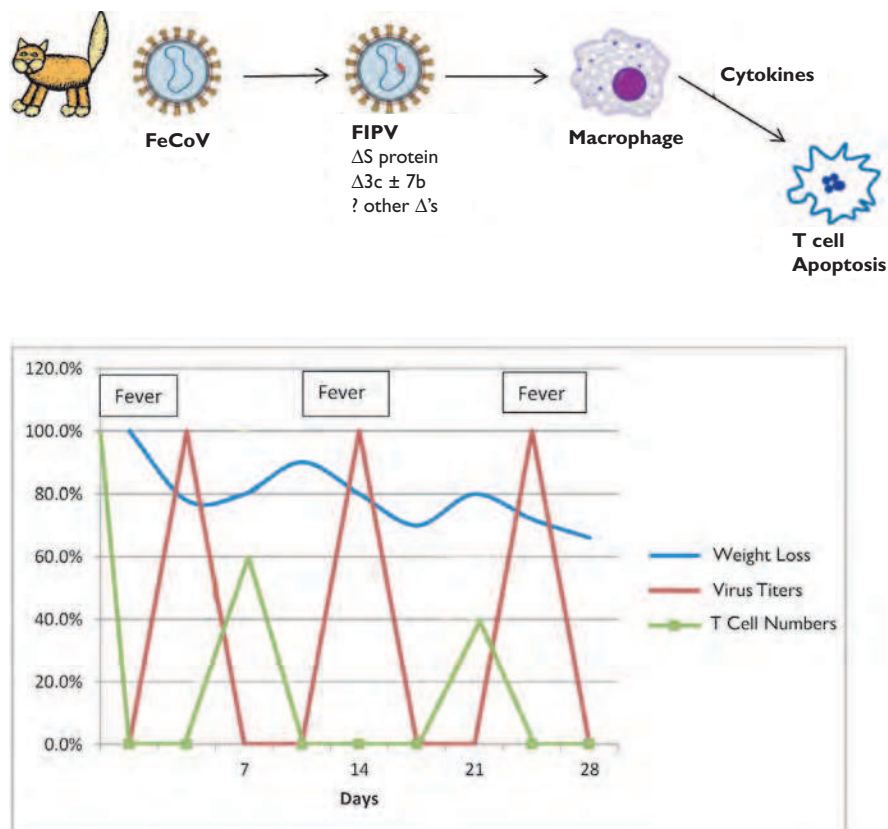
Feline Enteric Coronavirus and Feline Infectious Peritonitis Virus

Feline enteric coronavirus (FeCoV) commonly causes mild or asymptomatic infection in domestic cats and other felines. Two serotypes of FeCoV are recognized, with serotype II strains arising by recombination of serotype I FeCoV with CCoV in dually infected animals.²¹⁶ In some cats infected persistently with FeCoV, mutations in the virus occur, resulting in the development of a lethal disease called *feline infectious peritonitis* (FIP); FIPV is the virulent strain of FeCoV. Virulence correlates with the ability of the virus to replicate in macrophages.¹¹⁰ The nature of the mutations required for transition from FeCoV to FIPV is not well understood, although, at least for serotype II viruses, virulence maps in part to the surface glycoprotein.⁴⁸⁸ This was shown using reverse genetics, in which S proteins from virulent and avirulent strains were swapped and tested for their ability to cause severe disease in cats. FIPV causes a multiphasic

FIGURE 28.12. Recurrent feline infectious peritonitis (FIP). FIP virus—the etiologic agent of FIP—occurs in felines persistently infected with feline coronaviruses.

Upper panels: Mutations in the S glycoprotein and the ORF3b and 7b proteins occur as virus gains the ability to replicate in macrophages. Infected macrophages serve to transport the virus to sites in the host distant from the initial infection. These infected cells also express several cytokines that are believed to contribute to T-cell apoptosis.

Lower panel: Clinical disease is characterized by recurrent bouts of virus replication accompanied by fever and clinical disease. Lymphopenia subsequently occurs as disease progresses. The pattern of disease shown in the figure is representative of progressive disease; however, the rate and extent of recurrence of virus replication, as well as the rate of weight loss and of development of lymphopenia, are variable from animal to animal. (Based on De Groot-Mijnes JD, van Dun JM, van der Most RG, et al. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. *J Virol* 2005;79:1036–1044.)



disease with relapses that result, ultimately, in immunosuppression, weight loss, and death (Fig. 28.12). Each episode is characterized by increased virus replication, fever, and lymphopenia.¹¹² FIPV does not directly infect lymphocytes. Rather, lymphopenia is believed to be a consequence of infection and activation of macrophages and dendritic cells. Subsequent lymphocyte depletion occurs when cells are exposed to high levels of proinflammatory cytokines, such as tumor necrosis factor, released by these infected cells.²⁰⁵ Virus dissemination occurs when infected macrophages traffic throughout the body and are deposited in the vasculature. Infected macrophages provoke a pyogranulomatous reaction, which is responsible for many disease manifestations of FIP, such as peritonitis and serositis. Another consequence of immune dysregulation is hypergammaglobulinemia. Antibody-antigen complex formation commonly occurs in FIPV-infected cats and may contribute to vascular injury.²⁵² However, its precise role in pathogenesis remains uncertain because it is a late manifestation of disease and may make only a minor contribution to disease progression. Neutralizing antibody against the S glycoprotein enhances FIPV infection of macrophages. Enhanced macrophage infection is mediated by virus entry through Fcγ receptors, although virus binding to fAPN—the specific FIPV host cell receptor—is also likely required.¹¹⁰ This phenomenon has been demonstrated *in vitro* using isolated macrophages and also occurs in cats that have been previously immunized with vectors that express the S glycoprotein.⁵⁸¹ FIPV, but not FeCoV, uptake is augmented by neutralizing antibody that contributes to the propensity of FIPV strains to replicate in macrophages. Although the potential occurrence of antibody-enhanced dis-

ease has hindered vaccine development and was raised as a potential difficulty in development of a live attenuated SARS-CoV vaccine, it has never been demonstrated in the natural infection. In fact, cats infected with FeCoV often develop only low antiviral neutralizing antibody titers.²²⁶

Human Coronavirus Infections

Human Coronaviruses, Other Than Severe Acute Respiratory Syndrome Coronavirus, Associated with Respiratory and Enteric Disease

Prior to 2003, HCoVs were primarily considered to be agents of upper respiratory tract disease and to cause little mortality. In general, whereas coronaviruses were readily isolated from infected birds and other animal species, and serially propagated in continuous cell lines, isolation of HCoVs from infected individuals was only rarely achieved.³⁸⁹ HCoV-229E and HCoV-OC43 were isolated from patients with upper respiratory tract infections in the 1960s.^{210,390,570} There are striking differences in extent of genetic variability when isolates of HCoV-OC43 and HCoV-229E are compared. HCoV-229E isolated at geographically distinct locations show little evidence of variability.⁸⁷ In contrast, isolates of HCoV-OC43 isolated from the United States and from France differ in sequence, and virus from the same geographic area but isolated in different years show considerable sequence variations.⁵⁸⁷ The ability of HCoV-OC43 to tolerate mutations probably accounts for its ability to grow in mouse cells and infect the mouse brain³⁸⁹ as well as its ability to cross species (see the Epidemiology section). In contrast, HCoV-229E does not readily cross species

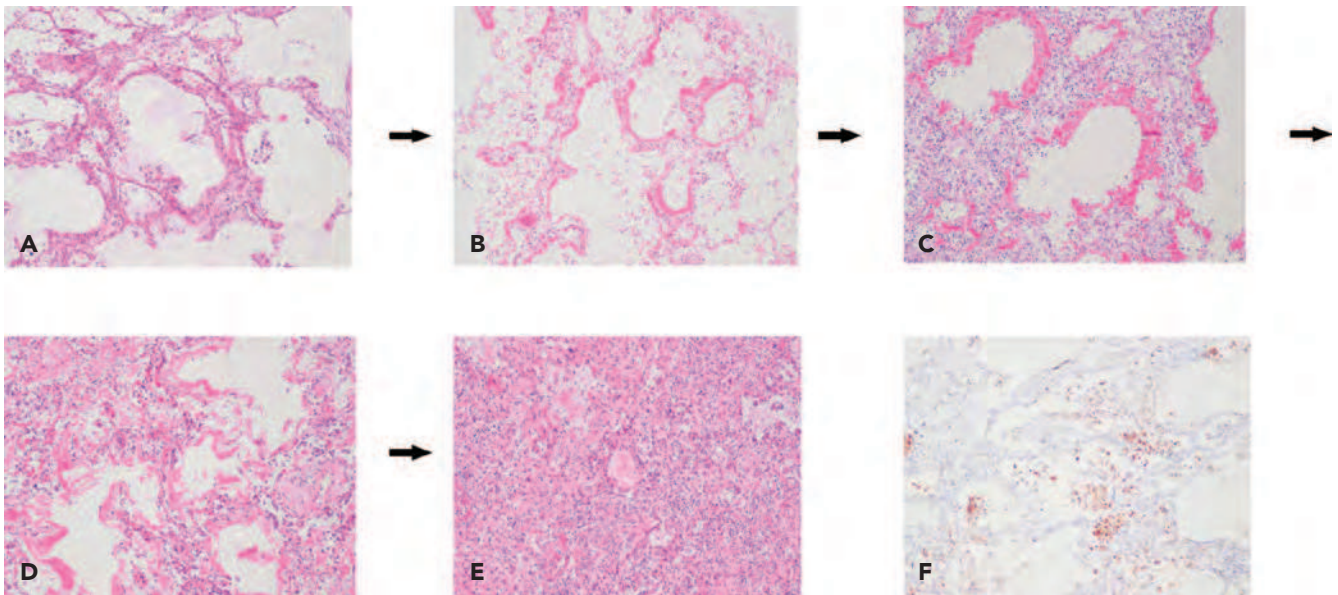


FIGURE 28.13. Pathologic changes in lungs of patients with SARS. Lung samples obtained on autopsy were examined for pathologic changes following SARS-CoV infection. **A–E:** Hematoxylin and eosin stain showing the progression of SARS pneumonia. Early stages of the SARS infection show edema and early hyaline membrane formation (**A**), hyaline membrane formation (**B**), and increased inflammatory cell infiltration and pneumocyte hyperplasia (**C**). As the disease progresses, fibrotic changes become apparent (**D**). Late manifestations include obliteration of the alveolar volume by fibrous tissue, reactive pneumocytes, and inflammatory cells (**E**). **F:** Viral antigen is detected most prominently during early stages of the infection in macrophages and alveolar pneumocytes. Magnification, $\times 100$. SARS, severe acute respiratory syndrome; SARS-CoV, severe acute respiratory syndrome coronavirus. (Courtesy of Dr. John Nicholls, University of Hong Kong.)

and does not infect mice. Even in mice that are transgenic for expression of the HCoV-229E host cell receptor (human aminopeptidase N [hAPN]), the virus does not grow unless mice are also rendered immunodeficient by genetic disruption of the *STAT1* gene.³⁰⁶

Several new HCoVs were isolated from the respiratory tracts of patients in the post-SARS era. HCoV-NL63, which causes mild respiratory disease, displays homology with HCoV-229E.⁴⁶⁰ Phylogenetic analyses suggest that HCoV-NL63 and HCoV-229E diverged approximately 1,000 years ago.⁴⁶¹ A novel feature of HCoV-NL63 is that unlike HCoV-229E, HCoV-NL63 does not use hAPN as a receptor. Rather, infection of cells is mediated by ACE2, the same molecule that is used by SARS-CoV, an unrelated betacoronavirus.^{219,331} However, unlike SARS-CoV, HCoV-NL63 does not use cathepsin L or require endosomal acidification to infect ACE2-expressing cells²³² and does not cause severe respiratory disease. HCoV-HKU1, isolated from an adult patient in Hong Kong with pneumonia,⁶¹⁵ also generally causes mild respiratory disease.

A role for HCoVs in the etiology of the human disease MS was postulated based on the ability of murine coronaviruses to cause chronic demyelinating diseases. Coronavirus-like particles have occasionally been detected in the CNS of patients with MS and have also been isolated from the brains of patients after passage in mice or murine cell lines. HCoV-229E RNA was detected in about 44% (40 of 90) of human brains tested, with similar frequencies in brains from MS patients and patients who died from other neurologic diseases or normal control subjects.¹³ HCoV-OC43 sequences were

detected in 23% (21 of 90) of brains tested, with 36% incidence in brains from MS patients and 14% in that of controls. Although these results are suggestive, the role of non-SARS-CoV HCoVs in diseases outside the respiratory tract, especially in those involving the CNS, is not proven and requires further investigation.

Severe Acute Respiratory Syndrome Coronavirus Infections

SARS-CoV causes the most severe disease of any HCoV.^{79,310,439,448,602} The virus infects both upper airway and alveolar epithelial cells, resulting in mild to severe lung injury. Virus or viral products are also detected in other organs, such as the kidney, liver, and small intestine, and in stool. Although the lung is recognized as the organ most severely affected by SARS-CoV, the exact mechanism of lung injury is controversial. Levels of infectious virus appear to diminish as clinical disease worsens, consistent with an immunopathologic mechanism.⁴³⁷ However, this conclusion must be tempered because patient samples were obtained from nasopharyngeal aspirates, not from the lungs or other organs. Thus, it is not known whether virus titers in the lung also decrease as virus is cleared. Furthermore, virus titers obtained from patients at autopsy do not provide longitudinal information about the relationship between viral load and disease. The SARS-CoV spike protein may also contribute to disease severity. Administration of the SARS-CoV S protein to mice with pre-existing lung injury enhanced disease severity.^{239,289} ACE2 appears to have a protective role in animals with lung injury, and S protein may exacerbate disease by causing its down-regulation.²⁸⁹

Pathologic findings are nonspecific in patients who died from SARS. Cells in the upper airway were initially infected, resulting in cell sloughing but relatively little epithelial cell damage. However, virus rapidly spread to the alveoli, causing diffuse alveolar damage. This was characterized by pneumocyte desquamation, alveolar edema, inflammatory cell infiltration, and hyaline membrane formation (Fig. 28.13). Over time, alveolar damage progressed, eventually resulting in pathologic signs of acute lung injury (ALI) and, in the most severe cases, acute respiratory distress syndrome (ARDS). Most notably, multinucleated giant cells, originating either from macrophages or respiratory epithelial cells, were detected in autopsy specimens. Although virus could be cultured from infected patients for several weeks, viral antigen was rarely detected in lung autopsy samples after 10 days postinfection.^{137,172,318,417}

Like other coronaviruses, such as MHV and FIPV, SARS-CoV infects macrophages and dendritic cells; however, unlike these two animal coronaviruses, it causes an abortive infection in these cells.^{314,436,533} Several proinflammatory cytokines and chemokines, such as interferon-inducible protein (IP)-10 (CXCL10), monocyte chemoattractant protein (MCP)-1 (CCL2), macrophage inflammatory protein (MIP)-1 α (CCL3), RANTES (regulated on activation normal T cell expressed and secreted) (CCL5), MCP-2 (CCL8), tumor necrosis factor (TNF), and interleukin (IL)-6, are expressed by infected dendritic cells; many of these molecules are also elevated in the serum of SARS-CoV-infected patients.³¹⁰ Lymphopenia and neutrophilia were detected in infected patients and were likely to be primarily cytokine driven.⁶¹³ A potentially confounding factor is that many patients with SARS in the 2003 epidemic were treated with corticosteroids,⁵³⁸ and steroid treatment is a well-known cause of lymphopenia.

An important unresolved issue is how SARS-CoV causes severe respiratory disease in humans. This question is virtually impossible to address in patients, because SARS has not recurred in humans since 2004. SARS-CoV infects several species of animals, including mice, ferrets, hamsters, cats, and monkeys,⁵⁴⁹ although most of these animals develop either mild or no clinical disease, making them not useful for studies of lethal SARS. However, serial passage of SARS-CoV in mice or rats resulted in the isolation of several rodent-adapted strains that cause severe disease in some strains of young mice and rats.^{400,401,478} Most importantly, these strains cause a fatal disease in all aged rodents, paralleling the age-dependent severity observed in infected patients.¹⁴⁰ An age-dependent increase in disease severity is also observed in aged animals experimentally infected with the original human isolates, although disease severity is less than that observed with the mouse-adapted strains.⁴⁷⁹ Animals with severe disease, whether infected with human isolates of SARS-CoV or rodent-adapted strains, show pathologic signs of ALI, increased levels of proinflammatory chemokines and cytokines, and diminished T-cell responses. These observations suggest that immune dysregulation contributes to severe disease in these animals, paralleling pathologic changes observed in infected humans.

Immune Response and Viral Evasion of the Immune Response

As in most viral infections, both the innate and adaptive arms of the immune response are required for successful virus clearance and must be appropriately controlled to minimize

bystander immunopathologic damage. One of the first steps in the host immune response to a coronavirus infection is the production of type I IFN (IFN- α/β). Plasmacytoid dendritic cells (pDCs) are the source for most IFN- α/β produced in coronavirus-infected hosts, although other cells, such as macrophages, also express IFN.^{63,484,657} pDC expression of IFN is mediated by signaling through a toll-like receptor (TLR) 7- and interferon regulatory factor (IRF) 7-dependent pathway. The importance of IFN signaling in the initial immune response to coronaviruses was shown using mice that are defective in expression of the IFN- α/β receptor (IFNAR^{-/-}).^{63,244} Infection of IFNAR^{-/-} mice with mildly virulent strains of MHV results in rapid and uniformly fatal diseases. Additionally, the importance of the IFN response is also evidenced by the multiple IFN evasive mechanisms that coronaviruses employ, as described later. Although the importance of the IFN response is well established, little is known about which specific IFN-induced proteins are most critical for protection. Ribonuclease L (RNase L) appears to have a role in the immune response to neurotropic strains of MHV²⁴³; however, whether this molecule is also important in the immune response to nonneurotropic strains of coronavirus remains to be determined.

Once the initial IFN response is induced, virus clearance requires expression of proinflammatory cytokines and chemokines and their receptors, such as CCL2, CXCL9, CXCL10, CCL3, to mediate T-cell and macrophage trafficking to sites of infection.³¹ Infection of the CNS also requires breakdown of the blood-brain barrier, which is partially neutrophil dependent. In the absence of neutrophils or of neutrophil chemoattractants, such as CXCL1 and CXCL2, breakdown does not occur, resulting in more severe disease.⁶⁵⁸ A robust T-cell response is required for destruction of infected cells and clearance of infectious virus. T-cell responses are poor in felines with progressive FIP (see Fig. 28.12) and in some strains of mice with severe SARS-CoV infections.^{112,653} Virus is not cleared in MHV- or SARS-CoV-infected mice that lack T cells, again demonstrating the importance of the response in clearance.⁶²¹ Both CD4 and CD8 T-cell epitopes have been identified in mice infected with MHV or SARS-CoV and in patients with SARS. Most epitopes are located on the N, M, and S proteins.^{78,345,444,447} Once virus has been cleared, the proinflammatory response must be controlled to prevent immunopathology. In MHV-infected mice, regulatory CD4 T cells, characterized by Foxp3 expression, are important for dampening a potentially pathogenic immune response.⁵⁶⁵ IL-10, another anti-inflammatory factor important for minimizing immunopathologic changes in MHV-infected mice, is expressed predominantly by virus-specific CD4 and CD8 T cells in the infected brain.^{339,566} As described earlier for MHV-infected mice, T cells are responsible for initial virus clearance; however, an effective antiviral antibody response is required to prevent virus recrudescence.³³⁸ Similarly, a robust neutralizing antibody response was detected in survivors during the 2002–2003 SARS outbreak.⁵⁶

Coronaviruses use several approaches, both active and passive, to evade the host IFN response and thereby establish a productive infection (Table 28.4). Coronaviruses replicate in DMVs (see Fig. 28.8), which may shield viral RNA from recognition by intracellular sensor molecules, such as RIG-I, MDA5, and TLR3. Thus, in fibroblasts or conventional DCs infected with MHV or SARS-CoV, no IFN is induced.^{173,586,656} However,

TABLE 28.4 Coronavirus Proteins with Immuno-evasive Properties

Protein	Virus source	Function	References
nsp1	MHV, SARS-CoV, SARSr-BatCoV Rp3, BatCoV HKU4, BatCoV HKU9, TGEV	a. Suppresses host protein expression through direct inhibition of translation or by promoting degradation of host mRNA, including IFN mRNA b. Inhibits IFN induction and signaling	230,258,259 598,666
nsp3 (PL ^{pro})	SARS-CoV, HCoV-NL63, MHV	Blocks IRF3 activation and NF- κ B signaling	91,136,174,654
nsp3 (ADRP)	SARS-CoV, HCoV-229E, MHV	a. Interferes with IFN-induced antiviral activity b. Enhances host proinflammatory cytokine expression	158,297
nsp16	MHV	Evades MDA5 activation, evades IFIT recognition	106,665
ORF 3b protein	SARS-CoV	Inhibits IFN synthesis and signaling	283
ORF 5a protein	MHV	Interferes with IFN-induced antiviral activity	278
ORF 6 protein	SARS-CoV	Inhibits STAT1 nuclear translocation	175
ORF 7 protein	TGEV	Interferes with PKR and 2'-5' OAS/RNase L activities	103
N protein	MHV, SARS-CoV	Inhibits IFN induction; interferes with 2'-5' OAS/RNase L activity	283,637
M protein	SARS-CoV	Inhibits IRF3 activation	518

nsp, nonstructural protein; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARSr, severe acute respiratory syndrome-related; BatCoV, bat coronavirus; TGEV, transmissible gastroenteritis virus; mRNA, messenger RNA; IFN, interferon; PL^{pro}, papain-like protease; HCoV, human coronavirus; IRF, interferon regulatory factor; NF- κ B, nuclear factor-kappaB; ADRP, adenosine diphosphate-ribose-1"-phosphatase; MDA5, melanoma differentiation-associated gene 5; IFIT, IFN-induced proteins with tetratricopeptide repeats; ORF, open reading frame; STAT, signal transducers and activators of transcription; PKR, double stranded RNA-dependent protein kinase; OAS/RNase L, oligoadenylate synthetase/ribonuclease L.

the IFN response does not appear to be actively blocked in these cells, because infection with Sendai virus or exposure to poly I-C induces IFN. In some cells, such as macrophages, microglia, and oligodendrocytes, coronaviruses induce an IFN response by signaling through MDA5, and in oligodendrocytes, RIG-I.^{328,484} To counter IFN induction through activation of MDA5, all coronaviruses express a 2'-O-methyltransferase (nsp16; see the Expression of the Replicase-Transcriptase Complex section). In the absence of 2'-O-methylation, viral RNA induces a potent MDA5-dependent IFN response, which limits replication in wild-type animals but not in those deficient in IFNAR expression⁶⁶⁵ (see Table 28.4). Additionally, SARS-CoV, but not MHV nsp3, inhibits IFN induction by antagonizing IRF3 and NF- κ B function.^{136,174}

Once IFNs are expressed, they bind to IFNAR, resulting in the up-regulation of a large number of interferon-stimulated genes (ISGs). Several coronaviral proteins inhibit either IFN signaling or specific ISGs (see Table 28.4). In addition to inhibiting IFN induction, the nsp16 2'-O-methyltransferase counters the ability of IFN-induced proteins IFIT1 and IFIT2 (also referred to as ISG56 and ISG54) to inhibit translation of viral mRNA.¹⁰⁶ N protein inhibits IFN signaling, as do SARS-CoV, MHV and TGEV nsp1, and SARS-CoV ORF3b and ORF6 proteins.¹⁷³ The mechanism of action of some of these proteins has been elucidated. The N protein interferes with 2',5'-oligoadenylate synthase-associated RNase L activity.⁶³⁷ Nsp1 appears to enhance host cell mRNA degradation and inhibit host cell protein synthesis, with specific effects on IFN signaling.^{259,598,666} The karyopherin complex is required for nuclear import of STAT1, a critical component of the IFN signaling pathway, as well as the import of many other host proteins. SARS-CoV ORF6, by binding karyopherin α 2, sequesters karyopherin β 1 in the cytoplasm, indirectly inhibiting nuclear translocation of STAT1.¹⁷⁵

EPIDEMIOLOGY

Human Coronaviruses Other Than Severe Acute Respiratory Syndrome Coronavirus

Four known coronaviruses—HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1—are endemic in human populations. HCoV-OC43 and HCoV-229E cause up to 30% of all upper respiratory tract infections, based on several prospective studies.^{245,389} The variable range of detection reflects year-to-year variability, detection methods, season, and age of subjects. These studies also suggest that peak activity occurs every 2 to 4 years.^{184,264,397} In temperate climates, infections occur predominantly in the winter and early spring. HCoV-OC43 and HCoV-229E have also been associated with severe pneumonia in neonates and aged populations, especially those with underlying illnesses, such as chronic obstructive pulmonary disease, or those requiring intensive care.^{163,198} The high rate of HCoV infections early in life and the pattern of infections during outbreaks demonstrate that HCoVs are efficiently transmitted in human populations, most likely via large and, to a lesser extent, small droplets. Serologic studies suggest that infection with HCoV-229E and HCoV-OC43 frequently occurs in young children and then repeatedly throughout life.^{245,264,556,557} Neutralizing antibodies against HCoV-OC43 or HCoV-229E have been detected in about 50% of school-age children and up to 80% of adults.^{264,389,458}

HCoV-NL63 and HCoV-HKU1 also have worldwide distributions, causing up to 10% of respiratory tract infections.^{1,460} Initial reports suggested that HCoV-NL63 was associated with severe respiratory disease; however, subsequent population-based studies showed that most patients developed mild disease, similar to those infected with HCoV-229E or HCoV-OC43. HCoV-NL63 is also an important etiologic agent of acute laryngotracheitis (croup).¹ HCoV-HKU1 was initially identified in an elderly patient with severe pneumonia,



FIGURE 28.14. SARS-CoV spread from infected bats to infect humans in wet markets in Guangdong Province, China.

SARS-related coronaviruses were detected in Chinese horseshoe bats and other bat species in China. The virus spread to human populations, likely animal handlers, in wet markets in Guangdong Province. Spread occurred either indirectly, via infection of exotic animals such as Himalayan palm civets, or directly, with subsequent human transmission to Himalayan palm civets and other exotic animals. This transmission occurred more than once, because a fraction of the animal handlers were positive for anti-SARS-CoV antibody.²⁰³ In one episode, a physician taking care of an animal handler became infected. He then flew to Hong Kong and stayed at Hotel M, where he inadvertently infected several other people staying at the hotel, probably via superspreading events. These infected individuals then flew to other countries, resulting in the international outbreak. SARS-CoV, severe acute respiratory syndrome coronavirus; SARS, severe acute respiratory syndrome.

although more recent studies suggest that it is associated with both mild and severe respiratory infections.^{460,615}

Severe Acute Respiratory Syndrome

During the 2002–2003 epidemic, SARS-CoV was isolated from several exotic animals, including Himalayan palm civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*), in wet markets in Guangdong Province in China²⁰³ (Fig. 28.14). Subsequent investigations showed that SARS-CoV could not be detected in these animals in the wild but that severe acute respiratory syndrome–related coronaviruses

(SARSr-CoV) could be isolated from wild bats in China^{308,332} (see Table 28.1). Bats are now considered to be the ultimate source for SARS-CoV, with probable infection of human populations occurring after initial adaptation to animals in Chinese wet markets. Sequences from several distinct SARSr-CoVs have been amplified from Chinese horseshoe bats from Hong Kong and several provinces in China, and 30% to 85% of bats of this genus (*Rhinolophus*) had serologic evidence of infection with a SARSr-CoV. *N* gene sequences for three SARSr bat coronaviruses (BatCoVs) differed by 3% to 6%, similar to the level of difference between the *N* proteins

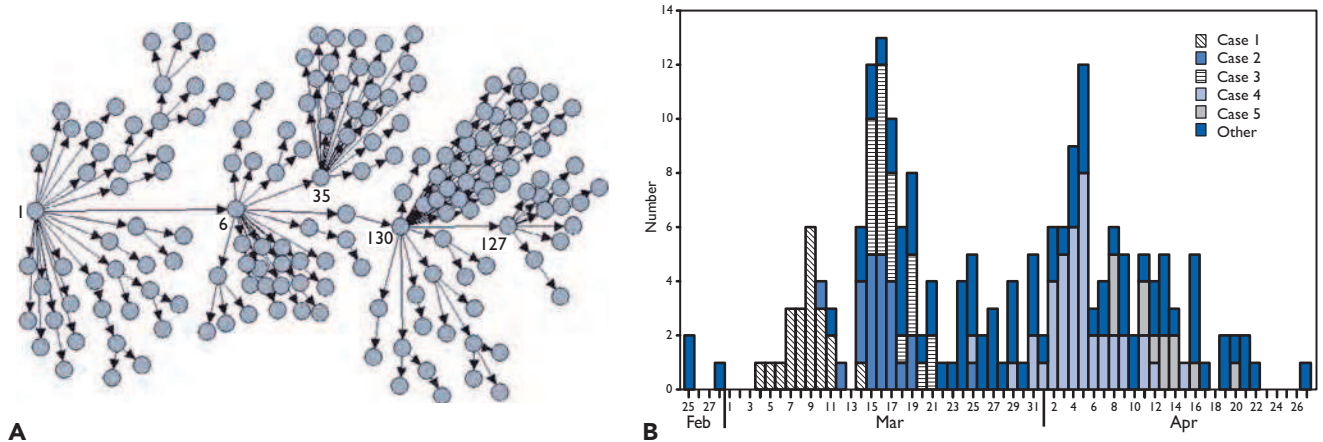


FIGURE 28.15. Role of superspreading events in SARS-CoV epidemics. SARS-CoV spread in Singapore in 2003, illustrated here, via superspreading and non-superspreading events. Most infected persons transmitted virus to fewer than five susceptible contacts. However, in a few instances, infected individuals were highly contagious, resulting in infection of larger numbers of contacts. The basis for superspreading events is not known but likely is a manifestation of larger virus burdens in a few infected patients. **A:** Probable cases of SARS by reported source of infection. **B:** Number of probable cases of SARS, by date of onset of fever and probable source of infection. SARS-CoV, severe acute respiratory syndrome coronavirus; SARS, severe acute respiratory syndrome. (From Leo YS, Chen M, Heng BH, et al. Severe Acute Respiratory Syndrome — Singapore, 2003. *Morb Mortal Wkly Rep* 2003;52:405–411.)

of each of these viruses and that of SARS-CoV. This degree of difference between SARS-CoV and the various SARSr-BatCoVs indicates that the precise source of the 2002–2003 SARS outbreak viruses remains unknown. Neither SARS-CoV nor reconstructed BatCoVs can use the Chinese horseshoe bat ACE2 protein to enter target cells, raising the possibility that the bat host receptor is unrelated to ACE2²⁶; alternatively, the virus that was the actual progenitor for SARS-CoV may have originated from a BatCoV distantly related to the SARSr-CoVs identified thus far.²²⁵

Serologic studies demonstrated that SARS-CoV had not circulated to a significant extent in humans prior to the outbreak in 2002–2003.^{64,320} However, some persons working in wild animal wet markets in China had serologic evidence of a SARS-CoV-like infection acquired before the 2003 outbreak but reported no SARS-like respiratory illness.²⁰³ Thus, virus may have circulated in these wild animal markets for a few years, with the SARS outbreak occurring only when a confluence of factors facilitated spread into larger populations. Although animals were the original source of SARS, its global spread occurred by human-to-human transmission. Transmission appeared to occur through close contact—that is, direct person-to-person contact, fomites, or infectious droplets and probably aerosols in some instances.⁴³⁸ Because transmission usually only occurred after onset of illness and most efficiently after the patient was sufficiently ill to be hospitalized, most spread occurred in household and healthcare settings but infrequently in other settings.⁴⁴⁰ There was also substantial patient-to-patient variation in efficiency of transmission, which, in part, was associated with the degree of illness severity. Many susceptible persons were infected in superspreading events; however, fortunately, only a minority of infected individuals were involved in this type of spread^{342,475} (Fig. 28.15). Superspreading events, which occurred when a single individual infected multiple susceptible contacts, may have resulted from high virus burdens or a tendency for these individuals to aerosolize virus more efficiently than most infected

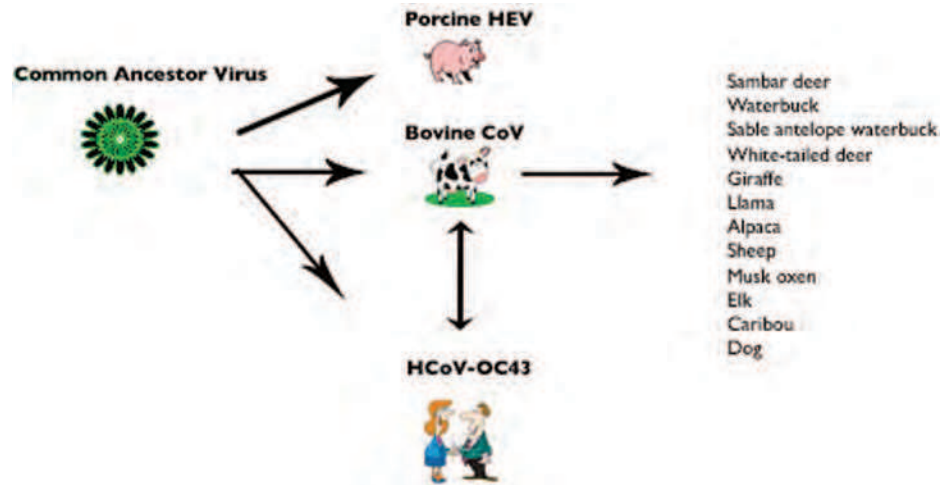
persons. Most infected individuals spread the virus to only one or a few susceptible persons, suggesting that virus spread was relatively inefficient.^{342,475} The outbreak was partly controlled using quarantining, and the lack of efficient spread contributed to the success of this approach. Because the SARS outbreak was controlled in June 2003, only 17 cases of SARS were subsequently confirmed, and none of these occurred after June 2004. Thirteen of these 17 cases resulted from laboratory exposures, including 7 secondary cases associated with one of the cases.³³⁶ The other 4 cases occurred in southern China and resulted from exposure in the community, presumably to SARS-CoV–infected animals from wild animal markets.³³⁴

Genetic Diversity of Coronaviruses

The SARS outbreak demonstrated the ability of coronaviruses to cross species, as the virus, naturally a bat virus, was able to infect small mammals, such as the Himalayan palm civet, and humans. Initially predicted from studies of coronavirus-infected cultured cells,²³ the ability of coronaviruses to cross species was also demonstrated when the betacoronaviruses HCoV-OC43, PHEV, and BCoV were analyzed¹⁵⁸⁸ (Fig. 28.16). It is estimated that PHEV diverged from HCoV-OC43 and BCoV 100 to 200 years ago, whereas HCoV-OC43 and BCoV diverged about 100 years ago. Whether the common ancestor of HCoV-OC43 and BCoV was a human or bovine virus is not known. More recently, BCoV has crossed species to infect many ruminants, including elk, giraffe, and antelope,⁴ and also canines.^{159,160} Other phylogenetic studies suggest that the porcine alphacoronavirus TGEV resulted from cross-species transmission of a CCoV.³⁵⁵

In addition to their ability to cross species, coronaviruses readily undergo recombination (see the Viral RNA Synthesis section). Recombination events between canine (CCoV-I) and feline (FeCoV-I) coronaviruses and an unknown coronavirus resulted in the appearance of two novel viruses (CCoV-II and FeCoV-II).³⁵⁵ In another illustration, new strains of IBV

FIGURE 28.16. Coronaviruses mutate and recombine to cross species barriers. Phylogenetic analyses indicate that HCoV-OC43, BCoV, and PHEV shared a common ancestor and diverged about 200 years ago. More recently (100–130 years ago), HCoV-OC43 and BCoV diverged; however, it is not known whether BCoV infected human populations or HCoV-OC43 crossed species barriers to infect bovids. BCoV then spread to many ruminants and to dogs, probably via contact with infected domesticated cows. HCoV, human coronavirus; BCoV, bovine coronavirus; PHEV, porcine hemagglutinating encephalomyelitis virus.



have been detected in chicken populations and appear to have resulted from recombination between circulating vaccine and wild-type IBV strains.²⁸⁴ This propensity for recombination has raised concerns about the use of live attenuated coronavirus vaccines (see the Prevention section).

CLINICAL FEATURES

Human Coronaviruses Other Than Severe Acute Respiratory Syndrome Coronavirus

In humans, coronaviruses have been clearly shown to cause respiratory disease, including its most severe manifestation—SARS. HCoVs have occasionally been implicated in enteric disease, particularly in newborns, using electron microscopy.^{185,270,365} Electron microscopy has been used in these studies, because efforts to propagate human enteric coronaviruses in tissue culture cells have thus far been unsuccessful, hindering further studies. Because other particles in stool specimens (e.g., cellular membranes) can have similar morphology to coronaviruses, electron microscopic detection of coronavirus particles in stools is not considered diagnostic of infection. However, polymerase chain reaction (PCR) assays designed to detect coronavirus RNA sequences in pathologic specimens will now make it possible to determine whether these viruses play a role in enteric diseases. It seems likely that coronaviruses will be the etiologic agent in a fraction of patients with gastroenteritis, given the ability of these viruses to cause enteritis in a variety of domestic and companion animals.

Clinical features of infections in humans follow two distinct patterns: one for the non-SARS-CoV coronaviruses (i.e., HCoV-229E, -NL63, -OC43, -HKU1), and one for the zoonotic coronavirus SARS-CoV. Among the HCoVs, HCoV-229E and HCoV-OC43 were extensively characterized in volunteer studies in the 1960s.³⁸⁹ Human volunteers inoculated intranasally with respiratory coronaviruses developed symptoms that included fever, headache, malaise, chills, rhinorrhea, sore throat, and cough, with peak infection observed 3 to 4 days following infection. About half of the volunteers challenged with virus developed illness, and approximately 30% were asymptotically infected, as indicated by detection of virus in the upper respiratory tract. Symptoms lasted for a mean of 7 days, with a range of 3 to 18 days. Natural infection in both adults and children is also usually associated with a common

cold-like illness.^{44,389} Natural infection is probably acquired in a fashion similar to that for many other respiratory viruses (i.e., inoculation of infectious secretions from infected persons or fomites onto mucous membranes of the upper respiratory tract or inhalation of infectious droplets), with primary infection of ciliated epithelial cells in the nasopharynx.³ Destruction of these cells, combined with exuberant production of chemokines and cytokines by resident and infiltrating cells, results in signs and symptoms of clinical illness.

HCoV infections are also occasionally associated with lower respiratory tract disease in children and adults. Coronaviruses have been detected in children hospitalized with lower respiratory tract disease at varying rates, although usually less than 8% of patients.^{88,155,179,556,557,572} One caveat is that coronaviruses are also sometimes detected in well, control patients; thus, the presence of virus may not be etiologically related to the illness.¹⁰⁸ Coronavirus infection has also been detected in adults with acute respiratory tract illness, including about 5% of those hospitalized with lower respiratory tract disease.^{108,155,163,182,198} Studies using PCR to detect viral RNA in middle ear fluids suggest that coronaviruses, like other respiratory viruses, can cause otitis media.^{454,455} In addition, HCoVs have been associated with wheezing and exacerbations of asthma.^{245,556} HCoV-NL63 and HCoV-HKU1 have also been detected in persons with acute upper and lower respiratory tract illness,^{1,108,182,556,557} and as described earlier, HCoV-NL63 is associated with croup in children younger than 3 years.⁵⁷⁴ Studies of natural infection and volunteer studies have shown that reinfection with coronaviruses is common, demonstrating that infection does not induce stable protective immunity.^{245,264,389} For example, previously infected volunteers developed symptomatic disease if infected 1 year later with the same strain of HCoV-229E.⁴⁷⁰

Severe Acute Respiratory Syndrome Coronavirus Infections

In contrast to the mild illness usually associated with HCoV infections, SARS-CoV have nearly always resulted in a serious lower respiratory tract illness that required hospitalization, often in an intensive care unit (up to 20% of infections)⁴³⁸ (Fig. 28.17). In the 2002–2003 epidemic, approximately 8,000 individuals were infected, with an overall mortality rate of 10%. Disease severity increased proportionally with age. Thus, no mortality occurred in patients younger than

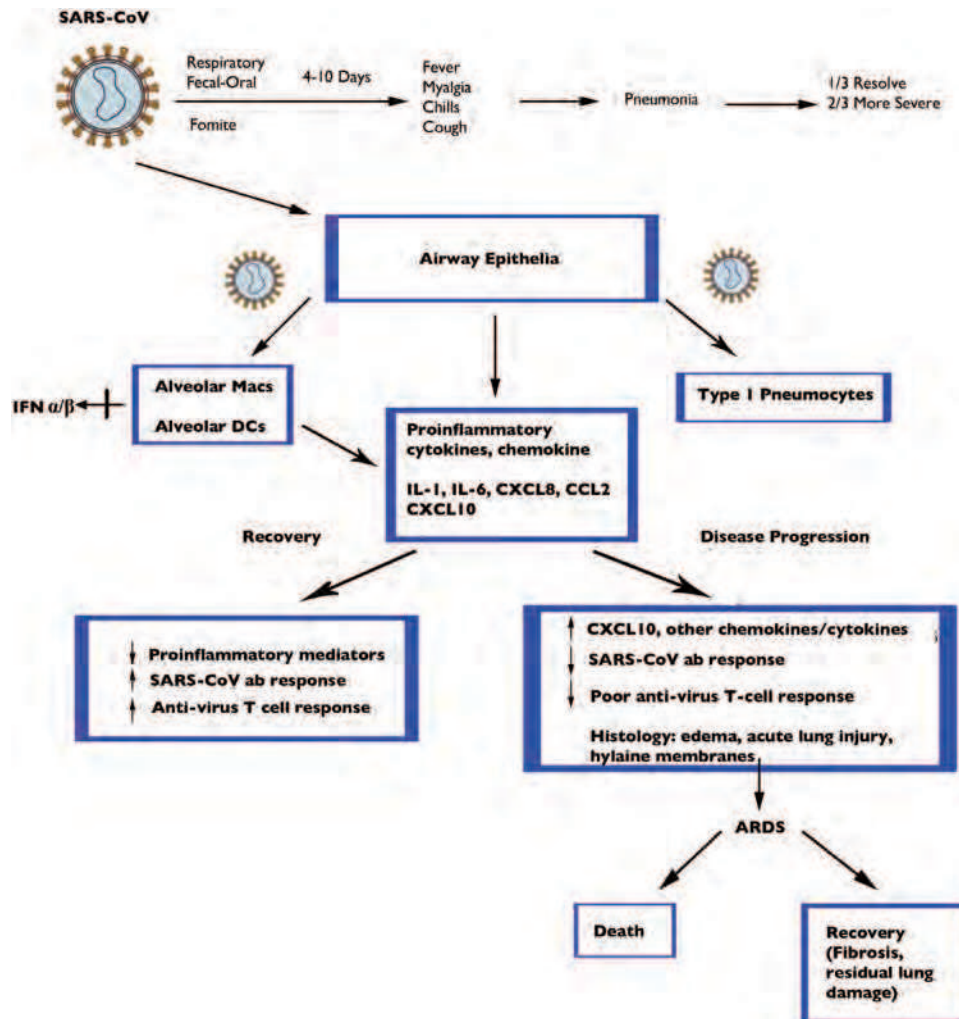


FIGURE 28.17. Clinical disease in patients infected with SARS-CoV. SARS-CoV spread to susceptible individuals via respiratory and fecal–oral routes and, less commonly, if at all, via fomites. Virus replication was initiated in the upper airway epithelial cells, based primarily on animal studies and *in vitro* studies using primary cultures of airway epithelial cells. Virus subsequently spread to the lower respiratory tract, with infection of type 1 pneumocytes and macrophages and dendritic cells most prominent. The infection of the latter two cell types was abortive, resulting in production of proinflammatory cytokines and chemokines such as CXCL10 and CXCL8 but not type 1 IFN. In patients who recovered, expression of proinflammatory cytokines diminished, and robust antiviral antibody responses were detected. In patients who developed progressively more severe disease, cytokine production continued and patients remained lymphopenic without developing an effective anti-SARS-CoV antibody response. Some of these patients died, and significant long-term morbidity was found in many of the survivors. SARS-CoV, severe acute respiratory syndrome coronavirus; IFN, interferon.

24 years, although about 50% of infected individuals older than 60 years succumbed to the infection. Mortality was also greater in patients with underlying disease. Clinical disease in patients with SARS was not diagnostic; however, some features were more common in SARS patients compared to those infected with other pathogens.^{35,318,437,438} Illness usually had an onset of 4 to 7 days, although occasionally an incubation period of as little as 2 days or as long as 10 to 14 days was observed. Disease was characterized by systemic symptoms such as fever, malaise, and myalgias. Unlike many other respiratory tract infections, upper respiratory tract signs and symptoms such as rhinorrhea, sore throat, and nasal congestion were not common, although they

still occurred in a minority of patients. The first lower respiratory tract symptoms (usually a nonproductive cough and shortness of breath) developed several days after onset of systemic symptoms. Respiratory symptoms were often accompanied by evidence of involvement of other organ systems. Thus, whereas diarrhea occurred at disease onset in fewer than 25% of patients, up to 70% developed gastrointestinal disease during the course of the illness. Most patients developed abnormal liver function tests (70%–90%) and lymphopenia (70%–95%), with a substantial drop in both CD4 and CD8 T-cell numbers.^{104,438} Patients who failed to resolve their illness often had progressive respiratory failure leading to ARDS and death weeks to months

after illness onset.^{56,171,321} In these patients, lymphocyte and platelet counts remained abnormally low, whereas neutrophilia and elevated titers of virus or viral RNA in clinical specimens for prolonged periods of time were common features. Asymptomatic or mild illness was uncommon, as illustrated by studies of exposed healthcare workers. In these studies, fewer than 1% of those without a SARS-like illness had serologic evidence of infection.^{70,218,466} Most survivors of SARS-CoV infection achieved full recovery, although pulmonary function abnormalities sometimes took months to subside.^{86,626} Some, however, had persistently abnormal pulmonary function. Curiously, a fraction of survivors showed more evidence of neurologic or psychiatric disease than expected based on the degree of respiratory illness or steroid use. Although brains were not commonly studied during the 2002–2003 epidemic, a few studies did demonstrate SARS-CoV infection of the brain, suggesting that CNS infection may have occurred in some cases.^{84,201,307,316}

DIAGNOSIS

Most HCoV infections, other than SARS-CoV, are not diagnosed because they cause mild, self-limited upper respiratory disease, and no specific therapy is available. Diagnosis is laboratory-based because coronavirus infections cannot be distinguished clinically from other causes of upper respiratory tract infections, such as rhinoviruses. However, in some clinical settings, such as in hospitalized patients with pneumonia and in epidemiologic studies, specific diagnosis is important. Coronavirus infections in animals and humans were initially diagnosed by isolation of infectious virus, by electron microscopy, and in serologic assays, with the caveat that some coronaviruses, especially those in the stool, are not easily cultured. HCoV-229E and related alphacoronaviruses have sometimes been isolated in human diploid cell lines. Other HCoVs, most notably HCoV-OC43, initially required cell organ culture systems for isolation,³⁸⁹ although this virus can now be grown in tissue culture cells. HCoV-NL63 can infect monkey kidney LLC-MK2 cells or Vero cells,^{170,460,573} whereas HCoV-HKU1 has been grown only in primary human airway epithelial cells.⁴⁶³ RT-PCR-based methods and immunofluorescence assays (IFA) for virus antigen have largely replaced these other methods for the diagnosis of respiratory coronavirus infections.^{108,156,163,198,392} PCR primers can be designed to be broadly reactive or strain specific, based on primer location and design. With a sensitive system to detect the PCR amplicon (e.g., a real-time assay), fewer than five RNA copies in the reaction mixture can be consistently detected.¹⁵⁶ A multiplex real-time RT-PCR assay has also been described that is able to detect all four respiratory coronaviruses and may become the diagnostic method of choice.¹⁸⁴

Electron microscopic examination of clinical material, although laborious, contributed to the identification and characterization of many coronaviruses, including SARS-CoV.^{143,288,389,439} At present, electron microscopy is used most commonly to identify coronaviruses in patients with enteritis,²⁷⁰ because none of these coronaviruses have been cultured; however, because other particles in clinical specimens can resemble coronaviruses and coronaviruses may be present without causing disease, identification of such particles does not confirm infection.

Various serologic assays have been used to detect coronavirus infections, including complement fixation, hemagglutination inhibition (HI) for viruses with an HE protein (i.e., some betacoronaviruses), neutralization, IFAs, and enzyme-linked immunoassays (EIAs). Initially, these assays used virus lysates or inactivated whole virus; more recently, cloned expressed proteins, synthesized peptides, and pseudoviruses have been used as antigens for serologic assays.^{319,357,389,418,458,558}

SARS or another coronavirus infection of equivalent severity presents a different diagnostic situation. A specific diagnosis is critical because a positive result will guide clinical management and have public health implications. However, testing should only be considered when, based on the likelihood of an exposure and clinical features of the illness, infection is plausible. SARS-CoV was initially isolated in fetal rhesus kidney cells and Vero cells; however, during the 2002–2003 epidemic, a combination of serologic and RT-PCR assays, not virus culture, were used to detect and confirm SARS-CoV infection.⁴⁴⁰ With very sensitive PCR assays (e.g., a nested or real-time PCR assay) and RNA extraction procedures that increased the amount of specimen available for the assay, the positivity rate in respiratory specimens obtained during the second and third days of illness increased from less than 40% to more than 80% as the epidemic progressed.⁴⁵⁹ SARS N protein EIA was positive in 50% to 80% of serum specimens collected during the first week of illness⁷⁴ and in more than 50% of respiratory and stool specimens collected during the second and third weeks of illness.³⁰⁹ SARS-CoV-specific antibodies were usually detected by 14 days into the illness, although sometimes not until 4 weeks after infection.^{229,233} Whereas RT-PCR provided the best way to make an early diagnosis, serologic assays were important in confirming or ruling out SARS-CoV as the cause of infection. Because serum specimens from persons not infected with SARS during the 2002–2003 outbreak have rarely tested positive for SARS-CoV antibodies,³²⁰ a single serum specimen positive for SARS-CoV antibodies was usually considered diagnostic; a negative test on a serum specimen collected late in the illness (28 days or later after onset of illness) could be used to rule out SARS-CoV infection.

TREATMENT

At present, there are no antiviral drugs for HCoV infections, and therapy is supportive. During the major part of the SARS epidemic, most patients were treated with ribavirin or high-dose steroids, based on the idea that the virus would be susceptible to ribavirin and steroids might diminish immune-mediated bystander damage.⁵³⁸ Late in the outbreak, based on their ability to inhibit SARS-CoV replication *in vitro* and/or in experimental animals, IFN- α , SARS convalescent-phase immune globulin, and lopinavir plus ritonavir (two protease inhibitors licensed for the treatment of HIV) were used to treat patients.^{65,85,89,356,530} However, a large-scale review of all of these therapies concluded that whereas some showed efficacy in inhibiting SARS-CoV replication in tissue culture cells, none showed a beneficial effect in patients.⁵³⁸ The molecular biology of coronavirus infection suggests several potential targets for antiviral drugs, including the viral RdRp, virus-encoded proteases, host cell receptors used by the virus for entry, and the viral S glycoprotein. Subsequent to the outbreak, several antiviral drugs targeting these

viral proteins or processes have been developed and evaluated for their ability to inhibit SARS-CoV replication *in vitro*. These include specific coronavirus protease inhibitors,⁴⁶⁸ monoclonal antibodies that inhibit SARS-CoV binding to cells,⁵⁵¹ peptides from the heptad repeat regions of the S protein or from ACE2 that inhibit receptor binding or fusion,^{40,211} and small interfering RNAs.⁷¹ If SARS or another severe coronavirus-mediated disease emerges, *in vitro* and animal model studies of antiviral drugs will be used to guide treatment.

PREVENTION

No vaccines are available to prevent HCoV infection; however, vaccines against common veterinary coronaviruses, such as IBV and CCoV, are routinely used to prevent serious disease in young animals. Efforts are ongoing to improve these vaccines and to enhance safety and efficacy while minimizing the likelihood of reversion to a virulent strain.⁴⁹² In addition, various SARS-CoV vaccines have been developed, including inactivated whole virus, live virus vectors expressing single viral proteins and recombinant proteins, and DNA vaccines.^{10,480} Nearly all of these vaccines express the surface glycoprotein and are designed to induce SARS-CoV neutralizing antibodies. For some of these vaccines, efficacy has been demonstrated in animal models. Large stocks of anti-SARS-CoV neutralizing antibody have been prepared and will be used for passive immunization of healthcare workers and other high-risk personnel if SARS recurs.

In general, live attenuated vaccines are likely to be most effective in inducing protective immune responses against coronaviruses. This has been illustrated elegantly in the case of TGEV—an important cause of neonatal diarrhea and death in swine. In the mid-1980s, a naturally occurring, attenuated variant of TGEV—PRCoV—was identified in pig populations. This virus, which causes mild disease and no enteritis, induces an immune response in pigs that is protective against TGEV and largely eliminated it from dually infected populations.³¹² Live attenuated vaccines induce not only neutralizing antibodies but also antiviral T-cell responses, which are required for virus clearance from infected cells in SARS and other coronavirus infections. However, the development of live coronavirus vaccines is challenging.⁴⁹² First, in many instances, natural infection does not prevent either subsequent infection or disease, therefore an effective vaccine would need to be superior to immunity induced naturally. Second, the genetic and antigenic variability of coronaviruses and their ability to readily recombine hinder vaccine development. Thus, a vaccine may not provide equal protection from all antigenic variants, and subsequent recombination with vaccine strains could increase the number of different strains circulating in the wild. As an example, recombinants of IBV vaccine strains with virulent wild-type strains have caused disease outbreaks in chicken flocks.^{249,596} In addition, the finding that immunization with an S protein-expressing FIPV vaccine led to more severe disease after subsequent natural infection raises the concern that other coronavirus vaccines might also enhance, rather than protect, from disease.⁵⁸¹ Several strategies to minimize the likelihood of recombination and to attenuate candidate vaccines without compromising efficacy have been recently described. These include engineering viruses with deletions in *nspl*, important for the anti-IFN response,⁶⁶⁶ or in E

protein, important for virus assembly.¹²⁴ In other approaches to minimizing the likelihood of recombination of vaccine viruses, the coronavirus genome has been reconstructed, changing the order of structural genes at the 3' end¹²¹ or modifying the leader and body TRSs (see the Viral RNA Synthesis section) to eliminate homology with natural virus sequences.⁶⁴⁴

In the absence of effective vaccines and antiviral drugs, the most important ways to prevent coronavirus infections are a highly active public health surveillance system and good infection control practices. This was demonstrated unequivocally during the SARS outbreak in 2002–2003, in which sharing of information by national public health agencies and governments and involvement of international agencies such as the World Health Organization resulted in the rapid identification of a coronavirus as the cause of SARS and implementation of measures that minimized spread. At the local level, strict attention to good isolation and infection control practices and identification and management of exposed persons (contacts) minimized human-to-human spread of the virus within a few months of its global spread. The low risk of SARS-CoV transmission before hospitalization and the low rate of asymptomatic infection facilitated the efficacy of these public health measures.^{70,218,466} The identification of cases of laboratory-acquired SARS-CoV, with subsequent transmission to others after one of these cases,^{334,336} reinforces the importance of strict attention to safe laboratory practices. These practices include handling the virus in the appropriate type of facility, using standardized operating procedures, and providing appropriate training and medical surveillance programs for staff.

PERSPECTIVES

Many important problems remain to be resolved by future studies of coronaviruses. One critical task will be to broaden our picture of how coronaviruses jump between species. We need to know whether cross-species viral trafficking events, both abortive and successful, are rare or common. Although there has been a recent expansion of our knowledge of spike protein interactions with receptors and associated proteases, we cannot yet fully gauge the height of the barrier preventing productive adaptation by a spike protein to new receptors and proteases. Such information will be directly relevant to forestalling or coping with the re-emergence of a SARS-related (or other) coronavirus from ubiquitous bat reservoirs. Related to this is the challenge of developing *in vitro* culture systems for virus species that are currently only known through their genomic sequences. A second area of crucial importance will be to further develop our understanding of the immunopathogenesis of the more severe human and animal coronaviruses and to more precisely delineate the correlates of immune protection. This will better inform the effective design and evaluation of vaccines for control of these agents. Finally, one of the most exciting areas of future research will be to address the many gaps in our basic knowledge of the intricacies of the coronavirus RTC—the largest and most complicated machinery of RNA synthesis found in any RNA virus. The past few years have seen tremendous advances in this field, particularly in structural and biochemical studies, and it is likely that progress will continue apace. A long-term goal will be the total *in vitro* reconstitution of coronavirus RNA synthesis, which would definitively demonstrate the roles

of the many viral replicase subunits as well as those of putative host factors. It can be expected that studies of this type will reveal fundamental principles common to all RNA-dependent RNA synthesis, in addition to mechanisms unique to the order *Nidovirales*. Knowledge derived from this enterprise will be critical for the design of antiviral drugs to combat diseases caused by existing and emerging coronaviruses.

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Arteriviruses

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HISTORY AND CLASSIFICATION OF ARTERIVIRUSES

The family *Arteriviridae*⁶⁸ was established in 1996 and currently comprises the following four enveloped, plus-stranded RNA viruses: equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, porcine reproductive and respiratory syndrome virus (PRRSV), and simian hemorrhagic fever virus (SHFV). Three of these (EAV, LDV, and SHFV) were first isolated and characterized about 50 years ago.^{62,161,189} The porcine arterivirus PRRSV emerged only about 20 years ago,^{36,227} causing vast epidemics of a previously unknown reproductive and respiratory disease in swine in both Europe (genotype I) and North America (genotype II). Remarkably, the subsequent molecular characterization of PRRSV strains from both continents revealed considerable genetic differences, suggesting that the two PRRSV genotypes evolved separately and are only distantly related to a common ancestor.^{152,170} PRRSV infection can cause high-mortality disease outbreaks and has developed into the most prevalent disease of swine worldwide. Recently, a large outbreak of highly virulent PRRSV affected the Asian pig industry, causing enormous economic losses.^{194,246}

In general, the consequences of arterivirus infection can range from an asymptomatic, persistent or acute infection to abortion or lethal hemorrhagic fever.^{199,226} EAV is capable of inducing a variety of symptoms, including necrosis of the small muscular arteries from which the name of the family prototype EAV was derived. The name of the mouse arterivirus LDV is derived from the increase in the level of lactate dehydrogenase (LDH) caused by LDV infection.¹⁶¹ The virus, which has been used extensively as an *in vivo* research model, is able to escape immune surveillance and establish a largely asymptomatic persistent infection.^{22,155} SHFV was isolated from outbreaks of fatal hemorrhagic fever in macaque colonies¹⁸⁹ that were probably caused by inadvertent transmission by humans from African monkeys to macaques.

The unification of the previously unclassified arteriviruses was the direct result of the sequence analysis of their genomes, which revealed an intriguing relationship with coronaviruses and toroviruses (discussed in Chapter 28). Despite striking differences in genome size and virion structure, the genome organization and expression strategy of these viruses were found to be comparable and their replicase genes were postulated to share common ancestry⁵⁴ (e-Fig. 29.1). One of the most prominent features of their genome expression strategy, the generation of a nested set of subgenomic (sg) messenger RNAs (mRNAs), provided the basis for the name *Nidovirales*

(*L. nidus* = nest) that was given to the novel virus order comprising the arterivirus and coronavirus families in 1996. Subsequently, the order was further expanded with the invertebrate virus family *Roniviridae* and the genus *Bafinivirus*, which contains fish nidoviruses.⁸³ Most recently, the isolation of the first insect nidoviruses (proposed family name *Mesoniviridae*) was reported,^{140,249} yet again expanding the exceptional host range of the order *Nidovirales*. Furthermore, on the basis of its partial genome sequence, a novel nidovirus isolated from Australian possums (wobbly possum disease virus) appears to represent yet another nidovirus lineage, which is relatively closely related to the *Arteriviridae*.^{66a}

Nidoviruses represent a distinct lineage among plus-strand RNA viruses (e-Fig. 29.1). The complex evolutionary relationship between arteriviruses and nidoviruses with a much larger genome has been reviewed extensively elsewhere.⁸³ Related replicase genes and replication strategies have been combined with seemingly unrelated sets of structural protein genes. RNA recombination likely was an important factor in these evolutionary events and was also invoked to explain some internal rearrangements of arterivirus genomes.^{52,81,103}

VIRION STRUCTURE

Arteriviruses have been observed as spherical particles, 50 to 60 nm in diameter, and possess a relatively smooth, mostly featureless surface, which is likely explained by the small

ectodomains of the two major envelope proteins (Fig. 29.1; see^{20,68,175,182} and references therein). The nucleocapsid structure has long been assumed to be isometric, but recent cryo-electron tomography studies of PRRSV revealed a rather pleomorphic and “disorganized” core structure (average diameter 39 nm). These findings are clearly incompatible with an icosahedral core and suggest a resemblance to the nucleocapsid structure proposed for coronaviruses, a helical coil, or an even more loosely organized filamentous structure.^{61,182}

The buoyant density of arteriviruses is 1.13 to 1.17 g/cm³ in sucrose, and their sedimentation coefficient ranges from 214S to 230S. Virions are highly unstable in solutions containing low concentrations of nonionic detergents or at a pH other than 6.0 to 7.5, and quickly lose their infectivity when stored at temperatures higher than 4°C.

The arterivirus nucleocapsid structure (Fig. 29.2, e-Fig. 29.2) is composed of the 12.7 to 15.7 kb RNA genome and the nucleocapsid protein (N). The crystal structure for the capsid-forming C-terminal domain of PRRSV N⁵⁹; e-Fig. 29.2B) suggested that it represents a new class of viral capsid-forming domains, a hypothesis further supported by cryo-EM studies.

Based on studies with EAV and PRRSV, the lipid bilayer that surrounds the nucleocapsid is now presumed to contain seven envelope proteins (Table 29.1, Fig. 29.2), an unusually large number compared to other plus-stranded RNA viruses. In this chapter, we refer to the glycoproteins as “GPx”, where x indicates the number of the corresponding open reading frame

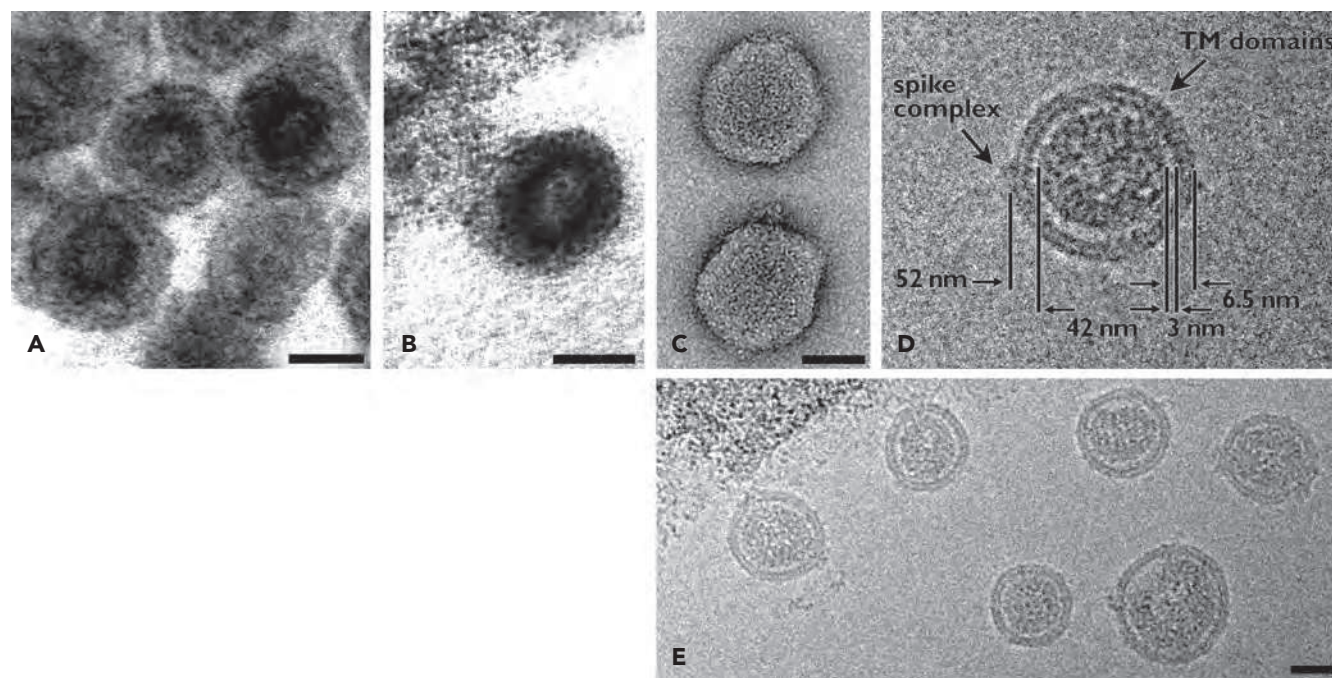


FIGURE 29.1. Electron micrographs of arterivirus particles. **A:** Transmission electron microscopic (EM) image of extracellular porcine reproductive and respiratory system virus (PRRSV) particles. **B:** Transmission EM image of an equine arteritis virus (EAV) particle budding from smooth intracellular membranes. **C:** Negatively stained, purified PRRSV particles. **D–E:** Cryo-EM of PRRSV particles in vitreous ice. **Panel D** shows a typical PRRSV particle with dimensions indicated. A possible spike protein complex and the striated appearance that most likely corresponds to the transmembrane domains of envelope proteins are visible. All bars are 25 nm. (A and B from Snijder EJ, Meulenbergh JJM. The molecular biology of arteriviruses. *J Gen Virol* 1998;79:961–979 with permission; **C–E** from Spilman MS, Welbon C, Nelson E, et al. Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. *J Gen Virol* 2009;90:527–535, with permission.)

TABLE 29.1 Molecular Properties of Arteriviruses

Virus ^a	Host	Genome size (kb)	Replicase proteins			Structural proteins ^c		
			ORF	Size (aa)	Nsps ^b	ORF	Protein name ^d	Size (aa)
EAV	Horse Donkey	12.7	1a	1,727	9	2a	E	67
			1ab	3,175	13	2b	GP2 (GP2b/G _S)	227
						3	GP3	163
						4	GP4	152
						5	GP5 (G _L)	255
						5a	ORF5a protein	59
						6	M	162
LDV	Mouse	14.1	1a	2,206	10	7	N	110
			1ab	3,616	14	2a	E	70
						2b	GP2 (VP3-M)	227
						3	GP3	191
						4	GP4	175
						5	GP5 (VP3-P)	199
						5a	ORF5a protein	47
PRRSV	Pig	15.1	1a	2,397	10	6	M (VP2)	171
			1ab	3,854	14	7	N (VP1)	115
						2a	GP2 (GP2a)	249
						2b	E	70
						3	GP3	265
						4	GP4	183
						5	GP5	201
SHFV	Monkey	15.7				5a	ORF5a protein	43
						6	M	173
						7	N	128
			1a	2,105	10?	2a'	ORF2a' protein	281
			1ab	3,594	14?	2b'	ORF2b' protein	94
						3'	ORF3' protein	204
						4'	ORF4' protein	205
						2a	E	80
						2b	GP2 (GP2b)	214
						3	GP3	179
						4	GP4	182
						5	GP5	278
						5a	ORF5a protein	64
						6	M	162
						7	N	111

ORF, open reading frame; EAV, equine arteritis virus; aa, amino acid; GP, glycoprotein; nsp, nonstructural protein; LDV, lactate dehydrogenase-elevating virus; PRRSV, porcine reproductive and respiratory syndrome virus; SHFV, simian hemorrhagic fever virus.

^aMolecular characteristics were based on the sequences of the EAV-Bucyrus (European Molecular Biology Laboratory (EMBL) database accession number NC_002532), PRRSV-Lelystad (accession number M96262), LDV-P (accession number U15146), and the SHFV-LVR (accession number NC_003092).

^bNumbers of nsps are based on the known (EAV) or predicted (LDV/PRRSV/SHFV) replicase processing schemes as depicted in Figure 29.3 and e-Figure 29.6. Nsp8 is identical to the N-terminal domain of nsp9.

^cNot all proteins listed here have been identified in all four arterivirus particles.

^dAlternative names used in other (older) publications are indicated in brackets; SHFV protein nomenclature has been adapted with the most recent recommendations of the *Arteriviridae* study group of the International Committee on Taxonomy of Viruses (ICTV).⁶⁸

(ORF) in the genome (Table 29.1, Figs. 29.2 and 29.3). For simplicity, the GP encoded by ORF2a (PRRSV) or ORF2b (EAV/LDV) will be called GP2. Among arterivirus envelope proteins two major and five minor species are discriminated. The two major species, the nonglycosylated triple-spanning membrane protein M and GP5, form a disulfide-linked heterodimer^{57,69,174} (Fig. 29.2). By separately knocking out the expression of each of the structural proteins, it was established that

all major and minor structural proteins are required for the production of infectious progeny,^{131,232} with the possible exception of the recently discovered ORF5a protein.^{75,94}

Studies of GP2, GP3, and GP4, which form a heterotrimer in the virion^{230,232} (Fig. 29.2), have further highlighted their importance. Knockout mutants for minor structural protein genes produced noninfectious subviral particles consisting of GP5, M, N, and the genome RNA.^{231,232,243} When one of the

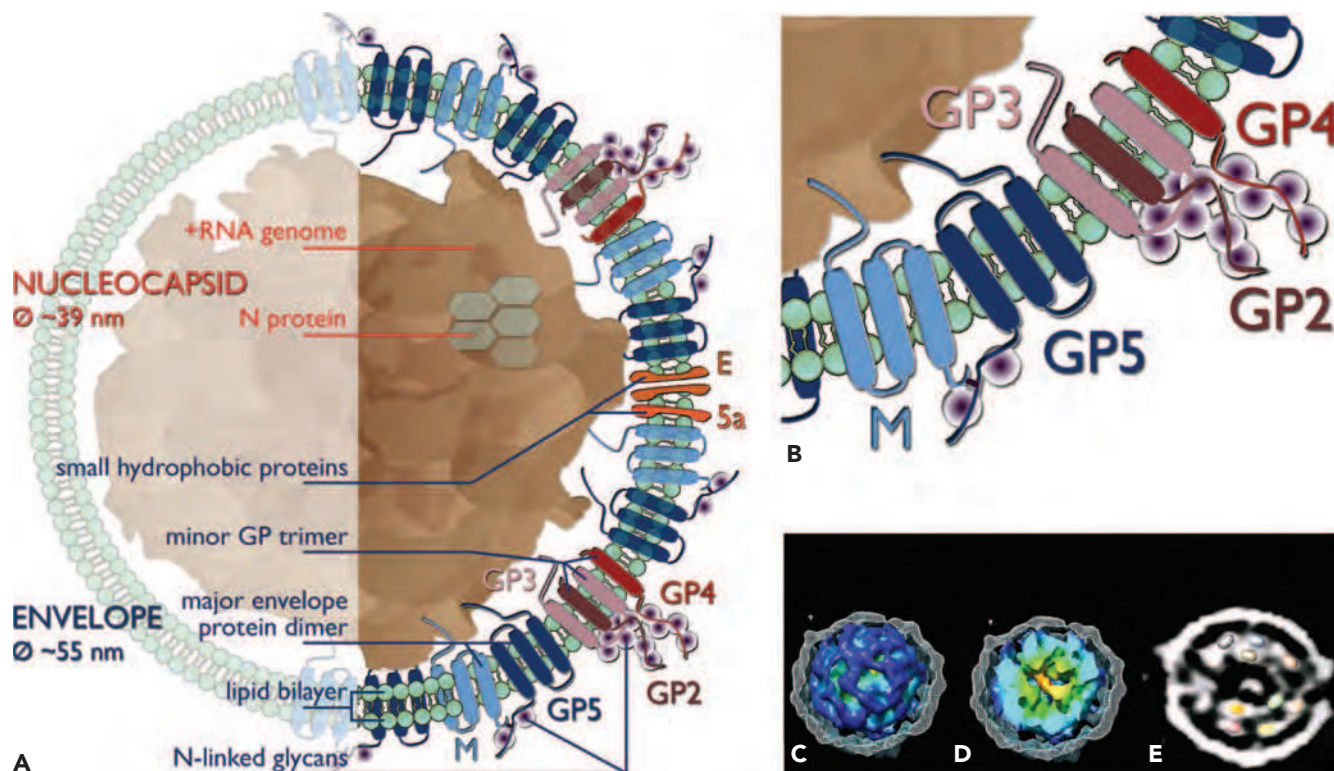


FIGURE 29.2. Arterivirus structure. **A:** The presumed location and topology of the envelope proteins GP2 to GP5, E, and M, the recently identified open reading frame (ORF)5a protein, and the N protein are shown (see also Table 29.1 and Fig. 29.3). The major envelope proteins GP5 and M form a disulfide-linked heterodimer. The minor glycoproteins GP2, GP3, and GP4 form a disulfide-linked heterotrimer. See **Panel B** for a close-up. In addition, also GP₂-GP₄ dimers (not depicted) have been identified in equine arteritis virus (EAV) particles. It should be noticed that not all proteins depicted here have been identified in all four arterivirus particles. **C–E:** Cryo-electron microscopy–based tomographic reconstruction of a porcine reproductive and respiratory syndrome virus (PRRSV) particle,¹⁸² revealing that the virion core is not solid, but consists of a two-layered shell that surrounds a hollow central cavity. **C:** Cutaway view of the internal core, obtained by peeling away the envelope (shown in mesh representation). The core, which is separated from the envelope by a 3-nm gap, appears disorganized and to consist of density strands that are bundled together into a ball. The data suggest a model for the core in which two layers of N dimers form a linked chain (see also **Panel E**). The core is shown as an isosurface, colored by the radius from the center of the particle (from red to blue). **D:** The core has been cut open to show the internal structure, including the central density (red-orange) typically seen in the tomograms. **E:** A 6.3-nm thick slab through the center of one PRRSV particle tomogram, with several copies of the crystal structure of the dimeric C-terminal domain of N, rendered at a comparable resolution and superimposed on the oblong densities in the core. (See also e-Fig. 29.2). (**C–E** from Spilman MS, Welbon C, Nelson E, et al. Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. *J Gen Virol* 2009;90:527–535, with permission.)

components of the GP2-GP3-GP4 trimer or the small non-glycosylated envelope protein (E)¹⁷⁶ was lacking, the incorporation of the three minor GPs into virions was blocked.²³¹ Taken together, these data indicate that the basic protein scaffold of the arterivirus particle consists of the three major structural polypeptides, N, M, and GP5. Whether the incorporation of (genome) RNA is essential for the formation of the nucleocapsid structure, and which RNA sequences/structures specifically interact with N, remains to be established.

GENOME STRUCTURE AND ORGANIZATION

The arterivirus genome is a plus-stranded, 3′-polyadenylated RNA molecule, likely containing a cap structure at its 5′ end.¹⁶⁵

Full-length genomic sequences (see also Table 29.1) have been obtained for European and North American isolates of EAV, a large number of European, North American, and Asian PRRSV isolates, two LDV strains, and three SHFV isolates. The arterivirus replicase gene consists of the large ORFs 1a and 1b and roughly occupies the 5′ three-fourths of the polycistronic genome (Fig. 29.3). In contrast to the more conserved ORF1b region, the size of ORF1a is variable (encoding between 1,727 [EAV] and about 2,500 amino acids [PRRSV]), which largely explains the genome size differences encountered among arteriviruses. The region downstream of the replicase gene contains 8 to 11 relatively small genes, most of which have both 5′- and 3′-terminal sequences that overlap with neighboring genes. These genes encode mostly (or exclusively) structural proteins and are translated from sg mRNAs (see below). Their organization is

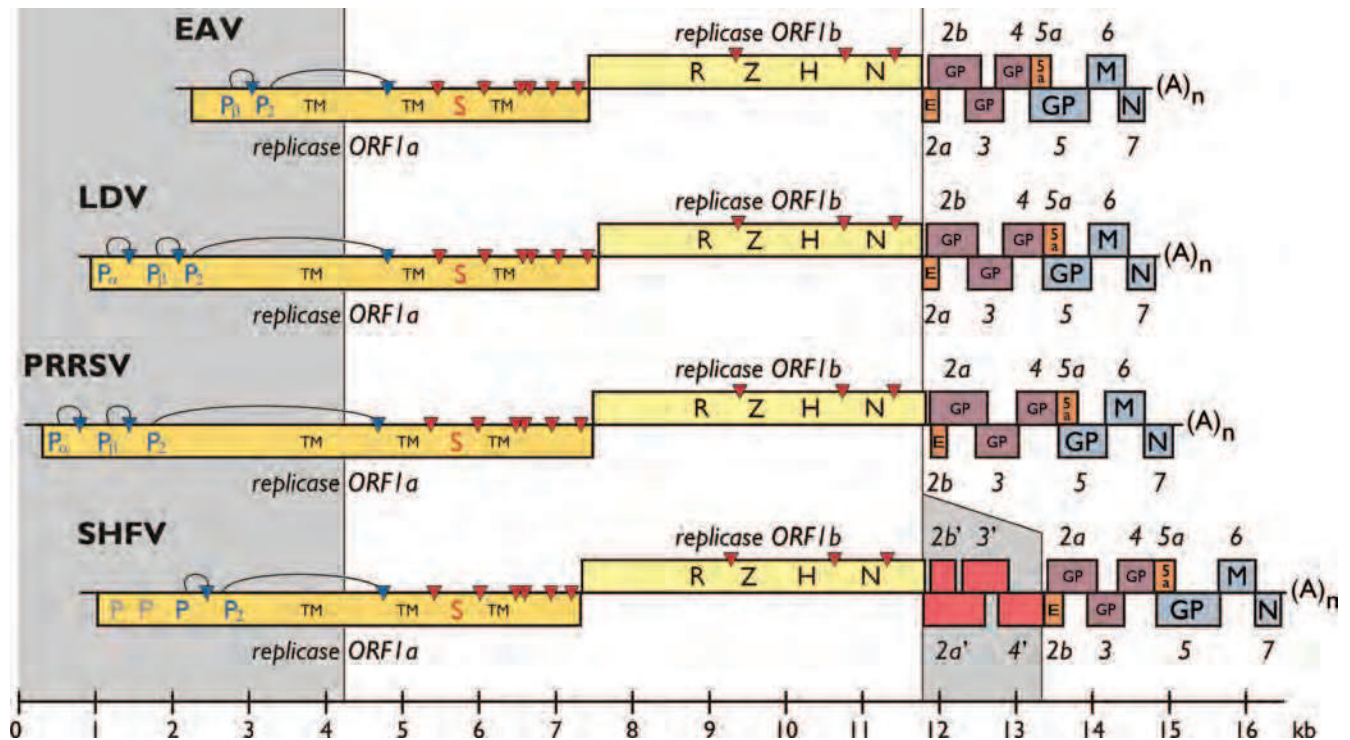


FIGURE 29.3. Arterivirus genome organization. The family prototype equine arteritis virus (EAV) is shown at the **top**. The replicase open reading frames (ORFs) 1a and 1b are followed by the gene encoding the E protein, three genes (ORFs 2a/b-4) encoding minor glycoproteins, the recently discovered ORF5a (presumably encoding a minor envelope protein), and the genes for the three major structural proteins GP5, M, and N (blue). The 3′-proximal region of the simian hemorrhagic fever virus (SHFV) genome carries a large insertion (highlighted in grey), containing four ORFs that may encode additional virion proteins. In the replicase ORFs, the positions corresponding to known or predicted cleavage sites in the encoded polyproteins is depicted. *Red arrowheads* represent sites cleaved by the nsp4 serine proteinase (S), the viral main protease. The papain-like proteinase domains (P) in the quite variable nsp1-nsp2 region and their (predicted) cleavage sites (*blue*) are also shown. The processing scheme of the SHFV nsp1 region remains to be elucidated. The three (putative) transmembrane domains (TM) in the ORF1a-encoded polyprotein are indicated (residing in nsp2, nsp3, nsp5). In ORF1b, the domains encoding the four most conserved nidovirus replicase domains are depicted: the RNA-dependent RNA polymerase (R), (putative) multinuclear zinc-binding domain (Z), RNA helicase (H), and the NendoU endoribonuclease domain (N).

generally well conserved in the arterivirus genome. An exception is the region downstream of the SHFV replicase gene, which contains four additional ORFs, comprising about 1.6 kb, which may have arisen from the duplication of ORFs 2a to 4.^{68,81}

THE ARTERIVIRUS REPLICATION CYCLE

Attachment and Entry

The entry of PRRSV and EAV requires a low pH, suggesting that it occurs via the standard endocytic route^{101,137,141} (Fig. 29.4). Clathrin heavy-chain knockdown suppressed EAV infection¹⁴¹ and electron microscopy revealed arterivirus particles contained in relatively small vesicles that appeared to be clathrin coated.^{100,101}

The host factors required for arterivirus entry have been studied in detail only for PRRSV (e-Fig. 29.3). Several viral and cellular players have been implicated in binding, entry, and uncoating, although their exact roles remain to be defined in more detail (for recent reviews see 203,224). Sialoadhesin (or sialic acid-binding immunoglobulin [Ig]-like lectin 1 [CD169]; 216), a macrophage-restricted membrane protein, mediates the

internalization of the virus by porcine alveolar macrophages (PAMs), the primary target cells of PRRSV.^{64,66} In addition, glycosaminoglycans (heparan sulfate) on the cell surface⁵¹ and sialic acids on the virion surface⁵⁰ were implicated in the initial binding step. It is believed that the virus initially binds heparin-like molecules on the cell surface and that subsequently internalization via clathrin-mediated endocytosis is triggered by the interaction of CD169 with sialic acids on the ectodomains of the GP5/M dimer.²⁰⁴ Expression of porcine CD169 in nonsusceptible cell lines can mediate PRRSV internalization, but not disassembly and productive infection,²¹⁶ indicating that additional factors must be required for successful infection. This notion is further supported by the fact that MARC-145 cells, which are commonly used to grow PRRSV, do not express CD169 on their surface.⁶⁴ In particular CD163, a member of the scavenger receptor cysteine-rich (SRCR) family, was implicated in the early stages of PRRSV infection.²⁶ Although normally a macrophage-specific antigen, CD163 is aberrantly expressed on MARC-145 cells, possibly explaining their unique susceptibility to PRRSV infection among nonengineered cell lines. Expression of CD163 from various species rendered a variety of nonpermissive cell lines susceptible to PRRSV infection, in the

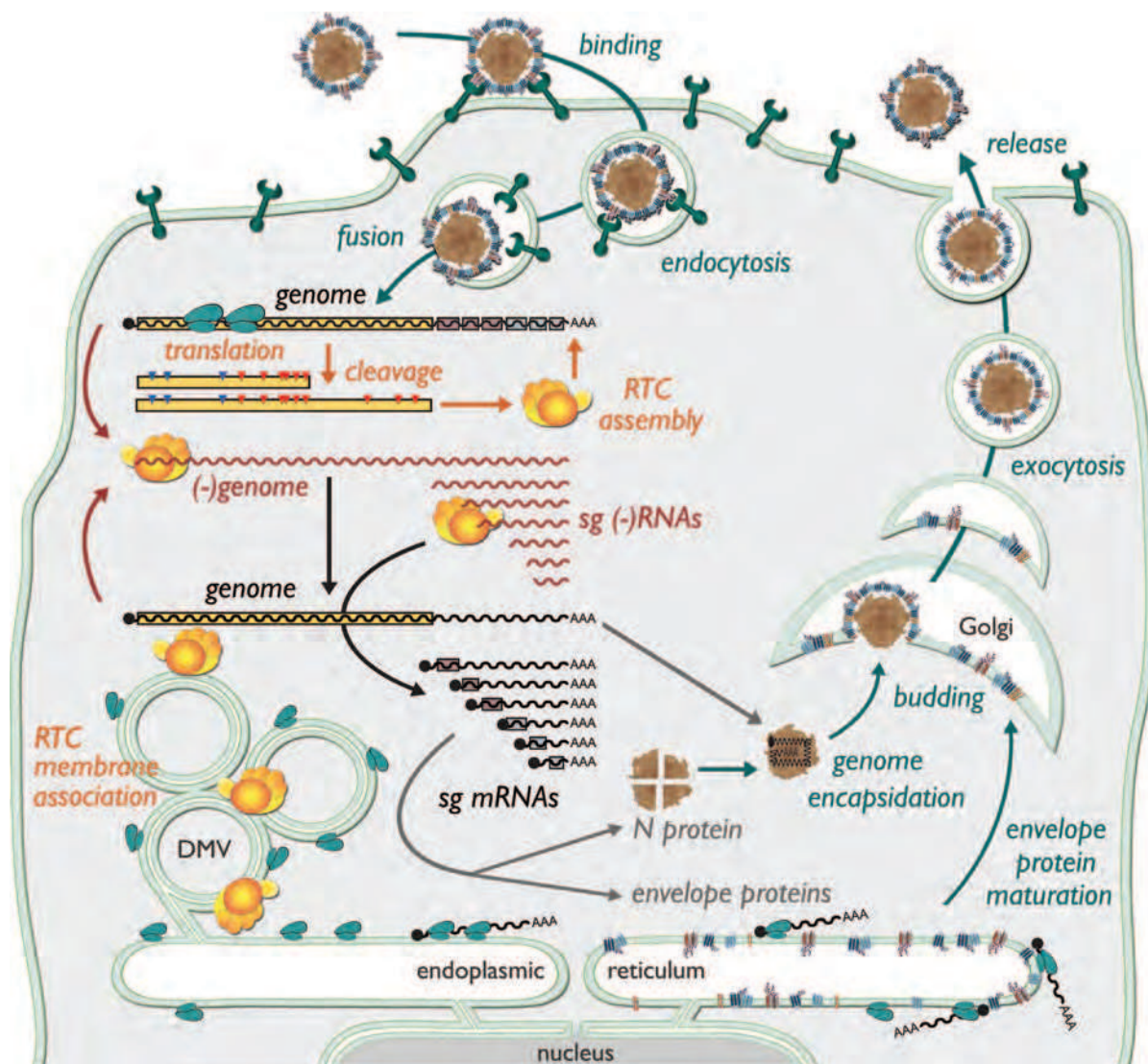


FIGURE 29.4. Overview of arterivirus replication. Following entry by receptor-mediated endocytosis and release of the genome into the cytosol, genome translation yields the pp1a and pp1ab replicase polyproteins (shown as yellow bars). Following polyprotein cleavage by multiple internal proteases, the viral nonstructural proteins assemble into a replication and transcription complex (RTC) that engages in minus-strand RNA synthesis. Both full-length and subgenome-length minus strands are produced, with the latter templating the synthesis of subgenomic messenger RNAs (mRNAs) required to express the structural protein genes in the 3'-proximal quarter of the genome. Ultimately, novel genomes are packaged into nucleocapsids that become enveloped by budding from smooth intracellular membranes, after which the new virions leave the cell by following the exocytic pathway. (See text for more details.)

absence of detectable CD169 expression. It was postulated that in PAM, CD169 and CD163 work together, with the former serving as receptor for internalization and the latter playing a key role in virus uncoating and genome release, which are thought to occur in association with the early endosome, following its acidification²¹¹ (e-Fig. 29.3).

Which virion proteins direct the fusion between viral envelope and endosomal membrane remains one of the key questions to be addressed. A role for the minor glycoproteins in arterivirus receptor recognition and tropism has not been rigorously excluded.⁴⁴ In fact, recent data show that a chimeric PRRSV carrying E, GP2, GP3, and GP4 of EAV acquired the broad tropism for cultured cells that is typical of the

latter virus.¹⁹³ These findings are in line with the previously observed phenotypes of recombinant viruses in which the GP5 or M ectodomain was replaced, which did not result in an altered tropism in cell culture.^{60,219} These studies, together with the identification of several other host factors as potential "PRRSV entry mediators",^{203,224} illustrate that several questions and controversies regarding arterivirus entry remain to be addressed.

Genome Translation and Replication

The arterivirus replication cycle (Fig. 29.4) is presumed to be entirely cytoplasmic, despite the fact that at least two viral proteins are (in part) targeted to the nucleus (see below). The

incoming genome is translated into the two large replicase polyproteins pp1a (1,727 to 2,502 amino acids) and pp1ab (3,175 to 3,959 amino acids), which comprise all functions required for viral RNA synthesis.¹³¹ Despite the relatively large 5' non-translated region (NTR), translation presumably initiates following “conventional” ribosomal scanning of the genomic 5' NTR.²⁰⁶ ORF1b translation requires a -1 ribosomal frame shift (estimated efficiency of 15% to 20%) just before ORF1a translation is terminated⁵⁴ (Fig. 29.3). The ORF1a/1b overlap region contains two signals that are assumed to promote this event: a so-called “slippery” sequence, which is the actual ribosomal frame shift site, and a downstream RNA pseudoknot structure.

Following proteolytic processing of the replicase polyproteins, a complex for viral RNA synthesis is formed that generates

a genome-length minus strand (or “anti-genome”), the template for genome replication. In addition, a complex transcription mechanism operates to produce complementary nested sets of sg-length minus-strand RNAs and sg mRNAs⁵⁵ (see below and Fig. 29.5). The RNA signals involved in arterivirus genome replication remain to be studied in detail. The coding regions of the genomes are flanked by 5' and 3' NTRs of 156 to 221 and 59 to 117 nucleotides, respectively. However, natural and synthetic defective interfering RNAs of EAV invariably require at least 300 nucleotides from both genome termini for efficient replication, indicating that replication signals extend into the coding sequences.^{130,198} Likewise, in the case of PRRSV, a so-called “kissing interaction” between the loop sequences of RNA hairpin structures in the 3' NTR and the N protein gene was found to be crucial for viral RNA synthesis.²¹⁸

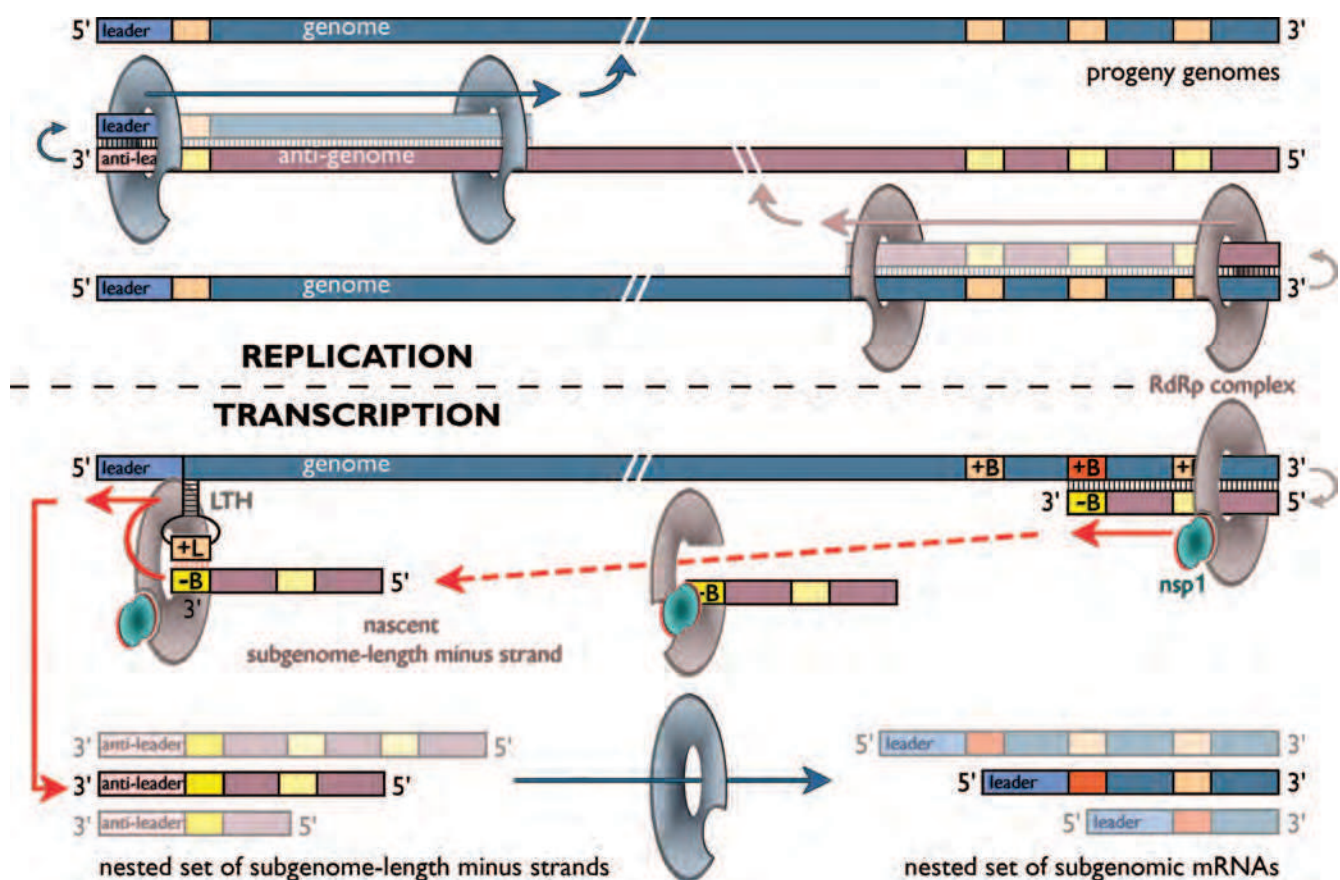


FIGURE 29.5. Arterivirus RNA synthesis. Model for arterivirus (and coronavirus) replication and transcription^{146,166–167,168} using a hypothetical arterivirus genome encoding three subgenomic messenger RNAs (mRNAs). The top half of the scheme depicts the replication of the genome by the viral RNA-dependent RNA polymerase (RdRp) complex, which requires a full-length minus-strand intermediate (anti-genome). The bottom half illustrates how minus-strand RNA synthesis can be interrupted at a body transcription-regulating sequence (TRS) (+B), after which the nascent minus strand, having a body TRS complement (-B) at its 3' end, is redirected to the leader TRS (+L) near the 5'-end of the genome. This +L sequence is thought to be “presented” for base pairing by a viral RNA structure, the leader TRS hairpin (LTH), that is critical for subgenomic RNA synthesis. Guided by a base-pairing interaction between the complementary -B and +L sequences, RNA synthesis is resumed to add the complement of the genomic leader sequence (anti-leader) to each nascent subgenome-length minus strand. Subsequently, the subgenome-length minus strand RNAs each serve as template to produce one of the subgenomic mRNAs. The RdRp complexes engaged in replication and transcription may be (partially) different. For example, in the equine arteritis virus (EAV) model, nsp1 has been identified as a regulatory factor that is dispensable for replication but required to regulate the accumulation levels of the different subgenomic RNAs, most likely by controlling a step during minus strand RNA synthesis. See text for more details.

Using a combination of approaches, detailed RNA secondary structure models were developed for the EAV 5' and 3' NTRs. In the 5' NTR, a region involved in translation, replication, and transcription,²⁰⁵ (e-Fig. 29.4B), one domain in particular was found to be crucial for sg RNA production (see below). This so-called “leader TRS [transcription-regulating sequence] hairpin” (LTH) is potentially conserved in the 5' NTR of all arteriviruses (e-Fig. 29.4C). The importance of the other structural features of the EAV 5' NTR and, for example, their involvement in RNA–protein interactions, remains to be investigated because few of these elements are conserved in other arteriviruses.²⁰⁵ A possible exception is EAV hairpin C (termed SL2 in PRRSV; 115) that was reported to be crucial for PRRSV replication and subgenomic RNA synthesis in particular (e-Fig. 29.4C).

The 3' NTR of the arterivirus genome does not contain obviously conserved primary sequences. For EAV, the 3'-terminal CC motif immediately upstream of the poly(A) tail plays a critical role in viral RNA synthesis.¹⁵ Furthermore, a stem-loop structure near the 3'-terminus of the EAV genome is also required for RNA synthesis¹⁴ (e-Fig. 29.5A) and its loop was implicated in an essential pseudoknot interaction with an upstream stem-loop structure residing in the N protein gene.¹⁵ This conformation was predicted to be conserved in all arteriviruses and proposed to constitute a molecular switch that could regulate the specificity or timing of viral (minus strand) RNA synthesis (e-Fig. 29.5B).

Various proteins from MA-104 cells bind to arterivirus-derived RNA sequences,^{90,122} and the *in vitro* RNA-synthesizing activity of semi-purified EAV replication and transcription complexes depends on the presence of a soluble host protein of 59 to 70 kD, which remains to be identified.²¹²

Synthesis and Translation of Subgenomic mRNAs

One of the hallmarks of the replication cycle of arteriviruses (and other nidoviruses) is the synthesis of a 3'-co-terminal nested set of sg mRNAs (Fig. 29.5) from which the genes in the 3' end of the genome are expressed. In the case of arteriviruses, all these genes encode structural proteins. Arterivirus sg mRNAs also have a common 5' end, the so-called “leader sequence,” which is derived from the 5' end of the genome.⁵⁵ This property is shared with coronaviruses, but—remarkably—not with some other nidoviruses (toroviruses and roniviruses; for reviews, see 83,146,168). Supported by the presumed common ancestry of the arterivirus and coronavirus replicase genes, leader-to-body fusion during arterivirus sg RNA synthesis was proposed to rely on a mechanism of discontinuous RNA synthesis similar to that previously proposed for coronaviruses. In both virus groups, short conserved TRSs are present at the 3' end of the leader sequence (“leader TRS”) and at the 5' end of each of the transcription units specifying a sg mRNA “body” (“body TRS”; reviewed in 146,168,175). The observation that arterivirus-infected cells contain a nested set of sg-length minus-strand RNAs, complementary to the sg mRNAs, is another important parallel with coronaviruses.^{30,53}

With the exception of the smallest species, the arterivirus sg mRNAs are structurally polycistronic, but most of them are assumed to be functionally monocistronic. Notable exceptions are mRNAs 2 and 5 (in EAV, LDV, and PRRSV; Fig. 29.3), which are functionally bicistronic transcripts from which the partially overlapping gene sets E/GP2 and ORF5a/GP5 are

expressed.^{75,94,176} The mRNAs tentatively numbered 4 and 6 are thought to be used to translate the corresponding SHFV gene sets, and also mRNA2 of this virus was proposed to be functionally bicistronic.⁸¹

A substantial number of models for coronavirus and arterivirus sg mRNA synthesis have been proposed and reviewed extensively (see also Chapter 28; 146,168,175, and references therein). The detection of sg-length minus strands indicated that the discontinuous step in sg RNA synthesis likely occurs during minus-strand RNA synthesis. This concept was subsequently supported by data from biochemical and genetic studies with coronaviruses and arteriviruses and resulted in a model (Fig. 29.5; e-Fig. 29.4) in which discontinuous extension of minus-strand RNA synthesis yields sg-length minus-strand templates for sg mRNA synthesis.^{166,168}

Direct proof for base-pairing between leader TRS and anti-body TRS was obtained from reverse genetics studies using an EAV infectious complementary DNA (cDNA) clone.^{147,214} The mechanism by which the transcriptase is translocated between the body and leader TRS in the genomic template, a step that may resemble copy-choice RNA recombination,^{19,147,214} remains to be elucidated. Arterivirus sg RNAs are produced in nonequimolar, but relatively constant amounts, thus providing a mechanism to regulate the expression of the various structural protein genes. EAV reverse genetics studies have rigorously demonstrated that transcription depends on duplex formation between leader TRS and anti-body TRS and that—in general—the relative amount of sg mRNA correlates with the calculated stability of this duplex.^{147,148,214} Sequences flanking the body TRS, the relative order and/or location of body TRSs in the genome, and possibly also higher order RNA structure were also shown or postulated to influence transcription.¹⁴⁵ Structural studies on the 5'-proximal part of the EAV genome²⁰⁵ placed the leader TRS in a single-stranded loop of the structure referred to as the “leader TRS hairpin” (or LTH; e-Fig. 29.4B,C), which was characterized as a critical player in transcription.²⁰⁶

At the protein level, transcription-specific functions have been attributed to several replicase subunits, in particular non-structural protein 1 (nsp1) and nsp10, for which mutations were described that resulted in the (near) complete inactivation of sg mRNA synthesis.^{138,196,198,208} EAV nsp1 controls the accumulation levels of viral genome and individual sg mRNAs in the infected cell by determining the levels at which the minus-strand templates for each of these molecules are produced.¹³⁸ An N-terminal zinc finger (ZF) domain was implicated in this function, but also other nsp1 domains appear to be important. Mutagenesis of nsp1 triggered the evolution of numerous nsp1 pseudorevertants with compensatory mutations that invariably rescued both balanced EAV mRNA accumulation and efficient virus production.¹³⁸ In the case of PRRSV, where nsp1 is internally cleaved into nsp1 α and nsp1 β , the ZF-containing nsp1 α subunit is presumed to fulfill a similar role in transcription regulation.¹⁰²

Arterivirus Proteinases and Posttranslational Processing of the Replicase

The proteolytic maturation of the arterivirus pp1a and pp1ab replicase polyproteins involves the rapid autoproteolytic release of three or two N-terminal nsps and the subsequent cleavage of the remaining part of both polyproteins by the viral nsp4 “main protease.” The posttranslational processing of the

replicase polyproteins has been studied most extensively for EAV (see 202,248, and references therein; e-Fig. 29.6A,B) for which pp1a and pp1ab are cleaved 8 and 11 times, respectively, by three ORF1a-encoded proteinases (see below). In combination with the ORF1a/1b ribosomal frame shift, this yields 13 processing end products (named nonstructural protein [nsp] 1 to 12, including nsp7 α and nsp7 β ; Fig. 29.3; e-Fig. 29.6E). Of these, nsp1-8 are generated from ORF1a, whereas nsp10-12 are entirely ORF1b-encoded and nsp9, due to the ribosomal frame shift consisting of a small, ORF1a-encoded N-terminal domain (identical to nsp8) and a large C-terminal part that is encoded by ORF1b and includes the viral RNA-dependent RNA polymerase (RdRp) domain. EAV reverse genetics studies with cleavage site mutants underscored the critical importance of replicase polyprotein processing for virus replication.^{202,209} The nsp3-8 region of pp1a (and likely also pp1ab) is subject to two alternative processing cascades, with the “major pathway” requiring an interaction with nsp2 as a cofactor, to mediate cleavage of the nsp4/5 site²²² (e-Fig. 29.6D).

The three EAV proteinase domains in nsp1, nsp2, and nsp4^{474,175,248} (e-Fig. 29.6) and their corresponding cleavage sites are well conserved in the other arteriviruses (Fig. 29.3). EAV nsp1 and nsp2 both contain a papain-like proteinase domain (PLP; formerly referred to as PCP or CP for [papain-like] cysteine protease) that mediates their rapid release from the polyprotein,¹⁷⁸ whereas nsp4 includes a chymotrypsin-like serine proteinase (SP), the arterivirus main proteinase.¹⁸⁰ PRRSV and LDV, in addition to having homologs of these three EAV proteinases, possess a fourth nonstructural proteinase,⁵² which mediates the rapid release of an additional N-terminal cleavage product. This PLP α possibly is a duplication of the proteinase (PLP β) present in the C-terminal domain of EAV nsp1 and appears to have become inactivated in EAV.⁵² The sequence analysis of the SHFV nsp1 region revealed an even more complex situation, with an array of three potential PLP domains present in the 480-residue region upstream of the (predicted) nsp1/nsp2 junction. The nsp4 SP combines the His-Asp-Ser catalytic triad of classical chymotrypsin-like proteinases with the substrate specificity of the so-called 3C-like cysteine proteinases, a subgroup of chymotrypsin-like enzymes named after the picornavirus 3C proteinases. Specific residues in the substrate-binding region of the SP are assumed to determine its specificity for cleavage sites containing Glu (or sometimes Gln) as the P1 residue and mainly Gly, Ala, or Ser at the P1' position. Nine such sites were identified in EAV pp1a/pp1ab, and they were all found to be conserved in the other family members.^{175,202,248}

Nsp4 structures have been obtained by x-ray crystallography for both EAV and PRRSV^{11,195} (e-Fig. 29.7). The protein consists of three domains, with domains I and II forming the typical chymotrypsin-like two- β -barrel fold of the SP. The C-terminal domain III is dispensable for proteolytic activity and may be involved in fine-tuning replicase polyprotein cleavage.^{200,201} Recent structural studies also elucidated the structures of PRRSV nsp1 α ¹⁸⁶ and nsp1 β ,²³⁸ including their respective PLP domains (e-Fig. 29.7), which—in line with previous studies—were both confirmed to employ a Cys-His tandem as active site residues. Both PLP α and PLP β appear to act exclusively *in cis* and the two structures indeed revealed the presence of the C-terminal region of the proteins in the PLP substrate-binding pocket, suggesting an intramolecular cleavage mechanism that

would preclude further proteolytic reactions. Both nsp1 α and nsp1 β of PRRSV have also been implicated in evasion of the host's immune response (see below), but this was most directly demonstrated for the PLP that resides in the N-terminal domain of the highly variable nsp2 subunit. This PLP2, which possesses both *cis* and *trans* cleavage activities,^{86,179} not only directs the critical cleavage of the nsp2/3 site in pp1a and pp1ab, but is also able to remove ubiquitin (Ub) and Ub-like modifiers like ISG15 from yet-to-be-identified substrates in the infected cell.²¹³ The protein is distantly related to the ovarian tumor domain (OTU) family of deubiquitinating enzymes.^{76,123,187}

Replicase Proteins and the Replication Complex

Although accelerated by research efforts following the emergence of severe acute respiratory syndrome (SARS)-coronavirus, the functional dissection of the complex array of nidovirus nonstructural protein functions is still in its infancy. Even the arterivirus replicase polyproteins are of extraordinary size and complexity, despite their twofold smaller size compared to nidoviruses with larger genomes like coronaviruses and roniviruses. Therefore, future studies will undoubtedly reveal both novel similarities and differences between these two groups. In arteriviruses, with the notable exception of the role of nsp1 in sg mRNA synthesis (see above), the ORF1a-encoded functions mainly appear important for the regulation of replicase gene expression (by proteolytic processing; see above) and formation of the membrane-anchored “scaffold” for the replication/transcription complex. The ORF1b-encoded proteins, on the other hand, appear to be more directly involved in viral RNA synthesis.

Except for the proteins from the nsp1 region,^{31,197} all replicase subunits localize to the perinuclear region of the infected cell (e-Fig. 29.8A–D; 207), where they are associated with intracellular membranes that are derived from the endoplasmic reticulum (ER). Upon arterivirus infection, these host cell membranes are modified into vesicular double-membrane structures that presumably carry the viral replication complex (e-Fig. 29.9A–F).^{149,177,212} The formation of closely paired membranes and double membrane vesicles (DMVs) is a typical feature of arterivirus-infected cells described many years ago.^{18,184,234} Recent electron tomography studies of EAV-infected cells revealed that these structures are in fact interconnected and form a network of modified ER⁹⁹ (e-Fig. 29.9E). Biochemical and electron microscopy studies have implicated ORF1a-encoded subunits that contain hydrophobic, probable trans-membrane domains (in particular nsp2, nsp3, and nsp5; e-Fig. 29.6C) in the formation of these membrane structures.^{71,149,158,177,207}

Replicase ORF1b is the most conserved part of the arterivirus genome and encodes the core enzymes for viral RNA synthesis—RdRp (nsp9) and helicase (nsp10).^{54,83,140} Recombinant EAV nsp9 is able to initiate RNA synthesis *de novo* in the absence of other viral or cellular proteins, but could not utilize sequences derived from the 3' end of the viral genome as a template,¹³ suggesting additional requirements for its activity *in vivo*. The predicted NTP binding and superfamily 1 helicase activities of arterivirus nsp10 were corroborated by *in vitro* assays with recombinant nsp10. These also revealed the 5'-to-3' polarity of the unwinding reaction, a property shared with coronaviruses,^{12,169} although it has not been reconciled with the protein's presumed role in unwinding local double-stranded

RNA structures that might hinder the RdRp during viral RNA synthesis, which proceeds in the opposite direction. As in all nidoviruses, the helicase is linked to an N-terminal zinc-binding domain that might assist the proper folding of nsp10 and/or mediate interactions of the protein with its substrate RNAs. This domain was also implicated in a remarkable transcription-specific defect.^{208,210}

Advanced bioinformatics studies provided the first evidence to suggest that nsp11, and its coronavirus homolog nsp15, contain a nidovirus-specific endoribonuclease activity (NendoU; 173). Subsequently, this prediction was experimentally verified in biochemical assays for recombinant coronavirus nsp15⁹¹ and EAV nsp11.¹³⁹ In the meantime, the site-directed mutagenesis of key NendoU residues had been found to exert pleiotropic effects on EAV RNA synthesis, including a complete block in some of the mutants and more moderate effects in others.¹⁵⁷ The exact function of the NendoU domain, a genetic marker of vertebrate nidoviruses,¹⁴⁰ remains to be elucidated, and in particular its substrate specificity in the infected cell is an enigma. Recombinant NendoU-containing proteins exhibit broad substrate specificity *in vitro*, processing both single-stranded and double-stranded RNA substrates 3' of pyrimidines.¹³⁹ However, in the context of the infected cell, NendoU activity or substrate specificity may be controlled via specific protein–protein interactions or compartmentalization of the enzyme as part of the membrane-associated replication and transcription complex.

The final ORF1b-encoded replicase subunit of arteriviruses, nsp12, has not been the subject of experimental studies, and sequence comparisons did not suggest a function or a relationship to any other known protein family.

Assembly and Release

EAV N partially co-localizes with the complex for viral RNA synthesis (197; e-Fig. 29.8D). Because N is not required for replication or transcription,^{131,208,232} this suggests that genome encapsidation may occur (or begin) at the site of viral RNA synthesis. Recent electron tomography studies of EAV-infected cells revealed a network of N-containing sheets and tubules in close vicinity of the membrane structures where viral RNA synthesis is thought to occur, but the functional significance of these N structures remains to be studied in more detail.⁹⁹

Arteriviruses acquire their envelope by budding of preformed nucleocapsids into the lumen of the smooth endoplasmic reticulum and/or the Golgi complex^{121,184,234} (e-Fig. 29.8E–F and e-Fig. 29.9G–H). Most arterivirus envelope proteins are retained in intracellular membranes (e-Fig. 29.8E–F), and the formation of the GP5-M heterodimer is a primary determinant of virus budding.^{231,232,243} The transport of GP5 and M to the Golgi complex appears to depend on complex formation and correlates with the production of infectious virus.^{60,174,219} After budding, virions accumulate in intracellular vesicles, which are transported to the plasma membrane and release the progeny virus.

Major Structural Proteins

The three major structural proteins GP5, M, and N are encoded—in this order—by the three most 3'-proximal ORFs in the arterivirus genome (Fig. 29.3 and Table 29.1). N is small (12 to 15 kD) and contains many basic residues, in particular in its presumably disordered N-terminal domain, which

is thought to interact with the genomic RNA during nucleocapsid assembly.^{59,61} The C-terminal “capsid-forming” half of N forms dimers in solution and is the basis for a proposed “nidovirus nucleocapsid fold” that is also encountered in the much larger coronavirus N protein.⁶¹ The EAV and PRRSV N proteins are phosphorylated.^{56,235} In the case of PRRSV N, phosphoserines mapped to both the RNA binding domain and the capsid-forming domain where they may modulate nucleic acid binding activity or protein–protein interactions.²³⁵

The nonglycosylated M protein (16 to 20 kD) resembles the coronavirus M protein in that its N-terminal half presumably traverses the membrane three times,^{56,70,127} resulting in an N_{exo}-C_{endo} configuration with a short ectodomain of only 10 to 18 residues exposed at the virion surface. One of the membrane-spanning fragments is thought to function as an internal signal sequence. The arterivirus M protein forms disulfide-linked heterodimers with the 24 to 54 kD GP5, a step probably driven by the formation of a disulfide bridge between a conserved Cys residue in the M ectodomain and a Cys residue in the GP5 ectodomain.^{57,69,174} EAV GP5-M heterodimers are thought to be essential for virus assembly, possibly by inducing the membrane curvature required for virus budding.^{57,60,174}

Despite considerable differences in primary structure, the major GPs of arteriviruses (GP5) share common structural features. They contain an N-terminal signal sequence that is assumed to be cleaved from a short ectodomain. The central hydrophobic region probably spans the membrane three times and is followed by a cytoplasmic domain of 50 to 75 amino acids. In the case of LDV and PRRSV, the ectodomain is only about 30 residues long and contains one to three N-linked glycans.^{70,127} The ectodomain of EAV GP5 is 95 residues long and usually possesses a single N-linked polylactosamine side chain,⁵⁶ although in some strains an additional N-linked glycan is present. In addition, in the case of LDV, GP5 glycosylation occurs by the addition of variable numbers of lactosamine repeats.¹¹⁰ Nonneuropathogenic and neuropathogenic strains typically contain three and one polylactosaminoglycan chain(s), respectively (see below).

Minor Structural Proteins

The minor arterivirus GP encoded by ORF2a (PRRSV) or ORF2b (EAV/LDV) is a conventional class I integral membrane protein with an N-terminal signal peptide, a C-terminal transmembrane segment, and 1 to 4 potential N-glycosylation sites in its ectodomain. GP2 occurs in EAV-infected cells in a variety of monomeric conformations and in complex with other minor GPs.^{228,231} Complex formation is a prerequisite for incorporation into virions.^{231,232} Cysteine residues in the EAV GP2 ectodomain form both intramolecular and intermolecular cysteine bridges, the latter involving a cysteine in the ectodomain of GP4, which also is a typical class I integral membrane protein.²²⁸

GP3 is a heavily glycosylated integral membrane protein with an uncleaved N-terminal signal sequence and a hydrophobic C-terminal domain, suggesting the protein is anchored in the membrane with both termini.^{87,229} Like GP2 and GP4, EAV GP3 localizes to the endoplasmic reticulum, both in infected cells and in expression systems.²²⁹ Following initial conflicting reports, PRRSV GP3 is now firmly believed to be present in virions,^{48,232} which is also in line with the data obtained for EAV. Following virus release, GP3 becomes disulfide-linked to

the GP2-GP4 heterodimers, and this postassembly maturation event yields a complex of three covalently bound minor GPs. As a result, EAV particles contain both GP2-GP4 heterodimers and GP2-GP3-GP4 heterotrimers.^{230,231}

Like GP2, GP4 is a predicted class I membrane protein with a cleaved signal sequence and multiple N-glycosylation sites in its ectodomain.^{129,215,229} Little is known about the properties or function of GP4, apart from the oligomerization described above and the finding that GP4 may be responsible for an interaction of the GP2-GP3-GP4 trimer with GP5.⁴⁴ Recent reverse genetics studies with PRRSV mutants lacking specific glycans in GP2, GP3, and GP4 documented the general importance of these posttranslational modifications for virus viability and interactions with CD163.⁴⁵

The higher order structure of the small E protein is unknown, but this protein is essential for virus infectivity¹⁷⁶ and may in fact be associated with trimer of minor GPs.²³¹ The protein has been proposed to oligomerize and form an ion channel that could play a role during viral entry.¹⁰⁶ Furthermore, its N-terminus contains a myristoylation signal that is conserved across arteriviruses and is functional in EAV and PRRSV. A block of the fatty acid addition was not lethal, but knockout mutants were crippled and displayed a small-plaque phenotype.^{63,190}

Finally, another gene encoding a small generally hydrophobic protein was recently identified in EAV⁷⁵ and PRRSV.⁹⁴ Bioinformatics analyses revealed a potential open reading frame (ORF5a) overlapping the 5' end of ORF5, and the ORF5a protein was identified in purified PRRSV virions. EAV reverse genetics revealed that the protein is not essential, but knockout mutants showed a significant reduction of progeny titers.

Replication in Cultured Cells and Host Cell Interactions

With the exception of EAV, arteriviruses have a very restricted host specificity (for reviews, see 155, 175 and references therein). LDV grows in primary cultures of mouse macrophages, but not in macrophage or other cell lines. In addition to primary macrophages from their respective hosts, SHFV and PRRSV also replicate in cell lines of African green monkey kidney cells (MA-104 and derivatives thereof, like the MARC-145 cell line discussed above). EAV replicates efficiently in primary cultures of horse macrophages and kidney cells, and—remarkably—also in a variety of cell lines such as baby hamster kidney, rabbit kidney, and African green monkey kidney and mouse C2C12 cells.²⁴⁵

One-step growth experiments have shown that maximum progeny virus titers are generally released by 10 to 15 h postinfection. The maximum titers obtained in cell culture are 10^6 to 10^7 tissue culture infectious dose (TCID)₅₀/ml for PRRSV, but may exceed 10^8 PFU/ml for EAV and SHFV.^{155,175} In general, arterivirus infection of macrophages and cell lines is highly cytotoxic, resulting in rounding of the cells and detachment from the culture plate surface, although recently a model for persistent noncytopathic infection with EAV in human HeLa cells was established.²⁴⁵

Arteriviruses interact with a variety of host factors and mechanisms. For example, arteriviruses were claimed to induce or modulate apoptosis in cell lines and/or cultured macrophages.^{3,39,185,251} PRRSV also induces apoptosis in germ cells *in vivo*.¹⁸⁸ In both macrophages and MARC-145 cells, PRRSV stimulated antiapoptotic pathways early in infection, but late in infection cells died from caspase-dependent apoptosis, culminating in secondary necrosis.³⁹

Despite the cytoplasmic replication cycle of arteriviruses, some viral proteins are directed (in part) to the nucleus of infected cells (e-Fig. 29.8), specifically nsp1 (see above) and the N protein. A nuclear localization signal, for interaction with the nuclear transporters importin α and β , was identified at positions 41–47 of PRRSV N,¹⁶⁴ which—like its EAV counterpart—accumulates in the nucleoli of infected cells.^{164,197} Remarkably, a block of CRM1-mediated nuclear export with the drug leptomycin B resulted in the nuclear accumulation of EAV N, indicating that the protein apparently shuttles between cytoplasm and nucleus before playing its role in cytoplasmic virus assembly.¹⁹⁷ Various nuclear host proteins interact with PRRSV N (reviewed by 240), including fibrillarin, nucleolin, and poly(A)-binding protein, but the functional implications of these findings remain to be unraveled. Using reverse genetics, a knockout mutation for the nuclear localization signal of PRRSV N was engineered and yielded a viable, although seriously attenuated, mutant virus.¹⁰⁵ Compared to the wild-type control, pigs infected with this mutant virus showed reduced viremia and significantly higher neutralizing antibody titers.

PATHOGENESIS AND PATHOLOGY OF ARTERIVIRUS INFECTIONS

The natural host range of arteriviruses is restricted to horses and donkeys (EAV), pigs (PRRSV), mice (LDV), and several genera of African and Asian monkeys (SHFV). Recent outbreaks of equine viral arteritis in New Mexico, United States (2006) and Normandy, France (2007) increased the interest of horse owners and veterinarians in EAV treatment and vaccines. Highly virulent PRRSV variants, causing the so-called “porcine high fever disease,” emerged in China in 2006 and still continue to cause problems in this and surrounding countries.^{107,194,246}

Site of Primary Replication, Spread, and Cell and Tissue Tropism

Macrophages appear to be the primary target cell for all arteriviruses.¹⁵⁵ Cell surface molecules mediating entry into these cells are only known for PRRSV and have been discussed above. Following respiratory transmission, EAV initially replicates in lung macrophages and endothelial cells.^{5,88} The virus then spreads to draining lymph nodes, from where it becomes disseminated throughout the body via the circulation. By the third day of infection, a viremia has developed and virus can be isolated from practically all tissues.

Reported primary target cells for PRRSV replication are fully differentiated porcine lung alveolar macrophages and other cells of the monocyte/macrophage lineage including pulmonary intravascular macrophages, subsets of macrophages in lymph nodes and spleen, and intravascular macrophages of the placenta and umbilical cord.^{65,104,191} After spreading through the circulation, PRRSV replicates persistently in tonsils, lungs, and lymphoid organs (reviewed in 192).

IMMUNE RESPONSES

Several arteriviruses cause persistent infections despite the presence of an adaptive immune response. Neither neutralizing antibodies nor effective helper and cytolytic T lymphocytes are

able to control virus replication in these persistently infected animals. Therefore, arteriviruses must have developed strategies to evade immune responses, underpinning the importance of studying immunity in natural and experimental infections in order to enhance our understanding of the underlying mechanisms. For a more detailed overview of the immune response to arterivirus infection, the reader is also referred to a variety of other review articles,^{5,43,114,125,134} and references therein.

Innate Immune Response

It is rather unclear at present which pattern-recognition receptors (PRRs) of the innate immune system recognize arteriviruses during infection of different cell types. Several reports have suggested or speculated on the involvement of toll-like receptor 3 (TLR3) during infection of PRRSV in macrophages and lymphoid tissue (see 43 and references therein). A study with EAV in knockout mouse embryonic fibroblasts suggested that of the cytosolic retinoic acid inducible I-like receptors (RLRs), melanoma differentiation-associated gene (MDA5) is predominantly involved in the recognition of this virus.²¹³ Experimental infection of TLR7^{-/-} mice with LDV revealed the importance of this TLR expressed by plasmacytoid dendritic cells (pDCs) for the induction of type I interferon (IFN) and the activation of lymphocytes during LDV infection.¹

In vitro studies using alveolar and blood-derived macrophages demonstrated that tumor necrosis factor (TNF)- α and other proinflammatory cytokines are induced following infection with virulent EAV strains.^{5,132} In the case of PRRSV, it is generally believed that a rather weak innate immune response is induced (see 5,43,132,134, and references therein). Induction of TNF- α as well as IFN- α by PRRSV and also the sensitivity to these innate cytokines, seems to depend on the isolate. Chen et al.³³ suggested that this may at least in part relate to variability in nsp2, in which abundant changes and deletions were found among different isolates, which seem to influence host immune responses. Multiple studies reported that PRRSV induces interleukin 8 (IL-8), whereas the induction of IL-6 as well as that of IL-10 is debated (see also below; 43, and references therein).

LDV induced natural killer (NK) cells in infected mice and as a consequence a large increase in serum IFN- γ was observed, but these responses were unable to control LDV replication.¹²⁴ LDV also elicits IFN- α induction through TLR7 activation in plasmacytoid dendritic cells, but the virus is not sensitive to a systemic IFN- α response in mice.¹

Humoral Immune Response

Antigenic cross-reactivity between different arterivirus species has not been demonstrated, with the exception of antibodies directed against the single linear neutralization site of LDV GP5, which do not only neutralize LDV, but also PRRSV.¹⁵⁶ Sera from EAV-infected horses recognize N, M, GP5, and GP2.^{34,118} In addition, antibodies against the nonstructural proteins nsp2, nsp4, nsp5, and nsp12 were found in experimentally or persistently infected horses, whereas animals vaccinated with a modified live virus (MLV) vaccine against EAV produced antibodies against nsp2 and nsp12, and much less against nsp4 and nsp5.⁷⁹ PRRSV-infected pigs produce antibodies directed against the structural proteins GP2 to GP5, M, and N, with the antibodies recognizing N being detected earliest and most abundantly.^{43,112,128} The early humoral response against PRRSV also

includes antibodies against nsp1 and particularly nsp2, which develop to titers as high as those of the anti-N antibodies.⁹⁵

It has been proposed that LDV- and PRRSV-specific antibodies contribute to antibody-dependent enhancement (ADE) of infection.^{25,241,242} For PRRSV, certain nonneutralizing epitopes in the N and GP5 proteins induce antibodies that seem responsible for ADE through opsonization, leading to enhanced internalization of the virus into macrophages.^{27,43,125}

Neutralizing antibodies (NAs) in arterivirus-infected animals are predominantly directed to the major glycoprotein GP5.^{6,24,34,82,143,153,154} The neutralization site in EAV, PRRSV, and LDV GP5 was mapped to the ectodomain of the protein.^{8,34,110,154} For EAV, its four major neutralization sites are conformation-dependent, and interaction of GP5 with M is critical for neutralization.⁸ For LDV and PRRSV, the primary neutralization site was mapped close to the GP5 N-terminus and exhibits 77% amino acid identity, probably explaining the observation that LDV neutralizing antibodies to GP5 also neutralize PRRSV.^{154,156} For PRRSV, a second NA-binding domain was identified in the GP4 ectodomain¹²⁹; however, neutralization by monoclonal antibodies directed against this region is less effective than neutralization by GP5 antibodies.²²³ The GP4 region is subject to immune selection, and it is therefore highly heterogeneous among different PRRSV strains.^{40,41}

The production of NA in EAV-infected horses coincides with virus clearance, suggesting that the humoral immune response plays an important role in recovery.⁷⁷ In contrast, NA against LDV and PRRSV are detected only very late, 1 to 2 months after infection, are produced at low levels, and are believed to not or hardly reduce viremia.^{24,58,112,241} One of the most common hypotheses for the delayed or absent NA production in PRRSV infection, or after vaccination, is the presence of an immuno-dominant decoy epitope, just upstream of the GP5 neutralization epitope, which induces a strong nonneutralizing antibody response.¹⁴³ Insertion of another epitope in between the neutralizing and decoy epitopes increased the neutralizing response, suggesting that the juxtapositioning of the two original epitopes indeed plays a role.⁷² In addition, the glycosylation state of the GP5 ectodomain in the vicinity of the neutralizing epitope and glycosylation of GP3 may influence the efficiency of NA production.²²⁰ Similarly, in the case of nonneuropathogenic strains of LDV, the GP5 ectodomain contains three polylactosaminoglycan chains, as opposed to a single polylactosaminoglycan in the ectodomain of neuropathogenic strains. It was postulated that the nonneuropathogenic strains can establish persistent infections because neutralizing antibodies bind less efficiently to the highly glycosylated ectodomain of their GP5. In contrast, neuropathogenic strains are not able to persist.^{32,110,155} Yet another explanation for inefficient neutralization was inspired by the observation that PRRSV infection in piglets manipulates the development of the B-cell repertoire.²¹ Together with the general idea that PRRSV suppresses (innate) immune responses (see below), this may explain the delayed and aberrant antibody production that is observed (reviewed in 43,89,97,114).

The humoral immune response against SHFV varies with the species of monkey and the virus isolate that is tested.^{84,85} The rapid death of macaques after SHFV infection precludes an effective host immune response. Virulent SHFV strains, which cause acute disease in patas monkeys, induced neutralizing antibodies at 7 days postinfection. The production of neutralizing antibodies correlated with the complete clearance of the virus from the circulation by 21 days postinfection.

On the other hand, SHFV strains that cause a persistent infection in these monkeys induce very low antibody titers.

Cell-Mediated Immune Response

Cell-mediated immunity (CMI) to arterivirus infection has not yet been characterized in great detail. Studies in ponies experimentally infected with EAV have shown that cytotoxicity induced by EAV-stimulated peripheral blood mononuclear cells (PBMC) was virus-specific, genetically restricted, mediated by CD8⁺ T cells, and that the precursors persist for at least 1 year after infection.^{5,28}

Cell-mediated responses in PRRSV-infected animals include CD4⁺, CD8⁺, and double positive T cells, which appear transiently between 2 and 8 weeks after experimental infection (reviewed in 43,134,237) or become more pronounced at later stages.¹²⁶ The abundance of PRRSV-specific T cells and IFN- γ -producing cells in both acute and persistently infected animals is highly variable and does not correlate to the level of virus in lymphoid tissue.^{58,126,237} Several studies indicate that the strongest CMI inducers of PRRSV are proteins M, N, and GP4.⁴³

Cytotoxic and helper T-cell responses were detected in LDV-infected mice, but did not reduce LDV replication.⁶⁷ Additional studies are required to determine whether there is a correlation between T-cell responses *in vitro* and protection *in vivo*, and the overall data suggest that the arteriviruses probably directly, or indirectly, manipulate CMI (see below).

Immune Evasion

Not much is known about the mechanistic details of immune evasion by the arteriviruses, but it is clear that modulation of the immune response occurs on different levels. An expanding body of data documents the suppression of innate immune responses (reviewed for PRRSV in 240) and several reports suggest that arteriviruses also manipulate CMI. Downregulation of major histocompatibility complex class I and II (MHC-I and MHC-II) molecules on the surface of antigen-presenting cells (APCs) was shown for PRRSV,^{43,125} and co-infection with LDV causes a delay of the CD8⁺ T-cell-mediated immune response to Friend virus in mice, suggesting manipulation of the MHC-I presentation pathway.¹⁶²

With regard to the molecular mechanisms underlying immune suppressive activities, few data are available at present. Several reports previously suggested that PRRSV significantly induces IL-10 production in pigs during the first 2 weeks of infection. This immunosuppressive cytokine interacts with a wide array of immune cells, including the PRRSV target cells from the monocyte/macrophage lineage, to downregulate in particular cell-mediated innate and adaptive immunity. Recent experiments suggest that PRRSV N may be responsible for IL-10 upregulation during infection, and this may be achieved through induction of specific regulatory T-cell populations.^{172,192,233,240}

In addition to N, three nonstructural proteins have been implicated in arterivirus immune evasion: nsp1 (nsp1 α and nsp1 β in PRRSV), nsp2 (PRRSV and EAV), and nsp11 (PRRSV). All three proteins were suggested to suppress innate immune signaling induced by the RLR- or TLR-innate sensors, or TNF- α , which lead to IFN- β and/or nuclear factor kappa B (NF- κ B) expression.^{16,240} PRRSV nsp1 α and nsp1 β both inhibit the expression of IFN- β after induction of innate responses by Sendai virus or double-stranded DNA (dsRNA),¹⁶ and nsp1 β

also inhibits signaling downstream of type 1 IFNs by inhibiting nuclear translocation of STAT1.³¹ Furthermore, both nsp1 α and nsp1 β suppress NF- κ B activation.^{16,181} Kim et al.⁹⁶ showed degradation of cAMP response element-binding (CREB)-binding protein induced by PRRSV nsp1 during infection, causing inhibition of interferon regulatory transcription factor 3 (IRF3) transcription factor activity in the nucleus. Besides localizing to the perinuclear region with other nsps, nsp1 is partially transported to the nucleus,^{96,197} which may be connected to at least some of its immune evasive activities.

Arterivirus nsp2 contains a papain-like cysteine protease (PLP2) in its N-terminal domain that cleaves the nsp2/nsp3 junction in the replicase polyproteins. This protease is distantly related to the OTU family of deubiquitinating enzymes (DUB).¹²³ Upon overexpression, EAV and PRRSV PLP2 showed a general DUB activity toward cellular ubiquitin conjugates, and also cleaved the IFN-induced ubiquitin homolog ISG15, which is thought to have antiviral activity.⁷⁶ Indications that these DUB activities could be functional in the suppression of innate immune responses were obtained from experiments in which the EAV PLP2-DUB suppressed TNF α -induced NF- κ B signaling in 293T cells. In addition, the PLP2-DUB of PRRSV inhibited the NF- κ B signaling pathway, as well as the IRF3-dependent IFN- β pathway induced by Sendai virus infection.^{109,187} PRRSV PLP2-DUB appears to remove K48-linked polyubiquitin from I κ B α to prevent its proteasomal degradation and thereby downstream signaling toward NF- κ B activation. Besides the PLP2 domain of PRRSV nsp2, also the variable regions in the central part of this large protein could influence antiviral responses.³³ Recent data suggest that PLP2 of all arteriviruses suppresses RLR-mediated IFN- β induction by removing K63-linked polyubiquitin from RIG-I, which results in inhibition of downstream signaling.²¹³

Probably too few data are available at present to firmly establish the suppression of IFN- β production by arterivirus nsp11.¹⁶ The RNase activity of the NendoU domain in PRRSV nsp11 has been implicated in this activity,^{171,240} but it is unclear whether nsp11 confers specific activity targeting certain innate immune responses or might attack the overall mRNA population of the cell on the basis of its RNase activity, thus inducing a translational shut-off.

RELEASE FROM THE HOST AND TRANSMISSION

In nature, EAV and PRRSV are transmitted primarily via the respiratory route.^{199,226} Both viruses may persist in the semen of infected male animals, and are shed in milk of infected female animals, making vertical transmission an important secondary route of infection. In addition, LDV is efficiently transmitted from mother to fetus,²⁵⁰ and also sexual transmission was reported.²³ PRRSV was shown to replicate in testicular germ cells such as spermatids and spermatocytes¹⁸⁸; infectious EAV is excreted in semen by persistently infected “carrier” stallions and can be transmitted to broodmares.^{5,199} Both EAV and PRRSV are furthermore shed in virtually all body secretions, including saliva, respiratory tract secretions, oropharyngeal secretions, urine, and feces (reviewed in 38,88). PRRSV can also be mechanically transmitted in pig herds through aerosols, infected needles, contaminated boots and coveralls, and carrier insects like

houseflies.^{38,144,151} In contrast to the other arteriviruses, SHFV is not transmitted transplacentally from mother to offspring.⁸⁵

VIRULENCE

Sequence comparisons revealed that the highly pathogenic PRRSV strain causing the “porcine high fever disease” in Asia since 2006 originated from Chinese domestic type II viruses (which are related to the VR-2332 genotype II prototype strain).²³⁶ They all have the same striking deletions of a conserved leucine and a 29-amino acid stretch in *nsp2*,^{107,170,194} but these typical differences were found to be unrelated to the increased virulence.²⁴⁷

Infectious full-length cDNA clones were constructed of both cell culture-adapted²⁰⁸ and virulent⁷ EAV isolates, which can be used to study the determinants of virulence. Comparative sequence analysis of EAV strains that differ in virulence identified potentially relevant amino acid substitutions in both structural and nonstructural proteins. Reverse genetics experiments then showed that substitutions in the structural proteins may lead to more severe attenuation than those in the non-structural proteins.²⁴⁴ Collectively, interactions of both major (GP5 and M) and minor (GP2, GP3, and GP4) envelope proteins seem to influence tropism, and mutations in these proteins can therefore affect virulence.⁸⁰

PERSISTENCE

Typical for natural EAV infections is the persistence that occurs in about 35% of the infected stallions.¹⁹⁹ The virus persists in the reproductive tract of these “carrier stallions” and is continuously shed into the semen for a long time. In contrast, persistent infection in mares generally does not last longer than one month.⁸⁸ The establishment and maintenance of persistent infection is testosterone-dependent in stallions, and high serum titers of neutralizing antibodies are insufficient to clear the virus.^{5,199} A study of EAV evolution in persistently infected stallions strongly suggested that neither defective interfering particles nor immune evasion from B-cell responses are involved in persistence.⁹

Persistence of PRRSV has been commonly observed for up to 150 days in pigs, and up to 210 days in congenitally infected piglets.³⁵ Although the mechanisms underlying the failure to promptly clear PRRSV infection are poorly understood, it appears that a major reason is the inability of pigs to develop effective protective immune responses, which is probably due to the concerted immune evasion strategies exploited by the virus.^{117,125}

SHFV appears to be endemic among several species of African monkeys, in which it causes asymptomatic acute or persistent infections depending on the virus strain.¹¹³ LDV is also able to establish a largely asymptomatic persistent infection.¹⁵⁵

EPIDEMIOLOGY

EAV

Despite its worldwide distribution, EAV has not caused many disease outbreaks. The first recognized and most severe epizootic occurred in 1953 in Bucyrus, Ohio, U.S.A.⁶² Milder out-

breaks have been reported from elsewhere in the United States, Canada, and a variety of European countries, with recent epizootics occurring in Normandy (France, 2006) and New Mexico (U.S.A., 2007).⁸⁸ The apparent discrepancy between the high incidence of the virus and the relatively low number of recorded disease outbreaks is explained by the predominantly subclinical course of infection.

A recent genome-wide association study pin-pointed genetic differences within and among horse populations that are associated with susceptibility of CD3+ T lymphocytes to EAV infection *in vitro*.⁷⁸ The genomic region identified encodes proteins (potentially) involved in virus entry, cytoskeletal organization, and antiviral innate responses, and the association of a specific haplotype (ECA11) with susceptibility to EAV infection will allow the development of a targeted molecular test for diagnostic purposes and large-scale studies.

PRRSV

PRRS was first detected in 1987 in the United States,³⁶ and the first outbreaks in Europe were recognized in Germany in 1990.^{225,227} Today, PRRSV infection is ubiquitous in all swine-producing areas of the world, including North and South America, Europe, and Asia. Severe abortion storms had a resurgence in 1996 to 1998 in the United States. Subsequently, the number of acute disease outbreaks decreased until atypical PRRSV variants emerged in China in 2006, causing outbreaks of fatal PRRSV that were unparalleled in severity. The novel variant usually spread through a herd within 3 to 5 days, causing morbidity between 50% and 100%. Mortality rates in the 2006 outbreak were high, with a mean around 20%, and they could be as high as 100% in suckling piglets, 70% in nursery pigs, and 20% in finishing pigs. More than 40% of pregnant sows suffered abortion and 10% of these sows themselves succumbed to the disease.^{111,194,246} In the following years, this highly pathogenic PRRSV spread to all Chinese swine-producing areas as well as surrounding Asian countries. About 60% of the Chinese pigs were infected with PRRSV over the 5 years after the virulent virus emerged.¹⁰⁷ To improve infection control, porcine genetic markers associated with PRRSV susceptibility are being sought, which can be used in breeding programs to optimize virus resistance, in balance with other traits of economic importance in pig production such as feeding efficiency, meat production, and leanness (reviewed in 116).

LDV

LDV was first discovered in laboratory mice.^{155,161} The virus was also isolated from wild mice in several countries, although the worldwide incidence is not known. Despite a life-long viremia and virus secretion in urine, feces, and saliva, horizontal transmission is inefficient, except in the case of fighting males. In contrast, transmission from mother to offspring is much more efficient, as long as anti-LDV immune responses have not yet been elicited.¹⁵⁵

SHFV

SHFV appears to be endemic among several species of African monkeys (*Erythrocebus patas*, *Ceropithecus aethiops*, *Papio anubis*, and *Papio cyanocephalus*).^{85,103,113} Nevertheless, the virus was first isolated from Asian macaques, during outbreaks of fatal hemorrhagic fever in research centers in the Soviet Union and the United States.¹⁸⁹ These epizootics were probably

caused by inadvertent transmission by humans from African monkeys to the macaques. During these outbreaks, SHFV was readily transmitted from the initially infected rhesus monkeys (*Macaca mulatta*) to other macaque species (*Macaca fascicularis* and *Macaca arctoides*), most likely by direct contact and via aerosols, whereas members of other monkey genera did not show clinical symptoms. Subsequently, similar epizootics among macaques occurred in various other primate centers.

CLINICAL FEATURES

EAV

The manifestations of EAV infection after an incubation period of 2 to 14 days range from subclinical to flu-like symptoms in adult animals, abortion in pregnant mares (e-Fig. 29.10A), persistent infection in stallions, and interstitial pneumonia in neonates.^{5,62,88} As with most infectious diseases, old, debilitated, or immunosuppressed horses and very young foals are predisposed to more severe disease.⁸⁸ Clinical features are characteristic vascular lesions, necrosis of small muscular arteries (from which the name of the family prototype EAV was derived), acute anorexia, and fever, usually accompanied by palpebral edema, conjunctivitis, nasal catarrh, and edema of legs, genitals, and abdomen (62, e-Fig. 29.10). Virulence and clinical signs are strain dependent, but the genetic basis for these differences has not been established.¹⁹⁹

PRRSV

At 12 to 24 hours after exposure to PRRSV, young pigs, sows, and boars become viremic, a state that can last from 1 to 2 weeks in mature animals to 8 weeks in young pigs. Clinical manifestations of PRRSV include occasional discoloring and blotching of the skin, most often on the ears (which gave PRRS the name “blue ear disease”) and vulva, and occasionally on the trunk. Further symptoms are fever, anorexia, breathing difficulties, lymphadenopathy, gross and microscopic lesions in the lung, and reproductive failure characterized by delivery of weak or stillborn piglets (e-Fig. 29.11A), or autolyzed fetuses.²²⁵

The clinical features of the highly pathogenic PRRSV variants that emerged in China in 2006 are strikingly more severe than those reported for the older isolates (e-Fig. 29.12). The new variant affected pigs of all ages and was characterized by high fever (40 to 42°C), depression, anorexia, lethargy, and rubefaction of the skin and ears. Most diseased pigs showed obvious respiratory distress, such as sneezing, coughing, and asthma, as well as intestinal problems including diarrhea. At autopsy, the severe lesions in skin, lung, gastrointestinal tract, and brain were considered unique for this atypical form of PRRS^{194,246} (e-Fig. 29.12F). Mortality rates ranged from 20% to 100%, depending on the age and health of the infected animals.

LDV

Infection of mice with LDV leads to a life-long viremia, but the infection is asymptomatic. It is maintained by continuous rounds of cytocidal virus replication in a renewable subpopulation of macrophages.¹⁴² By 24 h after infection, LDV titers of 10^{10} infectious dose (ID)₅₀/ml are present in the plasma, which then decrease to a level of 10^4 to 10^6 ID₅₀/ml. These titers remain present throughout the life of the mouse, together with elevated levels of lactate dehydrogenase and

other serum enzymes, which is due to the destruction of the macrophages that play a role in their clearance. LDV can be detected in the spleen, lymph nodes, thymus, and liver of persistently infected mice. Neurovirulent LDV variants can cause a fatal age-dependent poliomyelitis in certain inbred mouse strains that are of the Fv-1^{n/n} genotype and carry N-tropic, ecotropic murine leukemia virus (MuLV) proviruses³⁷ (e-Fig. 29.13). The replication of these ecotropic MuLVs in the glial cells of the spinal cord was proposed to render the anterior horn neurons susceptible to cytocidal LDV infection. Consequently, the development of age-dependent poliomyelitis may result from a combination of increased expression of ecotropic MuLVs and a decreasing ability to mount a motor neuron-protective anti-LDV response. LDV can also induce severe thrombocytopenia in animals that have been treated with anti-platelet antibodies at a dose that in itself was insufficient to induce clinical disease.^{135,136} The mechanism is unknown, but macrophage activation by virus-induced IFN- γ production is likely to play an important role.

SHFV

Depending on the virus strain, SHFV causes asymptomatic acute or persistent infections in several species of African monkeys,^{85,113} whereas in captive macaques fatal hemorrhagic fever was reported upon SHFV infection¹⁸⁹ (e-Fig. 29.14). Clinical signs in the latter animals consist of early fever, mild facial erythema, and edema, followed by anorexia, dehydration, and various hemorrhagic manifestations. The macaques usually die within 2 weeks, with mortality rates approaching 100%. Very little is known about SHFV pathogenesis in macaques. Macrophages are the primary target cells for SHFV, and a causal relationship exists between the cytocidal infection of these cells and the clinical symptoms of hemorrhagic fever.⁸⁴

PREVENTION AND CONTROL

Diagnosis

Diagnosis of EAV or PRRSV infections on the basis of clinical signs alone is generally very difficult, and therefore not reliable. This is due to the often subclinical or mild symptoms that resemble the symptoms of other respiratory diseases of horses and swine. Differential diagnosis of EAV- or PRRSV-induced abortions are also not straightforward, although these are generally characterized by (partial) autolysis of the fetuses and a lack of pathognomonic lesions, which is for example different for equine herpesvirus-induced abortions where aborted fetuses are usually fresh.⁸⁸

For laboratory diagnosis of EAV, nasopharyngeal swabs or washings, conjunctival swabs, and blood samples can be used. Several reverse transcriptase polymerase chain reaction (RT-PCR) assays are available for detection of EAV RNA in such clinical samples. In addition, immunohistochemistry using monoclonal antibodies to EAV proteins is a reliable method for EAV diagnosis in tissues. A virus neutralization assay remains the gold standard for detection of serum antibodies against EAV.⁸⁸

PRRSV infection can be diagnosed from pig serum or semen samples from boars, umbilical cords from piglets at birth, or serum samples from weaned sows, using fluorescence microscopy, enzyme-linked immunosorbent assay (ELISA), or an RT-PCR test. Recent studies established that PRRSV detection in

oral fluids of boars is an alternative to serum and semen sampling, since it gave very similar results, but with far less invasive sampling procedures. These oral samples were collected from cotton ropes impregnated with apple juice and sugar, which the animals were allowed to chew for approximately 20 minutes. Subsequently, fluids were mechanically extracted from the wet ropes and used to measure the presence of PRRSV RNA^{98,102} or PRRSV-specific antibodies.^{102a}

Disease Control

Equine viral arteritis is a manageable disease. Effective strategies for prevention and control have been designed, and uniform methods and rules have been published by the U.S. Department of Agriculture–Animal and Plant Health Detection Service (USDA-APHIS). These include directions to prevent spread of the virus in horse breeding populations, which usually suffice to suppress the further spread of infection.

Since 2006, the outbreaks of highly virulent PRRSV variants in Asia have boosted research aimed at the development of efficient control strategies against all variants of this virus, which continues to cause significant economic losses worldwide. Changes in swine management have been proven effective in preventing PRRSV outbreaks and are presently thought to be key to controlling the disease in the less intensive swine industry. The method of herd closure, for example, involves the uniform exposure of a confined herd to PRRSV, followed by a continued isolation of the herd for more than 200 days. This effectively eliminates PRRSV, as long as no new animals, and thereby possibly new PRRSV strains, are introduced from outside. In addition, strict biosecurity protocols, including air filtration, have been shown effective (reviewed in 38,46,133 and references therein). In areas with highly intensive pig farming this type of relatively costly strategies are often difficult to implement, and the need for better PRRSV vaccines than those currently available is high (see below).

Because arteriviruses generally infect production or laboratory animals, low priority has been given to the development of antiviral treatments. Infected animals either die quickly of the disease, or are culled as a way to prevent further spread.

Vaccines

For EAV, several genetically engineered candidate vaccines have been developed and tested in experimental infections. Some promising results were obtained with a vaccine based on Venezuelan equine encephalitis virus replicon particles expressing both EAV GP5 and M. Horses vaccinated with this recombinant vaccine produced neutralizing antibodies, shed little or no virus, and developed only mild symptoms after a challenge with virulent EAV.⁴ An EAV candidate live marker vaccine was developed on the basis of the deletion of the immunodominant domain of GP5, for which a peptide-specific ELISA is available.²⁹ This recombinant virus caused an asymptomatic infection in ponies and induced neutralizing antibodies, albeit only against the recombinant and not against the wild-type virus. The vaccinated animals were fully protected against disease following a challenge with virulent EAV.²⁹ The ELISA for the deleted immunodominant domain can be used to distinguish between vaccinated and naturally infected animals.

For PRRSV, a variety of live-attenuated and killed vaccines are commercially available. The MLV vaccines induce

long-lasting protection, but when derived from a single PRRSV vaccine strain they do not fully protect against heterologous PRRSV infection.⁹⁷ Furthermore, MLV vaccines do not completely prevent reinfection with wild-type virus and virus transmission. In some situations, it is impossible to discriminate between vaccinated and naturally infected animals. Either a subunit vaccine or a genetically modified live marker vaccine could overcome this problem, although the use of recombinant viruses in the field continues to be debated between vaccine developers, swine practitioners, and animal health authorities.

Adverse effects of vaccination of Danish pig herds with a modified live PRRS vaccine have been described, which were probably caused by reversion of the vaccine virus to virulence. Acute PRRS-like symptoms, including an increasing number of abortions and stillborn piglets, were experienced in vaccinated herds. Furthermore, vaccine virus was transmitted from vaccinated to nonvaccinated boars in several cases, resulting in viremia and shedding of vaccine virus in the semen.^{17,89,120,183} In addition, in Thai swine farms, vaccine-derived viruses were found to spread² and homologous recombination with circulating virus was observed in China.¹⁰⁸ In this respect, the killed vaccines are safer but they are less efficacious in the induction of protection.^{217,252} In general, it is believed that the strong immunomodulatory capabilities of PRRSV prevent the mounting of an efficient vaccine-induced immune response, and that the limited level of immunity that can be induced is insufficient to protect against challenging viruses.^{89,192}

Some promising results were obtained in DNA vaccination experiments with plasmids expressing PRRSV GP5.^{10,97,150} Neutralizing antibodies and lymphocyte proliferation were detected in DNA-vaccinated pigs, and the spread and clinical signs of challenge virus were reduced. This DNA immunization protocol was, however, not sufficient to prevent virus persistence and shedding in the respiratory tract. Combination with plasmids encoding M or GP3 in some cases increased the immune efficacy of candidate PRRSV DNA vaccines, as did co-delivery with plasmids encoding IFN- γ and IL-2.^{92,93,163,239} These results suggest that PRRSV GP5 may at least be a basis for a DNA-based subunit vaccine. Protection against clinical disease and reduction of pathogenic lesions were also observed with a recombinant pseudorabies virus vaccine expressing PRRSV GP5¹⁵⁹ and a recombinant transmissible gastroenteritis coronavirus expressing GP5 and M.⁴² Nevertheless, the genetic instability of heterologous genes inserted in these vaccine vectors remains to be solved. Using reverse genetic systems, chimeric infectious cDNA clones have been engineered aimed at developing attenuated modified live virus (marker) vaccines. Chimeric infectious clones in which sequences from virulent field strains were combined with attenuated vaccine strains gave some promising results.²²¹ The possibility to engineer marker vaccines was demonstrated by the removal of one or multiple conserved immunodominant B-cell epitopes from PRRSV nsp2, which resulted in viable marker viruses eliciting useful immune responses.⁴⁹ These deletions themselves, however, did not attenuate the virus, unless a green fluorescent protein marker gene was inserted at the site of the deletion. In this manner, an attenuated virus with both a negative and a positive marker was engineered, although the foreign insert proved to be genetically instable.⁷³

A novel approach to increase the immunogenicity of viral subunits is to facilitate their uptake into dendritic cells

(DCs) by inducing the expression of appropriate surface receptors (reviewed in 89). Proof of principle for this approach was recently obtained for CD169, one of the surface receptors for PRRSV, using anti-CD169 monoclonal antibodies as test ligands, which indeed induced *in vitro* T-cell proliferation at 100-fold lower concentrations than the nontargeting control ligand.¹⁶⁰ When more data will become available about the induction of regulatory T cells by PRRSV, this knowledge could be applied to improve vaccine efficacy as well. Removal of viral activities that suppresses innate immune responses is a strategy that is being developed for other viruses like influenza virus, where the innate immune “evasion” NS1 can be removed to produce a viable, attenuated vaccine virus. However, for arteriviruses, this may be far more difficult, since the viral proteins presently thought to suppress innate immunity are indispensable for virus viability.⁸⁹

PERSPECTIVES

A variety of important issues remain to be addressed in future studies of arteriviruses. Most of the viral proteins have been defined in basic terms only, and understanding the molecular details of their role in the viral life cycle is one of the major challenges for arterivirus research. For example, the characterization of the now eight structural proteins and their functional interactions during particle assembly and disassembly promises to be a highly complex issue, which also links to the many unanswered questions regarding host cell functions relevant for arterivirus attachment and entry.

In recent years, prompted in particular by the enormous PRRSV problems in Asia, determinants of arterivirus pathogenesis and virulence have received a lot of attention. The outline of a highly complex interplay between arterivirus and host is emerging, which will undoubtedly prove to be a critical factor in future vaccine development as well. Reverse genetics will continue to be a crucial tool for both basic and applied research in this area.

Our understanding of arterivirus epidemiology and evolution must be improved to prevent problems like the Asian PRRS outbreak in the future, and this field also connects to the interesting question of the potential for arterivirus cross-species transmission. On a different evolutionary level, that of the order *Nidovirales*, arteriviruses continue to be part of a unique group of positive-strand RNA viruses that is characterized by having the largest and most complicated replication machinery among currently known RNA viruses. In anticipation of systems allowing the complete *in vitro* reconstitution of arterivirus RNA synthesis, progress will continue to depend on successfully combining bioinformatics, biochemistry, and structural and molecular biology. This powerful approach has already provided detailed insights in some of the intricacies of arterivirus RNA synthesis, replication structures, and virus–host interactions, which will also be key to the design of antiviral strategies to combat diseases caused by known or currently unknown arteriviruses. Modern virus hunting techniques are increasingly likely to identify such additional family members in the years to come. This might compensate for the fact that—based on the inapparent and persistent infections frequently caused by currently known arteriviruses—clinical symptoms may not be the most direct indicator for arterivirus infections in other species.

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Mononegavirales

Host Range
Morphology
Lipids
Proteins
Nucleic Acid, Genome Organization, and Replication

The Eighth Report of the International Committee on the Taxonomy of Viruses (ICTV) (2005)¹ has recognized a hierarchy of viral taxa as follows: (order), family, (subfamily), genus, and species. Only three recognized orders of viruses are listed: *Caudovirales*, *Nidovirales*, and *Mononegavirales*. To avoid duplication of literature citations with the chapters on *Paramyxoviridae* (Chapter 33), *Rhabdoviridae* (Chapter 31), *Filoviridae* (Chapter 32), and *Bornaviridae* (Chapter 39) only the minimal list of references

that are unique to this chapter are cited. For full documentation of facts discussed here, see the specific chapters describing each family of the *Mononegavirales*. Table 30.1 lists nomenclature derivations relevant to this material.

The order *Mononegavirales* is composed of four families that have a phylogenetic relationship. Examples of the members of the *Mononegavirales* are shown in Table 30.2. These enveloped viruses possess linear, nonsegmented, negative-sense, single-stranded RNA (ssRNA) genomes. The four families are *Paramyxoviridae*, *Rhabdoviridae*, *Filoviridae*, and *Bornaviridae*. The common features of three families—*Paramyxoviridae*, *Rhabdoviridae*, and *Filoviridae*—include the negative strandedness of the monopartite RNA genome; a similar gene order (3'-untranslated region [UTR]-core protein genes-envelope protein genes—a large polymerase gene—5' UTR) (Table 30.3);

TABLE 30.1 Nomenclature Derivations

Borna	From Borna, a town in Saxony, Germany
Cyto	From Greek, <i>kytos</i> , "cell"
Ebola	From the river Ebola, Zaire
Ephemer	From Greek, <i>ephemeros</i> , "ephemeral"
Filo	From Latin, <i>filo</i> , "thread-like"
Lyssa	From Greek, <i>lyssa</i> , "rage, fury, canine madness"
Marburg	From the city of Marburg, Germany
Meta	From Greek, <i>meta</i> , "after"
Mono	From Greek, <i>monos</i> , "single"
Morbilli	From Latin <i>morbillus</i> , diminutive of <i>morbus</i> , "disease"
Nega	Modern invention from negative-sense RNA
Novi	Modern invention (no- and vi-) to describe a characteristic of the genus
Nucleo	From Latin <i>nux</i> , "nut"
Paramyxo	From Greek <i>para</i> , "by the side of," and <i>myxo</i> , "mucus"
Pneumo	From Greek, <i>pneuma</i> , "breathe"
Respiro	From Latin, <i>respirare</i> , "to breathe"
Rhabdo	From Latin, <i>rhabdos</i> , "rod"
Rubula	From Latin, <i>rubber</i> , "red"; <i>rubula inflans</i> was the old name for mumps.
Vesiculo	From Latin, <i>vesicula</i> , diminutive of <i>vesica</i> , "blister"
Virales	From Latin, "viruses"

TABLE 30.2 Taxonomic Structure of the Order *Mononegavirales*

Order	<i>Mononegavirales</i>
Family	<i>Bornaviridae</i>
Genus	<i>Bornavirus</i>
Family	<i>Rhabdoviridae</i>
Genus	<i>Vesiculovirus</i>
Genus	<i>Lyssavirus</i>
Genus	<i>Ephemerovirus</i>
Genus	<i>Novirhabdovirus</i>
Genus	<i>Cytorhabdovirus</i>
Genus	<i>Nucleorhabdovirus</i>
Family	<i>Filoviridae</i>
Genus	<i>Marburgvirus</i>
Genus	<i>Ebolavirus</i>
Family	<i>Paramyxoviridae</i>
Subfamily	<i>Paramyxovirinae</i>
Genus	<i>Rubulavirus</i>
Genus	<i>Avulavirus</i>
Genus	<i>Respirovirus</i>
Genus	<i>Aquaparamyxovirus</i>
Genus	<i>Ferlavirus</i>
Genus	<i>Henipavirus</i>
Genus	<i>Morbillivirus</i>
Subfamily	<i>Pneumovirinae</i>
Genus	<i>Pneumovirus</i>
Genus	<i>Metapneumovirus</i>

Adapted from Pringle CR. Mononegavirales. In: Fauquet CM, Mayo MA, Maniloff J, et al., eds. *Virus Taxonomy. Eighth Report of the International Committee on the Taxonomy of Viruses*. London: Elsevier/Academic Press; 2005:609–614, with permission.³

TABLE 30.3 Representation of the 3' to 5' Arrangement of the Transcriptional Units in the Genomes of the *Mononegavirales*

Family															
Subfamily	Genus	Virus 3'				Gene Order							5'		
<i>Bornaviridae</i>	<i>Bornavirus</i>	BDV	le		N	(P)	(M)				(G)			L	tr
<i>Rhabdoviridae</i>	<i>Vesiculovirus</i>	VSV	le		N	P	M				G			L	tr
	<i>Lyssavirus</i>	RV	le		N	P	M				G	Ps		L	tr
	<i>Cytorhabdovirus</i>	LNyV	le		N	P	4b	M			G			L	tr
	<i>Nucleorhabdovirus</i>	SYNV	le		N	P	Sc4	M			G			L	tr
	<i>Novirhabdovirus</i>	IHNV	le		N	P		M			G	NV		L	tr
	<i>Ephemerovirus</i>	BEFV	le		N	P		M			G	Gus ($\alpha 1, \alpha 2, \beta, \gamma$)		L	tr
	<i>Ephemerovirus</i>	ARV	le		N	P		M			G	Gus ($\alpha 1, \alpha 2, \beta$)		L	tr
<i>Filoviridae</i>	<i>Ebolavirus</i>	ZEBOV	le		N	P		(M1)			GP/SP	?	(M2)	L	tr
	<i>Marburgvirus</i>	MARV	le		N	P		(M1)			G	?	(M2)		
<i>Paramyxoviridae</i>															
<i>Parantyxovirinae</i>	<i>Avulavirus</i>	NDV	le		N	P/V	M	F			H			L	tr
	<i>Henipavirus</i>	HeV	le		N	P/C/V	M	F			H			L	tr
	<i>Morbillivirus</i>	MeV	le		N	P/C/V	M	F			H			L	tr
	<i>Respirovirus</i>	SeV	le		N	P/C/V	M	F			HN			L	tr
	<i>Rubulavirus</i>	MuV	le		N	P/V	M	F		SH	HN			L	tr
<i>Pneumovirinae</i>	<i>Metapneumovirus</i>	TRTV	le		N	P	M1	F	M2	SH	G			L	tr
	<i>Pnetunovirus</i>	HRSV	le	NS1	NS2	N	P	M1	SH	G	F	M2		L	tr

Genes encoding proteins of presumed homologous function are aligned vertically.

Virus abbreviations: ARV, Adelaide River virus; BDV, borna disease virus; BEFV, bovine ephemeral virus; HeV, Hendra virus; HRSV, human respiratory syncytial virus; IHNV, infectious hematopoietic necrosis virus; MARV, Lake Victoria Marburgvirus; MeV, measles virus; MuV, mumps virus; RV, rabies virus; SeV, Sendai virus; SYNV, Sonchus yellow net virus; TRTV, turkey rhinotrachitis virus; VSV, vesicular stomatitis virus; ZEBOV, Zaire ebolavirus.

Gene order abbreviations: Le, noncoding leader region; NS, nonstructural protein gene; N, nucleocapsid protein, P, phosphoprotein/N protein chaperone; V protein, interferon antagonist in most cases; C protein, interferon antagonist and involved in virus assembly; Sc4 and 4b, genes of unknown function, M and M1, matrix protein gene; F, fusion protein gene; SH, small integral membrane protein that may block apoptosis; G (or H or HN), attachment protein (hemagglutinin or hemagglutinin-neuraminidase; SP, secreted version of G; M2 envelope protein gene, Ps, pseudogene; NV, nonvirion protein gene; Gns, presumptive duplicated G sequence; L, large (polymerase) protein; tr, noncoding trailer region.

helical nucleocapsids; initiation of transcription by the virion-associated, RNA-dependent RNA polymerase (RdRp) from a single 3'-promoter; utilization of a stop-start transcription mechanism for each cistron; complementarity of the genome at the immediate 3' and 5' ends (to act as polymerase promoters); and 93% to 99% of the genome is protein encoding. The ribonucleoprotein (RNP) cores are infectious, but naked RNA is not infectious because it is not in the form of an RNP with its associated RdRp. Maturation is by budding from a cellular membrane and most members of the *Mononegavirales* bud from the plasma membrane, although rabies virus can bud into intracellular membranes and some plant rhabdoviruses are thought to bud from the inner nuclear membrane.

The family *Bornaviridae* has a unique pattern of messenger RNA (mRNA) processing among the *Mononegavirales*

as it utilizes the cellular splicing machinery to process precursor RNA to mRNA. The family *Bornaviridae* is included in the order *Mononegavirales* based on the negative strandedness of the monopartite genome ssRNA, similarity of the order of related genes, complementarity of the immediate 3' and 5' ends of the genome, and the relatedness of the transcription start and stop signals. Bornaviruses, however, are different from the other three families because their replication and transcription occurs in the nucleus, whereas replication of the families *Paramyxoviridae*, *Rhabdoviridae*, and *Filoviridae* (with the exception of plant viruses in the genus *Nucleorhabdovirus*) occurs in the cytoplasm. The phylogenetic relationship among the families *Paramyxoviridae*, *Rhabdoviridae*, *Filoviridae*, and *Bornaviridae* are illustrated in Figure 30.1. The phylogenetic relationship among members of the family *Paramyxoviridae* is shown in greater detail in Figure 30.2.

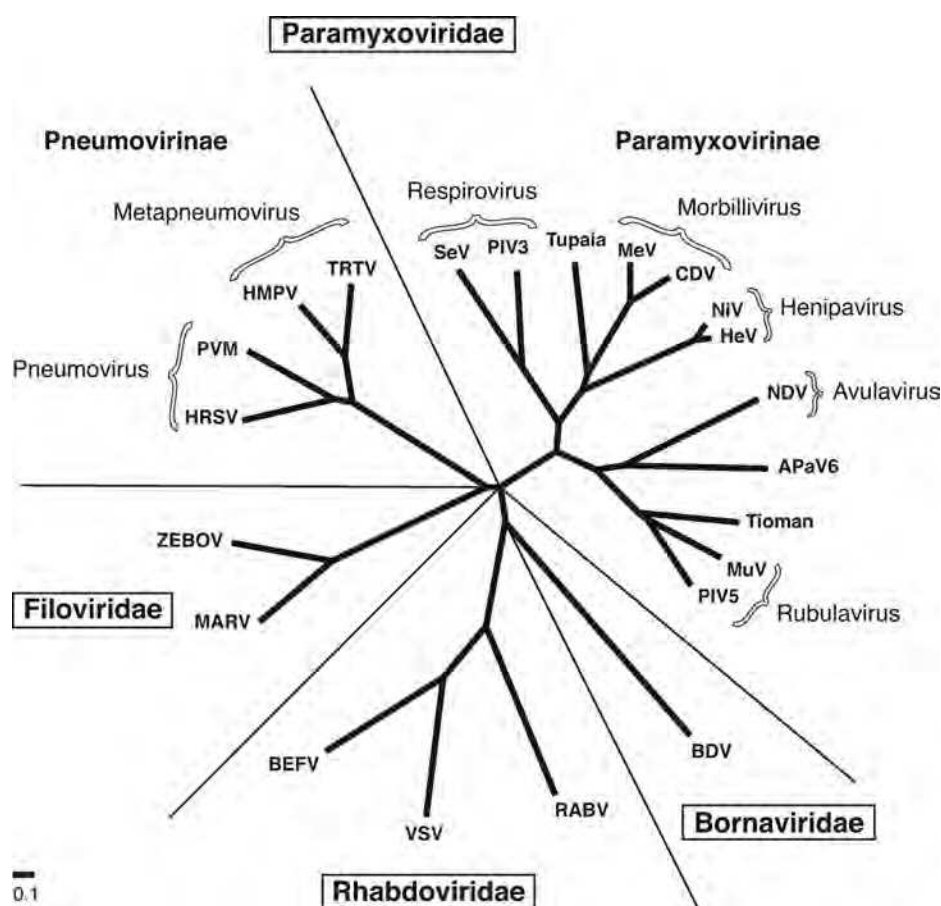


FIGURE 30.1. Unrooted phylogenetic tree of members of the order *Mononegavirales*. The tree was constructed using the CLUSTALX program with the sequences of the conserved domain III of the polymerase proteins. Three paramyxoviruses formerly unclassified by the International Committee on the Classification of Viruses (ICTV) are included: Tupaia paramyxovirus (Tupaia), avian parainfluenza virus type 6 (APaV6), and Tioman virus (Tioman). BDV—, Borna disease virus; BEFV—, bovine ephemeral fever virus; CDV—, canine distemper virus; HeV—, Hendra virus; HMPV—, human metapneumovirus; HRSV—, human respiratory syncytial virus; MARV—, Marburg virus; MeV—, measles virus; MuV—, mumps virus; NDV—, Newcastle disease virus; NiV—, Nipah virus; PIV3—, parainfluenza virus type 3; PVM—, pneumonia virus of mice; RABV—, rabies virus; SeV—, Sendai virus; PIV5—, parainfluenza virus 5, formerly known as simian virus 5 (SV5); TRTV—, turkey rhinotracheitis virus; VSV—, vesicular stomatitis Indiana virus; ZEBOV—, Zaire Ebola virus. (From Fauquet CM, Mayo MA, Maniloff J, et al., eds. *Virus Taxonomy. Eighth Report of the International Committee on the Taxonomy of Viruses*. London: Elsevier/Academic Press; 2005, with permission.)

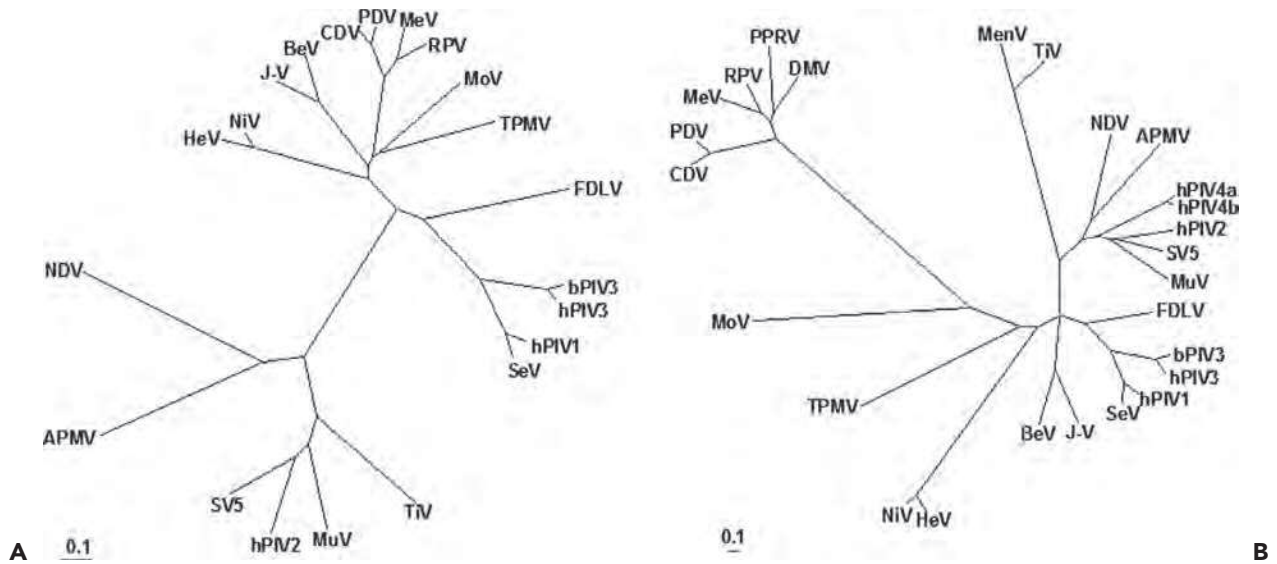


FIGURE 30.2. Unrooted phylogenetic trees based on complete L protein (A) and attachment protein (B) sequences of selected viruses within the subfamily *Paramyxovirinae*. The trees were generated from ClustalW (accurate) protein alignments using distance matrix programs (Protdist and Neighbor) within the PHYLIP software package and drawn in TreeView. Branch lengths represent relative genetic distances. GenBank accession number sequences used to generate the phylogenetic tree are listed below. For viruses in which a full-length genome sequence was not available, individual gene sequences were used and are indicated by the abbreviated gene letter in parentheses followed by the accession number. Avian paramyxovirus type 6 (APMV6) AY029299; BeV DQ100461; bovine parainfluenza virus 3 (bPIV3) AF178654; bovine respiratory syncytial virus (bRSV) AF092942; canine distemper virus (CDV) AF014953; Cetacean morbillivirus (CMV) strain Dolphin morbillivirus (DMV) X75961(N), Z47758(P/V/C), Z30087(M), Z30086(F), Z36978(H); Fer-de-Lance virus (FDLV) AY141760; Hendra virus (HeV) AF017149; human parainfluenza virus 1 (hPIV1) AF457102; human parainfluenza virus 2 (hPIV2) X57559; human parainfluenza virus 3 (hPIV3) AB012132; human parainfluenza virus 4a (hPIV4a) M32982(N), M55975(P/V), D10241(M), D49821(F), M34033(HN); human parainfluenza virus 4b (hPIV4b) M32983(N), M55976 (P/V) D10242(M), D49822(F), AB006958(HN); human respiratory syncytial virus (hRSV) AF013254; J virus (J-V), AY900001; measles virus (MeV) AB016162; Menangle virus (MenV) AF326114 (N,P/V,M,F,HN); Mossman virus (MoV) AY286409; mumps virus (MuV) AB040874; Newcastle disease virus (NDV) strain Beaudette C AF064091(N), X60599(P/V), X04687(M), X04719(F), X04355(HN), X05399(L); Nipah virus (NiV) AF212302; Peste-des-petits-ruminants virus (PPRV) X74443(N), AJ298897(P/V/C), Z47977(M), Z37017(F), Z81358(H); Phocine distemper virus (PDV) X75717(N), D10371(P/V/C, M, F, H), Y09630(L); Rinderpest virus (RPV) Z30697; Sendai virus (SeV) AB005795; simian virus 5 (SV5) AF052755; Tioman virus (TiV) AF298895; Tupaia paramyxovirus (TPMV) AF079780. (Adapted from Li Z, Yu M, Zhang H, et al. Beilong virus, a novel paramyxovirus with the largest genome of non-segmented negative-stranded RNA viruses. *Virology* 2006;346:219–228.) PHYLIP software package available from Joseph Felsenstein, Department of Genome Sciences, University of Washington, Seattle, Washington.

HOST RANGE

The host range of *Mononegavirales* varies from restricted to unrestricted. Filoviruses have been isolated from primates only. Paramyxoviruses are found only in vertebrates and there are no known vectors. In contrast, rhabdoviruses infect invertebrates, vertebrates, and plants. Some rhabdoviruses multiply in both invertebrates and vertebrates, some in invertebrates and plants, but no known example exists of a rhabdovirus that replicates in vertebrates and plants. In humans the families *Paramyxoviridae*, *Rhabdoviridae*, and *Filoviridae* can cause mild to severe morbidity and mortality, for example: hemorrhagic fever (Ebola and Marburg), neurologic disease (rabies virus), respiratory and neurologic disease (paramyxoviruses: measles virus, mumps virus, parainfluenza viruses 1 to 4, Nipah virus and Hendra virus, respiratory syncytial disease virus, and human metapneumovirus). The paramyxoviruses—parainfluenza virus 5, Newcastle disease virus, canine distemper virus, phocine distemper virus, Nipah virus, Hendra virus, pneumonia-like virus of mice, turkey rhinotrachitis virus and the rhabdoviruses,

vesicular stomatitis virus, and rabies virus—cause disease in animals. Bornaviruses have been isolated from horses, cattle, sheep, rabbits, rats, cats, and humans. Infection of some model animals is associated with behavioral disturbances to severe nonpurulent encephalomyelitis. There has been great debate concerning whether bornaviruses can cause psychological disease in humans.

MORPHOLOGY

The defining characteristic of all members of *Mononegavirales*, except the family *Bornaviridae*, is that the virions are large, enveloped structures containing a visible fringe of spike glycoproteins. The families exhibit very different shapes, however. Members of the family *Paramyxoviridae* are filamentous or pleomorphic (somewhat) spherical particles (200 to 300 nm in diameter), whereas members of the family *Filoviridae* are bacilliform, forming long threads (800 nm). Viruses within the family *Rhabdoviridae* are regular bullet-shaped particles or

bacilliform. The viral nucleocapsid (ribonucleoprotein [RNP]) is often observed in ruptured virion particles on an electron microscopy (EM) grid and the nucleocapsids have a diameter of 13 to 20 nm and characteristic morphologies, depending on the particular virus family.

LIPIDS

The lipid composition of the *Mononegavirales* reflects that of the host cell membrane from where the virions bud. Some of the spike glycoproteins have fatty acid covalently linked to their cytoplasmic tails.

PROTEINS

Members of the *Mononegavirales* contain five to seven structural proteins: the envelope glycoproteins; a matrix protein that underlies the lipid envelope; a major RNA binding protein often called the nucleocapsid protein (N or NP); other nucleocapsid-associated proteins; and a very large protein that has RdRp activity and capping, methylating, and polyadenylate transferase activities. The viruses also encode several nonstructural proteins, several of which are involved in antagonizing the innate immune system. Some viruses have a single glycoprotein that mediates both attachment of the virion to the cellular receptor and fusion of the viral envelope to a cellular membrane for viral entry into the cell. Other viruses have two major spike glycoproteins, one of which has attachment activity and the other of which has membrane fusion activity. When the receptor is sialic acid, the attachment protein usually has a neuraminidase activity that acts as a receptor-destroying activity.

NUCLEIC ACID, GENOME ORGANIZATION, AND REPLICATION

Members of the *Mononegavirales* contain one molecule of negative-sense ssRNA that varies from 8.9 to 19 kilobases (kb). The RNA is not infectious; by definition, a negative-sense RNA has to be copied to a plus-sense RNA to be translated to protein and the naked RNA of the *Mononegavirales* has to be packed into an RNP with its associated RdRp. The 5' end of the genome RNA is not modified by a cap structure or addition

of a covalently linked protein and the 3' end of the genome RNA is not polyadenylated. The immediate 5'- and 3'-termini exhibit inverse complementarity and are used as promoters by the RdRp for synthesis of both the antigenomic full-length positive RNA strand and the new genome RNA. The genome is composed of a series of genes with limited overlaps in some viruses (e.g., respiratory syncytial virus). The RdRp transcribes the genes in sequential order from the 3' end of the genome to make a series of mostly monocistronic mRNA. Transcription is polar with step-wise attenuation. Examples of the use of overlapping translational reading frames are found. Mostly, the genes have short 5' and 3' UTR (notable exceptions are found for Nipah virus, Hendra virus, J virus, and Beilong virus² of the paramyxovirus family). Conserved nucleotide sequence motifs define the transcriptional gene start with addition of a cap structure to the mRNA and conserved nucleotide sequence motifs that define the gene end and cause the addition of poly(A) to the mRNA in all families. A region of genome RNA between the gene-end and gene-start sequences is found, which is called the intercistronic region. This region is not transcribed into mRNA and can range from two nucleotides to hundreds of nucleotides. Members of the subfamily *Paramyxovirinae* undergo an insertion of nontemplated nucleotides at a pseudo-poly(A) addition site within their "P" gene to give rise to additional mRNA encoding extra proteins. A related event occurs in the glycoprotein gene of Ebola virus, giving rise to two versions of the glycoprotein. Splicing of mRNA only occurs for bornaviruses. In the subfamily *Paramyxovirinae* of the family *Paramyxoviridae*, the genome length in nucleotides has to be a number divisible by six (the so-called "rule of six"). It is thought that this constraint is because each nucleocapsid protein subunit binds to precisely six nucleotides to form the RNP.

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Rhabdoviridae

History

- Lyssaviruses
- Vesiculoviruses
- Ephemeroviruses
- Novirhabdoviruses
- Sigma Virus

Taxonomy

Virion Structure

Genome Structures

Stages of Replication

- Mechanism of Attachment
- Mechanism of Penetration
- Uncoating and Primary Transcription
- Genome RNA Replication
- Assembly of Progeny Virions

Molecular Genetics of Rhabdoviruses

- Rapid Evolution and Existence of Quasispecies
- Defective Interfering Particles
- Genetic Engineering of Rhabdoviruses

Molecular and Cellular Basis of Pathogenesis

- Induction and Suppression of Host
- Antiviral Responses
- Induction of Cytopathic Effects

Mouse Models of Rhabdovirus Infection

- Entry and Site of Initial Replication
- Virus Spread and Tissue Tropism
- Immune Responses Involved in Recovery
- From Rhabdovirus Infection
- Immune Response to Vesiculovirus Infection
- Immune Response to Lyssavirus Infection
- Determinants of Viral Virulence

Epidemiology of Rhabdovirus Infections

- Epidemiology of Lyssavirus Infections
- Epidemiology of Vesiculovirus Infections
- Epidemiology of Ephemerovirus Infections
- Epidemiology of Novirhabdovirus Infections

Clinical Features of Rhabdovirus Infections

- Lyssavirus Infections
- Vesiculovirus Infections
- Ephemerovirus Infections
- Novirhabdovirus Infections

Diagnosis of Rhabdovirus Infections

- Lyssavirus Infections
- Vesiculovirus Infections

Ephemerovirus Infections

Novirhabdovirus Infections

Prevention and Control of Rhabdovirus Infections

- Lyssavirus Infections in Humans
- Control of Rabies in Animals
- Control of Vesiculovirus Infections
- Control of Ephemerovirus Infections
- Control of Novirhabdovirus Infections

Perspectives

The family *Rhabdoviridae* consists of more than 185 different viruses isolated from both plants and animals. They are enveloped viruses that have helical nucleocapsids containing single-stranded, negative-sense RNA and share a common elongated, rod-like or bullet-like shape. This distinctive morphology separates rhabdoviruses from other taxa in the order *Mononegavirales*, the *Bornaviridae*, the *Filoviridae*, and the *Paramyxoviridae*. Rhabdoviruses can replicate in plants, invertebrates, or vertebrates. The family *Rhabdoviridae* contains many members that are significant medical, veterinary, and agricultural pathogens. Currently, animal rhabdoviruses include four genera: *Lyssavirus*, *Vesiculovirus*, *Ephemerovirus*, and *Novirhabdovirus* (Table 31.1). Many other rhabdoviruses have not received adequate study and are assigned to the family solely on the basis of morphology.

HISTORY

Lyssaviruses

Rabies is an archaic entity, one of the oldest recognized infectious diseases. The continuing biomedical preoccupation with rabies is understandable because of its “alarming manifestations in man and dog alike . . . and its almost inevitable progression to a fatal outcome have ensured unparalleled notoriety”.⁷⁷² These concerns extend beyond the material to the spiritual plane, as revealed by the following prayer: “San Roque, San Roque, que este perro no me toque!” This supplication for protection to the patron saint against pestilence, taught to children in both the Old and the New World for invocation whenever they encountered a dog on the street, literally translates to “St. Roque, St. Roque, do not allow this dog to touch me!”—classically linking dogs, bites, and resulting misfortune.

Ancient civilizations were familiar with rabies. An early passage mentions the dangers of dog bites, in the pre-Mosaic

TABLE 31.1 Taxonomy of *Rhabdoviridae*

Virus species	Example GenBank accession numbers for genome sequences	Virus species	Example GenBank accession numbers for genome sequences
Genus <i>Vesiculovirus</i>		Genus <i>Ephemerovirus</i>	
<i>Carajas virus</i>	FW339542	<i>Adelaide River virus</i>	L09206, ^a L09208, ^a U05987, ^a U10363 ^a
<i>Chandipura virus</i>	GU212856	<i>Berrimah virus</i>	
<i>Cocal virus</i>	EU373657	<i>Bovine ephemeral fever virus</i>	AF234533
<i>Isfahan virus</i>	AJ810084	Other related viruses that have not been approved as species	
<i>Maraba virus</i>		<i>Kimberley virus</i>	AY854637 ^a
<i>Piry virus</i>	Z15093, D26175	<i>Kotonkan virus</i>	AY854638, ^a DQ457009 ^a
<i>Spring viremia of carp virus</i>	AJ318079	<i>Malakal virus</i>	
<i>Vesicular stomatitis Alagoas virus</i>	EU373658	<i>Obodhiang virus</i>	DQ457098 ^a
<i>Vesicular stomatitis Indiana virus</i>	AF473864	<i>Puchong virus</i>	
<i>Vesicular stomatitis New Jersey virus</i>	K02379 ^a	Genus <i>Novirhabdovirus</i>	
Other related viruses that have not been approved as species		<i>Hirame rhabdovirus</i>	AF104985
<i>BeAn 157575 virus</i>		<i>Infectious hematopoietic necrosis virus</i>	L40883
<i>Boteke virus</i>	GU816014 ^a	<i>Snakehead virus</i>	AF147498
<i>Calchaqui virus</i>		<i>Viral hemorrhagic septicemia virus</i>	Y18263
<i>Eel virus American</i>		Other related viruses that have not been approved as species	
<i>Eel virus European X</i>	FN557213	<i>Eel virus B12</i>	
<i>Grass carp rhabdovirus</i>		<i>Eel virus C26</i>	
<i>Gray Lodge virus</i>		Genus <i>Cytorhabdovirus</i>	
<i>Jurona virus</i>	GU816024 ^a	<i>Barley yellow striate mosaic virus</i>	FJ665628 ^a
<i>Klamath virus</i>		<i>Broccoli necrotic yellows virus</i>	
<i>Kwatta virus</i>		<i>Festuca leaf streak virus</i>	
<i>La Joya virus</i>		<i>Lettuce necrotic yellows virus</i>	AJ867584
<i>Malpais Spring virus</i>		<i>Lettuce yellow mottle virus</i>	EF687738
<i>Perinet virus</i>	AY854652 ^a	<i>Northern cereal mosaic virus</i>	GU985153
<i>Pike fry rhabdovirus</i>	FJ872827	<i>Sonchus virus</i>	
<i>Porton virus</i>	GU816013 ^a	<i>Strawberry crinkle virus</i>	AY005146, ^a AY250986 ^a
<i>Radi virus</i>		<i>Wheat American striate mosaic virus</i>	
<i>Tench rhabdovirus</i>		Other related viruses that have not been approved as species	
<i>Ulcerative disease rhabdovirus</i>		<i>Wheat rosette stunt virus</i>	AF059602-04, ^a AF059677 ^a
<i>Yug Bogdanovac virus</i>		<i>Soybean blotchy mosaic virus</i>	EU877231 ^a
Genus <i>Lyssavirus</i>		<i>Ivy vein banding virus</i>	GQ249162, ^a GQ249163 ^a
<i>Aravan virus</i>	EF614259	Genus <i>Nucleorhabdovirus</i>	
<i>Australian bat lyssavirus</i>	AF418014	<i>Datura yellow vein virus</i>	
<i>Duvenhage virus</i>	EU293119	<i>Eggplant mottled dwarf virus</i>	AM922319, ^a AM922322 ^a
<i>European bat lyssavirus 1</i>	EU293112	<i>Maize fine streak virus</i>	AY618417
<i>European bat lyssavirus 2</i>	EU293114	<i>Maize mosaic virus</i>	AY618418 ^a
<i>Irkut virus</i>	EF614260	<i>Potato yellow dwarf virus</i>	GU734660
<i>Khujand virus</i>	EF614261	<i>Rice yellow stunt virus</i>	AB011257
<i>Lagos bat virus</i>	EU293108, EU293110, EF547454, ^a GU170202	<i>Sonchus yellow net virus</i>	L32603
<i>Mokola virus</i>	Y09762	<i>Sowthistle yellow vein virus</i>	
<i>Rabies virus</i>	M13215, M31046, AY705373, EU293115, EU293111, EU311738	<i>Taro vein chlorosis virus</i>	AY674964
<i>West Caucasian bat virus</i>	EF614258	Other related viruses that have not been approved as species	
Other related viruses which have not been approved as species		<i>Cereal chlorotic mottle virus</i>	
<i>Shimoni bat virus</i>	GU170201	<i>Cynodon rhabdovirus</i>	EU650683 ^a
		<i>Maize Iranian mosaic virus</i>	DQ186554
		<i>Sorghum stunt mosaic virus</i>	

^aSequences do not compose the complete genome.

Eshnunna Code of Mesopotamia, circa the 23rd century BC: “If a dog is mad and the authorities have brought the fact to the knowledge of its owner; if he does not keep it in, and it bites a man and causes his death, then the owner shall pay two-thirds of a mina [40 shekels] of silver”.⁴¹ In *The Iliad* (700 BC), Hector is compared to a rabid dog. Chinese scholars warned of the dangers of rabid dogs in 500 BC, and Aristotle (4th century BC) correctly associated the disease with animals but erroneously exempted humans from contracting it from a mad dog’s bite. In Rome, Cordamus guessed that a poison (i.e., a “virus”) was present in saliva. Similarly, in the 1st century AD, another Roman, Celsus, described clinical aspects of human infection: “The patient is tortured at the same time by thirst and by invincible repulsion toward water.” For prevention, he recommended immediate excision of the bitten tissue, cauterization of the wound by a hot iron, and dunking the victim into a pool. The Hebrew Talmud, also dating from the 1st century, makes several references to the disease. Throughout the ages, ingestion of a wide variety of substances (e.g., the liver from a mad dog, crayfish eyes, a cock’s brain or comb, or the cast slough of snakes pounded in wine with a male crab) and carrying sacred talismans or “madstones” were believed to be cures for rabies.⁴¹

The transition from the medieval era to the Renaissance period of pragmatism and experimentation resulted in a remarkable treatise in 1546, entitled “The Incurable Wound,” by Fracastoro. This Italian physician clearly stated that human beings are susceptible to rabies, and he vividly described a clinical case:

Its incubation [following a bite by a rabid animal] is so stealthy, slow and gradual that the infection is very rarely manifest before the 20th day, in most cases after the 30th, and in many cases not until four or six months have elapsed. There are cases recorded in which it became manifest a year after the bite. [Once the disease takes hold,] the patient can neither stand nor lie down; like a madman he flings himself hither and thither, tears his flesh with his hands, and feels intolerable thirst. This is the most distressing symptom, for he so shrinks from water and all liquids that he would rather die than drink or be brought near to water; it is then that they bite other persons, foam at the mouth, their eyes look twisted, and finally they are exhausted and painfully breathe their last.³⁶⁰

His portrayal of human rabies is accurate in that the incubation periods can extend from months to years after initial exposure,⁶⁶² but a biting attack on others by a rabid patient with resultant disease is an uncommon event.²¹⁸

Although rabies is known to have been widespread in the Old World for thousands of years, its occurrence in the New World is less understood because of a dearth of records before European arrival. Rabies in the Americas was reported by the Reverend Marmolejo in Mexico as early as 1709, but some suspect that it was present before Columbus’s arrival in the 15th century. For example, not long after the discovery of the Americas, the bishop Petrus Martyr-Anglerius wrote in his *De Rebus Oceanicis et de Orbi Novi* Decades Octo, “In several places bats not much smaller than turtle doves used to fly at them [the Spanish sailors and soldiers] in the early evening with brutal fury and with their venomous bites brought those injured to madness . . . [and] bats . . . come in from the marshes on the river and attack our men with deadly bite”.³⁸⁸ This may

have been one of the first descriptions of rabies transmission by vampire bats.

The bite of a rabid animal was considered a likely source of rabies infection by many, but it was only in 1804 that Zinke used dog saliva for transmission.³⁸⁶ Later in 1879, Galtier is credited with experimental rabies transmission and serial passage in rabbits.³⁸⁶ Clinical descriptions formed the basis for diagnosis until the advent of light microscopy. A clear description of viral and neuronal interactions was made by Negri in 1903, with the detection of cytoplasmic inclusions (Negri bodies) in neurons of rabid animals.³⁹³ Although the diagnostic value of Negri bodies was established by 1913, their viral composition had to wait until the later development of electron microscopy.

Pasteur’s research on rabies is perhaps the most well-known historical achievement in the field. First, through adaptation of “street” (wild-type) virus to laboratory animals, he was able to change its properties. Today, one could apply the term *attenuated* to his “fixed” virus strains. Second, Pasteur and his team developed concepts and experimental approaches to the first protective vaccination against rabies.³⁸⁸ Desiccated spinal cords from rabies virus–infected rabbits became the first rabies vaccine, and they were supposedly safe, although now it is known that the fixed viruses from which these vaccines were derived were not apathogenic but could actually cause the disease. July 6, 1885, is a milestone in the history of rabies. On that day, 9-year-old Joseph Meister was bitten at multiple sites by a rabid dog and received the first postexposure prophylaxis with Pasteur’s vaccine. Remarkably, Joseph survived.³⁸⁶ Pasteur’s vaccine, with all its modifications, became the accepted rabies prophylactic throughout the world in the early 20th century. Problems remained, however, because improperly inactivated virus caused rabies, and animal brain tissue induced allergic reactions leading to neuromuscular accidents. Moreover, the vaccine was not very effective in cases of severe bites, such as those inflicted on the face and neck by rabid wolves and dogs.

Postexposure prophylaxis against rabies through simultaneous administration of antirabies serum and vaccine was introduced in 1889 by Babes.²⁹ This approach found few adherents and languished until about 1940, when interest in the use of serum-containing rabies virus (RABV) antibodies was revived. In a trial organized by the World Health Organization in 1954, the combined use of serum and vaccine was found to be more protective than vaccine alone,²⁸⁸ an observation later corroborated by Chinese findings.²¹² Today, the combination of immune globulin and vaccine is the recommended standard for prophylaxis in human rabies exposure.

In the 1960s, an RABV grown in human diploid cells was used to produce a safe and efficacious inactivated vaccine,^{385,386} eliminating many of the problems connected with vaccines produced in brain tissue. This vaccine and others derived from cell culture are used widely throughout the world, although for economic reasons, several developing countries still use nervous tissue vaccines. Other RABV strains are used for vaccine production for human and animal use in addition to the original Pasteur virus (PV) strain. Given the progress in biotechnology, improved versions of rabies vaccines are currently under development.

Vesiculoviruses

Vesicular stomatitis virus (VSV) is the best-studied member of the genus *Vesiculovirus*. The extensive body of knowledge about

the replication of VSV reflects its status as a widely studied prototype for the nonsegmented, negative-strand RNA viruses. VSV produces an acute disease in cattle, horses, and pigs characterized by fever and vesicles in the mucosa of the oral cavity and in the skin of the coronary band and teat. Clinically, VS is very similar to foot-and-mouth disease (FMD). VSV can also cause an acute febrile disease in humans. Laboratory-adapted strains, however, are rarely pathogenic for humans.

Although VS was first reported in the United States in 1916 during an epidemic in cattle and horses,⁶⁹⁵ a clinically similar disease was previously described in army horses in 1862, during the U.S. Civil War.⁴⁸⁴ In 1915, French veterinarians described a disease clinically similar to VS in horses imported to Europe from the United States and Canada during World War I. At that time, the etiology of this disease could not be determined with certainty, but it could be transmitted from horse to horse by rubbing the saliva of a sick animal on the tongue of a healthy one, establishing the infectious nature of the disease.²⁹⁵ In 1925, cattle transported from Kansas City, Missouri, to Richmond, Indiana, initiated an outbreak of VS in the area. The disease was experimentally transmitted to horses and the infectious agent was maintained by serial passages in animals. This strain became the VS-Indiana virus (VSIV) strain.¹⁴⁹ In 1926, an outbreak of VS in cattle occurred in New Jersey. The causative agent was found to be a filterable agent that could infect cattle, horses, and guinea pigs. This virus, serologically different from the VSIV strain, is currently known as the VS-New Jersey virus (VSNJV) strain.^{148,149}

The VSIV and VSNJV viruses represent the two serotypes most commonly isolated in the Americas. Most of the commonly studied laboratory-adapted strains of VSV (e.g., Glasgow, Orsay, San Juan, Mudd-Summers) belong to the VSIV serotype. In the United States, the last reported outbreak of the VSIV serotype occurred in 1965.⁷⁵¹ The VSNJV serotype was responsible for outbreaks in the United States in 1944, 1949, 1957, 1959, 1963, 1982–1983, 1985, and 1995.⁸⁷ In 1997, isolated cases were diagnosed in several horses in New Mexico, but this did not initiate an outbreak.²¹ Between 1946 and 1954, during an outbreak of FMD in Mexico,²⁷⁰ the joint Mexico–American commission for the control of FMD developed techniques for the differential diagnosis of FMD and VS based on the isolation of the agent and complement fixation methods.²⁹⁵ The availability of a more efficient diagnostic methodology demonstrated that VS was prevalent throughout the year in the tropical areas of Mexico.²⁹⁵

In South America, the disease was reported in 1939 in La Plata, Argentina.²⁹⁵ Later, VSV was isolated in Barinas, Venezuela, in 1941, and in Colombia in 1943.²⁹⁵ Currently, VSV is endemic in many Latin American countries and is responsible for important economic losses in the livestock industry. Disease caused by VSV was reported in 11 countries of Latin America in 1996.⁷⁸⁸ Although the presence of VS was previously suggested in Africa in 1884 to 1887 and in Asia in 1944,²⁹⁵ presently the disease is considered enzootic only in the Americas.⁷⁸⁸

Other vesiculoviruses are endemic in the Americas, Asia, and Africa. Piry virus was isolated from an opossum (*Philander opossum*) in Brazil in 1960⁷⁰¹ and caused a febrile disease in humans.⁴⁷⁴ Cocal virus (COCV, or Indiana 2) was isolated from mites of the genus *Gigantolaelaps* from rice rats (*Oryzomys laticeps velutinus*) trapped during 1961, on Bush Bush Island in the Nariva swamp in eastern Trinidad.³⁵⁴ The VS Alagoas virus

(VSAV, or Indiana 3) was isolated from domestic animals in the state of Alagoas, Brazil, during a VS outbreak.⁷⁰⁰ Later it also was isolated from sand flies and seropositive (but otherwise healthy) livestock in Colombia.⁷⁰⁰ Maraba virus (MARAV) was isolated from sand flies (*Lutzomyia* sp.) collected in the state of Pará, Brazil. Although humans are infrequently infected based on serology, the actual public health significance of MARAV has not been assessed.⁷⁰⁹

Vesiculoviruses endemic in Asia include Chandipura virus (CHPV) and Isfahan virus (ISFV). ISFV was isolated from sand flies (*Phlebotomus papatasi*) collected in Dormian, Isfahan Province, Iran, in 1975.⁶⁹⁹ From serologic analyses, the presence of ISFV has been detected in India, Iran, Turkmenistan, and other Asian countries.⁴⁷⁴ CHPV was obtained from the sera of two patients with a febrile illness in Nagpur City, Maharashtra State, India, in 1965 during an epidemic of chikungunya and dengue.⁶⁶ This virus was also isolated from phlebotomine sand flies in West Africa in 1991.²⁴³ CHPV is now known to be a cause of viral encephalitis in children, following its identification as the cause of two recent outbreaks. One outbreak in 2003 in Andhra Pradesh State, India,⁵⁸⁰ included 329 cases (183 fatalities), and another in 2004 in Gujarat State, India,¹¹⁴ included 26 cases (at least 18 fatalities).

Several vesiculoviruses infect fish, and at least one of these, Spring viremia of carp virus (SVCV), has been recognized as a species of the *Vesiculovirus* genus. Dating back possibly to the Middle Ages, common carp *Cyprinus carpio* in European pond culture have been plagued by a complex of infectious diseases variously known as infectious dropsy, rubella, infectious ascites, hemorrhagic septicemia, and red contagious disease.^{59,316,621,706} These diseases proved to be of great economic importance, causing serious losses in carp pond fisheries of the central and eastern parts of Europe.^{227,228} The proposed causes (nutrition, environment, parasites, bacteria, viruses) for the acute and chronic forms of the epizootics remained controversial for a long time. However, a viral etiology for the acute form of infectious dropsy became evident when a cytopathic agent was isolated,⁷⁰⁶ and River's postulates were fulfilled using virus isolated from affected carp.²²⁹ In order to distinguish the viral disease from other etiologic entities within the infectious dropsy complex, the disease was renamed spring viremia of carp (SVC), and the causative virus was termed SVCV (or, initially, *Rhabdovirus carpio*).²²⁹

SVCV has been identified in different parts of Europe, Russia, and the Middle East, causing mortality of up to 70% of young carps.^{9,58,61,99,229,645,698,706} In 2002, SVCV was first reported in U.S. waters at a North Carolina koi hatchery. Unfortunately, there is evidence that koi had been distributed from this hatchery to most of the 48 contiguous states before being confirmed with SVC. The first common carp die-off of wild fish that tested positive for SVC occurred in 2002 at Cedar Lake, Wisconsin,¹⁸³ and the virus has rapidly disseminated to other states.

Ephemeroviruses

The first reference to bovine ephemeral fever (BEF) can be found in the book *The Heart of Africa*.⁶³⁶ Not until the 20th century was the disease reported among ruminants in much of its natural range throughout the tropical and subtropical regions of Africa, Asia, Australia, and the Middle East.⁶⁷¹ The apparent emergence and re-emergence of BEF over 125 years is likely due to the expansive growth of the cattle industry and

improved surveillance.⁷³⁵ Until 1966, when bovine ephemeral fever virus (BEFV) was grown in mice,⁷²³ research on the agent was restricted largely to transmission studies in cattle.¹³⁴ Characterization of ephemeroviruses is an ongoing process. For example, viruses Obodhiang (Sudan, 1963) and Kotonkan (Nigeria, 1967), isolated from mosquitoes, were initially suggested as “rabies related,” based on a limited antigenic cross-reactivity with lyssaviruses.⁶⁰ However, gene sequencing and phylogenetic reconstructions demonstrated that these viruses belong to ephemeroviruses.⁴⁰¹ Likely, more members of the genus will be recovered among other rhabdoviruses, isolated decades ago and awaiting molecular characterization.

Novirhabdoviruses

Infectious hematopoietic necrosis virus (IHNV) was first discovered in sockeye salmon (*Oncorhynchus nerka*) dying at hatcheries in Washington in 1953.⁶¹¹ Similar outbreaks among hatchery-reared salmonid fish in California were reported in the following decades.^{285,777} It was thought that IHNV was confined to salmonid fish in the Pacific coast of North America.⁴⁸² However, the virus spread during the 1970s to the eastern United States, Europe, Japan, Korea, Taiwan, and China by shipment of infected fish and eggs.^{85,418,617} Electron microscopy of IHNV particles along with physicochemical and serologic analysis demonstrated that IHNV is a member of the *Rhabdoviridae*.^{16,314,483} Gene sequencing demonstrated that IHNV has the five structural genes common to rhabdoviruses, with the addition of a nonstructural, nonvirion (NV) gene between the genes for the G and L proteins.³⁹⁹ Several more fish rhabdoviruses, which demonstrate similar pathobiology and have similar genome organization, have been described, including Hirame rhabdovirus, snakehead virus, and viral hemorrhagic septicemia virus. These viruses were identified not only in North America but also in eastern and southern Asia, where they appear to be endemic.^{363,374} These viruses were first assigned into the genus *Novirhabdovirus*, based on the presence of the NV gene, in the Seventh Report of the International Committee on Taxonomy of Viruses (ICTV).⁷³⁶

Sigma Virus

This virus, a natural pathogen of *Drosophila* spp. fruit flies, was described in 1937.⁴⁰⁷ Sigma virus appears to be distributed worldwide. This is the only arthropod-specific rhabdovirus described to date, with an unusual mode of transmission: it is only transmitted vertically through both eggs and sperm and does not move horizontally between hosts.¹⁴⁵ Sigma virus was initially placed in the *Rhabdoviridae* based on its bullet-shaped viral particles,^{64,696} and this has subsequently been confirmed using sequence data.⁷¹ Initially it was believed that Sigma virus infects only *D. melanogaster*. However, additional surveillance identified recently that related variants of Sigma virus infect *D. affinis* and *D. obscura*.⁴⁵¹

TAXONOMY

The rhabdoviruses share a variety of gross morphologic and functional attributes with other members of the order *Mononegavirales*. For example, the virions are large structures that mature by budding, with membrane-bound spikes and a helical nucleocapsid. They possess single-stranded, nonsegmented,

negative-polarity RNA, with a similar gene arrangement. Within the family, recent analyses support the concept of a unified phylogeny and suggest an evolutionary history influenced by host species and transmission dynamics.^{83,404} Currently, the ICTV recognizes four genera of animal rhabdoviruses and two genera of plant rhabdoviruses. Furthermore, several rhabdovirus species have been recognized without inclusion into any of the established genera.¹⁷⁷ Figure 31.1 shows phylogenetic relationships among rhabdoviruses.

Within the genus *Lyssavirus* only one major serogroup had been established, although various serotypes were defined.⁶¹³ Placement within the genus was determined by serologic cross-reactivity of viral antigens, primarily based on antigenic sites on the nucleoprotein (the N protein). Historically, placement of a species as a rabies or rabies-related virus was determined by recognition of antigenic sites of the glycoprotein (the G protein) via virus neutralization tests. As nucleotide sequence data became available for a number of other *Lyssavirus* species,^{44,84,173,377,400,402,405,663} a trend toward genetic classification was established. Currently ICTV recognizes 11 *Lyssavirus* species, and one more representative (*Shimoni bat virus* [SHIBV]) is included in the genus provisionally without established species status (Table 31.1).

In general, demarcation criteria for *Lyssavirus* species include the following: (1) Genetic distances, with the threshold of 80% to 82% nucleotide identity for the complete N gene or 80% to 81% nucleotide identity for concatenated coding regions of N+P+M+G+L genes. Globally, all isolates belonging to the same species have higher identity values than the threshold, except the viruses currently included into the *Lagos bat virus* (LBV) species. For that reason some authors suggested that LBV be subdivided into several genotypes.^{173,473} However, as these LBV representatives are segregated into a monophyletic cluster in the majority of phylogenetic reconstructions, in the absence of other sufficient demarcation characters there is currently no possibility to subdivide LBV into several viral species. (2) Topology and consistency of phylogenetic trees, obtained with various evolutionary models. (3) Antigenic patterns in reactions with antinucleocapsid monoclonal antibodies (preceded by serologic cross-reactivity and definition of *Lyssavirus* serotypes, using polyclonal antisera). (4) Whenever available, additional characteristics, such as ecological properties, host and geographic range, and pathologic features, are considered.¹⁷⁷ Moreover, based on genetic distances and serologic cross-reactivity, the genus has been subdivided into two phylogroups. Phylogroup I includes RABV, *European bat Lyssavirus type 1* (EBLV-1), EBLV-2, *Duvenhage virus* (DUVV), *Australian bat Lyssavirus* (ABLV), *Aravan virus* (ARAV), *Khujuand virus* (KHUV), and *Irkut virus* (IRKV). Phylogroup II includes LBV, *Mokola virus* (MOKV), and SHIBV. The remaining species of the genus, *West Caucasian bat virus* (WCBV), cannot be included in either of these phylogroups and is suggested to be considered as a representative of independent phylogroup III.^{400,402}

Based on the serologic cross-reactivity patterns and sequence analyses of the members of the genus *Vesiculovirus*, a unique VSV serogroup has been established. This serogroup includes VSIV (currently the type species of the genus), VSJNV, VSAV, *Carajas virus*, CHPV, COCV, ISFV, MARAV, and *Piry virus*.¹⁷⁷ Also recognized as a *Vesiculovirus* species is SVCV. Furthermore, 19 viruses are provisionally included in the genus without established species status (Table 31.1).

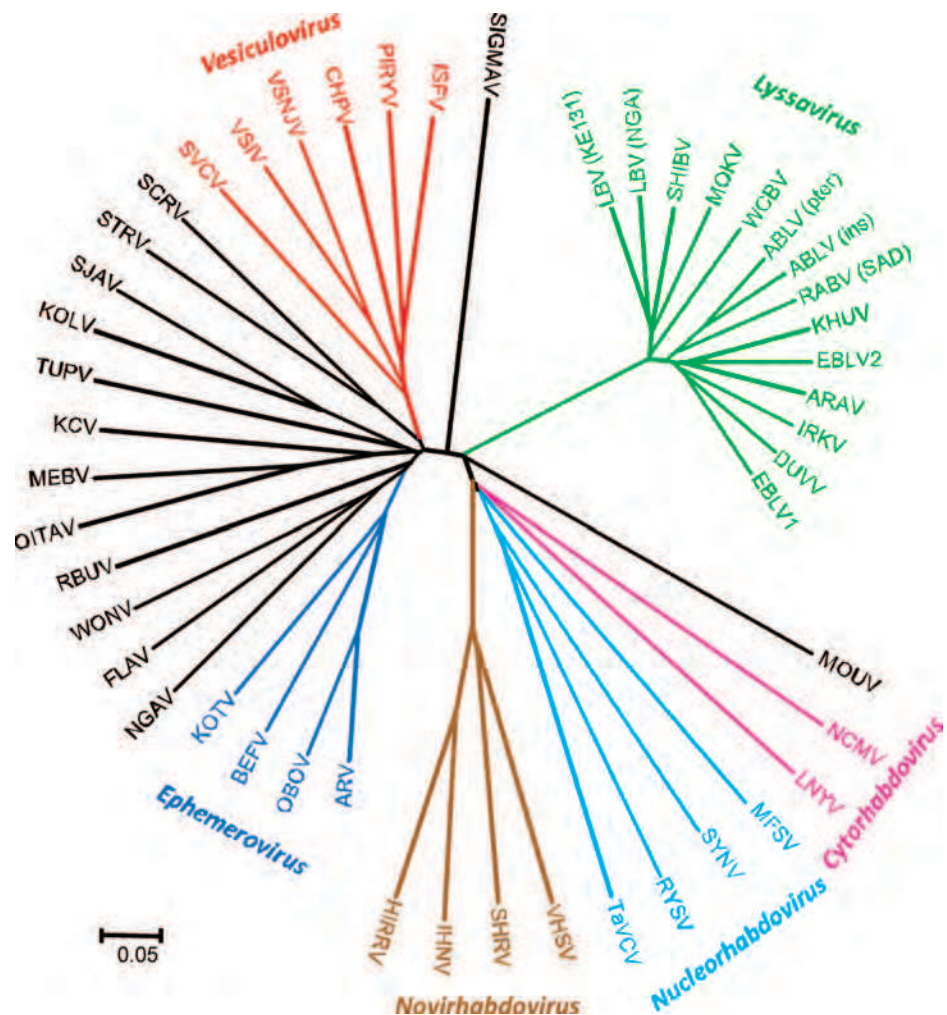


FIGURE 31.1. Phylogenetic relationships among rhabdoviruses.

The members of the genus *Ephemerovirus* show limited cross-neutralization reactivity, but they are highly cross-reactive in complement fixation or indirect immunofluorescence tests. They exhibit similar but distinct genome organization with the common feature of a nonstructural glycoprotein (G_{NS}) gene but variations in the number of accessory protein genes and the location of transcriptional control sequences. Different species may share up to 91% identity in N protein amino acid sequence. Currently the genus includes only three recognized species, but five more viruses are included provisionally¹⁷⁷ (Table 31.1), based on antigenic reactivity and phylogenetic analysis of limited gene fragments.^{83,401} Phylogenetic relationships indicate that several intercontinental translocations of ephemeroviruses are likely to have occurred. Adelaide River virus (ARV) (Australia) and Obodhiang virus (OBOV) (Africa) demonstrate more genetic identity to each other than is observed between ARV and BEFV, both circulating in Australia. BEFV also circulates broadly in Africa, the Middle East, and southern areas of Asia, without significant genetic diversity.^{401,404,735}

The genus *Novirhabdovirus* was established based on the presence a small NV protein of unknown function. The NV open reading frame (ORF) is located between the G and L genes and is preserved in diverse viruses and strains. The NV protein sequences are significantly less conserved between viruses in different species than sequences of the structural proteins.^{317,398}

Species within the genus have been distinguished serologically on the basis of cross-neutralization with polyclonal rabbit antisera. Thus, IHNV and hirmine rhabdovirus (HIRRV) each constitute single serotypes, and viral hemorrhagic septicemia virus (VHSV) has one major serotype with a small number of associated strains. Viruses from different species do not show cross-neutralization, but in some cases there is a low level of cross-reaction with specific proteins in western blot analyses. Nucleotide sequence data are available for most genes of these viruses and will undoubtedly contribute to the distinction of viral species in the future. For strains within a virus species, the nucleotide sequence divergence ranges up to a maximum of 8% for IHNV G and NV genes and 18% for the G genes of European and North American VHSV. N protein amino acid identity between IHNV and VHSV is approximately 34%.¹⁷⁷

Members of two genera of the *Rhabdoviridae* infect plants and are transmitted via arthropod vectors, such as leafhoppers, planthoppers, and aphids.³⁴² The cytorhabdoviruses and nucleorhabdoviruses are primarily distinguished based on their sites of virion maturation, in the cytoplasm and the nucleus, respectively. Genus classification based on sequence diversity has thus far correlated with classification by intracellular virus maturation. The genus *Cytorhabdovirus* currently includes nine recognized species and three provisional members, whereas genus *Nucleorhabdovirus* includes nine and four members, respectively

(Table 31.1). There is no significant sequence similarity (>50%) between analogous genes of the different species analyzed to date. However, nucleotide sequences are available for only a limited number of representatives and at the moment cannot be considered as sufficient to demarcate different species.¹⁷⁷

Recently, several rhabdoviruses, previously referred to as “unclassified”,⁷⁰⁷ were recognized by the ICTV as species, without assignment to any particular genus, based on their unique genome structure, phylogenetic and antigenic properties, and sufficient amount of knowledge on their ecology or pathobiology. These include *Flanders*, *Tupaia*, *Sigma*, *Ngaingan*, and *Wongabel* viruses.¹⁷⁷ The recently described *Moussa virus*⁵⁷⁵ is another candidate for the establishment of a viral species without inclusion in any recognized genus.

VIRION STRUCTURE

Rhabdoviruses are enveloped, rod- or cone-shaped particles (Fig. 31.2A, B), approximately 100 to 430 nm long and 45 to 100 nm in diameter. Animal rhabdoviruses are usually approximately 180 nm long and 80 nm wide, but those isolated from plants can be longer. The length of the virion is dictated by the length of the RNA genome, so that incorporation of additional genes into the viral genome results in correspondingly longer virions.⁶²⁹ Typically, mature virions appear either as bullet-shaped particles with one rounded and one flattened end or as bacilliform particles that appear hemispheric at both ends.

The genome RNAs of VSV and RABV, which are 11 to 12 kb, are encapsidated by approximately 1,200 copies of a

single major nucleoprotein (N protein), with each molecule of N protein covering nine bases.^{277,702} Unlike the paramyxovirus *rule of six*, there is no requirement that the genome size be a multiple of this number. The nucleocapsid also contains 466 copies of the phosphoprotein (P protein, formerly called NS protein)⁷⁰² and 50 copies of the large polymerase protein (L protein), which are responsible for the virion-associated RNA polymerase activity. The viral RNA polymerase cannot use naked RNA as a template but instead requires that the virion RNA template be encapsidated by N protein. P protein is responsible for binding L protein to the N protein–RNA template, and L protein is likely responsible for all of the enzymatic activities associated with RNA synthesis.

The structures of N protein–RNA complexes from RABV and VSV have been determined by x-ray crystallography.^{11,276} The N protein molecule consists of two lobes, with the RNA inserted between the two lobes (Fig. 31.3). In the nucleocapsid, an amino-terminal extension from each N protein subunit interacts both with the adjacent subunit and with the subunit two positions away (Fig. 31.3, inset). Contacts between the C-terminal lobes also contribute to the stability of the nucleocapsid. N protein forms a stable nucleocapsid-like structure even in the absence of RNA.^{278,810} The C-terminal lobes of two N protein molecules in the nucleocapsid form a binding site for the P protein polymerase subunit, which is proposed to bind and dissociate in a processive manner during RNA synthesis.²⁷⁶

P protein consists of three domains, an acidic N-terminal domain, a central domain, and a C-terminal domain.¹⁸⁵ P protein forms homo-oligomers, which are necessary for P protein to bind L protein to the nucleocapsid and for subsequent

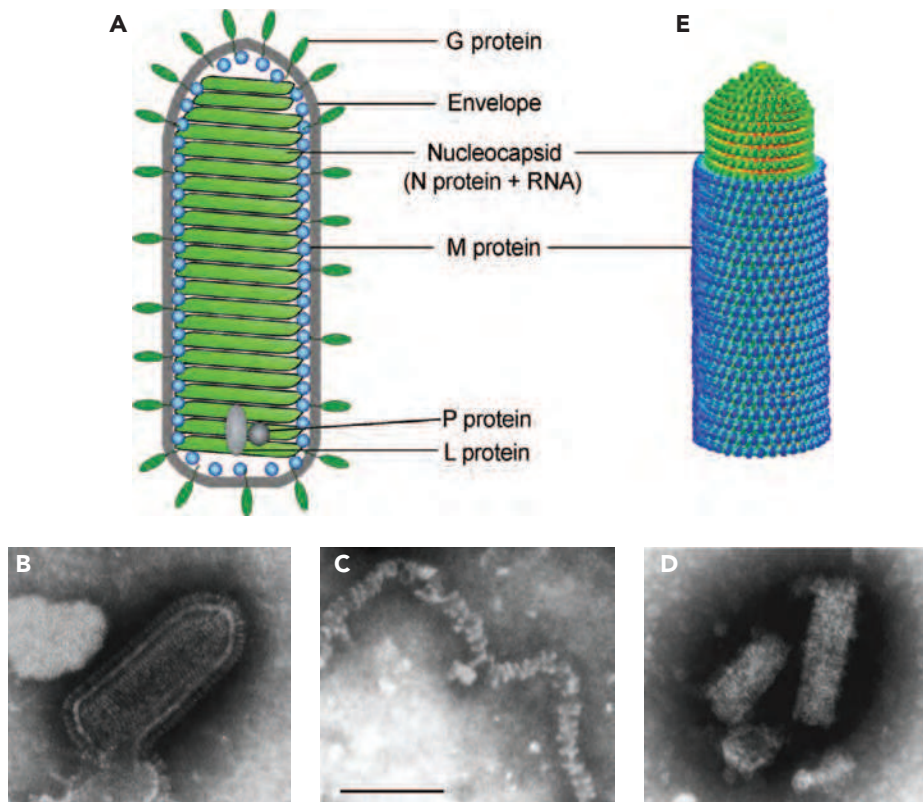


FIGURE 31.2. Structure of rhabdovirus virions. **A:** Diagram of virion. **B:** Negative stain electron micrograph of vesicular stomatitis virus (VSV) virion. **C:** VSV nucleocapsids prepared by solubilization of virion envelopes with triton X-100 in high-ionic-strength buffer. **D:** VSV nucleocapsid–M protein complexes prepared by solubilization of virion envelopes with triton X-100 in low-ionic-strength buffer. **E:** Model of the VSV nucleocapsid–M protein complex derived from cryoelectron microscopy data of Ge et al.²⁶⁴ Bar = 100 nm. (Negative stain electron micrographs by E. Alexander Flood, as described in [237].)

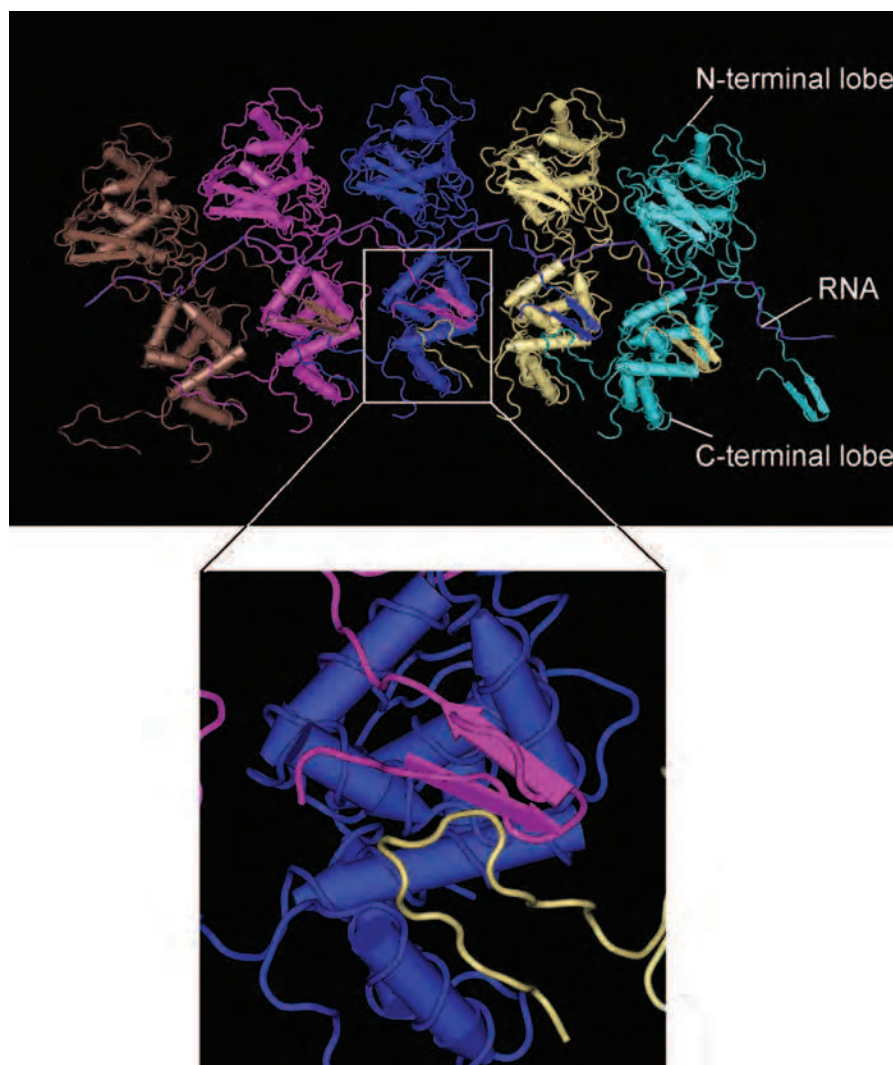


FIGURE 31.3. Structure of the vesicular stomatitis virus (VSV) nucleocapsid.

Model of N protein and RNA in the VSV nucleocapsid derived from x-ray crystallography²⁷⁹ and cryoelectron microscopy.²⁶⁴ **Inset** shows interaction of the N-terminal extension from the pink N protein subunit with the C-terminal domain of the adjacent subunit (blue) as well as the subunit two positions away (white). (Assembled from PDB file 2WYY (MMDB ID: 80066) using Cn3D4.2 software.)

transcriptase activity.^{256,257} The oligomerization is mediated by the P protein central domain, the structure of which has been determined by x-ray crystallography.¹⁸⁴ Both the isolated central domain and the unphosphorylated full-length P protein form dimers.^{184,267} The phosphorylated transcriptionally active form of P protein was originally considered to be a trimer, based on epitope dilution experiments.²⁵⁶ Reanalysis of those data,⁶⁹³ however, suggests that P protein forms tetramers, similar to the P proteins of paramyxoviruses. Much of the N-terminal domain of P protein appears to be intrinsically disordered, although it probably adopts a well-defined structure upon binding ligands such as the L protein or soluble N protein (N_0) involved in encapsidation of progeny genomes during genome replication.^{268,434} Two sites in the N-terminal domain must be phosphorylated by cellular casein kinase II for P protein to form oligomers and act in transcription.^{257,689} The C-terminal domain is responsible for binding the P protein to the nucleocapsid template, as described earlier. A basic region near the C-terminus of P protein is also necessary for interaction with L protein in viral transcription.¹⁶⁴ The structures of the C-terminal domains of RV and VSV P proteins have been determined by x-ray crystallography and NMR spectroscopy.^{276,481,588}

The organization of the L protein has been deduced by analysis of sequence homology among members of the order *Mononegavirales*, which identified six conserved regions designated CRI through CRVI. The RNA polymerase activity has been mapped to CRIII.⁶⁵⁵ The L protein also has messenger RNA (mRNA) capping and methylation activity, which map to CRV⁴³⁷ and CRVI,⁴³⁵ respectively. High-resolution structures of L protein have not been published thus far, but its domain organization has been determined by negative stain electron microscopy combined with proteolytic digestion and deletion mutagenesis.⁵⁷⁷ The protein is organized into a ring-like structure that contains the RNA polymerase and an appendage of three globular domains. The capping activity maps to a globular domain attached directly to the ring, and the methylation activity maps to a more distal and flexibly connected domain.

When released from virions by treatment with detergents at high ionic strength, the nucleocapsid is loosely coiled and flexible (Fig. 31.2C), with a total length of 3.6 μm .⁷⁰² In virions, however, the nucleocapsid is associated with the matrix (M) protein, which condenses the nucleocapsid into a tightly coiled helical nucleocapsid–M protein complex (Fig. 31.2D), sometimes referred to as the virus *skeleton*,^{54,514,515} which gives

the virion its bullet-like shape.^{54,463,514,515} The structure of the nucleocapsid–M protein complex has been determined to 10.6 Å resolution by analysis of cryoelectron micrographs of VSV virions (Fig. 31.2E).²⁶⁴ The N and M protein subunits in this structure were identified by fitting the electron density data from electron microscopy to that from x-ray crystallography. The N protein and RNA form an inner helical layer surrounded by an outer helical layer composed of M protein. The orientation of N protein subunits indicated that the 5′ end of the genome RNA is at the tip of the bullet, and the 3′ end is at the base. The conical tip of the bullet is formed by approximately seven successive turns of the N protein helix expanding gradually from 10 subunits per turn to 37.5 subunits per turn, with two turns forming a helical repeat. This pattern continues for approximately 29 turns to form the cylindrical trunk of the bullet. The M protein layer is formed by interaction of each M protein subunit with two successive turns of the N protein helix. The helical structure is held together by the M–N interactions as well as the interaction between M protein subunits in successive turns of the helix.

The amino-terminal 50 to 57 amino acids of M protein appear to be largely disordered in purified M protein.^{260,274,362} However, this sequence may form an ordered structure upon binding to the nucleocapsid.²⁶⁴ The remainder of the M protein sequence forms a compactly folded C-terminal domain, whose structure has been determined by x-ray crystallography.^{261,274} Sequences in the amino-terminal region are involved in interaction with N protein in the nucleocapsid–M protein complex, as well as interacting with sequences in the C-terminal domain in M–M interactions in the complex.^{144,160,264,274} The C-terminal domain of M protein also appears to interact with the virus envelope, perhaps with the cytoplasmic domain of the envelope glycoprotein.²⁶⁴

The structure derived from cryoelectron microscopy accounts for approximately 1,200 of the 1,800 copies of M protein in the virion. The remaining 600 M protein subunits are likely present in a nonhelical arrangement, thus rendering them undetectable in the analysis. One likely location is in association with the envelope lipid bilayer. M protein interacts with the lipid bilayer of the virus envelope, which was shown using lipophilic photoreactive probes.^{430,804} The M protein sequences involved in the interaction are present in the N-terminal region, partially overlapping the sequences involved in interaction with the nucleocapsid.^{160,430} This supports the idea that there are two populations of M protein in the virion, one involved in the nucleocapsid–M protein complex and the other involved in interaction with the envelope lipid bilayer.

The lipids of the envelope are derived from the host cell membrane during virus assembly by budding. The lipid composition of the envelope generally reflects that of the host membrane from which the virus buds, consisting primarily of phospholipids and cholesterol, although virus envelopes appear to be enriched in cholesterol and sphingomyelin compared with the host membranes from which they were derived.⁴⁵⁵ The virus envelope contains approximately 300 to 400 spike-like projections composed of a single species of viral glycoprotein (G protein). The individual spikes are trimers of G protein,^{188,262,765} which function in virus attachment and penetration by fusion of the virus envelope with endosome membranes. G protein is anchored in the envelope lipid bilayer by a 20–amino acid hydrophobic transmembrane domain near the

C-terminus, which is followed by a 29–amino acid *cytoplasmic domain*, which is inside the virus envelope.⁶⁰⁶ The structure of the 446–amino acid external domain (ectodomain) of the VSV G protein has been determined by x-ray crystallography in both the neutral pH (“prefusion”) and low pH (“postfusion”) conformations (Fig. 31.4).^{595,596} Like other viral fusion proteins, the two conformations are dramatically different, indicating that major structural rearrangements must occur during

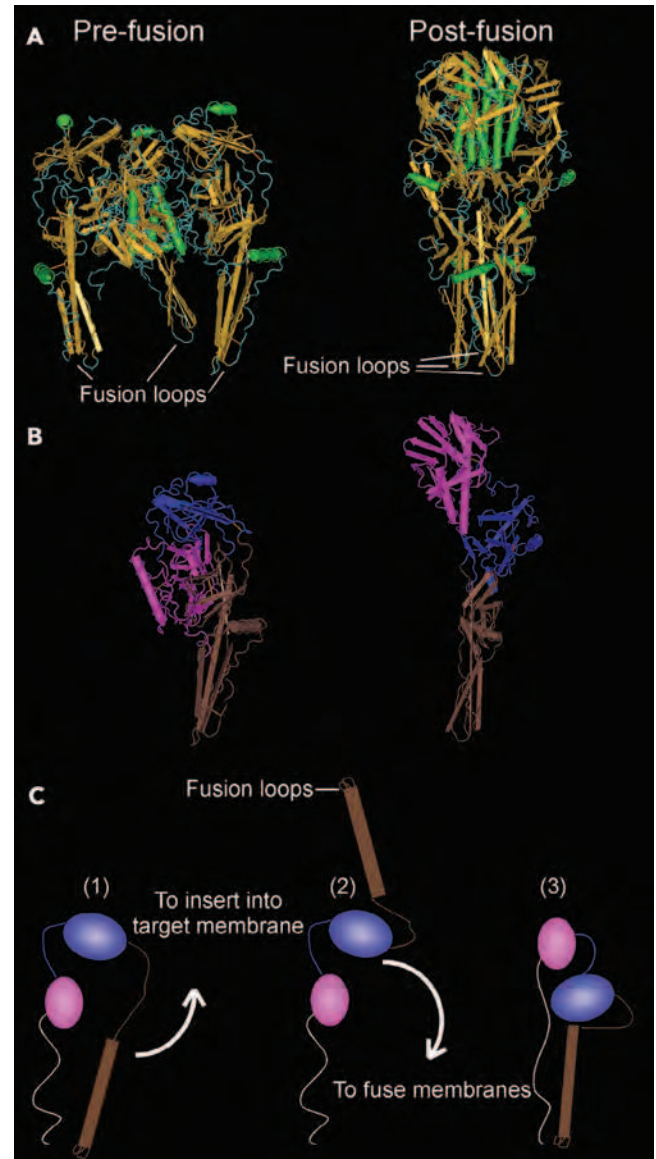


FIGURE 31.4. Structure of the vesicular stomatitis virus (VSV) G protein. **A:** G protein ectodomain trimers in the prefusion and postfusion state. G protein ectodomain (residues 1 to 422) was generated by limited proteolysis of virions with thermolysin.^{595,596} **B:** Comparison of domain organization of a single G protein subunit in the pre- and postfusion states. **C:** Diagram of domain rearrangements in transition from prefusion state (1) to proposed intermediate inserted into target membrane (2) to postfusion state (3). White line represents G stem leading to membrane anchor sequence that is missing from the crystal structure. (Assembled from PDB files 2CMZ and 2J6J using Cn3D4.2 software.)

the fusion process. The surprising result is that the folding of G protein bears no resemblance to the fusion proteins of other negative-strand or positive-strand RNA viruses that have been determined. Instead, the structure of the VSV G protein is homologous to that of the gB glycoprotein of herpesviruses.³⁰⁵ This raises interesting questions about the evolutionary origin of these proteins.

GENOME STRUCTURES

Genomes of rhabdoviruses are single-stranded, nonsegmented RNA of negative polarity. They lack 5' caps and 3' poly A, consistent with their inability to function as mRNA. Genomes of three genera of *Rhabdoviridae* are shown diagrammatically in Figure 31.5. Genomes of lyssaviruses and vesiculoviruses are similar to each other. The approximately 50 nucleotides at the 3' and 5' ends (the leader and trailer sequences, respectively) are partially complementary. They contain important *cis*-acting sequences that serve as promoters for transcription and replication and as signals for encapsidation of genomes and anti-genomes during replication, as described later. Although they do not encode proteins, short RNAs of unknown function are generated from these sequences. The five protein-encoding genes are in the order 3'-N-P/C-M-G-L-5', which is the order of the analogous genes in other nonsegmented, negative-strand RNA viruses, regardless of the number of additional viral genes. Each gene junction contains a conserved sequence specifying the end (E) of the upstream gene, a two-nucleotide intergenic (I) sequence, and the start (S) sequence for the downstream gene. These sequences control the activities of the viral RNA polymerase, which transcribes these genes according to a stop-start mechanism described later. In general, the 5' and 3' untranslated regions of the viral mRNA are short (10 to 50 nucleotides) and lack *cis*-acting sequences that control translation or mRNA turnover. The one exception is the P/C gene of vesiculoviruses, which contains alternate start codons. The upstream start codon initiates translation of the P protein, whereas two downstream start codons initiate translation of an alternate reading frame that encodes two small basic proteins, C and C'.^{392,667} Analogous proteins encoded by paramyxoviruses often play a role in pathogenesis by altering viral gene expression and suppressing host responses to virus infection. Mutation of the VSV P gene to introduce a stop codon in the C and C' open reading frame (without altering the sequence of the P protein), however, had no detectable effect on virus replication in cell culture or pathogenesis in mice.³⁹²

This still leaves open the possibility that the C and C' proteins play a role in replication in other hosts.

The genomes of ephemeroviruses are larger than those of most other rhabdoviruses. The genome of BEFV is 14.8 kb and contains 10 genes (3'-N-P-M-G-G_{NS}-α₁-α₂-β-γ-L-5') separated by intergenic regions of 26 and 53 nucleotides.^{177,735} The genome of the related Adelaide River virus is 14.6 kb in length and contains nine genes (3'-N-P-M-G-G_{NS}-α₁-α₂-β-L-5') separated by intergenic regions of one to four nucleotides.⁷⁴² The G_{NS} gene encodes a glycoprotein, which is synthesized in approximately the same amount as G protein during virus infection,⁷³⁷ but it is not found in the mature virion. Intracellularly, G_{NS} protein is localized in the endoplasmic reticulum-Golgi complex, and it is associated with amorphous structures in the cell surface but not with viruses in the budding process. It is highly glycosylated, with a molecular weight of 90 kD. G_{NS} protein shares significant amino acid sequence homology with the G protein, but it does not induce *protective* neutralizing antibodies.³¹² The function of G_{NS} protein is unknown. It has been proposed that the gene coding for this protein originated by gene duplication by a copy-choice mechanism involving relocation of the polymerase in an upstream position during viral replication.⁷⁴²

Genomes of novirhabdoviruses contain an NV gene between the G and L genes. The NV protein (12 to 14 kD) is expressed at variable levels in infected cells but is not detectable in purified virions. The NV protein sequences are significantly less conserved between viruses in different species than sequences of the other structural proteins, such that there is no significant amino acid sequence similarity between the NV proteins of IHNV and VHSV. The specific function of the NV protein is not yet defined, but it is required for efficient virus replication. Results of studies with NV gene deletion mutants generated by reverse genetics are inconsistent in that the NV appears to be required for pathogenicity in IHNV and VHSV but not snakehead virus (SHRV).^{317,398}

Rhabdoviruses that have not been assigned to a particular genus have a variety of additional transcription units. For example, the gene order of FLAV is 3'-N-P-pseudogene 1-19K-pseudogene 2-M-G-L-5'. The unique features include the gene encoding a 19-kD protein of unknown function, surrounded by two pseudogenes, about 500 nucleotides each, situated between the P and M genes.^{86,177} The gene order of TUPV is 3'-N-P/C-M-SH-G-L-5'. The unique small hydrophobic (SH) transcription unit between M and G genes encodes a protein with two hydrophobic amino acid stretches, including a potential signal sequence at the amino terminus

Vesiculovirus and lyssavirus



Ephemerovirus



Novirhabdovirus



FIGURE 31.5. Diagram of rhabdovirus genomes.

and a potential membrane-spanning sequence near the center. The C protein ORF that overlaps the TUPV P gene has the potential to encode a 221-amino acid basic protein that is more than three times larger than the VSV C protein.⁶⁶⁸

The genome of Ngaingan virus (NGAV) is over 15.7 kb, which is the largest genome yet described for any rhabdovirus, containing 13 ORFs in the order 3'-N-P-U1-U2-U3-M-U4-G-G_{NS}-U5-U6-U7-L-5'. The NGAV P gene contains two alternative ORFs designated P1' and P2', analogous to alternative P ORFs referred to as either C or P' in several other rhabdoviruses. The G_{NS} gene encodes a nonstructural glycoprotein (568 amino acids [aa]) analogous to that of ephemeroviruses. NGAV contains seven additional genes (U1 through U7) with the potential to encode small proteins of unknown function. Although similar in size (81 to 153 aa) to proteins encoded by ORFs located in similar positions in several other rhabdoviruses, they lack significant sequence or structural similarity to any known protein. However, none of the small unique NGAV proteins has yet been detected in infected cells.²⁸³

The gene order in WONV is 3'-N-U4-P-U1-U2-U3-M-G-U5-L-5'. WONV lacks an alternative ORF in the P gene but contains five additional genes (U1 through U5), each of which encodes a protein that lacks significant amino acid sequence identity with other known proteins. The U1 protein (179 aa) is hydrophilic with numerous potential phosphorylation sites, an N-glycosylation site, an amidation site, and two N-myristoylation sites. The U2 protein (192 aa) contains two predicted N-myristoylation sites and a highly hydrophobic domain of 10 amino acids followed by a mitochondrial energy transfer signature that is characteristic of carrier and transport proteins. The U4 protein (49 aa) contains a single putative N-myristoylation site and shares overall 49% identity with guanosine triphosphate (GTP)-binding proteins of several bacteria. The U5 protein (127 aa) contains a predicted N-terminal extracellular domain, 22-aa transmembrane domain, and highly basic cytoplasmic tail and has overall structural similarity to the α_1 proteins of ephemeroviruses, which have been suggested to be viroporins. Proteins of similar size

to the U1, U2, U3, and U5 proteins have been detected in WONV-infected cells by immunoblot analysis using polyclonal mouse ascitic fluid.²⁸⁴

The genome of Moussa virus is similar to those of the genera *Lyssavirus* and *Vesiculovirus*. However, the ORFs located in the position of the P (ORF 2) and M (ORF 3) genes in other rhabdoviruses show no nucleotide or amino acid homology to sequences of other rhabdoviruses.⁵⁷⁵

The genomes of Sigma virus and members in the genera *Cytorhabdovirus* and *Nucleorhabdovirus* contain an additional gene between the P and M genes, referred to as "X" or "a." The putative X protein is of unknown function but contains conserved domains found in reverse transcriptases. Another unusual feature is that M and G mRNAs overlap by 33 nucleotides.^{106,145}

STAGES OF REPLICATION

The replication cycle of rhabdoviruses is typical of that of most nonsegmented, negative-strand RNA viruses (Fig. 31.6). The initial events of attachment, penetration, and uncoating result in release of the viral nucleocapsid into the cytoplasm of the host cell. The encapsidated parental genome RNA serves as a template for primary transcription by the virion RNA-dependent RNA polymerase, resulting in synthesis of leader (le) RNA and all five viral mRNAs. The accumulation of viral proteins synthesized from primary transcripts leads to replication of the genome, which involves synthesis of full-length positive-strand RNA, or antigenomes. The antigenomes, in turn, serve as templates for synthesis of progeny negative-strand genomes. Encapsidation of genomes and antigenomes occurs concomitantly with their synthesis and, indeed, is a key signal for the RNA polymerase to function as a replicase versus a transcriptase. Progeny nucleocapsids are used for three different purposes: (a) as templates for further rounds of replication; (b) as templates for secondary transcription, which is the major amplification step for viral gene expression; and (c) for assembly into progeny virions, which occurs by budding

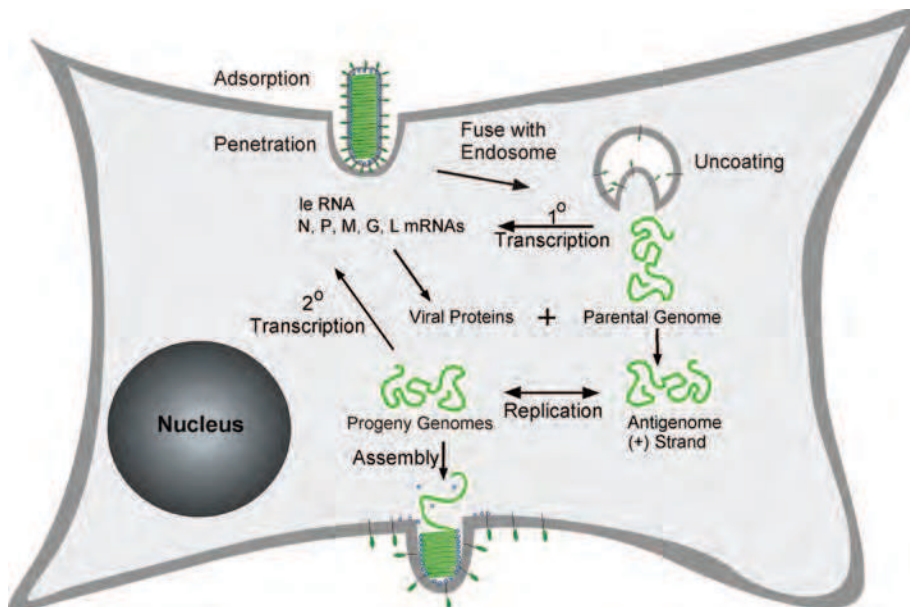


FIGURE 31.6. Diagram of rhabdovirus replication cycle. The steps illustrated are virus adsorption and penetration by endocytosis, envelope fusion and penetration by endocytosis, release of nucleocapsids containing parental genomes into the cytoplasm, primary (1-degree) transcription, genome replication to produce nucleocapsids containing antigenomes and progeny genomes, secondary (2-degree) transcription, and assembly by budding from host plasma membrane. (Drawn by E. Alexander Flood.)

from host membranes. In a single-cycle growth experiment, the early events including attachment, penetration, uncoating, and primary transcription occur within the first few hours postinfection. The processes of genome replication, secondary transcription, and virus assembly occur continuously throughout the remainder of the infectious cycle, which lasts for an additional 12 to 18 hours for VSV or several days for RABV.

Mechanism of Attachment

Rhabdoviruses appear to use a variety of different receptors for attachment to different types of host cells. The RABV G protein binds most effectively to cells of neuronal origin,⁷¹³ reflecting the neurotropism of RABV *in vivo*. Several different surface molecules expressed at high levels on neurons have been identified as potential receptors, including the nicotinic acetyl choline receptor,^{259,431} the neural cell adhesion molecule (CD56),⁷⁰⁴ and the low-affinity nerve-growth factor receptor p75^{NTR}.^{714,715} Expression of CD56 and p75^{NTR} has been shown to confer susceptibility to RABV on cells that are normally resistant to infection. Transgenic mice that lack CD56 show a delay in RABV spread through the central nervous system (CNS) and in RABV-induced mortality, but the mice still die following virus infection,⁷⁰⁴ indicating that other receptors are involved. RABV infection of transgenic mice that lack p75^{NTR} was found to be similar to that of wild-type mice of the same strain,³³⁹ initially indicating that p75^{NTR} was not an important receptor for RABV pathogenesis. However, RABV G protein binds to a region of p75^{NTR} that is present on a splice variant of p75^{NTR} that is still expressed in the transgenic mice.⁴¹⁴ Thus, further experiments are required to fully evaluate the role of p75^{NTR} in RABV pathogenesis.

In addition to the receptors that are enriched on cells of neuronal origin, RABV can also use receptors that are widely distributed among many cell types.^{582,685,793} These receptors appear to be of lower affinity than those on neuronal cell surfaces⁷¹³ and have been difficult to identify. A similar difficulty exists in identifying receptors for VSV, which also binds to many different cell types in culture by interactions that appear to be of low affinity and often are not easily saturable. For both RABV and VSV, negatively charged lipids have been proposed to be cellular receptors for virus attachment. In the case of RABV, neuraminic acid-containing glycolipids (gangliosides) have been implicated⁶⁸⁶ and, in the case of VSV, phosphatidyl serine has been proposed as a cellular receptor,⁶²⁴ although later experiments have indicated that phosphatidyl serine is not the receptor for VSV.¹³⁹ Instead, it seems likely that nonspecific electrostatic and hydrophobic interactions mediate attachment of VSV to cells. Treatment of cells with polycations and polyanions such as diethylaminoethyl-dextran (DEAE-dextran) and dextran sulfate can markedly enhance the efficiency of attachment and infection of cells by both VSV⁴⁵ and RABV.⁷⁹³

An interesting feature of attachment by both viruses is that binding is markedly enhanced at lower pH in the range from pH 6.5 to 5.6.^{248,793} The pH dependence of attachment is similar to that of envelope fusion with cellular membranes (discussed in the next section), although fusion occurs most efficiently at slightly lower pH than does virus attachment. Furthermore, G protein mutations that shift the pH dependence of fusion also shift the pH dependence of attachment.²⁴⁸ This suggests that attachment to many cell types is mediated by G protein in a conformation that is similar to the fusion-

active form of the viral G protein. This idea is supported by experiments with photoactivatable lipid probes, which indicate that the putative *fusion peptide* (described later) is inserted into target membranes under the optimal conditions for both attachment and fusion.^{197,542} This would also account for the affinity of the VSV G protein for phosphatidyl serine and other negatively charged phospholipids, because the presence of negatively charged lipids in the target membrane appears to be necessary for virus envelope fusion.^{105,199,796}

Mechanism of Penetration

VSV was one of the early examples of a virus shown to penetrate into cells by clathrin-dependent endocytosis.⁴⁷⁸ Following attachment to host cell surfaces, virions can either migrate to preformed clathrin-coated pits or nucleate the formation of new coated pits,^{157,348} where they undergo endocytosis into coated vesicles (Fig. 31.7). Because VSV virions are longer than the typical diameter of a coated vesicle, the final closure of the endocytic vesicle requires participation of the actin cytoskeleton.^{157,158} The endocytic vesicles lose their clathrin coats to become early endosomes. The contents of early endosomes are transported to late endosomes and lysosomes for degradation. During this process, the endosomal vesicles often invaginate to form multiple intraluminal vesicles.²⁸² Such membranes are referred to as *multivesicular bodies* (MVBs). As virions progress through the endocytic pathway, they are exposed to progressively lower pH. At a pH below 6.5, the G protein mediates fusion of the viral envelope with the endosome membrane. This fusion event releases the internal virion components into the cytoplasm (left side of Fig. 31.7). Most of the available evidence indicates that VSV virions fuse primarily with the membranes of early endosomes.^{348,498,648} Other evidence, however, suggests that many fusion events occur within MVBs (right side of Fig. 31.7), releasing the internal virion contents into the cytoplasmic contents trapped within the MVBs and requiring *back-fusion* of internal vesicles with the limiting membrane of the MVBs to release the viral nucleocapsid into the cytoplasm of the cell.^{422,460} Viral proteins that fail to be released into the cytoplasm are degraded by proteases and other enzymes in lysosomes.⁴⁷⁸

The mechanism by which rhabdovirus G proteins induce fusion of the virus envelope with cellular membranes shares many features with other viral envelope fusion proteins but is clearly distinct in several respects. The VSV G protein and the structurally similar fusion proteins of herpesviruses and baculoviruses are referred to as class III fusion proteins to distinguish them from class I proteins, which are structurally similar to the influenza virus hemagglutinin, and class II proteins, which include the envelope glycoproteins of the alphaviruses and flaviviruses.³² As with class I fusion proteins, rhabdovirus G proteins exist as a trimer of subunits held together by noncovalent bonds.^{188,262,765} Unlike most viral envelope proteins, however, the subunits of G protein are in a dynamic equilibrium between monomers and trimers because of the rapid dissociation and reassociation of subunits.^{464,801,802} As with most low pH-dependent fusion proteins, the effects of low pH are mediated by conformational changes in G protein. Unlike other viral fusion proteins, the conformational changes in G protein are reversible upon returning the pH to neutrality, whereas those of many other viral fusion proteins are not reversible.^{188,263,573}

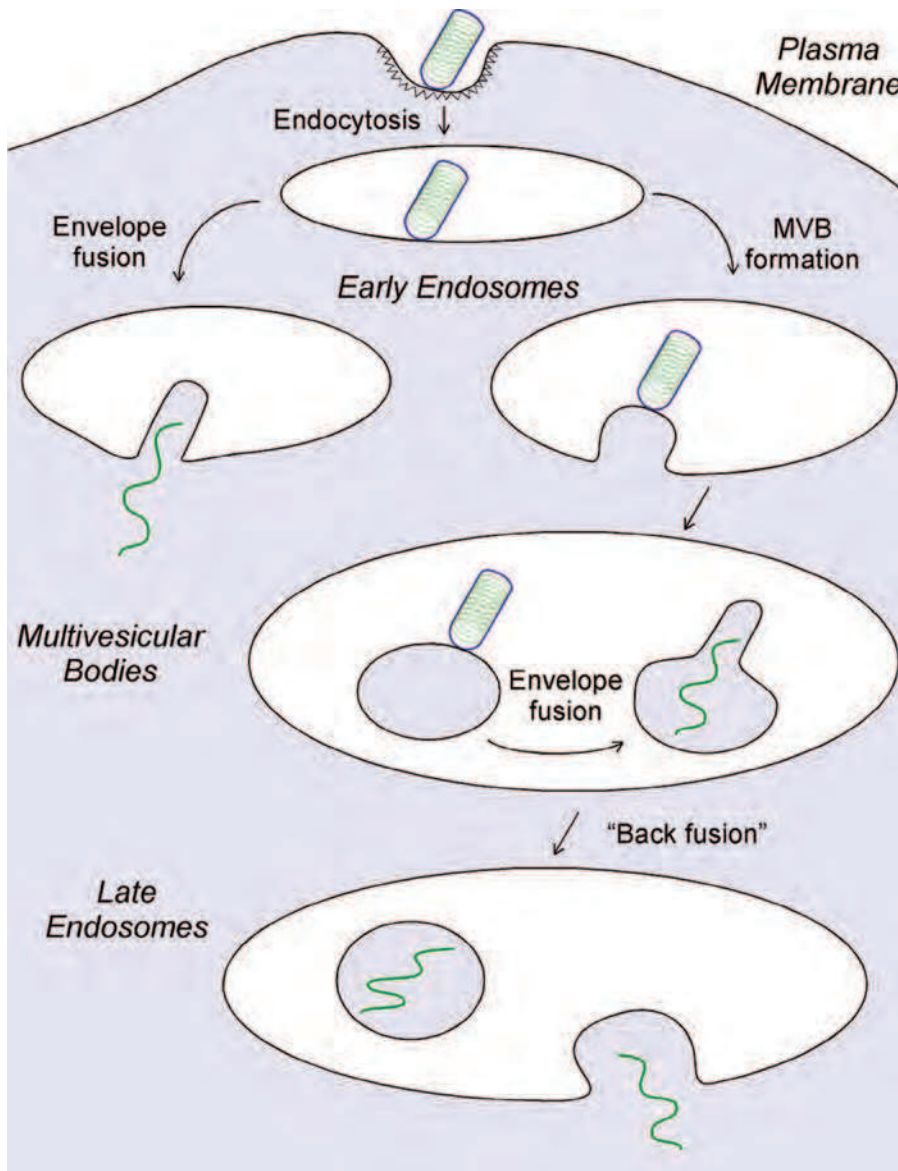


FIGURE 31.7. Diagram of rhabdovirus penetration by endocytosis. Pathway on the **left** shows virus envelope fusion with early endosomes. Pathway on the **right** shows virus envelope fusion with internal vesicles of multivesicular bodies (MVBs) and release of nucleocapsids into the cytoplasm by "back-fusion" with the MVB-limiting membrane.

A general principle by which viral envelope proteins promote fusion is that they must insert into the target membrane through a region of their sequence referred to as the *fusion peptide*. In the class I fusion proteins, such as the influenza virus hemagglutinin (HA) and the paramyxovirus F proteins, the fusion peptide resides at the N-terminus of one of the subunits (HA₂ or F₁, respectively) generated by proteolysis of an inactive precursor. In contrast, proteolysis of rhabdovirus G proteins is not involved in activating fusion. This is similar to the case of the class II fusion proteins, in which the fusion peptide appears to be an internal region of the protein sequence. The regions of G protein that insert into target membranes at low pH have been mapped using photoactivatable lipid probes and mutagenesis studies in both the RABV and VSV G proteins.^{197,249,441,684,808} These sequences form two loops containing hydrophobic amino acids that extend from the protein structure ("fusion loops," Fig. 31.4A). In the neutral pH "prefusion" state, the fusion loops are oriented toward the viral membrane (Fig. 31.4A, B). Upon lowering the pH, there is a

proposed intermediate, in which the domain containing the fusion loops is reoriented to insert into the target membrane (Fig. 31.4C). Fusion of the viral and target membrane involves another domain rearrangement that brings the two membranes together in the "postfusion" state.

A second region of the G protein sequence functionally involved in fusion is the membrane-proximal ectodomain sequence immediately N-terminal to the membrane anchor sequence. Most of this sequence is not visible in the x-ray structures, because it was cleaved to solubilize the G protein. Mutations in this region dramatically inhibit fusion.^{347,647} G protein truncations containing part of this region (amino acids 421 to 461) together with the membrane anchor sequence and the cytoplasmic domain (*G stems*) enhance the fusion activity of other membrane fusion proteins and are able to cause hemifusion (mixing of the outer phospholipid leaflets of the two membranes) in the absence of other fusion proteins.³⁴⁷ The cooperation of the fusion loops and the membrane-proximal sequence may be analogous to similarly separate sequences in

other viral fusion proteins in bringing the viral and host membranes together for fusion.

Uncoating and Primary Transcription

Following fusion of the virus envelope with endosome membranes, which releases the internal virion components into the cytoplasm of the host cell, the viral M protein dissociates from the nucleocapsid.^{162,498,590} This step is necessary for viral RNA synthesis to occur, because M protein inhibits viral transcription.^{107,136,443,543,775} Binding of most of the M protein to nucleocapsids is readily reversible,⁴⁶¹ and dissociation following envelope fusion is believed to occur spontaneously, although acidification of the virion interior appears to promote M protein dissociation from the nucleocapsid, similar to the M1 protein of influenza virus.⁴⁹⁷ Rhabdoviruses do not encode a separate ion channel protein analogous to the M2 protein of influenza viruses. Instead, the G protein is responsible for the permeability of the envelope to protons.⁴⁹⁷ Once the M protein has dissociated from the nucleocapsid, no further uncoating is necessary, because the encapsidated RNA is the template for the viral transcriptase complex.

The first biosynthetic step in the viral replicative cycle is primary transcription, mediated by the virion-associated, RNA-dependent RNA polymerase. The mechanism of primary transcription, defined as transcription from parental templates, appears to be identical to that of secondary transcription, or transcription from progeny templates following

genome replication. The principal differences are in the much larger quantity of secondary transcripts, because of the larger number of progeny templates, and the brief time of primary transcription, compared with the prolonged period of secondary transcription throughout most of the viral infectious cycle.

The viral RNA polymerase is fully competent to synthesize all of the viral mRNA without new synthesis of viral proteins, as shown by the transcriptase activity of virion cores following solubilization of the envelope.^{51,506} Indeed, the first demonstration of a viral RNA-dependent RNA polymerase was made with virions of VSV.⁵¹ This cell-free transcriptase system has been a major tool in determining the mechanisms of viral transcription, establishing the requirement for both the L and P proteins for RNA polymerase activity²⁰³ and the requirement that the template RNA be encapsidated.^{70,202} An early insight was that a single entry point exists for the viral RNA polymerase near the 3' end of the genome, and the viral mRNAs are transcribed sequentially in the order they appear in the genome: N–P–M–G–L. Thus, transcription of each gene depends on prior transcription of all upstream genes.^{1,50,201,336,337} This has since been found to be a general property of nonsegmented, negative-strand RNA viruses (see Chapter 30).

The mechanism of sequential transcription is generally considered to be a stop–start mechanism, in which *cis*-acting signals in the template RNA sequence govern the activities of the transcriptase complex at each gene junction (Fig. 31.8). With the exception of the junction between the leader and N

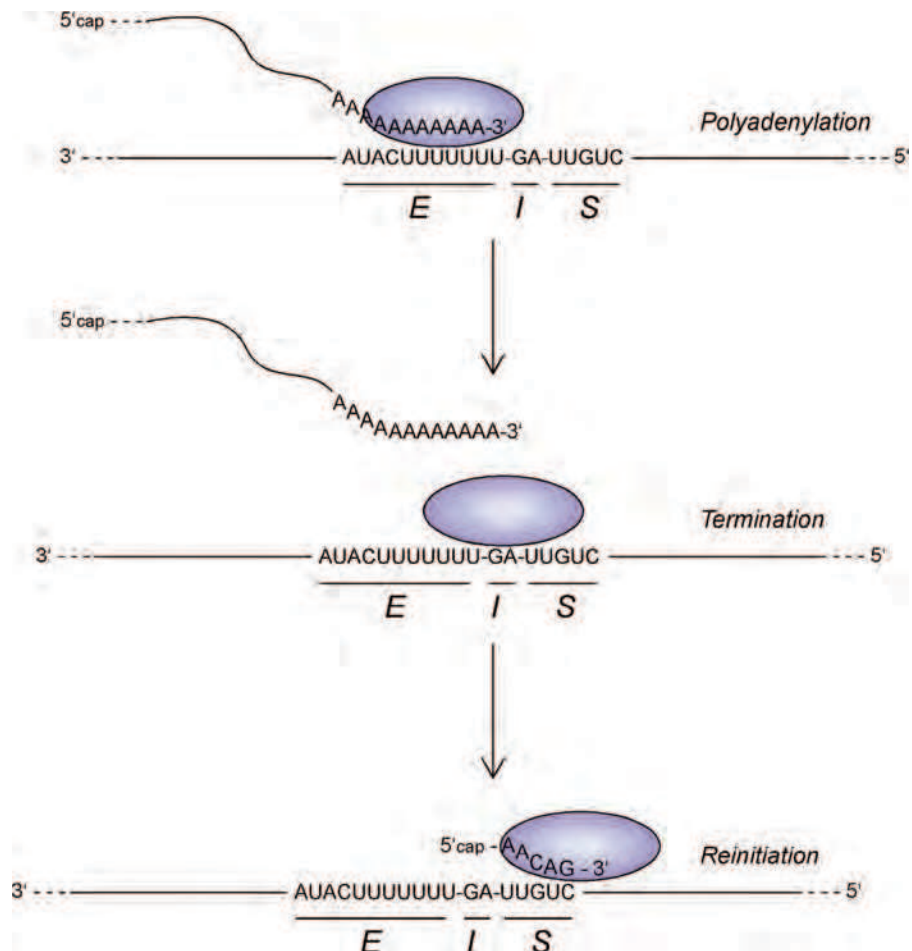


FIGURE 31.8. Diagram of rhabdovirus polymerase activities during transcription in response to gene end (E), intergenic (I), and gene start (S) sequences in the genomic RNA template.

genes (discussed later), each of the VSV gene junctions contains a gene end sequence for the upstream gene (3'AUACU-UUUUUU5'); an intergenic dinucleotide (G/CA), which is not transcribed; and a gene start sequence for the downstream gene (3'UUGUC5').⁶⁰⁴ These sequences at each gene junction function as a signal for polyadenylation and termination of the upstream mRNA and also as a signal for the initiation, capping, and methylation of the downstream mRNA.^{55–57,315,630,677–679} Whereas the *cis*-acting signals in the template and the resulting modifications to the mRNA have been well defined, the mechanism by which these signals alter the activities of the transcriptase complex to accomplish these different tasks is a major question that remains to be addressed.

Transcript initiation requires both proper termination of the upstream gene and the gene start sequence 3'UUGUC5'.^{56,57,315,329,677–679} The requirements of the individual nucleotides in the start sequence for 5' end modification are more rigorous than the requirements for correct initiation.^{679,741} Alterations of the capping and methylation of the transcripts affect the processivity of the polymerase and the extent of polyadenylation of the 3' end, supporting a model in which the correct initiation and modification of the viral mRNAs play a regulatory role in the subsequent activities of the polymerase.^{255,436,741} The mechanism of 5' end modification of VSV mRNAs and likely those of other negative-strand RNA viruses differs substantially from that of host mRNAs and mRNAs of other virus types. The viral mRNAs are capped by guanosine in a 5'-5' triphosphate linkage, as are host mRNAs.² The capping reaction differs, however, in that both the α - and β -phosphates are derived from the GTP donor, whereas for host capping enzymes, only the α -phosphate is derived from the GTP donor.^{2,533} This reaction occurs through an unusual covalent L protein–RNA intermediate.⁵³³ The host translation factor EF-1 is associated with the viral L and P proteins, and it has been proposed that the α -subunit of EF-1 plays a role in the capping reaction through its guanine nucleotide-binding activity.^{124,574}

The mechanism of methylation of the viral mRNA cap is also unusual. S-adenosyl methionine is used as a methyl donor,² as with host enzymes. However, instead of having separate enzymes that catalyze ribose 2'-O methylation versus guanine-N-7 methylation, both activities appear to reside in a single domain of L.^{254,275,435,438} This domain has a single binding site for S-adenosyl methionine and transfers the methyl groups in an unconventional order, in which 2'-O methylation precedes guanine-N-7 methylation.⁵⁷⁶

Following elongation of viral mRNA, the transcriptase complex encounters a termination signal at the end of each gene consisting of the sequence 3'AUACU-UUUUUU. This signals the polymerase to "stutter" over the seven Us in the template, resulting in polyadenylation of the viral mRNA.^{55,56} Occasionally, the termination signal is ignored, resulting in read-through by the polymerase to give a dicistronic transcript.^{56,57,311} Some nonsegmented, negative-strand viruses have gene junctions with a high degree of read-through, which plays a substantial role in regulating the relative levels of the different viral proteins. Because read-through transcripts are rather uncommon for VSV, they are not thought to play a significant role in regulation of viral gene expression.

Following the polyadenylation reaction, which stops after addition of approximately 200 As, two possible fates exist for the transcriptase complex at each gene junction. The most com-

mon outcome is that the transcriptase complex traverses the two intergenic nucleotides and resumes transcription at the initiation signal of the downstream gene. Approximately 20% to 30% of transcriptase complexes fail to resume transcription of the downstream gene, however, and presumably dissociate from the template, leading to a 20% to 30% attenuation of expression of the downstream gene at each gene junction.^{336,730,757} This transcription attenuation results in a gradient of mRNA and protein expression, such that the abundance of each gene product depends on its distance from the 3' end of the genome (i.e., $N > P > M > G > L$). The G–L gene junction is unusual in that the level of attenuation is much higher than that at the other gene junctions,⁴⁹ resulting in much lower levels of L protein relative to the other viral proteins. The basis for this difference is not known, because the sequence of the G–L gene junction does not differ from that of the other gene junctions.

Transcription attenuation is a general feature of nonsegmented, negative-strand RNA viruses and is the major mechanism regulating abundance of the individual mRNA. The importance of the gene order in regulating the relative levels of viral proteins was dramatically illustrated by genetic engineering experiments to change the order of the genes of VSV. The resulting changes in relative abundance of the viral proteins resulted in substantial reductions in viral replication and pathogenesis.^{49,757} The similarity of the basic mechanisms in virus replication among nonsegmented, negative-strand RNA viruses and their dependence on the relative levels of each viral protein presumably accounts for the conservation of the basic gene order among these viruses.

The initiation and termination of transcription of the leader RNA differs from that of the viral mRNAs. The leader RNA is encoded by the 47 3'-terminal nucleotides of the genome. The leader gene differs from the other genes both in terms of the *cis*-acting signals in the template that initiate transcription⁷⁶⁰ and the nature of the product leader RNA, which is phosphorylated at the 5' end and lacks a cap structure. In addition, the sequence at the leader–N gene junction is distinct from that of the other gene junctions and lacks the U₇ sequence that governs polyadenylation.⁷⁶⁰ Correspondingly, leader RNA is not polyadenylated. Another unusual feature of transcriptional regulation at this gene junction is that its behavior is different in the cell-free transcription system versus transcription in infected cells. In the cell-free system, synthesis of leader RNA is required to transcribe the downstream N gene, consistent with the single polymerase entry site and stop–start model.^{201,762} In infected cells, however, the viral RNA polymerase can initiate synthesis at the first downstream gene without prior synthesis of leader RNA. This has been shown by inserting a small gene between the leader and N genes and determining the target size for UV inactivation of the inserted gene.⁷⁶² This is the only gene junction that shows this behavior, because transcription initiation at all of the other genes requires prior transcription of the upstream gene both in the cell-free assay and in infected cells.

The difference in the site of initiation in infected cells versus that in the cell-free system indicates that host factors can influence the site of initiation. A viral transcriptase complex has been isolated from VSV-infected cells that contains, in addition to P and L proteins, the host proteins EF-1 α , heat shock protein 60 (hsp60), and smaller amounts of the host mRNA capping enzyme guanylyl transferase.⁵⁷⁴ Unlike the virion RNA polymerase, this complex initiates transcription at

the N gene and does not transcribe leader RNA.⁵⁷⁴ The ability of the polymerase to independently initiate at the first gene downstream of the leader gene appears to account for the phenotype of a VSV mutant (polR1) that synthesizes N mRNA in excess over leader RNA,¹²⁸ which would be difficult to achieve if transcription of the N mRNA required prior transcription of the leader RNA. The mutation responsible for the polR phenotype is in the N protein associated with the template,¹²⁷ indicating that the nature of the template can influence the site of initiation.

Genome RNA Replication

Requirement for Encapsidation of Newly Synthesized RNA

A fundamental principle in replication of nonsegmented, negative-strand RNA viruses is that the ability of the RNA polymerase to replicate the viral genome depends on new viral protein synthesis to encapsidate the newly synthesized RNA. For example, treating infected cells with inhibitors of protein synthesis (e.g., cycloheximide) allows synthesis of viral mRNA but inhibits replication of genome RNA.^{324,756} The critical viral protein required for replication is the N protein, as shown by its ability to support synthesis of genome RNA in the cell-free system.⁵⁵⁰ In infected cells, however, a complex of N protein with P protein (often referred to as N_0 -P) is likely to be the active complex in promoting genome replication.^{555,556} The role of P protein in this complex appears to be to maintain the solubility and proper folding of N protein so that the nascent RNA synthesized by the RNA polymerase can be encapsidated.^{167,470,476,477} Analysis of an N_0 -P complex expressed in insect cells indicates that the complex contains one N protein and two P proteins.⁴⁸⁰

Encapsidation of nascent RNA appears to constitute a signal for the viral RNA polymerase to ignore the sequences in the genome template at each gene junction that govern the stop–start mechanism for transcription, thereby generating full-length, encapsidated RNA that is complementary to the genome (i.e., antigenomes). Use of antigenomes as templates results in synthesis of progeny genomes. The mechanism of RNA repli-

cation appears to be the same regardless of whether genomes or antigenomes are used as templates. In particular, replication of both templates requires that the nascent product RNA be encapsidated to generate full-length products. In addition to serving as a template for progeny genomes, the antigenome can be used as a template to generate a short, noncapped, nonpolyadenylated RNA complementary to the 3' end of the antigenome that is analogous to the leader RNA. Various called the *minus strand leader* or *trailer* RNA, this RNA is found in small amounts in infected cells and is the primary product produced in the cell-free system when antigenomes are used as templates in the absence of a source of new viral proteins.^{432,755,776}

Cis-Acting Signals and RNA Polymerase Complexes that Govern Replication Versus Transcription

The critical *cis*-acting RNA sequences that govern replication are located at the 3' ends of the genome and antigenome (Fig. 31.9). These sequences in the templates serve as promoters to initiate RNA synthesis, and their complementary sequences at the 5' end of the product RNA serve as encapsidation signals, with the resulting encapsidation permitting elongation of the RNA into full-length products. The sequences required for encapsidation have been mapped using synthetic RNA in cell-free encapsidation assays and also in transfected cells using minigenomes (described later).^{163,507,548,760} The sequences in the templates that serve as promoters for replication and transcription have been defined by mutagenesis studies using minigenomes.^{439,440,548,758,760} The 3' termini of the genome and antigenome of VSV are identical at 15 of 18 positions. These 18 nucleotides are essential elements of both the genomic and antigenomic promoters. The near identity of the 3' termini of the genome and antigenome implies that both RNAs display terminal complementarity. This complementarity enhances the activity of these RNA as templates for replication,⁷⁵⁸ suggesting that base pairing of the termini is an important element of promoter recognition by the VSV RNA polymerase, similar to promoter recognition by the influenza virus RNA polymerase.

The genomic and antigenomic promoters of VSV differ substantially at positions 19 to 29 and 34 to 46. These

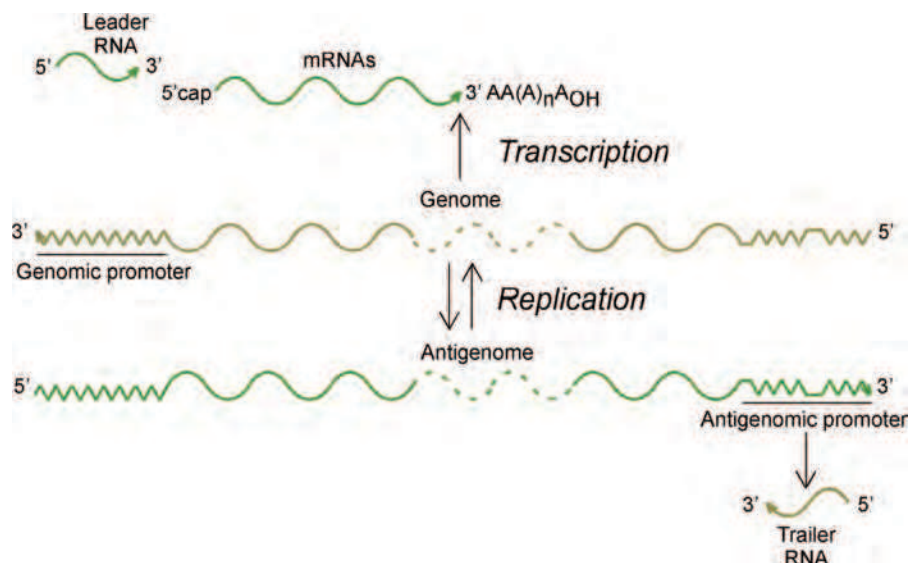


FIGURE 31.9. Diagram of activities of the rhabdovirus genomic and antigenomic promoters in transcription versus replication.

sequences in the genomic promoter are required for mRNA synthesis, but not for replication.^{439,760} In contrast, these sequences in the antigenomic promoter serve as an enhancer of replication.⁴⁴⁰ As a result of this enhancer activity in the antigenomic promoter, replication of genomes versus antigenomes is asymmetric: many more genomes than antigenomes are synthesized in virus-infected cells.^{231,650,666,754} The functional differences between the genomic and antigenomic promoters were dramatically illustrated by engineering an *ambisense* RABV that contained the sequence of the genomic promoter at the 3' ends of both the genome and antigenome.²³¹ The promoter in the antigenome of this virus was engineered to drive the transcription of a foreign gene. As a result, both the genome and antigenome of this virus were used as templates for mRNA synthesis—the genome as a template for the five viral mRNAs and the antigenome as a template for the foreign mRNA. Furthermore, the normal asymmetry of replication was abolished, so that genomes and antigenomes were synthesized in approximately equal amounts.

In addition to differences in the *cis*-acting sequences that govern replication versus transcription, differences in the nature of the polymerase complex and the structural requirements of the P protein are also important for replication versus transcription. The C-terminal basic region of P protein that is involved in interaction with L protein is required for transcription, but not replication.¹⁶⁴ Similarly, phosphorylation of serine and threonine residues in the N-terminal domain of P protein by cellular casein kinase II is required for transcription, but not for replication.⁵⁵⁰ In contrast, phosphorylation of sites in the C-terminal domain is required for replication, but not transcription.³²⁹ These differences in the structural requirements for P protein are consistent with the idea that the polymerase complex that carries out replication is distinct from that which carries out transcription. In particular, the transcriptase appears to be an L-(P₄) complex, whereas the replicase appears to be an L-N-P₄ complex.²⁸⁶ Establishing the structural basis for the differences in replication versus transcription, particularly the ability of the two polymerase complexes to respond to *cis*-acting signals in the template and the requirement for encapsidation of the nascent product RNA, is a key issue for understanding RNA synthesis by rhabdoviruses and other nonsegmented, negative-strand viruses.

Secondary Transcription

Once nucleocapsids containing progeny genomes begin to accumulate in infected cells, they are used as templates for secondary transcription, and they are assembled into progeny virions. In the case of VSV, most of the viral nucleocapsids that are made during the infectious cycle remain associated with infected cells and are not released in the form of progeny virions,^{379,666} suggesting that use of these nucleocapsids as templates predominates over their use for virion assembly. Although conceptually it is the last step in the virus replication cycle, virus assembly begins at approximately the same time as secondary transcription (for VSV, around 2 to 3 hours postinfection), reaches a maximum rate around 8 to 10 hours postinfection when viral protein synthesis is at its maximum, and declines concomitantly with a decline in viral protein synthesis toward the end of the infectious cycle around 16 to 20 hours postinfection.

Assembly of Progeny Virions

As with most viruses, the individual components of rhabdoviruses are assembled in separate cellular compartments and only come together in the final steps of virus assembly: the nucleocapsid is assembled during the process of RNA replication as described in the previous section, the G protein is assembled in the secretory pathway, and the M protein is synthesized as a soluble protein that then associates with the cytoplasmic surface of the host plasma membrane.

Assembly of G Protein

The assembly of the VSV G protein in the secretory pathway of host cells has been studied for many years by both virologists and cell biologists, not only for its importance for virus assembly, but also as a prototype for the assembly of other host and viral integral membrane proteins. G protein is synthesized by ribosomes bound to the rough endoplasmic reticulum (ER) and is inserted into the ER membrane in the typical *type I* orientation.^{605,606} An N-terminal signal sequence of 16 amino acids targets the protein for insertion and is cleaved from the nascent polypeptide.⁴⁴⁶ The new N-terminus and most of the protein sequence (446 amino acids) are transferred to the luminal side of the ER membrane to form the protein's ectodomain.³⁶⁷ A hydrophobic sequence of 20 amino acids near the C-terminus serves as a stop-transfer sequence and becomes the membrane anchor.⁶⁰⁶ The 29 C-terminal amino acids remain on the cytoplasmic side of the ER membrane and form the protein's cytoplasmic domain.³⁶⁷ Two asparagine residues in G protein are glycosylated during translation.³⁶⁸ The initial oligosaccharides added to G protein are of the high mannose type, which are later modified by enzymes in Golgi membranes to the complex type of oligosaccharides.⁵⁸¹

Following insertion into the ER, G protein associates with two molecular chaperones, BiP (GRP78) and calnexin,^{290,467} which assist in the formation of the proper disulfide bonds and correct folding of the ectodomain. Mutations that prevent correct folding of the ectodomain or that prevent glycosylation, which is required for calnexin binding, result in the formation of aggregates of misfolded G protein together with BiP, which are not transported from the ER.^{189,467,468} Therefore, the ability of a mutant protein to be transported from the ER is a minimal criterion by which it can be said to be properly folded. Shortly after release of the properly folded G protein from the chaperones, G protein monomers associate into trimers¹⁸⁹ and are transported to Golgi membranes by membrane vesicles that bud from the ER and subsequently fuse with Golgi membranes.^{394,705} G protein is one of the most rapidly transported integral membrane proteins, requiring approximately 15 minutes to be transported from ER to Golgi membranes.⁶³ This rapid transport is dependent on a six-amino acid sequence in the cytoplasmic domain, which can function to concentrate G protein at the sites of vesicle budding.^{520,641}

Once G protein is transported to Golgi membranes, it undergoes further posttranslational modifications, including conversion of its oligosaccharides from the high-mannose type to the complex type, containing additional N-acetyl glucosamine, galactose, and sialic acid.⁵⁸¹ Although these modifications are not required for G protein function, they provide a convenient and widely used marker for transport of G protein through successive Golgi membranes.⁶³⁵ Another G protein

modification that occurs in Golgi membranes is the addition of the fatty acid palmitate to a cysteine residue in the cytoplasmic domain.^{606,625} Again, this modification does not appear to be critical, because some strains of VSV lack this modification, and mutation of the target cysteine residue, which abolishes palmitoylation, does not affect G protein function.⁷⁶⁶

Transport of G protein from Golgi membranes to the plasma membrane requires approximately 15 minutes, so that the total time from synthesis in ER to appearance in the plasma membrane is about 30 minutes.⁶³ In polarized epithelial cells, G protein is selectively transported to the basolateral surface.^{82,271,676} The same amino acid sequence in the cytoplasmic domain that promotes the rapid transport of G protein from ER to Golgi membranes is also necessary for the selective transport to the basolateral surface of polarized epithelial cells.⁷⁰³ Sorting of G protein and other basolaterally targeted proteins from those destined for the apical surface occurs first in Golgi membranes, and from Golgi membranes G protein is transported to the recycling endosome compartment prior to transport to the basolateral plasma membrane.^{20,104,155} This intermediate step presumably reflects additional sorting steps by which cells regulate the protein composition of their plasma membranes.

At the plasma membrane of infected cells, G protein is organized into clusters or *microdomains* that are approximately 100 to 150 nm in diameter.^{91,92} These G protein-containing microdomains are formed independently of other viral components⁹² and appear to be similar to cholesterol- and sphingolipid-rich *lipid rafts* that serve as sites of assembly for other viruses, such as influenza viruses.^{455,561} Lipid rafts have been defined in part by their resistance to solubilization with detergents at low temperatures.⁹⁰ In contrast to envelope glycoproteins of influenza virus and other viruses that assemble at lipid rafts, G protein in host plasma membranes and in virion envelopes is detergent soluble.⁶²² Nonetheless, the plasma membrane microdomains (and virus envelopes) that contain G protein resemble lipid rafts in that they are enriched in cholesterol and sphingolipids,^{455,561} but these lipids must not be in sufficiently high amounts to confer detergent resistance.^{93,622}

The G protein-containing microdomains at the sites of virus budding are somewhat larger (300 to 400 nm) than the microdomains in the plasma membrane outside of virus budding sites (100 to 150 nm), implying that formation of virus budding sites involves clustering of membrane microdomains.^{91,93} An interesting feature of the budding process is that envelope glycoproteins from many unrelated viruses, as well as some host integral membrane proteins, can be incorporated into the envelopes of VSV or RABV in a process referred to as *pseudotype formation* or *phenotypic mixing*. Pseudotype formation was originally demonstrated by coinfection of cells with two different viruses.⁸⁰⁷ More recently the incorporation of heterologous glycoproteins into the envelopes of VSV and RABV has been demonstrated using recombinant viruses that express the foreign glycoprotein from the viral genome.^{101,240,358,391,479,629,690} Incorporation of heterologous glycoproteins into the virus envelope appears to result from clustering of microdomains containing the heterologous glycoprotein together with G protein-containing microdomains at the sites of virus assembly.⁹³ It is not known what causes microdomains containing G protein or other glycoproteins to cluster at the sites of virus budding, but a model has been proposed in

which clustering is driven by formation of the viral nucleocapsid–M protein complex.^{93,688}

The efficiency with which heterologous glycoproteins are incorporated into the virus envelope varies over a considerable range, although thus far, none has been found to be incorporated as efficiently as G protein. In the case of RABV, interaction of the G protein cytoplasmic domain with the internal virion components may promote incorporation into the virus budding site, because appending the G protein cytoplasmic domain to foreign glycoproteins enhances their incorporation into RABV virions.^{489,490} In the case of VSV, incorporation of glycoproteins into the envelope does not depend on the sequence of the cytoplasmic domain, however, because substituting foreign sequences for the cytoplasmic domain of G protein or deleting the cytoplasmic domain does not alter the efficiency of G protein incorporation,^{92,628} and, with one exception (the human immunodeficiency virus [HIV] envelope glycoprotein),^{351,539} substituting the G protein cytoplasmic domain into foreign glycoproteins does not promote their incorporation into the virus envelope.^{358,594,629} Instead, the ability of a foreign glycoprotein to be incorporated into the VSV envelope may depend on the composition or physical properties of the microdomains containing the foreign glycoprotein. For example, the influenza virus hemagglutinin, which is in detergent-insoluble lipid rafts, is incorporated into the VSV envelope less efficiently than the T-cell antigen CD4, which is present in microdomains that are primarily detergent soluble.^{93,391,629}

The presence of G protein in the plasma membrane is not essential for virus budding, as shown by studies with recombinant VSV and RABV in which the G gene has been mutated or deleted.^{378,491,594,632,690} In the absence of a complementing source of G protein, these viruses produce noninfectious particles that lack G protein but are otherwise indistinguishable from wild-type viruses. The efficiency of virus budding, however, is reduced by at least an order of magnitude in the absence of G protein, indicating that G protein plays a role in virus assembly to enhance the budding process. Thus, not only is it likely that internal virion components promote incorporation of G protein into the envelope as described earlier, but also it appears that G protein promotes assembly of internal virion components. As with the ability to be incorporated into the envelope, the ability of the VSV G protein to promote assembly is independent of the sequence of the cytoplasmic domain, although a minimal length of eight amino acids in the cytoplasmic domain does appear to be required.⁶²⁸ Instead of the cytoplasmic domain, the sequences in G protein that are responsible for promoting assembly appear to be in the membrane-proximal amino acids of the ectodomain.⁵⁹⁴ This has led to the suggestion that these sequences are responsible for introducing curvature in the membrane that promotes budding.⁵⁹⁴ This idea is supported by the observation that G protein expressed in the absence of other viral components is released from cells in membrane vesicles that may form by a process similar to virus budding.⁵⁹⁹

Role of M Protein in Virus Assembly

Unlike G protein, the viral M protein is synthesized as a soluble protein^{379,486} and associates with membranes in the manner of peripheral membrane proteins (i.e., through a combination of ionic and hydrophobic interactions and without spanning the membrane lipid bilayer).^{430,454,455,532,797,803,804} In virus-infected

cells, most of M protein is usually localized in the cytoplasm and is found in the soluble cytosolic fraction in subcellular fractionation experiments, with smaller amounts being membrane associated.^{238,379,535,537} This distribution is also observed in transfected cells that express M protein in the absence of other VSV components,^{123,124,797} indicating that association of M protein with membranes does not depend on other viral components. In addition, membrane association does not appear to require posttranslational modification, such as phosphorylation or covalent modification with lipids.^{361,430} Instead, M protein appears to spontaneously associate with membranes containing negatively charged phospholipids,^{454,455,532,797,803} which are enriched on the cytoplasmic surface of host plasma membranes. The N-terminal 20 amino acids of M protein, which are enriched in positively charged residues, appear to be responsible for membrane association, as shown using photoactivatable membrane probes.⁴³⁰ Mutational analysis has also implicated the N-terminal region of M protein in membrane binding.^{123,160,797} The membrane-bound M protein in infected cells is organized into membrane microdomains whose size is similar to G protein microdomains.⁶⁸⁸ However, M protein and G protein reside in separate microdomains except at the sites of virus budding.⁶⁸⁸

Both the cytosolic and membrane-bound M protein are recruited into nucleocapsid–M protein complexes at the site of virus budding from host plasma membranes.^{237,528} Nucleocapsids clearly get *selected* for assembly with M protein, because most of the intracellular nucleocapsids, which are being used as templates for viral RNA synthesis, are not able to bind M protein.^{237,535,537} The only place in infected cells where co-localization of nucleocapsids and M protein is observed is in the nucleocapsid–M protein complexes in the process of budding from the plasma membrane.^{485,528,535,537} In the case of VSV, nucleocapsids containing genome RNA are incorporated into virus particles much more efficiently than those containing antigenomes.^{610,650,666,756} An RNA sequence near the 5′ end of the genome has been identified that is required for nucleocapsids containing this RNA to be incorporated into virus particles.⁷⁶¹ If such a sequence is present in the RABV genome, it must also be present in the antigenome, because the recombinant ambisense RABV described earlier, which contains the genomic promoter in both the genome and antigenome, incorporates both genome and antigenome RNA into virions with equal efficiency.²³¹ One of the important questions about virus assembly that needs to be addressed is how this RNA sequence promotes incorporation into virions, because selection of nucleocapsids for virion assembly is a critical step.

Once assembly of nucleocapsid–M protein complexes has begun, the recruitment of M protein into the complex appears to occur spontaneously. This process can be recreated in a cell-free system using purified M protein and virion nucleocapsids that have been stripped of most of their M protein by treatment with high-ionic-strength buffers.^{54,461,514,515} Nucleocapsids, from which M protein has been completely removed, however, cannot rebind M protein with the same high affinity observed in virion nucleocapsid–M protein complexes.^{237,461} This suggests that addition of the initial one or a few molecules of M protein to nucleocapsids (and, therefore, their removal) is fundamentally different from that of most M protein molecules in the nucleocapsid–M protein complex. Although the basis for this difference has yet to be discovered, it is tempting

to think that it may be connected to the process of selection of intracellular nucleocapsids for virus assembly described in the previous paragraph.

Release of Assembled Virions

Following assembly of the nucleocapsid–M protein complex, the final step in virus assembly is release of the budding virion. This process is mediated by interaction of M protein with host proteins involved in MVB formation.^{298,299,331,332,345} The MVB machinery is also involved in release of retroviruses and filoviruses mediated by “late budding domains” in their Gag proteins or matrix proteins (VP40), respectively.⁵⁶⁴ In the formation of MVBs, vesicles derived from endosome membranes bud into the lumen of the endosome, carrying elements of the cytoplasm as their internal contents (Fig. 31.7). Modification of proteins on the cytoplasmic surface of the endosome membrane by covalent attachment of ubiquitin appears to be an important signal for incorporation of such *cargo* molecules into MVBs. The process of virus budding has the same membrane topology (cytoplasmic contents are internal), except that the process occurs at the plasma membrane rather than at the endosome membrane. A short sequence in M protein (PPPY in VSV, PPEY in RABV) appears to be responsible for redirecting this cellular machinery to the plasma membrane.^{298,299,331,332,345,785} No other viral components appear to be required, because expression of M protein in transfected cells in the absence of other viral components results in budding of membrane vesicles containing M protein.^{299,357,442} The PPPY sequence interacts with an E3 ubiquitin ligase called *Nedd4*, which is able to ubiquitinate M protein, in a cell-free assay.²⁹⁸ It has yet to be established whether *Nedd4* itself, or one of its numerous family members, ubiquitinate M protein in infected cells, because this is likely to involve only a small proportion of the total M protein. Nonetheless, it is likely that ubiquitination of M protein is critical for release of budding virions, because mutation of the PPPY motif in M protein, or depletion of free ubiquitin in infected cells, dramatically reduces the release of virions and causes the accumulation of budding particles at the plasma membrane because of inhibition of their release.^{298,345} One of the questions that needs to be resolved is which cellular factors involved in MVB formation are involved in virus budding? Tsg101, a protein involved in recognizing ubiquitinated cargo proteins, and Vps4a, an adenosine triphosphatase (ATPase) involved in recycling the membrane trafficking machinery, have been implicated in budding of HIV but were reported not to be involved in budding of VSV or RABV.^{331,332} However, a more recent report indicated that Vps4a is involved in VSV budding.⁶⁹⁴

MOLECULAR GENETICS OF RHABDOVIRUSES

Rapid Evolution and Existence of Quasispecies

Rhabdoviruses are classic examples of RNA viruses capable of undergoing rapid evolution. This is because of the high error rates of their RNA polymerases and their lack of proofreading activity. As a result, the rate of base substitutions during replication of genome RNA is approximately 1 in 10⁴.¹⁹⁰ Because their genomes are only slightly larger than 10⁴ bases, this implies that nearly every genome contains at least one base substitution. Thus, even clonal populations of these viruses are actually collections of viruses with closely related sequences

(i.e., they are quasispecies). Because of their diversity of genome sequences, these viruses are capable of rapid genetic adaptation when placed under selective pressure of replication under different conditions. These viruses are genetically reasonably stable, however, when replication is maintained under a constant set of conditions.⁵⁹⁷ This is because the collection of genome sequences quickly reaches a consensus sequence representing the sequence with the highest level of fitness within a few replication cycles in a new host. This rapid adaptability may be advantageous in nature for viruses that alternate replication among different hosts. For example, VSV replicates both in arthropod hosts, where it establishes persistent infection, and in mammalian hosts, where it causes an acute infection. Transfer from one type of host to the other requires substantial increases in viral fitness to maintain optimal replication in the new host.⁸⁰⁶ In principle, this adaptation can occur through random mutation of the consensus sequence from the original host to one with greater fitness in the new host. However, in a virus population undergoing periodic cycling between insect and mammalian hosts, rapid adaptation to the mammalian host likely involves maintenance of a minority population of genomes in the insect host that quickly became dominant during mammalian infection.⁵²⁴ Replication of RABV is restricted to mammalian hosts, but similar though less drastic adaptation can also occur when RABV is transferred between different hosts.

The high rate of spontaneous mutation makes it feasible to isolate a variety of different types of viral mutants in the laboratory. For example, several large collections of temperature-sensitive mutants of VSV have been isolated, which fall into five or more complementation groups, corresponding to mutants with defects in each of the five viral genes.^{234,320,571} Similarly, antigenic variants that escape neutralization with monoclonal antibodies are readily isolated in the laboratory.^{410,426,459} Unlike influenza viruses and HIV, however, relatively little *antigenic drift* is found in rhabdoviruses during outbreaks in nature. This may reflect the harmful effects on G protein function of accumulating the multiple mutations necessary to escape neutralization by a polyclonal antibody response in intact animal hosts.⁵²⁵

Despite the advantages for rapid adaptation, the high rate of mutation makes these viruses susceptible to the harmful effects of *genetic bottlenecks*, in which only one or a few genomes are selected for further replication. In a process known as *Muller's ratchet*, successive passage under conditions of limited genetic diversity, such as sequential passage by isolation of individual virus plaques, leads to progressive accumulation of base substitutions, most of which decrease virus replication, thus leading to progressively lower viral fitness¹⁹² (see Chapter 11).

Defective Interfering Particles

Besides point mutations generated by nucleotide substitutions during genome replication, the other major mechanism of genetic alteration of rhabdoviruses is the generation of defective interfering (DI) particles. DI particles appear to be generated when the viral RNA polymerase switches from copying one region of the template to copying an alternate template or an alternate region of the same template. Because this inevitably results in generation of defective genomes, DI particles can replicate only in cells co-infected with standard virus to provide a source of viral proteins. If the polymerase switches to copying a distant region of the same template, this generates large

deletions of the viral genome, referred to as *internal deletion* DI particles.¹⁴⁰ In other cases, the polymerase will switch to copying the terminal sequences of the nascent product RNA strand, generating RNA products with terminal complementarity, referred to as *panhandle* or *snap-back* DI particles.⁵⁵⁷ Panhandle DI particles derived during synthesis of progeny negative-strand RNA thus have the sequence of the 5' end of the viral genome, but the 3' region of terminal complementarity has the sequence of the antigenome, including the antigenomic promoter. Because this is the more powerful of the two viral promoters, such DI genomes have a substantial replicative advantage over standard viral genomes and interfere with the replication of standard virus, resulting in reductions in virus titer.¹⁴⁰ Although they have the same genomic promoter as standard virus, internal deletion DI particles also have a replicative advantage over standard virus, presumably because of their smaller size, and they also interfere with replication of standard virus.

Defective interfering particles are readily generated during virus replication in culture, so that repeated passage at high multiplicity leads to substantial reductions in virus titer because of accumulation of DI particles.¹⁴⁰ Because of the smaller size of their genomes, virions containing DI genomes are shorter than standard virions and can be separated from standard virus by centrifugation in density gradients. This ability to physically separate virions containing DI genomes from those containing standard genomes has proved to be useful in studies of the generation and replication of DI particles. In most experimental situations, however, the presence of DI particles is more of a nuisance, so that virus stocks are usually prepared to minimize the presence of DI particles. This can be accomplished by passaging virus at low multiplicity or by plaque isolation, both of which favor the replication of standard virus over DI particles.

In principle, rhabdoviruses should be able to generate recombinants between genetically distinct viruses in co-infected cells as a result of polymerase *copy choice*, similar to the mechanisms that give rise to DI particles, in which the viral RNA polymerase switches from copying one template to copying another, leading to production of progeny genomes that are recombinants between the two parental genomes. Despite many years of the genetic study of these viruses, no convincing evidence indicates that this type of RNA recombination occurs, although the possibility of rare occurrences in nature has been proposed.¹¹⁶

Genetic Engineering of Rhabdoviruses

The methods for genetically engineering viral genomes developed with rhabdoviruses have become the standard methods used for genetic modification of many nonsegmented, negative-strand RNA viruses. A major hurdle is that RNA transcribed from complementary DNA (cDNA) needs to be encapsidated to function as a viral genome. This process, which occurs so efficiently when RNA is replicated by the viral RNA polymerase, occurs inefficiently when RNA is transcribed from cDNA. Shorter RNAs expressed from cDNA appear to be encapsidated more efficiently than longer RNA. Thus, the first viral genomes to be recovered from cDNA were a VSV DI genome⁵⁴⁹ and an RABV *minigenome* containing the terminal sequences of the viral genome required for transcription and replication flanking a foreign gene.¹⁴⁶ These genomes were expressed from plasmid cDNA in transfected cells together with plasmids encoding the N, P, and L proteins required for

encapsidation and replication. Another hurdle to the recovery of viral genomes from cDNA was the requirement for the 3' end of the RNA to reflect precisely the sequence of the viral genome (or antigenome) without additional nucleotides, in order to be recognized and replicated by the viral RNA polymerase. This was addressed by incorporating into the cDNA the sequence of the hepatitis delta virus ribozyme engineered to cleave the RNA transcript to generate a precise 3' end.⁵⁴⁹ Apparently, the sequence requirements at the 5' end of the viral RNA are not as critical, because additional nucleotides derived from cDNA are removed during replication.

The experimental approach used to generate minigenomes from cDNA led to recovery of complete viral genomes from cDNA for RABV⁶³⁴ and VSV.^{421,759} A key insight was that the RNA transcribed from cDNA needed to be the antigenome rather than the genome. Otherwise, the mRNA derived from the helper plasmids encoding N, P, and L proteins would interfere with recovery by hybridization to the genomic RNA transcribed from cDNA. In most recovery experiments, high levels of expression of the antigenome RNA and the mRNA from the helper plasmids have been achieved by infecting cells with a recombinant vaccinia virus encoding T7 RNA polymerase and transfecting them with plasmids driven by T7 promoters.^{421,634,759} Because this requires isolation of the recovered virus from contaminating vaccinia virus, methods for isolating recombinant rhabdoviruses without the use of vaccinia virus have been developed.^{297,335} Although recovery of infectious recombinant viruses from cDNA has been a major breakthrough, minigenome systems continue to be widely used for studying various aspects of the viral replicative cycle, particularly analysis of mutations that are likely to prevent replication, so that it would be difficult or impossible to isolate viruses containing such mutations.

The ability to engineer specific mutations into viral genomes has become an important tool for studying the mechanistic aspects of virus replication. In addition, it has made possible the use of rhabdoviruses as vectors for expression of foreign genes for potential use as vaccines and therapeutic agents. Several reviews have appeared on the use of rhabdoviruses as potential recombinant vaccines and as cytolytic agents for the treatment of diseases such as cancer.^{52,444,488} The basic methodology to express foreign genes is to introduce a new transcription unit containing the gene stop–start signals and the foreign gene between two of the native viral genes, such as between the G and L genes.^{492,630} The foreign gene is subject to the same transcriptional attenuation as the other viral genes. Thus, incorporation of a new gene reduces the expression of downstream genes by approximately 20% to 30%. Although this attenuation has the potential to reduce the efficiency of virus replication, incorporation of a single foreign gene usually does not notably reduce virus yields unless the foreign gene product itself has the potential to interfere with virus replication.⁶²⁹ The processes of genome encapsidation and envelopment are sufficiently flexible that incorporation of new genes into rhabdovirus genomes simply leads to longer nucleocapsids and, therefore, longer virions.⁶²⁹ In principle, no limit exists for packaging new genetic information, so that multiple foreign genes can be incorporated into genomes of recombinant viruses. Reductions in virus yield owing to multiple new transcription attenuation sites, as well as the difficulty of recovering longer genomes, however, probably places practical limits on

the amount of new genetic information that can be incorporated into rhabdovirus genomes.

MOLECULAR AND CELLULAR BASIS OF PATHOGENESIS

Induction and Suppression of Host Antiviral Responses

Two of the major determinants of viral pathogenesis are the nature of the antiviral response mounted by the infected host and the mechanisms used by viruses to suppress or evade this response. In order for cells to mount an antiviral response, viral products have to be recognized by sensors known as pathogen pattern recognition receptors (PRRs). For most cell types, the major PRR that initiates the response to many negative-strand RNA viruses appears to be a cytoplasmic RNA helicase, RIG-I (retinoic acid–inducible gene I).^{365,366} The other major PRRs that have been implicated in the host response to these viruses are Toll-like receptors (TLRs). TLRs act either at the plasma membrane or in the endocytic compartment to recognize molecules that may be associated with infection by bacteria, fungi, and protozoa as well as viruses. Activation of RIG-I or TLRs results in formation of signaling complexes through a variety of adapter proteins, which activate protein kinases that turn on the expression of antiviral genes.^{512,691} Most of the recent research in this area has focused on the production of type I (α and β) interferons (IFNs) and IFN-stimulated gene products, although other cytokines produced by virus-infected cells also play a major role in innate antiviral responses (Fig. 31.10).

One of the principal ligands for RIG-I is 5' phosphorylated RNA that is part of a short double-stranded RNA (dsRNA).^{322,623} In the normal replication cycle of nonsegmented negative-strand RNA viruses, 5' phosphorylated RNAs are produced during the process of transcription in the form of leader and trailer RNAs and during genome replication as either genomic or antigenomic RNAs (see earlier). However, the 5' ends of these RNAs are shielded by the nucleocapsid protein. Thus, removal of the nucleocapsid protein would appear to be necessary to expose free 5' ends of viral RNA and formation of dsRNA for recognition by RIG-I.⁵⁸⁴ Another potential source of such RNAs would be aberrant transcription products that are not capped by the viral polymerase. Thus, the origin of the signals that activate RIG-I is not clear, but to a large extent these signals are coupled to the production of viral RNAs.

Unlike RIG-I–dependent signaling, which is widely distributed among many cell types, the distribution of TLRs and the relative importance of their signaling pathways is often cell type dependent. For example, TLR7 is a major PRR in the response to VSV in plasmacytoid dendritic cells,⁴⁵⁸ while TLR13 and perhaps TLR4 are major PRRs in the response to VSV of splenic conventional dendritic cells and macrophages.^{266,646} The signal that activates TLR7 appears to be the presence of single-stranded RNA in the endocytic compartment.¹⁷⁶ Such single-stranded RNA can arise during virus penetration by degradation of virions or can be generated from viral RNA in the cytoplasm, which enters the endocytic compartment by autophagy.⁴²⁵ The signal that activates TLR4 in macrophages appears to be the VSV G protein, which interacts with TLR4 during virus attachment and penetration.²⁶⁶ The

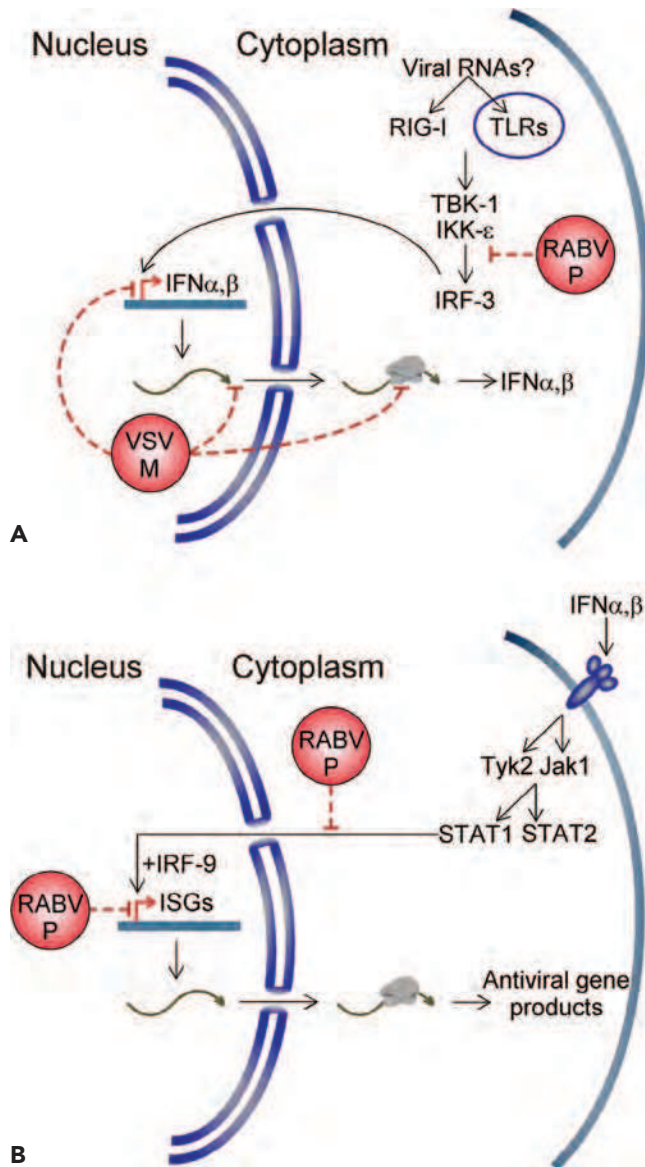


FIGURE 31.10. Induction and suppression of host interferon (IFN) responses by rabies virus (RABV) and vesicular stomatitis virus (VSV). **A:** Induction of synthesis of type I (α and β) IFN and its suppression by RABV P protein and VSV M protein. **B:** Response to IFN and its suppression by RABV P protein.

signal that activates TLR13 is not known but does not appear to be related to viral RNA.⁶⁴⁶

The only TLR that has been implicated thus far in the innate response to RABV is TLR3.^{341,569} TLR3 responds to the presence of dsRNA in the endocytic compartment.¹² As pointed out earlier, dsRNAs are not part of the normal replication cycle but may arise from abnormal replication products. TLR3 may play a role in the antiviral response to RABV, but surprisingly TLR3^{-/-} mice are less susceptible to RABV than their wild-type controls.⁴⁹³ This appears to be due to a role for TLR3 in the formation of Negri bodies (described later), which are viral inclusions that may play a role in enhancing virus replication.^{411,493}

Nearly all viruses have mechanisms to evade or suppress host antiviral responses. This is a critically important aspect of viral pathogenesis. Mutations in viruses that either increase the induction or decrease the suppression of antiviral responses almost inevitably decrease virus replication in susceptible hosts. Vesiculoviruses and lyssaviruses present strikingly different approaches to this aspect of virus replication. If it is possible to generalize, vesiculoviruses usually replicate rapidly and to high levels, generating high levels of potent inducers of host antiviral responses. Correspondingly, they have rapid and potent means of inhibiting these responses, involving the general inhibition of nearly all host gene expression. In contrast, lyssaviruses do not replicate as rapidly and likely are weaker inducers of host responses. As a result, they have more subtle means of inhibiting host responses that do not involve the general inhibition of host gene expression.

Suppression of Interferon Signaling by RABV

The P protein of RABV functions both as a subunit of the viral RNA-dependent RNA polymerase and as a suppressor of IFN production and IFN signaling. P protein inhibits IFN production by preventing the phosphorylation of the transcription factor IRF-3 by two cellular protein kinases, TBK1 and IKK- ϵ (Fig. 31.10A).⁹⁷ Mutations in P protein that inactivate its IFN inhibitory function without affecting its RNA synthesis function have been identified.⁵⁸⁹ Recombinant viruses that express either mutant P protein or lower levels of P protein than their wild-type controls^{97,230} are less effective in suppressing IFN production. These viruses are able to replicate in cell types that are defective in their IFN responses but are rapidly eliminated from IFN-competent cell types and are less virulent in mice.^{97,230,589}

In addition to inhibiting IFN production, RABV P protein inhibits signal transduction in response to IFN (Fig. 31.10B). RABV P is expressed not only as full-length P protein but also as four truncated forms (P2 through P5) that are synthesized from internal start codons.⁵⁴⁶ P3, P4, and P5 proteins are found only in the nucleus. The ability of P protein and its truncated derivatives to inhibit IFN signaling is due to their ability to interfere with the transcription factors that activate interferon-stimulated genes (ISGs). Type I IFNs bind to a common receptor that is coupled to two tyrosine kinases, Jak1 and Tyk2.⁵⁷⁹ Receptor activation leads to activation of these kinases, which in turn phosphorylate two cytoplasmic proteins, STAT1 and STAT2. Phospho-STAT1 and -STAT2 are transported to the nucleus, where they associate with IRF9 to form the ISGF-3 transcription factor that activates expression of ISGs. RABV P protein does not interfere with STAT phosphorylation. Instead, it binds to phosphorylated STAT1 and STAT2 and inhibits their translocation to the nucleus and binding to target DNAs (Fig. 31.10B).^{98,119,504,728,729} This appears to be due to association of the P protein–STAT complex with microtubules in the cytoplasm, which prevents transport to the nucleus.⁵⁰⁴ STAT1–STAT2 complexes that do get transported to the nucleus associate with P protein or its truncated derivatives, which interferes with DNA-binding activity.⁷²⁹

Inhibition of Host Gene Expression by VSV

VSV inhibits host gene expression at three different levels: (a) transcription of host mRNA, (b) transport of host mRNA from the nucleus to the cytoplasm, and (c) translation of host mRNA into proteins (Fig. 31.10A). The inhibition of all three

processes presumably reflects the fact that no single inhibitory mechanism is completely effective in suppressing host antiviral responses. The inhibition of both host transcription and translation generally occurs in parallel and is usually 80% to 90% complete by 4 to 6 hours postinfection.^{7,193} Some evidence indicates that the inhibition of nuclear-cytoplasmic RNA transport may occur earlier after infection, although a direct comparison with the inhibition at other levels has not been made.^{681,732}

The VSV gene product that is primarily responsible for inhibiting host gene expression is M protein. Expression of VSV M protein in transfected cells in the absence of other viral components inhibits expression of co-transfected genes driven by a wide variety of different promoters.^{4,72,225,462,541} This inhibitory activity of M protein is very potent and is evident even when M protein is expressed at 100 to 1,000 times lower levels than those in VSV-infected cells.⁴⁶² Viruses containing a variety of M protein mutations are defective in their ability to inhibit host gene expression.^{5,7,73,151,175,225,245,346,681} Most of these mutations that render M protein defective in its ability to inhibit host gene expression do not affect its functions in virus assembly. Conversely, M protein mutations such as truncation of the N-terminal sequences that are important for virus assembly do not affect the ability of M protein to inhibit host gene expression.^{73,346} Thus, the functions of M protein in the inhibition of host gene expression are genetically separable from its virus assembly functions. Although no separate M protein domains appear to mediate these two classes of functions, the point mutations that affect inhibition of host gene expression do map to one face of the M protein three-dimensional structure. Presumably, this face of M protein is involved in interaction with host components involved in the inhibition.

Because M protein lacks any enzymatic activity, it probably interferes with host gene expression by interacting with cellular proteins to alter their function. Thus far, the only host protein whose binding to M protein is correlated with the inhibition of host gene expression is Rae1.²¹³ Rae1 was originally implicated in mRNA transport, but more recent experiments suggest its principal function is in mitotic spindle assembly and mitotic checkpoint regulation.^{30,79,651,787} M protein and Rae1 form complexes with multiple proteins involved in mRNA transport and other cellular functions, such as Nup98, hnRNP-U, and E1B-AP5.^{115,213,732} However, deleting the *Rae1* gene in cultured mouse embryo cells or silencing its expression in *Drosophila* does not lead to mRNA transport inhibition.^{30,651} Given the observation that Rae1 is not essential for nuclear-cytoplasmic RNA transport, it is unlikely that the VSV M protein inhibits host gene expression simply by interfering with Rae1 function. Instead, it is more likely that the complex of M protein and Rae1 interferes with the function of other factors that are essential for host gene expression.

Because Rae1 is distributed throughout the cytoplasm and the nucleus of the cell, M protein-Rae1 complexes may be involved in inhibition of multiple steps in host gene expression. In support of this idea, the inhibition of transcription by host RNA polymerase II involves inactivation of the general transcription factor TFIID,⁸⁰⁰ which binds to the TATA box upstream of most RNA polymerase II-dependent promoters and recruits other general transcription factors to these promoters. M protein, however, does not appear to interact directly with TFIID,⁷⁹⁹ suggesting that the inactivation is indi-

rect. The mechanisms involved in such an indirect effect have yet to be discovered.

The inhibition of host translation in VSV-infected cells does not result from depletion of cellular mRNA secondary to the inhibition of mRNA transcription and transport. As described earlier, the inhibition of host translation occurs early in the infectious cycle on a time scale too rapid to be caused by turnover of cellular mRNA. Instead, the translation apparatus is reprogrammed such that only new mRNAs are translated.⁷⁶³ Pre-existing host mRNAs are incorporated into translationally inactive messenger ribonucleoproteins (mRNPs), where they are stably maintained in infected cells.^{608,764} Thus, the inhibition of host translation reflects the inhibition of translation of pre-existing mRNAs together with the lack of production of new host mRNAs due to the inhibition of transcription and transport by M protein. The reprogramming of the translation apparatus appears to be due at least in part to alterations in the cap-binding translation factor eIF4F.^{142,143,191} How the changes in the eIF4F complex result in the altered translation in VSV-infected cells is a major question that remains to be addressed.

Viral transcription provides a continuous supply of newly synthesized viral mRNAs, which are efficiently translated by the reprogrammed translation apparatus. Translation of viral mRNAs continues until late in the infectious cycle, when translation is inhibited because of phosphorylation of eIF2 α by the antiviral kinase PKR, as well as other mechanisms.^{142,143,764} Viral mRNAs do not appear to have *cis*-acting sequences that promote their translation analogous to the internal ribosome entry sites in picornavirus mRNAs.⁷⁶⁴ Regardless of their sequences, new mRNAs are translated more efficiently in VSV-infected cells than in uninfected cells.⁷⁶³ This enhancement of translation of new mRNAs appears to involve M protein, because M protein mutant viruses have been identified that inhibit host translation as effectively as their wild-type controls but are defective in promoting translation of viral mRNAs.^{144,499}

Induction of Cytopathic Effects

As in the case of suppression of host antiviral responses, lyssaviruses and vesiculoviruses present strikingly different abilities to induce cytopathic effects in infected cells. In the case of most RABV strains, usually few, if any, morphologic changes occur in infected cells that would be interpreted as cytopathic effects until several days after infection. Indeed, some cell types infected with RABV continue to divide and establish persistent infections.²²³ In contrast, many strains of VSV are among the most cytotoxic of animal viruses, at least in mammalian and avian cells. In many insect cells, however, VSV replication is attenuated and a persistent infection is established with little, if any, cytopathic effect.⁷⁹⁴ In most cases, cytopathic effects are a result of host responses to virus infection involving activation of programmed cell death or apoptosis.

Induction of Apoptosis by Vesicular Stomatitis Virus

It is widely appreciated that many viruses induce apoptosis in infected cells, and, in general, apoptosis is a form of antiviral response in which death of the host cell should reduce the number of progeny resulting from the infectious cycle. VSV was one of the early viruses shown to induce apoptosis in infected cells.³⁸⁹ In fact, most, if not all, of the cytopathic effects of VSV infection are caused by the induction of apoptosis. These effects include nearly all of the morphologic and biochemical

changes typical of apoptosis in cell culture.^{384,389} One of the earliest effects is cell rounding, followed by membrane blebbing, nuclear condensation and DNA fragmentation, cytoplasmic shrinkage, and cell lysis. Despite the general opinion that apoptosis of infected cells reduces virus yield, little, if any, difference exists in yield of VSV if apoptosis is delayed by overexpression of the antiapoptotic host protein Bcl-2,³⁸³ indicating that the VSV replication cycle is largely complete before the infected cell has a chance to die.

At least two distinct mechanisms exist by which VSV infection can induce apoptosis in infected cells.^{109,253,384,553} One appears to be a direct result of the inhibition of host gene expression by M protein, and thus is only activated by viruses with wild-type M protein. The other mechanism appears to be a cellular response to virus replication and is induced by both wild-type and M protein mutant viruses. The relative importance of these two mechanisms varies widely among different cell types, depending on the nature of the proteins that regulate apoptosis pre-existing in the cell before infection and the contribution of newly synthesized proapoptotic protein induced after infection.^{109,252,253,383,553,616} Such newly synthesized proapoptotic proteins can contribute to apoptosis induced by M protein mutant viruses but are suppressed by viruses with wild-type M protein.

Wild-type M protein induces apoptosis when expressed in transfected cells in the absence of other viral components.³⁸⁴ In contrast, mutant M proteins that are defective in their ability to inhibit host gene expression cannot induce apoptosis in the absence of other viral components.^{383,384} Induction of apoptosis by M protein appears to be similar to that induced by pharmacologic inhibitors of host gene expression (e.g., actinomycin D). As with most forms of intracellular damage, both M protein and pharmacologic inhibitors of host RNA synthesis activate the *mitochondrial pathway*, involving the release of cytochrome c and other proapoptotic proteins from mitochondria, which activate the upstream caspase, caspase-9.^{48,383} As a result, induction of apoptosis by M protein can be inhibited by overexpression of antiapoptotic proteins like Bcl-2 and Bcl-X_L, which prevent release of proapoptotic factors from mitochondria.

Despite the inability of mutant M proteins to induce apoptosis in the absence of other viral components, M protein mutant viruses, which are defective in their ability to inhibit host gene expression, are still very effective inducers of apoptosis in infected cells.^{109,252,253,383,384,644} The induction of apoptosis by M protein mutant viruses appears to be part of the antiviral response induced by virus replication. In contrast to apoptosis induced by M protein alone, the death receptor pathway is the major mechanism of cell death, in which caspase-8 is the major upstream caspase, rather than caspase-9.^{109,252} The Fas death receptor appears to be the major death receptor involved. In contrast to viruses such as influenza virus, in which Fas signaling is mediated through the adaptor protein FADD,⁴⁸ VSV infection appears to activate an alternative adapter protein Daxx, which is involved in the induction of apoptosis.²⁵² In some cell types, cross-talk between the death receptor and mitochondrial apoptotic pathways is required for efficient induction of cell death. This cross-talk is mediated through caspase-8 cleavage of the pro-apoptotic BH3-only protein Bid. Cleaved Bid (tBid) then promotes destabilization of the mitochondria through activation of pro-apoptotic mitochondrial proteins. Death receptor signaling has been classified as type I

or type II depending on whether signaling is independent of the mitochondrial pathway (type I) or depends on amplification by the mitochondrial pathway (type II).^{619,620} Cells that respond to death receptor ligands by a type II pathway also respond to VSV infection by a similar pathway.¹⁰⁹

Induction of Apoptosis by Lyssaviruses

As pointed out earlier, there are cell types in which RABV induces little, if any, cytopathic effect and establishes persistent infections.²²³ In other cell types, the infected cells eventually die as a result of induction of apoptosis.^{340,718} Furthermore, RABV induces apoptosis in infected neurons *in vivo* during experimentally induced encephalitis in mice.³⁴⁰ Inhibition of host gene expression does not play a role in the induction of apoptosis by RABV as it does in the case of VSV. Indeed, RABV infection induces the expression of host pro-apoptotic proteins, such as Bax,⁷¹⁸ and in this regard, resembles the induction of apoptosis by M protein mutants of VSV that do not inhibit host gene expression. Also analogous to VSV M protein mutants, the induction of apoptosis depends on activation of the death receptor pathway involving caspase-8, in the case of two other lyssaviruses, MOKV and LBV.³⁶⁴ In contrast to the activation of the Fas death receptor by VSV M protein mutants, however, MOKV and LBV activate apoptosis through interaction of the death ligand tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) with its receptor.³⁶⁴ Transfection of cells with plasmids encoding lyssavirus M proteins induced apoptosis by the same caspase-8-dependent and TRAIL-dependent mechanism and also through mitochondrial disruption.^{269,364} It is unlikely, however, that the lyssavirus M proteins induce apoptosis by a mechanism similar to VSV M protein, because they do not inhibit host gene expression. In addition to M protein, G proteins of some RABV strains also induce apoptosis.^{568,570} Thus, it is likely that multiple viral components are involved in the induction of apoptosis in cells infected with lyssaviruses.

MOUSE MODELS OF RHABDOVIRUS INFECTION

Both RABV and VSV are highly neurotropic in mice, and virulence is largely due to virus-induced encephalomyelitis. Laboratory rodents (e.g., mice) have been extensively used for rabies diagnosis, vaccine potency testing, and pathogenic studies, although these taxa are epidemiologically insignificant as lyssavirus vectors or reservoirs,^{121,396} compared with the families *Carnivora* and *Chiroptera*. Similarly, rodents are not known to be natural hosts for vesiculoviruses. In fact, the disease induced by VSV in rodents bears little resemblance to the disease in natural hosts, such as horses and cattle (described later). Nonetheless, much of what we know about the mechanisms of pathogenesis and immunity for these viruses is derived from experimental infection of mice.

Entry and Site of Initial Replication

Infection of mice with RABV is usually fatal regardless of the route of inoculation. Intramuscular inoculation is often used as a model for virus transmission by animal bites, and intranasal inoculation is used as a model for the occasional transmission

of RABV by inhalation. In contrast to RABV, the ability of VSV to invade the CNS is highly dependent on the age of the mice and route of inoculation,⁶¹⁵ as well as the strain of mice infected.^{196,244} In general, adult mice are relatively resistant to VSV inoculated intravenously or intraperitoneally, although systemic virus infection clearly occurs in these mice, as shown by the potent induction of immune responses (described later). In contrast to intravenous or intraperitoneal inoculation, mice are very sensitive to VSV introduced by intranasal or intracerebral inoculation. As an example of strain differences, mice of the 129 strain, which are often used in the generation of transgenic mice, were found to be five orders of magnitude more resistant than strains such as BALB/c, which are often used in studies of immunology and pathogenesis.¹⁹⁶

Virus entry into the host is accompanied by initial virus replication at the site of entry. In the case of RABV infection of mice, initial replication following intramuscular inoculation can occur in either sensory or motor neurons without apparent replication in muscle,^{150,642} although in natural hosts, virus can replicate in muscle tissue before progressing to the peripheral nervous tissue via neuromuscular connections.^{117,219,511} In the case of either RABV or VSV infection following intranasal inoculation (Fig. 31.11), the primary site of virus replication is in olfactory receptor neurons and other cells of the olfactory epithelium.^{409,563} In addition to olfactory epithelium, VSV can also infect cells of the respiratory epithelium and spread through the respiratory tract to the lungs, although little, if any, pathology is associated with virus replication in the lungs.²⁴⁴

Virus Spread and Tissue Tropism

Both RABV and VSV are transmitted to the CNS primarily by neural spread along the tracts served by the initially infected neurons. For example, following intramuscular inoculation,

RABV spreads from the initially infected sensory and motor neurons to the spinal cord and sensory ganglia in subsequent rounds of replication.¹⁵⁰ Similarly, following intranasal inoculation (Fig. 31.11), both VSV and RABV quickly spread to the glomerular cells of the olfactory bulb as well as the anterior olfactory nuclei.^{327,409} From these sites, the viruses spread to other parts of the CNS that are served by the neurons that innervate the olfactory bulb. These viruses have a clear preference for some classes of neurons over others. For example, RABV can also enter the CNS through neurons of the trigeminal ganglia,^{409,642} whereas VSV cannot.⁵⁶³ Similarly, RABV infects mitral cells of the olfactory bulb and spreads along tracts served by these cells,⁴⁰⁹ whereas VSV does not.³²⁷ In addition to neural spread, VSV infects cells lining the ventricular system, where it can be released into the cerebrospinal fluid (CSF) and can spread to other parts of the brain and spinal cord, leading to paralysis.^{244,327,563}

The pathology associated with infection of mice by either RABV or VSV is typical of viral encephalomyelitis, involving both death of infected cells and inflammation at the infection sites. The inflammatory changes include activation of resident inflammatory cells (e.g., microglia) and infiltration of inflammatory cells (e.g., monocytes, natural killer [NK] cells, and T cells).^{67,126,244,321} The morbidity and mortality associated with virus infection is usually attributed to virus-induced death of infected cells that are critical for the host, rather than to immunopathologic mechanisms, because nearly all experimental manipulations that reduce the immune response to virus infection either enhance mortality by allowing more virus replication or have little effect. For example, virus infection of T-cell-deficient mice results in more extensive spread of virus throughout the brain and higher mortality than in immunocompetent mice.^{321,326}

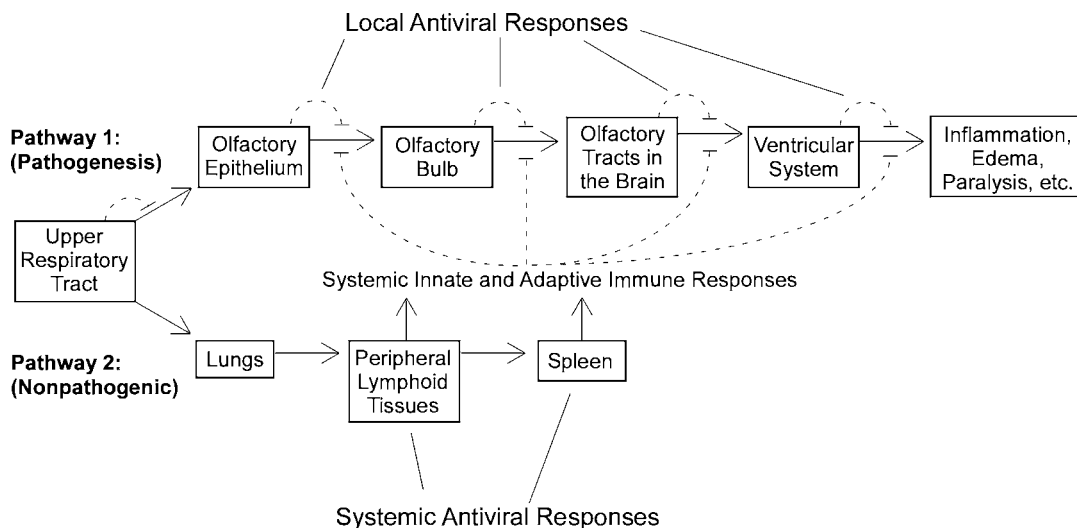


FIGURE 31.11. Diagram of pathogenesis and immune response in mice infected with vesicular stomatitis virus (VSV) by intranasal inoculation. Pathway 1 is the route of virus spread through the central nervous system by neuronal transmission leading to encephalitis.³²⁷ **Pathway 2** is a hypothetical route of spread from the respiratory tract to peripheral and central lymphoid organs. Also shown are sites at which local and systemic antiviral responses exert an inhibitory effect on spread of virus to the next stage. The outcome of infection depends on the relative ability of the virus to replicate and spread versus the ability of the host to inhibit virus replication at each step.

Immune Responses Involved in Recovery from Rhabdovirus Infection

The immune response to VSV infection in mice has been studied for many years by viral immunologists as a prototype immune response to virus infection. In addition, the development of recombinant VSV as a potential vaccine vector⁵⁹² has stimulated additional research into the anti-VSV immune response. In most cases, the infection resulting from intraperitoneal or intravenous inoculation has been studied, which often is asymptomatic and results in complete recovery in immunocompetent mice. Less frequently studied is the infection resulting from intranasal inoculation. In this case, nearly all mice develop clinical signs, and only about half of the mice survive. Clearly, differences in the immune responses resulting in resistance or recovery must exist between these two situations. In the case of RABV, there is little incidence of recovery from a productive CNS infection. The immune response to attenuated virus strains is often studied as a model for the immune response to potential live virus vaccine strains.

Immune Response to Vesiculovirus Infection

In the case of VSV infection, elements of both the innate and adaptive immune response are critical for survival (Fig. 31.11). One of the most striking effects on VSV pathogenesis occurs in the absence of a response to type I (α and β) IFN. Mice that lack the type I IFN receptor, or the STAT1 transcription factor that mediates many of the effects of type I IFN, are extremely susceptible to the lethal effects of VSV infection.^{195,510} In contrast to immunocompetent mice, in which virus replication occurs primarily in the CNS, in IFN receptor- or STAT1-deficient mice VSV replicates to high titer in all of the tissues tested.^{195,510} In fact, the brains of these mice had the lowest titers of any of the organs examined.⁵¹⁰ This result implies that the pronounced neurotropism of VSV in immunocompetent mice is not caused by the inherent ability of different tissues to support virus replication, but rather differences in their ability to produce or respond to type I IFN.

As in the case of most viruses, no single IFN-inducible gene product is responsible for the effect of IFN in protecting nonneural tissues from VSV infection. No deletion of a single IFN-inducible gene has as profound an effect as deficiency of IFN receptor or STAT1. The antiviral protein kinase PKR is notable, however, in that its deletion leads to enhanced virus replication in the lung following intranasal inoculation, leading to enhanced morbidity and mortality caused by respiratory infection.¹⁹⁶

The major source of type I IFN in mice following systemic inoculation appears to be a subclass of plasmacytoid dendritic cells residing in the marginal zone of the spleen.⁵³ This would be consistent with the ability of VSV to suppress IFN production by most other cell types, as described in the previous section. Dendritic cells containing TLR7, such as plasmacytoid dendritic cells, appear to be resistant to the inhibition of IFN production following VSV infection.^{8,458,734} Other cell types involved in innate immunity also appear to be involved in protecting neural and nonneural tissues from VSV infection. Following systemic inoculation, chemokine-secreting marginal zone macrophages in the spleen are particularly important,^{132,529} and following subcutaneous inoculation, subcapsular macrophages in draining lymph nodes are critical

for preventing VSV neuroinvasion through peripheral nerves in the lymph nodes.³³⁰

Whereas the host IFN response and other innate immune mechanisms can protect most nonneural tissues from VSV infection, they do not fully protect the CNS, particularly following intranasal or intracerebral inoculation. This inability to protect the CNS is not because of a failure of neurons to respond to IFN.⁷¹⁰ Instead, the problem appears to reside in the amount of IFN produced in the CNS and the timing of the peripheral IFN response relative to virus invasion.⁷¹¹ Indeed, treating mice with exogenous IFN can increase their resistance to CNS infection by VSV.^{168,281,708} In addition to the IFN response, other innate immune mechanisms affect the susceptibility of the CNS to VSV infection. For example, deficiency in the production of nitric oxide by neuronal nitric oxide synthase-1 (NOS-1) enhances the susceptibility of the CNS to infection.³⁸² In contrast, deficiency of the inducible NOS (NOS-2) or NOS-3 has little effect on VSV infection of the CNS.

In addition to innate immune responses, adaptive immune responses are critical for recovery from VSV infection. Particularly important is the production of neutralizing antibodies. As with most viruses, the envelope glycoprotein of VSV (G protein) is the viral antigen that elicits neutralizing antibodies.^{108,359,427} Induction of antibodies by G protein expressed on the surface of infected cells requires T cells.³¹ The high density of G protein in virions is able to induce a T-cell-independent IgM response, however, which is consistent with the induction of T-cell-independent responses by antigens with highly repetitive epitopes.³¹ T cells are required for isotype switching to produce immunoglobulin G (IgG) and other isotypes.^{428,471} VSV-infected dendritic cells appear to be responsible for transporting the virus to secondary lymphoid organs, such as the spleen (Fig. 31.11), where they present viral antigens to virus-specific T cells and B cells.^{131,132,456} The CD4+ T-helper cell (Th) response to VSV infection includes elicitation of both Th1 and Th2 cells. The response is predominantly of the Th1 type, resulting in secretion of IFN- γ and isotype switching in B cells to produce predominantly IgG2a antibodies. Isotype switching to IgG2a is also mediated by IFN- γ -producing γ - Δ T cells.⁴⁷¹ This polarization of the T-cell response presumably reflects secretion of IL-12 by dendritic cells and other antigen-presenting cells. Depletion of phagocytic cells, including marginal zone dendritic cells and macrophages, largely eliminates the Th1 response, although the Th2 response is left largely intact, suggesting that a different class of antigen-presenting cells is responsible for activation of Th2 cells.¹³²

VSV infection also effectively elicits CD8+ cytolytic T cells (Tc). In contrast to neutralizing antibodies, which are serotype specific, many of the Tc cells are cross-reactive between Indiana and New Jersey serotypes of VSV.⁶⁰⁹ These cells recognize peptides containing conserved sequences derived from G protein or N protein (and perhaps other viral proteins) presented in the context of class I major histocompatibility complex (MHC) molecules on virus-infected cells.^{572,722,798} In addition to CD8+ Tc, VSV also elicits CD4+ cytotoxic T cells, which recognize epitopes derived from G protein presented in the context of class II MHC molecules.⁹⁴

The importance of the antibody response in recovery from VSV infection is demonstrated by the observation that mice containing disruptions of the immunoglobulin μ gene are highly susceptible to VSV infection.⁹⁵ These mice die

from CNS infection, even when infected by intraperitoneal inoculation. In contrast, depletion of either CD4+ or CD8+ T cells has little effect on susceptibility to VSV infection by this route.⁴²⁸ Depletion of either T-cell subset or both subsets, however, enhances the susceptibility of mice to infection with VSV by intranasal inoculation,³²⁶ indicating that T cells are important for reducing virus replication once infection is established in the CNS.

Immune Response to Lyssavirus Infection

Antibodies induced by vaccination, particularly those with neutralizing activity, play a prominent role in immune defense against RABV infection.³²¹ On rare occasions, immunity can also be naturally acquired after multiple exposures to virus.²⁴² The G protein represents the only antigen that induces neutralizing antibodies and is able to confer immunity against a lethal challenge.¹⁵³ Antibodies can mediate viral clearance from the CNS without other immune effectors.¹⁸⁰ The presence of other immune mechanisms, including IFN responses, and both CD4+ and CD8+ T cell responses, however, hastens the clearance of virus from the CNS.³²¹ Although G protein is the only antigen that elicits neutralizing antibodies, the RNP is a major antigenic complex that induces a virus-specific antibody response, and antibodies directed against RNP can contribute to protection against infection.^{179,182,448} Animals treated with anti-N sera can be protected against a subsequent challenge with RABV, and anti-N sera can exhibit an antiviral activity *in vitro*.⁴⁴⁸ The mechanism by which anti-RNP antibodies inhibit viral replication, however, remains unclear.

Infection with RABV results in the generation of virus-specific CD8+ and CD4+ T cells. The G protein is one of the antigens that induces Tc responses.^{465,466} Some mouse strains infected with virus also develop strong Tc responses to the P protein.⁴¹⁷ The role of CD8+ T cells in immune defense is unclear, however. Some investigators report clearance of rabies virus after transfer of RABV-specific T cells and protection against rabies by a Tc clone, whereas other investigators showed that Tc are insufficient to protect against challenge, and *in vivo* depletion of CD8+ T cells had no effect on host resistance to street virus infection.^{369,417,558,566} In contrast, Tc may actually be involved in the immunopathology and have been implicated in neuritic paralysis.^{683,752} By comparison, the induction of CD4+ T cells is an integral part of the protective immune response against rabies.¹⁷⁸ Elimination of CD4+ cells abrogates the production of IgG neutralizing antibody in response to virus infection.⁵⁵⁸ The RNP contains major epitopes that induce CD4+ T-cell responses, and most of these T cells cross-react with other lyssaviruses.²⁰⁷ The RNP-specific T cells, which can augment the production of neutralizing antibody, are believed to be the major factor that mediates the protective immune response induced by internal viral antigens.^{181,251}

Determinants of Viral Virulence

In general, determinants of viral virulence among rhabdoviruses can be classified into those that enhance virus replication and those that enhance the suppression or evasion of host antiviral responses. Mutations in such virulence determinants are of considerable interest, because of their potential to generate live virus vaccines. In the case of RABV, much attention has focused on mutations in G protein that attenuate viral pathogenicity. For example, antigenic variants selected with neutralizing

monoclonal antibodies against G protein often display reduced neurovirulence in mice. These variants contain mutations that change R/K333 in G protein to other amino acids. These changes reduce the ability of G protein to attach to neuron-specific receptors,^{152,713} although virus replication in nonneuronal cells is not affected. Similarly, mutations in VSV that compromise its ability to replicate often result in attenuated virus strains. For example, truncation of the G protein cytoplasmic domain reduces the efficiency of virus budding, as described earlier. Such mutants are attenuated in their pathogenicity and form the basis for candidate recombinant viral vaccines.^{592,607} Likewise, recombinant viruses in which the order of the VSV genes has been altered usually display reduced pathogenicity as a result of reduced virus replication and are candidate recombinant vaccines.^{147,236}

An example of the second class of virulence determinants—those that lead to suppression of host antiviral responses—would be the VSV M protein. Mutations in M protein that render it defective in its ability to inhibit host gene expression attenuate viral virulence in mice without compromising the ability of the virus to replicate in cell culture.^{3,6,681} In this case, the attenuation is caused by the enhanced innate immune responses elicited in infected cells because of the failure of the virus to suppress host gene expression.⁷¹¹

Similar to VSV M protein mutants, RABV P protein mutants have been generated that are defective in their ability to block IRF-3 phosphorylation⁵⁸⁹ or STAT1 nuclear translocation.³³⁴ These viruses are also attenuated in their pathogenicity in mice, emphasizing the importance of P protein-mediated suppression of IFN responses as a virulence factor for RABV. However, equally important virulence factors for RABV are viral mechanisms that suppress production of the activators of innate antiviral responses. The difference in pathogenicity of attenuated viruses compared to field strains (i.e., street viruses) is correlated with lower levels of viral gene expression by the more virulent strains.^{502,743} This leads to correspondingly lower induction of antiviral responses by the more virulent viruses. Similarly, strain differences in the N protein have been linked to differences in RIG-I signaling and corresponding differences in virulence.⁴⁷⁵ Thus, the key to the pathogenicity of RABV is the combination of having a potent suppressive mechanism to inhibit IFN responses in susceptible cells together with a sufficiently low level of viral gene expression to reduce the responses generated in the cells of the innate immune system. In other words, RABV is said to “[use] stealth to reach the brain”.⁶³³

EPIDEMIOLOGY OF RHABDOVIRUS INFECTIONS

Epidemiology of Lyssavirus Infections

Lyssavirus epidemiology is partially influenced by host species distribution, abundance, demographics, behavioral ecology, dispersal, and interactions with humans.⁶¹⁴ Because of its consequences when ignored, rabies is a reportable disease in many countries, although surveillance is inadequate, particularly in sylvatic hosts. Biased epidemiologic information usually derives from clinical reports or the examination of suitable brain material submitted to public health or veterinary diagnostic laboratories only after infectious contact with animals is suspected.

Exposure is generally defined as transdermal contact, typically by a bite, or mucosal contamination with potentially infectious material (e.g., saliva or CNS tissue).⁷⁸⁹ The relative risk associated with other scenarios is difficult to define.

The domestic dog is the principal host and major vector of rabies throughout the world,^{217,740} currently most prominent in the tropical regions of Asia, Latin America, and Africa. International reporting of both human and animal rabies cases grossly underestimates the magnitude of the problem.⁴⁹⁵ Predominant wild reservoirs and maintenance hosts belong to the family *Carnivora*^{38,376} and include foxes^{74,154} in the Arctic (*Vulpes lagopus*), Canada, central and western Europe, and moderate latitudes of Asia (*V. vulpes*, *V. corsac*), and scattered foci elsewhere throughout North America (e.g., *Urocyon cinereoargenteus*); the raccoon dog (*Nyctereutes procyonoides*) in eastern Europe, Scandinavia, and portions of Asia^{120,395,526}; coyotes, jackals (*Canis* species), and other wild canids in North America, Asia, and Africa²⁵⁸; skunks (*Mephitis mephitis*, *Spilogale putorius*) in North America^{118,280}; procyonids, such as the raccoon (*Procyon lotor*), in eastern North America^{68,778}; and herpestids (e.g., the yellow mongoose, *Cynictis penicillata*; the small Asian mongoose, *Herpestes javanicus*) and their relatives throughout Africa, Asia, and the Middle East.⁷²⁵ Additionally, the ferret badger (*Melogale moschata*) was documented as a rabies reservoir in several regions of China.^{447,809} Rabies detection in rodents is uniformly rare.^{121,396,779}

Bat rabies predominates in the New World, described primarily among insectivorous bats of the United States and Canada (over 40 species) and the three hematophagous vampire species (principally, *Desmodus rotundus*) ranging from northern Mexico to Argentina.^{39,122} Many bat species may also be important throughout Latin America.^{169,170} Other lyssaviruses are transmitted by bats in Africa, Europe, Asia, and Australia.^{17,42,247,400,402,403}

Surveillance efforts in the United States follow changes in indigenous and translocated cases in space and over time. For example, in 2009, 49 states and Puerto Rico reported 6,690 rabid animals and 4 human rabies cases to the Centers for Disease Control and Prevention (CDC). Approximately 92% of reported rabid animals were wildlife. Relative contributions by the major animal groups were 34.8% raccoons, 24.3% bats, 24.0% skunks, 7.5% foxes, 4.5% cats, 1.2% dogs, and 1.1% cattle. Compared with 2008, reported numbers of rabid raccoons and bats decreased, whereas reported numbers of rabid skunks, foxes, cats, cattle, dogs, and horses increased.⁷⁵ Historically, Hawaii remained the only rabies-free state, never having reported a case of indigenously acquired rabies.^{233,618}

Combined with historical, temporal, and spatial disease surveillance data, antigenic characterization with monoclonal antibodies (MAbs) and nucleotide sequence analysis can assist in the assignment of isolates to different animal reservoirs.^{661,663,664} Arctic RABV circulates circumpolarly, although local variability was documented in several areas, such as Alaska and Ontario. Although *V. lagopus* historically has been recognized as the major reservoir of Arctic RABV, *V. vulpes* increasingly participates in circulation of this virus variant due to climate changes. Skunk rabies isolates appear to be distinct variants defining separate outbreaks in the north-central and south-central parts of North America, and California. Additionally, smaller independent foci involve foxes, dogs, and coyotes in Texas, as well as foxes in Arizona and portions of the southwestern United States.^{75,135,390,660,661,663}

Analysis of human rabies cases from the United States implicated viruses associated with insectivorous bats as the most frequent source of infection after elimination of canine rabies,^{75,390,522} and some bat isolates appear to possess unique pathogenic properties compared with isolates from the family *Carnivora*.⁵⁰⁵ During 2003, a first reported occurrence of rabies in a human infected with the raccoon rabies virus variant was documented in Virginia; however, the exposure history was unknown. During 2004, transplantation from an infected donor resulted in four human cases in the United States⁶⁶⁹ and three in Germany,³⁰⁶ demonstrating the devastating consequences when rabies is not suspected.

Host switching from bats to terrestrial mammals during the history of lyssavirus evolution has been inferred from RABV phylogeny.³⁵ Based on the relatedness of carnivore RABV variants to bat RABV variants, the switch is proposed to have occurred approximately 1,000 to 1,500 years ago. Moreover, relatively frequent spill-over cases and host shifts of bat RABV variants to terrestrial mammals have been documented repeatedly during recent years.^{23,433} More controversial is a proposed cross-species shift in lyssaviruses, given that a variety of non-RABV lyssaviruses, but not RABV, have been detected in bats in the Eastern Hemisphere, and only RABV detected in all reservoir hosts, including bats, in the Western Hemisphere.⁴⁰⁶

In western and central Europe, where fox rabies has been largely eliminated via oral vaccination, bat rabies still poses public health concerns. The EBLV-1, first isolated in 1954 in Germany, was later identified across Europe, from Spain to the Ukraine.^{17,401} About 95% of EBLV-1 cases have been observed in *E. serotinus* bats.^{17,627} However, it has also been reported in numerous other bat species.^{627,637,638} Spill-over infections of EBLV-1 were documented in sheep in Denmark,⁶⁰⁰ in stone marten in Germany,⁵⁰⁹ and in domestic cats in France.¹⁵⁹ In contrast to EBLV-1, distribution of EBLV-2 is limited to northwestern Europe.⁴⁰⁶ This virus circulates primarily among bats of the *Myotis* genus. Five human rabies cases of bat origin have been documented in Europe. In one case the virus was identified as EBLV-1, in two others EBLV-2 was identified, and in two cases the virus was not characterized.⁴⁰⁶

Bat lyssavirus surveillance in southeastern Europe and Asia is extremely limited. Nevertheless, such viruses as ARAV and KHUV were isolated from *Myotis* bats in Central Asia,⁴⁰⁵ IRKV was isolated from *Murina leucogaster* in Eastern Siberia⁸¹ and later caused a human rabies case in the Far East,⁶² and WCBV was isolated in the Caucasus region from *Miniopterus schreibersii*.⁴⁰⁰ Historical records indicate isolation of lyssaviruses from bats in India and Thailand,^{544,665} and serologic surveys demonstrated the presence of lyssavirus antibodies in bats from the Philippines, Cambodia, Thailand, and Bangladesh.^{24,457,587} Presumed human rabies of bat origin was reported from China, although no virological examination was performed.⁶⁹² Indeed, significant surveillance efforts are needed in this large part of the world to elucidate ecology and epidemiology of lyssaviruses.

In Africa several divergent lyssavirus species have been documented. Dog rabies is widely distributed and represents the major burden for humans and domestic animals. At least three phylogenetic lineages of dog RABV were described, along with a separate lineage associated with mongooses.^{165,473,725} Epizootics in dogs frequently spread to wildlife.⁴²⁹ Several outbreaks have been described that significantly reduced populations of such

endangered species as African wild dog (*Lycaon pictus*)^{258,318} and Ethiopian wolf (*Canis simensis*).^{353,578} Another African lyssavirus, MOKV, has been sporadically isolated from shrews, domestic cats and dogs, and a rodent in various localities of Sub-Saharan Africa.^{370,473,513} MOKV is the only lyssavirus species never documented in bats. However, the principal reservoir host of this virus is still unknown. Two human cases of MOKV infection were documented via active surveillance efforts, both with unknown exposure history.^{210,211} In contrast to MOKV, LBV is clearly associated with bats from the *Pteropidae* family, such as *Eidolon helvum*, *Rousettus aegyptiacus*, *Micropteropus pusillus*, *Epomophorus wahlbergi*, and likely others, with only infrequent spill-over infections into terrestrial mammals, such as cats, dogs, and a mongoose.^{403,472,473} LBV is broadly distributed in Sub-Saharan Africa and at least once was translocated to France with *R. aegyptiacus* fruit bats, imported from Togo or Egypt.²⁸ The other two African bat lyssaviruses are less studied. Of the four known isolates of DUVV, three came from humans, who died of rabies after bites of insectivorous bats in South Africa and Kenya, and only one was obtained from a bat, presumably of the *Miniopterus* genus, in Zimbabwe.^{375,494,552,724} The last member of the genus, SHIBV, is known by a single isolate, obtained from an insectivorous bat *Hipposideros commersonii* in Kenya.⁴⁰² Indeed, more studies are needed to understand ecology and epidemiology of African non-RABV lyssaviruses.

Prior to 1996, Australia had been considered free of rabies and rabies-like viruses. An outbreak of rabies involving several dogs occurred in the island state of Tasmania in 1867 but was quickly eradicated. Since then, only a few imported rabies cases were registered. Following the discovery that flying foxes were a reservoir of Hendra virus, surveillance of these animals was increased, which resulted in the discovery of ABLV in 1996.^{247,272} ABLV has been identified in all four flying fox species in continental Australia: *P. alecto*, *P. poliocephalus*, *P. scapulatus*, and *P. conspicillatus*, in locations along the eastern coastal territory of the continent, where the surveillance was enhanced.²⁸⁷ Further, a distinct ABLV variant was identified in insectivorous bats *Saccolaimus albiventris*.²⁷³ Two human cases of ABLV infection have been described to date. The first, documented in 1996, was caused by the insectivorous bat ABLV variant,^{14,273} and the other one was caused by the pteropid ABLV variant.⁷⁴⁸ Both cases were fatal, and clinical symptoms were compatible with rabies. The distribution range of *P. alecto* bats extends into Papua, New Guinea, and the eastern islands of Indonesia.²⁴⁷ There is no reason to expect that distribution of ABLV is limited to continental Australia. For example, the presence of antibodies to this virus was demonstrated in 9.5% bat serum samples collected in the Philippines.²⁴

Epidemiology of Vesiculovirus Infections

The mechanisms of VSV transmission are not completely understood. Ecologic factors and special conditions regarding the host and the etiologic agent have been implicated in the clinical presentation of the disease.^{294–296} Experimental transmission from animal to animal by direct contact has produced irregular results. The virus is unable to penetrate intact skin or mucosa; for a successful transmission, it needs to be introduced beneath the skin and mucous membranes via wounds and abrasions.^{295,356} The virus can also be transmitted by the bite of insect vectors such as mosquitoes (*Aedes* spp.),^{701,751} sand flies (*Lutzomyia* spp.),¹⁴¹ blackflies (*Simulium* spp.),¹⁵⁶ and other *Diptera*.²²⁶ Virus isola-

tions from nonbiting insects (e.g., *Musca domestica*) have been reported.²⁴⁶ Many potential biological vectors of VSV have been suggested, but the phlebotomine sand fly, *Lutzomyia shannoni*, is the only one confirmed in the United States.⁷⁵¹

The disease is present only in the Western Hemisphere, and it is enzootic in southern Mexico, in Central America, in some regions of South America,⁷⁵⁰ and on Ossabaw Island, off the coast of Georgia.⁶⁷³ In temperate zones, VSV outbreaks begin in late summer and end with the arrival of frost.²⁷⁰ In the United States, the outbreak during 1982–1983 was unusual because it continued throughout the winter months until the following spring.⁸⁷ In tropical areas, the disease appears at the end of the rainy season and disappears with the advent of the dry season.⁷⁵¹ Typically, the disease affects only horses, cattle, and swine. During outbreaks, morbidity rates in a herd usually range from 10% to 15%.⁷⁵⁰ Cattle generally recover in a few days, but horses and pigs can develop lameness.^{295,750} A broad spectrum of wild mammals can also be affected.⁷⁵⁰ Factors that influence the disease spread in dairy cattle include coarse roughage, hard pelleted concentrates, poor general and milking hygiene, and insufficient teat sanitation.²⁹⁴ In the southeastern United States, feral swine had 10% to 100% antibody prevalence from 1979 to 1985⁶⁷⁴ and on Ossabaw Island showed 12% and 60% seroconversion between June and September in 1982 and 1983, respectively.⁶⁷⁵ In some enzootic areas of Central America, over 80% of the cattle have antibodies against VSV, but only 9% of the animals may present clinical signs in a particular year. In the same regions, wildlife also have a high VSV seroprevalence. In tropical areas where the disease is enzootic, VSV seroprevalence in the human population can be as high as 48%.³⁵² Serologic studies during outbreaks in Panama demonstrated a seroprevalence of 71% and 34% in personnel working with infected and noninfected cattle, respectively.⁸⁹ A similar situation has been observed during VSV outbreaks in Colorado, where personnel (veterinarians, researchers, and regulatory staff) handling sick livestock showed an antibody prevalence of 13%, whereas unexposed humans had a 6% seroprevalence.⁵⁸⁶

The mechanism by which VSV is maintained in enzootic regions is not fully understood. Sand flies may transmit the virus from a reservoir (e.g., plants, wildlife, cattle) to livestock. Alternatively, VSV may be maintained in the sand fly population by transovarial transmission, and the insects infect susceptible animals during feeding.¹⁴¹ In some enzootic areas, feral swine have been suggested as a potential amplifying host.⁶⁷³ Molecular epidemiologic studies indicate that enzootic areas may be the origin of the virus responsible for outbreaks in epizootic zones.⁵¹⁷ VSV may be introduced in a particular area by the movement of infected animals, wildlife, or insects, but the actual mechanism is unknown. Viruses circulating in enzootic areas present a high genetic diversity, with several lineages coexisting in the same region.⁵¹⁶ Within enzootic areas, the viruses seem to adapt under selective pressures exerted by ecologic factors. Viruses from different ecologic areas within enzootic regions belong to different genotypes. Viral adaptation to different insect vectors or mammalian reservoirs might be determinant factors for this divergent evolution.⁵⁹⁷ Viruses obtained from a particular outbreak are genetically homogeneous.⁵¹⁷

Epidemiology of Ephemerovirus Infections

Bovine ephemeral fever is distributed throughout Africa, the Middle East, Southeast Asia, and northern Australia.⁶⁷¹ It has

never been reported in the Americas. The disease occurs during summer and autumn and disappears with the arrival of the first frosts in subtropical areas. In the tropics, yearly occurrence is associated with the rainy season.^{671,735} Although transmission of the virus occurs via insect vectors, the difficulty of isolating the virus from insects hampers the recognition of the vector species involved in its transmission. BEFV was isolated from *Culicoides* spp. and from mosquitoes. In Australia, the geographic range of the disease is greater than that of the *Culicoides* species from which the virus was isolated. Two species of mosquitoes, *Culex annulirostris* and *Anopheles annulipes*, may be implicated in transmission in these areas.⁶⁷¹ In general, morbidity is low, but in some outbreaks, all of the animals in a herd may be affected. In other instances, only 2% or 3% of the animals show clinical signs.^{671,735} Natural disease occurs only in cattle and water buffalo. Although seroprevalences of 13% to 38% were reported in cattle in enzootic areas, a higher prevalence of 64% has been observed during outbreaks.¹⁶¹ The role of wild ruminants in the maintenance of BEFV in nature is not understood. Seroprevalences between 28% and 54% are found in wild ruminants in Kenya, Zimbabwe, and Tanzania.^{18,166}

Epidemiology of Novirhabdovirus Infections

Novirhabdoviruses cause severe economic losses to the salmonid farming industries.^{317,786} As was described earlier, IHNV is endemic to western North America, and dispersal of the virus outside North America has occurred by inadvertent transport of infected eggs and juvenile fish.^{313,373,617} Within North America, dispersal of IHNV is thought to have involved the historical use of unpasteurized salmon viscera in feed for salmon hatcheries, and possibly the historically common practice of salmon transplantations.^{102,749,786} Following the introduction to the Eastern Hemisphere, European and Asian IHNV isolates demonstrate relatedness to specific phylogenetic lineages within the endemic area from which they were derived.^{372,521,540}

VHSV was first discovered in Western Europe.⁷⁸⁶ So far, VHSV has been isolated from over 60 fish species from both marine and freshwater habitats representing North America, Asia, and Europe.⁶⁵⁴ VHSV is endemic to numerous marine species in both the Atlantic and Pacific Oceans of the northern hemisphere and could have been introduced into freshwater habitats by marine fish species (e.g., herring, sprat, sand eel) that are used as fresh feed for commercial farming in some countries.⁶⁵⁴ The freshwater isolates of VHSV appear to be evolving ~2.5 times faster than the marine isolates.²⁰⁰ The successful recent viral adaptation in new hosts is one of the possible explanations for such higher evolutionary rates of VHSV in freshwater fish.⁵⁰⁵ Alternative explanations for the increased substitution rates in freshwater VHSV are the intensive aquaculture practices and the higher water temperature in culture ponds, which could cause an increase in virus replication rates.²⁰⁰ A similar pattern has also been observed for IHNV in North America, where the evolutionary rate was found to be three to four times higher in regions with intensive aquaculture, as compared with other regions.⁷¹²

HIRRV was first isolated in Japan from Japanese flounder³⁷⁴ and subsequently was also reported in Korea.^{371,534} It has recently been reported to infect several other fish species endemic to Japan.³⁷⁴ SHRv was first isolated from snakehead

fish in Thailand and has not been reported outside Southeast Asia to date.³⁶³

CLINICAL FEATURES OF RHABDOVIRUS INFECTIONS

Lyssavirus Infections

Rabies cases are almost always attributable to the bite of a rabid animal. For example, animal bites were the cause of 99.8% of 3,920 human rabies cases examined at various Pasteur Institutes between 1927 and 1946.⁴⁸⁷ Nonbite exposures, which rarely cause rabies, include inhalation of aerosols,^{325,333,781} licks,⁴²⁴ transdermal scratches, or other unusual events that lead to contamination of an open wound or mucous membrane,²³² such as tissue or organ transplantation.^{344,669} Bat RABV-associated human deaths in the United States may not have a reported exposure source,^{112,522} but these cases are most likely caused by bat bites in which either the risk was not appreciated or the bites were not immediately recognized by the patient. Disease development after exposure depends on the location and severity of a bite, the species of animal responsible for the exposure, and the virus variant.^{29,40,518,649,726} In the absence of vaccination, the highest mortality tends to occur in persons bitten on the head and face (40% to 80%), with intermediate mortality in those bitten on the hands or arms (15% to 40%), and least in those bitten on the trunk or legs (5% to 10%) or through clothing (less than 5%).^{29,637,652,726}

The incubation period (the length of time between exposure to virus and development of clinical signs) is usually 1 to 2 months.^{232,744} Because it can vary from less than a week^{307,551,560} to several years,^{10,33,662,770,774} rabies is one of the most variable infectious diseases. The length of the incubation period may depend on the bite site and relative proximity to the CNS,^{10,350,687} severity of the bite, type and quantity of virus introduced, host age, and immune status.^{10,19,194,205,301,304,487,519,649}

Development of clinical rabies in humans can be divided into three general phases: a prodromal period, the acute neurologic phase, and coma preceding death.^{232,307} During the prodromal period, lasting 2 to 10 days, symptoms are usually mild and almost entirely nonspecific; they include general malaise, chills, fever, headache, photophobia, anorexia, nausea, vomiting, diarrhea, sore throat, cough, and musculoskeletal pain. One specific early symptom is abnormal sensation around the bite site, such as itching, burning, numbness, or paresthesia.¹⁹⁴

During the acute neurologic phase, patients exhibit signs of nervous system dysfunction such as anxiety, agitation, dysphagia, hypersalivation, paralysis, and episodes of delirium. Occasionally, priapism or increased libido may be observed.¹⁹⁸ Cases in which hyperactivity is predominant are classified as *furious* rabies. When paralysis dominates, it is classified as paralytic or *dumb* rabies.^{125,380,727} From 17% to 80% of patients exhibit hydrophobia, a pathognomonic sign of rabies believed to be caused by an exaggerated respiratory tract protective reflex.^{10,19,746,774} Hydrophobic episodes, initially triggered by attempts to drink,^{307,774} can last from 1 to 5 minutes. In furious rabies, the neurologic period ends after 2 to 7 days with coma or sudden death from respiratory or cardiac arrest.⁷⁶

Paralytic rabies occurs in about 20% of patients and may be more frequent in persons exposed to certain strains, such

as vampire bat RABV.³²⁸ In marked contrast to furious rabies, the sensorium is largely spared.^{309,328} Patients initially develop paresthesia and weakness, and finally flaccid paralysis, usually in the bitten extremity.¹²⁵ Paralysis progresses to paraplegia and quadriplegia. In paralytic rabies, the course is usually less rapidly progressive, with some patients living up to 30 days without intensive care.⁴⁵³ The final stage of the disease is coma, which lasts 3 to 7 days and results in death.⁷⁶ In patients receiving respiratory assistance, survival may be prolonged for weeks,^{76,522} with death caused by other complications.^{65,204,301}

To date, six cases of human recovery from clinical rabies have been documented. Five had exposure to animal bites,^{15,300,565,773} and one occurred after suspected inhalation of rabies virus in the laboratory.¹¹⁰ Only one of these occurred in a patient who had never been vaccinated,⁷⁷³ whereas the other five cases were attributed as exposures and vaccination failures. In the nonvaccinated patient, an experimental treatment included induction of ketamine coma in conjunction with antiviral compounds and intensive care.⁷⁷³ Nevertheless, more than 10 attempts to repeat such experimental treatment (although with deviations and modifications) failed.³³⁸ However, in one vaccination failure case (immunoglobulin was not administered, although all five doses of vaccine were administered on time), the experimental treatment was implemented successfully.¹⁷⁴ In addition, this was the only survival case where the virus variant was identified (vampire bat RABV). In all other survivors neither antigen detection nor virus isolation nor RNA amplification was successful, and rabies diagnosis was based on the history of exposure, compatible incubation period and clinical signs, and serologic tests. Another case of presumptive abortive rabies infection in a human, who never required intensive care, and only once received rabies biologics after establishment of the diagnosis, was reported recently.¹¹³

Clinical disease in animals is not unlike that of humans, except for the absence of hydrophobia. Signs are variable but can include altered phonation, pica, cranial nerve deficits, altered activity patterns, and loss of fear of humans.³⁷

Vesiculovirus Infections

In natural infection, the incubation period of vesiculovirus varies from 2 to 9 days, but usually lesions develop between 2 and 5 days after exposure.^{303,356} The lesions of vesiculovirus are indistinguishable from those of FMD. In cattle, the initial lesions are characterized by pink to white papules in the mouth that progress in 1 to 2 days to vesicles. The vesicles can coalesce and rupture, leaving a denuded area that heals in 1 or 2 weeks if no secondary infections occur.³⁵⁶ These lesions can also occur in the dental pad, lips, gums, muzzle, nose, teats, and feet. In experimental inoculation of horses, vesicles appeared in the mouth 42 hours after inoculation, and 2 days later, part of the dorsal epithelial covering of the tongue sloughed off. Vesicles also appeared on the feet. At necropsy, the spleen was enlarged, but no other lesions were observed in the internal organs.¹⁴⁸ In natural infection of horses, lesions are found in the lips, corners of the mouth, muzzle, nostrils, ears, belly, prepuce, and udder.³⁰³ The lesions in pigs are similar to those described for cattle.²⁹⁶ Affected animals have increased salivation and a sharp reduction in milk production. Eating is difficult because of the sore mouth, with a consequent decline in physical condition. Lameness develops with foot lesions.

Although development of secondary lesions in places other than the point of inoculation is suggestive of viremia, the virus has not been isolated from blood even at 6 hours after the experimental inoculation of pigs.^{133,583,672} The virus is present at its highest titer in the vesicular fluid, which represents a transient but very efficient source of virus for contact transmission. The virus can be isolated from specimens taken from saliva, tonsils, vesicular fluids of feet, and, in some cases, feces.^{133,583,672}

Ephemerovirus Infections

After an incubation period of 2 to 4 days, the first clinical sign is fever (40°C to 42°C), accompanied by malaise and a severe drop in milk production. In 12 to 24 hours, fever remits, followed by a second febrile phase. During this second phase, the animals are depressed, are anorexic, and show muscle stiffness and lameness. Ruminal stasis, nasal and ocular discharges, and swelling of one or more joints are present. Subcutaneous emphysema can develop in some animals.^{671,735} The clinical signs persist for 1 or 2 days, followed by rapid recovery. Clinical signs are much more severe in adults than in calves. Calves under 6 months of age show no clinical signs.⁷⁸

Novirhabdovirus Infections

Viral hemorrhagic septicemia (VHS) generally occurs at temperatures between 4°C and 14°C. At water temperatures between 15°C and 18°C, the disease generally has a short course with a modest accumulated mortality. VHS rarely occurs at higher temperatures. Low water temperatures (1°C to 5°C) generally result in an extended disease course with low daily mortality but high accumulated mortality. For IHNV, the temperature optimum is slightly greater, 3°C to 18°C. VHS outbreaks occur during all seasons but are most common in spring when water temperatures are rising or fluctuating. For more detailed reviews of the condition see, Smail⁶⁵⁶ and Wolf.⁷⁸⁶

VHS progresses in three stages. The acute stage includes a rapid onset of high mortalities (up to 90%, particularly in young fish) often with severe clinical signs such as darkening of body color, exophthalmia (bulging eye), bleeding around eyes and fin bases, pale gills, and petechial (pinpoint) hemorrhaging on the surfaces of the gills and viscera and in the muscle. Virus multiplication in endothelial cells of blood capillaries, hematopoietic tissues, and cells of the kidney underlies the clinical signs. Gross pathology includes generalized petechial hemorrhaging in the skin, muscle tissue (especially in dorsal muscles), and internal organs.⁵³¹

During the second subacute, or chronic, stage, the body continues to darken and exophthalmia may become more pronounced, but hemorrhaging around the eyes and fin bases is often reduced. Fish are severely anemic and paleness is particularly evident in the abdomen. Fish may develop a spiraling swimming motion. The final, nervous stage involves reduced mortality and clinical signs are usually absent, but the corkscrew swimming motion becomes more pronounced. The disease is transmitted horizontally through contact with infected fish or water. Large amounts of virus are shed in the feces, urine, and sexual fluids. There is no vertical transmission of the VHSV. However, vertical transmission has been documented for IHNV.⁵³⁰ Virus is shed from infected fish via the urine⁶⁵⁶ and reproductive fluids and can also be transferred by piscivorous birds as external mechanical vectors.^{536,559} Incubation time is dependent on temperature and dose; it is 5 to 12 days at

higher temperatures. During and immediately following an outbreak, virus can be isolated readily from kidney, heart, and spleen tissues.

VHSV can also establish a carrier state in freshwater fish species.^{206,355} The virological status of such carriers will be dependent on a range of parameters including the length of time following initial exposure and geographical proximity to fish-farm outlets. Based on virus isolation in cell culture, the prevalence of VHSV in marine fish species has been found to be in the range of 0.0% to 16.7%.⁶⁵⁴

DIAGNOSIS OF RHABDOVIRUS INFECTIONS

Lyssavirus Infections

Clinical diagnosis of rabies is not difficult in cases of a documented history of exposure and subsequent compatible clinical signs or symptoms. Because an exposure history may be lacking, rabies should be considered in any acute, unexplained neurologic disease that rapidly progresses to coma and death.³⁸ Routine diagnosis is established by standard laboratory tests for specific virus isolates, antigens, nucleic acids, or neutralizing antibodies.^{293,659,745} Postmortem diagnosis should be performed on CNS specimens, especially the brainstem and cerebellum.⁶⁹⁷ The fluorescent antibody test⁶⁵⁹ and the avidin-biotin immunohistochemical technique²⁸⁹ are sensitive and specific methods for detecting virus antigen (Fig. 31.12).

Examination of skin biopsies from the face⁹⁶ or hair-covered occipital portions of the neck for virus antigen^{77,522} is

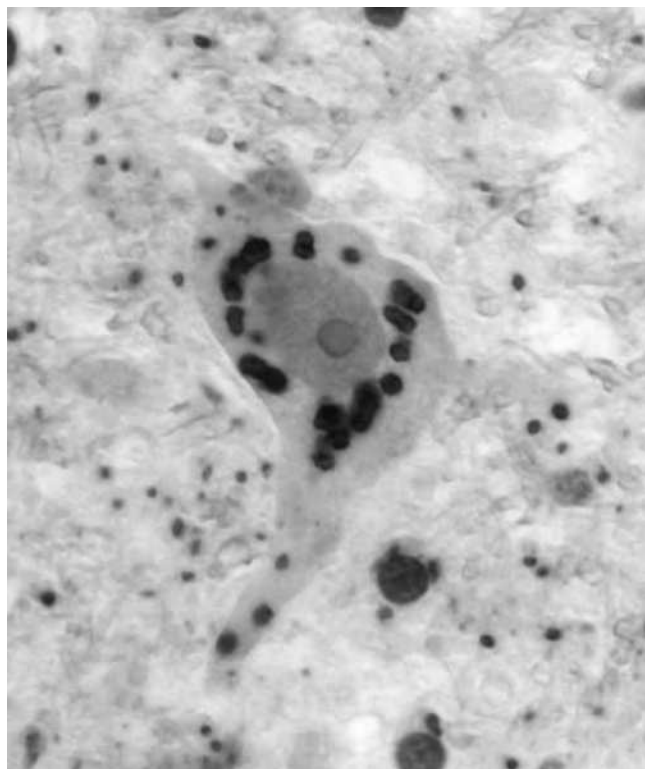


FIGURE 31.12. Immunohistochemical staining of intracytoplasmic viral inclusions in the neuron of a human rabies patient (630 \times). (Courtesy of M. Niezgodna, CDC/OID/NCEZID/DHPP/PRB.)

a rapid method to diagnose human rabies before death. Rabies virus can be isolated from saliva by direct intracerebral inoculation into mice³⁸⁷ or by infection of neuroblastoma cells.⁶⁵⁷ Fluorescent antibody examinations of corneal impressions may occasionally lead to the diagnosis of human rabies.³⁸¹ The reverse transcriptase-polymerase chain reaction (RT-PCR) assay has been used to amplify and sequence parts of the lyssavirus genome directly from brain, saliva, and other affected tissues.^{138,302,522,659} This allows detection of rabies virus-specific RNA and also permits insights into the identity of the virus variant by genetic sequencing. Detection of specific antibodies in serum^{522,662} late in the clinical course can be diagnostic for rabies, if the patient has not been previously vaccinated. Except for certain cases of postvaccinal encephalomyelitis, CSF antibodies are produced only in rabies-infected, not in vaccinated, individuals.⁷⁴⁷ Several diagnostic tests have been developed for detection of virus-neutralizing antibodies, such as the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody viral neutralization (FAVN) test, which are recommended by national and international authorities, such as the World Health Organization (WHO), Office International des Epizooties (OIE), and Advisory Commission on Immunization Practices (ACIP).^{137,323,658} Recently, lentiviral pseudotypes containing glycoproteins of different lyssaviruses have been developed for replacement of infectious RABV in such virus-neutralizing tests.⁷⁹¹ Several modifications of enzyme-linked immunosorbent assays (ELISAs) have been developed for capture and measure of antiglycoprotein antibodies of RABV.^{639,753,810} However, such ELISA-based tests are currently not recommended for the cases where diagnostic accuracy is critical.⁵⁰¹ Other serologic methods have been developed that detect antibodies against other components of RABV, primarily the nucleocapsid, which is most abundant in the infected cells. Of these, the best established is the indirect fluorescent antibody test (IFA). Antibodies detected by IFA appear earlier than virus-neutralizing antibodies and sometimes are the only positive result obtained antemortem.^{113,659,662}

Vesiculovirus Infections

Because vesiculovirus is clinically similar to FMD, differential diagnosis between the two diseases is of utmost importance, especially in countries free of the latter disease. VSV can be isolated from vesicular fluid or epithelium of the lesions by inoculation in mice, embryonated eggs, or cell culture.¹³⁴ The virus can be identified by virus neutralization, complement fixation, or immunofluorescence.²⁹⁶ Complement fixation provides a rapid, sensitive, and accurate method for the differentiation of VSV and FMD virus.²⁹⁵ A rise in virus neutralization or ELISA antibody titer in serum samples taken during the clinical and convalescent phases of the disease is evidence that the infection was caused by VSV.¹³ Recently, detection of VSV in clinical samples by RT-PCR has been described. This method is highly sensitive and specific, providing a rapid diagnosis and material for genetic characterization of the virus.^{319,598}

Ephemerovirus Infections

Clinical diagnosis of BEF is based on its rapidity of spread and transient nature.⁷⁸ For confirmation, virus isolation or the demonstration of an increase in virus neutralization or ELISA antibody in paired serum samples is needed.¹⁸⁷ A blocking ELISA compares favorably with neutralization and does not detect

cross-reacting antibodies to Kimberly or Berrimah viruses.⁸⁰⁵ Although impractical for routine diagnosis, cattle inoculation with blood from BEFV-affected animals is the most sensitive method for viral isolation. Isolation of BEFV in *Aedes albopictus* cells from the blood of infected animals, followed by direct immunofluorescence to detect the presence of viral antigens, has been used in experimental studies.⁷²⁰

Novirhabdovirus Infections

The occurrence of clinical signs of VHS described earlier should suggest the presence of IHNV and VHSV. Gross pathology includes generalized petechial hemorrhaging in the skin, muscle tissue (especially in dorsal muscles), and internal organs. Histopathologic findings reveal degenerative necrosis in the hematopoietic tissues, kidney, spleen, liver, pancreas, and digestive tract. Necrosis of eosinophilic granular cells in the intestinal wall is pathognomic of IHNV infection.⁸⁰ The kidney, liver, and spleen show extensive focal necrosis and degeneration—cytoplasmic vacuoles, pyknosis, karyolysis, and lymphocytic invasion. In case of VHSV, diagnosis can involve immunohistochemistry analysis of VHSV-positive endothelial cells in the vascular system.²⁰⁹ The standard surveillance method to detect carrier fish for IHNV and VHSV is based on direct isolation of the virus in cell culture followed by identification using antibody-based methods (IFA, ELISA) or nucleic acid-based methods (e.g., RT-PCR), followed by gene sequencing. PCR-based detection of viral genomes in fish tissue is still under development. The technique can be used for confirmation of overt infection in fish but has yet to be validated for use in direct surveillance programs.^{22,25,172,186,416,784}

PREVENTION AND CONTROL OF RHABDOVIRUS INFECTIONS

Lyssavirus Infections in Humans

Rabies has the highest case-to-fatality ratio of any infectious disease. With rare exceptions, comfort care, sedation, and life support measures may prolong life but do not prevent death. In most situations, use of the term *treatment* is a misnomer and refers to medical aid related to animal bite and disease prevention by postexposure prophylaxis.⁷⁶⁷ However, the establishment of a protocol for experimental treatment of clinical rabies⁷⁷³ has led to more attempts to combat the clinical disease. The majority of these have failed,³³⁸ although at least one positive result, with recovery of the patient, was reported.¹⁷⁴ Indeed, more studies in suitable animal models are needed to investigate different components of the protocol, potential ways of their modifications, and improvements.³³⁸ More than 12 million humans are exposed and may undergo antirabies prophylaxis annually, but in excess of an estimated 50,000 to 100,000 die, primarily from the bite of an infected dog.^{495,719} Regional epidemiologic surveillance and knowledge of viral pathogenesis, development of vaccination algorithms, and communication of risk to different occupational groups can significantly reduce human morbidity from inappropriate prophylaxis and rabies mortality.^{643,778} Eliminating primary exposure to rabid animals is a fundamental means of rabies prevention. Human rabies deaths are infrequent in regions with controlled canine rabies. Nevertheless, tens of thousands

of potential exposure cases are treated annually in Europe and North America because of enzootic wildlife rabies.²⁹³

Postexposure prophylaxis in humans includes proper wound care and the administration of rabies vaccine and antirabies immune globulin.^{111,790} Although the inclusion of antirabies serum or immune globulin in the prophylaxis protocol is not new,¹⁰³ it is infrequent. Most cases of human rabies prophylaxis in Africa, Asia, and Latin America are with vaccine only,⁴⁹⁶ often a nervous system tissue vaccine.^{43,349,415,523,562} Cell culture–based rabies vaccines (e.g., human diploid cell rabies vaccine [HDCV]) are used in much of the developed world and form the standard for historical comparison with the Pasteurian neural vaccines from the 19th century,^{731,767} including its later phenolized derivatives (Fermi, Semple, and others). Inactivated cell culture–based vaccines⁶⁸² and antirabies immune globulin, which are major improvements over cruder biologicals, decrease the adverse events related to anaphylaxis or serum sickness.^{412,769} Other major rabies vaccines are produced in avian embryo fibroblasts (e.g., purified chick embryo cell culture rabies vaccine [PCEC], Rabipur) or in rhesus monkey kidney cells (purified Vero cell rabies vaccine [PVRV], Verorab), with aluminum phosphate as an adjuvant.⁷⁹⁰ Production of HDCV is relatively difficult, with limited viral yields, resulting in high production costs. Primary hamster kidney cell vaccines are used in Russia, China, and other parts of Asia.⁴⁴⁵ Efficacy trials using reduced doses, different immunization schedules, and alternative routes (e.g., intradermal administration) have been conducted and have demonstrated both high efficacy and safety.^{129,130}

At present, no evidence suggests that prophylaxis failure is caused by antigenic variation of RABV.⁷⁹⁰ Rather, vaccine failures are usually associated with inadequate wound care, omission of potent serum, failure to infiltrate the wound with immune globulin, delay, or failure to follow recommended procedures.^{308,545,585,771} Future tactics for global human rabies prevention will continue to focus on the need for enhanced public health communications; continuing professional education; potent, inexpensive pre- and postexposure vaccines^{250,412,413} and new schedules; and viable alternatives to rabies immune globulin (e.g., monoclonal antibodies).⁴⁶ Based on the recognition that rabies at its source can be effectively controlled and sometimes eliminated, safer, more effective, and inexpensive veterinary vaccines are a necessity for animal reservoirs, vectors, or victims of the disease.^{38,100,220,222,235,780,790}

Although available rabies biologics provide reliable protection against phylogroup I lyssaviruses (RABV, DUVV, EBLV-1, EBLV-2, ABLV, ARAV, KHUV, DUVV), they do not protect against phylogroup II lyssaviruses (LBV, MOKV, SHIBV) or against WCBV, because of the significant antigenic differences.^{34,291} Given broad distribution of the latter divergent lyssaviruses in Africa, in southeastern Europe, and perhaps more widely in the world,^{81,402,403,473,513} there is a need to develop new biologicals, capable of providing reliable protection against them.

Control of Rabies in Animals

Rabies is not considered a serious candidate for disease eradication at this time because of numerous and diverse wild reservoirs.¹¹² The correlation between canine rabies and human fatalities, however, has led to the successful application of domestic animal vaccines, particularly in developed countries.¹⁰⁰

A comprehensive domestic animal program also requires responsible pet ownership. Such a program entails stray animal management; leash law amendments; humane population curtailment (e.g., early spay and neuter programs); animal importation, translocation, and quarantine regulations; schedules for early pre-exposure vaccination of companion animals (in light of potential maternal immune inhibition); and rational post-exposure management.¹¹¹ Unlike postexposure prophylaxis of humans, euthanasia is usually recommended for the naïve animal exposed to rabies, but this may eventually change with the development of safe and effective biologicals and protocols.

Current veterinary vaccines are more potent than earlier attenuated and inactivated vaccines.^{26,567} Because no vaccine is 100% effective, given poor cross-reactivity with some viral species,^{291,733} and because correct identification of the properly immunized animal may be confusing, the vaccinated dog or cat is not exempt from confinement and close observation. This strict period of observation of the biting animal applies to dogs, cats, and, in some countries, domestic ferrets.¹¹¹ Human prophylaxis may be delayed during this time in areas that are not enzootic for canine rabies.⁶⁵³ In addition, pet vaccination status does not necessarily alter the need for euthanasia of an offending animal, regardless of vaccine potency or efficacy, if rabies is suspected.

In the case of free-ranging, nondomestic mammals, population reduction of major rabies reservoirs has been practiced for centuries but has not been generally regarded as a humane, long-term, cost-effective, or ecologically sound tool to control widespread lyssavirus infection.^{171,293} Anticoagulants, however, have been used successfully to control hematophagous bats in Latin America. Anticoagulants have been applied topically to bite wounds on cattle, followed by systemic treatment of exposed cattle, and finally topical treatment of vampire bats themselves, exploiting their behavior of mutual grooming at the roost.²³⁹ These control efforts can avoid the destruction of beneficial nontarget bat species, perhaps some day to be augmented with novel vaccination strategies.⁶⁴⁰

For more than four decades, efforts have been made to protect free-ranging wildlife against virulent street virus by oral consumption of vaccine contained within bait.⁷⁸⁰ Millions of rabies virus vaccine-laden baits have been distributed over rural and urban areas in western Europe, eastern Canada, and the United States for wildlife rabies control.^{27,36,235,419,601,602,738} Historically, attenuated rabies virus strains (such as ERA, SAD) were broadly used for oral vaccination of wild carnivores in Western Europe and North America.^{626,739} However, sporadically these vaccine strains caused rabies in wildlife.^{216,508} A vaccinia-rabies glycoprotein (V-RG) vaccine was the first recombinant rabies vaccine to be constructed, field tested, and considered for regulation in Europe and North America for wildlife rabies control. This vaccine has been extensively reviewed to ensure safety (tested in more than 40 species of mammals and birds) and efficacy (proved against severe rabies challenge in target species). Thermostability of the vaccine has been demonstrated under laboratory and field conditions. Following the success of the V-RG vaccine against fox rabies in Belgium⁸⁸ and France, preliminary field trials suggest its potential utility for rabies control in raccoons, foxes, and coyotes in the United States.^{215,292,591,603} Other orthopoxviruses have been considered as vectors of lyssavirus antigens, but these have not yet been field tested.^{217,450} A number of

attenuated and recombinant rabies vaccines have been developed.^{36,69,235,408,423,547,612,613,768} Oral vaccines have been successfully developed for red, Arctic, and gray foxes; coyotes; raccoon dogs; raccoons; skunks; and domestic dogs.^{88,221,241,526,613} If future recombinant, replication-incompetent, inactivated, or DNA-based vaccines^{208,343,420,449,500,538,613,795} prove both efficacious and economical, they may render most previous biosafety concerns obsolete, paving the way for more widespread, free-ranging wildlife and dog rabies control, particularly in developing countries. Another promising approach is combination of rabies vaccination with immunocontraception, which can significantly reduce the population of the disease vectors, particularly stray dogs.⁷⁹²

Control of Vesiculovirus Infections

Supportive veterinary care of affected animals helps to prevent complications that can delay recovery from VSV infection. Vaccination against VSV has been practiced to only a limited extent. A modified live vaccine, attenuated in cell culture or chicken embryos, has been used in parts of the United States, Central America, and Peru. This vaccine, administered intramuscularly in cattle, protects from disease for at least 1 year.²⁹⁶ During the 1985 epizootic in Colorado, an inactivated VSNJV vaccine was used in the field and later tested in an experimental trial. It induced antibodies that lasted for 175 days, but viral challenge was not performed to assess protection.²⁶⁵ Recently, a recombinant vaccinia virus expressing the VSV-I glycoprotein was developed and used experimentally to immunize cattle. Inoculated animals developed antibodies and resisted intradermal lingual challenge.⁴⁶⁹

Control of Ephemerovirus Infections

Vector control of BEFV is very difficult. International efforts to prevent the introduction of disease and vaccination may be the only practical methods for prevention of BEF.⁷³⁵ Several attenuated vaccines, produced by serial passage in mouse or cell culture, provided protection against experimental challenge when mixed with adjuvants and given in several doses.⁶⁷⁰ Inactivated vaccines were developed in Japan and Australia, but they induced poor and unreliable immunity. Vaccinia virus expressing the G protein elicited neutralizing antibodies in cattle, which were resistant to a subsequent viral challenge.³¹² Additionally, an experimental subunit vaccine consisting of purified G protein mixed with Quil adjuvant conferred protection against viral challenge.⁷²¹

Control of Novirhabdovirus Infections

Control methods for IHNV currently rely on avoidance of exposure to the virus through the implementation of strict control policies and sound hygiene practices.⁷⁸³ The thorough disinfection of fertilized eggs, the use of virus-free water supplies for incubation and rearing, and the operation of facilities under established biosecurity measures are all critical for preventing infectious hematopoietic necrosis at a fish production site.

Vaccination of salmonids against IHNV is at an early stage of development; however, a range of vaccine preparations have shown promise in both laboratory and field trials.^{397,782} Both autogenous, killed vaccines and a DNA vaccine have been licensed for commercial use in Atlantic salmon net-pen aquaculture on the West Coast of North America, where such vaccines can be delivered economically by injection. Vaccines

against IHNV have not yet been licensed in other countries, where the application of vaccines to millions of small fish will require additional research on novel mass delivery methods. Although research on vaccine development against VHSV has been ongoing for more than three decades, a commercial vaccine is not yet available. DNA-based vaccines have proven to be very promising, inducing good protection from VHS.⁴⁵² Several immunostimulants, such as yeast-derived β -glucans, IL-1 β -derived peptides, and probiotics, have been assessed for enhancing protection against VHS.⁵⁵⁴ Disinfection of eggs is a highly effective method to block egg-associated transmission of novirhabdoviruses in aquaculture settings.⁷⁸³ The method is widely practiced in areas where the virus is endemic. Other experimental approaches include resistance breeding and restocking with a resistant fish species.^{310,783}

PERSPECTIVES

The foreseeable future for rhabdoviruses is for these viruses to occupy essentially the same positions in the science of virology that they have occupied for the past several decades—VSV as a well-studied prototype for the nonsegmented, negative-strand RNA viruses, RABV as a dreaded cause of disease in animals and humans, and the ephemeroviruses and novirhabdoviruses as important animal pathogens. Study of these viruses should continue to provide fundamental insights into the basis for virus–host interactions, neurotropism, and neuropathogenesis. In terms of the basic molecular biology of negative-strand RNA viruses, a number of important questions that have yet to be fully addressed have been pointed out through the course of this chapter. For example, the question of how the large, multifunctional L protein is able to respond to the many different *cis*-acting sequences that regulate its activity and the issue of how different intracellular nucleocapsids are selected for envelopment during the process of virus assembly are fundamental questions. In the area of virus–host interactions, the questions of viral virulence determinants and how rhabdoviruses suppress host responses among the different cell types involved in viral pathogenesis and immunity will be key questions for understanding the basis for viral pathogenesis in intact animals. In terms of the control of rabies, advances will come from the enhanced ability to control the spread of RABV among wild animal populations as well as the development of newer, more effective vaccine strategies.

One of the exciting areas of development with rhabdoviruses is the use of genetically engineered viruses as vaccine vectors or therapeutic agents. The use of recombinant VSV and RABV as vaccine vectors has been mentioned several times throughout the chapter.^{147,214,592,593,607,631} In addition, both VSV and RABV have potential use as cytolytic agents for therapeutic purposes. For example, genetically engineered strains of both VSV and RABV for cytolysis of HIV-infected cells have been generated, which lack G protein but express CD4 and chemokine receptors in the virus envelope.^{490,632} Another example of use of rhabdoviruses for cytolytic purposes is in the development of oncolytic viruses to treat patients with cancer. These viruses take advantage of the fact that many cancers appear to be defective in their ability to respond to antiviral cytokines, such as IFN.^{47,680} Such cancers are susceptible to viruses that induce host antiviral responses (e.g., M protein mutant viruses)^{3,681} or

viruses that encode antiviral cytokines (e.g., IFN- β , IL-4),^{224,527} whereas normal cells are largely resistant. Thus, these genetically engineered viruses have a greater selectivity for replication in cancers compared with normal tissues.

As a result of the efforts in developing recombinant rhabdoviruses as vaccine vectors and as cytolytic agents, it is likely that clinical trials of genetically engineered rhabdoviruses in humans will take place in the near future. For example, at least two genetically engineered VSVs have been considered by the National Institutes of Health Recombinant DNA Advisory Committee for potential oncolytic therapy in humans, a key step toward beginning clinical trials in a variety of cancer types.^{716,717} A number of issues need to be considered in the use of such agents, such as their safety for use in humans, as well as the protection of animal populations that may be exposed to such viruses. Nonetheless, the advances in understanding virus replication and pathogenesis should make it feasible to address these issues, so that these viruses that have long been a burden to humanity can instead be a benefit.

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Filoviridae: Marburg and Ebola Viruses

Classification

- Taxonomy
- Biosafety and Biosecurity

History

- Marburg Hemorrhagic Fever (MHF)
- Ebola Hemorrhagic Fever (EHF)
- EHF in Nonhuman Primates
- Laboratory Infections/Exposures

Virion Structure

Genome Structure and Organization

Virus Proteins

- Nucleoproteins
- Polymerase Complex Proteins
- Structural (Surface) Glycoprotein
- Nonstructural Glycoproteins
- Matrix Proteins

Stages of Replication

- Mechanism of Attachment
- Mechanism of Entry and Intracellular Trafficking
- Transcription and Translation
- Replication of Genomic Nucleic Acid
- Assembly and Release
- Effects on Host Cell Cultures

Pathology and Pathogenesis

- Entry into Host
- Host-Cell Pathology
- Host Immune Response
- Impairment of the Vascular System
- Impairment of the Coagulation System
- Virulence
- Host Genetics
- Persistence

Epidemiology

Ecology

Clinical Features

Diagnosis

- Clinical Diagnosis
- Laboratory Diagnosis

Prevention and Control

- Patient Management
- Therapeutic Options
- Prophylaxis

CLASSIFICATION

Taxonomy

Filoviruses are taxonomically classified within the order *Mono-negavirales*, a large group of enveloped viruses whose genomes are composed of a nonsegmented, negative-strand (NNS) RNA molecule.⁹⁸ Following their discovery, filoviruses were initially grouped with rhabdoviruses, based primarily on the appearance of virus particles. However, subsequent morphologic, genetic, physiochemical, and virologic studies of Marburg virus (MARV) and Ebola virus (EBOV) isolates revealed unique properties and led to their placement into a separate family, the *Filoviridae*.²²⁴ Further characterization of these agents demonstrated that EBOV and MARV represent divergent lineages of filoviruses; their differences were significant enough to warrant the formation of the genera *Marburgvirus* and *Ebolavirus*. According to the International Committee on Taxonomy of Viruses (ICTV) (<http://www.ictvonline.org/virusTaxonomy.asp?version=2009&xbhcp=1>), the *Marburgvirus* genus contains a single species, *Lake Victoria marburgvirus*, because strains exhibit only limited genetic variation. However, the appearance of distinct MARV lineages may lead to further speciation.⁴⁰¹ There is a greater divergence within the *Ebolavirus* genus, five species having been recognized: *Zaire ebolavirus* (type species; ZEBOV), *Sudan ebolavirus* (SEBOV), *Reston ebolavirus* (REBOV), *Tai Forest ebolavirus* (formerly Ivory Coast or *Cote d'Ivoire ebolavirus*, ICEBOV or CIEBOV), and *Bundibugyo ebolavirus* (BEBOV), of which BEBOV has still to be approved.⁴⁰³ A distinct filovirus sequence has recently been obtained from bats in Spain, but no virus has yet been isolated. This new "putative virus" (designated Lloviu virus) is proposed to represent the single species *Lloviu cuevavirus* in the new genus *Cuevavirus* of the *Filoviridae* family.²⁹⁹

Biosafety and Biosecurity

Because of their high mortality rate, their potential for person-to-person transmission, and a lack of an approved vaccine or antiviral therapy, MARV and EBOV are classified as biosafety level 4 (BSL-4; risk group 4) pathogens, for which maximum containment facilities are required when handling the infectious agent (<http://www.cdc.gov/biosafety/publications/bmbl5/BMBl.pdf>). Filovirus infectivity is quite stable at room temperature (20°C), but is largely inactivated in 30 minutes at 60°C; MARV is somewhat resistant to desiccation. Infectivity is greatly reduced or destroyed by high doses of ultraviolet light and gamma irradiation, lipid solvents, β -propiolactone, photo-induced alkylating probe 1,5-iodonaphthylazide, guanidinium isothiocyanate, and commercial hypochlorite and phenolic disinfectants.

The threat of bioterrorism in the aftermath of the September 11, 2001 and anthrax attacks against the United States has

prompted governments to implement countermeasures. This has led to greater restrictions on the acquisition and use of a variety of agents that pose serious threats to public health. Filoviruses have been classified as Centers for Disease Control and Prevention (CDC) Category A Agents (<http://www.bt.cdc.gov/agent/agentlist-category.asp#a>), as part of a system for prioritizing initial public health preparedness efforts and grading the potential of agents for large-scale dissemination. Filoviruses are also classified as “select agents” by the CDC Select Agent Program. This program is mandated by federal law to regulate

activities involving these agents within the United States and to register laboratories and entities handling one or more select agents (<http://www.cdc.gov/od/sap/>).

HISTORY

Table 32.1 lists the documented occurrences of MARV and EBOV disease, along with information regarding these outbreaks. Additional details of these episodes are described in the following sections.

TABLE 32.1 Outbreaks of Filovirus Disease

Filovirus (species)	Year	Outbreak location	Place of origin	Human cases (% mortality)
LVMARV	1967	Marburg/Frankfurt, Germany; Belgrade, Serbia	Uganda	32 (23)
	1975	Johannesburg, South Africa	Zimbabwe	3 (33)
	1980	Nzoia and Nairobi, Kenya	Western Kenya	2 (50)
	1987	Kisumu, Kenya	Western Kenya	1 (100)
	1998–2000	Durba/Watsa, DRC	DRC	154 (83)
	2004–2005	Uíge, Angola	Angola	252 (90)
	2007	Uganda (western)	Uganda	4 (25)
	2008	The Netherlands	Uganda	1 (100)
	2008	United States	Uganda	1 (0)
ZEBOV ^a	1976	Yambuku, DRC	DRC	318 (88)
	1977	Tandala, DRC	DRC	1 (100)
	1994	Ogooué-Invindo province, Gabon	Gabon	52 (60)
	1995	Kikwit, DRC	DRC	315 (79)
	1996	Mayibout, Gabon	Gabon	37 (57)
	1996	Booue, Gabon and Johannesburg, South Africa	Gabon	60 (75) ^b
	2001–2002	Ogooué-Invindo province, Gabon; Cuvette region, RC	Gabon?	124 (79)
	2002–2003	Cuvette region, RC; Ogooué-Invindo province, Gabon	RC?	143 (90)
	2003	Mboma and Mbandza, RC	RC	35 (83)
	2005	Etoumbi and Mbomo in Cuvette region, RC	RC	12 (75)
	2007	Kasai Occidental province, DRC	DRC	264 (71)
	2008–2009	Kasai Occidental province, DRC	DRC	32 (47)
SEBOV	1976	Nzara, Maridi, Tembura, Juba, Sudan	Southern Sudan	284 (53)
	1979	Nzara, Yambio, Sudan	Southern Sudan	34 (65)
	2000–2001	Gulu District, Mbarrara, and Masindi, Uganda	Uganda	425 (53)
	2004	Yambio County, Sudan	Southern Sudan	17 (41)
	2011	Uganda (central)	Uganda	1 (100)
BEBOV	2007	Bundibugyo district, Uganda	Uganda	149 (25) ^c
ICEBOV (CIEBOV)	1994	Tai Forest, Ivory Coast, and Basel, Switzerland	Ivory Coast	1 (0)
	1995	Liberia	Liberia?	1 (0)
REBOV	1989	Reston, Virginia (also Pennsylvania and Texas)	Philippines ^d	4 (0)
	1992	Siena, Italy	Philippines	0 (0)
	1996	Alice, Texas	Philippines	0 (0)
	2008	Philippines	Philippines	0 (0)

Note, in 2012, Uganda has reported two EHF and one MHF outbreak; another EHF outbreak was reported from DRC. The EHF outbreaks were caused by SEBOV and BEBOV. The border region between Uganda and DRC seems to be a new “hot spot” for filovirus HF. For more information, please see the CDC and WHO websites.

^aFrom approximately 1998 to the present time, there have been partially confirmed reports of transmission of ZEBOV among great apes in Gabon and the Republic of Congo (RC), which has severely impacted gorilla and chimpanzee populations.

^bIncluded an imported case in South Africa where an ill Gabonese physician (survivor) infected a nurse who died.

^cCase fatality rate (CFR) was much higher if one considered only laboratory-confirmed cases.

^dREBOV has only been traced to a single monkey-breeding facility in the city of Calamba, Philippines, which was depopulated in 1996 and is no longer in operation. DRC, Democratic Republic of Congo; RC, The Republic of Congo.

Marburg Hemorrhagic Fever (MHF)

The first identified instance of filovirus disease occurred in 1967, when MARV caused severe cases of hemorrhagic fever in Europe.^{274,275,276} The epidemic started in mid-August in Marburg, Germany with three laboratory workers who contracted the disease after processing organs from African green monkeys (*Cercopithecus aethiops*) imported from Uganda. Seventeen more patients were hospitalized and two medical personnel contracted the disease while attending to patients. The last patient, who apparently had been infected by her husband during the convalescent period, was admitted in November 1967.²⁷⁶ Six cases (including two secondary infections) occurred in Frankfurt, Germany concomitant with the Marburg infections.³⁷² In September, two cases were identified in Belgrade, former Yugoslavia, in which a veterinarian was infected while performing a necropsy on a dead monkey and transmitted the virus to his wife, who nursed him early in his illness.²⁷⁶ A total of 31 cases (including six secondary infections) were identified, with seven fatalities in primary infections (23%). Subsequent serologic investigations have suggested that there was one additional primary case in Marburg.³⁷⁰

MHF remained an obscure medical curiosity until 1975, when three cases were reported in Johannesburg, South Africa.¹²² The index case was a white male who just prior to his infection had traveled in Zimbabwe. Seven days after onset, his travel companion also became ill and transmitted the disease to an attending nurse; the index case was the only fatality. An investigation was conducted along the travel route of the index case, but the source of the virus was not determined.⁶⁶ Two further episodes of MHF were reported from Kenya in 1980 and 1987. The index case in 1980 became ill in western Kenya and died in Nairobi, where an attending physician was infected but survived the disease.³⁷¹ In 1987, a fatal case occurred in the same region of western Kenya.²¹⁸ The 1980 and 1987 index cases both traveled to the Mt. Elgon region, which is located close to Lake Victoria and was the source of the monkeys that initiated the original 1967 outbreak (trapped near Lake Kyogo, Uganda).

The first outbreak of MHF in a community setting of central Africa started in October 1998 in Durba/Watsa, located in the northeastern region of the Democratic Republic of Congo (DRC). Its remote location and the hazards of an ongoing armed conflict hampered efforts to study this outbreak, but an investigation was initiated after the death of an attending physician in 1999. Sporadic cases continued and were directly or indirectly linked to activity in the vicinity of an underground gold mine. Primary cases were mainly gold miners who started multiple, usually short chains of human-to-human transmission within their families. Overall, 154 cases were reported with a case fatality rate (CFR) of 83%. Analysis of viral sequences derived from clinical specimens and virus isolates showed nucleotide diversity up to approximately 20%.^{22,23,65,400,401} The largest outbreak of MHF (252 cases with 227 deaths; CFR of 90%) took place in northern Angola, Uige province. The first cases date back to October/November 2004, but initial diagnostic tests were negative for filoviruses. The main outbreak started in February/March 2005 and the last confirmed case died in July. Initial infections were linked to a Uige hospital and included a high number of pediatric cases. Sequence analysis of virus isolates suggested a single introduction into the community.^{151,211,401} The latest MHF episode dates to 2007, with four documented cases associated with a single mine in western Uganda.⁴⁰⁰ In addition, two imported cases were reported from the United

States (nonlethal) and the Netherlands (lethal), who independently visited the same cave in Uganda in 2008.^{6,396}

Ebola Hemorrhagic Fever (EHF)

EHF was first reported in 1976, when EBOV appeared simultaneously in the DRC (at that time Zaire) and Sudan with 318 (CFR of 88%) and 284 cases (CFR of 53%), respectively. These epidemics were determined to have been caused by two distinct species (ZEBOV and SEBOV), a fact not recognized until years later. Viruses were isolated from patients of both outbreaks and named after a small river in northwestern DRC.^{36,449,450}

No index case was clearly identified in the Sudan outbreak in 1976, although initial cases originating in Nzara, Sudan, involved six cotton factory workers and their close relatives. The epidemic was augmented by the spread of cases to neighboring areas (Maridi, Tembura, and Juba). High levels of transmission occurred in the hospital of Maridi (a teaching center for student nurses), primarily through the use of contaminated needles and a lack of barrier nursing practices. At the same time, a larger outbreak in the DRC, centered around a Belgian mission hospital in Yambuku, Equateur Region, was being fueled by similar circumstances. During a 7-week period of the outbreak, the single most significant factor in the spread of infection in the hospital was the reuse of contaminated syringes and needles, although secondary transmission to family members caused 45% of all recorded infections. The outbreak ended with closure of the hospital and quarantining of infected patients.

In 1977, a single fatal ZEBOV case was reported from Tandala, DRC, about 325 km from the original focus of the 1976 Yambuku outbreak.¹⁷⁹ SEBOV reemerged in 1979 in Nzara and Yambio, Sudan. The index case worked in the same textile factory cited as the potential source of infection in the 1976 Sudan outbreak. Hospitalization of the patient led to four nosocomial infections and further transmission to five families (34 cases with 22 fatalities).⁴⁵¹

No further cases of EHF were reported until 1994, when a novel EBOV (ICEBOV [CIEBOV]) was isolated from an ethnologist who had become ill while working in the Tai Forest reserve of Ivory Coast. The infection was determined to have occurred while performing a necropsy on a dead chimpanzee (whose troop had lost several members to EHF).^{114,243} Later, a single seroconversion suggested a second nonfatal human case in nearby Liberia. This episode extended the geographic distribution of known EBOV cases to include most of the African rain forest and was the first case in West Africa.

In 1995, a strain of ZEBOV very similar to the original 1976 virus reemerged in the DRC, causing a large hospital and community outbreak of EHF in and around Kikwit.^{223,453} The presumed index case was a charcoal worker, but transmission escalated following two consecutive laparotomies performed on an infected male laboratory worker at Kikwit General Hospital. About three-quarters of the first 70 patients within the subsequent developing epidemic were health care workers. In total, there were 315 cases and 250 deaths (CFR of 81%). Major risk factors for contracting disease were involvement in patient care in hospitals and households and preparations of bodies for burial.

Beginning in 1994, ZEBOV became active in or adjacent to the central African rain forest on both sites of the border between Gabon and the Republic of Congo (RC).^{5,148} Almost all outbreaks in this region described in this section were associated with hunting and butchering of wildlife, often

great apes. The first epidemic was reported in 1994 from the Ogooué-Ivindo Province in northeast Gabon with a total of 52 cases (CFR of 60%).¹⁴⁷ In 1996, two more outbreaks were reported from the same province.¹⁴⁷ The first epidemic started in early February and included 37 cases (CFR of 57%); the second episode began in July/August and resulted in 60 cases (CFR of 75%). The latter epidemic included an imported case in South Africa where an ill Gabonese physician infected a nurse who died with EHF (2 cases; CFR of 50%). The first reported epidemic that crossed the border into the RC began in late November of 2001 with the index case again reported from Ogooué-Ivindo Province in northeast Gabon. The epidemic spread to Mekambo and Makokou and from there into the RC by ill Gabonese who sought medical care by traditional healers. In total, there were 65 (CFR of 82%) and 59 (75%) cases from Gabon and the RC, respectively.²⁵¹ The next occurrence of EHF was a large epidemic reported from the districts of Mbomo and Kelle in Cuvette Ouest Region, RC, in late 2002 to May 2003 with 143 cases (CFR of 90%), followed in late 2003 by a smaller episode in the district of Mbomo with 35 cases (CFR of 83%).¹¹⁵ A neighboring area (Etoumbi) was affected in 2005 by a small outbreak of EHF with 12 cases (CFR of 75%) (http://www.who.int/csr/don/2005_06_16/en/index.html) (Table 32.1). This has so far been the last reported outbreak in this region.

In 2000 to 2001, the largest known epidemic of filovirus disease occurred in Uganda, with 425 cases and 224 deaths. The causative agent was closely related to SEBOV from the Sudan 1976 and 1979 outbreaks, and marked the first appearance of EBOV in Uganda. The CFR of 53% was in line with the generally lower mortality associated with the SEBOV species.⁴⁵⁴ The epidemic was mainly concentrated in the Gulu district, a savannah area located in the north of the country close to the Sudanese border, with person-to-person transmission including nosocomial infections. The index case was never identified. During the epidemic, the virus spread to the neighboring Masindi district and more distantly to the town of Mbarara in southwestern Uganda.³¹ During this outbreak a high number of health care workers were infected after barrier nursing procedures were instituted. It was also the first time that laboratory diagnostics were performed in the field to assist in outbreak management.⁴⁵⁴ In 2004, southern Sudan was again affected by a small SEBOV outbreak with 17 cases, of which 7 died (CFR of 41%).⁴⁵⁵ The index case had butchered a monkey and human-to-human transmission was mainly by contact.

The last reported ZEBOV outbreaks occurred in the Kasai Occidental province of the DRC in 2007 and 2008/09. The first larger outbreak included 264 reported cases with a CFR of 71%⁴⁵⁷; the second smaller outbreak had 32 cases, of which 15 died (CFR of 47%).⁴⁵⁸ Both outbreaks affected rural communities in the vicinity of the city of Luebo and are thought to be related to hunting and handling of migratory fruit bats.²⁴⁸

A new EBOV species, designated *Bundibugyo ebolavirus* (BEBOV), has been identified as the causative agent for an outbreak that occurred in the Bundibugyo district in western Uganda in 2007.^{403,423} In total, there were 149 reported cases, with 37 deaths (CFR of 25%); of these, 56 cases were laboratory confirmed. This single outbreak had the lowest reported CFR among all EBOV that have caused outbreaks in central Africa so far.

Most recently, a single case of SEBOV has been reported from Central Uganda.⁷ No further cases have been reported.

EHF in Nonhuman Primates

In November 1989, an EBOV with low or no apparent pathogenicity for humans was recognized in a shipment of cynomolgus monkeys (*Macaca fascicularis*) housed at a quarantine facility in Reston, Virginia. These monkeys were imported from a single supplier in the Philippines, and an unusually high mortality was observed in animals during transportation and quarantine. Simian hemorrhagic fever virus was also circulating in the facility; efforts to culture this virus led to the detection of a new species of EBOV that was named *Reston ebolavirus* (REBOV).^{72,203} The actual origin of this novel EBOV was never determined. Resumption of importation of monkeys led to new outbreaks of monkey disease in the United States in 1990 and 1996³³⁶ and in Italy in 1992.⁴⁵² Subsequent investigations have traced all shipments except one to a single supplier in the Philippines. The mode of contamination of this exporter's holding compound has never been ascertained, but whether the virus persisted in the facility or was reintroduced from wild-caught animals, the result was a continued movement of infected macaques. A few infected handlers were also identified by serologic methods without reports of severe illness or suspicious deaths among this cohort.²⁸⁵ Improved shipping, housing, and quarantine regulations regarding importation of monkeys have been implemented to protect the United States from future episodes of EBOV introductions.⁷⁴ Recently, REBOV emerged in pigs in the Philippines.¹⁴ The pigs were co-infected with porcine respiratory and reproductive virus (PRRS) and the actual pathogenic potential of REBOV in pigs remains unclear. This discovery certainly raises issues for food production. Six workers from pig farms and slaughterhouses developed antibodies to REBOV, indicating that they became infected but did not develop disease. The potential for REBOV as a human pathogen remains unanswered but should not be totally dismissed. As of yet, REBOV infections/exposures have never resulted in clinical disease in humans.

Note, in 2012, Uganda has reported two EHF and one MHF outbreak; another EHF outbreak was reported from DRC. The EHF outbreaks were caused by SEBOV and BEBOV. The border region between Uganda and DRC seems to be the new "hot spot" for filovirus infections. For more information, please see the CDC and WHO websites.

Laboratory Infections/Exposures

A single laboratory infection of EHF occurred in the United Kingdom in 1976. Treatment with human leukocyte interferon and human convalescent plasma was initiated and the patient survived.⁸⁷ In the past two decades there have been at least three laboratory infections with MARV (1 fatal) in Russia.²⁸² In 2004, accidental ZEBOV exposures via needlesticks while working with animals occurred in the United States and Russia, but only the latter became infected (fatally).^{196,233} In 2009, a German researcher had an accidental ZEBOV exposure via needlestick while working with animals. The person was treated with a recombinant vesicular stomatitis virus (VSV)-based vaccine expressing the ZEBOV glycoprotein. It could not be determined if the exposure resulted in infection.¹⁵⁸

VIRION STRUCTURE

Initial electron microscopic (EM) observations of filoviruses revealed distinctive bacilliform to filamentous virus particles; it was this highly characteristic morphology that inspired their name (Latin *filum*, *thread*).^{294,316} The virions of MARV and EBOV produced in tissue culture are pleomorphic, appearing as either U-shaped, 6-shaped, or circular (torus) configurations, or as elongated filamentous forms of varying length (up to 14,000 nm), all from the same culture fluid (Figs. 32.1A–C). The filamentous forms can also be seen to form branched structures (Fig. 32.1C, *arrow*). The unit length associated with peak infectivity for MARV and EBOV was measured to be 860 and 1,200 nm, respectively.¹³⁹ Virions have a uniform diameter of 80 nm, contain a helical ribonucleoprotein complex or nucleocapsid (NC) roughly 50 nm in diameter (Figs. 32.1 and 32.2), and have a central axial space (~20 nm in diameter) running the length of the particle. The NC has a helical periodicity of ~5 nm (Fig. 32.1G), and is surrounded by a matrix protein and a closely apposed outer envelope derived from the host-cell plasma membrane. The virion surface is studded with membrane-anchored peplomers projecting ~10 nm from the surface (Figs. 32.1E and 32.1F). Virions can often appear ragged or “moth-eaten” (Fig. 32.1D) (especially late in the infection). The density of virions has been determined to be 1.14 g/mL by centrifugation in a potassium tartrate gradient.

GENOME STRUCTURE AND ORGANIZATION

The single-stranded, negative-sense RNA molecule that makes up a filovirus genome constitutes ~1% of the virion mass.³³⁰ The genomes of filoviruses are very similar in their organization, which generally conform to those of paramyxoviruses and rhabdoviruses, but their complexity is more akin to those of paramyxoviruses. Filovirus genomes are approximately 19,000 bases in length, making them the largest in the order *Mono-negavirales* (Fig. 32.2), and contain seven sequentially arranged genes in the order nucleoprotein (NP)–virion protein (VP) 35–VP40—glycoprotein (GP)—VP30–VP24—polymerase (L). Genes are delineated by conserved transcriptional signals, and begin close to the 3′ end of the genomic sequence with a start site and end with a stop (polyadenylation) site. For rhabdo- and paramyxoviruses, genes are usually separated by short intergenic regions of one or more nucleotides, which are also seen in filovirus genomes. An unusual feature of all filovirus genomes is the presence of gene overlaps, which have been identified in the genomes of some paramyxo- and rhabdoviruses, but do not resemble those of filoviruses. As seen in Figure 32.2, the stop site of an upstream (3′) gene overlaps the start of the downstream gene, and overlapping sequences are limited to the conserved transcriptional signals and are centered on a 3′-UAAUU pentanucleotide sequence common to start and stop sites.^{49,98,103,157,192,347} There is one overlap in the MARV genome (VP30–VP24), but the characterized EBOV genomes contain at least two overlaps (VP35–VP40, GP–VP30, and VP24–L; REBOV lacks the GP–VP30 overlap). Intergenic regions of filovirus genomes are generally short, although all genomes have a single lengthy sequence (>120 bases) separating the GP and VP30 genes of MARV and the VP30 and VP24 genes of EBOV. The positioning of the MARV overlap and a

long intergenic region (that precedes the VP30 gene) appears to be shifted one gene (in the 3′ direction) with respect to the genome of the EBOV. The significance of this arrangement and how it may have been generated are unknown. The extragenic sequences at the 3′ end of all filovirus genomes (leader) are short, ranging in length from 50 to 70 bases, while the length of the 5′ end (trailer) sequences are variable. The extreme 3′ and 5′ ends of the filovirus genomes are conserved, show a high degree of complementarity, and potentially form stem-loop structures.^{67,290,352,414} Filovirus trailer sequences are more variable in length, the longest being that of ZEBOV (677 bases), followed by BEBOV (475 bases), ICEBOV (474 bases), SEBOV (381 bases), MARV (76–95 bases), and REBOV (25 bases).

The evolutionary profile of the family *Filoviridae* (Fig. 32.3) indicates that EBOV and MARV represent distinct filovirus lineages, the five species of EBOV also represent distinct lineages, and there is an extraordinary level of genetic stasis within the lineages of EBOV. Nucleotide and amino acid differences between MARV and EBOV are both approximately 55%, whereas EBOV species show 32% to 41% differences in nucleotide and amino acid sequences.^{98,353,400} These same levels of sequence variation are also seen when other genes are compared. Within species of EBOV, however, there is a remarkable degree of genetic stability, indicating that these viruses have most likely reached a high degree of fitness as they have adapted to their respective niches. MARV isolates have not shown the degree of variation seen among EBOV species, but two lineages of MARV have been described that are genetically distinct by more than 20% genetic diversity.^{98,354,400}

VIRUS PROTEINS

Filovirus structural proteins can be subdivided into two categories, those that form the NC and those that are associated with the envelope (Fig. 32.2 and Table 32.2). The NC-associated proteins are involved in the transcription and replication of the genome, whereas the envelope-associated proteins have a role in either the assembly of the virion or virus entry. Shown in Figure 32.4 are the characteristic migration patterns of purified filovirus proteins separated by SDS-PAGE.

Nucleoproteins

The NP and VP30 proteins of filoviruses are the major and minor nucleoproteins, respectively, are phosphorylated, and interact strongly with the genomic RNA molecule to form the viral NC (along with VP35 and L).^{27,261} Expression of recombinant NP alone in mammalian cells results in the formation of inclusions and nonspecific association with cellular RNA to form helical structures.^{306,435} Analysis of NP amino acid sequences has identified a conserved, hydrophobic N-terminal half that contains all the cysteine residues, and a divergent, hydrophilic C-terminal half that contains most of the proline residues and is extremely acidic.^{348,349} The N-terminal 450 amino acids of the ZEBOV NP have been linked with self-assembly of NP into tube-like structures that may function as a platform for NC formation.⁴³⁵ Predicted mass values for NP molecules are approximately 20 kd smaller than estimated sizes derived from SDS-PAGE migration, possibly as a result of reduced binding of SDS molecules to the negatively charged NP. This hypothesis is supported by a study of recombinant

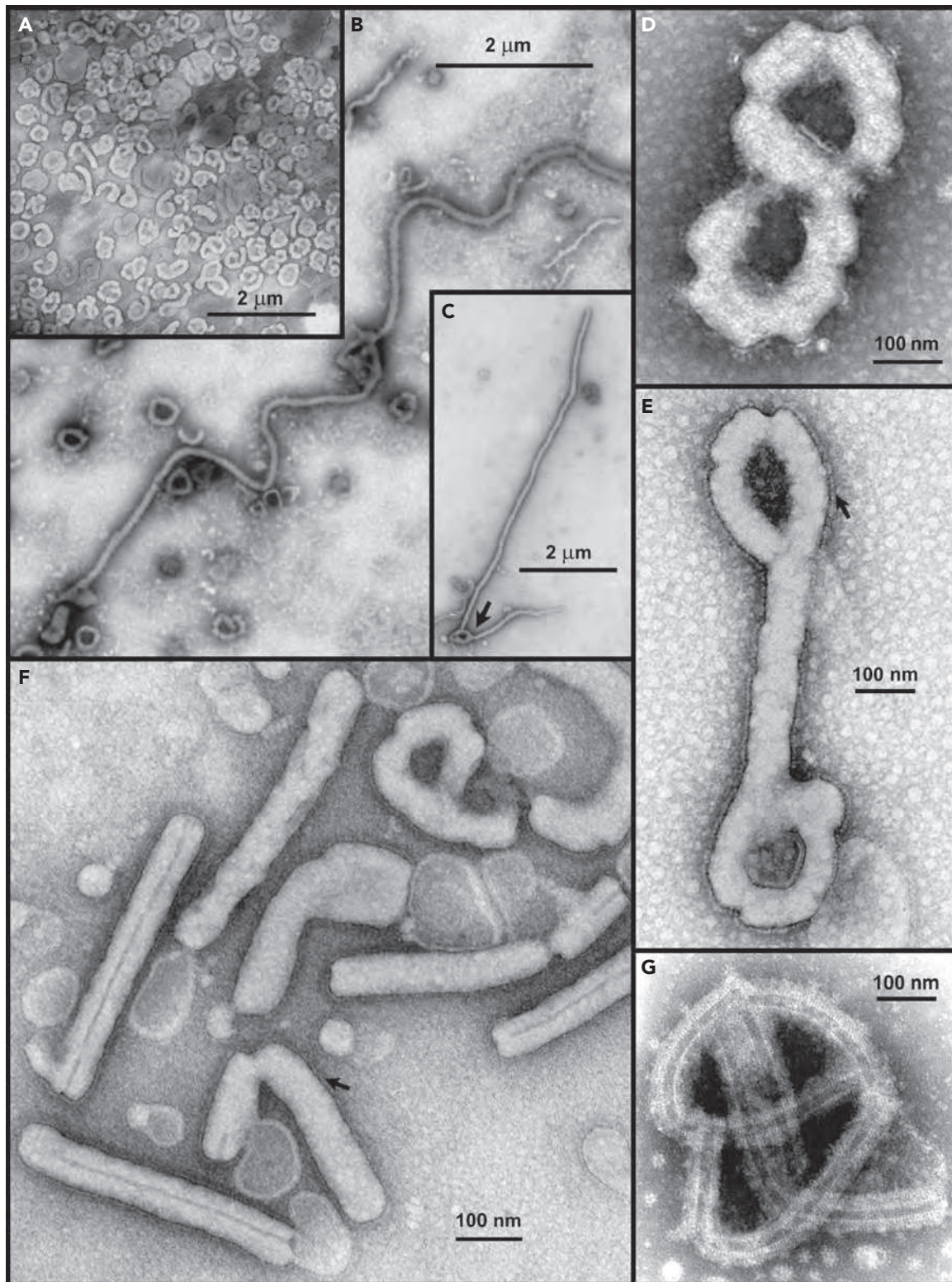


FIGURE 32.1. Transmission electron microscopy (negative stains) of filovirus virions (A–F) and nucleocapsids (G) derived from the culture medium of infected Vero E6 cells. Shown are low magnifications of MARV (strain Angola 2005) (A), SEBOV (strain Yambio 2004) (B), and MARV (strain Yambio 2005) (C), and higher magnification images of ZEBOV (strain Mayinga 1976) (D, E), MARV (strain Ravn 1987) (F), and SEBOV (strain Yambio 2004) (G). Arrows indicate a branch point in a filamentous particle (C) and peplomers on the surface of virions (E, F). (Courtesy of A. Sanchez and C. Humphrey, Centers for Disease Control and Prevention, Atlanta, GA.)

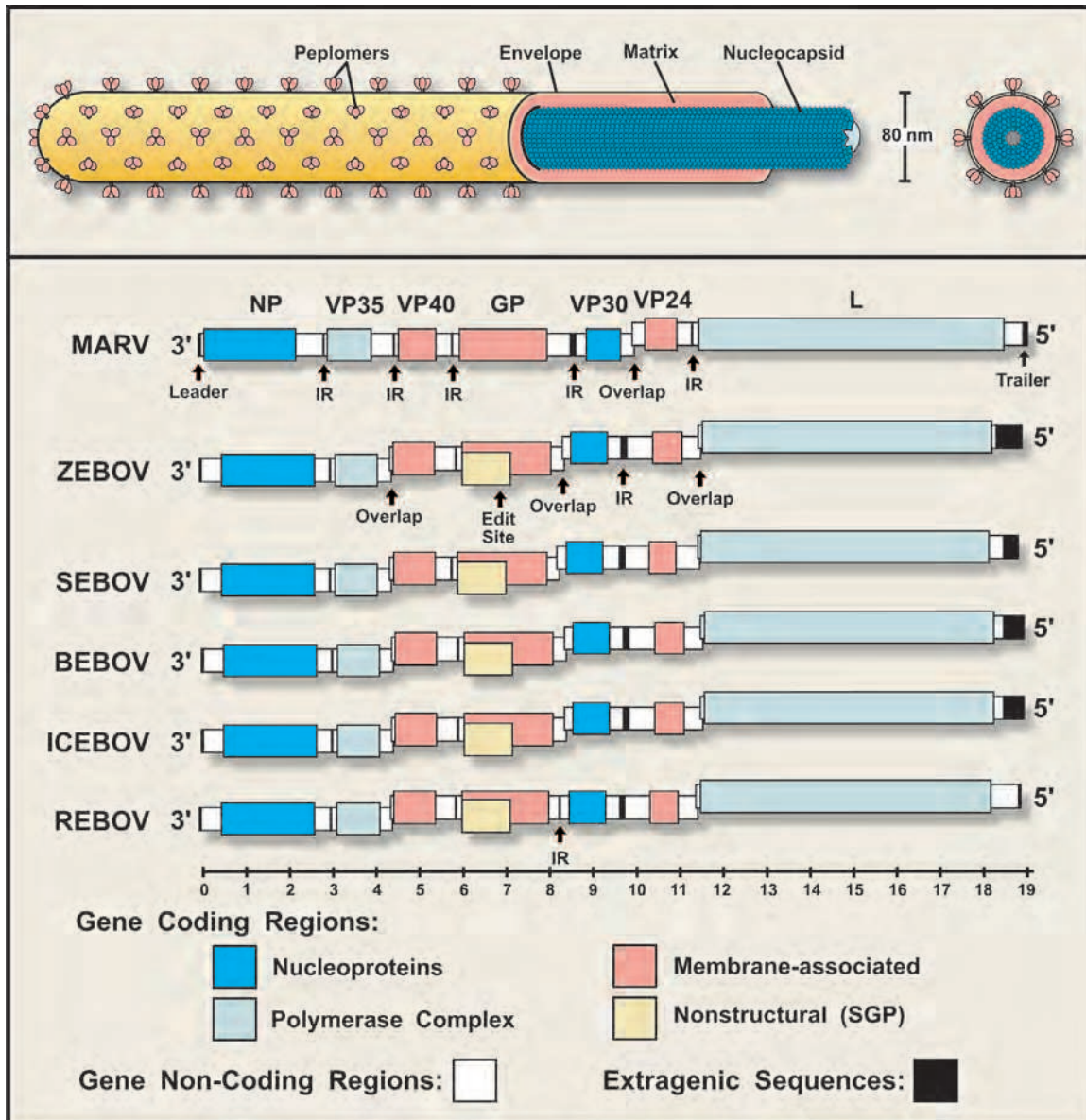


FIGURE 32.2. Schematic representation of a filovirus particle (*top*) and the organization of filovirus genomes (*bottom*).

ZEBOV NP³⁶³ that mapped this property to two C-terminal domains (aa 439–492 and 589–739). In the central region of the NPs of filoviruses is a highly conserved region that shows some homology with nucleoprotein sequences of paramyxoviruses, and to a lesser extent rhabdoviruses, and likely has a similar structure and function.^{183,349}

The VP30 protein of ZEBOV is also capable of binding RNA, particularly a stem-loop structure located near the leader sequence; this property mapped to residues 26 to 40 that is arginine rich.²¹⁴ This region may have an additional role of binding to the acidic C-terminal half of NP. VP30 contains a zinc-finger motif ~70 to 80 residues from the N-terminus that is highly conserved in filoviruses (consensus = CX₈CX₄CX₃H X₂D/E),²⁸⁶ and RNA binding activity is increased by Zn²⁺.²¹⁴ Immediately C-terminal to this sequence (separated by six residues) is a conserved tetraleucine sequence linked to co-trans-

lational homo-oligomerization of VP30.¹⁶⁸ Additional studies have shown that the C-terminal half of VP30 also contains a homo-oligomerization domain and that hexamerization occurs via an N-terminal domain.¹⁶⁹ A functional study of recombinant ZEBOV VP30 has revealed that it behaves as a transcription activator^{169,440} regulated by its phosphorylation state,²⁸⁷ but this property appears to be absent from the VP30 of MARV.²⁸⁹ The ZEBOV VP30 also interacts with L; a role of bridging NP and L in the NC complex has been postulated.¹⁵⁴ Recently, the ZEBOV VP30 (along with VP35 and VP40) has been implicated in suppressing antiviral immunity through its antagonistic effect on the host cellular RNA interference (RNAi) pathway.⁹¹

Polymerase Complex Proteins

The L and VP35 proteins form the polymerase complex, which transcribes and replicates the filovirus genome. The

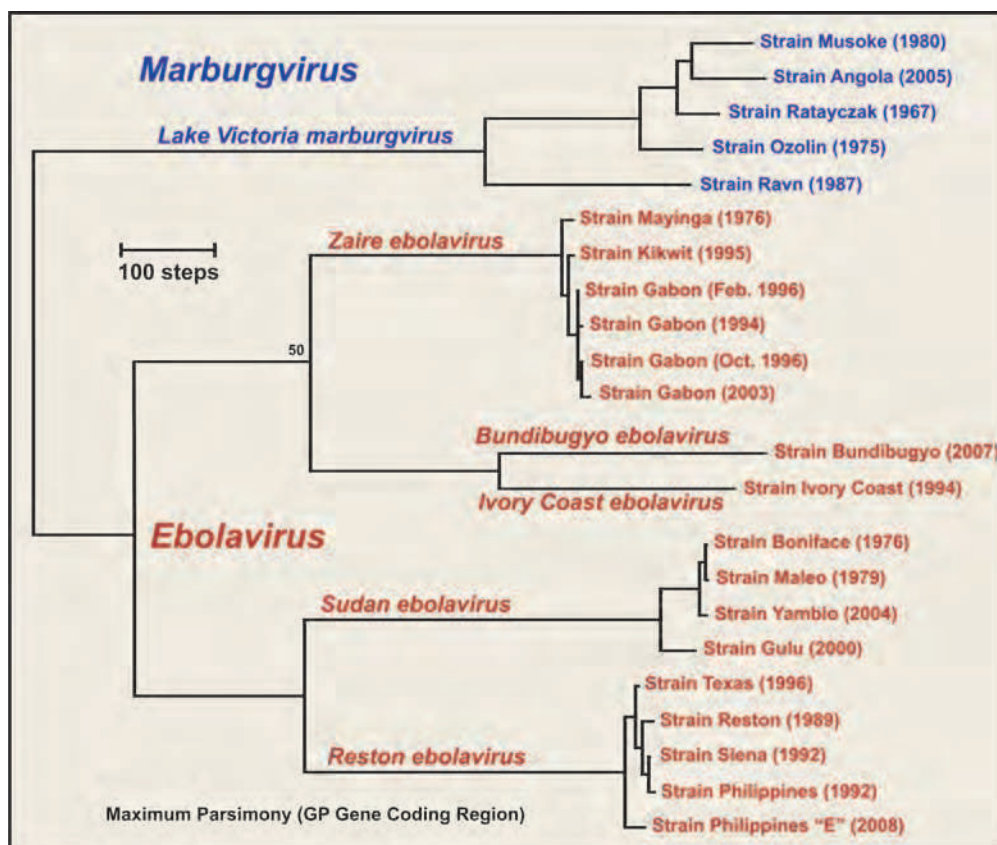


FIGURE 32.3. Phylogeny of the family Filoviridae. Nucleotide sequences for the GP gene coding region (ORF) of filovirus isolates were aligned using Clustal X version 2.0, and phylogenetic (maximum parsimony) analysis was performed using the MEGA5 computer program. Sequences were obtained from GenBank. The tree represents the most parsimonious tree, and confidence values at branch points were generated from 1,000 bootstrap replicates.

TABLE 32.2 Filovirus Genes and the Functions and Relative Molecular Weights of Their Gene Products

Gene order	Gene	Protein function	MW (kd) ^a
1	Nucleoprotein (NP)	Major nucleoprotein; RNA encapsidation	90–104
2	Virion protein (VP) 35	Polymerase complex cofactor; interferon antagonist	35
3	VP40	Matrix protein; virion assembly and budding; interferon antagonist ^e	35–40
4	Glycoprotein (GP)	Virus entry (surface peplomer); receptor binding and membrane fusion	150–170 ^b
	Soluble glycoprotein (sGP)	unknown	50–55 ^c
	Small soluble glycoprotein (ssGP)	unknown	50–55 ^c
5	VP30	Minor nucleoprotein; RNA encapsidation and transcription activation	27–30
6	VP24	Minor matrix protein; virion assembly; interferon antagonist ^d	24–25
7	Polymerase (L)	RNA-dependent RNA polymerase; enzymatic component of polymerase complex	~270

^aMolecular weights (MW) are approximated and based on SDS-PAGE migration.

^bMW is for the monomeric heterodimer.

^cExpressed only by EBOV; MW is for the monomeric form.

^dOnly shown for EBOV.

^eOnly shown for MARV.

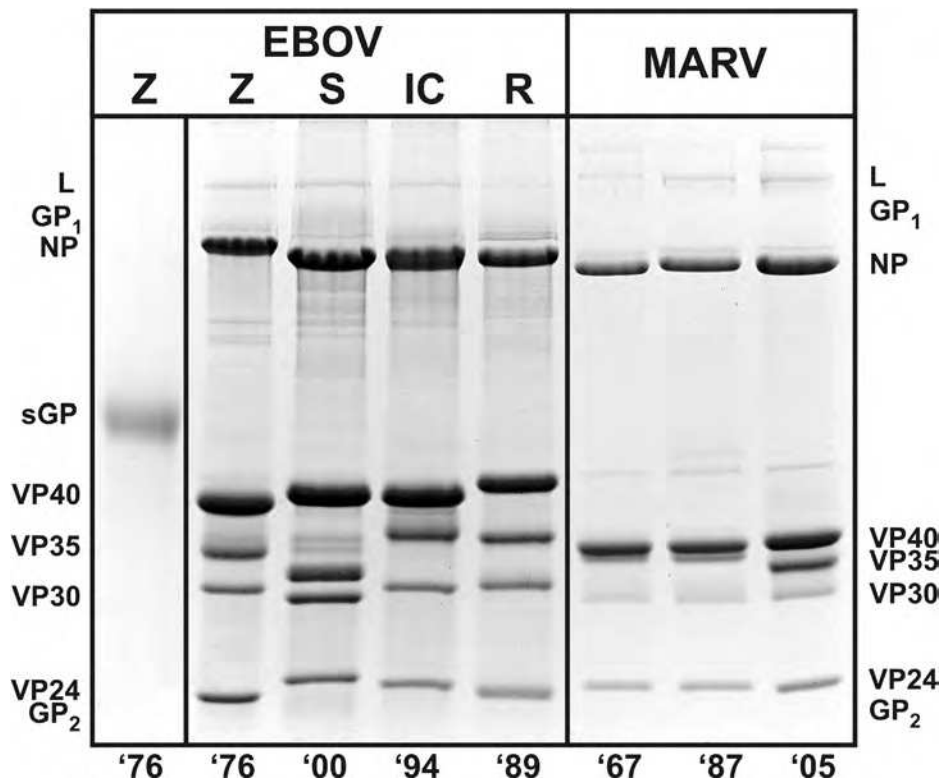


FIGURE 32.4. Migration patterns of filovirus proteins separated by SDS-PAGE on a 10% acrylamide gel (stained with Coomassie blue). The far left lane contains highly purified ZEBOV sGP, while the other lanes contain proteins from purified virion preparations from various filovirus isolates. Approximate migration locations for EBOV and MARV proteins are shown at the left and right margins, respectively, and year of isolate/outbreak is shown along the bottom.

L protein provides the RNA-dependent RNA polymerase activity of the complex; motifs linked to RNA (template) binding, phosphodiester bonding (catalytic site), and a ribonucleotide triphosphate binding have been described.^{290,414} Conserved regions or “blocks” of sequences have been identified in filovirus L proteins, but there are also areas of divergence (particularly within the C-terminal quarter of the molecule) and sequences that are unique to the larger MARV L protein.

VP35 has an essential role as a cofactor that affects the mode of RNA synthesis (transcription or replication), similar to that of the P proteins of other NNS viruses^{290,292,321,415}; VP35 acts as a link between L and NP.²⁸ Basic residues in the C-terminal region have been identified as critical to viral RNA synthesis.³²¹ The ZEBOV VP35 has also been shown to be a virulence factor through its inhibitory effect on the host innate immune system.²⁵⁵ VP35 has an antagonistic effect on the interferon type I pathway (see Host Immune Response section) by binding virus-generated double-stranded RNA (dsRNA) and by directly interfering with pathway kinases^{19,20,21,54,90,170,171,253,322,323}; as noted previously, it also acts as a suppressor of RNA silencing.^{91,162} A C-terminal domain in VP35 confers its dsRNA binding property¹⁷¹ and X-ray crystallography studies of ZEBOV and REBOV VP35 have shown that it forms an asymmetrical dimer, the units of which separately bind and “end-cap” dsRNA^{226,253,254,255} (Fig. 32.5). Additionally, the ZEBOV VP35 interacts with the 8kDa dynein light chain, a component of the microtubule transport system,²³⁷ which may have an effect on the virus life cycle.

Structural (Surface) Glycoprotein

As noted earlier, the surface of the filovirus virion is covered with peplomers (spike structures) composed of the structural

glycoprotein, GP, and is anchored in the envelope in a type I orientation (Figs. 32.1 and 32.2). GP has been the most studied of the filovirus proteins, due in large part to its role in virus entry, its influence on pathogenesis, its antigenicity, and its attractiveness as an immunogen in vaccine development. The GP of EBOV species is encoded in two reading frames and expressed through transcriptional editing (^{353,410}; see Stages of Replication, next section), while the GP of MARV is encoded in a single ORF.^{49,103} Despite this difference, the features of their amino acid sequences are very similar. A schematic representation of the EBOV GP is depicted in Figure 32.6 and illustrates the general characteristics of a filovirus GP molecule.

The glycoproteins of filoviruses are translocated into the endoplasmic reticulum (ER) by a signal sequence at the N-terminus of GP₀ (precursor molecule) and are anchored by a membrane-spanning sequence at the C-terminus (Fig. 32.6); the cytoplasmic tail is extremely short (3 residues for EBOV and 7 for MARV). As GP₀ is transported through the ER and Golgi apparatus, it is glycosylated with both N-linked glycans (hybrid and complex) and O-linked glycans.^{104,107,149,409} An extremely divergent, mucin-like region (rich in threonine, serine, and proline residues) is located in the middle of GP₀ and is heavily glycosylated; all O-linked glycans are located in this region. Analysis of the carbohydrate composition of GP has shown that MARV isolates lack terminal sialic acid when grown in Vero E6 or MA-104 cells, unlike the GP of EBOV species, which contain abundant $\alpha(2-6)$ and/or $\alpha(2-3)$ linked sialic acids.^{104,149} Differences in sialic acid addition may be caused by differences in targeting as they are directed though the *trans*-Golgi apparatus. In addition, no neuraminidase activity has been found with any filovirus. The MARV GP (Musoke strain) is also phosphorylated by Golgi protein

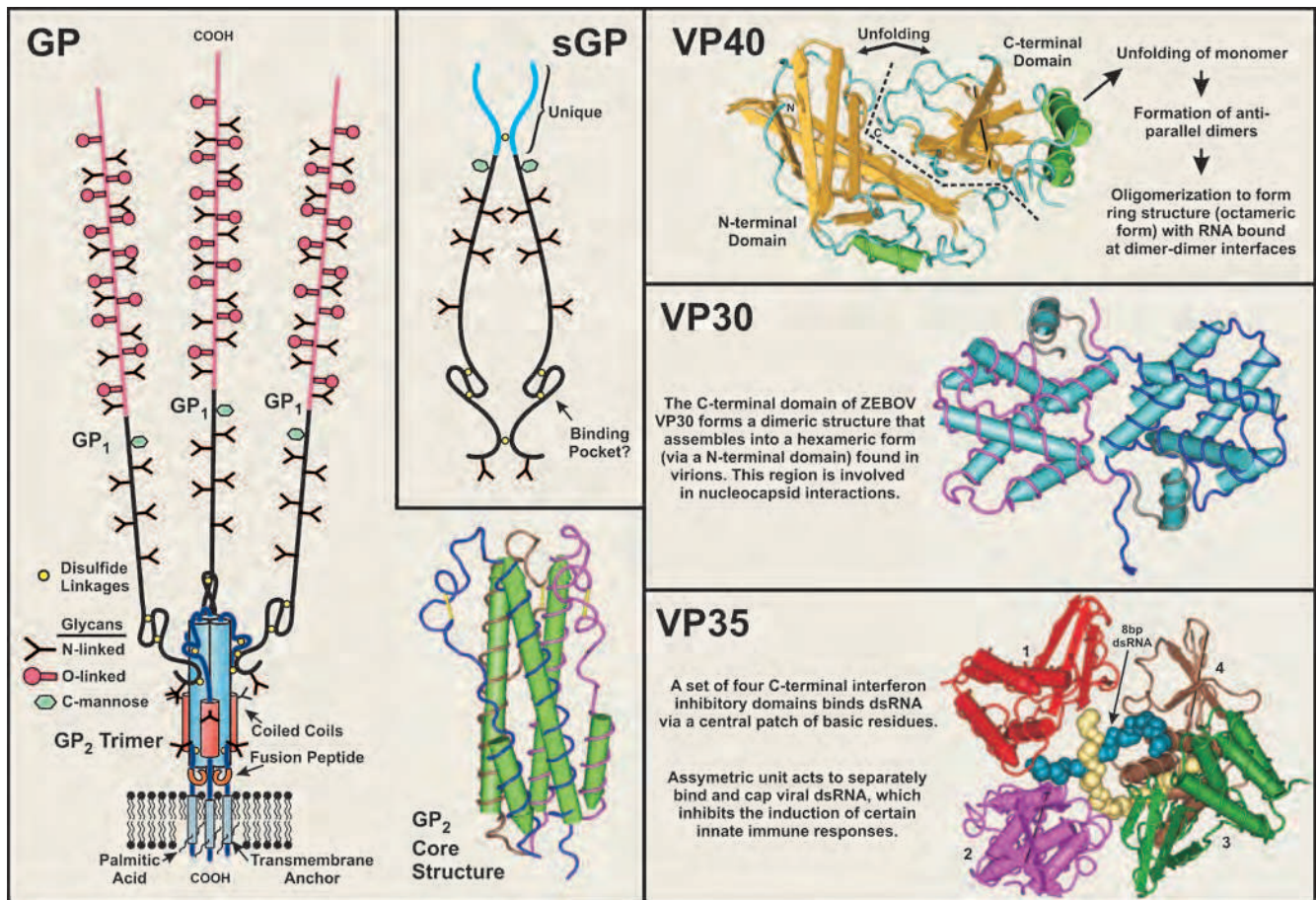


FIGURE 32.5. Structural features of the ZEBOV GP, sGP, VP40, VP30, and VP35 molecules. The left panel shows schematic structures for GP and sGP. The basic features of GP are essentially the same for all filoviruses, as are those of sGP for all EBOV species. Structural depictions of the GP₂ trimer and the C-termini of VP40, VP30 (dimer), and VP35 (four molecules bound to dsRNA) were generated from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank files (PDB ID = 2EBO, 1ES6, 2I8B, and 3L25, respectively; <http://www.rcsb.org/pdb>). Structures were rendered using Cn3D 4.1 (<http://www.ncbi.nlm.nih.gov/Structure/Cn3D/cn3d.shtml>)

kinases, putatively at serine residues near the center of GP₁ (²⁶⁰SSDDEDLATSGSGS²⁷³)³⁵⁷ the C-terminal set of 3 serines is conserved in MARV.³⁵⁴ The implications of this processing are unknown, but could influence the trafficking of MARV GP.

GP₀ is cleaved by furin, a subtilisin/kexin-like convertase localized in the *trans* Golgi, or a furin-like endoprotease at a site just C-terminal to a long, variable, mucin-like region.^{356,413,417} Cleavage leads to the formation of a GP_{1,2} heterodimer that is held together by a single disulfide bond formed between the most N-terminal cysteine of GP₁ (cysteine at position 53 in ZEBOV) and the fifth cysteine from the N-terminus GP₂ (predicted) (Fig. 32.6). The MARV cleavage site is located ~70 residues N-terminal to that of the EBOV site, and a second conserved furin/furin-like cleavage sequence is located immediately after the second cysteine from the N-terminus of GP₂ (just within the first heptad repeat), but there is no evidence that this sequence is cleaved. It should be noted that cleavage of GP₀ to form the GP_{1,2} heterodimer is not required for virus entry in tissue culture, as mutation of the furin cleavage site does not prevent entry by pseudotyped virus,^{199,448} nor does it significantly affect infection and subsequent spread by a recom-

binant ZEBOV or virulence in nonhuman primates.^{301,302} Nevertheless, cleavage may be required for efficient maintenance in the natural host.

Peplomers are composed of trimerized GP_{1,2} heterodimers, and X-ray crystallography studies of a recombinant-expressed portion of GP₂ have shown that trimerization occurs when heptad repeat sequences form coiled coils in a rod-shaped structure (Figs. 32.5 and 32.6) similar to those of the HA₂ of influenza, the transmembrane (TM) of retroviruses, and SNAREs.^{269,442,443} Two conserved cysteine residues at the C-terminal end of the membrane spanning sequence are palmitoylated,^{119,200} which could stabilize the anchorage of the peplomer and may influence virus entry, although GP pseudotyping studies suggests that these cysteines are not essential for infectivity.^{200,209} The TM region of GP₂ has been linked to increased permeability of infected cells with the 667ALF669 sequence particularly important.¹⁶⁵ A fusion peptide is internally positioned near the N-terminus of GP₂ and is flanked by two conserved cysteines that are predicted to form a disulfide bond; this arrangement is very similar to the TM of Rous sarcoma virus and avian leukosis virus.^{120,209} The fusion peptide of ZEBOV inserts efficiently

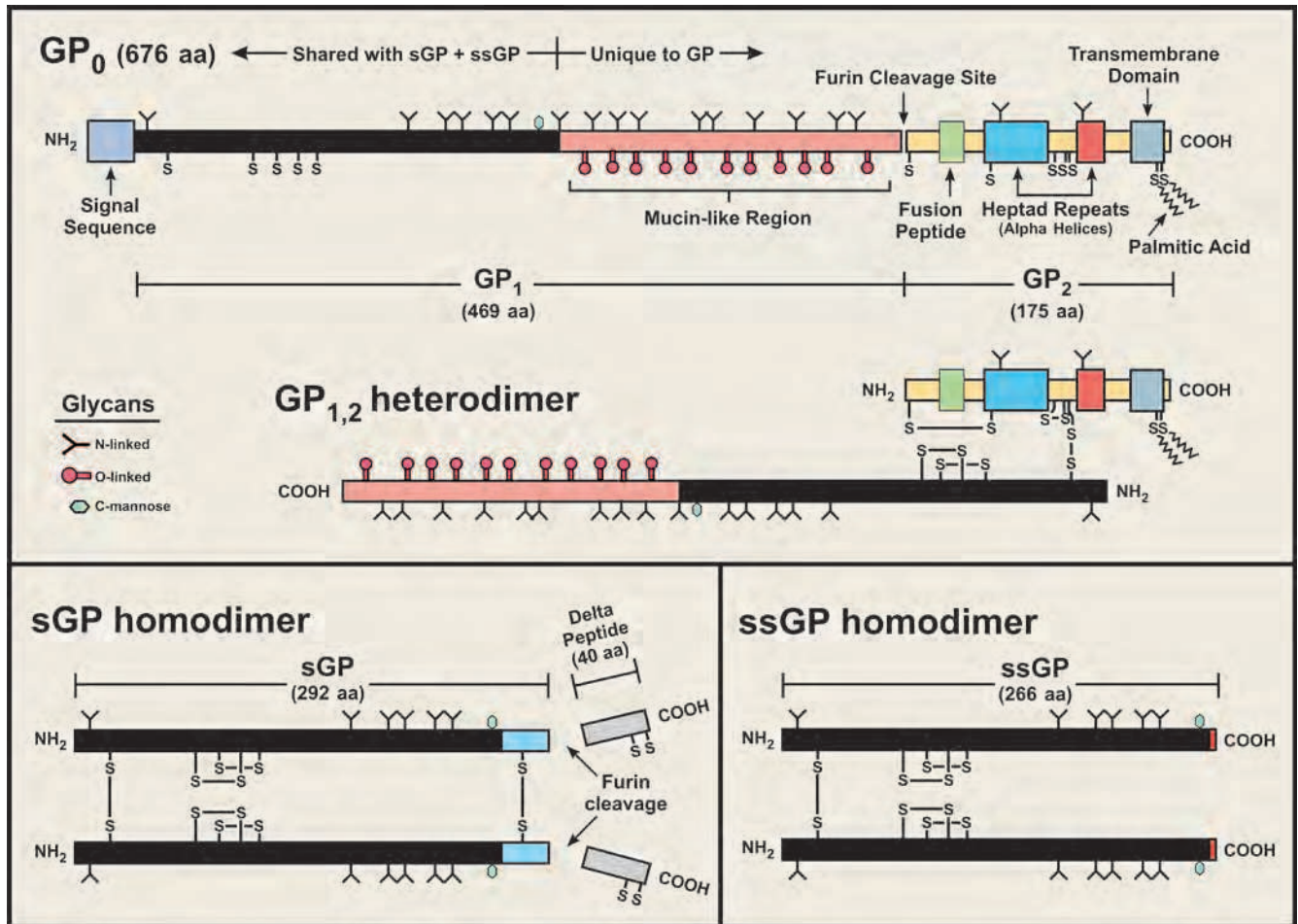


FIGURE 32.6. Features of the GP (top), sGP (bottom left) and ssGP (bottom right) proteins of ZEBOV. N-linked glycosylation sites (Y) and cysteine residues (S) are identified along the sequences. The basic features of GP are essentially the same for all filoviruses, as are those of sGP and ssGP for all EBOV species.

into synthetic membranes containing phosphatidylinositol and promotes fusion of lipid vesicles.^{1,200,341,375}

Sequence analysis of the GP gene coding regions indicates that the N-terminal end (~200 residues) of GP₁ and most of GP₂ are conserved and have regions of increased hydrophobicity. The N-terminal region of EBOV GP₁ contains conserved cysteine residues that are closely positioned and form intramolecular disulfide bonds (C108–C135, C121–C147), which are also found in the sGP molecule^{15,209,418} and likely form an important structural feature. The MARV GP has conserved cysteines that correspond to the C108–C135 linkage of EBOV, but the other two closely positioned cysteines appear to be shifted towards the center of the molecule (and likely form a disulfide bridge). The abundant O-glycans of the mucin-like region confers an extended structure and its heavy glycosylation makes it very hydrophilic. The mucin-like region is located at the C-terminus of GP₁, and was predicted to project away from the virion membrane (toward the aqueous environment) with the N-terminal end (linked to GP₂) contributing to the stalk structure of the peplomer (Fig. 32.5). X-ray crystallography of GP₁ has verified this prediction.²⁴¹ In tissue culture, it has been shown that the ectodomain por-

tion of the ZEBOV peplomer is released from cells (separate from virions) through proteolytic cleavage by tumor necrosis factor α -converting enzyme (TACE; zinc-dependent metalloprotease) near the transmembrane anchor (residue D637 of ZEBOV).⁷⁸ GP can also be released into the medium as peplomers anchored in vesicles extruded from the plasma membrane.⁴¹⁶

Nonstructural Glycoproteins

The expression of a nonstructural soluble glycoprotein (sGP) as the primary product of the GP gene of EBOV is unusual and an important distinction from MARV.^{353,410} The N-terminal ~300 amino acids of sGP are identical to those of the structural GP, but the C-terminus is unique in sequence (Fig. 32.6). sGP is produced from a precursor molecule that is also cleaved by furin (or a furin-like endoprotease) near the C-terminus to release a short peptide that seems to contain exclusively O-linked glycans and has been named delta peptide.^{409,419} No biologic activity has been attributed to delta peptide. Biochemical and antigenic analyses of the ZEBOV sGP have shown that it is structurally distinct from GP^{15,356,418,419} and is secreted from infected cells as a homodimer that is likely formed in

the ER. Initial structural studies indicated an antiparallel orientation for sGP molecules in the dimer by disulfide bonding between cysteine residues C53 and C306.⁴¹⁸ However, subsequent MALDI-TOF MS analysis of sGP peptide fragments have unequivocally demonstrated a parallel orientation for the homodimer, which is held together by disulfide bonds between the N-terminal (C53–C53′) and C-terminal (C306–C306′) cysteines that fix the orientation of the molecules.^{15,95} The intramolecular disulfide bonds are similar in topology and spacing to the fibronectin type II module (binding site for collagen and gelatin), and may form a binding pocket for an as-of-yet unidentified ligand. Biophysical characterization of ZEBOV sGP has also revealed that the tryptophan residue at position 288 is C-mannosylated,^{16,94} an unusual form of glycosylation, at the specific motif W-X-X-W (first W is C-mannosylated) that is in the shared N-terminal region and is conserved in all EBOV species. GP₁ is presumed to have this same type of glycosylation. It is possible that sGP could contribute to disease progression, because large amounts circulate through the blood of acutely infected humans,³⁵⁰ but there has been no evidence linking sGP to a role in pathogenesis.

Recently, another ZEBOV nonstructural glycoprotein, termed small soluble glycoprotein (ssGP), has been identified and partially characterized,²⁸¹ and outwardly appears to be a truncated version of sGP. As with GP, ssGP is expressed through transcriptional editing. This glycoprotein is expressed at a low level (~1/20 that of sGP+GP) and has structural properties similar to that of sGP, in that it has N-linked glycans (no O-linked) and exists as a homodimer (disulfide bond between cysteines at position 53). As with sGP, the function of ssGP has yet to be adequately defined, but ssGP appears to lack an anti-inflammatory property reported for sGP.²⁸¹

Matrix Proteins

The VP40 protein functions as the matrix protein and the VP24 protein may have a secondary/minor matrix protein function.¹⁶⁴ VP40 is the most abundant protein in the virion, while only small amounts of VP24 are incorporated into virus particles (Fig. 32.4). Both proteins have an affinity for membranes and are associated with the virion envelope (no membrane-spanning regions),^{208,340,362} and are easily released from virions by nonionic detergents under low-salt conditions.^{85,225} VP40 is critical to the budding process, as it initiates and drives the envelopment of the NC by the plasma membrane.²⁰⁷ In addition, it has been reported that both VP40 and VP24 of EBOV contribute to regulation of genome replication and transcription.¹⁸⁰

VP24 has a decidedly hydrophobic profile, and a study of a recombinant-expressed form (ZEBOV) indicates that it has an affinity for the plasma membrane and perinuclear region of infected cells.¹⁶⁴ VP24 is capable of forming homotetramers, which is influenced by pH and divalent cation changes. Because disulfide-bonded oligomers of VP24 are not evident in the virion,³⁵² the formation of multimers is likely due to ionic and/or hydrophobic interactions. The precise role of VP24 in the replication of filoviruses is still unclear and direct interactions with other virus proteins have not been described, but a role in formation of nucleocapsid-like structures has been described.¹⁶⁴ The VP24 of EBOV has also been reported to antagonize the interferon type I signalling pathway, similar to that of VP35 (see Host Immune Response section).²⁰

STAGES OF REPLICATION

Recent studies have provided valuable insights into filovirus entry into host cells and the mechanisms leading to the production and release of infectious progeny. Although very much incomplete, the details of this complicated series of molecular events are slowly being revealed. The current understanding of this process is illustrated in Figure 32.7 and described in the following sections.

Mechanism of Attachment

In filovirus infections a variety of host organs and cell types are involved; this broad tropism is related in large part to the binding properties of the peplomers that populate the surface of the virion. Because GP is the only filovirus protein involved in initiating infection, it has been intensely studied for its ability to bind cellular receptors. Much of the work directed at receptor binding (and subsequent entry processes) has utilized recombinant pseudotyping systems, which provide a safer and easier approach to characterizing these properties and events.^{56,58,200,390,447,448,467} However, these results need to be verified using infectious filoviruses, as interactions of GP with VP24 and/or VP40 need to be considered along with other properties that may be peculiar to filovirus virions.

Identification of attachment molecules (receptors?) involved in filovirus entry is complicated by the ability of GP trimers to specifically or nonspecifically bind a variety of host-cell surface molecules. The asialoglycoprotein receptor found on hepatocytes binds MARV,²⁹ yet EBOV also infects hepatocytes despite its GP having sialylated glycans. The β 1 group of integrins has been suggested to interact with ZEBOV GP on the cell surface and during intracellular trafficking (when co-expressed),³⁹³ although cells that express this molecule (such as Jurkat cells) are not easily infectible. The folate receptor alpha has been implicated as a cofactor in filovirus entry,⁵⁶ but virus entry independent of this molecule has been shown to take place.^{366,369} C-type lectins (DC-SIGN and DC-SIGNR; bind oligosaccharide ligands)—present on certain forms of dendritic cells, macrophages, and endothelial cells—are also capable of binding filovirus peplomers,^{3,12,279,365} especially when N-linked glycans contain high mannose carbohydrates.^{213,258} However, there are indications that DC-SIGN may not act as an EBOV receptor, but instead acts to promote attachment and other host factors are involved in entry.²⁸⁰ One study has shown that macrophages are more susceptible to virus entry by ZEBOV GP-pseudotyped HIV-1 particles than are monocytes, and that HUVEC cultures pretreated with TNF- α showed increased entry over untreated cells.⁴⁶⁷ These results imply that changes in cellular gene expression can alter the makeup of surface attachment molecules (and cofactors?). Antibody binding to peplomers might also enhance infectivity through its interaction with the Fc portion of the complement protein C1q bound to the surface of host cells.³⁹¹ Very recently, T-cell Ig and mucin domain 1 (TIM-1) has been described as a potential binding protein for EBOV GP and enhances virus entry into cells.²³²

Results of site-directed mutagenesis studies of ZEBOV GP have shown that individual glycosylation sites are not critical to virus entry,^{209,270} and deletion of the entire mucin-like region can actually increase virus entry *in vitro*.²⁰⁹ A role in receptor binding or increased binding has yet to be attributed to the mucin-like region. Further deletion of the GP₁ C-terminal

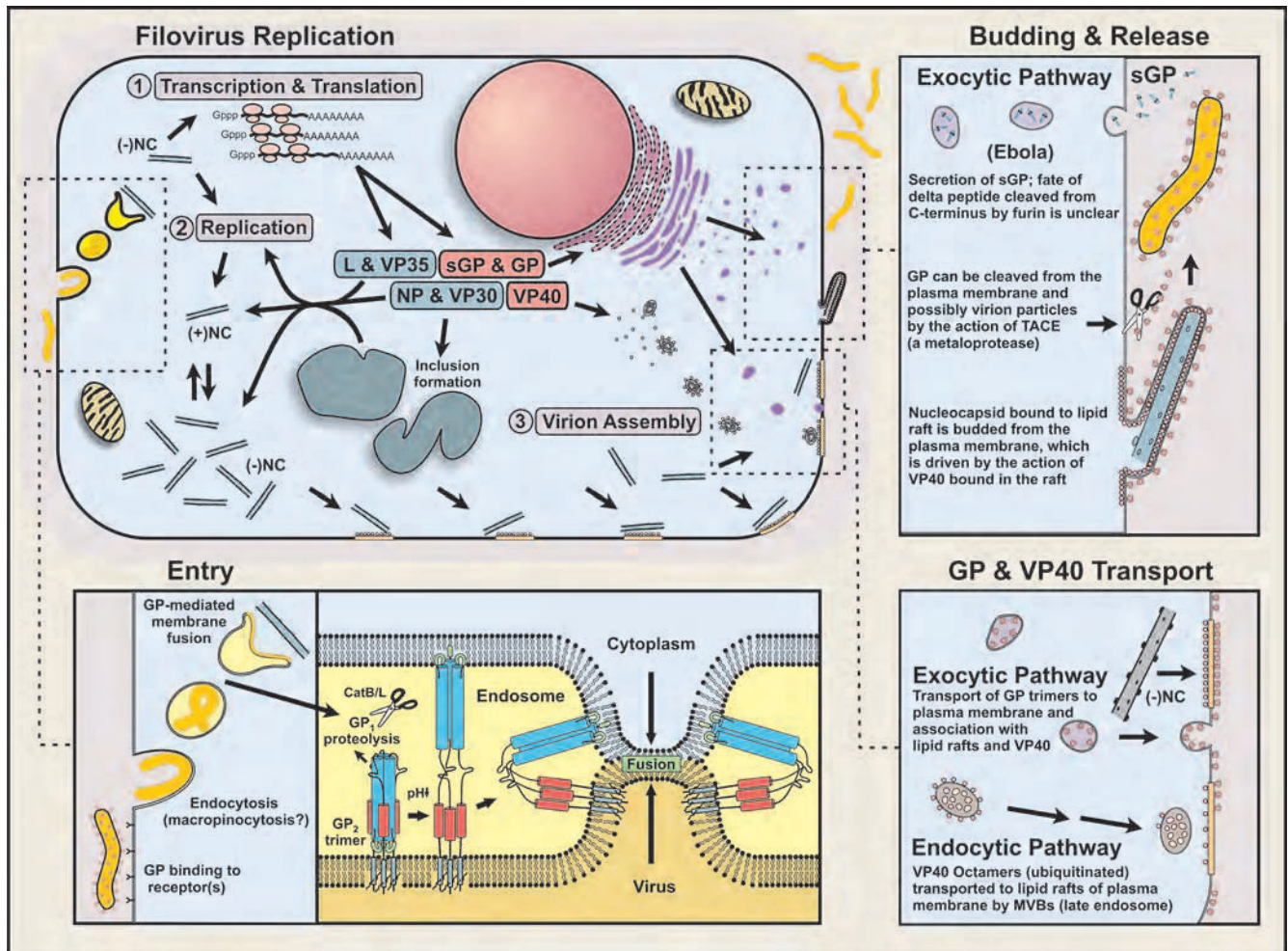


FIGURE 32.7. Schematic view of the processes associated with filovirus entry, synthesis of viral molecules, and the production of infectious virions in a susceptible eukaryotic cell.

sequences (past the mucin-like region), together with mutagenesis of N-terminal residues, has localized the entry function to ~150 residues at the N-terminus (residues 33–185).²⁷⁰ It should be noted that this region also contains the same intramolecular disulfide bonds as sGP, which potentially forms a binding pocket that is involved in cell attachment and/or receptor binding. If this is the case, then the corresponding region of MARV GP₁ would likely have distinctive binding characteristics due to a differing disulfide bonding pattern. In contrast, another study identified approximately the same region as the potential receptor binding site for both viruses, indicating that EBOV and MARV utilize a common receptor.²³⁹

Mechanism of Entry and Intracellular Trafficking

Following attachment, virions are presumed to enter the cell by a process of endocytosis, acidification of the endocytic vesicle, and fusion of virus and host membranes resulting in the release of the NC into the cytoplasm. ZEBOV GP-mediated entry and fusion are affected by the treatment of host cells with agents that disrupt microtubules or inhibit the function

of microfilaments.⁴⁶⁷ These cytoskeletal components are key to clathrin-dependent and caveolae-mediated internalization, and support the theory that filoviruses enter the cell through endocytosis. However, studies examining the type of endocytic pathway utilized by filoviruses are conflicting. One study has demonstrated that disruption of the caveola vesicular system (via cholesterol binding compounds) inhibited ZEBOV and MARV entry and that filovirus GP-pseudotyped virus co-localized with the caveolin-1 (cholesterol binding protein) marker.⁸⁸ However, cells lacking caveolae are infectible and co-expression of folate receptor alpha and caveolin-1 in a T-cell line did not increase infectivity.³⁶⁶ Clathrin-mediated endocytosis and GP-dependent macropinocytosis or a macropinocytosis-like mechanism for EBOV internalization and an involvement of lipid rafts.^{2,298,344} It should be noted that inhibitors of macropinocytosis (amiloride) and the lipid raft-caveolae endocytosis pathway did not significantly affect ZEBOV entry.³⁴⁵ Latest, it was reported that EBOV entry was dependent on Niemann-Pick C1 (NPC1), a protein known to function in cholesterol transport.⁵³ It thus appears that the entry of ZEBOV (and possibly other filoviruses) occurs through multiple routes.

A filovirus virion internalized in a vesicle at the plasma membrane traffics through the endosomal pathway, and at some point in time the NC is released into the cytoplasm by GP₂-mediated fusion of the virus envelope and endosomal membrane. Membrane fusion is dependent on endosomal acidification,^{392,447} and endosomal proteolysis of the GP₁ subunit peplomer by the cysteine proteases CatL and CatB (active in acidic pH environments) can enhance ZEBOV entry.^{44,59,222,345} CatL removes the glycan cap and mucin-like domain, exposing core residues of a recombinant peplomer and increasing infectivity.^{182,222} Removal of GP₁ is believed to set off a conformational change in the GP₂ trimer that triggers the deployment of the fusion machinery, resulting in the insertion of the GP₂ fusion peptides into the endosomal membrane. This event would link and draw viral and host membranes together to induce fusion and the release of the NC into the cytoplasm.⁴³⁶ The minimum number of peplomers needed to induce fusion has not been determined.

Transcription and Translation

Following filovirus entry, negative-strand RNA genetics dictates that transcription is the first (and obligatory) viral process, similar to paramyxoviruses and rhabdoviruses. Once the nucleocapsid is released into the cytoplasm, polyadenylated monocistronic messenger RNAs (mRNAs) are synthesized from virus genes in a 3' to 5' direction (with polar attenuation) from the encapsidated genomic RNA template. Transcription seems to involve a process of starting and stopping as the polymerase complex encounters conserved start (initiation) and stop (termination/polyadenylation) sites along the genome. Synthesis of the "leader" sequence is postulated to occur, but intergenic sequences and the "trailer" sequence seem to be ignored, although this has not been shown experimentally. NP mRNA can be detected as early as 7 hours postinfection, and peaks around 18 hours.³⁴⁶ It is assumed that transcripts are capped at the 5' end (⁷MeG⁵-ppp⁵-R) by the L protein, as it contains conserved motifs associated with this enzymatic activity.¹⁰⁸

Analyses of defective interfering particles of ZEBOV have shown that promoters for initiating RNA synthesis are contained within 156 and 177 nucleotide regions of the genomic and antigenomic RNA 3' termini, respectively.⁵² Subsequent to these studies, it was shown that a bipartite promoter is located within the first 128 nucleotides of the 3' end of the ZEBOV genome.⁴⁴¹ One element is located at the extreme 3' end and the other within the nontranslated region of the NP gene. These elements are separated by a nonspecific sequence (nucleotides 56–80) that acts to provide proper spacing and also contains the NP gene transcription start site. This bipartite promoter is similar to that of various paramyxoviruses (i.e., Sendai virus) and obeys the "rule of 6".⁴⁴¹

Transcriptional start sites are 12 or 14 nucleotides in length and end in the consensus sequence 3'-CUUCUAAUU for EBOV and 3'-CUURUAAUU for MARV, while stop sites are 11 or 12 nucleotides long with the conserved sequence 3'-UAAUUC(U)_{5/6}. Polyadenylation is believed to occur by slippage or stuttering of the polymerase at the 5 to 6 uridines ending the stop site. A characteristic that is unique to the transcriptional signals of filoviruses is a common pentanucleotide sequence, 3'-UAAUU, present at the 5' end of start sites and at the 3' end of stop sites¹⁰³; the stop sites of ZEBOV and

REBOV polymerase genes deviate slightly from this sequence (3'-UAAUA).

The mechanism initiating transcription of a downstream (5') gene involved in a gene overlap and the consequences of this arrangement are unknown. Because the overlaps are short (18–21 nucleotides), the proximity of the polymerase may not affect recognition of the start site as it finishes polyadenylating the upstream gene. The function of these overlaps remains unclear, but attenuation of transcription does not appear to take place, as the transcription of the VP40 and VP30 genes of ZEBOV is substantial and expression of VP40 is very strong.

Filovirus mRNA molecules have characteristics that make them somewhat unique. They contain long noncoding regions at their 3' and/or 5' ends, which contribute to the increased length of the genome and may function in the stability of transcripts. In addition, the 5' ends of filovirus transcripts have the potential to form stable, stem-loop structures, which might affect their stability and ribosome binding capacity/translation.^{291,347,352}

The ZEBOV VP30 has a transcription activation property that is linked to an RNA secondary structure formed at the 5' end of the NP gene transcript as it is synthesized^{168,169,286}; the presence of VP30 is required for transcription of downstream genes. This property is impaired by phosphorylation at six serines and one threonine at the N-terminus, and restored by the action of cellular phosphatases.²⁸⁶ Because ZEBOV VP30 in the virion is at least partly phosphorylated,⁸⁵ the action of phosphatases on the NC may be required before transcription proceeds efficiently. Thus, the phosphorylation state of VP30 may be a critical component in regulating EBOV RNA synthesis; a corresponding mechanism has not been shown for MARV.

The organization and transcription of the GP genes of EBOV are unusual and provide an important distinction between MARV and EBOV. The MARV GP gene encodes a single product, GP, in a conventional open reading frame (ORF), whereas all EBOV species encode their GP in two ORFs (-0 and -1 frames). Expression of the EBOV GP requires a transcriptional editing event^{281,353,410} comparable to the editing described for the phosphoprotein gene of certain paramyxoviruses. Translation of the unedited transcript of the EBOV GP gene results in the production of sGP, a smaller, nonstructural, secreted glycoprotein, the primary gene product (Figs. 32.4 and 32.6). The transcriptional editing event that leads to GP expression occurs at a series of seven uridines on the genomic RNA template and results in the insertion of an additional adenosine, which connects the GP open coding frames; approximately 20% to 25% of the transcripts are edited. The mechanism of insertion most likely evolved out of the polymerase's ability to polyadenylate by stuttering on a poly(U) template. However, insertion of a single nucleotide at the editing site appears to occur with a high degree of fidelity, but insertion of two adenines can occur (in ~5% of GP gene transcripts), which leads to the synthesis of low levels of ssGP.²⁸¹ The editing of EBOV GP gene transcripts is the only example of a virus glycoprotein that is expressed through this type of mechanism. Sequence analysis of the GP genes of MARV isolates indicates that a nucleotide sequence that corresponds to the editing region of EBOV GP genes is totally absent.^{49,103,356,445} The difference in filovirus GP gene

organization provides important evidence pointing to a divergent evolution for EBOV and MARV.

Replication of Genomic Nucleic Acid

In addition to transcription, the promoter at the 3' end of the genomic RNA also drives the synthesis of full-length complementary/antigenomic RNA from the encapsidated template. As with other NNS RNA viruses, the ends of the genome have a high degree of sequence complementarity,^{67,352} and stem-loop structures are predicted to form at the 3' and 5' ends of genomic and antigenomic RNAs. These structures are believed to be essential to the replication of filoviruses.⁶⁷ The initial expression of virus genes leads to a buildup of viral proteins (especially NP), which is thought to trigger a switch from transcription to replication. This switch results in the synthesis and encapsidation of antigenomic RNA molecules, which in turn serve as templates for genomic RNA that is also rapidly encapsidated. Depletion of capsid proteins is believed to cause a return to transcription, and eventually an equilibrium is established wherein transcription and replication are concurrent processes. As replication progresses in the infected cell, NC particles containing genomic RNA accumulate and are directed to the plasma membrane for virion assembly.

The development of reverse genetics systems based on EBOV and MARV genetics has provided significant advances in understanding filovirus replication^{32,89,156,289,292,301,395,415} and has allowed the production/reconstitution of recombinant ZEBOV and MARV (Musoke strain) from plasmid DNA.^{89,301,415} For MARV, the NP, VP35, and L proteins are all that is required to transcribe and replicate minigenomes,²⁹⁸ but systems developed for ZEBOV also required VP30.^{301,395,415} When components of minigenome reporter gene systems for REBOV and ZEBOV were switched, it was noted that *cis*-acting signals and nearly all combinations of proteins were exchangeable.³² Rescue of recombinant ZEBOV using NC-associated proteins from REBOV or MARV has also shown that exchanging of these heterologous proteins can lead to recovery of recombinant virus.³⁹⁵

Assembly and Release

When sufficient levels of negative-sense nucleocapsids and envelope-associated proteins are reached, a coalescing of these components occurs at the plasma membrane,²⁰⁷ or to a lesser extent at membranes forming intracellular vacuoles.⁹⁶ Filovirus-infected cells develop prominent inclusion bodies, easily visualized by light, immunofluorescent, and electron microscopy.^{183,294} Inclusions are induced by NP, but also contain other proteins that form the NC.²⁸ Inclusions may be a source of components for forming NCs, which can be seen associated with inclusions (Fig. 32.8A). Recombinant-derived, NC-like structures form in cells expressing NP, which may be facilitated by the expression of VP35 and VP24.^{183,305} NC particles are believed to interact with VP40 molecules in the budding process.

Membrane/lipid rafts have been identified as platforms for the assembly of filovirus virions.^{24,310} Membrane rafts are rigid microdomains (containing sphingolipids and cholesterol) present in biological membranes and are isolated from the fluid phospholipids surrounding them. GP trimers conveyed to the plasma membrane have an affinity for these lipid rafts, which is associated with palmitoylation of the membrane-spanning anchor sequence.²⁴

Structural and functional studies of VP40 have provided important insights into the assembly of filovirus virions.^{75,173,198,207,229,230,257,461} Posttranslational processing and intracellular trafficking of VP40 result in the deposition of VP40 at the plasma membrane via the vacuolar protein sorting/endosomal pathway. By itself, ZEBOV VP40 is capable of mediating its own release from mammalian cells to form enveloped virus-like particles (VLPs),^{208,256,307,399} which are more efficiently produced when GP and NP are present²⁵⁶; VP40 interacts with the C-terminal 50 amino acids of NP.²⁵⁷ ZEBOV VP40 determines VLP morphology and density,²¹⁹ and likely has the same influence on infectious filovirus particles. A structural study of ZEBOV VP40 demonstrated that it associates with lipid bilayers containing a high level of L- α -phosphatidyl-L-serine (abolished by 1 M NaCl).³⁴⁰ It was also found that this property maps to the C-terminal ~110 residues, which contains basic and hydrophobic regions that could bind membranes.³⁹⁷ In addition, the N-terminal region is involved in oligomerization, and deletion of the C-terminal region of VP40 allowed it to hexamerize into ring structures. The crystal structure of monomeric ZEBOV VP40 is composed of similar/related β sandwich domains (N-terminal and C-terminal) connected by a hinge region, which unfolds upon interaction with membranes and dimerizes in an antiparallel orientation (Fig. 32.5).³⁶² These dimers form octomeric rings (~84 Å diameter) with a central pore and RNA binding properties¹⁵² that may be essential for replication.¹⁸¹ Late (L) domain motifs are positioned near the N-terminus of filovirus VP40 molecules, and are important in posttranslational processing and tracking events that facilitate virus budding.^{173,405} The VP40 of EBOV contains overlapping PT/SAP and PPXY motifs (PTAPPE/AY), while MARV contains only the PPXY motif (PPPY). These L domain motifs on the ZEBOV VP40 interact with cellular proteins (with WW domains) associated with the endocytic pathway of mammalian cells^{229,230,257,272,398,465}; for ZEBOV the PPXY domain appears to have a greater role in budding efficiencies.³⁰⁰ Results of *in vitro* studies have suggested that VP40 is bound as an oligomeric form at its PPXY motif by Nedd4 and ubiquitinated, is subsequently targeted to endosomes or multivesicular bodies (MVB) by Tsg101 and VPS-4 (components of the vacuole sorting pathway), and is recruited to membrane rafts through Tsg101 interactions with VP40 and raft proteins (Fig. 32.7). The finding that small interfering RNA (siRNA)-silencing of Rab9, an enzyme important in late endosome transport, inhibits filovirus replication in Vero cells,^{60,297} as well as the observation that Rab11 is incorporated into MARV virions,²³¹ support the involvement of the endosomal sorting machinery in filovirus assembly. However, the details of VP40 transport from the late endosome to the plasma membrane have not been defined. In addition, mutation of L domains from VP40 did not prevent recovery of a recombinant ZEBOV, nor did it significantly reduce virus production in cell culture.³⁰⁰ This information suggests that VP40 can be transported to the plasma membrane through a process separate from endosomal trafficking. Raft-associated VP40 is believed to associate with NCs, drawing them tightly to the membrane where they are enveloped and extruded from the host cell as infectious virions (Figs. 32.7 and 32.8). Electron tomography studies of MARV budding indicate that the entire length of nucleocapsids associate laterally with the plasma membrane (much like a rising submarine), which is followed by its protrusion and release of

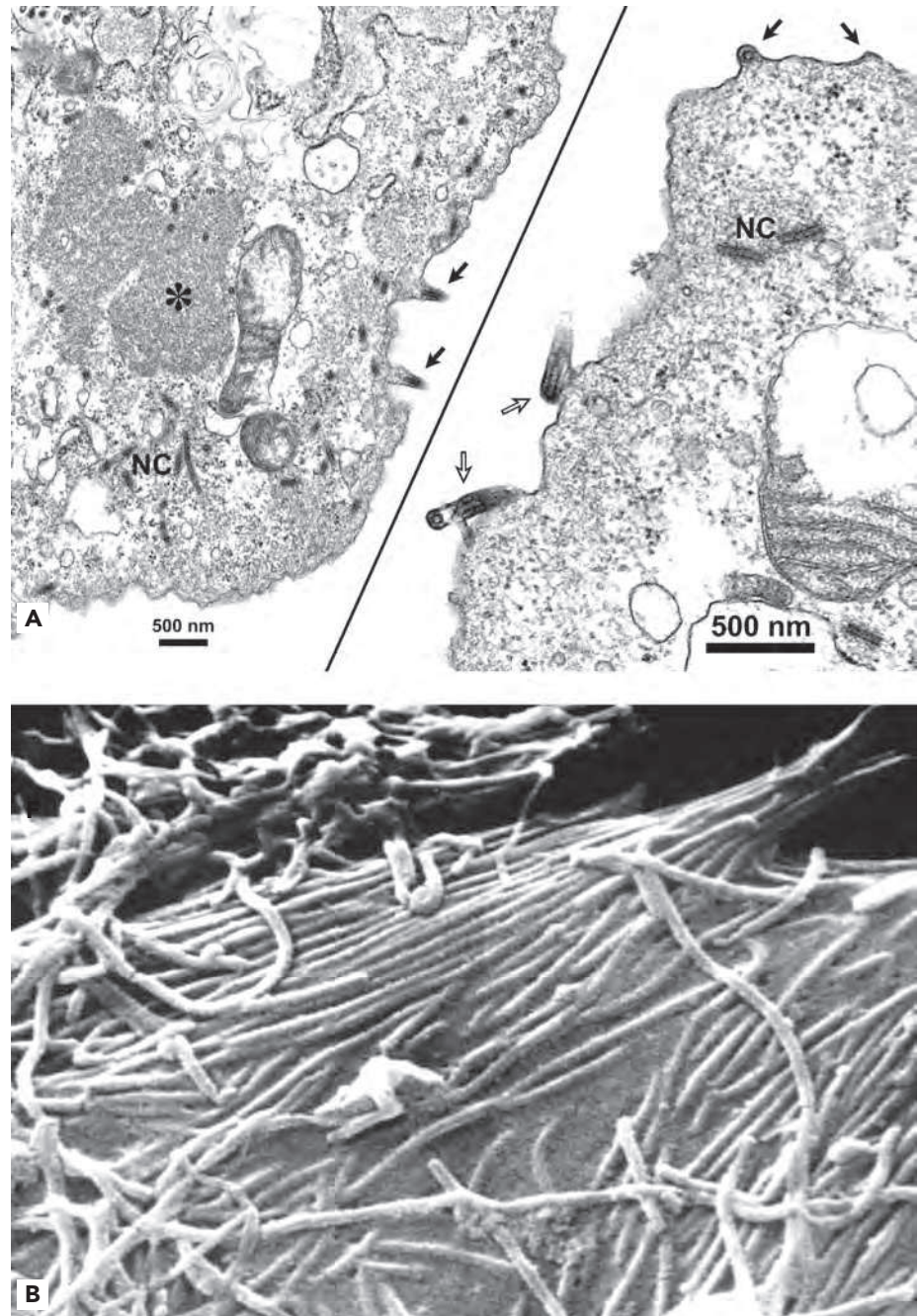


FIGURE 32.8. Transmission (A) and scanning (B) electron microscopy of Vero E6 cells infected with MARV (Angola 2005) and ZEBOV (1976), respectively. **A:** Low and higher magnification images of different cells with virus particles forming at and detaching from the plasma membrane (dark and white arrows, respectively). An inclusion body is marked with an asterisk and areas of nucleocapsid (NC) accumulation are identified. (**A** courtesy of A. Sanchez and C. Humphrey, Centers for Disease Control and Prevention, Atlanta, GA.) **B:** A multitude of filovirus particles (with varying lengths) are seen attached to and budding from the cell surface. (**B** courtesy of C. Goldsmith, Centers for Disease Control and Prevention, Atlanta, GA.)

the mature virion particle by being pinched off at the trailing end.^{405,444}

Effects on Host Cell Cultures

The growth of adapted strains of MARV and EBOV in cultured cells can be striking. Intracytoplasmic vesiculation and mitochondrial swelling are followed by a breakdown of organelles and terminal cytoplasmic rarification or condensation. In African green monkey kidney cell lines infected with filoviruses, cytopathic effects (CPE) are evidenced by a rounding and detachment of cells (without syncytia formation), which can result in a total loss of the monolayer (~5 days). However, replication of REBOV and ICEBOV is slow in tissue culture, and CPE is usually less evident and gener-

ally does not develop until after 7 to 9 days incubation. Persistent REBOV infection with continued production of large amounts of virus particles can be established in Vero E6 cells (A. Sanchez, unpublished observations) and ZEBOV can establish persistent infection under partial immunity.¹⁶¹ Filovirus infection does not lead to the shutdown of host-cell protein synthesis, but expression levels diminish as the infection progresses and virus proteins accumulate. The expression of GP has a cytotoxic effect that is associated with the mucin-like region.^{117,415,463} Elevated expression would likely impact the function of host-cell adhesion proteins by downregulating and/or displacing them,^{367,380,393} and could also cause cell detachment without cell death through a phosphorylation-dependent signal cascade.⁵⁷ Using a reverse genetics system,

it was further demonstrated that cytotoxicity depends on the level of GP expression, with overexpression leading to an early detachment and cytotoxicity of infected cells.⁴¹⁵ The effects of filovirus infections on endothelial cells and immunocompetent cells will be discussed in the next section.

PATHOLOGY AND PATHOGENESIS

Clinical investigations from episodes and outbreaks of human EBOV and MARV infections have provided important descriptive information on the pathology and pathogenesis of these agents; however, the available data are sparse, often fragmentary and sometimes paradoxical. Comprehensive studies have been carried out to a much greater extent in laboratory animals. Rodents—including guinea pigs, mice, and hamsters—have been employed to study viral hemorrhagic fever (VHF) caused by filoviruses.^{26,38,343,428,472} Because filovirus isolates derived from primates do not typically produce severe disease in rodents upon initial exposure, serial adaptation is required to produce a uniformly lethal infection. Mice and guinea pigs have served well as early screens for evaluating antiviral drugs and candidate vaccines, and genetically engineered mice clearly have utility for dissecting out specific host–pathogen interactions. However, the disease pathogenesis in rodent models is far less faithful in portraying the human condition than disease observed in nonhuman primates.^{40,143} As data derived from studies using rodents may not correlate with human disease or may be deficient in identifying certain processes, this section primarily focuses on data obtained from human clinical studies and experimental infections of nonhuman primates (Fig. 32.9).

Entry into Host

Little is known regarding what constitutes a typical dose and route of exposure in human filovirus infections. Viruses enter the host through mucosal surfaces, breaks or abrasions in the skin, or by parenteral introduction. While a recent study has suggested that exposure to fruit bats may have initiated a ZEBOV outbreak in the DRC in 2007,²⁴⁸ most cases that propagate outbreaks are thought to occur by direct contact with infected patients or cadavers.^{79,223,449,450} Infectious filoviruses and/or RNA have been isolated from semen and genital secretions^{275,333,338} and detected in skin in human cases⁴⁷⁰; they have also been demonstrated in skin, body fluids, and nasal secretions of nonhuman primates.^{137,205,368}

Laboratory exposure through needlestick and filovirus-infected blood has been reported.^{87,196} Reuse of contaminated needles played an important role in the 1976 EBOV outbreaks in Sudan and Zaire,^{449,450} and the question of whether reuse of contaminated needles contributed to some of the cases in the 2004 to 2005 outbreak of MARV in Angola was raised.⁴⁵⁶ A needlestick exposure involving an acute-phase patient would likely entail a dose of 1,000 plaque-forming units (pfu) or more if viremias associated with terminal patients are comparable to viremias in infected nonhuman primates, which often reach levels as high as 10^7 to 10^8 pfu/mL of serum.^{83,126,127,137,174,204,220,328} The generation of human viremia data has been notoriously problematic,²³⁵ but levels are thought to exceed 10^6 pfu/mL of serum in outbreaks of SEBOV and ZEBOV.^{402,450} The fact that circulating EBOV and MARV particles are readily observed by direct electron microscopic inspection of postmortem fluids and tissues^{76,86,138,294,468}

supports this view, considering that the lower limit for ultrastructural detection of virus particles is generally on the order of 10^6 pfu/mL of fluid or gram of tissue.

Butchering of a chimpanzee for food was linked to outbreaks of ZEBOV in Gabon¹⁴⁸ with contact exposure the likely route of transmission. While proper cooking of foods should inactivate infectious filoviruses, ingestion of contaminated foods cannot completely be ruled out as a possible route of exposure in natural infections. Organ infectivity titers in filovirus-infected nonhuman primates are frequently in the 10^7 to 10^9 pfu/g range^{126,127,137,174,204,220}; thus, it is likely that exposure through the oral route would invariably be associated with very high infectious doses. In fact, ZEBOV is highly lethal when orally administered to rhesus macaques.²⁰¹

The role of aerogenic transmission in outbreaks is unknown, but is thought to be rare.³¹⁵ Aerosol transmission in nonhuman primates was inferred in the 1989 to 1990 epizootic of REBOV,²⁰⁵ although it is thought that aerosols may have been created mechanically by workers cleaning the facility. High concentrations of REBOV in nasal secretions and ultrastructural detection of large numbers of viral particles in alveoli were reported.²⁰⁵ Filoviruses are reasonably stable in aerosols,^{25,61} and reports of interage transmission of ZEBOV and MARV between monkeys^{202,319,368} suggest that virus spread was mediated by small-particle aerosols. Moreover, ZEBOV and SEBOV are highly infectious by aerosol exposure in cynomolgus^{126,320,328} and rhesus^{217,328} macaques and African green monkeys,³²⁸ as is MARV in cynomolgus and rhesus macaques^{4,126,264} and African green monkeys.²⁵ Rhesus macaques were also lethally infected with ZEBOV by conjunctival exposure.²⁰¹

Host-Cell Pathology

The pathologic changes seen in patients dying with all filovirus infections seem similar, with extensive necrosis in parenchymal cells of many organs, including liver, spleen, kidney, and gonads; little inflammation is seen within infected tissues.^{76,294,295,468} The most characteristic histopathologic features are seen in the liver (Fig. 32.10), where hepatocellular necrosis is widespread with intact, hyalinized, ghost-like cells often remaining in place amid large amounts of karyorrhectic debris. Often, extraordinary numbers of virions are present in this debris. Characteristic intracytoplasmic inclusion bodies are present in intact hepatocytes. Light microscopic, electron microscopic, immunohistochemistry (IHC), and *in situ* hybridization studies show concordance between tissue damage, the presence of viral antigens and nucleic acid, and sites of virus replication, suggesting that direct viral damage is one major element in the pathogenesis of the disease (Figs. 32.9 and 32.10).

EBOV and MARV have a broad cell tropism, infecting a wide variety of cell types. IHC and *in situ* hybridization analyses of tissues from fatal human cases or experimentally infected nonhuman primates show that monocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, hepatocytes, adrenal cortical cells, and several types of epithelial cells all support replication of these viruses.^{4,18,73,127,137,138,141,146,174,201,296,342,468,470} The sequence of infection, however, has not been fully elucidated. Temporal studies in nonhuman primates experimentally infected with either ZEBOV or MARV suggest that monocytes, macrophages, and dendritic cells are early and preferred replication sites of these viruses.^{137,174} These cells appear to play pivotal roles in dissemination of the virus as it spreads

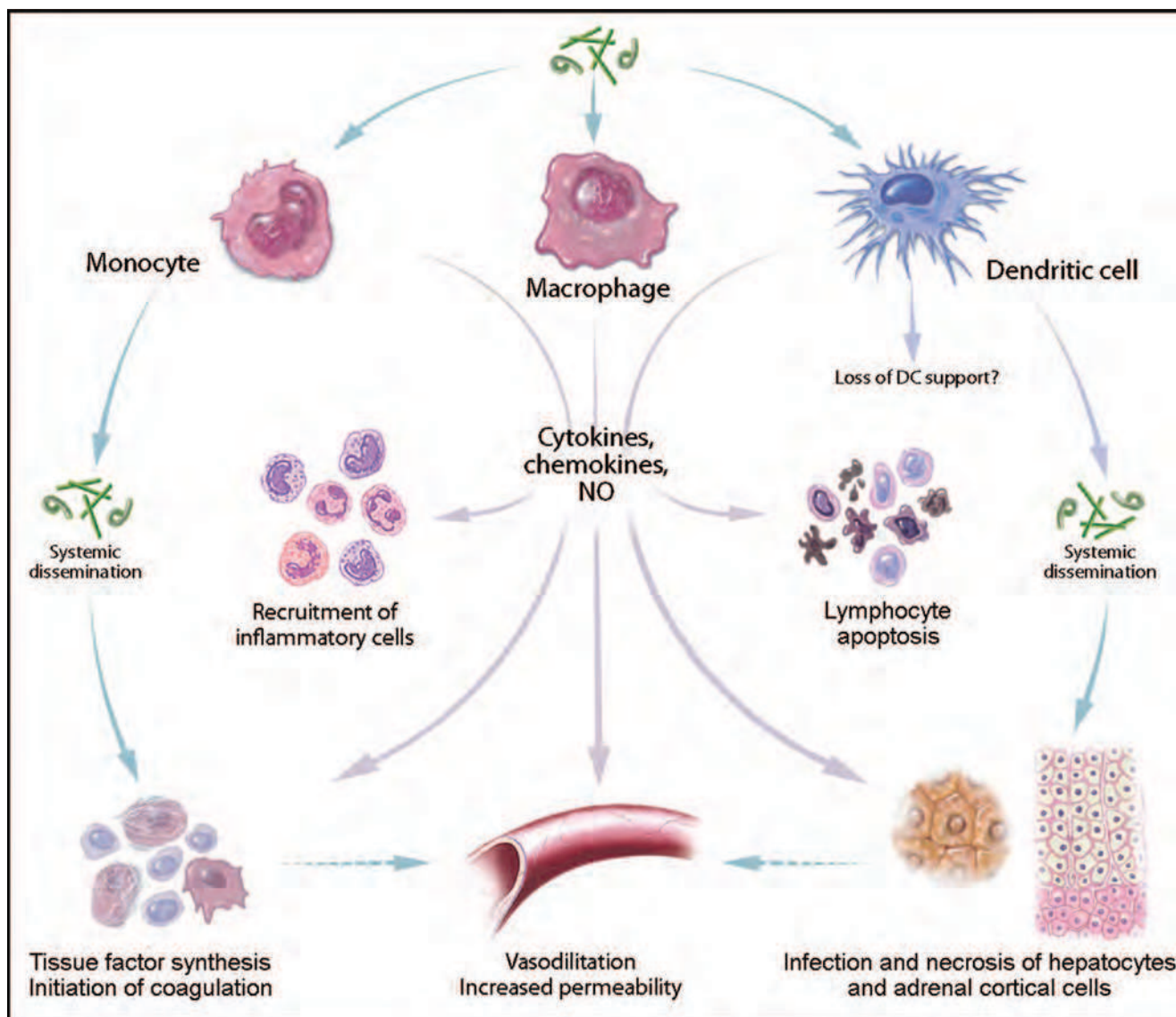


FIGURE 32.9. Model of filovirus pathogenesis in primates. Monocytes, tissue macrophages, and dendritic cells appear to be early and preferred sites of filovirus replication. Soluble factors released from virus-infected mononuclear cells act locally and systemically. Release of chemokines from these virus-infected cells recruits additional monocytes and macrophages to sites of infection, making more target cells available for viral exploitation and further amplifying an already dysregulated host response. In addition, these soluble factors contribute to the impairment of the vascular system. Although filoviruses do not productively infect lymphocytes, the rapid loss of lymphocytes by the process of apoptosis is a noted feature of disease. This lymphocyte loss is probably due to a combination of factors including virus-induced impairment of dendritic cell function, release of proapoptotic soluble factors from virus-infected monocytes and macrophages, and possibly direct interaction between viral antigens and lymphocytes. Coagulation abnormalities are consistent features of filovirus disease and are caused by a number of factors, particularly during the later stages of disease; recent data strongly implicate a role for tissue factor. The hemodynamic and coagulation disorders are exacerbated by infection of hepatocytes and adrenal cortical cells, resulting in impairment in the synthesis of important clotting factors. At the same time, impaired secretion of steroid-synthesizing enzymes by virus-infected adrenal cortical cells leads to hypotension. DC, dendritic cell; NO, nitric oxide. (Adapted from a prior publication by Bray M, Geisbert TW. Ebola virus: the role of macrophages and dendritic cells in the pathogenesis of Ebola hemorrhagic fever. *Int J Biochem Cell Biol* 2005;37:1560–1566.)

from the initial infection site via monocytes/macrophages and dendritic cells to regional lymph nodes, likely by way of lymphatics, and to the liver and spleen through blood. At these locations, filoviruses infect resident macrophages and dendritic cells. Several lines of evidence suggest that filovirus-infected monocytes/macrophages release various soluble factors that

then recruit additional monocytes/macrophages to areas of infection; this makes more target cells available for viral exploitation, further amplifying the infection.^{39,137,174}

In addition to the macrophage-rich lymphoid tissues such as spleen, the liver and adrenal gland also appear to be important target organs for both EBOV and MARV; this

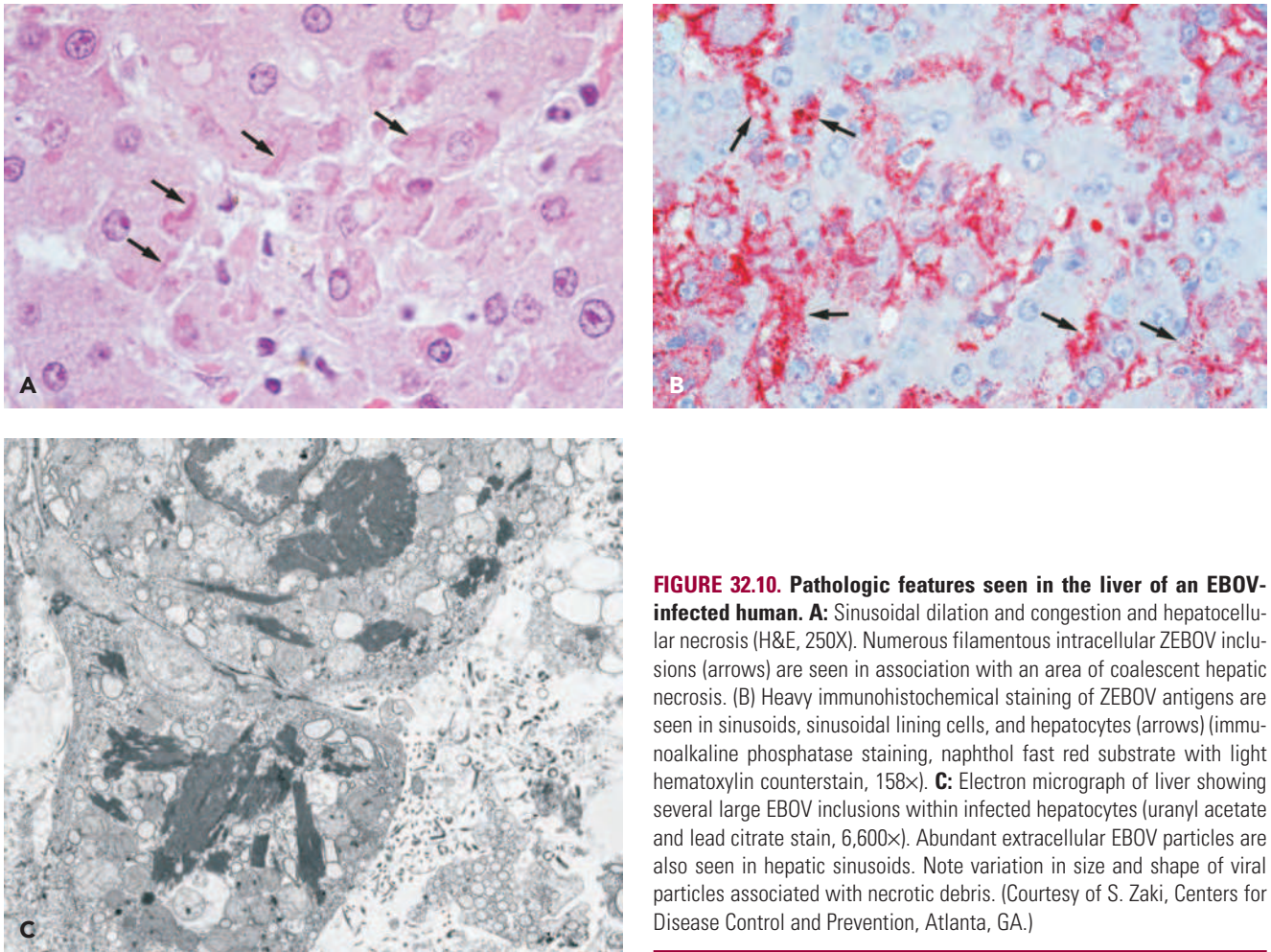


FIGURE 32.10. Pathologic features seen in the liver of an EBOV-infected human. **A:** Sinusoidal dilation and congestion and hepatocellular necrosis (H&E, 250X). Numerous filamentous intracellular ZEBOV inclusions (arrows) are seen in association with an area of coalescent hepatic necrosis. **(B)** Heavy immunohistochemical staining of ZEBOV antigens are seen in sinusoids, sinusoidal lining cells, and hepatocytes (arrows) (immunoalkaline phosphatase staining, naphthol fast red substrate with light hematoxylin counterstain, 158X). **C:** Electron micrograph of liver showing several large EBOV inclusions within infected hepatocytes (uranyl acetate and lead citrate stain, 6,600X). Abundant extracellular EBOV particles are also seen in hepatic sinusoids. Note variation in size and shape of viral particles associated with necrotic debris. (Courtesy of S. Zaki, Centers for Disease Control and Prevention, Atlanta, GA.)

tropism likely plays an equally important role in the disease pathogenesis. Elevations in liver enzymes are prominent findings in most filovirus infections.^{109,135,137,174,175,201,273,342} Various degrees of hepatocellular degeneration and necrosis have been reported in filovirus infections of humans and nonhuman primates.^{4,122,127,137,174,201,294,296,342,468,471} The hepatocellular lesions are generally not significant enough to explain the cause of death. However, impairment of the liver could contribute to the overall pathogenesis as hemorrhagic tendencies in some cases may be related to decreased synthesis of coagulation factors and other plasma proteins as a result of severe hepatocellular necrosis.

Adrenocortical infection and necrosis were also reported in filovirus infections of humans and nonhuman primates.^{127,137,138,174,342} The adrenal cortex plays an important role in blood pressure homeostasis. Impaired secretion of steroid-synthesizing enzymes leads to hypotension and sodium loss with hypovolemia, which are important elements that have been noted in nearly all cases of filovirus disease. This suggests that impairment of adrenocortical function by viral infection may contribute to the development of shock that typifies late stages of disease.

Host Immune Response

For both EBOV and MARV, lymphoid depletion and necrosis are commonly seen in spleen, thymus, and lymph nodes

of fatal cases and in experimentally infected nonhuman primates.^{4,118,122,123,137,138,174,296,342,468,471} Although lymphoid tissues are primary sites of filovirus infection, there is usually little inflammatory cellular response in these tissues or other infected tissues. Lymphopenia is a consistent finding among filovirus infections of humans and nonhuman primates.^{83,110,112,127,137,174,201,328,351,368} Despite the massive die off and loss of lymphocytes during filovirus infection, the lymphocytes themselves have not been shown to be infected. For both EBOV and MARV, large numbers of lymphocytes undergo apoptosis in humans and experimentally infected nonhuman primates^{4,9,11,134,137,174,327,438} in part, explaining the progressive lymphopenia and lymphoid depletion at death. In the 2000 outbreak of SEBOV in Uganda, numbers of T lymphocytes were lower in fatal cases than in nonfatal cases.³⁵¹ In the ZEBOV and MARV macaque models, the lymphocyte loss appears to be greatest among the T-lymphocyte and NK-cell populations.^{118,137,327}

The mechanism(s) for the underlying apoptosis and loss of “bystander” lymphocytes during the course of filovirus illness is unknown but is thought to be provoked through several different agonists or pathways. These pathways or processes may include the TNF-related apoptosis-inducing ligand (TRAIL) and Fas death receptor pathways,^{137,177,438} impairment of dendritic cell function,^{34,35,137,174,177,268} abnormal production of

soluble mediators such as nitric oxide (NO) that have proapoptotic properties,^{10,137,177,351} or possibly by direct interactions between lymphocytes and filovirus proteins. Severe cases of EHF have a prolonged high virus load, and one study of SEBOV-infected humans reported peripheral blood mononuclear cell unresponsiveness during the acute phase of disease.³⁵¹ The recognition of an immunosuppressive motif in the C-terminal region of the EBOV and MARV glycoproteins^{48,411,460} supports the premise that filovirus particles/proteins may contribute in part to the dysfunction and/or loss of lymphocytes.⁶³ A recent study has also suggested that the dramatic loss of lymphocytes that occurs during filovirus infection may be a result of the superantigen activity of filoviruses.²⁴⁷ In this study, human ZEBOV infection was associated with mRNA downregulation of three T-cell receptor (TCR) V β subsets, indicating either anergy or deletion of these T-lymphocyte populations.

Filovirus infection of humans and nonhuman primates triggers the expression of a number of inflammatory mediators including the interferons, interleukin (IL)-6, IL-8, IL-10, IL-12, interferon-inducible protein (IP)-10, monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T-cell expressed and secreted (RANTES), TNF- α , and reactive oxygen and nitrogen species.^{9,10,83,135,137,174,177,186,187,351,408,438} Infection of various primary human cells *in vitro* also shows that filovirus infection can trigger the production of many of these same inflammatory mediators.^{96,146,159,160,177,374} Overall, it appears that virus-induced expression of these mediators results in an immunologic imbalance that contributes to the progression of disease. However, information regarding the inflammatory response after filovirus infection has not been fully delineated and there are some differences in data among studies. For example, high levels of circulating interferon (IFN)- α were noted in acute-phase sera of patients infected with ZEBOV in one study⁴⁰⁸ but not detected in a subsequent similar study.¹⁰ Such differences complicate interpretation of some *in vitro* data as well. The differences in profiles of circulating cytokines and chemokines may be due to factors other than the differences among the filovirus species or strains assessed such as genetic differences among patient populations, and in particular, differences related to the disease phase when the samples were obtained.

For ZEBOV, there has been a report of patients with asymptomatic, nonfatal infections.^{244,246} It was proposed that these infections are controlled by an initial increase in cytokines including IL-1 β , IL-6, and TNF- α that is followed by a return to baseline levels. Results of this study suggest that protection from development of a fatal infection may depend on an early and robust cytokine response; however, this remains to be proven. On the other hand, disease severity may also be increased by an inappropriate proinflammatory response early in the course of infection; therefore, the balance between protective and detrimental proinflammatory responses remains to be defined.

Inhibition of the type I IFN response appears to be a feature of filovirus pathogenesis, and was initially indicated by studies of ZEBOV-infected endothelial cells.^{166,167} Subsequently, the EBOV protein VP35 was shown to function as a type I IFN antagonist^{19,20–21} by blocking interferon regulatory factor (IRF-3) activation and possibly preventing transcription of IFN- β .¹⁹ This activity of the ZEBOV VP35 has been linked to a C-terminal motif (³⁰⁵RACQKSLR³¹²)¹⁷² that is similar in sequence to the RNA-binding domain of the influenza A NS1 protein (interferon antagonist). In addition to VP35, other

studies suggest that EBOV VP24 expression interferes with type I IFN signaling^{20,329}; mutations in VP24 have also been linked to adaptation of ZEBOV to produce lethal disease in mice⁸⁴ and guinea pigs.⁴¹² Interestingly, MARV utilizes a different mechanism to evade the host IFN response. Recent studies have shown that MARV VP40 blocks the phosphorylation of Janus kinases and their target STAT proteins in response to type I and type II interferon and IL-6.⁴⁰⁶ Mutations in VP40 have been linked to adaptation of MARV to produce lethal disease in mice²⁶³ and guinea pigs.²⁶²

Several studies indicate an important role for reactive oxygen and nitrogen species in filovirus disease pathogenesis. Increased blood levels of NO were reported in nonhuman primates experimentally infected with ZEBOV^{137,177} and were also noted in ZEBOV- and SEBOV-infected patients.^{10,351} Significantly, increased blood levels of NO in patients was associated with mortality.³⁵¹ Abnormal NO production has been associated with a number of pathologic conditions including apoptosis of bystander lymphocytes (as noted previously), tissue damage, and loss of vascular integrity, which may contribute to virus-induced shock. NO is known to have both protective and caustic effects; this autotoxic overproduction may represent the host's endogenous counter-regulatory mechanism of protection against noxious agents, in this case the filoviruses. In general, microbes induce monocytes and macrophages to produce NO in an attempt to control infection. However, in the case of the filoviruses, monocytes and macrophages are preferred host cells for viral replication. Enhanced replication in these cells may in turn exacerbate disease by producing large amounts of NO, resulting in deleterious effects such as suppressive effects on lymphocyte proliferation and damage to other cells. NO is an important mediator of hypotension, a prominent finding in most VHF including those caused by filoviruses.¹⁴⁰

Together, the information collected to date suggests that an impaired and ineffective immune response leads to high levels of virus and proinflammatory mediators in the late stages of disease, which are important for the pathogenesis of hemorrhage and shock. Indeed, the prevailing hypothesis at this time is that infection and activation of monocytes/macrophages is fundamental to the development of EHF and MHF, and that it is the release of proinflammatory cytokines, chemokines, and other mediators that causes impairment of the vascular and coagulation systems (discussed in the following section) leading to multiple organ failure and a syndrome that in some ways resembles septic shock.^{39,41,135,137,140,145,160,174,177,267,358,359,360,374,420}

Impairment of the Vascular System

The endothelium is thought to play an important role in the pathogenesis of EBOV and MARV, although studies defining the molecular mechanisms of endothelial impairment are incomplete. It was speculated that EBOV GP is the primary determinant of vascular cell injury and that EBOV infection of endothelial cells induces structural damage,⁴⁶³ which could contribute to hemorrhagic diathesis. Human and nonhuman primate endothelial cells are susceptible to EBOV and MARV infection,^{135,166,167,360,361} but while *in vitro* studies have reported some cytopathic effects associated with filovirus replication, in general, filovirus replication in nonhuman primates did not induce overt cytopathology. In fact, in one study using primary human endothelial cells, ZEBOV infection induced an upregulation of protective antiapoptotic genes.¹⁴⁶

EBOV and MARV infection of endothelial cells *in vivo* has been documented, as noted previously, but human data is sparse. ZEBOV antigens were readily detected in endothelial cells of a variety of tissues during the 1995 Kikwit outbreak.⁴⁶⁸ On the other hand, an immunohistochemical survey of a fatal case of MARV infection showed infrequent infection of endothelial cells in the tissues examined.¹³⁸ Clearly, disturbance of the blood tissue barrier is an important component of filovirus disease, and direct infection and destruction of endothelial cells cannot completely be dismissed as contributing to the hemorrhagic diathesis. However, histologic observations of autopsy tissues from several of the early filovirus outbreaks failed to identify the presence of vascular lesions²⁹⁴ and there have been no reports of vascular lesions in any subsequent studies to date. There is also no evidence of significant vascular lesions in filovirus-infected nonhuman primates.^{17,18,73,135,174,201,342}

In temporal studies in nonhuman primates, ZEBOV and MARV infection of endothelial cells was infrequent and primarily restricted to the terminal stages of disease.^{135,174} In these animals, the endothelium remained relatively intact morphologically, although increased vascular permeability was observed. This is consistent with the imbalance of fluid between the intravascular and extravascular tissue spaces observed in patients. Using *in vitro* systems, increased endothelial permeability was associated temporally with the release of TNF- α from MARV-infected human monocytes/macrophages.⁹⁶ Subsequent studies showed that EBOV-induced cytokine release led to activation of the endothelium, as demonstrated by a breakdown of barrier function,⁴²¹ providing further evidence that endothelium may be affected indirectly by a mediator-induced inflammatory response of primary target cells more so than by direct filovirus replication-induced cytopathology. It is important to keep in mind, when comparing results among studies, that differences in findings could represent differences between macaque models and human disease possibly through the divergence of endothelial cell receptors such as DC-SIGNR. Nonetheless, and as noted previously, most studies indicate that changes in integrity of the endothelium are influenced primarily by local or systemic increases in levels of cytokines and other host-cell factors triggered by infection.

Impairment of the Coagulation System

Defects in blood coagulation and fibrinolysis during EBOV and MARV infections are manifested as petechiae (Fig. 32.11), ecchymoses, mucosal hemorrhages, congestion, and uncontrolled bleeding at venipuncture sites. However, massive loss of blood is infrequent and, when present, is primarily limited to the gastrointestinal tract. In fact, even in these cases, the amount of blood that is lost is not significant enough to account for death. Thrombocytopenia, consumption of clotting factors, and increased levels of fibrin degradation products are other indicators of the coagulopathy that characterizes EBOV and MARV infections.

Although disseminated intravascular coagulation (DIC) is often viewed as a prominent manifestation of filovirus infection in primates, evidence of DIC in human filovirus infections is sparse primarily due to difficulties encountered in performing studies in inaccessible geographic settings. Clinical laboratory data suggest that DIC is an important feature of human EHF.^{197,334,449} D-dimer levels were substantially increased in all patients with SEBOV infections but were four times higher in

patients with fatal disease than in patients who survived.³³⁴ The coagulation picture is clearer for nonhuman primates. Numerous studies have shown histologic and biochemical evidence of DIC syndrome during EBOV infection in a variety of nonhuman primate species.^{17,40,73,83,109,111,112,141,145,146,176,201,342} For MARV, histologic or biochemical evidence of DIC has been reported in a handful of available cases^{122,138} and in a few studies of experimentally infected monkeys.^{127,174,471}

Despite any differences between humans and nonhuman primates regarding DIC, impairment of coagulation ostensibly contributes to the disease pathogenesis of EHF and MHF (Figs. 32.9 and 32.11). The mechanism(s) responsible for triggering the coagulation disorders is not completely understood. Several studies suggest that development of coagulation abnormalities might occur much earlier than previously thought. For example, in one study, markedly elevated levels of D-dimers were detected one day after experimental infection of cynomolgus monkeys with ZEBOV, which occurred two days before the detection of viremia in these animals.¹⁴⁵ Although it is likely that the coagulopathy seen during filovirus infections is caused by a number of factors, particularly during the later stages of disease, data strongly implicate tissue factor expression/release from EBOV-infected monocytes/macrophages as a key factor that induces the development of coagulation irregularities.¹⁴⁵ Of course, as noted previously, other factors may also contribute to the coagulopathy associated with filovirus infections. For example, impairment of the fibrinolytic system was documented by rapid declines in plasma levels of protein C during the course of ZEBOV infection of cynomolgus and rhesus monkeys.^{83,145,176}

Virulence

The virulence of filoviruses in humans is highly variable depending primarily on the species or strain; a similar variability seems to recapitulate well in nonhuman primates. Among the EBOV species, ZEBOV is the most virulent and REBOV appears to be the least virulent. Infection of nonhuman primates with ZEBOV usually progresses rapidly and is uniformly lethal, with as little as one infectious unit being required to cause disease. The course of disease appears to be influenced by the dose of filovirus used. As an example, cynomolgus macaques exposed by intramuscular injection with a low challenge dose of ZEBOV (10 pfu) succumbed to infection 8 to 12 days after challenge,³⁷⁷ but when exposed to a high dose (1,000 pfu) died 5 to 8 days after challenge.^{137,143} Likewise, a similar protraction of disease course in nonhuman primates concurrent with serial dilution was noted for MARV.¹⁵³

In human cases, route of infection ostensibly affects the disease course and the outcome. The mean incubation period for cases of ZEBOV known to be due to injection was 6.3 days, versus 9.5 days for contact exposures.⁴³ Moreover, the CFR in this 1976 ZEBOV outbreak was 100% (85 of 85) in cases associated with injection compared with ~80% (119 of 149) in cases of known contact exposure.⁴³ Although the nonhuman primate models appear to be exquisitely sensitive to the filoviruses compared to humans, particularly for ZEBOV, this observation in part could relate to the fact that most nonhuman primate studies involve intramuscular injection with very high challenge doses.

Fewer studies have evaluated the pathogenesis of SEBOV in nonhuman primates.^{86,109} The disease course in experimentally infected rhesus and cynomolgus macaques appears much

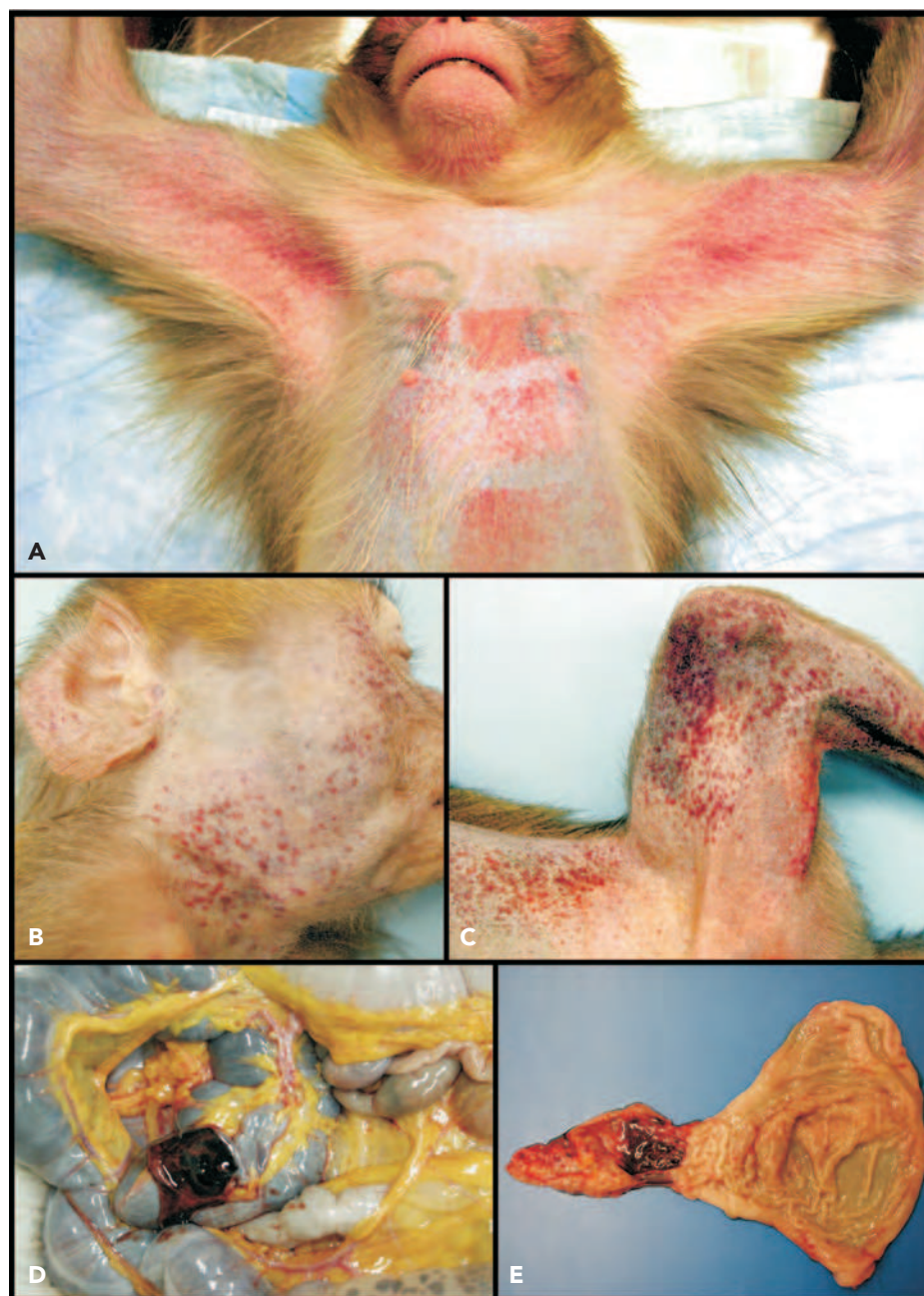


FIGURE 32.11. Hemorrhagic manifestations seen in nonhuman primates acutely infected with filoviruses.

Shown are examples of petechiae evident on (A) the upper torso and arms of a rhesus macaque infected with ZEBOV (strain Mayinga 1976) (A), the head and neck of a rhesus macaque infected with MARV (strain Angola 2005) (B), and the lower trunk and leg of a cynomolgus macaque infected with SEBOV (strain Gulu 2000) (C). Also shown are a gastroduodenal lesion (D) and hemorrhage in the ileum (E) of a SEBOV-infected cynomolgus macaque (Courtesy of A. Sanchez and P. Rollin (A), Centers for Disease Control, Atlanta; T.W. Geisbert (B, C) US Army Medical Research Institute for Infectious Diseases (USAMRIID), Frederick; and T. Larsen (D,E), USAMRIID, Frederick).

slower than that seen in ZEBOV infections, and the rates of survival appear consistent with human disease. SEBOV infection was not lethal in a small cohort of African green monkeys nor was REBOV.¹⁰⁹ Similar to SEBOV, the disease course in REBOV-infected cynomolgus monkeys is protracted.²⁰⁵ Experimental infection of cynomolgus macaques by intramuscular injection with 1,000 pfu of SEBOV results in 50% to 100% mortality, with deaths typically occurring 7 to 12 days after infection. In comparison, experimental infection of cynomolgus macaques with 1,000 pfu of REBOV results in 80% to 100% mortality, with deaths usually occurring 8 to 21 days after infection. Recent studies have shown similar results for CIEBOV and BEBOV. Specifically, experimental infection of cynomolgus macaques with 1,000 pfu of CIEBOV resulted in 60% mortality, with deaths occurring 12 to 14 days after

infection¹³¹ while infection with 1,000 pfu of BEBOV resulted in 75% mortality, with deaths occurring 11 to 14 days after infection.^{92,175}

There appears to be some difference in virulence among the strains of MARV. Historically, virulence of the MARV strains in humans has been comparable to SEBOV. However, virulence of the recently isolated Angola strain appears to be more consistent with ZEBOV. Most strains of MARV produce near uniformly lethal infections in cynomolgus and rhesus macaques. Among the MARV strains, infections of macaques with the Angola strain appear to progress more rapidly than other strains. For example, challenge of rhesus macaques by intramuscular injection with 1,000 pfu of the Musoke strain produces a uniformly lethal infection, with deaths occurring 10 to 12 days after infection, whereas an identical challenge of

rhesus macaques with 1,000 pfu of the Angola strain resulted in deaths occurring 6 to 8 days after challenge.¹²⁷

Currently, the variability in virulence in primates within and between species of filoviruses is unclear, but for EBOV there has been some speculation that the GP has a major influence on virulence.⁴⁶³ Studies have shown that unlike ZEBOV, expression of the GP from REBOV did not disrupt the vasculature of human blood vessels. It was initially reported that the expression of the EBOV GP caused significant cell death in cultured cells^{462,463}; however, subsequent studies showed that most of the detached cells (>90%) were still viable,^{57,367} suggesting that GP expression may interfere with cell attachment without triggering cell death. It has been speculated that EBOV may control GP cytotoxicity by regulating its expression through RNA editing,⁴¹⁵ but this mechanism needs to be studied in cells derived from the natural host and reconciled with the importance/role of sGP expression.

EBOV produces five soluble glycoproteins during infection: sGP, Δ -peptide, GP₁, GP_{1,2Δ}, and the newly identified ssGP.²⁸¹ MARV produces GP₁ and presumably GP_{1,2Δ}.¹⁰⁵ Upon the discovery of sGP, it was logical to attempt to correlate the higher pathogenicity of EBOV with its expression; however, the EBOV-like virulence and mortality rates associated with the Angola strain of MARV⁵⁵ dispel any such associations. Additionally, the lower virulence of REBOV and CIEBOV does not support a role in virulence for sGP.¹⁰⁵ The contribution of the secreted GPs to the disease pathogenesis of EBOV and MARV remains largely unknown, but recent studies have begun to examine the effects of EBOV GPs on the host response to infection. Initial studies suggested that the EBOV sGP interfered with innate immunity by binding to CD16b and inhibiting neutrophil activation.^{227,462} However, subsequent studies questioned these findings and in contrast showed that neutrophils do not express a receptor for EBOV sGP.³⁷⁶ Other studies have evaluated the role of the secreted GPs in activating macrophages and endothelial cells. For example, studies using primary human macrophages and endothelial cells concluded that the presentation of the EBOV GP_{1,2} in a membrane-bound form (on virions or VLPs) is sufficient for activation of these cells.^{420,421} However, these studies also showed that none of the four secreted EBOV GPs was capable of activating human macrophages, and neither sGP nor delta peptide were capable of activating endothelial cells. In fact, sGP protected endothelial cell barrier function⁴²⁰ and could counteract or lessen the cytotoxicity caused by EBOV GP. Furthermore, it has been proposed that soluble glycoproteins circulating in the blood of virus-infected animals may play an important role in pathogenesis by efficiently blocking the activity of virus-neutralizing antibodies.^{78,105,200}

Host Genetics

Recent studies have shown that the outcome of filovirus infection could in part be determined by host genetics. Sequence-based HLA-B typing was performed on patients from the 2000 outbreak of SEBOV in Uganda.³⁵⁵ In this study, statistically significant associations were found between certain sets of alleles and either fatal or nonfatal disease outcomes. Alleles B*67 and B*15 were associated with fatal outcomes, whereas B*07 and B*14 were associated with nonfatal outcomes. In a different study, the association of KIR genotype with disease outcome was determined by comparing genotypes of a Gabonese

control population, IgG+ contacts, survivors, and fatalities of ZEBOV infection.⁴³⁹ In this study, the activating KIR2DS1 and KIR2DS3 genes were associated with fatal outcome.

Persistence

As noted earlier, mortality rates for EBOV and MARV are high and few patients survive infection. In survivors, levels of circulating virus in the blood decline as the patient recovers.^{235,351} However, during the recovery phase several lines of evidence suggest that EBOV and MARV may persist in humans in immunologically privileged sites. In one laboratory-acquired infection, EBOV was isolated from semen samples 39 and 61 days after the onset of illness.⁸⁷ After the 1995 outbreak of ZEBOV in Kikwit, infectious virus was recovered from seminal fluid of one patient 82 days after disease onset, while viral RNA was detected in semen samples of three additional patients between 63 and 101 days after the onset of illness.^{333,338} For MARV, sexual transmission was reported in one case during the original outbreak in Marburg, Germany in 1967, with semen apparently containing infectious virus more than 12 weeks after clinical recovery.²⁷⁵

EPIDEMIOLOGY

Because much of the early serosurvey data has been based on the fluorescent antibody test, a subjective and unreliable assay, identification of the geographic range of filoviruses is more accurately determined from filovirus outbreaks. ZEBOV, SEBOV, and CIEBV and BEBOV are found in the African tropical forest or nearby savanna and occasionally emerge often during the rainy season.¹⁵⁵ REBOV has been linked only to a single export nonhuman primate facility in the Philippines and more recently to a few pig farms in the country.^{14,285} Based on current data and the new discovery of REBOV on pig farms in the Philippines,¹⁴ REBOV is most likely an Asian filovirus, possibly derived from certain fruit bat species in the forests of the Philippines.⁶⁹ Alternatively, the REBOV strains could represent derivatives from a single introduction (most likely Africa) through bat migration or importation, and subsequent establishment and circulation in the Philippines. MARV has apparently been contracted in forested and derived areas of Kenya, Uganda, Zimbabwe, the DRC, and recently Angola, but in several cases the epidemiologic information does not provide an adequate description of the environment where infections were suspected to have occurred. The European outbreak from 1967 was initiated through imported infected African green monkeys and could be traced to a source in Uganda. The animals were compounded in Entebbe (central holding station at Lake Victoria) and shipped via London (where they had potential contact with other animals) to Germany and the former Yugoslavia.

The epidemiology of human infections in nature, besides the internationally recognized outbreaks, is unknown. However, the time elapsed between occurrence of the index cases and the recognition of the subsequent large outbreaks suggests that sporadic cases of unrecognized filovirus infections could readily pass unnoticed.²¹² The number of such identified clusters in the past decade may represent a combination of unidentified ecologic factors and increasing diagnostic interest. Serologic surveys revealed EBOV antibody prevalence from 10.2% among gold panners in Gabon to 9.3% among rural villagers in the

DRC using enzyme-linked immunoabsorbent assay (ELISA)-based technology. EBOV infection, potentially with nonpathogenic strains/variants or strains/variants of low pathogenicity, may be frequent in select rural African populations.^{147,216}

Whatever the source of the initial index case, person-to-person transmission is the means by which human filovirus outbreaks have been propagated. This generally involves intimate contact; secondary attack rates have not exceeded 10% to 15%, indicating that transmission is not efficient. However, this risk increases as a function of contact. For example, during the 1976 SEBOV outbreak 23% of family members sleeping in the same room as the patient were infected, compared to 81% of persons providing active nursing care to a patient.¹³ The need for this intimacy is reflected in the relative paucity of infected children, who are less likely to be primary care givers for ill family members.⁷⁹ Nosocomial transmission is a special problem and hospitals have often served as a source of disease amplification into the community and to health care workers. A quarter of all cases during the 1995 ZEBOV outbreak were among health care workers. Extreme care should be taken with infected blood, secretions, excretions, tissues, and hospital materials and waste. Well-documented and surreptitious reuse of needles and syringes has also played a role in these outbreaks. No person whose contact was exclusively parenteral during the 1976 ZEBOV outbreak survived. Sexual transmission has been reported with MARV and can also be assumed for EBOV (discussed earlier).

There is a striking difference in the ZEBOV epidemics in Gabon/RC compared to those caused by most other filoviruses including ZEBOV outbreaks at other sites. Most of the epidemics in this area are limited in case numbers and are related to contact with wildlife (chimpanzees, gorillas, and other species). Epidemiologic and genetic investigations showed that outbreaks resulted from the introduction of distinct strains, indicating that multiple ZEBOV strains were co-circulating in this region. All index cases (mainly hunters) were infected by handling dead or wounded animals, and subsequently led to person-to-person transmission within their families. In many instances human infections have been preceded by disease in wildlife, and these infected animals acted as either dead-end hosts or interim/amplification hosts.^{106,250} Multiple introductions of MARV lineages were also noticed in the MARV outbreak in Durba/Watsa.^{22,23,65,400,401}

Filoviruses are transmissible to nonhuman primates in the laboratory by aerosols,^{25,217} and virions have been identified in alveoli of infected monkeys and humans.^{141,202,469,470} Furthermore, the outbreak caused by REBOV among quarantined monkeys in 1989/1990 was strongly suggestive of droplet and/or small-particle aerosol transmission. However, these animals were housed in a poorly ventilated building in which aerosols could have been generated by cleaning procedures.²⁰⁵ Aerosol transmission has not been unequivocally implicated in human outbreaks to date. Interestingly, extremely efficient person-to-person transmission has been attributed to two individuals who may have been the source of infection for over 50 cases in the 1995 ZEBOV outbreak.²²³ The mechanism of this heightened transmission was not identified, although contact with the patient and/or cadaver was strongly implicated. Despite little evidence for aerosol transmission in nature, this is the most likely route used for delivery of filoviruses in a deliberate act.

ECOLOGY

The natural reservoir(s) of filoviruses remains elusive despite increased numbers of outbreaks and opportunities to investigate their origins.^{106,155,318} As classical zoonotic agents, these viruses likely persist in an animal (or several animals) or arthropods, which transmit the virus directly to humans, great apes, nonhuman primates, or an interim amplifying host.^{113,288}

Lack of replication in arthropod cells or inoculated arthropods⁴⁰⁴ argues against such an intermediary for filoviruses, and extensive arthropod field surveys have failed to detect the presence of EBOV. It has been suggested that human contact with filovirus-infected bats may have initiated the early SEBOV outbreaks in Sudan,⁸ the ZEBOV outbreak in the DRC in 2007²⁴⁸ and the MARV infections in Kenya.^{218,371} In addition, the outbreak of MHF in the northeast region of the DRC had some connection, directly or indirectly, with a bat-infested gold mine.²² Interestingly, experimental infection of wild African fruit and insectivorous bats has shown that these animals are capable of supporting the replication of EBOV without becoming ill, despite high levels of circulating virus.³⁸³ Recent findings of asymptomatic EBOV and MARV infections in fruit bats are additional evidence that such animals are capable of harboring filoviruses and may serve as reservoir species; in particular *Rousettus aegyptiacus* for MARV.^{249,400} Because persistently infected hosts are postulated for zoonotic diseases, chronic infection in bats or other small animal species is likely involved in the ecology of filoviruses.^{317,318}

The epizootics caused by REBOV have raised the question as to whether nonhuman primates act as reservoirs for filoviruses.²⁸⁵ This seems unlikely for the African filoviruses, which are highly pathogenic for nonhuman primates, and this trait is generally incongruous with the concept of a reservoir host. In addition, there has been no evidence for latent virus infection in these animals.^{109,113} If monkeys are not the reservoir, they at least act to amplify the virus in the wild, and unexplained disease/mortality in these animals could be an indicator of impending transmission in humans. This is supported by reported deaths in monkey species prior to outbreaks of CIEBOV in the Tai Forest,²⁴³ several of the ZEBOV outbreaks occurring since 1996 in Gabon,^{250,252} and a more recent outbreak of ZEBOV in the RC.¹¹⁵ Investigations into the outbreaks in Gabon/RC confirmed this concept and showed multiple introductions of different ZEBOV strains from an unknown reservoir into wildlife that then served as sources of initial human infections.^{250,337} In contrast, filovirus sequences from patients involved in a distinct epidemic chain of human cases were conserved, indicating that these episodes were mainly caused by single-source introductions.^{245,250,333} Thus, it appears that distinct filovirus strains have evolved to occupy undefined ecologic niches throughout the forests of Central Africa, and that the potential for human contact with these agents is greatly increased when viruses are circulating in indigenous nonhuman primate populations.

Beginning in 1994, the frequency of filovirus outbreaks in Africa increased and shows no sign of diminishing. Future episodes of EBOV and MARV transmission are unavoidable, which poses a serious risk to human populations, but also threatens to decimate the world's largest populations of gorillas

and chimpanzees.^{100,250,422} This dangerous situation makes the identification of the natural reservoir an important objective for the scientific community, which needs to improve and formulate new working hypotheses and strategies. Efforts at ecologic niche modeling from data gleaned from outbreaks and sporadic cases have revealed a different Afrotropic distribution for filoviruses, with EBOV more likely to occur in the humid rain forests of Central and Western Africa and MARV in the drier and more open areas of Central and East Africa.^{155,317} Future surveillance at existing field sites (e.g., Tai Forest, Ivory Coast; Watsa/Durba, DRC; Gabon, DC; Uíge, Angola) should focus on affected animal populations, such as great apes, and possibly include the use of sentinel animals. Experimental studies of potential reservoir species should be initiated and/or intensified to better understand filovirus persistence and transmission.

CLINICAL FEATURES

Filovirus infections are generally the most severe of the VHF, but only limited information is derived from close observations of acute human cases. Differences in the clinical syndromes caused by filoviruses may exist, but there have been few opportunities for close observation of the diseases under favorable conditions.^{13,51,97,102,114,122,197,210,211,276,282,312} The abrupt onset follows an incubation period of 2 to 21 days, averaging 4 to 10 days, and is characterized by flu-like symptoms (fever, chills, malaise, and myalgia; Fig. 32.12). The subsequent signs and symptoms indicate multisystem involvement and include systemic (prostration), gastrointestinal (anorexia, nausea, vomiting, abdominal pain, diarrhea), respiratory (chest pain, shortness of breath, cough), vascular (conjunctival injection, postural hypotension, edema), and neurologic (headache, confusion, coma) manifestations. Hemorrhagic manifestations develop during the peak of the illness and include petechiae, ecchymoses, uncontrolled oozing from venipuncture sites, mucosal hemorrhages, and postmortem evidence of visceral hemorrhagic effusions. Often a macropapular rash associated with varying degrees of erythema

appears by days 5 to 7 of the illness; this is a valuable differential diagnostic feature and is usually followed by desquamation in survivors. Abdominal pain is sometimes associated with hyperamylasemia and true pancreatitis. In later stages, shock, convulsions, severe metabolic disturbances, and, in more than half the cases, diffuse coagulopathy supervenes (Fig. 32.12).

Laboratory parameters are less characteristic but the following findings are associated with the disease. There is an early leukopenia (as low as 1,000/ μ L) with lymphopenia and subsequent neutrophilia, left shift with atypical lymphocytes, thrombocytopenia (50,000–100,000/ μ L), markedly elevated serum transaminase levels (AST typically exceeding ALT), hyperproteinemia, and proteinuria. Prothrombin and partial thromboplastin times are prolonged and fibrin split products are detectable. In a later stage, secondary bacterial infection may lead to elevated white blood counts.

Nonfatal cases have fever for about 5 to 9 days and improvement typically occurs around days 7 to 11, about the time the humoral antibody response is noted.²³⁵ Convalescence is prolonged and sometimes associated with myelitis, recurrent hepatitis, psychosis or uveitis.^{97,102,276,282,338} There is an increased risk of abortion for pregnant women, and clinical observations indicate a high death rate for children of infected mothers. Fatal cases develop clinical signs early during infection and demise typically occurs between days 6 and 16, due to hemorrhage and hypovolemic shock. The mortality from ZEBOV infections is high (60–90%), SEBOV and BEBOV somewhat lower (50–60% and 25–35%, respectively), and MARV probably around 70% to 85%, with the exception of the outbreak in Europe (only 23%).^{97,282} The single observed Ivory Coast infection case survived, as did a second serologically diagnosed case. The few REBOV infections/exposures identified so far in Reston, Virginia and in the Philippines had no symptoms, but one patient (accidentally infected during a necropsy of an infected monkey) yielded a serum virus isolate. Thus, it is generally assumed that REBOV has a reduced pathogenicity or is apathogenic for humans, but this judgement may be premature and needs further investigation.

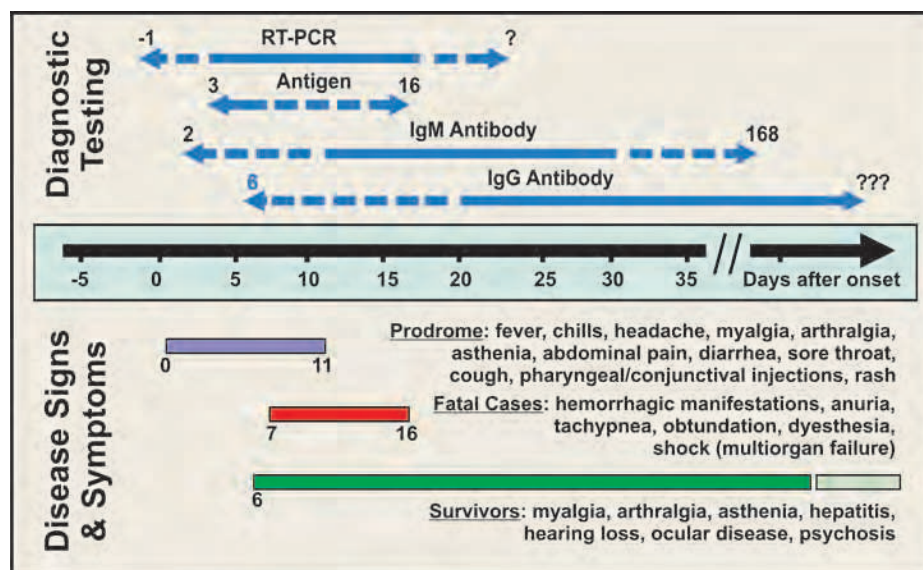


FIGURE 32.12. Graph showing time frames, relative to the time of disease onset, for the sensitivity of diagnostic assays and the development of signs and symptoms associated with severe filovirus infections. Dashed areas on arrows indicate approximate times where clinical features of disease or assay targets can be negative/absent or difficult to detect.

DIAGNOSIS

Clinical Diagnosis

Filovirus disease can be suspected in acute febrile patients with symptoms, as described earlier, and a history of travel to an endemic area. Identification may be difficult, due to a wide variety of infectious diseases causing similar clinical symptomatology. The most common causes of severe, acute, febrile diseases in filovirus-endemic areas are malaria and typhoid fever. A wide range of infectious diseases must also be considered, such as shigellosis, meningococcal septicemia, plague, leptospirosis, anthrax, relapsing fever, typhus, murine typhus, yellow fever, Chikungunya fever, and fulminant viral hepatitis. Rural travel, jungle or cave exposure, treatment in local hospitals, contact with sick persons or wild and domestic animals, particularly monkeys and apes, are useful historical features, especially in travelers returning from Africa. For patients with filovirus disease, prostration, lethargy, wasting, and diarrhea seem to be more severe than is seen with other VHF infections; the appearance of a characteristic rash is useful in narrowing the differential diagnosis. Diagnosis of single cases is extremely difficult, but the occurrence of clusters of cases with prodromal fever followed by hemorrhagic diatheses and person-to-person transmission is suggestive of VHF and require the implementation of containment procedures.

Laboratory Diagnosis

Despite the capabilities of laboratory diagnostics, it should be kept in mind that initial diagnosis of filovirus infections will be based on clinical assessment. Clinical microbiology and public health laboratories are generally ill equipped to diagnose VHF infections, particularly those caused by filoviruses, so specimens should be sent to national and/or international reference laboratories capable of performing the required testing. In addition, many nations encounter difficulties in sample transport, which can cause substantial delays in laboratory response. Once samples are received by appropriate reference laboratories, response is fairly efficient.

During outbreaks health care workers, who have direct contact with patients, are at high risk for infection; adequate barrier nursing precautions should be implemented in the collection of samples.^{314,373} Special care should be taken to avoid needle sticks and to immediately dispose of contaminated material in an appropriate manner. Collection of specimens should be done facilitating sterility and prevention of cross-contamination of specimens. This has become particularly important for ultra-sensitive techniques such as reverse transcriptase–polymerase chain reaction (RT-PCR).³⁷³ Filoviruses are relatively stable and infectious particles can survive less than favorable handling and shipping for months. Care should be taken to ensure the physical integrity for biosafety reasons and to maintain an adequate refrigerated or frozen state for biologic integrity of the sample to maximize the reliability of diagnostic results.

Laboratory diagnosis of filovirus infections can be achieved in two ways: measurement of host-specific immune responses to infection and detection of virus particles or particle components (RNA and protein) in infected individuals (Table 32.3). Today, RT-PCR^{81,151,350,373,402} and antigen detection ELISA^{193,234,235,265,266,304,373} are the primary test systems to diagnose an acute infection. For antibody detection the most commonly used assays are direct IgG and IgM ELISAs and IgM capture ELISA.^{195,235,236,373} RT-PCR, antigen detection, and serology can be performed on materials that have been rendered non-infectious by radiation or chemicals. Gamma irradiation is an efficient means of inactivating specimens prior to antigen detection and serology and is achieved by exposure to a cobalt-60 source. Samples for RT-PCR (nucleic acid extraction) can be treated with guanidinium isothiocyanate, a chaotropic agent that denatures proteins and renders the sample noninfectious. These methods of inactivation allow the safe manipulation of material outside of the containment laboratory, where work can be carried out more expeditiously.

Other serologic tests that have been used in filovirus diagnosis are the western blot assay (can be used as a confirmatory assay) and the indirect immunofluorescence assay (IFA; occasionally used as a screening assay) on gamma-inactivated, fixed

TABLE 32.3 Laboratory Assays Used in the Diagnosis of Filovirus Infections

Test	Target	Source	Remarks
A. Primary Assays			
Polymerase chain reaction (PCR)	Viral nucleic acid	Blood, serum, tissues	Rapid and sensitive; requires special equipment
Antigen enzyme-linked immunosorbent assay (ELISA)	Viral antigen	Blood, serum, tissues	Rapid and sensitive; requires special equipment, but capable of high throughput
ELISA (IgM capture, IgG)	Antiviral antibodies	Serum	Rapid, specific, and sensitive; slower than IFA when testing small numbers of specimens
B. Confirmatory Assays			
Indirect immunofluorescence assay (IFA)	Antiviral antibodies	Serum	Rapid and simple to perform, but prone to nonspecific positives and subjective interpretation
Western blot	Antiviral antibodies	Serum	Protein specific; interpretation sometimes difficult
Immunohistochemistry	Viral antigen	Tissues (e.g., skin, liver)	Slow; material inactivated
Fluorescence assay (FA)	Viral antigen	Tissues (e.g., liver)	Rapid and easy, but interpretation is subjective
Electron microscopy	Viral particle	Blood, tissues	Unique morphology (immunostaining possible); insensitive and requires expensive equipment
Virus isolation	Viral particle	Blood, tissues	Slow; virus isolate available for studies

cells infected with EBOV or MARV or containing expressed recombinant filovirus proteins.^{194,215,216,373} Due to relatively high viremia levels in humans, electron microscopy has been helpful in diagnosis of filovirus infections but is not generally used.^{139,144,203,294,364} Filovirus particles present in sera and cell culture fluids (primary isolation) can be directly visualized by negative staining, and can be easily detected in thin sections of infected tissues, especially the liver and spleen. IHC on formalin-fixed material and paraffin-embedded tissues can be used for detection of filoviruses^{203,470} as well as immunofluorescence (IF) on impression smears of tissues.³³⁵ IHC is a very useful surveillance assay especially when no other specimens are available during an outbreak. Advantages of IHC testing include its simplicity and specificity but also lack of a need for enhanced biocontainment,^{260,469,470} as formalin-fixed biopsy specimens are not infectious, are easily generated, and can be transported without special precautions or refrigeration.

Isolation of infectious virus from serum or other clinical material is a relatively simple and sensitive procedure and should always be attempted if BSL-4 containment is available. Filoviruses grow well in a large variety of cell lines, although Vero cells (or the Vero E6 clone) have been most used, but often upon primary isolation the development of CPE may be subtle or lacking. Guinea pigs can be used for primary isolation of those filoviruses that initially do not grow well in tissue culture, but repeated passaging is usually required to produce severe/fatal disease. In addition, broad clinical syndrome-based technologies have been developed on the basis of multiplex PCR and pan-microbial oligonucleotide array technologies.^{259,309} These assays, however, have yet to be implemented into common diagnostic settings.

Of the available techniques for diagnosis, antigen-capture ELISA and RT-PCR are today the most useful for making a diagnosis in an acute clinical setting. Viral antigen/nucleic acid can be detected in blood as early as day -1 until past day 16 post-onset of symptoms (Fig. 32.12).^{97,282,338} RT-PCR assays seem to be favored by many investigators because BSL-4 biocontainment is not necessary after proper inactivation, as well as the sensitivity/specificity and rapidity of the technique.^{151,373} However, the diagnosis of index cases of outbreaks or of single imported cases should not be solely based on RT-PCR. Confirmation by an independent assay such as antigen-capture ELISA should always be attempted. When case confirmatory techniques and biocontainment (virus isolation) are not available, RT-PCR on an independent target gene and/or independent sample should be the minimum confirmation.¹⁵¹ In such instances it may be useful to seek confirmation through another reference laboratory, which is always preferred.

Serology can be useful for confirmation, but it should be kept in mind that a negative serology is inconclusive because filovirus-infected individuals often die without seroconversion. Based on past investigations, IgM antibodies can appear as early as 2 days post-onset of symptoms and disappear between 30 and 168 days after infection. IgG-specific antibodies develop between days 6 and 18 after onset and persist for many years (Fig. 32.12).^{97,282,338} A rising IgM or IgG titer constitutes a strong presumptive diagnosis. However, a single positive result should be confirmed on a follow-up sample, preferably at least a week apart. Decreasing IgM and/or increasing IgG titers (fourfold) in successive paired sera are highly suggestive of a recent infection.

Standardization and evaluation of diagnostic procedures for filoviruses is difficult because of the restricted availability

of virologic and clinical material. The European Network for Imported Viral Diseases (ENIVD) provides external quality assurance for filovirus RT-PCR diagnostic procedures.³⁰³ Continued and extended quality assurance studies are required to maximize the robustness of filovirus diagnostic procedures.

Filovirus outbreaks usually occur in remote areas where sophisticated medical support systems are limited and timely diagnostic services are extremely difficult to provide. Provision of a fieldable laboratory offering basic diagnostics for filoviruses and other agents that may be confounding to the diagnosis could aid in the management of patients specifically and the outbreak in general. The development of truly portable real-time thermocyclers and fieldable immunologic assays has made the deployment of a field diagnostic laboratory a reasonable undertaking. In setting up field diagnostics, the initial and most important consideration is to minimize the exposure of workers to infectious materials. This can be accomplished by setting up a portable class III biosafety cabinet, but the use of personal protective equipment, such as powered air-purifying respirators (PAPRs) is perhaps a more convenient and realistic means of protecting workers processing infectious material in the field. Clinical and other specimens can be safely heat inactivated (together with an appropriate ionic or nonionic detergent) for serologic analysis and with guanidinium isothiocyanate buffers for RNA isolation.^{151,402}

PREVENTION AND CONTROL

Patient Management

Devising a strategy for the prevention of primary filovirus infections of humans is problematic, as the natural reservoirs and factors that affect filovirus movement in the wild are still largely unknown. Assuming that bats serve as a reservoir, proper education seems the most feasible way of prevention.²⁴⁸ Except for instances in which infected great apes have been the source of infections, it is difficult to identify a human index case, let alone the type of contact that initiated the infection. However, once it enters a human or nonhuman primate population, it is clear that the virus is spread through close contact with acutely infected members. Isolation of patients and use of strict barrier nursing procedures, including the use of protective clothing and respirators, have been sufficient to rapidly interrupt transmission in the hospital.^{65,211} Cadavers from fatal cases represent a residual risk for community members, and unprotected handling of corpses should be avoided.³³² Under specific circumstances, the use of full-face respirators or PAPRs for protection against aerosols may be indicated (79; <http://www.cdc.gov/ncidod/dvrd/spb/mnpages/vhfmanual.htm>); an N95/N100 mask with face shielding can provide a good level of protection. Methods for implementing barrier nursing, waste disposal, and other key elements (inexpensive and practical in Africa) have been devised, and a field-tested manual is available.²⁶⁰ One of the important elements is provision of sterile equipment for injections, which are remarkably and tragically lacking in Africa today.

Therapeutic Options

Filovirus infections are currently managed solely with supportive therapy, which is directed towards maintenance of effective blood volume and electrolyte balance. Shock, cerebral edema,

TABLE 32.4 Selected Promising Treatment Options

Treatment option	Success	Issues/concerns
Antibody therapy	Efficacy in rodents and nonhuman primates	Escape mutants; genetic variability; antibody-dependent enhancement (ADE)
Antisense oligonucleotides		
Phosphorodiamidate morpholino oligonucleotides (PMOs)	Efficacy in rodents and nonhuman primates (NHPs) (prophylactic only)	Genetic variation; delivery
Small interfering RNAs (siRNAs)	Efficacy in rodents and NHPs	Genetic variation; delivery
Inflammatory modulators		
Type I interferons	Efficacy in rodents but not in NHPs	Manipulation of immune system
S-adenosylhomocysteine hydrolase (SAH) inhibitors	Efficacy in rodents but not in NHPs	Manipulation of immune system
Coagulation modulators		
Heparin sulfate	Efficacy in humans questionable; not tested in animals	Manipulation of coagulation
Tissue factor pathway inhibitors	Not tested in rodents; partial protection in NHPs	Manipulation of coagulation
Activated protein C	Not tested in rodents; partial protection in NHPs	Manipulation of coagulation
Postexposure vaccination		
Vesicular stomatitis virus (VSV)	Efficacy in rodents and NHPs	Efficacy dependent on species and treatment start

Note: Only approaches that have shown *in vivo* efficacy have been listed.

renal failure, coagulation disorders, and secondary bacterial infection may be life threatening and have to be managed. Antipyretics and pain medication should be provided as needed. At present, there is no proof that any strategy has been successful; however, detailed knowledge of filovirus replication, pathogenesis, and host responses to infections has steadily identified new targets for therapeutic intervention (Table 32.4). Although no single treatment is likely to be sufficiently potent to offset the severe and rapid progression of EHF and MHF, a combination of therapies (with different mechanistic actions) may be a more effective approach to treating infections. Slowing disease progression may provide enough time for the adaptive immune response to develop enough momentum to clear the infection.^{42,97,101,282}

Over the years, several experimental therapeutic approaches that target the virus or the host immune response have been evaluated in different animal models.^{30,42,97,282} The use of specific antisera as a therapy for filovirus infections (Table 32.4) has been investigated since their discovery,^{33,238} but their development has been complicated by lack of efficacy and reports on the potential of antibody-dependent enhancement (ADE) of infection as demonstrated *in vitro*^{388,394} but not yet confirmed *in vivo*.¹³² Convalescent blood and serum have been administered to human patients,^{87,293} but any beneficial results from these treatments are either unsupported or conflicting. Antibody therapy with equine anti-EBOV immunoglobulin has failed to protect nonhuman primates from fatal outcomes.^{206,284} However, more recently, the first successful IgG transfer that protected rhesus macaques from lethal MARV and EBOV challenge was reported,⁸² which might indicate that antibody therapy could be more beneficial. *In vitro* neutralizing EBOV GP-specific monoclonal antibodies generated from different species, including human, showed distinct protective and therapeutic properties in rodent and nonhuman primate models.^{277,308,311,325a,389,446} Although no definite therapeutic

conclusion can yet be drawn from the current studies, passively acquired antibodies can to a certain extent reduce the viral burden during infection, which could be useful in combination with other pharmaceutical agents.

Because filoviruses inhibit the expression of type I interferons (discussed earlier), treatment with exogenous IFN would seem to be an obvious therapeutic approach (Table 32.4). However, despite efficacy in the mouse model, IFN- α treatment was less effective in guinea pigs and failed to increase survival rate in nonhuman primates, despite a delay in disease onset, viremia, and death.²⁰⁴ The beneficial effect of S-adenosylhomocysteine hydrolase (SAH) inhibitors on EBOV infection in mice¹⁸⁵ has been partially associated with a mechanistically unexplained strong increase of IFN- α production. Therefore, improved formulations, selective use of IFN types and IFN- α subtypes, and the combination with other treatment options may be useful.

The viral transcription and replication machinery is an important antiviral target (Table 32.4). Unfortunately, ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a broad-spectrum synthetic guanosine analog with virustatic activity against a number of RNA viruses including arenaviruses and bunyaviruses, has no *in vitro* or *in vivo* effect on filoviruses.^{184,190} Recent strategies to interfere with transcription and replication include antisense oligonucleotides, phosphorodiamidate morpholino oligomers (PMOs) and RNA interference (RNAi).^{90,116,136,142,387,431,433,434} These approaches are promising, but may be limited by sequence specificity (genetic variation of species), production (high costs) and the administration route (mainly intravenous).

Vaccine vectors based on recombinant vesicular stomatitis virus (VSV) expressing EBOV or MARV GP^{121,130} have shown remarkable utility when administered to nonhuman primates 30 minutes and up to 48 hours following lethal EBOV and MARV infection, respectively.^{71,99,129,133} This strategy was

used in a recent needle stick injury with high-risk exposure to ZEBOV. It is currently unclear whether the treatment prevented infection or the incident did not lead to infection, but there were no adverse effects noted with the administration of the vaccine vector.¹⁵⁸ Postexposure vaccination with VSV-based vectors is species specific due a lack of cross-protection among the various EBOV species.^{93,130}

The targeting of host gene products might also prove to be beneficial in treating filovirus disease. The production of TNF- α can develop into a deleterious host response during infection, and the therapeutic use of anti-TNF- α neutralizing antibodies has been partially successful in rodent models,^{189,190,191} but has not been evaluated in nonhuman primates. Furthermore, inhibitors of apoptosis of lymphocytes during EBOV infection^{134,177} might be a possible intervention strategy. There have been relatively few attempts to modulate the dysregulated cytokine/chemokine response that is a consistent feature of many VHF. Treating MARV-infected guinea pigs with Desferal, an IL-1 and TNF- α antagonist, partially protected these animals.^{190,191} In another study, treating MARV-infected guinea pigs with IL-1 receptor antagonist (IL-1RA) or anti-TNF- α serum decreased the concentration of circulating TNF- α and protected 50% of the animals from lethal infection.¹⁸⁹

Coagulation abnormalities are a hallmark of filovirus infections and considered a key factor in pathogenesis. The nematode-derived anticoagulation protein (rNAPc2) was used to treat ZEBOV-infected nonhuman primates and resulted in 33% survival in an otherwise uniformly lethal animal model.¹³⁵ The same treatment has shown reduced efficacy when administered to animals infected with MARV, Angola strain, but other less virulent strains have not been tested yet¹²⁷ (Table 32.4). D-dimer formation has been identified as an early event during EBOV infection in nonhuman primates and could be used as a marker for treatment.¹³⁵ As rNAPc2 primarily targets signaling through the extrinsic blood coagulation pathway, additional benefits might be realized by using inhibitors of Factor X, targeting the common pathway, thereby blocking signaling through both the extrinsic and intrinsic blood coagulation pathways. A recent study showed that treatment of ZEBOV-infected nonhuman primates with recombinant human activated protein C resulted in partial protection consistent with survival seen with rNAPc2.¹⁷⁶ Both drugs have been approved for different applications in humans and could be more easily and safely considered under emergency use protocols.

Given the severe and rapid progression of filovirus infections, monotherapy is unlikely to be effective versus combination approaches that interfere with disease progression to allow innate and adaptive immune responses to overcome infection.^{42,101} Viremia levels below $1 \times 10^{4.5}$ pfu/mL are strongly associated with survival of patients and experimentally infected nonhuman primates.⁹⁷

Prophylaxis

Protective EBOV and MARV vaccines would be extremely valuable for at-risk medical personnel, first responders, military personnel, researchers, and populations affected during a filovirus outbreak. Attempts to produce vaccines from cell culture-derived filovirus particles (inactivated with formalin, heat, or gamma irradiation) have not been effective in stimulating protective immune responses.⁹³ More recently, vaccine development has concentrated on the use of subunit vaccines

based on a single, or combination of, viral structural proteins to induce protective immunity against an EBOV challenge. Aside from minor efforts to use the viral structural proteins VP24, VP30, VP35, and VP40 as immunogens, GP and to a lesser extent NP are the key viral proteins used for vaccination approaches. The following different delivery/expression systems have been evaluated in established animal models³⁰: naked DNA^{80,271,283,407,459,464}; virus-like particles (VLPs)^{382,385,386,427,429,430,432,437,466}; replication-deficient vectors such as adenovirus^{68,125,228,240,313,320,331,339,377,378,381,424,425,426,464}; Venezuelan equine encephalitis virus (VEEV) replicons^{178,242,324,325}; and replication-competent live attenuated vectors such as vaccinia virus,^{62,150} vesicular stomatitis virus (VSV),^{70,126,128,131,220,221} human parainfluenza virus type 3 (HPIV3),^{45,46,47,50} and New Castle disease virus (NDV).⁷⁷ Recently, a new concept based on replication-deficient ZEBOV (lacking VP35) generated through “reverse genetics” has shown promising protective efficacy in rodent models,¹⁶³ but remaining safety issues need to be addressed prior to generating proper vaccine candidates.

Most of the vaccine approaches showed protective efficacy in rodent models, but several failed to protect nonhuman primates.^{93,143,130} Currently, at least five different vaccine systems (based on adenovirus serotype 5 [Ad5], VEEV, VSV, HPIV3, and VLPs) have demonstrated complete protection against lethal filovirus infections in nonhuman primates, the gold standard animal model. Of those, the replication-deficient Ad5 system is the furthest developed platform and has already been in phase I clinical trials.²⁴⁰ This platform has been further developed by others using a multivalent adenovirus technology for the development of a panfilovirus vaccine that provides protection against several filovirus species.³⁸⁴ In addition, effective mucosal delivery seems possible. The Ad5 platform seems safe and robust but suffers from preexisting immunity in the world population and the recent failure in an AIDS/HIV trial.^{37,64} Preexisting immunity might be bypassed through mucosal delivery, which would also be beneficial for mass vaccination and for administration in rural Africa.^{68,313} The VLP platform, generated by co-expression of the viral proteins VP40, NP, and GP^{429,432} seems to best address safety issues but may require adjuvant and booster immunization for potent efficacy in nonhuman primates, which is less favorable for emergency use. Other issues are associated with the costs and production of the VLP-based vaccine compared to viral vector-based platforms.

Live attenuated recombinant vaccine vectors may be of advantage over nonreplicating vectors because of their ease in production and their more potent stimulation of innate and adaptive (humoral and cellular) immune responses. It would be difficult to ensure the safety of live attenuated EBOV and MARV strains, because of the high level biohazard of filoviruses. However, promising live attenuated filovirus vaccine vectors have recently been developed based on the backgrounds of VSV and HPIV3.^{93,124,130} The VSV-based vectors are more advanced and have demonstrated efficacy in nonhuman primate models.^{93,124,130} However, the vectors are associated with safety issues despite having a clean record in experimental animal models including immunocompromised animals.^{93,128,130,221} As with Ad5 and HPIV3 vectors, preexisting immunity is negligible for VSV. Both the HPIV and VSV vaccine platforms may have potential for needleless delivery.^{47,326} The VSV-based platform has the potential for a multivalent vaccine protecting against several species of EBOV and MARV¹³¹ and is the only

filovirus vaccine platform with postexposure efficacy in the nonhuman primate model.^{71,99,129,133}

It should be noted here that vaccine efforts for filoviruses have been largely based on ZEBOV and MARV strain Musoke immunogens. In particular, cross-species protection has only been achieved in a few attempts.^{92,93,131,175,278}

Despite largely good to excellent protective efficacy in animal models, correlates and mechanisms of protection have not been well defined for most of the vaccine candidates. Current data on antibody responses, T-cell proliferation, and CTL responses indicate that antibody and T-helper cell memory are essential for protection and that cell-mediated immunity, while possibly important, is not an absolute requirement. Total antibody response is thought to be a correlate for protection for filovirus vaccines.³⁷⁹

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Paramyxoviridae

Classification

The Structure and Replication Strategy of the Paramyxoviridae

Virion Structure

The Paramyxoviridae Genomes and their Encoded Proteins

- The Nucleocapsid Protein
- The *P* Gene and Its Encoded Proteins
- The Large Protein
- The Matrix Protein
- Envelope Glycoproteins
- Other Envelope Proteins

Stages of Replication

- General Aspects
- Virus Adsorption and Entry
- Viral RNA Synthesis
- Genome Replication
- Virion Assembly and Release

Molecularly Engineered Genetics (Reverse Genetics)

Viral Accessory Genes and Their Interactions with the Host

- Antagonists of Interferon Synthesis
- Antagonists of Interferon Signaling Pathways

The *Paramyxoviridae* include some of the great and ubiquitous disease-causing viruses of humans and animals, including one of the most infectious viruses known (measles virus), some of the most prevalent viruses known (measles virus, parainfluenza viruses [PIVs], mumps virus, respiratory syncytial virus [RSV], and metapneumovirus), a virus that has been targeted by the World Health Organization for eradication (measles virus; however, to date, eradication has failed), a virus that has been eradicated (rinderpest virus), viruses that have a major economic impact on poultry rearing (Newcastle disease virus [NDV]), and many recently identified viruses (pinniped morbilliviruses, Hendra virus, Nipah virus, J virus and Beilong virus), some of which cause deadly diseases (Hendra and Nipah viruses). The *Paramyxoviridae* are enveloped negative-stranded RNA viruses that have special relationships with two other families of negative-strand RNA viruses, namely the *Orthomyxoviridae* (for the biological properties of the envelope glycoproteins) and the *Rhabdoviridae* (for the similarity of organization of the nonseg-

mented genome and its expression). The *Paramyxoviridae* are defined by having a protein (F) that causes viral–cell membrane fusion, in most cases at neutral pH. The genomic RNA of all negative-strand RNA viruses has to serve two functions: first as a template for synthesis of messenger RNAs (mRNAs) and second as a template for synthesis of the antigenome positive strand. Negative-strand RNA viruses encode and package their own RNA polymerase (RNAP); however, mRNAs are only synthesized once the virus has been uncoated in the infected cell. Viral replication occurs after synthesis of the mRNAs and requires the continuous synthesis of viral proteins. The newly synthesized antigenome positive strand serves as the template for further copies of the negative-strand genomic RNA.

CLASSIFICATION

The family *Paramyxoviridae* is classified into two subfamilies: the *Paramyxovirinae* and the *Pneumovirinae*. The *Paramyxovirinae* contains seven genera: *Respirovirus*, *Rubulavirus*, *Morbillivirus*, *Henipavirus*, *Aquaparamyxovirus*, *Avulavirus*, and *Ferlavirus*. The *Pneumovirinae* contains two genera *Pneumovirus* and *Metapneumovirus*. The classification is based on morphologic criteria, the organization of the genome, the biological activities of the proteins, and the sequence relationship of the encoded proteins now that all of the genome sequences have been obtained. The more recently identified tree shrew (*Tupaia*) paramyxovirus, J virus, Beilong virus, Salem virus, Menangle virus, Mossman virus, Fer-de-Lance virus, and Tioman virus have yet to be officially classified within the *Paramyxovirinae* by the International Committee on the Taxonomy of Viruses.

The morphologic distinguishing feature among enveloped viruses for the subfamily *Paramyxovirinae* is the size and shape of the nucleocapsids (diameter 18 nm, 1 μ m in length, a pitch of 5.5 nm), which have a left-handed helical symmetry. The biological criteria are (a) antigenic cross-reactivity between members of a genus and (b) the presence (*Respirovirus* and *Rubulavirus*) or absence (*Morbillivirus* and *Henipavirus*) of neuraminidase (NA) activity. In addition, the differing coding potentials of the *P* genes are considered, and there is the presence of an extra gene (*SH*) in some rubulaviruses as well as J virus and Beilong virus. The pneumoviruses can be distinguished from *Paramyxovirinae* morphologically, as they contain narrower nucleocapsids. In addition, the *Pneumovirinae* have major differences in the number of encoded proteins and an attachment protein that is very different from that of *Paramyxovirinae*. Examples of members of various genera are shown in Table 33.1.

TABLE 33.1 Examples of Members of the Family *Paramyxoviridae*

Family <i>Paramyxoviridae</i>
Subfamily <i>Paramyxovirinae</i>
Genus <i>Rubulavirus</i>
Mumps virus (Mu V)
Parainfluenza virus type 5 (previously called simian virus 5 [SV5] (PIV5))
Human parainfluenza virus type 2, types 4a and 4b (HPIV2/4a/4b)
Mapuera virus
Porcine rubulavirus (La-Piedad-Michoacan-Mexico virus)
Genus <i>Avulavirus</i>
Newcastle disease virus (avian paramyxovirus 1) (NDV)
Genus <i>Respirovirus</i>
Sendai virus (mouse parainfluenza virus type 1) (SeV)
Human parainfluenza virus type 1 and type 3 (HPIV1/3)
Bovine parainfluenza virus type 3 (bPIV3)
Genus <i>Henipaviruses</i>
Hendra virus (HeV)
Nipah virus (NiV)
Genus <i>Ferlavirus</i>
Fer-de-Lance virus (FDLV)
Genus <i>Aquaparamyxovirus</i>
Atlantic salmon paramyxovirus
Genus <i>Morbillivirus</i>
Measles virus (MeV)
Cetacean morbillivirus
Canine distemper virus (CDV)
Peste-des-petits-ruminants virus
Phocine distemper virus
Rinderpest virus
Subfamily <i>Pneumovirinae</i>
Genus <i>Pneumovirus</i>
Human respiratory syncytial virus A2, B1, S2 (HRSV)
Bovine respiratory syncytial virus (BRSV)
Pneumonia virus of mice (PVM)
Genus <i>Metapneumovirus</i>
Human metapneumovirus (HMPV)
Avian metapneumovirus
Unclassified paramyxoviruses
<i>Tupaia</i> paramyxovirus (TPMV)
Menangle virus (MenV)
Tioman virus (TiV)
Beilong virus
J virus
Mossman virus (MoV)
Salem virus (SaV)
Nariva virus

THE STRUCTURE AND REPLICATION STRATEGY OF THE *PARAMYXOVIRIDAE*

Paramyxoviruses contain nonsegmented single-stranded RNA genomes of negative polarity and replicate entirely in the cytoplasm. Their genomes are 15 to 19 kB in length, and the genomes contain 6 to 10 tandemly linked genes. A lipid envelope containing two surface glycoproteins (F and a second gly-

coprotein variously referred to as HN, or H or G) surrounds the virions. Inside the envelope lies a helical nucleocapsid core containing the RNA genome and the nucleocapsid (N), phospho- (P), and large (L) proteins, which initiate intracellular virus replication. Residing between the envelope and the core lies the viral matrix (M) protein that is important in virion architecture, and which is released from the core during virus entry. In addition to the genes encoding structural proteins, paramyxoviruses contain “accessory” genes that are found mostly as additional transcriptional units interspersed with the tandemly linked invariant genes. For the *Paramyxovirinae*, the accessory genes are found mostly as open reading frames (ORFs) that overlap within the *P* gene transcriptional unit.

Intracellular replication of paramyxoviruses begins with the viral RNA-dependent RNAP (minimally a homo-tetramer of P and a single L protein) transcribing the N-encapsidated genome RNA (N:RNA) into 5′ capped and 3′ polyadenylated mRNAs. The viral RNA-dependent polymerase (vRNAP) begins RNA synthesis at the 3′ end of the genome and transcribes the genes into mRNAs in a sequential (and polar) manner by terminating and reinitiating at each of the gene junctions. The junctions consist of a gene-end (GE) sequence, at which polyadenylation occurs by the reiterative synthesis of adenylates directed by a template of four to seven uridylates (followed by release of the mRNA), a short nontranscribed intergenic (IG) region, and a gene-start (GS) sequence that specifies mRNA initiation as well as capping. The vRNAP occasionally fails to reinitiate the downstream mRNA at each junction, leading to the loss of transcription of further downstream genes; hence, there is a gradient of mRNA synthesis that is inversely proportional to the distance of the gene from the 3′ end of the genome. After primary transcription and translation, when sufficient amounts of unassembled N protein are present, viral RNA synthesis becomes coupled to the concomitant encapsidation of the nascent [+] RNA chain. Under these conditions, vRNAP ignores all of the junctions (and editing sites) to produce an exact complementary antigenome chain in a fully assembled nucleocapsid.

VIRION STRUCTURE

The *Paramyxoviridae* contain a lipid bilayer envelope that is derived from the plasma membrane of the host cell in which the virus is grown.⁵⁹ *Paramyxoviridae* are generally spherical, 150 to 350 nm in diameter, but can be pleomorphic in shape, and filamentous forms can be observed. Inserted into the envelope are glycoprotein spikes that extend approximately 8 to 12 nm from the surface of the membrane, and that can be readily visualized by electron microscopy. Inside the viral membrane is the nucleocapsid core (sometimes referred to as the ribonucleoprotein [RNP] core) that contains the 15,000 to 19,000 nucleotide single-stranded RNA genome. Figure 33.1 shows a highly stylized schematic diagram of the virion. F and HN are trimers and tetramers, respectively. No attempt has been made to represent the real abundance of F, HN, SH, or N subunits in the virion. The pleomorphic nature of virus particles is illustrated in the electron micrograph in Figure 33.2, and a comparison of the RNPs of influenza virus, rabies virus, and Sendai virus is shown in Figure 33.3.

The helical nucleocapsid, rather than the free genome RNA, is the template for all RNA synthesis. For Sendai virus,

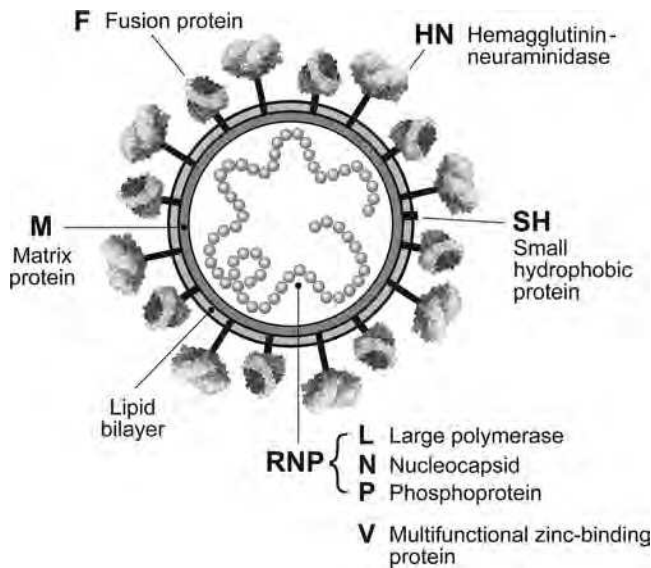


FIGURE 33.1. Schematic diagram of a paramyxovirus (not drawn to scale). The lipid bilayer is shown as the *gray concentric circle*, and underlying the lipid bilayer is the viral matrix protein shown as a *dark gray circle*. Inserted through the viral membrane are the hemagglutinin-neuraminidase (HN) attachment protein and the fusion (F) protein. The relative abundance of HN and F is not illustrated by the diagram. The small hydrophobic protein, SH, is found only in certain rubulaviruses, such as parainfluenza virus type 5 (PIV5). The HN protein is thought to have a stalk region and a globular head, and the F protein consists of two sulfide-linked chains F₁ and F₂. The HN protein is a tetramer, and the F protein a trimer. Inside the virus is the negative-strand virion RNA that is encapsidated with the nucleocapsid protein (N). Associated with the nucleocapsid are the L and P proteins, and together this complex has RNA-dependent RNA transcriptase activity (vRNAP). For the rubulaviruses, the cysteine-rich protein V is found as an internal component of the virion, whereas for other members of the family, the V protein is only found in virus-infected cells. The nature of possible interactions between the cytoplasmic tails of the glycoprotein spikes and the matrix protein, as well as the interactions between the matrix protein and the nucleocapsid, have not been fully elucidated, and no attempt has been made to illustrate them.

each nucleocapsid is composed of approximately 2,600 N, 300 P, and 50 L proteins.²¹⁴ The N and genome RNA together form a core structure, to which the P and L proteins are attached. This nucleocapsid core is remarkably stable, as it withstands the high salt and gravity forces of cesium chloride (CsCl) density gradient centrifugation. In the electron micrograph of nucleocapsids, the P and L proteins are not observed and have only been visualized with the aid of antibodies.³²⁷ Holo-nucleocapsids (N:RNA plus P and L) have the capacity to transcribe mRNAs *in vitro*, presumably mimicking primary transcription in infected cells, and they are thought to be the minimum unit of infectivity.

When negatively stained preparations of paramyxovirus nucleocapsids are viewed in the electron microscope, the most tightly coiled forms resemble the *Tobamovirus* tobacco mosaic virus (TMV)—a relatively rigid coiled rod 18 nm in diameter, with a central hollow core of 4 nm and a helical pitch of nearly 5 nm.^{68,115} Unlike TMV, however, in which the nucleocapsid must disassemble so its positive RNA genome can function as

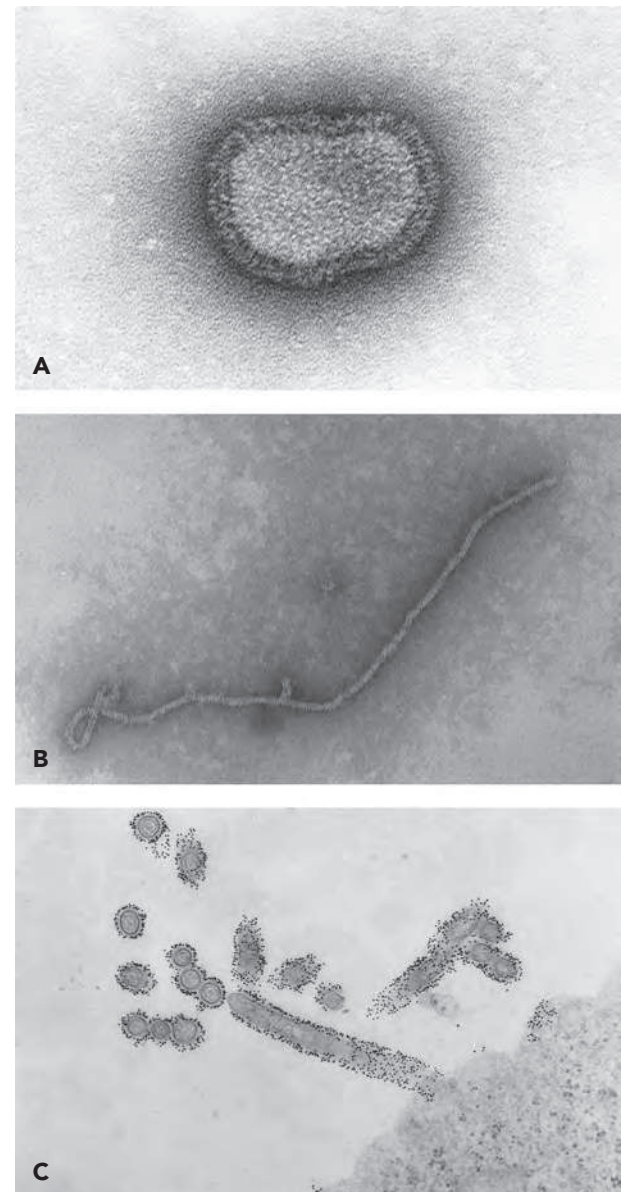


FIGURE 33.2. Ultrastructure of parainfluenza virus type 5 (PIV5; formerly simian virus type 5 [SV5]) virions revealed by negative staining. **A:** Negatively stained PIV5 particle: The glycoprotein spikes on intact 150- to 300-nm virus particles can be observed (226,280 x). **B:** Negatively stained PIV5 nucleocapsid (74,570 x). **C:** Budding PIV5 virions particles from the surface of CV-1 cells: Colloidal gold staining of hemagglutinin-neuraminidase (HN) is shown (24,700 x). (Micrographs courtesy of George Leser, Northwestern University. Copyright © G. D. Park and R. A. Lamb, 2006.)

a template, paramyxovirus nucleocapsids function without disassembling their nucleocapsids.

Sendai virus nucleocapsids exist in several distinct morphologic states at normal salt concentration.^{101,156} The most prevalent form in negatively stained preparations is the most tightly coiled one, with a helical pitch of 5.3 nm. Two other forms—one with a slightly larger pitch of 6.8 nm and another with a much larger pitch of 37.5 nm—have also been noted.

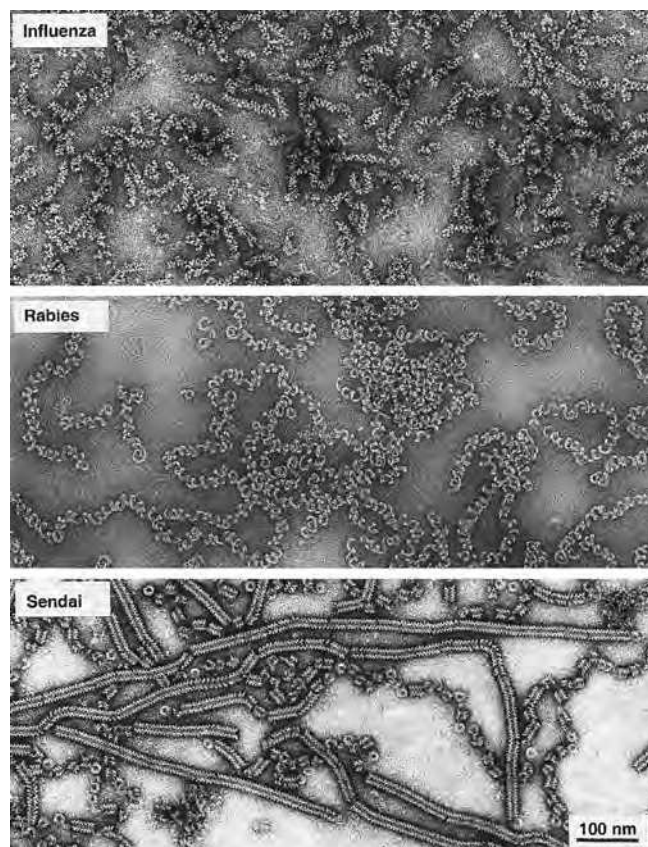


FIGURE 33.3. Nucleocapsids of negative-strand RNA viruses. Electron micrographs of the nucleocapsids of three negative-strand RNA viruses, negatively stained with 1% sodium silicotungstate. **Top:** Ribonucleoprotein particles of influenza virus with a stoichiometry of 24 nucleotides per NP monomer. **Middle:** Nucleocapsids of rabies virus with a stoichiometry of 9 nucleotides per N monomer. **Bottom:** Nucleocapsids of Sendai virus with a stoichiometry of 6 nucleotides per N monomer. All micrographs have the same magnification; bar = 100 nm. (Micrographs courtesy of Rob Ruigrok, EMBL, Grenoble, France.)

The fact that no structures of intermediate pitch have been found indicates that these are distinct states. It is thought that the template is copied without dissociation of N protein from the nucleocapsid and the uncoiling of the nucleocapsid may be necessary for the polymerase to gain access to the RNA bases. It is possible that the vRNAP traverses the nucleocapsid template by uncoiling the helix in front of it and recoiling it once the polymerase has passed a given position, much the same as cellular RNAP generates its template “bubble” in traversing double-stranded DNA (dsDNA).

As expected, the diameter of the nucleocapsid decreases as the pitch increases and the nucleocapsid lengthens; for Sendai virus, the diameter is 3.5 nm less for the 6.8 nm form than for the 5.3 nm pitch form. These latter values are very similar to those of *Pneumovirus* nucleocapsids, which also have a pitch of 7 nm. As discussed earlier, these differences in nucleocapsid morphology are used to distinguish the different *Paramyxoviridae*; however, they probably relate mainly to which form predominates in negatively stained preparations.

THE PARAMYXOVIRIDAE GENOMES AND THEIR ENCODED PROTEINS

The complete genome sequence for all known members of the *Paramyxoviridae* has been obtained (available at <http://www.ncbi.nlm.nih.gov/>). The 15,000 to 19,000 nucleotide genomic RNA contain a 3′ extracistronic region of approximately 50 nucleotides known as the leader and a 5′ extracistronic region of 50 to 161 nucleotides known as the trailer (or [–] leader). These control regions are essential for transcription and replication, and flank the six genes (seven for certain rubulaviruses and eight to ten for pneumoviruses). (Note: By the convention used for paramyxoviruses, the term *gene* refers to the genome sequence encoding a single mRNA, even if that mRNA contains more than one ORF and encodes more than one protein). The coding capacity of the genome of *Paramyxovirinae* is extended by the use of overlapping ORFs in the P gene. The gene order of a representative member of each subfamily is shown in Figure 33.4. At the beginning and end of each gene are conserved transcriptional control sequences that are copied into mRNA. Between the gene boundaries are intergenic regions (Fig. 33.5). These are precisely three nucleotides long for the respiroviruses and morbilliviruses but are quite variable in length for the rubulaviruses (1–47 nucleotides) and pneumoviruses (1–56 nucleotides) (see Fig. 33.5).

The Nucleocapsid Protein

The nucleocapsid (N) protein is present as the first transcribed gene in the viral genome for all paramyxoviruses except the pneumoviruses and ranges in size from 489 to 553 amino acids (molecular weight ~53–57 kDa). N is an RNA-binding protein that coats full-length viral negative sense genomic and positive sense antigenomic RNAs to form the helical nucleocapsid template, which is the only biologically active form of these viral RNAs. Electron microscopy and three-dimensional image reconstruction for Sendai virus nucleocapsids reveals that N binds approximately six consecutive nucleotides and 13 N subunits constitute each turn of the nucleocapsid helix.¹⁰¹ In general, these parameters apply to other paramyxovirus nucleocapsids as well, although there can be slight differences in the number of N subunits per helix turn and in the pitch of the helix.¹⁵ The binding of N to RNA to form a helical structure is thought to serve several functions, including protection from nuclease digestion, minimizing the annealing of mRNA to complementary genomic RNA, alignment of distal RNA segments to create a functional 3′-end promoter, and most likely providing interaction sites for assembly of progeny nucleocapsids into budding virions.

Expression of paramyxovirus N proteins in the absence of other viral components results in the formation of nucleocapsid-like structures, suggesting that N has inherent self-assembly properties and that N–N interactions drive nucleocapsid assembly.^{116,277,287} Biochemical and mutational studies have shown that the N protein can be generally divided into two main structural regions: Ncore, an N-terminal domain representing approximately three-fourths of the protein and is conserved in sequence among related viruses, and Ntail, a C-terminal nonconserved acidic domain. Approximately 400 residues of the Sendai virus Ncore are essential for self-assembly, RNA binding, and activity in RNA replication.⁷⁶

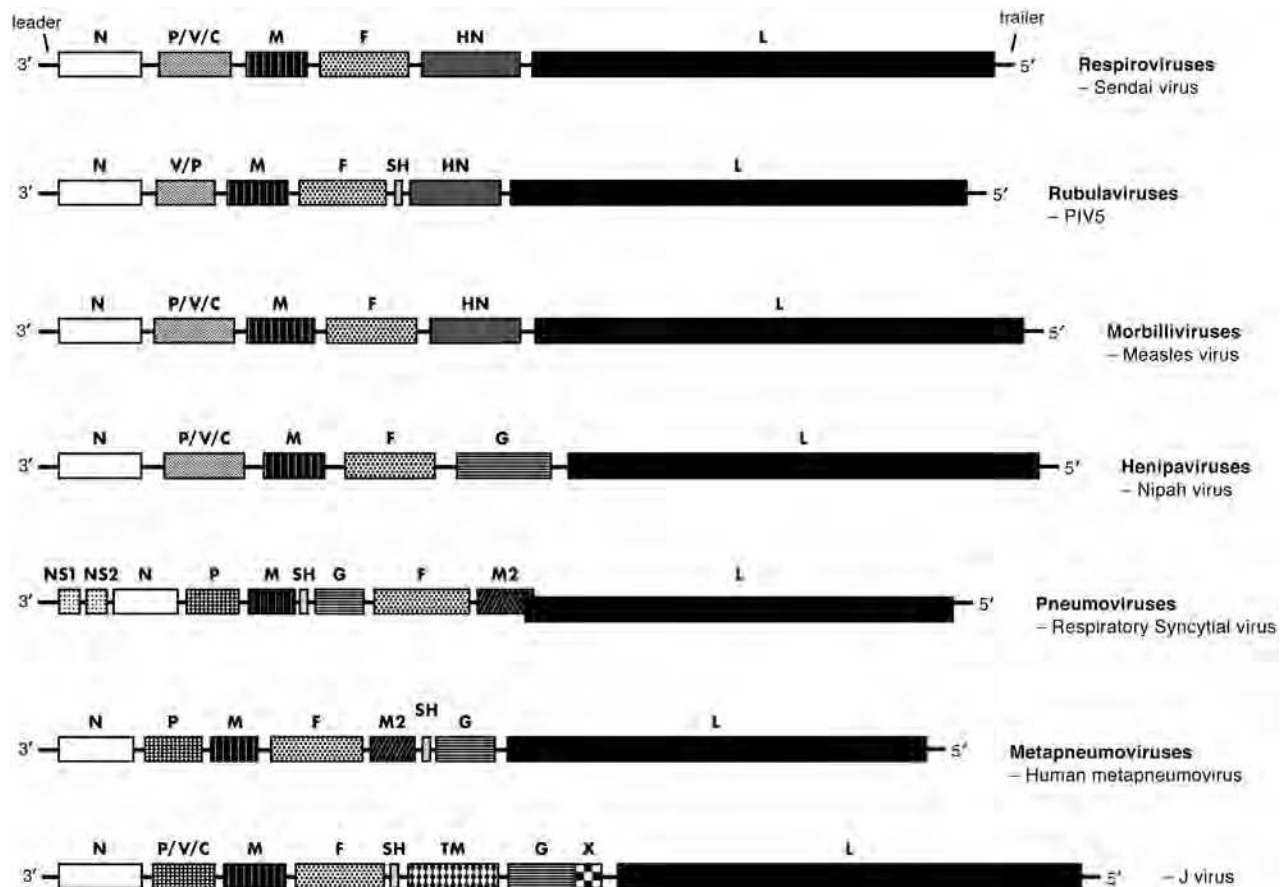


FIGURE 33.4. Genetic map of a typical member of six genera of the *Paramyxoviridae*. The gene sizes are shown as boxes that are drawn to approximate scale, with 3'-leader and 5'-trailer regions indicated for Sendai virus only. Gene boundaries are shown by thin horizontal lines. Note that the beginning of the human respiratory syncytial virus *L* gene overlaps the end of the *M2* gene by 68 nucleotides, whereas human metapneumoviruses do not have an *L*-gene overlap. For J virus, *X* denotes an internal open reading frame in the *G* gene of unknown function.

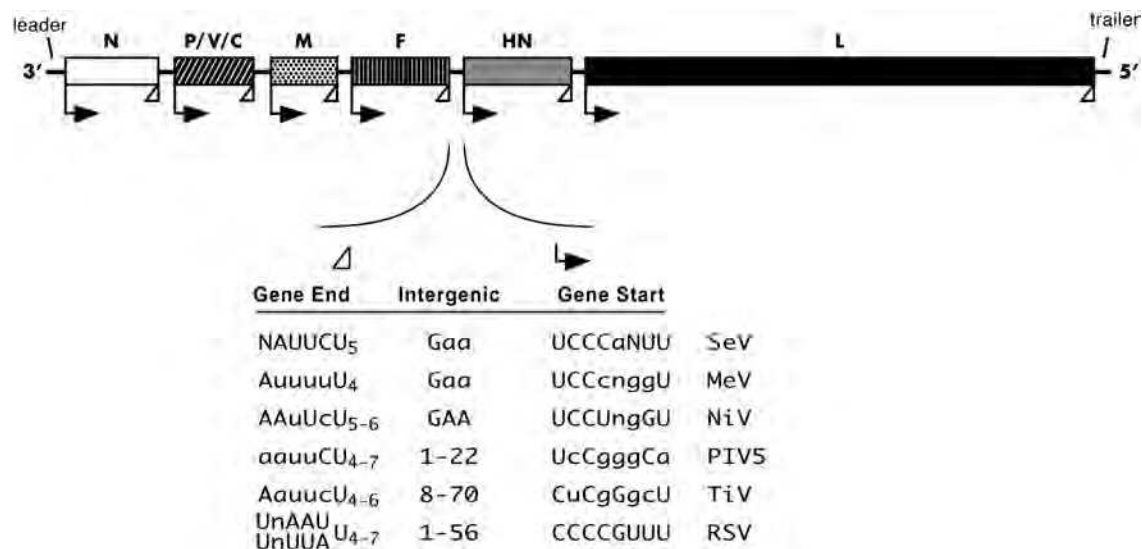


FIGURE 33.5. Schematic diagram of a paramyxovirus genome with the transcriptional gene-end, intergenic, and transcription gene-start sequences. The positions of the extragenic 3'-terminal leader, 5'-terminal trailer, and gene junctions are shown as thin horizontal lines. The conserved gene-end (open triangle) and gene-start (rightward arrow) transcription regulatory sequences at the boundaries between genes are indicated. Consensus sequences for the gene-end, intergenic, and gene-start regions of representative viruses are listed as negative sense genomic RNA. Nucleotides that are strictly conserved at each viral junction are shown as capital letters; nucleotides that are mostly conserved (3/6 junctions or better) are shown in lowercase letters. SeV, Sendai virus; MeV, measles virus; NiV, Nipah virus; PIV5, parainfluenza virus 5; TiV, Tioman virus; RSV, respiratory syncytial virus.

For RSV, the N-terminal 92 residues are sufficient for assembly with RNA.²⁷¹ A central region of Ncore that is highly conserved for all members of the *Paramyxovirinae* (residues 258–369 for Sendai virus) contains an F-X4-Y-X3- ϕ -S- ϕ -A-M motif (where X is any residue and ϕ is an aromatic amino acid). This region is essential for self-assembly of N with RNA²⁷⁶ and may be involved in N–N or N–RNA interactions.

The C-terminal Ntail region is less well conserved among related paramyxoviruses. Treatment of purified nucleocapsids with trypsin removes a portion of the C-terminal Ntail to yield a more rigid structure,¹⁵⁵ suggesting that this domain may confer flexibility in the coiling of the native nucleocapsid. In contrast to the essential role of the N-terminal Ncore in all N functions, the C-terminal Ntail (124 residues for Sendai virus N) is dispensable for binding RNA and for assembly of newly synthesized N–RNA complexes during replication of defective interfering particle RNAs.⁷⁶ However, nucleocapsids that are assembled with N that lacks this C-terminal Ntail are not functional as templates for the viral RNAP.⁷⁶ Structural studies have shown that the C-terminal Ntail is intrinsically disordered,²⁶ consistent with a proposed role for this domain in multiple protein–protein interactions. One of these essential interactions with P protein is thought to tether the L–P polymerase complex to the nucleocapsid template.³⁷ For example, the measles virus C-terminal Ntail has been shown to interact with a C-terminal domain of the P protein, undergoing an induced folding in some parts of this N segment.^{26,186} In the case of measles virus, Ntail has also been shown to bind to the cellular chaperone protein Hsp72⁴⁵⁵—an interaction that could influence nucleocapsid morphologies and the synthesis of viral RNAs.⁴⁵⁵

The paramyxovirus nucleocapsid protein is an unusual RNA-binding protein, as it has an overall acidic charge (net charge of –7 to –12, with exception of mumps virus [+2]) and does not contain conventional RNA-binding motifs that are

typically found on cellular RNA-binding proteins. The interactions of N with RNA are remarkably stable, and nucleocapsid-associated RNA is protected from nucleases even at very high salt concentrations, or when the hypersensitive C-terminal Ntail is removed by protease digestion.¹⁵⁵ N binding to RNA is thought to be independent of nucleotide sequence and, through interactions with the phosphodiester backbone,¹⁸¹ a mechanism that would leave the nucleoside bases accessible to the viral RNAP during RNA synthesis. The Sendai virus nucleocapsid-associated RNA shows hyperreactivity to chemical treatment at cytidine residues predicted to be at positions one and six of a hexamer of nucleotides.¹⁸¹ Together with the finding that the Sendai virus N protein binds six nucleotides,¹⁰¹ these results have led to the proposal that the accessibility of the viral RNAP to bases within the nucleocapsid-associated genomic RNA may be controlled by their position within a hexamer of N-bound nucleotides.^{181,203}

N protein exists in at least two forms in infected cells: one stably associated with RNA in a nucleocapsid structure and a second unassembled soluble form termed N⁰. This latter form of N has been found to be associated with P in several viruses, including Sendai virus,¹⁶⁹ PIV5 (formerly known as simian virus 5 [SV5]),³³¹ measles virus,³⁷⁹ and RSV.¹²¹ N⁰ is thought to be the functional form of N that encapsidates the nascent RNA strand during genome and antigenome replication.^{78,169} N-terminal regions of Ncore are important for formation of the N⁰–P complex,¹⁶⁶ and these domains are distinct from those involved in binding of P to N in the assembled nucleocapsid.

The P Gene and Its Encoded Proteins

The *Paramyxovirinae* P gene is a remarkable example of exploiting the coding capacity within a viral gene. The Sendai virus P/V/C gene is the most diverse of the paramyxovirus P genes,

TABLE 33.2 Examples of Identified P Gene Open Reading Frames

Paramyxovirus		mRNA insertion			Alternative ORFs
		0	+1G	+2G	
<i>Rubulavirus</i>	PIV5 (SV5); MuV; HPIV2; HPIV4	V	W	P	
<i>Avulavirus</i>	NDV	P	V	I	
<i>Respirovirus</i>	SeV	P	V	W	C' C Y1 Y2
	BPIV3	P	V	D	C
	HPIV3	P	V	D	C
	HPIV1	P			C' C
<i>Henipavirus</i>	NiV; HeV	P	V	W	C
<i>Morbillivirus</i>	MeV; CDV	P	V	W	C
Unclassified	J virus; TPMV; MoV	P	V	W	C
	MenV; TiV	V	W	P	
	FDLV	V	W	P	

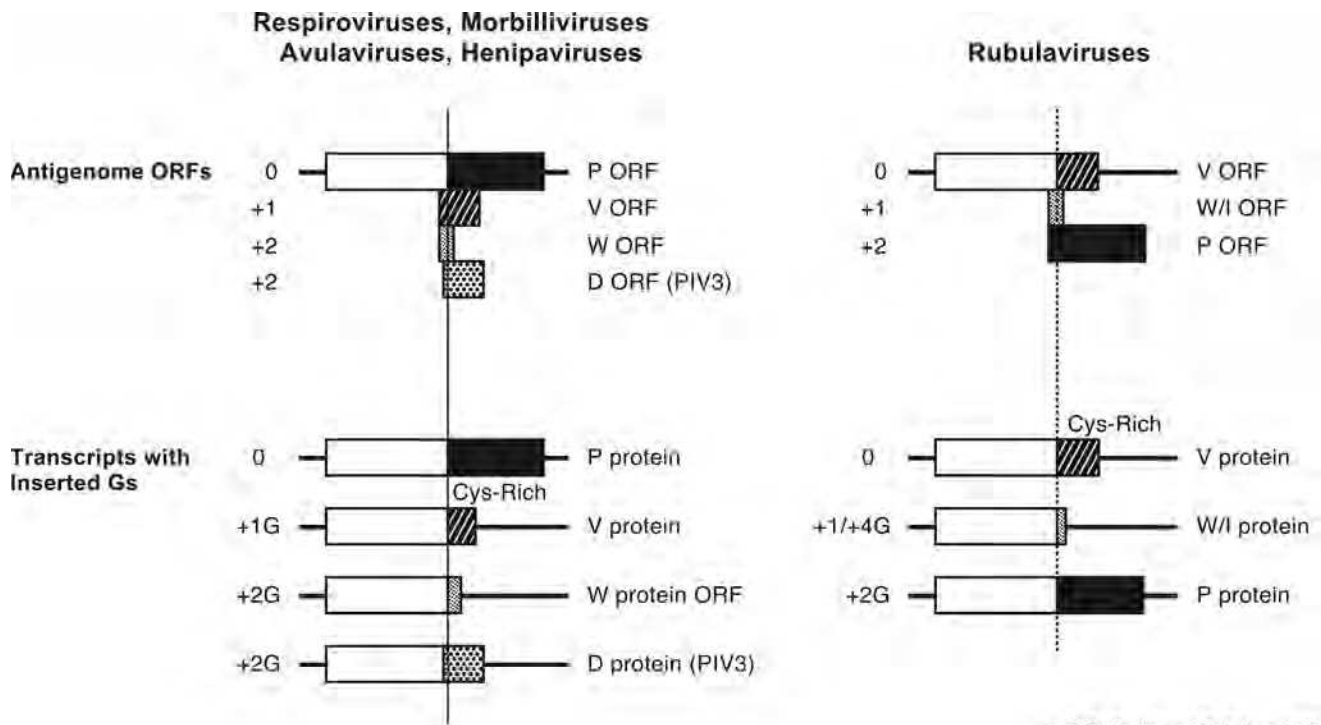
mRNA, messenger RNA; ORFs, open reading frames; PIV5, paramyxovirus type 5; SV5, simian virus 5; MuV, mumps virus; HPIV2, human parainfluenza virus type 2; HPIV4, human parainfluenza virus type 4; NDV, Newcastle disease virus; SeV, Sendai virus; BPIV3, bovine parainfluenza virus type 3; HPIV3, human parainfluenza virus type 3; HPIV1, human parainfluenza virus type 1; NiV, Nipah virus; HeV, Hendra virus; MeV, measles virus; CDV, canine distemper virus; TPMV, *Tupaia* paramyxovirus; MoV, Mossman virus; MenV, Menangle virus; TiV, Tioman virus; FDLV, Fer-de-Lance virus.

directing the expression of at least seven polypeptides, including the P, V, W, C', C, Y1, and Y2 proteins. Whereas other paramyxoviruses express fewer proteins from the *P/V/C* gene than Sendai virus, the *P* gene always produces more than one polypeptide species (Table 33.2). Expression of *P/V/C* proteins can involve two main mechanisms, with members of a paramyxovirus genus having a characteristic combination of these expression strategies. The first expression mechanism, which produces the P, V, and W/I/D proteins, has been termed *RNA editing* or pseudotemplated addition of nucleotides.^{307,408,419} This mechanism involves the production of mRNAs whose ORFs are altered by insertion of G residues at a specific position in the mRNA. As described later, the second expression mechanism involves ribosome initiation at alternative translation codons and produces the family of C proteins.

The P and V proteins, as well as virus-specific proteins variously referred to as W, I, and D, are produced as a co-N-terminal nested set of proteins. These polypeptides are translation products from distinct mRNAs that differ only by inserted G nucleotides that shift the translational reading frame at the site of insertion. As shown in Figure 33.6, the *P* gene of the respiro-,

morbilli-, and henipaviruses codes for a long N-terminal ORF shared by all three proteins and three shorter ORFs starting at approximately base 400 in the mRNA. During transcription of the nucleocapsid template, the viral RNAP is directed to make an accurate copy of the *P* gene template or to insert one or two G residues at a precise site in the nascent mRNA. The result is that the accurate transcription product encodes the full-length P ORF, whereas the mRNAs with insertions of +1G and +2G have a shift in the translational ORF such that the 5' end P ORF is fused at the site of insertion in the mRNA coding sequence to a more 3' ORF encoding V (+1G) or W (+2G). Thus, the P, V, and W/I/D proteins that are produced as a result of RNA editing share a common N-terminal region but differ in their C-terminal regions starting at the site of G insertion.

All viruses of the *Paramyxovirinae* (with the exception of human parainfluenza virus type 1 [HPIV1])²⁵⁰ encode a characteristic editing site in the *P* gene, and the number of inserted G residues, as well as the frequency of inserting G nucleotides, is determined by sequences surrounding and within the editing site. For example, Sendai virus encodes the P protein as the translation product from the unedited mRNA (+0 G; see Fig. 33.6). The V



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FIGURE 33.6. Schematic diagram of translational open reading frames (ORFs) generated by RNA editing during *P* gene transcription for representative paramyxoviruses. Antigenome ORFs, which span the editing site in the *P* gene, are indicated at the top by boxes. The shared N-terminal ORF is shown as a white box. The RNA editing site, at which nontemplated nucleotides are added to the messenger RNA (mRNA) is indicated by the vertical line. At the bottom, RNA transcripts are shown to contain insertions of zero, one, or two G residues at the editing site, with shaded boxes indicating unique C-terminal ORFs fused to the common N-terminal ORF shown in white. For the respiro-, morbilli-, avula-, and henipaviruses, the mRNA for the P protein is transcribed faithfully (unedited) from the viral genome and is shown as a white box fused to a black box. Transcriptional RNA editing with the addition of one G nucleotide at the editing site produces an mRNA that encodes the V protein, in which the common N-terminal domain shown in white is fused to a different ORF. Addition of two G nucleotides at the editing site produces an mRNA that encodes the W or D or I proteins (depending on the virus). For rubulaviruses, the unedited mRNA encodes the V protein, the addition of either one or four G nucleotides produces mRNA encoding the W or I protein, and the addition of two G nucleotides produces the mRNA encoding the P protein. The cysteine-rich domain of the V protein is indicated by a striped box.

protein is produced from a transcript containing a single G residue at the insertion site (+1 G), which fuses the common N-terminal ORF to the V-specific ORF. The Sendai virus transcript with two inserted G nucleotides codes for the W protein (+2 G). As shown in Figure 33.6, rubulaviruses differ from other paramyxoviruses in that V protein is produced by translation of the unedited mRNA (+0 G), and P is produced by translation of an mRNA containing a two-G insertion (+2 G).

Insertion of G residues into *P* gene mRNA transcripts is a co-transcriptional event catalyzed by the vRNAP⁴¹⁹ and is usually limited to insertions of between 0 and 2 nucleotides, depending on the virus. Human and bovine parainfluenza virus type 3 (HPIV3 and BPIV3) are exceptions to this general rule, and mRNAs with one to six inserted G residues are almost equally abundant.¹²⁰

The Phosphoprotein

The P protein is the only *P/VC* gene product that is essential for viral RNA synthesis.⁷⁸ P is generally 400 to 600 amino acids long and is heavily phosphorylated at serine and threonine residues, predominantly within the N-terminal region. P protein contains regions of high intrinsic disorder,²⁵ consistent with the requirement for interacting with multiple partners during the viral growth cycle. P is an essential component of both the vRNAP enzyme¹⁴¹ and the N⁰ nascent chain assembly complex that functions to encapsidate RNA during replication.¹⁶⁹ Extensive mutational analyses have identified distinct modular C- and N-terminal domains within the P protein that play essential roles as a polymerase cofactor and in nascent chain assembly, respectively.

The C-terminal polymerase cofactor module is relatively well conserved in predicted secondary structure for all viruses of the *Paramyxovirinae*, and all P proteins carry this essential module as the C-terminal segment of fusion protein with the shared P/V domain; this module is never naturally expressed by itself. The P protein C-terminal region contains domains for P-P multimerization, for interactions with L protein, and for binding to

the N:RNA template. P protein functions as a multimer, and structural analysis suggests that the Sendai virus P protein is a tetramer.⁴⁰² For Sendai virus, the P-carboxy region is sufficient for catalyzing viral RNA synthesis, as this protein fragment by itself (residues 325–568) can substitute for intact P protein in all aspects of mRNA transcription.⁷⁹ Although the L protein is thought to contain all vRNAP catalytic activities, L binds to the nucleocapsid template via the P protein.¹⁶⁹ This P–L interaction requires a domain in P that maps to the C-terminal end of the coiled-coil P–P multimerization region.^{32,80} At the end of the C-terminal domain, P also contains a region that binds to the N:RNA template,^{32,80} providing the bridge to link L with the N:RNA template. In the case of Sendai virus, structural data indicate that this C-terminal region of P binds through weak hydrophobic interactions to the C-terminal tail of N, inducing folding of the intrinsically disordered Ntail.²³² The ability of P and Ntail to form transient weak interactions between intrinsically disordered domains may be important for the dynamic functions of P during movement of the viral RNAP across the N:RNA template or in the flexibility of the nucleocapsid template.^{17,25,200}

In contrast to viral transcription, genome replication requires an N-terminal region of P (defined by deletion of Sendai virus residues 33–41). A short segment of the P protein N-terminal domain is thought to facilitate interactions with unassembled N⁰ to prevent N aggregation and to ensure specificity in assembly.^{78,169} The rest of the N-terminal domain of P protein is apparently dispensable for genome RNA synthesis and assembly, as a P protein in which residues 78 through 324 have been deleted is still active for minigenome replication in transfected cells.⁷⁴

The V Protein

The V protein is an approximately 25- to 30-kDa polypeptide that shares an N-terminal domain with the P protein but has a distinct C-terminal domain as a result of RNA editing.^{48,303,408,419} The C-terminal V-specific domain is highly conserved among related paramyxoviruses (Fig. 33.7), with invariantly

						*				*	*				*	*	*	*	*	
Respiro-	SeV	316...	KG	HRRE	HHIYER	DGYIV	DES	WCNPV	CS	RIRI	IPREL	CVCKT	CPKV	CKL	CRD...	367				
	BPIV3	346...	RG	HRRE	HHSIYR	EGDYI	ITES	WCNPI	CS	KIRP	VPRQES	CVCGE	CPKQ	CGY	CIE...	397				
Rubula-	PIV5	169...	GF	HRRE	YSIGW	VGVDEV	KVTE	WCNPS	CS	PITAA	AARRFE	CTCHQ	CPVT	CSE	CER...	220				
	HPIV2	172...	GN	HRRE	WSIAW	VGDQV	KVFE	WCNPR	CA	PVTAS	ARKFT	CTCGS	CPSI	CGE	CEG...	223				
	MuV	168...	GG	HRRE	WSLSW	VQGEV	RVFE	WCNPI	CS	PITAA	AARFHS	CKCGN	CPAK	CDQ	CER...	219				
Avula-	NDV	175...	PG	HRRE	HSISW	TMGGV	TTIS	WCNPS	CS	PIRAE	PRQYS	CTCGS	CPAT	CRL	CAS...	226				
Morbilli-	MeV	230...	KG	HRRE	ISLIW	DGDRV	FIDRW	WCNPM	CS	KVTL	LG	TIRAR	CTCGE	CPRV	CEQ	CRT...	281			
	CDV	230...	KG	HRRE	VSLTW	NGDSC	WIDK	WCNPI	CT	QVNW	GII	IRAK	CF	CGE	CPPT	CNE	CKD...	281		
	RPV	230...	KG	HRRE	IDLIW	NDGRV	FIDRW	WCNPT	CS	KVT	VG	TVRAK	CI	CGE	CPRV	CEQ	CIT...	281		
Henipa-	HeV	404...	KG	HRRE	VSICW	DGRRAW	VVEE	WCNPV	CS	RITP	QPRKQE	CY	CGE	CPTE	CSQ	CCH...	455			
	NiV	404...	KG	HRRE	ISICW	DGKRAW	VVEE	WCNPA	CS	RITPL	PRRQE	CQ	CGE	CPTE	CFH	CG...	456			
Unclassified	SalV	250...	SR	HRRE	YSIIW	DSEGI	QIES	WCNPV	CS	KVRST	PPREK	CR	CGK	CPAR	CSE	CGD...	301			
	TPMV	228...	KG	HRRE	YSMW	VSNDG	VFI	ES	WCNPM	CA	RIRPL	PIREI	CV	CGR	CPLK	CSK	CLL...	279		
	MenV	164...	GG	HRRE	IAIDW	IGGRP	RVTE	WCNPI	CH	PISQ	STFRGS	CR	CGN	CPGI	CSL	CER...	215			
	TiV	162...	GG	HRRE	IAISW	ATGTP	RVTE	WCNPI	CH	PISQ	STYRG	CR	CGC	CPDV	CSL	CER...	213			
	J virus	234...	KG	HRRE	FCIDN	FGGKTY	I	REW	WCNPO	CA	PITV	TP	TQSR	CT	CGE	CPKV	CARC	IK...	285	

FIGURE 33.7. Amino acid sequence alignment of the conserved cysteine-rich C-terminal region of selected paramyxovirus V proteins. Numbers indicate the amino acid position within the respective proteins. Positions of the conserved histidine and seven conserved cysteine residues that are involved in coordinating Zn^{2+} are indicated by **bold lettering**. Additional areas of sequence identity are *shaded*.

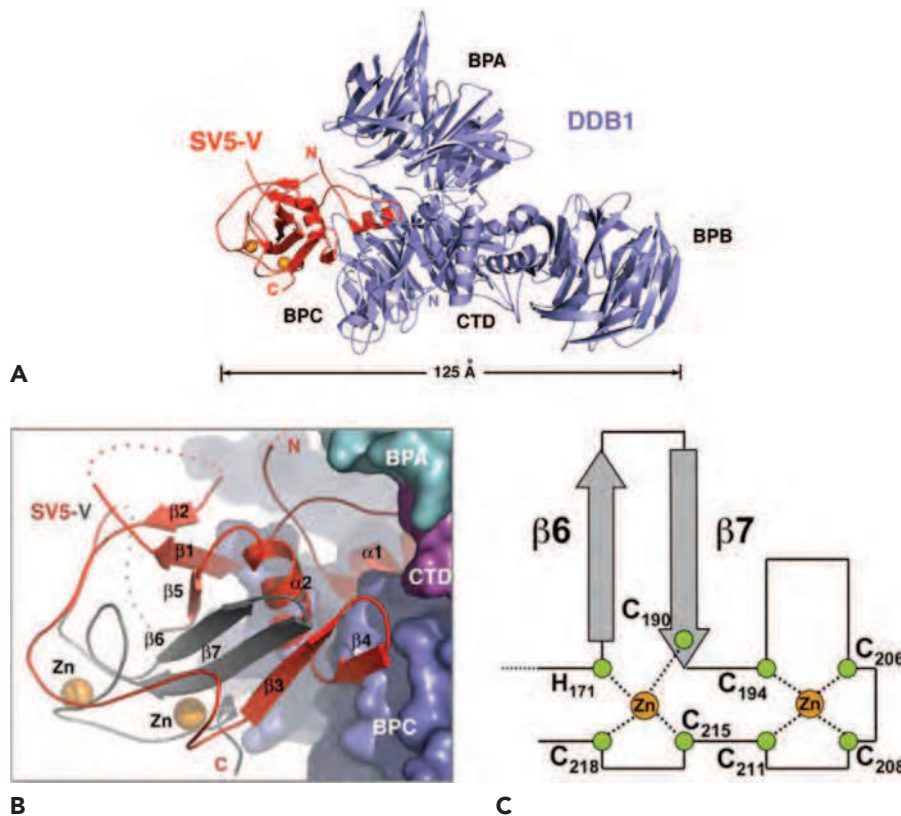


FIGURE 33.8. Atomic structure of the parainfluenza virus type 5 (PIV5) V protein in complex with damage-specific DNA-binding protein 1 (DDB1). The PIV5 V protein binds to DDB1, which adopts a four-domain structure consisting of a three-propeller cluster and a helical C-terminal domain. **A:** Overall view of the DDB1-simian virus 5 (SV5)-V complex with DDB1 in blue and the PIV5 (SV5) V protein in red. The zinc ions in SV5-V are shown as orange spheres. The four DDB1 domains are labeled BPA, BPB, BPC, and CTD. The longest dimension of the complex is indicated. **B:** The PIV5 (SV5) V protein adopts a bipartite structure upon interacting with the DDB1 BPC domain. DDB1 and SV5-V are shown in surface and ribbon representation, respectively. The N-terminal part of the V protein, which is also found in the viral P protein, is colored in red. The rest of the V protein, including the zinc-binding sequence, is colored in gray. **C:** A novel zinc-finger fold found in the SV5-V protein. (Adapted from Li T, Chen X, Garbutt KC, et al. Structure of DDB1 in complex with a paramyxovirus V protein: Viral hijack of a propeller cluster in ubiquitin ligase. *Cell* 2006;124:105–117.)

spaced histidine and cysteine residues forming a novel domain that binds two zinc molecules per V protein.^{118,225,231,304} Despite the high level of intracellular synthesis, paramyxovirus particles typically contain little V protein,³⁰⁴ and the degree of incorporation of V into virions varies among paramyxoviruses.^{75,431}

V protein plays several important roles in the virus replication cycle, as evidenced by recombinant viruses that have been engineered to disrupt expression of the V protein Cys-rich domain.^{12,153,191,412} In many cases, these mutant viruses display an elevated RNA synthesis phenotype, although they generally grow well in many tissue culture cell lines.^{82,192} However, in many cases, these viruses are severely attenuated for growth *in vivo* or are cleared more rapidly than wild-type viruses from lungs of infected animals.^{94,192,418} These results suggest that V is an accessory protein that plays a role in viral pathogenesis, perhaps involving a counteracting of host cell antiviral responses that occur early after infection and that can lead to enhanced clearance of virus.

V protein has also been shown to inhibit viral RNA synthesis in transfection experiments involving model RNA genomes.^{78,168,229} Recombinant viruses that are engineered with V protein mutations often show increased viral RNA synthesis.^{81,191,360,412,430} This has led to the proposal that V protein serves as a negative regulator of viral RNA synthesis. V protein shares the amino-terminal domain of P protein that can interact with N⁰ to form the assembly competent P-N⁰. Thus, the mechanism of V inhibition may involve interactions with N that result in a form of a V-N⁰ complex that is not competent to function during the RNA encapsidation step of replication. This V-N⁰ interaction has been detected

in the case of PIV5, Sendai virus, and measles virus,^{168,331,412} and a model whereby V and P compete for soluble N⁰ has been proposed.^{78,168} The V protein is also capable of binding RNA,²²⁶ and it has been proposed that this function is involved in inhibiting RNA synthesis for the Sendai virus V protein.³⁰⁰

In addition to binding viral components, V protein also has been detected in interactions with cellular proteins. For several paramyxoviruses, V protein interacts in the cytoplasm with the cellular damage-specific DNA-binding protein 1 (DDB1).^{4,227} In the case of the PIV5 V protein, interaction with DDB1 is important for the function of blocking signaling through the type I interferon pathway (see later discussion). Interaction of V with DDB1 and the ability of V to inhibit host cell antiviral responses depends on the C-terminal Cys-rich domain but can also be disrupted by alterations to the common N-terminal P/V region.^{4,227} The structural analysis of PIV5 V protein complexed with DDB1 shows that V protein has a bi-partite structure,²²⁵ with a core domain built around a central seven-stranded β sheet, which is in turn sandwiched between one α helix and two long loops (see Fig. 33.8). The unique C-terminal domain forms the middle two β sheets and part of the central core, and this structure is anchored through the Cys-rich zinc-binding region. Thus, despite sharing a 164 amino acid N-terminal domain, the PIV5 P and V proteins can adopt very different structures owing to the unique properties of the C-terminal Cys-rich region. V protein from several paramyxoviruses has been shown to interact through the Cys-rich domain with the cellular protein MDA-5, an IFN-inducible host cell DEXD/H box helicase that is involved in signaling to initiate host cell antiviral responses.

The W/D/I Proteins

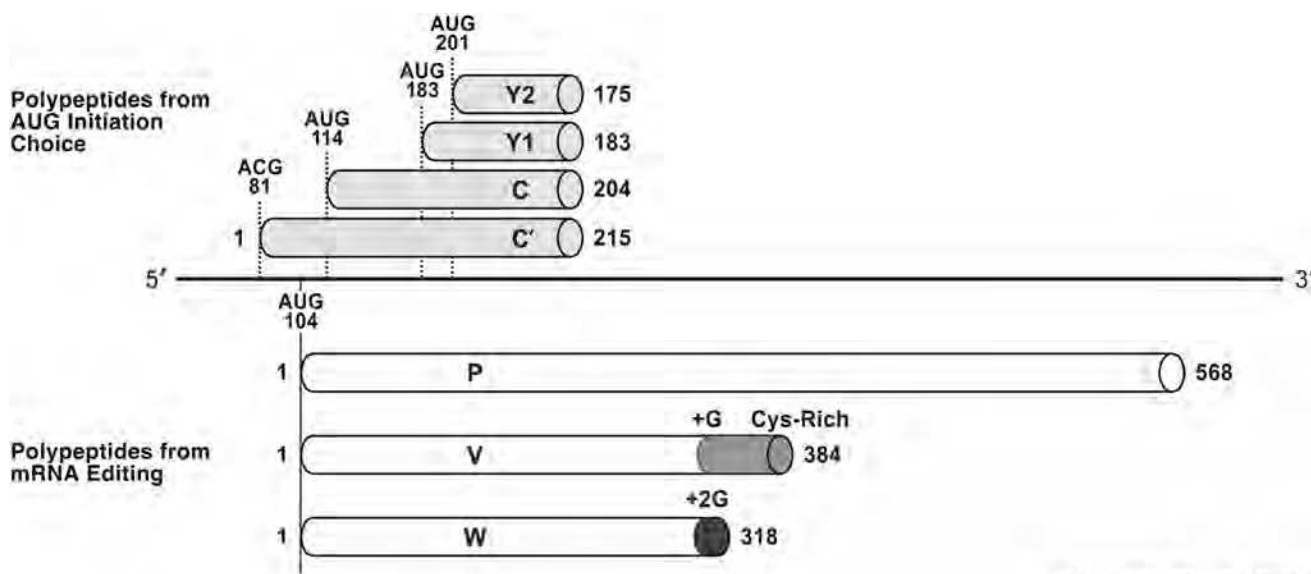
The W and D ORFs of respiro-, morbilli- and henipaviruses are expressed from mRNAs with two inserted G residues (see Fig. 33.6). For most of these viruses, the insertion of two G residues into the mRNA is relatively rare, and the ORF is closed by a stop codon shortly after the editing site, resulting in the ORF for the W protein. Thus, the W protein is essentially a truncated P protein, containing the N-terminal N⁰ assembly module of the P protein alone. W protein is abundantly expressed in Sendai virus-infected cells⁷³ and has been found to interact with unassembled N⁰, suggesting an inhibitory role in viral RNA synthesis.¹⁶⁸ In the case of BPIV3 and HPIV3, the +2 ORF extends for 131 residues from the editing site, and the protein that links the amino-terminal P domain to this ORF is called *D protein*.¹²⁰ The *Rubulavirus* I protein is generated when the upstream N-terminal P region is fused to a downstream ORF by the insertion of either one or four G residues during RNA editing.^{303,408} The role that the W/D/I proteins play in the viral growth cycle has not been established.

The C Proteins

In addition to RNA editing, some paramyxoviruses use a second mechanism to express *P* gene polypeptides that involves the use of alternative translation initiation codons to yield the C proteins (Fig. 33.9). The Sendai virus C', C, Y1, and Y2 proteins comprise a nested set of carboxy-co-terminal polypeptides that range in size from 175 to 215 residues. These proteins are expressed independently from a P/V mRNA through the use of alternative start codons (Fig. 33.9), with the C protein ORF being in the +1 reading frame relative to the P ORF. The C'

and C proteins are translated by a leaky scanning mechanism, being initiated at an unconventional ACG triplet at base 81 and AUG at base 114, respectively.⁷⁷ By contrast, translation of the Y1 and Y2 proteins occurs through a scanning-independent ribosome shunting mechanism that is directed by a 5' noncoding RNA segment, resulting in ribosomes initiating at AUG codon bases 183 and 201, respectively. Translation of each of the C', C, Y1, and Y2 ORFs is initiated at a different site, although translation is terminated at the same downstream stop codon; thus, these proteins share a common C-terminal region. The C protein is abundantly expressed in infected cells at levels higher than C', Y1, and Y2; however, virions contain only very low levels of these polypeptides.²¹⁷ Morbilliviruses express one C protein,¹³ as do the henipaviruses,⁴²⁷ whereas the respiroviruses such as Sendai virus and HPIV1 express all four C', C, Y1, and Y2 polypeptides.¹²⁸ Rubula- and avulaviruses do not express C proteins (see Table 33.2).

C proteins are small basic polypeptides that play multiple functions in the viral growth cycle, being involved in the control of viral RNA synthesis, counteracting host cell antiviral pathways, and facilitating release of virus from infected cells. Although nonessential for infectivity, Sendai virus mutants engineered to express only a subset of C proteins or lacking expression of all four proteins show defects in virus growth.²⁰⁹ The C proteins have been shown to inhibit mRNA transcription and suppress RNA replication in a promoter-specific manner.^{193,238,399} Consistent with this, viral mutants that are engineered to lack C protein expression show elevated synthesis of viral mRNA and protein.¹⁴⁷ The inhibition of RNA synthesis by C proteins correlates with the ability to bind to the



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FIGURE 33.9. Representation of the Sendai virus P messenger RNA (mRNA) to illustrate the mechanisms of producing P, V, and C proteins. The position of four unique initiation codons for the C', C, Y1, and Y2 open reading frames (ORFs) are shown above the horizontal black line representing the *P* gene mRNA. The position of the common initiation codon for the P, V, and W ORFs at base 104 is shown below the mRNA. The gray cylinder indicates the V protein Cys-rich C-terminal domain, which is fused to the shared P N-terminal domain by addition of a G residue during viral transcription; the black cylinder indicates the short W domain, which is accessed by insertion of two G residues. Numbers denote the amino acids contained within each polypeptide chain. Note that the initiation codon for C' is ACG.

L subunit of the viral polymerase,¹⁷⁰ and in the case of Sendai virus, naturally occurring variant C proteins can have differential effects on inhibition of virus RNA synthesis.¹⁰

The role of paramyxovirus C proteins in pathogenesis and in counteracting host cell IFN responses is best understood in the cases of Sendai virus and measles virus. For Sendai virus, the C', C, Y1, and Y2 proteins can antagonize IFN signaling when assayed in stably transfected HeLa cells¹⁹³; however, there may be more subtle differences in the functions of each polypeptide in the context of viral mutants.¹²⁴ In the case of measles virus, recombinant viruses defective for C protein expression grow well in certain culture cells but are defective for growth in peripheral blood mononuclear cells¹⁰⁴ and are less virulent *in vivo*.³¹⁰ Changes in pathogenesis of C-mutant viruses may be related to the ability of the C proteins to inhibit type I IFN responses.³⁶⁸ This proposal is further supported by a naturally occurring mutation in the Sendai virus C protein (phenylalanine 170 to serine) that eliminates the ability of C protein to block IFN signaling,¹²⁴ and a mutant Sendai virus harboring this altered C protein is attenuated for growth in mice. The mechanism by which C proteins attenuate IFN signaling has not been elicited but may involve binding of C to STAT1¹²⁴ or altering STAT1 phosphorylation patterns.²⁰⁵

An additional role for C proteins in virus release became evident with the analysis of mutant Sendai virus that cannot express any of the four C proteins.²⁰⁹ Whereas viral RNA and protein synthesis was high for this mutant virus, production of infectious virions was low, and heterogeneous noninfectious particles were produced.¹⁴⁷ C protein expression enhances release of virus-like particles (VLPs), possibly through interactions with AIP1/Alix—a cellular protein involved in apoptosis and endosomal trafficking.³⁴⁷

The Large Protein

The large (L) protein is an essential subunit of the paramyxovirus RNAP. Consistent with a catalytic role in viral RNA synthesis, the L protein is invariably encoded as the most promoter-distal gene in the paramyxovirus genome (see Fig. 33.4). L protein is generally found in only very low amounts in infected cells or associated with nucleocapsids and virions.²¹⁴ A paramyxovirus particle typically contains only about 50 copies of L,²¹⁴ where it is found on the nucleocapsid in clusters that co-localize with P protein.³²⁷ L is thought to possess all of the enzymatic activities needed for synthesis of functional viral mRNA, including nucleotide polymerization as well as 5'-end capping and methylation and 3'-end polyadenylation of mRNAs.^{137,158,289} Polyadenylation of viral mRNAs occurs co-transcriptionally, where L is thought to add poly A tails to nascent viral mRNAs through a mechanism that involves stuttering at a stretch of template U residues at the end of each viral gene (see Fig. 33.5). L protein is also responsible for the replication of viral genomic and antigenomic RNA; however, this form of RNA synthesis differs from mRNA transcription by having a strict requirement for soluble N⁰ to allow encapsidation of the nascent genomic RNA.^{139,169}

The paramyxovirus L protein is generally approximately 2,200 amino acids in length (~250 kDa). Although the N- and C-terminal regions of the L proteins are diverse, sequence comparisons among L proteins have identified six highly conserved domains (I–VI) near the middle of the polypeptide. It was originally proposed that these domains may be individually

responsible for each of the multiple L functions.³¹⁹ Domain II is proposed to be an RNA-binding domain owing to the high net positive charge. In domain III, mutational analyses are consistent with the proposal of a conserved GDNQ motif as the active site for nucleotide polymerization.²³⁷ Based on sequence homologies, domain VI of the rhabdovirus L protein has been implicated in playing a major role in 5' cap formation, perhaps as a methyltransferase domain.^{114,319} The precise roles of the remaining domains I, IV, and V in individual steps of RNAP activity are not clear; however, for Sendai virus, mutations in some of these domains result in L proteins that can transcribe viral mRNA but are defective in RNA replication.^{56,113} In the case of the L proteins of Sendai virus and rinderpest virus, sequence alignment has identified nonconserved hinge regions that can be modified by insertions of green fluorescent protein (GFP), and remarkably, viable recombinant viruses encoding these L-GFP hybrid proteins have been isolated and used to identify sites of L localization during infection.^{35,92}

L protein activity in RNA synthesis highly depends on protein–protein interactions, involving self-assembly as well as binding to other viral and cellular proteins. Biochemical evidence and genetic complementation studies indicate that the Sendai and measles virus L proteins function as homomultimers that interact through an N-terminal self-assembly domain.^{54,376} L also binds to the viral P protein—an interaction that is essential for formation of the active enzyme complex and can lead to enhanced stability of L.^{141,169} L–P interaction domains generally map to an N-terminal domain of L that is distinct from the L–L assembly domain.¹⁶⁵ Within the L–P complex, P protein serves as the bridge to link the L polymerase to the nucleocapsid template.^{80,169} In addition to L–L and L–P interactions, L protein also interacts with host cell proteins.^{269,375} In the case of measles virus and Sendai virus, L interactions with tubulin are thought to promote L activity.²⁶⁹ Other cellular proteins have also been shown to promote viral RNA synthesis (e.g., β -catenin for HPIV3),²² although the precise role that these proteins play in viral RNA synthesis has not been determined.

Whereas interactions of L with P are generally thought to promote activity, L protein can also interact with other viral components that inhibit the vRNAP. For both rinderpest virus and Sendai virus, L protein has been found to bind the viral C proteins.^{170,390} The Sendai virus L–C interactions are through a domain of L that maps to the first 895 residues (domains I–III),¹⁷⁰ and this binding correlates with inhibition of RNA synthesis.¹³⁸ Other proteins encoded in the viral *P/V/C* gene (C', Y1, and Y2) also interact with L and inhibit defective interfering RNA synthesis *in vitro*¹³⁸ and *in vivo*.¹⁹³

The Matrix Protein

The paramyxovirus matrix (M) protein is the most abundant protein in the virion. The M proteins contain 341 to 375 residues (M_r ~38,500–41,500), are quite basic proteins (net charge at neutral pH of +14 to +17), and are somewhat hydrophobic, although there are no domains of sufficient length to span a lipid bilayer. In electron micrographs of virions, an electron-dense layer is observed underlying the viral lipid bilayer, and this is thought to represent the location of this protein. Fractionation studies of virions indicate that the M protein is peripherally associated with membranes and is not an intrinsic membrane protein. Reconstitution studies of purified

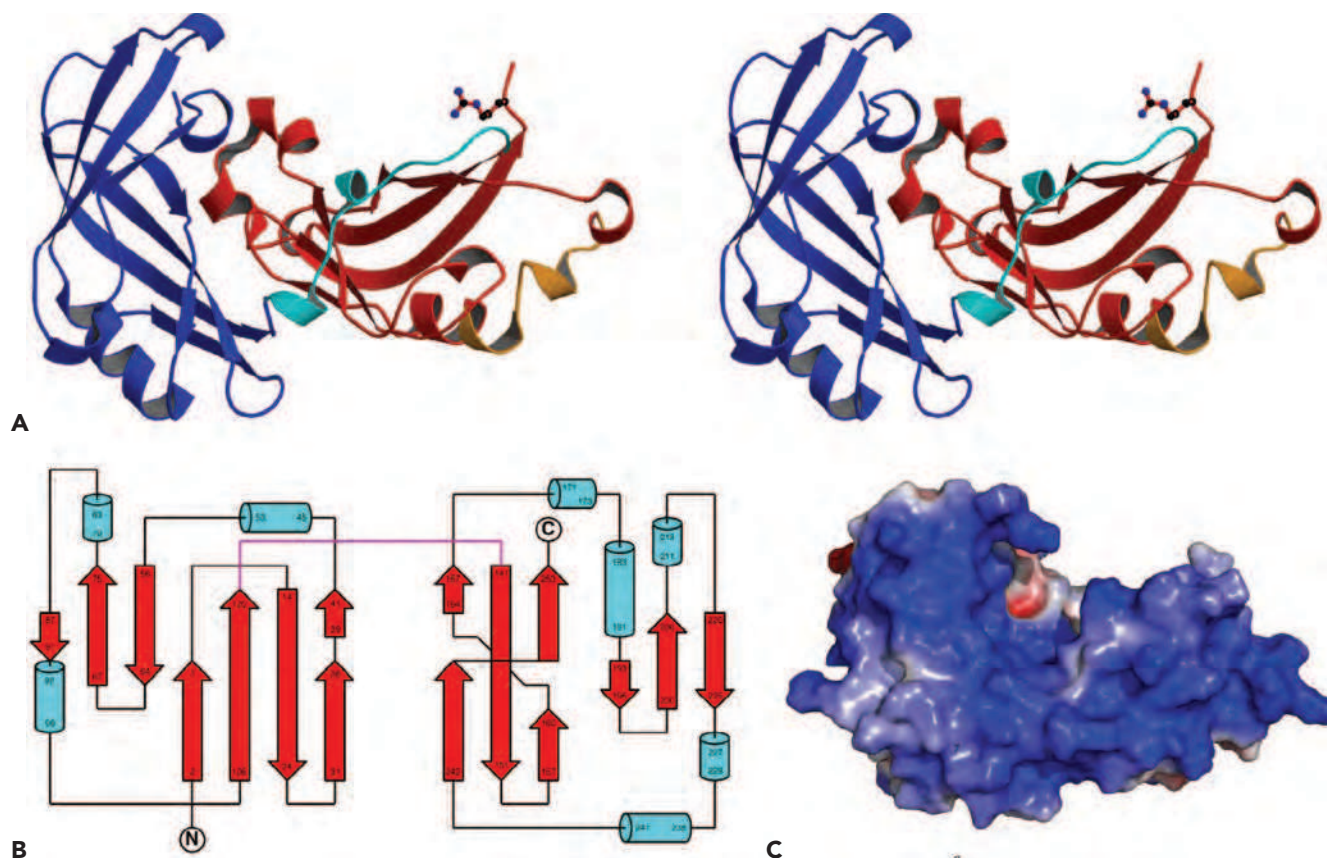


FIGURE 33.10. Three-dimensional structure of the RSV M protein. The crystal structure of M (resolution 1.6 Å) shows two domains composed largely of β -sheets. **A:** Divergent (wall-eyed) stereoview of M colored according to domain with the linker shown in cyan, the N-terminal domain in blue, and the C-terminal domain in red. Residue R254 is shown in ball-and-stick representation. **B:** A topology diagram of the protein. The linker between the N- and C-terminal domains is shown in magenta. Residues (numbers refer to Met as +1) in β -sheets are represented by broad arrows and helices as cylinders. **C:** Electrostatic surface potential (calculated with APBS) for M, presented in a color range from red to blue (−5 to +5 kT/e); uncharged residues are uncolored. (From Money VA, McPhee HK, Mosely JA, et al. Surface features of a Mononegavirales matrix protein indicate sites of membrane interaction. *Proc Natl Acad Sci U S A* 2009;106:4441–4446.)

M protein and fractionation studies of infected cells indicate that the M protein can associate with membranes.^{105,212,280}

As a purified protein, the Sendai virus M protein can self-associate and form two-dimensional paracrystalline arrays (sheets and tubes) in low salt conditions.^{7,157} There is a paracrystalline array of identical periodicity at the inner surface of the plasma membrane of infected cells when examined by freeze-fracture techniques in the electron microscopy.⁷ In addition, the M protein is associated with nucleocapsids.³⁸⁶ As of January 2011 the only atomic structure of M to be obtained is that of RSV M.²⁶⁴ It shows that the protein has extensive β -sheets and a continuously charged region covering approximately 600 Å, which probably interacts with a negatively charged surface on the RNP (Fig. 33.10). Genetically engineered recombinant measles virus and PIV5 that lack glycoprotein cytoplasmic tails show a subcellular redistribution of the matrix protein,^{46,357} which implies that there is an interaction of the F and HN cytoplasmic tails with the M protein. Thus, the M protein is considered to be the central organizer of viral morphogenesis interacting with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the nucleocapsids. The self-association of M and its

contact with the nucleocapsid may be the driving force in forming a budding virus particle.³¹¹ The relative abundance of basic residues in the M protein may reflect their importance in ionic interactions with the acidic N proteins.

For several enveloped viruses, it has been shown that budding occurs by using components of the endosomal sorting complexes required for transport (ESCRTs)—proteins involved in multivesiculate body formation. Protein–protein interaction domains called *late domains* have been identified in the matrix proteins of several viruses; for the paramyxoviruses, a late domain has been identified in PIV5 M protein.³⁵⁸ This topic is discussed further in the Assembly of the Envelope section.

Consistent with its central role in virus budding, M is often inactivated in persistent paramyxovirus infections where budding fails to occur. For example, in subacute sclerosing panencephalitis (SSPE)—a rare, progressive, and invariably fatal persistent measles virus infection of the brain—the M protein is either absent for various reasons⁴⁷ or, when present, is not associated with budding structures *in vivo* and is unable to bind to viral nucleocapsids *in vitro*.¹⁶² Although a genetically engineered recombinant measles virus that lacks a matrix

protein has been obtained,⁴⁵ it produces approximately 4 logs lower titer of released infectious particles than wild-type virus and remains mostly cell associated. Therefore, it is reasonable to conclude that the M protein does play a very important function in virus assembly. Moreover, in model systems of persistent Sendai virus infection in culture, the normally lytic infection is converted to a persistent one using defective interfering particles. This change correlates mainly with M protein instability and an absence of budding structures.³⁴¹

The M protein of several paramyxoviruses is phosphorylated. For Sendai virus, a large proportion of the M protein is phosphorylated, whereas the M protein found in virions is not phosphorylated.²¹² However, a Sendai virus could be rescued from an infectious complementary DNA (cDNA) in which the single phosphorylation site in Sendai virus M protein had been eliminated.³⁴⁸ This M protein phosphorylation-minus mutant did not show an altered phenotype from wild-type virus in either cultured cells or mice.

Envelope Glycoproteins

All *Paramyxoviridae* possess two integral membrane proteins, and some rubulaviruses and all pneumoviruses encode a third integral membrane protein (Fig. 33.11). One glycoprotein (HN, H, or G) is involved in cell attachment and the other glycoprotein (F) in mediating pH-independent fusion of the viral envelope with the plasma membrane of the host cell. The *Rubulavirus* and *Pneumovirus* third integral membrane protein is referred to as SH; for PIV5, this 44 amino acid integral membrane protein is thought to block virus-induced apoptosis. The assignment of specific biological activities of F and HN was originally made on the basis of purification and reconstitution studies, mainly for the Sendai virus and PIV5 proteins.^{353,354} The attachment proteins (HN, H, or G) are all

type II integral membrane proteins, and bioinformatics and structural predictions indicate that the proteins will all exhibit a related propeller-like fold despite having different receptors and the presence or absence of NA activity.

For the respiroviruses and rubulaviruses, the attachment glycoprotein binds to cellular sialic acid-containing receptors, and these can be glycoproteins or glycolipids. The binding is probably of fairly low affinity but of sufficiently high avidity that these viruses agglutinate erythrocytes (hemagglutination). The attachment proteins of respiroviruses and rubulaviruses also have NA activity (receptor-destroying activity), and the proteins have been designated hemagglutinin-neuraminidase (HN). However, a possible role of a specific protein-protein involvement in infection of host cells has not been ruled out.

The restricted host range of measles virus for primate cells and the lack of NA or esterase activity make it unlikely that sialic acid is the primary receptor for measles virus. Nonetheless, the *Morbillivirus* attachment protein (H) can cause agglutination of primate erythrocytes, most likely owing to receptor binding: the designation of measles and CDV glycoprotein as H is thus a misnomer. In 1993, human CD46 was identified as a cellular receptor for Edmonston and Halle strains of measles virus.^{91,282} Edmonston and Vero cell-isolated strains of measles virus are capable of infecting any CD46⁺ primate cell. However, viruses isolated from B- and T-cell lines do not grow in CD46⁺ cells. A second receptor was identified—human CD150 (SLAM), a membrane glycoprotein involved in lymphocyte activation.^{404,442} It is now thought that CD150 is the principle receptor for unadapted isolates of lymphotropic measles virus.²⁹¹

Very recently, a third receptor for measles virus has been identified, known as poliovirus receptor-like (PVRL4; Nectin 4) or adherens junction protein nectin 4. It is proposed that this new receptor is the epithelial receptor for measles virus that

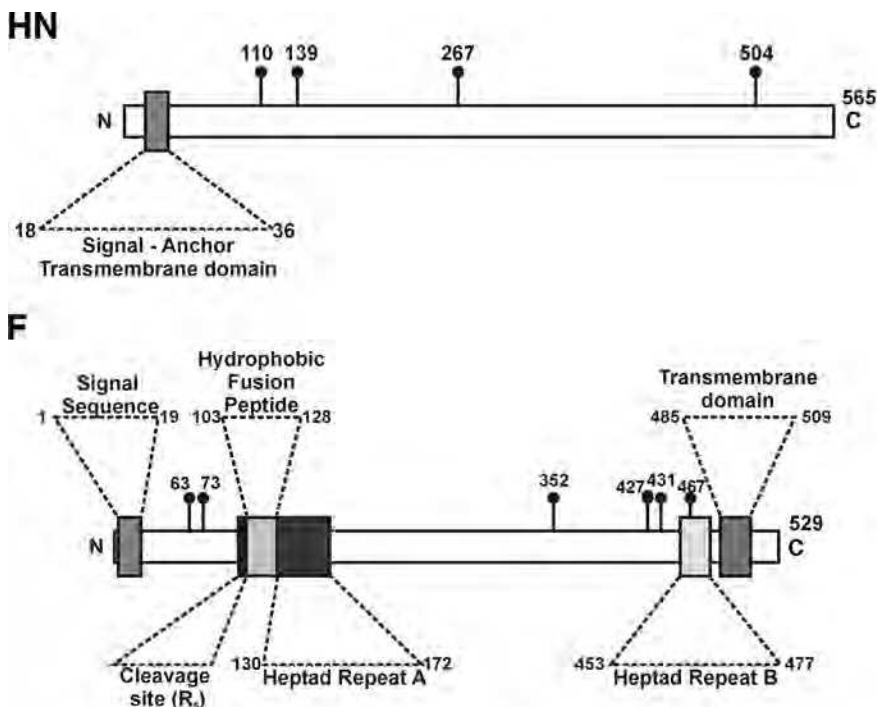


FIGURE 33.11. Schematic diagram showing the orientation and domains of paramyxovirus integral membrane proteins. A: Hemagglutinin-neuraminidase (HN) attachment protein (based on the predicted sequence of the parainfluenza virus type 5 [PIV5] HN gene.¹⁵⁹ The signal anchor transmembrane domain and the sites used for addition of N-linked carbohydrate (*lollipops*)²⁸⁵ are indicated. **B:** Fusion protein (based on the predicted sequence of the PIV5 F gene). The position of the signal sequence, the transmembrane domain, the cleavage site, the hydrophobic fusion peptide, and the heptad repeats A and B are indicated. The sites used for addition of N-linked carbohydrate (*lollipops*)⁸ are indicated. R₅ indicates the five arginine residues site for cleavage activation.

is used to transfer virus from the basolateral surface of epithelial cells to the luminal side of the airway.^{270,288}

The receptor for Hendra virus and Nipah virus G glycoprotein has been shown to be ephrin-B2 or ephrin-B3. In one approach, direct binding of Nipah G to receptor was obtained and the identity of the receptor determined by protein sequencing and bioinformatics.²⁸⁴ In another approach, microarray analysis was used to identify mRNAs that were expressed in henipavirus-susceptible cells and not in cells refractory to henipavirus infection.²⁰ Ephrin-B2 and -B3 are members of a family of cell surface glycoprotein ligands that bind to ephrin (Eph) receptors—a large family of tyrosine kinases. The identification of ephrin-B2/B3 as the cellular receptor for both Hendra virus and Nipah viruses and the widespread occurrence of ephrin-B2/3 in vertebrates, particularly in arterial endothelial cells and in neurons, provides an explanation for the wide host range of henipaviruses and their systemic infection.⁹⁸

The *Pneumovirus* RSV does not cause detectable hemagglutination, and the cellular receptor for RSV is not completely understood but involves interactions with heparan sulfate—a glycosaminoglycan that is part of the extracellular matrix. Interestingly, the G protein of RSV and human metapneumovirus (HMPV) can be deleted from the viral genome,¹⁸⁹ and Sendai virus-like particles devoid of HN can infect cells via the asialoglycoprotein receptor.²²⁴ Both of these cases suggest that some paramyxovirus F proteins may have a binding activity. After attachment of a *Paramyxoviridae* particle to the host cell receptor, the viral envelope fuses with the host cell plasma membrane, and the major viral protein involved in this process is the F glycoprotein.

Paramyxovirus Attachment Protein

The *Respirovirus* and *Rubulavirus* surface glycoprotein HN is a multifunctional protein and the major antigenic determinant of the paramyxoviruses. HN has three activities: (a) receptor binding to sialic acid; (b) cleavage of sialic acid from complex carbohydrate chains (NA activity); and (c) fusion promotion—that is, co-expression of HN and F is required for cell–cell fusion (see later discussion). By analogy to the role of influenza virus NA, it seems likely that the role of this NA activity is to prevent self-aggregation of viral particles during budding at the plasma membrane. These dual activities of HN can be modulated by pH.²⁵⁸ Whereas the pH of the extracellular environment is optimal for hemagglutination, paramyxovirus NAs have an acidic pH optima (pH 4.8–5.5), suggesting that NA acts in the acidic *trans*-Golgi network to remove sialic acid from the HN carbohydrate chains and from the F protein carbohydrate chains.

The HN polypeptide chain ranges from 565 to 582 residues. For some strains of NDV, HN is synthesized as a biologically inactive precursor (HN₀), and 44 residues from the C-terminus are removed to activate the molecule.^{278,279} HN is a type II integral membrane protein that spans the membrane once and contains an N-terminal cytoplasmic tail, a single N-terminal transmembrane (TM) domain, a membrane-proximal stalk domain, and a large C-terminal globular head domain.¹⁵⁹ The globular head domain contains the receptor-binding and enzymatic activity.^{301,353,410} HN is glycosylated and contains from four to six potential sites for the addition of N-linked carbohydrate chains. For PIV5 and NDV HN, it is known that four sites are used.^{253,285} HN is noncovalently associated to form a dimer of dimers, based on biochemical, cross-linking,

electron microscopy, and structural studies that, depending on the paramyxovirus, can be composed of two disulfide-linked dimers.^{72,148,218,252,285,286,409,450,451} The covalent linkage occurs through a cysteine residue at the C-terminal end of the stalk domain, just prior to the beginning of the head domain. The stalk domain appears to play an essential role in the formation of the tetramer,⁴⁵¹ and head domains when expressed without the stalk are often monomeric.^{72,218,451}

The structure of the enzymatically active head domain of HN is similar to other NAs or sialidases, such as influenza NA,¹⁰³ with the globular head composed of identical subunits. Each NA domain exhibits the six-blade propeller fold typical of other NA/sialidase structures from viral, protozoan, or bacterial origin.^{41,216,405} Atomic structures of soluble head domains of NDV, HPIV3, PIV5 (liganded and bound to a receptor/substrate sialyllactose), measles virus H (unliganded and bound to its receptor CD150/SLAM), and Hendra and Nipah virus G (unliganded and bound to its receptor ephrin-B2 or ephrin-B3) have been obtained,^{30,31,60,72,148,149,218,441,451,453} and it shows the typical sialidase fold consisting of six antiparallel β -strands organized as a super barrel with a centrally located active site (Fig. 33.12). The seven highly conserved active site residues found in NA and sialidases are found in the paramyxovirus HN structures. However, these key active site residues are mutated in the measles H and Nipah/Hendra G proteins, rendering these proteins enzymatically dead. Superimposition of the NDV, HPIV3, and PIV5 HN monomer structures indicates a high degree of conservation on one face of the molecule, with the other face containing more variability and additional protein loops.⁴⁵¹

It has long been debated whether the hemagglutinin and NA activities of HN involve one or two separate sialic acid binding sites.^{72,329,453} The disparate theories of one site with dual function or of two distinct sites that are intimately related are both consistent with the observation that sialic acid–derived NA inhibitors interfere with receptor binding.^{177,275,355} A single site can provide both hemagglutinin and NA activities by binding sialic acid tightly and hydrolyzing the molecules slowly.³⁵⁵ For NDV HN, two sialic acid sites have been observed in the x-ray structures: one is the active site, and a second site is located at the dimer interface.⁴⁵³ Strong biological evidence supports the notion of a second sialic acid binding site in NDV.^{29,323} Mutagenesis of a key residue involved in the dimer interface sialic acid binding site abolishes sialic acid binding to the second site.²⁹ However, virus containing this key residue mutation is only marginally affected in growth properties.²⁹ For HPIV3, the second sialic acid binding site is blocked by a carbohydrate chain that prevents its function,²⁶¹ although mutagenesis to ablate the carbohydrate chain allows the HPIV3 second site to bind sialic acid. The growth curve of HPIV3 with or without the carbohydrate chain that shields the second sialic acid binding site is very similar, suggesting no major biological importance.

For PIV5 HN, not only was a second sialic acid binding site not observed, the molecule could not form the second sialic acid binding site between two monomers owing to changes in sequence and conformation.^{218,451} Thus, the biological importance of the second sialic acid binding sites in NDV HN and the one created in HPIV3 by removal of the carbohydrate addition site are a conundrum.^{261,322,325}

From the structural studies of NDV HN, it was also suggested that the NA domain could form two distinct dimeric

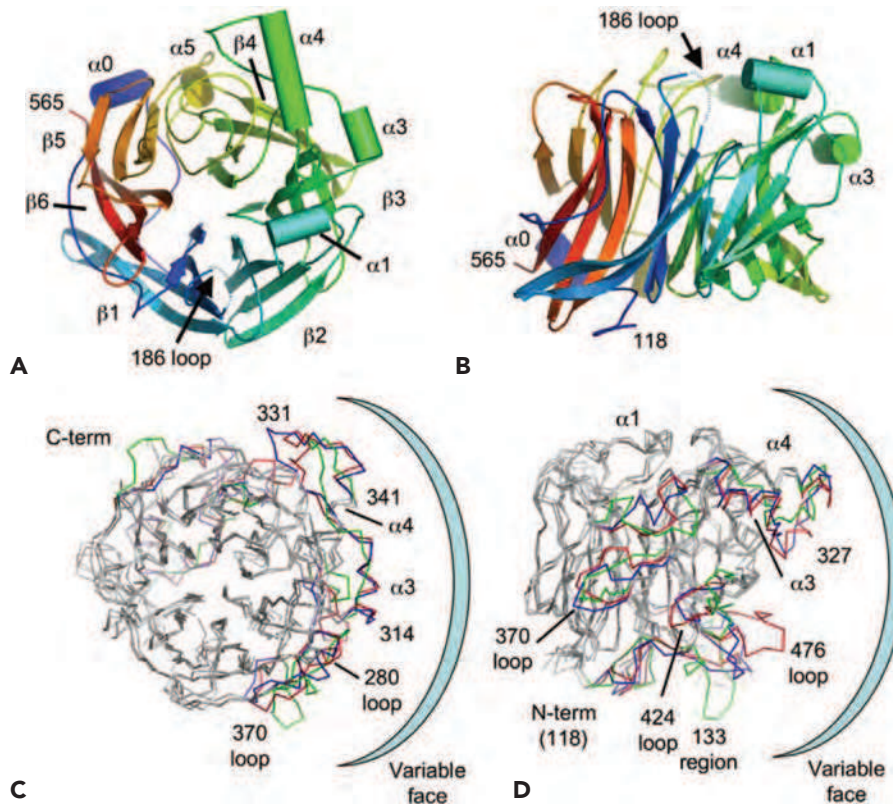


FIGURE 33.12. Parainfluenza virus type 5 (PIV5) hemagglutinin-neuraminidase (HN) monomer structure and comparison with Newcastle disease virus (NDV) HN and human parainfluenza virus type 3 (HPIV3) HN. **A, B:** Schematic cartoon diagrams showing top and side views of PIV5 HN. Helices are shown in *cylinders*, and β -strands are shown in *arrowed belts*. The N-terminus is shown in *blue*, and the C-terminus is shown in *red*. The missing loop from residues 186 through 190 is indicated as a *dashed blue line*. **C, D:** α ribbon diagram of the superposition of PIV5 HN with NDV and HPIV3 HN, shown in top and side views. Major differences in the PIV5, NDV, and human parainfluenza virus (HPIV) HN structures are colored *red*, *blue*, and *green*, respectively. Areas of major structural differences are labeled, and the highly variable face of the HN monomer is highlighted. (Adapted from Yuan P, Thompson T, Wurzberg BA, et al. Structural studies of the parainfluenza virus 5 hemagglutinin-neuraminidase tetramer in complex with its receptor, sialyllactose. *Structure* 2005;13:1–13.)

assemblies that were ligand dependent.⁷² One of the dimers, observed after co-crystallization with ligand, formed an extensive buried interface, whereas the second dimer, crystallized in the absence of ligand and at low pH, formed a much smaller interface. Conformational changes were observed in the active site of the HN protein upon ligand binding that were correlated with changes in the dimer interface, suggesting a possible mechanism for coupling ligand recognition to changes in the oligomeric assembly of the HN protein. However, engineered disulfide bonds block dimer dissociation and do not affect fusion, rendering major HN rearrangements unlikely.²³⁴

Structural studies of HPIV3 and PIV5 HN also do not support the notion that there are ligand-dependent conformational changes within the monomeric protein structure.^{218,451} The dimer of HN that is observed in the HPIV3 and PIV5 structures occurs in the absence of ligand binding, and there is no crystallographic evidence that monomeric ligand binding influences the oligomeric structure of these HN proteins.

The HN tetrameric arrangement^{451,453} is unusual, because rather than having fourfold rotational symmetry as might be anticipated, it is arranged with two twofold symmetry axes (Fig. 33.13) that are orientated at approximately 90 degrees

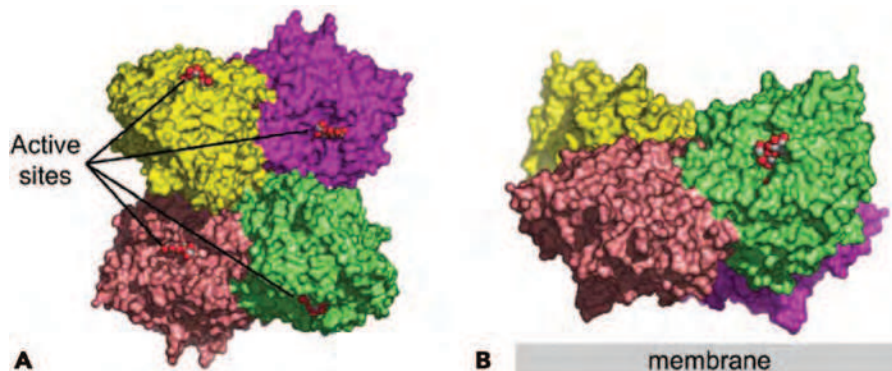
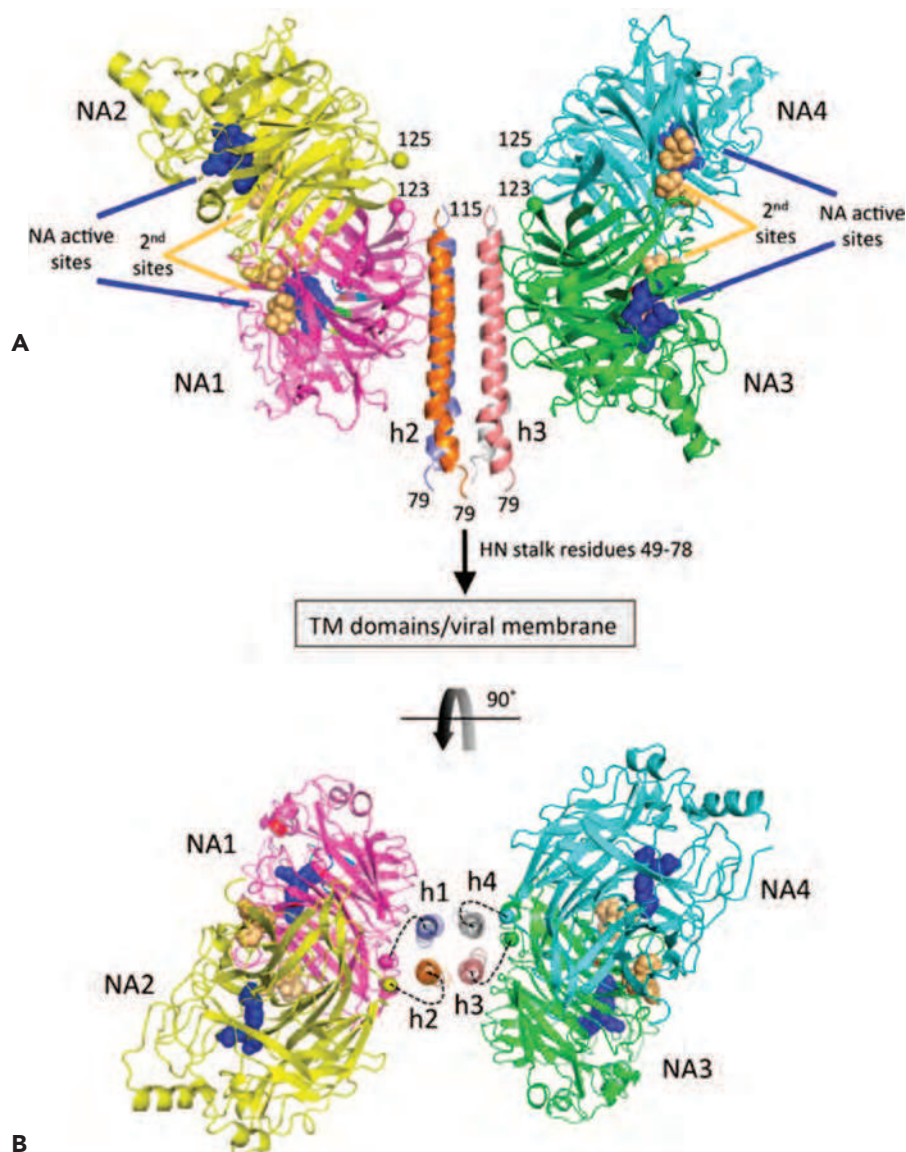


FIGURE 33.13. Parainfluenza virus type 5 (PIV5) hemagglutinin-neuraminidase (HN) tetramers. Active sites are marked by space-filling representations of the ligand sialyllactose. The four subunits are shown in different colors. **A:** Top view of the PIV5 HN tetramer arrangement. **B:** Side view of the PIV5 HN tetramer arrangement, with a 60-degree packing angle between dimers. (Adapted from Yuan P, Thompson T, Wurzberg BA, et al. Structural studies of the parainfluenza virus 5 hemagglutinin-neuraminidase tetramer in complex with its receptor, sialyllactose. *Structure* 2005;13:1–13.)

FIGURE 33.14. Structure of the Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) (Stain Australian–Victoria) ectodomain. A:

Two dimers of the NDV HN neuroaminidase (NA) domains flank the four-helix bundle in the stalk. The four NA domains are labeled NA1 through NA4. The active sites are marked by three residues shown as *blue* CPK spheres (E400, R415, and Y525) and labeled accordingly. The secondary sialic acid binding sites located at the NA domain dimer interfaces are marked by residues shown as *orange* CPK spheres and labeled (second sites). The N-termini of the four NA domains, residues 123 and 125, are labeled and indicated by their α atoms shown in CPK format colored by chain. The connections of the N-terminal region of the stalk to the HN transmembrane domains and viral membrane are indicated.

B: End-on view of the packing of the HN stalk tetramer between two NA domain dimers rotated through 90 degrees as indicated by the *curved arrow*. Although no electron density was observed to connect the HN stalk helices with the individual NA domains, the *dotted lines* indicate possible linkages between these domains, with NA1/NA2 and NA3/NA4 forming covalently linked dimers through C123. The four-stalk helices are indicated as h1 through h4. (Adapted from Yuan P, Swanson KA, Leser GP, et al. Structure of the Newcastle disease virus hemagglutinin-neuraminidase [HN] ectodomain reveals a four-helix bundle stalk. *Proc Natl Acad Sci U S A* 2011;108:14920–14925.)



to each other and in the crystal lattice, allowing neighboring dimers and tetramers to associate in infinitely long oligomers. The dimer places the two HN active sites at nearly 90 degrees to each other. The calculated buried surface area for each monomer in the PIV5 HN dimer is 1,818 Å². In contrast to the dimer interaction, the dimer-of-dimers interface is much smaller, involving only 10 residues and burying only 657 Å². The small surface of interaction suggests that the arrangement is not very strong and that the dimers may dissociate.

Recently, the atomic structure of the NDV head domain with a tetrameric stalk has been obtained⁴⁵⁰ (Fig. 33.14). The stalk forms a four-helix bundle, and on either side are dimers of head domains. One head domain of each dimer makes extensive interactions with the stalk. This structure, as compared with the head-only tetramer,⁴⁵¹ suggests plasticity in the stalk/head-connecting region.

The structure of the *Pneumovirus* attachment protein (G) is very different from the attachment protein of the *Paramyxovirinae*. The RSV G protein has neither hemagglutinating nor NA activity. The nucleotide sequence of the RSV G gene predicts

that the protein is of 289 to 299 amino acids (M_r)^{32,587} and is a type II integral membrane protein with a single N-terminal hydrophobic signal/anchor domain.^{352,435} The G protein is found in virus-infected cells in both membrane-bound and proteolytically cleaved soluble forms. The distinguishing feature of the RSV G protein is the extent of its carbohydrate modification. On SDS-PAGE, the protein migrates with an apparent M_r of approximately 84,000 to 90,000, and the dramatic increase in molecular weight over that predicted for the polypeptide chain is because 8 to 12 kDa is owing to addition of N-linked carbohydrate (four potential addition sites) and 40 to 50 kDa is owing to the addition of O-linked glycosylation (77 potential acceptor serine or threonine residues; 30% of total residues) (61 and references therein). Quite remarkably, it appears that the RSV G protein is not essential for virus assembly or growth in tissue culture or animals, although it does confer a growth advantage. A virus that had been extensively passaged in cells was found to contain a spontaneous deletion of the G and SH genes,¹⁸⁹ yet the virus replicated in Vero cells. In addition, the G gene has been deleted from recombinant virus recovered from

an infectious cDNA clone (see Chapter 38). These findings suggest that RSV has an alternate mechanism for attachment to cells that does not involve G protein, and evidence has been obtained that RSV lacking G protein can bind to heparan sulfate and possibly other molecules.^{111,140,406} Similar observations have been made for HMPV.⁵⁷

Paramyxovirus Fusion Protein

The paramyxovirus fusion (F) proteins mediate viral penetration by fusion between the virion envelope and the host cell plasma membrane, and this fusion event occurs at neutral pH for all family members except a few isolates of HMPV, where low pH appears to have some role in fusion activation.^{249,362} The consequence of the fusion reaction is that the nucleocapsid is delivered to the cytoplasm. Later in infection, the F proteins expressed at the plasma membrane of infected cells can mediate fusion with neighboring cells to form syncytia (giant cell formation), which is a cytopathic effect that can lead to tissue necrosis *in vivo* and might also be a mechanism of virus spread.

The F proteins are homotrimers^{58,346,444,445} that are synthesized as inactive precursors (F₀). To be biologically active, they have to be cleaved by a host cell protease at the cleavage activation site. Cleavage releases the new N-terminus of F₁, thus forming the biologically active protein consisting of the disulfide-linked chains F₁ and F₂.^{167,354} The paramyxovirus F genes encode 540 to 580 residues (see Fig. 33.11). The F proteins are type I integral membrane proteins that span the membrane once and contain at their N-terminus a cleavable signal sequence that targets the nascent polypeptide chain syn-

thesis to the membrane of the endoplasmic reticulum. At their C-termini, a hydrophobic stop-transfer domain (TM domain) anchors the protein in the membrane, leaving a short cytoplasmic tail (~20–40 residues). Sequences adjacent to the fusion peptide and the TM anchor domain typically reveal a 4–3 (heptad) pattern of hydrophobic repeats and are designated HRA and HRB, respectively. Approximately 250 residues separate HRA and HRB (Fig. 33.15A).

Evidence has been presented that there is a second polytopic form of the NDV F protein that is 10% to 50% of the total F protein.²⁵⁴ The proposed second polytopic form of F has not been found for other paramyxovirus F proteins, and it is unclear why NDV F protein would be different from other F proteins. Because the second form of NDV F is only partially membrane translocated, it would have a very different protein fold from prefusion F (see Fig. 33.15), and it is unclear why NDV would uniquely require this form of F for the viral replication cycle.

The F protein is thought to drive membrane fusion by coupling irreversible protein refolding to membrane juxtaposition, initially folding into a metastable form that subsequently undergoes discrete/stepwise conformational changes to a lower energy state.^{183,211} The F protein found on virions is considered to be in a prefusion form; after membrane fusion has occurred, the F protein is considered to be in a postfusion form. Cleavage of F₀ primes the protein for membrane fusion. The varying nature of the residues found at the cleavage site, the enzymes involved in cleavage, and the role of cleavage in pathogenesis will be discussed later.

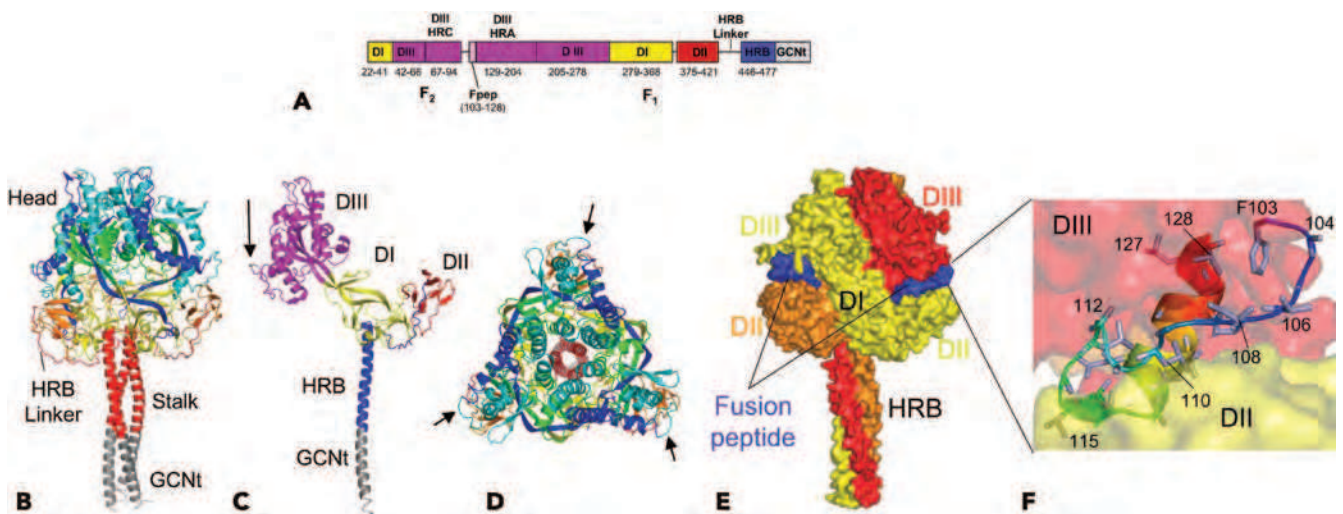


FIGURE 33.15. The fusion (F) protein prefusion structure. **A:** Schematic diagram of the F-GCNI domains. Important domains are colored and their corresponding residue ranges indicated. **B:** Ribbon diagram of the F trimer, with each chain colored by residue number in a gradient from blue (N-terminus) to red (C-terminus). The head and stalk regions are indicated. HRB linker residues 429 through 432 could not be modeled in one subunit and had high temperature factors in the other two. **C:** Ribbon diagram of one subunit of the F trimer colored by domain. The domains are labeled, and the colors correspond to those used in **A**. The cleavage/activation site is indicated with an arrow. **D:** Top view of the trimer colored as in **A**. Cleavage/activation sites are indicated by arrows. **E:** Surface representation of the F trimer colored by subunit. The fusion peptide exposed surface is colored blue. **F:** Close-up view of the fusion peptide (residues 103–128). The peptide is folded back on itself with a small hydrophobic core and contains a mixture of extended chain, one β -strand and a C-terminal α -helix. The fusion peptide is sandwiched between two subunits of the trimer, between DIII and DIII domains. (Adapted from Yin HS, Wen X, Paterson RG, et al. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. *Nature* 2006;439:38–44.)

Comparison of the amino acid sequences of paramyxovirus F proteins (reviewed in 267) does not show overall major regions of sequence identity, with the exception of the fusion peptide, which has a conserved sequence (up to 90% identity). However, the overall placement of cysteine, glycine, and proline residues suggests a similar structure for all F proteins. The *Respirovirus* and *Rubulavirus* F2 and F1 subunits are glycosylated, and there are a total of 3 to 6 potential sites for the addition of N-linked carbohydrate. For PIV5 F protein, it is known that all four potential sites for addition of N-linked carbohydrate are used.⁸ The measles virus F protein contains three sites in the F2 subunit for N-linked carbohydrate addition, and all three sites are used; there are no sites in F1 for N-linked carbohydrate addition.¹

CLASS I VIRAL FUSION PROTEINS

The paramyxovirus F proteins belong to the class I viral fusion protein type, of which the longest standing member is the influenza virus hemagglutinin. Class I also includes the fusion proteins from retroviruses including human immunodeficiency virus type 1 (HIV-1; Env/gp160), coronaviruses (S), and Ebola virus (G).^{67,96,97,183,213} Models for class I viral fusion protein-mediated membrane merger have been developed, until recently, primarily from the structural studies of hemagglutinin.³⁷³ The general mechanism for class I viral fusion proteins posits the folding of the uncleaved protein to a metastable state, which can be activated to undergo large conformational changes to a more stable fusogenic or postfusion state. The attainment of the prefusion conformation, its regulation, and relative free energy as compared to the postfusion form are all key to the process by which class I viral fusion proteins function.

CLASS I VIRAL FUSION PROTEINS AND THE HELICAL HAIRPIN (CORE TRIMER)

Biophysical data has indicated that HRA and HRB form a complex, and crystallographic studies have shown that HRA and HRB form a helical hairpin or six-helix bundle (6HB) structure (core trimer) that is related to that observed for the low-pH induced proteolytic fragment of hemagglutinin (TBHA2). For example, the core trimers of PIV5 and human RSV F,^{9,187,456} human immunodeficiency virus (HIV) gp41,^{42,55,396,432} Moloney murine leukemia virus envelope protein,¹⁰⁷ Ebola GP2,^{235,433} and human T-cell leukemia virus type 1 (HTLV-1)²⁰² fusion proteins all share this similarity in structure (Fig. 33.16A). Although the structural details vary, all reveal a trimeric, coiled-coil beginning near the C-terminal end of the hydrophobic fusion peptide. The C-terminal segment abutting the TM domain is also often helical and packs in an antiparallel direction along the outside of the N-terminal coiled-coil, placing the fusion peptides and TM anchors at the same end of a rod-like structure (for PIV5 6HB, see Fig. 33.16A). These 6HBs typically represent a relatively small fraction of the intact fusion protein, yet their structures are generally highly thermostable, with melting temperatures near 100°C. Intermediates along the pathway of membrane fusion can be trapped by the addition of peptides derived from either the N-terminal (HRA) or C-terminal (HRB) heptad repeat regions for many class I fusion proteins,^{97,100,106,344,449} indicating that the intact protein undergoes conformational changes that expose both HR regions prior to refolding to the final 6HB. The intermediates are thought to represent partially refolded forms of the fusion protein, with a hydrophobic fusion peptide anchored in the target cell membrane and the TM domains

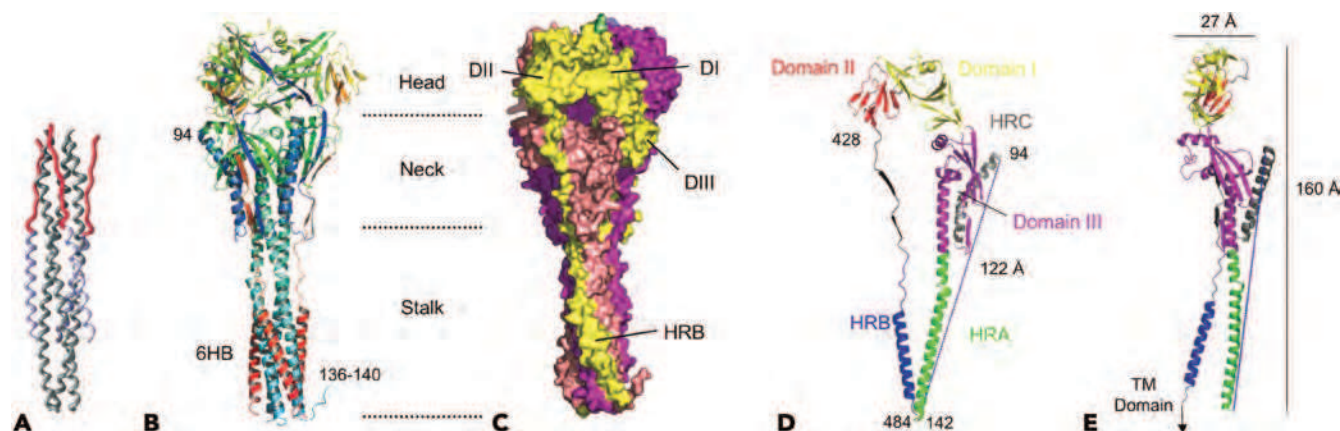


FIGURE 33.16. The F protein postfusion structure. **A:** The complete parainfluenza virus type 5 (PIV5) F1 core trimer is shown with the N1 helix colored gray and the C1 peptide colored blue except for the extended-chain N-terminal residues of C1 that are colored red. **B:** Ribbon diagram of the HPIV3 solF0 trimer. The three chains are colored similarly from blue (N-terminus) to red (C-terminus). Residues 95 through 135 are disordered in all chains. Residue 94 is labeled in one chain, and residues 136 through 140 at the base of the stalk are ordered in one chain owing to crystal packing interactions. **C:** Surface representation of the solF0 trimer. Each chain is a different color, and domains I through III and HRB for one chain (yellow) are indicated by the DI, DII, DIII, and HRB labels. One radial channel is readily apparent below domain I and II of the yellow chain and above domain III of the red chain. **D:** Ribbon diagram of the solF0 protein monomer colored by domain. The direct distance within one monomer between residue 94 at the end of HRC and residue 142 at the base of the stalk region is 122 Å. **E:** Ribbon diagram of the monomer rotated by 90 degrees, indicating the width and height of the solF0 monomer. An arrow at the C-terminus of the HRB segment points toward the likely position of the transmembrane anchor domain that would be present in the full length protein. (Adapted from Yin H-S, Paterson RG, Wen X, et al. Structure of the uncleaved ectodomain of the paramyxovirus [HPIV3] fusion protein. *Proc Natl Acad Sci U S A* 2005;102:9288–9293.)

anchored to the viral membrane. The formation of the 6HB is tightly linked to the merger of lipid bilayers and is thought likely to couple the free energy released on protein refolding to membrane fusion.^{257,344}

ATOMIC STRUCTURES OF THE PARAMYXOVIRUS F PROTEIN

Structure of the Prefusion F Protein. The atomic structure of the PIV5 F protein in its uncleaved metastable prefusion form has been determined.⁴⁴⁵ To solve the atomic structure, the secreted F protein was stabilized by the addition of a soluble trimeric TM domain (GCNt) that supplants the hydrophobic TM domain. The F trimer has a large globular head attached to a three-helix coiled-coil stalk formed by HRB (see Fig. 33.15B–E) orienting the head away from the viral membrane. The F head contains three domains (DI–DIII) per subunit that extend around the trimer axis, making extensive intersubunit contacts. A large cavity is present at the base of the head, with the bottom and sides formed by DI and DII. DIII (residues 42–278) covers the top of the cavity, HRA, and the fusion peptide (see Fig. 33.15B–D). At the C-terminus of DII, an extended linker to HRB wraps around the outside of the trimer and into the center of the base of the head where the stalk begins. The structure has three lateral vertices projecting from the trimer axis, exposing the cleavage/activation sites adjacent to the fusion peptides (see Fig. 33.15C,D). Helices line the central threefold axis at the top and bottom of the trimer. In DIII, two sets of six helices form rings sealing the top of the head, whereas the HRB three-helix bundle seals the bottom (see Fig. 33.15D).

In the prefusion PIV5 F structure, the hydrophobic fusion peptide (residues 103–128) is wedged between two subunits of the trimer (see Fig. 33.15E). The N-terminal end of the fusion peptide is exposed at the F surface and then proceeds inward, becoming more buried from solvent. The fusion peptide adopts a partly extended, partly β -sheet, and partly α -helical conformation and is sandwiched between DIII of its own

subunit and DII of another. Residues 107 through 117 pack against the hydrophobic edge of the neighboring DII domain. The fusion peptide folds back on itself, forming a small hydrophobic core between its N-terminal and C-terminal ends, making less extensive contacts with DIII (see Fig. 33.15E,F). Proteolytic cleavage of F0 might allow the N-terminus of the fusion peptide to make additional contacts with DII and to affect intersubunit interactions.

Structure of the Postfusion Form of the F Protein. The atomic structure of intact F protein in its postfusion form has been determined for HPIV3, NDV, and RSV.^{255,388,389,444} The structure of the HPIV3 F protein was solved by molecular replacement, using as a model the structure of a proteolytic fragment of NDV F,⁵⁸ now known to be in its postfusion form.

HPIV3 F forms a trimer, with distinct head, neck, and stalk regions (Fig. 33.17A–D). The only part of the structure lacking electron density is the fusion peptide and cleavage site; however, the residues would be draped flexibly on the exterior of the stalk region. Given that the uncleaved F ectodomain was secreted from cells by removal of the TM domain, it was initially unexpected that the structure contained a 6HB (see Fig. 33.17A–D) that represents the postfusion conformation of the protein. It had been widely anticipated that cleavage of F at the cleavage site was a requirement for conversion to the postfusion form. Nonetheless, many lines of evidence suggested that the observed HPIV3 conformation represented the postfusion form, although the polypeptide chains were intact in the crystal and the fusion peptide was not located at the appropriate end of the 6HB.

The observation that the soluble, secreted HPIV3, NDV F, and RSV F proteins were in the postfusion conformation was unexpected, and there are at least two possible explanations for this finding. First, the TM anchor (and potentially the cytoplasmic tail)⁴²⁸ could be an important determinant of the stability of the prefusion conformation, providing a

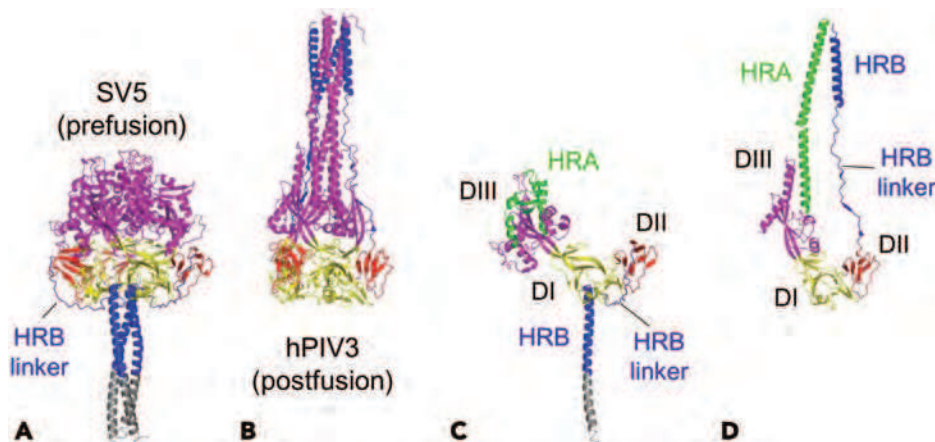


FIGURE 33.17. Structural changes between the pre- and postfusion F protein conformations.

A: Ribbon diagram of the parainfluenza virus type 5 (PIV5) F-GCNt trimer. DI is colored yellow, DII is colored red, DIII is colored magenta, HRB is colored blue, and GCNt is colored gray. **B:** Ribbon diagram of the human parainfluenza virus type 3 (HPIV3) (postfusion) trimer, colored as in **A**. **C:** Ribbon diagram of a single subunit of the PIV5 F-GCNt trimer, colored as in **A**, except for HRA residues, which are colored green. **D:** Ribbon diagram of a single subunit of the HPIV3 F trimer, colored as in **C**. (Adapted from Yin HS, Wen X, Paterson RG, et al. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. *Nature* 2006;439:38–44.)

significant fraction of the energy barrier that traps the protein in a metastable state. In this case, the secreted protein may fold to the prefusion form transiently but then refold to the postfusion form. A second possible explanation for the structural results is that the TM domain is important for the protein to attain the prefusion metastable state and that in the absence of this region, the soluble F protein folds directly to the final, most stable postfusion conformation. In either case, it appears that the amino acids comprising the intact F protein ectodomain are not sufficient for the protein to fold to and maintain a metastable conformation. Hence, to trap a soluble form of the F protein in its metastable form, the F protein was stabilized by the addition of a soluble trimeric TM domain (GCNt) that supplants the hydrophobic TM domain.

COMPARISON OF THE PREFUSION AND POSTFUSION F STRUCTURES

The PIV5 prefusion F and HPIV3 postfusion F structures are in strikingly different conformations (see Fig. 33.17), consistent with a transition from pre- to postfusion forms. None of the intersubunit contacts are conserved in the pre- and postfusion forms. The two F structures are related by flipping the stalk and TM domains relative to the F head. Substantial compacting of the head is observed in HPIV3 postfusion F compared to PIV5 prefusion F. DI domains pivot slightly inward, shearing intersubunit contacts, and DII domains swing across, contacting neighboring subunits. Individual DI and DII domains in the two structures remain similar. Potentially related forms of the F protein have been observed in electron micrographs of RSV F.^{44,130,342,343}

DIII undergoes major refolding between the two structures, projecting a new coiled coil (HRA) upward and away from DI, the prefusion stalk, and the viral membrane. The fusion peptide, located at the top of the HRA coiled coil, moves

approximately 115 Å from its initial position between subunits in the prefusion conformation, allowing DII domains to reposition. None of the postfusion HRA intersubunit coiled-coil contacts are observed in F-GCNt. Instead, they are replaced by two sets of six-helix rings at the DIII interfaces (see Fig. 33.15D). For the HRA coiled coil to form, DIII must rotate and collapse inward, further compacting the head.

The F protein refolding also requires the opening and translocation of the HRB stalk (see Fig. 33.17). In the prefusion form, HRB is located at the base of the head region. During the conversion to the postfusion conformation, HRB segments must separate and swing around the base of the head to pack against the HRA coiled coil. In the prefusion conformation, HRA is broken up into four helices, two β -strands, and five loop, kink, or turn segments. Thus, the conformational changes in HRA involve the refolding of 11 distinct segments into a single, extended α -helical conformation (Fig. 33.18).

THE MECHANISM OF PARAMYXOVIRUS-MEDIATED MEMBRANE FUSION

The prefusion and postfusion F structures suggest how discrete refolding intermediates are coupled to the activation and progression of F-mediated membrane fusion. Whereas proteolytic cleavage of the paramyxovirus F protein is required for membrane fusion activity, it is not required for the formation of the postfusion conformation. A model for membrane fusion is as follows. In the first step, the HRB helices melt (open-stalk form, Fig. 33.19), breaking interactions at the base of the head but leaving HRA in the prefusion conformation. This intermediate is consistent with effects of mutations of PIV5 residues 443, 447, and 449 as well as peptide inhibition data.^{305,344,345} HRA-derived peptides, which likely bind to the endogenous HRB segment, inhibit an early intermediate along the fusion

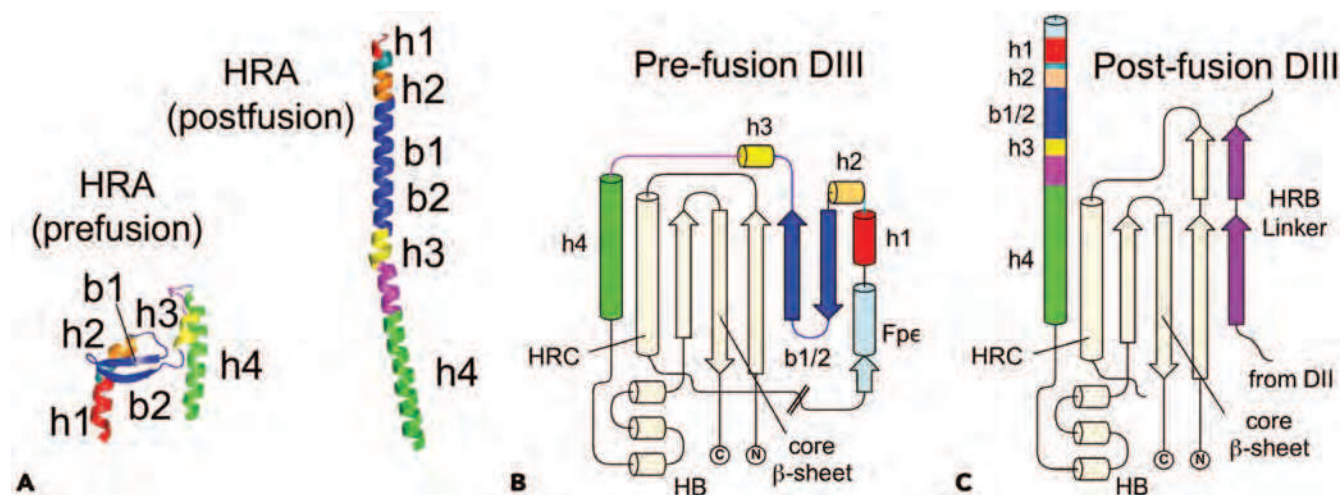


FIGURE 33.18. F protein refolding: the role of DIII in HRA folding and transformation. **A:** HRA refolds from 11 distinct segments (h1, h2, b1, b2, h3, h4, and the intervening residues) in the prefusion conformation into a single, nearly 120 Å long helix in the postfusion form. **B:** Secondary structure diagram for DIII in the prefusion (parainfluenza virus type 5) conformation. The “DIII core” includes three antiparallel strands, HRC, a helical bundle (HB), and h4 of HRA. HRA segments are colored as in **A**, and the cleavage site (//) and fusion peptide are indicated. The DIII core sheet is extended by the b1 and b2 strands from HRA. **C:** Secondary structure diagram for DIII in the postfusion (human parainfluenza virus type 3) conformation. The DIII core sheet is extended by one strand from HRB linker from a neighboring subunit (dark violet). (Adapted from Yin HS, Wen X, Paterson RG, et al. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. *Nature* 2006;439:38–44.)

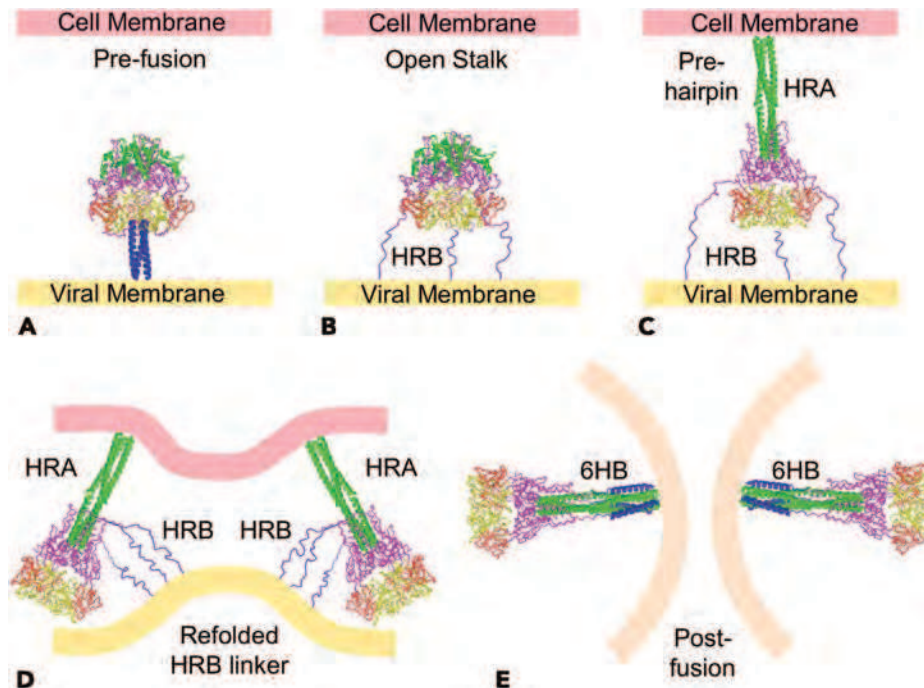


FIGURE 33.19. A model for F-mediated membrane fusion. **A:** Structure of the prefusion conformation. HRB is colored *blue*, HRA is colored *green*, and domains I, II, and III are colored *yellow*, *red*, and *magenta*, respectively. **B:** An open-stalk conformation, in which the HRB stalk melts and separates from the prefusion head region. HRB is shown as three extended chains because the individual segments are unlikely to be helical. This conformation is consistent with a low-temperature intermediate that is inhibited by HRA peptides but not HRB peptides. Mutations of the switch peptide residues 443, 447, and 449 would influence the formation of this intermediate by affecting stabilizing interactions between the prefusion stalk and head domains. **C:** A pre-hairpin intermediate can form by refolding of DIII, allowing the formation of the HRA coiled coil and insertion of the fusion peptide into the target cell membrane. This intermediate can be inhibited by peptides derived from both HRA and HRB regions. **D:** Prior to forming the final six-helix bundle, the close approach of viral and cellular membranes may be trapped by folding of the HRB linker onto the newly exposed DIII core, with the formation of two β -strands (see Fig. 33.15D,F). **E:** The formation of the postfusion six-helix bundle is tightly linked to membrane fusion and pore formation, juxtaposing the membrane interacting fusion peptide and transmembrane domains. (Adapted from Yin HS, Wen X, Paterson RG, et al. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. *Nature* 2006;439:38–44.)

pathway, whereas HRB-derived peptides inhibit a later intermediate by binding the endogenous HRA coiled coil. Opening of the HRB stalk could initiate further changes in F by affecting the packing of DII and the fusion peptide (through the HRB linker) and by affecting the stability of the head intersubunit contacts, which shift during the conformational transition. It seems possible that transient dissociation of the F trimer could occur, analogous to the dimer-to-trimer transition characterized in alpha- and flavivirus fusion proteins (Chapter 3). The open-stalk intermediate is then likely followed by refolding of DIII, the assembly of the HRA coiled coil, and the translocation of the fusion peptide toward the target cell membrane (see Fig. 33.19). This pre-hairpin intermediate has been trapped and co-precipitated with HRB peptides³⁴⁴ and imaged by electron microscopy.¹⁹⁸ Removal of the fusion peptide from the intersubunit interfaces would enable an inward swing of DII and the formation of new contacts with DI of a neighboring subunit, compacting the head. The refolding of DIII HRA would also expose its core β -sheet, and together with the inward movement of DII allow the HRB linker (at the C-terminus of DII) to form parallel β -strands with the DIII core, likely preceding and initiating the final positioning of HRB (see Fig. 33.19). The assembly of the final

6HB completes the conformational change and membrane merger.

HN ACTIVATES THE F PROTEIN FOR MEMBRANE FUSION

The triggering mechanism that regulates the F protein conformational changes such that it occurs at the right place and the right time is not fully understood, although as described later for most paramyxoviruses, there is a requirement for the receptor-binding protein (HN, H, or G) in mediating the fusion reaction.^{215,268} The precise role of the HN, H, or G protein in stimulating the F conformational change remains to be understood; however, the emerging picture indicates a regulated complex biological machine.^{178,222,318}

For all paramyxoviruses, co-expression of F and HN (H or G) is either required for fusion or co-expression of HN (H or G) makes fusion more efficient.^{51,99,173,176,266,349,438,443} Furthermore, the homotypic HN (i.e., of the same virus), not a heterotypic HN, has to be co-expressed in the same cell as the F protein to promote fusion.^{173,176,365} However, expression of the F of PIV5, measles virus, or RSV alone causes some syncytium formation,^{1,173,188,293,302,306} although it is important to note that it is likely that many more cells express the F protein than are found in multinucleated cells.³⁰⁶ Furthermore,

point mutations within NDV F render the protein HN independent for fusion.³⁶⁷ Thus, it seems likely that there are different activation energies for triggering fusion for the different paramyxovirus F proteins. This is highlighted by the observation that PIV5 F-GCnT soluble protein can be converted to the postfusion form by using heat (55°C) as a surrogate for HN activation.⁶⁹

It was hypothesized that a type-specific interaction would occur between the HN and F protein.^{176,211,366} Immunoprecipitation assays show that F and HN co-precipitate, indicating that they can associate^{86,256,381,443}; for HPIV3, F and HN undergo antibody-induced co-capping, indicative of a protein complex formation.⁴⁴³ A great deal of effort has been spent to map the regions of F and HN that interact. One of the difficulties in the work in studying HN is that mutations often affect more than one of the three known biological activities of hemadsorption, NA activity, and fusion promotion. Mutations have been identified in the HN globular domain,^{84,260} the HN stalk,^{23,86,256,295,324,366,382,397,452} and TM anchor^{28,252} that decrease or abolish fusogenic activity with no or little effect on receptor recognition. Analysis of the fusion-promoting activity of chimeric HN molecules derived from different paramyxoviruses largely suggests that the stalk domain and, in some cases, parts of the globular head impart F specificity.^{86,397,414} A point mutation was found in the NDV HN globular head that abolishes both its receptor recognition and NA activity, and that also abolishes its ability to interact with F in co-immunoprecipitation assays.⁸⁵ Based on the view that HRB in F mediates an interaction with HN, it was found that a peptide mimicking HRB bound to a fragment of HN (residues 124–152) when the HN fragment was expressed as an artificial fusion protein.¹³⁶ However, other biological data argues against HN residues 124 through 152 as being part of the F-interactive domain in HN.²⁵⁶

No one model for fusion activation has been universally embraced, and data obtained from studying measles virus and Nipah virus fusion suggest different mechanisms of F protein activation.^{222,318} There are two major models for F activation, with the constant factor being that the HN stalk is required for activation. The *clamp* model (or dissociation model) posits that F and HN/H/G associate with each other in the endoplasmic reticulum and reach the cell surface as a complex that holds F in its metastable prefusion state. Once the attachment protein binds to its receptor, the attachment protein undergoes a conformational change that causes release of F, enabling F to be fusion active. A corollary of the clamp model is that when F is expressed in cells from cDNA without HN/H/G expression, it should be in its postfusion form. The *provocateur* (or association model) posits that F and HN/H/G are transported to the cell surface independently, and on the HN/H/G-binding receptor, there is a conformational change in the receptor-binding protein that leads to complex formation with F, most likely through the HN/H/G stalk, hence triggering fusion activation.^{69,178} Although it is no longer thought that there is a conformational change within an HN/H/G monomer, there is accumulating evidence for more than one form of dimer-dimer interaction^{149,283,450,451} and evidence for the association of the NDV HN dimer with its stalk.⁴⁵⁰

CLEAVAGE ACTIVATION

As discussed previously, the precursor F0 molecule is biologically inactive and cleavage of F0 to the disulfide-linked chains

F1 and F2 activates the protein, rendering the molecule fusion active and permitting viral infectivity. It is important to note that F2 and F1 are not separate domains in the atomic structure of F and thus are not individual parts of the protein. Cleavage of F0 is a candidate to be a key determinant for infectivity and pathogenicity; for certain viruses, this appears to be the case. Proteolytic activation of F0 involves the sequential action of two enzymes: the host protease that cleaves at the carboxyl side of an arginine residue and a host carboxypeptidase that removes the basic residues. The *Paramyxoviridae* can be divided into two groups: those that have F proteins with multibasic residues at the cleavage site and those with F proteins that have a single basic residue at the cleavage site (see Table 33.3). Cleavage of F proteins containing multibasic residues at the cleavage site occurs intracellularly during transport of the protein through the *trans*-Golgi network.

Furin is a cellular protease localized to the *trans*-Golgi network, and its sequence specificity for cleavage is R-X-K/R-R. The available evidence suggests that furin, a subtilisin-like endoprotease, is the (or one of the) protease(s) that cleaves most F proteins intracellularly.^{201,294}

Paramyxoviruses that have F proteins with single basic residues in the cleavage site (e.g., Sendai virus) are not usually cleaved when grown in tissue culture, and thus only a single cycle of growth is obtained. However, the F0 precursor that is expressed at the cell surface and incorporated into released virions can be cleavage activated by the addition of exogenous protease,³⁵⁴ leading to multiple rounds of replication. Purification of a protease from the allantoic fluid of embryonated chicken eggs has indicated that the endoprotease responsible for Sendai virus activation is homologous to the blood clotting factor Xa, which is a member of the prothrombin family.^{132,134} A protease with a similar substrate specificity is secreted from Clara cells of the bronchial epithelium in rats and mice, and this enzyme is probably responsible for activating paramyxoviruses in the respiratory tract. For NDV, the nature of the cleavage site correlates with virulence of the virus. Those strains with multibasic residues in the F0 cleavage site are virulent strains and readily disseminate through the host, whereas those strains with F0 molecules having single basic residues are avirulent and tend to be restricted to the respiratory tracts where the necessary secreted protease can be found.²⁷⁸

A variation on the cleavage theme is found with Hendra virus, as its F protein does not contain a multibasic cleavage site, and yet Hendra F is cleaved in expressing cells at the sequence HDLVDGVK↓,⁷¹ but the K residue is not essential for cleavage.²⁶² In the search for the cleavage enzyme, it was found that inhibition of cathepsin L blocks cleavage,²⁹⁶ suggesting that cleavage occurs in the endocytic pathway.^{89,259} Recent evidence suggests that Hendra F protein is expressed at the cell surface in an uncleaved F0 form, internalized, cleaved by cathepsin in the late endosome, and recycled to the cell surface.³²¹

Another variation on the cleavage theme is found for RSV F protein. The RSV F protein contains two consensus sequences for furin cleavage. One is located at the F2-F1 junction and the other in F2, 27 residues N-terminal to the F2-F1 junction.¹³⁰ Cleavage at both sites is required for fusion activity.⁴⁵⁷

Other Envelope Proteins

The rubulaviruses PIV5 and mumps virus both contain a small gene located between F and HN designated SH.^{160,161} The PIV5

TABLE 33.3 Amino Acid Sequences Upstream of the F Protein Cleavage Site of Some Members of the *Paramyxoviridae*

Sendai virus	G-V-P-Q-S-R↓
HPIV1	D-N-P-Q-S-R↓
HPIV3	D-P- R-T-K-R ↓
PIV5	T-R- R-R-R-R ↓
Mumps	S-R- R-H-K-R ↓
	R
NDV (virulent strain)	G-R- R-Q —R↓
	K
	G K G
NDV (avirulent strain)	—G—Q—R↓
	E R S
Measles	S-R- R-H-K-R ↓
Hendra virus	HDLVDGVK↓
RSV	R-A-R-R ↓ 109 ELPRFMNYTLNNTKKTNTVLS KKRKRR ↓ 136

HPIV1, human parainfluenza virus type 1; HPIV3, human parainfluenza virus type 3; PIV5, parainfluenza virus type 5; NDV, Newcastle disease virus; RSV, respiratory syncytial virus.

Consensus sequence for furin protease cleavage is **R-X—R**↓
K

Adapted from Hosaka M, Nagahama M, Kim W-S, et al. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. *J Biol Chem* 1991;266:12127–12130.

SH protein is a 44-residue, type II integral membrane protein that is expressed at the plasma membrane and is packaged in virions. The mumps virus SH protein is a 57-residue integral membrane protein orientated in membranes in the opposite direction from the PIV5 SH protein with a C-terminal cytoplasmic domain.^{102,393} Owing to the variability in sequence among different strains of mumps virus, the *SH* gene sequence has been used as marker to identify mumps isolates.³⁹⁴ PIV5 lacking SH (PIV5ΔSH) grows as well as wild-type in tissue culture cells; however, the virus is attenuated *in vivo*.¹⁵¹ PIV5ΔSH induces apoptosis in L929 and MDCK cells (but not in HeLa cells) through a tumor necrosis factor alpha-mediated extrinsic apoptotic pathway in the PIV5ΔSH-infected cells.^{152,228} The *SH* gene has been found in all strains of mumps virus, although expression of the SH protein does not seem to be required for mumps virus replication in tissue culture,³⁹³ because in the Enders strain of mumps virus, a monocistronic mRNA encoding SH is not found. Mumps SH may have a similar role as PIV5 SH, considering replacement of PIV5 *SH* gene with the mumps *SH* gene behaves like wild-type PIV5.⁴³⁹ It has been proposed that the attenuating phenotype of viruses with deletions in SH (e.g., PIV5-ΔSH) reflects an altered gradient of transcription rather than a loss of critical function.²³⁶

Members of the *Pneumovirinae* encode a small hydrophobic protein, also designated SH protein. However, this does not necessarily mean that there is a commonality in function with the *Rubulavirus* SH protein. The RSV SH protein contains 64 amino acids and is expressed at the plasma membrane of RSV-infected cells as a type II integral membrane protein and is packaged in virions.^{66,252} In RSV-infected cells, four SH-related polypeptide species have been identified: M_r 4,800; M_r 7,500; M_r 13,000 to 15,000; and M_r 21,000 to 30,000. The M_r 4,800 species is thought to result from the initiation of protein synthesis at an internal AUG codon, the M_r 7,500 species is unglycosylated SH, the M_r 13,000 to 15,000 species is SH

containing one high-mannose N-linked carbohydrate chain, and the M_r 21,000 to 30,000 species is generated by the addition of polylactosaminoglycan to the N-linked carbohydrate chain.^{2,292} The *SH* gene was found to be deleted spontaneously from a virus passed extensively *in vitro*,¹⁸⁹ and it has been deleted from recombinant RSV⁴⁰ with only minor alterations in virus growth properties in tissue culture cells or the respiratory tract of mice or chimpanzees. Thus, the role of the SH protein in the RSV life cycle is not understood.

Pneumovirus M2 Gene

The RSV *M2* gene contains two partially overlapping ORFs, designated M2-1 and M2-2, which give rise to two proteins M2-1 (194 amino acids) and M2-2 (90 amino acids), respectively.⁶⁴ The mechanism for translating the M2-2 ORF is not clear but may involve a ribosomal stop-restart mechanism analogous to that used for synthesis of the influenza B virus BM2 protein¹⁷⁴ (see Chapter 40). The M2-1 protein is an essential transcriptional elongation factor,^{62,109} and in its absence, the polymerase does not transcribe beyond the *NS1* and *NS2* genes.¹⁰⁹ The *M2-1* gene also increases RNAP processivity across the gene junctions, attenuating transcriptional termination.^{109,142,143} The *M2-2* gene is not essential for RSV growth, as it can be deleted from a recombinant RSV.^{14,185} However, the ΔM2-2 virus grows slowly in tissue culture, and there is an increase in transcription and decrease in RNA replication,^{14,185} suggesting that M2-2 protein is involved in regulating transcription and RNA replication. The *M2* gene products of human metapneumoviruses also play a role in controlling viral RNA synthesis, as recombinant HMPV with deletions in the *M2-2* gene showed elevated levels of viral mRNAs.³⁸

Pneumovirus NS1 and NS2 Genes

RSV NS1 (139 amino acids) and NS2 (124 amino acids) are considered to be nonstructural proteins, although the difficulty

in purifying virions from contaminating infected cell debris for this poorly growing virus makes this assignment provisional. Neither protein is thought to be essential for virus growth in cultured cells or in chimpanzees, as the genes can be deleted from a recombinant RSV, although growth *in vitro* and *in vivo* is reduced substantially.^{39,407,437} In a minireplicon system, when NS1 was expressed, it was inhibitory to both transcription and replication,⁶ and expression of NS2 at high levels had a small inhibitory effect on transcription and replication.⁴⁰⁷ Thus, the role of these accessory proteins in controlling RNA synthesis remains to be fully understood. In addition, however,

the human RSV and bovine RSV NS1 and NS2 gene products have been shown to be important viral suppressors of type I IFN induction^{24,377} as described later.

STAGES OF REPLICATION

General Aspects

As far as is known, all aspects of the replication of *Paramyxoviridae* take place in the cytoplasm. An overview of the life cycle of the virus is shown schematically in Figure 33.20, and a

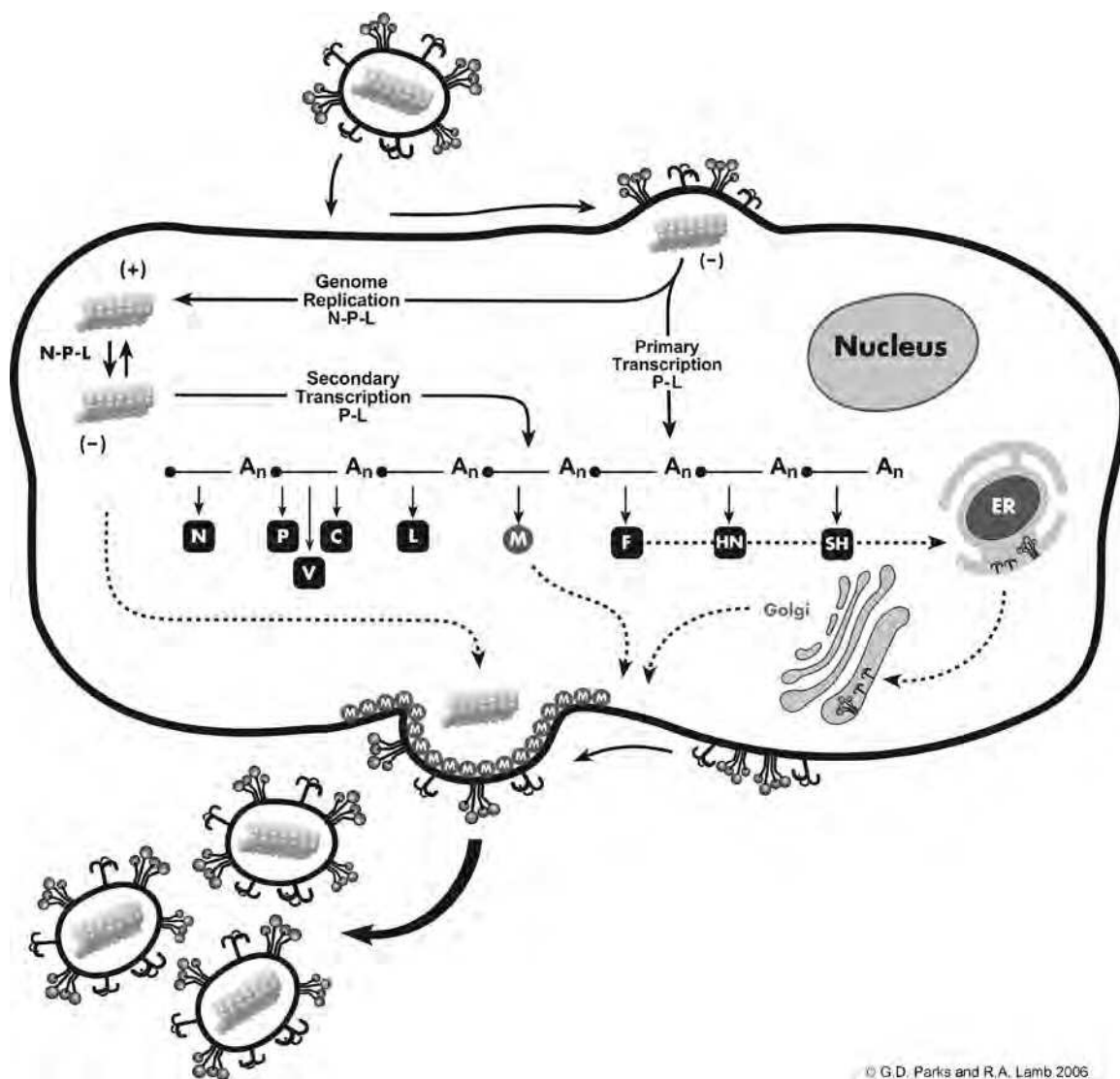
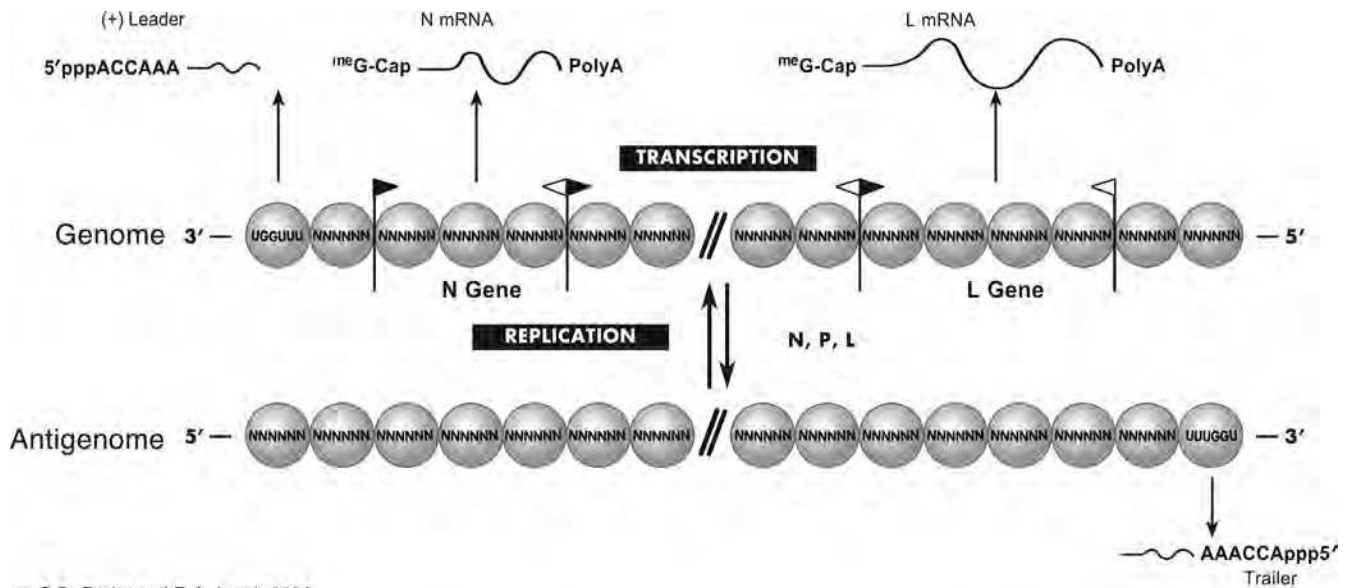


FIGURE 33.20. Schematic representation of the paramyxovirus life cycle. (Refer to the text for details of the viral life cycle.)

The top of the figure shows an incoming virion that fuses with the plasma membrane to release the negative sense nucleocapsid in the cytoplasm. Viral messenger RNAs (mRNAs) are indicated by *lines* with the 5' mRNA cap denoted by a *filled circle* and 3' poly A tail by A_n. The gradient of decreasing molar abundance of the mRNAs from N to L owing to polar transcription is not illustrated. Also not illustrated is the relative abundance of genomic (negative sense) nucleocapsid versus antigenomic (positive sense) nucleocapsid. *Solid lines* denote primary and secondary transcription carried out by a P-L complex and genome replication carried out by an N-P-L complex. *Dotted lines* denote intracellular transport of nucleocapsid and M protein to the plasma membrane and the viral glycoproteins F, HN, and SH from the endoplasmic reticulum to Golgi to plasma membrane. The *large arrow* denotes release of progeny virions from the plasma membrane by a budding process.



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FIGURE 33.21. Paramyxovirus RNA synthesis. Viral nucleocapsids—the templates for RNA synthesis—are shown as a linear array of N subunits (ovals), with arrowheads indicating the gene junctions. Note that N protein binds six nucleotides, resulting in complete encapsidation of the RNA if it has a chain length that is an even multiple of six. The viral polymerase (P-L) transcribes the genome template, starting at its 3' end, to generate the positive leader RNA and the successive capped (meG-Cap) and polyadenylated (PolyA) mRNAs, by stopping and restarting at each junction. Once these primary transcripts have generated sufficient viral proteins, unassembled N (as a P-N complex) begins to assemble the nascent leader chain, and the coordinate assembly and synthesis of the RNA causes the polymerase to ignore the junctions, yielding the antigenome nucleocapsid (**bottom**). The P-L polymerase can also initiate RNA synthesis at the 3' end of the antigenome in the absence of sufficient P-N; however, only a 5' trailer RNA is made in this case. Note that positive leader RNA is not capped or polyadenylated and that genomic and antigenomic RNAs never appear as naked RNAs.

diagram indicating the differences between transcription and replication is shown in Figure 33.21. Unlike the situation for influenza viruses, *Paramyxoviridae* mRNA synthesis is insensitive to DNA-intercalating drugs such as actinomycin D,⁵⁹ and the *Paramyxoviridae* can replicate in enucleated cells.³¹⁶ In cell culture, single-cycle growth curves are generally of 14 to 30 hours duration but can be as short as 10 hours for virulent strains of NDV. The effect of viral replication on host macromolecular synthesis is quite variable, ranging from almost complete shut-off late in infection for NDV to no obvious effect with PIV5.

Virus Adsorption and Entry

For the respiroviruses and rubulaviruses, it has long been accepted that molecules containing sialic acid (sialoglycoconjugates) serve as cell surface receptors. This is based on the fact that sialidase of *Vibrio cholerae* acted as a “receptor destroying enzyme” and protected the host cell from infection.²⁴⁴ Sialic acid, the acyl derivative of neuraminic acid, is found on both glycoproteins and on lipids (sialoglycolipids or gangliosides). For Sendai virus, gangliosides function as both the attachment factor and the receptor for the virus.^{243,245,246} As described earlier, the cellular receptor for the *Morbillivirus* measles virus is the cell surface protein CD150 (SLAM), and the cellular receptor for pneumoviruses, although not proven, seems to involve binding to glycosaminoglycans containing the disaccharide heparan sulfate and chondroitin sulfate B.¹¹² On adsorption

of the virus to the cellular receptor, the viral membrane fuses with the cellular plasma membrane at the neutral pH found at the cell surface, the consequence of which is the release into the cytoplasm of the helical nucleocapsids.

In the virus particle, the M protein shell is thought to make numerous contacts with the nucleocapsid. On fusion of the viral envelope with the cell plasma membrane and release of the nucleocapsid into the cytoplasm, a mechanism needs to exist to disrupt the M-N contacts. With influenza A virus, the factor that alters the equilibrium between self-assembly and disassembly is thought to be the difference in pH between the acidic uncoating compartment (endosomes) and the assembly site (plasma membrane). The driving force for paramyxovirus uncoating is not known.

Viral RNA Synthesis

Paramyxoviruses have evolved mechanisms to control both the level and type of viral RNA that is synthesized during the course of an infection, largely through the use of *cis*-acting RNA sequences. The relationship between RNA sequences and the vRNAP functions that they control is particularly complex, because these *cis*-acting signals are only recognized when they are in the context of the nucleocapsid structure (see Fig. 33.21). Paramyxoviruses also encode *trans*-acting accessory proteins that control activities of the viral RNA. Recently, experimental systems have been developed that reconstitute RNA synthesis from model synthetic minigenomes using cDNA-derived

viral components, allowing the functional analysis of *cis*-acting sequences or *trans*-acting proteins.

Viral Transcription (Messenger RNA Synthesis)

Early in virus infection, before the viral translation products have accumulated to high levels (or in the presence of drugs that inhibit protein synthesis at any stage of the infection), vRNAP is restricted to the production of leader RNAs and mRNAs from the incoming virion nucleocapsid in a growth phase called *primary transcription* (see Fig. 33.20). At later times following infection, this input nucleocapsid is used as a template to produce positive sense antigenomes that in turn are used as templates to produce new negative sense genomic RNA. When abundant progeny genomes have been produced, they can serve as additional templates in the growth phase, called *secondary transcription*, to produce much higher levels of viral mRNA transcripts (see Fig. 33.20).

The paramyxovirus RNAP is thought to gain access to the viral genes through a single entry site at or near the 3' end of the genome. For the *Paramyxovirinae*, the *N* gene is transcribed as the first coding gene, and more than 90% of vRNAP that have initiated the Sendai virus *N* mRNA complete transcription of the entire *N* mRNA.⁴²⁰ This processive vRNAP responds to the *cis*-acting sequences at the end of the *N* gene (open triangles in Fig. 33.21) to produce capped and polyadenylated viral mRNAs. The vRNAP then reinitiates mRNA synthesis at the start site of the next downstream gene (closed triangles in Fig. 33.21), and this sequential “stop-start” mechanism continues across the viral genome in a 3' to 5' direction.

The viral gene junctions that modulate transcription can be divided into three segments: a GE region at the 3' end of the upstream gene, the IG region between the two genes that is normally not transcribed, and a GS region for the downstream 5' gene (see Fig. 33.5). The GE region contains a signal directing the vRNAP to terminate transcription and a stretch of four to seven uridine residues (U tract) that acts as a template for polyadenylation of the nascent mRNA by a mechanism that involves stuttering by the vRNAP. After termination of transcription, the vRNAP is thought to remain attached to the template as it moves across the IG nucleotides. Reinitiation of transcription is directed by sequences at the downstream GS site, which also directs the addition of a methylated 5' guanine cap to the nascent mRNA. The frequency of reinitiation is not perfect, and not every vRNAP that terminates at a GE remains on the template to reinitiate transcription at the next GS. This imperfect reinitiation frequency leads to a gradient of mRNA abundance that decreases according to distance from the genome 3' end, with *N* mRNAs being found in higher abundance than *L* mRNA.⁵⁰ Initiation at a downstream GS site depends on termination at the upstream GE site,²⁰⁸ consistent with a single entry site for the vRNAP at the 3' end of the genome.

In addition to synthesizing monocistronic mRNAs, the vRNAP can also ignore the GE sequences for polyadenylation/termination and synthesize a transcriptional read-through product that consists of a fusion of the upstream and downstream mRNAs. While read-through transcription is generally an infrequent event, for several paramyxoviruses, such as HPIV types 1 through 3, measles virus, and PIV5, read-through transcription is highest at the M-F junction, and approximately 50% to 80% of the F mRNA is locked into an M-F read-through product.^{27,49,334,380} An extreme example of this is seen

with simian virus 41 (SV41), where the M mRNA is seen exclusively as an M-F read-through product owing to a deletion of the M GE,⁴¹³ and F mRNA is found in both mono- and dicistronic forms.⁴¹⁴ The basis for this elevated M-F read-through for some paramyxoviruses is owing to GE insertions³⁸⁰ or substitutions³³⁴ that alter the efficiency of termination signals. The selective pressure to maintain elevated M-F read-through for some paramyxoviruses is not known but could reflect a need to increase access of a transcribing polymerase to the more 3' distal genes (e.g., HN or L) or a mechanism to down-regulate F protein expression, as this ORF would be locked into a dicistronic mRNA that would presumably not be translated.^{190,334}

Paramyxoviruses can be divided into two groups based on whether the viral GE and IG sequences are highly conserved across the genome or have a high degree of variability. The GE and IG regions of Sendai virus, HPIV1, and HPIV3 have a high degree of genetic conservation.²⁰³ For example, each of the Sendai virus GE sequences consist of a 3'-AUUCU₅-5' motif, and the IG region is 3'-GAA-5' (except the HN-L junction, which is 3'-GGG-5'). By contrast, the GE and IG regions of RSV, human parainfluenza virus type 2 (HPIV2), mumps virus V, SV41, and PIV5 are highly diverse and provide an additional level of transcriptional control beyond that which results from the distance of a gene from the 3'-end promoter. This diversity is reflected in the combinations of GE and IG sequences that can act together to differentially control vRNAP activities.^{144,333} RSV GE sequences are more diverse than their GS sequences and operate at variable efficiency in transcription termination and read-through.^{142,207} Moreover, for RSV, the L GS sequence is actually located upstream of the GE sequence of the upstream *M2* gene⁶⁵ (see Fig. 33.4). Thus, the RSV polymerase terminates at the *M2* GE; however, it is thought to scan backward on the template to reinitiate at the upstream L GS site.¹⁰⁸

Trans-acting viral proteins can also contribute to the control of stop-start transcription. For example, the RSV M2-1 protein is an essential transcription elongation factor that is necessary for high processivity of the vRNAP.⁶³ M2-1 can also modulate vRNAP activities at the diverse gene junction sequences,¹⁴² resulting in junction-specific changes in transcriptional read-through versus termination and in the relative abundance of mono- and dicistronic mRNAs.

P Gene Messenger RNA Editing

Pseudo-templated addition of nucleotides, popularly known as RNA editing, is a mechanism for obtaining more coding potential from a gene, and it was first identified for PIV5.^{307,408} It is now known that most paramyxovirus *P* genes contain a functional editing site within the coding region of the *P* gene (see Fig. 33.6). In addition to a faithful (unedited) mRNA, transcription across of the *P/V/C* gene yields edited versions that contain variable numbers of inserted G residues. The number of G insertions can differ for each virus group and mirrors their requirements for mRNAs that encode the individual P/V/W/I/D proteins (see Fig. 33.6). For the morbilliviruses, respiroviruses, and NDV, a single G is added to transcripts as the predominant editing event, resulting in an mRNA that shifts from the genome-encoded P ORF to the V ORF (see Fig. 33.6). For the rubulaviruses, which encode the V ORF as the unedited faithful copy of the *P/V* gene, the insertion of 2 Gs constitutes a high proportion of editing events, producing an

mRNA that encodes the P protein subunit of the vRNAP. For BPIV3 and HPIV3, where both the V and D ORF overlap the middle of the genome-encoded P ORF, one to six G residues are added at roughly equal frequency so that mRNAs encoding all three overlapping ORFs are transcribed.

As the paramyxoviruses replicate in the cytoplasm, they must provide enzymes for all aspects of their mRNA synthesis. Paramyxovirus vRNAPs polyadenylate their mRNAs by stuttering on a short run of template U residues (four to seven nucleotides long) at the end of each gene. By analogy to the polyadenylation mechanism, it was suggested that the G insertions at the specific site in the P gene would occur similarly by pseudo-templated transcription,⁴⁰⁸ and there is now strong experimental evidence that the insertions occur by a co-transcriptional stuttering mechanism.^{150,419} The efficiency of G insertion by the stuttering polymerase depends on the relative position of an editing site within the N-bound hexamer of nucleotides,^{181,203} suggesting that polymerase function is directed by a combination of RNA sequence and N protein structure within the template.

Genome Replication

A schematic diagram of transcription and RNA replication in the paramyxovirus growth cycle is shown in Figure 33.20, and the role of genomes and antigenomes as templates for these phases of RNA synthesis is shown in Figure 33.21. At early times of infection, the genome directs the synthesis of positive leader and viral mRNAs. After translation of the primary transcripts and accumulation of the viral proteins, the negative sense genome is replicated to produce a full-length complementary copy, called the *antigenome*, which is found only in a form that is assembled with N protein. Here, it is thought that the same vRNAP copies the same template that had been used for transcription, although now all of the gene junction signals (and editing sites) are ignored and an exact complementary copy of the template is generated. In infected cells, antigenomes are typically found in lower levels than genomes, and they do not code for any known functional ORFs or mRNAs. The sole function of the antigenome is thought to be as an intermediate in genome replication; however, the short trailer RNAs expressed from the antigenome 3' end (see Fig. 33.21) may also play a role in preventing the host cell from undergoing programmed cell death.¹⁸²

It has long been known that when infected cells are treated with drugs that inhibit protein synthesis, mRNA synthesis continues normally but genome synthesis is lost very quickly. As genome synthesis and encapsidation appear to occur concomitantly,¹³⁹ this requirement for ongoing protein synthesis during RNA replication is thought to reflect the need for a continued supply of unassembled N for genome encapsidation. Similar to the model for vesicular stomatitis virus (VSV), this coupling of genome assembly and synthesis also leads to a self-regulatory system for controlling the relative levels of viral transcription and replication. Because the leader sequences contain the N encapsidation site, the leader must be separated from the body of the first mRNA (by termination and reinitiation at the leader-N junction) to prevent the first mRNA from ending up in an assembled and untranslatable form. Thus, when unassembled N is limiting, such as early times in the growth cycle, vRNAP is preferentially engaged in mRNA synthesis, and this results in an increase in intracellular levels of all the

viral proteins, including unassembled N. When unassembled N levels are sufficiently high, some vRNAP would be switched to replication, thereby lowering the levels of unassembled N, as each initiation of encapsidation would commit approximately 2,600 N monomers to finish the assembled genome chain.⁴²⁰ The level of unassembled N may not be the only mechanism controlling transcription versus RNA replication, because higher levels of N protein have not affected the ratio of replication to transcription for RSV minigenomes.¹¹⁰

Paramyxoviruses employ additional mechanisms to control genome and antigenome replication, including the expression of viral accessory proteins that are not essential for virus growth but play a role in control of viral RNA synthesis. For example, the Sendai virus V protein is thought to inhibit RNA replication through binding to N⁰, the assembly-competent form of nucleocapsid protein.¹⁶⁸ The RSV M2-2 protein appears to play a role in regulation of transcription versus replication,¹⁴ and the RSV NS1 protein is a potent inhibitor of RNA replication in a minigenome system.⁶

After synthesis, the antigenomic RNA is used as a template to direct synthesis of genomic RNA by a mechanism similar to that for antigenome synthesis, in that the promoter at the 3' end of the antigenome directs synthesis of the short trailer RNA, also referred to as negative leader (see Fig. 33.21). Under conditions of sufficient intracellular concentrations of unassembled N (and perhaps other viral proteins), encapsidation of the nascent trailer chain would quickly begin and lead to the synthesis of encapsidated minus-strand genomes. As shown in Figure 33.20, these progeny negative sense genomes can serve three subsequent functions: as a template for mRNA synthesis in a phase called *secondary transcription*, as a template to produce additional antigenomes, or for incorporation into progeny virions during the budding process.

Paramyxovirus Replication Promoter

The 3'-end promoter in the antigenome directs the synthesis of progeny genomes and is thought to be a stronger promoter for RNA replication than the genomic 3'-end promoter in the genome that directs the synthesis of both mRNAs and antigenomes (see Fig. 33.21). Thus, the relative strength of genomic and antigenomic promoters in RNA replication can contribute to the relative ratio of these full-length RNAs in infected cells.²²¹ Extensive mutagenesis studies with model minigenomes have identified three factors that contribute to promoter function: the rule of six chain length requirement, nucleotide sequences within the bipartite promoter, and proper spacing of promoter elements.

It was found that changes in the overall length of a paramyxovirus N-RNA complex can profoundly affect the efficiency of RNA replication.⁴⁰ This RNA chain length requirement, called the *rule of six*, dictates that efficient replication of a viral genomic or antigenomic RNA will only occur when the total number of nucleotides in the RNA is an even multiple of six.⁴³ This surprising requisite is thought to reflect the precise nature with which the genomic RNA must be encapsidated by N to form a nucleocapsid template for the viral polymerase.^{43,315} The hexamer requirement emerged from microscopy studies of Sendai virus nucleocapsids, which revealed that each Sendai virus N molecule contacts six bases of RNA.¹⁰¹ During replication, N-RNA assembly is thought to initiate with the 5' end of the nascent RNA chain as it emerges from the vRNAP

complex, and encapsidation proceeds in a 5' to 3' direction.^{139,420} As shown schematically in Figure 33.21, a genome whose length is an even multiple of six nucleotides will be precisely encapsidated by N, with no unencapsidated nucleotides protruding from the 3' end of the nucleocapsid.³¹⁵

There is large variability in the stringency to which various groups of paramyxoviruses adhere to the rule of six requirement. Sendai virus RNA replication highly depends on the rule of six,⁴³ whereas there is no replicative advantage to RSV genome analogs having genome lengths that are a multiple of any particular integer.³⁵⁰ RNA replication for PIV5, NDV, and HPIV3

is most efficient for 6N-length genomes, although this is not as stringent a requirement as found for Sendai virus.^{95,242,273}

The rule of six was originally proposed to reflect the need to have a functional nucleocapsid template with a precisely encapsidated 3' end and no "dangling" free bases. However, extensions to the 3' end of Sendai virus DI RNAs do not result in a decrease in RNA replication, regardless of whether these extensions were multiples of six or not.⁴²⁵ As shown in Figure 33.22B, an alternative hypothesis emerged by the observation that the phase of a particular base in the promoter can range from position 1 to 6 within a hexamer of N-bound sequences.

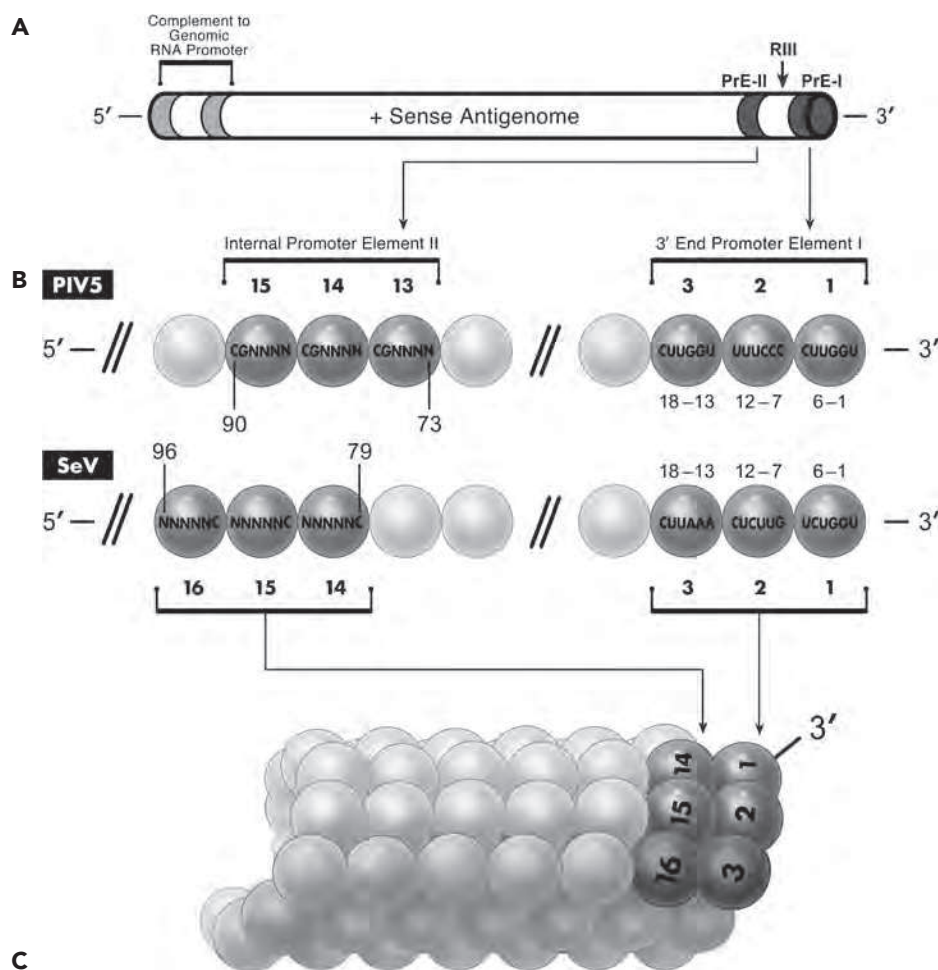


FIGURE 33.22. Nucleocapsid structure, hexamer phasing of nucleotide sequences, and the bipartite replication promoters of the Paramyxovirinae. **A:** Schematic of the paramyxovirus positive sense antigenomic RNA. The location of the 3'-end promoter element I (PrE-I) and internal promoter element II (PrE-II) are indicated by shaded areas, with location of the intervening nonessential region III replication element (RIII) shown by an arrow. The 5' end of the antigenome contains sequences that are the complement to the genomic RNA promoter and are partially complementary to the 3' region of the antigenomic RNA. **B:** Expanded view of a portion of the first 16 N protein subunits (drawn as ovals) of the antigenome nucleocapsid. Numbers above the ovals indicate the position of each N monomer, and numbers below the ovals refer to the nucleotide sequence relative to the 3' end of the RNA. The position of nucleotides within the 3'-end PrE-I and internal PrE-II elements for parainfluenza virus type 5 (PIV5) and Sendai virus are shown relative to the N subunits, with each subunit containing precisely six nucleotides. Note that for the internal PrE-II element, essential C residues are located in the first position of each hexamer for Sendai virus, whereas GC residues are located in the fifth and sixth positions for PIV5. **C:** A model for the Sendai virus and PIV5 nucleocapsid as an assembly of single N protein subunits (shaded spheres), in the form of a left-handed helix with N binding six nucleotides and 13 N subunits per turn. Numbers refer to the position of each N subunit from the RNA 3' end. Note that both PrE-I and PrE-II of the bipartite replication promoter are found on the same face of the helix.

Experimentally altering the predicted phase of nucleotides in the antigenomic promoter reduces RNA replication.³¹⁵ This finding is consistent with a model for the rule of six requirement based on critical *cis*-acting promoter sequences that can only be recognized when they are in the correct positions within their encapsidating N monomers.²⁰³ Further support for this hypothesis comes from the finding that the Sendai virus promoter can function when located at an internal position in a viral N-RNA complex, but only when the hexamer phase of promoter elements is correct.⁴²⁵

A second primary determinant of replication promoter strength resides in the nucleotide sequence in the leader RNA at the 3' terminus of the genome and in the trailer complement RNA at the 3' terminus of the antigenome.^{164,242,272,400,401} As shown in Figure 33.22, two discontinuous sequence-specific elements have been identified within the 3'-terminal 90 bases of the antigenomic promoter. In the two original descriptions of this requirement, these elements have been termed conserved region I (CRI) and conserved region II (CRII) for PIV5^{272,274} and promoter element I (PrE-I) and promoter element II (PrE-II) for Sendai virus.⁴⁰⁰

PrE-I is located at the 3' end of viral genomic and antigenomic RNA (see Fig. 33.22), and the specific sequence is highly conserved between members of a paramyxovirus genus. The internal PrE-II sequence is located within the L gene for the antigenomic promoter or within the N gene for the genomic promoter.^{164,272,400,426} Paramyxoviruses fall into two groups based on the distance of their critical PrE-II bases from the 3' end of the viral RNA and the sequence and the position of essential bases within an N hexamer. As diagrammed in Figure 33.22B, the internal PrE-II element for PIV5 and related rubulaviruses is located between 72 and 90 bases from the 3' end of the viral RNA.²⁷² By contrast, the PrE-II element for Sendai virus and related respiro- and morbilliviruses is located between 79 and 96 bases from the 3' end.^{400,426} The sequence requirements within PrE-II are remarkably simple; however, they differ for PIV5 and Sendai virus RNA replication. For PIV5, extensive mutational analyses²⁷⁴ indicated that the PrE-II requirements consisted of a CG motif located in the first two positions of three sequential hexamers of nucleotides (5'-CGGGAU CGAUGG CGAGAA-3', template sense, see Fig. 33.22B). By contrast, the Sendai virus PrE-II requirements consisted of three copies of a 5'-(NNNNNC)-3' motif (see Fig. 33.22B; shown as template sense). An additional element located between antigenomic PrE-I and the internal PrE-II has been identified for PIV5 (bases 51–66) and HPIV3 (bases 13–55), which is thought to act as a nonessential enhancer of replication.^{164,196}

A third factor in RNA replication was identified during mutational analyses of the PIV5 and Sendai virus antigenomic promoters, where it was found that deletions or insertions in the RNA segment located between the 3'-terminal PrE-I and the internal PrE-II resulted in templates that were not competent for RNA replication.^{272,400} The template defect was not attributed to disruption of an important *cis*-acting segment or the rule of six. As described in the next section, it is thought that the sensitivity of RNA replication to changes in the length of the region between PrE-I and PrE-II reflects a requirement for these two RNA segments to align to the same face of the nucleocapsid template.^{274,400}

Image reconstruction of electron micrographs indicates that Sendai virus N binds six nucleotides and that 13 N mon-

omers constitute a turn of the nucleocapsid helix.¹⁰¹ Together with extensive mutational analysis of minigenome analogs, these data suggest a model for the paramyxovirus promoters.^{220,272,400} As shown in Figure 33.22B,C, nucleotides within the 3' PrE-I and internal PrE-II elements are separated along the linear RNA sequence by 55 to 60 bases; however, in the N-encapsidated form, these RNA elements are aligned to the same face of the nucleocapsid template by the helical winding. Alignment of PrE-I and PrE-II may form a binding site for the polymerase complex to initiate RNA synthesis at the 3' end of the template, although formal proof of this hypothesis is not currently available. Taken together, the available evidence indicates that promoter strength is a major factor dictating level of RNA replication, and promoter strength is in turn affected by (a) changes in the phase of nucleotides within the N-induced hexamer phase of PrE-I or PrE-II (rule of six requirement); (b) changes in the RNA sequences themselves; or (c) changes in the spacer region between PrE-I and PrE-II, which alter their alignment on one face of the nucleocapsid template.

Virion Assembly and Release

Paramyxoviruses, like other enveloped viruses, are formed by a budding process. Buds emerge from sites on the plasma membrane where viral components have assembled, then pinch off resulting in the release of particles. Assembly of paramyxoviruses is thought to require coordinated localization of multiple but distinct virus components, including viral glycoproteins, which are transported to the plasma membrane by the exocytic pathway, and soluble viral components, such as the RNP. This coordination appears to be accomplished through a series of protein–protein and protein–lipid interactions, many of which involve the viral matrix protein that could potentially interact with both glycoproteins via their cytoplasmic tails and with the RNPs in the cytoplasm of the infected cell, and also interactions between viral components and the host machinery that allows bud formation and membrane fission. Those *Paramyxovirinae* that have NA activity contain glycoproteins that lack sialic modification of their carbohydrate chains, and it is thought that the HN NA activity serves the same purpose as NA of influenza virus—to prevent self-binding and to prevent reattachment to the infected cell.

Assembly of the Nucleocapsid

Nucleocapsids assemble in the cytoplasm in two steps: first, association of free N subunits with the genome or template RNA to form the helical RNP structure, and second, the association of the P-L protein complex.¹⁹⁹ By analogy to the mechanism of assembly of TMV nucleocapsid, which uses a defined nucleation site for the association of the first coat protein subunit with the RNA, and the observation that the paramyxovirus mRNAs are not encapsidated in contrast to antigenomes, it has been assumed that the positive leader (5' end of the antigenome) and negative trailer (5' end of the genome) regions contain specific sequences for initiating encapsidation.¹⁹

Assembly of the Envelope

The assembly of the second part of the virus—the envelope—is at the cell surface. In polarized epithelial cells, the *Paramyxovirinae* bud only from the apical surface. For a long time, it has been thought that the matrix proteins of negative-strand RNA viruses have important roles in virus assembly and budding.

Matrix proteins are positioned in virions beneath the lipid envelope, so that they have the potential to contact both RNP cores and envelope glycoprotein cytoplasmic tails, and are therefore likely to be the key organizers of virus assembly that induce separate viral components to concentrate together at defined budding sites on the plasma membranes of infected cells. Matrix proteins bind to viral RNPs *in vitro* and are found stably attached to RNPs when purified from virions, they bind to lipid membranes both *in vitro* and in living cells, and they self-assemble into ordered structures as purified proteins *in vitro* and in virus-infected cells. Studies using reverse genetics techniques have aided in understanding the role of the matrix protein in assembly. The M proteins of measles virus and Sendai virus had previously been implicated in budding based on analysis of viruses derived from persistent viral infections. Cells persistently infected with Sendai virus were found to express an unstable M protein, and lack of stable M protein correlated with a reduction in virus particle formation.³⁴¹ A role for the measles virus M protein in budding was suggested based on analysis of viruses isolated from patients with SSPE. SSPE viruses are defective for the production of progeny virus particles. Nucleotide sequence analysis has revealed extensive defects in the M genes of SSPE measles virus strains.^{53,440} Recombinant measles viruses were generated having defective or deleted M genes.^{45,309} Both of these viruses were shown to be severely defective in budding; Indeed, the infectivity was so low that the particles may represent adventitious vesicles with RNPs.

The Use of Virus-like Particles to Study Assembly

The importance of the matrix protein for virus budding has been investigated using the assembly of VLPs from proteins expressed from cDNAs. For PIV5, although M protein expressed by itself does not induce efficient budding of particles, when M is co-expressed with N protein and a viral glycoprotein (either F or HN), budding of particles becomes very efficient, approaching the budding efficiency observed in virus-infected cells.³⁵⁹ VLP budding that is normally observed on expression of the Sendai virus M protein alone can be made more efficient by co-expressing the Sendai virus F glycoprotein.³⁹⁵

Glycoprotein Cytoplasmic Tails and Assembly

It has long been thought that the glycoprotein cytoplasmic tails would be used in recognizing the M protein. Early studies using a temperature-sensitive mutant of Sendai virus (ts271) demonstrated that the Sendai virus HN protein is dispensable for budding of virus particles.^{326,328,387,415} VLP budding was observed on expression of M protein alone and the efficiency of budding was found to be stimulated on co-expression of F protein; however, co-expression of HN protein had no effect on budding efficiency.³⁹⁵ Recombinant Sendai viruses with altered glycoprotein cytoplasmic tails have been generated, and truncation of the F protein cytoplasmic tail resulted in poor budding. Release of particles on expression of F protein alone was found to depend on the amino acid sequence TYTLE, comprising amino acids 542 to 546 of the F protein cytoplasmic tail.³⁹⁵ Recombinant PIV5 were recovered harboring HN proteins with truncated cytoplasmic tails, and budding was found to be inefficient on HN protein cytoplasmic tail deletion.³⁵⁷ The same approach was used to define the role of the PIV5 F protein cytoplasmic tail in virus budding, and it was found that recombinant viruses with deleted F protein cytoplasmic

tails replicated in tissue culture and were released from infected cells with similar efficiency to wild-type virus.⁴²⁹ This result was surprising in light of work with Sendai virus suggesting that F protein is in fact quite important for proper paramyxovirus budding. To investigate further the relative roles of the HN and F glycoproteins for paramyxovirus budding, a VLP system was developed for PIV5.³⁵⁹ Here, efficient budding of VLPs from transfected cells was observed only on co-expression of multiple PIV5 proteins. Thus, expression of M protein alone did not lead to substantial particle budding, and neither of the PIV5 glycoproteins was found to have an autonomous exocytosis activity. However, co-expression of M protein with N protein and either the HN or F glycoprotein led to budding of VLPs with an efficiency comparable to that found in virus-infected cells. Budding decreased more than 25-fold when neither of the PIV5 glycoproteins were included, and the HN and F proteins were found to be completely interchangeable for VLP budding. This result suggested that the two PIV5 glycoproteins might have redundant functions for budding. Consistent with this idea, recombinant PIV5 lacking both HN and F protein cytoplasmic tails was found to have a greater defect in particle production and release than PIV5 lacking only the HN protein cytoplasmic tail.⁴²⁹ From VLP experiments, the importance of the glycoprotein cytoplasmic tails for efficient budding was confirmed; VLPs containing wild-type HN or wild-type F as the only glycoprotein bud efficiently, whereas those containing only cytoplasmic tail-deleted HN or cytoplasmic tail-deleted F protein bud poorly.³⁵⁹ SSPE measles virus strains contain drastic sequence alterations not only in their M genes, as discussed previously, but also in their F protein cytoplasmic tail sequences.⁵² Truncations or other alterations to the F protein cytoplasmic tail led to more rapid and extensive cell-to-cell fusion of virus-infected cells, consistent with a shift in the mode of virus spread to one that is independent of budding.^{46,263} Very recently, for RSV, it was shown that a critical phenylalanine residue in the F protein cytoplasmic tail mediates assembly of internal viral proteins into viral filaments and particles.³⁶⁹

Budding and Interactions with the Multivesiculate Body Formation Machinery

For several enveloped viruses, it has been shown that budding occurs in a way that requires the manipulation of host machinery. Protein-protein interaction domains called *late domains* have been defined in retroviral Gag proteins and in the matrix proteins of some negative-strand RNA viruses. These late domains function to recruit host factors to viral assembly sites where they assist in virus release.^{117,265} Disruption of viral late domains often leads to phenotypes in which virus particles assemble normally but fail to be released by membrane fission and instead accumulate as tethered particles on cellular membranes.^{83,127,135,184}

Several types of late domain have been identified: P(T/S)AP, PPxY, and YP(x)_nL. Each of these late domain sequences likely functions to bind with a different host factor to facilitate virus budding. P(T/S)AP late domains mediate binding to TSG101,^{127,248} and the host partner protein for YP(x)_nL late domains appears to be AIP1.^{247,383,421,424} Both TSG101 and AIP1 are part of the cellular vacuolar protein sorting (VPS) pathway (ESCRT) that allows formation of multivesicular bodies (MVBs), an observation that is significant owing to the fact that virus budding and vesicle budding into MVBs are similar

processes, in which cytoplasmic cargo is packed into vesicles that bud outward from the cytoplasm. PPxY-type late domains have been shown to interact with WW domains from a variety of proteins, such as Nedd4-related E3 ubiquitin ligases.^{146,197,411} It has been proposed that recruitment of Nedd4 family members may allow indirect recruitment of other host proteins, including those involved in MVB formation.³⁸⁴

Budding of PIV5 and PIV5-like particles was reduced by treatment of cells with the proteasome inhibitor MG-132.³⁵⁸ Proteasome inhibitors also reduce virus budding of retroviruses such as HIV-1³⁶³ and RSV³⁰⁸—viruses that use PTAP and PPxY late domains for budding. Inhibition of proteasome function prevents recycling of ubiquitin that is attached to proteins targeted for degradation, thereby depleting free ubiquitin levels in the cell.⁴²³ It has been found that the PIV5 M protein is targeted for monoubiquitination in transfected cells at three lysine residues. Mutation of the lysine residues leads to altered ubiquitination and impaired VLP production. Analysis of these mutations in recombinant viruses suggest that monoubiquitination of PIV5 M protein is needed for virus assembly and budding.¹⁴⁵

There have been several attempts to discover cell proteins that interact with viral proteins during budding. Production of PIV5 and PIV5-like particles is reduced on expression of a dominant-negative VPS4A adenosine triphosphatase (ATPase).³⁵⁸ Interestingly, substantial incorporation of the dominant negative VPS4A mutant into VLPs was noted, despite the relatively small amount of VLPs produced under these conditions. VPS4 mutants disrupt the cellular MVB formation pathway, likely because adenosine triphosphate (ATP) hydrolysis is required for release of class E proteins from late endosomal membranes. The PIV5 M protein lacks previously defined late domains (e.g., P[T/S]AP, PPxY, YPDL) to recruit cellular factors. However, a new late domain for budding (core sequence FPIV) that can compensate functionally for lack of a PTAP late domain in budding HIV-1 VLPs was identified.³⁵⁸ Mutagenesis experiments suggested the more general sequence Ø-P-x-V. The proline residue was found to be critically important for function of this late domain, as substitution of this proline in PIV5 M protein resulted in poor budding of PIV5 VLPs and failure of recombinant PIV5 virus to replicate normally. Adaptation of mutant virus harboring an altered FPIV domain occurred rapidly, resulting in new proline residues elsewhere in the M protein.³⁵⁸ Yeast two-hybrid screening identified angiomin-like 1 (AmotL1) as a host factor that interacts with PIV5 M protein. Overexpression of M-binding AmotL1-derived polypeptides potently inhibited production of PIV5 VLP budding and small interfering RNA (siRNA)-mediated depletion of AmotL1 reduced PIV5 budding.³¹³ Yeast two-hybrid screening also identified a protein designated as 14-3-3 as a binding partner of PIV5 M, and it was found that 14-3-3 negatively affects virus particle formation.³¹⁴ However, it is not known if AmotL1 and 14-3-3 are involved in the ESCRT pathway.

For Sendai virus, a sequence YLDL has been found in the M protein that acts as a late domain and is required for budding.¹⁸⁰ Curiously, YLDL of Sendai virus M protein is not replaceable by other late domains, perhaps because of structural constraints. It has been found that the Sendai virus M late domain interacts with the N-terminus of the cellular protein Alix/AIP1, a component of the ESCRT pathway. Mutagenesis of M protein YLDL to ALDA abolished budding and the interaction with

Alix/AIP1. A revertant virus was obtained with the sequence ALDV, and budding was restored together with the interaction of the M protein with Alix/AIP1.¹⁷⁹ The Sendai virus C protein also interacts with Alix/AIP1, and C protein expression enhances budding. It is thought that C protein recruits Alix/AIP1 to the plasma membrane and enhances the efficiency of the utilization of the ESCRT machinery for efficient VLP budding.

Budding of Paramyxoviruses from Membrane Rafts

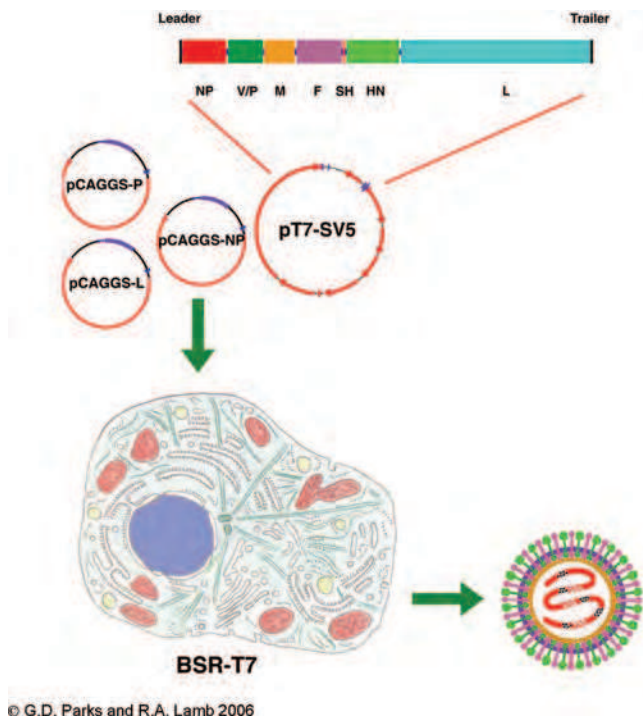
It has recently become clear that lipid molecules within the plasma membrane are not distributed homogeneously in each leaflet of the bilayer, but rather participate in lateral associations to form subcompartments within the membrane. One type of lipid microdomain is the membrane raft, which preferentially contains sphingolipids and cholesterol as well as certain integral membrane proteins.^{34,372} Membrane rafts can be separated biochemically from other membrane components based on their resistance to solubilization by certain nonionic detergents such as TX-100 at low temperatures. Some viral proteins have been found to be enriched within membrane rafts of infected cells, suggesting that virus assembly can occur on rafts. For example, in influenza A virus-infected cells, the glycoproteins hemagglutinin and NA, as well as the matrix protein, are found associated predominantly with TX-100-insoluble lipids.^{223,339,340,374,454} Assembly of viral proteins on raft membranes does not appear to be a universal strategy for negative-strand RNA virus assembly, however, as in the cases of VSV and rabies virus infections, the viral proteins are found excluded from raft membranes in the infected cells. Paramyxovirus proteins in many cases have been found to be associated with raft membranes in infected cells, including the HN and F glycoproteins of Sendai virus,³⁵¹ F and HN proteins of NDV,^{90,210} the measles virus proteins H, F, M, and N,^{239,422} and the RSV glycoproteins F and G.³⁶

Polarized Budding from Epithelial Cells

The paramyxoviruses Sendai virus, PIV5, and measles virus have been found to bud preferentially from the apical membranes of polarized cells.^{18,338} Polarized budding may have important consequences for viral pathogenesis, as budding from the apical surface could favor restriction of the infection to the epithelial cell layer, whereas budding from the basolateral surface allows viral access to underlying tissue and could favor development of a systemic infection. Consistent with this view, Sendai virus and PIV5 both produce localized infections of the respiratory tract *in vivo*, whereas VSV and Marburg virus both produce systemic infections *in vivo*. Furthermore, a mutant Sendai virus has been characterized in which polarized budding from the apical membrane is lost and virus is instead released in a nonpolar fashion from both the apical and basolateral cell surfaces, and this virus causes a systemic infection and is more virulent than wild-type Sendai virus.⁴⁰³ The correlation between pathogenicity and virus budding from the basolateral cell surface is not absolute, however, because measles virus is released apically yet produces a systemic infection *in vivo*.

MOLECULARLY ENGINEERED GENETICS (REVERSE GENETICS)

The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer



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FIGURE 33.23. Rescue of paramyxoviruses from cloned complementary DNA (cDNA). Nonsegmented negative-strand virus rescue involves the transfection of plasmids encoding the viral P, N, and L proteins (and sometimes other viral proteins depending on the virus), as well as the viral antigenome, all under control of the T7 promoter. The bacteriophage T7 RNA polymerase is provided by either infection with a vaccinia virus expressing T7 polymerase (in this case, modified vaccinia virus Ankara, MVA-T7) or by transfecting into cell lines that stably express the protein (e.g., BSR-T7 cells). pT7-SV5 contains a complete copy of the parainfluenza virus type 5 (PIV5) genome (15,246 nucleotides) and is flanked at one end by a bacteriophage T7 RNA polymerase (T7 RNAP) promoter and at the other end by a hepatitis delta virus ribozyme and T7 transcriptional terminator. The plasmids pT7-L, pT7-P, and pT7-N each contain the cDNA for the PIV5 L, P, and N proteins, respectively, under the control of T7 RNAP promoters such that messenger RNA transcripts encoding L, P, and N can be transcribed using T7 RNAP. (Adapted from He B, Paterson RG, Ward CD, et al. Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. *Virology* 1997;237:249–260.)

specific mutations into viral genomes—a technique known as reverse genetics.⁴³⁴ For RNA viruses, genome manipulation of the positive sense RNA bacteriophage Q β was the first to be performed.³⁹⁸ The negative-stranded RNA viruses, in contrast to positive sense RNA viruses, require that the virion RNA is assembled into an active transcriptase-replicase complex in order for the genome to initiate virus replication. Nonetheless, techniques to manipulate the genomes of nonsegmented negative-strand RNA viruses have now been developed.³³⁵ The development of the system originally proved quite frustrating. Several laboratories studying various rhabdoviruses and paramyxoviruses worked over a period of several years to establish the methods of reconstructing functional nucleocapsids from transfected cells. The concept of replicating minigenomes using support plasmids providing N, P, and L proteins in *trans* was key to the development of the technology. This culminated

in the successful recovery of infectious rabies virus in 1994, followed several months later by VSV, and several months later by Sendai, human RSV, and measles virus.

Rabies virus was rescued when plasmids encoding L, P, and N protein, as well as a plasmid containing the viral antigenome, all under control of the bacteriophage T7 RNAP promoter, were transfected into cells infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNAP protein (vac-T7).³⁶¹ As an example, the following viruses have been rescued: VSV,^{219,436} measles virus,³³² human RSV,⁶² Sendai virus,^{126,194} rinderpest virus,¹¹ HPIV3,^{93,163} PIV5 (SV5),¹⁵⁴ NDV,³¹² and bovine RSV.³⁹ A schematic diagram showing the general scheme for rescue is illustrated for PIV5 in Figure 33.23. Some refinements to the original technique have been made, such as the use of stably transfected cell lines expressing the bacteriophage T7 RNAP (in lieu of vac-T7 infection), or one or more of the viral proteins required for genome replication.^{39,332}

VIRAL ACCESSORY GENES AND THEIR INTERACTIONS WITH THE HOST

Type I IFN is one of the most important antiviral cytokines that can be a major determinant of tropism, pathogenesis, and viral dissemination. As shown in Figure 33.24, the cellular IFN response involves two general phases: the induction of IFN synthesis in a primary transcriptional phase and signaling through the type I IFN signaling pathway to activate a secondary transcriptional phase (see Chapter 8).¹⁶ A large body of work has emerged recently on the role of paramyxovirus accessory proteins in counteracting the host cell IFN pathways at the level of IFN synthesis or IFN signaling.^{70,122}

Antagonists of Interferon Synthesis

As shown in Figure 33.24, synthesis of IFN- β can be induced by by-products of virus replication such as viral double-stranded RNA (dsRNA) or 5'-triphosphate RNAs, which are recognized by cellular pattern recognition receptors (PRRs). These PRRs include the dsRNA-activated protein kinase R (PKR), toll-like receptors (TLRs), and two cytoplasmic RNA helicases retinoic acid inducible gene-I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5).^{3,446} Many PRRs signal through a mitochondrion-associated protein called *mitochondrial antiviral signaling protein* (MAVS) or IFN- β promoter stimulator 1 (IPS-1)¹⁹⁵ to activate cytoplasmic kinases including TANK-binding kinase 1 (TBK-1)/inhibitor of κ B kinase ϵ (IKK ϵ). Ultimately, this leads to dimerization and phosphorylation of latent transcription factors such as NF κ B and members of the family of interferon regulatory factors (IRFs), which can translocate to the nucleus and activate cellular promoters that drive expression of IRF responsive genes such as IFN- β . Results from transfection assays and the study of viruses that have been engineered to encode altered or deleted genes have identified viral antagonists of pathways that lead to IFN- β induction. These antagonists include the respirovirus and morbillivirus V and C proteins, the rubulavirus V protein, and the henipavirus V and W proteins.^{24,153,204,320,337,370,377} The pneumovirus NS1 and NS2 proteins also limit induction of IFN- β and are discussed elsewhere in this text.^{230,377}

For many paramyxoviruses, PKR is not activated to a large extent during infection except in the case where viral

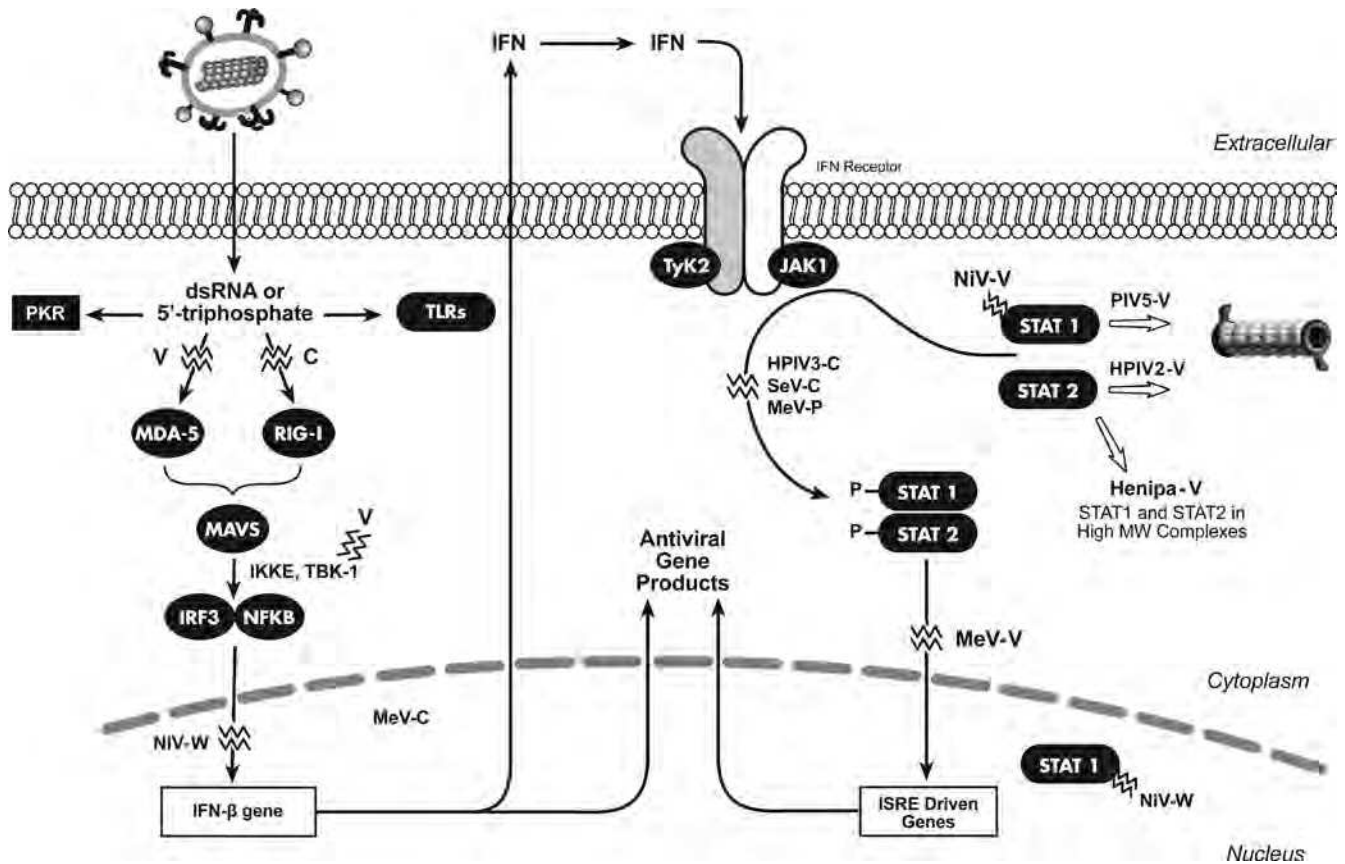


FIGURE 33.24. Interferon (IFN) induction, IFN signaling, and sites of antagonism by paramyxovirus accessory proteins. Schematic diagram of IFN induction (**left**), binding of IFN to extracellular receptors (**top and middle**), and signal transducers and activators of transcription (STAT)-mediated signaling (**right**) to activate transcription from cellular genes containing an interferon-stimulated response element. Sites of inhibition by a paramyxovirus antagonist are indicated by two wavy lines or by an open arrow.

antagonist proteins such as V and C proteins are altered.^{21,251,391} In the case of these viral mutants, PKR activation results in a global inhibition of both viral and cellular translation, and there is an amplification of IFN-β synthesis.²⁵¹ Expression of wild-type viral proteins *in trans* can in some cases suppress activation of the PKR. These results have been interpreted by some as evidence that the V and C proteins directly inhibit PKR activation. A more likely mechanism is that the wild-type proteins act to control viral RNA synthesis, which is deregulated in the case of the mutants, and this indirectly prevents PKR activation.^{21,251,391} This is further supported by the finding that PKR activation by a PIV5 P/V mutant is suppressed by expression of the wild-type P protein—a component of the viral RNAP with no known antiviral activity.¹¹⁹

The V protein has been shown to be an inhibitor of MDA-5-mediated IFN production for numerous paramyxoviruses.¹³¹ The highly conserved V protein Cys-rich domain is both necessary and sufficient (in transfection experiments) for V protein to limit dsRNA-induced activation of the IFN-β promoter.^{204,320} This inhibition is thought to occur through direct interaction of V protein with multiple sites on MDA-5, a competition with dsRNA for MDA-5 binding, and an inhibition of MDA-5 multimerization to the active form.³ Despite this well-documented inhibition, MDA-5 can still contribute to IFN induction *in vivo*.¹²⁹ In the case of Nipah virus, both V and

W proteins can block IFN-β promoter activation. This inhibition may result from targeting of different steps in the IFN induction pathway or different cellular sensors, because Nipah virus V and W proteins appear to localize to the cytoplasm and nucleus, respectively,³⁷⁰ and the W protein completely lacks the Cys-rich C-terminal domain found in the V protein.

In addition to targeting MDA-5, V proteins from a select group of paramyxoviruses have been reported to also act as a decoy substrate for the cellular kinases IKKε/TBK-1 (in the case of rubulaviruses²³³) or IKKα (in the case of measles virus³¹⁷), resulting in a second mechanism for blocking activation of IRF-3 and IRF-7 through a range of PRRs. This would predict that rubulaviruses would have the potential to inhibit many cellular pathways, including TLR3, TLR4, RIG-I, and other activators of IRFs that depend on TBK-1. While intriguing, this would be inconsistent with previous work showing that PIV5 is unable to block signaling through TLR3 and TLR4⁵ and that RIG-I function is very likely also not blocked in PIV5-infected cells.²⁴¹ An interesting possibility is that these kinases are targeted in a cell-type-specific manner, as evidenced by the finding that V protein acts as a decoy for IKKα kinase to facilitate measles virus inhibition of IRF-7 in plasmacytoid cells.³¹⁷ The measles virus V protein is reported to bind to the p65 subunit of NFκB³⁶⁴; however, it is unclear how widespread this is among other paramyxoviruses.

The C proteins that are expressed by some but not all paramyxoviruses can play a role in blocking IFN responses through mechanisms distinct from that of V protein.²⁸¹ Stable cell lines expressing the Sendai virus C proteins have reduced capacity to activate the IFN- β promoter in response to dsRNA.²⁰⁴ Sendai virus, measles virus, and HPIV1 mutants that are defective in C (or Y) protein expression are strong inducers of the IFN- β promoter.^{21,204,251} Whereas some data support a model whereby the C proteins directly block IFN induction,^{33,204,385} other studies indicate that C proteins modulate the viral polymerase to limit production of dsRNA^{21,391} and thus indirectly limit IFN induction. This latter model is supported by the finding of high levels of dsRNA in cells infected with C protein mutants compared to infections with wild-type virus.^{21,391} Inhibition of IFN- β synthesis by the measles virus C protein appears to depend on the ability of C protein to shuttle between the cytoplasm and nucleus,³⁷⁸ suggesting an additional mechanism to limit IFN- β transcription.

There is relatively little current available data on the role of RNA-activated TLRs in IFN responses to paramyxovirus infections. The Nipah virus W protein appears to block TLR3 signaling through a mechanism that depends on nuclear localization.³⁷⁰ Measles virus has been shown to block TLR7- and TLR9-mediated IFN production from human plasmacytoid dendritic cells³⁵⁶ through V protein targeting the cellular kinase IKK α to inhibit IRF-7 phosphorylation.³¹⁷ By contrast to the inhibition seen by measles virus, PIV5 is a potent activator of IFN- α secretion in human plasmacytoid dendritic cells through a mechanism that depends on TLR7 signaling and autophagy pathways.²⁴⁰

Antagonists of Interferon Signaling Pathways

As shown in Figure 33.24, IFN signaling is initiated when secreted IFN binds to its receptor on the cell surface, resulting in the phosphorylation of latent transcription factors STAT1 and STAT2 (signal transducers and activators of transcription) by the cellular Janus kinases (JAK) Tyk2 and Jak1. STAT1 and STAT2 heterodimerize and associate with IRF-9 to form the transcription factor ISGF3, which translocates to the nucleus to bind to interferon-stimulated response elements (ISRE) located in the promoter region of IFN-inducible genes.^{16,171} Paramyxoviruses employ a remarkably diverse range of mechanisms to circumvent IFN signaling.^{172,448}

For some paramyxoviruses, the V protein blocks IFN signaling by targeting one of the STAT proteins for degradation: the PIV5, mumps virus, and SV41 V proteins target STAT1 degradation, whereas the HPIV2 V protein directs STAT2 degradation.^{4,88,172,448} V-dependent targeting of STAT to the proteasome involves the assembly of a cytoplasmic ubiquitin ligase complex, which for PIV5 contains V protein, STAT1, STAT2, the ultraviolet (UV)-DNA damage repair binding protein DDB1, and a member of the Cullin family of ubiquitin ligase subunits.^{227,330,416,417} The PIV5 V protein specifically targets STAT1 and not STAT2 for degradation, although this STAT1 specificity requires the presence of the nontargeted STAT2 protein.²⁹⁸ PIV5 V protein degradation of STAT1 occurs in human cells but not mouse cells, and PIV5 growth in mouse cells is restricted.⁸⁸ However, efficient STAT1 degradation and higher PIV5 replication levels can be restored in mouse cells engineered to express human STAT2.²⁹⁸ Similarly, PIV5 replicated to higher levels in lungs of transgenic mice that were

engineered to express human STAT2.²⁰⁶ A single amino acid substitution in the PIV5 V protein N-terminal domain is sufficient to allow targeted degradation of mouse STAT1, leading to a block in IFN signaling.⁴⁴⁷ Thus, the ability to assemble specific STAT degradation complexes and disrupt IFN signaling may be a factor in determining the host range of some paramyxoviruses such as PIV5 and NDV,^{298,299} which can be restricted for growth in cells from particular species.

IFN signaling is blocked by some paramyxoviruses through mechanisms that do not involve targeted degradation, but rather by binding of V to STAT proteins and preventing phosphorylation or transport to the nucleus. For example, the Hendra virus and Nipah virus V proteins induce the formation in the cytoplasm of high molecular weight complexes consisting of STAT1, STAT2, and IRF-9,³³⁷ and these underphosphorylated complexes are unable to function in signaling. The Nipah virus V and W proteins are both capable of blocking IFN signaling through binding to STAT1 but apparently do so in the cytoplasm and nucleus, respectively.³⁷¹ Nipah virus V protein can also bind to STAT2³³⁶; however, the importance of this binding relative to STAT1 interactions is not entirely clear. By a different mechanism, the measles virus V protein prevents IFN signaling by blocking translocation of both STAT1 and STAT2 into the nucleus,²⁹⁷ and cytoplasmic aggregates can be detected where STATs co-localize with nucleic acids and viral nucleocapsid protein. Remarkably, the measles virus P protein binds to STAT1 to prevent phosphorylation⁸⁷ through interactions involving a specific tyrosine residue that is associated with some attenuated vaccine strains.²⁹⁰

The respiro- and morbilliviruses utilize the C proteins to block IFN signaling,^{123,133,193,368} and viral C protein mutants can be highly attenuated for growth.^{94,124,310} Recent evidence indicates that in different experimental systems, the C proteins can alter STAT phosphorylation patterns,¹³³ can be detected as an interacting partner with STAT1,^{123,392} and can induce ubiquitination and degradation of STAT1 in some types of cells.¹²⁵ Thus, the mechanisms by which the C proteins block IFN signaling are not completely understood and may differ depending on a particular cell type and virus.

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Parainfluenza Viruses

History

Infectious Agent

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HISTORY

The four serotypes of human parainfluenza virus types 1 to 4 (HPIV1 to HPIV4) were first recovered between 1956 and 1960, following the application of cell culture and hemadsorption techniques to the study of pediatric respiratory tract disease.^{48,49,175} HPIV1, HPIV2, and HPIV3 were initially isolated from infants and children with lower respiratory tract illness (LRI), and HPIV4 was recovered from children and young adults with mild upper respiratory tract illness (URI). Soon after their discovery, these viruses were shown to be a major cause of croup (HPIV1, 2, and 3) as well as pneumonia and

bronchiolitis (HPIV3).^{45,115,283} As a group, HPIV1, HPIV2, and HPIV3 are second only to human respiratory syncytial virus (HRSV) as a cause of serious viral respiratory tract disease in infants and children, whereas disease due to HPIV4 is less frequent and less serious.

There also are a number of parainfluenza viruses (PIVs) that infect animals. Indeed, the first PIV to be identified was the avian pathogen Newcastle disease virus (NDV). This virus was isolated following outbreaks in 1926 in Java, Indonesia, and Newcastle-upon-Tyne, England, of a seemingly new poultry disease with high mortality.⁹¹ The origin of this then-emerging pathogen remains obscure. This virus, or a close progenitor, may have been indigenous in wild birds, and its appearance or evolution as a “new” disease entity may have been associated with the increasing scale of poultry farming at that time. Nine distinct serotypes of avian PIVs (now usually called avian paramyxoviruses, or APMVs) are now recognized, of which NDV constitutes serotype 1.⁵ Another PIV was recovered in 1952 in Japan from mice inoculated with an autopsy specimen from an infant with respiratory disease.^{216,264} The natural history of this virus, Sendai virus (SeV), is not well understood, but it appears to be a murine virus that is closely related to HPIV1 but is not a human pathogen. Its antigenic relatedness to HPIV1 led to some confusion when it was used in serologic studies of patients with acute respiratory disease. Bovine parainfluenza type 3 (BPIV3), a close bovine relative of HPIV3, was isolated in 1959 from cattle with respiratory tract disease called shipping fever.^{1,2} PIV5, previously known as simian virus 5 (SV5), was first isolated in 1954 as a common contaminant of primary monkey kidney tissue cultures (hence its name).¹⁶³ This was at a time when these cultures were being used to prepare poliovirus vaccine material, and a number of new viruses were recovered and identified from the primary tissue. PIV5 was shown to be related to HPIV2 and was identified as a cause of croup (“kennel cough”) in dogs.^{21,22,44} Simian virus 41 (SV41) was isolated in 1961, also as a contaminant of primary monkey kidney cell culture.²⁵¹ SV41 was found to be even more closely related to HPIV2 than PIV5.³⁷⁷ Therefore, there are four known human PIVs (HPIV1–4) and 13 known animal PIVs (SeV, BPIV3, PIV5, SV41, NDV/APMV1, and APMV2–9). This number may increase: virus that appears to represent a 10th APMV serotype was recently isolated from penguins from the Falkland Islands.²⁵⁰

The name *parainfluenza* originally was coined because some of the disease signs are influenza-like and because, like influenza, the particle is medium-sized, has a lipid envelope, and has hemagglutination and neuraminidase activities. This name was first used in 1959 for the four viruses now known as HPIV1, HPIV2, HPIV3, and SeV.¹⁰ Therefore, the term parainfluenza refers to the four serotypes of HPIV and their

close animal relatives, and also includes the APMVs, even though these lack close human relatives. Another common human virus, mumps virus (MuV, Chapter 35), is related to the PIVs (most closely to HPIV2) and shares their physical and morphologic properties, but its hallmarks of parotitis and orchitis render it distinct.

Because they can readily be grown to high titer, the animal PIVs SeV, PIV5, and NDV have been used extensively in studies spanning several decades that have defined many of the basic molecular and biological properties of Family *Paramyxoviridae*; this information is described in detail in Chapter 33.

The present chapter focuses on PIV biology and in particular the HPIVs.

INFECTIOUS AGENT

Classification, Relationships, and Diversity

The PIVs are enveloped, cytoplasmic viruses (Fig. 34.1) with single-stranded, nonsegmented, negative-sense RNA genomes of 14.9 to 17.3 kb (Fig. 34.2). They are distributed among three genera (namely *Respirovirus*, *Rubulavirus*, and *Avulavirus*)

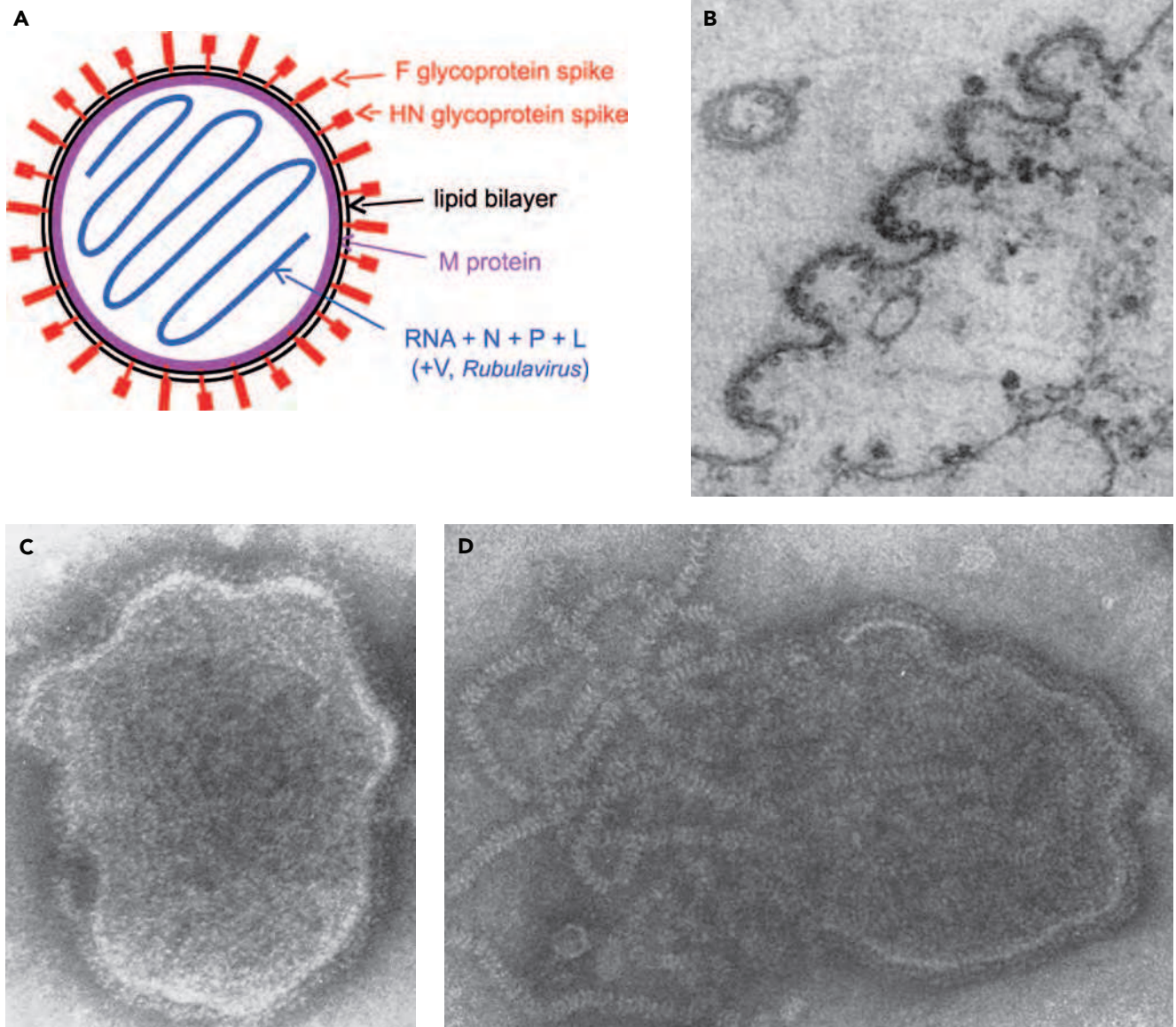


FIGURE 34.1. Schematic diagram (A) and electron photomicrographs (B–G) of parainfluenza virus (PIV) virions.

A: Idealized diagram of a PIV virion, not to scale and not intended to imply relative molar amounts or exact spatial relationships. The V protein (not shown) is found as a structural protein only in *Rubulavirus*.²⁸⁴ The C protein (not shown) of the *Respirovirus* Sendai virus (SeV) also has been reported to be present associated with the virion nucleocapsid (not shown).⁴⁰³ **B:** PIV5 virions budding from the surface of a cultured cell.⁵⁸ Intact **(C)** and disrupted **(D)** HPIV2 virions that were fixed and negatively stained; envelope spikes can be seen in both **C** and **D**, and the helical nucleocapsid is evident in **D**.¹⁵⁶ (continued)

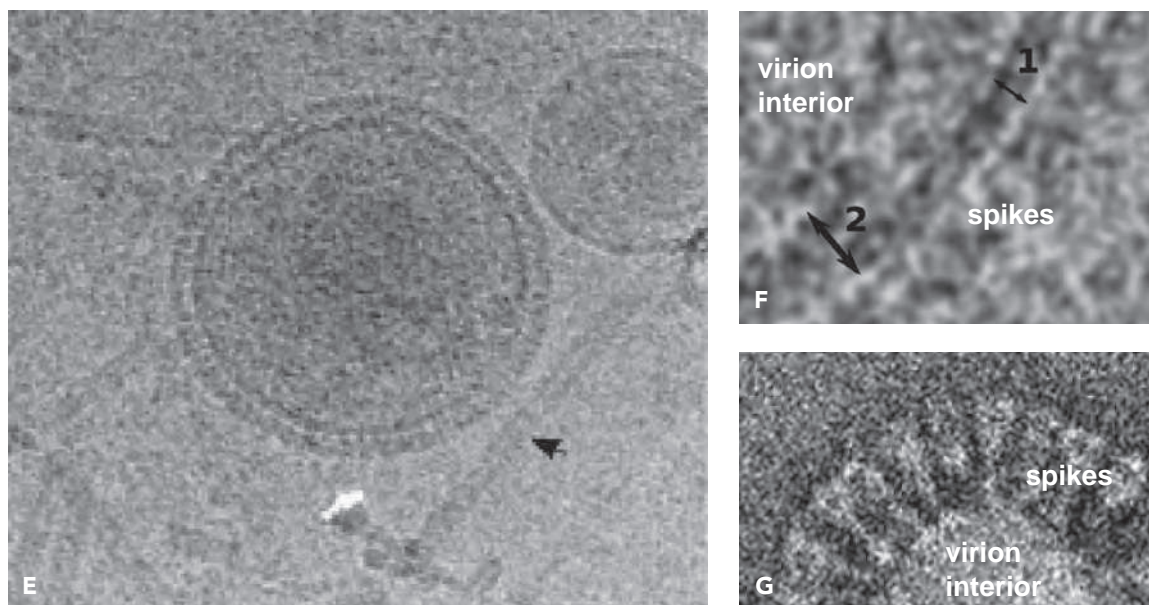


FIGURE 34.1. (continued) **E** and **F**: Cryomicrographs of ice-embedded PIV5.³⁶⁹ **E**: Intact PIV5 virions and free nucleocapsids (arrow), and **(F)** higher-magnification images showing the thickness of the lipid bilayer (double arrow 1) and areas of the lipid bilayer with underlying matrix M protein (double arrow 2).³⁶⁹ **G**: Negatively stained cryomicrographs of a portion of a PIV5 virion showing distinct envelope spikes.²³⁷

of subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales*.²¹⁸ (The other subfamily is *Pneumovirinae*, which contains HRSV, human metapneumovirus [HMPV], and their relatives). HPIV1 and HPIV3, and their respective murine and bovine relatives SeV and BPIV3, constitute the genus *Respirovirus* (Fig. 34.3). HPIV2, its relatives PIV5 and SV41, and HPIV4 are part of the genus *Rubulavirus*. *Rubulavirus* is a diverse genus that also contains related viruses that are not considered PIVs, such as MuV, Mapuera virus, and porcine rubulavirus. The various APMV serotypes constitute the genus *Avulavirus*.

The relationships between the PIVs are illustrated in Figure 34.3 by alignment of the amino acid sequences of the L proteins. Representative viruses from *Morbillivirus* and *Henipavirus*, two other genera of the subfamily *Paramyxovirinae*,

are included for comparison. This shows that the PIVs as a whole are broadly divergent and are not clearly demarcated by sequence relatedness as a group distinct from the non-PIV members of *Paramyxovirinae*. This comparison also shows the close relatedness between some of the human and animal PIVs, such as between HPIV1 and SeV and between HPIV3 and BPIV3. It is likely that these closely related viruses arose from transmission across host species.

The relationships between the PIVs also are illustrated in Tables 34.1 to 34.3 by the percent amino acid sequence identity for the two major surface antigens, namely the fusion F glycoprotein (Table 34.1) and the hemagglutinin-neuraminidase HN glycoprotein (Table 34.2), as well as for the large polymerase L protein (Table 34.3). This illustrates, for example, that the HPIV serotype distinctions are associated with

TABLE 34.1 Percent Amino Acid Sequence Identity Between the F Proteins of the Indicated PIVs^a

	HPIV1	SeV	HPIV3	BPIV3	HPIV2	SV41	HPIV4A	HPIV4B	NDV
HPIV1		67^a	43	42	22	23	24	23	23
SeV			41	41	23	23	24	23	23
HPIV3				82	24	23	25	25	23
BPIV3					24	23	24	24	23
HPIV2						59	34	35	29
SV41							33	33	27
HPIV4A								95	33
HPIV4B									33

^aComparisons within *Respirovirus* or *Rubulavirus* are in bold or bold italics, respectively.

PIV, parainfluenza virus; HPIV, human parainfluenza virus; SeV, Sendai virus; BPIV, bovine parainfluenza virus; SV41, Simian virus 41; NDV, Newcastle disease virus.

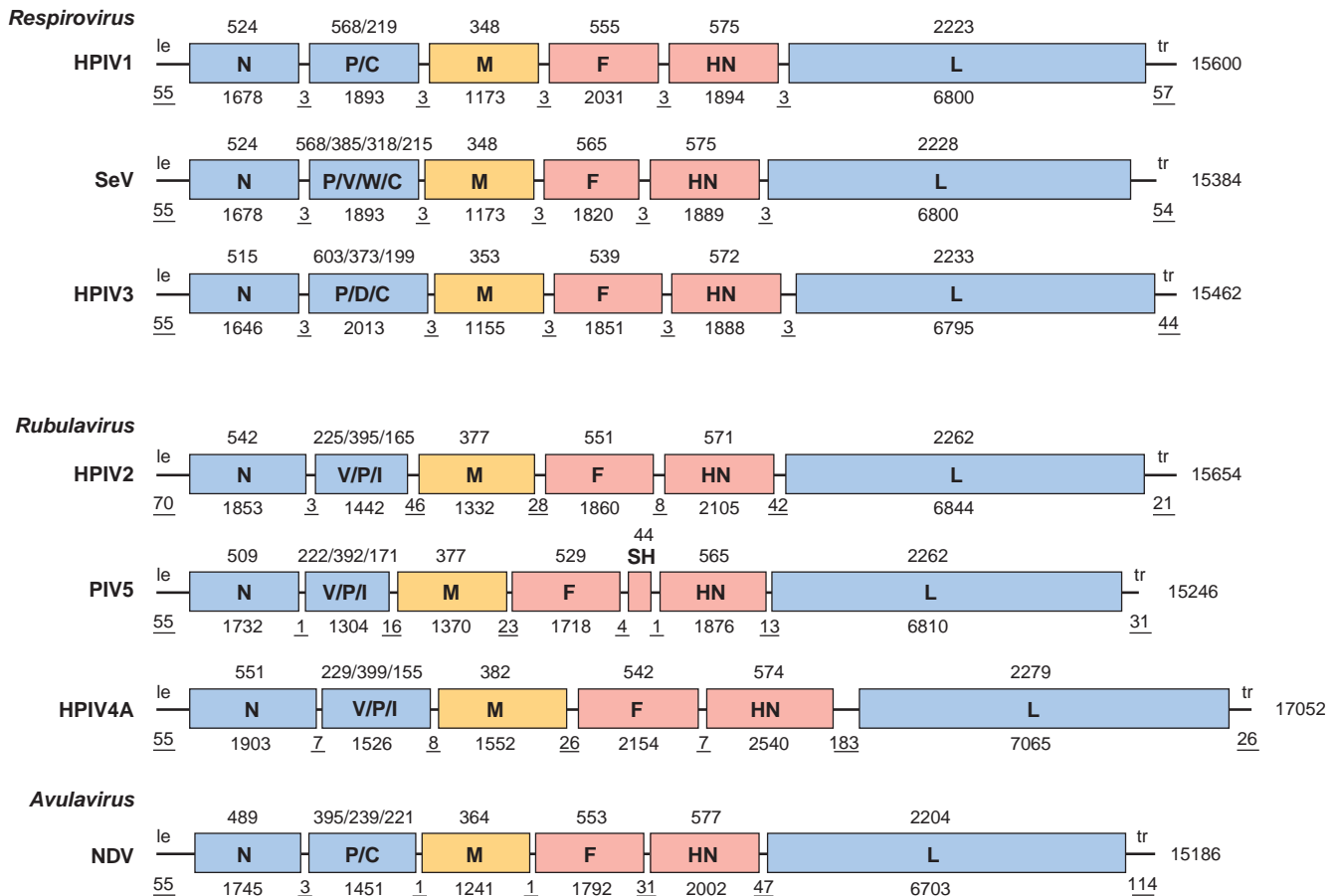


FIGURE 34.2. Gene maps of selected parainfluenza viruses (PIVs), not to exact scale. Each negative-sense genome is drawn 3' to 5', which is the direction of transcription. Genes are shown as rectangles: those encoding nucleocapsid-associated and accessory proteins are in blue; those encoding the transmembrane surface glycoproteins are in red; and those encoding the matrix protein of the inner envelope are in brown. The letters within each rectangle identify the encoded protein(s); for P and V, the protein encoded by the unedited messenger RNA (mRNA) is shown first. Nucleotide lengths are shown under each diagram; those representing the extragenic leader (le), trailer (tr), and intergenic regions are underlined. Amino acid sequence lengths are shown above the diagrams. The maps are generally similar, and there is general similarity in genome, gene, and protein lengths, although some differences exist: (a) the P and V proteins are encoded by unedited and edited mRNA, respectively, for *Respirovirus* and *Avulavirus*, whereas for *Rubulavirus* the situation is the converse; (b) there are differences among the PIVs in other proteins encoded by P gene; (c) PIV5 and APMV6 (the latter not shown here) have the additional SH gene; (d) the intergenic regions of *Respirovirus* are conserved trinucleotides, whereas those of the other genera are variable; (e) the P proteins of *Rubulavirus* and *Avulavirus* are shorter compared to those of *Respirovirus*; and (f) human parainfluenza virus 4 (HPIV4) has a substantially longer genome. Maps are based on the Washington/1964 strain of HPIV1 (NC_003461), the Z strain of Sendai virus (SeV) (M30202), the JS strain of HPIV3 (X11575), the V94 strain of HPIV2 (AF533010), PIV5 (NC_006430), the Toshiba/M-25/1966 strain of HPIV4A (AB543336), and the LaSota strain of Newcastle disease virus (NDV) (AF077761).

amino acid sequence identities of less than 50% for F and HN. The APMV serotypes are not shown in these tables, but they also usually (but not always) have less than 50% amino acid sequence identity between F and HN of the different serotypes.^{43,172,213,214,265,318,353,401,402} For example, the percent amino acid sequence identity between APMV5 versus serotypes 1, 2, 3, 4, 6, 7, 8, and 9 is, respectively: 41, 47, 31, 33, 55, 37, 46, and 37 for the F protein; and 35, 42, 33, 30, 56, 43, 41, and 31 for the HN protein.³¹⁹

Antigenic reactivity based on binding assays can be detected between PIVs within a genus with polyclonal sera and, less frequently, with monoclonal antibodies (MAbs).^{167,203,271} A lower

level of reactivity between genera sometimes is detected with polyclonal sera, although there is no group antigen encompassing the three PIV genera.

HPIV4 has been segregated into two variants, A and B, based on antigenic differences detected by hemadsorption-inhibition (HI) and MAb reactivity.²⁰⁴ Sequence analysis shows that these two subgroups are very closely related: the percent identity between the F, HN, and L proteins is 95, 87, and 97, respectively (Tables 34.1 to 34.3), and they likely would not be distinguishable in neutralization assays with postinfection sera. Variation within the other HPIV serotypes appears to be somewhat less. For HPIV2, the percent amino acid sequence

FIGURE 34.3. Phylogenetic analysis of the amino acid sequences of the L proteins of the parainfluenza viruses (PIVs) and other selected members of *Paramyxovirinae* (genera are indicated on the right). PIVs are boxed. The scale at the bottom indicates evolutionary distance as the number of substitutions per site. The analysis is based on the neighbor-joining method³¹³ and was performed with Molecular Evolutionary Genetics Analysis (MEGA)4.³⁶¹ The numbers at branch points indicate the percentage in which the associated taxa clustered together in the bootstrap test (500 replicates). The L protein sequence was chosen for analysis because it is one of the more conserved proteins, accounts for a substantial part of the viral coding sequence, and is similar in size for each virus. The sequences were as in Figure 34.2 or were from the following: avian paramyxovirus 2 (APMV2), EU338413; APMV3, EU403085; APMV4, EU877976; APMV5, GU206351; APMV6, EU622637; APMV7, FJ231524; APMV8, FJ215863; APMV9, EU910942; Mapuera virus, NC_009489; porcine rubulavirus, NC_009640; MuV, NC_002200; Simian virus 41 (SV41), NC_006428; BPIV3, NC_002161; SeV, NC_001552; CDV, canine distemper virus, NC_002728; MeV, measles virus, AF266288; HeV, hendra virus, NC_001906; NiV, Nipah virus, NC_001906. This analysis was kindly provided by Drs. Sachin Kumar and Siba Samal, University of Maryland at College Park.

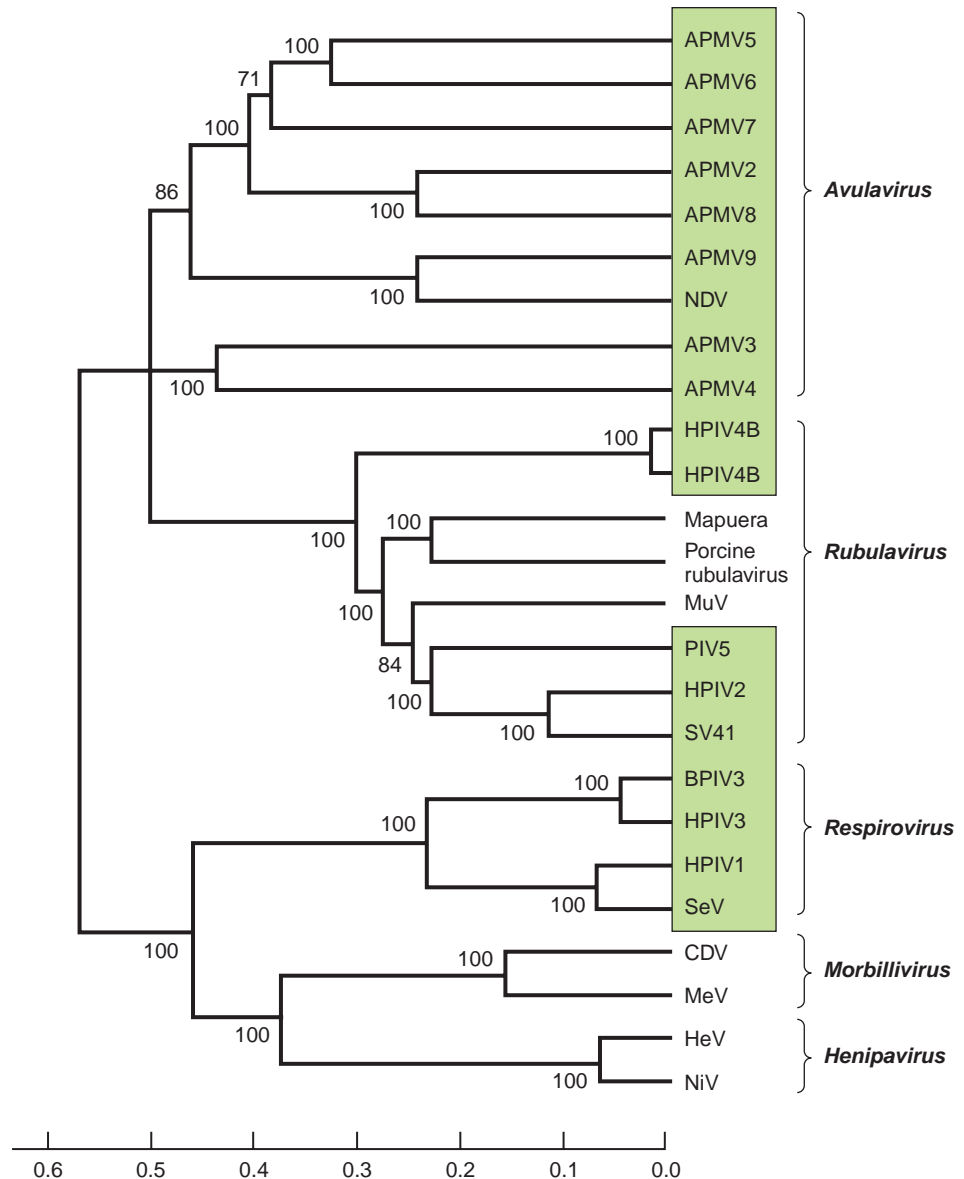


TABLE 34.2 Percent Amino Acid Sequence Identity Between the HN Proteins of the Indicated PIVs^a

	HPIV1	SeV	HPIV3	BPIV3	HPIV2	SV41	HPIV4A	HPIV4B	NDV
HPIV1		73^a	47	46	22	23	24	24	22
SeV			45	46	22	23	22	22	23
HPIV3				76	23	24	22	23	22
BPIV3					22	25	22	22	22
HPIV2						61	38	38	31
SV41							36	36	33
HPIV4A								87	29
HPIV4B									29

^aComparisons within *Respirovirus* or *Rubulavirus* are in bold or bold italics, respectively.

PIV, parainfluenza virus; HPIV, human parainfluenza virus; SeV, Sendai virus; BPIV, bovine parainfluenza virus; SV41, Simian virus 41; NDV, Newcastle disease virus.

TABLE 34.3 Percent Amino Acid Sequence Identity Between the L Proteins of the Indicated PIVs^a

	HPIV1	SeV	HPIV3	BPIV3	HPIV2	SV41	HPIV4A	HPIV4B	NDV
HPIV1		86^a	60	61	28	29	29	29	26
SeV			60	60	28	29	28	28	26
HPIV3				89	28	28	29	29	25
BPIV3					28	28	29	29	26
HPIV2						77	51	50	35
SV41							51	51	34
HPIV4A								97	35
HPIV4B									35

^aComparisons within *Respirovirus* or *Rubulavirus* are in bold or bold italics, respectively.

PIV, parainfluenza virus; HPIV, human parainfluenza virus; SeV, Sendai virus; BPIV, bovine parainfluenza virus; SV41, Simian virus 41; NDV, Newcastle disease virus.

identity between the V94 strain versus the V98 and Greer strains was, respectively, 98 and 99 for F, 95 and 96 for HN, and 99 and nearly 100 for L.³³⁷ For HPIV3, comparison of the F protein sequence of prototype strain Washington/47885/57 with seven clinical strains revealed 98% or more identity,⁶² and comparison of HN with six clinical strains revealed 97% or more identity.³⁸⁵ For HPIV1, comparison of 40 strains showed that the percent amino acid sequence identity for the HN protein was 95% or greater.¹⁴⁶ For NDV, comparison of 50 strains from various times and places of isolation showed that the percent identity between F and HN was 91% and 90% or greater, respectively.³¹⁷ Some of the other animal PIVs, such as APMV2,³⁵² APMV3,²¹⁴ APMV6,⁴⁰² and BPIV3,¹⁵² have been found to have somewhat greater diversity (i.e., intraserotype amino acid sequence identity for F and HN of 75% to 79% for APMV2, 70% to 73% for APMV3, 81% to 86% for APMV6, and 86% to 89% for BPIV3), resulting in distinct genotypes or subgroups within a serotype. In the case of APMV2, APMV3, and APMV6, this has been shown to be associated with a modest degree of antigenic difference detectable with postinfection sera.

NDV is notable because the many highly related naturally occurring isolates and strains that have been recovered for this single serotype exhibit a broad spectrum of virulence, ranging from nonvirulent or mildly virulent (lentogenic), to moderately virulent (mesogenic), to highly virulent (velogenic). Lentogenic strains are associated with subclinical infection or can cause mild respiratory tract disease, and the more attenuated natural isolates are used as live vaccines. At the other extreme, velogenic strains can be highly virulent and, depending on the strain, can cause hemorrhagic lesions in the intestines (viscerotropic) or neurologic disease (neurotropic).³¹⁷ In contrast, there is no evidence of differences in virulence among the various isolates of each of the four serotypes of HPIV, although this has not been studied extensively. Little is known about the possible diversity of disease within the other animal PIVs.

Virion Morphology and Activities

The virions of PIVs are medium-sized particles of 150 to 200 nm. Fixed, negatively stained virions typically appear in electron micrographs as pleomorphic (irregularly shaped) round particles (Fig. 34.1).^{58,156,253,324,369} Filamentous virions have been described in some cases, such as for HPIV2.⁴⁰⁵ Cryoelectron

microscopy of ice-embedded SeV and PIV5 provided images of virions as predominantly perfect spheres of varied diameters.^{153,233,369} This suggests that the irregular shapes of particles observed with conventional electron microscopy are artifacts of sample fixation and dehydration, whereas the variations in size were observed using both methods and thus may be authentic. Seventy-one percent of the ice-embedded PIV5 virions consisted of spheres of 129 to 360 nm (average 217 nm), and the remainder were elongated particles of up to 445 nm.³⁶⁹

PIVs replicate in the cytoplasm and bud through the plasma membrane (Fig. 34.1B). The virion consists of a nucleocapsid that is packaged in a lipid envelope derived from the host cell plasma membrane during budding (Fig. 34.1). In the nucleocapsid, the viral genome is tightly bound along its entire length with the nucleoprotein N at a ratio of one protein molecule per six nucleotides (2,484 to 2,877 protein molecules, depending on the length of the viral genome). Associated with the nucleocapsid in the virus particle are approximately 300 copies of the phosphoprotein P and approximately 40 copies of the major polymerase protein L, based on studies with SeV.^{200,219} *Rubulavirus* virions contain an additional nucleocapsid-associated protein called V,²⁸⁴ and the virion of the *Respirovirus* SeV has been reported to contain 40 copies of a small protein called C, also associated with the nucleocapsid.⁴⁰³ Electron micrographs of nucleocapsids released from PIV virions indicate a length of approximately 1.0 to 1.1 μm .^{65,233} The nucleocapsid with its associated proteins has RNA-dependent RNA polymerase activity.¹³⁴ Purified virions can be activated for cell-free transcription by disruption of the envelope with detergent and can transcribe the viral genome in its entirety into messenger RNAs (mRNAs).⁶⁴

The envelope bears spike-like surface projections composed of homotrimers and tetramers of the F and HN glycoproteins, respectively. Based on cryoelectron microscopy, PIV5 virions were estimated to contain approximately 2,000 glycoprotein spikes per 200 nm particle, with an average spike length of 14.2 nm.³⁶⁹ PIV5 and APMV6 have a third, small transmembrane protein, SH ("small hydrophobic"). The nonglycosylated matrix M protein is associated with the inner surface of the envelope. The hemagglutination activity of the HN protein mediates adsorption of virus to the host cell to initiate infection. The cellular receptor for the PIVs is N-acetylneuraminic

acid (sialic acid) in a terminal linkage to cellular glycoproteins and glycolipids.^{356,411} In the case of HPIV3, cell surface nucleolin also has been reported to serve as a receptor co-factor.²⁹ Viral attachment can be measured experimentally by the agglutination of erythrocytes by virus in suspension (hemagglutination) or by the adsorption of erythrocytes to infected cell monolayers expressing HN (hemadsorption) as was used in the original detection of the HPIVs. Late in infection, the neuraminidase activity of HN cleaves sialic acid to facilitate release of progeny virions. Neuraminidase activity can be quantified using sialic acid derivatives as substrates in a colorimetric or fluorometric assay. The F protein mediates fusion between the viral envelope and the host cell plasma membrane, an activity that can be measured *in vitro* by lysis of erythrocytes (hemolysis).

RNA

The PIV genome is a single strand of negative-sense RNA that ranges in length from 14,904 (APMV2) to 17,262 (APMV5) nucleotides (nt). The differences in genome length between the different PIVs are mostly due to differences in the lengths of noncoding sequences rather than substantial differences in the lengths of open reading frames (ORFs). The PIV genome is not capped or polyadenylated. It contains, in 3′ to 5′ order: a short 3′ extragenic leader region of 55 nt (except in the case of HPIV2, for which the leader region is 70 nt), followed by six genes encoding the N, P, M, F, HN, and L proteins, followed by an extragenic trailer region of 21 to 291 nt (Fig. 34.2; note that the longest, 291-nt trailer region is that of APMV-3 and is not shown in this figure). Sequences of the leader regions of selected PIVs are shown in e-Fig. 34.1A. As noted below, the P gene also encodes one or more accessory proteins—namely C, V, W, I, and D—depending on the virus (Fig. 34.2). PIV5 and APMV-6 each contain a seventh small gene that is located between F and HN and encodes the SH protein. In the case of *Respirovirus*, the PIV genes are separated by intergenic (IG) regions that are conserved trinucleotides (usually 3′-GAA in genome-sense); in the case of *Rubulavirus* and *Avulavirus* the IG regions have nonconserved sequences of variable length (0 to 183 nt) (Fig. 34.2; also, see e-Fig. 34.1B for gene junction sequences of selected PIVs).

Transcription and RNA replication occur in the cytoplasm and follow the *Mononegavirales* model. Briefly, the genes are transcribed sequentially in their 3′ to 5′ order to yield separate nonoverlapping mRNAs that are polyadenylated, capped, and methylated. RNA synthesis also yields short nonpolyadenylated and noncapped transcripts of the leader and trailer regions. Transcription is guided by short conserved gene-start (GS) and gene-end (GE) transcription signals that flank each gene (see e-Fig. 34.1B for gene junction sequences of selected PIVs). For RNA replication, the polymerase ignores the GS and GE signals and produces a complete positive-sense copy of the genome that is called the antigenome. Like the genome, the antigenome is not capped or polyadenylated. Both the genome and antigenome are completely bound with N protein.¹⁹⁹ Encapsidation of nascent genomes and antigenomes is thought to drive chain elongation during RNA replication. The tightly encapsidated nature of the nucleocapsid likely shields the uncapped and nonpolyadenylated genome/antigenome from degradation. It also likely shields the genome/antigenome from recognition by the cytoplasmic helicases retinoic acid-inducible gene 1 (RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA5),

which detect triphosphorylated RNA and double-stranded RNA (dsRNA) and initiate signaling to activate the cellular transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NF-κB) to induce type I interferon (IFN) and proinflammatory cytokines. This also reduces activation of protein kinase R (PKR), which is triggered through dsRNA to activate NF-κB as well as to phosphorylate eukaryotic translation initiation factor eIF-2α and thereby inhibit translational initiation as part of host defense. As another example of how viral RNA can affect host cell responses, one of the products of SeV RNA replication is a 55-nt aborted RNA representing the 5′ trailer region that contains a U-rich sequence that inhibits apoptosis by binding to the proapoptotic factor T-cell intracellular antigen 1 related (TIAR).¹⁶⁶

The nucleotide lengths of the genomes and antigenomes of the PIVs (and of all of subfamily *Paramyxovirinae*) are even multiples of six. This property is essential for efficient RNA replication and is called the “rule of six”.^{199–200,201,337} This is thought to reflect an obligatory nucleocapsid organization in which each N protein monomer associates with exactly six nucleotides. In experiments to recover recombinant HPIV2 and HPIV3 viruses whose nucleotide lengths were designed to not be even multiples of six, the recovered viruses contained genomes that had mutated to conform to the rule.^{336,337}

Each PIV gene encodes—via transcribed mRNA—a single major protein, with the exception of the P gene that can encode additional proteins in two ways that are described briefly here and in greater detail for the HPIVs in e-Fig. 34.2. First, all of the PIVs in the genus *Respirovirus* contain a C ORF that initiates near the 5′ end of the P mRNA, closely overlapping the start of the P ORF. Depending on the virus, the C ORF has from one to four different translational start sites that are utilized to give rise to up to four carboxy-co-terminal C proteins. *Rubulavirus* and *Avulavirus* do not have a C ORF. Second, the P genes of most PIVs encode additional proteins by “RNA editing”.^{199,201,388} This involves the co-transcriptional insertion of 1 or more G residues into the nascent mRNA by polymerase stuttering at an editing motif midway along the P gene. An array of mRNAs is produced: they include the unedited form as well as subpopulations that contain 1, 2, or more G residues inserted at the editing site. The insertion of 1 G residue (or 3+1, and so on) or 2 residues (or 3+2, and so on) creates frameshifts that access ORFs in the two other reading frames. For the PIVs of *Respirovirus* and *Avulavirus*, the unedited mRNA encodes the P protein,^{286,348,388} and the addition of a single G by RNA editing fuses the upstream half of the P ORF to an internal ORF encoding a domain with a conserved cysteine-rich domain: the resulting protein is called V. The addition of 2 G residues fuses the upstream half of the P ORF to an ORF in the third reading frame: this downstream ORF encodes only a few added amino acids and results in a protein called W, except in the case of HPIV3 and BPIV3 in which the number of added amino acids is substantially more and the resulting protein is called D (e-Fig. 34.2). HPIV1 is an exception because it does not appear to engage in RNA editing.^{245,307} In addition, although HPIV3 does engage in RNA editing, the V ORF is separated from the editing site by two or more (depending on the strain) stop codons in the same reading frame that may preclude expression of V (see e-Fig. 34.2).¹⁰⁸ For the PIVs of *Rubulavirus*, the exact-copy mRNA encodes the V protein, whereas an edited version containing

two inserted G residues encodes P.^{192,208,276,342,370} An edited version containing one inserted residue encodes the I protein, which is the *Rubulavirus* equivalent of W. [See the ebook for more information on coding assignments and RNA editing.]

Several factors control the relative efficiency of transcription of the various PIV genes. As is typical for *Mononegavirales*, there is a gradient of transcription in which promoter-proximal genes are expressed somewhat more efficiently than promoter-distal genes.^{64,138} This is thought to be due to polymerase fall-off at the gene junctions.¹⁶⁸ However, with the exception of L, the gradient of expression is not continuous or steep; in the case of SeV, for example, the P, M, F and HN mRNAs accumulate at 0.30, 1.15, 0.61, and 0.38 times the level of N.¹⁴⁸ Accumulation of the L mRNA is much lower (0.02 that of N). Differences in transcription signals also influence transcription. In PIV5, the efficiency of transcription across the different gene junctions, measured by the relative level of expression of the downstream versus upstream gene, was found to vary over a fourfold range, indicative of regulation at the level of the termination/re-initiation at the gene junctions.¹³⁸ However, the HN-L junction was not associated with a particularly high level of fall-off.¹³⁸ This suggests that the low level of expression of L relative to the other genes is due to some other factor such as polymerase fall-off during L gene transcription or instability of the L mRNA.

A number of PIVs have evolved mechanisms for downregulating expression of the F gene. The M GE signal of HPIV3 contains an apparent eight-nucleotide insertion that causes increased M-F readthrough (see e-Fig. 34.1B).³⁴⁴ The M GE signal of PIV5 contains a single nucleotide substitution that has the same effect.³⁰⁰ In SV41, the M GE signal is lacking altogether and M is expressed solely as an M-F readthrough mRNA.³⁷⁶ Interestingly, the F gene of SV41 also is expressed as a monocistronic mRNA by initiation at its GS signal, but this occurs at a reduced level because the majority of the polymerase molecules are already engaged in reading across the M-F junction. In HPIV1, the same effect of increased production of M-F mRNA at the expense of monocistronic F mRNA

was observed, and studies with recombinant viruses mapped the effect to a combination of features, namely the intergenic sequence, the F GS signal, and the long upstream nontranslated region of the F gene.³¹ Therefore, various features in these different viruses result in the synthesis of an M-F readthrough mRNA at the expense of a monocistronic F mRNA. In these M-F readthrough mRNAs, the F ORF would not be efficiently accessed by ribosomes due to its internal position.²¹¹ Finally, SeV downregulates expression of its F gene by yet another mechanism, namely through a suboptimal GS signal.¹⁸⁸ Therefore, each of these strategies results in reduced expression of this fusogenic factor. In the case of SeV, this was shown to reduce the virulence of the virus.¹⁸⁸ It might be that, by reducing morbidity and mortality in the host, the virus increases its opportunities for shedding and spread.

Proteins

All PIVs encode six common proteins: N, P, M, F, HN, and L, all of which are essential for virus replication. All members encode at least one additional protein from the P gene (C, V, D, W, and I, depending on the virus). PIV5 and APMV-6 also encode a small hydrophobic transmembrane SH protein.

HN Glycoprotein

The PIV HN glycoprotein (Fig. 34.4) mediates attachment by binding to host cell sialic acid. This activity is responsible for the ability of the virus to agglutinate erythrocytes. HN also functions late in infection to cleave sialic acid residues on the virus and nearby cell surface proteins to facilitate release of progeny virions. The dual hemagglutinin/neuraminidase functions of HN appear to be modulated by halide ion concentration and pH.²⁴⁸ Hemagglutination activity appears to be favored by the halide ion concentration and pH of the extracellular environment, consistent with the role of HN in binding to extracellular receptors, whereas neuraminidase activity is optimal at lower pH and halide ion concentration, consistent with the role of HN in stripping sialic acid from newly formed

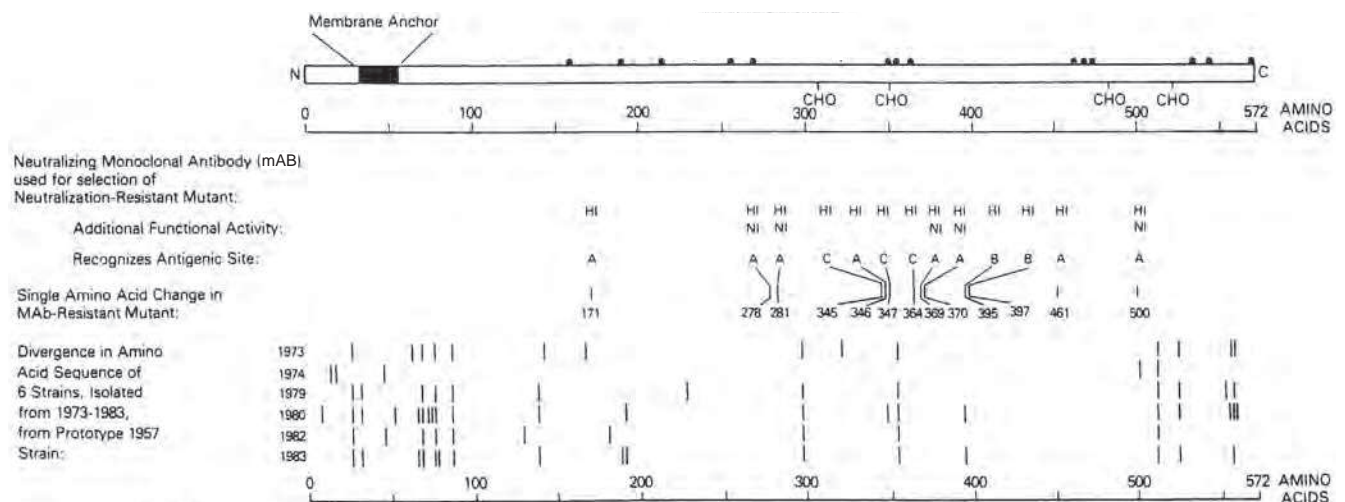


FIGURE 34.4. Linear diagram and antigenic organization of the human parainfluenza virus 3 (HPIV3) HN glycoprotein (strain 47885/5761). • Denotes cysteine; CHO denotes a potential site for N-glycan; HI (hemagglutinin-inhibiting) and NI (neuraminidase-inhibiting) denote positions of amino acid substitutions identified in neutralization-resistant mutants selected with HI and NI monoclonal antibodies (MAbs), and the amino acid positions and antigenic sites (A-C) are indicated; bars indicate positions of amino acid variability among natural isolates.

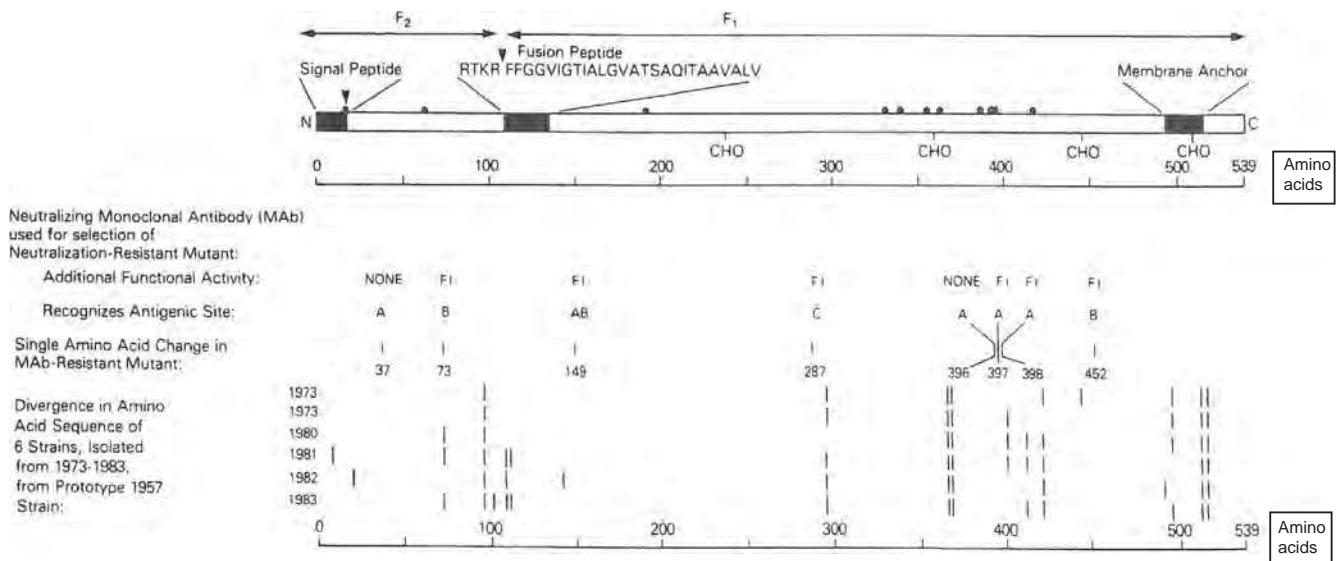


FIGURE 34.5. Linear diagram and antigenic organization of the human parainfluenza virus 3 (HPIV3) F protein (strain 47885/57⁶¹). • Denotes cysteine; CHO denotes a potential site for N-glycan; FI (fusion-inhibiting) denotes amino acid substitutions identified in neutralization-resistant mutants selected with FI MABs, and the amino acid positions and antigenic sites are indicated; bars indicate positions of amino acid variability among natural isolates.

viral and host cell glycoproteins in intracellular vesicles during transport to the cell surface. The HN proteins of PIVs and indeed most of *Paramyxovirinae* also play an essential role by interacting with the F protein to promote fusion.^{66,256,290,292,311}

HN is a type II glycoprotein that contains an uncleaved signal/anchor sequence located near the N-terminus (Fig. 34.4). HN assembles into homotetramers that contain a stalk that is sensitive to trypsin cleavage and a globular head that represents most of the extracellular domain. The globular head retains the HA and NA biologic activities and the major antigenic sites. On a gross level, the globular head has a box-shaped structure in which the four identical subunits exhibit fourfold symmetry. Crystal structures have been determined for the HN proteins of HPIV3, NDV, and PIV5, both free and complexed with its receptor or inhibitors^{32,69,222,312,409}; these are described in Chapter 33.

The HN protein of some avirulent strains of NDV is synthesized as a longer precursor, HN₀, in which the hemagglutinin and neuraminidase are inactive.^{263,316} Activation requires an endoproteolytic cleavage that results in the loss of a small, 9-kD glycopeptide from the carboxy terminus and a change in conformation.^{198,263} Like the F₀ precursor protein of the avirulent strains (see below), HN₀ is resistant to intracellular cleavage in most cell types and presumably is cleaved by extracellular secretory proteases, but unlike F₀ it does not have a marked trypsin-like specificity and can be activated *in vitro* by a variety of proteases.²⁶³ It might be that the shorter HN proteins of virulent NDV strains that lack this extension and do not require cleavage arose evolutionarily from longer cleaved ancestral ORFs by the introduction of translational stop codons. This is suggested by the finding that the ORFs of certain virulent NDV strains retain the apparent relic of an in-frame C-terminal extension beyond the nonsense codon terminating the current ORF.²⁴⁹ A counterpart to HN₀ has not been described for any

other PIV, although the sequence of the HN gene of HPIV4 has been interpreted as containing a relic of such an extension.¹³

F Glycoprotein

The fusion (F) glycoprotein (Fig. 34.5) mediates penetration of the host cell by fusion of the viral envelope to the plasma membrane. Late in infection, when newly synthesized F glycoprotein has accumulated on the surface of the infected cell, it also can mediate fusion with contiguous uninfected cells. This results in the formation of syncytia, a prominent cytopathic effect in monolayer cultures *in vitro*. At least in the case of SeV, the F protein also can act as an auxiliary attachment protein that binds to cells via the hepatocyte-specific asialoglycoprotein receptor,²⁴ although the significance of this *in vivo* is not known.

F is a typical type I glycoprotein (Fig. 34.5), with a cleaved N-terminal hydrophobic signal peptide and a C-proximal membrane anchor. The F protein is synthesized as an inactive precursor, F₀, which is converted into the fusogenic form by cleavage by a host endoprotease to yield two subunits: F₂, which contains the N-terminal 20% of the molecule, and F₁, which contains the remainder of the molecule and is anchored in the membrane. F₁ and F₂ remain linked by a disulfide bond.^{170,198} The F₁ amino terminus created by cleavage is a hydrophobic region called the fusion peptide that is thought to insert into the target membrane to initiate fusion. Crystal structures have been determined for the F proteins of HPIV3, NDV, and PIV5.^{52,357,406,407} The structure and function of F is described in detail in Chapter 33.

Cleavage of F₀ is a prerequisite for PIV infectivity and can be an important determinant of tissue tropism and pathogenesis for NDV and possibly other PIVs (see below and Pathogenesis and Pathology).^{262,374} Most velogenic (highly virulent) and mesogenic (moderately virulent) strains of NDV have a cleavage site with the sequence R/K-R-Q-R/K-R↓F (Table 34.4). This multibasic

TABLE 34.4 Cleavage Sites of the F₀ Proteins of Selected PIVs

Virus	Cleavage site sequence ^a							
HPIV1	Asp	Asn	Pro	Gln	Ser	Arg	↓	Phe
SeV	Gly	Val	Pro	Gln	Ser	Arg	↓	Phe
HPIV3 (prototype and five clinical isolates) ⁶²	Asp	Pro	Arg	Thr	Arg/Lys	Arg	↓	Phe
HPIV3 (two clinical isolates) ⁶²	Asp	Pro	Arg	Thr	Glu	Arg	↓	Ser
HPIV2 (prototype strains) ¹²	Thr/ Lys	Thr	Arg	Gln	Lys	Arg	↓	Phe
HPIV2 (nine clinical isolates) ¹²	Thr/Ala	Thr/Pro	Arg	Gln	Glu	Arg	↓	Phe
HPIV4	Ser	Glu	Ile	Gln	Ser	Arg	↓	Phe
NDV (virulent strains, consensus) ^{249,317,374}	Gly	Arg/Lys	Arg	Gln	Arg/Lys	Arg	↓	Phe
NDV (avirulent strains, consensus) ^{249,317,374}	Gly	Gly/Glu	Arg/Lys	Gln	Gly/Glu	Arg	↓	Leu

^aArg/Lys residues are bold, and Arg/Lys residues consistent with the preferred furin motif (Arg-X-Arg/Lys-Arg↓) are underlined. BPIV3, PIV5, and SV41 are not shown, but all contain the preferred furin motif. For the sources of the sequences, see the legends to Figures 34.2 and 34.3, references ^{26,62,158,202,247,346,367} and the other references cited in this table. PIV, parainfluenza virus; HPIV, human parainfluenza virus; SeV, Sendai virus; BPIV, bovine parainfluenza virus; SV41, Simian virus 41; NDV, Newcastle disease virus.

(basic residues are underlined) cleavage site conforms to the favored cleavage site R-X-R/K-R↓ for the ubiquitous intracellular protease furin,³⁰⁸ providing for efficient intracellular cleavage. Cleavage by furin or a furin-like protease allows the virus to replicate in cell culture without the need to supply exogenous protease in the culture medium. *In vivo*, it provides the potential for systemic spread and replication in a wide range of tissues, resulting in increased virulence. In comparison, the cleavage site sequence found in most avirulent NDV strains, G/E-K/R-Q-G/E-R↓, has fewer basic residues and does not conform to the furin cleavage site. These strains are not cleaved by furin and require added protease (typically trypsin or allantoic fluid added to the culture medium) for replication *in vitro*, and are restricted *in vivo* to mucosal tissue of the lungs or intestines where secreted protease capable of cleaving the F₀ precursor is found. Furin reportedly also may cleave at a “minimal” motif R-X-X-R↓,³⁰⁸ but apparently does not do so for the avirulent NDV strains.

Prototype strains of most of the PIVs have F₀ cleavage sites that contain the furin cleavage motif, including HPIV3, BPIV3, HPIV2, SV41, and PIV5 (examples are shown in Table 34.4), and these viruses do not require added protease for replication *in vitro*. On the other hand, the F₀ proteins of HPIV1, SeV, and HPIV4 lack the furin motif, and these viruses do require added protease *in vitro*. However, for viruses other than NDV, the lack of intracellular cleavage by furin does not necessarily indicate reduced virulence; for example HPIV1 and SeV can be highly virulent *in vivo* despite the lack of furin cleavage. Analysis of clinical isolates of HPIV3 showed that, although five of seven isolates contained the consensus furin motif observed in the prototype strains, two other isolates had the sequence D-P-R-T-E-R↓,⁶² which has the same arrangement of basic residues as avirulent NDV strains. However, these strains were fully competent for replication and the production of infectious virus *in vitro* without added protease, and did not exhibit any restriction for replication in the respiratory tract of rhesus monkeys. Similarly, several clinical isolates of

HPIV2 were found to have the cleavage site sequence T/A-T/P-R-Q-E-R↓,¹² which does not match the preferred furin motif. In this case, restricted growth by these clinical strains *in vitro* was observed in simian Vero cells but not in primary cultures of primate cells. Therefore, the presence of the preferred furin cleavage motif is not essential for intracellular cleavage or virulence in HPIVs.

Nucleocapsid-Associated N, P, and L Proteins

The N, P, and L proteins, together with the RNA genome, are the viral components that are necessary and sufficient to assemble the nucleocapsid and to direct transcription and RNA replication.^{94,134,365}

The N protein is one of the more conserved PIV proteins. It associates with genomic and antigenomic RNAs to form highly stable, RNase-resistant helical nucleocapsids. Monomeric N is maintained in a soluble complex with the P protein prior to assembly into nucleocapsids. The N-terminal 75% of N is the more highly conserved part and is involved in forming the soluble complex with P as well as in subsequently associating with other N monomers and with RNA to form the nucleocapsid. The more variable C-terminal 25% of the molecule is not required to form the nucleocapsid but is essential for it to function as a template.^{34,199}

The P protein is not highly conserved within a genus and has little or no significant sequence identity between genera. The P protein consists of N- and C-terminal functional modules separated by a divergent spacer that spans the RNA editing site.¹⁹⁹ P is found as a homotetramer.³⁶⁶ P is the most heavily phosphorylated viral protein, although the bulk of constitutive phosphorylation can be ablated by mutation in recombinant SeV without effect.¹⁵⁹ The N-terminal module of P is responsible for binding to free N protein and maintaining it as a soluble monomer necessary for nucleocapsid formation during RNA replication.^{74,149} The C-terminal module contains the homo-oligomerization domain and the polymerase co-factor domain, and is the only region of P necessary for transcription. This C-terminal module

mediates binding of P to the nucleocapsid. It also binds L protein and mediates its association with the nucleocapsid.^{149,199}

The L protein is a large multifunctional protein responsible for nucleotide polymerization and mRNA capping and methylation.²⁷⁵ The N-terminal half of L contains blocks of highly conserved amino acids that are thought to be polymerase domains.^{287,288} The L protein forms a complex with the P protein that appears to serve as the RNA polymerase.^{149,199}

The matrix M protein is a conserved, nonglycosylated species that is the most abundant virion protein and is located on the inner face of the virion envelope. In the infected cell, M associates with the inner face of the plasma membrane and plays key roles in virion assembly, budding, and release.^{326,360} Depending on the virus, expression of M alone (e.g., HPIV1) or together with N and HN or F (e.g., PIV5) triggers the formation and release of virus-like particles.^{67,354} The M protein of PIV5 was recently shown to contain a domain that mediates interaction with the host ubiquitin-proteasome pathway during the late stage of budding.³²⁶ M may also play a role in directing the transport of viral components to the plasma membrane.^{277,360}

Accessory C, V, D, W, and I Proteins

These are products of the P gene, and the various PIVs differ as to which of these proteins are expressed, with the general pattern being genus specific. These proteins are not essential for virus replication (and thus are termed accessory), although the C and V proteins in particular can substantially increase the efficiency of growth *in vitro* and *in vivo*. As noted, C is encoded by a separate ORF in the P gene of *Respirovirus*, and is not found in *Rubulavirus* or *Avulavirus*. The V, D, W, and I proteins are produced by various PIVs (see Fig. 34.2) by frame shifts introduced by RNA editing (except in the case of *Rubulavirus*, where the V protein is produced from unedited mRNA while P depends on editing). These proteins are summarized below, and additional information on the expression and functions of these proteins in HPIVs is in e-Fig. 34.2. (The complexity of the proteins encoded by the P gene is even greater for SeV, for which the last ~95 codons of the P ORF also are translated independently to yield a small nonstructural protein called X^{73,76}; this protein is almost equimolar to P in infected cells, but its function is unknown and it will not be considered further.)

The C protein is an abundant small basic protein whose sequence is not well conserved between viruses. C is expressed into one or more carboxy-co-terminal forms, depending on the virus, by utilization of one or more translational start sites in the ORF: for example, SeV and HPIV1 produce four C proteins (C', C, Y1, and Y2, in order of decreasing size), whereas HPIV3 produces one C protein (see e-Fig. 34.2). The different forms of the C proteins of SeV have been reported to have functional differences.^{75,111,112,220} C has historically been considered to be nonstructural, but the C protein of SeV was reported to co-localize with nucleocapsids in the infected cell and to be tightly associated with the virion-bound nucleocapsid, at 40 molecules per nucleocapsid.⁴⁰³ The functions of the C proteins have been investigated in detail for SeV. Deletion or mutation of the SeV C proteins results in strong induction of type I IFN and the establishment of an IFN-mediated antiviral state that restricts viral replication in IFN-competent cell culture and *in vivo*.^{110,215} The SeV C proteins were reported to inhibit activation of the transcription factors IRF-3 and NF- κ B that leads to induction of IFN- β .²⁰⁶ The C proteins also inhibit signaling

from the type I IFN receptor by binding to the signal transducer and activator of transcription protein 1 STAT1 and inhibiting phosphorylation of both STAT1 and STAT2.^{112,122,207,359} Another function of the SeV C proteins is to downregulate production of viral RNA at the level of transcription⁷⁵ and RNA replication.^{37,150,364} By preventing overly robust RNA synthesis, this regulatory activity appears to prevent the formation of dsRNA and unencapsidated triphosphorylated replicative RNAs during SeV infection, thus reducing activation of MDA-5/RIG-I and PKR involved in innate immunity.³⁵⁸ This regulatory activity also prevents the overproduction of antigenomes, which otherwise can result in the packaging of antigenomes into progeny virions that would be noninfectious.¹⁶⁵ The SeV C proteins inhibit apoptosis²⁰⁹ and have been reported to play a role in budding.^{136,315,354} Expression and functions of the C proteins of the HPIVs are described in e-Figure 34.2.

The V protein consists of the N-terminal half of P fused to a C-terminal V-specific domain that contains a sequence motif that is highly conserved in *Paramyxovirinae* and includes seven invariant cysteine residues (e-Fig. 34.2).^{140,272,299,322} The cysteine-rich domain has been shown to coordinate with two zinc atoms per protein molecule.^{227,284,347} V is a structural component of the nucleocapsid in the case of *Rubulavirus*, whereas V does not appear to be a structural component in *Respirovirus* virions and may be present in small amounts in *Avulavirus* virions.^{72,284,348} The clearest characterization of the functions of the V protein has come from studies with PIV5 and HPIV2, in which the absence of C protein facilitates evaluation. V has been shown to bind to MDA-5 and inhibit induction of IFN- β , whereas it did not appear to inhibit RIG-I.^{8,54,289,322} In addition, the V protein inhibits IFN-mediated signaling by mediating degradation of STAT1 or STAT2, depending on the virus and the host cell.^{9,161,272,320} PIV5-mediated degradation of STAT1 has been studied in detail and involves the V-protein binding to ubiquitin ligase and hijacking this cellular complex to target STAT1 for ubiquitination and proteasome-dependent degradation.^{85,227} The cysteine-rich domain must be present in order for V to inhibit IFN induction and signaling.¹⁴⁰ The V protein also delays apoptosis during viral infection,³⁵⁵ and downregulates viral transcription and RNA replication.²³⁰ The mechanism for the effect on RNA synthesis was studied with minireplicons of SeV and HPIV2 and was found to be different for the two viruses.^{151,270} With SeV, the presence of the N-terminal domain of P allows the V protein to bind to soluble N protein and thus interfere with nucleocapsid assembly,¹⁵¹ whereas with HPIV2, the inhibitory activity of the V protein was associated with binding to the L protein, and involved the unique C-terminal domain of V.²⁷⁰ The V protein of PIV5 also has been shown to slow progression of the cell cycle.²²⁸

Therefore, the PIV C and V proteins have a number of similarities in their general effects, even though they are completely distinct proteins that appear to operate by distinct mechanisms. Two major common functions involve interference with host innate immunity—especially the type I IFN response—and downregulation of viral RNA synthesis. These functions may be related: as noted above for the C protein, reducing viral RNA synthesis can reduce activation of MDA-5/RIG-I, PKR, and other sensors that trigger innate immunity. The V protein is particularly important for members of *Rubulavirus* and *Avulavirus* given their lack of C proteins. For *Respiroviruses*, which encode the potent C proteins, some of the

host-antagonist functions of the V protein may be redundant or less robust. For example, although the V protein of SeV has been shown to bind MDA-5 and inhibit induction of IFN- β ,^{53,54,206,289} the magnitude of this effect may be minor.^{123,314,350}

Nonetheless, loss of expression of the SeV V protein significantly reduces the efficiency of viral replication *in vivo*, indicating a contribution that is additional to that of the C proteins.^{189,190} Exactly what this contribution is remains unclear.³¹⁴ For the human *Respiroviruses*, V is more dispensable: as noted, HPIV1 does not encode a V protein due to a lack of RNA editing and the presence of translational stop codons within the V ORF, and HPIV3 likely expresses, at most, only low levels of V due to the presence of stop codons upstream of the V domain (see e-Fig. 34.2 for details on the expression and functions of the V proteins of the human PIVs). The presence of relict V ORFs interrupted by stop codons suggests that predecessors of the present HPIV1 and HPIV3 expressed V proteins, but that this ability became compromised by mutations that introduced these stop codons. Their animal relatives, SeV and BPIV3, respectively, retain the ability to efficiently express V.

The W (present in SeV and *Avulavirus*), I (*Rubulavirus*), and D (HPIV3 and BPIV3) proteins are created when RNA editing fuses the upstream end of the P ORF to a short internal ORF in the remaining reading frame. In the case of W and I, this internal ORF adds only a few amino acids; in the case of the D proteins of HPIV3 and BPIV3 the extension is longer (see e-Fig. 34.2). In general, the functions of the W, I, and D proteins are poorly understood. In the case of SeV, the W protein (like V, as noted above) was reported to downregulate viral genome replication in a reconstituted minireplicon system, an effect that was mediated by its P-related domain.^{71,151} The HPIV3 D protein was shown to accumulate in the nucleus of HPIV3-infected cells, but the significance of this is unclear.³⁹⁸

SH Protein

Among the PIVs, only PIV5 and AMPV6 encode SH proteins, which are 44 and 142 amino acids in length, respectively. MuV (Chapter 36) and all members of subfamily *Pneumovirinae* (Chapter 38) also encode SH proteins. In each case, SH is a transmembrane virion envelope protein with an externally oriented C-terminus. SH can be deleted without much effect on the magnitude of virus replication *in vitro*. However, deletion of SH from recombinant PIV5 resulted in increased cytopathology in cell culture due to increased apoptosis, although overall replication was not reduced, and the virus was attenuated *in vivo*.^{139,229} Further results indicated that infection with the Δ SH virus was associated with increased production of, and signaling by, tumor necrosis factor α , leading to the observed increase in apoptosis.²²⁹

Antigenic Composition and Determinants

Postinfection sera from animals and humans contain antibodies against most or all of the major PIV proteins. However, the HN and F proteins are the only antigens that have been shown to induce antibodies that neutralize infectivity, and they have been shown to be major independent protective antigens. *In vivo*, the parenteral administration of polyclonal or monoclonal antibodies specific to SeV HN or F mediated resistance to challenge with SeV.³⁰² Sera obtained from children following HPIV3 infection that contain antibodies specific to the HN and F proteins have been shown to have virus-neutralizing activity.¹⁸⁵ Infection of rodents with vaccinia virus recombinants expressing the HPIV3

HN or F glycoprotein, or immunization with purified HN and F glycoprotein, showed that either protein induced a high level of resistance to HPIV3 challenge, with HN being more protective than F.^{7,33,303,345}

The “internal” PIV proteins also induce a protective response. This was demonstrated in experiments in hamsters using a recombinant version of HPIV3 in which the HN and F surface antigen genes were replaced by those of HPIV1. This made it possible to compare the relative contributions of the “internal” proteins and the surface glycoproteins to protection.³⁶³ The HN and F proteins induced a high level of protection (in this case specific to HPIV1) that was long-lived. In contrast, the HPIV3-specific protection attributed to the internal proteins—which presumably was mediated by major histocompatibility class I-restricted, CD8+ cytotoxic T lymphocytes (CTLs)—was weaker and waned over a period of several months.³⁶³ This suggests that cellular immunity can contribute significantly to protection for a short period following infection, but is not effective in providing long-term protection.

Antibodies to HN can be measured by HI and neuraminidase-inhibition (NI) assays, which are based on the ability of the antibodies to block these activities of purified virions *in vitro*. Antibodies specific to the F protein can also be measured by inhibition of syncytium formation (fusion inhibition, FI) in cell culture, as well as by inhibition of hemolysis of erythrocytes by purified virions *in vitro*. However, since F activity also depends upon HN; as already noted, HN-specific antibodies also inhibit these activities.

The antigenic sites in the HN protein of HPIV3 were investigated by competitive-binding assays with pair-wise combinations of MAbs.³⁸⁴ Six antigenic sites were defined; five of the sites (A, B, D, E, and F) did not overlap, whereas site C overlapped sites A and B. Three of the sites (A, B, and C) reacted with MAbs that neutralize virus and inhibit hemagglutination.⁶¹ Amino acid residues important for the structures of the neutralization epitopes of HPIV3 HN were identified by sequence analysis of neutralization-resistant mutants selected with MAbs to sites A, B, or C (see Fig. 34.4).⁶⁰ Each of the mutants sustained a single amino acid substitution that in most cases was located in the C-terminal half of the molecule. Different MAbs directed to the same site selected amino acid sequence substitutions that were widely separated on the HN molecule, suggesting that these sites are formed by juxtaposition of distant regions in the folded structure. Consistent with this interpretation is the observation that boiling and reduction of the HN protein of HPIV3 or NDV markedly reduced reactivity with a panel of MAbs, and that neutralization epitopes could not be mimicked by synthetic peptides.¹⁴⁴ Many of the antigenic sites defined by the murine MAbs for HPIV3 HN (and F) are recognized by antibodies in postinfection human sera.³⁸⁶

The antigenic and functional organization of the HPIV3 F protein was elucidated by the same strategy. Competitive binding of a panel of HPIV3 F-specific MAbs identified seven nonoverlapping antigenic sites (A to G) and one site (AB) that bridged sites A and B.⁶¹ Neutralizing MAbs reacted with sites A, B, C, and AB, and at least some of the neutralizing MAbs represented by each site also inhibited fusion. The remaining sites reacted with MAbs that did not neutralize infectivity or inhibit fusion, and site A also reacted with a nonneutralizing MAb. Sequence analysis of neutralization-resistant mutants selected with individual MAbs showed that they contained single amino acid substitutions; those representing sites A or

B contained substitutions located in both the F₁ and F₂ subunits (Fig. 34.5).^{61,382} As was the case with the HN protein, this suggests that distant regions of the linear protein are folded into proximity to create antigenic sites. Consistent with this, F-specific MAbs that neutralize infectivity and inhibit fusion usually do not react efficiently with denatured F protein, such as in Western blots.

The primary antibody response to HPIV1, HPIV2, and HPIV3 is relatively specific to the infecting virus, consistent with their status as distinct serotypes.⁹² Although HPIV1, HPIV2, and HPIV3 each appear to be monotypic based on reactivity with postinfection sera, antigenic polymorphism within serotypes can be detected with MAbs. For example, analysis of 38 HPIV3 strains recovered over a 26-year interval in widely separated locations (United States and Australia) indicated that 6 of the 11 neutralization epitopes on HN, as well as 3 of 14 such epitopes on F, were completely conserved among all strains.^{60,61} The observed variation in HN and F neutralization epitopes did not seem to involve progressive accumulation of changes with time because variation detected in early isolates was not consistently conserved in later strains. Rather, it appears that the heterogeneity that exists results from random mutations that are not subject to strong immunologic selective pressure.⁶¹ Sequence analysis of HPIV1, HPIV2, and NDV has documented the apparent progressive accumulation of sequence differences, resulting in distinct “lineages,” but antigenic changes have been noncumulative and nonprogressive, and they do not correlate with the genetic lineages.^{147,301,316} Conversely, the detection of subgroups of APMV2, APMV3, and APMV6 that can be distinguished by reactivity with postinfection sera suggests that immune-driven divergent evolution can occur.^{214,352,402} The introduction of a vaccine against any of the HPIVs will provide an opportunity to evaluate the capability of circulating virus to accumulate antigenic differences compared to the vaccine strain.

Healthy adult humans tested for memory CD8⁺ CTL against HPIV1 demonstrated strong responses to HN, P, and N, a weak response to M, and an insignificant response to F in this particular subject group.⁷⁸ CTL lines that had been stimulated *in vitro* with HPIV1 showed high reactivity with the closely related SeV, and several cell lines recognized an N peptide that was conserved between HPIV1 and SeV. Remarkably, lower but clearly demonstrable reactivity also was detected against HPIV3 by the HPIV1-stimulated lines. Therefore, the human CTL response is directed against multiple HPIV proteins (as would be expected), and cross-reactivity between serotypes can occur.

Propagation and Assay of HPIVs in Cell Culture

The HPIVs grow well in primary simian or human kidney cell cultures, which allows efficient recovery of these viruses from clinical specimens. They also grow well and can be recovered in a number of established cell lines, including LLC-MK2 rhesus monkey kidney, Vero African green monkey kidney, and NCI-H292 human lung carcinoma cells.⁴¹ Growth of HPIV1 and HPIV4, but not HPIV2 or HPIV3, requires the addition of trypsin (1–5 µg/ml) to the medium for cleavage of the F₀ protein. Virus infection of cultured cells can be monitored by hemadsorption or by immunofluorescence staining. HPIV2 and HPIV3 produce a cytopathic effect that is characterized by syncytia formation, particularly in heteroploid cell lines, whereas

that of HPIV1 and HPIV4 is less. HPIV3 can readily be quantitated by plaque assay or by limiting dilution and direct observation of cytopathology, whereas plaque production or growth following limiting dilution by the other HPIVs usually is visualized by hemadsorption or immunostaining. Typical yields in tissue culture for HPIV1, HPIV2, and HPIV3 are 10⁷ to 10⁸ 50% tissue culture infectious dose (TCID₅₀) per milliliter of medium, whereas replication of HPIV4 is substantially less efficient.⁴¹

The ability of a PIV to replicate efficiently in a given cell culture depends in part on whether it can interfere with type I IFN production and signaling in that particular host. For example, SeV efficiently blocks IFN production and signaling in mouse cells and efficiently grows in those cells. In contrast, PIV5 does not efficiently antagonize the IFN system in murine cells, and growth is inefficient; however, efficient growth is achieved if IFN is depleted by adding IFN-specific antibodies to the medium or if the cells are from a genetically manipulated mouse that lacks the type I IFN receptor.⁸⁴ Typically, a virus can antagonize the IFN system in its native host but not necessarily in heterologous hosts.

The Nature of Cell Injury

The nature of cell injury *in vivo* is not fully understood and seems to involve different pathways for different PIVs. Some viruses, particularly PIV5, can cause a persistent, productive infection in primary cell culture that does not kill cells or shut off cellular RNA or protein synthesis.⁵⁷ In contrast, syncytia formation leading to cell death is a prominent feature of infection of monolayer cell cultures with HPIV2 or HPIV3. As noted, a number of viral products, including the C, V, and SH accessory proteins and the SeV trailer RNA, modulate and reduce cytopathology by inhibiting apoptosis and preventing activation of PKR that otherwise inhibits translation. In some situations, the persistence of PIV5 may also be related to its ability to form cytoplasmic bodies that sequester viral nucleocapsids and may provide for prolonged low-grade infection.⁴⁰

Recently, the characteristics of HPIV infection were studied in a culture system of primary human airway cells that are differentiated into a pseudostratified mucociliary epithelium that closely models the epithelium of the conducting airways (Fig. 34.6).⁴¹¹ HPIV3 infection was highly specific to ciliated cells on the apical surface of the tissue, and virus release occurred exclusively from the same face. Interestingly, there was no evidence of cell-to-cell fusion or spread to underlying cells, and the tissue remained intact over the 2-week duration of the experiment,⁴¹¹ in contrast to influenza A virus, which was rapidly cytopathic in this system.⁴¹² Therefore, HPIV3 is not inherently a highly cytotoxic virus. Similar observations have been made with HPIV1 and HPIV2 infections in this *in vitro* model,^{15,323} as well as with HRSV (Chapter 38).⁴¹² It may be that much of the cytopathology observed *in vivo* is the result of the host response to infected cells rather than direct viral damage. When the HPIV3-infected cultures were maintained over a course of 2 weeks, most of the infected cells were shed into the medium, possibly by an acceleration of the normal mechanism of cell shedding and replacement. In addition, there was a substantial increase in mucin-containing cells, which is consistent with the increased mucus production observed in infected individuals.

In the *in vitro* model of human airway epithelium, the lack of cell-to-cell fusion appeared to be a consequence of the

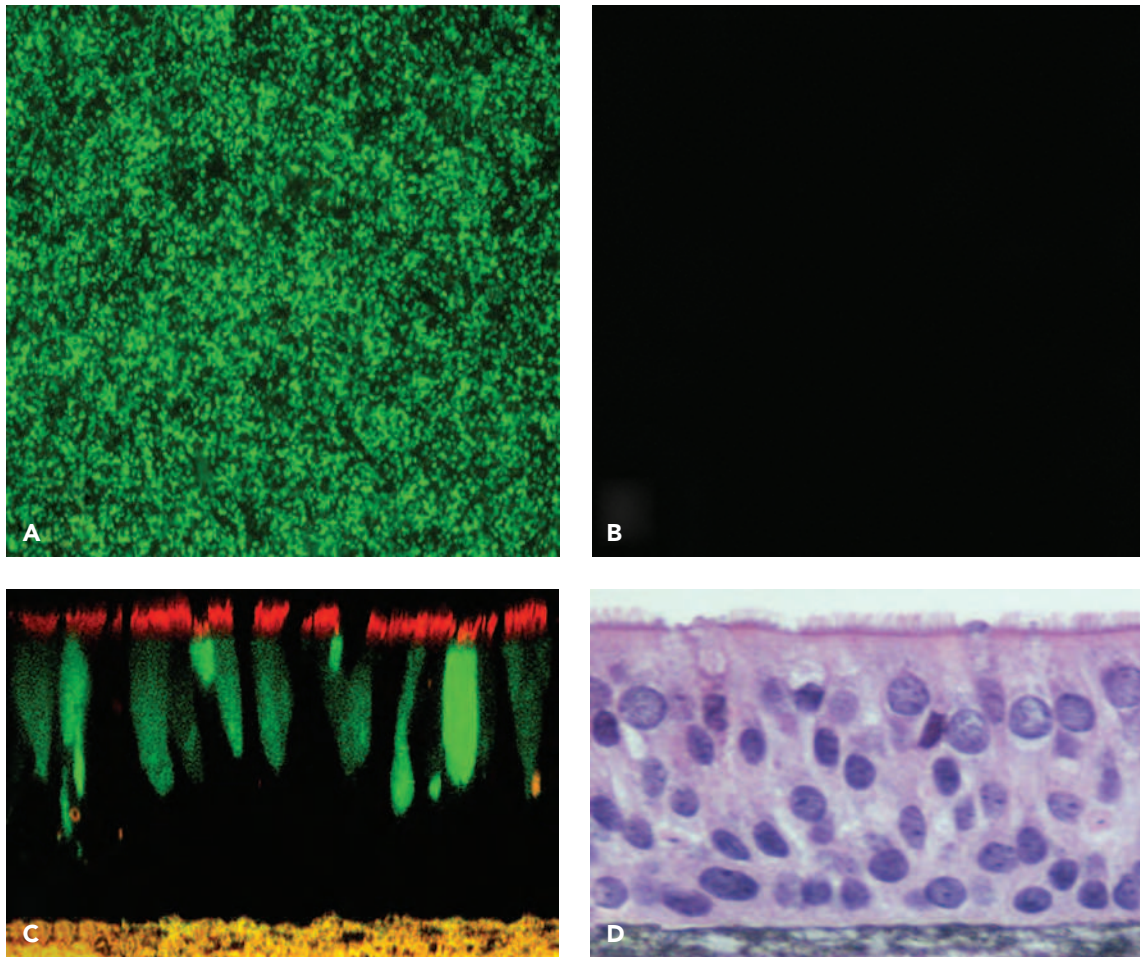


FIGURE 34.6. Infection of an *in vitro* model of human airway epithelium by HPIV3. Primary cultures of human airway epithelium, consisting of pseudostratified mucociliary tissue that closely resembles authentic airway epithelium, were infected with approximately 3 plaque-forming units (PFU) per cell of recombinant HPIV3 expressing green fluorescent protein (GFP) from an added gene and were viewed 48 hours postinfection. **A** and **B**: GFP expression in cultures that were infected on the **(A)** apical or **(B)** basolateral surface, viewed *en face* (i.e., from the top) by fluorescence microscopy at low magnification. **C** and **D**: Cross sectional images of infected cultures at higher magnification. **C**: Fluorescence microscopy reveals GFP-expressing parainfluenza virus 3 (PIV3)-infected cells, with counterstaining by antibodies against β -tubulin to identify cilia (*red*). **D**: Infected cells stained with hematoxylin and eosin, illustrating the lack of syncytia and cytopathic effects.⁴¹¹ Bar, 20 μ m.

tightly polarized nature of the apical cells: surface expression of the F glycoprotein was localized to the apical surface and probably was restricted from contact with neighboring cells. Therefore, the syncytium formation that is prominent in non-polarized monolayer cultures might not be significant in the airway epithelium. Whether it occurs in the alveolar epithelium is unknown. In humans, the pathology of fatal HPIV disease usually does not include giant-cell formation unless the patient has a severe defect in T-lymphocyte function^{82,171} or is profoundly immunosuppressed.³⁹⁵

Infection of HPIVs in Experimental Animals and Other Laboratory Hosts

Hamsters are readily infected by HPIV1, HPIV2, or HPIV3 and support moderate levels of virus replication. However, infection usually is asymptomatic, and pulmonary pathology is minimal or undetectable. Guinea pigs, cotton rats, and ferrets also undergo

a semipermissive silent infection with these viruses, but mice are poorly permissive. Chimpanzees and a variety of monkeys can be infected with HPIV1, HPIV2, and HPIV3, but only HPIV3 has been reported to cause symptomatic illness that sometimes occurs in both chimpanzees and African green monkeys.^{59,92} The absence of significant disease in most experimental animals is associated with limited virus replication.

Some strains of HPIV1, HPIV2, or HPIV3 replicate in the embryonated chicken egg, whereas others do not, and eggs are less reliable and sensitive for the isolation of virus from patients than are monkey kidney cells.

Genetics and Reverse Genetics

As is typical for RNA viruses, PIVs have a high rate of nucleotide misincorporation of approximately 10^{-3} to 10^{-4} . This provides the potential for rapid evolution and, indeed, PIVs readily “evolve” under selective pressure *in vitro*.¹³³ However,

these viruses appear to evolve very slowly in nature. For example, the HPIV3 F and HN glycoproteins have undergone little variation (1.5% to 2.4% amino acid sequence differences) since the first strain was recovered from humans.⁶¹ With NDV, sequence analysis of the F and HN genes from strains collected over a 50-year period identified what appeared to be the progressive accumulation of a small number of sequence changes, but these did not correlate with antigenic differences and overall the sequences remained highly conserved.^{316,373}

Like other paramyxoviruses, PIVs readily produce defective interfering (DI) particles when virus is passaged *in vitro* at high multiplicities of infection.³⁰⁴ DI genomes contain large deletions created by polymerase jumping during RNA replication. The polymerase can reinitiate further down the genome to produce an internally deleted molecule containing unaltered 3' and 5' ends, or, more commonly, can reinitiate on the nascent strand to produce a copy-back RNA in which the ends are exact complements. In either case, DI genomes lack most or all of the viral genes, are dependent on complementation by standard virus, and interfere with its replication. For this reason, care is taken to avoid high-multiplicity passage of PIVs. DI particles have been proposed to be a mechanism of down-regulating standard virus replication based on their effects in cell culture, but the significance of this for infections *in vivo* is unclear. Copy-back DI genomes of SeV have been shown to be highly effective inducers of type I IFN, and the presence of such DI particles in PIV vaccine preparations may increase immunogenicity due to the adjuvant effect of IFN.^{16,349} In addition, copy-back DI genomes typically have antigenomic promoters on both the positive and negative strands and are particularly active in expressing the anti-apoptotic trailer-RNA noted above, which may aid virus infection and viral persistence.

It is generally thought that recombination between co-infecting viruses to produce mosaic genomes containing segments from each parent is very rare for *Mononegavirales*.^{50,295,316} Early attempts to demonstrate the generation of a mosaic recombinant virus during mixed infections, such as with NDV, were unsuccessful.^{77,126,127} A mosaic virus has been produced experimentally only once for *Mononegavirales*, with HRSV.³⁴³ However, there have been reports of viral genomes with sequence discontinuities that may be indicative of RNA recombination.⁶³ Among the PIVs, this has been noted in particular for NDV, perhaps because the widespread use of live vaccines provides the potential for recombination with endemic wild strains.^{56,135,141,298} This evidence remains indirect, and at least some of the cases were found to be PCR artifacts rather than real mosaic genomes.³⁴¹ Recombination probably is a rare event that occurs mostly between closely related viruses, and there is no evidence that it is an important force in PIV evolution.^{50,63,316}

Complete infectious PIV can be produced entirely from cloned complementary DNA (cDNA) by the reverse genetics strategy that has been developed for *Mononegavirales*.^{93,138,267,285,325,337,351} This involves transfecting cells with plasmids that express an RNA copy of the genome or antigenome together with the N, P, and L proteins. These components assemble into a nucleocapsid that launches a productive infection. Reverse genetics provides a method to engineer predetermined changes into infectious virus for use in a variety of studies, including vaccine development.

A second type of reverse genetics system involves minireplicons, which are short cDNA-encoded versions of genomic or

antigenomic RNA. In some cases, the minireplicons are modeled after DI genomes; in other cases they resemble genomic RNA in which the viral genes have been replaced by one or more marker genes such as luciferase.^{94,365} When complemented by the appropriate mix of viral proteins supplied by co-transfected plasmids, the minireplicon is encapsidated and undergoes efficient RNA replication and transcription and packaging. A minireplicon system has advantages for detailed mutational analysis of *cis*-acting RNA signals or *trans*-acting viral proteins because of its smaller, simpler construction and because mutations that might have drastic effects on infectious virus can be studied in this transient system.

Natural Histories of the Animal PIVs

As noted, SeV was first detected in mice that had been inoculated with material from a fatal case of pediatric pneumonia, but the virus is recognized as a pathogen of rodents rather than humans.²⁶⁴ SeV replicates and causes disease in the respiratory tract of mice, and also readily infects hamsters, guinea pigs, and rats. SeV has been detected in mouse colonies worldwide, but is infrequently detected in wild mice, which makes its natural history somewhat unclear.^{101,281} In addition, SeV has been recovered from pigs experiencing outbreaks of influenza-like disease, but pigs are not thought to be a natural host.¹⁰¹ In experimental infections, SeV replicated in the respiratory tract of African green monkeys and chimpanzees with an efficiency similar to that of HPIV1, and thus may not have a substantial host range restriction in primates.³³⁵ This raises the possibility that SeV can initiate zoonotic infections in humans, which is one possible explanation for its original isolation from a human autopsy specimen. However, if this indeed ever occurred, it appears to be a rare event. The virus was well tolerated when administered experimentally to healthy adult humans,³³⁹ although it is not clear whether this reflects restriction by host range or by HPIV1-specific immunity present in most adults. SeV presently is being developed as a potential vaccine vector for human use,^{164,410} as a vector for gene therapy in the airway,¹²⁹ and as an oncolytic agent.¹⁷⁶

BPIV3 is a common cause of respiratory infections in cattle, and usually is associated with mild disease. However, it can promote secondary bacterial infection resulting in severe respiratory disease (shipping fever). Both inactivated and live attenuated vaccines against BPIV3 are available. BPIV3 is highly restricted in rhesus monkeys³³³ and in humans,¹²⁸ in which it has been evaluated as a potential live vaccine against HPIV3 (see Prevention and Control). The host range restriction of BPIV3 was investigated using reverse genetics to exchange each gene of HPIV3 with its counterpart from BPIV3. Evaluation of the resulting chimeras in rhesus monkeys showed that all of the BPIV3 genes contribute to the host range restriction, with N and P (the latter including all of the multiple ORFs) making the greatest contribution.³³³

PIV5 is a natural pathogen of dogs^{21,22} and causes acute self-limiting tracheobronchitis with the potential to progress to pneumonia, particularly since infection can promote opportunistic bacterial infection. In addition, PIV5 was recovered from a dog with posterior paralysis in 1978, and this isolate was neurotropic and caused acute encephalitis when inoculated intracerebrally in gnotobiotic puppies.¹⁷ Vaccination of puppies against PIV5 is routine. Although PIV5 was first isolated as a contaminant of primary rhesus monkey kidney cells, it does not appear to infect monkeys in the wild.¹⁵⁷ However, captive monkeys readily seroconvert, implying that they are

exposed to the virus during captivity, perhaps by human handlers. When inoculated intranasally into nonhuman primates, PIV5 was shed from the upper and lower respiratory tract for a week with mild or no illness. In some animals, the virus was reported to be isolated from the kidneys up to 113 days postinfection, although no viremia was detected.¹⁵⁷ PIV5 also has been isolated from the lungs of a stillborn piglet.¹⁴³

PIV5 has reportedly been isolated from a variety of human tissues, leading some researchers to suggest that the virus naturally infects humans and may establish persistent infections.^{51,120,121,157} PIV5 also has reportedly been detected in association with a number of human diseases ranging from the common cold to neurologic diseases (multiple sclerosis, subacute panencephalitis, and Creutzfeldt-Jakob disease). However, infection and possible persistence of PIV5 in humans remain to be clearly demonstrated, and the virus has not been clearly associated with any human disease.⁵¹ Detection of PIV5-specific antibodies in human populations is confounded by its antigenic relatedness to common human pathogens such as HPIV2 and MuV. In addition, the ability of PIV5 to readily contaminate cell cultures raises doubts about reports of its detection and possible association with various diseases. Therefore, the possible status of PIV5 as a common infectious agent in humans—either benign or pathogenic—remains unresolved but is generally considered unlikely. Comparison of partial genome sequences of PIV isolates from human, canine, simian, and porcine sources did not reveal any striking differences, and the isolates did not differ in the species specificity of their IFN antagonists.⁵¹ This suggests that these isolates represent a single viral species rather than a series of host-specific relatives. PIV5 is presently being investigated as a possible vaccine vector for human use,³⁷¹ although the unresolved issue of possible long-term infections in humans should be cause for caution.

Evidence of PIV infection in an unexpected host must be viewed critically. As noted, antigenic cross-reactivity between PIVs complicates serologic studies. The high prevalence of circulating PIVs in human and animal populations raises the possibility of contamination of tissue specimens and cell cultures, leading to false “isolations.” For example, antigens to HPIV1 and HPIV3—in addition to PIV5—were reported in a large fraction of bone marrow cell specimens,¹²⁰ indicating either that all three viruses persistently infect bone marrow cells (which seems unlikely), or that contamination had occurred. In addition, PIVs in general are highly infectious and sometimes can readily infect other species that are not necessarily natural hosts. An APMV2-like virus was isolated from cynomolgus monkeys with respiratory tract disease.²¹⁷ An APMV3-like virus was isolated from pigs in Israel.²³¹ Nonhuman primates are readily infected in captivity with HPIVs from their handlers. A paramyxovirus isolated from a wild Samango monkey was shown by sequence analysis to be HPIV3, and did not exhibit any evidence of divergence compared to a number of human isolates and thus likely was a virus of human rather than simian origin.²¹² HPIV3 was isolated from a pig with respiratory tract disease, and serologic studies provided evidence of infection on some farms.¹²⁵ More recently, analysis of PIV-like isolates from swine indicated that they were variants of BPIV3 that infected swine but did not become established in that host.²⁹⁷ Taken together, these incidents suggest that the PIVs, being highly prevalent and infectious viruses, sometimes can infect and cause disease in certain nonnatural hosts without becoming established.

As noted, SV41 is a relative of HPIV2 and PIV5 that was isolated from cynomolgus monkey kidney cell cultures. Its natural history remains unclear. When SV41 was inoculated intracerebrally into suckling mice, adult mice, hamsters, guinea pigs, 2-week-old chicks, and rhesus monkeys, it caused central nervous system disease signs in all of the animals and killed most of them.²⁵¹ Approximately 2% of tested human sera had antibodies that reacted with SV41, which was confirmed by immunoprecipitation of the SV41 HN protein by positive sera. This raises the possibility that the virus may sometimes infect humans.²⁷¹ The natural host(s) for SV41 remains unknown.

NDV (APMV1) is among the most important pathogens of poultry worldwide.³¹⁷ As noted, the many isolates or strains of NDV exhibit a broad spectrum of virulence. Vaccines against NDV are in widespread use, and North America, Australia, and New Zealand are relatively free of the disease.³¹⁷ Velogenic and mesogenic strains of NDV are classified by the Centers for Disease Control and Prevention (CDC) and the U.S. Department of Agriculture (USDA) as Select Agents—necessitating strict regulation of the possession and transfer of the virus—due to the potential risk for poultry farming. NDV infects more than 240 species of birds, with disease varying greatly depending on the virus strain and the host species. In addition, NDV can infect humans, particularly poultry farmers or laboratory workers working with the virus, but usually causes only mild conjunctivitis.³⁹ However, NDV was the apparent etiologic agent of fatal pneumonia in an adult who had been a recipient of a peripheral blood stem-cell transplant and as a consequence had increased susceptibility to infection.¹¹⁹ There are no reports of human-to-human infection. NDV can replicate in the respiratory tract of nonhuman primates, but is highly restricted.^{35,36} Lentogenic and mesogenic strains of NDV are being developed as vaccine vectors to express the protective antigens of other agricultural pathogens^{114,160,266,280} and human pathogens.^{35,36,86,87} In addition, NDV is being developed as a potential oncolytic agent.^{96,97,99,296,389}

Eight other serotypes (serotypes 2–9) of APMV have been identified based on antigenic differences measured by HI and NI assays,³¹⁷ and as noted there is new evidence for an additional, 10th serotype.²⁵⁰ Some serotypes exhibit limited cross-reaction and cross-protection, such as between serotypes 1 and 3. APMV serotypes 2 to 9 have been isolated worldwide from various wild and domesticated birds, although their natural histories are generally unknown. APMV serotypes 2, 3, 6, and 7 have been associated with mild disease in poultry, whereas the others have not been associated with poultry disease. There is serologic evidence in commercial poultry for all of the APMV serotypes except for 5 and 10, although this analysis may be complicated by cross-reactivity and the use of NDV vaccines.³⁹¹ As noted, complete genome sequences have been determined for one or more representatives of each APMV serotype.

PATHOGENESIS AND PATHOLOGY

The HPIVs replicate in epithelial cells that line the respiratory tract, causing rhinitis, pharyngitis, laryngitis, tracheobronchitis, bronchiolitis, and pneumonia (Table 34.5). Early during HPIV infection, the mucous membranes of the nose and throat are involved. Obstruction of the paranasal sinuses and eustachian tube may also occur and lead to sinusitis and otitis media. Many

TABLE 34.5 Infections^a Caused by Parainfluenza, Influenza, or Respiratory Syncytial Virus in Pediatric Inpatients^b

Illness	No. tested	Patients with evidence of infection with virus indicated (%)							
		HRSV	HPIV3	HPIV1	HPIV2	Any HPIV ^c	Flu A H2N2 ^c	Flu A ^d H3N2	Flu B
Pneumonia	1,162–1,742	25.0	11.2	3.5	1.6	14.4	3.5	5.4	1.0
Bronchiolitis	873–1,186	43.1	9.4	2.4	1.1	10.9	0.9	2.5	0.4
Croup	593–776	9.8	18.3	20.3	12.2	41.4	7.7	24.1	1.9
Pharyngitis/bronchitis	895–1,337	10.6	11.0	3.7	2.0	14.7	2.0	4.6	0.9
Total respiratory	3,523–5,104	23.3	11.5	6.0	3.2	17.9	3.2	7.1	1.0
Inpatient control	1,237–2,155	5.4	5.0	1.9	1.2	7.5	0.5	0.9	0.5

^aInfection documented by virus isolation and/or a complement-fixing antibody response.

^bStudies performed at Children's Hospital National Medical Center from 1957 to 1976.^{194,195,260} Data were summarized by Murphy et al.²⁶⁰

^cTested from 1957 to 1968.

^dTested from 1968 to 1976.

HPIV, human parainfluenza virus; HRSV, human respiratory syncytial virus.

patients with mild disease may have limited involvement of the bronchi as well. In more extensive infections there is a tendency for HPIV1 and HPIV2 to involve the larynx and upper trachea, resulting in the croup syndrome; such infections may extend also to the lower trachea and bronchi, with accumulation of inspissated mucus and resultant atelectasis and pneumonia.²⁸³ When HPIV3 produces severe disease, infection of the small air passages is likely, with the development of bronchopneumonia, bronchiolitis, and/or bronchitis.^{283,397}

An intensive longitudinal study of infants and children in a semiclosed nursery indicated that 80% of individuals undergoing primary infection with HPIV3 developed a febrile illness; in one third of the illnesses, there was involvement of the lower respiratory tract, resulting in either pneumonia or bronchiolitis.⁴⁶ When infants and children were studied less intensively in a family setting, the estimate for lower respiratory tract involvement during primary HPIV3 infection was 13%.¹¹⁸ Longitudinal nursery studies also indicated that one half of initial HPIV1 infections and two thirds of initial HPIV2 infections produced a febrile illness.⁴⁶ Lower respiratory tract involvement also occurs commonly during primary HPIV1 infection; about 25% of primary infections produced bronchitis or pneumonia.¹¹⁷ Severe acute laryngotracheobronchitis (croup), which is the most dramatic and serious manifestation of initial HPIV infection, was noted in only 2% to 3% of primary HPIV1 or HPIV2 infections in longitudinal studies of healthy children,^{46,116} although HPIV1 and HPIV2 are the major etiologic agents detected among children who develop croup.

The magnitude of viral replication *in vivo* in the natural host appears to be a major factor in pathogenesis. For example, clinical trials of two candidate live-attenuated HPIV3 vaccines indicated that a virus that was highly restricted in replication was well-tolerated in young children, whereas a virus that was less restricted in replication produced fever and LRI in some vaccinees.^{19,184} Similar observations have been made with HRSV vaccines.¹⁸²

A possible role of the immune response in pathogenesis was suggested by the observation that infants and children who develop croup associated with HPIV infection produce local, virus-specific immunoglobulin E (IgE) antibodies earlier and

in larger amount than patients of comparable age with HPIV URI.³⁹⁶ In addition, histamine is detected in nasopharyngeal secretions of croup patients more often than in secretions from patients with URI caused by the same virus.³⁹⁶ Based on these observations, it was proposed that more rapid and increased production of HPIV-specific IgE antibodies mediates histamine release in the trachea and the subglottic region, which in turn produces the symptoms of croup.³⁹⁶ However, it is not clear whether a more pronounced virus-specific IgE response plays a role in pathogenesis of croup or whether it merely reflects more extensive production of viral antigens and consequent increased antibody response during severe disease. Measurement of proinflammatory cytokines in nasal washes obtained from children with HPIV infections demonstrated increased levels of interleukin 6 (IL-6), CC chemokine ligand (CCL)3, CCL4, CCL5, CXC chemokine ligand (CXCL)8, and CXCL9 compared to nasal washes from control subjects, and increased levels of CXCL8 in children with HPIV LRI compared to those with URI.⁹⁵ Cell-mediated immune responses to HPIV antigens, as well as HPIV-specific IgE antibody responses, have also been reported to be greater among infants with HPIV bronchiolitis than among infected infants who developed only URI.³⁹⁶ These observations have been interpreted as evidence for a role of immunologic factors in HPIV bronchiolitis, but the caveat cited before also applies. Infection of epithelial cells with HPIV2 was reported to increase adhesion of, and concomitant cell damage by, neutrophils, suggesting another potential factor in immune-mediated pathology.³⁷²

PIVs have been used in a number of animal models to study airway responsiveness to various stimuli following infection. In the guinea pig, which undergoes a brief, self-limited, and asymptomatic bronchiolitis after intratracheal inoculation of BPIV3, hyperresponsiveness to both histamine and a choline receptor agonist, arecoline, was seen from day 4 until day 16 after infection.¹⁰³ The effect lasted through the full recovery of the epithelium but was accompanied by airway hypercellularity and depletion of mucosal mast cells. Similar responses to PIV5 have been observed in beagle puppies.²²⁶

Older children or adolescents who had severe croup as infants or young children may exhibit bronchial hyperactivity

following exercise or inhalation of methylcholine.^{131,234} It is not known whether heightened airway reactivity is a preexisting condition that contributes to the pathogenesis of croup or whether inflammatory damage during croup produces a prolonged state of increased reactivity. Clearly, an enhanced IgE and histamine response would assume greater importance if preexisting heightened airway reactivity, especially in the trachea and subglottic region, played a role in pathogenesis of croup.

The susceptibility of an individual to severe disease probably is influenced by that individual's genetic background. Studies with HRSV indicate that genetic differences in IL-4, IL-8, and other aspects of host immunity may be associated with a greater frequency of severe disease (see Chapter 38).²⁵² The same may be true for the HPIVs.

Microscopic pathology is not well defined, as very few fatal cases of HPIV disease have been studied. Syncytium formation was observed in the lungs of two infants with severe immune deficiency who died of HPIV3 pneumonia; however, syncytium formation is not a feature of fatal HPIV disease occurring in immunocompetent individuals.^{3,82,90} As with HRSV bronchiolitis, the pathogenesis of HPIV3 bronchiolitis in young infants may involve mechanical events, such as mucus plugging and air trapping in the distal airways, but this has not been clearly defined.

The cleavage-activation of the F₀ precursor can be a major determinant of virulence for NDV, with the presence of a polybasic sequence containing the favored furin cleavage site (R-X-R/K-R↓) being associated with the ability to spread beyond the respiratory and enteric tracts and cause increased disease.²⁶² For example, natural outbreaks of virulent NDV in Australia in 1998 to 2000 appeared to arise from a low-virulence strain by mutation at the F₀ cleavage site that introduced a furin motif, R-R-Q-G-R↓L to R-R-S/Q-R-R↓F.¹²⁴ Serial passage of avirulent NDV strains in chicken eggs and brain resulted in the progressive mutation to a polybasic/furin cleavage site and the acquisition of a highly virulent phenotype.^{81,329} Mutation by reverse genetics of the cleavage site in avirulent NDV to be polybasic with a furin motif resulted in a dramatic increase in viral virulence.^{279,285,309} However, in this latter case, the engineered strains did not gain the full velogenic phenotype, suggesting that other factors contributed to the difference between the lentogenic and velogenic phenotypes. Other studies have found instances where the cleavage site sequence did not predict the virulence phenotype. For example, highly virulent NDV strains from China have been described with the nonfurin cleavage sequence G-R-Q-G-R↓L.³⁶² Conversely, some strains from Africa had cleavage sequences containing the furin motif (R-R-Q-K-R↓F) and yet were isolated from healthy chickens,³²⁸ and NDV strains from pigeons with a polybasic/furin cleavage site R-R-K-K-R↓F were avirulent.^{88a} Recent studies of the other APMV serotypes have provided additional examples of incongruity between the F₀ protein cleavage site sequence and viral virulence.³¹⁷ For example, APMV2 has a nonfurin cleavage sequence (D-K-P-A-S-R↓F) and is avirulent in chickens, and mutation of this site into a variety of polybasic/furin sequences resulted in substantially increased replication *in vitro* but did not increase the virulence of the virus in chickens.³⁵¹ Therefore, the presence of a furin motif at the cleavage site can be a major determinant of APMV virulence in some situations but not others. The presence of phenylalanine versus leucine or isoleucine as the first

residue of the F1 subunit also has been suggested to be associated with intracellular cleavage²⁵⁴ and pathogenesis,¹⁹¹ but there are many exceptions to this association, some of which include examples noted above.

SeV is another PIV that depends on secreted protease for cleavage of its F₀ protein. SeV is considered to be strictly pneumotropic in mice, and the basis for this phenotype was investigated using a mutant, F1-R, which had acquired the ability to cause systemic infection.^{277,367,368} Characterization of F1-R and a series of related mutants identified the following three acquired abilities that were necessary in combination for the pantropic phenotype: (a) the ability of F₀ to be cleaved intracellularly, due to mutations near the cleavage site; (b) the ability of the virus to bud from the basolateral surface in addition to the normal apical budding, due to mutations in M; and (c) the ability of the virus to cause depolymerization of microtubules, which also mapped to M.^{277,367} The acquisition of intracellular cleavability was sufficient to confer the pantropic phenotype if the virus was administered systemically, confirming the idea that intracellular cleavability confers the ability to replicate widely. The further requirement for basolateral budding provides for delivery of the virus into subepithelial tissues and into the blood, providing for escape from the respiratory tract. It is not yet understood how microtubule depolymerization is related to the pantropic phenotype.

It is not clear how directly one can extrapolate from these observations made with animal PIVs to the HPIVs. For example, it is tempting to speculate that the ability of the F₀ protein of HPIV3 to be cleaved intracellularly, in contrast to HPIV1, might explain the greater predilection of HPIV3 to cause pneumonia. This could be evaluated experimentally by altering F₀ cleavability in recombinant virus. It also is not clear whether other determinants, such as polarity of budding, are significant for the HPIVs. HPIV3 buds exclusively from the apical surface and generally is pneumotropic. However, it readily spreads beyond the respiratory tract under suitable conditions. This was demonstrated experimentally by studies in which immunosuppression of HPIV3-infected cotton rats resulted in dissemination of the virus and productive infection in other organs (G. A. Prince, B. R. Murphy, and R. M. Chanock, unpublished observations). HPIV3 viremia also has been reported during infection of untreated hamsters.¹⁷³ Similarly, in children with severe combined immunodeficiency disease, systemic, fatal infection with HPIV3 has occurred, with dissemination to the liver, myocardium, and cerebrospinal fluid.^{102,106} The rare isolation of HPIV3 from cerebrospinal fluid associated with aseptic meningitis in infants and children,⁶⁸ and a single case report of HPIV3 viremia in humans, also indicates that these viruses can (rarely) disseminate from the respiratory tract in immunocompetent individuals. Therefore, host immunity, rather than viral tropism, seems to be a major factor in restricting HPIV3 to the respiratory tract.

Other factors involved in the differences in virulence among the NDV strains have also been identified. Reciprocal swaps of the HN protein between a virulent and an avirulent strain showed that the HN of the latter was associated with reduced receptor recognition, reduced neuraminidase activity, and reduced virulence, indicating that HN can contribute to tissue tropism and virulence.¹⁶² However, in another study, swaps involving HN from a velogenic strain did not confer increased virulence,⁹⁸ suggesting that the contribution of HN

depends on the strain. Other studies identified contributions to strain-specific differences in virulence by the NDV N, P, V, L, and, to a lesser extent, M protein.^{4,89,310} A limited comparison of virulent and avirulent strains of NDV indicated that reduced virulence was associated with reduced viral RNA synthesis.²³⁸

IMMUNITY

PIV infections of experimental animals and adults induce potent systemic and local humoral and cellular responses. However, immune responses to HPIV infections in infants and very young children are qualitatively and quantitatively deficient. For example, young infants undergoing primary infection with wild-type HPIV produce local nasal IgA antibodies that neutralize virus infectivity poorly or not at all,⁴⁰⁴ and infants infected with live-attenuated HPIV3 vaccines exhibit reduced serum antibody responses to the HN glycoprotein compared to older infants and children.¹⁸⁰ This is likely the result of immunologic immaturity, which is most evident during the first 6 months of life,⁷⁰ and suppression of the humoral response to virus infection by maternally derived, virus-specific serum antibodies present in young infants.⁷⁰ The production of poorly neutralizing local antibodies and the poor serum antibody response in this young age group may partially explain why symptomatic reinfection (especially with HPIV3) occurs during early childhood.

The ability of serum neutralizing antibodies to confer resistance to pulmonary replication by PIVs has been well documented in experimental animals, where parenteral administration of polyclonal or monoclonal virus-neutralizing antibodies conferred a high level of resistance to virus replication in the lower respiratory tract and a lower level of resistance in the upper respiratory tract.^{278,303} Studies of HRSV have demonstrated that significant resistance in the respiratory tract requires high titers of serum antibodies (approximately 1:390 and 1:3,500 for 99% reduction of virus titer in the lungs and

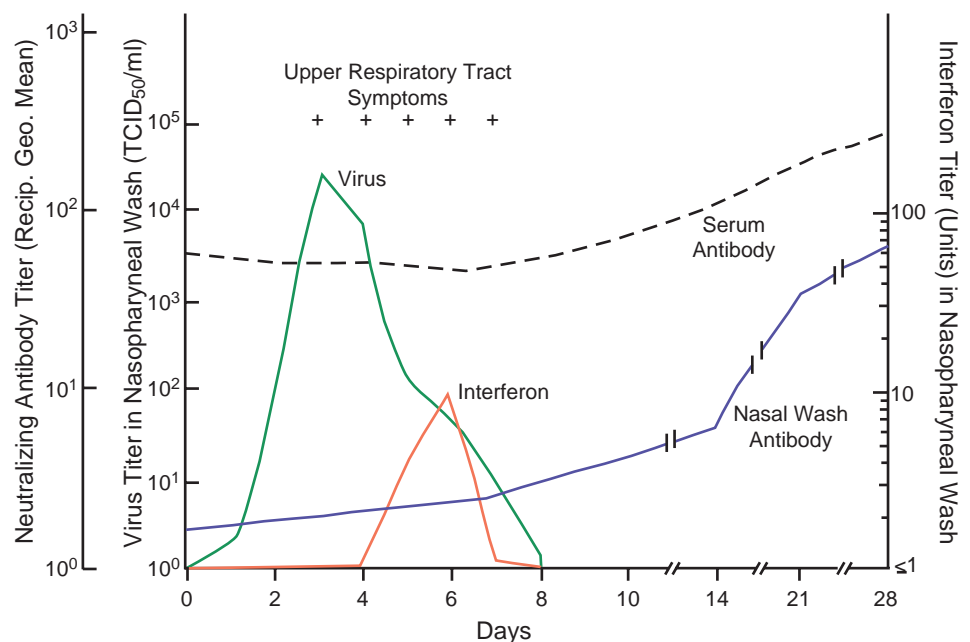
nasal turbinates, respectively, of cotton rats),^{294,330} and this likely also is the case for HPIVs. High titers of serum antibodies are likely required because there is no specific mechanism for transporting serum IgG to the mucosal surface of the lumen of the respiratory tract.

In a longitudinal study of HPIV3 outbreaks in infants and young children residing in a semiclosed nursery, preexisting serum neutralizing antibodies correlated with resistance to infection and illness.⁴⁶ However, resistance associated with serum neutralizing antibodies was only partial. One third of infants and children with a high serum antibody level became infected. Although reinfection occurred, a partial effect of immunity from previous infection was indicated by a shorter period of virus shedding compared to that observed during primary infection. Moderate levels of serum antibody were not associated with complete protection against febrile illness, which occurred approximately 40% as often as during first infection. A somewhat greater reduction of LRI was observed during second infections with HPIV3 in a longitudinal family study.¹¹⁸

The protective effect of serum antibodies against the HPIVs is also suggested by the relative sparing of young infants—who possess serum IgG antibodies acquired passively from their mother—from HPIV infection and associated disease. For example, the risk of infection with HPIV3 during the first 4 months of life is inversely related, in the aggregate, to the level of neutralizing antibodies present in cord blood.¹¹⁸ However, the protective effect of passive immunity to HPIV3 appears to be less than that observed for HPIV1 and HPIV2, as some infants with a moderately high level of maternally derived serum antibodies become infected with HPIV3 and develop illness.¹¹⁸

Experimental HPIV1 and HPIV2 infection of adults possessing varying levels of local nasal secretory IgA neutralizing antibodies and relatively high levels of serum neutralizing antibodies indicated that resistance to infection and URI was mainly a function of mucosal antibodies.^{340,375} Adults respond to reinfection by developing an increase in local nasal secretory IgA antibodies that neutralize virus infectivity (Fig. 34.7).³⁴⁰ In

FIGURE 34.7. Experimental infection of adults with wild-type human parainfluenza virus 1 (HPIV1). Adult volunteers, all of whom had evidence of previous natural infection with HPIV1, were administered 10^5 median tissue culture infective doses (TCID₅₀) of HPIV1 by the intranasal route, and the indicated parameters were monitored.²⁶¹



addition, the efficacy of IgA in mediating resistance has been demonstrated in studies in experimental animals. IgA and IgG were equally effective in providing resistance to SeV challenge in mice when administered directly into the respiratory tract.²⁴⁶ However, in the natural situation, IgA has the advantage of being specifically transported through the epithelium to the surface of the lumen as well as being able to neutralize virus within infected epithelial cells.²⁴⁶

The role of CD4+ and CD8+ T cells in recovery from pulmonary SeV infection was demonstrated in mice, the putative natural host.^{155,186,187} Intranasal infection resulted in a rapid increase in pulmonary CD8+ CTLs, whose appearance coincided with the decrease in titer of pulmonary virus and resolution of infection.¹⁵⁵ However, possible participation of other effectors such as secretory or serum antibodies was not defined. Immunization of mice with a peptide bearing a major epitope for CD8+ CTLs induced a high level of resistance to a short-term virus challenge.¹⁸⁷ Mice that had a mutant nonresponder class I restriction element failed to develop a virus-specific CD8+ CTL response upon infection, whereas serum antibody, delayed-type hypersensitivity, and natural killer (NK) cell responses were unimpaired. These mice cleared infection, but the LD₅₀ of SeV in these animals was 10-fold lower than that for wild-type congenic mice. In a situation in which infection was not lethal, removal of CD8+ cells, either through antibody depletion or disruption of the β 2-microglobulin gene, resulted in delayed viral clearance and 20% mortality.¹⁵⁵ In comparison, antibody-mediated depletion of CD4+ cells, which inhibited antibody production but did not affect the CD8+ CTL response, delayed viral clearance only marginally with no mortality. Removal of both subsets resulted in a failure to clear the virus and 100% mortality.¹⁵⁵ Pulmonary virus-specific CD8+ cells exhibited efficient cell-killing activity *in vitro*, suggesting that this is a likely effector function *in vivo*. CD4+ cells isolated from normal SeV-infected mice did not have significant *in vitro* cell-killing activity, nor did they seem to have a critical role in promoting the CD8+ response.¹⁵⁵ It seems likely that the major antiviral activity of CD4+ cells was to provide T-cell help for antibody-producing B cells.^{155,186}

The importance of virus-specific CD4+ and CD8+ T cells in recovery from HPIV infection has not been formally established. However, as already noted, immunization of hamsters with a recombinant chimeric HPIV3–HPIV1 virus containing the HN and F from HPIV1 and internal protein genes from HPIV3 afforded short-lived protection against HPIV3, presumably involving cell-mediated immunity.³⁶³ The importance of cell-mediated immunity in recovery from HPIV infections is also suggested by the experience of immunodeficient individuals (see Clinical Features).

Evaluation of the role of innate immunity in PIV infections has been limited mostly to identifying the mechanisms by which PIVs interfere with the induction of and signaling by IFN- α/β , as has already been described. As noted, these IFN-antagonist mechanisms are important for efficient infection, and the deletion of the viral proteins involved can be severely attenuating. However, IFN production and action usually are not completely blocked by these viral mechanisms and likely contribute to host defense. A significant proportion (30%) of young patients with HPIV1 infection develop a detectable IFN response (geometric mean titer of 23.5 units) during the acute stage of illness.¹³² This frequency is somewhat less than that

observed during influenza A virus infection of children (55%) but is decidedly greater than that observed during HRSV infection of infancy and childhood (4%).

In summary, innate immunity, antibodies, and cellular effectors such as CTLs play important independent roles in restricting and clearing an infection. With regard to virus-specific immunologic determinants that confer resistance against re-infection, local IgA plays a key role, but this protective effect can be relatively short-lived, especially following primary infection. Cellular immunity also confers short-lived resistance to reinfection. These short-lived effectors might be important in restricting reinfection during an ongoing epidemic season. Serum antibodies provide long-term protection that is more effective in the lower versus upper respiratory tract. Serum antibodies probably play the major role in resistance to lower respiratory tract infection observed in older children, adolescents and adults. Long-term resistance in the upper respiratory tract is less complete, and reinfection of the upper respiratory tract can occur throughout life. It also should be noted that the immunologic determinants of clearance and protection in the young infant, for whom severe PIV disease is most common, are not well understood.

EPIDEMIOLOGY

Morbidity, Mortality, Age

HPIV1, HPIV2, and HPIV3 have a worldwide distribution and cause acute respiratory illness in individuals of all ages, with the greatest impact in infants and young children. Historically, HPIV4 has been isolated less frequently, likely due in part to the greater difficulty of recovering this serotype in cell culture. However, serologic studies and recent studies using reverse transcription–polymerase chain reaction (RT-PCR) suggest that HPIV4 also is a ubiquitous pathogen that can infect and cause disease in both children and adults.^{100,137,306} Each of the four HPIV serotypes has been recovered more often from patients with respiratory diseases than from healthy individuals,^{45,46–47,193,282} and each has produced upper respiratory tract infection and illness when administered to adult volunteers.^{177,375,378,379}

HPIV infection generally occurs early in life. Serologic surveys indicate that at least 60% of children have been infected with HPIV3 by 2 years of age and that approximately 80% have been infected by 4 years of age.^{282,283} A longitudinal study of 121 infants and young children indicated that the incidence of HPIV3 infection was approximately 67 per 100 per year during the first 2 years of life.¹¹⁸ Infection with HPIV1 or HPIV2 generally occurs somewhat later, but by 5 years of age a majority of children have been infected with HPIV2, and more than 75% have been infected with HPIV1.^{282,283} Pneumonia and bronchiolitis associated with HPIV3 infection typically occur during the first 6 months of life, whereas severe illness caused by HPIV1 or 2 is rare in early infancy.^{45,46,115} The incidence of croup, which is principally caused by HPIV1 and HPIV2, peaks between 1 and 2 years of age, but croup remains an important cause of LRI in children up to 6 years of age.^{145,197}

In infants and young children, disease caused by HPIV1, HPIV2, and HPIV3 can range from mild URI to life-threatening LRI. Table 34.5 shows typical data on the frequency of detection of various respiratory viruses in pediatric inpatients with respiratory tract disease, as well as the association between

these viruses and various clinical manifestations, from a prospective study from 1957 to 1976 in Washington DC.²⁶⁰ In this study, the percentage of pediatric hospitalizations attributable to HPIV1, HPIV2, and HPIV3 was 6.0, 3.2, and 11.5, respectively, compared to 23.3 for HRSV. It should be noted that these data are more than 35 years old, and the now-routine treatment of croup with steroids in outpatient and emergency room settings has markedly reduced hospitalizations for this illness, which is mainly caused by HPIV1 and HPIV2. A more recent study in the United States estimated that HPIV1, HPIV2, and HPIV3 were responsible in aggregate for 6.8% of all hospitalizations for fever and/or acute respiratory illnesses in children younger than 5 years of age, with the different serotypes being responsible for 38%, 12%, and 50% of the HPIV-associated cases, respectively.³⁹³ Therefore, HPIV1, HPIV2, and HPIV3 differ in their contribution to severe respiratory tract disease, with HPIV3 having the greatest impact. One retrospective review of the pediatric medical burden of HPIV3 in the United States estimated that, in children younger than 5 years of age, the virus was responsible yearly for up to 3.24 million cases of medically attended acute respiratory illness (MAARI; rate of 16.9% in that age cohort), 1.08 million cases of LRI (rate of 5.6%), and 29,000 hospitalizations (rate of 0.15%),²²³ although a limitation of this study was that rates of outpatient MAARI were extrapolated from rates of LRI. The rate of medically attended HPIV3 disease and LRI was more than threefold greater for those younger than 2 years of age compared to 2 to 5 years of age, and the rate of HPIV3 hospitalization was nearly fivefold greater for those younger than 1 year of age compared to 2 to 5 years of age.

In addition, Table 34.5 illustrates that HPIV1, HPIV2, and HPIV3 each has diversity in its clinical manifestations, with considerable overlap between viruses. However, there are differences in the frequency of association of specific HPIVs with specific illnesses. HPIV1 is the principal cause of croup in children: studies that utilized virus isolation for diagnosis indicate that HPIV1, HPIV2, and HPIV3 are associated with approximately 50% to 75% of cases of croup,^{83,197,305} with HPIV1 responsible for 40% to 65% of all cases.⁸³ In contrast, the principal serious illnesses caused by HPIV3 are pneumonia and bronchiolitis.^{45,47,49,83,282} HPIV2 closely resembles HPIV1 in clinical manifestations, but severe illness occurs less frequently.

Pediatric illness associated with HPIV4 is generally thought to be less severe than with the other HPIV serotypes, although the virus has been found in association with a broad spectrum of clinical manifestations including severe LRI.^{100,306,338} HPIV4 may account for approximately 10% of pediatric HPIV infections, a prevalence that is less than that of HPIV1 and HPIV3 and may be similar to that of HPIV2.^{100,306,338}

The HPIVs commonly reinfect children and adults. However, illness in healthy older children and adults usually is limited to URI; LRI associated with re-infection is rare, although this does occur on occasion and can result in hospitalization.²⁴³ Although the frequency of reinfection is not known, it is probable that most individuals have repeated experience with HPIV1, HPIV2, and HPIV3. In a series of three outbreaks of HPIV3 infection in a semiclosed nursery population, it was observed that 17% of the children infected during one outbreak were reinfected during a subsequent outbreak, although the interval between the first and last outbreaks was only 9 months.⁴⁶ Reinfection by HPIV3 of preschool children living at home

also occurs with high frequency, and usually is associated with a lower incidence and severity of disease compared to primary infection.¹¹⁸ HPIV4 also has been detected in adults with medically attended influenza-like illness.¹³⁷

HPIVs are readily spread in the family setting. In a longitudinal study of families with young children, the overall rate of HPIV infection as estimated by serology (HI and complement-fixation assays) was 44.4 infections per 100 person-years.¹⁰⁴ The rate of HPIV infection varied from 59% for children 2 to 5 years of age to 40% for adults. Spread of virus within infected families was extensive, as indicated by serologic studies: following infection of an index case, 64% of family members developed a serum HI antibody response. HPIVs are typically introduced into the family by preschool children.¹⁰⁴

Nosocomial HPIV infections are particularly problematic in hospitalized children and in immunocompromised patients.²⁵⁹ Nucleic acid sequence analysis has been used to analyze hospital outbreaks, and it is clear that, although outbreaks caused by a single genotypically uniform strain can occur, much of the problem is due to sporadic spread of multiple strains within the hospital or community.¹⁸¹ Infection in hospital staff can occur and may be a factor in spread.³³¹ Transmission may also occur among immunocompromised patients in an outpatient department.²⁶⁹

Mortality due to the HPIVs has not been carefully studied but is thought to be uncommon in otherwise healthy individuals (see Clinical Features in this chapter for information on immunodeficient individuals). Although estimates of the fatality rates for the HPIVs are not available, the aggregate rate is thought to be substantially less than for HRSV, another common viral cause of severe pediatric respiratory tract disease (Chapter 38). In one study in Brazil, immunohistochemical analysis of necropsy samples from fatal respiratory infections (200 subjects, aged 1 month to 14 years, median age 7 months) detected HPIV1, HPIV2, and HPIV3; HRSV; and influenza A in 6.5, 8.0, 15.5, 21, and 10.5 percent of cases, with many cases having more than one virus.⁸⁸ Another report described a fatal case of HPIV1-confirmed laryngotracheitis, with no evidence of bacterial superinfection, in a 15-month-old toddler.²³⁶ Therefore, infection with the HPIVs can be fatal, although this is uncommon.

The seasonality of HPIV infections has been most clearly defined in temperate climates, principally in the United States. From 1957 to 1961, HPIV1 appeared to be endemic; infection occurred sporadically and without a definite seasonal pattern.^{45,46} Beginning in 1962, a different pattern developed, in which sharp outbreaks of HPIV1 occurred every 2 years in the autumn of even-numbered years. In contrast, HPIV2 caused outbreaks in the autumn of odd-numbered years.^{46,116} At present, HPIV1 epidemics occur during the fall of odd-numbered years. HPIV2 epidemics occur annually in the fall,¹⁶⁹ but infection with HPIV2 can also occur at other times.³⁹⁴ For many years, HPIV3 exhibited an endemic pattern, with infection occurring in all seasons of the year. Within the last 15 years, there has been a shift toward yearly epidemics of HPIV3 infection in the spring and summer. In North America, the seasonality of croup primarily mirrors the seasonality of HPIV1, with large peaks occurring in odd-numbered years in the fall^{83,244,327} and smaller peaks occurring in the winter, reflecting influenza and HRSV as additional causes of the syndrome.

One of the hallmarks of the HPIVs is that they persist in the population without undergoing significant antigenic

change. This persistence is likely the result of reinfection and subclinical infection in older children and adults throughout life. HPIV infections induce potent systemic and local humoral and cellular responses (except in young infants) that are thought to completely resolve the acute infection. Serum antibody responses are durable, but local respiratory tract IgA and cellular responses can be more transient, which may contribute to the ease of reinfection. In addition, reinfection is facilitated by the ability of the HPIVs to block the host IFN response. A number of PIVs readily establish persistent infection in cell culture^{57,113,257} raising the possibility that persistent infection might be another mechanism for maintaining virus in the population. However, although prolonged infection by HPIVs can occur in immunocompromised individuals, evidence for persistence of HPIVs in healthy hosts is lacking.

Spread and Infectivity

Transmission of HPIVs is by direct person-to-person contact or by large-droplet spread; however, the viruses do not persist long in the environment. These viruses spread rapidly following introduction into day care, family, and institutional settings. There is evidence from studies in adult volunteers that the infectious dose of HPIV1 is small. For example, in one study, two thirds of adults who possessed a moderately high level of preexisting serum-neutralizing antibodies became infected and developed a “common cold–like” illness following intranasal instillation of 80 TCID₅₀ of HPIV1.³⁴⁰ In addition, reinfected individuals appear to be infectious. HPIV3 appears to be the most efficient of the HPIVs in its ability to spread from person to person. HPIV3 generally infects all susceptible individuals in a semiclosed population (such as a nursery) in a relatively short time.⁴⁶ In contrast, HPIV1 and HPIV2 appear to be less effective in this regard, infecting 40% to 69% of susceptible individuals in semiclosed populations.⁴⁶ During experimental infection of adult volunteers, the interval between administration of HPIV1, HPIV2, or HPIV3 and onset of upper respiratory tract symptoms ranges from 3 to 6 days (see Fig. 34.7).^{177,340,378,379} The incubation period in pediatric infections has not been defined; however, in several institutional outbreaks of HPIV3 infection, the interval between exposure and the onset of virus shedding was 2 to 4 days.⁴⁶

The interval during which an individual infected with an HPIV can infect another person is not known. Longitudinal studies indicate that HPIV3 is usually shed from the oropharynx for 3 to 10 days (median of 8 days) during primary infection, whereas during reinfection the virus is detected during a shorter interval.⁴⁶ On occasion, infants and young children may shed HPIV3 during primary infection for as long as 3 to 4 weeks.¹⁰⁵ Prolonged shedding of HPIV3 has also been observed occasionally in adults with underlying chronic lower respiratory tract disease.¹³⁰ It is not clear whether chronic damage to the respiratory tract is responsible for this unusual manifestation of adult reinfection.

CLINICAL FEATURES

Most primary infections with the HPIVs result in respiratory illness. In children, the most common type of illness consists of rhinitis, pharyngitis, cough, and hoarseness, usually with fever.^{282,283} The cough may be croupy, but respiratory distress

is not present. Approximately three fourths of such ill children have a temperature above 100°F; fever usually lasts 2 to 3 days. Coarse breath sounds, rhonchi, erythema of the pharyngeal mucous membranes, and rhinitis are the characteristic physical findings. Cervical adenopathy is uncommon.²⁸³ Otitis media occurs frequently in HPIV infections, and virus can be detected in the middle ear fluids of patients with otitis media.¹⁴²

When acute laryngotracheobronchitis (LTB; croup) develops, the initial symptoms of rhinitis, pharyngitis, fever, and cough progress. After several days, the cough worsens and becomes brassy, “seal-like,” or barking, and stridor ensues. The illness typically varies in intensity, with the worst symptoms frequently observed in the evening and at night. Most children recover after 48 to 72 hours. In some children, however, air hunger develops, with cyanosis, sternal and intercostal retractions, and progressive airway obstruction. The anteroposterior (AP) radiograph of the neck (which should be obtained under carefully controlled medical supervision, if at all) shows glottic and subglottic narrowing (the “steeple sign”) and differentiates this disease from epiglottitis.

When bronchiolitis or pneumonia develops, fever persists and the cough progresses and becomes somewhat productive. It is accompanied by wheezing, tachypnea, retractions, and, in severe cases, cyanosis. The chest radiograph shows interstitial or perihilar infiltrates and air trapping. In some patients, a combined bronchopneumonia–croup syndrome occurs. There are also case reports of children who have developed severe pulmonary disease resembling the adult respiratory distress syndrome following HPIV infection.¹⁵⁴

HPIVs may cause prolonged and severe infections in patients with congenital and acquired immunodeficiencies, including those with severe combined immunodeficiency and those who have undergone hematopoietic stem cell transplantation (HSCT) or lung transplantation. In these patients, HPIV3 is the most frequently isolated serotype. In one large study of HSCT recipients, HPIV infection was documented in 7.1% of subjects: all four serotypes were detected, but HPIV3 accounted for 90% of the isolates.²⁶⁹ Of the HPIV3 cases, 24% developed pneumonia, and 35% of those died within 30 days. Steroid use was a risk factor for development of pneumonia. Co-pathogens were isolated from 53% of those with pneumonia and increased the risk of mortality.²⁶⁸ HPIV3 also causes LRI in lung transplant recipients and has reported associations with both acute allograft rejection and later development of bronchiolitis obliterans, an often fatal complication.³⁹⁰ In wealthy countries, prior infection with human immunodeficiency virus (HIV) does not appear to increase the morbidity and mortality associated with HPIV infection in children, although prolonged viral excretion has been reported.¹⁹⁶ In resource-limited countries, HPIVs have been reported to be associated with greater morbidity and mortality in HIV-infected than HIV-noninfected children, although the contribution of other illnesses cannot be excluded.²³⁹

DIAGNOSIS

Differential

In croup, other viral causes must be considered. Influenza A viruses (particularly H3N2 viruses) cause up to one third of croup cases (Table 34.5), but these are usually confined to epidemic periods. HRSV also occasionally causes croup. In

resource-limited settings, measles virus may also produce severe laryngeal inflammation. In regions where *Haemophilus influenzae* type B (HiB) vaccine is not used, epiglottitis becomes the most important differential diagnosis, although this disease is becoming increasingly rare with the global use of HiB vaccines. In contrast to croup, epiglottitis is usually of sudden onset, without a prodrome of rhinitis, hoarseness, or cough, and is accompanied by high fever, leukocytosis, a “toxic” appearance, and drooling. The AP radiograph of the neck is characteristic, as is the cherry-red enlargement of the epiglottis, which should be examined only as part of the intubation procedure or when preparations for nasotracheal intubation have been made. Another bacterial infection that must be differentiated from viral croup is bacterial tracheitis. This disease is usually caused by *Staphylococcus aureus* and may be a complication of viral croup rather than a primary bacterial infection. Intubation for bacterial tracheitis is frequently necessary, as well as antistaphylococcal antibiotic therapy. In bronchiolitis or pneumonia of infancy, other viruses (particularly HRSV and HMPV) and *Chlamydia trachomatis* are the most frequent etiologic agents to be considered.

Laboratory

Definitive diagnosis of HPIV infection requires viral isolation by conventional methods or spin-enhanced culture,²²⁴ identification of viral antigens in respiratory tract secretions by immunofluorescence, or detection of viral RNA in nasal secretions by reverse transcription followed by RT-PCR. For viral culture or immunofluorescence, specimens are best collected by nasal aspirate or nasal wash, but nose and throat swabs appear to be adequate for RT-PCR. To enhance recovery by culture, specimens should be transported to the lab on wet ice or “snap” frozen on dry ice and stored at -80°C. Increasingly, RT-PCR is being used as the diagnostic method of choice in both pediatric and adult populations because the sensitivity of these assays allows detection of even small quantities of virus (adults typically shed less virus than children). RT-PCR can be used in multiplex formats capable of detecting multiple pathogens, and several such assays are currently available. Immuno-compromised subjects may shed HPIVs for prolonged periods, which may make it difficult to correlate detection with specific disease states.

PREVENTION AND CONTROL

Treatment

Symptomatic treatment of croup usually includes humidification of air by ultrasonic nebulizer. Nebulized or systemic corticosteroids have been shown to decrease the frequency of intubation and hospitalization in moderate to severe croup.^{11,174} A single dose of oral dexamethasone in children with mild croup has been shown to shorten the duration of symptoms, improve patient sleep, and decrease parental stress.²⁵ Treatment with nebulized epinephrine is usually reserved for patients in severe respiratory distress.²⁵ Steroids act to reduce inflammation, whereas epinephrine relieves airway constriction through relaxation of smooth muscles.

Specific antiviral treatment is not available. The nucleoside analog ribavirin exhibits *in vitro* activity against the HPIVs, but is not recommended for use in otherwise healthy individuals

because its effectiveness is unclear; the same is true for HRSV. Ribavirin does find use in the treatment of HRSV and HPIV infections in HSCT recipients, although its effectiveness in this setting also is unclear.^{42,268} A number of potential inhibitors of the HPIVs that target various steps in the replicative cycle are in preclinical development. The neuraminidase inhibitor 4-GU-DANA (zanamivir) that was developed as an antiviral drug for influenza virus inhibits HPIV3 neuraminidase activity. However, it also interferes with HN binding to the cellular receptor and has the undesired effect of promoting HPIV3 release, which makes it unsuitable for therapy of HPIV infections.^{255,291} Using the crystal structure of NDV as a guide, neuraminidase inhibitors BCX 2798 and 2855 were developed and shown to be active in inhibiting HPIV1 in cell culture, and were effective in prophylaxis and treatment in mice against a recombinant SeV in which the SeV HN gene had been replaced with that of HPIV1.^{6,392} Another strategy has been to use recombinant sialidase to enzymatically remove sialic acid receptor molecules from target cells to block infection by HPIVs and influenza virus: this strategy has been shown to inhibit infection by HPIVs in cell culture and in cotton rats.²⁵⁸ Structure-based “virtual screening” for compounds predicted to interact with the active sites on the HPIV3 HN protein identified a small molecule that interacts with the HN protein and triggers premature activation of the F protein, with the result that entry is blocked.²⁵⁸ Fusion is driven by interaction between heptad repeats in the F protein (see Chapter 33), and synthetic peptides derived from these repeats have been shown to block this interaction and inhibit HPIV3 infection *in vitro*.²⁹³ This strategy is based on earlier work with HIV that resulted in the HIV fusion inhibitor enfuvirtide presently in use in anti-HIV therapy. The addition of cholesterol to HIV or HPIV3 peptides targeted them to the membrane and increased their antiviral activity.²⁹³ A novel small molecule LJ001 was recently described that intercalates into viral membrane and irreversibly blocks virus–cell fusion, and is active against a wide array of enveloped viruses including PIVs.³⁹⁹ Small molecules that inhibit HPIV3 transcription in cell culture have recently been described.²⁴² In addition, small interfering RNA (siRNA) targeting the HPIV3 N gene was reported to have antiviral activity in cell culture and in mice (with the caveat that HPIV3 replicates poorly in mice)²³; a similar strategy against HRSV has been shown to reduce virus infection and replication in clinical studies (Chapter 38). Recently, DAS 181, a recombinant sialidase containing the catalytic domain of *Actinomyces viscosus*, has been shown to have *in vitro* activity against all of the human PIVs²⁵⁸ and has been associated with viral clearance and clinical improvement when delivered via inhalation to stem cell or lung transplant patients with HPIV3 LRI.^{52a,91a,131a} Clinical evaluation of DAS181 is ongoing.

Vaccines

The primary need for HPIV vaccines is in the pediatric population, with the need being greatest for HPIV3 followed by HPIV1 and HPIV2. Given the epidemiology of the viruses, immunization against HPIV3 should begin by the second month of life (ideally given in combination with an HRSV vaccine), whereas immunization against HPIV1 and HPIV2 could begin in late infancy or early childhood. A major obstacle to developing pediatric vaccines is that antibody responses to live respiratory viral vaccines are frequently limited in infants at or below 6 months of age^{70,109} (see Immunity, above).

In early work, inactivated vaccines prepared with virus from infected embryonated eggs or primary monkey kidney cell culture were developed for HPIV1, HPIV2, and HPIV3 and shown to be immunogenic in children, as indicated by the development of serum HI and neutralizing antibodies. Unfortunately, parenteral administration of these vaccines failed to induce resistance to HPIV disease.^{55,107} Effective immunization against HPIV disease using a killed virus vaccine has not been demonstrated and is not an active area of research.

Subunit vaccines have also been evaluated, namely, preparations of the HPIV3 HN and F glycoproteins isolated from purified HPIV3 or from insect cells infected with recombinant baculoviruses expressing the HN or F protein or a chimeric form of HN and F encoded by an engineered cDNA.^{7,225,302,383} Parenteral immunization of cotton rats with these viral glycoproteins induced satisfactory levels of neutralizing, HI, and FI antibodies, and the immunized animals were highly resistant to intranasal challenge with HPIV3. In addition, intranasal administration of the purified glycoproteins was effective in inducing a local IgA response.³⁰³ However, none of these strategies has been validated in nonhuman primates or in clinical trials, which pose a more stringent test of vaccine efficacy, since HPIVs do not replicate efficiently in rodents and thus are readily restricted.

A major focus of current research is the development of live-attenuated intranasal vaccines for HPIV1, HPIV2 and HPIV3, either using attenuated versions of the native human HPIVs, or by using related nonhuman PIVs as vectors to express HPIV HN and F glycoproteins. Information about specific experimental vaccines is detailed below. Importantly, live attenuated respiratory viruses administered intranasally are infectious and moderately immunogenic even in the presence of maternal serum antibodies.⁴⁰⁰ Furthermore, these intranasally administered vaccines induce both local and systemic immunity.

HPIV3 Vaccines

Two biologically derived HPIV3 experimental vaccines that have been evaluated in clinical trials have served as prototypes for the development of live-attenuated HPIV3 vaccines. HPIV3 cp45 (cp45) was derived from the JS wild-type strain of HPIV3 by 45 passages in primary African green monkey kidney cells at low temperatures.¹⁸ This cp45 mutant exhibited three interesting properties: (a) cold adaptation (*ca*) (i.e., an ability to replicate efficiently at 20°C, a temperature restrictive for wild-type PIV3); (b) temperature sensitivity (*ts*) (i.e., a decreased ability to produce plaques at the high end of the normal temperature range); and (c) reduction in the level of replication ("attenuation" or *att*) in hamsters, monkeys, and humans.¹³³ The cp45 virus contains 20 point mutations that differentiate it from the JS wild-type strain, 10 of which are silent, without a change in amino acid sequence. Of the remaining 10 mutations, the ones associated with attenuation involve amino acid substitutions in the C, F, HN, and L proteins, with the L mutations playing the major role.^{332,334} The second candidate is BPIV3, which is naturally attenuated in humans, as already noted.⁶⁰ BPIV3 and HPIV3 are 25% related antigenically by cross-neutralization assays, which would reduce the effectiveness of BPIV3-specific immunity in restricting HPIV3. Both cp45 and BPIV3 have been evaluated in phase I and phase II trials in adults, HPIV3 seropositive children, HPIV3 seronegative children, and infants as young

as 1 month (cp45) or 2 months (BPIV3) of age. Both candidates were over-attenuated in adults and in seropositive children but were highly infectious in seronegative children and infants.^{20,59,178,183,184} There were no significant differences in the incidence of respiratory or febrile illnesses among seronegative vaccinees and placebo recipients, suggesting that the vaccines were well tolerated. Otitis media occurred more frequently among seronegative children vaccinated with HPIV3 cp45 in phase I trials than among placebo recipients, but this was not observed in infants vaccinated with HPIV3 cp45, or in phase II trials of HPIV3 cp45 in seronegative children. A recombinant form of the cp45 vaccine, designated rcp45, has been recently developed. rcp45 has the advantage of having a short, well-defined passage history using qualified cells and reagents. rcp45 was recently assessed in phase I and II trials in HPIV3-naïve children to determine the optimal dosage and dosing interval (clinicaltrials.gov NCT01021397, NCT01254175, and NCT01150799), and has been found to be comparable to the biologically derived cp45 vaccine with respect to tolerability, infectivity, and immunogenicity.¹⁷⁹ and unpublished observations

Although both HPIV3 cp45 and BPIV3 elicited HI antibody responses against HPIV3 in most vaccinated seronegative children, the magnitude of the HPIV3-specific response was lower in children who received BPIV3, consistent with the antigenic differences between BPIV3 and HPIV3.⁶⁰ For this reason, recombinant bovine/human PIV3 candidate vaccines containing the HPIV3 HN and F genes and one or more BPIV3 internal genes have been developed by reverse genetics (see below). These viruses were constructed either through replacement of the HN and F genes of BPIV3 with their counterparts from HPIV3³²⁵ or through replacement of individual "internal" protein genes (e.g., N or P genes) of HPIV3 with their counterparts from BPIV3. Two of these vaccines, rHPIV3-N_B (consisting of HPIV3 in which the N gene was replaced with its BPIV3 counterpart) and rB/HPIV3 (consisting of BPIV3 in which the F and HN genes were replaced with their HPIV3 counterparts), have recently been evaluated in phase I clinical trials (clinicaltrials.gov NCT00366782). Each candidate was highly attenuated in adults and seropositive children, but was infectious and immunogenic in seronegative children. An additional candidate, a chimeric vaccine designated MEDI-534, is a version of rB/HPIV3 that also expresses the HRSV F protein from an added gene placed between the N and P genes. This strategy thus provides a bivalent vaccine virus against HPIV3 and HRSV. MEDI-534 has been evaluated in a phase I trial in HRSV and HPIV3 doubly seronegative children ages 6 to 23 months (clinicaltrials.gov NCT00493285). Preliminary data from this trial indicate that all children who received the highest dose of vaccine (10⁶ TCID₅₀) were infected with vaccine virus, and that seroconversion to HRSV and HPIV3 occurred in 67% and 100%, respectively, of recipients of this vaccine dose.

HPIV1 and HPIV2 Vaccines

SeV, the murine relative of HPIV1, has been evaluated as a HPIV1 vaccine candidate in nonhuman primates and healthy adults. SeV was highly immunogenic and protective against HPIV1 challenge when administered intranasally to African green monkeys, and was well tolerated in adult human recipients and immunogenic in three of nine individuals.³³⁹

The replication of SeV was not significantly restricted in African green monkeys and chimpanzees compared to

HPIV1,³³⁵ which raises the possibility that it might not be satisfactorily attenuated in infants and children. However, SeV was attenuated in adults and is currently being evaluated as an experimental intranasal vaccine in young children (clinicaltrials.gov NCT00186927).

Live-attenuated candidate vaccines for HPIV1 and HPIV2 have also been developed based on the introduction of known attenuating mutations into cDNA-derived HPIV1 and HPIV2 by reverse genetics. In some cases, mutations were designed to have increased genetic stability, such as by choosing codons that could not revert by a single nucleotide substitution, or by deleting the relevant codon(s). One vaccine candidate, rHPIV1 84/del170/942A, contains mutations in the C, HN, and L proteins. Replication of this virus is highly restricted in adults and HPIV1 seropositive children. Evaluation of this vaccine in HPIV1 seronegative children is in progress (clinicaltrials.gov NCT00641017). Similarly, rHPIV2-15C/948L/Δ1724, an HPIV2 vaccine candidate, contains a mutation in the extragenic leader region of the genome, and an amino acid substitution and a deletion in the L gene. rHPIV2-15C/948L/Δ1724 is highly attenuated in African green monkeys yet protects against challenge with wt HPIV2.²⁷⁴ Replication of rHPIV2-15C/948L/Δ1724 is highly restricted in adults, and evaluation in HPIV2 seropositive children is in progress (clinicaltrials.gov NCT01139437).

PERSPECTIVE

The HPIVs continue to be an important cause of pediatric respiratory illness. There still are no approved antiviral therapies or vaccines for any of these viruses. The pathogenesis of serious HPIV disease such as croup, bronchiolitis, and pneumonia is still not well understood. Indeed, most of our understanding of HPIV infection, pathogenesis, and the host response is by inference from HRSV. In recent years, we have gained an understanding of the role of HPIV proteins in inhibiting the type I IFN response. Fewer advances have been made in characterizing the adaptive immune response to HPIVs, especially in infants and young children. However, significant progress has been made in the treatment of croup and in the development of HPIV vaccines. The natural histories of most of the animal PIVs remain poorly understood.

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Mumps Virus

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a highly neurotropic agent and a leading cause of virus-induced aseptic meningitis and encephalitis.^{20,42,190}

A number of laboratory investigations suggested that a filterable, transmissible agent was responsible for mumps^{141,281,423}; however, a viral etiology was not proven until 1935 when Johnson and Goodpasture, using bacteria-free parotid secretions, successfully transmitted the disease between monkeys and children and then back to naïve monkeys, fulfilling Koch's postulates.^{186,187}

The demonstration by Habel¹⁴⁵ and Enders¹⁰⁸ in 1945 that MuV could be isolated and propagated in embryonated eggs enabled the demonstration of the hemagglutinating, hemolytic,²⁶⁸ and neuraminidase¹⁸² properties of the virus, leading to the development of an inactivated vaccine in 1946¹⁴⁴ and to the first live virus vaccine in 1958.³⁵³ The introduction of tissue culture as a practical alternative for the propagation and study of the virus in 1948⁴¹⁴ was pivotal for advancing studies of the epidemiology and pathogenesis of the disease as well as the molecular biology of the virus, permitting the development of cell-based vaccines.

Although historically a benign disease of childhood, mumps was viewed as a major concern for the military, particularly in times of mobilization. Mumps was a notable issue during the Civil War of the United States,¹⁶⁶ World War I,¹³⁸ and during World War II,^{253,302} and continues to occur in military settings.^{13,218,316} During World War I, mumps was a leading cause of days lost from active duty in the United States Army in France, exceeded only by losses due to influenza and gonorrhea infections.

Use of live, attenuated mumps vaccines have nearly eliminated the disease from countries with high vaccine coverage rates of a two-dose regimen,^{126,301} although sporadic and sometimes large mumps outbreaks continue to occur even in highly vaccinated populations.⁹⁸

INFECTIOUS AGENT

Classification

MuV is a nonsegmented, negative-strand RNA virus in the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Rubulavirus*. See Chapter 33 for a detailed overview of the *Paramyxoviridae*.

Virion Morphology and Structure

Mumps virions are pleomorphic particles ranging from 100 to 600 nm in size, consisting of a helical ribonucleoprotein (RNP) core surrounded by a host cell-derived lipid envelope (Fig. 35.1). The RNP consists of a single-stranded RNA

HISTORY

In the 5th century BC, Hippocrates described a mild epidemic illness associated with nonsuppurative swelling near the ears and, variably, with painful swelling of one or both testes. These descriptions of parotitis and orchitis, respectively, are the hallmarks of mumps virus (MuV) infection. The name *mumps* may derive from an old English verb that means to grimace, grin, or mumble. Hamilton, a physician of the late 18th century, is credited as being the first to associate central nervous system (CNS) involvement with mumps in his description of the neuropathology of a fatal case. Later studies would reveal MuV as

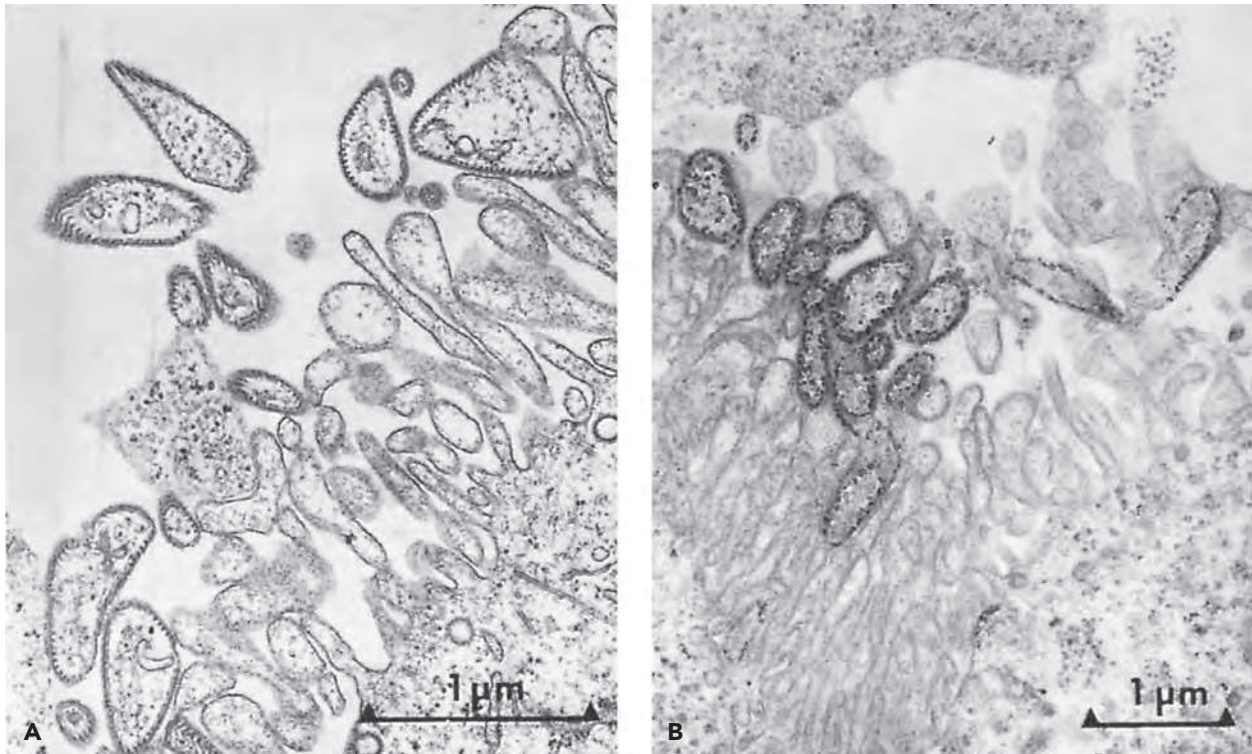


FIGURE 35.1. **A:** Pleomorphic mumps virions budding from a choroid plexus epithelial cell. **B:** A subjacent section processed for post-embedding localization of the major nucleocapsid-associated protein using monoclonal anti-N antibody. The N is localized by punctate, electron-dense reaction products below the surface membranes of the cell.

(ssRNA) molecule coated by the viral nucleoprotein.⁸² The RNP appears to be a hollow tube with a unit length of approximately 1 µm, a diameter of 17 to 20 nm, and a central core of 5 to 6 nm.^{168,169,251} The viral host cell-derived envelope contains the viral glycoproteins that project 12 to 15 nm from the virion surface.

Full-length genomic RNA (gRNA) is an unsegmented, single-stranded macromolecule of negative polarity that consists of 15,384 nucleotides. The presence of multiploid virions in MuV preparations has been reported, but only one of the genomes is believed to be biologically active.²⁵⁰

Genomic Organization

The MuV genome contains 7 tandemly linked transcription units: the nucleo- (N), V/phospho-/I (V/P/I), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) protein genes. The gene order is 3'-N-V/P/I-M-F-SH-HN-L-5'.^{105,107} The MuV genome is flanked at the 3' end by an extracistronic leader sequence of 55 nucleotides³⁷⁰ and at the 5' end by a trailer sequence of 24 nucleotides,²⁸⁸ of which the last 10 share inverse complementarity. These regions are essential for transcription and replication. Unlike most other paramyxoviruses, MuV does not have identical gene-start and gene-end sequences. The consensus gene-start sequence is 3'-U-U/C-C-G/U-G/U-N-C/U-U/C-U and that of the stop sequence is 3'-A-A/U-U/A-U-C/A-U-6-7,¹⁰⁵ separated from each other by intergenic sequences of 1 to 7 nucleotides.

Each gene encodes a single protein, with the exception of the V/P/I gene (conventionally referred to as the

P gene), which gives rise to additional mRNA species as a result of the cotranscriptional insertion of nontemplated G nucleotides between positions 461 to 466.^{107,297} Faithful transcription of the gene produces the V protein (formerly referred to as NS1 protein), whereas insertion of two G residues within the editing site produces an mRNA encoding the P protein and insertion of one or four G residues produces an mRNA encoding the I protein (formerly referred to as NS2 protein and analogous to the W protein reported in related paramyxoviruses). Thus, the V, P, and I proteins have the same amino-terminal segment but different C-terminal regions.

Genome transcription occurs by a stop-start mechanism in which the viral polymerase produces a decreasing gradient of monocistronic mRNA for genes located further from the 3'-end promoter (see Chapter 33). Due to the occasional failure of the viral RNA-dependent RNA polymerase (RdRp) to recognize the intragenic stop signals, bi-, tri-, tetra-, penta- and hexacistronic read-through transcripts can be detected in MuV-infected cells, from which only the first cistron is translated.^{5,89,106} The size of the monocistronic mRNAs as well as the number of amino acids and molecular weights of the MuV proteins are provided in Table 35.1.

Virus Proteins and Replication

N, P and L Proteins

The first translated transcriptional unit of the virus, the N protein, complexes with the gRNA to form the RNP, the template for RNA synthesis. Only encapsidated RNA, not naked

TABLE 35.1 Mumps Genes and Gene Products

Gene	mRNA ^a	Amino acids	MW in kd		Biological activity
			Predicted	Observed ^b	
<i>N</i>	1,845	549	61.4	69–73 ^c	<ul style="list-style-type: none"> • Encapsidates genomic RNA • Protects RNA from nucleases • Confers helical structure of RNP
<i>P</i>	1,314	391	41.6	45–47	<ul style="list-style-type: none"> • Binds to RdRp • Part of the RdRp
<i>V</i>	1,312	224	24.2	22–28	<ul style="list-style-type: none"> • Tethers RdRp to RNP • Antagonizes IFN-β induction • Inhibits IFN-α/β and INF-γ signaling
<i>I</i>	1,313/1,315	170–171	18.3	16–19	Unknown
<i>M</i>	1,253	375	41.6	39–42	Virus assembly and budding
<i>F</i> ₀	1,721	538	58.8	65–74	Fusion protein precursor
<i>F</i> ₁		436	47.4	58–61	Viral attachment and entry
<i>F</i> ₂		83 ^d	9.4	10–16	Viral attachment and entry
<i>SH</i>	310	57	6.71	6	Anti-apoptotic activity
<i>HN</i>	1,887	582	64	74–80	Viral attachment, entry, and release
<i>L</i>	6,925	2261	256.6	160–200	Part of the RdRp

^aWithout polyA tail.^bApproximate molecular weight as observed by gel electrophoresis.^cDifference between calculated and observed weight likely due to phosphorylation.^dWithout the cleaved 10 amino acid signal sequence.

RNA, can be transcribed. RNA synthesis begins with the binding of the RdRp, a complex of the P and L proteins, to the RNP, as inferred from other studies of highly homologous viruses.^{140,147}

The MuV N protein is a bipartite molecule consisting of a globular N-terminal assembly domain (amino acids 1 to 398) mediating RNA binding (to form the RNP), and an unstructured hypervariable C-terminal tail believed, based on studies of related viruses, to interact with the MuV M protein during virion assembly.^{35,88,342}

The MuV P protein forms homotrimers via a centrally located coiled-coil domain⁸⁷ and complexes with the L protein to form the RdRp. The C-terminal 48 amino acids of the P protein is a nucleocapsid-binding domain (NBD), responsible for tethering of the RdRp to the RNP by binding to the assembly domain of the N protein (amino acids 1 to 398).²⁰⁷ The three-dimensional structure of the NBD has been deciphered using X-ray crystallography and revealed formation of a compact bundle of three α -helices, a feature that appears to be conserved among paramyxovirinae.²⁰⁸ Unlike the NBD of measles virus and Sendai virus, that of MuV lacks defined tertiary structure and is fundamentally unstable.²⁰⁷ As indicated by its name, the MuV P protein is heavily phosphorylated,²⁷⁸ an action believed to regulate polymerase activity, but this has not been clearly demonstrated for MuV.^{123,361,362}

Six functional domains have been identified in the MuV L protein based on sequence similarity with L proteins from related viruses.^{308,350} These domains have been ascribed all catalytic functions such as execution of transcription and replication, as well as methylation, capping, and polyadenylation of

mRNAs (see Chapter 33). A detailed functional and structural analysis of the MuV L protein has not yet been carried out.

Matrix Protein

The MuV M protein orchestrates virus assembly and budding. Only the MuV M protein when expressed alone is sufficient for virus-like particle production, although in the absence of co-expression with other viral proteins, efficiency is low.²³⁴ Evidence indicates that the M protein binds to the cytoplasmic tails of the F and HN glycoproteins assembled at distinct locations on cellular membranes, presumably lipid raft microdomains. There, the M protein functions as an adapter, physically linking the region of host-cell membrane expressing the viral F and HN glycoproteins with the viral RNP via its interaction with the N protein.^{152,234} Budding appears to be mediated by an interaction between the MuV M protein and the cellular endosomal sorting complex for transport (ESCRT) machinery.²³⁴ The MuV M protein has also been shown to interact with host proteins angiomin-like 1 (AmotL1) and 14-3-3.^{298,299} Whereas the AmotL1–M protein interaction appears to promote MuV VLP production, based on studies of PIV5, M protein interaction with 14-3-3 decreases the efficiency of virus budding. Interestingly, the MuV M 14-3-3 binding site is adjacent to a sequence motif conserved among rubulaviruses, presumably functioning as a binding site for the host protein caveolin 1 (Cav-1), an essential structural component of caveolae that are considered a subset of lipid rafts. It is therefore likely that MuV budding, like that of PIV5, occurs from caveolae. The close proximity of the 14-3-3 and postulated Cav-1 binding sites raises the possibility that the MuV

M protein switches between either binding to 14-3-3 or Cav-1, thereby regulating the amount of M that can participate in virus budding.²⁹⁹

Surface Glycoproteins

The F and HN are transmembrane glycoproteins of types I and II, respectively. The F glycoprotein is synthesized as an inactive precursor, F₀, that is targeted to the rough endoplasmic reticulum via a 19 amino acid signal peptide, which is subsequently cleaved.⁴⁰⁸ Following N-glycosylation, the precursor is transported to the trans-Golgi network where it is proteolytically cleaved between amino acids 102 and 103 by the host cell protease furin at the R-R-H-K-R motif to produce two disulfide-linked heterodimers F₁ and F₂.^{162,257,278,409} Cleavage of F₀ is essential for virus-to-cell and cell-to-cell membrane fusion and for virus infectivity. The amino terminus of the F₁ subunit possesses the fusion peptide, a conserved hydrophobic domain exposed by the cleavage event (see Chapter 33). Evidence indicates that a second cleavage event occurs during which F₁ is processed into two subunits, F_{1a} and F_{1b}; this event is important in mediating fusion activity.⁴⁰⁰

At least two heptad repeat (HR) domains are found in the F1 ectodomain: HR1 at the amino terminus adjacent to the fusion peptide and HR2 at the carboxyl terminus adjacent to the transmembrane domain.²³⁹ The MuV F protein forms homotrimers and the HR1 and HR2 domains interact to form a stable six-helix bundle structure.^{239,249} The HR2 domain is also involved in the binding of F with HN. In related viruses, additional HR domains have been identified, although this has not been confirmed for MuV.^{131,270} The specific processes involved in MuV fusion have not been delineated; however, based on similarity with the six-helix bundle structural of the PIV5 F protein, the events that mediate MuV fusion are likely similar to those for PIV5 (see Chapter 34).

In its native state, the HN protein is a disulfide-bonded oligomer assembled as homotetramers. The protein is held in the lipid bilayer by a hydrophobic domain of 19 residues near the amino terminal, a domain that probably also serves as a signal sequence in a manner similar to other paramyxovirus HN proteins. HN monomers display a membrane proximal stalk that supports a globular head, forming a six-blade propeller structure.^{84,219} The globular head is responsible for attachment and neuraminidase activity^{182,291}; the stalk region, in conjunction with the F protein, mediates virus-to-cell and cell-to-cell fusion.^{371,382} Based on inference from related viruses, a single site is believed to mediate both neuraminidase and receptor binding activity using a protein conformational switch mechanism to toggle activity.^{77,84,226} There may also be a second sialic acid binding site within the globular head region, theorized to stabilize viral–cellular membrane interactions in addition to the large binding pocket that fluxes between neuraminidase and receptor binding activity.^{37,314,437} Two competing models on the HN-assisted mechanism of F protein activation have emerged⁷⁸ (see Chapter 34 for details). Briefly, the “clamp model” proposes that the HN protein associates with F intracellularly, stabilizing the prefusion conformation of the protein. Upon binding of HN protein to its receptor, the F protein is released, allowing for the conformational change required for fusion activity. In the “provocateur model,” a change in the structure of the HN itself is postulated to promote fusion activity. In this model, the HN

protein either is preassociated with the F protein or associates with the protein following receptor binding, at which point the HN protein undergoes a conformational change leading to destabilization of the F protein, which confers fusion activity. Results from recent studies using PIV5 HN and F proteins support the “provocateur model.” Whether this model applies to MuV awaits confirmation.

V Protein

The MuV V protein, as reported for other rubulaviruses, is involved in inhibiting IFN production and signaling (see Chapter 33). The 69 aa C-terminal cysteine-rich domain of the MuV V protein appears to be the key player in these activities. This region directly interacts with MDA5 (melanoma differentiation-associated gene 5), a pattern recognition receptor that recognizes cytosolic viral RNA, and with the TBK-1 (TANK-binding kinase 1)/IKKε (inhibitor of κB kinase-ε) kinases responsible for interferon regulatory factor-3 (IRF-3) phosphorylation. MuV V protein interaction with MDA5 inhibits its ability to induce transactivation of the IFN-β promoter,^{12,320} and in the case of TBK-1/IKKε, leads to their ubiquitination and subsequent proteasomal degradation,²⁴³ preventing IRF-3 phosphorylation, an event required for transcription of IFN and IFN-stimulated genes. The C-terminus of the MuV V protein also interacts with the cellular signal transducer and activator of transcription (STAT) proteins, STAT-1, STAT-2, and STAT-3.^{151,282,434,435} STAT-1 and STAT-2 play a central role in the IFN signal transduction pathway that eventually leads to activation of IFN-induced genes. STAT 3 has also been implicated in cellular antiviral responses.^{318,389} Binding of MuV V leads to the ubiquitination and subsequent degradation through the proteasomal pathway of STAT-1 and STAT-3, but not of STAT-2; however, the latter is required for targeting of STAT-1 for degradation.^{216,388,389,435} The C-terminus of the MuV V protein is not the only region important in these interactions, as exemplified in a study demonstrating that a single point mutation at amino acid position 95 abrogates the ability of the MuV V protein to degrade STAT3, while retaining the virus's ability to target STAT 1.³¹⁸

The observation that the MuV V protein can oligomerize and form spherical particles suggests that the MuV protein provides a scaffold for coordinating the assembly of the cellular components (e.g., UV-damaged DNA binding protein 1 [DDB1], cullin 4A [Cul4A], and regulator of cullin 1 [Roc1]) involved in ubiquitination, collectively referred to as V-dependent degradation complexes (VDC).³⁸⁸ For more details on VDC, see Chapter 33.

SH Protein

The SH protein consists of 57 amino acids, 25 of which are highly hydrophobic and clustered at the amino terminus serving as a membrane anchor region with its C-terminus facing the cytoplasm.^{104,106,367} The predicted SH mRNA exhibits two AUG start codons, the second of which represents the actual AUG for the SH open reading frame. The first AUG, located at positions 4 to 6, gives rise to a minicistron with a stop codon at nucleotides 19 to 21.^{106,367} Due to the immediate proximity of the first cistron to the cap of the mRNA, it is predicted that with a frequency of about 50%, the ribosomes will skip the first AUG and initiate at the second cistron,²¹² thus enabling translation of the SH protein, although at reduced efficiency. The

biological significance of this minicistron and its proposed role in reducing the amount of SH protein synthesized is unknown.

In certain MuV strains, such as the Enders and Rubini strains, a point mutation exists in the putative F gene polyadenylation signal resulting in an F-SH bicistronic mRNA,^{220,367,368} from which only the F protein is made. This demonstrates that the SH protein is not essential for virus replication, which has been confirmed for PIV5.¹⁵⁶

Studies of PIV5 demonstrated that the SH protein facilitates evasion of the host antiviral response via blocking the TNF- α -mediated apoptosis pathway.^{156,157} The MuV SH protein appears to be functionally similar, based on recombinant DNA studies in which PIV5 SH gene was then replaced with that of MuV.⁴¹⁸ Yeast two-hybrid and co-immunoprecipitation studies identified ataxin-1 ubiquitin-like interacting protein (A1Up) as a cellular target of the MuV SH protein. This protein plays a role in proteasomal degradation, but the biological significance of its interaction with the MuV SH protein is not clear.⁴²⁷

I Protein

Expression of the I protein in infected cells was confirmed, but its role in the life cycle of the virus is unknown.²⁹⁷

Virus Infection of Host Cells

Sialic acid, an acyl derivative of neuraminic acid, is found on cellular glycoproteins and lipids and serves as the receptor for MuV. Following attachment of the virus to its receptor, the viral and cellular membranes fuse, permitting entry of the viral RNP followed by its transcription and replication. These events occur in a manner common to most paramyxoviruses (see Chapter 33 for a detailed description of these and other events such as viral assembly and release).

Because the MuV receptor is ubiquitously expressed on mammalian cells, the virus infects most cell types. The presence of MuV is typically detected in cell cultures by the induction of syncytia, large homogeneous masses of cytoplasm enclosing numerous nuclei (Fig. 35.2), followed by lysis of infected monolayers; however, the cytopathic effects of MuV can vary considerably among isolates and substrates, and in some instances, there is little evident morphologic change.^{4,142,250} The type of cytopathic effect produced *in vitro* does not correlate with the *in vivo* behavior of the virus.

PATHOLOGY AND PATHOGENESIS

Infection in Experimental Animals

Humans are the only natural host of MuV, although experimental infection has been induced in laboratory animals, including monkeys,^{230,327,334} hamsters,^{192,295,419} mice,^{205,295} and rats.^{315,331} Experimental infection of animals has mostly been used to study the pathogenesis of MuV neurotropism and neurovirulence. Intracerebral inoculation of MuV into the suckling hamster results in a massive inflammatory response, including meningitis, encephalitis, and ventriculitis. Intraperitoneal inoculation of virulent strains into suckling hamsters also leads to CNS infection.⁴²⁰ Associated neuropathology includes hydrocephalus, Chiari type I cerebellar malformation, and neuronal necrosis.^{204,295,366,369,421} Many of these are reported features of CNS infection in humans, suggesting the applicability of the hamster model for studying the pathogenesis of MuV infection in man. Similar findings have also been reported in monkeys.^{70,241,326,334,436} Very little, if any, work has been accomplished in mice because MuV infection in this species tends to be abortive, thereby limiting the value of this model system.^{155,317,383} Early studies also indicated mumps infection to be abortive in rats, unless adapted by serial passage in brain.³¹⁵ Subsequent studies, however, determined that intracerebral inoculation of the virus into newborn rats resulted in inflammation of the ventricular system (choroiditis, ependymitis) and hydrocephalus, but not meningitis or encephalitis.³³¹ Interestingly, the severity of hydrocephalus in rats was found to correlate well with the virus strain-specific neurovirulence potential for humans, suggesting the relevance of such a model of disease in examining the pathogenesis and molecular basis of MuV neurovirulence.³³² The severity of virus-induced neuropathology in marmosets, but not other monkey species, was also found to correlate with virus neurovirulence potential for humans.³²⁸

Infection in Humans

Transmissibility after nasal or buccal mucosal inoculation of virus¹⁸⁷ suggests that natural infection is initiated by droplet spread. The incubation period is 16 to 18 days,^{167,259} during which the virus multiplies in the upper respiratory mucosa before spreading to draining lymph nodes. Based on studies involving experimental infection of hamsters, virus disseminates via a transient plasma viremia,⁴²⁰ potentially infecting multiple tissues and

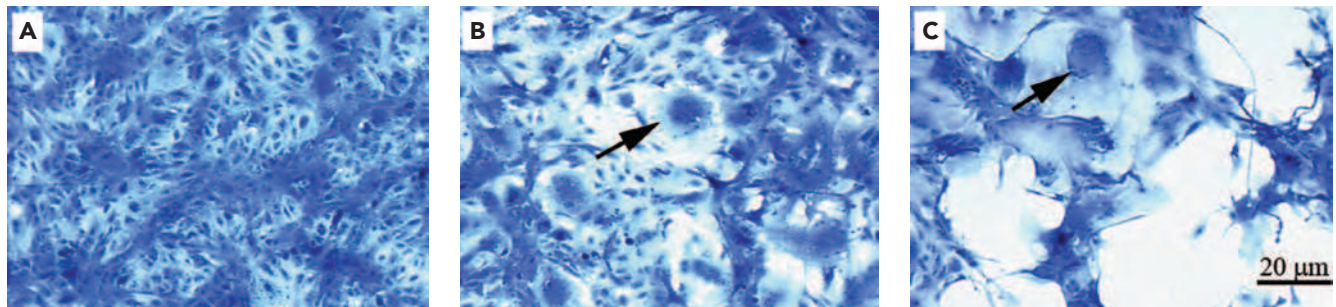


FIGURE 35.2. Phase contrast image showing the progression of typical cytopathic effects of a mumps virus clinical isolate after incubation on Vero cells for 1 (A), 4 (B), and 7 (C) days. The classic cytopathic effect of cell-to-cell fusion and syncytia formation (arrows) appear within a few days of culture, followed by cell lysis. By day 7 nearly the entire cell monolayer is consumed by syncytia and lysis. Acetone fixation, cresyl violet stain.

organ systems.¹⁷² The most common sites of virus dissemination are glandular tissues (parotid glands, testes, breasts, and pancreas), and the CNS. If viruria is used as an indication of kidney infection, then kidney involvement is common in mumps, although clinical nephritis is rarely diagnosed.^{392,394} In rare cases, MuV can be transmitted transplacentally.

Virus is shed in saliva as early as 6 days before the onset of parotitis.¹⁵⁹ Termination of viral shedding correlates with the local appearance of virus-specific secretory IgA and IgM, as early as a few days after disease onset.^{67,304} Fewer than 15% of patients continue to shed virus beyond day 4 of symptom onset.³¹⁰ Thus, patients with mumps are capable of spreading virus by the respiratory route over a 10-day interval.

Plasma viremia disappears coincident with the development of MuV-specific antibody, which can be detected in serum as early as 11 days after infection of humans.¹⁵⁹ Animal models suggest that circulating infected lymphocytes provide a means for the spread of virus in the face of mounting humoral immunity.⁴²⁰ Despite the apparent high frequency of viremia during mumps, MuV has only rarely been detected in blood.^{183,201,294}

Parotid Gland

Initial clinical symptoms usually relate to infection of the parotid gland, but viral involvement of this gland is neither a primary nor obligate step in the infection.²⁰² Virus infects the ductal epithelium, resulting in desquamation of involved cells, periductal interstitial edema, and a local inflammatory reaction primarily involving lymphocytes. Swelling, inflammation, and tissue damage in the parotid gland can produce elevation of serum and urine amylase levels.³⁴⁶

Central Nervous System

MuV CNS invasion, as demonstrated by cerebrospinal fluid (CSF) pleocytosis, occurs in greater than one third of patients presenting with clinical mumps.^{20,42,46,112} Symptomatic CNS infection (i.e., meningitis) is less common, occurring in approximately 10% of cases.^{20,149,198} Encephalitis occurs in less than 0.5% of cases. In the prevaccine era, MuV was a leading cause of viral meningitis and encephalitis in most developed countries and continues to be a leading cause in unvaccinated populations worldwide.^{18,125,160,258} Neurologic manifestations appear with a 3:1 or greater male–female ratio^{30,210,228} and are generally preceded by parotitis by 4 to 5 days but can occur before or in the total absence of detectable salivary gland swelling.

As inferred from animal studies, viral invasion of the CNS occurs across the choroid plexus.⁴²⁰ Blood-borne infected mononuclear cells, and possibly cell-free virus, can cross the fenestrated endothelium of the choroid plexus stroma and serve as a source for subsequent infection of the choroidal epithelium. Maturation of virus from the ventricular surfaces of choroidal cells provides progeny virions that are widely distributed through ventricular pathways and the subarachnoid space by CSF (Fig. 35.3). Virus can penetrate brain parenchyma and infect neurons by contiguous spread from infected ependymal cells that line the ventricular cavities of the brain. Once within neurons, virus most likely spreads along neuronal pathways, as reported in nonhuman primates.²³⁰ Although primary encephalitis is typically the response to direct viral invasion of neural cells, cases of postinfectious encephalitis, an autoimmune attack on CNS myelin sheaths, also occurs. Symptoms of primary encephalitis appear before or during the develop-

ment of parotitis; symptoms of postinfectious encephalitis and associated demyelination appear 1 to 3 weeks after the onset of parotitis.^{238,372}

Typical CNS pathology of MuV encephalitis includes edema and congestion throughout the brain with hemorrhage, lymphocytic perivascular infiltration, perivascular gliosis, and demyelination. Findings in the spinal cord include early degenerative changes in the anterior horn cells and perineuronal edema.^{102,264,372} When seen, the selective periventricular myelin loss with relative sparing of axons is typical of parainfectious autoimmune encephalitis.

Rarely is CNS infection fatal and most cases resolve without sequelae. In some instances, however, electroencephalographic changes, ataxia, and behavioral disturbances may take months to resolve^{173,210} and permanent neurologic damage—such as obstructive hydrocephalus,²⁸⁶ deafness,²³⁶ and myelitis^{284,395}—can occur.

Hydrocephalus can develop days to years after initial MuV infection and can lead to progressively worsening headaches, mental status changes, and gait abnormalities.^{71,137,286} The pathogenesis of hydrocephalus is inferred from animal studies that have suggested that desquamation of virus-infected ventricular ependymal cells blocks egress of CSF through the aqueduct of Sylvius. Abnormally restricted flow of CSF to adsorptive sites over the cerebral convexities results in progressive enlargement of the lateral and third ventricles.^{191,192} The presence of ependymal cell debris in the CSF of humans presenting with MuV CNS infection suggests a similar mechanism of hydrocephalus induction in humans.^{161,286,380} Hydrocephalus, however, has also been observed before, or in the total absence of, aqueductal stenosis.^{189,364,365,419} This indicates that stenosis of the aqueduct could be a secondary consequence of external compression by surrounding edematous tissue and not causally related to the pathogenesis of hydrocephalus.

MuV is the most frequent cause of acquired sensorineural hearing loss in children. Transient, high-frequency deafness is the most common form, occurring in approximately 4% of mumps cases.⁴⁰² Permanent deafness occurs in less than 1 per 20,000 cases^{27,153} and is usually unilateral. Deafness is believed to be the result of direct viral invasion of the cochlea, likely via the perilymph, which freely communicates with the CSF^{146,254,352,415}; however, evidence indicates a hematogenous route exists.²⁶⁵ Pathology includes degeneration of the stria vascularis, tectorial membrane, and organ of Corti and collapse of Reissner's membrane.¹¹¹ Hearing loss caused by indirect effects of virus infection (e.g., immune-mediated damage) have also been suggested.³⁹⁰ Deafness is not disproportionately seen with other complications, suggesting no specific pathogenic link with parotitis, meningitis, or other complications. Deafness has also been observed in otherwise asymptomatic patients.^{287,390}

Gonads

Orchitis, usually unilateral, occurs in approximately 20% of postpubertal men who develop mumps.^{16,124,224} Orchitis rarely occurs in children, suggesting that certain hormonal factors, such as receptors for luteinizing hormone and follicle-stimulating hormone expressed during adolescence, might promote testicular tropism of the virus.³⁷⁶ Virus has been isolated from testicular biopsies of the affected gland within the first 4 days of symptoms, and from semen,^{29,179} strongly suggesting that symptomatic gonadal involvement reflects local virus replication. The

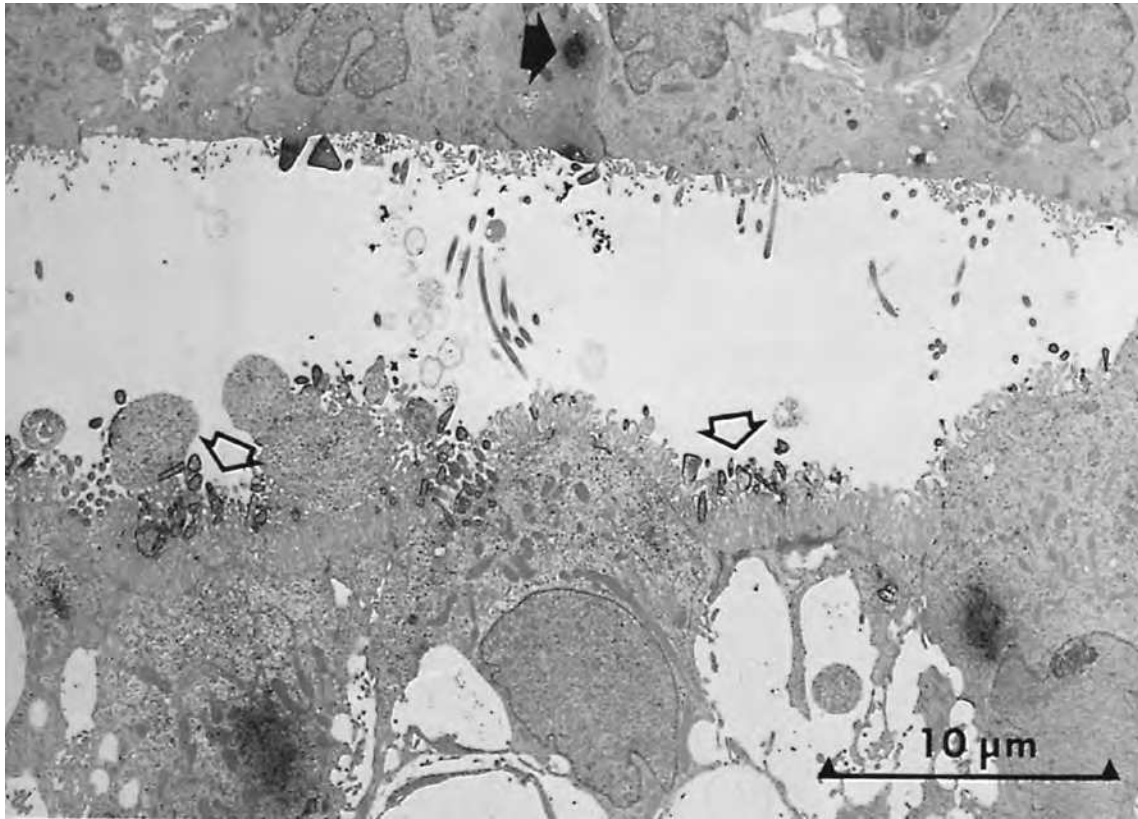


FIGURE 35.3. Post-embedding electron-microscopic immunocytochemical staining of a region of the lateral ventricle of a hamster 5 days after intraperitoneal infection with the Kilham strain of MuV. Anti-N monoclonal antibody was used as the primary reagent. The reaction product defines nucleocapsids located below the cytoplasmic membranes at sites of virion budding along both the ependymal and choroidal (unfilled arrows) surfaces. Intracytoplasmic nucleocapsid inclusions are also demarcated by the reaction product in both choroid plexus and individual ependymal (filled arrow) cells.

seminiferous tubules may be the primary site of viral replication, with local lymphocytic infiltration and edema of interstitial tissues.

Ovary infection is diagnosed in less than 5% of postpubertal women who develop mumps,^{38,64,97,199,248,360} which may be an underestimate because unless a pelvic examination is performed, abdominal or pelvic pain from inflamed ovaries might be attributed to pancreatic infection.

Kidneys

Based on the frequency of viruria, virus frequently disseminates to the kidneys, where epithelial cells of the distal tubules, calyces, and ureters appear to be primary sites of virus replication.⁴¹³ Viruria can be detected in most patients, sometimes for as long as 14 days after the onset of clinical symptoms.^{392,393} Mild abnormalities of renal function have been described, but they are usually of little clinical importance.³⁹² Although virus dissemination to, and replication in, the parotid gland and kidney can occur simultaneously, replication in renal tissue is more prolonged and continues well beyond the appearance of neutralizing antibody in serum.

Pancreas

Pancreatic involvement, diagnosed in 1% to 27% of cases,^{38,47,64,232,296,345,356,376,416} is usually expressed as mild epigastric pain,

but severe hemorrhagic pancreatitis¹¹⁴ and transient exocrine function abnormalities⁹² have been reported. MuV infects human pancreatic beta cells *in vitro*,⁴⁰³ and virus infection of the pancreas has been demonstrated in hamsters inoculated intraperitoneally.⁴²⁰ Viral infections have been considered a possible precipitating event leading to the onset of about one third of all cases of juvenile-onset or type I insulin-dependent diabetes mellitus (IDDM); however, whether MuV causes IDDM is unclear.^{83,127,136,344} No association has been found between mumps and type II diabetes.²⁴⁰

Heart and Joint Tissues

Myocardial invasion occurs frequently in mumps, as indicated by electrocardiographic abnormalities.¹⁴ Although it is seldom symptomatic, interstitial lymphocytic myocarditis and mild pericarditis may occur following mumps replication in cells of the myocardium and pericardium.⁴³ MuV myocarditis can lead to the rare but serious sequelae of endocardial fibroelastosis.²⁷⁹

Mild to moderately severe mono- or polyarticular and, often, migratory arthritis has rarely been associated with mumps.¹³⁹ MuV has not been isolated from joint fluids or synovial tissues, and no evidence exists for significant immune complex deposition.

Fetus and Newborn

MuV can be transmitted transplacentally as demonstrated in nonhuman primates³⁵⁷ and by the isolation of the virus from the human fetus following spontaneous first-trimester abortion during maternal mumps.^{221,433} The virus can produce a fetal wastage in humans, with or without subsequent spread of virus to involve fetal tissues directly.⁴³³ MuV has also been isolated from fetal tissues following planned therapeutic abortion of seronegative women 1 week after vaccination with live, attenuated MuV, although it is unclear if the virus detected was vaccine virus, or wild-type virus coincidentally contracted at or shortly before the time of vaccination.⁴³⁰ A proliferative necrotizing villitis with decidual cells containing intracytoplasmic inclusions has been described in the products of spontaneous and induced abortions.¹²⁹ Late-gestation intrauterine infection was reported in an infant born to a mother who developed mumps more than 4 weeks before delivery, diagnosed by reverse transcription polymerase chain reaction (RT-PCR) testing of the infant's cord blood cells.³⁶³ This infant developed severe pulmonary symptoms, including hypertension and hemorrhage.

MuV is excreted in breast milk,²⁰³ but few cases of perinatal mumps have been described^{195,222} and it is not clear if breast milk was responsible for these cases. There appears to be a somewhat different mode of pathogenesis of mumps in newborns. In the first year or two of life, infants may have only pulmonary involvement without evidence of parotitis.^{195,363} Split immunologic recognition in the infants can follow maternal parotitis, resulting in MuV-specific cell-mediated immune responses without a concomitant antibody response.^{1,357,378}

Molecular Basis of Virulence

While comprehensive studies on the molecular basis of MuV virulence have yet to be performed, it is clear that the genetic basis of MuV neurotropism and neurovirulence does not lie within any one gene^{7,329,339,348,428} and no simple pattern of genomic mutations capable of discriminating virulent from attenuated MuV strains has been identified.^{8,177,339}

CLINICAL FEATURES

The clinical features of mumps reflect the pathogenesis of the infection, as reviewed here. Approximately one third of all MuV infections occur without recognized symptoms.^{44,79,112,305,321} Moderate fever is often present at the onset of disease, with defervescence a few days later. The feature most characteristic of mumps is salivary gland swelling, particularly the parotid glands (Fig. 35.4),³⁰⁵ which constitutes the basis of a clinical diagnosis. Submaxillary gland enlargement and involvement of the sublingual glands also occur. Enlargement of individual glands is painful, usually lasting 2 to 3 days, but may persist 10 days or longer.¹⁵⁸ Virus is present in the saliva for several days before the onset of clinical disease¹⁵⁹ and for up to 5 days later.⁶⁷ The virus can be detected in urine for several weeks after the onset of mumps.³⁹³

Various organs and tissues can be symptomatically involved during mumps, including the testes, CNS, mammary glands, ovary, pancreas, kidneys, and heart.¹⁷² Parotitis usually precedes manifestations of involvement of other sites of virus infection, but the latter can be clinically evident before, during, or even in the absence of parotitis.

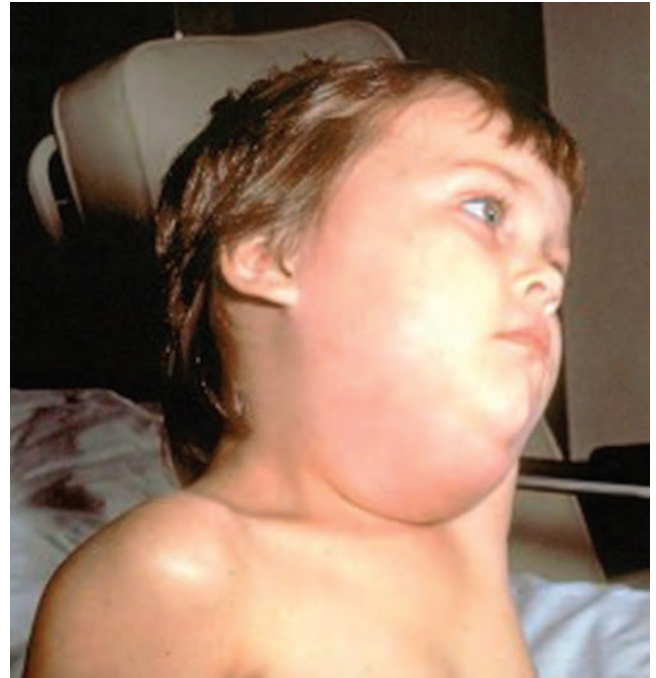


FIGURE 35.4. Child with parotitis. (Courtesy of Centers for Disease Control and Prevention, Atlanta, GA.)

The most common complication of mumps (aside from parotitis) is epididymo-orchitis. Testicular pain and swelling has a time course similar to that of the parotid gland. Mumps orchitis often leads to testicular atrophy of the involved and, occasionally, the clinically uninvolved gland.²⁵ Atrophy after mumps orchitis is rarely implicated as a cause of male sterility.³⁴⁹ About 15% of females complain of breast swelling and tenderness. As with orchitis in men, the incidence of mastitis is significantly higher after puberty.³⁰⁵

Nausea and vomiting with or without epigastric or left upper-quadrant pain are frequent features of mumps, occurring in approximately half of all cases during the St. Lawrence Island epidemic.³⁰⁵ It is unclear whether these symptoms reflect involvement of the pancreas or other viscera. Some of these generalized features of the illness may reflect the effects of circulating IFN, which can be found in serum early in the clinical course of mumps.^{269,404}

More than one third of mumps cases develop CSF pleocytosis, but symptomatic CNS involvement is far less frequent.^{20,42,305} Mumps meningitis may precede parotitis, but it typically develops 5 days following parotitis. However, as many as half of those with mumps meningitis may not have detectable salivary gland enlargement.^{193,202}

IMMUNE RESPONSES

Humoral Immunity

Virus-specific salivary IgM and IgA and serum IgA, IgM, IgG, and neutralizing antibody are all detectable at the time of symptom onset. During natural infection, salivary IgM appears to be the first detectable immunoglobulin class, followed by salivary

IgA. The latter is capable of virus neutralization and is detectable for approximately 10 weeks after symptom onset.^{68,119,120} Levels of serum IgA and IgM peak approximately 1 to 2 weeks after symptom onset and decline to undetectable levels by week 8 after symptom onset.^{41,387} MuV-specific IgG has been detected as early as 4 days before symptom development,²⁸⁰ peaking 4 to 8 weeks later and detectable for decades.^{41,387} In naïve individuals, IgG is of low avidity²⁷⁷ and IgG3 antibodies predominate early in the course of disease, whereas in later stages isotypes IgG1 and IgG2 predominate. Upon a second exposure to MuV, serum IgM and IgA antibodies are typically absent or are produced at very low levels; the IgG1 subclass, typically of high avidity, predominates.¹⁴³

Virus-neutralizing antibodies, which are a correlate of protection, are detectable as early as day 2 after symptom onset, peaking 4 to 8 weeks later, and are detectable for decades. Studies conducted during the prevaccine era indicate that neutralizing antibody titers in the range of 1:4 to 1:8 confer protection,^{23,44,109,260} however, it is not clear if such low levels of vaccine-induced neutralizing antibody are protective. Studies of the effectiveness of the Jeryl Lynn mumps vaccine strain conducted shortly after licensure in the United States indicate that neutralizing antibody titers as low as 1:2 are protective.^{410,411} However, in a more recent study conducted among Jeryl Lynn vaccinees involved in a mumps outbreak, many confirmed mumps cases had neutralizing antibody titers in excess of 1:8 in preoutbreak blood samples.⁸⁰

Only antibodies directed against the F and HN protein have been definitively shown to neutralize the virus and protect against infection.^{170,235,242,354} Use of polyclonal antisera in virus neutralization assays demonstrates a broad cross-reactivity among different MuV genotypes.^{176,291,293,333,431} However, neutralizing antibody titers can differ substantially against different MuV strains, suggesting the existence of virus strain-specific neutralizing epitopes. Despite such demonstrations in the laboratory, MuV appears serologically monotypic in clinical situations as suggested empirically by the dramatic reductions in mumps incidence in countries with high two-dose vaccine coverage.^{126,300,351,407}

The role of antibody in arresting the infection cannot be underestimated. Seropositive patients challenged intravenously with up to 10^9 plaque-forming units of MuV showed only transient fever and no symptoms of reinfection developed, although an anamnestic rise in antibody titer was seen 4 to 5 days later.²⁸⁹ Animals are protected from otherwise lethal viral challenge by neutralizing monoclonal antibody administered several days after primary virus inoculation.⁴²² The development of hemagglutination-inhibition and virus-neutralizing antibody responses in the hamster model correlates well with the fall in peak virus titers in infected organs. In hamsters, however, viral antigens continue to be expressed, and clinical CNS disease actually occurs, after virus titers have fallen considerably.⁴²⁰

Cell-Mediated Immunity

In vitro lymphocyte-proliferative responses to MuV antigens are readily measured in seropositive individuals.¹⁷⁴ CD8+ and γ/δ cytotoxic T lymphocytes have been demonstrated in blood and CSF following infection with wild-type or vaccine MuV strains.^{26,69,116,215} Mononuclear cell inflammation in MuV-infected tissues is well developed at the time of onset of clinical disease, suggesting that specific cellular responses develop

during the incubation period. The presence of major histocompatibility (MHC) antigen (e.g., MHC Ia)-restricted T lymphocytes and specifically sensitized cells in both the blood and the CSF of some patients with active mumps meningitis support this view.¹²¹ After immunization, peak cell-mediated immune responses are found 2 to 4 weeks later and can last for decades.^{69,150,194} Circulating cytotoxic T-cell responses restricted to autologous infected target cells are found within the first few weeks of mumps meningitis.

Although the presence or absence of MuV-specific cell-mediated responses often correlates with the presence or absence of virus-specific antibody, the magnitude of the two types of immune responses do not correlate^{66,324}; in some instances, cell-mediated immune responses have been detected in seronegative persons.^{150,194} Immunologic studies of related viruses (e.g., measles) indicate the importance of the cellular response in the recovery and long-term protection from disease,^{24,128,180,181,303,405} but this has not been established for MuV. For example, levels of interferon- γ induced by MuV infection do not correlate with illness severity^{223,404} and patients with severely compromised T-cell responses experience a course of disease that does not differ from that seen in healthy individuals.⁹⁹ Similarly, neither severe symptoms of mumps nor a protracted course of illness has been reported in persons with AIDS.

Considering data demonstrating that MuV targets mda-5 and STAT proteins for degradation (discussed earlier), MuV may impede cytotoxic T lymphocyte (CTL) activation, by suppressing IFN production and signaling, which regulate MHC class-I expression.⁴⁹

EPIDEMIOLOGY

Age

Before widespread vaccine use, mumps was most commonly seen in children between 5 and 9 years of age⁷⁶; by 15 years of age, greater than 90% of most populations had serologic evidence of infection with MuV.^{52,271} Despite a high seroprevalence in older individuals in the prevaccine era, large outbreaks were common, particularly in high-density, close-contact environments, such as military settings.^{138,302}

In the years following mumps vaccine licensure in 1967, a gradual shift was seen in the typical age of infection in the general U.S. population from young children towards those 10 to 19 years of age, likely reflecting protection of younger children by vaccination, while older children and young adults not eligible for vaccination at the time remained susceptible.^{54,396} By 1992, the highest age-specific incidence in the United States shifted back to the 5- to 9-year-old age group, where it remained until a large multi-state mumps outbreak occurred in 2006, predominantly on university campuses.^{255,256} In the years following the 2006 mumps outbreak, the age-specific incidence returned to the younger age group,^{58,59} until the recent mumps outbreaks in New York and New Jersey in 2009–2010, where again most cases occurred in young adults.⁶⁴ A similar pattern has been observed in many other countries and the occurrence of mumps predominantly in young adults is now common.

Mumps is rarely seen in children younger than 1 year of age, most likely because of acquisition of immunity by placental transfer of maternal antibody. In premature infants born to seropositive mothers, little maternal antibody can be detected

at birth; by 3 months of age, no antibody can be detected in these babies.¹³³ Thus, premature infants may be particularly susceptible to MuV infection.

Morbidity and Mortality

The major morbidity from mumps results from meningitis, encephalitis, and orchitis. These occur as age- and sex-specific hazards, with peak risk in postpubertal boys.¹⁰ With the case fatality ratio for mumps between 1.6 and 3.8 per 10,000,⁵¹ and with 0.5% to 2.3% of all mumps encephalitis cases being fatal,²⁶⁶ it is apparent that most fatal mumps cases have CNS infection.

Origin and Spread of Epidemics

Humans are the only natural host for MuV infection; there is no known animal reservoir. MuV, as with measles virus, requires a population of about 200,000 people to sustain its continued transmission. Such population densities were first achieved some four to five millennia ago,³¹ so it follows that MuV first evolved about 5,000 years ago. Presently, mumps is a geographically unrestricted disease, except for its absence among a few remote tribes or isolated small-island populations. In modern-day urban populations and in the absence of immunization, mumps is endemic, with peak incidence rates in the winter and spring months.²⁶⁶ In the prevaccine era, preschool-aged children were an important source of virus introduced into families, given that inapparent infections are common in children of this age.^{79,305}

In the present era of high vaccine coverage of children, most cases now occur among young adults, suggesting waning immunity. Indeed, levels of MuV-specific antibody decline significantly with time after vaccination,^{36,40,94,96,227,263} and time since last vaccination has been identified as a factor in decreased vaccine effectiveness^{75,122,345} and in increased odds of contracting disease.^{48,81,397}

Prevalence and Seroepidemiology

Data on the incidence of mumps are complicated by the fact that approximately 30% of all MuV infections are subclinical.^{44,79,112,233,246,305,321} Furthermore, only 25% of all cases are seen by a clinician and fewer than 30% are reported to public health agencies.^{112,233} Annual incidence rates vary, with an interepidemic period of approximately 3 years.^{10,22,283}

The highest incidence of mumps reported in the United States since 1922 was almost 250 per 100,000 population in 1941. In 1968, when mumps vaccine was introduced, the yearly incidence was 76 per 100,000 population, steadily declining to less than 1 case per 100,000 population by 1993 and then to an all-time low of less than 0.1 case per 100,000 population by 2003 (Fig. 35.5).^{51,56} Mumps incidence remained low until 2006 when the United States experienced its largest mumps outbreak in 19 years, with 6,584 cases reported, representing a 20-fold increase in disease incidence from the prior year. Mumps incidence rapidly returned to baseline, before another large outbreak occurred in 2009,⁶³ suggestive of a possible return to the 3-year interepidemic cycle.

In the prevaccine era, or in countries not incorporating mumps vaccine in national immunization programs, most persons become seropositive by adolescence. In the United States, where vaccination is required for school entry, by 1996, approximately 90% of 19- to 35-month-old children had received at least one dose of mumps-containing vaccine^{50,57,62} and among adolescents aged 13 to 17 years, by 2006 coverage with two or more doses was 89%.⁶¹ These figures are close to the herd immunity threshold, estimated to be 90% to 92% based on theoretical studies,^{11,306} but are lower than the 95% figure based on evidence from the experience in Finland where indigenous mumps has been eliminated.⁹⁵

MuV was the most common cause of viral encephalitis in the United States until 1975, following attainment of high coverage rates of vaccination.⁵⁵ In unvaccinated populations,

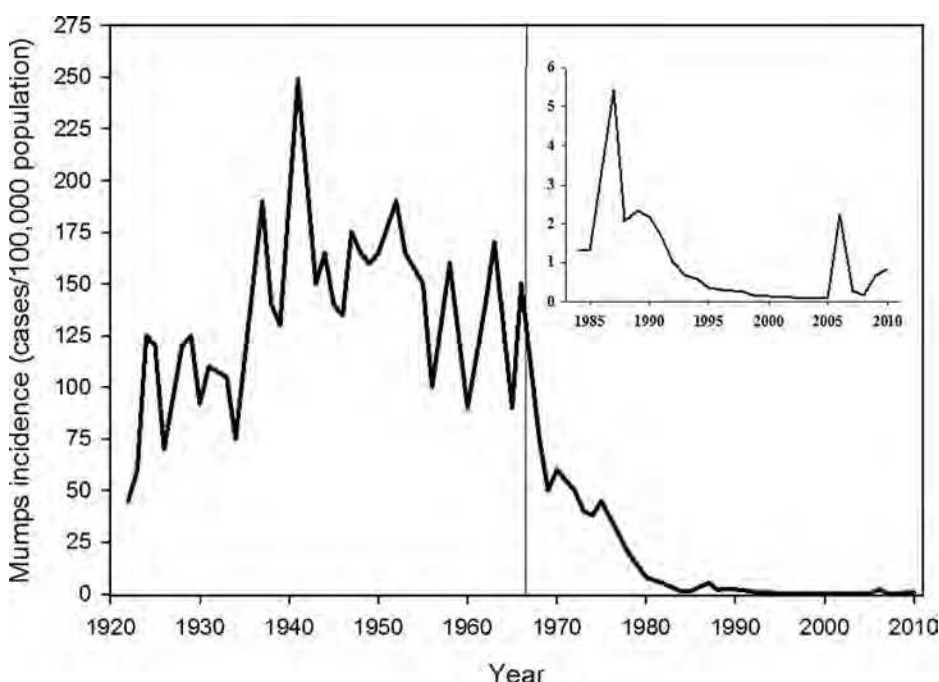


FIGURE 35.5. Mumps cases per 100,000 population in the United States from 1922 to 2010. Vertical line indicates year of vaccine introduction (1967). **Inset:** Larger scale showing disease incidence between 1985 and 2010, with large outbreaks in 1987–1989, 2006, and 2009–2010.

mumps accounts for up to half of all encephalitis cases.³¹¹ In an epidemic setting, 11% of clinical mumps cases have CNS disease, 90% with meningitis and 10% with encephalitis.³⁰⁵ Severe mumps meningitis or encephalitis is most common between the ages of 5 and 9 years, with a clear male predominance in all age groups.³⁰

New Epidemiologic Approaches

The most significant molecular epidemiology advance has been RT-PCR to identify and group individual virus strains by related genomic sequences. RT-PCR analysis has become a key element in mumps surveillance and is a definitive means of identifying transmission pathways. MuV genotyping is conventionally performed based on the nucleotide sequence of the *SH* gene, the most variable sequence in the genome. Phylogenetic analyses of these sequences place the virus strains into distinct clusters that form the basis for genotype assignment.⁴³² Virus strains belonging to the same SH genotype vary at the nucleotide level within the SH gene by up to 4%, whereas intergenotype variation is typically on the order of 8% to 19%.^{184,185} It has been suggested that establishment of new genotypes be based on variation from existing genotypes by greater than 5% at the nucleotide level, among other criteria.^{184,185} Currently, 12 MuV genotypes exist, designated A through L (Fig. 35.6). A provisional genotype M has been proposed.³³⁶ Because of the lack of a central body for assigning genotype designations, some confusion exists to which strains are members of genotype J and which are members of genotype K. Genotypes A, C, D, G, and H are predominantly observed in the Western hemisphere, whereas genotypes B, F, I, J/K, and L are typically found in countries of the Asia-Pacific region.²⁷² Cocirculation of multiple genotypes within a region, or even within an outbreak, is not uncommon. There is no established association between genotype designation and specific virus characteristics,^{8,177,339} although some have suggested that virus genotypes C, D, H, and J are more neurotropic than other genotypes.^{319,373,375}

Relationships among MuV strains have also been inferred from nucleotide sequence analyses of the *N*,¹⁷⁸ *P*,^{335,429} *F*,^{118,374,385} and *HN*^{229,330,431} genes. While these genes are more conserved in sequence and, thus, their use in discriminating among virus strains is not as powerful, phylogenetic analyses of these sequences may prove useful. For example, because the HN protein is the major cell surface target of neutralizing antibody,^{291,292,347} it stands to reason that HN-based genotyping will provide valuable information on antigenic diversity and protective immunity.^{91,211,293} Sequence analysis of this and other genes may also provide valuable information on the evolution of MuV.

RT-PCR and sequencing of viruses has proved particularly useful in investigation of possible vaccine-associated adverse events, such as aseptic meningitis. It was not until the availability of this technology that reports of this complication of vaccination could be confirmed.^{17,196,273,275,340,377,401}

DIAGNOSIS

Clinical Diagnosis

A clinical diagnosis of mumps is made on the basis of fever and constitutional symptoms (e.g., parotitis) usually developing within 3 weeks after known exposure. Meningeal symptoms

include headache, vomiting, fever, and nuchal rigidity, usually appearing 3 to 5 days after the onset of parotitis with a range of 1 week before to 2 weeks afterward.^{20,322} Seizures occur in 20% to 30% of patients with CNS symptoms.^{19,132} Meningeal involvement is also suggested by positive Brudzinski's and Kernig's signs and can be differentiated from encephalitis by a normal electroencephalogram (EEG), although the EEG findings can be normal in subjects with encephalitis, even when clinical seizures occur.¹³² In many cases, the diagnosis can be confirmed by an elevated CSF-to-serum antibody ratio.^{269,387} Mumps meningitis is generally a benign condition with complete recovery in 3 to 4 days. Frank delirium and focal neurologic deficits are uncommon. Patients with mumps meningitis and encephalitis without focal deficits, prolonged or recurrent focal seizures, or papilledema have marked clinical improvement of neurologic function within 2 to 4 days. Even with profound obtundation, patients recover with few sequelae.^{19,42,46}

Laboratory confirmation is often required because a number of infectious agents, drugs, and conditions can cause mumps-like symptoms (see Differential Diagnosis below).

Laboratory Diagnosis

Laboratory diagnosis presently rests on infectious virus isolation (typically using Vero cells), detection of viral genome by RT-PCR, and appropriate serologic studies of acute and convalescent sera.

Infectious virus and virus genome can be detected in several clinical fluid specimens, including oral fluid samples (e.g., saliva, throat swabs, and buccal swabs), CSF, and urine. Specimens should be collected as early in the disease as possible (within the first week) with storage at -70°C or below until testing can occur. Success rates are highest with oral fluid samples and lowest with urine and blood samples, despite the apparent high frequency of viruria and viremia.

Studies using oral fluid specimens demonstrate that MuV isolation by cell culture is as sensitive as detection by RT-PCR.^{33,213,267,325} For urine, however, success rates are higher for virus isolation as compared to RT-PCR detection, possibly due to the presence of PCR inhibitors in urine,^{3,154,213,267,426} and for CSF, for reasons that are not clear, success rates are higher for RT-PCR.^{309,384}

The relative success of virus detection also appears to depend on vaccination status. Whereas the success rate of virus detection in cases with a history of 0 or 1 dose of vaccine typically exceeds 70%,^{3,90,267} in persons with a history of 2 doses of vaccine, virus could only be detected in approximately 30% of clinically apparent cases.^{28,325}

Many of the viruses in the differential diagnosis of mumps cause cellular pathology indistinguishable from that induced by MuV, and not all MuV strains produce cytopathic effects *in vitro*. Therefore, cultures must be tested for the presence of virus antigen by immunocytochemistry on cells inoculated with clinical specimens, or for viral genome by RT-PCR. Direct testing of clinical specimens by RT-PCR without an intervening cell culture step is commonly practiced.

Mumps humoral immune responses are most commonly measured by ELISA or virus neutralization. Other once commonly performed serologic tests, including complement fixation, immunofluorescence assay, hemagglutination-inhibition and hemolysis-in-gel are no longer widely used due to inferior sensitivity and specificity (i.e., cross-reactivity with other

paramyxoviruses). Because most people either have a history of vaccination or natural infection, simple detection of MuV antibodies in patient serum is not sufficient for a diagnosis of acute infection or reinfection. Rather, a significant rise in antibody titer relative to that in serum drawn before or at the onset of symptoms (e.g., as neutralizing antibody or ELISA-based IgG) needs to be demonstrated in convalescent serum drawn 2 to 4 weeks after the onset of clinical symptoms. Alternatively, MuV IgM and IgG levels can be compared when only acute-phase serum is available. The MuV-specific IgM response will exceed the IgG response early in the infection before diminishing over the next few weeks to months.³⁸⁷ In the present era of high vaccine coverage, reliance only on IgM detection is no longer advised, given the difficulty of its detection in anamnestic immune responses.^{154,337,338} Thus, in vaccinees, the absence of MuV-specific IgM is not confirmation of the absence of MuV infection. Furthermore, testing of sera too early or too late in the course of disease can also yield false-negative IgM results.^{86,214,406}

Because of its ease of use and high throughput, ELISA is more often performed than the labor-intensive virus neutralization assay. However, antibodies without known biological activity (e.g., those directed against the MuV nonstructural proteins) as well as cross-reactive anti-parainfluenza virus antibodies yield a positive result in the MuV ELISA, raising concern with interpretation of ELISA-based results in certain settings.

White blood cell (WBC) and differential counts are usually normal although leukocytosis is common in cases presenting with meningitis, orchitis, or pancreatitis. Serum amylase levels are elevated in cases of parotitis or pancreatitis, the latter being differentiable from parotitis by isoenzyme analysis or serum pancreatic lipase determinations.²³⁸

In cases of CNS invasion, lymphocytes are the predominant cell type found in the CSF, with WBC counts averaging 250/mm³.^{202,424} Cells counts peak around the third day of neurologic symptoms and then gradually decline over a period of several weeks.^{121,417} In some instances, CSF pleocytosis can persist for as long as a year.³⁹⁸ The CSF is usually under normal pressure⁴⁶ and the protein content in the CSF is elevated in 60% to 70% of all cases.^{121,202} Protein levels up to 100 mg/dL can be seen; on occasion, they exceed 700 mg/dL. This appears to reflect damage to the blood-brain barrier, as indicated by elevated albumin indices that do not normalize for several weeks to months after the onset of CNS symptoms.²³⁷ The CSF glucose content is modestly depressed to 17% to 41% of the serum value in 6% to 29% of all cases.^{101,193,417} About one third of all patients have evidence of intrathecal IgG synthesis and demonstrable oligoclonal immunoglobulins during the first week of CNS symptoms; this oligoclonal antibody can persist for more than 2 years.¹²¹ More than one half show increased IgG indices by the third month following mumps meningitis.^{121,237,399} Up to 90% of patients with acute mumps meningitis produce virus-specific IgG within the CNS compartment,^{237,387,399} and one half show MuV-specific IgM.¹¹⁷ Because mumps antibodies are uncommon in the CSF during other CNS infections,¹⁰⁰ in many instances of mumps meningitis, a diagnosis can be confirmed by elevated CSF-to-serum antibody ratios using samples taken during illness.^{269,387}

Of all cases of mumps with symptoms sufficiently severe to warrant hospitalization, less than 10% can be expected to show transient electrocardiograph abnormalities consistent

with myocardial damage. These usually consist of ST-segment depression, but patients can show evidence of atrioventricular conduction defects, including complete heart block.¹⁴ Seldom is the cardiac involvement acutely symptomatic. MuV myocarditis can be linked, however, to serious long-term sequelae, such as endocardial fibroelastosis.²⁷⁹

DIFFERENTIAL DIAGNOSIS

When parotitis is present, the clinical diagnosis of mumps is straightforward, even if the disease is not epidemic. Nonetheless, other causes of parotitis should be considered. These include parainfluenza virus types 1 and 3, influenza A virus, coxsackievirus, lymphocytic choriomeningitis virus, human immunodeficiency virus (HIV), and suppurative infections including *Staphylococcus aureus* and atypical mycobacteria. All can be easily differentiated from MuV by serology or culture. Starch ingestion, drugs (e.g., phenylbutazone, thiouracil, iodides and phenothiazines), metabolic disorders (e.g., diabetes mellitus, cirrhosis, and uremia), and malnutrition can also cause parotitis. Rare conditions such as Mikulicz's, Parinaud's, and Sjogren's syndromes can also be confused with mumps.²³⁸ Other possible causes of parotid swelling include tumors, cysts, and salivary stones. Without parotitis or with inconspicuous salivary gland enlargement, symptoms of other visceral organ or CNS involvement may predominate, thus laboratory confirmation of the diagnosis is required, even during an epidemic.

TREATMENT

Treatment of mumps and its various complications is generally symptomatic. In a controlled study, adult men presenting with parotitis were alternatively given either an intramuscular injection of 20 mL of gammaglobulin prepared from human convalescent serum or simply confined to a hospital for routine, nonspecific symptomatic therapy.¹³⁰ Orchitis developed in 4 of 51 antibody-treated and 14 of 51 symptomatically treated patients ($P < 0.01$). No protective effect was seen with gammaglobulin obtained from a normal donor serum pool.¹³⁰ This study suggests that immunotherapy with high-titer polyvalent or monoclonal antibody preparations could be useful in selected cases but should be used very early in the course of the illness. Notably, immunoglobulin for the treatment of mumps is no longer available in most countries.

PREVENTION AND CONTROL

The apparent ineffectiveness of passive protection and the near impossibility of preventing virus spread by case isolation (considering that virus is shed before the appearance of clinical symptoms and a significant portion of infected individuals are asymptotically infected) leaves vaccination as the only practical control measure.

Vaccines and Adverse Events

Two general types of MuV vaccines have been used, formalin-inactivated (killed) vaccines and live, attenuated vaccines. Use of formalin-inactivated MuV vaccines (used in the

TABLE 35.2 Mumps Virus Vaccine Strains in Current Use

Vaccine strain	Genotype	Manufacturer	Vaccine name	Main area of use
Hoshino	B	Kitasato Institute	Hokken, Hoshino	Japan, Korea
NK M-46		Chiba Serum Institute	NK M-46	Japan
Torii		Takeda Pharmaceutical	Torii	Japan
Urabe		Sanofi-Pasteur	Trimovax® (trivalent)	Worldwide
Leningrad-3	Classification pending	Moscow State Facility for Bacterial Preparations	Leningrad-3	Russia
Leningrad-Zagreb	Classification pending	Inst. of Immunol., Zagreb	Leningrad-Zagreb	Croatia, Slovenia
Jeryl Lynn	A	Serum Institute of India	Tresivac® (trivalent)	Worldwide
		Merck/Aventis Pasteur MSD	MMRII® (trivalent)	Worldwide
			MMR-Vaxpro® (trivalent)	Europe
			ProQuad® (quadrivalent)	Worldwide
			Priorix® (trivalent)	Worldwide
			PriorixTetra® (quadrivalent)	Europe
			BMR vaccine® (trivalent)	Netherlands
			Pavivac®	Czech Republic
			Trivivac® (trivalent)	Slovak Republic
			S79	China
S-12	H	Dalian Jinjang-Andi Bioproducts	S-12	Iran
		Razi State Serum and Vaccine Institute	BBM-18 ^a	Europe
		Crucell Vaccines (formerly Berna Biotech)		

^aIn development

United States from 1950 to 1978) has been discontinued worldwide due to short-lived (<1 year) immunity and relatively poor efficacy.^{175,307} All mumps vaccines currently in use are composed of live, attenuated virus (Table 35.2) and have been responsible for the remarkable decline in the incidence of mumps and related sequelae.

Live, attenuated virus mumps vaccines were developed through continuous serial passage of wild-type isolates. At present, no clear distinguishing marker exists for attenuation of MuV strains apart from the failure of a passaged isolate to produce clinical symptoms in vaccinees, although various animal tests of mumps vaccine virulence are under evaluation. In the United States, the only licensed mumps vaccine is the Jeryl Lynn strain and currently is only available in combination with measles and rubella vaccines (MMR) or measles, rubella, and varicella vaccines (ProQuad [Merck, West Point, PA] in the United States). Monovalent Jeryl Lynn vaccine is no longer manufactured.

Because of demonstrated and theoretical considerations about spread of virus to placenta and fetal tissues, administration of vaccines containing live MuV three months before or during pregnancy is not advised.³⁰⁷

Prelicensure studies demonstrated that a single dose of the Jeryl Lynn vaccine strain induces protective levels of virus neutralizing antibodies in greater than 95% of recipients.^{165,359,412} Similar results have been found for other mumps vaccine strains licensed for use elsewhere.^{103,276} Although antibody titers after vaccination develop more slowly and are lower than those following natural infection, protection from natural mumps appears complete,^{45,110} and neutralizing antibody persists for decades.⁹⁴ However, waning and loss of neutralizing antibody has been reported as early as 4 years postvaccination.^{40,93,263}

The Jeryl Lynn vaccine is composed of two distinct but genetically related viruses, designated Jeryl Lynn-5 (JL-5) and Jeryl Lynn-2 (JL-2), existing in an approximate 5:1 ratio.⁶ These

two viruses differ from each other at 414 nucleotides (3%), leading to 87 amino acid substitutions.⁹ Over 500 million doses of the Jeryl Lynn–based MuV vaccines have been distributed worldwide, with few serious adverse effects noted.¹⁶⁴ Most reported complications (e.g., rash, pruritus, and purpura) have been allergic in nature; these complications are both uncommon and usually mild and self-limited.⁵⁵ Approaches for the immunization of egg-allergic children have been detailed.²²⁵ A comprehensive survey of the literature by the Vaccine Safety Committee of the Institute of Medicine failed to find adequate information to establish or reject a causal relationship between the administration of Jeryl Lynn MuV–containing vaccines and the development of encephalopathy, encephalitis, residual seizure disorders, optic neuritis, transverse myelitis, Guillain-Barré syndrome, IDDM, or sterility caused by orchitis, all of which have been extensively reviewed elsewhere.^{32,358} Parotitis following Jeryl Lynn vaccination occurs in approximately 1% of vaccinees.^{115,261,313}

Other widely distributed vaccine strains include the RIT 4385, Urabe AM9, Leningrad-Zagreb, and Rubini (discontinued). The RIT 4385 strain, produced by GlaxoSmithKline (Philadelphia, PA), was derived from the Jeryl Lynn strain by clonally isolating the JL-5 population.³⁷⁹ The Jeryl Lynn and RIT 4385 strains of vaccine appear to have similar safety and efficacy profiles.^{85,262,391}

In contrast to the Jeryl Lynn–based strains, most other strains have been linked to aseptic meningitis,^{72,171,200,206,247,274,285,386} occurring 15 to 21 days after vaccination, with a male predominance in those affected. The rate of vaccine-associated aseptic meningitis ranges from more than 1 case per 400 doses to less than 1 case per 100,000 doses, a range influenced by vaccine manufacturer, stringency of adverse event reporting, method of surveillance, study size, clinical definition, and background rates of aseptic meningitis.³⁴ The course of illness is mild, with no sequelae noted. Other, less frequent

adverse events following vaccination include orchitis^{2,73,217} and pancreatitis.^{113,381} Vaccine-associated sensorineural deafness has been reported but is exceedingly rare.¹⁵ Symptomatic transmission of certain vaccine viruses, including the Lenin-grad-3, Leningrad-Zagreb, and Urabe strains, has been reported.^{17,196,340} Although mumps and its complications are usually mild, most analyses of cost-to-benefit ratios favor an intensive vaccination program for developed nations.^{126,199,209,355,438}

The Rubini vaccine strain, which was extensively passaged in embryonated hens' eggs and WI-38 and MRC-5 human diploid cells,¹³⁴ was found to offer little protection against mumps.^{135,290,312,341} As of 2001, the World Health Organization (WHO) no longer recommended its use for national immunization programs.⁴²⁵

Vaccine Use

The Advisory Committee on Immunization Practices (ACIP) recommends administration of the first dose of MMR at 12 to 15 months of age and administration of a second dose at 4 to 6 years of age. The 1-dose schedule was instituted in 1977 and modified to a 2-dose schedule in 1989.⁵³ ProQuad (quadrivalent measles, mumps, rubella, and varicella vaccine) can be used in place of MMR for the second dose at any age, or for the first dose at 48 months of age or older.²⁴⁴ Studies suggest that vaccination as early as 9 months of age^{197,343} and a second dose at 9 to 13 years of age^{55,188,227} may be equally effective.

Vaccination in the face of recent chemotherapy or radiation therapy is unlikely to induce adequate serologic responses and should be delayed for at least 3 months following such treatment.³⁰⁷ Studies of MuV-containing vaccines in nonimmunocompromised HIV-infected patients, primarily in the form of trivalent mumps, measles, and rubella vaccines, have not documented serious or unusual adverse effects and therefore vaccination is recommended.^{60,252}

After many years of steadily declining disease incidence in the United States following the institution of mumps vaccination, a transient reversal in the number of reported cases of mumps occurred between 1986 and 1987 (Fig. 35.5). This appeared to be a reflection of the pool of susceptible children, teenagers, and young adults who were not immunized aggressively for mumps,^{39,65,74,163,199,355} especially during the first decade following introduction of the vaccine. Models of the effects of mass vaccination programs suggest that the average age of disease can be anticipated to rise with the level of induced immunity, with the additional paradoxical result of increased relative risk of serious consequences of mumps infection (e.g., orchitis in susceptible postpubertal males).¹⁰

Following the outbreaks in the late 1980s, the incidence of mumps rapidly returned to preoutbreak levels and then precipitously declined over the next two decades to record low levels before a resurgence in 2006 and then in 2009 (Fig. 35.5). In contrast to the 1986 and 1987 mumps outbreaks, most cases occurring in 2006 and 2009 involved persons with a history of 2 doses of vaccine.^{21,38,64,81,93,245,323,325} As discussed earlier, recent outbreaks appear to be facilitated by waning of vaccine-induced immune responses, which may be a consequence of the success of the mumps vaccination program itself in reducing wild-type virus circulation, resulting in reduced opportunities for periodic boosting, which in the past may have served to maintain immunity.⁹⁷

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Measles Virus

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Measles is a highly contagious disease characterized by a prodromal illness of fever, coryza, cough, and conjunctivitis followed by the appearance of a generalized maculopapular rash. Introduction of measles into virgin populations and endemic transmission in populations with inadequate medical care are associated with high mortality. Despite the development of a successful live attenuated vaccine, measles remains a major cause of mortality in children, particularly in developing countries, and a cause of continuing outbreaks in industrialized nations.

HISTORY

Measles is a relatively new disease of humans and probably evolved from an animal morbillivirus. Phylogenetically, measles virus (MeV) is most closely related to rinderpest virus (RPV), a pathogen of cattle (Fig. 36.1), and it is postulated that MeV evolved in an environment where cattle and humans lived in

close proximity.⁴⁴⁴ Because large numbers of people are required to generate sufficient susceptible individuals to maintain measles in a population, measles may have evolved in the early centers of civilization in the Middle East where populations attained sufficient densities to sustain continued transmission.⁴⁴⁴

Abu Becr, an Arab physician known as Rhazes of Baghdad, is generally credited with distinguishing smallpox from measles in the 9th century. He dated its first description to the 6th century. Rhazes referred to measles as *hasbah* (eruption) and regarded it as a modification of smallpox with the distinction that “anxiety of mind, sick qualms and heaviness of heart oppress more in the measles than in the smallpox”.⁵⁸⁵ Repeated epidemics of illnesses characterized by a rash are recorded in European and Far Eastern populations between 1 and 1200 AD.⁴⁴⁴ It appears that measles spread across the Pyrenees into France with the Saracen invasion of the 8th century.⁶⁰⁰ Repeated epidemics identified as measles were recorded in the 11th and 12th centuries, and it is first mentioned as a childhood disease in 1224.⁴⁴⁴

In the European literature the name applied was “morbilli,” derived from the Italian “little diseases” to distinguish it from plague, “il morbo,” but morbilli included several exanthemata. Sanvages in 1763 defined morbilli as measles but called it rubeola (derived from the Spanish⁶⁰⁰), leading to a common confusion with rubella that persists to the present. Introduction of measles into previously unexposed populations has been associated with high morbidity and mortality.^{68,600,655,667} Epidemics of rash illnesses were associated with episodes of depopulation in China, India, and the Mediterranean region. Introduction of measles into the Fiji Islands in 1875 resulted in 26% mortality.⁶⁰⁰ Approximately 56 million people died as a result of European exploration of the New World, largely due to the introduction into native Amerindian populations of Old World diseases, notably smallpox and measles. Decreases in population are likely to have facilitated the transfer of Spanish culture to South America.⁴⁴⁴

Many of the basic principles of measles epidemiology were elucidated by Peter Panum, a Danish physician who worked in the Faroe Islands during a large measles epidemic in 1846.⁵³⁰ Panum deduced the highly contagious nature of the disease, the 14-day incubation period, and the lifelong immunity present in older residents and postulated a respiratory route of transmission.

Complications of measles were first described in the 18th century. In 1790, James Lucas, an English surgeon, described the first case of postmeasles encephalomyelitis in a young woman who developed paraparesis as the rash was fading.⁴¹² Nineteenth-century medical textbooks associated measles with the exacerbation of tuberculosis, and in 1908, while working at a tuberculosis hospital in Vienna, von Pirquet recorded the disappearance of

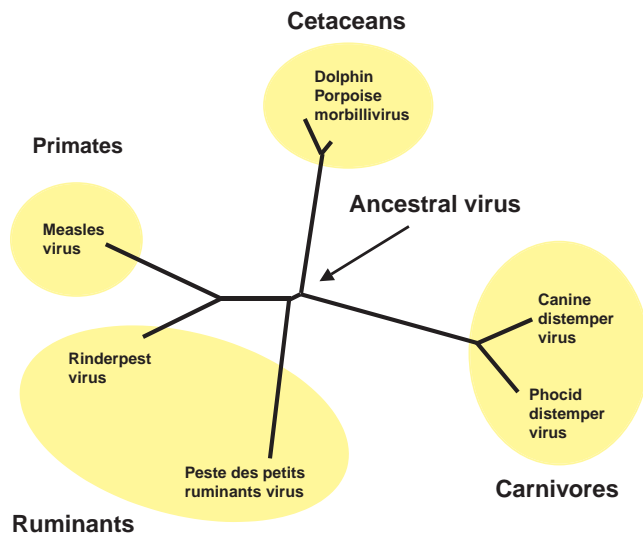


FIGURE 36.1. Genetic relationships between morbilliviruses based on comparison of the nucleotide sequences of the N genes. The tree was derived using PHILIP, DNADIST, and FITCH programs. The branch lengths are proportional to the mutational differences between the viruses and the hypothetical common ancestor that existed at the nodes in the tree. (From Barrett T. Morbillivirus infections, with special emphasis on morbilliviruses of carnivores. *Vet Microbiol* 1999;69:3–13, with permission.)

delayed-type hypersensitivity skin test responses to tuberculin,⁷⁵⁰ the first experimental evidence of measles-induced immune suppression.

In 1933, Dawson described subacute sclerosing panencephalitis (SSPE) in a 16-year-old boy with progressive neurologic deterioration. Histologic examination of the brain showed eosinophilic intranuclear and intracytoplasmic inclusions in neurons and glial cells.¹⁴² After reports of paramyxovirus-like particles in the inclusions,⁷¹⁷ observations of elevated levels of MeV antibody in serum and cerebrospinal fluid (CSF) and the reactivity of the inclusions with antibody to MeV identified the paramyxovirus as MeV.¹³⁰

INFECTIOUS AGENT

In 1757, an infectious agent was formally shown to be the cause of measles when the Scottish physician Francis Home, attempting immunization, transmitted the disease to naive individuals using blood taken from measles patients during the early stages of the rash.²⁹⁷ In 1905, Hektoen transmitted disease to volunteers with blood “free of bacteria” taken from measles cases in the acute stage and observed an incubation period of 13 days.²⁸⁰ In 1911, Goldberger and Anderson transmitted measles to rhesus macaques with filtered respiratory tract secretions from measles patients and successfully passed disease from one monkey to another.^{238,531}

Propagation and Assay in Cell Culture

Primary Isolation

In 1954, Enders and Peebles first isolated MeV in tissue culture by inoculating primary human kidney cells with the blood

of David Edmonston, a child with measles.¹⁸⁴ Isolates were also made using primary monkey kidney cells¹⁸⁴ and later continuous monkey kidney cell lines (e.g., Vero, CV-1).⁶⁷⁷ However, isolation of wild-type strains of MeV is most often successful using an Epstein-Barr virus-transformed marmoset B-lymphocyte line, B95-8³⁶⁵; a human T-cell line from cord blood, COBL-a³⁶⁴; or Vero cells engineered to express the MeV receptor signaling lymphocyte activation molecule (SLAM).⁵²¹ Generally, the first observable sign of virus growth is cell–cell fusion and syncytia formation (Fig. 36.2).

Laboratory Propagation and Assay

Growth of MeV led to the development of live-attenuated vaccine strains by adaptation of MeV to growth in cells from foreign hosts, such as the chick embryo and canine and bovine kidney cells.³⁴⁹ Most experimental work is done with tissue culture–adapted strains so virus stocks are generally grown in Vero cells using a low multiplicity of infection to avoid accumulation of defective interfering (DI) particles. Vero and Vero/SLAM cells are useful for virus titration by plaque formation.¹⁰ The virus replicates slowly and 3 to 5 days of culture are often needed for plaques to become visible. Wild-type strains can also be assayed by syncytia formation in B95-8 or human cord blood mononuclear cells.

Biological Characteristics

The morbilliviruses form two genetically distinct groups of viruses related either to canine distemper virus (CDV) or to RPV (Fig. 36.1)⁵⁰ and differ from other paramyxoviruses in formation of intranuclear inclusion bodies. Virions are pleomorphic and range in size from 100 to 300 nm. The envelope carries surface projections that are composed of the viral transmembrane hemagglutinin (H) and fusion (F) glycoproteins (Fig. 36.3). The matrix (M) protein lines the interior of the virion envelope. The helical ribonucleocapsid (total length of 1.2 μ m) formed from the 16 kb genomic RNA wrapped with the nucleocapsid (N) protein is packed within the envelope in the form of a symmetrical coil with the phosphoprotein (P) and large polymerase (L) proteins attached.

Proteins

NUCLEOCAPSID PROTEIN

The N protein messenger RNA (mRNA) is the first transcribed from the genome and the N protein (525 amino acids [aa]) is the most abundant of the viral proteins. N appears as a 60-kD band on polyacrylamide gels and can self-assemble but usually surrounds viral genomic or antigenomic RNAs to form helical ribonucleocapsid structures.¹⁰⁰ This conformationally flexible structure^{62,643} is the required template for both replication and transcription. Each N monomer binds six nucleotides, and viral genomes must be multiples of six for replication.³⁶⁷ Monomeric N (N⁰) can be transported into the nucleus but is usually retained in the cytoplasm by binding to P.^{309,679} Phosphorylation regulates oligomer formation and activation of transcription.^{243,267}

N is organized into two functionally distinct regions. The N-terminal portion of the protein (aa 1 to 400, N_{CORE}) is conserved and is required for self-assembly into nucleocapsids and for RNA binding^{44,242,342,403,679} (Fig. 36.4). N_{CORE} forms a globular domain located toward the helical axis of the nucleocapsid,⁶⁴³ includes a nuclear localization signal,⁶²⁸ and

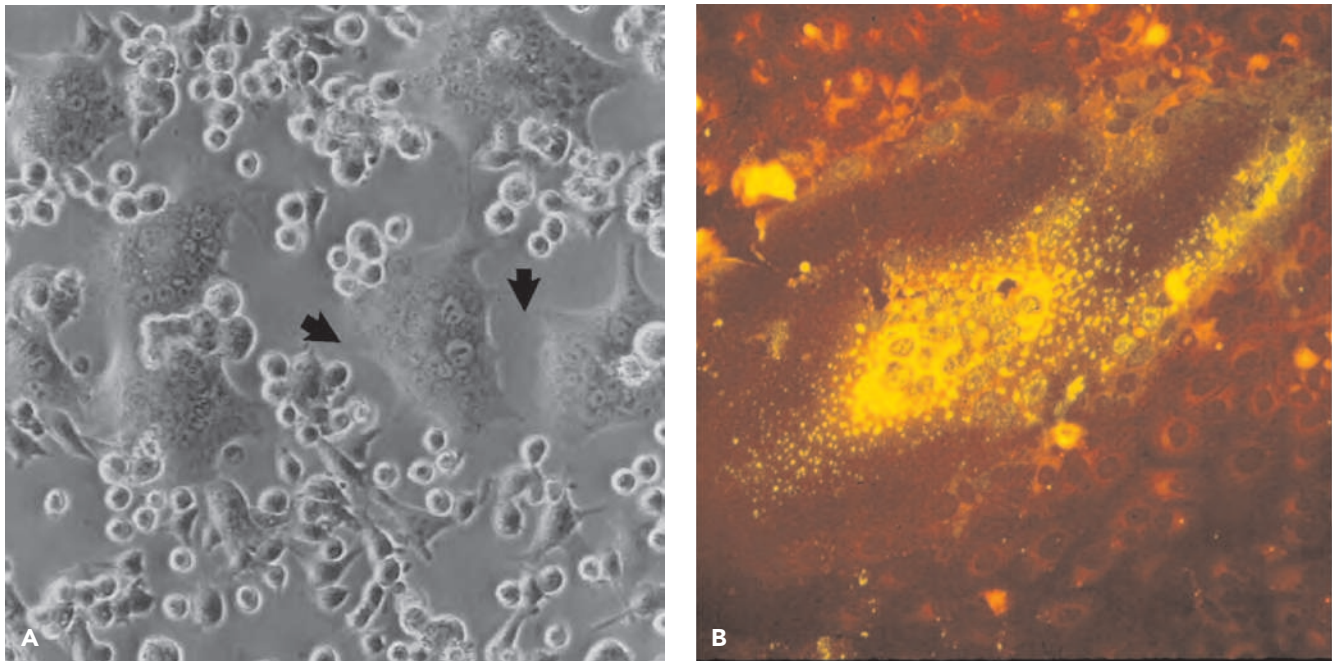


FIGURE 36.2. Typical cytopathic effects of syncytia formation associated with measles virus (MeV) replication in Vero cells. A: Unstained cells in culture. (A courtesy of William Bellini, Centers for Disease Control and Prevention, Atlanta, Georgia.) **B:** Cells stained with a fluorescently labeled antibody to MeV.

can also react with the cell surface through FcγRII.⁵⁷⁹ The C-terminal portion of the protein (aa 401 to 525, N_{TAIL}) is more variable, intrinsically disordered, acidic, and phosphorylated.^{40,110,267,341,408,567} N interacts with P through residues in N_{CORE} and N_{TAIL}.^{44,77,356,792} N_{TAIL} is required for nucleocapsid

flexibility,⁶² but its location in the nucleocapsid structure is not clear.¹⁵¹ N_{TAIL} contains an α-helical molecular recognition element (α-MoRE, aa 488 to 499) reversibly involved in induced α-helical folding upon interaction with the X domain (XD) in the C-terminus of P (PCT) that likely positions the polymerase

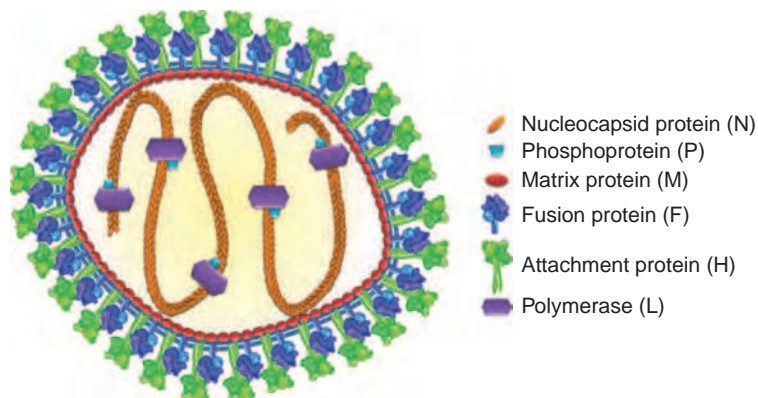


FIGURE 36.3. Schematic diagram of measles virus. The lipid bilayer of the pleomorphic particle is represented by blue lines, under which the matrix protein layer (red) resides. The viral membrane is densely packed with envelope glycoprotein complexes consisting of fusion protein trimers (shades of blue) and attachment protein tetramers (shades of green). The negative-strand RNA genome and the nucleocapsid protein (orange) form the nucleocapsid, which interacts with the phosphoprotein (light blue) and the polymerase (purple). In addition to contacts between the nucleocapsid and matrix proteins,³²⁵ the luminal tails of the glycoproteins are thought to contact the matrix layer. Individual viral components are not drawn to scale. Structural renderings of the glycoprotein complexes are based on original crystal structures (H head domains²⁷⁷); homology models of measles virus (MeV) F^{386,552} derived from coordinates reported for pre- and postfusion PIV5 and PIV3 F, respectively^{794,795}; or hypothetical structural models (F prehairpin intermediate). High-resolution structural models were aligned at the level of the transmembrane domain (viral envelope) as described⁵²⁷ and then morphed into low-resolution images using the Sculptor (resolution 12, voxel size 3) package.⁵⁴ (Adapted from Plemper RK, Brindley MA, Iorio RM. Structural and mechanistic studies of measles virus illuminate paramyxovirus entry. *PLoS Pathog* 2011;7:e1002058; courtesy of M. A. Brindley and R. K. Plemper.)

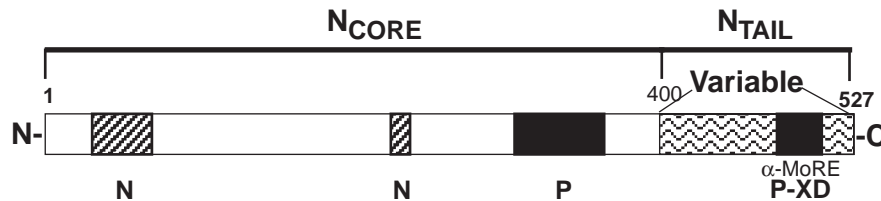


FIGURE 36.4. Schematic diagram of the nucleocapsid (N) protein. The N-terminal region (N_{CORE}) contains the RNA-binding domain and the oligomerization domain and is sufficient for nucleocapsid formation. The C-terminal region (N_{TAIL}) is unstructured and acidic, binds C-terminal domain of P (PCT), and contains the region of sequence variability that is used for identification of measles virus (MeV) clades. Regions of interaction with N and P are indicated.

complex near the RNA in the nucleocapsid.^{44,76,77,151,333,408,468} The intrinsic disorder of N_{TAIL} provides a structural plasticity that allows strain- and cell type-specific interactions with several host proteins including heat shock protein (Hsp) 72, interferon regulatory factor (IRF)-3, the cellular protein responsible for nuclear export of N, the p40 subunit of eukaryotic initiation factor 3, cyclophilins A and B, and an unidentified cell surface nucleocapsid receptor.^{128,379,627,718,760,803}

P, C AND V PROTEINS

The P (phospho) protein (507 aa) is a polymerase co-factor that is activated by phosphorylation, forms tetramers, and links L to N to form the replicase complex.¹³⁵ While the 72-kD P protein is abundant in the infected cell, only small amounts are present in the packaged virus. P is a multifunctional protein with a modular organization (Fig. 36.5). The N-terminus (PNT) is poorly conserved, intrinsically unstructured, acidic, phosphorylated, and required for replication. PNT binds to N_{CORE} and this interaction induces folding of PNT.³⁴³ PNT is a chaperone for N^0 that prevents binding to cellular RNAs, illegitimate N self-assembly, and nuclear translocation. PNT initiates encapsidation of genomic viral RNA by sequence-specific binding of the leader RNA.^{135,309} Elongation of the nucleocapsid structure is sequence independent. PNT also interacts with cellular proteins to regulate the response to interferon (IFN) (see discussion of V later).

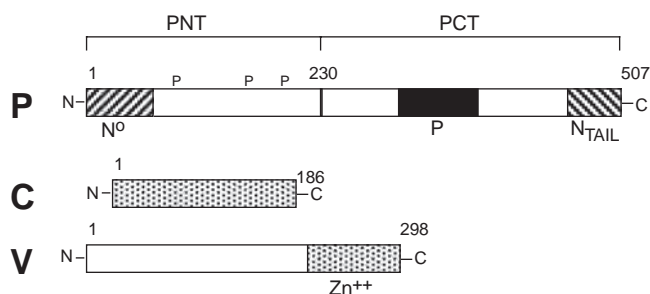


FIGURE 36.5. Schematic diagram of the phospho (P), C, and V proteins. Diagram of the proteins encoded in the P gene. P and C are initiated at AUGs in different reading frames. The N-terminal region of P (PNT) is acidic, unstructured, and phosphorylated on serines and binds N^0 . The C-terminal region of P (PCT) has the P oligomerization domain and the X domain that binds and induces folding of N_{TAIL} . V is translated from an edited RNA with a nontemplated G at nucleotide 751 that results in a distinct cysteine-rich zinc-binding C-terminus.

PCT is conserved⁴⁵ and contains all domains required for transcription. The region between amino acids 204 and 321 contains the α -helical domain that binds to N_{TAIL} as part of the nucleocapsid structure and is responsible for tethering the polymerase L to its template.^{309,356} A coiled-coil domain between aa 344 to 411 is sufficient for P oligomerization.¹³⁵ The unique XD portion of PCT (aa 459 to 507) has three α -helices arranged in an antiparallel triple helix bundle that binds the N_{TAIL} α -MoRE with 1:1 stoichiometry and induces its folding.^{65,229,336,356,408} Binding affinity is weak, consistent with a model in which XD is responsible for tethering L to the ribonucleocapsid in a way that allows it to progress during RNA synthesis.^{356,792} XD also interacts with and stabilizes the ubiquitin ligase p53-induced-RING-H2.¹¹⁸

The P gene of MeV, like other members of the *Paramyxoviridae*, encodes proteins in addition to P that, together with P, regulate the innate response to infection (Fig. 36.5). The C protein is a basic protein of 186 amino acids encoded by the same mRNA but translated using an initiator methionine codon 19 nucleotides downstream from that for P and an overlapping reading frame.⁶⁰ The V protein shares the initiator methionine and the amino terminal 231 amino acids of the P protein, but RNA editing adds an extra non-template-directed guanosine (G) residue at position 751. This shifts the reading frame and results in a different 68-amino acid cysteine-rich C-terminus with zinc-binding properties.^{105,404} Neither C nor V is necessary for MeV replication in Vero cells,^{572,636} but both C and V interact with cellular proteins and regulate the response to infection.^{155,405,646,722}

C interferes with innate immune responses by inhibition of IFN signaling, modulates viral polymerase activity, and has been implicated in prevention of cell death.^{47,584,654,711} Deletion of C decreases MeV replication in monkeys, peripheral blood mononuclear cells (PBMCs), and thymic epithelial cells and decreases neurovirulence for CD46 transgenic mice, suggesting that C has an important *in vivo* role.^{155,194,537,711,731} In some cells decreased replication is associated with inhibition of translation and induction of IFN.⁴⁹¹ C suppresses IFN induction by regulating viral RNA synthesis⁴⁹⁰ and preventing the activation of the cellular protein kinase PKR.^{435,723}

MATRIX (M) PROTEIN

The envelope of the virion (Fig. 36.3) consists of the M protein (335 aa) and the two transmembrane glycoproteins F and H. M is a basic protein with several conserved hydrophobic domains.⁵⁹ The mRNAs for morbillivirus M proteins contain approximately 400 nucleotides of noncoding sequence at the 3'

end that increases M protein production.^{59,705} In infected cells M is associated with nucleocapsids and with detergent-resistant regions of the inner layer of the plasma membrane where it regulates MeV RNA synthesis and assembly.^{291,325,556} M also interacts with the intracytoplasmic regions of one or both transmembrane glycoproteins, modulates the targeting and fusogenic capacity of the envelope glycoproteins, and directs release of virus from the apical surface of polarized epithelial cells.^{71,487,556,618} Deletion of M increases cell-to-cell fusion and decreases production of infectious virus.¹⁰² These properties are often defective in the mutated M proteins of viruses causing persistent infection.^{290,693}

FUSION (F) PROTEIN

F is a highly conserved type I transmembrane glycoprotein synthesized as an inactive precursor (F_0) of about 60 kD. The mRNAs for morbillivirus F_0 proteins contain unusually long (460 to 585 nucleotides) G-C rich 5' nontranslated regions (NTRs) that are predicted to have extensive secondary structure and are followed by clusters of three to four AUGs.⁵⁸⁸ The 5' NTR influences the choice of AUG and decreases translation of F, virus production, and cytopathogenicity.^{101,705} There is a 28-residue signal sequence and after translation, F is glycosylated and trimerized in the endoplasmic reticulum (ER).⁵⁵⁰ F_0 is cleaved at a multibasic site (108 to 112: Arg-Arg-His-Lys-Arg) by furin in the trans-Golgi to yield the 41-kD (F_1) and 18-kD (F_2) disulfide-linked fusion-competent mature protein.^{73,761} Mutation of Arg 112 results in a reduced rate of F transport to the cell surface, aberrant cleavage, and abolition of the fusogenic activity necessary for infection.^{13,761} Restricted processing of F is associated with persistent infection.⁴⁴⁷ The 33-residue cytoplasmic tail of F_1 possesses basolateral sorting

and endocytosis signals.⁴⁶¹ F_2 has all of the predicted N-linked glycosylation sites (aa 29, 61, and 67). Mutation of any of these asparagines decreases transport to the cell surface and impairs proteolytic cleavage, stability, and the fusion capacity of F, perhaps because F_2 is an integral part of the prefusion F head.^{14,746}

F_1 contains a highly conserved stretch of hydrophobic amino acid residues at the new N-terminus (aa 113 to 145) that constitutes the fusion peptide. Oligopeptides that mimic this segment of F_1 inhibit fusion.⁵⁸⁷ Mutants resistant to the fusion inhibitory effect of these oligopeptides have amino acid alterations in a cysteine-rich region (aa 337 to 381) of F_1 important for interaction with H^{311} (Fig. 36.6). There are two predicted heptad repeat amphipathic α -helices: one adjacent to the fusion peptide and another N-terminal to the transmembrane region. Partial membrane-proximal cleavage between the heptad repeat and the transmembrane region enhances fusion.⁷⁴⁸ Synthetic peptides representing the heptad repeat regions inhibit fusion,³⁸¹ as does mutagenesis of the leucines in the zipper region.⁸³ F_2 possesses a third heptad repeat region that modulates fusogenicity through a microdomain around residue 94.⁵⁴⁸

Fusion requires the expression of both H and F, with a predicted interaction of the F head with the H stalk (Fig. 36.6), and binding of H to a cell surface receptor (Fig. 36.7).^{385,527,802} Modeling of pre- and postfusion conformations of the F trimer indicates large conformational changes that result in formation of a six-helical bundle.⁵⁶⁸ Basolateral expression of H and F is important for syncytia formation and increases cell-to-cell spread *in vitro* and *in vivo*.⁴⁶¹ In polarized epithelial cells transport is directed by interaction of the F luminal tail with M to the apical surface where virus is released.^{71,487} F tail mutations in viruses causing persistent infection include premature stop codons, missense mutations, altered reading frames, and

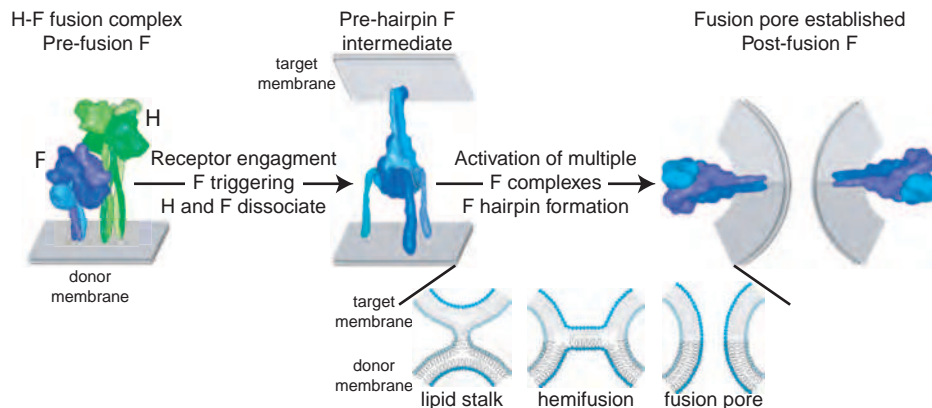


FIGURE 36.6. Schematic of measles virus (MeV) membrane fusion. MeV H and F envelope glycoproteins exist as hetero-oligomeric complexes on the surface of infectious viral particles (**left panel**). Receptor binding by the H protein triggers major conformational changes in prefusion F, resulting in insertion of the F fusion peptide domain into the target membrane in a hypothetical prehairpin intermediate conformation (**center**). Most likely, the concerted refolding of several prehairpin F complexes into the thermodynamically stable postfusion conformation is required to open a fusion pore and enable infection (**right panel**). For clarity, MeV H is represented as a single tetramer and F as a single trimer in the hetero-oligomeric fusion complex. More than one F trimer may interact with each individual H tetramer. The **insert** shows enlarged representations of hypothetical lipid mixing intermediates. Formation of a local fusion nipple is thought to be followed by merger of the outer lipid layers (lipid stalk; hemifusion stage) and ultimately merger of the inner lipid layers and the opening of a fusion pore. F complexes have been eliminated from the lipid mixing representation. Structural renderings were prepared as described in Figure 36.3. (Adapted from Plemper RK, Brindley MA, Iorio RM. Structural and mechanistic studies of measles virus illuminate paramyxovirus entry. *PLoS Pathog* 2011;7:e1002058; courtesy of M. A. Brindley and R. K. Plemper.)

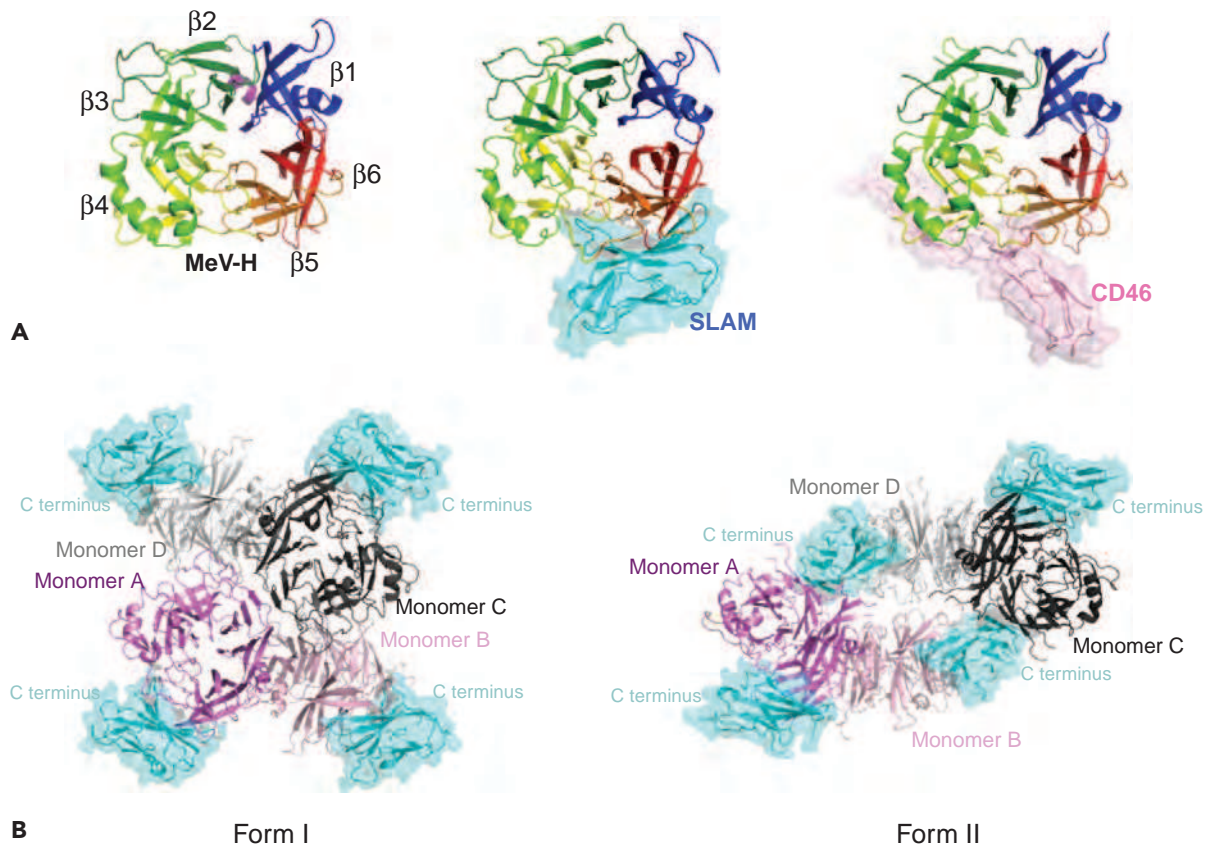


FIGURE 36.7. Crystal structures of the measles virus hemagglutinin (MeV-H) in complex with its receptors. **A:** The H head domain (monomer) unbound (**left**) and bound to signaling lymphocyte activation molecule (SLAM) (**middle**) or CD46 (**right**). The H head domain as viewed downward from top is illustrated as a cartoon model. It exhibits the six-bladed β -propeller fold ($\beta 1$ through $\beta 6$, rainbow colors). This structure is topologically similar to the hemagglutinin neuraminidase of other paramyxoviruses and the influenza virus neuraminidase. The SLAM V domain (cyan) and CD46 SCR1 and 2 domains (pink) are shown in surface models. **B:** Two forms of H tetramer (dimer of dimers) with each monomer bound to the SLAM V domain as observed in the crystals of the H-SLAM complex. H monomers A (violet) and B (light pink) form one dimer, while monomers C (dark gray) and D (light gray) form another. Each dimer (AB or CD) constituting a tetramer has essentially the same structure within and between the two forms and is likely the basic unit of MeV-H. In Form I (**left**), monomers A and C largely form the interface of two dimers, whereas in Form II (**right**), monomers B and D do so. SLAM is shown in cyan. PDB ID: MV-H receptor free form (2ZB6), MV-H-SLAM Form I (3ALZ) and Form II (3ALX), MV-H-CD46 (3INB). (Courtesy of Takao Tashiguchi, Katsumi Maenaka, and Yusuke Yanagi.)

nonconservative amino acid substitutions.^{104,635} These changes interfere with virus envelope assembly and budding and increase cell-to-cell fusion and neurovirulence in hamsters.^{36,103}

HEMAGGLUTININ (H) PROTEIN

H (617 aa) is the receptor-binding and hemagglutinating (HA) protein and an important determinant of morbillivirus cellular tropism. H is a type II transmembrane glycoprotein that resides on the surfaces of infected cells and virions as disulfide-linked homodimers that associate in the ER to form tetramers (Figs. 36.3 and 36.7).^{79,277,550} The mature H protein has a cytoplasmic tail of 34 amino acids preceding a single hydrophobic transmembrane region and a large C-terminal ectodomain with 13 strongly conserved cysteines. The cytoplasmic tail is essential for efficient transport to the cell surface and includes signals for basolateral sorting and endocytosis. However, H can be redirected to the apical surface for efficient particle formation and virus release.^{71,462,463}

The H protein of the Edmonston strain of MeV has five predicted N-linked glycosylation sites clustered between positions 168 and 238 (Fig. 36.8). The first four of these sites are used.³⁰⁵ More recent MeV isolates often have an additional glycosylation site at residue 416, and this correlates with a loss of HA activity.⁶¹⁵ Glycosylation is necessary for proper folding, antigenicity, dimerization, and export of H from the Golgi.³⁰⁵ H processing and intracellular transport is relatively slow, taking approximately 30 minutes for oligomerization and an hour to reach the medial Golgi.³⁰⁶ During persistent infections H proteins often accumulate mutations that affect glycosylation, oligomerization, and intracellular transport.¹⁰⁴

Structural studies of H indicate that the N-terminus forms an α -helical stalk supporting a cubic-shaped six-blade β -propeller head structure (Fig. 36.7).^{127,276,385} Each of the blade modules contains four antiparallel β -strands connected sequentially through extended loops. In the dimer, N-linked carbohydrates cover the top pocket of the head domain and

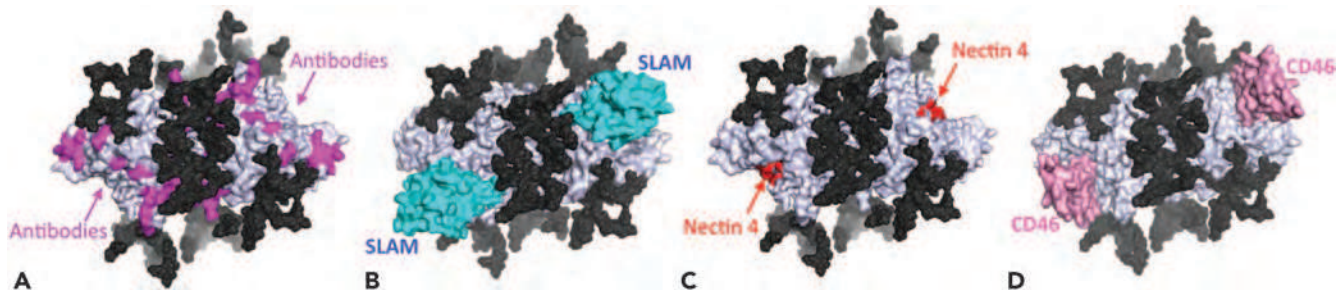


FIGURE 36.8. Structural relationships between glycans, antibody epitopes, and receptor binding sites on the hemagglutinin protein. Measles virus hemagglutinin (MeV-H) dimer (blue white) as viewed downward from top is illustrated as surface presentation with plausible N-linked sugars (black), which cover large surface areas of H. Epitopes for anti-H monoclonal antibodies (magenta) are mapped onto the sugar-uncovered surface of H (A). This area also includes binding sites for signaling lymphocyte activation molecule (SLAM) (cyan, B), nectin 4 (red, C), and CD46 (pink, D). Because the structure of the H-nectin 4 complex has not been determined, the putative binding sites for nectin 4 are indicated, based on mutagenesis analysis. Most neutralizing antibodies bind to sites that overlap or are located very close to the receptor binding sites, which are unlikely to mutate. This may explain why measles virus has never escaped immune responses induced by natural infection or vaccination and why there is only a single serotype. PDB ID: MV-H receptor free form (2ZB6), MV-H-SLAM (3ALX), MV-H-CD46 (3INB). (Courtesy of Takao Tashiguchi, Katsumi Maenaka, and Yusuke Yanagi.)

cause the two molecules to tilt away from each other, optimizing exposure of neutralizing epitopes and the receptor-binding sites away from the dimer interface on the lateral surface (Fig. 36.8)^{276,308,749,751} (see section on Cellular Receptors). Cysteine residues at 139 and 154 are responsible for intermolecular disulfide bonding of monomeric H glycoproteins.⁵⁴⁹

H acts in conjunction with F for budding and for cell-to-cell fusion and entry (Fig. 36.6). Fusion occurs through conformational changes in both proteins triggered by the binding of H to a cellular receptor (Fig. 36.7).^{277,321,499,500,624} Heterooligomerization occurs in the ER.⁵⁵⁰ Mutagenesis has identified separate regions in the H stalk required for interacting with F and for triggering F fusion.^{385,527,547} MeV fusogenicity correlates inversely with the strength of the interaction between F and H^{133,134,189,321,551} (see section on Entry).

L PROTEIN

The L (large) protein (2,183 aa) is a multidomain protein with several highly conserved regions. One contains the Gly-Asp-Asn-Gln motif common to the RNA polymerases of negative-strand viruses.^{174,669} L is present in small quantities in the infected cell, interacts with and functions in association with P, and is part of the viral nucleocapsid both in the cell and in the virion. A domain in the N-terminal 408 amino acids binds to a trihelical binding domain in PCT that links it to the nucleocapsid for transcription and replication.^{300,357}

Cellular Receptors

MeV can infect several types of cells and uses multiple receptors in a virus strain and cell type-specific manner. Three of these receptors have been identified: membrane co-factor protein or CD46,^{168,497} SLAM or CD150,⁷¹⁵ and polio virus receptor-related 4 (PVRL4) or nectin 4.^{479,508} The binding sites for these cellular receptors are all found on the lateral surface of the head structure of H^{277,622} (Figs. 36.7 and 36.8).

CD46

CD46 is a widely distributed human complement regulatory protein expressed on all nucleated cells and preferentially on

the apical surface of polarized epithelial cells.^{71,445} It normally acts as a co-factor in the proteolytic inactivation of C3b/C4b by factor I.⁵⁹¹ Monkeys have a CD46 homolog that is expressed on erythrocytes, but such a protein has not been identified in mice. Multiple mRNAs are produced by alternative splicing of CD46 transcripts. All code for proteins that contain an N-terminal signal peptide, four short consensus repeats (SCRs), a transmembrane region, and an anchor. Isoforms differ in the length and composition of an extracellular serine/threonine/proline domain near the transmembrane segment and in having one of two alternative cytoplasmic tails.⁵⁹¹ The cytoplasmic tail of CD46 is associated with intracellular kinases and adaptor proteins, and cross-linking of CD46 can induce autophagy and regulate inflammatory responses.^{338,591} The four isoforms common on human cells can all serve as receptors for MeV.^{231,423} SCR1 and SCR2 interact with the MeV H protein, while SCRs 2, 3, and 4 bind C3b/C4b.⁵⁹¹ MeV infection of cells or expression of the H protein alone can lead to rapid internalization of CD46 from the cell surface.³⁷³ In persistently infected cells CD46 down-regulation is accomplished through a membrane-proximal Tyr-X-X-Leu motif in the cytoplasmic domain.⁷⁹¹

The H binding site involves one planar face of SCR1 and SCR2 with an important role for the N-linked carbohydrates on SCR2.^{99,304,417} Most vaccine strains use CD46 efficiently, while wild-type strains often do not.^{191,790} A tyrosine at position 481 of H and glycine at 546 are key determinants of the affinity of H for CD46,^{53,660} but several additional residues are also important.^{432,603,637,695} The crystal structure of Edmonston H with SCR1 and SCR2 shows that CD46 binds to the side of the β -propeller through three contact regions on blades 4 and 5 (Figs. 36.7 and 36.8).⁶²²

SLAM

SLAM/CD150 is a 70-kD glycoprotein expressed on cells of the immune system including immature thymocytes, activated T and B lymphocytes, activated monocytes, and mature dendritic cells.¹²⁴ SLAM is a member of a family of immunomodulatory type I transmembrane proteins^{93,153} and is the most important receptor for MeV infection of lymphoid tissue.¹⁴³

A recombinant MeV that interacts inefficiently with SLAM is attenuated in macaques.³⁸⁹ CDV, RPV, and peste des petits ruminants virus also use SLAM as a receptor, suggesting that this is a common feature of morbilliviruses.^{5,49,716,749}

SLAM has two highly glycosylated immunoglobulin-like domains (V and C2) and structural features of the CD2 family of membrane proteins.^{670,790} The cytoplasmic domain has immunoreceptor tyrosine-based switch motifs that bind small SH2 (src homology 2) domain adaptor proteins, such as SLAM-associated protein (SAP) and Ewing sarcoma-associated transcript-2 (EAT-2), important for cell signaling.^{93,153,515,670,790} MeV H binds to the V domain of human, but not mouse, SLAM,⁵¹⁵ and this results in down-regulation of SLAM expression on the surface of infected cells.⁷⁶⁷ Mutagenesis studies have identified MeV H residues Ile194, Asp505, Asp507, Asp530, Arg533, Phe552, and Pro554 as important for binding SLAM.^{431,500,751} The crystal structure of H with the V domain of SLAM shows that these residues contribute to four components of the binding interface located primarily on the side of H blade 5 contiguous to the binding site for CD46²⁷⁷ (Fig. 36.7).

Studies of different strains of MeV have shown that both vaccine and wild-type strains can use SLAM as a receptor and that most H proteins can bind both CD46 and SLAM, but receptor affinity and efficiency of entry differ.^{191,422,432,521,621,637,751,790} In general, binding to SLAM is of higher affinity than binding to CD46.⁴³² Viruses with asparagine at H481 use SLAM and enter PBMCs more efficiently than viruses with tyrosine at this position.^{191,637} Differences in efficient receptor usage likely involve interactions with MeV proteins in addition to H.^{370,710}

NECTIN 4

The distributions of SLAM and CD46 in tissues do not account for MeV replication in epithelial cells *in vivo* or *in vitro*.^{18,197,278,460,519,661,696,708–710} Recently, poliovirus receptor-like 4/nectin 4, an adherens junction protein of the immunoglobulin superfamily, has been identified as a receptor on epithelial cells.^{479,508} This is consistent with previous studies that indicated that an epithelial receptor is expressed on the basolateral surface of polarized cells and involved in formation of tight junctions.^{665,674} Nectin 4 is a transmembrane protein with two C2-type immunoglobulin domains and a V domain that interacts with H.⁴⁷⁹

OTHER RECEPTORS

Several pieces of information suggest that MeV uses additional receptors. The currently known receptors do not account for the ability of MeV to infect endothelial cells in acute infections¹⁹⁷ or cells of the central nervous system in chronic infections.^{445,661} Receptors used by attenuated vaccine strains adapted to growth in cells from nonsusceptible hosts (e.g., chickens) probably represent an additional category of MeV receptors that have yet to be identified.¹⁹³

Other cell surface molecules interact with MeV but do not serve as entry receptors. For instance, MeV H can bind Toll-like receptor 2 (TLR2) and induce signaling.⁶³ Dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3) grabbing nonintegrin (DC-SIGN) is an attachment receptor that enhances infection and modulates function of DCs.^{31,147,263} Incorporation of cyclophilin B into MeV virions by binding to N leads to interaction with the cyclophilin ligand CD147/EMMPRIN, a multifunctional transmembrane protein expressed on epithelial and neural cells.⁷⁶⁰

Hemagglutination, Hemadsorption, and Hemolysis

Some strains of MeV bind to and agglutinate the erythrocytes of Old World monkeys, particularly African green, patas, and rhesus macaques. HA occurs optimally at physiologic pH and 37°C. Infected cells can also adsorb monkey erythrocytes (hemadsorption). Both HA and hemadsorption are properties of the H glycoprotein. Many wild-type MeV isolates require high salt or have little HA activity.⁵⁹⁴ HA of monkey erythrocytes is indicative of binding to CD46, is improved by adaptation to growth in Vero cells, and is dependent on the C-terminal 18 amino acids of H, amino acids 451 and 481, and absence of glycosylation at 416.^{616,630,660} This is consistent with the distribution of the CD46 molecule, which is not present on human red blood cells, and with the molecular characteristics of primate CD46.³⁰³ In baboons, lack of HA is due to an amino acid substitution in SCR2 and in New World monkeys to an absence of SCR1.³⁰³ HA is followed within a few hours by lysis of the agglutinated erythrocytes. Hemolysis is a consequence of fusion and dependent on F, as well as H.^{14,108,779}

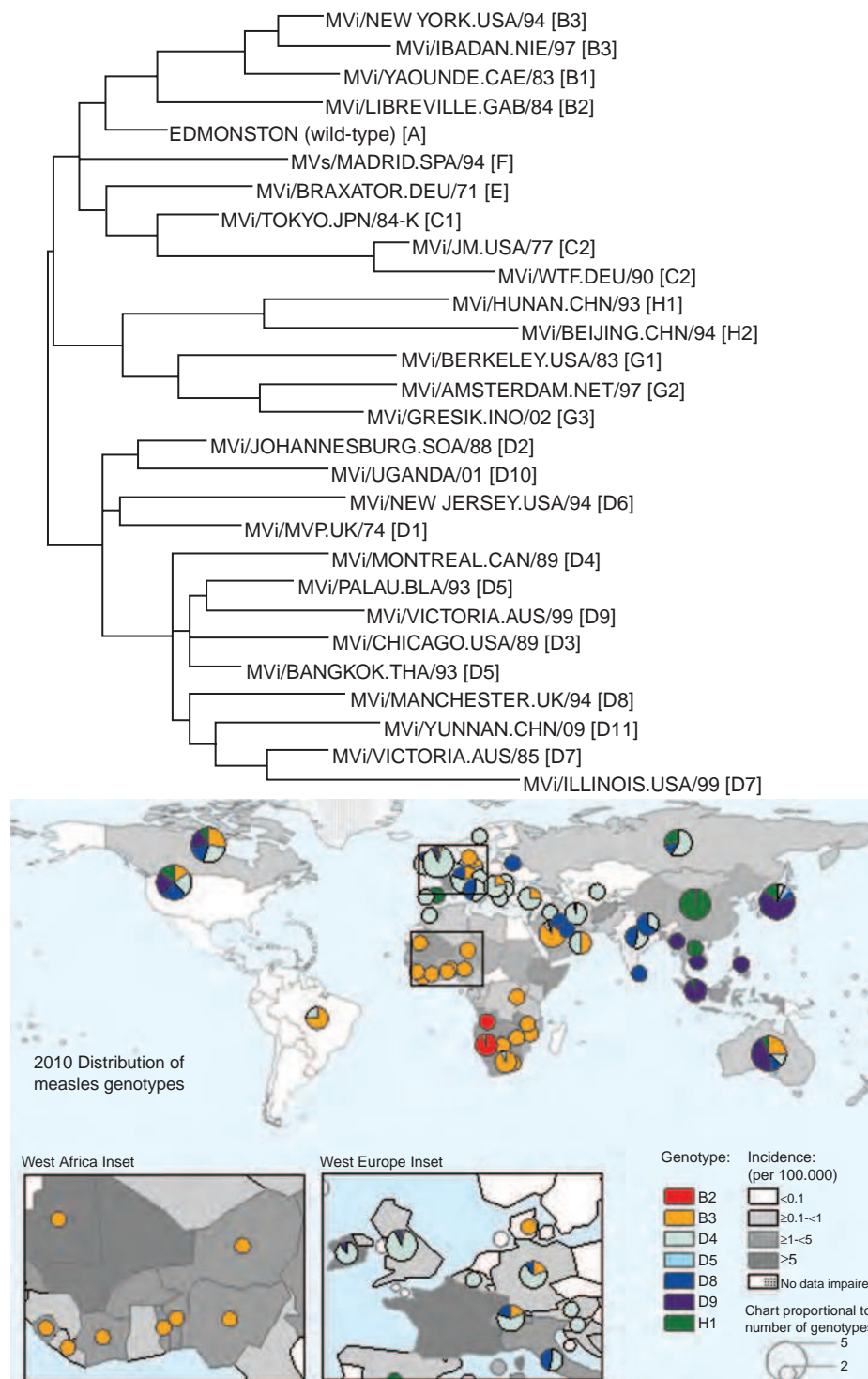
Entry

H attachment to a cellular receptor is followed by fusion of the virus envelope with the plasma membrane and delivery of the viral ribonucleocapsid into the cytoplasm for initiation of infection (Fig. 36.6). The H dimer of dimers (tetramer) associates with the prefusion F trimer in the secretory system of the host cell and exists as H-F hetero-oligomer on the virion surface (Fig. 36.3). The oligomers cooperate to induce fusion at neutral pH.⁵¹⁶ Fusion requires H and F to be from compatible virus species and prior cleavage of F₀ into F₁ and F₂.⁴⁷⁸ The MeV H protein stalk interacts directly with the MeV F protein head, suggesting that the metastable F trimer is shorter than the H tetramer, resulting in a staggered head domain arrangement on the virion surface (Figs. 36.3 and 36.6).^{385,527,547} The strength of H and F binding determines fusogenicity. More avid binding decreases fusion, indicating the need for H and F dissociation during the process of entry and productive infection.¹⁸⁹ Separate regions on the H stalk have been identified for F interaction and for F triggering, suggesting that these are discrete functions.^{79,547} It is postulated that interaction of H with its receptor on the cell membrane induces a reorganization of the dimer-dimer head domains that transmit receptor binding to the F contact zone in the H stalk to trigger refolding of F and membrane fusion.^{277,499,624} This H reorganization may be represented in the crystal structure of H with SLAM that shows two forms of the H dimer with the orientation shifted with respect to each other^{277,547} (Fig. 36.7).

Cytopathic Effects

MeV replication in cell culture results in cytopathic changes of three varieties: multinucleated giant cells (syncytia), altered cell shape, and inclusion bodies.¹⁸⁵ Cell-to-cell fusion occurs at neutral pH and syncytia formation occurs *in vitro* (Fig. 36.2) and *in vivo*¹⁸⁴ presumably using fusion mechanisms similar to those for virus entry (Fig. 36.6). Syncytia formation is facilitated by basolateral expression of H and F and the actin filament-plasma membrane cross-linker moesin and is inhibited by cytochalasin B.¹⁶⁷ Fusion of infected cells with uninfected cells may produce syncytia with 50 or more nuclei. Nuclei in the center of the syncytia have marginated chromatin¹⁸⁴ and are often undergoing apoptotic cell death¹⁹⁶ leading to plaque

FIGURE 36.9. Genetic variation in wild-type measles viruses (MeV) and geographic distribution of MeV genotypes. The World Health Organization (WHO) currently recognizes 23 genotypes and one provisional genotype of wild-type MeV. The phylogenetic tree (**top**) is based on the sequences of the N genes of the WHO reference strains for each genotype⁷⁷⁵ and the provisional genotype.⁸⁰⁴ Map (**bottom**) shows the global distribution of MeV genotypes and measles incidence in 2010. Colored circles indicate MeV genotypes reported to the WHO database for the year 2010, and the size of the circles is proportional to the number of genotypes reported for the indicated areas. Two areas, Western Africa and Eastern Europe, are also shown as inserts to provide more resolution. The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the WHO concerning the legal status of any country, territory, city, or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate borderlines for which there may not yet be full agreement. (Courtesy of David Featherstone, WHO, and Paul Rota, Centers for Disease Control and Prevention.)



formation *in vitro*. Infected cells may also change from a normal polygonal shape to a stellate, dendritic, or spindle shape with increased refractility to light. This type of “strand-forming” cytopathic effect appears after several passages and may be related to the production of DI particles.⁴⁴³

Both spindle-shaped cells and syncytial cells may contain intracytoplasmic and intranuclear inclusion bodies. Cytoplasmic inclusions are generally larger than nuclear inclusions and contain N-encapsidated RNA decorated with P, producing *fuzzy* or

granular nucleocapsids.⁷² Intranuclear Cowdry type A inclusion bodies are characteristic of morbillivirus infections and occur late in infection. CDV intranuclear inclusion bodies are usually complex nuclear bodies derived from nucleoli that contain N and a cellular heat shock protein.^{511,513} MeV N, when expressed alone, migrates to the nucleus^{309,680} where it can assemble into nucleocapsids that lack P and viral RNA and appear “smooth” by electron microscopy.^{140,512} Because binding of P to assembled nucleocapsids leads to cytoplasmic retention,³⁰⁹ it has been

postulated that the amount of P may be limiting late in infection, allowing N to move into the nucleus.^{72,309}

Budding

M plays a central role in virus assembly and release. In the absence of M, infectious particles are not released and expression of M alone leads to release of virus-like particles.^{102,109,556,611} To initiate virus assembly, M associates with the nucleocapsid, is co-transported to the plasma membrane,³²⁵ and interacts with the cytoplasmic domains of the F/H glycoprotein oligomers to promote virus budding.^{461,462,697} Proteins associate with detergent-resistant microdomains^{425,556,743} and budding is independent of the cellular endosomal sorting complex required for transport (ESCRT) system.⁶¹⁸ In polarized epithelial cells, budding is directed to the apical surface by the M protein despite the intrinsic glycoprotein targeting to the basolateral surface,^{418,487} and loss of apical targeting by M enhances cell-cell fusion at the expense of virus production.^{102,103,610}

Evolution, Antigenic Composition, and Strain Variation

Antigenically, MeV is a relatively stable virus. Antisera from individuals infected decades ago retain the ability to neutralize current wild-type strains of MeV and vice versa, although with varying efficiency.^{144,361,659} The observed rate of mutation of H in virus circulating in defined geographic locations is low, estimated at 5×10^{-4} per year for a given nucleotide,⁵⁹⁴ while the rate of mutation during growth *in vitro* is higher, estimated at 9×10^{-5} per replication for a nucleotide.⁶⁴⁴ Although historical accounts date the emergence of measles to approximately the 6th century (see History earlier), phylogenetic analysis of morbillivirus sequences suggests a more recent divergence from a common ancestor with RPV.²²² However, this more recent estimate may reflect the effects on sequence evolution of population bottlenecks after outbreaks and purifying selection to maintain protein function.^{222,561,769} The structure of H with carbohydrates masking the top surface and exposed receptor-binding sites on the side (Fig. 36.8) is postulated to constrain acquisition of mutations.^{276,608} Evidence of vaccine-induced selective pressure on wild-type strains of MeV has been identified in the noose and receptor-binding regions of H.^{209,236,623,658,659,713}

Nucleotide sequence variability, primarily in the N, P, and H genes, has been a useful tool for the MeV genotyping needed for molecular epidemiologic studies of transmission pathways.^{353,594,602} N genes differ by up to 7% in the C-terminal N_{TAIL} region, the region most often used for strain identification^{82,236} (Fig. 36.4). The P gene is most variable in the shared PV_{NTD},⁴⁵ and P gene sequencing has provided increased power to identify transmission routes when the N_{TAIL} sequences are identical.³⁵³ The H gene nucleotide sequence is most variable between residues 167 and 241 where the N-linked glycosylation sites are located, but can become regionally fixed.^{353,602}

Strains examined to date separate into eight different clades (A to H) and at least 24 different genotypes based on sequencing of the C-terminal 450 nucleotides of the N gene or the entire coding region of H.^{590,605,606,775,804} (Fig. 36.9). New genotypes are designated if the nucleotide sequence differs from the closest reference sequence by more than 2.5% in N or 2.0% in H.⁷⁷⁶ Some genotypes are found in one geographic region, others are co-circulating, while others are inactive and may be

extinct⁵⁹⁰ (Fig. 36.9). Live attenuated vaccines were all derived from genotype A wild-type strains and are quite similar.⁴⁶

PATHOGENESIS AND PATHOLOGY

Classic Measles

Measles is typically a childhood infection of humans spread by the respiratory route. Disease is characterized by a latent period of 10 to 14 days and a 2- to 3-day prodrome of fever, coryza, cough, and conjunctivitis followed by the appearance of a characteristic maculopapular rash (Fig. 36.10).³⁶² The onset of the rash coincides with the appearance of the immune response and initiation of virus clearance (Fig. 36.11). Recovery is accompanied by lifelong immunity to reinfection.⁵³⁰ Macaques exposed to infected humans or experimentally infected with wild-type strains of MeV develop a similar disease, and much of our more detailed understanding of pathogenesis, immune responses, and sites of virus replication come from studies of nonhuman primates, often facilitated by the use of engineered reporter viruses.^{29,143,146,441,735}

Entry and Sites of Primary Replication

MeV is efficiently transmitted over short distances by respiratory droplets and over longer distances by small-particle aerosols.^{119,617} High MeV infectivity suggests that the cellular sites of initial virus replication are very susceptible to infection. However, the nature of these cells is unclear because it has been difficult to identify MeV-positive cells in the respiratory tract at early times after infection.^{143,388} Although autopsy studies have shown abundant infection of respiratory epithelial cells,^{460,617} detailed studies of experimentally infected monkeys early after infection have only identified infected alveolar macrophages and subepithelial DCs.^{143,146,388} Because *in vitro* studies suggest that MeV infects epithelial cells from the basolateral



FIGURE 36.10. Measles virus rash.

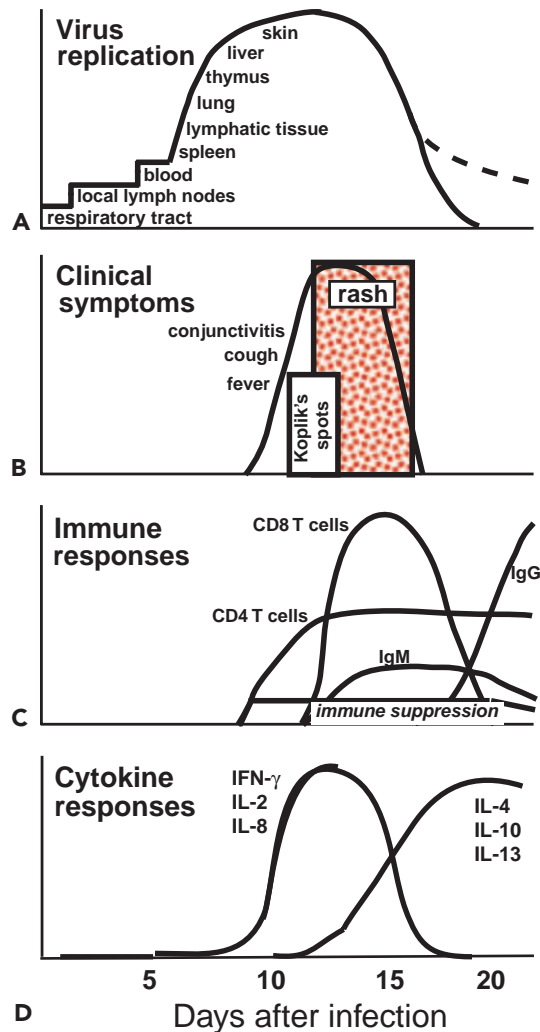


FIGURE 36.11. Pathogenesis of measles. Measles virus (MeV) is spread by the respiratory route and replication begins in the respiratory tract and spreads to lymphocytes, monocyte/macrophages, endothelial cells, and epithelial cells in the blood, thymus, spleen, lymph nodes, liver, skin, and lung and to the conjunctivae and mucosal surfaces of the gastrointestinal, respiratory, and genitourinary tracts. The rash appears at the time of the virus-specific immune response with activation of MeV-specific CD4+ and CD8+ T cells and synthesis of MeV-specific immunoglobulin M (IgM) and IgG antibody. Clearance of infectious virus is approximately coincident with fading of the rash, but clearance of RNA is slower. Cytokines produced are consistent with activation of Th1 CD4+ and CD8+ T cells, followed by Th2 CD4+ T cells and regulatory cells. Immune suppression is initiated during the rash and persists for weeks after its resolution.

surface^{414,674} and MeV engineered not to infect respiratory epithelial cells can still initiate infection after intranasal inoculation,³⁹⁰ epithelial cell infection has been postulated to be a late, rather than an early, event. It is possible that pulmonary macrophages and DCs take up and transport MeV to local lymphoid tissue where virus is amplified, leading to viremia and subsequent systemic spread of infection to many tissues, including the lung.⁷⁰³

Spread

MeV interaction with DC-SIGN leads to up-regulation of SLAM on DCs, MeV entry,³¹ and likely transport from the respiratory tract to local lymphatic tissues in lung and draining lymph nodes.^{339,652,663} *In vitro* and *in vivo* studies suggest that DCs can transfer infection to susceptible T cells.^{143,148,663} Replication in lymphatic tissue is efficient, and infected CD150-expressing monocytes, T cells, and B cells are detected in peripheral blood within 4 to 7 days after infection.^{29,198,264,439,736} Only rarely has infectious virus been isolated from plasma,⁵³⁸ but viral RNA can be detected by reverse transcriptase-polymerase chain reaction (RT-PCR). MeV-infected mononuclear cells increase expression of the integrins LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$) and transmembratory cups that promote adherence to endothelial cells and cell-to-cell transmission of infection.^{26,166,313,413} These properties likely facilitate virus dissemination. The viremia is accompanied by leukopenia due either to death of infected cells or to changes in leukocyte trafficking.^{29,517,613}

Target Cells and Tissues

From the blood, infection is spread to distal lymphoid tissue and to epithelial cells, endothelial cells, and macrophages in multiple organs.^{197,460} Transmigration across an endothelial barrier is impaired for MeV-infected lymphocytes,¹⁶⁶ so entry of MeV into tissues may occur primarily from endothelial cells infected by circulating leukocytes or by movement of other types of infected cells, such as monocytes, across blood vessel walls.⁴¹³ Once within tissue, spread is cell type and virus strain dependent and occurs by cell–cell fusion or by release of infectious virus. Tyrosine residues in the cytoplasmic tails of F (aa 549) and H (aa 12) are important for basolateral glycoprotein sorting and determine the fusogenic spread of MeV in epithelial cells.^{462,463} However, only the H sorting signal determines wild-type MeV release versus cell–cell fusion in lymphocytes.^{609,610}

Lymphoid organs and tissues (e.g., thymus, spleen, lymph nodes, appendix, and tonsils) are prominent sites of virus replication⁶¹⁷ where infection results in the appearance of lymphoid or reticuloendothelial giant cells first described by Warthin⁷⁵⁹ and Finkeldey.²⁰⁸ These cells can be 100 μ m or more in diameter and contain up to 100 nuclei aggregated near the center. Inclusion bodies are not generally present. Warthin-Finkeldey cells tend to be located in or near germinal centers, in the thymus, and in submucosal lymphoid tissue.^{279,509} In the thymus, infection of epithelial cells and thymocyte apoptosis lead to a prolonged decrease in the size of the thymic cortex, while other lymphoid tissues recover promptly.⁷⁷²

MeV also spreads to the skin, conjunctivae, kidney, lung, gastrointestinal tract, respiratory mucosa, genital mucosa, and liver (Fig. 36.11). In these nonlymphoid sites the virus replicates primarily in endothelial cells, epithelial cells, and macrophages.^{197,198,460,699} Endothelial cell infection may be accompanied by vascular dilatation, increased vascular permeability, mononuclear cell infiltration, and infection of surrounding tissue.¹⁵⁰ The histopathology of the measles rash suggests that the initial event is infection of dermal endothelial cells³⁵⁵ followed by spread of infection into the overlying epidermis with infection of keratinocytes in the stratum granulosum leading to focal keratosis and edema.⁶⁹⁹ Epithelial giant cells form and mononuclear cells accumulate around vessels.¹⁵⁰ Koplik's spots found on the oral mucosa are pathologically similar and involve the submucous glands.¹⁵⁰

On rare occasions there is spread to the nervous system. *In vitro* studies have demonstrated infection of brain microvascular endothelial cells by adherent MeV-infected T lymphocytes,¹⁶⁶ and infection of endothelial cells has been demonstrated in the brains of children dying of measles.^{197,358} Polarized endothelial cells can release virus from both the apical and basolateral cell surfaces, allowing access to the brain parenchyma, as well as the blood.¹⁶⁶ If neurons become infected, virus can spread through the central nervous system (CNS) from neuron to neuron without the release of infectious particles.¹⁷⁷ It has been suggested that the F protein interacts at the synapse with the substance P receptor neurokinin-1 to mediate transsynaptic spread.⁴¹⁹

Immune Responses

The immune responses to MeV are important for clearance of virus and recovery from infection and are directly responsible for several of the clinical manifestations of measles. Although infectious virus cannot be isolated after the rash is cleared, viral RNA can be detected for many weeks, indicating that complete viral clearance is a prolonged process.^{529,542,734} The roles of various components of the immune response in recovery from infection have been deduced from *experiments of nature* in which the outcome of MeV infection in patients with deficiencies of immunologic function has been documented and from the studies of monkeys depleted of specific components of the immune system.^{244,539,540,542} In general, deficits in antibody production permit recovery, while deficits in cellular immune responses may lead to slowed clearance and progressive disease (see Measles in the Immunocompromised Host later). In immunologically normal individuals, the onset of clinically apparent disease coincides with the appearance of the MeV-specific adaptive immune response. There is also marked activation of the immune system that is coincident with the appearance of immune suppression (Fig. 36.11). Immune suppression and immune activation continue for many weeks after apparent recovery.

EARLY INNATE RESPONSES

Innate responses may contribute to control of virus replication during the incubation period, but determining the role and importance of specific components of the innate response in measles has been complicated. *In vitro* studies have shown that innate responses triggered by interaction of MeV RNA or proteins with pathogen recognition receptors at the cell surface or in the cytoplasm to activate signaling pathways involving transcription factors nuclear factor- κ B (NF κ B) and IRF-3 differ with the strain of virus, are cell type specific, and are highly regulated by the viral P, C, and V proteins.^{63,172,284,345,625,646,718} Epithelial cells show activation of NF κ B and activator protein-1 (AP-1)³²⁰ and production of the chemokine CXCL8 (interleukin-8 [IL-8])⁶²⁹ after MeV infection. However, monocytes respond differently than epithelial cells, and interaction of H with TLR2 at the monocyte cell surface stimulates induction of IL-6 and increases surface expression of CD150,⁶³ while interaction with CD46 inhibits IL-12 production.³⁴⁴ The NF κ B pathway and tumor necrosis factor- α (TNF- α) production are suppressed in MeV-infected monocytes, potentially as a result of MeV P protein-induced up-regulation of the ubiquitin-modifying enzyme TNFAIP3 (A20), a negative regulator of NF κ B.^{393,629,756}

Some inflammatory cytokines and chemokines are induced *in vivo* during measles. Levels of IL-1 β and IL-8 are increased in plasma of children during measles,^{629,807} and infected macaques

show increases in IL-6 and IL-8.⁶²⁶ IL-1 β mRNA and protein are increased in MeV-infected monocyte-derived cells and in PBMCs cultured from patients after rash onset.^{393,756} Transcriptional analysis of PBMCs from children with measles has shown increases in mRNAs for cytokines IL-1 β and TNF- α and chemokines CCL4 (MIP-1 β), CXCL2 (MIP-2 α), and IL-8.⁸⁰⁷ The mRNA for CIAS-1 (NALP3), a component of the inflammasome responsible for processing proIL-1 β to its active form, is also increased.⁸⁰⁷

Type I IFN is an important component of the innate response to many virus infections, and MeV replication is sensitive to the inhibitory effects of IFN- α/β .^{391,640,714} MeV replication is required for induction of IFN- β transcription in most responsive cells.²⁸⁸ Two induction mechanisms have been identified. In epithelial cells, MeV leader RNA can interact with and activate RIG-I and, to a lesser extent, MDA5,^{318,555} and N can interact with and activate IRF-3 in concert with an unidentified cellular co-factor.^{128,718} Induction of IFN by MeV may also occur at the cell surface through interaction of the virus with CD46 or TLR2.^{63,345} *In vitro*, MeV infection of epithelial cells and DCs leads to rapid production of IFN- β and many IFN- α s followed by induction of IFN-responsive genes.^{463,625,633,714,808} On the other hand, MeV infection of mitogen-stimulated PBMCs does not usually stimulate IFN production.⁴⁹⁸ In fact, MeV suppresses type I IFN production and signaling in CD4+ T cells⁶²⁵ and has a variable effect on plasmacytoid DC IFN production.^{169,633}

Many of the reported effects of MeV on immune cell function *in vitro* are secondary to the effects of IFN. For instance, MeV induction of IFN inhibits development of DCs but stimulates maturation of immature DCs and terminal differentiation of cortical thymic epithelial cells.^{270,741} IFN also plays a role in suppressing proliferation of T cells in cultures of MeV-infected PBMCs.⁶¹⁹ However, interpretation of investigations related to IFN induction and its role in measles pathogenesis has been confounded by the frequent presence of 5' copy-back DI RNAs in the stocks of the virus strains studied.^{352,662} Vaccine strains are more likely to induce IFN- α/β than wild-type strains,⁴⁹⁸ but this may be related to the efficiency with which they generate DI RNAs,^{352,662,701} which are potent inducers of IFN through activation of MDA5.^{688,800}

It is not clear whether IFN- α/β is induced during MeV infection *in vivo*. Transcriptional analysis of PBMCs during measles shows no evidence of up-regulation of IFN-induced genes.⁸⁰⁷ No IFN- α/β has been detected during natural infection in humans or experimental infection of macaques.^{259,262,476,664,801} Biologically active IFN has been detected occasionally, but IFN- γ produced by T cells is produced in response to infection, and the protein responsible for IFN activity (type I or II) was not identified.^{543,626} A recombinant wild-type MeV that cannot interfere with STAT1 translocation is attenuated in macaques, suggesting some role for this signaling pathway in the response to infection.¹⁵⁶

Natural killer (NK) cells constitute another potentially important early defense mechanism, but studies of NK activity indicate that NK cell function is actually lower than normal during measles.²⁶² These studies were performed using samples collected at, or after, the rash, so they do not exclude NK cell activation at earlier stages of infection.

MeV and MeV-infected cells activate the factor B-dependent alternative complement pathway, rendering the cells susceptible to complement-mediated lysis.⁶⁷⁵ This is a property of F₁

and results in deposition of C3b on the virion and infected cell surface independent of virus use of the complement regulatory protein CD46 as a receptor.¹⁵⁴ In infected cells, the complement regulators CD46 and CD55 are segregated into separate membrane microdomains from F.²³³

ANTIBODY

Antibodies are first detectable when the rash appears^{55,75,249} (Fig. 36.11). The isotype of MeV-specific antibody is initially IgM followed by a switch first to immunoglobulin G2 (IgG2) and IgG3 and then, in the memory phase, to IgG1 and IgG4.^{75,322} IgG is initially of low avidity and this improves steadily over several months.^{488,726} IgA, IgM, and IgG antibodies to MeV are found in secretions and sampling of saliva has provided a noninvasive method for determining immune status.^{81,317}

Antibodies are eventually produced to most viral proteins (Fig. 36.12). The most abundant and most rapidly produced antibody is to N.²⁴⁹ Because of the abundance of anti-N antibody, absence of this antibody is an indicator of seronegativity. The M protein elicits only small amounts of antibody, except in atypical measles.^{249,415} Antibodies to H are the primary antibodies that neutralize virus infectivity.^{144,145,237} Neutralization is generally measured by plaque reduction of the Edmonston strain of MeV on CD46-expressing Vero cells,¹²⁵ but this assay may not reflect neutralization of the infection of wild-type MeV strains on SLAM-expressing cells.⁵⁵⁹ Neutralizing epitopes have been mapped by competitive binding of monoclonal antibodies and by analysis of different strains and escape mutants (Fig. 36.8).^{307,312,396,658} Human convalescent sera show reactivity to linear epitopes, as well as to epitopes dependent on conformation and glycosylation.^{305,451,482} A highly conserved linear neutralizing epitope is in the H noose (aa 379 to 410).⁵⁷⁰

Major conformational epitopes have been localized to regions between amino acids 368 and 396 and in the SLAM-binding region.^{192,396} Essentially all of these epitopes are on exposed surfaces on the sides of H (Fig. 36.8).^{276,622} Antibodies to F induced by regions encompassing amino acid 73 and amino acids 388 to 402 contribute to virus neutralization, probably by preventing fusion of the virus membrane with the cell membrane at the time of virus entry.^{25,144,201,420,420,557} Human sera also recognize linear epitopes in six to seven regions spread over much of the F protein frequently close to T-cell epitopes.^{481,778}

Antibody can protect from MeV infection, may contribute to recovery from infection, and may play a role in establishing persistent infection.^{9,186,577} Antibody-dependent cellular cytotoxicity correlates temporally with cessation of cell-associated viremia,²¹² and failure to mount an adequate antibody response carries a poor prognosis.⁷⁷⁰ However, in monkey studies transient depletion of B cells does not affect clearance of infectious virus.⁵³⁹ Antibody binding to infected cells alters intracellular virus replication and may contribute to control of infection.^{218,240,639} The role of antibody in protection from infection is discussed under Vaccination and the role in establishing persistence is discussed under Persistent Infection.

CELLULAR IMMUNITY

The ability to recover from measles was postulated by Burnet to be an indication of the adequacy of T-lymphocyte-mediated immune responses,⁸⁶ and depletion of CD8+ T cells in infected monkeys impairs control of virus replication and slows clearance.⁵⁴⁰ MeV-specific, proliferating, and clonally expanded CD8+ T cells are present in blood at the time of the rash and in bronchoalveolar lavage fluid during pneumonitis.^{331,464,476,485,732,757} IFN- γ , soluble CD8, and β_2 -microglobulin, a component of

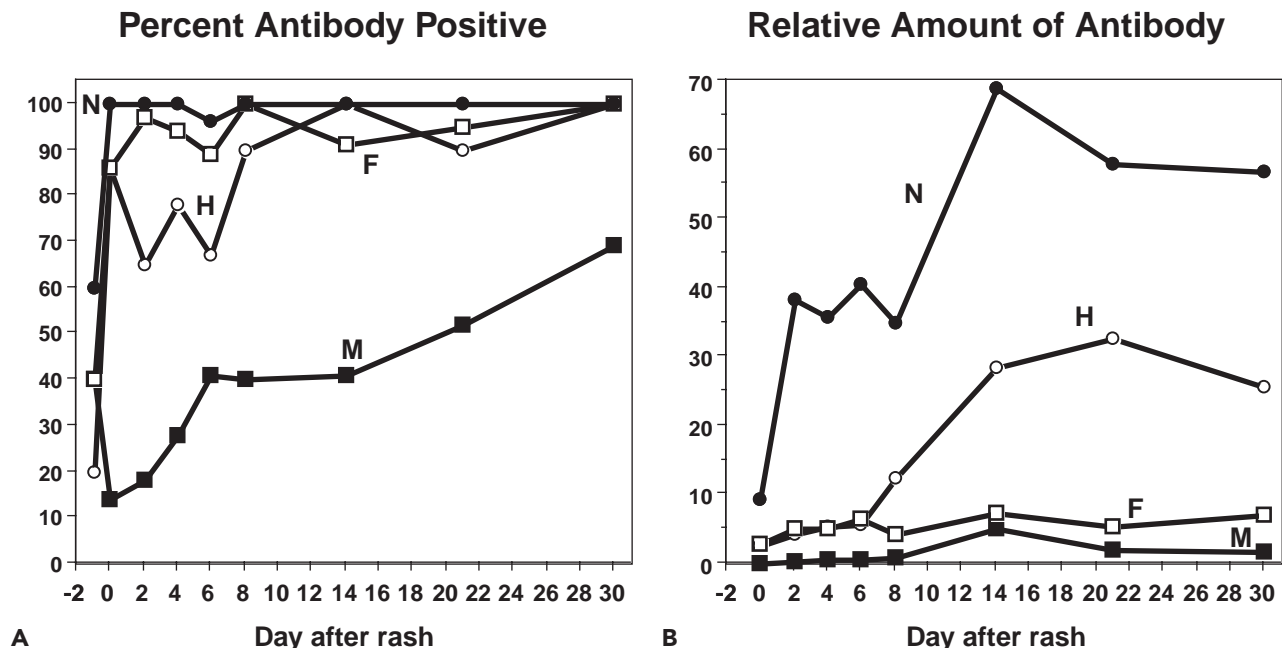


FIGURE 36.12. Production of antibody to measles virus (MeV) proteins during natural infection. A: Percent of individuals positive for antibody to each protein. **B:** Relative amounts of antibody to each protein. Antibody was measured by immunoprecipitation. (Adapted from Graves MC, Griffin DE, Johnson RT, et al. Development of antibody to measles virus polypeptides during complicated and uncomplicated measles virus infections. *J Virol* 1984;49:409–412.)

the major histocompatibility complex (MHC) class I molecule, are increased in plasma.^{259,260,261,476} The H and M proteins can be processed and presented to CD8+ T cells in a transporter associated with antigen processing (TAP)-independent fashion.⁵⁰² Cultures of PBMCs with autologous MeV-infected or MeV peptide-pulsed cells after recovery show expanded CD8+ T cells that are cytotoxic and produce IFN- γ , demonstrating that effector CD8+ T-cell memory is established by infection.^{331,332,492,732} CD8+ T-cell responses in humans show a broad pattern of reactivity with epitopes identified in all viral proteins, except V.^{289,330,331,523,737–739}

CD4+ T cells are also activated in response to MeV infection. *In vitro*, binding of H to CD46 targets virion proteins to an endosomal compartment for efficient presentation by MHC class II molecules.²³² CD4+ T cells are proliferating during the rash.⁷⁵⁷ Soluble CD4 becomes elevated in plasma and remains so for several weeks after recovery.²⁵⁸ Classic CD4+ T-cell responses, such as MeV-specific proliferation and production of cytokines, and regulatory T cells are stimulated during measles.^{476,732,801} In immune individuals, most MeV proteins can induce lymphocyte proliferation.^{430,480,737}

Cytokines produced by CD4+ T cells determine their function. Type 1 CD4+ T (Th1) cells produce IFN- γ that activates macrophages and IL-2 that promotes T-cell proliferation. These are the primary mediators of classical delayed-type hypersensitivity. Type 2 CD4+ T (Th2) cells produce IL-4, IL-5, IL-10, and IL-13 that are important for B-cell growth and differentiation and for macrophage deactivation. Th17 cells are associated with autoimmunity and produce IL-17, while T-regulatory cells produce IL-10 and transforming growth factor- β (TGF- β). In measles, IFN- γ , neopterin (a product of IFN- γ -activated macrophages), and soluble IL-2 receptor rise during the prodrome, prior to the appearance of the rash.^{259,261} This is followed by elevation of IL-2, soluble CD4, and soluble CD8 at the time of the rash.^{260,261,476} As the rash fades, IL-4, IL-10, and IL-13 increase, and elevation of these cytokines persists in some individuals for weeks^{260,476,801} (Fig. 36.11). This pattern of cytokine production suggests early activation of CD8+ (IFN- γ) and type 1 CD4+ (IFN- γ and IL-2) T cells during the rash followed by activation of type 2 CD4+ T cells (IL-4, IL-13) and regulatory T cells (IL-10) during recovery.

IFN- γ may have an important direct antiviral effect. IFN- γ can suppress MeV replication in epithelial and

endothelial cells *in vitro* through induction of indoleamine 2,3-dioxygenase⁵¹⁰ and inhibits MeV replication in the brains of infected rodents.^{534,764}

LONGEVITY OF THE IMMUNE RESPONSE

Epidemiologic studies have documented that long-term protection from reinfection does not require re-exposure.⁵³⁰ Immunologic memory includes both continued production of antibody and circulation of MeV-specific memory T cells.^{69,302,493,732,784} The role of the slow or potentially incomplete process of MeV clearance from lymphoid tissue in establishing lifelong immunity to reinfection is unknown.^{542,589} Extensive replication of MeV in lymphoid tissue may maximize the interaction of viral antigen with antigen-retaining follicular dendritic cells in germinal centers,⁴⁴¹ leading to long-term antibody production.

IMMUNE SUPPRESSION

MeV can suppress immune responses *in vivo* during measles, *in vitro* when immune cells are cultured with virus or viral proteins, and in some animal models. It has not yet been possible to synthesize information from these various sources into a coherent understanding of increased susceptibility to other infections, the important clinical correlate of immune suppression.

During Measles. Measles was the first disease recognized to increase susceptibility to other infections, and most measles deaths are caused by other infections.⁵⁶ Increased susceptibility continues for weeks after the rash has cleared.²⁴⁷ Clemens von Pirquet first quantified the immunosuppressive effects of measles in his study of tuberculin delayed-type hypersensitivity skin test responses during a measles outbreak in a tuberculosis sanitarium,⁷⁵⁰ and this response is suppressed for weeks after the rash has cleared and recovery appears complete⁷¹² (Fig. 36.13). Reactivation of tuberculosis and remission of immunologically mediated diseases such as nephrotic syndrome, juvenile rheumatoid arthritis, and idiopathic thrombocytopenic purpura have been reported to follow measles^{121,401} but not always confirmed.³⁸⁴ Production of antibody and cellular immune responses to new antigens is impaired.¹³¹ *In vitro*, PBMCs from patients with measles have suppressed lymphoproliferative responses to mitogens and abnormal lymphokine production.^{294,756}

Immune suppression is probably a multifactorial process. Evidence of immunosuppression begins during a period of intense immune activation associated with the onset of the

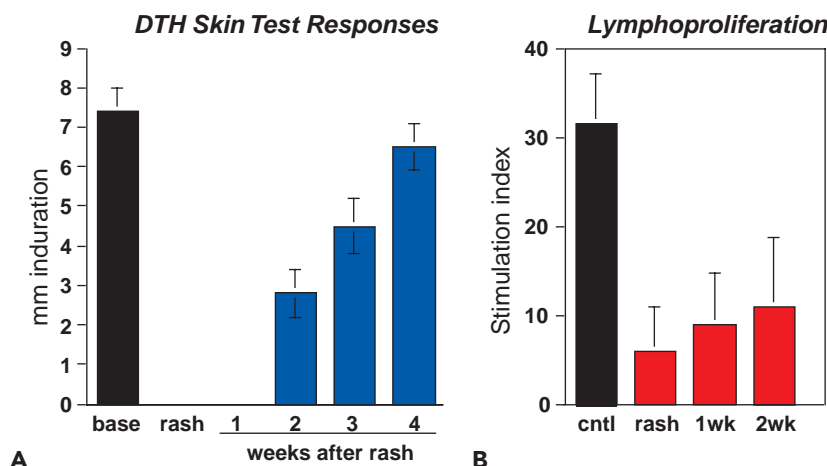


FIGURE 36.13. Immune suppression during measles. **A:** Changes during measles in tuberculin-induced delayed-type hypersensitivity (DTH) skin test responses in children with measles who had previously received bacille Calmette-Guérin (BCG) immunization against tuberculosis. **B:** Changes in proliferation of peripheral blood mononuclear cells to the mitogen phytohemagglutinin. (Data from Tamashiro VG, Perez HH, Griffin DE. Prospective study of the magnitude and duration of changes in tuberculin reactivity during complicated and uncomplicated measles. *Pediatr Infect Dis J* 1987;6:451–454; and Hirsch RL, Griffin DE, Johnson RT, et al. Cellular immune responses during complicated and uncomplicated measles virus infections of man. *Clin Immunol Immunopathol* 1984;31:1–12.)

measles rash and generation of the immune response to MeV that eventually results in virus clearance and in lifelong immunity to reinfection (Fig. 36.11). *In vitro* studies suggest defects in the responses of both monocytes and lymphocytes. Monocytes and macrophages are infected during measles,¹⁹⁶ and monocyte function is abnormal with low levels of IL-12 and TNF- α production during both acute and convalescent phases of the disease.^{24,255,546,560} B cells and T cells, particularly CD4+ memory T cells, are also infected,^{143,441} with decreased numbers in circulation and reduced CD4:CD8 ratios during the acute phase of measles.^{23,141,256,613,757,773} Lymphocytopenia may be due to death of infected cells or to fever-induced changes in lymphocyte trafficking and is more marked in girls and in malnourished children.^{3,196,613} More severe disease has been associated with decreased CD4+ T cells.³⁵⁴ Induction of bystander lymphocyte apoptosis by MeV-infected cells may contribute to lymphocyte loss.^{27,217,379,517,752} Ligation of CD150 also favors CD95-mediated apoptosis.^{187,670} However, numbers of T cells in circulation rapidly return to normal during recovery, and output of naive CD4+ and CD8+ T cells from the thymus is sustained,^{28,541,729} while other immunologic abnormalities persist.^{29,253,613,756}

One of the reasons for low T-cell proliferation to mitogens *in vitro*, especially during convalescence, is inadequate production of IL-2, and supplementation supports spontaneous proliferation and improves mitogen responses of lymphocytes.²⁵⁶ There is persistent suppression of IL-12 production, lymphocyte expression of CD30, and elevation of IL-4, IL-10, and IL-13 after resolution of the rash (Fig. 36.11).^{96,258,546,742} Lack of IL-12 and induction of IL-10 may contribute to the type 2 cytokine responses that develop during recovery.^{258,476} *In vitro* production of IFN- γ is low to normal and IL-4 is high compared to controls,^{258,756} suggesting that type 2 CD4+ T cells are preferentially activated. The suppressive effects of IL-4 and IL-10 on type 1 CD4+ T cells and macrophages may contribute to suppression of delayed-type hypersensitivity during measles. The role of persistent MeV RNA in these PBMC defects has not yet been evaluated.

Th2 cytokine predominance produces an environment favoring B-cell maturation that facilitates the establishment of humoral memory important for lifelong protection from reinfection while depressing macrophage activation and induction of type 1 responses that may be required for combating new pathogens. Infection of monkeys with an IL-12-producing recombinant MeV increased production of IFN- γ and suppressed production of MeV-specific antibody, but there was no improvement in lymphocyte proliferation.²⁹⁵ Similarly, infection of cotton rats with an IL-4-producing recombinant MeV did not affect mitogen-induced lymphocyte proliferation.⁹⁶ These data suggest that the MeV-induced Th2 cytokine milieu may alter responses to other pathogens and thus contribute to increased susceptibility to infection, but do not explain the characteristic measles-induced defect in lymphoproliferation.

***In vitro* Infection of Immune Cells.** *In vitro* infection of leukocytes and leukocyte cell lines with MeV induces abnormalities of DC, monocyte/macrophage, B-cell, T-cell, and epithelial cell function. T-cell proliferation to mitogens and soluble antigens,⁴³⁷ cytotoxic function,⁹⁸ and B-cell production of immunoglobulin^{98,438} are suppressed by *in vitro* infection, and bone marrow stromal cells do not support development of hematopoietic stem cells.⁴²⁴ In addition, both B and

T cells can produce a soluble factor that inhibits proliferation of uninfected cells.^{692,755} These abnormalities may be relevant to immune suppression during measles, although some can be attributed to production of type I IFN in the MeV-infected cultures.

Infection of monocytes or macrophage-lineage cells *in vitro* stimulates production of IFN- α/β , inhibits production of TNF- α and IL-12, and may interfere with expression of peptide-loaded MHC class II complexes.^{344,393,619,793} Virus production decreases as macrophages mature.^{217,283} MeV infection of immature DCs induces maturation,^{642,653} but MeV replication impairs CD40L signaling necessary for terminal differentiation.⁶⁵³ Mannose receptor-mediated endocytosis is not affected.²⁶⁵

Monocytes and DCs exposed to MeV or N protein have impaired production of IL-12 but preserved or increased production of the regulatory cytokine IL-10.^{217,269,344,427,653} MeV interaction with CD46 increases nitric oxide and decreases IL-12 production,^{292,344} and interaction with DC-SIGN during infection of DCs increases IL-10 transcription by inducing acetylation of TLR-activated NF κ B subunit p65.²⁶³ Suppression of IL-12 production in response to TLR4 ligation is facilitated by H interaction with SLAM.²⁶⁹ N_{CORE} interacts with Fc γ R2 on B cells to inhibit antibody production *in vitro* and can trigger apoptosis.⁵⁷⁹ N_{TAIL} binds to thymic epithelial cells and activated T cells through an unidentified receptor to induce cell cycle arrest.⁵⁷⁹

Analysis of antigen-presenting function has shown that MeV-infected monocytes can present MeV, but not an unrelated antigen, to T-cell clones.³⁹² DC antigen-presenting function has been assessed primarily with the mixed leukocyte reaction (MLR) that measures proliferation of heterologous CD4+ T cells.^{642,681} The ability to stimulate this allogeneic T-cell response is lost after MeV infection, and the cells become apoptotic when co-cultured with T cells.^{217,265} MeV-exposed T cells are recruited into conjugates with DCs *in vitro* but have impaired clustering and maintenance of immune synapse proteins needed for sustained T-cell activation,⁶⁶⁶ in part due to sphingomyelinase activation and accumulation of ceramide.^{227,483,725}

The importance of the *in vitro* deficiencies identified in DC function is unclear. Microarray analysis of the transcriptional changes in DCs infected with MeV in comparison with other pathogens revealed that many of the changes postulated to be responsible for MeV-induced immune suppression were also induced by pathogens that are not associated with immune suppression⁸⁰⁸ and may be associated with IFN production. A vigorous MeV-specific cellular and humoral immune response is mounted to infection. This response results in rapid clearance of infectious virus, gradual clearance of viral RNA, and establishment of lifelong protective immunity.

Lymphocytes infected with MeV *in vitro* can be activated by mitogen but proliferate poorly because entry into S phase and progression through the cell cycle are impaired.^{57,225,437,440,496,641,789} Suppression of T-cell proliferation can also be induced without infection through direct inhibitory signaling to T cells by the viral glycoprotein complex of H and F₁-F₂ on virions or infected cells.^{170,360,634,642,766} This contact-dependent inhibitory signal prevents S phase entry of T cells for several days with accumulation of cells in the G₀/G₁ phase^{503,634,766} and is not dependent on cell death, membrane fusion, production of soluble inhibitors, or T-cell infection.^{170,188,505,641,765} Blocking the interaction of H with antibody to SLAM or CD46 does not interfere with

suppression of lymphocyte proliferation,¹⁹⁰ but antibody to H or F can reverse the inhibition.¹⁷⁰

MeV interaction with lipid rafts on the surface of T cells affects association of signaling molecules and their regulators with lipid rafts and interferes with T-cell activation of phosphatidylinositol 3 (PI3) kinase/Akt necessary for cell cycle progression in response to ligation of the T-cell receptor or the IL-2 receptor.^{30,33} Binding of the MeV glycoprotein complex to lipid rafts on resting T cells inhibits degradation of the cytoplasmic inhibitory protein Cbl-b and recruitment of Akt kinase and Vav.³³ In addition, induction of SIP110 phosphatase decreases availability of phosphatidylinositol-3,4,5-trisphosphate (PIP3) needed for phospholipid signaling.³² Determination of the relevance of this process to *in vivo* suppression of lymphoproliferation requires further study, but ongoing interaction of T cells in lymphatic tissue or in circulation with MeV-infected cells could induce this refractory state and result in suppressed proliferation in response to stimulation *ex vivo*.

Animal Models. Small animal models, primarily cotton rats and CD46 and SLAM transgenic mice, have also been used to study immunosuppression *in vivo* after MeV infection. Respiratory infection of cotton rats results in decreased proliferation of cultured T cells.⁵⁰⁴ Intravenously or intraperitoneally infected CD46 transgenic mice have impaired T-cell cytotoxicity and antibody production, and this is associated with increased susceptibility to bacterial infection.^{520,676} MeV infection of SLAM transgenic mice inhibits differentiation of DCs from bone marrow precursors through STAT1-independent, STAT2-dependent actions of type I IFN.^{96,270} N_{CORE} interacts with FcγRII on DCs to inhibit cutaneous hypersensitivity in mice.⁵⁷⁹ The relevance of these observations to the immune suppression that occurs during human infection is a matter of continued investigation.

AUTOIMMUNITY

An autoimmune demyelinating disease, postinfectious or acute disseminated encephalomyelitis (ADEM), is an important complication of measles⁴⁵⁷ (Fig. 36.14) and is associated with an immune response to myelin basic protein³³⁷ similar to that seen in animals with experimental autoimmune encephalomyelitis. The similarity of these diseases and lack of MeV in the brain have led to the current understanding that ADEM is an autoimmune disease³³⁷ induced during measles.

The mechanism of induction of autoimmune disease is not clear. Hypotheses have included altered presentation of myelin antigens due to MeV infection of oligodendrocytes, “molecular mimicry” of myelin antigens by MeV, and dysregulation of immune responses.^{294,328} There is little evidence for MeV infection of cells in the nervous system.^{230,337,460,460} The possibility of molecular mimicry between myelin basic protein

and an MeV protein has been explored. Neither cross-reactive antibodies nor cross-reactive T-cell clones have been identified in humans or rats with demyelinating disease induced in conjunction with MeV infection.³⁹⁷ Genetic susceptibility to ADEM has been postulated, but the numbers of patients studied have been insufficient to clearly identify a link between MHC antigens or other genetic markers and disease.³⁸³

Studies of immune regulation have shown that patients with encephalomyelitis differ from patients with uncomplicated disease by having a distinct pattern of immunologic abnormalities. IgE is more persistently elevated and soluble IL-2 receptor is lower.^{254,261} The timing of autoimmune disease suggests that immune dysregulation during measles may play a role in allowing activation and expansion of autoreactive lymphocytes.

Release and Transmission

Epidemiologic data suggest that infected individuals become infectious for nonimmune contacts a few days before the onset of the rash.³⁶² At this time virus can be cultured from the mucous membranes of the nasopharynx, conjunctivae, and mouth,¹⁸⁰ suggesting that the respiratory tract is the site of virus release. Multinucleated epithelial giant cells are readily demonstrated in nasal secretions and the conjunctivae during the prodrome and first days of the rash and are also shed into the urine.^{400,632} A recombinant virus that cannot infect epithelial cells is not shed from the respiratory tract of infected macaques, further suggesting that epithelial cells are the source of virus that is transmitted.³⁹⁰

Virulence

Strains of MeV can clearly differ in virulence because attenuated vaccine strains cause little disease in humans and wild-type strains cause measles. Sequences of these strains have been compared and several changes that may contribute to attenuation have been identified, but *in vivo* testing of virulence is difficult because of the lack of a good small animal model.⁷⁰⁷ Because adaptation of wild-type MeV to growth in Vero cells selects for a virus that no longer causes a rash in monkeys,^{43,365} many studies of virulence have focused on the sequence changes required for wild-type viruses to grow in Vero cells. Vaccine strains tend to use the CD46 receptor efficiently, while wild-type strains do not (determined in part by H aa residues at 390, 416, 446, 481, and 492), but this alone does not determine virulence.⁶⁹⁵ In addition to changes in H, attenuation and improved growth in Vero cells or chicken embryo fibroblasts are also associated with amino acid changes in the P/C/V, M, F, and L genes.^{42,169,346,489,656,697,698,704,706,788}

Virulence has also been assessed by the study of replication of MeV in human tissues explanted into culture or implanted into immunodeficient mice. In thymic implants wild-type

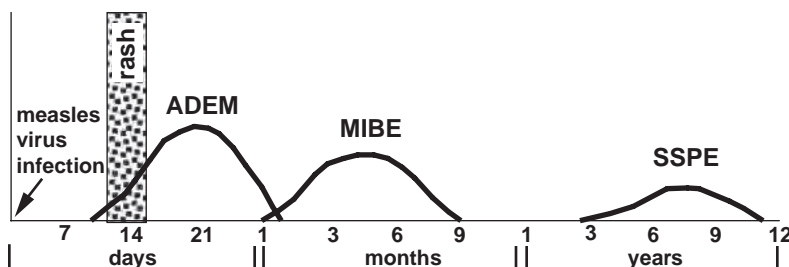


FIGURE 36.14. Time after infection of the occurrence of the three neurologic complications of measles: postinfectious or acute disseminated encephalomyelitis (ADEM), measles inclusion body encephalitis (MIBE), and subacute sclerosing panencephalitis (SSPE).

viruses grow more rapidly, to higher titer, and cause more extensive thymocyte apoptosis than attenuated strains.^{28,730} In human mononuclear cell implants and tonsil explants, wild-type strains grow better than vaccine strains.^{129,312} Attenuated vaccine strains infect naive T cells (express CD46, but little SLAMF7) more efficiently than virulent wild-type strains but infect B cells, macrophages, and NK cells less efficiently.¹²⁹ Again, the regions of the genome and the specific amino acid substitutions associated with these changes in replication and tissue destruction have not yet been defined.

Persistent Infection

MeV can establish persistent infection of cells *in vitro* and *in vivo*. A number of cell lines persistently infected with MeV have been established. Neurologic disease can be caused by persistent infection of neurons and glial cells.

Persistently Infected Cells in Culture

Replication of MeV usually causes death of cells in culture, but this is not necessarily the case *in vivo*. The elements determining persistent noncytopathic versus lytic infection are not completely understood, but properties of both the infected cell and the infecting virus contribute. Methods used to establish persistently infected cell lines include (a) passage of virus at high multiplicity with generation of DI particles,⁵⁹² (b) passage of infected cells in the presence of antibody,⁶¹² (c) cultivation of cells surviving lytic infection,^{204,612} and (d) co-cultivation of cells with MeV-infected brain cells from patients with SSPE or animals with persistent infection.⁴⁵⁰

Persistent noncytopathic infection is most easily established in neuronal cells but has also been established in lymphoid, epithelial, and glial cell lines.^{456,593} Cellular factors that influence persistence include expression of heat shock proteins, cyclic adenosine monophosphate (cAMP), IFN-inducible proteins, and altered regulation of lipid metabolism.^{456,593,597,640,700} Cellular protein synthesis is relatively unaffected by MeV infection, but specific cellular proteins (e.g., cell surface receptors) and functional responses (e.g., signal transduction, expression of transcription factors) may be altered, particularly in neural cells.^{54,210}

Strains of MeV and CDV differ in their ability to establish persistent nonlytic infection in the same host cell.²⁰⁴ Replication of variants likely to cause persistent infection is often temperature sensitive.⁵⁷⁴ Persistent infection is usually accompanied by a marked decrease in the amount of infectious virus released and by intranuclear and intracytoplasmic accumulation of nucleocapsids with decreased virus-induced cytopathic effect. Some cell lines produce no infectious virus,⁸⁷ and infection is maintained by passage of encapsidated viral RNA to daughter cells during cell division.

Limited expression of viral proteins on the surface of persistently infected cells has led to the suggestion that defects in synthesis of viral envelope proteins or processing of F may be an important component of persistent infection.^{450,797} Defects in glycoprotein expression may be due in part to limited mRNA production associated with steep transcriptional gradients and an increase in bicistronic messages.¹⁰⁷ However, mutations in these genes also lead to proteins with altered expression or function. For instance, defects in the M protein facilitate persistence and hinder association of N with the viral glycoproteins.⁵³⁵

Subacute Sclerosing Panencephalitis

SSPE is a rare (approximately 1 in 10,000) late complication of measles for which the pathogenesis remains poorly understood.^{61,702} The route of virus entry into the CNS is unknown, but infection of cerebral endothelial cells is one possibility.^{197,358} SSPE is most likely to occur when MeV infection occurs under the age of 2 years when the immune system is immature and maternal antibody may still be present.^{61,152,272,326,455,458}

There is no clustering of cases to suggest that the wild-type virus causing the initial infection is different from the virus causing uncomplicated disease. Extensive sequence analysis of viral RNA from various parts of the brain in SSPE suggests that the virus in the CNS is clonal,³⁷ implying that virus entered the brain at one time (presumably during the original acute infection), was not cleared, and then gradually spread throughout the nervous system. The typical incubation period is 7 to 10 years⁴⁵⁸ (Fig. 36.14). There may also be a genetic predisposition to developing persistent infection.^{323,324}

STUDIES OF BRAIN AND BRAIN-DERIVED CELL LINES

At the time that neurologic symptoms occur, neurons and glia contain nuclear and cytoplasmic viral inclusion bodies, antibody responses to virus are vigorous and evident both in serum and CSF, and there is an extensive mononuclear inflammatory reaction in the CNS.^{80,142,287} Both white matter and gray matter are affected.¹⁴² No virus is seen budding from the surface of infected cells, and nuclear inclusions are filled with “smooth” nucleocapsids.^{171,287} The cytoplasm contains “fuzzy” nucleocapsids that extend into neuronal processes, suggesting that virus can spread within the CNS by synaptic transmission of the ribonucleoprotein from cell to cell, a process that has been observed both *in vivo* and *in vitro*.^{175,177,382,631} Neurokinin-1, the substance P receptor, may interact with the MeV F protein at the synapse for neuronal transmission.⁴¹⁹ In CDV infection, astrocytes participate in rapid noncytolytic spread from cell to cell to establish persistence.⁷⁸⁷

Alterations in any of the MeV envelope proteins that in some way interfere with assembly and budding of infectious virus can be associated with persistent infection and SSPE.^{38,108,110,635} Extensive sequence analysis of viral RNAs from tissue has shown that SSPE viruses are related to wild-type strains circulating at the time of the primary infection but frequently have mutations in the viral genes encoding the M, F, and H proteins.^{38,110,335,635}

In general, expression of M, a protein particularly important for assembly of infectious virus, is low.³⁹⁵ This may be due either to lack of M protein synthesis or instability or mislocalization of the synthesized protein.^{334,657,682} A variety of defects have been encountered in the mRNAs encoding M extracted from SSPE brain.¹¹⁰ Mutations occur throughout the gene, and the frequent U-to-C sequence changes noted in some viruses may suggest mutation of double-stranded RNA in persistently infected cells by adenosine deaminase (biased or A/I hypermutation).^{104,783} M transcripts often lack initiator AUGs necessary for expression and, when expressed, the proteins have defects in binding to viral nucleocapsids and in down-regulating transcription.^{290,693} H proteins are often defective in intracellular transport and protein–protein interactions important for cell–cell fusion.¹⁰⁸ Truncations, mutations, and deletions in the cytoplasmic domain of F are almost universal and some enhance fusion and thus interfere with virus budding.^{35,108,635}

IMMUNE RESPONSES

The possibility that the development of SSPE is due to an immune defect has been investigated. The antibody response to MeV is accentuated with significant production of MeV-specific antibody by plasma cells residing in the CNS and present in CSF.^{85,526} Levels of CSF immunoglobulin, much of which is MeV specific, are elevated.^{195,724} Antibody produced in the CNS is of restricted heterogeneity, leading to the appearance of oligoclonal immunoglobulin bands on electrophoretic analysis of CSF. Antibodies against the N and P proteins present in the ribonucleoprotein complex are particularly abundant, and antibody against the M protein is particularly deficient.²⁷¹

Antibody to MeV has been postulated to play a role in establishing persistent CNS infection either through alteration of the induction of the primary immune response at the time of infection⁵⁷⁷ or through modulation of infection once virus is in the nervous system.²¹⁸ Treatment with antibody after intracerebral infection of small mammals with neuroadapted strains of MeV attenuates acute disease but increases the incidence of subacute or chronic encephalitis.⁵⁷⁶ Cases of SSPE have been associated with passive transfer of immune globulin, and persistent infection has been induced experimentally by passive transfer of antibody.⁵⁷⁷

Studies of cellular immunity have been less extensive. MeV-specific lymphoproliferation is not impaired, but MeV-specific cytotoxic T-cell activity and production of IFN- γ are decreased compared with healthy seropositive individuals.^{158,275} General measures of cellular immunity, such as skin test responses to various recall antigens and proliferative responses to mitogens, are normal. The mononuclear inflammatory response in the brain includes CD4+ and CD8+ T cells, as well as monocytes and Ig-secreting B cells.^{20,195} Class I and class II MHC expression is increased in brain, and β_2 -microglobulin, soluble IL-2 receptor, and soluble CD8 are increased in CSF.⁴⁴⁸ Thus, there is no evidence for a global defect in immune responses, but these immune responses are ineffective in clearing virus from the CNS.

Other Chronic Diseases

In addition to a clear role in SSPE, MeV in combination with genetic factors has also been implicated in the pathogenesis of Paget disease,^{216,376,673} otosclerosis,^{442,645} chronic active hepatitis,⁵⁹⁶ and multiple sclerosis.¹⁵ For most of these diseases an etiologic link to measles is controversial^{282,434,453,575,777} and for none has a definitive role for MeV been identified.

Veterinary Correlates

Canine Distemper

Canine distemper was first described by Carre in 1905 as an infectious disease of young dogs associated with gastroenteritis, pneumonitis, conjunctivitis, and encephalomyelitis.⁹⁴ The disease has been recognized for centuries and epidemics were recorded in the 17th century.⁷⁰ Canine distemper was shown to be of viral etiology in 1926,³⁷⁸ and the virus was isolated in primary canine kidney cells in 1959.⁵⁹⁸ CDV causes disease in both domestic dogs and wild canids.^{16,245,465} Through adaptive mutation in the H gene, CDV has acquired a broader host range than many morbilliviruses and can cause disease in monkeys and all families of terrestrial carnivores.^{149,436,465,571} Canine distemper has many similarities to measles in humans, but infection of the CNS and neurologic disease are more common

with glial cells as a primary target.⁷⁴⁰ CDV is spread by aerosol and the disease is characterized by fever, coryza, conjunctivitis, gastroenteritis, and pneumonitis.¹⁷³ Using ferrets as a model system, researchers showed that CDV appears first in bronchial lymph nodes followed by a cell-associated viremia and spread to multiple lymphoid organs.⁷⁴⁷ Later, infection spreads to epithelial tissues and enters the brain by infecting endothelial cells or through infiltration of infected monocytes.³⁴ Like measles, canine distemper is associated with profound short- and long-term immune suppression, and much of the mortality is due to secondary infections.

Dogs, ferrets, and lions may develop neurologic complications characterized by gait abnormalities and seizures.¹⁷³ Neurologic disease is most common in young animals³⁷² and can occur either early after infection or 3 to 4 weeks later associated with demyelination.⁷⁴⁰ Persistence of virus in neural cells and in epithelial cells of the feet leads to chronic diseases known as old dog encephalitis and hard pad disease.^{21,371} Some cases of old dog encephalitis have defective virus, with intranuclear inclusions in the CNS co-existing with high levels of antiviral antibody, features in common with SSPE.

Rinderpest

Rinderpest virus was first recognized as a distinct clinical entity causing severe gastroenteritis in cattle during a 4th-century European epizootic.⁵⁵³ The causal agent of this disease was reported to be filterable in 1902⁴ and was isolated in bovine embryonic kidney cells in 1957.⁵⁵⁴ RPV has been an important veterinary pathogen⁵¹ and causes a highly contagious disease of ruminants and swine characterized by inflammation, hemorrhage, necrosis, and erosion of the gastrointestinal tract.⁴ RPV replicates in lymphocytes and macrophages, leading to lymphoid necrosis, and in epithelial cells of the gastrointestinal, respiratory, and urinary tracts and endocrine and exocrine glands, but not the brain or spinal cord.^{687,780} Vaccination has recently succeeded in eradicating this disease from its last sites in Africa.^{301,467}

Peste des Petits Ruminants

Peste des petits ruminants virus (PPRV) causes an economically important disease of sheep, goats, buffalo, and camels in Africa, the Middle East, and Asia.^{157,377} The disease was first discovered in West Africa in 1942 and is characterized by fever, erosive stomatitis, pneumonitis, diarrhea, and lymphoid cell depletion.⁴⁷⁰ PPRV replicates in epithelial cells, pneumocytes, macrophages, and lymphocytes.³⁷⁵ PPRV is related to RPV⁴¹ (Fig. 36.1), and four lineages are recognized. Disease in northern and eastern Africa is increasing, perhaps due to the introduction of a more virulent Asian strain into the region.³⁷⁷

Morbillivirus Infections of Aquatic Mammals

In 1987, an epizootic of respiratory disease with high mortality due to secondary infections occurred in seals in the Baltic and North Seas.⁷⁴⁵ Serology suggested infection with a morbillivirus related to CDV, and the causal agent, phocid distemper virus (PDV), was isolated in 1988 (Fig. 36.1).³⁵¹ Infection has since spread to otters in the Pacific Ocean and seals along the East Coast of the United States.^{241,545} The disease is characterized by fever, nasal and ocular discharge, and severe respiratory, gastrointestinal, and CNS symptoms. In the CNS there is neuronal infection with inflammation.⁶⁸³ Disease is complicated by

a variety of secondary viral, bacterial, and parasitic infections, suggesting the type of immune suppression seen in measles and canine distemper virus.⁵²² Similar epizootics of severe respiratory disease were subsequently recognized in porpoises and dolphins, and distinct morbilliviruses (PMV and DMV, now grouped together as cetacean morbillivirus [CeMV]) were isolated from these animals⁵² (Fig. 36.1). PDV and CeMV cause giant cell pneumonia, encephalitis, and lymphoid depletion with virus demonstrable in lung, brain, spleen, and bladder.¹⁶¹

Experimental Infection in Animals

Nonhuman primates can be infected naturally or experimentally with MeV and provide the only experimental model for the pathogenesis of human disease. Small laboratory mammals generally are not susceptible to wild-type strains of MeV, although replication has been reported in the respiratory tract of cotton rats⁷⁸⁵ and in transgenic mice expressing the human MeV receptor CD46⁴²¹ and/or SLAM.⁶⁶³ Several neurotropic strains of MeV have been developed by repeated intracerebral passage in hamsters, mice, and rats. These strains do not produce acute disease similar to that seen in humans, but rather serve as models for neurologic diseases such as SSPE.

Nonhuman Primates

Natural MeV infection occurs only in humans. However, a number of species of monkeys are susceptible to measles and can contract the disease through contact with humans.^{583,735} Experimentally, monkeys can be infected by intranasal, intratracheal, or subcutaneous inoculation.^{29,735} Disease is generally similar to that seen in humans, and when deaths occur they are often caused by secondary infections.⁵⁸³ Rhesus and cynomolgus macaques have served as animal models for studies of measles pathogenesis, immune suppression, virus virulence, response to vaccines, and protective immunity.^{29,178,182,389,402,439,441,528,735}

Marmosets and tamarins are also susceptible to natural and experimental MeV infection by the respiratory route but develop a fulminant disease with high mortality characterized by interstitial pneumonitis, gastroenterocolitis, and bacteremia.⁴⁰⁹ Infection with vaccine strains produces little disease.⁴⁰⁹ Intracerebral inoculation with the most virulent strains of MeV produces encephalitis, while attenuated strains do not.¹¹

Cotton Rats

The respiratory tract of cotton rats (*Sigmodon hispidus*) can be infected with vaccine and wild-type strains of MeV.^{544,785} Virus replicates in the lungs and wild-type strains can spread to mediastinal lymph nodes and induce immunosuppression as measured by decreased proliferation of spleen cells to mitogen stimulation.⁵⁴⁴ Cotton rats have been used for studies of pathogenesis, immune suppression, vaccine-induced immune responses, and protection from infection.^{96,359,463,544,569,785,786}

Rats

The hamster neurotropic (HNT) strain of MeV can infect laboratory rats after intracerebral inoculation. The CAM strain of MeV was adapted by serial passage to replicate in rat brain and causes fatal encephalitis in newborn rats.³⁹⁹ The type of disease produced in older rats is determined by the strain of rat. In Brown Norway rats, 10% to 20% develop acute encephalitis and there is no late disease in survivors. In Lewis rats, 80% to 90% develop acute fatal encephalitis and the rest develop sub-

acute disease. Infectious, cell-free virus can be recovered during acute, but not subacute, disease.³⁹⁹ Unlike mice, transgenic expression of human CD46 does not increase the permissiveness of rats for MeV infection.⁵⁰⁶

Rats have been used to identify determinants of neurovirulence and the role of the immune response in MeV-associated neurologic disease and persistent infection.^{416,459,581} Acute encephalitis in newborn rats can be modulated by passive transfer of antibody to the H protein that results in persistent infection and a late onset of disease.³⁹⁶ Features of subacute disease that are similar to SSPE include an inability to recover virus by routine methods, restricted expression of MeV envelope protein mRNAs in the brain, and intrathecal synthesis of oligoclonal MeV-specific antibody.^{399,638} Demyelination is not a feature of subacute encephalitis; however, demyelinating disease can be more easily induced in previously infected animals.³⁹⁸

Hamsters

Newborn hamsters are susceptible to CNS infection with wild-type, vaccine, and SSPE strains of MeV; develop hyperirritability leading to death within a few days; and have been used to assess determinants of neurovirulence.^{36,329} Brains from these young animals show giant cell encephalitis with intranuclear and intracytoplasmic inclusion bodies and a polymorphonuclear inflammatory response.⁷⁵³ Older animals develop myoclonus with infection of cortical, thalamic, and hippocampal neurons without giant cell formation.^{691,753} Ependymal infection may lead to hydrocephalus.³²⁹ Cell-free virus can be easily recovered from the brains of newborn, but not older, hamsters.⁹⁰ Demyelination is uncommon,⁷⁵³ but some hamsters inoculated as newborns with the LEC SSPE strain develop myelitis weeks later.⁹⁵

Mice

Several strains of MeV have been adapted to grow in the brains of newborn mice and cause fatal disease after intracerebral, but not peripheral, inoculation.^{319,501} Histopathology and immunocytochemical staining show neuronal and glial infection and giant cell encephalitis.^{257,410,501} Maturation of CNS cells converts the production of MeV from the infectious form in newborn mice to a defective form in weanling mice.^{257,286} In weanling mice, MeV antigen-positive neurons have only smooth nucleocapsid structures and no detectable H, F, or M proteins.^{286,410} Disease consists of hyperexcitation and seizures associated with production of excitotoxic amino acids and destruction of pyramidal cells in the hippocampus that can be prevented by administration of glutamate receptor antagonists.^{257,410} The genetic background of the mouse influences susceptibility,⁷⁶³ with C3H/He mice most susceptible and SJL mice most resistant.⁵⁰¹ Mice with deficiencies in cellular immunity are more susceptible than immunocompetent mice.^{207,727}

A number of investigators have produced mice engineered, either by grafting human cells or by transgenic expression of human genes, to provide better murine systems for studying measles pathogenesis. Mice with severe combined immunodeficiency have been grafted with human PBMCs³¹⁵ or implanted with human fetal thymus and liver.²⁸ In thymic implants MeV shows strain-specific differences in virus growth and thymocyte apoptosis that have been useful for assessing virulence and determinants of replication *in vivo*.^{28,730,731} Immunologically

normal mice with transplanted human leukocytes develop graft versus host disease and can also be infected with MeV.³¹⁵

Transgenic mice expressing human CD46 or SLAM have been produced using genomic DNA or complementary DNA (cDNA) with promoters designed to provide generalized or cell type-specific expression of the receptor transgene.^{270,421,536,663,768} In general, only low levels of infectious virus can be recovered from the mice and this is age dependent, with neonatal mice much more susceptible than older mice.⁵³⁶ MeV replication in transgenic mice is enhanced by the absence of components of the innate or adaptive immune response,^{421,514,663} but blocks to MeV replication in mice are at multiple levels.⁷⁴⁴ These mice and cells from these mice have been used for studies of pathogenesis, virulence, immunosuppression, and CNS infection.^{205,268,270,366,518,649,650,706}

Ferrets

Intracerebral inoculation of ferrets with cells infected with certain SSPE strains of MeV causes acute encephalitis.³⁴⁷ Subacute or chronic encephalitis is established if animals are immunized prior to intracerebral injection with infected cells.⁷²⁰ Pathologic and clinical features in animals with chronic disease resemble SSPE with cerebral inflammation, gliosis and intranuclear inclusion bodies, electroencephalographic changes, bound immunoglobulin in brain, and high titers of antibody to MeV in serum and CSF.^{347,720}

EPIDEMIOLOGY

Classic Measles

In the Absence of Vaccination

The study of island populations was important in establishing many of the principles of measles epidemiology. Pioneering observations on the transmissibility, incubation period, and lifelong immunity of measles were made by Panum during the 1846 epidemic in the Faroe Islands.⁵³⁰ Measles is one of the most infectious of communicable diseases. It is estimated that 76% of household exposures of susceptible persons lead to measles²⁹⁹ and that the reproductive number (R_0), or average number of secondary cases produced by an infectious individual in a totally susceptible population, is approximately 12 to 18.^{466,694} Transmission is most efficient through direct exposure to an infected symptomatic individual, but MeV can survive for hours in respiratory droplets, and direct contact is not required.¹¹⁹ Individuals are most infectious from 4 to 5 days before through 4 days after the appearance of the rash.¹²¹

There is no animal reservoir or evidence of latent or epidemiologically significant persistent infection. Therefore, maintenance of MeV in the human population requires a continuous supply of susceptible individuals. Because older members of a community are immune through previous exposure to the virus, endemic measles is primarily a disease of childhood. If the population is too small to establish endemic transmission, the virus cannot be maintained.⁶⁶ Mathematical calculations and studies of islands and cities with populations of different sizes have shown a requirement for a population of 250,000 to 500,000 to establish measles as an endemic disease.^{67,350} This is approximately the population of the earliest urban civilizations in ancient Sumaria around 3000 BCE where measles is postulated to have emerged.

In large population centers, measles is endemic with occasional epidemics as the numbers of susceptible individuals increase. These epidemics spread in waves from large cities to smaller cities and then to rural areas over time.²⁵² In temperate climates measles is more frequent in the winter and early spring. The frequency of epidemics is determined by numbers of susceptible individuals, the duration of infectiousness, and patterns of population mixing.³⁵⁰ The size of the population is also a primary determinant of the age of seroconversion. The average age of infection is earlier in urban than in rural areas in both developed and developing countries.¹³⁹ In countries with high birth rates, infection occurs at an early age. Very young infants are protected from measles (and from response to vaccine) by maternal antibody.⁹ The duration of protective antibody in the infant is dependent on the level of maternal antibody. The source of maternal immunity (vaccine vs. natural measles), gestational age, and presence of maternal infection (e.g., human immunodeficiency virus [HIV], malaria) are determinants of the amount of antibody passively transferred and, therefore, the length of time required for initial levels of antibody to decay to the point that an infant will become susceptible to measles.⁹¹ In small isolated populations measles epidemics are controlled by extinction and chance reintroduction of the virus that leads to infection at older ages on average.^{121,586}

Effect of Vaccination

Immunization alters the epidemiology of measles by reducing the susceptible individuals in the population. In countries with high rates of vaccination, the average age for measles is increased because herd immunity reduces transmission and indirectly protects children from infection. Vaccination also lengthens the interepidemic period.¹³⁹ When outbreaks occur in areas of sustained high vaccine coverage, an increasingly large portion of the cases will be in older individuals who are susceptible due to primary or secondary vaccine failure. In contrast to prevaccination populations, outbreaks are increasingly likely to be local and dependent on social networks.⁷⁵⁴ Because of the infectiousness of MeV and the high R_0 , it is estimated that 95% or more of the population needs to be immune to interrupt endemic transmission.²²⁸ However, even highly immunized populations are vulnerable to localized outbreaks associated with importation from areas where measles remains endemic.^{228,607} These risks are increased in communities that include individuals who refuse vaccination for philosophical or religious reasons.²⁰²

Molecular Epidemiology

Sequence analysis of the variable C-terminus of the N (N_{TAIL}), P, and H genes has been useful for identification of genotypes and analysis of the molecular epidemiology of measles.⁶⁰⁵ Several lineages of MeV that have characteristic temporal and geographic distributions have been identified.^{595,604} Some are localized to specific regions of the world and some are extinct, but most are widely distributed. Currently 8 distinct clades with 24 genotypes are recognized (see section on antigenic composition and strain variation) (Fig. 36.9). Assembly of this increasingly large database of MeV genotypes has aided in the identification of global measles transmission pathways.^{353,606} Identification of the source of the MeV-causing disease in a particular location has become increasingly important as control programs are implemented, and identification of cases as imported or indigenous is necessary.⁶⁰⁷

Subacute Sclerosing Panencephalitis

SSPE occurs preferentially in boys from rural areas with a history of measles at an early age.^{272,326,458} In developing countries with high birth rates, measles often occurs in young infants,^{247,272,472,475} and these countries have a high burden of SSPE.^{426,614,702} This is further exacerbated when there is a high prevalence of HIV infection because children of HIV-infected mothers are more likely to acquire measles at an early age.^{179,475} Several studies have identified exposure to birds as a risk factor, but this relationship has not yet been explained.²⁷² The incidence has decreased dramatically with the introduction of measles vaccination,^{61,92} and there is no evidence that the vaccine virus can cause SSPE.⁴⁵⁸

CLINICAL FEATURES

Classic Measles and Its Complications

Measles has an incubation period of 10 to 14 days spanning the time from exposure to the appearance of clinical disease (Fig. 36.11). The first prodromal symptoms of measles are fever, malaise, and anorexia followed by cough, coryza, and conjunctivitis. The prodrome lasts 2 to 3 days, and during this time small bright red spots with a bluish-white speck at the center, Koplik spots, may become visible on the buccal mucosa, providing early diagnostic evidence of measles.^{368,721} The prodrome ends as the maculopapular rash appears, first on the face and behind the ears and then on the trunk and extremities (Fig. 36.10). It begins to fade within 3 to 4 days. In malnourished children the rash may desquamate.⁴⁶⁹ Generalized involvement of lymphoid tissue may result in lymphadenopathy, mild splenomegaly, and appendicitis. In uncomplicated measles clinical recovery begins soon after appearance of the rash.

Respiratory Disease

The prominent respiratory symptoms are manifestations of diffuse mucosal inflammation in response to widespread infection of epithelial cells. Interstitial pneumonitis due to MeV replication and inflammation in the lower respiratory tract is common in uncomplicated disease but frequently is detectable only by x-ray or by measuring the alveolar–arterial oxygen gradient.^{251,285} Pneumonitis is more likely to be clinically severe during pregnancy.¹² Symptomatic giant cell pneumonia is seen primarily in immunocompromised individuals.^{183,429} Most of the severe pneumonia that complicates measles and leads to chronic pulmonary disease is caused by secondary bacterial and viral infections.^{56,251} Other common respiratory complications caused by secondary infections are otitis media and laryngotracheobronchitis.⁵⁶ In addition to increasing susceptibility to new infections, previously latent viral and bacterial infections may be reactivated.^{121,690}

Gastrointestinal Disease

MeV replication in the liver, particularly the bile duct epithelium, is common in all ages, but clinically evident hepatitis is most frequent in adults.^{235,387} Diarrhea is a common complication of measles, particularly in young patients requiring hospitalization.²⁵⁰ Many epithelial surfaces are infected with MeV and this may lead directly to gastrointestinal symptoms. However, diarrhea is frequently associated with secondary bacterial

and protozoal infections²⁵⁰ that compound the borderline nutritional status of young children in developing countries.

Myocardial Disease

Electrocardiographic abnormalities, including prolongation of the P-R interval and ST-segment and T-wave changes, can be detected in 20% to 30% of children with uncomplicated measles.²³⁹ However, symptomatic cardiac disease is uncommon and is most often related to transient conduction abnormalities. In autopsy studies, myocardial and pericardial lesions are usually attributable to systemic bacterial infections.

Neurologic Disease

There is little evidence that the brain parenchyma is an important target tissue for MeV replication during acute disease in immunologically normal individuals.^{446,460} However, apparently uncomplicated measles is frequently accompanied by a CSF pleocytosis and changes in the electroencephalogram,^{234,274} suggesting the possibility of CNS infection. The occasional appearance of slowly progressive neurologic disease (measles inclusion body encephalitis [MIBE]) in immunosuppressed individuals^{8,214,484} and the occurrence of late neurologic disease (SSPE) in immunologically normal individuals infected at a young age (Fig. 36.14) attests to the ability of MeV occasionally to enter the brain and replicate in neurons and glial cells.

ADEM complicates 1 in 1,000 cases of measles, with the highest attack rate in children older than the age of 5 years,⁴⁵⁷ and usually occurs within 2 weeks after the onset of the rash.⁴⁰⁶ (Fig. 36.14). The disease is characterized by an abrupt onset of fever and obtundation, accompanied by seizures and multifocal neurologic signs. The majority of survivors have neurologic sequelae.^{337,406} Pathologic studies show that this is a perivenular demyelinating disease, not inclusion body encephalitis like MIBE and SSPE.⁴⁰⁶ The pattern of perivenular inflammation and demyelination suggests that it is immunologically mediated.^{230,799} There is little evidence for virus in the brain as assessed either directly by virus isolation or detection of viral antigen or RNA^{230,460} or indirectly by the appearance of MeV-specific antibody in the CSF.³³⁷ There is, however, the induction of an autoimmune response to myelin protein similar to that seen in animals with experimental autoimmune encephalomyelitis and humans with Semple rabies vaccine-induced encephalomyelitis.^{78,162,337,380}

Eye Disease

Measles is considered to be an important cause of childhood blindness associated with corneal lesions. In areas of vitamin A deficiency, the two problems are synergistic and corneal ulceration resembling keratomalacia is a frequent complication of measles.⁶²⁰

Determinants of Morbidity and Mortality

Measles remains one of the most important causes of child morbidity and mortality worldwide.^{473,494,781} Predictors of poor outcome are severe lymphopenia and poor antibody response.^{132,770} Mortality is highest at the extremes of age, in girls, in those with low socioeconomic status, and in those without access to medical care.^{121,163,226,599} Mortality is also increased in malnourished individuals⁴⁸ and secondary cases in a household.^{1,369} Malnutrition affects the immune responses to MeV, impairs virus clearance, and may lead to lower rates of

diagnosis.^{141,564} Vitamin A is a particularly important nutrient influencing outcome.⁴⁸ Low serum retinol is common in severe measles due either to prior dietary deficiency or to impaired mobilization from hepatic stores.^{48,89} Case-fatality rates range from 0.1% in places with good access to medical care to 6% in developing countries and up to 25% in refugee camps and virgin populations.^{139,247,471,494,655,781}

Atypical Measles

A severe form of measles with unusual clinical features was seen in individuals who had previously received an inactivated measles vaccine used in the mid-1960s. Atypical measles differs from typical measles by having higher and more prolonged fever, unusual skin lesions, and severe pneumonitis.^{221,486} The rash often is accompanied by evidence of hemorrhage or vesiculation and begins on the extremities and spreads to the trunk. Pneumonitis is associated with distinct nodular parenchymal lesions and hilar adenopathy.^{251,798} Abdominal pain, hepatic dysfunction, headache, eosinophilia, pleural effusions, and edema are also described. Cases of atypical measles were reported up to 16 years after administration of the inactivated vaccine. Administration of live virus vaccine after two to three doses of killed vaccine did not eliminate subsequent susceptibility to atypical measles and was often associated with severe local reactions.^{88,117,220}

Hypotheses about the pathogenesis of atypical measles included an abnormally intense cellular immune response,²²⁰ an inability of the inactivated vaccine to induce local respiratory tract immunity,⁵⁸ and a lack of production of antibody to F, which allowed virus to spread from cell to cell despite the development of antibody to H.^{452,507} In rhesus macaques the formalin-inactivated vaccine induces transient neutralizing and fusion-inhibiting antibodies, but there is a poor T-cell response and antibodies induced do not mature. Subsequent infection with MeV induces an anamnestic response of low-avidity antibody that cannot neutralize wild-type virus. This leads to immune complex deposition, vasculitis, and pneumonitis.⁵⁵⁹

Measles in the Immunocompromised Host

Children with hypogammaglobulinemia appear to recover uneventfully from MeV infection, while those with deficiencies in cellular immune responses may not.²⁴⁴ Virus clearance is delayed,⁵⁴² and two types of progressive disease, giant cell pneumonia and MIBE, may complicate infection, often in the absence of the typical rash or other characteristic features of measles.^{84,183,429}

Giant cell pneumonia is characterized by increasing respiratory insufficiency beginning 2 to 3 weeks after a history of exposure to measles. Virus can be isolated from lung tissue and bronchial and nasopharyngeal washings.¹⁸³ Pathology shows multinucleated epithelial alveolar and bronchiolar giant cells with intranuclear and intracytoplasmic inclusion bodies.²² There is often evidence of systemic disease with giant cells visualized in multiple organs.^{22,183}

MIBE occasionally accompanies giant cell pneumonia³⁴⁰ but is more often present as an isolated primary manifestation of progressive MeV infection in immunocompromised adults and children.^{8,214,310,484,782} Neurologic disease usually becomes evident within 6 to 12 months after exposure to measles (Fig. 36.14). Initial signs and symptoms include altered mental status, focal seizures or epilepsy partialis continua,

and occasionally blindness or hearing loss.^{6,8,273} The course is typically rapid with progression to coma and death within weeks to months.⁴⁸⁴ Pathology shows gliosis with inclusions in glial cells and neurons.²⁴⁶ Giant cell formation is unusual and little inflammation is present.^{6,484} MeV protein and RNA are abundant and detectable by immunocytochemical staining or *in situ* hybridization. MeV can occasionally be recovered from brain, but the virus is usually defective, as it is in SSPE.^{6,39,601} Viral nucleocapsids without budding particles are seen intracellularly by electron microscopy.^{6,601} The mRNAs for genes encoding the H, M, and F envelope proteins are reduced, and these proteins are often undetectable.^{39,106} MIBE may be fundamentally similar to SSPE, but clinical symptoms are manifested earlier after infection and are more rapidly progressive in the absence of an immune response.⁶⁰¹

Subacute Sclerosing Panencephalitis

SSPE most commonly occurs in individuals younger than the age of 20 who present many years after measles with neurologic disease. The average time to onset of SSPE after measles is 6 to 10 years (Fig. 36.14) but ranges from 1 to 24 years.^{92,458} The onset is insidious and the diagnosis is often not suspected early in disease.^{298,565} The typical presentation is with mental deterioration and personality changes (stage I). Subsequently, there is myoclonus and often seizures (stage II) followed by progressive neurologic deterioration marked by rigidity (stage III), optic atrophy, akinetic mutism, and coma (stage IV).³²⁷ Death occurs within months to years after onset.²¹⁵ Antibody to MeV is elevated in both serum and CSF, and inclusions are present in glial cells and neurons.²⁸⁷ Perinatal infection can be associated with SSPE that has an accelerated onset and a fulminant course.^{92,672}

DIAGNOSIS

The classical clinical features of measles—Koplik spots, fever, erythematous maculopapular rash, coryza, cough, and conjunctivitis—are generally sufficient to make the diagnosis, especially in the setting of a community outbreak, but laboratory confirmation should be obtained. The clinical case definition for measles includes (a) a generalized maculopapular rash of 3 days or more; (b) fever of 38.3°C (101°F); and (c) one of the following: cough, coryza, or conjunctivitis.¹¹¹ However, not all of these signs and symptoms may be present in measles and many are shared with other diseases. The differential diagnosis includes all causes of rash and fever including scarlet fever, rubella, alphavirus infection, parvovirus B19, human herpesvirus-6, human herpesvirus-7, meningococcemia, Kawasaki disease, toxic shock syndrome, and dengue.^{81,563} Fever and/or rash may be absent during measles in very young infants, immunocompromised patients, malnourished children, and previously immunized individuals.^{176,564}

Laboratory diagnostic procedures consist of isolation of virus; direct detection of the virus, viral RNA, or viral antigens in secretions; detection of IgM or low-avidity IgG antibody by enzyme immunoassay (EIA); or documentation of seroconversion using hemagglutination inhibition (HI), complement fixation (CF), virus neutralization, or IgG-specific EIA on serum taken during the acute and convalescent phases of disease. In immune-compromised patients, the diagnosis is difficult, often

not suspected, and usually dependent on biopsy to detect MeV in tissue.^{8,484,562}

Isolation or Detection of Virus

Virus can be cultured from PBMCs, respiratory secretions, conjunctival swabs, and urine, but culture is rarely used as the means of diagnosing acute disease. Cell lines vary in their susceptibility to infection by wild-type strains, with human cord blood leukocytes, the COBL-a cord blood T-cell line, marmoset B95-8 or B95a cells, and Vero cells expressing SLAM being most sensitive.^{211,364,365,521} Epithelial cells from the nasopharynx, buccal mucosa, conjunctivae, or urine can be used for direct cytologic examination for giant cells and inclusions and for antigen detection.^{400,677} Generally, the most useful antibody for staining is one directed to N because this protein is most abundant in the infected cell and N antibody reactivity is retained using a wide variety of fixation methods.⁶⁷⁷ Direct examination for virus is of particular importance for diagnosis in immunocompromised individuals where antibody responses may not be present. The detection of MeV RNA by RT-PCR using primers targeted to highly conserved regions of the N, M, or F genes has been successfully applied to a variety of clinical samples^{7,198,317,542,719} and to most morbilliviruses.^{248,394,668}

Detection of Antibody

The clinical diagnosis of measles is most often confirmed by serology. Samples ideally consist of acute and convalescent serum pairs, but detection of MeV-specific IgM in serum or saliva or low-avidity IgG in serum is diagnostic and may require only a single sample.^{81,206,317,726} IgM antibody appears at the time of the rash and can be detected by 3 days and for up to 4 weeks after the onset of the rash in most individuals.²⁸¹ MeV-specific IgG peaks approximately 2 weeks later and gradually increases in avidity. EIA allows for differential detection of IgM and IgG and is widely used because of its convenience. Plates may be coated with lysates of MeV-infected cells or with recombinant MeV proteins.^{74,314}

The HI test detects antibody to HA and correlates well with the neutralization test. The major limitations of the HI test are the requirement for fresh sensitive monkey erythrocytes, the difficulty of producing sufficient antigen for large numbers of tests, and the possible presence of nonspecific HA inhibitors in serum. The CF test has also been used to determine measles immunity, but titers are less stable over time. Neither of these tests is in common use. The virus plaque reduction neutralization assay remains the standard against which other tests are measured. It is more sensitive than HI or EIA tests^{10,126} and provides the best correlate for protection from infection¹²⁰ and therefore remains the best measure of response to vaccination.⁹

PREVENTION AND CONTROL

Treatment

There is no standard antiviral treatment for measles. Ribavirin inhibits MeV replication *in vitro*; however, immunocompromised patients with pneumonitis have shown no clear evidence of improvement after treatment with aerosolized ribavirin. Newer approaches to therapy that are effective *in vitro* include nonnucleoside polymerase inhibitors^{771,796} and fusion inhibitors.⁵⁶⁸ Administration of high doses of vitamin A dur-

ing acute measles decreases morbidity and mortality even in the absence of clinical evidence of vitamin A deficiency by an unknown mechanism.^{48,221,316} In areas of vitamin A deficiency and xerophthalmia, supplementation prevents blindness due to measles-induced corneal destruction. The World Health Organization recommends two doses of vitamin A for all children with measles.⁷⁷⁴

Numerous therapeutic agents, including amantadine, IFN, isoprinosine, ribavirin, and transfer factor, have been used for the treatment of SSPE. Evaluation of the efficacy of any of these regimens is difficult because the disease is rare, the course is variable, reports are anecdotal, and the benefits are at best short term.²⁶⁶

Vaccination

History

The earliest attempts at vaccination against measles by Home in 1749 were based on the principles of variolation²⁹⁷ with the reasoning that introduction of disease through the skin would lessen the effects on the lung. "Morbillization" was in general unsuccessful.²⁸⁰ Subsequent approaches between 1920 and 1940 designed to inactivate or attenuate the virus by culture in chick embryos also met with limited success.⁴²⁸ The isolation of MeV in tissue culture opened the way for a more concerted approach to vaccine development using the Edmonston strain of virus.¹⁸⁴ Killed virus vaccines were developed using formalin and tween-ether for inactivation.⁷⁵⁸ Simultaneously, attenuated vaccines based on adaptation of MeV to growth in chick cells were developed and are now widely used.³⁴⁹ The attenuated live virus vaccine has dramatically decreased the incidence of measles in all countries in which it has been effectively delivered.

The live attenuated MeV vaccine induces both neutralizing antibody and cellular immune responses that are long-lasting, but the immune responses necessary for protection from infection or disease have not been completely defined. Antibody is sufficient for protection because infants are protected by maternal antibody⁹ and passive transfer of immune serum can modify or interfere with measles vaccination and partially protect children from measles after exposure.⁵⁸² The role of the cellular immune response in protection has been more difficult to study but is induced more readily than antibody in young infants and in the presence of maternal antibody.²²⁴

Inactivated Vaccines

The alum-precipitated inactivated vaccine was used in a three-dose regimen.^{97,758} Recipients developed moderate levels of neutralizing and HI antibodies and low levels of CF antibody.^{97,203,507} Studies in rhesus macaques have shown that the inactivated vaccine induces no CD8+ T-cell response and that the avidity of the MeV antibody induced does not mature.^{558,559} The vaccine was protective when exposure to measles occurred soon after immunization.^{203,219} However, antibody titers declined rapidly, and recipients again became susceptible to measles^{219,578}; when infected, they had a tendency to develop the more severe disease, atypical measles,^{486,578} discussed previously, and the vaccine was withdrawn.

Attenuated Live Virus Vaccines

The first attenuated live measles vaccine was developed by adaptation of the Edmonston strain of MeV to chick embryos and subsequently to chick embryo fibroblasts after passage in

primary renal and amnion cells to produce the Edmonston B virus.¹⁸¹ Inoculation of this virus produced no detectable viremia and no spread to the respiratory tract or disease in monkeys, but it did induce antibody and protected from subsequent challenge. This vaccine was efficacious³⁷⁴ and licensed in 1963 but produced fever and rash in a large proportion of immunized children.³⁴⁸ Reactions were reduced when MeV antibody was given at the same time as the vaccine. Further passage of the Edmonston B virus in chick embryo fibroblasts produced a more attenuated virus that was licensed in 1965.⁶⁴⁷ The Moraten strain used in the United States was licensed in 1968 and is closely related to Schwarz.⁶⁰³ Other Edmonston-derived vaccine strains (e.g., Zagreb, AIK-C) and attenuated strains developed independently (e.g., CAM, Leningard-16, Shanghai-191) are also successful vaccines. Few differences have been described among MeV vaccine strains (all genotype A) regardless of the geographic origin of the parent virus.⁶⁰³ The lyophilized vaccine is relatively stable but rapidly loses infectivity at room temperature after reconstitution.⁴⁴⁹

The recommended age of initial vaccination varies from 6 to 15 months. The probability of seroconversion and the levels of antibody induced are determined by the level of persisting MeV-specific maternal antibody and by the age of the infant.^{137,223,224,580} The recommended age of immunization is determined by a region-specific balance between the optimum age for seroconversion and probability of acquiring measles before that age.¹³⁷ In areas where measles remains prevalent, measles vaccination is routinely performed at 9 months (85% response), whereas in areas with little measles, vaccination is often at 12 to 15 months of age (95% response).¹³⁹ The standard route of administration is either subcutaneous or intramuscular, but there is substantial interest in alternative routes that would avoid the need for needles and syringes. Transcutaneous delivery has not yet been successful,²⁰⁰ and intranasal administration of reconstituted liquid vaccine elicits a local, but not a systemic, immune response.⁶⁷¹ However, respiratory delivery of aerosolized liquid vaccine has shown promise in older children,^{136,164,165,293,411} and respiratory delivery of a small-particle dry powder vaccine induces protective immunity in macaques.⁴⁰²

Host genetic differences affect the likelihood of seroconversion and level of antibody induced,^{164,573} and several specific polymorphisms that affect responses have been identified.^{123,159,160,524,525} The effect of common childhood illnesses on seroconversion is unclear,⁴⁵⁴ and any potential decrease in seroconversion must be balanced against the loss of the opportunity for vaccination and the consequent risk of the child acquiring measles. Overall, the efficacy of a single dose of measles vaccine in infancy is estimated at 77% to 84% when administered at 9 to 11 months, 92% when administered at 12 months or older, and 94% after two doses.^{224,728} Compromises must also be considered with respect to immunizing individuals with HIV infection.¹³⁸ Progressive fatal infection has been occasionally associated with measles vaccine in severely immunocompromised children and adults.¹⁹ However, in general, measles vaccine has been well tolerated in HIV-seropositive children,⁶⁴⁸ although the seroconversion rate is lower, antibody is of lower avidity, and titers wane more quickly than in HIV-uninfected children.^{213,477,488} Because of the potential severity of measles in these individuals, the vaccine is recommended for routine administration to infants without respect to HIV

serostatus in most countries, unless the CD4+ T-cell count is known to be low.

The dose of MeV routinely used for immunization is between 10^3 and 10^4 plaque-forming units. Ten- to 100-fold higher doses improved seroconversion in younger infants, but subsequent follow-up of children receiving high-titered vaccines in countries with high childhood mortality showed an increased mortality in girls over the subsequent 2 to 3 years.^{296,363} Mortality was not due to measles, but rather to a relative increase in the deaths due to other infections.² The pathogenesis of delayed increased mortality after high-titered vaccine is not understood but occurred primarily in those who developed a rash after vaccination and may be related to long-term suppression of immune responses similar to that induced by measles.⁶⁵¹

The immune response to the live attenuated vaccine is similar to that induced by natural disease. However, the duration of vaccine-induced immunity appears to be more variable. In general, levels of antibody are lower after vaccination than after recovery from natural disease, and MeV-specific antibody and CD4+ T cells decay with time.^{122,495} Secondary vaccine failure rates have been estimated at approximately 5% 10 to 15 years after immunization but are probably lower when vaccination is given after 12 months.^{17,433} A second dose of vaccine is necessary to immunize persons who did not respond to the first dose.^{114,138,228} The second dose can be delivered as a part of a routine vaccination program or in national or regional supplementary immunization campaigns. The two-dose strategy has been credited with elimination of indigenous measles in a number of countries.¹³⁸

Experimental Vaccines

Development of new vaccines has been hampered by an incomplete understanding of protective immunity and of the priming for enhanced disease by the inactivated vaccine. Nevertheless, a number of experimental vaccines have been developed. The primary motivations for development of a new vaccine are to increase thermostability, to provide a vaccine that can be given to young infants, and to avoid the hazards associated with the use of needles and syringes. Vaccination with the H, F, and/or N protein expressed in bacterial, poxvirus, adenovirus, or bacille Calmette-Guérin (BCG) vectors or as DNA, peptides, or proteins can induce cellular and humoral immunity in mice and cotton rats.^{116,199,407,532,678,684,762} Monkeys are the most relevant model system, and studies have shown different degrees of protection after vaccination with H, F, and/or N expressed with BCG, alphavirus replicon particles, vaccinia virus, immune-stimulating complexes, and variously formulated DNAs.^{529,533,557,566,685,686,733,805,806}

Prospects for Eradication

Theoretically, measles is an ideal virus for eradication through vaccination: there is only one serotype; most cases are clinically identifiable; there is no animal reservoir; and a vaccine is available.⁴⁷³ However, measles remains a leading cause of vaccine-preventable childhood mortality.¹¹⁵ In 1979, eradication was identified as a goal for the United States, but it was 20 years before endemic transmission was interrupted.¹¹² In 2010, the World Health Assembly endorsed 2015 targets of greater than 90% national coverage with one dose of vaccine, reduction of measles incidence to less than 5 cases per million, and mortality reduction of greater than 95% compared to

2000 estimates. The highly communicable nature of the virus requires continued maintenance of high vaccine coverage to prevent outbreaks, with 95% to 98% seropositivity needed for herd immunity. The role of asymptomatic infection of partially immune individuals in maintaining transmission is unclear. Global programs, such as the Expanded Program for Immunization and the Measles Initiative, have increased vaccine coverage worldwide and have resulted in significant decreases in measles and measles mortality.^{139,474} However, delivery remains difficult in many areas due to civil strife, the need to maintain a cold chain, decreasing donor support, and religious or philosophical resistance to vaccination.^{113,474} Eradication remains a worthy but difficult goal.⁶⁸⁹

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suburb where the outbreak occurred. A second person died from HeV infection 13 months after the Brisbane outbreak, a farmer from Mackay, nearly 1000 km north of Brisbane. Unlike the first case, however, the man succumbed to encephalitis caused by HeV infection.¹⁰⁷ A forensic investigation found that the farmer had suffered a mild meningitic illness 14 months earlier after assisting at the necropsy of two horses that had died of severe respiratory distress—later found to have been caused by HeV¹²⁵—and that he became infected at that time. After initial serologic evidence suggested that fruit bats (flying foxes) of the genus *Pteropus* in the suborder *Megachiroptera* were the reservoir hosts,¹⁶⁵ HeV was isolated from two species of flying fox. In total, there have been 14 recognized occurrences of HeV in Australia between 1994–2010, with at least one occurrence per year since 2006.^{5,10} Every occurrence of HeV has involved horses as the initial infected host, causing lethal respiratory disease and encephalitis, along with a total of seven human cases arising from exposure to infected horses, among which four have been fatal and the most recent in 2009.^{6,10,115}

Nipah virus (NiV), the second known member of the genus *Henipavirus*, emerged as the cause of an outbreak of disease in pigs and humans in Peninsular Malaysia in 1998 through 1999. The epidemic started in Perak State as clusters of cases of encephalitis among pig farmers. It was initially believed to be caused by Japanese encephalitis virus; however, various features of the outbreak, including a high proportion of cases in direct contact with pigs and illness and deaths in pigs, differed from those expected with Japanese encephalitis.³⁰ Indeed, respiratory illness and encephalitis in pigs preceded human cases in the same district.⁹⁷ The epidemic spread south to the intensive pig-farming areas of Negeri Sembilan in December 1998 and subsequently peaked between February and April 1999. More than 1 million pigs were destroyed to halt the spread of the epidemic, and by late May, 265 human cases of acute encephalitis with 105 deaths were recorded.^{29,30} A cluster of 11 cases with 1 death occurred among abattoir workers in Singapore.¹¹⁴ In early March 1999, a virus was isolated from the cerebrospinal fluid (CSF) of a patient with encephalitis and identified as the etiologic agent.^{29,30} Named *Nipah virus* after the village from which the patient had come, it was shown to be closely related to HeV. NiV was subsequently isolated from the urine of Malaysian flying foxes.³³ The virus re-emerged in Bangladesh in 2001,⁶⁸ and outbreaks of NiV-related encephalitis have occurred in people from that country almost every year since, along with two reports of NiV encephalitis in India^{23,60} and the most recent occurrence in early 2011 in Bangladesh.⁷ The human case fatality rate of these occurrences of NiV has approached 75%.^{68,85}

HISTORY

Hendra virus (HeV), the first known member of the genus *Henipavirus* in the family *Paramyxoviridae*, came to light in September 1994 as the causative agent of a sudden outbreak of acute respiratory disease in thoroughbred horses at a stable in Brisbane, Australia. A total of 21 horses and 2 humans (a horse trainer and a stable hand) became infected. The horse trainer and 14 horses died, and 7 horses with mild or subclinical infection were killed.¹³⁰ A virus was isolated, called *equine morbillivirus* but later named *Hendra virus* after the Brisbane

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Classification

When HeV was first isolated in 1994, partial sequencing of the matrix gene (M) revealed that it most closely resembled members of the genus *Morbillivirus* in the subfamily *Paramyxovirinae*.¹⁰⁴ Subsequent characterization of the full-length genome, however, revealed that many of the genetic features of HeV were unique among paramyxoviruses and that the virus did not fit within any of the three genera existing at that time, *Morbillivirus*, *Respirovirus*, and *Rubulavirus*.^{147,148} When NiV was isolated in 1999, it was initially described as Hendra-like on the basis of its strong reactivity with anti-HeV antibodies. Later it was shown that sera raised against HeV were able to neutralize NiV and vice versa, and that both viruses shared a high degree of similarity in genome organization and protein size and sequence.^{29,58,59,146} In 2002, the genus *Henipavirus* was created to accommodate these novel paramyxoviruses, and HeV was designated the type species.⁷⁵ Currently, the genus *Henipavirus* contains two virus species and several strains isolated from humans, bats, horses, and pigs over a wide geographic area and spanning a period of 15 years (Table 37.1). The susceptibility

of humans, the virulence of the viruses, and absence of therapeutics and vaccines led to classification of HeV and NiV as biosafety level 4 (BSL4) pathogens.

Propagation in Cell Culture and Cytopathic Effect

The ultrastructural characteristics of henipavirus-infected cells resemble those found in cells infected by other members of the *Paramyxovirinae*. Shared features include generation of large syncytia and the presence of viral nucleocapsids in cytoplasmic inclusion bodies and underlying electron-dense areas of the plasma membrane.^{49,69} In Vero cells, NiV-induced syncytia are significantly larger than those generated by HeV and nuclei and nucleocapsids are frequently located at the cell periphery, compared with HeV-induced syncytia, where they tend to be more centrally located or distributed randomly throughout the cytoplasm (Fig. 37.1). Henipavirus-infected cells also contain structures that are not seen with other paramyxoviruses—specifically a network of membrane-like reticular structures in the cytoplasm and long tubules that appear to be continuous with the plasma membrane in NiV-infected cells. Tubules can also be observed in NiV virions (Fig. 37.2A). *In situ* hybridization suggests that

TABLE 37.1 Summary of Henipaviruses Isolated from Different Species and Geographic Locations

Virus	Isolate name and number	Isolation details			
		Year	Country	Host species/tissue	Reference
Hendra	Horse-1	1994	Australia	Horse/spleen, lung	104
	VR-1	1994	Australia	Human/lung, liver, kidney, spleen	104
	Bat-1-1	1996	Australia	Grey-headed flying fox (<i>Pteropus poliocephalus</i>)/uterine fluid	56
	Bat-1-2	1996	Australia	Grey-headed flying fox (<i>P. poliocephalus</i>)/fetus	56
	Bat-2	1996	Australia	Black flying fox (<i>Pteropus alecto</i>)/fetal lung	56
	Murwillumbah	2006	Australia	Horse/lung	90
	Clifton Beach	2007	Australia	Horse/lung	90
	Peachester	2008	Australia	Horse/blood	90
	Redlands	2008	Australia	Horse/lung	90
	Proserpine	2008	Australia	Horse/lung	90
Nipah	PKL	1999	Malaysia	Human/cerebral spinal fluid	29, 30
	EKK	1999	Malaysia	Human/cerebral spinal fluid	29, 30
	WWS	1999	Malaysia	Human/cerebral spinal fluid	29, 30
	UMMC1	1999	Malaysia	Human/cerebral spinal fluid	24
	UMMC2	1999	Malaysia	Human/throat secretion	24
	UM-0128	1999	Malaysia	Human	2
	VRI-0626	1999	Malaysia	Pig/lung	2
	VRI-1413	1999	Malaysia	Pig/lung	2
	VRI-2794	1999	Malaysia	Pig/lung	2
	B13/6-18	2000	Malaysia	Bat/pooled urine	33
	B13/6-43	2000	Malaysia	Bat/pooled urine	33
	JA13/6-4	2000	Malaysia	Bat/partially eaten jambu air fruit	33
	Rajbari-1	2004	Bangladesh	Human/oropharyngeal	57
	Rajbari-2	2004	Bangladesh	Human/cerebral spinal fluid	57
	Faridpur	2004	Bangladesh	Human/urine	57
	Rajshahi	2004	Bangladesh	Human/urine	57
	CSUR381	2004	Cambodia	Flying fox (<i>Pteropus lylei</i>)/urine	120
	CSUR382	2004	Cambodia	Flying fox (<i>P. lylei</i>)/urine	120

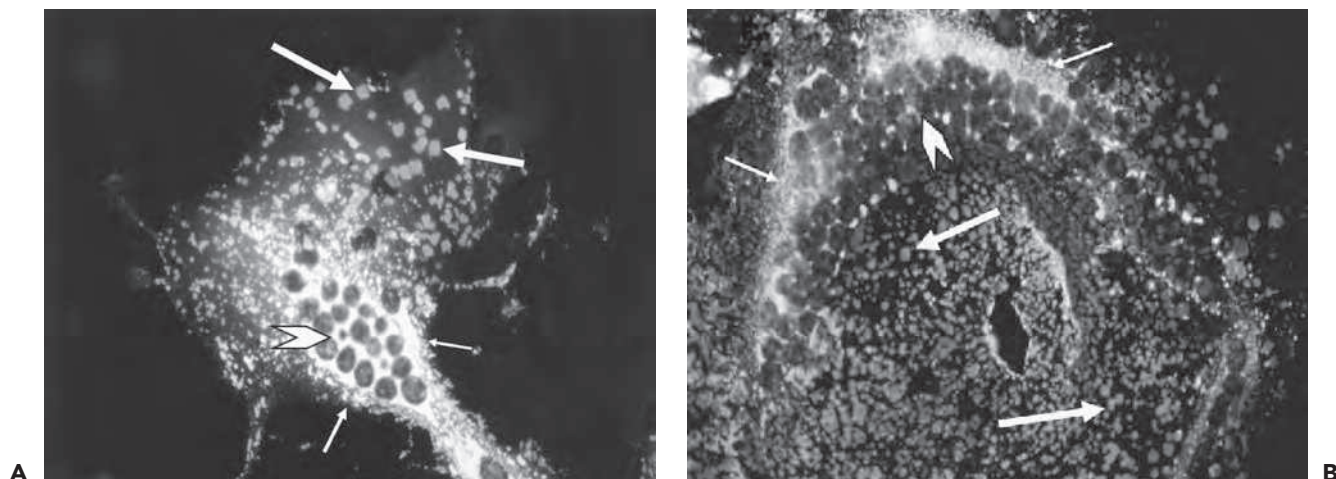


FIGURE 37.1. Syncytia induced in vero cells 24 hours after infection by Hendra virus (HeV) (A) and Nipah virus (NiV) (B). Methanol-fixed infected cells were labeled with rabbit monospecific antiserum to the HeV P protein and fluorescein-conjugated goat antirabbit immunoglobulin G. P protein is detected in extensive perinuclear ribonucleoprotein complexes (*small arrow*) and in discrete regularly shaped arrays (*large arrow*) distributed throughout the cytoplasm and believed to be sites of virus egress from the cell. Nuclei are indicated by *chevrons*.

these reticular structures contain viral RNA and may play a role in viral transcription.⁴⁹

Virus Morphology

Henipavirus particles are pleomorphic, varying from spherical to filamentous and ranging in size from 40 to 1,900 nm.^{49,69,104} Nucleocapsids have a diameter of 18 to 19 nm with an average pitch of 5 nm. When examined by electron microscopy (EM), HeV has a unique double-fringed appearance, caused by the presence of surface projections 15 ± 1 nm and 8 ± 1 nm in length (see Fig. 37.2B). Approximately 95% of virions contain the double fringe, and the remaining 5% display a uni-

form fringe length of 15 ± 1 nm. Unlike HeV, NiV possesses a single layer of surface projections with an average length of 17 ± 1 nm, and NiV particles released into the culture medium are difficult to image because they are routinely penetrated by negative stains. This suggests that the viruses may differ in the physical nature of their envelope.⁶⁹

Genome Length and Organization

In the subfamily *Paramyxovirinae*, the genome length of all characterized viruses is divisible by six, an observation caused by the requirement of each N protein in the viral ribonucleoprotein to bind 6 nucleotide (nt) residues.⁷⁶ This is also true

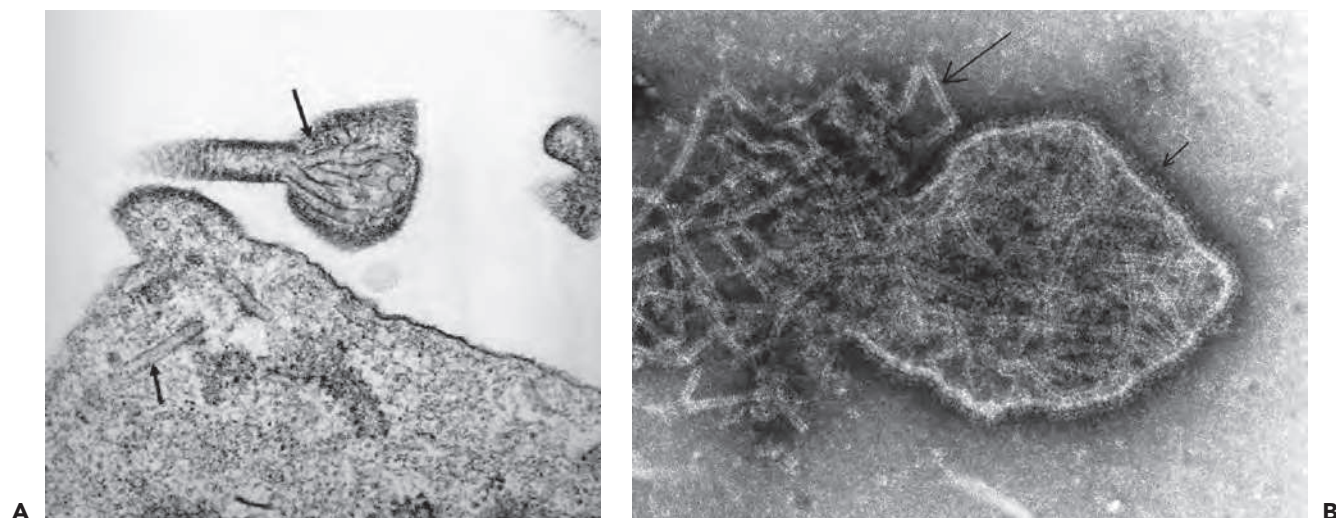


FIGURE 37.2. A: Electron micrograph of Nipah virus (NiV)-infected Vero cells showing tubule-like structures, both in the cytoplasm and in a maturing virus particle (*arrows*). **B:** Electron micrograph of negatively stained Hendra virus (HeV) displaying the double fringe at the virus envelope (*small arrow*) and the herringbone nucleocapsids (*large arrow*). (Courtesy of Dr. Alex Hyatt, CSIRO Australian Animal Health Laboratory.)

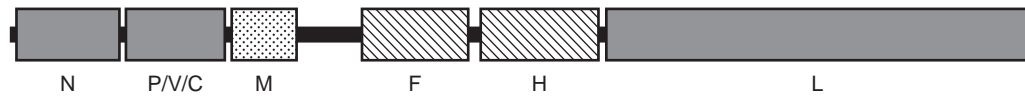
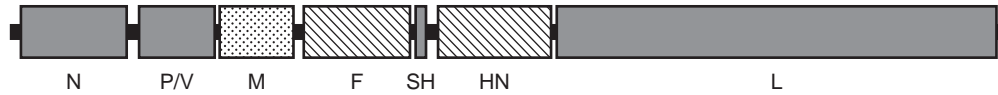
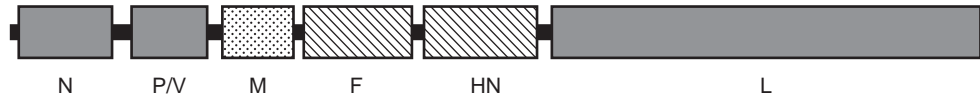
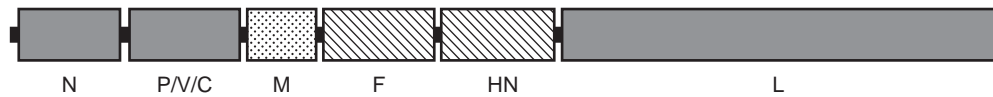
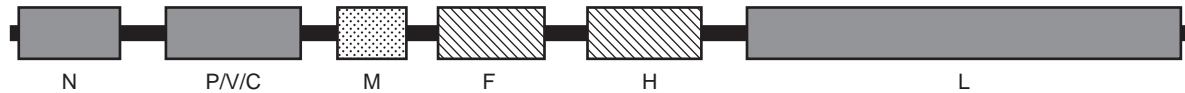
Morbillivirus - Measles virus (15894)**Rubulavirus** - Mumps virus (15384)**Avulavirus** - Newcastle disease virus (15186)**Respirovirus** - Sendai virus (15384)**Henipavirus** - Hendra virus (18234)

FIGURE 37.3. Genome size and organization of Hendra virus compared with type species of each of the other four classified genera in the subfamily *Paramyxovirinae*. Genome lengths (in nucleotides) are given in brackets after each virus.

for HeV and NiV despite their much larger genome sizes.¹⁴⁶ The genomes of the Malaysian and Bangladesh strains of NiV differ by 6 nt because of a 6-nt increase in the 3' untranslated region of the *F* gene.⁵⁷ A minigenome replicon study confirms that NiV complies with the rule of six.⁵⁵ When the complete genome sequence of HeV was determined, its length (18,234 nt) was more than 2,700 nt, or 15% longer than the genomes of all other paramyxoviruses known at that time.¹⁴⁸ The size of the NiV genome at 18,246 nt to 18,252 nt is slightly larger than that of HeV.^{57–59} The extra length of the henipavirus genome is in the form of unique, long untranslated sequences at the 3' end of five of the six genes. A comparison of genome length and untranslated regions of representative members of the *Paramyxovirinae* is shown in Figure 37.3.

The genome organization of henipaviruses resembles that in the genera *Respirovirus* and *Morbillivirus*. The first 12 nt of the 3' and 5' genomic terminal sequences of paramyxoviruses are highly conserved and complementary, containing promoter elements for replication and transcription.⁷⁶ The first 3 nt of the henipavirus genome termini are 5'-ACC-3'—a sequence that is absolutely conserved in members of the subfamily *Paramyxovirinae* but different from that found in the *Pneumovirinae*.

Genetic Diversity

Partial genome sequencing revealed that HeV isolated from equine and human sources during the outbreak in Brisbane appears identical and differs little from HeV isolated from flying foxes 2 years later.^{56,104} Sequencing of five additional horse isolates from five different locations from the 2006 to 2008 HeV occurrences has demonstrated a very high genetic

similarity.⁹⁰ Similar observations were made in Malaysia, where it was demonstrated that NiV isolated from pigs at the height of the outbreak and at its geographic focus were essentially identical to human isolates made at that time and isolates obtained from flying foxes several years later.^{2,24,33,58,59}

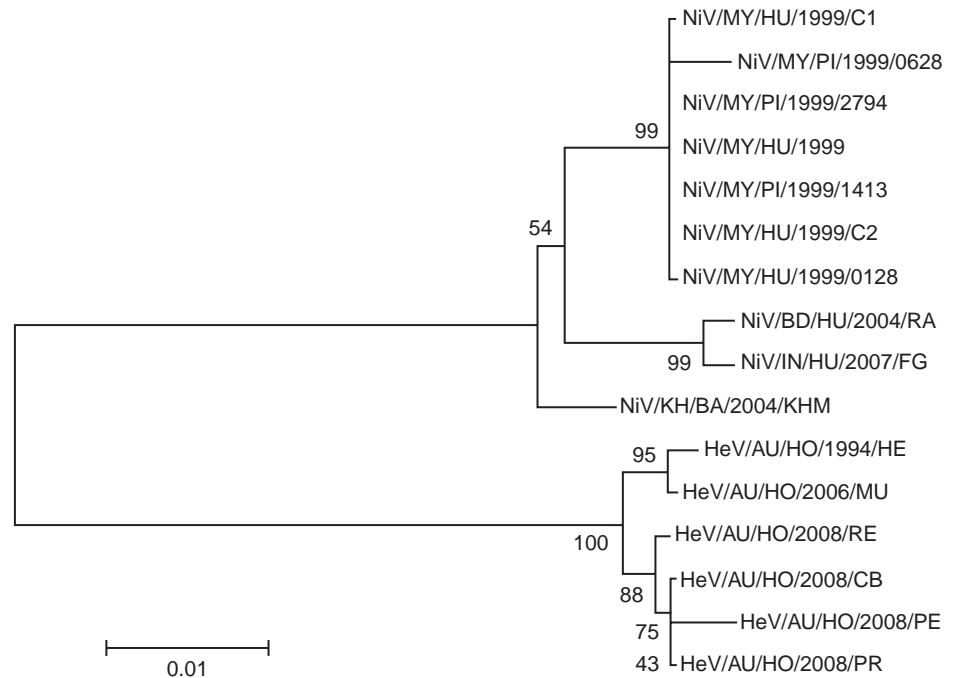
In Bangladesh, four human isolates obtained in 2004 demonstrate significant genetic heterogeneity and suggest multiple spillovers of NiV from flying foxes into the human population.⁵⁷ The NiV sequence detected from lung tissue of human patients in India was more related to NiV from Bangladesh than NiV from Malaysia.^{9,23} On the other hand, NiV isolated from the flying fox *Pteropus lylei* in Cambodia¹²⁰ represents an evolutionary lineage that is separated from the Malaysia or Bangladesh/India cluster (Fig. 37.4). Partial *N* gene sequences detected in *Pteropus lylei* in Thailand indicate the circulation of at least two lineages of NiV—one related to the Bangladesh NiV and the other more related to the Malaysian NiV.¹⁴⁴

The presence of henipavirus-reactive (but not neutralizing) antibodies in bats from other regions of the world (see Epidemiology section) predicts that a much greater genetic diversity of henipaviruses exists in different bat populations. This also suggests the existence of henipaviruses with different transmissibility and pathogenicity in nonbat hosts.

Virus Proteins and Their Properties

Analysis of purified viruses by polyacrylamide gel electrophoresis reveals L, P, G, F₀, N, F₁, and F₂ proteins,^{146,147} where F₀ is the uncleaved and F₁ and F₂ the cleaved products of the *F* gene. Interestingly, F₀ is more readily detected in HeV compared with other paramyxoviruses, including NiV.^{93,146} Overall, the proteins

FIGURE 37.4. Phylogenetic relationship between the N protein sequences of henipavirus isolates from different geographic locations. The virus nomenclature abbreviation follows the following format: virus/country origin/host/year/isolate name. For example, NiV/KH/BA/2004/KHM represents a Nipah virus isolated from bats in Cambodia in 2004 with KHM as its isolate name.



of henipaviruses are typical of those of the subfamily *Paramyxovirinae*, with the exception of the P protein, which is significantly larger than cognate proteins in the subfamily.¹⁴⁷ The P protein is translated from messenger RNA (mRNA) that is co-linear with genomic RNA. The P gene also encodes V and W proteins, produced from mRNA in which one and two nontemplated G residues, respectively, are inserted at the RNA editing site during transcription. The P, V, and W proteins, therefore, are identical for the first 405 amino acid residues. A C protein is encoded by the 5' end of the gene in an overlapping reading frame and is produced by an internal translational initiation mechanism, which is common to other members of *Paramyxovirinae*, except for rubulaviruses.⁷⁶ The P, V, and the C proteins predicted from the coding regions in the P gene are present in HeV-infected cells.¹⁴⁶ The functional expression of W in NiV-infected cells has recently been demonstrated.⁸⁴

Paramyxovirus N, P, and L proteins are necessary and sufficient for replication of viral RNA both *in vitro* and *in vivo*,⁷⁶ and this has been confirmed for henipaviruses by reverse genetics. Using a minigenome replicon containing leader and trailer sequences of the NiV genome with its entire coding region replaced with a reporter gene, it was shown that efficient genome replication was achieved only when all three proteins were expressed in the same cell.⁵⁵ NiV N, P, and L proteins were also able to rescue a minigenome constructed from the leader and trailer sequences of the HeV genome, further demonstrating the close genetic relationship between the viruses.

The L protein of nonsegmented, negative-stranded RNA (NNR) viruses in the order *Mononegavirales* contains a highly conserved GDNQ motif, believed to be important for polymerase activity.¹¹⁶ Henipaviruses were the first NNR viruses in which GDNQ was replaced by GDNE. It was speculated that this motif might be unique to paramyxoviruses with relatively large genomes^{146,148}; however, the GDNE motif has since been found in the L protein of Mossman virus that has a genome length of 16,650 nt.⁹⁶

The cell attachment proteins of the *Paramyxovirinae* display hemagglutination (H) and neuraminidase (N) activities in a predominantly genus-specific manner. Viruses in the genera *Respirovirus*, *Avulavirus*, and *Rubulavirus* possess both activities,⁷⁶ whereas viruses in the genus *Morbillivirus* do not behave uniformly and only some possess hemagglutination activity.⁷⁷ In contrast, henipavirus attachment proteins have neither of these activities^{146,166}; rather, they utilize the host cell expressed ephrin-B2 and ephrin-B3 molecules as attachment and entry receptors.^{11,13,105,106} Recent solution structures of the henipavirus G glycoprotein alone and in complex with the ephrin-B2 and ephrin-B3 receptors have revealed the details of the virus–host cell binding process, distinguishing it from other paramyxovirus receptor strategies.^{20,21,163}

Proteolytic processing of paramyxovirus F proteins is essential for the generation of a fusogenic form of the protein. For most paramyxoviruses that generate systemic infections, cleavage is catalyzed by the cellular protease furin at a multibasic cleavage site.⁷⁶ Surprisingly, henipavirus F proteins are cleaved without the involvement of furin and, although cleavage occurs at a single basic residue—lysine for HeV and arginine for NiV⁹⁸—activation of the NiV F protein does not require a basic amino acid at the cleavage site.⁹³ It has been shown that the lysosomal cysteine protease cathepsin L is responsible for the cleavage of the HeV F protein.¹⁰⁸ The cytoplasmic tails of henipavirus F proteins contain the endocytosis consensus motif YXXØ.^{92,143} Endocytosis is required for cleavage activation of the F protein,^{35,92} consistent with identification of cathepsin L as the enzyme responsible for cleavage.

Host Range

For most paramyxoviruses, host range is limited and interspecies transmission is rare. In contrast, henipaviruses display a broad species tropism. In addition to at least three flying fox species, two nonpteropid fruit bat species, and an insectivorous species, NiV has naturally infected pigs, humans, dogs,

horses, and cats,^{29,63,97,120,164} whereas HeV infects four Australian flying fox species and has naturally infected humans and horses.^{42,104} Confirmation of the wide host range of henipaviruses and the identical cell tropism of HeV and NiV were obtained early using an *in vitro* cell fusion system that relies on vaccinia virus-mediated cell surface expression of G and F glycoproteins.^{17,18,136} Guinea pigs, hamsters, ferrets, squirrel monkeys, and African green monkeys are also susceptible to experimental NiV infection.^{19,46,88,94,157} Laboratory studies have added cats, guinea pigs, hamsters, and African green monkeys to the list of HeV-susceptible species,^{53,121,151,156} along with ferrets (J. Pallister and L.-F. Wang, unpublished results).

PATHOGENESIS AND PATHOLOGY

Cell and Tissue Tropism

The primary site of NiV replication and the dynamics of virus spread in humans are unknown. However, the distribution and time of appearance of lesions throughout the vasculature and in the brain and lung in NiV encephalitis suggest that secondary infection probably arises via hematogenous spread of the virus, with secondary replication occurring in vascular endothelium.¹⁵⁹ Inflammation of blood vessels occurs in most organs but is particularly prominent in the brain, lung, heart, and kidney.^{30,159,160} Vasculitis is limited to small arteries, arterioles, and capillaries where NiV antigen is found in both endothelial cells and the smooth muscle of the tunica media. The pattern and time of appearance of vasculitis and viral antigen distribution are consistent with endothelial cell infection occurring before infection of the smooth muscle. Syncytial endothelial cells are present in blood vessels of various organs and represent a pathognomonic but insensitive feature of henipavirus infections found in only 25% of human NiV infections.¹⁵⁹ Syncytial endothelial cells were also observed in the single human case of encephalitis caused by HeV.¹⁰⁷ NiV antigen is also found in neurons and, less frequently, in bronchiolar and renal epithelial cells. The 5-day interval between maximal vasculitis in the brain and parenchymal infection in acute NiV encephalitis suggests that primary virus replication occurs in endothelial cells, with infection of neurons occurring as a result of vascular damage. The presence of inclusion bodies and viral antigen in neurons suggests that neurologic impairment in encephalitis may be caused by both the effects of ischemia and infarction and viral infection of neurons.^{30,114,159} Human HeV encephalitis has been described as widespread cortical, subcortical, and deep white matter involvement in two cases in 2008¹¹⁵ and similar to those described in a previous HeV encephalitis case^{82,107} and NiV encephalitis cases.^{82,127}

Although NiV antigen is found less frequently in bronchiolar and renal epithelial cells compared with vascular and neuronal locations, replication in epithelial locations may play a role in virus dissemination, because NiV is found in urine and in tracheal and nasopharyngeal secretions of infected patients in the early phase of their illness.^{31,48} Despite this, human-to-human transmission in Malaysia was extremely rare.¹⁰⁰

The identification of ephrin-B2 and ephrin-B3 as functional receptors for henipaviruses in cultured cells provides an explanation for the observed distribution of viral antigen in arterial endothelial cells, smooth muscle, neurons, and some epithelial cells.^{11,13,105,106} Ephrin-B2 is found in arteries, arterioles,

capillaries in multiple organs, and tissues including arterial smooth muscle and human bronchiolar epithelial cells¹³⁵ but is absent from venous components of the vasculature.⁴⁵ Ephrin-B3 is found predominantly in the nervous system as well as the vasculature.¹¹³ The ephrins engage Eph receptors and mediate bidirectional cell–cell signaling events and are modulators of cell remodeling events, especially within the nervous and vascular systems.¹¹³

Immune Response

In patients with encephalitis, anti-NiV antibodies were observed more frequently in the serum than the CSF. Immunoglobulin M (IgM) antibodies occurred more frequently than immunoglobulin G (IgG) antibodies in both locations.^{30,114,159} The appearance of specific IgM antibodies in serum preceded their appearance in the CSF, a sequence consistent with viremia preceding central nervous system (CNS) infection. Anti-NiV antibodies were present in most patients with clinical NiV encephalitis; however, no difference was observed in clinical features, laboratory results, or mortality between seropositive and seronegative patients.^{27,48} Seroconversion of IgG against HeV was seen in two human cases of HeV infection, one fatal, in 2008,¹¹⁵ following an influenza-like illness and before progressing to encephalitis.

Inhibition of the Interferon Response

In henipaviruses, as in other paramyxoviruses,⁶⁶ anti-interferon (IFN) activities are encoded by the *P* gene and *in vitro* studies indicate that *P* gene products inhibit both IFN induction and signaling.³⁷ In IFN induction, viral double-stranded RNA (dsRNA) is detected by intracellular RNA helicase enzymes^{3,79} and by toll-like receptor 3 (TLR-3).¹² Both of these dsRNA signaling pathways lead to activation of the pre-existing transcription factors interferon regulatory factor 3 (IRF-3) and nuclear factor kappa B (NF- κ B),⁵⁰ and the synthesis of IFN- α and a subset of IFN- β proteins.⁸⁹ HeV inhibits dsRNA signaling by using a strategy adopted by other paramyxoviruses in which the V protein binds to the intracellular RNA helicase sensor and prevents downstream signaling³ but does not abrogate dsRNA signaling through TLR-3.¹³² In an additional strategy unique to henipaviruses, the W protein, by virtue of a nuclear localization signal located in the unique carboxy terminal, inhibits dsRNA signaling in the nucleus by targeting a process that is part of both helicase-dependent and TLR-3-dependent signaling pathways.¹³²

In the IFN signaling pathway, IFN binds to cell surface receptors in a paracrine manner and initiates a signaling sequence that leads to activation of members of a family of proteins called *signal transducers and activators of transcription* (STAT).^{1,50} Henipaviruses inhibit IFN signaling by sequestering STAT proteins in high molecular weight complexes.^{123,124} The anti-IFN signaling activity is a property of the V protein, as has been observed for other paramyxoviruses, but also of the W and P proteins.^{123,124,133} The P, V, and W proteins of henipaviruses have an N-terminal extension of 100 to 200 amino acids compared with cognate proteins in the subfamily,^{59,147} and the STAT binding domain of NiV V maps to this region.¹²² The V and P proteins bind STAT in the cytoplasm, whereas the W protein, with its nuclear localization signal, co-localizes with STAT in the nucleus.^{132,133} The W protein is the most and P protein the least efficient IFN antagonist.¹¹² The NiV

C protein also displays a modest inhibition of IFN signaling, although the mechanism and target are unknown.¹¹²

It should be noted that most, if not all, studies described previously were carried out *in vitro* using single-gene transfection expression systems. A recent study conducted using live virus infection indicated that IFN signaling remains functional during henipavirus infection of human cell lines, whereas IFN production was inhibited.¹⁴²

Infections of Animals

Only limited data are available on the minimal lethal and infectious doses of henipaviruses. In golden hamsters (*Mesocricetus auratus*), the NiV lethal dose 50% (LD₅₀) following intraperitoneal and intranasal administration was 270 and 47,000 plaque-forming units (pfu), respectively,¹⁵⁷ and was calculated to be 12 pfu for HeV by intraperitoneal injection.⁵³ The NiV minimal infectious dose in hamsters is 100 pfu if the intraperitoneal route is used and 10³ pfu if the virus is administered intranasally. Guinea pigs and pigs are also more resistant to infection by HeV and NiV, respectively, when the viruses are administered by the oronasal route compared with the subcutaneous route.^{95,154} In contrast, both HeV and NiV appear to be equally infectious for cats following either parenteral (5,000 tissue culture infectious dose 50% [TCID₅₀] virus) or oronasal (50,000 TCID₅₀ virus) administration.^{64,91,95,101,151}

Experimental infection of horses with HeV by parenteral or oronasal routes is almost uniformly fatal, with death or euthanasia usually occurring 5 to 10 days after infection. Several horses in the original outbreak in Hendra survived infection, however, some asymptotically.^{103,104,156} In horses, HeV displays a predominantly respiratory tropism, and infection is characterized by pulmonary edema and congestion.^{64,104,156} In field cases, the airways are often filled with a blood-tinged frothy exudate. Neurologic signs do exist, but they have been observed infrequently in terminally ill horses and in horses that recovered from respiratory infection.^{125,156} Infection is associated with virus replication and with the appearance of viral antigen in endothelial cells in a wide variety of organs, including lungs, lymph nodes, kidneys, spleen, bladder, and meninges. The subsequent degeneration of small blood vessels is accompanied by the appearance of syncytial endothelial cells.⁶³ Virus can be recovered from several internal organs, including lung, and from saliva and urine.^{64,156}

In contrast, NiV infection of pigs is frequently asymptomatic, particularly following natural infection and after experimental administration of the virus by the ocular and oronasal route.^{95,97,150} When symptoms are present, they vary according to the age of the pig, with older animals presenting primarily with a neurologic syndrome, whereas a respiratory syndrome predominates in young animals. The virus manifests respiratory and neurologic tropisms in both asymptomatic and clinical infections.^{95,149} Neurologic signs include trembling and neurologic twitches, muscle spasms, and uncoordinated gait.⁹⁷ After experimental NiV infection of young pigs by the ocular and oronasal routes, virus replication occurs in the oropharynx and spreads sequentially to the upper respiratory tract and submandibular lymph nodes, the lower respiratory tract, and additional lymphoid tissues.¹⁴⁹ Here, viral antigen is widespread and syncytia are common in clinically affected animals as a result of replication in the endothelial and smooth muscle cells of medium to large veins as well as in the

arteries of the central nervous, lymphoid, and respiratory systems.^{63,95,149} NiV invades the CNS via the cranial nerves and by crossing the blood–brain barrier.¹⁴⁹ Virus is recovered from a range of tissues, including tonsil, nasal, and throat swabs and lung, but is recovered infrequently from urine.^{34,95,149} Experimental HeV infection of pigs (Landrace and Gottingen mini-pig breeds) via oronasal or nasal inoculations has shown both to be susceptible to infection,⁸⁰ with virus detected mainly in tissues from respiratory and lymphoid systems, and could be isolated from nasal, oral, and rectal swabs, indicating the possible routes for virus shedding.

Laboratory Animal Models

In hamsters, the pathologic features following NiV infection resemble those found in humans.¹⁵⁷ Hamsters die 5 to 9 days after intraperitoneal administration of 100 to 10,000 infectious particles and 24 hours after the appearance of tremors and limb paralysis. In contrast, hamsters inoculated intranasally with doses as high as 10³ to 10⁶ infectious particles die between 9 and 15 days later, displaying progressive deterioration with limb paralysis, lethargy, limb twitching, and breathing difficulties. Vascular pathology is observed in a range of organs, including brain, lung, liver, kidney, and heart, and viral antigen and genome are found in endothelial cells. The brain is the most severely affected organ, with the vascular and parenchyma lesions consistent with CNS-mediated clinical signs.¹⁵⁹ HeV infection of hamsters also resembled the pathology seen in acute human cases, including both respiratory and brain pathology.⁵³ HeV-induced pathology in the hamster was similar to that of NiV¹⁵⁷ and consisted of endothelial infection and vasculitis with thrombosis and microinfarction, with evidence of direct parenchymal cell infection, notably in the CNS.

In cats, the first clinical signs, which are observed on days 4 to 8 after parenteral or oronasal administration of HeV and NiV, include depression, fever, and an increased rate of respiration.^{65,101,151,152} Most infected animals die 1 day after the appearance of respiratory distress. The disease caused by HeV and NiV in cats closely resembles that seen in HeV-infected horses, with copious frothy sanguineous fluid in the bronchi and hemorrhage or congestion of the tracheal epithelium.⁶⁴ Vaculitis affects both arteries and arterioles, and syncytial cells are observed in endothelia, predominantly in the lungs but also in gastrointestinal, spleen, and lymphoid organs. A major difference between NiV and HeV infection of cats is the extensive degree to which NiV, but not HeV, infects the respiratory epithelium.⁹⁵ Henipaviruses are found in the urine and bladder of experimentally infected cats, and NiV can transplacentally infect and replicate in fetal tissues with high levels of recoverable virus from the placenta and uterine fluid.¹⁰²

In guinea pigs, the clinical response to henipavirus infection is frequently mild and often variable, ranging from inapparent to sudden death, with only a proportion of animals displaying signs such as transient weight loss, depression, ataxia, lethargy, and twitching.^{154,157} The vascular tropism of HeV in guinea pigs is evident in many organs.⁶⁵ Death appears to result from vascular disease in a variety of organs.⁶⁵ Only a proportion of infected animals develop encephalitis with virus observed in blood vessels and neurons.¹⁵⁴

A ferret model of henipavirus infection and pathogenesis has also been developed.^{19,110} The ferret exhibits both severe

respiratory and neurologic disease as well as generalized vasculitis following an oronasal challenge with NiV with doses as low as 500 TCID₅₀ within 6 to 10 days postinfection. Clinical signs in affected ferrets included various combinations of severe depression, cough, serous nasal discharge, dyspnea, subcutaneous edema of the head, cutaneous ecchymoses, and obtundation with tremor and hind limb paresis depending on the challenge dose. Clinical disease in the ferret included vascular fibrinoid necrosis in multiple organs, necrotizing alveolitis, and syncytia of endothelium and alveolar epithelium. Histopathologic lesions included severe focal necrotizing alveolitis, vasculitis, degeneration of glomerular tufts, and focal necrosis in a wide range of other tissues. High levels of viral antigen were noted in blood vessel walls, and syncytial cells were frequently present. Viral antigen was present in neurons, and infectious NiV was isolated from multiple organs including the brain. Overall, NiV-mediated disease observed in the ferret model manifested with all the hallmarks seen among NiV-infected humans, and essentially identical results have been observed with HeV infection of ferrets (J. Pallister and L.-F. Wang, unpublished results).

A nonhuman primate model of henipavirus infection has been developed using the African green monkey,^{46,121} which yields a uniformly lethal disease with doses as low as approximately 2×10^4 pfu (NiV) or 4×10^5 TCID₅₀ of HeV. Monkeys, following intratracheal inoculation with either NiV or HeV, reveal a rapid spread of the virus (3–4 days postinfection) to numerous organ systems. NiV-infected monkeys developed a severe acute respiratory distress syndrome (ARDS)-like disease, associated with copious amounts of sanguineous fluid and froth. The lungs are enlarged with multifocal areas of congestion and hemorrhage, and immunohistochemical and histopathologic examination revealed significant amounts of polymerized fibrin and NiV antigen.⁴⁶ Endothelial syncytial cells were prominent in most of the tissues, and vasculitis was widespread. NiV antigen was present in endothelial and arterial smooth muscle cells in most examined tissues. Respiratory disease development could be seen within 7 days postinfection with either NiV or HeV following intratracheal inoculation by radiologic examination^{46,121} progressing to severe congestion and infiltration in the lung fields.

NiV and HeV could be found in virtually every organ system sampled at the time of death in the African green monkey. Immunohistochemical and histopathologic analysis revealed the presence of NiV antigen, predominantly in endothelial cells and smooth muscle cells, along with associated pathology. Most animals showed evidence of henipavirus-induced neurologic disease,^{46,121} with severe congestion and evidence of meningeal hemorrhaging and edema. NiV and HeV antigen was detected in endothelial cells in brain, with infection of neurons often widespread in the brain stem.^{46,121} NiV infection of squirrel monkeys has also been examined.⁸⁸ Although some animals demonstrated limited similarities to NiV pathogenesis in humans, only 50% of challenged animals exhibited any clinical signs, with most remaining well even following intranasal or intravenous delivery of doses as high as 10^7 pfu of NiV.

Unlike the cat, guinea pig, hamster, and squirrel monkey models of NiV or HeV infection, severe respiratory pathology, neurologic disease, and generalized vasculitis all occur in henipavirus-infected African green monkeys, providing an accurate reflection of what is observed in henipavirus-infected humans.

EPIDEMIOLOGY

Age

The age of patients with encephalitis in the Malaysian outbreak ranged from 9 to 76 years, with almost 50% of cases occurring in those 40 to 44 years.^{27,28,48,111,159} The male-to-female ratio was approximately 3:1, and more than 80% of the patients were Chinese, with statistics reflecting the increased risk to those working with infected pigs.^{48,111} In Bangladesh, the age of patients ranged from 4 to 60 years, and males constituted 47% and 67% of the cases in the 2001 and 2003 outbreaks, respectively.^{43,68} The recent outbreak in early 2011 in Bangladesh claimed at least 35 lives, including many children and infants, with ages ranging from 2 to 56 years.⁸

Morbidity and Mortality

In Malaysia, between September 1998 and June 1999, 256 patients who developed acute NiV encephalitis were admitted to Malaysian hospitals and 105 died, a mortality rate of approximately 40%.^{30,111} The rate of subclinical infection in households and farms where cases of NiV encephalitis occurred was calculated to be 8% and 11%, respectively.^{111,139} In Singapore, where 11 patients were confirmed to have acute NiV encephalitis, a further 2 asymptomatic abattoir workers were serologically positive, representing a rate of subclinical infection of 15%.²⁵ Subsequently, 89 individuals were identified on the basis of positive serology as having experienced either an asymptomatic or mildly symptomatic NiV infection.¹³⁸ This increases the number of people infected with NiV to 345 and decreases the mortality rate to approximately 30%.¹⁶⁰ In Bangladesh, 98 of 135 patients died in eight outbreaks from 2001 to 2008, giving a combined case fatality rate of 73%.^{43,62,85} There have only been seven known human cases of HeV infection in Australia in the past 16 years, four of which have been fatal (three acute and one case of relapsed encephalitis).^{6,104,107,115}

Origin and Spread of Epidemics

Fruit bats (flying foxes) in the genus *Pteropus*, family *Pteropodidae*, suborder *Megachiroptera* are main reservoir hosts of HeV and NiV.^{33,56,164,165} In Australia, HeV has been shown to occur in four flying fox species, with the crude seroprevalence of 47%, indicating an endemic pattern of infection throughout Australia.⁴² Serologic tests show that NiV is widely dispersed in pteropid bats in Malaysia, especially the Island flying fox (*Pteropus hypomelanus*) and the Malayan flying fox (*Pteropus vampyrus*).¹⁶⁴ NiV was first isolated from the urine of Island flying foxes and from the saliva on partially eaten fruit³³ and has since been isolated from Lyle's flying foxes (*Pteropus lylei*) in Cambodia.¹²⁰ The Indian flying fox (*Pteropus giganteus*) is the only pteropid species throughout Bangladesh and the Indian subcontinent with a high seroprevalence of henipavirus-specific antibody.^{39,68,86,87} Additional serologic and limited nucleic acid evidence has suggested that related henipaviruses are circulating in other regions, including Thailand, Indonesia, China, Madagascar, and West Africa.^{36,61,70,81,131,145}

Neither HeV nor NiV appear to cause clinical disease in flying foxes infected naturally,^{42,43,119,164} and experimental infection with doses of HeV, consistently shown to be lethal in horses, generates only sporadic vasculitis in the lung, spleen, meninges, kidney, and gastrointestinal tract and only in a proportion of infected bats.¹⁵⁶ Viral antigen is detected in the tunica media

rather than endothelial cells, a fact that may spare the flying fox from the clinical effects associated with vasculitis.³⁷ In infected pregnant flying foxes, antigen was observed in similar locations and in the placenta.¹⁵⁵ The mode of transmission between flying foxes is unknown. Transplacental transmission has also been observed experimentally without apparent harm to the fetus.¹⁵⁵ Experimental infection of flying foxes with NiV produced a subclinical infection with a transient presence of virus within selected viscera along with periodic viral excretion in bat urine and seroconversion with neutralizing antibody present.⁹⁴

The spillover and epidemic hosts of HeV and NiV were horses in Australia and pigs in Malaysia. All human infections with HeV in Australia and NiV in Malaysia have only occurred through transmission from these domestic animal hosts.^{30,111} No evidence exists of direct transmission from pteropid bats to humans in Australia or Malaysia, despite many opportunities in Australia for transmission to bat carers.^{127,129} In contrast, flying foxes apparently play a direct role in the transmission of NiV to humans in the many recent outbreaks of disease in Bangladesh, where epidemiologic evidence in support of a role for pigs was lacking.^{43,68} Three pathways of NiV transmission from bats to people have been identified based on epidemiologic investigations in Bangladesh.⁸⁵ Consumption of fresh date palm sap appears to be the predominant risk factor, and infrared camera studies have confirmed that *P. giganteus* bats frequently visit date palm sap trees and consume sap during collection.¹²⁶ In the 2005 NiV outbreak in Tangail District, Bangladesh, drinking raw date palm sap was the only activity significantly associated with illness (64% among cases vs. 18% among controls).⁸⁷ Another route of transmission for NiV from bats to people in Bangladesh could be via domestic animals. Contact with a sick cow in Meherpur, Bangladesh, in 2001 was strongly associated with NiV infection,⁶⁸ and contact with pigs and diseased goats have also been implicated in other occurrences of NiV in Bangladesh.⁸⁵ Transmission via direct contact with NiV-infected bat secretions also appears possible from evidence in the Goalando

outbreak in 2004, where individuals who climbed trees were more likely to develop NiV infection than controls.⁹⁹

The mode of transmission from bats to spillover hosts in Australia and Malaysia remains to be determined. Three principal hypotheses exist. One is that masticated pellets of virus-contaminated, residual fruit pulp spat out by flying foxes are ingested by horses or pigs.¹⁶⁴ The second is that urine from infected animals contaminates pastures or pigsties. The third is that infected fetal tissues or fluids contaminate pastures or sties and are ingested. The latter is based largely on the fact that the HeV outbreaks have occurred during the birthing period of some species of flying fox and is supported by the isolation of virus from a pregnant flying fox and its fetus.⁵⁶

HeV has been transmitted from horse to man on seven occasions from 1994 to 2009, twice during the initial outbreak in Brisbane,⁴ twice during necropsy of horses that died in the field,^{125,130} twice during either daily nasal cavity lavage and participating in a necropsy,¹¹⁵ and once from performing an endoscopy on an infected horse.⁶ HeV is rarely found in the bronchi or bronchioles of infected horses, which suggests that aerosol transmission to either man or horses is less likely⁶⁴ and horse-to-horse transmission of HeV has not been demonstrated.¹⁵⁶ The presence of HeV in equine saliva, however, suggests that close contact with infected horses, such as might occur during manual feeding of the animals, may facilitate horse-to-human transmission.¹³⁰ The presence of virus in a wide range of tissues and in the nasal discharge commonly found at the terminal stage of infection offers a range of sources for virus transmission during necropsy.^{103,104} As shown in Figure 37.5A, high level of viral antigen can be detected in the nasal cavity of HeV-infected horses.

In the Malaysian NiV outbreak, contact with pigs or fresh pig products was required for transmission of the virus to humans, with greater likelihood of transfer to those in direct contact with sick or dying pigs on farms or in abattoirs.^{30,111} The presence of NiV in the respiratory epithelium of naturally and experimentally infected pigs (Fig. 37.5B) indicated that

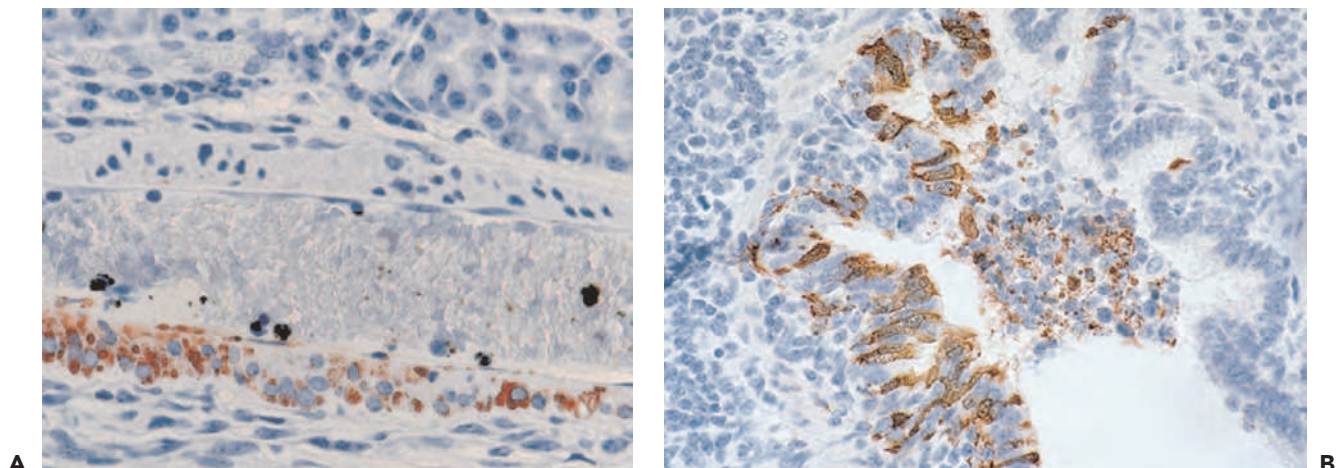


FIGURE 37.5. Immunoperoxidase detection of viral antigen in henipavirus-infected tissues. A: Staining of Hendra virus (HeV) antigen (N protein-specific antibody) within the wall of superficial arteriole in the submucosa of the nasal cavity in a HeV-infected horse. **B:** Immunolabeling (using anti-Nipah virus rabbit serum) within the lung of a pig infected with Nipah virus. Involvement of bronchial epithelium and airway debris is noted. (Courtesy of Dr. Deborah Middleton, CSIRO Australian Animal Health Laboratory.)

virus probably spread to humans and within the pig population by aerosol or by direct contact with oropharyngeal or nasal secretions.^{95,97,149} The presence of the virus in a wide range of organs indicates that humans may also have been infected during processes such as slaughtering or farrowing. In Bangladesh, pigs were excluded as potential sources of NiV on epidemiologic grounds, and human-to-human transmission was observed.^{54,62} The virus may have been transmitted to human index cases directly through contact with fruit bat secretions in contaminated fruit or date palm sap before circulation in the human population.^{33,43,68,85} Nosocomial transmission has been detected in some of the Bangladesh and India outbreaks.^{9,23,85}

CLINICAL FEATURES

Incubation Period

Based on the time interval between the last exposure to pigs and onset of disease, the incubation period for NiV ranged from 2 to 45 days; however, for 90% of patients, it was 2 weeks or less.^{27,28,48} An estimate of 2 to 3 weeks was made based on the time interval between importation of pigs from NiV-affected areas of Malaysia and development of human disease at a Singaporean abattoir.²⁵ A mean incubation period of 9.4 days was calculated for four patients who had a fixed period of exposure.²⁷ In the occurrence of HeV in Australia in 2008, a detailed examination of exposure histories from two infected patients (one fatal) suggested a likely incubation period of 9 to 16 days, with exposure occurring some 3 days before the onset of symptoms of HeV infection in the horse.¹¹⁵

Acute Clinical Features

The first two patients infected with HeV presented with myalgia, headaches, lethargy, and vertigo. One patient recovered; however, the other developed pneumonitis, respiratory failure, renal failure, and arterial thrombosis, and died of cardiac arrest 7 days after admission to the hospital. Findings at autopsy were consistent with a viral infection; both lungs were congested, hemorrhagic, and filled with serous fluid, and the histology revealed focal necrotizing alveolitis with many giant cells, some syncytial formation, and viral inclusions.¹³⁰ A third case of HeV infection presented first with meningitis and a 12-day history of sore throat, headache, drowsiness, vomiting, and neck stiffness. After an apparent full recovery, this patient developed fatal encephalitis 13 months later and was admitted to the hospital with a generalized tonic-clonic seizure after 2 weeks of irritable mood and low back pain. Recurrent focal motor seizures occurred over the next 7 days, as did secondarily generalized seizures and low-grade fever, followed by dense right hemiplegia, signs of brain stem involvement, and depressed consciousness requiring intubation. The patient remained comatose and died 25 days after admission.¹⁰⁷ Two patients in 2008¹¹⁵ presented with initial influenza-like illness, although soon after apparent clinical improvement and an absence of fever, encephalitis developed in both. Magnetic resonance imaging (MRI) revealed widespread cortical, subcortical, and deep white matter involvement, similar to the previous late-onset case of HeV encephalitis¹⁰⁷ and to NiV encephalitis cases.^{78,127}

With NiV, the mean duration of illness from the onset of symptoms to the nadir was 3 to 31 days, with an average of 6.9 days.⁴⁸ Most patients presented with acute encephalitis

characterized by fever, headache, drowsiness, dizziness, myalgia, and vomiting, and more than 50% had a reduced level of consciousness.^{27,30,48,114} The major clinical signs included drowsiness, areflexia, segmental myoclonus, tachycardia, hypertension, pinpoint pupils, and an abnormal doll's eye reflex. Such clinical features as these suggested involvement of the brain stem and upper cervical spinal cord, and were observed more frequently in patients with a reduced level of consciousness.^{48,139} Patients who retained normal levels of consciousness throughout their illness recovered fully; however, only 15% with reduced levels of consciousness survived. Such neurologic manifestations are consistent with vasculitis-induced thrombosis in the brain and the direct infection of neurons.^{29,30,114,159} The multiple discrete lesions 1 to 5 mm in diameter in the cerebral white matter detected by MRI may be the site of such microinfarctions and are distinct from lesions caused by other viruses.^{30,82,114,127,159} Although the predominant clinical features of NiV encephalitis derive from CNS involvement, a proportion of patients displayed pulmonary involvement, which presented as an atypical pneumonia with fever, cough, and headache.^{27,48,114} The clinical presentation of NiV infections in Bangladesh is also predominantly a severe respiratory disease.⁶⁷

Outcome of Infection

Most patients who survived acute NiV encephalitis made a full recovery; however, approximately 20% had residual neurologic deficits.^{27,48,83} Neurologic sequelae included cognitive difficulties, tetraparesis, cerebellar signs, nerve palsies, and clinical depression. A few patients remained in a vegetative state. In patients with encephalitis who recovered, most brain lesions revealed by MRI disappeared or became smaller over a period of 12 to 18 months, although some remained unchanged during this period.⁸³ Approximately 7.5% of patients who recovered from acute encephalitis and 3.4% of those who experienced nonencephalitic or asymptomatic infection developed late neurologic disease.^{48,127,138,161} Relapse encephalitis and late-onset encephalitis presented several months to 4 years after the initial infection. Relapsed cases had elevated IgG, but not IgM, and no vasculitis, and unlike the situation in acute encephalitis, virus was not isolated from throat and nasal secretions.^{30-32,127,159,161}

The clinical features associated with relapse and late-onset encephalitis resembled those found with acute NiV encephalitis, although decreased incidence was seen of fever, coma, segmental myoclonus, and meningism and an increased occurrence of seizures and focal cortical signs compared with the acute manifestation of the disease.¹³⁸ The clinical, radiologic, and pathologic features of relapse NiV encephalitis resembled those of the patient who became infected with HeV, suffered mild, transient aseptic meningitis, and recovered but died of a fatal meningoencephalitis 13 months later.^{107,158} Most patients with relapse and late-onset encephalitis had only one neurologic episode, although some patients experienced two episodes separated by a mean of 7.6 months (6 weeks to 1 year).¹³⁸ The mortality rate associated with relapse and late-onset encephalitis at 18% was lower than that associated with acute encephalitis, at 30% to 40%. However, 61% of patients with relapse and late onset had further neurologic sequelae compared with 22% after acute encephalitis. Among NiV survivors in Bangladesh, some 30% have moderate to severe persistent neurologic dysfunction for years following acute infection.¹²⁸

The demographics, clinical features, serology, and MRI of patients with relapsed and late-onset encephalitis were similar, suggesting that the two diseases have identical pathogenesis¹³⁸ and that the initial infection in late-onset encephalitis patients may not have been sufficiently severe to cause neurologic symptoms. MRI abnormalities similar to those observed in patients with acute encephalitis were also seen in 16% of asymptomatic patients, although the lesions were fewer in number.¹³⁹ The involvement of the cortex in relapse and late-onset encephalitis suggests a different pathologic mechanism compared with acute encephalitis. Relapse and late-onset encephalitis are considered to be caused by the recrudescent and rapid replication of virus that had persisted following acute or asymptomatic NiV infection.¹³⁸ NiV, however, was not isolated from CSF and brain tissue of patients with relapse and late-onset encephalitis.¹³⁸

DIAGNOSIS

Laboratory Diagnosis

Virus isolation, EM, immuno-EM, immunohistochemistry (IHC), serology, and polymerase chain reaction (PCR) played key roles in the initial discovery of HeV¹⁰⁴ and NiV,²⁹ and they remain essential elements in a repertoire of procedures for the rapid and specific diagnosis of henipavirus infections in humans and animals.

During investigation of a suspected disease outbreak, attempts to grow henipaviruses may be initiated in a BSL3 laboratory. However, if a cytopathic effect (CPE) is observed and the growth of henipavirus is confirmed by PCR or immune staining, infected cultures should be handled under BSL4 conditions and subsequent work with live virus restricted to BSL4. Both HeV and NiV replicate in various cell lines—a feature that contributed to the efficiency with which they were isolated during the initial disease outbreak investigations.^{29,104} Vero cells are commonly used, generating titers of virus as high as 10^8 infectious virions per milliliter.^{28,34} In fatal cases, attempts should be made to isolate virus from brain, lung, kidney, and spleen.³⁴ For tissue specimens containing a high virus load, direct examination by immuno-EM and IHC can be very useful in providing early diagnosis. Various antibody reagents have been developed for this purpose, including polyclonal antisera, monospecific antibodies raised against recombinant antigens,¹⁴⁷ and monoclonal antibodies (mAb) raised against whole virions or vaccinia virus-expressed viral proteins.^{71,140,141,153} Using HeV- or NiV-specific mAb, it is possible to differentiate between the two viruses.^{141,153} Quantitative real-time PCR (TaqMan assay) has been the method of choice to detect viral materials in infected tissues because of its speed, specificity, and sensitivity. The first-generation henipavirus TaqMan assays are either HeV specific¹⁵⁴ or NiV specific.⁵² Recently, several consensus henipavirus real-time PCR assays have been developed that target different conserved regions of the viral genome.⁴¹ It should be cautioned that the current PCR tests may not work with new henipaviruses yet to be discovered, especially those from African bats, owing to expected greater genetic divergence than those detected in Australia and Asia.

For henipaviruses, serologic tests are important both during outbreak investigation and for disease surveillance. The virus neutralization test (VNT) is accepted as the reference standard.³⁴ Few laboratories, however, can conduct neutralization

tests because of the requirement to handle live virus at BSL4. For surveillance and diagnostic purposes, three types of tests that do not require BSL4 containment have been developed:

1. *Enzyme-linked immunosorbent assay (ELISA)*: Several ELISA-based tests have been reported for the detection of henipavirus antibodies.^{34,40,73} For diagnosis of human infections, two different ELISA tests have been applied: an IgM capture ELISA for early diagnosis of infection and an indirect ELISA for detection of IgG antibodies.³⁴
2. *Liquid protein array multiplex test*: A Luminex-based test based on recombinant soluble G proteins of HeV and NiV was developed that is capable of mimicking VNT with great sensitivity and differentiating between antibody responses of HeV versus NiV infection.¹⁵
3. *Pseudotype virus*: Different pseudotype systems carrying the henipavirus F and G proteins have been developed as a surrogate VNT for detecting henipavirus-specific antibodies.^{72,74,137} Incorporation of reporter genes in these systems resulted in greater sensitivity and reproducibility.^{72,137}

PREVENTION AND CONTROL

Treatment

Ribavirin, which inhibits replication of HeV *in vitro*,¹⁶² was used during the NiV outbreak in Malaysia in an open-label study in which 140 patients with encephalitis were given the drug, and 54 patients who presented before ribavirin became available or who refused treatment acted as controls.²⁶ Mortality in the treated group was 32% compared with 54% in the control group, representing a 35% reduction ($P = 0.011$). Duration of ventilation and total hospital stay were both significantly shorter in the ribavirin group ($P = 0.0002$ and <0.0001 , respectively). In the absence of other therapies, ribavirin may be an option for treatment of henipavirus infections. However, two HeV-infected patients in 2008¹¹⁵ were given a high-dose intravenous regimen of ribavirin, although basal concentrations appeared inadequate given the results of *in vitro* susceptibility testing of HeV, and the efficacy of ribavirin as therapy or prophylaxis in people remains at best uncertain. Chloroquine, an antimalarial drug, was first demonstrated to block the critical proteolytic processing needed for HeV F maturation and function.¹⁰⁹ Not surprisingly, the drug was later shown to inhibit NiV and HeV infection in cell culture experiments.¹¹⁷ Chloroquine was administered along with ribavirin to one HeV-infected individual in 2009⁶ with no apparent clinical benefit.

In vivo, ribavirin only delayed but did not prevent deaths caused by NiV and had no effect on HeV infection in a hamster model.^{44,47} Ribavirin treatment also only delayed disease onset by 1 to 2 days in African green monkeys challenged with HeV with no significant benefit for disease progression or outcome.¹²¹ Chloroquine administration, either alone or in combination with ribavirin, had no therapeutic benefit in ferrets challenged with NiV or hamsters challenged with either NiV or HeV.^{44,110}

Vaccines and Passive Immunotherapy

No henipavirus vaccines are available at this time; however, studies suggest that vaccination may offer several viable anti-henipavirus strategies.^{22,38} NiV F and G glycoproteins expressed from vaccinia virus elicit neutralizing antibodies in mice and

hamsters,^{51,136} with higher antibody titers generated in response to the G glycoprotein compared with the F glycoprotein. Hamsters were protected from lethal challenge with NiV following vaccination with vaccinia virus expressing either NiV F or G glycoprotein.⁵¹ The challenge virus was capable of hyperimmunizing vaccinated animals, indicating that although the virus replicated, the presence of neutralizing antibodies ameliorated replication to an extent that limited infection and prevented clinical disease. Further, the protection afforded hamsters by passive transfer of anti-G and anti-F antibodies before a lethal NiV or HeV challenge confirmed both the importance of a humoral protective immune response to NiV.^{51,53}

HeV G glycoprotein has been expressed in a soluble form (sG-HeV) that retains many native characteristics and can elicit a potent cross-reactive neutralizing antibody response in rabbits.¹⁴ Rabbit anti-HeV G antibodies neutralize both HeV and NiV in cell culture, displaying a slightly higher titer against the homologous virus. The nature and location of the neutralizing epitopes on the F glycoprotein have not been reported, although preliminary information is available for the HeV G glycoprotein.¹⁵³ Four neutralizing epitopes have been mapped on the globular head of the HeV G protein. Two are located on the base of the head and two on the top, in locations resembling those identified as neutralizing sites in other paramyxoviruses.

Immunization and challenge studies using recombinant sG-HeV in the cat model have demonstrated that the protein can illicit a completely protective immune response against NiV challenge,¹⁰¹ even at a low-dose formulation with CpG and Alhydrogel and a two-dose protocol followed by oronasal challenge with 50,000 TCID₅₀ of NiV.⁹¹ Further sG-HeV immunization studies have been completed in the ferret with a HeV challenge (J. Pallister and L-F. Wang, in submission) and in the African green monkey with a NiV challenge (T. Geisbert and C. Broder, in submission). In both of these investigations, complete protection from henipavirus-induced disease was achieved. The potential application of sG-HeV as an equine vaccine is being evaluated in Australia (D. Middleton, personal communication). These data suggest that a single vaccine (sG-HeV) may be effective against both HeV and NiV. Analysis of the antibody responses in sera from naturally infected or immunized sources has also shown that HeV-infected sources had high levels of NiV G cross-reactive antibodies, whereas NiV-infected individuals had limited cross-reactive antibodies to HeV G. Together, these data suggested that the HeV G stimulates a more cross-reactive immune response.¹⁵

The sG-HeV glycoprotein was also used to isolate HeV G-specific human mAbs. One human mAb (m102.4) was HeV and NiV cross-reactive and possessed extremely potent virus neutralizing activity.^{167,168} *In vivo* studies have demonstrated that m102.4 can protect animals from a lethal challenge with henipavirus as a postexposure application in the ferret model with NiV¹⁹ and in the African green monkey model with HeV (T. Geisbert and C. Broder, in submission). In August 2009, m102.4 was used on a compassionate basis to save the life of an HeV-infected individual while in a coma (G. Playford, personal communication). Unfortunately, delivery and intravenous administration of only 100 mg of available antibody occurred after the onset of encephalitis, and the individual died shortly thereafter. During the 2010 HeV emergence, 11 people had potential exposure and 2 individuals considered at high risk. In this instance, the m102.4 antibody was given to 2 individu-

als prior to HeV diagnosis or the onset of clinical disease⁵ at doses sufficient to achieve a high serum concentration. Both individuals have remained healthy. Altogether, these findings highlight the therapeutic potential of antibody-based passive transfer modalities for treating henipavirus exposure.

Peptide Inhibitors

The first potential henipavirus-specific therapeutic was shown to be a heptad peptide-based fusion inhibitor¹⁷ analogous to the human immunodeficiency virus type 1 (HIV-1)-specific peptide, enfuvirtide (Fuzeon) approved by the Food and Drug Administration (FDA) in March 2003. The henipavirus F₁ glycoprotein resembles other fusion glycoproteins in having α -helical heptad repeat (HR) domains proximal to both the fusion peptide at the amino (N) terminus and the transmembrane domain near the carboxy (C) terminus of the protein. The HR domains are involved in the formation of a trimer-of-hairpins structure during or immediately following the fusion of virus and cell membranes that occurs during infection. Addition of exogenous peptide from either HR domain blocks formation of the trimer-of-hairpins and abrogates membrane fusion and entry of the viral genome into the cell.^{16,17,38} These observations were followed up with testing cholesterol tagged HR-derived peptides in the hamster model of NiV infection.¹¹⁸ The *in vivo* efficacy of peptide fusion inhibitors of henipavirus infection merits further investigation.

PERSPECTIVE AND GEOGRAPHIC CONSIDERATIONS

The high virulence of the henipaviruses and the requirement for BSL4 facilities have hampered investigations into the biology and pathogenesis of these novel paramyxoviruses. Recent investigations into the structure and function of henipavirus proteins expressed from cloned genes have provided insight into the functions of many henipavirus proteins in infected cells. It remains to be determined if all of the functional characteristics of the henipavirus proteins determined *in vitro* accurately reflect the role that they play in the cells of both terrestrial and chiropteran hosts.

Many questions relating to the ecology and biology of henipaviruses remain unanswered. Little doubt exists that *Pteropus* species of fruit bats are the major reservoir host of these viruses. With the wide geographic range of *Pteropus* species as overlapping populations, extending from islands in the South Pacific through Australia, and southern Asia to Pakistan, and with additional species on islands off the eastern coast of Africa, together with the cross-reactive serologic evidence of henipavirus presence,^{61,81} it would seem that several other related viruses may remain to be identified.^{43,56} The emergence of these and related viruses is probably associated with the destruction of the flying fox native habitats, driving the animals to seek food from orchards and ornamental trees in urban and periurban areas. Thus, with continued deforestation, undoubtedly further outbreaks of HeV, NiV, and novel related members of the genus will occur. The mechanisms by which henipaviruses are transmitted between fruit bats and maintained within their colonies, as well as the pathways leading to the infection of spillover hosts, remain to be elucidated.

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Respiratory Syncytial Virus and Metapneumovirus

History

Infectious Agent

- Classification
- Virion
- RNA
- Proteins
- Replicative Cycle
- Propagation *In Vitro*
- Genetics and Reverse Genetics
- Infection of Experimental Animals
- Antigenic Subgroups and Diversity
- Animal Counterparts

Pathogenesis and Pathology

Immune Response

- Antigens
- Innate Immunity and Inflammation
- Antibodies
- T Lymphocytes
- Viral Inhibition and Evasion of Host Immunity

Epidemiology

- Infection of Infants and Young Children
- Infection of Adults
- Other High-Risk Populations
- Epidemics

Clinical Features

Diagnosis

- Differential Diagnosis
- Laboratory Diagnosis

Treatment and Prevention

- Treatment
- Prevention

Perspective

Acknowledgments

from infants with respiratory illness, and serologic studies indicated that infection in infants and children was common.^{86,87} HRSV is now recognized as the most important viral agent of pediatric lower respiratory tract illness (LRI) worldwide. In many areas it outranks other microbial pathogens as a cause of pneumonia and bronchiolitis in infants. In addition, HRSV can infect and cause disease in individuals of all ages and severe disease in the elderly and in profoundly immunosuppressed individuals.^{146,163,233} Worldwide, acute respiratory infection (ARI) is the leading cause of mortality due to infectious disease, and HRSV remains one of the pathogens deemed most important for vaccine development. HRSV research is hampered by its poor growth *in vitro* and its physical instability. HRSV has a single serotype with two antigenic subgroups A and B.

Human metapneumovirus (HMPV) was first described in 2001 following its isolation from infants and children experiencing HRSV-like disease of unknown etiology.⁵⁷² There is serologic evidence of extensive pediatric infection dating back more than 50 years, and thus HMPV is newly discovered rather than newly emerged.^{572,608,610} The virus had been overlooked because it grows slowly *in vitro*, has a delayed cytopathic effect, and usually requires added trypsin for activation of the fusion F protein. HMPV is recognized as an important agent of respiratory tract disease worldwide, especially in the pediatric and elderly populations, although its impact is less than that of HRSV.⁶⁰⁸ HMPV also has a single serotype with two subgroups A and B.⁵¹⁵

INFECTIOUS AGENT

Classification

HRSV and HMPV are enveloped, cytoplasmic viruses with single-stranded nonsegmented negative-sense RNA genomes. They are members of the family *Paramyxoviridae* (Chapter 33) of the order *Mononegavirales* (Chapter 30). *Paramyxoviridae* has two subfamilies: *Paramyxovirinae*, which includes the human and animal parainfluenza viruses (PIVs, Chapter 34), mumps virus (Chapter 35), and measles virus (Chapter 36) among others, and *Pneumovirinae*, which consists of HRSV and HMPV and their animal relatives. There are two genera in *Pneumovirinae*: Genus *Pneumovirus* consists of HRSV, bovine RSV (BRSV), and pneumonia virus of mice (PVM), and genus *Metapneumovirus* consists of HMPV and avian metapneumovirus (AMPV, formerly called turkey rhinotracheitis virus [TRTV] or avian pneumovirus [APV]). Comparisons of

HISTORY

Human respiratory syncytial virus (HRSV) was first isolated in 1955 from a laboratory chimpanzee with illness resembling the common cold.³⁹⁸ Shortly thereafter, the same virus was recovered

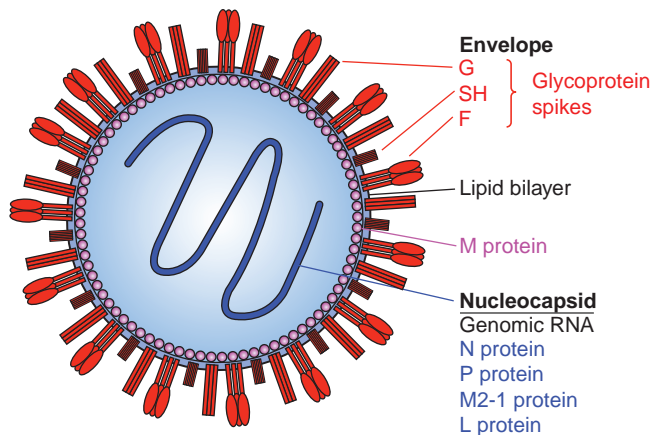


FIGURE 38.1. Idealized diagram of the human respiratory syncytial (HRSV) particle. The G, SH, and F proteins are present in homo-oligomers that constitute the glycoprotein spikes. The M protein underlies the lipid bilayer. The proteins of the nucleocapsid are not depicted individually. The human metapneumovirus (HMPV) particle is similar.

these viruses based on gene maps and nucleotide sequences are shown [e-Figures 38.1](#) and [38.2](#).

(Note that the descriptions in the following sections usually will begin with HRSV followed by HMPV.)

Virion

The HRSV virion consists of a nucleocapsid that is packaged in a lipid envelope derived from the host cell plasma membrane during budding (Fig. 38.1 and Fig. 38.2C). When visualized by electron microscopy (Fig. 38.2C and E), virions appear as irregular spherical particles of 100 to 350 nm in diameter and long filamentous forms that often predominate and are 60 to 200 nm in diameter and up to 10 μ m in length. Despite the variability in size and shape, the ultraviolet (UV) inactivation kinetics of infectivity indicates that most particles contain a single functional genome.¹⁴⁰ Both forms of the virion mostly remain cell associated. The filaments can be visualized as projections from the surface of infected cells by fluorescence photomicroscopy^{19,270,423,475} (Fig. 38.2B). The nucleocapsid appears in electron micrographs as a herringbone structure that is characteristic of *Paramyxoviridae*. However, the HRSV nucleocapsid is narrower than those of prototype members of *Paramyxovirinae* (12 to 16 nm compared to 17 to 20 nm) and has a steeper pitch.^{19,42,423}

The HRSV envelope contains three virally encoded transmembrane surface glycoproteins: the major attachment protein G, the fusion protein F, and the small hydrophobic (SH) protein (Figs. 38.3A and 38.4). In addition, there is a nonglycosylated matrix M protein that is thought to form a layer on the inner face of the envelope. The viral glycoproteins are present as transmembrane homooligomers that are visualized as short (11 to 20 nm), closely spaced (intervals of 6 to 10 nm) surface projections or “spikes.” HRSV lacks a neuraminidase or a hemagglutinin; PVM alone agglutinates murine erythrocytes, via its G protein.³³⁹

The viral RNA is associated with four nucleocapsid/polymerase proteins: the nucleoprotein N, the phosphoprotein P, the transcription processivity factor M2-1, and the large polymerase subunit L (Fig. 38.3A). However, polymerase activity in purified virions, an activity found in prototypic

members of *Mononegavirales*, has not been demonstrated for HRSV preparations.

HRSV virions can readily lose infectivity during handling, presumably reflecting particle instability. This can be partly overcome by agents such as sugars that reduce aggregation and improve thermal stability.¹⁷ When recombinant HRSV was engineered to contain a foreign attachment protein from baculovirus in place of the HRSV glycoproteins, the stability of infectivity was improved,⁴⁹¹ suggesting that the lability of the particle may reside in one or more of the glycoproteins. Other data have implicated the F protein in thermo instability.⁴⁶⁹ The long filamentous shape of the particle may also contribute to fragility and loss of infectivity.

HMPV virions were visualized by electron microscopy as pleomorphic spheres and filaments that appear to have general similarity to those of HRSV^{443,572} (Fig. 38.2F). The spherical particles had a reported diameter of 150 to 600 nm with envelope spikes of 13 to 17 nm. The nucleocapsid diameter was reported as 17 nm, suggesting a possible difference compared to HRSV.⁴⁴³ HMPV appears to lack a hemagglutinin⁵⁷²; other virion-associated activities have not been reported. HMPV has the same array of structural proteins as HRSV (Fig. 38.3B). The infectivity of HMPV particles is markedly more stable than that of HRSV.⁵⁵⁷

RNA

The HRSV genome (Fig. 38.3C) is a single negative-sense strand of RNA ranging in length from 15,191 to 15,226 nucleotides for six sequenced strains including the subgroup A strains A2 (15,222 nucleotides; GenBank accession number M74568), Long (15,226 nucleotides; AY911262), S2 (15,191 nucleotides; NC_001803), and line 19 (15,191 nucleotides; FJ614813), and the subgroup B strains B1 (15,225 nucleotides; NC_001781) and 9320 (15,225 nucleotides; AY353550). More recently, complete sequences were reported and analyzed for 60 and four additional subgroup A and B strains, respectively.^{313a,491a,538a} In addition, extensive sequence information and extensive inter-subgroup comparison is available for subgroup B strain 18537.^{274,276,278} The genome is neither capped nor polyadenylated. Both in virions and intracellularly, the genome is tightly and completely bound by N protein to create an RNase-resistant nucleocapsid, as is typical of *Mononegavirales*. This tight encapsidation likely protects the genome, which lacks stabilizing features of capping and polyadenylation, from degradation. It also likely shields the genome from recognition by host cell pattern recognition receptors, especially (a) the cytoplasmic helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein (MDA-5), which detect triphosphorylated RNA and double-stranded RNA (dsRNA) and activate the cellular transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NF- κ B) to induce type I interferon (IFN) and proinflammatory cytokines, and (b) RNA-inducible protein kinase R (PKR), which also activates NF- κ B and phosphorylates eukaryotic translation initiation factor 2a (eIF-2a) to inhibit translational initiation as part of antiviral defense.

The HRSV genome contains 10 genes in the order 3' NS1-NS2-N-P-M-SH-G-F-M2-L (Fig. 38.3C) that are transcribed sequentially into 10 separate messenger RNAs (mRNAs).^{95,99,107,140,388} Each gene begins with a highly conserved nine-nucleotide gene-start (GS) transcription signal and ends with a moderately conserved 12- to 13-nucleotide

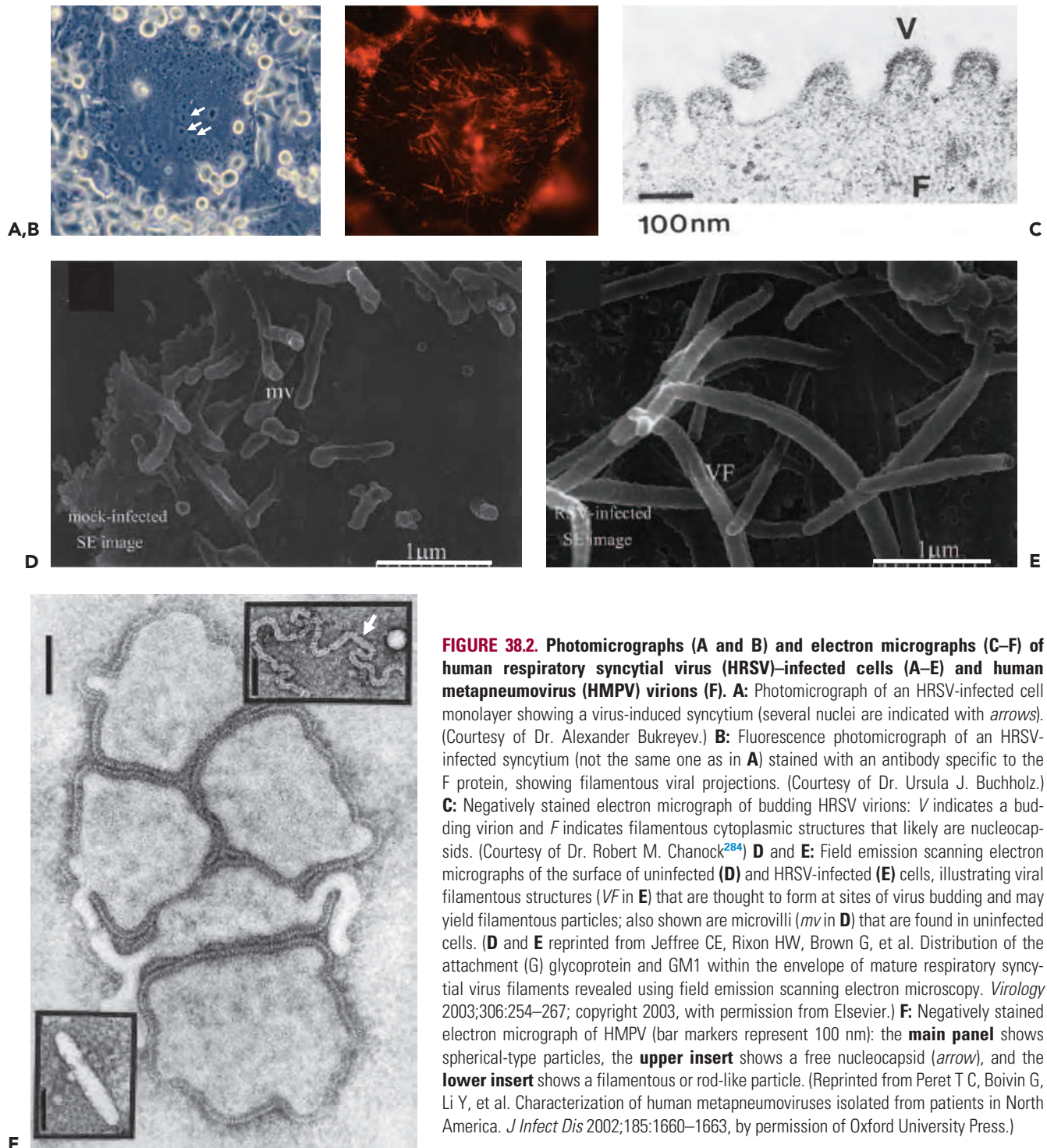


FIGURE 38.2. Photomicrographs (A and B) and electron micrographs (C–F) of human respiratory syncytial virus (HRSV)–infected cells (A–E) and human metapneumovirus (HMPV) virions (F). **A:** Photomicrograph of an HRSV-infected cell monolayer showing a virus-induced syncytium (several nuclei are indicated with arrows). (Courtesy of Dr. Alexander Bukreyev.) **B:** Fluorescence photomicrograph of an HRSV-infected syncytium (not the same one as in **A**) stained with an antibody specific to the F protein, showing filamentous viral projections. (Courtesy of Dr. Ursula J. Buchholz.) **C:** Negatively stained electron micrograph of budding HRSV virions: V indicates a budding virion and F indicates filamentous cytoplasmic structures that likely are nucleocapsids. (Courtesy of Dr. Robert M. Chanock²⁸⁴) **D** and **E:** Field emission scanning electron micrographs of the surface of uninfected (**D**) and HRSV-infected (**E**) cells, illustrating viral filamentous structures (VF in **E**) that are thought to form at sites of virus budding and may yield filamentous particles; also shown are microvilli (mv in **D**) that are found in uninfected cells. (**D** and **E** reprinted from Jeffree CE, Rixon HW, Brown G, et al. Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. *Virology* 2003;306:254–267; copyright 2003, with permission from Elsevier.) **F:** Negatively stained electron micrograph of HMPV (bar markers represent 100 nm): the **main panel** shows spherical-type particles, the **upper insert** shows a free nucleocapsid (arrow), and the **lower insert** shows a filamentous or rod-like particle. (Reprinted from Peret T C, Boivin G, Li Y, et al. Characterization of human metapneumoviruses isolated from patients in North America. *J Infect Dis* 2002;185:1660–1663, by permission of Oxford University Press.)

gene-end (GE) signal^{275,315} (e-Fig. 38.3A). The first nine genes are separated by intergenic regions that vary in length from 1 to 58 nucleotides for the strains sequenced to date.²⁷⁵ These lack any conserved motifs, are poorly conserved between strains, and appear to be unimportant spacers. The last two HRSV genes, M2 and L, overlap by 68 nucleotides¹⁰⁶ (Fig. 38.3C and e-Fig. 38.3A). Specifically, the GS signal for the L gene is located upstream, rather than downstream, of the M2 GE signal. The same overlap occurs in BRSV; overlapping genes are not found in any other members of *Paramyxoviridae*, but sometimes are

found in *Rhabdoviridae* and *Filoviridae*. The 3′ and 5′ ends of the genome consist of short extragenic leader and trailer regions (44 and 155 nucleotides long, respectively, in strain A2).

The HRSV mRNAs contain a methylated 5′ cap structure m⁷G[5′]ppp[5′]Gp²⁴ and are polyadenylated by reiterative copying on a U tract in the GE signal. Each HRSV mRNA encodes a single major protein except for M2, which has separate open reading frames (ORFs) for the M2-1 and M2-2 proteins. The M2-1 ORF is located in the upstream part of the mRNA, whereas the M2-2 ORF is located downstream and

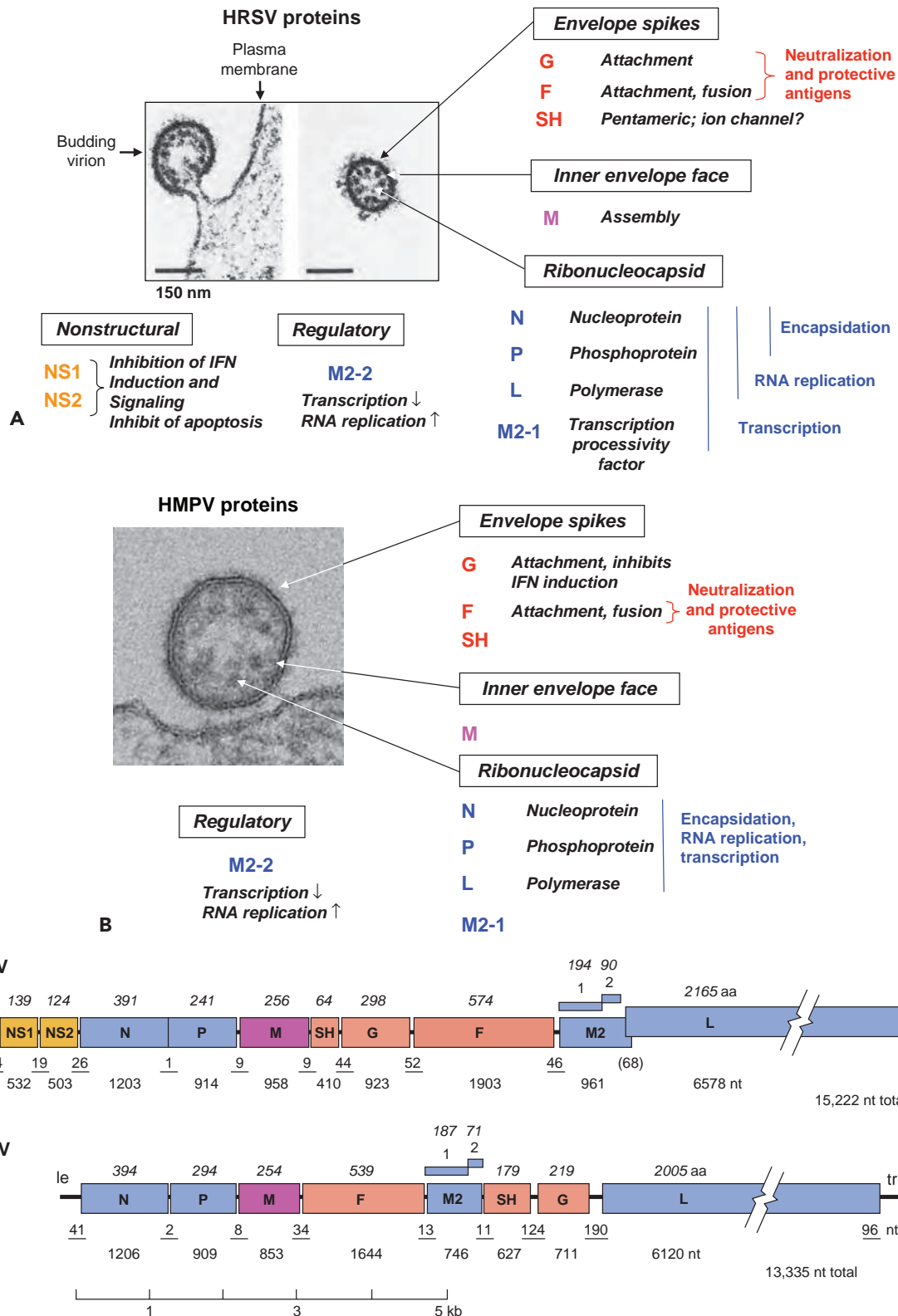


FIGURE 38.3. The proteins of human respiratory syncytial virus (HRSV) (A) and human metapneumovirus (HMPV) (B), and maps of the viral genomic RNAs (C). **A** and **B**: Locations of the proteins in the virus particle and major functions are indicated when known. **A–C**: Color-coded: proteins/genes of the nucleocapsid and polymerase complex or that are involved in RNA synthesis are in blue, surface glycoproteins in red, matrix protein in magenta, and the two HRSV nonstructural proteins in brown. The maps in **panel C** are approximately to scale and show the 3' to 5' negative-sense genomes of HRSV strain A2 and HMPV strain CAN97-83. The overlapping open reading frames (ORFs) of the M2 messenger RNAs (mRNAs) are illustrated over the M2 genes. Numbers beneath each map indicate nucleotide (nt) lengths; those of the extragenic leader (le), trailer (tr), and intergenic regions are underlined, and that of the HRSV gene overlap is in parentheses. *Italicized numbers* above each map indicate amino acid (aa) lengths.

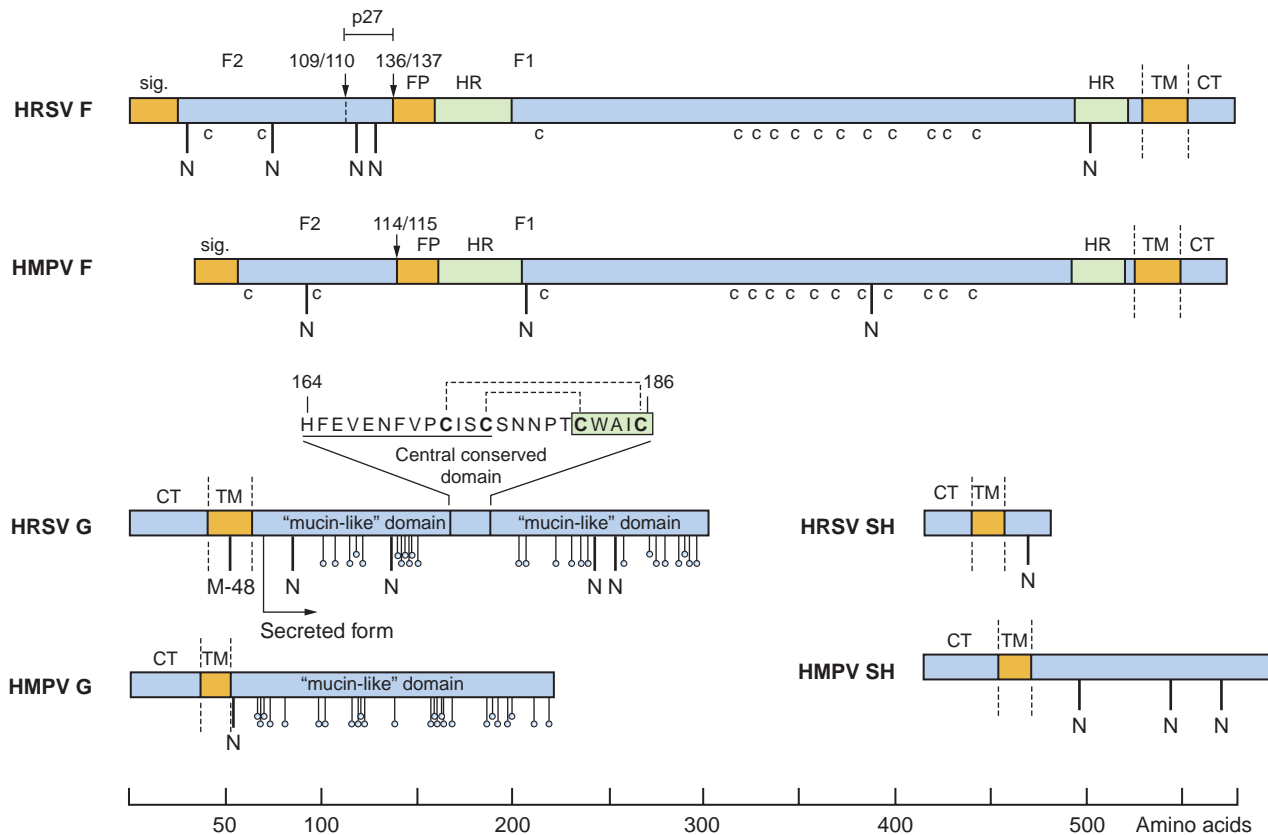


FIGURE 38.4. Primary structures (approximately to scale) of the F, G, and SH surface glycoproteins of human respiratory syncytial virus (HRSV) strain A2 and human metapneumovirus (HMPV) strain CAN97-83. Hydrophobic domains are *brown bars*: Sig., signal peptide; FP, fusion peptide; TM, transmembrane anchor; CT, cytoplasmic tail. Heptad repeats (HR) in the F protein are *green* and cysteine residues conserved between the F proteins of HRSV and HMPV are indicated underneath (indicated as c). Downward-facing arrows identify the cleavage-activation site(s) in the F protein. Potential acceptor sites for N-linked carbohydrate are indicated as *downward facing stalks with N*. For each G protein, the 25 potential acceptor sites for O-linked sugars predicted by NetOGlyc 2.0 to be the most likely to be utilized are indicated as *downward facing stalks with small circles*. The sequence excerpt above the HRSV G protein shows the conserved segment (*underlined*) and cystine noose; cysteine residues are *bold*; the disulfide bonding pattern is indicated by *dotted lines*²⁰¹; and the fractalkine CX3C motif is *boxed*. M-48 in the HRSV G protein is the translational start site for the secreted form, and the mature secreted form is indicated.⁴⁷⁶

overlaps by 32 nucleotides in strain A2. Translation of the downstream M2-2 ORF depends on reinitiation by ribosomes exiting the upstream M2-1 ORF, a process that appears to be influenced by the structure of a region of RNA located ~150 nucleotides upstream of the M2-2 translational start site.^{202,203} Whereas the P genes of *Paramyxovirinae* encode additional accessory proteins by overlapping ORFs, alternative translational start sites, and RNA editing (Chapter 33), the P gene of HRSV (and HMPV) encodes only P.

HRSV transcription follows the general *Mononegavirales* model^{111,140,316} (Chapters 31 and 33), involving initiation at a single 3' promoter and a start-stop-restart sequential mechanism guided by the GS and GE signals. RNA sequences important in transcriptional initiation are shown in *e-Fig. 38.4*. Capping seems to be an essential step for efficient mRNA elongation: when capping was blocked using a novel HRSV-specific inhibitor, transcription produced uncapped abortive RNAs of ~45 to 50 nt.³⁴³ Termination at the various GE signals typically is somewhat inefficient, resulting in readthrough transcription that creates mRNAs representing two or more adjacent genes

and their intervening intergenic regions.¹⁰⁷ These readthrough mRNAs account for approximately 10% of total mRNA.

The M2/L gene overlap raises two questions for the model of sequential transcription: (a) how do polymerases that exit the M2 gene find the upstream L GS signal (or does polymerase enter independently at that site), and (b) how do polymerases that initiate at the L GS signal avoid premature termination when they cross the M2 GE signal? Studies with a minireplicon system showed that when polymerase completes transcription of the M2 gene, it efficiently gains access to the L gene by retrograde scanning.¹⁷⁰ The polymerase appeared to scan in both directions. This led to the realization that scanning may be a common function of the polymerase, and may occur at each gene junction as well as prior to the initiation of transcription and RNA replication. The M2 GE signal within the L gene indeed causes premature termination for 90% of L gene transcripts, producing a 68-nucleotide polyadenylated RNA that does not appear to encode a protein and is not known to have any further significance.¹⁰⁶ The synthesis of full-length L mRNA depends on polymerase readthrough at

the M2 GE signal. Therefore, the “error” of reading through a GE signal is necessary for synthesis of this essential mRNA.¹⁰⁶ Premature termination of 90% of L gene transcripts might be expected to severely downregulate the production of full-length mRNA, but the ability of the polymerase to recycle back to the L GS signal apparently relieves much of this effect, and the amount of L mRNA produced for HRSV relative to the other mRNAs appears to be about the same as for other members of *Paramyxoviridae*. The gene overlap does not seem to be of any particular benefit to the virus, and may be an accidental arrangement that can be tolerated due to the scanning function of the polymerase.

RNA replication by HRSV also follows the general *Mononegavirales* model (Chapters 31 and 33). RNA sequences important in the initiation of RNA replication are shown in e-Fig. 38.4. The replicating polymerase ignores the GS and GE signals and produces a complete positive-sense copy of the viral genome that is called the antigenome and that also is tightly encapsidated. The antigenome serves in turn as the template for producing progeny genomes. Chain elongation of nascent genomes and antigenomes depends on concurrent encapsidation.³⁷³ For viruses in the subfamily *Paramyxovirinae*, the nucleotide length of the genome must be an even multiple of six in order for efficient RNA replication to occur (“rule of six”; Chapter 33), reflecting a requirement for nucleocapsid organization, but there appears to be no comparable requirement for members of *Pneumovirinae*.^{303,488}

The HMPV genome is approximately 13.2 kb—nearly 2 kb shorter than that of HRSV—and lacks the NS1 and NS2 genes (Fig. 38.3C). In addition, the order of the SH, G, F, and M2 genes differs between HRSV (SH-G-F-M2) and HMPV (F-M2-SH-G). Correcting for the lack of NS1 and NS2 for HMPV and the difference in gene order, the genomes of HRSV and HMPV share 50% nucleotide sequence identity. Strains for which complete sequences have been reported include the following: CAN97-83 (13,335 nt, GenBank accession AY297749) and NL/00/1 (13,350, AF371337) of subgroup A, and CAN98-75 (13,280, AY297748) and NL/1/99 (13,293, AY525843) of subgroup B.^{46,259}

Features of the structure, encapsidation, transcription, and replication of the HMPV genome are generally similar to those described above for HRSV. The cis-acting signals of HMPV have considerable similarity to those of HRSV^{46,571} (e-Fig. 38.3B). The HMPV intergenic regions can be longer than those of HRSV, up to 190 nt in the case of CAN97-83 (Fig. 38.3C and e-Fig. 38.3B). Unlike HRSV, the HMPV M2-2 protein appears to be translated independently of the upstream M2-1 ORF.⁶⁶

Proteins

HRSV encodes 11 separate proteins (Fig. 38.3A). HMPV encodes nine proteins (Fig. 38.3B) that generally correspond to those of HRSV except for the lack of NS1 and NS2.^{46,571} Table 38.1 shows amino acid sequence relatedness between viruses within subfamily *Pneumovirinae*.

Fusion F Glycoprotein

The HRSV F and L proteins are the ones that most closely resemble their counterparts in *Paramyxovirinae*. As is typical for *Paramyxoviridae*, the HRSV F protein directs viral penetration by fusion between the virion envelope and the host cell plasma membrane. Later in infection, F protein expressed on the cell surface can mediate fusion with neighboring cells to form syncytia (Fig. 38.2A). Recent findings suggest that the HRSV F protein also plays a major role in viral attachment involving interaction with the cellular protein nucleolin.⁵⁴³

F is a type I transmembrane surface protein that has a cleaved signal peptide at the N-terminus and a membrane anchor near the C-terminus¹⁰⁰ (Fig. 38.4). A predicted three-dimensional structure of the prefusion form of the HRSV F protein has been described based on homology modeling with the crystal structure of the Newcastle disease virus F protein,⁵¹⁷ and crystal structures representing the postfusion form have recently been published.^{378,536} The structure of the *Paramyxoviridae* F protein is described in detail in Chapter 33.

As is typical for *Paramyxoviridae*, F is synthesized as an inactive F0 precursor that assembles into a homotrimer.⁷⁴ HRSV F is activated by cleavage in the trans-Golgi complex by furin or a furin-like cellular endoprotease to yield two

TABLE 38.1 Percent Amino Acid Sequence Identity between the Proteins of HRSV Subgroup A (HRSV-A) or HMPV Subgroup A (HMPV-A) and the Indicated Viruses^a

Viruses compared		% Amino acid sequence identity for the indicated protein										
		NS1	NS2	N	P	M	SH	G	F	M2-1	M2-2	L
HRSV-A versus:	HRSV-B	87	92	96	91	91	76	53	89	92	72	93
	BRSV	69	84	93	81	89	38	30	81	80	42	84
	PVM	16	20	60	33	42	23	12	43	43	10	53
	HMPV-A	^{—b}	^{—b}	42	35	38	23	15	33	36	17	45
	AMPV-A	^{—b}	^{—b}	41	32	38	19	16	35	37	12	43
HMPV-A versus:	HMPV-B	^{—b}	^{—b}	96	85	97	59	37	95	96	89	94
	AMPV-C	^{—b}	^{—b}	88	68	87	24	23	81	83	56	80
	AMPV-A	^{—b}	^{—b}	70	58	77	20	12	68	73	25	64
	AMPV-B	^{—b}	^{—b}	69	53	76	20	13	67	71	27	ND ^c

HRSV, human respiratory syncytial virus; BRSV, bovine RSV; PVM, pneumovirus of mice; HMPV, human metapneumovirus; AMPV, avian metapneumovirus.

^aViruses are listed in order of decreasing relatedness to HRSV-A or HMPV-A.

^bDoes not encode NS1 or NS2.

^cND, not done.

disulfide-linked subunits: NH₂-F₂-F₁-COOH.^{103,531} The N-terminus of the F₁ subunit that is created by this cleavage contains a hydrophobic domain (the fusion peptide) that inserts directly into the target membrane to initiate fusion. The F₁ subunit also contains two areas of heptad repeats that associate during fusion, driving a conformational shift that brings the viral and cellular membranes into proximity^{100,633} (Chapter 33). HRSV F protein can direct efficient fusion independent of other viral proteins.²⁸³ This differs from the situation for most members of *Paramyxovirinae*, for which efficient fusion requires interaction with the homologous attachment protein (Chapter 33).

The F proteins of HRSV and BRSV are unique in *Paramyxoviridae* in having two cleavage sites, rather than one cleavage site^{200,636} (Fig. 38.4). The site that is immediately upstream of the fusion peptide (with cleavage between residues 136/137 in HRSV strain A2) is the one that corresponds to other *Paramyxoviridae*. This site in HRSV contains six tandem Arg and Lys residues (Lys-Lys-Arg-Lys-Arg-Arg↓). The second, novel site is located 27 amino acids upstream (with cleavage between residues 109/110) and has the sequence Arg-Ala-Arg-Arg↓. Therefore, both sites contain the preferred furin cleavage motif (Arg-X-Arg/Lys-Arg↓). HRSV F appears to be readily cleaved during intracellular processing, although some of the F protein packaged in virions remained uncleaved at site 109/110. There is indirect evidence that the double-cleavage site in the F protein is associated with a hyperfusogenic character that is linked to reduced thermostability and the ability to fuse independent of a cognate attachment protein.⁴⁶⁹

Cleavage at the two sites in F₀ releases a short peptide of 27 amino acids (p27) that contains two (strain A2) or three (strain Long) potential acceptor sites for N-linked sugars, of which at least two are utilized in strain Long.⁶³⁶ (Note that the remainder of the F protein has three acceptor sites for N-linked sugars, two in F₂ and one in F₁, all of which are utilized.⁶³⁹) For BRSV, this peptide contains a tachykinin sequence motif and is processed and released from BRSV-infected cells to yield a virokinin that induced smooth muscle contraction *in vitro*, which is a property of tachykinins.⁶³⁸ Tachykinins also have proinflammatory and immunomodulatory activities. Recombinant BRSV in which most of p27 was deleted, or in which the upstream cleavage site was mutated, replicated as efficiently as the wild-type parent in calves, but induced less pulmonary inflammation and a somewhat lower titer of serum neutralizing antibody.^{567,637} Therefore, p27 of BRSV has the potential to augment viral disease by effects on smooth muscle contraction and pulmonary inflammation, and might also stimulate the host immune response. In contrast, the sequence of p27 of HRSV does not resemble a tachykinin, and HRSV p27 did not have tachykinin-like properties *in vitro*.⁶³⁸

The HMPV F protein shares a moderate level of amino acid sequence identity with HRSV (33%)^{46,571} and is similar in general organization (Fig. 38.4). As with HRSV, HMPV F directs fusion involved in penetration and syncytium formation, and does so efficiently without need of the G and SH proteins.⁴⁸ HMPV F also appears to play a role in attachment via interaction with cellular $\alpha\beta 1$ integrin.¹¹⁸ This integrin characteristically binds to a specific recognition sequence, Arg-Gly-Asp, and this motif is present in the HMPV F protein and mediates its binding to cells.

The HMPV F₀ precursor contains only a single cleavage activation site (Fig. 38.4). The sequence at this site, Arg-Gln-

Ser-Arg↓, does not conform to the consensus furin motif and, consistent with this, clinical isolates typically require exogenous trypsin for growth *in vitro*. *In vivo*, cleavage of the HMPV F protein presumably depends on secreted protease present in the lumen of the respiratory tract. Several observations indicate that this is not a limiting factor in pathogenesis. For example, in some cases, serial passage of HMPV clinical isolates in cell culture resulted in the emergence of mutants that no longer required added trypsin, but these did not exhibit increased replication in hamsters.⁴⁹² These mutants contained the substitution of Pro in place of Ser in the -2 position of the cleavage activation site (Arg-Gln-Pro-Arg↓), which conferred intracellular cleavability even though it did not create a furin motif. Similarly, when the F cleavage site of recombinant HMPV was engineered so that it was multibasic and cleaved intracellularly, this did not increase its replication in African green monkeys (AGMs).⁴⁵

Glycoprotein G

The HRSV G protein was originally thought to be the sole viral attachment protein,³³⁴ but it now appears that the F protein also plays a role, as noted. The HRSV G protein has no apparent relatedness by sequence or structure to the attachment HN, H, and G proteins of *Paramyxovirinae* and has only half the amino acid length.^{278,597} HRSV G is a type II transmembrane glycoprotein, with a hydrophobic signal/anchor located near the N-terminus (amino acids 38-63 in strain A2) and the C-terminal two thirds of the molecule oriented extracellularly (Fig. 38.4). A secreted version of G is produced by translational initiation at the second ATG in the ORF (codon 48), which lies within the signal/anchor sequence.^{255,476} This truncated form is then trimmed by proteolysis, removing the remainder of the signal/anchor and creating a new N-terminus at Asn-66 for the final secreted form. The secreted form constituted approximately 20% of the total G protein expressed in HRSV-infected cells *in vitro*, but because of its rapid secretion it accounted for 80% of the G protein released in cell culture by 24 h post infection, the remainder being virion associated.²⁵⁵

The polypeptide backbone of HRSV G has an Mr of approximately 32,000. In the case of strain A2, an estimated four N-linked sugar side chains are added co-translationally, increasing the Mr to 45,000.^{102,598} G assembles in the endoplasmic reticulum into oligomers that probably are trimers or tetramers, and O-linked sugars are added subsequently in the trans-Golgi compartment or network.^{102,598} Mature G migrates in gel electrophoresis as a broad, seemingly heterogeneous band of Mr 80,000-90,000. Analysis of an F/G fusion protein (created as a potential vaccine) expressed in insect cells by a recombinant baculovirus indicated that G contains approximately 24-25 O-linked side chains.⁵⁹² However, when HRSV was grown in an *in vitro* model of human airway epithelium (HAE)—specifically, a differentiated pseudostratified mucociliary tissue that closely resembles the authentic airway epithelium⁶³¹—the Mr observed for the G protein was 180,000.³¹⁹ Because these cells would be considered to be a more authentic substrate than typical immortalized monolayer cultures, this suggests that the amount of carbohydrate present on G is substantially greater than previously thought. The presence of a sheath of host-specified sugars might shield the G protein from immune recognition, and might interfere with antigen processing and presentation. The secreted form of G appears to be

processed in a similar fashion to the membrane-anchored form except that it is secreted as a monomer, and no antigenic differences were detected when the secreted and membrane-bound forms were analyzed with an extensive panel of monoclonal antibodies (MAbs).¹⁵⁵

Most of the ectodomain of HRSV G consists of two large domains that are highly divergent between strains and have a “mucin-like” structure. Like mucin, these domains have a high content of serine, threonine, and proline residues; are heavily glycosylated; and are thought to have an extended, nonglobular secondary structure. Because there are more than 75 serine and threonine residues in the mucin-like domains as potential acceptor sites for O-linked sugars, there may be heterogeneity in site usage that could provide additional antigenic heterogeneity. The significance of this mucin-like character remains unknown. One possibility is that it somehow helps the virus penetrate the protective mucous layer overlying the respiratory epithelium.

The two mucin-like domains flank a central conserved domain that includes a 13-residue segment (positions 164–176 in strain A2) that is completely conserved among HRSV strains, as well as an overlapping segment containing four closely spaced invariant cysteine residues (positions 173, 176, 182, and 186)²⁷⁸ (Fig. 38.4). Disulfide linkages occur between Cys-173 and Cys-186, and between Cys-176 and Cys-182, to create a cystine noose.²⁰¹ The cysteine-rich segment contains a Cys-X₃-Cys (CX3C) motif involving Cys-182 and Cys-186 embedded in a region of limited sequence relatedness with the CX3C chemokine called fractalkine.⁵⁶¹ A peptide containing this sequence mimicked the leukocyte chemoattractant activity of fractalkine in an *in vitro* assay.

The HRSV G protein participates in viral attachment *in vitro* by binding to glycosaminoglycans (GAGs), which are long unbranched chains of repeating disaccharide subunits that are part of the glycocalyx present on the outer surface of the cell. The conserved central domain of G seemed like an obvious candidate to be involved in attachment. However, this domain can be deleted from recombinant HRSV with little effect on virus replication in HEp-2 cells or in mice.⁵⁴⁸ A study that probed for possible GAG-binding domains by evaluating interaction between peptides spanning the ectodomain of G and the GAG heparin identified a potential binding site shortly downstream from the central conserved domain,¹⁷⁵ but this also could be deleted with little effect on HRSV infection and replication *in vitro* or in mice.⁵⁴⁸ More recently, cleavage of the C-terminal domain of G was found to result in virus that bound less efficiently to GAGs and had reduced infectivity in HAE cultures, suggesting that this domain is important for the attachment function of G.³¹⁹

Extensive passage of a subgroup B strain of HRSV in AGM Vero cells—in an attempt to attenuate the virus—resulted in mutants with various spontaneous deletions involving most of the G and SH genes.²⁸⁸ One such mutant virus that was evaluated as a potential vaccine replicated efficiently in Vero cells but was highly restricted in humans. Recombinant HRSV strain A2 from which the G gene was deleted (Δ G) also replicated efficiently in Vero cells. However, the Δ G virus was restricted in HEp-2 cells, with defects at the levels of virus binding, fusion, and assembly.^{544,545,547,550} In addition, the Δ G virus was highly restricted in mice and might not have replicated at all.⁵⁵⁰ Therefore, HRSV G is dispensable for replication in Vero cells but it is essential for efficient replication in HEp-2 cells and *in vivo*.

The HMPV G protein (Fig. 38.4) has a number of similarities with HRSV G, although it lacks significant sequence identity^{46,571} (Table 38.1). HMPV G has a comparable N-terminal proximal signal/anchor domain and a high content of serine/threonine/proline residues concentrated in the ectodomain.^{46,571} In addition it is modified by the addition of N-linked and O-linked sugars to yield a mature form that migrates in gel electrophoresis as a diffuse band of Mr 97,000.³⁴¹ Its amino acid sequence also is highly divergent between the HMPV subgroups. However, HMPV G lacks the conserved central domain and cystine noose mentioned above for HRSV and is correspondingly shorter in length. A secreted form of HMPV G has not been described, and the amino acid sequence gives no suggestion that such a species exists. As with HRSV, the HMPV G protein binds to cell surface GAGs, which may reflect its role in attachment.⁵⁵¹ Peptide binding studies suggested that two closely spaced clusters of basic amino acids at positions 149–155 and 159–166 may mediate binding to GAGs.⁵⁵¹

Deletion of G from recombinant HMPV was less attenuating than for HRSV. Deletion of G had little effect on HMPV replication *in vitro*, and the HMPV Δ G mutant had a low-to-moderate level of replication in hamsters and AGMs.^{44,48} Interestingly, infection of epithelial cells *in vitro* with the Δ G mutant resulted in increased activation of the transcription factors IRF3 and NF- κ B and increased expression of type I IFN and proinflammatory cytokines compared to wild-type HMPV, implying that the G protein otherwise inhibits these responses.²¹ The HMPV G protein was found to bind to the RIG-I cytoplasmic RNA recognition receptor, which explains its inhibitory effect.

Small Hydrophobic SH Protein

The HRSV SH protein (Fig. 38.4) is a short (64 amino acids for strain A2) transmembrane protein that is anchored by a hydrophobic signal-anchor sequence near the N-terminus, with the C-terminus oriented extracellularly.^{101,427} Most of the SH protein remains unglycosylated (Mr ~7,500), but there are also forms that contain a single N-linked side chain (resulting in Mr ~13,000 to 15,000), as well as the further addition of polylactosaminoglycan (Mr ~21,000 to 60,000 or more), as well as N-terminally truncated forms that arise from translational initiation at the second methionine codon in the ORF.^{9,427} However, the significance of these multiple forms is not known.

Chemical cross-linking studies indicated that the HRSV SH protein associates into pentamers.¹⁰¹ When SH was expressed in bacteria, it was incorporated into the membrane, resulting in increased membrane permeability.⁴⁴⁶ Incorporation of partial or full-length SH into artificial membranes *in vitro* resulted in the formation of pentameric and hexameric pore-like structures and the acquisition of cation-selective channel-like activity.^{81,183} Therefore, the SH protein may have properties of a viroporin, which typically are small proteins that modify membrane permeability and can have roles in budding and apoptosis.

Recombinant HRSV from which SH was deleted replicates with wild-type efficiency *in vitro* and appears to be fully fusogenic.^{69,544} The SH protein was reported to reduce apoptosis, but the effect was small.¹⁸¹ SH also appeared to inhibit signaling from tumor necrosis factor (TNF)- α .¹⁸¹ The Δ SH virus was slightly attenuated in mice and chimpanzees,^{69,603} but deletion of the SH gene in an experimental live vaccine strain did not increase its level of attenuation in seronegative children.²⁹⁰ Therefore, the function(s) and impact of the HRSV SH protein seem unclear.

The SH protein of HMPV is nearly three times longer (177–183 amino acids) than its HRSV counterpart^{46,571} (Fig. 38.4). It has the same membrane orientation and a similar array of nonglycosylated and glycosylated forms. Passage of HMPV frequently results in mutations that ablate SH expression,⁴³ suggesting that the protein may be somewhat toxic in cell culture. Infection of epithelial cells and mice with the Δ SH virus resulted in a modest increase in NF- κ B activation and expression of proinflammatory proteins compared to wild-type virus, suggesting that the SH protein downregulates the innate response, but the effect may be small.²⁰ *In vivo*, deletion of SH had little or no effect on HMPV replication in hamsters or in AGMs.^{44,48}

Matrix M Protein

The HRSV M protein is a nonglycosylated internal virion component that is smaller than its *Paramyxovirinae* counterparts (256 amino acids versus 335–375 amino acids), with little apparent amino acid sequence relatedness. HRSV M appears to play two roles typical for *Mononegavirales*: it helps organize virion components at the plasma membrane for budding,^{254,547} and it may silence viral RNA synthesis in preparation for packaging into the virus particle.¹⁹¹ A crystal structure determined for the HRSV M protein revealed a monomer that is organized into compact N-terminal (residues 1 to 126) and C-terminal (residues 140–255) domains joined by a 13-residue linker.³⁹⁴ The surface of M was found to contain a large positively charged area that extends across the two domains and the linker and may mediate association with the negatively charged membrane as well as with nucleocapsids.³⁹⁴ The HMPV M protein (254 amino acids) likely is a close functional counterpart.

Nucleocapsid/Polymerase Proteins N, P, L, and M2-1

The N, P, and L proteins of HRSV appear to be close functional analogs of their counterparts in other members of *Paramyxoviridae*. The N protein binds tightly along the entire length of genomic RNA and antigenomic RNA to form separate RNase-resistant nucleocapsids that are templates for RNA synthesis. The P protein is a multifunctional adapter that helps mediate interactions between components of the nucleocapsid/polymerase complex. The L protein contains the polymerase catalytic domains. Studies with HRSV minireplicons showed that N, P, and L are necessary and sufficient to direct RNA replication.^{98,215,626} N, P, and L alone also have transcriptase activity, but fully processive transcription requires in addition the M2-1 protein.^{98,171}

The 391 amino acid HRSV N protein is shorter than its counterparts in *Paramyxovirinae* (approximately 490–555 amino acids), and sequence relatedness is limited to several conserved segments located towards the C-terminus.^{27,542} HRSV N protein produced in bacteria was recovered in decamer rings bound to bacterial RNA, and a crystal structure was obtained⁵⁴² that is described in e-Figure 38.5.

The HRSV P protein (241 amino acids) is shorter than its *Paramyxovirinae* counterparts (approximately 390–605 amino acids) and lacks evident sequence relatedness. However, it is thought to have the same general array of functions. Like its counterparts in *Paramyxovirinae*, HRSV P operates as a stable homotetramer formed through a multimerization domain in the middle of the molecule.^{15,82,344} The C-terminal region of the P tetramer interacts with N protein in the nucleocapsid by binding to a hydrophobic pocket surrounded by positively charged

residues made up from discontinuous segments within amino acids 46–151 of the N protein, a structure distinct from that of other members of *Mononegavirales*^{182a} (e-Fig. 38.5). Soluble P also binds to free N protein monomers—probably through the N-terminal domain of P—and delivers N to nascent genomes/antigenomes during RNA replication. P thus prevents N from self-aggregating or binding to nonviral RNA.⁸² In addition, P binds to the L²⁹⁴ and M2-1^{12,559} proteins and helps mediate their interactions with the nucleocapsid. P is an essential polymerase co-factor. It may contribute to conformational changes that help the polymerase access the RNA template⁸² and appears to be necessary for promoter clearance and chain elongation by the viral polymerase.¹⁴⁷ It also appears to have a role in dissociating the M protein from the nucleocapsid during uncoating to initiate infection.¹³

P is the major phosphorylated HRSV protein and contains phosphate 10 to 12 or more sites, with different sites exhibiting differing rates of turnover: the C-terminal domain contains low-turnover phosphates and accounts for most of the total phosphate, the middle domain contains intermediate-turnover phosphates, and the N-terminal domain contains high-turnover phosphates.^{14,418} Many of the activities of P described above appear to be directed by dynamic phosphorylation and dephosphorylation of P at a subset of these sites, usually involving a small percentage of the total phosphate content.^{12,13,14–15,580} Most of the constitutive phosphorylation, involving five sites, could be ablated in recombinant HRSV with only modest effects on virus growth.³⁵⁰

The 194 amino acid HRSV M2-1 protein is an essential transcription processivity factor; in its absence, the viral polymerase terminates prematurely and nonspecifically within several hundred nucleotides of the 3' end of the genome, and downstream genes are not significantly transcribed.^{94,97,98,171} M2-1 also decreases the efficiency of termination at the GE transcription signals—possibly a reflection of the same processivity activity—resulting in increased production of readthrough mRNAs.²⁴⁵ HRSV M2-1 accumulates in phosphorylated and nonphosphorylated forms²⁴⁴ and forms a homotetramer via an oligomerization domain at residues 32 to 63.⁵⁵⁹ M2-1 contains a cysteine–histidine zinc finger motif (C-X₇-C-X₅-C-X₃-H) near its N-terminus (residues 7 to 25) that is essential for its activity²⁴⁴; this motif is conserved in *Pneumovirinae*.⁵⁷¹ The HRSV M2-1 protein binds RNA, and may be delivered to the RNA template by the P protein.^{80,120,559} M2-1 is unique to *Pneumovirinae*, although VP30 of Family *Filoviridae* has some similarities.

The 2,165 amino acid HRSV L protein is similar in length to its *Paramyxovirinae* counterparts and has low but unambiguous sequence relatedness along nearly its entire length.⁵²⁷ Specific segments of L are conserved within and beyond *Mononegavirales* and appear to include polymerase motifs.⁴⁵¹ A putative nucleotide-binding domain involved in capping mRNA was identified in the central region of the L protein based on sequence analysis of HRSV mutants selected for resistance to novel capping inhibitors, in which resistance was associated with an amino acid substitution at position 1381, 1269, or 1421.³⁴³ In separate work, an amino acid substitution at position 1049 or 1169 in the L protein was associated with reduced efficiency of termination at the GE signals, resulting in increased synthesis of polycistronic mRNAs and reduced growth efficiency.^{79,282}

The HMPV N, P, L, and M2-1 proteins are similar in size to their HRSV counterparts and share significant amino acid

sequence identity^{46,571} (Table 38.1). One notable difference is that, whereas M2-1 appears to be essential for HRSV, recombinant HMPV in which the M2-1 ORF has been silenced is viable and replicates *in vitro* with an efficiency that is only marginally reduced.⁶⁶ HMPV lacking M2-1 appeared to execute processive transcription efficiently, although the level of RNA accumulation was somewhat reduced. Therefore, in contrast to HRSV, HMPV M2-1 appears to be a nonessential accessory protein of unknown function.⁶⁶ However, in the hamster model, replication of HMPV lacking M2-1 could not be detected, indicating that M2-1 is important for replication *in vivo*.

M2-2 Protein

HRSV M2-2 is a small (90 amino acids for strain A2) protein that is expressed at a low level that may reflect inefficiency of the stop–restart mechanism of translation noted previously.⁵ It is not known whether M2-2 is packaged in the virion. Recombinant HRSV in which the M2-2 ORF has been silenced grows more slowly *in vitro* than wild-type HRSV, although it eventually achieves a similar titer.^{41,271} In Δ M2-2 virus–infected cells, the accumulation of mRNA was increased, whereas that of the genome and antigenome was decreased compared to wild-type virus. Conversely, when M2-2 was overexpressed during HRSV infection—using a co-transfected plasmid or a recombinant HRSV in which expression of M2-2 was upregulated by engineering it to be a separate gene⁸⁹—HRSV replication was inhibited. Expression of M2-2 also was inhibitory to HRSV RNA synthesis by a minireplicon.⁹⁸ These observations indicate that M2-2 plays a role in shifting RNA synthesis from transcription to RNA replication, that M2-2 can be inhibitory to RNA synthesis, and that the inhibitory activity occurs with increased M2-2 expression. The Δ M2-2 virus retained the ability to replicate in mice and chimpanzees, but it was attenuated approximately 500- to 1,000-fold compared to wild-type HRSV.^{41,271,549}

HMPV encodes a 71 amino acid M2-2 protein from a comparable internal ORF in its M2 mRNA^{46,571} (Fig. 38.3C). The HMPV M2-2 protein co-immunoprecipitated with the L protein and inhibited RNA synthesis by a mini-replicon, an activity that was lost by short deletions at the N- or C-terminus of M2-2.³⁰¹ Deletion of HMPV M2-2 from recombinant virus resulted in an attenuated virus in which transcription is increased and RNA replication decreased.⁶⁶

Nonstructural Proteins NS1 and NS2

The NS1 and NS2 proteins (139 and 124 amino acids, respectively) are unique to the genus *Pneumovirus*. NS1 and NS2 can be co-immunoprecipitated⁵³⁷ and may occur in complexes of various stoichiometries.¹⁵⁶ Monomeric NS2 was unstable with a half-life of 30 minutes.¹⁵⁶

NS1 and NS2 strongly interfere with the induction and signaling of type I IFN and type III IFN (the latter comprising λ 1, λ 2, and λ 3; also known as IL-29, IL-28A, and IL-28B, respectively) in human epithelial cells, macrophages, and dendritic cells. This suppresses a major component of host innate defense. The steps at which NS1 and NS2 act are summarized in e-Figure 38.6.

Deletion of NS2 from recombinant HRSV decreased its ability to induce activation of transcription factor NF- κ B,⁵²⁴ and small interfering RNA (siRNA)–mediated knockdown of the expression of either NS1 or NS2 in HRSV-infected cells

reduced activation of NF- κ B and the serine-threonine kinase AKT (also known as protein kinase B), with the effect of speeding the onset of apoptosis.⁵⁰ Therefore, the two NS proteins appear to activate prosurvival pathways, thereby prolonging the life of the cell and increasing the viral yield.⁵⁰

In a minireplicon system, coexpression of NS1—and, to a lesser extent, NS2—inhibited transcription and RNA replication, affecting both the genomic and antigenomic promoters.¹⁶ These effects remain to be defined. The V and C accessory proteins of some members of *Paramyxovirinae* also have been shown to downregulate viral RNA synthesis (Chapters 33 and 34). This may be a general viral mechanism to prevent excessive RNA synthesis and avoid the accumulation of naked genomic/antigenomic RNA and the formation of dsRNA, which otherwise would activate the RIG-I/MDA5/PKR pattern recognition molecules.

As might be expected from the results described above, HRSV lacking NS1 and/or NS2 replicates to reduced titer in cultured cells competent for producing type I IFN, as well as in experimental animals, with the level of attenuation increasing in the order Δ NS2 < Δ NS1 \leq Δ NS1 + NS2.^{272,523,546,549,603}

Replicative Cycle

Efficient infection of cell lines *in vitro* by HRSV involves binding to cellular GAGs, especially heparan sulfate and chondroitin sulfate B.²³⁸ The G and F proteins each appear able to bind to GAGs.^{174,175,239,545} A number of additional potential receptor molecules for HRSV have been tentatively identified, including intracellular adhesion molecule (ICAM)-1,³³ RhoA,⁴³⁸ the CX3CR1 fractalkine receptor,⁵⁶¹ and annexin II.³⁵⁸ More recently, efficient HRSV infection *in vitro* and in the mouse model was shown to depend on binding to the cellular protein nucleolin,⁵⁴³ which also has been identified as a co-receptor for human parainfluenza virus 3 (HPIV3).⁵⁸ Somewhat unexpectedly, binding to nucleolin is mediated by the F protein rather than by the G protein. This suggests that efficient attachment and infection by HRSV depends on two different binding events mediated by G and F, but the details of this process remain unclear.

HRSV entry occurs by fusion of the viral envelope with the cell plasma membrane^{283,526} or with endosomal membranes following clathrin-mediated endocytosis.³⁰⁴ However, HRSV entry does not require endosomal acidification, suggesting that there is no mechanistic difference between entry at the plasma versus endosomal membrane.^{304,526} When infection was monitored by video microscopy, the initiation of fusion by attached virions appeared to be a slow step, but once started the process was rapid.¹⁸ Genome transcription and replication occur in the cytoplasm and the virus can grow in enucleated cells and in the presence of actinomycin D, indicating a lack of essential nuclear involvement.

HRSV mRNAs and proteins can be detected intracellularly at 4 to 6 h after infection and reach a peak accumulation by 15 to 20 hours. The release of progeny virus begins by 10 to 12 h postinfection, reaches a peak after 24 hours, and continues until the cells deteriorate by 30 to 48 hours. Transcription and RNA replication occur concurrently but, as noted, the low-abundance M2-2 protein accumulates during infection and shifts the balance of RNA synthesis from transcription to RNA replication.⁴¹ In minireplicon experiments, increasing the level of accumulation of the N and P proteins did not shift the balance between RNA replication and transcription, indicating

that the availability of protein to encapsidate replicative RNA does not control this balance.¹⁷²

Several factors influence the relative levels of expression of the various HRSV genes. Like other *Mononegavirales*, sequential transcription has a polar gradient due to polymerase fall-off, and thus promoter-proximal genes are expressed more efficiently. This gradient of expression is not very steep, with the exception of the L mRNA.^{107,307} The greatly reduced accumulation of L mRNA compared to the other mRNAs appears to be due to a posttranscriptional effect rather than polymerase fall-off, with one possibility being mRNA stability.³¹⁸ Differences in the termination efficiency of the various GE signals may also have effects on the relative levels of gene expression.^{246,399} For example, if a GE signal is particularly inefficient, a greater fraction of the polymerase continues synthesis into the next downstream gene to produce a readthrough transcript, rather than terminating and reinitiating to produce an individual transcript of the next gene. Because ORFs in internal positions in eukaryotic mRNAs generally are not efficiently translated,³⁰⁶ this would have the effect of down-regulating expression of the protein from the downstream gene. HMPV in particular has considerable variation in the efficiency of its GE signals, and in particular this appears to sharply down-regulate the production of monocistronic SH mRNA, contributing to the observed low level of expression of SH protein.

HRSV assembly and budding occur at the plasma membrane. In polarized cells, this occurs at the apical surface.^{475,631} Video microscopy showed that budding occurs within circumscribed regions on the cell surface and appeared visually to be the reverse of fusion.¹⁸ These regions contain localized virus-modified lipid rafts involving all three viral surface proteins and the M protein.^{254,270,371,372,623} The minimum viral protein requirements for the formation of virus-like particles capable of delivering the viral genome to target cells are the F, M, N, and P proteins,⁵⁴⁷ and expression of these proteins induced the formation of viral filaments.⁵⁶⁴

As has been observed for other members of *Paramyxoviridae*, HRSV utilizes the host cytoskeleton in its replicative cycle. Viral substructures are associated with polymerized actin throughout the infectious cycle.²⁶⁹ Actin is packaged in the virion and was required, in combination with profilin (an actin-binding protein involved in restructuring actin polymers), for efficient RNA synthesis by intracellular nucleocapsids in a cell-free reaction.⁷² HRSV appears to hijack cellular apical recycling endosomes (ACEs) for budding, a pathway that is distinct from that described for a number of other enveloped RNA viruses.^{65,564}

Efficient infection by HMPV *in vitro* depends on binding to cell surface GAGs and $\alpha\beta 1$ integrin.^{118,551} The F proteins of some strains of HMPV depend on acidification for activation, indicating a dependence on endosomal uptake rather than fusion at the plasma membrane: this requirement mapped to the presence of glycine at position 294.^{260,496,497} However, this was observed for only 6% of subgroup A strains and not for subgroup B, indicating an unexpected diversity in entry mechanisms. Finally, one notable difference between HRSV and HMPV is that the kinetics of infection of the latter in cell culture are slower, with the peak of intracellular protein expression occurring at 48 to 72 hours postinfection.

Propagation In Vitro

HRSV replicates most efficiently *in vitro* in immortalized cell lines derived from human epithelial cells, but can infect and replicate

to a significant extent in a wide variety of cell lines representing various tissues from various hosts, including humans, monkeys, bovines, hamsters, and mice. The most commonly used cell line is human HEP-2, now thought to have been contaminated historically with, and outgrown by, the HeLa cell line.³⁶⁸ Virus is usually quantified by plaque titration; immunostaining is often used to enhance visualization of plaques. Fresh clinical isolates of HRSV may undergo some sort of adaptation to cell culture,³⁶¹ but this is poorly understood. However, passaged laboratory strains retain their virulence for chimpanzees and humans.^{291,606}

In cell culture, 90% of progeny HRSV virions remain associated with the cells, attached in a manner that suggests a failure to complete the budding process. To make virus stocks, cell-associated virus is dislodged by freeze-thawing, sonication, or vortexing. The yield is low, typically ten plaque-forming units (pfu) per cell. Virion preparations commonly have substantial contamination by cellular debris. A large fraction of released virions appeared to be empty and presumably noninfectious.¹⁹ Less than 5% of the infectivity of a preparation of HRSV passed through a 0.45- μ m filter, whereas more than 85% passed through a 3- μ m filter, consistent with the infectious particles being filaments⁴⁷⁵; similar findings had been reported for BRSV.⁴³³

HMPV is more restricted in its *in vitro* host range than HRSV, but is readily propagated in rhesus LLC-MK2 or AGM Vero cells.^{134,557} HMPV typically requires added trypsin to support cleavage of the F protein, although some strains mutate during passage to become trypsin independent, as noted.⁴⁹² HMPV replicates more slowly than HRSV, its cytopathic effect is less prominent, and the yield of infectious virus is similarly low. Like HRSV, HMPV tends to remain cell-associated but is more stable.

Genetics and Reverse Genetics

HRSV (and presumably HMPV) has a high rate of nucleotide substitution, as is typical for RNA viruses. The rate of nucleotide substitution in RSV was estimated to be 6.47×10^{-4} substitutions/site/year, and the G gene had the elevated rate of $10^{-2.7}$ substitutions/site/year, reflecting relaxed selective constraints, as already noted.^{538a,641} HRSV and HMPV, like other *Mononegavirales*, also engage in nonhomologous recombination caused when the polymerase jumps from one template to another during synthesis. This can create defective interfering (DI) genomes,⁵⁶⁹ or deletion of parts of genes,²⁸⁸ or sequence duplications.⁵⁶⁰ Foreign sequence also can be acquired, as evidenced by a 1,015-nucleotide insert of unknown origin in the G gene of certain AMPV-C isolates.³⁸ Recombination between co-infecting viruses appears to be rare, although potential mosaic genomes within a viral species are identified occasionally by sequence analysis.⁶³⁴ One study detected a single case of recombination between two co-infecting HRSV mutants in cell culture, involving both homologous (i.e., guided by sequence relatedness) and non-homologous recombination⁵²²; this is the only such report for *Mononegavirales*. In addition, the difference in the gene orders of HRSV and HMPV may be evidence of past recombination, within one or the other species, resulting in gene rearrangement.

As with other *Mononegavirales*, HRSV and HMPV can be produced entirely from cloned cDNA by reverse genetics, involving co-transfection of plasmids encoding a copy of the genome or antigenome as well as the proteins of the nucleocapsid/polymerase complex. The viral protein requirements for recovering HRSV are N, P, L, and M2-1, but M2-1 is not

required for HMPV.^{47,66,97,259} A second type of reverse genetics system that has been used extensively for HRSV in particular is based on cDNA-encoded minireplicons in which the viral genes in the genome or antigenome cDNA have been replaced by one or more foreign marker genes, with the system driven by viral proteins supplied from co-transfecting plasmids. Depending on the supplied proteins, this system can recreate genome encapsidation, transcription, RNA replication, and particle morphogenesis.^{98,170,373,424,547} This system is ideal for detailed structure-function studies. In addition, mutations that might have drastic or lethal effects on infectious virus can readily be studied in the transient context.

Infection of Experimental Animals

A number of animal species can be infected in the respiratory tract by intranasal administration of HRSV, including cotton rats, mice, ferrets, guinea pigs, hamsters, marmosets, lambs, and various nonhuman primates.^{35,73,210,460,521,606} Among experimental animals, only the chimpanzee approaches the human in being highly susceptible to infection by contact, in supporting moderate to high levels of virus replication, and in exhibiting rhinorrhea and cough resembling that of humans. The most widely used experimental animals, cotton rats and mice, support a low to moderate level of virus replication that peaks on day 4 and is cleared quickly. *In situ* hybridization of lung tissue from cotton rats at the peak of virus replication showed that only scattered cells were infected.⁴¹³ Inbred strains of mice can vary 100-fold in permissiveness for replication.⁴⁶⁰ The BALB/c mouse is one of the more permissive strains, but it is less permissive than cotton rats. Rodents do not exhibit overt HRSV respiratory tract disease, although pulmonary histopathologic changes are evident and disease can be monitored by weight loss and changes in pulmonary function. More recently, newborn lambs have been used as models in detailed studies of neonatal HRSV disease.⁵²¹

Intranasal administration of HMPV has been reported to infect guinea pigs, ferrets, mice, cotton rats, and hamsters, and several species of nonhuman primates.^{6,241,353} BALB/c mice and hamsters may be more permissive for HMPV than for HRSV. Among nonhuman primates, cynomolgus macaques and AGMs support moderate levels of virus replication that peak on days 5 to 6 and are mostly resolved by day 10.^{311,515} Captive cynomolgus macaques, AGMs, and chimpanzees have a high seroprevalence for HMPV, suggesting that they can be infected readily from their handlers and possibly by animal-to-animal transmission.

Antigenic Subgroups and Diversity

HRSV has a single serotype with two antigenic subgroups, A and B. These exhibit a three- to fourfold reciprocal difference in neutralization by polyclonal convalescent serum.⁹² Studies with MAbs have demonstrated extensive antigenic difference in the G protein with substantially less difference in the other viral proteins.^{10,400} Analysis of glycoprotein-specific responses in cotton rats or human infants by enzyme-linked immunosorbent assay (ELISA) with purified F and G glycoproteins (the HRSV neutralization antigens) showed that F has 50% antigenic relatedness between subgroups compared to 1% to 7% relatedness for G.²⁵⁶ Consistent with this, F protein expressed from a recombinant vaccinia virus was equally protective in cotton rats against infection with either subgroup, whereas the

G protein was 13-fold less effective against the heterologous subgroup virus.²⁷⁷ Therefore, the F protein is responsible for most of the observed HRSV cross-subgroup neutralization and protection.

The genomes of the two HRSV subgroups share 81% nucleotide identity. The various proteins vary considerably in their level of subgroup divergence, with M2-2, SH, and G being the most divergent (Table 38.1). The divergence is greatest for the ectodomains of SH and G, which exhibit only 50% and 44% sequence identity between subgroups, respectively.^{105,278} The genome-wide nature of the sequence differences indicates that the two subgroups represent two lines of divergent evolution, rather than being variants that differ only at a few major antigenic sites. The divergence of the two HRSV subgroups has been estimated to have occurred approximately 350 years ago.⁶⁴⁰ In addition, there is considerable variation within each subgroup: for example, the G glycoprotein can have 20% difference between strains from the same subgroup.

The HRSV F protein is relatively stable antigenically, consistent with its high level of sequence conservation between the two HRSV subgroups. For example, analysis of 18 subgroup A strains and five subgroup B strains recovered from geographically diverse regions over 30 years using F-specific murine MAbs representing 16 separate neutralization epitopes in four antigenic sites showed that seven epitopes were conserved in all but one of the strains.³² Similarly, a major epitope in the N protein for CD8⁺ cytotoxic T lymphocytes (CTLs) was highly conserved in field strains.⁵⁷⁸

The G protein appears to be subject to greater change, although this occurs incrementally over a period of years. In one study, analysis of HRSV isolates collected over 47 years suggested that positive selection occurred at 13 codons in the G ectodomain, a number of which involved known epitopes.⁶⁴¹ A second study of isolates collected over 38 years provided evidence of progressive amino acid changes at an average rate of 0.25% per year that were paralleled by changes in reactivity with MAbs.⁷⁶ The mucin-like domains in G are unusual in that they are more divergent at the amino acid level than at the nucleotide level,²⁷⁸ which is the converse of what is observed for most proteins. This suggests that there is a selective pressure for amino acid substitutions, presumably driven by immune pressure. These regions in G may be relatively tolerant of amino acid change because of their proposed extended, nonglobular structure. In contrast, although F would be subjected to the same selective immune pressure, it likely is less tolerant of amino acid substitutions due to constraints from its folded structure and functional requirements. Analysis of BRSV isolates, including those from countries in which vaccination was in wide use, provided evidence of modest progressive changes not only in G, but also in N and F.⁵⁶⁸

HMPV also has a single serotype with two antigenic subgroups, A and B, which have extensive cross-reactivity and cross-protection.^{55,259,442,572,574} The level of genome nucleotide sequence identity (80%) and the relatedness between the various proteins is similar to that described for HRSV (Table 38.1). The HMPV F protein is somewhat more conserved than that of HRSV and plays a major role in the high level of cross-neutralization and protection between the two subgroups.⁵¹⁵ Vaccination against AMPV appears to drive antigenic drift in the field, especially in the G and SH genes, resulting in virus

that is sufficiently divergent from the vaccine strain that it is less restricted by vaccination.⁸⁴

Animal Counterparts

The BRSV gene map is very similar to that of HRSV.^{67,627} The genome of BRSV strain A51908 (NC_001989 and AF295543) contains 15,140 nucleotides and has 73% nucleotide identity with HRSV. Amino acid sequence identity ranges from 30% (G protein) to 93% (N protein) (Table 38.1), and there is extensive antigenic cross-reactivity. BRSV and HRSV have broadly overlapping host ranges in cell culture; however, *in vivo*, BRSV is highly restricted in nonhuman primates.⁶⁸ Ovine and caprine RSV also have been described. Sequence analysis suggests that ovine RSV and BRSV represent two branches of ruminant RSV with a degree of divergence similar to that between the HRSV antigenic subgroups. Caprine RSV appears to be more closely related to BRSV than to ovine RSV.¹¹²

PVM was first identified during experiments in which clinical material was passaged in mice in an effort to identify new human pathogens, and an apparent mouse virus present in the animals became evident.²⁶⁴ PVM is a respiratory pathogen that readily infects mice and other rodents and can be highly virulent. However, the natural host of PVM is unclear: the virus occasionally appears in colonies of laboratory mice, but serologic studies usually find little evidence of PVM-specific antibodies in wild rodents under conditions where there is extensive seropositivity for other common rodent viruses.^{31,516} Serologic studies suggested that a substantial proportion of the human population has antibodies that react with PVM.⁴⁶⁴ However, further analysis suggested that infection of humans by PVM or a closely related virus is unlikely.^{64a} A virus that was recently isolated from dogs with acute respiratory disease appears to be a strain of PVM (e.g., the percent amino acid sequence identity between the isolate and PVM strain 15 ranged from a low of 90.2% [SH] to a high of 98.1% [M]).^{473,474} Whether PVM commonly infects and causes respiratory tract disease in dogs remains unknown. The PVM gene map³⁰⁸ is essentially the same as that of HRSV and BRSV except that (a) the M2 and L genes of PVM do not overlap, and (b) the PVM P gene contains a second ORF that encodes a 137-amino acid product that represents a novel, 12th PVM protein.²⁶ The complete nucleotide sequence of PVM strain 15 (AY729016) is 14,886 nucleotides in length and has 52% identity with that of HRSV.³⁰⁸ The level of amino acid sequence identity ranges from 10% (M2-2 protein) to 60% (N protein) (Table 38.1).

BRSV and PVM in their respective hosts are used as models for HRSV,^{190,285,441,481,614} although a review of these studies is beyond the scope of this chapter. The BRSV model is limited by the inconvenient nature of the large host. PVM differs from HRSV in that lethal infections are typical rather than an infrequent outcome, but it can serve as a model for severe pneumovirus disease and has the advantage of the many available murine immunologic reagents and inbred mouse strains.

AMPV causes respiratory tract disease of economic importance in turkeys and also infects chickens and other birds.⁴²¹ Four AMPV antigenic subgroups have been described: subgroups A and B have been found in South Africa, Europe, Israel and Asia; subgroup C in North America; and subgroup D in France.⁴²¹ Complete genomic sequences have been determined for strains of subgroup A (AY640317) and subgroup C (AY590688 and AY579780). These range in length from 13,134 to 14,150 nucle-

otides, have the same general gene map as HMPV, and share 61% to 68% nucleotide sequence identity with HMPV subgroup A. Subgroups A, B, and D are more closely related to each other than to C^{205,504} (e-Fig. 38.2B). Surprisingly, AMPV-C is more closely related to HMPV than to the other AMPV subgroups^{571,572} (e-Fig. 38.2B). Sequence analysis of isolates collected over a 25-year period suggested that the most recent common ancestor for AMPV-C and HMPV existed approximately 200 years ago, implying a cross-species jump.¹²⁷ There is substantial host range restriction between human and avian MPV. AMPV-C (the subgroup most closely related to HMPV) is highly restricted in nonhuman primates.⁴⁴⁷ HMPV has been reported to be unable to infect chickens and turkeys,⁵⁷² or to transiently infect turkeys.⁵⁷⁷

PATHOGENESIS AND PATHOLOGY

In typical monolayer cultures of immortalized cells, infection with HRSV or HMPV results in long surface filaments that bear viral antigen and probably give rise to filamentous virus particles⁴⁸⁹ (Fig. 38.2B and E). The formation of HRSV filaments and filamentous virus depends on activation of RhoA and actin rearrangement: when activation is blocked, the production of infectious virus shifted to the nonfilamentous form.^{71,206,371} HRSV-infected cells develop large electron-dense cytoplasmic inclusion bodies of up to several microns in diameter that include the N, P, M2-1, and L proteins and presumably contain active nucleocapsids^{78,185}; HMPV forms similar inclusions.¹³⁶ Recently, the HRSV inclusion bodies were found to sequester a number of cellular proteins involved in intracellular signaling pathways, with the effect of inhibiting innate immunity and stress responses.^{179a} HRSV infection has slight inhibitory effects on cellular DNA and RNA synthesis and little effect on gross protein synthesis.³³⁵ As one factor in the lack of inhibition of protein synthesis, the HRSV N protein binds to PKR and prevents it from phosphorylating eIF-2α and inhibiting protein synthesis.²¹⁶ Apoptosis of HRSV-infected cells occurs slowly and is inhibited by the NS1, NS2, and SH proteins, as already noted. HRSV blocks the formation of stress granules, which may otherwise restrict HRSV replication.^{179a, 242} The formation of syncytia (Fig. 38.2A) is a major factor in cell death in typical nonpolarized monolayer cell cultures, but usually is not a prominent histopathologic finding *in vivo* (below).

HRSV and HMPV are highly infectious viruses. Humans are their only natural host, although HRSV and HMPV can readily spread to nonhuman primates and are pathogenic in some situations.³⁰⁵ The major mode of spread by HRSV is by large droplets or through contaminated objects and depends on close contact with infected individuals or contact of contaminated hands to nasal or conjunctival mucosa (self-inoculation). It is generally thought that small-particle aerosols (which can remain airborne for extended periods and also can be inhaled deeply into the respiratory tract) are not an important mode of HRSV transmission,^{222,224} although there are contrary data.³³⁸ The incubation period from time of infection to onset of illness for HRSV is about 3 to 5 days^{137,286,291} (Fig. 38.5). The incubation period for HMPV is not defined, but is thought to be similar.

HRSV replicates initially in the nasopharynx. Signs of LRI, when it occurs, usually appear 1 to 3 days following the onset of rhinorrhea (Fig. 38.5). Viral spread to the lower respiratory tract likely involves aspiration of secretions. Infants hospitalized

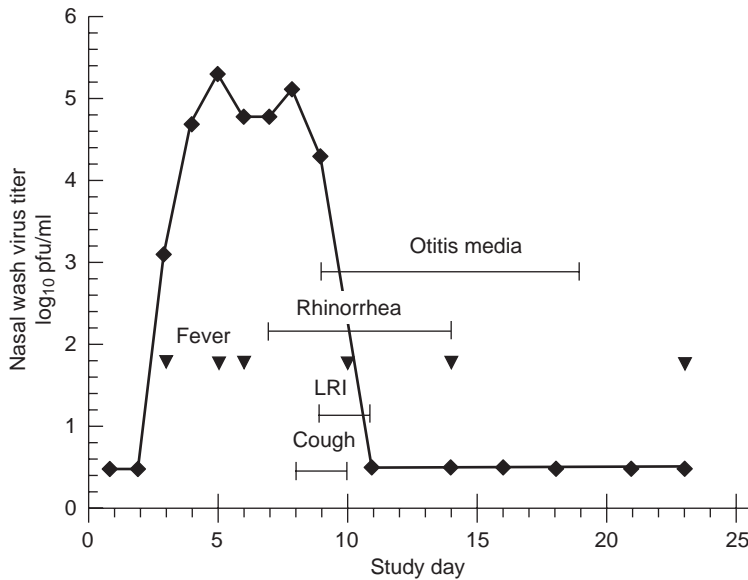


FIGURE 38.5. Time course of viral shedding and disease signs following experimental infection of a young seronegative vaccinee with 10^4 pfu of an investigational live partially attenuated human respiratory syncytial virus (HRSV) vaccine called *cpts248/955*. Fever was intermittent. (Adapted from Karron RA, Wright PF, Crowe JE, Jr, Clements ML, et al. Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus (RSV) vaccines in chimpanzees, adults, infants and children. *J Infect Dis* 1997;176:1428–1436.)

for HRSV disease shed infectious virus in nasal secretions with peak titers of 10^4 to 10^6 infectious units per ml.^{138,139,225,615} Many infants continue to shed virus at hospital discharge and a few can continue to shed virus for weeks following clinical recovery. With the use of quantitative reverse transcription polymerase chain reaction (RT-PCR), the period of detection of HRSV shedding is prolonged, suggesting that virus is present but is neutralized by secretory antibodies made in response to the infection.^{137,351} Limited data suggest that the titer of virus in the lower respiratory tract, sampled in bronchoalveolar lavage fluids of ventilated patients, is similar to that in the upper respiratory tract. Infectious virus has been difficult to recover from adults during symptomatic infection despite positive serologic or RT-PCR tests, probably due to the presence of neutralizing antibodies.

HRSV principally exhibits tropism for the airways and lung tissues. Studies in human adenoid or HAE epithelium models *in vitro* showed that HRSV preferentially infected cili-

ated cells. Infection was limited to the apical surface and did not spread to underlying cells, and virus was released from the apical surface^{562,616,631} (Fig. 38.6). In experimental HAE infections lasting several weeks, infected cells were shed and replaced with little visible alteration of the microscopic appearance of the tissue, although ciliary beating usually was impaired.^{616,631} Syncytia were not observed, probably because the F glycoprotein was expressed on the apical surface and did not contact adjacent cells. HRSV contrasted sharply with influenza A virus examined in parallel, which was rapidly destructive and quickly spread to underlying cells.^{616,631} HRSV infection of epithelial cells induces a rapid inhibition of Na^+ transport, resulting in apical fluid accumulation.³¹⁴ This might be a host mechanism to dilute and remove irritants, but also might contribute to excess fluid and virus spread. This effect was not unique to HRSV, and was observed with parainfluenza and influenza viruses as well as with bacterial pathogens.

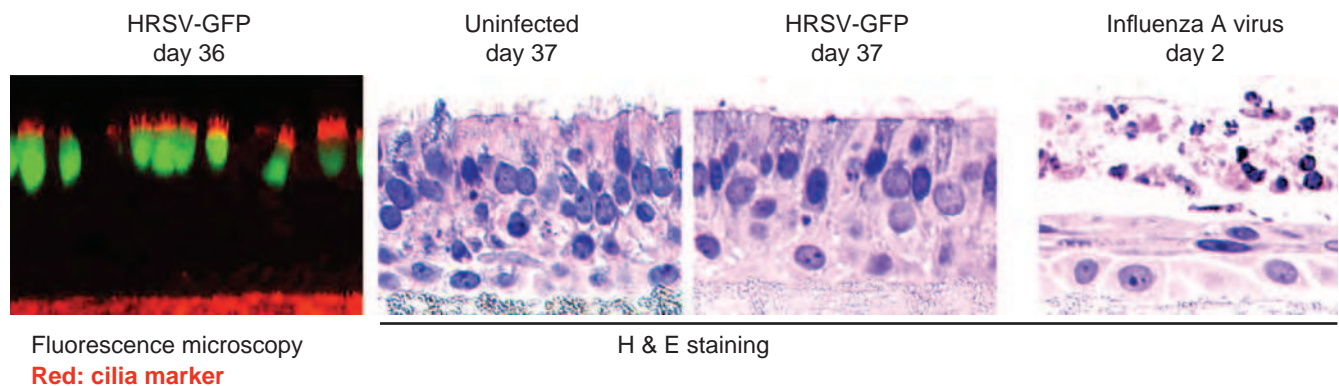


FIGURE 38.6. Infection of an *in vitro* model of human airway epithelium (HAE) with human respiratory syncytial virus (HRSV) and influenza A virus, shown in cross-section. The left hand panel is a fluorescence photomicrograph of cells 36 days following infection with HRSV expressing green fluorescent protein (GFP), and also stained with antibody specific to cilia (*red*; note that this antibody also stained the filter support underlying the cells). The three panels to the right show mock-infected, HRSV-infected, and influenza A virus-infected cultures visualized 37 (mock and HRSV) or 2 (influenza) days postinoculation.⁶³¹ In the three right-hand panels, the cells were stained with hematoxylin and eosin.

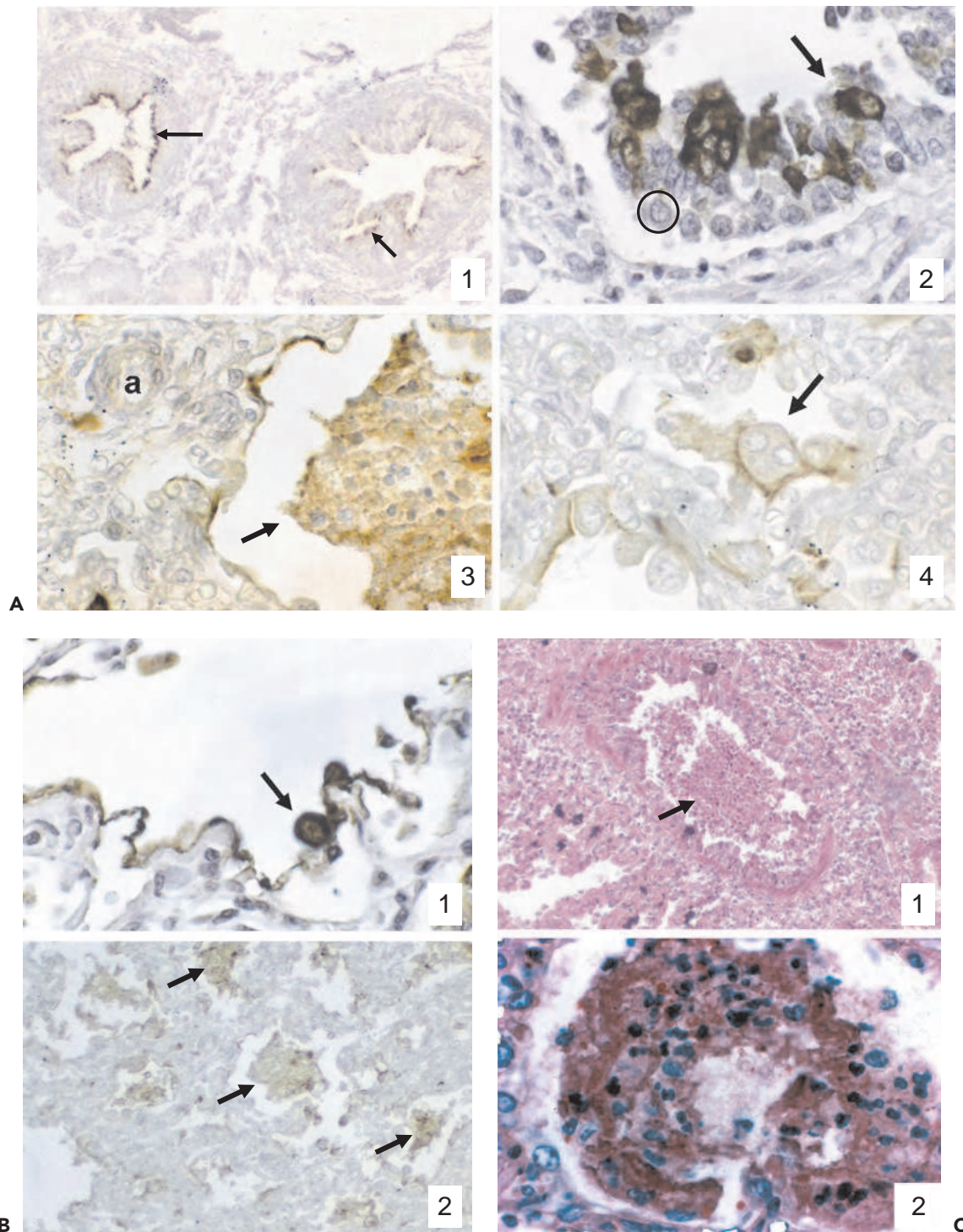


FIGURE 38.7. Histopathology of human respiratory syncytial virus (HRSV) infection in children. **A:** Bronchiolar localization of HRSV antigen (stained brown) detected by immunohistochemistry. (1) HRSV antigen localized in the luminal epithelium of two bronchioles (arrows indicate examples of staining). (2) Higher magnification of infected ciliated cells of the luminal surface of the bronchiole (an example is indicated with the arrow), also showing that the basal progenitor cells (an example is circled) are not infected. (3) Intraluminal debris (the arrow indicates a large debris plug) in a small airway, staining positively for HRSV antigen (an arteriole is marked with "a"). (4) A small syncytium (arrow). **B (left):** Alveolar localization of HRSV antigen (brown) detected by immunohistochemistry. (1) HRSV-infected alveolar cells (the arrow indicates an infected cell). (2) Alveolar lumens and small airways clogged with debris (examples are indicated with the arrows) that stains positively for HRSV antigen. **C (right):** Histopathologic features of bronchiolar inflammation, with periodic acid-Schiff (PAS) staining of carbohydrate macromolecules. (1) Bronchiole occluded with an intraluminal plug of debris and inflammatory cells (arrow). (2) Higher magnification image of intraluminal debris. (Reprinted by permission from Macmillan Publishers Ltd. From Johnson JE, Gonzales RA, Olson SJ, et al. The histopathology of fatal untreated human respiratory syncytial virus infection. *Mod Pathol* 2007;20:108–119; copyright 2007.)

Histopathology of fatal HRSV infections indicates that infection is limited to the superficial cells of the respiratory tract, consistent with the findings in HAE cultures, although it is likely that nonciliated cells also are infected^{4,187,273,596} (Fig. 38.7). There is necrosis and destruction of ciliated cells and occasional proliferation of the bronchiolar epithelium, but basal cells are spared. Ciliated epithelial cells rarely reappear before 2 weeks postinfection, and complete restoration requires 4 to 8 weeks,²²⁰ similar to the duration of postinfection altered lung function and airway reactivity in adults.²³⁷ There is an early influx of polymorphonuclear cells, and a peribronchiolar infiltrate of lymphocytes, plasma cells, and macrophages develops, with migration of the lymphocytes among the mucosal epithelial cells. Submucosal and adventitial tissues become edematous, and secretion of mucus is excessive, which combines with cell debris and inflammatory cells to obstruct the bronchioles and alveoli, causing either collapse or emphysema of distal portions of the airway. HRSV infects both type I and type II alveolar cells.²⁷³ In those instances in which pneumonia occurs, the interalveolar walls thicken as a result of mononuclear cell infiltration, and the alveolar spaces may fill with fluid. There is usually a patchy appearance of these pathologic changes, even though disease may be widespread. Immunostaining identified virus-infected cells in the bronchial, bronchiolar, and alveolar epithelium.^{273,419} Syncytia are sometimes observed, but are not prominent. However, syncytium formation and giant cell pneumonia are hallmarks of infection in individuals with extreme T-cell deficiency. HRSV antigen can be relatively abundant in lower respiratory tract infection, although the staining is usually focal, and in some cases of fatal HRSV bronchiolitis, antigen was present only in small amounts.^{187,419} The histopathology of HMPV infection is not well known but has been reported to involve a characteristic enlarged, darkly staining pneumocyte or “smudge cell” that is not seen with other respiratory paramyxoviruses.⁵³² Studies in nonhuman primates indicate that HMPV has a similar tropism for the superficial cells of the respiratory epithelium.³¹¹

Infection by HRSV and HMPV is largely limited to the respiratory tract. Infectious virus has not been recovered from the blood of HRSV- or HMPV-infected humans. However, viral nucleic acid can frequently be detected in the blood of HRSV-infected infants,⁴⁷⁸ and there are isolated reports of HRSV RNA in cerebrospinal fluid and myocardium.¹⁴⁹ HRSV antigen has been detected in circulating mononuclear leukocytes.¹⁴³ HRSV, HMPV, and influenza viral RNA have been detected in sera of immunosuppressed individuals infected with the respective viruses with high viral loads in the respiratory tract.⁷⁵ When immunosuppressed cotton rats are infected with HRSV, infectious virus was cultured from the liver and kidneys in a small proportion of animals.²⁷⁹ HRSV has been cultured from the myocardium of an infected infant with combined immunodeficiency as well as from a liver biopsy from an infected immunocompetent infant.^{149,178} This suggests that HRSV (and these other respiratory viruses) has some potential to spread beyond the respiratory tract, but usually is restricted by host immunity. A possible role in extrapulmonary disease manifestations has been suggested.¹⁴⁹

HRSV is not thought to cause a latent or persistent infection, and the virus is rarely recovered in the absence of respiratory disease. However, some reports suggest the prolonged persistence of virus or viral material. Infectious virus has been recovered

from guinea pigs 60 to 100 days postinfection,¹²⁴ and was recovered sporadically from mice 100 days postinfection upon T-cell depletion.⁴⁹⁹ BRSV RNA and proteins have been detected in bovine pulmonary lymph nodes 71 days following infection, and there was indirect evidence for *in vitro* infectivity from isolated bovine B lymphocytes.⁵⁶⁵ There is conflicting evidence as to whether HRSV might persist in some individuals with stable chronic obstructive pulmonary disease.^{161,511} HRSV was shed for 199 days, largely without disease, in a child infected with human immunodeficiency virus (HIV),²⁹⁹ and persistent symptomless shedding of HMPV has been described in a number of hematopoietic stem cell transplant (HSCT) recipients.¹³³

The available information suggests that there are not marked differences in replication or virulence among HRSV isolates. Some reports have suggested that subgroup A, or a particular genotype of subgroup A, is associated with increased replication and virulence, but a number of studies have not found such a link.^{63,251,363,518} Recently, an HRSV strain called line 19 was shown to induce increased lung IL-13 expression and mucous secretion in BALB/c mice, an effect that mapped to the F gene,³⁹⁵ raising the possibility that clinically relevant heterogeneity in strain virulence may be uncovered by further investigation. With HMPV, there presently is no indication that specific genotypes are associated with increased disease.^{1,60}

Primary HRSV disease is usually symptomatic, but disease manifestations can vary greatly, and may include upper respiratory tract illness (URI), fever, otitis media, LRI ranging from mild disease to bronchiolitis and/or pneumonia with or without subsequent long-term abnormalities in pulmonary function (see *Clinical Features*), and death in rare cases. Infection in immunosuppressed individuals ranges from asymptomatic to highly lethal, probably reflecting the extent of T cell deficiency. In otherwise healthy individuals, a number of factors are thought to contribute to HRSV (and HMPV) pathogenesis and the observed heterogeneity of disease, including direct viral damage, the host immune response, and other host factors including age, immune status, underlying disease, and genetic polymorphisms affecting host defense.⁹⁶ These are discussed below and in subsequent sections.

Because HRSV can readily infect and cause disease during the first months of life, young age must be considered as a factor in pathogenesis (e-Fig. 38.7). Maternal antibody and immunologic immaturity result in reduced immune responses during infancy (see Immunity section in this chapter), reducing the ability to control the infection. Immune responses in the neonate and young infant can have a reduced Th1 component, which is protective for the fetus prior to birth but can contribute to a Th2 bias in early infancy,^{3,121} and which may contribute to an inflammatory response in the lower airways. The small diameter of bronchioles in infants makes them particularly susceptible to obstruction by edema, secretions, and immune and exfoliated cells.²⁶² Infancy is a time of considerable lung growth and development.¹⁸⁹ Developing lungs may be more susceptible to disease and to long-term effects on lung function. For example, infants with severe HRSV infection express increased levels of neurotrophic factors and receptors.⁵⁵⁸ Studies in a rodent model suggest that this can lead to remodeling of neural networks that innervate the respiratory mucosa, contributing to recurrent airway inflammation and hyperreactivity.⁴⁴⁹ Children with a history of HRSV bronchiolitis early in life were found to have higher obstructive sleep

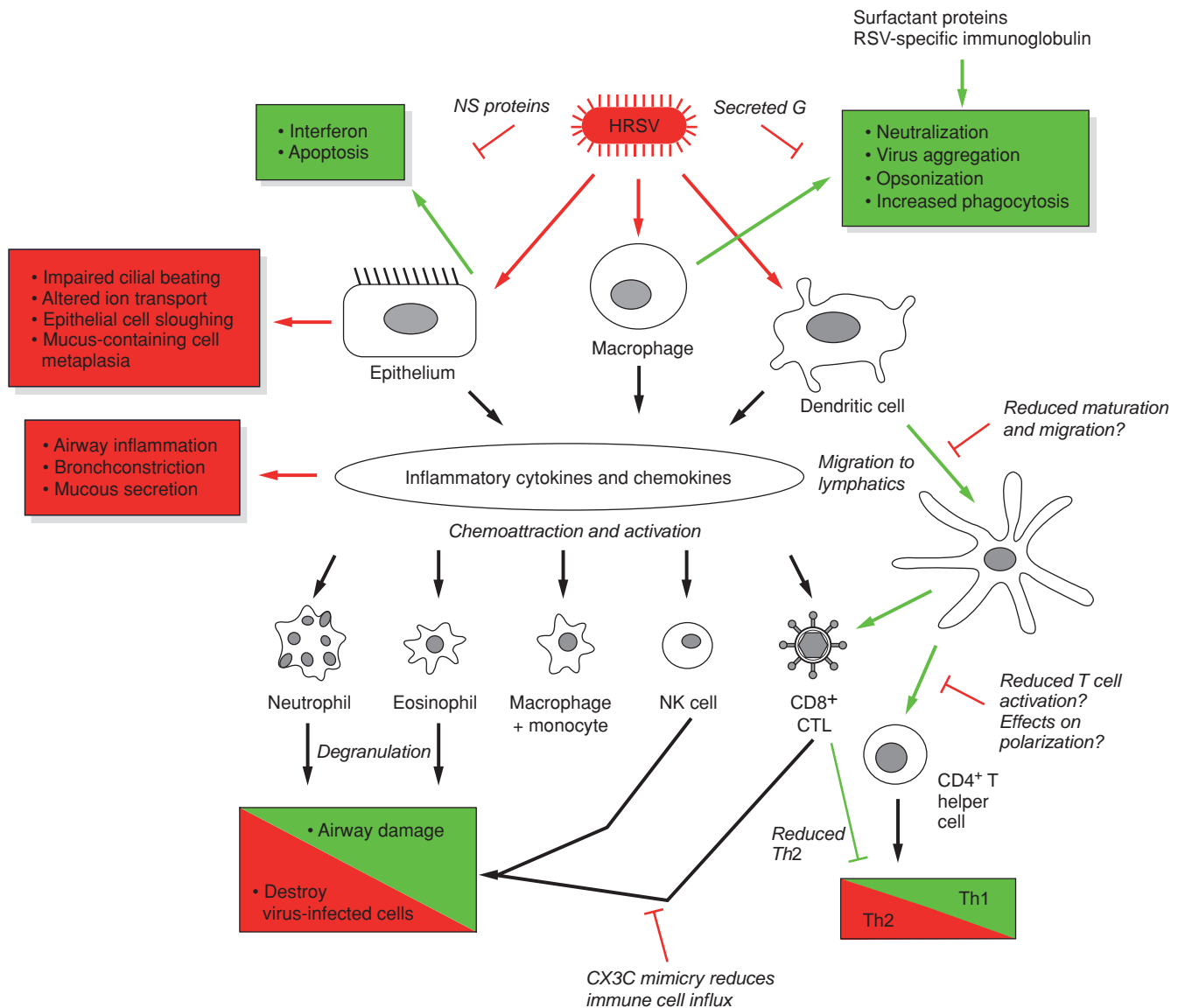


FIGURE 38.8. Factors in human respiratory syncytial virus (HRSV) infection, disease, and immune evasion. In the respiratory tract, the virus (**top**) infects epithelial cells (the major site of virus replication), macrophages, and dendritic cells. Effects that increase virus replication and pathogenesis and/or decrease immune protection are in red; factors favoring protection are in green. **Top right:** surfactant proteins, virus-specific antibodies, and macrophages help restrict infection and spread; antibody-mediated clearance is inhibited by secreted G protein. **Top left:** in infected cells, the viral NS proteins inhibit IFN production and signaling and delay apoptosis. **Center left:** virus infection in epithelial cells impairs their function in maintaining airway fluid level and mucous flow and eventually leads to cell sloughing. **Center:** infected epithelial cells, macrophages, and dendritic cells produce inflammatory cytokines and chemokines that promote airway reactivity and mucous production, attract and activate immune cells, and in some cases may directly induce antiviral effects. **Bottom:** immune cell influx is reduced by viral CX3C fractalkine mimicry. Degranulation by neutrophils (a major inflammatory cell in the airway) and eosinophils, and cell killing by natural killer (NK) cells and CD8⁺ T cells, help restrict infection but also may contribute to disease. **Right:** antigen encounter by dendritic cells leads to their maturation and migration to secondary lymphatic tissue: infection of dendritic cells by HRSV is inefficient and subsequent maturation and migration may be suboptimal. **Bottom right:** dendritic cells induce CD4⁺ and CD8⁺ T-cell activation, polarization, and proliferation: these steps may be reduced due to HRSV exposure. Th2 responses may be suppressed by CD8⁺ T cells and NK cells. (Adapted from Brearey SP, Symth RL. Pathogenesis of RSV in children, In Cane P, ed. *Respiratory Syncytial Virus*. Elsevier: Amsterdam; 2007;141–162.)

apnea and hypopnea indices than control children, which also is associated with increased expression of neurotrophic factors and receptors.⁵¹⁹ There also is evidence from the mouse model that HRSV infection early in life can result in skewed primary and recall immune responses.^{121,541} Other host risk factors are described in the section entitled Epidemiology.

A number of studies, including prospective studies of natural infection, experimental infection of adults with wild-type HRSV, and clinical studies with live HRSV vaccine candidates, have indicated a positive correlation between virus load and disease severity.^{137,138,291,362} although there also are contradictory data.^{37,225,226,615} High nasopharyngeal viral load also was associated with disease severity for HMPV.^{60,362} However, it is not clear whether this correlation reflects pathogenesis due to direct viral damage or a heightened immune response. As noted, studies with *in vitro* human epithelium models indicate that HRSV is not very cytopathic or invasive, compared to influenza virus for example (Fig. 38.6). However, impaired ciliary function and increased cell shedding resulting from HRSV infection would facilitate clogging of bronchioles and alveoli. In experimental infection in adults, the timing of HRSV disease coincided with the timing of viral shedding, suggesting that direct viral damage contributes to disease.¹³⁷ The observation that HRSV (and HMPV) can cause severe disease in immunocompromised cotton rats⁶¹³ and humans^{146,163,233} indicates that severe disease does not depend on an intact adaptive immune system. However, in humans with HRSV LRI, treatment with virus-neutralizing antibodies or the antiviral drug ribavirin that reduced virus replication resulted in limited improvement (see Treatment section), suggesting that factors in addition to direct viral damage are important in pathogenesis, especially by the time that LRI occurs.

It is widely thought that host immunity plays a major role in HRSV (and presumably HMPV) disease. In HRSV-infected cotton rats, treatment with HRSV-neutralizing antibodies rapidly reduced pulmonary titers but had little effect on lung histopathology.⁴⁶³ In contrast, treatment with antiinflammatory glucocorticoids reduced lung histopathology, even though clearance of the virus was slowed, suggesting that the host immune response played the major role in pathogenesis in this model.⁴⁶³ However, the situation is less clear in humans, where treatment with corticosteroids does not significantly reduce disease^{309,439} (see Treatment section). Immune factors that have been suggested to contribute to HRSV disease include (a) an excessive inflammatory response, (b) an overly robust CD8⁺ T-cell response, resulting in excessive tissue damage, and (c) a Th2-biased CD4⁺ T-cell response that is not optimally effective against intracellular pathogens and results in excessive mucous production, airway reactivity, and other effects (see Immune Response). There are supporting and contrary data in each case, as will be shown, and it may be that the contributions of various factors vary in different individuals. Host and viral factors that are suspected to contribute to restriction of HRSV replication and/or to disease pathogenesis, as well as some of the viral mechanisms that subvert or inhibit host immunity, are depicted in Figure 38.8 and discussed throughout this chapter. These general themes likely also apply to HMPV.

IMMUNE RESPONSE

HRSV and HMPV cause acute infections that are restricted and resolved by innate and adaptive immunity. Immune responses

to HRSV and HMPV are broadly similar to those against other respiratory viruses such as influenza and the HPIVs. Infection induces mucosal and systemic virus-neutralizing antibody responses as well as cell-mediated responses. Cellular immunity is particularly important in resolving infection. Secretory and serum antibodies play the primary role in protection against reinfection. With the exception of passively acquired HRSV-neutralizing antibodies, correlates of protection in young infants are difficult to measure and are poorly understood.

Antigens

Postinfection sera from experimental animals or humans contain antibodies that recognize a number of HRSV proteins. However, the F and G proteins are the only virus neutralization antigens and are major, independent protective antigens.¹⁰⁸ This was demonstrated in experiments in which cotton rats or mice were immunized with vaccinia virus recombinants expressing individual HRSV proteins and subsequently challenged with HRSV.^{108,428} F and G were the only proteins that induced neutralizing antibody responses and conferred long-lasting protection. F was more immunogenic and protective than G^{108,428}; the apparent poorer immunogenicity of G may be a consequence of its unusual structure and heavy glycosylation.

A large fraction of MABs raised against the HRSV F protein neutralize infectivity efficiently, and many inhibit fusion.^{32,349} Analysis of viral mutants selected to be resistant to individual MABs mapped five major antigenic sites that mainly involve the F1 subunit.^{113,349} In some cases, mapping has been extended by visualization of antigen-antibody complexes by electron microscopy⁷⁴ or by crystallographic analysis,³⁷⁷ and sites have been mapped on a predicted three-dimensional model of the F protein.⁵¹⁷ Operationally, most of the epitopes of F appear to be conformation dependent, since most monoclonal and polyclonal antibodies raised against native F react poorly with denatured protein, such as in Western blots, and synthetic peptides designed from F are inefficient in inducing neutralizing antibodies or protective immunity.

In contrast, HRSV G has a very different antigenic nature. Most G-specific MABs bind efficiently to denatured G, such as in Western blots, and many bind efficiently to synthetic peptides, suggesting that reactivity does not depend on a complex folded structure.¹⁸⁶ Most MABs against the G protein are inefficient in neutralizing RSV infectivity *in vitro* when tested individually, although neutralization can be achieved with mixtures of MABs.^{365,367} Most G-specific MABs react with some HRSV strains but not others within an antigenic subgroup and thus are “strain-specific”: these MABs often react only with glycosylated G, and their epitopes mostly are in the mucin-like domain in the C-terminal third of the molecule. Other MABs react broadly within a subgroup, and a small number react with viruses from both subgroups: these MABs typically do not depend on glycosylation of G for binding, and their epitopes usually are located in the central conserved region of G.^{365,384} A synthetic peptide representing this central region was protective in mice and calves,²⁹ confirming its importance in immunogenicity and antigenicity. Postinfection human serum antibodies to the G protein were found to be enriched for the immunoglobulin G2 (IgG2) subclass, consistent with G being recognized in part as a polysaccharide antigen.⁵⁸³

Most of the HRSV proteins stimulated memory CD8⁺ CTL from seropositive humans.⁹⁰ However, in both mice and

humans, the G protein is a poor CTL antigen, possibly due to its skewed amino acid content and extensive glycosylation. In the commonly used BALB/c mouse model, the F, N, and M2-1 proteins (of HRSV strain A2) have been shown to induce CD8⁺ CTL responses: the dominant CTL epitope is contained in amino acids 82 to 90 of the M2-1 protein, accounting for approximately 40% of the primary CTL response.³¹² Immunization of mice with vaccinia virus recombinants expressing the N or M2-1 proteins provided protective immunity that was not mediated by neutralizing antibodies and, in the case of M2-1, was confirmed to be mediated by CD8⁺ CTLs.^{108,312} However, protection waned within weeks, suggesting that pulmonary protection by CTLs is short-lived.

For HMPV, the F protein also is a major neutralization and protective antigen, which was shown in studies in which F was expressed by a HPIV1 vector in rodents and nonhuman primates,^{515,539} or by an alphavirus replicon evaluated in mice and cotton rats.³⁹² Surprisingly, vectors that individually expressed the G and SH proteins did not induce detectable neutralizing antibodies or protection in rodents.^{392,514} Similarly, purified HMPV G protein administered to cotton rats did not induce neutralizing antibodies or protection.⁴⁸⁵ Therefore, HMPV may differ from other members of *Paramyxoviridae* in having only a single surface glycoprotein as a major neutralization and protective antigen.

Innate Immunity and Inflammation

The first line of host defense includes the physical barriers of the glycocalyx of the superficial epithelial cells, the secreted mucous layer, and ciliary sweeping. A number of studies have highlighted the role of pulmonary surfactant proteins (SPs), specifically SP-A, SP-C, and SP-D, in restricting HRSV. SPs are lipoprotein complexes produced by type II alveolar cells. SP-A and SP-D have been shown to bind to HRSV, neutralize infectivity (in the case of SP-A), and promote phagocytosis.^{25,192,332} Mice with targeted disruption of SP-A, SP-C, or SP-D exhibited increased pulmonary HRSV titers and disease, which was ameliorated by topical administration of the missing surfactant.^{195,332,333} Genetic polymorphisms in SP-A, SP-B, and SP-D have been associated with severe pediatric HRSV disease.^{150,389,466} Infants with severe HRSV disease were found to have reduced surfactant concentration and function, although it is not known whether this was a cause or a result of severe disease.²⁹³

Toll-like receptors (TLRs) present on the plasma membrane and endosomes³⁰² play a significant role in the host response to HRSV. The HRSV F protein stimulates TLR4 on leukocytes independent of viral replication, leading to activation of the NF- κ B pathway to produce cytokines such as IL-6 and IL-8 and activation of IRF3 to induce type I IFN.³¹⁷ TLR4-deficient mice are less efficient than normal mice in resolving HRSV infection.^{302,317} A number of studies have linked severe pediatric HRSV disease with genetic polymorphisms in TLR4 that reduce its signaling capacity, although other studies did not detect this association.³⁰² HRSV has also been shown to activate signaling from the TLR2/TLR6 heterodimer complex on the surface of leukocytes, resulting in the expression of proinflammatory cytokines, neutrophil influx, and dendritic cell activation in mice.⁴⁰⁷ Mice lacking TLR2 or TLR6 were less able to restrict HRSV challenge compared to normal mice. HRSV also activates TLR3 in epithelial cells, resulting in the production of inflammatory cytokine and chemokines.⁴⁸³ Stud-

ies in TLR3 knockout mice indicated that TLR3 did not make a significant contribution to restricting HRSV replication, but helped prevent IL-13 upregulation and increased mucous production.⁴⁸⁴ Excessive stimulation of TLRs can potentially contribute to pathogenesis: viral infection increases the expression of TLR3 and TLR4, promoting sensitivity both to HRSV and to possible co-infecting viruses or bacteria. In addition to TLRs, the cytoplasmic receptor RIG-I plays a major role in innate responses.^{348,625} Of course, as noted, HRSV and HMPV have mechanisms that suppress activation of these signaling pathways (e-Fig. 38.6).

Infection of epithelial cells by HRSV (or HMPV) alters the cell transcriptional profile markedly, resulting in changes in expression (mostly upregulation) of 900 genes or more.^{22,266,366,632} This was mostly dependent on viral replication. These genes represented multiple biological pathways, showing a broad effect of viral infection. Proteomic profiling of the proteins expressed in A549 epithelial cell cultures following infection with HRSV identified changes in expression for more than 100 cellular proteins, similarly indicating a complex cellular response to infection (the greater number of responding species detected by transcriptional versus proteomic profiling reflects the greater sensitivity of the former method).^{81,402} Comparison of the responses in A549 epithelial cells infected with HRSV, HMPV, HPIV3, and measles virus identified a common core response that mainly involved defense against endoplasmic reticulum stress and induction of apoptosis.⁵⁷⁶

Airway epithelial cells and macrophages exposed to HRSV *in vitro* produce a broad array of proinflammatory cytokines, including IL-1, IL-8/CXCL8, RANTES/CCL5, IL-10, MIP-1 α /CCL3, MCP-1/CCL2, IP-10/CXCL10, IL-6, TNF α , and type I and type III IFNs.^{30,385,386,523} Many of these cytokines have been found in secretions from the upper and lower respiratory tracts of infants with HRSV bronchiolitis.^{56,248} One consequence is the recruitment of immune cells to the infected lung (Fig. 38.8). The total number of immune cells recovered in washes from the upper and lower respiratory tracts of infants was several fold higher in cases of severe HRSV disease compared with uninfected controls.^{157,249,380} Neutrophils were by far the most abundant immune cells present in the airway, comprising 76% to 93% of recovered cells from the upper and lower respiratory tracts, with lymphocytes and mononuclear cells being present at $\leq 10\%$ each and eosinophils at $< 1\%$.^{157,380} Incoming immune cells become activated to express cytokines and other proinflammatory molecules such as cysteinyl leukotrienes that can heighten the inflammatory response.

Inflammation helps control infection but may also contribute to disease (Fig. 38.8), although the picture remains unclear. Studies in mice showed that natural killer (NK) cells are prominent in the early response to infection, are an important source of IFN γ , and have cytotoxic activity against infected cells.²⁶⁷ Granulocytes release factors, such as neutrophil elastase and eosinophil-associated ribonucleases, which can be antimicrobial but also may damage tissue.^{57,480} Increased levels of eosinophil cationic protein and neutrophil elastase have been observed in respiratory washes from infants with severe HRSV disease, indicating the presence and activation of these granulocytes.^{2,188} Mice with eosinophilia due to increased IL-5 expression had increased viral clearance, indicating that eosinophils can be protective.⁴⁴⁸ The amount of mRNA for IL-8—a cytokine that promotes neutrophil chemotaxis and survival—measured in

nasal aspirates of children with bronchiolitis correlated with the severity of disease,⁵¹⁸ and an IL-8 haplotype characterized by increased IL-8 gene transcription was associated with increased susceptibility to HRSV disease.²¹⁹ Bronchoalveolar washes from infants with severe HRSV disease had increased levels of mRNA for IL-9, which is secreted by neutrophils and other cells and promotes inflammation and increased mucous production.³⁷⁹ In infants with severe HRSV disease, the appearance of neutrophil precursors in peripheral blood, preceding their influx into the airways, closely followed the peak of viral load and was coincident with clinical symptoms³⁵¹ (e-Fig. 38.8). These findings suggest that neutrophils in particular have the potential to contribute to both viral clearance and disease. Conversely, however, there also is evidence that, although HRSV may indeed induce high levels of inflammatory cytokines in children hospitalized with HRSV disease, this may not be the dominant factor in severe disease and indeed may be protective.^{37,503} Furthermore, a prospective study of primary HRSV and HMPV infections in infants showed that, although infants infected with either virus had similar disease signs, the levels of inflammatory cytokines induced by HMPV were significantly lower than with HRSV.³²¹ Therefore, although these viruses exhibited similar patterns of disease, they do not share a common pattern of overly robust innate immune responses that might be expected if that is the major determinant of disease.

Macrophages are abundant in the respiratory tract and play important roles in restricting virus replication and modulating the host response. Mice that are genetically deficient in macrophage function, or in which macrophages have been depleted, are less able to restrict HRSV infection and exhibit increased lung inflammation.⁴⁷² This illustrates a key role for these phagocytic cells in restricting the virus as well as in clearing the lung of debris that can cause further damage and inflammation. Macrophages also play an important role in the early response to HRSV infection by producing an immediate release of proinflammatory cytokines.⁴⁵⁷ Studies in mice suggest that macrophages are the primary producers of type I IFN in response to viral respiratory infection in general, with myeloid dendritic cells (DCs) also contributing, whereas pDCs can play a role later in infection if virus replication is not controlled.³¹³

Type I IFN has well-known antiviral effects mediated by signaling through its ubiquitous receptor IFNAR. Type I IFN also is considered to stimulate both innate and adaptive immunity, although these effects can be complex.⁴⁰³ Studies in which bovines were immunized with mutants of BRSV provided evidence of increased humoral and cellular immune responses associated with deletion of NS2, which is the major IFN antagonist of BRSV.⁵⁶⁶ Type III IFN is less well characterized but also has antiviral effects. It generally is produced from the same cell types that produce type I IFN. However, its receptor is expressed mainly on the epithelial cells of the respiratory and gastrointestinal tracts, and thus its effects may be largely limited to those sites.^{144,396} Studies in mice lacking the receptor for either or both type I and III IFN showed that each is involved in restricting replication of respiratory viruses including HRSV,³⁹⁶ although a study with PVM suggested that type I IFN has a greater antiviral role.²⁵²

IFN α/β were detected less frequently and at lower levels in nasal secretions from infants, young children, and adults infected with HRSV, compared to influenza and parainfluenza viruses.^{228,229,374} HRSV infection and replication are

more resistant to prophylaxis or treatment with IFN- α compared to influenza and parainfluenza viruses.^{184,534} Therefore, HRSV may be particularly effective at inhibiting the host IFN response. HMPV induces higher levels of IFN- α/β in mice and is more sensitive to IFN- α than HRSV.²¹⁷

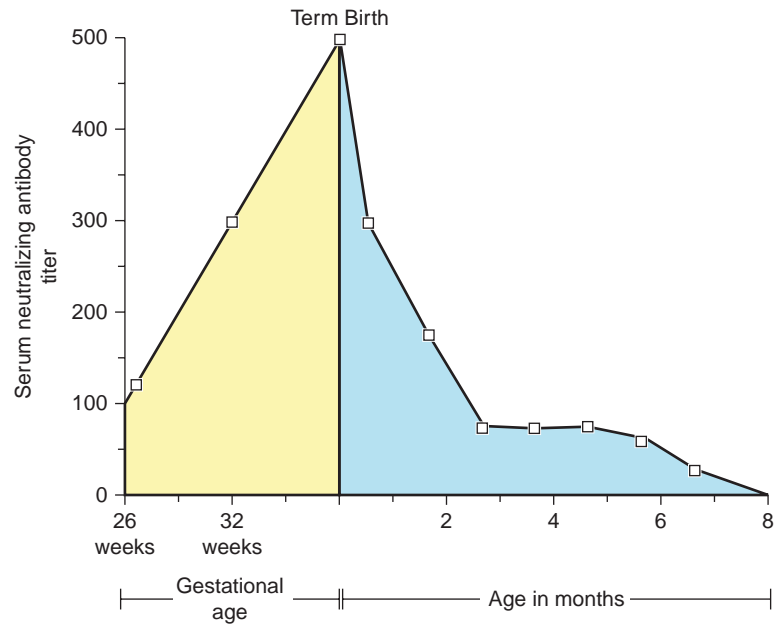
Antibodies

Both serum and secretory antibodies are made in response to HRSV infection. In young infants, the appearance of secretory IgA antibodies usually corresponds with a decrease in HRSV shedding during infection, suggesting a contribution to resolving infection.³⁷⁶ Secretory IgA antibodies are thought to be particularly efficient in restricting HRSV replication. The secretory antibody response is not long-lasting following primary infection, but as individuals grow older and are reinfected, the response is more sustained.²⁹² Experimental infections in adults showed that resistance to reinfection correlated with the titer of HRSV-specific secretory or serum antibodies,^{235,387} and there are similar findings for natural infection.⁵⁸⁵

Studies of prophylaxis in high-risk infants with either intravenous immunoglobulin containing high titers of HRSV-neutralizing antibodies (RSV-IGIV) or HRSV-neutralizing F MAb (palivizumab, motavizumab) have demonstrated the protective effect of serum HRSV-neutralizing antibodies against severe HRSV disease.^{77,214,434} (see Prevention, below). Studies in experimental animals and observations with maternal serum antibodies also illustrate the protective effect of HRSV-neutralizing antibodies (below). The serum antibody response is long-lived (except early in life, as noted below), and peak titers of antibodies detected by ELISA were reached following the second infection in life.⁵⁸² However, serum IgG antibodies gain access to the respiratory tract primarily by the relatively inefficient process of passive transudation. As a consequence, high serum antibody titers are necessary to achieve protection in the respiratory tract. For example, in studies of passively transferred antibodies in the cotton rat model, neutralizing serum titers of 1:390 and 1:3,500 were required to achieve a 99% reduction in HRSV replication in the lower and upper respiratory tracts, respectively.⁴⁶² As another indication of the concentration gradient between the circulation and the lumen of the respiratory tract, the therapeutic effect of passive antibodies in reducing HRSV replication in cotton rats was 160-fold greater when administered directly to the respiratory tract versus systemically.⁴⁵⁹ Studies in humans suggested an antibody concentration gradient of approximately 350:1 between sera and nasal washes.⁵⁸¹ Therefore, protection conferred by serum antibodies obtained by maternal transfer, or prior infection, or passive antibody prophylaxis, depends on a high titer of HRSV-neutralizing antibodies and is less efficient in the upper versus the lower respiratory tract.

Young infants possess maternally derived serum IgG antibodies against HRSV, HMPV, and other common pathogens.¹¹⁴ Epidemiologic studies indicate that HRSV disease in infants born with higher levels of maternal HRSV-neutralizing antibodies was milder and occurred at an older age than in infants with lower antibody levels, suggesting a protective effect.¹⁹⁶ Maternal antibodies are obtained from the mother during gestation by active transport across the placenta beginning at ~26 weeks of gestation and continuing until birth (Fig. 38.9). Infants who are born prematurely have lower titers, increasing their susceptibility to infection and disease. The IgG1 subclass is preferentially

FIGURE 38.9. An idealized diagram based on experimental data illustrating the titer of human respiratory syncytial virus (HRSV)–neutralizing maternal serum antibodies in the fetus¹²⁹ and newborn human infant⁴³⁷ as a function of gestational and postnatal age. This illustration depicts the transplacental transfer of serum antibodies and the subsequent decrease in titer following birth; this diagram shows only the HRSV-neutralizing component of the multivalent maternal antibodies. Courtesy of Dr. James E. Crowe, Jr. Note that absolute neutralizing antibody titers achieved at birth can vary substantially between individual infants depending upon maternal titers.



transferred, but otherwise the titer and specificity of maternally derived IgG antibodies in the full-term neonate are similar to or slightly higher than those of the mother. Thereafter, these antibodies decay with a half-life of approximately 21 to 26 days and protection diminishes. Breast milk may also contribute protective antibodies, although this has not been clearly documented and likely is a minor effect.

In hospitalized infants, HRSV-specific serum IgA and IgG responses were detected 10 days following the onset of illness, and peak titers were achieved at 3 to 4 weeks.⁵⁹⁴ The magnitude of serum and secretory antibody responses to HRSV in young infants typically is reduced compared to older individuals. For example, in a study of infants and young children with a primary HRSV infection, the titers of serum antibodies that bound to the F or G glycoprotein or that neutralized HRSV were 8- to 10-fold lower in individuals of 4 to 8 months versus 9 to 21 months of age.⁴⁰⁹ The frequency of a detectable secretory IgA response to HRSV in infected infants was directly related to age, and these antibodies often were nonneutralizing *in vitro*.^{292,376} The effect of age on the response of serum and nasal wash antibodies to a live attenuated HRSV vaccine is illustrated in e-Figure 38.9. In addition, antibody responses in the young infant are short-lived compared to older individuals: the increases in HRSV-specific IgA and IgG antibodies observed shortly after infection in infants were absent or at very low titer 1 year later.⁵⁹⁴ Therefore, secretory and serum antibody responses are reduced in young infants (<~6 months of age) compared to older individuals with respect to magnitude, neutralizing activity, and longevity. This is particularly relevant to HRSV, since HRSV infection is very common in infancy (*Epidemiology*). Responses to HMPV are assumed to be similar.

Reduced immune responses to HRSV (and HMPV) early in life are due to immunologic immaturity^{3,336} and the immunosuppressive effects of maternal antibodies.^{114,507} Immunologic immaturity has been characterized for a variety of antigens and has been shown to limit innate, antibody, and cellular responses during the first year of life, although these effects are not absolute and adult-like responses can be elicited under

some circumstances.^{3,336} Examination of the human neonatal B cell response to HRSV revealed a bias in the repertoire of antibody gene expression and a dramatically reduced frequency of somatic mutations compared to individuals older than 3 months, which likely contributes to the poorly neutralizing nature of the response in the young infant.⁶¹¹ Immunosuppression by HRSV-specific maternal or passive serum antibodies has been documented in natural infection,⁴⁰⁸ in experimental animals,^{410,411} and in the setting of clinical evaluation of live HRSV vaccines in infants.⁶¹⁷ For example, in the case of live-attenuated vaccines, the titer of maternal antibody had little effect on virus replication in the upper respiratory tract, but suppressed the serum and secretory antibody responses.⁶¹⁷ In mice, passive HRSV-specific serum antibodies suppressed both serum and secretory antibody responses to infection with wild-type HRSV but did not suppress the cell-mediated response and priming for a secondary antibody response.¹¹⁷ Surprisingly, in a cohort of infants younger than 6 months of age who were identified as having very low titers of HRSV-specific maternal antibodies, natural wild-type HRSV infection induced neutralizing serum antibody titers that were indistinguishable from those of individuals aged 6 to 24 months.⁵⁰⁵ This indicates that young infants are capable of mounting a substantial antibody response when maternal antibodies are not present to exert their suppressive role. Therefore, maternal antibodies are beneficial in providing some disease sparing early in life, but also suppress antibody responses.

In adults, natural HRSV infection induced an approximately eightfold rise in serum HRSV-neutralizing antibodies, which declined fourfold during the subsequent year.¹⁶⁵ Titers in uninfected control subjects did not decline during this period, suggesting that there may be a relatively stable set-point, whereas postinfection rises are more short-lived. Interestingly, elderly individuals exhibited a more robust antibody response to infection with HRSV or HMPV compared to younger adults.^{162,584} This indicates that the increased susceptibility that comes with aging is not due to a defect in the ability to produce virus-neutralizing antibodies.

It has been suggested that, in some individuals, HRSV infection induces IgE that may contribute to HRSV disease. IgE—whose production is promoted by the Th2 cytokines IL-4 and IL-13—is bound by receptors on mast cells, and contact with antigen induces the release of mediators including histamine and leukotrienes that stimulate inflammation, rhinorrhea, cough, and wheezing. Increased HRSV-specific IgE and histamine were detected in respiratory secretions from HRSV-infected infants with wheezing in concentrations that correlated with the degree of hypoxia.⁵⁹⁵ However, other groups have not found convincing evidence of HRSV-specific IgE,¹²⁶ and its significance remains uncertain.

T Lymphocytes

Experience with patients with deficiencies in cell-mediated immunity has demonstrated its importance in restricting HRSV and other enveloped respiratory viruses.^{178,233,299,600} Immuno-deficient children fail to clear HRSV and can shed virus for months rather than the typical interval of 1 to 3 weeks. Severely immunocompromised adults, such as HSCT recipients or individuals with leukemia, are readily infected with HRSV and have a high incidence of serious disease and death. In addition, HMPV can cause severe disease in HSCT recipients,⁷⁵ although there also can be prolonged shedding without disease.¹³³

In the mouse model for HRSV, depletion of the CD4⁺ and CD8⁺ T-lymphocyte subsets individually or together showed that both are important in clearing a primary infection.²⁰⁹ Both subsets also contributed to disease, with CD8⁺ cells having the greater effect.²⁰⁹ In contrast, depletion of B lymphocytes indicated that HRSV-specific antibodies are not required for clearance of a primary infection in mice, although they reduced disease and were important for restricting secondary infection.²⁰⁷ Although treatment with HRSV-neutralizing antibodies can greatly reduce viral replication in HRSV-infected mice, it was inefficient in eliminating the virus, illustrating the importance of cellular immunity for viral clearance.⁴³¹

In humans, robust CD8⁺ T-cell responses have been documented in the peripheral blood of infants and children during primary and secondary HRSV infections (e-Fig. 38.8), which precedes the migration of these cells to the respiratory tract.^{249,250} Infants with severe HRSV disease have increased numbers of T lymphocytes in the airways, with a greater abundance of the CD8⁺ versus CD4⁺ subset, and a greater proportion of airway CD8⁺ T cells compared with rhinovirus-infected children.^{157,250,380} However, although the number of CD8⁺ T cells was increased by infection, they did not constitute a particularly abundant cell population either in fluid recovered from the airways of infants with severe HRSV disease^{157,250,380} or in pulmonary tissue from infants that experienced severe or fatal RSV infection.^{273,596} Increased disease was observed in a severely immunocompromised child with a long-term HRSV infection following transfer of donor lymphocytes.¹⁵¹ However, in infants and children with HRSV LRI, the timing of the CD8⁺ T-cell response did not correlate with disease: specifically, the appearance of virus-specific CD8⁺ T cells in peripheral blood peaked during recovery 9 to 15 days following the onset of symptoms.^{249,351} (e-Fig. 38.8). In addition, the magnitude of the systemic virus-specific CD8⁺ T-cell response did not correlate with disease severity.²⁴⁹ Therefore, although studies in mice suggest that CD8⁺ T cells make an important contribution to HRSV disease, this is less evident from observations in humans.

An increased response of Th2 versus Th1 CD4⁺ lymphocytes (defined by the signature cytokines IL-4 and IFN γ , respectively) may contribute to pathogenic responses to HRSV infection and reinfection. The clinical picture of severe HRSV disease, including airway plugging, wheezing, and long-lasting effects on lung function, has some similarity with asthma, which involves a Th2 bias. As noted, young infants can have a Th2 bias lingering from the prenatal period. In addition, studies in rodents indicate that a Th2-biased response was involved in the enhanced HRSV disease that was associated with a formalin-inactivated HRSV vaccine evaluated in the 1960s (see *Prevention*). However, analogies to asthma and the formalin-inactivated vaccine are inexact because disease in those examples depends on prior sensitization, whereas HRSV disease during natural infection is most severe during the initial exposure and is less severe with repeat exposures.

The evidence for Th2-biased responses in HRSV disease is mixed. In some studies, peripheral blood mononuclear cells from infants hospitalized with HRSV disease exhibited a Th2-biased HRSV-specific recall response when stimulated *in vitro*.^{36,479} However, other studies documented Th1-biased responses⁶² or heterogeneity in responses, with some infants having Th1-biased responses and others Th2.^{328,390} Th2-biased cytokine responses detected in respiratory washes from infants infected with HRSV were significantly higher than from influenza virus-infected infants.⁵³³ A positive association was found between HRSV disease and a genetic polymorphism in the IL-4 gene that increases gene expression.³⁸⁹ Other studies detected Th2-biased responses predominantly in individuals with a history of asthma or allergy, suggesting that Th2 involvement is linked to those with this predisposition.^{296,330} In another study, Th2-biased responses were observed with influenza and parainfluenza virus in addition to HRSV and were greater in individuals ≤ 3 months of age compared to those > 3 months of age, suggesting that this reflects a Th2 bias during the first few months of life but is not specific to HRSV.³¹⁰

Studies in mice suggest that NK cells and HRSV-specific CD8⁺ CTLs play an important role in enhancing Th1 and limiting Th2 responses to HRSV antigens during infection, an effect mediated by secretion of IFN γ and probably other regulatory molecules.^{267,429,525} IFN- α/β also appeared to enhance Th1 and suppress Th2 responses.^{148,403} In addition, HRSV-specific CD8⁺ T cells may reduce virus-induced inflammation through the secretion of IL-10.⁵²⁹

Viral Inhibition and Evasion of Host Immunity

HRSV is able to readily reinfect symptomatically throughout life without need for significant antigenic change, and symptomatic reinfection by HMPV also appears to be common (*Epidemiology*). This is in contrast to influenza A virus, for which symptomatic reinfection usually depends on significant antigenic change. This is widely interpreted as evidence that HRSV (and HMPV) inhibits or skews the development of long-term protective immunity. However, the evidence for this is unclear.

HRSV and HMPV indeed have a number of mechanisms that inhibit host innate and adaptive immunity. Some of these have already been noted for HRSV in particular, including very effective (for HRSV) inhibition of type I and type III IFN production and signaling, inhibition of apoptosis, inhibition of TNF α and NF- κ B signaling, and inhibition of PKR activation

and stress granule formation. HRSV also inhibits signaling from type II IFN (IFN γ) in macrophages.⁵⁰⁰

In addition, studies in the mouse model with HRSV mutants showed that the presence of the fractalkine motif in the G protein has the effect of reducing the pulmonary influx of CX3CR1-bearing leukocytes, including subsets of NK cells and CD4⁺ and CD8⁺ T cells, and thus reduces both innate and adaptive responses to infection.²⁴³ In a separate effect, the central conserved domain of G was shown to reduce activation of TLR2, TLR4, and TLR9 in human monocytes, thus suppressing innate immune responses.⁴⁵³

As another example, presentation of HRSV and HMPV antigens by myeloid dendritic cells (mDCs) may be inefficient. HRSV and HMPV infect human mDCs inefficiently *in vitro* and induce a low-to-moderate level of mDC maturation.^{128,218,325} In the case of HRSV, maturation was partly suppressed by the NS proteins,⁴⁰⁴ but reduced maturation also appeared to be due to insufficient stimulation rather than strong inhibition.³²⁵ In addition, expression of NS1 was associated with a shift toward Th2 polarization, reduced Th17 polarization, and reduced activation of CD8⁺ T cells bearing CD103, a homing integrin that directs CD8⁺ T cells to mucosal epithelial cells.⁴⁰³ In mDCs infected with HRSV or HMPV, upregulation of the CCR7 receptor that is necessary for mDC migration to lymphatic tissue was inefficient.³²⁴ Some studies demonstrated reduced activation and altered polarization of CD4⁺ T lymphocytes *in vitro* in response to HRSV-exposed human mDCs,^{128,218,482} although other results suggest that HRSV does not differ significantly from HMPV, HPIV3, and influenza A virus in this regard.^{323,325} HRSV also can suppress T-cell proliferative responses *in vitro* by direct contact mediated by the F protein.⁴⁹⁴ These observations suggest a variety of mechanisms that might reduce/alter antigen presentation and activation, polarization, and proliferation of CD4⁺ and CD8⁺ T cells, but also suggest that some of these effects may not be unique to HRSV.

However, although HRSV has mechanisms that inhibit host immune responses, this likely is true of most viruses, and it is not clear that this is more pronounced with HRSV. It also is not clear that protective responses to HRSV and HMPV are inherently weak or skewed. As noted, the weak and short-lived immune responses observed in young infants are particular to that population rather than to the virus, and brisk serum antibody responses were observed when the suppressive effect of maternal antibodies was minimized.⁵⁰⁵ Primary infection of seronegative mice and cotton rats with HRSV induces robust antibody and cellular immune responses and long-lived protective immunity.^{208,461} Infection of seronegative chimpanzees with wild-type HRSV or with strongly attenuated live vaccine candidates induced very robust serum antibody titers and protection.^{115,549,603,606} With HMPV, infection of mice or cotton rats results in a brisk neutralizing serum antibody response and clearance of the virus, although there was one report of prolonged virus replication for 60 days.^{6,241} In another report, infection of cynomolgus macaques with HMPV resulted in neutralizing serum antibody responses that waned substantially over the course of several months, and provided poor protection against a homologous challenge 8 months later.⁵⁷³ However, in human adults, titers of neutralizing antibodies to HMPV and HRSV are quite high.^{162,165} Importantly, reinfection with either virus usually is associated with substantially reduced disease (see *Epidemiology* section), indicating that

prior infections induce substantial protection against clinically significant disease. Therefore, the extent to which HRSV and HMPV inhibit or subvert the host protective response remains unclear.

The ability of HRSV and HMPV to reinfect might also reflect evasion of protective immunity. For example, the secreted form of the HRSV G protein was shown to help the virus escape antibody-mediated neutralization, serving as an antigen decoy to spare the virus from neutralizing antibodies, and acting to reduce antibody-mediated clearance by immune cells.⁷⁰ Because other common respiratory viruses do not appear to encode secreted forms of a major protective antigen, this may help explain the relative insensitivity of HRSV to restriction by maternal antibodies in HRSV-naïve young infants, and also would facilitate reinfection later in life.

Features of the major viral protective antigens may help evade immunity. As noted, HRSV G is less efficient than F as a neutralization antigen, and HMPV G does not appear to induce neutralizing antibodies or protection. HRSV G also generally appears to be a poor inducer of CD8⁺ CTLs. Clonal analysis of the human antibody response to HRSV F showed that half of the repertoire was against nonnative protein, suggesting that a substantial proportion of F is produced in an unfolded form that may divert and reduce the immune response to the folded, functional form that is packaged in the virion.⁴⁸⁷ Although antigenic differences in HRSV or HMPV are not necessary for reinfection, they do increase its efficiency.⁵⁸⁹

The tropism of HRSV (and HMPV) also may help evade immunity. Restriction of virus replication to the superficial layer of the epithelium, apical budding, and low invasiveness and low cytopathogenicity likely delay and reduce the exposure of viral antigen to the host immune system⁶³¹ (Fig. 38.6). Dendritic cells can sample antigen in the respiratory lumen, but poor infectivity and poor induction of CCR7, as noted, would reduce antigen presentation. As noted, serum antibodies are not efficiently transported to the lumen, and local secretory antibodies can be short-lived. In addition, a number of reports have indicated that CD8⁺ CTLs are functionally downregulated in the lung. This was originally suggested to reflect immune inhibition by HRSV,⁸⁵ but further studies showed that this effect does not appear to be specific to HRSV but rather is a property of the tissue.^{141,211,570} This probably is a mechanism to reduce lung injury, but would also reduce immune protection. Therefore, evasion of host immune responses likely contributes to infection and reinfection by HRSV and HMPV.

EPIDEMIOLOGY

Infection of Infants and Young Children

HRSV is the most important global cause of severe acute viral LRI in infants and young children. In a benchmark surveillance study from 1957 to 1976 in Washington, DC, HRSV was detected in 23.3% of hospitalizations for respiratory tract disease in infants and young children, compared with 11.5%, 6%, 3.2%, and 5.2% of hospitalizations with HPIV3, HPIV1, HPIV2, and influenza A virus, respectively.^{297,412} Similar values for the proportion of ARI hospitalizations attributable to HRSV in infants and young children were obtained in more recent studies.^{258,263} HRSV infection was detected in 43% of

those hospitalized with a diagnosis of bronchiolitis and in 25% of those with pneumonia, compared to 11% of those hospitalized with bronchitis and 10% of those with croup.^{297,412} HRSV infection is more likely to result in pediatric LRI compared to influenza or HPIV1, HPIV2, or HPIV3.¹⁷⁹ Shay et al.⁵⁰² estimated that there are 73,400 to 126,306 annual hospitalizations for HRSV pneumonia and bronchiolitis in infants younger than 1 year of age in the United States; more recent estimates by Hall et al.²³⁶ and Lee et al.³²⁹ for annual hospitalizations for HRSV disease in children younger than 5 years of age were 57,527 and 113,000, respectively. The burden of HRSV disease is not confined to hospitalization. In the United States, an estimated 2.1 to 4.2 million children younger than the age of 5 years (representing 10% to 20% of that age group) receive medical care each year for HRSV-related illness.^{236,329} Hall et al.²³⁶ estimated that this involves 517,747 (1 of 38) visits to the emergency room and 1,534,064 (1 of 13) primary care office visits, in addition to the hospitalizations already noted.

Although less is known about the burden of HMPV disease, a prospective study of infants hospitalized for ARI or fever without localizing symptoms in Nashville, TN, and Rochester, NY, found that 3.8% of 1,104 such hospitalizations were associated with HMPV.⁶⁰⁷ Other retrospective studies at referral hospitals suggested that approximately 5% to 10% of specimens from children hospitalized with ARI or fever were positive for HMPV RNA by RT-PCR. In a cohort of more than 2,000 subjects aged 0 to 5 years followed during a 25-year period at Vanderbilt University Medical Center, approximately 12% of outpatient LRI was associated with HMPV infection, which was second only to HRSV in this population.⁶⁰⁸

Worldwide, HRSV was estimated to cause 33.8 million new cases of LRI in children younger than 5 years of age in 2005, accounting for 22% of all LRI in that age group.^{221,417} This resulted in an estimated 3.4 million hospitalizations and 66,000 to 199,000 deaths due to HRSV-associated LRI. These are probably underestimates, since HRSV testing was incomplete and community LRI was undercounted. Although the contribution of HRSV to morbidity and mortality in resource-limited settings is less well understood, a recent study in coastal

Kenya showed that HRSV was the predominant virus detected among hospitalized children with severe pneumonia.³⁹

HRSV infection is more common in infancy than is infection with other respiratory viruses. Prospective studies have demonstrated that 50% to 69% of infants are infected during the first year of life,^{197,253,297} and virtually all are infected by age 2.¹⁹⁷ Primary infection is usually symptomatic, and 25% to 40% of primary infections result in LRI.^{197,286} Hospitalization for severe HRSV disease is most frequent between 6 weeks and 6 months of life, with a peak incidence at 1 to 3 months of life (Fig. 38.10). HRSV also causes a substantial disease burden in children beyond the first year of life: of the primary care visits for HRSV disease in infants and children younger than 5 years of age, 61% occurred between the ages of 2 and 5 years.²³⁶ Primary infections with HMPV typically occur slightly later than HRSV, with peak hospitalization occurring at about 6 months of life.^{59,401,575} HMPV seropositivity is almost universal by age 5 years.⁵⁷²

Reinfection with HRSV is frequent during the first few years of life, more so than with other respiratory viruses. For example, in the prospective study noted above,¹⁹⁷ 47% and 45% of the children during the second and third years of life, respectively, were re-infections. In a day care center study in which 98% of HRSV-naïve infants and young children were infected when exposed to HRSV during an outbreak, the frequency of reinfection during the two subsequent yearly epidemics was 74% and 65%, respectively.²⁵³ Therefore, children often are infected two or more times with HRSV during the first few years of life. Reinfection is usually symptomatic. One study found that LRI could occur during either the first or second infection early in life, whereas for subsequent infections there was a considerable reduction in disease severity reflecting increasing protective immunity.²⁵³ Recurrent infection with HMPV also is thought to be common. In infants, the second HMPV infection can present with upper or lower respiratory tract disease.⁶¹⁰

Mortality due to HRSV is uncommon in children in developed countries, although mortality is increased substantially in infants with congenital cardiac or pulmonary disease, or immunodeficiency or immunosuppression. There are no

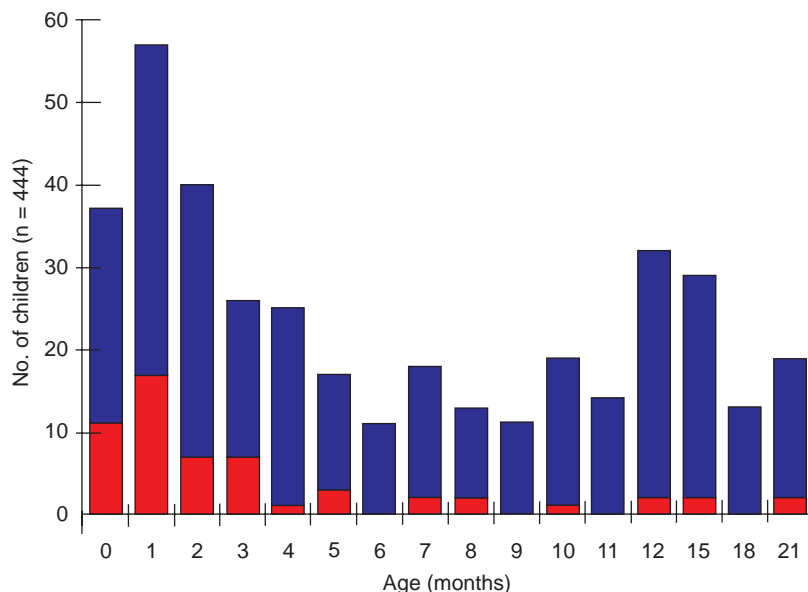


FIGURE 38.10. Age at time of hospitalization for human respiratory syncytial virus (HRSV) disease at the Johns Hopkins Hospital during 1993–1996. The red bars indicate more severe disease. (Adapted from Karron RA, Singleton RJ, et al. Severe respiratory syncytial virus disease in Alaska native children. RSV Alaska Study Group. *J Infect Dis* 1999;180:41–49.)

exact determinations of the overall death rate, but the estimates appear to have dropped over time. A survey from the mid-1970s estimated the fatality rate at 0.5% to 2.5% of hospitalized children with HRSV infection,⁹¹ and in 1985 HRSV was estimated to be responsible for 4,500 pediatric deaths annually in the United States.³⁸¹ More recent estimates are as low as 0.3% of hospitalized children. In the United States, the pediatric mortality rate was estimated to be 5.3 and 0.9 per 100,000 per year for individuals younger than 1 year of age and 1 to 4 years of age, respectively, totaling approximately 300 deaths per year.⁵⁵³ This likely reflects improvement in supportive care.

Major risk factors for pediatric HRSV infection and disease include increased exposure (day care attendance, siblings younger than 5 years of age, and admission to the hospital during HRSV season), low titers of maternal antibodies, lack of previous HRSV infection, premature birth (<36 months gestation, and especially <28 months),^{40,122} young age, chronic lung disease,²¹² congenital heart disease,³⁵² neuromuscular diseases, and primary immunodeficiency disorders.^{178,375} Other factors include asthma or a family history of asthma, poverty, exposure to tobacco smoke, and male gender. In one study, the estimated number of HRSV hospitalizations per 1,000 during the first year of life was 388 for infants with chronic lung disease, 92 for those with congenital heart disease, 66 for those born at 29 to less than 33 weeks, and 30 for term infants with no underlying disease.⁶¹ Fatality rates for children born prematurely or having underlying pulmonary or cardiac disease were similarly elevated.⁵⁹³ However, it is important to note that 50% to 70% of HRSV hospitalizations occur in previously healthy full-term infants.^{61,455}

Very high rates of hospitalization can occur in certain populations. In American Indian and Alaska Native children, the risk of hospitalization for HRSV is 3 to 5 times greater than that of the general United States pediatric population,^{51,289} and high rates of severe HRSV disease have been observed in other aboriginal populations.

Various host genetic factors have been implicated in susceptibility to pediatric HRSV disease. A recent study found an increased concordance of severe pediatric HRSV infection in identical versus fraternal twins.⁵⁵⁴ A number of studies have described positive or negative associations of severe pediatric HRSV disease with polymorphisms in a variety of host genes, notably ones involved in innate immunity and the Th2 subclass of CD4⁺ T lymphocytes. These include genes for surfactant proteins, TLR4, IL-4, IL-8, IL-9, IL-10, IL-13, IL-18, RANTES (CCL5), CX3CR1, vitamin D receptor, nitric oxide synthetase, transcription factor Jun, IFN α 5, and other innate immunity genes.^{268,346,389,498,508} These gene-association studies remain preliminary and are sometimes contradictory, probably because the study sizes were too small to validate associations, but they suggest that a number of genes contribute to resistance or susceptibility to severe HRSV disease.

Infection of Adults

HRSV reinfects adults at a rate of approximately 5% to 10% per year.^{159,163} Reinfection is more frequent in adults with increased exposure to the virus: during a typical HRSV season, 25% to 50% of health care workers are reinfecting, and family members of sick children are readily reinfecting. It is likely that adults become susceptible to re-infection as titers of secretory and serum HRSV-neutralizing antibodies from previous

exposure decline with time.^{166,235,387} HRSV is considered to be second to influenza as a cause of medically significant respiratory tract disease in adulthood. For example, in a study in the United Kingdom using RT-PCR to identify pathogens in various age groups with medically attended respiratory disease, in individuals 15 years of age or older, HRSV was identified in 11% to 22% (depending on the year) of cases compared to 16% to 43% with influenza virus.⁶²⁹ Hospitalization of healthy, nonelderly adults for HRSV disease is rare. However, severe disease and even death due to HRSV can rarely occur in young, previously healthy adults.⁵⁰¹ HMPV reinfection occurs in 1% to 9% of adults each year, with a wide disease spectrum ranging from asymptomatic to severe respiratory disease.^{158,586}

HRSV is an important cause of morbidity and mortality in the elderly (>65 years of age). In one study, HRSV infection was associated with 10.6% of hospitalizations in the elderly for pneumonia, 11.4% for chronic obstructive pulmonary disease, 5.4% for congestive heart failure, and 7.2% for asthma.¹⁶³ Low serum neutralizing antibody titer was associated with increased risk of hospitalization.⁵⁸⁷ HRSV is estimated to cause on average 17,358 deaths annually in the United States, with 78% of these deaths in adults older than age 65.⁵⁵³ Therefore, in more affluent countries, deaths due to HRSV are much more frequent in the elderly than in the pediatric population, whereas in less affluent countries the pediatric burden is likely to be greater. HMPV also afflicts the elderly. In one study of adult patients hospitalized for cardiopulmonary conditions, 11% had evidence of HMPV infection, and the frail elderly appeared to be at increased risk of severe disease.¹⁶⁰ In another study of elderly adults hospitalized for respiratory tract disease, HMPV was identified in 8% of cases (ranging from 4.4% to 13.2%, depending on the year), compared to 10.5% for influenza A and 9.6% for HRSV.¹⁵⁸ Immune senescence,⁴⁴⁰ resulting in a reduced ability to control infection, presumably is an important factor in the increased severity of HRSV and HMPV disease in the elderly.

Other High-Risk Populations

HRSV is important in adults and older children with underlying pulmonary or cardiac disease or who are severely immunocompromised, especially with T-cell deficiencies.^{406,600} Patients with congenital immunodeficiencies such as severe combined immunodeficiency diseases are at high risk. The mortality rate associated with severe HRSV disease in adults with profound immunosuppression due to leukemia or HSCT can be as high as 80% to 100%.⁶⁰⁰ The severity of disease depends on the type and magnitude of immunosuppression.^{247,599} For HSCT recipients, HRSV infection that occurs preengraftment is associated with the highest risk of pneumonia and death, but mortality also is high in those who develop pneumonia postengraftment.²⁴⁷ HMPV also can cause severe disease in individuals with hematologic malignancies and in HSCT recipients,⁶⁰⁹ although mild or asymptomatic infections also can occur. Both HRSV and HMPV can cause severe LRI in lung transplant recipients, and HRSV has also been associated with chronic rejection and the development of bronchiolitis obliterans in these patients.⁶¹² HRSV is the leading viral cause of hospitalization and reduction in pulmonary function for children with cystic fibrosis.^{11,220,261} The relationship between HIV infection and HRSV disease appears to vary by setting. In the United States, prior infection with HIV does not appear

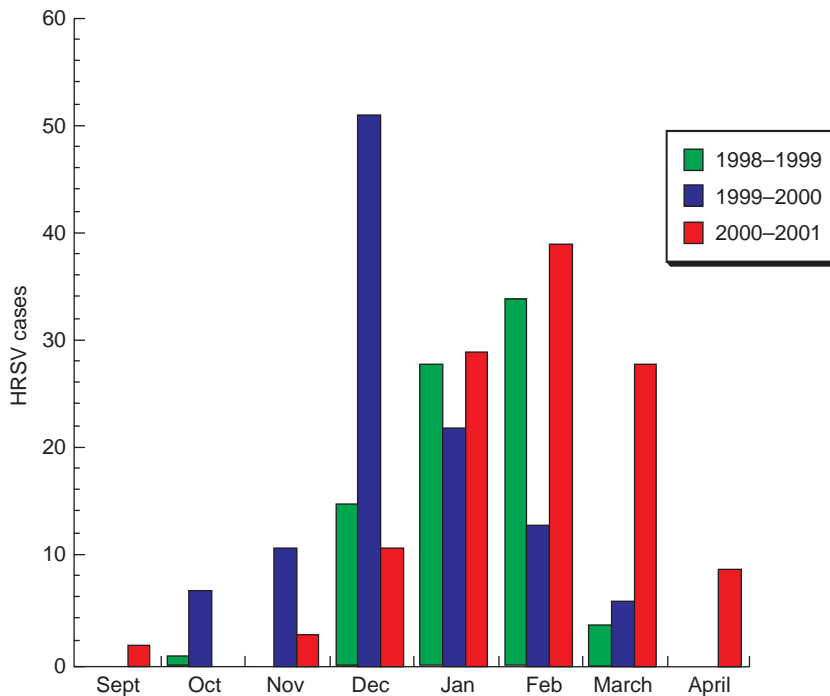


FIGURE 38.11. Variation in human respiratory syncytial virus (HRSV) epidemics during three successive years at the Johns Hopkins Hospital, based on pediatric hospitalizations for HRSV disease.

to increase the morbidity and mortality associated with HRSV infections in children, although prolonged viral shedding has been reported.²⁹⁹ However, in South Africa, HRSV has been reported to cause greater morbidity and mortality in HIV-infected than HIV-uninfected children, although the contribution of other illnesses cannot be excluded.³⁵⁷

Epidemics

In temperate climates, HRSV occurs primarily in yearly epidemics of 4 to 5 months duration in the winter and early spring (Fig. 38.11), although continuous activity also has been reported.²⁴⁰ Elsewhere in the world, the HRSV season varies from location to location.⁶²⁸ Globally, HRSV activity is greatest during periods of moderate humidity and either cool (2°C to 6°C) or warm (24°C to 30°C) temperature.⁶²⁸ HMPV also occurs primarily in annual epidemics during the late winter and early spring in temperate climates, often overlapping in part or in whole with the annual HRSV epidemic.^{1,426} However, long-term studies have shown that sporadic HMPV infection occurs year round in temperate areas.^{426,608}

HRSV is highly infectious and easily spread by contact, especially in settings of close interaction such as day care facilities, hospitals, and families. HRSV is introduced into families primarily by a young school-aged child, after which infection spreads to siblings and adults with high frequency. In one prospective study, approximately 40% of all family members older than 1 year were infected after the introduction of HRSV.²³⁰ HRSV is a major cause of nosocomial infection. The rate of hospital-acquired infection for infants and children during an HRSV season was reported to range from 26% to 47% in newborn units and from 20% to 40% for older children. Hospital staff members appear to play a major role in nosocomial spread of HRSV infection. The likelihood that an individual infant or child will acquire nosocomial HRSV infection increases with the duration of stay and the number of individuals housed in his or her room.²²⁷ Outbreaks in units caring for immunocom-

promised patients can be devastating.³⁷⁰ Outbreaks producing LRI in facilities housing institutionalized adults also have been reported.¹⁷⁷

An evaluation of the HRSV strains circulating in distinct geographic regions during the same years found that five to seven distinct lineages or genotypes representing both antigenic subgroups circulated during the same season in a given location.^{444,445} In a given year, the pattern of genotypes frequently is different in different locations. The pattern of local strains gradually changes in successive years. There typically are one or two dominant local genotypes that are replaced in dominance in successive years. In addition, there can be shifts in the predominance of subgroup A versus B occurring in 1- or 2-year cycles.⁵⁸⁹ This presumably reflects an advantage of the heterologous strain in evading previously induced immunity,⁶⁰² but reinfection by the same subgroup also frequently occurs. Therefore, HRSV epidemics do not involve spread of a predominant new strain, as is the case with influenza A virus. Rather, epidemics appear to involve local endemic strains as well as new genotypes introduced from other regions. The introduction and spread of new strains were illustrated by the appearance in 1998 of a new subgroup B strain called BA that contains a 60-nucleotide duplication in the G gene, and which subsequently disseminated across five continents and underwent further evolution.⁵⁶⁰ The pattern of circulation of HMPV is similar: strains from the two subgroups can co-circulate, and there are several reports of an alternating pattern of dominance by the different subgroups.^{1,426}

CLINICAL FEATURES

In the previously healthy term infant who encounters HRSV for the first time at age 6 weeks to 9 months, HRSV infection usually causes URI, sometimes accompanied by fever. In 25% to 40% of such infections, however, the respiratory tract below the larynx also becomes involved.¹⁹⁷ Bronchiolitis

and pneumonia are the primary manifestations of HRSV and HMPV LRI, with laryngotracheobronchitis (croup) occurring less frequently. In those infants in whom LRI develops, there is a prodromal phase of rhinorrhea often accompanied by a decrease in appetite. Cough may appear simultaneously, but occurs more often after an interval of 1 to 3 days. At that time, there also may be sneezing and a low-grade fever. Soon after the cough has developed, the child may begin to wheeze; if the disease is mild, the symptoms may not progress beyond this stage. Examination usually shows moderate tachypnea, diffuse inspiratory crackles, and expiratory wheezes. There is profuse rhinorrhea, intermittent fever, and frequently otitis media.²⁵¹ In most instances, uneventful recovery occurs after an illness of 7 to 12 days (Fig. 38.5). Among infants and children who require hospitalization with HRSV or HMPV, clinical manifestations range from bronchiolitis or asthma exacerbation to severe pneumonia.^{54,401,435} Cough is present in most, fever in 52% to 86%, and wheezing in about half of cases.⁶⁰⁸ In more severe cases, the coughing and wheezing progress, and the child becomes dyspneic. Hyperexpansion of the chest is evident, and there may be intercostal and subcostal retractions. Infants often feed poorly, because they are obligate nose breathers and nasal obstruction and tachypnea prevent adequate respiration during feedings. Severe tachypnea is common, even in the absence of visible cyanosis. In advanced disease, the child tires and hypoxia becomes more extreme, and then listlessness and respiratory failure occur.

Bacterial sepsis and lobar pneumonia are unusual complications of HRSV²³⁴ or HMPV infection, although simultaneous bacterial–viral co-infection does occur.⁵⁵⁵ Trials of pneumococcal vaccine in infants significantly reduced the incidence of severe HRSV LRI³⁵⁵ and HMPV LRI,³⁵⁶ suggesting that there are bacterial–viral interactions that may not be appreciated clinically. In contrast, bacterial otitis media is a common complication of HRSV and HMPV upper respiratory tract infection. HRSV and HMPV are two of the most common precipitating factors associated with otitis media. Most of this disease is due to eustachian tube dysfunction, resulting in bacterial stasis in the middle ear and subsequent otitis media. HRSV and HMPV antigens and nucleic acids have been reported in middle ear fluids.^{425,490}

Most infants who require hospitalization for HRSV or HMPV infection are hypoxemic on admission; in fact, a low reading on pulse oximeter is often cited as the criterion for admission. This hypoxemia is probably due to an abnormally low ventilation/perfusion ratio. In those with underlying cardiac or respiratory disease, the progression of symptoms may be rapid, especially in those with cyanotic congenital heart disease or chronic lung disease.²¹² In these instances, respiratory failure requiring intubation and ventilation may develop on the second or third day of illness. In children younger than 2 years with cystic fibrosis, the consequence of HRSV infection may be prolonged respiratory morbidity.²⁶¹

In the newborn infant, most HRSV infections produce only URI,²³² probably reflecting the protective effects of maternal HRSV-specific serum antibodies against LRI. Bronchiolitis is rare, and severe infection is more often characterized by lethargy, irritability, and fever or temperature instability than by specific respiratory signs.²³² Symptomatic HMPV infection is rare in newborns. In infants who were born prematurely, and sometimes in normal infants younger than 6 weeks, frequent apneic spells may occur in response to HRSV infection.⁵⁵⁶

Apnea often is seen even in the presence of minimal respiratory signs and may be the predominant symptom bringing the infant to medical attention. Such apneic spells, although often recurrent during the acute infection, are usually self-limited and rarely cause neurologic or systemic damage. Exceptions to this occur, however, and such episodes should be an indication for hospitalization and careful medical supervision with respiratory monitoring.

HRSV has been detected in the lungs of a percentage of children dying suddenly and unexpectedly (sudden infant death syndrome [SIDS]). However, there is no statistically significant association between HRSV and SIDS, and the detection of HRSV in some SIDS cases likely reflects its prevalence as well as the historic concurrence of the peak incidence of both HRSV infection and SIDS in winter months.¹¹⁹ An association between HMPV and SIDS has not been reported.

Abnormalities in pulmonary function are common after HRSV bronchiolitis or pneumonia and may persist for 7 to 10 years or even into adulthood.^{231,280,347,422,509,528} Recurrent wheezing may be common after HRSV bronchiolitis or pneumonia and has been reported in 10% to 50% of former patients,⁸³ but is rarely of great severity. A large case–control study of 200 children hospitalized for bronchiolitis or pneumonia (half of which was due to HRSV) showed that 7 years later there was a significant tendency toward decreased pulmonary function, recurrent cough, wheezing, school absenteeism, asthma, and bronchitis in both of the previously hospitalized groups.³⁹³ Postexercise or pharmacologically induced bronchial lability also is increased, despite an absence of symptoms.⁵¹³ The pulmonary function deficit is partially responsive to bronchodilators, indicating a bronchospastic component.⁸³ Most of these studies did not observe a link between these lingering respiratory abnormalities and allergic sensitization and the development of persistent asthma, but other studies have suggested such a link,^{509,510} and this remains an area of controversy. To date, no such epidemiologic links have been reported for HMPV.

It remains unclear whether infection with HRSV (and possibly HMPV) causes subsequent abnormal pulmonary function, or whether the abnormalities observed existed before infection and indeed may have contributed to disease. For example, Martinez et al. measured pulmonary function in infants at birth or shortly thereafter and found a strong correlation between prior reduced function and the development of bronchiolitis with wheezing on HRSV infection.³⁶⁴ This correlation persisted in children in whom at least one additional lower respiratory infection developed during the first 3 years of life.³⁶⁴ It is likely that respiratory disease and recurrent abnormalities in children are due to multiple factors. These include prior underlying anatomic or functional abnormality that might predispose to initial and recurrent acute bronchiolitis, a tendency to airway hyperreactivity that might predispose to recurrent wheezing, and infection by HRSV or other airway pathogens. Environmental factors such as family smoking may trigger episodes of lower respiratory dysfunction and aggravate long-term or recurrent airway pathology. There is evidence that preventing severe HRSV infections early in life reduces the incidence of recurrent wheezing, suggesting that HRSV can play a causal role.⁵¹² Most pulmonary abnormalities associated with prior respiratory tract infection resolve by 8 to 12 years of age.

Symptomatic HRSV infections are common in normal adults, particularly in medical personnel or in individuals caring

for small children.^{227,230} Such episodes are usually characterized by rhinorrhea, pharyngitis, cough, bronchitis, constitutional symptoms of headache and fatigue, and fever. Disease usually lasts about 5 days but may be more prolonged. In contrast, HMPV infection in adults is often asymptomatic, although the spectrum of clinical illness in symptomatic individuals is similar to HRSV, with hoarseness being particularly prominent in young adults.⁵⁸⁶ In older persons, particularly those with underlying disease, severe LRI may occur with HRSV or HMPV, requiring hospitalization and intensive care. HRSV infection in severely immunocompromised individuals usually starts with URI for 5 or 6 days, with symptoms similar to those of a normal host, but frequently evolves into LRI.

DIAGNOSIS

Differential Diagnosis

The differential diagnosis of HRSV and HMPV infection in young infants must include all other causes of acute LRI. This includes parainfluenza viruses, particularly HPIV3, adenoviruses, influenza viruses, rhinoviruses, coronaviruses, and enteroviruses. In infants younger than 4 months, *Chlamydia trachomatis* also must be considered, which causes afebrile interstitial pneumonia with cough and, in some cases, wheezing. In HIV-infected children, infection with *Pneumocystis carinii* should be considered. Differentiation from bacterial pneumonia may occasionally be difficult, although wheezing is almost never present during infection with pyogenic bacteria, and infants with bacterial pneumonia typically have lobar pneumonia and may have systemic illness associated with bacteremia. In older infants, infections with HRSV or HMPV must be differentiated from other causes of mild respiratory tract infection as well as causes of acute bronchospastic disease, including environmental allergens and aspiration of a foreign body.

In the elderly, HRSV or HMPV infection should be suspected in any patient (with or without underlying cardiopulmonary disease) who has acute bronchitis, pneumonia, or wheezing accompanied with or without a low-grade fever, particularly when such an event occurs as part of a cluster of cases in the winter season.

Presumptive diagnosis of HRSV infection in infants can often be made on the basis of the clinical syndrome combined with the time of year and other epidemiologic features. It is common to observe that other members of the family have respiratory illness at the time of, or just preceding, an episode of HRSV bronchiolitis or pneumonia in an infant. Usually the infant has far more serious illness than other members of the family.²³⁰

Radiographic findings for HRSV and HMPV are common but relatively nonspecific. The radiologic findings on chest radiographs of HRSV and HMPV pneumonia are similar to those of bronchiolitis and reactive airways disease and include hyperaeration, diffuse prominent lung markings due to bronchial wall thickening, and focal areas of atelectasis.

Laboratory Diagnosis

Definitive diagnosis of HRSV and HMPV depends on laboratory tests. Rapid detection of HRSV or HMPV is desirable to guide the use of appropriate infection-control measures and to potentially limit unnecessary antibiotic use. In practice, however, while diagnostic testing is typically performed in aca-

demie centers, it may be used less frequently in community hospitals even in wealthy countries. Few hospitals in resource-limited settings have the capacity for such testing.

The gold standard is isolation of virus in cell culture from nasal swabs, nasal washes, or nasopharyngeal aspirates; however, the lability of HRSV, the slow growth characteristics of HMPV, the expense of the technique, and the increasing availability of assays that rely on nucleic acid detection have made cell culture an increasingly infrequent choice, even for diagnostic virology laboratories in academic centers.

More rapid diagnosis can be made by the detection of viral antigen in nasal swabs or washes. In the direct immunofluorescence assay (DFA), exfoliated cells and other debris are concentrated from secretions by centrifugation, reacted with virus-specific fluorescence-labeled antibodies, and visualized by microscopy. This has the advantage that a knowledgeable technician can confirm that the signal has the pattern expected for infected cells, but the method is time-consuming and requires expertise and a fluorescence microscope. Alternatively, antigen in secretions can be detected by antigen-capture enzyme-linked immunoassay (EIA), in which the sample is incubated with immobilized virus-specific antibodies to capture viral antigen, which is then detected with a second, enzyme-linked antibody. Both DFA and EIA for HRSV are commercially available (with the former being more sensitive than the latter), and DFA is commercially available for HMPV. Another HRSV rapid antigen test available commercially is based on lateral flow immunochromatography.^{194,337} The test sample of secretions is solubilized, mixed with a colored gold-labeled HRSV MAb, and applied to a test membrane on which antibody-antigen complexes migrate laterally and are captured by a line of immobilized HRSV-specific antibody to produce a visible band. The sensitivity of these rapid commercial tests, which produce results in less than 30 minutes, generally is lower than that of DFA, and negative results should be confirmed by DFA. In addition, antigen-based tests in general often have reduced sensitivity in adults due to low levels of viral shedding.¹⁵⁹

RT-PCR of viral RNA for detection of HRSV or HMPV in nasal swabs, nasopharyngeal aspirates, or nasal washes is a useful method of diagnosis for both pediatric and adult populations, because the sensitivity of these assays allows detection of even small quantities of virus. RT-PCR can be used in multiplex formats capable of detecting multiple pathogens. HRSV and HMPV RT-PCR multiplex assays are commercially available, although the procedure is somewhat time-consuming (3 to 6 hours) and requires technical expertise and specialized equipment.^{337,360} RT-PCR is more sensitive than virus culture or antigen assay.²⁵⁷

Serologic methods are used infrequently and provide only retrospective information, since antibody rises are detected by analysis of sera collected 2 to 4 weeks postinfection. Typically, serum antibodies are detected by ELISA or microneutralization assay, with fourfold or greater increases considered indicative of infection.

TREATMENT AND PREVENTION

Treatment

Most infants and children with mild HRSV LRI can be managed as outpatients. Treatment of more severe LRI can be

guided by measurement of oxygenation, monitoring respiratory rate and the trend in respiratory rate, and evaluation of dehydration. Severe respiratory distress, hypoxia, and dehydration are among the indications for hospitalization.

The American Academy of Pediatrics (AAP) has issued evidence-based recommendations for the diagnosis and management of bronchiolitis,⁷ most cases of which can likely be attributed to HRSV or HMPV. Where appropriate, AAP recommendations are cited below.

Symptomatic Interventions and Supportive Care

Inpatient treatment of HRSV infection requires considerable supportive care: mechanical removal of secretions, proper positioning of the infant, administration of humidified oxygen, intravenous fluids, and, in the most severe cases, respiratory assistance with mechanical ventilation. Antibiotic therapy does not improve the outcome in viral pneumonia, does not alter the risk of bacterial complication of viral pneumonia as a superinfection, and should not be used unless there are indications that a bacterial co-infection is present.⁷ The youngest infants infected with HRSV may require monitoring for apnea. Improvements in supportive care clearly have had a major impact on mortality from severe HRSV disease, resulting in the current overall low mortality rate in high-resource settings. Current AAP guidelines advocate the use of supplemental oxygen if the oxyhemoglobin saturation (SpO₂) falls below 90%.⁷ Of all the potential therapeutic modalities described here for severe HRSV disease, supplemental oxygen may be the most accessible for infants in resource-limited settings.⁵⁵²

Corticosteroids have been used in therapy for HRSV bronchiolitis since the 1960s, based in part on their effectiveness in the treatment of acute asthma and on the premise that much of HRSV disease is immune-mediated. However, a meta-analysis of placebo-controlled studies indicated that systemic glucocorticoids on their own provided no improvement in oxygenation, respiratory rate, or length of hospitalization,⁴³⁹ and a large controlled study indicated that inhaled glucocorticoids provided no improvement in wheezing following HRSV bronchiolitis.¹⁵⁴ Current AAP guidance suggests that corticosteroids should not be routinely used in the management of bronchiolitis.⁷

Other antiinflammatory approaches for the treatment of HRSV have also been considered. Acute HRSV bronchiolitis is associated with elevations of cysteinyl leukotrienes in respiratory secretions¹²³; however, the leukotriene receptor antagonist montelukast was not shown to be effective in ameliorating acute bronchiolitis.⁸ Assessment of the utility of montelukast for prevention of post-HRSV wheezing episodes has yielded conflicting results^{49,295,465} and further studies may be warranted.

Drugs used to treat reversible airway smooth muscle constriction in asthma have also been used to treat HRSV infection. Hospitalized infants in the United States frequently are treated with inhaled bronchodilators, but systematic reviews indicate that they provide only modest short-term improvement.^{182,331} This suggests that, in some infants, increased airway resistance is not predominantly due to reversible smooth muscle constriction, but rather is a consequence of obstruction of small airways with mucus, immune cells, and sloughed epithelial cells. Current AAP recommendations are that trial of a bronchodilator may be considered, but that inhaled bron-

chodilators should be used only if there is a demonstrated objective benefit in an individual child.⁷

Antiviral Interventions

Ribavirin, a nucleoside analog, is a broad spectrum antiviral compound the mode of action of which remains unclear,⁵⁰⁶ but which exhibits potent activity against HRSV and HMPV in cell culture and experimental animals.⁶²¹ Ribavirin was approved in 1986 in the United States for use in the treatment of HRSV infection.⁵⁷⁹ A recent review stated that some improvement in subjectively or objectively measured outcome was observed in 7 of 11 published randomized clinical trials of ribavirin, although the size and quality of the trials were highly variable.³⁰⁰ In addition, the drug is difficult to administer via aerosol, can cause anemia, and concerns have been raised about health risks for caregivers.⁴⁷⁷ Currently, the AAP recommends that ribavirin not be used routinely for the management of bronchiolitis, although it could be considered for use in children at risk with severe disease (e.g., those who are immunocompromised or who have "hemodynamically significant cardiopulmonary disease").⁷ Ribavirin has been used empirically for the management of HRSV LRI in HSCT or lung transplant patients,⁵² and was administered intravenously to a lung transplant patient with HMPV.⁴⁷⁰

HRSV disease in young infants can be reduced by prophylaxis with HRSV-neutralizing antibodies (see *Prevention*). Therefore, these antibodies also have been evaluated for therapy of established infection. The most recent studies have utilized the HRSV F MAb palivizumab and the more potent motavizumab administered intravenously.^{320,359} In these studies, palivizumab and motavizumab reduced viral shedding by 10-fold, but a clear effect on clinical outcome has not been demonstrated.²⁶⁵ It is possible that these small studies were not sufficiently powered to observe differences in clinical outcome; alternatively, it may be that much of the LRI observed by the time infants reach the hospital (usually several days into their illnesses) is immune mediated and will not be ameliorated by antiviral treatment. However, antibodies sometimes are used empirically for treating severely immunocompromised individuals, in whom infection can be prolonged,^{164,322} although definite evidence of efficacy is lacking.

Small molecule antiviral drugs specific for HRSV have been developed that target the F, G, N, or L proteins.^{153,343,430,506,530} Four different small molecule inhibitors are undergoing clinical trials for HRSV (www.clinicaltrials.gov, accessed June, 2011). A drug specific to the N protein (AA-60444, previously RSV604)⁸⁸ was evaluated in 2006 in a phase 2 clinical trial in HRSV-infected adult HSCT recipients (clinicaltrials.gov NCT00232635), although the results have not been published. An siRNA that targets the N gene (ALN-RSV01) was evaluated in a phase 2 challenge study in 85 healthy adults (clinicaltrials.gov NCT00496821).¹³⁷ The drug was administered daily by nasal spray for 2 days before and 3 days after HRSV challenge. This resulted in a 38% decrease in the number of individuals infected, and modest but insignificant decreases in viral load in those who were infected.¹³⁷ It will be important to evaluate efficacy in a situation where the drug is administered after, rather than beginning before, infection. This drug also was evaluated in a phase 2 trial of 24 lung transplant recipients with HRSV infection, in which three daily doses were associated with reduced symptom scores and significantly reduced

incidence of bronchiolitis obliterans syndrome.⁶³⁰ An orally administered inhibitor specific to the F protein (BTA9881) was recently evaluated for safety and tolerability (clinicaltrials.govNCT00504907). Reports in the lay press indicated that its safety was unsatisfactory and that further preclinical development is planned. A phase 1 study is planned for 2011 to assess safety, tolerability, and pharmacokinetics for another F protein-specific inhibitor (MDT-637, previously VP14637)^{145,622} that is delivered by a dry powder inhaler (clinicaltrials.govNCT01355016). Small molecule inhibitors are under preclinical development for HMPV.

Other antisense strategies also have been explored against HRSV. Conventional antisense oligonucleotides probably have little potential when administered on their own, but they also have been chemically linked to the 2-5A molecule of the host IFN system.³²⁶ This serves to recruit the cellular antiviral nuclease RNase L to cleave the hybridized RNA. A 2-5A-antisense molecule that targets the conserved GS signals in the viral genome reduced nasal HRSV replication up to 10,000-fold in AGMs when the drug was administered intranasally shortly after infection and during the following 5 days,³²⁶ but further development has not been reported.

Combination therapies also are being developed, involving either the combination of two different antiviral drugs to achieve a higher level of inhibition of viral replication, or the combination of one or more antiviral drugs with an anti-inflammatory drug to restrict replication and reduce immune-mediated disease. This latter combination strategy effectively resolved viral replication and disease in the cotton rat model.⁴⁶³ Similarly, early and aggressive combined therapy involving ribavirin, corticosteroids, and antibodies is being explored for severely immunocompromised adults.^{342,601} In the more usual setting of acute HRSV infection of otherwise healthy individuals, antiviral therapy of a rapidly progressing infection such as HRSV is challenging because by the time the viral agent is identified, it may be too late to control disease solely by inhibiting virus replication.

Prevention Infection Control

Infection by HRSV (and HMPV) can be reduced by hand washing, limiting exposure to infected individuals, and avoiding self-inoculation of nasal and conjunctival mucosa. Nosocomial spread of HRSV has been shown to be reduced by the use of gloves and gowns by caregivers, strict observance of hand washing, active surveillance for HRSV infection, limiting visitors during the HRSV season, and cohorting of infected patients and caregivers.^{223,327,354,416,520}

Passive Immunoprophylaxis Against HRSV

Based on experimental data in animals^{461,462,588} and epidemiologic data in humans¹⁹⁷ indicating that HRSV-neutralizing serum antibodies protected against HRSV LRI, products containing high titers of HRSV-neutralizing antibodies were developed for clinical administration. These products do not necessarily prevent HRSV infection completely, but can proactively restrict replication sufficient to reduce disease. The first product, RSV Immune Globulin Intravenous (RSV-IGIV; RespiGamTM, MedImmune), consisted of immunoglobulin purified from human donor sera that had been screened for high HRSV-neutralizing activity.

It was licensed in 1996 for use in infants and young children at high risk for severe HRSV disease due to prematurity or underlying disease. Administered in monthly intravenous infusions during the HRSV season, RSV-IGIV reduced the frequency of hospitalization for HRSV disease by 55% or more and reduced days spent in intensive care by 97%.^{214,471} However, it had the disadvantages of involving an intravenous infusion, the theoretical risk of adventitious agents, and potential interference with live pediatric vaccines due to the presence of antibodies against common pathogens. RSV-IGIV (RespiGamTM) has been superseded by the development of the MAb palivizumab (see below) and has not been commercially available since 2004.

Palivizumab (SynagisTM; MedImmune) is an HRSV-neutralizing MAb directed against the F glycoprotein that was licensed in 1998. Palivizumab is based on a murine MAb³² that was “humanized” by recombinantly transferring its complementarity-determining regions (~5% of the molecule) onto a human IgG1 backbone (95% of the molecule). Palivizumab is 50- to 100-fold more effective on a weight basis than RSV-IGIV, and accordingly is administered in a much smaller volume by monthly intramuscular injection. Its clinical efficacy is similar to that of RSV-IGIV.⁴³⁴ In infants with cyanotic heart disease, RSV-IGIV was associated with an increased incidence of adverse events²¹⁴—likely due to its large volume and high protein content—but a phase III study demonstrated that palivizumab was safe and effective in preventing hospitalizations for HRSV in children with congenital heart disease.¹⁷⁶ Currently the AAP recommends that high-risk infants receive prophylaxis with palivizumab during the HRSV season. Phase 1 trials also showed that palivizumab was well tolerated based on evaluation in a small number of HSCT recipients.⁵³ There is continued discussion of the cost-effectiveness of palivizumab due to its expense,⁴⁵⁶ but antibodies remain the only effective prophylaxis available for HRSV disease other than infection control. The emergence of antibody-resistant mutants in association with the use of palivizumab has not been a significant problem: they are detected in ~5% of treated individuals from whom HRSV was recovered and appear to have a modest reduction in growth fitness *in vitro*.⁶³⁵

More recently, palivizumab was modified by *in vitro* affinity maturation to create a more potent derivative called motavizumab (MEDI-524 or Numax, MedImmune). Compared to palivizumab, motavizumab differs at 13 amino acid positions. It exhibits a 70-fold increase in antigen binding and a 20-fold increase in neutralization activity *in vitro*, and is substantially more protective in cotton rats and also protects the upper respiratory tract.^{382,620} In a clinical study comparing palivizumab and motavizumab in a group of 6635 preterm infants, infants who received motavizumab had 26% and 55% reductions in HRSV hospitalization and medically attended HRSV LRI (MALRI), respectively, relative to palivizumab.^{77,193} Motavizumab also was evaluated in a placebo-controlled trial in healthy Native American populations (clinicaltrials.govNCT00121108). In this study of more than 2,000 infants, motavizumab was found to reduce HRSV hospitalizations by 83% and outpatient MALRIs by 71%. However, this product was associated with a slight increase in the incidence of hypersensitivity reactions and antidrug antibodies in clinical trials, and its further development for HRSV prophylaxis has been suspended as of this writing. Motavizumab also was evaluated

for the treatment of infants hospitalized for HRSV disease (clinicaltrials.gov/NCT00421304). Prior to the problematic clinical data, motavizumab had been engineered to increase its serum half-life by increasing its affinity for a receptor involved in recycling and sparing antibodies that have been taken up intracellularly for degradation.¹²⁵ This increased the antibody's serum half-life in cynomolgus monkeys fourfold, but it seems unlikely that this derivative will be developed further. Other approaches have sought to develop fully human HRSV-neutralizing MABs using recombinatorial phage display libraries,^{23,397} or from libraries of transformed B cells.⁹³ This latter study is noteworthy because it was able to produce G-specific MABs with high neutralizing activities.

Vaccines

The need for an HRSV vaccine is greatest for infants and the elderly, although a vaccine for toddlers and preschool children might have a substantial impact on emergency room and primary care visits for respiratory illness.^{236,329} Immunization with a pediatric HRSV vaccine ideally should be initiated during the first weeks of life. Because the reduced immune responses characteristic of infancy likely will reduce vaccine immunogenicity and efficacy, multiple vaccine doses will likely be required.

Development of a pediatric HRSV vaccine has been complicated by the phenomenon of enhanced disease that occurred in association with a formalin-inactivated HRSV (FI-RSV) vaccine evaluated in infants and children in the 1960s.^{287,458} This vaccine consisted of concentrated, formalin-inactivated virus that was mixed with alum adjuvant and administered intramuscularly. Immunization with FI-RSV was well-tolerated but proved to be poorly protective. Unexpectedly, upon subsequent natural infection, vaccinees experienced much greater frequency and severity of HRSV disease: 80% of FI-RSV vaccinees required hospitalization compared to 5% in the control group, and two toddlers (ages 14 and 16 months) died of enhanced RSV disease. Autopsies of the two fatalities provided evidence of HRSV replication and pulmonary inflammation.⁴⁵⁸

Subsequent studies, which had the advantage of improved methods and the development of experimental animals models, showed that the immune response to FI-RSV differed markedly from that to HRSV infection. The serum antibodies induced by FI-RSV in the original vaccinees or in experimental animals bound efficiently to HRSV antigen in ELISA but were inefficient in neutralizing infectivity.⁴¹⁵ Studies in experimental animals showed that FI-RSV did not induce a significant CTL response, reflecting its noninfectious nature. Peripheral blood lymphocytes from the original vaccinees exhibited an exaggerated proliferative response to HRSV antigen *in vitro*, suggestive of a heightened CD4⁺ T-cell response to FI-RSV compared to natural infection.²⁹⁸ Consistent with this, subsequent studies in experimental animals showed that FI-RSV induces a disproportionately increased stimulation of the Th2 subset of CD4⁺ T cells compared with HRSV infection.^{109,130,591} These findings indicated that FI-RSV primed for an aberrant, pathologic secondary immune response that occurred upon subsequent infection and viral antigen expression.

The poor neutralizing activity of the serum antibodies induced by FI-RSV likely was a major factor in the lack of protective efficacy, and probably reflects denaturation of neutralization epitopes in the vaccine. It also may reflect deficient antibody affinity maturation due to poor TLR stimulation

by the nonreplicating vaccine.¹³⁵ The formation of antibody-antigen complexes and complement fixation also appears to have contributed to disease, a consequence of the induction of antibodies that did not restrict infection but bound efficiently to antigen.³⁸³ The Th2-biased response also played a key role, demonstrated by the finding that disease enhancement was not observed when FI-RSV-immunized rodents were treated with antibodies to deplete either CD4⁺ T cells or selected Th2 cytokines prior to HRSV challenge.¹¹⁰ The bias toward Th2 appeared to be favored by the lack of stimulation of NK cells and CD8⁺ CTLs, which otherwise downregulate Th2 responses to HRSV antigens, at least in the mouse model.^{267,429} and may also have other immunomodulatory effects.^{429,529} The Th2-bias may also have been enhanced by the presence in FI-RSV of carbonyl groups arising from the formalin treatment.³⁹¹ Therefore, the major elements of disease enhancement include an inadequate protective response, an altered antibody response, and a heightened Th2 response. Similar phenomena of vaccine-related disease enhancement have been described in the clinical use of formalin-inactivated measles virus vaccine⁴⁵² and in experimental animals receiving formalin-inactivated HPIV3⁴³² and HMPV^{131,624} vaccines, and thus does not appear to be specific to HRSV or to *Pneumovirinae*.

Disease enhancement has also been observed in experimental animals that have been immunized with purified HRSV F and G glycoproteins as experimental vaccines.⁴¹⁴ This cautionary finding needs to be strongly considered if nonreplicating HRSV vaccines are developed as potential immunogens for HRSV-naïve infants and children. In contrast, disease enhancement is not observed with natural HRSV infection and reinfection, or in most cases with viral or DNA vectors expressing HRSV antigens. This distinction between killed-virus/subunit HRSV vaccines versus live/vectored HRSV vaccines likely reflects the greater efficiency of the latter in broadly stimulating innate and adaptive immunity, including stimulation of TLRs, NK cells, and CD8⁺ CTLs important in regulating HRSV-specific immune responses. It also is noteworthy that priming for enhanced disease appears to be limited to HRSV-naïve infants and children and has not been observed when nonlive vaccines are used in HRSV-experienced individuals. This has been demonstrated in experimental animals⁵⁹⁰ and in clinical studies described below. Therefore, although a subunit vaccine is contraindicated for RSV-naïve recipients, it could be safely used to boost immunity in older children and adults.

Because of disease enhancement, efforts in recent years to develop an HRSV vaccine for HRSV-naïve infants and young children have focused on live attenuated vaccines. The absence of disease enhancement by live HRSV vaccines has been confirmed in clinical trials in HRSV-naïve infants.⁶¹⁸ Studies in experimental animals including chimpanzees showed that the intranasal route of administration is surprisingly immunogenic and highly protective. It also partially avoids the immunosuppressive effects of serum antibodies.¹¹⁶ Beginning in the 1960s, a series of live attenuated HRSV strains was developed by classic biological methods, notably multiple passages at increasingly low, suboptimal temperature¹⁸⁰ followed by chemical mutagenesis and selection for temperature-sensitive mutants.¹¹⁵ None of the viruses produced by these methods had a satisfactory level of attenuation in 1- to 2-month-old HRSV-naïve infants, although one virus (called *cpts248/404*) was well-tolerated and

immunogenic in an older age group, namely seronegative infants and children 6 months of age or older.⁶¹⁷ Further efforts to obtain satisfactory derivatives by biological means were unsuccessful, and all subsequent vaccine candidates have been made by reverse genetics.

Reverse genetics provides a number of advantages for developing live vaccines, including the means of identifying attenuating mutations in existing viruses,^{104,604} developing new mutations or strategies for attenuation,^{28,41,68,420,549,603} producing viruses with short, well-defined passage histories important for safety,⁵³⁵ increasing genetic stability,³⁶⁹ introducing mutations in desired combinations to incrementally increase attenuation,⁶⁰⁵ and other possibilities such as rearranging the gene order to increase the expression of protective antigens.³⁰⁷

Several attenuated HRSV strains produced by reverse genetics have been evaluated in clinical trials.^{290,619} The lead candidate, a virus called rA2cp248/404/1030ΔSH (e-Fig. 38.10), was well-tolerated in 1- to 2-month-old infants and was moderately immunogenic and highly protective against a second vaccine dose.²⁹⁰ This virus contains a series of point mutations and deletion of the SH gene, is strongly temperature sensitive, and does not form plaques at temperatures higher than 35°C. This degree of temperature sensitivity would preferentially restrict replication in the warmer lower respiratory tract. This virus presently is in phase 1/2 clinical trials. Other attenuated HRSV strains also are in development. One example is the ΔM2-2 virus, which lacks most of the coding sequence for the M2-2 protein. This virus exhibits reduced RNA replication and increased gene transcription and antigen production, which may provide for increased immunogenicity.⁴¹

Another type of live attenuated HRSV vaccine recently evaluated in clinical trials (clinicaltrials.gov NCT00686075, NCT00686075 NCT00493285) uses an attenuated chimera of bovine PIV3, in which the F and HN genes are derived from HPIV3, as a vector to express the HRSV F and/or G proteins from one or two added genes^{495,540} (e-Fig. 38.10). This provides a bivalent intranasal vaccine against these two important pediatric pathogens. Importantly, BPIV3 and HPIV3 replicate more efficiently and are more stable physically than HRSV. This might facilitate vaccine manufacture and use. However, recent data indicate that the antibody response to RSV F was inferior to the antibody response to HPIV3,^{41a} perhaps resulting from sequence variation in the RSV F transgene (unpublished observations). An analogous approach is being pursued using Sendai virus as vector.²⁸¹ Sendai virus is a murine relative of HPIV1 that has sufficient antigenic cross-reactivity that intranasal immunization protects AGMs against HPIV1 challenge. Sendai virus was well-tolerated in adults and has been engineered to express HRSV antigens as a potential bivalent vaccine against HPIV1 and HRSV.²⁸¹ Other vectored approaches, such as using replication-defective adenoviruses or alphaviruses, also may have promise for pediatric use.

There also is a need for an HRSV vaccine in older children and the elderly, especially for individuals at increased risk due to underlying disease or old age. Live vaccines appear to be too restricted in replication in HRSV-experienced older individuals due to existing immunity.¹⁹⁹ Vectors might not be suitable for repeated vaccinations due to the development of vector-neutralizing antibodies. However, subunit protein vaccines could be suitable, since they do not prime for enhanced disease in RSV-experienced individuals. In addition, prior immunity

can be partially overcome by increased dose.⁴¹¹ A number of HRSV-subunit vaccines have been evaluated in clinical trials. A series of preparations of purified F protein (PFP) isolated from HRSV-infected cells has been evaluated in healthy adults, in children older than 12 months of age with and without underlying chronic pulmonary disease (chronic lung disease of prematurity or cystic fibrosis), in institutionalized and ambulatory elderly subjects, and in pregnant women (immunization during pregnancy had the goal of increasing the titer of HRSV-neutralizing maternal antibodies in the newborn).^{34,167,168,213,405,436,450,563} The PFP vaccines were well tolerated in these populations: acute reactions were minimal, and enhanced disease was not observed. However, HRSV-neutralizing antibody responses were unsatisfactorily low. More recently, a vaccine based on the HRSV F, G, and M proteins purified from HRSV-infected cells was evaluated in an elderly population in conjunction with the inactivated seasonal influenza virus vaccine.¹⁶⁹ This vaccine was moderately immunogenic with or without an alum adjuvant, but is no longer in clinical development.

Another experimental protein-based vaccine, called BBG2Na, consists of a bacterially expressed fragment containing the central conserved domain of the HRSV G protein fused to the albumin-binding domain of the streptococcal G protein. However, in HRSV-naïve infant macaques it was associated with a low level of pulmonary eosinophilia in some animals upon challenge,¹³² and in clinical trials in adults it induced low levels of HRSV-neutralizing antibodies,⁴⁵⁴ with hypersensitivity observed in some vaccine recipients.

Increasing knowledge of the structure and antigenic properties of the HRSV F protein and improved methods of expression and purification may allow for the production of more stable and more immunogenic HRSV F vaccine preparations. As of this writing, the only HRSV subunit vaccine currently being evaluated in clinical trials is an F protein particle vaccine developed by Novavax (clinicaltrials.gov NCT01290419), which is currently undergoing phase 1 evaluation in healthy adults. In other recent work, the HRSV F protein was engineered to remove the fusion peptide, transmembrane region, and cytoplasmic tail, yielding an expressed protein that formed a postfusion trimeric structure that was homogeneous, stable, and highly immunogenic.^{378,536}

Numerous additional approaches to an HRSV vaccine continue to be evaluated in preclinical studies, including synthetic peptides, engineered multiepitope vaccines, antigen expressed by recombinant baculoviruses or produced in plants, antigen fused to carrier proteins such as cholera toxin B subunit, immune-stimulating complexes containing HRSV antigens, liposome-encapsidated HRSV antigen, the use of adjuvants such as CpG oligonucleotides or biopolymer nanoparticles, and vectors including vesicular stomatitis virus, vaccinia virus (Modified Vaccinia Ankara), rhinovirus, Newcastle disease virus, *Mycobacterium bovis* BCG (Bacillus Calmette-Guerin), and *Salmonella typhimurium*. Whether any of these offer advantages remains to be seen. Realistic evaluation of candidate vaccines for HRSV (and HMPV) is complicated by the semipermissive nature of infection of convenient experimental rodent models, which thus gives overly optimistic appraisals of efficacy.

Recombinant HMPVs that lack the M2-2 ORF or G gene have been developed by reverse genetics. These viruses were highly attenuated in rodents and AGMs while retaining a satisfactory level of immunogenicity.^{44,48,66} The ΔM2-2 and

ΔG viruses have levels of attenuation and immunogenicity suitable to be evaluated clinically as candidate live intranasal vaccines. Another approach has been to replace individual genes of HMPV with their AMPV counterparts to create chimeric viruses that are attenuated in primates due to host incompatibility effects of the AMPV gene. A chimera in which the HMPV P gene was replaced by its AMPV counterpart replicated efficiently *in vitro* and appeared to have a satisfactory level of attenuation in AGMs,⁴⁴⁷ and presently is being evaluated in phase 1 clinical trials for safety and immunogenicity (clinicaltrials.govNCT01255410). An attenuated HPIV3 vaccine virus also has been used as a vector to express the F protein of HMPV as a bivalent HPIV3-HMPV vaccine in preclinical studies.^{515,539}

PERSPECTIVE

HRSV is one of the more complex members of *Mononegavirales*. We have general information on many of the roles of the viral proteins and RNAs in the viral replicative cycle, and have some information on how they affect the host cell and host immune system. Continued research is needed to identify and elucidate these mechanisms and their significance to viral biology and the host response.

HRSV is one of the most common and widespread human viruses. HRSV is notable for a historic and tragic vaccine failure, namely the formalin-inactivated vaccine. It also is notable for the development of the successful strategy of antibody immunoprophylaxis against HRSV, which contributed to widespread interest in the development of products based on MAbs. Although the importance of HRSV has been known for more than 50 years, we lack effective vaccines or antiviral therapeutic drugs. There is a need for both, but developing these products for young infants is challenging. A substantial reduction in serious HRSV disease would be a major advance for human health. Vaccines may be improved by new adjuvants, and antiviral drugs may be improved by combination with antiinflammatory therapy.

HRSV is unusual in its ability to efficiently infect and cause disease in early infancy even in the presence of maternal antibodies. The consequences of severe HRSV infection in the context of the immature lung and immature immune system are poorly understood. Severe HRSV disease early in life frequently is associated with lingering abnormalities in pulmonary function, although the causal relationship is uncertain. It also has been speculated that early infection in life can result in long-term deficiencies in the immune response to this virus in some individuals. Further studies of infants who receive passive or (when available) active immunoprophylaxis against HRSV may help to resolve these issues. The relative contributions of direct viral damage and immune factors to HRSV disease remain unclear and likely will vary among individuals, due in part to genetic differences that are being identified. HRSV can reinfect symptomatically throughout life in the absence of significant antigenic change. The extent to which this reflects virus-mediated inhibition or subversion of protective responses remains controversial.

HMPV is a close relative to HRSV that infects somewhat less early in life and causes severe disease less frequently. Many of

the questions regarding disease mechanisms, immune response, and long-term consequences of HRSV infection early in life also apply to HMPV. Research into these important causes of severe LRI in infants and the elderly should yield new insights into pathogenesis and host responses at both extremes of age.

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Bornaviridae

History

The Virus

- Taxonomy
- Morphology and Physical Characteristics
- Genome
- Genetic Diversity
- Proteins
- Cycle of Infection

Epidemiology of BDV Infection

- Host Range
- Geographic Distribution and Potential Reservoir
- Prevalence and Seroepidemiology
- Route of Infection and Transmission

Epidemiology of ABV Infection

Human Infection

BDV Neuropathogenesis

- Experimental Infection of Animals with BDV
- Tissue Culture Models

Natural Infection with BDV

- Clinical Signs
- Pathogenesis

Natural Infection with ABV

- Clinical Signs
- Pathogenesis

Diagnosis

- Differential Diagnoses
- Intra Vitam Diagnosis
- Postmortem Diagnosis

Therapy and Control

- Vaccination
- Therapeutics

Perspectives and Public Health Considerations

1894–1896 in the district around the town of Borna in Saxony, Germany.³⁵⁸ The name contains the “classifying” letters “RNA,” a classification that was only justified at the end of the 20th century, when the etiologic agent Borna disease virus (BDV) was identified as an RNA virus. BD predominantly affects horses and sheep but other Equidae, certain farm and zoo animals, or companion animals are occasionally also diagnosed with natural BD (reviewed in^{67,113,253,262,310}).

During the first decades of the 20th century, studies of BD focused primarily on defining the etiology, pathology, and pathophysiology of the disease. Initial evidence for a viral etiology was presented by Zwick³⁵⁸ by reproducing the disease with bacteria-free filtrates of brain homogenates from affected horses. Histopathologic studies demonstrated a nonpurulent encephalomyelitis characterized by massive lymphohistocytic infiltrates affecting the gray, and to a lesser extent, the white matter of the central nervous system (CNS), reactive astrogliosis, and intranuclear eosinophilic, so-called “Joest-Degen,” inclusion bodies (described in detail in^{88,141,142,294}). Pathologic changes were preferentially localized in the limbic system, most likely resulting in the observed behavioral disturbances.^{135,262} Detailed studies have been performed on the spectrum of susceptible host species and on the manifestations of the infection. Transmission experiments between naturally infected horses and sheep as well as other host species—including rabbits, guinea pigs, rats, chickens, and monkeys—established the infectious nature of the BD agent and confirmed that the same agent afflicted horses and sheep.^{113,181,205,223,262,358,359}

Interest in BD and its causative virus lapsed until the late 1970s/early 1980s, when the optimization of tissue and cell-culture techniques for propagating the agent paved the way for work on the identification of the agent and on mechanisms of its pathogenesis in rabbit, rat, and tree shrew models.^{177,191,203,308} Milestones in pathogenesis included the adaptation to Lewis and Wistar rats, the demonstration of an age-dependent outcome in experimentally infected rats,^{128,203,206} and the recognition of T-cell–dependent immunopathology.^{128,203,204,257,258,313,318} Narayan’s observations of a biphasic disease in adult-infected rats characterized by initial hyperactivity and followed by hypoactivity prompted efforts to determine whether humans were infected with a related agent. Serologic findings suggestive of a potential role for BDV in affective disorders²⁶³ intrigued many investigators (including the authors of this chapter), resulting in new efforts to identify the causative agent and explore the pathobiology and epidemiology/epizootiology of BDV infection.

However, the causative agent remained elusive until the late 1990s, when BDV was isolated and classified as a negative, single-stranded, nonsegmented RNA virus, the first

HISTORY

The syndrome we know as Borna disease (BD) was first described in European veterinary textbooks in the 1700s^{331,358} as a disease of farm horses using various names like Hitzige Kopfkrankheit (German; “hot-headed disease”) or Seuchenhafte Gehirn-Rückenmarksentzündung (German; “epidemic encephalomyelitis”). The contemporary name Borna disease was coined after the occurrence of major outbreaks in the years

member of the new family *Bornaviridae* in the order *Mononegavirales*.^{27,44,51,176,258,277,281,326} With the advent of reverse genetics systems to produce infectious cDNA clones, detailed molecular analyses of the BDV genome and its gene products, regulation of their expression, and detailed pathogenicity studies using constructs stably expressing fluorescent proteins became possible.^{1,52,220,282,286,353}

In the first decade of the 21st century, a novel BDV, now designated avian bornavirus (ABV), was identified in parrots with proventricular dilatation disease (PDD).^{131,152} PDD is a progressive, variably contagious and often fatal disease of domesticated and wild psittacine birds worldwide. Typical clinical signs such as gastrointestinal (GI) dysfunction and associated wasting with or without neurologic symptoms are caused by nonpurulent inflammation of the enteric, autonomic, and central nervous system (CNS). A viral etiology for PDD has been assumed for over 40 years; recent work provides evidence for the etiologic role of ABV in the development of clinically manifest PDD. ABV has been detected worldwide in many captive parrots but also in other nonpsittacine species.^{55,112,132,217,343}

Until recently, it has been believed that no endogenous nonretroviral viruses exist in animal genomes. Surprisingly, endogenous elements homologous to BDV genes were detected in the genomes of bats, elephants, fish, lemurs, rodents, squirrels, primates, and humans.^{11,133} Although phylogenetic analyses indicate that bornaviruses infected primates at least 40 million years ago, there is only controversial data to support current infection of humans. Indeed, a recently published multicenter study used a wide range of molecular and serologic methods to analyze well characterized samples from subjects with schizophrenia and affective disorders, finding no evidence for human infection with a virus similar to either of the two currently known bornaviruses, BDV and ABV.¹³⁴

THE VIRUS

Taxonomy

Although the syndrome known as BD has been described since the 1700s, its causative agent, BDV, eluded characterization until the late 1980s, when the application of a novel technique, subtractive cDNA cloning, yielded the first cDNA clones of the agent.^{176,326} Thereafter, analysis of concentrated, partially purified virus preparations^{24,251} led to the identification of BDV as a nonsegmented, negative-strand RNA virus, distantly related to rhabdo-, paramyxo-, and filoviruses.^{27,44} Identification of distinctive features including nuclear replication and transcription,^{24,42,258} differential use of transcription initiation and termination signals,^{27,276} and the use of alternative mRNA splicing^{27,45,281} resulted in the creation of a new family *Bornaviridae* within the order *Mononegavirales* in 1996.²⁴³ BDV is the prototype of the family and was its sole member until a new species, ABV, was discovered in 2008.^{131,152}

Morphology and Physical Characteristics

Spherical, enveloped particles of 80 to 100 nm with an electron dense core have been visualized by electron microscopy (EM) in extracts of BDV-infected cultured cells.^{159,356} ABV viral particles of 83 to 104 nm in diameter have been detected in the brain, eye, or small intestine of ABV-infected birds and detection of

comparable particles 25 years ago led to the conclusion that PDD is due to a virus infection.^{132,185,352} Smaller structures also identified in these extracts presumably represent defective particles. No similar structures have been reported in tissues or fluids from infected mammals.^{3,40,270} The virion M_r and the $S_{20,w}$ are not known; partially purified virus has a buoyant density of 1.15 to 1.22 g/cm³ in CsCl, 1.18 to 1.22 g/cm³ in sucrose, and 1.13 g/cm³ in renografin.^{47,84,216,251,356} Virus infectivity is only marginally affected after 24 hours in serum or by incubation at 37°C. In tissues and cell-free virus preparations the virus can be more stable than in culture extracts, and depending on the mode of desiccation, dried preparations can remain infectious for months (tissue at ambient temperature) to years (brain suspension under vacuum).^{47,177,179,205,358,360} At 4°C BDV infectivity is stable for more than 3 months. BDV can withstand both alkaline and acidic environments, but is most stable at neutral pH.^{47,70,114} Heating to 56°C for more than 3 hours inactivates the virus and common disinfection methods are appropriate as BDV is sensitive to organic solvents, detergents, pH below 4, and to UV light.^{47,63,113,114,203,205,216,358}

Genome

The bornaviral genome is a negative sense, single-stranded, nonsegmented RNA comprising approximately 8,900 nucleotides (nt) that includes six major open reading frames (ORF) with structural proteins in a 3' and the viral polymerase in a 5' position.^{27,131,152,208,230} Short noncoding complementary sequences are found at the termini. Unlike other nonsegmented negative-strand (NNS) RNA viruses, bornaviruses lack specific intergenic regions and instead have mostly overlapping ORFs (Fig. 39.1). The first transcription unit encodes the nucleocapsid protein (N). N exists in two isoforms, p40 (40 kDa) and p38 (38 kDa), that differ in the presence or absence of an amino terminal basic sequence that mediates nuclear localization.^{156,245} Although nucleocytoplasmic shuttling can be deduced from immunohistochemical and *in situ* hybridization results for ABV,^{340,341} there is no proof for the existence of N isoforms in ABV.^{92,327,328} The viral phosphoprotein (P, p23) and the regulatory X protein (X, p10) are encoded by the second transcription unit.³³⁹ The 5' end of the P ORF overlaps with the 3' portion of the X ORF in the +1 reading frame, an organization that resembles the P/C/C' organization of the second gene of vesiculoviruses.^{167,307} However, BDV X is the first ORF in the RNA transcript and there is no evidence of co-transcriptional mRNA editing, a mechanism used by some paramyxoviruses to regulate expression of multiple reading frames from their second transcription unit.^{160,321} The first and second transcription units of bornaviruses overlap, because the transcription start S2 is located upstream of the termination/polyadenylation signal T1 (Fig. 39.1).²⁷⁶ The start S3 of the third transcription unit is located 2 nt downstream of T2 and generates multiple transcripts for the matrix protein (M, p16), the type I surface glycoprotein (G, p57), and the L-polymerase (L, p190). The G ORF overlaps the M ORF in the +1 reading frame. The L gene initiates with a short ORF of 6 amino acids (aa) that is spliced to the large 5' ORF (Fig. 39.1).^{281,334}

Bornaviruses are phylogenetically distinct from other taxa. Sequence divergence between strains of BDV and ABV is less than 20%, while divergence between BDV and ABV is greater than 30%; thus, they are classified as different species.^{131,152} The only region where bornaviruses have significant sequence

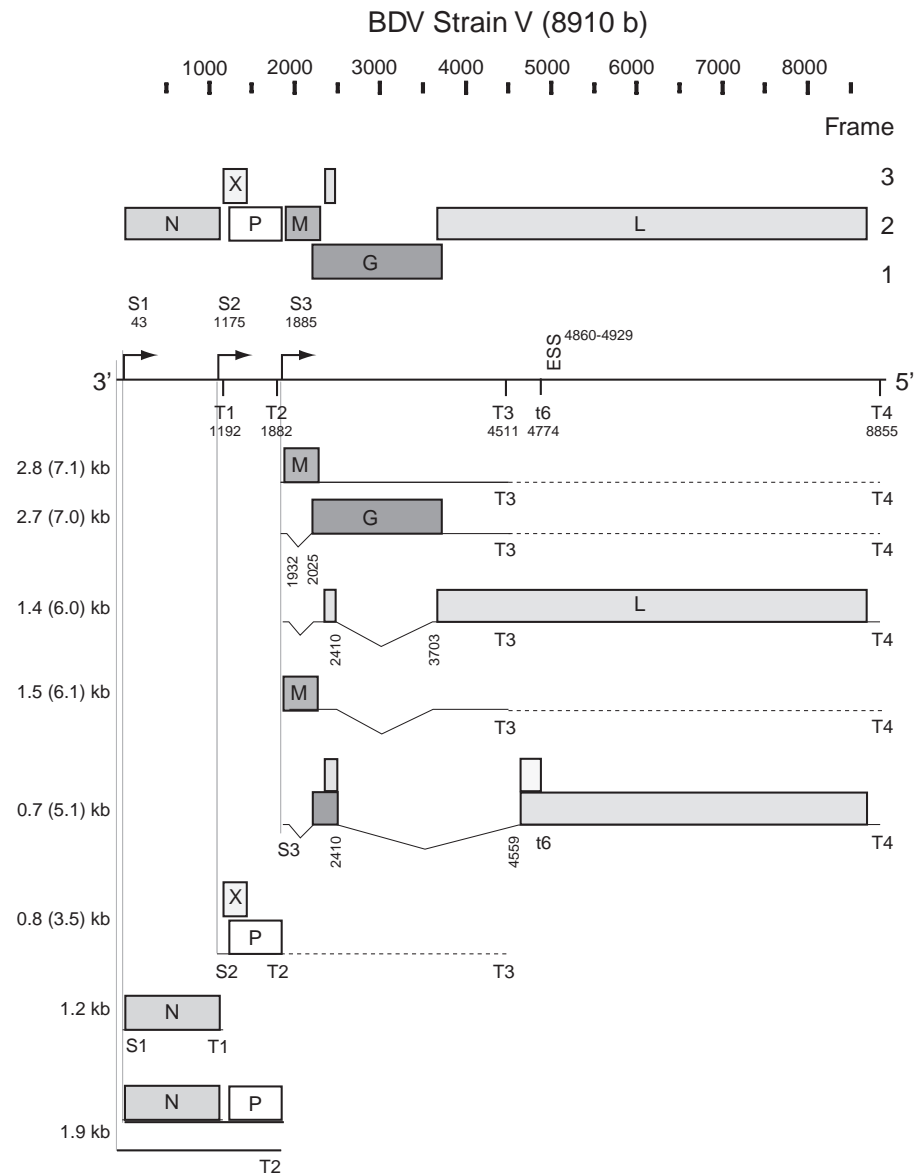


FIGURE 39.1. Genome and subgenomic transcript map of BDV. S1 through S3, transcriptional initiation sites; T1 through T4, and t6, transcriptional termination sites; ESS, exon-splicing suppressor. Dashed lines indicate readthrough at termination sites T2 and T3. See text for details.

similarity to other known viruses is in the conserved signature motifs of RNA-dependent RNA polymerases (RdRp).^{234,235} The closest phylogenetic relations exist to rhabdo- and paramyxoviruses,^{27,44} with the N-terminal half of the L sequence being closer to rhabdoviruses, whereas the C-terminal half is more closely related to paramyxoviruses.

Sequences distantly related to BDV L, M, and N sequences were recently identified in the genomes of several animal species, including bats, elephants, fish, lemurs, rodents, squirrels, primates, and man.^{11,133} Detailed phylogenetic analyses suggest multiple ancient independent integration events. An intriguing example is a BDV N-related sequence that likely integrated before the separation of marmosets and macaques 40 million years ago.^{11,133} In some instances, the endogenous Borna-like (EBL) element comprises a complete ORF that includes BDV-like transcription initiation and termination signals. The finding of mRNA transcripts of such EBL elements suggests potential functional roles that may include protection from BDV infection.^{11,81}

Genetic Diversity

BDV isolates reveal a remarkably high degree of genetic stability and homology. Among wild-type and experimentally host-adapted viruses, sequence identity is about 95% at the nucleotide level, and 1.5% to 3% at the predicted amino acid level.^{18,27,44,123,161,208,230,278,310} Phylogenetic analysis of wild-type and laboratory strains of BDV indicates distinct clusters, which correspond to the different endemic areas in Central Europe.¹⁶¹ Geographical virus clusters exhibited a higher degree of identity to each other than to BDV isolates from distant regions, independent of host species or year of isolation. There is only one highly divergent BDV strain, No/98, which originated from Styria in Austria where Borna disease is not endemic.²⁰⁸

ABV displays remarkable genetic variability in contrast to the high genome conservation of BDV. At present, 7 different genotypes (ABV1, ABV2, ABV3, ABV4, ABV5, ABV6, ABV of canaries) have been identified that share less than 70% sequence identity to any of the described BDV isolates.^{131,152,217,340,343} The ABV genotypes vary considerably in their gene sequences with

a homology range of 50% to 90% without clustering according to country of origin or avian species.^{131,132,152,153,259,309,340,343} ABV4 appears to be the most abundant genotype in natural PDD cases but also in healthy carriers. Recently, a distinct ABV genotype has been detected in wild geese and trumpeter swans.^{55,217}

Proteins

Nucleocapsid Protein (N)

In BDV, N exists as a 40-kDa and 38-kDa isoform;^{26,156,245} N isoforms have not yet been observed in ABV.^{92,327,328} The 40-kDa variant (p40) is derived from the full length ORF, while the 38-kDa variant (p38) initiates at a second in-frame AUG, resulting in the lack of 13 aa at the amino terminus (Fig. 39.2). Although an RNA with coding information for p38 has been found that starts downstream of S1,²⁴⁵ it is unknown whether p40 and p38 can both be translated from mRNA transcripts starting at the S1 transcriptional initiation site. The 13 aa amino terminal sequence present in p40 contains a nuclear localization signal (NLS; P₃KRRLVDDA₁₁) compatible with the differential cellular distribution of the two isoforms seen in cells transfected with constructs expressing only one of the isoforms; while p40 is primarily nuclear, p38 is primarily cytoplasmic.^{156,245} However, both, p38 and p40 bind to P. As P contains potent NLS (Fig. 39.2), the *in vivo* significance of the two N isoforms is unknown; p38 may enter the nucleus through

interaction with P. Experimental evidence with expression constructs indicates that p38 can accumulate in the nucleus to levels similar to those of p40; however, p38 alone cannot support transcription/replication of BDV (mini-)genomes.²²⁰ Thus, the amino terminal sequence of p40 may have functions in addition to N protein translocation. Both p40 and p38 bind to P through two motifs (K₅₁–Y₁₀₀, and L₁₃₁–I₁₅₈; Fig. 39.2).^{13,155} p38 appears to regulate cellular levels of free p40 by blocking the respective binding site on P, thus modulating cellular ratios of free p40 to P.²⁸⁴ In addition, p38 and p40 contain a nuclear export signal (NES; L₁₂₈TELEISSIFSHCC₁₄₁)¹⁵⁵ that overlaps the binding motif for P (Fig. 39.2). It is therefore hypothesized that p38, which lacks the NLS motif, may redistribute to the cytosol after dissociation from bound P, possibly once assembled in ribonucleoprotein (RNP) complexes.¹⁵⁵ As indicated by purification experiments and co-localization studies, both p38 and p40, as well as P and M, are included with genomic RNA in the RNP.^{35,189} Association of genomic RNA with N relies on basic amino acid residues located in a cleft formed between the amino- and carboxy-terminal helical domains of N.^{129,267} However, binding to N in the multimeric RNP appears not to shield the genomic RNA from enzymatic attack.

Together with P, N constitutes the BDV s-antigen, a complex found in the noninfectious supernatant fluid obtained after high-speed centrifugation of sonicated infected brain tissue or cultured cells. Characterization of the s-antigen provided the

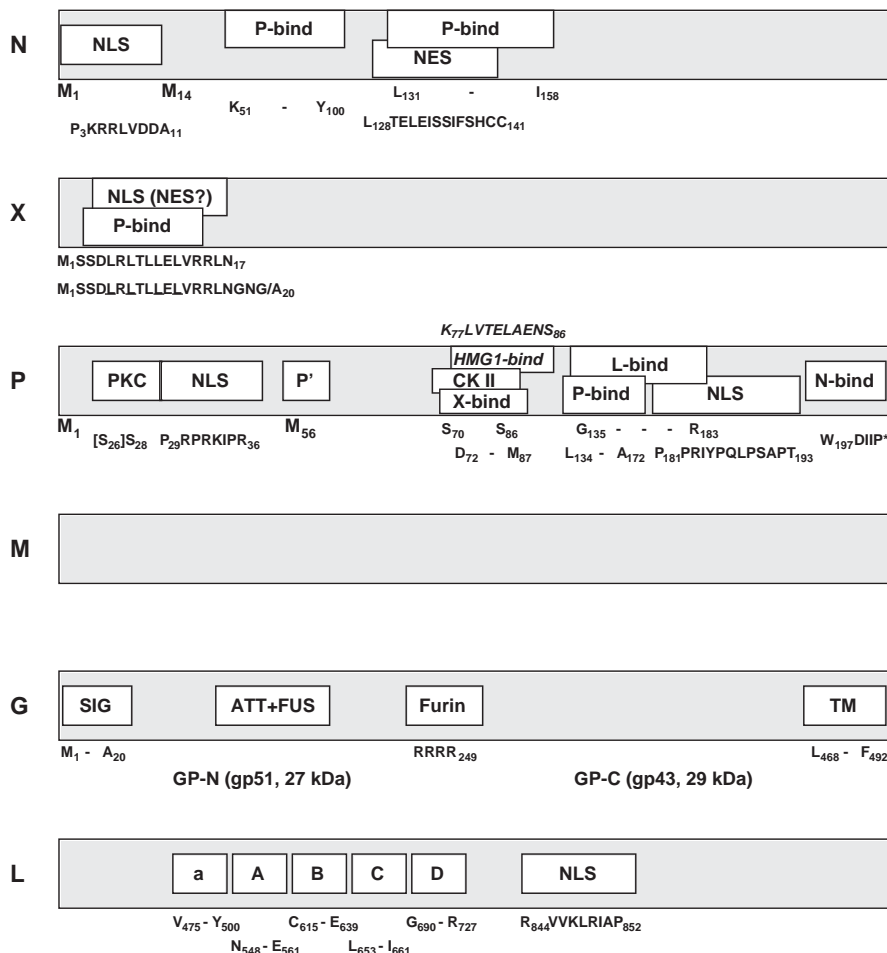


FIGURE 39.2. Map of motifs identified in BDV proteins. M1 and M14 in N, and M1 and M56 in P indicate start sites of p40 and p38, or P and P', respectively; NLS, nuclear localization signal; NES, nuclear export signal; P-bind, site of interaction with P; X-bind, site of interaction with X; L-bind, site of interaction with L; N-bind, site of interaction with N; HMG1-bind, site of interaction with host-cell protein amphotericin; PKC, protein kinase Cε phosphorylation; CK II, casein kinase II phosphorylation; SIG, signal peptide; TM, transmembrane domain; ATT+FUS, attachment and fusion domain; Furin, Furin cleavage site; "++", stop codon a, A, B, C, D, conserved L-polymerase motifs. See text for further details.

first evidence of protein-protein interactions,^{8,96,177} and until the development of molecular assays, served as the critical diagnostic marker for infection.^{211,332,333}

X Protein (X)

BDV X or p10³³⁹ is a nonstructural protein^{189,288} that, together with P, modulates BDV polymerase activity as a function of the relative abundance of the two proteins.^{237,288} X effects appear to be differently pronounced in different cell types.³⁵³ Although X is not essential for the formation of infectious particles,²¹⁹ it does perform crucial functions during the BDV life cycle because recombinant BDV constructs carrying a nonfunctional X ORF are not viable.²³⁹ Mammalian two-hybrid and co-immunoprecipitation experiments indicate an interaction between X and P,²⁹³ and recombinant BDV systems showed that X inhibited BDV RNA replication and transcription through binding to P^{220,238,285} in the absence of viral M and G.²¹⁹ The site of X interaction with P has been mapped to the N-terminal motif S₃DLRLTLLELVRRRL₁₆, with aa 7 through 15 being probably most essential.^{184,350} X is small (10 kDa) and can be found in the nucleus and cytoplasm. A leucine-rich amino-terminal motif with primary sequence similarity to the NES of cellular and viral export proteins like HIV-1 Rev or PKI (Fig. 39.2, underlined leucines) led to the speculation that X may mediate nucleocytoplasmic shuttling through its interaction with the viral RNP via P. However, X has not been shown to be part of the RNP,^{35,189,219} and other data suggest that this motif functions as an NLS rather than an NES (R₆LTLELVRRNGN₁₉).³⁵¹ These experiments also showed that transport of X through the nuclear pore complex is mediated by direct binding to importin- α .

Phosphoprotein (P)

P, a cofactor of the L viral polymerase, is phosphorylated at multiple serine residues by two different cellular kinases.^{289,320} P is phosphorylated predominantly by protein kinase C ϵ (PKC ϵ) at Ser28 (and Ser26, which is not present in all BDV strains), and to a lesser extent by casein kinase II (CK II) at Ser 70 and Ser 86 (Fig. 39.2). As in other NNS virus phosphoproteins, phosphorylation status may regulate P's ability to form homomultimers, bind to other viral proteins, and serve as a transcriptional activator. P interacts with itself, X, N, M, and L as shown by mammalian two-hybrid and co-immunoprecipitation analyses. Regions of interaction of P with P (aa 135–172), with N (aa 197–201),²⁹³ with M (aa 1–11),³⁵ and with X (aa 72–87)¹⁵⁸ were mapped through analysis of truncation mutants of P. A region of interaction with L (aa 135–183) overlaps that identified for homo-oligomerization; however, the sites are functionally separated as shown by analyses of P mutants that bound to L but had lost the ability to oligomerize.²⁸³ These analyses also demonstrated that P-oligomerization is essential for polymerase activity. Overlap also exists between the CK II phosphorylation sites and the region of interaction with X, as well as between the PKC ϵ phosphorylation site(s) and the amino-terminal NLS of P; two NLS have been mapped at the amino- and carboxyl-terminus of P (Fig. 39.2).^{292,295} Thus, it is conceivable that P phosphorylation may influence nuclear trafficking of P (and possibly of X through its interaction with P). In this context it is intriguing that PKC ϵ is highly concentrated in limbic circuitry,²⁶⁸ as it suggests that PKC ϵ phosphorylation may be important to the limbic distribution of BDV. BDV

infection of neurons interferes with synaptic vesicle recycling through blockade of PKC-phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) and mammalian uncoordinated-18 (Munc-18). There is speculation that P may contribute to BDV pathogenesis by competing with neuronal substrates for phosphorylation by PKC.^{241,330}

Matrix Protein (M)

Like the BDV s-antigen, a 14.5-kDa protein purified from infected brain homogenate had been linked to the unidentified BD agent prior to molecular characterization of BDV.²⁷⁴ Once genome sequence data became available, microsequencing indicated that this protein is the product of the 16-kDa ORF of BDV (p16).¹⁵⁴ Subsequent analyses showed that p16 forms noncovalently linked tetramers and constitutes a non-glycosylated viral matrix protein.^{165,166} M is a component of the viral RNP and can bind to P. However, in contrast to other NNS RNA virus M proteins, BDV M appears to have no inhibitory effect on polymerase activity.^{35,189}

Glycoprotein (G)

The BDV glycoprotein is a classical type I membrane protein that is generated from the 57-kDa ORF and posttranslationally modified by N-glycosylation to yield a 94-kDa primary product (gp94).^{252,279} The primary product is processed by cellular furin protease into an amino-terminal GP-N (27 kDa, gp51) and a carboxy-terminal GP-C (29 kDa, gp43) through cleavage after arginine₂₄₉.^{151,252} (Fig. 39.2). Whereas the gp94 precursor contains only high-mannose glycans, analysis of the cleaved fragments indicated glycan maturation by showing mixtures of high-mannose and complex-type glycans on both GP-N and GP-C.¹⁵¹ Anchored by its transmembrane domain, GP-C is transferred to the cell membrane, while the gp94 precursor predominantly accumulates in the endoplasmic reticulum. The final composition of mature virions is not clear; gp94 as well as GP-C and GP-N have been found in infectious particles,^{38,68,84} but GP-N and GP-C were also demonstrated in infectious particles that lacked the gp94 precursor after virus purification.¹⁵¹ Computational analyses indicate an arrangement of structural features of BDV G that is co-linear to rhabdoviral G, suggesting that BDV G also belongs to the class III viral fusion proteins.⁸⁰

RNA-Dependent RNA Polymerase (L)

The large polymerase protein (L) of BDV is the product of alternative splicing, a mechanism unique among NNS RNA viruses.^{27,281} The generated continuous ORF translates a 190-kDa protein³³⁴ that displays motifs characteristic of RNA-dependent RNA polymerases (RdRp). L interacts with P and, analogous to other NNS L-polymerases, it is phosphorylated by cellular kinases (Fig. 39.2).³³⁴ Plasmid constructs directing expression of the continuous 190-kDa protein that supported replication of recombinant BDV genomes confirmed the polymerase activity of the protein.^{187,220,285,286,353} Translocation of L to the nucleus of the infected cell appears to be promoted by an NLS motif (R₈₄₄VVKLRIP₈₅₂; Fig. 39.2) located toward the center of L,³³⁵ or in association with BDV P.²⁸⁵

Cycle of Infection

Attachment and Entry

BDV attachment and entry appear to be analogous to the pH-dependent entry via intracellular vesicles described for

rhabdo- and filoviruses, as opposed to the pH-independent surface fusion mechanism used by paramyxoviruses.^{229,260,269} BDV G binds to one or more still unidentified cellular surface receptor(s).^{84,279} Receptor interaction of G triggers BDV internalization through energy-dependent, clathrin-mediated endocytosis and subsequent pH-dependent membrane fusion leads to release of the RNP from intracellular vesicles into the cytosol.^{37,83} Protease inhibitor studies indicate that cleavage of the precursor gp94 is essential for infectivity.²⁵² Whereas the amino-terminal 244 aa of gp94 and/or GP-N are involved in receptor binding, the hydrophobic amino-terminus of GP-C is hypothesized to initiate membrane fusion upon a conformational change induced by acidification in the early to intermediate endosome.^{37,68,83,221,279} No data are available regarding trafficking of the released nucleocapsid after membrane fusion.

Despite its predilection for neuronal cell types *in vivo*, BDV has the capacity to infect a wide variety of cultured neuronal as well as nonneuronal cell types from many species. Only hematopoietic cells²⁶⁴ and mouse cell lines⁷⁴ have been reported as resistant to infection *in vitro*. This may indicate potential exploitation of secondary receptors, and has also led to hypotheses about a nonreceptor-mediated cell-to-cell spread of BDV that is supported by *in vitro* as well as *in vivo* observations.^{86,180} More recent studies with a CHO cell line that apparently was resistant to infection by BDV virions, and with furin protease-deficient CHO cells, indicated that BDV can disseminate by G-receptor-independent pathways.³⁸ However, correct G maturation enhanced the proposed receptor independent cell-to-cell spread, and is mandatory for the formation of infectious progeny virions.

Transcription, Replication and Gene Expression

Recombinant virus systems confirm that BDV N, P, and L are essential and sufficient for transcription and replication of the viral genome.^{187,219,220,285,286,353} As with other negative-strand RNA viruses, genomic RNA packaged by N constitutes the RNP that serves as a template for the associated polymerase complex components L and P.^{129,189} The BDV X protein, although not part of the incoming RNP complex,^{189,288} appears to modulate the formation and activity of functional polymerase complexes later in infection by buffering the crucial N-to-P ratio and likely attenuating the enzymatic activity of the polymerase.^{219,237,337} Furthermore, BDV is unique among known animal NNS RNA viruses in its nuclear location for transcription and replication.^{24,42} To generate its proteins, BDV uses predominantly polycistronic mRNAs that are transcribed from three transcriptional initiation sites characterized by a CUU consensus sequence and terminate at four AU₆ termination/polyadenylation sites (Fig. 39.1).^{27,276}

The first and second transcription units overlap such that the initiation signal S2 lies upstream of the termination signal T1.^{27,44,240,276} A similar organization was postulated to serve as an attenuation signal for the control of polymerase expression in respiratory syncytial virus.³⁹ However, attenuation appears not to take place in BDV as the two transcription units are found at similar levels,²⁷ in contrast to the usual transcriptional gradient observed in the other viruses of the order. Other factors may modify mRNA levels beyond effects attributable to the typical 3'-to-5' transcriptional gradient, including the incorporation of regulatory sequences in spliced introns.²⁹⁶

Read-through at termination/polyadenylation signals is a vital feature of BDV transcription, leading to primary, sub-genomic RNA transcripts of 0.8, 1.2, 1.9, 2.8, 3.5, and 7.1 kb. The 1.2-kb transcript is the only monocistronic product. It is co-linear with the p40 ORF and directs translation of the p40 and p38 isoforms of BDV N from alternative in-frame AUG codons.^{156,245} The second transcription unit generates the 0.8-kb transcript that codes for the X and the P protein in overlapping ORFs. There is no evidence of splicing to eliminate the AUG initiating translation of X.^{27,276,339} Thus, it is likely that P is expressed through a leaky scanning mechanism, possibly analogous to the termination-mediated reinitiation of X translation from the small upstream ORF included in the 0.8-kb transcript. The expression of X may be further modulated by cellular protein interactions with the long 5'-untranslated region (UTR) of this BDV transcript.³³⁸ The long 5'-UTR region also includes poorly defined regulatory elements controlling polymerase read-through at the T1 signal.^{237,240} Interestingly, the region containing the AUG of the upstream small ORF has been found deleted in sequenced psittacine ABV genomes but not in sequences derived from geese.^{217,259} In BDV, a truncated 16-kDa P' product of undefined function may result from initiation at a downstream in-frame AUG.¹⁵⁷

Read-through at T2 produces an elongated 0.8-kb derivative of 3.5 kb. Two primary transcripts of 2.8 and 7.1 kb are generated from the third transcription unit through differential read-through at T3. The 2.8- and 7.1-kb transcripts include the p16 and p57, or the p16, p57, and pol ORF, respectively; in addition, several secondary transcripts are derived from these RNAs through alternative splicing (Fig. 39.1).

Transcripts of the third transcription unit include two intron sequences, intron-1 and intron-2, that are subject to alternative splicing.²⁸¹ Whereas the 2.8- and 7.1-kb transcripts direct translation of M, the expression of G likely requires removal of intron-1. Although G may also be expressed *in vitro* from unspliced transcripts by leaky ribosomal scanning, splicing of intron-1 creates a stop-codon after the 13th aa of the p16 ORF that facilitates translational initiation at the AUG of G.²⁸⁰ Splicing of intron-2 removes almost the complete p57 ORF and fuses a small 17 nt ORF located upstream of the splice donor site to the large downstream ORF that, in the case of the 2.8-kb RNA, terminates at T3, generating a truncated L protein of unknown function (Fig. 39.1). Expression of L is, analogous to G, likely facilitated by splicing of intron-1. In addition, an alternative splice acceptor site, controlled by a downstream exon-splicing suppressor, has been identified at nt 4559.³²² Splicing of this potential intron-3 (nt 2410–4559), and transcriptional read-through at t6,²⁷ may result in expression of two additional BDV-specified proteins.^{43,322} However, the splice acceptor site at nt 4559 is not strictly conserved throughout sequenced BDV isolates. The lack of conservation in BDV No/98²³⁰ as well as in ABV isolates indicates that the potential gene products are unlikely to fulfill essential functions in the bornavirus life cycle.

There have been reports of multiple types of BDV 1.9-kb RNA transcripts with unspecified function. Analyses based on RNA circularization and sequencing over the junction indicated the presence of a noncapped RNA complementary to position 1 to 1882 of the BDV genome that interacted with oligo(dT)-beads, but was not fully polyadenylated.²⁷⁶ Such transcripts may represent abortive replication intermediates or subgenomic

RNAs analogous to leader RNAs found in other mononegaviruses. On the other hand, studies employing precipitation with a cap-specific antibody characterized a capped 1.9-kb mRNA with a long poly(A)-tail, extending from S1 to T2²⁴⁰ (Fig. 39.1).

Initiation of transcription and replication in negative-strand RNA viruses is commonly mediated by sequences located in the UTR. A single transcriptional promoter located in the 3'-UTR of the genome generates the usual transcriptional gradient, and promoter sequences driving genome replication are located in both the complementary 3'- and 5'-genomic termini. However, BDV analyses indicated remarkable terminal heterogeneity.²³⁰ Kinetic analyses comparing the genomic termini in acute and persistent BDV infection showed an accumulation of terminal truncations in the course of infection, which in several cases resulted in strongly attenuated replicational and transcriptional promoter activity, possibly contributing to BDV's persistent lifestyle.²⁶¹ In addition, rescue of infectious recombinant BDV constructs demonstrated trimmed 5' genomic termini generated from originally perfectly complementary constructs.²⁸⁶ In this system, when recessed 5' ends aligned to the 3' terminus were generated, there was strong attenuation of replication while transcriptional activity appeared to be unaffected. This finding is compatible with the high antigen levels in conjunction with low levels of infectious virus that are observed during persistent BDV infection. The four 5' terminal bases of genome and antigenome appear to be copied from internal template motifs through backfolding of the termini followed by specific elongation and termination on the template motifs.¹⁸⁶ This allows later cleavage of these terminal bases to generate monophosphorylated 5' termini of progeny strands without the loss of genetic information. Trimming of the termini in BDV may support its persistent infection by escape from innate immune responses through RIG-I-mediated recognition of triphosphorylated genomic termini.⁹⁷ It is not known whether viral and/or host functions are responsible for the terminal trimming. Further characterization of these unique BDV transcriptional and replicational promoter sequences may help to resolve results obtained in various recombinant test systems.

Assembly and Release

As with other negative-strand RNA viruses, a first step in the production of BDV progeny is packaging of the replicated nascent genomic RNA by N. The 5'-trailer RNA specifically promotes its association with N via basic residues in the cleft between the amino- and carboxy-terminal domains of the protein.^{129,267} Formation of the RNP complex includes association of P via its carboxy-terminal aa residues, and the inclusion of L is hypothesized to occur via its characterized protein-protein interaction sites (Fig. 39.2). Based on NLS located in N, P, L, and X, and NES in the two N isotypes and possibly X, various hypotheses concerning nucleocytoplasmic shuttling of BDV RNP have been proposed, but experimental confirmation is lacking. Colocalization studies suggest that M is also an integral part of the BDV RNP.³⁵ M, but not X, has been demonstrated in purified RNP,¹⁸⁹ a finding consistent with the observation that in recombinant BDV mini-genome systems the expression of M and G, but not X, is required for the formation of infectious BDV-like particles.²¹⁹ The processing of BDV G by protease digestion appears to be crucial to the assembly of infectious virions.^{5,38} Distinct packaging signals are not defined.

EPIDEMIOLOGY OF BDV INFECTION

Host Range

BD is reported most commonly in horses and sheep; but disease has also been reported in other *Equidae*, farm animals (cattle and goats), rabbits, lynx, zoo animals (alpacas, sloths, various monkeys, hippopotamuses), and rarely in companion animals (dogs and cats).^{21,31,46,54,67,113,138,140,182,192,262,310,344,358} Experimental infections have been achieved in various animal species ranging from chickens to nonhuman primates.^{4,46,87,113,148,188,201,212,255,262,266,308,314} Infection in horses leads to death 1 to 4 weeks after onset of signs in 80% to 90% of animals.^{67,89,91,253} In 72% of stables with equine BD cases, only individual animals develop clinically manifested BD. In cattle and sheep, death was noted after 1 to 6 weeks or 1 to 3 weeks in more than 50% of animals, respectively.^{21,255}

Geographic Distribution and Potential Reservoir

Natural BD is endemic in areas of central Europe such as southern and eastern Germany, Switzerland, Liechtenstein, and Austria.^{30,46,66,113,120,193,344,358} Reports of natural BD outside these endemic areas suggest a wider distribution of the disease.^{78,98,99,144,147,178,253,262,354} Virus-specific serum antibodies and/or nucleic acids in absence of disease or in association with unusual clinical signs have been reported in animals from different geographic areas, including European countries, such as France, Sweden, Finland, and Italy, as well as Turkey, Israel, Japan, Iran, China, Australia, and the United States.^{66,78,98,99,144,147,178,253,262,354} However, as some of these data are debated and require more confirmation, further epidemiologic studies are warranted.

A seasonal accumulation of BD cases in April, May, and June—with a significant decrease in late fall and winter—is quite characteristic and argues, in combination with the geographically limited occurrence, for a natural reservoir.^{66,67,89,113,181,325} Recently, BDV infection has been detected in bicolored shrews (*Crocidura leucodon*) in endemic areas in Switzerland. These animals had a disseminated virus distribution in the absence of overt disease and represent a potential reservoir for BDV.^{127,244} Whether other species can serve as reservoir species is currently unknown.

Prevalence and Seroepidemiology

In contrast to the epidemic course of BD at the end of the 19th century, the incidence of BD decreased significantly during recent decades; usually less than 100 horses or sheep are diagnosed with BD per year.^{66,120,199} However, BDV infections in horses and sheep can be inapparent as indicated by seroepidemiologic surveys in Germany. The average seroprevalence of BDV-specific antibodies in clinically healthy horses in Germany is approximately 11.5%¹²⁰ and increases in endemic areas up to 22.5%, reaching 50% in stables with a history of clinical BD.⁹¹ There is a higher frequency of BD on farms with mixed stock of horse, sheep, and cattle, operating under lower hygiene standards.⁶⁶ Repeated outbreaks of BD within the same premises have been noted but usually spaced several months or years apart.^{91,253} The reason for the discrepancy between the high BDV seroprevalence and the low BD incidence remains unknown but may relate to age, immune status, genetic background, virus strain, and/or dose of infection.

Route of Infection and Transmission

There is evidence that nerve endings in the nasal and pharyngeal mucosa represent the most likely natural route of entry.^{142,188,197} Experimental BDV infection of neonatal rats results in virus persistence and disseminated virus distribution with presence of viral gene products and infectious virus in saliva, urine, and feces.^{188,197} Such secretions or excreta are important in transmission of other pathogenic viruses (e.g., lymphocytic choriomeningitis virus and hantaviruses). This finding further supports the concept of a natural reservoir of BDV, which is substantiated by stable geographic virus clusters,^{18,161,325} despite substantial horse movement and trade. This was recently confirmed by a case of natural BD in Great Britain that was traced back to a likely origin of infection in Germany.²⁴² It seems that widespread horse-to-horse or sheep-to-sheep transmissions do not occur.^{16,66,254,310} The infectious dose for natural infections is unknown.

EPIDEMIOLOGY OF ABV INFECTION

The epidemiology of ABV infections is less well understood. Since its first description in the United States and Israel in 2008, ABV infections have been reported from various European countries, for example, Germany, Austria, Switzerland, Hungary, Spain, Italy, United Kingdom, and Denmark, but also from Canada, Australia, and Japan,^{112,131,152,172,209,246,259,340} indicating a worldwide distribution of ABV.

Within the order *Psittaciformes*, ABV infections have been reported in captive psittacines of 34 different genera as well as in a canary (*Serinus canaria*), and recently in a toco tucan (*Ramphastos toco*) and wild waterfowl (Canada geese, [*Branta canadensis*], and trumpeter swans, [*Cygnus buccinator*]) in Canada and the United States.^{55,112,132,218,343} Whether wild birds might serve as a natural reservoir for ABV remains to be further investigated. The transmissibility of ABV in natural infections has also been confirmed.^{111,153} The natural route of ABV infection is unclear but detection of ABV RNA in feces, cloacal and crop swabs argues for oronasal entry and bird-to-bird transmission. The possibility of vertical transmission has to be further investigated.¹⁷³

HUMAN INFECTION

Much of the impetus for characterization of BDV came from concerns that it infected humans and might be implicated in psychiatric syndromes, including major depressive disorder, bipolar disorder, schizophrenia, and autism, as well as chronic fatigue syndrome, AIDS encephalopathy, multiple sclerosis, motor neuron disease, and brain tumors (glioblastoma multiforme) (Tables 39.1 and 39.2). Over a period of three decades investigators reported evidence for human infection using primarily PCR and serologic assays, including the detection of circulating immune complexes.²³ However, there were also reports of infectious virus isolated from humans or BDV gene products detected in human brain by *in situ* hybridization and immunohistochemistry.^{53,202} Failure to independently replicate positive results in the majority of laboratories undermined confidence in the association of BDV with human disease.^{175,349} Another concern was the possibility of cross-contamination suggested

by sequence similarity of putative human BDV sequences with those of the laboratory strains and field isolates handled in the laboratories reporting the human sequences.^{65,134,250,291,310,349} A recent blinded multicenter analysis failed to find either molecular or serologic evidence for human BDV infection using methods established by investigators reporting links between BDV and human neuropsychiatric disease.¹³⁴ Thus, although the potential for human infection has not been excluded it is exceedingly unlikely that a bornavirus similar to those identified to date is responsible for a significant burden of human disease.

BDV NEUROPATHOGENESIS

Experimental Infection of Animals with BDV

Early virus detection and isolation experiments by Zwick and colleagues were performed in rabbits,³⁵⁸ which has served since then as the most sensitive small-animal model for BDV infection. Rabbits are highly susceptible and after early weight loss develop neurologic disease about 3 weeks after infection with BDV-infected brain homogenates. Symptoms include slow movement, depression, and somnolence followed by flaccid back musculature, paresis starting from the hind limbs, and trismus.¹⁷⁹ Comparable to BD in ungulates, infection of rabbits is not characterized by excessive hyperactivity, as seen in infected rats. Nevertheless, most insights into pathogenesis have been obtained after adaptation of BDV to the rat.^{128,203,204,206} Susceptibility to disease in rats is genetically determined. Wistar rats and black-hooded rats show less severe disease than Lewis rats, a strain with deficiencies in the hypothalamic–pituitary–adrenal axis associated with enhanced susceptibility to immune-mediated disorders.^{36,128,312} Resistance to BD is inherited as a dominant trait independent of MHC genes in black-hooded and Lewis hybrids.^{121,128} Serial passage in rat brain may enhance the virulence of BDV strains for rats.²⁰⁶ BDV infection of susceptible adult rats results in a biphasic disorder that manifests approximately 10 to 20 days after intracerebral infection as an acute immunopathologic disease, presenting clinically as hyperactivity and exaggerated startle responses.^{203,204} The onset of acute disease coincides with infiltration of mononuclear cells into the brain, particularly in areas of high viral burden such as the hippocampus, amygdala, and other limbic structures.³² After the initial acute phase, the animals enter a chronic disease phase characterized by somnolence, apathy, paralysis, dystonias, dyskinesias, stereotyped and self-mutilation behaviors, and blindness.^{203,204,299,301} Chronic disease is paralleled by widespread distribution of virus in the limbic system and prefrontal cortex, and 5% to 10% of animals become obese, developing up to 3 times the weight of normal rats.¹⁷⁹ BDV variant strains have been described that cause primarily an obesity syndrome without obvious neurologic disease. The obese phenotype is correlated with inflammation and viral antigen expression in the septum, hippocampus, amygdala, and ventromedian tuberal hypothalamus.¹¹⁶

Initially, the unique neuropathogenesis of BDV was mostly studied in the adult rat model, in which infection is associated with behavioral changes and disturbances in monoamine neurotransmitter systems and limbic circuitry. However, disease in immunocompetent adults is characterized by marked CNS inflammation, loss of brain mass, and gliosis. In contrast, neonatally infected rats present with hippocampal and cerebellar

TABLE 39.1 Serum Immunoreactivity to Borna Disease Virus in Subjects with Various Diseases

Disease	Prevalence		Assay	Reference
	Disease (%)	Control (%)		
Psychiatric (various)	0.6 (4/694)	0 (0/200)	IFA	Rott et al. (1985) <i>Science</i> 228:755
	2 (13/642)	2 (11/540)	IFA	Bode et al. (1988) <i>Lancet</i> 2:689
	4–7 (200–350/5000)	1 (10/1000)	WB/IFA	Rott et al. (1991) <i>Arch Virol</i> 118:143
	12 (6/49)		IFA	Bode et al. (1993) <i>Arch Virol</i> S7:159
	30 (18/60)		WB	Kishi et al. (1995) <i>FEBS Lett</i> 364:293
	14 (18/132)	1.5 (3/203)	WB	Sauder et al. (1996) <i>J Virol</i> 70:7713
	24 (13/55)	11 (4/36)	IFA	Igata-Yi et al. (1996) <i>Nat Med</i> 2:948
	0 (0/44)	0 (0/70)	IFA/WB	Kubo et al. (1997) <i>Clin Diagn Lab Immunol</i> 4:189
	2.8 (35/1260)	1.1 (10/917)	ECLIA	Yamaguchi et al. (1999) <i>Clin Diagn Lab Immunol</i> 6:696
	9.8 (4/41)		IFA	Bachmann et al. (1999) <i>J Neurovirol</i> 5:190
	15 (4/27)	0 (0/13)	IFA	Vahlenkamp et al. (2000) <i>Vet Microbiol</i> 76:229
	0 (0/89)	0 (0/210)	IFA/WB	Tsuji et al. (2000) <i>J Med Virol</i> 61:336
	5.5 (5/90)	0 (0/45)	WB (N ^a)	Fukuda et al. (2001) <i>J Clin Microbiol</i> 39:419
	2.1 (17/816)		ECLIA	Rybakowski et al. (2001) <i>Eur Psychiatry</i> 16:191
	2.4 (23/946)	1.0 (4/412)	ECLIA	Rybakowski et al. (2002) <i>Med Sci Monit</i> 8:CR642
				Rybakowski et al. (2001) <i>Psychiatr Pol</i> 35: 819
	13 (11/87)	16 (45/290)	IFA	Lebain et al. (2002) <i>Schizophr Res</i> 57:303
	15 (26/171)	2 (1/50)	RLA	Matsunaga et al. (2005) <i>Clin Diagn Lab Immunol</i> 12:671
	23 (39/171)	0 (0/9)	WB	Matsunaga et al. (2005) <i>Clin Diagn Lab Immunol</i> 12:671
	29 (24/84)	20 (77/378)	RLA	Matsunaga et al. (2008) <i>J Clin Virol</i> 43:317
	67 (26/39)	22 (28/126)	CIC	Rackova et al. (2009) <i>Neuro Endocrinol Lett</i> 30:414
	4.5 (12/265)	0 (0/105)	IFA	Amsterdam et al. (1985) <i>Arch Gen Psych</i> 42:1093
Affective disorders	4.2 (12/285)	0 (0/200)	IFA	Rott et al. (1985) <i>Science</i> 228:755
	38 (53/138)	16 (19/117)	WB (P ^a)	Fu et al. (1993) <i>J Affect Disord</i> 27:61
	37 (10/27)		IFA	Bode et al. (1993) <i>Arch Virol</i> S7:159
	12 (6/52)	1.5 (3/203)	WB	Sauder et al. (1996) <i>J Virol</i> 70:7713
	0–0.8 (0–1/122)	0 (0/70)	IFA/WB	Kubo et al. (1997) <i>Clin Diagn Lab Immunol</i> 4:189
	2.2 (1/45)	0 (0/45)	WB	Fukuda et al. (2001) <i>J Clin Microbiol</i> 39:419
	93 (26/28)	32 (21/65)	CIC	Bode et al. (2001) <i>Mol Psychiatry</i> 6:481
	27 (9/33)	4 (1/25)	WB	Terayama et al. (2003) <i>Psychiatry Res</i> 120:201
	19 (25/129)	20 (77/378)	RLA	Matsunaga et al. (2008) <i>J Clin Virol</i> 43:317
	4.8 (5/104)	0 (0/42)	ELISA	Flower et al. (2008) <i>APMIS Suppl</i> (124):89
	0 (0/138)	0 (0/60)	IFA	Na et al. (2009) <i>Psychiatry Investig</i> 6:306
Schizophrenia	25 (1/4)		IFA	Bode et al. (1993) <i>Arch Virol</i> S7:159
	32 (29/90)	20 (4/20)	WB	Waltrip et al. (1995) <i>Psychiat Res</i> 56:33
	17 (15/90)	15 (3/20)	IFA	Waltrip et al. (1995) <i>Psychiat Res</i> 56:33
	14 (16/114)	1.5 (3/203)	WB	Sauder et al. (1996) <i>J Virol</i> 70:7713
	20 (2/10)		WB	Richt et al. (1997) <i>J Neurovirol</i> 3:174
	0–1 (0–2/167)	0 (0/70)	IFA/WB	Kubo et al. (1997) <i>Clin Diagn Lab Immunol</i> 4:189
	14 (9/64)	0 (0/20)	WB	Waltrip et al. (1997) <i>Schizophr Res</i> 23:253
	36 (24/67)	0 (0/26)	WB (P ^a)	Iwahashi et al. (1997) <i>Acta Psych Scand</i> 96:412
	12 (38/276)		WB	Chen et al. (1999) <i>Mol Psychiatry</i> 4:33
	10 (3/29)	23 (6/26)	IFA	Selten et al. (2000) <i>Med Microbiol Immunol</i> 189:55
	8.9 (4/45)	0 (0/45)	WB	Fukuda et al. (2001) <i>J Clin Microbiol</i> 39:419
	13 (11/87)	16 (45/290)	IFA	Lebain et al. (2002) <i>Schizophr Res</i> 57:303
	8.6 (10/116)	0 (0/54)	WB	Yang et al. (2003) <i>Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi</i> 1:85
	22 (7/32)	4 (1/25)	WB	Terayama et al. (2003) <i>Psychiatry Res</i> 120:201
	23 (21/91)	20 (77/378)	RLA	Matsunaga et al. (2008) <i>J Clin Virol</i> 43:317
	0 (0/60)	0 (0/60)	IFA	Na et al. (2009) <i>Psychiatry Investig</i> 6:306
Childhood neuro-psychiatric disorder	56 (93/166)	51 (50/98)	CIC	Donfrancesco et al. (2008) <i>APMIS Suppl</i> (124):80

TABLE 39.1 Serum Immunoreactivity to Borna Disease Virus in Subjects with Various Diseases (continued)

Disease	Prevalence		Assay	Reference
	Disease (%)	Control (%)		
CFS	24 (6/25)		WB	Nakaya et al. (1996) <i>FEBS Lett</i> 378:145
	34 (30/89)		WB	Kitani et al. (1996) <i>Microbiol Immunol</i> 40: 459
				Nakaya et al. (1997) <i>Nippon Rinsho</i> 55: 3064
	0 (0/69)	0 (0/62)	WB	Evengard et al. (1999) <i>J Neuroviro</i> 5:495
	100 (7/7)	33 (1/3)	WB	Nakaya et al. (1999) <i>Microbiol Immunol</i> 43:679
MS	11 (7/61)	0 (0/73)	WB	Li et al. (2003) <i>Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi</i> 17:330
	21 (17/82)	0 (0/73)	WB	Li et al. (2005) <i>Zhonghua Yi Xue Za Zhi</i> 85:701
	13 (15/114)	2.3 (11/483)	IP/IFA	Bode et al. (1992) <i>J Med Virol</i> 36:309
	0 (0/50)		IFA	Kitze et al. (1996) <i>J Neurol</i> 243:660
	7.8 (36/460)	2.0 (11/540)	IFA	Bode et al. (1988) <i>Lancet</i> ii:689
HIV-positive	8.1 (61/751)	2.3 (11/483)	IP/IFA	Bode et al. (1992) <i>J Med Virol</i> 36:309
HIV-early	14 (34/244)	2.3 (11/483)	IP/IFA	Bode et al. (1992) <i>J Me. Virol</i> 36:309
HIV-LAP	9.8 (19/193)	2.3 (11/483)	IP/IFA	Bode et al. (1992) <i>J Med Virol</i> 36:309
Schisto/malaria	22 (39/174)	23 (39/173) ^a	ELISA	Güngör et al. (2005) <i>Pediatr Infect Dis J</i> 24: 833
SSPE associated BDV antibody	9.8 (8/82)	2.9 (8/277)	WB	Chen et al. (1999) <i>Mol Psychiatry</i> 4:33
Mental healthcare workers	12 (16/132)	2.9 (8/277)	WB	Chen et al. (1999) <i>Mol Psychiatry</i> 4:33
Family of schizophrenic patients	15 (16/108)	1 (1/100)	ELISA	Takahashi et al. (1997) <i>J Med Virol</i> 52:330
Living near horse farms	46 (19/41)	10 (4/41)	ELISA	Weisman et al. (1994) <i>Lancet</i> 344:1232
Ostrich exposure	0.7 (1/138)		IFA	Kinnunen et al. (2007) <i>J Clin Virol</i> 38: 64
Veterinarians	0.2 (1/361)		IFA	Kinnunen et al. (2007) <i>J Clin Virol</i> 38:64
Suspected hanta virus infection	37 (15/41)	37 (47/126)	CIC	Rackova et al. (2010) <i>BMC Psychiatry</i> 10: 70
Alcohol and drug addiction	8.3 (14/168)	0 (0/42)	ELISA	Flower et al. (2008) <i>APMIS Suppl.</i> (124):89
Multi-transfused	0.9 (2/214)		ELISA	Flower et al. (2008) <i>APMIS Suppl.</i> (124):89
Pregnant women	2.3 (5/219)		ELISA	Flower et al. (2008) <i>APMIS Suppl.</i> (124):89
Blood donors	59 (1204/2101)		TELISA	Patti et al. (2008) <i>APMIS Suppl.</i> (124):70
Normal population	37 (591/1588)		TELISA	Patti et al. (2008) <i>APMIS Suppl.</i> (124):74
	50 (130/258)		TELISA	Patti et al. (2008) <i>APMIS Suppl.</i> (124):77

CFS, chronic fatigue syndrome; CIC, circulating immune complexes; ECLIA, enhanced chemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; IFA, immunofluorescence assay; IP, immunoprecipitation; LAP, lymphadenopathy; MS, multiple sclerosis; RLA, radioligand assay; Schisto/malaria, schistosomiasis and malaria; SSPE, subacute sclerosing panencephalitis; TELISA, triple ELISA–CIC, Ab, Ag; WB, western immunoblot.

^aImmunoreactivity to BDV N and P was measured and the higher prevalence is given.

^bEpilepsy, headache, and cerebral palsy.

dysgenesis and display behavioral changes in the absence of appreciable inflammation, which offers an alternative, potentially more relevant model to study consequences of viral infection of the CNS. Rats inoculated as neonates with low passage virus during the first day of life become persistently infected, are smaller than uninfected littermates, and display only mild behavioral disturbances manifest as cognitive, emotional or social deficits.^{10,33,61,135,169,231,232,265}

In addition to rats, BDV has been experimentally transmitted to a variety of other animal species. Susceptibility to infection varies according to species and virus strain. Compared to the highly susceptible rabbits, guinea pigs, gerbils, and rats, other species, such as mice, cattle, chickens, monkeys, and tree shrews, appear to be less susceptible to disease.^{4,46,87,148,179,188,201,255,262,266,308,314}

Ferrets, pigeons, dogs, and hamsters develop no disease symptoms although they usually become persistently infected.^{46,262}

BDV Infection of the Adult Rat

Intracranial, intraocular, or intranasal inoculation routes have been used to infect adult rats. However, all routes that give the virus access to nerve terminals appear successful and produce infection of the CNS and symptomatic disease after varying incubation periods. Onset of disease correlates with distance from the inoculation site to the CNS,³² indicating that BDV spreads primarily through neural networks. This is supported by the finding that sciatic nerve transection after footpad infection prevents CNS infection.³² Further, after olfactory, ophthalmic, or intraperitoneal inoculation, viral proteins or nucleic

TABLE 39.2 Borna Disease Virus Nucleic Acid in Subjects with Various Diseases

Disease	Tissue	Prevalence		Divergence ^a	Reference
		Disease (%)	Control (%)		
Psychiatric (various)	PBMC	67 (4/6)	0 (0/10)	0–3.6	Bode et al. (1995) <i>Nat Med</i> 1:232
	PBMC	37 (22/60)	6.5 (5/77)	4.2–9.3	Kishi et al. (1995) <i>FEBS Lett</i> 364:293
					Kishi et al. (1996) <i>J Virol</i> 70:635
	PBMC-coculture	9.1 (3/33)	0 (0/5)	0.07–0.83	Bode et al. (1996) <i>Mol Psych</i> 1:200
					de la Torre et al. (1996) <i>Virus Res</i> 44:33
	PBMC	1.9 (2/106)	0 (0/12)		Kubo et al. (1997) <i>Clin Diagn Lab Immunol</i> 4:189
	PBMC	0 (0/24)	0 (0/4)		Richt et al. (1997) <i>J Neurovirol</i> 3:174
	PB	0 (0/159)			Lieb et al. (1997) <i>Lancet</i> 350:1002
	Blood	(1/1)			Planz et al. (1998) <i>Lancet</i> 352:623
	PBMC	4 (5/126)	2.4 (2/84)		Iwata et al. (1998) <i>J Virol</i> 72:10044
	PBMC	20 (3/15)	0 (0/3)		Planz et al. (1999) <i>J Virol</i> 73:6251
	PBMC	0 (0/81)			Kim et al. (1999) <i>J Neurovirol</i> 5:196
	PBMC	0 (0/27)			Bachmann et al. (1999) <i>J Neurovirol</i> 5:190
	CSF	0 (0/27)			Bachmann et al. (1999) <i>J Neurovirol</i> 5:190
	PBMC	1.8 (1/56)	0.6 (1/173)		Tsuji et al. (2000) <i>J Med Virol</i> 61:336
	PBMC	37 (10/27)	15 (2/13)		Vahlenkamp et al. (2000) <i>Vet Microbiol</i> 76:229
	PBMC	1.1 (1/90)	0 (0/45)		Fukuda et al. (2001) <i>J Clin Microbiol</i> 39:419
	PBMC	33 (10/30)	13 (4/30)		Miranda et al. (2006) <i>J Affect Disord</i> 90:43
Affective disorders	PBMC	33 (1/3)	0 (0/23)		Sauder et al. (1996) <i>J Virol</i> 70:7713
	PBMC	17 (1/6)	0 (0/36)		Igata-Yi et al. (1996) <i>Nat Med</i> 2:948
	PBMC	0 (0/9)			Richt et al. (1997) <i>J Neurovirol</i> 3:174
	Brain	40 (2/5)	0 (0/10)		Salvatore et al. (1997) <i>Lancet</i> 349:1813
	PBMC	4.1 (2/49)	2.4 (2/84)	0–5.1	Iwata et al. (1998) <i>J Virol</i> 72:10044
	CSF	4.6 (3/65)	0 (0/69)	[Protein]	Deuschle et al. (1998) <i>Lancet</i> 352:1828
	PBMC	2.2 (1/45)	0 (0/45)		Fukuda et al. (2001) <i>J Clin Microbiol</i> 39:419
	PBMC	11 (6/53)	0 (0/32)		Wang et al. (2006) <i>Zhonghua Liu Xing Bing Xue Za Zhi</i> 27(6):479
					Na et al. (2009) <i>Psychiatry Investig</i> 6:306
					Sierra-Honigman et al. (1995) <i>Br J Psych</i> 166:55
Schizophrenia	Brain	0 (0/3)	0 (0/3)		Sierra-Honigman et al. (1995) <i>Br J Psych</i> 166:55
	CSF	0 (0/48)	0 (0/9)		Sierra-Honigman et al. (1995) <i>Br J Psych</i> 166:55
	PBMC	0 (0/9)	0 (0/9)		Sierra-Honigman et al. (1995) <i>Br J Psych</i> 166:55
	PBMC	64 (7/11)	0 (0/23)		Sauder et al. (1996) <i>J Virol</i> 70:7713
	PBMC	10 (5/49)	0 (0/36)		Igata-Yi et al. (1996) <i>Nat Med</i> 2:948
	PBMC	0 (0/26)	0 (0/14)		Richt et al. (1997) <i>J Neurovirol</i> 3:174
	Brain	53 (9/17)	0 (0/10)		Salvatore et al. (1997) <i>Lancet</i> 349:1813
	PBMC	9.8 (6/61)	0 (0/26)		Iwahashi et al. (1997) <i>Acta Psych Scand</i> 96:412
					Iwata et al. (1998) <i>J Virol</i> 72:10044
	PBMC	3.9 (3/77)	2.4 (2/84)	0–5.1	Chen et al. (1999) <i>Mol Psychiatry</i> 4:566
	PBMC	14 (10/74)	1.4 (1/69)		Nakamura et al. (2000) <i>J Virol</i> 74:4601
	Brain	25 (1/4)		[RNA, virus, and protein]	
	PBMC	14 (4/29)	35 (9/26)		Selten et al. (2000) <i>Med Microbiol Immunol</i> 189:55
	PBMC	0 (0/45)	0 (0/45)		Fukuda et al. (2001) <i>J Clin Microbiol</i> 39:419
	PBMC	12 (3/25)		6.0–14	Nakaya et al. (1996) <i>FEBS Lett</i> 378:145
					Kitani et al. (1996) <i>Microbiol Immunol</i> 40:459
	PBMC	12 (7/57)	4.9 (8/172)		Nakaya et al. (1997) <i>Nippon Rinsho</i> 55:3064
	PBMC	0 (0/18)			Evengard et al. (1999) <i>J Neurovirol</i> 5:495
	PBMC	0 (0/60)	0 (0/60)		Na et al. (2009) <i>Psychiatry Investig</i> 6:306

TABLE 39.2 Borna Disease Virus Nucleic Acid in Subjects with Various Diseases (continued)

Disease	Tissue	Prevalence		Divergence ^a	Reference
		Disease (%)	Control (%)		
Schizoaffective	PBMC	44 (12/27)	15 (4/27)		Nunes et al. (2008) <i>J Clin Lab Analysis</i> 22: 314
Viral encephalitis	CSFMC	12 (6/52)	0 (0/32)		Wang et al. (2006) <i>Zhonghua Liu Xing Bing Xue Za Zhi</i> 27(6): 479
	PBMC	14 (6/43)	0 (0/98)	2.3–4.5	Wang et al. (2008) <i>Zhonghua Liu Xing Bing Xue Za Zhi</i> 29:1213
	PBMC	15 (6/40)	0 (0/46)		Li et al. (2009) <i>Eur J Neurol</i> 16:399
	PBMC	10 (6/59)	0 (0/60)	4.7	Ma et al. (2009) <i>Zhonghua Liu Xing Bing Xue Za Zhi</i> 30:1284
FMS	CSF	0 (0/18)	0 (0/6)		Wittrup et al. (2000) <i>Scand J Rheumatol</i> 29:387
CFS	PBMC	12 (3/25)		6.0–14	Nakaya et al. (1996) <i>FEBS Lett</i> 378:145
	Brain	80 (4/5)			de la Torre et al. (1996) <i>Virus Res</i> 44:33
Hippocampal sclerosis	Brain	15 (3/20)	0 (0/85)		Czygan et al. (1999) <i>J Infect Dis</i> 180:1695
Epilepsy	Brain	0 (0/106)			Hofer et al. (2006) <i>J Clin Virol</i> 36:84
MS	CSF	11 (2/19)	0 (0/69)	[Protein]	Deuschle et al. (1998) <i>Lancet</i> 352:1828
	PBMC	0 (0/34)	0 (0/40)		Haase et al. (2001) <i>Ann Neurol</i> 50:423
	PBMC	22 (2/9)	0 (0/98)	2.3–4.5	Wang et al. (2008) <i>Zhonghua Liu Xing Bing Xue Za Zhi</i> 29:1213
	PBMC	0 (0/9)	0 (0/46)		Li et al. (2009) <i>Eur J Neurol</i> 16:399
Peripheral neuropathy	PBMC	0 (0/7)	0 (0/98)		Wang et al. (2008) <i>Zhonghua Liu Xing Bing Xue Za Zhi</i> 29:1213
	PBMC	0 (0/16)	0 (0/46)		Li et al. (2009) <i>Eur J Neurol</i> 16:399
Parkinson's disease	PBMC	0 (0/5)	0 (0/98)		Wang et al. (2008) <i>Zhonghua Liu Xing Bing Xue Za Zhi</i> 29:1213
	PBMC				Cotto et al. (2003) <i>J Clin Microbiol</i> 41:5577
HIV infection	PBMC	13 (11/82)			Cotto et al. (2003) <i>J Clin Microbiol</i> 41:5577
Immunosuppressive treatment	PBMC	1.3 (1/80)			
Multiple transfusions	PBMC	0.8 (1/127)	2 (2/200)		Lefrere et al. (2004) <i>Transfusion</i> 44:1396
Mental health-care workers	PBMC	15 (7/45)	1.4 (1/69)		Chen et al. (1999) <i>Mol Psychiatry</i> 4:566
Normal controls	PBMC		4.7 (8/172)		Kishi et al. (1995) <i>Med Microbiol Immunol</i> 184:135
	Brain		6.7 (2/30)		Haga et al. (1997) <i>Brain Res</i> 770:307
	PBMC		0 (0/100)		Davidson et al. (2004) <i>Vox Sang</i> 86:148
	Plasma		0 (0/275 ^b)		Davidson et al. (2004) <i>Vox Sang</i> 86:148

CFS, Chronic fatigue syndrome; CSF, cerebrospinal fluid; PBMC, peripheral blood mononuclear cells; PB, peripheral blood; FMS, fibromyalgia syndrome; MS, multiple sclerosis.

^aDivergence of P gene nucleotide sequence from Borna disease virus strain V and He/80.

^bPlasma minipools of 91 individual samples.

acids move centripetally via synaptic connections.³² Analogous to rabies virus, spread of viral RNPs instead of mature virus within neural networks has been hypothesized for BDV⁸⁶; and the possibility of G-receptor-independent dissemination of BDV has been described *in vitro*.^{38,180} Although viral RNA can be found in peripheral blood mononuclear cells (PBMC) of infected animals, viremia is currently not considered to play a significant role in the pathogenesis and dissemination of BDV.

BDV infection of adult Lewis rats results in severe CNS immunopathology and pronounced behavioral disturbances.^{203,204,257,313} Monocyte infiltration, glial activation and Th₁-type cytokines in limbic structures coincide with the onset

of hyperactivity and exaggerated startle responses 2–3 weeks after infection and persist until the animals enter the chronic phase approximately 12 weeks later. This is associated with a switch of the Th1 to a Th2 response.¹⁰⁴ Microglial activation precedes astroglial reaction but seems to depend on persistent infection of neurons and activation of astrocytes.^{118,214} In many other viral CNS infections, the presence of an intact immune response results in viral clearance or death of the host. However, in BDV infection the virus persists at high titer in the presence of a robust cellular immune response during the acute phase, and also during the chronic phase when Th₂-type cytokines increase while immune cell infiltrates decline in the

presence of almost unchanged viral load in the CNS and continued glial activation.¹⁰⁴ While lymphocytes isolated from the brains of acutely infected rats had potent cytolytic activity *in vitro* and can recreate BDV-specific pathology after adoptive transfer into healthy, immunosuppressed BDV-infected rats, the lymphocytes isolated from brains of chronically infected rats failed to lyse BDV-infected target cells *in vitro* or to induce pathology after adoptive transfer.²⁹⁷ Furthermore, levels of IgG antibodies produced intrathecally increase during the chronic disease phase, accompanied by high titer neutralizing antibodies in peripheral blood,^{105,106} in addition to the nonneutralizing anti-N and anti-P serum antibodies generated during the acute phase.^{25,77} Although the increasing titer of neutralizing antibodies does not result in viral clearance, it is likely restricting the virus to the nervous system. Passive immunization of immunoincompetent rats restricts viral replication to cells of the central, peripheral, and autonomic nervous system, whereas rats not passively immunized showed viral replication outside of the nervous system.³¹⁵

Infection of immunoincompetent, immunosuppressed, or athymic animals does not result in overt disease, and combined with the fact that BDV does not show cytopathic effects in cultured cells suggested a primarily immune-mediated pathology in immunocompetent adult rats.^{125,203,204,318} This was further supported by the finding that adoptive transfer of spleen cells from diseased rats, or of BDV-specific CD4⁺ T-cell lines, triggered classical disease in such animals.^{203,226,249,256,257,297} Infiltrating cells in infected animals include CD4⁺ and CD8⁺ T cells, macrophages and B cells, with CD4⁺ cells accumulating primarily in perivascular cuffs and CD8⁺ cells distributing in the parenchyma.^{17,57,225} A crucial role for CD8⁺ cells has been deduced from the effects of antibody treatment against CD4⁺ and CD8⁺ cells, which led in both cases to neuroprotection and abrogation of severe immunopathology.^{17,226,317} However, the ultimate mechanisms of immune-mediated pathologic cell death remain undefined. Both CD4⁺ cell lines possessing or lacking cytotoxic capacity have been established, and although current evidence is compatible with an involvement of classical cytotoxic T-cell action,²⁰⁷ a role of indirect mechanisms elaborated through proinflammatory cytokines and their potentially cytotoxic effects, particularly in neuronal cell death, is also possible.²¹⁵ The immunopathology in adult infected rats represents a delayed-type hypersensitivity response with contribution of both CD8⁺ and CD4⁺ T cells in the immunopathologic events.

Behavioral and movement disturbances in adult infected rats have been linked to dysfunction of the dopamine (DA) neurotransmitter system.^{299,300,301–302,303} Both pre- and postsynaptic sites appear to be damaged. DA reuptake sites are reduced in the caudate-putamen and nucleus accumbens.^{302,303} DA receptor losses vary by receptor subtype and brain region. D2-receptor binding is markedly reduced in the caudate-putamen. Both D2 and D3 receptor binding are reduced in nucleus accumbens.^{300,301–302} In contrast, postsynaptic DA receptors (D1, D2, D3) are intact in the prefrontal cortex.³⁰⁰ Selective losses of D2 receptors and resultant D1 receptor hypersensitivity may be implicated in behavioral disturbances. Whereas treatment with D1 receptor antagonists such as SCH23390, or clozapine—an atypical antipsychotic with mixed D1, D2, D3 and D4 antagonist activity—reduces repetitive and self-injurious behaviors, D2-selective antagonists such as raclopride do not.³⁰¹ Neuro-

chemical studies indicate a lesion in DA transmission consistent with partial DA deafferentation and compensatory metabolic hyperactivity in nigrostriatal and mesolimbic DA systems.

In addition to disturbances in the DA system, decreased mRNA levels for somatostatin, cholecystokinin, and glutamic acid decarboxylase have been recorded in adult infected rats during the acute phase of disease, while recovery to normal levels was observed during the chronic phase.¹⁷⁴ Decreases are also evident in choline acetyltransferase-positive fibers of the cholinergic system involved in learning and memory. Losses in cholinergic fibers become evident by 6 days postinfection (dpi), and almost complete absence of choline acetyltransferase-positive fibers is reported in the hippocampus and neocortex by 15 dpi.⁸² Infected animals also respond abnormally to the opiate antagonist naloxone with hyperkinesia and seizures.³⁰⁵ Normal rats show increased levels of the endogenous cannabinoid anandamide in hippocampus and amygdala after naloxone treatment, and develop no seizures. In virus-infected rats seizures develop after naloxone treatment, while anandamide levels remain comparable to baseline levels recorded in the same structures of mock-treated BDV-infected or normal rats.²⁹⁸ Blockade of anandamide transport in infected rats prevented naloxone-induced seizures. Furthermore, levels of mRNA encoding the opioid precursor preproenkephalin were elevated in striatum of infected rats 14, 21, and 45 dpi, and virus and met-enkephalin co-localized in a high percentage of cells.^{75,304} Induction of enkephalin expression in infected cells may relate to increased levels of phosphorylated cyclic AMP response element binding (phosphoCREB) protein due to activation of the mitogen-activated protein kinase (MAPK) pathway.¹⁶² Indeed, interaction of BDV with MAPK signaling has been demonstrated in cell culture systems.^{103,227} The marked CNS inflammation in adult infected rats makes it difficult to determine to what extent monoamine, cholinergic, and opiateergic dysfunction results from direct effects of the virus, indirect effects on resident cells of the CNS, or cellular immune responses to viral gene products.

BDV Infection of the Neonatal Rat

The neonatal rat model is not characterized by overt immunopathology as described for the adult rat; instead, lifelong persistence of high virus load in the brain correlates with only mild behavioral disturbances that are compatible with the observed cerebellar and hippocampal dysgenesis.^{33,136,204} Infected animals exhibit learning deficits, increased motor activity, decreased anxiety responses, stereotypic behaviors, reduced initiation of and response to play interactions, and a preference for salt solutions.^{10,61,136,233,265} Compared to normal littermates, rats neonatally infected with BDV show an altered circadian rhythm, and have a smaller size, although food ingestion and levels of glucose, growth hormone, and insulin-like growth factor 1 appear to remain at normal levels.^{9,10,33} Neuropathologic, physiologic, and neurobehavioral features of the neonatal BDV infection are therefore suitable for exploring the mechanisms by which viral and immune factors may damage developing neurocircuitry.

Despite significant astrocytosis and microgliosis, the overall CNS architecture of neonatally infected rats appears maintained, although losses in distinct structures such as the granule cells of the dentate gyrus after formation of mossy fibers, Purkinje cell subsets of the cerebellum in a temporal pattern, and pyramidal

neurons of the cortex, are recorded.^{69,136,265,290,342,346,347} Consistent with Purkinje cell loss in the cerebellum, testing of cerebellar function demonstrated deficits in motor coordination and postural stability.¹³⁶ Neuronal loss in the dentate gyrus correlates with the severity of the spatial learning and memory deficiencies observed.^{231,265}

BDV infection in neonates does not cause immunopathology, but transient and modest immune responses are observed. Serum antibody titers measured in an immunofluorescence assay are low in comparison to adult infected rats, but persist for more than 10 months.³³ In the CNS a brief surge of T-cell infiltration is observed starting at 4 weeks and resolving by 6 weeks postinfection, paralleled by elevated expression of proinflammatory cytokine, chemokine, and chemokine receptor transcripts,^{136,248,271,272,357} whereas the observed neuropathology parallels regions and time course of microglial proliferation and expression of MHC class I and class II, ICAM, CD4 and CD8 molecules.³⁴²

Abnormal regulation of apoptosis also contributes to the disturbance of CNS architecture in neonatal BDV infection. Excitotoxic stimulation, including activation of glutamatergic circuitry, is one factor that might trigger neuronal apoptosis. There are complex alterations in mRNAs for apoptosis mediators in the hippocampus, amygdala, prefrontal cortex, nucleus accumbens, and cerebellum consistent with prolonged promotion of apoptosis throughout the brains of rats neonatally infected with BDV.¹³⁶ Levels of mRNAs for FAS and ICE (caspase-1), two promoters of apoptosis, were increased. Levels of mRNA for bcl-x, a factor that inhibits apoptosis, were decreased. Maximal shifts were observed at 4 and 6 weeks postinfection, closely paralleling the increases in proinflammatory cytokines noted earlier. Terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling (TUNEL) was shown in cerebral cortex and dentate gyrus, and in the granule cell layer of the cerebellum.^{136,342} Degeneration of cells in the hippocampal formation is related to activation of caspase 3, PARP-1, and deregulation of zinc homeostasis.³⁴⁶ Although apoptosis is described in the normally developing rat hippocampus as late as days 7 to 10 of postnatal life, it is normally not found at later time points.³²³ The anatomic location of apoptotic cells and the absence of inflammatory cells in the hippocampus and cerebellum suggest that apoptotic processes may play a more important role than cell-mediated immunity to BDV in neonatal BDV infections.

CNS dysfunction in neonatally infected animals may be linked to direct viral effects on morphogenesis of the hippocampus and cerebellum, two structures that continue to mature postnatally in rodents. BDV-induced down-regulation of the neuronal gap junction protein connexin 36 occurs first throughout the hippocampal formation and at 8 weeks postinfection also in the cerebellum; this indicates reduced electrical coupling and impaired neuronal functioning in these structures.¹⁶³ Further studies are needed to evaluate the mechanisms by which early postnatal exposure to BDV induces apoptotic losses and morphogenic damage in cerebellar and limbic circuitry.

BDV Infection of Tree Shrews

Nonacute infection in a phylogenetically higher species has been reported in the prosimian tree shrew (*Tupaia glis*). In this model, intracerebral inoculation establishes a persistent infection and

transient mild encephalitis, resulting in a disorder characterized primarily by hyperactivity and alterations in sociosexual behavior rather than motor dysfunction.³⁰⁸ Housing of animals as mating pairs revealed pronounced disturbances in social and breeding behavior.³⁰⁸ Females, not males, initiated mating and despite increased sexual activity the infected animals failed to reproduce. The behavioral changes were attributed to dysfunction of the limbic system, although neuroanatomic analyses were not performed.

BDV Infection of Nonhuman Primates

The only reported studies of experimentally infected primates employed adult immunocompetent rhesus macaques (*Macaca mulatta*). Following intracerebral infection, an acute neurologic syndrome ensued, during which animals were initially hyperactive and subsequently became apathetic and hypokinetic, similar to BDV-infected adult rats. Pathologic changes were remarkable and severe meningoencephalitis and retinopathy were observed.^{34,205,223,314,358}

BDV Infection of Mice

Adult mice develop high virus titers in the CNS after BDV inoculation, but most strains do not develop encephalitis.¹⁴⁸ However, disease may be induced by infection of certain mouse strains during the neonatal period¹⁰⁰ or by adaptation of virus through multiple passages in mice.²⁶⁶ Serial passage results in adaptive mutations in the viral polymerase that may contribute to pathogenicity.² The reason for the different course of experimental BDV infection in adult vs. newborn mice, and in rats, remains unclear, but might be associated with the time point of antigen presentation in the periphery.¹⁰⁷

The incidence and severity of BDV-induced clinical manifestations varies considerably between different mouse strains. Similar to the adult rat model, occurrence of clinical signs is associated with immune cell infiltration in the brain and glial activation, mainly in the cerebral cortex and hippocampus. An immunopathologic syndrome mediated by MHC class I-restricted CD8⁺ T cells in a CD4⁺ T-cell-dependent manner is most pronounced in mouse strains such as MRL with the MHC H-2k allele.^{100,108} This MHC I-haplotype is associated with severity of clinical disease, but the incidence of disease is most likely associated with other, to date unknown, genetic factors.¹⁰⁰ The BDV N peptide T₁₂₉ELEISSI was the dominant epitope shown to sensitize cytotoxic T cells.²⁷⁵ Transgenic expression of BDV-N in neurons or astrocytes in B10.BR mice did not result in clinical disease and prevented induction of BDV-N specific CD8⁺ T-cells.²⁴⁷ Moreover, downregulation of the functional avidity of virus-specific CD8⁺ T cells in experimentally infected mice seemed to be involved in controlling the inflammatory reaction and facilitating viral persistence.⁷¹ Overexpression of cytokines such as IL-12 or TNF in mice less susceptible to BDV-induced disease sensitize the mice to develop an immune-mediated disorder and clinical disease, for example, epileptic seizures in the case of TNF overexpression.^{73,100,130,164} The IL-12 effect seems to be mediated via IFN- γ , which also exerts neuroprotective effects in the mouse model.^{109,130}

A behavioral syndrome similar to that of neonatally infected rats has been described after expression of an individual BDV gene product in transgenic mice. High glial expression of BDV-P decreases synaptic density, serotonin receptors, and levels of brain-derived neurotrophic factor (BDNF), resulting

in behavioral disturbances.¹⁴⁵ Thus, at least one viral product interferes with neural function (see below), but in natural disease, replication of the virus itself and other viral components are also likely to contribute.²³⁶

Tissue Culture Models

Studies in cell culture systems are beginning to provide further insights into the mechanisms by which BDV interferes with cellular functions and induces neuropathology. Interference with basic cellular signalling pathways and modulation of cellular protein functions through direct binding by, or indirect interaction with, individual viral proteins has been recognized. The N-terminal portion (aa 13–171) of BDV N binds to the Cdc2-cyclin B1 complex and delays cell cycle progression in primary rat and mouse fibroblasts.²²⁸ Direct protein-protein interaction was also shown between BDV P and the neurite outgrowth factor amphoterin (also designated HMG1).¹⁴⁶ Complex formation between amphoterin's A-box and BDV P (aa 77–86) leads to competitive inhibition of p53 binding to amphoterin, resulting in downregulation of cyclin G promoter activity.³⁵⁵ Through interference with the transcriptional activity of p53, BDV may affect cell-cycle regulation as well as apoptosis. BDV P interaction with amphoterin also causes altered intracellular distribution of amphoterin in infected cells, leading to an inhibition of neurite outgrowth and migration that has been ascribed to interference with the normal interaction between amphoterin and its receptor RAGE (Receptor for Advanced Glycation End-products).¹⁴⁶ Studies of neuronal differentiation of PC12 cells indicate an interaction of BDV with cellular MAPK signaling pathways.¹⁰³ Persistently infected PC12 cells demonstrate constitutive phosphorylation of MAPK/ERK kinase (MEK), extracellular signal-regulated kinase (ERK), and the E 26 (ETS)-like transcription factor 1 (Elk-1); however, nuclear translocation of ERK is impaired, contributing to the failure of the cells to differentiate with nerve growth factor (NGF) treatment. Activation of MAPK signaling is evident within 1 hour after acute infection, suggesting that gene expression is not required for BDV's activation of the MAPK cascade, whereas sustained MAPK activation appears essential to virus transmission. Chemical blockade of MEK inhibited virus spread to neighboring cells.²²⁷ BDV impairment of ERK-mediated neurotrophin signaling also modulates synaptic functioning by interfering with synaptogenesis and synaptic protein synthesis.¹⁰² Analogous to findings in the neonatal rat,⁸⁵ BDV infection of neuronal cells specifically downregulates the expression of proteins related to synaptic plasticity, such as synaptophysin and growth-associated protein 43 (GAP-43).¹⁰³ In addition, infection interfered with synaptic activity by inhibiting synaptic vesicle recycling in response to stimulus-induced potentiation in hippocampal neuronal cultures.³³⁰ Several cellular kinases participate in the phosphorylation events involved in synaptic vesicle recycling, including protein kinase C (PKC), the epsilon isoform of which phosphorylates BDV P.²⁸⁹ Thus, it is conceivable that the high levels of BDV P that are expressed in the cytoplasm as well as in the nucleus of BDV-infected cells may act as a decoy substrate for PKC, analogous to the BDV P phosphorylation by Traf family member-associated NF- κ B (TANK)-binding kinase 1 (TBK-1) that results in the suppression of TBK-1-dependent interferon (IFN) expression in non-neuronal cells.³²⁴ However, species-specific differences appear to exist in the susceptibility of BDV to the antiviral action of

IFN γ . Human IFN γ efficiently prevented BDV infection of human and monkey cell lines, whereas rat IFN interfered only ineffectively with the infection of rat cell lines or rat hippocampal slice cultures.²⁷³ Moreover, experiments in mouse brain slice cultures indicated a tissue-specific action of IFN γ , with a more pronounced effect of IFN γ on BDV proliferation in cerebellar than in hippocampal cultures.⁷⁴

NATURAL INFECTION WITH BDV

Clinical Signs

In the main natural hosts, horse and sheep, infection with BDV typically causes a severe neurologic disorder.^{29,30,67,89,91,113,139,179,181,193,253,262} Death within 1 to 4 weeks after onset of clinical signs is common. However, inapparent infections occur more frequently in both horses and sheep.^{120,199,253} Clinical recovery or recurrence of acute disease is observed only rarely.^{46,67,89,91,113,149,190,193,223,253} A few animals may also develop a chronic disease course with low-level viral persistence. The incubation period for natural BD has been estimated to range from 2 weeks to several months.^{181,262} This is substantiated by recent cases of natural BD in a horse in the United Kingdom and an alpaca in Germany that had likely been infected in their endemic home areas before transport to the new housing areas outside endemic regions, where clinical signs were noted approximately 3 to 4 months later.^{140,242} In experimental studies, the incubation period for 3 ponies after intracerebral inoculation was 15 to 26 days.¹⁴⁹

Neurologic signs of BD in horses may vary between individual animals depending on the brain area affected by inflammatory lesions and on the course of the disease (Fig. 39.3).^{67,89,91,181,253,358} Clinical hallmarks are simultaneous or consecutive changes in psyche, sensorium, sensibility, motility, and in the autonomous nervous system.^{67,89,91,113,181,253,262,358,361} Recurrent therapy-resistant fever, lethargy, somnolence and stupor, hyperexcitability, fearfulness, and aggressiveness accompanied by repetitive motor activities, slow-motion eating, and “Pfeifenrauchen” (pipe smoking, eating arrest with chewing movements, Fig. 39.3A) are typically recorded. Disturbances of mental status could be attributed to impairment of the limbic system, mainly the hippocampus, which usually shows the most pronounced inflammatory lesions.^{15,89,91,117,253} With disease progression, postural instability, unawareness, hyporeflexia, hypokinesia, head tilt, hypoesthesia with disturbances in proprioceptive sensory functions, and ataxia can appear (Fig. 39.3B). Finally, food intake ceases; torticollis, compulsive circular walking, head tremor with subsequent convulsions and head pressing, coma, and possibly blindness are characteristic (Fig. 39.3C).^{15,91,126} Depending on brain inflammation localization, cranial nerve disturbances might lead to dysphagia, salivation, trismus, and facial nerve paresis, as well as nystagmus, strabismus, or miosis.^{91,253} Besides neurologic signs, a variety of atypical symptoms have been reported, for example, mental, behavioral or gait disturbances, recurrent colic, emaciation, and chronic lameness.^{12,22,58,66,178}

Sheep can develop similar clinical signs as horses, with disturbances in behavior and movement.³⁰ The few cases of natural BD in cattle exhibited a classical neurologic disorder analogous to horses.^{21,31} Rabbits, analogous to experimental



FIGURE 39.3. Clinical signs of Borna disease in horses in different stages of disease. A: Early stage of BD. The BDV-infected horse exhibits somnolence and eating arrest with chewing movements (so-called “Pfeifenrauchen” or “pipe smoking”). **B:** More advanced stage of BD. The BDV-infected horse displays abnormal posture as sign of disturbed proprioception and facial nerve paralysis. **C:** Final stage of BD. The BDV-infected horse presents neurogenic torticollis and compulsive circular walking. (From Richt JA, Grabner A, Herzog S, et al. Borna disease in horses. *Vet Clin North Am Equine Pract* 2000;16:579–595, with permission.)

infection, develop an acute and fatal paralytic disease accompanied by blindness.^{181,192,212,262}

Pathogenesis

In horses, gross findings are mostly sparse and consist of leptomeningeal hyperemia, brain edema, or hydrocephalus internus in later disease stages.^{113,149,358,361} Viral antigen and RNA are present in neurons, astrocytes, and occasionally in ependymal cells and oligodendrocytes as described for experimental infections of rodents (Fig. 39.4). In contrast to the variability of lesion severity and affected organs in ABV infections (see below), histopathologic changes after BDV infection are similar in all mammalian species and mainly restricted to gray matter areas of the CNS, spinal cord, and retina. A severe nonpurulent poliomeningoencephalomyelitis with perivascular and parenchymal immune cell infiltrates accompanied by activation of astrocytes and microglia is characteristic. Diagnosis can be completed if pathognomonic intranuclear “Joest-Degen” inclusion bodies are present in neurons (Fig. 39.5). A loss of pyramidal cells of the hippocampus is noted occasionally but neuronal necrosis and neuronophagia are not regular features. The main affected brain areas are the olfactory bulb, basal cortex, caudate nucleus, thalamus, hippocampus, and periventricular areas of the medulla oblongata.

Despite the detection of viral antigen and infectious BDV in the retina in most affected horses, the retina is microscopically mostly unaffected, indicating central blindness.^{15,117} However, degeneration of retinal neurons resulting in blindness has been reported.⁶⁰ In contrast, BDV-infected rats and rabbits typically develop blindness caused by a nonpurulent chorioretinitis with degeneration of rods and cones.^{168,203,204}

Naturally infected horses display a similar composition of infiltrating immune cells (macrophages, CD4⁺ and CD8⁺ T cells, and plasma cells in chronic stages) and increased expression of MHC class I- and class II-antigens as described for experimentally infected rats.^{16,29,57,117} Thus, it can be assumed that the underlying neuropathogenesis is also immunopathologic in naturally infected hosts.

NATURAL INFECTION WITH ABV

Clinical Signs

Reports of natural cases and experimental studies provide evidence that ABV infections are the cause of PDD; ABV has been detected in 60% to 100% of PDD-affected birds investigated.^{79,92,112,131,132,152,213,218,246,259,309,340} Interestingly, ABV infections, as shown either by detection of viral RNA and/or

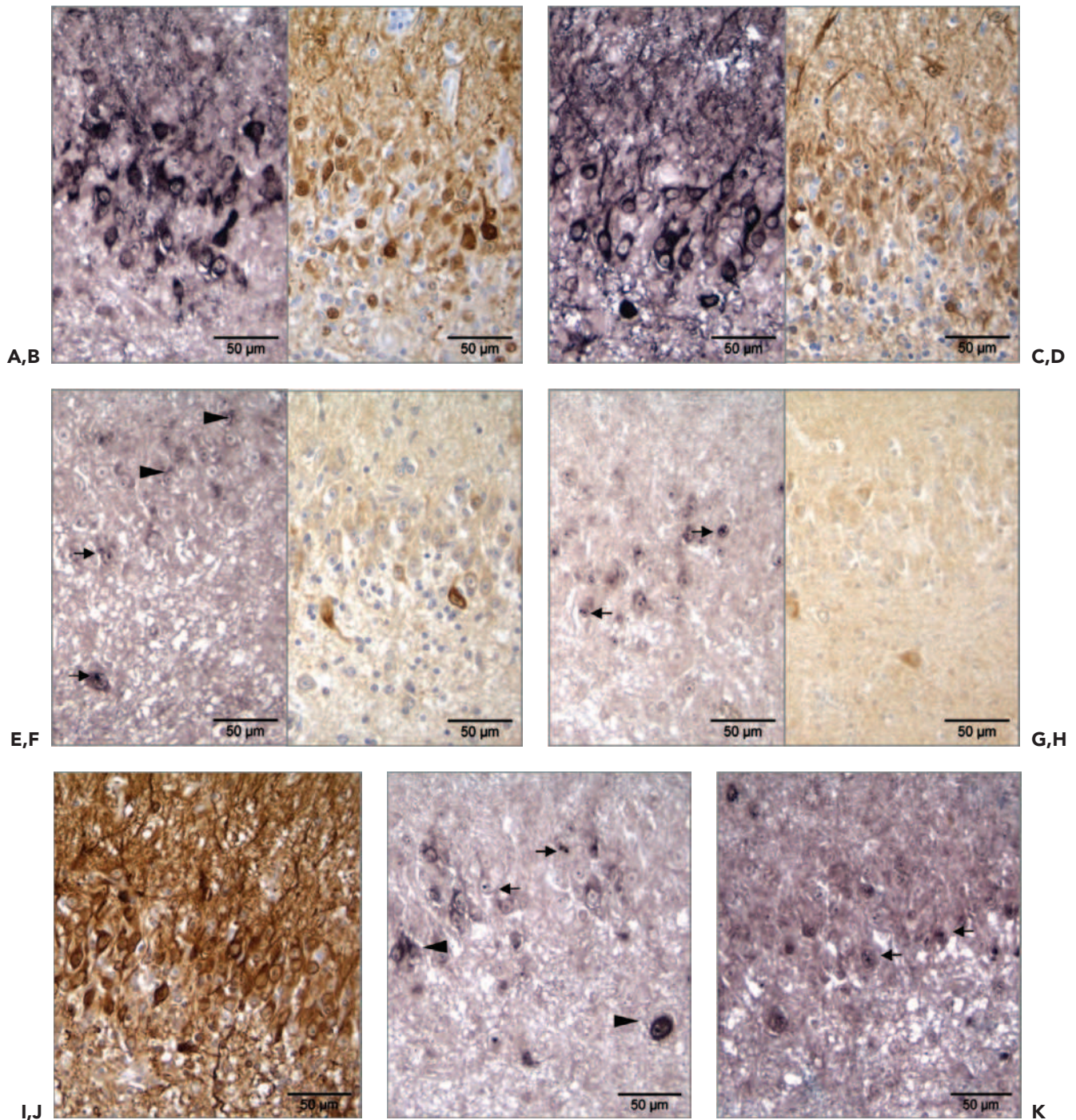


FIGURE 39.4. Demonstration of BDV RNAs and proteins in a horse with typical BD by *in situ* hybridization and immunohistochemistry. **A, C, E, G, J, K:** Demonstration of BDV-specific RNAs by *in situ* hybridization (ISH). **B, D, F, H, I:** Demonstration of BDV-specific proteins by immunohistochemistry (IHC). **A:** Widespread BDV N mRNA mainly in the cytoplasm and processes of neurons. **B:** Widespread BDV N (monoclonal antibody Bo 18) in the cytoplasm and nuclei of infected neurons and in the neuropil. **C:** Widespread BDV P mRNA mainly in the cytoplasm and processes of neurons. **D:** Widespread BDV-P (polyclonal monospecific anti-BDV P antibody) in the cytoplasm and a few nuclei of neurons and in the neuropil. **E:** Demonstration of BDV M mRNA in the cytoplasm (*arrowhead*) and few nuclei as dot-like signal (*arrow*) in some neurons. **F:** Detection of the BDV M (polyclonal anti-BDV M antibody) mainly in the cytoplasm of some neurons. **G:** Demonstration of BDV G mRNA mainly in the nuclei as dot-like signal (*arrow*) in some neurons. **H:** Detection of the BDV G (monospecific polyclonal anti-BDV G antibody) only in the cytoplasm of a few neurons. **I:** Detection of BDV X (polyclonal anti-BDV X antibody) mainly in the cytoplasm of neurons and the neuropil. **J:** Demonstration of BDV L mRNA in the cytoplasm (*arrowhead*) and nuclei as dot-like signal (*arrow*) in some neurons. **K:** Demonstration of genomic BDV RNA mainly in the nuclei as dot-like signal (*arrow*) in some neurons. N, nucleoprotein; P, phosphoprotein; X, X protein; M, matrix protein; G, glycoprotein; L, polymerase; Bar, 50 μm . (From Herden C, Richt JA. Equine Borna disease. *Equine Vet Educ Manual* 2009;8:113–127, with permission.)

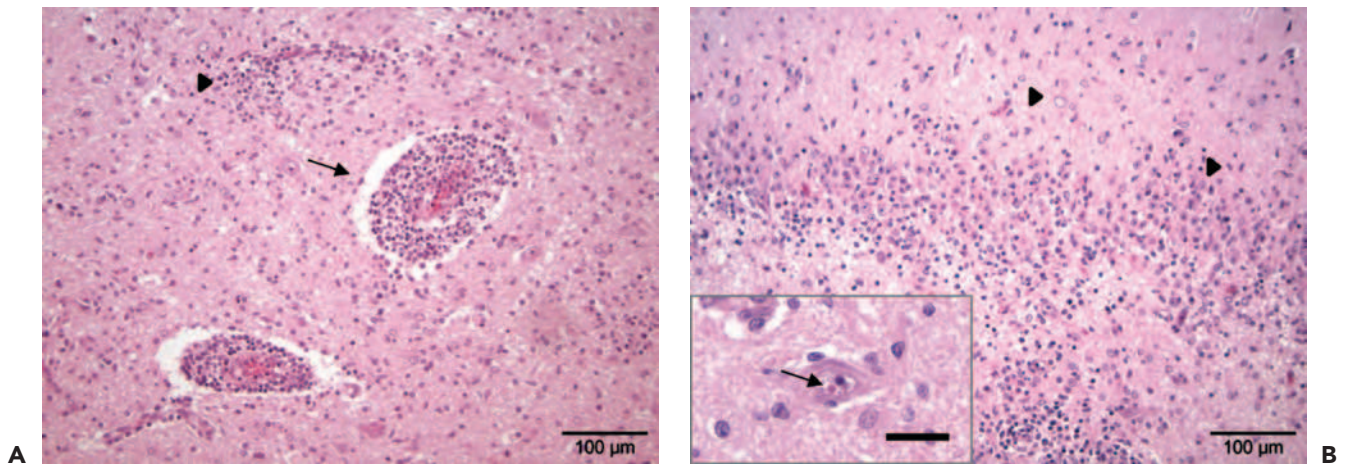


FIGURE 39.5. Characteristic histopathologic lesions in a horse suffering from BD. **A:** Severe perivascular (arrow) and moderate parenchymal (arrowhead) mononuclear immune cell infiltrates in the CNS. **B:** Moderate to severe parenchymal immune cell infiltrates with astrogial and microglial activation (arrowhead) in a more advanced stage of BD. **Insert:** Intranuclear Joest-Degen inclusion body (arrow). **Bar:** a, b: 100 μ m, **Insert:** 25 μ m. (From Herden C, Richt JA. Equine Borna disease. *Equine Vet Educ Manual* 2009;8:113–127, with permission.)

viral antigen, were also found in psittacines without clinical signs of PDD.^{50,111,119,132,172,218,327,352} Clinically healthy animals that only show ABV-specific antibodies are also described.

PDD was initially reported in macaws in the late 1970s and several synonyms—for example, macaw wasting disease, neuropathic gastric dilatation of psittacines, and myenteric ganglioneuritis—have been used.^{14,62,95,185} Up to now, PDD has been found in approximately 60 psittacine species that belong to 20 different genera, but also in some nonpsittacine species.^{48,55,95,132,222,343} Large parrots, including endangered species, seem to be affected most frequently and severely. Typical clinical signs of PPD are GI dysfunction and associated wasting with or without neurologic signs.^{14,95,132,185,309} Affected animals develop decreasing GI motility until stasis resulting in anorexia, lethargy, undigested seeds in the feces, regurgitation, diarrhea, weight loss leading to cachexia, and vomiting. Finally, affected birds die. Interestingly, there are also cases displaying only neurologic signs such as depression, ataxia, tremor, seizures, and motor or proprioceptive deficits. Blindness has been described in a few cases,³¹¹ and the mortality rate approaches 100%.⁹⁵

Pathogenesis

The incubation period of natural ABV infection is unknown and varied in experimental infections from approximately 20 to 60 days, up to 200 days.^{79,92,218,224} In these experiments, ABV-specific serum antibodies were detected between 7 and approximately 60 DPI and ABV RNA was detected in swabs between 20 and 72 DPI, depending on the route of inoculation (intracerebral, intramuscular, intravenous) and the bird species used, with the earliest detection of virus after intracerebral infection in cockatiels.^{79,92,218,224} Surveillance studies in aviaries with PDD cases revealed that birds displaying high viral RNA loads and high ABV-specific antibody titers are more likely to develop clinical PDD.¹¹¹ It should be noted that, similar to BDV infection, ABV-specific antibodies do not have any protective effect.^{111,218,224} A wide variation in the

clinical status of infected animals has been noted, including healthy animals that show typical histologic lesions at necropsy, clinically inconspicuous birds with widespread ABV distribution or restriction of viral RNA to the nervous tissue and any variation thereof.^{50,172,224,246,327,352} Asymptomatic carriers may contribute to virus dissemination. However, animals that did not become infected despite close contact to ABV-infected birds also exist.^{111,246}

Typical gross findings consist of emaciation with atrophy of pectoral muscles, dilatation or rupture of the proventriculus, and atrophy of the proventricular muscle (Fig. 39.6).^{95,185} Clinically manifest PDD is associated with characteristic mononuclear (lymphocytes, macrophages, plasma cells) infiltrates in ganglia and nerves of the enteric autonomous nervous system of crop, proventriculus, gizzard, and duodenum and, in most cases, non-purulent encephalitis, myelitis and/or ganglioradiculoneuritis of spinal nerves (Fig. 39.7).^{14,95,132,150,153,213,224,246,352} Gliosis and inflammation in peripheral nerves, myocardium, cardiac conductive tissue, and adrenal glands or eyes may also occur.

ABV shows a strong affinity for nervous tissue comparable to BDV, but broad peripheral tissue distribution is also seen. ABV RNA or antigen is most frequently found in the brain, spinal cord, and GI tract (crop, proventriculus, gizzard, small intestine) but often also in many other organs, for example, adrenal gland, eye, heart, liver, kidney, spleen, pancreas, lungs, gonads, thyroid, and skin (Fig. 39.8).^{119,153,218,224,246,259,340,341,352}

Many factors may contribute to the variability of the clinical picture and *in vivo* dissemination of ABV. Viral properties and host-specific features such as species, genetic background, age, immune competence, and the extent of contact with ABV-infected animals may influence the outcome of infection. It is unknown whether clinically apparent disease is due to similar immunopathologic processes as those in BD. Although similar inflammatory infiltrates and possibly virus persistence occurs, striking differences between ABV and BDV infection exist regarding the variability of clinical outcome, tissue tropism, and virus distribution.



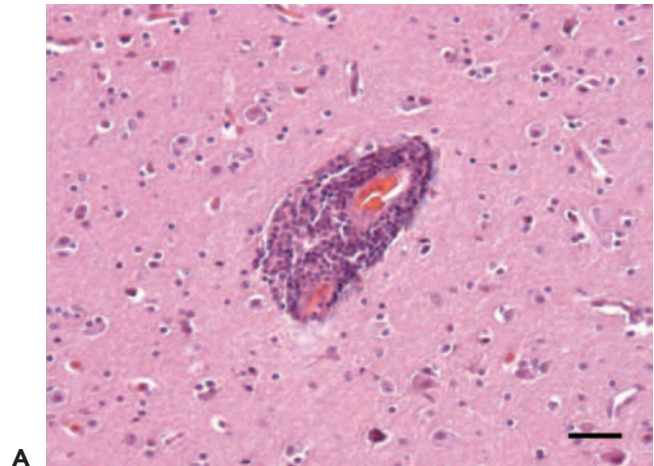
FIGURE 39.6. Gross findings in an African grey parrot suffering from proventricular dilation disease (PDD). Note the thin-walled, filled and dilated proventriculus (arrow). (Courtesy of A. Piepenbring and M. Lierz, Clinic for Birds, Reptiles, Amphibians and Fish, Faculty of Veterinary Medicine, Justus Liebig University Gießen, Germany.)

DIAGNOSIS

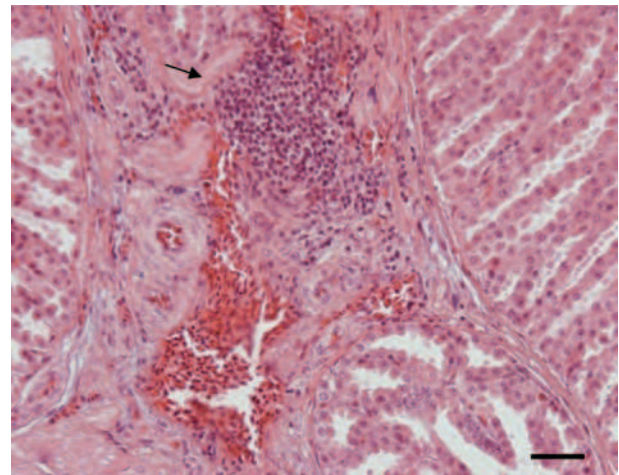
Differential Diagnoses

Neurologic signs of BD of mammals can be complex and variable so that a variety of CNS infections can result in a comparable clinical picture. Due to this lack of specificity, further laboratory tests are needed for a confirmed diagnosis. The differential diagnosis includes other neurotropic virus infections such as equine herpesviruses,³⁴⁸ rabies,⁹³ tick-borne encephalitis,¹⁹⁸ as well as bacterial diseases such as botulism³⁴⁵ and bacterial meningitis,⁷² and parasitic infections such as verminous myeloencephalitis⁶⁴ and equine protozoal myeloencephalitis (EPM).¹⁸³ In certain geographic regions, arthropod-borne flaviviruses (e.g., West Nile virus) and alphaviruses (e.g. western, eastern, Venezuelan equine encephalitis viruses) must also be considered.

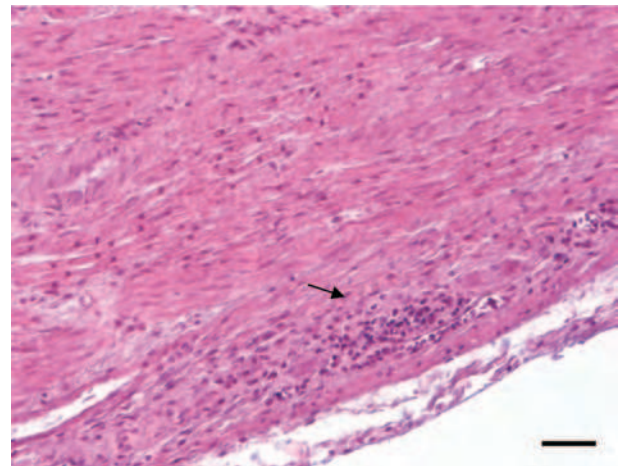
Reliable *ante mortem* diagnosis of PDD and ABV infection has been problematic since clinical signs resembling PDD can also occur in other diseases such as bacterial, parasitic, or mycotic infection of the GI tract, ingestion of foreign bodies, intoxications or neoplasia, and the detection of ABV might be only intermittently possible.^{95,112,132,218} Thus, ABV infection and a diagnosis of PDD have to be confirmed by laboratory investigation.



A



B



C

FIGURE 39.7. Typical histopathologic lesions in PDD. **A:** Perivascular lymphohistiocytic infiltrate in the brain. **B:** Severe lymphohistiocytic infiltrate in the proventriculus (arrow). **C:** Lymphohistiocytic infiltrate in and around ganglia of the gizzard (arrow). Bar: 50 μ m.

Intra Vitam Diagnosis

BD can be confirmed by the demonstration of BDV-specific antibodies in the serum and CSF,^{89,91} or BDV-specific antigens, RNA, or virus in blood or CSF specimens. As serologic test

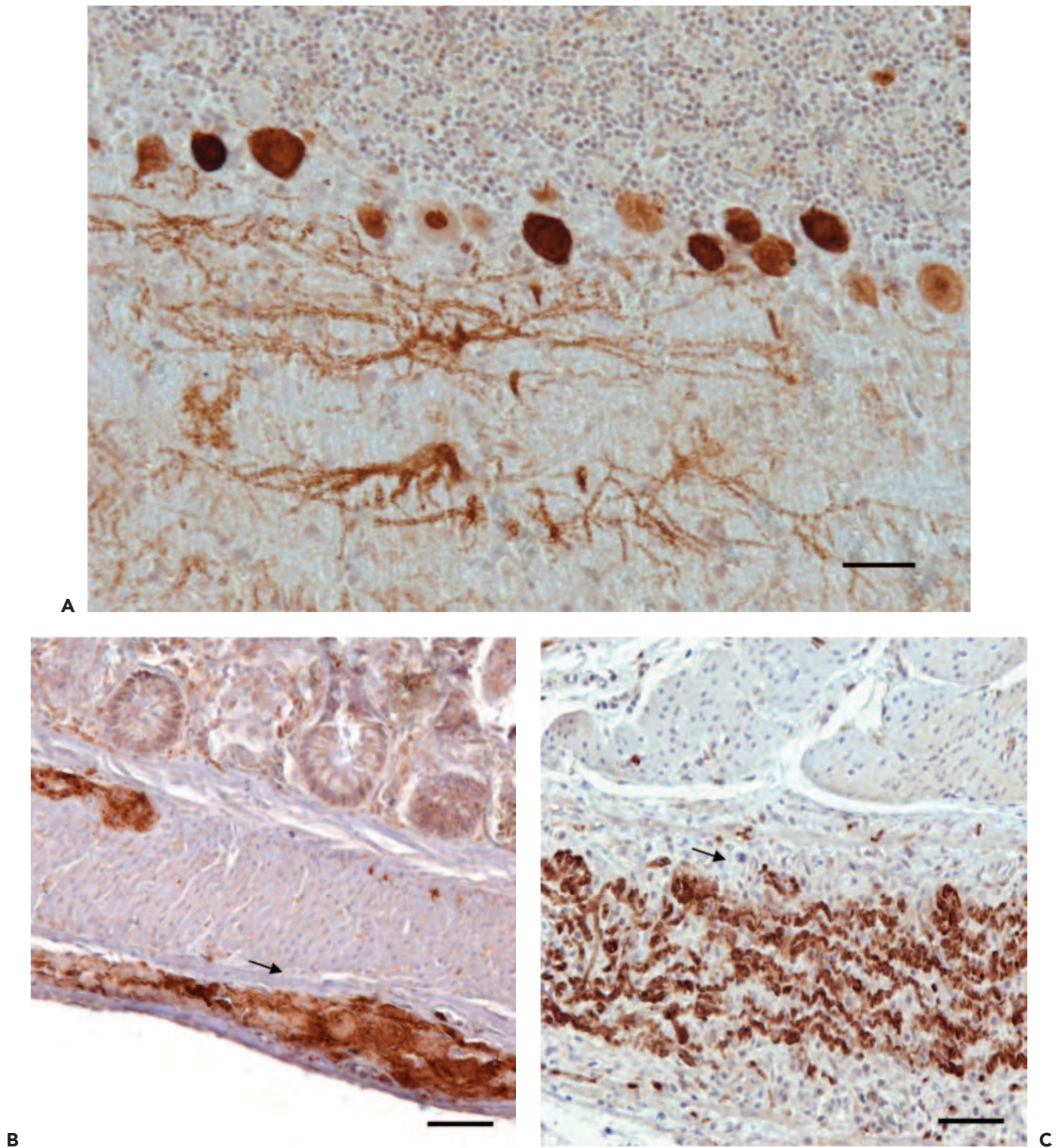


FIGURE 39.8. Demonstration of ABV phosphoprotein (P) by immunohistochemistry (IHC) in psittacines. A: Expression in the nucleus, cytoplasm, and processes of numerous Purkinje cells and a few granule cells in the cerebellum. **B:** Expression in ganglia (arrow) of the small intestine. **C:** Expression in nerve fibers (arrow) of the gizzard. **Bar:** 50 μ m.

systems, Western blot (WB) analysis, enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescence assay (IFA) have been established (Fig. 39.9).^{23,91,120,122,124} IFA with BDV-infected and control cells is considered to be the most reliable method for the detection of BDV-specific

antibodies with high sensitivity and specificity.¹²² Titers of BDV-specific antibodies vary widely from 1:2 to 1:1280 in serum and CSF, and do not correlate with the clinical course of the infection.^{91,120} In very early stages of acute BD, or after treatment with corticosteroids, BDV-specific antibodies may

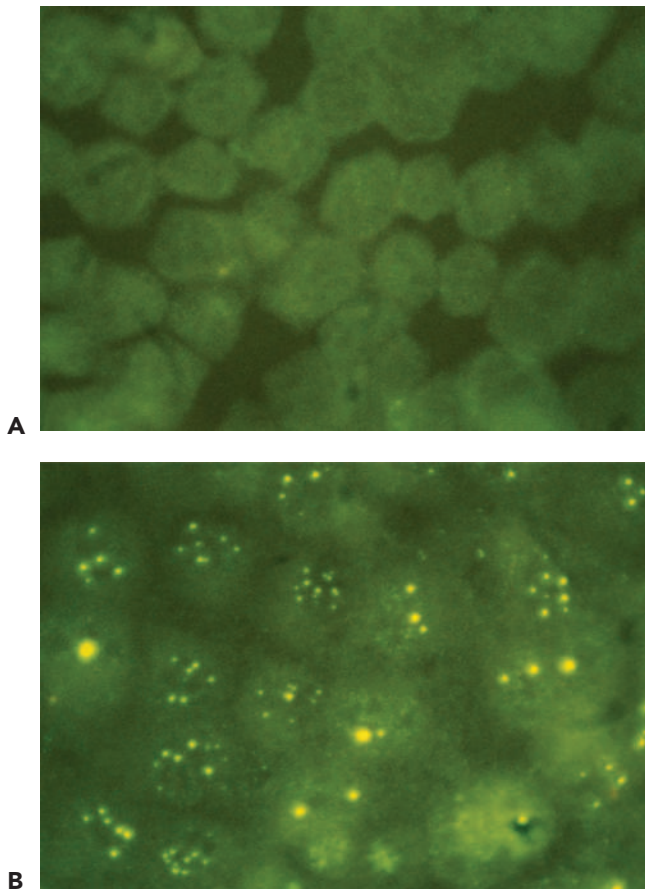


FIGURE 39.9. Indirect immunofluorescence assay (IFA) for the demonstration of BDV-specific antibodies in horse sera employing Madin-Darby canine kidney (MDCK) cells. **A:** BDV-positive serum incubated with uninfected MDCK cells. **B:** BDV-positive serum incubated with BDV-infected MDCK cells. (From Richt JA, Grabner A, Herzog S, et al. Borna disease in Equines. In: Sellon DC, Long M, eds. *Equine Infectious Diseases*, St Louis: Saunders Elsevier, 2007:201–216, with permission.)

not be detectable. Clinically healthy horses can have BDV-specific antibodies in the serum but not in the CSF.^{91,120} In acute BD, the quantity of CSF protein content can be elevated and a mononuclear pleocytosis is regularly present.⁹¹ Evidence of infection including BDV RNA or BDV antigen in PBMC, or circulating immune complexes, has been presented by some investigators,^{23,58,200} but has been questioned by others.^{91,122} BD is reliably diagnosed *ante mortem* by neurologic signs combined with CSF pleocytosis and BDV-specific antibodies in serum and/or CSF.

As with BD in horses and sheep, signs and symptoms of PDD are not pathognomonic of ABV infection. Dilatation of the proventriculus and possibly other parts of the upper GI tract can be visualized by radiography/imaging techniques.⁵⁶ A definite diagnosis of PDD has to be substantiated by histopathologic examination of upper GI tract biopsies, which is hampered by the inconsistent distribution of lesions. Only the presence of mononuclear infiltrates of ganglia are confirmatory. In one study, crop biopsies revealed false negative

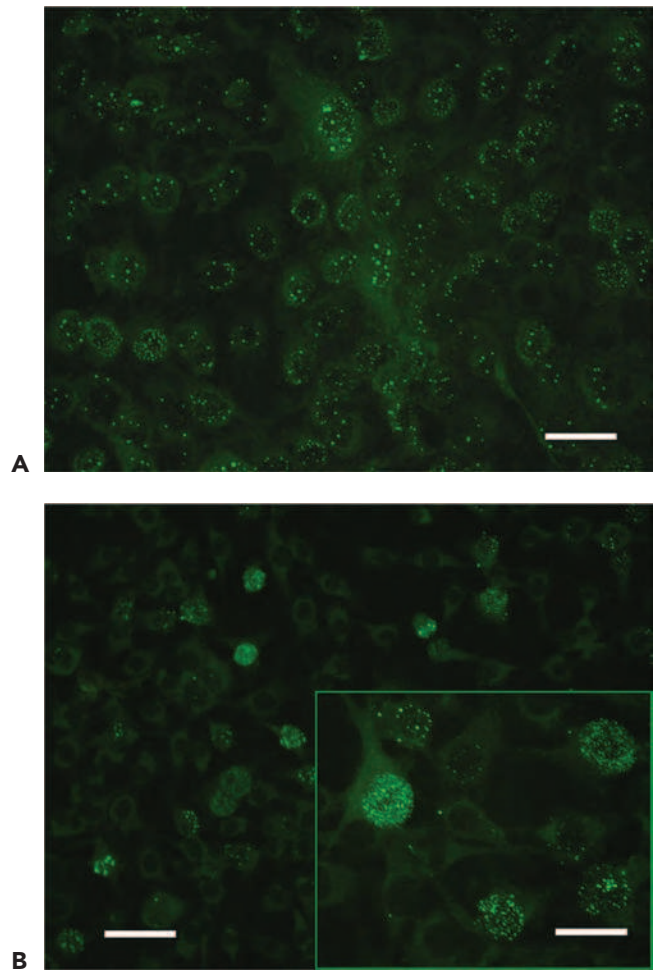


FIGURE 39.10. Indirect immunofluorescence assay for the demonstration of ABV-specific antibodies. **A:** IFA for demonstration of ABV-specific antibodies employing BDV-infected MDCK cells. Note the brilliant granular fluorescence in the nucleus. **Bar:** 50 μ m. **B:** IFA for demonstration of ABV-specific antibodies employing ABV-infected CEC cells. Note the brilliant granular fluorescence in the nucleus. **Bar:** 100 μ m; **Insert:** 50 μ m. (From Herzog S, Enderlein D, Heffels-Redmann U, et al. Indirect immunofluorescence assay for intra vitam diagnosis of avian bornavirus infection in psittacine birds. *J Clin Microbiol* 2010;48:2282–2284, with permission.)

results in approximately 24% of cases.⁹⁴ ABV-specific serum antibodies can be demonstrated by WB assay,^{50,172,327} ELISA,⁵⁰ and IFA (Fig. 39.10).^{92,119} ABV infection has also been diagnosed by detection of ABV RNA in feces, swabs of crop and cloaca, blood, and feather calami using RT-PCR.^{49,92,153,172,259} The diversity of different ABV genotypes may require several RT-PCR assays. ABV genotypes 4 and 2 have been detected most frequently.^{79,92,153,194,224} Due to the possible intermittent presence of RNA in swabs and presence of ABV RNA and/or ABV-specific antibodies in clinically healthy virus carriers, repeated testing and the combined demonstration of ABV-specific serum antibodies and ABV RNA in crop and cloacal swabs currently represent the most reliable diagnostic approach.^{50,111,119,153,172,327}

Postmortem Diagnosis

For the postmortem diagnosis of natural BD, typical histopathologic lesions (Fig. 39.5), infectious virus and/or viral proteins and RNA in the CNS are usually demonstrated. Viral proteins are demonstrated by monoclonal or polyclonal antibodies recognizing the BDV N, P, M, X, or G proteins in immunohistochemical (IHC) approaches (Fig. 39.4) or WB analysis.^{15,91} Histopathology, IHC, WB, and nested RT-PCR gave identical diagnostic results in a comparative study of over 150 horses with or without BD.¹¹⁷ Furthermore, isolation of infectious BDV and demonstration of BDV RNA by *in situ* hybridization (ISH) (Fig. 39.4) can be used with adequately preserved tissue specimens.

Postmortem investigations in PDD show typical histopathologic lesions predominantly in the GI tract and CNS (Fig. 39.7). ABV RNA can be demonstrated not only in the nervous system and GI tract, but often in many other tissues.^{92,112,131,152,153,172,246,259,327,352} ABV RNA may be visualized by ISH³⁴¹ and ABV protein by IHC, applying either cross-reactive anti-BDV antibodies against the N, P, or X-protein, or ABV-specific antibodies against the N protein (Fig. 39.8).^{119,213,224,246,259,340,352} However, the monoclonal anti-BDV N antibody Bo18 that is widely used for diagnosis of mammalian BDV infections consistently gives negative results with ABV. Virus isolation has been successful in the quail cell lines CEC-32 and QM7^{119,259} or duck embryo fibroblasts.⁹²

THERAPY AND CONTROL

Vaccination

Although there is only limited experience with vaccines in BD, better success has been reported with live attenuated than with killed virus vaccines.^{46,210} A lapinized live vaccine³⁶² was used for many years in the eastern federal states of Germany. Because efficacy was questionable, and there were concerns regarding the possible post-vaccination shedding of infectious virus as well as potential establishment of a persistent virus reservoir, the vaccine was abandoned in 1992.^{196,287} High virus titer may be important for the successful implementation of live vaccines.^{196,287} Cell-culture attenuated BDV protected rats against intracerebral challenge with a virulent inoculum only when administered at high titer (10^5 – 10^6 vs. 10^2 – 10^4).²¹⁰ Similarly, a high dose of another extensively passaged virus resulted in strong humoral and cellular immunity. A lower dose inoculum did not provide this protection.⁷⁶ Neither of these studies investigated virus shedding by the vaccinated animals or the biology of progeny virus.

There have been several efforts to develop recombinant BDV vaccines. A vaccinia virus recombinant expressing BDV N primed rats for enhanced viral clearance after challenge, but also for aggravated disease due to increased immunopathology.¹⁷¹ More recently, expression of BDV N by a parapoxvirus vector system was reported to protect rats from challenge.¹¹⁵ No data are available on vaccines based on BDV G; however, the observation that a monoclonal anti-G antibody was protective in rats suggests that such an approach could be successful.⁷⁷

Therapeutics

Amantadine sulfate (AS), a drug with antiviral activity against influenza A,¹¹⁰ inhibits replication of certain human and horse

BDV isolates in cell culture and improves the clinical course in some subjects with affective disorders.^{19,20,59} However, the efficacy of AS for treatment of persistently BDV-infected cell cultures and animals is controversial.^{41,101,316} In a small clinical study of horses with acute BD, AS (2 mg/kg orally) had no effect in 8 out of 9 animals.⁹⁰

Ribavirin inhibits transcription and replication of both He/80 and strain V BDV in a variety of cell lines.^{143,195} In addition to its antiviral effects through depletion of cellular GTP pools, interference with mRNA capping, and direct interaction with viral polymerases, ribavirin can promote a Th₁-type immune response.^{137,319} This led to concerns that enhancement of cellular immunity might aggravate immunopathology of BD.^{104,171,297} In one study, the efficacy of ribavirin administered directly to the brain by intraventricular injection was assessed in the rat model. Although ribavirin had no effect on viral load, treated animals had less inflammation and milder disease presumably due to antimitotic effects on microglia.³⁰⁶ In another animal model, the Mongolian gerbil, in which BDV can cause direct neuronal damage independent of immunopathology,³³⁶ ribavirin at a higher dosage than tolerated in the rat model reduced virus load in the brain.¹⁷⁰

1- β -D-arabinofuranosylcytosine (Ara-C) inhibits BDV replication in cultured cells and inhibits viral replication, improving clinical outcome in infected rats.^{6,47} Ara-C is a DNA polymerase inhibitor and has no effect on polymerases of influenza or measles viruses, but has antiviral activity against rabies virus.²⁸ In BDV, Ara-C appears to act as a competitive inhibitor of cytidine.³²⁹ A related cytosine nucleoside, 2'-fluoro-2'-deoxycytidine, shows similar antiviral activity *in vitro* and may be preferable due to its reduced cytotoxicity.⁷

At present, there is no curative therapy for PDD or ABV infection; symptomatic treatment is the only option. Methods for ABV infection control and prevention include appropriate management practices in aviaries such as isolation of ABV-infected birds, sanitation, and disinfection.¹³² Control of traffic and trading may help to impede spread in the pet bird population.

PERSPECTIVES AND PUBLIC HEALTH CONSIDERATIONS

In the last 20 years, substantial progress in BD research has been achieved regarding the molecular characterization of the causative agent, underlying immunopathogenesis, mechanism of viral persistence, and natural distribution of BD. However, we still know little about its ecology and epidemiology, especially the identity of reservoirs and mechanisms for natural transmission. Also, a novel bornavirus, ABV, was discovered in a variety of avian species. The worldwide epidemiology of BD in various warm-blooded animals remains controversial. Virulence of the virus and genetic predisposition of the host or other exogenous and endogenous factors might play a role in the outcome of infection. Although the genomic fossil record indicates infection of primates, including human ancestors up to 40 million years ago, there is no convincing evidence of human infection. The intriguing properties of BDV are providing insights into cellular trafficking, neural circuitry, neural

development, and immunopathology of CNS infections. Further investigations will improve our understanding of the complexity of the disease mechanisms, BDV-associated disorders, and the epidemiology of BD.

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Orthomyxoviridae

Classification

Virion Structure

Genome Structure and Organization

Influenza Viruses

Thogoto Virus

Infectious Salmon Anemia Virus

Quarantilla Virus

Stages of Viral Replication

Mechanism of Attachment

Mechanism of Entry

Mechanism of Fusion and Uncoating

Influenza Virus Transcription and Replication

Initiation of Messenger RNA Synthesis

Polyadenylation

Splicing

Replication Products: cRNA and vRNA

The Switch from Transcription to Replication

Regulation of Viral Gene Expression

Virus Assembly and Release

Interactions of Influenza Virus with the Host Cell

Reverse Genetics

Inhibitors of Influenza Viruses

Perspectives

Influenza viruses were probably responsible for the disease described by Hippocrates in 412 BC,²⁷⁵ and thus they have been with us for a long, long time. Influenza remains a major cause of morbidity and mortality worldwide, and large segments of the human population are affected every year. In addition, many animal species can be infected by influenza viruses, and some of these viruses may give rise to pandemic strains in humans, as in the case of the 2009 H1N1 pandemic. Most threatening is the possibility of another pandemic similar to that experienced in 1918, which is estimated to have caused on the order of 50 million deaths worldwide.³¹⁹

CLASSIFICATION

The family of *Orthomyxoviridae* is defined by viruses that have a negative-sense, single-stranded, and segmented RNA genome. The definition of negative-sense RNA viruses came

from work by David Baltimore, who showed that the packaged genome of this class of viruses is complementary to the messenger RNA (mRNA), which is defined as positive.²¹ There are six different genera in the family of *Orthomyxoviridae*: the *Influenzaviruses A, B, and C*; *Thogotovirus*; *Isavirus*; and a new genus, *Quarantilla virus*⁵²⁶ (Fig. 40.1). Members belonging to any of the three different genera of influenza viruses can undergo genetic reassortment (see below), and thus readily exchange genetic information. However, reassortment between members of different genera (types) has never been reported. This absence of genetic exchange between viruses of different genera (types) is one manifestation of speciation as a result of evolutionary divergence.

Different influenza virus strains are named according to their genus (type), the species from which the virus was isolated (omitted if human), location of the isolate, the number of the isolate, the year of isolation, and, in the case of the influenza A viruses, the hemagglutinin (H) and neuraminidase (N) subtypes. For example, the 220th isolate of an H5N1 subtype virus isolated from chickens in Hong Kong in 1997 is designated influenza A/chicken/Hong Kong/220/97(H5N1) virus. There are now 17 different hemagglutinin (H1 to H17) subtypes and 9 different neuraminidase (N1 to N9) subtypes for influenza A viruses as well as a new N10 neuraminidase,^{186,668,721} while the influenza B virus hemagglutinins and neuraminidases are each classified into two lineages (Fig. 40.2).

VIRION STRUCTURE

Influenza A viruses have a complex structure and possess a lipid membrane derived from the host cell (Fig. 40.3A). This envelope harbors the hemagglutinin (HA), the neuraminidase (NA), and the M2 proteins that project from the surface of the virus. The matrix protein (M1) lies just beneath the envelope, and the core of the virus particle is made up of the RNP (ribonucleoprotein) complex, consisting of the viral RNA segments, the polymerase proteins (PB1 [polymerase basic 1], PB2 [polymerase basic 2], and PA [polymerase acid]), and the nucleoprotein (NP).⁶⁰¹ The NEP/NS2 (nuclear export protein/nonstructural protein 2) protein is also present in purified viral preparations.⁵⁵⁶ The overall composition of virus particles is about 1% RNA, 5% to 8% carbohydrate, 20% lipid, and approximately 70% protein.^{2,116,188} However, these results will have to be revisited using more quantitative approaches. Specifically, it will be important to get more quantitative data on the presence of individual viral components as well as cellular components that are packaged into the virus.⁶⁰² Excellent analyses have already been performed using electron

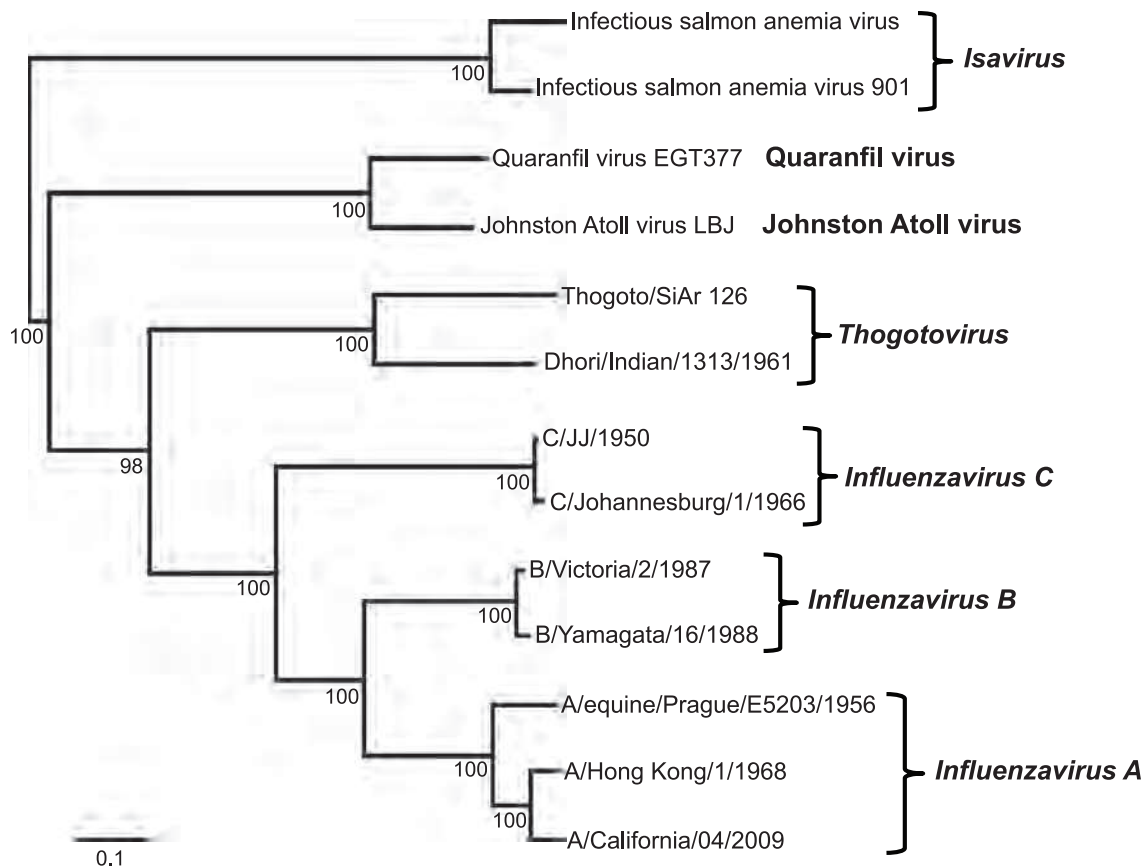


FIGURE 40.1. Phylogenetic relationships within the family *Orthomyxoviridae*. Nucleotide sequences of the polymerase basic 1 proteins (PB1) were aligned using transAlign and CLUSTAL W, and their phylogenetic relationships were determined by the neighbor-joining method (HKY model) using PAUP* (version 4.0b). The tree was midpoint rooted and bootstrap values (1,000 replicates) are indicated on the branches. The GenBank accession numbers for the sequences used for comparison were (top to bottom) AF404346, GU830904, FJ861695, FJ861697, AF004985, M65866, M28060, AF170575, CY018763, CY018771, GU053121, CY044267, and FJ966080. (Adapted from Perez D, Rimstad E, Smith G, et al. *Orthomyxoviridae*. In: King AMQ, Adams MJ, Carstens EB, et al, eds. *Virus Taxonomy*. Oxford, UK: Elsevier, 2011:749–762.)

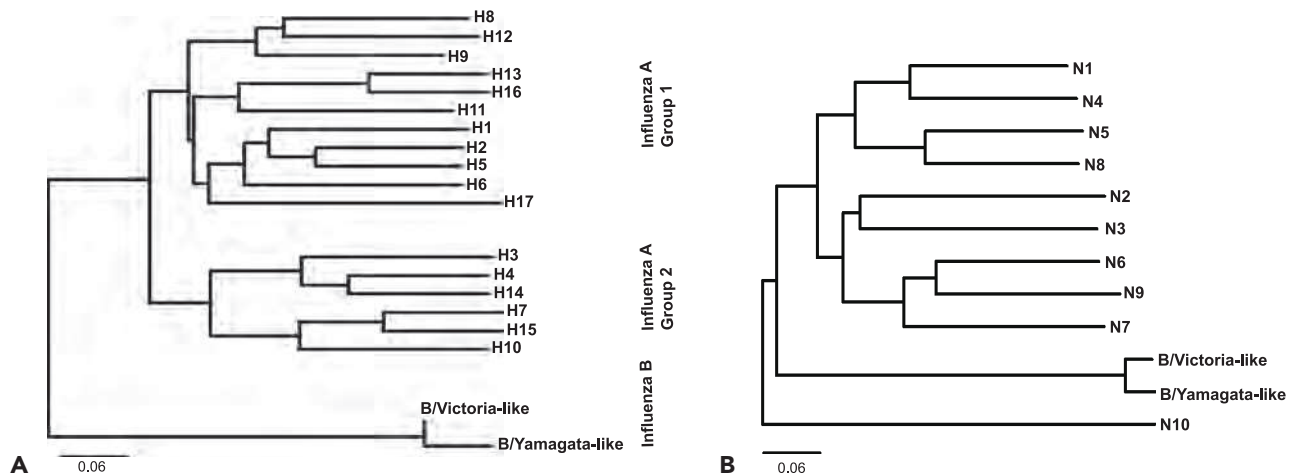


FIGURE 40.2. Phylogeny of influenza A and B virus hemagglutinins (HAs) and neuraminidases (NAs). Rooted phylogenetic trees are based on amino acid sequences of HA (A) and NA (B) segments from influenza A and B viruses. Representative viruses were selected from GenBank and then aligned using ClustalW. Phylogenetic trees were constructed using FigTree software. The scale bars represent approximately 6% amino acid changes between close relatives. (Courtesy of Natalie Pica.)

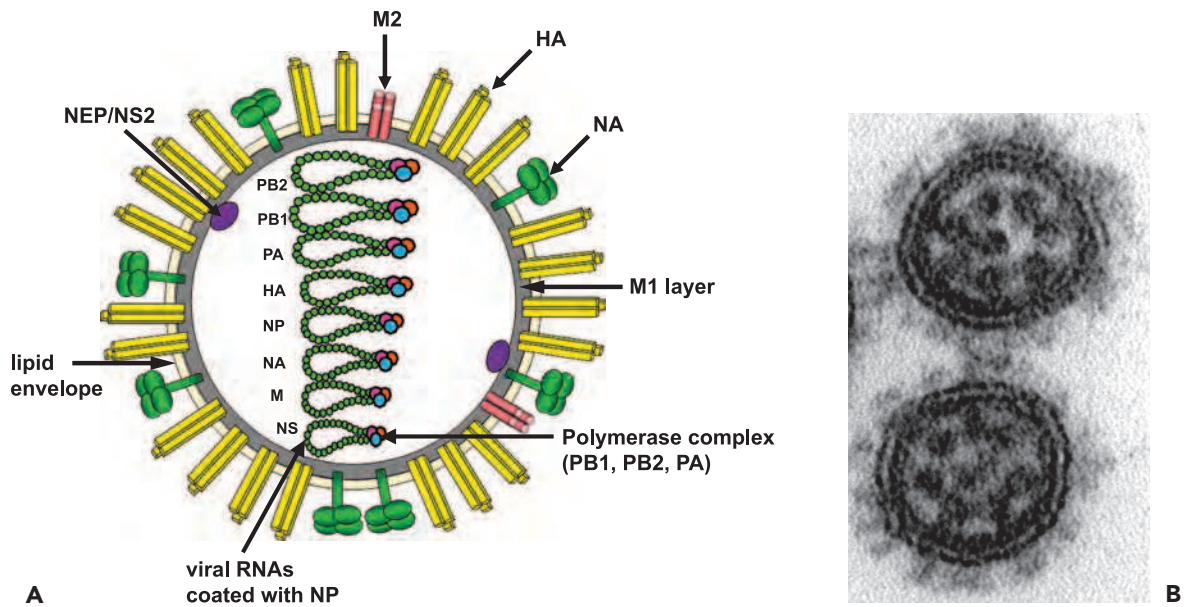


FIGURE 40.3. Schematic diagram and electron micrograph of influenza virus particles. **A:** The hemagglutinin (HA), neuraminidase (NA), and M2 proteins are inserted into the host-derived lipid envelope. HA is found as a trimer and NA and M2 both as tetramers. The matrix (M1) protein underlies the lipid envelope. A nuclear export protein (NEP/NS2) is also associated with the virus. The viral RNA segments are coated with nucleoprotein and are bound by the polymerase complex. **B:** Electron micrograph thin section image of influenza virus particles (diameter ~100 nm) with the HA and NA spikes visible on the surface and the eight ribonucleoprotein (RNP) segments visible in the interior of each particle. (Courtesy of Yi-ying Chou.)

microscopy (EM) of negatively stained or frozen-hydrated (cryoelectron microscopy) particles and tomographic reconstructions.^{80,192,256,464,489} The morphology of influenza A virus particles is characterized by distinctive spikes that are readily observable in electron micrographs of negatively stained virus particles (Fig. 40.3B). These spikes, made up of HA and NA, have lengths of ~10 to 14 nm, with an approximate ratio of four HA to one NA. Influenza viruses are pleomorphic. The spherical particles have a diameter of about 100 nm, but filamentous particles with elongated viral structures (more than 300 nm) have frequently been observed, particularly in fresh clinical isolates¹⁰² and in preparations of viruses with specific M1 or M2 proteins (e-Fig. 40.1).^{55,77,159,571}

Less is known about the internal structures of influenza viruses. However, the underlying M1 layer can be visualized and reveals a helical superstructure.^{80,575,577} The RNP complexes were first separated by Duesberg¹⁴⁷ on sucrose gradients and were visualized by electron microscopy using positive staining with uranyl acetate.¹¹⁵ These RNP structures appear to consist of a strand that is folded back on itself to form a double-helical arrangement.¹¹⁵ Most recently, attempts have been made to visualize RNPs or individual RNA segments by electron microscopy of thin sectioned virus particles⁴⁸⁹ (for review see (488)).

Influenza B viruses are mostly indistinguishable from the A viruses by electron microscopy. They have four proteins inserted in their lipid envelopes: the HA, NA, NB, and BM2.^{38,62,491,757} The M1 and the RNP complexes make up the interior of the particle. It has also been shown that the influenza B virus NEP/NS2 is associated with purified virus preparations.³⁰⁴

Influenza C viruses have been found to possess hexagonal reticular (net-like) structures on the surface¹⁰ and to form

unusually long (500 μ m) cord-like structures on the surface of infected cells.^{461,484} Influenza C viruses also contain a core of three polymerase proteins and the NP, which are associated with seven RNA segments. The influenza C virus M1 appears to have a similar role to those of influenza A and B viruses. The major glycoprotein, HEF (hemagglutinin-esterase-fusion), combines the functions of the HA and NA (and thus influenza C viruses contain one less RNA segment than do the A and B viruses). The HEF is inserted into the lipid membrane, as is the glycosylated CM2, which is structurally analogous to the M2 of influenza A viruses and the NB of influenza B viruses.^{466,467,524}

GENOME STRUCTURE AND ORGANIZATION

Influenza Viruses

All A- and B-type influenza viruses possess eight RNA segments, whereas influenza C viruses only have seven RNAs (Fig. 40.4 and e-Figs. 40.2 to 40.4). This was first shown by using polyacrylamide gel electrophoresis of isolated RNAs from two parent influenza A virus strains and their reassortants. Identifying the derivation of an RNA segment (by gel electrophoresis) in a reassortant and simultaneous protein analysis (by serologic or gel analysis) allowed the assignment of individual RNAs to specific viral proteins^{508,513,514} (for review, see (509)) (Fig. 40.5 and e-Fig. 40.4). Interestingly, influenza A viruses increase the coding capacity of their genomes via both splicing and use of alternative open reading frames. The M and NS genes each give rise to a spliced mRNA encoding the M2 and the NEP/NS2 proteins, respectively.^{354,355} The PB1-F2 and PB1-N40 proteins are expressed from alternative open reading frames

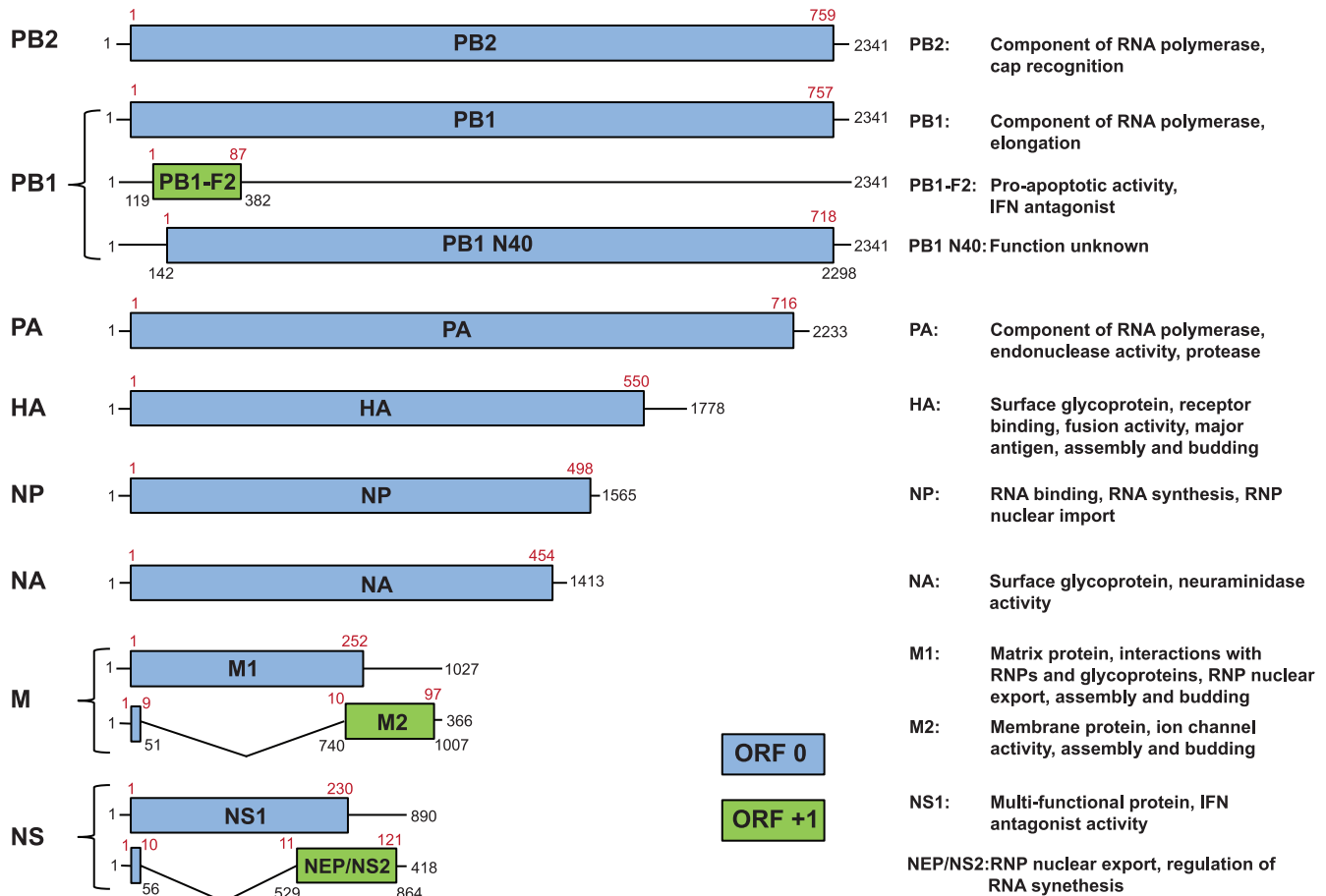


FIGURE 40.4. Genome structure of influenza A/Puerto Rico/8/34 virus. RNA segments (nucleotides in black) shown in positive sense and their encoded proteins (amino acids in red). The lines at the 5' and 3' termini represent the noncoding regions. The polymerase basic 1 protein (PB1) segment contains a second open reading frame (ORF) in the +1 frame resulting in the PB1-F2 protein and a third ORF in the 0 frame resulting in the PB1 N40 protein. The M2 and nuclear export protein (NEP/NS2) proteins are encoded by spliced messenger RNAs (mRNAs) (the introns are indicated by the V-shaped lines). (Courtesy of Heinrich Hoffmann.)

within the *PB1* gene^{93,726} (Fig. 40.4), although not all influenza A virus strains encode these proteins, making them true accessory proteins. Each viral segment contains noncoding regions at both the 5' and 3' ends. The extreme ends are conserved among all segments of influenza A viruses and this is followed by a segment-specific noncoding region.

The influenza B virus genome is similar to that of influenza A virus. Again, eight RNA segments code for one or more viral proteins (e-Figs. 40.2 and 40.4) with the three largest RNAs coding for the polymerase proteins, the fourth RNA for the HA, and the fifth and sixth RNAs for the NP and NA, respectively.⁵⁵⁰ The NA gene codes for the NB protein as well as for the NA. The NB protein is encoded by a -1 open reading frame seven nucleotides upstream (...AUGAACA AUG...) of the NA coding frame.⁶⁰³ The seventh RNA codes for the M1 protein (248 amino acids in length). Its termination codon (...UAAUG) overlaps with the initiation codon (UAAUG...) for the BM2 (109 amino acids in length), which allows for a "stop-start" translation mechanism.²⁸⁸ The eighth RNA codes for the NS1 as well as for the NEP/NS2 protein, the latter via a

spliced mRNA. Cognate PB1-F2 and PB1 N40 proteins have not yet been identified in influenza B viruses. The noncoding regions of the influenza B virus genome are longer than those in influenza A virus.

The genome of influenza C viruses has only seven RNA segments, with the three largest RNAs each coding for one of the polymerase proteins (e-Figs. 40.3 and 40.4). The PB1 and PB2 proteins are homologous to the corresponding influenza A and B virus proteins. The third influenza C virus polymerase protein is named P3 because it does not display acid charge features at neutral pH, as do the corresponding PA proteins of influenza A and B viruses.⁷⁴⁰ The fourth RNA codes for the HEF protein,²⁷¹ combining the hemagglutinin, receptor-destroying, and fusion activities. The NP is encoded by the fifth RNA. The sixth RNA codes for the M protein, which is expressed from a spliced mRNA, and from the unspliced mRNA a long precursor is translated (p42), which is then processed by signal peptide cleavage into CM2. This 115-amino acid (aa)-long protein consists of an amino-terminal extracellular domain (with a carbohydrate chain), a hydrophobic transmembrane domain, and an

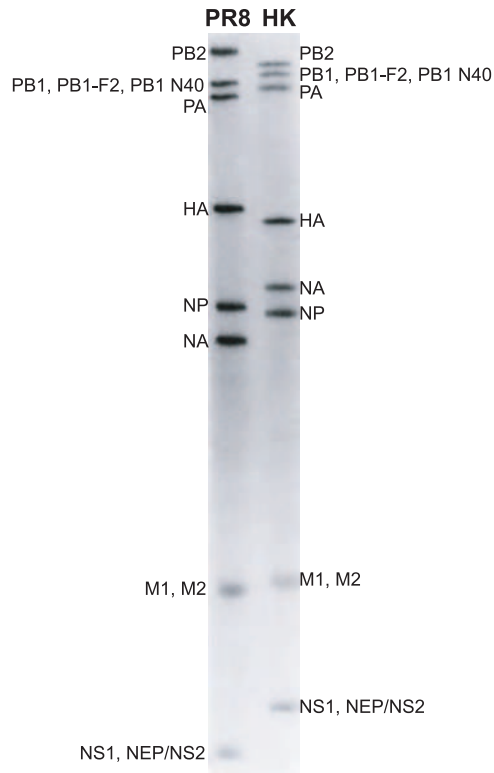


FIGURE 40.5. RNA segments of influenza A/Puerto Rico/8/34 (H1N1) and A/Hong Kong/8/68 (H3N2) viruses. The viral RNAs are separated on a polyacrylamide gel and the proteins encoded by the RNAs are indicated. (Adapted from Palese P. The genes of influenza virus. *Cell* 1977;10[1]:1–10.)

intracellular cytoplasmic tail.^{523,524,741} Finally, RNA 7 codes for the NS1 protein (246 amino acids),⁴⁶⁷ and via a spliced mRNA, for the NEP/NS2 protein (182 amino acids).^{5,284}

Evolutionarily, influenza A, B, and C viruses have a common precursor, and it is also likely that influenza A and B viruses diverged from each other more recently than influenza C viruses. Based on comparative sequencing studies using the hemagglutinin molecules, it has been postulated that the influenza A viruses diverged about 2,000 years ago, and the influenza B and C viruses about 4,000 and 8,000 years ago, respectively.⁶⁵³ Clearly, these numbers are based on a series of unprovable assumptions, including a steady rate of evolution over time, and therefore must be taken *cum grano salis*.

Thogoto Virus

The genomes of Thogoto viruses possess only six single-stranded RNA segments of negative polarity, with a total coding capacity for seven proteins. As with the influenza viruses, three proteins make up the RNA-dependent RNA polymerase complex. The NP, the glycoprotein (G), the matrix protein (M), and one non-essential accessory protein (ML) are coded for by the remaining three RNAs. The M and ML proteins are both encoded by the shortest RNA, with the M protein being derived from a spliced mRNA.^{242,339} The 304-aa-long ML protein has been shown to possess interferon antagonist activity^{69,242,316} and is virion associated.²⁴¹ It appears that Thogoto viruses do not possess a nonstructural protein.

Infectious Salmon Anemia Virus

The genome of infectious salmon anemia virus consists of eight negative-sense, single-stranded RNA segments.^{122,447} Segment 1 most likely encodes a protein analog of the influenza virus PB2.⁶²⁶ The second segment appears to code for a PB1 analog because it carries the PB1-specific polymerase motifs.⁶²⁶ Segments 3 and 4 code for the NP and PA proteins, respectively.^{15,169} Segment 5 encodes the 50-kD F (fusion) protein, and segment 6 encodes the HA (hemagglutinin-acetylesterase) protein.^{16,169,526} The 42-kD HA has been demonstrated to bind to 4-O-acetylated sialic acid (i.e., to use it as a receptor) and also to hydrolyze the acetyl group.²⁶⁷ This activity is similar to the binding and receptor-destroying enzyme (RDE) activities previously observed for the HEF protein of influenza C viruses and the HE glycoprotein of coronaviruses.^{267,271,685–687} Segment 7 encodes two proteins via an alternative splicing mechanism.⁴⁰ The larger protein is expressed from the unspliced transcript and has interferon antagonist activity.^{211,430} Segment 8 encodes two proteins of 27.6 kD and 22 kD, the larger of which has interferon antagonist activity.^{122,211} The smaller protein is a structural protein, presumed to be the equivalent of the influenza virus matrix protein.¹⁶⁹

Quarantfil Virus

The genome of *Quarantfil virus*, which is the type species of the new *Quarantfilvirus* genus, consists of six negative-sense, single-stranded RNA segments.⁵²⁶ The ends of each segment are conserved and partially complementary. Segments 1, 2, and 3 encode the PB2, PA, and PB1 polymerase subunits, respectively, while segment 5 codes for a protein that is distantly related to the Thogoto virus glycoprotein, and thus is likely the attachment protein.⁵⁴⁴ The predicted protein products of segments 4 and 6 do not show any homology with known proteins.

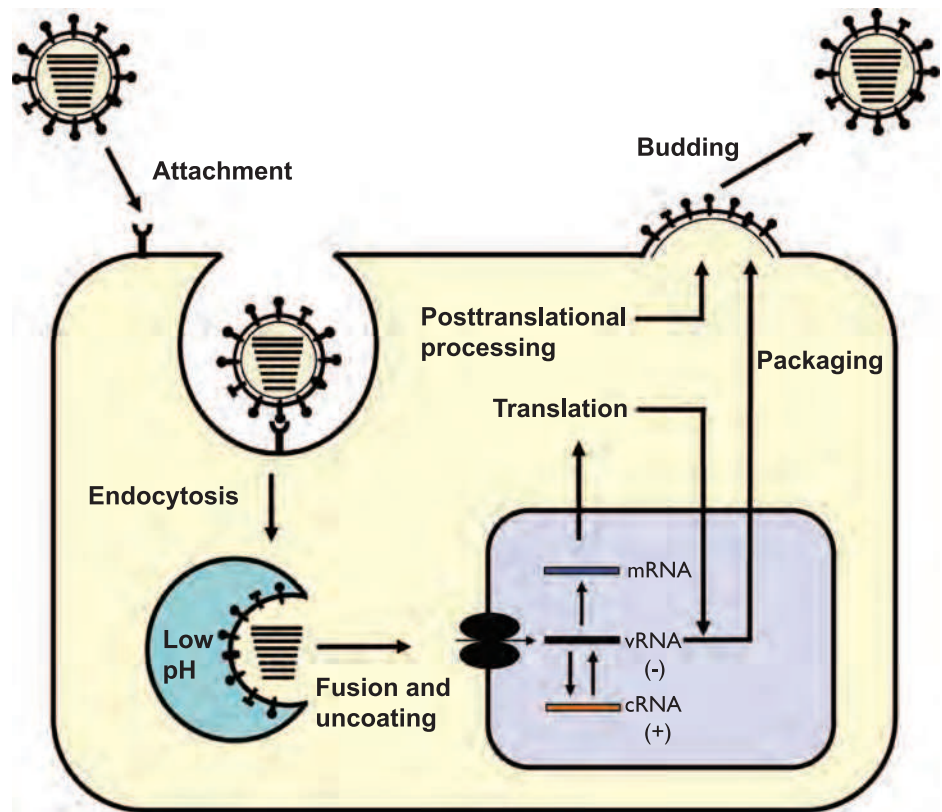
STAGES OF VIRAL REPLICATION

An overview of the influenza virus life cycle is illustrated in Figure 40.6 and in the following pages we will discuss each stage of this life cycle in order.

Mechanism of Attachment

Influenza viruses bind to neuraminic acids (sialic acids) on the surface of cells to initiate infection and replication. The interaction of influenza viruses with a ubiquitous molecule such as sialic acid is constrained by the fact that the HAs of viruses that replicate in different species show specificity toward sialic acids with different linkages. Human viruses preferentially bind to N-acetylneuraminic acid attached to the penultimate galactose sugar by an $\alpha 2,6$ linkage (SA $\alpha 2,6$ Gal), whereas avian viruses mostly bind to sialic acid with an $\alpha 2,3$ linkage¹²⁰ (e-Fig. 40.5). In agreement with this finding is the fact that human tracheal epithelial cells contain mostly SA $\alpha 2,6$ Gal, while the gut epithelium from ducks possesses mostly SA $\alpha 2,3$ Gal sugar moieties.^{123,307} It should be noted, however, that this viral specificity is not absolute and that avian and human cells can contain both neuraminic acid linkages (2,3 as well as 2,6). Studies on ciliated cells in the human airway epithelium have shown that sialylated proteins with $\alpha 2,3$ linkages are present and that these cells can be infected with avian influenza viruses.⁴²⁵ Furthermore, glycan structure is far more complex than just the terminal sialic acid

FIGURE 40.6. Illustration of the influenza virus replication cycle. Upon binding at the cell surface, the virus is internalized by receptor-mediated endocytosis. The low pH in the endosome triggers fusion of the viral and endosomal membranes, releasing the viral ribonucleoproteins (vRNPs) into the cytoplasm. vRNPs are imported into the nucleus where they serve as the template for transcription. New proteins are synthesized from viral mRNA. The viral genome (vRNA) is replicated through a positive-sense intermediate (complementary RNA [cRNA]). Newly synthesized viral RNPs are exported from the nucleus to the assembly site at the apical plasma membrane, where virus particles bud and are released.



linkage, and evidence suggests that factors such as the type of backbone, chain length, and branching pattern as well as sulfation and fucosylation may also influence the interactions with HA.^{99,639} Glycan microarrays that contain a wide spectrum of glycan structures are now being used as tools to better understand the specificity of receptor binding.⁶³⁸ Also, when viruses are passaged in a particular host, they can adapt to that host by mutating the receptor-binding site in the viral HA.^{202,448} In a study of the A/New York/1/1918 virus HA, it was shown that the binding specificity can be changed by a single amino acid mutation (D190E) to a preference for α 2,3-linked sialic acids. It is thus hypothesized that the HA gene of the 1918 influenza virus has its origin in avian species and that a single amino acid change (E190D) allowed the hemagglutinin to recognize the α 2,6-linked sialic acids prevalent in human cells.^{222,637}

Mechanism of Entry

While some viruses (e.g., paramyxoviruses and herpes viruses) can enter cells directly through the plasma membrane by a pH-independent fusion process, influenza viruses require a low pH to initiate fusion and are therefore internalized by endocytic compartments. There are four internalization mechanisms: (a) via clathrin-coated pits; (b) via caveolae; (c) through nonclathrin, noncaveolae pathways; and (d) through macropinocytosis (for review, see (119,353,443,613)). Clathrin-mediated endocytosis has traditionally been the model for influenza virus entry.⁴²⁴ However, a non-clathrin, non-caveolae-mediated internalization mechanism has also been described for influenza viruses.⁶¹² The latter pathway is dependent on low pH and trafficking to late endosomes, as it requires protein kinase C, Rab5, and Rab7 functions.⁶¹¹ More recently, through the use of specific inhibitors and RNA interference (RNAi), it has been shown that in addition

to entering via a dynamin-dependent, clathrin-driven pathway, influenza viruses can also enter via a dynamin-independent pathway that is characteristic of macropinocytosis.¹³³ Potential differences in the entry pathways defined in polarized versus nonpolarized cells should also be appreciated, as, for example, the actin cytoskeleton appears to be critical for uptake of influenza viruses into polarized cells but not nonpolarized cells.⁶⁵¹ The requirement of specific host proteins during influenza virus entry will help to further define the cellular pathways utilized by the virus. Genome-wide RNAi screens have identified multiple factors that are required for efficient entry mediated by the influenza virus glycoproteins³⁴¹; however, the ability of the virus to enter via different routes means that this approach is unlikely to capture factors that are specific to one endocytic route. A more focused approach, such as that which shows the requirement of epsin 1 for clathrin-mediated uptake of influenza virus, is needed.⁹⁰ The epidermal growth factor receptor (EGFR) has also been demonstrated to play a role during influenza virus entry,¹⁵⁴ and it is thought that virus attachment to the cell stimulates EGFR, which leads to activation of signaling cascades such as phosphatidylinositol 3 kinase (PI3K) signaling, which is known to promote influenza virus entry.¹⁵² There are also questions over whether sialic acid is the only attachment molecule. Lec-1 cells, which are deficient in N-linked glycosylation (but still contain sialylated proteins), are unable to internalize influenza viruses.¹⁰³ Similarly, cells deficient in sialic acid can be made to support influenza virus entry if they express one of the C-type lectins, DC-SIGN or L-SIGN.³⁹⁸

Mechanism of Fusion and Uncoating

Influenza viruses and other enveloped viruses (including rabdo-, flavi-, bunya-, and filoviruses) require low pH to fuse

with endosomal membranes. After binding to the target cell surface and endocytosis, the low pH of the endosome activates fusion of the viral membrane with that of the endosome. This fusion activity is induced by a structural change in the HA of influenza viruses, but in order for this to occur, the HA0 precursor must first be cleaved into two subunits, HA1 and HA2. Once in the acid environment of the endosome, the cleaved HA molecule undergoes a conformational change and this exposes the fusion peptide at the N-terminus of the HA2 subunit, enabling it to interact with the membrane of the endosome (for review, see (128,258,632) and for details see the following section on Hemagglutinin). The transmembrane domain of the HA2 (inserted into the viral membrane) and the fusion peptide (inserted into the endosomal membrane) are in juxtaposition in the low pH-induced HA structure. The concerted structural change of several hemagglutinin molecules then opens up a pore, which releases the contents of the virion (i.e., viral RNPs) into the cytoplasm of the cell. The precise timing and the location of uncoating (maturity of the endosome) depends on the pH-mediated transition of the specific HA molecule involved.

The uncoating of influenza viruses in endosomes is blocked by changes in pH caused by weak bases (e.g., ammonium chloride or chloroquine) or ionophores (e.g., monensin) (for review, see (418)). Effective uncoating is also dependent on the presence of the M2 protein, which has ion channel activity.^{532,534} Early on it was recognized that amantadine and rimantadine inhibit replication immediately following virus infection.⁶¹⁷ Later it was found that the virus-associated M2 protein allows the influx of H⁺ ions from the endosome into the virus particle, which disrupts protein–protein interactions and results in the release of RNP free of the M1 protein.^{424,767} (for review, see (534)). Amantadine and rimantadine have been shown to block the ion channel activity of the M2 protein and thus uncoating.^{100,281,532,648,699} The HA-mediated fusion of the viral membrane with the endosomal membrane and the M2-mediated release of the RNP result in the appearance of free RNP complexes in the cytoplasm. This completes the uncoating process.⁴²¹ The time frame for the uncoating process was examined by inhibiting virus penetration with ammonium chloride. The majority of virus particles showed a half time for penetration of about 25 minutes (after adsorption). Barely 10 minutes later (half time of 34 minutes after adsorption) RNP complexes are found in the nucleus.⁴²¹ The process for uptake of RNP molecules through nuclear pores is an active one, involving the nucleocytoplasmic trafficking machinery of the host cell (for details, see Nuclear Import of RNPs).

Much less is known about the uptake and uncoating of influenza B and C viruses. Influenza B viruses are more like influenza A viruses as both recognize N-acetylneuraminic acid as receptors, while influenza C viruses bind to 9-O-acetylated neuraminic acid derivatives.²⁷² Like influenza A viruses, the B- and C-type viruses go through an endosome-mediated uncoating process, which requires proteolytic activation (cleavage) of the HA or HEF proteins and subsequent fusion of the viral and endosomal membranes. Although the viral glycoproteins of both viruses are dependent on a low pH-triggered fusion process, the influenza C virus HEF-mediated fusion/uncoating may occur at a higher pH in early endosomes.^{184,767} At this point, the role of CM2 in uncoating of influenza C viruses is less well established than that of the BM2 protein of influenza

B viruses, which is the homolog of the influenza A virus M2 protein.^{193,253,262,283,455,701}

The Hemagglutinin

STRUCTURAL FEATURES

Much is known about the HA molecule and excellent reviews are available.^{150,204,258,613,618,633,658} In fact, the influenza virus HA has become a model for studies of protein folding and trafficking, protein quality control, membrane fusion, protein–receptor interactions, and antigen–antibody complexes and, last but not least, for investigating how the immune system reacts to a foreign protein. The major functions of the HA are the receptor-binding and fusion activities, but there may also be a structural role for the HA in budding and particle formation. The HA is a trimeric rod-shaped molecule with the carboxy terminus inserted into the viral membrane and the hydrophilic end projecting as a spike away from the viral surface (i.e., a type I integral membrane protein). Early on it was shown that (a) posttranslational modifications of the precursor molecule (i.e., glycosylation and palmitoylation), (b) cleavage of the signal peptide in the endoplasmic reticulum (ER), and (c) cleavage of the HA0 precursor into HA1 and HA2 subunits are required for the full activity of the molecule.^{335,363,542}

The first x-ray crystallographic structure of an HA (the ectodomain released from the virus by bromelain treatment) was resolved in 1981 by Wilson et al.⁷²⁵ At that time the HA (from A/Aichi/68 [H3N2] virus) was the largest biological molecule for which a structure had been resolved, and it started an unprecedented drive to study the structure/function relationships of biologically important molecules, which continues unabated to this day. The structures of numerous HAs have now been resolved, including the subtype 1 HA molecules of the 1918 and 2009 pandemic influenza viruses^{203,640,735} and that of an H2 subtype HA⁷³⁶ (Fig. 40.7). Remarkably, even though the overall amino acid sequence identity can be less than 50%, the structure and functions of these HAs are highly conserved (e-Fig. 40.6). This represents a case of evolution and sequence variation proceeding to an extreme level while structure and function have remained conserved. Even more surprising is that the structure of the influenza B virus HA is similar to that of influenza A virus HA despite sharing only 25% sequence identity.^{703,704}

The crystallographic structure of the uncleaved influenza A virus HA is superimposable onto that of the cleaved HA1 and HA2, with the exception of the amino acids adjacent to the cleavage site. The major features of the structure are (a) a long fibrous stem, which is made up of a triple-stranded coiled coil of α -helices derived from the three HA2 parts of the molecule (helix A and helix B; Fig. 40.7 and e-Fig. 40.6), and (b) the globular head, which is also made up of three identical domains whose sequences are derived from the HA1 portions of the three monomers. The *first major function* of HA is binding to receptors and the receptor-binding site lies within the globular head of the molecule. This site has been defined through crystallization and structure analysis of HA–receptor complexes, as well as by mutational analysis. In the H3-subtype viruses, it appears that within the receptor-binding pocket of an avian HA, a glutamine in position 226 preferentially accommodates the 2,3-linked sialic acid, whereas a leucine in that position in human H3 HAs preferentially accommodates the 2,6-linked sialic acid²³⁹ (e-Fig. 40.7). (See the previous section on Attachment for more details).

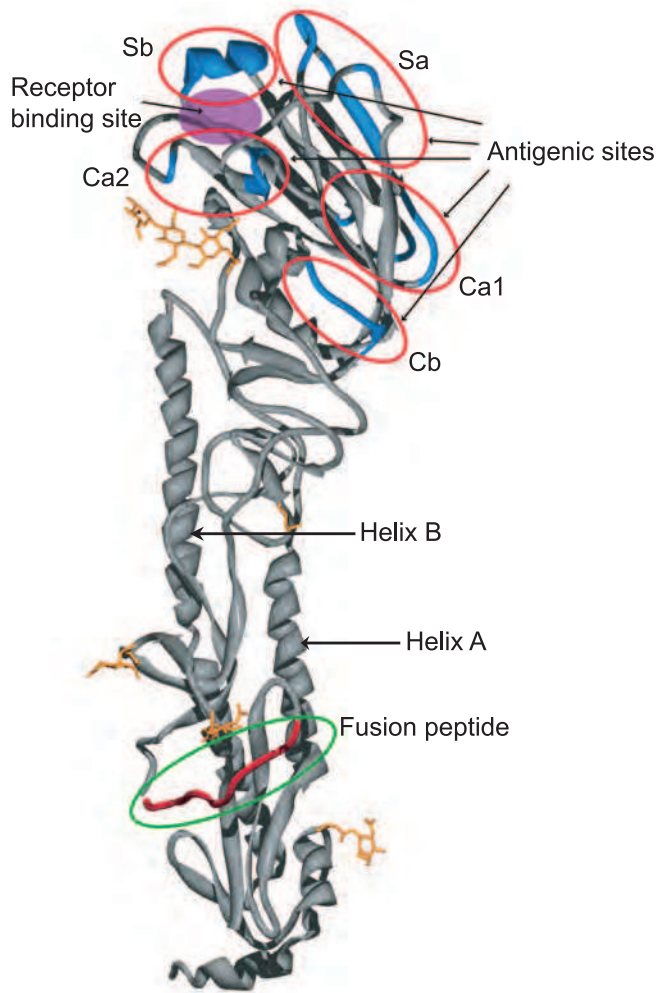


FIGURE 40.7. Ribbon representation of the uncleaved hemagglutinin monomer from the 1918 influenza virus based on x-ray diffraction analysis. The five predicted antigenic sites (Ca2, Sb, Sa, Ca1, and Cb) surround the sialic acid receptor-binding site. Toward the membrane proximal end (bottom) is the fusion peptide and helices A and B are indicated. For details see (640). (Courtesy of James Stevens and Ian Wilson.)

The *second major function* of the HA is acid pH-triggered fusion, which is required for the uncoating process. Low pH treatment changes the structure of the HA dramatically. The molecule becomes susceptible to protease digestion, and the disulfide bond linking the HA1 and HA2 subunits becomes susceptible to mercaptoethanol.^{232,615} However, the important feature of the acid pH-mediated change is that the fusion peptide becomes aligned antiparallel to the membrane anchor of the HA2 (Fig. 40.8). The end result is that the fusion peptide brings the endosomal membrane into juxtaposition with the viral membrane, leading to fusion. The presence of more than one hemagglutinin then leads to the formation of a fusion pore through which the RNP can enter the cytoplasm (Fig. 40.9). Structures of the postfusion HA as well as an early fusion intermediate have helped to reveal the molecular details of the changes that occur during the transition from pre- to postfusion state.^{75,737} Structures of the fusion peptide in lipid environments have been resolved by nuclear magnetic resonance

(NMR) and show that the peptide forms a tight helical hairpin structure that angles back on itself^{252,351,399} (e-Fig. 40.8). This hook structure may help to pull the endosomal membrane close to the viral membrane, resulting in the initiation of the actual fusion process. These studies represent the first foray into the characterization of the transmembrane region, which so far has proved difficult to analyze.

The third major structural element of the HA, the cytoplasmic tail, is highly conserved among all subtypes. There are three cysteines that are palmitoylated (with one of them located in the transmembrane domain). The role of this cytoplasmic tail (and the palmitates attached to the cysteines) is not entirely clear due to subtype- and cell/host-specific differences.^{89,318,439,696,774}

ANTIGENIC DETERMINANTS

In addition to having an important role in receptor binding, fusion, and assembly, the influenza virus HA is also the *major determinant* recognized by the adaptive immune system of the host. Following infection and replication, a vigorous immune response is induced, which usually results in the formation of neutralizing antibodies. These antibodies then lead to the selection of “antibody escape” variants. The amino acids undergoing change are almost exclusively on the HA1 (and on the outside of the molecule). Many of these changes get fixed (accumulate over time), defining the antigenic drift of influenza viruses (e-Fig. 40.9). Fab antibody fragments have been shown to bind to different regions of the HA1 (e-Fig. 40.10) and, interestingly, not in all cases do three Fabs bind to one trimeric spike (3:1 ratio). Examples have been found where only one Fab molecule binds to one HA spike (1:1 ratio) or where just two Fab molecules bind to a trimeric spike (2:1 ratio) (e-Fig. 40.10).³³⁶ In the latter case, the two Fab fragments cross-link the two monomers so that the HA molecule cannot undergo an acid pH-induced conformational change.³³⁶ Attempts have also been made to measure antigenic evolution of influenza virus strains by pairwise comparison of hemagglutination inhibition assays.^{187,622}

Unexpectedly, broadly cross-reactive monoclonal antibodies have been identified, which do not bind to the tip of the HA molecules but also have neutralizing activity.^{121,157,327,495,650,663,730} These antibodies are directed against the conserved stem region of the HA spike and recognize the membrane-proximal part of HA1 in combination with HA2 or the HA2 alone (Fig. 40.10).⁷⁰⁷ In general, these antibodies recognize the HAs within either group 1 (for review see (706)) or group 2,^{158,707} but recently a human monoclonal antibody was described that binds to the stem of both group 1 and group 2 HAs.¹²¹ The most likely mechanism by which these cross-reactive antistem antibodies neutralize influenza viruses is by blocking conformational rearrangements associated with membrane fusion (antifusion vs. hemagglutination inhibition activity). A broadly neutralizing human monoclonal antibody that recognizes the highly conserved sialic acid-binding site of H1 HAs has also been described.³⁴³ It is hoped that the conserved epitopes identified by these cross-protective antibodies could be utilized as immunogens with the possibility of developing novel vaccine constructs that will result in effective and safe universal influenza virus vaccines.^{47,631,708}

The HEF of Influenza C Viruses

In contrast to the HA of influenza A and B viruses, the major glycoprotein of the C viruses has a receptor-destroying activity.

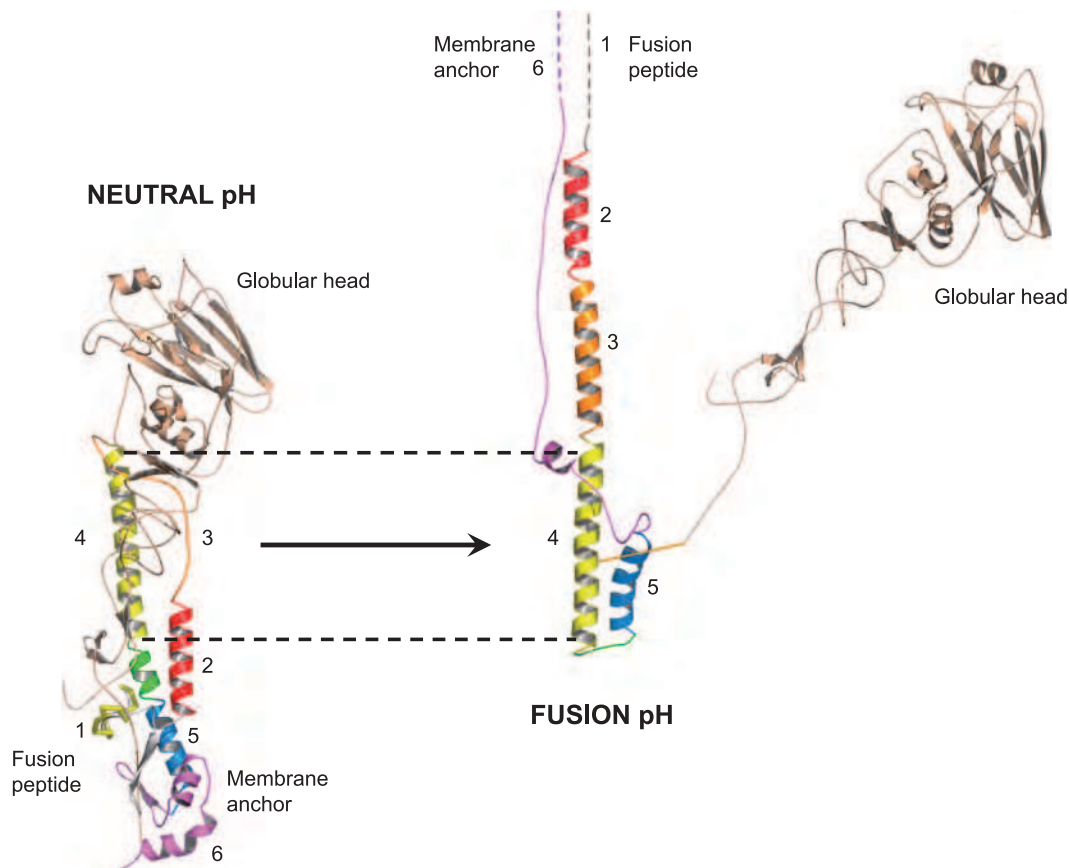


FIGURE 40.8. Ribbon representation of the structural changes that occur in hemagglutinin (HA) at low pH. Bromelain-treated HA monomer with regions of HA2 undergoing conformational changes at low pH as indicated by numbered domains (**left**). The domains, starting at the fusion peptide (domain 1), are numbered sequentially until the membrane anchor is reached (domain 6). The structure and the position of the region comprising residues 75 to 106 (domain 4) are the same before and after the conformational change (denoted by the dotted lines). The globular head domains retain their structures but detrimers (falling to the right, away from the HA2 portion). For details see (92) and (616). (Courtesy of John Skehel and Rupert Russell.)

In contrast to the neuraminidase activity of the NA proteins of influenza A and B viruses (see Neuraminidase section), HEF has esterase activity, which cleaves off an acetyl group at position 9 of the neuraminic (sialic) acid receptor, eliminating the ligand (for review, see (644)). This activity is important for entry of the virus, implying a role in releasing the incoming virus from the receptor so that the uncoating process can begin.⁶⁸⁸ Thus, in addition to receptor-binding (hemagglutination) and fusion activities, the molecule also has esterase activity, hence the name HEF. Although there is only about a 12% sequence identity between HAs and HEF, the overall structure of the molecule is similar, as was shown by x-ray crystallographic analysis.⁵⁶⁹ Even more surprising are structural and sequence similarities between the esterases of influenza C viruses and some coronaviruses.⁷⁵⁸

The M2 Protein

The M2 protein of influenza A viruses is a tetrameric type III (lacking a signal peptide sequence) integral membrane protein. It has a short ecto-domain, a transmembrane domain, and a cytoplasmic domain with palmitate and phosphate modifications (for review, see (531,534)). M2 has been shown to

possess ion channel activity, and its major role is thought to be that of conducting protons from the acidified endosomes into the interior of the virus to dissociate the RNP complex from the rest of the viral components, thus facilitating the uncoating process (see earlier section on Fusion and Uncoating). The structural and genetic analysis of the M2 protein has revealed that the ion channel is acid gated (but not voltage gated) and highly selective for H^+ ions.^{86,100,453,454,485} The structures of the transmembrane regions of M2 and of those that include the cytoplasmic sequences reveal a good understanding of the mechanism of proton conductance, which is controlled by the histidine-37 and tryptophan-41 cluster.^{1,79,291,482,589,599,643} The transmembrane region, when viewed from the top, shows four helices that sit at an angle in the lipid bilayer, forming a pore (Fig. 40.11). Structural studies on M2 in complex with adamantane drugs indicate two potential sites of interaction. In the x-ray structure, a single drug molecule binds to the core of the pore.⁶⁴³ In contrast, the NMR structure shows four drug molecules binding to the lipid-exposed surface of the channel close to the cytoplasmic ends of the helices.⁵⁸⁹ More recent data confirm the existence of these two interaction sites and propose that both drug-binding mechanisms may have physiologic

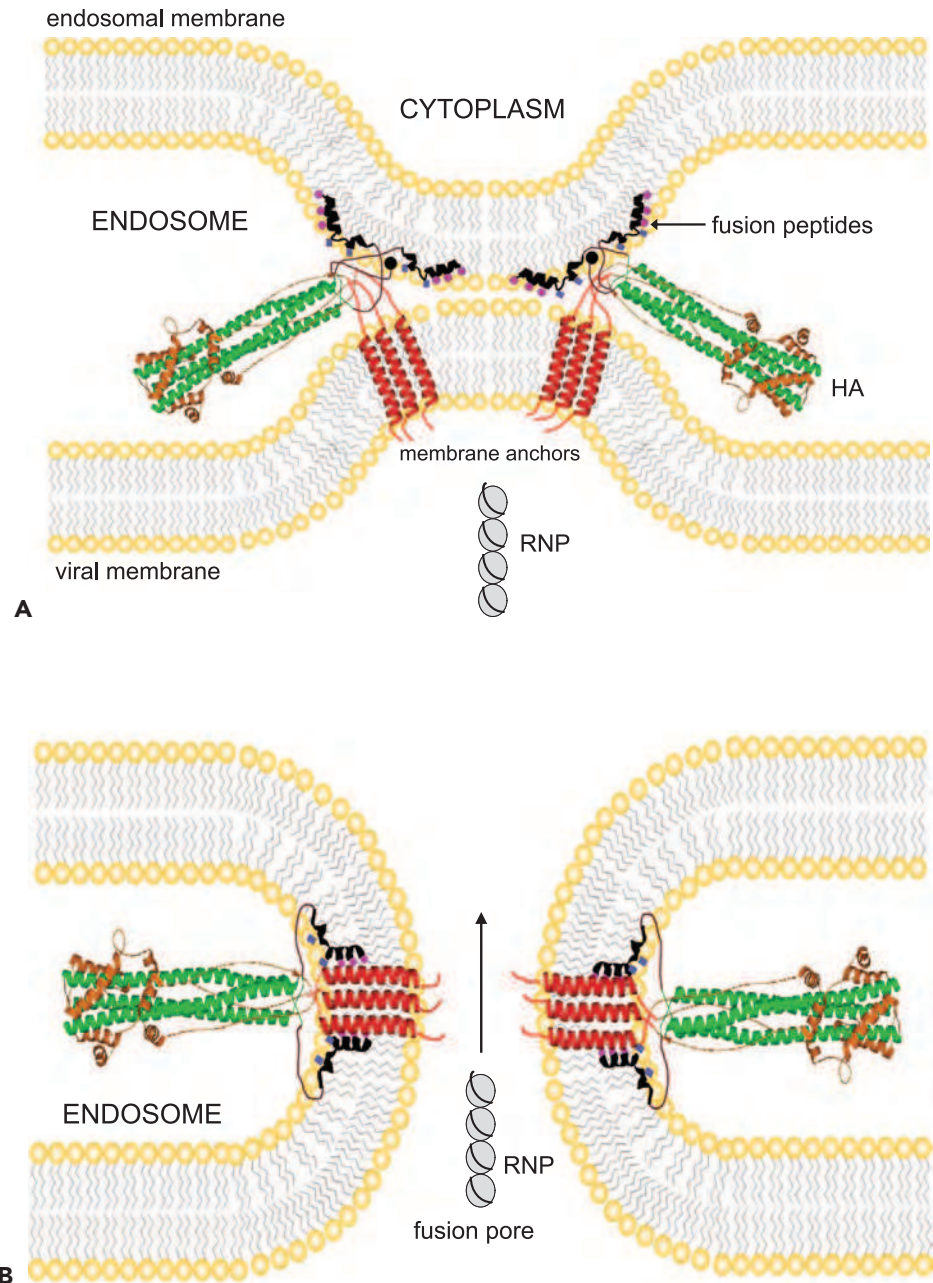


FIGURE 40.9. Model for juxtaposition of viral and endosomal membranes resulting in formation of a fusion pore and release of ribonucleoproteins (RNPs). Structures of influenza virus hemagglutinins in their postfusion state modeled into a possible fusion intermediate (A) and into a fusion pore (B). The fusion peptides are shown inserted into the endosomal membrane, while the trans-membrane domains remain anchored in the viral membrane. Note the large conformational changes of the ecto- and fusion domains when compared to their prefusion structures (see Figs. 40.7 and 40.8). The small spheres (pink) on the fusion peptide denote glycines that may mediate helix interactions and the small squares (blue) denote glutamates that may be responsible for the pH dependence of the fusion peptide penetration into lipid bilayers. Following the formation of a pore, the RNPs are released from the interior of the virus particle into the cytoplasm, completing the uncoating process. (Courtesy of Lukas Tamm.)

significance.^{79,342} The structure and the precise function of the extracellular portion of the M2 protein remain to be resolved. This external portion of M2 has been considered as the basis of a universal influenza virus vaccine approach because the M2 protein maintains a highly conserved sequence over long periods of time.⁵⁹⁰

The ion channel activity of M2 has also been implicated in stabilizing HAs from premature low pH transitions in the trans-Golgi network, but this second function may only come into play for viruses carrying highly acid-sensitive HAs.¹⁰⁴ This is the case for H5 and H7 HAs, which have a multibasic cleavage site that can be cleaved by ubiquitous proteases and are therefore more susceptible to a premature low pH-induced conformational change. Further functions attributed to the M2 protein include roles in particle morphology,^{231,310,564,571}

genome packaging,^{231,310,432} membrane scission⁵⁷² (see Assembly and Release), and inhibition of autophagy.²⁰⁵

Influenza Virus Transcription and Replication Overview

After uncoating, the viral ribonucleoproteins (vRNPs) are transported into the nucleus and the incoming negative-sense viral RNAs (vRNAs) are transcribed into mRNA by a primer-dependent mechanism. These mRNA products are incomplete copies of the vRNA templates and are capped and polyadenylated, unlike vRNA. Replication occurs via a two-step process. A full-length, positive-sense copy of the vRNA is first made, which is referred to as complementary RNA (cRNA), and is in turn used as a template to produce more vRNA (Fig. 40.12).

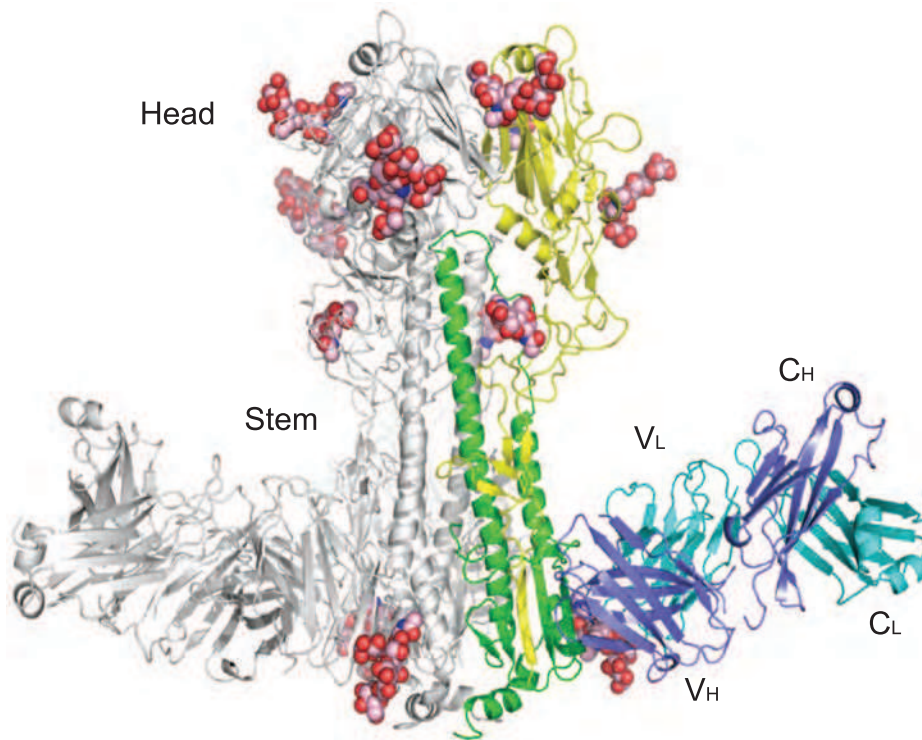


FIGURE 40.10. Crystal structure of anti-body CR8020 bound to the H3 hemagglutinin. CR8020 binds an epitope on the hemagglutinin (HA) stem and has broad neutralizing activity against multiple group 2 influenza A viruses, including H3, H7, and H10. One monomer from the HA trimer is depicted in yellow and green (HA1 and HA2 subunits, respectively) and CR8020 is colored blue and cyan (heavy chain and light chain, respectively). N-linked carbohydrates are represented as pink van der Waals spheres. See (158) for details. (Courtesy of Damian Ekiert and Ian Wilson.)

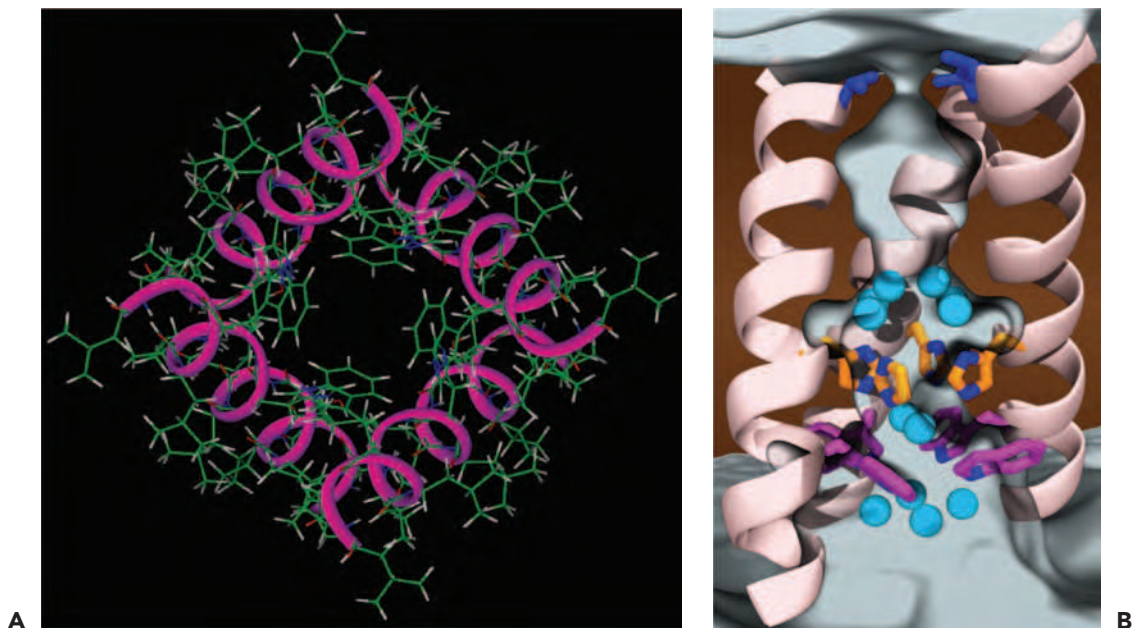


FIGURE 40.11. Structure of the tetrameric M2 ion channel. **A:** As seen from the top, four helices sit at an angle in the lipid membrane forming a pore. The backbone structure was determined by solid-state nuclear magnetic resonance (NMR) spectroscopy of the aligned bilayers. The histidine 37 and the tryptophan 41 side chains form the bottom of the pore in the closed state at neutral pH. For details see (485). (Courtesy of Tim Cross.) **B:** X-ray structure of the transmembrane section of the M2 proton channel. The protein backbone is shown as a cartoon, viewed from across the viral membrane (lipid molecules and one of the monomers are hidden). Cyan spheres represent crystallographically resolved water molecules, which are stepping stones in possible proton conduction pathways. The three most important groups of side chains (the Val27-valve [blue], the His37-box [orange], and the Trp41-basket [magenta]) are shown in sticks. The three-dimensional density of water at 37°C, calculated from molecular dynamics simulations, is drawn as a white contour. See (1) for details. (Courtesy of Giacomo Fiorin.)

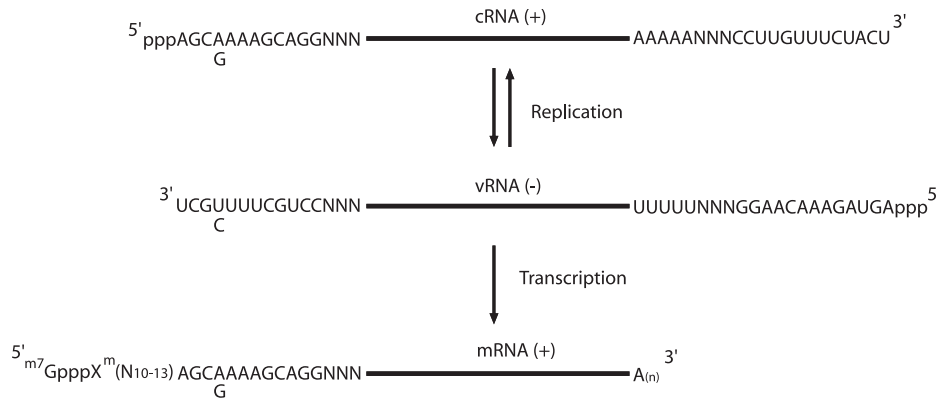


FIGURE 40.12. Influenza virus RNA synthesis. The incoming negative-sense RNA is shown in the middle with the conserved noncoding sequences at either end, adjacent to the segment-specific nucleotides (NNN). The polyadenylation signal consisting of a poly-uridine stretch is at the 5' end of the viral RNA (vRNA). The messenger RNA (mRNA) derives its cap and 10 to 13 5' nucleotides from host mRNAs and has a 3' poly(A) tail. The complementary RNA (cRNA) is an exact positive-sense copy of the incoming virion vRNA. The variation at position 4 in the 3' vRNA noncoding region is indicated.

Nuclear Import of Ribonucleoproteins

One of the characteristics of the influenza virus life cycle, and one that is unusual for an RNA virus, is its dependence on nuclear functions. All viral RNA synthesis occurs in the nucleus, and the trafficking of the viral genome into and out of the nucleus is a tightly regulated process (reviewed in (54,127)). The eight influenza virus genome segments never exist as naked RNA but are associated with four viral proteins to form vRNP complexes. The major viral protein in the RNP complex is the nucleocapsid protein (NP), which coats the RNA. The remaining proteins are the three polymerase proteins (PB1, PB2, and PA), which bind to the partially complementary ends of the viral RNA, creating the distinctive panhandle structure.²⁹⁰ These RNPs (10 to 20 nm wide)^{115,419} are considered too large to allow for passive diffusion into the nucleus and therefore, once released from an incoming particle, they must rely on an active nuclear import mechanism. All proteins in the RNP complex possess nuclear localization signals (NLSs), which mediate their interaction with the nuclear import machinery.^{320,456,470,483,625,702,720} However, it is the signals on NP that have been shown to be both sufficient and necessary for the import of viral RNA^{73,126,496,503} (e-Fig. 40.11).

NP INTERACTIONS WITH KARYOPHERIN α

The transport of proteins across the nuclear membrane is an energy-driven process that is initiated upon recognition of an NLS-containing cargo protein by members of the karyopherin α (also called importin α) family. Karyopherin α binds directly to the NLS and then recruits karyopherin β into a trimeric complex, which docks at the nuclear pore (e-Fig. 40.12). Prior to the identification of a definitive NLS within NP, the human homolog of yeast SRP-1 was identified as an NP-interacting partner.⁴⁹⁷ This uncharacterized protein was subsequently identified as karyopherin $\alpha 1$ and NP was also shown to interact with another family member, karyopherin $\alpha 2$.⁴⁹⁶ The minimal karyopherin α -binding site was used to identify the unconventional NLS in NP, so named because it does not contain a cluster of basic residues.⁷⁰² As described earlier,¹²⁶ this NLS has been shown to be essential for RNP import, and this implicates

karyopherin α as a critical component of RNP nuclear import. Interestingly, the NP NLS-binding site on karyopherin α is distinct from that of classical NLS-containing proteins.^{126,437} One could postulate that this serves to avoid competition for karyopherin α binding with host proteins and may explain the use of an unconventional NLS. There is also evidence that differential interactions with human versus avian karyopherin α proteins may determine species specificity of influenza viruses.^{197,198}

The Viral Ribonucleoprotein Template

Each viral RNA segment exists as an RNP complex in which the RNA is coated with NP and forms a helical hairpin that is bound on one end by the heterotrimeric polymerase complex (reviewed in (554)). NP is an arginine-rich protein and has a net positive charge (at pH 6.5), which reflects its RNA-binding activity and its primary role in encapsidation (reviewed in (541)). The RNA/NP interaction is thought to be mediated by the positively charged residues on NP and the negatively charged phosphate backbone of the RNA, and thus there is no apparent sequence specificity to the interaction.^{30,161} The RNA within the influenza virus RNP also remains sensitive to digestion with RNase,¹⁴⁷ supporting the model that the RNA is wrapped around the outside of the NP with its bases exposed so that they can be accessed by the polymerase without disrupting the RNP structure.³⁰ Approximately 24 nucleotides of RNA are bound by each NP monomer,⁵⁰² and NP also has homooligomerization properties,⁵⁴⁷ which adds a higher-order structure to the RNP complex. This is maintained even in the absence of RNA^{147,576} and has been shown to be crucial for maintaining the RNP in a transcriptionally active form.^{83,160} Crystal structures of NP show that it is composed of a head domain and body domain and that a flexible tail loop mediates oligomerization.^{481,745} A potential RNA-binding groove, which is highly positively charged, has been identified between the head and body domains.⁷⁴⁵ Structural data based on electron microscopy also provide evidence that NP makes direct contact with the bound polymerase complex on the RNP^{13,113,419} (e-Fig. 40.13A), which may reflect the previously reported interaction of free NP with both PB1 and PB2.⁴¹ Detailed mutagenesis of conserved

residues in NP indicates regions that are involved in genome replication/transcription and also genome packaging.³⁸⁸

The RNA Polymerase Complex

The influenza virus RNA-dependent RNA polymerase is a 250-kD complex of three proteins: PB1, PB2 and PA.⁵⁹ A three-dimensional image of the complex obtained by electron microscopy indicates that the three subunits are tightly associated to form a compact structure^{13,669} (e-Fig. 40.13B). Protein interaction studies have shown that PB1 binds to both PA and PB2, through its N- and C-terminal domains, respectively,^{228,492} and that the N-terminus of PA interacts with PB2.²⁶⁸ The details of how the newly synthesized polymerase is assembled are still under debate. One model proposes that PB1 and PA enter the nucleus as a dimer through interactions with RanBP5 and then bind to PB2, which is imported independently via a karyopherin α interaction.^{135,183,300,555,661} This is supported by fluorescent spectroscopy data in live cells.²⁹⁵ However, another model suggests that PB1-PB2 dimer is transported into the nucleus (via the chaperone Hsp90) and that PA traffics separately.⁴⁶⁵ It has also been shown that vRNA can bind to the PB1-PA dimer *in vitro* prior to PB2 binding as well as to the preformed trimeric complex.¹³⁶

THE PB1 PROTEIN

The PB1 protein catalyzes the sequential addition of nucleotides during RNA chain elongation⁵⁹ and contains the conserved motifs characteristic of RNA-dependent RNA polymerases.⁴² The active site for the polymerization activity is an S-D-D motif at positions 444 to 446,⁴² but we currently lack structural information for this region. In fact, only small regions encompassing the extreme N- and C-termini of PB1 have been crystallized in complex with portions of PA and PB2, respectively. Two studies have provided x-ray structures of either residues 1 to 81 or 1 to 25 of PB1 in complex with the C-terminal portion of PA,^{265,490} and it has been shown that synthetic peptides corresponding to this region of PB1 can compete with full-length PB1 for PA binding and inhibit virus replication.²¹⁹ The PB1 C-terminus (residues 678 to 757) has been shown to mediate an interaction with residues 1 through 37 of PB2, and structural analysis of this complex indicates that both peptides form three helices whose folding is dependent on the presence of each partner.⁶⁴⁶ PB1 is also responsible for binding to the terminal ends of both vRNA^{226,377} and cRNA²²⁷ for initiation of transcription and replication.

THE PB2 PROTEIN

The PB2 protein plays a critical role in the initiation of transcription as it is responsible for binding the cap on host pre-mRNA molecules.^{43,674} Despite earlier discrepancy in the position of the binding site, it has now been shown that a domain encompassing PB2 residues 318 to 483 is sufficient for cap binding²³⁶ and confirms the findings of a mutagenesis study that identified two aromatic residues (F363 and F404) as being important for the interaction.¹⁷¹ An x-ray structure of this minimal domain bound to cap analog m⁷GTP reveals that the cap is sandwiched between phenylalanine 404 and histidine 357 in a mode similar to that described for other cap-binding proteins, although the involvement of a histidine is unique.²³⁶ In fact, in influenza B and C virus PB2 proteins, the histidine is replaced with a more traditional tryptophan.

Both NMR and x-ray structures of the C-terminal domain (aa 678 to 759) of PB2 have been obtained, the latter in complex with karyopherin $\alpha 1$.⁶⁶¹ Within this domain the authors report the presence of a bipartite nuclear localization signal, and in the co-crystal it is shown how this region unfolds to allow for interaction with karyopherin $\alpha 1$. PB2 has also been shown to localize to the mitochondria, and this is determined by an N-terminal mitochondrial-targeting signal.⁸¹ However, avian influenza viruses have a polymorphism in this signal, so it appears that mitochondrial localization of PB2 is unique to human influenza viruses.²²⁹ Finally, as well as interacting with PB1 via its N-terminus (see PB1 section earlier), PB2 is also reported to interact with PA; however, the region of PB2 involved has not yet been defined.²⁶⁸ PB2 also participates in genome replication as mutations affecting this activity but not transcription have been reported.²¹⁵

THE PA PROTEIN

Until recently, the specific function of PA was unknown, but crystal structures of the N-terminal domain revealed that the endonuclease activity of the polymerase, which is required to generate the capped primer, resides in the PA protein.^{139,752} Previous work had mistakenly attributed this function to the PB1 protein. In the structure, the fold and position of the active site identifies the PA endonuclease as a member of the PD-(D/E)XK family of nucleases. The catalytic site involves residues His 41, Glu 80, Asp 108, Glu 119, and Lys 134 and harbors two Mn⁽²⁺⁾ ions.^{124,139} Mutation of these residues abolishes the transcriptional activity of the trimeric polymerase but replication activity is unaffected, confirming the specific role of the endonuclease in viral transcription.^{124,752} PA does, however, participate in genome replication as mutations affecting this process have been described.^{179,294} In addition to encoding nuclease function, the N-terminus of PA (aa 1 to 100) is also reported to be involved in an interaction with PB2,²⁶⁸ while the C-terminus makes contact with PB1. Structures of PA residues 257 to 716 show this region forming a “dragon-like head” with the N-terminal peptide of PB1 inserted into the mouth.^{265,490} Another function ascribed to PA is that it possesses proteolytic activity.⁵⁸² Two residues, S624²⁵⁵ and T157,⁵²⁵ have been reported to be involved in the proteolytic function, although viruses containing mutations at position 157 appear to be more severely affected than those mutated at position 624.^{294,670} PA has also been shown to be a target for casein kinase II and to be phosphorylated at serine and threonine residues.⁵⁸³

The vRNA Promoter

All influenza virus RNA segments contain noncoding sequences at their 5' and 3' ends, which flank the coding region. Some of this sequence is segment specific,⁷⁶⁶ but the terminal ends are conserved between all segments in all influenza viruses. These conserved 13 nucleotides at the 5' end and 12 nucleotides at the 3' end display partial and inverted complementarity, which led to the proposal of a panhandle structure created by base-pairing of the 5' and 3' ends.^{138,565} This is supported by cross-linking experiments that demonstrated a circular configuration for virion RNAs²⁹⁰ as well as by more recent structural analysis.^{19,419} Studies using *in vitro* transcription of model RNA templates or reporter gene expression *in vivo* have shown that both 5' and 3' terminal ends are necessary for promoter activity and that base-pairing is required (reviewed in (177,475)). Furthermore,

it has been demonstrated that the polymerase can interact with both 5' and 3' ends and that the binding affinity decreases when the duplex is disrupted.^{182,226,240,373,664} These data define the vRNA promoter as a double-stranded element formed by the conserved 5' and 3' terminal ends of the vRNA molecule.⁴⁸⁷ While the need for base-pairing is clear, several models for the secondary structure of the promoter have been proposed based on mutational analyses (e-Fig. 40.14). The original panhandle model predicts extensive Watson-Crick base-pairing, whereas the RNA-fork model proposes that the extreme termini remain single stranded.^{181,182,332} The corkscrew model suggests that these single-stranded regions in fact base-pair within themselves to form 5' and 3' hairpin loops.¹⁷⁶ The presence of both 5' and 3' stem-loops has been shown to be critical for endonuclease activity, and the 5' stem-loop is also required for polyadenylation.^{364,365,545} This favors a model where the stem-loop structures are involved in binding and stabilizing the polymerase complex.⁶⁴

Initiation of Messenger RNA Synthesis

Influenza virus mRNA synthesis is dependent on cellular RNA polymerase II activity. This is because it requires a 5'-capped primer, which it steals from host pre-mRNA transcripts, to initiate its own mRNA synthesis. This process is known as cap snatching and involves the cap-binding function of the PB2 protein and endonuclease function of the PA protein. The

initiation of transcription commences with binding of the 5' end of the vRNA to the PB1 subunit (Fig. 40.13). This induces an allosteric change in the polymerase, which allows the PB2 protein to recognize and bind the cap structure on host pre-mRNAs^{106,377} (reviewed in (170)). The change in the polymerase also increases its affinity for the 3' vRNA end, which is bound by PB1. Binding of the 3' terminus stabilizes the polymerase complex⁶⁴ and also serves to activate the endonuclease function.^{106,240,364,377} However, in contrast to this model, one report states that endonuclease activation only requires a bound 5' end,⁵⁵¹ the difference being that this study used capped RNA fragments with "CA" 3' termini as primers. Primers with this specific end have previously been shown to be used preferentially for transcription initiation in infected cells.^{32,604} Another study indicates that both primer-binding and endonuclease activities are greatly enhanced when the polymerase binds simultaneously to the 5' and 3' ends (i.e., in a preformed duplex).³⁷³ Endonuclease activation leads to cleavage of the bound pre-mRNAs. This occurs approximately 10 to 13 nucleotides from their 5' caps, usually after a purine residue.^{32,537} Transcription is then initiated by the addition of a "G" residue to the primer, directed by the penultimate "C" nucleotide at the 3' end of the vRNA template,³² although in some instances the incorporation of a "C" that is directed by the "G" at position 3 in the vRNA has also been observed.¹⁸¹ Unlike influenza viruses, Thogoto viruses lack host-derived sequences at the 5' end of their capped mRNAs.⁷¹⁹ RNA chain elongation is

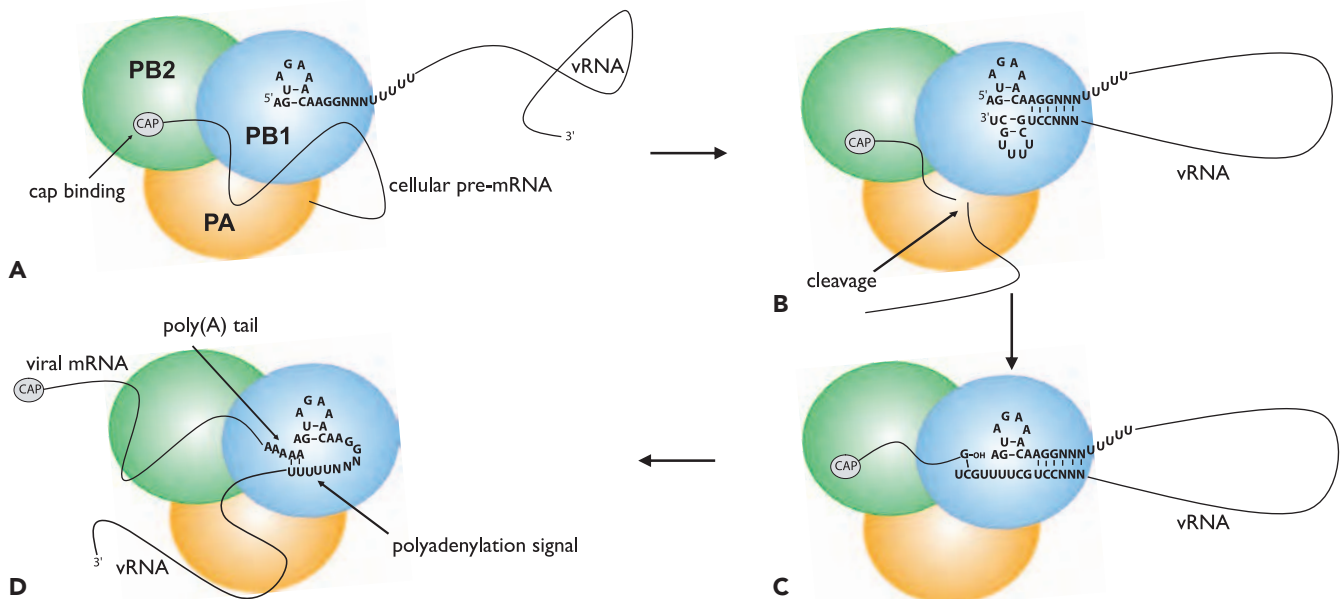


FIGURE 40.13. Proposed model for transcription initiation, elongation, and polyadenylation of influenza virus messenger RNA (mRNA). **A:** The 5' end of the viral RNA (vRNA) is shown in the corkscrew configuration bound to the polymerase basic 1 (PB1) subunit of the polymerase complex. This activates the cap-binding activity of the PB2 subunit. **B:** The 3' end of the vRNA binds to PB1 and forms a duplex with the 5' end. The endonuclease activity of the polymerase acid (PA) then cleaves the pre-mRNA 10 to 13 nucleotides downstream of the cap structure. **C:** A guanosine residue is added to the 3' end of the capped primer and base-pairs with the penultimate C residue at the 3' end of the vRNA. This initiates transcription and chain elongation is catalyzed by the polymerase function of the PB1 subunit. **D:** During elongation the cap detaches from the polymerase. However, the 5' end of the vRNA remains bound while the template vRNA is read in a 3' to 5' direction and consequently the polymerase is unable to read beyond the poly-uridine stretch due to steric hindrance. This causes it to stutter and a poly(A) tail is added to the 3' end of the nascent mRNA. (Adapted from Fodor E, Brownlee GG. Influenza virus replication. In: Potter CW, ed. *Influenza*. Amsterdam, The Netherlands: Elsevier, 2002:1–29.)

catalyzed by the polymerase function of PB1 and continues until a stretch of uridine residues is encountered approximately 16 nucleotides before the 5' end of the vRNA.^{383,407,566} This is the signal for polyadenylation (Fig. 40.13).

Polyadenylation

Unlike host cells, which use a specific poly(A) polymerase for generating the poly(A) tail on mRNA transcripts, polyadenylation of influenza virus mRNAs is catalyzed by the same polymerase that is used for transcription. This activity is dependent on an uninterrupted stretch of five to seven “U” residues and the adjacent double-stranded region of the vRNA promoter.^{383,407,566} The current model proposes that the 5' end of the vRNA remains bound to the polymerase during elongation while the template is threaded through in a 3' to 5' direction (Fig. 40.13). When the polymerase nears the 5' end to which it is bound, it is blocked by steric hindrance and consequently it stutters on the preceding stretch of uridines, which it repeatedly copies to produce a poly(A) tail.^{182,240,539,765} In support of this model, mutations introduced into the 5' end of the vRNA that prevent or weaken polymerase binding have been shown to also inhibit polyadenylation.^{180,540,545,546} The polyadenylation signal is vital for gene expression as replacement of the uridines with adenosines has been shown to result in transcripts with poly(U) tails, which fail to be properly exported from the nucleus.⁵³⁸

Splicing

Members of the *Orthomyxovirus* family can extend the coding capacity of their genomes by producing two proteins from one gene via an alternative splicing mechanism. Genome segments that encode proteins from both spliced and unspliced mRNA transcripts are segments 7 and 8 of influenza A virus,^{358,360} segment 8 of influenza B virus,⁶³ segments 6 and 7 of influenza C virus,^{468,741} segment 6 of Thogoto virus,³³⁹ and segment 7 of isavirus⁴⁰ (see Genome Structure and Organization section). The primary transcripts from these segments have 5' and 3' splice sites, which (more or less) fit the consensus sequence for the exon/intron boundaries of cellular transcripts. This, combined with the fact that splicing can be demonstrated in the absence of any viral proteins,^{357,359} indicates that the virus is using the cellular splicing machinery. However, unlike cellular splicing, which is extremely efficient, splicing of viral mRNA has to be relatively inefficient because proteins must be expressed from both spliced and unspliced mRNAs. In influenza virus–infected cells, splicing is tightly regulated such that the steady-state level of spliced viral transcripts is only 10% that of the unspliced viral transcripts.^{356,360} These control mechanisms may act on several different levels. The rate of nuclear export of the unspliced transcript is certainly crucial as this determines its availability for splicing. It has been proposed that the NS1 protein inhibits both the splicing and nuclear export of NS1 transcripts via negative feedback,^{8,209} but contradictory reports⁵⁵⁹ suggest that alternative mechanisms may exist for regulating splicing of viral transcripts. Potentially these may involve *cis*-acting sequences in the NS1 transcript that negatively control the rate of splicing.^{7,474} Strangely, the influenza C virus NS1 protein has been reported to up-regulate viral mRNA splicing.⁴⁵⁸ Splicing of the influenza A virus M1 transcript is controlled by the aforementioned rate of nuclear export⁶⁷⁵ as well as by the viral polymerase and a cellular splic-

ing factor, SF2/ASF. The polymerase determines the time at which splicing (and hence production of M2) occurs, and SF2/ASF is required to activate splicing.^{605,606}

Replication Products: cRNA and vRNA

Full-length copies of the incoming vRNA have to be made, and these positive-sense cRNAs serve as templates for the synthesis of new negative-sense genomic vRNA. *In vitro* evidence suggests that the *de novo* initiation mode for vRNA synthesis may occur via terminal initiation and elongation, whereas for cRNA synthesis it involves internal initiation and realignment.¹³⁷ This is a primer-independent model; however, a primer-dependent mode of vRNA initiation has also been proposed.⁵²⁸ The cRNA promoter is complementary to the vRNA promoter and has also been reported to assume a corkscrew configuration, albeit with subtle differences.^{18,129,519,766} This variation has been implicated in determining whether or not the endonuclease function of the polymerase is activated and therefore may play an important regulatory role.³⁶⁶

The Switch from Transcription to Replication

The vRNA serves as a template for both mRNA and cRNA synthesis, and yet the means of initiation and termination for the generation of these two molecules are quite different. In contrast to the primer-dependent mechanism of initiation of mRNA synthesis, initiation of cRNA synthesis occurs without a capped primer and cRNA molecules are full-length copies of the vRNA and thus are not prematurely terminated and polyadenylated as are mRNAs. The different initiation and termination reactions therefore have to be coordinated, but exactly how the polymerase switches between these two modes is not well understood. It has been proposed that the transcription-competent polymerase is structurally different from the replication-competent polymerase, and support for this theory comes from evidence that different domains of PB1 are involved in binding vRNA versus cRNA and that PA is more critical for binding the cRNA than the vRNA promoter.^{227,412} One obvious difference is that the cap-binding and endonuclease functions of PB2 and PA are not required when the polymerase is in replication mode.

In contrast to mRNAs, newly synthesized cRNAs and vRNAs are encapsidated, and it has been proposed that the availability of soluble NP (i.e., not associated with RNPs) controls the switch between mRNA and cRNA synthesis. This hypothesis arose from the observation that replication is dependent on *de novo* protein synthesis, which means that the incoming RNPs are only capable of transcription.²⁶³ Indeed, free NP has been shown to be required for production of full-length cRNA (antitermination),³³ and this is consistent with data from temperature-sensitive (ts) NP mutants,^{345,435,598} which show that cRNA but not mRNA synthesis is affected at the nonpermissive temperature. However, this model has been challenged by a report demonstrating that overexpressed NP does not promote replication.⁴⁵⁷ Another study disputes the existence of a switch, rather suggesting a stabilization role for NP and the polymerase.⁶⁹⁴ It claims that the incoming polymerase is able to synthesize both mRNA and cRNA,⁶⁹⁰ but until there is a sufficient pool of polymerase and NP to encapsidate the cRNA, it is degraded and therefore at early times postinfection there is a bias toward mRNA accumulation. Also, requirements for higher nucleotide concentrations

to initiate cRNA synthesis may determine the timing of transcription versus replication.⁶⁹³ Furthermore, the accumulation of NEP/NS2 is associated with a decrease in transcription and an increase in replication, suggesting a regulatory role.^{74,560} Interestingly, NEP/NS2 is also required for the generation of small viral RNAs (svRNAs), which have been implicated in the initiation of vRNA synthesis.⁵²⁸ The svRNAs are 22 to 27 nt in length and correspond to the 5' end of each viral RNA segment. These segment-specific svRNAs are needed for vRNA but not cRNA synthesis, so according to this model the polymerase is in replication mode when these svRNAs are present. It has also been proposed that the switch from transcription to replication is the result of accumulation of a newly synthesized free polymerase complex, which enhances cRNA to vRNA synthesis (and vice versa) over mRNA synthesis.³²² The role of host factors in regulating influenza virus replication, including posttranslational modification of viral proteins, should also not be excluded.^{51,323,392,427,449,450}

Regulation of Viral Gene Expression

Early studies have provided evidence for temporal regulation of viral gene expression,^{263,624} but the mechanism(s) is still unresolved. Disproportionate accumulation of mRNAs from the eight segments has been observed, but whether this represents specific up-regulation of transcription for these segments^{164,260} or reflects different rates of vRNA synthesis^{597,624} or RNA stability is unclear. Suffice to say that the synthesis of NP and NS1 mRNAs and protein is favored at early stages, whereas the synthesis of HA, NA, and particularly M1 mRNAs and proteins is delayed.^{260,263,597,624} This differential expression is mirrored by the roles these proteins play at different points in the virus life cycle. As discussed earlier, NP is required for replication, and NS1 plays a crucial role in combating the host immune response; thus, both these proteins are needed early in the virus life cycle. M1 has been found to inhibit viral transcription,^{527,713} which demands its delayed expression, and at later stages M1 accumulation probably dictates the arrest of viral mRNA synthesis. M1 is also involved in the export of RNPs from the nucleus,⁴²⁰ which must only occur once replication is complete.

Another control mechanism for differential gene expression resides in the vRNA promoter. A natural variation is found at position 4 from the 3' vRNA end in an otherwise totally conserved region. The PB1, PB2, and PA RNA segments have a "C" at this position, while the remaining segments usually have a "U." The C4-containing promoter is associated with a down-regulation in transcription and an up-regulation in replication compared to the U4 promoter,³⁷⁰ which correlates with the lower amounts of polymerase mRNAs and proteins found in infected cells.^{263,624} A structural analysis of the C4 and U4 promoters has revealed differences that may alter their interaction with the polymerase and thereby regulate gene expression.³⁷²

As observed with many other viruses, influenza virus gene expression is also controlled at the level of translation. This is achieved via numerous mechanisms and results in the selective translation of viral genes and suppression of host protein synthesis (reviewed in (200,692,743)). These mechanisms include (a) degradation of host pre-mRNAs following cleavage (due to cap-snatching), (b) inhibition of host mRNA processing, (c) degradation of cellular RNA polymerase II, and (d) preferential translation of viral mRNA transcripts. Several of these processes involve the influenza virus NS1 protein. The NS1-mediated

effect on mRNA processing is discussed in a later section (see The Actions of Influenza Virus Nonstructural Proteins on the Host Cell). NS1 is also involved in the specific translational enhancement of viral mRNAs through its association with the 5' noncoding region of viral mRNA transcripts and with cellular proteins involved in translation initiation.^{11,76,520} These include the translation initiation factor eIF4GI and poly(A)-binding protein 1, and it has been proposed that this protein complex acts to specifically recruit ribosomes to the 5' end of viral mRNA transcript. Another cellular protein that may play a role is GRSF-1, an RNA-binding protein that has been reported to interact with the 5' end of the NP transcript and to stimulate the specific translation of a template driven by the NP 5' non-coding region in a cell-free translation system.^{326,521} Whether this interaction is relevant *in vivo* remains to be determined.

An interesting model explaining the selective translation of viral mRNAs has been proposed suggesting that the viral polymerase complex remains associated with the viral mRNA transcript in the cytoplasm. It is thought that this interaction eliminates the need for complex formation with eIF4E. As eIF4E is inactivated in influenza virus-infected cells,¹⁷² this would explain the selective translation of viral transcripts over cellular transcripts. Other components of the translation machinery, such as eIF4A and eIF4G, are required for influenza viral protein translation.⁷⁴² Although this model is compelling in its simplicity, this mechanism is questioned by the finding that viral mRNAs in the cytoplasm are devoid of viral polymerase but are associated with cellular cap-binding proteins, including eIF4E.³⁹ Clearly, further investigation is required to explain the selective translation of viral transcripts in infected cells.

An additional host shut-off mechanism is controlled by the viral polymerase at the level of host transcription. It has been shown that the influenza virus polymerase interacts with the C-terminal domain of the large subunit of cellular RNA polymerase II¹⁶⁷ and that this interaction mediates the degradation of RNA polymerase II at late times postinfection.^{568,691} This may play a role in viral pathogenicity because attenuated influenza viruses have been shown not to induce RNA polymerase II degradation.⁵⁶⁷

Virus Assembly and Release

Nuclear Export of Ribonucleoproteins

ASSOCIATION OF RNP WITH M1

Following virus replication, newly formed RNP complexes are assembled in the nucleus from where they are exported into the cytoplasm. Two viral proteins, the matrix protein (M1) and the nuclear export protein (NEP/NS2), are involved in directing the nuclear export of RNPs (reviewed in (54,127)). Our present understanding of this process indicates that M1 associates with RNPs in the nucleus and may actually promote the formation of RNP complexes.²⁹³ M1 makes contact with both the vRNA and NP^{31,746} (reviewed in (127,472)), and evidence that M1 also binds to nucleosomes^{210,769} has led to the hypothesis that M1 interactions cause the dissociation of RNP from the nuclear matrix. This agrees with the significant finding that nuclear import of M1 is required for subsequent export of RNP complexes.^{72,420} Furthermore, at high temperatures, heat shock protein 70 is found bound to RNP, which prevents association with M1 and results in a block in RNP export.^{273,580} Recently it has been reported that sumoylation of M1 is essential for its nuclear export function.⁷³²

NEP/NS2 INTERACTS WITH THE CELLULAR EXPORT MACHINERY

Initially these data suggested that M1 alone could control RNP export, but as is the case for import, nuclear export of large molecules involves direct interactions with the cellular export machinery and as yet no such interactions with M1 have been demonstrated. However, NEP/NS2 has been found to interact with the export receptor Crm1⁴⁷⁷ and several nucleoporins.^{91,498} NEP/NS2 also associates with M1,^{4,608,744} so the current model is that an RNP–M1–NEP/NS2 complex is formed in the nucleus and that NEP/NS2 is responsible for recruiting the export machinery and directing export of the complex (e-Fig. 40.15). In support of this role, it has been shown that injection of anti-NEP/NS2 antibodies into the nucleus of infected cells inhibits RNP export,⁴⁹⁸ which is also seen in a system using recombinant virus-like particles lacking NEP/NS2.⁴⁷⁷ A methionine/leucine-rich nuclear export signal (NES) has been identified in the N-terminus of NEP/NS2⁴⁹⁸ and shown to be critical for RNP export and virus growth^{309,477}; however, this NES does not appear to mediate the interaction of NEP/NS2 with Crm1.⁴⁷⁷ Yet treatment with leptomycin B, a Crm1 inhibitor, completely inhibits RNP export in infected cells, which indicates that export does occur in a Crm1-dependent manner.^{162,309,714}

Another viral protein, NP, has also been shown to bind to Crm1¹⁶² and therefore is proposed to play a role in export as well as import. Both this study and another also revealed that RNPs are localized to the periphery of the nucleus after leptomycin B treatment but that M1 and NEP/NS2 retain diffuse nuclear staining.^{162,410} The RNPs were shown to co-localize with nuclear lamins just beneath the nuclear pore complex, which may represent an intermediate step prior to export through the pore.⁴¹⁰ The roles of M1 and NEP/NS2 are obviously called into question by their differing staining pattern compared to RNP,^{162,410} but this could be explained if only a fraction of the total M1 and NEP/NS2 pool was used for export. Nevertheless, it raises the possibility that redundant export mechanisms may exist.

REGULATION OF RNP EXPORT

As discussed earlier, the late expression of M1 determines that export takes place only after a full round of replication has occurred, therefore preventing premature exit of RNPs from the nucleus. Similarly, control mechanisms must also exist to stop the re-entry of RNPs into the nucleus following export. Studies with a temperature-sensitive virus (ts51) showed that when grown at the nonpermissive temperature, this virus is unable to retain its RNPs in the cytoplasm following export.⁷²³ This defect mapped to M1,⁷²⁴ once again demonstrating the vital role of this protein in regulating the nucleocytoplasmic transport of RNPs. It is interesting to note that the NEP/NS2-binding site on M1 maps to the M1 NLS⁴ which suggests that NEP/NS2 may act to mask the NLS on M1 and therefore prevent the complex from re-entering the nucleus. NP is also proposed to cause the cytoplasmic retention of RNPs by binding to filamentous actin and thereby anchoring the RNPs in the cytoplasm.¹⁴⁰ All these processes are likely to be regulated at some level, and protein modification by phosphorylation is probably involved as several studies have reported alterations in M1, NEP/NS2, and NP trafficking in the presence of kinase inhibitors.^{70–72,536,553,724}

RNP EXPORT IN INFLUENZA B AND C VIRUSES

Few studies concerning export in influenza B and C viruses have been performed, but it has been shown that as with influ-

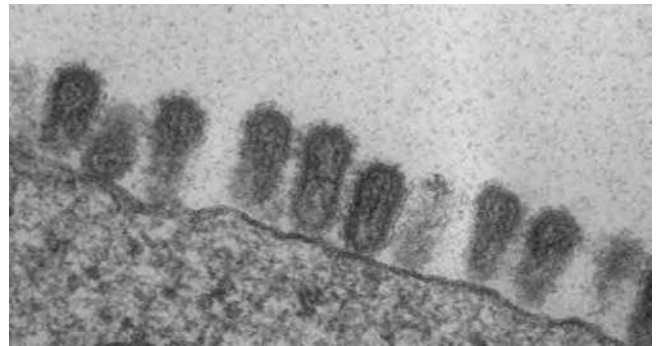


FIGURE 40.14. Budding influenza virus particles. Electron micrograph thin section image of influenza virus particles budding from the apical surface of an infected cell. (Courtesy of Yi-ying Chou.)

enza A viruses, the NEP/NS2 proteins possess nuclear export activities.⁵¹⁸ They have both been shown to interact with Crm1 (and a subset of nucleoporins), and NES motifs have been defined in each protein. Interestingly, the influenza C virus NES is composed of two separate leucine-rich domains, both of which are required for full activity.⁵¹⁸ In contrast to influenza A virus where M1 acts as a bridge between NEP/NS2 and the RNP complex, the influenza B NEP/NS2 has been proposed to bind directly to RNP as well as to M1,³⁰⁴ which suggests that the model for export of influenza B virus RNPs may be slightly different.

The Site of Virus Assembly and Budding

Influenza viruses assemble and bud from the apical plasma membrane of polarized cells (e.g., lung epithelial cells of the infected host)⁵³ (reviewed in (573,587)) (Fig. 40.14). This asymmetrical process (i.e., apical vs. basolateral) is thought to have an important role in viral pathogenesis and tissue tropism in that viruses that bud from the internal cell surface (e.g., Marburg virus) tend to cause systemic disease, whereas viruses such as influenza virus that bud from the external cell surface generally have a more restricted tissue tropism.¹⁷³ The influenza virus HA, NA, and M2 have all been shown to localize to the apical surface of polarized cells when expressed alone,^{296,321,574} and apical sorting signals have been identified within the transmembrane domains of HA and NA.

THE M1 PROTEIN

M1 is the most abundant virion protein and lies just beneath the lipid envelope where it is believed to make contact with the cytoplasmic tails of the glycoproteins and with the RNPs, thereby forming a bridge between the inner core components and the membrane proteins (reviewed in (471,587)). Structural analyses indicate that the M1 protein consists of two globular helical domains that are linked by a protease-sensitive region.^{14,257,595} Rods (6 nm in length) corresponding to M1 monomers have been observed by negative stain electron microscopy of virions with one end in contact with the membrane and the other end pointing toward the interior of the particle.⁵⁷⁵ These rods form an ordered structure consistent with the homo-oligomerization properties of M1^{80,764} and are arranged such that the positive and negatively charged residues are on opposite sides of the oligomer.^{14,257} Several reports have documented the ability of M1 to associate with lipid membranes^{163,575,759} (reviewed in (471,587)),

and as mentioned previously (see section on Nuclear Export of Ribonucleoproteins), M1 interacts with both RNP and NEP/NS2. M1 also interacts with the cytoplasmic tail of M2.⁸⁷ Therefore, it is proposed that M1 plays a vital role in assembly by recruiting the viral components to the site of assembly at the plasma membrane.

Assembly of Viral Components

Following synthesis on membrane-bound ribosomes, the three integral membrane proteins, HA, NA, and M2, enter the ER where they are folded and glycosylated (except for M2) and where HA is assembled into a trimer and NA and M2 into tetramers (reviewed in (141)). They are subsequently transported to the Golgi apparatus where cysteine residues on HA and M2 are palmitoylated in the *cis*-Golgi network.^{635,647,681–683} For those HAs that have a multibasic cleavage site (i.e., some H5 and H7 subtypes), furin cleavage of HA into HA1 and HA2 subunits may occur in the *trans*-Golgi network.⁶⁴² From here HA, NA, and M2 are all directed to the virus assembly site on the apical plasma membrane via their apical sorting signals. The signals for HA and NA have been described to reside in their transmembrane domains (TMDs).^{26,350,393} The TMDs of HA and NA also contain the determinants for association with lipid rafts^{26,393} (reviewed in (25)). Lipid rafts are nonionic detergent-resistant lipid microdomains within the plasma membrane that are rich in sphingolipids and cholesterol (reviewed in (499)), and examination of the lipid content of purified virus particles indicates that influenza virus buds preferentially from these domains.^{585,760} HA and NA individually also selectively accumulate at and are incorporated into rafts.^{350,586} Although the signals for apical sorting and raft association both lie within the TMD, they are not mutually exclusive (reviewed in (25,472)). Raft association of HA has been shown to be essential for efficient virus replication.⁶⁵⁵ This is thought to be because of a requirement for concentrated “patches” of HA at the plasma membrane, which governs the level of HA incorporation into budding particles and hence affects fusion. A similar explanation holds for raft association of NA, as an optimal amount of NA must be incorporated to allow for efficient virus release.²⁴ In contrast to HA and NA, the majority of M2 protein is excluded from lipid rafts,⁷⁶⁰ which may reflect its low abundance in virus particles. M2 has been shown to bind cholesterol and this property is suggested to target M2 to the raft periphery where it may act to bridge several raft domains.⁵⁹¹ Mutation of the cholesterol recognition/interaction amino acid consensus (CRAC) motif in M2 is reported to affect membrane targeting but not raft association,⁶⁶² and in the context of a recombinant virus it is shown to attenuate the virus *in vivo* but not in tissue culture.⁶⁴¹ There is also evidence that M2 is involved in capturing the RNPs at the assembly site. Experimental evidence for this mechanism was first demonstrated with an influenza B virus that lacked BM2 expression and produced particles devoid of RNPs.³⁰³ Subsequently, mutation or truncation of the influenza A virus M2 cytoplasmic tail has been shown to correspond with decreased incorporation of genome segments into virions.^{231,310,431,432}

In comparison to the integral membrane proteins, relatively little is known about how the remaining viral components reach the assembly site. A long-standing hypothesis is that M1 acts as the master recruiter as dictated by its position between the viral envelope and the RNP core. This is supported

by evidence that the availability of M1 affects the timing of assembly and maturation, as seen with a virus engineered to express reduced levels of protein from the M segment.⁵⁶ This virus showed no defects in virion protein composition but displayed delayed growth kinetics, suggesting that a minimum amount of M1 protein must accumulate before assembly can begin. The association of M1 with the RNP–NEP/NS2 complex is well described (see section on Nuclear Export of Ribonucleoproteins), but the specific interaction of M1 with the membrane-bound glycoproteins has been difficult to prove because of its intrinsic membrane-binding properties and initially resulted in some conflicting reports.^{163,344,759} However, it was noted that in influenza virus-infected cells M1 becomes resistant to extraction with Triton X-100 (a marker for lipid raft association), whereas M1 expressed alone remains soluble.^{6,759} This suggested a role for other viral proteins, and indeed, co-expression of HA and NA together with M1 has been shown to promote raft association of M1.⁶ This requires the TMDs and cytoplasmic tails of HA and NA,^{6,760} and in the absence of the cytoplasmic tails of these two proteins, virus particles have been found to be grossly distorted, which perhaps indicates reduced M1 association.³¹⁷ The hypothetical model therefore proposes that M1 becomes associated with the glycoproteins during their passage through the exocytic pathway and “hitches a ride” to raft domains in the apical membrane, taking the RNP–NEP/NS2 complex with it. However, alternative models have been suggested, and these include the possibility that the M1/RNP complex may use the cytoskeleton to reach the virus assembly site. This is fueled by the finding that both NP and M1 interact with cytoskeletal components.¹⁷ The M1 interaction is dependent on the presence of RNP and is most likely mediated by direct binding of F-actin by NP.^{17,140} However, an intact cytoskeleton has only been found to be necessary for the production of filamentous virus particles,^{563,614} so a specific role in assembly *per se* is debatable, although actin does appear to be involved in the organization of lipid rafts.⁶¹⁴ Finally, there is evidence that NP alone is intrinsically targeted to the apical plasma membrane and associates with lipid rafts in a cholesterol-dependent manner, which suggests that RNPs could reach the assembly site independently of the other viral components.⁸² Recent studies have shown that the Rab11-dependent recycling endosome is critical for the delivery of RNPs to the plasma membrane.^{9,155,451} as is another endosomal protein, human immunodeficiency virus rev-binding protein (HRB), which interacts with NEP.¹⁵⁶

Packaging of Eight RNA Segments

Correct assembly and packaging of a full complement of RNA genome segments is a requirement for a fully infectious virion. The precise mechanism of packaging of the eight viral RNA segments is not well understood, although two different models have been proposed. The first model, the *random incorporation model*, assumes that a common structural feature is present on all vRNAs (vRNPs), which enables them to be randomly incorporated into budding virions. This model is supported by evidence that virions may possess more than eight vRNPs, ensuring the presence of a full complement of eight vRNPs in a significant percentage of virus particles.^{22,166,207} Mathematical analysis of packaging suggests that if eight RNA segments were randomly packaged into budding virions, only 0.24% of released virus particles would be infectious.¹⁶⁶ However, if a

greater number of RNA segments were randomly packaged, then the percentage of infectious particles increases (reviewed in (114)). If 12 RNA molecules are packaged per virion, then approximately 10% of the virus particles would be infectious,¹⁶⁶ a number that is compatible with experimental data ((142) and reviewed in (114)).

The second model, *the selective incorporation model*, suggests that each vRNA segment acts independently, allowing each segment to be packaged selectively. A similar model has been reported for the packaging of the 3 double-stranded RNA (dsRNA) segments of bacteriophage $\phi 6$ (reviewed in (446)) and for the packaging of the 11 dsRNA segments of rotavirus (RV) (reviewed in (433)). This model suggests that each vRNA segment contains a unique “packaging signal” and predicts that every virion possesses a full complement of the eight vRNP segments. There is increasing evidence to support this model. First, the precise number of RNAs packaged in a single virion has been determined by imaging of serially sectioned budding virions using electron microscopy.⁴⁸⁹ Each virion appears to contain exactly eight vRNPs organized in a distinct pattern: one in the center and seven in the surrounding positions. The eight vRNPs are oriented perpendicular to the budding tip. Second, the existence of packaging signals within the noncoding and coding regions at both the 5′ and 3′ ends of the genomic RNAs has been confirmed (e-Fig. 40.16). Coding regions of the NA¹⁹¹; HA⁷¹⁸; NS¹⁸⁹; PB2, PB1, and PA^{390,462}; NP⁵⁰³; and M⁵⁰⁵ segments have all been demonstrated to increase the ability of a reporter sequence to be incorporated within assembling virions. Both the coding and noncoding regions of the packaging signals are relatively conserved compared to other parts of the sequences.^{194,224} Mutations introduced into the packaging signal region of one segment can result in a decrease in packaging efficiency of the segment itself and other segments,^{299,302,391,416,417} suggesting the existence of specific interactions among genomic segments. Interestingly, data also show that efficient packaging of the NS segment does not absolutely require the original sequences of the packaging signal.¹⁹⁰ Segment-specific packaging is hypothesized to occur via specific RNA–RNA or protein–RNA interactions, but exactly how the packaging signals participate during the genome packaging process is yet to be determined. Another piece of evidence supporting the specific packaging model is the generation of a rewired influenza virus carrying the HA packaging signal on the NS segment and the NS packaging signal on the HA segment.²⁰⁸ The modified virus grew well; however, it lost its ability to independently reassort its rewired HA or NS segment with a wild-type virus, indicating that only viruses containing a full complement of all eight packaging signals will grow to high yields. This model is also supported by data showing specific interference of deleted RNA segments with packaging of the corresponding wild-type RNA segment but not with any other genome segment.^{148,149} It will be interesting to define the precise sequences or structures that determine the specific packaging of each segment. Such conserved features must also be compatible with the divergent sequences observed among influenza viruses. For further details about the influenza genome packaging process, please see the reviews by Noda et al.⁴⁸⁸ and Hutchinson et al.³⁰¹

The Budding Process

Initiation of bud formation requires outward curvature of the plasma membrane. The virus bud is then extruded until

the inner core is enveloped. The budding process is completed when the membranes fuse at the base of the bud and the enveloped virus particle is released following fission from the cell membrane (reviewed in (573,587)). It is likely that several of the influenza virus structural proteins contribute to the budding process. HA, NA, and M2, when expressed alone in transfected cells, are all competent to form virus-like particles (VLPs).^{88,352,750} Although M1 obviously participates in the formation of virus particles from infected cells, unlike other viral matrix proteins, it does not appear to possess a late domain sequence that mediates interaction with the cellular ESCRT pathway.^{66,572,715} It has also been demonstrated that in the absence of other viral proteins, M1 does not associate with membranes.⁷⁰⁰ Thus, it appears that there are redundant mechanisms for the initiation of bud formation, and in the context of an infected cell it is presently unclear which of these are dominant. The extent to which the membrane is extruded before pinching off occurs affects the size and shape of the virus particle. Generally, influenza virus particles are either spherical or filamentous, and this characteristic morphology is genetically linked to the M segment^{55,159,564,621} (e-Fig. 40.1). It has been further shown that the determinants for a particular filamentous isolate (A/Udorn/72[H3N2]) map to two residues (R95 and E204) in the M1 protein,⁵⁵ although other residues in M1 as well as in the cytoplasmic tail of M2 are also involved in regulating morphology.^{231,310,564,571} Host factors such as polarization and an intact actin cytoskeleton also play a critical role in determining the morphology of virus particles.^{563,614} The final step of the budding process is membrane scission, which may be facilitated by both viral and cellular factors. The amphipathic helix in the cytoplasmic tail of M2 has been demonstrated to mediate membrane curvature and scission.⁵⁷² Also, the small guanosine triphosphate (GTP)-binding protein, Rab11, has been implicated in this process.⁶⁵

Release

Influenza virus particles have to be actively released after the viral envelope has separated from the cell membrane during the completion of budding. This is because the HA anchors the virus to the cell by binding to sialic acid-containing receptors on the cell surface. The enzymatic activity of the NA protein is required to remove the sialic acid and thereby releases the virus from its host cell. NA activity is also required to remove sialic acid from the carbohydrates present on the viral glycoproteins themselves so that the individual virus particles do not aggregate. The essential function of NA in particle release has been demonstrated through the use of NA inhibitors,^{404,510} ts NA mutant viruses,⁵¹⁶ and NA-deficient viruses.³⁹⁵ In all cases, the absence of NA enzymatic activity was seen to cause viral particles to amass in clumps at the cell surface (Fig. 40.15), resulting in a loss in infectivity that could be restored by addition of exogenous sialidase.

Due to the fact that both HA and NA recognize the same molecule (sialic acid) but have opposing effects (receptor binding vs. receptor destroying), a delicate balance exists between the HA and NA functions.⁶⁹⁷ This is optimized for individual viruses but if disturbed can result in attenuation.⁷⁴⁸

THE NEURAMINIDASE

The NA is the second major glycoprotein of influenza A and B viruses and is a type II integral membrane protein with its

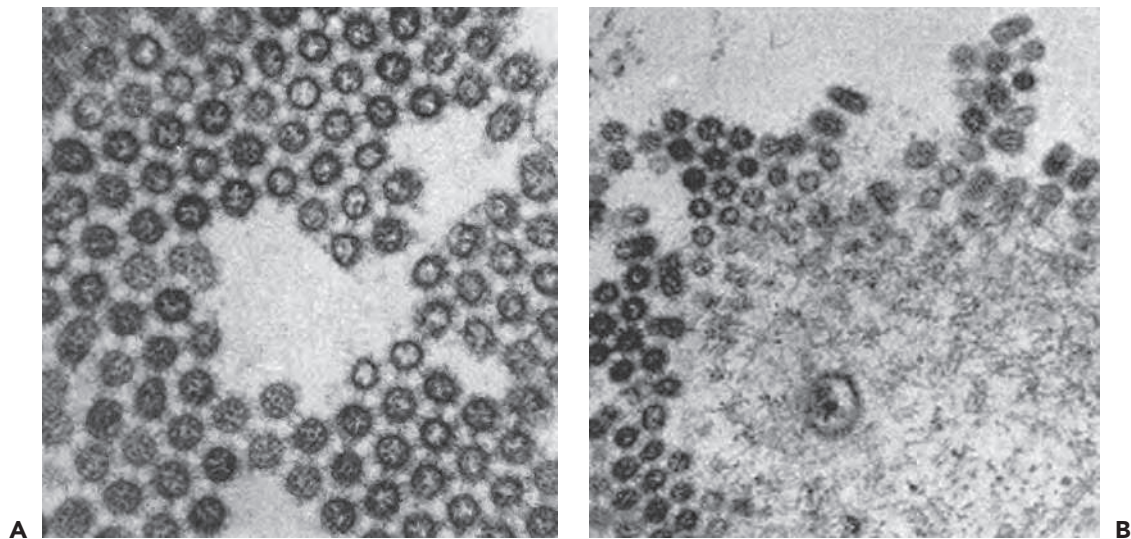


FIGURE 40.15. Aggregate formation of influenza virus particles in the absence of neuraminidase activity. Electron micrograph thin section images showing aggregates of temperature-sensitive neuraminidase (NA) mutant influenza virus grown at nonpermissive temperature (**A**) or grown in the presence of the neuraminidase inhibitor FANA (**B**). For details see (516) and (510).

N-terminus oriented toward the interior of the virus¹⁰⁹ (for review see (204) and for an interesting personal account see (362)). The nine subtypes of the A virus NA fall into two major groups (N1, N4, N5, N8 and N2, N3, N6, N7, N9) based on sequence comparisons¹⁸⁶ (Fig. 40.2). No subtypes have been found for the NAs of B viruses, possibly because these viruses do not have an animal reservoir. The influenza A virus NAs have a highly conserved short cytoplasmic tail and a hydrophobic transmembrane region, which provides the anchor for the stalk and the head domains. The purified head domain of an N2 NA (obtained by pronase treatment of whole virus) was first crystallized by Graeme Laver and its x-ray crystallographic structure was solved by Peter Colman.¹¹² The structure of the N1 NA from the 1918 pandemic virus has also been determined.⁷³⁸ The head of the NA is a homotetramer, each monomer of which is composed of six topologically identical β sheets arranged in a propeller formation. Sugar residues are attached to four of the five potential glycosylation sites in the head (e-Fig. 40.17). The structure of the influenza B virus neuraminidase, similar to that of the A virus, is characterized by the interaction of the sialic acid ligand with nine conserved active site residues.⁷⁸ The enzymatic activity of NA was first recognized by George Hirst, who found that red blood cells treated with virus were refractory to reagglutination by another virus preparation.²⁷⁴ The enzyme was also found to cleave (at position 2 of neuraminic acid) ketosidically bound sugars of alcohols (for review, see (68)), and neuraminidases from different subtypes are described to have different substrate specificities.^{385,543} Transition state inhibitors such as 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid, which mimic the enzymatic substrate, were shown early on to inhibit influenza virus replication,⁵¹⁵ and compounds with the same mechanism of action were later developed for use as highly effective antivirals in humans (for review, see (214,315,434)). Early work had also elucidated the function of the NA in virus replication. Cells infected by temperature-sensitive mutants with defects in the

NA were shown by electron microscopy to have large aggregates of intact virus particles accumulating near the cell surface (Fig. 40.15). This finding was interpreted to mean that the viral NA must remove the sialic/neuraminic acid receptor from the surface of the cell as well as from the virus particles to prevent recognition by the HA of the virus. The NA thus has a role in releasing the virus from the infected cell and in cleansing the environment (e.g., mucus and cell surfaces in the respiratory tract) of sialic acid receptors to allow for virus spread.^{510,516} In addition, it has been shown that the viral NA may also play a role early in infection, possibly facilitating entry of the virus^{426,493} and/or enhancing late endosome/lysosome trafficking.⁶⁵² As described earlier (see Budding Process), the NA can also mediate virus budding^{352,750} and the cellular restriction factor, tetherin, can influence this step.^{716,750} Another function for at least one subtype NA has also been reported. In the case of an N9 NA, a hemadsorption activity was found to be associated with the purified molecule, and x-ray structure analysis revealed a second independent binding site for sialic acid.⁶⁷⁸ This activity is only associated with avian neuraminidases and appears to be lost upon adaption to humans.⁶⁷³

It is assumed that the function of the influenza B virus NA is similar, if not identical, to that of the A virus NA,^{220,405} and the active sites of influenza A and B virus NAs are conserved, which allows for broad-spectrum activity of NA inhibitors. In influenza C viruses, the receptor-destroying role of the NA is played by the esterase activity of the viral HEF. By removing the acetyl group from 9-O-acetylneuraminic acid, the HEF facilitates the release and spread of virus from infected cells. In addition, it appears that the enzyme is needed for virus entry, suggesting that there is a need for the HEF to be released from cell receptors during the endosomal uptake and fusion/uncoating process.⁶⁸⁸

Like the HA, NA molecules are antigenic and variants are selected in nature. Antibodies directed against the NA are usually not neutralizing, but immunization with NA preparations

has been proposed as an infection-permissive, disease-suppressive vaccine approach against influenza.^{330,654}

Interactions of Influenza Virus with the Host Cell Cellular Functions Required for Influenza Virus Replication

A virus with a small coding capacity, such as influenza virus, relies on numerous host cell functions in order to complete its replication cycle. In comparison to our understanding of the role of each viral protein in the influenza virus life cycle, we know relatively little about the contribution of host cell proteins. Some well-characterized interactions between viral and host proteins are noted in the sections covering specific viral proteins (e.g., NP and karyopherin α , NEP/NS2 and Crm1, NS1 and CPSF30), but these probably represent only a small fraction of the molecular interactions that occur between influenza virus and its host cell during the viral life cycle. More recent efforts to expand our knowledge of these cellular binding partners have involved a comprehensive yeast two-hybrid analysis of 10 influenza virus proteins (all except PB1-F2), which identified interactions with 87 human proteins.⁵⁹⁶ These interactions exist in a tightly connected network, as 24 of the human proteins interact with two or more viral proteins and there are 51 known interactions occurring between the 87 human proteins. Other studies have examined interacting partners of viral protein complexes rather than individual proteins, and particularly those that retain functionality such as the RNP or trimeric polymerase complexes. Forty-one human proteins were reported to interact with the viral RNP complex of influenza A/WSN/33 (H1N1) virus,⁴²⁷ and 13 interacting proteins were identified in two studies on the WSN polymerase complex.^{323,427} A recent large-scale proteomic analysis of an H5N1 influenza virus polymerase complex has revealed an astonishing 859 human proteins that are associated with either the full polymerase complex or PA-containing subcomponents thereof.⁶⁰ In their analyses, the authors also distinguished between those interactions that are dependent on RNA and those that are not. In summary, 166 PA interacting proteins, 23 that bind to the PB1-PA dimer and 10 that associate with the full polymerase, were identified irrespective of the presence of RNA. Notably, a number of the PA-interacting host proteins are localized to the mitochondria and may be linked to apoptosis. Functional studies are now required to determine if these interacting proteins are required for efficient replication of influenza virus or whether they perhaps play an antiviral role. Using RNAi, 31 proteins known for interacting with the vRNP or polymerase complex were assessed for their role in polymerase activity.⁵¹ Eighteen were shown to facilitate the activities of both H1N1 and H5N1 polymerases, while two antagonized both polymerases, supporting the idea that interacting proteins are likely to play functional roles. Moreover, Bortz et al.⁵¹ showed that an interaction with human DDX17 is specifically associated with promoting activity of the mammalian adapted H5N1 polymerase (PB2 627K), while the chicken homolog is required by the avian H5N1 polymerase (PB2 627E). Thus, knowledge of these interactions provides a molecular basis for host adaptation and contributes to our understanding of differing pathogenicity phenotypes.

Completion of the human genome and the discovery of RNAi have made it possible to query the participation of each

human gene product in functional assays using genome-wide small interfering (siRNA) libraries. Using this powerful tool, genome-wide RNAi screens have been performed on influenza virus-infected cells to identify those genes that are required for efficient virus growth (reviewed in (636,717)). These five studies^{61,254,325,341,596} identified a total of 1,077 unique genes that when targeted by siRNAs lead to decreased influenza virus replication. Each study employed different assay conditions and this likely contributes to the finding that only 85 genes were common to two or more of the screens (34 of these were validated with at least two different siRNAs and with wild-type influenza virus [Fig. 40.16]). However, a greater degree of concordance is seen when one analyzes the results at the level of cellular function rather than gene name.^{600,636,717} Host factors involved in kinase-mediated signaling, pre-mRNA processing, nucleocytoplasmic transport, the COPI complex, and the vacuolar-type H⁺ ATPase (vATPase) complex are all significantly enriched (Fig. 40.16), indicating that these processes are critical for influenza virus replication. Depending on the design of the assay used for the RNAi screen, it is also possible to detect factors that have antiviral activity and thus enhance virus replication when they are depleted. The interferon-inducible transmembrane (IFITM) proteins were identified in this manner and are thought to inhibit entry of influenza virus as well as several other viruses.^{61,292}

Future progress in this area will depend on integrating the data obtained from these global approaches (e.g., proteomics, RNAi, microarray) to build a clearer picture of the cellular networks that govern efficient influenza virus growth. Such information may address questions concerning species specificity and also provide new avenues to explore for drug discovery (see section on Inhibition of Cellular Factors).

The Actions of the NS1 Protein

When a virus infects a cell, it has to contend with the rapid onset of the host innate immune response, whose mission it is to establish an antiviral state within the cell and prevent virus replication. A critical component of this response is type I interferon (IFN- α/β), which is secreted from virus-infected cells. A characteristic feature of all orthomyxoviruses is their sensitivity to the inhibitory effect of IFN-inducible Mx GTPases.²⁵¹ In fact, IFN was first described as a factor induced by heat-inactivated influenza virus,³⁰⁵ although interestingly, live influenza virus was found to inhibit the induction of IFN by inactivated virus.³⁹⁴ The reason for this observation did not become clear until almost 40 years later when, with the benefit of reverse genetics technology, it was possible to engineer an influenza A virus that lacked the *NS1* gene (delNS1).²¹³ This virus displayed unusual growth properties as it was severely attenuated in IFN-competent systems but grew well in IFN-deficient systems such as Vero cells and 6-day-old embryonated eggs and was lethal in STAT1^{-/-} mice^{213,657} (e-Fig. 40.18). Thus, in the absence of an IFN response, NS1 appears to be dispensable, whereas in the context of an immune-competent host, it is essential. Microarray analysis has demonstrated that infection with delNS1 virus leads to enhanced expression of IFN and IFN-regulated genes compared to wild-type influenza virus infection,²¹⁸ and NS1 is therefore termed an *IFN antagonist* because it acts to suppress the virus-induced host IFN response (reviewed in (245,727)). These findings have given rise to a new concept for the design of live-attenuated vaccines based on mutations in the NS1 gene⁶⁵⁷ (reviewed in

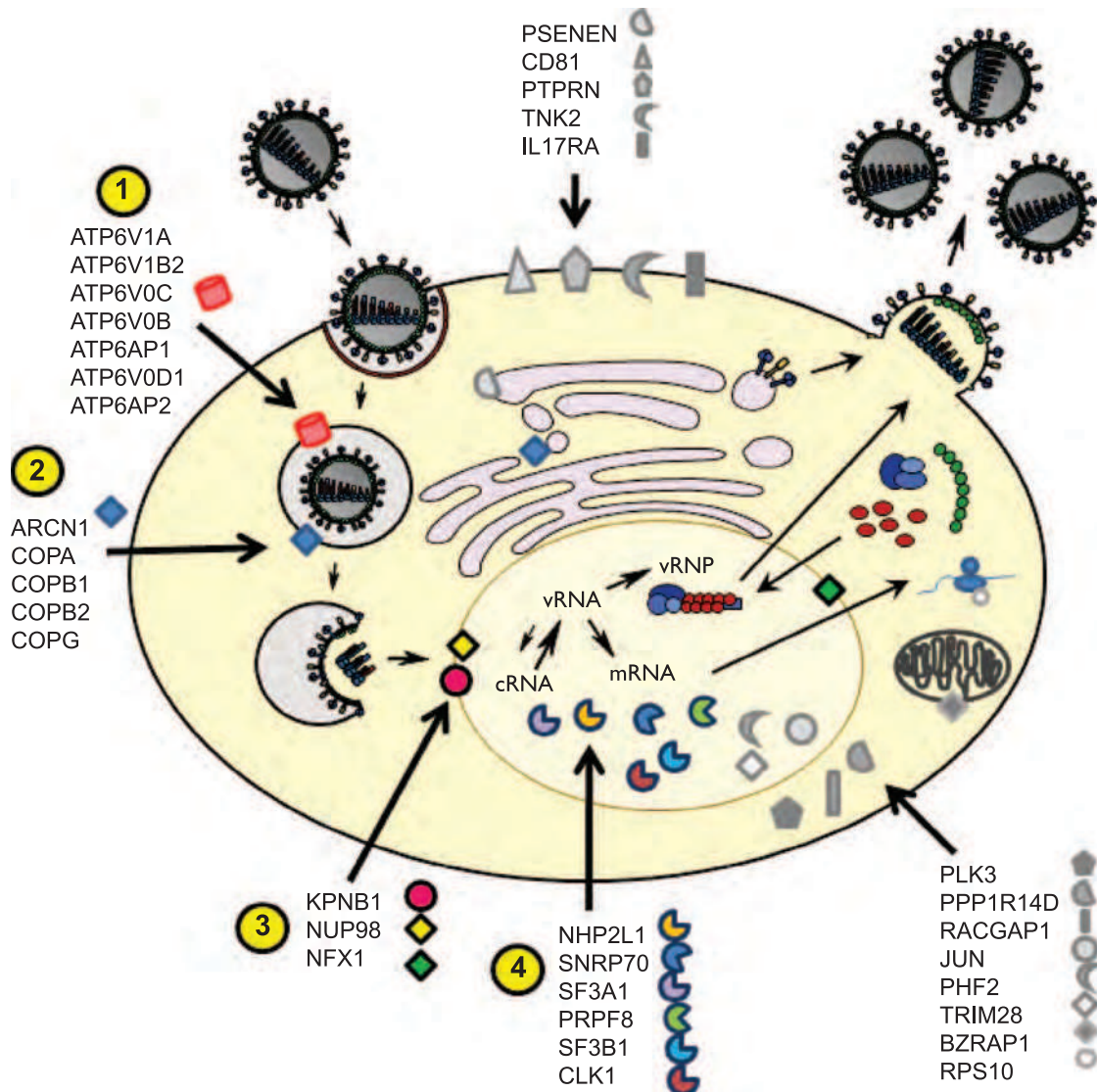


FIGURE 40.16. Graphical representation of the 34 best validated host factors identified in the influenza virus RNA interference (RNAi) screens. Analysis of the validated hits from the RNAi screens by Brass et al,⁶¹ Karlas et al,³²⁵ and König et al³⁴¹ resulted in a list of 34 genes that were identified in at least two screens. These 34 host cell factors are shown with regards to their localization within the cell and in context of the viral replication cycle. The four different groups of host proteins with known function in the viral life cycle are depicted in colored symbols: (1) components of the viral adenosine triphosphatase (vATPase) complex, (2) COPI proteins, (3) nucleocytoplasmic transport factors, and (4) members of the splicing machinery. The host factors with as yet unknown function are illustrated with gray symbols. See (636) for details. (Reprinted from Stertz S, Shaw ML. Uncovering the global host cell requirements for influenza virus replication via RNAi screening. *Microbes Infect* 2011;13[5]:516–525; with permission from Elsevier.)

(511,512,557)). Studies on viruses expressing truncated forms of NS1 have shown that the level of attenuation is determined by the amount of IFN induced by the virus (i.e., highly attenuated viruses induce larger quantities of IFN),^{27,174,549,627,657} and immunization with these mutant viruses produced protective immunity in mice, chickens, swine, horses, and macaques (reviewed in (557)).

STRUCTURAL FEATURES OF THE NS1 PROTEIN

NS1 is a nuclear, dimeric protein that is highly expressed in infected cells and has a dsRNA-binding domain, an effector domain, and a disordered tail (reviewed in (250)). The RNA-

binding domain lies within the N-terminal 73 amino acids (548), for which both NMR and crystal structures have been obtained.^{96,97,397,749} These data indicate that the NS1 RNA-binding domain forms a symmetric homodimer with a six-helical fold and that conserved tracks consisting of basic and hydrophilic residues on each monomer mediate interactions with dsRNA. Mutational analysis has further demonstrated that dimer formation is crucial for RNA binding as are residues R38, R35, and R46.^{96,710} Residue K41 strongly enhances the binding affinity⁷¹⁰ and residues S42 and T49 also participate in dsRNA binding.⁹⁶ It is suggested that the basic residues make contact with the phosphate backbone of the RNA via electrostatic

interactions,^{98,710,749} which is consistent with the observed lack of sequence specificity.^{259,548} Structural data indicate that the NS1 dimer spans the major groove of canonical A-form dsRNA in a length-independent mode.⁹⁶ Structures of the influenza B virus NS1 RNA-binding domain indicate a similar binding mode.⁷⁴⁹

The remaining portion of NS1 has been termed the effector domain and includes binding sites for several host factors (as reviewed in (250)) (e-Fig. 40.19). Crystallographic structures of the NS1 effector domain have been determined for several influenza A virus strains. While the monomer conformation is very similar, different dimer interfaces have been determined, specifically, one that is mediated by a helix–helix interaction^{12,246,328,733} and another mediated by a strand–strand interaction.^{49,329} The helix–helix interaction is dependent on residue W187 in each monomer,^{246,734} and it has been proposed that the monomer interface can twist and therefore the dimer can exist in both open and closed conformations.³²⁸ Finally, one structure of a full-length NS1 protein has been reported.⁵⁰ Strikingly, instead of individual dimers, NS1 is shown to form a chain with alternating interactions occurring via the dsRNA-binding and effector domains. Moreover, three of these chains are shown to interact with one another to form a tubular structure that can accommodate dsRNA in its center. This chain-

like structure opens up questions regarding how interactions between NS1 and its cellular partners can be accommodated, but models for how this may occur in the context of an individual NS1 dimer have been proposed.³²⁸

INHIBITION OF INTERFERON SYNTHESIS

Wild-type (WT) influenza virus infection induces far less IFN than does delNS1 virus, and this difference lies at the level of mRNA molecules.^{218,656,712} This implies that NS1 either acts to prevent the synthesis of IFN mRNA or destabilizes IFN mRNA. The transcriptional activation of IFN- β in response to virus infection is regulated by transcription factors including interferon regulatory factor-3 (IRF-3), nuclear factor- κ B (NF- κ B), and activator protein-1 (AP-1). Each one of these transcription factors has been shown to be activated in delNS1 virus-infected cells but not in WT virus-infected cells,^{403,656,712} which corresponds with the differential induction of IFN- β by these viruses. Moreover, expression of NS1 alone inhibits the activation of the IFN- β promoter in response to infection with a heterologous virus or even delNS1 virus.^{403,712} Substantial progress has been made to understand the precise mechanism by which NS1 suppresses IFN synthesis (reviewed in (245,250)) (Fig. 40.17). First, dsRNA binding is important as

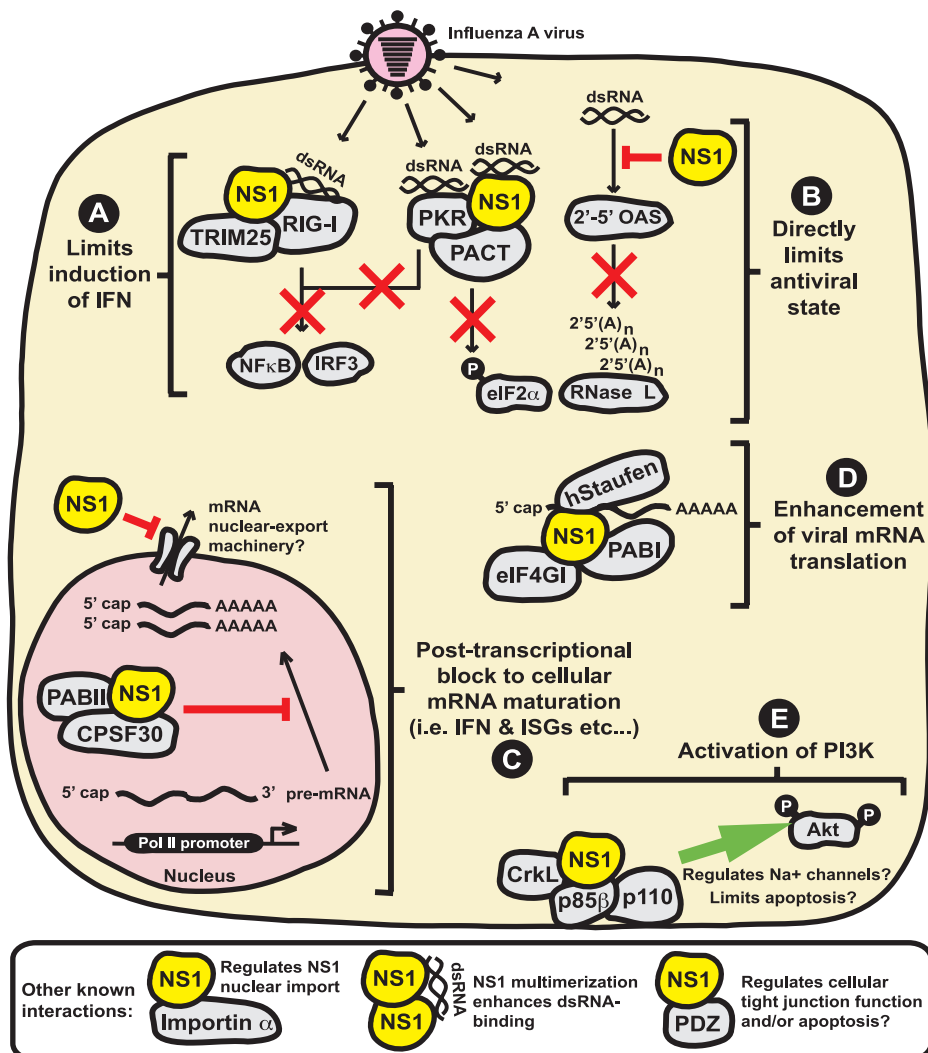


FIGURE 40.17. Schematic diagram of the multiple functions of NS1 within infected cells. A: Pretranscriptional limitation of interferon- β (IFN- β) induction. **B:** Inhibition of the antiviral properties of protein kinase R (PKR) and 2'-5'-oligoadenylate synthetase (OAS)/RNase L. **C:** Posttranscriptional block to processing and nuclear export of all cellular messenger RNAs (mRNAs). **D:** Enhancement of viral mRNA translation. **E:** Activation of phosphatidylinositol 3 kinase (PI3K). Some other interactions that have been characterized are detailed in the **lower box**. See (250) for details. (Adapted from Hale BG, Randall RE, Ortin J, et al. The multifunctional NS1 protein of influenza A viruses. *J Gen Virol* 2008;89[Pt 10]:2359–2376; courtesy of Ben Hale.)

expression of the RNA-binding domain alone is sufficient to block virus induction of IFN.⁷¹² However, a virus expressing only the first 73 residues is still attenuated in mice (with a phenotype intermediate to that of delNS1 and WT virus), pointing to a role for the effector domain *in vivo*.^{174,657,711} Alanine substitution of the residues involved in RNA binding (R38 and K41) was found to significantly reduce the ability of NS1 to inhibit IRF-3 and NF- κ B activation^{656,712} and, hence, IFN- β synthesis.¹⁴³ In the context of a virus, these mutations resulted in increased IFN- β production and, therefore, an attenuated phenotype in mice.¹⁴³ Second, NS1 is found in complex with the cytoplasmic sensor RIG-I and acts to prevent RIG-I signaling and IFN- β production.^{238,444,501,530} This interaction appears to depend on the same residues implicated in dsRNA binding, indicating that it is potentially mediated by RNA.⁵³⁰ Third, NS1 interacts with TRIM25 via residues E96 and E97.¹⁹⁹ TRIM25 is responsible for ubiquitinating and activating RIG-I and the presence of NS1 blocks this activity.

INHIBITION OF PROTEIN KINASE R AND THE RNASE L PATHWAY

Protein kinase R (PKR) is activated in the presence of dsRNA (i.e., during virus infection) and is responsible for the phosphorylation of the eIF2 α subunit, which causes protein translation to cease and thus prevents further viral replication. Not surprisingly, many viruses have devised ways of blocking the activation of PKR, and influenza virus does so via two different mechanisms. PKR expression is induced by IFN and therefore the NS1-induced block in IFN synthesis will reduce the levels of PKR in infected cells. In addition, NS1 has been shown to inhibit PKR activation by sequestering its activator, dsRNA,^{261,401} and NS1 has also been observed to form a complex with PKR,⁶⁵⁹ which inhibits its activation in response to both dsRNA and the cellular PACT protein.³⁷⁸ The role of NS1 in PKR inhibition has been demonstrated *in vivo*, as seen by restoration of delNS1 virus replication in PKR^{-/-} mice with an accompanying increase in pathogenicity.³⁷ Via a similar mechanism (i.e., sequestration of dsRNA), NS1 can prevent activation of the 2'-5'-oligoadenylate synthetase (OAS)/RNase L pathway.⁴⁴⁵

INHIBITION OF HOST mRNA PROCESSING

Influenza virus-infected cells harbor pre-mRNAs in their nuclei that do not undergo efficient 3'-end processing and therefore cannot be exported.^{94,607} This is thought to be an NS1-mediated effect that occurs via interaction of the NS1 effector domain with two components of the 3'-end processing machinery: the 30-kD subunit of the cleavage and polyadenylation specificity factor (CPSF)^{131,338,473} and the poly(A)-binding protein II (PABII)⁹⁴ (reviewed in (245,250,346)). The NS1 interaction effectively inhibits these processing factors and results in pre-mRNAs that either remain uncleaved^{473,607} or only acquire short poly(A) tails.⁹⁴ NS1 also inhibits splicing of pre-mRNAs, which also results in their retention in the nucleus,^{185,400} and evidence for the requirement of CPSF in splicing suggests that both of these mRNA-processing defects in influenza virus-infected cells may be related to the inhibition of CPSF.³⁸⁶ NS1 also interferes with mRNA export via complex formation with several components of the nuclear export machinery.⁵⁸⁴ As the induction of the host antiviral response relies so heavily on transcriptional up-regulation of genes, this global posttranscriptional inhibition may aid in aborting or at least delaying the onset of this response. This is

reflected in data from a CPSF-binding mutant virus, which is attenuated in tissue culture and which causes an earlier induction of antiviral gene products compared to WT virus.⁴⁸⁶ It should be noted, however, that the delNS1 virus shows shutoff of host protein synthesis at levels similar to that of wild-type virus.⁵⁸¹ This suggests that other (or additional) factors may be responsible for inhibition of general gene expression.

ADDITIONAL NS1 INTERACTIONS WITH HOST CELL FACTORS

The influenza A virus NS1 protein binds directly to the p85 β regulatory subunit of PI3K via its C-terminal effector domain, and NS1 expression is sufficient to activate PI3K signaling.^{153,248,610} Specifically, residues Y89, M93, P164, P167, L141, and E142 in the NS1 protein, which are located in predicted src homology 2 (SH2)- and SH3-binding motifs, have been implicated in the interaction with p85 β .^{248,384,609,610} The NS1-binding site on p85 β is located in an inter-SH2 domain,²⁴⁷ and a co-crystal of the NS1 effector domain in complex with this region of p85 β shows that residues Y89 and P167 of NS1 are at the binding interface.²⁴⁹ Furthermore, a model of a heterotrimeric complex consisting of NS1-p85 β -p110 predicts that the presence of NS1 would disrupt the inhibitory contact between p85 β and p110.²⁴⁹ This explains the PI3K-activating properties of NS1, which theoretically serve to delay apoptosis at late stages of infection.¹⁵¹ However, recombinant viruses expressing NS1 proteins that fail to activate PI3K were not seen to induce any more apoptosis than wild-type virus, so the biological significance of NS1-mediated PI3K activation remains unclear.³¹⁴ It also should be noted that a pro-apoptotic activity of NS1 has been reported.⁵⁹²

NS1 has also been reported to interact with several other host factors (e-Fig. 40.19), including the eukaryotic translation initiation factor 4GI, poly (A)-binding protein I, stauferin, NS1-I and NS1-BP, nucleolin, hnRNP-F, E1B-AP5, Herc5, PAB-II, importin α , CrkL, scribble, Dlg1, PDLim2, p15, NXF1, Rae1, PACT, and Ubc9.^{11,76,94,168,225,266,289,368,396,438,463,584,588,660,728,729,751,763} It has been shown that disruption of the stauferin-1/NS1 interaction inhibits influenza virus replication, indicating that this virus-host interaction is required for optimal virus growth.³⁶⁹

ANTI-INTERFERON PROTEINS OF INFLUENZA B AND C VIRUSES, THOGOTO VIRUS, AND ISAVIRUS

Like its A virus counterpart, the NS1 protein of influenza B virus (B/NS1) exists as a dimer and has RNA-binding activity in its N-terminal domain (residues 1 to 93).^{144,709,753} A virus lacking B/NS1 has also been demonstrated to induce larger amounts of IFN than WT virus,¹³² and the B/NS1 protein can complement the growth of influenza A delNS1 virus,¹⁴⁴ indicating that A/NS1 and B/NS1 are functional equivalents. Therefore, as described for influenza A virus, recombinant influenza B viruses either lacking NS1 or expressing truncated NS1 proteins have been proposed as vaccine candidates.^{244,731} Expression of B/NS1 has been shown to inhibit virus activation of the IFN- β promoter,^{132,144} but interestingly, both N- and C-terminal domains of the protein encode this inhibitory activity and hence RNA binding was found not to be essential for inhibition of IFN- β synthesis.¹⁴⁴ However, in the context of the N-terminal domain alone, RNA binding was required. Similarly, both portions of B/NS1 were shown to independently inhibit virus activation of the transcription factor, IRF-3.¹⁴⁴ B/NS1 is also an inhibitor

of PKR, but unlike A/NS1 it does not interfere with mRNA processing.⁷⁰⁹ B/NS1 does possess the unique ability to bind to *ISG15* (an IFN-inducible gene) and prevent its conjugation to target proteins,⁷⁵⁴ which it does through its N-terminal domain independently of RNA binding.⁷⁵³ Structural analysis shows that a dimer of the B/NS1 N-terminal domain interacts with two ISG15 molecules, with each ISG15 binding distinct regions of each NS1 monomer.²³³ ISG15 conjugation or ISGylation has been shown to regulate the IFN signaling pathway and to be critical for host antiviral defense.^{413,558,762} Interestingly, B/NS1 inhibits ISGylation in a species-specific manner, binding only to human and nonhuman primate ISG15.^{628,684} This likely contributes to the fact that influenza B infections are restricted to humans. The influenza B virus NS1 protein may also have a role in virus replication as the virus lacking B/NS1 grows to lower titers, even in IFN-deficient cells.¹³² It has also been shown that B/NS1 associates with nuclear speckles and that this is linked to residues in its N-terminal region.⁵⁸⁸

The influenza C virus NS1 protein (C/NS1) has been shown to inhibit RIG-I-mediated activation of the IFN- β promoter through a region located in its C-terminus.⁵⁰⁷ C/NS1 has also been shown to up-regulate splicing of viral mRNAs.⁴⁵⁸

Unlike the influenza viruses, Thogoto virus does not have an NS segment and instead its anti-IFN activity is encoded by the M segment.²⁴² The M segment produces the M protein from a spliced transcript and the ML protein from an unspliced transcript, of which the latter has been shown to be an IFN antagonist protein.^{242,339} As seen with the delNS1 influenza virus, a recombinant Thogoto virus lacking the ML protein was shown to induce far greater levels of IFN in infected cells than WT virus²⁴² and was remarkably attenuated in mice expressing functional Mx1 (an IFN-inducible protein that protects against orthomyxovirus infection).⁵²⁹ ML has also been demonstrated to inhibit virus-induced activation of IRF-3 but in a manner distinct from that of NS1.³¹⁶ Another striking difference between ML and NS1 is that ML is a structural protein.²⁴¹ Because expression of ML is controlled by the same promoter as that of the M protein (which is expressed late in infection), this strategy ensures that ML is present at the initial stages of virus infection when it can most effectively exert its effect on the host immune response.

The isavirus IFN antagonist proteins are expressed from the unspliced transcript of RNA segment 7 and the larger open reading frame (ORF) of RNA segment 8.^{122,211}

The Actions of the PB1-F2 Protein

Influenza A viruses can express an 11th protein, PB1-F2, which is encoded by the +1 alternate ORF in the *PB1* gene.⁹³ PB1-F2 is 87 to 90 aa long, depending on the virus strain, and is expressed by most human H3N2 viruses, while a large number of human H1N1 isolates have a premature stop codon in the PB1-F2 ORF. Of note, the pandemics that occurred in 1918, 1957, and 1968 were all caused by influenza viruses that express full-length PB1-F2. The protein has been shown to contribute to influenza virus pathogenicity through several mechanisms. Initially, a pro-apoptotic function was described for PB1-F2. It was found to localize to mitochondria and disturb the mitochondrial membrane potential, leading to the efflux of cytochrome c into the cytoplasm.^{93,221} The induction of apoptosis by PB1-F2 is thought to occur specifically in immune cells in a strain-dependent manner and thus contribute to immune

evasion by influenza viruses.^{93,428,756} It was demonstrated that PB1-F2 triggers an apoptotic response by interacting with the mitochondrial adenine nucleotide translocase 3 (ANT3) and voltage-dependent anion channel 1 (VDAC1) proteins⁷⁵⁵ and/or form pores via self-oligomerization^{67,84,269} (Fig. 40.18).

In addition to its pro-apoptotic activity, PB1-F2 was reported to have pro-inflammatory properties. Specifically, it was observed that PB1-F2-expressing viruses increase the levels of several cytokines and chemokines, enhance cell infiltration, and exacerbate lung injury in infected mice.^{117,118,428,429} Notably, it was found that a serine (S) at position 66 in the PB1-F2 protein dramatically increases immunopathology and mortality caused by the 1918 pandemic strain and by highly pathogenic H5N1 viruses.¹¹⁸ Transcriptional profiling of mice infected with a PB1-F2 N66S-expressing virus revealed an early suppression of interferon-stimulated genes (ISGs)¹¹⁷ and *in vitro* studies demonstrated an anti-interferon activity of PB1-F2 at the level of the MAVS adaptor protein⁶⁷⁷ (Fig. 40.18). Interestingly, PB1-F2 N66S, which is associated with increased pathogenicity, inhibited the induction of IFN more efficiently than a wild-type PB1-F2 protein.⁶⁷⁷ It is hypothesized that there may be a possible link between the pro-apoptotic and anti-interferon functions of PB1-F2 through the MAVS protein.⁶⁷⁶

REVERSE GENETICS

Because the *Orthomyxoviridae* are negative-strand RNA viruses, introduction of the genomic RNAs into cells does not result in the formation of infectious virus (as it does in the case of positive-strand RNA viruses). Initial experiments eventually leading to the genetic engineering of influenza viruses involved the reconstitution of functional RNP complexes *in vitro*^{282,522} and transfection of functional RNPs into cells. In these experiments, cDNA-derived RNA for a specific segment was mixed with purified virion NP and polymerase proteins and transfected into cells before or after infection with a helper influenza virus in order to provide the remaining vRNP segments.⁴⁰⁹ Rescue of infectious virus containing the cDNA-derived RNA required selection of the novel virus against the helper virus.¹⁶⁵ Alternatively, cells were transfected with a plasmid construct containing the gene of interest flanked by an RNA polymerase I promoter and terminator sequences. Cellular RNA polymerase I normally transcribes rRNA (which lacks a 5' cap and 3' poly[A] tail), and therefore the RNA synthesized from the plasmid construct is an exact replica of the vRNA. The viral polymerase proteins were supplied by transfection with polymerase II-driven expression plasmids, and the remaining genomic segments were provided by infection with a helper influenza virus.^{480,535} A disadvantage of these early systems was the need for helper virus, which must be selected against in order to isolate the rescued virus.

In 1999, a decade after the initial influenza reverse genetics system had been described, Fodor et al.¹⁷⁸ and Neumann et al.⁴⁷⁹ reported the generation of influenza viruses entirely from cloned cDNAs. In the system reported by Fodor et al, cDNA from each of the eight genome segments was cloned in negative orientation between a truncated human RNA polymerase I promoter and the hepatitis delta virus ribozyme.¹⁷⁸ Transfection of the eight vRNA-encoding plasmids into Vero cells along with four polymerase II-driven plasmids expressing NP and the polymerase complex (PB1, PB2, PA) resulted in

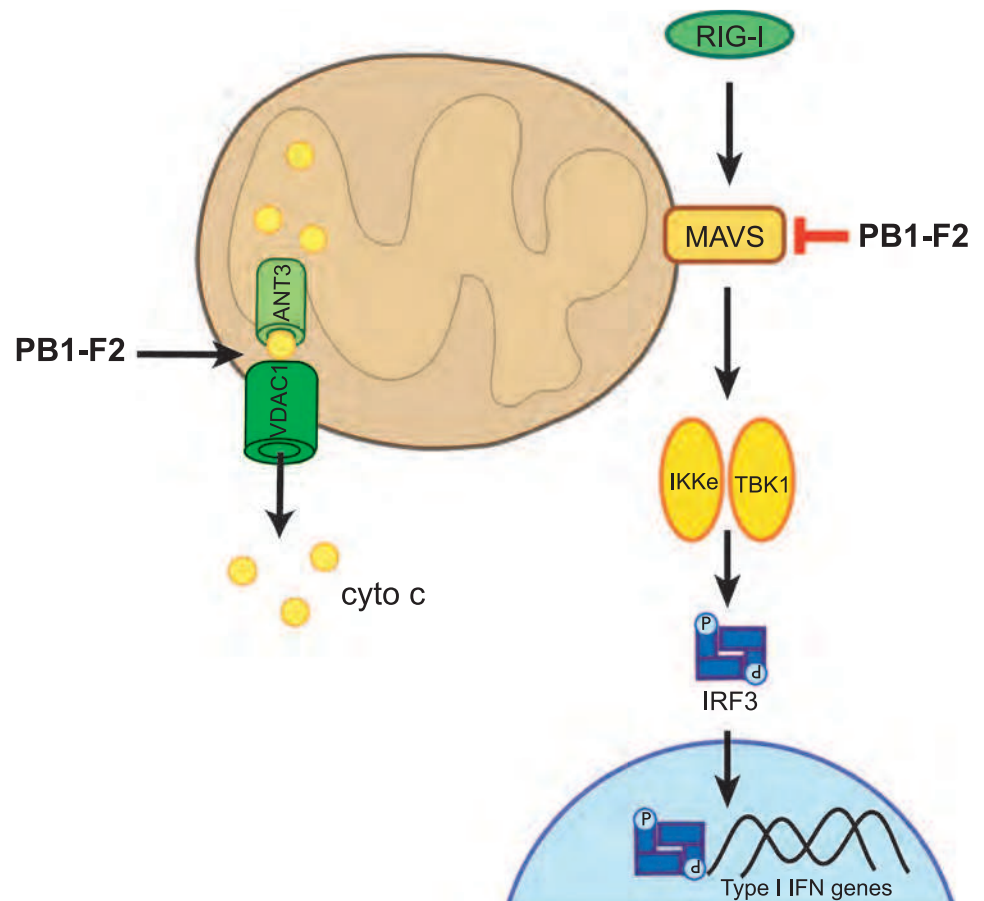


FIGURE 40.18. The pro-apoptotic and anti-interferon activities of the influenza A virus protein PB1-F2. The protein promotes apoptosis by interacting with the mitochondrial VDAC1 and ANT3 proteins and interferes with the induction of interferon at the level of the MAVS adaptor protein. Cyto c: cytochrome c. (Courtesy of Zsuzsanna T. Varga.)

recovery of infectious virus (Fig. 40.19). As helper virus is not required for the generation of recombinant virus, the cumbersome selection process was unnecessary. Improvements to this system now include the transfection of co-cultured 293T cells (necessary due to the human RNA polymerase I promoter) and

Madin Darby Canine Kidney (MDCK) cells, which support high levels of virus replication (for review, see (478)).

Further improvements to these systems were reported in which only eight plasmids were required.^{278,279} The plasmids contained cDNAs of genomic segments cloned in negative

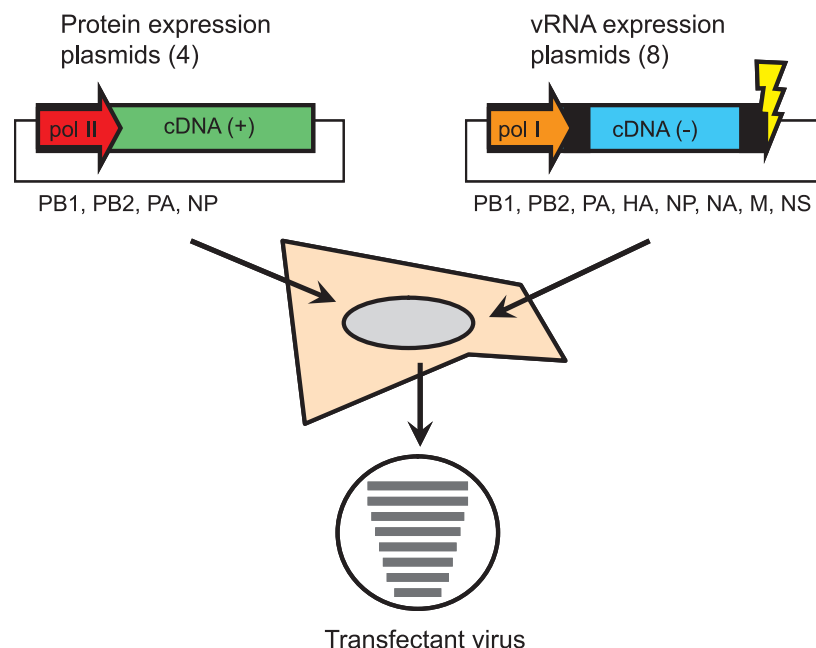


FIGURE 40.19. Schematic representation of the plasmid-based rescue system for influenza A virus. The negative-sense complementary DNA (cDNA) for each viral segment is cloned between a polymerase I promoter and the hepatitis delta virus ribozyme or polymerase I terminator. These eight plasmids are transfected into mammalian cells along with four expression plasmids for the polymerase proteins and nucleoprotein (NP). The resulting transfectant virus is then passaged on fresh cells. For details see (178). (Courtesy of Adolfo García-Sastre.)

orientation with a human RNA polymerase I promoter at the 5' end and the mouse RNA polymerase I terminator at the 3' end. The cellular RNA polymerase I was responsible for the copying of the cDNA into vRNA. Downstream of the RNA polymerase I terminator was a CMV immediate-early promoter. A polyadenylation sequence was inserted at the other end, giving rise to a polymerase II-driven mRNA transcript from the opposite DNA strand. Expressed viral proteins and vRNAs then assembled in the transfected cells and resulted in the formation of infectious virus derived entirely from only eight plasmids. A single plasmid containing the cDNAs of all eight RNAs resulted in the generation of infectious virus when transfected into human cells.⁴⁷⁶ Most likely, transcription of mRNA-like molecules occurred from this plasmid, which then gave rise to the formation of the complementing viral polymerase proteins. These proteins, together with the full-length vRNA segments (also transcribed from the plasmid), allowed rescue of fully infectious virus. Another one-plasmid system was developed for the rescue of influenza A viruses in chicken cells.⁷⁶¹ Other modifications of the rescue system involve the use of uncloned PCR-amplified products, which obviates possible problems in cloning toxic sequences,^{772,773} and the use of adenovirus as a vector to deliver the required plasmid constructs.⁵⁰⁴ Finally, de Wit et al¹³⁴ designed a rescue system built on transcription by the T7 polymerase, which allows the rescue of influenza viruses in practically all cells independent of the species origin.

Influenza viruses expressing foreign genes have also been generated, demonstrating the use of influenza virus as a vector to deliver foreign antigens to the immune system. Numerous approaches have been successful for the expression of foreign antigens by influenza viruses. These include (a) replacement of the antigenic domains of either the influenza HA or NA glycoproteins with epitopes from foreign proteins.^{379,380} (b) Modification of existing viral genomic segments to express influenza viral proteins fused to foreign proteins. These polyproteins can subsequently be cleaved into two proteins. Also reported was the rescue of an influenza virus that expresses an uncleaved chimeric HA with a 140-amino acid insertion of the receptor-binding domain of the *Bacillus anthracis* protective antigen (PA).³⁸⁹ (c) Replacement of ectodomains of surface glycoproteins with those of foreign glycoproteins.¹⁷⁵ (d) Preparation of viruses with foreign antigens encoded by a ninth RNA segment (for review, see (212,478)).

Advantages of influenza viruses over other viruses for the expression of foreign proteins include the fact that influenza virus is extremely safe as a nonintegrating, nononcogenic virus. Infection with influenza viruses also elicits a strong and long-lasting immune response, and thus recombinant influenza viruses may be useful vaccine vectors in the future. The limitations related to the use of influenza viruses for expression of foreign antigens include the limited (but not yet well-defined) capacity of influenza viruses to express foreign sequences and the requirement of packaging signals on both the 5' and 3' ends of the vRNA, which may interfere with the expression of foreign genes.

The advances of reverse genetics techniques have been of great benefit to the study of structure/function of different influenza virus genes and their proteins. In many cases, the definitive role of a gene or of a domain (or even of a single amino acid) can only be explored by introducing appropriate mutations into the genome of the virus and then analyzing the phenotype of the rescued virus. As discussed, rescue systems have been described

for influenza A viruses (for review see (212,367,422)) and B viruses^{277,312} (for review see (313)). Also, rescue systems have now been developed for influenza C viruses^{125,460,506} (for review see (459)). Influenza viruses have been generated that express chimeric (type A/B) HAs and NAs. For example, such viruses may express, in a type A genetic background, the extracellular portion (ectodomain) of an influenza B HA and/or NA.^{175,285,287} These findings show that the HA and NA of an influenza A virus can be functionally replaced with the corresponding protein from an influenza B virus. In turn, viruses have been made in the influenza B virus background expressing proteins derived from influenza A viruses.²⁴³ By taking advantage of the knowledge about packaging sequences, influenza A viruses have been made to contain nine segments expressing an H1 and an H3 HA²⁰⁷ or only seven segments.²⁰⁶ In the latter virus the HA and the NA have been replaced by the HEF protein of an influenza C virus. Whole organ imaging and analysis of infected cells is now facilitated by chimeric influenza viruses that express a green fluorescent protein (GFP) molecule for visualization of influenza virus-infected cells.⁴¹⁴ Reverse genetics has also been successfully used to rescue an influenza virus expressing all eight genes of the "extinct" 1918 pandemic virus, which has allowed its extraordinary virulence to be studied.^{517,671}

Reverse genetics has also helped in designing improved influenza virus vaccines. The live attenuated pH1N1 2009 vaccine was made from a plasmid-generated strain, into which HA gene mutations were introduced to give high yields without changing the antigenicity of the strain.⁹⁵ Also, killed and live pandemic H5N1 virus vaccines were prepared using reverse genetics, allowing the removal of the basic peptide from the HA cleavage site, in order to make the strains used for manufacturing less virulent (for review (286)).

Thogoto virus, which has six negative-strand RNA segments, has also been rescued by a reverse genetics system.^{337,695} The viral RNAs were transcribed from the plasmids under the control of a polymerase I promoter and the structural proteins were expressed from six plasmids driven by a T7 polymerase promoter in the presence of a T7 vaccinia recombinant.

INHIBITORS OF INFLUENZA VIRUSES

Because influenza viruses remain a constant health threat, major efforts have been directed at discovering effective antivirals over the past several decades. Presently there are four Food and Drug Administration (FDA)-approved drugs available for use in humans: amantadine, rimantadine, oseltamivir, and zanamivir (Fig. 40.20). Past and current approaches to antiviral therapy are briefly discussed here (according to the step in virus replication targeted by each drug). Extensive reviews cover the vast literature on this subject.^{28,36,46,110,216,264,315,406,408,562}

Inhibition of Attachment and Uncoating

While vaccination, in essence, targets the HA so that specific antibodies are generated that block attachment of the virus to the receptor, drugs that interfere with the HA-sialic acid interaction have not been successfully developed. This is perhaps surprising because the x-ray crystallographic structures of the HA and of HA-ligand complexes have now been known for more than two decades. In principle, such an approach could work,^{101,223} but this strategy, using sialic acid analogs (or polymers bearing sialic acids), has not led to an FDA-approved

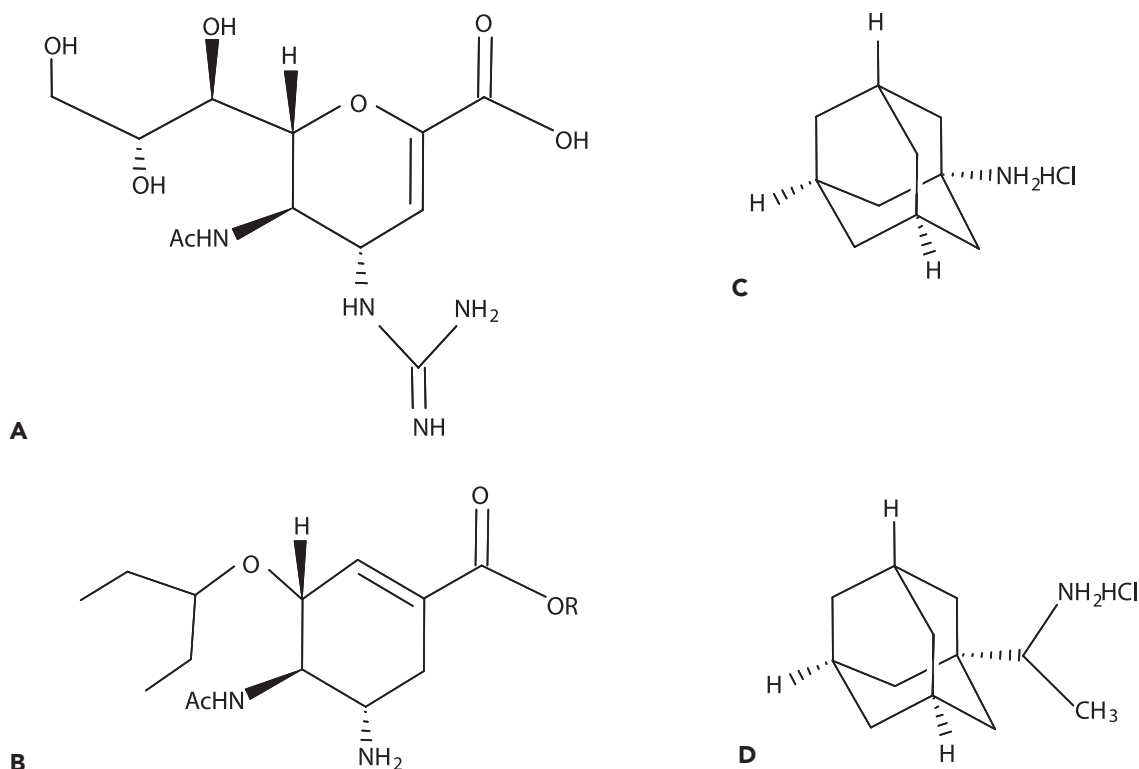


FIGURE 40.20. Anti-influenza virus compounds. The chemical structures of (A) zanamivir (Relenza), (B) oseltamivir (Tamiflu), (C) amantadine (Symmetrel), and (D) rimantadine (Flumadine) are shown.

drug. Whether removal of receptor molecules from the respiratory tract by administration of exogenous sialidase/neuraminidase is a viable antiviral approach remains to be seen. A sialidase fusion protein, DAS181, has been shown to be an effective antiviral strategy in tissue culture and animal models.^{35,361} However, it seems doubtful that receptors could be (safely) denuded for extended periods of time in order to prevent infection by influenza viruses.

Quinone derivatives that prevent the first stage of the conformational change of the HA and thus inhibit infection were discovered in 1993,⁴⁵ and other compounds with a similar mechanism showed inhibition for some strains but not for others.^{106,630} Compounds have also been identified that appear to push the HA into an inactive state²⁷⁶ or that associate with the N-terminal heptad-repeat trimer, thus interfering with the *trimer of hairpins* (helix bundle) formation.¹⁰⁵ This latter approach would be similar to that successfully applied for human immunodeficiency virus (HIV) using the fusion inhibitor T-20.³³¹ In addition, several other approaches aimed at preventing virus attachment have been reported.^{201,237,374,579,620,629} Nitazoxanide (a thiazolide) is reported to prevent terminal glycosylation of HA and thereby impair HA maturation and trafficking to the cell surface.⁵⁷⁰ So far, none of the influenza virus HA inhibitors has advanced beyond the experimental stage.

Another approach concerns the inhibition of the post-translational cleavage of the HA, which results in a molecule unable to undergo the conformational change required for fusion/uncoating. Several exogenous protease inhibitors have been investigated,^{34,52,371,770,771} of which aprotinin has been

found to be effective in humans.⁷⁶⁸ Drugs belonging to this general class have been successful against HIV but have not been further developed for widespread therapeutic or prophylactic use against influenza in humans.

Amantadine, which has been known for many decades to inhibit most influenza A viruses, has been found to target the M2 ion channel (for details see M2 Protein). During uptake, virus enters endosomes where the acid pH activates the ion channel, resulting in the transport of protons into the viral interior. This process, which is required for the dissociation of the RNP complex from the M1 protein and subsequent release of the RNP into the cytoplasm, is blocked by amantadine and its derivatives (including rimantadine),⁷²² (for review see (533)). In addition, amantadine can affect the pH regulation of vesicles involved in the transport of viral glycoproteins to the cell surface during assembly.⁶³⁴ Thus, there are two possible steps at which amantadine can exert an antiviral effect: uncoating and HA stability (in some strains) during transport in vesicles. Unfortunately, resistance to amantadine and to its 10-fold more active derivative, rimantadine, develops with increased use in humans and animals (for review see 452). In fact, according to Centers for Disease Control and Prevention (CDC) guidelines, adamantanes are not recommended for clinical use as of 2010/2011.

Inhibitors of the Viral Replication Complex

The viral RNA-dependent RNA polymerase is a good antiviral target as it possesses unique features not found in the cell. Tomassini et al.^{665,666} reported that 2,4-dioxobutanoic acid and

2,6-diketopiperazine derivatives selectively inhibit the endonuclease activity (PA) of the influenza virus polymerase. Recent knowledge of the PA structure will encourage investigation of endonuclease inhibitors.¹³⁰

Ribavirin (which is approved for treatment of hepatitis C) and several other nucleotide analogs are known to inhibit influenza in humans, but toxicity remains a problem (for review see (376,406)). For RNA viruses, most of the antiviral effects of ribavirin are likely due to incorporation as a purine analog, resulting in lethal mutations. Similarly, T-705 (favipiravir) inhibits influenza virus RNA polymerase activity by acting as a purine analog and has been shown to be effective against several RNA viruses.^{195,196,334} Also, capped and uncapped RNA fragments interfering with cap binding, capped-RNA primed transcription, or panhandle formation have been reported (for review see (406)), but these compounds would have pharmacologic limitations because of difficulties in getting charged molecules into cells. Recent high-throughput screens of small molecules have uncovered novel inhibitors of influenza virus replication that target the NP and PB1 proteins.^{324,645}

Antisense Oligonucleotides and siRNAs

Synthetic oligodeoxynucleotides in the phosphorothioate series corresponding to sequences in the *PB1* gene of influenza A and C viruses were found to inhibit virus replication,³⁷⁵ possibly mediated through an antisense RNA mechanism (for review see (408)). More recently, siRNA inhibition has become a promising route to interfere with influenza virus replication in tissue culture and in animals (for review see (23,36,216,594)). RNAi is a process by which small molecules of double-stranded RNA direct the sequence-specific degradation of mRNA molecules. Theoretically, viral mRNA as well as cRNA and vRNA could be targets for an siRNA approach, and several siRNA molecules directed against specific influenza virus genes have been shown to successfully inhibit influenza virus replication in tissue culture and mice.^{217,297,382,649,667} Challenges of this approach remain: First, the effective delivery of these molecules defies standard approaches. Second, contradictory results have been reported regarding the induction of interferons and cytokines by siRNAs, which may represent an obstacle to their use as specific and nontoxic inhibitors.^{411,561,619}

NS1 Inhibitors

The NS1 protein is being considered as a possible antiviral target due to its role as a pathogenicity factor. Loss of NS1 activity should restore normal immune function to influenza virus-infected cells and promote viral clearance. For this reason small-molecule inhibitors that are able to reverse NS1-mediated inhibition of the innate immune response are being sought. One such molecule has been described that is dependent on an intact IFN response and, specifically, RNase L.^{29,698} Some screens have focused specifically on compounds that may act by disrupting the interaction of NS1 with RNA,^{3,415} while others have examined NS1-mediated inhibition of host gene expression.⁴²³

Neuraminidase Inhibitors

The study of temperature-sensitive mutants with defects in the NA of influenza viruses has shown that the function of this enzyme is to release the newly formed virus from the cell surface.⁵¹⁶ Also, it was shown that neuraminic acid analogs inhibit influenza virus replication in tissue culture and that aggregates

of virus are formed at the cell surface in the presence of these drugs^{510,515} (Fig. 40.15). By relying on the three-dimensional x-ray structure, von Itzstein et al.⁶⁸⁹ designed a derivative of neuraminic acid that had a guanidino group at C atom 4 instead of the OH group of the previously studied neuraminidase inhibitor, DANA (e-Fig. 40.20).⁴³⁶ This compound, zanamivir (Fig. 40.20), is not orally bioavailable and is FDA approved for administration by inhalation or by nasal spray. Most recently intravenous administration has been investigated.⁵⁵² In numerous studies, this compound has been shown to be a potent anti-influenza drug, both prophylactically and therapeutically (for review, see (108,214,315,434)). Peramivir is another neuraminidase inhibitor that can be administered intravenously.³⁴⁰ It received emergency approval in the United States during the 2009 H1N1 pandemic and is already approved in several Asian countries. A long-acting neuraminidase inhibitor (laninamivir), which requires a single administration for the entire course of treatment, was recently developed.⁷³⁹ A search for compounds that are orally active led to oseltamivir.³⁸¹ Its prodrug is an ethyl ester of a compound that has the three OH groups of C atoms 7, 8, and 9 of sialic acid replaced by a hydrophobic side chain, thus making the drug pass through the gut into the bloodstream (Fig. 40.20). This systemically active compound has been shown to be highly effective against both influenza A and B viruses, including strains containing the NA gene of the 1918 pandemic virus,⁶⁷² (for review see (306,315,434)). Although oseltamivir-resistant variants had been described with escape mutations in the HA as well as the NA,^{20,111,234,311,333} it was still unexpected that such widespread resistance would be seen among the seasonal H1N1 viruses by the 2009 season.^{298,500} Prior to 2007, the presence of the NA H274Y mutation was associated with a cost to viral fitness.^{270,308} By 2008/2009, this mutation had a fitness advantage even in the absence of oseltamivir and these resistant viruses were shown to be highly transmissible in a guinea pig model.⁵⁸ It has been proposed that compensating mutations in the NA resulted in a more stable molecule with enhanced expression at the cell surface than was observed for a mutant with only the H274Y mutation.⁴⁴ Interestingly, for the H3N2 viruses, oseltamivir resistance is associated with a loss of fitness and is detrimental to transmissibility of these viruses.^{57,747} However, resistant H3N2 viruses have been isolated particularly from immunocompromised patients undergoing therapy.^{442,494} Similarly, oseltamivir-resistant isolates of the 2009 pandemic virus have been observed in these patients but not in the community.^{230,441} Based on experiments in animal models, it is predicted that an H275Y change in the NA of the 2009 pandemic virus would not be associated with any substantial loss in fitness or transmissibility.^{146,440,593} With this virus already being resistant to the adamantanes, acquisition of oseltamivir resistance would make it a multidrug-resistant virus and a significant threat. Fortunately, mutations associated with oseltamivir resistance do not generally confer zanamivir resistance, and there are rare reports of zanamivir resistance in patients.²³⁵ Structures of oseltamivir-resistant NAs that remain sensitive to zanamivir show that this is due to an altered hydrophobic pocket in the active site that affects oseltamivir but not zanamivir binding.¹⁰⁷

Inhibition of Cellular Factors

The identification of host factors that are required for optimal influenza virus replication (see section Cellular Functions Required for Influenza Virus Replication) provides additional

targets that can be explored for potential antiviral development (reviewed in (402,600)). There are distinct advantages and disadvantages to this approach. First, the obvious disadvantage is that inhibition of a cellular activity that is essential for cell survival or growth may be detrimental to the host, so such proteins may not be suitable as antiviral targets. However, particularly with acute infections such as influenza, the short duration of therapy may allow for temporary loss of a cellular function without harming the host. The major advantage of targeting a host factor over a viral factor is that resistance is much less likely to develop. Also, there is greater opportunity for host-directed compounds to have broad-spectrum activity as many viruses may rely on the same host function. For example, HSP90 inhibitors have been shown to inhibit influenza virus⁸⁵ and other viruses including hepatitis C virus and Ebola virus.^{469,623} Several inhibitors of enzymes in the *de novo* pyrimidine synthesis pathway have been shown to inhibit the replication of a wide range of viruses, including influenza virus,^{48,280,705} presumably because virus replication is particularly dependent on large pyrimidine pools. Inhibitors of receptor tyrosine kinases have also been shown to inhibit influenza virus replication as well as other viruses.^{348,349} The identification of the target of a novel compound is not always easy, but a cellular target is strongly suggested if the compound can inhibit viruses belonging to different families and/or shows species-specific activity. This is the case with a compound shown to block replication of both influenza viruses and several paramyxoviruses,³⁴⁷ and future identification of the target may reveal an important virus–host interaction. Likewise, known inhibitors of host factors or signaling pathways identified as critical for influenza virus replication could be repurposed as antivirals or at least chemical probes for investigating the function of the virus–host interaction. Inhibitors of the following cellular factors have all been shown to inhibit influenza virus growth: MEK, CAMK2B, vATPase, and CLK1.^{145,325,341,536} Rather than inhibiting the activity of a required host protein, it is also feasible for an antiviral compound to act by activating a cellular factor with antiviral activity, as shown by chemical activation of REDD1 expression, which decreases growth of both influenza and vesicular stomatitis viruses.⁴²³

PERSPECTIVES

Human influenza viruses were first isolated in 1933, and since that time they have been studied extensively. Extraordinary progress has been made in elucidating the components of the virus and in understanding the medical consequences of an influenza virus infection. Many of these discoveries have had implications far beyond the influenza virus field and have sparked new developments in disciplines such as immunology and protein structure as well as furthering our basic understanding of viruses in general. For example, the ability of virus to agglutinate red blood cells (hemagglutination) was first recognized as a property associated with influenza virus, hence the name of its major surface glycoprotein. With the discovery that this phenomenon extends to other viruses (e.g., measles virus, rubella virus), it became the basis for viral diagnostic tests, allowing for easy detection of virus or of protective antibody (hemagglutination inhibition) in patient sera. The influenza virus neuraminidase was also the first enzyme found to be

associated with any animal virus, even before it was recognized that viruses encode their own polymerases, including those with reverse transcriptase activity. The discovery of interferon by Isaacs and Lindenmann in 1957 was as a result of studying infection with heat-inactivated influenza virus, and structural analyses of the HA and NA proteins helped to lay the foundation for the exploration of structure/function relationships of large, biologically active proteins. Today, the intensity of studying influenza and influenza viruses has not diminished and a PubMed search yields approximately 65,000 entries (at the time this chapter was written).

Many approaches that served us well in the past have now been superseded by newer techniques, but their contribution to our current knowledge should not be overlooked. The superb collections of temperature-sensitive mutants obtained and characterized by Akira Sugiura in Japan and by Christoph Scholtissek and Rudolf Rott in Germany made it possible to study the genetics of the virus on a gene-by-gene level, and thus allowed the field to take a giant step forward. The sequencing of these influenza virus proteins and RNAs was an effort of months, if not years. Now this can be done in a matter of hours, and since the last edition of this book, there has been a 20-fold increase in the number of influenza virus sequences submitted to GenBank, which now number almost 200,000. We also have exciting new molecular technologies (including reverse genetics) that have allowed us to obtain an excellent understanding of the virus on a molecular level and to learn how it has changed over the years. Using this technology, we have even been able to resurrect the 1918 pandemic influenza virus from sequenced RNA fragments and to study its pathogenicity in an animal model.

What are the challenges for the future? With the threat of yet another pandemic influenza virus emerging, a detailed molecular understanding of virus–host interactions is needed in order to know how best to disable the virus. Perhaps one of the most pressing questions is, what makes an influenza virus transmissible from human to human and from animal to animal? This aspect has been notoriously difficult to study and will require the use of complex animal models. On a molecular level it will be important to learn more about the cell's signaling pathways and how they are modulated during influenza virus replication. What makes the virus a pathogen in one species and not in another? How does the virus affect the host immune response, and is it in turn affected in any way? What role does the age of a person (child, adult, elderly) and conditions/diseases (pregnancy, obesity, diabetes) play in influenza virus replication? What are the complicating factors (coinfection with other viral/bacterial agents, environmental changes) in an influenza virus infection? And last but not least, we need to address the question of host genetics in influenza virus infection and in virus infections in general. Which genetic makeup (polymorphisms and gene expression profiles) determines susceptibility to (and recovery from) influenza virus infection in humans and animals?

These efforts will need to be accompanied by the development of reliable and rapid diagnostic tests and safe and broadly effective antivirals. As we begin to obtain a better understanding of the host factors involved in influenza virus replication, it presents the opportunity to use these host factors as new antiviral drug targets. Potentially, this approach can lead to the development of broad-spectrum antivirals that can be used to treat

not only influenza but also other viral diseases. Since the isolation and characterization of broadly protective monoclonal antibodies directed against conserved portions of the HA, it is now possible to design new immunogens that may serve as vaccine constructs. Such universal influenza vaccines may avoid annual revaccinations against influenza and provide protection against intrasubtype variants and possibly even against strains belonging to different subtypes. In a new pandemic outbreak the availability of such tools will be imperative. It is likely that answers to these challenges will come from a vigorous basic science enterprise, which has brought us a long way in the past several decades of influenza virus research.

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INTRODUCTION

Influenza viruses (family *Orthomyxoviridae*) cause highly contagious respiratory disease with potentially fatal outcomes. Symptoms include fever, headache, cough, sore throat, nasal congestion, sneezing, and body aches. Influenza viruses also cause local epidemics or pandemics (worldwide outbreaks) with significant infection rates. Although the economic burden of influenza is most prominent during pandemics, the combined annual costs of seasonal epidemics due to sick days, emergency department visits, and medications are significant. With the realization that avian influenza viruses can be directly transmitted to humans, influenza viruses are now considered a major, global health threat.

Technologies such as reverse genetics¹⁴⁹⁶ have allowed the routine manipulation of influenza viral genomes. In addition, large-scale sequencing of viral genomes in combination with improved tools for sequence analysis, and other technologies—including yeast two-hybrid screens, small interfering RNA (siRNA)-mediated screens, and transcriptomics, proteomics, metabolomics, and lipidomics studies—are being used to discover the viral and cellular factors that control influenza virus replication, interspecies transmission, and pathogenesis. Despite recent advances, much still needs to be learned about the molecular determinants of these events.

NOMENCLATURE

Influenza viruses belong to the *Orthomyxoviridae* family. This family comprises five genera: *Influenzavirus A*; *Influenzavirus B*; *Influenzavirus C*; *Thogotovirus*, which includes *Thogoto virus* and *Dhori virus*; and *Isavirus*, which includes infectious salmon anemia virus (ISAV; see the International Committee on Taxonomy of Viruses website: <http://www.ictvonline.org>).^{520,1358}

Influenza A viruses are further classified into subtypes based on the antigenicity of their HA and NA molecules; currently, 17 HA subtypes (H1–H17) and 9 NA subtypes (N1–N9) are known. The present nomenclature system¹ includes type of virus, host of origin (except for humans), geographic site of isolation, strain number, and year of isolation, followed by the antigenic description of the HA and NA subtypes in parenthesis; for viruses isolated before 2000, the year should be given as two digits; for viruses isolated in 2000 or later, the year should be given as four digits. For example, A/swine/Iowa/15/30 (H1N1) describes an influenza A virus isolated from a pig in Iowa in 1930 with a strain number of 15 and an H1N1 subtype. Antigenic subtypes have not been identified for influenza

B and C viruses. Thogoto virus, Dhori virus, and ISAV do not cross-react antigenically.

Seroarcheology

Retrospective seroepidemiologic analysis, or seroarcheology, has provided information about influenza virus outbreaks that preceded the virologic techniques currently used to unequivocally identify infectious agents. Early studies suggested that the pandemic of 1889 to 1891 was caused by a virus of the H2N2 subtype, whereas that of 1900 had been attributed to an H3N8 strain.^{1326,1327,1435,1531,1719} More recent re-evaluation of the data indicates that the 1889 to 1891 pandemic was caused by an H3-like virus, and there is no compelling evidence that links the H2 subtype to a pandemic other than that of 1957.⁴⁴² The latter conclusion is substantiated by the lack of protection among those who were at least 80 years old during the 1957 H2 pandemic. Seroarcheology has also linked the 1918/1919 pandemic to an H1 virus, a finding that has been confirmed by sequence determination of influenza virus RNA from the lung tissues of victims.¹⁷¹² Studies using antibodies to the NA protein suggest that in the late 1800s, viruses of the N8 subtype were circulating and later replaced by N1 and N2 subtype viruses. Thus, during the 1900s, only a limited number of virus subtypes (H1N1, H2N2, H3N2, H3N8) was established in humans. Reassortant H1N2 viruses emerged in humans in 2001 and circulated in 2002 and 2003, but have not been isolated from humans since early 2004.

Virus Isolation

In 1930, the first swine influenza virus, A/swine/Iowa/30, was isolated,¹⁸⁶³ but it was not until 1933 that the first human virus was isolated by Wilson Smith, Sir Christopher Andrewes, and Sir Patrick Laidlaw of the National Institute for Medical Research in London, England. These investigators inoculated ferrets intranasally with human nasopharyngeal washes from an influenza patient. The animals exhibited an influenza-like disease, and the virus was transmitted to cage mates. One of their junior colleagues (later Sir Charles Stuart-Harris) became infected by these experimentally infected animals; the virus was subsequently isolated from him.¹⁹⁰⁷ Because it was the first human influenza virus, it was named influenza A virus. In 1940, an antigenically distinct virus was isolated and named type B virus (B/Lee/40).⁵⁵¹ The first influenza C virus was isolated in 1947.²⁰⁵⁴ “Fowl plague” was first described in 1878 as a disease that affected chickens in Italy. The causative agent was isolated in 1902 (A/chicken/Brescia/1902 [H7N7]); however, it was not until 1955 that Schafer recognized fowl plague virus as an influenza virus.¹⁷⁹²

Virus Propagation

Influenza viruses were first propagated in embryonated hens' eggs,²⁰² which continue to be the most widely used system for vaccine production, although cell-culture systems are now also in use (see later discussion). Avian and equine strains of influenza A viruses can be isolated from the allantoic cavity of 10- to 11-day-old embryonated eggs after 2 to 3 days of incubation at 33°C to 37°C. Human influenza viruses have also been isolated from clinical samples inoculated into the allantoic or amniotic cavity of eggs and incubation at 33°C to 34°C. However, recent human viruses are difficult to isolate from embryonated eggs. Influenza virus growth in embryonated eggs leads

to the selection of antigenic variants that are characterized by mutations in the HA protein^{202,992,1738,1739,1800} (see also Host Cell-Mediated Selection of Antigenic Variants section). Influenza C viruses amplify in the amniotic, but not allantoic, cavity of eggs and are usually grown for 5 days in 7- to 8-day-old embryonated eggs.

Influenza viruses can also be propagated in cell culture. Madin-Darby canine kidney (MDCK) cells support the efficient replication of many influenza A and B viruses and are used to isolate viruses from humans.^{2071,2072} Although many influenza viruses can grow in African green monkey kidney (Vero) cells,⁶⁶⁰ they do so less efficiently than in MDCK cells. Cell culture systems based on MDCK,⁷³⁰ Vero,¹⁰⁵⁵ and PER.C6 (human primary embryonic retinoblast)^{1197,1620} cells have been developed for influenza virus vaccine production, and MDCK and Vero cell-based vaccines are approved in Europe for use in humans; MDCK cell-based vaccines are now also approved in the U.S. Influenza viruses also replicate in a number of primary cell cultures, including monkey, calf, hamster, and chicken kidney cells, as well as in chicken embryo fibroblasts and primary human epithelial cells. With the exception of primary human airway epithelium and kidney cells, most cell culture systems require the addition of trypsin to cleave the HA protein of human viruses (except highly pathogenic H5N1 viruses), a prerequisite for efficient replication.

Replication of influenza viruses in eggs or cell culture is measured by testing the ability of the viruses to agglutinate erythrocytes⁸¹⁴ or by use of molecular biology techniques, such as reverse transcriptase (RT)-polymerase chain reaction (PCR).

EVOLUTION OF INFLUENZA VIRUSES

Influenza viruses evolve via a complex process that involves the accumulation of mutations over time and the rearrangement of viral RNA segments in cells infected with two (or more) different viruses (known as “reassortment”). In wild aquatic birds, avian influenza viruses evolve slowly; while mutations occur, most are not sustained in viral populations since they

do not provide an evolutionary advantage. The exceptions are avian viruses in terrestrial poultry, including highly pathogenic H5N1 viruses, which evolve rapidly [see Outbreaks of Highly Pathogenic Avian Influenza (HPAI) Virus section]. In contrast to most avian influenza viruses, human influenza viruses show detectable net evolution over time.

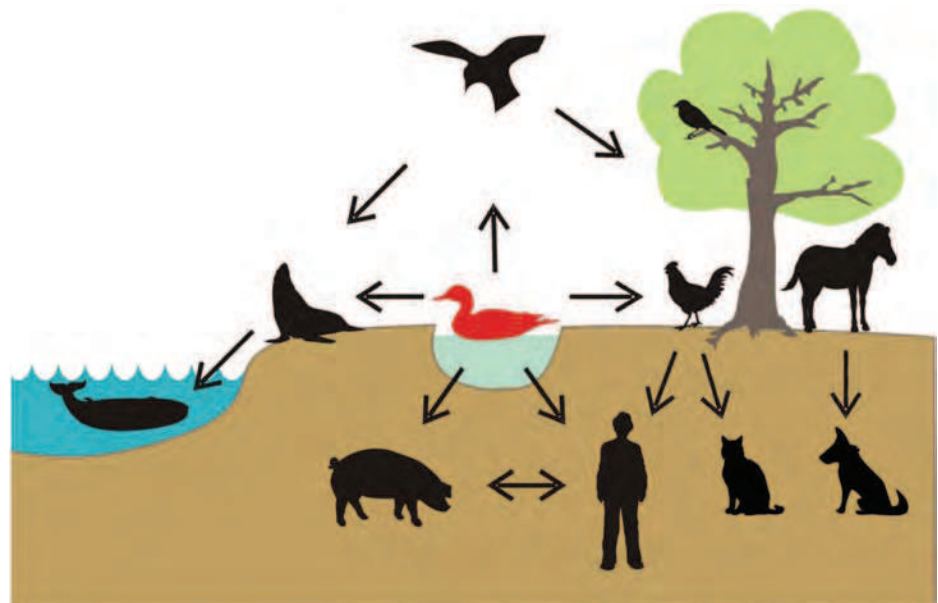
Evolutionary Rates of Influenza A Viruses

Phylogenetic analyses, together with the finding that viruses of all known HA and NA subtypes are maintained in wild aquatic birds, led to the hypothesis that all mammalian influenza A viruses are derived from one avian influenza virus pool^{647,648–649,881,1864,2214,2221} (Fig. 41.1). At the nucleotide level, the reported mutation rates range from $\sim 5 \times 10^{-4}$ to $\sim 8 \times 10^{-3}$ nucleotide substitutions per site per year.^{198,288,397,538,548,562,648,1233,1870,1985,2214,2221,2289,2359}

At the amino acid level, viruses from wild aquatic birds evolve slower than those from terrestrial poultry, swine, or humans.^{562,647,648–649,1870,1995,2214,2246} The fact that in wild aquatic birds most avian influenza A viruses seem to evolve slowly suggests that they are well adapted to their hosts.^{91,2214} Thus, although mutations may occur with similar frequency compared to other hosts, they do not result in many amino acid changes.⁶⁴⁷ Among avian influenza A viruses, the evolutionary rates are highest for the HA, NA, and NS1 proteins,^{288,1110} possibly reflecting their immunogenic or immunomodulatory functions.

Proteins of mammalian and terrestrial poultry viruses continuously accumulate amino acid substitutions. For human influenza A viruses, the evolutionary rates differ among the proteins, likely reflecting differences in the selective pressure of the host.^{527,1870,2214} For example, the HA protein evolves faster than the PB2, PB1, PA, NP, and M1 proteins, because HA variants may confer a selective advantage by allowing the virus to evade the host immune response. The human M1 and M2 proteins, encoded by overlapping reading frames, are under different selective pressures: For the M1 protein, a higher percentage of changes is silent than for the M2 protein.^{562,881,2214} The M1 protein thus appears to be well adapted to its mammalian

FIGURE 41.1. Influenza A virus reservoir. Wild aquatic birds are the main reservoir of influenza A viruses. Virus transmission has been reported from wild waterfowl to poultry, sea mammals, pigs, horses, and humans. Viruses are also transmitted between pigs and humans, and from poultry to humans. Equine influenza viruses have been transmitted to dogs.



hosts, whereas the M2 protein is under stronger selective pressure. The biologic reason for the selective pressure on the M2 protein is unknown. The two proteins encoded by the NS gene also differ in their evolutionary rates, with NS1 showing more variation between alleles than NS2.^{1000,1987} High evolutionary rates have been reported during the establishment of new virus lineages, for example, the introduction of avian H1N1 influenza viruses into European pigs in 1977,¹²⁷⁴ the emergence of highly pathogenic avian H5N2 influenza viruses in poultry in Mexico in 1993/1994,⁶⁰³ and the emergence of highly pathogenic avian H5N1 influenza viruses in Hong Kong in 1997,²³⁷⁵ which may reflect preferential selection of mutants that provide an advantage in a new host. This process may be facilitated by so-called mutator mutants that lead to increased error rates in the viral replication complex.^{1274,1812,1984,1990}

Host-Specific Lineages, and Geographic Segregation of Influenza A Viruses

Extensive phylogenetic analyses have revealed host-specific virus lineages for several viral genes (e-Fig. 41.1). The phylogenetic trees of the PB2, PA, NP, M, and NS genes are similar in that they can be divided into two major branches consisting of avian and avian-like swine, or classic swine and human influenza viruses, respectively.^{90,562,592,647,648,682,881,1559,1901,2214}

The phylogenetic tree of the PB1 gene differs from those of other influenza virus genes. The PB1 genes of human H1N1 viruses cluster with classic swine viruses, whereas the PB1 genes of human H2N2 and H3N2 viruses form a different sublineage that reflects the introduction in 1957 and 1968 of avian virus PB1 genes into human influenza viruses.^{682,1001,1813,1901,2214} The genes other than the HA and NA of equine H7N7 viruses do not cluster with avian, human, or swine influenza viruses,¹⁹⁰¹ suggesting their early separation into a separate lineage.

The phylogenetic tree of the NS gene is divided into two alleles: A and B.^{1000,1987} All mammalian virus NS genes belong to allele A, whereas avian influenza virus NS genes can belong to allele A or B.^{1000,2091}

The H1 HA genes can be separated into a branch consisting of avian and avian-like swine influenza viruses versus a branch consisting of human and classic swine viruses.^{961,1901} The phylogenetic tree of the H3 HA gene consists of two major branches¹⁹⁰¹: one branch splits into two major subbranches that represent equine/canine and North American avian virus isolates. The second branch can be separated into Eurasian avian viruses, and human and swine H3 HAs that separated from the avian viruses in the 1960s. The human H3 gene has evolved in a single lineage since its introduction into the human population in 1968.^{527,682,2214}

The phylogenetic tree of N1 NA genes shows two major branches that separate into human and classic swine, or Eurasian swine and avian N1 NA genes, respectively.^{516,548,1901} The phylogenetic tree of the N2 NA gene can be divided into a North American avian clade, and a second clade that evolved into Eurasian avian and human virus genes at the beginning of the last century.¹⁹⁰¹

These analyses also reveal that influenza virus genes can be separated by their geographic origin, with a North American and a Eurasian gene pool.^{438,456,1446,1545,1569,2246} These gene pools appear to evolve largely independently, although reassortment between North American and Eurasian viruses has been reported.^{456,1050,1094,1248,1305,2181,2210,2246,2263}

Host-Specific Amino Acids

Recent large-scale sequencing efforts have generated thousands of full-genome influenza viral sequences⁵⁴⁵ (www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html; www.fludb.org; www.gisaid.org). The comparison of viral proteins derived from different host species has revealed signature amino acids at specific positions that distinguish human, avian, and swine virus isolates.^{10,19,276,516,533,562,603,648,881,1037,1205,1233,1399,1400,1559,1603,1686,1812,1840,1986,2214} In particular, comparative studies have identified a number of human-like signature amino acids in highly pathogenic avian H5N1 influenza viruses,^{19,276,533,1037,1205,1400,1686,1840} the pandemic 1918 virus^{276,533,1399,1603,1713,2049} and 2009 pandemic H1N1 viruses,¹⁶⁰³ which may play a role in adaptation to humans. For some of these signature amino acids, a role in pathogenicity has been demonstrated (see Molecular Determinants of Host Range Restriction and Pathogenesis section).

Computational analyses of viral sequences have also identified differences in mutation patterns and codon usage between human and avian influenza viruses.^{460,663,927,1698,2258} Such approaches may improve our understanding of influenza virus evolution.

Quasispecies

The high error rate of the replication complex of RNA viruses results in the generation and co-circulation of different genetic variants within a host organism.⁴³³ If the biologic fitness of a quasispecies is comparable to that of the predominant variant, the minor variant may be maintained in virus populations at low frequencies.¹¹⁴⁹ In the event of host or environmental pressure (including innate and adaptive immune responses, and selective pressure resulting from a host change or antiviral pressure), greater genetic diversity may increase the probability for variants that are better adapted to the changed environment. In such a scenario, quasispecies may be selected and become the dominant virus population. In the past, the detection of quasispecies was cumbersome due to the detection limits of conventional sequencing techniques. With advances in deep sequencing and mass spectrometry analyses, minor sequence variants can be detected more easily. These techniques are now used to assess the levels of quasispecies and mixed infections,^{620,1118,1786,2318} which may provide critical information on the emergence of novel variants. For example, deep sequencing revealed that variants of the 2009 pandemic H1N1 virus possessed mutations in the antigenic site¹¹¹⁸; it will be interesting to assess whether these variants become dominant in the future.

Evolution in Influenza B and C Viruses

Significant differences in evolutionary rates exist for influenza A, B, and C viruses.^{289,527,812,1000,1100,1236,1534} Type B viruses, and especially type C viruses, evolve more slowly than influenza A viruses. Type B and C viruses seem to be near or at an evolutionary equilibrium in humans; in contrast, the genes of type A human viruses were introduced from birds²²¹⁴ and have not reached an equilibrium in humans. Influenza A viruses in humans evolve along single lineages, which suggests evolution by clonal selection¹⁹⁶ and limited co-circulation of sublineages. Co-circulation of sublineages has been shown over only limited periods.^{372,527} In contrast, the evolution of influenza B and C viruses is characterized by the co-circulation of antigenically and genetically distinct lineages over extended periods of time.^{197,2305} For influenza B viruses, two lineages—B/Victoria

(represented by B/Victoria/2/87) and B/Yamagata (represented by B/Yamagata/16/88)—have been co-circulating for about 25 years with changing patterns of prevalence and geographic distribution.^{812,960,1236,1360,1488,1623,1757,1759}

INFLUENZA VIRUS GENETICS

Reassortment

Reassortment is the rearrangement of viral gene segments in cells infected with two (or more) different influenza viruses (Fig. 41.2). Reassortment between two viruses can theoretically result in $256 \cdot 2^8$ different gene variations (i.e., the two parental genotypes and 254 new gene combinations). Reassortment occurs for influenza A, B, and C viruses, but has not been observed among the different types of influenza viruses.

The importance of reassortment to the generation of new influenza virus strains is highlighted by the last three pandemics: reassortant viruses that contained HA, PB1, and NA, or HA and PB1 segments of avian virus origin in a human genetic background, caused the so-called Asian Influenza in 1957 and

Hong Kong Influenza in 1968.^{1001,1155,1813} The H1N1 pandemic in 2009 was caused by a reassortant virus that possessed North American avian-like PB2 and PA segments, Eurasian avian-like swine NA and M segments, a human H3N2-like PB1 segment, and classic swine H1N1-like HA, NP, and NS segments.^{388,604,1903,2094} Six of these segments (all but the NA and M segments) were derived from triple human/avian/swine reassortant viruses that emerged in 1997/1998 and have spread throughout the North American pig populations^{972,1570,2209,2373} (see Influenza in Swine section). These viruses caused a few cases of self-sustained human infections without further spread.^{78,2055,2163} Hence, at least two sequential reassortment events were critical in the emergence of the new pandemic strain: first, the generation of a triple reassortment swine virus; and second, the introduction into this virus of Eurasian avian-like NA and M segments. In addition, the highly pathogenic H5N1 viruses currently circulating in Southeast Asia arose from multiple reassortment events among avian influenza viruses, and continue to reassort.^{278,279,281,449,677,678,681,826,1089,1205,1247,1296,1491,1902,2157,2290} [see Outbreaks of Highly Pathogenic Avian Influenza (HPAI) Viruses section].

In addition to reassortment events that create new lineages or pandemics, reassortment can create novel viruses that circulate for a limited time period.^{752,846,873,1034,1174,1182,2005,2187,2277,2285,2358}

For human influenza viruses, intrasubtypic reassortment may be more important than previously thought,^{829,1235,1479,1481,1482,1700} and may have led to the epidemics observed with reassorted H1N1 viruses in 1947 and 1951,^{1030,1482} reassorted H2N2 viruses in 1967,¹²³⁵ and reassorted H3N2 viruses in 1997 and 2003.^{829,1700}

In experimental settings, multiple reassortants can be generated between highly pathogenic avian H5N1 and human H3N2^{284,905,1198,1301} or 2009 pandemic H1N1 viruses¹⁵⁴⁸; between 2009 pandemic H1N1 and seasonal H1N1¹⁵⁴⁷ or avian H9N2 viruses²⁰⁰⁶; or between human H3N2 and genetically distant equine H7N7 viruses.¹¹⁹⁹ However, not all gene combinations can be generated experimentally, and others may not be compatible in nature. Nonetheless, these studies demonstrate the propensity of influenza viruses for reassortment.

Recombination

Recombination has been detected in influenza virus segments that contain genetic material from more than one origin. For negative-sense RNA viruses, homologous recombination is uncommon; however, recombination by “template switching” can occur and lead to increased biologic fitness of the virus. For example, the insertion of 54 nucleotides of 28S ribosomal RNA into the A/turkey/Oregon/71 HA gene increased HA cleavability.¹⁰²⁰ Similarly, an A/seal/Massachusetts/1/80 variant contained a 60-nucleotide insertion (likely derived from the NP gene) in the HA gene, which also enhanced HA cleavability.¹⁵⁷⁶ Avian influenza viruses of low pathogenicity have converted to high pathogenicity following the insertion of 21 nucleotides of the M segment^{161,815,1614} or 30 nucleotides of the NP segment¹⁹⁸⁹ into the HA segment. Serial egg passages of an A/WSN/33 virus containing a 24-amino-acid deletion in the NA stalk led to variants that replicated efficiently in eggs.¹⁴⁰¹ The NA stalk of these variants contained sequences that originated from the PB1, PB2, and NP genes. In another example, a virus contained an NP gene that likely resulted from intracistronic recombination between two NP

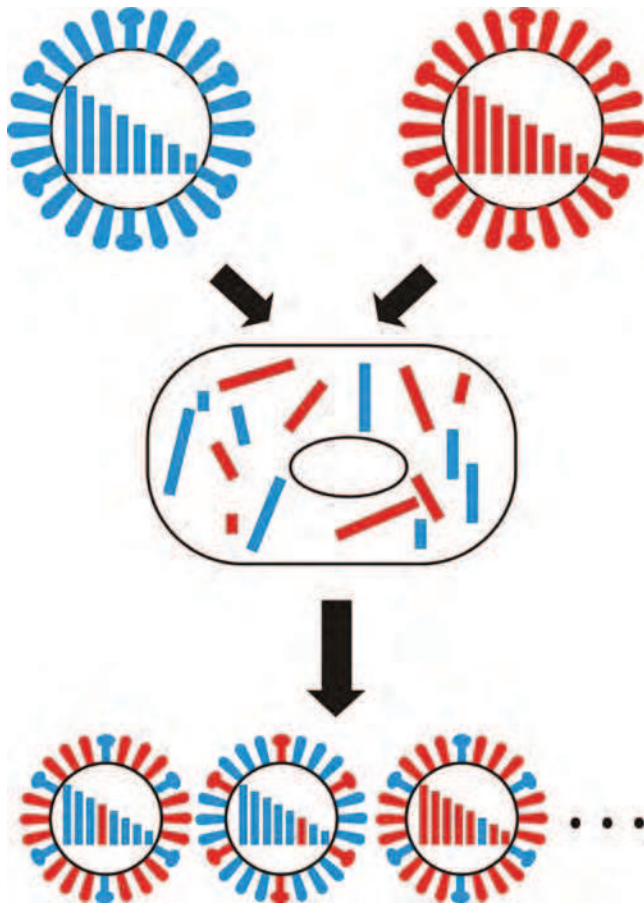


FIGURE 41.2. Reassortment. Co-infection of cells with two different influenza A viruses can theoretically result in 256 different genotypes (2^8 , i.e., the two parental genotypes and 254 new genotypes). Reassortment is a major mechanism for the generation of pandemic influenza viruses, as demonstrated in 1957 (Asian influenza), 1968 (Hong Kong influenza), and 2009 [A(H1N1)pdm09].

segments.¹⁷⁴⁷ In this case, it is unclear whether the recombination event provided a selective advantage to the virus. Moreover, attempts to generate influenza virus by providing an *in vitro* synthesized RNA encoding a viral segment yielded several recombinant viruses.¹²¹ Recently, computational analyses of influenza viral sequences identified several potential recombination events among influenza viral genes^{152,770,771}; however, some of these may in fact represent laboratory-generated artifacts.

Reverse Genetics

Highly efficient systems are now in place for the artificial generation of influenza A,^{542,1496} B,^{824,897} and C^{362,1448} viruses, and of Thogotovirus.²¹⁷³ These systems rely on the intracellular synthesis of influenza viral RNAs by a cellular enzyme, RNA polymerase I, that transcribes ribosomal RNA in the nucleus of eukaryotic cells. The influenza viral segments are encoded by cDNAs flanked by the RNA polymerase I promoter and the RNA polymerase I terminator or a ribozyme sequence. RNA polymerase I transcription in transfected cells results in the efficient synthesis of RNA transcripts with defined 5' ends, whereas the integrity of the 3' ends is achieved by using the nucleotide-specific RNA polymerase I terminator¹⁴⁹⁶ or a self-cleaving ribozyme.⁵⁴² To generate influenza viruses, cells are transfected with eight plasmids to provide all eight viral RNAs, as well as with four plasmids for the expression of the polymerase and NP proteins that are required to initiate viral replication. Although this approach requires the co-transfection of cells with 12 plasmids, it is highly efficient, routinely yielding 10⁸ plaque-forming units of influenza A virus per mL of cell culture supernatant. In one modification, both the RNA polymerase I transcripts (for viral RNA synthesis) and the RNA polymerase II transcripts (for mRNA synthesis) are derived from the same template,⁸²⁵ which reduces the number of plasmids required for virus generation to eight. In another modification, the eight RNA polymerase I transcription units for the eight viral RNAs are combined,¹⁴⁹⁰ allowing the generation of the entire viral genome from a single plasmid. In addition, a reverse genetics system based on T7 RNA polymerase has been established.⁴⁰⁷ These systems have revolutionized influenza virus research [reviewed in^{1492–1494,1497}] in that they allow researchers to study the functions of viral proteins in the viral life cycle, as well as their roles in pathogenesis and host range restriction. Moreover, these systems are now invaluable tools for the generation of influenza virus vaccines and vaccine vectors. In fact, reverse genetics has permitted the generation of inactivated and live vaccine strains for H5N1 viruses that could not have been produced by conventional approaches.

INFLUENZA IN HUMANS—PAST PANDEMICS AND THE H5N1 EPIDEMIC

Pandemics are outbreaks that impact large geographic areas and large portions of the population in a short period of time. Pandemics are the most dramatic manifestation of influenza, attacking 20% to 40% of the world population and causing significant mortality. Influenza pandemics have occurred in 10- to 40-year intervals, although reliable records only date back to the 1918/1919 pandemic (Fig. 41.3). The cumulative death

toll of epidemics in interpandemic periods, although less dramatic, parallel those of pandemics.

The Pandemic of 1918/1919—Spanish Influenza (H1N1)

The pandemic of 1918/1919 remains unprecedented in its severity. It killed more people than World War I and reduced life expectancy in the United States by 10 years. AIDS has killed 25 million people in its first 25 years—the Spanish influenza killed an equal number in 25 weeks (from September 1918 to March 1919). This pandemic occurred in three waves. In the spring of 1918, a mild respiratory disease started at Fort Funston, Kansas (now Fort Riley), attributed to a soldier that had been cleaning pig pens.³⁶⁶ There is no mention of the presence of poultry in the camp at that time. The disease spread among soldiers from Fort Funston along the rail lines to other military bases and cities in the United States and on troopships to Europe.¹⁶¹⁹ This first wave was highly contagious but caused few deaths and received limited attention in most parts of the world. In Spain, a neutral country without news censorship, the outbreak was covered extensively by news media and was soon referred to as the “Spanish influenza.” In late August, a second wave with a higher mortality rate started, probably in western France, from where it spread around the world; this wave peaked between September and November. During that time, death tolls reached more than 10,000 people per week in some US cities. About one-third of the US population became sick,⁵⁵⁷ and the mortality rate was estimated to be over 2.5%, compared to less than 0.1% in typical influenza outbreaks. These figures reflect the impact of the pandemic on the developed world; death rates are believed to have been significantly higher in African and Asian countries. In some isolated populations, the mortality rate reached 70%, likely because of the lack of previous exposure to influenza virus. A third wave of similar impact to that of the second wave struck in late 1918/early 1919.

Typically, the onset of symptoms was sudden, with high fever, severe headache and myalgia, cough, pharyngitis, and coryza. Pathologic findings were mostly restricted to the respiratory tract; death was due to pneumonia and respiratory failure.²²⁵⁵ There was no evidence of systemic viral infection.²²⁵⁵ Most patients died of secondary bacterial pneumonia,^{190,302,1272,1420} but some showed massive acute pulmonary hemorrhage or pulmonary edema,²²⁵⁵ indicating the extreme virulence of the virus. The high rate of bacterial complications may be attributed to the lack of antibiotics in 1918 and 1919.

Age-specific morbidity was similar to that of other pandemics, with children younger than 15 years experiencing the highest infection rates.⁹⁴⁰ The mortality pattern, however, differed significantly from that of other influenza virus outbreaks. In typical influenza outbreaks, the highest death rates are observed in very young children and in the elderly. In 1918 and 1919, many deaths occurred among young adults. The death rate for the 15- to 35-year-old age group was 20 times higher in 1918 than in previous years^{1229,1880} and persons younger than 65 years accounted for more than 99% of excess deaths.¹⁸⁸⁰

The origin of the 1918/1919 virus remains an enigma. In 1927, E. Jordan⁹⁴⁰ published a comprehensive review of the origin of the pandemic. He found no evidence that the disease had originated in China. He also evaluated two reports that described local outbreaks of respiratory infections associated with high mortality and heliotrope cyanosis, which was

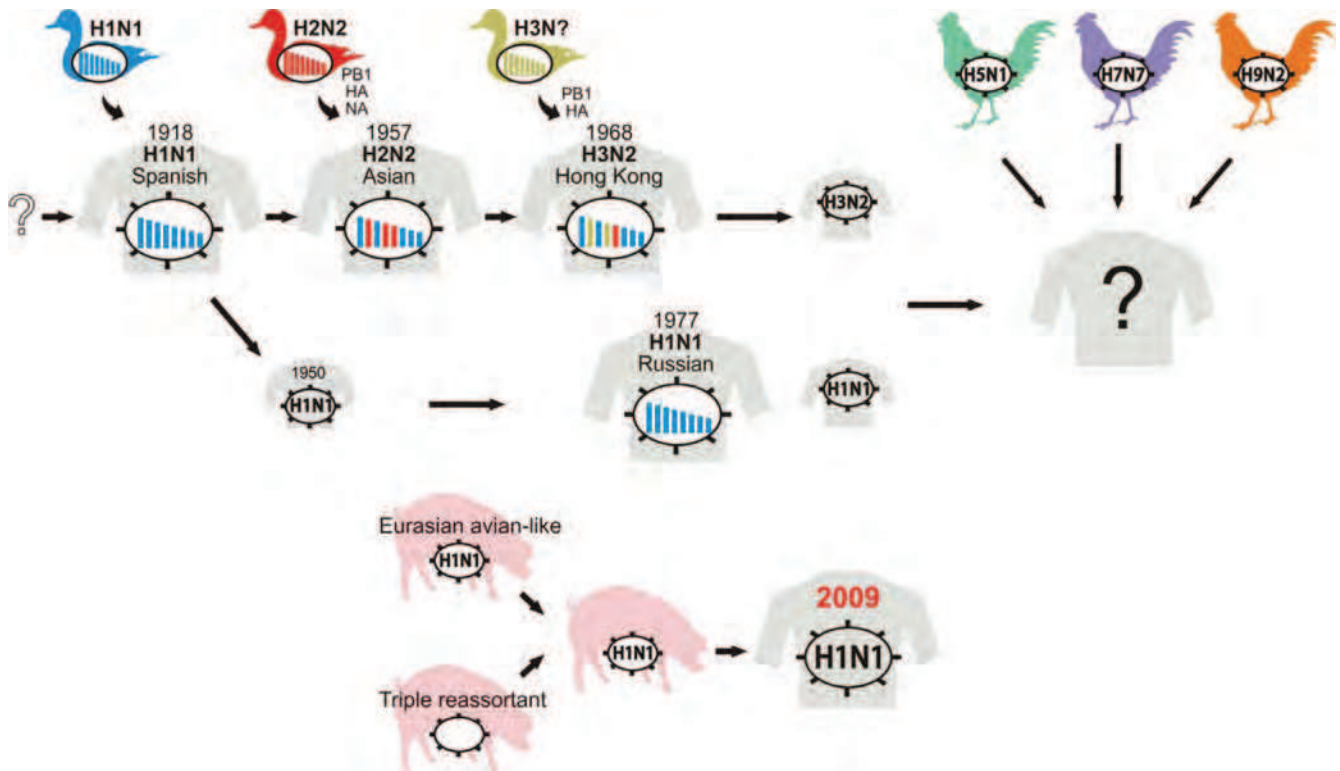


FIGURE 41.3. Evolution of influenza A viruses circulating in humans. An avian H1N1 virus caused the Spanish influenza in 1918. Its descendants circulated until the mid-1950s and reemerged in 1977, causing the Russian influenza. Viruses of this lineage continued to circulate in human populations until 2009. The Asian influenza in 1957 was caused by an H2N2 virus that acquired its HA, NA, and PB1 genes from an avian H2N2 virus. A similar reassortment event in 1968 resulted in the introduction of avian virus HA and PB1 genes into the human population, causing the Hong Kong influenza. H3N2 viruses circulate in humans to this day. In 2009, reassortment of triple-reassortant swine viruses and Eurasian avian-like swine viruses (which donated the NA and M segments) resulted in the A(H1N1)pdm09 virus (see Fig. 41.5 for more details), which replaced the then-circulating H1N1 viruses.

observed during the 1918/1919 outbreak, in army camps in Étaples in Northern France in the winter of 1916 and in Aldershot barracks in March 1917. He dismissed both reports because the disease did not spread but disappeared after short episodes. The most likely origin of the pandemic was Haskell County, Kansas, where Dr. L. Miner noticed an outbreak of influenza in early February 1918 that differed from other influenza outbreaks in that it attacked young, healthy adults, who developed pneumonia that often led to sudden death.⁷⁵ Dr. Miner's observations were published in *Public Health Reports* (now *Morbidity and Mortality Weekly Reports*) and appear to be the first reference to the 1918/1919 pandemic. Men from Haskell County reported to Fort Funston for military training, where they arrived between February 26 and March 2, 1918. On March 4, the first soldier at the camp was reported ill; within a 3-week period, more than 1,100 soldiers at the camp required hospitalization.

Seroarchaeology suggests that the causative agent was an H1N1 virus. This was confirmed by Taubenberger et al., who recovered viral RNA from formalin-fixed, paraffin-embedded tissues from two soldiers who died in 1918,^{1712,2048} and from an Inuit female of unknown age whose body was exhumed from a mass grave in the permafrost of Alaska.¹⁷¹² RT-PCR amplification of the viral RNAs provided viral gene sequences.²⁰⁴⁸ Phylogenetic analyses revealed that 1918 Spanish influenza

virus proteins contain both “avian-like” and “human-like” signature amino acids (see Host-Specific Amino Acids section).²⁰⁴⁹ Further analysis suggested that the 1918 virus genes were not directly transmitted from an avian species, but likely circulated in a mammalian host for several years before causing the pandemic outbreak in 1918.^{33,1901,2258}

Reconstitution of the 1918 Spanish influenza virus by use of reverse genetics demonstrated its high pathogenicity in mice, ferrets, and nonhuman primates.^{1068,2102,2107,2206} In nonhuman primates, the virus caused severe respiratory disease with extensive edema and hemorrhagic exudates,¹⁰⁶⁸ similar to reports of human infections. Rapid recruitment of macrophages and neutrophils was observed in the lungs of infected mice,^{1068,1639,2105} in line with findings of altered immune responses in infected mice⁹⁸³ and nonhuman primates.¹⁰⁶⁸ Further studies indicated a role in virulence for several viral proteins (see later discussion and Molecular Determinants of Host Range Restriction and Pathogenesis section): HA,^{981,1070,1607,2103,2104–2105} the polymerase proteins, particularly PB1,^{1607,2102,2131,2206} NS1,^{132,612} and PB1-F2.¹³⁵⁶ Two viral proteins, HA and PB2, are critical for the transmissibility of this virus in ferrets.²¹³¹ In contrast to its high virulence in humans, mice, ferrets, and nonhuman primates, the 1918 virus is of low pathogenicity in pigs,²²³¹ chickens, and mallard ducks.⁵³

Unlike contemporary human viruses, reassortants possessing the 1918 HA, or HA and NA, genes are highly pathogenic in mice.^{981,1070,1607,2103,2104–2105} Reassortant viruses containing the 1918 HA gene induce high levels of macrophage-derived cytokines and chemokines, which stimulate inflammatory cell infiltration and hemorrhage^{981,1070,2105}—hallmarks of Spanish influenza infection. The underlying mechanism is unknown. Structural analysis of the HA protein revealed that although most of the amino acids of the receptor-binding pocket are avian-like, the amino acid at position 190 (an aspartic acid as found in human viruses) is responsible for HA binding to human respiratory cell-surface receptors,^{590,1972} as previously predicted.¹³³⁰ Consistent with this structural finding, the 1918 HA protein preferentially recognizes human-type receptors in receptor-binding assays.⁶²⁸ The replacement of two amino acids in the 1918 HA (D190E and D225G) abolished respiratory droplet transmission in ferrets, although the mutant virus maintained its lethal phenotype in infected animals.²¹⁰⁷

The NS1 protein is an interferon antagonist and, as such, is considered a determinant of pathogenicity. A recombinant virus containing the 1918 virus NS gene in the background of A/WSN/33 (H1N1) virus⁷⁷ is attenuated in mice; however, microarray studies of samples obtained from mice infected with this virus indicate that the 1918 virus NS gene blocks the expression of interferon (IFN)-regulated genes more efficiently than does the parental A/WSN/33,^{132,612} suggesting a role for NS1 in viral pathogenicity. This is further supported by the finding that the PDZ ligand domain motif of the 1918 NS1 protein (formed by the four C-terminal amino acids of this protein; see Molecular Determinants of Host Range Restriction and Pathogenesis section) increased the virulence in the background of A/WSN/33 virus.⁸⁹⁸ In addition, the PB1-F2 protein (see Molecular Determinants of Host Range Restriction and Pathogenesis section for more information) of the 1918 virus contributes to virulence.^{340,1356} Further studies using recombinant viruses found no significant contributions of the 1918 M and NP genes to viral pathogenicity.^{2103,2104}

The Pandemic of 1957—Asian Influenza (H2N2)

This pandemic originated in the Southern Chinese province of Guizhou in February 1957 and spread to Hunan Province and to Singapore and Hong Kong in March and April, respectively.¹⁹⁸⁰ In May 1957, the causative agent of the outbreak, an influenza A virus of the H2N2 subtype, was isolated in Japan. A first wave struck the United States and United Kingdom in October 1957, and was followed by a second wave in January 1958. The infection rate was highest in 5- to 19-year-olds,⁶³⁰ where it exceeded 50%. Both waves were characterized by heightened mortality, with about 70,000 deaths in the United States^{1531,1880} and more than 1 million deaths worldwide.

Genetic and biochemical analyses indicated that the 1957 pandemic virus originated from reassortment between human and avian viruses (Fig. 41.3). It contained H2 HA and N2 NA genes of avian virus origin.^{1791,1813} Because the pandemic virus did not appear to be extraordinarily pathogenic, the increased mortality is attributed to the lack of preexisting immunity among humans to the new surface glycoproteins of this virus. In addition to avian virus HA and NA genes, the 1957 pandemic virus also possessed a PB1 gene of avian virus origin.¹⁰⁰¹ The contribution of this gene segment to the pathogenicity of the pandemic 1957 virus is unknown.

Influenza viruses of the H2 subtype continue to be isolated from avian species, and were isolated from pigs in 2006.¹²⁹² Since vaccination against H2N2 viruses was discontinued in the late 1960s, only individuals 40 years of age or older have protective antibodies against this subtype,¹⁴⁶¹ suggesting that a new pandemic by an H2 virus would cause appreciable excess morbidity and mortality in a large section of the population. However, avian H2N2 viruses have evolved slowly, so that the pandemic H2N2 vaccine from 1957 still protects mice against recently circulating avian or swine H2 strains^{274,997}; this vaccine may thus provide a first line of defense in the event of an H2N2 pandemic.

The Pandemic of 1968—Hong Kong Influenza (H3N2)

Eleven years after their emergence, viruses of the H2N2 subtype were completely replaced by those of the H3N2 subtype (Fig. 41.3). The first signs of a new pandemic emerged in southern Asia in the summer of 1968.³³⁰ A virus of the H3N2 subtype was isolated in Hong Kong in July 1968, which soon spread around the world. The attack rates reached 40% and were highest in 10- to 14-year-olds. The excess mortality was estimated to be 33,800 in the United States.¹⁵³¹

The 1968 pandemic virus contained an avian virus HA protein of the H3 subtype that shared less than 30% sequence homology with its predecessor. However, preexisting antibodies to the N2 protein in human populations likely accounted for the moderate severity of the outbreak. In addition to an avian H3 gene,¹⁸¹³ the 1968 pandemic strain also acquired an avian virus PB1 gene,¹⁰⁰¹ as did the 1957 pandemic strain. It is unknown whether the introduction of an avian virus PB1 gene into the human population contributed to the pathogenicity of the 1968 pandemic virus. The HA and PB1 genes originated from viruses of the Eurasian avian lineage, consistent with epidemiologic findings that southern China was the likely origin of the pandemic.

The Reemergence of H1N1 Viruses in 1977—Russian Influenza

The first signs of a new influenza virus outbreak were noted in Tianjin, China, in May 1977. From November 1977 through the end of 1978, young adults around the world suffered from an influenza virus outbreak in the Union of Soviet Socialist Republics and in China.^{1114,2361} The United States experienced a similar outbreak in mid-January 1978, and outbreaks in other countries occurred during the following winter. Among school-age children, the attack rates were more than 50%. Morbidity was almost exclusively limited to persons younger than 25 years, suggesting that older individuals were protected by preexisting immunity. This assumption was proven when the causative agent was identified as an influenza H1N1 virus (A/USSR/77) closely related to strains that had circulated in the early 1950s.^{1016,1091,1469,1814} (Fig. 41.3). This close relationship and the lack of mutations that are typically acquired during replication argue against maintenance of the virus in a nonhuman species. It is now believed that accidental release of this virus started the pandemic. In contrast to 1968, when the newly emerging H3N2 viruses replaced the circulating H2N2 viruses, replacement of H3N2 viruses did not occur in 1977 with the reemergence

of H1N1 viruses. Instead, both H1N1 and H3N2 viruses continue to circulate to this day.

The H5N1 Outbreak

Although not yet a pandemic, the outbreak of H5N1 viruses across Asia and the Middle East deserves discussion here because of its socioeconomic implications and clinical threat. The virus first emerged on geese farms in 1996 in Guangdong Province, China.^{1883,2290} In May 1997 in Hong Kong, a 3-year-old boy was infected and succumbed to the infection.^{320,322,1993}

The causative agent was an H5N1 virus of entirely avian origin. This incident marked the first reported transmission of a wholly avian influenza virus to a human with fatal outcome.^{320,322} In November and December 1997, 17 additional cases were reported, 5 of which had fatal outcomes. No conclusive evidence of human-to-human transmission exists. These human cases accompanied an outbreak of influenza in live bird markets in Hong Kong. Because most infected individuals had contact with poultry prior to their illness, officials ordered the culling of all poultry in Hong Kong's live bird markets, resulting in appreciable economic losses. This intervention proved successful, and no further cases were reported until 2003. Further human infections were probably prevented when poultry stocks were depopulated again in May 2001 and February and April 2002, after highly pathogenic H5N1 viruses reemerged in live bird markets. However, in February 2003, two Hong Kong residents were infected with H5N1 virus.¹⁶²⁵ A 9-year-old boy became sick when his family traveled to Fujian Province in mainland China and was hospitalized in Hong Kong. He recovered from an H5N1 virus infection, but his 33-year-old father succumbed to the disease. The 7-year-old daughter died in a hospital in China during the family's travels; the cause of her death was not determined.

A new outbreak of H5N1 virus started in July 2003 in poultry in Vietnam, Indonesia, and Thailand, although it lacked official recognition at the time. It has since spread to more than 60 Asian, European, Middle Eastern, and African countries, and has led to the depopulation or death of more than 100 million poultry. H5N1 viruses are now enzootic in poultry populations in China, Indonesia, Vietnam, Egypt, India, and Bangladesh⁴³² (<http://www.fao.org/news/story/en/item/66118/icode/>), and continue to cause outbreaks in the Middle East and sporadic infections in Europe. In addition to far-reaching economic consequences, direct avian-to-human transmission of highly pathogenic avian viruses has caused widespread public apprehension. As of February 1, 2013, 615 cases with 364 fatalities had been reported, resulting in a mortality rate of ~60% (http://www.who.int/influenza/human_animal_interface/EN_GIP_20130201CumulativeNumberH5N1cases.pdf). Most human infections have occurred in Indonesia, Egypt, and Vietnam. The number of reported human infections was highest in 2006 (115 infections that resulted in 79 fatalities), and has declined to 48 cases (with 24 fatalities) in 2010. It is not known if this decline can be attributed to changes in human behavior and/or viral properties, or represents a random fluctuation.

Although H5N1 viruses have not yet acquired the ability to spread efficiently among humans, isolated cases of human-to-human transmission may have occurred.^{626,958,959,1573,1574,1829,2084,2116,2118,2191,2245} However, most of these clusters involved family members living in the same household, and infection may have occurred through exposure to a common source (such

as sick or dead poultry), rather than human-to-human transmission. Overall, transmission of H5N1 viruses to humans appears to be rare and primarily associated with contact with sick or dead poultry,^{32,38,283,426,519,957,1429,2084,2133,2371} although other modes of infection (for example, through virus-contaminated feces or water) have been considered as well.^{398,958,1829,2169,2170,2244}

During the 1997 and 2003 H5N1 outbreaks in Hong Kong, there was no evidence of systemic viral infection in infected individuals.^{1625,2069} More recent H5N1 viruses, however, appear to cause systemic infection in humans^{398,399,676,2112,2113}; virus has been recovered from not only respiratory organs, but also stool and cerebrospinal fluid (CSF), while viral sequences and/or antigens have been detected in brain neurons; in epithelial cells of the intestinal tract; in heart, spleen, kidney, and liver; and in the placenta and fetus of a pregnant woman infected with an H5N1 virus. The detection of viral sequences and antigen in the intestinal tract is consistent with the expression of avian-like virus receptors in the human gut,¹⁸⁷³ the *ex vivo* infection of gut tissue with an H5N1 virus,¹⁸⁷³ and reports of gastrointestinal symptoms.^{37,797,2084,2345} In H5N1 virus-infected patients, acute respiratory distress syndrome (ARDS) with diffuse alveolar damage is common.^{38,95,310,591,676,1087,1509,1650,1662,2069,2084,2112,2331,2357} Virus and/or antigen binding has been detected to nonciliated epithelial cells in the bronchioles, and type II pneumocytes and macrophages in the alveoli.^{413,676,1219,1521,1662,2113,2139} Type II pneumocytes differentiate to type I pneumocytes, which are critical for gas exchange in the alveoli; the infection of type II pneumocytes may thus result in more severe lung damage. The observed pattern of virus binding is also in accordance with the detection of avian-type receptors on some bronchiolar and alveolar cells.^{1521,1662,1854,2139}

However, H5N1 viruses also replicate in *ex vivo* organ cultures of the upper respiratory tract.¹⁵²¹ The levels of proinflammatory cytokines are higher in individuals infected with H5N1 viruses than in those infected with seasonal influenza virus, and higher in fatal than in nonfatal cases of H5N1 infection.^{399,1625} The pathology in humans is in line with cell-culture studies that demonstrate increased levels of proinflammatory cytokines upon H5N1 infection,^{266,298,300,611,848,1131,1180,2190,2368} animal infection studies that show increased pathology, viral targeting of type II pneumocytes,^{1064,1733,2139} and stronger innate immune responses upon H5N1 virus infection compared to infection with control viruses [see Outbreaks of Highly Pathogenic Avian Influenza (HPAI) Virus section]. Collectively, the available data suggest that the high mortality in humans results from a combination of high virus loads and the induction of high levels of proinflammatory pathways, which may culminate in extensive alveolar damage.

Some of the currently circulating H5N1 viruses are resistant to the antiviral drugs amantadine and rimantadine.^{299,1686} Most H5N1 viruses are sensitive to the NA inhibitors oseltamivir and zanamivir¹¹⁸⁶; however, oseltamivir-resistant viruses have been isolated from H5N1 virus-infected patients treated with this drug^{400,1164} (http://www.emro.who.int/csr/media/pdf/ai_press_22_01_07.pdf). These viruses contain an amino acid substitution at position 274 or 294 of the NA protein. It is of major concern that two patients treated with oseltamivir subsequently shed drug-resistant viruses and eventually died.⁴⁰⁰

The H1N1 Pandemic in 2009

The first reports of an influenza-like outbreak in a small Mexican town can be dated to mid-February 2009 (<http://www.guardian.co.uk/world/2009/apr/27/swine-flu-search-outbreak-source>).

In early April, Mexican public health authorities began investigating and informed international agencies of a possible outbreak. In mid-April, genetically similar swine-origin H1N1 influenza A viruses were detected in several specimens collected in southern California^{253,388} and Mexico.^{249,258,331,332} The novel virus spread rapidly among humans across different continents, prompting the World Health Organization (WHO) to declare Phase 6 (pandemic phase, which is characterized by community-level outbreaks with human-to-human spread in at least two countries in more than one WHO region) on June 12, 2009.^{333,2351} The WHO soon adopted the name “pandemic (H1N1) 2009” for the novel virus (<http://www.who.int/csr/disease/swineflu/en/index.html>) but now suggests A(H1N1)pdm09 (<http://afludiarly.blogspot.com/2011/10/who-call-it-ah1n1pdm09.html>). This outbreak marked the first pandemic in more than 4 decades. Viruses of the H1N1 subtype have circulated in humans since 1977; hence, pandemics are not limited to viruses with novel HA subtypes (i.e., those not recently circulating in humans), but can be caused by viruses possessing HA subtypes that are circulating in human populations, as long as the novel HA is antigenically distant enough from its predecessor to escape human immune responses.

The pandemic virus spread rapidly.¹⁸⁹ The southern hemisphere (where the influenza season lasts from May to September) experienced significant pandemic influenza activity from May to mid-July of 2009; the United States experienced a first wave in May and June, and a second wave that started in late August and peaked during the second week of October. The novel virus soon dominated the previously circulating seasonal H1N1 viruses; in fact, more than 99% of influenza A viruses subtyped during the winter of 2009/2010 were novel A(H1N1)pdm09 viruses.²⁵⁶ For the United States, the Centers for Disease Control and Prevention (CDC) estimated a total of 61 million infections with 274,000 hospitalizations and 12,470 deaths between April 2009 and April 2010 (http://www.cdc.gov/h1n1flu/estimates_2009_h1n1.htm). However, about 30,000 influenza-related deaths occur in the United States during each interpandemic season, indicating a low case-fatality rate for infections with the A(H1N1)pdm09 virus. Morbidity and mortality rates differed significantly between age groups^{311,469,556,705,1676,1677,1770,2310}; children experienced a low case-fatality rate. Nonetheless, an appreciable number of small children died from infection with the A(H1N1)pdm09 virus. In contrast to seasonal influenza epidemics, the elderly experienced a low infection, but high case-fatality rate. The low infection rate among the elderly can be explained by serum cross-reactivity between A(H1N1)pdm09 viruses and close descendants of the pandemic 1918 virus,^{252,311,705,734,864,890} which is a consequence of shared antigenic epitopes between the HA proteins of these two viruses.^{604,664,860,890,1093,1309,1903,2229,2287,2307} Those aged 5–59 years accounted for the highest absolute numbers of deaths and cases of pneumonia, in contrast to seasonal outbreaks.^{311,469} Epidemiologic data identified several factors associated with an increased risk of severe disease, including pregnancy (particularly in the last trimester), underlying chronic conditions, and obesity.^{57,244,246,259,388,469,705,706,909,910,1254,1261,2351}

Human infections with A(H1N1)pdm09 viruses typically caused mild upper respiratory tract illnesses with fever, cough, sore throat, shortness of breath, headache, and rhinorrhea.^{223,244,259,388,469,705,706,1635} In addition, gastrointestinal

symptoms (which are unusual with seasonal influenza infections) were reported in some cases. In some patients, respiratory and multiorgan failure occurred, leading to death. These severe infections caused diffuse alveolar damage, hemorrhagic interstitial pneumonitis, and peribronchiolar and perivascular lymphocytic infiltrates.^{86,625,684,705,706,1635,1847,1927} (Fig. 41.4), similar to human infections with avian H5N1 viruses (see The H5N1 Outbreak section). These findings are in line with animal infection studies that demonstrated more severe lung lesions and higher lung virus titers in mice, ferrets, and nonhuman primates infected with A(H1N1)pdm09 virus compared with seasonal influenza virus infections.^{101,890,1302,1442,1637,1772,2126} In nonhuman primates, viral antigen was detected in type I and II pneumocytes,⁸⁹⁰ as has been reported for some human cases of A(H1N1)pdm09 infection,⁶⁸⁴ and for nonhuman primates infected with avian H5N1 influenza virus.⁷⁶ Efficient replication in the lungs with infection of type I and II pneumocytes (which likely contributes to the observed alveolar damage) may thus be a hallmark of severe influenza virus infections. In many human A(H1N1)pdm09 cases, bacterial co-infections were detected^{243,625,705,706,914,1254,1345,1599,1635} (Fig. 41.4), a finding that has rekindled interest in the contribution of bacterial infections to influenza-related morbidity and mortality.

Sequence and phylogenetic analyses revealed that the A(H1N1)pdm09 virus possesses PB2 and PA genes of North American avian virus origin; a PB1 gene of human H3N2 virus origin; HA (H1), NP, and NS genes of classic swine virus origin; and NA (N1) and M genes of Eurasian avian virus origin.^{388,604,1903,2094} (Figs. 41.3 and 41.5). A(H1N1)pdm09 viruses do not possess amino acids associated with high virulence in mammals.^{388,556,604,1495,1903,1931} In line with its presumed porcine origin, the virus replicates efficiently, but without symptoms, in experimentally infected miniature pigs⁸⁹⁰ and transmits efficiently among pigs.¹⁷⁸ Other studies have demonstrated efficient transmission of A(H1N1)pdm09 viruses in ferrets.^{890,1302,1442,1637} In nature, A(H1N1)pdm09 viruses have also infected turkeys,¹²³ cats,^{1256,1362,1935} dogs,⁴⁵⁹ and ferrets.²⁰¹⁵ The widespread circulation of A(H1N1)pdm09 viruses may lead to reassortment with other human, swine, or avian influenza viruses. In fact, the pandemic virus has infected pigs^{511,827,1417} and reassortment with swine influenza viruses has been reported.^{1418,1949,2158} Experimental studies demonstrated ready reassortment of A(H1N1)pdm09 viruses with avian H5N1,^{1548,1921} avian H9N2,²⁰⁰⁶ or contemporary human influenza viruses^{1547,1819,1921}; some of these reassortants showed increased replicative ability than the A(H1N1)pdm09 parental viruses.^{1547,1819,1921}

Concerns over a potentially severe pandemic spurred the development of vaccines for the new pandemic virus. In the United States and Europe, the first vaccines were approved in September 2009.^{251,254,933} These vaccines proved to be safe and efficacious. Protective antibody titers were obtained with a single dose of vaccine, despite earlier concerns that two doses may be required; however, two doses were recommended for children younger than 10 years of age, due to lack of preexisting immunity.^{1215,1664,2376} A(H1N1)pdm09 viruses have now replaced seasonal H1N1 virus as part of annual, trivalent influenza vaccines (see also Vaccines section). On August 10, 2011, the WHO declared an end to the pandemic (http://www.who.int/mediacentre/news/statements/2010/h1n1_ypc_20100810/en/index.html). The A(H1N1)pdm09

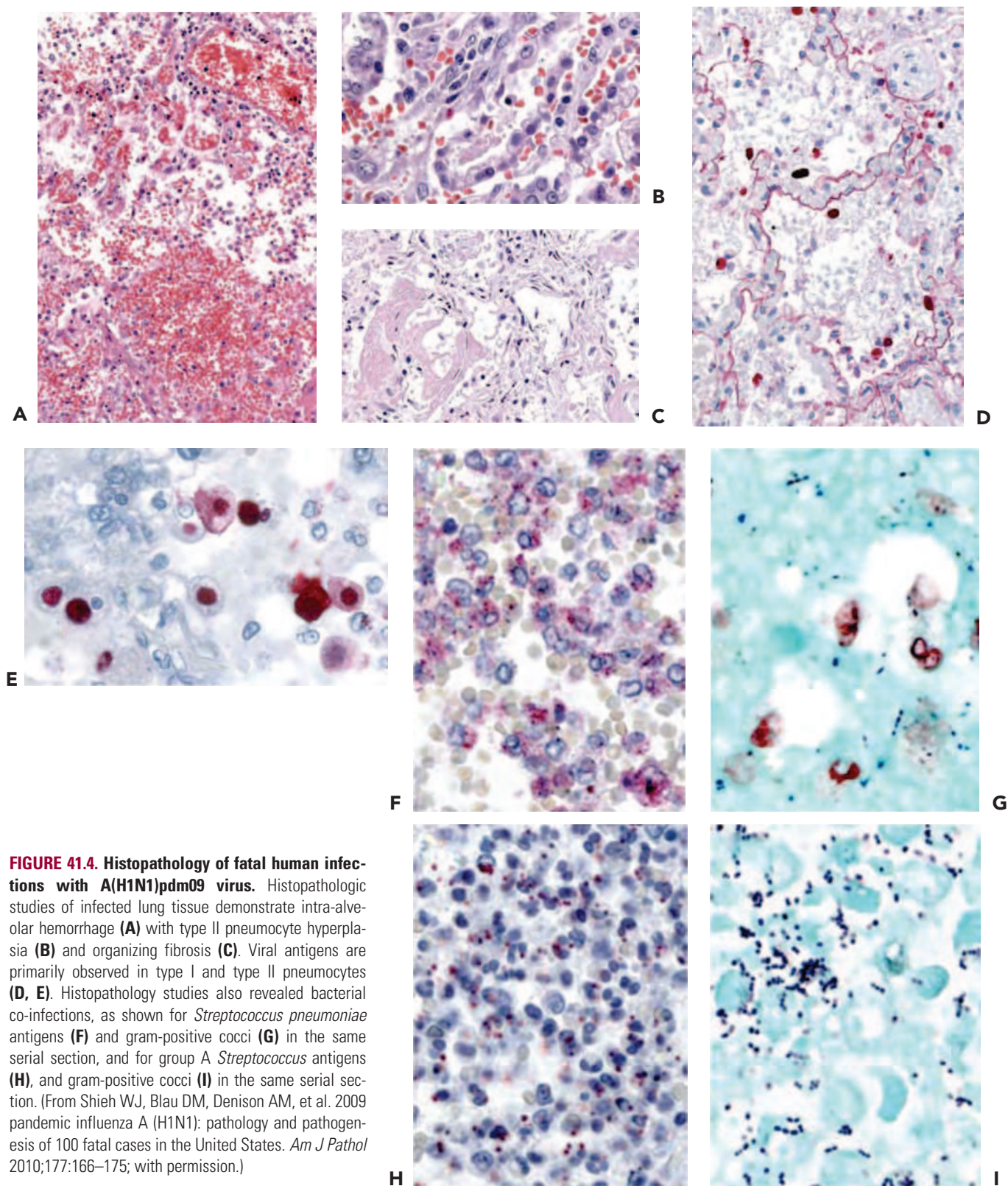


FIGURE 41.4. Histopathology of fatal human infections with A(H1N1)pdm09 virus. Histopathologic studies of infected lung tissue demonstrate intra-alveolar hemorrhage (**A**) with type II pneumocyte hyperplasia (**B**) and organizing fibrosis (**C**). Viral antigens are primarily observed in type I and type II pneumocytes (**D**, **E**). Histopathology studies also revealed bacterial co-infections, as shown for *Streptococcus pneumoniae* antigens (**F**) and gram-positive cocci (**G**) in the same serial section, and for group A *Streptococcus* antigens (**H**), and gram-positive cocci (**I**) in the same serial section. (From Shieh WJ, Blau DM, Denison AM, et al. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. *Am J Pathol* 2010;177:166–175; with permission.)

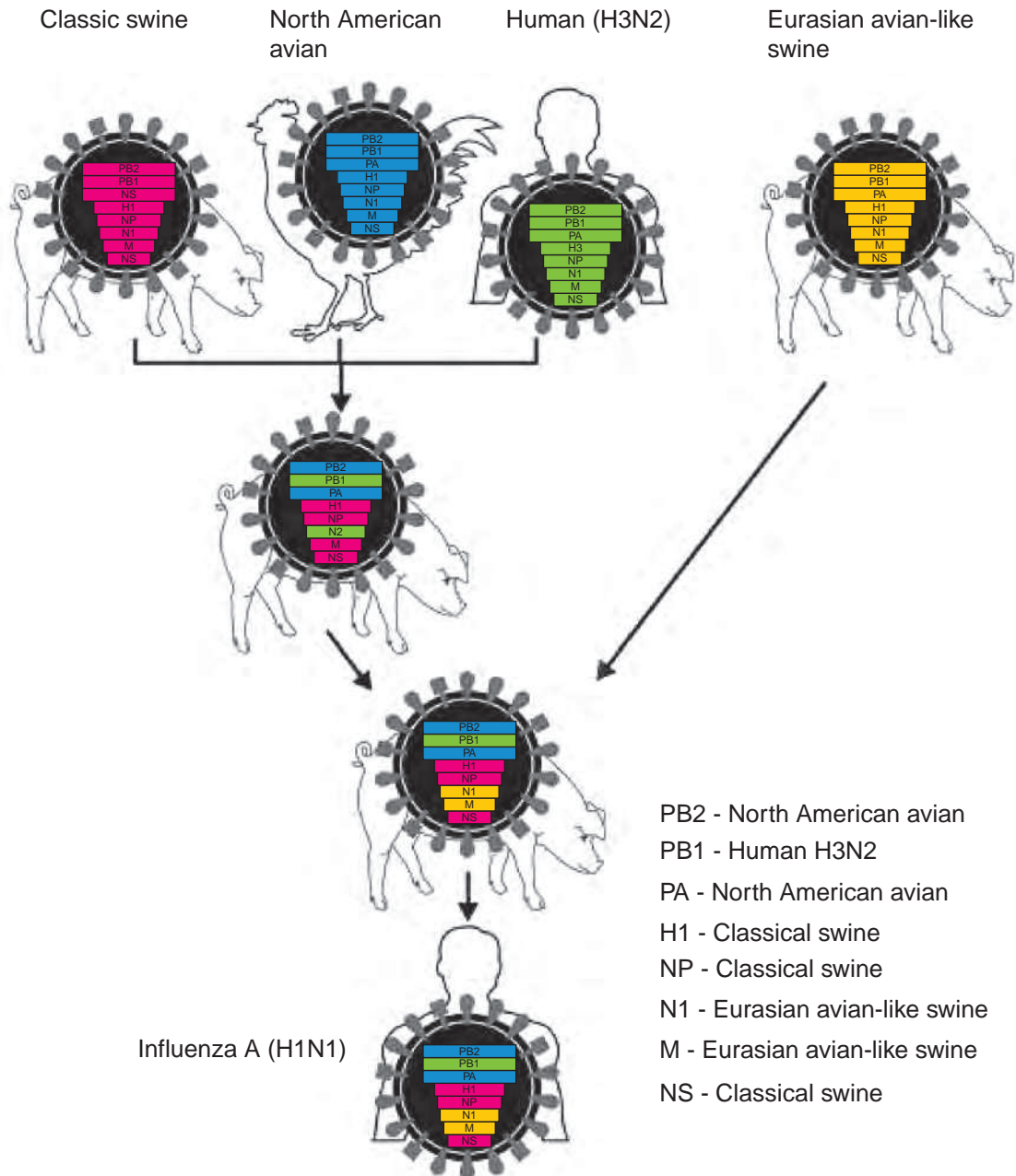


FIGURE 41.5. Genesis of A(H1N1)pdm09 virus. In the late 1990s, reassortment between classical swine, North American avian, and human H3N2 viruses resulted in triple-reassortant H3N2 and H1N2 swine viruses that became established in North American pig populations (see Influenza in Swine—North America section). Reassortment of these viruses with a Eurasian avian-like swine virus led to the emergence of the A(H1N1)pdm09 virus. (From Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* 2009;459:931–939, with permission.)

virus will likely continue to circulate as a seasonal influenza virus.

INFLUENZA IN HUMANS—EPIDEMIOLOGY

Since 1977, seasonal H1N1 and H3N2 viruses have been circulating together with influenza B viruses; in 2009, the seasonal H1N1 viruses were largely replaced by A(H1N1)pdm09

viruses. The prevalence of these groups of viruses varies geographically and temporally, making influenza virus epidemiology complex.

Several studies have assessed the global circulation patterns of influenza viruses^{532,1480,1700}; some analyses indicate that human H3N2 and H1N1 epidemic strains originate from Southeast Asia, from where they are seeded into temperate regions.^{94,263,1766} Temporally overlapping epidemics in Southeast Asia result in continuous virus circulation.¹⁷⁶⁶ Even

though influenza viruses circulate in tropical regions throughout the year,^{1766,2155,2257} seasonality has been observed in these areas, although it is less pronounced than in temperate climates.^{1430,1610,1766} In the temperate regions of North America and Europe, multiple variants may circulate during the early epidemic period, which are replaced by a dominant variant at the peak of an epidemic.³⁶¹

The epidemiology of human influenza viruses is defined by their constant antigenic variation to escape the host immune response. In contrast to most other respiratory viruses, influenza viruses possess two different mechanisms that allow them to reinfect humans and cause disease—antigenic drift and antigenic shift.

Antigenic Drift

Antigenic drift occurs as a result of point mutations in influenza A and B viruses and refers to minor, gradual, antigenic changes in the HA or NA proteins. Influenza A virus drift variants result from the positive selection of spontaneous mutants by neutralizing antibodies.^{204,1666} These variants can then no longer be neutralized by antibodies to the “parental” strains. Antigenic drift has also been observed among influenza viruses in terrestrial poultry, although to a lesser extent than in humans.¹¹⁷⁰

Mutations in the human virus HA or NA amino acid sequence occur at a frequency of less than 1% per year. Nevertheless, antigenic drift variants can cause epidemics and typically prevail for 1 to 5 years before being replaced by a different variant.

Antigenic Drift of the HA Protein

The HA protein is the major antigenic component of the virus. Its function and structure are described in more detail in Chapter 40. X-ray crystallography, comparative sequence analysis, and the characterization of escape mutants have identified five antigenic domains (A–E) for H3 HAs^{619,1066,2223,2247,2248} (Fig. 41.6A): Site A is formed by a protruding loop (amino acids 140–146); site B is formed by another loop (amino acids 155–160) and an α -helix (amino acids 188–198) and is situated at the membrane distal end of HA; site C is located at the base of the globular domain in the antiparallel sheet of HA1; site D is situated near the trimeric interface of the globular head domains; and site E lies near the bottom of the globular distal domain between sites C and A. For H1 viruses, the antigenic sites are designated Ca1, Ca2, Cb, Sa, and Sb²³⁹ (Fig. 41.6B). Some overlap exists among antigenic sites.^{239,374} For H5 HAs, X-ray crystallographic structures have been resolved for the HA proteins of an H5N1¹⁹⁷⁰ and an H5N3⁷⁰⁷ virus, which allowed the mapping of antigenic escape variants obtained with H5N2,^{995,996} H5N9,¹⁶⁴⁹ and H5N1^{995,1204,1762} viruses. Depending on the virus and antibodies used for the analysis, three to five antigenic sites that confer neutralization were identified, some of which overlap with antigenic sites in H3 or H1 HA proteins.

Numerous studies have analyzed antigenic drift in nature.^{156,157,395,1100,1150,1387,1472,1634,1799,1801,1892,2293,2294} Minor antigenic heterogeneity among the viruses is detectable at any time,^{64,395,1586,1888,1967} whereas larger differences, detectable in hemagglutination-inhibition tests, usually require the accumulation of mutations over a 1- to 5-year period.^{930,1363,2150} However, single point mutations in one HA antigenic site can

be sufficient for antigenic variation.^{2247,2248,2252} The H3 HA has drifted more rapidly than the H1 and H2 HAs,⁵²⁷ resulting in the frequent replacement of antigenic variants. Although human H1 and H3 HAs evolve in single lineages,^{204,2085} H1 antigenic drift variants co-circulate, yielding a phylogenetic tree with more side branches.^{527,2085} The faster evolution of H3 HAs has necessitated the update of the H3 vaccine component 23 times since 1972, whereas the H1 component has been replaced only eight times^{236,756} (<http://www.who.int/csr/disease/influenza/vaccinerecommendations1/en/index.html>). The HA of A(H1N1)pdm09 viruses has not yet drifted significantly,⁵⁶³ presumably due to the lack of selective pressure in a predominantly naïve population.

In the laboratory, antigenic drift can be mimicked by virus propagation in the presence of monoclonal antibodies to a single site, with a frequency of variant selection of 1 in 10⁵.^{328,618,1137,1151–1153,1470,1471,2144,2215,2222,2223,2224} The selection of variants in the presence of antibodies to several antigenic sites is likely a rare event. Accordingly, the selection of drift variants in humans who have polyclonal responses is difficult to imagine. Postinfection human sera have a limited antibody repertoire,²¹⁹³ and some animal sera are essentially monoclonal.¹¹³⁶ Therefore, the selection of antigenic drift variants may be a sequential event with the stepwise accumulation of mutations through different individuals.

Recently, a new hypothesis has been proposed for antigenic drift.⁷⁸⁴ Escape variants selected with neutralizing antibodies showed increased affinity to cellular receptors; passage of such variants in naïve mice resulted in the loss of high-affinity receptor binding, while this property was maintained in immune mice. These findings suggest interplay between selection by antibody and receptor recognition, at least in the mouse model.

Over the past decade, computational bioinformatics approaches have become an invaluable tool for studying and predicting antigenic drift. Most notably, antigenic cartography now provides an interpretation of antigenic clusters and their relationships, as well as the extent and directionality of antigenic drift.^{547,1900} (Fig. 41.7). Antigenic cartography suggests that antigenic drift of human H3N2 viruses occurs in clusters: while nucleotide changes continue to occur, clusters of antigenically similar variants exist for several years until they are replaced by a new cluster, founded by an antigenic variant that necessitates an update of the vaccine strain.^{58,1077,1078,1766,1900} Hence, the genetic evolution of H3 HA genes is continuing, while its antigenic evolution is punctuated. The same pattern of antigenic jumps likely also exists for human H1 viruses. Antigenic cartography as developed by Smith et al. is now routinely used by the WHO for the selection of vaccine strains.⁴⁹⁹ Other groups have developed similar approaches.^{212,843,2279}

Antigenic drift has also led to the diversification of recent H5 HA proteins into several antigenically and genetically distinguishable clades and subclades; these are described in the Outbreaks of Highly Pathogenic Avian Influenza (HPAI) Virus section.

Antigenic cartography and computational modeling have provided new insight into antigenic drift of human influenza viruses. Computational approaches have also been used to analyze mutation patterns in HA, and have demonstrated that most positively selected amino acid changes map to the antigenic sites.^{41,140,204,638,844,1842,1843,1848}

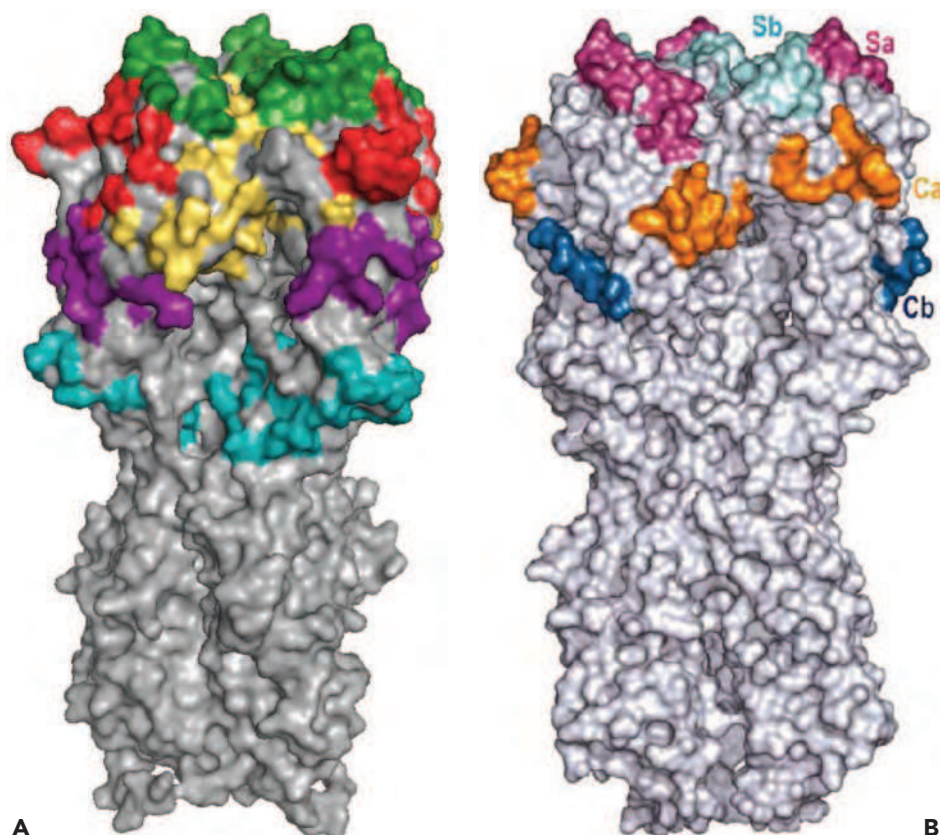


FIGURE 41.6. Crystallographic structures of influenza A virus H3 and H1 HA proteins. **A:** Crystallographic structure of the trimer complex of influenza A virus H3 HA protein (Protein Data Bank #1HGD) showing the locations of the five antigenic epitopes: antigenic site A (amino acids 122, 124, 126, 130–133, 135, 137, 138, 140, 142–146, 150, 152, 168), *red*; antigenic site B (amino acids 128, 129, 155–160, 163–165, 186–190, 196–198), *green*; antigenic site C (amino acids 44–48, 50, 51, 53, 54, 273, 275, 276, 278–280, 294, 297, 299, 300, 304, 305, 307–312), *blue*; antigenic site D (amino acids 96, 102, 103, 117, 121, 167, 170–177, 179, 182, 201, 203, 207–209, 212–219, 226–230, 238, 240, 242, 244, 246–248), *yellow*; antigenic site E (amino acids 57, 59, 62, 63, 67, 75, 78, 80–83, 91, 92, 94, 109, 260–262, 265), *purple*. **B:** Crystallographic structure of the HA protein of A(H1N1)pdm09 virus. Shown is the trimer complex with the antigenic sites Ca (amino acids 140–145, 169–173, 206–208, 224, 225, 238–240), *orange*; Cb (amino acids 79–84), *dark blue*; Sa (amino acids 128, 129, 156–160, 162–167), *red*; Sb (amino acids 187–198), *light blue*. (**B** reproduced from Xu R, Ekiert DC, Krause JC, et al. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. *Science* 2010;328:357–360, with permission.)

Antigenic Drift of the NA Protein

The function and structure of this protein are discussed in detail in Chapter 40. In addition to HA, antigenic drift has also been reported for NA^{367,1286,1350,1605,1802} and correlated with amino acid differences in the molecule.³³⁸ Studies with monoclonal antibodies and amino acid sequence analyses have revealed two to three antigenic sites.¹¹ The NA of most influenza A virus subtypes and of influenza B viruses has been crystallized.^{12,1226,2050,2291} The structure contains two major antigenic sites located on the upper surface of the molecule, where they flank the sialic acid-binding site. A possible third antigenic site resides on the bottom of the head; however, it is difficult to imagine how antibodies binding to this site would result in the selection of escape mutants. A more detailed structural analysis of the Fab fragment of a monoclonal antibody (NC41) to N9 NA showed that five peptide loops, located at the rim of the enzyme active site, constitute the epitope.²¹⁰⁰

The second epitope, characterized with monoclonal antibodies to N8 NA, is located at the interface of two adjacent monomers in the tetrameric NA and involves peptide loops on both monomers.¹⁷⁷⁹ Antibodies with this epitope bind only to NA tetramers.

Antigenic Shift

Antigenic shift involves major antigenic changes in which an HA or NA that is antigenically distinct from the circulating variant is introduced into the human population. Typically, antigenic shift is caused by an HA or NA of a new subtype, that is, one that did not circulate in humans prior to the pandemic. The H1N1 pandemic in 2009 was a notable exception since it was caused by a virus of the H1N1 subtype, even though viruses of this subtype had been circulating in humans since 1977. These newly introduced proteins are immunologically distinct from the previously circulating strains and result in

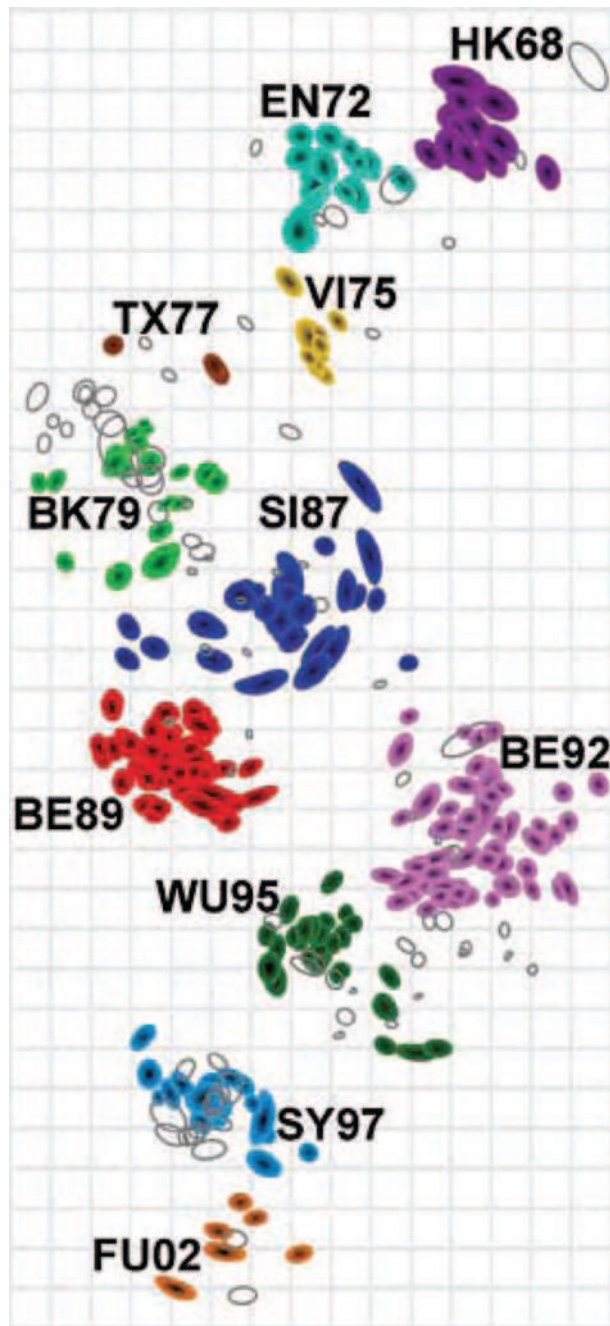


FIGURE 41.7. Antigenic cartography. The map shows seasonal H3N2 viruses from 1968 to 2003. Each *open circle* represents an antiserum used for analysis. Each *colored circle* represents an H3N2 strain tested by using the hemagglutination inhibition assay against the selected antisera. The distances between strains and/or antisera are relative to their antigenic distances (in HI units). The spacing between the grid lines is equivalent to a twofold dilution of antiserum in the HI assay. Typically, fourfold changes in HI titers (i.e., two gridlines in the map) require an update of the vaccine. The different colors represent the antigenic clusters to which the strains belong (HK68, Hong Kong 1968; EN72, England 1972; VI75, Victoria 1975; TX77, Texas 1977; BK79, Bangkok 1979; SI87, Sichuan 1987; BE89, Beijing 1989; BE92, Beijing 1992; WU95, Wuhan 1995; SY97, Sydney 1997; FU02, Fujian 2002). (From Smith DJ, Lapedes AS, de Jong JC, et al. Mapping the antigenic and genetic evolution of influenza virus. *Science* 2004;305:371–376, with permission.)

high infection rates of the novel virus in the immunologically naïve population, leading to pandemics.

Since the beginning of the last century, five antigenic shifts have occurred: in 1918, with the appearance of H1N1 viruses that caused the Spanish influenza; in 1957, when the H1N1 subtype was replaced with H2N2 viruses, causing the Asian influenza; in 1968, when H3N2 viruses replaced the H2N2 subtype, leading to the Hong Kong influenza; in 1977, when the H1N1 subtype reappeared (Russian influenza); and in 2009, when a novel, antigenically distinct H1N1 virus caused a pandemic that largely replaced seasonal H1N1 viruses (see The H1N1 Pandemic in 2009 section). These new subtypes occurred suddenly and at irregular and unpredictable intervals.

The antigenic shift that caused the pandemics in 1957 and 1968 resulted from reassortment between human and avian viruses. By contrast, the A(H1N1)pdm09 virus resulted from reassortment events among swine, avian, and human influenza viruses (see The H1N1 Pandemic in 2009 section). Although not conclusive, phylogenetic evidence suggests that the Spanish influenza was caused by the introduction of an avian-origin virus into the human population.^{77,1712,1713–1715,2048,2049}

Transmission of Avian Influenza Viruses to Humans

Prior to 1997, the direct transmission of avian influenza viruses to humans was not considered a serious human health threat. This assumption was based on findings that avian viruses do not replicate efficiently in experimentally infected humans⁹² and that no severe cases of human infections had been reported during any outbreaks of highly pathogenic avian influenza (HPAI). The differences in receptor-binding specificities between human and avian viruses (for details, see Molecular Determinants of Host Range Restriction and Pathogenesis section) were believed to provide a host range barrier that limited the transmission of avian viruses to humans. Until 1997, only three cases of direct avian-to-human transmission of influenza viruses had been described: (1) an HPAI virus of the H7N5 subtype isolated from a patient with hepatitis in 1959,^{219,409} (2) an H7N7 HPAI virus isolated from a laboratory worker who developed conjunctivitis,²⁰⁵³ and (3) an H7N7 virus of low pathogenicity isolated from a woman who developed conjunctivitis and was likely infected from ducks she kept.^{60,1119} These cases, together with serologic studies,^{1864,1872,2345,2372} demonstrated the potential for avian influenza viruses to be transmitted to humans; however, the true threat of avian-to-human virus transmission was not fully realized until 1997, when avian H5N1 viruses were transmitted to humans in Hong Kong and killed 6 of 18 patients.^{320,322,394,1993,2345}

In 1998 and 1999, H9N2 viruses transmitted from birds to pigs and humans. Five human infections were reported in southern China⁶⁹⁸ and, in March 1999, two children were infected in Hong Kong.^{1227,1626} Both presented with symptoms of typical influenza and recovered. The isolates were genetically similar to those from quail (A/quail/Hong Kong/G1/97 [H9N2]). There was, however, no evidence of human-to-human transmission.²¹¹⁹ During an outbreak of HPAI H7N7 viruses in poultry in the Netherlands in February/March 2003, 89 people were infected, 83 of whom developed conjunctivitis.^{546,1085} Most cases were mild and self-limiting, although a veterinarian developed acute respiratory distress and an ultimately

fatal pneumonia.²¹³⁵ In three cases, human-to-human transmission was documented and antibodies were detected in 59% of those who had contact with infected poultry workers. Also in 2003, a young child in Hong Kong was infected with an H9N2 virus but recovered uneventfully.^{207,297} In November 2003, an H7N2 virus was isolated from an adult in New York who was hospitalized for upper and lower respiratory tract illness, but eventually recovered.^{255,463} In 2004, two people developed conjunctivitis and mild respiratory symptoms after an outbreak of an HPAI H7N3 virus in poultry in Canada.^{815,2111} Additional cases of human infection with avian influenza viruses were detected in the United Kingdom in 2006 and 2007, when H7N3 and H7N2 viruses caused conjunctivitis in one¹⁵¹⁰ and four individuals,⁴⁶³ and in Hong Kong in 2008 and 2009, when genetically different H9N2 viruses were isolated from two immunocompromised patients.²⁹⁷ These findings underscore the potential of H7 and H9 viruses to infect humans, although human-to-human transmission has thus far been limited. In 2004, two cases of human infection with H10N7 virus were reported in Egypt (<http://www.paho.org/english/ad/dpc/cd/eid-eer-07-may-2004.htm>); this subtype has not been isolated in humans before or since.

Highly pathogenic H5N1 viruses have thus far claimed 364 lives in several countries (for more details see The H5N1 Outbreak section). The recent increase in the number of human infections with avian influenza viruses may be a consequence of better surveillance and reporting infrastructure, and/or increased human contacts with poultry and other bird populations.

Vaccines for use in humans for H5 (see Vaccines section), H7,^{275,357,405,906,944,1608} and H9^{275,977} viruses are currently in development. In the United States, the first H5N1 vaccine for human use was approved in April 2007 (<http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm112838.htm>). However, due to the antigenic drift of the H5 HA, the cross-protection among the antigenic clades is not optimal without an adjuvant. Likewise, human infections with H7 viruses have been caused by antigenically distinct viruses of the North American and Eurasian lineages, which may necessitate the development of several vaccines based on antigenically different viruses.

Transmission Among Humans

Influenza viruses do not cause persistent or latent infections in immunocompetent individuals; they are maintained in human populations by direct person-to-person spread during acute infections. Seasonal disease patterns have been reported for temperate climate regions^{444,1242,1255}; in the northern hemisphere, epidemics generally peak between January and April, but may flare up as early as December or as late as May.^{532,830,1531} In the southern hemisphere, outbreaks occur between May and September. The low relative indoor humidity during the winter months may prolong the survival of influenza in aerosols^{735,780,781,1263,1793} and contribute the seasonal pattern in the northern hemisphere. However, seasonality is also observed in tropical and subtropical climates^{23,376,402,1181,1431,1766,2155,2308} where increased influenza activity appears to coincide with the onset of the rainy season.^{301,402,440,1431,1703,1704} On a global scale, influenza virus activity is detectable throughout the year,^{1531,1766} and viruses can be isolated in large cities year-round.

The incubation period is 1 to 3 days for influenza A viruses and 1 to 4 days for influenza B viruses.^{550,1191} The most effective means of spread among humans are aerosols.^{1428,2056} Most aerosol droplets formed during sneezing or coughing are less than 2 μm in diameter and are preferentially deposited in the lower airways of the lung^{46,290,1231,1232,1513,2309}; however, normal breathing also produces aerosols.^{471,512,529} Volunteers are readily infected by aerosol transmission¹⁷; the human infectious dose of influenza A virus infection is 0.6 to 3 TCID₅₀ (dose required to infect 50% of tissue culture) when delivered by aerosol,¹⁷ but 127 to 320 TCID₅₀ when delivered intranasally.³⁴⁷ Ferrets are an established animal model for influenza virus transmission studies (reviewed in^{1341,1542}). Recently, guinea pigs have been established as a model for aerosol transmission,^{1265,1434} and have been used to assess the importance of temperature and humidity for influenza virus transmission.^{1264,1266}

Influenza-Related Morbidity and Mortality

The collective burden of interpandemics can be substantial and depends on a variety of factors, as indicated in this section. The average annual death toll during interpandemic seasons is about 20,000 in the United States.

Morbidity

Influenza virus is estimated to cause about 50 million illnesses annually in the United States. Excess hospitalizations of between 50,000 and 200,000 Americans per season have been reported.^{670,1436,1879,1882,2063} The H1N1 pandemic in 2009 is estimated to have caused 274,000 hospitalizations in the United States alone. Direct costs include hospitalizations, medical fees, drugs, and testing, and were estimated in 1986 to be about \$1 billion annually, while indirect costs such as loss of productivity reached \$2 to \$4 billion annually.¹⁸⁰⁶ The temporal curves of individual pandemics are similar in that virus introduction into a community is followed by a relatively sharp, single peak that represents school and workplace absenteeism, which is followed by excess mortality slightly later.¹⁵³¹ Notable exceptions are the pandemics in 1918 and 1957, both of which were characterized by a second, more severe wave.^{941,2047,2185}

A number of studies have documented how influenza virus epidemics reduce school attendance and work productivity, increase doctor's visits and hospitalizations, and can cause increased mortality, particularly among high-risk groups.^{1504,1505,1506} Children younger than two years of age and the elderly have the highest hospitalization rates,^{631,895,1506,1641} reaching 1 per 270 for those older than 65 years, compared to 1 per 2,900 for the 1- to 44-year-old age group.^{68,69} The impact of influenza on the elderly extends beyond influenza and pneumonia to all respiratory conditions as well as to congestive heart failure.¹⁵¹⁶ It is an important cause of respiratory infections in nursing homes.⁶⁹ Among children, 14% to 16% of those seeking medical care for acute respiratory illness or fever are infected with influenza virus.^{635,1668,2272}

During interpandemic seasons, the overall infection rates range from 10% to 20% but can reach 50% in selected age groups or populations. The rate and severity of infection depend on the level of preexisting immunity, age of an individual, and virulence of the virus, all of which vary greatly among outbreaks.^{304,549,629,942,1724} Age-specific attack rates are highest in school-age children, and symptoms

in this age group are usually more severe than in young adults.^{549,629,632,1411,2018} Often, increases in school absenteeism mark the beginning of a new epidemic,⁶³¹ suggesting that school-age children play a critical role in disseminating influenza viruses. Increases in school absenteeism are typically followed by increases in workplace absenteeism. Influenza A viruses of the H1N1 and H3N2 subtypes, as well as influenza B viruses, all cause similar symptoms^{555,1522,1934}; however, the frequency of severe infections is higher with H3N2 viruses.²⁰⁶³ Reinfection with a closely related variant can occur,^{384,385,549,553,554,1924} although the symptoms are usually less severe than those that follow the initial encounter with a particular virus strain.^{384,385,629,1924}

The H1N1 pandemic in 2009 showed a similar pattern to previous pandemics since elderly people (who typically suffer high attack rates) were infected in relatively small numbers; their exposure to the descendants of the 1918 pandemic virus which circulated until 1957 may have provided partial protection against the A(H1N1)pdm09 virus^{252,311,705,734,864,890} (see The H1N1 Pandemic in 2009 section).

Mortality

The increase in mortality during pandemics and epidemics is a hallmark of influenza virus infection. The term “excess mortality” was introduced by William Farr and describes the number of deaths observed during an outbreak in excess of the number of expected mortalities. The number of influenza virus–related deaths, however, is difficult to determine because the death certificate may not indicate influenza as a primary cause of death or because a laboratory diagnosis may not have been performed.

Excess mortality was estimated to be between 20 and 50 million deaths for Spanish influenza worldwide,¹⁶¹⁹ and 70,000, 33,800, and 12,470 deaths in the United States for the Asian influenza, Hong Kong influenza, and H1N1 pandemic in 2009, respectively. Although excess mortality is highest during a pandemic, the cumulative deaths of interpandemic seasons usually exceed those of pandemic years^{1531,1881} (Fig. 41.8). During epidemics, excess mortality is estimated to be more than 20,000 in the United States alone but can exceed 40,000 deaths. In the United States, the annual excess mortality appears to have increased over the last decades, perhaps because of an increasing number of elderly and/or immunocompromised individuals.²⁰⁶³ Excess mortality is typically highest with H3N2 virus infections, whereas H1N1 and type B viruses contribute to smaller extents.^{168,667,1881,2063}

Excess mortality affects all age groups but is highest in those older than 65 years, who account for approximately 90% of excess mortalities during interpandemic seasons.^{69,71,483,1936} This age group has a 100-fold higher mortality rate than those younger than age 65.¹⁸⁷⁹ During the 1968 pandemic, however, people younger than 65 accounted for 50% of influenza virus–related deaths,³⁵⁴ and during the 1918 pandemic, excess mortality rates were extremely high not only in the elderly, but also in young adults.¹⁵³¹ The reasons for these unusual patterns are unknown. The highest risk for pneumonia is seen in the elderly who have cardiovascular and pulmonary conditions⁷¹; other risk factors include metabolic or neoplastic diseases and pregnancy.^{69,70,665,1505} Several other factors associated with increased risk of severe disease have been identified for infections with

A(H1N1)pdm09 viruses (see The H1N1 Pandemic in 2009 section).

EPIZOOLOGY AND PATHOGENESIS IN ANIMALS

Influenza A viruses infect a variety of animals, including humans, birds, swine, horses, and dogs. Occasionally, influenza viruses have been isolated from cats, tigers and leopards, stone martens and Owston civets, whales, seals, mink, and camels. Serologic evidence also suggests exposure of several ruminant, reptile, and amphibian species to immunogens related to influenza viruses (reviewed in¹⁷²³).

Viruses of all known HA and NA subtypes are maintained in aquatic birds, generally without causing disease; therefore, aquatic birds are considered the natural reservoir of influenza A viruses.^{465,807,809,1749,1755,1864,1894,2214,2221} Nonpathogenic viruses of the H5 and H7 subtypes can evolve into highly pathogenic avian strains that cause significant losses to the poultry industry.^{62,225,834,836,1002,1748} Viruses of the H5, H6, and H9 subtypes have established or may be establishing lineages in chickens.^{432,1182,2220} Although human pandemics have been associated with viruses of the H1 to H3 subtypes, the ability of avian viruses of the H5, H7, and H9 subtypes to infect humans has flagged viruses of these subtypes as potential candidates for future influenza pandemics. Influenza B viruses typically replicate in humans but have been isolated from seals.^{1550,1582} Influenza C virus infects humans, swine,^{701,1558,2303,2344} and dogs.^{1310,1558}

Influenza in Birds

The natural reservoir of influenza A viruses are the orders *Anseriformes* (ducks, geese, swans) and *Charadriiformes* (gulls, terns, shorebirds),^{445,1894,2214} although influenza A viruses have been isolated from at least 105 different avian species from 26 different families.^{466,1340,1489,1569,1943,1945} Almost all HA and NA subtypes have been detected in dabbling ducks (*Anas* spp.),^{999,1095,1147,1560,1569,1837,1838,1947,2182,2225} suggesting that these species are the major reservoir of influenza A viruses. In mallard ducks (*Anas platyrhynchos*), viruses of the H3, H4, and H6 subtypes are isolated most frequently; the most prevalent subtypes are H4N6 and H6N2.^{737,1095,1441,1838,2182} In addition, several HA subtypes (including H1, H2, H5, H7, and H9–H12), in combination with several NA subtypes, have been detected in waders of the *Charadriidae* family, suggesting a role for these species in the perpetuation of influenza A viruses. Viruses of the H13 and H16 subtypes appear to circulate only in *Laridae* (gull and tern) species.^{262,999,1094,1441,1569,1633,2142} This observation is consistent with the finding that a gull influenza virus did not replicate efficiently in ducks,⁸⁰² perhaps due to differences in receptor-binding preferences between viruses derived from ducks or *Laridae* species.²³⁰⁶ For the NA gene, N2, N6, and N8 subtypes predominate in ducks, whereas N6 and N9 are prevalent in shorebirds and gulls. Poultry play a critical role in the perpetuation of influenza A viruses; thus, most HA and NA subtypes can be isolated from poultry in live-bird markets.^{195,308,1249,1511,1932,1986,2214,2319}

Surveillance studies have demonstrated seasonal patterns in prevalence; the infection rate of mallard ducks reaches 60%

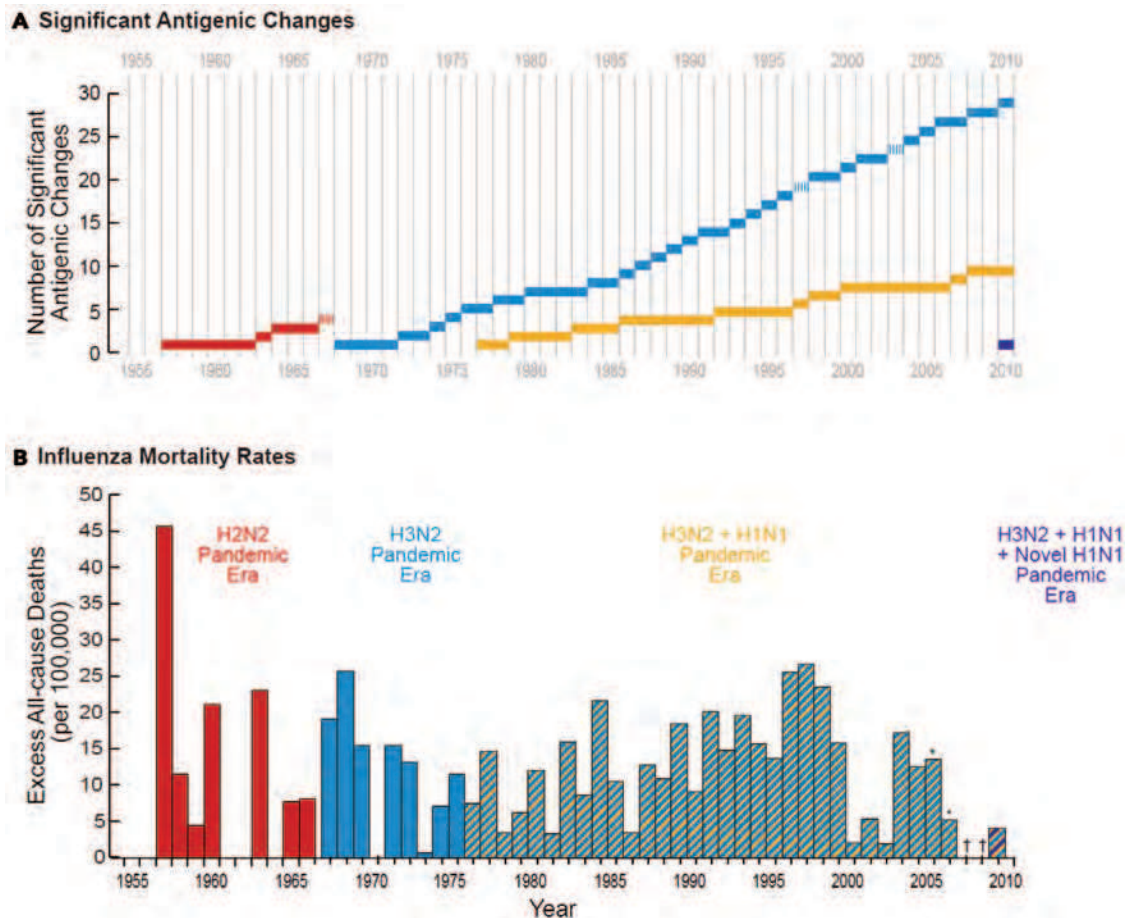


FIGURE 41.8. Influenza virus mortality rates in the United States from 1957 to 2010. Shown are the excess mortality rates per 100,000 persons. *Estimates are given since data are unavailable; †, data unavailable. (Modified from Morens, DM, Taubenberger JK, Fauci AS. 2010. The 2009 H1N1 Pandemic Influenza Virus: What Next? *MBio* 1(4):e00211–10; with permission.)

before the autumn migration, and declines to less than 10% in spring and summer.^{809,811,1095,1441,1560,1569,1945,2182,2214} This pattern may represent the influx of immunologically naïve juveniles every summer, and/or the high population density in marshalling areas before the migration. By contrast, in North American shorebirds, the infection rate is highest in late spring and early summer.¹⁰⁹⁵ Since the prevalence of infection declines along the migration route and is relatively low at the wintering grounds of mallard ducks, geographic patterns in prevalence can be observed (i.e., infection rates are higher at the marshalling areas in the North than at the wintering grounds in the South). Moreover, the prevalence of virus subtypes in the same species changes from year to year.^{811,1095,1838,1945} In particular, cyclic patterns exist in which high rates of prevalence for one subtype in a certain population may be followed by low detection rates in this population in subsequent seasons,^{1095,1838} perhaps due to herd immunity to viruses of the respective subtype.

Reassortment may play a critical role in the evolution of avian influenza viruses with significant contributions of both intrasubtypic reassortment (i.e., reassortment between viruses of the same subtype) and intersubtypic reassortment (i.e., reassortment between viruses of different subtypes),^{278,449,677,678,752,805,1089,1205,1296,1491,2157,2187} and of

reassortment between the North American and Eurasian gene pools, which evolve largely independently, as described earlier.^{388,456,752,1050,1094,1248,1305,2181,2210,2246,2263}

Avian Influenza Viruses of High or Low Pathogenicity

Based on their pathogenicity in chickens, avian influenza viruses are classified as highly pathogenic avian influenza (HPAI) or low pathogenicity avian influenza (LPAI) viruses. LPAI viruses cause mild respiratory disease, depression, and/or a decrease in egg production. For outbreak control purposes, the Office International des Epizooties (OIE) classifies an avian influenza virus as HPAI if it is “lethal for six, seven, or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid” (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2010; accessible at: <http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/>), or if it has an intravenous pathogenicity index (IVPI) greater than 1.2 (the IVPI is the mean clinical score of ten 6-week-old chickens intravenously infected). All H5 and H7 viruses of low pathogenicity in chickens with a multibasic sequence at the HA cleavage site are considered highly pathogenic.

Most HPAI viruses have a series of basic amino acids at the HA cleavage site; however, exceptions exist in which viruses with multiple basic amino acids at this site are not highly pathogenic in chickens,^{1002,1171,1257,2012} or highly pathogenic viruses do not possess a conventional HA cleavage site.^{1614,1989} All HPAI viruses known to date belong to the H5 or H7 subtype; however, only a small proportion of all H5 and H7 viruses are highly pathogenic. Historically, disease caused by HPAI viruses was called “Fowl plague.” Viruses that are highly pathogenic for chickens often cause high mortality in turkeys and Japanese quail, but are usually nonpathogenic for ducks and geese, with the exception of some of the recently circulating HPAI H5N1 viruses.

The infection of aquatic birds with LPAI is typically asymptomatic, although even LPAI viruses can cause mild disease in mallards^{945,1147} and swans.²¹²⁸ In ducks, avian viruses replicate in the epithelial cells in the intestinal tract and, to a substantially lesser extent, in cells of the respiratory tract.^{489,2218,2228} These viruses are resistant to the low pH environment they encounter during their passage through the digestive tract. Avian species shed influenza viruses in high concentrations in feces.^{1023,1893,2225,2228} The viruses are relatively stable in water^{1944,1946} and have been isolated from water samples of lakes where wild birds have nested or congregated before migration.^{731,809,810,886} Contaminated water and feces may, therefore, serve as major routes of transmission of LPAI among feral birds.^{810,1885} By contrast, highly pathogenic H5N1 viruses replicate efficiently in the upper respiratory tract of infected birds.^{1006,1982} Human influenza viruses also replicate in the upper respiratory, but not in the intestinal, tract of ducks,²²¹⁸ because they do not recognize efficiently the receptors in duck intestine [sialic acid (Sia) linked to galactose by α 2,3 linkages (Sia α 2,3Gal)].¹⁷⁴⁴

Infection of ducks with some highly pathogenic H5N1 influenza viruses results in systemic infections with high virus titers in the respiratory organs; neurologic symptoms may also be observed.^{488,1049,1982,2148} However, infected birds may die without clinical symptoms.⁴⁸⁸ In experimental infections of several bird species, HPAI H5N1 were not uniformly lethal, and infected animals shed appreciable amounts of viruses, which could facilitate virus spread.^{185,279,1006,1147,1983} A characteristic feature of HPAI H5N1 infection in ducks is that virus titers in oropharyngeal swabs are higher than those in cloacal swabs.^{1006,1983} In chickens, influenza virus infections can result in mortality rates of up to 100%.

Occasionally, avian influenza viruses infect mammalian species. Most of these infections are self-limiting and stable lineages have emerged rarely, such as in pigs in 1979 with the introduction of an avian H1N1 virus.¹⁶³⁰ In mammals, LPAI viruses cause bronchitis, bronchiolitis, and/or pneumonia. Infection with HPAI results in severe, systemic infection (see following discussion and The H5N1 Outbreak section).

Outbreaks of Highly Pathogenic Avian Influenza (HPAI) Viruses

With only three exceptions [A/tern/South Africa/61(H5N3),⁹³ a highly pathogenic H7N1 virus that caused an outbreak in a backyard flock of geese and Muscovy ducks in Italy in 1999/2000,²²⁸ and the so-called Qinghai Lake HPAI H5N1 viruses,^{279,281,1247}], all HPAI outbreaks have occurred in

poultry (Table 41.1). However, H5N2 viruses isolated from an apparently healthy duck and goose possessed an HA cleavage sequence characteristic of HPAI viruses,⁵⁷³ demonstrating that HPAI viruses may emerge in aquatic birds without apparent signs of infection. HPAI is controlled by depopulation and vaccination. Although LPAI viruses had long been considered of negligible risk, it is now clear that HPAI arise from LPAI by mutations.^{62,225,834,836,1002,1748} The major recent outbreaks of HPAI are discussed in the sections that follow (Table 41.1).

H5N1

HPAI H5N1 viruses were first isolated from sick domestic geese in 1996 in Guangdong province.^{1883,2290} Outbreaks of H5N1 HPAI occurred in poultry in Hong Kong from March to May 1997 and again in November 1997.^{1865,1869} These viruses caused high mortality rates (70–100%) in experimentally infected chickens.^{1988,1993} The causative agent was identified as an H5N1 reassortant virus that had acquired its HA gene from an A/goose/Guangdong/1/96 (H5N1,Gs/Gd)-like virus,²²⁹⁰ its NA gene from another avian N1 virus, and its remaining six genes (i.e., PB2, PB1, PA, NP, M, and NS) from other avian viruses.^{681,826} H5N1 HPAI viruses have in common an HA protein with a multibasic sequence at the cleavage site (a characteristic of highly pathogenic viruses).¹⁸⁶⁹ By the same token, many H5N1 isolates possess a shortened NA stalk,²³⁶⁷ a characteristic of viruses adapted in terrestrial poultry.^{116,1331,1869,1988} The H5N1 viruses isolated from Hong Kong patients in 1997 fell into two groups of either high or low pathogenicity in mice.^{593,990,1269,1869} The outbreaks in poultry were accompanied by the first transmissions of wholly avian influenza viruses to humans with fatal outcomes.^{320,322,394,1993} These events, together with the finding that H5N1 infections were widespread in poultry markets in Hong Kong (close to 20% in chickens)¹⁸⁶⁵ prompted the slaughter of all poultry in Hong Kong in late December 1997/early January 1998. The mass slaughter of poultry eliminated HPAI H5N1 viruses from the Hong Kong poultry markets, and no further cases of human infections were reported. However, HPAI H5N1 viruses with an HA gene belonging to the Gs/Gd lineage continued to circulate in avian species in mainland China.¹²⁰⁵ They reappeared in poultry in Hong Kong in 2001 and 2002, and have been isolated from both aquatic and terrestrial poultry since 2001.^{677,1205} The outbreaks in poultry in Hong Kong in 2001 and 2002 were controlled by depopulation, and by vaccination with an H5N2 strain in April 2002. These strict surveillance and vaccination measures have reduced the number of outbreaks of HPAI H5N1 viruses in Hong Kong.

Waterfowl are usually resistant to HPAI viruses; however, in late 2002, waterfowl in Kowloon and Penfold Park, Hong Kong, showed signs of neurologic disease as they succumbed to H5N1 influenza virus infection.^{488,1982} With the exception of A/tern/South Africa/61(H5N3)⁹³ and a highly pathogenic H7N1 virus that caused an outbreak in Muscovy ducks in Italy in 1999/2000,²²⁸ influenza viruses were not known to cause disease in aquatic birds. At the same time as the Kowloon Park outbreak, H5N1 viruses were isolated from dead chickens in retail poultry markets and a local chicken farm.

In April/May 2005, more than 6,000 birds—mostly bar-headed geese, but also brown- and black-headed gulls, ruddy

TABLE 41.1 Outbreaks of Highly Pathogenic Avian Influenza Viruses Since 1955

Virus ^a	Subtype	Comments
A/chicken/Scotland/59	H5N1	Two chicken flocks
A/tern/South Africa/61	H5N3	1,300 common terns
A/turkey/England/63	H7N3	29,000 breeder turkeys
A/turkey/Ontario/7732/66	H5N9	8,100 breeder turkeys
A/chicken/Victoria/76	H7N7	25,000 laying chickens, 17,000 broilers, and 16,000 ducks
A/chicken/Germany/79	H7N7	Outbreaks in former East Germany; numbers unknown
A/turkey/England/199/79	H7N7	3 commercial turkey farms
A/chicken/Pennsylvania/1370/83	H5N2	17 million birds, mostly chickens or turkeys
A/turkey/Ireland/1378/83	H5N8	800 infected turkeys died; 8,640 turkeys, 28,020 chickens and 270,000 ducks were culled
A/chicken/Victoria/85	H7N7	24,000 broiler breeders, 27,000 laying chickens, 69,000 broilers, and 118,518 unspecified chickens
A/turkey/England/50-92/91	H5N1	8,000 turkeys
A/chicken/Victoria/92	H7N3	12,700 broiler breeders and 5,700 ducks
A/chicken/Queensland/95	H7N3	22,000 laying chickens
A/chicken/Puebla/8623-607/94	H5N2	Millions of birds died or were culled; exact numbers are not available
A/chicken/Queretaro/14588-19/95		
A/chicken/Pakistan/447/95	H7N3	3.2 million broilers and broiler breeder chickens
A/chicken/Pakistan/1369-CR2/95		
A/chicken/Hong Kong/220/97	H5N1	1.5 million chickens and other domestic birds; 6 human fatalities (among 18 infected individuals)
A/chicken/New South Wales/1651/97	H7N4	128,000 broiler breeders, 33,000 broilers, and 261 emus
A/chicken/Italy/330/97	H5N2	Chickens, turkeys, guinea fowl, ducks, quail, pigeons, geese, pheasant (all in small numbers)
A/turkey/Italy/4580/99	H7N1	8.1 million laying chickens, 2.7 million meat and breeder turkeys, 2.4 million broiler breeders and broilers, 247,000 guinea fowl, 260,000 quail, ducks, and pheasants; also backyard poultry and ostriches
A/chicken/Chile/4957/2002	H7N2	2 million birds died or were culled
A/grey heron/Hong Kong/861.1/2002	H5N1	Outbreak in wild birds in Hong Kong; over 800,000 domestic birds were culled
A/chicken/Netherlands/1/2003	H7N7	Virus was isolated from 241 poultry farms in The Netherlands, two farms in Belgium, and one farm in Germany; outbreak was controlled by killing more than 30 million birds; one human fatality
A/chicken/Canada/AVFV1/2004	H7N3	Spread to more than 40 commercial poultry farms; outbreak was controlled by culling all 19 million domestic birds in Fraser Valley, British Columbia
A/chicken/Canada/AVFV2/2004		
A/ostrich/South Africa/2004	H5N2	Outbreak in ostriches; 26,000 ostriches were culled to control virus spread
Various H5N1 viruses (since 2003)	H5N1	Outbreak started in July 2003 in poultry in Vietnam, Indonesia, and Thailand and has since spread to a number of Southeast Asian, European, Middle Eastern, and African countries; more than 100 million domestic birds have died or have been culled; mortality has also been observed among wild birds; as of February 1, 2013, 364 human fatalities had been reported (among 615 infected individuals)
A/chicken/North Korea/2005	H7N7	Approximate number of birds affected: 219,000
A/chicken/Saskatchewan/HR-00011/2007	H7N3	Outbreak on a broiler breeding farm in Saskatchewan, Canada; depopulation of all animals on the premise (53,000)
A/chicken/England/1158-11406/2008	H7N7	Outbreak in a laying flock of chickens; death or depopulation of 25,000 animals
A/chicken/Spain/6279-2/2009	H7N7	Outbreak in a layer hen farm; outbreak was stopped through death (~30,000) and depopulation (~278,000) of all animals on the farm

^aOutbreaks that caused significant economic losses (defined as outbreaks that killed or resulted in the slaughter of more than 1 million birds) are shown in bold.

(Modified from Swayne DE, Halvorson DA. Influenza. In: Saif YM, Barnes HJ, Fadly AM, et al., eds. *Diseases of Poultry*. 11th ed. Ames: Iowa State University Press; 2003:135–160.)

shelducks, and great cormorants—died from HPAI H5N1 virus infection at the Qinghai Lake Nature Reserve in Gangcha County, Qinghai Province, China.^{279,281,1247} Although viruses of several genotypes were detected during the outbreak, one genotype was dominant.^{279,281,1247,2370} HPAI H5N1 viruses closely related to the Qinghai Lake isolates have spread through Russia, into Europe, the Middle East, and several African countries. Two factors likely contributed to the dissemination of these viruses: movement of infected poultry and virus spread through migratory birds.^{280,522,1031,1444,1645,1680,1722,1876,2125,2212,2253}

Recent HPAI H5N1 viruses have expanded their geographic range, become enzootic in poultry populations in different parts of the world, more pathogenic in ducks than isolates from 1997,^{1606,1647,1982} and have a broad host range. While HPAI infections are typically limited to terrestrial poultry, the currently circulating H5N1 viruses have been isolated from a number of different wild birds including water birds (order *Anseriformes*) such as ducks, geese, and swans; shore birds (order *Charadriiformes*) such as gulls and waders; small songbirds (order *Passeriformes*) such as sparrows and crows; large wading birds (order *Ciconiiformes*) such as herons, storks, and egrets; several *Ratites* species including ostriches,³⁹⁰ emu, and rhea; and species of several other birds orders.^{279,281,454,488,1082,1221,1247,1287,1839,1982} In addition, HPAI H5N1 viruses have been isolated from carnivores including dogs,^{29,206,325,624,1923} cats,^{29,1064,1104,1105,1733,1922,2120} tigers,^{28,2058} leopards,¹⁰⁰⁵ a stone marten,^{1065,1735} *Owston civets*¹⁷³⁵ (www.promedmail.org, Archive no. 20080312.0991), and raccoon dogs.¹⁶⁸⁸ Moreover, HPAI H5N1 viruses have been isolated from pigs on several occasions^{1202,1526,1846,2378}; these animals can also be infected experimentally with HPAI H5N1 viruses.^{306,1241,1869} However, pigs do not show symptoms upon infection with HPAI H5N1 viruses.¹²⁴¹ The experimental infection of calves was limited to subclinical seroconversion,⁹⁵⁵ and no HPAI H5N1 infections have been reported in horses to this point.

Past outbreaks of HPAI originated from one virus strain. However, antigenic drift and reassortment have resulted in a diversification of HPAI H5N1 viruses, both genetically and antigenically. Based on the HA sequence, ten clades (clade 0–9) are currently recognized, some of which are divided into second- and third-order clades.^{547,674,675} Recently, only viruses of clades 1, 2, and 7 have been isolated (http://www.who.int/influenza/gisrs_laboratory/201101_h5n1evoconceptualdiagram.pdf): clade 2.1 represents Indonesian H5N1 viruses, whereas Qinghai Lake H5N1 viruses^{279,281,1247} and their European, Middle Eastern, and African descendants form clade 2.2. Clade 2.3 viruses have become dominant in southern China, but have also been isolated in Hong Kong, Vietnam, Thailand, Laos, Malaysia, and Japan. Other Japanese H5N1 viruses belong to clade 2.5, which also includes viruses from Korea and China. Clade 2.4 is formed predominantly by chicken viruses from southern China (Yunnan and Guangxi Provinces). Human infections have been caused by viruses of clades 0, 1, 2.1, 2.2, 2.3, and 7. Viruses of clade 2.2 have spread to three continents and continue to infect humans. The evolution of these viruses has been studied intensively.^{281,451–454,1054,1409,1785,1948} The NA genes of HPAI H5N1 viruses can be divided into two lineages: one possesses a 19-amino-acid deletion in the stalk region (amino acids 54–72); the second comprises viruses with a full-length NA stalk or with a 20-amino-acid deletion spanning amino acids 49–68.

Since their first appearance, multiple genotypes have been isolated that are indicative of frequent reassortment with viruses of other genotypes or subtypes.^{278,449,677,678,1089,1205,1296,1491,2157} One genotype became dominant in 2002^{280,1129,1205}; its descendants continue to circulate in Indonesia and have spread westward into the Middle East. In southern China and other parts of Southeast Asia, however, this genotype was replaced by viruses of another genotype (the Fujian-like viruses).¹⁹⁰²

Reassortment between HPAI H5N1 and circulating human influenza viruses could create highly pathogenic viruses that transmit among humans. While such an event (which would likely result in a devastating pandemic) has not occurred yet, experimental studies have demonstrated a high propensity of HPAI H5N1 viruses for reassortment with A(H1N1) pdm09¹⁵⁴⁸ or with seasonal H3N2 viruses.^{284,905,1198,1301} While viruses possessing the HPAI HA and NA genes in combination with human H3N2 internal genes failed to transmit among ferrets,¹³⁰¹ other reassortants were more pathogenic than either parent virus.¹¹⁹⁸

HPAI H5N1 viruses have been extensively studied in primary human cells^{264,265–266,300,369,611,848,1180,1405,1410,1639,1780,2354,2368,2369} and in several animal models including mice, ferrets, guinea pigs, and nonhuman primates.^{76,186,279,292,319,515,1097,1106,1731,1825,2075} In alveolar epithelial cells^{264,266,2354} and macrophages,^{300,611,848,1180,1410,1639,1780,2368,2369} HPAI H5N1 viruses typically—but not always—elicit higher levels of proinflammatory cytokines than are observed upon infection with contemporary human viruses. Upregulated factors include IFN- β , IP-10, RANTES, and IL-6 in alveolar epithelial cells, and TNF- α , IFN- α and - β , IP-10, RANTES, MIP-1, and MCP-1 in macrophages. Many of these factors are also upregulated in the lungs of infected mice,^{232,318,339,1148,1239,1640,1784,2017,2106} ferrets,²¹⁶ and nonhuman primates.^{76,186,319} In these animal models, HPAI are highly pathogenic and cause diffuse alveolar damage with massive infiltration of immune cells and infection of pneumocytes. In nonhuman primates, HPAI H5N1 viruses are more pathogenic than the 1918 virus, or reassortants possessing 1918 virus genes (HA and NA, or HA, NA, and NS) in the background of a contemporary human virus.^{76,186,2075}

H5N2

Pennsylvania Outbreak. In April 1983, a low virulent H5N2 virus (A/chicken/Pennsylvania/1/83) emerged in chickens in Pennsylvania. By October, this virus had mutated into a highly pathogenic variant (A/chicken/Pennsylvania/1370/83) with a mortality rate of more than 80% in chickens. The virus was eventually eradicated by the slaughter of more than 17 million birds. The avirulent predecessor was unusual in that it had multiple basic amino acids at the HA cleavage site. The virulent strain differed from its predecessor by only a few nucleotides¹⁰⁰² including one that caused the loss of the HA glycosylation site,^{415,1002} thereby exposing the multibasic HA cleavage site to ubiquitous cellular proteases, furin, and PC6.¹⁰⁰²

Mexican Outbreak. In May 1994, a mildly pathogenic H5N2 virus was isolated from Mexican chickens (A/chicken/Mexico/26654-1374/94). This virus was not eradicated by mass slaughter because it had already spread widely. Stepwise accumulation of mutations over several months yielded moderately (A/chicken/Puebla/8624-602/94) and highly (A/chicken/Queretaro/14588-19/95) pathogenic strains with

a series of basic residues at the HA cleavage site.^{602,836,2011,2013} Vaccination was implemented in 1995 and by 2001, more than 1 billion doses of inactivated vaccine had been used. Between 1998 and 2001, 459 million doses of recombinant fowl pox-vector vaccine were also administered. Cases of HPAI have not occurred since 1996; however, low pathogenic H5N2 strains continue to circulate, as do genetically related viruses in the neighboring countries of Guatemala and El Salvador. In 2005, an H5N2 virus with low pathogenicity emerged in chickens in Japan. This virus is a descendant of the virus responsible for the Mexican outbreak and is closely related to a virus isolated from Guatemala. The origin of the Japanese strain remains unknown.

H7N1

In March 1999, an LPAI virus of the H7N1 subtype was isolated from a poultry farm in Italy.^{226,227,229} The virus was not eradicated and the infection spread, resulting, in December, in the emergence of a highly pathogenic isolate. More than 13 million birds were destroyed to control the outbreak. The reappearance of this LPAI in August 2000 was controlled by additional depopulation followed by a vaccination campaign from November 2000 to May 2002. The vaccine was based on inactivated H7N3 virus to allow differentiation of infected and vaccinated animals (DIVA).²³¹ Vaccination in combination with intensive monitoring led to the eradication of this H7N1 virus.

H7N3

An outbreak of HPAI H7N3 viruses occurred in poultry farms in Pakistan in 1995. This outbreak killed 3.2 million birds and was brought under control by vaccination.¹⁴⁶² In 2001, H7N3 viruses of both high and low pathogenicity were again identified, and despite further vaccination, HPAI H7N3 viruses reappeared in 2003 and 2004, causing the death of an estimated 10 million birds. Outbreaks of LPAI and HPAI H7N3 viruses also occurred in Chile in 2002¹⁷⁵⁰ and Canada in 2004,^{122,161,815,1613} leading to the slaughter of 2 million and 19 million birds, respectively. In Canada, two workers involved in the depopulation developed symptoms of influenza virus infection, including conjunctivitis, headache, and coryza.^{815,2111} H7N3 viruses isolated from these individuals had increased binding affinity for human-type receptors (as did other recent North American H7 viruses tested in this study).⁹⁹ Both the Chilean and 2004 Canadian isolates arose from LPAI viruses by recombination events that inserted 10 amino acids from the NP protein¹⁹⁸⁹ (Chilean virus) or 7 amino acids from the M1 protein^{161,815,1614} (Canadian virus) into the HA cleavage site.

Again in Canada, an HPAI H7N3 virus caused another outbreak in 2007 among roosters in a broiler hatching egg farm. After hundreds of roosters in one of the barns died, all animals on the premise (53,000) were culled.^{122,1613} The source of the outbreaks is not known, although serologic data indicate that a low pathogenic H7N3 had circulated in some birds on the premise prior to the outbreak. Phylogenetic analysis indicates that the HPAI H7N3 virus was similar to North American H7 viruses isolated from waterfowl.

H7N7

In 2003, an HPAI of the H7N7 subtype caused outbreaks in layer farms in The Netherlands, resulting in the death or culling of more than 30 million birds.¹⁹⁵⁵ Experimental infection of chickens confirmed the highly pathogenic phenotype of the virus.^{486,487,2141} The outbreak spread to Belgium and Germany

but was brought under control by mass slaughtering. In The Netherlands, the outbreak was associated with one fatal human case, a veterinarian who contracted the disease, and with conjunctivitis in 78 individuals who either directly handled affected poultry or had family members who did.^{546,1085} These data suggest human-to-human transmission, and experimental infection of cats demonstrated that the virus isolated from the fatal case caused alveolar damage with infection of type II pneumocytes and nonciliated bronchial cells, comparable to infection with an HPAI H5N1 virus.²¹⁴⁰ The virus isolated from the fatal case differed by 14 amino acids from a virus isolated from an individual with conjunctivitis; the acquisition of PB2-627K (a known determinant of pathogenicity in mammals; see Molecular Determinants of Host Range Restriction and Pathogenesis section) was critical for increased pathogenicity and tissue tropism.¹⁴⁴³ Depopulation of infected poultry and the treatment of at-risk individuals with NA inhibitors likely prevented further spread to or among humans.

Further outbreaks of H7N7 HPAI occurred in the United Kingdom in 2008 and in Spain in 2009. In the United Kingdom in 2008, mild disease was first noted in a laying flock of chickens, followed by the death of approximately 10,000 birds (http://www.oie.int/wahid-prod/public.php?page=weekly_report_index&admin=0). The remaining 15,000 chickens in the flock were culled. In Spain in 2009, an outbreak of H7N7 HPAI in a layer hen farm resulted in the death of approximately 30,000 birds, while the remaining 278,640 birds on the farm were culled (http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=8521).⁸⁶¹

Despite the typically mild symptoms of human infection with H7 viruses, these viruses cause systemic infections in experimentally infected mice and ferrets without prior adaptation.^{100,405}

Low Pathogenic H9N2 Avian Influenza Viruses

LPAI viruses of the H9N2 subtype became panzootic in the mid-1980s among chickens, ducks, turkeys, pheasants, quail, and ostriches in Europe, Africa, North America, Asia, and the Middle East.^{14,15,61,224,1200,1636,1864} Since the mid-1990s, several sublineages have become established in Asia, represented by the prototype viruses A/chicken/Beijing/1/94, A/duck/Hong Kong/Y280/97, and A/quail/Hong Kong/G1/97.^{307,679,1206} These viruses appear to evolve rapidly through reassortment and antigenic drift.^{846,873,1034,1174,1182,2005,2277,2285,2358} A recent computational analysis of available H9N2 sequences revealed multiple different genotypes that likely originated from frequent reassortment with viruses of various subtypes.⁴³⁷

H9N2 viruses were detected in approximately 5% of birds in live poultry markets in Hong Kong in 1997. These viruses were not highly pathogenic but caused respiratory symptoms and decreased egg production. Between 2001 and 2003, H9N2 viruses were the most prevalent subtype in live poultry markets in Hong Kong, presenting in various different genotypes.³⁰⁷ Viruses of the A/quail/Hong Kong/G1/97 lineage share six internal genes with the H5N1 viruses isolated from humans in Hong Kong in 1997, and reassortants between H9N2 and H5N1 viruses have been described.^{771,873,2285,2358} On several occasions, H9N2 viruses of the A/duck/Hong Kong/Y280/97 lineage have been isolated from pigs in China,^{342,1624,2332} and seem to reassort frequently in these animals.²³³⁷ In an experimental study, multiple reassortants were generated between

avian H9N2 and A(H1N1)pdm09 viruses, some of which were more pathogenic in mice than the parental viruses,²⁰⁰⁶ as has been observed for reassortants between HPAI H5N1 and seasonal H3N2 viruses.¹¹⁹⁸

In animal experiments, H9N2 viruses replicate in chickens and mice without adaptation^{307,2277}; subsets of these viruses cause acute respiratory distress syndrome with high lethality in mice.^{307,412}

Some H9N2 viruses bind to Sia α 2,3Gal (preferentially recognized by most avian viruses) and also to Sia α 2,6Gal (preferentially recognized by human viruses).^{1335,2332} This is consistent with the finding that a virus possessing avian H9N2-derived HA and NA genes in the genetic background of a human H3N2 virus requires little adaptation for transmission among ferrets.¹⁹²⁶

Continued circulation of H9N2 viruses in poultry populations, combined with frequent reassortment, occasional transmission to pigs, and recognition of human-type receptors, emphasize the pandemic potential of the currently circulating H9N2 viruses.

Vaccines for Avian Influenza Viruses

The increasing number of outbreaks caused by HPAI H5 and H7 viruses, the fact that H5 and H9 viruses are now enzootic in poultry populations in parts of the world, and the increasing number of human infections with H5, H7, and H9 viruses have spurred the development of avian vaccines for these viruses, and the vaccination of poultry flocks. Most approved vaccines for H5, H7, and H9 viruses are based on inactivated whole-virus preparations, although some live recombinant vaccines based on Newcastle disease and fowl pox virus are in use (reviewed in^{230,277,432,559,1072,1763}; for an overview of currently available avian influenza vaccines, see <ftp://ftp.fao.org/docrep/fao/011/ai326e/ai326e00.pdf>).

Official vaccination programs against H5N1 viruses have been carried out in Hong Kong, Indonesia, China, Vietnam, Russia, India, Pakistan, Egypt, and Côte d'Ivoire, but failed to eradicate H5N1 viruses in some of these countries. Possible reasons include limited coverage of vaccination campaigns, failure to induce sterilizing immunity that may result in undetected virus spread and evolution, and limited cross-reactivity with viruses of different clades.

Influenza in Swine

Since the isolation of the first influenza virus from pigs in 1930 (A/swine/Iowa/15/30 [H1N1]),¹⁸⁶³ swine influenza has become enzootic and is a prevalent respiratory disease in these animals. Avian and human viruses of several subtypes (or reassortants thereof) have caused local outbreaks or become enzootic in pigs (Table 41.2). Until 2009, human influenza viruses contributed to the establishment of several new lineages in pigs (Table 41.2), whereas swine influenza viruses only infected humans occasionally, and without sustained human-to-human transmission (reviewed in¹⁴⁵⁹; see also^{78,1081,2055,2163}). This situation changed dramatically with the H1N1 pandemic in 2009, which originated from pigs.^{388,604,1903}

Collectively, the prominent role for pigs in the emergence of pandemic influenza viruses is supported by several findings: (1) pigs can be naturally (Table 41.2) or experimentally^{306,876,1021,1869,2180} infected with avian or human viruses; (2) swine influenza viruses can play a critical role in the emergence

of new pandemic viruses,^{388,556,604,1903} although most (reassortant) swine viruses cause self-limiting infections in humans (see previous discussion), which, however, can be fatal^{315,607,1042,1618,1758,1906,2235,2236}; (3) epithelial cells in pig trachea contain both human- and avian-type receptors (i.e., Sia α 2,6Gal- and Sia α 2,3Gal, respectively)⁸⁸⁰; (4) frequent reassortment (both intra- and intersubtypic) has been described for viruses isolated from pigs, suggesting repeated mixed infections of swine, avian, and/or human viruses; and (5) in nature, continued replication of an avian virus in pigs leads to variants that preferentially recognize human-type receptors.⁸⁸⁰ These observations support the “mixing vessel” hypothesis that pigs are simultaneously infected with avian and human influenza viruses, which allow for the generation of reassortants capable of causing pandemics.¹⁸¹⁰

In pigs, influenza virus infections cause high morbidity, but typically low mortality. Signs of infection include inactivity, nasal discharge, coughing, fever, labored breathing, weight loss, and conjunctivitis. Infections are limited to the respiratory tract with tracheobronchial lesions. During the Spanish influenza of 1918/1919,¹⁰⁷⁹ pigs presented with symptoms similar to those observed in humans.¹⁸⁶³ Phylogenetic analyses indicate that the 1918/1919 human and swine viruses were genetically similar and likely originated from a common ancestor.^{647,1717}

Influenza in Swine—North America

The descendants of the H1N1 1918/1919 isolates, now referred to as “classic swine viruses,” circulated in pig populations for more than 6 decades.^{181,1285,1532,1572,1841,2161} However, serologic studies in 1988 and 1989 also indicated a low prevalence of H3N2 viruses.²⁶⁰ In North America, this changed in 1997 and 1998, when H3N2 double human/swine reassortant and triple human/avian/swine reassortant viruses emerged²³⁷³ (Fig. 41.9). The double reassortant virus possessed human-like HA, NA, and PB1 genes, while the remaining genes were of classic swine virus origin. This virus did not establish a stable lineage but was soon replaced by human/avian/swine triple reassortant viruses that contain HA, NA, and PB1 polymerase genes of human virus origin; PB2 and PA polymerase genes of avian virus origin; and NP, matrix (M), and nonstructural (NS) genes of classic H1N1 swine virus origin.^{972,1570,2209,2373} These triple reassortants have spread widely throughout the swine population,^{972,1570,2209,2373} and have been linked to respiratory disease in pigs and abortion in pregnant sows. The major difference between the double and triple reassortant viruses was the presence of avian-origin PB2 and PA genes, suggesting that these genes may have contributed to the genetic stability of the novel reassortant virus; however, no mechanistic explanation is currently available. Although the biologic significance remains unknown, it is interesting that the HA and PB1 polymerase genes share the same origin, because this occurred with the 1957 and 1968 human pandemic viruses.¹⁰⁰¹ The triple reassortant H3N2 viruses evolved into four HA clades and continue to circulate in North American pig populations along with several reassortants that carry the same internal genes (PB2, PB1, PA, NP, M, NS), but different HA and NA genes (Table 41.2). These reassortants include several different H3N2 viruses possessing human-like HA genes^{1729,2208,2209}; an H1N1 virus possessing classic swine HA and NA genes²²⁰⁸ (cH1N1); an H1N2 virus possessing a classic swine HA gene (cH1N2)^{305,970,971}; an H1N2 virus possessing a human-like HA gene⁹⁶⁹ (huH1N2); and an H1N1 virus possessing human-like

TABLE 41.2 Overview of Major Swine Virus Lineages

Geographic distribution	Subtype	Designation	Date	Currently circulating	Origin of HA	Origin of NA	Origin of internal genes	Comments
North America	H1N1	Classic swine	1918	Yes	Classic swine	Classic swine	Classic swine	Descendants of 1918 pandemic virus PB1: human; PB2, PA: avian; NP, M, NS: classic swine (triple reassortant internal genes = TRIG) Reassortants of classic swine H1N1 × triple reassortant H3N2 viruses Reassortants of classic swine H1N1 × triple reassortant H3N2 viruses Reassortants of human H1N1 × TRIG virus Reassortants of human H1N1 × TRIG virus
	H3N2	Triple reassortant H3N2	1997/1998	Yes	Human H3	Human N2	Avian, human, swine (TRIG)	
	H1N1	cH1N1	1999	Yes	Classic swine	Classic swine	TRIG	
	H1N2	cH1N2	1999	Yes	Classic swine	Human N2	TRIG	
	H1N2	huH1N2	2003–2005	Yes	Human H1	Human N2	TRIG	
Europe	H1N1	huH1N1	2003–2005	Yes	Human H1	Human N1	TRIG	Reassortants of human H1N1 × TRIG virus
	H1N1	Classic swine	1976	No	Classic swine	Classic swine	Classic swine	Descendants of 1918 pandemic virus Wholly avian virus
	H1N1	Eurasian avian-like swine H1N1	1979	Yes	Avian	Avian	Avian	
	H1N1	Reassortant swine H1N1	2001, 2006	Unknown	Human	Avian	Avian	Reassortants of Eurasian avian-like swine H1N1 × human-like swine H3N2
	H3N2	Human-like swine H3N2	1970s	No	Human	Human	Human	Wholly human viruses
	H3N2	Eurasian reassortant human-like swine H3N2	1984	Yes	Human	Human	Avian	Reassortants of human-like swine H3N2 × Eurasian avian-like swine H1N1
Asia	H1N2	Reassortant human-like swine H1N2	1994	Yes	Human	Human	Avian	Reassortants of Eurasian reassortant human-like swine H3N2 × human-like swine H1N1
	H1N2	Reassortant human-like swine H1N2	2000	Yes	Human	Human	Avian	Reassortants of reassortant human-like swine H1N1 × Eurasian avian-like swine H1N1
	H3N2	Human-like swine H3N2	Early 1970s	Yes	Human	Human	Human	Wholly human viruses
	H1N1	Classic swine	Late 1970s	Yes	Classic swine	Classic swine	Classic swine	Descendants of 1918 pandemic virus
	H1N2	cH1N2	1978	Yes	Classic swine	Human N2	Classic swine	Reassortants of classic swine H1N1 × human H3N2
	H1N1	Eurasian avian-like swine H1N1	1993	Yes	Avian	Avian	Avian	Wholly avian virus
	H3N2	Double reassortant H3N2	1999	Unknown	Human	Human	Avian	PB2, PB1, PA, M, NS: avian; NP: swine Wholly avian virus
	H3N2	Triple reassortant H3N2	2001	Unknown	Human	Human	Avian, swine	
	H9N2		2002	Yes	Avian	Avian	Avian	

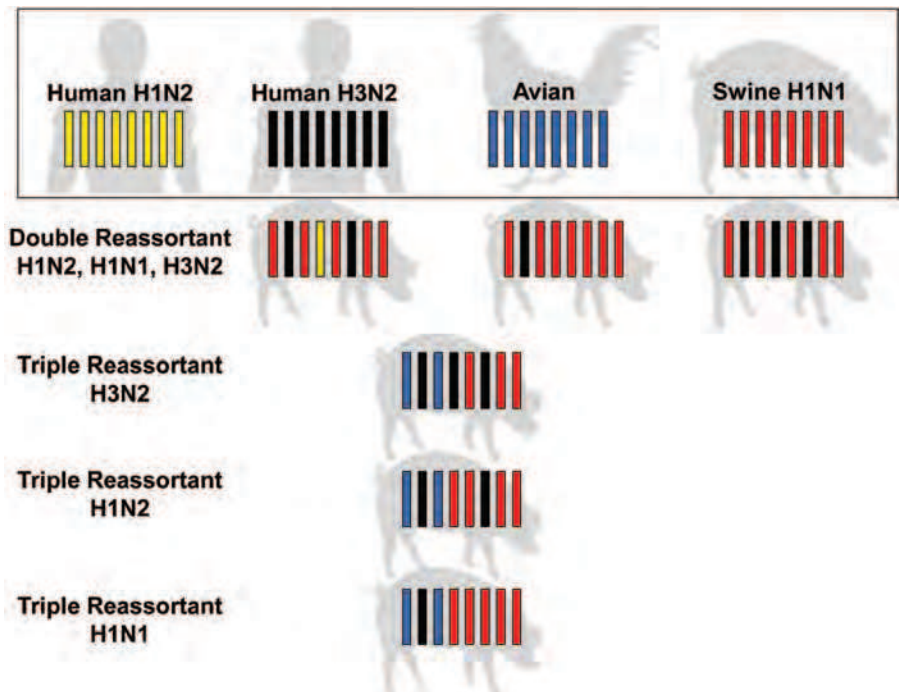


FIGURE 41.9. Recent reassortment events among North American swine viruses. In 1998, triple reassortant viruses emerged in North American pig populations that contained PB2 and PA genes of avian origin; PB1, HA, and NA genes of human origin; and NP, M, and NS genes that originated from classical H1N1 swine viruses. Subsequent reassortment events resulted in triple reassortant H1N2 and H1N1 viruses. In addition, human/swine reassortants of different genotypes have been isolated from North American pigs since 1998. The eight viral RNA segments are arranged from left to right according to their lengths, starting with the longest segment (i.e., PB2, PB1, PA, HA, NP, NA, M, and NS).

HA and NA genes²¹⁶² (huH1N1). Hence, while the HA and NA genes have been replaced frequently with genes of human or swine origin (further supporting the role of pigs as “mixing vessels”), the constellation of the internal genes (now referred to as TRIG—triple reassortant internal genes) has been stable. Moreover, experimental co-infection of pigs with classic H1N1 and triple reassortant H3N2 viruses resulted in the selection of the TRIG cassette in most viruses characterized.¹²⁹¹ The reason for the biologic fitness of the TRIG cassette in pigs is not known. In 2009, the introduction of NA and M genes of Eurasian avian-like swine virus origin into a triple reassortant swine virus created the A(H1N1)pdm09 virus. In addition, a reassortant between triple reassortant and seasonal H1N1 viruses infected three individuals in Canada in 2009, but did not spread further.⁷⁸

Several other wholly avian or reassortant viruses have been isolated from pigs in North America, but did not establish new lineages; these include avian viruses of the H1N1,⁹⁷³ H3N3,⁹⁷³ and H4N6⁹⁶⁸ subtypes, and reassortant viruses of the H1N1 and H1N2,⁹⁶⁹ H3N2,²³⁷⁴ H2N3,¹²⁹² and H3N1^{1185,1290} subtypes. The H4N6 virus isolated from pigs in Canada possessed two amino acid changes,⁹⁶⁸ one of which confers binding to Sia α 2,6Gal.⁷⁹ The H2N3 virus isolated from pigs in the United States was transmitted among experimentally infected pigs and ferrets, and its HA amino acid sequence suggests the ability to recognize mammalian-type receptors.¹²⁹²

Influenza in Swine—Europe

In Europe, three virus subtypes—H1N1, H3N2, and H1N2—are currently circulating in pig populations.^{1121,2137} Influenza viruses were first isolated from European pigs in 1938 and 1940.^{141,1139} Classic swine H1N1 viruses were detected in pigs in Czechoslovakia in 1950.⁴³⁵ These viruses were reintroduced in 1976 into pigs in Italy (Table 41.2), probably by pigs imported from the United States.¹⁴⁷⁶ In 1979, a wholly avian

H1N1 virus, closely related to a duck virus, was introduced into European pigs¹⁶³⁰ and replaced the classic H1N1 viruses in the 1990s.^{237,1274,1809,1824} These so-called Eurasian avian-like swine H1N1 viruses still circulate in European pig populations. New antigenic variants of this lineage have caused multiple outbreaks throughout European pig populations.^{183,396,1319} In 2001 and 2006, reassortant swine H1N1 viruses were isolated in France that arose from the reassortment of Eurasian avian-like swine H1N1 viruses with reassortant human-like swine H1N2 viruses (see later discussion¹¹¹⁶).

Human H3N2 viruses were introduced into pig populations in the early 1970s, where they established a new lineage.^{741,1670,1787} Multiple introductions of human H3N2 viruses into pigs occurred,^{51,237,1319,1388,1583,2067} and these so-called human-like swine H3N2 viruses circulated over the next decade. In 1984, severe disease among Italian and French pigs was traced to a novel reassortant H3N2 strain that possessed human-like HA and NA genes, while the internal genes were derived from Eurasian avian-like H1N1 swine viruses.^{220,238,1736,2101} The novel virus (referred to as Eurasian reassortant human-like swine H3N2 virus, Table 41.2) soon became enzootic and replaced the human-like swine H3N2 viruses. Viruses of this lineage continue to circulate to this day. Also, Eurasian reassortant human-like H3N2 swine viruses were transmitted to two children in The Netherlands,³²¹ and to a child in Hong Kong.⁶⁶⁸

Since the mid-1990s, H1N2 viruses have been isolated frequently that contain an HA gene similar to a human H1N1 virus introduced into pigs in 1977 (human-like swine H1N1, Table 41.2), and the remaining genes from Eurasian reassortant human-like swine H3N2 viruses.¹⁸⁴ These so-called reassortant human-like swine H1N2 viruses spread throughout European pig populations and established a new lineage.^{182,184,656,1306,1319,1817,2138} Since their emergence, the reassortant human-like swine H1N2 viruses have reassorted with avian-like swine H1N1 viruses or reassortant human-like swine H3N2 viruses.^{1319,2353}

Influenza in Swine—Asia

Some of the viruses detected in European and North American pig populations have also been isolated from swine in Asia. In the early 1970s, human H3N2 viruses were isolated from pig populations in Asia^{1113,1868} (Table 41.2), where they continue to circulate.^{1624,2332,2333} From the late 1970s onwards, human H3N2 viruses have co-circulated with classic swine H1N1 viruses in pig populations in several Southeast Asian countries.^{40,948,1867,1871,2301} During this time, multiple introductions of human H3N2 virus into Asian pig populations must have occurred, since the human H3N2 viruses detected in pigs typically resembled human epidemic viruses circulating at the time.^{652,956,989,1403,1485,1705,1866,1871,1872} Influenza H1N2 viruses (cH1N2) that resulted from reassortment of classic H1N1 swine viruses and human H3N2 viruses were first isolated in Japan in 1978^{1487,1994} and have since spread in the Japanese swine population.^{39,885,1484,1486,1584,1778,2316} H1N2 viruses of the same and different genotypes also circulate in other parts of Asia, including Taiwan, China, and Korea.^{932,946,948,1689,2096} Eurasian avian-like H1N1 viruses were first detected in Asian pigs in 1993⁶⁸⁰ and may be enzootic in pig populations in parts of Asia.^{1245,2027} A recent study also demonstrated the circulation of double and triple reassortant H3N2 viruses in pigs in China.²³³³ These viruses possess human-like HA and NA genes in combination with avian-like internal genes (double reassortants), while the NP gene of the triple reassortant viruses is of swine virus origin.²³³³ The gene constellation of these viruses is thus different from that of the North American double and triple reassortant swine viruses.

In 1998, avian H9N2 viruses were first detected in swine in Hong Kong but did not establish a stable lineage.¹⁶²⁴ However, different H9N2 viruses may now be circulating in pigs in Asia.^{342,2282,2332,2337} (Table 41.2). Based on their HA sequences, some of the swine H9N2 viruses may bind to human-type receptors.²³³²

HPAI H5N1 viruses have been isolated from pigs in Asia on several occasions,^{306,1526,1846,2026} although the prevalence of HPAI H5N1 viruses in pigs appears to be low.³⁰⁶ Moreover, H9N2 viruses have been isolated from pigs that possess HPAI H5N1-like sequences, suggesting reassortment between H5N1 and H9N2 viruses.³⁴¹

In addition to the established lineages, human H1N1 viruses have been isolated occasionally from pigs in Japan⁹⁸⁸ and China.^{2335,2338} In Korea in 2006, a novel H3N1 virus was isolated from pigs¹⁸⁵⁰ that possessed an HA gene from a human-like H3N2 virus, an NA gene from a swine H1N1 virus, and internal genes from swine H1N2 viruses. The H3N1 virus likely circulated for some time, since it donated the PB2, PA, NP, and M segments to an H5N2 virus isolated from pigs in 2008.¹¹⁷⁶ Another H5N2 virus isolated in the same study was a wholly avian virus. In addition, numerous reassortant viruses have been isolated from pigs in Asia,^{2002,2027,2158,2283,2336} suggesting frequent reassortment between swine, avian, and human influenza viruses.

Interspecies Transmission

The first report of interspecies transmission involving pigs dates back to 1938, when Shope presented serologic evidence for the transmission of a human virus to pigs.¹⁸⁶² Further evidence for virus transmission between these two species came in 1976, when an H1N1 swine virus was isolated from a soldier

who had died of influenza at Fort Dix, New Jersey.^{607,642,819,1013} This virus was subsequently isolated from five other soldiers; serologic studies suggest that more than 500 personnel were infected.^{443,607,819} As stated earlier, more than 50 cases of human infection with swine influenza virus had occurred until 2009 (reviewed in^{78,1081,1459,2055,2163}), some of which resulted in fatal outcomes.^{315,607,1042,1618,1758,1906,2235,2236} Although the A(H1N1)pdm09 virus was not isolated from pigs prior to the 2009 pandemic, sequence and phylogenetic analyses strongly suggest that this virus originated from pigs.^{388,604,1903} Recently, A(H1N1)pdm09 viruses have been reintroduced into pigs in several countries (http://www.oie.int/wahis/public.php?page=weekly_report_index&admin=0),^{1615,1919,2158,2232} and have reassorted with swine viruses.^{1304,1949,2158} It remains to be seen if these viruses will establish novel virus lineages in pigs.

Several other reports indicate the sporadic transmission of swine viruses to avian species, or *vice versa*: classic swine H1N1^{1404,2259} or triple reassortant H3N2 viruses have been isolated from turkeys²³¹⁴; surveillance studies suggest infection of ducks with swine H1N1²⁰⁸ or H1N2 viruses¹⁵⁷¹; avian H3N2 viruses likely transmitted to pigs^{1022,2315}; and serologic evidence also suggests the infection of Chinese pigs with H4, H5, and H9 influenza viruses.¹⁵²⁹ Moreover, two equine H3N8 viruses were isolated from pigs in China.²⁰⁹⁸

Influenza in Horses

Two different subtypes of influenza A viruses are recognized in horses: H7N7, historically referred to as equine 1, and H3N8,²¹⁷² referred to as equine 2. Equine influenza is typically associated with fever, nasal discharge, dry hacking cough, loss of appetite, muscular soreness, and tracheobronchitis. The disease is usually more severe with equine 2 (i.e., H3N8) viruses and can include inflammation of the heart muscle. Secondary bacterial infections can be fatal.

The first equine influenza virus (A/equine/Prague/56 [H7N7]) was isolated in 1956 during a widespread pandemic of respiratory disease among horses in Eastern Europe. The last confirmed outbreak of this subtype occurred in 1979.²²¹³ Anecdotal reports of equine H7N7 outbreaks in Egypt⁸⁷⁵ and India,¹⁸⁸⁴ and the sporadic detection of H7-specific antibodies in reportedly unvaccinated horses, suggest that the viruses may have still circulated in small geographic pockets and/or in a subclinical form since their last isolation. In 1963, an equine influenza virus of the H3N8 subtype (A/equine/Miami/63 [H3N8]) was isolated in the United States²¹⁷² and caused a major epidemic. It was later found to have been introduced into North American horses via the importation of horses from Argentina. Another enzootic with an H3N8 virus occurred in China in 1989. This virus caused morbidity rates of up to 80% and mortality rates of up to 20%.⁷⁰² Based on sequence analysis, the virus was of avian origin and unrelated to the H3N8 virus already established in horse populations.^{699,2217} It remained confined to the Chinese horse population and was not isolated after the mid-1990s.⁷⁰⁰ Several studies suggested reassortment between H7N7 and H3N8 horse viruses while they were co-circulating,^{8,90,884,1450} and intrasubtypic reassortment among H3N8 horse viruses.¹⁴⁵⁰ Moreover, phylogenetic separation of the Eurasian and American lineages has been observed in the equine H3 HA proteins since 1987; within the American lineage, three clades (original strains, Florida clade 1, Florida clade 2) are currently recognized (reviewed in³⁷¹).

An H3N8 virus isolated from a horse did not replicate in ducks but caused disease in experimentally infected mice and ferrets.⁶⁹⁹ More importantly, an equine H3N8 horse virus transmitted to dogs in North America in 2004,³⁵⁹ and has been circulating in domestic dogs since then. Inactivated vaccines against the H7N7 and H3N8 subtypes are widely used; however, with H7N7 viruses seemingly extinct in horses, the H7N7 vaccine component is no longer critical. Studies suggested that immunity conferred by inactivated vaccines is short lived.¹⁴³⁷ Therefore, between 1978 and 1981, and again in 1989, widespread epidemics of H3N8 viruses occurred in Europe and North America, affecting both unvaccinated and vaccinated horses. Outbreaks of equine H3N8 continue to occur throughout the world, probably supported by the international transportation of vaccinated horses, in which virus replicates in a subclinical form. For example, international transportation of vaccinated horses likely led to the introduction of equine influenza into Australia in 2007.²¹⁵ A cold-adapted, temperature-sensitive, modified live equine influenza virus vaccine has been licensed for use in the United States. Although this vaccine is safe and efficacious, it does not provide sterilizing immunity.^{261,1284,2082,2328} In addition, a recombinant canarypox virus vaccine expressing H3 HA is now licensed in several countries.⁴⁷⁰

Influenza in Dogs and Cats

Two reports demonstrated the natural infection of dogs with human influenza viruses.^{269,1753} However, these viruses did not establish stable lineages in dogs, in line with surveillance studies that show low levels of seropositivity to influenza viruses in dogs.^{459,1195,1651}

In 2004, outbreaks of respiratory disease occurred in racing greyhounds in Florida.³⁵⁹ The causative agent was an influenza A virus (A/canine/Florida/43/04 [H3N8]) closely related to contemporary H3N8 equine viruses, suggesting interspecies transmission. The virus spread among greyhound populations, indicating that the equine influenza viruses replicated in and transmitted among greyhounds. Serologic studies indicate that the virus has spread to other breeds, although it has not spread widely within the general dog population. The canine H3N8 influenza virus appears to be largely confined to animal shelters in some large cities in the United States where the turnover of dogs and introduction of new susceptibles has allowed this apparently inefficiently spread virus to be maintained for the past 11 years.³⁵⁹ The H3N8 viruses circulating in horses and dogs in North America have become genetically distinguishable and no interspecies transmission from horses to dogs or back to horses has been reported.^{1734,2300} Infection of dogs with an equine H3N8 virus was also reported in Australia in 2007.¹⁰⁴⁶ A retrospective study suggested that an outbreak of respiratory disease in English foxhounds in the United Kingdom in 2002 was also caused by an equine H3N8 virus.³⁷⁰ In experimental studies, horses infected with the canine virus seroconverted but showed little signs of disease.^{2299,2300} The infection of dogs with equine influenza viruses may be facilitated by the presence on canine respiratory epithelium cells of Sia α 2,3Gal receptors, which are preferentially bound by equine influenza viruses.^{370,1449} Experimental infection of chickens, turkeys, and ducks with the canine virus did not cause infection.¹³⁶⁹

In 2007, three genetically similar avian H3N2 influenza viruses were isolated in Korea from domestic dogs with severe respiratory symptoms.¹⁹¹⁵ Experimental testing showed

that these viruses cause tracheobronchitis and bronchiolitis in dogs,^{947,1916} and are transmitted to contact animals.¹⁹¹⁶ Surveillance studies demonstrated that avian H3N2 viruses circulate at low but detectable levels in dog populations in Korea^{31,1169} and China.¹²⁰⁹

Recently, domestic cats and zoo tigers died after eating poultry infected with HPAI H5N1 viruses,^{28,500,1005} and probable virus transmission was reported among tigers.²⁰⁵⁸ Several other reports now also indicate the infection of dogs^{206,325,624,1923} and cats^{1064,1104,1105,1733,1922,2120} with HPAI H5N1 viruses. Cats and dogs can also be infected with HPAI H5N1 viruses in experimental settings.^{624,1105,1733}

Influenza in Seals and Whales

The most significant epizootic in seals occurred in 1979 and 1980, when approximately 20% of the harbor seal (*Phoca vitulina*) population of the northeast coast of the United States died of a severe respiratory infection with consolidation of the lungs typical of primary viral pneumonia.⁶¹³ Antigenic and genetic analyses^{1143,2219} revealed that the influenza virus isolated from the lungs and brains of dead animals (A/seal/Massachusetts/1/80 [H7N7]) was of avian origin. This virus replicated to high titers in ferrets, cats, and pigs,²²¹⁹ and caused conjunctivitis in humans.^{2216,2219} The death of an experimentally infected squirrel monkey¹⁴⁵⁴ and recovery of the virus from several of its organs demonstrated systemic spread of this virus. From June 1982 to March 1983, harbor seals along the New England coastline again died of viral pneumonia,⁸⁰⁴ which was caused by an avian H4N5 virus that was antigenically and genetically related to avian viruses and replicated in the intestinal tracts of ducks, a characteristic of avian influenza viruses. Serology and direct virus isolation revealed infection of seals with influenza A viruses of the H3N3²¹⁴ and H4N6 subtypes,²¹⁴ as well as influenza B virus.^{1550,1582} Further surveillance studies identified antibodies to influenza A^{375,391,1527,1549,1550,1981} and B viruses in several seal species.¹⁵⁵⁰

Influenza viruses of the H13N2 and H13N9 subtypes were isolated from the lungs and hilar nodes of a stranded pilot whale.⁸⁰³ It is not known whether the influenza virus infection caused or contributed to the stranding of this whale. In addition, H1N3 viruses have been isolated from lung and liver samples of whales from the South Pacific.¹²⁸⁸

Influenza in Mink

Mink are naturally susceptible to infection with human and avian influenza viruses.^{1063,1562} Avian influenza viruses of the H10N4 subtype killed mink in Sweden and spread to contact animals.^{118,494,1063} In 2006, respiratory problems were noticed among mink in mink ranches in Canada; subsequently, an influenza A virus was isolated from one animal with clinical signs. Further analysis identified the virus as a swine triple reassortant H3N2 virus.⁵⁷² Influenza viruses of human, swine, avian, and equine origin replicate in experimentally infected mink,^{118,494,495,1343} and transmission to cage mates has been observed for human and avian viruses,^{118,1343,1561,2292} as well as for equine and swine viruses.²²⁹²

Experimental Infections

Mice

Although mice are not naturally infected, they can be experimentally infected with influenza A or B viruses. Most human

influenza viruses cause indiscernible infection of the upper and lower respiratory tract and do not cause lethal disease without adaptation, although A(H1N1)pdm09 viruses are more pathogenic than other human viruses.^{101,890,1302} By contrast, most avian influenza viruses will replicate in respiratory organs (but not cause lethal infections) without prior adaptation (reviewed in¹⁵⁴²), most likely because of the presence of Sia α 2,3Gal receptors in murine respiratory tissues.^{856,1528} Notable exceptions include the recently isolated H5N1 viruses, which can cause lethal infections without prior adaptation.^{593,1269,2106} Most laboratory strains are susceptible to infection with influenza A viruses and have been used widely to study the pathogenesis of and the immune response to influenza viruses. Knock-out mice are useful tools for deciphering the molecular basis of virus–host interactions. However, most laboratory strains lack the Mx gene, which plays a role in the innate host defense,^{838,1941} and data obtained from laboratory strains may not therefore be strictly comparable to natural virus infections. Recent studies have identified appreciable differences in susceptibility between mouse strains.^{153,1938} The comparison of influenza virulence and pathogenicity in different mouse strains may lead to a better understanding of the contribution of host genetics to infections with influenza viruses.

Guinea Pigs

Influenza virus propagation in guinea pigs was first reported in 1938,²²⁶⁴ then in the 1970s.^{1648,2014} Recently, guinea pigs have been established as a transmission model for influenza viruses.^{1264–1266,1434,1830,2004} Human, swine, and avian influenza viruses can be isolated from the lungs and nasal turbinates of infected animals, suggesting efficient virus replication; however, signs of disease such as weight loss and increased temperature are largely missing,¹²⁶⁵ and no mortality was observed upon infection with HPAI H5N1 or pandemic 1918 virus.²¹³⁰ The nasal tract and trachea of guinea pigs possess both avian-type (Sia α 2,3Gal) and human-type (Sia α 2,6Gal) receptors, whereas the lungs contain predominately avian-type receptors.²⁰⁰⁴

Ferrets

Ferrets infected with influenza A or B viruses develop a febrile rhinitis. Upon infection with human influenza viruses, the lesions are usually confined to the nasal mucosa, but infection of the lower respiratory tract has been demonstrated. The pathologic changes of bronchitis and pneumonia resemble those seen in humans,¹⁹⁰⁴ which makes ferrets a valuable animal model to study various aspects of influenza virus infection. Unlike mice, ferrets can also be used to study the transmissibility of influenza viruses. The high susceptibility of ferrets to human influenza viruses may be due to the predominance of Sia α 2,6Gal receptors in their upper respiratory tract.¹¹⁸⁴

Nonhuman Primates

Influenza viruses infect various Old and New World primates, such as chimpanzees, gibbons, and baboons, as well as rhesus, squirrel, African green, and cynomolgus monkeys.¹⁴⁵⁸ Several species (e.g., gibbons, baboons) can be infected and develop illness.⁹³⁴ Illness has also been observed in other species (e.g., cynomolgus, rhesus) experimentally infected with influenza virus.⁶⁷¹ Several human viruses known to be virulent in humans also produce illness in squirrel monkeys,^{1455,1458} indicating that nonhuman primates may be a useful animal model for

the study of influenza virus infections. Nonhuman primates have been used to test the pathogenicity of (reassortant) 1918 viruses,^{76,186,319,1068} HPAI H5N1 viruses,^{76,186,279,292,319,1106,1731,2075} and A(H1N1)pdm09 virus.^{890,1772}

MOLECULAR DETERMINANTS OF HOST RANGE RESTRICTION AND PATHOGENESIS

Four influenza virus proteins—HA, PB2, NS1, and PB1-F2—are known determinants of host-range restriction and pathogenicity. Other viral proteins also participate in these events.

The HA Protein

The HA protein is responsible for virus attachment and the subsequent fusion of viral and cellular membranes. It is synthesized as a single polypeptide chain (HA0) that undergoes posttranslational cleavage by cellular proteases. This cleavage is essential for infectivity because it exposes the hydrophobic N-terminus of HA2, which mediates fusion between the viral envelope and the endosomal membrane.^{1060,1159,2241}

HA Cleavage

HA is a critical determinant of the pathogenicity of avian influenza viruses, with a clear link between HA cleavability and virulence.^{833,1059,1959,2227} The HA proteins of highly pathogenic H5 and H7 viruses contain multiple basic amino acids at the cleavage site (Table 41.3), which are recognized by ubiquitous proteases such as furin,¹⁹⁷³ PC6,⁸³⁵ mosaic serine protease large (MSPL)^{1025,1564} and transmembrane protease 13 (TMPRSS13).^{1025,1564} For this reason, these viruses can cause systemic infections in poultry. In cell culture, the HAs of these viruses do not need exogenous proteases to form plaques. In contrast, the HA proteins of avirulent avian and nonavian influenza A viruses, with the exception of H7N7 equine influenza viruses,⁹⁹⁸ contain a single arginine residue at the HA cleavage site^{154,155} and are cleaved in only a few organs. These viruses, therefore, produce localized infection of the respiratory and/or intestinal tract that is usually asymptomatic or mild. The tissue tropism of viruses is thus partly determined by the availability of host proteases to recognize and cleave the two types of amino acid sequences found at the HA cleavage site. The significance of HA cleavability for pathogenicity of avian influenza viruses is underscored by the finding that the acquisition of a highly cleavable HA can convert avirulent strains to virulent ones, as occurred in Pennsylvania in 1983 (H5N2),¹⁰⁰² in Mexico in 1994 (H5N2),⁶⁰² in Italy in 1999 (H7N1),⁶² in Chile in 2002 (H7N3),¹⁹⁸⁹ and in Canada in 2004 (H7N3)¹⁶¹⁴ (Table 41.3).

Two groups of proteases are responsible for HA cleavage. The first group recognizes a single arginine and cleaves all HAs. Members of this group include plasmin,¹¹⁶⁰ blood-clotting factor X-like proteases,⁶⁵⁴ tryptase Clara,¹⁰²⁶ mast-cell tryptase,²⁹³ ectopic anionic trypsin I,²⁰⁸¹ tryptase TC30,¹⁷⁸⁹ miniplasmin,¹⁰²⁴ HAT (human airway trypsin-like protease),¹⁵⁹ TMPRSS2 (transmembrane protease serine S1 member 2),¹⁵⁹ and bacterial proteases.^{1577,2043} *In ovo*, a protease similar to the blood-clotting factor Xa that is present in allantoic fluid cleaves HA, which explains why influenza viruses grow efficiently in eggs.⁶⁵⁴ Tryptase Clara is secreted from specialized respiratory

TABLE 41.3 Sequence at HA Cleavage Site Determines Pathogenicity

Virus isolate	Pathogenicity	Sequence at HA cleavage site	Reference
A/chicken/Pennsylvania/1/83 (H5N2)	Avirulent	P Q ----- K K K R/G	(1002)
A/chicken/Pennsylvania/1370/83 (H5N2)	Virulent	P Q ----- K K K R/G ^a	(1002)
A/chicken/Mexico/31381-7/94 (H5N2)	Avirulent	P Q ----- R E T R/G	(602,836)
A/chicken/Queretaro/14588-19/95 (H5N2)	Virulent	P Q ----- R K R K T R/G	(602,836)
A/turkey/Italy/99 (H7N1) consensus	Avirulent	P E I P K G ----- R/G	(62)
A/turkey/Italy/99 (H7N1) consensus	Virulent	P E I P K G ----- S R V R R/G	(62)
A/chicken/Chile/176822/02 (H7N3)	Avirulent	P E K P K ----- T R/G	(1989)
A/chicken/Chile/4957/02 (H7N3)	Virulent	P E K P K T C S P L S R C R K T R/G	(1989)
A/chicken/Chile/4322/02 (H7N3)	Virulent	P E K P K T C S P L S R C R E T R/G	(1989)
Isolate CN6/04	Avirulent	P E N P K ----- T R/G	(1614)
A/chicken/BC/CN12/04 (H7N3)	Virulent	P E N P K --- Q A Y Q K R M T R/G	(1614)
A/chicken/BC/NS1337-1/04 (H7N3)	Virulent	P E N P K --- Q A Y K K R M T R/G	(1614)
A/chicken/BC/NS-1319-2/04 (H7N3)	Virulent	P E N P K --- Q A Y H K R M T R/G	(1614)
A/chicken/BC/CN7-3/04 (H7N3)	Virulent	P E N P K --- Q A Y R K R M T R/G	(1614)
A/chicken/BC/NS-1390-2/04 (H7N3)	Virulent	P E N P K --- Q A H Q K R M T R/G	(1614)
A/chicken/BC/NS-2035-12/04 (H7N3)	Virulent	P E N P K --- Q A C Q K R M T R/G	(1614)
A/goose/Guangdong/1/96 (H5N1)	Virulent	P Q R E ----- R R R K K R/G	(2290)
A/Hong Kong/156/97 (H5N1)	Virulent	P Q R E ----- T R R K K R/G	(322,1993)
A/Hong Kong/486/97 (H5N1)	Virulent	P Q R E ----- R R R K K R/G	(322,1993)
H5N1 HPAI ^b	Virulent	P Q R E ----- R R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P Q G E ----- R R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P Q R E ----- R R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P L R E ----- R R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P Q R E ----- G R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P Q R E ----- S R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P Q G E ----- R R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P L R E ----- R R R K K R/G	Influenza Sequence Databases
H5N1 HPAI	Virulent	P Q R E R E G G ---- R R R K R/G	Influenza Sequence Databases

/, HA cleavage site.

^aHA cleavability is enhanced by a single amino acid substitution that abrogates glycosylation near the HA cleavage site.

^bFor H5N1 HPAI viruses, there are numerous cleavage site motifs; several examples are listed here.

epithelial cells in rats and mice¹⁰²⁶; mast-cell tryptase is found in mast cells,²⁹³ whereas ectopic anionic trypsin I is present in stromal cells in the peribronchiolar region.²⁰⁸¹ The tissue tropism of tryptase TC30 is currently unknown. The type II transmembrane serine proteases HAT and TMPRSS2 localize to human airways and support influenza virus replication in cell culture.^{125,158,159} Miniplasmin is a trypsin-type serine protease in the epithelial cells of the bronchia that cleaves HA downstream of the consensus motif Gln(Glu)-X-Arg.^{1024,1447} Cleavage of HA by plasmin can be augmented by the ability of the A/WSN/33 (H1N1) NA protein to sequester its protease precursor, plasminogen.⁶⁵¹ Bacterial proteases can also activate HA, either directly or indirectly by activating plasminogen, a property that may explain the development of pneumonia after mixed infections with viruses and bacteria.²⁰⁴³

The second group of proteases that cleaves HA proteins^{834,1973} comprises the ubiquitous intracellular subtilisin-related endoproteases furin and PC6.^{835,1973,2178} These enzymes are calcium dependent, have an acidic pH optimum, and are located in the Golgi and/or *trans*-Golgi network.^{1058,1061,2179} Recently, two other ubiquitous type II membrane serine proteases (mosaic serine protease large form, MSPL, and transmembrane protease 13, TMPRSS13) were identified that do

not require calcium for enzymatic activity,¹⁰²⁵ and support the replication of an HPAI H5N1 virus in cell culture.¹⁵⁶⁴ The cleavage efficiency of these ubiquitous proteases is determined by the sequence at the cleavage site and absence or presence of a nearby carbohydrate chain on the HA molecule.^{415,640,831,832,834,1002,1003,1025,1426,1556,1577,2003,2154,2177} The proposed sequence required for HA cleavage is Q-R/K-X-R/K-R (X, nonbasic amino acid) in the absence of a nearby carbohydrate chain. The presence of a nearby carbohydrate chain requires insertion of two additional residues, Q-X-X-R-X-R/K-R, or alteration of the conserved glutamine at position -5 or the proline at position -6, B(X)-X(B)-R/K-X-R/K-R (B, basic residue). The presence of direct repeats of basic amino acid insertions of various lengths in the HA proteins of several H5 and H7 viruses suggests that these sequences arose from polymerase stuttering,¹⁶³¹ likely caused by secondary structure in the template RNA. HA cleavage efficiency can also be affected by the nature of the amino acid immediately downstream of the cleavage site, that is, the N-terminal amino acid of HA2.⁸³²

Introduction of multibasic HA cleavage sites into low-pathogenic avian H6N1¹⁴⁴⁵ or H9N2⁶³⁹ viruses created highly pathogenic variants. However, this finding is not universally applicable, as the introduction of multibasic HA cleavage sites

into low-pathogenic H5N1¹⁴⁹ or H3N8 viruses,¹⁹⁵¹ or into a human H3N2¹⁸¹⁸ virus did not create highly pathogenic viruses. Thus, a multibasic HA cleavage site seems to be necessary, but not always sufficient for high pathogenicity.

Sialic Acid Receptors

Influenza viruses bind to sialic acids, that is, negatively charged 9-carbon sugars that typically occupy the terminal positions of glycoproteins or glycolipids. The term sialic acid is now usually used for the most common member of the group, *N*-acetylneuraminic acid (NeuAc). Another sialic acid species that is not synthesized in humans, *N*-glycolylneuraminic acid (NeuGc), is also recognized by some influenza viruses; in fact, influenza viruses differ in their recognition of these two sialic acid species.⁷⁹⁸ Most sialic acids are linked to galactose (Gal) or *N*-acetylgalactosamine (GalNAc) by α 2,3- or α 2,6-linkages (Sia α 2,3/6Gal, Sia α 2,3/6GalNAc), or to *N*-acetylglucosamine (GlcNAc) by α 2,6-linkages (Sia α 2,6GlcNAc).^{1795,2147}

Sialic Acid Receptors in Humans

Early studies demonstrated that epithelial cells in human trachea contain Sia α 2,6Gal sialyloligosaccharides on their cell surface but lack those with α 2,3-linkage.³⁴⁵ Consequently, viruses with Sia α 2,6Gal specificity (i.e., human virus isolates, which preferentially bind to Sia α 2,6Gal), but not those with Sia α 2,3Gal specificity (i.e., avian virus isolates, which preferentially bind to Sia α 2,3Gal), bind to the epithelial cells lining the human trachea.³⁴⁵ Recent data obtained with *in vitro* differentiated human epithelial cells from tracheal/bronchial tissues suggest a more complex situation: Nonciliated epithelial cells express Sia α 2,6Gal oligosaccharides and are predominantly infected by human virus isolates, whereas ciliated cells contain Sia α 2,3Gal oligosaccharides and are preferentially infected by avian virus isolates.¹³³⁶ Additional studies of human respiratory tissue have shown that while Sia α 2,6Gal oligosaccharides are dominant on epithelial cells in nasal mucosa, paranasal sinuses, pharynx, trachea, and bronchi, Sia α 2,3Gal oligosaccharides are found on nonciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus, and on type II cells lining the alveolar wall^{85,1521,1854,2311} (Fig. 41.10).

Attachment and infection data for viruses of known receptor specificity are consistent with this distribution of Sia α 2,3Gal and Sia α 2,6Gal oligosaccharides.^{1854,2139} These findings offer an explanation for the severe pneumonia observed after infection of humans with some avian influenza viruses^{320,322,546,1993} and suggest that the limited human-to-human transmission of highly pathogenic avian H5N1 viruses likely reflects the restrictive replicative efficiency of these viruses in the upper portion of the respiratory tract, where transmission could occur via droplets generated by coughing and sneezing (Fig. 41.11). Conversion to preferential recognition of the human-type receptor thus is required for efficient human-to-human transmission, an assumption supported by the finding that the earliest isolates in the 1918, 1957, and 1968 pandemics preferentially recognized Sia α 2,6Gal, rather than Sia α 2,3Gal, sialyloligosaccharides.¹³³⁰ One study found higher amounts of Sia α 2,3Gal in the respiratory tract of children compared to adults,¹⁵²⁰ a finding that may suggest higher susceptibility of children to infection with avian influenza viruses. Another study reported predominant expression of Sia α 2,3Gal in epithelial cells of the human eye,¹⁵⁶⁷ which may explain the conjunctivitis associated with H7 influenza virus infections.

SIALIC ACID RECEPTORS IN OTHER MAMMALS

Epithelial cells in pig trachea contain both Sia α 2,3Gal and Sia α 2,6Gal,^{80,880,1478,1939,2136} which may explain why this species can be infected by both avian and human influenza viruses.¹⁰²¹ For mice, one study reported the expression of both types of receptors in the trachea, lung, and other organs,¹⁵²⁸ while another study did not detect human-type receptors in these animals.⁸⁵⁶ Ferrets express predominantly Sia α 2,6Gal on cells of the upper respiratory tract.^{85,1184} Equine viruses prefer Sia α 2,3Gal sialyloligosaccharides, which predominate in horse trachea.^{887,1449,2010} These studies also demonstrated that epithelial cells in horse trachea express NeuGc in addition to NeuAc, and that influenza viruses isolated from horses bind to oligosaccharides possessing NeuGc. Sia α 2,3Gal is also found on epithelial cells in the respiratory tract of dogs,^{370,1293,1449} and in the lungs of seals and whales.⁸⁸³ The nasal tract and trachea of guinea pigs possess avian-type (Sia α 2,3Gal) and human-type (Sia α 2,6Gal)

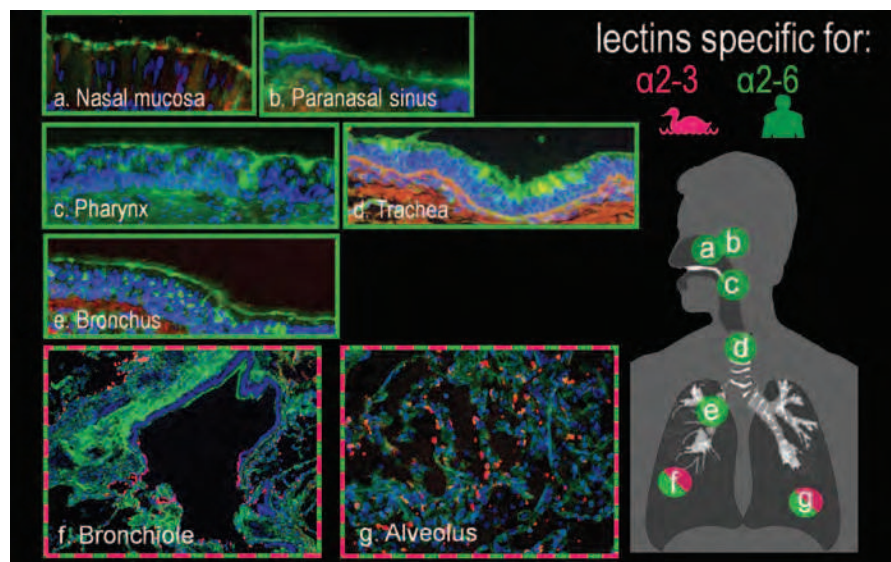


FIGURE 41.10. Expression of human virus (Sia α 2,6Gal) and avian virus (Sia α 2,3Gal) receptors in human respiratory tissue. The indicated tissues were tested with *Sambucus nigra* lectin (green), indicating the presence of sialic acid linked to galactose by an α 2,6-linkage (Sia α 2,6Gal), or with *Maackia amurensis* lectin (red), indicating the presence of Sia α 2,3Gal. Cells were counterstained with DAPI (4,6-diamidino-2-phenylindole; blue). In the nasal mucosa, paranasal sinuses, pharynx, trachea, and bronchus, Sia α 2,6Gal dominated. In the bronchiole and alveolus, both Sia α 2,6Gal and Sia α 2,3Gal were detected. (From Shinya K, Ebina M, Yamada S, et al. Avian flu: influenza virus receptors in the human airway. *Nature* 2006;440:435–436, with permission.)

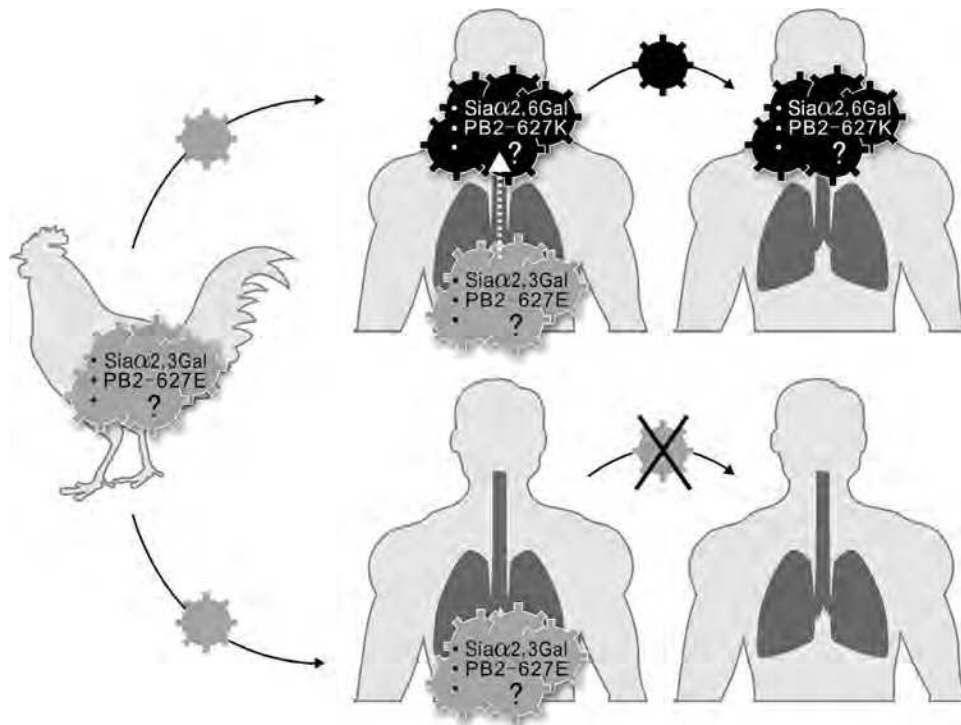


FIGURE 41.11. Model for the emergence of avian-derived influenza A viruses capable of being transmitted efficiently among humans. Avian influenza viruses preferentially bind to Sia α 2,3Gal oligosaccharides and contain Glu at position 627 of their PB2 protein (see The Replication Complex section). After transmission to humans, these viruses can infect epithelial cells in the lower respiratory tract that contain Sia α 2,3Gal oligosaccharides on their surface. However, due to their limited replication in mammalian cells (determined by PB2-627E) and their restriction to the lower respiratory tract, viruses do not efficiently spread among humans. The current working model predicts at least two changes for the generation of avian-derived viruses that are efficiently transmitted among humans: **(A)** mutations (e.g., PB2-627E to PB2-627K) that increase the viruses' replicative ability in mammalian cells and also provide a growth advantage at the lower temperature in the upper respiratory tract, and **(B)** changes in HA that allow the virus to bind to Sia α 2,6Gal oligosaccharides that are prevalent on epithelial cells of the upper respiratory tract. These two changes are likely required but not sufficient for the generation of a pandemic H5N1 virus. Additional, yet unknown, mutations may be required for efficient transmission among humans.

receptors, whereas the lungs contain predominately avian-type receptors.²⁰⁰⁴

SIALIC ACID RECEPTORS IN AVIAN SPECIES

The epithelial cells of duck intestine (where avian influenza viruses replicate) express predominantly Sia α 2,3Gal,⁸⁸² although small amounts of Sia α 2,6Gal have been detected in the intestinal and respiratory tract.^{578,580,586,1041,1103,1654,2339}

The duck intestinal tract also possesses NeuGc, which appears to be absent in chickens.⁸⁸⁸ In the respiratory and intestinal tract of chickens, Sia α 2,6Gal and Sia α 2,3Gal are expressed.^{578,580,586,695,1036,1103,1654,2339} Similarly, both Sia α 2,3Gal and Sia α 2,6Gal are expressed on tracheal and intestinal cells of quail, turkey, pheasant, and guinea fowl,^{580,695,1041,1654,2186,2339} that is, species that may play a role in the adaptation of avian influenza viruses to mammalian species (see Role of Terrestrial Poultry in the Emergence of New Influenza Viruses section). However, differences exist among poultry species in the relative abundances of influenza virus receptors in the different organs and cell types tested.^{578,586,695,1036,1041,1103,1654,2339} Substantial amounts of Sia α 2,6Gal were detected in the respiratory tract of pigeons,¹²⁵⁰ which are not known to play a significant role in influenza virus ecology.

Receptor Specificity of Influenza Viruses

The specificity of HA for the different sialyloligosaccharides is responsible for the host-range restriction of influenza virus. Human and classical H1N1 swine influenza viruses bind preferentially to Sia α 2,6Gal, whereas most avian and equine viruses have higher binding affinity for Sia α 2,3Gal.^{343,589,882,1330,1333,1533,1743,1744,1746,1889,1969}

Receptor specificity is determined by the amino acids that form the receptor-binding pocket. For H2 and H3 HAs, glutamine at position 226 and glycine at position 228 (found in avian viruses) determine the specificity for Sia α 2,3Gal oligosaccharides, whereas leucine and serine at these positions (found in human viruses) confer Sia α 2,6Gal specificity in H2 and H3 viruses.^{343,1330,1463,1745,2034,2164} (Fig. 41.12). For H1 viruses, aspartate at positions 190 and 225 (found in human viruses) confers binding to α 2,6, while aspartate and glycine at these positions (found in swine viruses) allow binding to both α 2,6 and α 2,3 linkages; glutamate and glycine at positions 190 and 225 (found in avian viruses) are responsible for the interaction with α 2,3-linked sialic acids.^{590,1070,1330,1333,1716,1937,1969,1972,2034,2107}

In addition to these key residues, the amino acids at several other positions also affect receptor-binding

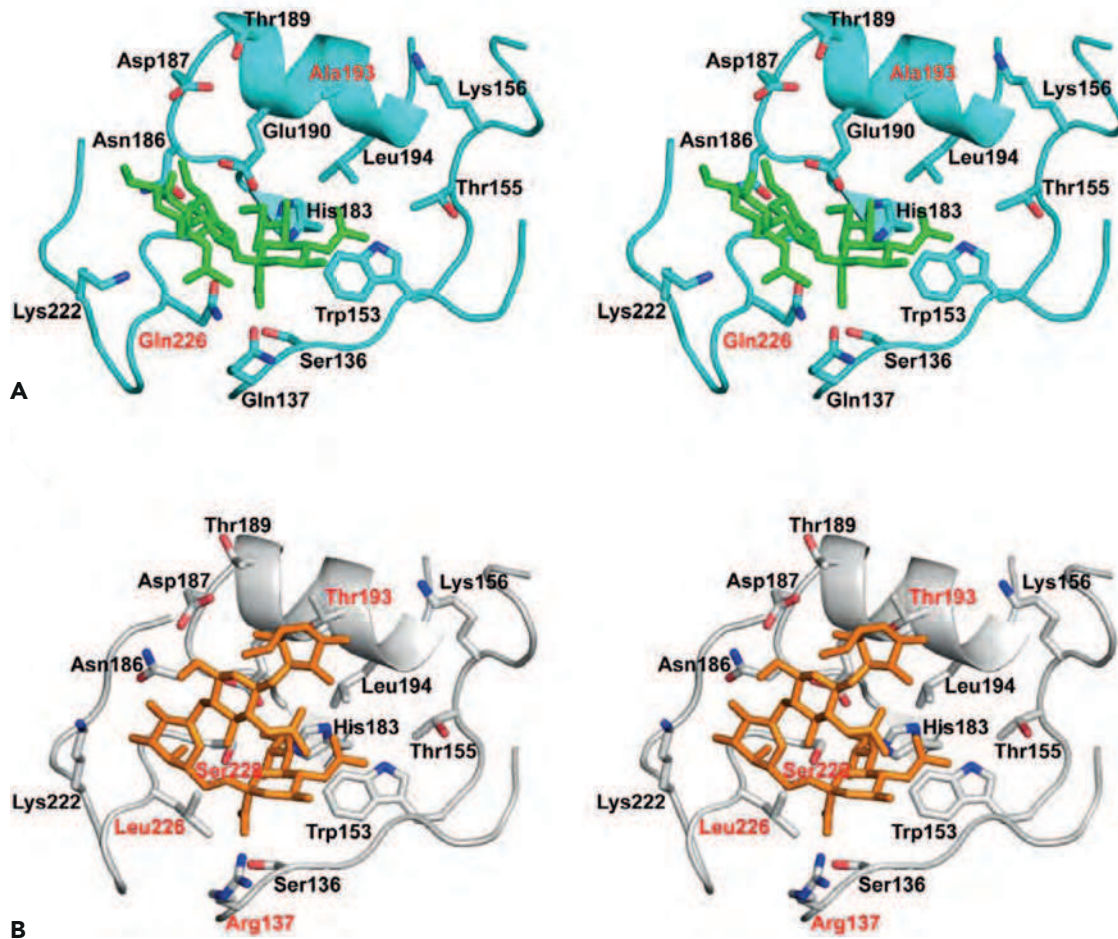


FIGURE 41.12. Structural models of HA–glycan receptor complexes. A: Interaction of avian H2 HA receptor-binding site (magenta) with avian-type receptor (green). **B:** Interaction of human H2 HA receptor-binding site (gray) with human-type receptor (orange). (From Viswanathan K, Koh X, Chandrasekaran A, et al. Determinants of glycan receptor specificity of H2N2 influenza A virus hemagglutinin. *PLoS One* 5:e13768, with permission.)

properties.^{1330,1333,1372,1380,1627,1743} X-ray crystallographic structures of HA proteins in complex with receptor analogs are available for human H1–H3 HAs,^{484,590,1246} avian H1–H3 and H5 HAs,^{590,708,709,1246} and swine H1 and H9 HAs,^{590,709} and provide detailed insights into the role of individual HA amino acids in receptor binding.

With the use of synthetic sialylglycopolymers,⁵⁸⁴ glycan arrays that allow the simultaneous testing of hundreds of carbohydrates and glycoproteins,¹⁹⁶⁹ and surface plasmon resonance assays,²⁰²⁹ a more complex picture of influenza viral receptor specificities has emerged. Binding of influenza viruses to their receptor is not only determined by the linkage between the sialic acid and the penultimate sugar residue (α 2,3 vs. α 2,6) but also by the nature of the penultimate sugar (Gal, GalNAc, or GlcNAc), the length of the carbohydrate chain, and the inner core of the carbohydrate (such as the linkage between the second and third sugar residue, modifications such as sulfation, or other sugar residues such as fucose).^{268,579–581,586–588,795,1112,1216,1332–1334,1768,1937,1969,1971,2306} Noteworthy differences exist between human and avian influenza viruses, and among avian viruses. For example, viruses

isolated from ducks, chickens, and gulls differ in their preference for the inner core structure of the receptor.⁵⁸¹ Avian influenza viruses favor NeuAc α 2,3 attached to shorter carbohydrate chains over the same sialic acid attached to a long chain; by contrast, seasonal human H1 and H3 influenza viruses bind to α 2,6 linkages preferentially in the context of long oligosaccharides^{795,1112,1969}; thus, avian virus HAs bind to their receptors in a narrow cone-like topology, whereas human virus HAs bind in a more flexible umbrella-like topology.^{268,1937} These differences in receptor-binding specificities can be correlated with specific differences in the HA sequence and/or structure.^{343,373,583,585,740,1330,1333,1533,1745,1968–1969,1970,2288}

Receptor Specificity of Avian Influenza Viruses

H5N1 viruses bind efficiently to Sia α 2,3Gal.^{579,1331} Mutations at position 190 or 225 that change the receptor-binding specificity of H1 HAs do not confer H5N1 virus binding to Sia α 2,6Gal; however, the introduction of human-type amino acids at positions 226 and 228 increases binding to Sia α 2,6Gal, while retaining substantial binding to Sia α 2,3Gal.^{746,1970}

H5N1 viruses isolated from infected individuals typically retain specificity for Sia α 2,3Gal.^{579,1331,1969,2297} However, several H5N1 viruses have been isolated that recognize Sia α 2,6Gal and Sia α 2,3Gal.^{48,579,1856,1968,2196,2297,2320} This change in binding properties can be linked to several specific amino acid changes in HA, most notably a serine-to-asparagine mutation at position 227, with or without a mutation at position 158 that results in the loss of a glycosylation site. Passage in ducks of a virus possessing asparagine at position 227 resulted in reversion to serine and reduced human-type receptor-binding specificity,¹⁸⁵⁷ suggesting that the human-adapting mutation is not stably maintained in ducks. One study tested all naturally or experimentally identified mutations that alter H5 receptor-binding specificity in the same genetic background and noted differences in their effect on binding to Sia α 2,6Gal.³¹⁴

Poultry H9N2 viruses isolated in China in 1999 possessed mutations at positions 190 and 226¹²²⁷ that conferred binding to Sia α 2,6Gal.^{1335,1776} These viruses, which also infected humans,^{1227,1626} demonstrated that variants with human-type receptor-binding properties can emerge in avian species. Similarly, several low pathogenic H7 viruses isolated from avian species and humans in the United States in 2002 to 2003 and in Canada in 2004 showed increased binding to Sia α 2,6Gal, although they retained their ability to bind to avian-type receptors.⁹⁹

Receptor Specificity of Human Influenza Viruses

The HA proteins of the pandemic 1918, 1957, and 1968 viruses originated from avian influenza viruses; nonetheless, the earliest available human isolates of these pandemic viruses already possessed Sia α 2,6Gal receptor-binding specificity,^{343,628,1330,1969} indicative of strong selective pressure. However, early pandemic H3N2 viruses differ from more recent isolates in their amino acid sequence and their binding affinity to nonciliated cells.^{1234,1329,1333,2061}

For the pandemic 1918 virus, two variants have been identified that differ at amino acids 190 and 225: A/South Carolina/1/18 (possessing aspartate at positions 190 and 225 as typically found in human influenza viruses) binds to Sia α 2,6Gal and transmits efficiently among ferrets, whereas A/New York/1/18 (possessing aspartate at position 190 but glycine at position 225) binds to both Sia α 2,6Gal and Sia α 2,3Gal and transmits with reduced efficiency compared to A/South Carolina/1/18.^{628,1937,1969,2107} Replacement of aspartate at positions 190 and 225 with the avian-like amino acids at these position—that is, glutamate and glycine—abolishes binding to Sia α 2,6Gal and transmission in ferrets.²¹⁰⁷ The X-ray crystallographic structure of a 1918 HA protein in complex with receptor analogs demonstrates how this overall avian-like protein interacts with human-type receptors.⁵⁹⁰

The A(H1N1)pdm09 viruses possess aspartate at positions 190 and 225 and bind efficiently to Sia α 2,6Gal,^{285,1302,2307} although one study reported dual recognition of both Sia α 2,6Gal and Sia α 2,3Gal.³⁰³ Molecular dynamics simulation of H1 proteins of the 1918 pandemic, a swine virus from 1930, a seasonal human H1N1 virus from 2005, and the A(H1N1)pdm09 virus showed that lysine at position 145 and glutamate at position 227 [found in the A(H1N1)pdm09 virus] increased the binding affinity to a human-type receptor, and that aspartate 225 increased the number of hydrogen-bonding interactions compared to glycine at this position.¹⁵⁴⁰ An aspartate-to-glycine

change at position 225 (H3 numbering; position 222 in H1 numbering) has been found in some A(H1N1)pdm09 viruses and appears to correlate with more severe disease outcomes in humans.^{34,282,448,637,863,1027,1168,1303,1389,1593,1671} Few transmission events have been reported for this variant,¹⁶⁸⁷ suggesting that it does not transmit efficiently among humans. The aspartate-to-glycine change at position 225 arises during passage in eggs or adaptation to mice and increases virulence *in vitro* and *in vivo*.^{868,1781,2286,2362} Experimental infection of pigs with a mixed population of viruses encoding glycine or glutamate at position 225 resulted in the selection of glutamate in viruses isolated from nasal secretions, but glycine in viruses isolated from the lower respiratory tract.¹⁷⁸

Glycan arrays in combination with X-ray crystallographic structures of pandemic 1957 H2 HAs highlight the significance of position 226 for H2 HA receptor-binding specificity.²²⁸⁸ This is further supported by the finding that A/El Salvador/2/57, possessing glutamine and glycine at positions 226 and 228, preferentially binds avian-type receptors and transmits poorly among ferrets, while A/Albany/6/58, possessing leucine and serine at position 226 and 228, recognizes human- and avian-type receptors and transmits efficiently in ferrets.¹⁶⁰⁹ Moreover, a mutational study demonstrated a critical role for the amino acids at positions 226 and 228 in converting an avian H2N2 virus into one that binds human-type receptors.²¹⁶⁶ The amino acids at position 137 and 193 also contributed to receptor-binding specificity.²¹⁶⁶

Glycosylation

Influenza virulence and host range are also affected by the number and location of oligosaccharide side chains, which are not conserved among strains or subtypes.^{859,1533} HAs typically contain 5 to 11 glycosylation sites that affect receptor-binding affinity and/or specificity,^{404,583,694,1216,1331,1555,2097,2189} antigenicity,^{3,52,379,1656,1777,2196,2200} innate immune responses,^{298,1709,2045} replication,^{59,2174} fusion activity,¹⁵⁵⁷ virulence,^{744,1342,1463,1632,1708,2156} and host range.¹⁷⁵² For efficient virus replication, a functional balance between HA and NA is critical.^{55,2175} Growth restrictions due to the lack of HA glycosylation site(s) can be partially overcome by amino acid substitutions in NA.^{55,2175}

Role of Terrestrial Poultry in the Emergence of New Influenza Viruses

Terrestrial poultry, such as chickens and quail, are susceptible to infection with influenza viruses. Most influenza viruses circulating in wild birds are typically asymptomatic in poultry, but their continued circulation among terrestrial poultry could lead to viruses with increased virulence in these birds.

Adaptation of waterfowl viruses in terrestrial poultry may lead to the emergence of viruses that are better able to replicate in humans or pigs than their original counterparts.^{580,695,1041,1654,2186,2339} due to altered receptor specificity.^{841,1331,1335,1925} Support for this hypothesis comes from the finding that viruses isolated from terrestrial poultry resemble human viruses in their low affinity for Sia α 2,3Gal compared to viruses isolated from wild birds¹³³¹ and that the receptor specificity of H9N2 viruses isolated from terrestrial poultry, but not that of viruses isolated from aquatic birds, is similar to the receptor specificity of human isolates.^{1335,1776} The H5N1 viruses isolated from terrestrial poultry are characterized by an additional glycosylation site in HA that reduces the affinity for the

receptor and, in some instances, by a deletion in the NA stalk that reduces NA functionality and balances the functions of these two proteins. The presence of additional glycosylation sites and a deletion in the NA stalk are typical for human viruses¹³³¹ and have also been found in different virus subtypes isolated from terrestrial poultry.^{55,62,116,145,146,221,622,1331,1355,1401,1776,1932} Collectively, these data suggest that waterfowl viruses acquire certain mutations in terrestrial poultry, such as NA stalk deletions and additional HA glycosylation sites that may facilitate their transmission to humans or other mammals such as pigs.

Host Cell-Mediated Selection of Antigenic Variants

In 1942, Burnet and Clarke²⁰³ first described “O” (original) and “D” (derived) variants that differed in their ability to agglutinate human or guinea pig erythrocytes after passage in eggs. Subsequent studies established that the antigenicity of influenza A and B viruses grown in embryonated chicken eggs differs from that of viruses propagated in cell culture, including chicken embryo fibroblasts.^{583,991,993,1335,1738,1800} For minor populations of egg isolates, however, the antigenic properties remained identical to those of cell culture isolates.¹⁵⁸⁹ The HA sequences of cell culture–grown but not egg-grown viruses are identical to those of the original isolates, demonstrating that HA variants arise during virus replication in eggs.^{202,296,582,583,585,589,605,992,1277,1278,1699,1739,1800,1971,2028} Moreover, human isolates tend to grow better in cell culture than in eggs,^{458,1415} further suggesting that human viruses undergo selection in eggs. Historically, human viruses were isolated by inoculation of samples into the amniotic cavity of embryonated eggs, followed by virus amplification in the allantoic cavity. Some recent human viruses do not grow in eggs when inoculated into the allantoic cavity. Mutations in the HA of egg-grown viruses cluster around the receptor-binding pocket, likely reflecting an adaptation to selective pressure in eggs. This selective pressure can be attributed to the fact that in addition to Sia α 2,3 sialyloligosaccharides, Sia α 2,6 sialyloligosaccharides exist on amniotic cells, whereas only Sia α 2,3 sialyloligosaccharides are found on allantoic cells.⁸⁸⁹

Currently, influenza vaccine viruses are propagated in embryonated chicken eggs. In animal models, however, vaccines prepared from cell culture–grown viruses induce better protective immunity than those grown in eggs,^{26,993,994,1394,1508,2261} a finding that was substantiated when these vaccines were tested in humans.^{27,1508} Influenza vaccine production in cell culture rather than eggs thus may be preferable.

The NS1 Protein

The NS1 protein functions as an IFN antagonist that allows efficient virus replication in IFN-competent hosts,^{599–600,601,1406,1978} in addition to roles in the splicing and nuclear export of viral mRNAs^{22,513,596} and the enhancement of viral mRNA translation.^{401,490} NS1 targets the production of type I IFN (IFN- α , IFN- β), and interferes with IFN-induced antiviral factors. These functions are described in the Innate Immune Responses section.

The Replication Complex

The influenza viral replication complex comprises the three polymerase proteins—PB2, PB1, and PA—and the nucleoprotein NP. The PB2 protein recognizes and binds to type I cap structures of cellular mRNAs. It has emerged as an impor-

tant determinant of virulence and host-range restriction. Early studies suggested that the PB2 segment,^{21,327,1991} in particular the amino acid at position 627,¹⁹⁹¹ was involved in host-range restriction.^{21,327,1991} The significance of this finding in the context of interspecies transmission was not recognized, however, until 2001, when a substitution at position 627 of PB2 from glutamic acid (found in most avian isolates) to lysine (found in all contemporary human isolates) was shown to enhance the pathogenicity of an HPAI H5N1 virus in mice.⁷⁵³

Multiple lines of evidence now suggest that PB2-627K is a major determinant of pathogenicity in mammals: (1) PB2-627K leads to increased virulence of HPAI H5N1 viruses^{593,753,1037,1783,1855}; (2) PB2-627K is selected during virus replication in humans¹¹⁶⁵; (3) PB2-627K was critical for the pathogenicity of an H7N7 virus isolated from a fatal infection in The Netherlands in 2003^{406,546,1443}; by contrast, viruses isolated from nonfatal cases and chickens contained glutamic acid; (4) HPAI H5N1 viruses with PB2-627K have been isolated from tigers (A/tiger/Suphanburi/Thailand/Ti-1/04 [H5N1])²⁸ and a cat in Thailand in 2004 (A/cat/Thailand/KU-02/04 [H5N1]); (5) viruses possessing PB2-627K were isolated after adaptation of H5N1, H7N7, and H9N2 viruses to mice,^{841,1203,1322,1860,2278} and after virus replication in pigs¹³¹²; (6) PB2-627K increases virus transmission in the guinea pig model¹⁹⁵²; (7) PB2-627K is selected during HPAI H5N1 virus replication in ostrich cells and ostriches¹⁸⁵⁸ and has been found in viruses isolated from turkeys, ostriches, emu, rhea, and quail, suggesting that PB2-627K selection can occur in some avian species, which may facilitate the adaptation of avian influenza viruses to mammals (see Role of Terrestrial Poultry in the Emergence of New Influenza Viruses section); and (8) PB2-627K increases replicative ability in mammalian cells,^{363,1324,1464,1855} particularly at the lower temperatures of the upper respiratory tract⁷⁵⁴ (Fig. 41.11). Collectively, these findings suggest that PB2-627K provides a replicative advantage in mammals and is hence selected in these species. However, PB2-627K also emerged in HPAI H5N1 viruses isolated from wild waterfowl at Qinghai Lake in 2005 (see The H5N1 Outbreak section)^{279,281,1247} and is maintained in descendants of the Qinghai Lake viruses to this day.

Considering the significance of PB2-627K for influenza virus replication in humans, the finding of PB2-627E (i.e., the avian-type amino acid) in A(H1N1)pdm09 viruses was unexpected. Two studies demonstrated that the lack of PB2-627K is compensated for by a basic amino acid at position 591.^{1373,2296} In fact, PB2-627K does not provide a replicative advantage in the background of A(H1N1)pdm09 viruses.^{785,907,2296,2377} Structural analyses demonstrated that positions 591 and 627 are in close proximity,^{1120,2041} and that a basic amino acid at position 591 alters both the shape and charges on the surface of the protein,²²⁹⁶ which may affect the interaction of PB2 with other host and/or viral factors.^{543,1374,1407} For example, one study suggested that the amino acid at PB2-627 affects the association of the polymerase complex with NP.^{1125,1701}

A second amino acid in PB2—at position 701—was first identified as a virulence factor of HPAI H5N1 viruses in mice,¹²¹² and is now known to facilitate viral adaptation to mammalian species. Replacement of aspartic acid (found in most avian influenza viruses) with asparagine at residue 701 enhances binding of PB2 to the cellular nuclear import factor importin α and facilitates PB2 nuclear import and replicative

ability in mammalian cells.^{566,568,569} This amino acid change was found upon adaptation of an avian H7N7 virus to mice⁵⁶⁷; it also proved to be critical for virus transmission in guinea pigs.^{595,1952} In addition, mouse-adapted human H3N2 viruses possessed the PB2-D701N mutation.¹⁶⁵⁵ Like PB2-627K, PB2-701N is selected during HPAI H5N1 virus replication in humans¹¹⁶⁵ and ostriches,¹⁸⁵⁸ but does not provide a replicative advantage to A(H1N1)pdm09 viruses.^{785,2296}

Replacement of the avian-type threonine residue at position 271 of PB2 with alanine (i.e., the residue typically found in human influenza viruses) increases virus replication in mammalian cells and mice.²⁰⁵ The PB2 protein of the A(H1N1)pdm09 virus (which is of avian-virus origin) encodes alanine at position 271, suggestive of human adaptation.

The PB1 protein possesses conserved motifs found in all RNA-dependent RNA polymerases¹³⁷ and is considered critical for the polymerase enzymatic function. In a minireplicon system, reporter gene expression was more efficient with avian than with human virus PB1 protein.¹⁴⁶⁴ Avian virus PB1 protein may hence provide a replicative advantage over its human counterpart, an attractive hypothesis since both the 1957 and 1968 pandemic strains contained avian PB1 genes (in combination with avian HA, or HA and NA genes^{1001,1813}). Moreover, in reassortment studies between HPAI H5N1 and human H3N2 viruses, one of the most pathogenic reassortants possessed the avian PB1 gene.²⁸⁴ Reassortment studies also demonstrated a critical role for the pandemic 1918 PB1 gene in virulence in animal models.^{1607,2206}

The PA protein is an integral part of the influenza viral replication complex with a role in the cap-snatching process.⁴¹⁸ Recent studies now also suggest a role for PA in the virulence of the A(H1N1)pdm09 virus^{1781,1921,2006} and in the adaptation of an avian H5N2 virus to mice.¹⁹²⁰

In addition to the polymerase genes, the NP gene may play a role in host range restriction.^{592,1810,1811,2066}

A large body of information demonstrates that the composition of the replication complex affects viral pathogenicity.^{284,905,1192,1198,1199,1300,1464,1548,1607,1819,2006,2019,2203,2206} As a general trend, more efficient replication in minireplicon assays translates into increased pathogenicity in animal models.

The PB1-F2 Protein

PB1-F2 is a short protein of 87 to 90 amino acids that was discovered in 2001.²⁹¹ It is expressed from the +1 reading frame of the PB1 gene of most avian and human influenza viruses; however, human H1N1 viruses isolated after 1950 encode a truncated version of 57 amino acids.^{291,2352} Most swine virus isolates (particularly classical H1N1 swine influenza viruses) do not encode a functional PB1-F2 peptide, due to several in-frame stop codons. These stop codons are also found in the reading frames of A(H1N1)pdm09 virus PB1-F2s, which likely originated from pigs; therefore, human pandemic viruses do not encode a functional PB1-F2 protein. Reconstitution of full-length PB1-F2 expression in the background of A(H1N1)pdm09 viruses had only minor effects on replicative ability and virulence in mice and ferrets,^{273,711,1592} suggesting that PB1-F2 is not critical for the pathogenicity of A(H1N1)pdm09 viruses. Phosphorylation of PB1-F2 may be critical for its function.¹⁴⁰² The role of PB1-F2 in viral pathogenicity is discussed in more detail in the Innate Immune Responses section.

The NA Protein

The sialidase activity of the NA protein removes sialic acid from sialyloligosaccharides, thereby serving two functions: (1) the removal of sialic acid from HA, NA, and the cell surface, facilitating virus release; and (2) the removal of sialic acid from the mucin layer, which likely allows the virus to reach the surface of the epithelial cells.^{1331,1602} The NA protein may also have a role in host-range restriction⁸⁰⁸ and pathogenicity.^{651,1607,1819} Notably, the NA activity of some avian viruses is more resistant to the low pH of the upper digestive tract than that of human- or swine-derived NA,^{2009,2025} a feature that may contribute to host-range restriction. Interestingly, low-pH stability has also been reported for the NA protein of the pandemic 1918 virus.²⁰²⁴ The NA protein of A/WSN/33 (H1N1) virus is critical for plaque formation in Madin-Darby bovine kidney cells and for neurovirulence^{1473,1821,1996}; these two phenotypes are linked to the loss of a carbohydrate chain on NA and the presence of a C-terminal lysine residue.¹²⁰⁸ The lack of the carbohydrate chain at position 146 of NA (N2 numbering) allows the NA protein to bind to and sequester plasminogen, a plasmin precursor. This function facilitates HA cleavage and, thereby, virus pathogenicity in mice.^{651,653,1208}

Multiple studies now indicate that the length of the NA stalk affects virulence and pathogenicity^{55,62,116,145,146,221,622,1331,1335,1342,1401,1439,1776,1869,1932,1988,2367}; deletions in the NA stalk have been observed after virus replication in eggs and in poultry, and among recent HPAI H5N1 viruses.

Like HA, the NA protein shows preference for certain types of sialyloligosaccharides according to the host species. Avian virus NAs cleave α 2,3-linked, but not α 2,6-linked, sialic acids. After their introduction into the human population, N2 NAs acquired the ability to cleave α 2,6-linked sialic acids in addition to α 2,3-linked sialic acids⁸⁴; however, the enzymatic activity for α 2,6-linked sialic acids is significantly lower than that for α 2,3-linked sialic acids. This acquired ability likely represents adaptation to the respective recognition pattern of the HA protein. NA substrate specificity is determined by the amino acid at position 275.¹⁰⁶⁹

CLINICAL FEATURES AND PATHOGENESIS IN HUMANS

Pattern of Virus Shedding

Human influenza viruses replicate almost exclusively in superficial cells of the respiratory tract. Influenza virus is released from the apical surface of the cell, which may limit more systemic spread but facilitate accumulation of virus in the lumen of the respiratory tract for transmission to the next susceptible host. Alveolar macrophages and dendritic cells can also be infected. Their role is in initiating the innate and cognate immune response to influenza virus by processing antigens and presenting them for recognition (see Innate Immune Cells and Cellular Immunity sections). Influenza virus replicates throughout the respiratory tract, with virus being recoverable from the upper and lower tracts of people naturally or experimentally infected with virus. The site of optimal growth in the respiratory tract for influenza viruses is, in part, determined by the prevalence of the Sia α -2,3Gal or Sia α -2,6Gal receptors (see the HA Protein section).

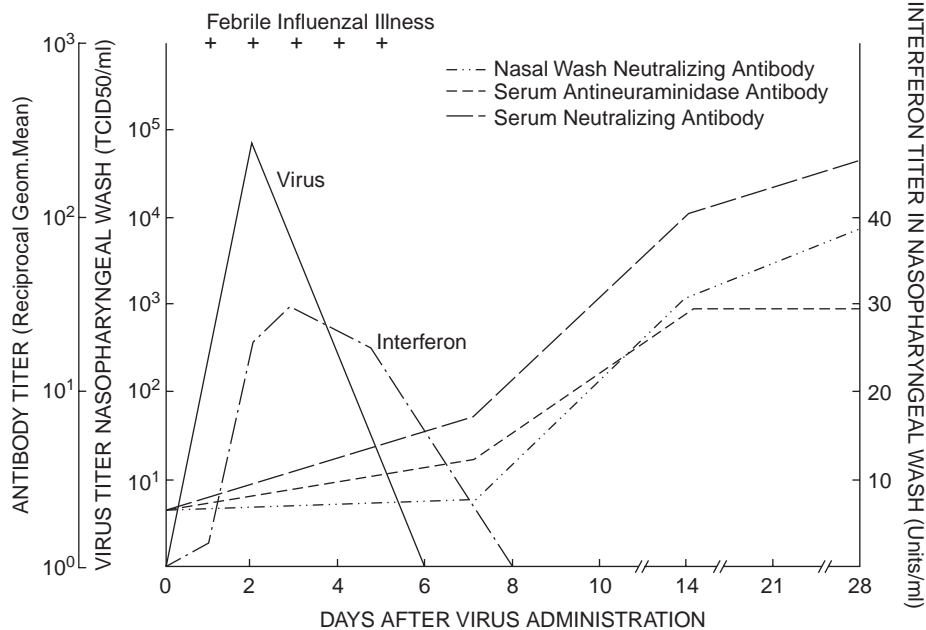


FIGURE 41.13. Six seronegative volunteers received $10^{4.0}$ TCID₅₀ of wild-type A/Bethesda/1015/68 virus intranasally on day 0.

The pattern of virus replication in six adult volunteers administered an influenza A/Hong Kong/68-like H3N2 virus, in relation to the onset of clinical symptoms, IFN response, and serum and nasal wash antibody responses, is presented in Figure 41.13.¹⁷²⁸ More recent work has defined the patterns of local and systemic chemokine and cytokine responses seen with experimental influenza infection.⁷⁶⁴

Virus replication peaks about 48 hours after inoculation and declines slowly thereafter, with little shedding after days 6 to 8. Peak virus titers in symptomatic adult volunteers range from $10^{3.0}$ to $10^{7.0}$ TCID₅₀/mL of nasopharyngeal wash. There was a positive correlation between the amount of virus shed and the severity of the clinical illness. Individuals who shed less than $10^{3.0}$ TCID₅₀/mL were either asymptomatic or had only minor upper respiratory tract symptoms. Even after infectious virus can no longer be recovered, viral antigen can be detected for several days in cells and secretions of infected individuals.^{119,467,2046} Viral antigen is detectable in conjunctival cells and secretions.²⁰⁴⁶ In children, virus can be found for up to 13 days after the onset of symptoms.⁵⁵⁵ The higher titers and more prolonged shedding in children contribute to the important role of this population in the spread of influenza.

The duration of shedding of A(H1N1)pdm09 virus is similar to that of seasonal influenza viruses. In immunocompetent patients, the A(H1N1)pdm09 virus is typically shed from the day prior to the onset of symptoms to 5 to 7 days after the onset of illness.^{131,2070,2334} The highest amounts of virus are shed within the first two days of illness. In a study of elementary school children (median age, 8 years), A(H1N1)pdm09 virus was isolated for a median 2 days after fever resolution.¹³¹ Early oseltamivir treatment within 48 hours of symptom onset significantly reduced the duration of shedding.^{1237,2334} More prolonged shedding of A(H1N1)pdm09 virus can occur in young children,¹²⁰¹ immunocompromised individuals,¹¹⁴⁵ and in patients with severe disease such as acute respiratory distress syndrome or fatal infection.²⁰⁷⁰

Pathology

Influenza A virus induces changes throughout the respiratory tract, but the most clinically important pathology develops in the lower respiratory tract.^{688,791–792,793,2183} During bronchoscopy in uncomplicated influenza infections, acute diffuse inflammation of the larynx, trachea, and bronchi are observed with mucosal inflammation and edema. Light microscopic studies of infected cells show that columnar ciliated cells become vacuolated, edematous, and lose cilia before desquamating down to a one-cell-thick basal layer. Submucosal edema and hyperemia occur with the infiltration of neutrophils and mononuclear cells.¹³²⁰ In more severe primary viral pneumonia, there is an interstitial pneumonitis with marked hyperemia and broadening of the alveolar walls, with a predominantly mononuclear leukocyte infiltration and capillary dilation and thrombosis. Influenza virus-specific antigen is present in types 1 and 2 alveolar epithelial cells, as well as in intra-alveolar macrophages^{685,1847} (Fig. 41.4). The pathologic changes associated with HPAI H5N1 viruses include a hemophagocytic syndrome, renal tubular necrosis, lymphoid depletion, and diffuse alveolar damage with interstitial fibrosis.^{267,310,1087,1101,1625,1993,2069,2116} Similarly, infection with A(H1N1)pdm09 virus leads to diffuse alveolar damage, hemorrhagic interstitial pneumonitis, and peribronchiolar and perivascular lymphocytic infiltrates^{86,625,684,705,706,1635,1927} (see The H5N1 Outbreak section).

Necrotizing changes may occur with rupture of alveoli and bronchiole walls. Influenza virus has been obtained from lungs at autopsy in titers of $10^{2.0}$ to $10^{5.7}$ 50% egg infectious doses per gram of tissue.¹²⁶²

At the cellular level, influenza virus shuts off cell protein synthesis and induces apoptosis as an additional mechanism of cell destruction.^{806,1130,1267,1424,1822,2355,2356} From the third to fifth day after onset of illness, mitoses appear in the basal cell layer, and regeneration of the epithelium begins. During this time, reparative and destructive processes may be present simultaneously. Complete healing of the epithelial damage takes up to 1 month.

Clinical Features

Adults

Infection with influenza A viruses results in clinical responses ranging from asymptomatic infection to primary viral pneumonia that rapidly progresses to a fatal outcome. The typical uncomplicated influenza syndrome is a tracheobronchitis with some involvement of small airways.^{200,441} The incubation period ranges from 1 to 5 days (reviewed in⁴⁶⁸). The onset of illness is usually abrupt, with headache, chills, and dry cough, which are rapidly followed by high fever, myalgias, malaise, and anorexia. Substernal tightness and soreness can accompany the cough. The most prominent sign of infection is fever that often peaks within 24 hours at 38°C to 40°C. The fever begins to decline on the second or third day of illness and has usually abated by the sixth day.⁴⁴¹ The elderly can have high fever, lassitude, and confusion without respiratory signs.

Physical findings in influenza are confined to the respiratory tract. Nasal obstruction, rhinorrhea, sneezing, and pharyngeal inflammation without exudate are common. Conjunctival inflammation and excessive tearing may occur. Conjunctivitis was the hallmark of an H7N7 outbreak in poultry workers in the Netherlands.⁵⁴⁶ Small cervical nodes can be felt in a minority of cases. Chest radiograph and auscultatory findings are usually normal, although occasionally patchy rales and rhonchi are heard.

As the fever declines, the respiratory signs and symptoms may become more prominent. The cough frequently changes from dry and hacking to one that produces small amounts of mucoid or purulent sputum. After the fever and upper respiratory tract symptoms resolve, cough and weakness can persist for 1 to 2 additional weeks. Illness is more frequent and more severe in cigarette smokers.⁹⁷⁴ The loss of the mucociliary blanket is a factor in a predisposition to secondary sinusitis and bacterial pneumonia. Although airflow in large airways is usually unaltered in uncomplicated influenza, there is an increase in bronchial reactivity to chemical and particulate stimuli during infection.²¹¹⁷ Small peripheral airways are often affected even in otherwise uncomplicated influenza A virus infections.⁷²¹ This small airway abnormality can persist after symptomatic illness has subsided. Alterations in pulmonary gas exchange are seen, with a depression of the diffusing capacity of carbon dioxide and an increase in the alveolar-arterial oxygen tension gradient.⁸³⁹ Tracheobronchial clearance of radiolabeled particles is depressed during acute influenza virus infections but returns to normal levels about 1 month later.²¹⁷ Although significant abnormalities in large and small airways can be demonstrated during acute infection and early convalescence, uncomplicated influenza appears to cause little permanent damage in the lung, even in patients with chronic obstructive lung disease.^{336,1167}

Clinical symptoms of A(H1N1)pdm09 virus infections in adults were similar to those of seasonal influenza, although gastrointestinal manifestations—including vomiting and diarrhea—occurred at higher rates (approximately 20–30%)^{86,1261} (see The H1N1 Pandemic in 2009 section). In addition, adults with A(H1N1)pdm09 infection were more likely to have radiographically confirmed pneumonia (4.0%) compared with those with seasonal H1N1 (2.3%) or H3N2 (1.1%) infection.⁹⁷

Children

The clinical manifestations of influenza in children are similar to those in adults, but there are some distinct differences. There is a proportionally greater burden of illness in children than in adults.¹⁶⁶⁸ Children have higher fevers that may be accompanied by febrile convulsions.¹⁶⁷⁸ At least 14% of fevers with respiratory tract symptoms that result in a pediatrician visit are caused by influenza viruses.^{629,633,635,2268,2271}

Otitis media, croup, pneumonia, and myositis are more frequent in children than in adults.^{782,1035} Otitis media develops in approximately 12% of influenza-infected seronegative children.²²⁷² Between 24% and 33% of children hospitalized with influenza A virus infection have otitis media.¹⁵⁹⁵ Although less well documented in children than in adults, bacterial sinusitis may follow acute influenza.

Studies in children have shown that some influenza A viruses are important causes of laryngotracheobronchitis (croup).¹⁰³⁵ Croup occurs predominantly in children younger than 1 year. Influenza-associated croup can be especially severe and occasionally requires intubation of the patient.¹⁹³ In a prospective study of 121 susceptible (seronegative) young children seen during an H3N2 virus outbreak, 5 of 60 infected infants had clinical and radiographic evidence of pneumonia.²²⁷⁰ Influenza A virus infection has been shown to exacerbate asthma.^{1083,1395} Children (especially those younger than 3 years) have a higher incidence of gastrointestinal manifestations such as vomiting and abdominal pain. Infection of neonates can present as unexplained fever¹³⁷⁵ and can be life threatening.^{81,1375} With HPAI H5N1 infections, acute respiratory distress syndrome is observed frequently (see The H5N1 Outbreak section).⁹⁵ Influenza A and B viruses are significant causes of serious lower respiratory tract disease that often lead to hospitalization of children^{398,634,1238,1503,1581,2062} and rarely to death.^{130,353}

Lower Respiratory Tract Complications

Three distinct syndromes of severe pneumonia can follow influenza infection in children or adults. Complications are most common in the elderly.¹²⁷⁹

Primary Viral Pneumonia

Primary influenza virus pneumonia was first described in fatal cases caused by the 1957 Asian influenza A virus (H2N2),^{1297,1320} although retrospectively, it occurred during previous influenza A epidemics.⁶⁴⁵ Primary viral pneumonia occurs predominantly in individuals at high risk for the complications of influenza virus infection (i.e., the elderly or patients with cardiopulmonary disease), but 25% of cases are in individuals without risk factors and an additional 13% are in pregnant women. The typical case of primary viral pneumonia develops abruptly after the onset of influenza illness. It progresses within 6 to 24 hours to a severe pneumonia with rapid respiration rate (30 to 60 respirations per minute), tachycardia (greater than 120 beats/min), cyanosis (in 80% of patients), high fever (average of 39°C), and hypotension. The illness may progress rapidly to hypoxemia and death in 1 to 4 days; the presence of frothy hemoptysis, tachypnea, and cyanosis portends a fatal outcome. Auscultatory findings include bilateral crepitant inspiratory rales. Examination by radiograph reveals mottled densities in two or more lobes. Diffuse symmetric interstitial infiltrates or areas of consolidation, cavitation, or pleural effusion suggest

bacterial superinfection. Pathologic findings in the trachea and bronchi are similar to those previously seen during acute uncomplicated disease, but bronchiolitis and alveolitis are also present.¹³²⁰ The laboratory findings in primary viral pneumonia are not specific. The erythrocyte sedimentation rate and the complete blood count are usually within normal ranges.

In nonfatal cases, initial improvement occurs from 5 to 16 days after onset of pneumonia. Resolution of radiographic changes can take up to 4 months.²²⁵⁴ Survivors can develop diffuse interstitial fibrosis with decreases in diffusion capacity of carbon dioxide and in arterial oxygen tension.^{1146,1657,2254} Influenza B virus can cause severe disease but does not seem to be associated with fatal primary viral pneumonia in normal individuals.^{534,2095}

Avian H5N1 influenza appears to follow a clinical pathway of primary viral pneumonia, although with a striking mortality of ~60% (updated regularly at http://www.who.int/csr/disease/avian_influenza/en/). As of February 1, 2013, 615 cases with 364 fatalities had been reported, resulting in a mortality rate of ~60%.⁹⁵

During the H1N1 pandemic in 2009, a small number of patients developed rapidly progressive primary viral pneumonia that led to acute respiratory distress syndrome and multiple organ failure, which was associated with high mortality.^{86,625,684,705,706,1635,1927} (see The H1N1 Pandemic in 2009 section). Histopathologic findings included diffuse alveolar damage (DAD) with hyaline membranes and septal edema, DAD with intense alveolar hemorrhage, and necrotizing bronchiolitis.^{625,1345,1847}

Combined Viral–Bacterial Pneumonia

Combined viral–bacterial pneumonia is quite common. The bacteria most often involved are *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*, although other micro-organisms can play a role.^{614,1320,1644,1827} (Fig. 41.4). Clinically, this syndrome may be indistinguishable from primary viral pneumonia, except that the symptoms of pneumonia appear somewhat after the influenza symptoms and chest radiographs are more likely to show pleural effusions. Virus has been recovered from the lungs and pleural fluid.^{1320,1347} The erythrocyte sedimentation rate is more frequently elevated than in primary viral pneumonia. Because there is no effective distinction between the two syndromes, the diagnosis depends solely on the demonstration of bacteria in the sputum, in fluid obtained at bronchoscopy, or in the pleural fluid. The case fatality rate for combined viral–bacterial pneumonia is 10% to 12%.^{136,510,1262,1644} Co-infection with influenza and *S. aureus* can have a fatality rate of up to 42%.¹⁷⁴¹

The available data suggest that bacterial pneumonia contributed significantly to the high mortality rate associated with the 1918 pandemic^{190,302,1272,1420} (see The Pandemic of 1918/1919—Spanish Influenza (H1N1) section). Bacterial superinfections were frequently observed with A(H1N1)pdm09 infections, in particular with fatal cases^{243,625,705,706,914,1254,1345,1599,1635,1847} (Fig. 41.4). The most common bacterial pathogens were *S. pneumoniae*, *S. aureus*, *H. influenzae*, and group A *Streptococcus*, similar to those found with seasonal influenza.

Influenza virus–infected mice experience a higher mortality rate upon *S. aureus* infection compared to mice not infected with influenza viruses.⁸⁹¹ In ferrets, influenza virus infections

increase the susceptibility to *S. pneumoniae* infection.¹³⁵⁹ The magnitude of this effect may vary among the influenza (sub) types: 9 out of 10 ferrets infected with an H3N2 virus and subsequently infected with pneumococcus developed sinusitis or otitis media, in contrast to only 1 of 11 ferrets infected with an influenza A H1N1 or influenza B virus.¹⁶²⁸

There are strains of *S. aureus* that secrete proteases capable of activating the infectivity of influenza virus by proteolytic cleavage of the HA. These strains play a synergistic role in experimental pneumonia in mice.^{2043,2044} Such protease-secreting bacterial strains could be an added pathogenic factor in combined viral–staphylococcal pneumonias in humans. An apparent increase in combined viral–bacterial pneumonias was being noted with the 2003–2004 influenza A H3N2 epidemic, perhaps coincident with the rapidly increasing impact of staphylococcal disease and emergence of methicillin-resistant *S. aureus*.¹⁶⁶⁷

Secondary Bacterial Pneumonia

In this syndrome, an individual recovering from a typical influenza illness develops shaking chills, pleuritic chest pain, and an increase in cough productive of bloody or purulent sputum.¹²⁶² Cyanosis and a marked increase in respiratory rate are less common than with primary viral pneumonia. Radiographic examination reveals local areas of lung consolidation but not diffuse pneumonia. Often, influenza virus is no longer recoverable, leukocytosis is common, and the erythrocyte sedimentation rate is elevated. This condition is generally amenable to antibiotic therapy, although a case fatality rate of about 7% has been described.¹²⁶²

Extrapulmonary Manifestations

Viremia

Viremia is highly unusual in influenza virus infections.^{1028,1397} Virus was isolated from the blood at low levels (present only in undiluted blood specimens) from a patient on the fourth day of influenza illness.¹⁴⁶⁵ Virus was present in barely detectable amounts in the blood of two patients with fatal primary viral pneumonia on the sixth day after the onset of illness and was recovered at necropsy from the liver and spleen of one patient.¹¹⁸³ In addition, the presence of viremia has been inferred because virus was present in low titer in extrapulmonary tissues such as heart, liver, spleen, kidney, adrenal glands, and meninges of patients dying of pneumonia.^{954,1737}

During the H1N1 pandemic in 2009, viremia (detected by RT-PCR) occurred rarely and was only observed in severe and fatal cases.^{1585,2070} Viremia and systemic spread is more common with avian H5N1 influenza, with PCR detection of virus from the blood in each of six patients tested.⁹⁵

Myositis

In adults, a diffuse myositis can occur with generalized pain, tenderness, and weakness of muscles, increased serum levels of muscle enzymes, myoglobinemia, and myoglobinuria.¹³⁹⁶ Acute renal failure sometimes follows. The pathogenesis of myositis remains uncertain.^{1018,1396} Although influenza virus has been recovered from muscle, the relation between the virus and myositis remains unclear. In children, myositis is usually localized to the gastrocnemius and soleus muscles and is characterized by pain on walking. Onset of myositis occurs as

respiratory illness wanes—the muscles are tender and swollen, serum levels of muscle enzymes are elevated, the course is usually benign and reversible, and light microscopic changes of muscle necrosis and inflammatory cell infiltrates are seen.⁴²¹

Cardiac Involvement

Influenza recovery from the heart of patients with myocarditis associated with fatal pulmonary infection is rare.^{1320,1581} Clinical findings and cardiac function studies in patients with severe pulmonary infection suggest that myocardial dysfunction is not a direct result of influenza A virus infection.^{200,1262,2070}

Reye Syndrome

Reye syndrome is a rapidly progressive noninflammatory encephalopathy and fatty infiltration of the viscera, especially the liver, which results in severe hepatic dysfunction with elevated serum transaminase and ammonia levels. This syndrome is seen following respiratory, varicella, and gastrointestinal viral infections. The onset of the central nervous system (CNS) and hepatic symptoms usually begins as respiratory tract symptoms wane. The case fatality rate varied between 22% and 42%.⁸⁵⁵ The etiology and pathogenesis of this syndrome are unknown. There is electron microscopic evidence of mitochondrial swelling.¹⁶¹² Influenza B virus infection is the most common antecedent infection. The disease associated with influenza B virus infection occurs in children ages 0 to 18 years (median age, 11 years) and is more frequently seen in rural than in urban areas.⁸⁵⁵ Salicylate administration is a critical cofactor in the development of Reye syndrome. The incidence of Reye syndrome after influenza virus infection in the salicylate era was estimated to be between 0.37 and 0.88 cases per 100,000 children younger than 18 years. This may have been underestimated because milder forms of Reye syndrome have been described.¹²¹⁸ There has been a dramatic decrease in Reye syndrome cases in the United States associated with reduced use of salicylates, which are now widely recognized to be contraindicated in influenza virus infection.

Central Nervous System Involvement

A wide spectrum of CNS disease has been observed during influenza A and B virus infections in humans,^{635,759} ranging from irritability, drowsiness, boisterousness, and confusion to serious manifestations such as psychosis, delirium, and coma. Febrile convulsions leading to hospitalization occur in children with and without underlying CNS abnormalities. The pathogenesis of these CNS symptoms is unclear. Nonspecific metabolic effects such as hypoxia resulting from severe pulmonary infection may contribute to the CNS signs and symptoms.

Two specific CNS syndromes accompany influenza infection: influenzal encephalopathy and postinfluenzal encephalitis.⁵⁴¹ Encephalopathy occurs at the height of influenza illness and may be fatal.⁴¹⁰ The cerebrospinal fluid (CSF) is usually normal, the brain at autopsy shows severe congestion, and histologic changes are minimal. Lungs of such patients show changes typical of influenza and yield virus in high titer. A subset of influenza encephalopathy has been described extensively in Japan and seen in other countries as an acute necrotizing encephalopathy with bilateral thalamic and cerebellar involvement.²²³⁴

The postinfluenzal encephalitic syndrome is extremely rare, occurring from 2 to 3 weeks after recovery from influenza.

Recovery is achieved in most cases. The association of postencephalitic syndrome with influenza virus infection is less certain because virus is no longer recoverable and initial serum specimens may already reflect a rising antibody titer.⁵⁴¹ Influenza A virus has only rarely been recovered from the brain or cerebrospinal fluid, and attempts to isolate it from the CNS of individuals dying of primary viral pneumonia have been unsuccessful.¹³²⁰

The syndrome of encephalitis lethargica followed by postinfluenzal encephalitic Parkinson disease was associated with the influenza epidemics of 1918 and the epidemics that followed.¹⁷⁰⁷ The epidemics of encephalitis lethargica followed the epidemics of influenza, which were followed, in turn, by postencephalitic Parkinson disease about 1 decade later.¹⁷⁰⁷ Experiments in animals suggest that avian H5N1 viruses can cause pathology similar to Parkinson disease.⁹¹¹

An increase in the incidence of Guillain-Barré syndrome (GBS), an acute neurologic disorder that causes muscle weakness and partial paralysis, has not been seen after influenza A or B virus epidemics (see the Reactogenicity of Seasonal Influenza Vaccines and Vaccines to A(H1N1)pdm09 Virus sections).

Infection During Pregnancy

Pregnant women in the second or third trimester have an increased risk of developing fatal influenza disease.¹⁵⁰⁵ The increased mortality is generally seen during the years after introduction of a new pandemic strain, as in 1918, 1957, and 2009,^{665,1807,2134,2265} although using a large data set, the impact can be appreciated in the interpandemic period.¹⁵⁰⁵ During the H1N1 pandemic in 2009, an increased risk of complication, hospitalization, and death was observed among pregnant and postpartum women, when compared to the general population.^{1261,1427,1887} Based on a systematic review, pregnant women infected with A(H1N1)pdm09 virus accounted for 6.3%, 5.9%, and 5.7% of hospitalizations, intensive care unit admissions, and deaths, respectively, although only approximately 1% of the population is pregnant at any given time.¹⁴²⁷ The risk for complications is greater during the later stages in pregnancy; among 56 pregnant women who died from A(H1N1)pdm09 virus infection in the United States from April through December 2009, four (7.1%) were in the first trimester of pregnancy, 15 (26.8%) in the second, and 36 (64.3%) in the third.¹⁸⁸⁷ Increased risks of adverse neonatal outcomes including preterm delivery and low birth weight were observed, particularly with severe maternal illness due to A(H1N1)pdm09 influenza.^{360,1887}

Virus is typically not recovered from the fetus, although in one case viral RNA was detected in the fetus of a woman who succumbed to infection with HPAI H5N1.⁶⁷⁶ Influenza virus itself has not been implicated as a cause of congenital defects.^{975,1297,1408} Although an increase in congenital anomalies and hematologic malignancies has been reported after influenza virus infection in pregnancy, no consistent association between specific defects or malignancies and influenza epidemics has emerged.^{49,669,712,1702}

Infection in Immunosuppressed Patients

Influenza viruses can cause severe disease in immunocompromised individuals,^{348,1251,1742,2240} but more often the illness resembles that seen in immunocompetent persons.¹³⁹³ Somewhat prolonged shedding of virus occurs in immunosuppressed persons.^{877,878,1742,2240}

In adults infected with human immunodeficiency virus (HIV)—most of whom were on antiretroviral therapy and had well controlled-HIV infections—the clinical outcomes of infection with A(H1N1)pdm09 virus (including the frequency of severe illness and death) were similar between HIV-infected and noninfected patients.^{1643,1730} In the United States, however, the hospitalization rate among patients co-infected with HIV and A(H1N1)pdm09 virus (3.4%) was higher compared to the HIV prevalence rate in the US adult population (0.45%).¹⁶⁴³ Among recipients of hematopoietic stem cell transplants or solid organ transplants, A(H1N1)pdm09 influenza caused an increased risk of severe illness with a high incidence of lower respiratory tract complications, and an increased risk of mortality.^{508,1109,1911}

Influenza B and C Virus Infections

Influenza B virus causes the same spectrum of disease as influenza A but there are less frequent epidemics so that its total impact in a pediatric population is roughly one-third that of influenza.⁹⁸⁶ Severe illness can occur^{56,1035,2268}; however, the frequency of serious influenza B virus infections requiring hospitalization is about fourfold less than that of influenza A virus. Influenza B virus illness predominantly involves adolescents and school-age children, but adults and the elderly can also be affected.^{56,323,629,633,635,722,1536} Myositis, Reye syndrome, and gastrointestinal symptoms appear to occur more commonly with influenza B than A virus infection.^{323,518,1017,1140}

Influenza C virus causes sporadic upper respiratory tract illness and is rarely associated with severe lower respiratory tract disease.¹⁴²² By early adulthood, most individuals (96%) have antibody to influenza C virus.¹⁵⁴¹ Administration of influenza C virus to volunteers induced mild coryza with some systemic symptoms.⁹³⁸ Of 170 children infected with influenza C virus, most (92%) were younger than 6 years of age.¹³⁴⁴ In infants and young children (particularly <2 years of age), complications of bronchitis, bronchiolitis, and bronchopneumonia that may require hospitalization have been reported^{655,1344}; nevertheless, influenza C virus is not a major cause of lower respiratory tract infection in children.

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The respiratory tract has multiple nonspecific protective mechanisms against influenza virus infection—including the mucin layer, ciliary action, and protease inhibitors—that may prevent effective cell entry and virus uncoating. The extremely short incubation period between infection and clinical illness implies that innate immunity or preformed cognate recognition components are important. Much of what we know about the protective components of immunity and events that terminate primary influenza infection is from murine models. To better understand immune responses to influenza infection and vaccination, systems biology approaches are now employed.^{169,192,738,939,976,1084,1348,1467,1468,1474,1836,1998,2205}

INNATE IMMUNE RESPONSES

In vertebrates, innate immune responses are a critical first line of defense against microbes. Innate immune responses can

broadly be divided into three steps: (1) microbe recognition by a pathogen recognition receptor (PRR), resulting in the production of type I IFN, chemokines, and cytokines; (2) activation of IFN-signaling pathways leading to the upregulation of IFN-stimulated genes, many of which have antimicrobial functions; and (3) the actions of cellular proteins with antimicrobial functions. Recent data also demonstrate high induction of IFN- λ upon influenza virus infection,⁹²⁴ suggesting a role for type III IFN in innate immune responses to influenza virus infections.

Pathogen Recognition Receptors (PRRs)

Microbes are recognized by PRRs, including Toll-like receptors (TLRs), RIG-I-like receptors, and NOD-like receptors (NLRs). Recognition of pathogen-associated molecular patterns by PRRs results in the activation of signaling pathways that ultimately converge on the expression of antimicrobial genes. Influenza virus infection is sensed by TLR3 in airway epithelium^{693,1161,1386,2281} and TLR7 in plasmacytoid dendritic cells,^{419,610,1214,1283,1386,2281} upregulating their expression. For example, high levels of TLR3 were found in the lungs of individuals who died after infection with A(H1N1)pdm09 virus.¹³⁴⁵

TLR3 (gene name: *TLR3*) senses double-stranded RNA (dsRNA) and acts through the adaptor molecule TRIF, thereby leading to the activation of IRF3, NF κ B, and the activator protein 1 (AP1) transcription factors. These three factors are critical in stimulating the expression of type I IFN and of pro-inflammatory cytokines. TLR7 (gene name: *TLR7*) recognizes single-stranded RNA (ssRNA), and relies on the adaptor protein MyD88 to induce NF κ B and IRF7, which subsequently induce the expression of type I IFN and pro-inflammatory cytokines. TLR7 is expressed in high amounts in plasmacytoid dendritic cells, and hence, may be critical for abundant IFN- α secretion, which is observed in response to influenza virus infection.^{419,951,1338,1339} By contrast, TLR3 may play a more significant role in the induction of NF κ B-dependent proinflammatory cytokines.¹¹⁶² Compared to wild-type mice, TLR3^{-/-} mice infected with influenza virus showed reduced levels of proinflammatory cytokines and increased virus titers in the lungs, but reduced mortality.¹¹⁶¹ This suggests that TLR3-dependent gene expression, potentiated in airway epithelium, may contribute to immunopathology associated with influenza virus infections.

Stimulation with TLR2 and TLR4 prior to influenza virus infection increases resistance to virus infection,¹⁸⁵⁹ and TLR4 signaling has been implicated in influenza viral pathogenicity.^{329,870} Immunization of mice with nanoparticles containing influenza HA protein in combination with ligands that signal through TLR4 and TLR7 induced higher levels of neutralizing antibodies and better protection against viral challenge than treatment with antigen and one ligand only⁹⁸⁵; hence, the interplay of B-cell responses and TLR4/7 signaling seemed to confer robust protection from influenza virus infection. TLR7 has also been shown to play a critical role in Th1-mediated immunogenicity triggered by inactivated whole influenza virus vaccine.⁶¹⁰ Despite these findings, the exact roles of TLRs in the induction of B- and T-cell responses to influenza virus infection remain somewhat controversial.^{774,1090,1260,1833}

RIG-I (gene name: *DDX58*) is activated by 5'-triphosphate groups on influenza viral RNAs.^{82,83,1652,1711} Once activated,

RIG-I interacts with the interferon β promoter stimulator-1 (IPS-1) protein at the mitochondrial membrane,¹⁵⁵¹ which leads to the stimulation of IRF3, IRF7, and NF- κ B activity. RIG-I expression increases in response to influenza virus infection; the expression of both RIG-I and IPS-1 is essential for the induction of type I IFN and upregulation of IFN-stimulated antiviral genes in mouse embryo fibroblasts.¹²⁵⁹ RIG-I is critical for virus recognition and type I IFN production in fibroblasts, conventional dendritic cells, and epithelial cells.^{987,1575}

The inflammasome, a complex of a NOD-like receptor and the ASC adaptor protein, is also activated by influenza virus infection, in a mechanism that may involve M2 ion channel activity.^{18,857,858,2059} Inflammasome activation leads to the cleavage and activation of procaspase-1, which in turn cleaves and activates certain cytokines, particularly IL-18 and IL-1 β . In human macrophages infected with influenza virus, IL-18 and IL-1 β release occurs in a caspase-1–dependent manner, consistent with inflammasome activation in response to infection.¹⁶⁶¹ In addition, these two cytokines are upregulated in bronchoalveolar lavage fluids of mice infected with influenza virus, an effect that was not observed in mice deficient in NLRP3 (the prototype NLR), ASC, or caspase-1.^{18,857,2059} Mice lacking different components of the inflammasome show increased mortality and delayed viral clearance after influenza virus infection.^{18,857,858,2059}

NS1 Interferes with the Activation of PRRs

The activation of PRRs stimulates the synthesis of type I IFN, chemokines, and cytokines. Influenza viruses, primarily through their NS1 protein, have evolved mechanisms to counteract the induction of antiviral responses. Viruses with truncated, deleted, or mutated NS1 proteins induce higher levels of IFN than their wild-type counterparts.^{241,380,381,474,703,767,1074,1075,1385,1507,1575,1652,1914,2032} Early studies suggested that NS1 may accomplish this function by sequestering dsRNA, which activates transcription factors such as ATF-2/c-Jun, NF κ B, and IFN regulatory factors (IRFs) that stimulate IFN- β production.^{1271,1275,2032,2198} However, little dsRNA seems to be produced in influenza virus infected cells,^{1652,2211} and mutation of the NS1 amino acids that are critical for dsRNA binding have only limited effects on IFN levels.³⁸¹ Rather, NS1 seems to regulate IFN production through two other mechanisms: interference with RIG-I activation and signaling, and interference with the processing of cellular pre-mRNAs.

The NS1 protein interferes with RIG-I activation,^{703,1385,1575,1652} most likely by interacting with RIG-I directly,^{1385,1652} and/or by interacting with TRIM25,⁵⁷⁰ a factor that is required for RIG-I ubiquitination and activation. Moreover, influenza virus infection prevents the activation of IRF-3^{436,1073,1575,2032} and blocks IPS-1 (a function that is carried out by the viral polymerase complex⁸⁹⁴). Interestingly, chickens seem to lack RIG-I,⁶⁵ which may, at least in part, explain their high susceptibility to influenza virus infections.

NS1 also controls IFN levels by blocking splicing,^{545,1270,1694} polyadenylation, and nuclear export of cellular pre-mRNAs (including IFN- β mRNA).^{545,1530,1690,1693} This function requires the interaction of NS1 with the 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF; gene name: *CPSF4*).^{295,378,1073,1483,1530} Two amino acids in NS1 at positions 103 and 106 are critical for CPSF binding and affect virulence.^{378,952,1073} Strain-specific differences in

the ability to interfere with the maturation of pre-mRNA^{952,1073} may contribute to viral pathogenicity. For example, the NS1 of the A(H1N1)pdm09 virus does not efficiently block host gene expression,^{719,952} a function that was restored upon the introduction of three human virus-like amino acids at positions 108, 125, and 189, which also increased binding to CPSF.⁷¹⁹ Interestingly, however, these mutations attenuated the virus in mice.⁷¹⁹

IFN Signaling

Type I IFN acts in an autocrine and paracrine manner through the ubiquitously expressed IFN- α/β receptor (IFNAR). IFNAR loss results in high susceptibility to influenza virus infection^{600,1080} but, interestingly, increases survival of mice to secondary bacterial infections.¹⁸³⁵ Activation of IFNAR by type I IFN stimulates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway. JAK/STAT signaling induces the formation of a transcription factor complex that upregulates the expression of IFN-stimulated genes. The suppressor of cytokines signaling (SOCS) proteins 1 and 3 are upregulated upon influenza virus infection^{1621,1672} and counteract type I IFN signaling, likely by interfering with the JAK/STAT pathway. As a consequence, reduced levels of SOCS-3 or lack of this protein reduce influenza virus titers in cell culture.¹⁶²¹

Proteins with Antiviral Activity

IFN-induced stimulation of the JAK-STAT pathway results in the induction of IFN-stimulated genes, including those encoding TLRs (suggesting positive feedback loops), PKR, OAS, RNaseL, ISG15, Mx proteins, Viperin, and IFITM.

PKR

Activation of dsRNA-activated protein kinase R (PKR; gene name: *EIF2AK2*) leads to the phosphorylation of the translation initiation factor eIF2 α and the subsequent shutdown of protein synthesis. Influenza viruses inhibit PKR activation,^{120,749,1271,1280} and a mutant virus lacking NS1 was virulent in PKR^{-/-} mice, but not in wild-type mice.¹²⁰ NS1 may block PKR activation through direct binding^{1207,1391,2037} and/or sequestration of dsRNA.^{749,1271} However, as stated earlier, little dsRNA has been detected in influenza virus–infected cells; in addition, PKR is now also known to be activated by ssRNA with 5′-triphosphate groups,¹⁴⁷⁵ suggesting that influenza viral RNAs may activate both RIG-I and PKR. PKR activity may also be affected by the influenza virus M2 protein, which binds to the cellular PKR inhibitor p58(IPK), resulting in increased PKR phosphorylation.⁶⁸³ The role of p58(IPK) in the regulation of PKR activity and influenza pathogenesis is underscored by the finding of increased lung pathology and mortality in influenza virus–infected p58(IPK)^{-/-} mice, compared to wild-type mice.⁶⁴⁴

OAS and RNase L

Two members of the 2′-5′-oligoadenylate synthetase (OAS) family, the OAS1 (gene name: *OAS1*) and OAS-like (gene name: *OASL*) genes are induced upon influenza virus infection.^{1381,1390} Activated OAS stimulates RNase L (gene name: *RNASEL*), a single-strand-specific nuclease that degrades cellular and viral RNA, resulting in antiviral activity. Pretreatment of cells with IFN- β had no significant effect on a wild-type virus, but restricted a virus with a mutation in the RNA-binding

domain of NS1.¹³⁹⁰ This restriction was relieved in RNase L^{-/-} mouse cells or in cells treated with siRNA to RNase L.¹³⁹⁰ These findings demonstrate that the RNA-binding domain in NS1 controls the antiviral functions of OAS/RNase L.

ISG15

Interferon-stimulated gene 15 (ISG15; gene name: *ISG15*) is conjugated (ISGylated) to its target proteins; this alteration may interfere with the normal (enzymatic) function of the target protein. For example, ISG conjugation promotes IRF3 activation by preventing ubiquitination and subsequent degradation of the transcription factor.¹⁸⁴⁵ ISG15^{-/-} mice are more susceptible to infection with influenza A and B viruses than are control mice,^{1127,1187} and treatment of cells with siRNAs to ISG15 partially impairs IFN-induced antiviral activity.⁸⁴² Mechanistically, conjugation of ISG15 to NS1 appears to interfere with NS1 function in several ways: it impairs dimerization of the NS1 RNA-binding domain; disrupts NS1 binding to dsRNA, U6 snRNA, and PKR; interferes with NS1-mediated disruption of antiviral gene expression; and inhibits NS1 association with importin α .^{2038,2360} In contrast to the influenza A virus NS1 protein, the influenza B virus NS1 protein blocks the antiviral activity of ISG15 by interfering with E1 ligase for ISG15 modification activity.^{2342,2343}

Mx Proteins

Mx proteins are IFN-induced GTPases of the dynamin superfamily. They confer resistance to influenza virus infection^{598,723,1228,1942} by interfering with viral replication.^{725,727,2383} In particular, they interfere with the replicative activity of the viral ribonucleoprotein complex,^{427,2110} perhaps by interacting with and wrapping around the nucleoprotein.^{724,2110} Mx proteins have been identified in several mammalian and avian species; most, but not all, inhibit influenza viruses (reviewed in⁷²⁶).

Of note, influenza viruses differ in their sensitivity to Mx proteins: in general, avian influenza viruses are more sensitive to inhibition by Mx proteins than human influenza viruses.⁴²⁷ The replication complex of an HPAI H5N1 virus was significantly affected by human MxA protein,⁴²⁷ and mice expressing murine Mx1 protein were protected against infection with HPAI H5N1.²¹⁰⁸

Most conventional laboratory mice, including most commercially available knock-out mice, lack a functional Mx gene.¹⁹⁴⁰ This fact should be taken into account when interpreting data obtained with such mice.

IFITM

The interferon-induced transmembrane (IFITM) proteins 2 and 3 (gene names: *IFITM2* and *IFITM3*) are ubiquitously expressed cellular proteins with roles in immune cell signaling, oncogenesis, and cell adhesion. Overexpression of IFITM2 and 3 inhibits influenza virus, most likely during cell entry.¹⁶⁹ IFITM3 is modified by palmitoylation; deletion of its palmitoylation site abrogates its antiviral activity against influenza virus.²³²⁹ The underlying mechanism is currently not known.

Viperin

Viperin (gene name: *RSAD2*) is an IFN-stimulated, ER-associated protein expressed in many species from fish to mammals. It inhibits several viruses, including influenza

virus, most likely by disrupting lipid rafts, the sites of influenza virus budding.²¹⁹⁷

Other Strategies to Counteract Innate Immune Responses

The PDZ Domain of the NS1 Protein

A large-scale sequencing project of avian influenza viruses identified a PDZ ligand domain motif at the carboxy-terminal four amino acids of NS1.¹⁵⁴⁵ This motif is recognized by PDZ domain proteins, a large family of proteins that play multiple roles in cellular processes. Most human influenza viruses possess an RSKV motif, in contrast to the ESEV motif found in most avian influenza viruses. When tested in the background of a laboratory-adapted human H1N1 virus (A/WSN/33), the PDZ ligand domain motifs of pandemic 1918 and HPAI H5N1 viruses conferred increased virulence to viruses in mice,⁸⁹⁸ demonstrating the significance of this sequence for influenza viral pathogenicity. Interestingly, the observed differences in virulence did not correlate with IFN expression levels,⁸⁹⁸ suggesting that the PDZ ligand domain motif in NS1 affects pathogenicity through a currently unknown mechanism. By contrast, the PDZ ligand domain motif had little effect on the virulence of an HPAI H5N1 in mice and chickens.²³⁸⁰ Replacement of the avian virus ESEV motif with the human virus RSKV motif in a low pathogenic avian H7N1 virus attenuated the virus in mice.¹⁹³⁰ The ESEV motif binds to the PDZ domain proteins Scribble, Dlg1, and MAGI-1, -2, and -3.¹²⁴⁴ Scribble possesses proapoptotic function and interaction of NS1 with Scribble reduces apoptosis,¹²⁴⁴ suggesting that the PDZ ligand domain motif may regulate the induction of apoptosis in virus-infected cells. Moreover, the PDZ ligand domain motif affects Mx levels,¹⁹³⁰ suggesting a role in the regulation of innate immune responses.

The A(H1N1)pdm09 viruses encode a truncated NS1 protein that lacks the 11 C-terminal amino acids. Reconstitution of this region and thus the PDZ domain does not significantly affect replication, pathogenicity, or transmission in animal models.^{718,1592}

NS1 Activates the PI3K/Akt Pathway

PI3K (phosphoinositide-3-kinase) and its most prominent substrate, the serine/threonine protein kinase Akt, regulate many different cellular events including apoptosis, cell metabolism, and proliferation. In addition, PI3K plays a role in the transcriptional regulation of IFN-stimulated genes, and in the regulation of TLR-mediated cytokine production.

The NS1 proteins of influenza A,^{475-477,713,714,717,1211,1852,1853} but not influenza B viruses,⁴⁷⁶ activate the PI3K/Akt pathway. Although this pathway was believed to have antiviral function, its activation is also required for efficient influenza virus replication.^{475,1852} NS1 binds to p85 β , a regulatory subunit of PI3K; several residues in NS1 have been identified that are critical for this interaction.^{713-715,1211,1851,1853} One study demonstrated that NS1 interacts with phosphorylated Akt, resulting in increased Akt activity.¹³³⁷ Activation of the PI3K/Akt pathway may delay the induction of apoptosis,^{476,1268,1851,2363,2365} although another study suggested that this process is not regulated through the PI3K pathway.⁸⁹⁹ Two recent studies also suggest roles for PI3K in clathrin-independent endocytosis⁵⁶⁰ and endosomal acidification,¹³¹⁶ suggesting that PI3K activation may facilitate virus infection through these processes.

Additional Roles of NS1 in Pathogenicity

In addition to the described functions and key amino acids, several studies have identified amino acids in NS1 that contribute to virulence and possibly also innate immune responses through currently unknown mechanisms.^{869,926,1213,1681,1788,1831,1832,1956} For example, the differences in pathogenicity in pigs between NS genes derived from HPAI H5N1 or a non-H5N1 virus were traced to a single amino acid at position 92 of NS1, with glutamic acid at this position conferring higher pathogenicity than aspartic acid.^{1831,1832}

Two internal deletions in NS1 affect pathogenicity. Since 2000, most HPAI H5N1 viruses possess a deletion of amino acids 80 to 84 in their NS1 proteins, which enhances virulence.¹²⁵⁸ Of note, during the generation of viruses possessing this deletion, the D92E mutation was co-selected,¹²⁵⁸ suggesting a functional relationship between these two potential virulence determinants. In addition, HPAI H5N1 viruses isolated in 2004 to 2005 in Thailand possessed both the 5-amino-acid deletion at positions 80 to 84 and the D92E mutation.²⁰⁰⁷ In virus-infected cells, the 5-amino-acid deletion and the D92E mutation affected responses to TNF- α , or the induction of IFN, respectively.¹²¹⁰ The other deletion in NS at nucleotides 612 to 626 (resulting in amino acid deletions in both the NS1 and NS2 proteins) also affects IFN induction and virulence.²³⁷⁸

Differences Between NS1 Proteins in Their Ability to Affect Innate Immune Responses

NS proteins differ in their ability to counteract cellular IFN responses. Compared to the NS1 proteins of human viruses, the NS1 proteins of HPAI H5N1 viruses induce higher levels of proinflammatory cytokines, such as TNF- α and IFN- β ,³⁰⁰ and confer higher pathogenicity in pigs.¹⁸³¹ These findings suggest that the NS1 protein of highly pathogenic viruses may cause the cytokine imbalance that was observed in victims of H5N1 infection in Hong Kong in 1997.^{95,399,2069,2345} The NS gene of the 1918 pandemic virus interfered with the transcription of IFN-regulated genes more efficiently than did a control virus possessing an NS gene derived from a human virus.^{132,612}

Role of Mitogen-Activated Protein Kinase (MAPK) Signaling Pathways in Influenza Virus Infection

Currently, four MAPK signaling pathways are recognized including the extracellular signal regulated kinases 1/2 (ERK1/2), the c-jun-N-terminal kinase (JNK), p38, and ERK5. Activation by external stimuli leads to the phosphorylation of kinases in the signaling cascades, and the phosphorylation of transcription factors such as c-Jun or ATF-2, which are critical for the upregulation of IFN- β , RANTES, and other factors with functions in innate immune responses and apoptosis.

All four MAPK pathways are activated upon influenza virus infection.^{611,794,1107,1223,1273,1321,1421,1665,2280} The p38 and ERK1/2 MAPK pathways are strongly activated by HPAI H5N1 viruses, in contrast to H1N1 viruses.¹¹⁷² ERK1/2 is important for efficient viral replication^{1276,1316,1568,1665,2280} since the influenza HA protein-mediated activation of ERK1/2^{1315,1317} appears to be critical for efficient nuclear export of viral ribonucleoprotein complexes.¹⁶⁶⁵ In addition, ERK1/2 may phosphorylate NS1, which might be critical for efficient virus replication.⁷¹⁶ By contrast, JNK, p38, and ERK5 have antiviral activity through their roles in the regulation of apoptosis and the expression of

cytokines and chemokines; p38 may also play a role in influenza virus internalization.¹³¹⁴

The PB1-F2 Protein

For some influenza viruses, PB1-F2 increases viral pathogenicity, inflammation, and the frequency and severity of bacterial co-infections in mice.^{340,891,1356,1899,2349} These phenotypic differences likely result from the currently known functions of PB1-F2 in the induction of apoptosis and replicative ability.

PB1-F2 localizes to mitochondria,^{291,623,2295} where it interacts with the ANT3 (adenine nucleotide translocator 3) and VDAC1 (voltage-dependent anion channel 1) proteins,²³⁴⁸ and induces the formation of membrane pores with subsequent changes in mitochondrial permeability.^{271,783} The resulting induction of apoptosis, which is primarily observed in immune cells,^{291,623,2348} may contribute to the increased virulence upon PB1-F2 expression. However, one study suggests that this induction of apoptosis is strain specific and may not be the major function of PB1-F2.¹³⁵⁵

PB1-F2 enhances inflammatory responses, as demonstrated by an increased influx of inflammatory cells in lungs of mice infected with an influenza virus expressing the PB1-F2 protein from the 1918 pandemic influenza virus.¹³⁵⁶ Enhanced inflammatory responses were observed with PB1-F2 peptides (encompassing the 27 C-terminal amino acids) of an HPAI H5N1 virus and the pandemic 1918, 1957, and 1968 viruses, but not for that of a recent human H3N2 virus.¹³⁵⁵ The PB1-F2 protein of the 1918 pandemic virus encodes serine at position 66, instead of the asparagine commonly found among human influenza viruses at this position. Serine at position 66 of PB1-F2 increased virus titers and pathogenicity, immunopathology, cytokine levels, and the severity of secondary bacterial pneumonia in mice, resulting in higher mortality.^{339,340} The increased pathogenicity of the serine variant likely resulted from delayed immune responses, as assessed by transcriptional profiling studies.³³⁹

PB1-F2 co-localizes and interacts with the polymerase PB1 protein. Lack of PB1-F2 expression results in altered intracellular localization of PB1 and decreased viral polymerase activity,¹³⁵² which may affect virulence. These effects are strain specific and cell type specific.¹³⁵⁷

Innate Immune Cells

Innate immune cells—including macrophages, neutrophils, dendritic cells, and natural killer (NK) cells—play an important role in the control of influenza virus infections. These cells contribute to virus clearance through phagocytosis or lysis of infected cells, the production of cytokines and chemokines, and/or the stimulation of adaptive immune response. Macrophages and neutrophils phagocytose infected cells and produce chemokines and cytokines. Macrophages also act as professional antigen-presenting cells.^{561,748,1033} Infection with highly pathogenic influenza viruses leads to pronounced infiltration of macrophages and neutrophils into the lungs of infected individuals^{95,2345} or animals.^{76,1068,1639,2105} Inhibition of macrophage and neutrophil phagocytosis,⁷⁴⁸ or depletion of alveolar macrophages,²¹⁰⁵ increases virus titers and mortality in mice. By contrast, mice lacking CXCR2 (a receptor on neutrophils) can clear influenza viruses, although they fail to recruit neutrophils to the lung.²²⁰² Macrophages may also contribute to excessive inflammation and immunopathology since they produce high

levels of cytokines/chemokines upon infection with highly pathogenic influenza viruses.^{300,848,1180,1405,2340,2368} In addition, macrophages produce nitric oxide synthetase 2 (NOS2), which exacerbates influenza virus infections.^{913,1912}

Dendritic cells (DC) present antigens to other immune cells, acting as messengers between the innate and adaptive arms of the immune system. Upon influenza virus infection, they are the major professional antigen-presenting cell and, hence, CD4 and CD8 T cell-stimulating cell population.^{128,129,1538} DCs can acquire antigen through direct infection by influenza viruses^{114,128,733,1538} or through the phagocytosis of infected epithelial cells.^{13,128,739,1790} DCs also produce type I IFN and various cytokines and chemokines.⁷³⁹ In particular, large amounts of type I IFNs are produced by plasmacytoid dendritic cells, a subset of DCs that is rapidly recruited into the lungs of mice infected with influenza viruses.²²⁵⁶ The maturation and type I IFN production of respiratory dendritic cells are affected by the NS1 protein.^{420,528,767}

NK cells release perforin and granzymes that lead to the lysis of infected cells; in addition, they produce chemokines and cytokines. A large body of data indicates a role for NK cells in innate immunity to influenza virus infections,^{7,43,446,608,697,817,1088,1243,1308,1384,1886,1957,1958} and NK cell depletion renders mice and hamsters more susceptible to influenza infections.¹⁹⁵⁸ NK cells detect influenza viruses through interaction of the NK receptor NKp46 with HA,^{7,43,1308,1384} and mice lacking NKp46 show increased morbidity and mortality upon influenza virus infection.⁶⁰⁸ The HA proteins of different subtypes may trigger different activation programs in NKs: while an H1 HA triggered NK cell-mediated lysis, additional activation signals for killing were required with an H5 HA.⁷ Influenza viruses appear to interfere with NK functions by infecting them^{696,1313}; infected NK cells have reduced killing capacity, produce lower amounts of chemokines and cytokines, and eventually die. In several cases of influenza virus infection, low levels of peripheral NK cells were detected^{414,779}; however, it is not clear if these cells were killed by influenza viruses or migrated to respiratory organs.

In summary, several immune cell populations have functions that may in parallel dampen and aggravate influenza virus infection. Most likely, a fine balance between temporal, spatial, and quantitative regulation of these cell populations and regulatory factors will contribute to infection outcome.

ADAPTIVE IMMUNE RESPONSES

Mice that lack both B and T cells succumb to influenza virus infection,^{428,2060,2230} whereas those lacking either immune cell type can control, although not necessarily clear, influenza virus infections.^{115,480,481,1222,1726} These findings demonstrate that adaptive antibody- and T cell-mediated immune responses are important to clear influenza virus infections.

Strain-specific immunity to influenza can be of long duration as reflected in the age-specific attack rates of pandemics with reemergence of a strain, as in the A/USSR H1N1 pandemic in 1977, recall cellular immunity to distant infection,¹¹⁵⁸ and neutralizing antibodies to the pandemic 1918 virus or its descendants that were detectable 90 years later.^{734,890,1070,1309,1890,2341}

Although murine models clearly demonstrate immune responses that are cross-reactive among influenza strains of the

same subtype, the classic teaching in humans based on experiencing recurrent influenza infections is that adaptive immunity is quite strain specific. The rapid drift of B-cell epitopes in influenza viruses due to immune selection suggests that there is strong evolutionary pressure exerted by the collective population immunity. Similar pressure on T-cell epitopes has been suggested, but there appears to be less variability in these sites.²¹⁶⁷

However, two recent observations suggest immunity that is cross-reactive among influenza viruses of different subtypes in humans. The first is the demonstration of common neutralizing epitopes in the stem of the hemagglutinin.^{485,1565,1897,1999,2230} The second is a strong resistance to infection of adults with live, attenuated vaccines with the HA and NA of potentially pandemic strains—for example, H5N1, H7N7, H9N2—that vaccinees have never been infected with.⁹⁷⁸

Humoral Immunity

Serum antibodies play an important role in resistance to and recovery from influenza illness in humans, or at the very least are a strong correlate of immunity.^{346,349,616,617,1282,1452} Indicative of the importance of antibody is that immunization of mothers during pregnancy provides protection in infancy through placental transfer of antibody.²³⁴⁷ Upon infection with influenza viruses, antibodies to the HA, NA, NP, and M proteins are produced, of which antibodies to HA are the most important for virus neutralization.^{364,615,616,1674} HA and NA are the primary protective antigens, and antibodies against these glycoproteins are the main mediators of resistance to virus challenge.^{346,349,503,1452}

The level of serum antibody to HA and NA correlates with resistance to illness and with restriction of influenza virus replication in the respiratory tract of humans.^{326,351,923,980,1456} In addition to the levels of serum antibodies, their avidity is likely important for optimal protection.^{187,525,900,1067,1808}

HA antibodies can prevent infection by neutralizing the infectivity of the virus, typically by interfering with HA-mediated receptor binding or fusion.^{66,485,1067,1999,2035,2051,2052,2165,2326} Most neutralizing antibodies are believed to bind conformational epitopes. The X-ray crystallographic structures of several HA/antibody complexes have been resolved now^{138,485,540,1999} and allow the structural interpretation of antibody binding to HA (for more information on the HA antigenic sites, refer to the Antigenic Drift of the HA Protein section). Although antibodies to HA play the primary role in protection against influenza virus infections, NA antibodies mediate an antiviral effect by limiting virus release and hence restricting virus spread.^{350,1029,1456,1675,2226} NA-specific antibodies may have thus ameliorated the severity of the Hong Kong influenza in 1968,¹⁴¹⁴ when an H3N2 influenza virus emerged following a reassortment event with a previously circulating H2N2 virus.

Passively transferred monoclonal antibodies to M2 are protective in mice^{1433,2077} and M2 antibodies develop in naturally infected humans,^{139,526,2092} although the titers are typically low. M2 is highly conserved among influenza A viruses, suggesting its potential as a universal influenza vaccine (see later discussion and Vaccines section). Antibodies to NP have been detected in individuals,^{498,685,2000,2302} and vaccination with NP leads to faster virus clearance and reduced mortality in mice.^{67,491,502,504,558,1138,1252,1796,2036,2068,2114,2115,2266,2366} Information on the three-dimensional structure of NP²³¹⁷ allowed the mapping of several monoclonal antibodies onto NP.²¹⁴⁶ Infection with influenza viruses also elicits antibodies to M1.^{364,1019}

A protective effect of transplacentally acquired antibody has been inferred from the correlation between age at the time of symptomatic influenza A virus infection in infants and level of maternally transmitted antibody measured in cord serum.¹⁶⁸² In addition, vaccination of pregnant women against A(H1N1)pdm09 virus increased the percentage of newborns with protective immunity to influenza virus compared to babies born to unvaccinated mothers,^{1683,2382} further suggesting vertically acquired passive immunity.

A vaccine that elicits heterosubtypic immunity against influenza A viruses of different subtypes would no longer require frequent changes in the composition of influenza vaccines. Heterosubtypic immunity was first demonstrated in the 1960s when infection with an influenza virus was shown to reduce virus titers and pathology of a challenge virus belonging to a different subtype in mice.¹⁸²⁰ In contrast, sequential heterotypic infections have been demonstrated in children in a single season²²⁷¹; the prior receipt of a heterotypic cold-adapted vaccine did not alter the pattern of virus shedding with subsequent vaccination while the second dose of the same strain shows no evidence of reinfection. Broadly neutralizing antibodies to HA have been described^{344,485,984,1565,1566,1898,1999,2065,2195,2230} that bind to a highly conserved region in the stem region of HA2, and block the low pH-induced conformational changes that mediate membrane fusion. Their broad neutralizing activities and the apparently low rate of escape mutants^{344,1999,2195} make HA2 an interesting target for a universal vaccine (see Vaccines section). Cross-protective antibodies against the ectodomain of M2 have also been described.^{393,506,514,530,849,1432,1477,2077} Although M2 is immunogenic and antibodies to it are protective in mice,^{393,506,514,849,1432,1477,2077} the potential of M2 vaccines in other animal models^{514,1706} and humans¹⁸¹⁶ is less well established.

A potent antibody-dependent cellular cytotoxicity (ADCC) response has been demonstrated following influenza infection,⁷⁴⁷ but it has not been integrated into our conceptualization of the protective immune response to influenza.

Cellular Immunity

The role of cellular immunity in clearance of influenza virus has been well defined in the murine model but is less well understood in humans.^{979,1370}

There are two major subsets of effector lymphocytes in humans. The first subset mediates cytotoxicity that is restricted by class I histocompatibility antigens and has the CD8⁺ phenotype. CD8⁺ T cells (cytotoxic lymphocytes, CTLs) eliminate virus through two mechanisms: direct killing of virus-infected cells, and the production of proinflammatory cytokines.¹¹²⁴ CTLs appear in the blood of infected individuals or those vaccinated with a live virus on days 6 to 14 and largely disappear by day 21.⁴⁹⁷ Most studies have examined memory CTLs, which are detected *in vitro* by stimulation of peripheral blood lymphocytes with antigen. These CTLs can exhibit a cross-reactive pattern of virus specificity (i.e., they lyse target cells infected with influenza A viruses belonging to any subtype, but not target cells infected with influenza B virus).^{165,473,501,1096,1370,2313,2325,2385} The cross-reactivity of CTL responses likely contributed to the lower infection rate of the elderly with A(H1N1)pdm09 virus, as 50% of influenza-derived T cell epitopes are similar between the pandemic 1918 and 2009 viruses.^{662,664} Indeed, both CD8⁺²⁰⁹⁹ and CD4⁺⁶⁰⁹ cells reactive with A(H1N1)

pdm09 virus were present prior to infection with this virus. However, a more specific subpopulation of CTLs lyses only cells infected with homologous virus. Depending on human leukocyte antigen (HLA) haplotype, individuals differ considerably in the peptides that their CTLs can recognize.¹³⁷¹ Memory CTLs characterized after infection or vaccination of humans have the cross-reactive pattern of cytotoxicity. The pre-challenge level of memory CTLs did not correlate with susceptibility to infection or illness after experimental administration of wild-type virus, but did correlate with accelerated clearance of virus from the respiratory tract of humans.¹³⁷⁰

The other class of T cells is restricted by histocompatibility class II antigens and has the CD4⁺ phenotype^{965,1966}; thus, it belongs to the T-helper class of T cells. CD4⁺ T cells are helper T cells, providing help to B cells for antibody production and to class I-restricted CTLs for their proliferation.^{1132-1134,2325} Helper T cells specific for M or NP antigen can provide help to B cells secreting HA antibody^{1132-1134,1798} and, in this manner, can augment the antibody response to protective antigens. CD4⁺ T cells also have cytolytic activity^{661,1979} that is most likely mediated through perforin, rather than Fas:FasL.^{179,180}

The important role of CD4⁺ T cells in immunity to influenza virus infection has been defined by studies in CD8⁺ T cell-deficient animals, which can clear influenza A virus by a CD4⁺ T cell-dependent mechanism.^{115,480,481,1222} Passively transferred influenza virus-specific CD4⁺ T-cell clones require functional B cells to clear an ongoing influenza virus infection. Thus, the primary antiviral activity of the CD4⁺ T cell is probably to help B cells produce antiviral antibodies. Because CD4⁺ T cells recognize many epitopes on influenza viral proteins, a large number of T cells are available to provide such help.²⁴⁰

CD8⁺ T cells can mediate clearance of influenza A virus infection in CD4⁺ T cell-deficient animals.^{481,1726} In mice lacking CD4⁺ T cells, primary CD8⁺ T cell responses are not significantly affected,¹⁷²⁶ in contrast to CD8⁺ T cell memory formation and recall.¹¹² Animals deficient in both CD4⁺ and CD8⁺ T cells succumb to infection,^{428,2060} indicating that nonspecific mediators of immunity are not sufficient in the absence of T cells to contain and clear influenza A virus infection. The number of CD8⁺ T cell epitopes on viral proteins is much more restricted than the number of B-cell or CD4⁺ T cell epitopes. Cytolysis of influenza virus-infected cells can also be mediated by antibody plus complement and antibody-armed lymphocytes.^{747,1697,1760} While CD8⁺ T cells play a critical role in virus elimination, they may induce significant immunopathology.^{429,1123}

Lymphoproliferation is another measure of the cellular response to influenza. Lymphocytes responsive to influenza virus antigens have been isolated from the blood and lower respiratory tract secretions of influenza virus-infected humans, but only the former have been studied extensively.^{949,2325} Lymphocyte blastogenic responses to influenza antigens in humans increase after influenza A virus infection, beginning between the third and sixth day after infection,⁴³⁰ and return to baseline levels by day 28.

Invariant NKT (iNKT) cells (also referred to as type I NKT cells) are the most prevalent NKT cells in mice. Their name reflects the invariant T cell receptor α chain expressed by these cells. Activated iNKT cells appear to play roles in both cell- and antibody-mediated immunity,¹¹⁰⁸ and produce large amounts of interferons and cytokines such as IFN- γ and

IL-4.²¹⁰⁹ Stimulation of iNKT in mice reduced virus titers compared to control mice,⁸¹⁸ and mice lacking iNKT cells show a higher mortality rate than wild-type mice.⁴⁰³ Moreover, use of an iNKT activator as an adjuvant for influenza vaccination results in increased immunogenicity and protection.¹⁰⁸⁶ These findings suggest a contribution of iNKT cells to immunity against influenza viruses.

Influenza A virus infection can depress skin test reactivity to common antigens used to assess cutaneous delayed hypersensitivity.¹⁷¹⁰ In addition, depression of the peripheral blood lymphocyte blastogenic responses to mitogenic and antigenic stimulation^{430,1196} is seen during the acute stage of infection and early convalescence. Despite this transient depression of delayed hypersensitivity and blastogenic responses to noninfluenza antigens or mitogens, influenza virus-specific cellular responses develop. The mechanisms underlying the depression in the function of lymphocytes in influenza virus infection indicated earlier have not been defined, but influenza A virus infection can abortively infect human lymphocytes *in vitro*¹⁸⁸ and possibly *in vivo*.²²⁵¹

Mucosal Antibody Response

Francis³⁵² was the first to detect neutralizing activity to influenza in nasal secretions. He subsequently showed that resistance to experimental influenza A virus infection in mice was mediated by an antibody in bronchial secretions. The neutralizing antibody in the nasal secretions of humans is primarily locally produced IgA.^{44,1756} Antibody to both HA and NA can be detected in local secretions, but the former is easier to detect.³⁴⁹ It has been proposed that IgA antibodies can act intracellularly during transcytosis to the apical surface to inhibit virus replication and thus participate in clearance of infectious virus from the epithelial cells and lumen of the respiratory tract.^{1351,1720} IgA and IgG may switch in relative importance between the upper and lower respiratory tract as shown in a murine model of the protective role of passive neutralizing antibody.¹⁷²¹

During primary viral infection, IgA, IgG, and IgM hemagglutinin-specific antibodies can be detected in nasal washes by use of an enzyme-linked immunosorbent assay (ELISA). IgA and IgM antibodies are detected more frequently than IgG antibody.¹⁴⁵⁷ Most of the HA-specific IgA and IgM antibodies are actively secreted locally, whereas local secretion has been demonstrated only infrequently for IgG antibody, although a receptor for IgG transport is present on bronchial epithelial cells.²³²⁷ Individuals with a local IgA response also have a serum IgA response. Local IgA antibody stimulated by natural infection is detectable for 3 to 5 months after infection, and there is local IgA memory for influenza antigen.¹⁴⁵² After secondary infection, local antibody is also primarily of the IgA isotype, those with a local IgA response also have a serum IgA response, and the magnitude of the serum IgA HA antibody response correlates with that of the local response.

The presence of local IgA antibody induced by infection with an attenuated virus correlates with resistance of volunteers to infection and illness after challenge with a virulent wild-type virus.⁹³⁵ Challenge of children with an attenuated live vaccine shows similar findings.^{162,163} Resistance to experimental wild-type infection also correlates with the level of local HA antibody present at the time of virus administration, with IgG and IgA each contributing to resistance.³²⁶

SURVEILLANCE

Surveillance efforts in humans have increased considerably in recent years. These efforts are coordinated by the WHO through the Global Influenza Surveillance and Response System (GISRS; formerly the Global Influenza Surveillance Network, GISN) that was founded in 1952 and currently includes five Collaborating Centres (in Atlanta, Beijing, London, Melbourne, and Tokyo), 136 National Influenza Centres in 106 countries, 11 H5 Reference Laboratories, and four Essential Regulatory Laboratories (http://influenzacentre.org/centre_GISN.htm). The National Influenza Centres collect more than 175,000 samples each year from patients with influenza-like illness and submit approximately 2,000 representative samples per year to the Collaborating Centres for antigenic and genetic characterization. These data are used to monitor antigenic drift and resistance to antiviral compounds. Based on surveillance, antigenic, and genetic data, vaccine viruses are recommended by the WHO each February and September for the Northern and Southern Hemispheres, respectively.

Summaries of influenza activity are published in the *Morbidity and Mortality Weekly Report* from the Centers for Disease Control and Prevention (CDC) during the influenza season and in the *Weekly Epidemiologic Record* published by the WHO. An even more current report of influenza epidemiology is available at the CDC FluView website (www.cdc.gov/flu/weekly). These reports are linked with reports of pneumonia and influenza deaths in 121 US cities to monitor the emergence of an influenza epidemic. Surveillance in the face of highly pathogenic H5N1 viruses has focused not only on human illness, but also on causes of mortality in domestic and wild birds. There continues to be a strengthening of routine surveillance particularly in China and Southeast Asia to identify the emerging strains that inform the choice of vaccine components for the coming influenza season. The threat has propelled countries around the world to prepare for an influenza pandemic. The US plan can be found at www.hhs.gov/nvpo/pandemicplan; since its release in 2005, the plan has been updated to reflect the guidelines established during the H1N1 pandemic in 2009.

DIAGNOSIS

Several diagnostic tests are available to detect influenza A and B viruses, including viral culture, reverse transcriptase-polymerase chain reaction (RT-PCR), immunofluorescence assays, and rapid antigen testing. Growth of samples in eukaryotic cells is still considered the gold standard but requires 3 to 10 days (pertinent cell lines are described in the Virus Propagation section). This time can be reduced to 1 to 3 days. Influenza virus in culture can be detected by using immunoassays or RT-PCR. PCR-based diagnosis is significantly more sensitive than culture. RT-PCR is now the method of choice in many diagnostic labs since it combines several attractive features: it is highly sensitive (which, on the other hand, requires good laboratory practices and stringent controls to avoid or monitor for cross-contamination); it has reasonable test times since data can be obtained within 1 day; and it can be designed to distinguish between different types and subtypes, depending on the sets of oligonucleotides used. In addition, multiplexing is possible to test multiple samples at once, or to test for more than one viral RNA segment in one reaction.

In recent years, multiple rapid influenza diagnostic tests have been developed and approved for the analysis of human samples. Most of these are immunoassays that detect the NP antigen. Some of these can distinguish between influenza A and B viruses. Currently available rapid influenza diagnostic tests provide results within 5 to 30 minutes, but have a low sensitivity rate of about 40% to 70%. Indirect immunofluorescence assays are not widely used for routine diagnostics since they are not conducive to high-throughput testing or multiplexing. Overviews of currently recommended diagnostic tests and rapid tests can be found at <http://www.cdc.gov/flu/professionals/diagnosis/>, <http://www.cdc.gov/flu/professionals/diagnosis/rapidlab.htm>, and http://www.who.int/csr/disease/avian_influenza/guidelines/RapidTestInfluenza_web.pdf. The standard protocols for the diagnosis of influenza viruses from avian species (some of which are also used for the diagnosis of human samples) can be found in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2010 (<http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/>).

The available tests can be carried out with nasopharyngeal or throat swabs, bronchial washes, nasal or endotracheal aspirate, or sputum. The tests can differ in their sensitivity and specificity for different types of specimens. In general, nasopharyngeal specimens collected with a swab are more sensitive than throat swab samples. Samples should be collected as soon as possible after the onset of symptoms. In adults, virus shedding typically declines on days 4 to 5 after the onset of symptoms, making virus detection difficult. Children typically shed virus for longer periods of time.

Serologic testing to assess influenza A virus subtypes and HA antigenic drift variants is typically carried out by using hemagglutination inhibition (HI) or microneutralization assays. These assays are used by the WHO because of their reliability, but are not recommended for standard diagnostic testing because of their complexity.

ANTIVIRALS

Four antiviral compounds—the M2 ion channel inhibitors amantadine and rimantadine, and the neuraminidase inhibitors oseltamivir and zanamivir—are currently approved in many countries for use in humans. Additional neuraminidase inhibitors such as peramivir and laninamivir received temporary emergency approval during the H1N1 pandemic in 2009, and/or are licensed in Japan and Korea. Most H1N1 and H3N2 viruses currently circulating in humans are sensitive to neuraminidase inhibitors; in contrast, more than 90% of currently circulating H1N1 and H3N2 viruses are resistant to the ion channel inhibitors. Accordingly, this latter class of compounds is no longer recommended for use in humans.⁵³⁵

Amantadine and Rimantadine

Amantadine hydrochloride and rimantadine, an analog of amantadine, were licensed for prophylactic and therapeutic use against influenza A virus in humans in the United States in the 1960s. These compounds are active against all subtypes of influenza A virus, but not against influenza B or C viruses.²⁰⁹

Amantadine and rimantadine are adamantane derivatives. Both compounds have a tricyclic structure with an amine side

group. They inhibit virus replication by blocking the acid-activated ion channel formed by the virion-associated M2 protein.^{110,757,758,872,1659,1660,1997,2192,2239} The primary antiviral action of these compounds results from blocking the flow of H⁺ ions from the acidified endosome into the interior of the virion, a process necessary for release of ribonucleoprotein complexes into the cytosol for transport to the nucleus. These compounds also inhibit the replication of viruses that have multiple basic amino acids at their HA cleavage site by inhibiting M2 ion channel activity in the *trans*-Golgi network, which prevents the premature low pH-induced conformational change of HAs cleaved by furin.^{316,1764,1782}

Until 2004, amantadine and rimantadine were used to treat infections caused by seasonal influenza A viruses.^{25,575,720,1056,1057} Symptomatic improvement, including accelerated clearance of local symptoms and fever, occurs about 1 day earlier in treated than in untreated patients, and peripheral airway dysfunction also resolves more quickly. Accelerated clearance of symptoms was seen in children infected with an H3N2 virus treated with rimantadine compared with control children who received an antipyretic.⁷²⁰

Amantadine and rimantadine have also been used for prophylaxis against seasonal influenza A virus infections.^{25,36,387,431,915,1141,1412,1622,1646,1725,1828,1908–1910,2073,2074,2381} During an epidemic involving both influenza A H1N1 and H3N2 viruses, amantadine and rimantadine protected against influenza-like illness (78% and 65%, respectively), documented influenza illness (91% and 85%, respectively), and influenza A virus infection (74% and 66%, respectively).⁴³¹ Lower efficacy rates against documented illness (70%) and infection (39%) were observed for amantadine prophylaxis during an H1N1 virus epidemic in 1977.¹⁴¹²

At the recommended adult dose of amantadine or rimantadine (i.e., 100 mg twice daily orally), significant adverse effects have not been reported (reviewed in⁹⁰⁴).

Resistance to adamantanes is conferred by mutations at position 26, 27, 30, 31, 34, or 38 of M2, with mutations at position 27, 30, or 31 found most frequently.^{110,757,758,1658,2008} Structural studies of the M2 ion channel in the absence and presence of amantadine^{210,1805,1976} show that the channel is relatively narrow at position Val27, but opens into a wider cavity that is lined by Ala30 and Ser31 (among other amino acids), providing structural information on the effect of mutations at these critical positions.

In experimental settings, resistant variants emerge rapidly and frequently.^{788,1587,1588,1590,2176} Until 2004, the rates of resistance among seasonal influenza viruses remained low,^{102,389,2379} although adamantane-resistant variants had been isolated from infected individuals.^{408,493,765,776,893,1325,1587,1629,1774,1775,1803,1861} During the 2003 to 2004 season, the rate of adamantane-resistant seasonal H3N2 viruses increased to 12.3%.¹⁷⁵ One year later, more than 90% of seasonal H3N2 viruses had acquired adamantane resistance,^{176,416,761,799,1175,1383,1773} typically conferred by a Ser-to-Asn mutation at position 31 of M2. At that time, many seasonal H1N1 viruses remained sensitive to ion channel inhibitors.^{73,416,799} In 2009, the seasonal H1N1 viruses were largely replaced by the novel A(H1N1)pdm09 viruses, which are resistant to adamantanes.²⁵³ Hence, the H1N1 and H3N2 viruses circulating in humans since 2009 are resistant to adamantanes, thus use of these compounds is no longer recommended.^{535,537}

Several avian H5 viruses isolated before the emergence of the currently circulating HPAI H5N1 viruses were found to be resistant to amantadine.²¹⁷⁶ For HPAI H5N1 viruses, the rate of adamantane resistance varies greatly depending on their geographic origin.^{199,299,772,801,853,866,1163,1686} Based on sequence analysis, avian H6 viruses should be sensitive to amantadine⁸⁶⁶; among H7 and H9 viruses, some were identified with mutations known to confer resistance to adamantanes.⁸⁶⁶ Many European swine viruses of several lineages are resistant to adamantanes,^{1099,1804} likely because their M genes belong to the same phylogenetic lineage.¹⁰⁹⁹ The pandemic 1918 virus is sensitive to ion channel inhibitors.^{1714,2103}

Neuraminidase Inhibitors

The neuraminidase inhibitors oseltamivir and zanamivir were approved in the United States in 1999.¹⁴⁶⁰ They are effective against influenza A and B viruses and are currently recommended for “all persons with suspected or confirmed influenza requiring hospitalization or who have progressive, severe or complicated illness regardless of previous health or vaccination status”.⁵³⁵

The recommended treatment differs by age group (summarized in⁵³⁵). Five-day courses are recommended for therapeutic treatment, while longer courses may be used for prophylactic treatment. Oseltamivir is administered orally as a granulated powder in capsules. The standard dose for adults is 75 mg, twice daily. Oseltamivir is currently approved for persons aged 1 year and older. During the H1N1 pandemic in 2009, emergency approval was granted in the United States (<http://www.cdc.gov/h1n1flu/recommendations.htm>), Europe (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000402/WC500033106.pdf), and several other countries for the use of oseltamivir in infants younger than 1 year of age. Zanamivir is provided as a powder and administered by inhalation. For adults, two daily inhalations (5 mg each) are recommended for the treatment of infections, whereas one daily inhalation of 10 mg is recommended for prophylactic purposes. Zanamivir is approved for persons aged 7 years or older. Adverse effects to neuraminidase inhibitors include vomiting, abdominal pain, and nausea, but are generally mild^{762,763,1642,2171} (reviewed in⁴⁶²).

The first NA inhibitors included DANA (2-deoxy-2,3-dihydro-*N*-acetylneuraminic acid; Neu5Ac2en) and its *N*-trifluoroacetyl analog FANA, which were effective *in vitro*^{1379,1600} but did not inhibit replication of influenza viruses in animals.¹⁶⁰¹ After resolution of the NA structure,^{338,2145} DANA served as the lead compound in the rational design of drugs targeting the NA protein. Replacement of the 4-hydroxyl group on DANA with a guanidino group filled the unoccupied pocket in the NA active site with an inhibition constant (K_i) of 2×10^{-10} mol/L,^{2168,2262} resulting in zanamivir (5-(acetylamino)-4-[(aminoiminomethyl)-amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid, also called 4-guanidino-Neu5Ac2en).

An additional NA inhibitor, oseltamivir, was developed during the following years. This was achieved through the discovery and use of a hydrophobic pocket in the enzyme-active center that could accommodate lipophilic groups necessary to improve the inhibitor's oral bioavailability.^{1032,1834} The resulting compound, oseltamivir, is administered in the form of a pro-drug, oseltamivir phosphate; in the liver, oseltamivir phosphate

is converted into the active form, oseltamivir carboxylate (reviewed in³⁸³). Finally, an intravenously administered NA inhibitor, peramivir, was approved for emergency use in hospitalized patients during the H1N1 pandemic in 2009 but is not presently considered an option (except for Japan and Korea).⁹⁰⁸

The efficacy of oseltamivir and zanamivir in the treatment and prevention of influenza A and B virus infections have been assessed in a number of studies.^{25,201,461,535,904,916-918,1328,1875,2040} Both compounds are also effective against A(H1N1)pdm09 viruses.^{704,909,1887} In general, the initiation of treatment within 48 hours of the onset of symptoms is critical^{360,434,517,1177,1179,1261,1887,2334}; however, treatment later in infection may still provide some benefit.^{1173,1177-1179,1365,1887}

For uncomplicated infections with seasonal influenza viruses, both drugs reduce the duration of illness by about 1 day (if treatment is started early). In addition, the amount of virus shed appears to be reduced. Several studies suggest that neuraminidase inhibitors also reduce the risk of severe complications.^{272,736,778,936,953,1177-1179,2243}

Oseltamivir is effective against HPAI H5N1 viruses. It improves the outcome of mice^{659,1186,2324} and ferrets^{151,658} infected with highly pathogenic viruses. Data from infected individuals suggest that treatment with NA inhibitors improves survival rates; early treatment was again noted as a critical factor.^{2,796,957,959,1220,1829} Based on these findings, NA inhibitors are recommended by the WHO as the primary treatment for human HPAI H5N1 virus infections (http://www.who.int/csr/disease/avian_influenza/guidelines/pharmamanagement/en/index.html). Although treatment with NA inhibitors improves the outcome of HPAI H5N1 infections in humans, a significant percentage of individuals still succumb to the infection; in one example, even early treatment of infected individuals resulted in the emergence of resistant variants (see later discussion) and death of the treated patients.⁴⁰⁰ Higher doses and prolonged treatment with neuraminidase inhibitors have shown additional benefits in animal studies^{151,2324} and also in infected individuals.²

Oseltamivir may also be efficacious in the prophylaxis or treatment of human infections with avian viruses of the H7 subtype. During the H7N7 virus outbreak in The Netherlands in 2003 (see Transmission of Avian Influenza Viruses to Humans section), health care workers and their close contacts were prophylactically treated with oseltamivir, which likely prevented severe infections¹³⁷⁶; the reported cases of conjunctivitis^{546,1085} and death²¹³⁵ were limited to individuals who did not take oseltamivir.

Treatment with neuraminidase inhibitors does not appear to prevent the development of humoral antibodies, which is critical to protect against reinfection with antigenically similar viruses. Ferret studies have demonstrated protection against reinfection,^{151,658} and seroconversion has also been noted in individuals.^{171,257}

Resistance to Neuraminidase Inhibitors

Variants with reduced sensitivity to oseltamivir or zanamivir can be selected experimentally,^{72,143,334,687,691,1368,1383,1950,2021} and have been isolated from patients treated with neuraminidase inhibitors (see later discussion). Mutations that confer resistance to neuraminidase inhibitors are summarized in [e-Table 41.1](#); most of these mutations map to catalytic sites in NA (R118, D151, R152, R224, E276, R292, R371, and Y406;

N2 numbering),²¹⁴⁵ or framework sites (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425; N2 numbering)²¹⁴⁵ that stabilize the active site. The most frequently identified mutations are R292K or E119V for H3N2 viruses,^{35,686,1367,2064} and H274Y for H1N1 viruses.^{35,686,1367,2064} Most oseltamivir-resistant viruses remain sensitive to zanamivir.

Resistance to zanamivir is extremely rare in treated patients,⁶⁹⁰ likely because of the structural differences between zanamivir and oseltamivir.^{337,1767} However, several viruses have been isolated that are resistant to both zanamivir and oseltamivir (e-Table 41.1), including these following 5 viruses. (1) An A(H1N1)pdm09 virus isolated from an immunocompromised child possessing H274Y and I222R mutations that were acquired during treatment with oseltamivir, and subsequent treatment with zanamivir, respectively; the I222R mutation rendered the virus less susceptible to zanamivir, oseltamivir, and peramivir (see Other Antivirals Against Influenza section).²¹²⁷ (2) Another A(H1N1)pdm09 virus with H274Y and I222R mutations was isolated from an immunocompromised child sequentially treated with oseltamivir and zanamivir; retrospective analysis revealed that both mutations had emerged before the initiation of zanamivir treatment.¹⁵¹² (3) H274Y/I222V mutations were reported for oseltamivir-resistant viruses isolated from two individuals who developed A(H1N1)pdm09 virus infection after prophylactic treatment with oseltamivir.²⁴⁷ (4) In an infant with no history of neuraminidase inhibitor treatment, an influenza B variant possessing a D197E (D198E in N2 numbering) mutation in NA was detected, in addition to wild-type virus.¹⁵⁴⁴ While viruses with a D197N mutation are known to confer resistance to oseltamivir,^{686,751,879} the D197E mutation had not been described. Of note, the D197E mutation conferred resistance to oseltamivir, zanamivir, and peramivir (see Other Antivirals Against Influenza section).¹⁵⁴⁴ The crystal structures of the D197 (wild-type) and D197E (resistant) variants have been resolved with and without inhibitor,¹⁵⁴⁴ and revealed that the D197E mutation affects the interaction with the *N*-acetyl group of sialic acid and NA inhibitors. (5) Zanamivir treatment of a ferret experimentally infected with an HPAI H5N1 virus yielded a zanamivir-resistant variant possessing a Q136L mutation in NA.⁸⁵² This mutation also conferred reduced sensitivity to oseltamivir.⁸⁵²

Overall, the rate of resistance to oseltamivir remained relatively low until 2007 to 2008, and resistance was observed more often with H1N1 viruses^{689,1961,2233} than with H3N2^{88,377,879,1051,1563,1961} or influenza B viruses.⁸⁷⁹ Only 8 of 2287 influenza A and B isolated from humans during routine surveillance between 1999 and 2002 showed reduced susceptibility to oseltamivir.¹⁴¹⁶ Another analysis of 1050 viruses isolated during surveillance in 2000 to 2002 found no mutations at positions known to confer resistance to neuraminidase inhibitors.¹⁴³⁸ A Japanese study reported resistance rates of 0.3% to 2.2% for viruses isolated in 2003 to 2007,¹²⁸⁹ whereas a global surveillance study of viruses isolated in 2004 to 2007 reported a resistance rate of 0.4% (12/3261).¹⁸⁴⁴ Another study of almost 2,000 patients treated with oseltamivir found resistance rates of 0.33% and 4.0% for viruses isolated from adults or children, respectively.³⁵ Resistance emerges frequently after prolonged treatment of immunocompromised patients, in whom the viruses replicate for extended periods of time.^{88,248,352,457,690,800,879,1382,1543,1563,2233}

During the 2007 to 2008 season, an increase in the number of oseltamivir-resistant H1N1 viruses was noted: in the

United States, 6.4% of isolates were resistant,¹⁸⁴⁴ while this number was even higher in Europe (with a resistance rate of ~20%) (http://ecdc.europa.eu/en/activities/surveillance/EISN/Newsletter/SUN_EISN_INFL_Bulletin_2008week16.pdf). In Europe, large differences were reported from country to country, with resistance rates between 1% in Italy and 68% in Norway.^{1092,1377} In 2008, 64% of seasonal H1N1 viruses tested in the Southern Hemisphere were resistant to oseltamivir,⁸⁵¹ and by March 2009, 1291 of 1362 seasonal H1N1 viruses analyzed had acquired resistance (http://www.who.int/csr/disease/influenza/H1N1webupdate20090318%20ed_ns.pdf). Clinical data indicate that oseltamivir-resistant H1N1 viruses are comparable in their severity to oseltamivir-sensitive viruses,^{194,317,417,755,1377} a finding that is consistent with studies in ferrets.⁷³²

In 2009, the oseltamivir-resistant H1N1 viruses were largely replaced by A(H1N1)pdm09 viruses. Most A(H1N1)pdm09 viruses are sensitive to neuraminidase inhibitors; however, up to 1% of viruses are oseltamivir resistant.^{89,218,222,247,248,352,457,745,871,1166,1382,1512,1933,2057,2064,2083,2127,2188}

Currently, most HPAI H5N1 viruses are sensitive to neuraminidase inhibitors, although several resistant variants have been isolated.^{400,464,606,636,800,801,853,1164} Of particular concern, avian surveillance studies have identified a number of HPAI H5N1 viruses with NA mutations that are known to confer resistance to neuraminidase inhibitors.¹⁵⁷⁸

Early studies indicated that viruses resistant to oseltamivir are attenuated in animal models, as demonstrated for lab-adapted H1N1 viruses,^{5,142} seasonal H1N1^{787,892} and H3N2 viruses,^{160,234,786,2021} an avian H4N2 virus,⁶⁹¹ and artificially generated viruses.²³²¹ Based on these findings, viruses resistant to neuraminidase inhibitors were not expected to out-compete the viruses circulating at the time. This assumption was consistent with the finding that oseltamivir-resistant viruses isolated from treated individuals did not transmit efficiently among humans (exceptions include an oseltamivir-resistant influenza B virus that may have arisen spontaneously or was transmitted from a treated patient,⁷⁵¹ and a community cluster of oseltamivir-resistant A(H1N1)pdm09 viruses.¹¹⁶⁶ Therefore, the rapid spread of oseltamivir-resistant seasonal H1N1 viruses in 2007 to 2008 was unexpected. However, these viruses possess additional mutations in NA (V234M and R222Q) that compensate for the loss of viral fitness due to the H274Y mutation.¹⁴⁷

In animal models, some oseltamivir-resistant A(H1N1)pdm09 viruses appear attenuated,⁴⁵⁰ while others are comparable in their pathogenicity and transmissibility to oseltamivir-sensitive A(H1N1)pdm09 viruses^{732,1052,1830}; the latter finding raises concerns that the resistant variants may supersede sensitive viruses, as was observed in 2007 to 2008 with seasonal H1N1 viruses. This concern is potentiated by increasing numbers of A(H1N1)pdm09 viruses possessing an S247N mutation that confers reduced sensitivity to oseltamivir and zanamivir.⁸⁵⁰ In an oseltamivir-treated patient, this mutation was found in combination with the H274Y mutation, resulting in extremely high resistance to oseltamivir.⁸⁵⁰

Combination Therapy

Although a number of influenza viruses have acquired resistance to adamantane or neuraminidase inhibitors, HPAI H5N1 viruses remain largely sensitive to both classes of compounds. Therefore, the combined use of adamantanes and neuraminidase inhibitors has been assessed in several *in vitro* and *in vivo*

studies.^{574,657,865,867,1323,1425} The combination of amantadine and oseltamivir was well tolerated in volunteers,¹⁴²⁵ and superior to monotherapy in terms of efficacy in mice.^{867,1323} Similarly, the combination of rimantadine with oseltamivir, zanamivir, or peramivir provided additive effects in cell culture⁶⁵⁷ and mice.⁵⁷⁴ Combination therapy could reduce the rate of emergence of resistant variants, as demonstrated in one study in cell culture.⁸⁶⁵

Other Antivirals Against Influenza

Peramivir, a neuraminidase inhibitor that is administered intravenously, received market authorization in Japan and Korea in 2010. In several other countries, including the United States, peramivir is currently in clinical trials⁵⁴ (<http://www.clinicaltrials.gov/ct2/show/NCT00958776?term=biocryst%2C+peramivir&rank=1>; accessed 06/05/2011). During the H1N1 pandemic in 2009, peramivir was available in the United States by Emergency Use Authorization (which expired in June 2010).^{135,789} Its efficacy has been assessed in mice and ferrets,^{1878,2346} and limited data on peramivir use in humans suggest a reduction in the duration of symptoms.^{74,213,789} Several mutations that confer resistance to oseltamivir also render those viruses resistant to peramivir (e-Table 41.1).

Laninamivir, a long-lasting inhaled NA inhibitor, is effective against oseltamivir-resistant influenza viruses.^{2204,2304} It is licensed in Japan^{1102,2304} and is in clinical trials in other countries.^{2204,2304}

T-705 (favipiravir) is an antiviral compound currently in phase II clinical trials in the United States (http://www.fujifilmholdings.com/en/pdf/investors/library/ff_announcement_20100215_001.pdf); in Japan, a phase III clinical trial has been completed (<http://www.clinicaltrials.jp/user/showCteDetailE.jsp?japicId=JapicCTI-090934>). It has favorable pharmacokinetics in cell culture¹⁸⁹⁵ and is effective against influenza A, B, and C viruses,^{564,1877,2023} including HPAI H5N1 viruses sensitive or resistant to oseltamivir.^{1053,1891,1896} T-705 inhibits the viral polymerase⁵⁶⁵ but, unlike ribavirin, does not affect cellular DNA or RNA polymerases.⁵⁶⁵ T-705 may be attractive for combination therapy with a neuraminidase inhibitor.¹⁸⁹⁶ Other targets include both viral and host pathways involved in influenza replication.⁷⁶⁰ An example would be the host-encoded proteases that cleave the influenza HA²³⁶⁴ (see HA cleavage section).

VACCINES

Vaccination is one of the most effective methods for preventing influenza virus infections and complications.³⁵⁵ Both inactivated and live attenuated vaccines are available against seasonal influenza viruses. These vaccines are currently trivalent, that is, they contain influenza A virus components of the H1N1 and H3N2 subtypes, and an influenza B virus component. Trivalent inactivated vaccines can be used for persons older than 6 months of age, while live attenuated vaccine is currently approved in the United States for healthy, nonpregnant individuals 2 to 49 years of age. In 2009, monovalent vaccines to A(H1N1)pdm09 virus were used widely. In the 2010 to 2011 season, A(H1N1)pdm09 virus became part of the trivalent vaccine and replaced the former seasonal H1N1 component. In addition, several vaccines to HPAI H5N1 viruses have been

approved. Other vaccines to H5, H7, and H9 viruses are in development.

Seasonal Inactivated Vaccines

Based on the immune status of the populations, and on antigenic and genetic information about circulating viruses (obtained through surveillance studies), the vaccine strains are recommended each year by the WHO (see Surveillance section). Since this decision has to be made more than 6 months prior to the influenza season, the selected vaccine strains occasionally differ antigenically from the viruses circulating during the subsequent influenza season. Limited antigenic match between the selected vaccine strains and the actually circulating strains may result in low efficacy (see Efficacy of Seasonal Influenza Vaccines section). After vaccine virus selection, so-called seed strains are generated that possess at least the HA and NA genes of the selected vaccine strains, and the remaining genes from the A/Puerto Rico/8/34 (A/PR/8/34; H1N1) virus, which confers a high level of growth in eggs.¹⁷⁴⁰ Traditionally, these seed viruses were generated by co-infecting cells with A/PR/8/34 virus and the donor virus of the HA and NA genes, and subsequent selection with antibodies to select viruses with the desired gene constellation. Although not yet used widely, reverse genetics methods (see Reverse Genetics section) can be used to accomplish this task faster and in a more controlled manner. As an alternative to these reassortant vaccine viruses, some countries use the WHO-recommended wild-type viruses for vaccination.

Most vaccine viruses are grown in the allantoic cavity of embryonated chicken eggs; in this system, however, they may acquire mutations in HA that can change the antigenic properties of the virus (see Virus Propagation and Host Cell-Mediated Selection of Antigenic Variants sections). In Europe and in the U.S., vaccines produced in MDCK cells are now also marketed. Several theoretical advantages exist for preparation of vaccine in mammalian cells including maintaining the mammalian HA phenotype and relative easiness of capacity expansion compared to egg-based vaccine production.²²⁶⁷

For vaccine virus production in embryonated chicken eggs, virus present in the allantoic fluid is purified and concentrated by zonal centrifugation or column chromatography and inactivated with formalin or beta-propiolactone. The resulting preparations are referred to as whole virus vaccine; due to their reactogenicity, whole virus vaccines are no longer used widely. Vaccine virus preparations may be further disrupted by treatment with chemicals (split vaccine) and partially purified to remove viral ribonucleoprotein complexes (subunit vaccine). Split and subunit vaccines are often collectively referred to as subvirion vaccines. In addition, so-called virosomal subunit vaccines can be generated by treating viruses with detergent, followed by ultracentrifugation to remove viral ribonucleoprotein complexes. After the extraction of detergent, membrane vesicles form that contain the HA and NA proteins.²²⁵⁰ Collectively, these purification procedures have greatly reduced the incidence of local and systemic reactions.

The quantity of immunoreactive HA in each dose is standardized to contain the amount recommended by the Advisory Committee on Immunization Practices, which is usually 15 μ g per component for adults and older children or 7.5 μ g for children younger than 3 years. The quantity of NA is not standardized because this glycoprotein is highly labile during

purification and storage.^{1012,1015} Each 0.5-mL dose of vaccine contains approximately 10 billion virus particles, and one egg yields one to three doses of vaccine. Vaccine also contains variable but small quantities of endotoxin, egg-derived protein, free formaldehyde, and most have thiomersal preservative, all of which do not appear to contribute to the reactogenicity or toxicity of the vaccines for humans. Inactivated vaccines are administered intramuscularly or, less often, subcutaneously or intradermally.

Seasonal Live Attenuated Vaccines

A live attenuated vaccine based on the A/Ann Arbor/6/60 (A/AA/6/60; H2N2) and B/Ann Arbor/1/66 (B/AA/1/66)^{108,472,1294,1451} viruses was developed in the United States and approved in 2003.^{108,1517,2080} A different live attenuated vaccine based on the A/Leningrad/134/17/57 and B/USSR/60/69 viruses has been used in the Russian Federation since the 1960s.^{16,1011,1014}

The A/AA/6/60 master donor virus was developed by passage of the wild-type virus at progressively lower temperatures in primary chicken kidney cell cultures until a mutant was identified that replicated efficiently at 25°C, a temperature restrictive for the replication of wild-type virus.^{1294,1295} The donor virus is temperature sensitive (*ts*; i.e., restricted in its replication at 38°C to 39°C, temperatures permissive for the replication of wild-type virus); cold adapted (*ca*; i.e., replicates efficiently at 25°C); and attenuated (*att*; i.e., restricted in its replication in the upper and lower respiratory tract of ferrets). The attenuated B/AA/1/66 master donor virus was developed by using a similar approach.

For the A/AA/6/60 master donor virus, the *ts* and *att* phenotypes are each defined by mutations in the PB2, PB1, and NP proteins,^{929,931} while the mutations that define the *ca* phenotype have not been identified. For the B/AA/1/66 master donor virus, the *att*, *ts*, and *ca* phenotypes are defined by mutations in the PA, NP, and M1 proteins⁸²³; the PA and NP⁸²³ proteins; or the PB2, PA, and NP proteins,²⁹⁴ respectively. The type A and type B master donor viruses differ by only seven or eight amino acids from the respective wild-type viruses; however, each of the characterized phenotypes is conferred by at least two mutations in different proteins, and reversion to wild type has not been observed.^{1010,1453,2152}

The attenuated A/Leningrad/134/17/57 virus was developed through 17 sequential passages in embryonated chicken eggs at 25°C. It differs by eight amino acids from the parental virus, which map to the PB2, M1, M2, and NS2 proteins (one amino acid change each), and the PB1 and PA proteins (two amino acid changes each).¹⁰⁶² The *ts* phenotype is conferred by mutations in the polymerase proteins.^{874,1047} The *ts* phenotype of the attenuated B/USSR/60/69 virus is defined by mutations in the PB2 and PA genes.¹⁰⁴⁸

Originally, seed viruses for vaccine production were generated by co-infecting, for example, the A/AA/6/60 master donor virus with the recommended vaccine viruses, followed by selection of reassortant progeny viruses at 25°C (restrictive for replication of wild-type virus) in the presence of an H2N2 antiserum, which inhibits replication of viruses bearing the surface antigens of the attenuated A/AA/6/60 master donor virus. In the United States, live attenuated vaccine viruses are now generated by using reverse genetics methods. Vaccine viruses are then grown in embryonated eggs, filtered, and concentrated. The vaccine is administered intranasally by spray.

Shedding of live attenuated viruses on days 1 to 2 post-vaccination is frequent in young children, but decreases with age.^{111,144,1043,1045,1194,1307,2031,2152} So far, only one case of virus transmission has been reported.²¹⁵²

Reactogenicity of Seasonal Influenza Vaccines

For inactivated vaccines, local or systemic allergic reactions to vaccine components are rare,^{126,1460,1594} and are primarily due to residual egg protein.^{1460,1594} Reactions generally occur within the first 24 hours and last for 1 to 2 days; they include systemic manifestations such as fever, malaise, myalgia, and headache, as well as local manifestations such as pain, erythema, induration, and tenderness at the inoculation site. The reactogenicity of influenza B virus vaccine is similar to that of influenza A virus.^{20,643,1156,1546} In children, whole virus vaccines cause more systemic reactions than subvirion vaccines^{127,166,382,1154,2273}; for children younger than 12 years of age, subvirion vaccines are therefore recommended. Cell culture-grown vaccines are comparable in their reactogenicity to egg-grown vaccines.^{27,673,1597,1598,1718} More recently developed adjuvants, MF59 and AS03, have increased local reactogenicity. For live attenuated vaccines, mild symptoms including stuffy or runny nose, sore throat, and/or mild fever have been reported.^{103,108,536,902,1517,1553,1554,2080}

An increase in reported cases of GBS (see Central Nervous System Involvement section) occurred in the United States in 1976 to 1977 during vaccination to the swine H1N1 virus that infected recruits in New Jersey^{1318,1754,1771,1815,2260} (see Interspecies Transmission section). About 1 in 100,000 individuals who received the vaccine against the 1976 swine H1N1 influenza virus developed GBS, with a relative risk in vaccine recipients about four- to eightfold greater than that in unvaccinated subjects^{1318,1771,1815}; these findings led to the cessation of the vaccination program. Typically, the risk of GBS after influenza virus vaccination is very small.^{710,950,966,1928,1929} Preliminary data from the A(H1N1)pdm09 vaccination program in 2009 suggests a definable but minimal risk of GBS.²⁵⁰

In vaccinated individuals, exacerbated illness (as observed after measles or respiratory syncytial virus vaccination) does not occur. There has been a single instance in which vaccinated school-aged children had more influenza-related lower respiratory tract illness than their unvaccinated counterparts.⁹¹² Inactivated influenza virus vaccines otherwise have a consistent record of efficacy and safety.

Immunogenicity of Seasonal Influenza Vaccines

The immunogenicity of a vaccine is significantly affected by prior exposure to the antigen. In unprimed individuals (such as children or persons with no or limited previous exposure to the respective antigen), higher amounts of antigen are required to elicit satisfactory levels of antibody. Thus, two doses of inactivated vaccine are recommended for children in their first year of vaccination.^{537,1500,1502,2184} In the elderly, serum antibody responses are typically lower than in younger subjects,^{646,1364,1596} which spurred the development of a high-dose vaccine (containing 60 µg of HA per component) that is now available for adults 65 years of age and older.^{245,537,2001}

Whole virion vaccines are more immunogenic and cross-protective than subvirion vaccines,^{127,170,496,610,1224,1579,1673,1964,2269,2273} likely because the RNA content of whole virus preparations stimulates TLR7⁶¹⁰ (see Pathogen Recognition

Receptors section); however, due to their higher reactogenicity, they are not used widely.

Live attenuated vaccine viruses replicate primarily in epithelial cells of the nasopharyngeal mucosa where they induce mucosal IgA antibodies, serum IgG antibodies, and cellular immunity, overall stimulating better cross-protection than inactivated viruses^{30,106,107,356,544,773,2076,2129} (reviewed in²⁰⁸⁰). Compared to inactivated vaccines, vaccination with live attenuated vaccine more closely resembles a natural infection, stimulating both arms of the immune system and providing better heterosubtypic immunity.^{356,1519,1962}

The addition of adjuvants to seasonal vaccines [and to avian virus vaccines and A(H1N1)pdm09 vaccines] increases their immunogenicity, particularly in immunologically naïve individuals^{126,577,1524} [see also Vaccines to Avian Influenza Viruses and Vaccines to A(H1N1)pdm09 Virus sections].

Controlled studies comparing the antibody response after intradermal and parenteral administration of vaccine containing comparable amounts of HA antigen indicated that intradermal administration offers a dose-sparing advantage over parenteral administration.^{1117,1695,1696,2143} Such approaches may be particularly important in the face of a pandemic when estimates indicate that vaccine supply will be a rate-limiting step.

The duration of immunity after inactivated influenza vaccination has not been evaluated systematically, but a relatively rapid decline in antibody levels suggests that protective immunity is of short duration. At any rate, antigenic changes in the influenza virus predicate annual immunization with current vaccines.

Efficacy of Seasonal Influenza Vaccines

Vaccination with influenza A and B viruses reduces infection and influenza-related illness.^{50,472,523,921,1378,1422,1423} The timing of vaccination is important since protective levels of antibodies are usually not reached until 2 weeks after vaccination.^{177,1157}

For inactivated vaccines, the levels of neutralizing antibodies against the infecting strain correlate well with the level of protection.^{326,813,1499,1591,1674} For live attenuated vaccines, the hemagglutination inhibition antibody test is not an optimal correlate, as seroconversion rates often remain low,^{109,544,978,2086} and protection has been observed in the absence of significant levels of antibody responses.^{472,2089}

Vaccine efficacy, defined as the reduction of cell culture–confirmed influenza illness, ranges from 70% to 90% in years in which the vaccine strains closely match the circulating viruses, and 50% to 80% in years when the vaccine is not well matched.^{98,172,411,536,790,919,1515,1517,1553,1874,1977,2160} Several meta-analyses have clearly established the benefits of vaccination.^{790,920,1515,2160} In healthy adults, inactivated and live attenuated vaccines provide similar levels of protection.^{172,411,472,482,1514,1515,1553,1554,1977,2080,2089,2199}

In the elderly, vaccine efficacy is significantly lower than that in young adults,^{69,70,672,1769} ranging from 20% to 40% in years of good antigenic match, and little protection in years of poor antigenic match.^{335,903,1217,1413,1518,1552} Limited data suggest that live attenuated and inactivated vaccines are of similar efficacy in the elderly.^{650,2042}

In children, influenza vaccines are efficacious.^{105,108,164} In this age group, live attenuated vaccine is more efficacious than inactivated vaccine,^{45,103,539,919,1514,2080,2151} and also provides better protection against antigenically mismatched strains.^{106,571,728,729}

Compliance with the currently recommended two doses of vaccine for children may be low; however, several studies demonstrated that even a single dose of live attenuated vaccine elicits protective immune responses in more than half of the children tested.^{108,164,2033}

Vaccination protects the vaccine recipient, but unvaccinated individuals can also benefit if the extent of vaccination is sufficient to dampen the epidemic spread of virus in the community (herd effect).^{386,509,621,854,1039,1044,1411,1501,1653,2201,2238}

Vaccination of high-risk groups, including pregnant women,^{742,895,1230,1440,1505,2347} patients with impaired immunity due to cancer,^{191,348,524,1115,1253,1353,1580,1797,1849,1974,2330} organ transplants,^{9,133,134,148,173,242,455,492,597,769,1004,1040,1111,1115,1346,1354,1794,1913,2159} HIV infections,^{1043,1045,1115,1194,1225,1398,1498,2298} or patients with chronic heart disease^{392,1298} is efficacious, although the antibody responses may be lower than those in healthy individuals.

Recommendations for Seasonal Influenza Vaccines

Recommendations for vaccination in the United States have continued to expand. Currently, annual vaccination is recommended for all over 6 months of age.⁵³⁷ Protection of persons at higher risk for influenza-related complications should continue to be a focus of vaccination efforts as providers and programs transition to routine vaccination of all persons aged 6 months or older. A summary of current influenza vaccination recommendations follows (modified after⁵³⁷):

When vaccine supply is limited, vaccination efforts should focus on delivering vaccination to person who:

- Are aged 6 months to 4 years (59 months)
- Are aged ≥50 years
- Have chronic pulmonary (including asthma), cardiovascular (except hypertension), renal, hepatic, neurologic, hematologic, or metabolic disorder (including diabetes mellitus)
- Are immunosuppressed (including immunosuppression caused by medications or by human immunodeficiency virus)
- Are or will be pregnant during the influenza season
- Are aged 6 months to 18 years and receiving long-term aspirin therapy and who therefore might be at risk for experiencing Reye syndrome after influenza virus infection
- Are residents of nursing homes and other chronic-care facilities
- Are American Indians/Alaska Natives
- Are morbidly obese (body mass index ≥ 40)
- Are health-care personnel
- Are household contacts and caregivers of children aged less than 5 years and adults aged 50 years or older, with particular emphasis on vaccinating contacts of children aged <6 months
- Are household contacts and caregivers of persons with medical conditions that put them at higher risk for severe complications from influenza

Vaccines to A(H1N1)pdm09 Virus

Based on the initial characterization of A(H1N1)pdm2009 viruses, the WHO recommended a vaccine strain (A/California/07/2009) on May 26, 2009 (http://www.who.int/csr/resources/publications/swineflu/H1N1Vaccinevirus_recommendation26May2009.pdf). Early studies indicated that the

candidate vaccines grew poorly in eggs (<http://www.thelancet.com/H1N1-flu/egmn/0c03c805>), and that two doses of vaccine might be needed to elicit protection against the novel virus.^{439,1071} However, several egg- and cell culture-grown, inactivated and live attenuated vaccines became available that grew to reasonable titers and, more importantly, triggered robust immune responses after one vaccination^{254,324,666,1215,1535,1664,1751,2122,2124,2249,2376} (for an overview of clinical trials, see⁶²⁷). Overall, seroconversion rates were lower in children,^{1215,1664,2376} although one dose of vaccine induced protective immune responses in some children.¹⁵³⁵ The robust immune responses in adults likely resulted from partial immunity due to prior infection with an antigenically similar strain (see The H1N1 Pandemic in 2009 section); in the United States, vaccination with the 1976 swine H1N1 virus also contributed to partial immunity to the A(H1N1)pdm09 virus.^{982,1361}

Vaccines against A(H1N1)pdm09 virus became available in China, Europe, Australia, and the United States in the fall of 2009^{251,254,933,1975} (<http://www.ema.europa.eu/influenza/vaccines/home.htm>; <http://www.tga.gov.au/alerts/medicines/h1n1vaccine.htm>). By February 2010, about 30 A(H1N1)pdm09 vaccines had been approved.⁶²⁷ In the United States, nonadjuvanted subvirion vaccines and a live attenuated vaccine were available; one dose was recommended for adults, and two doses for children between the age of 6 months and 9 years. In Europe, only inactivated (whole virus and subvirion) vaccines have been approved, and two doses are recommended for all age groups; both nonadjuvanted vaccines and preparations adjuvanted with MF-59 or ASO3 received market authorization. MF-59 greatly enhanced the responses to A(H1N1)pdm09³²⁴ and HPAI H5N1¹⁹⁶⁰ vaccines. A similar dose-sparing and enhanced response to novel antigens has been seen with ASO3 in adults² and children.²³³

The safety of the A(H1N1)pdm09 vaccines has been monitored closely, particularly for cases of GBS (see Reactogenicity of Seasonal Influenza Vaccines section). While a few cases of GBS have been reported after vaccination with A(H1N1)pdm09 vaccines,⁸⁴⁵ the frequency of reported GBS cases does not exceed the baseline number of cases reported annually (http://vaers.hhs.gov/resources/2010H1N1Summary_June03.pdf).

Vaccines to Avian Influenza Viruses

For most vaccines to highly pathogenic avian influenza viruses, the multibasic cleavage site in the HA protein is replaced with a low-pathogenic motif^{834,837,1525,1992,2207} (see HA Cleavage section) to generate attenuated vaccine viruses. This step is critical to increase the biosafety of vaccine production, and to avoid low vaccine virus yields in eggs resulting from the killing of the chicken embryo by the highly pathogenic avian viruses.

The first clinical trials of vaccines to HPAI H5N1 viruses indicated that very high amounts of antigen²⁰⁸⁷ and/or two doses of antigen, possibly in combination with adjuvant,¹⁷⁰ may be required to elicit protective immune responses. Since then, a number of candidate vaccines have been tested in clinical trials, including inactivated whole virus vaccines,^{478,479,521,862,1224,2020,2121,2123,2275,2276} inactivated subvirion vaccines,^{63,96,104,117,124,167,309,312,313,422,777,840,1007,1008,1144,1188-1190,1193,1466,1537,1617,1761,1765,1826,1905,2153,2275,2276,2350} and live attenuated viruses.^{978,1761} Most of these candidate vaccines were

grown in eggs; however, several were propagated in Vero,⁴⁷⁹ MDCK,¹⁰⁰⁷ or primary monkey kidney cells.⁷⁶⁶ In addition, a DNA vaccine,¹⁹⁰⁵ virus-like particle vaccine (<http://www.medicalnewstoday.com/articles/139352.php>), and vaccines composed of recombinant M2 protein expressed in *Escherichia coli* (http://www.who.int/vaccine_research/immunogenicity/immunogenicity_table.xls2009), or recombinant HA protein expressed in insect cells,^{766,2093} have been tested in clinical trials. For an overview of clinical trials, see http://www.who.int/vaccine_research/diseases/influenza/flu_trials_tables/en/ (last modified: 06/08/2010; accessed 05/30/2011).

These studies tested various virus strains, vaccine preparations, and vaccination regimens, including different amounts of antigen administered in one to three doses at different intervals, with or without adjuvants. Generally, nonadjuvanted vaccines are of low antigenicity in humans^{124,167,170,309,1008,1189,1193,1311,2087}; the addition of aluminum salts as adjuvants (currently the only adjuvant approved in the United States for use in influenza vaccines) provided little or no increase in immunogenicity,^{124,170,1009,1189,1190,1311} whereas oil-in-water emulsions such as MF-59 (approved in Europe for use with inactivated vaccines) and ASO3 [approved in Europe for use with A(H1N1)pdm09 vaccine] increased the immunogenicity of experimental H5N1 vaccines more noticeably.^{63,124,1189,1193,1311,1765} By 2010, several egg-derived, inactivated H5N1 influenza vaccines had been approved in different countries, including four whole virus vaccines adjuvanted with aluminum and one split virus vaccine adjuvanted with ASO3 (reviewed in¹⁶⁷⁹).

Other vaccine approaches are in different stages of development. These include inactivated low-pathogenic surrogate H5N3^{576,1269,1524,1960,1963} or H5N4^{1240,2022} virus; H5 HA or NP protein expressed by baculovirus,^{641,2090,2093} adenovirus,^{594,820-822,828,1616,1706,2132,2149} alphavirus,^{847,1823} poxviruses,^{24,967,1097,1098,1122,1349,1669,1691,1692,1732,1954} or Newcastle disease virus.^{423,424} Virus-like particles generated by expressing the influenza virus HA, NA, M1, and/or M2 proteins have been tested,^{42,174,365,368,425,766,768,962-964,1142,1299,1638,1917,2016,2039,2274} and a VLP vaccine is now in clinical trials (<http://www.medicalnewstoday.com/articles/139352.php>). DNA vaccines to H5 HA, NP, NA, and/or M2^{270,286,287,425,502,505,743,1038,1076,1126,1128,1281,1604,1706,2079,2284,2312} have been tested, but often suffered from suboptimal immune responses; however, several DNA vaccines are now in clinical trials.^{447,928,937,1905} Delivery of a DNA construct by small-particle aerosol has proven highly immunogenic in nonhuman primates.¹⁹¹⁸

The rapid evolution of HPAI H5N1 viruses might result in suboptimal cross-protection against viruses from different clades, and several H5N1 vaccines may have to be prepared to cover the currently circulating clades (for an overview of recently circulating clades and their antigenic properties, and an overview of current candidate vaccines, see http://www.who.int/csr/disease/avian_influenza/guidelines/2011_02_h5_h9_vaccinevirusupdate.pdf). However, recent data indicate that vaccination with an antigenically distinct H5N1 vaccine has a priming effect that leads to a faster and stronger response upon revaccination.^{576,1965} Hence, prepandemic priming,⁹²² potentially limited to high-risk groups, may be considered to prepare for a possible H5N1 pandemic.

Similar vaccine strategies are being applied to viruses of the H7^{358,405,906,925,943,944,1392,1608,1611,2016,2078,2242} and H9 subtypes.^{47,211,775,977,1684,1685,1964} Clinical trials have been conducted

for live attenuated H7N3²⁰³⁰ and H9N2⁹⁷⁷ viruses. A cell culture-grown H7N1 split virus vaccine,³⁵⁷ and H9N2 whole virus and subunit vaccines¹⁹⁶⁴ have also been evaluated.

Universal Vaccines and Other Approaches to Influenza Vaccination

Universal influenza A vaccines that protect against viruses of all subtypes are highly desirable. Antibodies against the highly conserved ectodomain of the M2 ion channel protein are broadly cross-reactive (see Humoral Immunity section), but a DNA/adenovirus vaccine expressing M2 is suboptimal in ferrets.¹⁷⁰⁶ A vaccine in which the M2 ectodomain is expressed as a fusion peptide of the hepatitis B virus core protein is now in clinical trials.^{531,1816} Broadly neutralizing antibodies to a conserved region in HA2 (see Humoral Immunity section) are attractive as broad-acting therapeutics, while the HA2 stalk region may be an attractive vaccine candidate because of its broad protective efficacy.^{1953,2194} These approaches have not been tested yet in clinical trials. In another approach, a universal vaccine composed of a synthetic protein encoding conserved linear epitopes from the HA, NP, and M1 proteins is undergoing trials in humans.¹¹³ Other approaches to improving our current influenza vaccines include changes in the substrate for vaccine preparation, vector-based vaccines, DNA vaccines, recombinant proteins, virus-like particles (VLPs) and newer adjuvants (see review in¹¹³⁵).

PERSPECTIVES

Over the last decade, we have witnessed several important developments in influenza virology. Highly pathogenic avian influenza viruses, which in the past caused transient, local outbreaks, have now become enzootic in parts of Asia in the form of H5N1 viruses. It is unlikely that these viruses will be eradicated in the near future. More likely, they will continue to circulate in—and transmit between—wild bird and domestic poultry populations, with ample opportunity for reassortment with other avian influenza viruses. In 2007 to 2008, oseltamivir-resistant H1N1 viruses became dominant around the world. This event reminds us that novel antiviral compounds, ideally targeted at different viral proteins and/or steps in the viral life cycle, are urgently needed. Finally, the H1N1 pandemic in 2009 caught most researchers by surprise, since the next pandemic was believed to be caused by a virus of an HA subtype that is not circulating in humans. Hence, while the next pandemic was thought to be caused by a virus of the H5, H7, or H9 subtype (scenarios that should not be dismissed), the H1N1 pandemic in 2009 has renewed the awareness for the pandemic potential of H1, H2, and H3 viruses. In particular, H2 viruses, which have not circulated in humans since 1968, are now receiving more attention.

Techniques are in place to rapidly sequence, analyze, generate, and test novel influenza virus strains and potential vaccine candidates. The development of additional adjuvants and the transition to cell culture systems for vaccine production will provide more flexibility for scale-up in case of a pandemic. However, significant administrative and regulatory challenges in the development of novel vaccines and antivirals remain.

In summary, the most important lesson may be that influenza pandemics do occur, will continue to occur, and will continue to challenge our ability to respond.

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Bunyaviridae

History

Classification

Orthobunyavirus Genus
Phlebovirus Genus
Nairovirus Genus
Hantavirus Genus
Tospovirus Genus

Virion Structure

Morphology
 Biochemical and Biophysical Properties

Genome Structure and Organization

Viral Genome
 Coding Strategies of Viral Genes

Stages of Replication

Attachment and Entry
 Transcription and Replication
 Translation and Processing of Viral Proteins
 Morphogenesis

Effects of Viral Replication on Host Cells

Cytopathic Effects
 Host-Cell Metabolism
 Host-Cell Responses and Viral Suppression

Pathogenesis and Pathology

Orthobunyavirus Genus
Phlebovirus Genus
Nairovirus Genus
Hantavirus Genus

Epidemiology and Ecology

Orthobunyavirus Genus
Phlebovirus Genus
Nairovirus Genus
Hantavirus Genus

Clinical Features

Orthobunyavirus Genus
Phlebovirus Genus
Nairovirus Genus
Hantavirus Genus

Diagnosis

Orthobunyavirus Genus
Phlebovirus Genus
Nairovirus Genus
Hantavirus Genus

Prevention and Control

Orthobunyavirus Genus

Phlebovirus Genus

Nairovirus Genus

Hantavirus Genus

Tospovirus General Features Perspectives

HISTORY

The family *Bunyaviridae* takes its name from Bunyamwera virus (BUNV), which was isolated originally from *Aedes* mosquitoes in the Semliki Forest, Uganda, during a yellow fever study in 1943.⁴⁹⁹ Following the isolation of BUNV, several additional arboviruses were discovered that clearly did not fit into the classic arbovirus group A and B antigenic groups (which now are included in the *Flaviviridae* family or the *Alphavirus* genus of the *Togaviridae* family) and were assigned to what became known as group C arboviruses.⁸³ During the next decade, further virus discovery and detailed serologic studies, together with biochemical and morphologic analyses,³⁶³ led to the concept of a Bunyamwera supergroup consisting of groups of viruses that could be linked by repeatable serologic cross-reactions. Serogroups (viruses related by their reactivity in any serologic test) and complexes (closely related members of a serogroup) were further defined for these viruses.⁷³

The family *Bunyaviridae* was formally established in 1975.⁴²³ The International Committee on Taxonomy of Viruses (ICTV) approved the creation of the *Bunyavirus* genus in 1980, along with viruses morphologically and biochemically similar but antigenically distinct, that formed the *Uukuvirus*, *Phlebovirus*, and *Nairovirus* genera.⁴⁶ The discovery of a novel group of rodent-borne viruses and another group of plant infecting viruses with conforming molecular properties respectively led to the additions of the *Hantavirus* genus in 1985, and the *Tospovirus* genus in 1991. Further studies demonstrated a close biochemical similarity between uukuviruses and phleboviruses, resulting in their combination into the *Phlebovirus* genus in 1991.⁷¹ To prevent confusion when discussing viruses in the *Bunyavirus* genus as opposed to the family as a whole, the former was renamed *Orthobunyavirus* in 1995.¹⁴² Viruses within each genus are thus referred to as hantaviruses, nairoviruses, orthobunyaviruses, and so on, whereas the term “bunyavirus” is used to refer to any member of the family. Currently more than 350 named viruses are included in the family, making it one of the largest families of RNA viruses.

Except for the hantaviruses, which are transmitted in aerosolized rodent excreta, bunyaviruses are carried and

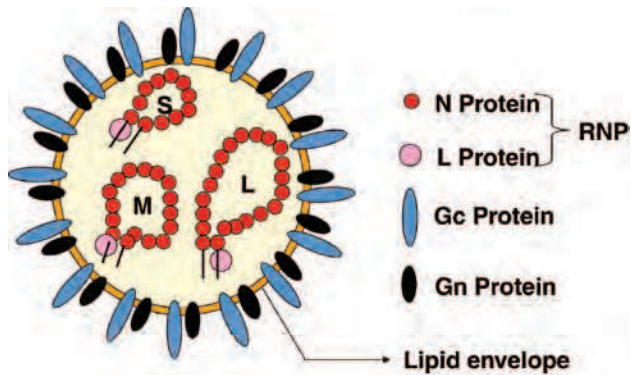


FIGURE 42.1. Schematic of a bunyavirus virion. The three genome segments (L, M, and S) are encapsidated by nucleocapsid protein to form ribonucleoprotein complexes (RNPs), and together with the viral L protein (RNA dependent RNA polymerase, RdRp) are packaged within a host cell–derived lipid envelope modified by insertion of the viral glycoproteins Gn and Gc. Note that there is no matrix protein.

transmitted by arthropods, primarily mosquitoes, ticks, sand flies, or thrips.

BUNV is the prototype of the family, and remains an important research model. It was the first bunyavirus whose genome was completely sequenced,^{141,306} and significantly was the first segmented genome negative-sense RNA virus that was recovered (rescued) entirely from cloned complementary DNA (cDNA).^{62,450}

CLASSIFICATION

The essential criteria for inclusion in the family are enveloped virions of 80 to 120 nm diameter (Fig. 42.1), containing a tripartite, single-stranded RNA genome of negative- or ambi-sense polarity, which replicate in the cytoplasm, and usually assemble at the Golgi complex. Assignment to one of the five genera is based on lack of serologic cross-reactivity with members of other genera, the patterns of sizes of virion proteins (Table 42.2) and genome segments (Table 42.1), gene expression strategy (Fig. 42.2), and conserved terminal nucleotide sequences of the genomic RNAs (Table 42.3). The ICTV, through the *Bunyaviridae* Study Group, describes criteria for the designation of individual species within each genus.³⁷² These designations, which are based on rather limited molecular data, should be treated with caution and as being fluid. The field requires large-scale, organized sequencing projects to more accurately understand the

relationships between these viruses, and in particular to investigate the extent of genome segment reassortment among isolated viruses.^{65,378,390,391} A potential prototype of a sixth genus, Gouleako virus, has recently been described.³²⁹ This virus is most similar to phleboviruses but appears restricted to mosquitoes and could not infect vertebrate cells in culture. Table 42.4 lists the most notable members in the currently defined genera, and a brief introduction to each genus follows.

Orthobunyavirus Genus

The largest genus of bunyaviruses is the *Orthobunyavirus* genus, which contains more than 170 named viruses that are found throughout the world. Almost all of these viruses are transmitted by mosquitoes and have amplification cycles in a variety of vertebrate hosts.^{73,372} Among important orthobunyavirus pathogens in humans are La Crosse virus (LACV) that causes pediatric encephalitis, Oropouche virus (OROV) that causes a debilitating febrile illness, and Ngari virus that causes hemorrhagic fever, whereas Aino, Akabane, and Cache Valley viruses are examples of viruses causing disease in domestic animals (Table 42.4).

Classification of orthobunyaviruses has proven to be a complex issue. The majority of viruses have been placed in one of 18 serogroups based on serologic relatedness of complement fixing antibodies (mediated by the N protein) and hemagglutinating and neutralizing antibodies (mediated by the glycoproteins), although a number of viruses classified into the *Orthobunyavirus* genus are currently not assigned to any of these serogroups.^{73,372} The 18 serogroups are Anopheles A, Anopheles B, Bakau, Bunyamwera, Bwamba, Group C, Capim, California, Gamboa, Guama, Koongol, Minatitlan, Nyando, Olifanstlei, Patois, Simbu, Tete, and Turlock. Serologic relatedness varies within a serogroup and is further complicated by the occurrence of natural reassortant viruses (e-Fig. 42.1), such that viruses may be more related to members of one group or another depending on the assay used.⁷²

The latest report of the ICTV delineates the orthobunyaviruses into 44 species,³⁷² although as described above such classification should be regarded as fluid because of the paucity of molecular data. Comprehensive molecular genetic studies have involved viruses in only 4 serogroups, namely Bunyamwera, Group C, California, and Simbu,^{57,126,378,457} but S segment nucleotide sequences have been obtained for one or two representatives of the Anopheles A, Anopheles B, Bakau, Bwamba, Nyando, Tete, and Turlock serogroups.^{292,351,588} In most cases the S genome segment encodes two proteins, N and, in an overlapping reading frame, a small nonstructural protein termed NSs. N and NSs proteins are translated from the same

TABLE 42.1 Pattern of Sizes of Viral RNA Segments in *Bunyaviridae* Genera

Genus RNA segment	<i>Orthobunyavirus</i>	<i>Hantavirus</i>	<i>Nairovirus</i>	<i>Phlebovirus</i>	<i>Tospovirus</i>
L	6.9	6.5	12.2	6.4	8.9
M	4.5	3.6	4.9	3.5	4.8
S	1.0	1.7	1.7	1.7	2.9
Total	12.4	11.8	18.8	11.6	16.6

Sizes given in kb.

TABLE 42.2 Pattern of Sizes of Viral Structural Proteins in *Bunyaviridae* Genera

Genus protein	<i>Orthobunyavirus</i>	<i>Hantavirus</i>	<i>Nairovirus</i>	<i>Phlebovirus</i>	<i>Tospovirus</i>
L	260	250	460	250	330
Gc	110	55	75	65	75
Gn	35	70	35	55–70	50
N	25	50	50	30	30

Sizes given in kD.

viral messenger RNA (mRNA) species as the result of alternate AUG-initiation codon selection.^{48,144} However, viruses in the Anopheles A, Anopheles B, and Tete serogroups do not show evidence of having an NSs ORF. In addition, these viruses have rather longer N proteins than those in the other serogroups.³⁵¹

Phlebovirus Genus

The *Phlebovirus* genus contains more than 80 viruses, with about half classified into nine antigenic complexes that are regarded as species, whereas 33 are considered as tentative species in the genus. The viruses of the genus *Phlebovirus* are present throughout the world, with the exception of Australia, and are more diverse in terms of arthropod vector than those of the other arthropod-borne genera. Most virus members are associated with phlebotomine sandflies, hence the genus name *Phlebovirus* (see Table 42.4). However, there are prominent exceptions, such as Rift Valley fever virus (RVFV), a medically and agriculturally important virus in Africa, which is primarily associated with *Aedes* species mosquitoes. In addition, Uukuniemi virus (UUKV), which has been used in a number of laboratory studies, is associated with the tick *Ixodes ricinus*. Recently a newly emerged phlebovirus was described in China (Huaiyangshan virus or severe fever with thrombocytopenia syndrome virus) that is associated with significant human mortality, and is transmitted by *Haemaphysalis* ticks.⁵⁹⁵

RVFV was first isolated in 1930 by Daubney et al. from an infected newborn lamb as part of an investigation of a large epizootic of disease causing abortion and high mortality in sheep.¹¹³ Large RVFV epizootics in various areas of sub-Saharan Africa have been noted since that time, and clinically compatible outbreaks have been retrospectively identified as far back as 1912.¹³³ Several decades passed before serologic and molecular similarities to phlebotomine fever viruses were noted. Sandfly fever Sicilian and Naples viruses (SFSV, SFNV)

were first isolated from American troops with febrile illness in the Palermo region of Sicily, Italy in 1943 and Naples, Italy in 1944, respectively, by inoculation of acute-phase sera into human volunteers and passage in newborn mice.⁴⁵⁶ Outbreaks of compatible human illness in the Mediterranean region date back to the time of the Napoleonic Wars, and association of the disease with sandflies was suggested as early as 1905.²⁰³

Nairovirus Genus

The nairoviruses are almost exclusively tick-borne viruses, although a few isolations have been made from *Culicoides* flies and mosquitoes (Table 42.4). Several serogroups exist, but the most important ones are the Crimean-Congo hemorrhagic fever (CCHF) group, which includes CCHF virus (CCHFV) and Hazara virus, and the Nairobi sheep disease (NSD) group, which includes NSD virus (NSDV) and Dugbe virus (DUGV).

The *Nairovirus* genus was named after NSDV, which was originally isolated in Nairobi, Kenya in 1910 by inoculation of sheep with the blood of sheep with acute gastroenteritis.³⁵⁴ The virus is now known to be present throughout various parts of Africa, and possibly India. CCHF was first recognized in the Crimean peninsula in the mid-1940s, when a large outbreak of severe hemorrhagic fever among agricultural workers was identified. The outbreak included more than 200 cases and a case fatality of about 10%.⁹⁹ Similar disease cases were later reported throughout the European and central Asian republics of the former Soviet Union, Romania, and Bulgaria. However, the virus was first isolated from a patient with a one-day fever in Kisangani, Democratic Republic of Congo, in 1956.⁴⁹⁴ It was some years before the connection was established, but subsequent serologic studies and virus isolations from Asia and Europe revealed that the viruses from the different outbreaks and geographic regions were essentially the same virus, which then became named CCHFV.^{82,100}

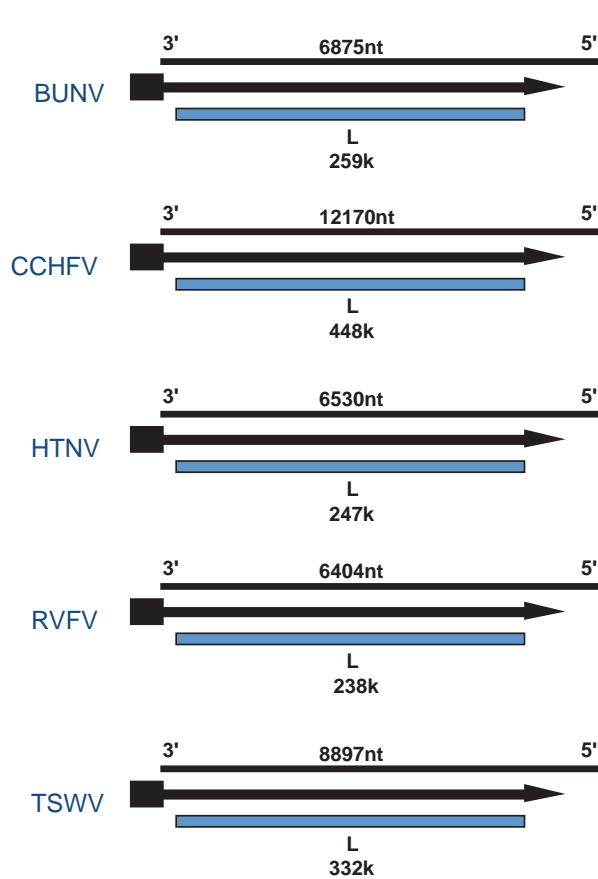
Hantavirus Genus

The discovery of hantaviruses traces back to 1951 to 1953, when United Nations troops were deployed during the border conflict between North and South Korea. More than 3,000 cases of an acute febrile illness were seen among the troops, about one third of which exhibited hemorrhagic manifestations, and an overall mortality of 5% to 10% was seen.^{129,305} The disease was initially termed Korean hemorrhagic fever but is now referred to as hemorrhagic fever with renal syndrome (HFRS). Despite considerable efforts, it took about 25 years until the field mouse, *Apodemus agrarius*, was identified as the rodent reservoir, and the virus was eventually isolated. At that

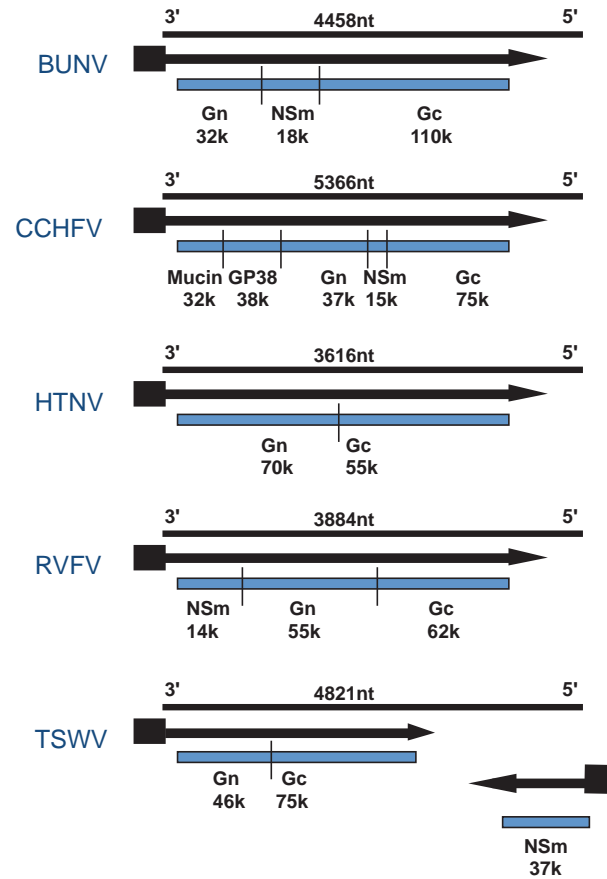
TABLE 42.3 Consensus 3' and 5' Terminal Nucleotide Sequences of Genome RNAs

<i>Orthobunyavirus</i>	3' UCAUCAUGA.....UCGUGUGAUGA 5'
<i>Hantavirus</i>	3' AUCAUCAUCUG.....AUGAUGAU 5'
<i>Nairovirus</i>	3' AGAGUUUCU.....AGAAACUCU 5'
<i>Phlebovirus</i>	3' UGUGUUUC.....GAAACACA 5'
<i>Tospovirus</i>	3' UCUCGUUAG.....CUAACGAGA 5'

L segment coding strategy



M segment coding strategy



S segment coding strategy

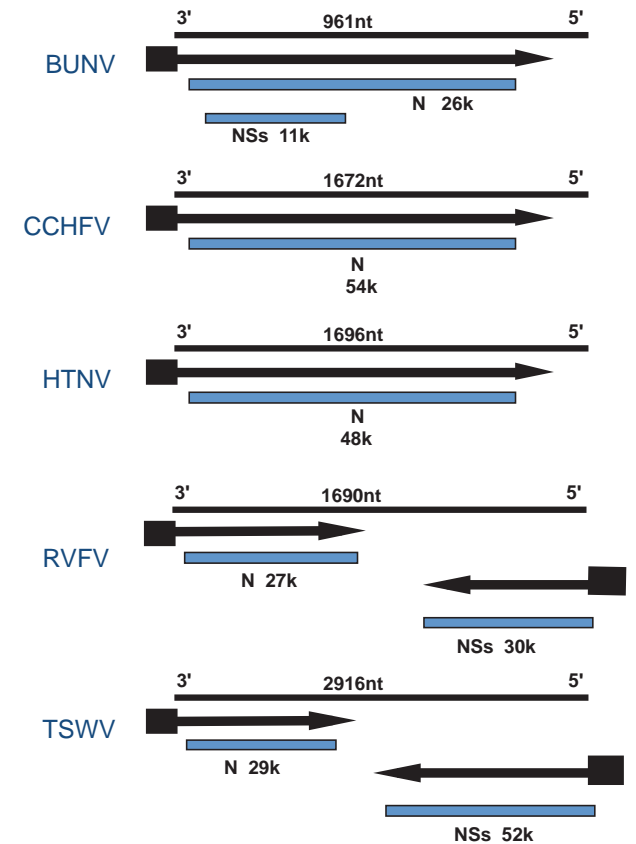


FIGURE 42.2. Coding strategies of *Bunyaviridae* genome segments. Genomic RNAs are represented by *thin lines* (the length in nucleotides is given above each segment) and the mRNAs are shown as *arrows* (■ indicates host-derived sequences at 5' end). Gene products, with their apparent M_r , are represented by colored boxes. BUNV, Bunyamwera orthobunyavirus; CCHFV, Crimean-Congo hemorrhagic fever nairovirus; HTNV, Hantaan hantavirus; RVFV, Rift Valley fever phlebovirus; TSWV, tomato spotted wilt tospovirus. In the CCHFV M segment, mucin represents mucin-like region.

TABLE 42.4 Species in the Family *Bunyaviridae*

Genus	Species	Notable virus	Geographic distribution	Principal vector	Disease
<i>Orthobunyavirus</i>	<i>Acara virus</i>	Acara virus	SA/ NA	Mosquitoes	
	<i>Akabane virus</i>	Akabane virus	Africa, Asia, Australia	Mosquitoes, culicoid flies	Cattle
	<i>Alajuela virus</i>	Alajuela virus	NA	Mosquitoes	
	<i>Anopheles A virus</i>	Anopheles A virus	SA	Mosquitoes	
	<i>Anopheles B virus</i>	Anopheles B virus	SA	Mosquitoes	
	<i>Bakau virus</i>	Bakau virus	Asia	Mosquitoes	
	<i>Batama virus</i>	Batama virus	Africa	N.D.	
	<i>Benevides virus</i>	Benevides virus	SA	Mosquitoes	
	<i>Bertioga virus</i>	Bertioga virus	SA	N.D.	
	<i>Bimiti virus</i>	Bimiti virus	SA	Mosquitoes	
	<i>Botambi virus</i>	Botambi virus	Africa	Mosquitoes	
	<i>Bunyamwera virus</i>	Bunyamwera virus	Africa	Mosquitoes	Human
		Batai virus	Asia, Europe	Mosquitoes	Human
		Cache Valley virus	NA	Mosquitoes	Sheep, Cattle, Human
		Fort Sherman virus	SA	Mosquitoes	Human
		Germiston virus	Africa	Mosquitoes	Human
		Ilesha virus	Africa	Mosquitoes	Human
		Ngari virus	Africa	Mosquitoes	Human
		Shokwe virus	Africa	Mosquitoes	Human
		Xingu virus	SA	Mosquitoes	Human
	<i>Bushbush virus</i>	Bushbush virus	SA	Mosquitoes	
	<i>Bwamba virus</i>	Bwamba virus	Africa	Mosquitoes	Human
		Pongola virus	Africa	Mosquitoes	Human
		California encephalitis virus	NA	Mosquitoes	Human
		Inkoo virus	Europe	Mosquitoes	Human
		Jamestown Canyon virus	NA	Mosquitoes	Human
		La Crosse virus	NA	Mosquitoes	Human
		Lumbo virus	Africa	Mosquitoes	Human
		Snowshoe hare virus	NA	Mosquitoes	Human
		Tahyna virus	Europe	Mosquitoes	Human
	<i>Capim virus</i>	Capim virus	SA	Mosquitoes	
	<i>Caraparu virus</i>	Caraparu virus	SA, NA	Mosquitoes	Human
		Apeu virus	SA	Mosquitoes	Human
		Ossa virus	NA	Mosquitoes	Human
		Catu virus	SA	Mosquitoes	Human
	<i>Estero Real virus</i>	Estero Real virus	NA	Ticks	
	<i>Gamboa virus</i>	Gamboa virus	NA	Mosquitoes	
	<i>Guajara virus</i>	Guajara virus	SA, NA	Mosquitoes	
	<i>Guama virus</i>	Guama virus	SA, NA	Mosquitoes	Human
	<i>Guaroa virus</i>	Guaroa virus	SA, NA	Mosquitoes	Human
	<i>Kairi virus</i>	Kairi virus	SA	Mosquitoes	Horse
	<i>Kaeng Khoi virus</i>	Kaeng Khoi virus	Asia	Nest bugs	
	<i>Koongol virus</i>	Koongol virus	Australia	Mosquitoes	
	<i>Madrid virus</i>	Madrid virus	NA	Mosquitoes	Human
	<i>Main Drain virus</i>	Main Drain virus	NA	Mosquitoes, culicoid flies	Horse
	<i>Manzanilla virus</i>	Ingwavuma virus	Africa, Asia	Mosquitoes	Pig
	<i>Marituba virus</i>	Marituba virus	SA	Mosquitoes	Human
		Murutucu virus	SA	Mosquitoes	Human
		Nepuyo virus	SA, NA	Mosquitoes	Human
		Restan virus	SA	Mosquitoes	Human

TABLE 42.4 Species in the Family *Bunyaviridae* (continued)

Genus	Species	Notable virus	Geographic distribution	Principal vector	Disease
<i>Hantavirus</i>	<i>Minatitlan virus</i>	Minatitlan virus	NA	Mosquitoes	
	<i>M'Poko virus</i>	M'Poko virus	Africa	Mosquitoes	
	<i>Nyando virus</i>	Nyando virus	Africa	Mosquitoes	Human
	<i>Olifantsvlei virus</i>	Olifantsvlei virus	Africa	Mosquitoes	
	<i>Oriboca virus</i>	Oriboca virus	SA	Mosquitoes	Human
		Itaqui virus	SA	Mosquitoes	Human
	<i>Oropouche virus</i>	Oropouche virus	SA	Mosquitoes, culicoid flies	Human
	<i>Patois virus</i>	Patois virus	NA	Mosquitoes	
	<i>Sathuperi virus</i>	Sathuperi virus	Africa, Asia	Mosquitoes, culicoid flies	
	<i>Simbu virus</i>	Simbu virus	Africa	Mosquitoes, culicoid flies	
	<i>Shamonda virus</i>	Shamonda virus	Africa	Culicoid flies	
	<i>Shuni virus</i>	Shuni virus	Africa	Mosquitoes, culicoid flies	
	<i>Tacaiuma virus</i>	Tacaiuma virus	SA	Mosquitoes	Human
	<i>Tete virus</i>	Tete virus	Africa	N.D.	
		Weldona virus	NA	culicoid flies	
	<i>Thimiri virus</i>	Thimiri virus	Africa, Asia	N.D.	
	<i>Timboteua virus</i>	Timboteua virus	SA	Mosquitoes	
	<i>Turlock virus</i>	Turlock virus	NA, SA	Mosquitoes	
	<i>Wyeomyia virus</i>	Wyeomyia virus	SA	Mosquitoes	Human
	<i>Zegla virus</i>	Zegla virus	NA	N.D.	
	<i>Andes virus</i>	Andes virus	SA	<i>Oligoryzomys longicaudatus</i>	Human
		Bermejo virus	SA	<i>Oligoryzomys chacoensis</i>	Human
		Lechiguanas virus	SA	<i>Oligoryzomys flavescens</i>	Human
		Maciel virus	SA	<i>Bolomys obscurus</i>	
		Oran virus	SA	<i>Oligoryzomys longicaudatus</i>	Human
		Pergamino virus	SA	<i>Akadon azarae</i>	
	<i>Bayou virus</i>	Bayou virus	NA	<i>Oryzomys palustris</i>	Human
	<i>Black Creek Canal virus</i>	Black Creek Canal virus	NA	<i>Sigmodon hispidus</i>	Human
	<i>Cano Delgadito virus</i>	Cano Delgadito virus	SA	<i>Sigmodon alstoni</i>	
	<i>Dobrava-Belgrade virus</i>	Dobrava virus	Europe	<i>Apodemus flavicollis</i>	Human
	<i>El Moro Canyon virus</i>	El Moro Canyon virus	NA	<i>Reithrodontomys megalotis</i>	
	<i>Hantaan virus</i>	Hantaan virus	Asia	<i>Apodemus agrarius coreae</i>	Human
	<i>Isla Vista virus</i>	Isla Vista virus	NA	<i>Microtus californicus</i>	
	<i>Khabarovsk virus</i>	Khabarovsk virus	Asia	<i>Microtus maximowiczii</i> , <i>Microtus fortis</i>	
	<i>Laguna Negra virus</i>	Laguna Negra virus	SA	<i>Calomys laucha</i>	Human
	<i>Muleshoe virus</i>	Muleshoe virus	NA	<i>Sigmodon hispidus</i>	
	<i>New York virus</i>	New York virus	NA	<i>Peromyscus leucopus</i>	Human
	<i>Prospect Hill virus</i>	Bloodland Lake virus	NA	<i>Microtus ochrogaster</i>	
		Prospect Hill virus	NA	<i>Microtus pennsylvanicus</i>	
	<i>Puumala virus</i>	Puumala virus	Europe, Asia	<i>Myodes glareolus</i>	Human
	<i>Rio Mamore virus</i>	Rio Mamore virus	SA	<i>Oligoryzomys microtis</i>	
	<i>Rio Segundo virus</i>	Rio Segundo virus	NA, SA	<i>Reithrodontomys mexicanus</i>	
	<i>Saaremaa virus</i>	Saaremaa virus	Europe	<i>Apodemus agrarius agrarius</i>	Human
	<i>Seoul virus</i>	Seoul virus	Worldwide	<i>Rattus norvegicus</i>	Human
	<i>Sin Nombre virus</i>	Blue River virus	NA	<i>Peromyscus leucopus</i>	
		Monongahela virus	NA	<i>Peromyscus maniculatus</i>	Human
		Sin Nombre virus	NA	<i>Peromyscus maniculatus</i>	Human
	<i>Thailand virus</i>	Thailand virus	Asia	<i>Bandicota indica</i>	
	<i>Thottapalayam virus</i>	Thottapalayam virus	India	<i>Suncus murinus</i>	
	<i>Topografov virus</i>	Topografov virus	Asia, Europe	<i>Lemmus sibiricus</i>	
	<i>Tula virus</i>	Tula virus	Europe	<i>Microtus arvalis</i> , <i>M. rossiaemeridionalis</i>	

(continued)

TABLE 42.4 Species in the Family *Bunyaviridae* (continued)

Genus	Species	Notable virus	Geographic distribution	Principal vector	Disease
<i>Nairovirus</i>	<i>Crimean-Congo hemorrhagic fever virus</i>	Crimean-Congo hemorrhagic fever virus	Africa, Asia, Europe	Culicoid flies, ticks	Human
	<i>Dera Ghazi Khan virus</i>	Hazara virus	Asia	Ticks	
	<i>Dugbe virus</i>	Dera Ghazi Khan virus	Asia	Ticks	
		Dugbe virus	Africa	Ticks	Human, cattle
		Nairobi sheep disease virus (Ganjam virus)	Africa, Asia	Ticks, culicoid flies, mosquitoes	Human, cattle
	<i>Hughes virus</i>	Hughes virus	NA, SA	Ticks	Seabirds
	<i>Qalyub virus</i>	Qalyub virus		Ticks	
	<i>Sakhalin virus</i>	Sakhalin virus	Asia	Ticks	
	<i>Thiafora virus</i>	Erve virus	Europe	N.D.	Human
<i>Phlebovirus</i>	<i>Bujaru virus</i>	Bujaru virus	SA	N.D.	
	<i>Candiru virus</i>	Alenquer virus	SA	N.D.	Human
		Candiru virus	SA	N.D.	Human
	<i>Chilibre virus</i>	Chilibre virus		Phlebotomines	
	<i>Frijoles virus</i>	Frijoles virus		Phlebotomines	
	<i>Punta Toro virus</i>	Punta Toro virus	NA, SA	Phlebotomines	Human
	<i>Rift Valley fever virus</i>	Rift Valley fever virus	Africa	Mosquitoes	Human, cattle
	<i>Salehabad virus</i>	Salehabad virus	Asia	Phlebotomines	
	<i>Sandfly fever Naples virus</i>	Sandfly fever Naples virus	Europe, Africa, Asia	Phlebotomines	Human
		Sandfly fever Sicilian virus	Europe	Phlebotomines	Human
<i>Tospovirus</i>	<i>Uukuniemi virus</i>	Toscana virus	Europe	Phlebotomines	Human
		Uukuniemi virus	Europe	Ticks	Seabirds
	<i>Groundnut bud necrosis virus</i>	Groundnut bud necrosis virus (Peanut bud necrosis virus)	Asia	<i>Frankliniella schultzei</i> , <i>Thrips palmi</i>	Plant
	<i>Groundnut ringspot virus</i>	Groundnut ringspot virus	SA, Africa	<i>F. occidentalis</i> , <i>F. schultzei</i> , <i>F. gemina</i>	Plant
	<i>Groundnut yellow spot virus</i>	Groundnut yellow spot virus (Peanut yellow spot virus)	Asia	N.D.	
	<i>Impatiens necrotic spot virus</i>	Impatiens necrotic spot virus	NA, Europe	<i>F. occidentalis</i>	Plant
	<i>Tomato spotted wilt virus</i>	Tomato spotted wilt virus	Worldwide	<i>F. bispinosa</i> , <i>F. cephalica</i> , <i>F. gemina</i> , <i>F. fusca</i> , <i>F. intonsa</i> , <i>F. occidentalis</i> , <i>F. schultzei</i> , <i>F. setosus</i> , <i>T. tabaci</i>	Plant
	<i>Watermelon silver mottle virus</i>	Watermelon silver mottle virus	Asia	<i>T. palmi</i>	Plant
	<i>Zucchini lethal chlorosis virus</i>	Zucchini lethal chlorosis virus	SA	<i>F. zucchini</i>	Plant

NA, North America; SA, South America; N.D., not determined.

time, all identified viruses of the family *Bunyaviridae* were known to be arthropod-borne viruses. Therefore, it was a big surprise when this rodent-borne virus was shown to share characteristics of viruses of the family *Bunyaviridae*. The virus was named Hantaan virus (HTNV), after the Hantaan river close to the location of some of the initial Korean cases, and became the prototype of the *Hantavirus* genus.³⁰⁵

During the course of the early studies in Korea, it became clear that HFRS cases were also occurring in urban areas. Years of investigation finally demonstrated that the urban cases in Korea, China, and Japan were associated with infection with Seoul virus, which is hosted by the rats *Rattus norvegicus* and *R. rattus*.^{300,305,514} For more than 50 years, a similar, but generally milder, disease termed nephropathia epidemica (NE) was

described in various parts of Scandinavia.³⁷⁵ Following the isolation of HTNV, the virus was shown to react with sera from patients with convalescent phase NE.³⁷⁵ This led to the discovery of Puumala virus (PUUV) as the cause of this form of HFRS and the bank vole, *Clethrionomys glareolus*, as the virus host.

The most recent major event in the history of hantaviruses was the discovery of hantavirus pulmonary syndrome (HPS) in the southwestern United States in 1993.³⁷³ A cluster of cases was identified in the Four Corners region, which presented with a flu-like illness (i.e., fever, headache, muscle aches, chills, and so on), but rapidly progressed to a more severe respiratory disease with bilateral pulmonary infiltrates, respiratory failure, shock, and death, occurring approximately 2 to 10 days after onset of illness in almost 50% of the cases.²⁵⁷ Within a couple of weeks of the initial outbreak, a newly identified virus, Sin Nombre virus (SNV), was shown to be the cause of HPS, and the rodent reservoir was shown to be the common deer mouse, *Peromyscus maniculatus*.^{96,373} Within the next several years, HPS was shown to occur throughout the Americas from Canada to Patagonia, and was found to be caused by at least 10 hantaviruses, each associated with a different rodent species. During the study of the hantaviruses associated with HPS and HFRS, many additional hantaviruses not known to be associated with human illness were discovered in a wide variety of rodent hosts (Table 42.4).

In addition to rodents, a number of hantaviruses have been isolated from insectivores. In fact the first hantavirus isolated was Thottapalayam virus, from the Asian house shrew, in Southern India,⁷⁹ although it was not recognized as a hantavirus until several years later.⁵⁹⁴ Very recently a number of other insectivore-associated hantaviruses have been identified; interestingly, prevalence in these animals is much higher than in rodents. The insectivore-borne viruses are widely distributed across the globe and display greater genetic diversity than the rodent-borne hantaviruses.⁴³⁴

Tospovirus Genus

The tospoviruses are plant viruses that are transmitted propagatively by thrips.⁵⁷⁹ At least 13 species of thrips, in the

Frankliniella and *Thrips* genera, are reported to transmit these viruses.^{384,575} Several of these viruses are now recognized as being of significant agricultural importance.⁴²⁷

The history of the tospoviruses goes back to 1915, with the recognition of spotted wilt of tomatoes in Australia,⁶⁶ and then the subsequent isolation in 1930 of tomato spotted wilt virus (TSWV),⁴⁶⁰ from which the genus gained its name.²⁷⁵ By the late 1940s, TSWV infections were greatly reduced in the United States and Western Europe due to pesticide use controlling the onion thrips, *Thrips tabaci*, which was likely the primary vector at that time. The geographic spread during the 1960s and 1970s of the Western flower thrips, *Frankliniella occidentalis*, another efficient vector of TSWV, led to a rapid expansion of TSWV.^{543,575} The virus is now known to be global in distribution, and it is found throughout agricultural areas in warmer climate zones and prevalent in greenhouse cultivations in more temperate areas. In contrast to the other genus members, TSWV has an unusually wide host range, with more than 925 plant species, including 82 botanical families, reported to be susceptible to infection. These include several important crops such as peanuts, peppers, tobacco, potatoes, peas, tomatoes, celery, lettuce, and ornamental flowers, with crop losses amounting to more than a billion dollars.³⁹³ There are 21 viruses assigned to the *Tospovirus* genus, with currently eight recognized species.^{200,372}

VIRION STRUCTURE

Morphology

Under the electron microscope, negatively stained bunyaviruses appear spherical or pleomorphic, 80 to 120 nm in diameter, and display surface glycoprotein (GP) projections of 5 to 10 nm, which are embedded in a lipid bilayered envelope approximately 5 to 7 nm thick. (Fig. 42.3). With use of cryoelectron microscopic techniques, which more accurately preserve particle integrity, LACV particles were reported to be 75 to 115 nm in diameter,⁵²² whereas particles of RVFV and Uukuniemi

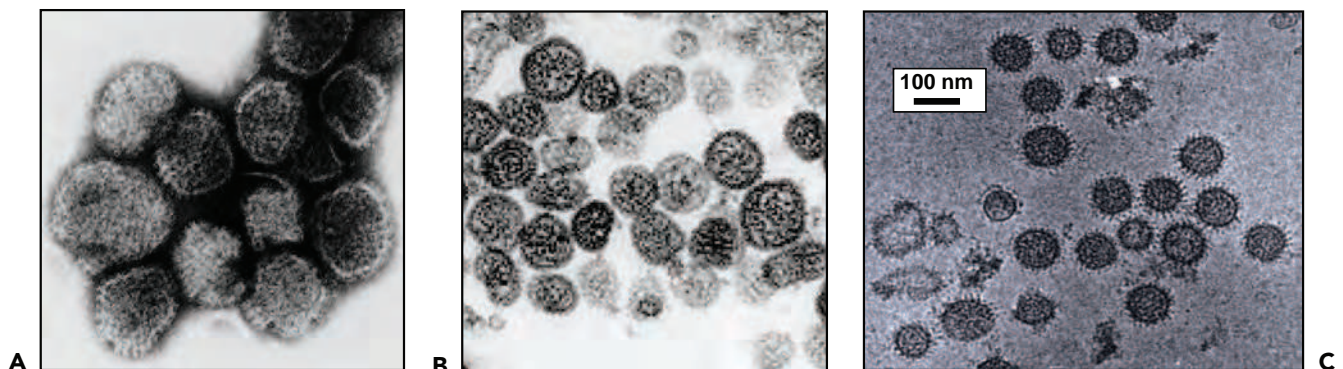


FIGURE 42.3. Morphology of bunyaviruses. **A:** Electron micrograph of glutaraldehyde fixed negatively stained Hantaan virions (*Hantavirus* genus). The morphologic units on the surface form a grid-like pattern. As described in the text, viruses in other genera show different surface characteristics. **B:** Thin-section electron micrograph of Puumala virus (*Hantavirus* genus). The interior of the virion has a filamentous or coiled bead appearance, presumably due to the presence of the ribonucleoprotein complexes (RNPs). **C:** Cryoelectron micrographs of purified vitrified-hydrated La Crosse virions (*Orthobunyavirus* genus). The glycoprotein spikes are clearly visible. (**A** and **B**, courtesy of Geisbert T, Kuhl K, White JS, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD; **C**, courtesy of Prasad BVV, Baylor College of Medicine, Houston, TX.)

(UUKV) phleboviruses had mean diameters of 100 nm and 125 nm, respectively,^{162,386} and Tula and Hantaan hantaviruses had mean diameters of 130 to 135 nm.^{35,216} The spikes are thought to mostly consist of heterodimers of the two viral GPs, and biochemical analysis of purified LACV particles indicated that the two GP species were equimolar and present at about 650 copies per virion. The GPs interact to form surface morphologic units that vary among viruses in different genera.³³³ Virions in the *Orthobunyavirus* genus have surfaces covered with closely packed, knoblike morphologic units with no detectable order. Similarly, no obvious order was found for the small surface structures with central cavities observed on viruses in the *Nairovirus* genus.³³³ The appearances of viruses in the *Tospovirus* genus have been likened to that of the nairoviruses.³⁴⁴ Other than the presence of GP projections, which are observed as a fringe on negatively stained virions, distinctive surface structure has not been noted for these viruses.

Viruses in the *Phlebovirus* genus have round, closely packed morphologic units, approximately 10 to 11 nm in diameter, with central cavities approximately 5 nm in diameter.³³³ Negative staining of glutaraldehyde-fixed particles, freeze-etching techniques, and/or cryoelectron tomography demonstrated that the glycoprotein spikes of UUKV and RVFV are organized on a T-12 icosahedral lattice, an arrangement so far unique to phleboviruses.^{162,386,557} For UUKV, two pH-dependent conformations of the glycoproteins were observed: tall spikes at pH7 and flat spikes at pH6. It is suggested that at low pH the conformational change exposes hydrophobic regions on the GP, possibly on Gc, that facilitate fusion between the viral and endosomal membrane during viral entry,³⁸⁶ a mechanism observed for other enveloped viruses that enter cells via the endocytic pathway.

The surface structure of viruses in the *Hantavirus* genus also are distinctly ordered but have a square grid-like appearance (Fig. 42.3).⁵⁷¹ Electron cryotomography^{35,216} confirms that the Gn-Gc spike complex has fourfold symmetry. Each spike is likely composed of four Gn-Gc heterodimers that form lattices on the virion surface. Specific lateral interactions between spike complexes are thought to induce membrane curvature during the budding process.

The interior of virions, as observed by thin-section electron microscopy, has a filamentous or coiled bead appearance, presumably due to the presence of the ribonucleocapsids (Fig. 42.3).¹²² From single particle reconstructions of tomograms, internally virions contained parallel thread or rod-like structures assumed to be ribonucleoprotein complex (RNP); some were located very close to the membrane suggesting interaction with the cytoplasmic tails of one or both of the glycoproteins.^{35,216,386,440}

Biochemical and Biophysical Properties

The composition and structure of virions has been inferred from biochemical and morphologic studies. An overall chemical content of 2% RNA, 58% protein, 33% lipid, and 7% carbohydrate was estimated for UUKV.³⁸⁰ Sedimentation coefficients of virions range from 400 to 500 S, and their buoyant densities in sucrose are 1.16 to 1.18 g/cm³, and in CsCl, 1.20 to 1.21 g/cm³. Treatment with lipid solvents or nonionic detergents removes the viral envelope and results in loss of infectivity for arthropods and mammals.³⁸⁰ For plants, the envelope is not required for infectivity, as demonstrated in studies with

TSWV, for which repeated mechanical passage among plants resulted in a defective virus that was unable to produce enveloped particles but was able to replicate in plant cells.¹¹⁶

GENOME STRUCTURE AND ORGANIZATION

Viral Genome

Virions contain three single-stranded RNA genome segments designated as large (L), medium (M), and small (S). All three RNA segments of a virus have the same complementary nucleotides at their 3' and 5' termini. The terminal nucleotide sequences are highly conserved among viruses within a genus, but differ from those of viruses in other genera (Table 42.3). Base-pairing of the terminal nucleotides is predicted to form stable panhandle structures and noncovalently closed circular RNAs. Direct support for base-pairing comes from electron microscopy of RNA extracted from virions, in which three sizes of circular RNAs were evident.²⁰⁴

The RNA segments are complexed with N to form individual L, M, and S nucleocapsids, which were generally assumed to have helical symmetry,³⁷⁹ but analysis of isolated RVFV nucleocapsids did not show this pattern but rather a string-like appearance.⁴⁴⁰ Nucleocapsids released by nonionic detergent treatment of virions often also appear as circular structures in electron micrographs,⁴¹¹ suggesting that the complementary RNAs can base-pair even when complexed with protein in an estimated ratio of 4% RNA to 96% protein.⁴³¹ This hypothesis is supported by data showing cross-linking of the ends of nucleocapsid-enclosed RNAs by treatment with psoralens, which are photoreactive, nucleic acid, cross-linking agents.⁴³¹

At least one each of the L, M, and S ribonucleocapsids must be contained in a virion for infectivity; however, equal numbers of nucleocapsids may not always be packaged in mature virions, as suggested by various reports of equimolar or nonequimolar ratios of L, M, and S RNAs.^{49,218} Unequal complements of the ribonucleocapsids may contribute to the size differences of virions observed by electron microscopy.^{35,522}

In addition to ribonucleocapsids containing virion-sense RNA (vRNA), certain viruses in the *Phlebovirus* and *Tospovirus* genera encapsidate small amounts of complementary sense (cRNA or antigenome). The phlebovirus UUKV was found to have S but not M segment cRNA, and the tospovirus TSWV had both M and S cRNAs in virions.^{274,491} For the orthobunyavirus, LACV, S segment cRNA was detected in virions synthesized in insect cells, but not in mammalian cells.⁴³¹ Interestingly, the phlebovirus RVFV encapsidated all three cRNA gene segments, and, as will be described later (see "S segment products"), at least one of the cRNA genes may have a role in early replication processes.²²⁵

Coding Strategies of Viral Genes

Both similarities and noteworthy differences in the coding strategies of viruses in the family *Bunyaviridae* are known. Viruses in each genus encode all structural proteins in their cRNA. Certain viruses also encode nonstructural proteins in their cRNA or in their vRNA. Therefore, some viruses in the family *Bunyaviridae* use only a negative-sense coding strategy and others use a combination of negative-sense and ambisense coding strategies (Fig. 42.2).

S Segment Strategies

Viruses in the *Orthobunyavirus* genus have smaller S segments than those of viruses in other genera (Table 42.1). Some orthobunyaviruses encode two polypeptides, the nucleocapsid protein (N) and a nonstructural protein (NSs), in ORFs in cRNA⁴⁸ (Fig. 42.2), whereas others only encode N protein.³⁵¹ The presence of NSs in virus-infected cells was demonstrated for several members of the genus.¹⁴⁰ Only one S segment mRNA species can be found in orthobunyavirus-infected cells⁸⁴; therefore, N and NSs are generated by alternative start codon recognition by ribosomes.^{126,144}

Hantaviruses andairoviruses encode larger N proteins than viruses in other genera, (Table 42.2, Fig. 42.2).^{332,470} Some hantaviruses (e.g., SNV, PUUV, Tula [TULV] and Prospect Hill [PHV] viruses), have ORFs within the N ORF, and an NSs protein has been detected in PUUV- and TULV-infected cells.²²⁷ No NSs protein has been detected inairovirus infected cells. Only one S-segment mRNA, similar in size to the coding region for N, was identified in hantavirus- orairovirus-infected cells, indicating that transcription termination occurs shortly after the translation stop codon.^{469,563} Certain hantaviruses (e.g., SNV) have long 3' noncoding regions (of greater than 700 nucleotides) containing numerous repeated sequences. These repeats may result from polymerase slippage on the vRNA template.⁵⁰⁵

The ambisense coding strategy of the S segments of phleboviruses and tospoviruses produces N from a subgenomic mRNA that is complementary to vRNA, and NSs from a subgenomic mRNA of the same polarity as vRNA (Fig. 42.2).^{118,220} Evidence that the mRNA for NSs is copied from cRNA (after genome replication) comes from time-course studies. For the phlebovirus UUKV, N was detected at 4 to 6 hours after infection, whereas NSs did not appear until 8 hours after infection.^{492,542} Likewise, the mRNA for NSs of the tospovirus TSWV was detected in infected plant cells 15 hours later than for N.⁵⁰⁷ In addition, studies with the phlebovirus Punta Toro virus (PTV) demonstrated that protein synthesis inhibitors arrest production of NSs mRNA, but not N mRNA.²²² These results suggest that protein synthesis must occur before the NSs mRNA can be made. In contrast, studies with the phlebovirus RVFV demonstrated the presence of cRNAs to all three RNA segments in purified virions. Moreover, mRNA for the NSs protein was detected as early as 20 minutes after infection, concomitant with the appearance of mRNA for N, suggesting that the ambisense-encoded NSs mRNA are transcribed from the incoming antiviral sense RNA.²²⁵

M Segment Strategies

Sizes of M segments range from ~3,600 nucleotides to ~4,900 nucleotides (Table 42.1). All bunyavirus M segments encode the two envelope GPs in a single ORF of cRNA (Fig. 42.2). Previous designations of G1 and G2, which were based on relative migration of the proteins in polyacrylamide gels, have been replaced by designations of Gn and Gc, referring to the amino-terminal or carboxy-terminal coding of the proteins.²⁹⁴ As described below, it is becoming increasingly clearer that the functions of the Gn and Gc proteins are conserved among the five genera.

Some viruses encode NSm proteins, and others do not. Except for the tospoviruses, which use an ambisense strategy to generate NSm from a subgenomic mRNA, a single mRNA,

nearly equivalent in size to the cRNA ORF has been detected in virus-infected cells. For tospoviruses, separate, subgenomic messages for the Gn-Gc precursor and for NSm were identified.^{273,295} NSm is readily detected in infected plants and is the only M segment nonstructural protein in the *Bunyaviridae* family to have a clearly defined role (i.e., it is a movement protein [see “M Segment Products,” below]). Another possible role for NSm for some viruses may be as a virulence factor. For example, a genetically engineered RVFV lacking NSm induced more extensive apoptosis than did one with NSm and the expression of NSm significantly inhibited the cleavage of caspase 8 and 9 induced by staurosporine, indicating that the NSm protein suppresses apoptosis.⁵⁸²

L Segment Strategies

The L segments of hantaviruses, orthobunyaviruses, and phleboviruses are of similar size, (~6,500 nucleotides), whereas those of tospoviruses andairoviruses are considerably larger (~9,000 and 12,000 nucleotides, respectively; Table 42.1). All L segments of viruses in the family use conventional negative-sense coding strategies (Fig. 42.2). For each L segment described thus far, there are fewer than 200 nucleotides of total noncoding information, and there is no evidence for additional coding regions in either the cRNA or vRNA.^{117,141,143,209,360,465}

STAGES OF REPLICATION

The principal stages of the replication process for viruses in the *Bunyaviridae* are illustrated in Figure 42.4 and are summarized in the following:

1. Attachment, mediated by an interaction of viral proteins and host receptors
2. Entry, by receptor-mediated endocytosis
3. Uncoating, by acidification of endocytic vesicles, and fusion of viral membranes with endosomal membranes
4. Primary transcription of viral-complementary mRNA species from genome templates using host-cell-derived primers and the virion-associated polymerase
5. Translation of L, M, and S mRNAs
 - co-translational cleavage of M-segment polyprotein and posttranslational cleavage of precursors for some viruses
 - dimerization of Gn and Gc in the endoplasmic reticulum (ER)
6. Membrane-associated RNA replication
 - synthesis and encapsidation of cRNA to serve as templates for vRNA or, for ambisense genes, templates for subgenomic mRNA
 - genome replication
7. Morphogenesis
 - localization of N in budding compartments
 - transport of dimerized Gn and Gc to the Golgi
 - glycosylation
 - acquisition of modified host membranes, generally by budding into the Golgi cisternae
8. Fusion of cytoplasmic vesicles containing viruses with the plasma membrane and release of mature virions
 - more rarely, some viruses in some cell types have been observed to bud directly from the host cell's plasma membrane

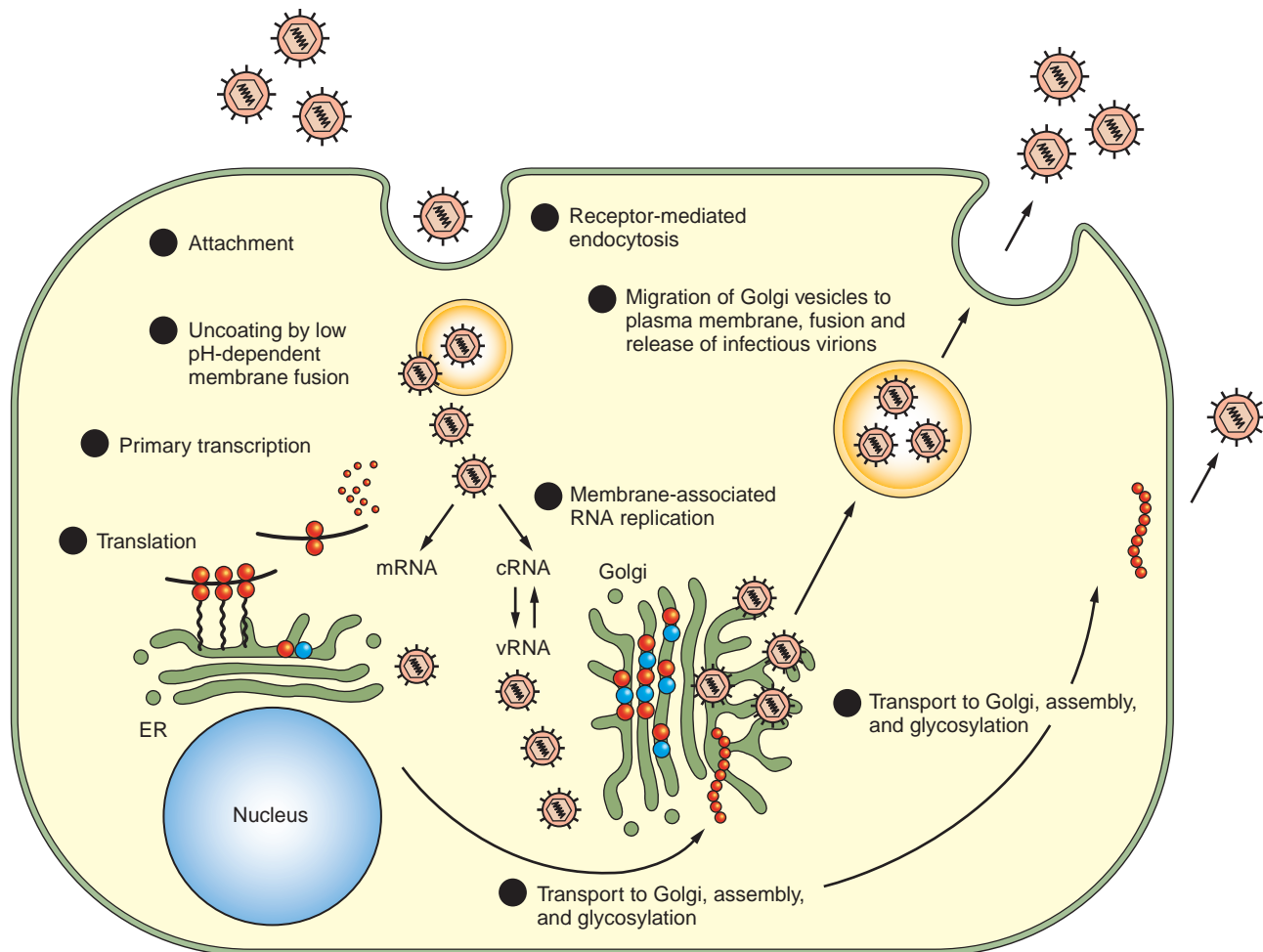


FIGURE 42.4. Replication cycle of viruses in the family *Bunyaviridae*. Steps in the replication cycle are the following: 1. Attachment, mediated by an interaction of viral proteins and host receptors; 2. Receptor-mediated endocytosis; 3. Uncoating, by acidification of endocytic vesicles, and fusion of viral membranes with endosomal membranes; 4. Primary transcription of viral-complementary messenger RNA (mRNA) species from genome templates using host-cell-derived primers and the virion-associated polymerase; 5. Translation of L, M, and S mRNAs, co-translational cleavage of the M-segment polyprotein, dimerization of Gn and Gc in the endoplasmic reticulum (ER); 6. Membrane-associated RNA replication, synthesis, and encapsidation of cRNA to serve as templates for vRNA or, for ambisense genes, templates for subgenomic mRNA, genome replication; 7. Morphogenesis including transport of the structural proteins to the Golgi, glycosylation of Gn and Gc, budding into the Golgi cisternae; 8. Migration of Golgi vesicles containing viruses to the cell surface, fusion of vesicular membranes with the plasma membrane, and release of infectious virions. Some viruses in some cell types can bud both into intracellular vesicles and also from the plasma membrane.

Attachment and Entry

Viral Attachment Proteins and Cellular Receptors

The mechanisms by which members of the family *Bunyaviridae* gain access to the host cell's cytoplasm appear similar to those reported for many other enveloped viruses. The first step involves an interaction between cell surface receptors and viral attachment proteins, Gn and/or Gc. The presence of neutralizing and hemagglutination-inhibiting sites on both the Gn and Gc proteins of phleboviruses and hantaviruses^{27,252} suggests that both proteins may be involved in attachment. Although it is possible that both are directly involved, it is more likely that both are required due to conformational requirements that depend on dimerization of Gn and Gc.

In general, Gc appears to be the primary attachment protein for orthobunyaviruses in mammalian cells, mosquito cells, and mosquitoes.^{193,261,515} Consistent with this view, expression of M-segment products of three orthobunyaviruses demonstrated that Gc, but not Gn, could effect attachment and entry when tested in a cell-to-cell fusion assay and a pseudotype transduction assay.⁴¹⁹ However, the amino terminal half of BUNV Gc ectodomain is not required for infection of cultured mammalian cells,⁴⁸³ and this region can be replaced by green fluorescent protein (GFP) allowing the creation of viable viruses expressing chimeric Gc-GFP in their virions.⁴⁸⁶

For tospoviruses, the envelope GPs are required only for infection of their arthropod vectors, as evidenced by the ability of envelope-deficient mutants to replicate in plants after

mechanical transmission, but their inability to infect thrips.⁴⁴⁵ In addition, analysis of reassortant viruses demonstrated that mutations that disrupted the Gn-Gc ORF of TSWV ablated transmissibility by thrips.⁴⁹⁵ These studies provide indirect evidence that the L-polymerase protein remains associated with ribonucleocapsids and is active despite the absence of intact virions.

Although there are no specific reports of Gc being the attachment protein of tospoviruses, functional homology to the Gc proteins of other bunyaviruses was proposed based on amino acid sequence homologies detected for iris yellow spot virus.¹⁰⁶ In contrast, however, a study with a soluble, truncated form of TSWV Gn expressed in baculoviruses showed binding of this protein to epithelial cells in the midguts of thrips, suggesting that Gn could mediate attachment in thrips.⁵⁷³ Similar results were reported earlier with the orthobunyavirus LACV, in that virions treated with a protease that cleaved Gc but not Gn exhibited increased binding to the insect vector midgut, but reduced binding to cultured mammalian or mosquito cells.³²⁴ Given that both of these systems use a Gn protein in the absence of Gc, it remains to be determined whether Gn functions for attachment when in its native conformation on virions.

Host cell receptors have not been identified for most viruses in the family; however, pathogenic and nonpathogenic hantaviruses were shown to use $\beta 3$ and $\beta 1$ integrins, respectively, to enter endothelial cells.¹⁷⁰ This finding was suggested to relate to pathogenesis, in that binding of hantaviruses to integrins might disrupt their ability to regulate cell-to-cell adhesion and result in the vascular permeability characteristic of hantaviral diseases.¹⁷¹ In addition, decay accelerating factor (DAF)/CD55 a glycosylphosphatidylinositol (GPI)-anchored protein, has been shown to mediate HTNV and PUUV entry across the apical membrane of polarized epithelial cells,²⁷⁷ and gC1qR/p32, a 32-kD glycoprotein that interacts with complement protein C1q, binds HTNV and mediates infection of cultured A549 lung cells.⁹⁸

Entry of the Viral Genome into the Host Cytoplasm

Shortly after attachment, viruses in the *Phlebovirus* and *Nairovirus* genera were observed in phagocytic vacuoles.^{145,455} This suggested a mode of viral entry similar to that first described for alphaviruses in which the virus is endocytosed in coated vesicles, and inhibitor studies have confirmed this to be so for the animal-infecting bunyaviruses. Orthobunyaviruses, hantaviruses, and nairoviruses enter by endocytosis into clathrin-coated pits,^{239,463,490} and HTNV proteins were shown to colocalize with clathrin in confocal immunofluorescence studies.²³⁹ In addition, CCHFV requires functional microtubules for infection.⁴⁸⁹ By contrast, entry of UUKV phlebovirus is predominately in a clathrin-independent manner, and viruses enter Rab5a+ early endosomes and subsequently Rab7+ and LAMP-1+ late endosomes.³²³ After endocytosis, acidification of the endocytic vesicles is thought to promote a conformational change in Gn and/or Gc that facilitates fusion of the viral and cellular membranes, thereby allowing the viral genome and polymerase access to the cytoplasm. Infection is blocked if cells were treated with ammonium chloride, which prevents acidification.^{229,419,463,484} Additional indirect support for membrane fusion as a mode of entry came from experiments demonstrating the ability of viruses in the family to mediate syncytia formation at low pH.^{153,183,229,383,419,484,574}

Computational studies revealed that the Gc proteins have characteristics similar to those of class II fusion proteins identified for viruses in the *Flaviviridae* and *Togaviridae* families.¹⁶⁹ Such proteins have internal fusion domains, as opposed to terminal domains found for the class I fusion proteins. Experimental support for this finding was obtained by demonstrating interaction of the fusion peptide postulated for the Gc protein of the hantavirus, Andes virus (ANDV), with artificial membranes.⁵³⁷ In addition, several lines of evidence indicate that the Gc of orthobunyaviruses is involved in membrane fusion. Protease sensitivity assays, detergent partitioning experiments, and antibody-binding studies suggest that Gc undergoes a conformational change at pH conditions where fusion is observed.¹⁸² Mutational analyses of a hydrophobic region (residues 1,066–1,087) of LACV Gc that is well conserved among different orthobunyaviruses¹⁰⁶ supported this notion,^{229,420} and the residues flanking the predicted fusion peptide in BUNV Gc (residues 1,058–1,079) were shown to be structurally critical for the conformational change in Gc that occurs during the fusion process.⁴⁸³ LACV Gc alone, however, when expressed from recombinant vaccinia viruses, could not cause cell fusion, suggesting that an association of the two GPs may be needed for membrane fusion,²²⁹ and mutations in the cytoplasmic tail of BUNV Gn severely affected membrane fusion indicating that Gn must also play an important role in the fusion process.⁴⁸⁴ Likewise for HTNV, both Gn and Gc were required to achieve surface expression and cell-to-cell fusion activity.³⁸³ Recombinant LACV carrying mutations in the Gc fusion peptide was impaired for growth in both mammalian and insect cells but were still neurotoxic to neuronal cells, implying that the fusion peptide is a determinate of neuroinvasiveness but not necessarily neurovirulence.⁵⁰¹

Transcription and Replication

After uncoating of viral genomes, primary transcription of negative-sense vRNA to mRNA is initiated by interaction of the virion-associated L protein, which is an RNA-dependent RNA polymerase (RdRp), and the three viral ribonucleocapsids.⁵⁵ Studies with hantavirus N protein suggest that N participates in transcription initiation by facilitating dissociation of the RNA panhandle and by subsequently remaining attached to the 5' terminus of the RNA, thereby freeing the 3' terminus for RdRp interactions. Furthermore, N was suggested to be important for replication by acting as an RNA chaperone and transiently and continuously unfolding the RNA to allow it to form more stable structures.³⁴⁷ A role for N in both transcription and replication is also evident by analysis of the behavior BUNV N proteins carrying specific mutations, either in a minigenome assay or in the context of virus infection.^{135,560} Four BUNV temperature sensitive mutants, carrying single amino acid substitutions in N, could be divided into two groups, those that were defective in antigenome synthesis but not mRNA transcription, and those that were replication defective but transcription competent, suggesting that different domains within N are associated with different RNA synthesis activities.¹³⁵ The mechanism by which residues in N modulate template activity requires elucidation; one possibility is that transcription and replication require different cellular cofactors that bind to distinct regions on the N protein.

Because only L and N are needed for RNA synthesis,¹²⁷ the location of these proteins must correlate with the site of RNA

synthesis. For nairoviruses and hantaviruses, N (or L and N) proteins were found to localize to the perinuclear region and to have a peripheral association with perinuclear membranes.²⁸³ In cells infected with a recombinant BUNV expressing an epitope-tagged L protein immunofluorescence microscopy showed L to have a punctate to reticular staining pattern, with concentration of staining in the perinuclear region, whereas cell fractionation studies showed L to be distributed in both cytosolic and microsomal fractions.⁴⁸¹ Together, these data suggest that bunyavirus RNA replication is membrane-associated, similar to that described for flaviviruses.

The L Protein

To mediate replication, the RdRp must affect numerous enzymatic functions. For primed synthesis of mRNA from genomic templates and nonprimed synthesis of vRNA from cRNA templates (as will be described), the RdRp performs endonuclease, transcriptase, replicase, and probably RNA helicase activities. Motifs common to polymerases in general are conserved in the bunyaviral RdRps, most notably a catalytic core motif.^{26,237,282,360} The N-terminal domain of orthobunyavirus L proteins contains a conserved PD-(D/E)xK amino acid nuclease motif and the isolated domain of LACV L exhibited nuclease activity *in vitro*; furthermore, bioinformatic analyses indicated that viruses in all the other *Bunyaviridae* genera contain a similar domain.⁴⁴² The nuclease domain of LACV bears structural similarities to that of the PA subunit of the influenza A virus polymerase, suggesting a common origin of the cap-snatching process of segmented negative sense viruses. None of the other functions has been definitively localized to a region of the RdRp gene or gene product; however, comparison of two nairovirus L proteins with functionally defined regions of other polymerases revealed potential helicase, topoisomerase, and gyrase coding regions as well as an N-terminal cysteine-protease motif typical of the ovarian tumor (OTU) protein superfamily and protease.²⁶² Biochemical and structural analyses demonstrate that in addition to the deubiquitinase activity as shown by cellular OTU proteases, the nairovirus OTU also targets ISG15 modification and thus enhances its role in overcoming host innate immune defenses.^{5,77,165,230} The OTU domain is not required for the RNA polymerase activity by CCHFV in a minigenome system.⁴³

Genome Promoters

The template for bunyavirus transcription and replication is not naked RNA but RNA in the form of ribonucleocapsid.^{127,319} The 3' and 5' nontranslated complementary nucleotides contain signals for both mRNA synthesis and antigenome synthesis, and thus are the genome promoters. For the phleboviruses RVFV and UUKV, the first 13 or the first 10 nucleotides, respectively, of the 3' end of the genome are sufficient for RNA synthesis activity.^{156,424}

For the orthobunyavirus BUNV, a minigenome reporter system was used to demonstrate that optimal transcription required exact complementarity at the 3' and 5' termini; however, one to three nucleotide deletions could be tolerated at the 3' terminus, but not the 5' terminus.²⁶⁸ Although sequence changes at several positions could be tolerated as long as complementarity was maintained, some changes did reduce transcription. Interestingly, mutagenesis experiments indicated that the single exception to complementarity (a U residue at 3' position 9)

was critical for transcription from the genomic promoter, whereas the corresponding 5' position 9 could be changed without influencing transcription.³³ Therefore, the mismatch itself is not important, but the nucleotide is. This nucleotide, however, does not have to be maintained for transcription activity of the antigenomic promoter.³³ Together, these data suggest that the structure of the panhandles is more important than the sequence, but that the sequence also plays a role in promoter strength and activity.

To further investigate promoter regions in an authentic replication system, S segments with shortened complementary termini were introduced into a BUNV reverse genetics system, and rescued viruses were screened to determine the minimal terminal complementary region needed for virus production.³²² The minimal 3' and 5' deletion mutant viable in this system maintained 29 of the 85 untranslated nucleotides on the 3' end and 112 of the 174 untranslated nucleotides on the 5' end, whereas those with shorter untranslated regions were not viable. Therefore, although only the complementary regions are needed for promoter activity *in vitro*, actual virus production also requires some of the unique sequences in the untranslated regions at the 3' and 5' termini of BUNV.³²²

The *in vivo* importance of intact termini was also suggested by a study in which terminally deleted RNAs were shown to accumulate during the establishment of persistent infections with the hantavirus, Seoul virus (SEOV). It was hypothesized that as deletions accrued, fewer replication-competent genomes were present, leading to a downregulation of the replication processes, and possibly to persistence.³⁴² Similar deletions were found on the termini of ANDV M and L, but not S genes, leading to a speculation that differences observed in the relative abundance of Gn and Gc compared to N could reflect down regulation of M-segment expression from genes without intact termini.³⁸⁹

Primed mRNA Synthesis (Cap Snatching)

Like influenza viruses, viruses in the family *Bunyaviridae* prime mRNA synthesis with capped oligonucleotides that are scavenged from host mRNAs (Fig. 42.5). Cleavage of the capped primers is accomplished by endonuclease activity found in virions and associated with the L protein.^{398,442} Unlike influenza viruses, which take primers from newly synthesized mRNAs in the host cell's nucleus, members of the *Bunyaviridae* family use primers cleaved from cytoplasmic host cell mRNAs. Evidence has been presented that the hantavirus N protein binds to the 5' cap of cellular mRNAs to protect them from cell-mediated degradation, and that N accumulates in cytoplasmic processing bodies (P bodies) where protected 5' caps are sequestered, and hence can serve as a pool of primers to initiate mRNA synthesis.³⁴⁶ A result of this mode of mRNA transcription is the presence of 5' terminal extensions of approximately 10 to 20 heterogeneous nucleotides that are not found in vRNA.⁴⁷ Studies using anticap antibodies to immunoselect mRNAs¹⁹² have provided direct proof for the presence of caps on the scavenged primers. Further evidence for capped extensions of mRNAs was provided by a study of tospoviruses, in which plants were co-infected with TSWV and with a positive-strand RNA virus (alfalfa mosaic virus). The tospovirus acquired 5' mRNA extensions with nucleotide sequences that matched those of the other virus with a preference for cleaving at an A residue.¹²⁵ It was suggested that this

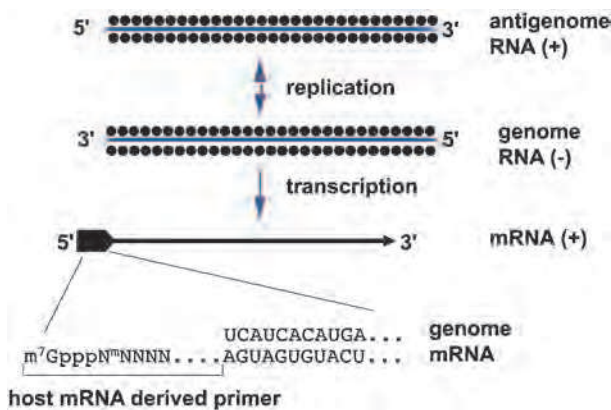


FIGURE 42.5. Transcription and replication scheme of negative-sense bunyavirus genome segments. The genome RNA and the positive-sense complementary RNA known as the antigenome RNA are found only as ribonucleoprotein complexes and are encapsidated by N protein (●). The messenger RNA (mRNA) species contain host-derived primer sequences at their 5' ends (■) and are truncated at the 3' end relative to the virion RNA (vRNA) template; the mRNAs are neither polyadenylated nor encapsidated by N protein. The sequence at the 5' end of an orthobunyavirus mRNA is shown.

preference is due to a need for base-pairing at the ultimate U residue of the gene segments. Consistent with this, later studies indicated that double and triple base complementarity to nucleotides at the ends of the tospovirus gene segments were preferred, even more than the single complementary residue.⁵⁴⁵ Interestingly, it was also found that newly synthesized mRNAs can serve as cap donors *in vitro* for tospoviruses, suggesting a “resnatching” mechanism.⁵⁴⁴

Other viruses in the family also have nucleotide or nucleotide motif preferences for endonuclease cleavage of capped primers. These preferences vary among the genera, and sometimes among viruses within a genus. A preferred primer sequence, or a favored nucleotide at the site of cleavage due to a need for limited base pairing with the viral genome, appears to be a common feature of primed transcription for bunyaviruses. In one study, the 3'-terminal nucleotides of the scavenged host primers often were similar to the 5'-terminal viral nucleotides.²³⁸ It was proposed that after transcription of two or three nucleotides of the nascent mRNA, the viral polymerase might slip backward on the template before further elongation, resulting in a partial reiteration of the 5'-terminal sequence. An extension of this concept, termed “prime and realign,” was proposed for mRNA transcription of hantaviruses.¹⁶⁸ According to this model (e-Fig. 42.2), priming by host oligonucleotides with a terminal G residue would initiate transcription by aligning at the third nucleotide of the viral RNA template (C residue). After synthesis of a few oligonucleotides, the nascent RNA could realign by slipping backward two nucleotides on the repeated terminal sequences (AUCAUCAUC) (Table 42.3), such that the G becomes the first nucleotide of the nontemplated 5' extensions (e-Fig. 42.2). The frequent deletion of one or two of the triplet repeats in hantaviral mRNA supports this sort of slippage mechanism and suggests that sometimes the initial priming might start at the C residue of the third triplet in the conserved sequence rather than at the C of the second triplet.¹⁶⁸

Bunyavirus transcription is unique among negative-sense RNA viruses in that functional viral mRNA synthesis requires ongoing protein synthesis,^{31,400,430,432,433,553} a finding that at face value appears incompatible with the presence of a virion transcriptase. In the absence of protein synthesis, only short transcripts are produced *in vivo* and *in vitro*. If the *in vitro* reaction is supplemented with rabbit reticulocyte lysate, however, full-length RNAs are synthesized. The translational requirement is not at the level of mRNA initiation, but rather during elongation or, more precisely, to prevent the transcriptase from terminating prematurely. A model to account for these observations proposed that in the absence of ribosome binding and protein translation, the nascent mRNA chain and its template can base-pair, thereby preventing progression of the transcriptase enzyme.³⁷ Recently this model (e-Fig. 42.3) has been tested using BUNV model templates containing translational stop codons,³¹ and the results showed that translation of nascent mRNAs prevented transcription termination. Thus orthobunyaviruses couple transcription and translation, a feature commonly found in prokaryotes, but rare in eukaryotic cells.

Transcription Termination

For gene segments with simple negative-sense coding, synthesis of M- and S-segment mRNAs terminates about 40 to 100 nucleotides before the end of the genome RNA template.^{109,148,235,399} Although most other negative-strand RNA viruses use a stretch of nucleotides rich in U residues to signal transcription termination and polyadenylation, no such tract has been consistently identified for members of the family *Bunyaviridae*. This is supported by experimental evidence showing that bunyavirus mRNAs are not 3' polyadenylated,^{1,400,542} but many have the potential to form stem-loop structures that are probably involved in enhancing translation of viral mRNAs.^{53,551}

By using a reporter system that involved mutated S segments, the transcription termination signal for the orthobunyavirus BUNV was mapped to a 33-nucleotide region within the 5' nontranslated region of the S segment.³² Within this region, a 6-nucleotide motif, 3'-GUCGAC-5' was critical to transcription termination. In addition, changing the nucleotides in this transcription termination signal revealed a second downstream transcription termination signal, which had a 5-nucleotide motif, 3'-UGUCG'-5', that was also found within the 33-nucleotide region of the upstream site, and that partially overlapped the critical 6-nucleotide motif. The finding that there are no U-rich regions in the transcription termination signal of BUNV is consistent with the absence of poly-A tails on the mRNAs.

Comparison of these S-segment sequences with those of other orthobunyaviruses revealed a high degree of conservation, suggesting that similar motifs also function for transcription termination throughout the genus, at least for S segments. A motif similar to that on the BUNV S segment was observed within the BUNV L, but not the M segment.³² Experimental mapping of BUNV L and M mRNA termination sites has not been reported.

Analysis of the L mRNAs of SNV hantavirus²¹⁸ and RVFV and Toscana (TOSV) phleboviruses⁷ showed they were co-terminal with the L vRNA template, suggesting L mRNA synthesis terminates by run-off of the RNA template.

A U-rich motif was proposed as the site of termination for the M mRNA and a C-rich motif (CCCACCC) as termination site for the S mRNA of SNV hantavirus.²¹⁸ Fine mapping

of M-segment mRNA termination sites for three phleboviruses (RVFV, TOSV, and SFSV) showed that termination occurred immediately following a C-rich domain in the template at a conserved motif 3'-C₁₋₃ GUCG/A-5'.⁷ Although previously it was thought the C-rich region itself was involved in transcript termination,¹⁷⁸ deletion of this region in template RNA did not affect specific termination at the identified motif.⁷

The mechanism of transcription termination of the ambisense genes of phleboviruses and tospoviruses was initially thought to involve RNA secondary structure in the intergenic region.^{103,118,146,187,188,493} However, reanalysis of computer predictions of hairpin formation in these intergenic regions suggest that they are unlikely to form because of their complexity or low energy.⁷ Detailed mapping of mRNA termination sites for the N and NSs mRNA of RVFV, TOSV, and SFSV phleboviruses showed that the 3' ends of the mRNAs contained most of the intergenic sequence and indeed overlapped each other.⁷ Furthermore, termination occurred for both messages at the same motif, 3'-C₁₋₃ GUCG/A-5', as in the M segment. Two copies of this motif are thus present, one in vRNA (genome) and one in the cRNA (antigenome). The conservation of this motif in viral genomes that display considerable sequence diversity suggests a similar mechanism for transcription-termination in the M and S segments. Deletion of either copy of the motif in RVFV S segment did not prevent correct termination of unaffected N or NSs mRNA, but the mutant viruses were attenuated in growth compared to wild-type.⁷

Genome Replication

The change from primary transcription to replication requires that the RdRp, either acting alone or in concert with undefined viral or cellular factors, must at some point, switch from primed mRNA synthesis to independently initiating transcription at the precise 3' end of the template to produce a full-length transcript. The processes involved in making that switch from primary transcription to genome replication have not been defined completely for any member of the family. Presumably, some viral or host factor is required to signal a suppression of the transcription termination signal responsible for generation of truncated mRNA and also to prevent the addition of the capped and methylated structures to the 5' termini of the cRNAs. There is no question that genome replication and subsequent secondary transcription are prevented by transla-

tional inhibitors such as cycloheximide. These results indicate that continuous protein synthesis is required for replication of the genome. Although not proven, it is likely that synthesis of N is required for genome replication, as described for other negative-strand RNA viruses such as the rhabdovirus, vesicular stomatitis virus, and the paramyxovirus Sendai virus. For these viruses, encapsidation by N seems to serve as an antitermination signal, thus allowing full-length genome synthesis.

A prime-and-realign model was also postulated for the nonprimed transcription of hantaviral vRNA and cRNA, except that transcription would initiate with pppG alignment at the third nucleotide (C residue) of the template RNA. After synthesis of several nucleotides, polymerase slipping would realign the nascent RNA such that the initial priming G residue would overhang the template. It was further theorized that nucleolytic activity of the L protein might remove the overhanging G, leaving a monophosphorylated U residue at the nascent 5' end. The presence of the monophosphorylated U on HTNV RNA was experimentally demonstrated¹⁶⁸ (e-Fig. 42.2).

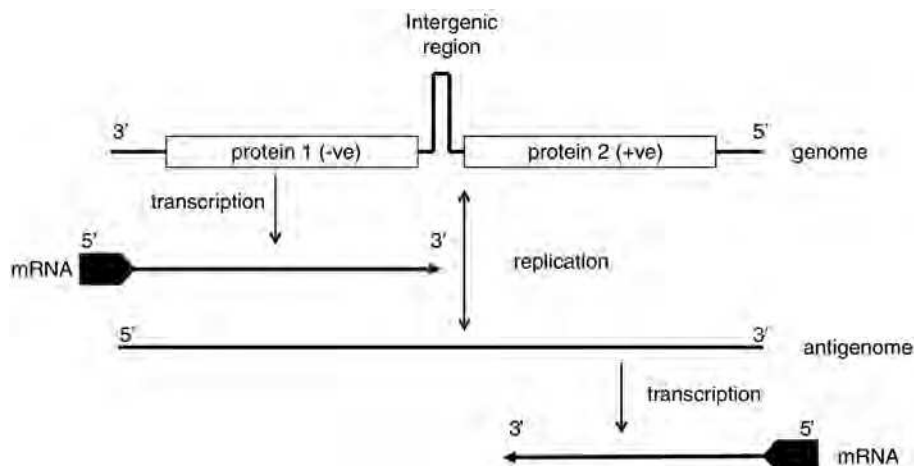
Indirect evidence suggesting that a prime-and-realign method of initiation is used by phleboviruses was obtained by using a reconstituted transcription system to study the polymerase recognition sequence at the 3' termini of the ambisense S segment RNA of RVFV. In those studies, mutational analysis of the terminal nucleotides revealed that the first 13 nucleotides are required for polymerase recognition, but that one of the two terminal dinucleotides (UGUG) could be removed without deleterious effects on transcription.⁴²⁴ These data also suggested that realignment is not a prerequisite for transcription initiation. In contrast, a recent study using the BUNV minigenome system showed that the viral polymerase was able to repair both insertions and deletions in model template RNAs, although this did not appear to involve the prime-and-realign mechanism.⁵⁵⁹

Schematics of transcription and replication based on information above are presented in Figures 42.5 and 42.6.

Encapsidation Signals

Signals for the encapsidation of the vRNAs and cRNAs of orthobunyaviruses, phleboviruses, nairoviruses, and hantaviruses were found entirely within the noncoding 3' and 5' regions of minireplicon reporters.^{52,154,155,157,321,345,348,382} Mutational

FIGURE 42.6. Transcription and replication scheme of ambisense-sense bunyavirus genome segments. The genome RNA encodes proteins in both negative- and positive-sense orientations, separated by an intergenic region that for some viruses has the potential to form a hairpin structure. The proteins are translated from specific sub-genomic messenger RNAs (mRNAs), with the mRNA encoding protein 2 transcribed from the antigenome RNA after the onset of genome replication.



analysis of these terminal regions for BUNV revealed a conserved sequence within nucleotides 20–33 of the 5′ end of vRNA that was critical for packaging.²⁷⁰ Variable packaging efficiencies were noted for the L, M, and S segment–derived minireplicons, and packaging appeared to be independent for each.

Host Factors

Beyond the need for host-derived primers for initiating mRNA synthesis, little is known about the role that host factors might play in viral replication. One putative host transcription factor is an ~40-kD protein isolated from the main insect vector of TSWV, and it was shown to bind to the viral RdRp at its C-terminus and to the viral RNA at its N-terminus.¹¹⁹ Both this protein and reticulocyte lysates were needed for RNA synthesis *in vitro*, indicating that other cellular factors are also required. Expression of this protein in mammalian cells rendered them permissive to TSWV replication.¹¹⁹ As is the case for a number of other negative-strand RNA viruses, heat-shock protein 90 (Hsp90) appeared essential for La Crosse virus replication in that virus yields were diminished in cells treated with Hsp90 inhibitors like geldanamycin due to destabilization of the viral L protein.¹⁰⁵

Translation and Processing of Viral Proteins

Viral polypeptides are synthesized shortly after infection, with S and L mRNAs translated on free ribosomes and M mRNAs translated on membrane-bound ribosomes. Expression products vary among the genera, and even within a genus.

S Segment Products: N and NSs

The N protein is the most abundant viral product in virions and infected cells. N plays several important roles in viral replication. In addition to protecting the RNA from degradation, N also interacts with L and Gn and Gc, although the exact interactions have not been defined. A critical step in N interaction with RNA appears to be the ability of the proteins to oligomerize. Oligomerization characteristics of the N proteins are likely different among the genera, which is probably not surprising, given the size differences of the N proteins, which range from approximately 19kD for orthobunyaviruses to 54kD for hantaviruses and nairoviruses (Table 42.2).

Chemical cross-linking studies indicated that the ribonucleocapsids of the phlebovirus RVFV, either from authentic virions or reconstituted from bacterially expressed protein, contain dimers of N as the basic oligomer.^{297,440} The crystal structure of RVFV N has been determined to 1.93Å resolution, which revealed that it has a novel all-helical fold without a positively charged surface RNA binding cleft as seen for other negative-sense RNA virus nucleocapsid proteins.⁴⁴⁰ Extensive ribonuclease digestion of authentic or reconstituted RNAs released heterogeneous N-RNA multimers, consistent with the lack of observed helical symmetry. In contrast, hantaviral N proteins form stable trimers, which are postulated to assemble on the viral RNA, followed by further protein–protein interactions that encapsidate the entire RNA.²⁴⁹ The N–N interactions are likely to be electrostatic, with both amino- and carboxy-terminal residues participating.²⁵⁰ The N-terminal residues of hantaviral N proteins are predicted to form a coiled coil domain, providing a trigger for trimerization, and containing charged residues important for intermolecular interactions.^{9,10,13,562} For

TULV, carboxy-terminal amino acids 393 to 398 were shown to be crucial for trimerization, and amino acids 1 to 43 provided a secondary interaction region.²⁴⁹ These data are in agreement with those obtained with other hantaviruses (e.g., SNV and HTNV, for which carboxy-terminal and amino-terminal regions were shown to be involved in the homotypic interactions of N).^{9,589}

Orthobunyaviruses and tospoviruses, in contrast to hantaviruses, do not appear to assemble preformed trimers, but instead multimerize by adding one N protein at a time. This hypothesis comes from chemical cross-linking experiments, which showed that the N protein of the orthobunyavirus BUNV was able to form a range of multimers from dimers to high-molecular-weight structures, with a preponderance of tetramers.³⁰⁸ Deletion of N- or C-terminal portions of N prevented multimerization and also resulted in loss of functionality in a minireplicon system. The data were interpreted to indicate a head-to-head and tail-to-tail multimerization model of individual N proteins. Likewise, for the tospovirus TSWV, analysis of deletion mutants identified binding regions both at the amino- and carboxy-termini of N, and although the type of multimers formed was not examined closely, it appears to be a continuous addition process, like that proposed for the orthobunyaviruses.^{242,540}

In addition to protein–protein interaction, the amino- and carboxy-terminal regions of the tospovirus N were also implicated in RNA binding.⁴⁴⁷ In contrast, the RNA-binding site of hantaviral N was localized to a central, conserved region of the protein.⁴⁷⁴ For hantaviruses and bunyaviruses, RNA-binding studies with bacterially expressed, hexahistidine-tagged N proteins indicated that N preferentially interacts with the 5′ end of the vRNA.^{385,475} In another study, high-affinity binding of hantaviral N to panhandle RNA was demonstrated, and it was suggested that such binding is related specifically to the trimers, which were able to discriminate viral from nonviral RNA, whereas monomer or dimer subunits bound nonspecifically to RNA.³⁴⁸ These data suggest a requirement for multimerization of N for effective RNA binding and encapsidation, and implicate the panhandle structure as the trigger for encapsidation. Hantavirus N is also able to bind 5′ capped mRNAs (to generate a source of primers for transcription initiation; see above), and the cap and vRNA binding sites have been mapped to separate domains within the protein.³⁴⁹ Furthermore, binding to the mRNA cap induces a conformational change in N, and the structurally altered N loaded with the capped primer binds specifically to the conserved 3′ terminus of vRNA.

The S segments of viruses in the *Phlebovirus* and *Tospovirus* genera and some viruses in the *Orthobunyavirus* and *Hantavirus* genera produce NSs proteins as well as N proteins. Sizes of NSs range from 10 kD for orthobunyaviruses and hantaviruses to more than 50 kD for tospoviruses (Fig. 42.2). The NSs protein of the phlebovirus RVFV is phosphorylated and accumulates in the nuclei of infected cells, where it forms fibrillar structures (e-Fig. 42.4).⁵¹³ The major phosphorylation sites of the RVFV NSs were mapped to serine residues located near the carboxy-terminus of the protein.²⁶⁷ Expression studies revealed that fibrils could form in the absence of other viral proteins; however, if the carboxy-terminal region of NSs was removed, NSs were still transported to the nuclei, but did not coalesce into fibrils.⁵⁸⁴ Similar fibrillar structures of NSs were observed for some strains of TSWV, but the structures appeared only in the cytoplasm.

Comparing the deduced amino acid sequences of NSs for five different phleboviruses revealed homologies of only 17% to 30%.¹⁷⁸ However, comparison of the NSs gene sequences for a number of strains of a single phlebovirus RVFV showed that certain areas were highly conserved.⁴⁵⁹ These data suggest that there may be a strong evolutionary pressure to maintain distinct portions of the NSs for individual viruses, but that the remainder of the protein can diverge without affecting the function of NSs.

Earlier work suggested that the NSs of phleboviruses and tospoviruses were only synthesized after viral replication; that is, after the ambisense vRNA has been copied to cRNA and mRNA was transcribed from cRNA. Recent work with RVFV, however, indicates that at least for this phlebovirus, virions can encapsidate cRNA and that the incoming cRNA ribonucleocapsids can serve as templates for NSs mRNA.²²⁵ The outcome of this is that NSs are present early in infection and might have a role in early replication. The NSs of three orthobunyaviruses all inhibited BUNV RNA synthesis in a dose-dependent manner in a minireplicon system.⁵⁶⁷ The results were suggested to indicate a function for NSs in regulating the activity of the RdRp. In addition to a possible role for NSs in early replication processes, NSs proteins have other important roles in viral infection, such as interferon antagonism, host-range determination, and gene silencing. These effects will be described in the context of effects on host cells.

M Segment Products: Gn, Gc, and NSm

The viral envelope GPs Gn and Gc are translated from a single mRNA complementary to vRNA. The polyprotein precursor of Gn and Gc is not seen in infected cells, and has been observed only by *in vitro* translation of RNA transcripts in the absence of microsomal membranes.⁵⁴² For most viruses, both Gn and Gc are preceded by signal sequences; therefore, cleavage of the polyprotein precursor is likely mediated by host signalase.¹⁵⁰ A pentapeptide motif, WAASA, which is highly conserved among hantaviruses, was shown to be the polyprotein cleavage site for HTNV, by mutational analysis of the signal sequence preceding Gc.³¹⁸

M-segment gene products have a cysteine content of approximately 4% to 7%. Positions of these residues are highly conserved in the M-segment products of related viruses, suggesting that extensive disulfide-bridge formation may occur and that the positions may be crucial for determining correct polypeptide folding. The secondary structure of the proteins is also involved in immunogenicity, as indicated by the finding that neutralizing or protective epitopes are often nonlinear.

All M-segment polyproteins display variable numbers of predicted transmembrane regions, and a hydrophobic sequence at the carboxy-terminus, indicative of a membrane anchor region. Therefore, the M-segment translation products of viruses in the family *Bunyaviridae* are typical class 1 membrane proteins, with the amino terminus exposed on the surface of the virion and the carboxy-terminus anchored in the membrane.

The Gn and Gc proteins of all viruses in the family possess asparagine-linked oligosaccharides. Examination of the oligosaccharides attached to the Gn and Gc proteins of orthobunyaviruses revealed that Gc has mostly high-mannose glycans, whereas Gn contains both complex and a novel intermediate-type oligosaccharide.^{326,377,479} In contrast, the GPs of hantaviruses were found to be mostly of the high-mannose type.⁴⁶⁷ These

findings indicate that the proteins are incompletely processed through the Golgi. This is likely related to retention of Gn and Gc in the Golgi, where assembly occurs, and which will be discussed more fully.

Nairoviruses appear unique among viruses in the family in that they have both N-linked and a region of heavily O-linked carbohydrates (a mucin-like domain, as will be described).⁴⁶¹

The M-segment gene-product processing events differ among the genera. Hantaviruses produce only Gn and Gc,⁴⁷¹ whereas some viruses in the *Phlebovirus* and *Orthobunyavirus* genera produce NSm from the same M-segment mRNA as Gn and Gc. The NSm protein of orthobunyaviruses is encoded between the Gn and Gc proteins.¹⁴⁹ Its hydropathy profile suggests that it is a membrane-bound protein, and expression studies demonstrated that it localizes to the Golgi along with Gn and Gc.³⁰¹ Although a function for this protein has not been identified, it was suggested that it might be involved in facilitating virion assembly in the Golgi.³⁰¹

For the phlebovirus RVFV, a NSm of approximately 14 kD is cleaved from the amino-terminus of the polyprotein translation product (e-Fig. 42.5). Sequence studies revealed five in-frame translation initiation codons (AUG) upstream of the amino-terminus of Gn. Mutational analysis of those codons indicated the fourth and the fifth AUGs to translate Gn and Gc. Whilst NSm was found to originate from the second AUG and a 78-kD polypeptide, representing uncleaved NSm and Gn, was translated from the first AUG.¹⁹⁸ Pulse-chase experiments revealed no precursor-product relationship between the 78- and 14-kD proteins; therefore, it appears that use of the first and second AUGs, respectively, is what dictates generation of these proteins.⁷⁷ Both the 14-kD NSm and the 78-kD polypeptide are found in abundance in RVFV-infected cells,⁷⁸ suggesting that they might play a role in replication or morphogenesis.

An even larger NSm (~30 kD) is cleaved from the amino terminus of the M-segment polyprotein of the phlebovirus, PTV (e-Fig. 42.5). *In vitro* expression studies indicated that both envelope GPs could be produced in the absence of the NSm coding region,³³⁵ but other studies to evaluate the use of the 13 potential translation initiation codons present in the NSm coding information have not been reported. No homology between the NSm proteins of PTV and RVFV was apparent.²²¹

In contrast to the mosquito-borne phleboviruses PTV and RVFV, the tick-borne phlebovirus UUKV does not produce an NSm (e-Fig. 42.5). The first (and only) initiation codon preceding sequences of the envelope GPs is located 17 amino acids upstream of the amino terminus of Gn⁴⁴⁹; hence, the Gn protein of UUKV appears to be analogous to the 78-kD NSm-Gn fusion product produced by RVFV (although there is no obvious sequence homology of these predicted products). Until a function can be assigned to NSm, it is impossible to determine whether UUKV replicates in the absence of such a function or accomplishes whatever function is required without removal of a portion of the amino terminus of Gn.

M-segment polyprotein processing events for nairoviruses are more complicated and appear to differ from those of other viruses in the family (e-Fig. 42.6). Nucleotide sequences of DUGV, CCHFV, and NSDV revealed a coding strategy similar to other viruses in the family, that is, a single ORF.³³¹ Studies on CCHFV demonstrated the polyprotein is co-translationally

cleaved into 140-kD PreGn and 85-kD PreGc precursors and a 15-kD NSm protein.^{14,44,461,462,555} PreGn is processed to the mature 37-kD Gn by cleavage with a cellular secretory pathway protease, SKI-1, following a consensus motif, RRLL, early in the secretory pathway.⁴⁶¹ SKI-1 cleavage generates a subprecursor of the other two polypeptides that is cleaved by a furin-like protease late in the secretory pathway.⁴⁶¹ This cleavage site is completely conserved among several CCHFV strains, suggesting that it has importance for viral replication or pathogenicity.⁴⁶¹ The amino-terminal portion of this subprecursor is a highly variable (among CCHFV strains) polypeptide with mucin-like characteristics, including numerous serines, threonines, and prolines and predicted extensive O-linked glycosylation. The carboxy-terminal portion of the subprecursor is a 38-kD nonstructural polypeptide. After furin cleavage, secreted proteins GP38, GP85, GP160, and the mucin-like protein are produced; presumably these products reflect extensive O-linked glycosylation differences. Although the PreGc precursor also possesses a SKI-1-like cleavage motif, SKI-1 cleavage could not be demonstrated for this protein, so it is likely that a related enzyme is responsible for the processing to yield mature Gc.⁴⁶¹

Unlike all other viruses in the family, the NSm protein of tospoviruses is translated from an ambisense mRNA.²⁷⁴ It is also the only NSm protein in the family to have a definitively assigned function. By subcellular fractionation of infected plants, or in thin-section immunoelectron microscopy studies, the NSm of TSWV was found to be present in cell wall-containing fractions or associated with aggregates of nucleocapsids and with the plasmodesmata.²⁷⁵ Expression of the NSm protein in plant cells or insect cells revealed that the protein first appeared near the cell surface and later as tubular structures protruding from the cell surface. In infected leaf tissues, the tubules were observed only in the plasmodesmata.⁵¹⁰ These data are characteristic of proteins able to aggregate into plasmodesmata-penetrating tubules that allow cell-to-cell movement of the virus across the cell walls in infected plants. In several other studies, the TSWV NSm protein was shown to have additional characteristics of plant movement proteins, including sequence similarity, expression during the early stages of infection, and RNA-binding activity. Formal proof of this function was obtained from experiments in which NSm of TSWV was able to complement cell-to-cell movement of a movement-defective tobacco mosaic virus vector.⁵¹¹ This expression system was also used to show that NSm alone induced tubule formation in protoplasts and induced TSWV-like symptoms in *Nicotiana benthamiana*. Separate domains within NSm have been mapped for each of these functions.⁵¹² NSm was found to be expressed in its natural thrip insect vector, but did not aggregate into tubules, leading to the suggestion that NSm might only have a role in the plant portion of the tospovirus replication cycle.⁵¹¹

L Segment Product: L Polymerase Protein

The L proteins of bunyaviruses range in size from about 237 kD for phleboviruses, to 459 kD for nairoviruses (Fig. 42.2). There are no known processing or posttranslational modifications to the L proteins.⁵⁴⁶ Although structural analysis of an L protein from the family *Bunyaviridae* is not yet available, several features common to other polymerase proteins have been observed.²⁸²

The overall shape of the polymerase is compared to that of a right hand, with fingers, palm, and thumb domains. Four major conserved motifs in the palm region, designated as A–D are shared by many viral polymerases. Motifs A and C are involved in divalent cation use, whereas motifs A and B may be involved in sugar and nucleoside triphosphate selection. Motif C contains the catalytic core of the protein. For BUNV, site-directed mutagenesis demonstrated the importance of amino acids in motif C for polymerase activity.²³⁷ Alignment of the amino acid sequences of L proteins of several viruses in the family *Bunyaviridae* demonstrate the presence of A–D motifs in these proteins. In addition, two novel regions, found at the amino-terminus of the L protein of the phlebovirus RVFV, were shown to be conserved only in the polymerases of viruses in the *Bunyaviridae* and *Arenaviridae* families.³⁶¹

The function of the L protein as an RNA-dependent RNA polymerase was first confirmed by using L protein expressed from vaccinia virus recombinants to transcribe authentic orthobunyavirus ribonucleocapsid templates.²³⁶ Endonuclease activity was demonstrated *in vitro* with expressed BUNV L protein, providing evidence that the L protein is also responsible for generating the capped primers needed for transcription,²³⁶ and this was confirmed by expression of an N-terminal fragment of LACV L protein.⁴⁴²

Morphogenesis

Transport of Viral Proteins

In contrast to other negative-strand RNA viruses, bunyaviruses usually mature within cells by budding at smooth membranes of the Golgi (e-Fig. 42.7). The plant-infecting members of the family, the tospoviruses, also appear to assemble in the Golgi; however, it was suggested that instead of budding, there is a coalescence of Golgi membranes around the ribonucleocapsids.²⁵⁸ Budding at membranes other than those associated with the Golgi have been reported for some viruses; for example, the hantaviruses, SNV, and Black Creek Canal virus (BCCV), and the phlebovirus RVFV were found to bud from the plasma membrane as well as in the Golgi in some cells.^{17,437} The reason(s) for maturation of viruses in the family *Bunyaviridae* in the Golgi complex as opposed to the more usual mode of viral morphogenesis (budding at the plasma membrane) are not understood completely; however, important clues have been obtained by studying the viral proteins, transport to and retention in the Golgi.

Golgi Targeting and Retention

Expression of M gene segments of representative bunyaviruses demonstrated that Gn and Gc are targeted to the Golgi in the absence of other viral components. When expressed individually, Gn is able to exit the ER, but Gc remains in the ER for the orthobunyaviruses, phleboviruses, nairoviruses, and tospoviruses.^{45,262,294,334} For hantaviruses, neither protein exits the ER when expressed separately.^{454,482} For all studies to date, the signal for Golgi transport has been localized to Gn and complexing of Gn and Gc in the ER is necessary for efficient transport of Gc to the Golgi.

Although ER retention signals have not been identified on Gc for most viruses, the phleboviruses have a characteristic carboxy-terminal ER retention motif (KKXX, where K = lysine and X = any amino acid).¹⁷⁶ Evidently, this signal can be overcome when Gn and Gc oligomerize.

To map the Golgi targeting and retention signal(s) on Gn of orthobunyaviruses or phleboviruses, a series of deleted and/or chimeric genes were constructed and their expression products were examined for Golgi targeting and retention. For BUNV, the Golgi localization and retention signal was mapped to the transmembrane domain of Gn.⁴⁸⁵ The Golgi localization signal for the phleboviruses PTV and RVFV were identified within the transmembrane domain plus 10 (PTV) or 28 (RVFV) amino acids of the cytoplasmic tail.^{176,334} The Golgi localization signal of another phlebovirus, UUKV, was found to be within a 30 amino acid peptide of the cytoplasmic tail.¹⁸

Similar expression studies with M-segment constructs of HTNV indicated that the ability of Gn to transport Gc to the Golgi requires the presence of the complete signal sequence of Gc. This suggests that the Gc signal peptide remains attached to the Gn cytoplasmic tail. This signal alone, however, is apparently not sufficient for Golgi targeting because M segment constructs that maintained the Gc signal peptide but had other internal deletions in Gn or Gc failed to reach the Golgi. These results were interpreted to indicate that the correct conformation of the oligomerized Gn and Gc is also important for Golgi targeting.⁴⁷¹ Consistent with this is the observation that overexpression of SNV Gn resulted in the apparent accumulation of misfolded proteins in aggresomes.⁵⁰⁴ In addition, the cytoplasmic tail of Gn was found to be polyubiquitinated when expressed alone, suggesting that it would undergo proteasomal degradation if not complexed with Gc.¹⁷²

For the nairovirus, CCHFV, removal of the transmembrane domain and cytoplasmic tail of Gn did not prevent Golgi targeting, and this soluble truncated Gn was still able to dimerize with Gc and transport both proteins to the Golgi.⁴⁵ In another study, Golgi targeting was dependent both on a signal found in the hydrophobic region of the cytoplasmic tail, as well as in the ectodomain¹⁹⁶; therefore, unlike other bunyaviruses, nairoviruses appear to have at least part of their Golgi-targeting signal in the ectodomain, rather than only in the transmembrane or cytoplasmic tail regions of Gn.

Clearly, although there are definite signals targeting the viral glycoproteins to the Golgi, there is no consensus motif or even region of Gn conserved among viruses in the family.

Trafficking Through the Golgi

The Golgi complex consists of several subcompartments, including the cis-, medial-, and trans-Golgi.²⁶⁴ By using the fungal antibacterial reagent Brefeldin A, which inhibits transport of proteins out of the ER and causes a redistribution of the Golgi component to the ER, the GPs of the phlebovirus PTV were found to be localized in the cis and medial Golgi membranes.⁹³ Similar redistribution of Gn and Gc from the Golgi to the ER were observed following Brefeldin A treatment of cells infected with vaccinia virus recombinants expressing the M segment of BUNV.³⁶⁶ Immunohistochemical and electron microscopy studies of the phlebovirus UUKV demonstrated that budding may begin as early as the pre-Golgi intermediate compartment and that virus budding continues in the Golgi stack.^{232,458}

Examination of the type and amount of oligosaccharides attached to viral proteins influences trafficking and provides clues regarding the transit of the proteins through the Golgi compartments. For example, shortly after primary glycosylation of nascent proteins at the ER, oligosaccharides are susceptible

to cleavage by endoglycosidase H (endo-H), an enzyme that cleaves only high-mannose residues. Later, after removal of glucose residues at the rough ER, migration of the GPs to the smooth ER and Golgi, trimming of residues, and attachment of peripheral sugars, the oligosaccharides are no longer susceptible to endo-H cleavage. Therefore, acquired resistance to endo-H generally indicates that the proteins have been processed through specific Golgi compartments as described earlier (see "M segment products"). For the nairovirus CCHFV and the hantavirus HTNV the GPs remain endo-H sensitive, suggesting that they have not been processed through the medial Golgi.^{45,480} In contrast, the GPs of the orthobunyavirus, BUNV become endo-H-resistant, so they can be assumed to have moved through the trans-Golgi.³⁷⁷

To identify the roles of specific glycosylation sites on trafficking of HTNV GPs, a series of expression constructs were created in which each of the four glycosylation sites that are used on Gn and the single site used on Gc were mutated. Removal of the glycosylation site closest to the amino-terminus of Gn resulted in its inability to exit the ER and for a loss of reactivity with monoclonal antibodies to native Gn.⁴⁸⁰ Mutating the third glycosylation site in Gn was also poorly tolerated, and resulted in inefficient Golgi targeting and loss of monoclonal antibody reactivity. In contrast, mutating the single site on Gc or two of the other three sites of Gn were well tolerated. A similar study with BUNV indicated that the single Gn glycosylation site is absolutely required for correct protein folding, trafficking, and infectivity.⁴⁷⁹ These data are consistent with results from another study, which will be described below, indicating that the processing of sugars plays a key role in maturation of BUNV.³⁷⁷

The time required to convert between endo-H susceptibility and resistance correlates with the time needed for protein transport from the ER to the Golgi. For the phlebovirus PTV, heterodimerization occurred between newly synthesized Gn and Gc within 3 minutes after protein synthesis, and the dimers were found to be linked by disulfide bonds. The dimeric Gn-Gc proteins were observed both during transport and after accumulation in the Golgi complex.⁹³ For another phlebovirus, UUKV, it was found that the transport of Gn and Gc from their site of synthesis on the rough ER through the Golgi occurred at an estimated two to three times slower rate than that of most viral membrane GPs destined to be transported to the plasma membrane.¹⁶⁶ That is, endo-H resistance was achieved at 45 and 90 to 150 minutes for Gn and Gc of UUKV,²⁸¹ compared to 15 to 20 minutes for the hemagglutinin protein of influenza virus or the G protein of vesicular stomatitis virus. The finding that UUKV Gn and Gc have different transport kinetics (i.e., Gn is incorporated into virions 20 minutes faster than Gc) suggests that the dimers may arise from different precursor proteins, possibly because faster-folding Gn cannot dimerize with slower-folding Gc until Gc has reached its correct conformation.⁵⁸⁰ In this same study, the Gn and Gc proteins of UUKV were found to exit the ER quickly, but did not enter the Golgi for 15 to 20 minutes. These findings suggest that the Gn and Gc proteins may dimerize in an intermediate compartment between the ER and Golgi.⁵⁸⁰

The NSm proteins are not known to play a role in transport or Golgi retention. For representative phleboviruses and orthobunyaviruses, expression of Gn and Gc in the absence of NSm had no effect on their transport to the Golgi; however,

when the entire M segments were expressed, NSm co-localized to the Golgi with Gn and Gc, suggesting an interaction of these proteins before their exit from the ER.

Assembly

For assembly to occur, N as well as Gn and Gc must move to the same intracellular location. For the hantavirus, SEOV, N could first be observed approximately 2 hours after infection and accumulated as scattered granules in the cytoplasm. Although N was not observed in the Golgi, it could be observed to surround the Golgi by 24 hours after infection.²⁴⁷ The N proteins of theairovirus CCHFV and the hantavirus BCCV were both observed in the perinuclear region of infected cells, and both were found to bind to actin.^{21,436} Disrupting the cellular actin network by treatment with cytochalasin D, reduced the assembly of infectious CCHFV, suggesting a role for actin in transporting N to the site of virion assembly. For BCCV, the N protein was found to be peripherally associated with Golgi membranes, suggesting that the ribonucleocapsids are recruited to the Golgi for assembly.⁵⁰⁴ In contrast, the CCHFV N protein was not found in association with Golgi membranes; therefore, it is unclear where the actual assembly site is forairoviruses, and it was suggested that some sort of novel structure close to the nucleus might be involved in bunyavirus assembly.²¹

This was confirmed by detailed electron microscopic analysis of BUNV-infected cells, which identified viral replication

occurring in a “viral factory” that is built around the Golgi complex (Fig. 42.7) and comprises repetitive units of Golgi stacks, mitochondria, components of the rough ER, and virus-derived tubular structures with a globular head.⁴⁵⁸ The viral polymerase and N protein were found mainly in the globular head, and viral RNPs were released from disrupted, purified tubes.¹⁵⁹ Viral tubes contain cellular proteins such as actin and myosin I, and both tube assembly and viral morphogenesis were sensitive to drugs that affect actin. Advanced imaging and three-dimensional (3D) reconstruction of infected cells showed that the tubes link cellular organelles, for example, mitochondria, to the Golgi and interact with intracellular viral forms, thereby providing a route for cellular factors required for genome replication. Based on these observations a model has been proposed (e-Fig.42.8) whereby viral RNPs are transported from sites of replication in the globular domain to the cytoplasm where they condense on Golgi membranes modified by the insertion of Gn and Gc, and promote budding of immature particles into Golgi-derived vesicles.

Unlike most other negative-strand RNA viruses, members of the *Bunyaviridae* do not have an M protein to link the integral viral envelope proteins and their nucleocapsids and to act as the nucleating step for assembly. The absence of M protein suggests a direct interaction between the ribonucleocapsids, which accumulate on the cytoplasmic side of vesicular membranes, and viral envelope proteins, which are displayed on the

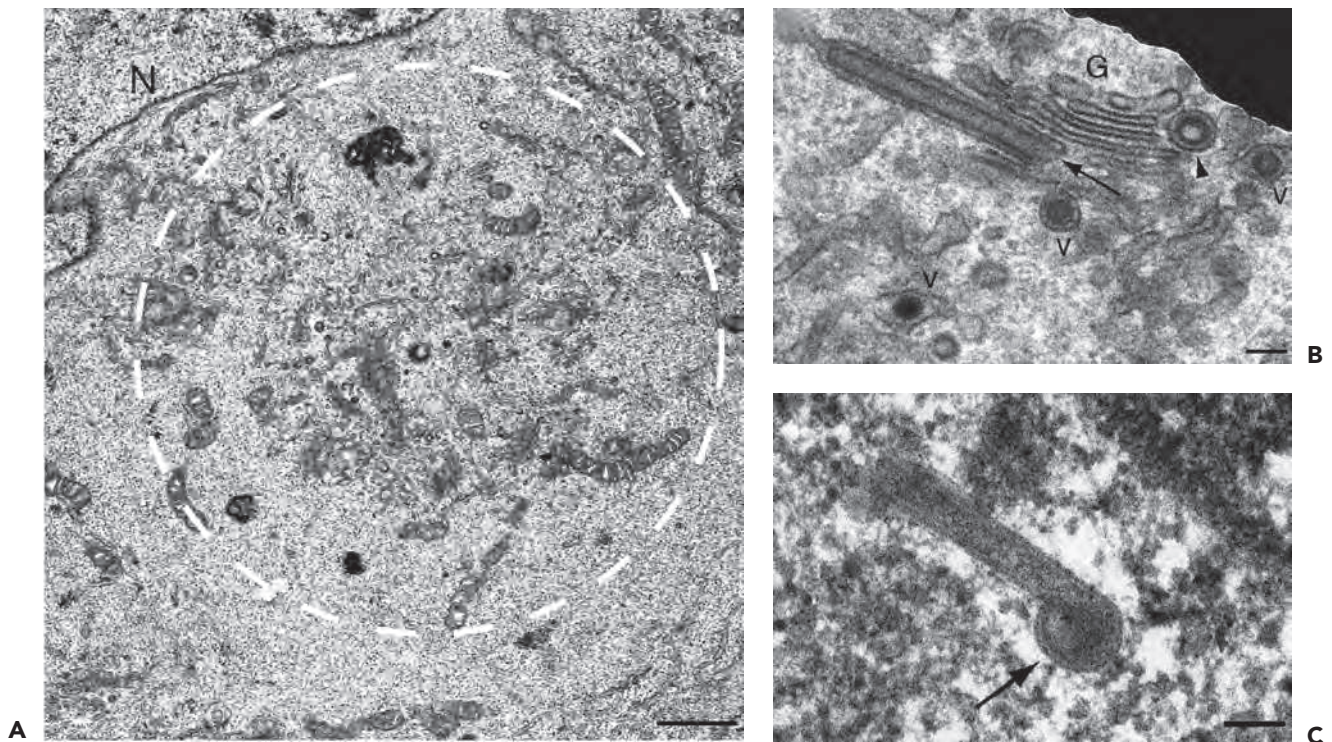


FIGURE 42.7. Visualization of viral factory in Bunyamwera virus (BUNV)-infected BHK cells by electron microscopy.

A: Viral factory (dashed white circle) shows groups of organelles near the nucleus (N). **B:** Higher magnification shows longitudinal (arrow) and transverse (arrowhead) sections of tubular structures in Golgi stacks (G). Virus particles (V) are also seen. **C:** Tubular assembly with a bigger globular domain (arrow). N, nucleus; G, Golgi stack; V, virus particle; scale bar represents 1 μm in (A). (Modified from Fontana J, Lopez-Montero N, Elliott RM, et al. The unique architecture of Bunyamwera virus factories around the Golgi complex. *Cell Microbiol* 2008;10:2012–2028.)

luminal side. Electron microscopy of cells infected with the phleboviruses PTV or Karimabad virus, revealed that ribonucleocapsids and spike structures (i.e., viral envelope GPs) were present only in regions of Golgi membranes where budding appeared to be occurring, and not on adjacent areas of the same membrane, suggesting a transmembranal interaction of N with Gn or Gc.⁴⁹⁷ Direct evidence for the requirement of the cytoplasmic tails of both glycoproteins has been obtained using virus-like particle production assays.⁴⁸⁴

The signal directing the ribonucleocapsids to the budding compartment has not been identified for most viruses in the family. For hantaviruses, however, N was shown to interact with the cellular proteins, SUMO-1 (small ubiquitin-like modifier-1) and Ubc9 (SUMO-1 conjugating enzyme 9) in yeast two-hybrid systems.^{251,301,327} For HTNV, the interaction occurred at a four amino acid motif, MAKE, located at amino acid positions 188 and 189 of N, and mutation of this motif prevented transport of N to the perinuclear region, suggesting a role for this interaction in directing N to the site of assembly.

Excess ribonucleocapsids of hantaviruses, tospoviruses, and nairoviruses were found to accumulate in large cytoplasmic inclusions, suggesting that only ribonucleocapsids that interact with the envelope proteins are transported to the Golgi. Although not yet defined, it is likely that the transmembrane domains of Gn or Gc that are exposed on the cytoplasmic face of the membrane are involved in this interaction. Candidate transmembrane regions have been predicted from hydrophobic characteristics of derived amino acid sequences representing the envelope proteins of all bunyaviruses examined to date. Direct examination of a phlebovirus by enzymatic digestion of exposed proteins embedded in intracellular membranes, demonstrated that approximately 12% of Gn or Gc was exposed on the cytoplasmic face of membranes in infected cells and was accessible to digestion. A large protease-resistant fragment was identified, which was presumably sequestered in the membrane in a manner that rendered it safe from enzymatic digestion.⁴⁹⁷ These enzyme-resistant fragments may therefore represent transmembrane regions of proteins, which could provide the interaction between ribonucleocapsids and the cellular membranes required for envelopment. The predicted cytoplasmic tails on Gn have also been suggested to be logical candidates for interacting with the ribonucleocapsids.⁴⁴⁴

Tospovirus assembly and release in plants may differ from those processes of other viruses in the family. A model was proposed in which Golgi membranes with integral viral envelope proteins wrap around ribonucleocapsids. These particles may then fuse with each other or with ER membranes to release single enveloped particles into the cisternae.²⁵⁸ Mature virions accumulated within the ER cisternae likely remain there until ingested by thrips.

For BUNV, the complete suite of L, M, and S packaging signals was not required for generation of infectious virus as a virus lacking the L segment UTRs (the L ORF was flanked by the complete UTRs from the M segment) was created by reverse genetics.³²¹ In contrast, a recent study on RVFV packaging suggested M-segment UTRs, particularly a region in the 5'UTR, was critical for co-packaging of the L and S segments.⁵²⁶

Transport and Release

After budding into the Golgi cisternae, virions apparently are transported to the cell surface within vesicles analogous to those

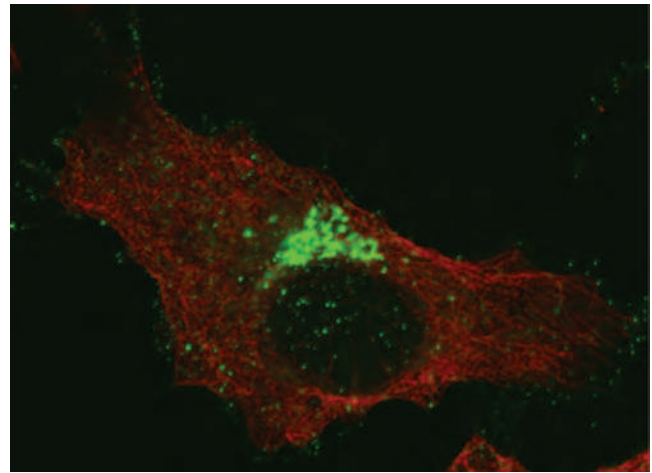


FIGURE 42.8. Bunyavirus transport by exocytosis. BSR-T7/5 cells were infected for 8 h with a recombinant Bunyamwera virus (BUNV) expressing a Gc-GFP (green fluorescent protein) fusion protein and co-stained with an anti-tubulin antibody (*in red*). Progeny virions in vesicles en route to the plasma membrane are visible as *green dots*. (From Shi X, van Mierlo JT, French A, et al. Visualizing the replication cycle of Bunyamwera orthobunyavirus expressing fluorescent protein-tagged Gc glycoprotein. *J Virol* 2010;84:8460–8469.)

in the secretory pathway. The release of virus from infected cells presumably occurs when the virus-containing vesicles fuse with the cellular plasma membrane (i.e., by normal exocytosis). Numerous viruses in the family have been observed late in infection within vesicles or in the process of exocytosis by electron microscopy, and more recently by light microscopy of BUNV expressing a Gc-GFP fusion protein (Fig. 42.8). In polarized cells, the phleboviruses PTV and RVFV show differing release characteristics, in that no marked polarized release could be detected for RVFV, whereas PTV was released primarily from the basolateral surfaces.^{94,177} In contrast to these phleboviruses, the hantavirus BCCV was released from the apical surface.⁴³⁷ Such polarized release of viruses might be important for disseminating virus during natural infection to produce a systemic disease. Release of tospoviruses from insect cells probably occurs via secretory exocytosis similar to that of animal-infecting members of the family.⁵⁷⁹

EFFECTS OF VIRAL REPLICATION ON HOST CELLS

Cytopathic Effects

The cytopathic effects observed in cultured cells infected with members of the family *Bunyaviridae* vary widely, depending both on the virus and the type of host cell studied. Viruses in all genera except for the *Hantavirus* genus are capable of alternately replicating in vertebrates (or plants for tospoviruses) and arthropods, and generally they are cytolytic for their vertebrate/plant hosts but cause little or no cytopathogenicity in their invertebrate hosts. Some viruses display a narrow host range, especially for arthropod vectors. Although the reason for this has not been defined completely, studies on variant and revertant LACV orthobunyaviruses have suggested that

the specificity was related to Gc, probably at the level of viral attachment to susceptible cells.⁵¹⁵ In natural infections of mammals, viruses are often targeted to a particular organ or cell type. For example, orthobunyaviruses such as LACV appear to be neurotropic³⁹⁶; the phlebovirus RVFV is primarily hepatotropic⁴⁰⁷; and the hantavirus HTNV persists in rodent lungs.³⁰⁴ It will be interesting to determine whether this targeting is due solely to host-cell receptors or to other factors such as differences in effects on host-cell metabolism in targeted cell types versus the unnatural situation in cultured vertebrate cell lines.

Host-Cell Metabolism

In vertebrate cells, orthobunyaviruses and some phleboviruses were found to cause a reduction in host-cell protein synthesis, which became more prominent as the infection progressed. For example, by 5 hours after infection, cells infected with BUNV at high multiplicity showed reduced levels of host protein synthesis, and by 7 hours there was almost no synthesis.⁴⁰² RVFV-infected cells displayed reduced host protein synthesis, which gradually became more pronounced from 4 to 20 hours after infection.³⁹⁴ In contrast, a reduction in host protein synthesis did not occur, even late in infection, with another phlebovirus, UUKV, or with theairovirus DUGV,^{410,542} both of which are transmitted by ticks rather than mosquitoes. Hantaviruses not only cause no detectable reduction in host macromolecular synthesis,⁴⁶⁶ but routinely establish persistent, noncytolytic infections in susceptible mammalian host cells, a finding consistent with their nonpathogenic persistence in their natural rodent hosts.³⁰⁴

The arthropod-borne members of the family, like most other arboviruses, cause little detectable cytopathology in mosquito cell cultures, and viral persistence is readily established. Unlike cultured vertebrate cells, mosquito cells infected with the orthobunyavirus, Marituba virus, displayed no reduction in host macromolecular synthesis; therefore, viral infection apparently does not drastically interfere with normal cellular processes.⁸¹ One suggested reason for this is that, in arthropod cells, excess viral proteins do not accumulate in the cells but rather are more efficiently processed into mature virions.³⁶⁹ Another possibility is that viral transcriptase may be less active in arthropod cells than in mammalian cells and that the endonuclease activity of the polymerase (which is used to acquire transcriptional primers) is detrimental to host-cell messages. A reduced level of activity of the viral transcriptase would, therefore, produce less damage to host-cell messages and consequently to protein synthesis.⁴⁵¹

Persistence, both in insect and mammalian cells, can be mediated by defective interfering (DI) viruses. Conventional DI particles, which displayed deletions only in L, were described for orthobunyaviruses and tospoviruses. The L deletions identified both in the TSWV and BUNV DI particles were in-frame, thus allowing translation of truncated L polypeptides.^{116,397} Persistent infections of viruses in the family have also been described that do not involve typical DI particles, but instead are caused by infection with temperature-sensitive and plaque morphology mutants.

Host-Cell Responses and Viral Suppression Interferon-Stimulated Genes and Gene Products

A first line of host defense against viruses is the innate immunity mechanism mediated by the type I interferon (IFN) pathway. Type I IFN (also called IFN α/β) is produced in,

and secreted from, infected cells, and in neighboring cells activates the expression of hundreds of IFN-stimulated genes (ISGs) whose gene products directly or indirectly inhibit virus replication. IFN induction is mediated by the recognition of pathogen-associated molecular patterns (PAMPs) by cellular receptors.⁴¹² Two RNA helicases, RIG-I and MDA5, are intracellular receptors that are activated by RNA, though the double-stranded RNA (dsRNA)-binding protein kinase PKR may also assist.^{413,435,443,473} RIG-I binds short dsRNAs and also the 5' triphosphate groups on uncapped viral single-stranded RNA (ssRNA), whereas MDA5 recognizes long dsRNA. Negative-sense RNA viruses do not produce much dsRNA,⁵⁶⁸ although they are strong activators of RIG-I, probably because of the 5' triphosphate groups on their genomes. Activation of RIG-I by binding of RNA initiates a signaling cascade that leads to phosphorylation of the transcription factor IFN regulator factor 3 (IRF-3)²⁰⁵; phosphorylated IRF-3 dimerizes, enters the nucleus, and initiates IFN- β mRNA synthesis. Virus replication also activates other transcription factors such as IRF-7, NF- κ B, and AP-1, which enhance IFN gene expression. IFN-induced proteins have a myriad of functions, some of which have been characterized with respect to their specific antiviral activity. The three major antiviral defence mechanisms are the Mx protein, PKR, and the 2'-5' oligoadenylate synthase (2'-5'-OAS)/RNaseL system.

The Mx proteins are interferon-induced cytoplasmic proteins, which belong to a family of large GTPases that function in intracellular trafficking. After viral infection, Mx proteins are rapidly induced and accumulate in the cytoplasm. Human MxA has been shown to inhibit the replication of representative members of the *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, and *Phlebovirus* genera.^{61,163,245,254} Both inhibition of primary transcription and genome replication have been observed.^{163,190,444} The MxA protein was shown to bind to the N proteins of LACV, BUNV, RVFV, ANDV, and CCHFV^{19,254,265,444} and in LACV infected cells to redistribute it into membrane-associated perinuclear cytoplasmic complexes, thus removing the protein from use in viral replication^{265,444}). For the orthobunyavirus LACV, MxA inhibits replication in mammalian cell cultures, in mosquito cells expressing the human MxA gene, and in MxA-transgenic mice that lack a functional IFN system.^{163,202,350}

Inhibition of the replication of several hantaviruses by MxA protein has been described. ANDV infection of human vein endothelial cells (HUVECs) was found to upregulate transcription of MxA RNA and expression of MxA protein. Virus replication was required for the induction of MxA, and the virus was found to replicate best in cells with low or no expressed MxA. Comparison of induction of MxA protein for pathogenic and nonpathogenic hantaviruses in HUVECs demonstrated that the nonpathogenic hantaviruses (PHV or TULV) induced an early and vigorous onset of MxA expression, whereas the pathogenic hantaviruses (ANDV or HTNV) induced MxA relatively late (48 hours) after infection.^{174,276} In contrast, Vero E6 cells, which lack IFN genes, supported the growth of both viruses. These results suggest that pathogenic, but not nonpathogenic hantaviruses, can delay the IFN- β induced antiviral MxA response and allow efficient viral replication early in infection.²⁷⁶

To determine whether MxA would be active against tospoviruses in plants, transgenic tobacco plants expressing MxA were created and then challenged with TSWV. No increased resistance to viral replication was observed in the transgenic

plants, indicating that expression of human MxA alone is not sufficient to impart virus resistance to plants.¹⁶⁴

In MxA-deficient systems bunyavirus replication can still be impaired, implying that other ISGs have anti-bunyaviral activity.^{20,56,191,381} PKR was shown to contribute to host resistance to BUNV, whereas 2'-5' OAS and RNaseL had no effect,⁵¹² and RVFV lacking its NSs protein is sensitive to PKR *in vitro* and *in vivo*.^{191,224} Other IFN-stimulated genes have been identified in hantavirus-infected cells. These studies were conducted mostly with the idea of understanding pathogenic mechanisms involved in disease and are discussed in more detail later. In one study, gene microarray analysis and reverse transcription-PCR revealed that a variety of ISGs, but not IFN genes, were turned on in response to infection or to treatment with ultraviolet-inactivated SNV.⁴²⁶ Because there was no IFN gene upregulation, it was suggested that IFN-independent pathways mediated the induction of the ISG, perhaps by binding of the viral particles to the $\beta 3$ integrin receptor that has been shown to be used by SNV and other pathogenic hantaviruses for entry via receptor-mediated endocytosis. Consistent with this, and with the aforementioned MxA study, differences in ISGs were observed at early times after infection of human endothelial cells with pathogenic or nonpathogenic hantaviruses, with the nonpathogenic hantavirus inducing much higher levels of ISGs as compared to pathogenic hantaviruses.¹⁷⁴

Interferon Antagonism

Because the IFN response induces an antiviral state in cells, most or all viruses encode one or more IFN antagonistic proteins, which allows them to overcome the effects of IFN early in infection. Evasion of the IFN response by viruses can occur by preventing IFN release or by inhibiting IFN-signaling and/or the activity of ISGs.

IFN antagonism has been demonstrated for the NSs proteins of the phlebovirus RVFV, the orthobunyaviruses BUNV and LACV, and the hantaviruses PUUV and TULV.²²⁷ For the phleboviruses and orthobunyaviruses, NSs were identified as IFN antagonists by comparing wild-type and mutant viruses that do not express NSs. A RVFV mutant, called Clone 13, was naturally occurring, whereas the BUNV and LACV mutants were created by reverse genetics.^{51,63,362} The wild-type viruses induced little IFN and were virulent in mice, whereas the NSs-defective viruses were potent inducers of IFN and attenuated in mice. In genetically altered mice that are nonresponsive to type 1 IFN, the mutant viruses were just as virulent as the wild-type parents, indicating that type 1 IFN is the target of NSs.^{53,63,265,566}

Although the orthobunyavirus and phlebovirus NSs have no sequence similarity and are expressed by different coding strategies, they both appear to antagonize host IFN at the level of cellular transcription. For BUNV and LACV, NSs was shown to inhibit phosphorylation of the C-terminal domain of the RNA polymerase II complex, leading to a downregulation of host mRNA synthesis.^{266,533,552} This effect was seen only in mammalian cells, not in insect cells, suggesting that IFN antagonism by NSs might be involved in the lytic infections observed in mammalian cells as opposed to viral persistence in insects.²⁶⁹ BUNV NSs was shown to interact with the protein MED8, a component of the Mediator complex that is essential for mRNA synthesis.³⁰⁷ LACV NSs selectively targets the RPB1 subunit of the elongating form of RNA polymerase II for proteasome-mediated degradation, an event similar to that

seen during the cellular DNA damage response.⁵⁵² RVFV NSs inhibits transcription of IFN mRNA in two ways, firstly by interacting with, and degrading, the p44 subunit of the TFIIF transcription factor,²⁹⁶ and secondly by recruiting the SAP30 repressor factor to the IFN- β promoter.²⁹⁸ The NSs proteins of the phleboviruses PTV and SFSV also block IFN induction^{191,404} whereas that of TOSV only blocked induction when expressed in a heterologous context.¹⁸⁴ In addition to interfering with the IFN response, BUNV NSs was also reported to delay apoptosis, probably by inhibiting some downstream effect of IRF-3 activation. This effect, however, was independent of the IFN response, in that the NSs-deleted BUNV also induced rapid apoptosis in a cell line that was not responsive to IFN. In a cell line that produces low levels of IRF-3, induction of apoptotic cell death by the wild-type and NSs deletion mutant was similar, suggesting that IRF-3 is involved in the BUNV-induced apoptotic pathways and that NSs somehow counteracts this effect.²⁶⁶ Orthobunyaviruses in the Anopheles A, Anopheles B, and Tete serogroups do not encode an NSs protein, and most behaved like a recombinant BUNV with the NSs gene deleted in failing to prevent induction of IFN- β mRNA.³⁵¹ However, Tacaiuma virus in the Anopheles A serogroup inhibited IFN induction similar to wild-type BUNV, suggesting that TCMV has evolved an alternative mechanism, not involving a typical NSs protein, to antagonize the host innate immune response.

Some hantaviruses have an additional ORF in the S segment,⁴²¹ and evidence for the expression of an NSs protein has been obtained for PUUV and TULV.²²⁷ The NSs protein was shown to be weakly antagonistic to IFN when overexpressed in cell culture, and the presence of a full-length NSs gave a growth advantage to TULV strains in IFN-competent cells.²²⁸ Both hantaviruses and nairoviruses have 5' monophosphorylated nucleotides on their genomes, thereby avoiding recognition by RIG-I and hence inducing IFN,¹⁸⁹ although the viruses also encode other countermeasures to innate immunity. The OTU domain in the nairovirus L protein deconjugates ubiquitin and ISG15 from cellular targets, thus antagonizing the antiviral effects of ISG15 and inhibiting NF- κ B dependent signaling.^{77,165,230} Certain hantaviruses have also been shown to interfere with TNF- α induced activation of NF- κ B signaling through an interaction between the viral N protein and karyopherin molecules that normally transport the NF- κ B subunits from the cytoplasm to the nucleus, thus preventing their use to activate ISG.⁵²³

As described above, it has been reported that pathogenic hantaviruses do not efficiently activate the IFN response as compared to nonpathogenic hantaviruses, suggesting the presence of an IFN antagonistic gene in pathogenic hantaviruses.¹⁷⁴ The cytoplasmic tail of the Gn glycoprotein (Gn-T) from pathogenic hantaviruses has been shown to downregulate IFN induction by interacting with TNF receptor-associated factor 3 (TRAF3), an adaptor protein for IRF-3 and NF- κ B signaling, whereas Gn-T from PHV fail to inhibit IFN induction.^{11,12} However, a recent paper demonstrates that the Gn-T of nonpathogenic TULV can also downregulate IFN induction, but does so in a manner not involving interaction with TRAF3.³³⁶ These data suggest that the pathogenic potential of hantaviruses does not depend on their ability to downregulate IFN alone. The situation is further complicated by the demonstration that for ANDV, IFN induction is inhibited by co-expression of the glycoprotein

precursor (GPC) and N protein, whereas downstream IFN signaling may be inhibited by either protein.³⁰⁹ In addition, hantavirus infection of VeroE6 cells was reported to result in induction and secretion of type III IFN (IFN- γ).^{425,509} Furthermore, the induction of ISGs in epithelial cell lines (e.g., A549 cells) infected with Vero cell grown virus was found to be due to the presence of IFN- γ rather than to virus infection.⁴²⁵

Cytokines/Chemokines/ITAMs

The host cells' responses to hantaviral infection have been studied in an attempt to understand the mechanism of disease. Hantaviruses cause two serious human disease syndromes: HFRS and HPS. Both HFRS and HPS are believed to result from host immune responses to viral infection, rather than damage caused by the viruses themselves.²⁵⁵ In both syndromes, vascular endothelial cells show increased permeability, which is believed to contribute greatly to the diseases. Several studies have measured the types of cytokines and chemokines released in response to disease, and these are discussed in the context of viral pathogenesis below. Of note is the presence of immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic tails of the Gn protein of hantaviruses. ITAMs are cell-signaling elements involved in regulating endothelial cell function. The presence of these elements in hantaviruses has also been suggested to relate to the dysregulation of endothelial cells during hantaviral infection.^{172,173}

Apoptosis

Apoptosis, or programmed cell death has been described for hantaviruses, orthobunyaviruses, and phleboviruses. Apoptosis usually results from activation of a proteolytic system involving caspases, a group of cysteine proteases that cleave cellular substrate proteins.

Apoptosis caused by a virus in the family *Bunyaviridae* was first noted in cultured cells and brains of newborn mice infected with the orthobunyavirus LACV.⁴⁰¹ The NSs proteins of LACV were found to have an amino acid sequence similar to that of a *Drosophila* protein, Reaper, which is involved in regulating caspase activity and can induce apoptosis.¹⁰⁴ Reaper is one of several proteins that are able to bind to a group of proteins, known as inhibitors of apoptosis, which function to control caspase activity.⁴⁸⁷ Like Reaper, BUNV NSs proteins were shown to both inhibit cellular protein translation and activate caspase in cell-free extracts. To demonstrate *in vivo* apoptosis, a Sindbis replicon expressing NSs was injected into the brains of young mice. The mice developed neuronal apoptosis and died 6 days after infection. A similar mechanism of action for inducing apoptosis by Reaper and orthobunyavirus NSs was proposed because both were shown to bind to and counteract the effects of a protein known as Scythe, which is an apoptosis regulator.¹⁰⁴ Mosquito cells persistently infected by LACV do not undergo apoptosis.⁵¹ Oropouche orthobunyavirus (OROV) induces apoptosis in HeLa cells that is dependent on virus uncoating and replication, and treatment of cells with a pan-caspase inhibitor did not affect virus production although apoptosis was prevented.³ The results indicated that the intrinsic apoptosis pathway was triggered by virus replication but apoptosis was not necessary for efficient virus production.

Apoptosis was also observed in the brains of adult Balb/c mice infected with a neurotropic strain of the phlebovirus

TOSV, but the mechanism of apoptotic triggering was not investigated.¹¹⁰

For several hantaviruses, apoptosis was observed in infected cultured monkey kidney (Vero E6) cells,^{246,313} in cultured human embryonic kidney cells (HEK-293),³³⁰ and in lymphocytes of HFRS patients.⁴ Unlike most infections with hantaviruses, which are generally noncytolytic and persistent, cytopathic effects were observed in the HEK-293 cells infected with hantaviruses, and apoptosis was observed almost entirely in cells adjacent to those actively infected with the hantavirus.³³⁰ In another report, apoptosis was not seen in confluent Vero E6 cells infected with various hantaviruses, and only a few apoptotic cells could be seen when subconfluent cells were infected.¹⁹⁸ Likewise, infection of primary immature dendritic cells or HUVECs with HTNV did not induce cell lysis or apoptosis.⁴²⁹ These differing results remain to be resolved, but it is likely that they can be attributed to differences in the cells or the condition and passage histories of the cells.

The factors triggering apoptosis for hantaviruses are not generally known. In TULV-infected Vero E6 cells, caspase 8 activation was observed and apoptosis could be inhibited with a caspase inhibitor.³¹³ Caspase 8 is one of several caspases that can be induced by the binding of a specific ligand to "death receptors," such as tumor necrosis factor 1 (TNF1) and Fas (also called CD95), which are found on certain cells.²⁹ Consistent with this, TNF1 was upregulated at times when apoptosis was apparent in the TULV-infected cells and a Fas-mediated apoptosis enhancer, Daxx, was found to bind to PUUV N proteins.³¹⁵ However, in another study, no significant increase in the mRNAs of the TNF superfamily was observed in hantavirus-infected HEK-293 cells.³³⁰

Stress on the host cell ER is another cellular condition that can trigger apoptosis. TULV infection of Vero E6 cells was noted to activate markers of ER stress, including induction of the chaperone protein, Grp78/bip, which was suggested to be induced by the accumulation of misfolded proteins in the ER.³¹⁴ Another cellular stress-response protein, heat shock protein 70, was found to be abundant in postmortem tissues of hantavirus-infected patients and to be upregulated in VeroE6 cells infected with HTNV.⁵⁸⁹ In this study, it was not clear what role the stress protein played in host-cell response to infection. Additional work is needed to identify the many possible factors that can contribute to cellular stress and apoptosis in bunyavirus-infected cells.

RNA Silencing

RNA silencing, or RNA interference (RNAi) was first described for plants as a mechanism to defend against viral infection, but is now known to occur in most eukaryotes. The gene silencing is mediated by short interfering RNAs (siRNAs), which arise from cleavage of dsRNAs, including the replication intermediates of RNA viruses by a host enzyme known as Dicer. The 21 to 25 nucleotide siRNAs become part of a protein complex known as the RNA-induced silencing complex, which recognizes and degrades sequence-specific mRNAs. Some viruses have been shown to produce suppressors to counteract the gene silencing mechanism. In the family *Bunyaviridae*, the NSs protein of the tospovirus TSWV was found to act as a suppressor of gene silencing.^{68,521} In addition to the plant-infecting bunyaviruses, siRNA suppression has been observed with the orthobunyavirus LACV, both in mammalian and insect cells,

and with the nairovirus Hazara virus (HAZV) in tick cells. As for the tospoviruses, the LACV-suppressing activity was localized to NSs, despite the completely different coding strategy used for this protein (i.e., ambisense vs. ORFs).⁵⁰² For HAZV, which does not produce an NSs protein, the activity was observed with the S-segment gene in either the sense or antisense orientation.¹⁶⁷

Although not yet demonstrated, it was suggested that the similarities of the ambisense-encoded NSs proteins of tospoviruses and phleboviruses would suggest similar functions in viral replication. It will be interesting to see whether the NSs of phleboviruses will have both interferon antagonistic activity (as described earlier) as well as RNAi activity. Such dual-pathogenic activity is known to occur for other viral proteins, for example, the NS1 protein of influenza virus.¹⁹⁷

PATHOGENESIS AND PATHOLOGY

Orthobunyavirus Genus

Human infections with California encephalitis, LACV, or Jamestown Canyon (JCV) viruses are initiated by the bite of a virus-infected mosquito. The course of the infection has been extensively modeled in mice.³⁹⁶ Similar to the human infection, the outcome of the infection in mice is dependent on the age of the animal and strain of virus used, with subcutaneous challenge of newborn mice most closely mimicking the natural human infection. The virus initially spreads from the site of inoculation into striated muscle, which is the major site of replication. It spreads to the plasma, presumably through the lymphatic channels, and the resulting high viremia allows the virus to cross the blood–brain barrier.²³¹ Mice inoculated intraperitoneally with LACV showed high levels of replication in nasal turbinates, suggesting the virus could enter the central nervous system (CNS) by olfactory neurons.³⁸ Once in the CNS, the virus replicates in neurons and glial cells, causing considerable neuronal necrosis. Death occurs 3 or 4 days postinfection. In contrast, although rhesus monkeys were highly susceptible to LACV infection, the animals remained asymptomatic and developed neutralizing antibodies.³⁸ High viremia is essential for neuroinvasion. A monoclonal antibody escape mutant of LACV (V22) showed decreased replication in striated muscle, and hence did not generate sufficient viremia to permit neuroinvasion, but V22 was as neurovirulent as wild-type LACV when inoculated intracranially.¹⁸² A similar distinction between neurovirulence and neuroinvasiveness is implied from observations on recombinant LACV with mutations in the fusion peptide domain of Gc, which showed reduced replication in muscle cells but retained the ability to cause neuronal loss in culture.⁵⁰¹ The lesions observed in the brains of fatal La Crosse encephalitis cases differ from those in infected suckling mice. In humans, and to a large extent in adult mice infected intracerebrally, cerebral edema, perivascular cuffing, glial nodules, mild leptomeningitis, and occasional areas of focal necrosis that are typical of acute severe viral encephalitis are observed. Lesions are mostly in the cerebral cortex, with some in the brainstem.²⁴³ Studies with Tahyna virus and JCV showed strain differences in neuroinvasiveness and neurovirulence in a Swiss Webster mouse model, whereas in rhesus monkeys no clinical disease was observed but the animals mounted strong neutralizing antibody responses.^{39,40}

Infection of natural vertebrate amplifying hosts of these viruses, such as adult chipmunks (LACV) or snowshoe hares (snowshoe hare virus), results in inapparent infection and viremia sufficient to infect mosquitoes.⁴⁷⁷ *Aedes triseriatus* mosquitoes are the principal vector of LACV, and their experimental infection with California encephalitis serogroup viruses has been studied in detail.^{36,529,535,564} Viruses ingested via a viremic bloodmeal infect the epithelial layer surrounding the mosquito midgut. Replication of the virus results in release of the virus across the basal lamina into the hemocoel, allowing transport of the virus to tissues throughout the body. Virus infection of mosquitoes other than the principal vector species usually results in poor penetration of the virus across this midgut barrier. Experimental infections using reassortant viruses have shown that virus M genomic segment (encoding the glycoproteins and NSm protein) is an important component in the correct match between virus and host mosquito, which allows efficient transit across the midgut barrier.³⁶ Once across the barrier, virus replication occurs in a wide variety of tissues, including the ovaries and salivary glands.⁵²⁹ Release of virus from the salivary glands allows virus transmission from the female mosquito to the vertebrate host during feeding. Mosquitoes begin to be infectious approximately 1 or 2 weeks after ingestion of virus, an interval referred to as the extrinsic incubation period.

The infection of the ovaries is thought to be crucial for virus maintenance in mosquito populations, in that it results in transovarial transmission of the virus from the female to the offspring and allows the virus to overwinter in infected eggs.⁴⁶¹ In addition, LACV-infected females appear to mate more efficiently than uninfected mosquitoes.⁴⁴¹ Male mosquitoes play no role in the vertical transovarial transmission of the virus, or in the horizontal transmission via amplifying vertebrate hosts, as they do not take a bloodmeal. However, the virus is detected in the gonads of transovarially infected males and can result in venereal transmission of the virus horizontally within the mosquito population.⁵³⁵ This combination of transmission mechanisms allows efficient maintenance of the LACV in *A. triseriatus* populations. Similar processes are thought to function in host mosquito infections with other California serogroup viruses,⁵³⁹ and results from studies with these viruses and their arthropod hosts serve as a model for understanding virus maintenance and transmission mechanisms for other family *Bunyaviridae* viruses.

Aino and Akabane virus (AKAV) are important causes of disease in livestock, causing epizootics of abortions and congenital defects in cattle, sheep, and goats.^{201,259,538} Experimental infections of pregnant cattle or ewes with either virus have been shown to produce viremia, followed by virus replication in the placenta and fetal tissues, and resulting in congenital defects such as microencephaly, and hydrocephalus.^{201,286,395,538} Similarly, experimental infection of pregnant hamsters with AKAV results in death of the fetus.¹⁶

Phlebovirus Genus

RVFV pathogenesis and disease has been reviewed in-depth recently.^{54,226,403} RVFV infections of livestock are often recognized by the onset of “abortion storms,” which sweep through livestock-producing areas, with simultaneous acute febrile disease in humans. Most human infections with RVFV result in a mild febrile illness. The incubation period is approximately 2 to 6 days, and is followed by an abrupt onset of fever, chills,

and general malaise. Susceptibility of livestock to RVFV infection varies considerably, depending on a variety of factors, including livestock species and age and the strain of the virus. Based on data from experimental infections and analogy with other arbovirus infections, the general pattern of infection likely follows the same course as that following inoculation of the virus by mosquito bite: the virus is transported by lymphatic drainage to the regional lymph node where local replication takes place. The virus spills over into the circulation, causing the primary viremia and spread of the virus to the major organs. Replication in the lymph nodes, spleen, liver, adrenals, lungs, and kidney tissues results in high viremia, and, in severe cases, hepatic necrosis is prominent and necrotic foci can be observed in the brains of cases exhibiting the less frequent encephalitic form of the disease.³⁵⁵ RVFV replication in cells is highly cytotoxic, suggesting that most cellular destruction in acute illness is likely due to direct virus killing of host cells.

Pathologic features of the disease in livestock vary considerably. Leukopenia is frequently seen during the first 3 or 4 days of infection, when fever and viremia are usually at their highest. Altered serum enzyme levels (e.g., aspartate aminotransferase and sorbitol dehydrogenases) indicative of hepatocyte destruction are often seen during the acute phase. Leukocytosis often occurs in the early phase of recovery. Thrombocytopenia and fibrin thrombi in several organs suggest that disseminated intravascular coagulopathy (DIC) may be a feature of severe disease in livestock, as is seen in hemorrhagic infections in humans.^{107,520}

Infection of pregnant animals frequently results in abortion of the fetus. Abortions early in the course of infection are probably the result of the high fever associated with the acute phase of illness; later abortions are more commonly the result of direct infection of the fetus with resulting hepatic necrosis. No clear correlation between RVFV infection and abortion has been seen in human infections.³⁴⁰

Nairovirus Genus **Vertebrate Hosts**

CCHF and NSD viruses are maintained in nature predominantly in their respective tick hosts. Although the viruses can persist in the ticks through the various life stages (transstadial transmission) and can be passed to the offspring (transovarial transmission), vertebrates are needed to provide blood meals for the ticks, and they can become infected and develop viremia capable of supporting virus transmission to uninfected ticks.¹¹⁵ In this manner, vertebrates may play an important amplifying role in the virus natural cycle. In the case of CCHFV, a variety of livestock (e.g., sheep, goats, cattle, ostriches), large wild herbivores, hares, and hedgehogs may become infected, resulting in an inapparent or subclinical disease.^{210,519} Sheep and goats are implicated as being important vertebrate hosts of NSD virus, and infection often results in severe disease.¹¹⁵ These animals are presumably subcutaneously infected when bitten by infected ticks. Studies of CCHFV pathogenesis have been hampered by lack of a suitable animal model, and most nonhuman primates do show evidence of disease. Recently, two mouse models have been described, one using type I IFN-receptor knockout mice,⁴² the other using Signal Transducer and Activator of Transcription (STAT1) knockout mice⁴¹; the animals are highly susceptible to infection, and the STAT1 knockout mice display many features of human disease including fever, leukopenia, thrombocytopenia, and highly elevated liver enzymes.

In experimental NSD virus infections of sheep, goats, and suckling mice, the virus replicates to high titers in the lung, liver, spleen, and other organs of the reticuloendothelial system.⁵²⁷ Vascular endothelium appears to be the primary cell target. Virus replication in the endothelial cells results in edema and necrosis of the capillary walls of the mucosal surfaces of the intestine, gall bladder, and female genital tract, leading to congestion, hemorrhage, and catarrhal inflammation.

Human Infections

In contrast to the inapparent infection characteristic of CCHFV infection of other vertebrate hosts, human infections often result in severe hemorrhagic fevers. On introduction of the virus, there is likely local replication followed by blood- and lymph-borne spread of the virus to the major organs, including the liver, where high levels of replication take place.⁵¹⁸ Congestion, edema, and focal hemorrhage and necrosis are seen in most organs. DIC is evident early, with thrombocytopenia, elevated prothrombin time ratio and activated partial thromboplastin time (APTT) present during the first few days of illness, and likely plays a central role in disease progression. Little is known regarding host-cell receptor use or factors influencing virus virulence. Both IgM and IgG antibodies are usually detectable approximately 7 days after the onset of illness and are detectable in all survivors by day 9.^{87,158} Antibody responses are rarely detectable in fatal cases.

NSD virus has been isolated from sick patients in Uganda, as has the identical or closely related Ganjam virus from febrile patients in India, but their importance in terms of human disease is currently unclear.

Hantavirus Genus **Reservoirs**

Hantaviruses have been detected in numerous rodents and insectivores (reviewed in 241). To date, all known pathogenic viruses are harbored by rodents, but additional study of the disease potential of the many hantaviruses carried by scrod and talpid insectivores is needed. Hantaviruses are horizontally transmitted among rodents primarily through exposure to excreta and saliva. Infection of natural reservoirs results in an acute phase with high viremia followed by prolonged or persistent infection with variable durations of virus shedding in the urine feces and/or saliva from days to months or even for the life of the rodent. Hantavirus persistence in rodents was found to relate to increased regulatory T cells that modulate the immune response and prevent clearance of infected cells.^{130,131,472} Experimental infection of host rodents indicates that viremia peaks approximately 2 weeks postinfection, and results in dissemination of the virus throughout the animal (e-Fig. 42.9).³⁷¹ The cessation of viremia correlates with the induction of hantavirus-specific antibodies, which first become detectable around 14 days postinfection, peak at approximately 50 days postinfection, and remain detectable for the life of the rodent.³⁰⁴ Although naturally infected rodent reservoirs do not display apparent disease symptoms, reduced winter survival and reduced body weights of infected rodents have been reported.^{95,123,244} Aggressive behavior has been correlated with increased infection among rodents in that the highest seropositive rates are seen in older males and animals with more scars.¹⁷⁹ Hantaviruses appear to target primarily

endothelial cells of rodents, with the highest concentrations of virus antigen being observed in the lungs and kidneys.³⁶⁸

Human Infections

In stark contrast to the asymptomatic hantavirus infection of primary rodent reservoir species, human infections frequently result in HFRS or HPS. Humans are infected by inhalation of aerosols produced from the infected rodent excreta. Virus RNA is usually detectable in patient blood during the early stages of disease. At the time of death, virus antigens are detectable in endothelial cell layers throughout the body, but predominantly in lung endothelial cells in the case of HPS, or kidney endothelial cells in the case of HFRS.^{217,591} Pathologic findings in HFRS patient kidney biopsies include mild to moderate interstitial infiltration of lymphocytes, plasma cells, monocytes/macrophages, and polymorphonuclear leukocytes (mainly eosinophilic granulocytes and neutrophils), although the exact mechanism of kidney failure in HFRS patients is unclear.⁵²⁵ The lung is the primary target organ of HPS. Patients usually develop pulmonary edema, plural effusions, and interstitial mononuclear cell infiltrate, edema, and focal hyaline membranes. Viral antigen is plentiful in lung endothelial cell.^{409,591}

In general, both HFRS and HPS result from capillary leakage and fluid loss. A number of studies have implicated immune-modulated processes rather than virus-induced cell death as the primary cause of disease. Hantavirus-specific antibodies and T cells are detectable at the time of onset of disease symptoms, consistent with immune-pathogenesis (reviewed in 241). For example, in HFRS, the expression of the cytokines tumor necrosis factor α (TNF- α), TGF- β , and platelet-derived growth factor has been observed at the peritubular area of the distal nephron. Similarly, high numbers of cytokine-producing cells can be seen in the lungs (but not the kidneys and livers) of fatal HPS cases, suggesting that local cytokine production may play an important role in pulmonary edema and the high case-fatality rate. Severity of illness in humans has been correlated with specific human lymphocyte antigen (HLA) haplotypes, both HFRS and HPS.^{328,365,548,561}

EPIDEMIOLOGY AND ECOLOGY

Orthobunyavirus Genus

Most orthobunyaviruses are transmitted by mosquitoes, although a few have been isolated from tabanids, phlebotomines, tick, and bedbugs, and have been found in every continent except Antarctica.⁵⁰

California Serogroup Viruses

LACV is the most significant of the California encephalitis serogroup viruses in terms of causing human disease in the United States, with an average of 79 cases per year, predominantly in children younger than 15 years of age. There is, however, severe underreporting.^{195,240} The primary vector is the forest-dwelling, tree-hole-breeding mosquito, *Aedes triseriatus*, although more recently, and concerning, the virus has also been isolated from the aggressive day-feeding Asian tiger-mosquito, *A. albopictus*.²⁹⁰ *A. triseriatus* is found throughout the northern Midwestern and Northeastern United States, and LACV is maintained in these

mosquitoes by transovarial transmission, which allows overwintering of the virus in mosquito eggs.⁵⁶⁵ During the summer months, squirrels, chipmunks, foxes, and woodchucks become viremic following LACV infection, and are important amplifying hosts.^{534,590} The majority of La Crosse encephalitis cases occur in the summer and early fall months when risk of bite from infected female mosquitoes is highest. Historically LACV infections mostly occurred in the Mississippi and Ohio river basins, with more than 90% of cases coming from Wisconsin, Minnesota, Iowa, Indiana, Ohio, and Illinois,⁵⁰ but since the 1980s, more cases have been reported from Appalachia and eastern Tennessee, and between 1987 to 2009 more than 30% of total cases in the United States were from West Virginia.¹⁹⁴

Jamestown Canyon virus (including the closely related Jerry Slough variety) is also associated with arboviral encephalitis in the United States. The virus is vectored by *Culex inornata* mosquitoes, and several species of *Aedes* mosquitoes, and is broadly distributed across much of North America. Vertical transmission of the virus has been demonstrated in several *Aedes* species mosquitoes.¹⁹⁹ White-tailed deer are the most likely vertebrate amplifying host. Unlike LACV, which mainly causes encephalitis in children, Jamestown Canyon virus appears to predominantly cause disease in adults, although only 15 human cases have been reported since 2004.²³

Bunyamwera Serogroup Viruses

Bunyamwera virus is present throughout much of sub-Saharan Africa, and appears to be an important cause of acute febrile illness in humans. The virus has been isolated from humans in Uganda, Nigeria, and South Africa.^{181,271,532} More recently, Ngari virus has been identified as a reassortant BUNV, with its M segment derived from the Batai virus (i.e., S_{BUN} M_{BAT} L_{BUN}). This reassortant virus has been associated with hemorrhagic fever cases in Somalia and Kenya and a large outbreak of acute febrile illness in Sudan.^{58,64,175,587} In addition, human antibodies reactive with BUNV have been detected in most of sub-Saharan Africa, with high prevalence (up to 82%) being recorded in some locations.⁴⁹⁹ Isolation of the virus from several *Aedes* species mosquitoes has implicated them as the primary vector.^{271,499} Antibodies reactive with BUNV have been detected in domestic animals, nonhuman primates, rodents, and birds, and viremias capable of supporting mosquito transmission have been recorded in experimentally infected rodents, bats, and primates.^{499,576} However, the role of a potential vertebrate amplifying host is currently unclear.

Several additional Bunyamwera serogroup viruses are present in the Americas³⁷⁰ and are infrequently reported to cause acute febrile illness in humans. Cache Valley virus is found throughout the United States, Canada, and Mexico, and often infects sheep and possibly all ruminants; infections have been associated with embryonic and fetal death, stillbirths, and multiple congenital malformations in sheep.^{134,337} The virus has caused a fatal encephalitis in a human.⁴⁷⁶ It remains unclear whether Cache Valley and serologically closely related viruses may play a role in syndromes of congenital malformations and embryonic losses in humans in North America.⁷⁵

Simbu Serogroup Viruses

Simbu serogroup viruses are global in distribution, and are principally vectored by biting midges of the genus *Culicoides*. Since the original isolation of OROV from a febrile patient in

Trinidad in the 1950s, the medical importance of the virus, particularly in the Amazon basin regions of northern Brazil and Peru, has become increasingly evident.⁴¹⁸ Between 1960 and 2009, at least 500,000 people are thought to have been infected in more than 30 outbreaks.⁵⁵⁰ Most of these outbreaks were in relatively urban areas, leading to the suggestion that there are likely separate urban and sylvatic cycles.^{121,448}

The principal urban vector in Brazil appears to be the tiny biting midge, *Culicoides paraensis*,^{415,448} which breeds in rotting vegetative matter, and seasonal populations can become high in agricultural areas, where build-up of debris such as banana tree stalks or cacao husks may occur.⁴¹⁷ These midges feed predominantly in the early evening hours and are quite anthropophilic. Infected humans develop viremia sufficiently high to transmit the virus to uninfected midges, and appear capable of serving as the vertebrate amplifying host during urban epidemics.⁴¹⁷ Serologic data also suggest that primates or sloths, or even birds, may be potential hosts in the sylvatic cycle. Recent phylogenetic studies indicate that four distinct OROV lineages are present in different geographic areas, and that OROV emerged in Brazil in around 1790.⁵⁵⁰

AKAV is widely distributed throughout Australasia (Australia, Japan, Korea, Taiwan), the Middle East (occasionally extending as far north as Turkey), and sub-Saharan Africa.^{8,15,111,352,524} The virus is an important cause of disease in livestock, with periodic outbreaks of abortions, stillbirths, and congenital malformations recorded in cattle, sheep, and goats in Australia, Japan, Korea, Taiwan, Israel, and Turkey, although intriguingly no disease outbreaks have been observed in Africa, despite the widespread presence of the virus.⁸ The virus is vectored by midges of the genus *Culicoides*, and although *C. brevitarsis* is the primary vector in Australia,³⁶⁴ different species are important in Asia and the Middle East.^{6,234,285}

Phlebovirus Genus

The geographic distribution of SFSV and SFNV Naples viruses closely follows that of their sandfly host, *Phlebotomus papatasi*.⁵²⁸ This distribution extends from the Mediterranean basin throughout the Middle East and Arabian peninsula, north up into areas around the Caucasus mountains, and as far east as Pakistan and India. Sandflies are most numerous in the warmer months, are found at ground level, and feed in the early evenings. Unfortunately, their small size allows them to pass through untreated mosquito netting.

A related phlebovirus, TOSV, is hosted by *P. perniciosus* and is found in central Italy, Cyprus, Portugal, and Spain. Relatively high (20% to 25%) antibody prevalence rates suggest that human infections with TOSV are widespread and frequent in endemic areas.^{60,136} In addition, many acute lymphocytic meningitis and meningoencephalitis cases, particularly those in children, occurring in the summer months are attributable to TOSV infection.^{59,136,341,374} These viruses replicate in their sandfly hosts, and virus transstadial and transovarial transmission have been demonstrated.⁵³¹ Sexual transmission of TOSV among sandflies has also been shown.⁵³⁰ The relatively low efficiency of transovarial transmission demonstrated in experimental infections suggests that vertebrate amplifying hosts are required to maintain these viruses in endemic areas. However, although antibodies to TOSV are present in many domestic animals, virus isolation has been unsuccessful, suggesting that these animals do act as reservoirs,³⁶⁷ and the identity of

amplifying hosts remains uncertain. Data showing that viremic humans can infect *P. papatasi*, together with attack rates as high as 75% and records of urban outbreaks of sandfly fever, suggest that humans can on occasion serve as the amplifying vertebrate host.⁴⁵⁶

The ecology and epidemiology of RVFV is complex and poorly understood. The geographic distribution of RVFV covers much of Africa, from Senegal to Madagascar and from Egypt to South Africa, with most reported epizootics in livestock being reported in East and Southern Africa.^{288,403} Mosquitoes have long been known to play an important role in RVFV epizootics, with epizootics frequently occurring at times of unusually high precipitation.³¹⁶ More recent data indicate that floodwater *Aedes* mosquitoes of the subgenera *Aedimorphus* and *Neomelaniconion* are likely the principal vectors. The ecology of RVFV has been best studied in Kenya, Zimbabwe, and South Africa. In these regions, damboes or vleis, which are shallow depressions up to several hundred meters across near streams and fed by ground water, are thought to play a central role.⁵¹⁶ These damboes flood at times of unusually heavy rainfall, which triggers a population explosion in floodwater *Aedes* mosquitoes.

Isolation of RVFV from unfed male and female *A. mcintoshi* mosquitoes hatched at a dambo in the endemic area of Kenya during an interepidemic period demonstrated the maintenance of the virus between epidemics and the transovarial transmission of the virus in such mosquitoes.³¹⁷ *A. mcintoshi* is thought to be the principal maintenance vector in Kenya and Zimbabwe.⁵¹⁷ The biology of these mosquitoes is such that they are among the earliest mosquitoes to hatch from eggs following flooding. If the eggs contain virus (via transovarial transmission), infected mosquitoes hatch and feed on nearby livestock, potentially initiating a local epizootic. Viremic livestock act as amplifying hosts that transmit the virus to other floodwater *Aedes*, and also other species of mosquitoes including culicines and anophelines, promoting further amplification and spread of the virus. When such a cycle gets initiated at damboes throughout a livestock producing area, it could give rise to a regional, relatively synchronous eruption of RVFV activity with an associated abortion storm, such as those observed in livestock at various intervals in East and Southern Africa.⁴⁰³ Although such a scenario is an attractive explanation of the dynamics of RVFV epizootics, more data are necessary to confirm this is the case.

Hemorrhagic fever cases in humans are usually seen 1 or 2 weeks after the appearance of abortions and disease in livestock. Cases are usually among farmers and others living in proximity to livestock. Humans likely acquire infection through a bite from an infected mosquito when vector densities are high. However, contact transmission seems to be most important with RVFV. People involved in the birth or abortions of livestock, butchering of animals, abattoir workers, and so on, are at high risk of infection during epizootics.^{2,88} RVFV is also highly infectious by aerosol, having resulted in many infections of laboratory personnel working with the virus.^{161,500} The relative contribution of infectious aerosols or fomites to transmission is unclear. Although less than 1% of human infections result in severe disease (hemorrhagic fever, encephalitis, retinitis), this can add up to a substantial number of cases given the large scale of some epizootics. For instance, an estimated 27,500 RVFV infections occurred during the 1997/1998 outbreak in the Garissa district of Kenya,⁵⁸³ and

approximately 200,000 human cases with 600 deaths were estimated to have occurred during the outbreak in Egypt in 1977 and 1978.³³⁹ The 2008 outbreak of RVFV in Sudan resulted in 698 human cases with 222 deaths, a case fatality rate of 31.8%.⁵⁷⁷ The economic effects of RVFV epidemics are huge, with the 2006/2007 outbreak in Kenya resulting in losses from livestock deaths valued at more than 7.6 million US dollars.⁴⁴⁶

In contrast to the long history of periodic RVFV epizootics in East and Southern Africa, the recent large RVFV outbreaks in Egypt in 1977 and 1978 and in West Africa in Mauritania/Senegal in 1987 were different situations.⁵⁹³ In Egypt, retrospective studies showed that RVFV had not been enzootic prior to 1977.³⁴⁰ Virus activity disappeared after 1981, only to be reintroduced in 1993.²⁸ These data suggest that although mosquitoes capable of acting as epizootic vectors (e.g., *Culex* sp.) exist in Egypt, mosquitoes capable of RVFV transovarial transmission are probably lacking. It is thought that RVFV was most likely introduced into Egypt from enzootic areas in Sudan.³³⁹

The first confirmed RVFV outbreak outside Africa was reported September 2000, in the western coastal plains of Saudi Arabia and Yemen.⁴⁸⁸ Unusually heavy rains and flooding of the foothills of the Asir mountains appeared responsible for increased mosquito populations and the explosive outbreak in naïve livestock. Eight hundred eighty-four hospitalized patients were identified in Saudi Arabia, with 124 deaths. In Yemen, 1,087 cases were estimated to have occurred, with 121 deaths. The virus associated with this outbreak was almost identical to those associated with earlier RVFV epidemics in East Africa, consistent with the recent introduction of RVFV into the Arabian Peninsula from East Africa.⁴⁸⁸ More recent outbreaks in Madagascar have been linked to importations of infected animals from on-going epidemics in East Africa.⁸⁰

Nairovirus Genus

The epidemiology of CCHFV reflects the complex ecology and geographic distribution of the *ixodid* tick hosts of the virus, particularly those of genus *Hyalomma*.^{147,210} Although CCHFV has been isolated from approximately 30 different tick species, evidence is lacking as to whether they truly represent vectors, or merely reflect the isolation of virus from engorged ticks that have fed on a viremic vertebrate host. Only some ticks of three genera, *Hyalomma*, *Dermacentor*, and *Rhipicephalus*, have actually been shown to be capable of transstadial transmission of CCHFV (i.e., passing the virus through the various stages of the life cycle, from larva to nymph to adult) following feeding on a viremic host. Transovarial transmission of CCHFV has also been shown to occur with some of the tick species in these genera. Moreover, the overall global pattern of geographic distribution of CCHFV cases corresponds most closely with the distribution of *Hyalomma* ticks, suggesting their principal role as vector.

There is evidence that CCHFV is present throughout much of sub-Saharan Africa (from Egypt to South Africa, and from Senegal to Madagascar), eastern Europe, the Middle East, and parts of Asia, particularly the former Central Asian republics, and Xinjiang province of NW China.¹⁴⁷ Of note is the emergence of CCHFV in Turkey since 2002,³⁸⁷ which may be related to climate change influencing tick distribution.^{139,558}

Most human infections are acquired by bite from infected ticks, contact with infected ticks during their removal from animals or human body, or contact with blood or tissues of

infected livestock. As one would expect, most cases are among individuals (e.g., shepherds, ranchers, and abattoir workers) living or working in close contact with livestock (e.g., sheep, goats, cattle, or ostriches) in endemic areas. Nosocomial outbreaks have also been reported in several regions, with infection of medical personnel following treatment or surgery on an unsuspected CCHFV case.¹⁴⁷

The distribution of NSD virus mirrors the distribution of the principal tick host, *Rhipicephalus appendiculatus*. The virus is present throughout much of Africa, from as far north as Ethiopia and Somalia, extending through much of East and Central Africa, and as far south as the Mozambique coastal plain.^{114,585} The distribution likely extends through parts of the Middle East and into India, based on the detection of the closely related NSD virus Ganjam variant from *Haemaphysalis intermedia* ticks.¹¹²

Hantavirus Genus

The geographic distribution of hantaviruses and epidemiologic patterns observed for HFRS and HPS reflect the distribution and natural history features of the hantavirus-infected rodent hosts of these viruses. This is because hantavirus infection of the rodent primary reservoir species produces a persistent infection with prolonged shedding of the virus, whereas infection of humans results in an acute infection with quick clearance of virus and recovery in survivors. Epidemiologic data accumulated since the 1950s for a variety of hantaviruses, including HPS-associated hantaviruses, suggest that human-to-human virus transmission does not occur, and that virtually all human infections are acquired by exposure to infected rodent excreta, with the one exception of an ANDV-associated HPS outbreak in Patagonia, Argentina, which clearly demonstrated human-to-human transmission between a patient and her physician, and circumstantial evidence suggested several additional human-to-human virus transmissions during this particular outbreak.^{388,570} This does not appear to be a common property of ANDV, as no health care workers became infected in the course of handling ANDV-associated HPS cases in a subsequent large HPS outbreak in Chile.⁸⁹

HTNV is found in various parts of eastern China and Korea and Far Eastern Russia, and is present in *Apodemus agrarius mantchuricus*, a mouse that is common in agricultural fields. Adults in rural areas (e.g., farmers, forest workers, or troops stationed in the field) are the most at risk of infection, and HFRS cases are seen to peak in the fall. This likely reflects a mixture of factors including the increased abundance of virus-infected mice in the fall coinciding with increased human activity in the fields associated with harvest of crops, and movement of rodents into the houses as winter approaches.²⁹⁹ Most cases are 20 to 50 years in age, and cases in children younger than age 10 are uncommon.^{91,303} A greater number of male than female cases is seen, consistent with predominant exposure being in the fields rather than in the homes. HTNV-related HFRS is probably most important in China, where approximately 100,000 cases are reported each year. A range of 300 to 900 HFRS cases are estimated to occur in Korea each year, and approximately the same number of cases are thought to occur in Far Eastern Russia annually.

SEOV is associated with the domestic rats, *R. norvegicus* and *R. rattus*,^{305,514} and is worldwide in distribution due to the recent global spread of rats to port cities and beyond through

international shipping.³⁰⁰ Seoul is the only hantavirus known to cause disease in urban areas, as the other hantaviruses are associated with rodents that are predominantly rural in their distribution. SEOV-related HFRS cases appear to be rare outside of China and Korea and institutions housing laboratory rat colonies, the exact reason for which is unclear. SEOV-related HFRS cases in general tend to be more moderate than those associated with HTNV, although mortality rates still range up to approximately 1% to 5%. Although most cases are in cities, SEOV-related HFRS cases are also seen in some rural areas in China. The seasonality of SEOV-related outbreaks are different from HTNV-related HFRS, with most cases occurring in spring and early summer as opposed to fall and early winter.⁹² The annual number of cases is unknown, as figures for SEOV- and HTNV-related HFRS are often not separated. However, Chinese reports suggest that SEOV-related HFRS in urban areas of several provinces has been on the increase since its discovery in 1981.⁹⁰ A genetically related virus, Thailand virus, is found in *Bandicota indica* rodents in Thailand, but is not known to cause human disease.

In Europe and Scandinavia most cases of HFRS are caused by infection with PUUV. Particularly in Scandinavia, disease is referred to as Nephropathia epidemica (NE). NE is usually milder than HTNV-associated HFRS. The virus is hosted by the bank vole, *Myodes glareolus*. In Sweden and Finland, the number of cases in rural residents usually peaks in November and January, although cases in urban residents appear to peak in August, which likely correlates with vacationing in summer cabins in rural areas. A male-to-female case ratio of approximately 2:1 is seen, and the disease is rare in children.⁶⁷ Local epidemics usually reflect increased local bank vole densities, which tend to cycle with a 3- to 4-year periodicity. Approximately 30% of PUUV infections result in reported disease, based on data from areas of Finland with high surveillance for disease.⁶⁷ In addition, antibody prevalence rates of up to 20% can be seen in known endemic areas in northern Sweden.²¹² Hantaviral disease in Belgium, northeastern France, and Germany occurs mainly in autumn and spring, and the extent of activity reflects the alterations in the size and structure of bank vole communities.⁴¹⁴ Large PUUV-associated HFRS outbreaks are periodically recorded in several areas of European Russia, particularly Udmurtia and Bashkortostan. A genetically distinct PUUV-like virus has also been identified in *C. rufocanus* voles in Far Eastern Russia and on Hokkaido Island, Japan, but is not known to be associated with human disease.²⁴⁸ Several additional hantaviruses are known to be associated with other subfamily *Arvicolinae* rodents but are not known to cause human disease. Topografov virus is present in lemmings in Arctic regions of Russia, and at least five hantaviruses are associated with various Old and New World rodents of the *Microtus* genus.

Dobrava virus (DOBV) also causes a considerable number of severe HFRS cases in Europe, particularly in the Balkan countries.^{30,392} The primary rodent reservoir is *A. flavicollis*, which is widely abundant in this region. However, a variant of DOBV, Saaremaa virus, can be found in *Apodemus agrarius* (a subspecies distinct from that which carries HTNV in Asia), which is of greater abundance in more northern areas.⁴⁹⁶ Although *A. flavicollis* does not frequent houses and other manmade structures, it will often enter campsites or places where food may be present. Unlike HTNV-associated HFRS, most DOBV-

associated HFRS cases occur in the late spring and summer months when activity in the rural areas is greatest. Epidemics of disease are seen at times when rodent populations are high, and have also been noted to increase in years when military conflicts are ongoing.³²⁵ Seroprevalence rates can be high in rural areas; for instance, antibody positivity rates of up to 14% were seen in mountainous areas of northern and western Greece.²⁴

Since the discovery of SNV and HPS in 1993, numerous other hantaviruses have been detected in New World subfamily Sigmodontinae rodents. In North America, SNV is by far the most important cause of HPS.³⁵³ SNV is present in deer mice (*P. maniculatus*, grasslands form) throughout Western and Central United States and Canada.³⁵³ The initial 1993 cluster of HPS cases occurred in the Four Corners region (meeting point of New Mexico, Arizona, Colorado, and Utah), and the majority of subsequent cases have been identified in the southwestern United States. However, HPS cases have now been identified in 32 states in the United States and four provinces of Canada, and SNV-infected deer mice have been detected throughout most of its range, which stretches from the Yukon to Mexico and from California to the Appalachian mountains in the eastern United States and Canada.^{343,353} Through December 2010, a total of 560 cases of hantavirus pulmonary syndrome have been reported in the United States with a case fatality rate of 36%. A survey of 2,500 individuals in risk groups, such as those with occupations that would bring them in close contact with rural rodents, found hantavirus-specific antibodies in only approximately 0.5% of those sampled,^{556,592} suggesting infection of humans with SNV is rare. It appears that clusters of cases are generally associated with local increases in infected deer mice populations. The peak of HPS cases occurs late spring–early summer, and as of December 2009, in the United States, 63% of cases have been male and 37% female and the mean age of patients is 37 years (range 6 to 83 years). Retrospective studies suggest the disease had gone unrecognized since at least 1959.¹⁶⁰ Several other hantaviruses are capable of causing HPS in the United States. The forest form of deer mice (*P. maniculatus nubiterrae*), which are found predominantly throughout the Appalachian mountain range extending from Georgia to eastern Canada, harbor a genetically distinct SNV-like virus referred to as Monongahela virus.^{353,503} This virus has been associated with several HPS cases in the Eastern United States. The white-footed mouse, *P. leucopus* (eastern haplotype) hosts another SNV-like virus, named New York virus. This virus has been responsible for some HPS cases in the Northeastern United States.^{208,213} A different hantavirus, Blue River virus, is present in *P. leucopus* (western haplotypes), in the Central and Western United States, but this virus is not known to be associated with human disease.³⁵⁸ Mitochondrial DNA analysis of the different rodent hosts shows that each of these rodent hosts is genetically distinct.³⁵⁸ This is in keeping with the general pattern for hantaviruses, whereby each specific hantavirus is hosted by a single different species or subspecies of rodent, and each specific rodent species or subspecies serves as the primary reservoir for only a single specific hantavirus.^{371,422}

Other sigmodontine genera harbor HPS-associated hantaviruses in the United States, such as BCCV, which was associated with a single HPS case in southern Florida and is hosted by the cotton rat, *Sigmodon hispidus* (eastern form).⁴³⁸ The genetically distinct western form of *S. hispidus* hosts a genetically distinct hantavirus, Muleshoe virus.⁴³⁹ Bayou virus

was discovered in 1994, associated with a fatal HPS case in Louisiana.³⁵⁷ Subsequently the virus was shown to be hosted by the rice rat *Oligoryzomys palustris*, to be present throughout the range of the rodent,²⁷⁹ and associated with a nonfatal HPS case in Texas.²⁰⁶ Although the HPS associated with BCCV and Bayou virus is still predominantly a pulmonary disease, there does appear to be more renal involvement than seen in classic SNV-associated HPS.^{206,256}

The confirmation of an HPS case in Brazil in late 1993 marked the beginning of a wave of discovery of HPS cases and associated hantaviruses throughout South and Central America. In addition to Brazil, Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Panama, Uruguay and Venezuela have also reported HPS. Rodents carrying viruses similar SNV have been found in Colombia, Costa Rica, and Mexico, but have not been associated with disease in humans.

The finding of high prevalence of hantavirus-specific antibodies in the general population in some areas of South and Central America differs considerably from the epidemiologic picture seen in North America, where seropositivity rates of 0.2% or less are seen. For instance, high rates of hantavirus-specific antibodies have been seen on surveillance of general populations in regions of Paraguay (40%), Salta province in Argentina (17%), and Los Santos province in Panama (13%).^{152,581} These data suggest more frequent exposure of people to hantaviruses in these areas and more inapparent or subclinical infections than seen with North American hantavirus infections.

Phylogenetic analysis of hantaviruses and their rodent reservoirs have indicated that their evolutionary trees overlap with only a few exceptions, suggesting that these viruses have co-evolved with their rodent hosts over 20 to 30 million years.^{371,373,422} These data show that although HPS and many hantaviruses were only recently discovered, they are actually quite ancient.

CLINICAL FEATURES

Orthobunyavirus Genus

The California serogroup viruses, LACV, Jamestown Canyon, and the prototype California encephalitis virus, all cause a similar encephalitic disease in humans.⁴⁵³ Indeed, due to serologic cross reactivity, many of the early disease descriptions of California encephalitis cases were likely due to Jamestown Canyon virus or LACV rather than California encephalitis virus, which is now known to be less common.^{76,120} The major difference between the viruses is in age-dependence of the spectrum of clinical disease observed, in that LACV infections tend to be more severe in children, whereas Jamestown Canyon virus infection is more severe in adults. The spectrum of disease ranges from inapparent or mild febrile disease through to fatal encephalitis.^{101,338,536}

The incubation period is approximately 3 to 7 days. Most cases report sudden onset of fever, followed by stiff neck, lethargy, headache, and nausea and vomiting, and symptoms usually end within 7 days. Approximately half the cases exhibit seizures, and up to 30% develop coma and exhibit a longer disease course. About 65% of patients have meningitis on presentation, with both mononuclear and polymorphonuclear cells present in cerebrospinal fluid. Despite mild neurological symptoms often present at the time of patient discharge, surprisingly

little residua is found. The most important sequela is epilepsy, which is observed in approximately 10% to 15% of children, and these are almost always patients who had seizures during acute phase of illness. In addition, about 2% of patients have persistent paresis.^{101,194,338}

Several thousand cases of acute febrile illness can occur during Oropouche virus epidemics observed throughout the Amazon basin regions of Brazil and Peru. Humans acquire the infection by bite from infected midges. The incubation period is approximately 4 to 8 days, followed by sudden onset of fever, with arthralgia, myalgia, severe headache, chills, photophobia, and prostration. Occasionally, rash, meningitis, or meningismus are seen.^{359,416} Most of the symptoms resolve within 3 to 5 days, although the myalgia may last 1 or 2 weeks. Viremia is detectable in the majority of patients 2 to 3 days after onset of illness. Approximately half of the patients can exhibit recurrence of some disease symptoms 1 to 10 days after initial recovery.⁵⁴⁹ Oropouche virus is also suspected to be infectious by aerosol, based on reported laboratory infections.⁴¹⁸

Phlebovirus Genus

Human infections with sandfly fever viruses results in an acute febrile illness with rapid onset. The incubation period last 2 to 6 days, followed by fever and general malaise, often accompanied by headache, photophobia, and back and joint pain.^{34,456} The disease is self-limiting, lasting 2 to 4 days before rapid and complete recovery.

Experimental RVFV infection of newborn lambs and kids suggests that the incubation period is usually 24 to 36 hours, but can be as short as 12 hours.¹³² Fever, which is often biphasic, is accompanied by listlessness, lack of appetite, abdominal pain, increased respiration rate, and lack of movement. Mortality rates of greater than 90% can be seen in animals younger than 1 or 2 weeks of age, with animals rarely surviving beyond 24 to 36 hours after onset of illness. Older lambs and kids, and adult sheep and goats are often less susceptible to RVFV infection, ranging from peracute to inapparent infection. Most older animals develop a febrile acute illness similar to the disease seen in 1- to 2-week-old animals, but with morality ranging from 5% to 60%, and a longer incubation period of 1 to 6 days. High and prolonged viremias, widespread tissue damage, vasculitis, and hepatic necrosis are seen in the more severe cases. Abortions in pregnant animals are frequently observed, with rates varying from 40% to 100%.

Human infections rarely lead to severe illness, with most infections resulting in a self-limiting influenza-like illness. However, approximately 0.5% of infections lead to severe hemorrhagic fever. In such cases, it is likely that the reduction of the antithrombotic function of the endothelial cells initiates intravascular coagulation and extensive necrosis of hepatocytes and other infected cells. The release of procoagulants into the circulation, together with the extensive liver damage, which severely impairs synthesis of coagulation factors and removal of circulating activated coagulation factors, are likely important factors resulting in DIC in the hemorrhagic fever cases.^{406,407}

In less than 0.5% of human RVFV infections, retinal vasculitis or encephalitis can be observed 1 to 4 weeks after recovery from the acute illness. Encephalitis begins with headache, meningismus, and confusion. Often fever reappears, and symptoms of severe cases include hallucinations, stupor, coma, and death. Focal necrosis, which most likely involves direct cell

destruction by the virus, is seen in the brain. In addition, that the onset of encephalitis after viremia has ceased and RVFV-specific antibodies are readily detectable suggests that there may be an immunopathologic component to these late complications.⁴⁰⁵ Indeed, polymorphonuclear and mononuclear cell infiltration associated with necrotic lesions implicates delayed hypersensitivity or cytotoxic T cells.⁴⁰⁵

Nairovirus Genus

The incubation period for CCHFV infections is commonly 3 to 7 days, but it varies depending on type of the exposure. Symptoms usually appear abruptly, and can include severe headache, dizziness, nausea, fever, neck and abdominal pain, and chills. Overall myalgia and malaise occur, and hepatomegaly may be seen. Hemorrhage frequently appears by 3 to 6 days postonset of illness, with a petechial rash observable on the trunk and limbs. Patients are often apathetic or obtunded by this stage, with delirium or coma occurring in the more severe cases. Blood may be seen in the urine, and bleeding from the nose, gums, vagina, and other mucosal surfaces or needle puncture sites may occur approximately 5 days after onset. The case fatality rate is approximately 30%, with most deaths occurring 5 to 14 days after onset of illness.^{147,518,572}

Hantavirus Genus

Severe HFRS begins with a phase lasting for 3 to 5 days typified by high fever, headache, malaise, chills, and prostration. Flushing of the face and neck, and conjunctival and pharyngeal injection (indicating capillary dilation) are frequently observed. Increased capillary permeability is likely responsible for the retroperitoneal edema and lower back pain often described. This febrile phase is followed by a hypotensive phase lasting several hours to days, characterized by marked thrombocytopenia, and often by petechial hemorrhage. Many patients exhibit low-grade DIC, and approximately 10% to 15% of patients show some degree of shock around this time. Usually shock improves within 12 to 48 hours and blood pressure returns to normal, or the patient becomes hypertensive. Oliguria may occur, and, historically, renal failure contributes to about half of the deaths.¹²⁹ Recovering patients will then go through a phase of diuresis that may last several months. Obviously, not all patients go through all the stages described, with some of the more severe cases rapidly progressing through to death in a few days, whereas other cases will skip some phases of the disease or exhibit very mild symptoms and not be recognizable as HFRS. However this tends to be the most severe form of HFRS, with a 5% to 15% case fatality. SEOV-associated HFRS tends to be milder, with a mortality rate of 1% or 2%. In general, the disease course resembles that observed with milder HTNV- and DOVB-associated cases, although more prominent liver involvement often occurs.²⁶⁰ PUUV-associated HFRS (often referred to as NE), is the mildest form of the disease, with a mortality rate of less than 1%. The phases of the illness are generally less pronounced, and symptoms are milder, with hypotension rather than shock, petechiae instead of more frank hemorrhage, and relatively mild renal involvement.

Similar to HFRS, in HPS there is also a 2- to 3-week incubation period followed by rapid onset of acute disease. The first 4 days often include fever, myalgia, malaise, headache, and gastrointestinal symptoms. Patients frequently present for medical attention with the onset of pulmonary edema, dyspnea, and

hypoxemia. These features, together with the hemoconcentration that is frequently observed, are presumably linked to the hantavirus infection of the microvascular endothelial cells surrounding the lungs, which leads to increased permeability and fluid leakage into the lungs. Bilateral pulmonary infiltrates are often visible on chest x-rays. Deterioration of patient condition is often rapid, with death occurring in almost 50% of the cases within 1 to 3 days of hospital admission. Clinical laboratory tests frequently reveal thrombocytopenia and prolonged PTT. Hypotension and shock can be prominent at this stage. Recovery of surviving patients is often remarkably quick, with rapid resolution of the lung lesion and shock within 3 to 6 days. Very similar clinical pictures are seen with HPS cases associated with other viruses found in rodents of the subfamily *Sigmodontinae*. HPS cases have also been associated with SNV-related Monongahela, and New York viruses, Bayou virus, BCCV, ANDV, and related viruses Oran, Lechiguanas and Hu39694, Laguna Negra, Juititaba, Araraquara, and Castelo dos Sonhos. The clinical picture seen in HPS cases associated with the BCCV and Bayou viruses differs slightly, in that more renal involvement and elevated serum creatine phosphokinase observed. Renal failure also appeared to be common with Oran virus-associated HPS in northern Argentina.³¹⁰ Flushing and petechiae and frank hemorrhage were seen in some HPS patients infected with ANDV in Argentina and Chile, respectively.²²

DIAGNOSIS

Orthobunyavirus Genus

A wide variety of assays have been used in the diagnosis of infections with viruses of the genus *Orthobunyavirus*, including complement fixation (CF), hemagglutination inhibition (HI), neutralization (NT), immunofluorescence assay, IgM capture enzyme-linked immunosorbent assay (ELISA), and reverse transcriptase-polymerase chain reaction (RT-PCR).^{74,128,186,284,293,428} Diagnosis of California serogroup virus infections relies on serologic methods, as the virus is generally absent from blood or secretions during the phase of CNS disease. The HI test is considerably more sensitive for these viruses than CF, but NT assay or RT-PCR coupled with multiplex nucleotide sequencing²⁹¹ is need for confirmation and subtype identification. The IgM capture ELISA works well for diagnosis of most infections by viruses of the genus *Orthobunyavirus*, but has not been widely applied to date.^{128,219,476} Virus isolation attempts complement serologic and genetic assays, and is best achieved by intracerebral (i.c.) inoculation of suckling mice or infection of susceptible cells such as Vero cells.

Phlebovirus Genus

Sandfly fever should be suspected when patients present with a classic “3 day fever” in areas where phlebotomine sandflies are numerous.^{136,456} Similarly, TOSV infection should be suspected in patients with acute CNS disease during the summer months in rural or semirural areas where phlebotomine sandflies are present.^{60,374} Diagnostic confirmation using acute or early convalescent phase samples is readily achieved by virus isolation by i.c. inoculation of suckling mouse or susceptible tissue culture cells, or IgM capture ELISA.

Differential clinical diagnosis of RVFV varies depending on the region in question. The disease should be suspected in

RVFV-endemic regions following abnormally high precipitation, increased rates of abortion in livestock, and appearance of acute influenza-like illness in persons in close contact with potentially infected livestock (e.g., farmers, veterinarians, and abattoir workers). Virologic diagnosis is usually simple given the high viremia present throughout the acute phase of illness and the ease of growth of the virus when inoculated i.c. into suckling mice, or in a wide variety of susceptible tissue culture cells.⁵¹⁸ Serologic testing is also straightforward, particularly if paired sera (one taken acute and the other 1 or 2 weeks later) are available. IgM and IgG ELISA tests using inactivated RVFV-infected cell lysates or slurries have proven particularly useful in outbreak investigations.^{278,356,547,583} RT-PCR protocols have also been developed, although their utility in clinical settings requires further investigation.⁵⁶⁹

Nairovirus Genus

Clinical differential diagnosis of CCHFV varies in the different regions where the disease is known to occur and can be difficult in early stages. In general, the disease can be suspected for patients presenting with a rapid onset of severe influenza-like symptoms, and known exposure to tick bite or contact with blood or tissues of potentially infected livestock or humans. The early stages can be confused with sandfly fevers in regions where the diseases overlap, and leptospirosis, Omsk hemorrhagic fever, other viral hemorrhagic fevers (yellow fever, HFRS, Ebola), tick typhus, and mycotoxicoses can appear similar in the more advanced hemorrhagic phase of the illness. Clinical values can be helpful in diagnosis, with leukopenia and thrombocytopenia both frequently being present early in the disease.^{147,518}

Rapid laboratory diagnosis of CCHFV can be achieved by RT-PCR,^{70,124} but virus isolation or antigen detection ELISA from patient acute phase sera^{69,147} are also performed. The virus and antigen are usually detectable up to 1 to 2 weeks after onset of illness. For CCHFV isolation, i.c. or intraperitoneal inoculation of patient acute phase blood samples into newborn mice is probably most sensitive, although quicker results can be obtained by inoculation into susceptible tissue culture cells, such as LLC-MK2, Vero, and baby hamster kidney (BHK)-21 cells, followed by fluorescent antibody staining.⁴⁷⁸ Fatal cases rarely show significant antibody responses. However, in those destined to survive, IgM and IgG antibodies are frequently detectable after about a week of illness.

Hantavirus Genus

Clinical differential diagnosis of HFRS and HPS cases can be difficult due to the nonspecific early symptoms in both types of cases. In areas of China, Korea, Far Eastern Russia, Europe, and Scandinavia where HFRS is known to occur, a febrile patient presenting with thrombocytopenia and renal symptoms should be questioned about potential rodent exposure and tested for evidence of hantavirus infection. The early signs of severe HFRS can be easily confused with those of leptospirosis, typhus, pyelonephritis, poststreptococcal glomerulonephritis, an acute abdominal emergency, or other hemorrhagic fevers. Mild HFRS can be confused with hepatitis A, mild leptospirosis, streptococcal pharyngitis, influenza, or nonsteroidal anti-inflammatory toxicity.

In the Americas, the early stages of HPS are extremely difficult to recognize, being easily confused with influenza.

However, fever and myalgia and a history of potential rural rodent exposure, together with the appearance of shortness of breath, and findings of thrombocytopenia, leukocytosis with left shift (higher ratio of immature-to-mature neutrophils), and atypical lymphocytes would be strongly suggestive of HPS.³⁷⁶

Antibodies are invariably seen in patient sera around the time of onset of illness. Laboratory analysis of the presence of virus-specific antibodies has historically included indirect fluorescent antibody (IFA) testing. This has good sensitivity, and hantavirus cross-reactivity and use of a representative virus from each of the main rodent subfamilies harboring hantaviruses (i.e., *Murinae*, *Arvicolinae*, and *Sigmodontinae*) will identify infections caused by virtually all hantaviruses. However, false positives are not uncommon, due to rheumatoid factor problems and overall nonspecific “stickiness” of some patient sera. Some of these difficulties can be controlled for by the inclusion of appropriate negative antigens to detect and remove the effects of problem sera. Although of some utility for acute diagnosis, the inherent nonspecific background and subjectivity problems make the IFA test unsuitable for serologic survey studies. Acute diagnosis is best achieved by IgM capture ELISA using hantavirus-infected cell slurry as antigen and incorporating an uninfected cell slurry control.²⁸⁰ The use of recombinant nucleocapsid proteins with appropriate negative control protein fractions have also proved useful as antigens in this assay format.^{137,151,185} Such assays have been shown to be highly sensitivity, rapid, and inexpensive. Considerable cross-reactivity among related hantaviruses is seen, such that inclusion of a limited number of representative virus antigens (e.g., SNV, PUUV, and HTNV) allow detection of virtually all acute hantavirus infections. The disadvantage of the cross-reactivity of the assay is the inability to precisely identify the virus based on a positive result. Western blot assays utilizing recombinant nucleocapsid proteins or GPs have also been effectively used for acute diagnosis, particularly with New World hantaviruses, and can provide additional insights into virus identity.^{207,233}

Virus isolation attempts from acute clinical specimens are almost always negative. The exact reason for difficulty of isolation of hantaviruses is unknown, but it is likely related to the presence of a strong immune response at the time of onset of illness, including serum-neutralizing antibodies. Demonstration of the lack of amino acid substitutions during the isolation of Sin Nombre virus from infected lung material would suggest that virus adaptation to growth in tissue culture is not a major element in the difficulty of isolation of these viruses.⁹⁷ Given the frequent difficulty in obtaining infectious virus, RT-PCR assay has been used effectively to complement immunodiagnostic assays and allow detailed genetic comparison of the hantaviruses.^{320,373,392,468} Given the potential for false positives due to cross-contamination, RT-PCR is not recommended as the sole means of hantavirus diagnosis.

PREVENTION AND CONTROL

Orthobunyavirus Genus

Prevention and control is difficult for the mosquito-borne California and Bunyamwera serogroup viruses due to the widespread presence of the mosquito vector hosts and relatively sporadic nature of the associated human or livestock diseases. No vaccines are available for human-infecting orthobunyaviruses.

Broad application of insecticides, as carried out in earlier decades, is no longer ecologically acceptable. Relative to human disease, use of fine mesh netting and personal protectants containing N,N-diethyl-meta-toluamide (DEET) are highly recommended in areas with high risk of people being bitten by virus-infected mosquitoes.

Similar vector control challenges exist for the Simbu serogroup viruses vectored by *Culicoides* midges. With the Oro-pouche virus disease in Brazil and Peru, avoiding the build-up of rotting organic debris such as banana tree stalks or cacao husks in agricultural areas may help curtail population levels of *C. paraensis* and reduce the risk of seasonal epidemics.⁴¹⁷ Avoiding exposure (treated netting, DEET repellents) to these midges during their early evening feeding hours is also recommended. Similar protective measures are recommended for AKAV. In addition, a successful formalin-inactivated AKAV vaccine has been developed in Japan using virus-infected hamster lung cell cultures,²⁸⁷ and a trivalent-inactivated vaccine protective against AKAV, Aino virus (AINOV), and Chuzan orbivirus has been successfully trialed in Korea.²⁵⁹ With knowledge of the distribution and seasonality of AKAV disease outbreaks, vaccination of at risk animals prior to pregnancy is feasible.

Phlebovirus Genus

The sandfly hosts of SFSV, SFNV, and TOSV have short flight range, and locomotion is often by hopping, making localized use of insecticide sprays particularly effective around residences. In addition, the use of repellants and treated bed nets and screens is recommended in such areas.

Immunization of livestock remains the most effective way to prevent RVFV epizootics and human cases. Several vaccines exist. Live attenuated viruses based on the mouse neuroadapted Smithburn RVFV strain are used in Kenya and South Africa.^{78,498} Formalin-inactivated wild-type viruses have also been used in Egypt and South Africa.¹³⁸ The modified live Smithburn vaccine has good efficacy in sheep (but not cattle), with a single dose inducing lasting immunity in sheep 6 to 7 days after vaccination.¹⁰² However, it appears to be only partially attenuated, causing abortions or teratology in some pregnant animals.^{223,289} The formalin-inactivated vaccines are safer, but are expensive and induce only short-lived immunity in sheep and cattle. Other candidate live-attenuated vaccines such the mutagen-derived MP12 strain, the naturally occurring clone 13 (which has a large deletion in NSs), and genetically engineered recombinant viruses lacking NSs protein, and perhaps also NSm, are in various stages of evaluation.^{54,223,289,403} Other approaches include DNA vaccines, nonreplicating virus-like particles and subunit vaccines. The main livestock vaccination problem is the long periods between epizootics and their irregular appearance, which makes it difficult to convince livestock owners to vaccinate regularly. Recent advances in the use of satellite imagery and surface ocean temperatures to predict regions at higher than usual risk of RVFV activity, may allow the timely vaccination of animals prior to potential epizootics.^{25,316,554}

In an African setting, humans are probably best protected by vaccination of livestock to prevent amplification of virus, thus limiting human exposure and epizootic potential. Formalin-inactivated RVFV vaccines have been developed for immunization of laboratory and field workers at risk of exposure^{223,288} but are unlikely to be used on a larger scale, given their limited commercial potential. RVFV is highly infectious by aerosol, as

shown by numerous infections of laboratory personnel. Workers at risk of exposure wear protective clothing to avoid contact with potentially infectious materials and aerosols. The antiviral drug ribavirin may be of some benefit in the treatment of sandfly fevers and RVFV, although human efficacy data are still lacking.^{108,408}

Nairovirus Genus

The best means of prevention is to minimize exposure to the virus. Treatment of clothing with pyrethroid preparations, which repels and even kills ticks, is highly recommended for those persons likely to come into contact with CCHFV-infected ticks (e.g., slaughterhouse workers, sheep shearers, veterinarians, and others involved with livestock). In addition, habits that avoid virus-contaminated blood or tissues contacting the skin should be practiced (e.g., wearing of gloves and avoiding crushing ticks with fingers). Medical personnel should practice standard barrier-nursing techniques during care of suspected CCHFV patients. Formalin-inactivated vaccines have been prepared from virus-infected suckling mouse brains and used in Bulgaria and other parts of Eastern Europe and the former Soviet Union, but their efficacy is currently unclear.²⁵³

Treatment of patients is by means of supportive and replacement therapy with blood products. Administration of immune plasma has been tried on numerous occasions, but its usefulness remains unclear. Based on the susceptibility of the virus in experimental tissue culture and mouse infections to the antiviral drug ribavirin, several CCHFV cases have been treated with ribavirin and shown encouraging results.^{147,253}

Hantavirus Genus

The primary means of prevention is the reduction of rodent exposure. Effective measures include rodent-proofing of homes, correct storage of food, airing of seasonally closed cabins, and disinfection and removal of trapped rodents and rodent droppings.^{85,86,180} The lack of nosocomial transmission or person-to-person transmission reported for virtually all hantavirus outbreaks suggests that spread to medical personnel or contacts is usually not a concern. The one exception is the limited person-to-person spread that can occur during ANDV-associated HPS outbreaks.^{388,570}

Several different inactivated virus vaccines for HTNV and SEOV have been developed in Asia.²¹¹ These include virus-infected rodent brain-derived antigens inactivated with formalin or beta-propiolactone.^{302,586} Although such products would not be suitable for use outside the region, good efficacy has been reported.²¹¹ Several monovalent and bivalent HTNV- and SEOV-tissue culture-derived inactivated vaccines have also been produced in China,²¹¹ some of which may have high efficacy. In addition, a number of recombinant DNA approaches to vaccine development have been investigated, some of which show promise in experimental systems (reviewed in 464). Of these, the only study in humans to date was with DNA vaccines for HTNV and PUUV delivered by gene gun, which showed both vaccines to be immunogenic when delivered alone or in combination (C. S. Schmaljohn, unpublished). The sensitivity of HTNV to the inhibitory effects of the antiviral drug ribavirin on virus replication in tissue culture or in animal models²¹⁵ has promoted clinical trials both with HFRS patients and HPS patients, with positive results reported in the case of HFRS.^{214,452}

TOSPOVIRUS GENERAL FEATURES

In infected plant cells, several types of aggregates of virus proteins are observed, and mature virus particles accumulate in the endoplasmic reticulum.²⁶³ Although the spread of virus from plant to plant is by ingestion of the cell contents by thrips, acquisition of a virus envelope membrane containing the virus GPs is still thought to be important for virus transmission by thrips. However, once inside the plant, complete virus maturation may be less critical as movement from cell to cell likely involves virus nucleocapsid movement through plasmodesmata cytoplasmic channels.^{275,510} In addition to the structural proteins, nucleocapsid (N), glycoproteins (Gn and Gc), and RNA polymerase (L), the tripartite genome of these viruses encode two nonstructural proteins, one from the S genome segment, NSs, and one from the M segment, NSm.²⁷³ The NSs protein accumulates in the salivary gland of infected thrips, suggesting a role in virus transmission.⁵⁷⁹ The NSm associates with virus nucleocapsids and localizes to plasmodesmata, where it forms tubular structures thought to be involved in virus cell-to-cell movement.^{275,510}

In contrast to the other arthropod-borne members of the family *Bunyaviridae*, adults of the vector thrips do not acquire the virus, only larvae becoming infected when feeding on virus-infected plants.^{541,575} Infected male *F. occidentalis* thrips were shown to feed more actively than uninfected thrips, thus increasing the opportunities for virus transmission.⁵⁰⁶ TSWV has been shown to be trans-stadially transmitted through molting, pupation, and emergence to the adult stage, and both larvae and adults can transmit the virus to plants.⁵⁷⁸ Unlike many of the other arthropod-borne bunyaviruses, there is no evidence of transovarial transmission for any of the tospoviruses. This presumably reflects the widespread and year-round replication of thrips in most climates and the enormous number of susceptible plants, making vertical transmission of the virus in the vector thrips of lesser importance.

Control of tospovirus infections in a field setting is difficult due to problems in eliminating thrips and the fact that many weed species surrounding cultivated fields will serve as a virus reservoirs. Several breeding programs have attempted to increase natural host resistance of plants to tospovirus infection, but with little success to date.⁵⁰⁸ Attempts to create genetically engineered host-plant resistance are showing promising results and have been reviewed in detail recently.²⁷²

PERSPECTIVES

Although unified by shared characteristics such as tripartite ssRNA genome, virion composed of four structural polypeptides, and cytoplasmic site of replication, the bunyaviruses exhibit considerable diversity in RNA and encoded protein sequences, their genome expression strategies, and biological properties. The degree of understanding of different bunyaviruses varies greatly, dependent on their clinical, veterinary, or agricultural importance, or ease of handling. The family affords considerable research opportunities for the future, three of which are considered of immediate importance highlighted in the following. (a) Large scale sequence analyses of bunyavirus genomes to begin to understand their relationships and evolution, with practical implications for robust classification and improved diagnostics. (b) The development of reverse genetic systems that allow rescue of infectious hanta-, nairo-, and tospo-

viruses, which will enhance the pace of research on these viruses (as evidenced from progress with orthobunya- and phleboviruses for which the technology is well-established) and should provide new opportunities in the generation of vaccine and antivirals. (c) An understanding of how bunyaviruses persist in either their arthropod or rodent hosts. What are the cellular interactions that occur that permit efficient viral replication without damage to the host or clearance from it?

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Stephen E. Straus, 1946–2007

Steve Straus was the consummate physician–scientist with broad interests in the basic science and clinical aspects of viral and immunological diseases and therefore was an ideal person to serve as clinical virology editor for *Fields Virology*. We were fortunate to work with him in his role as associate editor for the third through fifth editions of *Fields Virology*. However, unfortunately, with Steve's premature death in 2007, we lost our friend, colleague, and fellow editor. Steve's medical training and accomplishments are detailed elsewhere (*J Infect Dis* 2007;196:963–964). His research interests were broad and included the molecular biology and pathogenesis of varicella-zoster and herpes simplex viruses, acyclovir suppression of oral and genital herpes simplex viruses, antiviral drug resistance, clinical testing of herpes simplex virus and varicella zoster virus vaccines, chronic active Epstein–Barr virus, chronic fatigue syndrome, and autoimmune lymphoproliferative syndrome. Steve was one of the leading scientists in the National Institutes of Health intramural program, serving as chief of the Laboratory of Clinical Investigation at the National Institute of Allergy and Infectious Diseases and the founding director of the National Center for Complementary and Alternative Medicine.

Steve cowrote the chapter on varicella zoster virus, and additionally worked effectively as an associate editor, for the third to fifth editions of *Fields Virology*. He seemed to read and edit the chapters immediately upon their submission, amazing us with his ability to do all of this on top of his other responsibilities. Steve was diagnosed with brain cancer in 2004 but insisted on editing chapters for the fifth edition right through the compiling of the chapters. The book was published in early 2007, not long before his death in May 2007.

On behalf of everyone who contributed to the sixth edition of *Fields Virology*, we dedicate this book to the memory of Stephen E. Straus, MD.

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In the early 1980s, Bernie Fields originated the idea of a virology reference textbook that combined the molecular aspects of viral replication with the medical features of viral infections. This broad view of virology reflected Bernie's own research, which applied molecular and genetic analyses to the study of viral pathogenesis, providing an important part of the foundation for the field of molecular pathogenesis. Bernie led the publication of the first three editions of *Virology* but unfortunately died soon after the third edition went into production. The third edition became *Fields Virology* in his memory, and it is fitting that the book continues to carry his name.

We are pleased that the printed book of the sixth edition of *Fields Virology* contains four-color art throughout and that an e-book version accompanies the printed book as well. We have increased the numbers of figures in each chapter, and with the color and availability of the figures from the e-book for use as slides, most chapters should have sufficient figures for slides for one lecture. There have been continued significant advances in virology since the previous edition 6 years ago, and all of the chapters have been updated to reflect these advances. Our increased knowledge of virology has caused us to use shortened lists of key references (up to 200 in most cases) in the printed book to save space, whereas complete reference lists appear as part of the e-book. We have retained the general organization of the earlier editions for the sixth edition of *Fields Virology*. Section I contains chapters on general aspects of virology, and Section II contains chapters on replication and medical aspects of specific virus families and specific viruses of medical importance. In Section I, we have added a new emphasis on virus discovery in the Diagnostic Virology chapter and emerging viruses in the Epidemiology chapter to address the interest in discovery of new viruses and emerging viruses. In Section II, we have added new chapters on circoviruses and mimiviruses and have added a new section on Chikungunya virus to the alphavirus chapter.

Numerous chapters have been updated to include the latest information on outbreaks during the past 5 years, including pandemic H1N1 influenza, new adenovirus serotypes, noroviruses, human polyomaviruses, the re-emergence of West Nile virus in North America, novel coronaviruses, novel Coxsackie and rhino viruses, and other emerging and re-emerging viruses. Important advances in antivirals, including new hepatitis C virus protease inhibitors and HIV integrase inhibitors, have been described. As with the previous edition, we have continued to combine the medical and replication chapters into a single chapter to eliminate duplication and to present a more coherent presentation of that specific virus or virus family. The main emphasis continues to be on viruses of medical importance and interest; however, other viruses are described in specific cases where more is known about their mechanisms of replication or pathogenesis. Although not formally viruses, prions are still included in this edition for historical reasons and because of the intense interest in the infectious spongiform encephalopathies.

We wish to thank Lisa Holik of Harvard Medical School, Richard Lampert of Lampert Consultancy, Grace Caputo of Dovetail Content Solutions, Chris Miller of Aptara, and Leanne Vandetty and Tom Gibbons and all of the editorial staff members of Lippincott Williams & Wilkins for all their important contributions to the preparation of this book.

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Arenaviridae

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HISTORY

Arenaviruses cause chronic infections of rodents indigenous to Europe, Africa, the Americas, and perhaps other continents. These asymptotically infected animals move freely in their natural habitat and may invade human habitation; when humans come in contact with excreted viruses, disease may result. Arenavirus infections of humans are common and in some cases severe; several members of the group are responsible for severe acute infections termed *hemorrhagic fevers*. The mechanisms that have evolved to ensure chronic infection and intergenerational survival of the virus in rodents are complex and specific to the particular rodent–virus combination, and their elucidation over the past 60 years has led to a deeper understanding of immunology and immunopathology.¹⁶⁴

Lymphocytic choriomeningitis virus (LCMV) was the first isolated arenavirus, discovered in 1933 during the study of samples from a St. Louis encephalitis epidemic.¹⁸⁵ It was soon

found to be a cause of aseptic meningitis²⁰⁶ and to be identical to an agent that chronically infected mouse colonies.²²² This launched a series of studies that showed LCMV to be a common human pathogen and a fascinating virus with many lessons for biologists.^{31,32,164}

By the 1960s, several other viruses had been discovered and found to share common morphology,¹⁶⁰ serology,¹⁹² biochemical features, and a natural history that depended on chronic infection of rodent reservoirs, leading to the recognition of the *Arenaviridae* family, named after the sandy (Latin, *arenosus*) appearance of the ribosomes originally seen in thin sections of virions.¹⁹² Some of these viruses caused hemorrhagic fevers (Table 43.1), whereas others were apparently not pathogenic for or rarely infected humans (Table 43.2). Since the isolation of Tacaribe virus (TACV),⁵⁵ new arenaviruses have continued to be discovered in the Americas on the average of one every 3 years, including three lethal new viruses: Guanarito,¹⁹⁸ Sabia in 1990,¹³⁰ and Whitewater Arroyo in 1996.⁷⁹ As a result of new technologies of deep sequencing and genome discovery, several new arenaviruses have been revealed in the past decade in both the New World (NW) and Old World (OW) branches of the *Arenaviridae*.

CLASSIFICATION OF VIRUSES WITHIN FAMILY

The *Arenaviridae* consists of a unique genus (*Arenavirus*) containing 24 unique species currently recognized by the International Committee on Taxonomy of Viruses (ICTV), as well as several newly discovered viruses whose taxonomic status has not yet been determined by the ICTV (see Table 43.1). The original classification of arenaviruses, based mainly on virus antigenic properties, identified two groups: the NW (also referred to as Tacaribe serocomplex) and the OW (also referred to as Lassa-Lymphocytic choriomeningitis serocomplex) including viruses from Africa and the globally distributed prototypic arenavirus, LCMV. Results derived from molecular genetic studies are consistent with findings from comparative serology.

The OW group consists of a single lineage composed of five species (*Lassa virus*, *Mobala virus*, *Ippy virus*, *Mopeia virus*, and *Lymphocytic choriomeningitis virus*), and several newly discovered viruses including Kodoko, Morogoro, Dandenong and Lujo,^{39,119,166} whereas the NW group is composed of four lineages: clades A, B, C, and A/Rec (Fig. 43.1). Clade A contains five species (*Flexal virus*, *Parana virus*, *Pichinde virus*, *Pirital virus*, and *Allpahuayo virus*), whose internal genetic relationships are not well established.³⁹ Clade B contains eight species

TABLE 43.1 Arenaviruses That Are Known to Be Human Pathogens

Virus	Host in nature	Geographic distribution	Disease	Clinical picture	Case fatality
Lymphocytic choriomeningitis	<i>Mus domesticus</i> ; <i>Mus musculus</i>	Europe, Americas, perhaps elsewhere	Lymphocytic choriomeningitis	Usually recognized as aseptic meningitis; more severe central nervous system disease occasionally occurs. Most common clinical form, rarely diagnosed, is probably febrile illness without neurologic involvement. May cause transient hydrocephalus during acute infection or congenital hydrocephalus and chorioretinitis after fetal infection	<1%
Lassa	<i>Mastomys</i> spp.	West Africa	Lassa fever	Severe systemic illness with changes in vascular permeability and vasoregulation. Worst cases often associated with bleeding	15%
Junin	<i>Calomys musculus</i>	Argentina Pampas	AHF	Classical viral hemorrhagic fever. Similar to Lassa, except thrombocytopenia, florid bleeding, and neurologic manifestations much more common	15%–30%
Machupo	<i>Calomys callosus</i>	Beni region of Bolivia	Bolivian hemorrhagic fever	As in AHF	25%
Guanarito	<i>Sigmodon alstoni</i> ; <i>Zygodontomys brevicauda</i>	Venezuela	Venezuelan hemorrhagic fever	Probably similar to AHF	25%
Sabia	Unknown	Brazil	Not yet named	Probably similar to AHF. Extensive hepatic necrosis seen in one of the two cases not treated with ribavirin	1/3
Whitewater Arroyo	<i>Neotoma albigula</i>	New Mexico, California, United States	Not yet named	Hemorrhagic fever with liver failure	3/3

AHF, Argentine hemorrhagic fever.

(*Sabia virus*, *Cupixi virus*, *Guanarito virus*, *Amapari virus*, *Junin virus*, *Machupo virus*, *Tacaribe virus*, and *Chapare virus*), which form three well-supported lineages. Clade C is composed of only two established species, *Oliveros virus* and *Latino virus*, and a potential third member, Pinhal virus, for which little information is available. Clade A/Rec comprises all North American arenaviruses: *Whitewater Arroyo virus*, *Tamiami virus*, *Bear Canyon virus*, and the newly discovered Skinner Tank and Catarina viruses. As with clade A, intraclade A/Rec relationships are poorly resolved, owing to limited sequencing data and the incongruence of phylogenetic data derived from different genes. The geographic distribution of each arenavirus is determined by the range of its natural specific rodent res-

ervoir, with the exception of TACV, which was isolated from fruit-eating bats.^{59,84}

Recently, high-throughput sequencing and metagenomic pathogen discovery approaches have led to the identification and isolation of two highly divergent arenaviruses from cases of inclusion body disease, a common infectious disease of captive snakes.²¹⁵ These viruses show typical ambisense arenavirus genome organization but have a GP2 reminiscent of filoviruses. These findings indicate that arenavirus natural hosts are broader than previously thought and not restricted to mammals. In addition, significant genetic divergence between these newly identified reptile arenaviruses and known mammalian arenaviruses together with biological differences between rep-

TABLE 43.2 Arenaviruses Not Known to Be Significant Human Pathogens

Virus	Host in nature	Geographic distribution	Comments	Year isolated
Tacaribe	Unknown	Trinidad	Originally isolated from <i>Artibeus</i> bats in 1956–58 and not found in subsequent attempts; bats may be reservoir	1956
Amapari	<i>Oryzomys goeldi</i> , <i>Neacomys guianae</i>	Brazil	Apparently has two rodent reservoirs	1964
Parana	<i>Oryzomys buccinatus</i> ?	Paraguay	Reservoir not well established	1965
Tamiami	<i>Sigmodon hispidus</i>	United States	Antibodies detected in a few humans without disease	1963, 1965
Pichinde	<i>Oryzomys albigularis</i>	Colombia	Several asymptomatic laboratory infections	1967
Latino	<i>Calomys callosus</i>	Bolivia	Thought to be nonpathogenic for humans	1965
Flexal	<i>Oryzomys</i> spp.?	Brazil	Two laboratory infections with disease	1975
Oliveros	<i>Bolomys obscurus</i>	Argentina	Found during field studies of Junin virus; still under evaluation	1989
Ippy	<i>Arvicanthis</i> spp.	Central African Republic	Found to be an arenavirus in 1985; see Mopeia	1970
Mopeia	<i>Mastomys natalensis</i>	Mozambique, Zimbabwe	One of several non-Lassa arenaviruses from <i>Mastomys</i> spp. in Africa	?
Mobala	<i>Praomys</i>	Central African Republic	See Mopeia	?

tiles and mammals have raised new questions about the biology and evolution of arenaviruses.

The originally established species demarcation criteria included (a) association with a specific host species or group of species, (b) geographic niche, (c) association with human disease, (d) differences in antigenic cross-reactivity, and (e) sig-

nificant sequence difference from other species in the genus. However, often limited information and difficulties associated with its analysis regarding these criteria have led more recently to emphasize mainly genetic criteria to define *Arenavirus* species. This approach, however, faces the problem of how to correctly assign a cutoff value for species demarcation owing to

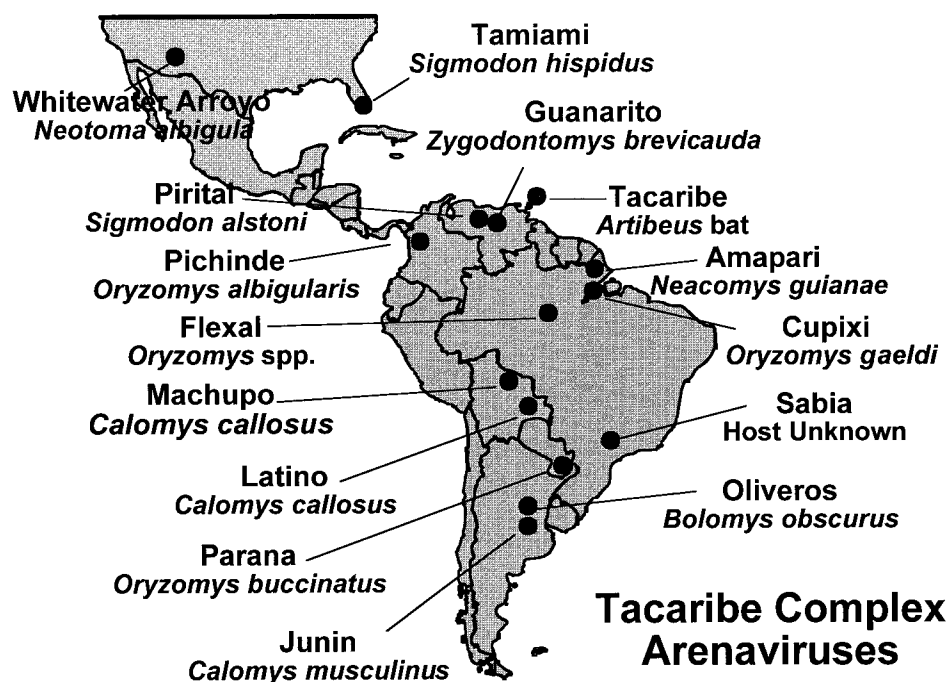


FIGURE 43.1. Location of American arenaviruses with their natural host. (Courtesy of Dr. S. Nichol, Centers for Disease Control and Prevention.)

biased sequence availability. There are many fewer OW than NW arenavirus sequences; however, OW sequences include most complete intraspecies sequences. Therefore, intraspecies diversity is likely underestimated among NW arenaviruses, whereas the interspecies diversity could be underestimated for OW arenaviruses. Notably, sequence comparison of the same genome region for a large number of isolates revealed similar degree of diversity in an OW arenavirus (Lassa virus [LASV]) and two NW arenaviruses (Whitewater Arroyo virus [WWAV] and Pirital virus [PIRV]).³⁹ These findings suggest that accurate classification of a new arenavirus isolate may require to follow the *Arenavirus* species definition together with comparing the sequence of the new isolate with the larger available set of sequences.

Virion Structure

Arenavirus virions are pleomorphic when examined by cryo-electron microscopy, ranging in size from 40 to more than 200 nm in diameter^{39,103} (Fig. 43.2). Virions contain a surface envelope that is studded with evenly spaced spikes that correspond

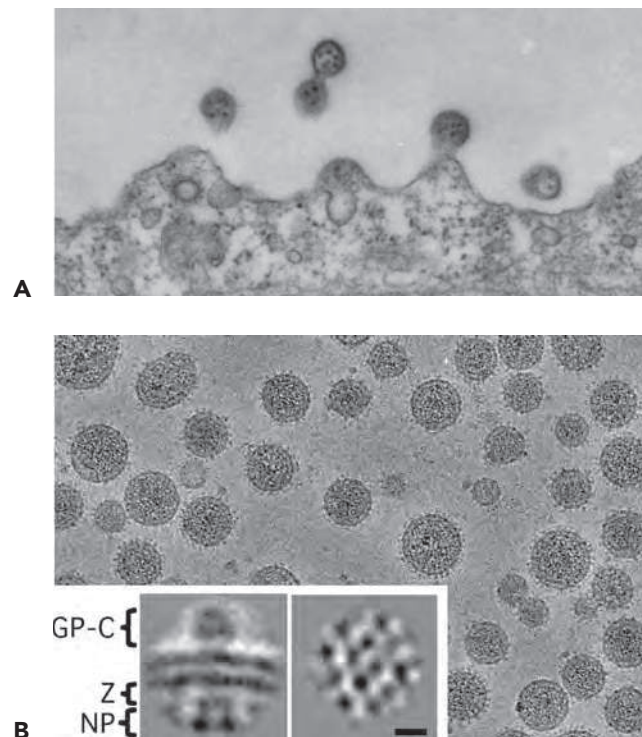


FIGURE 43.2. Electron microscopy of arenaviruses. **A:** Thin section showing lymphocytic choriomeningitis virus budding from infected BHK-21 cells. Typical 110-nm particles contain numerous electron-dense 20-nm ribosomes that were the basis for the family name. **B:** Cryo-electron microscopy of unstained, purified virions at $-4\ \mu\text{m}$ defocus to illustrate surface topography. Refined images of arenavirus glycoprotein spikes, Z protein, and ribonucleoprotein densities were obtained by single-particle image analysis of Pichinde glycoprotein complexes from particle edges (inset, **left**). Nucleoprotein packing is evident in refined images of ribonucleoprotein from virion centers (inset, **right**). Bar indicates 10 nm. (Courtesy of B. W. Neuman, J. W. Burns, R. Milligan, and M. J. Buchmeier.)

to glycoprotein (GP) projections consisting of complexes of the viral GPs—GP1 and GP2. The surface GPs are aligned with subjacent Z protein and ribonucleoprotein (RNP) densities, which are packed into a two-dimensional lattice at the inner surface of the viral membrane.^{103,161} Virions contain the L and S genomic RNAs as helical nucleocapsid (NC) structures that are organized into circular configurations, with lengths ranging from 400 to 1,300 nm.²⁴⁰ The L and S genomic RNA species are not present in equimolar amounts within virions (L:S ratios $\sim 1:2$), and low levels of both L and S antigenomic RNA species are also present within virions. In addition, it has been documented that host ribosomes can be incorporated into virions, although the biological significance of this remains to be determined.^{28,159}

Genome Structure and Organization

Arenaviruses are enveloped viruses with a bisegmented negative-sense single-stranded RNA genome and a life cycle restricted to the cell cytoplasm. Individual arenaviruses exhibit some variability in the lengths of the two genomic RNA segment, L (ca 7.2 kb) and S (ca 3.5 kb); however, their overall organization is well conserved across the virus family.³⁰ Arenavirus coding strategy has unique features compared to other NS RNA viruses. Each arenavirus genome segment uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by a noncoding intergenic region (IGR) with a predicted folding of a stable hairpin structure (Fig. 43.3). The S RNA encodes the viral glycoprotein precursor (GPC; ca 75 kDa) and the nucleoprotein (NP; ca 63 kDa),

LCMV Genome Organization

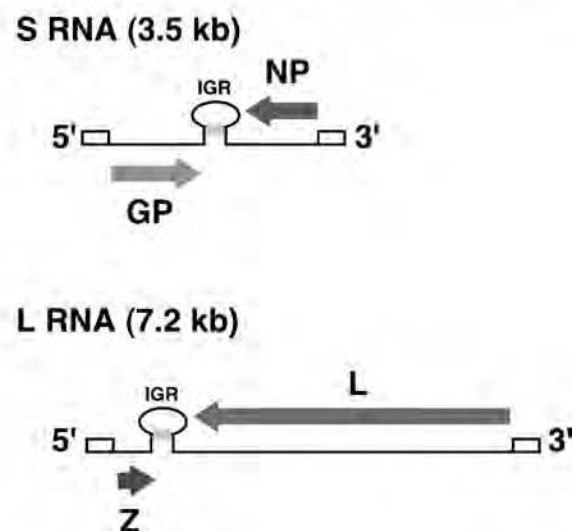


FIGURE 43.3. Schematic diagram showing coding strategy for the L and S RNAs of the arenaviruses. Stem-loop structures indicate noncoding regions of each genomic segment. The lower portion of the diagram summarizes the transcription and replication events for the S genomic segment. As a consequence of this arrangement, the GPC and Z open reading frames can be transcribed only after initiation of replication of viral genomic RNA.

whereas the L RNA encodes the viral RNA-dependent RNA polymerase (RdRp, or L polymerase) (ca 200 kDa) and a small (ca 11 kDa) RING finger protein (Z) that functions as a matrix protein.³⁰ This situation—where in one region the S and L RNA are negative sense and in a second, nonoverlapping region they are pseudo-positive sense—led investigators to coin the term *ambisense* to describe the arenavirus coding strategy. The qualifier *pseudo* is used because there is no evidence that the genomic S and L RNAs can function as messenger RNAs (mRNAs) and be directly translated into GPC and Z proteins, respectively.

Arenaviruses exhibit high degree of sequence conservation at the 3' end of the L and S RNA segments (17 out of 19 nucleotides [nt] are identical), suggesting that this conserved terminal sequence element constitutes the virus promoter for polymerase entry.^{30,151} Arenavirus genomes and antigenomes exhibit a high degree of complementarity between their 5' and 3' termini, and the 5' and 3' ends of both L and S genome segments are predicted to form panhandle structures.^{30,151,215} This prediction is supported by electron microscopy data showing the existence of circular RNP complexes within arenavirus virion particles.²⁴⁰ This terminal complementarity may reflect the presence at the 5' ends of *cis*-acting signals sequences that provide a nucleation site for RNA encapsidation, which is required to generate the NC templates recognized by the virus polymerase. Terminal complementarity may be also a consequence of strong similarities between the genome and antigenome promoters used by the virus polymerases. This terminal complementarity has been proposed to favor the formation of both intra- and intermolecular L and S duplexes that might be part of the replication initiation complex; however, its mechanistic aspects remain undefined.¹⁹⁹ For several arenaviruses, an additional nontemplated G residue has been detected on the 5' end of their genome RNAs.⁸²

Arenavirus IGRs are predicted to fold into a stable hairpin structure. Transcription termination of the S-derived NP and GP occurs at multiple sites within the predicted stem of the IGR, suggesting that a structural motif rather than a sequence-specific signal promotes the release of the arenavirus polymerase from the template RNA.^{30,151} There are significant differences in sequence and predicted folded structure between the S and L IGR; however, among isolates and strains of the same *Arenavirus* species, the S and L IGR sequences are highly conserved. Some arenaviruses, including LCMV, LASV, and Pichinde virus (PICV), contain a single predicted stem loop within the S IGR, whereas the S IGR of others, such as TACV and Mopeia virus (MOPV), are predicted to contain two distinct stem loops.^{151,151,214}

ARENAVIRUS LIFE CYCLE

Cell Attachment and Entry

Consistent with a broad host range and cell-type tropism, a highly conserved and widely expressed cell surface receptor for extracellular matrix (ECM) proteins—alpha-dystroglycan (α -DG)—has been identified as a main receptor for LCMV, LASV, and several other arenaviruses.^{35,115,151} Posttranslational modifications of α -DG, including O-mannosylation and LARGE-dependent glycosylation, are critical for α -DG's function as both an ECM and OW and clade C NW arenavirus receptor.^{151,190} These findings indicate that similar α -DG-derived glycan structures are recognized by both ECM proteins

and surface arenavirus GPs. Intriguingly, recent genome-wide studies have uncovered evidence of positive selection of specific LARGE alleles within the Yoruba people in West Africa where LASV is endemic.¹⁹⁵ However, many other arenaviruses appear to use an alternative receptor and human transferrin receptor 1 (TfR1) was identified as a cellular receptor used for cell entry of NW hemorrhagic fever arenaviruses Junin virus (JUNV) and Machupo virus (MACV).¹⁸⁴

Upon receptor binding, arenavirus virions are internalized by smooth-walled vesicles into a low-pH subcellular environment that triggers a pH-dependent membrane fusion step between cell and viral membranes that is mediated by GP2 and results in the release of the virus RNP into the cytoplasm of the cell where viral RNA synthesis, both transcription of replication, take place. Consistent with the use of TfR1 as a primary receptor for cell entry, JUNV was found to follow a clathrin-mediated endocytosis as its main route of entry.¹³⁸ Nevertheless, although TfR1 is internalized and recycles through early endosomes (pH ~6), optimal fusion activity of JUNV GP requires a significantly lower (<5.5) pH, suggesting that JUNV may redirect TfR1 from its normal recycling pathway into the late endosomes, a process likely influenced by the multivalent nature of the virus particle. In contrast, cell entry of the OW arenaviruses LCMV and LASV appears to use a rather unique endocytic pathway independent of clathrin, caveolin, dynamin, and actin.^{187,188,191,208,227} (Fig. 43.4). Consistent with these findings, endosomal trafficking of LASV and LCMV was only minimally affected by dominant negative forms of Rab5 and Rab7, which are small guanine triphosphatases (GTPases) involved in vesicular trafficking into early and late, respectively, endosomes. Moreover, LCMV was found to circumvent Rab5/EEA-1 early endosomes. LCMV and LASV GP have a high binding affinity for α -DG and thereby the unusual endocytic pathway followed by these viruses may reflect the natural cellular trafficking of α -DG. Nevertheless, it cannot be ruled out that binding of GP to α -DG could trigger a novel route of entry for dystroglycan that is tailored specifically to benefit virus cell entry as previously documented for other pathogens.

Expression and Replication of the Viral Genome

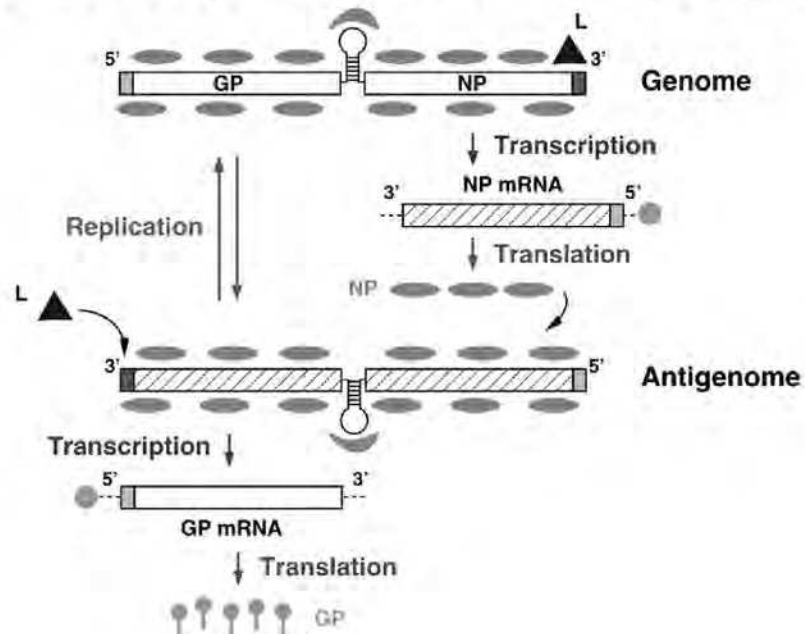
The fusion between viral and cellular membranes releases the viral RNP into the cytoplasm, which is ensued by the onset of viral RNA synthesis. Transcription termination of subgenomic nonpolyadenylated viral mRNAs were mapped to multiple sites within the distal side of the IGR, which suggested that the IGR acts as a bona fide transcription termination signal for the virus polymerase.

The NP and L coding regions are transcribed into a genomic complementary mRNA, whereas the GPC and Z coding regions are not translated directly from genomic RNA, but rather from genomic sense mRNAs that are transcribed using as templates the corresponding antigenome RNA species, which also function as replicative intermediates (Fig. 43.5). LCMV mRNAs have four to five nontemplated nucleotides and a cap structure at their 5' ends, which are likely obtained from cellular mRNAs via cap-snatching mechanisms whose details remain to be determined. A recently described endonuclease activity associated with the arenavirus L polymerase could play a critical role in this process.¹⁵⁷

The NP—the most abundant viral polypeptide both in infected cells and virions—is the main structural element of

LCMV Replication and Transcription

FIGURE 43.4. Basic aspects of arenavirus RNA replication and gene transcription illustrated for the S segment. Once the virus ribonucleoprotein (RNP) is delivered into the cytoplasm of the infected cell, the polymerase associated with the virus RNP initiates transcription from the genome promoter located at the genome 3' end. Primary transcription results in synthesis of nucleoprotein and L messenger RNA (mRNA) from the S and L segments, respectively. Subsequently, the virus polymerase can adopt a replicase mode and move across the intergenic region to generate a copy of the full-length antigenome RNA (agRNA). This agRNA will serve as template for the synthesis of the glycoprotein (agS) and Z (agL) mRNAs. The agRNA species also serve as templates for the amplification of the corresponding genome RNA species.



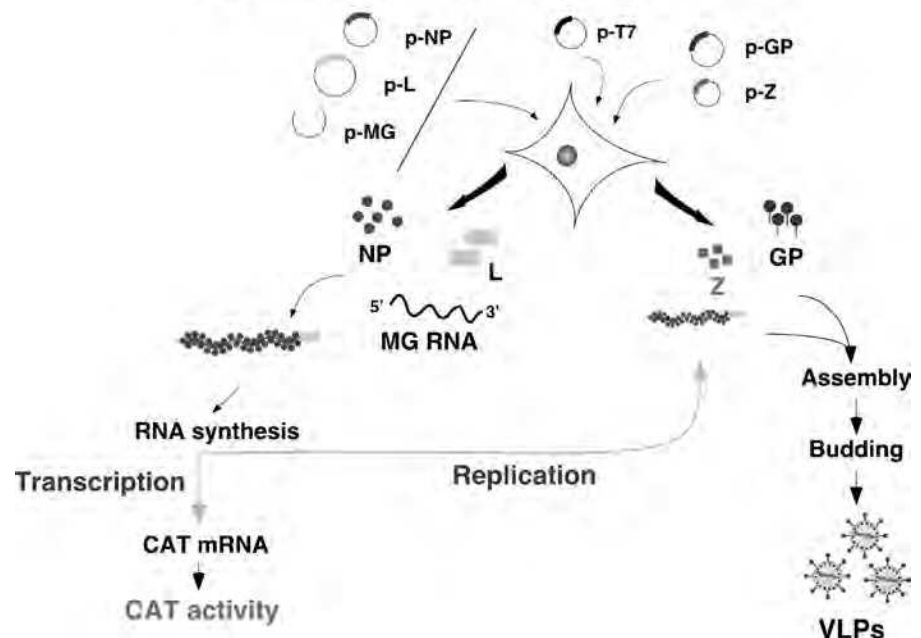
the viral RNP and plays an essential role in viral RNA synthesis. Recent evidence indicates that NP exhibits interferon type I (IFN-I) counteracting activity that was mapped to the C-terminus of NP.^{140,141,143} Two recently determined crystal structures of LASV NP at 1.80¹⁸³ and 1.5⁹⁰ Å resolution identified distinct N- and C-terminal domains connected by a flexible linker. The N-terminal domain was proposed to contain a potential cap-binding site that could provide the host-derived primers to initiate transcription by the virus polymerase.¹⁸³

However, analysis of the structure of the N-terminal domain of LASV NP in complex with single-strand RNA (ssRNA) suggest that this originally proposed cap-binding site likely corresponds to a binding site for the viral genome.

The C-terminal domain has a folding that mimics that of the DEDDH family of 3'-5' exoribonucleases like the one associated with severe acute respiratory syndrome (SARS) coronavirus nsp14 protein.¹⁵⁵ Functional studies confirmed the 3'-5' exoribonuclease activity of LASV NP, which was proposed to

LCMV Reverse Genetics

FIGURE 43.5. Scheme of the lymphocytic choriomeningitis virus (LCMV) mini-genome (MG) rescue system. This system allows us to re-create the steps of the virus life cycle intracellularly. LCMV RNA analogs, or MG with correct termini, are synthesized intracellularly using either a T7 RNA polymerase or an RNA pol-I expression system. Co-transfection with plasmids expressing the viral *trans*-acting factors L and nucleoprotein (NP) allow for the generation of viral ribonucleoprotein (MG RNA encapsidated by NP) that is substrate for the plasmid-supplied virus L polymerase. The virus polymerase directs the synthesis of two RNA species: the MG RNA (replicative species) and a subgenomic messenger RNA that directs expression of the reporter gene of choice, *CAT*, in this case. Incorporation of plasmids expressing Z and glycoprotein results in assembly and budding of infectious LCMV virus-like particles.



be critical for the anti-interferon (IFN) activity of NP but dispensable for the role of NP on replication and transcription of the viral genome.⁹⁰ This assertion, however, is difficult to reconcile with the finding that TACV NP, despite containing the conserved DEDDH residues at the active site of the exoribonuclease domain, has a dramatically reduced anti-IFN activity,¹⁴² and the finding that an LCMV with a mutant NP lacking the 3'-5' exoribonuclease had a large decrease in fitness during its replication in IFN-deficient Vero cells.¹⁴⁰

The arenavirus L protein has the characteristic sequence motifs conserved among the RdRp (L proteins) of NS RNA viruses.¹⁸¹ Detailed sequence analysis and secondary structure predictions done with the LASV L polymerase identified several regions of strong alpha-helical content and a putative coiled-coil domain at the N-terminus.²²⁸ Subsequent bioinformatic analysis together with biochemical and minigenome (MG)-based functional studies have shown that LASV L protein is likely organized into three distinct structural domains, and that at specific amino acid positions, LASV L can be split into N- and C-terminal parts that are able to functionally *trans*-complement each other.²⁷ Notably, the recent electron microscopy characterization of a functional MACV L protein has revealed a core ring domain decorated by appendages, which may reflect a modular organization of the arenavirus polymerase.¹¹⁴

Assembly and Budding

Assembly and cell release of infectious arenavirus progeny requires both Z and GPC, as well as the correct processing by the cellular site 1 protease (S1P) of GPC into GP1 (40–46 kDa) and GP-2 (35 kDa)^{116,125,178} Oligomers of GP1 and GP2 form the spikes that decorate the virus surface. GP1 located at the top of the spike and held in place by ionic interactions with the N-terminus of the transmembrane GP-2, mediates virus interaction with host cell surface receptors, whereas GP2 exhibits similarities with fusion active domains of other viral envelope proteins.^{66,80,236} GPC contains a 58-amino-acid signal peptide (SSP) that is expressed as a stable polypeptide in infected cells and it remains associated to the GP complex (GPCx). This SSP has been implicated in different aspects of the trafficking and function of the viral envelope GPs including the GP-2-mediated pH-dependent fusion process.^{2,203,237–239}

For most enveloped viruses, a matrix (M) protein is involved in organizing the virion components prior to assembly. Interestingly, arenaviruses do not have an obvious counterpart of M. The arenavirus RING finger protein Z is a structural component of the virion that has no homologue among other known NS RNA viruses. In LCMV-infected cells Z has been shown to interact with several cellular proteins including the promyelocytic leukemia (PML) protein and the eukaryote translation initiation factor 4E (eIF4E), which have been proposed to contribute to the noncytolytic nature of LCMV infection and repression of cap-dependent translation, respectively.^{54,229} Z has been shown to be the main driving force of arenavirus budding,^{169,218} a process mediated by the Z late (L) domain motifs: PTAP and PPPY similar to those known to control budding of several other viruses including HIV and Ebola virus, via interaction with specific host cell proteins.⁷⁸ Consistent with this observation Z exhibited features characteristic of bona fide budding proteins: 1) ability to bud from cells by itself, and 2) substituted

efficiently for other L domain. Targeting of Z to the plasma membrane, the location of arenavirus budding, strictly required its myristoylation.^{171,219} Results derived from cryo-electron microscopy of arenavirus particles¹⁶¹ were also consistent with the role of Z as a functional M protein.

ARENAVIRUS REVERSE GENETICS: IMPLICATIONS FOR THE INVESTIGATION OF ARENAVIRUS MOLECULAR AND CELL BIOLOGY

The generation of an infectious progeny of NS RNA viruses from cloned cDNA, referred to as reverse genetics, has revolutionized the analysis of cis-acting sequences and trans-acting proteins required for virus replication, transcription, maturation and budding. In addition, the possibility to generate predetermined specific mutations within the virus genome and analyze their phenotypic expression in the context of the virus natural infection provides investigator with novel and powerful approaches to investigate cellular and molecular mechanisms underlying virus-host interactions and their implications in viral pathogenesis. In addition, these developments have also paved the way for engineering recombinant viruses for vaccine and gene therapy purposes.

In contrast to positive-strand RNA viruses, deproteinized genomic and antigenomic RNAs of NS RNA viruses cannot function as mRNAs and are not infectious. Generation of biologically active synthetic NS viruses from cDNA requires trans complementation by all viral proteins involved in virus replication and transcription, and polymerases of NS RNA viruses recognize as templates an NC consisting of the genomic RNA tightly encapsidated by the NP, which associated with the virus polymerase proteins forms an RNP complex. These considerations hindered the application of recombinant DNA technology to the genetic analysis of these viruses. However, significant progress has been made in this area and for all NS RNA viruses, short model genomes (aka minigenomes (MG)) could be encapsidated and expressed by plasmid-encoded proteins. Moreover, it has become possible to rescue infectious virus entirely from cloned cDNAs for members of all families of NS RNA viruses.^{108,162}

Arenavirus Minigenome Systems

The first arenavirus MG rescue system was developed for LCMV.¹²² Subsequently MG systems were developed for LASV⁸⁷ and the NW arenaviruses PICV,¹¹⁷ TACV,¹³¹ and JUNV.³ Results derived from MG-based assays identified NP and L as the minimal viral trans-acting factors required for efficient RNA synthesis mediated by the virus polymerase. For LCMV both genetic and biochemical evidence indicated that oligomerization of L is required for the activity of the arenavirus polymerase.²⁰¹ Consistent with this finding, biochemical and MG-based functional studies have shown that LASV L protein contains both N- and C-termini sites that mediate L–L interaction. The use of MG-based assays facilitated mutation-function studies involving conserved acidic and basic amino acid residues within the N- and C-termini of LASV L protein uncovered several residues within the N-terminus of L that played a critical role in synthesis of viral mRNA but not in

RNA replication.^{88,124,201} The recent publication of a 2.13 Å resolution crystal structure and functional characterization of the N-terminal 196 residues (NL1) of the LCMV L protein uncovered an endonuclease functional domain similar to the one found in the N-terminus of the influenza virus PA protein.¹⁵⁷ MG-based assays have shown the endonuclease activity of NL1 to be critical for arenavirus transcription.

Mutation-function analysis of the genome 5′- and 3′-termini using the LCMV and LASV MG-based assays indicated that the activity of the arenavirus genomic promoter requires both sequence specificity within the highly conserved 3′-terminal 19 nt of arenavirus genomes and the integrity of the predicted panhandle structure formed via sequence complementarity between the 5′- and 3′-termini of viral genome RNAs.^{89,157,170} These studies revealed that arenavirus RNA replication and transcription are regulated in a coordinated manner. Likewise, MG-based assays provided direct experimental confirmation that the IGR is a bona fide transcription termination signal,¹⁷⁷ and that intracellular levels of NP do not determine the balance between virus RNA replication and transcription^{68,178,179}—a finding conceptually similar to that reported for the paramyxovirus respiratory syncytial virus.⁶⁸

Z was not required for intracellular transcription and replication of a LCMV MG; rather, Z exhibited a dose-dependent inhibitory effect on both transcription and replication of

LCMV MG.^{43–45,68} This inhibitory effect of Z has been also reported for TACV¹³¹ and LASV.^{87,131}

Generation of Recombinant Arenaviruses

Generation of infectious arenaviruses from cloned complementary DNAs (cDNAs) has been reported for LCMV,^{73,151,202} PICV,¹¹⁷ and JUNV.^{3,62,117} Both T7 RNA polymerase (RP)- and pol-I RP-based systems^{73,202} have been documented to launch intracellular synthesis of S and L genome, or antigenome, RNA species that were subsequently replicated and transcribed by the virus polymerase complex reconstituted intracellularly via plasmid-supplied L and NP (Fig. 43.6). Notably, rescue of PICV and JUNV using a T7RP-based system was efficiently achieved without requiring plasmid-supplied viral NP and L proteins,^{3,73,117} indicating that T7RP-mediated RNA synthesis produced viral antigenome RNA species that were substrate for encapsidation and replication as well as mRNAs that serve as a template to produce levels of NP and L sufficiently high to facilitate virus rescue. This finding parallels those reported for several other negative sense RNA viruses, including bunyaviruses,^{20,132} filoviruses,⁸⁵ and bornaviruses.²⁰⁵

The ability to generate recombinant arenaviruses with predetermined specific mutations and analyze their phenotypic expression in the context of the natural course of infection has opened new opportunities to investigate arenavirus–host

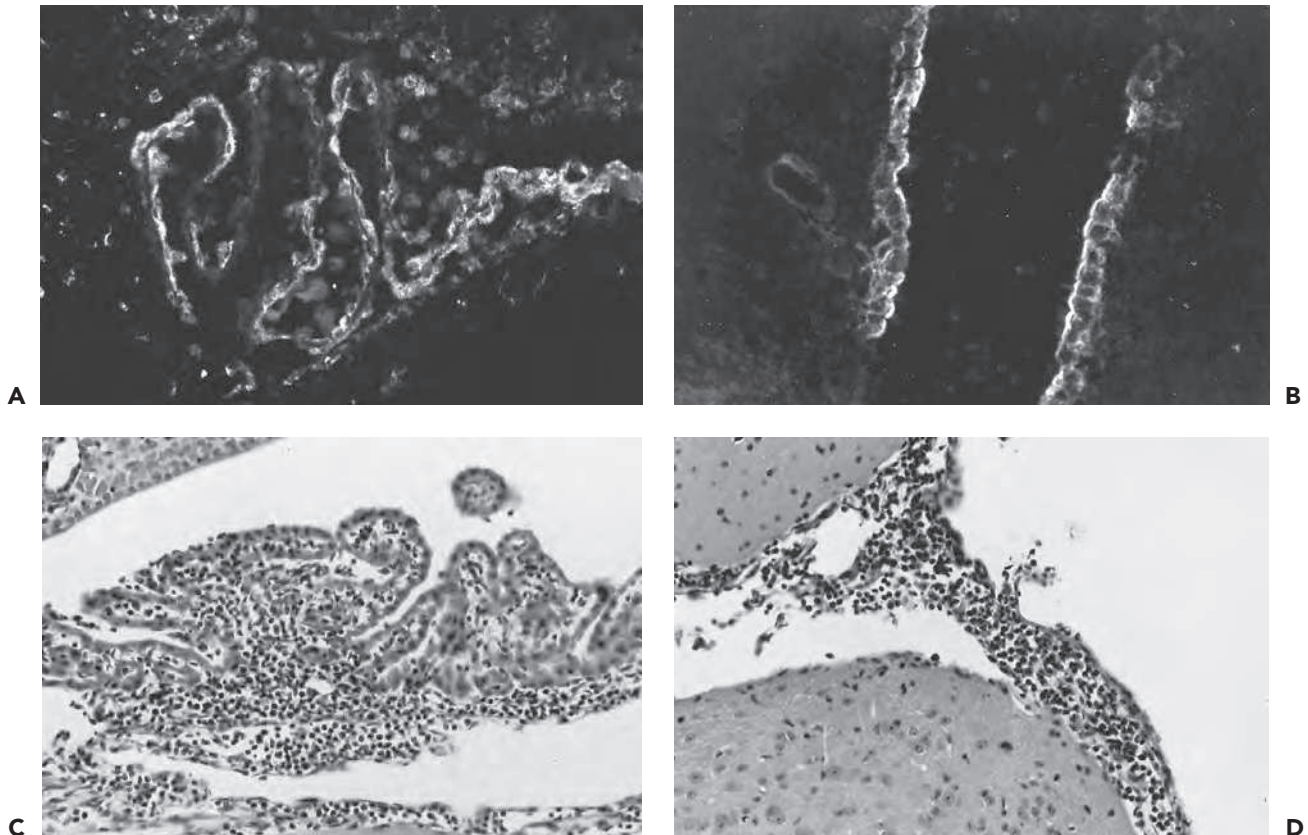


FIGURE 43.6. Brain of a BALB/c mouse inoculated intracranially with lymphocytic choriomeningitis virus (LCMV) Armstrong strain, 6 days previously. Abundant viral expression is seen in the choroid plexus (A) and in ependymal cells lining the third ventricle (B) when stained by monoclonal antibody 2-11.10 that detects glycoprotein 1 followed by fluorescein-labeled sheep antimouse immunoglobulin G. Mononuclear cell infiltration is prominent in the choroid plexus (C) and the meninges (D).

interactions that influence a variable infection outcome, ranging from virus control and clearance by the host defenses to long-term chronic infection associated with subclinical disease, and severe acute disease including hemorrhagic fever. Thus, studies with recombinant lymphocytic choriomeningitis virus (rLCMV)/vesicular stomatitis virus glycoprotein (VSVG) uncovered the arenavirus GP as a viral Achilles' heel and provided the foundations for a novel strategy to develop safe and effective live attenuated arenavirus vaccines.^{178,179} Likewise, the use of rLCMV/VSVG facilitated studies aimed at understanding the regulation of CD8⁺ T-cell function within the infected brain,¹⁸⁰ and how viruses can induce organ-specific immune disease in the absence of molecular mimicry and without disruption of self-tolerance.¹⁴⁹ Other rLCMVs have been used to investigate pathways of LASV cell entry¹⁸⁷ and the role of NP in the inhibition of induction of IFN-I by LCMV.¹⁴⁰ Likewise, the use of recombinant arenaviruses where a canonical furin cleavage site substituted for the S1P cleavage site within GPC illustrated the critical role played by the S1P-mediated processing of arenavirus GPC in the virus life cycle.^{3,189,224} The rescue of attenuated and virulent forms of PICV^{117,128} or the docile and aggressive strains of LCMV^{41,60,61} have allowed for the identification of specific genetic determinants of virus virulence. However, despite these successes, the ability to rescue recombinant arenaviruses expressing additional genes of interest posed unexpected difficulties. Approaches successfully employed with other NS RNA viruses including the use of an internal ribosome entry site (IRES), or of the picornavirus self-cleaving 2A sequence, did not work when applied to LCMV.⁶¹ This problem was finally overcome by the generation of trisegmented rLCMV (r3LCMV) containing one L and two S segments. For this, each of the two S segments was altered to replace one of the two viral open reading frames (ORFs), GPC or NP, by a gene of interest (GOI).⁶¹ The rationale for this approach was that the physical separation of GP and NP into two different S segments would represent a strong selective pressure to select and maintain a virus capable of packaging 1L and 2S segments (Fig. 43.7). Various r3LCMV have been rescued that express one or two additional GOI. Depending on the GOI expressed, with gene size and function being critical parameters, these r3LCMV have shown little or no attenuation

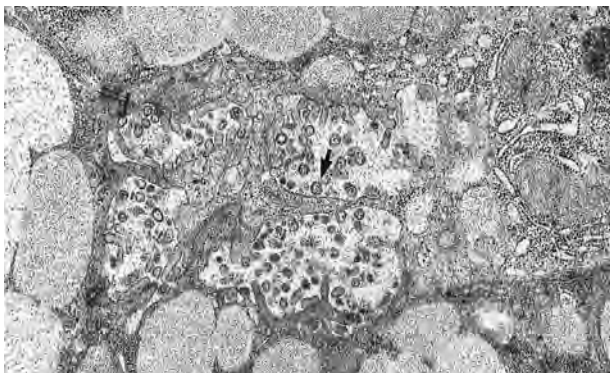


FIGURE 43.7. Accumulation of Machupo virus (arrow) budding from acinar cells of the salivary gland of a virus-infected large vesper mouse (*Calomys callosus*). Abundant virions in the lumen readily contaminate saliva (22,000 \times). (Courtesy of F. A. Murphy.)

and long-term genetic stability in cultured cell. In contrast, the use of r3LCMV to study virus–host interactions in mice has encountered some limitations owing to the observation that even r3LCMV with wild-type growth properties in cultured cells exhibited an attenuated phenotype in the mouse owing to reasons that remain to be determined.

PREVENTION AND CONTROL

Lymphocytic Choriomeningitis Virus Infection

LCMV infections in humans are rarely fatal. Two fatalities resulting from overwhelming systemic disease resembled hemorrhagic fever cases. A single well-studied encephalitis case showed mononuclear cell infiltrates in the meninges and around vessels and glial nodules in the deeper structures.²³² Fluorescent antibodies detected viral antigen in meninges and in cortical neurons.

The gross and light microscopic lesions found postmortem in arenaviral hemorrhagic fever patients rarely account for their demise.²³¹ Petechial hemorrhages of the skin and internal organs, cutaneous ecchymoses, and evidence of mucosal bleeding are common. Serous effusions, interstitial pneumonitis and pulmonary edema, and evidence of secondary bacterial infection are sometimes seen. Microscopic evidence of disseminated intravascular coagulation or vasculitis is uncommonly found and is not a regular feature of these diseases. Adequate studies of vascular endothelium are not available.

Focal necrosis may be present in several organs, including the liver, kidney, and adrenal cortex; fluorescent antigen tracings or virus titrations generally correspond to these lesions,^{135,230} as they do in experimental animal studies.⁴² These lesions usually contain a few mononuclear phagocytes as the only inflammatory cell contribution. Lymphoid depletion or necrosis in the marginal zone of the splenic periarteriolar lymphoid sheath is also present.²³⁰ Secondary changes associated with shock are usually found and may be responsible for the frequent presence of some degree of myocardial damage or myocarditis; however, virus has not been detected in the limited cardiac tissue studied.

Hemorrhages are common in the South American hemorrhagic fevers, in keeping with the low platelet counts. Thrombocytopenia is probably caused, at least in part, by maturation arrest of the megakaryocytes attributable to the high levels of IFN present in these patients.¹²⁶ In Lassa fever, thrombocytopenia is less marked, and liver involvement is more extensive. Although death does not appear to be caused by hepatic failure, the liver is an important site of virus replication, and the levels of aspartate transaminase (AST) in serum are proportional to the risk of dying.^{146,147}

Arenavirus diseases begin with a gradual onset of fever and myalgia, followed by systemic and hematopoietic cell abnormalities. In the viral hemorrhagic fevers (VHFs), a period of viremia lasting 1 to 3 weeks is accompanied by intense systemic symptoms; the occurrence of an effective immune response is concordant with clinical recovery and cessation of viremia. In LCMV infection, rare fatal cases have occurred with a pattern resembling that of VHF,²¹¹ although usually LCMV infection results in a febrile disease with accompanying viremia, often followed by central nervous system (CNS) disease. During the CNS disease, there is no viremia; however, virus is still detectable

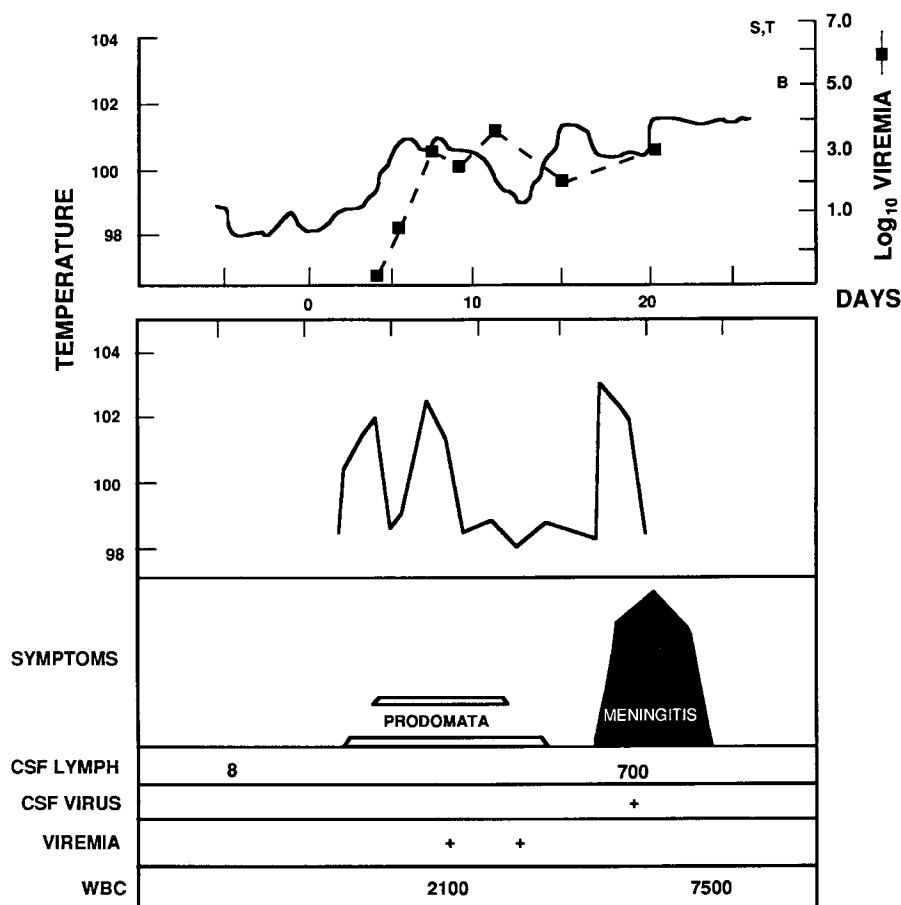


FIGURE 43.8. Probable pathogenesis of human lymphocytic choriomeningitis (LCM). In the **bottom panel**, results of inoculation of a normal human volunteer with lymphocytic choriomeningitis virus (LCMV) are shown. These and other human data show an initial period of 1 to 2 weeks' viremia associated with fever, leukopenia, and systemic symptoms. This is followed by the onset of the cellular immune response with meningeal inflammation leading to mild transient central nervous system (CNS) disease. Virus was isolated from the cerebrospinal fluid. In the **top panel**, the course of infection in a patient with Hodgkin disease, immunosuppressed by disease and chemotherapy, is shown. LCMV was inoculated in hopes of favorably influencing the underlying disease. Viremia continued as long as measured; no immunofluorescence assay, complementation fixation, or neutralizing antibodies developed; and there was no overt CNS disease. At the time of death, possibly secondary to bacterial pneumonia, there were high titers of virus present in brain (B), spleen (S), and tumor tissue (T). WBC, white blood cell. (Adapted from Genovesi EV, Johnson AJ, Peters CJ. Susceptibility and resistance of inbred strains of Syrian golden hamsters [*Mesocricetus auratus*] to wasting disease caused by lymphocytic choriomeningitis virus: pathogenesis of lethal and non-lethal infections. *J Gen Virol* 1988;69[Pt 9]:2209–2220.)

in cerebrospinal fluid (CSF), and the pathogenesis is believed to be immunopathologic.

Although LCMV is usually believed to be as a viral meningitis, deeper neurologic involvement is observed in a minority of cases, perhaps 10% or fewer in naturally observed outbreaks that provide some possibility of case ascertainment.^{52,95,225} If a febrile prodrome is present, meningeal signs usually begin about 10 days later, often after a brief hiatus in symptoms and fever (Fig. 43.8). Encephalitis has been diagnosed in 5% to 34% of hospitalized patients with documented LCMV.^{1,69,152} Full recovery is usual, although occasional deaths do occur.

The clinical laboratory abnormalities at the time of CNS involvement are usually confined to the CSF, which shows an increase in cells into the hundreds or low thousands, predominantly mononuclear in type. Protein is usually modestly elevated (50–150 mg%), and sugar may be low in

one-fourth of the patients, sometimes reaching values below 20 mg/dL.¹⁹

Rare neurologic presentations attributed to LCMV include ascending myelitis, transverse myelitis, and bulbar paralysis.¹ The course of acute LCMV has also been complicated by hydrocephalus. One case was believed to have posterior fossa arachnoiditis and was treated with ventriculomastoidostomy.²²⁰ Another was transient,⁹⁵ and a third required the placement of a ventricular reservoir.¹¹⁸ The latter case had evidence (by computed tomography) of periventricular edema and lateral ventricular enlargement, suggesting that the major lesion was ependymal inflammation. Both ependymal and meningeal inflammation are prominent lesions in experimental infections and in fatal LCMV encephalitis.²³² Nerve deafness has been associated with LCMV infection in two cases.⁹⁴ The occurrence of eighth nerve involvement in another OW arenavirus

disease (Lassa fever) suggests that these observations may represent a true causal relationship.

A surprising and illuminating recent finding has been the discovery of fatal LCMV infection in transplant patients.^{6,70,166,172} Two donors died of CNS hemorrhage and infected eight solid organ recipients; all recipients died, except one who received intravenous ribavirin and decreased immunosuppression. The cases were remarkably similar to three earlier fatal cases inoculated with LCMV in an attempt to cause regression in their lymphomas refractory to chemotherapy.⁹⁶ The clinical syndrome consisted of a systemic illness with thrombocytopenia, elevated transaminases, coagulation disorders, and involvement of lung, liver, and kidney. CNS abnormalities were seen but overshadowed by the severe systemic illness that resembled Lassa fever. The illness resembled the rare cases of VHF-like disease described previously²¹¹ and the milder illnesses observed in a carefully studied hospital outbreak.^{223,225} Presumably, the lack of an effective T-cell response allowed rampant virus replication and also prevented the development of the typical immunopathologic meningitis.

Lassa Fever

Infection with LASV leads to the gradual onset of fever and malaise after an incubation period of about 10 days (range, 5–21 days). As the process develops, there is an increase in fever and myalgia, with severe prostration. Gastrointestinal manifestations such as abdominal pain, nausea and vomiting, diarrhea, or constipation are common. Sore throat occurs in two-thirds of cases and is usually accompanied by objective inflammatory or exudative pharyngitis. Retrosternal pain and cough are frequent, and pleural effusions may develop. Bleeding manifestations are seen in fewer than one-third of patients but signal an unfavorable prognosis. Signs of increased vascular permeability such as facial edema or pleural effusions are present in a minority of patients and also suggest a poor prognosis. Mortality in hospitality patients is 15% to 20%.^{15,75–77,144,223}

A careful case-control study comparing Lassa fever to other febrile diseases seen in a West African hospital found several features significantly associated with Lassa fever, including bleeding, edema, exudative pharyngitis, conjunctivitis, and pharyngitis; however, the positive predictive values were only 0.61 to 0.74.²²³ The same study also found vomiting, sore throat, tachypnea, or bleeding to predict a 2.5-fold or higher increased risk of death. Despite the relative nonspecificity of the clinical findings, more than three-fourths of patients believed to have severe Lassa fever are confirmed virologically. Lassa fever is a major pediatric problem as well.^{150,156} Disease is more difficult to diagnose clinically. Occasional cases of infants developing anasarca have been described.

The course of fatal Lassa fever is relentless, with progression of the signs and symptoms culminating in the onset of shock and death. In survivors, symptoms and viremia persist until 2 to 3 weeks after onset when there is defervescence accompanied by the disappearance of virus from the blood. Pericarditis may occur in early convalescence, particularly in male patients. A case of polyserositis and recurrent pericarditis with constriction has been reported, which suggests that such complications should be sought more carefully.⁹³

Neurologic disease is not usually a dominant clinical manifestation in Lassa fever, although aseptic meningitis, encephalitis, global encephalopathy with seizures, and more subtle neurologic

problems are well described.^{46,47,213} Cerebellar ataxia in convalescence is an uncommon but interesting occurrence. In convalescence, deafness is common; this is an important feature of Lassa fever because it impacts the community and provides an important diagnostic clue.⁷⁵ Late in the course of disease or early in convalescence, unilateral or bilateral hearing loss was noted in 29% of prospectively studied patients.⁴⁷ No treatment is available, and the effects may be transitory or often permanent. The auditory patterns and clinical course resemble idiopathic nerve deafness.¹²⁹

The clinical laboratory provides few clues to the diagnosis. The leukocyte count can be low, normal, or modestly elevated. Platelet counts are generally normal but may be modestly decreased.⁹³ Albuminuria is common. AST is usually at least mildly elevated, and the degree of elevation, which parallels the viremia, is a useful predictor of mortality.¹⁰⁵ Patients with AST values in the hundreds or thousands are at considerable risk of dying, even with ribavirin treatment. Chest radiography may show infiltrates, pleural effusions, or, more commonly, no abnormalities. Electrocardiographic findings are often nonspecifically abnormal.⁴⁶

Lassa fever should be suspected in virtually any febrile patient returning from endemic areas of Africa, particularly after a rural sojourn in Nigeria, Sierra Leone, Liberia, Guinea, or another West African country. Lassa fever continues to be the most often imported VHF into Europe and the United States.^{5,204} Within a few days of onset, classical Lassa fever progresses to prostration and the other symptoms described previously, often including pharyngitis, retrosternal pain, gastrointestinal complaints, and proteinuria. However, mild or atypical cases can be diagnosed or excluded only with appropriate laboratory tests. Differential diagnoses include malaria, shigellosis, leptospirosis, rickettsial disease, relapsing fever, and typhoid; if doubt exists, these entities should be treated empirically.

Argentine Hemorrhagic Fever

Argentine hemorrhagic fever (AHF) begins insidiously with fever and malaise. Headache, myalgia, epigastric pain, and anorexia are common. Within 3 to 4 days, these symptoms give way to more severe prostration, nausea and vomiting, dizziness, and early indicators of vascular damage. The earliest signs are conjunctival injection, flushing over the head and sometimes upper torso, petechiae, and mild hypotension. Tremor of the tongue and hands is common and may resemble cerebellar disease. In the more severe cases, mucous membrane hemorrhages and ecchymoses at needle puncture points develop; shock supervenes and, with the hemorrhages, is an indicator of a poor prognosis. In some cases, there are extensive neurologic signs, including coma and convulsions; these cases also have a high mortality.¹⁹³ Usually around days 10 to 13, patients begin to improve as the appearance of immunofluorescence assays (IFAs) and neutralizing antibodies signals the onset of the immune response.⁴⁹ Overall case fatality is 30% without therapy. Convalescence often lasts several weeks with fatigue, dizziness, and hair loss.

Clinical laboratory findings are quite helpful in the diagnosis of AHF. Leukopenia, thrombocytopenia, and proteinuria are present in virtually all patients with the disease and may be early manifestations. AST and alanine transaminase are usually normal or slightly elevated. The electrocardiogram may be

nonspecifically abnormal, and chest radiography is usually normal in the absence of secondary infections.

Bolivian and Venezuelan Hemorrhagic Fevers

Bolivian hemorrhagic fever (BHF) resembles AHF clinically, just as the virus is closely related phylogenetically to JUNV.^{104,217} Mortality is about 25% to 35%. Early information from the San Joaquin epidemic suggested that most MACV infections resulted in disease sufficiently severe to be remembered as BHF¹³⁴; however, surveys in the endemic region of the Beni have found enzyme-linked immunosorbent assay (ELISA) immunoglobulin G (IgG) antibodies in persons with no previous history of BHF.

Venezuelan hemorrhagic fever also has a clinical spectrum similar to that of AHF, with thrombocytopenia, bleeding, and neurologic involvement being prominent.⁵⁰ Sore throat and pharyngitis were noted with some frequency among initial cases, and deafness has occurred in convalescence. Although reported cases have had a somewhat higher mortality (34%), it seems likely that with increased case finding, milder cases may be detected. Normal inhabitants of the endemic area have antibody prevalence rates of 0.1% to 3% and those of household contacts at 10.5%.

Sabia Virus Infection

Sabia virus was isolated from a fatal case of hemorrhagic fever presenting in Sao Paulo, Brazil, in 1990.¹³⁰ Because of the extensive liver necrosis associated with the clinical presentation, yellow fever was a leading diagnosis; however, it was excluded virologically. Later, the virus was shown to be an arenavirus, and a laboratory worker performing the studies developed severe disease resembling AHF.²²⁶ This virus once again infected a virologist in 1994, resulting in a febrile, leukopenic, thrombocytopenic syndrome that was promptly treated with intravenous ribavirin. There was an apparent clinical and virologic response. The reservoir remains unknown; it is presumed to be a rodent found around the small community of Sabia outside Sao Paulo, where the only known natural infection occurred. Without additional information, it must be assumed that this virus has a high morbidity and mortality as well as the aerosol infectivity expected from an arenavirus.

Whitewater Arroyo Virus

WWAV was first described as a zoonotic persistent infection in woodrats (*Neotoma albigula*) in New Mexico.⁷⁹ This and related reservoirs are widely distributed throughout the southwestern United States. The virus is most closely related to Tamiami virus, an arenavirus originally found in cotton rats (*Sigmodon hispidus*) in South Florida.³⁴ Little evidence for human infection or disease with either of these North American arenaviruses was found until the period from June 1999 to May 2000. Two fatal cases with a clinical picture suggesting acute hemorrhagic fever were reported to have preliminary evidence of acute WWAV infection.³³

Other Arenavirus Infections

Surveys of potentially exposed laboratory workers and residents of endemic areas indicate that most arenaviruses are infectious for humans. In most cases, actual infections have not been observed for their clinical effects, thus information about the consequences is relatively limited. The newly described Argentine arenavirus—Oliveros—provides an example.¹⁵³ This virus

TABLE 43.3 Duration of Viremia After Infection of Rodent Reservoirs with Arenaviruses

Virus	Age of infection and subsequent viremia		
	Fetal	Neonatal	Adult
LCM, Lassa	Lifelong	Lifelong	Transient
Junin, Machupo	Fetal death	Lifelong	50% lifelong 50% transient
Latino, Tamiami	?	Transient	Transient

LCM, lymphocytic choriomeningitis.

circulates in the same agricultural zones as JUNV and LCMV; however, the dark bolo mouse (*Bolomys obscurus*) is its natural reservoir. Even though these rodents comprise about 4% of the captures during longitudinal studies of small mammals in agricultural areas and one-fourth are infected, no antibodies were detected in 200 normal residents of the area or in 100 patients suspected of having AHF but found to be seronegative to JUNV. Nevertheless, it remains an open question whether this virus might not be pathogenic for humans.

Laboratory workers provide another source of information concerning arenavirus pathogenicity, because these viruses are quite infectious as small-particle aerosols. PICV has caused numerous laboratory infections without any overt disease.²⁹ Flexal virus has resulted in two symptomatic laboratory infections and should be regarded as potentially dangerous. TACV has also resulted in a single example of febrile disease with mild CNS symptoms (J. Casals, personal communication).

Little is known about the pathogenicity of OW arenaviruses other than LCMV and LASV. They are usually assumed to be less pathogenic for humans than LASV, based on their low virulence for monkeys and guinea pigs.^{173,230} They are studied at biosafety level 3 (BSL3) but with additional precautions to avoid aerosols of the substantial amounts of virus shed from infected animals.

Maternal and Fetal Infections

Several arenaviruses infect the fetus of the natural host when pregnant rodents are viremic. In some cases, this results in no damage to the developing embryo; however, in other situations, there may be fetal death (Table 43.3). Thus, it is not unexpected that maternal infections of nonreservoir rodent species may also lead to fetal infections. The extent of fetal invasion in infected humans is surprisingly high as well, and the impact on the fetus is often severe (Fig. 43.9). LCMV infection of the fetus has been associated with both fetal death at early stages of development and fatal neonatal infection.^{112,235} An association between maternal infection with LCMV and neonatal hydrocephalus with chorioretinitis was identified in Lithuania²⁰⁹; isolated cases have also been reported from Germany and France.⁴⁰

It is becoming increasingly apparent that LCMV is an important cause of fetal abnormalities in the United States. One report described the unusual occurrence of this syndrome in twins.¹² Additional cases have been reported, primarily from the midwestern and southwestern states.^{7,13,14,17,235} The emerging picture from recent observations in the United States and Europe is of a congenital infection that follows symptomatic acute febrile disease or asymptomatic infection of the mother



FIGURE 43.9. T1-weighted magnetic resonance imaging (MRI) scan of the brain of a 10-month-old child with congenital lymphocytic choriomeningitis virus infection. The child has severe developmental delay, spastic quadriplegia, and medically intractable epilepsy. The MRI scan reveals microcephaly, a lissencephalic frontal lobe (A), and hypoplasia of the corpus callosum (B), the brain stem (C), and the cerebellum (D). (Courtesy of D. Bonthius, University of Iowa.)

in mid- to late pregnancy. The fetus is severely affected with combinations of psychomotor retardation, microcephaly, hydrocephaly, visual loss, and seizures, often with evidence of aqueductal stenosis, periventricular calcifications, and chorioretinitis. The infants are not known to be chronically infected but may have a somewhat unusual antibody pattern.²¹⁰ Any child with CNS or ocular disease who does not have clear-cut evidence of intrauterine infection with one of the TORCH pathogens (toxoplasmosis, rubella, cytomegalovirus, herpes) should be evaluated for LCMV infection.

South American Hemorrhagic Fevers

Any patient from known endemic areas in South America should be under suspicion if fever, dizziness, and myalgia are accompanied by leukopenia, thrombocytopenia, and proteinuria. The relatively frequent discovery of “new” arenaviruses in the Americas implies that suggestive findings in patients with rural exposure or rodent contact anywhere in the Americas should lead to the consideration of arenavirus disease. Influenza, hantavirus pulmonary syndrome, and other febrile, myalgic diseases, as well as the more severe conditions noted for Lassa fever, enter into the differential diagnosis.

LABORATORY DIAGNOSIS

Lymphocytic Choriomeningitis

During the acute febrile phase, virus isolation from blood is the best approach. Viremia may also be detectable during the meningitic phase, although then the CSF may be more productive.^{21,67} Most clinical isolates of LCMV will be lethal for intracranially inoculated mice or intraperitoneally inoculated guinea pigs; however, some strains will require back challenge with a known virulent LCMV strain or antibody measurement to demonstrate the presence of LCMV in the original

inoculum; mice can also be injected with endotoxin to provoke lethality.⁹⁷ Isolation in cell culture (e.g., Vero cells) is less expensive, less hazardous, and less likely to suffer cross-contamination, although it is probably slightly less sensitive than animal inoculation. Polymerase chain reaction (PCR) tests applied to CSF have also shown promise.¹⁶⁷ Antibody is often present in serum and/or CSF during acute cases, and ELISA and IFA for immunoglobulin M (IgM) have been widely used to detect it.^{51,69,123,127} Neutralizing antibodies appear late after onset and are technically demanding to demonstrate, lessening their diagnostic importance.^{1,123} Complement fixation (CF) tests are relatively insensitive, and antibodies disappear quickly; high CF titers are believed to indicate recent infection. The optimal test for serosurveys needs to be established by careful comparison of the major candidates: ELISA, IFA, and neutralization.

Lassa Fever

LASV is easily isolated from blood or serum during the febrile phase of the disease up to 14 or more days after onset, even after the appearance of IFA antibody. Virus can also be detected in necropsy tissues.^{77,105} Vero cell cultures examined by IFA allow a diagnosis in 5 to 7 days or sooner. LASV antigen can be detected by ELISA capture in serum within 4 hours of beginning testing, and as it becomes negative, IgM antibodies appear.¹⁶ Antigen detection by ELISA is robust and reliable in rapidly fatal cases, even if the specimens are not handled properly for virus isolation. Reverse transcriptase PCR is also a sensitive test for virus RNA, being positive in the blood of 23 of 29 patients at admission and of 29 of 29 patients by the third day of hospitalization.^{53,221}

Antibody can be detected by either CF, IFA, or ELISA. IFA utilizing LASV-infected Vero cells as substrate is widely used. Interpretation is subjective, and discrepancies between laboratories are common. IFA IgG “seroreversion” has been reported and believed to represent loss of antibody by previously seropositive individuals.^{16,100,145} Lassa IgG and IgM can also be detected by ELISA.¹⁶³ ELISA IgM titers appear earlier and persist longer than IFA IgM titers. IgG ELISA antibody persists for long periods, whereas IFA antibody appears to wane below detectable limits within several years.

South American Hemorrhagic Fevers

Similar to LASV, Junin, Machupo, Guanarito, and Sabia viruses are infectious by aerosol, and human and rodent specimens should be processed with appropriate precautions in BSL4 laboratories. Virus can be isolated from blood or serum during the acute febrile phase until 10 to 12 days after onset by inoculation of newborn hamsters or mice.²⁵ Inoculation of cell cultures (Vero, BHK-21) is safer and more easily performed under the required BSL4 conditions. Co-cultivation of peripheral blood mononuclear cells with Vero cells seems to be more sensitive than suckling mouse or Vero cell inoculation for the isolation of JUNV from AHF patients.⁴ Virus can also be isolated from necropsy tissues, except from the brain. In rodent studies, virus is readily isolated from blood, urine, throat swabs, and autopsy tissues.^{106,107} Antigen-capture ELISA allows detection of viral antigen in blood, serum, or tissue homogenates. These tests could be used for acute diagnosis of patients suspected of having Junin, Machupo, Sabia, or Guanarito, and they are often the first available diagnostic result in rapidly fatal cases when

patients die before the appearance of antibody. Antigen detection is also possible in rodent urine, blood, and throat swabs during ecologic surveys.¹⁵⁴

Serologic diagnosis of AHF and BHF is usually made by CF^{134,233} and IFA.^{139,165,176} The lack of specificity and/or sensitivity of these tests is often a major problem. The plaque neutralization test can distinguish between antibodies to MACV and JUNV and is used for evaluation of convalescent plasma units intended for therapeutic use in AHF.⁶⁴ The ELISA test is the most useful and practical for rapid detection of IgG and IgM antibodies in a clinical setting and seroepidemiologic surveys.^{81,98} The antibodies detected by ELISA persist more than 30 years in some cases. Guanarito and Sabia IgG and IgM antibodies can also be detected by IFA and/or ELISA.

TREATMENT

Medical Management

Supportive therapy is important in the management of patients with arenaviral hemorrhagic fevers.^{174,175} Avoidance of travel and general trauma, gentle sedation and pain relief with conservative doses of opiates, the usual precautions for patients with bleeding diatheses (e.g., avoiding intramuscular injections and acetylsalicylic acid), and careful maintenance of hydration are indicated. Bleeding should be managed by platelet transfusions and factor replacement as indicated by clinical judgment and laboratory studies.

Management of shock is difficult. Modest increases in hematocrit indicate a generalized vascular permeability problem, although not of the magnitude seen in diseases such as hantavirus pulmonary syndrome⁵⁶; nevertheless, vigorous infusion of crystalloid carries a high risk of pulmonary edema. Cautious administration of fluids and early use of pressors is indicated. Because of the low cardiac output seen in the Pichinde guinea pig model of arenaviral hemorrhagic fever, and based on clinical experience with the human disease, careful monitoring is important and Swan-Ganz catheterization is desirable.

Containment

Patients with arenaviral hemorrhagic fevers are believed to pose a minimal risk of contagion in the early stages of disease. As the process continues, more extensive viral infection of target organs and increasing blood virus titers may increase the opportunities for dissemination. Nosocomial outbreaks and infection of multiple contacts have occurred; however, they are distinctly uncommon and the index patient has generally been severely ill at the time of the incident.^{36,111} The most dangerous exposure is parenteral and must be avoided through staff training. Thus, patients with these diseases should be treated in mask, gown, and glove isolation. Protection to caregivers and other patients should be enhanced by the addition of respiratory protection against small-particle aerosols.^{10,71} Close personal contacts should be monitored for fever for a period of 3 weeks. The patient may continue to excrete virus in urine or semen for weeks after recovery, thus body fluids should be monitored for infectivity before the patient is released. Meanwhile, a program of counseling that emphasizes the addition of disinfectant to toilets before use and protection used with sexual partners should be followed. Special precautions are indicated when blood and other body fluids are handled in the clinical laboratory.⁵⁸

Antiviral Drugs

Although several compounds have shown *in vitro* efficacy, only the guanosine analog ribavirin has had practical application. Although the mechanism of action is unknown, all arenaviruses tested have been inhibited in cell culture by similar concentrations of ribavirin, and the drug is effective in all arenaviral hemorrhagic fever models in which it has been studied.^{99,109,111,204,212} Ribavirin-treated experimental infections with MACV or JUNV have sometimes led to a late neurologic disease; however, activity against the visceral form of the disease has been a uniform finding. The drug is efficacious in Lassa fever and is the therapeutic agent of choice in that disease. Ribavirin has been of use principally in Lassa fever patients with a poor prognosis, usually being reserved for those with an AST value higher than 150. Preliminary results with ribavirin have been positive in AHF.⁶⁵ Anecdotal data on early treatment of human Machupo and Sabia infections have been promising.^{11,111}

Ribavirin has been considered for the prophylaxis of persons exposed to arenaviral hemorrhagic fevers. In experimental studies of Pichinde- or Lassa-infected guinea pigs, prophylaxis for periods such as 7 to 10 days only delayed the onset of acute disease by a similar period of time. However, drug administration for 14 days prevented disease.^{133,212,216} In the case of JUNV, prophylaxis of guinea pigs for 14 to 21 days prevented acute disease, although it did not prevent JUNV entering the CNS and causing fatal encephalitis.¹⁰⁹ Thus, high-risk exposures to arenaviruses are probably best managed by expectant treatment at the first sign of fever rather than dealing with the uncertainties of prophylaxis and its small but significant risk.

Passive Antibody

Therapy of JUNV infections with convalescent plasma has been carefully studied in humans and experimental animals and is quite efficacious, with reduction of mortality from 15% to 30% and to 1% to 2% if initiated within the first 8 days of illness.^{64,136} The administration of convalescent plasma (usually two to three units, depending on the neutralizing titer) is the treatment of choice. About 10% of these patients will return 3 to 6 weeks later with neurologic signs such as fever, headache, cerebellar tremor, and cranial nerve palsies. This late neurologic syndrome is usually self-limited. Serum antibody titers peak later and at a higher level, and serum-to-CSF antibody ratios are increased in comparison with those in patients without this syndrome, suggesting the prior invasion of the CNS by virus. Late eradication of virus by T cells may be a factor in its pathogenesis. The syndrome resembles other experimental arenavirus neurologic diseases seen after passive antibody therapy.^{57,110} The *in vitro* neutralization characteristics and limited studies in animal models suggest that passive antibody therapy would probably be useful in Machupo and Guanarito virus infections.⁵⁷

LASV infections are more difficult prospects for antibody therapy because, based on animal studies, the volumes of plasma needed are large; experimental studies of IgG for intravenous administration indicate that this could be a useful means of treatment only if highly active preparations were available.^{101,102,173} The future of antibody therapy in any of these diseases lies in development of standardized monoclonal antibody preparations of proven efficacy.^{194,200} Experimental studies of passive protection by monoclonal antibodies have been successful in LCMV.^{8,9,234}

Central Nervous System Disease

Ribavirin penetrates poorly into the CSF, and no established antiviral therapy exists for arenavirus infection in the CNS. LCMV ependymal infection and inflammation may cause acute hydrocephalus and a need for surgical shunting.¹¹⁸ Steroids have not been evaluated in this condition. The late neurologic syndrome seen after immune plasma treatment of AHF is generally self-limited and should be treated expectantly.⁶⁴

Vaccines

Novel Approaches for Vaccine Development

The JUNV live attenuated Candid #1 strain was generated by serial passages of the XJ strain of JUNV in mouse brain. The safety, immunogenicity, and protective efficacy of Candid #1 was demonstrated in preclinical studies in both guinea pigs and rhesus macaques.¹⁴⁸ Vaccination campaigns targeting agricultural workers in the JUNV endemic area have shown Candid #1 to be an effective and safe vaccine in humans,¹³⁷ and it was licensed in 2006 for use exclusively in Argentina. There are, however, some concerns related to the limited information about the genetic composition of the Candid #1 vaccine and still poor understanding regarding the viral genetic determinants of JUNV attenuation. The potential genetic instability of Candid #1 vaccine was illustrated by a 1,000-fold range of virulence among Candid #1 clones isolated from blood of vaccinated rhesus monkeys.⁶³ The lack of clearly established genetic markers associated with Candid #1 attenuation complicates the assessment of the emergence of potentially pathogenic variants during the preparation of Candid #1 vaccine. Reverse genetics approaches should facilitate the generation of molecular clones of genetically well-defined live attenuated vaccine strains with the ability to induce strong protective immunity.^{18,61,62}

Antiviral Drugs

The observation that a chimeric Vesicular Stomatitis Virus-LCMV vaccine was highly attenuated but induced a strong protective immune response¹⁸ led to examine the potential of LCMV as a viral vector system for immunization against non-LCMV antigens of interest. Thus, rLCM viruses where GPC was replaced by a protein antigen of interest were able to induce good immune responses against the foreign protein while immunized mice remained free of disease symptoms.⁷⁴ However, pre-existing immunity against LCMV in human populations may limit the use of LCMV as a vaccine platform. Notably, trisegmented rLCMV expressing high levels of non-LCMV GOIs have been found to exhibit attenuation *in vivo* while exhibiting sufficient levels of replication to induce strong protective immunity against a subsequent LCMV lethal challenge.⁶¹ This approach could be implemented to increase the safety of Candid #1. Moreover, the geographic restricted distribution of JUNV should eliminate pre-existing immunity as a barrier for the use of Candid #1 as a vaccine platform against other pathogens.

LASV poses the main concern among arenaviruses known to cause disease in humans. This is owing to its vast endemic region and size of the population at risk. Therefore, vaccination of the population at risk of LASV infection would be an important task in public health. Currently, there are not well-established attenuated strains of LASV, which prevents a vaccination approach similar to the one based on Candid #1 against Junin Virus. Nevertheless, several approaches have been pursued,

aimed at the development of an LASV vaccine including DNA immunization approaches¹⁸⁶ and different vector-based vaccines. Recombinant vaccinia viruses expressing LASV NP or GP have been shown to provide cell-mediated immunity against LASV in guinea pigs and nonhuman primates.⁷² However, the high prevalence of human immunodeficiency virus (HIV) within the LASV endemic region would pose some risk concerns of using a vaccinia virus-based vectors. Both alphavirus- and vesicular stomatitis virus (VSV)-based vectors^{83,182} have been used to induce protective immunity against LASV. Likewise, Venezuelan equine encephalitis virus¹²¹ and the 17D yellow fever vaccine²⁶ have shown promising results as potential candidate vectors for LASV vaccines. In addition, the induction of heterologous immunity by using a closely related but less pathogenic virus has been explored for the development of an LASV vaccine. Thus, a reassortant (called ML29) between MOPV, an OW arenavirus considered to be nonpathogenic to humans, and LASV has shown some promising results³⁸; however, detailed safety and efficacy studies have not been completed. An alternative approach for the development of a Lassa fever vaccine would be the use of reverse genetics to generate biologically contained versions of LASV in a similar way as described for Ebola virus⁸⁶ and influenza.¹⁴¹ Recent work with LCMV has provided strong support for the feasibility of this approach⁷⁴ to generate a safe and effective LASV vaccine. Likewise, the use of live attenuated trisegmented Candid #1 expressing relevant LASV T-cell epitopes could be used to induce protective immunity against JUNV and LASV. Finally, a fully synthetic approach to vaccine design has recently been proposed that builds on the observation of conserved peptide epitopes recognized by cytotoxic T lymphocytes directed against LCMV antigens.^{23,24} This system, if successfully established, offers the potential for a broadly cross-reactive arenavirus vaccine.¹¹³

Novel Targeting Strategies

The prophylactic and therapeutic value of the nucleoside analog ribavirin against several arenaviruses is well supported by both cell culture and whole organism studies. Thus, ribavirin reduced both morbidity and mortality in humans associated with LASV infection, and experimentally in MACV and JUNV infections, if given early in the course of clinical disease.⁴⁸ However, the need for intravenous administration for optimal efficacy and significant side effects including anemia and congenital disorders pose some significant limitations to the use of ribavirin. In addition, the mechanisms by which ribavirin exerts its antiarenaviral action remain poorly understood but are likely to involve different steps of the virus life cycle, which may complicate rational design to improve drug specificity.¹⁶⁸ Recent high-throughput screening (HTS) identified a potent small molecule inhibitor of TACV and several other NW arenaviruses.²² Likewise, cell-based HTS based on the use of pseudotyped virion particles bearing the GP of highly pathogenic arenaviruses identified several small molecule inhibitors of virus cell entry mediated by LASV GP.¹²⁰ These findings illustrate how screening of complex chemical libraries using appropriate assays represent a powerful tool to identify candidate antiviral drugs with highly specific activities. Progress in arenavirus reverse genetics is allowing investigators to dissect each of the steps of the virus life cycle to uncover novel targets and develop screens to identify drugs directed against specific steps of the arenavirus life cycle. Two

particularly promising possibilities are targeting virus budding and the S1P-mediated processing of arenavirus GPC.

For many characterized viral budding proteins, their budding activity requires interaction, via its L domains, with specific host cellular factors including members of the endosomal sorting complex required for transport (ESCRT) machinery within the endosomal/multivesicular body pathway of the cell. The generation of recombinant arenaviruses expressing appropriate tagged versions of Z should facilitate studies aimed at the identification and characterization of the Z–host cell protein interactions involved in arenavirus budding in the context of the natural course of infection of the virus. Knowledge from these studies may uncover novel targets and facilitate the development of screening processes to identify small molecules capable of disrupting these interactions and thereby interfering with virus propagation. The ESCRT machinery participates in various processes required for normal cell physiology^{37,158}; therefore, long-term disruption of the normal function of ESCRT components is likely to result in unacceptable levels of toxicity. However, arenavirus-induced hemorrhagic fevers are acute disease processes, and it is plausible that short-term inhibition of ESCRT members to combat an acute hemorrhagic fever arenaviral disease may cause only limited toxicity.

S1P-mediated processing of arenavirus GPC is strictly required for production of infectious progeny and cell-to-cell virus propagation, and thereby for both intra- and interhost virus propagation.¹¹⁶ Notably, studies with LCMV and JUNV indicated that the appearance of viral variants capable of growing independently of S1P-mediated processing of GPC appears to be a highly unlikely event. These findings strongly support the idea that inhibitors of S1P-mediated processing of GPC would represent promising antiarenaviral drug candidates.^{188,189} S1P is encoded by the membrane-bound transcription factor protease site 1 gene and is an endoplasmic reticulum/early Golgi membrane-anchored serine protease.^{196,197,207} S1P is involved in proteolytic processing of a defined set of cellular proteins. The key role of S1P in the regulation of lipid metabolism and cholesterol biosynthesis has raised significant interest in developing specific inhibitors of S1P activity. Several peptide and nonpeptide-based S1P inhibitors have been documented; however, their lack of cell permeability would pose severe limitations to their use as antiviral drugs. Recently, decanoylated chloromethylketone-derivatized peptides containing the RRLI recognition sequence of S1P have been shown to act as potent suicide inhibitors of S1P catalytic activity. These drugs cause irreversible inhibition of the catalytic activity of S1P against host cell and pathogen-derived targets, which might result in unacceptable levels of cellular toxicity. Recently, the small molecule PF-429242 was reported to be a potent S1P inhibitor both *in vitro* and in cell-based assay.^{91,92} In addition, PF-429242 was shown to inhibit efficiently S1P-mediated processing of arenavirus GPC, which correlated with the drug's ability to interfere with propagation of both LCMV and LASV in cultured cells.²²⁴

PERSPECTIVES

Since the fortuitous discovery of LCMV more than 70 years ago, arenaviruses have provided important model systems for the study of host–virus interactions. The study of these models has illuminated a wide range of principles, including persistent

infection, virus-induced immunopathologic disease, cytotoxic T-cell recognition, and immunological memory. Despite this wealth of knowledge, and even though arenavirus infections of humans remain a significant public health risk in much of the world, the prospects for control of these diseases remain remote because of poor diagnostic protocols and sparse resource allocation to vaccine development and other control measures. Recent laboratory developments have advanced our understanding of arenavirus structure and biology, and new vistas have been opened into arenavirus prevalence and distribution as a result of the application of deep sequencing protocols applied to this interesting group of agents. The application of new approaches to antivirals and vaccines targeting arenaviruses may provide a useful model for many localized viral diseases where the knowledge but not the financial incentive for control or eradication exists.

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Acknowledgments

INTRODUCTION AND HISTORY

Basic Features of the *Reoviridae*

Members of the *Reoviridae* form nonenveloped virions composed of 1 to 3 concentric protein shells. The genomes of these viruses consist of 9 to 12 discrete segments of double-stranded RNA (dsRNA). Much of what is known about the *Reoviridae* comes from studies of orbiviruses, orthoreoviruses, and rotaviruses. Although these and other *Reoviridae* members display important structural and biological differences, there are several properties held in common across the family.

The dsRNA gene segments of the *Reoviridae* viruses are not thought to leave the inner capsid particle. Enzymes that catalyze RNA transcription, capping, and replication are contained within the inner capsid, which serves as the site for the RNA synthetic activities. The messenger RNA (mRNA) transcripts are capped but not polyadenylated. The infectious cycle is entirely cytoplasmic and occurs for the most part in neooorganelles that are termed viral factories, viral inclusion bodies, or viroplasms. These viruses are sensed by the innate immune system and activate signaling cascades that induce an antiviral state or evoke apoptosis.

Discovery of Mammalian Reoviruses

Reoviruses were first isolated from stool specimens of children during the 1950s by Albert Sabin, Leon Rosen, and their colleagues.^{412,423} Reovirus is an acronym for respiratory enteric orphan virus; infections of humans by reovirus usually involve the respiratory and intestinal tracts with minimal or no associated disease symptoms (therefore the virus is an “orphan”). In the early 1960s, Peter Gomatos and Igor Tamm noticed that the inclusion bodies in reovirus-infected cells fluoresce greenish-yellow when stained with acridine orange, whereas single-stranded RNA (ssRNA) fluoresces orange.¹⁹⁸ Using chemical

TABLE 44.1 Reoviridae Viruses

Genus	Number of gene segments	Hosts
Subfamily Sedoreovirinae		
<i>Cardoreovirus</i>	12	Crustaceans
<i>Mimoreovirus</i>	11	Marine protists
<i>Orbivirus</i>	10	Mammals, birds, arthropods
<i>Phytoreovirus</i>	12	Plants, insects
<i>Rotavirus</i>	11	Mammals, birds
<i>Seadornavirus</i>	12	Humans, insects
Subfamily Spinareovirinae		
<i>Aquareovirus</i>	11	Fish, mollusks
<i>Coltivirus</i>	12	Mammals, arthropods
<i>Cypovirus</i>	10	Insects
<i>Dinovernavirus</i>	9	Insects
<i>Fijivirus</i>	10	Plants, insects
<i>Idnoreovirus</i>	10	Insects
<i>Mycoreovirus</i>	11–12	Fungi
<i>Orthoreovirus</i>	10	Mammals, birds, reptiles
<i>Oryzavirus</i>	10	Plants, insects

and physical studies, the viral genome was demonstrated to consist of dsRNA.^{40,197,347} Reoviruses were the first dsRNA viruses to be described.

The *Reoviridae* now includes 15 genera of dsRNA viruses that infect a wide variety of plants, animals, fungi, and protozoa (Table 44.1). The mammalian orthoreoviruses are the type species of the *Orthoreovirus* genus, which also contains viruses that infect birds and reptiles. The term “reovirus” refers to the mammalian orthoreoviruses and will be used as such in this chapter.

Pathogenic Reoviridae Viruses

Some members of the *Reoviridae* are pathogenic. Rotaviruses are responsible for gastroenteritis in animals and humans; they cause a large proportion of childhood gastroenteritis and are a major cause of infant illness and death in the developing world (see Chapter 45). Orbiviruses are transmitted by arthropod vectors and replicate in both mammalian and arthropod hosts (see Chapter 46). The best-studied orbivirus is bluetongue virus, an economically significant pathogen of sheep and cows named for a symptom affecting sick animals. Coltiviruses are also transmitted by arthropod vectors, and the prototype member, Colorado tick fever virus, can cause neurologic disease in humans. Aquareoviruses infect fish and mollusks. Fusogenic orthoreoviruses appear to cause pulmonary disease in humans.

Reovirus as a Model for Studies of dsRNA Virus Replication and Pathogenesis

Reoviruses are useful experimental models for studies of viral replication and pathogenesis. Studies of reovirus RNA synthesis were the first to describe the methylated cap structure that modifies most eukaryotic mRNA molecules and is required for normal translation;^{178–180} this work was done using RNAs synthesized *in vitro* from reovirus cores. Genetic approaches used to study the pathogenesis of reovirus in mouse models of infection and disease led to the discovery that phenotypic differences in

specific steps in the virus–host encounter segregate with specific gene segments.^{254,498,515} Reovirus was the first virus shown to traverse the intestinal mucosa by transcytosis across microfold (M) cells⁵³⁴ and undergo proteolytic disassembly catalyzed by endosomal cathepsins.¹⁵⁶ More recent developments have led to the deployment of reovirus as an oncolytic adjunct to cancer therapy^{475,493} and the potential use of reovirus as a vaccine vector through the application of plasmid-based reverse genetics.²⁶¹

TAXONOMY

dsRNA Viruses

There are nine taxonomic families of dsRNA viruses: the *Birnaviridae*, *Chrysovriidae*, *Cystoviridae*, *Endornaviridae*, *Hypoviridae*, *Partitiviridae*, *Picobirnaviridae*, *Reoviridae*, and *Totiviridae*. The *Birnaviridae*, *Picobirnaviridae*, and *Reoviridae* infect vertebrates; the *Picobirnaviridae* and *Reoviridae* infect mammals, including humans. The nine families are distinguished by genome organization, coding strategy, particle structure, and host range, among other properties. Differences in RNA-dependent RNA polymerase (RdRp) sequences suggest independent evolutionary lineages of dsRNA viruses from positive-sense RNA virus ancestors.²⁶⁷ Similarities beyond the existence of dsRNA genomes also suggest common ancestry, most notably in the organization of the capsid components. Interestingly, not all dsRNA viruses encode capsids. Members of the *Hypoviridae* transfer their genomes vertically during propagation of their fungal hosts.¹²⁶

Family Reoviridae

The *Reoviridae* is the largest and most diverse family of dsRNA viruses. The viral particles display icosahedral symmetry with a diameter of 65 to 80 nm. The protein capsid is organized as one, two, or three concentric capsid layers, which surround the dsRNA segments of the viral genome. Members of some genera have filamentous attachment proteins, but most do not. The viral RNA species are usually monocistronic, although some segments have two or three in-frame initiation codons that lead to expression of additional open-reading frames (ORFs). Alternative out of frame ORFs or nonoverlapping ORFs are occasionally present. Proteins are encoded by only one strand of each duplex (the mRNA species).

The 15 genera of the *Reoviridae* are divided into two groups based on particle morphology (Table 44.1). The *Sedoreovirinae* subfamily includes genera containing “smooth” viruses that are almost spherical in appearance, including *Cardoreovirus*, *Mimoreovirus*, *Orbivirus*, *Phytoreovirus*, *Rotavirus*, and *Seadornavirus*. The *Spinareovirinae* subfamily includes genera containing viruses with large “spikes” or “turrets” at the 12 icosahedral vertices of the virus or core particle, including *Aquareovirus*, *Coltivirus*, *Cypovirus*, *Dinovernavirus*, *Fijivirus*, *Idnoreovirus*, *Mycoreovirus*, *Orthoreovirus*, and *Oryzavirus*. The overall fold of several of the major inner and outer capsid proteins of *Reoviridae* members is similar, despite the absence of primary sequence identity.

The inner protein layer of the *Sedoreovirinae* (smooth) viruses has T=2 symmetry and is relatively fragile. This structure is surrounded by an additional protein layer, which has T=13 symmetry, to form the transcriptionally active core particle. These double-layered particles are surrounded by an

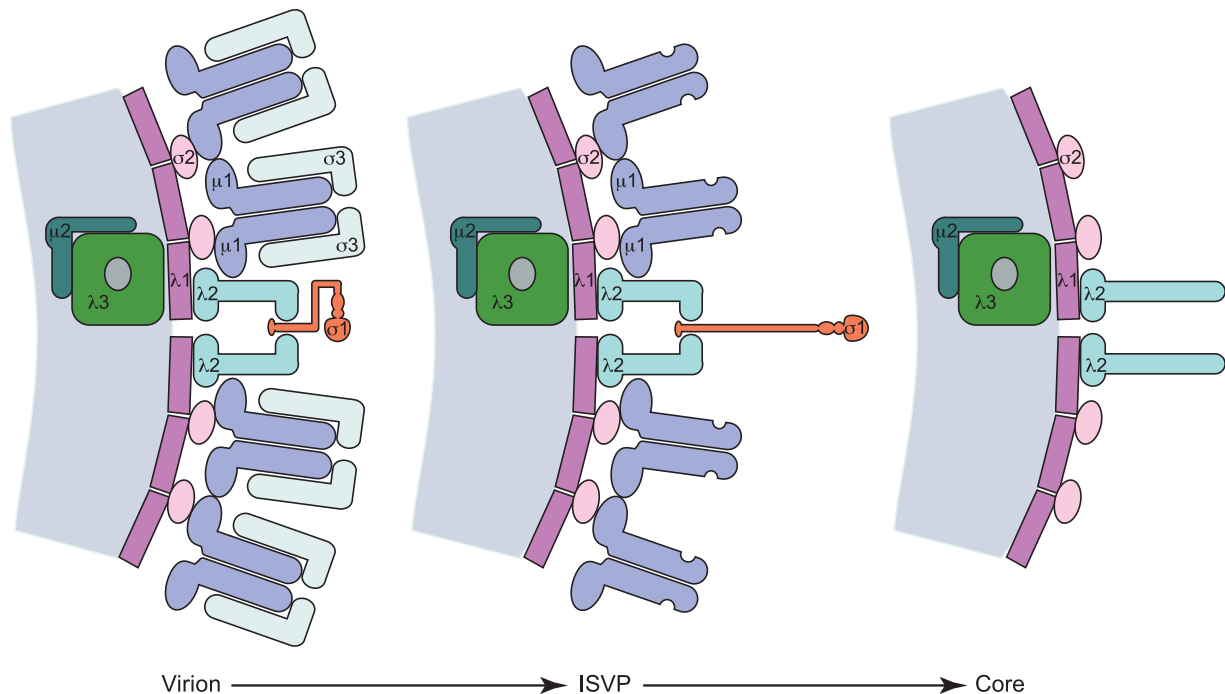


FIGURE 44.1. Schematic of reovirus particles with protein composition. Diagram of a cross-section from virions, infectious subviral particles (ISVPs), and core particles showing the arrangement of the viral structural proteins in the double-layered shells of virions, the formation of ISVPs by removal of $\sigma 3$, cleavage of $\mu 1$, and rearrangement of $\sigma 1$; and the conversion to core particles by removal of the $\mu 1$ fragments, loss of $\sigma 1$, and opening of the $\lambda 2$ turret.

outer-capsid shell, giving rise to triple-layered particles. In contrast, the transcriptionally active core particle of the *Spinareovirinae* (turreted) viruses appears to contain only a single complete capsid layer, which has been interpreted as having T=1 or T=2 symmetry, to which the spikes are attached. Despite these different interpretations, the structure of the innermost complete capsid layer is almost identical for members of both the *Sedoreovirinae* and *Spinareovirinae*. In most cases, the core is surrounded by an additional protein layer with T=13 symmetry that forms the outer capsid, which is penetrated by the spikes that arise from the core surface. These virus particles are therefore usually regarded as double shelled (Fig. 44.1).

The innermost protein layer of *Reoviridae* viruses has an internal diameter of approximately 50 to 60 nm surrounding the 9 to 12 linear dsRNA gene segments. In the *Sedoreovirinae* viruses, the enzymatically active proteins of the virion, RdRp, NTPase, guanylyltransferase, and transmethylease, are also situated within this central structure attached to the inner surface at the fivefold symmetry axes. In the *Spinareovirinae* viruses, some of these enzymes form the turrets on the surface of the core. These hollow projections also act as conduits for the exit of nascent mRNAs synthesized by the core-associated enzymes.

Genus *Orthoreovirus*

The genus *Orthoreovirus* contains the nonfusogenic mammalian orthoreoviruses and the fusogenic orthoreoviruses, which infect a variety of vertebrate hosts. Common features of the members of the genus *Orthoreovirus* include: (1) two protein shells with core spikes at the icosahedral vertices; (2) 10 dsRNA

segments including three large (L), three medium (M), and four small (S) size-class RNA gene segments; (3) three λ , three μ , and four σ primary translation products; (4) additional small gene products encoded by a polycistronic *S1* gene segment; and (5) common serologic characteristics. Related members of the genus *Orthoreovirus* can exchange gene segments following co-infection of cells, producing viral progeny termed “reassortant viruses” containing mixtures of gene segments derived from both parents. The capacity of two strains to produce viable reassortant progeny can serve as the basis for designating species of virus within the genus.^{153,322} Nonfusogenic isolates can exchange gene segments with each other, as can fusogenic isolates, but reassortment between nonfusogenic and fusogenic viruses has not been reported.

Mammalian orthoreovirus was the first species of the family *Reoviridae* to be isolated and identified.⁴²³ The prefix “ortho-” was added to the name of the initial isolates (orthoreoviruses) and the corresponding genus (*Orthoreovirus*) to distinguish them from other members of the family, which also could be called “reoviruses.” However, in common usage the term “reovirus” refers to the mammalian orthoreoviruses.

Nonfusogenic Orthoreoviruses

Reoviruses have a wide geographic distribution, and virtually all mammals, including humans, serve as hosts for infection. Based on sequence analysis of reovirus strains isolated from a variety of mammalian hosts, there is no evidence that any of the gene segments confer host range restriction.^{88,510} Because of their near ubiquity, reoviruses are used as a marker for mammalian fecal contamination of water sources.³¹⁴ Human infections

with reovirus are common, with most children demonstrating serologic evidence of infection by the age of 5 years.⁴⁸² However, reovirus is rarely associated with disease, except in the very young. There is evidence suggesting an association between reovirus and infantile biliary atresia,^{393,499} but a causal link has not been demonstrated. Newborn mice are exquisitely sensitive to reovirus infection and have been used as the preferred experimental model for studies of reovirus pathogenesis.⁵¹⁰

Three mammalian reovirus serotypes have been identified by classical neutralization and hemagglutination-inhibition (HAI) tests. Four virus strains isolated from children in the 1950s—type 1 Lang (T1L), type 2 Jones (T2J), type 3 Abney (T3A), and type 3 Dearing (T3D)—are used most frequently as prototype strains.⁴²³ A distinct reovirus strain was isolated from a mouse in the Cameroon¹⁸ and may represent a fourth mammalian reovirus serotype. Nucleotide sequences indicate that nonfusogenic mammalian isolates are a distinct phylogenetic group within the genus *Orthoreovirus*.¹⁵³ General features of the nonfusogenic mammalian orthoreoviruses are provided in Table 44.2.

Fusogenic Orthoreoviruses

The fusogenic orthoreoviruses infect birds, mammals, and reptiles. In contrast to the nonfusogenic orthoreoviruses, fusogenic reoviruses induce the formation of large, multinucleated syncytia.¹²⁷ Fusion activity is mediated by virus-encoded, fusion-associated small transmembrane (FAST) proteins, which are not capsid components but instead expressed on the surface of infected cells.⁴⁵³ FAST proteins are small (<20 kDa), basic, acylated proteins that induce fusion in transfected cells in the absence of other viral proteins.⁶² Fusogenic members of the related genus, *Aquareovirus*, also encode FAST proteins.³⁸⁶ Extensive syncytium formation results in apoptosis and enhanced release of infectious virions.⁴²⁶ Expression of the p14 FAST protein by a recombinant vesicular stomatitis virus increases neuropathology.⁶⁹ The FAST proteins therefore likely contribute to the natural pathogenicity of the fusogenic orthoreoviruses. Other than this fusion activity, the replication strategies of fusogenic and nonfusogenic orthoreoviruses are similar.⁴²

The fusogenic orthoreoviruses are divided into four species: avian reovirus (ARV), Nelson Bay reovirus (NBV), reptilian reovirus (RRV), and baboon reovirus (BRV).⁸⁸ ARVs infect a variety of avian hosts and are important pathogens in the poultry industry, causing gastroenteritis, hepatitis, malabsorption, myocarditis, and pneumonia.²⁴⁹ Birds that survive acute systemic infection can develop joint and tendon inflammation that resembles the pathology of human rheumatoid arthritis. RRVs have been isolated from numerous reptile species, and infected animals often develop pneumonia and neurologic symptoms.⁵¹⁹ BRV was isolated from baboons with meningoencephalomyelitis in a colony in Texas.²⁸⁰ Therefore, it is an example of a fusogenic mammalian orthoreovirus. BRV virions, like those of the aquareoviruses, lack the classical sigma-class fiber protein used for cell adhesion by other reoviruses.⁵³⁷ NBV is the prototype orthoreovirus species isolated from bats; it is more closely related to ARV than to BRV, the other fusogenic mammalian orthoreovirus species.¹²⁷

Additional fusogenic bat reoviruses have been isolated,³⁸¹ one of which represents a new species that also lacks a sigma-class fiber attachment protein.⁴⁸⁷ Three fusogenic reoviruses

TABLE 44.2 General Features of Nonfusogenic Mammalian Orthoreoviruses

Genome

Double-stranded RNA (dsRNA)
Ten gene segments in three size classes (L, M, S)
Total size ~ 23,500 base pairs
Gene segments each encode one or two proteins.
Gene segments are transcribed into full-length mRNAs.
Plus strands of gene segments have 5' caps.
Nontranslated regions at segment termini are short.
Gene segments can undergo reassortment if two or more strains infect a single cell (antigenic shift).

Particles

Nonenveloped
Spherical with icosahedral symmetry
Total diameter ~ 85 nm (excluding $\sigma 1$ fibers)
Two concentric protein capsids: outer-capsid subunits in T = 13 *laevo* lattice and arrangement of inner-capsid subunits in T = 1 lattice
Eight structural proteins: four proteins in the outer capsid ($\lambda 2$, $\mu 1$, $\sigma 1$, and $\sigma 3$) and five proteins in the core ($\lambda 1$, $\lambda 2$, $\lambda 3$, $\mu 2$, and $\sigma 2$)
Subviral particles (ISVPs and cores) can be generated from fully intact particles (virions) by proteolysis.
Protein $\lambda 2$ forms pentamers that protrude from the core surface.

Replication

Occurs in the cytoplasm of host cells
Viral attachment protein $\sigma 1$ binds to two cell-surface receptors, sialylated glycans and junctional adhesion molecule-A.
Proteolysis of outer-capsid proteins $\sigma 3$ and $\mu 1$ is required for infection and can occur either extracellularly or in the endocytic compartment.
Genomic dsRNA does not exit the viral core.
Transcription and capping of viral mRNAs occur within cores; both processes are catalyzed by particle-associated enzymes.
Single-stranded, positive-sense RNAs are assorted into progeny cores.
Minus-strand synthesis occurs within assembling particles.
Mature virions are released from infected cells by mechanisms that are not well understood.
Many reovirus strains induce apoptosis of host cells.

isolated from humans with acute respiratory infections also may be of bat origin.^{96–98} These viruses appear capable of human-to-human transmission, raising concern that fusogenic bat reoviruses may be a potential source of emerging infections in humans.

PARTICLES

Particle Function

Reovirus virions are spherical, nonenveloped particles approximately 85 nm in diameter as assessed by cryoelectron microscopy (cryo-EM) image reconstructions¹⁵¹ (Fig. 44.2). Virions are formed by two concentric capsid protein shells that surround and protect the dsRNA genome. The inner-capsid shell or core particle encloses the genome, which is present in a

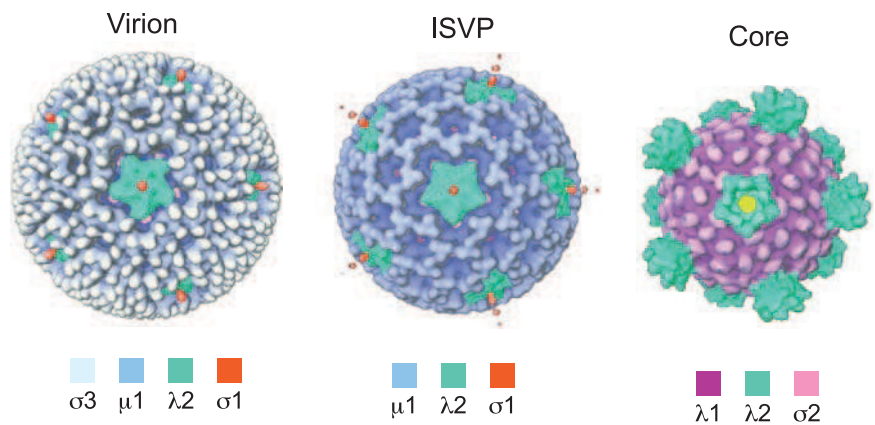


FIGURE 44.2. Cryo-EM image reconstructions of reovirus particles. Surface-shaded representations of cryo-EM image reconstructions of reovirus virions, infectious subviral particles (ISVPs), and cores viewed along a fivefold axis of symmetry. The color coding is based on radial density and roughly approximates the individual capsid proteins as depicted in the legend. *Yellow* corresponds to the interior of the particle. (Adapted from Dryden KA, Wang G, Yeager M, et al. Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J Cell Biol* 1993;122:1023–1041.)

condensed liquid crystalline form.³⁹⁴ The outer-capsid shell surrounds the core particle, which protrudes radially into the outer-capsid layer at each of the fivefold axes of symmetry.¹⁵¹

Several different morphotypes of reovirus particles are found within infected cells or can be generated *in vitro*. These include genome-containing virions, infectious or intermediate subviral particles (ISVPs), and core particles (Figs. 44.1 and 44.2) as well as their genome-lacking counterparts. In addition, less well-defined particles have been purified from infected cells and are characterized by the capacity to transcribe the genome *in vitro* (“transcriptase” particles) or synthesize a negative-stranded RNA copy of particle-associated viral mRNA (“replicase” particles).^{338,557} Physical properties of virions, ISVPs, and cores are described in Table 44.3.

Virions

Reovirus virions are relatively stable in the environment^{198,208} and maintain infectivity for years when stored below 4°C.⁴⁴ Large quantities of infectious virions can be purified from infected mouse L929 cells by Freon extraction and centrifugation in CsCl gradients.⁴⁴

Virions are composed of a genome-containing inner T=1 icosahedral core surrounded by a T=13 laevo outer-capsid layer (Fig. 44.2). The outer capsid consists of 200 heterohexameric complexes of the M2-encoded $\mu 1$ (76 kDa, 708 aa) and S4-encoded $\sigma 3$ (41 kDa, 365 aa) proteins. These complexes form a fenestrated shell that overlies the core. The outer-capsid shell is perforated at the icosahedral fivefold symmetry axes by turret-like structures formed by pentamers of the L2-encoded

TABLE 44.3 Characteristics of Common Reovirus Particle Forms			
Feature or property	Virion	Infectious subviral particle (ISVP)	Core
Buoyant density in CsCl (g/cm ³)	1.36	1.38	1.43
Sedimentation value (S)	730	630	470
Molecular Weight (MDa)	127	103	49
Particles per mL at 1 OD ₂₆₀ (×10 ¹²)	2.1	2.7	4.4
Diameter (nm)			
By negative-stain electron microscopy	73	64	51
By cryoelectron microscopy	85	80	60
Outer-capsid protein composition			
$\sigma 3$	Present	Absent	Absent
$\sigma 1$	Present	Present as extended conformer	Absent
$\mu 1/\mu 1C$	Present	Present as $\mu 1\delta/\delta$ plus ϕ fragments	Absent
$\lambda 2$	Closed conformer	Closed conformer	Open conformer
Particle-associated oligonucleotides	Present	Variable	Absent
Infectivity	High	High	Low
Transcription			
Initiation and capping	Yes	Yes	Yes
Elongation	No	No	Yes

$\lambda 2$ protein (144 kDa, 1288–1289 aa). The S1-encoded $\sigma 1$ attachment protein (49–51 kDa, 455–470 aa) forms filamentous trimers that protrude from each of the $\lambda 2$ turrets. In addition to encapsidating the 10 dsRNA gene segments, virions also contain short ssRNA oligonucleotides that can constitute up to 25% of the RNA in purified virions.⁴⁴³ Most of these oligonucleotides (70%) are 2- to 9-residue products of abortive transcription and terminate with 5'-GC(U)(A).⁵³⁶ The remainder (approximately 30%) are oligoadenylates that range in length from 2 to 20 residues.⁴⁵⁵ Complete or partial atomic resolution structures are available for $\sigma 1$ trimers,^{92,395} $\sigma 3$ dimers,³⁶⁸ and $\mu 1:\sigma 3$ heterohexamers.²⁸⁹ Cryo-EM image reconstructions of reovirus virions, ISVPs, and cores¹⁵¹ (Fig. 44.2), and an atomic resolution structure of the core³⁹⁴ have been determined.

Infectious Subviral Particles (ISVPs)

Treatment of virions with chymotrypsin or trypsin using controlled conditions *in vitro*⁵⁸ or virion disassembly during infection of cells⁴⁸⁰ or in the murine intestine^{29,54} generates ISVPs (Figs. 44.1 and 44.2). These particles lack $\sigma 3$ but retain $\sigma 1$ and a proteolytically cleaved version of $\mu 1$ (see Replication—Disassembly). The loss of $\sigma 3$ and cleavage of $\mu 1$ imparts a twofold increase in infectivity of ISVPs relative to virions for reovirus strain T1L. In contrast, *in vitro* generation of ISVPs from strain T3D virions leads to a 10-fold decrease in infectivity as a consequence of additional proteolytic cleavage of $\sigma 1$.³⁴⁹ This cleavage is controlled by a sequence polymorphism in $\sigma 1$ at amino acid 249⁸⁷ and likely explains the decreased growth in the intestine and avirulence of T3D after peroral inoculation of newborn mice.⁵³ An additional intermediate particle, the ISVP*, is generated from ISVPs during infection of cells.⁸¹ ISVP*s lack the $\sigma 1$ attachment protein, have an altered conformer of $\mu 1$, and tend to aggregate. In contrast to virions and ISVPs, ISVP*s are transcriptionally active.⁷⁹ ISVP*s are formed following generation of ISVPs and thought to interact with cell membranes leading to membrane penetration and release of the core particle into the cytosol (see Replication—Membrane Penetration).

Core Particles

Like ISVPs, cores can be generated *in vitro* by treating virions with chymotrypsin or trypsin using specific conditions.⁶⁰ Such treatment removes all of the outer-capsid proteins ($\mu 1$, $\sigma 3$, and $\sigma 1$) and exposes prominent turrets formed by pentamers of $\lambda 2$. These turrets protrude from the capsid shell at each of the fivefold symmetry axes (Figs. 44.1 and 44.2) and form hollow cylinders that open to the interior of the particle. In cryo-EM image reconstructions of virions and ISVPs, the cavity within each turret is partially filled by a bulb-like structure that corresponds to a portion of the $\sigma 1$ protein¹⁵¹ (Fig. 44.2). The $\sigma 1$ bulb is held in place by flap domains present on each of the five $\lambda 2$ proteins. In cores, the flap domains of $\lambda 2$ open outwards. The interior surface of the pore formed by the $\lambda 2$ turret possesses guanylyltransferase and methyltransferase activities, which allow the turrets to function as mRNA capping complexes³⁹⁴ (see Replication—Transcription). Cores are approximately 1,000,000-fold less infectious than virions⁸⁴ as a consequence of the loss of the outer-capsid proteins, which are required for virus attachment, entry, and membrane penetration.

The T=1 core shell is formed by 120 copies of the L3-encoded $\lambda 1$ protein (142 kDa, 1275 aa) arranged in parallel

asymmetric dimers (sometimes referred to as T=2). Five copies of $\lambda 1$ (conformer $\lambda 1A$) radiate from the fivefold axes of symmetry. These molecules interdigitate with a second set of five $\lambda 1$ proteins (conformer $\lambda 1B$) to form decamers; 12 decamers form the core shell.³⁹⁴ The $\lambda 1$ shell is stabilized by 150 copies of the S2-encoded $\sigma 2$ protein (47 kDa, 418 aa), which clamps onto $\lambda 1$ at three distinct sites within each icosahedral asymmetric unit of the shell.³⁹⁴ Channels permeate the shell, with those at the fivefold symmetry axes allowing entry of NTP substrates and exit of newly synthesized viral mRNAs.

The reovirus core is described as a “molecular machine” owing to its capacity to synthesize capped viral mRNA transcripts *in vitro* when incubated with suitable substrates. During infection, particles thought to be similar to *in vitro*-generated cores initiate viral transcription after being deposited in the cytoplasm. Core particles contain 12 copies of the L1-encoded $\lambda 3$ polymerase (142 kDa, 1267 aa). Each $\lambda 3$ protein is anchored to three $\lambda 1$ proteins on the inner surface of the core and overlaps with, but is eccentric from, each of the icosahedral symmetry axes.⁵⁵⁰ In addition, cores contain approximately 24 copies of the M1-encoded $\mu 2$ protein (83 kDa, 736 aa),¹¹⁴ a polymerase co-factor. The precise location of $\mu 2$ within the core particle is not known, but one or two copies of $\mu 2$ are thought to associate with each copy of $\lambda 3$.

Empty Particles (“Top Component” Particles)

Purification of reovirus particles from infected cells *in vitro* yields a substantial proportion of particles that lack genomic dsRNA.⁴⁶³ These empty particles have a lower buoyant density in CsCl gradients and band closer to the top of the gradient in comparison to genome-containing particles, hence the designation “top component”.⁴⁴ Protease treatment of such particles can generate “empty” ISVPs and cores.

Recombinant Particles

Recombinant core-shell particles can be formed by co-expression of the $\lambda 1$ and $\sigma 2$ proteins or the $\lambda 1$, $\lambda 2$, and $\sigma 2$ proteins.⁵³⁵ Purified viral core particles prepared by *in vitro* digestion of virions with chymotrypsin or trypsin can be recoated with outer-capsid proteins $\mu 1$, $\sigma 3$, and $\sigma 1$ prepared using baculovirus expression systems.⁸⁴ Such recoated particles display infectivity similar to native virions and are useful tools for defining the consequences of lethal mutations in outer-capsid proteins on early steps in viral infection (see Genome and Coding Strategies—Genetic Complementation).

GENOME AND CODING STRATEGIES

Physical Characteristics

The reovirus genome consists of 10 discontinuous segments of dsRNA. The [+] and [–] strands of genomic dsRNA are collinear and complementary.^{40,197} The genomic dsRNA has an A-form structure with a right-handed double helix (10 base pairs per turn, 30 Å pitch, nucleotides oriented at 75° to 80° to the long axis).¹⁷

Gene Segments and Nomenclature

The 10 reovirus dsRNA gene segments are present in equimolar amounts in viral particles.⁴⁴⁴ The segments are grouped by size into large (L), medium (M), and small (S) classes according

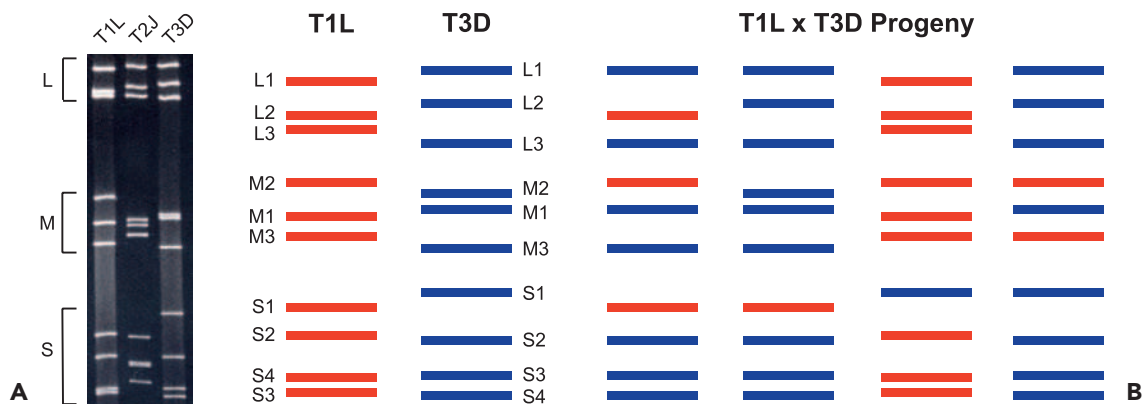


FIGURE 44.3. The segmented reovirus genome. A: Electrophoretic profiles of genomic double-stranded RNA (dsRNA) of prototype reovirus strains type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D). Equal numbers of reovirus particles were resolved by polyacrylamide gel electrophoresis. Viral dsRNA gene segments were visualized following ethidium bromide staining. Gene segment size classes, large (L), medium (M), and small (S), are indicated. **B:** Generation of reovirus reassortant viruses. Co-infection of cells with different reovirus strains produces a collection of progeny viruses containing various combinations of gene segments from each parent. Such viruses are generally termed “reassortant viruses.” Shown here are schematic diagrams of electrophoretic profiles of *T1L* and *T3D* gene segments along with some reassortant progeny. Gene segment identities of both strains are shown.

to their migration in polyacrylamide gels.^{195,330,444,513} There are three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, S4) segments. Homologous gene segments from different reovirus isolates often migrate with different mobilities in polyacrylamide gels despite being of similar or identical length³⁸⁸ (Fig. 44.3). This property allows gene segments from different isolates to be unambiguously distinguished, which is essential for the assignment of gene segments in reassortant viruses to specific parental strains (see Genome and Coding Strategies—Reassortment). The gene segments of prototype strain T3D are numbered according to their relative mobilities in Tris/acetate-buffered gels.⁴⁴¹ Each gene segment encodes at least one protein product (Table 44.4).

Modifications to the Genomic RNA

The [+] strand RNA of each gene segment has a dimethylated cap-1 structure on its 5′ terminus,^{95,179,332} which is added by viral guanylyltransferase and methyltransferase activities present in the λ2 protein.^{106,295} The viral mRNA cap is recognized by translation initiation factor eIF4E^{61,346} and protects the free 5′ ends of the viral mRNA from 5′ exonucleases.¹⁷⁷ The 5′ terminus of the [−] strand RNA of each gene segment has an unblocked diphosphate.^{24,95} The terminal γ-phosphate of the [−] strand is removed by a phosphohydrolase activity (possibly mediated by the μ2 or λ1 protein) within reovirus particles.^{24,51,95,256} The 3′ end of each gene segment has an unblocked hydroxyl group.³³⁰ The viral [+] strand RNA does not contain a polyadenylate tract.

Sequence Features

Full-length sequences of the entire genomes of prototype mammalian orthoreovirus strains T1L, T2J, and T3D as well as those of several fusogenic orthoreovirus isolates have been determined (National Center for Biotechnology Information). The total length of the genome sequence is approximately 23,500 base pairs. The length of individual gene segments varies from 1,196 (T3D S4 gene segment) to 3,916 (T3D L2 gene

segment) nucleotides. Most homologous gene segments of different isolates display little (± 4 base pairs) or no variation in segment length. However, the S1 gene segment varies in length to a greater extent (1,463, 1,440, and 1,416 base pairs for the T1L, T2J, and T3D S1 gene segments, respectively).^{154,350}

Protein-Coding Strategies and Nomenclature

Twelve proteins are translated from the 10 T3D reovirus gene segments (Table 44.4). Eight of these segments encode a single protein, and two encode two proteins each. Eight of the 12 proteins are structural components of virions (λ1, λ2, λ3, μ1, μ2, σ1, σ2, and σ3), and four are nonstructural (μNS, μNSC, σ1s, and σNS). The nonstructural protein μNS was originally designated μ0.⁵⁵⁸ The assignment of individual proteins to specific gene segments was determined by analysis of viral reassortants^{344,345,390,518} and *in vitro* translation of viral mRNAs.³¹⁵

The names of the proteins encoded by each of the gene segments are designated by a Greek symbol that corresponds to large (λ), medium (μ), and small (σ) sizes, which are encoded by gene segments of the L, M, and S classes, respectively. For historical reasons, the numbering of gene segments does not always correspond to the numbering of the proteins (Table 44.4).

During reovirus infection, the [−] strand of each gene segment is transcribed to yield a full-length [+] strand RNA copy that is capped as it is released from the transcribing core particle. The viral mRNAs are not polyadenylated, but they are efficiently translated. ORFs of each gene segment are flanked by short nontranslated regions (NTRs). The AUG start codon for each gene segment is usually the first to be encountered by a scanning ribosome and, in most cases, it is found in an excellent context for translation initiation.²⁶⁹ The S1 gene segment encodes a second protein, σ1s, in an ORF overlapping that of the larger σ1 protein.^{160,241,429} The σ1s protein is translated from the first out-of-frame start codon within the σ1 ORF.³⁴³ Like the S1 gene segment, the M3 gene segment encodes a second protein, which is called μNSC. Translation of μNSC initiates from the second or third in-frame start codon of the

TABLE 44.4 Functions of Reovirus Proteins

Gene segment	Protein	Mass (Da)	Copy number per virion	Function
L1	$\lambda 3$	142,300	12	Minor inner-capsid protein; catalytic subunit of the viral RNA-dependent RNA polymerase; associates with nonstructural protein μ NS
L2	$\lambda 2$	144,000	60	Core spike protein; forms pentameric turret for insertion of attachment protein $\sigma 1$; associates with nonstructural protein μ NS; adds a 5' methylated cap to mRNAs
L3	$\lambda 1$	142,000	120	Major inner-capsid protein; associates with nonstructural protein μ NS; possible RNA helicase; may remove terminal phosphate from newly synthesized mRNA in preparation for capping
M1	$\mu 2$	83,300	24 ^a	Minor inner-capsid protein; probable RNA polymerase subunit; associates with nonstructural protein μ NS; stabilizes microtubules; nonspecific RNA-binding protein; may remove terminal phosphate from newly synthesized mRNA in preparation for capping; interferon antagonist
M2	$\mu 1$	76,300	600	Major outer-capsid protein; important for penetration of endosomal membrane by the viral core; probably regulates late mRNA synthesis from secondary transcriptase particles; influences efficiency of reovirus-induced apoptosis
M3	μ NS	80,000	NS ^b	Nonstructural replication protein; forms cytoplasmic inclusion structures; associates with nonstructural protein σ NS and core proteins $\lambda 1$, $\lambda 2$, $\lambda 3$, $\mu 2$, and $\sigma 2$; first viral protein found in association with viral mRNA
M3	μ NSC	75,000	NS	Nonstructural replication protein; function unknown; dispensable for replication in cell culture
S1	$\sigma 1$	49,200 (T3D) – 51,400 (T1L)	36	Attachment protein that binds to cell-surface glycans and junctional adhesion molecule A (JAM-A); determines virus serotype; determines cell and tissue tropism and route of virus spread in the host
S1	$\sigma 1s$	14,000	NS	Nonstructural replication protein; required for reovirus-induced cell-cycle arrest; required for reovirus hematogenous dissemination
S2	$\sigma 2$	47,200	150	Major inner-capsid protein; associates with nonstructural protein μ NS; binds weakly to dsRNA
S3	σ NS	41,000	NS	Nonstructural replication protein found early in viral inclusions; associates with nonstructural protein μ NS; nonspecific RNA-binding protein with high affinity for single-stranded RNA
S4	$\sigma 3$	41,500	600	Major outer-capsid protein; forms protective cap over $\mu 1$; nonspecific dsRNA-binding protein; influences efficiency of translation; associated with viral mRNA early in replication cycle; inhibits PKR activation

^aStoichiometry not known with certainty.

^bNS, nonstructural, not a component of viral particles.

m3 RNA.³¹⁶ There is no evidence that any reovirus protein is translated from a genomic [–] strand.

Terminal Nontranslated Regions

The reovirus gene segments contain short terminal NTRs (Fig. 44.4). The 5' NTRs range in length from 12 to 32 nucleotides, and their relative sizes may affect their translation efficiency.⁴⁰⁶ The 3' NTRs are a little longer and vary in length from 35 to 83 nucleotides. In general, NTR length of homologous gene segments is conserved between different virus isolates.³¹⁶

The [+] strand of all reovirus gene segments contains the 5' sequence GpCpUpA and the 3' sequence UpCpApUpC.¹⁴ Longer regions of sequence near the RNA termini and extending into the ORFs display less variability between isolates than sequences farther from the ends^{90,202,253} (Fig. 44.4). RNA-folding predictions suggest that sequences near the ends of the reovirus [+] strands can form panhandle structures, which may

be important for certain RNA functions.^{90,286,553} For example, the 5' and 3' NTRs include sequences and structural elements important for RNA packaging.^{405,408,409}

Genetics

Reassortment

Gene segment exchange (or reassortment) among reovirus strains occurs during mixed infections of cultured cells or animal hosts. The progeny of such mixed infections are commonly referred to as reassortant viruses. Many reovirus strains are distinguishable by signature electrophoretic profiles of their dsRNA gene segments in acrylamide gels (Fig. 44.3A). Co-infection of cells with such strains produces a collection of reassortant viruses in which the parental origin of each gene segment can be determined following electrophoresis of viral genomic dsRNA (Fig. 44.3B). Studies of reassortant viruses have been used to assign biological polymorphisms displayed



FIGURE 44.4. Reovirus gene segment coding strategies. The 10 reovirus gene segments demonstrate no significant sequence similarity except for the 5'-terminal four nucleotides ([+] 5'-GCUA-3') and 3'-terminal five nucleotides ([+] 5'-UCAUC-3'), which are invariant among all 10 segments. However, sizable regions of subterminal sequence are conserved among homologous gene segments across individual strains. Each of the reovirus gene segments is monocistronic with the exception of the M3 gene segment, which encodes μ NS and μ NSC in the same reading frame (μ NSC is an N-terminally truncated version of μ NS), and the S1 gene segment, which encodes σ 1 and σ 1s in overlapping reading frames. Nucleotide sequence conservation extends inward from both nontranslated regions (NTRs) into the gene-segment open reading frames and is maintained even at synonymous positions. Only the plus strand is shown here. Sequence ranges are based on strain type 3 Dearing and include the larger of the two reading frames in the S1 gene segment.

by different reovirus strains to specific viral gene segments. Thus, a phenotypic difference between two parental strains can be genetically mapped by screening the reassortant viruses in appropriate assays and correlating expression of the phenotype with a specific parental gene segment.

Reassortment of reovirus gene segments also occurs *in vivo*. Infection of mice perorally with strains T1L and T3D yields reassortant progeny in the stool and at sites of secondary replication.⁵²¹ In at least one case, a reassortant virus arising during such a mixed infection displays virulence properties that differ from both parental strains,⁴⁵¹ suggesting the selection of new mutations *in vivo*. Beyond experimental infection of animals, additional support for the hypothesis that reovirus gene segments reassort in nature is provided by analysis of nucleotide sequences of the L1,²⁷⁷ M1,⁵⁴¹ S1,¹³⁷ S2,⁹⁰ S3,²⁰² and S4²⁵³ gene segments derived from a panel of field-isolate strains isolated in the 1950s and 1960s by Leon Rosen.^{413,414,417} The topologies of phylogenetic trees constructed from protein-coding sequences of these gene segments are distinct, providing convincing evidence that these segments reassort in nature.

Reassortment is not entirely random. In a study of 83 reassortant viruses derived from strains T1L and T3D, statistically significant nonrandom associations of parental alleles were observed in the L1–L2, L1–M1, L1–S1, and L3–S1 gene segment pairs.³⁵² It is possible that these gene segments or their protein products cannot accommodate exchange with gene segments from another strain without the selection of fitness-compromising mutations. Additionally, the majority of progeny viruses resulting from mixed infections retains the genome constellation of the parental strains,¹⁶⁹ suggesting that viral replication is compartmentalized in some way.

Selection of Mutants: “Forward Genetics”

TEMPERATURE-SENSITIVE MUTANTS

Reovirus mutants with reduced capacity to replicate at elevated temperature, usually defined as 39°C or 40°C^{115,169,214} as opposed to 31°C, have been selected using a variety of conditions. Temperature-sensitive mutants mapping to seven of the 10 genetic groups (gene segments) of strain T3D were isolated after chemical mutagenesis with nitrous acid (groups B [L2], D [L1], and E [S3]), nitroso-guanidine (groups C [S2],

F [M3], and G [S4]), or proflavin (group A [M2]).^{169,233} Mutants from the other three groups were isolated from T3D stocks passaged serially at high multiplicity of infection (MOI) (group H [M1]) or from persistently infected cultures (groups I [L3] and J [S1]).^{5,6,8} The genetic groups were assigned to discrete gene segments by reassortant analyses or the capacity to complement temperature-sensitive mutants from other groups.^{344,387,390} Although the prototype isolates of groups F, H, and J have not been confirmed to be temperature-sensitive,¹¹³ a new strain for temperature-sensitive group H (M1 gene segment) has been isolated.¹¹³ Sequence analysis reveals coding changes in mutants from groups A,²¹⁴ C,⁵²⁷ E,⁵²⁶ G,¹²⁰ and H.¹¹³ Reversion of reovirus temperature-sensitive mutants has been documented to occur through a change at the site of the original mutation (true reversion)¹¹⁵ or as a consequence of a mutation in a second gene that suppresses the defect produced by the original mutation (extragenic suppression).^{389,391}

DELETION MUTANTS

Some reovirus isolates lose portions of their genomes during serial high-MOI passage.^{7,70,358,360,434} Deletions in the L1 and L3 gene segments occur most commonly, but L2 and M1 deletions also occur. Analysis of a series of internally deleted M1 gene segments indicates that the minimum sizes for retained 5'- and 3'-terminal regions of message-sense RNA are 132–135 and 182–185 bases, respectively,⁵⁵³ suggesting that these regions contain signals required for RNA synthesis or gene segment packaging. Differences in the capacity of strains T1L and T3D to accumulate deletions segregates with the L2 gene segment, while the M3 gene segment is a determinant of which viral gene segments incur deletions.⁷⁰ Functions of the L2 and M3 gene products λ 2 and μ NS, respectively, in synthesis or packaging of reovirus RNA may influence the generation or amplification of deletion mutations.

CELL-ADAPTED MUTANTS

Although usually cytolytic, reoviruses are capable of establishing persistent infections in many types of cells in culture.¹³⁶ These cultures are maintained by horizontal transmission of virus from cell to cell and can be cured of persistent infection by passage in the presence of reovirus-specific antibodies. During maintenance of L-cell cultures persistently infected with

reovirus, mutations are selected in both cells and viruses.^{4,139} Mutant cells selected during persistent infection have alterations in the expression of cathepsin L and do not support disassembly of wild-type virus.²² These cells in turn select mutant viruses (termed PI viruses) that have gained the capacity to bypass cellular blocks to infection by virtue of mutations in outer-capsid proteins $\sigma 1$ ⁵³² and $\sigma 3$,⁵²⁴ which promote more efficient disassembly. Studies of the virus-cell co-evolution that occurs during persistent reovirus infections have thus enhanced an understanding of reovirus entry (see Replication—Disassembly).

Nonsialic acid-binding reovirus strains have been adapted to growth in murine erythroleukemia (MEL) cells by serial passage.⁹¹ These variants have single amino-acid substitutions in the sialic acid-binding domain of $\sigma 1$ protein and have gained the capacity to bind sialylated glycans.⁹¹ Serial passage also has been used to select reovirus mutants for resistance to ammonium chloride,⁹⁹ which inhibits endosomal acidification, and E64,¹⁵⁷ which inhibits cysteine proteases. These mutants have alterations in $\sigma 3$ protein that enhance kinetics of viral disassembly. Other reovirus mutants have been selected during chronic infections of immunocompromised mice; these viruses are adapted to replicate more efficiently in the organs from which they were isolated.²⁰⁶

MUTANTS RESISTANT TO DENATURANTS

Treatment of reovirus with harsh denaturants (e.g., heat, extremes of pH, ethanol, phenol, or SDS) results in substantial losses in infectivity.¹⁴⁸ Strain-specific differences in susceptibility to inactivation by heat and ethanol have been mapped using reassortant viruses to the $\sigma 3$ -encoding S4 gene and $\mu 1$ -encoding M2 gene,¹⁴⁹ respectively. Concordantly, reovirus mutants selected for resistance to ethanol have mutations in the M2 gene.^{226,522} These mutants display increased thermostability and a decreased capacity to penetrate host-cell membranes.²²⁶

Directed Mutagenesis of the Genome: “Reverse Genetics”

The ability to engineer viruses that contain specific sequence modifications was first accomplished for mammalian reoviruses using a strategy involving message-sense RNA transcribed *in vitro* and a helper virus.⁴¹⁰ This system has been used to recover infectious particles containing gene segments derived from cloned DNA copies of single reovirus gene segments.^{405,407} Development of a plasmid-based reverse genetics system for members of the *Birnaviridae* family,⁵³⁹ which contain two genomic dsRNA segments, suggested that delivery of viral positive-strand RNA alone could launch successful production of viral progeny for a dsRNA virus. This approach proved possible with the development of an entirely plasmid-based reverse genetics system for reovirus in which viable viruses are generated from a set of 10 cloned cDNAs representing the complete viral genome²⁶¹ (Fig. 44.5). Gene segment cDNAs corresponding to strains T1L²⁶⁴ and T3D²⁶¹ were introduced into plasmids at sites flanked by the promoter sequence for T7 RNA polymerase and the hepatitis delta virus (HDV) ribozyme (Fig. 44.5A). Neither helper virus nor co-expression of viral replication proteins is required for recovery of infectious virus.

Reovirus can be recovered from cells in which T7 RNA polymerase is delivered transiently by infection with a recombinant vaccinia virus²³⁶ or from cells that constitutively express

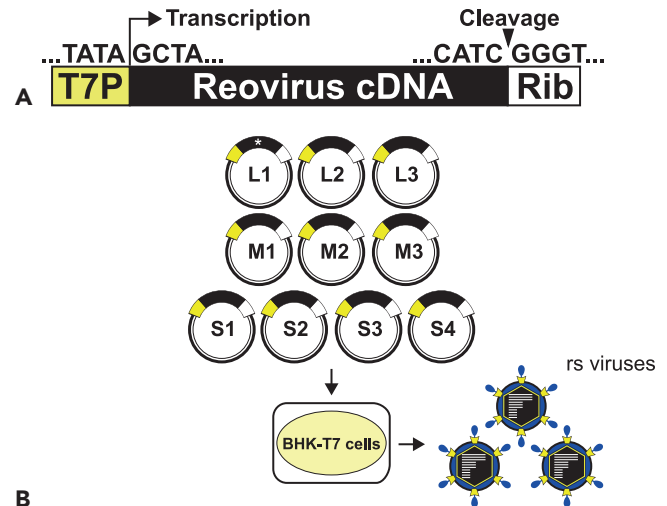


FIGURE 44.5. Recovery of reovirus from cDNA. **A:** Prototype reovirus gene segment cDNA in plasmid. Cloned cDNAs representing the 10 reovirus dsRNA gene segments are flanked by the bacteriophage T7 RNA polymerase promoter (T7P) and hepatitis delta virus ribozyme (Rib) sequences. **B:** The 10 plasmid constructs are transfected into BHK cells expressing T7 RNA polymerase. Transcription within the cells generates the 10 different viral mRNAs, each with its authentic 5' end. Self-cleavage by the ribozyme generates authentic viral 3' ends. These mRNAs are translated and produce all 12 reovirus proteins, which can then direct a productive infectious cycle. Following 1 to 2 days of incubation, transfected cells are lysed by freeze-thaw, and viable virus is isolated by a plaque assay using L929 cells. An engineered silent substitution in one of the cloned cDNAs (indicated by an asterisk in the L1 gene segment) creates a unique molecular signature to confirm the plasmid origin of the recombinant strain (rs) viruses.

the enzyme⁷³ (Fig. 44.5B). Since T7 RNA polymerase initiates transcription at a defined guanosine residue³²⁹ and all reovirus RNAs contain the nucleotide sequence, GCUA, at the 5' terminus of the message-sense strand, T7 RNA polymerase produces transcripts with native reovirus 5' termini. Self-cleavage of the HDV ribozyme generates RNAs with native 3' termini.⁴⁰⁷ In cells transfected with plasmids encoding all 10 reovirus gene segments, viral gene products join with reovirus RNAs to form viral replication complexes, which mediate negative-strand synthesis and ultimately generate infectious particles. Inclusion of expression plasmids encoding reovirus proteins does not enhance recovery of recombinant reoviruses.⁵⁷ However, the efficiency of virus recovery is increased by using baby hamster kidney cells constitutively expressing T7 RNA polymerase⁷³ and reducing the plasmid number from 10 to four by combining reovirus gene segment cDNAs into multicistronic vectors.²⁶⁴

Plasmid-derived wild-type virus recapitulates properties of native virus in all cell-culture and *in vivo* models of reovirus infection studied to date.^{261,264} This reverse genetics approach has been used to introduce changes into the virion capsid and replication proteins to define the roles of individual amino acids, functional domains, and structural motifs in receptor utilization,^{259,395} virion disassembly,^{147,261} membrane penetration,^{121,123,430} replication biochemistry,³⁶⁹ inclusion

formation,^{239,263} interferon (IFN) expression,^{235,556} apoptosis induction,^{121,123} viral growth and spread *in vivo*,^{55,56,261} neurovirulence,^{121,123} and myocardial injury.²³⁵ The same strategy also can be exploited to engineer recombinant reoviruses for vaccine and oncolytic applications.

Genetic Complementation

A variety of complementation strategies have been developed to enable studies of viral mutations that would otherwise compromise viral viability. Stable expression in cultured cells of wild-type forms of temperature-sensitive mutant proteins allows replication of the corresponding temperature-sensitive mutant virus at nonpermissive temperature. This approach has been used to complement the replication of tsE (which segregates with the σ NS-encoding S3 gene segment) and tsH (which segregates with the μ 2-encoding M1 gene segment) by stable expression of the σ NS³³ and μ 2⁵⁵⁴ proteins, respectively. These complementing cell lines and temperature-sensitive mutant viruses could be used to define sequences in σ NS and μ 2 required for viral replication.

Recombinant reovirus outer-capsid proteins μ 1, σ 1, and σ 3 can bind and “recoat” purified subviral particles (ISVPs or cores) *in vitro* to generate infectious virion-like particles.^{83,84,244} These recoated particles have been used to identify sequences in μ 1, σ 1, and σ 3 proteins that function during cell entry and disassembly.^{81,84,243,531}

Reovirus replication is blocked in cell lines stably expressing small interfering RNAs (siRNAs) specific for reovirus transcripts.²⁶² RNAi-mediated inhibition of viral gene expression can be complemented by vectored expression of viral genes in which the siRNA target sequence has been altered to prevent siRNA-mediated degradation. This approach has been used to identify sequences in μ 2, μ NS, and σ NS proteins that function in viral inclusion formation and RNA synthesis.^{16,77,262,263} Limitations of this strategy include the efficiency of viral RNA knockdown and transfection efficiency of the complementation plasmids.

REPLICATION

Attachment

Reovirus Attachment Protein σ 1

The S1-encoded σ 1 protein (49–51 kDa, 455–470 aa) mediates viral binding to cellular receptors^{27,89,279,514} and influences target-cell selection in the infected host.^{28,515,517} It is a homotrimer present in 36 copies per virion.^{114,478} Structural information is available for the σ 1 protein of prototype type 3 strain, T3D. The 455 amino acids of T3D σ 1 fold into a filamentous trimer approximately 480 Å long and 90 Å wide at its broadest point, with a globular C-terminal head, a central body, and a slender N-terminal tail^{92,174,395} (Fig. 44.6A). Residues 310 to 455 comprise the head, which is constructed from two Greek-key motifs that assemble into an eight-stranded β -barrel.^{92,431} With the exception of the loop connecting β -strands D and E (D-E loop), which contains a 3_{10} helix, loops connecting individual strands of the β -barrel are very short. Residues 170 to 309 form the body domain, which is constructed primarily from repeating units of two antiparallel β -strands connected by short loops. Three such units assemble into a triple β -spiral, which is a motif observed to date only in viral fibers, including the

adenovirus fiber,⁵⁰⁵ bacteriophage PRD1 P5,³²¹ avian reovirus attachment protein σ C,²⁰³ and mammalian reovirus T3D σ 1.^{92,395} The body domain features four β -spiral repeats at the N-terminus (β 1– β 4, residues 170–235), a short α -helical coiled-coil (residues 236–251), and three additional β -spiral repeats (β 5– β 7, residues 252–309) at the C-terminus (Fig. 44.6A). The short α -helical coiled-coil corresponds to a narrowing in the body domain in a composite image of negative-stained electron micrographs of purified σ 1.¹⁷⁴ Residues 1 to 160 form the tail domain, the structure of which is unknown. However, a repeating heptad sequence motif suggests that this region forms an amphipathic α -helix, which likely assembles into an α -helical coiled-coil in the trimer.^{92,350} The extreme N-terminus of σ 1 does not contain any obvious sequence motifs. It is hydrophobic and anchors the protein into the pentameric turret formed by λ 2 in the reovirus virion.^{151,176,283} This symmetry mismatch (a trimer of σ 1 engaged to a pentamer of λ 2) suggests an interaction of limited strength, which may aid in σ 1 release during viral disassembly.⁴⁷²

The σ 1 molecule possesses discrete regions of flexibility^{92,174,395} (Fig. 44.6A, arrows). One site of substantial flexibility in T3D σ 1 is contributed by a four-residue insertion between the two most C-terminal β -spiral repeats (β 6 and β 7).^{78,92} Sequence alignments suggest that σ 1 of reovirus prototype strains T1L and T2J each contain a six-residue insertion at the same position.⁹² This insertion appears to correspond to a region of flexibility observed just below the σ 1 head in electron micrographic images.¹⁷⁴ A second region of flexibility is observed at the midpoint of σ 1 and corresponds to the transition between the predicted α -helical coiled-coil region of the tail and the β -spiral-containing body. A final region of flexibility close to the N-terminus likely represents the virion insertion domain.^{92,174,350}

Glycan Binding by σ 1

Reoviruses display the capacity to agglutinate erythrocytes of several mammalian species.²⁸⁴ For type 3 reoviruses, hemagglutination is mediated by interactions of the σ 1 protein with terminal α -linked sialic acid residues on several glycosylated erythrocyte proteins such as glycophorin A.^{183,376} Strain T3D exhibits a reduced capacity to agglutinate erythrocytes following treatment with neuraminidase, which removes terminal sialic acid moieties.¹⁸³ Preincubation of L cells with neuraminidase or virus with sialosides also significantly diminishes T3D binding.^{184,375} Sialic acid residues linked in α 2,3 or α 2,6 configurations effectively block type 3 reovirus binding to L cells.³⁷⁵ Reovirus T3D binds to sialoglycophorin, but not to asialoglycophorin, with an avidity of approximately 5×10^{-9} M,²⁶ which is a property mediated by the σ 1 protein.⁸⁹

Binding to sialic acid is essential for reovirus infection of some types of cultured cells, such as MEL cells.^{91,422} Sequence polymorphisms within the σ 1 body domain at residues 198, 202, and 204 determine the capacity of field-isolate reovirus strains to bind sialic acid and infect MEL cells.^{138,421} Furthermore, nonsialic acid-binding type 3 variants can be adapted to growth in MEL cells during serial passage. These variants have gained the capacity to bind sialic acid and contain sequence changes within the polymorphic region of the σ 1 body. In addition, sialic acid binding also serves an important role in reovirus tropism and pathogenesis *in vivo*. A sialic acid-binding strain of reovirus, but not a nonsialic acid-binding strain, causes bile

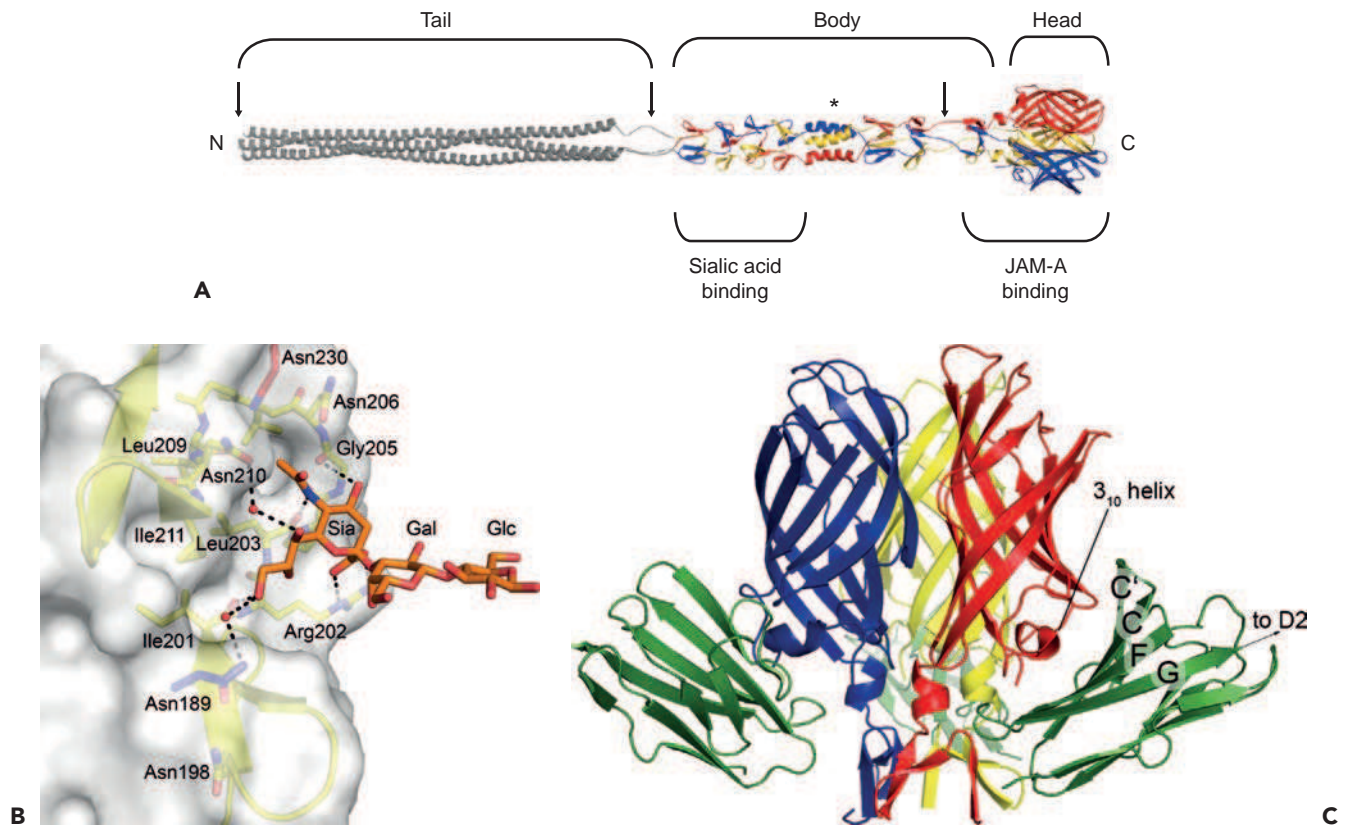


FIGURE 44.6. Structure of reovirus $\sigma 1$ in complex with its receptors sialic acid and junctional adhesion molecule-A.

A: Full-length model of $\sigma 1$. A full-length depiction of $\sigma 1$ was generated by modeling a predicted trimeric α -helical coiled coil and linking it to the N-terminus of the largest crystallized fragment of $\sigma 1$ (residues 170–455 of strain type 3 Dearing [T3D] $\sigma 1$).³⁹⁵ The three monomers of the crystallized fragment are shown in *blue*, *red*, and *yellow*; the model is shown in *grey*. Tail, body, and head regions are indicated. Regions of flexibility are marked by *arrows*. A sequence polymorphism that confers susceptibility to cleavage by intestinal proteases is indicated with an *asterisk*. The approximate locations of binding sites for sialic acid and junctional adhesion molecule-A (JAM-A) are shown. Amino (N)- and carboxy (C)-termini are indicated. **B:** The T3D $\sigma 1$ protein in complex with α -2,3-sialyllactose.³⁹⁵ Residues in the binding region are drawn in *stick representation*, while the remainder of the protein is shown as a *ribbon drawing*. The side chain of Asn189 (*blue*) is contributed by a neighboring $\sigma 1$ monomer. The α -2,3-sialyllactose is shown in *stick representation*, with carbons in *orange*, oxygens in *red*, and nitrogens in *blue*. The sugar moieties are labeled Sia (sialic acid), Gal (galactose), and Glc (glucose). Bridging waters are shown as *orange spheres*. Hydrogen bonds and salt bridges are represented with *broken lines*. **C:** The T3D $\sigma 1$ protein in complex with JAM-A. *Ribbon drawing* of a complex formed between the trimeric $\sigma 1$ head domain and monomeric JAM-A D1, viewed perpendicular to the threefold symmetry axis.²⁵⁹ Beta-strands C, C', F, and G of JAM-A D1 are indicated on the right. Many of the residues in $\sigma 1$ that engage JAM-A reside in the loop that connects β -strands D and E and its 3_{10} helix.

duct injury in newborn mice and exhibits 1,000-fold greater binding capacity for human cholangiocarcinoma cells²⁸ (see Pathogenesis and Immunity—Hepatobiliary System).

The structure of a T3D $\sigma 1$ construct encompassing the body and head domains in complex with α -2,3-sialyllactose has been determined.³⁹⁵ The oligosaccharide binds in a shallow groove next to the loop connecting the second and third β -spiral repeats ($\beta 2$ and $\beta 3$). The $\sigma 1$ protein contains three identical binding sites, one on each chain, and all three are occupied by α -2,3-sialyllactose molecules in the crystal structure, with the sialic acid making identical and extensive contacts in each chain (Fig. 44.6B). Remarkably, $\sigma 1$ also can form complexes with α -2,6-sialyllactose and α -2,8-di-sialyllactose.³⁹⁵ In each case, the terminal sialic acid forms almost all of the contacts with $\sigma 1$ in an identical manner, while the remaining compo-

nents of the oligosaccharides make little or no contacts. Studies using point-mutant viruses indicate that Asn198, Arg202, Leu203, Pro204, and Gly205 are required for hemagglutination and infection of MEL cells,³⁹⁵ suggesting that these residues serve a functional role in T3D $\sigma 1$ –sialic acid interactions.

Type 1 reoviruses also appear to bind sialic acid in some contexts. T1L, but not T3D, binds the apical surface of microfold (M) cells, but not enterocytes, in tissue sections of rabbit Peyer patches.²¹⁷ Binding is inhibited by preincubation of the tissue sections with neuraminidase or with lectins that specifically recognize α -2-3-linked sialic acid. The capacity of T1L to bind the apical surface of M cells segregates with the S1 gene segment using reassortant genetics and with reovirus particles recoated with recombinant $\sigma 1$ protein. The interaction between T1L $\sigma 1$ and sialic acid is especially intriguing as type 1 reoviruses are

incapable of infecting MEL cells, a property dependent on sialic acid binding that segregates with the S1 gene segment,⁴²² and are insensitive to the growth-inhibitory effects of neuraminidase treatment of L cells.³⁴⁹ These findings suggest that the functional glycans engaged by types 1 and 3 reoviruses differ, even though such glycans appear to terminate in α -2,3-linked sialic acid. Concordantly, studies using expressed protein suggest that T1L engages carbohydrates using a domain that differs from that used by T3D to bind carbohydrates.⁸⁹

Type 2 reoviruses also are capable of producing hemagglutination,²⁸⁴ but little is known about the basis for this property.

JAM-A Binding by σ 1

Reovirus strains of all three serotypes use junctional adhesion molecule-A (JAM-A, also known as F11R/JAM/JAM1), a member of the immunoglobulin superfamily,^{27,309,529} as a receptor.^{27,76,382} JAM-A was identified as a reovirus receptor using a genetic screen and subsequently shown to bind directly to the σ 1 head domain with nanomolar affinity.^{27,431} Human and murine homologs of JAM-A, but not JAM family members JAM-B or JAM-C, serve as receptors for all reovirus serotypes and strains tested to date.^{27,76,382} The role of JAM-A as a reovirus receptor *in vivo* has been examined using JAM-A-null mice¹³ (see Pathogenesis and Immunity – Spread of Virus). Following peroral inoculation, JAM-A is dispensable for reovirus growth in the intestine. However, it is required for infection of vascular endothelial cells and promotes efficient hematogenous dissemination of reovirus to sites of secondary infection. Thus, JAM-A serves as a high-affinity reovirus receptor in cultured cells and *in vivo*. Receptors for reovirus at other sites within the host, including the central nervous system (CNS), have not been conclusively identified.

Structural and biochemical studies highlight the regions and specific interactions that mediate reovirus engagement of JAM-A.^{76,92,173,204,259,382} (Fig. 44.6C). The largest area of conserved residues in σ 1 forms the D-E and F-G loops in the head domain.^{76,92} The crystal structure of the σ 1 head domain in complex with the JAM-A D1 domain reveals that residues in this region, centered at the D-E loop and its 3_{10} helix, form the largest area of JAM-A contact.²⁵⁹ Interactions in this area are polar and involve residues Thr380, Gly381, and Asp382. A second area of JAM-A contact includes residues within the σ 1 body, just N-terminal to the head domain. Interactions in this region are largely hydrophobic and involve Arg316 in β -strand A of the head, β -spiral residues Arg297 and Tyr298, the α -helical turn that connects the β -spiral with the β -barrel, and Pro377.

The D1 domain of JAM-A is required for high-affinity binding to σ 1.^{173,204,382} Mutation of individual JAM-A D1 domain residues Arg59, Glu61, Lys63, Leu72, Tyr75, and Asn76 that lie in or adjacent to the dimer interface diminishes or abolishes σ 1 binding and reovirus infectivity.²⁰⁴ Concordantly, the structure of the σ 1–JAM-A complex shows that each σ 1 trimer binds three independent JAM-A monomers. Contacts primarily involve the JAM-A dimer interface and a conserved region at the base of the σ 1 head^{92,259} (Fig. 44.6C). In addition, the structure of the σ 1–JAM-A complex also identifies residues bound by σ 1 that are found just outside the dimer interface of JAM-A.²⁵⁹ These residues may serve as initial contact points for σ 1 and facilitate disruption of the JAM-A homodimer to allow interaction of σ 1 with the JAM-A dimer

interface. It is also possible that a cavity in the JAM-A dimer interface renders the homodimer intrinsically unstable, thereby promoting its disruption by σ 1. Regardless of the mechanism, the σ 1–JAM-A interaction is thermodynamically favored, as the K_D is $\sim 1,000$ -fold lower than the K_D of the JAM-A homodimer interaction.^{204,259,506}

The presence of discrete receptor-binding domains in σ 1 suggests that reoviruses employ a multiple-step binding process similar to that used by some herpesviruses^{109,465} and retroviruses.^{46,503} Binding studies using isogenic point-mutant viruses T3SA+ and T3SA–, which vary only in the capacity to engage sialic acid,²⁶ support this hypothesis. Kinetic analyses using inhibitors of sialic acid and JAM-A binding demonstrate that sialic acid is engaged first in the adsorption process, as the inhibitory effect of sialic acid analogs on infection by T3SA+ occurs at early but not late time points.²⁶ However, a σ 1-specific monoclonal antibody that blocks virus binding to JAM-A inhibits viral infectivity at both early and late times during adsorption.²⁶ Thus, reovirus binding to sialic acid enhances virus attachment through rapid adhesion of virus to the cell surface where access to JAM-A is thermodynamically favored. It is not known whether binding to sialic acid induces structural changes in σ 1 that affect its capacity to interact with JAM-A. However, it is clear that sialic acid binding is not a necessary prerequisite for JAM-A binding, as reoviruses incapable of binding sialic acid are capable of binding JAM-A.²⁷ It is possible that engagement of JAM-A by the σ 1 head leads to conformational changes in other regions of σ 1 or in other capsid components.¹⁶⁸

Cleavage of σ 1 by Intestinal Proteases

Treatment of virions of some reovirus strains with intestinal proteases *in vitro* to generate ISVPs leads to cleavage of the σ 1 protein.^{87,349} Strain-specific differences in cleavage susceptibility segregate with a single amino acid polymorphism in the short α -helical coiled-coil in the body domain of σ 1 (Fig. 44.6A, asterisk). Strains like T3D with a threonine at position 249 in σ 1 are susceptible to cleavage by trypsin after Arg245, whereas those with an isoleucine at position 249 are resistant to cleavage.⁸⁷ It is possible that Thr249 disrupts the α -helical coiled-coil and allows access to sites that otherwise would be shielded from protease.^{87,349} Conversion of T3D virions to ISVPs by intestinal proteases leads to loss of the σ 1 head domain and a resultant 90% reduction in infectivity.³⁴⁹ Since cleavage of σ 1 occurs C-terminal to the sialic acid-binding pocket, residual infectivity of T3D ISVPs is dependent on this carbohydrate.³⁴⁹ Susceptibility of the T3D σ 1 protein to cleavage likely accounts for the attenuated virulence of this strain after oral inoculation.^{53,254,419}

Determinant of Neutralizing Antibody Responses

In addition to its function in receptor engagement, the σ 1 protein is the target of serotype-specific neutralizing antibodies.^{75,469,516} For example, serotype-specific monoclonal antibodies 5C6⁵⁰⁸ and 9BG5⁷⁵ neutralize infectivity of type 1 and type 3 reovirus strains, respectively, and bind the σ 1 head domain in a serotype-specific fashion.⁸⁹ Variants of T1L selected for resistance to neutralizing monoclonal antibodies have mutations at Ala415, Gln417, Asn445, and Gly447,²¹⁶ which are located in the σ 1 head. Similarly, variants of T3D selected for resistance to neutralizing monoclonal antibodies have mutations at

Asp340 and Glu419,³⁰ which are also located in the $\sigma 1$ head. Interestingly, for both T1L and T3D, the sites in $\sigma 1$ altered in the neutralization-resistant variants are in close proximity on adjacent monomers,^{92,216} suggesting an epitope that spans two monomers in the $\sigma 1$ trimer. Neutralization-resistant variants of T3D display diminished neurovirulence and altered CNS tropism in mice,^{467,468} perhaps as a consequence of altered $\sigma 1$ receptor recognition in the murine CNS.

Entry

Internalization by Clathrin-Dependent Endocytosis

Following attachment to cell-surface glycans and JAM-A, reovirus is internalized by receptor-mediated endocytosis (Fig. 44.7). Thin-section electron micrographs show virions in structures

that appear to be clathrin-coated pits on the cell surface and in clathrin-coated vesicles in the cytoplasm,^{59,60,298,421,480} suggesting clathrin-dependent uptake. This finding was confirmed using video fluorescence microscopy in which reovirus virions and clathrin are observed to colocalize during internalization.¹⁵⁸ Treatment of cells with chlorpromazine, which impairs clathrin-mediated endocytosis, inhibits reovirus internalization and infection,²⁹⁸ suggesting a functional role for clathrin in reovirus entry. However, there is mounting evidence that both clathrin- and caveolin-dependent mechanisms can be employed by some viruses to enter host cells.^{275,385} Although there are no published reports of clathrin-independent uptake strategies for reovirus, a role for caveolae or other entry mechanisms in reovirus cell entry has not been conclusively excluded.

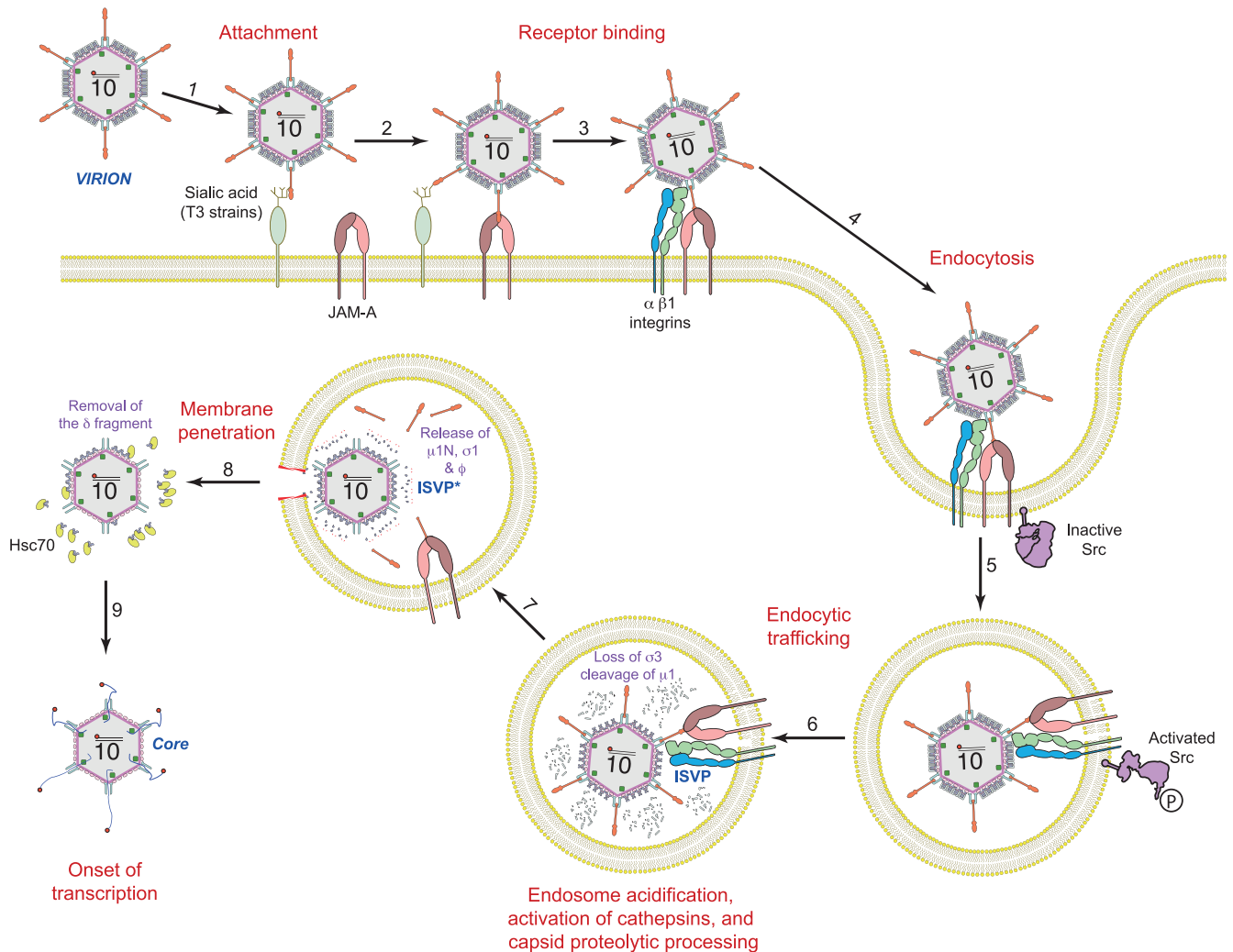


FIGURE 44.7. Schematic of reovirus cell entry and disassembly. Following attachment to (1) cell-surface carbohydrate (α -linked sialic acid for serotype 3 reoviruses) and (2) junctional adhesion molecule-A (JAM-A), (3 and 4) virions enter cells by receptor-mediated endocytosis using a mechanism that requires $\beta 1$ integrin. Following endocytic uptake (5), activation of Src is required for (6) correct trafficking of virions to an endocytic compartment where the viral outer-capsid undergoes acid-dependent proteolysis. The first disassembly intermediate is the infectious subviral particle (ISVP), which is characterized by loss of $\sigma 3$ and cleavage of $\mu 1C$ into particle-associated fragments δ and ϕ . The ISVP then undergoes further conformational changes (7) to form the ISVP*. The ISVP* is characterized by conformational rearrangements of the $\mu 1$ fragments to expose hydrophobic residues, release of $\mu 1N$ and ϕ , and loss of attachment protein $\sigma 1$. The $\mu 1$ cleavage fragments mediate penetration (8) through the endosomal membrane releasing the transcriptionally active core (9) into the cytoplasm. The cellular chaperone protein Hsc70 participates in the membrane penetration process.

Regardless of the internalization mechanism, reovirus particles are sorted into vacuoles resembling endosomes and lysosomes during the entry process.^{59,60,158,298,421,480}

β 1 Integrins Promote Entry and Sorting in the Endocytic Compartment

Expression of a JAM-A truncation mutant lacking a cytoplasmic tail allows reovirus to infect nonpermissive cells,²⁹⁷ suggesting that molecules other than JAM-A mobilize the internalization apparatus that promotes reovirus cell entry. The L2-encoded λ 2 protein (144 kDa, 1288–1289 aa) contains conserved integrin-binding motifs, RGD and KGE,^{64,438} suggesting that reovirus employs integrin-dependent internalization mechanisms to enter cells. The RGD and KGE sequences are displayed on surface-exposed loops of λ 2,³⁹⁴ where they could interact with integrins to promote internalization. However, direct interactions between λ 2 and integrins have not been reported. Interestingly, the L2 gene segment is genetically linked to viral shedding in infected mice and spread to littermates,²⁵⁴ suggesting a role for λ 2 in reovirus-induced disease.

Treatment of cells with antibodies specific for β 1 integrin reduces reovirus infection, while antibodies specific for other integrin subunits expressed on permissive cells, including those specific for α integrin subunits, have no effect.²⁹⁷ However, antibodies specific for β 1 integrin do not alter infection by *in vitro*-generated ISVPs,²⁹⁷ which directly penetrate the plasma membrane and do not require endocytosis.^{226,294} Concordantly, β 1 integrin-specific antibodies do not alter the binding of reovirus to JAM-A.²⁹⁷ These findings suggest that β 1 integrin blockade inhibits endocytic uptake of virions following JAM-A engagement. In comparison to β 1 integrin-expressing cells, β 1-null cells are substantially less susceptible to infection by reovirus virions, while infection by ISVPs is equivalent in both cell types.²⁹⁷ Diminished reovirus replication in β 1-null cells correlates with diminished viral uptake, indicating that β 1 integrin is required for efficient reovirus cell entry.

NPXY motifs in the β 1 integrin cytoplasmic tail play a key role in sorting reovirus within the endocytic compartment. NPXY motifs are found in the cytoplasmic domains of many receptors^{94,125,367} and recruit adaptor protein 2 or disabled protein 2^{341,367} to initiate clathrin assembly at the plasma membrane. Substitution of a tyrosine with a phenylalanine residue in either or both β 1 integrin NPXY motifs (NPXF) results in inefficient internalization of reovirus virions and diminished infectivity.²⁹⁸ Infection of cells expressing NPXF β 1 integrin results in distribution of virions to lysosomes where they are degraded, suggesting that the β 1 integrin NPXY motifs target reovirus to the precise endocytic organelle that permits functional disassembly.

Src Kinase Regulates Endosomal Sorting of Reovirus

The Src family of kinases contains eight members, Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, and Yes, three of which—Fyn, Src, and Yes—are expressed in most cell types.⁴⁹¹ Src-family kinases regulate numerous cellular processes including proliferation, differentiation, migration, adhesion, and cytoskeletal rearrangements⁴⁹¹ and transduce signals from a variety of receptors.^{145,182,308,427} In a small-molecule inhibitor screen, genistein, a broad-spectrum

tyrosine-kinase inhibitor, and PP2, a specific Src-family-kinase inhibitor, were found to diminish reovirus infectivity by inhibiting an early step in the viral life cycle.²⁹⁹ Although neither inhibitor impedes internalization of reovirus virions, both inhibitors target virions to lysosomes. At early times following infection, Src co-localizes with reovirus virions and is phosphorylated at the activation residue, Tyr416, suggesting that reovirus induces activation of Src during entry and traffics with Src during sorting in the endocytic compartment. Reduction of Src expression by RNAi decreases reovirus infectivity.²⁹⁹ These findings suggest that Src is part of a signaling network that targets reovirus to endocytic organelles for viral disassembly and thus promotes functional entry into host cells. The precise mechanism by which Src mediates these effects is unknown.

Disassembly

Cleavage of σ 3: The First Step in the Reovirus Disassembly Cascade

In cellular endosomes, reovirus virions undergo stepwise disassembly to form discrete intermediates, the first of which is the ISVP^{21,60,85,456,480} (Fig. 44.7). ISVPs are characterized by the loss of σ 3, a conformational change in σ 1, and cleavage of μ 1 to form δ and ϕ . The rate-limiting step in reovirus disassembly is the proteolytic removal of the S4-encoded σ 3 protein (41 kDa, 365 aa).^{21,480} In some cell types, proteolysis of σ 3 is dependent on acidic pH^{139,480} and endocytic cysteine proteases.²¹ Murine L929 fibroblast cells treated with inhibitors of endosomal acidification, such as ammonium chloride⁴⁸⁰ or bafilomycin A1,³¹⁰ or inhibitors of cysteine proteases, such as E64,²¹ do not support reovirus replication when infection is initiated by virions but do so when infection is initiated by ISVPs. Thus, the block to reovirus replication imposed by these inhibitors in murine L929 cells occurs following internalization but prior to disassembly, which is coincident with the proteolysis of σ 3.

Cathepsins B and L, which reside in late endosomes and lysosomes,⁸⁶ catalyze reovirus disassembly in fibroblasts.¹⁵⁶ Both enzymes are optimally active at acidic pH and serve functions in extracellular matrix formation, antigen presentation, and apoptosis.⁸⁶ These enzymes also mediate cell entry of several other viruses, including Ebola virus⁸² and SARS coronavirus.²²⁹ Cathepsin S, an acid-independent cysteine protease required for processing internalized antigens,⁴⁰¹ and the acid-independent serine protease neutrophil elastase can mediate uncoating of some reovirus strains in monocyte and macrophage cell lines.^{191,193} It is possible that the broad tissue tropism displayed by reovirus is determined in part by the multiple host proteases capable of mediating its disassembly.²⁴⁷

Proteolytic enzymes also are required for reovirus infection following peroral inoculation of mice^{29,54} (see Pathogenesis and Immunity—Entry into the Host). Reovirus virions are converted to ISVPs in the intestinal lumen by the resident serine proteases chymotrypsin and trypsin. ISVPs produced in this fashion enter intestinal M cells to allow systemic dissemination of reovirus in the host.¹² ISVPs generated by chymotrypsin or trypsin *in vitro* or in the gut lumen^{29,54} display a protein profile (loss of σ 3 and cleavage of μ 1) indistinguishable from that of ISVPs generated in the endocytic compartment of cells.^{22,156}

Sequences in σ 3 that influence its susceptibility to proteolysis have been identified through studies of viruses selected

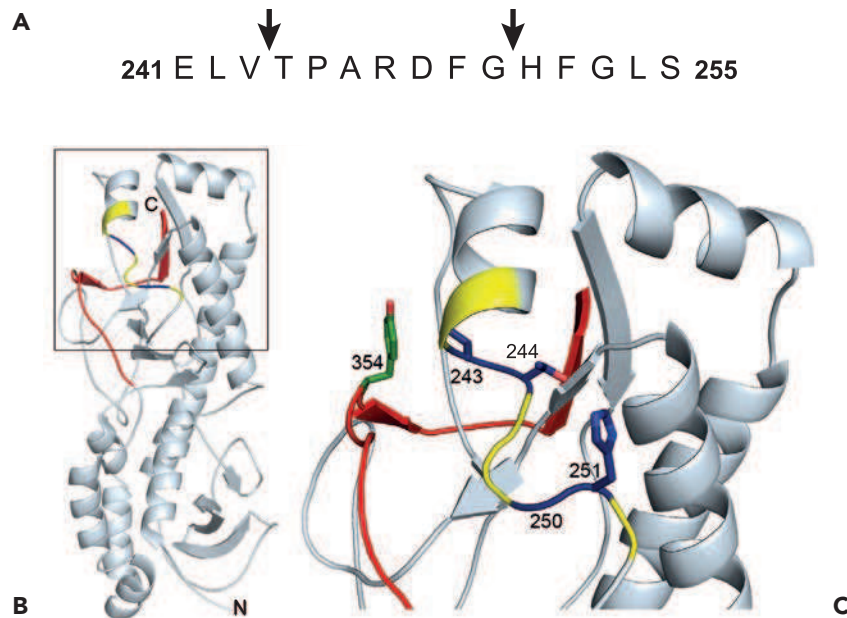


FIGURE 44.8. Structure of reovirus $\sigma 3$ with cleavage sites. **A:** The amino acid sequence of $\sigma 3$ from amino acids 241 to 255 is shown. Arrows highlight cathepsin L cleavage sites identified by N-terminal sequencing of $\sigma 3$ cleavage products following treatment of reovirus strain type 1 Lang (T1L) with cathepsin L *in vitro*. **B:** Cathepsin L cleavage sites are highlighted in the crystal structure of $\sigma 3$. A ribbon diagram of the crystal structure of type 3 Dearing $\sigma 3$ ³⁶⁸ is displayed on the left. The cathepsin L cleavage sites in T1L are depicted in *blue* between amino acids 243 and 244 and between 250 and 251. Surrounding residues, from amino acids 241 to 253, are shown in *yellow*. The C-terminal residues of $\sigma 3$, from amino acids 340 to 365, are in *red*. Amino acid 354, which is altered in a variety of mutant viruses selected for accelerated disassembly kinetics, is in *green*. The virion-distal end of $\sigma 3$ is at the top of the figure, and the virion-proximal end and N-terminus are at the bottom. **C:** An enlarged view of the boxed region of $\sigma 3$ indicated in **B** is shown using the same color scheme. Side chains of amino acids 243, 244, 250, 251, and 354 are depicted in *stick representation*.

during persistent infection (PI viruses) or mutant viruses selected for resistance to either cysteine protease inhibitor E64 (D-EA viruses)¹⁵⁷ or ammonium chloride (ACA-D viruses).⁹⁹ These viruses exhibit accelerated kinetics of disassembly and harbor a tyrosine-to-histidine mutation at amino acid 354 (Y354H) near the C-terminus of the protein^{99,157,524} (Fig. 44.8). Cryo-EM image analysis of a PI virus with an isolated Y354H mutation reveals a structural alteration in $\sigma 3$ at a hinge region located between its two major domains.⁵³¹ These findings suggest that the C-terminus of $\sigma 3$ influences susceptibility of the protein to cleavage. Interestingly, the $\sigma 3$ amino acid sequence of strain T3A differs from that of T3D at eight positions, including Y354H. However, a glycine-to-glutamate substitution at position 198 in T3A $\sigma 3$ suppresses the disassembly-enhancing effects of His354,¹⁴⁷ suggesting the existence of an allosteric network regulating $\sigma 3$ cleavage.

The $\sigma 3$ C-terminus also dictates strain-specific differences in the susceptibility of $\sigma 3$ to proteolytic attack.^{243,244} The $\sigma 3$ protein of strain T1L is cleaved more rapidly than that of T3D. Analysis of ISVPs recoated with chimeric $\sigma 3$ proteins generated from T1L and T3D revealed that the C-terminus is primarily responsible for the rate of $\sigma 3$ proteolysis. Moreover, sequence polymorphisms at residues 344, 347, and 353 in $\sigma 3$ contribute to this effect.²⁴³

Treatment of reovirus virions *in vitro* with either cathepsin B or cathepsin L leads to an initial cleavage of $\sigma 3$ at a terminus.¹⁵⁶ Since sequence polymorphisms in the $\sigma 3$ C-terminus

determine susceptibility to proteolysis, the initial cleavage of $\sigma 3$ probably occurs in this region. During proteolysis by cathepsin L, subsequent cleavages occur between residues 243 to 244 and 250 to 251¹⁵⁶ (Fig. 44.8A). These cleavage sites are physically located near the C-terminus in the $\sigma 3$ crystal structure³⁶⁸ (Fig. 44.8B and C). Because of this proximity, the small end fragment released following initial cathepsin L cleavage likely exposes the cleavage sites between residues 243 to 244 and 250 to 251, rendering them sensitive to proteolysis. The C-terminus therefore appears to control access to internal, proteolytically sensitive sites in $\sigma 3$. Because reovirus disassembly in some cell types is acid-dependent,^{139,480} the C-terminus might be primed for movement at acidic pH. Mutations near the C-terminus, like Y354H, may alter the conformation of the protein to allow improved access to these cleavage sites and thus accelerate outer capsid disassembly.⁵³¹

Conformational Changes in $\sigma 1$

The disassembly of reovirus virions to ISVPs is accompanied by a dramatic conformational change in $\sigma 1$. Electron micrographs of negatively stained reovirus virions and ISVPs reveal filamentous projections extending up to 400 Å from the surface of ISVPs but not virions.¹⁷⁶ These images suggest that $\sigma 1$ adopts a compact form in the virion and a more extended one in the ISVP. Cryo-EM image reconstructions of virions and cores lack a discernable density corresponding to $\sigma 1$ at the icosahedral vertices^{151,323} (Fig. 44.2). However, cryo-EM image analysis of

ISVPs demonstrates discontinuous density for $\sigma 1$ extending ~ 100 Å from each vertex (Fig. 44.2). Presumably, the full length of $\sigma 1$ is not visible in reovirus particles because the molecule is flexible and icosahedral averaging was employed for the cryo-EM image reconstructions. As further evidence of the conformational alterations in $\sigma 1$ during viral disassembly, proteolytic cleavage of T3D $\sigma 1$ during virion-to-ISVP conversion increases viral hemagglutination capacity,³⁴⁹ suggesting enhanced access to the sialic acid-binding region as a consequence of extension of the $\sigma 1$ tail and body domains. Interdomain regions of flexibility in $\sigma 1$ ¹⁷⁴ (Fig. 44.6) may be required to allow such conformational rearrangements.

Electron micrographs of purified $\sigma 1$ show molecules with either single- or multilobed head domains,¹⁷⁴ suggesting another type of structural alteration in $\sigma 1$ in which the head can exist in both “open” and “closed” conformations. Although neither the mechanism nor the functional significance of this $\sigma 1$ conformational change is understood, structural studies of $\sigma 1$ provide clues about how this change might occur. A cluster of six conserved aspartic acid residues on a rigid β -hairpin at the base of the $\sigma 1$ head, sandwiched between hydrophobic residues that block access to solvent, forms the main contact area between monomers in the trimer.^{92,431} Of the two aspartic acid residues contributed by each monomer, one (Asp346) is neutralized by a salt-bridge interaction with a nearby residue, while the other (Asp345) is not.^{92,431} The three Asp345 side chains closely appose each other at the center of the trimer in an otherwise hydrophobic environment. Since accumulation of negative charge in this region is predicted to destabilize the trimer,⁷⁸ and an aspartate-to-asparagine mutation results in $\sigma 1$ trimers with a structure indistinguishable from wild-type,⁴³¹ it is likely that Asp345 is protonated in the $\sigma 1$ crystal structure,⁹² thus representing the “closed” conformation of $\sigma 1$. This conformation might form during crystallization at near-neutral pH and physiologically in conditions of low pH, similar to those encountered in the endocytic compartment during reovirus entry.⁴³¹ Thus, the aspartic acid sandwich motif may contribute to $\sigma 1$ conformational rearrangements by acting as a molecular switch that

mediates the oligomeric state of the $\sigma 1$ head, depending on environmental pH.⁴³¹

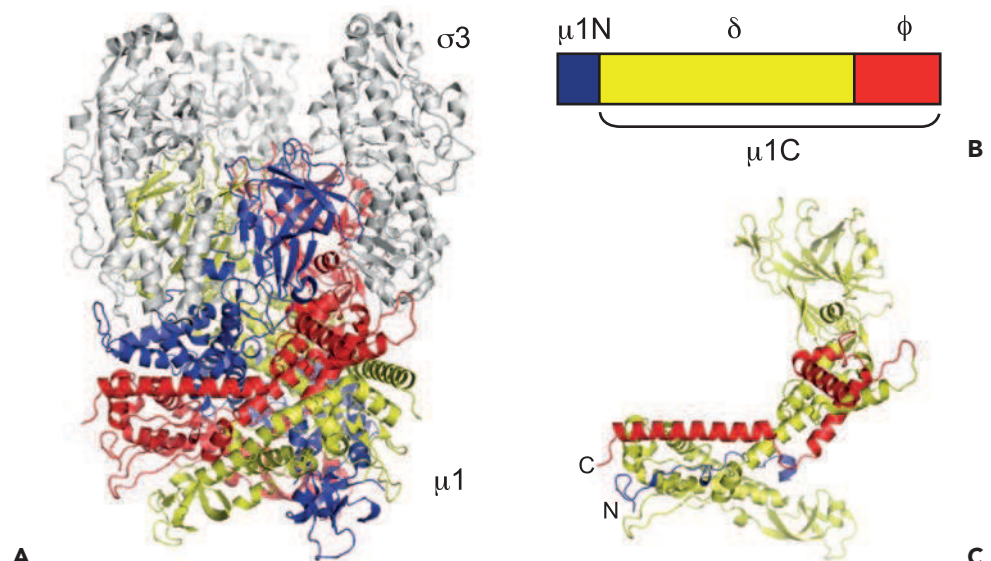
Membrane Penetration

Critical Function of the $\mu 1$ Protein

Studies to assess the capacity of reovirus entry intermediates to penetrate artificial lipid bilayers, model membranes of erythrocytes, or membranes of cells that support reovirus infection indicate that ISVPs but not virions or cores mediate membrane penetration.^{59,80,83,84,226,294,492} Such studies led to the idea that ISVPs or related subviral particles are the membrane-active intermediates in the reovirus entry pathway. Since ISVPs differ from cores by the presence of outer-capsid proteins $\sigma 1$ and $\mu 1$,^{114,151} and because cores recoated with $\mu 1$ and $\sigma 3$ *in vitro* and then treated with chymotrypsin to remove $\sigma 3$ are capable of membrane penetration,⁸³ these findings point to a role for the M2-encoded $\mu 1$ protein (76 kDa, 708 aa) in membrane penetration. This biochemical evidence also is supported by several genetic studies. Differences in membrane-penetration efficiency displayed by reovirus strains T1L and T3D segregate with the M2 gene segment.^{79,294,430} Additionally, viruses selected for resistance to denaturants such as ethanol contain mutations within the M2 gene segment and display alterations in membrane penetration capacity.^{79,123,226,522} Together, these data demonstrate a function for the $\mu 1$ protein in membrane penetration.

The $\mu 1$ protein is N-terminally myristoylated³⁵⁴ and folds into four distinct domains²⁸⁹ (Fig. 44.9). Domains I, II, and III are primarily α -helical and serve as the base of the protein. Domain IV forms a jelly-roll β -barrel commonly found in the capsid proteins of many nonenveloped viruses.²¹¹ This domain extends distally and interacts extensively with similar domains of the neighboring $\mu 1$ molecules and with $\sigma 3$. The $\mu 1$ protein also contains three proteolytic cleavage sites (Fig. 44.9B). These include an autocatalytic cleavage site at amino acid 42, which separates $\mu 1N$ and $\mu 1C$, a cleavage site at approximately amino acid 580, which releases the δ and ϕ fragments, and a cleavage site at the C-terminus that releases a ~ 10 amino acid peptide.^{81,320,351,366} While the physiologic roles of both the δ - ϕ and the C-terminal cleavages are unclear, studies using reovirus

FIGURE 44.9. Structure of reovirus $\mu 1$ with cleavage sites. A: Ribbon diagram of the crystal structure of the $\mu 1$ - $\sigma 3$ heterohexamer from strain type 1 Lang (T1L).²⁸⁹ The $\mu 1$ monomers are in blue, red, and yellow; the $\sigma 3$ monomers are in grey. **B:** Cleavage products of the $\mu 1$ protein. (The C-terminal cleavage is not depicted.) **C:** Ribbon diagram of the crystal structure of the T1L $\mu 1$ monomer showing regions corresponding to the cleavage products. The domain color code is as depicted in B.



cores recoated with a $\mu 1N$ - $\mu 1C$ cleavage-resistant $\mu 1$ mutant indicate that cleavage of $\mu 1$ to generate $\mu 1N$ and $\mu 1C$ is required for membrane penetration and virion infectivity.³⁶⁶ Since $\mu 1N$ is released from viral particles, it is postulated that cleavage of $\mu 1$ is required for release of $\mu 1N$, which then interacts with membranes as a function of its myristoyl moiety to effect membrane penetration.²³⁸

Formation of the ISVP*

In the native $\mu 1$ structure present in virions and ISVPs, the myristoylated $\mu 1N$ fragment is buried inside a hydrophobic cavity in the α -helical pedestal formed by portions of domains I, II, and III.^{289,549} Based on this observation, massive conformational rearrangements resulting in unwinding of the $\mu 1$ trimer must be required to release $\mu 1N$ during cell entry.^{289,547} Evidence for conformational changes in particle-associated $\mu 1$ following interaction of ISVPs with membranes or when exposed to high salt concentrations has led to the identification of an ISVP-like entry intermediate in the reovirus cell entry pathway.⁷⁹ This intermediate, referred to as the ISVP*, is characterized by changes in the conformation of the $\mu 1$ δ fragment, loss of the $\sigma 1$ protein, and an increase in the overall hydrophobicity of the particle.⁷⁹ The autocatalytic cleavage of $\mu 1$ to form $\mu 1N$ and $\mu 1C$ also occurs in part during ISVP* formation.³⁵³ Thus, the $\mu 1$ protein associated with ISVPs is in a metastable state primed to undergo conformational changes and autocatalytic cleavage to assume a more hydrophobic structure capable of interaction with membranes. While it is not understood how these conformational changes in $\mu 1$ are triggered, it is thought that interaction of an anion-binding site in domain IV with phospholipid head groups in endosomal membranes might trigger the requisite rearrangements in $\mu 1$ that reveal the myristoylated $\mu 1N$ and the internal hydrophobic residues.²⁸⁹ At high particle concentrations, ISVP* conversion is regulated by a positive feedback mechanism in which $\mu 1N$, which is released during ISVP* formation, promotes ISVP-to-ISVP* conversion of the remaining particles.³ Acceleration of ISVP* formation by $\mu 1N$ is dependent on temperature and target flexibility, suggesting that particle dynamics are required to expose a $\mu 1N$ interaction domain.³ Since such particle concentrations are unlikely to be achieved following a low-multiplicity viral infection, it remains unclear how these findings translate to ISVP* formation in cellular endosomes during viral entry.

Genetic studies using ethanol-resistant or thermostable mutants indicate that $\mu 1$ residues affecting the overall stability of the virus also regulate membrane-penetration efficiency.^{79,123,226,522} These and other stability-altering residues identified in thermostable reovirus mutants³²⁴ are located between residues 383 and 612 of $\mu 1$ and map to either domain IV that forms the jelly-roll β -barrel or the α -helical portions of domain III that lie just below the β -barrel structure. Since these $\mu 1$ domains participate in interactions between neighboring $\mu 1$ monomers, these residues are thought to modulate viral stability by preventing unwinding of the $\mu 1$ trimer.²⁸⁹ Consistent with an increase in $\mu 1$ protein rigidity in ethanol-resistant and thermostable mutants, viral cores recoated with mutant $\mu 1$ proteins, or recombinant reoviruses containing single amino acid substitutions in $\mu 1$ in an otherwise wild-type background, display diminished ISVP-to-ISVP* conversion and have defects in membrane penetration.^{79,121,123,226,324,430,522} These studies suggest that a central region of $\mu 1$ involved

in intermolecular interactions is an important regulator of the ISVP-to-ISVP* transition. In addition to these residues, changes in the C-terminal ϕ fragment also control viral stability³²⁴ and affect membrane penetration by reducing the efficiency of ISVP-to-ISVP* conversion.¹²¹ While it is not clear how ϕ residues modulate these properties, since both $\mu 1N$ and ϕ are released from the virus particle during ISVP* formation,²³⁸ it is likely that conformational rearrangements in $\mu 1$ during ISVP* formation are not restricted to the δ domain but also involve the $\mu 1N$ and ϕ domains. Therefore, amino acid substitutions within ϕ that negatively affect its conformational flexibility would likely prevent the $\mu 1$ reorganization required for ISVP* formation.

Pore Formation Mediated by $\mu 1N$ and ϕ

Reovirus forms small, size-selective pores in erythrocyte model membranes.² Both $\mu 1N$ and ISVP* associate with erythrocyte membranes,^{2,238} but $\mu 1N$ is capable of pore formation in the absence of other viral components.²³⁸ While ϕ also associates with membranes,²³⁸ its recruitment does not result in membrane penetration. These findings are consistent with the observation that viruses incapable of δ - ϕ cleavage can penetrate membranes and are fully infectious.^{80,83} Since pore formation by $\mu 1N$ is enhanced by the presence of ϕ , it is possible that ϕ functions as a $\mu 1N$ chaperone and facilitates membrane penetration by reovirus.²³⁸ Pores formed by released $\mu 1N$ fragments are considerably smaller than those required to allow the viral intermediate to traverse the membrane.² Therefore, it is not clear how pore formation in model membranes relates to membrane penetration during cell entry. Analogous to erythrocyte membrane rupture, pore formation may result in osmotic lysis of endosomes in which viral particles are present. Alternatively, the initial small pore formed by the virus might recruit cellular factors that produce larger pores or channels through which the viral intermediate can translocate.

Host Proteins Required for Membrane Penetration

Both the viral core and the δ fragment of $\mu 1$ are found in the cytoplasm following reovirus entry into host cells.⁸¹ While δ is found distributed diffusely throughout the cytosol, viral cores display a more punctuate cytoplasmic localization.⁸¹ These observations suggest that the δ fragment disassociates from the ISVP* during or immediately after membrane penetration. This idea is supported by evidence that reovirus cores are transcriptionally active in the cytoplasm and that activation of transcription requires removal of most of the $\mu 1$ fragments. Removal of δ from cores is thought to be accomplished by direct interaction of δ with the host chaperone Hsc70 via an ATP-dependent process.²³⁷ Based on evidence that chaperones can translocate proteins across membranes,⁵⁴² it is possible that concomitant with removal of particle-associated δ , Hsc70 also aids in transport of the viral core across membranes.²³⁷

Transcription

Reovirus core particles produced *in vitro* by proteolytic removal of outer-capsid proteins synthesize viral mRNAs representing each of the 10 gene segments when incubated with ribonucleotides and an ATP-regeneration system. During infection, transcriptionally active viral core particles produced following proteolytic disassembly and endosomal membrane penetration gain access to the cytosol and produce the initial wave of viral transcripts (primary transcripts) that encode new viral proteins. However,

in contrast to *in vitro*-generated transcripts, the primary viral transcriptome synthesized by entering viral particles during the first 4 to 5 hours of infection is biased towards transcripts from the L1, M3, S3, and S4 gene segments.⁵¹² One possible explanation for these observations is that transcriptionally active core particles in cells retain remnants of outer-capsid proteins or associate with cellular proteins that differentially repress transcription of some viral gene segments (presumably via allosteric effects). Alternatively, the stability of some viral transcripts in cells may depend on the presence of one or more of the $\lambda 3$, μ NS, σ NS, or $\sigma 3$ proteins encoded by the L1, M3, S3, and S4 gene segments, respectively. Notwithstanding this early difference in viral transcription, by 10 hours postinfection all of the viral transcripts are synthesized at frequencies proportional to their length.^{359,442,458} Primary transcription from particles entering cells peaks around 6 hours postinfection and is replaced by secondary transcription that results from newly assembled transcriptase particles. The large majority of viral mRNA is synthesized as a consequence of secondary transcription.

Cores as Molecular Machines

The viral core particle contains the genome and enzymes required to produce capped viral mRNAs. Each core contains 12 copies of the viral RdRp, $\lambda 3$, and ~ 24 copies of the polymerase co-factor, $\mu 2$. Electron micrographs of transcribing cores show viral mRNAs exiting at each of the fivefold symmetry axes.¹⁸⁷ Concordantly, all 10 gene segments can be transcribed simultaneously.⁴⁵⁸ Isolated $\lambda 3$ polymerase has limited enzymatic activity and likely must interact with its co-factor $\mu 2$ and the $\lambda 1$ shell protein to mediate native catalysis. RNA transcripts synthesized from the [–] strand template of the genomic dsRNA are extruded through a peripentonal channel in the $\lambda 1$ shell and enter a cavity formed by the pentameric $\lambda 2$ protein. As the nascent viral transcripts are extruded into and through the $\lambda 2$ turret, they are capped by guanylyltransferase and methyltransferase activities associated with $\lambda 2$ and then gain access to the cytosol.

Activation of Transcriptase Function

Transcription in intact, double-shelled virions is repressed. In the repressed state, the 3' end of each [–] strand template is probably unwound from the complementary [+] strand and held in a preinitiation complex with a polymerase molecule at the active site. In this conformation, the polymerase can only synthesize short abortive transcripts that represent the first 2 to 4 bases complementary to the 3' end of the [–] strand template. Indeed, $\sim 25\%$ of the total RNA found in reovirus virions represents short ssRNA oligonucleotides that are either abortive transcripts or nontemplated oligoadenylates that are likely synthesized during the last stages of virion morphogenesis as transcription is shut off by assembly of the outer capsid.^{39,41,248} The block appears to be at the stage of clearance of the promoter from the polymerase active site. Removal of outer-capsid proteins $\sigma 3$ and $\mu 1$ by proteolysis allows a conformational change in the core particle that permits opening of the C-terminal flaps of the $\lambda 2$ turret and release of $\sigma 1$.^{151,459} These changes are accompanied by activation of the transcriptase, which allows promoter escape and transcript elongation. However, 90% of cores generated *in vitro* synthesize only short abortive transcripts and are incapable of

transitioning from initiation to elongation.¹³⁴ The remaining 10% of cores (so-called C-fraction cores) synthesize full-length transcripts and, in these particles, elongation and not promoter clearance is the rate-limiting step in transcription. Mechanisms underlying these differences are not known, but the capacity of small molecules that modify the stability of RNA duplexes (e.g., DMSO and spermidine) to alter the percentage of cores capable of transcribing full-length transcripts suggests that global changes in the conformation of the RNA gene segments are required for activation of transcription.¹³⁴ This hypothesis is supported by the observation that the liquid crystalline RNA within transcriptionally active cores is less closely packed than genomic RNA within inactive cores, ISVPs, or virions.³¹⁹

The Transcriptional Cycle

Transcription of reovirus dsRNA gene segments is iterative and involves a well-orchestrated series of steps. The dsRNA gene segment first must be unwound to allow the 3' end of the [–] strand template to enter the catalytic site of the polymerase. The 3' end of the template strand is then specifically recognized and placed in register for priming and initiation of RNA synthesis. After RNA synthesis is initiated, the promoter region of the template must be cleared from the active site to allow the nascent RNA transcript to be elongated. As elongation proceeds, the transcript is separated from the template and extruded from the polymerase. The template exits the polymerase through a different polymerase channel and is likely rewound with its cognate [+] strand before having its 3' end guided back towards the template entry portal to allow efficient reinitiation of synthesis. Immediately after the nascent transcript exits the polymerase, the terminal γ phosphate at its 5' end is removed by an RNA triphosphate phosphohydrolase, and the transcript is passed into a cavity enclosed by the pentameric $\lambda 2$ turret where it is guanylated by a guanylyltransferase domain and 7'-N and 2'-O methylated by two independent methyltransferase domains. Finally, the capped RNA transcript exits the core particle into the cytosol.

Enzymatic Activities Associated with Transcription RNA-DEPENDENT RNA POLYMERASE ($\lambda 3$)

Twelve copies of the L1-encoded $\lambda 3$ RdRp (142 kDa, 1,267 aa) are encapsidated within each core particle.^{150,394,550} A 2.5 Å atomic resolution structure of $\lambda 3$ ⁴⁸³ reveals a central RdRp domain (aa 381–890) with canonical right-handed “thumb,” “palm,” and “fingers” subdomains (Fig. 44.10A). The catalytic cleft is enclosed (or “caged”) by an N-terminal domain (aa 1–380), which bridges the “fingers” and “thumb” subdomains, and by a C-terminal “bracelet” domain (aa 891–1267) (Fig. 44.10A). The $\lambda 3$ protein can be modeled as a rounded cube ~ 65 Å in diameter with channels on its front, bottom, rear, and left sides that allow access to the active site (Fig. 44.10B). These channels allow entry of the template and nucleoside triphosphates (NTPs) and exit of the minus-strand template or dsRNA product and the plus-strand transcript (Figs. 44.10C and D).

Crystal-soaking experiments with a cap analog identified a putative cap-binding site on the outside of the polymerase⁴⁸³ (Fig. 44.10B). The 5' cap of the [+] strand RNA likely remains anchored in this position close to the template entry channel (Fig. 44.10C). This placement would retain the 3' end of the [–] strand template RNA in a position where it could easily

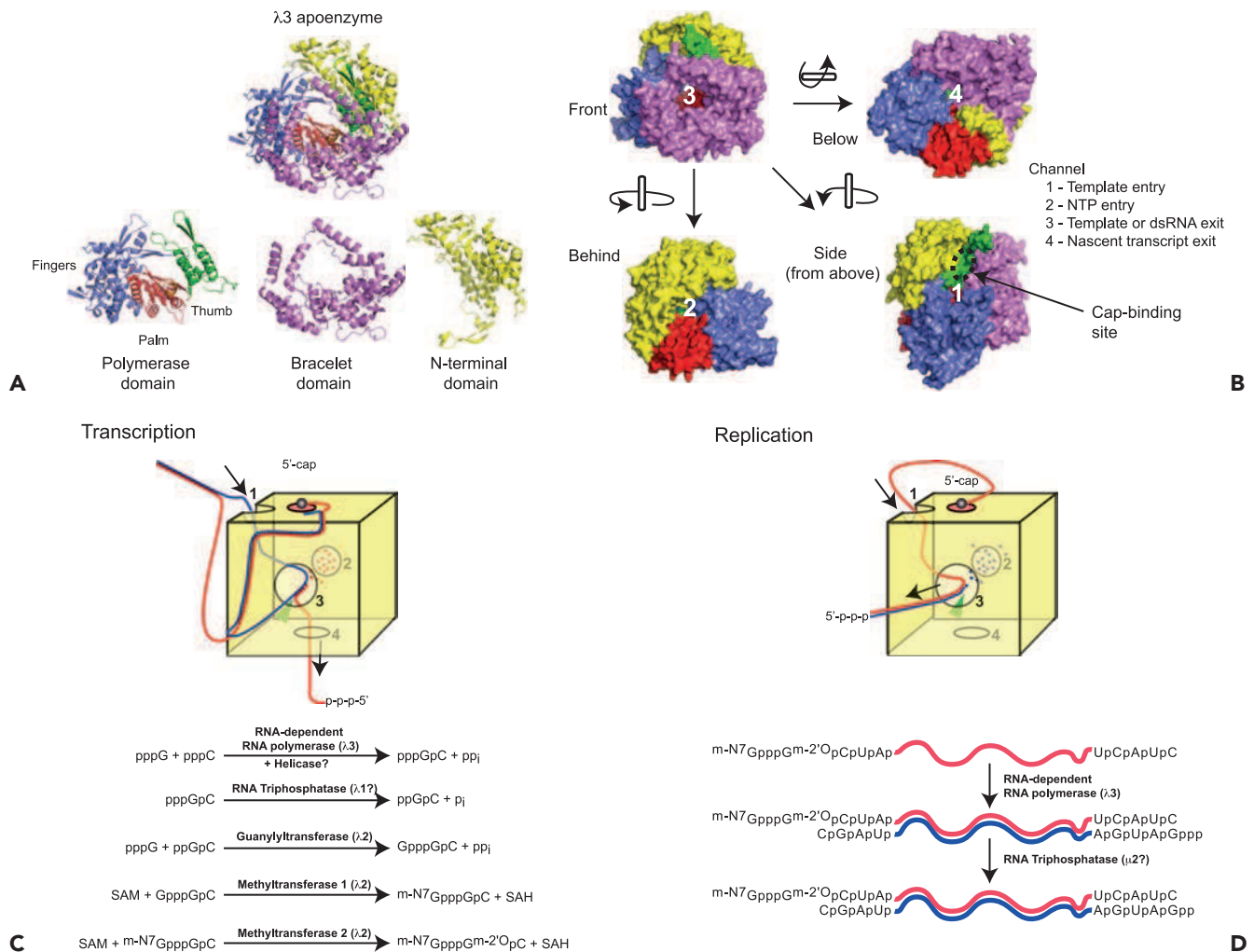


FIGURE 44.10. The reovirus $\lambda 3$ RNA-dependent RNA polymerase and its enzymatic actions. **A:** Ribbon diagram of the crystal structure of the $\lambda 3$ apoenzyme.⁴⁸³ Access to the central conserved polymerase domain is limited by the N-terminal domain (yellow) and C-terminal bracelet domain (purple), which form channels. **B:** Space-filling model of the polymerase with the channels that allow (1) template entry, (2) NTP entry, (3) template or dsRNA exit, and (4) nascent transcript exit indicated. The 5' cap of the template mRNA binds to the polymerase close to the template entry site. The enzymatic activities of $\lambda 3$, transcription (**C**) and replication (**D**), and the movement of the template minus-strand RNA (blue) during transcription or template positive-strand mRNA (red) during replication are shown in the schematic. Other enzymatic activities associated with transcription and replication are indicated below each schematic.

access the polymerase active site. An unusual feature of $\lambda 3$ that is also shared by the rotavirus VP1 polymerase is the presence of a priming loop that engages the incoming priming NTP during initiation. This loop is displaced toward the “palm” sub-domain during elongation. Failure of the loop to move would prevent elongation and might explain the large number of abortive transcripts within viral cores. Thus, the priming loop may be a kinetic barrier to the transition from initiation to elongation during transcription.^{93,134,318}

Comparison of the rotavirus VP1 and reovirus $\lambda 3$ polymerase structures reveals a difference in the location of the priming loop of VP1, which is bent away from the active site, and in this conformation would not be capable of stabilizing an incoming NTP and initiating RNA synthesis. The VP1 polymerase also differs from $\lambda 3$ in that it specifically recognizes and binds the 3'

end of the [+] strand RNA but not the 3' end of the [−] strand RNA. The $\lambda 3$ protein displays limited specificity for binding the 3' end of the RNA. However, the RNA template is held in precise register for the initiation of synthesis from the end of the template.³¹⁸ This arrangement contrasts with the specific recognition of the 3' end of the [+] strand RNA by VP1 that paradoxically places the template out of register.²⁹³ One attractive hypothesis is that the two structures represent different conformers, with VP1 being in the inactive state found prior to packaging of viral mRNA into cores. In this case, specific recognition of the 3' end together with cap binding would create mRNA–polymerase complexes, but initiation of synthesis of the partner dsRNA presumably would be prevented until a conformational change in the polymerase occurs concurrent with assortment and packaging of a complete set of viral

mRNAs.³¹⁷ The $\lambda 3$ structure would represent the active form of the polymerase found within transcriptionally competent cores, where specific recognition is not absolutely required, but it is imperative that initiation begins at the first residue of the template. RNA synthesis is fully conservative²¹³ and initiates with entry and correct registration of the 3' end of a template RNA at the active site.⁴⁸³

HELICASE ACTIVITY

An unwinding, or helicase, activity is required to separate the genomic dsRNA [+] and [-] strands prior to transcription and to facilitate rewinding of the template [-] strand with the [+] strand after it exits the polymerase. Although semipurified $\lambda 1$ protein has helicase activity *in vitro*,⁵² the viral protein responsible for helicase activity during infection is thought to be $\lambda 3$.⁴⁸³ The presence of a cap-binding site on $\lambda 3$ would allow strand separation to occur. In addition, a loop present in the bracelet subdomain of the $\lambda 3$ polymerase may aid in separation of the template and transcript during transcription.⁴⁸³

RNA TRIPHOSPHATE PHOSPHOHYDROLASE ACTIVITY

Following transcription, the terminal γ phosphate at the 5' end of the transcript is removed, yielding a diphosphate to initiate capping. The $\lambda 1$ -encoded $\lambda 1$ protein (142 kDa, 1275 aa) has phosphohydrolase activity,⁵¹ and removal of the γ phosphate likely occurs as the transcript is directed out of the core particle through pores in the $\lambda 1$ shell.⁵⁵⁰ The passage of the transcript through $\lambda 1$ may be facilitated by the RNA-binding activity associated with a $\lambda 1$ N-terminal domain.⁵² Alternatively, or in addition, the capacity of $\lambda 1$ to bind RNA may be important during assortment of viral mRNAs for packaging.

GUANYLYLTRANSFERASE AND METHYLTRANSFERASE ACTIVITY

Following removal of the 5' γ -phosphate from the transcript, a guanylyltransferase associated with the N-terminal domain of the $\lambda 2$ -encoded $\lambda 2$ protein (144 kDa, 1288–1289 aa) adds a guanylate cap to the 5' diphosphate of the transcript using GTP as a substrate.^{106,296} The transcript is then further modified by the addition of 7-*N* and 2'-*O* methyl groups by the two methyltransferase subdomains of $\lambda 2$.⁴⁸³ The methyl donor comes from S-adenosyl methionine (SAM), which is converted to S-adenosyl-L-homocysteine after the methyl group is transferred to the cap.²⁹⁵ Five $\lambda 2$ monomers form a turret-like structure that projects distally from the $\lambda 1$ shell. The long axis of each $\lambda 2$ monomer lies at an angle of approximately 45° to the fivefold axis of symmetry. The $\lambda 2$ monomers wrap around each other to form a hollow cylinder, approximately 70 Å in diameter at its base, with C-terminal flaps at the top that narrow to approximately 15 Å.³⁹⁴ The N-terminal guanylyltransferase domains (residues 1–385) of the five $\lambda 2$ monomers are located at the base of the turret. Lysine residues at positions 171 and 190 in the $\lambda 2$ sequence are required for enzymatic function and project into the active site.²⁹⁶ The guanylyltransferase domain is linked by a small domain (residues 386–433 and 690–802) to two methyltransferase domains (methylase 1, residues 434–691, and methylase 2, residues 804–1022), each possessing a SAM-binding site.³⁹⁴ Based on their location, it is hypothesized that methylase 1 catalyzes guanosine-7-*N*-methylation and methylase 2 catalyzes guanosine-2'-*O*-methylation. The flap of the $\lambda 2$ turret is formed by the C-terminal 250 residues that form three immunoglobulin-like folds.³⁹⁴

Location of $\lambda 3$ Within Core Particles

A 7.6 Å cryo-EM image reconstruction of viral core particles shows that each $\lambda 3$ polymerase lies close to but slightly adjacent from the fivefold symmetry axes.⁵⁵⁰ The transcript-exit channel (Fig. 44.10B) faces towards a peripentonal channel in $\lambda 1$, suggesting that the phosphohydrolase activity of $\lambda 1$ is responsible for removal of the γ phosphate from nascent transcripts. The location of $\mu 2$ has not been resolved, but two copies of $\mu 2$ are thought to associate with each $\lambda 3$ polymerase.⁵⁵⁰

Role of $\mu 2$ in Transcription

The M1-encoded $\mu 2$ protein (83 kDa, 736 aa) is found in approximately 24 copies within each core particle. It interacts with the $\lambda 3$ polymerase and may also interact with $\lambda 1$. Like many reovirus core proteins, $\mu 2$ binds ssRNA and dsRNA.⁶³ It has NTPase and RNA 5' triphosphatase activity and contains an A-box NTP-binding motif. The $\mu 2$ protein is thought to be a co-factor for the $\lambda 3$ polymerase. The $\mu 2$ RNA 5' triphosphatase activity may be required for removal of the 5' γ phosphate from newly synthesized [-] strand RNA during dsRNA synthesis. A role for $\mu 2$ in transcription is inferred from genetic studies indicating that the $\mu 2$ -encoding M1 gene segment segregates with strain-specific differences in the optimum temperature of transcription.⁵⁴⁰ The $\mu 2$ NTPase and RTPase activities are required for viral replication, as ectopic expression of mutant $\mu 2$ proteins lacking NTPase or RTPase activity (K415A or K419A) fails to complement siRNA-mediated knockdown of wild-type $\mu 2$.²⁶³

Translation and Viral Factory Formation

Translation of Reovirus mRNAs and Viral Protein Synthesis

Early studies of eukaryotic translation initiation used reovirus mRNAs synthesized by viral core particles *in vitro* to show an association of mRNA with ribosomes.^{268,270,271} From these studies, reovirus mRNAs were found to have 5' terminal sequences blocked or capped by a 7-*N*-methylguanosine linked 5' to 5' with a conserved GpCpUpA sequence.^{268,271} The 7-*N*-methylguanosine cap is required for efficient recognition of reovirus mRNAs by 40S ribosomal subunits.²⁷¹ However, nucleotide sequences that vary between gene segments also contain structural information required for efficient association of reovirus mRNAs with ribosomes.⁴⁰⁶ The structural information contained in the 5' sequences of reovirus mRNAs also may be important determinants of viral host range, as avian reovirus transcripts do not initiate protein synthesis in mammalian cells.^{43,304}

Unlike host mRNAs, reovirus mRNAs lack poly(A) tails and have relatively short 3' NTRs (35–83 nt) that terminate with UpCpApUpC-3'. A poly(A) tail is thought to promote translation by stabilizing mRNA and facilitating translational reinitiation by ribosomes as a consequence of mRNA circularization via interactions of the poly(A)-binding protein with the 3' poly(A) sequence and eIF-4G, which binds the cap-binding protein, eIF-4E.⁵²⁰ A similar mechanism for reovirus translation has not been found but is postulated.³⁴⁸ Translation enhancing and repressing elements are present in the 3' NTR of the *s4* mRNA,³³⁶ which is the most efficiently translated viral transcript.¹⁹ It is likely that such elements also are present in the 3' NTRs of other segments. The 3' NTR of *s4* mRNA is bound by cellular proteins that may be involved in translational control.³³⁶

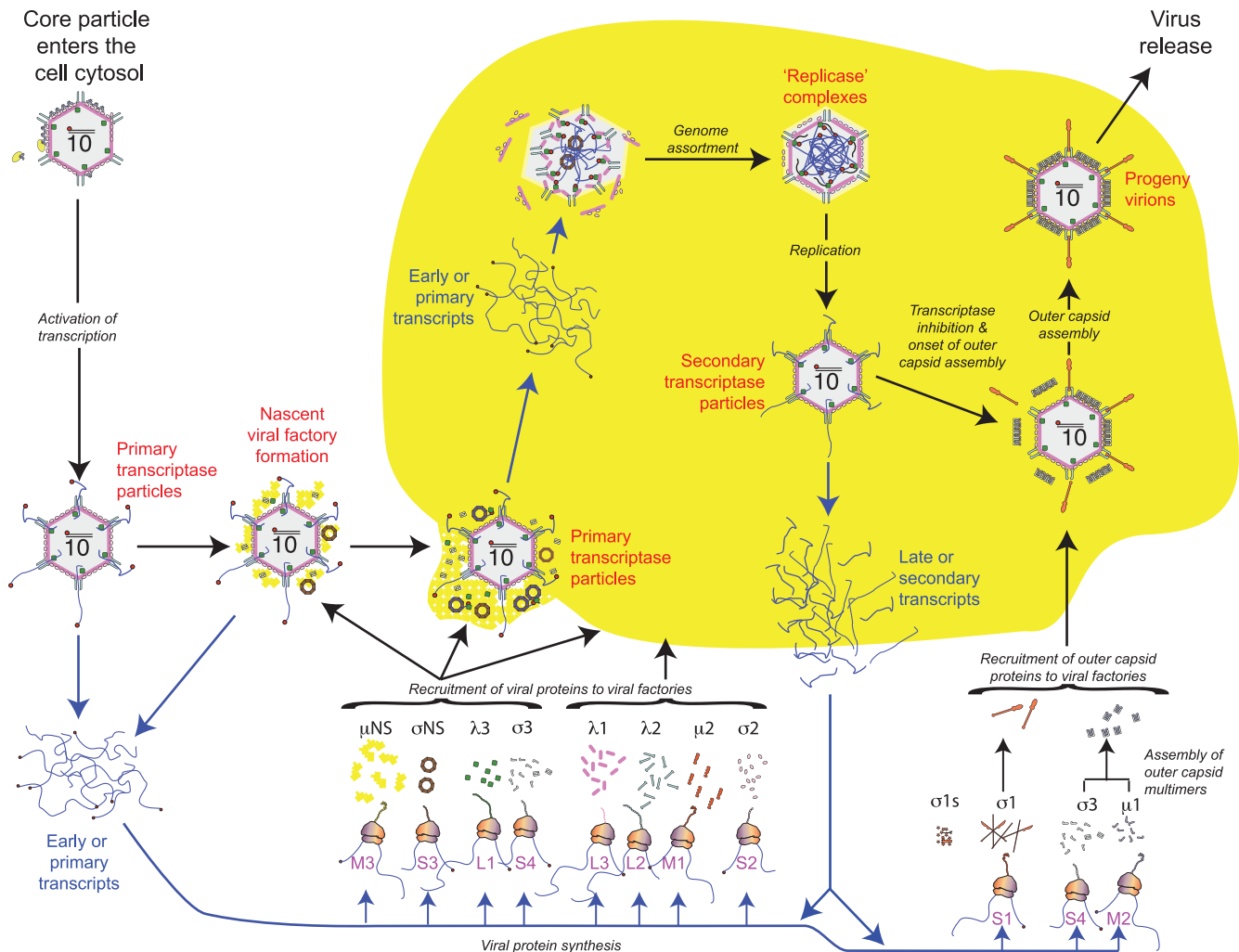


FIGURE 44.11. Schematic of reovirus genome replication and assembly. Following virus entry, a transcriptionally active sub-viral particle (primary transcriptase particle) is deposited in the cytoplasm and begins synthesis of capped viral transcripts (early or primary transcripts). The viral transcripts encode viral proteins, including the nonstructural protein μ NS, which surround and enclose the primary transcriptase complex to form a nascent viral factory (VF) also called a viral inclusion body (indicated in yellow). A subset of viral proteins (μ NS, σ NS, λ 3, and σ 3) may be synthesized early and are recruited to VFs. Assembly of new virions begins with the assortment of each of the 10 primary transcripts together with the viral structural proteins that form core particles (λ 1, λ 2, λ 3, μ 2, and σ 2). The details of this process are unknown but may involve binding of the 5' cap of the viral mRNA and its 3' end to a viral replicase complex, which may be formed by λ 1, λ 2, λ 3, and μ 2. Stoichiometric assortment of each of the 10 viral mRNAs into a complex is thought to require specific RNA–RNA interactions. Only single molecules of each of the 10 mRNA species are packaged. Following assortment, a putative “replicase” complex forms that rapidly replicates each of the viral mRNAs to form the 10 dsRNA genome segments. This process is accompanied by condensation of the viral core proteins to form a nuclease-resistant secondary transcriptase particle. During infection, the majority of viral transcripts are produced by secondary transcriptase particles. These transcripts are not incorporated into new virus particles, perhaps because they lack a cap. In a poorly understood process, the transcriptase activity of the secondary transcriptase particles is inhibited, and the viral outer-capsid proteins μ 1, σ 3, and σ 1 are added to the core particles to form progeny virions. Progeny virions are then released from the infected cells using a poorly understood process that may involve lysis of some cell types. Blue lines and arrows indicate movement of viral RNA. Black arrows indicate sequential steps.

Reovirus core particles synthesize m^7G -capped viral mRNAs both *in vitro* and in infected cells. Capped viral mRNAs are synthesized early after infection by core particles introduced into the cytosol following disassembly (Fig. 44.11). However, as infection progresses in at least some types of cells, such as mouse L929 cells, there is a transition from capped to uncapped viral mRNAs that have the structure [5'-pGpC...]

with uncapped viral mRNAs being first detected approximately 4 to 6 hours postinfection and peaking approximately 12 hours postinfection.⁴⁶⁰ The uncapped viral mRNAs are translated much more efficiently than capped viral or host mRNAs in extracts from infected L929 cells collected 10 hours postinfection.⁴⁶⁰ Addition of the σ 3 protein to lysates prepared from uninfected L929 cells substantially increases the efficiency

of uncapped viral mRNA translation.²⁸¹ The mechanism by which $\sigma 3$ enhances the preferential translation of uncapped viral mRNAs is not known, but $\sigma 3$ may function to modify the activity of a translational initiation factor such that it recognizes uncapped viral mRNAs. Core particles also transcribe uncapped viral mRNAs *in vitro* to varying extents depending on the reaction conditions.¹⁸⁰ It is possible that uncapped mRNAs are synthesized in cells by progeny viral core particles.⁵⁴⁵ Unlike the core particles that initiate infection, progeny core particles have repressed guanylyltransferase and methyltransferase activities. Interestingly, the guanylyltransferase and methyltransferase activities of progeny core particles can be stimulated by chymotrypsin,⁴⁵⁹ suggesting that these activities are triggered by viral disassembly. Although the evidence for uncapped viral mRNAs in L929 cells is compelling, uncapped viral mRNAs are not found in SC-1 cells.¹⁴¹ These differences may be attributable to differences in methodology or to cell type-specific effects on viral transcription.

The 10 reovirus gene segments encode mRNAs for synthesis of 12 viral proteins (Table 44.4 and Fig. 44.11). Viral protein synthesis gradually increases during infection with a concomitant decrease in cellular protein synthesis⁴⁴⁰ (see Responses of the Host Cell to Infection—Inhibition of Cellular RNA and Protein Synthesis and Induction of Cellular Stress Responses). Synthesis of viral proteins can be detected as early as 2 to 3 hours after infection. However, because the L1, M3, S3, and S4 gene segments are transcribed before the other gene segments, the $\lambda 3$, μ NS, σ NS, and $\sigma 3$ proteins encoded by these gene segments will be synthesized early.²⁷⁶ By approximately 10 hours postinfection, most of the proteins synthesized within infected cells are viral in origin. The rates of synthesis of the individual proteins vary, but their relative levels remain constant throughout infection.

For example, the $\mu 1$ protein, the major outer-capsid protein, is synthesized at 20-fold greater levels than the $\sigma 1$ protein despite only a twofold difference in mRNA levels.

Translation of reovirus transcripts is tightly regulated.²⁴⁸ Some of the factors that influence the differential rates and frequencies of translation of reovirus mRNAs include: (1) nucleotide sequences at the -3 and $+4$ positions flanking the AUG start codon; (2) length of the 5' NTRs, which may affect translation rate (those with the longest 5' NTRs are translated most efficiently); (3) conserved sequence motifs that extend beyond the 5' and 3' NTRs within the mRNA, which may recruit specific translation initiation factors; (4) transcript length, which influences translational frequency (shorter transcripts are translated at higher levels); and (5) cellular and viral RNA-binding proteins. The viral σ NS,¹⁸⁶ μ NS,¹⁵ and $\sigma 3$ ²³¹ proteins bind ssRNA, and within viral assembly sites, all or a subset of these proteins may preferentially retain viral transcripts. In addition, stress granules are induced early after reovirus infection³⁸⁴ (see Responses of the Host Cell to Infection—Inhibition of Cellular RNA and Protein Synthesis and Induction of Cellular Stress Responses). The induction of stress granules requires disassembly of virions but does not require *de novo* synthesis of viral RNA or protein. However, the recruitment of entering core particles to stress granules requires viral transcription.³⁸⁴ It is not clear whether transcripts synthesized within stress granules are eventually translated.

Viral Factories

Reovirus replication and assembly occur within cytoplasmic structures called viral factories (VFs) or viral inclusion bodies (VIBs) (Figs. 44.11 and 44.12). VFs are first observed as round punctate structures 2 to 4 hours postinfection, then increase in

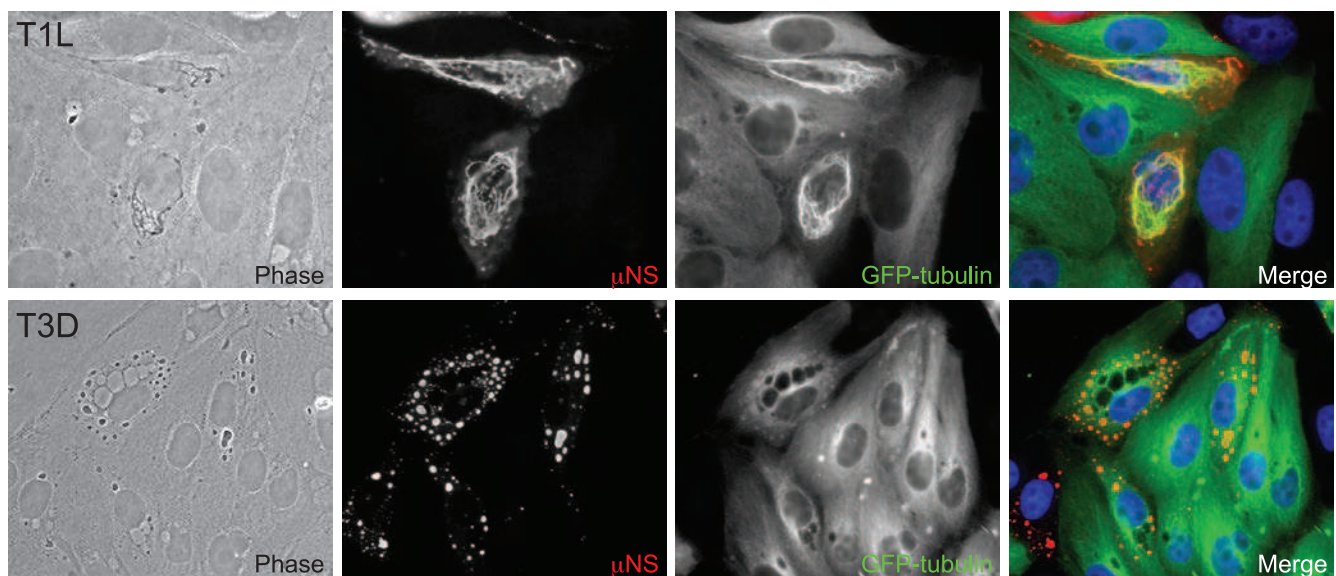


FIGURE 44.12. Reovirus forms inclusion complexes in infected cells. Most reovirus strains (e.g., type 1 Lang [T1L], *top*) form filamentous inclusions (also called viral factories or viral inclusion bodies) within infected cells that are associated with the microtubular cytoskeleton. However, a few strains (e.g., type 3 Dearing [T3D], *bottom*) form globular inclusions due to a temperature-sensitive mutation in the $\mu 2$ protein (P208S). To obtain these images, LLPCk cells expressing GFP-tubulin were stained with an antiserum specific for viral nonstructural protein μ NS, which localizes to inclusions, at 24 hours postinfection. The μ NS protein is shown in *red*, and GFP-tubulin, which marks microtubules, is shown in *green*.

size over time.^{33,68,373} In some infected cells, VFs occupy greater than 50% of the cytosol by 24 to 48 hours postinfection and completely surround the nucleus. Reovirus VFs initially appear as globular structures (Parker and Nibert, unpublished). However, VFs of most strains appear filamentous in morphology by ~12 hours postinfection as a consequence of their association with stabilized microtubules (373; Parker and Nibert, unpublished). In thin-section electron micrographs, VFs contain both assembling and complete viral particles embedded within a matrix composed of thin, kinked fibrils.¹¹⁹ VFs exclude ribosomes and most cellular organelles and are not surrounded by membranes. Microtubules are incorporated into VFs and responsible for the filamentous appearance of VFs formed by most reovirus strains.³⁷³

The VF matrix is formed by the M3-encoded nonstructural protein, μ NS (80 kDa, 721 aa) (Fig. 44.12). Ectopic expression of μ NS leads to formation of globular structures in the cytoplasm that are similar to the VFs formed by reovirus strain T3D.⁶⁸ The C-terminal 250 amino acids (aa 471–721) of μ NS is the minimal region required for formation of these structures.⁶⁵ This region of μ NS contains two predicted coiled-coil regions flanked by a linker that contains a predicted CCHC Zn-binding motif. This motif is required for formation of structures with the morphology of VFs. The μ NS protein specifically interacts with and recruits the viral structural proteins found in core particles (λ 1, λ 2, λ 3, μ 2, and σ 2) and the RNA-binding nonstructural protein σ NS^{66,68,325,326} (Fig. 44.11). These activities may promote efficient assembly of nascent viral transcriptase particles. The capacity of σ NS to be recruited to VFs is partially dependent on its capacity to bind RNA.³²⁶ Newly synthesized viral RNAs are localized to VFs.³²⁵ The regions of μ NS responsible for interacting with viral core proteins have been mapped to specific nonoverlapping regions of the protein.³²⁵

VFs are thought to promote virus replication and assembly by sequestering and concentrating viral RNA and structural proteins (Fig. 44.11). In addition, VFs may protect viral RNA from recognition by the intracellular pattern-recognition receptors, RIG-I and Mda-5. The N-terminal region of μ NS (aa 1–41), which interacts with viral structural protein μ 2^{68,325} and nonstructural protein σ NS,³²⁶ is required for viral replication, as a truncated version of μ NS lacking these residues does not complement siRNA-mediated knockdown of wild-type μ NS.²⁶³ An N-terminally shortened version of μ NS called μ NSC that lacks the first 40 amino acids of μ NS is synthesized in infected cells.²⁷⁸ It is proposed that μ NSC is synthesized as a consequence of translation initiation from an alternative, in-frame start codon in the *m3* mRNA.⁵²⁵ The function of μ NSC in infected cells is not known, but it is not required for viral replication.²⁶³ It also is not known how outer-capsid proteins μ 1, σ 3, and σ 1 are recruited to VFs. However, it is apparent from immunofluorescence images that these proteins are present in these structures (33,34; Coffey and Parker, unpublished).

The vast majority of viral transcripts are likely synthesized and extruded from transcriptionally active viral particles buried within the μ NS matrix of VFs (Fig. 44.11). When purified viral core particles and μ NS are mixed *in vitro*, the μ NS protein polymerizes around the core particles and prevents them from being coated with outer-capsid proteins.⁶⁷ Recoating of cores with outer-capsid proteins inhibits transcription.¹⁶⁷ However, cores buried within the μ NS matrix are capable of transcription when provided with suitable substrates and an energy regeneration

system.⁶⁷ It is likely that incoming transcriptionally active core particles become embedded within nascent VFs following synthesis of μ NS, as genome-lacking cores rapidly become embedded within μ NS VF-like structures when they enter the cytosol of cells ectopically expressing μ NS.⁶⁶ Thus, μ NS appears to protect the incoming viral cores that catalyze primary transcription from being prematurely inactivated by the addition of *de novo* synthesized outer-capsid proteins.

The VF matrix protein μ NS contains a conserved C-terminal clathrin-box motif (711-LIDFS-715) that recruits clathrin heavy and light chains to VFs. Cellular functions dependent on clathrin, including endocytosis and secretion, are inhibited in cells infected with wild-type reovirus but not a recombinant reovirus mutant with a leucine-to-alanine substitution at position 711 of μ NS (μ NS-L711A).²³⁹ The μ NS-L711A mutant displays only mildly impaired replication efficiency and normal VF formation. Reovirus-induced inhibition of viral endocytosis and secretion is modest and correlates with VF size in infected cells, and thus occurs late in infection. It is possible that sequestration of clathrin in VFs and concomitant inhibition of endocytosis prevents superinfection of cells.²³⁹

The viral μ 2 protein binds to and stabilizes microtubules.^{256,373} In addition, μ 2 binds to μ NS, which links the VF matrix to the microtubule cytoskeleton.^{68,373} One isolate of T3D used in many laboratories does not form filamentous VFs but instead forms globular VFs⁶⁸ (Fig. 44.12). This change in VF morphology is a consequence of a temperature-sensitive mutation in μ 2 (P208S). In cells grown at 31°C, T3D forms filamentous VFs. However, at 37°C, T3D VFs are globular, and the μ 2 protein is ubiquitinated and targeted for proteasomal degradation.³²⁷ Despite this defect, the T3D μ 2 allele appears to function adequately as a co-factor for the λ 3 polymerase within core particles, as there is no significant impairment in the capacity of this strain to infect cultured cells or cause disease in mice. The association of μ 2 with microtubules is not required for VF formation, as dissolution of microtubules by treatment with nocodazole does not reduce viral titer in tissue culture.³⁷³

Genome Assortment and Replication

The process by which the reovirus gene segments are assorted and packaged into nascent viral cores is a major mystery. The fidelity of packaging and the lack of evidence of mispackaging suggest a specific mechanism. It is thought that equimolar amounts of each of the viral mRNAs associate with nonstructural proteins and structural proteins of the core, then condense to form a replicase particle that copies the [+] strand mRNA yielding a [–] strand partner for each of the 10 RNA gene segments.¹⁵ Soon after synthesis, viral [+] strands associate with μ NS, σ NS, and σ 3. The association of core protein λ 2 with these RNA-protein complexes occurs concurrently with the onset of [–] strand synthesis.¹⁵ It is possible that mRNAs representing each gene segment are complexed with the λ 3 polymerase to form circular RNAs via interactions of the 5′ cap with the polymerase cap-binding site and the 3′ terminus with the polymerase template-binding site. Specific assortment and condensation of the RNAs would then presumably be driven by RNA–RNA interactions, perhaps aided by viral core proteins λ 1, λ 2, and μ 2.

Assembly and Release

Little is known about how reovirus particles are assembled in infected cells. Complete and incomplete viral particles are

observed in VFs by electron microscopy. Synthesis of dsRNA likely occurs in parallel with core assembly, as conditions that inhibit polymerase activity in cells yield core-like particles that contain less than complete dsRNA genome complements.^{1,425} Assembly of the outer capsid likely occurs following assembly of the core (Fig. 44.11). However, outer-capsid shells can assemble in the absence of fully formed cores, as such shells are observed by electron microscopy following replication of some temperature-sensitive reovirus mutants at restrictive temperatures.¹⁷⁰ It is not understood how the outer-capsid proteins condense onto cores in cells. Recoating of core particles with outer-capsid proteins *in vitro* inhibits transcription at the level of elongation.¹⁶⁷ Assembly of the outer capsid onto cores may be regulated in cells to prevent premature termination of transcription. Cellular chaperones distributed to VFs may act to promote specific viral assembly steps.

Empty core-like particles form in cells that express the $\lambda 1$, $\lambda 2$, and $\sigma 2$ proteins or the $\lambda 1$, $\lambda 2$, $\lambda 3$, and $\sigma 2$ proteins. Minimally, a shell will form if $\lambda 1$ and $\sigma 2$ are co-expressed; $\lambda 3$ can be incorporated if it is co-expressed with $\lambda 1$ and $\sigma 2$.⁵³⁵ The pentameric $\lambda 2$ turret cannot assemble when $\lambda 2$ is expressed alone,³⁰⁵ suggesting that formation of a shell or partial shell by the $\lambda 1$ and $\sigma 2$ proteins is required to precipitate oligomerization of $\lambda 2$. “Spikeless” shells that lack the $\lambda 2$ protein assemble in cells infected with temperature-sensitive mutant tsA279, which carries a mutation in the $\lambda 2$ -encoding L2 gene segment, at the restrictive temperature.²¹⁵ Since the $\lambda 2$ protein also can interact with $\lambda 3$,⁴⁷⁰ it is possible that assembly of the core involves a complex of $\lambda 3$ (associated with viral [+] strand RNA) with a pentamer of $\lambda 2$ interacting with a partial shell of $\lambda 1$ and $\sigma 2$.⁵³⁵

The reovirus outer capsid consists of 200 heterohexameric assemblies of the $\mu 1$ and $\sigma 3$ proteins arranged in a T = 13 *laevo* lattice.²⁸⁹ Co-expression of $\mu 1$ and $\sigma 3$ in insect cells leads to formation of $\mu 1:\sigma 3$ heterohexamers that can assemble onto cores *in vitro*.⁸³ Isolated $\sigma 3$ crystallizes as a dimer and may bind to dsRNA in that conformation.³⁶⁸ The $\mu 1:\sigma 3$ heterohexamer structure (Fig. 44.9) suggests that $\mu 1$ monomers must form trimers before addition of $\sigma 3$ monomers to each of the $\mu 1:\mu 1$ interfaces.²⁸⁹ This structural evidence suggests that $\sigma 3$ can exist in two conformations, dimers and monomers. Of note, $\sigma 3$ becomes sensitive to proteolysis when complexed with $\mu 1$ to form $\mu 1:\sigma 3$ heterohexamers,⁴⁴⁶ suggesting that such conformers exist.

The trimeric $\sigma 1$ attachment protein requires cellular chaperones Hsc70 and Hsp90 for assembly.^{188,282} Assembly of the trimeric $\sigma 1$ protein as part of the outer capsid requires that the $\lambda 2$ turret initially be open and then close to lock the $\sigma 1$ tail into place. This assembly reaction also may require cellular chaperones. The encapsidation of $\sigma 1$ onto virions presumably occurs within VFs, but this is not known. Interestingly, the cellular chaperone Hsc70 also specifically associates with the μ NS viral matrix protein in a manner that does not require its chaperone activity.²⁵¹

Mechanisms governing reovirus egress from infected cells are not understood. It is possible that virus is released from some types of cells following lysis, but how this occurs is not known. A large quantity of infectious virus remains associated with cellular debris following cell death,⁴⁶⁶ perhaps in association with VF matrices. Reovirus-induced apoptosis also

may promote virus release from infected cells,³⁰⁷ although the mechanism of such an egress pathway is unclear.

RESPONSES OF THE HOST CELL TO INFECTION

Interferon Induction and Effects on Viral Replication

Activation of cellular sensors during infection by many types of viruses leads to recruitment of adaptor proteins and consequent stimulation of transcription factors ATF-2/c-Jun, interferon regulatory factor-3 (IRF3), and NF- κ B that induce transcription and secretion of type I IFNs (IFN- α 4 and IFN- β) (Fig. 44.13). Secreted IFN binds to IFN- α/β receptors on the surface of infected and surrounding cells and, by so doing, activates Jak1 and Tyk2 kinases that phosphorylate and activate the transcription factors STAT1 and STAT2. Activated STAT1 and STAT2 associate with IRF9 to form the heterotrimeric ISGF3 transcription factor complex, which translocates to the nucleus to induce transcription of hundreds of interferon-stimulated genes (ISGs), some of which are antiviral, and one of which, IRF7, is a latent transcription factor (Fig. 44.13). When IRF7 is activated by virus-induced phosphorylation, it can form homodimers or heterodimers with IRF3 to induce multiple IFN- α subtypes and further induce IFN- α 4 and IFN- β in a positive amplification loop.

Reovirus induction of IFN requires virion RNA, as fully assembled double-layered viral particles lacking the dsRNA genome (empty particles) fail to activate IRF3/7 or induce IFN.^{225,274} However, viral replication is dispensable for activation of IRF3/7.²²⁵ IRF3 is required for reovirus induction of IFN- β in at least some cell types,^{224,357} while IRF1, a related IRF, is not.²⁰ While one study demonstrated that reovirus activation of IRF3/7 requires the sensor RIG-I and its adaptor IPS-1,²²⁵ other studies demonstrated that reovirus-induced expression of ISGs can be mediated by either RIG-I or the related sensor MDA-5.^{250,291} This discrepancy may reflect cell type-specific differences or the assessment of different indicators (IRF3/7 vs. ISGs). Indeed, in myeloid dendritic cells, IFN induction by reovirus requires the helicases DHX9⁵⁵² and a complex of the helicases DDX1, DDX21, and DHX36, and the adaptor molecule TRIF.⁵⁵¹ Activation of NF- κ B requires IKK α and IKK γ /Nemo²⁰⁹ and is not dependent on RIG-I or IPS-1,²²⁵ suggesting a different trigger for stimulation of this innate immune response transcription factor. RIG-I and MDA-5 can be activated by reovirus dsRNA gene segments of different lengths.²⁵⁰ However, it is not clear how the viral gene segments might be exposed during reovirus replication. It is possible that RIG-I is activated by release into the cytosol of the short oligonucleotides present in reovirus particles. At least a portion of these oligonucleotides contain an unmodified 5' triphosphate group,³⁸ which might stimulate RIG-I.³⁷⁷

Reovirus induces secretion of IFN- β in immortalized cells^{274,461} and primary cell cultures.^{199,452} However, the degree to which reovirus induces IFN is in part dependent on cell type. For example, reovirus induces significantly more IFN- β in cardiac myocytes than in cardiac fibroblasts *in vitro*⁴⁷⁴ and *in vivo*,²⁸⁸ likely as a consequence of differences in the basal expression of components of the IFN response in these cells.⁵⁵⁵

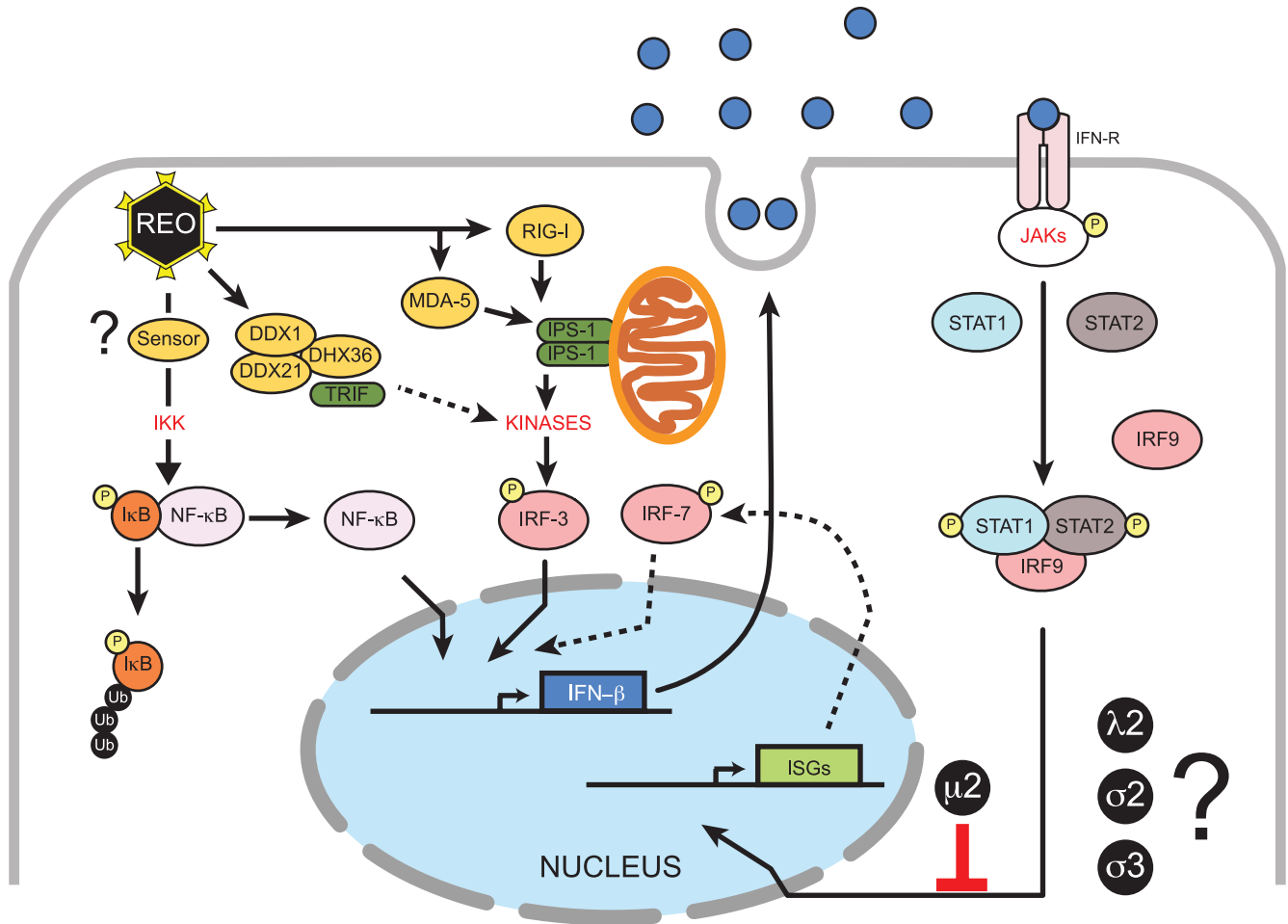


FIGURE 44.13. Reovirus induces interferon expression and represses interferon signaling. Reovirus activation of IRF3 and NF-κB for induction of interferon (IFN) is cell type–specific and utilizes several pathways. Reovirus activates RIG-I or Mda-5, which then stimulate IPS-1 to induce phosphorylation and activation of transcription factor IRF3. Reovirus activation of NF-κB does not require RIG-I or IPS-1. Reovirus also activates DDX1/DDX21/DHX36 and TRIF to induce IFN. Reovirus strain-specific differences in the induction of IFN are associated with the *M1*, *L2*, and *S2* gene segments, which encode μ2, λ2, and σ2, respectively. Reovirus strain-specific differences in sensitivity to IFN are associated with these same gene segments. A single amino acid polymorphism in μ2 determines the capacity of this protein to repress IFN signaling, which affects induction of antiviral genes and further induction of IFN through a positive amplification loop. The σ3 protein is an RNA-binding protein that influences viral induction of IFN. Dotted arrows indicate indirect pathways (TRIF, kinases) or pathways that occur later in infection for induction of additional IFN (amplification loop). Question marks indicate unknown proteins or unknown functions.

Reovirus induces detectable levels of only a subset of IFN-α subtypes in cardiac cells.²⁸⁸

Differences in the induction of IFN by reovirus are also strain dependent. Reovirus T3D induces significantly more IFN than does T1L in cardiac myocytes; this difference segregates with the *M1*, *S2*, and *L2* gene segments.⁴⁵² The *M1*-encoded μ2 protein from T1L but not from T3D represses IFN signaling.⁵⁵⁶ Repression is associated with alterations in the transcription factor IRF9,⁵⁵⁶ but the mechanism underlying the repression is not known. A single amino acid polymorphism at μ2 residue 208 (proline in T1L, serine in T3D) determines the capacity of these viruses to repress IFN signaling.²³⁵ Interestingly, this polymorphism in μ2 also is responsible for strain-dependent differences in the morphology of VFs and levels of ubiquitylation of μ2³²⁷ (See Replication—Viral Factories). The μ2 protein may modulate IFN levels by repressing IFN-induced IRF7, thus

preventing further induction of IFN. Indeed, mutation of this repressor function alters reovirus induction of IFN-β.²³⁵ Possible roles for the *S2* and *L2* gene segments in IFN induction are unclear. The sensitivity of reovirus to the antiviral effects of IFN is also strain dependent. Reovirus T3D is more sensitive than T1L to the antiviral effects of IFN^{242,452}; this phenotype also segregates with the *M1*, *S2*, and *L2* gene segments.⁴⁵² It is possible that μ2 modulates RNA-mediated activation of antiviral ISGs as a consequence of its capacity to bind RNA.⁶³ However, it is more likely that by repressing IFN signaling μ2 allows the virus to evade the effects of IFN.

The reovirus *S4*-encoded σ3 protein binds dsRNA^{135,328,368,432,511} and can modulate the type I IFN response.⁴⁷ Several antiviral ISGs are activated either directly or indirectly by dsRNA. For example, dsRNA binds to and activates protein kinase R (PKR), which phosphorylates and inactivates eukaryotic

translation initiation factor eIF2 α , thereby inhibiting translation and potentially viral replication. Although there is no direct evidence that $\sigma 3$ blocks reovirus dsRNA induction of IFN during infection, multiple lines of evidence suggest that this is likely the case. The reovirus $\sigma 3$ protein prevents dsRNA-mediated activation of PKR *in vitro*²³⁴ and in cells.⁵⁴³ Moreover, $\sigma 3$ can substitute for analogous dsRNA-binding proteins or repressors of PKR in other viruses.^{32,181,290} PKR is activated by reovirus in IFN-treated cells²⁰⁵ and is required to protect mice against reovirus-induced disease.⁴⁷³ However, activation of PKR does not inhibit reovirus replication or limit its cytopathic effect.⁵⁴⁸ In fact, reovirus may actually benefit from PKR activation.⁴⁶² Viral dsRNA also binds to and activates 2',5'-oligoadenylate synthetase, whose products then activate the ssRNA endonuclease RNase L, another antiviral ISG. Reovirus activates RNase L in IFN-treated cells,³⁵⁶ and RNase L can cleave reovirus mRNA *in vitro*.²³ However, paradoxically, RNase L does not inhibit reovirus replication or cell damage and, as is the case for PKR, some reovirus strains produce lower titers in cells that lack RNase L.⁴⁶²

Inhibition of Cellular RNA and Protein Synthesis and Induction of Cellular Stress Responses

Some strains of reovirus inhibit the synthesis of cellular RNA and protein. The mechanism by which reovirus modulates host-cell RNA and protein synthesis is not known, but viral replication is required. Differences in the capacity of reovirus strains to inhibit cellular RNA and protein synthesis segregate with the $\sigma 3$ -encoding S4 gene segment.⁴⁴⁰ Reovirus inhibition of protein synthesis involves both PKR and RNase L and likely other IFN-stimulated gene products.⁴⁶² Virus strain-specific differences in inhibition of cellular protein synthesis⁴⁴⁰ likely reflect the capacity of the $\sigma 3$ protein to repress dsRNA-mediated activation of PKR.^{234,290,355,543} The $\sigma 3$ protein is incapable of binding dsRNA when it assembles into heterohexameric complexes with the $\mu 1$ protein during capsid morphogenesis.^{47,289,368,433,447,543} Thus, strain-specific differences in the efficiency of assembly of $\mu 1$: $\sigma 3$ complexes could determine the bioavailability of $\sigma 3$ conformers capable of binding dsRNA. Ectopically expressed $\sigma 3$ is distributed in the nucleus and cytoplasm. The subcellular distribution of $\sigma 3$ in infected cells correlates with the capacity of different strains to inhibit cellular protein synthesis.^{433,544} One attractive hypothesis is that $\sigma 3$ modulation of PKR activity and inhibition of cellular protein synthesis is determined by differences in the subcellular distribution of free $\sigma 3$, which is in turn determined by the kinetics of assembly of $\mu 1$: $\sigma 3$ complexes. Accordingly, protein synthesis inhibition may be restricted by subcellular localization of free $\sigma 3$.⁴³³

In addition to inhibition of cellular protein synthesis, the preferential translation of reovirus mRNAs likely requires other as yet unidentified mechanisms. In some infected cells, reovirus transcripts produced later in infection lack 5' caps (see Replication—Translation and Viral Factory Formation). However, it is not clear whether the absence of caps functions in the preferential translation of reovirus transcripts. Reovirus infection induces the formation of stress granules,^{383,384,461} which are discrete cytoplasmic structures that sequester mRNAs and prevent their translation. Reovirus induces stress granules early in infection using a mechanism that is dependent on phosphorylation of eIF2 α .³⁸⁴ Later in infection, stress granules in infected cells are disrupted by a mechanism independent of PKR and eIF2 α phosphorylation status and which correlates with the release

of viral mRNAs from translational inhibition.³⁸³ Under conditions in which eIF2 α is phosphorylated and concentrations of ternary complexes required for translational initiation are limited, it is likely that the preferential translation of reovirus mRNAs requires other mechanisms. However, the dissolution of stress granules may be necessary for efficient translation of viral mRNAs.

Inhibition of Cellular DNA Synthesis and Cell-Cycle Progression

Some reovirus strains inhibit cellular DNA synthesis, which is noted by 8 hours postinfection.¹⁹⁶ Early studies showed that UV-inactivated virus⁴⁴⁵ but not empty virus particles²⁷³ inhibits cellular DNA synthesis, suggesting that virion-associated RNA but not viral transcription is required for cellular DNA synthesis inhibition. However, expression of nonstructural protein $\sigma 1s$ is required for cell-cycle arrest at the G₂/M checkpoint, raising the possibility that the UV treatment used in the initial experiments may have been insufficient to completely shut-down viral transcription. Differences in the capacity of strains T1L and T3D to inhibit cellular DNA synthesis segregate with the S1 gene segment,⁴³⁹ which encodes the $\sigma 1$ and $\sigma 1s$ proteins. Purified $\sigma 1$ can arrest G₁-to-S progression, presumably by transducing signals following ligation of cell-surface receptors.⁴²⁸ Strain-specific differences in G₂/M arrest similarly segregate with the S1 gene segment.³⁸⁰ However, $\sigma 1s$ rather than $\sigma 1$ stimulates the inhibitory hyperphosphorylation of cell cycle control kinase p34 (cdc2) to bring about G₂/M arrest.³⁷⁹ Reovirus-induced G₂/M arrest also may reflect modulation of the mitotic spindle checkpoint as a consequence of virus association with microtubules and disruption of their association with the kinetochore.¹¹⁸ Analysis of strain-specific differences in reovirus-induced cellular gene expression by transcriptional profiling identified several genes that may be involved in reovirus modulation of G₁-to-S, G₂-to-M, and the mitotic spindle checkpoint.³⁷⁸ The benefit of blocking cell-cycle progression for reovirus replication is not known.

Induction of Apoptosis and Activation of NF- κ B

Apoptosis induced by reovirus is a major pathogenic mechanism underlying disease in infected mice (see Tissue Damage and Disease). Reovirus infection leads to apoptosis in many cell lines^{102,112,403,500} and primary cultures of differentiated cells.^{131,396} However, there is frequently little correlation between apoptosis induction and yield of progeny virus.^{131,403,500} Many studies of apoptosis induced by reovirus assessed viral yield by artificially lysing infected cultures, perhaps obscuring a role for apoptosis in facilitating release. Indeed, when assessed directly, apoptosis facilitates release from Ras-transformed cells,³⁰⁷ suggesting a possible function for apoptosis in the reovirus replication cycle.

Reovirus strains vary in the capacity to induce apoptosis in cell lines and primary cultures. For example, strains T3A and T3D induce significantly higher levels of apoptosis than does T1L; this difference segregates with the S1 and M2 gene segments.^{403,500,501} A variant virus that fails to express the S1-encoded $\sigma 1s$ protein induces apoptosis, implicating $\sigma 1$ in the cell-death response.⁴⁰⁴ However, $\sigma 1s$ appears to function in reovirus-induced apoptosis in the murine brain and heart,²²⁷ suggesting that there may be cell type-specific requirements for the S1 gene products in evoking cell death. Reovirus induces apoptosis in ribavirin-treated cells,¹¹¹ and UV-inactivated

virus⁵⁰⁰ and empty particles¹¹¹ induce apoptosis, demonstrating that neither viral RNA synthesis nor virion genomic dsRNA are required for apoptosis, at least in some contexts. Together, these data suggest that attachment, disassembly, and penetration are important steps in cell-death induction by reovirus.

The S1-encoded $\sigma 1$ protein of some reovirus strains binds to sialic acid as a first step in cell engagement,²⁶ which is followed by subsequent binding to JAM-A²⁷ (see Replication-Attachment). For at least some of these strains, sialic acid binding is required for inducing maximum levels of apoptosis.¹¹⁰ Regardless of sialic acid-binding capacity, reovirus binding to JAM-A is required for apoptosis during infection.²⁷ However, uptake of antibody-coated reovirus into cells expressing Fc receptors leads to apoptosis, demonstrating that JAM-A signaling is not required for this form of cell death.¹²² These observations suggest that $\sigma 1$ functions in reovirus-induced apoptosis by providing strong cell binding or routing the virus to an endocytic compartment where apoptotic signaling is initiated.

Apoptosis induced by reovirus virions is blocked by drugs that inhibit acid-dependent proteolytic disassembly, an effect that can be bypassed by *in vitro*-generated ISVPs.¹¹¹ These findings indicate that apoptosis induction requires virion-to-ISVP disassembly. Membrane penetration by the reovirus disassembly intermediate also is required for apoptosis.¹²³ However, membrane penetration likely serves primarily to deliver the $\mu 1$ effectors of apoptosis to the cytoplasm. Indeed, a mutation in the ϕ domain of $\mu 1$ that does not affect membrane penetration decreases the strength of the proapoptotic signal, indicating that the ϕ domain of $\mu 1$ regulates apoptosis following penetration.¹²¹ Concordantly, plasmid-based expression of the $\mu 1$ ϕ domain is sufficient to induce apoptosis.¹⁰⁷

Apoptosis can be elicited by mitochondrial damage via the intrinsic pathway or activation of death receptors via the extrinsic pathway. In many cell types, reovirus infection activates both intrinsic and extrinsic apoptotic pathways (Fig. 44.14), although in some cell types, one mechanism may predominate.¹³⁰ The intrinsic apoptotic pathway requires activation of pro-apoptotic Bcl-2 family members Bak and Bax, which induce permeabilization of the outer mitochondrial membrane, resulting in release into the cytosol of cytochrome *c* and Smac/DIABLO. Cytochrome *c* couples with dATP in the cytosol to trigger oligomerization of Apaf-1 to form a complex called the apoptosome, which recruits and activates caspase 9. Caspase-9 can subsequently proteolytically process and activate effector caspases 3 and 7. Smac/DIABLO represses inhibitor of apoptosis proteins (IAPs), which prevent activation of effector caspases. The extrinsic pathway is stimulated by ligand binding to cell-surface death receptors. Death receptor oligomerization following ligand binding leads to recruitment of adaptor proteins (such as FADD) that form the death-inducing signaling complex (DISC). Once formed, the DISC leads to activation of initiator caspase 8, which in turn activates effector caspases 3 and 7. In some cells, activated caspase-8 also activates the intrinsic pathway by cleaving pro-apoptotic Bcl-2 family member Bid to form a truncated version of the molecule, called tBid, which associates with the outer mitochondrial membrane and stimulates oligomerization of Bak and Bax.

Reovirus infection induces the release of cytochrome *c* and Smac/DIABLO,²⁶⁶ indicating intrinsic pathway activation. Moreover, overexpression of the antiapoptotic mitochondrial protein Bcl-2 blocks reovirus-induced apoptosis.^{265,403} Reovirus infection also induces expression of TRAIL (a death receptor

ligand) and DR5, a TRAIL receptor,¹⁰² indicating extrinsic pathway activation. Treatment of cells with soluble TRAIL receptor or overexpression of a dominant-negative form of the adaptor protein FADD inhibits reovirus-induced apoptosis.^{102,265} However, activation of the extrinsic pathway alone is insufficient for induction of apoptosis during reovirus infection and must be accompanied by activation of the intrinsic pathway to elicit a full response.^{124,265}

The $\mu 1$ ϕ domain associates with the endoplasmic reticulum and mitochondria and can induce apoptosis in the absence of reovirus infection.¹⁰⁷ Apoptosis induced by either $\mu 1$ or reovirus infection occurs in cells lacking Bak and Bax, suggesting that in the presence of $\mu 1$, tBid is sufficient to disrupt mitochondrial membranes.⁵³³ Interestingly, a peptide corresponding to the minimal region of ϕ responsible for apoptosis can autonomously destabilize membranes,²⁵⁷ raising the possibility that ϕ together with tBid directly mediates mitochondrial release of pro-apoptotic factors.

Reovirus-induced apoptosis requires the activation of NF- κ B in most cell types¹¹² (Fig. 44.14). Interestingly, reovirus strains that induce apoptosis efficiently (e.g., T3A) and poorly (e.g., T1L) activate NF- κ B early in infection, but efficiently apoptotic strains inhibit NF- κ B activation late in infection,¹⁰¹ at least in some cells. Moreover, a poorly apoptotic strain (T1L) can induce apoptosis efficiently when NF- κ B is inhibited late in infection.¹⁰³ Inhibition of NF- κ B late in infection sensitizes cells to TRAIL-mediated apoptosis, linking NF- κ B activation to the extrinsic pathway of apoptosis induction.¹⁰³ Additionally, NF- κ B-induced expression of cFLIP, which inhibits caspase 8 activity, parallels reovirus early stimulation and late inhibition of NF- κ B activation and attenuates apoptosis induced by T1L.¹⁰¹ However, NF- κ B also is required for reovirus-induced cleavage of Bid, which provides a plausible mechanism to connect NF- κ B activation to the intrinsic pathway for reovirus-induced apoptosis.¹²⁴ Reovirus activation of NF- κ B also likely regulates other events in both the extrinsic and intrinsic pathways.^{129,364}

The mechanism by which reovirus activates NF- κ B is likely dependent on virus strain and cell type. In unstimulated cells, NF- κ B is sequestered in the cytoplasm by one of several isoforms of the repressor I κ B. Following stimulation of the classical pathway of NF- κ B activation, the heterotrimeric I κ B kinase (IKK) complex, which is composed of IKK α , IKK β , and IKK γ (Nemo), phosphorylates I κ B α , leading to its ubiquitylation and degradation for release of active NF- κ B.⁴⁸¹ Following stimulation of the alternative pathway of NF- κ B activation, a complex composed of IKK α and NIK phosphorylate an NF- κ B latent component, p100, resulting in its cleavage to the activated NF- κ B moiety p52.⁴⁸¹ Expression of dominant-negative forms of I κ B inhibit NF- κ B activation and apoptosis induced by reovirus,^{103,112} suggesting stimulation of the classical NF- κ B activation pathway. However, reovirus-induced NF- κ B activation and apoptosis require IKK α and IKK γ /Nemo and not the third component of the classical pathway, IKK β ,²⁰⁹ suggesting a novel mechanism for regulation of NF- κ B by reovirus. The ϕ domain of $\mu 1$ is thought to play a role in the activation of NF- κ B, as recombinant viruses with mutations in ϕ (K594D and I595K) have reduced capacity to activate NF- κ B.¹²¹ These mutant viruses are also deficient in apoptosis induction. The mechanism by which the $\mu 1$ ϕ domain activates NF- κ B is unknown.

In contrast to most cells, reovirus-induced apoptosis in cardiac myocytes does not require NF- κ B. Levels of apoptosis

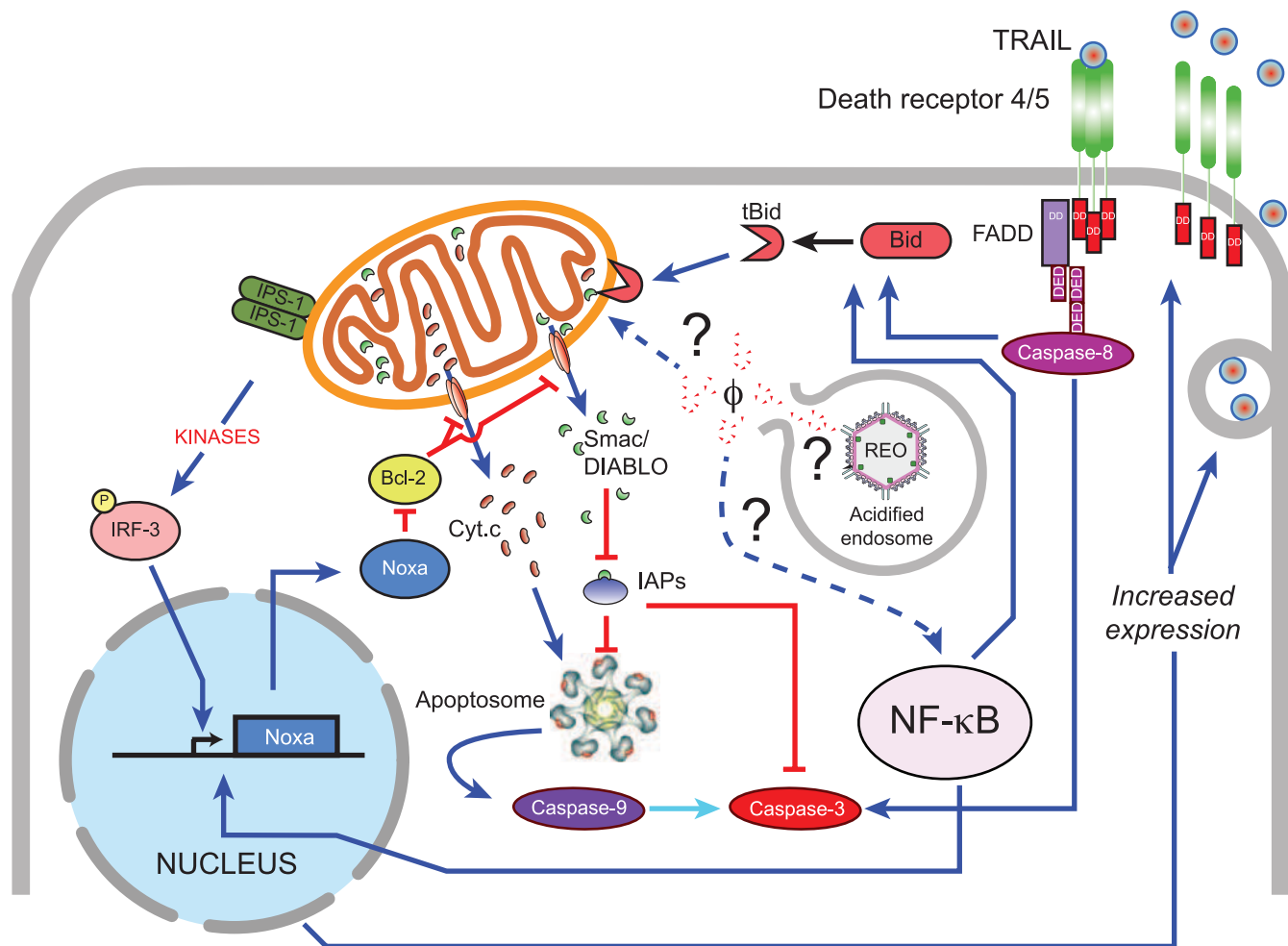


FIGURE 44.14. Reovirus activates both intrinsic and extrinsic apoptotic pathways. Reovirus-induced apoptosis requires disassembly of virions to infectious subviral particles, which leads to release of the $\mu 1$ ϕ fragment. The ϕ fragment may associate with tBid to permeabilize mitochondria. Reovirus stimulates mitochondrial release of cytochrome *c* and Smac/DIABLO, which together activate caspase 9 as part of the intrinsic apoptotic pathway. However, activation of caspase 9 is not required for apoptosis induced by reovirus infection. Instead, the critical event may be release of Smac/DIABLO to repress IAP inhibition of caspase 3. Reovirus activates IPS-1 and IRF3 as well as NF- κ B, resulting in induction of the proapoptotic protein, NOXA, which can inhibit antiapoptotic Bcl-2 family members. The $\mu 1$ ϕ fragment and other reovirus components can activate NF- κ B. Reovirus also induces expression of DR5 and TRAIL, which together activate caspase 8 as part of the extrinsic apoptotic pathway. Reovirus activation of NF- κ B is required for cleavage of Bid. Known stimulatory pathways are indicated by dark blue arrows (light blue arrows if not required); known inhibitory pathways are indicated by red bars. Possible stimulatory pathways are indicated by dashed blue arrows or question marks.

induced by reovirus strains T1L and 8B increase in primary cultures of cardiac myocytes when NF- κ B is inhibited.¹⁰¹ This increase likely reflects a requirement for NF- κ B in the induction of the antiviral cytokine IFN- β by reovirus. Similarly, apoptosis induced by reovirus strain T3SA+ increases in the heart of NF- κ B p50-null mice relative to wild-type mice.³⁶³ This effect can be ameliorated by treatment of infected animals with IFN- β .³⁶³ However, apoptosis induced by T3SA+ is decreased in the brain of NF- κ B p50-null mice, indicating that the role of NF- κ B in reovirus-induced apoptosis *in vivo* is tissue specific.³⁶³

Reovirus-induced apoptosis is regulated by additional cellular factors. Reovirus stimulates MEKK1 to activate c-Jun N-terminal kinase (JNK), resulting in phosphorylation and activation of transcription factor c-Jun.^{104,105} JNK activation is

required for efficient release of pro-apoptotic factors cytochrome *c* and Smac/DIABLO from mitochondria. Inhibitors of JNK block reovirus-induced apoptosis at low MOI and significantly delay the onset of apoptosis at high MOI.¹⁰⁴ However, the c-Jun transcription factor is not required for reovirus-induced apoptosis, indicating that JNK functions to induce apoptosis independent of its role in transcriptional activation of c-Jun. Activated JNK can directly modulate the activities of mitochondrial pro-apoptotic and antiapoptotic proteins such as Bad, Bid, and Bim.¹⁴² Thus, JNK may directly influence intrinsic apoptotic pathway activation in reovirus-infected cells. Reovirus also stimulates the innate immune response sensor IPS-1 for activation of transcription factor IRF3; these signaling events are required for induction of maximum levels of apoptosis.²²⁵ IRF3 and NF- κ B, independently of IFN- β , induce Noxa, a proapoptotic

BH3-only-domain Bcl-2 family protein; Noxa expression markedly enhances reovirus-induced apoptosis.²⁶⁰ Reovirus activates the cellular protease calpain, and apoptosis is reduced by a calpain inhibitor.¹³² Finally, reovirus stimulates TGF- β signaling, and inhibition of this pathway reduces apoptosis.³⁶ These pathways also are modulated *in vivo*.

PATHOGENESIS AND IMMUNITY

Reovirus pathogenesis and host immune responses have been studied most extensively using neonatal mice (Fig. 44.15). These studies provide most of the information in this section.

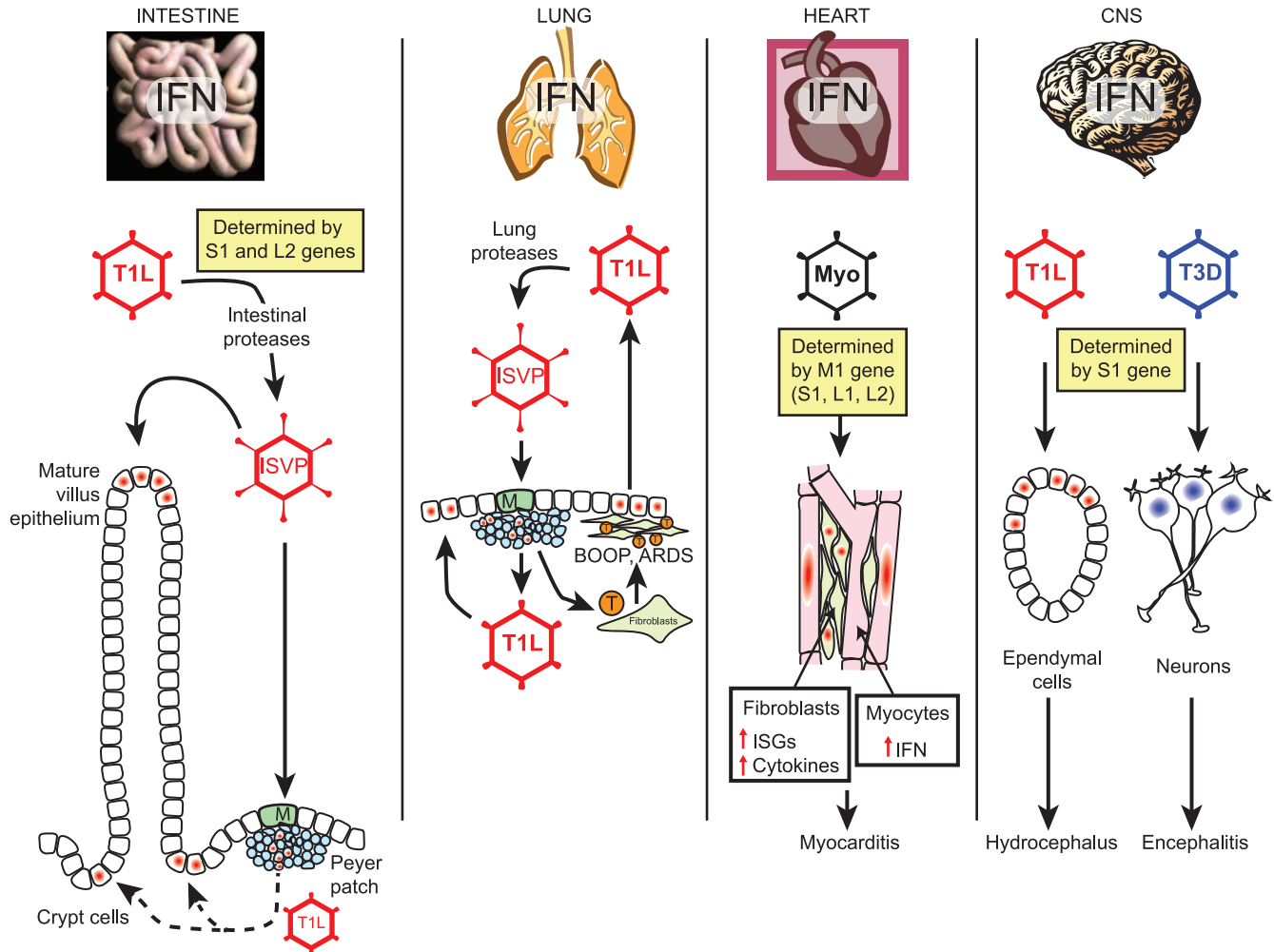


FIGURE 44.15. Viral and host factors determine reovirus tissue tropism. **Intestine.** Reovirus replication in the intestine requires conversion of virions to infectious subviral particles (ISVPs), which is catalyzed by intestinal proteases. ISVPs can infect epithelial cells at the villus tips or transit through M cells to underlying Peyer patches to infect epithelial cells in the villus crypts. Virus can spread systemically by hematogenous or neural routes from Peyer patch cells. Strain-specific differences in reovirus replication in the intestine segregate with the S1 and L2 genes. Virus-induced type I interferon (IFN) is essential for protection of intestinal tissue against reovirus infection. **Lung.** Reovirus replication in the lung also requires virion-to-ISVP disassembly, which is catalyzed by airway-resident proteases. Following transport through M cells to underlying bronchus-associated lymphoid tissue, virus infects epithelial cells basally and is released apically. Virus-stimulated T cells induce epithelial cell apoptosis and fibrosis, resulting in either bronchiolitis obliterans organizing pneumonia (BOOP) or acute respiratory distress syndrome (ARDS). **Heart.** Some reovirus strains infect and damage cardiac myocytes, leading to myocarditis. Strain-specific polymorphisms in the M1 gene segment lead to differences in virus induction of, and sensitivity to, type I IFN, which consequently influence the capacity of reovirus to cause myocarditis. Cardiac myocytes synthesize high levels of IFN, while cardiac fibroblasts synthesize high levels of antiviral IFN-stimulated genes (ISGs) and cytokines, offering an integrated network for organ protection. The S1, L1, and L2 genes are also determinants of strain-specific differences in myocarditic potential. **Central Nervous System (CNS).** Reovirus infects the CNS, but strains of different serotypes vary in the capacity to infect discrete populations of cells in the brain. The reovirus S1 gene segment, which encodes attachment protein $\sigma 1$ and nonstructural protein $\sigma 1s$, determines cell tropism within the CNS. The capacity of reovirus to infect ependymal cells and neurons, leading to hydrocephalus or encephalitis, respectively, is likely influenced by both $\sigma 1$ and $\sigma 1s$.

Entry into the Host

Reovirus infections in nature initiate in the enteric and respiratory tracts. Reoviruses bind to intestinal M (microfold) cells⁵³⁴ or pulmonary M cells³³⁹ in the epithelia and transit through these cells without replicating to reach underlying intestinal Peyer patches or bronchus-associated lymphoid tissue. Reovirus binding to intestinal M cells requires interactions of the $\sigma 1$ protein with glycoconjugates containing $\alpha 2,3$ -linked sialic acid.²¹⁷ However, viral replication in the intestine is not dependent on binding to sialic acid²⁸ or JAM-A.¹³ Binding to intestinal M cells by reovirus requires conversion of virions to ISVPs by intestinal proteases¹² (Fig. 44.15). Interestingly, while ISVPs are generated efficiently in the intestine,⁵⁴ proteases resident in the lung capable of converting virions to ISVPs *in vitro* do so inefficiently *in vivo*, perhaps because of protease inhibitors present at that site.³⁶² In comparison to intact virions, ISVPs inoculated perorally or intranasally lead to higher viral titers in the intestine²⁹ and the lung,³⁶² respectively. Moreover, pharmacologic inhibition of intestinal proteases blocks reovirus replication in the intestine.^{12,29} However, while proteolytic enzymes resident in host tissues facilitate infection by many reovirus strains,^{12,29,54,192,193} tissue proteases cleave the $\sigma 1$ protein of some strains, including T3D, and thereby compromise infectivity.³⁴⁹ Sequence polymorphisms that govern $\sigma 1$ cleavage offer one likely mechanism for strain-specific differences in reovirus replication in the intestine⁸⁷ (see Replication—Attachment), although polymorphisms in the L2-encoded $\lambda 2$ protein are also involved.⁵³ The L2 gene segment is similarly associated with the efficiency of reovirus shedding from the intestine and transmission to new hosts.²⁵⁴

Reovirus replicates in epithelial cells in the ileum⁴²⁰ and the respiratory tract.¹⁶⁴ One study suggests that intestinal epithelial cells in villus crypts are infected basolaterally from Peyer patches.⁴¹⁸ However, other studies indicate that virus infects intestinal epithelial cells at the villus tips^{13,56} (Fig. 44.15). Reovirus preferentially infects respiratory epithelial cells basolaterally and is released apically,¹⁶⁴ ensuring egress of virus into the environment for host-to-host dissemination.

Antibiotic treatment of mice reduces reovirus replication in the intestine, suggesting that the intestinal microbiota enhance reovirus replication.²⁷² Parallel studies with poliovirus suggest that the mechanism involves interactions of the virus with bacterial surface polysaccharides, which augment viral binding and infection of cells.²⁷²

Spread of Virus

Reovirus can disseminate from a site of entry to distant sites within the host by hematogenous or neural routes. Access to lymphoid cells in Peyer patches and bronchus-associated lymphoid tissue offers one potential mechanism for virus spread, as reovirus can bind to B and T lymphocytes.¹⁵⁹ Indeed, type 1 reoviruses access the CNS and other tissues primarily by hematogenous routes, perhaps by adhering to the lymphocyte surface,⁴⁹⁸ although these viruses can infect and replicate in sensory neurons in some circumstances.¹⁷¹ Reoviruses also can spread directly from Peyer patches to the CNS. Reovirus strain type 3 clone 9 (T3C9) transits from Peyer patches to adjacent neurons of the myenteric plexus, then through vagal autonomic nerves to the vagal dorsal motor nucleus in the brainstem.³⁴² In addition,

type 3 reoviruses can use hematogenous routes to access the CNS and other tissues.^{13,55}

Neural spread of type 3 reoviruses from the site of intramuscular inoculation to the spinal cord is inhibited by surgical transection of the sciatic nerve.^{55,498} Interestingly, sciatic nerve section delays, but does not inhibit, type 3 reovirus invasion of the brain,⁵⁵ indicating an important role for hematogenous dissemination to that site. Reovirus neural spread is diminished by colchicine, which inhibits fast axonal transport, but not by β - β' -iminodipropionitrile, which inhibits slow axonal transport.⁴⁹⁸ However, the interval between inoculation into the hind limb muscle and detection of virus in the brain is not uniformly consistent with either fast or slow axonal transport.^{55,498} Therefore, the precise mode of reovirus neural dissemination awaits further clarification.

Preference for hematogenous or neural routes of spread following intramuscular inoculation is determined by the viral S1 gene segment,⁴⁹⁸ which encodes viral attachment protein $\sigma 1$ and nonstructural protein $\sigma 1s$. The $\sigma 1$ proteins of both type 1 and type 3 reovirus strains use JAM-A as a receptor,^{27,76} which is required for infection of endothelial cells by both serotypes.¹³ Therefore, a requirement for JAM-A for reovirus infection of endothelial cells and hematogenous spread does not explain virus strain-specific differences in preference for that dissemination route. Similarly, preference for hematogenous routes by viruses with a type 1 S1 gene segment is not explained by the greater replication of T1L than T3D in cultured endothelial cells, as that capacity segregates with the T1L M1 rather than the S1 gene segment.³¹² The $\sigma 1s$ protein is required for viremia and hematogenous spread of both type 1 and type 3 reoviruses^{55,56} but dispensable for neural spread.⁵⁵ Therefore, $\sigma 1s$ polymorphisms are not likely to explain preference for hematogenous spread. Instead, it is possible that differential binding of type 1 and type 3 reoviruses to a neural receptor yet to be identified determines preference for neural spread. This idea is consistent with the observation that viral strain-specific differences in CNS tropism segregate with the S1 gene segment.⁵¹⁴ The capacity of type 3 $\sigma 1$ to bind sialic acid enhances the efficiency of viral dissemination.²⁸ However, it is not clear whether $\sigma 1$ interactions with sialylated glycans influence spread by hematogenous or neural routes.

Tissue Tropism, Damage, and Disease

Depending on the route of inoculation, reoviruses first replicate in the intestine, lung, or skeletal muscle and then spread from those primary sites to replicate in the liver, spleen, heart, brain, and other organs. Host factors required for reovirus replication influence pathogenesis. For example, reovirus intracellular disassembly is mediated by endosomal cathepsins B,¹⁵⁶ L,¹⁵⁶ or S,¹⁹¹ depending on the cell type (see Replication—Disassembly). In comparison to wild-type mice, peak reovirus titers in cathepsin B-null mice following peroral inoculation are similar in the intestine but lower in the heart and brain.²⁴⁷ Accordingly, survival of reovirus-infected cathepsin B-null mice is prolonged and mortality decreased,²⁴⁷ consistent with tissue-specific augmentation of viral replication by cathepsin B. Similarly, compared with wild-type mice, peak reovirus titers in perorally inoculated mice lacking cathepsins L or S also are diminished at sites of secondary replication.²⁴⁷ However, while survival is prolonged, mortality in these animals is increased,²⁴⁷ consistent with a defect in cell-mediated immunity in mice

deficient in either of these enzymes.⁸⁶ Treatment of infected mice with a cathepsin L inhibitor decreases viral titers and increases survival,²⁴⁷ suggesting that transient abrogation of cathepsin L activity can inhibit viral replication with minimal compromise of cell-mediated immunity.

Central Nervous System

Type 1 reoviruses infect the ependymal cells lining the ventricles in neonatal mice, which results in ependymitis and hydrocephalus.⁵¹⁵ In contrast, type 3 reoviruses infect neurons in these animals, replicate to high titers in the brain, and induce a lethal meningoencephalitis.⁵¹⁵ Type 3 reoviruses also damage spinal cord neurons resulting in acute flaccid paralysis.²⁰¹ The capacity to infect ependymal cells⁴⁸⁴ or neurons¹⁴³ and the resulting disease phenotypes⁵¹⁴ are determined by the S1 gene segment (Fig. 44.15). S1-determined differences in infection of ependymal cells correlate with differences in virus attachment to these cells,⁴⁸⁴ implicating $\sigma 1$ rather than $\sigma 1s$ in this tropism difference. However, analogous binding studies using primary cultures of neurons have not been reported. Infection of neurons in the brain does not require JAM-A¹³ or sialic acid,²⁸ and the viral receptor on those cells remains unidentified. The $\sigma 1s$ protein is dispensable for reovirus replication in the brain,^{56,227} but it influences apoptosis and tissue damage in that organ.²²⁷ Therefore, it is likely that both $\sigma 1$ and $\sigma 1s$ contribute to S1-determined differences in reovirus-induced CNS disease.

Type 3 reovirus field-isolate strains differ in virulence and peak titers achieved in the brain, but they induce similar patterns of tissue damage.²²⁸ However, variants of T3D selected for resistance to $\sigma 1$ -specific monoclonal antibodies are attenuated.^{398,467} One particularly well-studied variant, variant K, produces peak titers and cell damage in the hippocampus comparable to T3D. However, unlike T3D, this variant produces 1,000-fold lower titers and induces minimal neuronal damage in the cortex, patterns that are reproduced in primary cultures of hippocampal and cortical neurons.³⁹⁸ Variant K differs from T3D by a single amino acid substitution in the globular head of $\sigma 1$ ³⁰ (see Replication–Attachment), but the mechanism of differential replication and damage in the brain is not known.

Type 3 reoviruses induce apoptosis in neuronal cultures and in the brain.^{35,123,124,199,200,227,365,396,397–398} Cell killing by apoptosis plays a critical role in reovirus-induced CNS disease. A reovirus mutant with a single amino acid substitution in the ϕ domain of the M2-encoded $\mu 1$ protein does not affect membrane penetration but does diminish pro-apoptotic signaling.¹²¹ This mutant is less neurovirulent compared with wild-type virus,¹²¹ which provides one possible explanation for the genetic association of the reovirus M2 gene segment with neurovirulence.²²⁸ Although the majority of apoptotic cells in the CNS are infected, suggesting direct effects of the virus, nearby uninfected apoptotic cells suggest the existence of additional “bystander” mechanisms of cell death.^{365,396}

Reovirus-induced apoptosis involves both extrinsic and intrinsic pathways (see Responses of the Host Cell to Infection—Induction of Apoptosis and Activation of NF- κ B). Reovirus activates NF- κ B in the brain, and apoptosis is significantly diminished in the CNS of NF- κ B p50-null mice.³⁶³ Activation of NF- κ B is required for reovirus-induced cleavage and activation of the proapoptotic protein, Bid; reovirus replication and

histopathologic injury in the brain are substantially reduced in Bid-deficient mice.¹²⁴ Additionally, reovirus-induced apoptosis, but not viral replication, is diminished in the CNS of mice lacking the proapoptotic Bcl-2 protein, Bax.⁴⁵ Reovirus replication, CNS injury, and spread within the CNS are also reduced and survival enhanced in mice lacking effector caspase 3.³⁷ Reovirus also activates JNK, which upregulates Fas, and chemical inhibitors of JNK attenuate reovirus-induced encephalitis.^{35,100}

Reoviruses induce multiple protective responses in the CNS. Gene expression associated with IFN signaling is induced early in infection, but reovirus stimulates other cytokines and apoptotic pathways later in infection.⁴⁹⁶ Experiments using brain-slice cultures also indicate that reovirus induces cytokines and apoptotic pathways in neural tissues.¹⁴⁴ Reovirus activates the JAK-STAT pathway in the brain,¹⁹⁹ and when type I IFN signaling is ablated, reovirus tropism in the brain is extended to new neuronal populations with a concomitant increase in lethality.^{144,199} Interestingly, neurons in which STAT1 is activated are often uninfected,¹⁹⁹ suggesting that reovirus infection is prevented by IFN-mediated antiviral effects and consistent with reovirus repression of IFN signaling in infected cells.^{235,556} Reovirus also increases nitric oxide (NO) production and expression of inducible nitric oxide synthase (iNOS) in primary mouse cortical neuron cultures and brains of infected mice.²⁰⁰ Inhibition of iNOS increases reovirus replication in neurons, while an NO donor decreases reovirus replication in these cells.²⁰⁰

Following infection of the brain, reovirus activates TGF- β receptor I and its signaling effector SMAD3 as well as another member of the TGF- β superfamily, bone morphogenetic protein (BMP) receptor I, and its effector SMAD1.³⁶ Inhibition of TGF- β signaling *in vivo* decreases SMAD3 activation and increases reovirus-induced apoptosis.³⁶ Similarly, inhibition of BMP signaling in primary mouse cortical neuron cultures decreases SMAD1 activation and increases neuronal apoptosis, while viral replication remains unchanged. Treatment with a BMP agonist has the opposite protective effect.³⁶ Interestingly, SMAD1 activation occurs predominantly in adjacent uninfected neurons,³⁶ similar to the pattern observed for the activation of STAT1.¹⁹⁹ Thus, reovirus appears to selectively suppress protective responses in infected neurons.

Heart

Reovirus-induced cardiac damage in neonatal mice is caused by a direct cytopathic effect on cardiac cells^{31,450,451} (Fig. 44.15). Focal lesions are evident throughout the endocardium, myocardium, and epicardium, associated with cardiac myocyte loss and visible dystrophic calcification on the surface of the heart in the absence of significant inflammatory infiltrate.⁴⁵¹ Reovirus myocarditis is not immune mediated, as reovirus infection causes myocarditis in nude mice, which lack T cells,⁴⁵¹ and in severe combined immunodeficiency (SCID) mice, which lack both B and T cells.⁴⁵⁰ The capacity to induce myocarditis is virus strain specific. Studies of reoviruses that vary in myocarditic potential indicate that the extent of myocardial damage correlates with cytopathic effect in primary cultures of cardiac myocytes.³¹ A protease inhibitor that blocks reovirus replication at an early step inhibits reovirus-induced myocarditis, indicating that viral replication is required for disease.²⁵⁸ Interestingly, reoviruses that fail to induce myocarditis in immunocompetent mice also fail to do so in SCID mice,⁴⁵⁰ providing

additional evidence that development of cardiac lesions is not determined by the adaptive immune response but instead reflects virus interactions with cardiac cells.

The type I IFN response regulates reovirus-induced myocarditis. The capacity of reovirus strains to induce myocarditis correlates inversely with induction of and sensitivity to type I IFN in cardiac myocytes.⁴⁵² Moreover, nonmyocarditic reovirus strains induce cardiac lesions in mice when IFN induction^{224,363,452} or IFN-induced gene expression^{20,473} is inhibited. Myocarditic capacity segregates primarily with the reovirus M1 gene segment but also is influenced by the S1, L1, and L2 gene segments.^{448,449} The M1 gene segment determines reovirus strain-specific differences in replication in cardiac myocytes,³¹³ and nonmyocarditic reovirus strains achieve titers comparable to those of myocarditic reoviruses in cardiac myocytes when the IFN response is inhibited.^{235,452} The M1-encoded $\mu 2$ protein can repress IFN signaling,⁵⁵⁶ a property determined by a single sequence polymorphism in $\mu 2$ at amino acid 208²³⁵ (see Responses of the Host Cell to Infection—Interferon Induction and Effects on Viral Replication). Substitution at that position modulates reovirus induction of IFN, replication in cardiac myocytes, and myocardial injury, although other reovirus determinants also influence these properties.²³⁵ Therefore, the strength of the IFN response and viral subversion of IFN effects are critical determinants of virus strain-specific differences in damage to the heart.

The potentially myocarditic reovirus strain 8B induces apoptosis in primary cultures of cardiac myocytes,^{101,128,130,131,333} suggesting a link between apoptosis and myocardial injury. Concordantly, reovirus strain-specific differences in induction of apoptosis in primary cardiac myocyte cultures correlate with the capacity to induce myocarditis.³³³ Myocarditic reovirus strains activate caspase 8, which is associated with the death receptor-initiated extrinsic apoptotic pathway, and caspase 3, an effector caspase, in primary cultures of cardiac myocytes and in the heart.¹³⁰ In contrast, caspase 9, which is associated with the intrinsic mitochondrial apoptotic pathway, is only weakly activated, and the mitochondrial transmembrane potential is unaltered.¹³⁰ Pretreatment of neonatal mice with calpain or caspase inhibitors dramatically reduces cardiac damage caused by potentially myocarditic reovirus strains.^{128,131} Finally, as is the case in the CNS, the $\sigma 1$ s protein is dispensable for reovirus replication in the heart, but it influences apoptosis and tissue damage in that organ.²²⁷ Thus, death of cardiac myocytes during reovirus-induced myocarditis appears to occur as a consequence of apoptosis.

Patterns of reovirus-induced gene expression in the heart vary according to virus strain and cardiac cell type. The capacity to induce cardiac lesions correlates with cytopathic effect in primary cultures of cardiac myocytes but not cardiac fibroblasts.³¹ Indeed, myocarditic reovirus strains preferentially induce apoptosis of cardiac myocytes relative to cardiac fibroblasts, while nonmyocarditic reoviruses induce the converse.³³³ Nonmyocarditic reoviruses also induce expression of cytokines in cardiac fibroblasts that likely contribute to disease outcome *in vivo*.³³³ Reovirus induction of type I IFN and ISG expression also differs between cardiac myocytes and cardiac fibroblasts.^{288,474,555} (Fig. 44.15). In keeping with the differences in gene expression, reovirus-induced changes in the cardiac myocyte proteome also differ between virus strains and correlate with the capacity to induce myocarditis.²⁸⁷ Thus, reovirus

cardiac damage reflects an array of host responses determined by both viral and cardiac cell factors.

Hepatobiliary System and Diabetes

Neonatal mice infected with some type 3 reovirus strains develop oily fur syndrome, appearing as though they have been dipped in oil.^{28,140,530} This syndrome is associated with viral replication in intrahepatic bile duct epithelium, leading to biliary obstruction and fat malabsorption, which mimic in some respects human infantile biliary atresia. Differences in the capacity of reovirus strains to induce oily fur syndrome segregate with the S1 gene segment.⁵³⁰ A sialic acid-binding reovirus strain (T3SA+), but not a congenic strain that does not bind sialic acid (T3SA-), infects bile duct epithelial cells following peroral inoculation and induces periportal inflammation and bile duct necrosis, resulting in oily fur syndrome.²⁸ Moreover, T3SA+ but not T3SA- binds a human biliary epithelial cell line *in vitro*. Thus, reovirus binding to sialic acid is required for biliary tract destruction and oily fur syndrome.²⁸ Mutations in the M2 gene segment can abrogate the capacity to induce this disease, although the mechanism underlying this effect is unclear.¹⁴⁰

Nonobese diabetic (NOD) mice provide a model system for investigation of type I diabetes mellitus, an autoimmune disease resulting from destruction of pancreatic β cells. Following inoculation of neonatal NOD mice, reovirus strain T3A infects pancreatic islet cells, which prevents the development of diabetes but not the insulinitis (the recruitment of CD8+ T cells and macrophages to pancreatic islets by autoreactive CD4+ T cells).⁵²³ These findings suggest that T3A-induced cytopathic effect and release of antigens from β cells leads to tolerance by induction of regulatory T cells, thereby delaying the onset of diabetes.

Lung

Reovirus strain T1L inoculated intranasally into adult mice induces two different types of pulmonary tissue damage and fibrosis, which model distinct forms of lung disease in humans (Fig. 44.15). T1L can induce bronchiolitis obliterans organizing pneumonia (BOOP), which is characterized by inflammation and intra-alveolar fibrosis.³⁰⁰ However, when inoculated at a 10-fold higher dose, T1L induces acute respiratory distress syndrome (ARDS), which is characterized by an acute phase with severe inflammation and a chronic phase with persistent intra-alveolar and interstitial fibrosis.³⁰⁰ T1L inoculated into adult mice thymectomized as neonates induces ARDS but not BOOP, suggesting a requirement for T cells in BOOP pathogenesis.³⁰⁰ Moreover, corticosteroid treatment or depletion of CD4+ T cells, CD8+ T cells, or IFN- γ prior to inoculation inhibits the development of fibrotic lesions, consistent with a pathologic role for T cells in this disease.³⁰¹ T1L induces apoptosis of epithelial cells in both BOOP and ARDS. However, apoptosis as a direct consequence of T1L infection is unlikely to contribute to lung injury. Instead, Fas and FasL upregulated on the surface of infiltrating T cells likely mediate pulmonary epithelial cell apoptosis.²⁹² Pulmonary inflammation and fibrosis induced by T1L are diminished when caspase 8 is inhibited. However, T1L induces ARDS but not BOOP in mice expressing inactive Fas.²⁹² Thus, while apoptosis may contribute to the pathogenesis of both diseases, the Fas/FasL pathway is required only for the development of BOOP. Collectively, these

findings suggest that infiltrating T cells stimulated by reovirus T1L induce alveolar epithelial cell apoptosis and fibrosis and that apoptosis in BOOP but not ARDS is dependent on the Fas/FasL pathway.

Juvenile rats inoculated intratracheally with T1L also develop an acute pneumonia, characterized by degeneration of terminally differentiated type I alveolar epithelial cells, type II alveolar epithelial cell hyperplasia to replace the destroyed type I cells, and infiltration of leukocytes.³⁴⁰ Inoculation with T3D induces a similar pattern of pneumonia but with a more prominent neutrophil infiltrate. Both reovirus strains replicate in type I epithelial cells, but T1L achieves higher titers. The capacity to induce neutrophil infiltration segregates with the T3D S1 gene segment, while replication to high titers segregates with the T1L S1 gene segment.³⁴⁰

Reovirus infection of primary human airway epithelial cell cultures is more efficient following basolateral as opposed to apical adsorption of virus and is dependent on JAM-A.¹⁶⁴ Despite a preference for basolateral entry, reovirus is released from the apical surface of respiratory epithelial cells and does not disrupt the tight junctions.¹⁶⁴ These results suggest that epithelial cells are not the primary cell type responsible for systemic spread of reovirus from the respiratory tract to distant sites within the host.

Airway-resident proteases capable of catalyzing reovirus disassembly enhance infection in the lung. Type II transmembrane serine proteases that are expressed in human airways facilitate reovirus uncoating and infection *in vitro*. However, murine homologs of these enzymes are inefficient *in vivo*, likely reflecting repression by endogenous protease inhibitors.³⁶² Reovirus induces neutrophil infiltration in the lung,³⁴⁰ and the serine protease neutrophil elastase¹⁹³ as well as other inflammatory proteases facilitate reovirus uncoating and infection *in vitro*.³⁶² Treatment of mice with agents that induce pulmonary inflammation prior to intranasal inoculation of reovirus increases conversion of virions to ISVPs and enhances viral replication in the lung,³⁶² suggesting that inflammatory proteases facilitate reovirus infection at that site.

Reovirus Genetic Determinants of Tropism and Tissue Injury

Reovirus strains differ in the capacity to infect specific organs and produce tissue-specific disease in neonatal mice. Gene segments that segregate with such differences in reovirus pathogenesis have been identified in many cases (Fig. 44.15). The S1 and L2 gene segments determine the magnitude of reovirus replication in the intestine and efficiency of viral shedding in the stool. The S1 gene segment determines whether reovirus infects CNS neurons resulting in encephalitis or ependymal cells causing hydrocephalus, although the M2 gene segment influences neurovirulence. The M1 gene segment is the predominant determinant of the capacity of reovirus to cause myocarditis, although the S1, L1, and L2 gene segments influence the extent of cardiac damage.

Adult SCID mice, which lack B cells and T cells, are susceptible to reovirus infection and provide an alternative approach to identifying viral determinants of tissue tropism and disease. Results of experiments using these animals are largely consistent with those obtained in studies of neonatal mice, but they provide several additional associations between specific reovirus gene segments and infection in the intestine,

heart, and CNS.²⁰⁷ Importantly, results gathered in studies of adult SCID mice provided the first identification of reovirus genetic determinants of liver damage, which include the M1, L1, and L2 gene segments.²⁰⁷

Innate Immune Responses

Cells infected by reovirus express an array of innate protective responses, including cytokines, chemokines, and iNOS (see Responses of the Host Cell to Infection). Natural killer (NK) cells also are recruited to reovirus-infected tissues, but NK cells are neither necessary²⁴⁵ nor sufficient^{206,485} to clear the virus. Neutrophils are recruited to reovirus-infected tissues,³⁴⁰ but their importance in reovirus clearance has not been defined.

The type I IFN response is essential for protection against reovirus infection (see Responses of the Host Cell to Infection—Interferon Induction and Effects on Viral Replication). Following peroral inoculation, strain T1L induces type I IFN in Peyer patches and is cleared from the intestines of wild-type mice.²⁴⁵ In contrast, T1L damages the intestinal mucosa in IFN- α/β -receptor-null mice, depletes lymphoid cells, is not cleared, and causes a lethal outcome.²⁴⁵ The majority of T1L-induced type I IFN in Peyer patches is generated by dendritic cells (DCs). Consistent with studies implicating RIG-I and MDA-5 for reovirus induction of IFN,^{225,250,291} mice lacking TLR3 or its effector molecule MyD88 do not differ from wild-type mice in the response to T1L infection.²⁴⁵

Activation of NF- κ B by reovirus is required for the induction of IFN- β , which in turn protects against reovirus-induced encephalitis^{144,199} and myocarditis.^{224,235,363,452} However, in reovirus-infected mice, the role of NF- κ B in disease pathogenesis is organ specific. Apoptosis following reovirus infection is reduced in the brains of mice lacking the NF- κ B p50 subunit,³⁶³ consistent with a proapoptotic role for NF- κ B in reovirus infection of the CNS. In contrast, reovirus replication and apoptosis are increased in the hearts of p50-deficient mice, and these effects are prevented by treatment with IFN- β .³⁶³ These observations are consistent with NF- κ B-independent apoptosis by reovirus in cardiac myocytes¹⁰¹ and the importance of IFN- β in protection against reovirus-induced myocarditis.⁴⁵² In transformed cells, Ras enhances reovirus replication at least in part by suppressing RIG-I-mediated induction of IFN- β ⁴⁵⁴ (see Reovirus Oncolytics).

Adaptive Immune Responses

Reovirus replicates to high titers in neonatal mice, but replication in adult immunocompetent mice is modest and requires inoculation with high doses of virus.⁴²⁰ In contrast, reovirus replicates to high titers and is lethal in adult SCID mice (B cell- and T cell-deficient), and passive transfer of lymphocytes protects these animals against lethal infection.^{25,185,207,485} Together, these results suggest that the adaptive immune response influences the age-dependent restriction of reovirus replication and disease in mice. However, reovirus can induce immune cell-mediated pathology, as noted in cases of reovirus-associated lung disease.

Reovirus strains T1L and T3D do not replicate in peripheral blood mononuclear cells (PBMCs) or DCs derived from PBMCs *in vitro*,^{146,161,172} although these strains do replicate in THP-1 human monocyte cells.¹⁶⁶ Instead, DCs adsorbed with reovirus *in vitro* become loaded with viral antigen¹⁷² and express cell-surface markers and cytokine patterns characteristic of DC activation.¹⁶¹ Reovirus-exposed DCs, but not reovirus alone,

activate a majority of T cells for antigen-independent cytolytic activity and NK cells for perforin/granzyme-mediated cytotoxicity.¹⁶¹ Activation of T cells requires direct cell-to-cell contact with DCs, while activation of NK cells is mediated by DC-secreted soluble factors.¹⁶¹

The immune system of the intestine includes inductive (Peyer patches and mesenteric lymph nodes) and effector (lamina propria and intraepithelial lymphocytes [IELs]) sites. Following peroral inoculation, reovirus T1L infects epithelial cells in the intestine, resulting in activation of DCs in Peyer patches.²⁴⁵ Peyer patch DCs exposed to reovirus T1L *in vivo* can activate reovirus-primed CD4+ T cells *in vitro*¹⁷² and likely also activate T cells in the intestine. Indeed, reovirus T1L-activated T cells in Peyer patches migrate to the lamina propria and intraepithelial sites.⁵⁰ These activated T cells utilize perforin, Fas-FasL, and TRAIL pathways for cytotoxicity, but pathway preference is dependent on the mucosal site from which the T cells are harvested.⁵⁰ IELs induced by T1L express the $\alpha\beta$ T-cell receptor (TCR) and CD4, CD8, or both CD4 and CD8, and express IFN- γ characteristic of activated effector cells.⁴⁹ Depletion experiments indicate that CD8+Thy-1+ T cells expressing the $\alpha\beta$ TCR mediate IEL CTL activity.¹¹⁷

The humoral immune response to reovirus is important in protection of the intestine, mediating clearance following primary infection and prevention of secondary infection. Reovirus is cleared from the intestines of adult wild-type and $\beta 2$ microglobulin-null (CD8+ T cell-deficient) mice but not SCID (B cell- and T cell-deficient) and MuMT (B cell- and antibody-deficient) mice.^{25,185,207,303,485} Reovirus T1L inoculated perorally into adult mice induces intestinal IgA and serum IgG.⁴⁵⁷ Viable or UV-inactivated reovirus T3D induces polymeric immunoglobulin receptor expression in H29 human intestinal epithelial cells,³⁷¹ suggesting a possible mechanism for intestinal IgA secretion. During primary infection, reovirus T1L is cleared equally well from the intestines of IgA-null mice and wild-type mice. However, following subsequent challenge, reovirus T1L invades Peyer patches of IgA-null but not wild-type animals,⁴⁵⁷ consistent with a protective role for IgA during reinfection.

Studies using exogenous effectors offer additional insights into the importance of antibodies in protection of the intestine against reovirus infection. Oral introduction of IgA or IgG specific for the $\sigma 1$ protein protects Peyer patches from infection by reovirus T1L.^{232,457} Adoptive transfer of splenic lymphocytes derived from reovirus-immune mice inhibits reovirus strain T3C9 replication in the intestine, but efficacy is diminished if B cells are depleted prior to transfer, indicating that antibody responses are important.²⁵ Intraperitoneal introduction of reovirus-specific polyclonal antiserum or IgG2a monoclonal antibody into MuMT mice leads to clearance of T3C9.²⁵

Antibody and immune cells protect against reovirus infection at distinct stages of the virus-host encounter. Following systemic administration, neutralizing monoclonal antibodies specific for the $\sigma 1$ protein do not inhibit primary replication of reovirus T3C9 in the intestine or T3D in skeletal muscle. However, these antibodies potentially inhibit viral spread from both of those sites to the spinal cord.⁵⁰² Even when administered after virus has reached the spinal cord, antibody prevents viral spread to the brain. Antibody also inhibits replication in the brain following intracranial inoculation of T3D^{502,508} and spread from the brain to the eye, spinal cord, and muscle.⁵⁰²

Similarly, monoclonal antibodies that do not inhibit T1L replication in skeletal muscle inhibit viremia and replication in the brain.⁴⁹⁷ Together, these results indicate that antibody can mediate protection by blocking steps in viral pathogenesis following primary replication.

Adoptive transfer of adult spleen cells protects neonatal mice from T3C9 inoculated perorally, T3D inoculated parenterally, and T1L (but not T3D) inoculated intracranially.⁵⁰⁹ Depletion of either CD4+ or CD8+ T cells leads to significantly increased replication of both T3C9 and T3D. Adoptive transfer of immune cells is more effective than monoclonal antibodies in inhibiting viral replication, while the converse is true for inhibition of neural spread of virus.⁵⁰⁹

The route of reovirus inoculation is an important determinant of both humoral and cell-mediated immune responses. Peroral inoculation of reovirus T1L induces predominantly IgA in Peyer patches, while parenteral inoculation of that strain induces predominantly IgG2a in peripheral lymph nodes.³⁰² However, peroral inoculation of T1L induces IFN- γ and other Th1 cytokines but minimal Th2 cytokines.¹⁶⁵ Peroral inoculation of T1L also induces serum IgG2a, characteristic of a Th1 response, while parenteral inoculation induces both IgG1 and IgG2a, although this effect is MHC haplotype specific.³⁰² Finally, peroral inoculation of T1L suppresses the expression of Th2 cytokines IL-4 and IL-10 in draining lymphatic tissues early in infection, and treatment with IL-4 or IL-10 decreases serum IgG2a relative to IgG1.³¹¹ Thus, peroral inoculation of reovirus suppresses Th2 cytokines, resulting in a Th1-biased cell-mediated immune response. Concordantly, PBMCs from volunteers presumed to have been infected with reovirus in the past express recall responses when exposed to reovirus that are dominated by strong Th1-like and minimal Th2-like cytokine expression.¹⁴⁶ Interestingly, the requirements for specific PBMC subsets and ligand interactions to elicit cytokine responses are dependent on the virus strain used for stimulation.¹⁴⁶ The route of inoculation also is a determinant of TCR usage by CD8+ T cells. Inoculation of reovirus T1L perorally or parenterally induces preferential usage of TCR variable gene segment V $\beta 6$. However, peroral inoculation additionally restricts TCR β gene segment usage, suggesting an even greater limitation on TCR specificity.¹⁷⁵

EPIDEMIOLOGY AND HOST RANGE

Epidemiology

Epidemiologic surveys for the presence of reovirus antibodies in diverse populations suggest that most humans have been exposed to reovirus and that infection occurs early in childhood.²⁸⁵ However, reovirus infections are rarely symptomatic. In a 1994 study using ELISA to examine 850 serum samples from healthy European volunteers ranging in age from 6 months to over 60 years, the frequency of individuals with detectable IgG antibodies specific for reovirus rose with increasing age.⁴³⁷ Prevalence was approximately 35% in infants 6 months to one year, approximately 60% in individuals 11 to 19 years, and approximately 75% to 85% in individuals 20 years and older with no apparent decline after 60 years of age. In a 2005 study using ELISA to examine 272 serum samples from healthy US children ranging in age from birth to 6 years, there was rapid loss of maternal antibody as evidenced by declining seroprevalence

from 75% (6 of 8) in infants 0 to 3 months of age to 11% (1 of 9) in those from 3 to 6 months of age.⁴⁸² In contrast to results from the 1994 study, none of the 25 individuals 6 months to 1 year of age were seropositive. Seroprevalence increased in the remaining 181 individuals tested, ranging from 8% (4/49) in 1 to 2 year olds to 50% (11/22) in 5 to 6 year olds. Thus, while results from individual studies vary, reovirus infections are common before adulthood.

Host Range

Nonfusogenic mammalian orthoreoviruses infect a wide variety of species⁴¹¹ including swine^{116,411,546} and cattle.^{116,411} However, the significance of reovirus as a food animal pathogen is unclear. Reovirus infections also are common in dogs,¹³³ and reoviruses can be isolated from dogs with respiratory or enteric symptoms. However, experimental infections have not provided conclusive evidence that these viruses are pathogenic in canine species,⁷⁴ suggesting that concomitant infections with other agents are required to produce disease. Reovirus infections are common in both wild³⁷⁴ and domesticated^{116,435} cats, and reoviruses can be mildly pathogenic in experimentally infected feline species.^{335,435}

Fusogenic orthoreoviruses infect birds, mammals, and reptiles. Fusogenic bat reoviruses can be isolated from humans with clinical symptoms⁹⁶⁻⁹⁸ and, in one study, 13% of individuals frequently exposed to fruit bats were seropositive⁹⁶ (see Clinical Features—Pneumonia). Additional studies will be required to clarify the prevalence and clinical importance of these zoonotic events.

CLINICAL FEATURES

Reovirus Infections of Humans

Despite its near ubiquity, reovirus infections in humans and other animals are rarely symptomatic. When reoviruses do produce symptoms, the most common manifestations are coryza, pharyngitis, and cough²⁴⁰ as well as gastroenteritis.¹⁸⁹ Human-challenge studies conducted in the 1960s demonstrated that inoculation of reovirus strains representing each of the three serotypes by nasal instillation led to seroconversion in most cases, but only type 1 reovirus was associated with significant disease.⁴¹⁵ Three of eight previously seronegative individuals infected with type 1 reovirus developed headache, pharyngitis, sneezing, rhinorrhea, cough, and malaise. A fourth individual developed loose stools but was otherwise asymptomatic. Eight of nine individuals infected with type 3 reovirus had positive stool cultures.⁴¹⁵ One infected individual developed mild rhinitis, but the others remained well.

A large outbreak of type 1 reovirus infection was documented in institutionalized children in Washington, DC.⁴¹⁶ During a 9-week period in the months of January, February, and March, 22 of 73 children had at least one stool culture positive for type 1 reovirus. Stool shedding lasted for at least 1 week in the majority of the children. During the interval of virus isolation, the children had rhinorrhea (81%), pharyngitis (56%), diarrhea (19%), and otitis media (19%). However, the frequency and nature of these complaints did not differ significantly from those in the pre- and postisolation period. Therefore, it is not clear that the symptoms were associated with reovirus infection.

Biliary Atresia

Perhaps the strongest association of reovirus with human disease occurs in neonatal biliary atresia. This illness manifests in about 1 in 10,000 live births in the United States and is characterized by progressive inflammatory obliteration of extrahepatic and intrahepatic bile ducts.⁴⁸ Biliary atresia is thought to be precipitated by an environmental insult like a viral infection that triggers inflammatory destruction of bile duct epithelium. In a study performed in Denver, Colorado, reovirus RNA was detected by RT-PCR in fresh-frozen liver biopsy specimens in 78% (7 of 9) of tissue specimens from infants with choledochal cysts, a condition related to biliary atresia, and 55% (11 of 20) of those from infants with extrahepatic biliary atresia. However, reovirus RNA was detected in only 21% (7 of 33) of those with other liver diseases and 12% (2 of 17) of autopsy controls without known hepatic or biliary disease.⁴⁹⁹ The differences were statistically significant and support an association between reovirus infection and choledochal cysts and biliary atresia. A second study conducted in Hanover, Germany detected reovirus RNA in 33% (21 of 64) of fresh liver biopsies taken during Kasai portoenterostomy procedures.³⁹³ No other virus was detected in this study at a frequency of greater than 11%. However, other studies using different RT-PCR techniques failed to detect reovirus RNA in formalin-fixed tissue⁴⁷¹ or fecal samples.⁴²⁴ It is possible that these discordant findings are attributable to differences in the patient populations tested or dissimilar assay conditions (e.g., PCR primers and use of tissue fixatives).

Studies to examine the prevalence of reovirus-specific antibodies in infants with biliary atresia have yielded conflicting results. Some have found increased reovirus seroprevalence in such children,^{190,337,400} while others have not.^{71,72,155,331} Since reovirus infections are nearly universal by adulthood^{285,400,437,482} (see Epidemiology and Host Range), most infants will acquire reovirus-specific antibodies passively from their mothers. Therefore, it is not surprising that levels of reovirus-specific antibodies might not differ between infants with biliary atresia and healthy controls.

It is possible that only certain reovirus strains possess the appropriate combination of virulence determinants to induce biliary atresia. Following peroral inoculation of newborn mice, strain T3SA+, which binds sialic acid, causes bile duct injury. However, strain T3SA–, which does not bind sialic acid, does not harm bile ducts²⁸ (see Pathogenesis and Immunity—Hepatobiliary System). These strains also differ in the capacity to bind cholangiocarcinoma cells,²⁸ suggesting that sialic acid-binding capacity serves as a reovirus tropism determinant for biliary epithelium. It is possible that biliary atresia results only when an infant born to a reovirus antibody-negative mother is infected with a bile duct-tropic reovirus strain in the peripartum period. Additionally, a genetic predisposition toward aberrant inflammatory responses may be a prerequisite for development of the characteristic biliary fibrosis that follows viral infection.⁴⁸

Central Nervous System Disease

There are isolated case reports linking reovirus strains of all three serotypes to neurologic disease in children. For example, a healthy 3-month-old infant developed meningitis associated with a rise in titer of reovirus-specific antibodies and the isolation of type 1 reovirus from the cerebrospinal fluid (CSF).²⁴⁶

In another case, type 2 reovirus was isolated from CSF and stool of an 8-week-old girl with meningitis.²¹⁸ This child was subsequently found to have a congenital immunodeficiency with hypogammaglobulinemia. In a third case of reovirus-associated meningitis, type 3 reovirus was isolated from CSF of a 7-week-old child without other known morbidities.⁴⁹⁴ This virus was shown to produce encephalitis in mice.

Reovirus also has been associated with acute necrotizing encephalopathy (ANE), a CNS inflammatory condition that occurs a few days after the development of respiratory symptoms, often in association with influenza.^{230,334} Two cousins, a 6-year-old boy and a 22-month-old girl, were hospitalized in Tours, France with fever and neurologic symptoms following brief illnesses characterized by headache and rhinorrhea in the boy and girl, respectively.³⁷⁰ MRI scans of both children demonstrated characteristic abnormalities of ANE: high signal intensity on T2-weighted images in the thalami bilaterally, brainstem tegmentum, and cerebral white matter in external capsules. Type 2 reovirus was isolated from urine of both children and a throat swab from the girl.³⁷⁰ Culture and PCR tests were negative for other pathogens. Both children also developed reovirus-specific antibodies. Sequence analysis indicated that the reovirus isolates from these patients were identical and that most gene segments closely resembled strains circulating in China. Interestingly, a family member had returned from Indonesia a few days before the onset of symptoms in the children. No illness was reported in the returning traveler.

Myocarditis

Although certain reovirus strains cause myocarditis in newborn mice,^{363,448,451} reovirus is an unusual cause of myocarditis in humans. There is one case report of a possible association of reovirus infection with myocarditis in an adult.⁴⁸⁶ A 28-year-old man developed fever associated with transient paroxysmal atrial fibrillation and premature ventricular contractions in the absence of cardiac enzyme elevation. Stool culture yielded a reovirus isolate of unspecified serotype. Serologic studies and cardiac biopsy were not performed. It is possible that the reovirus strain isolated from stool contributed to the cardiac abnormalities, but this inference remains unproven.

Pneumonia

The fusogenic reoviruses have been associated with respiratory infections in humans. Melaka virus was isolated from a 39-year-old man in Melaka, Malaysia with fever and pneumonia.⁹⁶ Two family members developed similar symptoms 1 week after his illness and had serologic evidence of infection with the same virus. It is thought that Melaka virus was transmitted to the index case by an infected bat. Melaka virus encodes a FAST protein and produces syncytia in mammalian cells. The genome organization of the Melaka virus polycistronic S1 gene segment most closely resembles that of Pulau virus (another bat-borne fusogenic orthoreovirus) and NBV.⁹⁶ A closely related virus, Sikamat virus, was isolated from a 46-year-old man in Sikamat, Malaysia with fever, pharyngitis, and myalgia.⁹⁸ The man and his family had regularly visited an orchard frequented by fruit bats. Two family members had serologic evidence of Sikamat virus infection, but neither became ill. These cases illustrate the propensity of orthoreoviruses to spillover from bats to humans and suggest that targeted surveillance will reveal additional bat-borne zoonotic reovirus infections.

Diagnosis

Reovirus infections can be diagnosed by isolating virus from tissues or body fluids, detecting viral protein or RNA in patient samples, or demonstrating increases in reovirus-specific antibody titer. Reovirus has been isolated from nasal washings, throat swabs, urine, stool, and CSF. Monkey kidney cells have generally been considered optimum for isolating reovirus from clinical specimens, but reovirus infects most cells that are routinely maintained in clinical microbiology laboratories.⁴⁹⁵ In addition to characteristic cytopathic effect, the presence of reovirus proteins can be confirmed using either immunofluorescence or immunocytochemistry to detect reovirus proteins in infected cell cultures.⁴⁹⁵ Immunohistochemical techniques also can be employed for detection of reovirus proteins in tissue specimens.⁴⁹⁵ Determination of viral serotype can be accomplished using plaque-reduction neutralization or hemagglutination-inhibition assays with type-specific antisera. Sequence analysis of the S1 gene segment can be used to confirm the isolate serotype.^{137,494}

A variety of RT-PCR protocols have been established to detect reovirus RNA in blood or tissue samples. Primers specific for the L1,⁴⁹⁹ L3,⁴²⁴ M3,⁴⁷¹ and S4⁵⁵ gene segments have been used for this purpose. Primers specific for these and other gene segments also have been used to detect reovirus in cultured cells. The sensitivity and specificity of these protocols have not been established.

Reovirus-specific antibodies in serum can be detected by enzyme-linked immunosorbent assays^{399,437,482} and immunoblotting.^{370,437} Results can be confirmed by plaque-reduction neutralization or hemagglutination-inhibition assays.

Treatment

There are no established therapeutic modalities for reovirus infections in humans. Studies using cultured cells and experimental animals suggest that inhibition of either viral RNA synthetic capacity or host functions required for viral infectivity may have efficacy. Ribavirin, a broad-spectrum inhibitor of viral RNA synthesis, has antiviral activity against reovirus in cultured cells,^{111,392} but this drug has not been tested in animals. The inosine monophosphate dehydrogenase inhibitor, mycophenolic acid, inhibits the replication of both mammalian and avian reovirus strains in cultured cells by blocking viral transcription.^{219,402} There are no reports of this drug being used to treat reovirus infections *in vivo*.

A variety of pharmacologic inhibitors of host functions required for reovirus internalization and disassembly decrease reovirus yields in cultured cells. For example, chlorpromazine, which impedes clathrin-dependent uptake, chloroquine, which blocks endosomal acidification, and CLIK-148, which inhibits cathepsin L, arrest reovirus infection.^{247,298,306} Of these inhibitors, only CLIK-148 has been tested in animals; this drug diminishes reovirus loads and enhances survival in infected mice.²⁴⁷

Pharmacologic manipulation of host innate immune and apoptotic pathways dampens disease in mice infected with reovirus. Type 1 IFNs diminish reovirus infection in cultured cells^{242,452} and ameliorate reovirus-induced myocarditis³⁶³ and encephalitis in mice.¹⁴⁴ Minocycline delays disease onset and mortality in reovirus encephalitis in association with diminished viral loads and virus-induced apoptosis.³⁹⁷ Caspase inhibitors^{131,398} and a cell-permeating JNK inhibitor³⁵ have similar effects. Such strategies

may have efficacy in the treatment of other viral infections in which apoptosis is a major contributor to tissue pathology.

Passive administration of antiviral antibodies^{497,502,508} or virus-specific immune cells^{450,509} can protect mice infected with otherwise lethal doses of reovirus. Remarkably, both humoral and cell-mediated immune effectors can protect against lethal challenge even when administered after viral inoculation.

REOVIRUS ONCOLYTICS

The capacity of mammalian reoviruses to specifically infect and replicate in transformed cells was first noted in the late 1970s.^{152,212} Subsequent work showed that cells overexpressing native or truncated forms of the epidermal growth factor receptor are more susceptible to reovirus infection.^{477,479} This observation led to the discovery that cells with activated Ras signaling pathways are similarly more susceptible to reovirus infection.¹⁰⁸ In the last 15 years, several viruses have been tested in clinical trials for efficacy in the treatment of cancer. Reovirus strain T3D (Reolysin®, Oncolytics Biotech Inc.) is being tested for antineoplastic efficacy in phase I, II, and III clinical trials in humans (Table 44.5). However, other reovirus strains also appear to infect and kill transformed cells.¹¹

Although transformed cells with activated Ras signaling pathways are more susceptible to reovirus infection,⁴⁷⁶ those in which Ras pathways are not activated are also susceptible.⁴⁶⁴ The underlying mechanism for preferential replication of reovirus in transformed cells is not fully understood. However, a variety of factors enhance the susceptibility of transformed cells to reovirus-induced cell killing. These include a failure of transformed cells to mount a normal IFN response,⁴⁵⁴ enhanced proteolytic disassembly of the virus in the tumor microenvironment,^{10,307} increased receptor availability in tumor tissue,⁵⁰⁴ decreased particle-to-infectivity ratios of virus produced in

transformed cells, and increased virus release associated with caspase-mediated apoptosis.³⁰⁷ How cellular transformation enhances these processes is not known. However, stabilization of the p53 tumor suppressor protein enhances reovirus-induced apoptosis as a consequence of increased activation of NF-κB.³⁷² Increased reovirus-induced apoptosis in transformed cells also is associated with increased virus release and spread.^{307,372}

Reovirus T3D has been evaluated for oncolytic activity against a variety of tumors in animal models and humans (Table 44.5). Early studies of antitumor efficacy showed that human malignant glioma cell lines are susceptible to killing by reovirus.^{108,528} Subsequent work using tumor cell lines and animal models showed that reovirus can infect human bladder cancer,²⁵⁵ breast cancer,³⁶¹ colonic and ovarian tumors,²²² gastric cancers,²⁵² lymphoid tumors,⁹ medulloblastoma,⁵³⁸ melanoma,¹⁶² non-small-cell lung cancer,⁴³⁶ pancreatic cancer,¹⁶³ pediatric sarcomas,²²¹ and prostate cancer.⁴⁹⁰ In clinical trials performed thus far, reovirus is well tolerated in humans when given at doses of up to 3×10^{10} TCID₅₀ intravenously.¹⁹⁴ However, safety in immunodeficient persons has not been reported. Reovirus can systemically target metastatic tumors in animal models^{220,223} and humans⁵⁰⁷ and also is being evaluated for use as an *ex vivo* purgative for autologous stem cell transplantation.^{488,489} Reovirus oncolytic activity is increased when combined with chemotherapy or radiation (reviewed in 210).

PERSPECTIVES AND NEXT STEPS

Since its discovery more than 50 years ago, reovirus has been used to make numerous important contributions to the field of virology. The ability to manipulate the genome of both virus and host makes reovirus a tractable experimental system for studies to precisely define viral and host determinants of pathogenesis. The application of genetics, biochemistry,

TABLE 44.5 Completed Clinical Trials of Reovirus for the Treatment of Human Cancers^a

Cancer indication and phase	Method of delivery and therapeutic combination
Sarcomas metastatic to the lung (phase II)	Intravenous Reolysin ^b monotherapy
Metastatic melanoma (phase II)	Intravenous Reolysin monotherapy
Advanced head and neck cancers (phase II)	Intravenous Reolysin combined with paclitaxel and carboplatin
Advanced malignancies including melanoma and lung and ovarian tumors (phase I/II)	Intravenous Reolysin combined with paclitaxel and carboplatin
Advanced malignancies including bladder, prostate, lung, and upper gastrointestinal tumors (phase I)	Intravenous Reolysin combined with docetaxel
Advanced malignancies including pancreatic, lung, and ovarian tumors (phase I)	Intravenous Reolysin combined with gemcitabine
Various metastatic tumors, including head and neck tumors (phase II)	Intratumoral administration of Reolysin combined with low-dose radiotherapy
Recurrent malignant glioma (phase I/II)	Intratumoral infusion of Reolysin
Various metastatic tumors (phase I)	Intratumoral administration of Reolysin combined with radiotherapy
Various metastatic tumors (phase I)	Intravenous Reolysin monotherapy
Recurrent malignant glioma (phase I)	Intratumoral administration of Reolysin
Cancer confined to the prostate (phase I)	Intratumoral administration of Reolysin
Various progressive subcutaneous tumors (phase I)	Intratumoral administration of Reolysin

^aInformation obtained from the Oncolytics Biotech Inc. website.

^bReolysin® is reovirus strain type 3 Dearing.

TABLE 44.6 Key Unanswered Questions in Studies of Mammalian Reoviruses

1. What host molecules are used as reovirus receptors at sites of primary and secondary replication?
2. How does reovirus sort in the endocytic pathway?
3. How does reovirus penetrate membranes?
4. How are nonpolyadenylated reovirus mRNAs efficiently translated?
5. How do reovirus replication complexes form and function?
6. How are reovirus core particles and virions assembled?
7. How does reovirus disseminate systemically?
8. How does reovirus cause tissue injury?
9. How does reovirus activate and evade host innate and adaptive immune responses?
10. What is the basis and efficacy of reovirus oncolysis?

cell biology, and structural analyses has yielded a wealth of information about mechanisms that govern reovirus tropism, cell entry, replication, and disease. However, important questions remain (Table 44.6). Perhaps most enigmatic is why reovirus infection is so infrequently associated with disease. A crucial corollary question is whether mounting reports of fusogenic orthoreovirus infections of humans in Asia portend the emergence of more virulent reovirus strains in the future. As reovirus shares a number of features with more pathogenic viruses, it is likely that answers to these questions will reveal broadly conserved principles of virus biology and may illuminate new targets for broad-spectrum antiviral therapeutics and vaccines.

Rapidly advancing clinical applications will set future directions for reovirus research. Reovirus infects transformed cells more efficiently than it does nontransformed cells.¹⁵² Based on initial success in using reovirus for tumor killing in animal models,^{108,222} reovirus is currently undergoing evaluation in clinical trials as a virotherapeutic for aggressive and treatment-refractory human cancers.⁴⁷⁵ Reovirus undergoes primary replication in intestinal tissue with few or no symptoms in humans and is now amenable to genetic modification.²⁶¹ Therefore, it also is an excellent candidate for development of multifunctional vaccines to elicit mucosal immunity. Ongoing research to understand mechanisms by which reovirus engages host cells and initiates an infectious cycle will guide strategic retargeting of reovirus to selected host cells and tissues to maximize oncolytic and immunostimulatory potential.

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diseases (a) are one of the most common illnesses in this age group throughout the world; (b) are one of the six leading causes of 10.6 million deaths that occur annually in children younger than 5 years of age; and (c) account for 18% (i.e., greater than 2 million) of the 10.6 million deaths,⁸⁹ with the greatest toll being in developing countries.^{89,788}

Viruses were first discovered to be significant causes of diarrheal illness in the 1970s, with Norwalk virus being the first agent discovered in 1972 by Kapikian et al⁴⁰⁰ from an outbreak of gastroenteritis in a school in Norwalk, Ohio. The Norwalk and related viruses, now known as noroviruses, are members of the genus *Norovirus* of the family *Caliciviridae* (see Chapter 20). Human rotaviruses were discovered in 1973, when particles were visualized by Bishop et al^{59,60} in electron micrographs of thin sections of duodenal mucosa and later virus was identified in feces by electron microscopy (EM).^{58,246,398,505} Both of these fastidious gastroenteritis viruses were discovered without the benefit of tissue culture technology; their identification relied on direct visualization by EM. The 70-nm particles (Fig. 45.1)^{388,398} from children's feces were subsequently designated rotavirus²⁴⁷ and documented to be an important etiologic agent of severe diarrhea of infants and young children during the first 2 years of life^{56,391} in both developed and developing countries (Fig. 45.2); rotaviruses consistently outranked in importance other known etiologic agents of severe diarrhea.³⁹⁰

Although the human rotaviruses were discovered in 1973, several animal viruses were described during the previous 10 years that were later found to be rotaviruses based on exhibiting characteristic rotavirus morphology and sharing a group antigen with human rotaviruses.^{247,393} These animal agents included (a) the epizootic diarrhea of infant mice (EDIM) virus seen by Adams and Kraft,⁴ using thin-section EM in intestinal tissue of mice infected with EDIM virus; (b) a 70-nm simian agent 11 (SA11) that was cultivated in vervet monkey kidney cells from a rectal swab obtained from a healthy vervet monkey⁴⁷⁰; (c) the O (offal) agent isolated in vervet monkey kidney cell culture from the mixed washings of intestines of cattle and sheep⁴⁷¹; and (d) 70-nm virus particles in stools from calves with a diarrheal illness that could be passaged serially in calves and produce disease,⁴⁹⁸ were also cultivated in primary fetal bovine cell cultures, and were named the Nebraska calf diarrhea virus (NCDV).⁴⁹⁹ Thus, rotaviruses were excreted in stools of many species and frequently associated with diarrheal disease.

INTRODUCTION AND HISTORY

Rotaviruses are the single most important cause of severe diarrheal illness in infants and young children in both developed and developing countries worldwide, accounting for 30% to 50% of these illnesses.^{45,562,563–564} Regardless of cause, diarrheal

CLASSIFICATION

Rotaviruses are members of the genus *Rotavirus* within the family *Reoviridae*, and rotaviruses share common morphologic

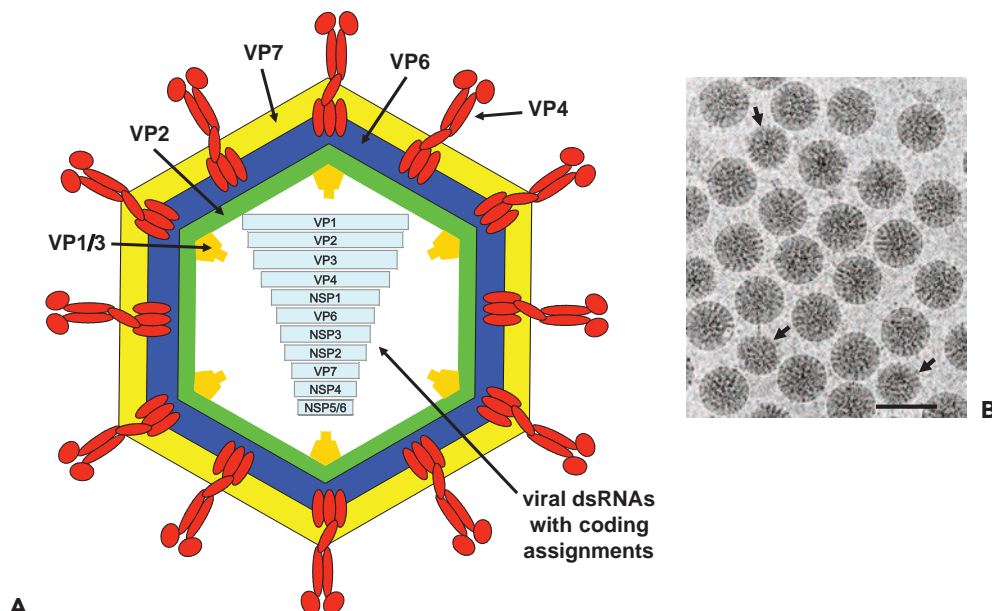


FIGURE 45.1. Schematic diagram and electron micrograph of rotavirus particles. A: The particle is composed of three concentric protein shells (VP7, VP6, and VP2, shown in different colors) and the spike protein VP4 that spans the VP6 and VP7 layers and extends out from the particle. A transcription complex of VP1 and VP3 is inside the VP2 layer. The viral double-stranded RNA (dsRNA) genome is segmented. **B:** Rotavirus triple-layered particles (TLPs) and a few double-layered particles (DLPs) (arrows) are easily visualized by electron microscopy (**right panel**). Bar, 100 nm.

and biochemical properties (Table 45.1). Early studies using negative-stain EM techniques underestimated the particle diameter, and the subsequent cryo-EM studies, in which no stains are used, established the particle diameter to be 100 nm including the spikes. Salient features are that (a) mature virus particles, including spikes, are about 100 nm (1,000 Å) in diameter and possess a triple-layered icosahedral protein capsid composed of an outer layer, an intermediate layer, and an inner core layer; (b) 60 protein spikes extend from the smooth surface of the outer shell; (c) outer capsid integrity requires calcium; (d) particles contain an RNA-dependent RNA

polymerase and other enzymes capable of producing capped RNA transcripts; (e) the virus genome contains 11 segments of double-stranded RNA (dsRNA); (f) rotaviruses of the same group (see later) are capable of genetic reassortment; (g) virus replication occurs in the cytoplasm of infected cells; (h) virus cultivation *in vitro* is facilitated by treatment with proteolytic enzymes enhancing viral infectivity by cleavage of the outer capsid spike polypeptide; and (i) the viruses exhibit a unique morphogenic pathway (transiently enveloped virus particles are formed by budding into the endoplasmic reticulum [ER]) during morphogenesis. Mature particles are nonenveloped,

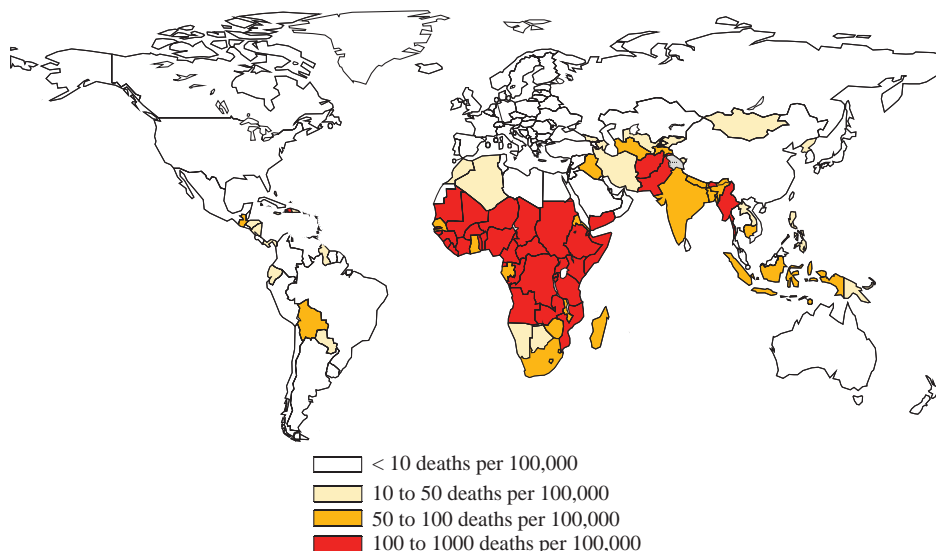


FIGURE 45.2. Rates of rotavirus mortality among children younger than 5 years of age by country. Colors indicate number of deaths per 100,000. (Data for 2008 from Umesh Parashar, Centers for Disease Control and Prevention.)

TABLE 45.1 General Characteristics of Rotaviruses

Structure
100-nm icosahedral particles (including the spikes)
Triple-layered protein capsid
Nonenveloped (resistant to lipid solvents)
Capsid contains all enzymes for mRNA production
Genome
11 segments of dsRNA
Purified RNA segments are not infectious
Each RNA segment codes for at least one protein
RNA segments from different viruses reassort at high frequency during dual infections of cells
Replication
Cultivation facilitated by proteases
Cytoplasmic replication
Inclusion body formation
Unique morphogenesis involves transient enveloped particles
Levels of intracellular calcium important for virus assembly and stability
Virus particles released by cell lysis or by nonclassical vesicular transport in polarized epithelial cells

mRNA, messenger RNA; dsRNA, double-stranded RNA.

and virions are liberated from infected cells by cell lysis or by a nonclassic vesicular transport in polarized epithelial cells.

Rotaviruses are classified serologically by a scheme that allows for the presence of multiple groups (serogroups, based on VP6 reactivity) and of multiple serotypes within each group (based on VP4 and VP7 neutralizing epitopes). Rotaviruses are composed of seven distinct groups (A to G, now designated RVA, RVB, RVC, etc.). RVA, RVB, and RVC strains are found in both humans and animals, whereas rotaviruses of groups D, E, F, and G have been found only in animals to date. Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses in different groups, and thus RV groups are considered unique species.⁸⁰⁶ A rotavirus group includes viruses that share cross-reacting antigens detectable by a number of serologic methods, such as immunofluorescence, enzyme-linked immunosorbent assay (ELISA), and immune electron microscopy (IEM). Cross-reactive epitopes on the inner capsid protein (VP6) are those usually detected by diagnostic ELISA, primarily because this protein is highly antigenic and it represents the largest mass of the particle. However, common antigens are found on most (if not all) of the structural proteins and probably on many of the nonstructural proteins as well. This is documented by observing that monospecific antisera and some monoclonal antibodies (mAbs) specific for individual polypeptides cross-react with strains other than those to which they were made.

RVAs cause significant diarrheal disease in infants and in the young of various mammalian and avian species. RVBs have been associated with epidemics of severe diarrhea primarily in adults in Asia.^{355,655,700} RVCs have been sporadically reported in fecal specimens from children with diarrhea and in several family outbreaks.⁵⁵⁷ Rapid diagnostic tests (ELISA), mAbs, and polymerase chain reaction (PCR) assays to detect non-

group A rotaviruses are available mainly in research laboratories, and these facilitate determining the clinical importance of these viruses.^{534,807} Very few non-group A rotavirus strains have been successfully propagated in cell culture. The inability to grow most non-group A viruses has hampered obtaining detailed information on these viruses, although gene-coding assignments and sequence data are available. Unless noted otherwise, this chapter focuses on information about the RVA viruses. Reviews on the non-group A rotaviruses and comparisons between the proteins of the group A and non-group A viruses have been published elsewhere.^{80,374,486,655}

Within RVA, viruses are classified into serotypes defined by reactivity in plaque reduction (or fluorescent foci reduction) neutralization assays using hyperimmune serum prepared in antibody-negative animals. With such assays, 27 VP7 (or G [for glycoprotein]) serotypes have been identified (Table 45.2), and strains of animal and human origin may fall within the same G serotype. Neutralization assays can measure reactivity of antibody against the two outer capsid neutralizing antigens (VP7 and VP4, Tables 45.2 and 45.3). In most cases, however, the predominant reactivity measured when using hyperimmune antisera is against the glycoprotein VP7. This may be because VP7 makes up a greater percentage of the virion outer capsid than VP4 does, or alternatively, with hyperimmunization, VP7 selectively induces highly specific antibodies. The protein specificity of neutralizing antibodies after primary and secondary infection is less well defined. Identical classification of the same virus isolates using mAbs to VP7 unequivocally demonstrates that plaque-reduction neutralization assays with hyperimmune serum *primarily* measure reactivities with VP7.^{54,155,708}

In some cases, a rotavirus strain will not react clearly in reciprocal neutralization assays with hyperimmune antiserum. This is usually because the two viruses being compared possess distinct immunologic forms of VP4 (the second outer capsid protein), which is also a neutralization antigen (Table 45.3). Many mAbs to VP4 possess neutralization activity. Because the genes encoding these two distinct neutralization antigens can segregate (reassort) independently, it is not surprising that some virus isolates possess heterologous neutralization (VP4, VP7) antigens.²⁴⁹ Rotaviruses are classified by a binary system (similar to that used for influenza viruses) in which distinct types of VP4 and VP7 are recognized.^{296,345,634} A lack of readily available typing serum or mAbs to different VP4 types, however, has hampered classification of VP4 (or P [for protease-sensitive protein]) serotypes. Instead, properties of VP4 have been studied primarily by sequence analysis, and current evidence indicates the existence of at least 35 different genotypes of VP4 (Table 45.3). Genotypes of VP4 and VP7 are determined by sequence analysis, whereas serotypes are determined by reactivity of individual strains or selected reassortants with polyclonal or monoclonal antisera.^{216,343} For VP7, a correlation between genotype and serotype has been established. Such a correlation is much less clear for VP4, although a variable region on VP8* that spans amino acid (aa) 71 to 204 can define P type-specific epitopes.²⁸⁹ Serotype designation, thus, reflects the expression of neutralization epitopes on both VP4 and VP7. The serology of the epitopes in proteins that interact in the capsid is complicated but is beginning to be understood with the availability of high-resolution structural data on these outer capsid proteins.

In 2008, a comprehensive nucleotide sequence-based, complete genome classification system was developed for

TABLE 45.2 Group A Rotavirus VP4 [P] Genotypes and Serotypes (P)

Genotype ^a	Serotype	Strain (bold) followed by species of origin	
		Human ^b	Animal
1	6		Bo/RF , Bo/C486, Bo/NCDV, Bo/BRV033, Bo/BRV16 Bo/A5, Bo/CR231/39, Bo/J2538 Si/SA11 4fm, Si/SA11-FEM, Eq/26/94, Ov/LRV1
2	5B		Si/SA11-H96 , Si/SA11 Cl3, Si/SA11-SEM
3	5B		Si/RRV, Fe/FRV64; Bu/10733; Ca/GRV
4	5A	Ro1845, HCR3a	Ca/K9 , Ca/CU-1, Ca/RS15, Fe/Cat97
4	1B	DS-1 , S2, L26, KUN, RV-5, E210, E201, CHW17, AU64, 107EIB, MW333, TB-Chen	
5	7	A64	Bo/UK , Bo/WC3, Bo/B641, Bo/61A, Bo/VMRI, Bo/678, Bo/V1005, Bo/IND, Po/PRV 4S, Po/P343 Po/134-04-8, Po/134/4-10
6	2A	ST3 , M37, 1076, RV-3, SC2, BrB McN13, US1205, MW023, US585	
	2B		Po/Gottfried, Po/BEN-144, Po/S5, Po/S7, Po/SB-2A
	2C	AU19	Po/JP3-6, Po/JP29-6
7	9		Po/OSU , Po/TFR-41, Po/C60, Po/JL94, Po/C134, Po/C95, Po/A821, Po/A138, Po/YM, Po/A253, Po/C91, Po/BMI-1, Po/AT/76, Po/SB- 2A, Po/SW20/21, Po/SB-1A, Po/CRW-8, Po/BEN-307, Po/CN117, Po/CC117, Po/A131, Po/EE, Po/ISU-64, Eq/H-1 Po/S8A, Po/S8B, Ov/LRV2c
8	1A	Wa , KU, P, YO, MO, VA70, D, AU32, CH-32, CH-55, CHW2, CH927A, WI61, F45, Ai-75, Hochi, Hosokawa, BR1054, WI78, WI79	
9	3	AU-1 , K8, PA151, M318, AU228, 02/92, T152, 0264	Fe/FRV-1, Fe/Cat2
10	4	69M , 57M, B37	
11	8	116E , I321, 157C	Bo/B223, Bo/A44, Bo/KK3, Bo/B-11, Bo/Cr, Bo/KN-4, Bo/CR129, Bo/BR65/255, Eq/R-22, Ov/LRV2a
12	4		Eq/E30 , Eq/H-2, Eq/Fl14, Eq/Fl23, Eq/FR4, Eq/FR5, Eq/FR8, Eq/K311, Eq/K1673, Eq/CH3, Eq/69, Eq/124, Eq/EQ431 Po/MDR-13, Po/A46, Po/Clon8
13	ND	MP130	
14	11	A64 , PA169, HAL1166, HAL1271, HAL8590, Mc35, MG6, GR475/87, B4106	La/ALA, La/C-11, La/BAP-2, La/R-2, La/30/96, La/MV1, La/MV3, Gu/Río Negro, Gu/Chubut Cap/Cap455, Ov/OVR762
15	ND		OV/Lamb-NT , Ov/Ip14, Ov/Ip16, Ov/K923
16	10		Mu/ETD-822 , Mu/Eb, Mu/EW, Mu/EC
17	ND		Pi/PO-13 , Bo/993/83, Ch/Ch-1, Ty/Ty-1, Ty/Ty-3
18	ND		Eq/L338
19	12	RMC321 , Mc323, Mc345	Po/PRV 4F
20	13		Mu/EHP
21	ND		Bo/Hg18
22	ND		La/160/01 , La/229/01, La/308/01, La/MV2, La/MV4, La/MV5
23	14		Po/A34 , Po/34461-4
24	ND		Si/TUCH
25	ND	Dhaka6 , KTM368	
26	ND		Po/134/04-15
27	ND		Po/CMP034 , Po/344/04-1, Po/P21-5
28	ND	Ecu534	
29	ND		Bo/AzuK-1
30	ND		Ch/02V0002G3 , Ch/Ch-2G3
31	ND		Ch/Ch-06V0661
32	ND		Po/61/07
33	ND		Bo/Dai-10
34	ND		Po/FGP51
35	ND		Ty/03V0002E10

Note: Serotypes P1A and P1B are two distinct serotypes, while serotypes P5A and P5B are subtypes of the same serotype, as are P2A, P2B, and P2C.

Si, simian; La, lapine; Po, porcine; Ca, canine; Mu, murine; Pi, pigeon; Bu, buffalo; Eq, equine; Fe, feline; Bo, bovine; Ov, ovine; Cap, caprine; Ty, turkey; Ch, chicken; Gu, guanaco; ND, not determined.

^aGenotypes 4, 6, 8, 9, 10, 11, 14, and 25 are epidemiologically important for humans.

^bFirst strain listed and bolded is reference strain for that genotype.⁴⁸³ For complete nomenclature guidelines of rotavirus strains refer to Matthijnssens et al.⁴⁸³

Adapted from Estes and Kapikian²¹⁷ and updated with data from Matthijnssens et al.,^{482,483} Solberg et al.,⁶⁹⁰ Trojnar et al.,⁷³⁵ and Schumann et al.⁶⁶⁸

TABLE 45.3 Group A Rotavirus VP7 (G) Serotypes/Genotypes

Serotype ^a	Strain followed by species of origin	
	Human ^b	Animal
1	Wa , KU, RV-4, K8, D, M37, Mont, AU64, WI79, AU19, AU32, HN-1, GR475/87, 89-12, GR67/91	Bo/T449, Po/C60, Po/C91, Po/C95, Po/CN117, Po/C86, Po/S7, Po/S8A
2	DS-1 , SC2, S2, RV-5, RV-6, KUN, HN-126, 1076, E201, E210, HU5, 7PE/89, BS3585/99, TB-Chen	Po/34461-4 ^c
3	Au-1 , Ito, YO, P, M, Nemoto, RV-3, WI78, AK-35, ST8, MO, McN13, McN14, HCR3a, CH927A, Ro1845, O264, AU228, PA710, O2/92, CH-32, CHW2, CHW17, CH-55, Ai-75, J-12, TK08, TK28, 107EIB, 157C, B4106	Si/SA11 Cl3, Si/SA11 4fm, Si/SA11-SEM, Si/SA11-FEM, Si/RRV, Ca/K9, Ca/CU-1, Ca/A79-10, Ca/LSU79C-36, Ca/RS15, Eq/H-2, Eq/FI14, Eq/HO-5, Eq/69, Eq/124, Eq/K1673, Eq/311, Fe/Cat97, Fe/Cat2, Fe/Cat22, Fe/Taka, Fe/FRV64, Fe/FRV-1, La/ALA, La/C-11, La/R-2, La/BAP-2, La/160/01, La/30/96, Po/CRW-8, Po/A131, Po/A138, Po/LCA843, Po/A821, Po/A411, Po/BEN-307, Po/PRV 4F, Po/AT/76, Po/PRV 4S, Po/MDR-13, ^d Ov/LRV1
4	ST3 , Hochi, Hosokawa, 57M, BrB, ST4, VA70 BR1054, IAL28 ^e	Po/Gottfried, Po/SB-1A, Po/SB-2A, Po/BEN-144, Po/BMI-1
5		Po/OSU , Po/EE, Po/TFR-41, Po/A34, Po/A46, Po/C134, Po/CC117, Po/S8B, Po/134/04-15, Eq/H-1
6	PA151, PA169, MG6, Hun4, Hun5, ASG6.02	Bo/RF , Bo/NCDV-Lincoln, Bo/UK, Bo/B641, Bo/C486, Bo/WC3, Bo/KN-4, Bo/IND, Bo/BRV033, Bo/B-60, Bo/CR231/39, Bo/Q17, Ov/LRV2a, Cap/Cap455
7	69M , B37, HAL1166, HAL1271, HAL8590, EGY2295, GRV570, DG8, QEH14262, HMG89, MW333, MW023, EGY1850	Ty/Ty-3 , Ch/Ch-2, Ty/Ty-2
8		Bo/678, Bo/A5, Bo/NCDV-Cody, Bo/J2538, Bo/BRV16, Bo/Sun9, Bo/Niigata, Eq/26/94, Ov/OVR762, Gu/Rio Negro, Gu/Chubut
9		Po/ISU-64, Po/S5, Ov/LRV2c
10	A64 , I321, Mc35, A28	Bo/B223, Bo/61A, Bo/V1005, Bo/KK3, Bo/B-11, Bo/A44, Bo/CR129, Bo/BR65/255, Eq/R-22, Ov/Ip14, Ov/K923, Po/P343
11	Dhaka6, KYM368	Po/YM , Po/A253
12	L26 , L27, US585, T152, Se585, K12, ISO-1, ISO-2, ISO-5, CP727, CP1030	Po/RU172
13	Ecu534	Eq/L338
14		Eq/403 , Eq/FI23, Eq/FR4, Eq/FR5, Eq/FR8, Eq/EQ431, Eq/CH3
15		Bo/383, Bo/Hg18
16		Mu/ETD , Mu/EW, Mu/EB, Mu/EL, Mu/EC, Mu/EHP, Mu/YR-1
17		Ty/Ty-1
18		Pi/PO-13 , Bo/993-83
19		Ch/02V0002G3 , Ch/Ch-1, Ch/Ch-2G3
20		
21		Bo/AzuK-1
22		Ty/Tu-03V0002E10 , Ty/Tu-03V0001E10
23		Ph/Phea14246 , Ph/Phea17655, Ph/Phea15871
24		Bo/Dai-10
25		Bt/KE4852/07
26		Po/TJ4-1

Si, simian; La, lapine; Po, porcine; Ca, canine; Mu, murine; Ty, turkey; Ch, chicken; Pi, pigeon; Eq, equine; Fe, feline; Bo, bovine; Ov, ovine; Cap, caprine; Gu, guanaco; Ph, pheasant; Bt, bat.

^aSerotypes 1, 2, 3, 4, 9, and 12 are epidemiologically important for humans.

^bFirst strain listed and bolded is reference strain for that genotype.⁴⁸³ For complete nomenclature guidelines of rotavirus strains refer to Matthijnsens et al.⁴⁸³

^cPo/34461-4 is considered a G2-like virus because it has borderline amino acid sequence identity with G2 human viruses.

^dPo/MDR-13 has dual serotype reactivity (G3 and G5).

^eHu/IAL28 has dual serotype reactivity (G5 and G11).

Adapted from Estes and Kapikian²¹⁷ and updated with data from Matthijnsens et al,⁴⁸³ Solberg et al,⁶⁹⁰ Trojnar et al,⁷³⁵ Schumann et al,⁶⁸⁸ and Ursu et al.⁷⁴¹

TABLE 45.4 Nucleotide Percentage Identity Cut-off Values Defining Genotypes for 11 Rotavirus Gene Segments

Gene product	Percentage identity	Genotypes	Name of genotypes
VP7	80	27G	Glycosylated
VP4	80	35P	Protease sensitive
VP6	85	16I	Inner capsid
VP1	83	9R	RNA-dependent RNA polymerase
VP2	84	9C	Core protein
VP3	81	8M	Methyltransferase
NSP1	79	16A	Interferon Antagonist
NSP2	85	9N	NTase
NSP3	85	12T	Translation enhancer
NSP4	85	14E	Enterotoxin
NSP5	91	11H	PHosphoprotein

Modified from Matthijnsens J, Ciarlet M, Rahmanet M, et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Arch Virol* 2008;153:1621–1629.

RVAs.⁴⁸³ This system assigns a specific genotype to each of the 11 RV genome segments according to established nucleotide percent cutoff values. The *VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6* genes of RV strains are described using the abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x = Arabic numbers starting from 1), respectively (Table 45.4). A Rotavirus Classification Working Group that includes researchers worldwide maintains and evaluates this system. Recent updates are published and new guidelines recommend uniform nomenclature for individual strains to be RV group/species of origin/country of identification/common name/year of identification/G and P type.⁴⁸² The prototype simian agent 11 is designated RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] and the full descriptor of genes is

indicated by G3-P[2]-I2-R2-C5-M5-A5-N5-T5-E2-H5 using this new system.

VIRION STRUCTURE

The morphologic appearance of rotavirus particles is distinctive, and three types of particles can be observed by EM (Fig. 45.3). The complete particles resemble a wheel with short spokes and a well-defined, smooth outer rim. The name *rotavirus* (from the Latin *rota*, meaning “wheel”) was coined based on this morphology.²⁴⁷ The complete infectious particles (virions) are also called *triple-layered particles* (TLPs). These particles are ~100 nm in diameter, which is relatively large for a nonenveloped, icosahedral virus. Double-layered particles (DLPs) lacking the outer shell are described as *rough particles* because their periphery shows projecting trimeric subunits of the inner capsid. Single-layered particles (SLPs or cores) are seen infrequently; they usually lack genomic RNA and are aggregated.

The structures of triple- and double-layered rotavirus particles are solved to near-atomic resolution based on x-ray crystallography and particle reconstructions of cryo-electron microscopy (cryo-EM) images, and these provide a detailed description of particles^{121,434,435,440,488,605,675,679,803,828} (Fig. 45.4). Particles possess icosahedral symmetry with a T = 13I (levo) icosahedral surface lattice for the two outer layers, while the innermost layer exhibits a unique T = 1 icosahedral organization. Distinguishing features of the TLP structure include 132 large aqueous channels and 60 spikes.

The channels span the two outer layers and link the outer surface with the inner core. Three types of channels are distinguishable based on their position and size. Twelve type I channels are located at the icosahedral fivefold axes, 60 type II channels are at each of the pentavalent positions surrounding the fivefold axes, and a second set of 60 type III channels are at the six-coordinated positions surrounding the icosahedral threefold axes. Type III channels are about 140 Å in depth and about 55 Å wide at the outer surface of the virus. On entering the particle, these channels constrict before widening to their

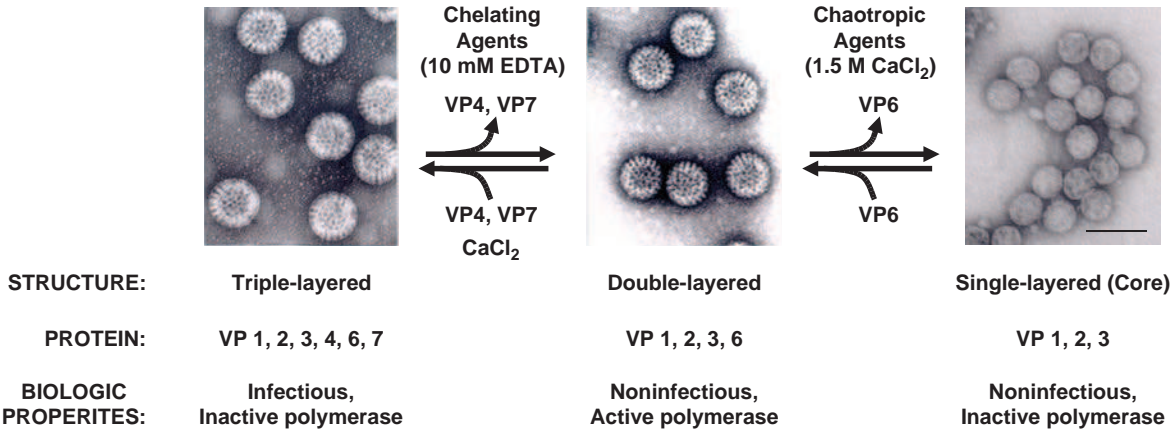


FIGURE 45.3. Structural and biological properties of rotavirus particles. Electron micrographs show typical triple-layered particles (TLPs), double-layered particles (DLPs), and single-layered particles (SLPs) (core) after staining with 1% ammonium molybdate. TLPs, DLPs, and core particles can be produced by sequential capsid protein removal (top arrows) or addition (bottom arrows), as shown. The proteins and biological properties of the particles are detailed in the text. Bar, 100 nm.

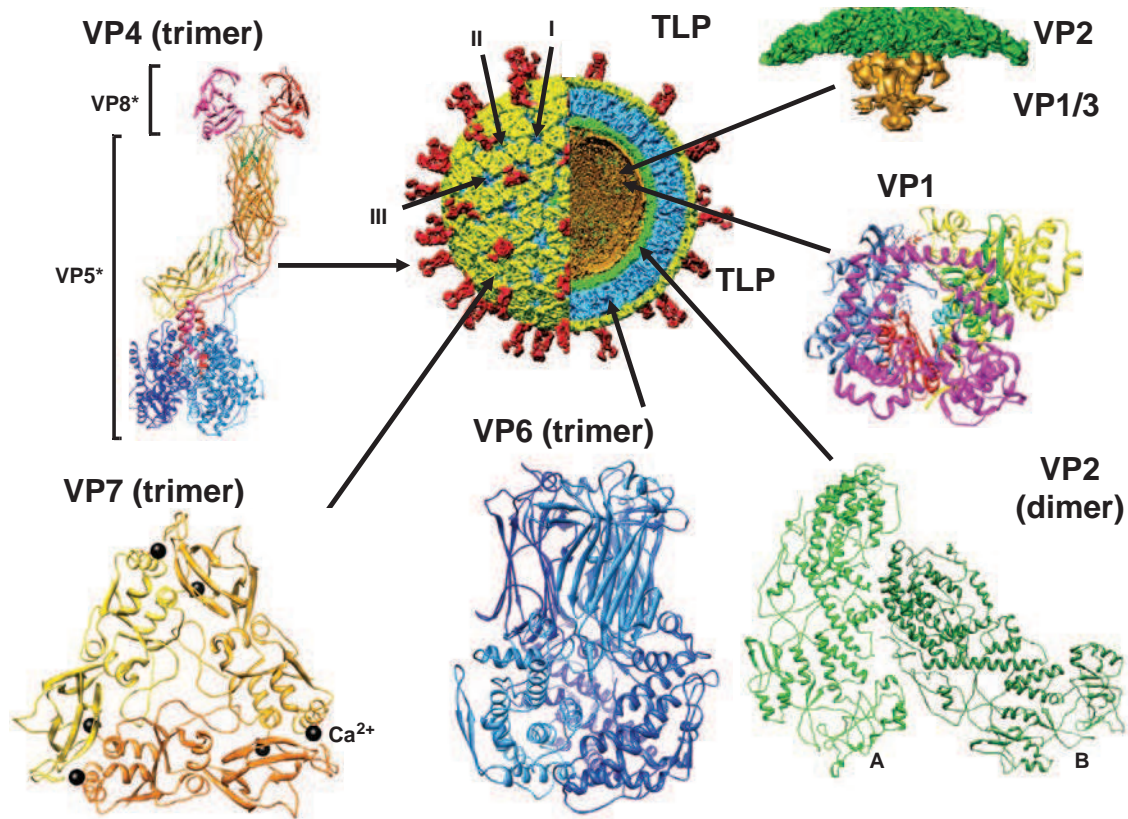


FIGURE 45.4. Rotavirus structures and locations of protein components. A cutaway view of a 9.5-angstrom cryo-electron microscopy (cryo-EM) reconstruction of the mature triple-layered rotavirus particle (TLP) shows surface and internal structural features. The TLP is colored with the VP4 spikes (60 trimers) in red, the VP7 (780 trimers) surface glycoprotein in yellow, the internal (middle) VP6 (780 trimers) layer in blue, and the core VP2 (120 dimers) layer in green. Atomic structures of the individual proteins also are shown along with their locations in the virion. Structures made from Protein Data Bank (PDB) IDs: 2R7R (VP1), 3KZ4 (VP2), 3IYU (VP4), VP6 (3KZ4), 3FMG (VP7). The two calcium (Ca^{2+}) molecules coordinated between each of the VP7 monomers are shown in black.

maximal width, which is close to the surface of the inner shell. Similar features and dimensions are seen in the other two types of channels, except that type I channels have a narrower (~ 40 Å) opening at the outer surface of the virus. The type I channels are conduits for the export of messenger RNA (mRNA) that first interacts with the enzyme complexes composed of VP1, the RNA-dependent RNA polymerase, and VP3, the methyltransferase and guanylyltransferase, that are present at the inner surface of the fivefold axes of the SLP^{432,435,604} (see Fig. 45.4). Atomic structures of the rotavirus polymerase, alone and in complex with RNA, show VP1 is a compact, globular protein of ~ 70 Å in diameter that has three domains: an N-terminal domain, a polymerase domain, and a C-terminal domain.⁴⁶⁰ The polymerase domain exhibits the right-handed architecture (fingers-palm-thumb) typical of polymerases in general as well as canonical motifs (A to F) involved in various aspects of phosphodiester bond formation^{461,546} (Fig. 45.4). The N-terminal and C-terminal domains envelop the polymerase domain, creating a cage-like enzyme with a hollow catalytic center. Four tunnels lead into the center, serving as conduits for the entry and exit of free nucleotides (nucleoside triphosphates [NTPs]), template RNAs, and RNA products. This solved structure represents a catalytically inactive form of the polymerase captured prior to the initiation of dsRNA synthesis. Models of how

this enzyme functions in coordinated genome replication and packaging are discussed later.

The single-layered particle exhibits a unique $T = 1$ symmetry and is composed of 120 molecules of VP2 arranged as 60 dimers that surround the genomic dsRNA that is highly ordered^{434,488,604} (Fig. 45.4). X-ray and cryo-EM structures of DLPs show that VP2 has two structural isoforms that interact extensively. One of the subunits in the asymmetric unit (VP2A) packs around the icosahedral fivefold axis forming a star-shaped complex with a small pore in the middle lined by conserved basic residues. The other subunit (VP2B) fills in space between the VP2A subunits forming a decameric cap structure at the fivefold axis. Twelve of these decameric complexes make up the VP2 layer that is 25 to 30 Å thick. Many segmented dsRNA viruses contain 120 molecules of a core protein (VP2 for rotavirus, VP3 for bluetongue virus, lambda 1 for reoviruses, the single capsid protein for cypovirus) that surrounds an ordered genome.^{307,337,825} Although the inner shell protein shares similar features among these dsRNA viruses, rotaviruses and orbiviruses are distinguished by housing their enzymatic functions entirely *within* the inner shell, leading them to be called *nonturreted viruses*, and nascent mRNA transcripts are released through channels penetrating the two capsid layers at the icosahedral vertices.^{337,433,434,536} This

capsid architecture contrasts with the structure of the *turreted viruses*, reoviruses,^{624,829} aquareovirus,⁸²⁷ and cypovirus,^{434,536,815} where the polymerase enzyme is housed within the core, but the capping enzymes are incorporated as pentameric turret-like projections that extend through the inner capsid layer at each icosahedral vertex.³³⁷

The VP2 layer is surrounded by 260 trimers of VP6 that form a $T = 13$ icosahedral lattice (Fig. 45.4). These trimers are located right below the VP7 trimers in the outer layer so that the channels are in register. The double-layered particle is about 705 Å in diameter, and the structure of the VP6 subunit has two domains with an overall structure similar to the VP7 of bluetongue virus^{307,478} (Fig. 45.4) and to the $\mu 1$ protein of orthoreovirus.⁴⁴² The distal domain of rotavirus VP6 has an eight-stranded jelly-roll β -barrel fold that makes contacts with VP7 and VP4, whereas the proximal domain with a cluster of eight α -helices and a conformationally flexible loop structure in VP6 is involved in establishing optimal contacts with the underlying VP2 subunits.^{488,828} Interactions of VP6 with the VP7 layer at the top and the VP2 layer at the bottom are important in stabilizing the entire rotavirus capsid and integrating the two essential functions of particles: cell entry and endogenous transcription. Structural integrity of the DLP is an essential requirement for endogenous transcription that takes place within the confines of the DLP with capped transcripts exiting through the aqueous type I channels at the fivefold axes.⁴³⁴

Sixty trimeric spikes extend from the smooth surface of the outer shell (Fig. 45.4). These protein spikes are situated at an edge of the type II channels surrounding the fivefold icosahedral axes. The spikes are composed of the protein VP4, as initially shown by seeing that two Fab subunits of mAb to VP4 bind on the sides near the tips of the spikes.⁶⁰⁶ Subsequent cryo-EM studies,^{158,192,587,679} including the most recent study at near-atomic resolution,⁶⁷⁵ confirmed that VP4 is the spike protein and showed that the spike is multidomained with a unique trimeric organization that projects about 120 Å from the surface of the virus with a total radial length of 200 Å. Spikes with well-defined structural features, two distal globular domains, a central body with an approximate twofold symmetry, and a globular domain called the foot domain are only visible in rotavirus particles grown in the presence of trypsin that enhances virus yield.^{158,679,802} Proteolysis cleaves VP4 (88K) into VP8* (28 kD, aa 1 to 247) and VP5* (60 kD, aa 248 to 776), and the cleavage products remain noncovalently associated in the virion (Fig. 45.4 and Table 45.5).

Recent crystallographic structures of VP8* and portions of VP5* as well as cryo-EM analyses at about 4.3 Å resolution indicate that the overall conformation of each VP4 subunit is that of a high loop, with the N-terminal helical segment of VP8* anchored against the C-terminal domain of VP5* in the foot domain of the same polypeptide chain.^{192,194,675} Different conformations including dimers, trimers, and asymmetric domains make up the unique trimeric configuration of the cleaved VP4 spike. Two β -barrel domains of VP5*, at the central body of the spike, adopt a dimeric appearance above the capsid surface, while another VP5* β -barrel of the third VP4 molecule is positioned closer to the capsid surface interacting with the VP7 capsid layer; the globular domains of each of the VP5* polypeptides form a trimeric base anchored inside the type II channels between the VP7 and VP6 capsid layers.^{440,587,675,802} Two VP8* molecules are present at the top of two upright VP5* molecules

and the third VP8* is disordered or may be removed by trypsin cleavage.⁶⁷⁵ Thus, VP4 subunits undergo extensive rearrangements that resemble conformational transitions of membrane fusion proteins of enveloped viruses during entry into cells.¹⁹²

The spike is held in place by interactions with both VP6 and VP7. The spike extends inward about 80 Å where it inserts into the lattice of VP6 trimers at the type II channels that surround the icosahedral fivefold axes. This interaction may template trimerization of cytosolic VP4.⁶⁷⁵ The VP7 shell partly covers the base of the VP4 spike and appears to lock VP4 onto the virion within the type II channel.

The outer 35-Å-thick capsid layer of rotavirus is formed by 260 trimers of the glycoprotein VP7 (37 kD) (Fig. 45.4). VP7 is a calcium-binding protein^{262,648} and consists of two domains: domain I with a disulphide bridge exhibits a Ross-mann fold and domain II with three disulfide bridges exhibits a jelly-roll β -sandwich fold. Two Ca^{2+} ions are bound at each subunit interface in the trimer.¹⁷ Three VP7 subunits interact with each other to form a plate-like trimer that sits on top of the VP6 trimers and the N-terminal arms of three VP7 subunits grip the underlying VP6 trimers and intrude into the VP4 foot cavity. These interactions imply that the VP4 spikes must first be attached to the DLPs prior to the addition of VP7 during virus assembly, and addition of VP7 results in a shift in the underlying VP6 trimers.⁶⁷⁵ This order of outer capsid assembly is supported by *in vitro* reconstitution studies where sequential addition of recombinant VP4 followed by VP7 onto DLPs can produce infectious virus.⁷³¹

It remains to be determined whether the helical heptad repeat or the putative fusion domain in the rotavirus spike protein is important only for virus entry or also during viral morphogenesis in cells, a unique process that involves a budding of particles through the membrane of the ER (see later).

GENOME STRUCTURE AND ORGANIZATION

The viral genome of 11 segments of dsRNA is contained within the virus core capsid. Deproteinized rotavirus genomic dsRNA is not infectious, reflecting that virus particles contain their own RNA-dependent RNA polymerase to transcribe the individual RNA segments into active mRNA. Hydrodynamic studies of the flexibility or “stiffness” of isolated rotavirus RNA segments in solution indicate that packaging of these RNA segments into the rotavirus capsid requires intimate protein–RNA interactions.³⁸⁷ The proteins directly responsible for segment packaging remain unknown; the structural proteins present in core particles (VP1, VP2, and VP3) are obvious candidates, but nonstructural proteins may play a role (see later). The genome RNA is highly ordered within the particle, with about 25% of the genome making up a dodecahedral structure, and VP2 interacts with the RNA. Several points of contact between the inwardly protruding portion of VP2 as well as VP1 and VP3 interact with the RNA surrounding each fivefold axis^{430,604} (Fig. 45.4), and VP2 interactions with the VP1 polymerase are required for replicase activity.^{492,573,824}

The nucleotide sequence of all 11 rotavirus RNA segments for many rotavirus strains are known, and this forms the basis for the new classification system discussed earlier.⁴⁸³ The prototype simian SA11 strain was the first genome completely sequenced. The sequences from different rotavirus strains show

TABLE 45.5 Rotavirus Proteins

Genome segment	Protein product ^a	Nascent polypeptide (Mol. Wt. × 1000) ^b	Mature protein modified	Location in virus particles	Number of molecules per virion ^c	Ts mutant group (mutation) ^d	Function ^e
1	VP1	125	—	Core	12	C (L138P)	RNA-dependent RNA polymerase, ssRNA binding, complex with VP3
2	VP2	102.4	—	Core	120	F (A387D)	RNA binding, required for replicase activity of VP1
3	VP3	98.1	—	Core	12	B (G527D)	Guanylyltransferase, methyltransferase, ssRNA binding, complex with VP1
4	VP4	86.8	Cleaved VP5* (529) VP8* (247) ^f	Outer capsid	120	A (A401T)	P-type neutralization antigen, viral attachment, homotrimer, protease-enhanced infectivity, virulence, putative fusion region
5	NSP1 (NS53)	58.7	—	Nonstructural	780	D (L140V)	Interferon antagonist, putative viral E3 ligase, RNA binding
6	VP6	44.8	—	Inner capsid		G (T10S, D13H)	Subgroup antigen, trimer, subgroup antigen, protection (intracellular neutralization ?); required for transcription
7	NSP3 (NS34)	34.6	—	Nonstructural		NA	Acidic dimer, binds 3' end of viral mRNAs, cellular eIF4G, Hsp90, surrogate of PABP, inhibits host translation
8	NSP2 (NS35)	36.7	—	Nonstructural	780	E (A152V)	Basic, octamer, RNA binding, NTPase, NDP kinase, RTPase, helix-destabilizing activities, forms viroplasms with VP1 and NSP5
9	VP7	37.4	Cleaved signal peptide glycosylation	Outer capsid		K (T280I)	G-type neutralization antigen, glycoprotein calcium-dependent trimer,
10	NSP4 (NS20)	20.3	NS29–NS28 uncleaved signal peptide high mannose glycosylation and trimming	Nonstructural		NA	RER transmembrane glycoprotein, viroporin, intracellular receptor for DLPs, role in morphogenesis of TLPs, interacts with viroplasms and autophagy pathway, modulates intracellular calcium and RNA replication, enterotoxin secreted from cells, virulence
11	NSP5 (NS26)	21.7	28K, O-glycosylated, phosphorylated (different forms)	Nonstructural		J (A182G)	Basic, phosphoprotein, RNA binding, protein kinase, forms viroplasms with NSP2, interacts with VP2 and NSP6
	NSP6	12	Product of second ORF	Nonstructural			Interacts with NSP5, present in viroplasms and most virus strains, RNA binding

ssRNA, single stranded RNA; mRNA, messenger RNA; PABP, poly(A)-binding protein; NTPase, nucleoside triphosphatase; NDP, nucleoside diphosphate; RTPase, RNA triphosphatase; RER, rough endoplasmic reticulum; DLP, double-layered particle; TLP, triple-layered particle.

^aThe virion polypeptides are designated as proposed by Mason et al.⁴⁷⁶ and modified by Liu et al.⁴⁴⁸ and Mattion et al.⁴⁸⁶ VP3 is the protein product of gene segment 3, and VP4 is the protein product of gene segment 4. Early studies failed to unequivocally identify a protein product from genome segment 3; hence, the protein product of genome segment 4 was called VP3 in early publications. When the protein product of genome segment 3 was confirmed to be a structural protein located in the inner core, the genome 3 product was designated VP3 and the genome 4 product was renamed VP4.⁴⁷⁶ In 1994, the nonstructural proteins were renamed NSP1 to NSP5 to facilitate comparisons among these proteins of different virus strains,⁴⁸⁶ and this nomenclature is now accepted. The parentheses in this table show the names of the nonstructural proteins as designated previously (NS followed by a number indicating their apparent molecular weight in thousands determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate). NSP6 has been characterized since 1994.^{287,729}

^bMolecular weights are for simian agent 11 (SA11) proteins and are calculated from the deduced amino acid sequences from the nucleotide sequence and from the longest potential open reading frame. The gene-protein assignment of segments 7 to 9 is for the SA11 strain.

^cCalculated from structural studies of purified virions.^{607,608,803}

^dNA, no mutant assignment. Two groups of temperature-sensitive mutants (tsH and tsI) are not yet mapped to a genome segment. Data compiled from the following sources.^{161,285,472,619}

^eSee text for references.

^fThere are three trypsin cleavage sites in SA11 VP4 at amino acids 231, 241, and 247. The indicated mature products are those based on use of only the preferred second cleavage site.^{451,452}

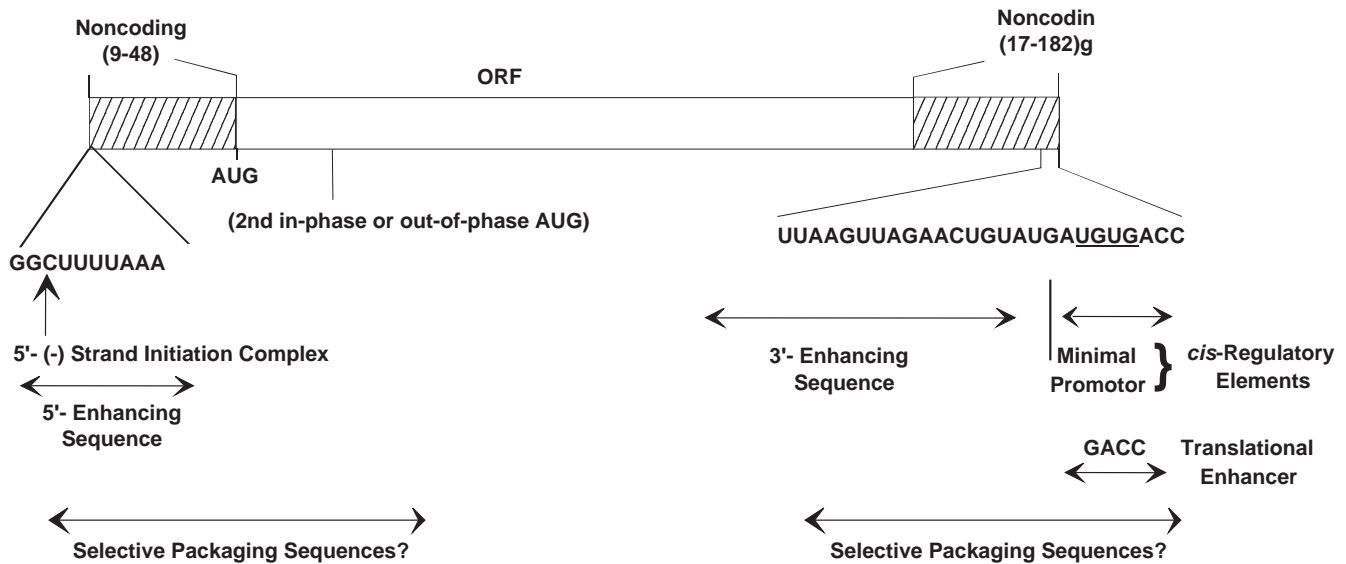


FIGURE 45.5. Major features of rotavirus gene structure. Schematic shows the overall structure of rotavirus genes derived from published sequences of genes 1 through 11. All 11 rotavirus genes lack a polyadenylation signal, are A + U rich, and contain conserved consensus sequences at their 5' and 3' ends. Variations in the conserved ends are also shown. The prototype simian agent 11 (SA11) genome segments range in size from 667 (segment 11) to 3302 (segment 1) with a total of 18,556 base pairs. The bottom arrows show *cis*-regulatory elements of rotavirus messenger RNA (mRNA) required for replication of transcripts assayed in a cell-free replication system.^{404,576,730,786} The study of viruses with variations in the sequence at the 3' termini indicates the minimal promoter is URN_{0.5}CC. The 5' and 3' noncoding regions at the termini of the mRNA are predicted to interact and stably base-pair to form a panhandle structure possibly stabilized by the viral polymerase,^{359,576,730} and interactions between the 3' terminus with the nonstructural protein NSP3 may promote translation of viral mRNA.⁷⁴⁸ The penultimate 5'-GACC-3' is a translation enhancer.¹²⁶

general features (Fig. 45.5) of the structure of each genome segment. Each positive-sense RNA segment starts with a 5'-guanine followed by a set of conserved sequences that are part of the 5' noncoding sequences. An open reading frame (ORF) coding for the protein product and ending with the stop codon follows, and then another set of noncoding sequences is found containing a subset of conserved terminal 3' sequences and ending with two 3' terminal cytidines. Almost all mRNAs end with the consensus sequence 5'-UGUGACC-3', and these sequences contain important signals for gene expression and genome replication. The last four nucleotides of the mRNAs function as translation enhancers.¹²⁶ The lengths of the 3' and 5' noncoding sequences vary for different genes, but the noncoding sequences of homologous strains are highly conserved. No polyadenylation signal is found at the 3' end of the genes. All of the sequenced genes possess at least one long ORF after the first initiation codon. This is usually a strong initiation codon based on Kozak's rules.⁴²⁵ Although some of the genes possess additional in-phase (genes 7, 9, and 10) or out-of-phase (gene 11) ORFs, current evidence indicates that all the genes are monocistronic, except gene 11.⁴⁸⁷

The rotavirus gene sequences are A+U rich (58% to 67%), and this bias against CGN and NCC codons is shared with many eukaryotic and viral genes. The dsRNA segments are base-paired end to end, and the positive-sense strand contains a 5' cap sequence m⁷GpppG^(m)GC.^{359,580} Similar features of the RNA termini (capped structures and 5' and 3' conserved sequences) are found in the genomes of other segmented viruses (e.g., reovirus, cytoplasmic polyhedrosis virus, orbivirus) in the family *Reoviridae* and in other virus families with segmented RNA genomes

(*Orthomyxoviridae*, *Arenaviridae*, and *Bunyaviridae*). One of the most intriguing aspects of the replication cycle of rotaviruses and all segmented dsRNA viruses relates to mechanism(s) of how these viruses coordinately replicate and package the 11 viral mRNAs. The 11 mRNAs must share common *cis*-acting signals because they are all replicated by the same polymerase, and the UGUG sequence of the consensus sequence is recognized in a base-specific manner by the polymerase.^{460,461} In addition, each mRNA must also contain a signal that is unique to it alone, because the 11 mRNAs must be distinguished from one another during packaging. Generally, the conserved terminal sequences in genome segments contain *cis*-acting signals that are important for transcription, RNA translation, RNA transport, replication, assembly, or encapsidation of the viral genome segments. Some of the *cis*-acting signals for rotavirus RNA replication and translation have been identified (Fig. 45.5), but assembly or encapsidation signals remain unknown.⁵⁷⁴ The highly conserved noncoding regions of the RNA may contain the gene-specific packaging signals. Sequence comparisons between RV strains have contributed to identifying conserved sequences and/or secondary structures in the RV genome.⁴³⁹ Conserved secondary structures in the positive-sense RNAs, including long-range interactions at the 5' and 3' terminal regions present in all segments, may facilitate RNA circularization, although such structures remain to be detected in cells.⁴³⁹ Computer modeling and RNase mapping experiments predict that viral (+)RNAs fold into panhandles through 5' and 3' base pairing and the 3' consensus sequence extends from the panhandle as a single-stranded tail.^{116,439,730} Interactions of this extended RNA with different rotavirus and cellular proteins are thought to regulate whether

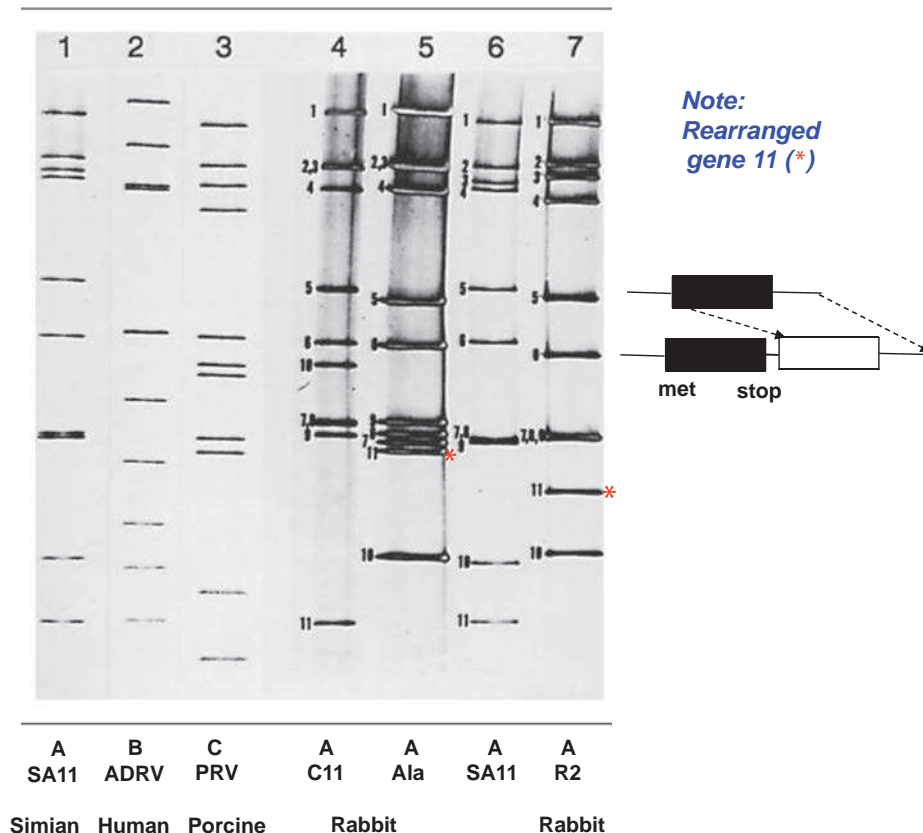


FIGURE 45.6. Electropherogram of rotavirus RNA segments. The RNA segments were separated by electrophoresis in a 10% polyacrylamide gel and visualized by staining with silver nitrate. The RNA patterns of a group A rotavirus (simian agent 11 [SA11], lane 1), a group B rotavirus (adult diarrhea rotavirus isolate from China, lane 2), and a group C rotavirus (lane 3) are shown. The rearranged RNA patterns of three group A rabbit rotavirus strains (C11, lane 4; Ala, lane 5; and R2, lane 7) and their cognate segments compared with those of SA11 (lane 6) are also shown. The cognate genes were identified by hybridization with complementary DNAs (cDNAs) for each SA11 RNA segment. Red asterisks show rearranged gene 11s in the electropherotypes of Ala and R2 viruses. Schematic on right illustrates features of a rearranged gene 11 that has a duplicated open reading frame but would encode a normal protein because it lacks an initiation site in the duplicated ORF. (From Tanaka et al.⁷⁰⁶ et al. Molecular characterization of three rabbit rotavirus strains. *Arch Virol* 1988;98: 253–265.)

the (+)RNA functions in translation, genome replication, or assortment and packaging.⁴⁹¹

Rotaviruses are the only known agents of mammals or birds that contain 11 segments of dsRNA. In most cases, the electrophoretic pattern of the genome of the group A viruses is composed of four high-molecular-weight dsRNA segments (numbered 1 to 4), two middle-sized segments (5 and 6), a distinctive triplet of segments (7 to 9), and two smaller segments (10 and 11). When this basic pattern is not seen, the rotavirus being analyzed may be a group A avian virus, a non-group A virus, a group A virus that contains rearrangements within individual genome segments (Fig. 45.6), or a new unique group A virus. Analysis of genomic electropherotypes is a relatively easy, rapid, and popular technique for virus detection and for molecular epidemiology studies to monitor virus outbreaks and transmission. However, because distinct RNA patterns can arise by different mechanisms (reassortment, mutation, rearrangements) and RNA segments of different sequences may co-migrate, these profiles are not useful as a definitive criterion for classification of a virus strain.^{109,220} (See also Molecular Epidemiology, later). Nucleic acid hybridization combined with northern blot was initially used to classify viruses based on the relatedness of genome segments²²⁵ (e.g., to classify genetically related viruses into different genogroups)⁵³¹ and to identify the origin of specific RNA segments in virus reassortants. This method characterized viruses involved in cross-species transmission^{529,530} but is now being replaced by complete genome sequencing, which has clearly identified zoonotic transmission of many rotavirus genes and reassortment.⁴⁷³

In viruses with genome rearrangements, typical RNA segments are missing or are decreased in concentration in an elec-

trophoretic profile and are replaced by additional, more slowly (or rarely more rapidly) migrating bands of dsRNA (Fig. 45.6, lanes 5 and 7). The slowly migrating bands represent concatemeric forms of dsRNA-containing sequences specific for the missing RNA segments, which has been reviewed elsewhere.¹⁸⁴ The more rapidly migrating bands appear to represent deletions. Viruses with genome rearrangements of this type have been isolated most frequently during infection from immunodeficient, chronically infected children, asymptotically infected immunocompetent children, and animals (calves, pigs, or rabbits). Viruses with rearranged genomes have also been obtained *in vitro* after serial high multiplicity of infection passage of tissue culture–adapted rotaviruses. Virus isolates with rearrangements in segments 5, 6, 8, 10, and 11 have been characterized, with the greatest number having rearrangements detected in segments 5 and 11. Viruses with a rearranged segment 11 may have some selective advantage (better growth *in vitro* or stability), so they are detected more easily, rather than occurring more frequently.

Viruses that contain rearranged genome segments are generally not defective, and the rearranged segments can reassort and replace normal RNA segments structurally and functionally. These viruses do not have a growth advantage, but they exhibit a selective advantage for being incorporated in viral progeny, indicating a preferential packaging of rearranged segments into progeny.⁷³⁷ Biophysical characterization of such particles has shown that up to 1,800 additional base pairs can be packaged in particles without causing detectable changes in particle diameter or apparent sedimentation values. The density of particles containing rearranged genomes may be increased, however, and the increase in density is directly proportional to

the number of additionally packaged base pairs.⁴⁹³ Thus, rotaviruses have considerable capacity to package additional genomic RNA, although the upper limit is unknown. Whereas a total of 11 RNA segments are invariably packaged, much less constraint appears to exist on the length of individual RNA segments assembled into the maturing virus particle. Because the amount of viral RNA present in virions can vary by a significant amount (~1,800 nucleotides), a headfull mechanism for RNA packaging, as seen for the dsRNA phage phi6,⁵¹⁰ may not operate for rotaviruses.

In most cases, the profiles of virus-encoded proteins in cells infected with rotaviruses with rearranged genomes are similar to those seen in cells infected with standard rotavirus strains, indicating that the rearrangement of the sequences in a segment leaves the normal reading frames and their expression unaltered. Sequence analyses of rearranged genome segments confirm this and reveal mechanisms by which the rearrangements arise. In most cases, the rearrangements result from a head-to-tail duplication that occurs immediately downstream from the normal ORF and, hence, the rearranged segment retains the capacity to express its normal protein product. In some cases, gene rearrangements result in truncated proteins. In one case, a gene rearrangement in genome segment 5 introduced a point mutation in the ORF and produced a truncated NSP1 lacking the C-terminal half of the protein.³⁵² This rearranged virus is nondefective *in vitro*, indicating the C-terminal

half of NSP1 is nonessential for rotavirus replication, at least in cell culture.³⁵⁰ Another mutant lacking the cysteine-rich zinc finger motif in the genome segment 5 protein, NSP1, is also viable in cell culture.⁷⁰⁷ Rearrangements have also been identified that affect the ORF for NSP3, and analysis of these viruses suggests a mechanism for gene rearrangements in which secondary structures facilitate and direct the transfer of the RNA polymerase from the 5' to the neighboring 3' end of the template during the replication step.²⁶⁶ Genome rearrangements (concatemerization and deletion) are a third mechanism of evolution (in addition to reassortment and mutation) of rotaviruses. The discovery that rearranged segments are preferentially segregated into progeny virions is helpful to identify sequences critical for replication and RNA packaging.¹⁸⁴ While the exact sequences required for packaging remain to be identified, rearranged genomes that are selectively packaged have parts of the 5' ends duplicated, suggesting packaging signals including coding sequences are located in the 5' region of the RNAs.⁷³⁷

CODING ASSIGNMENTS

The coding assignments and many properties of the proteins encoded in each of the 11 genome segments are now well established (Fig. 45.7 and Table 45.5), although new protein functions continue to be identified. Initially protein assignments

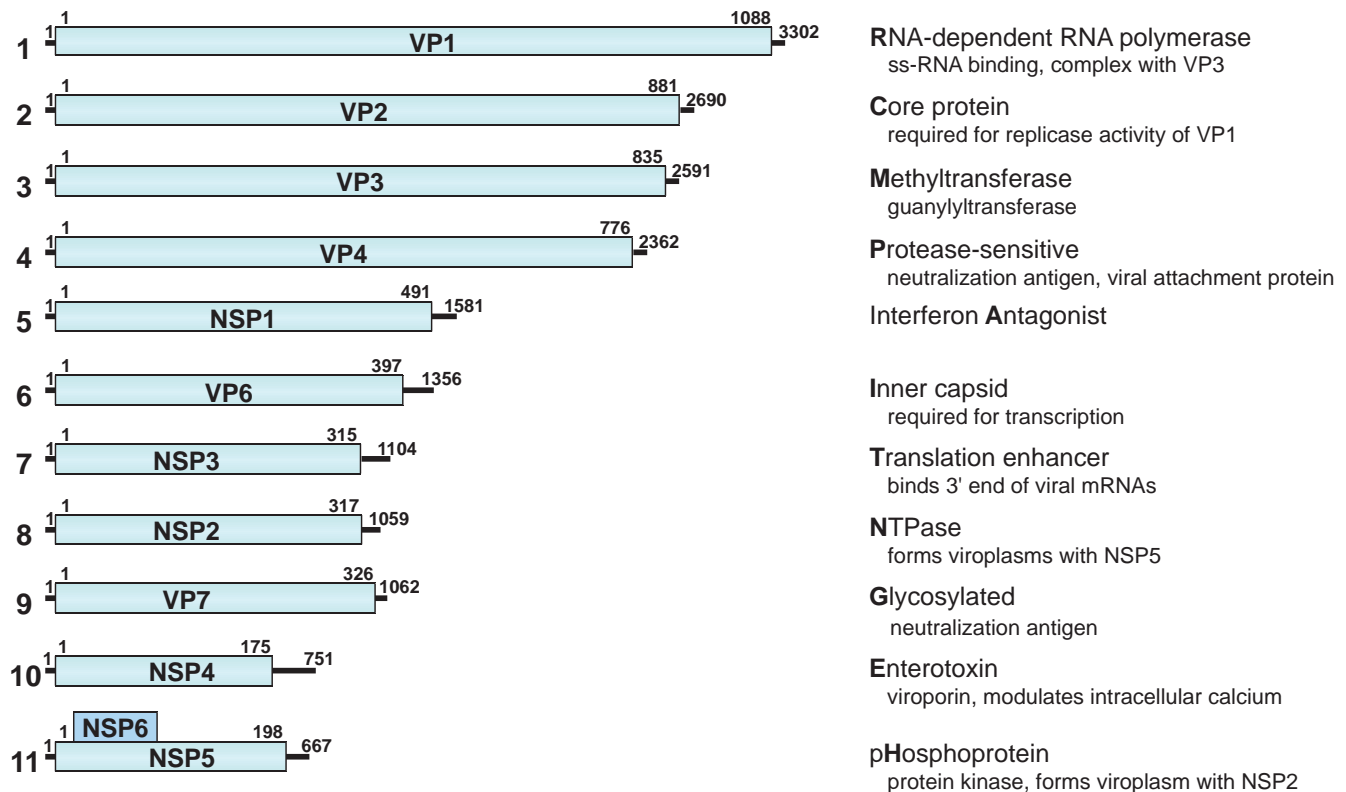


FIGURE 45.7. Genome structure of rotavirus simian agent 11 (SA11) virus. RNA segments (in nucleotides) shown in the positive sense and their encoded proteins (in amino acids). The lines at the 5' and 3' termini represent the noncoding regions. A few key functions of the proteins are listed with the letter bolded that is used in a new classification system based on the entire genome. See the text and Tables 45.4 and 45.5 for information on the classification system and more details on gene-coding assignments and locations of known temperature-sensitive mutations.

were determined by *in vitro* translation using mRNA or denatured dsRNA and by analyses of reassortant viruses. Complete coding assignments were known first for SA11, the type species of the *Rotavirus* genus. Comparative studies of other rotavirus strains have shown that the absolute order of migration in gels of cognate genes may differ among virus strains. Identification of cognate genes, therefore, is now routinely achieved from sequence information obtained directly from dsRNA or single-stranded RNA (ssRNA) following reverse transcription-polymerase chain reaction (RT-PCR) amplification followed by analysis of sequence homology with accumulating nucleic acid sequence databases.

The rotavirus genome segments code for structural proteins found in virus particles and nonstructural proteins found in infected cells but not present in mature particles (Table 45.5 and Fig. 45.7). Six of the genome segments code for structural proteins found in virus particles. Another six proteins are nonstructural, with a few tissue culture adapted virus strains lacking NSP6. Early studies often presented conflicting conclusions concerning the numbers and locations of the rotavirus proteins. Many of these conflicts were resolved, as reviewed elsewhere,⁴⁸⁶ when it was recognized that posttranslational modifications (glycosylation, trimming of carbohydrate residues, and proteolytic cleavages) occur after polypeptide synthesis. In addition, strain variations (e.g., the presence of more than one glycosylation site on VP7 in some bovine, equine, porcine, and human rotavirus strains) provide explanations for other differences in polypeptide patterns.

The nomenclature of the viral proteins (as originally proposed for SA11 proteins) designates structural proteins as viral protein (VP) followed by a number, with VP1 being the highest-molecular-weight protein, and proteins generated by cleavage of a larger precursor being indicated by an asterisk (VP4 is cleaved to produce VP5* and VP8*^{212,221}). Initial studies referred to the nonstructural proteins as NS followed by a number indicating the protein's molecular weight. This nomenclature has been replaced by NSP1 to NSP6 to facilitate comparisons among cognate nonstructural proteins of different molecular weights⁴⁸⁶ (Table 45.5). In fact, much of the literature before 1988 refers to the genome segment 4 product as "VP3"; before 1994, NS53 and NS35 were the designations used for what is now referred to as NSP1 and NSP2, and so forth (Table 45.5). The new nomenclature is used throughout this chapter.

STAGES OF REPLICATION

Overview of the Replication Cycle

Figure 45.8 shows a schematic of the rotavirus replication cycle. Most details of this cycle have been obtained from studies of rotaviruses infecting monkey kidney cell monolayers or polarized intestinal epithelial cells. Other information has come from assays to probe specific steps in the replication cycle based on the expression and interaction of individual wild-type or mutated proteins and RNA in *in vitro* systems, and conclusions from these studies are generally confirmed in the context of virus replication systems using confocal microscopy and small interfering RNA (siRNA). One limitation to some current conclusions on protein function is that an efficient reverse genetics system to test results by incorporating any desired

mutation into any gene in the rotavirus genome remains to be established. However, some progress has been achieved as discussed under Genetics and Reverse Genetics.

In vivo, the natural cell tropism for rotaviruses is the differentiated enterocyte in the small intestine, suggesting these cells express specific receptor(s) for virus attachment and entry into cells. However, extraintestinal spread of rotavirus also occurs in humans and all animal models studied,^{27,63,64-66,125,159,230,239,519,569,611,701} demonstrating a wider range of host cells than previously thought and possible additional receptors. Rotavirus replication in continuous cell cultures derived from monkey kidneys is fairly rapid, with maximal yields of virus being found after 10 to 12 hours at 37°C or 18 hours at 33°C when cells are infected at high multiplicities of infection (10 to 20 plaque-forming units [pfu]/cell).^{137,489,615} Rotavirus replication in differentiated human intestinal cell lines (Caco-2 cells) grown on permeable filter membranes is slower, with maximal yields of virus detected on the apical surface of cells 20 to 24 hours after infection.^{131,132,381} Significantly, EM studies of virus replication in polarized intestinal cells indicate that the replication process in these cells has some distinct differences from the virus replication cycle in nonpolarized cell cultures. These emerging differences are described further later.

The general features of rotavirus replication (based on studies in cultures of monkey kidney cells) are as follows:

1. Cultivation of most virus strains requires the addition of exogenous proteases to the culture medium. This ensures activation of viral infectivity by cleaving the outer capsid protein VP4.
2. Replication is totally cytoplasmic.
3. Cells do not contain enzymes to replicate dsRNA; hence, the virus must supply the necessary enzymes.
4. Transcripts function both to produce proteins and as a template for production of negative-strand RNA. Once the complementary negative strand is synthesized, it remains associated with the positive strand.
5. The dsRNA segments are formed within nascent subviral particles, and free dsRNA or free negative-stranded ssRNA is generally not found in infected cells.
6. RNA replication occurs within cytoplasmic viroplasms.
7. Subviral particles form in association with viroplasms, and these particles mature by budding through the membrane of the ER. In this process, particles acquire their outer capsid proteins.
8. Levels of intracellular calcium are important for controlling virus assembly and particle integrity.
9. Cell lysis releases particles from infected cells grown on monolayers.

In polarized intestinal cells, virus entry occurs almost exclusively through the apical membrane, although some strains enter through both the apical and basolateral membranes (see later). In addition, virus replication in polarized enterocytes alters differentiated enterocyte cell function by perturbing cellular protein trafficking, the cytoskeleton, and tight junctions, and by triggering epithelial cell signaling pathways that can activate innate responses and secretion of various chemokines or cytokines^{86,380,550} (see later). Finally, virus is released apically from polarized enterocytes by a novel, Golgi-independent, vesicular transport that does not result in extensive cytopathic effects or cell lysis.³⁸¹

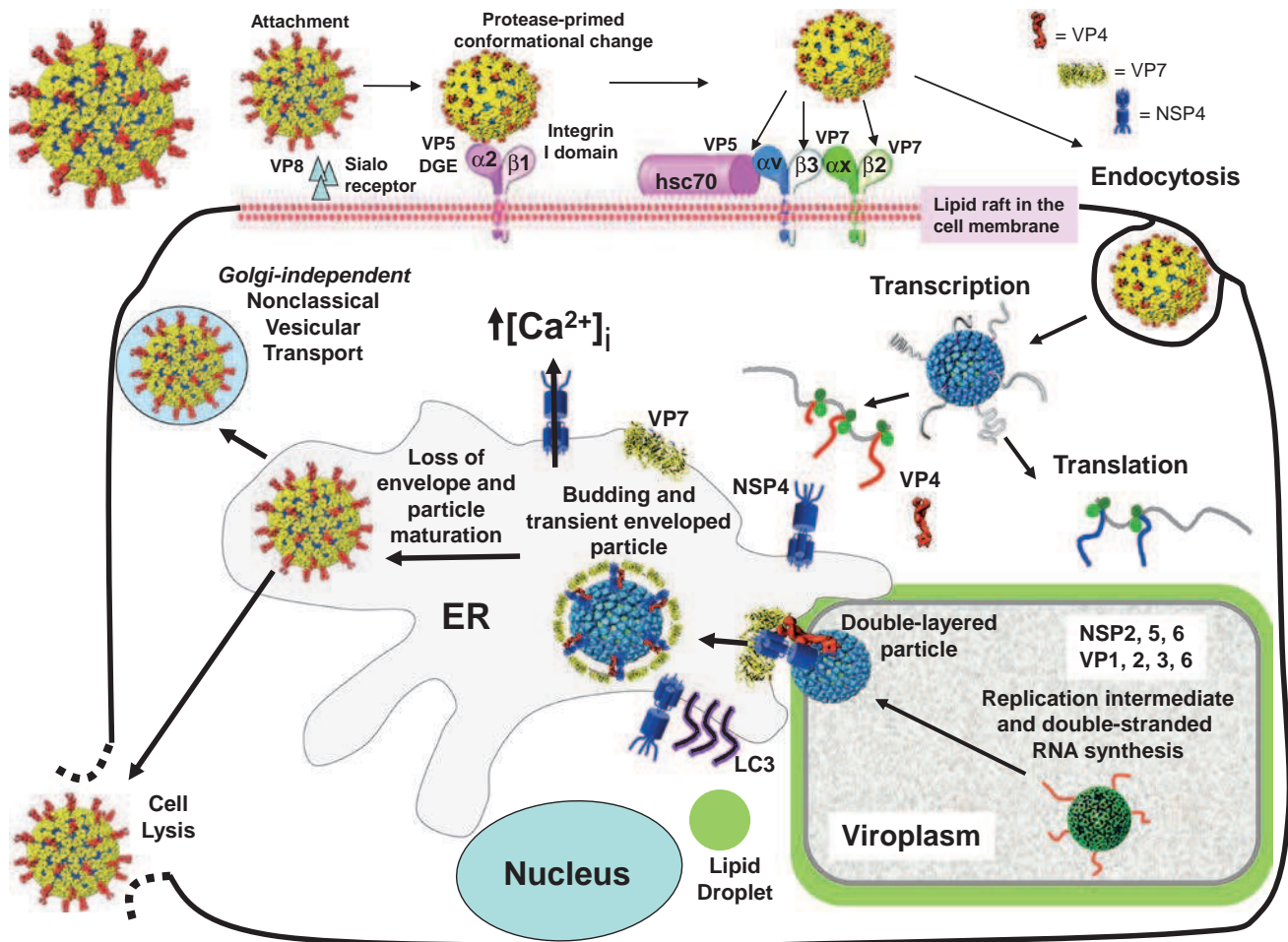


FIGURE 45.8. Schematic of the rotavirus replication cycle. Major features of the rotavirus replication cycle. For details, see section on the replication cycle. Efficient replication requires cleavage of the outer capsid spike protein VP4, which allows the structurally flexible spike protein, VP4, to undergo conformational changes to interact with a series of cellular receptors. The virus is internalized by receptor-mediated endocytosis. The low calcium of the endosome releases outer capsid VP7 trimers, resulting in a conformational change in the VP4 spike protein that releases the transcriptionally active double-layered particles into the cytoplasm. Viral messenger RNAs (mRNAs) are used to translate proteins and as templates for RNA genome replication and packaging into newly made double-layered particles (DLPs) that occurs in specialized structures called viroplasm that co-localize and require components of lipid droplets for formation. Triple-layered particle (TLP) assembly is completed by a unique process involving binding of newly made DLPs to NSP4 that serves as an intracellular receptor, followed by particles budding into the endoplasmic reticulum. During this process, transient enveloped particles are seen, the outer capsid proteins VP4 and VP7 are assembled, and the transient envelope is lost. The viral glycoproteins do not traffic to the Golgi. In polarized epithelial cells, particles are released both by viral lysis and by a nonclassical vesicular transport mechanism.

Attachment

The molecular details of rotavirus adsorption, entry, and uncoating are complex and remain incompletely understood, but progress is being made because of new molecular and structural information on the outer capsid proteins and an understanding of differences in virus strains. Rotavirus attachment and entry, highlighted here primarily based on extensive structural and molecular studies using the rhesus rotavirus strain RRV, is a multistep process that involves sialic acid-containing receptors in the initial cell attachment step and coordinated interactions with multiple receptors during the postattachment steps^{31,194,450,650} (Fig. 45.8). As expected from their locations in the virus structure, VP4 and VP7 are implicated in initial inter-

actions with host cells. A broad range of cells bind rotaviruses and are infected with different efficiencies,^{133,159} suggesting that the initial binding (attachment) is a generally promiscuous interaction with a common receptor and postattachment co-receptors are critical for virus entry into the cell. Virus attachment is by VP4^{157,644} or its cleavage product VP8* on TLPs. Binding to cells does not require cleaved VP4^{138,259} or glycosylated VP7,⁵⁸⁹ but efficient cell entry requires proteolytic cleavage of VP4. Subsequent cell entry is mediated by VP5.⁸²¹

RRV and some other rotavirus strains hemagglutinate red blood cells, and neuraminidase treatment of red blood cells reduces virus binding, indicating a role of sialic acid (SA) in virus attachment.^{42,693} Neuraminidase (NA) treatment of cultured

cells reduces the infectivity of hemagglutinin (HA)-positive virus strains, SA-containing compounds such as fetuin and mucin inhibit virus binding to cells,^{406,812} and such strains are named NA sensitive.³⁶⁰ The most easily cultivatable animal rotaviruses bind SA and can infect both surfaces of polarized cells, but they preferentially infect polarized cells apically because of the presence of terminal SA.^{131,622} Many animal rotaviruses and most strains isolated from humans do not hemagglutinate RBCs, bind to and infect NA-treated cells, and are now called NA-resistant viruses¹³²; these were previously called *SA-independent viruses* but were renamed after some NA-resistant strains were shown to bind to NA-insensitive internal SA moieties on glycolipids or to modified SA moieties in oligosaccharide structures, such as those present in the GM1 ganglioside, that are resistant to NA treatment.^{179,321} NA-resistant viruses preferentially infect polarized intestinal cells through the basolateral surface.^{132,622} The consequences of efficient infection through the basolateral membrane are not fully understood, but further studies on rotavirus entry into cells likely will reveal other virus–host interactions that are relevant for pathogenesis, especially given the extraintestinal spread of virus.

VP4 is the HA based on studies of (a) reassortants showing that HA activity segregates with the rotavirus gene 4, (b) mutants that lose their HA activity are selected with VP4 monoclonal antibodies, and (c) recombinant VP4 produced in insect cells agglutinates red blood cells. X-ray crystallographic structures of RRV VP8* complexed with SA show the VP8* domain exhibits a β -sandwich fold of the galactins, a family of proteins whose natural ligands are carbohydrates, despite a lack of sequence similarity¹⁹³ (Figs. 45.4 and 45.9). The SA moiety binds within a shallow groove on the VP8* surface using residues that are conserved in SA-binding strains. The VP8* structure represents one of the first observed cases of a rotavirus protein taking on a fold seen among host proteins and, based on this structural result, it is proposed that VP4 arose from the insertion of a host carbohydrate-binding domain into a viral membrane interaction protein.¹⁹³ The structures of the VP8* core from two NA-resistant human rotaviruses (DS-1 and Wa strains) show conservation of the same galactin-like fold but exhibit other structural differences including a widening of the cleft.^{62,514} It has been suggested from NMR, infectivity assays, and modeling that such a wider cleft accommodates cellular glycans with an internal sialic acid moiety.³³⁰

Recent data indicate that at least one NA-resistant rotavirus has a narrower VP8* glycan-binding cleft that cannot bind to SA, but instead binds to a nonsialylated histo-blood group antigen.³⁴⁸ This binding may be associated with the ability of this virus to cause zoonotic infections by switching receptors; these results indicate that our understanding of rotavirus–glycan interactions remains incomplete and glycan binding may differ significantly among human rotaviruses.

The identification of cellular co-receptor(s) for rotavirus is an active area of research. Early studies and conflicting results are explained by the use of different receptors by different viruses on different cell types.^{31,194,451,650} Several integrins, including $\alpha 2\beta 1$, $\alpha \nu \beta 3$, $\alpha \nu \beta 2$, and $\alpha 4\beta 1$, are implicated as possible receptors for rotavirus cell entry, with evidence for $\alpha 2\beta 1$ being the strongest.^{153,297,323,820,822} Antibodies to these integrins or synthetic peptides that represent integrin–ligand motifs that are present in VP5* or VP7 block virus infectivity, and some integrins influence cell binding in an additive manner, sug-

gesting they may play a role in different stages of the cell entry process.³¹⁹ Neutralizing antibodies to VP5* and VP7, but not to VP8* and VP6, inhibit virus binding to integrins.²⁴⁵ Involvement of integrins at a postattachment step is consistent with observations that integrin expression increases infectivity of rotaviruses in poorly permissive cells.^{133,335} Currently, rotaviruses are characterized by their integrin usage in addition to their NA sensitivity.^{297,323,360} Integrin usage has been characterized in nonpolarized monkey kidney cells, endothelial and polarized kidney cells, and intestinal epithelial cells. Integrin and postbinding receptor usage can differ depending on the cell type(s) analyzed, and the interactions between NA-sensitive and NA-resistant rotaviruses with intestinal or extraintestinal cells are distinct and may affect the pathogenesis and outcome of infections. Integrins have a polarized distribution and are located at the basolateral plasma membrane, so this may be why NA-resistant rotaviruses preferentially infect through the basolateral surface.^{131,622} Interesting studies suggested that the addition of VP8* peptides to polarized cells can trigger the movement of basolateral proteins to the apical surface, resulting in another possible mechanism for how rotaviruses might interact with integrins.⁵³⁷ A DGE sequence in VP5* binds to the α -subunit I domain on activated $\alpha 2\beta 1$, and rotavirus binding is eliminated by mutations in activation-responsive helices of this integrin.²⁴⁴ The residues that bind to rotavirus overlap with those used by type I collagen but are distinct from those that bind echovirus 1. However, rotavirus is distinguished from collagen by its specific $\alpha 2\beta 1$ binding site requirements, and rotavirus does not activate $\alpha 2\beta 1$ or induce p38 signaling as occurs with collagen–integrin binding.²⁴⁴ Despite demonstrated binding of VP5* to integrins, the lack of signaling after this binding leaves open the question of how virus actually penetrates the membrane. VP7 is proposed to also interact with $\alpha X\beta 2$ and $\alpha \nu \beta 3$ though GPR and CNP motifs, respectively,^{297,822} but the proposed integrin-binding motif (GPR, residues 253 to 255 on VP7) is questionable because it is located on the inward-facing surface of the trimer and would not be available to interact with integrins until after uncoating.^{17,297} However, the CPN residues are potentially available for integrin binding. Evaluation of the role of integrin $\alpha 2$ and $\beta 3$ in rotavirus cell entry using RNA silencing in permissive cells also concluded that these integrins may not play a major role in the rotavirus cell entry process.³⁶² Other receptors remain to be identified as a porcine rotavirus CRW-8 does not use human or monkey $\alpha 2\beta 1$ as a cellular receptor.²⁹⁸ Thus, the crucial molecules involved in cell entry may still remain to be identified.

Heat shock cognate protein 70 (hsc70) is another putative co-receptor proposed to bind virus and mediate virus entry into cells based on experiments in which rotavirus entry is blocked by a VP5* synthetic peptide and by antibodies against human recombinant hsc70.^{318,451,584,819} The role of hsc70 is proposed based on observing enhanced virus entry into cells and subsequent enhanced virus infectivity after heating some cells at 45°C.⁴⁵⁴ Binding of purified hsc70 to two domains in VP5* as well as to a domain in VP6 are also detected in cell-free assays and may modify the conformation of virus particles to help the virus enter cells.^{313,584} To date, however, binding affinities to the proposed post-SA binding receptor molecules have not been determined, and no structures of rotavirus–protein co-receptor complexes are solved. All the implicated RV receptors on cells (GM1 ganglioside, integrin subunits, and hsc70)

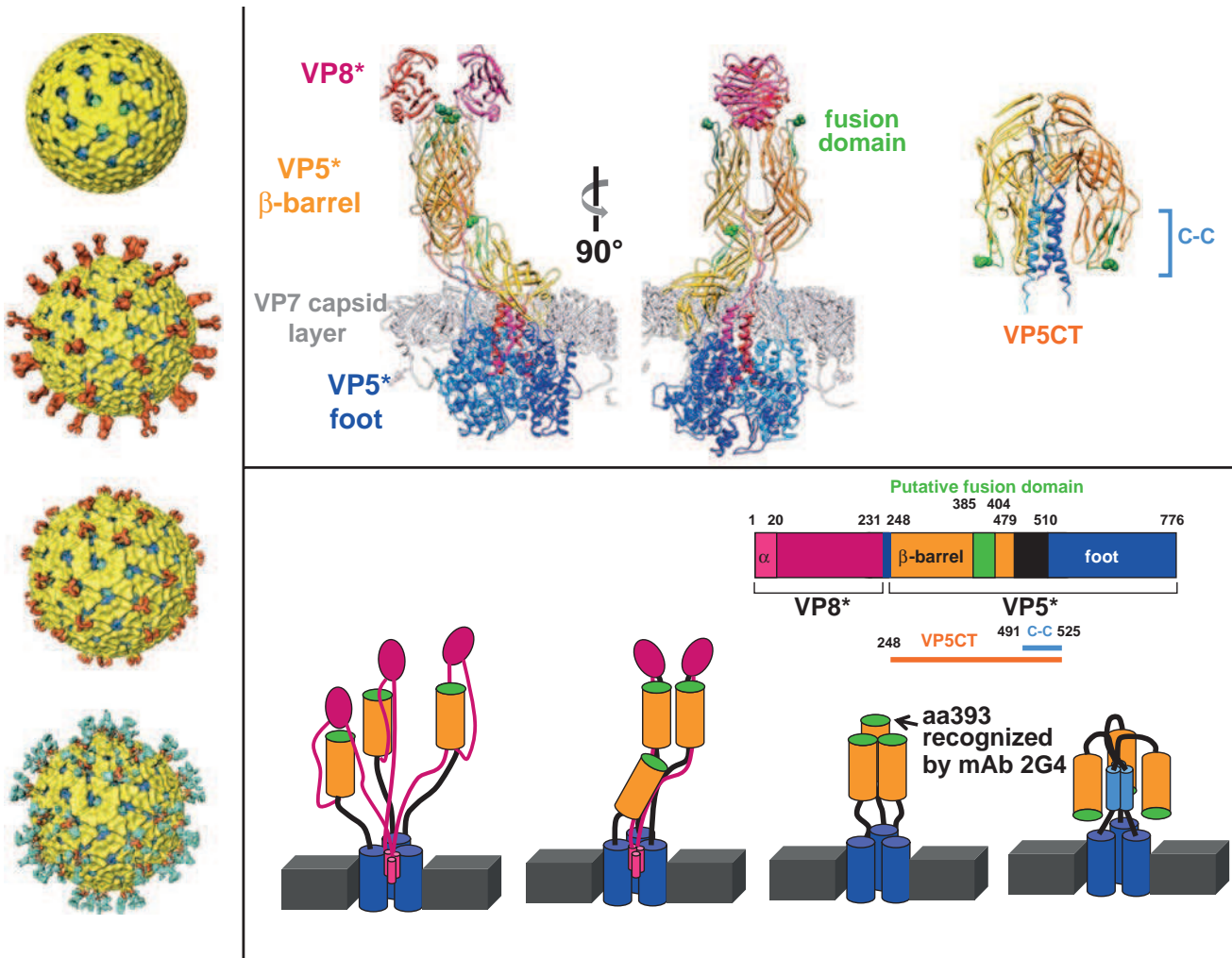


FIGURE 45.9. Conformational transitions of rotavirus particles and the spike protein VP4. **Left-side panel:** Images of cryo-electron microscopy (cryo-EM) reconstructions of rotavirus particles grown in the absence of trypsin (**top**), grown in the presence of trypsin (**second from the top**), and treated with high pH (**third from the top**), and high-pH-treated virus with a monoclonal antibody (2G4) bound to VP5* (**bottom**). Trypsin treatment causes a conformational change in the spikes and primes the cleavage products for further conformational changes. pH-treated particles provided initial evidence that the spike is a trimer and this structure may represent an intermediate that binds to co-receptors. Escape mutants indicate that mAb 2G4 binds to amino acid 393. **Top right panel:** High-resolution structures illustrating different domains and conformations of VP4. Domains in VP4 are color coded and show VP8* (magenta) and subdomains within VP5* including the β -barrel (orange), the fusion domain (green), and the foot domain (blue). The VP7 capsid layer is illustrated in gray. The mAb 2G4 binding site is indicated by green balls within the fusion domain. The **left two figures** show two orientations of an atomic structure of a trypsinized spike with dimeric and trimeric and asymmetric domains. Two of the subunits of VP5* associate to form a dimer-looking central body and the third VP5* subunit traverses the VP7 layer, forming a stalk between the body and the foot domains. Rotation of the body clearly illustrates its dimeric structure above the VP7 layer. The **right figure** shows a structure of the three color-coded domains of a cleaved form of VP5* (VP5*CT, solved from a cleaved, recombinant VP5*). These subunits associate into a coiled-coil (C-C) stabilized trimer that is shaped like an umbrella; this fold-back is thought to be the stable postmembrane penetration conformation of VP5*. **Bottom panel:** **Top row:** Schematic of the linear structure of VP4 showing color-coded domains. **Bottom row:** A color-coded linear diagram and cartoon representation of the proposed conformations of VP4. The colors depicted in the linear diagram correspond to the VP4 domains in the cartoon representation; VP8* (the α -helix [α] located within the foot domain [pink] and the rest of the VP8* domain [magenta]) and VP5* (the β -barrel domain [orange] containing the putative membrane fusion domain [green] and the stretch of amino acids [black] that link the β -barrel to the foot domain [blue]). The VP5*CT and coil-coil domain indicated in the panel above are indicated below the linear diagram. The numbers indicate amino acids. The cartoon represents the disordered VP4 spike on particles grown in the absence of trypsin; the ordered VP4 spike on virus grown in the presence of trypsin; the membrane-penetrating VP5* (VP8* may be retained on the particle and interact with cellular receptors but is not shown in the cartoon); and the postmembrane penetration, fold-back conformation of VP5*.

412,675,732,791

are associated with detergent-resistant lipid microdomains, and infectious rotavirus associates with these domains. Thus, lipid rafts are thought to provide a platform to facilitate efficient interaction of rotavirus receptors with virus particles.³⁶¹

Penetration and Uncoating

After initial binding to cells, rotavirus cell entry is a coordinated, multistep process involving a series of conformational changes in the capsid proteins (Figs. 45.8 and 45.9) and involves endocytic pathways. Because the initial VP8* glycan binding has a relatively low affinity, the virus entry event is probably responsible, in part, for cell type and host specificity.^{193,360,501} Crystallographic structures of VP8*–SA complexes indicate minimal conformational changes in VP8* upon SA binding,⁵¹⁴ but whether this is true in the context of the entire rotavirus structure remains to be determined. Instead, postattachment conformational changes involve VP5* and VP7.

Efficient entry of rotavirus into cells requires conformational rearrangements of the spike protein that facilitate membrane penetration. Trypsinized particles enter cells more rapidly than those not trypsinized.^{385,405} The body of the spike (VP5*) has lipophilic activity thought to be important for entry into cells,^{84,180,196,644,647,820,821} and cell permeabilization properties in the C-terminal VP5* are thought to depend on the exposure of three hydrophobic loops in the VP5* apex normally located below the VP8* lobes.⁴¹² Before trypsin cleavage, VP4 is flexible and spikes are not visible by cryo-EM, although they are present on particles¹⁵⁸ (Fig. 45.9). Trypsinization of VP4 stabilizes the spike structure, inducing an initial disorder-to-order or flexible form-to-rigid spike transition that results in a unique, elongated, asymmetrical shape and primes VP4 for additional conformational changes¹⁵⁸ (Fig. 45.9). Exposure of the specific cleavage site in the asymmetric spike structure and masking of nonspecific trypsin sites in VP4 by VP7 regulates the cleavage of VP4.⁶⁷⁵ The trypsin-primed VP4 intermediate is held within the particle and ready to undergo further molecular rearrangements during virus entry into cells that includes promoting membrane permeability associated with virus entry.⁶⁷⁵ The 3.2-Å crystal structure of the main part of the VP5* stalk generated *in vitro* reveals a folded-back rearrangement that translocates three clustered hydrophobic loops from one end of the molecule to the other to form a coiled-coil stabilized trimer that is shaped like an umbrella; this fold-back form is thought to be the stable “postpenetration” conformation of VP5*¹⁹² (Fig. 45.9). An entry-associated event, proposed to be dissociation of VP7 by exposure to low calcium levels in the endosome, triggers the transition of the trypsin-primed VP5* spike to the fold-back trimeric umbrella conformation that interacts with the uncoating membrane and refolds to destabilize the membrane.^{192,514,805} The VP5* fold-back depends on both membrane interactions and virus uncoating, suggesting that this rearrangement is one of the final steps of RV entry.^{732,805}

Studies with pH treatment of a NA-sensitive virus provide support for this proposal.^{192,514,586,587,805} At elevated pH, the spike undergoes a dramatic irreversible conformational change and becomes stunted with a pronounced trilobed appearance, although the amount of VP4 on particles remains unchanged (Fig. 45.9). Three Fab fragments of the VP5*-specific mAb, 2G4, can then bind to these altered spikes, indicating that VP4 has undergone a dimer-to-trimer transition (Fig. 45.9). Particles with altered spikes no longer hemagglutinate red

blood cells or infect mammalian cells. They retain the ability to bind to mammalian cells, but in an NA-resistant manner, different from untreated particles that bind cells in an NA-sensitive manner. High-pH treatment may trigger a conformational change that mimics the transition in VP4 that occurs with the post-SA attachment step. These particles resemble a mutant virus of an NA-sensitive rhesus rotavirus that exhibits NA-resistant cell binding, in contrast to its parental strain, and attaches to cells by interacting with the integrin $\alpha 2\beta 1$ through a DGE motif in VP5*.⁸²⁰ Of interest, the conformational rearrangements of VP5* translocate this DGE motif to the external surface of the trypsin-primed structural forms of VP5*, making it accessible to bind to an integrin.⁸⁰⁵ Recent analysis of integrin binding of virus strains with distinct VP5* sequences has identified sequence variation in VP5* amino acids that parallel rotavirus strain-specific differences in the effects of virus binding to the $\alpha 2$ I domain. These results indicate VP5* amino acids 335 to 380 that are surface exposed and near the DGE sequence may also influence rotavirus recognition of $\alpha 2\beta 1$ in addition to the DGE site.²⁴⁴

Internalization does not take place at 0°C to 4°C, indicating that this step requires active cellular processes.^{406,590} All virus is internalized by 60 to 90 minutes after binding.⁴⁰⁶ The mechanism of internalization (penetration) into cells remains unclear. Both morphologic and biochemical approaches have been used to investigate the mode of entry of rotaviruses into cells, and both receptor-mediated endocytosis and direct membrane penetration have been suggested as mechanisms of rotavirus entry into cells; trypsin-treated and non-trypsin-treated virus may enter cells by different mechanisms as reviewed previously.²¹⁵ Recent studies indicate that different rotavirus strains enter cells through different endocytic pathways.³²³

Other viruses that initiate infection by mechanisms involving receptor-mediated endocytosis often depend on the acidification of endosomes for partial uncoating or entry into the cell. The importance of acidification of endosomes for the initiation of infection of rotaviruses has been studied by several groups.^{260,385,405,462,791} In all cases, lysosomotropic agents (ammonium chloride, chloroquine, methylamine, and amantadine) do not affect rotavirus entry. Energy inhibitors (sodium azide and dinitrophenol) have a minimal effect on rotavirus infection, and this has been taken to suggest that rotaviruses do not use endocytosis to enter cells. Other endocytosis inhibitors, such as dansylcadaverine and cytochalasin D, and in some, but not all, cases the vacuolar proton-adenosine triphosphatase (ATPase) inhibitor bafilomycin A1, also do not block rotavirus entry. These results indicate that neither endocytosis nor an intraendosomal acidic pH or a proton gradient is required for rotavirus entry into cells.

While the passage of rotaviruses from endocytic vesicles to the cytoplasm does not occur by a pH-dependent fusion mechanism, other data indicate that rotaviruses are still taken up by endocytosis. Direct demonstration of virus fusion with membranes or hemolysis is lacking. Protease cleavage of VP4 is important for rapid entry into cells, and particles containing cleaved VP4 possess lipophilic activity and can affect release of fluorescent dyes from liposomes and isolated membrane vesicles. Rotavirus entry into cells can also be monitored by co-entry of toxins, such as α -sarcin, into cells^{162,446} and by a cell-to-cell fusion from without assay.^{207,227} Most observations are consistent with the hypothesis that virus enters cells by

endocytoses after direct interactions with a series of receptors on the plasma membrane.^{535,647}

The outer capsid proteins of rotavirus that are solubilized from virus particles are able to permeabilize cellular membranes,⁶⁴⁶ and it has been proposed that the outer capsid proteins are solubilized within an endocytic vesicle because of low Ca^{2+} concentrations. The decrease in calcium concentrations within the endosomal vesicle might trigger conformational changes in the capsid, capsid solubilization, and vesicle lysis.⁶⁴⁶ In this Ca^{2+} -dependent endocytosis model, acidification of the endosome would not be needed for the infectious process. Use of the calcium ionophore A23187 to increase the intracellular Ca^{2+} concentration during the early stages of replication can block uncoating.⁴⁶² These results support the hypothesis that low Ca^{2+} concentrations in the intracellular microenvironment may be responsible for uncoating. This idea was originally proposed because it was known that removal of the outer capsid of particles and activation of the endogenous polymerase could be accomplished by calcium chelation.^{143,341}

It is also possible that more than one mechanism, including endocytosis and direct entry, is operative for rotaviruses, as has been proposed for polioviruses and reoviruses.^{68,189} Further studies are needed to determine whether the common endocytosis-mediated entry pathway exists for all rotaviruses and in all cell types. Studies with drugs and dominant-negative mutants suggest that virus enters cells through a non-clathrin-, non-caveolin-dependent mechanism that depends on the presence of cholesterol on the cell membrane and on a functional dynamin.⁶⁵⁹ Trypsin also has been detected associated with the rotavirus outer capsid and is activated by solubilization of the outer capsid proteins.⁴³ This activated trypsin is proposed to cleave VP7 and VP4 into fragments capable of disrupting membranes, and this may allow DLPs to gain access to the cytoplasm to begin actively transcribing viral mRNA to complete the next step in the viral life cycle.

The entry of RRV, which is NA sensitive and binds to $\alpha 2\beta 1$ and of $\alpha \nu \beta 3$ integrins and hsp70, is the most extensively studied by biochemical, structural, and molecular methods. RRV entry into polarized cells is through an endocytic pathway but reportedly does not require cholesterol or a functional dynamin.⁷⁹¹ Use of imaging and unique mAbs that detect regional or conformation-dependent epitopes on VP8* and VP5* or soluble, unassembled protein to follow the proteins on incoming RRV particles at very early time points prior to the onset of viral replication indicates that internalization and decapsidation occur directly after cell membrane penetration as assessed by disappearance of trimeric VP7.⁷⁹¹ In addition, virus entry into cells involves endocytosis, calcium-dependent uncoating, and several VP4 conformational changes; VP8* staining is lost at the time of cell penetration and is not found in the cytoplasm, while VP5* is detected in the cytoplasm within 1 hour of infection. The fold-back conformation of VP5* is only detectable at the entry step.⁷⁹¹ VP5* and VP7 co-localize with early endosome markers Rab4 and 5, indicating RRV uses an endocytic route limited to the early endosomes to enter cells. Bafilomycin A1 and concanamycin A, two pharmacologic inhibitors of the vacuolar-type H^+ -ATPase, reduced cytoplasmic staining of VP6 indicative of blocking entry and reduced the appearance of the folded-back VP5*, suggesting that the appearance of this epitope is specific to entry. Elevating endosomal Ca^{2+} concentration also blocked entry. This study supports a model of RRV entry in which, after

membrane binding and internalization, the low Ca^{2+} concentration in the endosome triggers VP7 decapsidation and the appearance of the VP5* fold-back, ultimately leading to the release of DLPs into the cell cytoplasm. The results also suggest that the primary effect of BafA1 on RRV infection is mediated indirectly through changes in the endosomal Ca^{2+} gradient.⁷⁹¹ Unexpectedly, these studies did not find a role for dynamin or cholesterol in RRV entry, which had been implicated in RRV entry into nonpolarized cells through a non-clathrin-, non-caveolin-mediated endocytosis pathway that depends on a functional dynamin and on the presence of cholesterol on the cell surface.^{323,659} Entry of other RV strains with different NA sensitivity and integrin dependence into MA104 cells is reported to be dependent on hsc70, dynamin, and cholesterol, but these distinct strains enter cells through clathrin-mediated endocytosis pathways.³²³ The reasons for these differences are not known but might result from the different virus strains or the heterogeneity of raft-type membrane microdomains on different cell types in different differentiation states.¹⁷⁷

RNA Synthesis

Overview

Incoming RV particles containing the dsRNA genome segments must synthesize mRNAs that direct the synthesis of viral proteins and also serve as templates for the synthesis of the dsRNA genome that becomes encapsidated into newly made particles. The virion polymerase performs these functions as a transcriptase and as a replicase at different times during the replication cycle. Synthesis of viral transcripts is mediated by the endogenous viral RNA-dependent RNA polymerase complex (PC), consisting of VP1 and VP3, which is latent in the virion, where it appears as a flower-shaped feature in the icosahedrally averaged cryo-EM reconstruction of the virion attached to the inner surface of the VP2 layer at all the fivefold axes (Fig. 45.4). The PC contains the enzymatic activities needed for synthesis of capped messenger RNA, including transcriptase, nucleotide phosphohydrolase, guanylyltransferase, and methylases. Each genome segment is transcribed simultaneously and repeatedly by a specific polymerase complex within the confines of the capsid architecture, and the resulting transcripts exit through the type I channel system at the axis adjacent to its site of synthesis. This mechanism of transcription offers an explanation of why no dsRNA virus contains more than 12 genome segments.

The PC must be activated for transcription to occur. Transcription begins following removal of the VP7 outer layer and can also be studied *in vitro* by treatment of TLPs with a chelating agent or by heat shock treatment that removes the outer capsid proteins.^{143,694} Transcribing particles will continuously synthesize milligram quantities of mRNAs *in vitro* as long as fresh precursors and an energy-generating system are provided. Rotavirus transcription requires a hydrolyzable form of adenosine triphosphate (ATP). Studies with analogs that inhibit transcription suggest that ATP is required in reactions other than polymerization⁶⁹⁴ and may be used for initiation or elongation of RNA molecules.

Ultimately, transcription must be inhibited to allow RNA replication to proceed and virus assembly to be completed. While not completely understood, transcription can be inhibited by several mechanisms. Cryo-EM studies of DLPs complexed with

some monoclonal antibodies to VP6, or the addition of VP7 onto DLPs, indicate a conformational change at the interface of the VP2–VP6 layers or in the VP6 trimers can inhibit sustained elongation and translocation of transcripts.^{235,435,723} It is also possible that binding of VP6 to NSP4, which serves as an intracellular receptor for particle assembly (see later), is the key interaction that inhibits transcription. This hypothesis is consistent with the observation that knockdown of NSP4 by siRNA increases viral mRNA synthesis.⁶⁸⁴ NSP4 can form concentration-dependent pentamers, and such structures in the ER may interact with VP6 molecules by a fivefold axis on the surface of the DLPs.¹⁰⁶

The structure of the polymerase must provide mechanisms for transcription and subsequent RNA replication where the minus-strand RNA as well as duplex formation is achieved to produce new infectious virions. Transcription to produce (+) mRNAs requires several functions, including unwinding the genomic dsRNA, entry of the (–)RNA templates for synthesis

of nascent (+)RNA, exit of the (–)RNA for subsequent annealing and further rounds of transcription, and exit of the nascent (+)RNA for capping and extrusion from the DLP. In the replication mode, the polymerase must provide a mechanism for entry of (+)RNA template and exit of the duplex. There also should be easy access for the NTPs into the catalytic center of VP1 for (–)RNA synthesis.

The Viral RNA Polymerase, (+) Strand, and (–) Strand RNA Synthesis

Insight into how the rotavirus polymerase mediates both transcription and replication is now available based on the structure of VP1.⁴⁶⁰ VP1 has a compact cage-like structure with three domains similar to that of the reovirus polymerase.⁷⁰⁹ VP1 contains a putative cap-binding site to anchor the capped 5' end of the (+)RNA, and the structure has four distinct tunnels that lead to the central catalytic core of VP1 (Fig. 45.10). Having a

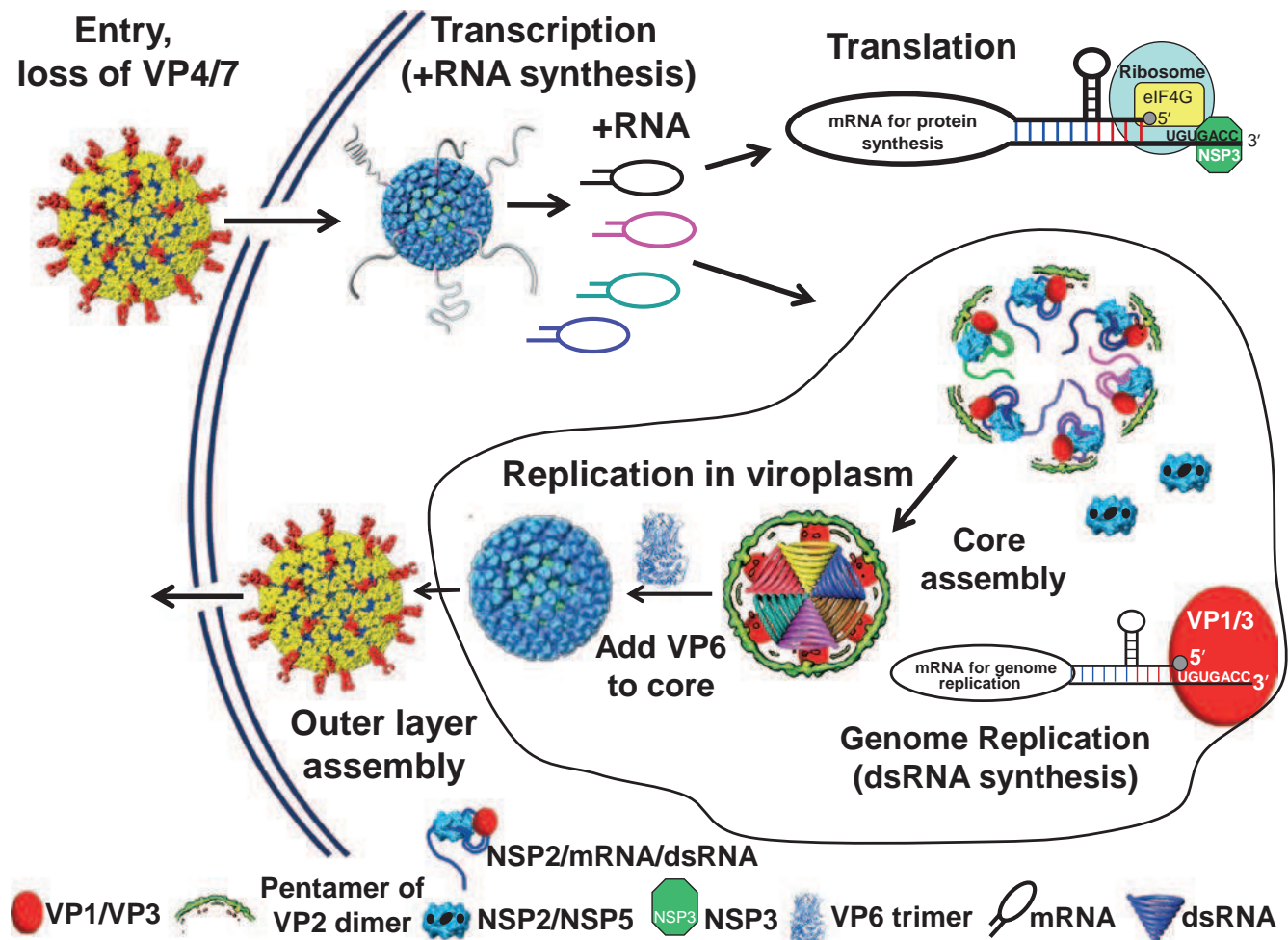


FIGURE 45.10. Virion disassembly and functions of RNAs for transcription, translation, genome replication, and packaging.

With virus entry into cells, the double-layered particles (DLPs) lose the outer layer proteins and the DLPs become transcriptionally active and release (+)RNAs that are thought to form panhandle structures that are translated into viral proteins and replicated into genomic double-stranded RNA (dsRNA) in viroplasms. A pentamer of VP2 dimers and VP1/VP3 form the initial assembly unit. NSP2 is likely involved in feeding (+)RNA into the enzyme complex for (–)RNA synthesis and formation of the duplex RNA.³²⁰ Twelve such units assemble together to encapsidate the genome segments to form the virion core, which subsequently is coated with VP6 to make new DLPs. (Modified from Hu L, Crawford SE, Estes MK, et al. 2010. Rotavirus structure and functional implications. In Hein NT, Baldacci G, Haenni AL, Benedetti EL, eds. *Viruses Responsible for Emerging Diseases in South-East Asia*, Universite Paris Diderot-Paris 7, 2011.)

fourth tunnel, a distinction from other known RNA-dependent RNA polymerases, is needed for the exit site of the (+)RNA transcripts. Based on information from reovirus polymerase-RNA elongation complexes,⁷⁰⁹ the four distinct tunnels in VP1 are implicated in (a) the entry of the templates ([+]RNA or [-]RNA), (b) the entry of NTPs, (c) the exit of dsRNA/(-)RNA, and (d) the exit of the (+)RNA. Having two distinct product exit tunnels ensures that (+)RNAs are effectively shuttled out of the core, while nascent dsRNA gene segments are directed toward the particle interior. In addition, the (-)RNA exits proximal to the template entry tunnel, which should facilitate its reuse in subsequent rounds of (+)RNA synthesis. Finally, the exiting of the (-)RNA template and (+)RNA product of transcription through separate tunnels suggests that the capping enzyme and polymerase are capable of internally separating dsRNA duplexes.

Crystallographic structures of VP1 in complex with the consensus sequences (CSs) in the 3' end of the minus strand (template for [+]RNA synthesis) and in the 3' end of the (+)RNA (template for [-]RNA synthesis) are also available.⁴⁶⁰ These structures indicate that for transcription, sequence-specific recognition of the 3' CS of the minus strand for positive-strand synthesis is not critical as it takes place within the confines of the capsid.⁴⁶⁰ In contrast, sequence-specific interactions are made only with the 3' CS of the plus strand that is anchored to the template entry site in VP1. This sequence-specific interaction involving the UGUG motif in the 3' CS (+)RNA confirms biochemical studies that showed this motif is the polymerase recognition signal and is required for high-affinity interactions with VP1 and subsequent replicase activity.^{112,115,116} Of interest, the VP1 3' CS (+)RNA complex structure is in an auto-inhibited state with a single nucleotide at the 3' end overshooting the initiation register. For VP1 to initiate (-)RNA synthesis, the overshoot 3' end of the template must be realigned, a priming loop must be repositioned to allow binding of the priming nucleotide, and a plug formed from the C-terminal domain of the polymerase that is in the dsRNA exit tunnel must be dislodged. These conformational changes in VP1 require VP2, GTP, the template, and Mg²⁺. Interactions with VP2 are known based on biochemical studies showing that interaction of RNA with VP2 is necessary for the initiation of rotavirus genome replication,^{576,824} but the details of how VP2 interacts with VP1 and how this interaction activates VP1 to initiate genome replication remain to be understood. They may require interactions with other viral proteins within assembling replication intermediates. Genome replication, RNA assortment and encapsidation, and particle assembly are highly coordinated events.

Kinetics and Cellular Sites of Transcription and Replication

Transcription in cells occurs following the release of DLPs from the endosome. Consistent with this idea, cells are susceptible to infection by liposome-mediated transfection of DLPs, indicating that simple delivery of these particles into the cell cytoplasm permits transcription to proceed.⁴¹ Transcription is asymmetric, and all transcripts are full-length positive strands made off the dsRNA negative strand.⁴⁹⁰ Primary transcription must occur before RNA replication. The synthesis of negative-strand RNA occurs in perinuclear nonmembranous, electron-dense cytoplasmic inclusions known as *viroplasm*s (see later),

concurrently with the packaging of positive-strand RNA into core replication intermediate (RI) particles.^{26,683}

The kinetics of synthesis of positive- and negative-stranded RNAs has been studied in rotavirus-infected cells,⁶⁹⁷ in a cell-free system using extracts from infected cells,⁵⁷⁰ in an electrophoretic system that allows separation of the positive and negative strands of rotavirus RNAs in acid urea agarose gels,^{571,575} and by quantitative RT-PCR.²⁶ Positive- and negative-stranded RNAs are initially detected during the first 4 hours after infection.^{26,697} A small linear increment of plus- and minus-strand RNA synthesis is detected followed by a logarithmic increase at later times of infection. This quantitation indicates that the entering DLPs produce a small amount of mRNA, which is then translated and replicated, producing new DLPs. When new DLPs are assembled, these particles then transcribe their genomes, initiating a secondary wave of transcription that significantly increases the amount of viral mRNA and dsRNA. Newly assembled DLPs are required for this second wave of transcription as knock-down of any of the viral proteins that constitute the DLP (VP1, VP2, VP3, and VP6) ablates the logarithmic increase in RNA synthesis.²⁶ The amount of mRNA accumulated at late time points postinfection is significantly greater (at least six times more) than the dsRNA accumulated during the same period of time. The assembly of infectious virus particles parallels the replication of the viral genome.

Viroplasms, the sites of incorporation of (+)RNA into replication intermediates and virus assembly, first appear 2 to 3 hours after infection. The number of viroplasms initially increases and then decreases with time after infection, whereas the area of each viroplasm increases, suggesting fusions of viroplasms.^{204,226} Viroplasms contain viral proteins (VP1, VP2, VP3, VP6, NSP2, NSP5, and in some strains NSP6).^{287,591,592,628} NSP2 and NSP5 are major components of viroplasms, and expression of these two proteins alone is sufficient to induce the formation of *empty* viroplasm-like structures. Viroplasm nucleation may be due to NSP2-tubulin interactions as NSP2 sequesters free tubulin molecules and induces microtubule depolymerization in RV-infected cells.⁴⁷⁴ Microtubules also may play a role in the growth and fusion of viroplasms later in infection based on inhibition of these processes by nocodazole.⁹⁵ Viroplasms associate with lipids and proteins (perilipin, adipocyte differentiation-related protein [ADRP]) characteristic of cytoplasmic lipid droplets (LDs), and blocking or interfering with LD formation reduces the number of functional viroplasms and production of infectious virus.¹²² The proteasome is also essential for early assembly of viroplasms.^{147,456} Inhibition of proteasome activity following virus entry and uncoating reduces accumulation of virus proteins, viroplasm formation, and RNA replication. The requirements of LDs and proteasomes for viroplasm formation represent examples of a virus hijacking cellular pathways for its own replication, and further information of how RV proteins interact or regulate these pathways should help understand the early stages of viroplasm formation and particle assembly.

The key role of NSP2 and NSP5 in viroplasm functions of genome replication and packaging has been demonstrated by studies of temperature-sensitive mutants or knocking down the expression of NSP2 or NSP5 by RNA interference or antibody treatment, which results in inhibition of viroplasm formation, genome replication, virion assembly, and a general decrease of viral protein synthesis.^{96,455,619,684,714,744}

NSP2 is an essential multifunctional protein with sequence-independent ssRNA binding as well as enzymatic activities including nucleoside triphosphatase (NTPase), nucleoside diphosphate (NDP) kinase,⁴²⁸ RNA triphosphatase (RTPase),⁷⁴⁵ and nucleic acid helix destabilizing activities.^{710,711} NSP2 is the most abundant protein of viroplasms,⁵⁹² is essential for viroplasm formation,⁶¹⁹ and exists as an octamer. The monomeric subunit has two distinct domains separated by a deep, catalytic cleft. The association of monomers results in a doughnut-shaped octamer with a 35-Å central hole along the fourfold axes and grooves that run diagonally across the twofold axes and are lined by basic residues³⁷⁰ (Fig. 45.11). The C-terminal domain of NSP2 resembles the cellular histidine triad (HIT) family of proteins that hydrolyze nucleotides. The NTP-binding residues are located within the cleft between the two domains.¹⁰⁰ Mutation of the catalytic residue (H225A) abrogates hydrolysis of the γ -phosphate from the 5' end of RNA and dsRNA synthesis.^{712,746} Although the NTPase activity is

localized in the monomeric subunit, the ability to bind RNA and other proteins requires the formation of the octamer.⁷⁴⁶ Cryo-EM structures of NSP2 octamer complexes show that both ssRNA and NSP5 (the other key component required for viroplasm formation) share the same binding site, the grooves in the NSP2 octamer, and NSP5 competes with ssRNA binding.³⁷⁶ Tubulin also binds to these charged grooves.⁴⁷⁵ In virus-infected cells, NSP2 is associated with polymerase complexes, including VP1 and VP2 and partially replicated viral RNA.¹⁸ Taken together, these results indicate that NSP2 is critical for RNA replication and suggest that competitive binding of different ligands to the groove may regulate NSP2 function during genome replication and virus assembly (Fig. 45.11).

NSP5 is a dimeric phosphoprotein rich in Ser and Thr residues that undergoes O-linked glycosylation,^{6,601,785} as well as phosphorylation that occurs when NSP5 is co-expressed with VP2.¹⁴⁶ NSP5 reportedly exists in several oligomeric forms, and biophysical and structural analyses suggest that the

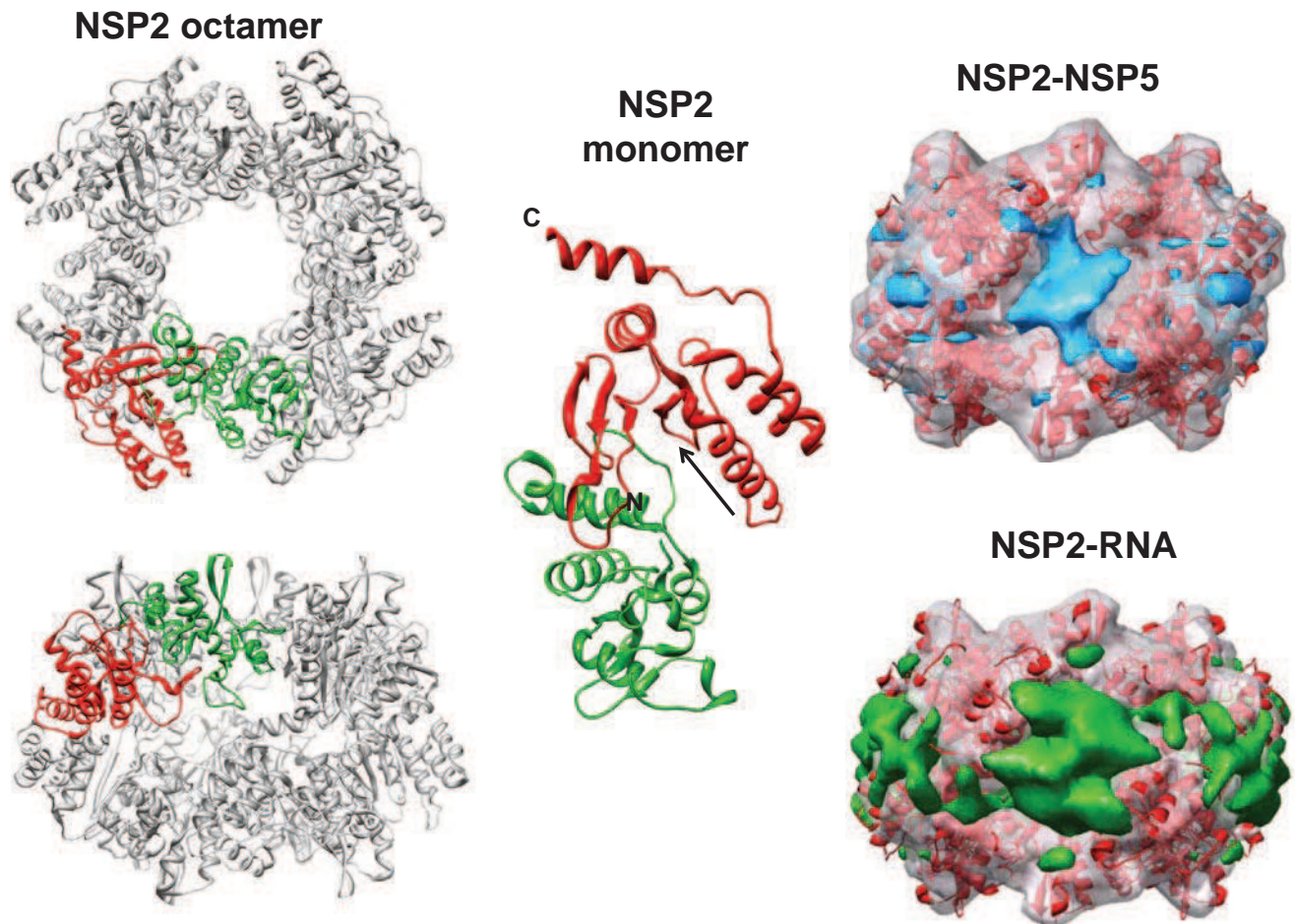


FIGURE 45.11. Structures of NSP2 and interactions with RNA and NSP5. **Left:** Octameric structure of NSP2. The N- and C-terminal domains of two subunits are colored green and red, respectively, and the rest of the subunits in the functional octamer are shown in gray. The donut-shaped octamer is viewed along the fourfold axis (**top left**) and along one of the two twofold axes perpendicular to the groove lined by positively charged residues (**bottom left**).³⁷⁰ **Middle:** NSP2 monomer showing the two subunits and catalytic cleft indicated by arrow. **Right:** Cryo-electron microscopy (cryo-EM) reconstruction of NSP2 binding to NSP5 (**top** with NSP5 in blue) and NSP2 binding to RNA (**bottom** with RNA in green) viewed perpendicular to the groove. These two ligands both bind to the grooves of the NSP2 octamer. (From Jiang et al.³⁷⁶ Cryoelectron microscopy structures of rotavirus NSP2-NSP5 and NSP2-RNA complexes: implications for genome replication. *J Virol* 2006;80:10829–10835.)

biologically relevant form in cells required for viroplasm formation is a decamer.⁴⁷⁵ The self-association of NSP5 may be regulated by NSP6, which is encoded by an alternative reading frame of gene segment 11 in most virus strains and is also associated with viroplasms.⁷²⁹ The precise roles of the modifications in NSP5 function remain unknown. However, the formation of viroplasm-like structures is also calcium regulated, and a pseudo-EF-hand motif in NSP5 (DxDxD) located upstream of a C-terminal helical domain possibly triggers calcium-dependent viroplasm formation.⁶⁷² Although NSP5 binds to the grooves in NSP2, steric hindrance is proposed to prevent NSP2 from binding to all NSP5 promoters, and therefore, some NSP2 is thought to remain free to interact with other binding partners such as viral RNAs and the viral polymerase to perform functions other than viroplasm organization.⁴⁷⁵

Viroplasm maturation and function also require NSP4, the only nonstructural protein that does not bind RNA. Based on siRNA experiments that knocked down NSP4 expression, NSP4 plays a role in the intracellular accumulation and the cellular distribution of several viral proteins, influencing the development of viroplasms, linking genome packaging with particle assembly, and acting as a modulator of viral transcription.^{453,684} A vesicular form of NSP4 is modulated by the levels of intracellular calcium, and that NSP4 also forms caps on viroplasms and co-localizes with the autophagy protein LC3.⁴⁴ Precisely how NSP4 regulates RNA synthesis remains to be determined. Formation of the punctate vesicular structures requires the elevation of cytoplasmic calcium (Ca^{2+} cyto), which is mediated by NSP4 functioning as a viroporin in the ER membrane.³⁵⁶ NSP4 regulation of Ca^{2+} cyto may serve as a viroplasm assembly trigger that directs NSP5 to form viroplasms during viral infection, and later NSP4 binding to VP6 functions to inhibit polymerase activity, leading to a switch to complete genome replication and encapsidation.

Genomic RNA Replication and Encapsidation (Packaging)

After its synthesis, dsRNA remains associated with subviral particles, suggesting that free dsRNA is not found in cells. Because of its inherent stiffness,³⁸⁷ dsRNA is not packaged. Instead, (+)RNAs are assorted, replicated, and packaged within complexes that remain poorly understood, but subviral particles (complexes separable by sedimentation through sucrose gradients and by equilibrium centrifugation in CsCl gradients) in which dsRNA synthesis occurs have been characterized both in infected cells and in a cell-free system. Based on structural and biochemical studies, a model of rotavirus replication includes genome encapsidation and DLP assembly occurring concurrently with the formation of a replication intermediate composed of a pentamer of VP2 interacting with VP1 and VP3.^{488,585,586} In this model, 12 units each composed of pentamers of VP2 dimers, a VP1/VP3 complex, and a dsRNA segment associate to form the VP2 capsid layer, which provides a platform for the subsequent addition of VP6 trimers resulting in the formation of the DLP. Protein components in these units perhaps represent the replication complex in which the (+)RNA, brought in with the aid of NSP2/NSP5, is fed into the enzyme complex for (–)RNA synthesis and the formation of the duplex RNA, which is spooled around the enzyme complex. VP2 may interact with NSP5⁴⁹ and NSP5 with the NSP2 octamer. This multimeric complex would then provide a platform or scaffold for

the replication complex.³⁶⁹ The NSP2 octamer binds (+)RNA and mediates unwinding of the RNA secondary structure via its nucleic acid helix destabilizing activity. The NSP2 octamer also binds the viral polymerase VP1 and may feed unwound RNA to the polymerase.⁴⁰⁵ The octamer is also an NTPase, but how this activity is used in rotavirus genome replication remains unclear. Structural studies to investigate how NSP2 interacts with nucleotides and hydrolyzes NTP indicate that the NTPase activity of NSP2 is associated with a phosphoryl-transfer function similar to that seen for cellular nucleoside diphosphate kinases. This kinase-like activity of NSP2 may have a role in the homeostasis of nucleotide pools within the viroplasm during genome replication.⁴²⁸ Overall, VP1 replicates the RNA, and the (–)RNA exits VP1 and interacts with an octamer of NSP2 so that NSP2, functioning as an RTPase, cleaves the γ -phosphate from the 5' (–)RNA.⁷⁴⁵ This accounts for the absence of the γ -phosphate at the 5' end of the double-stranded genomic RNA.^{359,490} However, how NSP2 recognizes the 5' CS of the (–)RNA of the dsRNA products exiting from VP1 is not understood, and this knowledge may be important to understand how NSP2 may play a role in facilitating genome packaging.

The selective packaging mechanism that leads to the presence of equimolar genome segments within rotaviruses, or any of the other members of the family *Reoviridae*, remains a challenging puzzle. Several models have been proposed.^{574,586} A currently favored model proposed earlier is based on structural data indicating that the core represents a collection of functionally separate pentameric units, with each unit containing its own RNA-dependent RNA polymerase activity and capping enzyme complex and being responsible for transcription of one of the genome segments.⁴³³ In this model, encapsidation would be concurrent with capsid assembly, and each VP1–VP3 enzymatic complex would associate with a specific mRNA and attract the VP2 core protein and assemble into pentamers (Fig. 45.12). RNA–RNA interactions between the mRNA of the distinct pentameric units would then drive the assembly of the icosahedral core from the pentameric units. Structural changes in the core lattice protein VP2, as a consequence of pentamer–pentamer binding, may activate the RNA-dependent RNA polymerase and stimulate negative-stranded RNA synthesis to form the genome. Another model, based on the ability of the rotavirus capsid proteins to self-assemble into empty VLPs^{157,431} and on data on the dsRNA bacteriophage phi6,⁵¹⁰ suggests that empty cores are first made and that mRNAs would be replicated and subsequently inserted into these cores. Future work will determine which of these models, if any, may be correct.

Virion Maturation

A distinctive feature of rotavirus morphogenesis is that subviral particles, which assemble in the cytoplasmic viroplasms, bud through the membrane of the ER, and maturing particles are transiently enveloped (Fig. 45.8). This is one of the most interesting aspects of rotavirus replication, differing from members of other genera in the family *Reoviridae* and from any other virus. The envelope acquired in this process is lost as particles move toward the interior of the ER, and the envelope is replaced by a thin layer of protein that ultimately constitutes the outer capsid of mature virions. Rotavirus particle transport, maturation, and assembly remain an interesting model to understand the transport of protein complexes across the ER membrane as well as envelope particle formation.

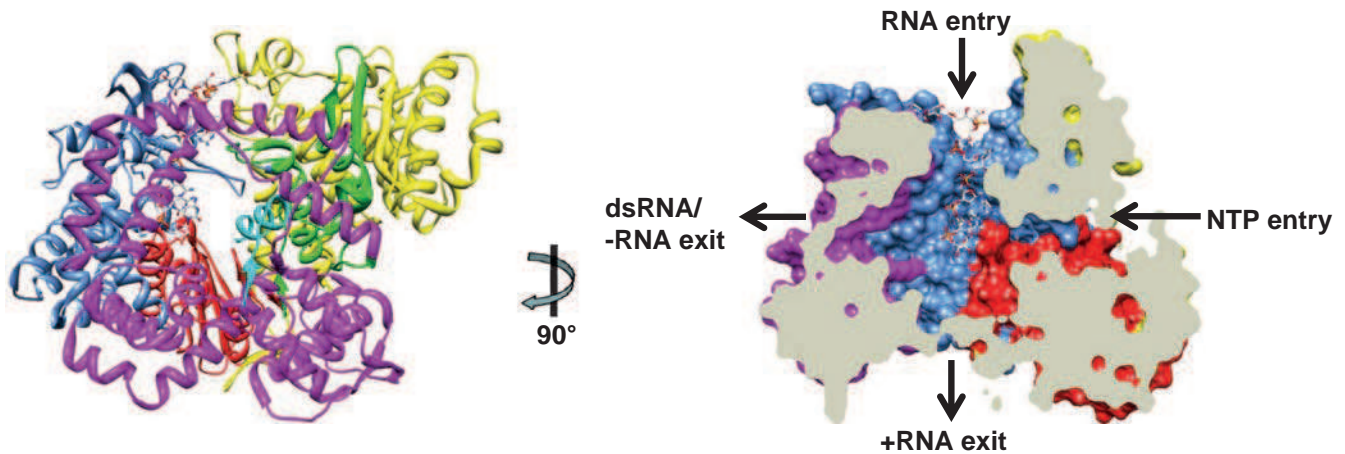


FIGURE 45.12. Structural features of VP1 polymerase. On the **left**, the structure of VP1 in complex with RNA oligonucleotide is shown.⁴⁶⁰ Ribbon diagram of VP1/RNA complex (PDB ID: 2R7R) is shown on the **left**. The N-terminal domain is in yellow, the C-terminal domain is in magenta, and the C-terminal plug is in cyan. The subdomains of the polymerase domain are in light blue (fingers), red (palm), and green (thumb). On the **right** is a cutaway of the surface rendering of the complex showing the four tunnels extending into the central cavity, the catalytic center.

Morphologic and biochemical data are consistent with rapidly assembling DLPs serving as an intermediate stage in the formation of triple-layered virions. The sites and precise details of RNA replication are beginning to be understood, and the viroplasms are the sites of synthesis of the double-layered particles that contain RNA (see earlier). This conclusion is based on the localization of several of the viral proteins (VP2, NSP2, NSP5, NSP6) to viroplasms, and of VP4 and VP6 to the space between the periphery of the viroplasm and the outside of the ER,^{287,591} and on the observation that particles emerging from these viroplasms bud directly into the ER that contains the glycoproteins VP7 and NSP4.

A fraction of VP4 can be detected in a filamentous array²⁸⁷ and at the plasma membrane associated with microtubules.^{178,538} Although the function of VP4 in lipid rafts at the plasma membrane in viral morphogenesis remains unclear, it has been proposed that VP4 is added to particles as an extra-ER event.¹⁷⁸ Particles lacking VP4 can be formed in cells treated with siRNA to gene 4, suggesting that VP4 is not essential for assembly or for release of DLPs from the ER.¹⁷⁶ Infectious particles are found associated with lipid rafts at the cell surface.^{163,165,361,664} Silencing VP4 and NSP4 (but not VP7) reduces rotavirus–raft interaction associations apparently by reducing targeting of VP4 to the rafts; these results support the idea that the primary association of VP4 with rafts occurs during the initial stages of particle assembly in the ER.¹⁶³ Taken together, two pools of VP4 appear to exist, with one associating with particles in the ER and one being found independently at the plasma membrane.

In the ER of SA11-infected cells, two pools of VP7 exist that can be distinguished using two classes of antibodies.³⁸⁴ One pool, found only on intact particles, is detected only by a neutralizing mAb. The second pool of VP7 is unassembled, remains associated with the ER membrane, and is detected by a polyclonal antibody made to denatured VP7.³⁸⁴ Distinction of these two forms of VP7 permitted a kinetic study of the assembly of VP7 and of other structural proteins into particles. The incorporation of the inner capsid proteins into double-

layered particles was found to occur rapidly, whereas VP4 and VP7 appear in mature TLPs with a lag time of 10 to 15 minutes. Kinetic analyses of the processing of the oligosaccharides on the two pools of VP7 show that the virus-associated VP7 oligosaccharides have a 15-minute lag compared with that of the membrane-associated form, suggesting that the latter is the precursor to virion VP7. This lag appears to represent the time required for virus budding and outer capsid assembly.³⁸⁴

NSP4 plays a key role in the assembly of TLPs. NSP4 is the only nonstructural protein that does not bind to RNA. NSP4 has been studied extensively because it plays a role in viral morphogenesis and functions as an enterotoxin (see later). NSP4 has multiple domains and an increasing number of functions.^{32,215} NSP4 is a 20K primary translation product; it is co-translationally glycosylated to become a 29K species, and oligosaccharide processing yields the mature 28K protein that is a transmembrane protein of the ER.^{209,383} The 175-aa polypeptide backbone of NSP4 consists of an uncleaved signal sequence, three hydrophobic domains with two N-linked high mannose glycosylation sites being in the first hydrophobic domain, a predicted amphipathic α -helix (AAH) that overlaps a folded coiled-coil region, the H2 transmembrane domain that traverses the ER bilayer, and the C-terminus, which is hydrophilic and forms an extended cytoplasmic domain.^{71,108,210,383} The carbohydrate moieties remain sensitive to endoglycosidase H digestion, and processing of the Man9-GlcNAc carbohydrate added to NSP4 stops at Man8GlcNAc with the mannose-9 species predominating,^{71,383} indicating that no further trimming occurs in the Golgi apparatus.

The C-terminal cytoplasmic domain (aa 161 to 175) of NSP4 functions in viral morphogenesis by acting as an intracellular receptor on the ER membrane.^{24,718,719} NSP4 binds newly made DLPs and mediates the budding of these particles into the ER lumen (see later). A receptor role for NSP4 is supported by the observation that DLPs bind to ER membranes containing only NSP4.^{23,24,502} The AAH region, distinct from the receptor domain, is predicted to adopt an α -helical coiled-coil structure

and is thought to mediate oligomerization of the virus-binding domains into a homotetramer.⁷²⁰ A crystal structure of the oligomerization domain of NSP4, which spans aa residues 95 to 137 (NSP4 95 to 137), self-associates into a homotetrameric coiled-coil, with the hydrophobic core interrupted by three polar layers and two of the four Glu120 residues coordinating a divalent cation⁷² (Fig. 45.11). Recently, crystallization of a similar domain revealed a pentamer that lacks a cation-binding site.¹⁰⁶ Sequence analyses have identified 14 types (14E types) of NSP4 in RVA strains (Table 45.4). The highest sequence diversity of NSP4 is located in the cytoplasmic domain. It is unclear whether this sequence diversity is important and driven by interactions with specific residues on VP6 or divergent regions on VP4, or both. However, specific combinations of types of VP6 and NSP4 have been found in natural reassortants, suggesting these interactions are biologically important.³⁶⁵ There also may be host-specific interactions important for function, but these remain to be fully characterized.

Glycosylation of NSP4 is not required for its binding activity to DLP or for oligomerization, but it is required for interaction with calnexin.^{24,511,719} NSP4 also has a binding site for VP5' within VP4^{24,357} and may play a role in removing the transient envelope.⁷²⁵ Heterooligomers of NSP4, VP4, and VP7 have been detected in enveloped particles,^{465,602} and calcium has been shown to be important for oligomerization of these proteins in the ER⁶⁰³ as well as for proper folding of VP7 epitopes and outer capsid assembly.^{17,190,677} The precise mechanisms of how (a) the envelope on particles is removed, (b) the hetero-oligomeric complexes function in particle budding through the ER, and (c) the outer capsid is assembled onto the newly made DLPs remain poorly understood. However, siRNA experiments indicate that VP4 and VP7 are assembled onto the particle in the ER and VP7 is involved in removal of the transient envelope.^{163,453}

Rotavirus maturation is a calcium-dependent process, and virus yields are decreased when virus is produced in cells maintained in calcium-depleted medium.⁶⁷⁸ Viruses produced in the absence of calcium are almost exclusively DLPs, and budding of virus particles into the ER is not observed.⁶⁷⁷ The crystal structure of VP7 provides new understanding of the requirement of calcium in forming TLPs. VP7 forms calcium-dependent trimers (Fig. 45.4); two calcium ions coordinate between each VP7 monomer and stabilize the trimeric interactions. Electron cryomicroscopy and single-particle reconstruction of VP7-recoated DLPs show that the N-termini (residues 51 to 70) of VP7 interact with VP6 and the VP7 trimers clamp onto the VP6 trimer.¹²¹ Interestingly, unglycosylated VP7 made in the presence of tunicamycin is relatively stable in a calcium-free environment. The budding process can occur in the absence of calcium, but VP7 is retained within the ER.⁸ VP7 does not fold properly unless it is expressed with other rotavirus proteins, and calcium must be present in cells for correct epitope formation.^{190,191} Outer capsid assembly also requires proper formation of disulfide bonds on VP7.⁷⁰³ Earlier studies showed treatment of cells with various agents (tunicamycin, dithiothreitol, or calcium-blocking drugs, such as thapsigargin) results in a build-up of enveloped particles in the ER.^{341,503,589} These agents may disrupt the proper folding of VP7 that is required for removing the envelope.

NSP4 is a novel calcium agonist; it mobilizes intracellular calcium when expressed intracellularly by a phospholipase

C (PLC)-independent mechanism or by a PLC-dependent mechanism when it is added to cells from the outside.⁵¹⁸ Mobilization of intracellular calcium by a PLC-independent mechanism occurs through NSP4 functioning as a viroporin³⁵⁶ that is important for viral replication and assembly. Binding of VP6 on DLPs to NSP4 that occurs when viroplasm are capped by NSP4 is thought to trigger the budding process. Recent reports indicate that siRNA silencing of NSP4 expression in rotavirus-infected cells affects the distribution of other viral proteins, mRNA synthesis, and the formation of viroplasms where viral RNA replicates, suggesting global regulatory functions of NSP4 in rotavirus replication^{453,684} with molecular mechanisms that remain to be understood.

At least three pools of intracellular NSP4 exist in rotavirus-infected cells dependent on the level of NSP4 protein expression.⁴⁴ The first pool is represented by NSP4 localized in the ER membrane and is present throughout the course of infection. This pool serves as a receptor for the budding of immature viral particles into the ER, as described earlier, at the peak of viral infection, when all viral proteins are abundant (after 6 hours after infection). A second minor pool of NSP4 molecules enters the ER-Golgi intermediate compartment (ERGIC) and can be recycled back to the ER or may be a part of the nonclassical secretion pathway for delivery and cleavage of an NSP4 peptide into the medium of infected cells at early time points after infection⁸²⁶ when the levels of viral proteins are relatively low. The third pool of NSP4 molecules, distributed in cytoplasmic vesicular structures associated with the autophagosomal marker LC3 and viroplasms, is regulated by calcium levels and appears at 6 hours after infection, when there is an increase of intracellular calcium levels because of increased expression of viral proteins.⁵⁰⁴ NSP4 also interacts with calveolin-1, and this may be involved in the secretion of NSP4.^{566,826} Inhibition of NSP4 expression interferes with the formation of large viroplasms and affects viral protein expression⁴⁵⁵ and secondary viral mRNA synthesis in rotavirus-infected cells.⁶⁸⁴ The NSP4- and autophagic marker LC3-positive vesicles may serve as a lipid membrane scaffold for the formation of large viroplasms by recruiting early viroplasms or viroplasm-like structures formed by NSP2 and NSP5.²²⁶ These NSP4-positive membranes may also function to regulate packaging of the rotavirus genome and transcription through NSP4 association with VP6 on DLPs. The calcium-dependent compartmentalization of NSP4 into an autophagosomal pathway raises questions regarding the involvement of autophagosomal membranes in rotavirus replication and release of infectious virus from cells.

Understanding viral morphogenesis has been facilitated by the expression of the rotavirus structural proteins individually or in combinations in insect cells using recombinant baculoviruses.^{157,431,623,823} This approach first showed that the single-layered VP2 particle shell self-assembles when VP2 is expressed alone, and that all of the other capsid proteins can self-assemble into virus-like particles when co-expressed in the proper combinations. Virus-like particles composed of VP2, VP1/2, VP1/2/3, VP2/3, VP2/6, VP2/6/7, VP2/4/6/7, and VP1/2/3/6 can be made.^{157,823,824} The outer and inner capsid proteins of different virus strains can also reassort and are able to be transcapsidated onto other virus strains^{117,118} and infectious virus is produced.⁷³¹ These results demonstrate that the structural proteins contain the intrinsic information required to form particles and that co-expression of mutant

proteins is a feasible approach to analyze the domains responsible for the structural interactions between the proteins composing the virus particles. These particles have been useful to (a) analyze the role of cleavage sites in the spike protein in infectivity,²⁷² (b) investigate the role of individual structural proteins in inducing protective immunity,^{130,156,371,543,544} (c) probe the inner structure of particles by analyzing difference maps of particles with distinct protein compositions or by x-ray analysis,^{211,604,675} and (d) analyze RNA transcription and replication^{110,114,434,435} and RNA packaging and assembly. Future studies should address questions of which mechanisms control packaging of the viral genome and virus assembly.

Virus Release

Electron microscopy studies have shown that the infectious cycle ends when progeny virus is released by host cell lysis in nonpolarized cells.^{12,111,497} Extensive cytolysis late during infection and drastic alterations in the permeability of the plasma membrane of infected cells result in the release of cellular and viral proteins.⁵²⁷ Despite cell lysis, most DLPs and many TLPs remain associated with the cellular debris, suggesting that these particles interact with structures within cells. Interactions with cell membranes and the cell cytoskeleton have been suggested,⁵²⁷ and virus purification procedures generally use Freon extraction to release particles from cellular debris. Whether the cytoskeleton provides a means of transport of viral proteins and particles to discrete sites in the cell for assembly or acts as a stabilizing element at the assembly site and in the newly budded virions or whether particles are simply trapped by the cytoskeleton remains to be determined. VP4 interacts with actin and lipid rafts and can remodel microfilaments, and this has been suggested as a mechanism by which the brush border membrane of polarized epithelial cells is destabilized to facilitate rotavirus exit from cells.²⁶⁴

Rotavirus Effects on the Host Cell

Inhibition of Translation of Cellular mRNAs by NSP3

Viral mRNAs are capped but not polyadenylated, and viral proteins are translated by the cellular translation machinery. Most of the rotavirus structural proteins and the nonstructural proteins are synthesized on free ribosomes, although nascent proteins on free ribosomes have not been analyzed. Instead, this conclusion has been drawn based on the absence of signal sequences that would indicate targeting to the ER and lack of protection from digestion in *in vitro* protease protection studies.^{210,383} The viral glycoproteins VP7 and NSP4 are synthesized on ribosomes associated with the membrane of the ER and are co-translationally inserted into the ER membrane as a result of signal sequences at their N-termini. VP7 has a signal sequence that is co-translationally cleaved, whereas the signal sequence on NSP4 is not cleaved.

Translation of the viral mRNAs that are capped but not polyadenylated is facilitated by the action of the nonstructural protein NSP3, which is one of the five nonstructural proteins (NSP1, 2, 3, 5, and 6) that bind nucleic acid.^{671,713} NSP3 function parallels that of the cellular poly(A)-binding protein (PABP). The N-terminus of NSP3 interacts with the 3' consensus sequence (UGACC) of viral mRNAs and the C-terminus of NSP3 interacts with eIF4G as does PABP, but with higher affinity.^{311,596,597,600,748} These events lead to NSP3

evicting PABP from eIF4G, to the enhancement of translation of rotavirus mRNAs, and to the concomitant impairment of translation of cellular mRNAs.^{558,748} PABP evicted from eIF4G accumulates in the nucleus of rotavirus-infected cells, and this relocalization of PABP from the cytoplasm to the nucleus occurs relatively early in the infection cycle (~3 hpi) and requires a limited amount of NSP3.^{328,516} The complete depletion of PABP from the cell cytoplasm can reinforce the shutoff of translation of cellular polyadenylated mRNAs and may also enhance translation of viral mRNAs by making available other cellular factors involved in translation termination, RNA stability, or subcellular localization of host mRNAs that would normally be bound to cytoplasmic PABP.³²⁸

In vivo, NSP3 stimulates the translation of mRNAs in synergy with the cap structure, possibly enabling circularization of viral mRNAs and its delivery to ribosomes for viral protein synthesis^{126,748} (Fig. 45.12). Atomic structures of both domains of NSP3 complexed with bound ligands indicate that both domains have novel folds and NSP3 functions as a dimer (Fig. 45.13).^{182,311} Whereas the RNA-binding domain forms a rod-shaped symmetric dimer, the N-terminal domain tightly binds to the consensus 3' end of the mRNAs inside a tunnel formed at the dimeric interface. NSP3 and eIF4G also interact with a novel cellular protein, named *RoXaN*, which has a role in translation regulation that remains to be completely understood.⁷⁶⁵ However, the nuclear localization of PABP seen in rotavirus-infected cells is dependent on the capacity of NSP3 to interact with eIF4G and also requires the interaction of NSP3 with a specific region in *RoXaN*. This domain functions as a nuclear export signal and *RoXaN* tethers PABP with RNA. Thus, *RoXaN* is a cellular partner of NSP3 involved in the nucleocytoplasmic localization of PABP.³²⁸ The cellular chaperone heat shock protein 90 also binds to NSP3, prevents proteosomal degradation of NSP3, and increases dimerization of NSP3 required for its functioning to bind eIF4G and effect translocation of PABP to the nucleus.¹⁹⁹ This chaperone is induced by virus infection though activation of phosphoinositide 3-kinase (PI3K)/Akt and nuclear factor- κ B (NF- κ B) signaling that positively regulate rotavirus infection.^{198,199}

The binding of NSP3 to viral mRNAs has also been proposed as a possible mechanism to transport newly made mRNAs to viroplasms for subsequent replication. The cytoskeleton-binding function of NSP3^{288,487} might be involved in this process directly or NSP3 may interact with other host proteins that link the translational machinery and the cytoskeleton.

While NSP3 indeed inhibits translation of cellular mRNAs, a second mechanism of rotavirus-induced inhibition of cellular mRNA translation involves eIF2 α that is phosphorylated early after infection and is maintained throughout the virus replication cycle.^{515,516} This phosphorylation depends on the synthesis of VP2, NSP2, and NSP5, and the continuous phosphorylated status of eIF2 α is beneficial for the virus because viral mRNAs are preferentially translated efficiently while translation of most cellular proteins is stopped. Protein kinase R (PKR) is apparently responsible for this phosphorylation event that is triggered by viral double-stranded RNA detected in the cell cytoplasm outside viroplasms, which is a paradigm-shifting result as traditionally it has been assumed that rotaviral dsRNA is hidden from the interferon system by ensuring that genome replication takes place within replicative intermediate particles, such that single-stranded RNA is replicated as it enters these

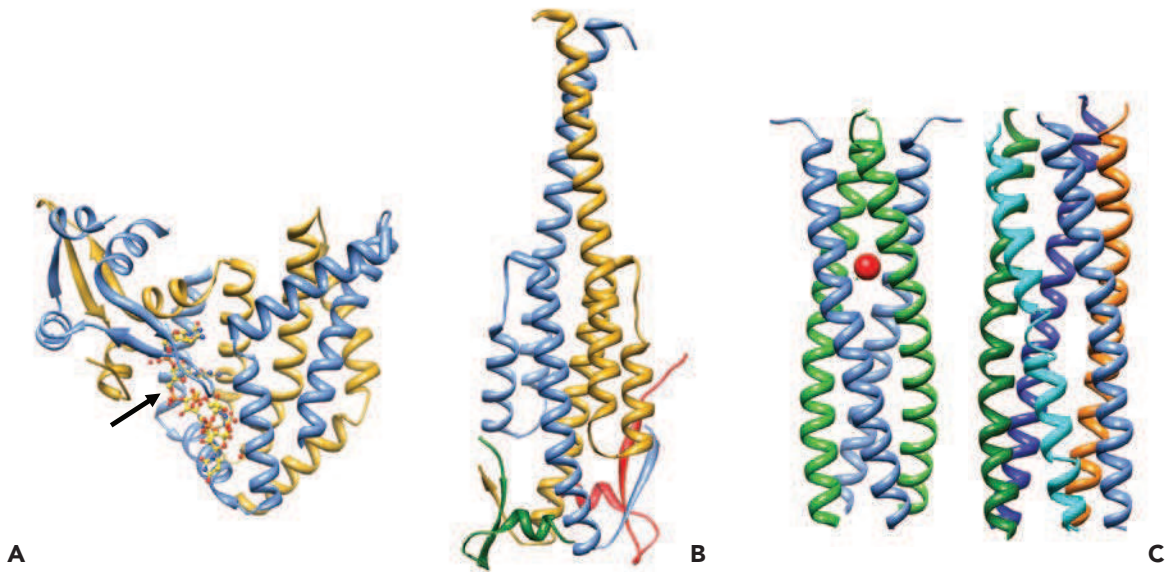


FIGURE 45.13. X-ray structures of NSP3 and NSP4. **A:** Structure of the NSP3 N-terminal domain homodimer in complex with the 3' consensus sequence of rotavirus messenger RNA (mRNA).¹⁸² The dimeric subunits are shown in blue and yellow. A single 5' RNA segment (shown in ball and stick representation and indicated by an arrow) is buried within a basic deep tunnel formed in the asymmetric homodimer (shown in blue and golden yellow). Each subunit participates in different interactions with the mRNA segments. **B:** Structure of NSP3 C-terminal domain homodimer (shown in blue and golden yellow) in complex with the dimer of the short peptide of eIF4G (shown in green and red).³¹¹ **C:** Ribbon representation of the homotetramer (**left**) with each dimer shown in blue and green and the homopentamer (**right**) of NSP4 95-137. The red circle indicates the location of a divalent metal binding site, possibly binding calcium in the tetramer.^{72,106}

particles.⁵⁷² While further work is needed to characterize the nature of the viral dsRNA present in the cytoplasm of infected cells, the multiple mechanisms used by rotaviruses to remodel the host translation machinery in novel ways to ensure efficient translation of viral proteins will likely continue to reveal unique regulatory systems.⁶³⁶

Rotavirus Effects on Cellular Signaling Pathways and Apoptosis

The effects of rotavirus replication on cultured cells are cell-type specific. In nonpolarized tissue-cultured cells, rotaviruses are normally cytocidal viruses that rapidly kill the permissive cells they infect. Adaptation of rotaviruses to culture can be difficult and may require initial passage of virus in primary monkey kidney cells before adaptation to growth in continuous monkey kidney cell lines. Fully permissive cells generally exhibit cytopathic effect, and cell death is preceded by the shut-off of host RNA, DNA, and protein synthesis.^{99,209,489} Cell death seems to result from the function of a viral gene on a specific target rather than from cumulative effects on host metabolism, because certain ts mutants (ts groups F [VP2], G [VP6], H, and I [unassigned NSP3 and NSP4] [Table 45.4]) do not efficiently shut off host cell protein synthesis.^{216,619} NSP4 is a viroporin and a novel calcium agonist that mediates cell death by causing intracellular calcium levels to increase as well as by affecting the plasma membrane permeability and tight junctions of cells.^{504,540,704,726} Rotavirus induces the unfolded protein response of the cell but also controls it through NSP3 that reduces host protein synthesis.^{597,738} Rotavirus has been

reported to induce apoptosis in polarized epithelial cell lines as well as in mice infected with a murine strain of virus.^{69,107,702} Replication-competent virus is required to induce apoptosis, indicating that viral gene expression is a prerequisite and, when examined, intracellular calcium levels play a role, suggesting an effect of NSP4.¹⁰⁷

After infection of nonpolarized cells, rotavirus induces early alterations in vimentin,⁷⁸² in the cytoskeleton organization,⁷⁸³ and in the biochemistry of the host cell⁵⁹⁷ (Fig. 45.14). A dramatic decrease in the level of host cell proteins and a high level of viral proteins is observed.²⁰⁹ Rotavirus NSP1 has been shown to interact with IRF3, which results in its degradation; this is a mechanism by which rotavirus interferes with the innate host response of producing type 1 interferons^{38,294} (see Immunity section). Four proteins that are specifically up-regulated in rotavirus-infected cells have been identified, two of which correspond to the ER chaperones BiP and endoplasmic reticulum chaperone; however, the role of these proteins in the replication and morphogenesis of rotavirus particles remains to be established.⁷⁹⁹ In general, little is known about the involvement of host proteins in the diverse steps of the rotavirus replication cycle, except for in translation.⁷⁶⁵ A cascade of signals that the virus elicits in the host cell, the induced cytoskeletal changes, and the temporal program of cell gene expression after virus infection is being dissected with the application of microarray analysis to infected cells *in vivo* or in cultured cells.^{101,164,437}

Rotavirus infection in polarized human intestinal epithelial (Caco-2) cells leads to the alteration of many cell functions. Expression of enterocyte-specific genes is down-regulated at

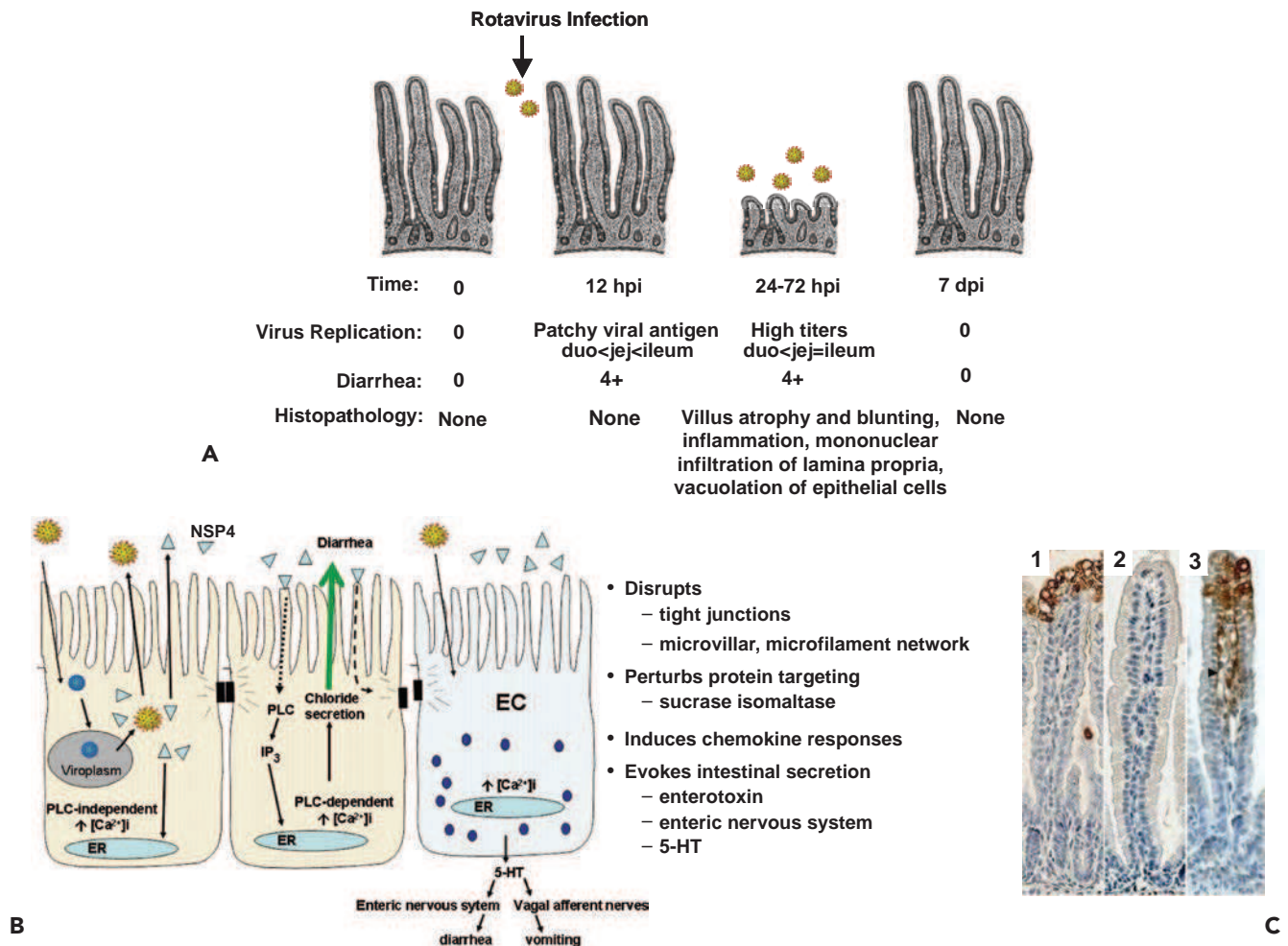


FIGURE 45.14. Rotavirus pathogenesis. **A:** Schematic of the histopathologic and clinical effects of rotavirus infection of intestinal cells. Rotavirus-induced diarrheal disease begins early after infection prior to significant histopathology of the infected mature enterocytes of the intestine. Diagram based on studies in piglets infected with virulent porcine rotavirus.⁷⁷⁰ It is thought that the early, profuse, secretory diarrhea is caused by the action of the rotavirus enterotoxin, NSP4 (see text and²²⁴). Histologic changes are seen at later time points when virus titers are high and loss of the differentiated enterocytes leads to malabsorption. The degree of villus atrophy can vary from species to species. The acute infection and disease normally resolves within 7 days in immunocompetent hosts. **B:** Model of rotavirus-induced diarrhea. Rotavirus infection of a polarized epithelial cell triggers many effects that contribute to pathogenesis. Infection of an initial cell (**left side**) results in virus entry and replication, formation of viroplasm, and release of virus and viral proteins. Intracellular NSP4 mobilizes intracellular calcium from internal stores, primarily the endoplasmic reticulum (ER), by a phospholipase C (PLC)-independent mechanism that involves NSP4 acting as a viroporin.³⁵⁶ An NSP4 or NSP4-derived polypeptide (amino acids 112 to 175) (triangles) are secreted from cells by a nonclassical secretory pathway. Enterocyte function is altered with many cellular messenger RNAs (mRNAs) being shut down and protein targeting is disrupted.^{69,617} Released NSP4-derived polypeptide affects uninfected cells, resulting in mobilization of intracellular calcium by a PLC-dependent pathway that activates or regulates chloride secretion. Tight junctions can also be disrupted by rotavirus infection as well as by NSP4 addition to uninfected cells. Finally, the enteric nervous system can be activated by mediators such as serotonin released from enterochromaffin (EC) cells infected with rotavirus or stimulated by NSP4.^{325,464} Disruption of epithelial cell structure and function occurs slowly (at 18 to 24 hours postinfection) and may lead to cell death and alterations of the paracellular pathway of fluid movement.^{86,131,379,381,504,550,704} Rotavirus infection of epithelial cells activates epithelial cell signaling that triggers crosstalk among the diverse types of cells that make up the villus architecture. This results in activation of the enteric nervous system, intestinal secretion, and immune responses.^{224,325,463,639} **C:** Viral antigen detection in mouse jejunum. Virus structural proteins are detected in enterocytes at the tips of intestinal villi at 4 days postinfection during murine rotavirus infection of neonatal mice (C1), while at the same time NSP4 is detected in epithelial cells at the tips of the villi, at the epithelial basement membrane, and in noninfected cells further down the villus (arrow in C3). NSP4 protein is not detected in uninfected mice (C2).⁷⁰

mRNA and protein levels,⁶⁹ and microvillar F-actin disassembly occurs,³⁷⁹ possibly due to the remodeling of actin by VP4 or NSP4.^{86,264} In addition, rotavirus infection causes alterations in tight junctions independent of virus-induced cytoskeletal rearrangements.^{188,550} Madin-Darby canine kidney (MDCK) cells support only restricted replication of rotavirus, but in both Caco-2 and MDCK cells, infected cells show transmembrane leaks or opening of tight junctions before the development of cytopathic effect and extensive virus release. Attachment of virus to, and entry into, Caco-2 cells does not cause any measurable transmembrane leak during the first hour of infection. Both VP8* and NSP4 have been shown to modulate tight junctions of polarized MDCK cells.^{537,704} It is possible that infected epithelial cells are sloughed from the intestine owing to the loss of tight junctions during the viral replication cycle and before development of cytopathic effect.

The epithelial cell response to rotavirus infection has also been examined in nonpolarized human intestinal cell lines, Caco-2 and HT-29 cells.^{102,682} Interleukin-8, growth-related peptide- α , RANTES (regulated on activation, normal T-cell expressed and secreted), and osteopontin are secreted from rotavirus-infected cells, and chemokine production is time and dose dependent. Interferons and granulocyte-macrophage colony-stimulating factor have also been detected in mice infected with rotavirus.⁶³⁹ The chemokine secretion response by enterocytes is an active component of the host response to rotavirus infection and may play a role in viral pathogenesis or in the immune response to rotavirus infection. Rotavirus infection of nonpolarized Caco-2 cells also increases mitochondrial superoxide dismutase expression and does not lead to induction of oxidative stress, possibly to prolong cell survival and allow for accumulation of viral particles before cell destruction and particle release.²⁶¹

Finally, rotavirus replication has been examined in polarized neuronal cells, where cytopathic effect again is not obvious. These infections are of interest because expression of VP7 is targeted to axons and dendrites by a pathway that does not involve the Golgi apparatus.⁷⁸¹ In contrast, NSP4 is localized solely in the cell body. Rotavirus infection stimulates alterations of specific neuronal proteins. Thus, the distribution of microtubule-associated protein 2, which is normally restricted to nerve cell bodies and dendrites, is altered and found in axons of cultured dorsal root ganglia and spinal cord neurons. This study indicates that selective interactions between certain viral and neuronal cytoskeletal proteins can occur and that noncytolytic viral infection can cause alteration in the polarized sorting of neuronal proteins.⁷⁸³ These studies are of interest because they highlight the usefulness of monitoring rotavirus protein transport to understand new intracellular trafficking and signaling pathways. It is not known if rotaviruses naturally infect neurons or if such infections result in activation of the enteric nervous system, which is a component of the pathogenesis of diarrheal disease.^{325,463}

Rotaviruses also have the ability to establish persistent infections in cell cultures^{28,123,219,520} and in mice with severe combined immunodeficiency disease (SCID).⁶³⁰ Analyses of these cells show that both viruses and cells co-evolve, and mutations in the virus confer trypsin-independent growth, suggesting that a protease requirement for efficient viral growth is a point of balance for viral growth and cellular survival during maintenance of persistent infection.⁵²⁰ Recognition that rota-

viruses cause persistent infections in SCID mice suggests that the *in vitro* observations of carrier cultures may have clinical relevance. The prolonged excretion and occasional extraintestinal spread of rotaviruses in children who are immunocompromised suggests that cell-mediated and innate immunity is important in limiting and clearing virus infections.^{273,353,580,792} Antibody, however, also plays a role in overcoming persistent infections in animals and children.^{91,315,317} The detailed mechanisms that regulate persistent virus–host relationships remain to be elucidated.

Genetics and Reverse Genetics

Genetic studies have been exploited to understand rotavirus gene structure and function. Temperature-sensitive mutant collections have been isolated from several rotaviruses, including the strains SA11, UK, and RRV.^{229,304,614–616} The most extensive ts mutant collection exists for rotavirus SA11, in which 10 of the expected 11 reassortment groups have been identified; 9 ts mutants have been assigned to genome segments (Table 45.5).¹⁶¹ Further biochemical and sequence analyses of these mutants or establishment of complementation systems with expressed gene products may permit dissection and localization of specific functional domains for the mutant genes.

Characterization of the genetic interactions between ts mutants has shown that recombination between pairs of ts mutants of SA11 occurs in the all-or-none fashion expected for recombination by a mechanism of reassortment of genome segments.⁶¹⁵ Genetic interactions of rotaviruses are influenced by factors similar to those that affect such interactions of other segmented viruses, including multiplicity of infection and the time of infection.⁶¹³ Maximal, or near-maximal, reassortment frequency is obtained at the earliest times when reassortment can be detected. This suggests that reassortment occurs early in the infectious cycle and that a single round of reshuffling of genes occurs, an observation consistent with the particle association of progeny dsRNA. Reassortment is efficient at nonpermissive temperatures and mutants from all reassortment groups can interfere with the growth of wild-type virus at both permissive and nonpermissive temperatures. These results are important to consider when trying to produce reassortants.

Naturally occurring rotavirus variants have also been found to be useful probes of gene function. Studies of a variant of the simian rotavirus SA11, which lacks glycosylated VP7, showed that glycosylation is not essential for virus infectivity or hemagglutination^{222,589} but that glycosylation can affect rotavirus antigenicity.¹⁰³ Another variant of SA11 with an altered genome segment 4 showed that the phenotype of certain reassortant constellations of genome segments depends on the parental strains used for analysis.¹¹³ This phenomenon has been confirmed with other reassortants containing specific combinations of VP4 and VP7, and this is beginning to explain some unexpected antigenic properties of some virus strains.^{114,197,414,422,528,800,831}

A reverse genetic system for rotavirus has been described in which a VP4 gene expressed from a complementary DNA (cDNA) was able to be rescued in a viable virus using conditions of strong selection.⁴¹⁸ This groundbreaking result proved that gene rescue is possible. Although the system remains inefficient, this breakthrough has allowed modifications of at least the VP4 gene to be studied. For example, a recombinant rotavirus with an antigenic mosaic of cross-reactive neutralization epitopes has

shown the feasibility of engineering novel vaccines.⁴¹⁷ In addition, the trypsin cleavage site of VP4 has been modified to a furin-sensitive site to determine if this would further enhance infectivity.⁴¹⁹ The mutant VP4 on virus was efficiently cleaved by furin, but the virus showed impaired infectivity, and infectious virus release into the extracellular medium was impaired, suggesting that intracellular cleavage of VP4 by furin is disadvantageous for RV infectivity, possibly due to an inefficient virus release process.⁴¹⁹ The efficiency of this system has been improved by using two independent strategies (use of a ts mutant and RNA interference [RNAi]-mediated degradation of the wild-type target mRNA) to drive the selection of recombinant viruses.⁷³³ A reverse genetics system for rotaviruses also has been achieved by another modified approach based on the preferential packaging of rearranged genomic RNA segments.⁷³⁶ In this case, an *in vitro* engineered form of a rearranged segment from a human rotavirus derived from cloned cDNA and transcribed in the cytoplasm of cells by using the T7RNA polymerase replaced the wild-type segment of a bovine helper virus. This system was used to enable the introduction of a mutated gene expressing a modified nonstructural protein into an engineered mono-reassortant infectious rotavirus without the need for any selective pressure other than selection inherent to cell culture. Continued improvements of these approaches and their use to study specific functions of the viral proteins and the biology of viral replication will be required to truly understand the molecular basis of viral pathogenesis.

PATHOGENESIS AND PATHOLOGY

Our understanding of rotavirus pathogenesis is based primarily on work with animal models. Such studies allow the use of characterized homologous (viruses initially isolated from the experimental host species) and heterologous (viruses initially isolated from a host species distinct from the experimental species) viruses. In animals, virus replication can be analyzed in different tissues to obtain precise information about the sites of virus replication and host response to infection. Rotaviruses replicate primarily, but not exclusively, in the nondividing, mature enterocytes near the tips of the small intestinal villi, suggesting that differentiated enterocytes express factors required for efficient infection and replication. Although the small intestine is clearly the site of optimal viral replication, several recent studies have demonstrated antigenemia, viremia, and limited systemic replication likely occurs in a variety of sites, although there is little evidence that this systemic spread and replication is responsible for any specific pathologic findings in normal hosts.^{63,64,230,239,533,611,618} Studies have demonstrated, however, that in severely immunocompromised infants, rotaviruses can replicate and cause abnormalities in the liver and other organs,²⁷³ and that in suckling mice without an intact interferon signaling system, some strains of rotavirus replicate very efficiently in the biliary tree and pancreas and cause biliary atresia and pancreatic disease.²³⁴ This strain-specific predilection to replicate in the biliary tree and pancreas of mice deficient in interferon signaling has been linked to specific phenotypic characteristics of VP4 and NSP1 and involves viral binding and interferon antagonism.²³⁷ The relationship between the ability of certain rotavirus strains to spread and replicate systemically and the association of rotavirus infection

or vaccination with several systemic manifestations such as fever or intussusception is presently unknown.

The severity, localization, and histological findings of intestinal infection vary among animal species,^{69,206,617} between strains in a single species, and between studies; however, in virtually all cases pathologic changes due to rotavirus infection are primarily limited to the small intestine.^{302,617} In animal models, infection is associated with a few visible lesions in the intestine, with some lesions such as enterocyte vacuolization and loss, or with significant histopathology changes such as villus blunting and crypt hyperplasia. Inflammation is mild compared with that observed with many other intestinal pathogens, especially bacterial pathogens. In many cases, no clear correlation exists between the degree of histopathology changes and the severity of diarrheal disease. Even in species where histopathology is significant, such as cows and pigs, frequently significant diarrhea occurs before the signs of intestinal pathology. This dichotomy has been explained by the existence of a variety of mechanisms that cause disease symptoms (primarily vomiting, diarrhea, and fever in humans) that are briefly reviewed here. Several of these mechanisms are not dependent on either tissue disruption or inflammation. More extensive reviews are available.^{32,90,145,218,303,463,617,649}

Pathogenesis of rotavirus infection is multifactorial, and both host and viral factors affect the outcome of disease. For example, the age of inoculation impacts severity of symptoms: in newborn or suckling interferon signaling-deficient mice, infection can result in biliary atresia; in 0- to 14-day-old mice and rats, diarrhea and some extraintestinal replication of virus occur; in adult mice, infection occurs in the absence of diarrhea. Genetic analysis of selected virus reassortants identified several viral proteins that are involved in virulence (VP3, VP4, NSP1, VP6, VP7, NSP2, NSP3, and NSP4).^{82,237,344,552,617} These proteins are thought to play roles in the efficiency of virus replication (VP3, NSP2, VP6, and NSP3), shut-off of host protein synthesis (NSP3), extraintestinal spread and replication of virus (NSP1 and NSP3), virus entry into cells (VP4 and VP7), regulation of the induction of interferon (NSP1), and the induction of diarrhea (NSP4).

A major confounder in interpreting the various genetic studies of rotavirus pathogenesis and virulence is the general failure by many investigators to carefully define what component of pathogenesis is being examined. For example, some studies have simply examined disease phenotype (diarrhea/no diarrhea), whereas others have attempted to quantify the level of diarrhea. Some studies have focused on the quantity of viral replication in the intestine or shedding in the feces as a surrogate of pathogenesis, and other studies have examined transmissibility between susceptible hosts. Finally, in some studies, disease due to extraintestinal replication was examined as a measure of virulence. Depending on which one of these various pathogenicity-associated phenotypes is under study, both the host-associated mechanisms and the viral genes involved are likely to vary.

As a more specific example, analysis of the most prominent aspect of rotavirus pathogenesis, rotavirus diarrhea-inducing capacity, has yielded discrepant results. Rotavirus diarrhea-inducing capacity has been attributed to several different mechanisms, including malabsorption secondary to destruction of enterocytes or disruption of enterocyte absorptive functions, villus ischemia, a virus-encoded enterotoxin (NSP4), and activation of the enteric nervous system.^{33,325,463,464,556} Each of these

distinct mechanisms would likely be affected by a reduction in viral replication capacity of any cause. In fact, several studies in people have linked the level of rotavirus shedding in the feces with the diarrheal severity,³⁸⁶ although recent studies in India of neonates symptomatically or asymptotically infected with a similar rotavirus strain failed to find a correlation between diarrheal severity and either the amount or the duration of viral shedding.⁶¹² Hence, it is important when studying the basis of rotavirus virulence to be cognizant of the constraints and limitations associated with the specific choice of experimental system or aspect of pathogenesis under examination.

In regard to pathogenesis, perhaps the most critical determining factor differentiating the virulence potential of one rotaviral strain from another is whether the virus examined is homologous or heterologous for the infected host. Homologous strains (strains generally isolated from the host species in question) tend to replicate efficiently, often causing diarrhea at a very low inoculation dose, and spread efficiently in that host. In general, heterologous strains (not routinely isolated from that host in question but found frequent in another host species) replicate poorly compared to the homologous strains, only cause diarrhea with large inoculation doses, and do not spread efficiently to other susceptibles. Host-range restriction barriers to rotavirus infection, while substantial, are not absolute, and there are numerous examples of zoonotic transmission to people.⁴⁷³ The mechanistic and genetic basis for host-range restrictions are one set of factors that often play a role in virulence. Host-range restriction also forms the mechanistic basis for the attenuation of several rotavirus vaccines and vaccine candidates. Viral determinants of host-range restriction have not been as thoroughly investigated as one might imagine, and studies have been basically limited to experimental infections of reassortant viruses in pigs and mice. In mice it appears that NSP1, a gene product involved in suppressing innate immune responses, and probably VP4, the viral attachment protein, are both important factors governing host-range restriction in the gut based on genetic studies⁸² (H. Greenberg, unpublished data). In pigs NSP1 may not be involved.^{207,410}

There are factors other than replication potential that likely affect virulence. The discovery of NSP4 as the first viral enterotoxin is of interest in this regard, because it shows this protein has pleiotropic properties in addition to its intracellular role in viral replication and morphogenesis (see earlier). NSP4 has been shown to induce age-dependent diarrhea in mice that mimics disease caused by rotavirus infection; this has been confirmed for the NSP4 proteins from several group A and non-group A viruses.^{303,342} How NSP4 might function as a virulence factor has been determined by analyzing reassortant viruses in gnotobiotic pigs,³⁴⁴ although other genetic studies have not directly linked NSP4 with diarrhea,⁸² and virulent and avirulent strains of rotavirus often have similar or identical NSP4 genes. Several features of a model for the function of NSP4 as an enterotoxin have been confirmed²¹⁴ (Fig. 45.14). The predictions that NSP4 would be released from rotavirus-infected cells and have paracrine effects on adjacent uninfected cells have been confirmed *in vitro* and *in vivo*. The mechanism of NSP4 enterotoxin function has been shown by extracellular administration of NSP4 to the intestinal mucosa and to crypt cells from mice and to human intestinal cell lines. The extracellular administration triggers a signal transduction pathway that leads to mobilization of intracellular calcium, $[Ca^{2+}]_i$,

through a PLC-dependent pathway and chloride secretion (Fig. 45.14). *In vivo*, in mice infected with a murine rotavirus strain, NSP4 is detected both in cells at the tips of the intestinal villi (together with other structural proteins) as well as by itself at the basolateral surface of cells and in cells in the lower part of intestinal villi (Fig. 45.14). The basolateral localization of NSP4 is consistent with the observation that NSP4 interacts with laminin β -3 and fibronectin.⁷⁰ Basolateral integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are receptors for the rotavirus enterotoxin.⁶⁷⁴ An antibody to NSP4 and siRNAs to NSP4 attenuate rotavirus-induced diarrhea in mice,^{119,127,346} but levels of antibody to NSP4 in children have not been directly correlated with protection, and thus the precise mechanism through which NSP4 induces diarrhea in people remains to be characterized. An assay to measure functional antibody may be needed.

A role for the enteric nervous system in rotavirus diarrhea has been shown by the ability to attenuate rotavirus-induced diarrhea in mice and children with drugs that block this pathway.^{423,463,656} NSP4 or other factors released from virus-infected cells may mediate this effect directly or by stimulating responses from enterochromaffin cells. These results suggest common mechanisms between diarrheas induced by viruses and bacteria not previously appreciated. Recent studies using human enterochromaffin cells (ECs) as well as *in vivo* analyses in the mouse model indicate that rotaviruses can infect ECs and that this infection induces secretion of serotonin. Serotonin release is also stimulated by NSP4 but not by inactivated virions. The release of serotonin, which acts through the enteric nervous system, could be responsible for the well-documented association of rotavirus infection with vomiting.³²⁵

EPIDEMIOLOGY

Morbidity and Mortality in Children

Until the discovery of the rotaviruses, only a small proportion of severe diarrheal illnesses of infants and young children could be linked to a specific etiologic agent.⁸¹⁴ As data from epidemiologic studies in developed and developing countries accumulated, it became clear that rotaviruses were the major etiologic agents of serious diarrheal illness in infants and young children throughout the world.⁵⁶ The impact of rotavirus infection on infant mortality was far greater, however, in the poorest regions of Asia and Africa (Fig. 45.2).

Although rotavirus diarrhea occurs with high frequency in developed countries, mortality is low. In the United States, rotaviruses cause about 5% to 10% of all diarrheal episodes in infants and children younger than 5 years of age, and prior to the advent of widespread vaccination (see Prevention and Control Section later), these viruses accounted for 30% to 50% of the severe diarrheal episodes.^{469,563,564} In this age group in the United States in the prevaccination era, rotaviruses were responsible annually for more than 3 million episodes of diarrheal illness, 500,000 visits to a medical practitioner, 60,000 to 70,000 hospitalizations (including those with nosocomial rotavirus discharge diagnoses), and 20 to 40 deaths.^{183,213,242,277} Similar epidemiologic studies from Europe demonstrated that before the institution of rotavirus vaccines, rotavirus infection was responsible for approximately 3.6 million episodes of diarrhea, over 87,000 hospitalizations, 231 deaths, between 28% and 52% of acute gastroenteritis cases, and up to two-thirds of

related hospitalizations and emergency room visits.^{692,742} There is not yet reliable data available on how the widespread use of rotavirus vaccines has affected total diarrheal illness numbers in the United States, but the number of severe illnesses and hospitalizations due to rotavirus has decreased by more than 85% for fully vaccinated children in the United States, and a recent study from Mexico indicates that vaccination resulted in a significant decrease in rotavirus-associated mortality.⁶⁹⁵

Prior to the implementation of widespread vaccination, practically every child younger than 5 years of age in the United States experienced a rotavirus infection of severity ranging from asymptomatic to life threatening; 1 in 100,000 children in this age group died from rotavirus infection.²⁴⁰ Directly calculated population-based rates for rotavirus hospitalizations and emergency department visits were 22.5 hospitalizations and 301.0 emergency department visits per 10,000 children younger than 3 years of age.⁵⁷⁸ In the United States, rotavirus diarrhea was estimated to result in more than \$400 million in direct medical costs (primarily for hospitalization) and more than \$1 billion in total societal costs (primarily for loss of work time of parents or caregivers) prior to the introduction of widespread vaccination.²⁴⁰ The high morbidity rate but low mortality rate was due to the development during the 1940s of effective means for replacement of fluid and electrolytes lost during acute illness and their routine use in developed countries.^{203,652} Widespread use of oral rehydration therapy was associated with a marked decrease in mortality from all forms of infantile diarrhea in the United States and other developed countries since the mid-1900s.

In developed countries, the widespread distribution of rotaviruses in the community is documented by the universal acquisition of serum antibodies to these viruses at an early age. Prior to rotavirus vaccine use, more than 90% of infants and young children in the Washington, DC area possessed rotavirus antibodies by the end of the third year of life, a pattern similar to that observed for respiratory syncytial and parainfluenza type 3 viruses.³⁹⁵ The high prevalence of rotavirus antibodies is maintained into adult life and periodically undergoes significant boosts, suggesting that subclinical rotavirus reinfection occurs relatively commonly throughout life. A similar pattern of rapid acquisition and maintenance of rotavirus antibody has also been observed in many other parts of the world. The widespread vaccination of children starting at 2 months of age in the United States means that acquisition of serum antibody can no longer be used as a surrogate for natural infection.

Cross-sectional studies of hospitalized infants and young children with diarrheal illnesses have yielded the most compelling evidence for the importance of rotaviruses as etiologic agents of severe diarrhea of early life. For example, during a period of over 8 years, 34.5% of 1,537 infants and young children admitted with diarrhea to a Washington, DC hospital shed rotavirus in their feces on admission.⁷⁴ In a similar study in Japan of over 6 years' duration, 45% of 1,910 pediatric patients with diarrhea were rotavirus positive.⁴²⁰ This pattern has been shown in other studies over shorter time periods.⁷⁵⁸ A 13-year, cross-sectional study of hospitalized children with gastroenteritis reported from Australia found that 39.6% of 3,785 children shed rotavirus in feces on admission.³⁴ Notably, 55% of children (12 to 23 months of age) with gastroenteritis were rotavirus positive. Rotavirus gastroenteritis was also often observed in the younger than 6 months of age group (18.7%

of cases) as well as the group consisting of 5- to 13-year-olds (16% of cases).

In developing countries, rotaviruses are consistently the leading cause of life-threatening diarrhea.^{78,562} The global burden of rotavirus diarrheal disease in infants and children younger than 5 years of age worldwide is estimated to be more than 100 million episodes, over 20 million outpatient visits, and a staggering more than 500,000 deaths (range 475,000 to 580,000)^{562,563} (Fig. 45.2). Hospital-based studies in Asia, Africa, and Latin America indicate that 25% to 55% of hospitalizations for diarrheal illnesses in children younger than 5 years of age are linked to rotavirus infection,²⁷⁷ with more than 80% of all rotavirus deaths occurring in low-income countries of South Asia and Sub-Saharan Africa.^{78,562,563} For example, recent estimates indicate that India, China, and Pakistan have approximately 122,000, 27,000, and 20,000 rotavirus-associated deaths, respectively, whereas the African countries of Nigeria, Democratic Republic of the Congo, and Ethiopia have approximately 50,000, 30,000, and 27,000 deaths per year, respectively.^{78,562} Thus, during the first 5 years of life in both developed and developing countries, in the absence of vaccination, every child will experience an episode of rotavirus infection and many will have an associated diarrheal illness, but the consequences of the rotavirus infection are likely to be quite different depending on where the child lives.

Although rotavirus vaccines have been widely deployed in a number of middle-income countries in Central and South America, vaccines have not yet been broadly available in the poorest countries of Asia and Africa. Widespread vaccination would dramatically lower the worldwide mortality and economic burden of rotavirus infection if the difficulties in financing and delivering the rotavirus vaccine could be overcome.⁵²²

Rotavirus Serotypes

The serotype classification of various rotavirus strains and the structural and genetic basis for this classification scheme is covered in the earlier Classification section of this chapter. Basically both surface proteins, VP4 (P types) and VP7 (G types), are targets of neutralizing antibodies and serotypic classification is based on a binary system including both proteins. There are currently 27 distinct G serotypes and 35 P serotypes found among group A rotaviruses of humans and animals.⁴⁸² Fortunately, the number of G and P combinations commonly found in people is substantially lower than the number of possible combinations, although approximately 12 G types (1 to 6, 8 to 12, and 20) and 15 P types (1 to 6, 8 to 11, 13, 14, 19, 25, and 28) have been isolated at one time or another from people.^{482,660} The development of the monoclonal antibody-based ELISA for identification of the VP7 type of clinical isolates and the subsequent application of RT-PCR genotyping and nucleotide sequencing have expanded our understanding of the epidemiology of rotavirus serotypes with regard to infection, disease, and immunity.^{241,267,660}

The global distribution of common human rotavirus serotypes has been consistent from 1989 to the present with the VP7 "G" serotypes 1, 2, 3, and 4 continuing to make up the great majority of strains detected, especially in the developed world. In the last decade, G9 strains have emerged as important serotypes in some parts of the world, such as Australia, the United States, Ghana, India, and Brazil^{2,21,267,413,660}; G8 strains have emerged occasionally in parts of Africa.^{267,660,728} A comprehensive

review encompassing 124 studies published between 1989 and 2004 of the global distribution of rotavirus serotypes and genotypes in 52 countries on five continents, which included 45,571 typed strains, showed that G1 strains were the most frequently detected serotype in each of the continents. G1, G2, G3, and G4 strains accounted for 97.5% of all rotavirus infections studied in Asia, North America, and Europe and 83.5% to 90.4% in South America, Africa, and Australia. G5, G8, G12, and G9 strains appeared focally in several areas, with G9 strains occasionally being predominant.⁶⁶⁰ More recent reviews emphasize the persistent frequency of G1 to G4 strains over the past decade and the emergence of G9 and G12 strains in a variety of locations around the world.^{484,547,658}

Serotype analysis of clinical isolates for their VP4 serologic specificity has not been widely performed because of the paucity of appropriate, monospecific serologic reagents. RT-PCR for VP4 genotyping has made it possible, however, to characterize rotavirus-positive specimens from epidemiologic surveys.^{267,660} Based on sequence analysis, VP4-type P1A[8] strains are the most frequently observed strains in humans followed in frequency by P1B[4] strains. This is to be expected because the P1A[8] genotype is almost always found in association with G1, G3, G4, and G9 rotavirus strains. The other major G serotype, G2, characteristically has VP4 P1B[4] specificity. Although various combinations of G and P serotypes or genotypes have been described, a review of 16,474 strains evaluated for both VP7 and VP4 specificities in 124 studies indicated that four predominant P-G combinations, P[8]G1, P[4]G2, P[8]G3, and P[8]G4, composed 88.5% of all the strains.⁶⁶⁰ A more recent analysis indicates that P[8]G9 strains are also frequently isolated in humans.²

The reason serotypic classification studies have received so much attention is because it is assumed that such classifications will be relevant to and correlate with the induction of protective immunity. With the recent demonstration that a G and P monotypic vaccine (see Prevention and Control section later) appears to efficiently protect against all rotavirus-associated illness in humans, the intense focus on human rotavirus serotypes must be called into question. Additional time will be required to determine the precise and long-term relationship between human rotavirus serotypes and protective immunity, but initial indications are that heterotypic immunity plays a larger role in the epidemiology of disease during rotavirus infection than in some other viral infections, and that despite the very large potential serotypic diversity of rotaviruses, immunity is generally generated rapidly after a limited exposure to a restricted number of serotypes, at least in developed countries.

Rotavirus Infections in Adults

Adults are frequently reinfected with rotavirus, but characteristically with only minimal to moderate or no clinical manifestations. For example, 22 of 40 adult household contacts of children hospitalized with rotavirus gastroenteritis had serologic evidence of rotavirus reinfection at the time of their child's admission to the hospital, although the adults were asymptomatic.⁴¹¹ When symptomatic, infection in adults is similar to that seen in infants, although diarrhea, vomiting, and dehydration are not usually severe.¹⁴ Although subclinical rotavirus infection is the most common outcome in adults, rotavirus gastroenteritis in adults has been described in army recruits in Finland as well as in patients (some geriatric) and staff in hospitals in

various countries.³⁴⁷ In a recent study from China, rotavirus was second to noroviruses as a cause of viral gastroenteritis in adults.⁴⁴⁷ Outbreaks in adults have been described, but these are considered to be unusual, isolated events.^{251,444} Rotavirus infections have also been associated with traveler's diarrhea in certain settings, and a recent large review of the global etiologies of traveler's diarrhea found that rotavirus infection was responsible for approximately 4% to 7% of cases depending on the location.⁶⁷⁶ Symptomatic rotavirus gastroenteritis can also be seen in adults undergoing bone marrow and other transplantations^{5,833} as well as in some patients with malignant disease undergoing chemotherapy.⁴⁴¹

Group B rotaviruses (adult diarrheal rotaviruses [ADRVs]) have been implicated in several large outbreaks (involving up to 20,000 individuals) of severe gastroenteritis in adults in various parts of China as well as smaller outbreaks and endemic disease in Bangladesh and India.^{354,407} Affected individuals often had cholera-like, severe, watery diarrhea, and a few elderly patients died. To date, group B rotavirus infections appear to occur primarily as isolated epidemics or sporadic cases, possibly due to contamination of water sources.

Nosocomial Infections

Nosocomial rotavirus infections occur frequently. For example, 10 (17%) of 60 children hospitalized for nondiarrheal disease during a period of rotavirus prevalence developed a diarrheal illness associated with a rotavirus infection.⁶⁵¹ In another study, approximately one of every five rotavirus infections appeared to be hospital acquired.⁵⁰⁶ The important contribution of nosocomial infections to the composition of hospital discharge diagnoses attributed to rotaviruses has been documented in various studies: a median of 27% of patients in developed countries and 32% in developing countries discharged with a diagnosis of rotavirus diarrhea were likely to have acquired the rotavirus infection while in the hospital.²⁴⁰ A review of nosocomial infections in European countries showed that infections are mainly associated with young infants (0 to 5 months of age) as opposed to older age groups in the community and that a sizeable proportion of the infections are asymptomatic.²⁸² The introduction of rotavirus vaccines leads to a significant reduction in hospital-acquired as well as community-acquired rotavirus infections.¹³ Nosocomial infections have also been described in various neonatal nurseries around the world.^{55,168,443} These infections are usually subclinical, although nosocomial outbreaks of rotavirus gastroenteritis have been described in a newborn nursery in Italy²⁷⁰ and in various neonatal intensive care units in the United States.²⁴⁰

Transmission

Rotaviruses are very contagious,²⁹⁵ with as little as one tissue culture infectious dose able to cause illness in a fully susceptible host. Further enhancing transmission potential, the virus is very stable in the environment and is shed in very large amounts in the feces.²²³ Rotaviruses are generally transmitted by the fecal-oral route. Oral administration of rotavirus-positive stool material induces diarrheal illness and viral shedding in adult volunteers.^{402,775} There has been speculation that rotaviruses are also transmitted by the respiratory route.³²² Evidence for this is circumstantial and rests on the following observations: (a) the rapid acquisition of rotavirus antibodies in the first few years of life in all settings, regardless of hygienic standards; (b) a few

large outbreaks in which a fecal–oral spread could not be documented²⁵¹; and (c) the occurrence of respiratory symptoms in a proportion of patients with rotavirus gastroenteritis.³²² Scattered reports have appeared of rotavirus detection in the upper respiratory tract; however, the predominance of data suggests that the respiratory route is not the usual mode of transmission.^{766,830} The source of rotavirus infection for the young infant who is normally not in contact with other infants and young children with gastroenteritis is not well understood. Most likely, infection is acquired from an older sibling or parent with subclinical infection.^{208,308} Shedding of rotavirus from the intestinal tract before onset of diarrhea or following cessation of diarrhea has been well documented.⁵⁹⁵ The duration of rotavirus shedding in 37 hospitalized children ranged from 4 to 29 days, with a median of 7 days, as measured by ELISA, whereas by the more sensitive PCR assay shedding was detected for up to 57 days, with a median of 10 days.⁶²⁷ Shedding in severely immunocompromised children can be even more prolonged.²⁷³

Resistance to physical inactivation may also contribute to the efficient transmission of the human rotaviruses. This inference is drawn from the observed stability of various human and animal rotaviruses at ambient temperatures and from the low doses required for infection of humans and animals.^{223,409,775} For example, porcine rotavirus present in feces retained infectivity for over 2 years when refrigerated.⁶²⁰ Other observations that suggest that environmental contaminations are viable sources of infection include (a) the persistence of rotavirus infection in certain newborn nurseries and (b) the high frequency of nosocomial rotavirus infection in hospitals (see earlier). The ability of rotaviruses to survive on various surfaces under different conditions may contribute to its rapid spread.⁷⁹⁰ Although rotaviruses have been detected in both raw and treated sewage, it is unlikely that contaminated water (which is important in group B rotavirus outbreaks) plays an important role in transmission of group A rotaviruses.^{354,688} However, an apparent community-wide water-borne epidemic of group A rotavirus was documented in Turkey in 2011.⁴²⁴ Effective disinfection of contaminated material and careful hand washing constitute important measures to contain rotavirus infection, especially in hospitals and institutional settings.^{61,657,705,790} Rotavirus has been detected on moist surfaces in daycare centers and, in one controlled study, rotavirus transmission to volunteers was prevented if a surface first contaminated with rotavirus was sprayed with a disinfectant (Lysol) prior to contact with the volunteer.^{94,773}

There has been speculation on the role of animals as a source of rotavirus infection of humans. This idea gained support with the observation that certain animal rotaviruses share a neutralization antigen with human rotaviruses and that certain naturally occurring animal rotavirus strains may infect humans or form reassortants with human rotaviruses.^{485,530} Nonetheless, interspecies transmission and persistence of animal strains in the human population appears to be rare. It should be noted, however, that human-bovine reassortant strains (I321, 116E) and a human–porcine reassortant strain (G5) have been endemic in selected areas in India and Brazil, respectively.^{11,169,276} Human–simian rotavirus reassortants have also been detected in India.²⁵ Recent large-scale full genomic sequencing of rotavirus isolates from around the world indicates that genome segments of animal rotavirus origin are present in human isolates at higher frequency than had been expected.⁴⁷³ However, to date, such studies have focused on sequencing of isolates that were unusual

from a serologic or electrophoretic perspective, so the actual frequency of such human/animal reassortment in the field is not clear. In a large unpublished sequencing study of rotavirus isolates collected over several seasons from the United States, the rate of human/animal reassortants was very low (John Patton, personal communication).

Incubation Period

The incubation period of rotavirus diarrheal illness has been estimated to be less than 48 hours.¹⁷¹ The onset of experimentally induced rotavirus diarrhea in adult volunteers occurred 2 to 4 days after challenge.^{401,402} One of the volunteers who developed diarrhea 3 days after virus administration developed a fever and vomited on the first day after challenge, thus indicating from such studies that the incubation period of the illness under experimental conditions was 1 to 4 days.

Distribution

Geographic Distribution and Seasonal Patterns

Rotaviruses have been detected throughout the world wherever they have been sought.^{78,279,563,610,767} These viruses consistently constitute the major etiologic agents of severe infantile diarrhea in every country where this disease has been studied. Prior to the widespread introduction of rotavirus vaccines, rotaviruses displayed a marked seasonal pattern of infection in developed countries, with epidemic peaks occurring in the cooler months of each year.⁵⁶⁰ This recurring pattern has been observed in the United States, Europe, Australia, and Japan.^{34,74,166,420} The cause for this striking seasonal pattern is not known, but the influence of low relative humidity in the home has been suggested in some but not all studies as a factor facilitating the survival of rotaviruses on surfaces.^{73,420,512}

An analysis of hospitalizations for diarrhea in the post-neonatal period and the temporal distribution of rotavirus isolations from various centers suggested that rotavirus diarrhea spreads from the west to the east in the United States, appearing in the fall in the southwest and spreading progressively across the country, so that by late winter and spring it has reached the northeast and maritime Canada.⁴³⁶ Of note, following the introduction of rotavirus vaccination, the marked seasonality of rotavirus infection declined substantially, and the initiation of the annual epidemic occurs later in the season.^{166,717} The usual seasonal pattern of rotavirus infection observed in the temperate climates does not occur uniformly in other areas of the world; many locations in the tropics show no, or a diminished, seasonal trend.^{336,438} Recent modeling studies have suggested that the relative lack of rotavirus seasonality observed in many tropical countries may be due to the high birth rates and transmission rates typical of developing countries.¹⁶⁶

Age, Sex, Race, and Socioeconomic Status

In developed countries, rotavirus gastroenteritis of sufficient severity to require hospitalization characteristically occurs most frequently in infants and young children from approximately 6 months to 2 years of age.⁵⁵ Infants younger than 6 months of age experience the next highest frequency of such illness. In certain studies, especially in less developed countries, the younger than 6 month group had the highest frequency of severe disease.^{160,208,699,740} In one study from the United States, the age distribution of patients admitted to the hospital with gastroenteritis of any cause was different for black and nonblack

patients; 59% of all black patients admitted for gastroenteritis were younger than 6 months of age, whereas most non-black patients were older.⁷⁶ This difference was also reflected in admissions for rotavirus diarrhea. Because the children were predominantly from inner-city areas, these differences may have reflected the effects of crowded living conditions, which may have allowed earlier and more efficient transmission of the virus.

In an Australian study of 4,637 children from 0 to 14 years of age hospitalized with gastroenteritis, the peak incidence of rotavirus gastroenteritis was in children 12 to 23 months of age. Rotavirus was also a frequent cause of gastroenteritis in children younger than 6 months of age (18.7%). There were also rotavirus-associated gastroenteric illnesses in children 5 to 13 years of age, and rotavirus was identified as the sole pathogen in 16% of patients in this age group. The low frequency of clinical illness in most (but not all) normal neonates who shed rotavirus remains unexplained.⁵⁶ It seems clear, however, that such asymptomatic neonatal infection is able to induce a protective immune response in the infected children.²⁷⁶ Malnutrition is considered to play an important role in increasing the severity of clinical manifestations of human rotavirus infections.^{85,167} This phenomenon has been reproduced in experimental mouse and piglet models.^{739,832} It has also been suggested that repeated diarrheal infections may be a precipitating factor in the development of malnutrition as these infections damage the intestinal mucosa so that absorptive cells are compromised over an extended period.⁴⁷⁷ It is striking that the World Health Organization (WHO) estimates that of the 10.6 million children younger than 5 years of age who died from 2000 to 2003, the underlying cause of death in 53% of cases was undernutrition, and in 18% of these children deaths were attributed to diarrhea.⁸⁹

Molecular Epidemiologic Studies

Initial investigations of the molecular epidemiology of human rotaviruses utilized gel electrophoresis and evaluated the individual genomic migration patterns of the segmented genomic dsRNA for strain identification (electropherotype), comparison, and epidemiologic correlations.³⁴⁰ For example, analysis of rotaviruses isolated from 116 children hospitalized with gastroenteritis in Melbourne, Australia, between 1973 and 1979 identified 17 different electropherotypes.⁶³³ In this study, a single electropherotype was predominant at any given time but was accompanied by less common types, and these predominant electropherotypes tended to change over time during a season and between seasons. The major exception to this pattern was the finding that the electropherotypes present in newborn nurseries tended to remain constant for years.¹³⁴ In addition to providing evidence for genetic diversity of human rotaviruses and heterogeneity of circulating rotaviruses, analysis of electropherotypes has also provided a simple and sensitive method for tracing the spread of rotavirus through a population group. The electropherotype cannot be used, however, to predict virulence or for serotypic classification.^{19,269}

Another early molecular approach to epidemiologic studies was RNA/RNA hybridization; this technique employs labeled ssRNA viral transcripts as probes for genomic dsRNA. These studies identified two major families (genogroups) of human rotaviruses and showed that human strains had a general lack of homology with animal strains. Occasional human/human and human/animal genogroup reassortants and some rare human genogroups have been observed using hybridization.^{250,529,531}

It was proposed that the term *genogroup* be adopted in lieu of *family* to conform to taxonomic hierarchy.⁵³⁰ Recent advances in the speed of full genomic sequencing as well as its reduction in cost make it likely that electropherotyping and RNA/RNA hybridization approaches will be replaced with genome sequencing in future epidemiologic studies.

IMMUNITY

The mechanisms responsible for generating protective immunity to rotavirus infections and illness following vaccination or natural infection are not completely understood, especially in humans where detailed examination of the acquired cellular immune response (B cells, T cells) in young children has been limited because of the difficulty in obtaining timely and sufficient specimens. The contribution of innate immunity to the control of rotavirus infection has only recently been examined, and these studies have been primarily in animal models. In general, a correlation has been found between the level of serum antibody in animals and people and protection from illness, but this correlation has not been absolute and it seems likely that serum antibody levels generally reflect other, less easily measured immune effector functions such as mucosal antibody titers or levels of virus-specific memory B cells in the gut.^{15,252,373} The one circumstance where serum antibody levels are likely to be directly related to protection is in newborn children where transplacentally acquired humoral immunity appears to offer some protection from illness early in life.⁶²¹

Because of the complexities inherent in studying the effector mechanisms responsible for the induction and persistence of protective immunity in young children, animal models have been particularly helpful. Animal models have been used to identify the respective roles of the humoral and cellular immune responses, in dissecting the relative importance of systemic and local immunity, in identifying the viral targets of protective immunity to rotavirus infection and/or disease, and in identifying the role of innate immunity.^{185,252,254,681,771,772} There are, however, several important considerations to keep in mind when evaluating experiments on rotavirus immunity in animal models. Many common laboratory and domestic animals undergo natural homologous rotavirus infection with strains that specifically infect that species and generally cause diarrheal disease in the young. In addition, many animal species can be experimentally infected with heterologous rotavirus strains (strains initially isolated from another animal species), and in many cases heterologous rotavirus infection, at high inoculation titer, can also cause diarrheal disease in the heterologous host. In general, heterologous strains do not spread efficiently in a heterologous host, do not cause disease at low inoculation titer, replicate less well than homologous strains in the gastrointestinal tract, and are usually attenuated compared to homologous rotavirus strains in the homologous host. Studies of the determinants of protective immunity in animal models have used both homologous and heterologous strains as challenges to examine the basis for inducing and measuring protective immunity. Unfortunately, it is not entirely clear whether information derived from such studies varies qualitatively or quantitatively or how much the response depends on whether the challenge was homologous or heterologous. Certainly in nature, virtually all disease in humans is caused by homologous

viral infection, at least in developed countries. In addition, most animal models of immunization and subsequent protection are short term (lasting weeks or a month or 2 months at the most) because of the difficulty of maintaining animals in a nonimmune and susceptible state over long periods of time (years), as is the case with rotavirus disease in humans where some children remain unexposed to rotavirus for at least 2 years. In addition, once a small animal (mouse or rabbit) is infected, it is highly or completely resistant to subsequent symptomatic disease and, in some cases, reinfection. Humans, on the other hand, remain susceptible to primary infection and disease over a long period of time (at least 3 years) and are susceptible to multiple reinfections and multiple bouts of rotavirus-associated disease in childhood and, to a lesser degree, in later life. Hence, one must approach animal model data on rotavirus immunity with some caution and realize that it may not be directly applicable to the human condition.

A variety of animal models (mice, rats, rabbits, pigs, calves, lambs, primates, and others) have been used to study rotavirus infection. The mouse and pig models have been the most extensively used, especially from the standpoint of evaluation of immunity, and they are the focus of this review.^{232,254,640,817}

The ability of serum antibody to rotavirus to provide protection from challenge has also been evaluated in animal studies.^{15,185,252,254,373,787}

In various studies, serum antibody was derived transplacentally, via systemic absorption of colostrum, via passive parenteral inoculation with immune serum or purified polyclonal or monoclonal immunoglobulins, or via systemic immunization with inactivated virus, virus-like-particles, or recombinant viral proteins with or without an adjuvant. For example, in an early study, it was observed that newborn calves frequently developed rotavirus diarrhea despite a moderately high level of circulating rotavirus antibodies derived from colostrum⁷⁹⁵; neither the challenge dose nor the relative titer of neutralizing activity in the absorbed colostrum antibodies were well quantified in this study. Passively acquired high levels of serum antibody have been associated with protection in a variety of animal studies, however,^{50,363,373} and if the level of systemic serum antibody is not sufficiently high, protection is limited.⁵⁵³

When examined *in toto*, these systemic antibody studies imply that if the level of circulating antibody is sufficiently high, enough will find its way into the gut lumen and mediate protection. The data are most compatible with the interpretation that it is the ability of systemic antibody to get into the gut lumen that is the critical effector mechanism of protection and not its isotype or its presence in the serum. These studies have generally not taken into account the fact that the efficiency of transfer of serum antibody into the gut (either active or passive) varies from species to species and also with the age of the animal at the time of antibody transfer. Recent studies have clearly demonstrated that in several animal species, including humans, there is an active transport mechanism to move systemic immunoglobulin G (IgG) into the intestinal lumen.³⁰ Of note, the relative sparing of rotavirus infection in the first 2 months of life in children and the correlation of protection with maternal antibody titers at birth provide indirect support for the notion that serum antibody can, under some circumstances, directly mediate local effects in humans as well as animals.⁶²¹

A number of studies in mice have delineated the immune effectors that mediate protection from reinfection and clearance of primary infection. These studies have been facilitated

by the availability of a large number of immunologic reagents to characterize the mouse immune response, by the availability of many useful knockout mouse models, and by the fact that mice have their own homologous rotavirus that causes natural diarrheal disease in suckling mice. Mice are also susceptible, in varying degrees, to a number of heterologous rotaviruses. B cells, and hence antibodies, are critical for the maintenance of long-term, high-level protection from reinfection in mice, and CD8+ T cells mediate short-term reduced susceptibility to reinfection.^{255,256} A protective B-cell response can occur in the absence of T-cell help, although it is substantially reduced compared to the response seen in wild-type mice,²⁵³ and T cells can mediate their antirotavirus effects in the absence of perforin, Fas, and interferon- γ .²⁵⁶ Both B and T cells can clear primary infection, but T cells appear to do this more quickly and, in some studies, more efficiently than B cells. CD8+ T cells can mediate almost complete protection (up to 2 weeks after primary infection) or partial protection (up to 3 months or less after primary infection) from reinfection; however, this protection diminishes greatly 8 months after primary infection.^{187,231,494,496} An important role for CD8+ T cells in the control of primary rotavirus infection was also demonstrated in a homologous gnotobiotic calf model.⁵⁵⁵

Studies using wild-type and various knockout mice have highlighted the importance of intestinal tract homing of both B and T cells, mediated by the $\alpha_4\beta_7$ integrin and CCR9 and CCR10, in promoting rotavirus immunity in the mouse model.^{233,426,427,641,813} Additional studies using IgA-deficient mice showed that in the absence of IgA, compensatory IgG protective immunity is generated,⁵⁴⁵ and that the IgG immunity is also dependent on the ability of B cells to traffic to the gut.⁴²⁷ Consistent with the animal findings, people with selective IgA deficiency resolve rotavirus infection normally and appear to develop compensatory increases in antirotavirus IgG during infection.³⁶⁴ Dietary factors have also been investigated for their influence on the immune response. For example, vitamin A deficiency impairs both serum antibody and cell-mediated immune responses to rotavirus in infant mice.⁹

Several experimental approaches have been used to identify the viral targets of protective humoral immunity. The most definitive of these have been carried out *in vitro* or in animal models. *In vitro* neutralization studies using monospecific hyperimmune or monoclonal antibodies generally have found that VP4 and VP7 are the only two targets of neutralizing antibody, although some data indicate that some antibodies to VP6 can also mediate *in vitro* and *in vivo* neutralization.^{15,91,185,263,305,776}

As discussed elsewhere in this chapter, both VP4 and VP7 come in a variety of antigenic specificities (called P and G serotypes, respectively) that are capable of segregating independently by gene reassortment. Many monoclonal and polyclonal antibodies directed at these two surface proteins preferentially react in a serotype-specific manner. On the other hand, both VP4 and VP7 contain heterotypic epitopes, and antibodies to either protein can have heterotypic specificity as well.⁴⁸¹

The rules governing the induction of homotypic versus heterotypic immunity following natural infection or vaccination are poorly understood for rotavirus as they are for most viral infections. This issue has been difficult to evaluate carefully in animal models because of the relatively short window of susceptibility to infection in the experimental setting, and both homotypic and heterotypic protection have been

shown in animal models.^{81,551,798,817} Recent studies in humans make clear that exposure to infection with a very limited number of VP4 and VP7 serotypes (either from natural infection or vaccination) provides substantial protective immunity to most heterotypic strains. What is still not clear is the basis for this heterotypic immunity.

Two other proteins have been implicated as targets of protective immunity following infection. Although rare, selected antibodies to VP6 appear to be capable of mediating classic viral neutralization.²⁶³ In addition, in a mouse model, selected IgA anti-VP6 monoclonal antibodies were protective despite the fact that they did not have traditional *in vitro* neutralization activity. These antibodies appeared to function intracellularly during transcytosis through gut epithelial cells.^{91,150,235,669} In other studies in the mouse model, CD4+ T cells directed at VP6 were able to mediate protection, and in mice and rabbits, but not pigs, systemic immunization with double-layered (VP2 and VP6) particles or VLPs induced varying levels of resistance to challenge.⁷⁷⁸ Whether VP6 immunity plays any role in the heterotypic nature of immunity seen in people is currently unknown. Finally, the NSP4 virally encoded enterotoxin is a potential target of protective immunity, and studies in mice have demonstrated protection from disease following administration of antibody to this protein. Again, the relevance of this data to people is not known.³⁴⁶

A variety of epidemiologic and serologic studies have provided compelling demonstrations of the development of acquired immunity to rotavirus infection and disease in people over time as well as the recurrent nature of rotavirus infection over a lifetime (Fig. 45.15B). These studies demonstrated that asymptomatic neonatal infection was associated with a subsequent reduction in severe rotavirus disease, but not with lack of reinfection. A large 2-year prospective study in Mexico showed that one or two natural rotavirus infections were highly effective at preventing further severe infections.^{57,747} This protection was both homo- and heterotypic in nature, although

the homotypic response appeared stronger following the first rotavirus exposure than after subsequent infections. These epidemiologic studies did not, however, identify the immunologic determinants of protective immunity.

Some experimental information regarding correlates of rotavirus immunity in people was obtained in an adult volunteer study in which a human rotavirus VP7 (G) serotype 1 (D strain) was orally administered to 18 individuals.^{401,402} Of the 18 volunteers, 5 shed rotavirus and 4 of those 5 developed diarrheal illness. The pre-existing level of serum neutralizing antibodies to the homotypic challenge virus or to a heterotypic VP7 (G) serotype 2 human rotavirus correlated with resistance to illness. The correlation of levels of neutralizing activity in intestinal fluid to resistance was not significant, however. Each of the five ill volunteers developed a serum neutralizing antibody response to the challenge strain and to the heterotypic human rotavirus DS-1 strain (serotype G2) as well as to a totally heterotypic bovine rotavirus NCDV strain (serotype G6). The prechallenge sera from this study were later re-examined by a competitive epitope-blocking immunoassay for antibody levels against several well-defined VP7 and VP4 neutralization epitopes.^{300,680} These studies correlated both homo- and heterotypic blocking activity to VP7 with resistance to disease. Confirmation of the importance of antibodies to protection in people was provided during a later series of challenge and rechallenge studies in which serum rotavirus IgG or jejunal neutralizing antibodies were observed to correlate with protection against infection and illness.^{401,402} In a third series of studies, however, serum and jejunal rotavirus antibodies did not correlate with resistance.^{774,775,777}

In other studies, salivary IgA antibodies, as measured by ELISA, were proposed as a surrogate for the level of intestinal rotavirus antibodies.⁷⁷⁹ Rotavirus serum IgA levels were also shown to be correlated with resistance to severe rotavirus illness.³³⁸ During a study of infants 1 to 24 months of age and young children residing in an orphanage, a significant

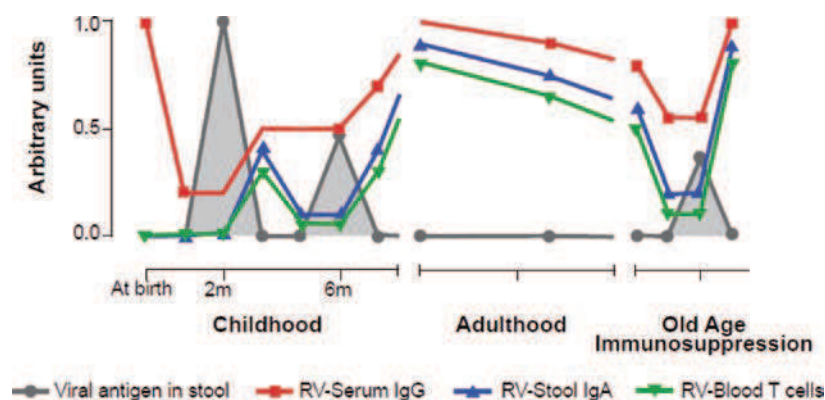


FIGURE 45.15. Relative quantities of rotavirus (RV)-specific antibodies and T cells during the natural history of RV infections. In people, rotavirus infections generally occur multiple times over a life span with decreasing frequency as exposures increase. Late in life when exposures are often reduced, increased susceptibility can occur. Protection from severe disease can be mediated by passively acquired immunoglobulin G (IgG) antibody at birth or from actively acquired IgA or IgG antibody following subsequent infection or vaccination. Protection from subsequent infections or mild disease (indicated by diminished viral shedding and immune boosting) is relative and likely depends on a number of factors but primarily the level of local and systemic antibodies. CD8+ T cells play a role in the timely resolution of rotavirus infection in animal models, but their role in humans is less well documented. Other critical immune factors like intestinal B and T cells have not been fully studied. (Lines represent a typical set of responses but inter-individual variations are frequent.)

correlation was observed between homotypic serum neutralizing antibodies and resistance to disease.¹²⁴ The association between fecal (IgA) and serum (IgG) antirotavirus antibody titers and protection against infection and illness was also investigated in 100 children younger than 18 months of age attending daycare centers in the United States for one or two rotavirus seasons.^{480,549} During the two seasons, seven outbreaks of G1 serotype and one of G3 serotype were detected. These studies concluded that (a) a rotavirus-specific fecal IgA titer of at least 1:80 correlated with protection against infection and a titer of at least 1:20 correlated with protection against illness; (b) a pre-existing serum antirotavirus IgA titer of greater than 1:200 or an IgG titer of greater than 1:800 was associated with protection against infection; and (c) a high level of pre-existing G type-specific blocking antibody was associated with protection against infection. Similar results were obtained from a study conducted in Australia: a direct correlation was observed between high levels of antirotavirus intestinal IgA antibody and protection against natural rotavirus infection and illness.¹⁵¹ In a heterologous gnotobiotic piglet model, a direct association was observed between the degree of protection and the level of intestinal IgA antibody-secreting cell response as well as serum IgA, intestinal IgA, and intestinal IgG titers.^{727,816} In mice, antirotavirus intestinal or serum IgA correlates with protection against homologous or heterologous rotavirus infection.^{231,495}

The development of homotypic versus heterotypic immunity in people remains a complicated and incompletely understood phenomenon. In recent years the highly successful implementation of the monovalent (G1P8) attenuated human rotavirus Rotarix vaccine (RV1) has provided definitive proof that immunization of young children with a single strain of rotavirus can provide substantial protective immunity from infection with multiple other strains and serotypes (see Prevention and Control section later). The lack of correlation between the rather low levels of serotype-specific neutralization responses following vaccination with either RV1 or the bovine reassortant pentavalent RotaTaq vaccine (RV5) and the high levels of protective efficacy induced by these vaccines imply that immunity is not exclusively homotypic and may not be directly linked to neutralizing antibody, at least in the serum.

Large numbers of animal studies have been carried out that either support or refute the hypothesis that rotavirus immunity is fundamentally homotypic in nature. There may be multiple reasons for these contradictory findings including species-specific differences in responses to infection, differences in measures of protection that are dependent on the challenge dose and strain, and differences in measurements of protection (i.e., lack of infection, lack of disease, and reduced disease severity are all possible end points). In any case, from a practical standpoint, it seems clear that some level of heterotypic immunity is frequently induced following primary rotavirus infection in people. Although serotype-specific neutralizing antibodies have been demonstrated in breast milk, the effect of breastfeeding on the occurrence of rotavirus gastroenteritis is controversial, but the weight of the evidence would indicate a real but relatively modest effect that is greatest early in infancy.^{139,283,517,539,599}

It is becoming clear that the innate immune system is also deeply involved in the early events of rotavirus immunity and may help shape the outcome of the B-cell-mediated antibody response to infection (Fig. 45.16).¹⁷⁵ Recent interest in the innate immune system and rotavirus immunity stems from

several important observations. First, one of the rotavirus proteins, NSP1, functions as an inhibitor of interferon production via the degradation of IRF3, 5, and 7 as well as β -transducin repeat containing protein (β -TrCP). Second, this degradation appears to function in a host and cell type-restricted manner. Third, inhibition of interferon signaling in murine models is associated with several enhanced and unusual disease phenotypes. Finally, plasmacytoid dendritic cell (pDC)-secreted interferon in response to rotavirus infection likely enhances the host B-cell response. The bulk of our recent knowledge regarding innate immunity has been derived from *in vitro* and animal studies. A type I interferon response can be detected during active viral replication both *in vivo* and *in vitro*.^{286,429,769} The early host innate immune response to rotavirus infection is dependent on transcription-mediated interferon regulatory factors (IRFs). Rotavirus activation of the innate immune response appears to vary depending on the cell type and viral strain. In human pDCs, interferon production is generally triggered by intact triple-layered particles independently of virus replication and is dependent on the particles containing dsRNA. In the minority of pDCs that are permissive for rotavirus replication, interferon production is down-regulated.¹⁷⁴ In other studies of myeloid dendritic cells (mDCs) from IRF3^{-/-} mice, type I IFN induction is reduced, indicating that IRF3 mediates the response in mDCs. Exposure of mDCs to UV-treated rotavirus induces significantly higher type I IFN levels, however, suggesting that rotavirus-encoded functions also antagonize the interferon response in mDCs, as is the case in fibroblasts and epithelial cells but not in pDCs.¹⁹⁵ There are little data on the innate response to rotavirus in other animal species, but in calves acute rotavirus infection is also associated with down-regulation of interferon-associated pathways.¹⁰

The rotavirus NSP1 protein is responsible for the proteosomal degradation of IRF3, 5, and/or 7 depending on the strain, and IRF degradation is associated with the ability of rotaviruses to suppress the host antiviral interferon response.^{38,39,293,294,404} NSP1 has been proposed to function as an E3 ligase in this regard, although definitive support for this hypothesis is lacking. The role of IRF3 is essential in mediating the interferon responses to rotavirus infection *in vitro* as shown using knock-out murine fibroblasts.⁶⁷¹ NSP1 proteins from selected rotavirus strains can also mediate the degradation of β -TrCP and thereby block activation of NF- κ B and interferon induction; however, a direct demonstration of the role of NF- κ B in mediating interferon responses during rotavirus infection using gene knockouts is lacking.²⁹² Recent studies revealed that the early induction of IRF- and NF- κ B-mediated interferon responses occurs primarily via the cytosolic adaptor MAVS and involves the viral pattern recognition receptors RIG-I and MDA-5 as well as the dsRNA dependent kinase PKR.⁶⁷¹ Additional studies also implicate membrane-associated RIG-I, MDA-5, and MAVS, but not PKR, in rotavirus-mediated innate immunity. Further studies are needed to better understand the cell- and strain-specific roles of these factors and the bases for these differences.⁸³ Rotaviruses may also inhibit STAT1 and 2 nuclear translocation and thereby inhibit the establishment of a complete antiviral state downstream of interferon induction, although the viral factors involved in this function are not yet known.³³⁹

Many of these innate immunity effects appear to be strain specific, and some are clearly cell-type specific as well.²³⁶ For example, in mouse embryonic fibroblasts (MEFs), heterologous

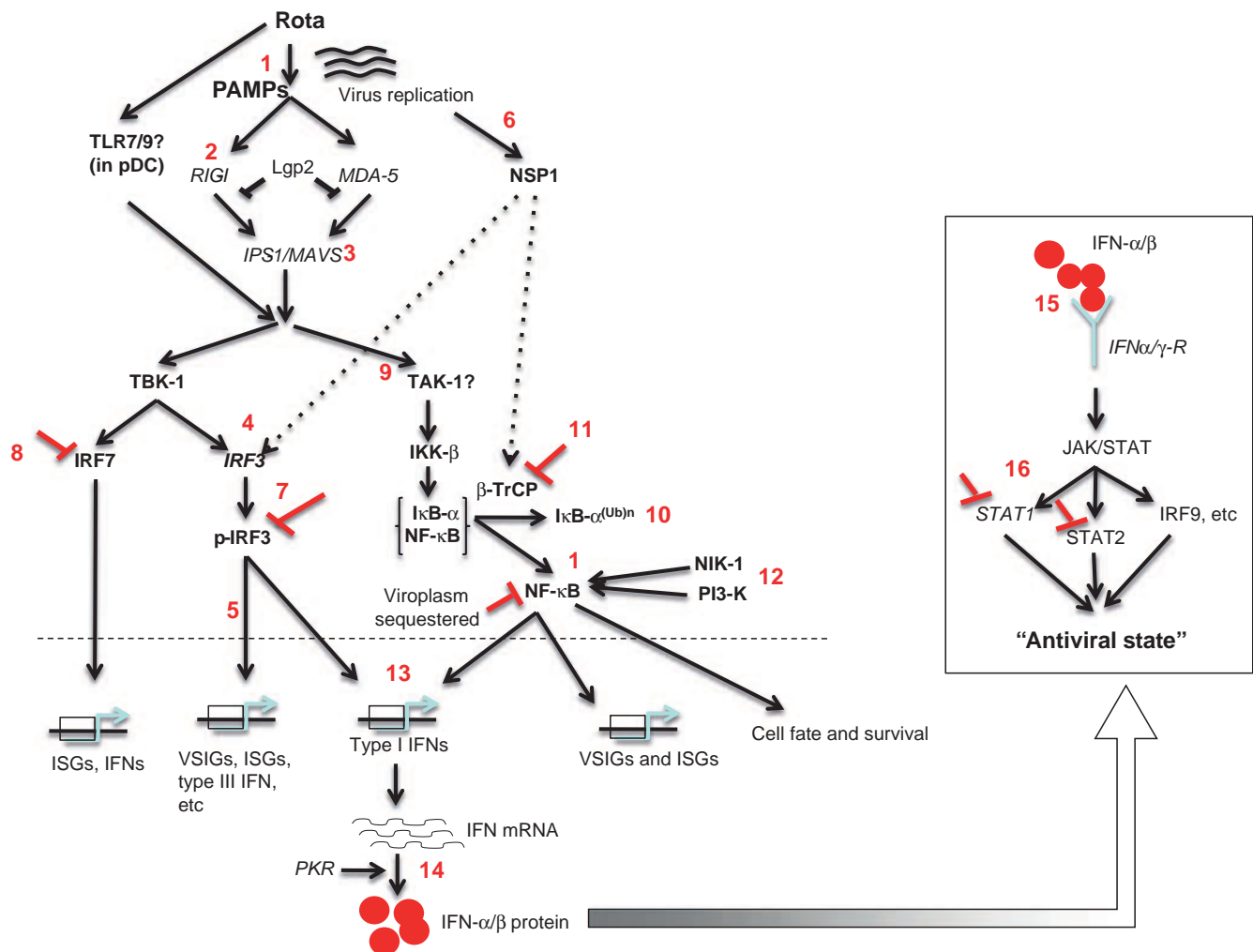


FIGURE 45.16. Rotavirus interactions with the host innate immune system. The numbers refer to interactions discussed below and red lines indicate points of viral interference with indicated pathways; the role of components in *italics* has been validated using genetic knockouts. Viral entry into cells is followed by the generation of pathogen-associated molecular pathways (PAMPs) (1) that are likely by-products of early viral replication. These viral ligands activate the cytosolic pathogen recognition receptors (PRRs) RIG-I and MDA-5 (2), leading to mitochondrial-associated adaptor protein IPS-1/MAVS-dependent activation (3) of the transcription factor IRF3 by the kinase TBK-1 (4). Activated IRF3 translocates to the nucleus where it induces the transcription of ISGs including ISG15, ISG54, ISG56, and so forth early in the replication cycle (5). Viral replication results in the expression of NSP1 (6). NSP1 interacts with and induces proteasomal degradation of IRF3. NSP1 degradation of IRF3 occurs following phosphorylation of the IRF3 carboxyl-terminus in a TBK-1-dependent manner (7) and is IRF3 host origin and cell type dependent. Other IRFs, including IRF5 and IRF7, can also be proteasomally degraded by NSP1 (8), influencing the quality and magnitude of the interferon response. Apart from IRF3, early induction of IFN in RV-infected cells also requires nuclear factor- κ B (NF- κ B), which is transcriptionally activated by a distinct signaling pathway (9) following the proteasomal degradation of its inhibitory partner, I κ B- α (10). Rotavirus strains can also block this part of the induction pathway by NSP1-mediated proteasomal degradation of β -TrCP, an essential co-factor for I κ B- α degradation (11), and for some viral strains this is the primary mechanism used to block early IFN secretion. Under certain conditions, activation of NF- κ B as well as its relocalization into viroplasm in infected cells has been reported (12), although the relevance to early IFN induction is not clear. Following IRF3- and NF- κ B-dependent transcription of IFNs (13), the double-stranded RNA (dsRNA)-dependent protein kinase PKR mediates IFN secretion by an unknown mechanism (14). In pDCs, RV triggers IFN secretion by a process that is likely a result of viral genomic dsRNA-mediated TLR7/9 signaling (steps 1 and 2). The result of IFN secretion from RV-infected cells is the establishment of an antiviral state in bystander cells mediated by signaling through the transcription factors STAT1, STAT2, and IRF9 (15). A second viral strategy exists to counter this phase of the IFN response by sequestration of STAT1 and STAT2 (16), although the viral factors involved are not known. Other interferons including type II and III IFNs may further restrict virus replication and dissemination in the host and may exert different effects depending on the tissue, strain, and stage of pathogenesis.

bovine and porcine rotaviruses fail to effectively degrade cellular IRF3, resulting in IRF3 activation and IFN- β secretion. As a consequence, replication of these viruses is severely restricted in IFN-competent, but not in IFN-deficient, MEFs. On the other hand, homologous murine rotaviruses and a heterologous simian RRV rotavirus efficiently degrade cellular IRF3, diminish IRF3 activation and IFN- β secretion, and are not replication restricted in wild-type MEFs. Mice deficient in interferon signaling (either lacking the interferon type I and II receptors or STAT1) are more susceptible to rotavirus replication and, in some cases, enhanced disease.^{234,743} In a mouse model, lethal biliary disease and enhanced rotavirus systemic replication were seen in interferon signaling-deficient mice infected with the simian RRV strain, and this strain-specific virulence was mapped to rotavirus genes 4 and 5 encoding VP4 and NSP1, respectively.²³⁷ In other studies using a distinct biliary tract disease model dependent on RRV systemic inoculation into newborn mice, analysis of viral reassortants linked only VP4 with the systemic disease phenotype.^{768,769}

The precise mechanism by which the innate immune response restricts rotavirus infection in some species and in some organs remains to be elucidated, but preliminary studies indicate that interferon per se may be more active in suppressing systemic than mucosal replication. Despite the fact that rotaviruses have evolved a strategy to inhibit the interferon response, interferon is actively produced during rotavirus infection *in vivo* in humans, pigs, and other animals.^{286,429,769} Although interferon is clearly present during rotavirus infection in several species, its antiviral effects *in vivo* are not entirely clear. Diarrheal disease in mice appears to be refractory to exogenous interferon therapy, whereas calves treated with recombinant type I interferon appear to have attenuated gastrointestinal disease.^{16,670} Interferon receptor knockout suckling mice do not have enhanced

gastrointestinal disease when infected with a murine rotavirus, but adult STAT1 knockout mice have enhanced replication compared to wild-type mice.²³⁴

Recent studies indicate that primary human pDCs are highly resistant to rotavirus infection but they respond to rotavirus exposure with a brisk and substantial interferon response that is likely initiated by the dsRNA rotavirus genome. This pDC-mediated response might play an important role beyond direct antiviral effects by enhancing the mucosal B-cell response to infection by stimulating B-cell proliferation and enhancing resolution of infection.^{174,175}

There has been less extensive study of primary T- or B-cell responsiveness to rotavirus infection in people than in animal models, and the functional role of these cells or their correlation to protection has been difficult to evaluate in people. Interferon- γ -secreting circulating RV-specific CD4+ and CD8+ T cells are present in very low amounts in children and relatively low amounts in adults, and these cells express the gut homing receptor $\alpha_4\beta_7$.^{366,637} Similarly, primary circulating human B cells have been examined in a number of studies using flow cytometry and ELISpot-based assays.^{367,638,724} These studies show that rotavirus-specific B cells also express intestine-specific homing receptors, and that both naïve and memory rotavirus-specific B cells directed at VP6 are enriched for a CD27+, IgD+, IgM+ subset of cells. The biological role of RV-specific circulating IgM memory B cells is not well understood.

CLINICAL FEATURES

Rotavirus infections produce a spectrum of responses that varies from subclinical infection to mild diarrhea to severe and potentially fatal dehydrating illness (Table 45.6). In one study,

TABLE 45.6 Clinical and Epidemiological Features of Rotavirus Infections

Variable	Description
Age predisposition	Primarily affects young children 3 months to 2 years old in developed countries; younger children affected in developing countries. Adults frequently infected, mostly asymptomatic
Seasonality	Seasonal infection in developed countries with epidemic peaks in cooler months of each year. No seasonality in tropical climates. Vaccination has diminished and delayed seasonal epidemic in United States
Settings	Households, daycare centers, hospitals, schools, favoring person-to-person spread. Occasionally water-borne, especially group B rotaviruses
Asymptomatic infections	Most common in adults and newborns. Can frequently occur in all age groups
Incubation period	Generally 24–48 hours
Symptoms	Sudden onset of vomiting and diarrhea. Diarrheal stools lack blood, mucus, or leukocytes. Fever common
Severity of illness	Overall, more severe than many other diarrheal etiologies, leading to dehydration and hospitalization. Malnutrition increases disease severity
Duration of illness	Typically 3–5 days. Longer illness in immunocompromised
Viral shedding	Peaks 1–3 days after illness onset. Shedding can be prolonged in immunocompromised individuals. Antigenemia and viremia detectable early after infection
Mode of transmission and vehicles	Fecal–oral; aerosol/vomit; contact with fomites Food, water, environmental contamination
Immunity	Repeated infections with or without illness can occur with the same or different strains. Increasing levels of immunity occur with repeated exposures or vaccination
Treatment	Supportive rehydration (oral preferable) therapy to prevent dehydration. Live, attenuated vaccines are available and highly effective in developed countries; vaccine effectiveness is lower in children in developing countries
Reservoir	Humans primarily and animal reservoirs rarely

a comparison was made between (a) the clinical manifestations of 78 patients hospitalized with rotavirus diarrhea and (b) 72 patients hospitalized with a diarrheal illness that was not associated with rotavirus.⁶³⁵ The majority of both rotavirus- and non-rotavirus-infected children had a temperature of 37.9°C or above. Those with rotavirus vomited and became dehydrated significantly more often than those who did not have rotavirus. The mean duration of vomiting was longer in the rotavirus-positive group than in those who did not have rotavirus (2.6 days versus 0.9 days). Rotavirus diarrhea started later than vomiting but lasted longer (mean duration, 5 days versus 2.6 days). After infants and children were hospitalized, diarrhea continued for a mean of 2.6 days (range, 1 to 9 days) in the rotavirus group and 3.8 days (range, 1 to 16 days) in the rotavirus-negative group. The duration of hospitalization ranged from 2 to 14 days, with a mean of 4 days, for the patients infected with rotavirus and for a mean of 6 days (range, 2 to 27 days) for the rotavirus-negative group.

Laboratory findings reflect the high frequency of vomiting and dehydration associated with rotaviral illness.⁶³⁵ Blood urea nitrogen values of greater than 18 mg/dL were observed in 58% of the rotavirus-positive group, and a urine-specific gravity of 1.025 or greater was noted in 71%. A review of liver function tests in children with rotavirus gastroenteritis found that 20% had elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels.⁷²¹ The pathophysiologic basis for these liver test elevations remains unknown. Over a 5-year period in a Toronto hospital in the early 1970s, rotavirus gastroenteritis was responsible for the death of 21 infants and young children between 4 and 30 months of age.⁹⁸ On arrival at the hospital, 10 of these children were dead, and 10 were moribund and could not be resuscitated. With the exception of two patients, each of the children who died had been healthy previously. Death occurred within 1 to 3 days of onset of symptoms. The major factor causing death was believed to be dehydration and electrolyte imbalance in 16 individuals and aspiration of vomitus in 3. In the remaining two patients, seizures were a contributing factor. Each of the 16 patients tested had a sodium level (serum or vitreous humor) in excess of 150 mEq/L. The rapidly fatal course of untreated severe rotavirus gastroenteritis was underscored by the fact that the parents of 16 of the 20 children who were brought to the hospital had some contact with a physician during the course of the illness. It is important to note that in the more than three decades since this study was published, the mortality rate associated with rotavirus infection in developing and in developed countries has declined substantially primarily due to more aggressive and early rehydration interventions.

Rotaviruses rarely produce a chronic symptomatic infection that involves diarrhea or systemic illness and includes hepatic and renal involvement in children who are severely immunodeficient.^{273,368,554,667,792} Recently, pentavalent vaccine-acquired chronic rotavirus infection and prolonged illness were observed in infants with severe combined immunodeficiency who were inadvertently vaccinated.^{29,569} During chronic infection of immunodeficient children, rotavirus can undergo genomic rearrangements, presumably because of multiple passages at high multiplicity *in vivo*.^{184,353}

Rotaviruses pose a special threat to individuals who are immunosuppressed for bone marrow, stem cell, or small bowel transplantation. In one study of gastroenteritis in a bone mar-

row transplant unit, 8 of 78 patients with gastroenteritis shed rotavirus as the sole pathogen, and 5 of these individuals died.⁸⁰⁹ Rotavirus infections acquired nosocomially have also been associated with severe diarrhea in adult renal transplant recipients.⁵⁸¹ Rotavirus may cause gastroenteritis in small bowel transplant recipients, often in association with acute episodes of rejection.^{5,833} Rotaviruses do not appear to play a disproportionately important role in diarrhea occurring in adults or children infected with human immunodeficiency virus (HIV), reinforcing the findings that rotavirus immunity is most directly influenced by B cells and antibody-related mechanisms.^{312,698}

A temporal association of rotavirus infection with a variety of other disease conditions, both as isolated illnesses or in a single outbreak, has been described.^{331,408,687,780} Because the occurrence of most of these conditions is extremely rare in comparison to the very high incidence of rotavirus infection, it appears that, with the exception of severe disease manifestations in immunocompromised patients, necrotizing enterocolitis and hemorrhagic gastroenteritis in neonates, and pneumato-sis intestinalis in infancy, the association of rotavirus infection with most of the reported conditions is likely temporal and not etiologic.^{97,643,801} Rotavirus infection has been associated with a number of central nervous system conditions, and the virus has been detected in the cerebrospinal fluid on a number of occasions.^{416,449,500,542} However, because viremia is now established to be relatively common, the finding of rotavirus RNA in cerebrospinal fluid does not necessarily imply direct central nervous system replication. It appears that acute rotavirus infection is associated relatively frequently with benign seizures in young children, perhaps in association with the elevated temperature seen in many rotavirus infections.⁵⁰⁰

An unexpected outcome of the effective RV vaccination program has been reports of intussusception after oral administration of the various live attenuated rotavirus vaccines^{92,301,525,568} (see Prevention and Control section); the significance of the temporal association of wild-type rotavirus infection with intussusception deserves further attention. Rotavirus was detected in 20 (33%) of 61 cases of intussusception in two studies, suggesting that wild-type rotavirus might have been the cause^{421,532}; however, three subsequent studies in Australia, France, and Germany failed to find a significant association of intussusception with rotavirus infection.^{521,541,696} Ultrasound examinations of infants with wild-type rotavirus diarrheal illnesses and controls indicate that wild-type rotavirus infections induce a significantly greater number of aggregates of lymph nodes and/or thickening of the ileal wall, both of which may be a prelude to intussusception.^{40,632} Finally, as referenced above and discussed further below, all three of the live attenuated rotavirus vaccines that have been licensed by the Food and Drug Administration, one of which is an attenuated human rotavirus, have been associated with intussusception at low frequencies. Hence, at this juncture, it seems reasonable to hypothesize that virtually any rotavirus infection in children will be etiologically associated with intussusception but that the relatively low rate of etiologic association that might vary from strain to strain or vaccine to vaccine is often so low that it is extremely difficult to identify except in the context of a large vaccination campaign and close monitoring. Recent studies have clearly demonstrated that rotavirus infection is not strictly restricted to the gut and that both viremia and systemic replication are a frequent consequence of natural infection with wild-type rotavirus in both humans and

other animals.^{63,230,618} It is also clear that this systemic phase of infection can be associated with clinical manifestations if the host is immunosuppressed. It is not clear currently whether this systemic phase of rotavirus infection is etiologically associated with any clinical syndromes, like intussusception, in the immunocompetent host.

DIAGNOSIS

The clinical and epidemiologic manifestations of rotavirus illnesses (severe diarrhea, vomiting, dehydration, fever in young children, and seasonality in nontropical areas) are not sufficiently distinctive to permit diagnosis even during a rotavirus “season.” The accurate diagnosis, therefore, requires detection of virus or viral antigen or a virus-specific serologic response. The epidemiologic pattern of rotavirus disease at any one time may strongly suggest this diagnosis, but laboratory confirmation is required to be definitive. Of note, because the primary treatment, rehydration, should be administered on clinical grounds alone and is not dependent on the specific identification of rotavirus infection, the clinical need for definitive etiologic diagnosis is not strong.

Since 1973, many assays have been developed for the detection of rotavirus in stools,⁸⁰⁸ although at present solid-phase immunoassays and RT-PCR-based assays are the primary diagnostic tools. Specimens from the first to the fourth day of illness are optimal for virus detection using traditional assays (e.g., EM or ELISA); however, shedding can continue for up to 3 weeks, depending on the duration of symptoms, and may be detected longer by RT-PCR.^{55,284} Viral shedding as detected by conventional techniques, such as ELISA, usually coincides with the duration of diarrhea, but diarrhea can continue for an additional 2 to 3 days and ELISA positivity can continue past the end of diarrhea.⁶³¹ Initially, direct visualization of stool material by EM was employed for rotavirus detection.^{58,75,398} EM has the advantage of high specificity because rotaviruses have a distinctive morphologic appearance, but there are substantial disadvantages of being relatively low throughput and requiring very expensive equipment. In early studies, direct EM examination of stools permitted detection of rotavirus in 80% to 90% of the virus-positive specimens when compared to immunoassay techniques.⁷⁵ Various methods with higher throughput than EM are now available for the detection of rotaviruses in stool specimens.²⁸⁴

The method of choice in many laboratories is ELISA, because it is sensitive, does not require specialized equipment, is available commercially in validated formats, is relatively inexpensive, and often has a built-in control for nonspecific reactions. Other methods for virus detection, such as counter-immunoelectrophoresis, gel electrophoresis of rotavirus RNA, reverse passive hemagglutination assay (RPHA), and latex agglutination, have been used in the past.⁸⁰⁸ A variety of generally sensitive and specific commercial kits for rotavirus detection are now available primarily using some form of ELISA solid-phase immunoassay format. Enzyme immunoassays have also been developed in research laboratories for detection of group B or group C rotavirus and for measurement of antibodies directed against these viruses.^{258,466}

A dot blot hybridization assay for detection of rotaviruses was developed based on *in situ* hybridization of labeled rotavi-

rus ssRNA transcripts to heat-denatured rotavirus RNA immobilized on a nitrocellulose membrane.²³⁸ The method is highly specific, yielding results concordant with those obtained with other tests such as EM, dsRNA detection, and ELISA.²⁰⁵ In a comparative study of ELISA and dot hybridization for the detection of rotavirus in various dilutions of fecal specimens, the dot hybridization method was 10 to 100 times more sensitive than the confirmatory ELISA but much less practical and has not been generally employed since RT-PCR-based assays became available.

Of the variety of research laboratory techniques that have been used for the detection of group A rotaviruses, the most important, after the solid-phase immunoassay, is RT-PCR, which is highly sensitive and specific and has the added advantage of being able to determine genotypes.²⁸⁴ For example, using an RT-PCR-based assay, virus shedding was detected from 4 to 57 days after diarrhea onset, and 30% of children rotavirus positive at early time points by ELISA shed virus detectable by the assay for 25 to 57 days.⁶²⁷ PCR has also been applied to the detection of group B and C rotaviruses.²⁹¹

It is also possible to recover human rotaviruses from stool specimens directly in cell culture with reasonable efficiency, but this approach is not practical except for specific experimental purposes. This method appears to be approximately 75% as efficient as antigen detection assays using conventional procedures.³²⁹ The efficiency of recovery of virus from rectal swabs using cell culture is considerably less. Growth of rotavirus in tissue culture allows the determination of its serotype by neutralization assay, and rotavirus serotype can now also be reliably predicted by nucleotide sequence analysis of the *VP7* and *VP4* genes. The use of RT-PCR has also enabled the genotyping of rotavirus-positive specimens that could not be typed by ELISA or cultivated directly.²⁴¹

A variety of techniques have been used in the past to measure a serologic response to rotavirus infection, such as IEM, complement fixation (CF) tests, immunofluorescence tests, immune adherence hemagglutination assay, ELISA, neutralization, hemagglutination inhibition, inhibition of reverse passive hemagglutination, enzyme-linked immunospot (ELISpot) assay, and immunocytochemical staining assays. Currently, the most useful assays are the IgA and IgG ELISAs and neutralization assays. The CF method is about as efficient as the other methods for detecting an antibody response in patients between 6 and 24 months of age, but it is not as efficient in adults or in infants younger than 6 months of age and is not generally used today. In infants and adults, IF and IgG, IgA, and IgM ELISAs are more efficient and sensitive. Because IgA does not cross the placenta, a rotavirus IgA ELISA has been the assay of choice in demonstrating serologic responses in infants younger than the age of 9 months who generally possess passively acquired maternal IgG antibodies.^{458,459} This assay can also be used for measuring antibodies in saliva, duodenal fluids, and stools⁴⁵⁹ and is the assay employed in most studies of seroresponse rates to rotavirus vaccines.¹⁸¹ Fecal rotavirus IgA antibody levels exhibit a direct relationship with duodenal IgA antibody levels³¹⁰ but are difficult to standardize. In addition, in one study a rise in fecal rotavirus IgA antibodies measured by ELISA correlated with an intestinal neutralizing antibody response.¹⁵⁴

It has been suggested that copro-IgA conversion in feces obtained weekly would be a more sensitive indicator of rotavirus reinfection than seroconversion or detection of virus

shedding,¹⁵² but difficulties in accurately and reproducibly monitoring fecal IgA levels have restricted the use of this assay. Based on observations from a clinical study in infants and children, rotavirus IgA in serum has been suggested to reflect the immunologic status of the intestinal lumen with respect to rotavirus antibody.^{172,338} Several studies have correlated levels of antirotavirus serum IgA with protection, although this association is not absolute.^{338,549,747} Early diagnosis can also be made by ELISA, which can detect a specific serum IgM response during the acute phase of illness.¹⁷²

Neutralizing antibodies can be detected by plaque reduction, neutralization of fluorescent foci, neutralization of virus as determined by quantitation of viral antigen by ELISA, or inhibition of cytopathic effect in roller-tube cultures.³⁹² Neutralization assays provide the serotype of infecting rotavirus and information about the development of a serotype-specific or heterotypic antibody response. A competition solid-phase immunoassay that measures epitope-specific immune responses to individual rotavirus epitopes associated with serotypes was shown to be useful in evaluating immune responses at the level of individual epitopes. This technique uses the test serum as the blocking reagent and the individual mAbs as the detecting reagent.⁶⁸⁰

Treatment

The primary aim of treatment of rotaviral gastroenteritis is to replace fluids and electrolytes lost by vomiting and diarrhea. Intravenous fluid administration has been used successfully for many years in treating dehydration from diarrhea. Because facilities and equipment for parenteral administration of fluids and electrolytes are not readily available in many parts of the world, intensive efforts have been made to evaluate the efficacy of oral fluid replacement therapy, which is mechanistically based on the specific coupling of sodium and glucose transport in the intestine.^{7,22,652,661} Various formulations of oral rehydration salts (ORSs) with added glucose or a glucose substitute are effective in the treatment of dehydration caused by rotavirus diarrhea, with some minor variations in their efficacy. For example, in a double-blind study, oral rehydration therapies with electrolyte solutions containing either glucose or sucrose were highly effective for treatment of rotavirus gastroenteritis.⁶⁵³

The standard WHO oral glucose electrolyte formula is composed of the following: 90 mM sodium, 80 mM chloride, 20 mM potassium, 10 mM citrate, and 111 mM glucose, resulting in an overall osmolality of 310 mOsm/L.^{1,22} Bicarbonate (30 mM) can be substituted for citrate. An ORS solution containing a reduced sodium concentration was found to be more effective for treatment of infants with noncholera diarrhea than the standard WHO solution in a multicenter trial.¹²⁸ The reduced-sodium solution contains 75 mM sodium, 65 mM chloride, 20 mM potassium, 10 mM citrate, and 75 mM glucose, resulting in an overall osmolality of 245 mOsm/L (reduced osmolality ORS solution). After correction of the initial calculated fluid loss by oral rehydration solution, either water or fluids without added electrolytes (e.g., breast milk or some other form of low-solute feeding) should also be administered orally in addition to the oral rehydration solution. When this regimen is used, both continued diarrheal fluid and electrolyte losses will be replaced and normal daily fluid requirements will be maintained.²²

Rice-based solutions have been compared with glucose-based oral rehydration solutions in infants and young children

hospitalized with mild to moderately dehydrating diarrheal illnesses associated with various pathogens, including rotavirus.⁵⁹⁸ Each was found to be effective, but the rice-based solution was more effective in decreasing total stool output and increasing absorption and retention of fluid and electrolytes. Both glucose-based and rice-based oral rehydration solutions were effective in treating mild to moderate dehydration caused by various pathogens, including rotavirus, in another study.⁵¹³ Recent studies from a number of investigators have demonstrated that zinc supplementation improves the therapeutic value of ORS, and this addition is now recommended by the WHO for children with acute diarrhea.²² Several other additives to the ORS formulation are currently under investigation; these include lactoferrin and lysozyme and various amino acids including glycine, alanine, and glutamine. When/if oral rehydration does not correct the fluid and electrolyte loss, or if the patient is severely dehydrated or in shock, intravenous fluids must be given immediately. Oral rehydration therapy should not be given to patients with depressed consciousness because of the possibility of fluid aspiration.

The American Academy of Pediatrics (AAP) does not currently recommend the use of loperamide, anticholinergic agents, bismuth subsalicylate, adsorbents, or lactobacillus-containing compounds for the management of the acute diarrhea in children 1 month to 5 years of age. The use of opiates, as well as opiate and atropine combination drugs, for treatment of children with acute diarrhea is also contraindicated by the AAP.¹

Human milk, containing rotavirus antibodies, has been used successfully for treatment of children who are immunodeficient and who have chronic rotavirus infection and illness.⁶⁶⁷ In contrast, colostrum or milk concentrate from cows immunized with human rotavirus was not effective for the treatment of acute rotavirus gastroenteritis in children, although a decrease in the duration of virus shedding was observed.²⁰² Daily oral administration of rotavirus antibody-containing bovine colostrum, however, appeared to exert a preventative effect during an orphanage outbreak of rotavirus diarrhea.²⁰² The effect of a single oral dose (300 mg/kg body weight) of gamma globulin in infants hospitalized for gastroenteritis (70% rotavirus associated) was evaluated in a double-blind, placebo-controlled trial. The treatment group had a significantly shorter duration of diarrhea, viral excretion, and hospital stay.³¹⁴ In another study, the oral administration of human serum immunoglobulin to two infants with prolonged rotavirus diarrhea of 4 or 7 months' duration was effective in clearing the virus and the associated diarrhea.³¹⁶ Also, bovine colostrum prepared by immunizing pregnant cows with human rotavirus strains of G1, G2, G3, or G4 specificity was given orally to patients 4 to 6 months of age with rotavirus diarrhea and was found to significantly reduce the duration and frequency of diarrhea as well as the need for oral rehydration solution.^{326,666} A randomized double-blind study in rotavirus-infected young children using antirotavirus immunoglobulin produced in eggs showed a modest improvement of diarrhea that was associated with earlier clearance of rotavirus from stools.⁶⁶⁵ Despite the generally positive results observed with immunotherapy for rotavirus disease, the cost considerations versus vaccination make this approach impractical except perhaps in the case of chronic rotavirus infection in the severely immunocompromised.

Recently, in murine rotavirus model studies, lactobacillus-expressed, llama-derived immunoglobulin domains, which

would be much easier to produce than immune bovine colostrum, were shown to have modest therapeutic as well as preventative effects on acute infection.⁵⁵⁹ It is noteworthy that despite the fact that (a) the first studies on oral immunotherapy for rotavirus diarrhea were done over 25 years ago and (b) a variety of studies have shown at least some degree of efficacy, this therapeutic approach has remained experimental and has never gained broad acceptance. The reasons for this are multifactorial, but most likely the primary reason is the lack of a commercial product, probably due to unacceptable cost and production issues.

Bismuth subsalicylate (BSS) given orally five times a day at 20 mg/kg for 5 days as an adjunct to rehydration therapy shortened the course of disease in children 4 to 28 months of age hospitalized with acute rotavirus diarrhea in a prospective double-blind, placebo-controlled treatment trial.⁶⁹¹ Because of the association between the use of salicylates and Reye syndrome, the possibility of an association with BSS or other nonacetylsalicylic acid salicylates and the disease was reviewed and none was found. However, the AAP does not recommend the use of bismuth subsalicylate for the management of acute diarrhea in the 1 month to 5 year age group.¹

The efficacy of several broad-spectrum antiviral agents has been examined as inhibitors of rotavirus replication *in vitro*. In one survey, various adenosine analogs were found to have antirotavirus activity, and it was suggested that this activity resulted from inhibition of S-adenosylhomocysteine hydrolase, an enzyme involved in regulating methylation required for production of functional viral mRNA.⁴¹⁵ Double-blind, placebo-controlled studies of racecadotril, an enkephalinase inhibitor (not available in the United States but available in a variety of European and South American countries) found the compound was effective in the treatment of hospitalized pediatric patients with watery diarrhea (including rotavirus diarrhea) when used in conjunction with oral rehydration therapy.⁶⁵⁶ Orally administered nitazoxanide (a broad-spectrum anti-infective drug) reduced the duration of severe rotavirus diarrhea in two studies of hospitalized pediatric patients.^{642,722} Several recent studies have supported a beneficial role for probiotic therapy for rotavirus diarrhea, and one direct comparison of probiotic with nitazoxanide showed that both had modest therapeutic effects, with nitazoxanide being somewhat more effective.^{148,228,299,722} Each of these preliminary therapeutic studies needs confirmation in a variety of clinical settings as well as distinct subject population groups. Importantly, the recent advent and widespread use of several safe and effective rotavirus vaccines has substantially diminished the need for an effective rotavirus therapy except in specialized circumstances such as severely immunocompromised infants or transplant patients.

PREVENTION AND CONTROL

Vaccines

General Principles and Background

Epidemiologic and hospital-based studies worldwide over the past 3 decades clearly demonstrate the substantial burden of disease associated with rotavirus infection and strongly support the need for prevention in both developed and less developed countries.^{275,469,692} The primary aim of a rotavirus vaccine should be to prevent severe rotavirus gastroenteritis during the

first 2 to 3 years of life, the period when rotavirus disease is most serious and takes its greatest toll. This is a realistic goal because, while natural infection does not provide complete protection against a subsequent reinfection and disease, in general disease severity is substantially reduced during secondary and subsequent infections.^{57,747} Considerable evidence from studies in animals indicates that the presence and quantity of rotavirus-specific antibody in the lumen of the gut plays a critical role in resistance to rotavirus disease.²⁵² Other studies from children have associated elevated levels of fecal antirotavirus IgA with resistance to illness.¹⁵¹ These observations suggest that the effectiveness of a rotavirus vaccine largely depends on its ability to stimulate transport of antibodies into the gut lumen or to stimulate local production of antibodies. Currently, perhaps the most reliable and well-documented method of stimulating local intestinal immunity is thought to be infection or immunization in the intestine. For this reason, most efforts to date have focused on live attenuated vaccines that are administered orally.

Longitudinal studies have shed light on the resistance to disease induced by prior, naturally acquired rotavirus infection. Reinfection within the first few years of life is common, and an infant who has had a primarily rotavirus infection, whether severe, mild, or asymptomatic, usually experiences a milder illness during reinfection. The level of protection induced by primary and subsequent reinfections may be less in some poor countries than in the developed world.^{47,57,275,749,750} In one epidemiologic study, neonates who were infected in a newborn nursery during the first 14 days of life experienced almost 50% fewer rotavirus diarrheal episodes during the next 3 years compared to a cohort of infants who had not been infected with rotavirus in the nursery.⁵⁷ In a large cohort study in Mexico in which 200 infants were followed from birth to 24 months of age, 96%, 69%, 42%, 22%, and 13% of them experienced one, two, three, four, or five rotavirus infections, respectively. Significantly, no child developed a moderate to severe diarrheal illness after the second infection, indicating that wild-type infection effectively “vaccinated” these young children against severe subsequent disease. Protection was greatest against severe disease and considerably less against mild disease or simple reinfection.^{746,747} In general, second infections were caused by a different G serotype ($P = 0.054$), but any primary infection appeared to offer substantial protective efficacy against severe disease on reinfection, irrespective of serotype. Recently, a similar prospective study from India demonstrated a substantially smaller protective effect from primary natural infection on subsequent reinfection, mirroring what has been observed with rotavirus vaccination in parts of Asia and Africa.²⁷⁴ In this study primary rotavirus infection occurred early in life, the levels of reinfection were high, and protection against moderate or severe disease increased with the order of infection but was only 79% after three infections. Unexpectedly, there was no evidence of homotypic protection.

Because wild-type infection has been shown to be a moderate to relatively effective inducer of immunity against subsequent severe diarrheal illness, the key issue that needed to be resolved in designing a live viral vaccine was an effective means of attenuating virulence while retaining immunogenicity. Rotavirus infection primarily involves the mature villus tip epithelium of the small intestine. Experience with several other viruses that cause mucosal infections (e.g., influenza A

virus and respiratory syncytial virus) indicated that attenuation is frequently a reflection of decreased viral replication in the target organ, which is also the site at which infection is initiated. However, decreased replication is frequently associated with decreased immunogenicity. Hence a delicate balance must be achieved between satisfactory attenuation and satisfactory immunogenicity in the design of live viral vaccines, especially those that immunize at the primary site of natural infection.

Epidemiologic studies worldwide have revealed that a very wide range of serotypically distinct human rotaviruses are currently in circulation but the great majority of infections (over 80%) are caused by viruses with just five G serotypes (1, 2, 3, 4, and 9) and two or three P serotypes.^{267,660,797} As discussed earlier, the relationship between this serotype diversity and protective immunity has been an area of intense investigation almost since the discovery of human rotaviruses. Obviously, the development of an effective vaccine is dependent on its ability to provide significant protection from many, if not all, rotavirus serotypes that infants are likely to encounter. Epidemiologic studies in children clearly provided evidence to support the development of both homotypic and heterotypic immunity following natural infection with the biggest point of contention being the breadth and duration of the heterotypic response.^{15,47,124,185,252,747,771} Unfortunately, experimental studies in animals, which have demonstrated both homotypic and heterotypic immunity depending on the experimental design, viral strain, immunogen examined, and species under study, have not provided a definitive answer vis à vis the design of an optimal human vaccine.²⁵² In gnotobiotic calves and piglets, heterotypic immunity is seen between an animal rotavirus (bovine, serotype G6) and a human rotavirus (serotype G1).^{798,834} Calves infected *in utero* with bovine rotavirus resist challenge with a heterotypic and heterologous human rotavirus at birth. In other studies in piglets and calves, however, only homotypic immunity was demonstrated, although in certain instances the severity of diarrhea was reduced following heterotypic challenge.^{265,523,794,796} Of note, many of these early cross-protection studies did not carefully and quantitatively evaluate the ability of heterotypic immunization to diminish disease severity; instead, success was defined as total elimination of disease following challenge. Given that the goal for human vaccines is the reduction of disease severity, this represents an important design defect in some of the investigations and restricts the utility of these studies to inform the human vaccine design approach.

The early development of live attenuated rotavirus vaccines was deeply influenced by considerations as to whether an effective vaccine would need to contain multiple serotypes or whether a monotypic vaccine could generate sufficient heterotypic immunity to be effective. As the subsequent discussion will indicate, after multiple studies with multiple vaccine candidates, we now have field efficacy evidence from around the world that both monotypic and multitypic live attenuated rotavirus vaccines can be successfully used in people and that both approaches lead to successful vaccines that substantially reduce rotavirus morbidity and mortality.^{181,186,281,309,375}

Initial Monovalent Animal Rotavirus ("Jennerian") Vaccine Candidates

Current production of licensed rotavirus vaccines is based on strategies that ranged from cell culture cultivation of strains

obtained from either humans or animals to the application of genetic techniques to produce reassortants between human and animal rotaviruses. The initial evaluated approach was based on the method first reported by Edward Jenner in 1798 for vaccination of humans against smallpox (see chapter on Smallpox). Jenner used a live, antigenically related animal virus, cowpox, to immunize humans against variola virus, the etiologic agent of smallpox. This strategy is based on the well-known tendency of many microbial pathogens to be host-range restricted. As such, viral strains isolated from one species frequently replicate inefficiently in other species; however, in some cases, this restricted replication capacity is sufficient to induce protective immunity. The usefulness of a *Jennerian* strategy for human rotavirus vaccination was suggested by the early observation that human and animal rotaviruses share a major common antigen.^{247,398,793} The potential feasibility of this approach was shown during animal studies in which calves administered a bovine rotavirus (serotype G6) *in utero* were protected from illness following challenge at birth with an entirely heterotypic human rotavirus of serotype G1 specificity.⁷⁹⁸ It should always be kept in mind, however, that all *Jennerian* or modified *Jennerian* rotavirus vaccines are based on cell culture–adapted animal strains and that the actual cell culture adaptation likely also contributes to their attenuated phenotypes in humans.

The initial rotavirus vaccine studies in people involved a cell culture–passaged, cold-adapted, serotype G6 bovine rotavirus (strain NCDV [RIT 4237]).^{749,752} This highly attenuated, orally administered G6 monovalent vaccine induced over 80% protection to clinically significant diarrhea from serotypically distinct human strains in infants and young children in two separate efficacy trials in Finland.^{750,751} In another study, vaccination of neonates with the candidate vaccine modified the severity of rotavirus diarrhea during the 16-month follow-up period.⁷⁶¹ Following two doses—one in the neonatal period and a second dose 7 months later—the vaccine had a protective efficacy of 43% against any rotavirus-caused diarrhea and 89% against severe rotavirus-induced diarrhea.⁷⁶² However, the RIT 4237 vaccine candidate failed to induce significant protection against rotavirus diarrhea in trials in several developing countries and, as a consequence, further evaluation of this vaccine was not pursued.^{173,327,662,673}

Another serotype G6 bovine rotavirus strain, designated WC3 (Wistar calf), also demonstrated variable protective efficacy in several studies in the United States and overseas.^{48,77,135,136} For example, in infants 3 to 12 months of age in Philadelphia, its protective efficacy (when serotype 1 human strains were predominant) was 76% against any rotavirus-caused diarrhea and 100% against moderate to severe rotavirus diarrhea.^{135,136} In contrast, during later studies, the vaccine was less effective: in subjects from Cincinnati and the Central African Republic, the vaccine exhibited a protective efficacy against moderate to severe rotavirus diarrhea of 35% to 41%; and in China protective efficacy was 50% against any rotavirus diarrhea.^{48,268} Because of the relatively low and variable levels of protection, the vaccine was withdrawn from further study in its monovalent formulation. However, this strain was subsequently used to provide the genetic backbone for a safe and effective pentavalent human-bovine reassortant rotavirus vaccine (RotaTeq, RV5, see later) that was first licensed in the United States in 2006 and is now used worldwide.

Another animal rotavirus strain, the simian G3 RRV strain MMU 18006, was studied extensively as a monovalent *Jennerian* vaccine candidate.^{396,509} The RRV vaccine has been evaluated in both developed and developing countries. Although in some settings (such as in children older than 3 months of age on initial vaccination) it induced a mild to moderate transient febrile response in about one-third of the target population of infant vaccinees, it appeared to be more antigenic than the NCDV (RIT 4237) bovine rotavirus vaccine candidate.^{458,753} The RRV vaccine, however, was much less reactogenic in neonates or children younger than 3 months of age, presumably because the higher levels of maternal antibody inhibited vaccine replication.²⁴⁸ The RRV vaccine, like the other candidates, was administered orally, usually following ingestion of buffered formula, or buffer alone, to protect the virus from partial or total inactivation by stomach acid.⁵⁹⁴ In a field trial in Venezuela, the protective efficacy of the RRV vaccine in infants 1 to 4 months of age was 83% against any rotavirus diarrhea and 90% against the most severe illnesses. Results of efficacy trials were also encouraging in Maryland, Sweden, and Finland, but the vaccine failed to protect young infants in New York State or in Arizona; furthermore, it induced only a low level of protection (29%) in infants 2 to 5 months of age in Maryland.^{129,290,626,662,760} The basis for the variable protective efficacy in these studies was never clearly demonstrated, but the vaccine appeared to work best against homologous G3 strains and less well against heterologous strains.

Another pure *Jennerian* vaccine, a lamb rotavirus strain LLR (G10P[12]), is currently produced by the Lanzhou Institute and was licensed for human use in China in 2000. Postlicensure prospective, double-blind, and randomized evaluations of efficacy have not been completed for this vaccine. However, a retrospective case-control study concluded that one dose of the LLR vaccine was moderately effective (81%) in preventing rotavirus gastroenteritis in children 2 to 11 months of age but not effective when administered to children older than 12 months of age. The vaccine is currently not included in the national immunization program in China, and its efficacy level has not been studied outside China.^{79,257}

Initial Quadrivalent RRV-based Reassortant “Jennerian” Rotavirus Vaccine

Failure of the RRV vaccine to reproducibly induce high levels of protection in young infants against heterotypic rotavirus strains suggested that the initial strategy for development of a monovalent *Jennerian* rotavirus vaccine might require modification. As a consequence, the *Jennerian* approach to rotavirus immunoprophylaxis was altered. The *modified Jennerian* approach was used to produce a quadrivalent (RV4) RRV-based vaccine that incorporated the VP7 specificity of each of the four epidemiologically important human G serotypes coupled to the attenuation phenotype of RRV.^{394,396,509} This approach was also used to create the bovine WC3 vaccine (see later). This approach is enabled by the segmented genome of rotaviruses and by the ability of these viruses to undergo gene reassortment with high frequency during mixed infection.^{304,306} Virus reassortants for use as vaccine candidates have been isolated from cell cultures co-infected with a cultivatable, wild-type animal virus (RRV, bovine WC3 or bovine UK rotavirus [see later]) and selected human rotavirus.^{332,399,507,508} Antisera against the animal rotavirus parent were sometimes used to select specific

virus reassortants with the appropriate human VP7 or VP4. It was generally possible to isolate reassortants that derived only one gene segment encoding VP7 or VP4 (in the case of WC3) from the human rotavirus parent and the 10 other genes from the animal parent. In this manner, starting with the simian RRV strain, single human rotavirus gene substitution reassortants were isolated that possessed the human (or rhesus in the case of G3) rotavirus gene for VP7 of serotypes G1, G2, G3, or G4 on a background of 10 animal rotavirus genes.^{507,508}

As expected, the RRV-human reassortants exhibited G1, G2, G3, or G4 neutralization specificity, similar to the most common human rotavirus serotypes.⁵⁰⁷ Phase I studies indicated that each of the RRV-human rotavirus reassortants alone or in a quadrivalent formulation were similar to its RRV parent with respect to attenuation and immunogenicity.^{397,582} Individual reassortants or the quadrivalent vaccine formulation has been evaluated for efficacy in more than 9,500 infants and young children worldwide. The individual reassortants, DxRRV (serotype G1) or DS-1xRRV (serotype G2), each induced 67% protection against serotype G1 diarrhea in Finland.⁷⁶³ In Rochester, New York, the DxRRV vaccine induced 77% protection and the RRV vaccine induced 66% protection against predominantly serotype G1 diarrhea.⁴⁶⁸ Protection against serotype G1, following immunization with serotype G3 or serotype G2 reassortants, strongly suggested that heterotypic immunity could be induced following monovalent heterotypic immunization. Despite the fact that several studies supported the use of a monovalent *modified Jennerian* RRV vaccine candidate, the weight of the evidence from multiple studies implied that a multivalent formulation was more likely to be reproducibly efficacious. Therefore, several pivotal, randomized, double-blinded phase III trials were carried out in developed and less developed populations with the quadrivalent RRV-based reassortant formulation (RV4).

In three large-scale efficacy and safety trials of RV4 in Finland, Native Americans in the United States, and a poor urban population in Venezuela, vaccine efficacy ranged from 48% to 68% against any rotavirus-caused diarrhea. Importantly, efficacy against severe rotavirus diarrhea reached 91% in Finland, 88% in Venezuela, and 69% in Native Americans.^{377,378,583,663} A transient low-grade fever of greater than 38°C (rectally) was observed in up to 29% of the vaccinees, but there was no statistically significant increase in intussusception seen in patients who received vaccine compared to controls in these large phase III trials.

In 1998, based on the safety and efficacy evidence from the major clinical trials of RV4, the FDA granted a biologics license to Wyeth Laboratories for the manufacture and distribution of RV4 (RotaShield) for the immunization of infants at 2, 4, and 6 months of age. The vaccine was recommended for universal vaccination in the United States and given to almost 1 million young children. In 1999, less than a year after licensure, the Centers for Disease Control and Prevention (CDC) reported that between September 1, 1998, and July 7, 1999, 15 cases of intussusception had been reported to the Vaccine Adverse Events Reporting System (VAERS) following administration of RV4.¹⁰⁴ Based on this initial signal, the CDC and the AAP recommended that administration of rotavirus vaccine be postponed pending availability of more data and analysis.¹⁰⁴ Subsequently, the CDC reported that in case-control and case series studies, there was a very significant temporal association

between RV4 vaccination and intussusception during the first 2 weeks after the first vaccine dose. This observation was translated to an estimate of attributable risk of intussusception following vaccination of up to 1 in 2,500, or approximately 1,600 excess cases of intussusception in a full national vaccination program.^{3,525} In 1999, after reviewing the available risk estimate data at the time, the Advisory Committee on Immunization Practices (ACIP) withdrew its recommendation¹⁰⁵ and the RV4 vaccine rapidly disappeared from the marketplace.

Twelve years after the withdrawal of RotaShield, continuing controversy exists in scientific and lay publications on its fate and the level of actual risk associated with vaccine administration.^{52,142,275,324,389,479,524,526,625,784} Central to this discussion is the lack of agreement on the actual attributable risk of intussusception from RV4 as well as the estimate of the effect of the RV4 withdrawal decision on developing countries where more than 500,000 infants and children younger than 5 years of age die annually from rotavirus diarrhea.^{278,301} Recent findings demonstrate that the two currently licensed rotavirus vaccines (RV1 and RV5, see later) also are associated with a transient increased risk of intussusception further and so call into question the advisability of the RV4 vaccine withdrawal. Since the RV4 withdrawal, the risk estimate associated with this vaccine has decreased substantially, with a consensus estimate of risk of intussusception now at 1:10,000 excess cases.^{524,525,588} After RV4's withdrawal, other investigators analyzed population-based hospital discharge data and reported an attributable risk of 1:32,000 to 1:302,000 excess cases of intussusception among the target population of infants 45 to 210 days of age, far less than initial estimates.^{524,685} Further complicating the analysis is the observation that age at vaccination was likely an important confounding factor in the rare occurrence of intussusception following RV4 use.^{399,686} Thus, the use of *catch-up vaccination* of older infants who received the first dose of RV4 later than the recommended age of 2 months was associated with a disproportionate number of vaccine-associated cases of intussusception that were observed in the CDC case-control study.⁶⁸⁶ Avoidance of initial vaccination of infants older than 3 months of age and vaccination during the relatively refractory period of the first 2 months of life may have substantially reduced or eliminated the risk of intussusception with the RV4 vaccine, although the issue of age dependence of intussusception following RotaShield administration remains controversial.^{53,120,399,565,686}

The Two Major Currently Licensed Live Rotavirus Vaccines

Despite the issues raised by the withdrawal of the RotaShield vaccine, work on other alternative live, attenuated vaccine candidates continued with vigor.^{280,332,548} Two separate but parallel approaches were pursued. Initial studies with a monovalent rotavirus vaccine (RV1) derived from a virulent human rotavirus strain 89-12 with G1:P1A[8] serotype specificity were continued. Unlike the *Jennerian* candidates, this strain's attenuation was based only on multiple tissue culture passages.^{46,593,645} In a pivotal efficacy study that included more than 17,000 infants and young children who received two oral doses of vaccine or placebo at 2 and 4 months of age, the vaccine was highly effective (84.7%) at preventing severe rotavirus gastroenteritis and hospitalization (85%).⁶⁴⁵ This vaccine provided a high level of protection against G-P homotypic G1P[8] strains (90.8%) and G heterotypic, P homotypic

G3P[8], G4P[8], and G9P[8] strains as a group. It has also, in a number of separate, postlicensure studies, generally afforded substantial protection against the entirely heterotypic G2P[4] strains.^{149,382,445,756} There are reports, however, showing a lack of protection against G2 strains.⁶⁸⁹

Because of the previous experience with RV4 and the increased incidence of intussusception in the 2 weeks after initial vaccination, the safety arm of the RV1 pivotal phase III trials included more than 60,000 infants, of whom one-half received the vaccine and one-half the placebo. A temporal link with intussusception was not observed in this very large randomized vaccine safety study.^{445,645} Of note, the mean age of the vaccinees at the time of the first dose with RV1 was 8.2 weeks, an age when the background rate of intussusception is very low. Given the high level of efficacy and safety observed in pivotal phase III trials around the world, this monovalent attenuated human rotavirus vaccine, manufactured by GlaxoSmithKline under the trade name Rotarix, was first licensed for use in Mexico, the Dominican Republic, and Brazil in 2005 and in the European common market countries in early 2006, and is currently licensed in more than 110 countries around the world.

At the same time when Rotarix was under development, the *Jennerian* bovine rotavirus candidate strain WC3 (G6:P7[5]), described earlier, was being used to provide the genetic backbone for a series of reassortants with selected human strains to create a pentavalent *modified Jennerian* vaccine RV5. This approach was taken because of the variable protective efficacy achieved using WC3 alone or using monoreassortants of WC3. Five individual WC3 reassortants were formulated into a pentavalent vaccine containing G1 through G4 VP7s and a single P1A[8] VP4.^{332,333} This approach was similar to that taken for the development of RotaShield except that the genetic backbone was derived from a bovine rotavirus rather than a monkey strain and the vaccine contained an additional component strain that expressed a human VP4 protein as well as the four component reassortant viruses expressing various serotypically distinct (G1 to G4) VP7 proteins.

In a pivotal efficacy study that included more than 5,000 infants and young children who received three oral doses of RV5 or placebo at 2, 4, and 6 months of age, the vaccine was highly effective (74%) in preventing any rotavirus gastroenteritis as well as severe gastroenteritis (98%).⁷⁵⁹ It provided a high level of protection against G1, G2, G3, G4, and G9 serotypes. Because of the previous experience with RotaShield and intussusception, a very large safety component of the phase III trial was undertaken that included more than 68,000 infants, of whom one-half received the vaccine and one-half the placebo. A temporal link with intussusception was not observed. Of note, in this safety study the mean and median ages of vaccinees were 9.8 and 10 weeks, respectively, at the time of the first dose; in this age range the background rate of intussusception is very low. This *modified Jennerian* pentavalent vaccine (RotaTeq) is manufactured by Merck and was licensed for use in the United States in early 2006. RotaTeq is recommended for routine use in infants at 2, 4, and 6 months of age by the ACIP.⁵⁶¹ It is now licensed in over 100 countries around the world.

Postlicensure Safety and Efficacy Studies of Rotarix and RotaTeq

In the 5 years since the RV5 and RV1 vaccines have been licensed, these vaccines have been used broadly across the

United States, Central and South America, and, to a somewhat more variable extent, Western Europe.²⁷¹ This has allowed investigators to examine the performance in terms of both safety and efficacy of the two vaccines in an actual practice as opposed to controlled clinical trial settings. These phase IV postlicensure studies in high- and moderate-income countries have confirmed and extended the highly promising results from the phase III trials.^{13,67,93,567,579,609,715,716,754,755} In the United States following the introduction of RV5 in 2006, there has been a continued and sustained decline in rotavirus detection through 2010 as vaccine coverage has increased. This decline has been seen in all regions and has been associated with substantial decreases in hospitalizations and emergency department and outpatient visits and a progressive delay in the timing of the peak week of the annual rotavirus epidemic.^{67,579,715,716} A cost-effectiveness study concluded that although, at current vaccine prices, routine vaccination is unlikely to be cost saving, it should still be considered a highly cost-effective intervention.⁷⁸⁹ A similar beneficial effect has been reported from Australia where both RV1 and RV5 are used.⁹³ Vaccination has diminished the rate of hospital-acquired rotavirus infection in Australia as well.¹³ Follow-up studies on vaccinees in Finland have shown that the protective effect of RV5 is sustained for over 3 years following vaccination, and the reduction in hospitalizations and emergency department visits due to rotavirus infection has been over 90%.^{754,755} Of interest, in these and other postlicensure rotavirus vaccine studies, an unanticipated but clear-cut “herd immunity” effect has been observed.⁵⁷⁷ In a recent study from the United States, rotavirus vaccination was associated with substantial reductions in hospitalization rates and medical costs among unvaccinated individuals 5 to 24 years of age.⁴⁵⁷ That is to say, children who did not receive a rotavirus vaccination also experienced a decrease in rotavirus-associated morbidity following the introduction of these vaccines into the population. This population-level benefit is likely due to lowered transmission rates from vaccinated children, although the spread of vaccine virus from vaccinated to unvaccinated children is also possible in some cases. To date, no clear-cut difference in vaccine efficacy or safety at the population level has been observed between RV1 and RV5, and

there is not yet any compelling data at the population level to indicate that vaccine introduction alters the genotype distribution among circulating rotavirus strains.³⁵¹ Both RV1 and RV5 appear to be highly efficacious and safe in developed countries (Table 45.7).

Studies from middle- and low-income Latin America also show substantial decreases in hospitalizations in both Mexico and Nicaragua, although the effectiveness of RV5 in Nicaragua was less (58% against severe diarrhea) than in developed countries.^{567,609} Most striking are data from a study of RV1 in Mexico, which revealed a 41% decrease in diarrhea-related infant mortality in infants younger than 11 months of age that was clearly associated with vaccination.⁶²⁹ Follow-up studies in Latin America show that RV1 remained highly efficacious when co-administered with other childhood vaccines including oral polio.⁷³⁴

Morbidity and mortality due to rotavirus infections are higher in the poorest countries of Africa and Asia than in the developed world, and investigators predicted that a reproducible effect on severe morbidity would translate into a direct effect on mortality.²⁴³ In support of this prediction, in a recent report from Mexico, a middle-income country where RV1 is primarily used, diarrhea-related mortality, especially among younger children most likely to have been vaccinated, fell by over 40%.⁶²⁹ There is considerable evidence that a variety of orally administered vaccines, such as polio and some cholera vaccines, preform less well in very poor countries, such as India and Sub-Saharan Africa. Recent randomized controlled trials indicate that this is also true for both RV1 and RV5.^{20,467,818} The two current vaccines prevent severe rotavirus-associated gastroenteritis with an overall efficacy of over 90% in the United States and Western Europe, over 85% in regions of Asia where rotavirus results in low mortality, and 80% in Latin America. In contrast, studies of RV1 efficacy in South Africa and Malawi demonstrated significantly reduced efficacy of 77% and 49%, respectively.⁴⁶⁷ Similarly, a study of RV5 in Ghana, Kenya, and Mali revealed efficacy against severe diarrhea of only 39% but substantially higher overall efficacy during the first year of life (64%); efficacy rates varied by country, being highest in Kenya and lowest in Mali.²⁰ Finally, a double-blind, placebo-controlled trial of RV5 against

TABLE 45.7 Comparison of the Three Licensed Rotavirus Vaccines

	RotaTeq (RV5)	Rotarix (RV1)	RotaShield (RV4)
Manufacturer	Merck Vaccine Division	GlaxoSmithKline	Wyeth
Genetic backbone	Bovine rotavirus-WC3	Human rotavirus-89-12	Simian rotavirus-RRV
Composition	5 human; bovine reassortant	Single human rotavirus	4 human; simian reassortants
Human VP7/VP4 types	G1, 2, 3, and 4 and [P8]	G1 [P8]	G1, 2, 3, and 4
Dosage schedule	3 doses (2, 4, 6 months)	2 doses (6 weeks, 10+ weeks)	3 doses (2, 4, 6 months)
Administration	Oral	Oral	Oral
Presentation	Liquid	Liquid	Lyophilized-reconstituted
Protection against severe disease in: ^a			
Developed countries	85% (72-92)	95% (91-97) ^a	85% (69-91)
Low-income countries in Asia/Africa	48% (43-64)	61% (49-77)	NA
Virus shedding	21%	≥50%	50%
Rare intussusceptions	Yes	Yes	Yes

^aDifferent scoring systems, analyses, and populations were used so efficacy results are not directly comparable.

Adapted from Greenberg HB, Estes MK. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* 2009;136:1939–1951.

severe rotavirus gastroenteritis in developing countries of Asia (rural Bangladesh and urban Vietnam) demonstrated an overall efficacy rate of 48%, with rates in Bangladesh being lower (43%) than those in Vietnam (64%).⁸¹⁸

In summary, large randomized studies have confirmed that overall efficacy of the two currently licensed live, attenuated rotavirus vaccines (RV1 and RV5) is significantly lower in the poorer countries of Asia and Africa than it is in high- or middle-income countries. That said, analysis of the impact of rotavirus vaccination on the burden of rotavirus disease by the WHO and others indicates that the current two licensed rotavirus vaccines will actually have their greatest impact on morbidity and likely mortality in the poorest countries because, despite their lower efficacy in these settings, the burden of disease is so much higher. Because of this substantial beneficial effect, in 2009 the WHO's Strategic Advisory Group of Experts recommended that health authorities in all nations routinely vaccinate young children against rotavirus.

The widespread use of RV1 and RV5 allows the opportunity to better understand the safety profile of these two vaccines. Both vaccines are shed in appreciable amounts following vaccination (with RV1 shed at higher levels than RV5), but, to date, no adverse effects have been associated with vaccine transmission.⁸⁰⁴ As mentioned earlier, in the initial, very large safety trials, serious reactogenicity was not observed and there was no association of either RV1 or RV5 with intussusception. However, despite the sizes of these phase III safety studies, they were underpowered to detect very rare safety problems and would not have detected adverse events occurring at rates below 1 in 25,000 or so. Recent population-based studies from Mexico, Brazil, and Australia now are providing new data that indicate very rare temporal associations of both RV1 and RV5 (as was the case with RV4 vaccine, see earlier) with intussusception.^{92,568} The rates of vaccine-associated intussusception appear to be very low, less than 1 in 50,000, and it seems clear that the benefits of these two vaccines substantially exceed the risks based on the data currently available.³⁰¹

Several other potential safety issues have also arisen during the postlicensure period. Deep sequencing analysis of the RV1 vaccine revealed that it was heavily contaminated with a previously undetected infectious porcine circovirus, likely introduced from a porcine trypsin additive used in manufacture.⁷⁶⁴ The FDA evaluated the risks and benefits associated with this adventitious agent contamination and recommended that the real benefits of the vaccine outweighed the potential risks and that the vaccine should remain available for use but that the manufacturer should work expeditiously to derive new seed stock that was not contaminated. In addition, recent reports demonstrate that severely immunocompromised infants are at risk for vaccine-acquired chronic infection and illness, despite the attenuated nature of the rotavirus vaccines,⁵⁶⁹ and this led to a change in the vaccine label to exclude administration to children with SCID. In summary, in the past 5 years, two highly safe and effective live, attenuated rotavirus vaccines have been widely introduced. Both appear to be associated with very rare safety risks, but both are highly beneficial. At the time of this review, both of the new vaccines were relatively expensive and the costs exceed the ability of many countries to purchase and deliver these vaccines widely in a sustainable manner. For this reason, the ready availability of less expensive, safe, and effective vaccines would be desirable.

Other Live, Attenuated Rotavirus Vaccines in Clinical Development

The bovine rotavirus UK strain constitutes the backbone of a tetravalent human-bovine rotavirus reassortant vaccine candidate, composed of individual human-bovine rotavirus reassortants with VP7 specificity for G1, G2, G3, and G4 in a background of 10 bovine rotavirus genes.^{507,508} Additional G-type UK monoreassortants are also available for inclusion in a vaccine if needed.³⁹⁹ Each component alone was nonreactogenic and immunogenic in clinical trials.¹⁴¹ The four reassortant strains were combined into a tetravalent formulation that also proved to be safe and immunogenic.¹⁴⁰ In an efficacy trial in Finland, the UK-based tetravalent vaccine had a protective efficacy of more than 80% against severe rotavirus diarrhea.⁷⁵⁷ The UK-based reassortant vaccine has now been licensed by the National Institutes of Health to several vaccine manufacturers in Brazil, China, and India and is currently undergoing development and clinical testing in those countries.¹⁸¹ If proven to be safe and efficacious, it is hoped that manufacture of this vaccine in less developed countries will lead to an affordable rotavirus vaccine.

Another approach to rotavirus immunoprophylaxis involves the use of live, orally administered neonatal human rotavirus strains that appear to be naturally attenuated. The impetus for evaluating neonatal strains came from the early observations that neonates who experienced subclinical rotavirus infections in Australian or Indian nurseries during the first 14 days of life were protected against clinically significant rotavirus diarrhea for up to 3 years.^{51,57} Clinical studies with neonatal strain RV3 (G3, P2A), isolated from the Australian nursery study described earlier, are under way to determine if the vaccine candidate is immunogenic, safe, and efficacious.^{36,37} In addition, a large phase III randomized, double-blind, placebo-controlled clinical trial of an asymptomatic neonatal nursery rotavirus strain isolated in India is now under way. This strain (116E, G9P8[11]) is a naturally occurring human-bovine rotavirus reassortant originally isolated in New Delhi.²⁷⁶ The 116E strain has 10 human rotavirus genes and a single bovine rotavirus gene encoding VP4. Preliminary phase I and II clinical trials indicate that it is attenuated but highly immunogenic in Indian infants. The 116E vaccine, if safe and effective, will be available at a substantially reduced cost compared to the two currently licensed vaccines.

Finally, the RRV-based tetravalent RotaShield vaccine (RV4) has been reformulated and is currently being tested in phase III safety and efficacy trials in Africa, premised on the ideas that it appears to be somewhat more immunogenic than either RV1 or RV5 and this increased immunogenicity might translate into improved efficacy in very poor countries. This plan is coupled with the rationale that if RV4 is given to newborns or children younger than 2 months of age at the time of the first dose, risks of intussusception will be very low.

Other Approaches to Vaccination

Due to the relatively poor efficacies of RV1 and RV5 in very poor countries of Africa and Asia, other strategies for rotavirus immunization are being explored. Among the most actively pursued approaches is the use of inactivated virus or recombinant virus-like particles administered parenterally.³⁷² Several types of animal studies in pigs and primates support the potential

of these approaches to induce protective immunity, and studies in primates have shown that systemically administered, passively transferred serum antibodies can protect from infectious rotavirus challenge.⁷⁸⁷ Other much less thoroughly examined approaches include the use of (a) synthetic viral proteins, (b) viral proteins expressed from cloned rotavirus cDNA, (c) synthetic peptides, and (d) DNA vaccines.^{87,144,334,358,778} None of these alternative strategies have yet advanced to the stage of initial testing in people.

Passive Immunization

Passive immunization with some form of rotavirus-enriched antibody preparation has been shown to be effective in preventing or modifying rotavirus illness in animals.^{200,326,666,787} Similar studies have now been carried out in infants and young children, with promising results in most, but not all, instances.^{35,88,170,201,202,316} Various sources of passively administered rotavirus antibodies (e.g., eggs from immunized hens, or milk formula containing bovine milk immunoglobulin from milk of cows hyperimmunized with human rotaviruses) have been considered.^{170,810,811} However, except in very special circumstances, such as in immunocompromised individuals with severe or chronic rotavirus infection, passive immunization is simply not currently practical for protection against rotavirus illness due to cost and lack of commercially available licensed product.

PERSPECTIVES

Rotavirus disease has become vaccine preventable with clear and substantial evidence of efficacy for children in developed and developing countries and reductions of mortality in children in developing countries. This is an exciting achievement, yet other questions remain to be answered regarding vaccines: Can the efficacy of the current oral vaccines be improved in children in some developing countries based on understanding why such children do not respond with high rates of induced protective immunity? Is this due to malnutrition, intestinal microbiota, frequent infection with other intestinal pathogens, or other health factors of the children, or to different circulating rotavirus strains that are not part of the current vaccines? Will worldwide vaccination induce changes in the epidemiology of circulating virus strains? Will epidemiology studies be able to show that vaccination reduces rare childhood diseases, such as biliary atresia, that might be associated with rotavirus infections but where causation has been difficult to prove in children? Can a correlate of protection be identified to facilitate the future development of third-generation vaccines?

Even with successful vaccines, rotaviruses will continue to serve as very useful models to understand mucosal virus–cell interactions. The interactions of viral and cellular proteins that influence virus entry into cells, trigger epithelial cell responses, and modulate function of the differentiated epithelial cells or neighboring cells in the complex architecture of the intestine remain to be understood. This will require new studies of virus pathogenesis *in vivo*, and such studies will require multidisciplinary efforts to understand fully the complex biochemical and physiologic consequences of infection. Rotaviruses also provide a prototypical infection for the study of the local intestinal innate and acquired immune response. Some questions to be asked include the following: Why do rotavirus infections

cause minimal intestinal inflammation? How do rotaviruses escape the intestine and cause extraintestinal infections? Do such infections result in currently unrecognized disease in a subset of individuals with specific genetic properties? What is the basis for host-range and organ-specific replication restriction? What factors govern the development of homotypic versus heterotypic humoral immunity? Why is the circulating T-cell response so small following rotavirus infection? What role does innate immunity play in determining the clinical outcome of rotavirus infection in people? Continued analysis of cellular responses (both epithelial and immune) by using microarray and proteomics analyses will help define pathways of cellular signaling that influence the outcome of infection as well as cellular genes important for replication. More studies on rotaviruses that represent most human and animal rotavirus strains that infect cells in a neuraminidase-resistant fashion and that replicate less efficiently in cultured cells clearly are needed to understand how these viruses differ from the well-studied neuraminidase-sensitive viruses. Will study of these viruses discover that they have other unexpected receptors or unusual properties that affect their stability or transmission? The first reports of success with reverse genetics systems for the rotaviruses should facilitate future probing of viral gene function, which is especially needed to fully understand pathogenesis. Achieving a more efficient and tractable reverse genetics system may require more knowledge about the unique processes by which rotaviruses inhibit host cell translation, enhance virus-specific RNA replication, and achieve genome encapsidation that may be linked and compartmentalized within the cytoplasm of cells. The application of new techniques in cell biology to understand the structural and biochemical basis of the unusual processes of rotavirus entry into, and exit from, cells can be expected to unravel new mechanisms of intracellular protein and vesicle trafficking. Structural knowledge of rotavirus and its proteins should help lead to new methods and inhibitors to interrupt virus transmission, prevent virus replication, and treat disease.

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Orbiviruses

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parts of the world, including Palestine, Turkey, Israel, Pakistan, and the Indian subcontinent.^{27,66}

In the late 18th century, BT disease was observed in domestic animals as well as in wild ruminants (e.g., Blesbuck, white-tailed deer, elk, and pronghorn antelope). As with AHS, BT disease was confined to Africa for many decades. Distinctive lesions in the mouths of the infected animals with a dark blue tongue were the characteristic symptoms. A detailed description of the disease was first published in 1905,¹¹² when it was named *malarial catarrhal fever*,²⁶ and Theiler demonstrated that the agent was a filterable virus.¹²⁵ The first confirmed outbreak outside of Africa occurred in Cyprus in 1924, followed by outbreaks in 1943 to 1944 when about 2,500 sheep died with a mortality rate reaching 70%. In the United States, BT disease in sheep was first recognized in 1948, and in 1956 a major epizootic began in Portugal and extended into Spain. Outbreaks of BT disease in the Middle East, Southeast Asia, southern Europe, and the United States in the early 1940s and 1950s led to its description as an *emerging disease*.⁵² Virus isolates, of 26 different serotypes, have been made in tropical, semitropical, and temperate zones of the world, including North and South America, Australia, southern Europe, Israel, Africa, and Southeast Asia. In 1998, significant outbreaks of BT occurred in Europe, initially in Greece and then in many other Mediterranean countries, causing the death of over a million sheep by 2005.⁹⁶ In 2006 and 2007, BTV emerged in North-western Europe eventually expanding into the United Kingdom, Denmark, and the Czech Republic. An important factor in the distribution of BTV worldwide is the availability of suitable vectors, usually biting midges (gnats) of the *Culicoides* species.

HISTORY

Two important orbivirus diseases are African horse sickness (AHS) and Bluetongue (BT) disease of sheep and cattle, both recognized first on the African continent.^{47,124} The first major outbreak of AHS was observed in 1719, when approximately 1,700 animals died and the most significant outbreak occurred in 1854 to 1855, affecting some 70,000 horses. AHS mainly appeared in years of unusually high rainfall and warm weather, and it was speculated that insect vectors were involved in spreading of the disease. Only in 1900 was it demonstrated that AHS was caused by a virus, when M'Fadyean succeeded in transmitting the disease by a bacteria-free filtrate from an infected animal to a healthy one.⁸⁸ The major vertebrate reservoir for the virus was believed to be zebra.³ To date, AHS virus occurs in multiple serotypes (AHSV-1 to 9) and is endemic in eastern and central Africa and many parts of sub-Saharan Africa. From 1943 onward, AHS outbreaks have been reported in different

CLASSIFICATION

The *Orbivirus* genus is one of 15 genera within the *Reoviridae* family, which includes vertebrate, arthropod, and plant pathogens. Despite a basic similarity, orbiviruses differ greatly in their structure, physicochemical properties, replication cycle, pathogenesis, and epidemiology. Unlike the reoviruses and rotaviruses, orbiviruses are arthropod-borne, and 22 serogroups or virus species of orbiviruses are distinguished in addition to several unclassified isolates.⁸⁷ Within each serogroup, multiple serotypes are differentiated by neutralization tests. Different orbiviruses infect a wide range of vertebrate hosts, including ruminants (domesticated and wild), equids (domesticated and wild), rodents, bats, marsupials, birds, sloths, and primates, including humans. The orbivirus serogroups, serotypes, principal vectors, and main hosts are listed in e-Table 46.1 with their assigned abbreviations.⁸⁷

VIRION STRUCTURE

Orbiviruses are nonenveloped, icosahedral particles containing segmented, double-stranded RNA (dsRNA) genomes. BTV, the prototype of the genus, is the main subject of this chapter. BTV virions (550S) are architecturally complex structures composed of seven discrete proteins that are organized into two concentric shells, the inner and outer capsids¹³⁸ (Fig. 46.1). The virion proteins encapsidate a genome of 10 dsRNA segments that can be divided into three different size classes (large, medium, and small). Unlike reovirus and rotavirus particles, mature BTV particles are relatively fragile, and the infectivity of BTV is easily lost in mildly acidic conditions. The outer capsid of BTV consists of two major proteins, VP2 and VP5, which are removed shortly after infection, to yield a transcriptionally active core (470S) particle (Fig. 46.1) which, in contrast to virions, is fairly robust. Cores contain two major proteins (VP7 and VP3), three minor proteins (VP1, VP4, and VP6), and the dsRNA genome.¹³⁸ They may be further uncoated to form sub-core particles (390S) that lack VP7. Cores can also be derived from virions *in vitro* by removal of the outer capsid.^{56,134}

Virion Particle and Outer Capsid

Early electron microscopy (EM) visualizations were of characteristically fuzzy particles, suggesting BTV is highly sensitive to preparation conditions.²⁴ However, cryo-electron microscopy (cryo-EM) and image reconstruction of BTV particles revealed an icosahedral symmetry of the virions with a diameter of ~880 Å and an accurate positioning of VP2 and VP5 proteins in relation to each other and to the underlying core surface.^{49,91} The images revealed a total of 60 triskelion spike-like structures formed by VP2 trimers and 120 globular structures of VP5 trimers, both of which attach to the underlying core surface layer independently of each other. A recent 7 Å resolution cryo-EM structure of virion (Fig. 46.2) further identified the secondary structural elements and the topological arrangement of these two proteins.¹⁴⁴ Each VP2 monomer has two distinct domains, a tip domain and a lower region, three of which form a hub domain at the base of the triskelion spike. The top of the VP2 tip domain is rich in β sheets and projects upward from the surface of the virion. The hub has a distinct β -barrel fold (Fig. 46.2C) and each monomer contains a sialic acid (SA) binding pocket (Fig. 46.2D).

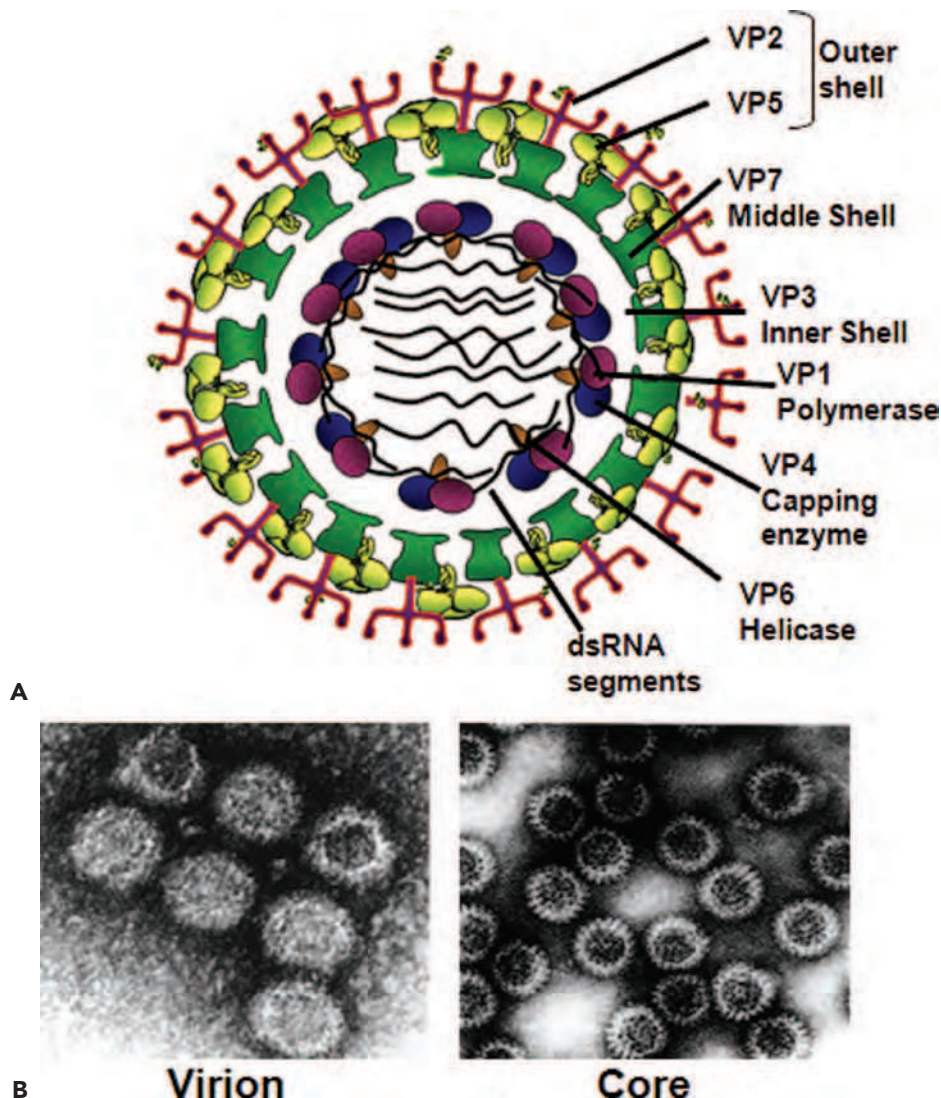


FIGURE 46.1. Bluetongue virus particles and proteins. **A:** Schematic diagram of bluetongue virus (BTV) showing the positions and structural organization of BTV components. **B:** Electron micrographs of negatively stained purified BTV particles and cores.

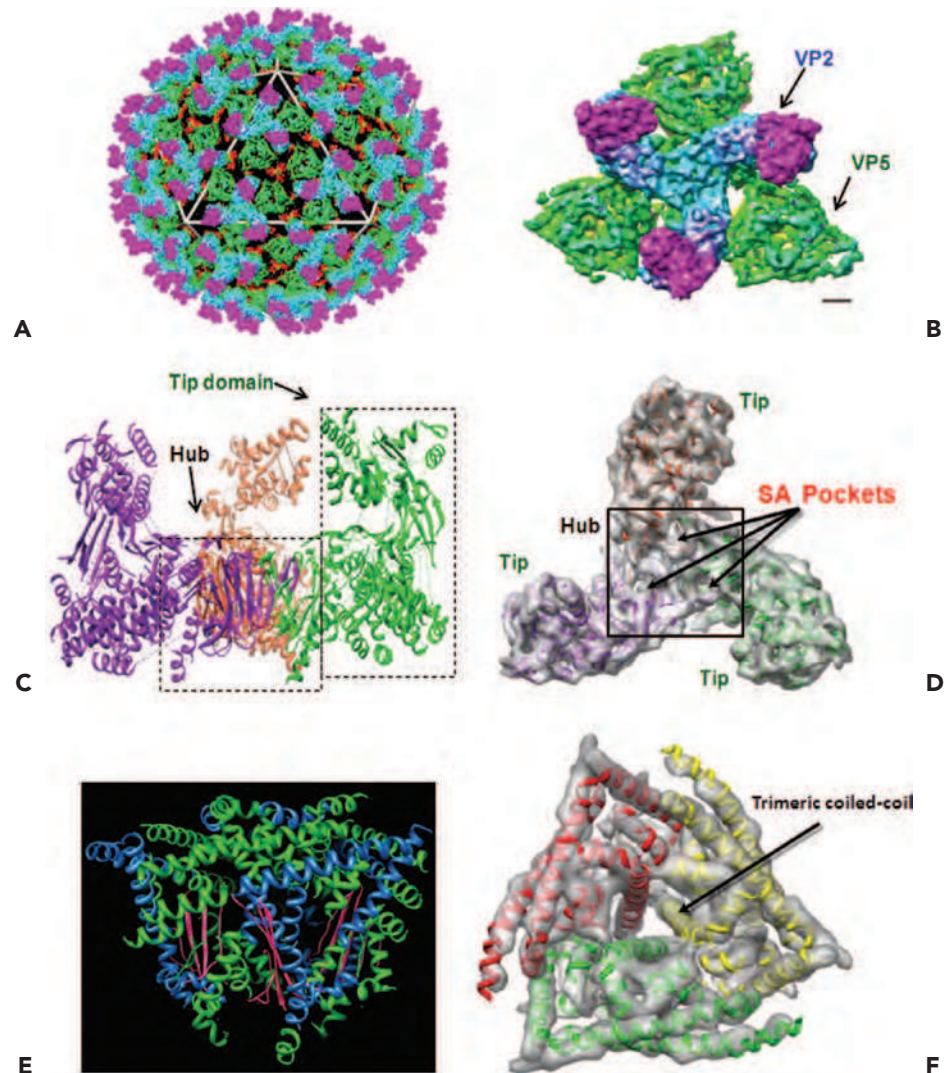


FIGURE 46.2. Cryo-electron microscopy (cryo-EM) structure of complete bluetongue virus 1 (BTV-1) particle at 7 Å resolution. **A:** Surface representation of whole particle showing VP2 (magenta/cyan) and VP5 (green) of outer capsid, and VP7 (red) of core. **B:** A cut-off section showing the arrangement of VP2 and VP5 trimers. **C:** VP2 triskelion formed by three VP2 monomers, each consisting of a tip domain and a part of the hub domain. **D:** A putative sialic acid-binding pocket is located at the hub domain. **E:** Side view of a VP5 trimer as a helical globular complex; the amphipathic helices (blue), nonamphipathic helices (green), and the single B-sheet (red). **F:** Top view of VP5 with an embedded ribbon model showing the central coiled-coil helix bundle.

In contrast to VP2, the VP5 trimer is globular, very rich in helices, and possesses only one β sheet (Fig. 46.2E). Five distinct amphipathic α -helical regions lie on the exterior surface of the protein, including an N-terminal α helical region that is positioned on the exposed surface of VP5.¹⁴⁴ A cluster of three copies of one amphipathic helix are well positioned to expose their hydrophobic undersides to the endosomal membrane (Fig. 46.2E). Other amphipathic helices are similarly located on the exposed surface, where they may mimic the arrangement seen in other fusion proteins such as the fusion protein of the enveloped HIV (Fig. 46.2F). The gaps among the VP2 triskelions are filled by three VP5 trimers. Density maps suggest that VP5 trimers interact very weakly with the VP2 hub domains and with the underlying core surface, but both the VP2 tip and hub domains connect to their underlying VP7 trimers by stronger forces.

Core Particle and Proteins

Core particles derived from purified virus *in vitro* are stable and have been studied extensively at the structural level, initially by cryo-EM (at various resolutions between 22 Å and 40 Å) and subsequently by establishing a 3.6-Å atomic structure from crystals, which has revealed four distinct features.

Surface Layer of the Core and VP7 Trimers

The core surface has a diameter of 73 nm with an icosahedral symmetry triangulation number of 13 ($T = 13$) in a left-handed configuration. The surface layer of VP7 trimers (appear as prominent triangular shapes) occupies the space between radii of 260 Å and 345 Å. The trimers are arranged around 132 distinctive channels as six-member rings, with five-member rings at the vertices (Fig. 46.3A). A total of 780 VP7 (38 kD) molecules per particle form 260 quasi-equivalent trimers the protomeric unit (P, Q, R, S, T), which can be seen clearly. Aqueous channels surrounded by these trimers are about 8 nm wide and 8 nm deep. Based on their location with respect to the icosahedral symmetry axes, they can be grouped into three types; type I channels run along the icosahedral fivefold axes, type II channels surround the fivefold axes, and type III channels are located around the icosahedral threefold axes. The arrangement of each monomer within the VP7 trimer was shown by x-ray crystallography.^{4,41} Each monomer (349 amino acids for BTV-10) consists of two distinct domains, “upper” and “lower,” which are twisted in such a way that the top domain of one monomer rests upon the lower domain of another (Fig. 46.3B). The smaller upper domain of VP7 forms the “head

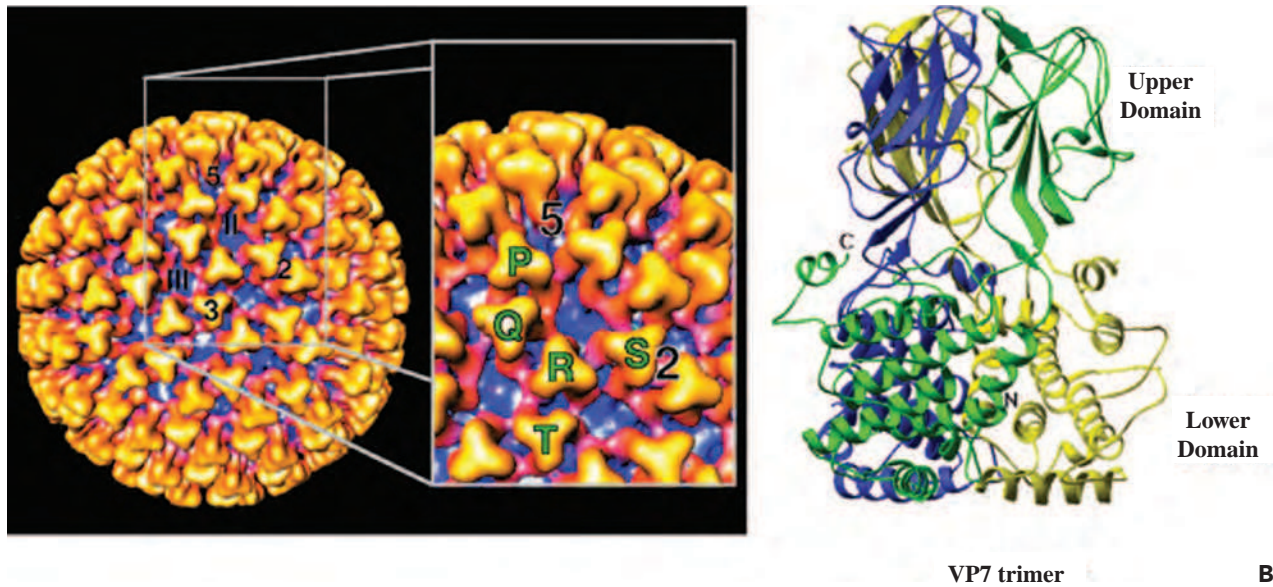


FIGURE 46.3. Three-dimensional structures of bluetongue virus (BTV) core and VP7 protein. **A:** Surface representations of the three-dimensional (3D) cryo-electron microscopy (cryo-EM) structures of BTV-10 core (700 Å in diameter) viewed along the icosahedral threefold axis showing the trimers of VP7 (in blue). The five quasi-equivalent trimers (P, Q, R, S, and T) and the locations of channels II and III are marked. **B:** The trimer image of the VP7 atomic structure solved at 2.8 Å resolution. Two domains of the molecule, the carboxyl and amino termini, are indicated. The view is shown from the side. Note the flat base of the trimer lies in a horizontal plane in this view.

region” of the trimer, which appears as a “knobbly” projection on the surface of the core and contains the central one-third of the polypeptide chain of the molecule (amino acids [aa] 121–249) folded into an anti-parallel β -sandwich. Two other x-ray structures of upper VP7 domains, one from BTV-10 (aa 123–253) and one from AHSV type 4, also revealed a similar structural arrangement. The lower, broader domain of the monomer is composed of nine α -helices and long extended loops, and is formed by both the N (aa 1–120) and the C (aa 250–349) termini of the molecule. A single lysine residue, prone to proteolysis, is situated at the junction between the two domains, and its mutation has a deleterious effect on core assembly.⁶⁹ A low resolution (5.4 Å) VP7 structure indicated a very different orientation of the helical lower domain from that of the high-resolution structure, suggesting an “unwound” or “open” form compared to the tightly packed helices found in the high-resolution form.⁴ These two very different structures indicate that VP7 has the potential for internal flexibility in the lower domain and suggest that substantial conformational change in VP7 occurs at some stage in the viral life cycle.

X-ray crystallographic structures for core particles of two different BTV serotypes (BTV-1 and BTV-10) have confirmed the precise organization of the 260 VP7 trimers⁴² (Fig. 46.4A). The channels, or “pores,” are small (7 Å diameter) at the icosahedral threefold axes (class III) and slightly larger (9 Å diameter) at the fivefold axes (class I), where they act as “portals” to release the newly synthesized transcripts. Although the “T” trimers of VP7 sit tightly on the pores at the threefold axes, the “P” trimers are located loosely above the pores at the fivefold axes. At the interface between VP7 and VP3, the VP7 trimer layer is principally formed by side-to-side packing of

short hydrophobic side chains, whereas interaction with the VP3 underneath layer is extensive and relatively tight. The lower domains of VP7 pack down against six corners of each VP3 molecule such that 12 monomers of VP7 (4 trimers) are in direct association with VP3 molecules. The thirteenth monomer of VP7 (organized in the icosahedron $T = 13$) is not directly in contact with a VP3 molecule, but is slightly raised and is in contact with monomers of adjacent trimers.

Arrangement of VP3 Molecules in the Inner Layer

The inner core layer, beneath the VP7 layer, is made up solely of VP3 proteins and occupies the space between radii 230 and 260 Å. Unlike the VP7 layer, the VP3 layer appears as an almost spherical structure as revealed by high-resolution x-ray structure of the cores.^{42,43,48,94}

Sixty dimers of VP3 (103 kD) serve as the unit building block to form the disc-shaped VP3 shell of 59 nm diameter and with an internal diameter of 38 nm that contains both the genome, as well as the minor core proteins.⁴² The 120 molecules of the VP3 shell are organized as an icosahedral lattice with $T = 2$ symmetry (Fig. 46.4B) and are arranged as two sets (A and B) of 60 subunits (Fig. 46.4C). Five of the “A” group of molecules are arranged as a five-pointed star around each fivefold axis, and five “B” group molecules are positioned, one between each of the points of the star, but at a further distance from the fivefold axis to produce a decamer. Such icosahedral organization of the inner shell is shared by all members of *Reoviridae* and other viruses with segmented dsRNA genomes. The inner surface of the VP3 shell has relatively few charged residues and has a series of shallow grooves.

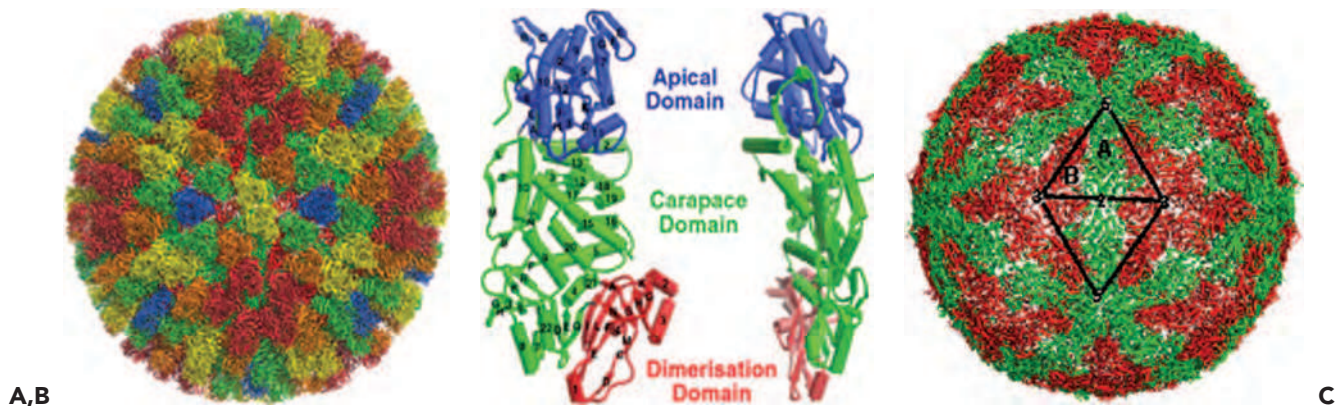


FIGURE 46.4. The x-ray structure of the blue tongue virus 1 (BTV-1) core. **A:** Surface of the core structure showing the arrangement of 260 trimers. The structurally equivalent trimers have been given the same color. **B:** The structure of the VP3 layer showing the arrangement of 120 copies of two conformationally distinct types of VP3 molecules, “A” shown in green and “B” shown in red. Sixty copies of each of the two molecules together form 12 decamers that are linked together forming a thin protein shell. The icosahedral fivefold, threefold, and symmetry axes are indicated. **C:** Structures of two types of VP3 molecule, A (left) and B (right), as observed in the BTV-1 core. The molecules are very similar in overall shape of thin triangular plates, with slight differences in conformation.

Structural Arrangement of Internal Minor Proteins

A clear density internal to the VP3 layer, in both the virion and the core cryo-EM reconstructions and in the x-ray structure, was assigned to the internal minor proteins (VP1, VP4, and VP6). In the absence of the viral genome, cryo-EM reconstruction shows the complex as a flower-shaped density directly beneath the icosahedral fivefold axes and attached to the underside of the VP3 layer⁹¹ (Fig. 46.5). It is not clear whether the VP6 protein is complexed with VP1 and VP4 at the fivefold vertices of the core; from *in vitro* studies, it can be deduced that the VP6 is tightly associated with the genomic RNA.

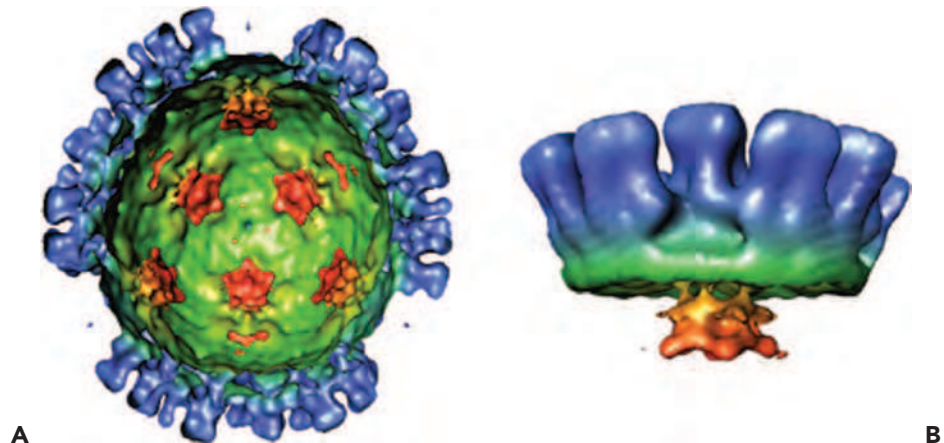
A modeled structure of VP1 has revealed a typical RNA dependent RNA polymerase (RdRp) organization with a distinct central polymerase domain (PD), an N-terminal domain (NTD), and a C-terminal (CTD) domain similar to that of Reovirus and rotavirus.¹⁴⁰ The model was supported by mutagenesis combined with dissection of biological activity of each

domain, and it is likely that all *Reoviridae* polymerases share similar structural organization.

An atomic structure is available for VP4, the capping enzyme of the virus.^{80,97,119} It shows an elongated morphology composed of four domains, which includes an N-terminal kinase-like domain (KL); a center methyltransferase-1 domain (N7MT) and O2-methyltransferase-2 domain (O2MT), and a C-terminal Guanylyltransferase (GTase) domain (GT) (Fig. 46.6). The 2'-O-MT structure is similar to the catalytic domains of class I S-Adenosyl L-methionine (SAM) SAM-dependent methyltransferases and in particular to vaccinia virus VP39. A Lysine Aspartic Lysine Glutamic (KDKE) tetrad in the active site seems to be characteristic of RNA2'-O-MTases. The N-terminal 108 residues of VP4 show a kinase-like fold (KL domain), and is the least conserved domain among the capping enzymes of different orbivirus species, whereas the GT domain is the most highly conserved region of VP4 between different orbivirus species. Functional analyses indicate that

FIGURE 46.5. Organization of internal proteins (transcription complex) and RNA genome.

A: Cryo-electron microscopy (cryo-EM) structure of a recombinant core-like particle showing the inside view of the core-like particle (CLP) reconstruction with VP3, VP7, VP1, and VP4. A flower-shaped density features (red) attached to the inside surface of VP3 (green) at all the fivefold axes is clearly seen. **B:** Conical cut-away from the reconstruction providing close-up views of the flower-shaped structure (red) and its interaction with the VP3 layer (green).



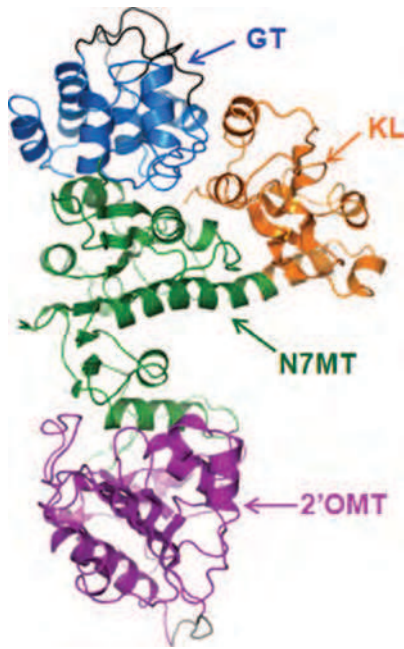


FIGURE 46.6. Cartoon representation of the structure of the VP4 molecule: Ribbon diagram of the VP4 structure with enzymatic domains coloured: guanylyl transferase (GT, blue), kinase-like guk domain (KL, orange), guanine-N7-methyltransferase (N7MT, green), and (nucleoside-2'-O)-methyltransferase (2'OMT, purple) domain.

catalytic residues of the GT lie with the 98 aa of the C-terminus where a number of conserved lysine and histidine residues could form the guanine adduct required for GTase activity.

RNA Genome Seen in X-ray Structure of the Core

The electron density in the x-ray structure of the core exhibited features consistent with layers of highly ordered RNA.⁴⁰ Approximately 80% of the genome can be modeled as four concentric layers that have center-to-center spacing between RNA strands of 26 to 30 Å. Grooves in the inner surface of the VP3 shell form a spiral around the fivefold axis with which the dsRNA layers appear to interact. The topography of the RNA molecules is uncertain, as the density detected in the inner layers of RNA gets progressively weaker, although each layer maintains an overall spiral organization.⁴⁰

GENOME STRUCTURE AND ORGANIZATION

The complete sequences of all 10 dsRNA segments were initially determined for BTV-10³⁷ and subsequently for a number of BTV serotypes as well as for many other orbiviruses (see review, 87,108). For BTV-10, the sizes of the RNA segments range from 3,954 (segment 1) to 822 base pairs (bp) (segment 10) (e-Fig. 46.1) and the total genome is 19,220 bp long³⁷ (Table 46.1). The 10 dsRNA segments of BTV have conserved terminal sequences with conserved 5' (8 nucleotides) and 3' (6 nucleotides) termini.⁹⁹ The 5' terminus of the coding strand of each duplex is capped and methylated, as are the BTV messenger RNA (mRNA) transcripts.

TABLE 46.1 Bluetongue Virus (BTV-10) Coding Assignments

Segment No.	Protein	No. of amino acids	Estimate No. molecules per virion	Location in virion particle (VP1–VP7)	Function
1	VP1	1,302	12	Inner core	RNA polymerase
2	VP2	956	180	Outer shell (spike)	Receptor binding, virus entry, hemagglutinin, type-specific, neutralization
3	VP3	901	120	Subcore layer (scaffold)	Forms scaffold for VP7 trimers, interacts with transcription complex and genomic RNA
4	VP4	654	~24	Inner core	Capping enzymes-guanylyltransferase, methyltransferases 1 and 2, RNA 5' triphosphatase, inorganic pyrophosphatase, NTPase
5	VP5	526	360	Outer shell (globular)	Virus penetration, fusogenic
6	NS1	552	High level	Nonstructural	Forms tubules, binds BTV transcripts and enhances protein synthesis
7	VP7	349	780	Core surface layer	Forms surface of core, responsible for core entry into insect cells
8	NS2	357	NA	Nonstructural	Phosphorylated, recruits BTV single-stranded RNA (ssRNA), forms cytoplasmic inclusion bodies, site for core assembly
9	VP6	328	60–72	Inner core	Viral helicase, adenosine triphosphatase (ATPase), form hexamer in presence of BTV RNA
10	NSP4	77–79	NA	Nonstructural	Interact with animal host
	NS3	229	NA	Nonstructural	Glycoproteins, membrane proteins, interact with host
	NS3A	216	Low level		membrane proteins calpactin, Tsg101 and outer capsid proteins, aids virus trafficking and release. NS3A is essential for virus maturation in vector insect cells

Apart from segment 1, the first AUG initiation codon in the mRNA strand of each segment initiates a single open reading frame (ORF). There are two methionine codons in the same reading frame in the S10 RNA sequence, coding for two related proteins: NS3 and NS3A.^{34,85} Recently an additional small non-structural protein has been identified, NSP4 encoded by S9, which also encodes the structured protein VP6.¹⁴⁵ The encoded structural proteins are numbered VP1 to VP7 in the order of their migration on sodium dodecyl sulfate (SDS)–polyacrylamide gels. In addition to the seven structural proteins in virion particles, four nonstructural proteins (NS1, NS2, NS3, and NS3A) are synthesized in infected cells.¹⁰⁸

MOLECULAR GENETICS

As early as 1981, the genetic diversity of the different BTV genome segments was identified by RNA oligonucleotide fingerprint analyses of field samples, and both genetic drift and shift were shown to contribute to BTV evolution.¹⁰⁸ In some segments, variability was clearly linked to serotype (e.g., segments 2 and 5 encoding outer capsid proteins VP2 and VP5). In addition, oligonucleotide fingerprinting revealed that genome segment reassortment occurs readily between different BTV serotypes and within other orbivirus serogroups, but not between viruses belonging to different groups.^{39,107} Partly as a result of these data, individual orbivirus serogroups are now recognized as distinct virus species.⁸⁷

Early molecular hybridization studies confirmed that genome segments 2 and 5 exhibited little or no cross-hybridization between serotypes, whereas the other eight RNA segments consistently showed some level of relatedness. Segments 1, 3, 4, 6, and 8 are relatively conserved, whereas segments 7 and 10 vary somewhat. Apart from segment 10 of AHSV, which appears to be even more variable than that of BTV, similar results have been obtained for AHSV.^{18,135}

Recently, an extensive phylogenetic analysis of segments 2 and 5 from >300 different BTV isolates has shown that the

nucleotide sequence of segment 2 varies from 29% to 59% between different serotypes.^{76,101} Based on this analysis, 26 serotypes were identified forming 10 distinct groups based on the fact that some serotypes are more related than others^{74,76} (e-Fig. 46.2A). Isolates within each serotype can also be separated into two major geographical groups, *group E*, from the East (India, Indonesia, China, Australia), and *group W*, from the West (Africa and North and South America).⁷⁴ Far less variation occurs in segment 5, which encodes VP5, although it too shows some correlation with serotypes.⁷⁶ A close genetic relationship has also been demonstrated among BTV, EHDV, and AHSV, three *Culicoides* transmitted Orbiviruses. Segments encoding the core proteins, for example, VP3, are the most conserved, whereas VP2 is the most variable^{60,102,141} (e-Fig. 46.2B). Despite as little as 17% to 23% identity, however, the VP2 protein of the three orbiviruses has a similar predicted secondary structure. Unlike VP2, the other outer capsid protein VP5 is more conserved among the three viruses, clearly indicating these viruses share strong phylogenetic relationships.

STAGES OF VIRUS REPLICATION

The basic features of replication cycle of orbiviruses are similar to those of reoviruses and rotaviruses. Unlike reoviruses or rotaviruses, however, orbiviruses multiply in arthropod as well as in vertebrate hosts, and thus some stages of orbivirus replication and morphogenesis are unique. The overall information available on the BTV replication cycle and assembly is summarized in a schematic diagram (Fig. 46.7).

Virus Entry

Of the two outer capsid proteins, the larger VP2 (110 kD) is the receptor binding protein responsible for eliciting neutralizing antibodies and also has hemagglutination activity. Furthermore, recent structural data revealed that the VP2 trimer has two putative ligand-binding domains, one at the tip of the monomer and the other, an SA-binding pocket, at the base

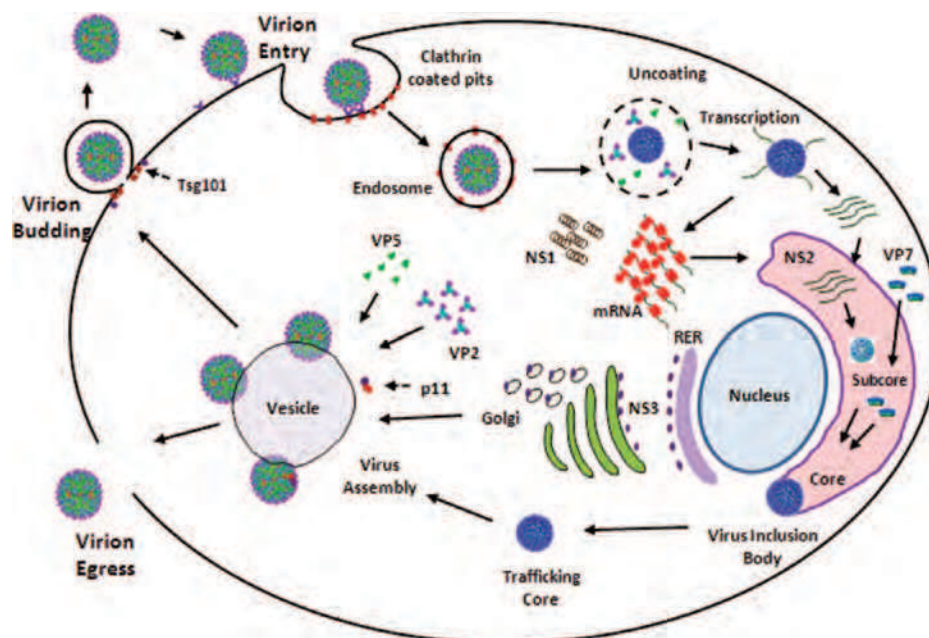


FIGURE 46.7. Replication cycle of bluetongue virus (BTV). Schematic diagram of an orbivirus replication cycle (deduced from bluetongue virus data).

of each trimer that binds type 2 oligosaccharide structures (Gal α 14GlcNAc β 13) in glycan arrays.^{9,144} In BTV VP2 the SA binding domain is about 40 Å inward from the outer end of the tip domain, a distance well within the range of lengths of chains of sugars on glycoproteins.¹⁴⁴ Because it is known that BTV particles agglutinate erythrocytes of ruminants²⁰ and VP2 alone is responsible for this activity,⁴⁶ it can be postulated that SA binding may promote adsorption of the virus onto the surface of erythrocytes, thereby increasing the probability of ingestion by the blood-feeding *Culicoides* vector.

The attachment of VP2 leads to receptor-mediated endocytosis of virions in clathrin-coated vesicles (Fig. 46.7). Clathrin is subsequently lost, and large endocytic vesicles are formed. Both VP2 and VP5 proteins can be identified within the early endosome where either one or both proteins are degraded.^{22,31,45} Compounds that raise the lysosomal or endosomal pH (e.g., ammonium chloride) prevent endocytosis of virus particle and the subsequent release of the uncoated cores into the cytoplasm.³⁰

As discussed above, VP5 shares certain structural features with the fusion proteins of enveloped viruses, consistent with membrane penetration activity.¹⁴⁴ Peptides representing the two amino terminal amphipathic helices of VP5 cause leakiness of the membrane, and the full-length protein triggers strong syncytia and multinuclear cells when localized to the plasma membrane and treated with low pH^{31,46} (Fig. 46.8). Therefore, VP5 functionally substitutes for a typical viral fusion protein, although it does not require proteolytic activation to render it fully functional as in the case of reovirus protein.

Combining the available structural and biochemical data, a plausible hypothesis would be that upon receptor binding and entry VP2 trimers undergo rearrangement or degradation, allowing the exposure of VP5 trimers and thereby allowing the VP5 amphipathic helices to freely interact with membranes and initiate the permeabilization process.

In summary, VP2 makes an initial contact with the mammalian host cell and VP5 mediates the penetration of the host cell membrane by destabilizing the endosomal membrane. Core particles lacking both VP2 and VP5 proteins are released into the cytoplasm from the endosomal vesicles. In contrast to mammalian host, BTV cores lacking VP2 and VP5 are infectious for invertebrate cells⁸⁶ with the Arginine Glycine Aspartic (RGD) motif of VP7 involved in entry into cells of *Culicoides* species.¹²²

Transcription and Replication

After cell entry, the outer capsid is removed to release a transcriptionally active core particle, which provides a compartment within which the 10 genome segments are repeatedly transcribed by the core-associated enzymes VP1, VP4, and VP6.^{134,139} Ten capped nonpolyadenylated mRNAs are synthesized from the 10 genome segments and are released from the core particle into the host cell cytoplasm where they act as templates both for translation and for negative-strand viral RNA synthesis to generate genomic dsRNAs.^{85,133,134} Transfection of mammalian cells with *in vitro* BTV transcripts also leads to generation of viral proteins and infectious virus particles, as demonstrated by the passage of infectivity in Baby Hamster Kidney cells (BHK) cells.¹² These findings have allowed the development of a helper virus-independent reverse genetics system for BTV and other orbiviruses and for the generation of targeted mutant viruses.^{12,81}

When intact cores isolated from virus particles are activated *in vitro* in the presence of magnesium ions and NTP substrates, distinct conformational changes can be seen around the fivefold axis.⁴⁰ This is interpreted as an outward movement of VP3 and VP7, allowing opening of a pore in the VP3 layer at the fivefold axes through which the mRNAs are extruded. The structural integrity of the core particle appears to be essential for efficient transcriptional activity *in vivo*, but it has been possible to use *in vitro* assays with the three minor proteins of the core to delineate the specific roles of each (see review in 103; Fig. 46.9).

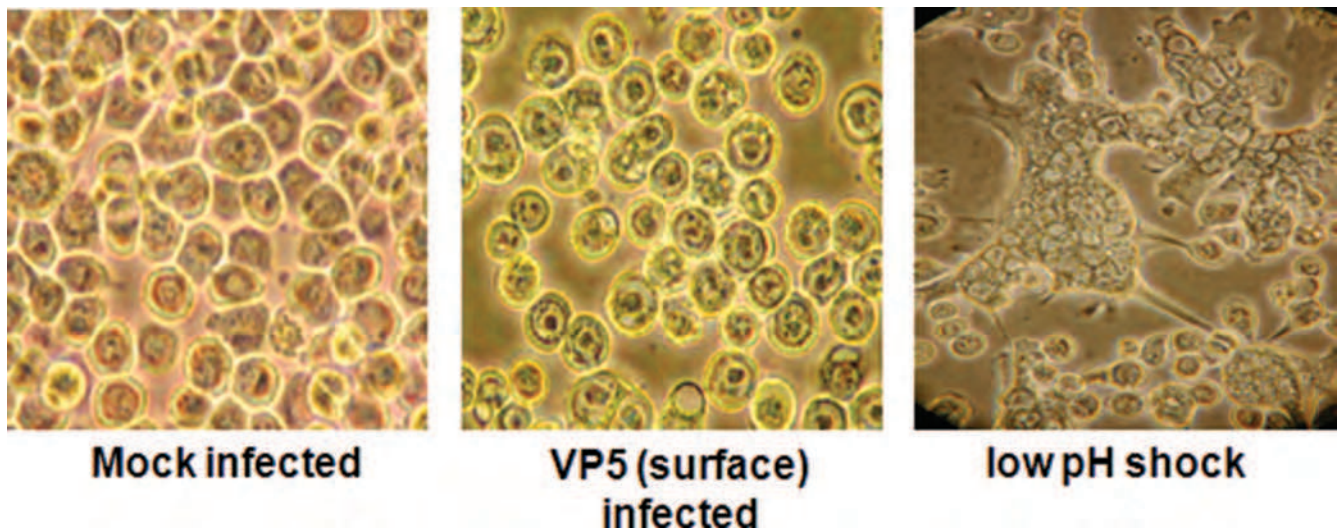


FIGURE 46.8. Fusion activity of VP5: *Spodoptera fugiperda* cells infected for 48 hours with a recombinant baculovirus expressing a chimeric VP5 that is flanked by the baculovirus gp64 signal peptide and the Vesicular Stomatitis Virus (VSV) G anchor domain. Note the formation of syncytium (seen by inverted light microscope) in Sf9 cells after 7 hours low pH shift, but no fusion activity before the pH shift.

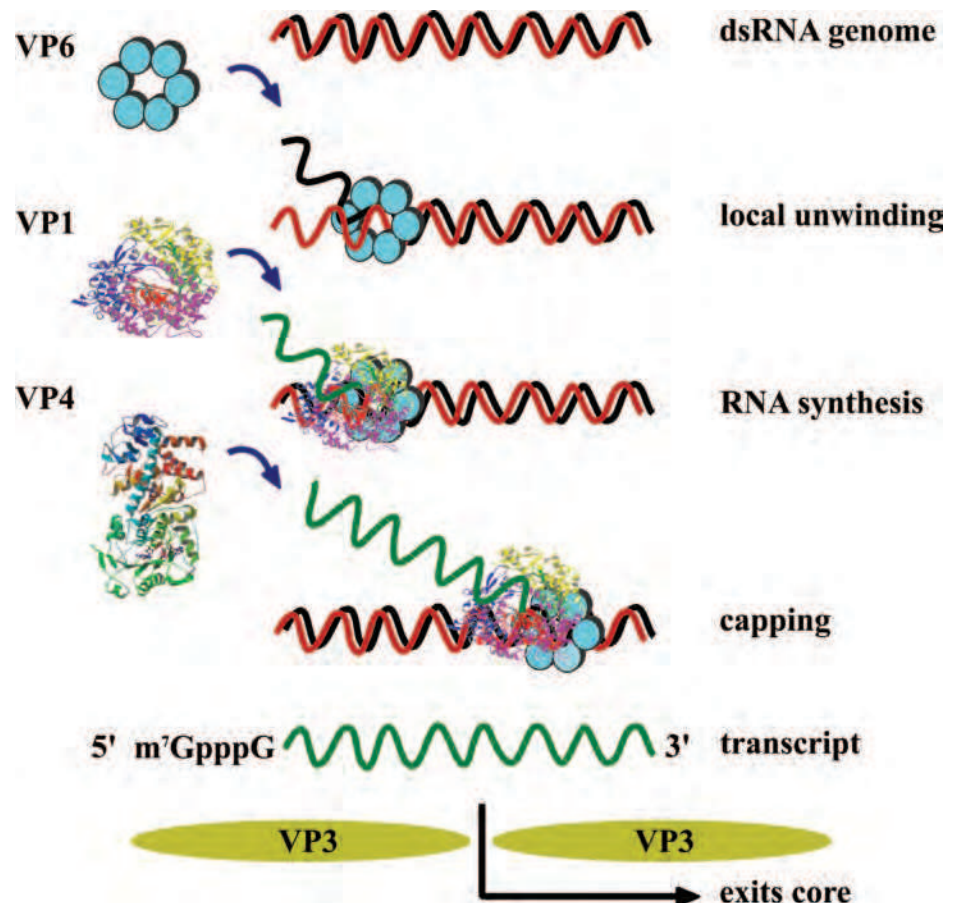


FIGURE 46.9. Schematic diagram summarizing the overall mechanisms of viral RNA synthesis and the role of VP1, VP4, and VP6 proteins in RNA synthesis.

The Largest Protein VP1 Is the RNA-Dependent RNA Polymerase

The first indication that the largest BTV protein, VP1 (149.5 kD) is the virus polymerase protein came from sequence comparison with other DNA and RNA polymerases and from an *in vitro* polymerase assay that used a crude extract of insect cells infected with a recombinant baculovirus expressing only BTV VP1.^{37,131} Subsequent *in vitro* studies have used purified recombinant protein and have shown that VP1 exhibits a processive replicase activity in the absence of any other viral proteins and initiates BTV minus-strand synthesis *de novo*.¹³ Recombinant VP1 copies each of the plus-strand RNA segments fully, from the smallest (822 nt) to the largest BTV plus-strand RNA of 3,954 nt, either individually or simultaneously in a single *in vitro* reaction, to produce a dsRNA duplex that is RNase I resistant, and RNase III sensitive. Synthesis of dsRNAs from capped single stranded RNA (ssRNA) templates is significantly higher than from uncapped ssRNA templates and could be further enhanced by priming with complementary terminal dinucleotide. Some evidence for template specificity was suggested by the fact dsRNAs were not synthesized from nonviral ssRNA templates unless they were fused to specific BTV sequences. These data contrast those of rotavirus and reovirus; in rotavirus the assigned polymerase protein (VP1) is only active in the context of the inner shell protein VP2,¹²⁸ and recombinant reovirus polymerase $\lambda 3$ is generally incapable of synthesizing *de novo* reovirus RNA, although short RNA transcripts were observed.^{113,123}

Three distinct domains of VP1, PD, NTD, and CTD have been synthesized in bacteria and purified in soluble form. The purified PD fragment, but not the NTD or CTD fragments, was able to bind NTP, but none had catalytic activity when tested individually.¹⁴⁰ When all three were mixed together *in vitro*, however, RdRp activity was reconstituted and was shown to depend on the signature catalytic Glycine Aspartic Aspartic (GDD) motif (aa 763–765) within the PD domain. Plausibly, the PD possesses catalytic activity but requires the other two domains to stabilize the overall structure. It is noteworthy that in reovirus $\lambda 3$, the large N- and C-terminal domains form a cage around the centrally placed polymerase domain, holding it rigid and preventing movement during the catalytic cycle.¹²³

The Minor Protein VP4 Is the mRNA Capping Enzyme

Although VP1 has been shown to be active as the viral RdRp, it does not form the methylated cap structure found at the 5' end of BTV mRNAs. Formation of the cap structure requires at least three key enzymatic activities: (1) an RNA triphosphatase (RTase) that hydrolyzes the 5'-triphosphate terminus of the mRNA to a diphosphate; (2) a (GTase) that caps the diphosphate terminus with GMP via a 5'-5' triphosphate linkage, and (3) a guanine-N7-methyltransferase (N7MTase) that adds a methyl group to the N7 position of the blocking guanosine. An additional nucleoside-2'-O-methyltransferase (2'OMTase) is also required for BTV and reovirus transcripts, to methylate

the 2'-hydroxyl group of the ribose of the first nucleotide (namely type 1 cap).

Recombinant, purified VP4 (76.4 kD) synthesizes type 1-like “cap” structures *in vitro* that are identical to those found on authentic BTV mRNA. VP4 is unique as it possesses methyltransferases, guanylyltransferase, and RNA triphosphatase activities in the absence of any other viral protein.^{80,97,98} This is notably different from other viral capping enzymes, for example, those of vaccinia virus, where completion of capping is dependent on a complex of three proteins.¹³⁷ The atomic structure of the protein has revealed how a single protein orchestrates all of these activities.¹¹⁹

VP6 Helicase

Genome replication of the *Reoviridae* is fully conservative and logically requires helicase activity, either to unwind the dsRNA ahead of the transcriptase protein or to separate the parental and newly synthesized RNAs following transcription. For BTV, VP6 has a strong binding affinity for both ssRNAs and dsRNAs, possesses nucleoside triphosphatase activity, and in isolation has the ability to unwind dsRNA substrates *in vitro*.¹¹⁴ Similar to other helicases, VP6 is hexameric and forms ring-like structures in the presence of BTV RNAs.⁶⁵ Using reverse genetics it has been shown that VP6 is an integral part of the transcription complex that is necessary for primary replication and requires the *cis*-acting sequences for replication or packaging.⁸³ How substrates pass from one component of the transcription complex to the next remains a significant question however.

Core transcripts are not produced at equimolar amounts from the 10 segments of BTV⁵⁶; the smaller genome segments are generally the most frequently transcribed, although segment 6 RNA (encoding NS1) is synthesized more abundantly than the smallest segment, segment 10 RNA (encoding NS3).

In vivo, the replication of BTV, like other members of the family, uses a packaged plus-strand RNA as template for the synthesis of a “minus” strand, and then a dsRNA, which remains within nascent progeny particles. The basis of copy choice between the plus- and minus-strand RNAs and the full roles of each protein in the process remain to be defined.

Protein Synthesis and Virus Replication

BTV-specific proteins are detectable within 2 to 4 hours postinfection, and the rate of protein synthesis increases rapidly until 11 to 13 hours postinfection.⁵⁵ In addition to the seven structural proteins of the virion, four virus-encoded nonstructural proteins, NS1, NS2, NS3, and NS3A, are synthesized in BTV-infected cells. Infection of mammalian cells with BTV, in contrast to insect cells, leads to a rapid inhibition of cellular macromolecular synthesis and the induction of a robust apoptotic response triggered by multiple apoptotic pathways.^{90,116}

The large nonstructural proteins NS1 (64 kD) and NS2 (41 kD) are highly conserved (96%) among BTV serotypes (see review 101,108), and they are synthesized abundantly early in infection, suggesting that they are essential for virus replication. The synthesis of NS1 and NS2 in BTV-infected cells coincides with that of two virus-specific intracellular structures, tubules and the viral inclusion bodies (VIBs), respectively, which are characteristics of orbivirus-infected cells.²² In contrast to the amounts of NS1 and NS2 produced, the steady-state levels of the small viral nonstructural proteins, NS3 and NS3A (26

and 25 kD, respectively) are highly variable (from being barely detectable to being highly expressed) and are dependent on the host cell species.

NS1 and Tubules

The 64-kD protein NS1 of BTV constitutes up to 25% of the virus-specified proteins in virus-infected cells and assembles into tubular structures within the cytoplasm.^{53,132} Tubules are characteristic of all orbivirus infection (e-Fig. 46.3) and are formed by helically coiled ribbons of NS1 dimers.⁵⁰

NS1 appears to regulate BTV protein synthesis through binding specifically to BTV mRNAs at the conserved extreme 3' hexanucleotide sequence (5' ACUUAC 3').^{12a} NS1 is essential for virus replication, as NS1 knockout and point mutants introduced by reverse genetics are lethal. NS1 thus appears to be a regulator of viral gene expression, which distinguishes between cellular RNAs with a 3' poly A tail and viral mRNAs by binding directly to the 3' end of viral mRNAs. However, the relationship of this activity to tubule formation, if any, remains to be established.

Virus Assembly Site, Inclusion Bodies, and Role of NS2

Shortly after release from the endosome, core particles become very rapidly associated within “virus inclusion bodies” (VIBs). VIBs initially appear as granular material scattered throughout the cell, but then coalesce to form a prominent perinuclear inclusion.^{14,21,57,63} The major component of VIBs is the NS2, and expression of NS2 alone results in the formation of inclusion bodies that are indistinguishable from the VIBs.^{89,127} Co-expression of core structural proteins has shown that although VP1, VP3, VP4, and VP6 all co-localize with NS2 inclusions in the absence of viral replication, the core surface protein VP7 requires the co-expression of VP3 to be recruited (Fig. 46.10A,B). Electron microscopy using recombinant NS2 and virion proteins in different combination has suggested NS2 associates with assembling particles.^{63,89}

NS2 is phosphorylated in BTV-infected cells by casein kinase II (CKII).⁸⁹ Phosphorylation sites of NS2 have been mapped to two serine residues at positions 249 and 259, and furthermore it was shown that the unmodified NS2 failed to assemble into VIBs, although smaller oligomeric forms readily assembled.⁸⁹ NS2 phosphorylation was not necessary for either its RNA-binding properties or its ability to interact with the viral core proteins, but it may control the level of VIBs formation to provide the matrix for viral assembly.

Consistent with this, when a cell line constitutively expressing NS2 was infected with BTV followed by first treatment with actinomycin D and subsequent addition of bromouridine, the newly synthesized labeled BTV transcripts were identified within the VIBs.⁶³ Purified recombinant NS2 has strong ssRNA binding activity *in vitro*, and BTV RNAs are preferentially bound over nonspecific RNAs^{73,79,126} via *cis*-acting sequences distributed throughout the coding and noncoding regions of the different genome segments.⁷³ Chemical and enzymatic structure probing of the preferentially bound regions has suggested NS2 recognition of unique hairpin-loop secondary structures.⁷³ The RNA binding activity of NS2 could explain how BTV mRNAs are selected from the pool of cellular messages for incorporation into assembling virus particles, but it remains unclear how only a single copy of each genome segment is included in newly formed

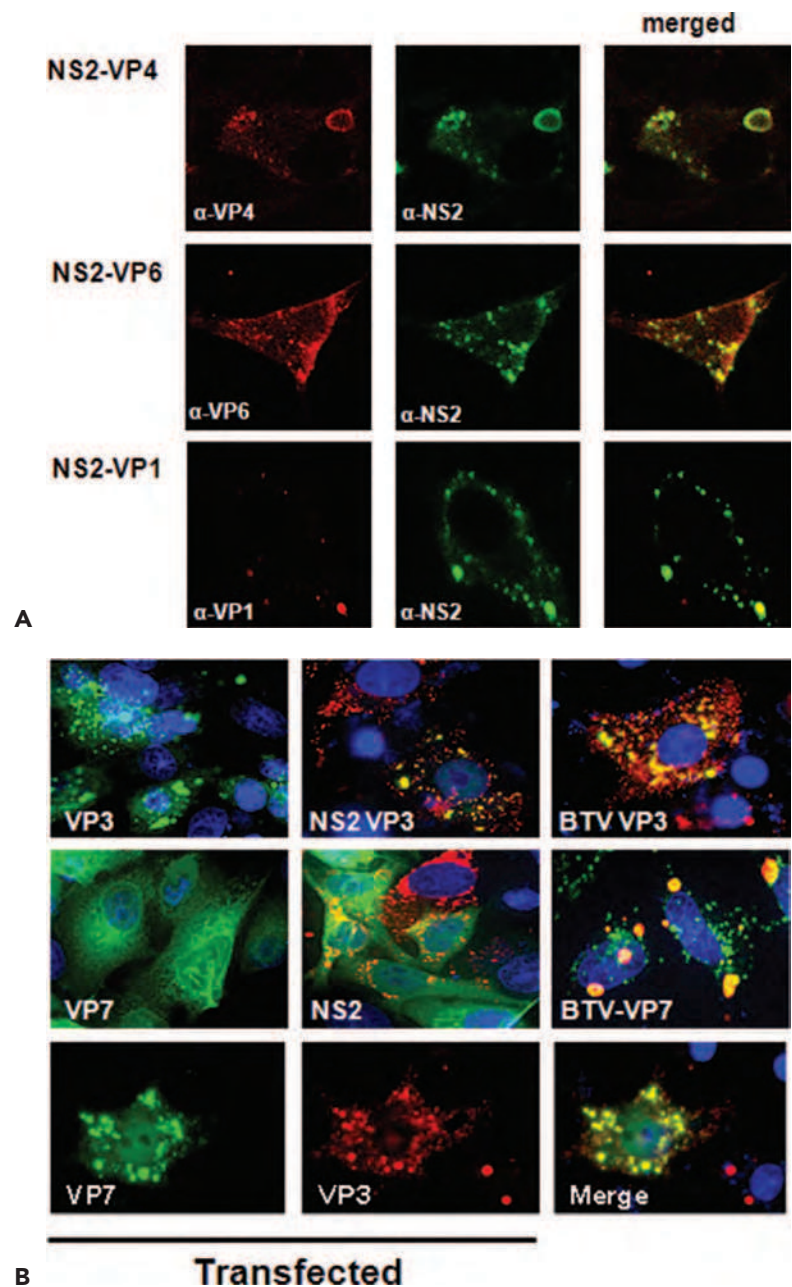


FIGURE 46.10. Interaction of NS2 and viral proteins.

A: Confocal microscopy showing the co-localization of three polymerase complex proteins and NS2 in BTV-infected cells.

B: Same with two major core proteins, VP3 and VP7 with NS2. Note that VP7 co-localizes with NS2 only in the presence of VP3.

C: Schematic showing the interaction of NS2/VIB with BTV proteins and the bluetongue virus (BTV) single-stranded RNA (ssRNA). "P" represents phosphorylation of NS2, which is responsible for formation of viral inclusion bodies (VIBs).

core particles. Plausibly NS2 could bring the viral RNAs into proximity via the formation of decameric complexes.^{15,29} Phosphorylation of NS2 may modulate these activities, VIB formation, and the interaction with other viral proteins or transcripts⁸⁹ (Fig. 46.10C), and following core assembly dephosphorylation could allow disassembly of VIBs and the release of immature virus particles into the cytoplasm.

Requirement for Primary Replication Versus Secondary Replication by Reverse Genetics System

The assembly of a distinct early replicase complex in BTV-infected cells was discovered by using a reverse genetics system. It was shown that repeated transfection of BTV *in vitro* generated transcripts improved the recovery of infectious BTV by approximately 50-fold when compared to single transfection.¹² Stimulation did not require the complete set of transcripts; those encoding VP1, VP3, VP4, VP6, NS1, and NS2 representing the presumptive transcriptase complex (VP1, VP4 and VP6), the major subcore protein (VP3), and the two NS proteins associated with VIBs and BTV protein synthesis sufficed (Fig. 46.11). The same study also suggested that helicase VP6 acted early in replication and that “capped” transcripts were not essential for packaging, as the second transfection could use capped or uncapped transcripts with the same level of rescue. Interestingly, the addition of a transcript encoding VP7 to the primary transfection mix, which would allow completion of the core particle, did not stimulate virus recovery, suggesting that a fully competent replicase complex requires only a VP3-based substructure. These data may indicate that an *in vitro* model of BTV core assembly is possible.

Capsid Assembly

BTV structural proteins have an inherent capacity to self-assemble into core-like particles (CLPs) and virus-like particles (VLPs) that lack the viral genome^{35,36} (e-Fig. 46.4), and their assembly by expression of viral structural proteins in insect cells (see review by 103) has shed much light on the assembly pathway.

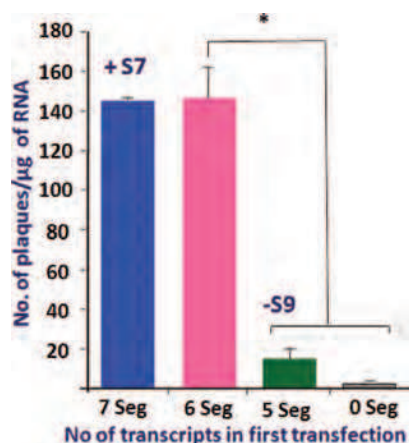


FIGURE 46.11. The recovery of bluetongue virus (BTV) with T7 transcripts. Number of plaques per μg RNA recovered when BHK cells were pretransfected with either 7 transcripts (S1, S3, S4, S6–S9), 6 transcripts (lacking S7), 5 transcripts (lacking S6 and S7), or no (0) transcripts, followed by transfection with all 10 capped transcripts.

VP3 Assembly and Subcore Formation

A key question in VP3 assembly is whether the decamer observed in the core structure⁴² is an identifiable intermediate in the assembly process. Deletion of the dimerization domain of VP3 abolished subcore formation, but decamer formation was not affected.⁶⁴ This suggests that decamers are the first stable assembly intermediate and that decamer-decimer interactions via the dimerization domain drive the assembly of the complete VP3 subcore.

Based on CLP assembly, both VP1 and VP4 have been shown to independently associate with the VP3 layer,^{71,91} and a VP1+VP4 complex also interacts directly with the VP3 decamer in solution.⁶⁴ By contrast, BTV RNAs interact with VP3 *in vitro* very efficiently but fail to associate with VP3 decamers under the same conditions. BTV cores may thus initiate with the complex formed by VP1 and VP4 with VP3 decamers and subsequently recruit the polymerase complexes prior to completion of the assembly of the VP3 subcore (Fig. 46.12). Unlike VP1 and VP4, it has not been possible to confirm the location of VP6 in the core, although it is likely to be at the fivefold axes together with VP1 and VP4. The newly synthesized mRNAs are also probably packaged during the assembly of subcores, and it is likely that RNA replication takes place after subcore or core formation. Recently, using a cell-free *in vitro* translation system, evidence has been obtained of the assembly of BTV RNA-protein complexes *de novo*. Following translation of the core replicase components, the addition of 10 ssRNAs representing noncapped positive-strand copies of each genomic segment leads to assembly into a complete BTV core. Such *in vitro* assembled cores were functional, as the 10 packaged ssRNA molecules were converted to 10 dsRNA genomic segments. Furthermore, reconstituted cores isolated from sucrose velocity gradients were infectious in *Culicoides* insect vector cell culture, generating all the viral proteins and subsequently leading to the recovery of infectious particles.⁷² Using this *in vitro* virus assembly system it should be possible to delineate the role and order of each component in the assembly pathway as well as the requirements for genome packaging, an exciting future prospect.

VP7 Assembly and Core Formation

The mismatch between the number of subunits in the VP3 and VP7 layers of the core poses an interesting problem for an icosahedral structure. Core assembly has been shown to depend on trimer formation, the precise “shape” of the trimers being sufficient to drive the formation of the tight lattice of 260 trimers on the core surface.^{69,70} Sheets of VP7 probably form around different nucleation sites instead of a cascade of trimer associations originating from a single nucleation site, as suggested by structural studies.⁴² A likely pathway of core assembly is therefore that a number of strong VP7 trimer-VP3 contacts act as multiple equivalent initiation sites and that a second set of weaker interactions then “fill in the gaps” to complete the outer layer of the core. The “T” trimers (of the P, Q, R, S, and T trimers) at the threefold axes of symmetry act as nucleation, whereas “P” trimers that are farthest from the threefold axis and closer to the fivefold axis are the last to attach.⁷⁰

Assembly of the Outer Capsid

Following the assembly of the core particle, the viral outer capsid proteins, VP2 and VP5, are added. Cryo-EM experiments have aided our understanding of how the outer capsid

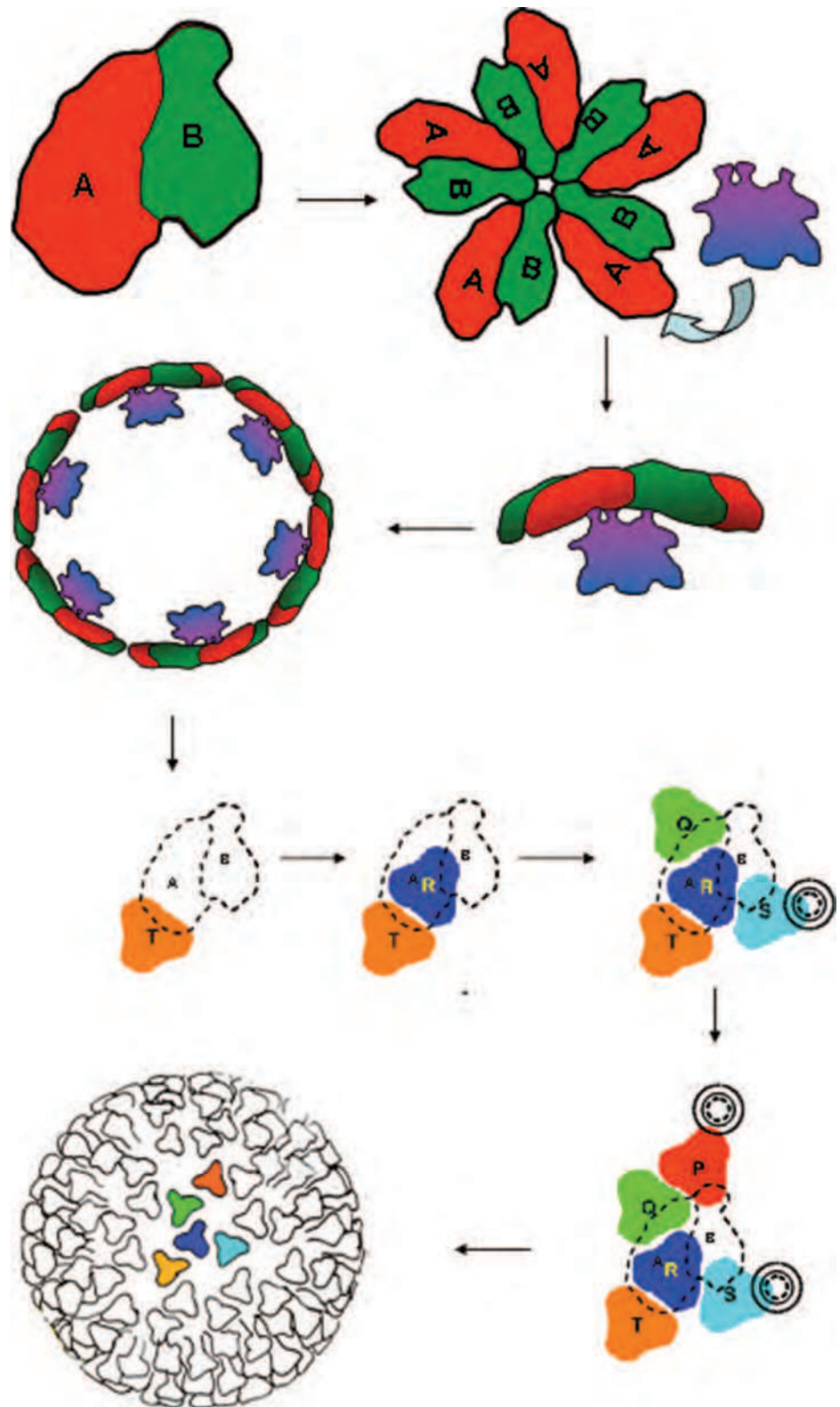


FIGURE 46.12. Schematic diagrams showing the growing VP3 decamers and assembly of polymerase complex with the VP3 decamer prior to the formation of VP3 protein shell and showing the possible pathway of VP7 trimer assembly: **Left**, the positions of VP7 protomeric unit and position of VP3 layer, and **right**, the sequential assembly of various VP7 trimers on VP3 scaffolds.

proteins interact with the outer VP7 layer of the core,^{91,144} but our understanding of where in the cell these proteins are added to the core is still limited. The addition of VP2 and VP5 abolishes transcription activity so the addition of the outer capsid is likely to be a highly regulated process to prevent premature shut-off of transcription. Baculovirus expression of cores with VP5 and VP2 leads

to assembly of a VLP mimicking the authentic virions.³⁵ The VP7 trimers of the core serve as the platform for the deposition of the VP2 and VP5¹⁴⁴ (e-Fig. 46.5). Each triskelion VP2 trimer interacts with four VP7 trimers at its underside and via the hub that connects to the VP7 “Q” trimer by a wall of density. Most VP7 molecules, the exception being the VP7 at the icosahedral

threefold axis (T type), are covered by the connections, and the top of the T trimer is clearly exposed to the exterior.

The globular-shaped VP5 trimers fill the gaps created by the three legs of the VP2 triskelion and sit right above the type II and III channels of the core (e-Fig. 46.6). The VP5 trimer makes relatively few weak interactions with its adjacent VP7 trimers, possibly to permit conformational changes of VP5 during the penetration process.

Overall, the principal protein–protein contacts of two outer capsid proteins of the BTV appear to be with the outer layer of the core rather than with each other, and this may be related to the distinct steps mediated by each protein during virus entry into uninfected cells.

Trafficking, Maturation, and Egress of Progeny Virions

Although the core particles are assembled in VIBs, the assembly of VP2 and VP5 onto the core to form mature infectious virus particles does not occur within these structures.^{39,40} Biochemical evidence has confirmed that the outer capsid proteins VP2 and VP5, an internal core protein VP6 and the nonstructural glycosylated NS3 protein, all interacts with membrane raft domains.

Interaction of Raft with BTV Proteins

Raft domains occur in both internal vesicular membranes, and the plasma membrane of cells and their disruption with methyl beta cyclodextrin alters the distribution of VP5 and NS3 and decreases the relative BTV viral titer.⁸ VP5 possesses a highly conserved sequence (WHXL) that is present in the Soluble N-ethylmaleimide sensitive attachment protein receptors (SNAREs) regulatory protein Synaptotagmin I (Syt1), and disruption of this sequence by alanine scan perturbed the association of VP5 with lipid rafts.⁸ The SNARE domains of cellular proteins interact with the negatively charged lipid phosphatidylinositol (4,5) bisphosphate [PI(4,5)P₂], present in membrane rafts,⁵¹ and depletion of cellular PI(4,5)P₂ by expression of phos-

phatase 5ptaseIV or its relocation to endosomal-like structures by Arf6/Q67L expression inhibited normal BTV maturation despite not altering BTV protein expression levels.¹⁰ EM showed the attachment of viral particles to the outer surface of vesicle-like structures in the cytoplasm that were absent in PI(4,5)P₂-depleted cells. NS3 is normally localized to the Golgi complex, and it is likely that reduction in cellular PI(4,5)P₂ perturbs the generation of vesicles from the distal Golgi stack. NS3 also interacts with both outer capsid proteins and core surface protein VP7,¹⁰ suggesting that NS3 might bring the newly assembled BTV core and the outer capsid components together for formation of mature virion particles. PI(4,5)P₂ plays an important role in the generation and trafficking of intracytoplasmic vesicles via the cytoskeleton, and in BTV-infected cells virus particles are found associated with the vimentin intermediate filaments,²² the disruption of which prevents normal virus release.^{7,9} The data are consistent with a role of internal vesicular membranes in providing a scaffold for virus assembly and the use of those vesicles for trafficking assembled virus to the plasma membrane.

NS3 and Cellular Exocytic Pathways in BTV Release

The canonical view of the release of nonenveloped viruses from infected cells is that of cell lysis. However, there is an increasing appreciation that complex trafficking and budding strategies are also involved. In the case of BTV, both mechanisms appear to act with a preponderance of lysis during mammalian cell infection, but essentially none during infection of vector insect cells.^{16,17,58}

The only membrane proteins that are encoded by BTV are NS3 (229 aa) and its shorter form NS3A (216 aa), which lacks the N-terminal 13 amino acids^{34,68} (Fig. 46.13). Both proteins have been found associated with smooth-surfaced, intracellular vesicles.⁵⁹ NS3/NS3A proteins comprise a long N-terminal and a shorter C-terminal cytoplasmic domain, which are connected by two transmembrane domains and a short extracellular domain.^{2,6,143} Co-expression of NS3 and NS3A with baculovirus-expressed VLPs (acting as surrogates

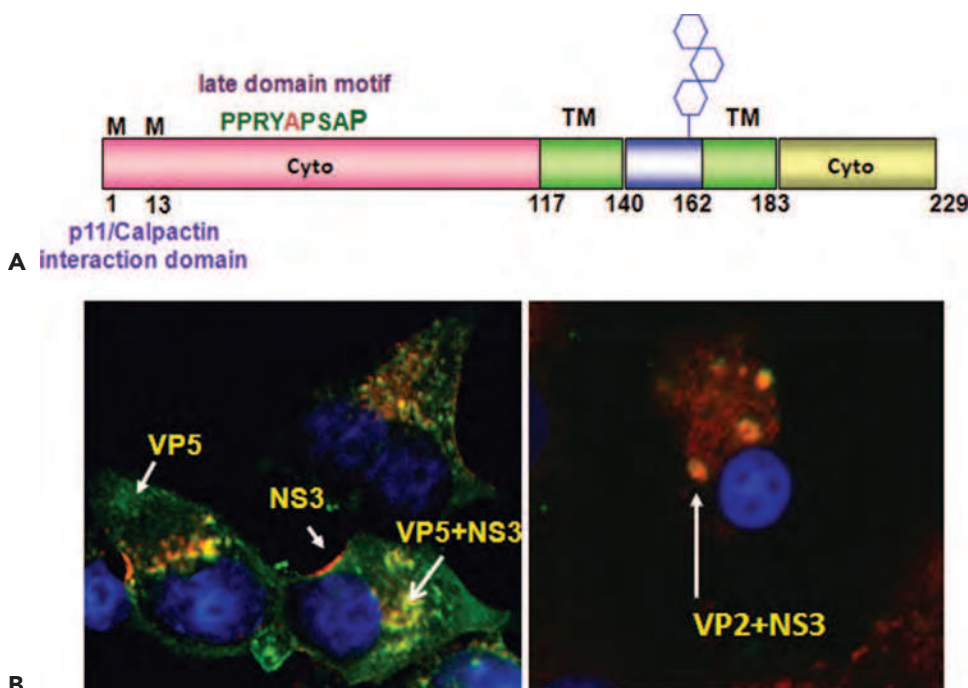


FIGURE 46.13. BTV NS3 protein. **A:** A schematic diagram showing the organization of NS3 protein domains. BTV NS3 is an integral membrane protein with two transmembrane domains (TMs) and contains the two late domain motifs PPXY and PS/TAP that recruit the components of cellular ESCRT complexes and N-terminal residues that recruit cellular calpactin p11. **B:** Confocal microscopy showing the co-localization of NS3 and two outer capsid proteins.

for authentic virions) stimulated VLP release, and NS3 protein has been observed at the site of VLPs release.⁵⁹ The NS3 protein of BTV and AHSV is cytotoxic when expressed in mammalian or insect cells,^{34,136} and it has been suggested that it may function as a viroporin, facilitating virus release by inducing membrane permeabilization.⁴⁴

Using the yeast two-hybrid analysis, the cytoplasmic N-terminal region of NS3 specifically interacts with S100A10,⁶ a Ca^{2+} insensitive member of the S100 family that forms a heterotetrameric complex with two heavy chains of annexin II (also known as annexin A2) to form the calpactin complex in cells. Calpactin

is involved in trafficking of proteins and membrane targeting (reviewed in 100). The cytoplasmic domain of NS3, situated at the C-terminus specifically with the BTV outer capsid protein VP2 suggesting that NS3 facilitates virus engagement with host cell membrane trafficking machinery (Fig. 46.13).

Using reverse genetics, which allows the introduction of mutations into the virus genome it was possible to investigate the roles of NS3 and NS3A in the virus replication.¹⁷ A virus synthesizing only NS3 but not NS3A was able to propagate in and release from mammalian cells efficiently (Fig. 46.14A–C). However, growth of a mutant virus expressing only NS3A was

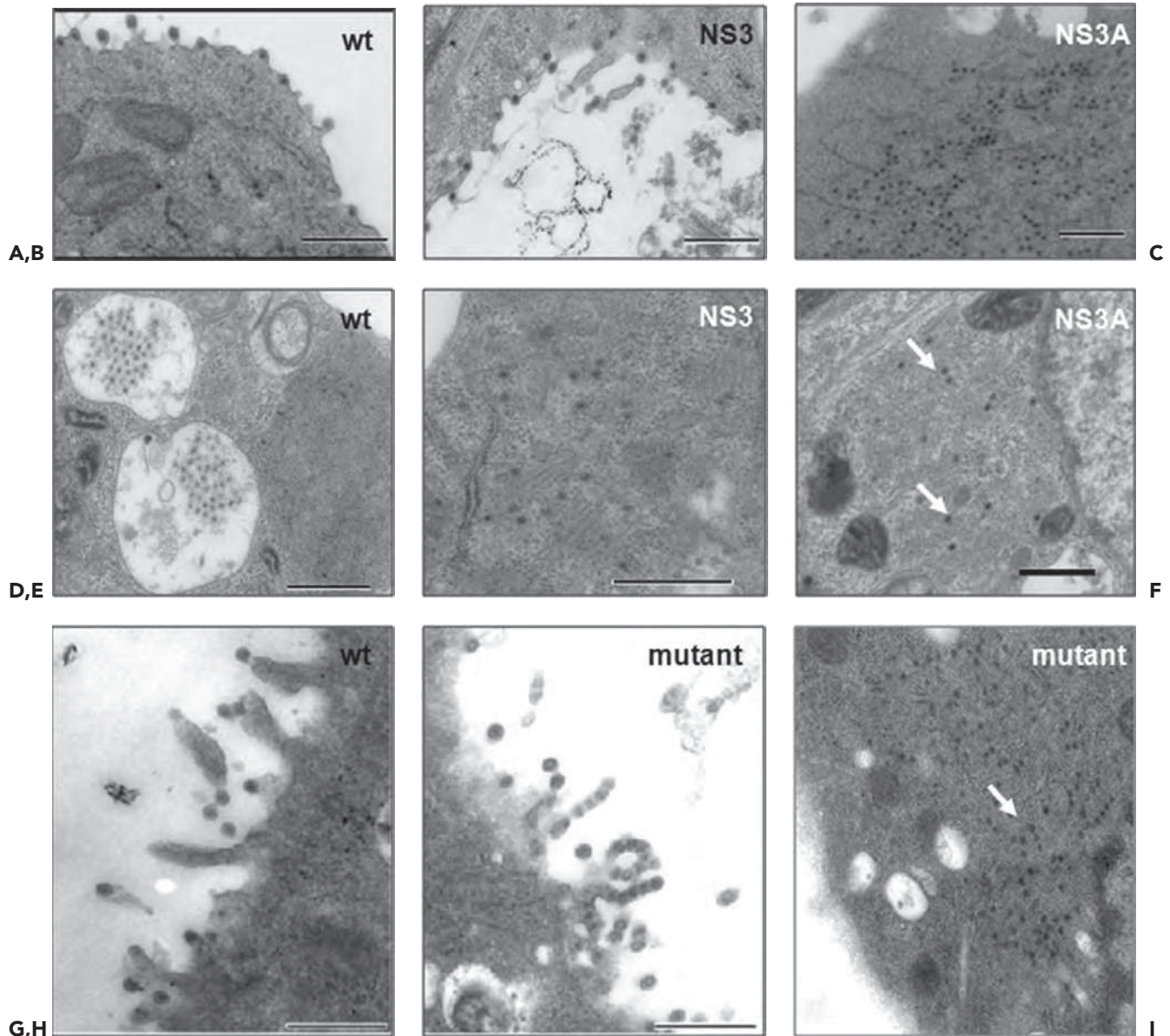


FIGURE 46.14. NS3 mutant viruses affect virus trafficking and egress. Electron micrograph of Baby Hamster Kidney cells (BHK) cell section infected with wild-type (A), or mutant BTV expressing only NS3 not NS3A (B), or only NS3A not NS3 (C). Parallel infections in vector insect cells with wild-type (D), NS3 (E), or NS3A (F) mutant viruses. Election micrographs of infected Baby Hamster Kidney cells (BHK) cells sections with wild-type (G), mutations in Tsg101 (H) and VP2 (I) binding domains of NS3. Note the changes in virus trafficking and egress in each mutant virus versus wild-type virus infection. (Reprinted from Celma CC, Roy P. A viral nonstructural protein regulates bluetongue virus trafficking and release. *J Virol.* 2009;83(13):6806–6816, with permission.)

severely attenuated, although protein expression, dsRNA synthesis, and particle assembly in the cytoplasm were observed. Two of three single amino acid substitutions in the N-terminal 13 residues of NS3 that interact with S100A10/p11 showed phenotypically similar effects. In insect cells virus replication was apparent, albeit with reduced titer, but whereas wild-type particles were found predominantly in cytoplasmic vesicles in insect cells, mutant viruses were scattered throughout the cytoplasm and not confined to vesicles (Fig. 46.14D–F). Therefore, the extreme amino terminus of NS3 is required for trafficking in mammalian cells via interaction with S100A10/p11, whereas both NS3 and NS3A were required for efficient BTV growth in insect cells.

NS3 also possesses a late (L) domain motif (PSAP) similar to those found in budding enveloped viruses (see Fig. 46.13).^{16,142} L domains engage with the vacuolar protein sorting pathway, a cellular budding network that gives rise to multivesicular bodies (MVBs).³³ BTV NS3 interacts with human tumor-susceptibility gene 101 protein (Tsg101), and both NS3 and NS3A of BTV and AHSV bind *in vitro* with similar affinity to human Tsg101 and also to its ortholog from *Drosophila melanogaster*. This interaction is mediated by the conserved PSAP motif in NS3. Mutations at PSAP motif by RG systems left nascent BTV particles tethered to the cellular membrane (Fig. 46.14G,H).¹⁶ Similar effects were seen with a mutant virus in which VP2 interacting C terminal sequences of NS3 were altered (Fig. 46.14I). BTV morphogenesis apparently makes use of multiple egress pathways, one of which is the NS3-Tsg101 interaction, whereas the second is NS3-mediated virus trafficking via S100A10/p11. Presumably these were acquired by the virus during replication in mammalian cells with the virus using NS3 as a multifunctional adaptor protein to bridge the gap between newly formed virus particle and the cell components that enable virus egress.^{6,16}

EFFECTS ON HOST CELLS

BTV infection causes hemorrhagic disease in ruminants and induces cell death through apoptosis. In BTV-infected mammalian cells, both the biochemical and morphological hallmarks of apoptosis are observed within 24 hours of infection, including activation of nuclear factor kappa B (NF- κ B), caspase-3, DNA fragmentation, membrane blebbing, and cellular shrinkage (e-Fig. 46.7). Those changes are not observed in insect (*Culicoides*) cells, although these cells are efficient for BTV replication.⁹⁰ In cell culture, both extrinsic (caspase-8 activation) and intrinsic (caspase-9 activation) pathways play roles in BTV apoptosis. BTV-induced cell death appears to be predominantly due to apoptosis rather than necrosis, and a correlation may exist between CPE, apoptosis of host cells, and pathogenesis.¹¹⁶ Microscopic analysis of primary cultures of endothelial cells from sheep and bovine lungs infected with BTV suggests that apoptosis may contribute to the pathogenesis of bluetongue disease in the mammal.¹⁹ BTV infection induces an early and transient NF- κ B response through the degradation of I κ B α , and virus titers are higher in the presence of an NF- κ B inhibitor (SN50), indicating that NF- κ B has a role in initiating the antiviral environment. BTV infection also induces the translocation of interferon regulatory factors (IRF-3 and IRF-7) into the nucleus, although as with the NF- κ B response, they are not sustained.

The morphology of cellular microfilaments changes extensively during infection (see review 20) in keeping with observations that BTV proteins, virus particles, VIB, and tubules are intimately associated with the intermediate filaments of cytoskeleton.^{7,22,63}

CLINICIAN SIGNS AND PATHOGENESIS

Orbiviruses produce a spectrum of disease, from subclinical infection to high morbidity and mortality, depending on virus strain and host species.⁷⁷ Following introduction by insect bite, virus migrates to local lymph nodes and then subsequently spreads to spleen, thymus, and other lymph nodes (Fig. 46.15). Orbivirus infections can induce prolonged cell-associated viremias in vertebrate hosts. Prolonged bluetongue viremias are particularly notable in cattle, which can serve as asymptomatic virus reservoirs. After experimental inoculation of calves, infectious virus can be detected in blood for up to 8 weeks and viral nucleic acid for up to 20 weeks.⁷⁷ BTV binds to glycoporphins on the surface of bovine and ovine erythrocytes,²⁰ where it can remain in an infectious state for prolonged periods, possibly for the lifetime of the red blood cell.⁷⁷

The role of leukocytes in cell-associated viremias is less certain. BTV has been recovered from bovine mononuclear cells during the early stage of infection.⁷⁷ Active replication of virus in these cells, however, appears to be restricted to proliferating cells. *In vitro* studies of bovine lymphocytes have shown multiple subpopulations to be susceptible to infection. Helper T-cell subsets replicate BTV to high titer and undergo cytopathic infection, whereas cytotoxic suppressor and null cells become persistently infected without lysis. Monocyte-macrophages appear to be targets for infection with AHSV.

BTV and related orbiviruses (e.g., AHSV and Epizootic Hemorrhagic Disease Virus (EHDV)) result in disease with pathophysiologic features resembling other viral hemorrhagic fevers.^{67,78,129} Infection of endothelial cells is followed by infection of vascular smooth muscle cells and

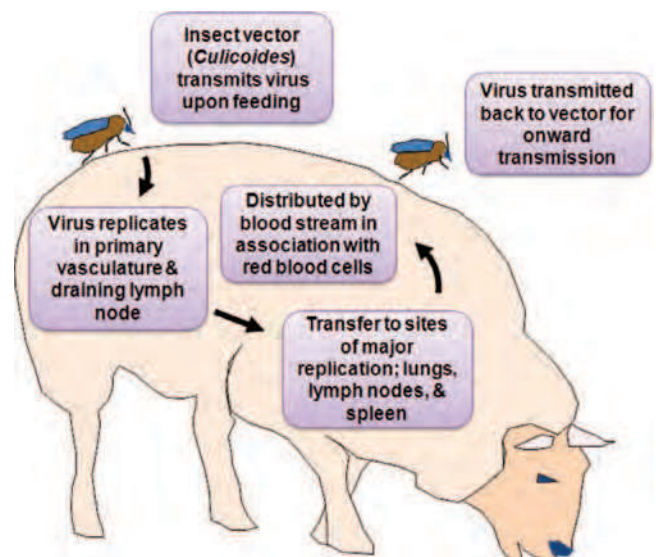


FIGURE 46.15. Transmission cycle of bluetongue virus (BTV) between the ruminant host and *Culicoides* vector.

pericytes, which then undergo lytic infections, resulting in virus-induced vascular injury and a cascade of pathophysiologic events characterized by capillary leakage, hemorrhage, and disseminated intravascular coagulation. Clinically these events are manifested by edemas, hemorrhages of blood vessels, conjunctiva and skin, hypotension, and shock. Other clinical features include fever, lameness, excessive salivation, and congestion. However, the swollen cyanotic tongue that gives bluetongue its name is rare. Mortality rate is variable and dependent on breed and species. There are some correlations between endothelial infection and severity of disease; sheep show the most severe clinical signs and generally have extensive endothelial infections, but cattle, where infections are often subclinical, generally have only minimal endothelial.⁷⁷

Both BTV and EHDV can cross the placenta, and infection of pregnant cattle and sheep can result in fetal infection, abortion, or congenital anomalies.⁷⁷ Live-attenuated BTV vaccines and BTV contamination of vaccines for other animals have been shown to be a principal cause of teratogenesis.⁹³

Similar to the differences in pathology seen when BTV and EHDV infect sheep and cattle, AHSV affects members of the Equidae family differently; horses are the most severe disease, whereas donkeys and mules generally show mild clinical signs. Zebras, a natural reservoir for AHSV, exhibit viremia but do not manifest clinical signs of infection. Severity of AHS disease in horses ranges from a pulmonary form with close to 100% mortality to African horse sickness fever, which is characterized by high temperature for 1 to 3 days but 100% recovery. The pulmonary form is most common in outbreaks among naïve populations of animals.

EPIDEMIOLOGY

Evidence for antigenically different types of BTV with strain variation in virulence was first reported in 1948.⁹² For a long time the epidemiology of BTV was focused on animals with clinical signs. However the introduction of simple serologic tests in the 1970s led to the recognition of the high prevalence of subclinical infections of BTV and other orbiviruses in ruminants throughout the tropics and subtropics. As with many arbovirus transmitted diseases, outbreaks are highly dependent on the density of vector and vertebrate hosts and whether the vertebrate host is immunologically naïve. In endemic zones, infections are generally subclinical, but in naïve populations large epizootic outbreaks with severe disease are observed. More recently, sequencing studies reveal accumulation of point mutations that suggest that BTV strains currently circulating in Asia and Africa may have become separated a long time ago.⁹⁵

Although BTV has been detected worldwide, in the past Europe has experienced only infrequent and short-lived incursions of the disease. However, since 1998, several different BTV serotypes have emerged in Europe, some of which can now be considered endemic. The repeated introduction and establishment of these viruses, together with their persistence over several years, has resulted in the deaths of several million animals and provides evidence for an epidemiologic *step-change*.⁸⁴ The later outbreak of BTV serotype 8 in Northern Europe, which started in 2006, was the result of a separate introduction of the virus. The route of introduction is unclear, but the outbreak

strain shows most sequence similarity to one found in Sub-Saharan Africa.⁷⁵

Earlier serologic surveys have shown that many BTV serotypes (including those that have recently caused outbreaks in the Mediterranean region) have been present on the periphery of Europe for several decades, most notably in sub-Saharan Africa, Cyprus, Turkey, and the Middle East.⁹⁶ The reason for the concentrated emergence around 1998 is therefore unclear. Sequence data indicate separate origins and routes of introduction for each of the BTV strains that emerged in Europe; therefore, a single change in the virus or trade patterns can be ruled out. One possibility that has been raised is that climate change may have contributed to changes in the period of activity and size of vector populations.³⁸ Certainly the assumption that *Culicoides obsoletus* and *C. pulicaris*, which are more common in North and Central Europe than the African vector *Culicoides imicola*, were unable to transmit virus sufficiently to sustain a large outbreak has proved wrong.

AHSV is confined mainly to the African continent, although as with BTV prior to 1998, AHSV has caused sporadic, short-lived outbreaks of disease in Europe and the Middle East. A previous outbreak of AHS (1987–1991), caused by an infected zebra, was limited to the south west of the Iberian Peninsula.

In conclusion, molecular epidemiology has provided us with tools for the analyses of geographic variants in orbiviruses and their relationship to ecosystems and arthropod vectors. From recent studies based on a European BTV scenario, it could be concluded that (a) weather patterns play an important role in transmission and (b) BTV can be transmitted by a wider range of vector species than previously realized.

IMMUNE RESPONSE

Neutralizing antibodies are produced within 8 to 14 days of natural BTV infections.³² Very good correlation is found between the induction of neutralizing antibodies against the major outer capsid protein VP2 and protection of sheep against homologous challenge.^{54,109} Infection of sheep and cattle with two serotypes confers protection against other serotypes that had not been included in the inoculums.¹¹⁸ Similarly, antibodies raised with a mixture of recombinant VP2 proteins of various serotypes not only neutralized the infectivity of the homologous strains but also cross-neutralized certain heterologous viruses.¹³⁰ It is possible that cross-reactive epitopes exist on VP2, but other mechanisms may also be involved in this cross protection.

In addition to antibody response, cell-mediated immunity (CMI) also plays a role in recovery from infection and protection against reinfection. Cytotoxic (CD8+) cells responsive *in vitro* to BTV antigen were demonstrated in the blood of cattle and sheep during the first week of infection and peaked at 2 weeks after infection.²³ Immunity was restricted by the major histocompatibility complex (MHC) and thus was host specific. Further evidence for CMI in sheep has been obtained by demonstrating cytotoxic T-lymphocyte (CTL) responses and protection of recipient twins by passive transfer of immune lymphocytes depleted of B cells.¹¹⁸ These data were also supported by *in vitro* studies, and BTV antigen-specific ovine T-cell lines have been shown to become stimulated by a number of

different BTV serotypes.¹²¹ Despite this, the specificity and location of antigenic sites for T cells are still poorly understood. Studies of BTV in mice identified cross-reactive CTL, but other studies have suggested that serotype-specific CTL responses may occur as well.¹¹⁸ Ovine T-cell lines established from sheep immune to BTV-1 showed serotype specificity and were directed against antigenic sites on the VP2 outer capsid protein,¹²⁰ but other T-cell lines reacted with antigens localized on VP5 and inner core proteins. Indeed, when expressed individually, each of the structural and nonstructural BTV proteins has been shown to induce a CTL response.⁶² Antibody-dependent, cell-mediated cytotoxicity may also play a role in protective immunity.⁶²

VACCINES

Live-attenuated BTV vaccines have been developed by serial passage in embryonated chicken eggs; because of the number of multiple serotypes, multivalent vaccination has generally been used in endemic areas.¹ Similarly, live-attenuated virus vaccines for nine serotypes of AHSV have been developed.²⁵ These are administered as doses of two polyvalent vaccines consisting of a total of seven serotypes of the nine serotypes and are considered to induce cross-protective immunity to remaining one or two serotypes. Optimal immunity and protection from disease, however, is best provided by serotype-specific vaccines.²⁵ This was demonstrated in an AHSV outbreak in Spain, where polyvalent vaccines failed to give protective immunity against AHSV-4, the serotype responsible for the disease.²⁵ Despite their success in endemic areas, the use of attenuated vaccines has some drawbacks. Teratologic effects as a result of vaccination with attenuated BTV are well documented.^{61,93,111} Furthermore, viremia following vaccination in both laboratory experiments and in the field has been sufficient for the vaccine strain to be transmitted.^{28,111} Due to the segmented nature of the virus genome, reassortment of genes between strains that co-infect the same animal is possible.¹¹⁷ This has also been observed in the field: A BTV-16 strain circulating in Italy in 2002 was found to be a reassortment between BTV-2 and BTV-16 live-attenuated vaccine strains.⁵ Therefore, although attenuated vaccines for BTV may offer a route to control disease, they are not suitable for a program designed to eradicate the disease in an area.

Due to the proven effectiveness of naturally attenuated vaccines, reverse genetics technology has now been exploited to rationally design vaccines for BTV and AHSV. To this end, BTV mutants with deletions in essential genes have been recovered by growth in complementing cell lines. The growth properties of these mutant viruses were consistent with the necessary characteristics for a disabled infectious single cycle (DISC) vaccine.⁸³ Vaccination with the DISC virus strains abolished viremia, and detectable clinical disease in sheep following the virulent virus challenge.⁸² The advantage of a DISC vaccine over traditionally attenuated viruses is that the mutations limiting virulence are defined and the challenge virus results in no detectable viremia in infected animals; therefore, the possibility of reassortment is reduced and it is possible to distinguish between vaccinated and convalescent animals. Although still experimental, DISC vaccines could offer a promising alternative to currently available BTV vaccines.

Recently, chemically inactivated BTV vaccines were used for mass vaccination of sheep and cattle in many countries in

Northern Europe. These inactivated virus vaccines have been demonstrated to be safe and immunogenic in animals but are currently only available for a limited number of serotypes.¹¹⁰ Inactivated vaccines, provided quality control is rigorously implemented, are a significant advance on the attenuated vaccines. However, vaccine production involves the growth of large amounts of infectious virus prior to inactivation.

The recent European BTV outbreaks and the need to distinguish vaccinated and convalescent animals have also led to development of pox virus-based vaccines for BTV. These vaccines are based on the expression of the virus VP2 protein, and the canarypox vector has been efficient at eliciting protective response in sheep.¹¹

Extensive clinical trials have been carried out in recent years with baculovirus-generated recombinant BTV proteins, assembled as CLP and VLP.¹⁰⁴ Initial studies using unpurified VP2 and VP5 proteins of BTV-10 have demonstrated that VP2 alone at high doses ($>100 \mu\text{g}/\text{dose}$) could give protection in sheep against virulent virus challenges, whereas much less VP2 was needed for complete protection when mixed with a low amount of VP5 ($\sim 20 \mu\text{g}/\text{dose}$), suggesting that the two proteins acting synergistically are probably sufficient for vaccine development. Complete protection against AHSV infection was achieved when horses were vaccinated with as little as $5 \mu\text{g}$ of baculovirus expressed recombinant AHSV-4 VP2.¹⁰⁵

Recombinant VLPs, consisting of VP2, VP3, VP5, and VP7, were assessed in vaccination trials with the rationale that VLPs are biologically and immunologically similar to virions and elicit high titers of neutralizing activity against the homologous and, to some extent, heterologous BTV serotypes. The protective activities of VLP vaccination have been assessed in a number of vaccination trials using susceptible Merino sheep. The data demonstrated that long-lasting protection against homologous BTV challenge could be achieved by vaccination with VLP (using as little as $10 \mu\text{g}$ of VLP, of which VP2 is only $1\text{--}2 \mu\text{g}$). Some cross-protection was also achieved, depending on the challenge virus and the amounts of antigen used for vaccination.¹⁰⁶ More recently, a number of VLPs for European serotypes have been prepared and strong protection against virulent virus challenge in a variety of European sheep breeds.¹¹⁵ In contrast, sheep trials with CLPs which consists of only VP3 and VP7 indicated that only partial protection (with only slight fever) against virus challenge could be achieved. Animals showed strong group-specific antibody responses but, as expected, no neutralizing antibodies. Because CLP are conserved across the various serotypes, they could have potential as a candidate universal vaccine, which could at least mitigate bluetongue disease and inhibit virus spread.

PERSPECTIVE

Significant progress has been made in recent years in understanding the structure and biochemistry of the BTV replication cycle. The function of BTV proteins as enzymes in the transcription complex, as structural proteins in capsid assembly, and as mediators of host-virus interaction—especially in virus entry, egress, and pathogenicity—have all been described. BTV is thus very well characterized, genetically and structurally, and it is reasonable to suppose that other Orbiviruses will have similar core functions. A recent development of some significance

for the future has been the development of a reverse genetics system for the virus, which will allow these molecular functions to be confirmed and refined in the context of a replicating virus. It should also allow the development of precisely engineered attenuated vaccines capable of inducing a vigorous immune response thereby preventing infection while excluding the possibility of reversion or reassortments that limit current live vaccines. Therefore, the application of the findings of basic virology should result in vaccine improvement, disease prevention, and management. Significant challenges, however, remain. Most of what has been learned of BTV and related Orbiviruses has focused on the replication in mammalian cells, principally cells in culture. The replication and spread of BTV in whole animals is less studied, and the replication and spread in *Culicoides* species even less so. These should improve with the development of a tractable small animal model for the former and the whole genome sequence of *Culicoides* for the latter. The identification of host cell factors critical to BTV replication may be important here as they could inform the construction of a suitable transgenic model and indicate why certain species of *Culicoides* are preferred for transmission. Assuming developments in these areas, the whole biology of BTV infection, including pathogenesis, breed susceptibility, and transmission should become as well understood as the molecular aspects described. When they do, the mechanisms of virus evolution and emergence, and the roles played by each host in the process should become apparent. As for the application of basic virology, it is likely that these developments too will result in better control of orbivirus disease in the future.

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Retroviridae

Taxonomic Classification

- Alpharetroviruses
- Betaretroviruses
- Gammaretroviruses
- Deltaretroviruses
- Epsilonretroviruses
- Lentiviruses
- Spumaviruses
- Evolutionary Relationships
- Transforming Viruses

Virion Structure

- Virion Proteins

Organization of the RNA Genome

Overview of the Life Cycle

- Changes in the Viral Genome

The Virus Receptors

- Alpharetrovirus Receptors
- Betaretrovirus Receptors
- Gammaretrovirus Receptors
- Deltaretrovirus Receptors
- Lentivirus Receptors

Penetration and Uncoating

Reverse Transcription

- Steps in Reverse Transcription of the Retroviral Genome
- Biochemistry and Structure of Reverse Transcriptase

Recombination

- Models for Recombination

Integration of Proviral DNA

- Unintegrated DNA Forms
- Entry into the Nucleus
- Structure of the Provirus
- Biochemistry of Integration
- Viral *att* Sites
- Structure of the Integrase
- Preintegration Complex
- Host Proteins and Integration
- Distribution of Integration Sites

Expression of Viral RNAs

- Overview of Viral RNA Synthesis
- Initiation of Transcription
- Beginning and Ending the RNA
- RNA Processing

Translation and Protein Processing

- Gag* Gene Expression
- pro* Gene Expression
- pol* Gene Expression
- env* Gene Expression
- Other Viral Gene Products

Virion Assembly

- Assembly of C-Type Virions
- Assembly of B- and D-Type Virions
- Gag and Virion Assembly
- Virion Assembly *In Vitro*
- Virion Size
- Incorporation of Other Proteins into Assembling Virions
- Host Proteins in the Virion

RNA Packaging

- Gag Sequences Important for Packaging
- RNA Sequences Important for Packaging
- Dimerization of the Viral Genome
- Incorporation of tRNA Primer
- tRNA Primer Placement

Protein Processing and Virion Maturation

- Activation of the Protease
- Protease Structure and Function
- Protease Inhibitors
- Processing of the Gag Precursor
- Processing of the Gag-Pro-Pol Precursor
- Processing of the Env Precursor
- Morphological Changes upon Virion Maturation
- Structure of Virion Core: CA Packing

Resistance to Retrovirus Infection: Host

Restriction Factors

- Receptor Blockade by *Fv4*
- Early Block to Infection by *Fv1*
- Early Block to Infection by Trim5a
- Deamination of Viral DNA by the APOBECs
- Blocking Early Events in Monocyte Lineage Cells by SAMHD1
- Elimination of Viral RNAs by ZAP
- Trapping Virion Particles on the Cell Surface by Tetherin
- MicroRNA or siRNA-Mediated Inhibition of Viral Gene Expression

Retroviral Diseases

The Varied Effects of Retroviral Infection
Diseases Caused by the Replication-Competent Retroviruses

Other Retroviral Diseases

Host Determinants of Retroviral Disease

Acute Transforming Retroviruses: Transduction of Cellular Proto-Oncogenes

Endogenous Viruses and Virus-Like Sequences

Endogenous Elements in Chickens, Mice, Pigs, and Humans

Properties of the Endogenous Provirus-Like Elements

Retroviral Vectors, Packaging Lines, and Gene Therapy Perspectives

The retrovirus family, the *Retroviridae*, are a large and diverse group of viruses found in all vertebrates. These viruses replicate through an extraordinary and unique life cycle, differentiating them sharply from other viruses. The virion particles generally contain a genomic RNA, but upon entry into the host cell, this RNA is reverse transcribed into a DNA form of the genome that is integrated into the host chromosomal DNA. The integrated form of the viral DNA, the provirus, then serves as the template for the formation of viral RNAs and proteins that assemble progeny virions. These features of life cycle—especially the reverse flow of genetic information from RNA to DNA, and the establishment of the DNA in an integrated form in the host genome—are the defining hallmarks of the retroviruses. This life cycle also accounts for many of their diverse biological activities. The creation of the proviral DNA confers on the viruses a powerful ability to maintain a persistent infection in the face of a host immune response and to enter the germ line, permitting the vertical transmission of virus.

The retroviruses have played a unique role in the history of molecular biology. They have attracted attention on several grounds.

- **Biochemistry:** The viral replication enzymes, including the reverse transcriptase (RT) and integrase (IN), are extraordinarily useful tools in manipulating nucleic acids *in vitro* and *in vivo*. Through the preparation of complementary DNAs (cDNAs), RT has been crucial for studies of messenger RNA (mRNA) synthesis and gene regulation.
- **Pathogenicity:** Retroviruses are known as major pathogens affecting nearly all vertebrates. HIV-1, the agent of the AIDS pandemic, will probably cause more human death and suffering than all but a handful of pathogens in recorded history.
- **Markers of evolutionary history:** The insertion of a provirus into the germ line provides a Mendelian tag that marks an event at a particular time in evolution. The inheritance of that tag can then be used to follow speciation, population migrations, and evolution of species.
- **Insertional activation of oncogenes:** The integration of retroviral DNA is inherently mutagenic; retrovirus replication thus

causes gross alterations of host genes and patterns of gene expression. When insertions lead to tumor formation, the locations serve to identify new oncogenes.

- **Transduction:** Retroviruses can acquire host sequences in the formation of acutely transforming genomes. The identity, structure, and expression of these genes has provided much of our current knowledge of the routes by which normal growth control can be subverted by genetic alterations.
- **Gene delivery vectors:** The structure of transforming viruses provided a model for the use of retroviruses to deliver therapeutic genes efficiently and cleanly into cells. Retroviruses now serve as major tools in the medical black bag of gene therapists.

This chapter will describe the replication and molecular biology of the retroviruses, concentrating on the most broadly conserved aspects of the life cycle. Because of the magnitude of the retroviral literature, citations here cannot be comprehensive, and referencing has been selective and concentrated on more recent publications. The distinctive features of the human retroviruses, especially the lentiviruses and spumaviruses, will be addressed in much more detail in other chapters. A comprehensive review of retroviral biology (called the *Retroviruses*; [108]) is still current, and should be consulted for additional details of almost all aspects of their replication.

TAXONOMIC CLASSIFICATION

The retroviruses were originally classified by the morphology of the virion core as visualized in the electron microscope. Examples of the appearance of the virions in these micrographs are presented in Figure 47.1. The virion particles are spherical, and are surrounded by an envelope consisting of a lipid membrane bilayer. The surface is studded by projections of an envelope glycoprotein. There is a spherical layer of protein under the membrane, and an internal nucleocapsid (or nucleoid) whose shape varies characteristically from virus to virus. The shape and position of the nucleocapsid core was historically used as the major classifying feature of the retroviral genera. A-type viruses were defined as those forming intracellular structures with a characteristic morphology, a thick shell with a hollow, electron-lucent center. These particles are now appreciated as representing an immature capsid on route toward the formation of other structures. This term is thus no longer in use to denote a virus classification, though it is used to describe the structures formed by some virus-related intracellular retrotransposons (the intracisternal A-type particles, or IAPs).^{307,349} B-type viruses show a round but eccentrically positioned inner core. C-type viruses assemble at the plasma membrane, and contain a central, symmetrically placed, spherical inner core. The D-type viruses assemble in the cytoplasm, via an A-type intermediate, and upon budding exhibit a distinctive cylindrical core.

These older classifications have been useful in partially defining the various genera of the family *Retroviridae*, but the number of genera have now been expanded on the basis of new criteria. The genera have recently been formalized and given new names by the International Committee on Taxonomy of Viruses. The alpharetroviruses, betaretroviruses, and gammaretroviruses are considered “simple” retroviruses, while the deltaretroviruses, epsilonretroviruses, lentiviruses, and spumaviruses are

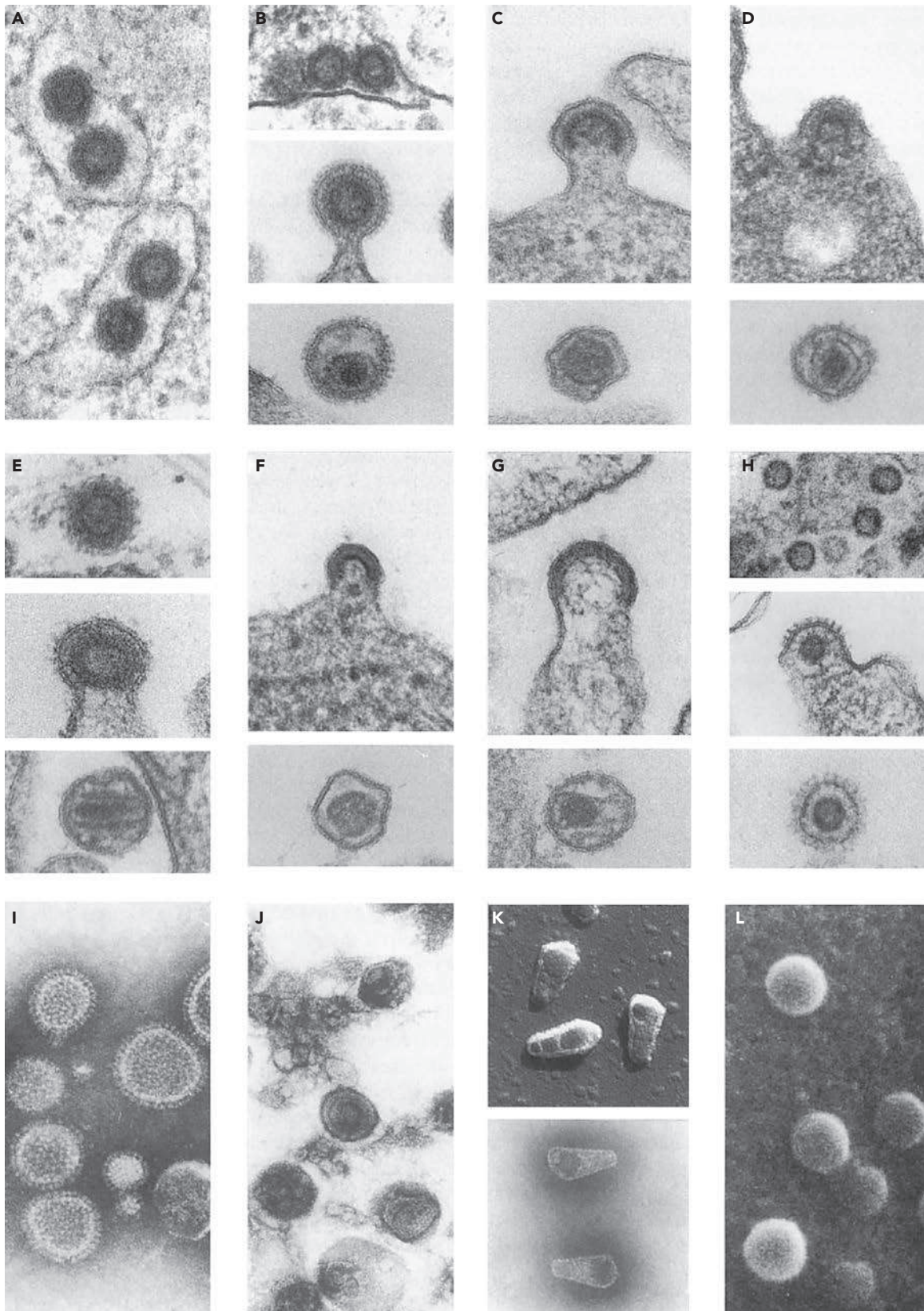


TABLE 47.1 Retrovirus Genera

Name	Examples	Morphology
Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus	C type
Betaretrovirus	Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (M-PMV) Jaagsiekte sheep retrovirus	B, D type
Gammaretrovirus	Murine leukemia viruses (MuLV) Feline leukemia virus (FeLV) Gibbon ape leukemia virus (GaLV) Reticuloendotheliosis virus (RevT)	C type
Deltaretrovirus	Human T-lymphotropic virus type 1, 2 Bovine leukemia virus (BLV) Simian T-lymphotropic virus type 1, 2, 3	Rod-shaped core
Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1	—
Lentivirus	Human immunodeficiency virus type 1 Human immunodeficiency virus type 2 Simian immunodeficiency virus (SIV) Equine infectious anemia virus (EIAV) Feline immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus	Rod/Cone-shaped cores
Spumavirus	Human foamy virus	Immature

considered “complex.” The simple viruses encode only the Gag, Pro, Pol, and Env gene products; the complex viruses encode these same gene products but also an array of small regulatory proteins with a range of functions. The properties of the viruses belonging to each of these genera are summarized briefly in the following section. Representative members of each genus are listed in Table 47.1.

Alpharetroviruses

The alpharetroviruses are simple retroviruses characterized by a C-type morphology, and are typified by the avian sarcoma and leukosis viruses (ALSV). The genome contains *gag*, *pro*, *pol*, and *env* genes, with no additional known genes; *pro* is at the 3′ end of *gag* and in the same reading frame. The transfer RNA (tRNA) primer is tRNA^{trp}. The viruses are widespread

in many avian host species. The ALSV members are classified into 10 subgroups (termed A–J) by their distinct receptor utilization. The first four subgroups represent exogenous viruses of chickens; the subgroup E includes a family of endogenous chicken viruses; and subgroups F and G include endogenous viruses of pheasants.

Betaretroviruses

The betaretroviruses are simple retroviruses characterized by either a “B-type” morphology, with a round eccentric core, or “D-type” morphology, with a cylindrical core. The best-known examples are the mouse mammary tumor virus (MMTV) and the Mason-Pfizer monkey virus (MPMV). Assembly occurs in the cytoplasm via an “A-type” intermediate, and the completed immature particle is then transported to the plasma membrane

FIGURE 47.1. Electron micrographs of representative virion particles. The diameters of all the particles are approximately 100 nm. **A:** Type A particles. Intracisternal A particles in the endoplasmic reticulum. **B:** Betaretrovirus. Mouse mammary tumor virus, MMTV; type B morphology (*top*, intracytoplasmic particles; *middle*, budding particles; *bottom*, mature extracellular particles). **C:** Gammaretrovirus. Murine leukemia virus, MLV; type C morphology (*top*, budding; *bottom*, mature extracellular particles). **D:** Alpharetrovirus. Avian leukosis virus; type C morphology (*top*, budding; *bottom*, mature extracellular particles). **E:** Betaretrovirus. Mason-Pfizer monkey virus, MPMV; type D morphology (*top*, intracytoplasmic A-type particles; *middle*, budding; *bottom*, mature extracellular particles). **F:** Deltaretrovirus. Bovine leukemia virus, BLV (*top*, budding; *bottom*, mature extracellular particles). **G:** Lentivirus. Bovine immunodeficiency virus (*top*, budding; *bottom*, mature extracellular particles). **H:** Spumavirus. Bovine syncytial virus (*top*, intracytoplasmic particles; *middle*, budding; *bottom*, mature extracellular particles). **I:** Betaretrovirus. Mouse mammary tumor virus, MMTV; type B morphology, visualized by negative staining with phosphotungstic acid. **J:** Gammaretrovirus, visualized as pseudoreplica stained with uranyl acetate. **K:** Lentivirus. Purified cone-shaped cores of equine infectious anemia virus (*top*, cores visualized by shadow casting technique; *bottom*, cores visualized by negative staining with phosphotungstic acid). **L:** Budding retroviral particles visualized by scanning electron microscopy. (Micrographs courtesy of Dr. Matthew Gonda, and reproduced from Coffin JM, Hughes SH, Varmus HE, eds. *Retroviruses*. Cold Spring Harbor, NY: Cold Spring Harbor Press; 1997).

and budded. The genomes contain *gag*, *pro*, *pol*, and *env* genes, and the *gag*, *pro*, and *pol* genes are all in different reading frames. The genome of MMTV contains an additional gene termed the *sag* gene for superantigen. The viruses also contain a dUTPase region as part of the *pro* open reading frame (ORF).¹⁵⁶ The tRNA primer is tRNA^{Lys}-3 or tRNA^{Lys}-1,2. There are both exogenous and endogenous viruses in this genus. Examples are found in mice, primates, and sheep.

Gammaretroviruses

The gammaretroviruses are simple viruses characterized by a C-type morphology. This genus has the largest number of members known, including the murine leukemia viruses (MuLVs), the feline leukemia viruses (FeLVs), and the gibbon ape leukemia virus (GALV). The genome contains only *gag*, *pro*, *pol*, and *env* genes; the *gag*, *pro*, and *pol* sequences are in the same reading frame, and the Gag-Pro-Pol protein is expressed by translational readthrough of a stop codon at the end of *gag*. The genome primer is most often tRNA^{Pro} or tRNA^{Glu}. The murine viruses are divided into subgroups by their distinct receptor utilization. Many exogenous and endogenous viruses are found in diverse mammals; examples have been isolated from reptiles and birds. A novel gammaretrovirus termed XMRV (for xenotropic murine leukemia virus-like virus) was identified in human prostate cancer tumors,⁶⁰⁴ but recent work strongly suggests that the virus was a recombinant derived during tumor passage in nude mice.⁴⁵²

Deltaretroviruses

The deltaretroviruses are complex viruses characterized by a C-type morphology. The most famous examples are the human T-lymphotropic viruses (HTLVs) and the bovine leukemia virus (BLV). The genome contains *gag*, *pro*, *pol*, and *env* genes; the *gag*, *pro*, and *pol* genes are present in three different reading frames, and expression of the Gag-Pro-Pol protein requires two successive frameshifts. In addition, the genomes contain regulatory genes termed *rex* and *tax* that are expressed from an alternatively spliced mRNA. These gene products control the synthesis and processing of the viral RNAs. The tRNA primer is tRNA^{Pro}. No closely related endogenous viruses are known, and the exogenous viruses are only rarely found in a few mammals.

Epsilonretroviruses

The epsilonretroviruses are complex viruses characterized by a C-type morphology. The prototype is the walleye dermal sarcoma virus (WDSV). The genomes contain *gag*, *pro*, *pol*, and *env* genes; the *gag*, *pro*, and *pol* genes are in the same reading frame. They also contain one to three additional genes termed *ORFs A*, *B*, and *C*. The *ORF A* gene is a viral homolog of the host cyclin *D* gene, and so may regulate the cell cycle. The viruses use tRNA^{His} or Arg as primers. The only known examples are exogenous viruses in fish and reptiles.

Lentiviruses

The lentiviruses are complex viruses characterized by a unique virion morphology, with cylindrical or conical cores. The most important example is the human immunodeficiency virus type 1 (HIV-1), but nonprimate viruses in the genus include the caprine arthritis encephalitis virus (CAEV) and visna virus. The genomes express *gag*, *pro*, *pol*, and *env* genes; *gag* is in one reading frame, and *pro-pol* in another. A single frameshift is

used to express Gag-Pro-Pol. The Pol region of the nonprimate lentiviruses includes a domain for dUTPase. A number of accessory genes are also expressed. In HIV-1, these genes are *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*; these genes control transcription, RNA processing, virion assembly, and host gene expression, and inactivate host restriction systems. The tRNA primer is tRNA^{Lys}1,2. A large number of exogenous viruses in this genus have been found in diverse mammals, but the only endogenous sequences are relatively distant from these viruses.

Spumaviruses

The spumaviruses are complex viruses with a unique virion morphology, containing prominent spikes on the surface and a central but uncondensed core. The prototype example is the human foamy virus. The virion is assembled in the cytoplasm and budded into the ER and plasma membrane. There is probably only a single cleavage of the Gag protein near the C-terminus, and no major change in morphology during maturation. The genomes express *gag*, *pro*, *pol*, and *env* genes, and also at least two accessory genes known as *tas/bel-1*, and *bet*.^{177,380} The *tas* gene encodes a transcriptional transactivator. Unique features are the separate expression of the Pol protein from a spliced mRNA and the presence of large amounts of reverse transcribed DNA in the virion.³⁹⁰ The genome contains a second transcriptional start site near the 3' end of the *env* gene. The tRNA primer is tRNA^{Lys}1,2. A number of exogenous viruses have been found in diverse mammals, and distantly related sequences are present as endogenous elements in the human genome.

Evolutionary Relationships

The sequences of the various retroviral genomes have been compared and used to determine the relatedness of any pair.³⁷⁵ A number of phylogenetic trees can be constructed using *gag*, *pro*, *pol*, or *env* genes, and in most aspects these trees are similar. A tree based on comparisons of the *pol* gene (Fig. 47.2) shows the clustering of viruses within each of the main genera. However, it is important to realize that a phylogenetic tree is not necessarily identical to an evolutionary history, and that the history that led to the formation of the known genera is not necessarily simple. It is noteworthy that there is no obvious clustering of all the simple viruses into a group apart from all the complex viruses. Thus, complex viruses probably arose from the simple ones more than once, with many evolving through the independent acquisition of separate genes.

The retroviruses are related to viruses of other families. The retroviral RTs show close sequence similarity to the polymerases of the hepadnaviruses and the caulimoviruses, which also replicate by reverse transcription. The retroviruses also show extensive similarity in both *gag* and *pol* gene sequences to the retrotransposons, endogenous mobile elements with long terminal repeats (LTRs), and to retroposons, elements without LTRs. Retroviral RTs show more distant similarity to proteins encoded by the group II mitochondrial introns and by the retrons, elements in myxobacteria and rare isolates of *E. coli*; to telomerase, an RT responsible for maintenance of the chromosomal termini in eukaryotes; and slight similarity to the DNA polymerases of viruses and hosts.³⁷⁴

Transforming Viruses

During the replication of any retrovirus, replication-defective variants can arise through deletion or recombination events.

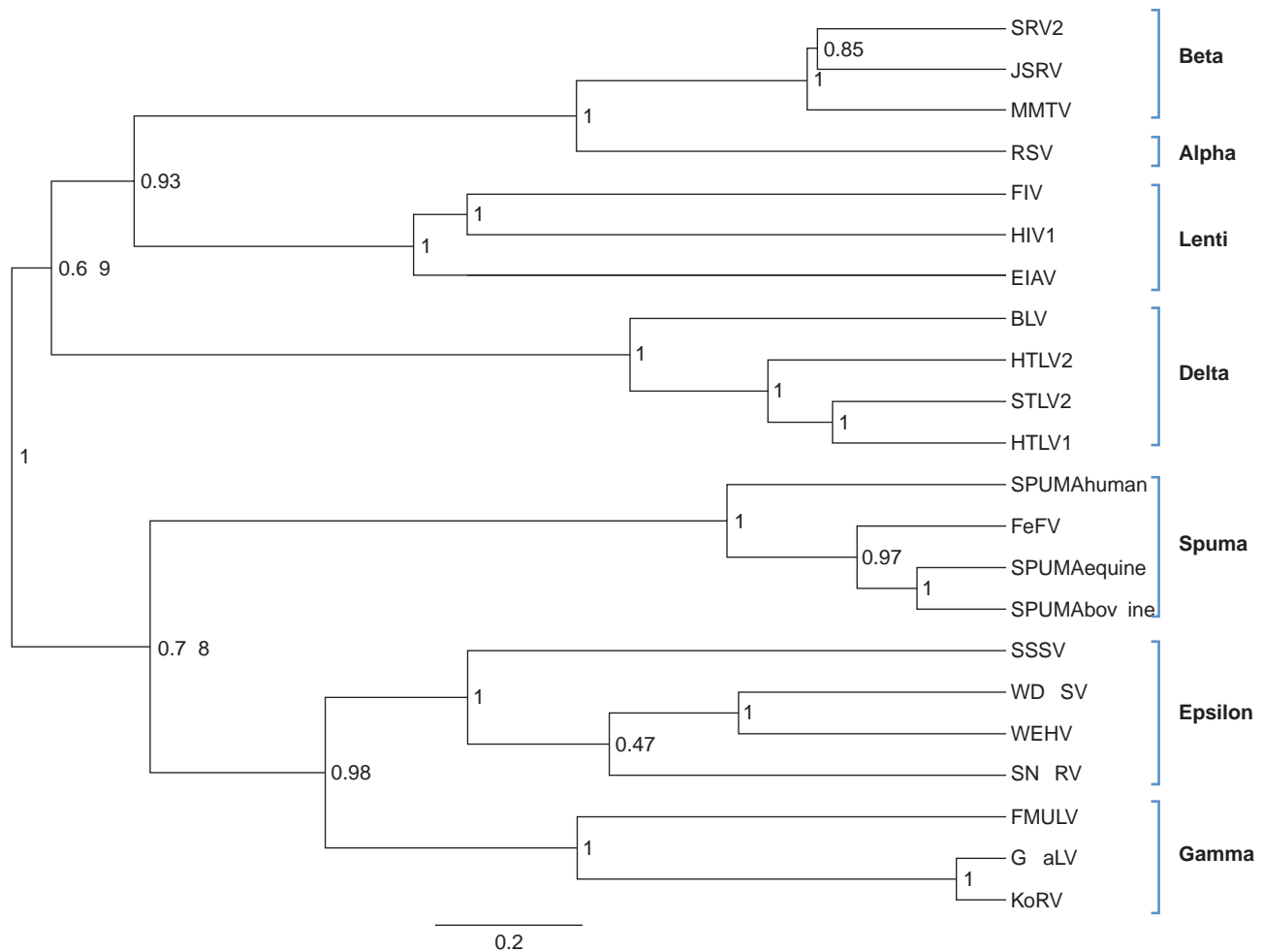


FIGURE 47.2. Phylogenetic reconstruction of representative exogenous retroviruses using reverse transcriptase sequences. The BEASTv1.6.1 tree¹⁴⁴ was created using two independent Bayesian MCMC chains (length of 1 million, 20% burn) run under relaxed clock (uncorrelated exp; 143) and rate heterogeneity among sites (gamma distribution with 8 categories). Monophyletic taxon sets consisting of alpha, beta, delta, epsilon, gamma, lenti, and spuma were also used in the model. The posterior probabilities label each node and branch lengths are scaled to expected substitutions per site. (Prepared by Marcella McClure, Montana State University, Bozeman, MT.)

Such mutants or variants can be propagated as a mixed virus culture along with the wild-type parent. In these mixtures of two genomes, the replication-competent parent acts as a helper virus to provide the missing replication functions in *trans* for the replication-defective virus. If a newly acquired gene product is mitogenic or antiapoptotic for the host cell, or in more subtle ways alters the growth of the cell, the recombinant may become a potent oncogenic virus. A large number of such transducing viruses have been isolated and characterized as derivatives of one or another of the replication-competent parent viruses. A partial listing of the most intensely studied of these viruses is presented in Table 47.2.

VIRION STRUCTURE

Retrovirus virions are initially assembled and released from infected cells as immature particles containing unprocessed Gag and Gag-Pol precursors of the proteins that eventually

make up the mature virus. The immature virion morphology is spherical, with a characteristic electron-lucent center. The virions have been described as a “protein vesicle,” to suggest some fluidity in the interactions between the individual Gag proteins that make up the particle. Upon maturation, the precursor proteins are cleaved, and the structure and morphology of the virion change drastically. The mature retrovirus particle is a spherical structure, roughly 100 nm in diameter. The size of the virions in a given preparation is not highly homogeneous but rather varies over a fairly wide range, suggesting that a discrete, highly ordered structure may not exist. After processing of the Gag precursor during virion maturation, the CA protein collapses to form a more ordered paracrystalline core, but even then the overall diameter of the virion is heterogeneous and suggestive of considerable disorder. The virions exhibit a buoyant density in sucrose in the range of 1.16 to 1.18 g/ml. The sedimentation rate of the particles is typically about 600 S. The virions are sensitive to heat, detergent, and formaldehyde.

TABLE 47.2 Examples of Acute Transforming Retroviruses

Parental virus	Transforming virus	Transduced gene(s)
ALV	Rous sarcoma virus	<i>c-src</i>
	Avian myeloblastosis virus	<i>c-myb</i>
	Avian erythroblastosis virus	<i>c-erbA,B</i>
	Avian sarcoma virus CT10	<i>c-crk</i>
	Fujinami sarcoma virus	<i>c-fps</i>
	Y73 avian sarcoma virus	<i>c-yes</i>
	Avian sarcoma virus 17	<i>c-jun</i>
Moloney MuLV	Abelson murine leukemia virus	<i>c-abl</i>
	Harvey sarcoma virus	<i>H-ras</i>
	Kirsten sarcoma virus	<i>Ki-ras</i>
	Moloney murine sarcoma virus	<i>c-mos</i>
	FBJ murine sarcoma virus	<i>c-fos</i>
Feline leukemia virus	3611-MSV	<i>c-raf</i>
	Snyder-Theilen feline sarcoma virus	<i>c-fes</i>
	Gardner-Arnstein feline sarcoma virus	<i>c-fes</i>
	McDonough feline sarcoma virus	<i>c-fms</i>
Simian sarcoma-associated virus	Woolly monkey sarcoma virus	<i>c-sis</i>

ALV, avian leukosis virus; MSV, murine sarcoma virus; MuLV, murine leukemia virus.

Virion Proteins

The stoichiometry of the various viral gene products in the virion is not very firmly established, but estimates suggest that about 1500 Gag precursors are present per particle. After processing, all cleavage products are thought to be retained, suggesting equimolar presence of these proteins in the mature virions. The levels of the Pol proteins are typically about one-tenth to one-twentieth those of the Gag proteins, corresponding to about 100 to 200 molecules per virion. The levels of the Env proteins are highly variable among the viruses. For the gammaretroviruses, the levels of Env are close to that of Gag;

perhaps 1200 monomers, or 400 trimers, are present per virion. For the lentiviruses, the levels of Env per virion are much lower, possibly as low as 10 trimers per virion.⁶⁷¹

Nomenclature

The cleavage of Gag, Pol and Env precursors forms the products in the mature infectious virions. These proteins are named by convention by a two-letter code: MA for matrix or membrane-associated protein; CA for capsid; NC for nucleocapsid; PR for protease; DU for dUTPase; RT for reverse transcriptase; IN for integrase; SU for surface protein; and TM for transmembrane protein.³²³ The localization of these proteins in the mature virion is not known with great precision, but a highly schematic version of the generic retrovirion can be drawn (Fig. 47.3).

Arrangement of Virion Components

The genomic RNA is highly condensed in the virion by its association with the nucleocapsid protein, NC. The complex is contained within a protein core largely composed of the capsid protein CA, another Gag gene product. The shape of the core is different among the various retroviral genera, and is a distinguishing feature of the genera. In most of the viruses the core is roughly spherical, but in some cases can be either conical or cylindrical. In all the viruses the core is surrounded by a roughly spherical shell consisting of MA, which in turn is surrounded by the lipid bilayer of the virion envelope. The virion membrane contains the envelope glycoprotein, with the TM subunit present as a single-pass transmembrane protein anchor, and the SU subunit as an entirely extravirion protein bound to TM. The envelope proteins for those viruses examined closely have been found to reside in the membrane as trimers.

ORGANIZATION OF THE RNA GENOME

The viral genome is a dimer of linear, positive-sense, single-stranded RNA (ssRNA), with each monomer 7 to 13 kb in size. The viral genomic RNA is present as a homodimer of two identical sequences, and thus the virions are functionally diploid. The dimer is maintained by interactions between the two 5' ends of the RNAs in a self-complementary region termed the dimer linkage structure (DLS). The RNA genome is generated by normal host transcriptional machinery, and thus exhibits many of the features of a normal mRNA. The RNA is capped at the 5' end, using the common m7G5'ppp5'G_{mp}

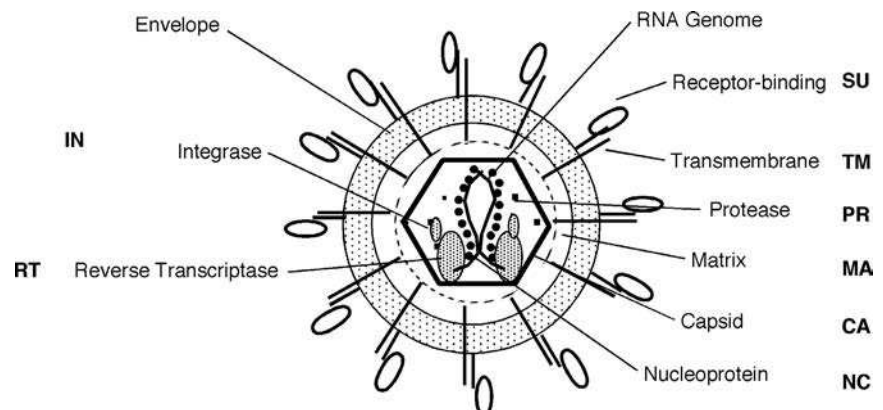


FIGURE 47.3. Generalized retrovirion structure and components. A highly schematic view of the arrangement of viral gene products within the virion particle. The two-letter nomenclature for each protein is indicated.

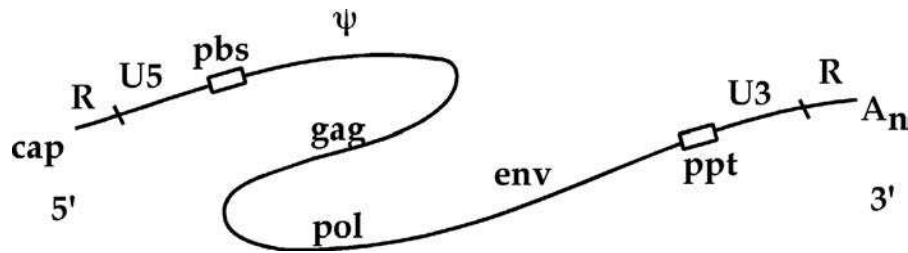


FIGURE 47.4. The organization of the retroviral RNA genome. The single-stranded RNA genome is depicted as a curved line. From 5' to 3' along the RNA, the features include a 5' cap structure; R, a sequence block repeated at both 5' and 3' ends; U5, a unique 5' sequence block; pbs, the primer binding site and site of initiation of minus strand DNA synthesis; Ψ, the major recognition site for the packaging of the viral RNA into the virion particle; the *gag*, *pol*, and *env* genes; ppt, the polypurine tract and site of initiation of the plus strand DNA synthesis; U3, a unique 3' sequence block; the second copy of the R sequence; and finally, a 3' poly(A) sequence.

structure; and contains a string of poly(A) sequence, about 200 long, at the 3' end.

A number of sequence blocks are so important that they have been named to facilitate descriptions of their functions in the life cycle (Fig. 47.4). These key sequences are clustered at the termini of the RNA. A short sequence, the R (for repeated) region, is so called because it is present twice in the RNA: once immediately after the cap at the 5' end and again at the 3' end, just before the poly(A) tail. Downstream of the 5' R lies another sequence, termed U5 for unique 5' sequence, which includes one of the att sites required for proviral integration. The U5 region is followed by the primer binding site, an 18-nt sequence at which a host tRNA is hybridized to the genome and the site of initiation of minus-strand DNA (msDNA) synthesis.

The region downstream from the primer binding site (pbs) often contains the major signals for the encapsidation of viral RNA into the virion particle, in sequences called the Psi element. The region also often contains a major splice donor site for the formation of subgenomic mRNAs. The bulk of the RNA sequences that follow are coding regions for the viral proteins. The genomes of all the replication-competent retroviruses contain at a minimum three large genes, or open reading frames: from 5' to 3' along the genome, the genes are termed *gag*, for group-specific antigen; *pol*, for polymerase; and *env*, for envelope. The three genes in the simple retroviruses occupy nearly all the available space in the center of the genome.

Downstream of the genes lies a short polypurine tract (ppt), a run of at least nine A and G residues. The ppt is the site of initiation of plus strand DNA (psDNA) synthesis. The ppt is followed by a sequence block termed U3 for unique 3' sequence; this region contains a number of key *cis*-acting elements for viral gene expression, and one of the att sites required for DNA integration. The U3 abuts the 3' copy of the R region, which is followed by the poly(A) tail. As will be demonstrated, the R, U5, U3, pbs, and ppt sequences all play important roles in reverse transcription.

OVERVIEW OF THE LIFE CYCLE

The retroviruses replicate through a complex life cycle. A short summary of the steps of the cycle is as follows (a schematic view is shown in Fig. 47.5):

- Receptor binding and membrane fusion
- Internalization and uncoating
- Reverse transcription of the RNA genome to form double-stranded linear DNA
- Nuclear entry of the DNA
- Integration of the linear DNA to form the provirus
- Transcription of the provirus to form viral RNAs
- Splicing and nuclear export of the RNAs
- Translation of the RNAs to form precursor proteins
- Assembly of the virion and packaging of the viral RNA genome
- Budding and release of the virions
- Proteolytic processing of the precursors and maturation of the virions

Changes in the Viral Genome

A quick perusal of this list reveals that the life cycle begins with an RNA genome, passes through an intracellular DNA intermediate, and is completed with a return to an RNA form in the progeny virus particle. An overview of the structures of the genome at various times in this cycle is presented in Figure 47.6. The RNA genome of the virion contains short terminal repeats (the R region) at its termini. During reverse transcription, to be seen below, sequence blocks termed U5 and U3 are duplicated, so that the resulting dsDNA is longer at both ends than the RNA template. This DNA thus contains long terminal repeats (the LTRs, consisting of sequence blocks U3, R, and U5) at both ends. The next step is the integration of the DNA to form the provirus; the integrated provirus is collinear with the preintegrative DNA, and retains the LTRs (except for one or two base pairs lost at the termini during the course of integration). Finally, the DNA is forward transcribed by the RNA polymerase II system to produce the progeny RNA genome. Transcription is initiated at the U3-R boundary of the 5' LTR, and the transcripts are processed and polyadenylated at the R-U5 boundary of the 3' LTR, recreating the exact structure of the input RNA, complete with its short terminal repeats. This RNA is packaged and exported in virion particles. Each step is described in more detail in the next section.

THE VIRUS RECEPTORS

To enter a cell and initiate infection, all retroviruses require an interaction between a cell surface molecule—a receptor—and

Retroviral Replication Cycle

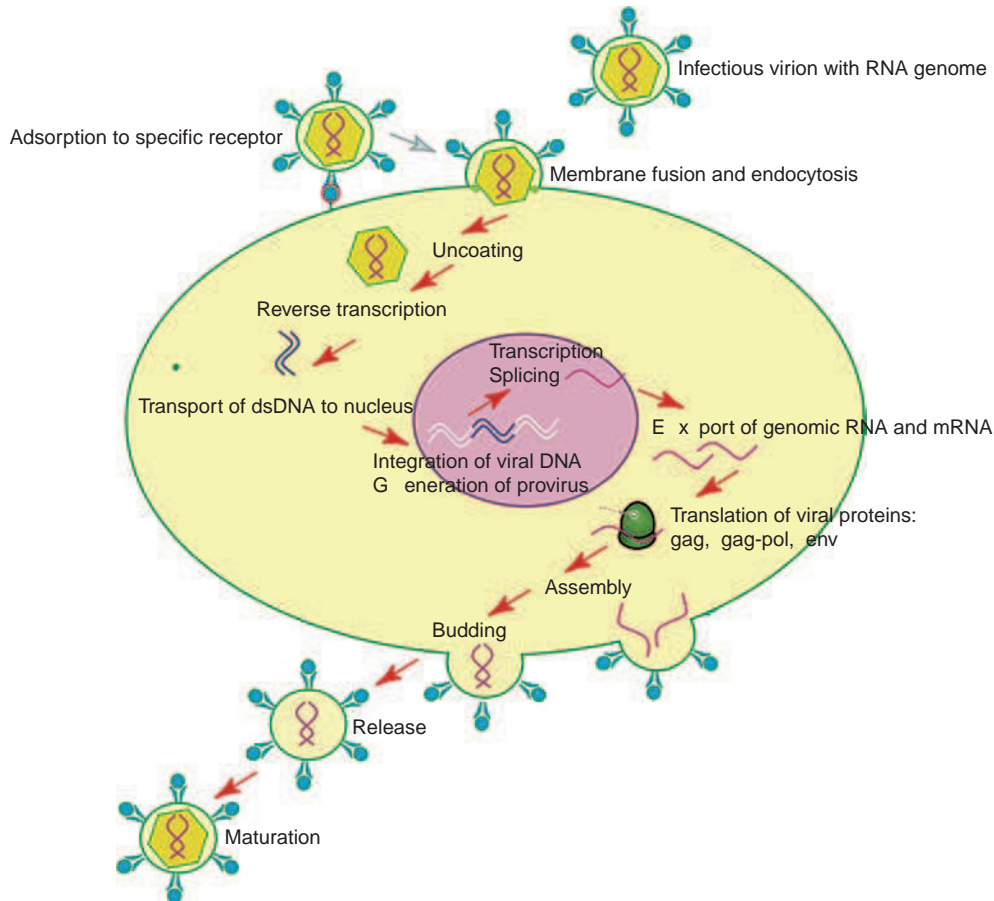


FIGURE 47.5. A schematic view of the retrovirus life cycle. The major steps in the replication of a typical retrovirus are indicated, including those in the early phase of the life cycle, extending from the infecting virion (*top left*) to the formation of the integrated provirus, and those in the late phase of the life cycle, extending from the provirus to the formation of mature progeny virus (*right*).

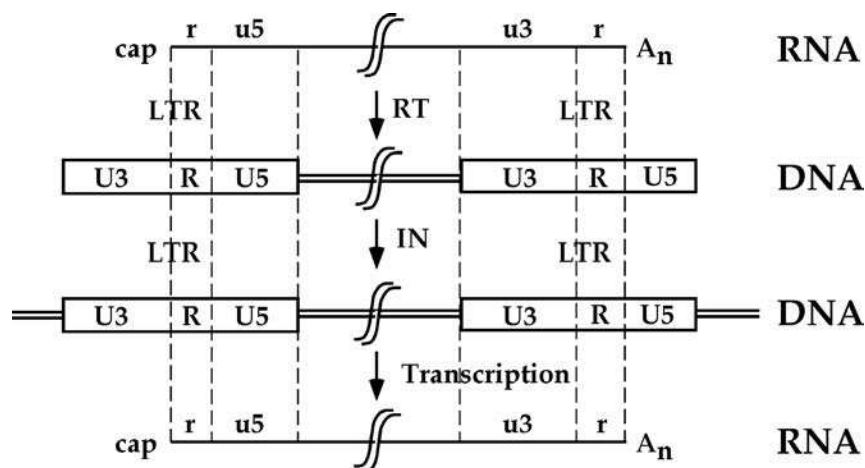


FIGURE 47.6. Structures of the termini of the viral RNA and DNA genomes at various stages of the viral life cycle. Sequence blocks in RNA are indicated by lower case, and those in DNA by upper case. The structure of the RNA genome in the virion particle is indicated at the top. Reverse transcription of the RNA soon after infection involves the duplication and translocation of u5 and u3 sequence blocks, and results in the formation of a double-stranded DNA molecule containing two terminal LTRs. The integration of the DNA genome occurs at the terminal sequences, establishing a provirus that is collinear with the preintegrative DNA. The forward transcription of the provirus is initiated at the U3/R border in the provirus; the resulting RNAs are cleaved and polyadenylated at the r/u5 border, recreating a viral RNA genome (*bottom*) identical to the infecting RNA.

TABLE 47.3 Retrovirus Receptors

Virus(es)	Receptor name(s)	Function	References
MuLV, ecotropic	CAT-1	Basic amino acid transporter	(154,282,386,428,607)
MuLV, amphotropic	Ram-1/GLVR2/PiT-2	Phosphate transporter	(154,282,386,428,607)
MuLV 10A1; FeLV-B	GLVR1/PiT-1	Phosphate transporter	(14,265,634)
MuLV, xenotropic; polytropic	Rmc1/XPR1	G-coupled receptor?	(34,582,650)
M813 ecotropic	SMIT-1	Na/inositol transporter	(233,488)
FeLV-C	Flvr	Organic anion transporter	(494)
MMTV	Tfr1	Transferrin receptor	(520)
ASLV-A	tv-a	LDLR-like	(33,110,653)
ALV-B,D,E	tv-b, -e	Fas receptor-like	(3,4,72,73,555)
ALV-C	tv-c	Butyrophilin-like	(157)
Perv-A	HuPAR-1, -2	G-coupled receptor?	(160)
RD114, BaEV, MPMV, HERV-W	RDR, RDR2/ASCT1,2	Neutral amino acid transporter	(316,498,583)
BLV	Blvr	AP-3 delta subunit-like	(26,27,576,652)
JSRV	HYAL2	Hyaluronidase receptor	(384,496)
HTLV-1	GLUT-1	Glucose transporter	(363)
HIV-1, HIV-2, SIVs	CD4 plus CCR5, CXCR4	T-cell differentiation markers	(152,171,294,357,552)

ALV, avian leukosis virus; ASLV, avian sarcoma and leukosis virus; BaEV, baboon endogenous virus; BLV, bovine leukemia virus; FeLV, feline leukemia virus; HERV, human endogenous retrovirus; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; JSRV, Jaagsiekte sheep retrovirus; LDLR, low-density lipoprotein receptor; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; MuLV, murine leukemia virus; Perv, porcine endogenous retrovirus; RD114, feline endogenous virus; SIV, simian immunodeficiency virus.

the envelope protein on the virion surface. The interactions are complex, involving an initial binding, drastic conformational changes in the envelope protein, an induced fusion of the viral and cellular membranes, and the internalization of the virion core into the cytoplasm. The SU subunit of Env is thought to make the major initial contacts with receptor, and the TM subunit is thought to be most important for membrane fusion. The reorganization of the two lipid bilayers—one on the virion and one on the cell—to join them and evert the core into the cell is a remarkable process. The details of these complex processes are not understood for any retrovirus, and the whole Env protein is likely to be involved in efficient entry. However, there is a great deal of information about the identity and structures of the receptors used by various retroviruses. It is apparent that these viruses utilize an extraordinarily diverse set of cell surface molecules as receptors (Table 47.3; see 41,581,624 for reviews).

An important tool in the analysis of receptor utilization is the phenomenon of virus interference, or superinfection resistance. Cells chronically infected by a particular virus cannot be infected by any virus that must enter by the same receptor as used by the first virus though they are readily infected by viruses that utilize a distinct receptor. The reason is that the expression of Env protein by the first virus binds to the receptor intracellularly, preventing its export to the cell surface or its function as a receptor for newly applied virus. The phenomenon allows for the rapid classification of those viruses that use a common receptor.

The properties of the receptors of the major retroviral genera are summarized in the following section.

Alpharetrovirus Receptors

The receptor for the A subgroup of avian viruses was identified as encoding a membrane-anchored glycoprotein with sequence

similarity to the ligand-binding repeat of the low-density lipoprotein receptor (LDLR).^{33,653} Its identity as the true receptor has been confirmed by correlating its genetic map position with the *tv-a* locus.³² The *tv-b* locus, encoding the receptor for both the B and D subgroups of the ASLV, encodes a protein termed CAR1, unrelated to *tv-a* but with sequence similarity to the receptors for tumor necrosis factor (TNF) and the Fas death receptors.⁷³ The intracellular portion of the molecule contains the sequence of a “death domain,” present on other cytotoxic receptors, and can trigger the apoptotic death of the cell upon ligand binding. The *tv-c* locus is closely linked to *tv-a* but encodes an unrelated surface protein, one with strong sequence similarity to mammalian butyrophilins, members of the immunoglobulin family.¹⁵⁷ The *tv-e* locus is present in turkey but not chicken, and allows for infection by the subgroup E viruses. The gene was cloned by its sequence similarity to the chicken *tv-b* locus.⁴

Betaretrovirus Receptors

The receptor for MMTV was cloned by co-segregation of DNA markers with virus susceptibility in mouse/hamster radiation chimeric cell lines, and so identified as the transferrin receptor *tfr1* on mouse chromosome 16.⁵²⁰ A second receptor for the betaretroviruses was also identified. The type D simian viruses, including MPMV and SRV-1, -2, -4, and -5, show cross-interference with three type-C viruses: feline endogenous virus (RD114), baboon endogenous virus (BaEV), and avian reticuloendotheliosis virus (REV), suggesting that they all utilize a common cell-surface receptor. Gene transfer of a human cDNA library into nonpermissive mouse cells was used to identify a gene that conferred susceptibility to infection by RD114.⁵⁸³ The cDNA encoded a protein nearly identical to the previously cloned human Na⁺-dependent neutral-amino-acid transporter named B^o.^{288,498} Consistent with this similarity, expression of

the RD114 receptor in NIH 3T3 cells resulted in enhanced cellular uptake of L-³H]alanine and L-³H]glutamine.

Gammaretrovirus Receptors

Several receptors for various gammaretroviruses are known.⁵⁸¹ The first example, the mouse receptor used by the ecotropic MuLVs, was identified by gene transfer to nonpermissive human cells, selecting for susceptibility to MuLV infection.⁸ The gene encodes a membrane glycoprotein of 67 kDa containing a total of 14 membrane spanning domains. The normal function of the protein has been identified as a transporter or permease for cationic, basic amino acids.²⁹² The receptor, termed mCAT-1, was shown to be identical to γ^+ , the previously characterized transporter in mammalian cells. The gene for mCAT-1 is now known as *Atrc1*.

The amphotropic receptor is utilized by a group of MuLVs derived from wild mice able to infect a wide range of mammalian species, including humans. The receptor was cloned by selection for susceptibility to virus infection after transfection of cDNA libraries into nonpermissive CHO cells,^{154,386} and by its homology to the gene for the previously identified GALV receptor.⁶⁰⁷ The gene, known variously as *Ram1* or *GLVR2* or *rPiT-2*, encodes a 652-amino acid protein that functions as a sodium-dependent phosphate symporter.²⁸² The synthesis and stability of the receptor is regulated by phosphate levels, and its downregulation by virus infection results in substantial reduction in phosphate uptake by cells.

The receptor utilized in common by GALV, simian sarcoma-associated helper virus (SSAV), and FeLV-B is widely expressed in many mammals, including primates, cat, dog, mink, rabbit, and rat (but not mouse), as well as in some avian species. The human receptor is termed GLVR1 or hPiT-1.^{265,426} The sequence of the gene predicts the existence of 10 membrane-spanning segments, and a large third intracellular loop. The protein is a sodium-dependent phosphate symporter.^{282,428} Specific amino acid changes introduced into the fourth extracellular loop can block FeLV-B and SSAV infection without affecting GALV, suggesting that these various viruses interact in slightly different ways with the receptor. A remarkable feature of infection by FeLV-B via feline PiT-1 is a requirement for the co-expression of an endogenous Env-like protein dubbed FeLIX.¹³

The xenotropic MuLVs are viruses present as proviruses in the mouse germ line but unable to infect inbred mouse cells. The polytropic MuLVs are also endogenous viruses with a wide host range that includes many mammalian species. Xenotropic and polytropic MuLVs cross-interfere to various extents in nonmouse species and in wild Asian mice, suggesting that they might use a common receptor for infection. The mouse receptor for the polytropic viruses was cloned by gene transfer, and was identified with the *Rmc1* gene.⁶⁵⁰ The human xenotropic receptor mediates infection by both the xenotropic and polytropic viruses, as well by the XMRV isolate.⁶⁴⁷ The gene encodes a membrane protein related to the yeast Syg1p protein (suppressor of yeast G alpha deletion). Its function is unknown, but its multiple membrane-spanning segments and its sequence suggests that it may act as a G-coupled receptor.

The receptor utilized by the subgroup C feline leukemia viruses (FeLV-C) encodes a protein with 12 membrane-spanning domains with significant sequence similarity to the D-glucarate transporters of bacteria and nematodes.⁴⁹⁴ The binding of virus

to this receptor may be responsible for its pathogenesis, a block in erythroid differentiation.

Additional receptors for other gammaretroviruses are known to exist. Three newly characterized porcine endogenous retroviruses (PERV-A, -B, and C) have been tested in interference assays with each other and with murine viruses using the known receptors; all three apparently utilize distinct and novel receptors.⁵⁸⁵ The PERV-A receptor has been identified and is likely a G protein-coupled receptor.¹⁶⁰

Deltaretrovirus Receptors

The receptor for the bovine leukemia virus (BLV) is highly similar to the delta subunit of the AP-3 complex.^{26,576} AP-3 is involved in intracellular trafficking of clathrin-coated vesicles and is not thought to be present on the cell surface. The properties of the receptor are not yet well established.

Lentivirus Receptors

The first receptor identified for any retrovirus was the CD4 molecule, established as essential for infection by HIV-1.^{122,294,357} CD4 is an important surface protein on T cells, and with few exceptions serves to define the helper subset of T cells. CD4 is also expressed at significant levels on dendritic cells, macrophages, and on certain cells in the brain, likely astrocytes rather than cells of neural origin. The limited distribution of expression of CD4 accounts well for the tropism of HIV-1, largely restricted to helper T cells and macrophages. There may be other routes of entry utilized at lower efficiency: antibody to virus, for example, can promote virus entry into cells by the Fc receptor. Receptor-negative dendritic cells can take up virions via binding to the DC-SIGN molecule and deliver them efficiently to T cells to promote their infection, but even here infection of the recipient cells requires their expression of the CD4 receptor.^{199,309}

Early work established that although CD4 was sufficient to mediate virus binding to a cell surface, it was not sufficient to mediate virus infection and entry. For example, rodent cells and other cells of nonprimate origin could not be successfully infected by HIV-1 even if they were engineered to express human CD4. Searches for genes that would render such cells sensitive to virus infection ultimately led to the identification of various members of the chemokine receptor family, notably CCR5 and CXCR4, as coreceptors needed to mediate the postbinding steps of membrane fusion and virus entry.^{152,171,552} Antibodies to the coreceptor as well as the natural ligand for these molecules, the chemokines themselves, can block virus entry. Variants of SIV and HIV-1 have been identified that are CD4-independent, needing only a chemokine receptor for infection; the existence of these viruses suggests that the chemokine receptors might have been the primary receptor for a primordial virus. Further proof of the importance of the chemokine receptor is the existence of a mutant allele of the gene encoding CCR5 in the human population, a 32-bp deletion, that confers dramatic virus resistance to homozygous individuals. More discussion of the roles of CD4 and the co-receptors in virus entry will be presented in Chapter 49 on HIV-1.

PENETRATION AND UNCOATING

Once virus particles have bound to the receptor, the virion and host membranes fuse together, and the virion core is delivered

into the cytoplasm of the infected cell. Entry may require, or be promoted by, membrane regions of special lipid composition termed lipid “rafts”.^{334,364,484} Virus particles may “surf” or slide across the outside of the cell to preferred locations where fusion or entry inside the cell can occur.³²² For most retroviruses, the processes of fusion and entry are thought to be pH independent: that is, they are not dependent on an endosomal acidification step to induce a pH-dependent change in the conformation of the envelope. Thus, for these viruses fusion can occur at the cell surface. However, the ecotropic and amphotropic MuLVs and the subgroup A avian viruses are inhibited by drugs that block acidification; these viruses thus likely enter by passage through endosomes.

The process of fusion involves major rearrangements of the Env proteins, and especially includes the exchange of disulfide bonds that exist within or between the TM and SU subunits of Env. The process for the MuLVs seems to be controlled by Ca²⁺ levels, and involves TM–SU intersubunit disulphide-bond isomerization and SU dissociation.⁶¹⁷ Entry by HIV-1 probably also involves the removal or shedding of SU.

The processes of uncoating or opening of the core to permit reverse transcription to begin are poorly understood. It is clear that the previous processing of the Gag precursor to the mature Gag proteins is required; immature virions are uninfected and cannot initiate reverse transcription, and mutants that prevent particular cleavages of the Gag protein are similarly blocked. A large number of mutant viruses with other alterations in the *gag* gene have been shown to be defective in early steps of infection, before reverse transcription, but the functions of Gag proteins at this stage remain uncertain. Mutant virions that are fragile and uncoat prematurely or, conversely, are resistant to disassembly, are poorly infectious, suggesting that the timing of uncoating may be critical.¹⁷⁸ There are indications that host factors are important in these early stages. In the case of HIV-1, the host protein cyclophilin A, which interacts with CA, is required for the efficient initiation of reverse transcription.⁵⁵⁹ A plausible role for this protein is to facilitate virion disassembly.²²⁹ The TRIM proteins restrict virus infection at this time (see Early block to infection by Trim5a section).

Small molecule inhibitors have been used to demonstrate a role of the cytoskeleton in virus entry, and furthermore to suggest that viruses may utilize different entry pathways in different cell lines.²⁹³ Biochemical analyses of these early events are made difficult by the presence of large numbers of defective particles that are probably not on the infectious pathway and that tend to obscure the properties of the rare particles that are on this pathway. Nevertheless, examination by fluorescence microscopy of GFP-tagged virion particles during infection has indicated that intracellular movement likely occurs along cytoskeletal fibers.³⁷⁷

REVERSE TRANSCRIPTION

The reverse transcription of the viral RNA genome into a dsDNA form is the defining hallmark of the retroviruses, and the step from which these viruses derive their name. The course of reverse transcription is complex and highly ordered, involving the initiation of DNA synthesis at precise positions and translocations of DNA intermediates that result in duplication of sequence blocks in the final product (for reviews see 201,590).

The major steps in the reaction are relatively well established, largely through the analysis of reactions carried out *in vitro* in purified virion particles (the so-called “endogenous reaction”).

Reverse transcription normally begins soon after entry of the virion core into the cytoplasm of the infected cell. The reaction takes place in a large complex, roughly resembling the virion core, and containing Gag proteins including NC, RT, IN, and the viral RNA.⁶⁶ The signal that triggers the onset of DNA synthesis is not known, though it may be as simple as the exposure of the viral core to the relatively high levels of deoxyribonucleotides present in the cytoplasm. This notion is consistent with the observation that simply stripping or permeabilizing the virion membrane with detergents in the presence of deoxyribonucleotides is sufficient to induce DNA synthesis. This may also be at least part of the explanation for the difficulty HIV has in completing reverse transcription and infection in quiescent cells. In some cells, notably cells arrested by starvation, triphosphate levels may be low and limiting for RT, so that addition of exogenous nucleosides can stimulate viral DNA synthesis. But the signal may be more complicated. Conformational changes in the RNA genome at the tRNA primer site may trigger DNA synthesis.³⁷

DNA synthesis can be initiated prematurely during virion assembly and release, such that virion preparations can be shown to contain small amounts of the early DNA intermediates, such as minus-strand strong-stop DNA. In most cases the levels of these DNAs are very low, indicating that only a very small minority of the virion particles have carried out any significant synthesis. However, some circumstances affecting the rate of production and release of virions may enhance this synthesis. In addition, in some particular retroviruses, notably the spumaviruses, substantial DNA synthesis occurs during assembly such that the major form of the genome found in mature virions is a partially or even completely reverse transcribed DNA molecule.^{390,656} These viruses thus resemble the hepadnaviruses more closely than the conventional retroviruses in the relative timing of assembly and reverse transcription.

Steps in Reverse Transcription of the Retroviral Genome

The course of reverse transcription is complex. The reaction can be broken down into a series of discrete steps,²⁰¹ as presented in Figure 47.7.

Formation of Minus-Strand Strong-Stop DNA

The process of reverse transcription is initiated from the paired 3' OH of a primer tRNA annealed to the viral RNA genome at a complementary sequence termed the primer binding site (pbs). DNA is first synthesized from this primer, using the plus strand RNA genome as template, to form minus strand DNA sequences. Synthesis occurs toward the 5' end of the RNA to generate U5 and R sequences. The intermediate formed in this step is termed minus-strand strong-stop DNA. The primer tRNA remains attached to its 5' end.

First Translocation

The next step involves the translocation, or “jump,” of the strong-stop DNA from the 5' to the 3' end of the genome. This translocation requires the degradation of those 5' RNA sequences that were placed in RNA:DNA hybrid form by the formation of strong-stop DNA. The degradation is mediated

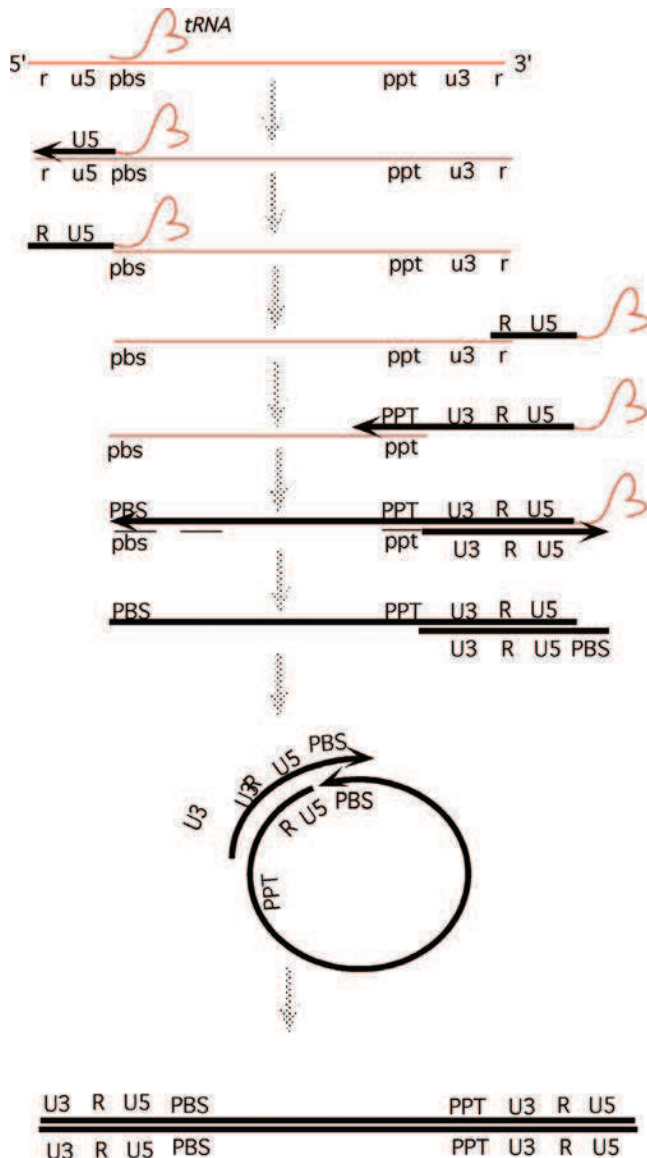


FIGURE 47.7. The reverse transcription of the retroviral genome. Thin lines represent RNA; thick lines represent DNA. See text for details. (Drawing courtesy of A. Telesnitsky.)

by the RNase H activity of RT; mutants with altered RNase H activity do not mediate the translocation. This step exposes the ssDNA and facilitates its annealing to the r sequences at the 3' end of the genome.⁹⁵ Normally a full-length strong-stop DNA, synthesized by copying to the 5' cap of the RNA, performs the translocation, though incomplete molecules can jump at low efficiency. The NC protein may facilitate the transfer step. Although there have been reports that jumping is always in *trans*, from one RNA template to the other RNA in the virion, the best evidence is that minus-strand strong-stop jumping goes randomly to either RNA.

Long Minus-Strand DNA Synthesis

The annealing of minus-strand strong-stop DNA recreates a suitable primer-template structure for DNA synthesis, and RT

can now continue to elongate the minus-strand strong-stop DNA to form long minus-strand products. Synthesis ends in the vicinity of the pbs. As the genome enters RNA:DNA hybrid form, the RNA becomes susceptible to RNase H action and is degraded.

Initiation of Plus Strand DNA Synthesis

The primer for plus-strand synthesis is created by the digestion of the genomic RNA by RNase H. A particular short purine-rich sequence near the 3' end of the genome, the polypurine tract or ppt, is relatively resistant to the activity of RNase H. The oligonucleotide remains hybridized to minus strand DNA and serves as the primer for synthesis of plus strand DNA, using minus strand DNA as template. The sequence of the PPT, an unusual structure of the nucleic acid at the PPT, and residues of the RNase H domain of RT have all been implicated in defining the cleavages that form the primer. Sequences upstream of the polypurine tract, an AT-rich region called the T-box, are also important for proper priming. The primer, once it has served to initiate DNA synthesis, is removed from the DNA. Synthesis proceeds toward the 5' end of the minus strand, first copying the U3, R, and U5 sequences, then extending further to copy a portion of the primer tRNA still present at its 5' end. Elongation stops at a modified base normally found at position 19 of the tRNA. The resulting intermediate is termed plus-strand strong-stop DNA.

In some viruses, secondary plus-strand initiation sites are used. There may be multiple RNA primers generated from the RNA genome by the nuclease action of RNase H that can initiate DNA synthesis at dispersed heterogeneous sites. In the case of the lentiviruses and spumaviruses, a second copy of the ppt sequence near the center of the genome is used at high efficiency, and is important for proper completion of reverse transcription.⁹¹

Removal of tRNA

In the next step, the primer tRNA at the 5' end of the minus strand DNA is removed by RNase H. Its removal may occur in two stages: with an initial cleavage near the RNA–DNA junction and a second one within the tRNA. The cleavage need not occur exactly at the RNA–DNA junction, and a single ribonucleotide base (A) is normally left on the 5' terminus of the HIV-1 minus strand without affecting subsequent processes. The posttranscriptional modifications present in natural tRNA are probably important for proper recognition by RT and for plus-strand strong-stop translocation.

The Second Translocation

The removal of tRNA exposes the 3' end of the plus-strand strong-stop DNA to permit its pairing with the 3' end of the msDNA. The sequences anneal via the shared pbs sequences. This annealing forms a circular intermediate, with both 3' termini in a suitable structure for elongation.

Completion of Both Strands

Both strands are now elongated. The final extension of minus strand DNA is coupled to displacement of the plus-strand strong-stop DNA from the 5' end of the minus strand; as minus-strand elongation occurs, the plus-strand strong-stop is peeled away and transferred to the 3' end of the minus

strand. At the end of this elongation, the circle is opened up into a linear DNA. The plus strands are then extended. When multiple plus-strand initiation events have occurred, the completed plus strand will consist of adjacent fragments and contain nicks or discontinuities. Displacement synthesis by an upstream fragment can slowly displace downstream RNAs and DNAs, leading to longer plus strands. However, some nicks or gaps may persist in the final double-stranded product. These breaks may be at heterogeneous positions, though strong sites of plus-strand initiation, such as the one at the central ppt of lentiviruses, can lead to specific sites for such discontinuities. Sequences near the central ppt of the lentiviruses cause termination of synthesis during elongation from upstream primers, ensuring the maintenance of a discontinuity at this site.⁹² This site retains a partially displaced sequence or overlap of a few nucleotides: 99 nt in the case of HIV-1. The structure has been shown to persist even to the time of integration of the DNA into the cell. Host DNA repair processes ultimately correct all such discontinuities.

Although most of the viral DNA is made in the cytoplasm, it may not always be completed in the cytoplasm. For some viruses, completion of the two DNA strands may occur only after entry into the nucleus. Specific mutants with alterations in the Cys-His residues of the NC protein show an interesting phenotype: the formation of linear DNA with heterogeneous and truncated ends.²⁰⁸ These experiments suggest that NC plays a role in the completion, or the stabilization of the ends, of the viral DNA.

A key consequence of the two translocation events that occur during reverse transcription is the duplication of sequences: duplication of U5 during minus-strand strong-stop DNA translocation and of U3 during plus-strand strong-stop DNA translocation. The resulting DNA thus contains two LTRs that have been assembled during reverse transcription. Each LTR consists of the sequence blocks U3-R-U5. The positions of the LTR edges—the left edge of U3, and the right edge of U5—are determined by the sites of initiation of DNA synthesis for the two DNA strands. Thus, the terminal sequences of the complete DNA molecule are also determined by these sites of initiation. These sequences for most viruses are perfect or imperfect inverted repeats, and serve an important role during integration of the DNA (see the Viral *att* sites section).

Biochemistry and Structure of Reverse Transcriptase

The enzyme that mediates the complex series of events outlined in the previous section is RT, one of the most famous of the viral polymerases (25; for review, see 553). All RTs contain two separate activities present in two separate domains: a DNA polymerase able to incorporate deoxyribonucleotides on either an RNA or a DNA template, and an RNase H activity able to degrade RNA only in duplex form. These two activities are responsible for the various steps of reverse transcription. Two distinct domains of the enzyme contain these two activities: an aminoterminal domain contains the DNA polymerase, and a carboxyterminal domain contains the RNase H activity.⁵⁸⁷ While isolated domains can be shown to exhibit either one of the two activities separately, an intact enzyme is required for full activity and specificity. However, the two functions can be provided by two mutant RT molecules so long as they are co-incorporated into a single virion.

DNA Polymerase

DNA polymerase activity is similar to that of all host and viral polymerases in requiring a primer, which can be either RNA or DNA, and a template, which can also be either RNA or DNA. RTs incorporate dXTPs to a growing 3'OH end with release of PP_i, and require divalent cations, usually Mg⁺⁺. The primer must contain a 3'OH end that is paired with the template. RTs cannot perform nick-translation reactions, but they can efficiently perform strand displacement synthesis. The only fundamental way in which RTs are unusual among the DNA polymerases is that they exhibit comparable specific activity on either DNA or RNA templates.

RTs are readily isolated from purified virion particles, and can be even more easily prepared as recombinant proteins expressed in bacteria. RTs are relatively slow DNA polymerases, under standard conditions only incorporating 1 to 100 nucleotides per second, depending on the template. Further, they exhibit poor processivity, and tend to release primer-template frequently *in vitro*. The enzyme must then rebind to the substrate to continue synthesis. Secondary structures in RNA templates can strongly enhance the pausing of RT and its tendency to release from the template.²²⁶ The enzyme also exhibits low fidelity, and though the values of the error rate vary widely with the primer, template and type of assay, the misincorporation rate of most RTs under physiologic conditions is on the order of 10⁻⁴ errors per base incorporated. This rate suggests that during replication there would be approximately one mutation per genome per reverse transcription cycle. The mutation rate observed *in vivo* is roughly consistent with this high error rate, though fidelity *in vivo* may be somewhat better than *in vitro*. Drug-resistant variants that do not incorporate chain-terminating analogs are often found to exhibit higher fidelity, perhaps because they require a more precise fit for the correct incoming triphosphate to allow for discrimination against the analog. A wide range of types of mutations are created by RT errors, and both the type and the frequency of appearance of each type of mutation exhibit a complex dependence on sequences and structures in the template.

RTs do not generally exhibit a proofreading nuclease activity,³⁵ and misincorporated bases are not removed as efficiently by most RTs as they are by host DNA polymerases. However, mutants of the HIV-1 RT resistant to AZT have been shown to exhibit an enhanced ability to remove the incorporated AZT moiety at the 3' end through a pyrophosphorolysis reaction.³⁸² Thus, it is possible for RT to remove some such analogs and rescue a terminated chain for continued elongation.

RNase H

The RNase H activity of RT is an endonuclease that releases oligonucleotides with a 3'OH and a 5'PO₄. This property allows the products of RNase H action to serve as primers for initiation of DNA synthesis by the DNA polymerase function of RT. There is an obligate requirement that the RNA be in duplex form, normally an RNA–DNA hybrid. However, retroviral RTs are also able to degrade RNA–RNA duplexes, an activity termed RNaseH*.²⁴³ The RNase H enzyme is capable of acting on the RNA of a template in concert with the polymerase as it moves along a nucleic acid, and as it does so its active site is located about 17 to 18 bp behind the growing 3' end.²⁰⁶ RNase H can also act independently of polymerization. All RNase H activity requires a divalent cation.

Subunit Structures

RT is incorporated into the virion particle during assembly in the form of a large Gag-Pol precursor (see below), and is released by proteolytic processing of the precursor during virion maturation. Different viruses make somewhat different cleavages in the precursor, and thus the RTs exhibit several different subunit structures (see below). In the gammaretroviruses, RT is a simple monomer in solution, corresponding only to the aminoterminal DNA polymerase and the carboxyterminal RNase H domains. These two domains can be expressed separately,⁵⁸⁷ and the isolated proteins exhibit their respective activities,⁵⁸⁷ though the specificity of the RNase H is affected by this separation. In the avian viruses, the RT is present as an $\alpha\beta$ heterodimer, comprised of a smaller subunit containing the DNA polymerase and RNase H domains; and a larger β subunit containing these two domains but also retaining the integrase domain. In the lentiviruses, RT is again a heterodimer with a larger subunit (p66) containing the DNA polymerase and RNase H domains, and a smaller subunit (p51) lacking RNase H. The properties of the different enzymes as DNA polymerases are very similar in spite of these different subunit structures, and thus the significance of these various compositions for RT function is unclear. A curious observation was made that some RT inhibitors—the so-called nonnucleoside RT inhibitors—can potentially enhance the association of p66 and p51, locking them into an inactive dimer.⁵⁸⁰

Crystal Structures

The three-dimensional structure of a number of RTs have been determined by X-ray crystallographic studies. Structures of the unliganded HIV-1 RT,^{246,515} RT bound to nonnucleoside RT inhibitors,^{127,135,300,504} RT bound to an RNA pseudoknot inhibitor,²⁶⁰ RT bound to a duplex oligonucleotide,^{17,248,258,259} and RT bound to a polypurine tract RNA:DNA hybrid,⁵³¹ as well as the isolated RNase H domain,¹²⁸ have all been reported. The two subunits are folded very differently so that the overall structure is highly asymmetric. The structure of the p66 is similar to that of a right hand, with fingers, palm, and thumb domains named on the basis of their position in the structure (Fig. 47.8). The nucleic acid lies in the grip of the hand, held

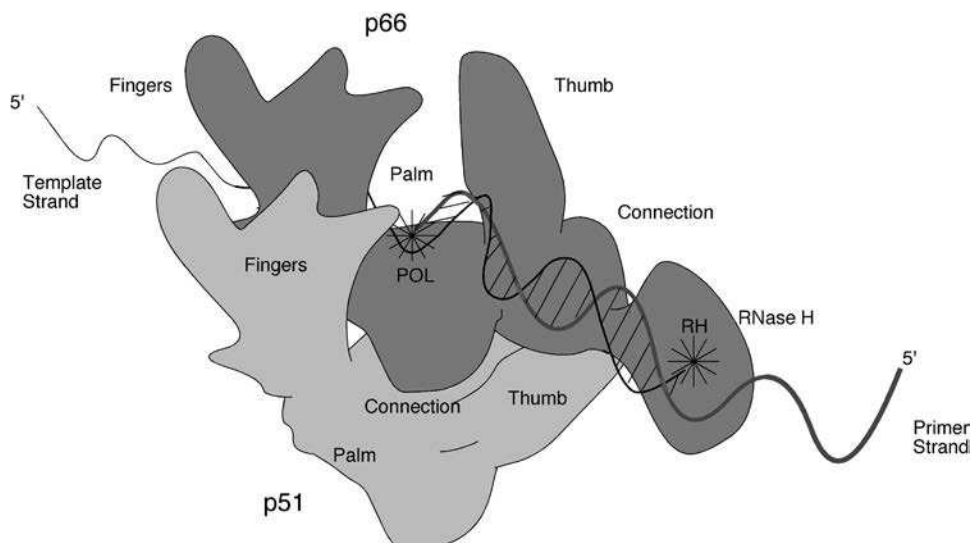
by the fingers and thumb. The YXDD motif present at the active site for the DNA polymerase lies at the base of the palm. The RNase H domain is attached to the hand at the wrist. The p51 subunit, while made up of the same domains as the aminoterminal part of p66, is folded differently and lies under the hand, not making direct contact to the nucleic acid and thus not thought to participate in chemistry. The structure of p66 with and without a liganded nucleic acid is very different, with the thumb domain flexing to allow substrate binding. A surprising aspect of the structures is that the nucleic acid helix can be highly bent, perhaps accounting for the enzyme's ability to sense conformationally strained substrates.⁵³¹ Theoretical considerations suggest that the thumb may move during elongation.

Structures of the fingers and palm subdomain and of the complete Moloney MuLV RT at very high resolution have also been determined.^{126,200} The monomeric protein is broadly similar to the p66 subunit of HIV-1 RT.

Inhibitors

RT is a major target of antiviral drugs useful in the treatment of retroviral diseases such as AIDS. All such drugs used to date are inhibitors of the DNA polymerase activity of RT, and fall into two classes: nucleoside analog inhibitors (chain terminators), and nonnucleoside RT inhibitors (NNRTIs). The nucleoside analogs are typically prodrugs, and need to be activated by phosphorylation to the triphosphate form. These are then incorporated by RT into the growing chain, and serve to block further elongation. Examples include AZT, ddC, ddI, d4T, and 3TC. The NNRTIs are a group of compounds that are structurally diverse, but nevertheless interact with a common binding pocket in RT to prevent its normal activity.⁶⁰⁰ There are indications that the binding may inhibit the enzyme's flexibility. For both classes of inhibitors, monotherapy with a single drug selects for drug-resistant variants that quickly predominate in the virus population, and for each drug, a pattern of mutations has been identified that serves to indicate the appearance of drug resistance.³¹⁵ In many cases these mutations alter the binding side for the nucleoside or NNRTI such that the drug cannot bind and therefore cannot inhibit the enzyme. In the case of AZT, however, the mutations do

FIGURE 47.8. Schematic image of the heterodimeric reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1), showing the p66 (top, dark gray) and p51 (bottom, light gray) subunits. The molecule is arranged in the conventional orientation to show its similarity to the human right hand, palm up. Fingers, thumb, palm, connection, and RNase H domains of each subunit are indicated. An RNA template strand (*thin line*) and a DNA primer strand (*heavy line*) are modeled into the polymerase (Pol) and RNase H (RH) active sites.



not prevent the binding and incorporation of AZTTP into the growing chain, but rather seem to activate a reverse reaction in which the AZT nucleotide is removed from the chain, subsequently permitting normal elongation.³⁸² Combination therapy, typically involving the simultaneous treatment with three different drugs, can suppress virus replication to such an extent that variants resistant to all the drugs do not appear, at least for months or years.

RECOMBINATION

The process of reverse transcription could in principle take place using a single template RNA molecule. In fact, however, retrovirions contain two copies of the RNA genome co-packaged into one particle, and the course of reverse transcription typically makes use of both RNAs.^{247,573} Recombination occurs between homologous sequences in the two RNAs, happening at surprisingly high frequencies, more than once per replication event per genome on average.^{511,667} Normally the two RNAs in a virion are identical, so that homologous recombination events are invisible and without consequence. When the two RNAs are distinct, however, as when they derive from two viruses or viral strains, the result is a very high frequency of recombination between them among the resulting proviral DNAs. Thus, physical markers and genetic markers recombine rapidly whenever the two genomes are co-packaged into one virion and thus are co-extant during a single round of reverse transcription. The frequency is highly dependent on the sequence and structure of the RNA in the region undergoing recombination. Similar recombination does not occur at high frequency when cells are co-infected simultaneously with two separate virus preparations, suggesting that each incoming virus particle performs its own reverse transcription reaction in the cytoplasm in *cis*, and does not freely exchange RNAs with other reactions happening in the same cell.

Models for Recombination

Two mechanisms provide for recombination between two genomes. In one, the copy choice model, recombination occurs during minus-strand synthesis. As RT proceeds along an RNA, it has the potential to carry out a template switch in which an incomplete DNA copied from one template serves to prime further elongation on the other RNA molecule.^{351,465} Pausing may enhance this transfer, and secondary structures in the RNA may act as hot spots for such recombination. Breaks in the RNA genome, which may be encountered often, cause a “forced copy choice”: transfer to the other RNA. This rescues an otherwise dead virus, and may represent the major evolutionary basis for high-frequency recombination in the viruses. The RNase H activity of RT may help release an incomplete DNA, promoting its serving as primer on the new template; NC also facilitates the reaction.⁴¹³ This mechanism is likely the more important one of the two.⁶⁶⁶

In the other mechanism, strand-displacement assimilation, recombination occurs when at least portions of two minus strands have been synthesized in one virion. While multiple plus-strand fragments are elongating on one minus-strand template, strand displacement can expose the 5′ end of such fragments, which can then pair with the other minus-strand DNA to form a bridged “H” structure as intermediate. Further

synthesis and repair of these structures leads to the transfer of sequences to the new DNA.²⁷⁴

When a recombination event occurs, there is a nonrandom increase in the probability that another recombination will occur nearby, a phenomenon called negative interference. This suggests that RT or the genomes may become recombination prone at specific times. When multiple recombination events occur, the resulting DNA is a patchwork of the sequences derived from the two input RNAs.

The translocation of the two strong-stop DNAs provides a special opportunity for recombination between the two viral genomes. When the minus-strand strong-stop DNA is formed, it has the potential to translocate from the 5′ end of its template to the 3′ end of either RNA molecule; though this event has been reported to occur strictly in *cis*, or strictly in *trans*, it most likely occurs randomly. Similarly, when plus-strand strong-stop DNA is formed, it too could in principle translocate to the 3′ of either minus strand. However, this translocation seems most often to occur in *cis*, perhaps simply because the frequency with which two long minus-strand DNAs are successfully formed, and thus are available to serve as acceptors, is low.

Recombination between two RNAs during reverse transcription can also occur between nonhomologous sites at lower frequency. Reconstructions suggest that these events are perhaps 100 to 1000 times less frequent than homologous recombination. These events can result in duplications or deletions in the DNA product of the reaction. Furthermore, if nonviral RNAs or chimeric RNAs containing viral and nonviral sequences are packaged into virions, such nonhomologous recombination events can create new joints and link a viral sequence to the nonviral sequences. These events are thought to play a central role in the process of transduction of cellular genes, most importantly during the formation of acute oncogenic retroviral genomes (see below).

INTEGRATION OF PROVIRAL DNA

The integration of linear retroviral DNA, like reverse transcription, is a crucial and defining feature of the retroviral life cycle. Integration is required for efficient replication of most retroviruses; mutants that are unable to integrate do not establish a spreading infection. The orderly and efficient integration of viral DNA is unique to the retroviruses. Although infection by some DNA viruses can result in the integration of viral DNA fragments into the host genome at low efficiency, these events are not the result of specific viral functions. Further, the establishment of the integrated provirus is responsible for much of retroviral biology. It accounts for the ability of the viruses to persist in the infected cell; for their ability to permanently enter the germ line; and for the mutagenic and oncogenic activities of the leukemia viruses. It also establishes a reservoir of latently infected cells in AIDS patients that resists antiviral drug therapy and that can be reactivated to induce virus replication.

Once the provirus is established, the DNA is permanently incorporated into the genome of the infected cell. There is no mechanism by which it can be efficiently eliminated. At very low frequencies, homologous recombination between the two LTRs can delete most of the provirus, but even here a single (“solo”) LTR remains.⁶⁰⁹ As the host cell divides, the provirus is transmitted to daughter cells as a new Mendelian locus. Thus,

it is likely to persist in the cell for its normal life span and to convert the cell permanently to a chronic producer of progeny virus.

Unintegrated DNA Forms

The product of the reverse transcription reaction, as outlined in the previous section, is a full-length double-stranded linear DNA version of the genome, flanked at each end by copies of the LTR. The next step is the movement of the DNA into the nucleus, and the appearance of two new DNA forms: closed circular molecules containing either one or two tandem copies of the LTR (Fig. 47.9). A small amount of the one-LTR circle may be formed during reverse transcription (see the Steps in reverse transcription of the retroviral genome section), but the bulk is thought to be formed by homologous recombination between the two LTRs of the linear DNA. The tandem two-LTR circles are apparently formed by the blunt-end ligation of the termini of the linear DNA. This event creates a unique sequence, termed the LTR–LTR junction, that is often used as a hallmark of nuclear entry of the viral DNA. The joints are often imperfect, with loss of nucleotides from one or both termini at the joint.^{556,626} There are also some circles that arise by autointegration of the ends of

the linear DNA into internal sites, forming DNAs with deletions or inversions⁵⁵¹; these circles are generally nonfunctional in terms of generating progeny virus.

Since three distinct unintegrated DNA forms—one linear and two circular—coexist in the nucleus, it was uncertain for many years which form might serve as the precursor for establishment of the integrated provirus. In spite of prejudices based on such precedents as phage lambda, it is now clear that circles are not efficient substrates in the integration reaction and that the immediate precursor for the integration reaction is the linear duplex DNA. The circles are apparently dead-end products of a side reaction, formed by host enzymes acting on linear DNAs that have failed to integrate. There are settings and cell types in which unintegrated viral DNAs are observed to accumulate to high levels; various tissues in human HIV disease show considerable circular DNAs. While this DNA may reflect some unusual processing of the DNA, much of it is probably formed simply by massive infection occurring shortly before the DNA is harvested.

Unintegrated DNA is not a good substrate for forward transcription,⁵²⁷ perhaps because it is still retained in a complex that is poorly accessible to RNA polymerase. Mutant viruses that cannot integrate are unable to establish an efficient spreading infection, although low levels of virus can be produced.⁵⁴¹ A very small subset of cells infected with such integration-defective mutants do integrate viral sequences through nonviral means, creating oligomeric tandem repeats similar to those formed after naked DNA-mediated transformation.²²²

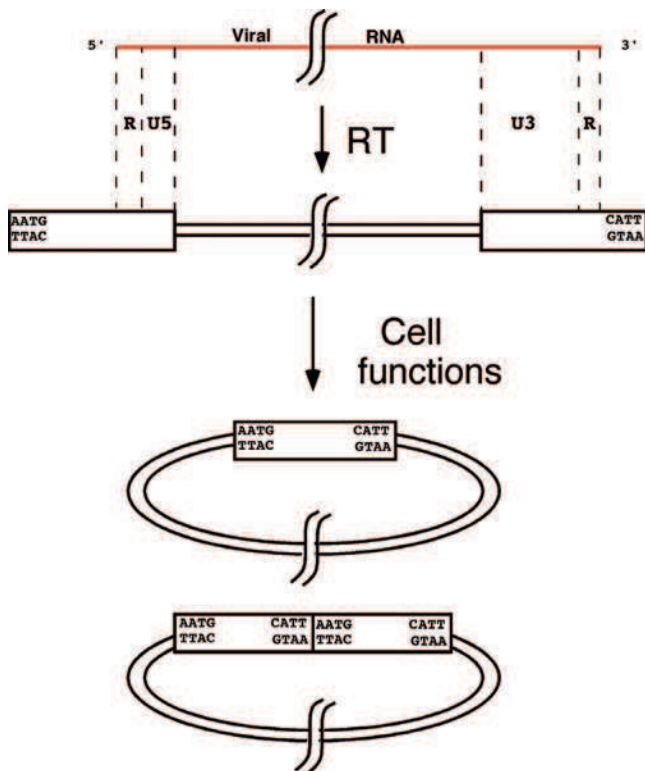


FIGURE 47.9. Unintegrated DNA structures formed after retroviral infection. The incoming RNA genome (*top*) is converted by RT to a double-stranded linear DNA containing two LTRs (*boxes*) in the cytoplasm. The termini of the DNA consist of short, inverted repeats, and always contain a conserved CA dinucleotide near the 3' ends; the 3' terminal sequences of the MLVs (CATT) are shown. The linear DNA is then localized to the nucleus, and two circular double-stranded DNAs are formed: a circle containing one LTR, and a circle containing two tandem LTRs. The LTR–LTR junction contains a unique inverted repeat sequence.

Entry into the Nucleus

A key step that must take place before integration can occur is the entry of viral DNA into the nucleus. The mechanisms of nuclear entry are largely unknown, but there are probably at least two distinct routes used by different retroviruses. Simple retroviruses show a profound requirement for passage through mitosis for successful establishment of the integrated provirus,^{326,385,517,608} and the block in nondividing cells is at or close to the step of nuclear entry. Tests of the state of the viral DNA in nondividing cells are consistent with the notion that the pre-integration complex must await the breakdown of the nuclear membrane in order to have access to the cellular DNA. Infection of nondividing cells results in the accumulation of linear dsDNA in the cytoplasm, and no further signs of infection. The viral DNA will persist in the cell for some time, and if the cell is stimulated to undergo division, the viral DNA will integrate and infection will proceed. However, the DNA loses its capacity to become activated in this way fairly rapidly.^{15,385} Some simple retroviruses are not strongly dependent on mitosis,²²⁸ and some postmitotic cell types may be susceptible to infection.³³⁸ For many viruses the restriction is quantitatively very significant, and profoundly limits the utility of simple retroviral vectors for gene therapy.

In contrast, lentiviruses and spumaviruses are able to successfully infect nondividing cells, suggesting that there must be an active transport of viral DNA through an intact nuclear membrane.^{77,325,326,409,623} This capability has made lentiviruses very attractive as gene delivery vectors for gene therapy applications. The molecular basis for this capability is a subject of great controversy. The lentiviral MA protein has been argued as essential for the infection of nondividing cells, and the phosphorylation of MA has been argued as necessary to promote

dissociation from the membrane and allow nuclear import, but these findings were discounted in later studies. Similarly, it has been shown that the Vpr protein is present in the preintegration complex, and can bind to nucleoporin components that may mediate nuclear import. DNA structures present at the second internal copy of the polypurine tract have also been suggested as important for infection of nondividing cells, but this notion has also been discounted. Another attractive model is that the IN protein might be involved in the nuclear import of the complex. IN itself contains nuclear localization signals that can function to target ectopically expressed IN to the nucleus, but these seem not to mediate PIC nuclear import or nuclear retention.

Recent experiments suggest that the CA protein of the incoming PIC may define competence for nuclear import.⁶⁴⁶ The lentiviral CA may serve to deliver the PIC to particular Nups, nuclear pore components, to initiate import. Studies of HIV-1 mutants with single changes in CA suggest that PICs can be imported via either of two alternative pathways, with wild-type virus using Nup153 and TNP03, and the N74D mutant using Nup155.^{99,306,320} Other studies have implicated Nup98 in HIV-1 PIC entry into the nucleus.¹⁵¹ Another study of import *in vitro* has suggested that a specific importer protein, importin 7, is required for PIC entry,^{167,661} though this has been disputed.⁶⁷² Fractionation of extracts using similar *in vitro* import assays showed, remarkably, that tRNAs can promote uptake of PICs into nuclei.⁶⁶² Whether tRNAs mediate import *in vivo* remains uncertain.

Foamy viruses may have a distinctive route of nuclear entry involving microtubular transport by dynein and centrisomal association, but the mechanism is not yet well understood.^{474,526}

Structure of the Provirus

An important aspect of retroviral integration that distinguishes the process from nonviral or other viral mechanisms of DNA integration is the fact that the insertions create a consistent provirus structure. The integrated provirus is collinear with the product of reverse transcription, and consists of a 5' LTR, the intervening viral sequences, and a 3' LTR, inserted cleanly into host sequences. The joints between host and viral DNA are always at the same sites, very near the edges of the viral LTRs. As compared to the unintegrated linear DNA, there is a loss of a small number of base pairs, usually two, from each terminus of the viral DNA. There is also a duplication of a small number of base pairs of host DNA initially present once at the site of insertion that flank the provirus (Fig. 47.10). The number of

base pairs duplicated is characteristic of each virus, and ranges from 4 to 6 bp.

Biochemistry of Integration

The actual integration of viral DNA into a target is mediated *in vivo* by the viral integrase protein IN,^{450,495,541} which is brought into the cell inside the virion, and acts to insert the linear DNA into the host chromosome. Some aspects of IN function have been studied by analysis of viral DNA formed *in vivo*.⁵²¹ Most of our understanding of IN function, however, has been obtained through analysis of *in vitro* integration reactions, first using complexes extracted from infected cells,^{74,186} and later using recombinant IN protein. The reaction proceeds in two steps: 3' end processing and strand transfer. A schematic view of these reactions is shown in Figure 47.11.

3' End Processing

In the first step, the two terminal nucleotides at the 3' ends of the blunt-ended linear DNA are removed by the integrase to produce recessed 3' ends and correspondingly protruding 5' ends. This cleavage occurs endonucleolytically at a highly conserved CA sequence, and releases a dinucleotide. For most viruses the terminal sequence is such that a TT dinucleotide is released, though this rule has exceptions. The ends do not remain covalently bound to protein, and the energy of the hydrolyzed phosphodiester bond is not retained.

Strand Transfer

In the second step, the 3'OH ends created by processing are used in a strand transfer reaction to attack the phosphodiester bonds of the target DNA.¹⁸⁶ The attack occurs by an Sn2-type reaction, with inversion of the phosphorus center as detected with chiral labeling of the phosphate.¹⁵⁸ The formation of the new phosphodiester bond between the viral end and host DNA displaces one of the phosphodiester bonds in the host DNA, leaving a nick. The protruding 5' end of the viral DNA is not joined to the host DNA by IN. The reaction is a direct transesterification, and thus no ATP or other energy source is required. Mutational studies strongly suggest that the two activities—processing and joining—utilize the same active site residues. In fact, the two steps involve similar chemistry: 3' end processing is an attack on DNA by a hydroxyl residue of water, while joining is an attack on DNA by a 3' hydroxyl residue of another DNA. It should be noted that other hydroxyl residues can participate; alcohols such as glycerol can be utilized, and

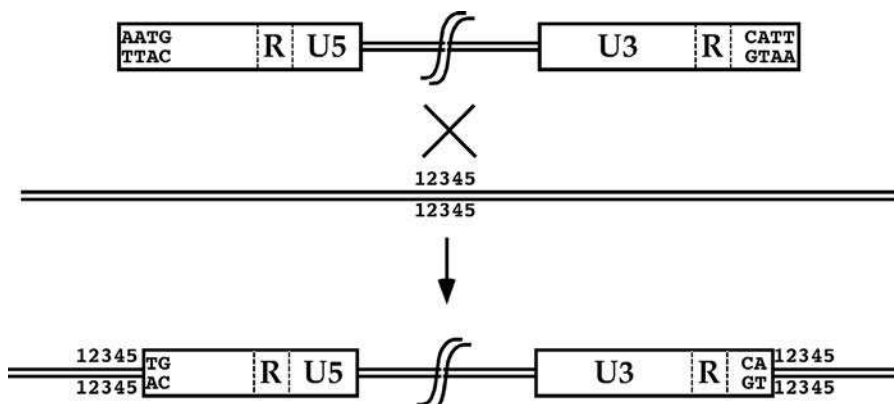


FIGURE 47.10. Integration of the viral DNA to form the provirus. The precursor for the formation of the provirus is a linear double-stranded DNA containing two LTRs (boxes) and with inverted repeat sequences at the termini. The target site in the host DNA is indicated by the arbitrary sequence block denoted 12345. Integration occurs by joining the 3' CA dinucleotides near the termini to the target DNA. The reaction is associated with loss of two base pairs at the termini of the viral DNA, and with duplication of a small number of base pairs (5 shown here) initially present only once in the target DNA.

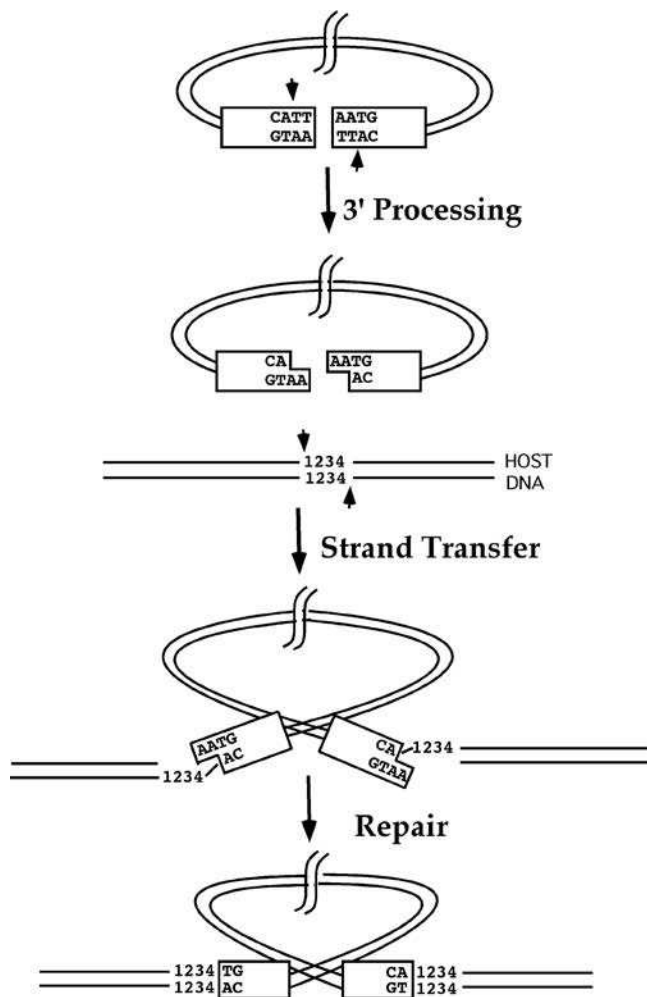


FIGURE 47.11. Steps in the integration of the viral DNA. The full-length linear DNA (top) is processed by the viral integrase with the endonucleolytic removal of dinucleotides at the 3' termini. The resulting DNA is then used in a strand transfer reaction in which the 3' OH ends attack phosphodiester bonds of the target site DNA to make staggered breaks in the two strands. The resulting gapped intermediate is subsequently repaired by host enzymes.

the 3'OH of a DNA can even attack a phosphodiester bond on the same DNA, forming a cyclic product.¹⁵⁸

Disintegration

The IN protein exhibits a third enzymatic activity *in vitro*: a reversal of the integration reaction known as disintegration.⁹⁸ This activity releases DNA from a branched structure and seals the nick at the site of the branch. The significance of the activity *in vivo* is uncertain.

Target Site Duplication

In a wild-type virus, when the two ends are joined to the two strands of the target DNA, the two sites of attack are staggered by a few base pairs. After the joining, the resulting structure contains short gaps in the host DNA and unpaired bases from each 5' end of the viral DNA. The 5' ends of the viral DNA are not joined to host DNA by any known activities of IN.

However, the 5' ends are very quickly repaired *in vivo*, almost as quickly as the initial integration reaction.⁵¹⁶ These discontinuities are presumed to be repaired by the host repair enzymes, though it is possible that the viral RT or IN could participate. The processing and filling in the gaps creates a short duplication of sequence that was present only once at the target site; these duplications flank the integrated provirus. The number of bases duplicated is characteristic of each virus. Thus, the murine and feline viruses cause a 4-bp duplication, HIV-1 causes a 5-bp duplication, and the avian viruses cause a 6-bp duplication.

Viral *att* Sites

The sequences at the termini of the viral DNA, the *att* sites, are recognized by the viral IN protein and are important for end processing and joining.^{79,104,109,610} These terminal sequences are imperfect inverted repeats. The most conserved residues are a CA dinucleotide pair that lies near the 3' terminus and determines the site of 3' processing. Sequences upstream from the CA for perhaps 10 to 12 bp are needed for efficient integration, but these sequences are different for different viruses, with no indication of broadly conserved sequence motifs. Since the two termini of any given virus are somewhat different, they usually show differential efficiency of utilization in various assays. The fact that two distinct ends are bound together in a complex may be important for the concerted integration of these ends into the target.⁶¹⁵

The sequence-specific binding to the *att* site is probably performed by the core domain of IN. The nonspecific DNA binding activity of IN has made it difficult to detect sequence-specific binding to these regions, though under some conditions preferential binding to the authentic sequences can be demonstrated.¹⁶¹ The observation that an IN mutation can compensate for a mutation in the DNA termini provides evidence for the delicate interaction between IN and the DNA termini.

Both 3' processing and strand transfer reactions are concerted reactions *in vivo*. The processing step occurs simultaneously at both termini of the viral DNA and requires the correct sequences at both termini. Thus, a mutation altering the sequence at one end of the viral DNA of MuLV blocks the processing reaction at both ends.⁴⁰⁵ This result suggests strongly that the reaction requires both termini to be loaded into a complex before hydrolysis proceeds. Similarly, the strand transfer reaction normally occurs so that both ends are joined to the target DNA, and at a fixed spacing between the two sites along the DNA helix. The 3' processing and strand transfer reactions can both be carried out *in vitro* using native PICs, extracted from recently infected cells, and these reactions reconstruct the concerted nature of the *in vivo* reactions. Alternatively, integration can be performed using artificial DNA constructs and recombinant IN protein. However, these systems typically only mediate a half-reaction: that is, the uncoupled processing of one viral terminus and its joining into a single target DNA. Efforts have led to the identification of conditions and factors that mediate formation of a complex and that enhance concerted joining.^{7,175,616} Once such a protein–nucleic acid complex is formed, it is very stable.

Structure of the Integrase

The IN protein consists of three distinct domains: an N-terminal region containing an HHCC zinc-finger motif; a

central catalytic core containing the so-called D,D-35-E motif; and a less well-conserved C-terminal region. The IN protein is a multimer: it readily dimerizes, and at high concentration forms tetramers as well. All three regions may be involved in the multimerization of IN and in DNA binding. Many of the residues important for enzymatic activities have been identified by mutagenesis. The most crucial residues for catalysis are the acidic amino acids in the D,D-35-E motif, a highly conserved array of three residues in the core region of many integrases and transposases.³⁰⁸ Mutants indicate that both the N- and C-terminus are also important for function. Surprisingly, pairs of IN mutants with alterations in different regions of the molecule can often complement to restore normal function. The separate N-terminal domain can even complement a nonoverlapping fragment, suggesting that these domains can still co-assemble into a functional oligomeric complex.

Early X-ray crystallography work first defined the structures of the HIV-1 and avian virus IN core domains, and NMR methods defined the structures of the N- and C-terminal domains. Very recently a crystal structure of the complete integrase from a foamy virus in complex with a model target DNA oligonucleotide has been obtained.³⁵⁹ This structure reveals a tetramer, arranged as a dimer of dimers, holding the target

DNA in a strongly bent conformation. The catalytic sites for strand transfer are nicely positioned to hold the viral DNA termini for attack of the target DNA (Fig. 47.12).

Preintegration Complex

Integrase does not normally act alone; a large complex of proteins and nucleic acid is responsible for mediating the formation of the provirus *in vivo*.^{66,94} The nature and components of the preintegration complex (PIC), or intasome, are not known in any detail for either the simple or the complex viruses. The PICs of the simple gammaretroviruses contain p12, CA, RT, and IN, but other viral proteins may be present.^{66,489} The PICs of the complex viruses contain only lower levels of CA, but contain MA, NC, Vpr, RT, and IN.³⁸⁸ Thus, the PICs of the complex viruses may be very different from those of the simple viruses, consistent with their distinctive ability to infect nondividing cells.^{165,166} Many of these proteins probably stay with the DNA even after entry into the nucleus. The PICs contain a large structure protecting the two ends of the DNA, and perhaps holding them in proximity. The formation of this structure, detected as a footprint in a modified nuclease sensitivity assay,⁶²¹ requires both IN and the correct sequences at the termini of the DNA.⁶²²

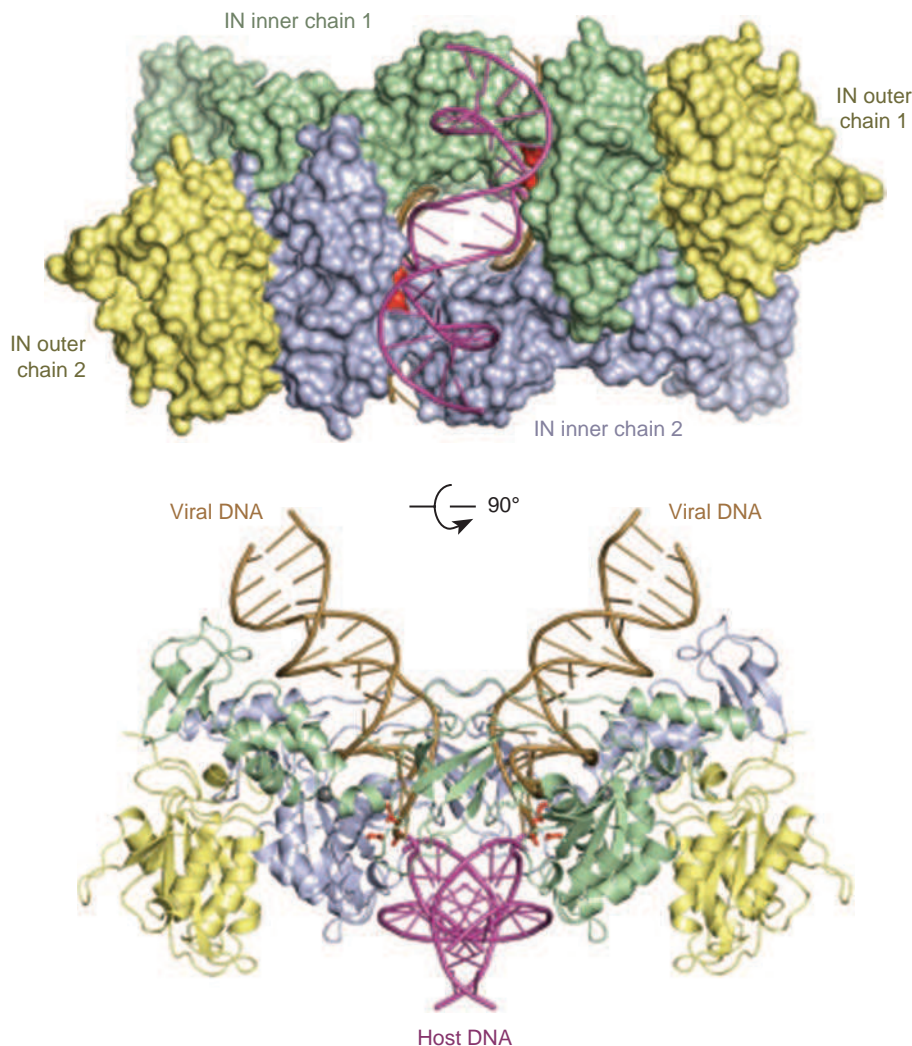


FIGURE 47.12. Structure of the integrase of a prototype foamy virus in complex with a short target DNA oligonucleotide.

Top, space filling model of integrase tetramer bound to the DNA. Domains are indicated: NTD, N-terminal domain; NED, NTD extension domain; CTD, C-terminal domain; CCD, catalytic core domain. *Bottom*, Ribbon diagram of protein. Position of domains, target DNA (tDNA) and modeled viral DNAs (vDNA) are indicated. (Courtesy of Peter Cherepanov, Division of Infectious Diseases, Imperial College London, London, UK.)

Host Proteins and Integration

A number of host proteins have been identified as potentially involved in the establishment of the provirus. One such protein is BAF-1, a low-molecular-weight protein recovered from the MuLV PIC for its ability to inhibit autointegration of the LTR edges into internal sites in the viral DNA.³²¹ By inhibiting this reaction, BAF-1 can enhance normal integration into target DNAs in *trans*. However, infection of BAF-1-deficient cells occurs normally, suggesting that BAF-1 is not an essential player in the early events of the viral life cycle. Another such partner is LEDGF (lens epithelial-derived growth factor, a misnomer), a nuclear protein of uncertain function, which binds directly to the HIV-1 IN and dramatically enhances its integration activity.^{96,339,358}

The integration of retroviral DNA has been shown to activate an apoptotic program in cells deficient in DNA-stimulated protein kinase (DNA-PK), an enzyme implicated in the DNA damage response¹²⁵; the related kinase ATR and other components of the nonhomologous end joining repair machinery may also be involved.^{123,124} While it is not clear whether these kinases play any direct role in integration, they are likely involved in sensing the products of active integrase and responding to the damage. Their absence leads to substantial cell death in cells taking up the PIC.

Distribution of Integration Sites

An important issue affecting the ability of the retroviruses to create mutations is the distribution of integration sites in the host genome. Proviruses are inserted at very approximately random locations in the genome, and thus have the opportunity to create mutations in any gene. Various studies, however, have uncovered significant deviations from a completely random distribution. At the sequence level, examination of large numbers of integration sites has revealed weak but statistically highly significant preferences for symmetrical target sequences.^{212,240,643} Large-scale surveys of thousands of integration sites cloned from pools of infected cells have allowed analysis of the frequency of insertions into the 5' upstream regions of genes, into transcribed regions, and into nontranscribed regions. The results show that different viruses show distinct biases for their target sites.^{30,389,535,642} HIV-1 tends to insert into transcribed regions, more or less equally along such regions; MuLV tends to selectively insert its DNA in sequences upstream from the 5' end of transcribed regions, near transcriptional start sites; ASLV shows only very weak preference for active genes and none for 5' regions. Activation of transcription *per se* can apparently, in some circumstances, inhibit avian retroviral integration at specific genes.³⁷² These studies collectively show that various retroviruses have evolved mechanisms to choose aspects of their integration sites, presumably in support of their chosen life styles during infection. The biases are presumably determined largely by their respective IN proteins, but could also involve other viral proteins.

EXPRESSION OF VIRAL RNAs

The integration of the provirus signals a dramatic change in the life style of retroviruses; it marks the end of the early phase of the life cycle and the beginning of the late phase. The early phase is driven by viral enzymes performing abnormal events such as reverse transcription and DNA integration, while the

late phase is mediated by host enzymes performing such relatively normal processes as transcription and translation. This late phase of gene expression may begin immediately with the synthesis of viral RNAs and proteins, and the assembly of progeny virions (see Fig. 47.13 for an overview). For many viruses, the transcriptional promoters that drive this expression are constitutively active and cause the production of virions in a relatively unregulated way. In other viruses the activity of the promoter may be regulated, either by viral or host factors. The basic phenomenology of proviral gene expression will be reviewed, and the regulation exhibited by the complex retroviruses will be mentioned briefly.

Overview of Viral RNA Synthesis

The synthesis of viral RNA from viral DNA leads to the formation of a long primary transcript, which is then processed and may be spliced to form a small number of stable transcripts. The U3 region of the LTR contains a promoter recognized by the RNA polymerase II system; these sequences direct the initiation of transcription starting at the U3-R border. Cellular machinery then caps the 5' end of the RNA with m⁷G5'ppp5'G_mp. The first G residue after the cap is a templated base in the provirus. Transcription proceeds through the genome, and continues through the 3' LTR and into the downstream flanking host DNA. Finally, the RNA is cleaved and polyadenylated at the R-U5 border of the 3' LTR, generating a complete, unspliced viral genomic RNA suitable for incorporation into the virion particle. Most genomes contain an AAUAAA sequence acting as the signal for this 3' processing. The sequence normally lies in the R region, but the complete sequence needed for recognition can be complex, lying upstream or downstream, and may even be discontinuous, brought together by RNA folding to create the functional signal. The exact site of polyadenylation is not critical for virus replication; mutants in which the polyadenylation signal is inactivated generate longer RNAs that extend into downstream flanking sequences.⁶⁶⁸ These RNAs very efficiently mediate normal replication.⁵⁷⁸ A subset of the RNA is spliced to give rise to one or more subgenomic RNAs. The patterns of spliced mRNAs can be simple or exceedingly complex. Both the unspliced and spliced RNAs are then exported from the nucleus for translation.

Initiation of Transcription

The efficiency of initiation of transcription at the 5' LTR is the major determinant of the levels of viral RNA formed in the cell. The promoter in the LTR is typically a very potent one, and the levels of viral RNA are often constitutively high. However, the cell type, the physiologic state, and the integration site¹⁶⁹ can all result in substantial variation in the efficiency of transcription. In some viruses, the promoter is not constitutively active but depends on the activity of specific transcription factors such as the glucocorticoid receptors.

Positive Regulatory Elements in U3

The transcriptional elements in the U3 region of the simple viruses contain both core promoter sequences and enhancers. The core promoters contain a TATA box, bound by TFIIB; a CCAAT box, bound by CEBP⁵²⁵; and sometimes an initiator sequence near the U3-R border. The U3 regions of even closely related retroviruses are very diverse, and can evolve rapidly during viral replication. The enhancers are similar to those found

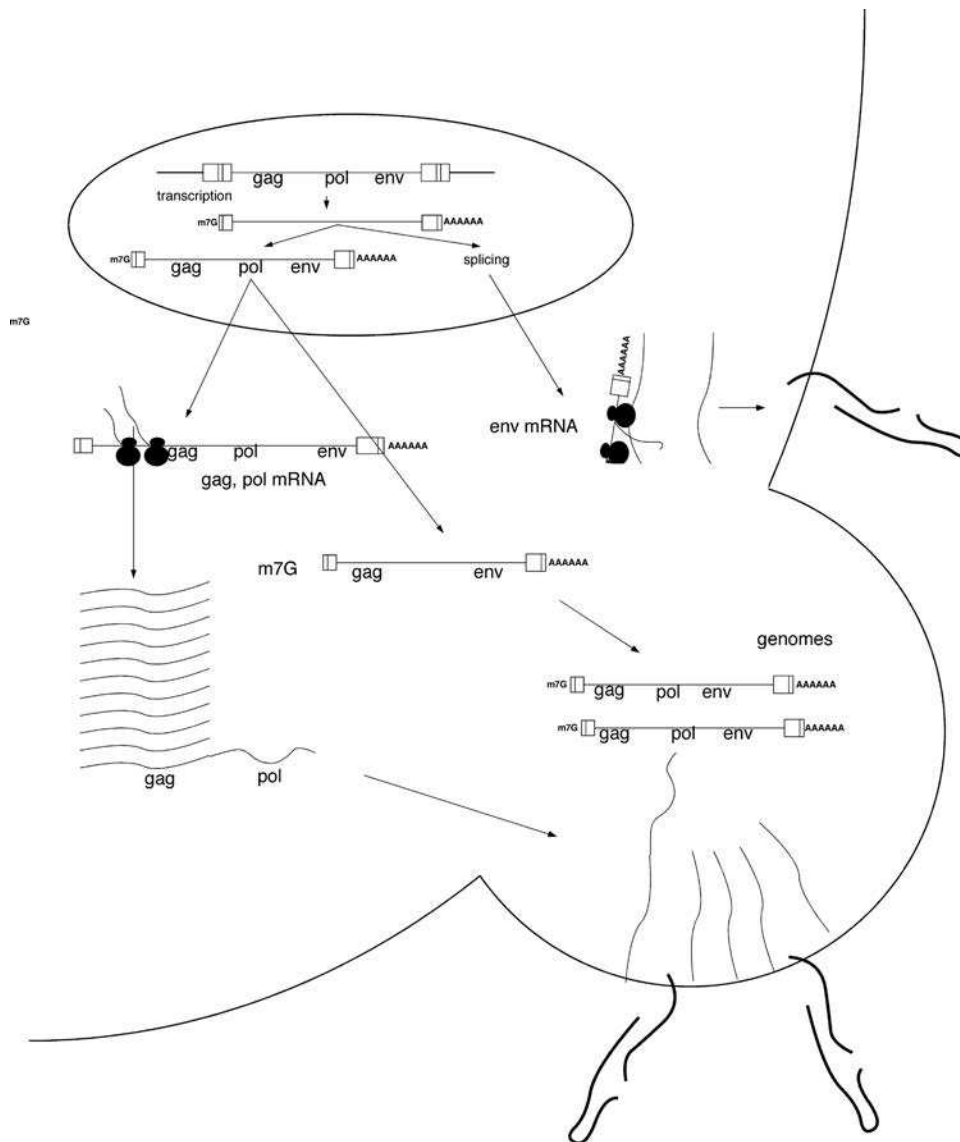


FIGURE 47.13. The late stages of the retroviral life cycle. The integrated provirus is used as the template (*top*) for the expression of viral RNAs. A subset of the transcripts are spliced, and the unspliced and spliced mRNAs are exported to the cytoplasm. The unspliced RNA is used to make Gag and Gag-Pol proteins, and also serves as the genome; spliced mRNA is used to make Env proteins. The proteins and RNA associate under the membrane to form the budding progeny virion.

in many host promoters in containing multiple short-sequence motifs, arranged in very close packing; often there are tandemly repeated copies of some of these motifs. These short sequences are the binding sites for a large number of host factors that regulate transcription (e.g., see 562). Different cells and cell types will make use of distinct arrays of these factors to mediate transcription from a given viral LTR.²¹³ The factors are not simply additive but may interact in complex ways on particular viral sequences. A partial list of these factors used by various retrovirus LTRs includes: Sp1; USF-1; the Ets family of factors, which include more than 20 members in vertebrates; the core-binding factor (CBF), consisting of an α - β heterodimer; nuclear factor 1 (NF1); and a mammalian type C retrovirus enhancer factor (MCREF-1). Specific viruses may often contain recognition sites for other more specific positive regulatory factors. Major examples of such factors include the glucocorticoid receptors, driving expression of the MMTV genome, and to a much lesser extent, other MuLVs; NF- κ B, important for expression from the HIV-1 LTR in certain cell types; the GATA factors for Cas-BR-E and other viruses; and the myb

protein. Evidence has been obtained that the STAT factors, DNA binding proteins normally activated the Janus kinases (Jaks) may also be important for MMTV transcription.⁴⁹³

Negative Regulatory Elements

A number of negative regulatory factors that reduce viral expression have been identified. Embryonic carcinoma cells, and true embryonic cells, are the best-characterized examples of cell types that strongly repress LTR-mediated transcription through expression of negative regulatory proteins. The MuLVs are silenced via a stem-cell specific repressor that binds to a site, curiously, overlapping almost perfectly with the proline tRNA primer binding site.^{289,473} The proteins responsible for this silencing in mouse embryonic stem cells have recently been identified as TRIM28 (Kap-1) and the zinc-finger protein ZFP809.^{637,638} Viruses that use an alternate primer tRNA and thus lack the pbs recognition site for these proteins can escape the repression.²³⁹ Other negative factors include one known variously as UCRBP, NF-E1, or YY1,¹⁷⁶ and a cellular embryonal LTR-binding protein (ELP; 598).

trans-Acting Viral Regulatory Factors

The complex retroviruses encode an array of small regulatory proteins that can activate transcription from the viral LTR in *trans*. Examples of these transactivators include the HTLV-1 Tax protein¹³² and the HIV-1 Tat protein.¹²⁰ The Tax protein acts in concert with a complex of host proteins, the activating transcription factor/CRE-binding protein (ATF/CREB), and binds to three cAMP response elements in the viral LTR. Tax thus sets up a positive feedback loop that results in high levels of viral transcripts. The Tat protein is unusual among transcriptional activators in that it binds to a structure in the 5' end of nascent viral RNA, rather than to DNA.^{136,548} Tat binds to a bulged hairpin structure, the TAR element, and recruits a pair of host proteins, cyclinT/cdk9, to the RNA. These proteins enhance the ability of RNA polymerase to elongate beyond the LTR and down the genome with high processivity, probably by phosphorylation of the C-terminal repeat domain (CTD) of the polymerase. Again, the result is a strong positive feedback loop that results in high levels of viral RNA. (For more detailed discussion of *tat* function, see 120, and Chapter 49 of this book.)

Beginning and Ending the RNA

Because proviruses contain two identical LTRs, transcription can be initiated at both 5' LTR and 3' LTR. However, the 5' LTR is generally much more efficiently utilized than the 3' LTR.²³⁵ One possible mechanism is promoter interference, in which the upstream promoter being active suppresses the utilization of the downstream promoter. It is possible that elements near the 3' LTR may restrict use of the downstream LTR, so that generally transcripts initiating at the 5' LTR predominate. These restraints may be lost in tumors, in which transcription from the 3' LTR can be significantly enhanced.⁵⁹ Similarly, since there are two LTRs, transcripts might in principle be subject to 3' end processing at either the 5' LTR or the 3' LTR, but most of the RNAs formed extend from the 5' LTR to the 3' LTR.

RNA Processing

The full-length transcript of the retroviral genome is directed into several pathways. A portion of the transcripts is exported directly from the nucleus and serves as the genome to be packaged into the progeny virion particle, assembling either at the plasma membrane or in the cytoplasm. Another portion with identical structure is also exported and used for translation to form the Gag and Gag-Pol polyproteins. It is not yet clear if these two subsets are truly distinct, whether there can be interchange between the pools, or whether there is a single pool of such molecules used for both purposes. A third portion is spliced to yield subgenomic mRNAs. For the simple retroviruses, there is a single spliced mRNA encoding the Env glycoprotein. For the complex viruses, there can be multiple alternatively spliced mRNAs, encoding both Env and an array of auxiliary proteins. Examples of the complicated array of mRNAs that are formed for both simple and complex viruses are shown in Figure 47.14. The protein products of these multiply spliced mRNAs will be discussed in Chapters 48–52.

The splicing and subsequent export from the nucleus of only a portion of an initially transcribed RNA is an extraordinary process; normally splicing of cellular mRNA precursors goes to completion, and only then is the mRNA exported. The export of a precursor mRNA is prevented until splicing is complete.

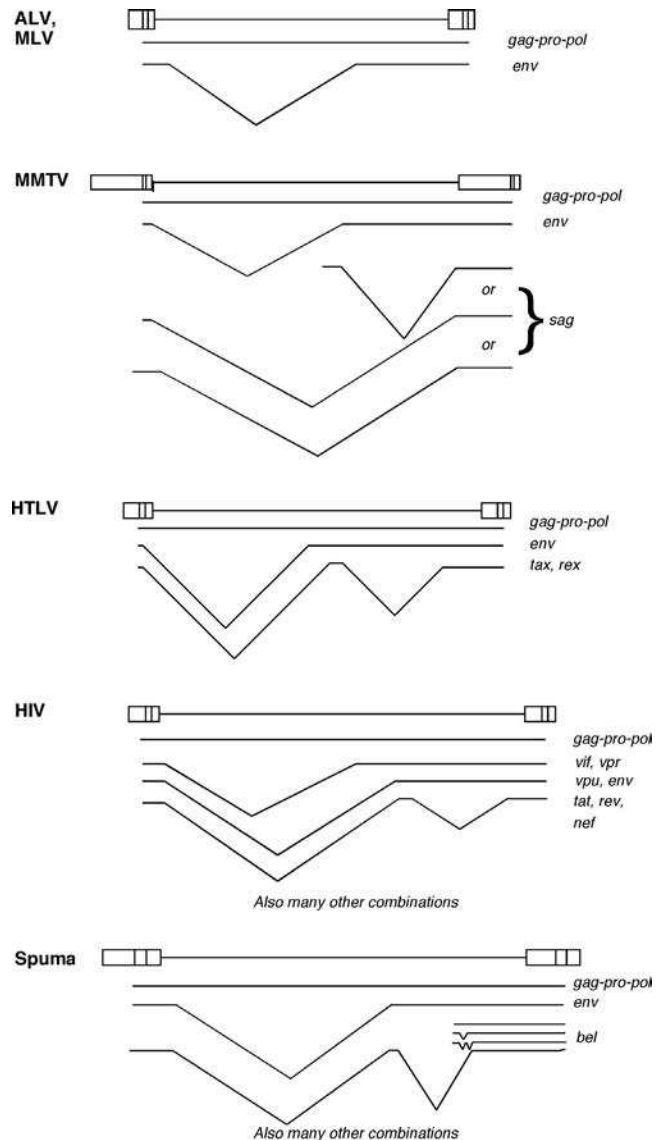


FIGURE 47.14. Splicing patterns of representative retroviral RNAs.

All retroviruses direct the synthesis of an unspliced RNA transcript, as well as a variable array of subgenomic mRNAs. Examples of the splicing patterns of the mRNAs of various retroviruses are shown. The complex viruses such as HIV-1 also encode a larger array of mRNAs containing various combinations of exons.

At least three aspects of the retroviral genome may promote the export of unspliced mRNAs. First, the splice sites of the viral RNA may have poor overall efficiency of utilization by the splicing machinery in the cell.²⁸⁰ The sequences at the splice donor and acceptor regions are often poor matches to the consensus sequences for splice sites, and mutations that make the sites better matches increase splicing and are actually deleterious to virus replication. These mutations can be suppressed by secondary mutations that reduce splicing efficiency. The overall folding of the RNA may affect the efficiency of splicing; thus, sequences at some distance, as in the *gag* gene, may modulate splicing.⁵⁶⁵

Second, studies of ASLV have identified specific sequences that act as negative regulators of splicing (NRS) through their

interaction with host factors.^{11,378,379} These elements can be important for the expression of transduced genes in some viruses.⁵⁵⁸ Similar signals may exist in other viruses; mutations in the Gag region of MuLV can affect RNA processing in complex ways.

In addition, unspliced mRNAs contain *cis*-acting elements that promote the export of the RNA out of the nucleus, the so-called constitutive transport elements (CTEs).⁶⁸ These sequences are located near the 3' end of the genomic RNA of MPMV, and possibly in similar regions of ASLV. The CTE is recognized by one or more host proteins that assemble a complex onto the RNA to mediate its export, including Tap and its cofactor Nxt. In the complex viruses, RNA export is regulated through complex interactions of the Rex or Rev gene products with *cis*-acting sites, the RRE elements that promote RNA export; and of various host factors with the CRS/INS elements that prevent it (see 121 for review). The key players include Crm1, a cellular nuclear export factor, and DDX3, an RNA helicase (see Chapter 49 for detailed discussion of the mechanism of Rev action).

Viral RNAs are subject to other modifications common to cellular mRNAs. Like cellular mRNAs, the N6 position on specific A residues can be methylated, and other sites can be modified by dsRNA adenosine deaminase. The significance of these modifications is uncertain.

TRANSLATION AND PROTEIN PROCESSING

All retroviral genomes, at a minimum, contain ORFs designated the *gag*, *pro*, *pol*, and *env* genes. These genes are expressed by complex mechanisms to form precursor proteins, which are then processed during and after virion assembly to form the mature, infectious virus particle. The expression of the various proteins as large precursors that are subsequently cleaved provides several advantages: it allows for many proteins to be made from one ORF; it ensures that the proteins are made at proper ratios; and it allows for many proteins to be targeted to the virion during assembly as a single entity. The *gag*, *pro*, and *pol* genes are expressed in a complex way from the full-length unspliced mRNA. The arrangement of these genes, and especially the way *pro* is expressed, are different in different viruses. A summary of the arrangement of the ORFs of various viruses is shown in Figure 47.15.

Gag Gene Expression

The *gag* gene is present at the 5' proximal position on all retroviral genomes. A full-length mRNA, identical in sequence to the genomic RNA, is translated in the cytoplasm to form a Gag precursor protein, in the 50 to 80 kDa range. Translation begins with an AUG initiator codon and proceeds to a terminator codon at the 3' end of the ORF. The viral RNA typically contains a relatively long 5' untranslated region, and there has been uncertainty regarding whether ribosomes could scan from the 5' cap to the start codon for Gag translation. These 5' RNA sequences are predicted to contain stable secondary structures that would inhibit scanning. Furthermore, the long 5' UTRs often contain AUG codons in contexts that are favorable for translation, that are not in frame with the *gag* ORF, and presumably would inhibit successful translation of Gag. Experiments suggest that for the MuLVs and related endog-

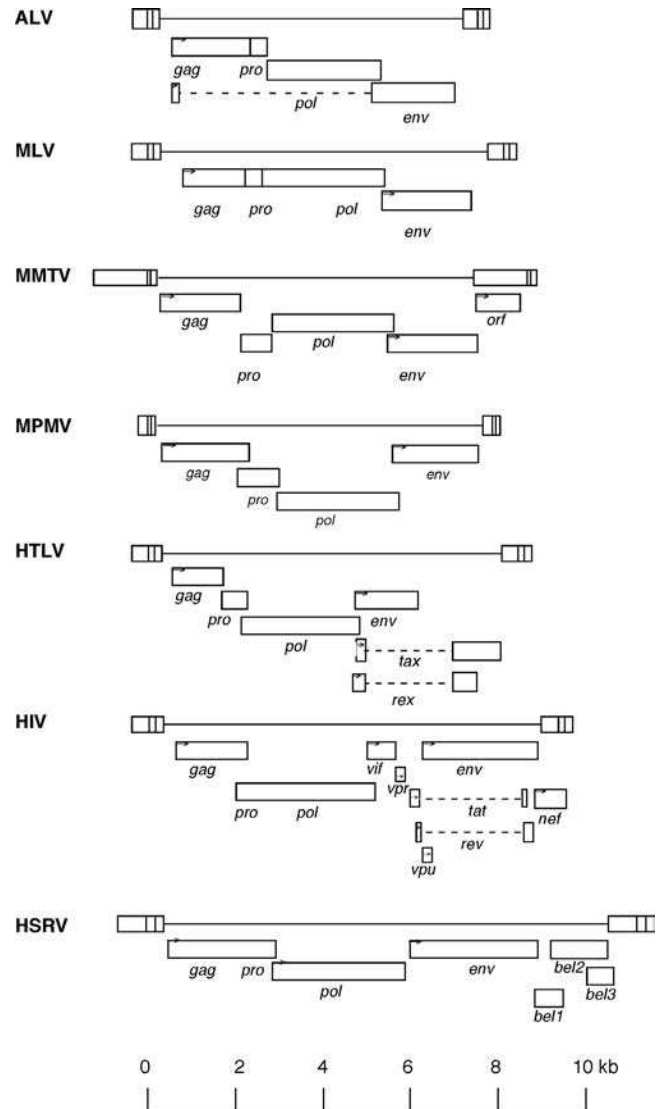


FIGURE 47.15. Arrangements of the open reading frames (ORFs) encoded by various retroviruses. The major ORFs of each virus are indicated by the open boxes. ORFs in the same reading frame are in the same line, and ORFs in different frames are on different lines. Translational starts are indicated by small arrows. Spliced introns are indicated by dashed lines.

enous RNAs, an internal ribosome entry site (IRES) is present near the start of the *gag* ORF and is used to initiate translation in a cap-independent mechanism.^{45,46,344} Thus, at least in these viruses, ribosomes can bind directly near the *gag* gene and do not need to scan the mRNA. Although the suggestion is not without controversy,³⁸³ it is likely that many other viruses, including HIV-1, also utilize IRES elements for translation of Gag.^{130,131,427} In the case of HIV-1, the IRES is remarkable in that critical sequences extend downstream of the AUG, lying within the Gag coding region.⁷⁶

Some retroviruses encode an additional Gag protein besides the major product, termed gPr80^{gag} or “glycoGag.” This Gag protein is longer than the major product and derives from translational initiation at a nonconventional CUG codon

upstream from the initiating AUG codon. Translation beginning at this codon first forms an N-terminal leader sequence and then proceeds in the same reading frame through the normal AUG and the rest of the Gag protein. Thus, where the proteins overlap their sequences are identical. The leader sequence contains a functional signal peptide directing the translation machinery to the endoplasmic reticulum, and specifying that the Gag protein be co-translationally inserted into the secretory pathway. The Gag become glycosylated at several sites, is transported via the golgi to the cell surface, and persists for some time as a membrane-bound glycoprotein, with the carboxyterminal domain exposed on the cell surface.⁴⁷⁶ The protein is processed into several fragments and has a relatively short half-life. It is not required for virus replication in some cells.⁵⁴² However, the protein can facilitate release of virus at lipid rafts,⁴²⁰ apparently acting in concert with the host La protein,⁴²¹ and can replace the function of the HIV-1 Nef protein in promoting virion release.⁴⁷⁹ Very recent work suggests that GlycoGag serves to inhibit the Apobec3 restriction factor.

The major Gag product is often modified by the addition of myristic acid, a relatively rare 14-carbon fatty acid, to the penultimate aminoterminal residue, a glycine.²³⁴ The addition is mediated by a myristyl CoA transferase that co-translationally transfers myristate from a myristyl CoA donor to the amino group of the glycine residue, forming an amide bond. The fatty acid is important for the membrane localization and binding of the Gag precursor, increasing the hydrophobicity of the aminoterminal domain. Mutant Gags in which the glycine is altered are not modified; these Gags do not associate with membrane properly and do not aggregate to form virions.^{75,210,500} It should be noted that although the myristate is important, it is not sufficient for membrane targeting; hydrophobic residues in the MA domain are also required. Furthermore, basic residues further downstream in the MA of some viruses form a patch of positive charge that interacts with negatively charged phospholipids in the membrane.

An aminoterminal myristate is not found on the Gags of BIV, EIAV, visna, or ASLV. For the avian retroviruses, the aminoterminal is not myristylated but rather acetylated. The Gag protein of these viruses is apparently sufficiently hydrophobic to be targeted to the membrane without the fatty acid in avian cells, though, curiously, not for ASLV in mammalian cells. Alteration of the avian Gag to allow its myristylation permits virion assembly in mammalian cells⁶³³ and does not block its function in avian cells.

pro Gene Expression

The relative position of the *pro* gene on retroviral genomes is always similar—in between *gag* and *pol*. However, the *pro* gene is expressed in very different ways in different viruses. Sometimes it is fused in frame onto the 3' end of *gag*, sometimes it is fused to the 5' end of *pol*, and sometimes it is present as a separate reading frame. These various patterns have led to considerable confusion in the literature; sometimes *pro* is considered a portion of *gag*, or sometimes of *pol*. Because of these different patterns of expression, it is best to consider this ORF as a separate gene.

The various arrangements of the *pro* gene and its mode of expression are as follows. For the alpharetroviruses, *gag* and *pro* are fused and expressed as a single protein; *pol* is in a different reading frame, and a frameshift is used to express the Gag-Pro-Pol polyprotein. For the betaretroviruses and deltaret-

roviruses, *gag*, *pro*, and *pol* are all in different frames and successive frameshifts are used to express Gag-Pro and Gag-Pro-Pol polyproteins. For the gammaretroviruses and epsilon-retroviruses, *gag* and a *pro-pol* fusion are in the same reading frame and separated by a stop codon, and translational readthrough is used to make Gag-Pro-Pol. For the lentiviruses, *gag* and a *pro-pol* fusion are in different reading frames, and frameshifting is used to make Gag-Pro-Pol. Finally, for the spumaviruses, *pro* is fused to *pol*, and the Pro-Pol protein is expressed without Gag, from a spliced mRNA. More about these varied mechanisms of expression is presented in the following section.

pol Gene Expression

The *pol* gene encodes several proteins needed at lower levels for the replication of the virus, including the reverse transcriptase and integrase enzymes. The *pol* ORF is not expressed as a separate protein in most retroviruses, but rather is expressed as a part of a larger Gag-Pro-Pol fusion protein. The Gag-Pro-Pol protein must be made at the correct abundance, in proportion to the amount of Gag protein, for efficient assembly of infectious virus; expression of only Gag-Pro-Pol does not result in virion assembly.^{170,458} The formation of this protein is mediated by one of two mechanisms, depending on the virus.

Translational Readthrough

In the gammaretroviruses and epsilon-retroviruses, the Gag and Pro-Pol ORFs are in the same reading frame, separated by a single UAG stop codon at the boundary between Gag and Pro-Pol. The translation of Gag-Pro-Pol in these viruses occurs by translational readthrough—that is, by suppression of termination—at the UAG stop codon.⁶⁵¹ Most of the time, translation of the RNA results simply in the formation of the Gag protein. But approximately 5% to 10% of the time, ribosomes translating the RNA do not terminate at the UAG codon, and instead utilize a normal aminoacyl tRNA, usually a glutamine tRNA, to insert an amino acid at the position of the stop codon. Translation then continues, in frame, through the entire long *pro-pol* ORF, resulting in the formation of a long Gag-Pro-Pol precursor protein.

The high-level suppression of termination is specified by a specific structure in the RNA immediately downstream of the UAG stop codon.^{241,449} The precise features of this structure that are required for suppression are not completely known, but they include a purine-rich sequence immediately downstream of the stop codon, and a pseudoknot formed from the next 60 or so nucleotides.¹⁷² The structure may slow translation, and it may also in some other way alter the balance between termination, which requires binding of termination factors eRF1 and eRF3 by the ribosome, versus incorporation of an amino acid, which requires misreading of the codon by an aminoacyl tRNA. No changes in the tRNA pool occur during infection. The signals in the RNA can operate to mediate suppression of both UAA and UGA termination codons as well as UAG.

A screen for proteins interacting with the MuLV RT resulted in the identification of the eukaryotic termination factor eRF1, and subsequent studies showed that overexpression of RT could inhibit termination and promote translational readthrough of the Gag stop codon *in vivo*.⁴³⁴ Mutant viruses with point mutations in RT blocking the interaction with eRF1 were unable to express normal levels of Gag-Pol and failed to replicate. These results suggest that RT, likely in the context of the nascent

Gag-Pol protein, can bind and inhibit eRF1, increasing the level of readthrough to increase its own synthesis. The final level of Gag-Pol produced in this positive feedback loop presumably is ultimately limited by other factors.

Translational Frameshifting

In the alpharetroviruses and lentiviruses, the *gag* and *pol* ORFs lie in different reading frames, and the formation of the Gag-Pro-Pol fusion is mediated by a translational frameshift mechanism.²⁵⁷ Most of the time, translation again results in the simple formation of the Gag protein. But approximately 10% of the time, as the translation approaches a specific site near the end of the *gag* ORF, the ribosome slips back one nucleotide (a -1 frameshift) and proceeds onward in the new reading frame. The ribosome passes through the stop codon out of frame and continues to synthesize protein using the codons of the *pol* ORF. As for readthrough, the determinants of frameshifting lie in the RNA sequence and structure near the site of the event. The requirements for frameshifting include a “slippery site,” a string of homopolymeric bases where the frameshift occurs; these are oligo U or oligo A in different viruses. In addition, the frameshifting requires either a very large and near-perfect hairpin or stem-loop structure (as for HIV-1 group M viruses); or a large pseudoknot structure (as for HIV-1 group O viruses), similar to those used in readthrough, though apparently containing a distinctive bend at the junction of the two paired sequences. As for readthrough, the proper frameshifting efficiency is crucial for normal virus replication.

In the betaretroviruses (e.g., MMTV) and deltaretroviruses (e.g., BLV, HTLV-1), the *pro* gene is present as a separate ORF, in a different reading frame from that of *gag* or *pol*. Two successive frameshifts are utilized to make the long Gag-Pro-Pol fusion protein. Near the 3′ end of the *gag* ORF, ribosomes carry out a first (-1) frameshift and continue into the *pro* ORF; near the 3′ end of the *pro* ORF, they perform a second (-1) frameshift and continue on into the *pol* ORF. These two frameshifts occur at extremely high frequencies—as much as 30% of the time that the ribosome transits through each site—so that the overall frequency of formation of the Gag-Pro-Pol protein is perhaps 10% that of formation of Gag.

Separate Pol Expression

The spumaviruses are unique among the retroviruses in that the synthesis of the Pol protein is not mediated by the formation of a Gag-Pol fusion protein. Instead, a subgenomic spliced mRNA is translated directly to form a separate Pro-Pol protein.^{159,340} This protein must be directed to the assembling virion by distinct domains rather than by the Gag portion of a Gag-Pol fusion.

env Gene Expression

In all retroviruses the *env* gene is expressed from a subgenomic mRNA. The *env* message is a singly spliced mRNA, in which a 5′ leader is joined to the coding region of *env*. Thus, the bulk of the *gag* and *pol* genes are removed as an intron from the mRNA. The resulting message is exported to the cytoplasm and translated from a conventional AUG initiator codon. In the alpharetroviruses, the AUG is actually the same one used for Gag translation; it lies in the leader, and the splicing brings this AUG and the first six codons into frame with the *env* coding region. The first translated amino acids constitute a hydro-

phobic signal peptide, and direct the nascent protein to the rough endoplasmic reticulum. The leader is removed by a cellular protease (the signal protease) in the ER, and the protein is heavily glycosylated by transfer of oligosaccharide from a dolichol carrier to asparagine residues on Env. These residues lie in the conventional Asn-X-Ser/Thr motifs recognized by the modification enzymes. Near the end of the co-translational insertion of Env into the ER, a highly hydrophobic sequence acts as a stop transfer signal to anchor the protein in the membrane. The remaining C-terminal portion of the protein stays on the cytoplasmic side of the membrane.

Before the Env proteins are transported to the cell surface, they are folded and oligomerized in the ER. The formation of oligomers is required for stable expression of the protein, and is sensitive to overall conformation; many mutants of Env show defects in oligomerization.⁵⁹⁹ Envelope proteins generally form trimers in the mature virus.³⁴⁷ The most studied envelope proteins (ASLV and HIV-1) may pass through dimeric or tetrameric intermediates, but the nature of these intermediates is not clear. The folding of the protein is presumably catalyzed by chaperone proteins in the ER and the formation of disulfide bonds between various pairs of cysteine residues by disulfide interchange enzymes.

The Env protein is then exported to the Golgi and cleaved by furin proteases to form the separate SU and TM subunits. This cleavage is essential for the normal function of the Env protein. The cleavage occurs at a dibasic pair of amino acids,¹³⁹ producing a hydrophobic N-terminus for the TM protein that is required to mediate fusion of the viral and host membranes during virus entry. In the Golgi the sugar residues are modified by the sequential removal of mannose residues and addition of N-acetyl glucosamine and other sugars to many of the oligosaccharide. O-linked glycosylation and sulfation of Env glycoproteins have also been documented.⁴⁷⁸ The pathway by which Env is transported to the cell surface is not fully understood, but presumably host vesicular transport systems are utilized. There is evidence that clathrin adaptor complexes interact with the cytoplasmic tail of Env and direct its movement to the plasma membrane. The protein typically becomes a prominent cell-surface protein on the infected cell.

In polarized epithelial cells, Env proteins are often restricted to the basolateral surface of the cell.⁴⁴¹ This localization is mediated by a tyrosine-based motif, Yxxf, present in the cytoplasmic tail of Env⁴⁴² (x, any amino acid; f, hydrophobic residue). Remarkably, this targeting of Env can redirect the budding of Gag proteins to this surface.

Other Viral Gene Products

The complex retroviruses express a number of small proteins with a range of functions. The proteins are translated from subgenomic mRNAs, usually resulting from multiple splicing events that join a 5′ LTR to a number of small exons encoding the protein. These gene products will be discussed in greater detail in Chapters 48–52 (see 120,215,482 for reviews).

Betaretroviruses

- *Sag*: The MMTV genome encodes a small protein whose function seems to be to act as an antigen to stimulate lymphocyte activation, providing a suitable tissue for virus replication.⁴⁹²
- Rev-like protein: MMTV encodes a protein involved in RNA metabolism.²⁵³

Deltaretroviruses

- *Tax*: The Tax gene product is a positive regulator of transcription from the viral LTR. Tax functions in association with the activating transcription factor/CRE-binding protein (ATF/CREB) by binding to three cAMP response elements in the viral LTR. Tax also plays a role in transformation, perhaps through Rb destruction, E2F-1 activation, or through effects on the cell cycle.²⁸⁷
- *Rex*: The Rex gene product facilitates the export of unspliced and singly spliced viral mRNAs from the nucleus. Its action is probably similar to that of the lentiviral Rev protein.

Epsilonretroviruses

- *Orf A*: The Orf A product of the piscine retroviruses is a cyclin D homolog that functions as a cyclin in yeast.³¹⁴ The function of the protein in virus replication or tumor formation is uncertain.
- *Orfs B, C*: The function of these Orfs is unknown.

Lentiviruses

- *Tat*: The Tat protein is a potent transactivator of transcription from the viral LTR. The protein acts by binding to a hairpin structure, the TAR element, encoded in the R region of nascent viral RNA, and recruiting host factors cyclinT and Cdk9 to the RNA. Tat does not increase the rate of RNA polymerase II initiation, but seems to enhance its processivity or elongation, perhaps by phosphorylation of the CTD of Pol II.
- *Rev*: The Rev protein mediates the export of the unspliced and singly spliced viral RNAs from the nucleus, thus permitting the expression of the Gag, Pol, and Env gene products. Viral RNAs contain multiple sequences, called CRS or INS elements, which bind several proteins—PTB/hnRNP I, hnRNP A1, PABP1, and p54nrb/PSF—that retain the RNAs in the nucleus.^{5,674} Rev binds to the Rev-responsive element (RRE) present in the HIV-1 *env* gene and by interacting with the importin Crm1 acts to export the viral RNAs through the nuclear pore, overriding the retention signals.
- *Nef*: The Nef protein is a multifunctional protein not essential for replication in some cells in culture, but important for replication *in vivo*. Nef-defective viruses do not induce high-level viremia in infected animals, and progression to disease is delayed or prevented. Nef downregulates the CD4 receptor from the cell surface,¹⁹⁷ facilitating virus release, probably by bridging CD4 to adapter proteins (APs).⁴⁷⁵ Nef also downregulates MHC class I levels, thereby inhibiting the CTL-mediated lysis of HIV-1-infected cells. The Nef of SIV can promote virion assembly and release by antagonizing the antiviral protein tetherin.⁶⁶⁴
- *Vpr*: The Vpr protein, as noted below, is packaged at high levels into virion particles through an interaction with the p6 domain of Gag.^{23,263,545} Vpr may facilitate the import of the preintegration complex into the nucleus in nondividing cells. Vpr also causes a strong cell-cycle arrest in the G2 stage of the cell cycle, perhaps through an indirect inhibition of Cdc25 phosphatase activity. Vpr binds via VprBP/DCAF1 to a ubiquitin ligase complex containing Cullin 4A and DDB1, presumably to promote the ubiquitinylation and degradation of unknown targets, perhaps including the cell-cycle regulator Ctd1.^{244,536}

- *Vif*: The Vif protein is expressed at high levels in the cytoplasm, and is packaged into virion particles of both homologous and heterologous viruses. Vif enhances infectivity by degrading or sequestering the APOBECs, a family of cytidine deaminases that attack the msDNA during reverse transcription.^{54,224}
- *Vpu*: The Vpu gene product, found only in HIV-1, is a membrane protein that enhances virion production by antagonizing tetherin, which traps virions on the cell surface and prevents their spread to neighboring cells.⁴¹⁴ Vpu also mediates the degradation of CD4 by the ubiquitin-conjugating pathway.⁵³⁸
- *Vpx*: Some SIVs encode a small protein, Vpx, which enhances early steps of infection and overcomes a block to infection of monocyte-derived macrophages and dendritic cells. Vpx acts by targeting the antiviral protein SAMHD1, a nuclease, for degradation.^{245,311}

Spumaviruses

- *Tas*: The Tas (or Bel1) protein is a transactivator of transcription from the viral LTR, acting at sequences near the 5' end of the genome. Its mechanism of action may be similar to that of the lentiviral Tat protein.
- *Bet, Bel2, and Bel3*: The functions of the *bet* (and overlapping *bel2*) and *bel3* genes are uncertain. Bet, like HIV-1 Vif, inhibits APOBEC3G,^{341,524} and the Bel3 protein may be a negative regulator of replication.

VIRION ASSEMBLY

As the Gag, Gag-Pro-Pol, and Env proteins are synthesized, they come together to form progeny virions (for reviews, see 251,528,579,632). The assembly of the retrovirus particle is driven primarily by the Gag precursor protein. Gag is required for the formation of a virion, and is sufficient to mediate the assembly and release of an immature “bald” particle—lacking infectivity and the “hair” of the Env protein. The Gag protein that is responsible for assembly is the uncleaved Gag precursor. This form of the protein is thus targeted for assembly and export—the “way out” of the cell. The trafficking routes that deliver Gag to the site of assembly and budding are not established with certainty for any retrovirus. Once the Gag proteins are processed by the viral protease, changes in virion structure occur to promote virus entry—the “way in” to the next cell.

There are two major routes by which the various retroviruses assemble their virions, discussed in the next section.

Assembly of C-Type Virions

For most of the retroviruses, those with C-type morphology, assembly occurs at the plasma membrane. In these cases the Gag precursor protein is targeted to the cytoplasmic face of the plasma membrane by hydrophobic sequences, basic residues, and sometimes by a myristic acid moiety,²³⁴ present at the aminoterminal. It is not clear if monomeric, dimeric, or higher-order structures of Gag are transported to the membrane to begin assembly. The Gag proteins aggregate, presumably by side-to-side contacts, and create a patch under the membrane. As the patch of protein grows, curvature is induced in the membrane, causing the nascent virus to bud outward. The bud eventually grows to a complete sphere, attached to the

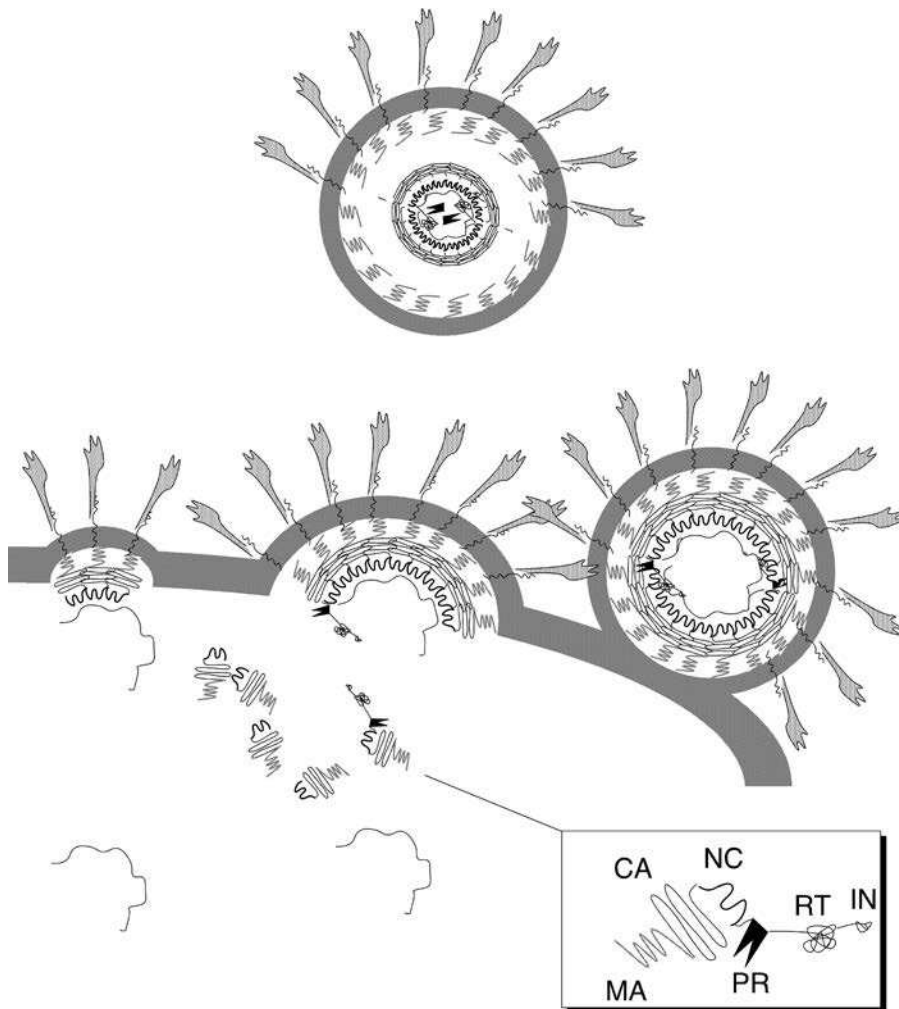


FIGURE 47.16. Schematic diagram of the process of virion assembly. The Gag precursor, containing the MA, CA, and NC domains, and the Gag-Pol precursor, containing the MA, CA, NC, PR, RT, and IN domains (see *magnification*), are transported to the inner leaflet of the plasma membrane. The proteins bind the viral genomic RNA (*thin line*). Curvature is induced in the membrane as the virion grows, and the roughly spherical particle is finally pinched off and released from the cell. The virion proteins are reorganized upon processing by the viral protease to form the mature, infectious virus (*top*).

cell by a narrow stalk. The stalk is then pinched off, the virion is released, and the host membrane is sealed. The structure of the immature virion is roughly spherical, with Gag arranged radially.⁶²⁹ The various steps are depicted in Figure 47.16.

The route of transport of Gag from the cytoplasm to the cell membrane may not be simple or direct. A substantial amount of Gag protein is found in the nucleus,^{411,533} and the Gag in this compartment may be a precursor to the molecules on the plasma membrane.⁴⁵⁵ Whether this is an obligatory step in virion assembly is unclear, but genomic RNA for packaging may be bound in the nucleus.¹⁹⁶ There is also evidence that Gags, perhaps bound to the genomic RNAs that they will package into particles, are trafficked to the plasma membrane on endosomal vesicles.³¹

Assembly of B- and D-Type Virions

In the alternative lifestyle exhibited by viruses with B- and D-type morphology, the betaretroviruses and the spumaviruses assemble in the cytoplasm, then are subsequently transported to the plasma membrane for envelopment and release.⁵⁰⁷ These two pathways would seem relatively distinct, and one might have supposed that the two groups of viruses would have evolved very different requirements for assembly, and that the details of the Gag–Gag interactions would be different. But these two mechanisms are not so far apart. A single amino

acid substitution in the MA protein of M-PMV can change the morphogenetic pathway of the virus from a cytoplasmic site of assembly to a membrane site of assembly.⁵⁰⁹ Thus, the main difference may be the timing of exposure of determinants for membrane transport: in the C-types, such a determinant might be constitutively available, while for the B- and D-types, the determinant may not be exposed until assembly occurs. For both mechanisms, the nascent virions consist of a spherical particle surrounded by a lipid bilayer that is pinched off from the cell and then released into the extracellular space.

MPMV Gag does not seem to assemble at the site of translation; the protein apparently first travels in a microtubule-dependent process to the pericentriolar region of the cytoplasm through interactions between a short peptide signal, known as the cytoplasmic targeting-retention signal, and the dynein/dynactin motor. The Gag precursors are assembled to form immature capsids in pericentriolar microdomains. Env may affect release of Gag from the centriolar region. Mutants of Rab11 that inhibit efflux of transferrin from the recycling endosome and Env localization also inhibited Gag transport.^{546,547} The mechanism of the subsequent movement to the cell surface is uncertain.

Gag and Virion Assembly

For most retroviruses, the expression of the Gag precursor is sufficient to mediate virion assembly and release, earning the

protein the name of the “particle-making machine.” (An exception to this rule is the foamy viruses, which also require the presence of the Env glycoprotein for efficient budding.) Because of their central role in virion assembly, the Gag proteins have been subjected to intense mutational analyses to define the domains required for various steps in the process.^{223,632} Surprisingly small portions of Gag, containing only a few critical regions, can still assemble virions.⁶¹⁸ Three domains, at least, seem to be crucial: a membrane-binding (M) domain; an interaction (I) domain; and a late-assembly (L) domain. It is important to remember that the form of the Gag protein that is mediating assembly is the precursor; thus, the assembly domains need not lie nearly within any of the cleavage products that form later and can span cleavage sites.

The M Domain

The M domain, or membrane-binding domain, ranging from 30 to 90 residues in length, is located in MA at the amino terminus of Gag. Mutations affecting this domain abolish assembly, but M mutants retain their ability to interact with other Gags and can be rescued into particles by the co-expression of a wild-type protein. The region seems to contain both hydrophobic and basic residues that are needed for proper interaction with lipid and with the acidic moieties of phospholipids. Structural information for the isolated M domain is consistent with this role. How Gag finds specific membranes is unclear, but M domains seem to be involved.⁵³⁴ There is evidence that budding is enhanced at membrane regions of unusual lipid composition termed lipid “rafts”,^{84,410,417,418,431} defined by high levels of phosphatidylinositol (4,5) bisphosphate.⁴²⁹

For many retroviruses, myristoylation of Gag, along with specific residues in MA, is required for membrane binding. This interaction with membrane, in turn, is important for virion assembly of the C-type viruses and for their proper subsequent Gag processing.⁵³⁹ Mutational studies have led to the notion of a “myristyl switch,” in which the myristic acid is exposed to mediate plasma membrane binding during virion assembly, but then can be sequestered in the compact globular core of MA after Gag processing.^{430,444,561,670} Although this region is generally considered important for virion assembly, surprisingly, much of the RSV MA and the entire HIV-1 MA domain can be deleted from Gag without preventing assembly, as long as a functional membrane-binding signal is retained. In the latter case, there are some effects on assembly: virions are budded indiscriminately into both intracellular membranes as well as at the cell surface. The aminoterminal sequences of Gag can be replaced with a heterologous membrane binding signal, such as that present at the aminoterminal of the Src kinase. It should be noted that the interaction of Gag with membrane is not required for assembly of the B- or D-type viruses *per se*. For these viruses, mutations in the myristate addition signals do not affect the cytoplasmic assembly of the virions, but rather block the transport of the assembled particles to the plasma membrane.⁵⁰⁸

The I Domain

The I (or interaction) domain is defined as a major region of Gag–Gag interaction, largely contained in the NC region. Although the major I domain has been suggested to lie in NC, some analyses have suggested that the C-terminal half of CA and NC are equally important for normal assembly. Mutations in the I domain block or reduce assembly,⁴⁰² and those particles

produced by these mutants have aberrantly low density, indicating fewer and poorly packed Gag proteins. The key feature of the I domains is not the zinc-binding residues of the cys-his box, but rather basic residues flanking the boxes that interact with nucleic acid. RNA bridging between NCs is likely a critical step in virion assembly. The assembly functions of NC can be replaced by foreign proteins, and the key activity seems to be the formation of protein–protein contacts.^{266,669} Mutations in this region can affect particle size as well as yield.

The L Domain

The third assembly domain is the L (or late) domain.^{631,645} Mutants affected in this function fail to produce and release particles efficiently, and though the mutant Gag proteins form spherical structures, they accumulate under the membrane and do not progress normally. The buds remain tethered to the cell surface by a membrane stalk, suggesting that the function of the L domain is to mediate virus–cell separation. L domains lie at different locations in the Gag proteins of different viruses. In ASLV, MPMV, and the MuLVs, the L domain lies in the amino terminal third of the protein, and its critical residues are PPPY. In HIV-1, the domain lies in p6, at the C-terminus, and instead contains the motif PTAP. In EIAV, the domain lies in p9, and contains the motif YPDL. Many viruses contain more than one L domain, and in such cases each domain can provide partial function. Remarkably, many (though not all) of these L domain motifs are interchangeable among the various retroviruses, and show a substantial position independence for their function.^{327,437,456,658} L domains have been shown to be important for the release of many budding viruses, including VSV, rabies virus, and Ebola.

The L domains are now appreciated as serving as the binding sites for various components of a host machinery normally involved in protein sorting and delivery into late endosomal compartments, the multivesicular bodies.^{198,394,407,572,588} The complex that carries out these trafficking events contains more than 20 distinct proteins held together through a network of protein–protein interactions.⁶¹⁴ Most show strong similarity to the so-called VPS proteins of the yeast ESCRT (endosomal sorting complexes required for transport) complexes, identified genetically as involved in vacuolar protein sorting in yeast.^{21,22} The proteins have been divided into three groups: the ESCRT-I complex, containing Vps28, Vps37C,¹⁵⁰ and *Tsg101*, a gene first identified as a tumor suppressor locus³²⁸; ESCRT-II, containing Alix/AIP-1 and several other proteins; and ESCRT-III, containing Vps4A and a large number of CHMPs (for Charged MVB proteins). The PTAP/PSAP class of L domains is bound by Tsg101; the PPPY class of L domains is recognized by various members of the Nedd4 family, a group of ubiquitin ligases that interact with Tsg101; and the YPxL class is bound by Alix/AIP-1. Other proteins associated with the ESCRT complexes, including the vesicle-associated endophilins, may also bind Gag and play a role in virus budding.⁶¹⁹ Depletion of many of the ESCRT homologs, or overexpression of dominant-acting negative fragments of these proteins, can potentially inhibit retroviral budding and release.^{198,267} The biochemical steps of virion release that are actually promoted by the complex are not clear, but may involve membrane targeting, membrane bending, or lipid destabilization and reorganization. The process may involve the covalent transfer of ubiquitin to Gag—Nedd4 can act as a ubiquitin ligase, Tsg101 is a catalytically inactive version of a ubiquitin ligase,

and Gag is indeed ubiquitinated at low levels^{436,463,569}—but it remains controversial at this point as to whether the transfer itself is actually required.^{211,371,613} There is hope that the process of virion budding could be interrupted by a new class of antiviral drugs that target the Gag–ESCRT interactions.

Virion Assembly *In Vitro*

Gag proteins and fragments of Gag have been shown competent to assemble *in vitro* to form various structures that more or less closely resemble virion cores.^{155,296,529} The CA–NC portions of ASLV and HIV-1 expressed as recombinant proteins can assemble to form particles or long, hollow tubes. The formation of these structures is dramatically enhanced by addition of RNA or oligonucleotides,^{355,356} and in some settings the length of the tubes can be determined by the length of the RNA.⁸³ The arrangement of Gag proteins is clearly hexameric, with critical contacts between N- and C-terminal domains of CA.³³⁰ The aminoterminal residue of CA, a proline, is critical for proper folding and assembly.⁵²³

Larger Gag fragments that include more aminoterminal regions can assemble into spherical particles^{218,272}; this assembly is stimulated by RNA^{174,355,356} and host cell extracts.⁸² A critical component in these extracts, curiously, has been identified as inositol hexaphosphate,⁸¹ which interacts with MA. HIV-1 Gag CA–NC fragments can assemble into conical structures,¹⁹¹ with a pitch that falls into discrete values. Image reconstruction of these cones has allowed the formation of a model for the packing of the protein into hexagonal arrays.³³⁰ Virus-like particles have also been formed with the Gag proteins of MPMV in cell-free protein synthesis systems, and in bacteria.⁴¹⁵

Virion Size

The number of Gag proteins per virion particle is estimated to be in the range of 1,200 to 1,800, though this number may vary somewhat from virus to virus (and has been disputed).⁶⁹ The number of Gag–Pol proteins is roughly 10 to 20 times lower, approximately 100 to 200 per virion. It is unlikely that these proteins in the immature virus form a completely homogeneous, ordered crystalline array, but rather they may form a “protein micelle” that is somewhat fluid, like a lipid micelle. The diameter of even wild-type virus preparations is not tightly homogeneous but shows a distribution that suggests some flexibility in the structures during assembly. However, the average size of the particle is determined by the Gag protein, and mutants with alterations in Gag often show abnormal or excessively heterogeneous diameters.³⁰⁵ Mutations in the CA domain commonly show this phenotype. Thus, CA–CA contacts may play a role in determining the angle between Gags during their packing into a spherical shape.

Gag proteins of one virus are sometimes able to interact with the Gags of another virus to co-assemble and form mixed virion particles. Various mutants with alterations in the Gag proteins of the MuLVs can co-assemble into particles that show phenotypes of both parental Gags.^{277,499} Viruses of very different genera can even form mixed particles in some cases.

Incorporation of Other Proteins into Assembling Virions

During assembly, other proteins are incorporated into the particle by contacts to Gag; these include Gag–Pol, Env, and auxiliary proteins encoded by the complex viruses. The Gag–Pol

precursor is thought to be incorporated into the assembling bud by virtue of the Gag protein present at the aminoterminal. Gag to Gag–Pol contacts can in some cases lead to the incorporation of mutants of Gag–Pol that do not retain the myristate modification to the aminoterminal,⁴⁵⁹ suggesting that the interaction is very strong. Gag fusions to foreign proteins can be similarly incorporated into particles formed by Gag²⁷¹; this process can even be used to target antiviral proteins into virions. Consistent with this notion, many mutations that block assembly of Gag, when tested in the context of Gag–Pol, are found to have similar effects on the incorporation of Gag–Pol.^{119,543} However, some mutations in HIV-1 Gag have also been identified that specifically affect the incorporation of Gag–Pol, suggesting that Gag–Pol utilizes some distinctive contacts not important for Gag–Gag interactions.⁵⁵⁴ Further, in the spumaviruses, Pol is incorporated without an appended Gag region, suggesting that distinct interactions must be utilized for its incorporation.¹⁵⁹

The Env protein is thought to be concentrated at the sites of budding and incorporated into the virions by virtue of contacts between the cytoplasmic tail of Env and the aminoterminal portion of Gag. These interactions have been difficult to document directly, though there is some biochemical^{113,644} and cross-linking studies in support of these contacts. Genetics has provided good evidence for this interaction. Selected mutants of MA show defects in Env incorporation,^{141,181} and some mutants of the cytoplasmic tail of TM are not efficiently incorporated.^{400,401,657} In addition, Env proteins that are specifically directed to the basolateral surface of polarized epithelial cells can redirect the sites of budding of Gag from a nonspecific assembly on both membranes to the exclusive assembly at basolateral membranes, and can similarly redirect Gag in neurons. Finally, mutants and revertants of these mutants with second-site suppressors in the binding partner have provided strong evidence for these interactions.^{181,182} However, it should be noted that the envelope proteins of viruses very distant from retroviruses, including VSV and influenza, can be functionally incorporated into retrovirus particles without any obvious sequence similarity in their cytoplasmic tails. Furthermore, truncating the tail of ASLV Env does not prevent its incorporation or function.⁴⁶⁶ Thus, there may be mechanisms to direct Env proteins to assembling virions without these specific contacts to Gag—a default pathway, or a pathway using other interactions. Other distinct parts of Gag, including the p6 region of HIV-1 Gag, have been implicated in Env incorporation.

The HIV-1 protein Vpr is efficiently incorporated into assembling virions at very high levels, approaching equimolarity with Gag. This incorporation requires the presence of the p6 domain of Gag⁵⁴⁵ and may be mediated by a direct interaction.²³ The binding can be used to direct foreign proteins into the particle; a fusion between Vpr and a foreign protein will be targeted to virions. Furthermore, Vpr can be used to direct separately expressed versions of RT or IN to particles in a functional form, to complement mutations in the RT or IN domains of the Gag–Pol fusion.

Host Proteins in the Virion

A number of host proteins have been shown to be present inside the virion particle; in most cases the significance of the protein is unknown. Prominent among the virion-associated factors are a number of cytoskeletal proteins. These include

actin^{19,439,628} and various members of the ezrin-radixin-moesin (ERM) family, specifically including ezrin, moesin, and cofilin.^{438,439} Gag and especially the nucleocapsid protein of HIV-1 have been shown to directly bind to actin,^{506,628} perhaps offering a mechanism for its incorporation into the particle. A complication in analyzing virion-associated proteins is that virion preparations tend to be contaminated with substantial amounts of microvesicles, entities released by cells that exhibit a density and size very similar to that of virions, and containing an array of host proteins.⁴⁹

The virions of HIV-1 contain substantial levels of cyclophilin A, a protein proline isomerase of uncertain function but implicated in protein folding and signal transduction.^{179,348,593} The role of the virion-associated form is uncertain. Several other proteins have also been found in virions: a translational elongation factor, eIF-1a,¹⁰⁰ and a protein known as H03,³¹² with similarity to histidyl tRNA synthetase, are additional examples. An intriguing protein present in HIV-1 virions is lysyl tRNA synthetase (LysRS), the cognate synthetase responsible for the charging of tRNA (Lys), the primer tRNA for HIV-1.^{88,90,261} The incorporation of lysyl tRNA synthetase is mediated by a direct interaction with Gag, specifically the C-terminal portion of CA, and does not require the tRNA itself. The synthetase may facilitate tRNA incorporation.⁸⁸

Host proteins may also be attracted into virion cores by mechanisms other than Gag. The host uracil DNA glycosidase, responsible for removing uracil bases from DNA, was shown to be incorporated into virions by contacts to IN.⁶³⁰ The Ini1/Snf5 protein is also incorporated into virions through binding to IN.⁶⁶⁰ Another protein, the RNA transporter staufen, is incorporated into virions, perhaps through contact with viral RNA.³⁹⁷

There are also substantial levels of host membrane proteins in the virion envelope. The mechanism of incorporation of these proteins into the virion is not clear, and in most cases again the significance is uncertain. However, one such molecule, MHC class I, is present at levels approaching those of the Env protein, and can be functionally significant in that xenogenic antibodies targeted to MHC can neutralize the infectivity of viruses such as HIV-1.¹⁹

RNA PACKAGING

The RNA genome is incorporated into virions by virtue of interactions between specific RNA sequences near the 5' end of the genome, termed the packaging or Psi sequences, and specific residues in the NC domain of Gag (see 43,147). Direct binding is readily observed *in vitro* (e.g., see 44,146). Both partners in this interaction have been intensively studied.

Gag Sequences Important for Packaging

The Gag precursor is the form of the protein that is responsible for packaging viral RNA⁴²⁵; the NC portion of the precursor plays the largest role. Mutations affecting the NC protein often reduce the incorporation of the genomic RNA into the virion particle (see 43 for review). The most crucial sequences are the Cys-His boxes, short-sequence blocks resembling zinc fingers and containing the motif Cys-X2-Cys-X4-His-X4-Cys²⁰⁹; but basic residues elsewhere in the NC molecule are also important. Structures of the HIV-1 NC bound to various RNAs have

been resolved by NMR, revealing specific contacts between both hydrophobic and basic residues of NC and nucleotides in the stem-loop of the RNA.^{10,129} The NC protein of various viruses contains one or two copies of the Cys-His box. When two copies are present, they are not equivalent or interchangeable, suggesting that they mediate distinct interactions with RNA.²⁰⁷ Some viral cores can cross-package heterologous viral RNAs, suggesting good binding to the heterologous Psi region, and sometimes there is a strong preference for the homologous RNA. Exchanging the NC domains between viruses can sometimes transfer the preferential selectivity of a Gag protein for its cognate RNA, though the specificity of these hybrid Gags is often poor, and in some cases other sequences in Gag can determine the preference for RNA packaging by chimeric Gags.

Although Gags can obviously package RNAs in *trans*—that is, RNAs such as vector genomes that do not encode Gag—there may be enhanced encapsidation of the RNAs that encode Gag in *cis*, perhaps by the interaction of nascent Gag with RNA during its translation.^{216,283,331,483}

RNA Sequences Important for Packaging

The packaging or Psi regions on the viral RNA genome that are recognized for incorporation are distinct in nucleic acid sequence among the various viruses (e.g., see 406). The key Psi regions lie near the 5' end of the RNA, generally between the LTR and Gag.^{284,368,373,663} However, other regions of the genome can affect RNA packaging, including sequences upstream in R and in U5, downstream in Gag coding regions, and even near the 3' end of the genome. In the case of ASLV, a region of 270 nt is necessary and sufficient to mediate the packaging of a foreign RNA.²⁸¹ In the case of the MuLVs, sequences that are at least partially sufficient to mediate selective packaging have been similarly identified.^{1,36} These Psi regions are relatively autonomous; Psi can be moved to ectopic positions in the genome with at least some retention of function.³⁶⁷

Considerable effort has been focused on the structures of the 5' RNA of various viruses (e.g., 28,101–103,317,443,654, 663). The various Psi sequences have been predicted or shown to form a number of stem-loops, often containing GACG in the loops.^{129,145,317} Reversion analysis of mutants with alterations in these loops confirms the importance of the stem-loop structure. Mutational studies show that several such loops may incrementally contribute to the efficiency of packaging of the RNA, though one or two are often found to be most important.^{173,396} One of the stem-loop structures of the HIV-1 Psi was replaced by a completely foreign sequence that was selected on the basis of its binding activity with NC; the resulting RNA was efficiently packaged and utilized for replication, strongly suggesting that the binding to Gag is the key function of Psi.¹⁰²

Many cells contain vast arrays of endogenous proviruses and retrovirus-like elements, a subset of which can be expressed constitutively or under various conditions of stress to produce large amounts of genomic RNA. If such a cell is infected by an exogenous virus or has been engineered by expression constructs to produce virions, the particles will incorporate the endogenous RNAs along with the viral RNA.^{47,460} The endogenous retroviral RNAs, notably the VL30 RNAs of rodents, contain highly efficient Psi sequences,^{381,595} presumably because they were selected to compete with the homologous genomes of exogenous viruses for packaging.

Virions also contain a number of host RNAs of uncertain significance. There are substantial levels of 7S RNA, a low-molecular-weight RNA thought to function in host RNA splicing.²⁸⁶ In addition, there are low levels of host mRNA. Particles released without efficient packaging of the viral genome (as are produced by Psi-mutant genomes) may carry enhanced levels of host RNAs; various mutants with alterations in NC can show selective enhancement of both endogenous viral and host RNAs,³⁸¹ including ribosomal RNA and even intact ribosomes.⁴⁰⁴ A variant avian leukosis virus, SE21Q1b, packages unusually high levels of host RNA,^{187,337} and is capable of transducing these host sequences into new cells by reverse transcription.³⁵⁰ This phenotype of high-efficiency transduction is associated with an unusually high level of proviral expression and particle production rather than any specific alteration in a viral protein.¹²

Dimerization of the Viral Genome

Mature virions contain a dimeric RNA that is highly condensed into a stable, compactly folded structure referred to as the 70S dimer on the basis of its sedimentation rate. Specific sequences in the 5' end of the RNA,²³⁸ termed dimerization or dimer linkage sequences (DLS), are required for RNA dimerization *in vitro*, and for the formation of the dimeric virion RNA *in vivo*.^{42,353,398} These DLS structures are in close proximity or even intermingled with sequences required for packaging of the RNA, often making it difficult to determine their separate contributions to these processes.⁵³⁰ A model for the process of dimerization, the “kissing-loop” model, suggests that duplex formation between two RNAs is initiated between loops on the two RNAs and propagates outward through the stems through the action of NC.^{103,137,221,352,391,403,445,446,513,584}

Viral and even virus-host chimeric RNAs are normally always packaged as a dimer.²³⁷ Recent structural studies suggest that a high-affinity binding site for NC is sequestered by base pairing in the monomeric RNA, and that dimerization of the RNA exposes this and other binding sites, allowing tight binding by NC.^{134,148} This strongly suggests that dimerization might be a prerequisite for packaging. Further studies support this idea, and suggest that many mutations in the virus affect packaging by altering the monomer-dimer equilibrium and thus the amount of dimer available for NC binding.⁴³³ However, some ASLV mutants can apparently package monomeric RNA.^{435,457} Even here it remains possible that dimers are packaged but dissociate later.

The viral RNA in newly budded virions is present as a relatively unstable dimer, dissociated by heat at relatively low temperatures, and becomes condensed to a more stable dimer during virion maturation.^{185,481} This condensation requires the proteolytic processing of Gag¹⁸³ and may be mediated by the free NC upon its release from the precursor. It is likely that the paired regions of an unstable dimer are extended by NC.^{153,513,636} Dimerization may sometimes, but not always, require mature Pol proteins; RT and IN seem to be required for stable dimerization of HIV-1 RNA, but not MuLV or MPMV.^{80,550} The dimerization of viral RNAs can be induced *in vitro*, and is stimulated by addition of NC or the Gag precursor. However, it is uncertain to what extent these reactions reflect dimerization *in vivo*.

Incorporation of tRNA Primer

A key aspect of RNA packaging is the incorporation of a host tRNA along with the genome to serve as the initiating primer

for msDNA synthesis (for review, see 360). Virions contain a substantial pool of free tRNA, perhaps 50 to 100 copies per particle. The bulk of these tRNAs is not associated with the genomic RNA, and is present in virions that lack the genome. In some viruses these tRNAs are largely representative of the pool of tRNAs in the cell, while in others they are highly enriched for the tRNAs needed for priming DNA synthesis, though even here many other tRNAs are present. Viruses prepared without the Pol proteins do not show this enrichment, suggesting that Pol, and most probably the RT protein, are responsible for bringing these tRNAs into the virion.^{290,472} In accord with this notion, the RT of ASLV has been shown to preferentially bind tRNA trp from a mixture of tRNAs, accounting for its enrichment in the virion.⁴⁴⁸ Similarly, HIV-1 RT preferentially binds tRNA^{lys3}, and the interaction domain has been shown to at least include the anticodon loop of the tRNA. The incorporation of tRNA^{lys3}, and its placement onto the HIV-1 genome, are likely also catalyzed by the co-packaged lysyl tRNA synthetase.^{88,295} However, no similar preference for the natural primer tRNA^{pro} has been detected for the MuLV RT, nor is a tRNA synthetase apparently co-packaged in MuLV particles.⁸⁷ It may be significant that the MuLVs have been shown to be able to utilize a range of different primer tRNAs when only the complementary sequence in the genome (the pbs) is altered to promote their use.

tRNA Primer Placement

A very small subset of these tRNAs—two per virion—are annealed to the pbs, an 18-nt sequence near the 5' end of the genome with perfect complementarity to the 3' sequences of a specific primer tRNA. The pbs sequences are, as one would expect, essential for normal reverse transcription of the virus.⁵¹⁰ The sequence of the pbs can determine the primer tRNA that is utilized,⁶⁵⁵ but changes in the pbs tend to revert back to the wild type,⁶²⁷ suggesting that alternate tRNAs do not function well. An interesting aspect of reverse transcription provides for an efficient mechanism for this reversion: the use of the original tRNA even once during replication will convert the pbs back to the original sequence, because the tRNA itself is the template for the DNA copy of the pbs. Other sequence blocks of the tRNA are also paired with complementary sequences in R and U5 to form a large, complex structure required for proper tRNA primer placement and utilization.^{6,105,250,254,395} These other sequences are presumably responsible for the selectivity for the natural tRNA primer. In the alpharetroviruses, *pol* gene products are required to mediate the placement of the tRNA on the genome; but in the gammaretroviruses, *pol* is not required.¹⁸⁴ In the case of HIV-1, Gag and Pol proteins and the co-packaged lysyl tRNA synthetase are all required.^{86,89,250,290,332,519} The Gag precursor, especially the NC domain, are thought to play a major role in promoting the annealing of the tRNA to the genome. While NC can promote annealing of complementary RNAs and DNAs *in vitro*, its role and the mechanism by which it may act *in vivo* remain uncertain.

PROTEIN PROCESSING AND VIRION MATURATION

As retrovirions are budded from the cell surface, the Gag and Gag-Pro-Pol precursor proteins are proteolytically cleaved to

release the smaller proteins present in the infectious virions (for review, see 612). The cleavage of Gag and Gag-Pro-Pol is mediated by the viral protease PR, which is expressed either in Gag, Gag-Pol, or Gag-Pro-Pol fusion proteins. Thus, PR is responsible for cleaving itself out of a precursor protein, then making a number of other cleavages in these proteins.

Activation of the Protease

The processing of Gag and Gag-Pro-Pol precursors is intimately linked to assembly and budding, and is controlled so that the precursors are not cleaved until they are assembled. It is not certain how PR is regulated during assembly to begin cleaving its substrates. The structure of PR has revealed that the active enzyme is a homodimer (see Protease Structure and Function section), and thus its activation could be promoted by dimerization of the Gag or Gag-Pro-Pol precursor associated with assembly. As the virions form, one could imagine the high concentrations of the protein generating an active PR that would begin to cleave Gag and Gag-Pro-Pol, and would release the mature PR dimer as well. However, for the betaretroviruses like MPMV, this mechanism cannot explain the delay in processing. For these viruses, assembly occurs in the cytoplasm and should result in the establishment of a high concentration of Gag-Pro-Pol at that time. Yet cleavage does not begin in the cytoplasm, but rather is restrained until budding and export of the pre-formed virion particle. Thus, other unknown mechanisms, perhaps coupled to membrane association, must be responsible.

Various domains of Gag have been suggested to inhibit PR; conformational changes could relieve this inhibition. In the alpharetroviruses, a cleavage at the NC-PR boundary is required to release active PR, and thus activating this cleavage could serve as a trigger.⁷⁸ Similar cleavages at the p6*-PR boundary are important for full activation of the HIV-1 PR. Another possibility is the activation of the PR by a drop in the pH associated with virion release. It should be noted that the overexpression of PR in many artificial settings, both in bacteria and in animal cells, as a Gag-PR fusion or alone, can result in formation of highly active enzyme. The high level expression of PR is often toxic for cells, presumably due to its inappropriate action on many host proteins.

Protease Structure and Function

The retroviral proteases are aspartyl proteases with clear sequence similarity to members of the cellular family of aspartyl proteases.^{278,342} The three-dimensional structure of many proteases, including those from ASLV, HIV-1, HIV-2, SIV, FIV, and EIAV, have been determined by X-ray crystallography.^{313,387,412,620} The viral enzymes are small, typically containing about 100 amino acids, and are homodimers as isolated from virions. Each subunit contributes to the active site a single aspartate residue, lying in a loop near the center of the molecule. There is a long cleft at the interface between the subunits where the substrate lies; there are pockets to interact with each of the side chains of the substrate, conferring specificity to the enzyme. Each subunit has a flap consisting of an antiparallel sheet with a β -turn that covers the cleft. This flap moves out of the way to permit the binding of the substrate into the active site.

Retroviral proteases have a complex specificity for substrate peptides. The enzyme makes contact with approximately seven or eight side chains on the substrate, and thus can select its cleavage sites on the basis of at least these amino acids. The

cleavage sites tend to be within hydrophobic sequences, yet must lie in accessible and extended conformations. Some analyses of the various sites in Gag and Gag-Pol that are recognized by PR suggest that either one of two sequence motifs constitute a consensus site: one set has an aromatic residue or proline flanking the cleavage site, and the other set has aliphatic residues at these positions. Mutational analyses have allowed further definition of the residues on PR that make specific contacts to the substrate.

Protease Inhibitors

Studies of mutant viruses lacking PR demonstrated that the protease is essential for virus replication. Viruses lacking a functional PR can still express Gag and Gag-Pol precursors, and can mediate the assembly and release of immature virion particles. Thus, PR is not required for the process of virion assembly *per se*. However, these particles are noninfectious, and are blocked at an early step prior to the initiation of reverse transcription.^{118,279,299} Because of its essential role in virus infectivity, PR was appreciated early in the course of the AIDS epidemic as an attractive target for antiviral therapy. A number of molecules have been generated that can bind and inhibit PR, including peptide mimetics with uncleavable, nonsessile bonds at the cleavage site. Some are transition state analogs, and may have inhibition constants (K_i) in the nanomolar or subnanomolar range. These inhibitors have been extremely effective antiviral agents, and because they target a distinct enzyme and distinct step in the life cycle from the RT inhibitors, they have been particularly effective in combination with earlier drugs targeted at RT. The combination of three drugs that include a protease inhibitor is now the standard treatment for AIDS, and such highly active antiretroviral therapy (HAART) can keep virus loads below detectable levels in some patients for many years. Ultimately, however, point mutations in PR that confer resistance to the drugs can arise, allowing some virus replication in spite of therapy.

Processing of the Gag Precursor

During and after release from the cell, the Gag precursor is cleaved by the protease into a series of products present at equimolar levels in the virion. The number and size of the products vary considerably among the various viruses; the spumaretroviral Gag is exceptional in undergoing the fewest cleavages. A summary of the Gag products of some representative viruses are indicated in Table 47.4. There are many features of these products common to most of the retroviruses.

The Matrix Protein, MA

Beginning at the amino terminus, most Gags are processed to form a membrane-associated or matrix protein termed MA. The MA protein is thought to remain bound to the inner face of the membrane as a peripheral membrane protein, and can be crosslinked to lipid. MA may make contacts with the cytoplasmic tail of the envelope protein. When the precursor Gag is myristoylated at the amino terminus, the corresponding MA protein retains that myristate and is presumably bound tightly into the membrane. The compact structure of the MPMV MA protein has been elucidated by NMR.¹¹¹ The MA proteins of HIV-1 and SIV have been shown to form trimers in crystallization studies,^{38,497} and can contribute to the ability of a larger Gag precursor to form trimers in solution.³⁹³ The protein can

TABLE 47.4 Virion Proteins Found in Mature Particles of Various Retroviruses

Protein	ASLV	MLV	MMTV	MPMV	HTLV-1	HIV-1	HFV
MA	p19	p15	p10	p10	p19/15	p17	—
?	p10	p12	p21	p24	—	—	—
CA	p27	p30	p27	p27	p24	p24	p33
NC	p12	p10	p14	p14	p12	p7	p15
DU	—	—	p30	—	p15	—	—
PR	p15	p14	p13	?	p14	p14	p10
RT	$\alpha\beta$	p80	?	?	?	p66/51	p80
IN	pp32	p46	?	?	?	p31	p40
SU	gp85	gp70	gp52	gp70	gp60	gp120	gp130
TM	gp37	p15E	gp36	gp22	gp30	gp40	gp48

ASLV, avian sarcoma and leukemia virus; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; MLV, Moloney leukemia virus; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus.

form extended sheets of trimers, with a large opening in the network. If similar structures were to form in a sphere, the surface could have openings into which the envelope tail may fit.

The Capsid Protein, CA

Gag proteins are cleaved to generate a large product serving as the major capsid protein, CA, in the virion core. The CA protein is relatively well conserved among Gags, and contains the only highly conserved motif among Gags, the so-called major homology region (MHR). The function of this motif remains uncertain; although mutations in the region affect virion assembly in some viruses,^{117,362,490,570} it is not absolutely required for this process, since the entire CA domain of ASLV can be deleted without blocking assembly. CA is thought to form the shell of the condensed inner core of the mature virus, making either a spherical, cylindrical, or conical structure, depending on the virion morphology. Image reconstruction of electron micrographs, coupled to the subdomain structures, have led to models for the packing of CA to form these large assemblies. The major CA–CA contacts must form after processing during the condensation of the virion core, and may be very different from the contacts that exist in the immature virion particle.

The CA protein can form dimers in solution, and recombinant proteins containing CA, or CA plus NC, can assemble to form higher-order structures consisting of either tubes, spheres, and in the case of HIV-1, cones.¹⁹¹ CA has also been studied after tethering sheets of the protein to membrane.²⁹ The CA protein has proved difficult to crystallize. Structures of the N-terminal and C-terminal fragments of the HIV-1, RSV, and EIAV CA were first determined,^{189,202,264,302} and only later were complete CA proteins visualized.¹⁹³ Mutants of HIV-1 CA with engineered potential to form disulfide crosslinks have allowed isolation and crystallization of stable hexamers, revealing a flower-arrangement with N-termini near the center and C-termini in radial extensions (Fig. 47.17).⁴⁸⁵ Very recent work has defined a similar arrangement of CA proteins in the pentamers that introduce curvature into the hexamers array.⁴⁸⁶

The Nucleocapsid Protein, NC

All Gag proteins except for those of the spumaviruses are cleaved to produce a nucleocapsid protein, NC, located near the carboxyterminus of the precursor. NC proteins are small,

highly basic proteins containing one or two copies of the Cys-His motif, Cys-X₂-Cys-X₄-His-X₄-Cys. These sequences bind a single Zn⁺⁺ ion avidly, and fold around the ion into a characteristic structure that is smaller and rather different from the better-known zinc finger structure. The structures of NC proteins in solution have been studied by NMR, revealing a tightly folded knuckle with disordered flanking sequences.^{298,392} The interaction with zinc results in the incorporation of substantial levels of Zn⁺⁺ into all retrovirus virion particles.

The NC protein in virions is closely associated with the viral RNA, probably coating the entire RNA molecule; the stoichiometry of binding is such that each NC molecule can bind to about 6 nucleotides of RNA. NC proteins bind non-specifically to heteropolymeric single-stranded nucleic acid

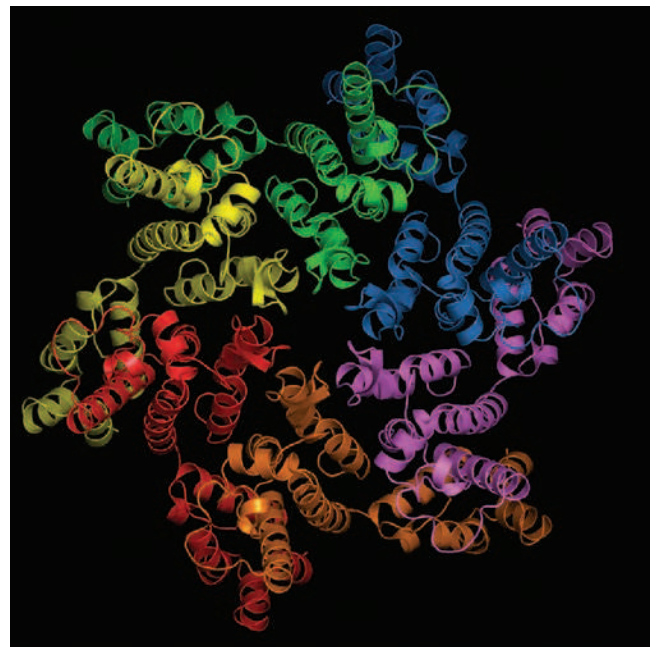


FIGURE 47.17. Structure of HIV-1 Gag CA hexamer. Top view of hexamers. Each subunit is colored distinctly. (Courtesy of Owen Pornillos, University of Virginia School of Medicine, Charlottesville, VA.)

with moderate affinity.⁶⁰³ However, NCs also exhibit specificity. Tests of binding to nucleic acids of defined sequence have shown that NCs bind poorly to poly(A), and most tightly to nucleic acids containing GT dinucleotides, especially alternating (GT)_n polymers.¹⁷⁴ In addition, NC has been shown to exhibit sequence-specific binding activity *in vitro* for nucleic acids containing the Psi region, required for packaging of the viral RNA.⁴⁴ A specific complex of the HIV-1 NC with a stem-loop derived from Psi has been studied by NMR. The resulting structure shows a number of specific contacts between hydrophobic residues of NC and bases in the four-nucleotide loop, and between basic residues and specific phosphates in the stem and loop.¹²⁹

NC proteins change the base-pairing properties of nucleic acids, and thus can have profound effects on the kinetics and thermodynamics of annealing. Under various conditions *in vitro*, NC can stimulate the dimerization of RNAs and duplex formation between tRNA and its complementary sequences at the primer binding site.⁶¹ Thus, NC can help promote primer tRNA placement during virion assembly.⁵⁰³ NC can also help melt out secondary structures, and may facilitate the movement of RT along the template during reverse transcription. In addition, it is clear that NC can bind to double-stranded nucleic acid, and is probably retained on the viral DNA after its synthesis by RT. NC mutants have been found that affect the course of DNA synthesis or DNA stability during the early stages of virus infection, suggesting a role in the processing of the DNA and protection of DNA from degradation.^{208,586} Finally, NC has been shown to promote the concerted integration of the two termini of the viral DNA into a target sequence (see Viral *att* sites section;⁸⁵).

An important class of inhibitors of virus infectivity and replication that act by targeting the NC protein have been identified.^{512,601} These compounds, disulfide-substituted benzamides (DIBAs), eject the zinc ion from NC and cross-link the cysteines via disulfide bonds. Virions treated with these compounds are potentially inactivated without disrupting the virion structure, and the course of virion assembly in infected cells is similarly blocked. Drug-resistant variants are not readily recovered.

Other Gag Products

Some retroviral Gag proteins, including those of the alpharetroviruses, betaretroviruses, and gammaretroviruses, contain one or more poorly conserved domains of 10 to 24 kDa lying in between MA and CA. The functions of these proteins is unclear. The ASLV p2 protein, the MuLV p12 protein, and the MPMV p24 protein contain a PPPY motif that plays an important role in late stages of virion assembly (see The L domain section). The MuLV p12 protein has also been shown to play a role in the early stages of infection.^{119,659}

In the lentiviruses, a p6 domain is present at the carboxy-terminus. The role of p6 is unclear, though it contains the late or L domain and thus may be important in virion release. It also is required to mediate the incorporation of Vpr into virion particles, perhaps by providing a direct docking site. Proteins can be targeted to virions by generating Vpr-X fusions, which are incorporated into lentiviral virions in a p6-dependent manner.

Processing of the Gag-Pro-Pol Precursor

At the same time that the Gag precursors are cleaved during virion maturation, the Pro and Pol region of the Gag-Pro-Pol

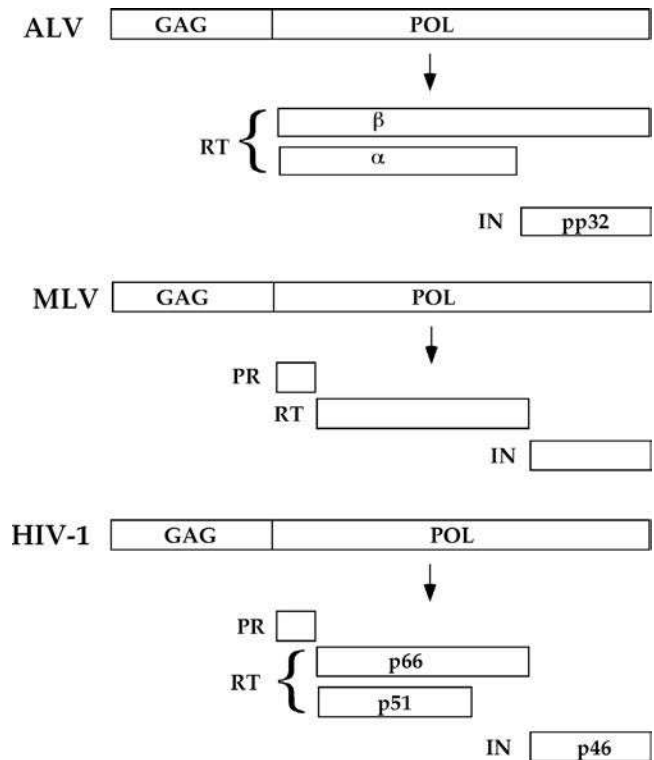


FIGURE 47.18. Cleavage patterns during the processing of the Gag-Pol fusion proteins of various retroviruses. The structure of the mature cleavage products found in the virion particles are shown aligned with their location in the precursor.

precursor is also cleaved, giving rise to the PR, RT, and IN products. The Pro- and Pol-containing precursors of different viruses are cleaved in diverse patterns (Fig. 47.18). In the gammaretroviruses, the Pol region is processed by complete digestion to form PR, RT, and IN. In the alpharetroviruses, the Pol region is cleaved to produce a heterodimeric RT with a larger β subunit and a smaller α subunit. The larger β subunit contains both RT and IN domains. It is not clear whether the IN domain in the context of this subunit performs an important function, although it is responsible for a weak nuclease activity associated with RT.²⁰⁵ A portion of the Pol precursor undergoes an additional cleavage to produce the α subunit of RT (an aminoterminal fragment of the β subunit), and the separate IN protein. In the lentiviruses, Pol is processed to give rise to PR, a heterodimeric RT, and IN. However, the RT of these viruses is not identical to the heterodimeric RT of the alpharetroviruses. Here the IN domain is fully removed from RT. One RT subunit remains intact (for HIV-1, this is the p66 subunit), and the other subunit undergoes an additional cleavage to remove a carboxyterminal domain (to form the p51 subunit). The functional significance of the different subunit structures of these various RTs is unclear, since they all perform a very similar set of reactions during virus replication. The processing of Pol precursors may be associated with the activation of the DNA polymerase of RT. In the alpharetroviruses, the immature Gag-Pol protein has very low DNA polymerase activity; its maturation results in a large increase in activity.^{116,564} However, the immature Gag-Pol protein of MuLV and HIV-1 has high DNA

polymerase activity, and there is only a very modest increase upon maturation.¹¹⁸

The Gag-Pro-Pol precursor of the betaretroviruses and the nonprimate lentiviruses is also processed to produce the dUTPase protein, DU. In the betaretroviruses, the *pro* ORF encodes both DU and PR; in the nonprimate lentiviruses the enzyme is encoded in the *pol* ORF, and DU lies in between RT and IN in the polyprotein. This enzyme acts to reduce the levels of dUTP that could otherwise be incorporated into viral DNA. Mutants of FIV lacking the function show increased rates of mutation during replication,³²⁴ and similar mutants of CAEV tend to accumulate G-to-A substitution mutations⁶⁰² presumably due to incorporation of dU residues that are subsequently read as dT.

Processing of the Env Precursor

The major proteolytic cleavage of the Env protein to form the SU and TM subunits is performed during its transport through the ER and golgi by host proteases termed furins. This cleavage is essential for virus infectivity,^{376,467} and is thought to induce substantial rearrangements of the polypeptide chain. The TM subunit remains embedded in the membrane, consisting of an extracellular domain, a membrane-spanning segment, and a cytoplasmic tail. The SU subunit lies wholly outside the cell, and after its incorporation into the virion particle, wholly on the extravirion surface. It is held onto the virion by contacts to TM, most often by noncovalent bonds, though disulfide links may occur in some viruses. SU is heavily glycosylated; the presence of at least some of these sugars is important for virus infectivity. Perhaps the most important function of this heavy glycosylation is to hide the peptides on the surface of Env from neutralizing antibodies that would otherwise have access to the virion surface. In addition, palmitoylation of the Env proteins of many viruses is essential for function.^{329,424,522}

The Surface Subunit SU

For most viruses the major receptor-binding site is located in hypervariable sequences on the SU subunit, so that SU is a major determinant of host range. Chimeric SU proteins can be generated to demonstrate that the receptor utilization function maps to specific regions of the protein. The key regions of the avian retroviral Env proteins have been similarly defined by selecting for changes in host range *in vivo*; these studies show that very small changes can result in the use of new receptors. The structures of two SU proteins have been determined at high resolution: a fragment of MuLV SU,¹⁶³ and a fragment of the HIV-1 SU bound to its receptor CD4.^{310,514} These structures suggest that the receptors make contacts to the envelope in shallow pockets that may not be readily bound by antibodies.

The Transmembrane Subunit TM

The TM subunit contains the so-called fusion peptide at its aminoterminal. TM is thought to play the major role in fusion of the virion and host membrane. Many TM mutations are defective for membrane fusion. However, mutations blocking fusion can lie in SU as well. The entire Env protein probably acts as a unified machine to mediate fusion, with complex interactions between the subunits and with major movements of the subunits during the fusion process. The fusion peptide of TM may simply insert into the host membrane or it may make contacts to proteins. The major contacts for oligomer

formation of Env are thought to lie in TM; isolated TM proteins form trimers in solution and in crystals.^{163,164,297,361} The trimer is held together by a modified leucine zipper motif that bridges the monomers via hydrophobic interactions.

It is possible to separate the two major functions of the Env protein onto two different molecules that cooperate to mediate these steps. Thus, the receptor binding function can be mediated by one Env protein, and the membrane fusion function can be mediated by another Env. This is apparent in the ability of two Env proteins to complement in mixed oligomers.⁵⁰² It is also demonstrated by the ability of a wild-type Env to provide the membrane fusion function for a chimeric Env that on its own can only mediate cell-surface binding.

The TM subunit of the MuLVs undergoes a second cleavage during virion assembly that is mediated by the viral protease, PR. This step removes a short sequence called p2E, or the R peptide, from the carboxyterminus of TM.²¹⁴ The cleavage step may require presentation of the tail to the protease, or some conformational change in the tail, that is mediated by Gag proteins; alterations in the MA or p12 Gag proteins can modulate the cleavage of TM.^{291,659} Astonishingly, the cleavage is necessary to activate the fusogenic activity of the envelope protein and thus for virus entry.^{71,501} Mutants in which the tail is truncated at the site of cleavage are constitutively activated for fusion, and these viruses induce dramatic syncytia in receptor-positive cells. Mutants in which the tail is not removed are inhibited for fusion, and particular residues can be shown to be required.⁶⁴⁸ How the cytoplasmic tail inhibits the fusogenic activity of Env is very much unclear.

In a similar way, the cytoplasmic tails of the TM of M-PMV and EIAV are processed by the protease. In the case of M-PMV, the presence of the intact tail is necessary for efficient incorporation of Env into the virion. The replication of some viruses in host cells of foreign species can select for alterations and truncations of the TM tails. The selective advantage conferred by this truncation is not well understood, though various aspects of Env function seem to be enhanced by this truncation.⁶⁷³

Morphological Changes upon Virion Maturation

The maturation of retrovirus particles is a complex process required for the formation of an infectious virus. The particles that are initially assembled either at the plasma membrane (by most retroviruses) or in the cytoplasm (by the betaretroviruses) have a characteristic immature morphology: the particles are round, and stain with an electron dense ring and a relatively electron lucent center. After release from the cell, the morphology changes to a more condensed structure, with a central core largely detached from the surrounding envelope. In the alpharetroviruses, gammaretroviruses, and deltaretroviruses, the core is spherical and concentric with the envelope; in the betaretrovirus the core is spherical but eccentrically placed within the envelope; in the lentiviruses the core is cylindrical or conical, with thin connections to the surrounding shell. In the spumaviruses the morphology does not change dramatically after assembly.

Mutant viruses lacking the protease show little change in morphology. Thus, cleavage of Gag and Gag-Pol is required to restructure the virion into the mature form.²⁷⁹ The changes in morphology visible in electron micrographs are probably associated with major rearrangements of the Gag proteins. The physical properties of the virus change dramatically upon maturation. Whereas the immature core is very stable to nonionic detergents

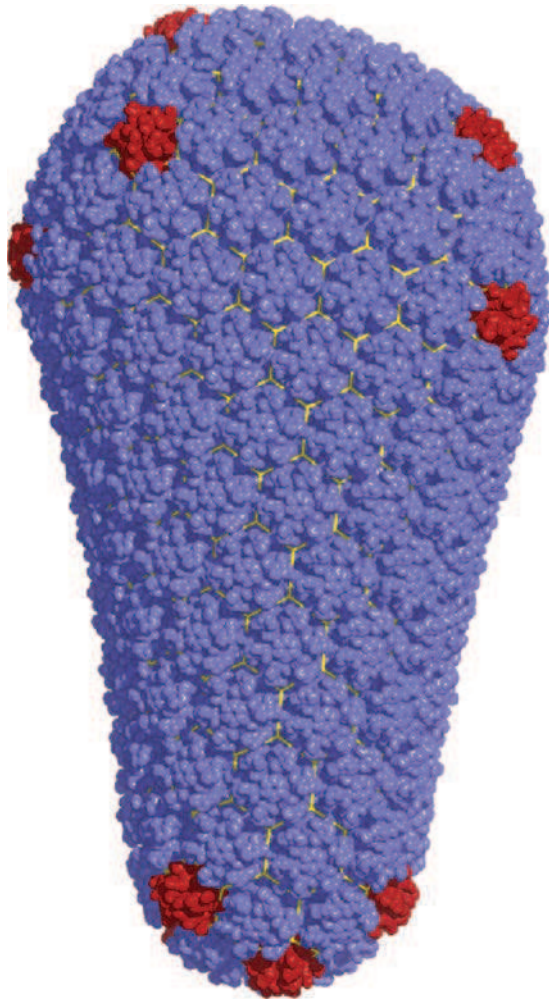


FIGURE 47.19. Reconstruction of the conical core of a single HIV-1 virion. Hexamers of the HIV-1 CA protein (purple) were organized in a folded array. Curvature was introduced by the asymmetric placement of pentameric defects (red) at each end of the cone. (Courtesy of Wes Sundquist, University of Utah, Salt Lake City, UT.)

and harsh conditions, the mature virion core is relatively labile. This change may reflect the inability of the immature virion, and the acquired ability of the mature virion, to uncoat upon infection of new cells and initiate reverse transcription.

Structure of Virion Core: CA Packing

After maturation, the Gag proteins rearrange to form the distinctive virion core, comprised of the CA protein surrounding the dimeric viral RNA condensed with the NC protein. The core is visible by electron microscopy as an electron-dense structure inside an electron-lucent area surrounded by a spherical shell and lipid envelope. The shape of the core is characteristic of the virus genera: round for the alpha- and gammaretroviruses, cylindrical for the betaretroviruses, and conical for the lentiviruses. Image reconstruction and X-ray crystallography studies of CA assemblies suggest that CA forms a hexameric array.^{191,302,330} The hexamers can form a two-dimensional lattice,¹⁹⁰ or long helical tubes or cones¹⁹¹ or spheres²¹⁸ *in vitro*. The N-terminal domains form external hexameric rings, and

the C-terminal domain forms internal dimer contacts to link together adjacent hexamers. While hexamers can form tubes without distortion, the curvature needed to close the ends of a cylinder or cone, or to form a sphere, is thought to be generated by introducing pentamers into the otherwise hexameric array.^{194,330} Asymmetric placement of the pentamers can create the cone-shaped core of the HIV-1 virion (Fig. 47.19).

RESISTANCE TO RETROVIRUS INFECTION: HOST RESTRICTION FACTORS

Several loci in the mouse genome have long been known to provide dominant resistance to the MuLVs, including *Fv1*^{336,477} and *Fv4*.⁵⁷⁴ Recently a number of novel host genes have been identified that also confer virus resistance to otherwise sensitive cells (for reviews, see 52,203). In some cases, these genes were identified as the targets of viral proteins that serve to inactivate the host restriction system; mutation of the viral functions then revealed the underlying restriction. In other cases, they were identified as the basis for a species-specific virus resistance: for example, the resistance to HIV-1 exhibited by various nonhuman primates. Collectively, these restriction systems target nearly all steps in the virus life cycle (Fig. 47.20). Although

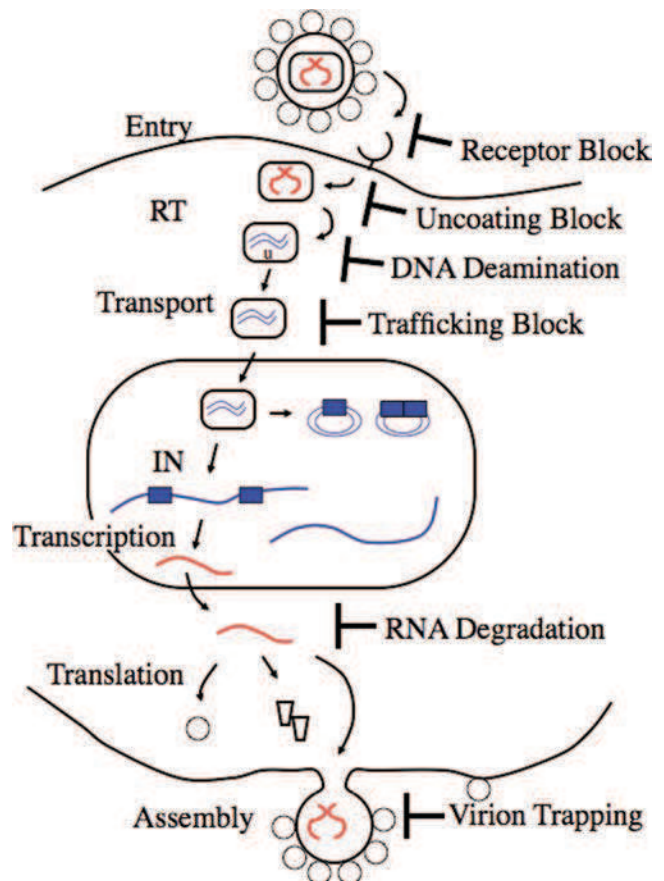


FIGURE 47.20. Sites of inhibition of the virus life cycle imposed by various restriction factors. A schematic of the virus life cycle is shown with steps in replication indicated on the left. Timing of blocks by different restriction factors is indicated on the right.

these factors are typically not effective in blocking wild-type viruses in humans, there is hope that they can somehow be activated or enhanced to provide antiviral protection.

Receptor Blockade by *Fv4*

One gene present in Japanese wild mice, identified as conferring resistance to Friend MuLV, has a simple mode of action: the *Fv4* gene restricts virus replication by blocking the ecotropic virus receptor.^{574,589} The *Fv4* locus corresponds to a defective endogenous provirus that encodes an Env protein fragment; the product downregulates the receptor and renders mice resistant to infection by exogenous viruses.^{252,303}

Early Block to Infection by *Fv1*

The *Fv1* gene was identified in several inbred mice in the early 1970s as mediating resistance to leukemogenesis by the Friend MuLV.^{336,477} Two naturally-occurring alleles provide resistance: the *Fv1*^b allele (in Balb/c mice) allows replication of so-called B-tropic viruses but blocks N-tropic viruses, while the *Fv1*ⁿ allele (in NIH swiss mice) allows replication of N-tropic viruses but blocks B-tropic viruses. Resistance is dominant in heterozygous animals. The tropism of the MuLVs can be characterized by their ability to replicate on cells of particular genotypes: N-tropic viruses grow only on *Fv1*ⁿⁿ cells, B-tropic viruses grow only on *Fv1*^{bb} cells, and NB-tropic viruses grow on both. The determinants of viral tropism lie in the *gag* gene, and affect a small sequence of the CA protein.^{63,242,440,518,563} The block to infection in resistant cells is at an interesting stage: largely after reverse transcription and before nuclear entry and provirus integration.^{270,649} Curiously, the block to integration that is observed for a particular virus and cell combination *in vivo* is lost when the PIC is extracted and tested for its ability to integrate *in vitro*.⁴⁹¹ The *Fv1* gene was identified as a unique member of an endogenous retrovirus gene family, with close similarity to the *gag* genes of the HERV-L family.⁵⁰ The two alleles differ by a few point mutations and a different carboxyterminal region. Thus, the intracellular expression of this variant Gag protein can somehow interact with the incoming PIC and its associated CA protein to block infection.

Early Block to Infection by Trim5a

Human and many other mammals are resistant to N-tropic MuLVs via an activity dubbed Ref1.^{48,596} This block is similar to that induced by the murine *Fv1*^b gene (both are determined by residue 110 of the CA protein), although it acts earlier, before viral DNA synthesis. Rhesus macaques and other non-human primates manifest a similar block to HIV-1 infection in the early steps of the life cycle, also determined by CA, and originally called Lv1.^{115,399} These blocks were saturable: exposing cells to high levels of a restricted virus could overcome, or “abrogate,” the block to infection by a second virus.^{48,227,301} The gene responsible for these blocks encodes TRIM5a, a member of a large protein family known as the RBCC (Ring, B box, coiled-coil) or TRIM (tripartite sequence motif) proteins.^{230,285,469,571} The mechanism of action of TRIM5a in blocking virus infection is not clear, but likely involves binding to the CA protein of the incoming virus.⁵⁴⁴ Recently a structure of a TRIM5a lattice superimposed over a hexameric sheet of CA protein has been visualized, suggesting that a highly multimeric form of TRIM5a might recognize the multimeric form of CA present in the virion core.¹⁹² Trim5 activity can be enhanced

by overexpression of SUMO-1, the small ubiquitin-like modifier protein, and requires the presence of two SIMs, or SUMO-interaction motifs.¹⁸ A simple model explaining these findings is that TRIM5 binding to CA is enhanced by CA SUMOylation.

The critical residues of HIV-1 CA for TRIM5a recognition lie in the cyclophilin A binding loop,^{188,605} and complex interactions between CA, TRIM5a and cyclophilin A may determine virus sensitivity.^{229,597} A remarkable confirmation of the functional interrelationships between these proteins is the finding that in a new world primate, the owl monkey, the TRIM5 gene is interrupted by the transposition of a cyclophilin A pseudogene and expresses a TRIM-Cyp fusion protein.^{419,532} A similar, but independently arising, gene fusion has been found in other primate lineages.^{333,611} These fusion proteins confer potent resistance to viruses that retain a cyclophilin A binding site in their CA proteins. A plausible model for the action of all these factors is a premature disruption of the capsid soon after viral entry. Restriction by TRIM5a may occur in two steps, with an initial proteasome-dependent step before reverse transcription, then a proteasome-independent one after reverse transcription.⁶⁴¹

Recently, TRIM5a has been shown to play a role in the induction of interferon production by stimulating synthesis of unlinked polyubiquitin chains.⁴⁷¹ Thus, another antiviral function of TRIM5a may be to signal interferon production in response to an incoming viral core; this function seems to be independent of its direct antiviral activity.

Deamination of Viral DNA by the APOBECs

A major mechanism of resistance to HIV-1 is provided by the deamination of cytosine residues in the minus strand of the viral DNA formed during reverse transcription.^{225,549} The main enzyme responsible for this activity in primates is APOBEC3G, one of a family of cytidine deaminases that includes APOBEC1 (a regulator of ApoB mRNA expression) and AID (activation-induced cytidine deaminase, used in immunoglobulin class switching and hypermutation of immunoglobulin genes during affinity maturation). The family member APOBEC3F is also effective at blocking infection. APOBEC3G is packaged into virions and during subsequent infection can deaminate as many as 4% of the C residues of the viral DNA minus strand, resulting in both DNA destruction and G-to-A hypermutation of the surviving plus strand DNAs.^{224,319,366,665} In addition to its deaminase activity, APOBEC3G may independently trap or inhibit viral DNA synthesis during infection.⁴¹⁶ APOBEC3G is apparently targeted to the virions by interactions with NC and/or viral RNA.

This potent block to infection mediated by the APOBECs is counteracted in human cells by the HIV-1 Vif protein, which drastically reduces the levels of APOBEC3G and F in the infected cell and prevents their incorporation into virions. Vif binds APOBEC3G and F, and blocks them either by inducing their proteosomal degradation via the Cullin5-SCF ubiquitin ligase complex, by inhibiting them directly, or by blocking their translation. The APOBECs of many nonhuman primates are not recognized by HIV-1 Vif; as a result, these species can block HIV-1 infection. In addition, the family member APOBEC3B, which has potent antiviral activity in cell lines, is resistant to HIV-1 Vif.¹³⁸ It is not clear why this isoform is not expressed adequately in lymphocytes to protect humans from infection.

Another host enzyme, uracil N-glycosylase (UNG), may collaborate with the APOBECs to promote degradation of the

viral DNA. This enzyme is also packaged into virions, and recognizes and removes uracils in the DNA, the product of deamination of cytidines, leaving an abasic site. This would block normal reverse transcription and lead to destruction of the viral DNA. However, the HIV-1 gene product Vpr can mediate the inactivation of UNG and the related SMUG enzymes, again probably via ubiquitin ligase-triggered proteosomal degradation.⁵³⁷

Blocking Early Events in Monocyte Lineage Cells by SAMHD1

Dendritic and myeloid cells exhibit a potent restriction of HIV-1 replication that prevents the normal accumulation of viral DNAs in the cytoplasm in the early phase of infection. The block can be counteracted by delivery of the Vpx protein encoded by certain strains of SIVs. Recent work has identified SAMHD1 as the mediator of the block.^{245,311} Vpx induces proteosomal degradation of SAMHD1 and thereby allows virus infection.

Elimination of Viral RNAs by ZAP

A block to MuLV infection was initially identified in a screen of cDNA overexpression libraries for genes that confer virus resistance. The product of a rat gene, dubbed ZAP (for zinc finger antiviral protein), blocks viral gene expression by eliminating viral RNAs from the cytoplasm of the infected cell.¹⁹⁵ ZAP contains four CCH-type zinc fingers that bind directly to viral RNA²¹⁹ and targets the RNA for destruction by the RNA exosome.²²⁰ Remarkably, ZAP expression also renders cells resistant to infection by a number of alphaviruses, including Sindbis, Semliki Forest virus, and Venezuelan equine encephalitis virus,⁵¹ and by Ebola virus.

Trapping Virion Particles on the Cell Surface by Tetherin

HIV-1 mutants lacking the *Vpu* gene are poorly able to replicate in certain cell lines, with the major block being at the time of virion release from the infected cell surface. The inhibition was traced to the cell-surface expression of a protein dubbed tetherin, which traps the virion particles and prevents viral spread to neighboring cells.^{414,468} Tetherin can similarly inhibit spreading infections by many enveloped viruses.²⁷³ The Vpu protein of wild-type HIV-1 binds to tetherin and inactivates it; this may be achieved either by sequestering it, preventing its delivery to the cell surface, or by directing its ubiquitinylation and degradation.^{142,149,231,256,365}

MicroRNA or siRNA-Mediated Inhibition of Viral Gene Expression

Retroviruses may be inhibited by host microRNAs,^{114,318} and HIV-1 has further been suggested to encode microRNAs that suppress the RNA silencing machinery of the cell,⁴⁰ though these observations are controversial. The significance of these observations for replication *in vivo* remains to be determined.

RETROVIRAL DISEASES

The Varied Effects of Retroviral Infection

Retroviruses cause an extremely wide range of responses in infected animal hosts. Discussion of retroviral pathogenesis begins with a little-appreciated but important point: retroviruses in general are surprisingly benign. The vast majority of

the replication-competent retroviruses are not cytopathic, and the infection of cells cause remarkably little impact on their replication or physiology. The morphology, control of cell division, and doubling time of cells in culture are not significantly changed after infection. Once a chronic infection is established, only a relatively small amount of the cellular metabolism is committed to virus expression: typically a few percent of the cellular mRNA and protein are viral, and thus the cell can perform its normal functions and survive for its normal lifespan. Animals show few acute effects upon infection. Animals do become viremic, and a vigorous immune response is often mounted that can reduce the levels of virus production. However, infected mice or birds may live relatively normal lives for many months or years; it therefore is appropriate to consider the viruses as relatively benign parasites. It is noteworthy that the virus is not eliminated but only suppressed by the immune response, and low-level viremia usually persists in infected animals for life.

Retroviruses often do, however, cause disease. The chronic viremia of the replication-competent retroviruses is tantamount to high-level mutagenesis of infected cells, for each infection event is associated with a proviral insertion that constitutes a mutation. Eventually the odds are that a cell will suffer an insertion that alters the normal control of cell division or cell survival, and abnormal proliferation of this cell results in tumorigenesis. Many retroviruses cause disease in this way, including the so-called “slow leukemia viruses” and agents such as MMTV. A few retroviruses, however, are more pathogenic: a small minority of the retroviruses are directly cytopathic, and many of the infected cells are killed. These agents can thus destroy the infected tissues and directly damage their function. These include the cytopathic avian viruses and, probably, the AIDS virus, HIV-1. Finally, a special class of retroviruses exists, the so-called “acute transforming viruses,” that can induce a rapid tumor formation. These viruses were among the first filterable oncogenic agents ever discovered; their dramatic effects were a major motivation for the intense study of all the tumor viruses throughout the twentieth century. We now understand that these agents are transducing viruses; the replication of retroviruses allows for recombination events between viral and host sequences that move genes onto the viral genome. These viruses carry and express host genes at inappropriate levels, in inappropriate cells, and often with drastic alterations in gene structure. If the gene product so expressed by the virus is mitogenic or antiapoptotic, the result can be a potent alteration in the physiology of the infected cell. These acute transforming viruses can thus initiate a highly aggressive tumor very efficiently and with minimal latency, because each infection of a cell has the high potential to initiate an oncogenic transformation event. Most often the acquisition of the host gene comes with a loss of a viral gene essential for its replication; as a result, these viruses are often replication defective and depend on a helper virus, usually a replication-competent leukemia virus, for their transmission to new cells. Each of these classes of pathogenic viruses will be discussed in the following sections.

Diseases Caused by the Replication-Competent Retroviruses

The typical pathology of many of the simple replication-competent retroviruses is the development of leukemia or lymphoma after a very long latency. For this reason these agents are often called the slow leukemia viruses; examples are found in rodents, including the many MuLVs, and in birds, including

the avian leukemia-leukosis viruses. The symptoms eventually begin with a lymphoid hyperplasia,⁵⁶⁶ which may be directly attributed to the immune response. Not all affected cells are infected, and the proliferating cells may be stimulated by cytokines that are released in response to the infection.⁷⁰ These cells may include a preleukemic state of partially transformed cells. There may be some cell killing due to enhanced apoptosis in these early stages,⁶² though the mechanism of the apoptosis and the relationship to tumorigenesis is unclear. A subset of these expanding cells progresses to frank leukemia, which ultimately can be fatal in susceptible animals. These observations strongly suggest that leukemogenesis is a multistep phenomenon, and it is also likely that the virus plays a role at more than one of these steps. The cell type transformed by the virus can be very narrowly defined, or more broadly variable, but will depend strongly on viral determinants. For example, the ASLV group of viruses typically causes a bursal or B-cell lymphoma in birds; the Moloney MuLV causes a T-cell leukemia; the Friend helper MuLV causes an erythroleukemia; and MMTV causes a mammary epithelial tumor.

In some species and settings, the infecting virus is the proximal agent of disease; such is the case with infection of rats by the Moloney MuLV. However, the course of leukemogenesis in mice and other animals is often associated with the appearance of recombinant retroviruses derived from the parental infecting virus and endogenous sequences present in the germ line.^{93,162} The recombinant viruses are often the true or proximal pathogens. These viruses are heterogeneous in structure and phenotype, but most contain substitutions of the *env* gene and LTR that confer novel properties to the initial virus. Some of the viruses arising in mice can be detected through an expanded host range, as an ecotropic virus acquires *env* sequences that allow infection through the xenotropic or dual-tropic receptors; these viruses are often termed MCF viruses, for mink cell focus forming viruses. The range of cell types and the replication ability of the input virus can be expanded by recombination to significantly enhance the incidence of leukemia and shorten the latency period to disease. The donor sequences for these recombination events are not all universally present in a given species but are highly variable from strain to strain. The presence or absence of suitable endogenous proviruses in the germline that provide the sequences needed for recombination can control the severity and course of disease.

Leukemogenesis by Insertional Activation

The most common mechanism of action of the replication-competent viruses in initiating tumors is termed proviral insertional mutagenesis, leading to the activation of endogenous proto-oncogenes.^{232,422,464} During replication in the infected animal, an enormous number of cells are infected, acquiring new proviral DNA insertions at near-random sites. Each of these insertions constitutes a somatic mutation, and thus retrovirus infection can be thought of as similar to a massive exposure to a potent mutagen. The vast majority of the insertions are harmless, causing no significant change in host gene expression. The majority of those that do disrupt genes simply create a recessive mutation at one allele out of two present in the cell, again causing no significant change in the overall pattern of gene expression. But very rarely, a provirus insertion can create a dominant-acting mutation that profoundly alters the physiology of the cell. When a provirus integrates near a gene that controls growth, altering its expression, the cell may

proliferate and ultimately form a clonal tumor in which all cells contain the provirus integrated at the same site.

A large number of cellular genes have been identified as potential targets for insertional activation in retrovirus-induced tumors. Among the most notable are an array of transcription factors, including *c-myc*, *N-myc*, *c-myb*, *Fli1*, *Fli2*, *Ets1* (*Tp11*), *Evi-1* (*Fim3*), *Bmi1* (*Flvi2*), and *Spi1* (*PU.1*); a number of secreted growth factors, such as *Wnt1* (*Int1*), *Wnt3* (*Int4*), *Int2* (*Fgf3*), and *Fgf8*; growth factor receptors, including *c-erbB*, *Int3* (*Notch4*), *Mis6* (*Notch1*), *c-fms* (*Fim2*), the prolactin receptor, and *Fit1*; and genes implicated in intracellular signal transduction pathways, such as the serine/threonine kinases *Pim1* and *Pim2*. Many of these genes are also known to be involved in or implicated in tumorigenesis in other settings, either when transduced on retroviral genomes or when activated by more conventional mutations. However, a number of the proto-oncogenes have been identified only by virtue of having served as target sites during tumorigenesis by leukemia viruses; thus, this route has made important contributions to the list of known proto-oncogenes.

The patterns of activation of these proto-oncogenes by retroviral insertion are highly varied. At least four distinct mechanisms have been observed (Fig. 47.21).

- **Promoter insertion:** The provirus may insert upstream of the gene or within the gene, and in the same transcriptional orientation as the gene. Transcription beginning in the 3' LTR reads into the gene and results in high-level expression of a transcript with R-U5 sequences at the 5' end. The resulting transcripts may be similar to the natural transcripts, but may be longer or truncated relative to the normal mRNAs.
- **Enhancer insertion:** The provirus may insert either upstream or downstream of the gene, and in either orientation relative to the gene. The insertion brings the powerful transcriptional enhancers present in the U3 regions of the two LTRs into close proximity of the gene, activating the endogenous promoter elements. While the levels are inappropriately high, the structure of the resulting transcript is normal.
- **Posttranscriptional stimulation of expression:** The provirus may insert downstream of the coding region and stabilize the formation of an mRNA. The provirus may provide a polyadenylation signal that enhances the formation of stable transcripts; or the insertion may remove RNA destabilization signals in the 3' UTR that would normally mediate the rapid turnover of the RNA. These mechanisms can result in inappropriately high steady-state levels of the mRNA and protein products.
- **Readthrough transcription:** The provirus inserts upstream or in the gene, but transcription initiates in the 5' LTR, reads through the provirus, and continues into the gene. The formation of such transcripts is often enhanced by mutations in the provirus, such as loss of the 3' LTR. The transcripts may be spliced aberrantly in complex patterns.

Insertional activation of a proto-oncogene by a provirus is not sufficient on its own to fully transform a cell, but represents only one step in a progression to a frank leukemia or tumor. Other mutations are usually required; these mutations can be point mutations in other proto-oncogenes or loss of function mutations of tumor suppressor genes. In some retroviral tumors, more than one oncogene can be activated by insertion of separate proviruses. Similarly, an acute transforming genome is usually not sufficient to transform a cell in one

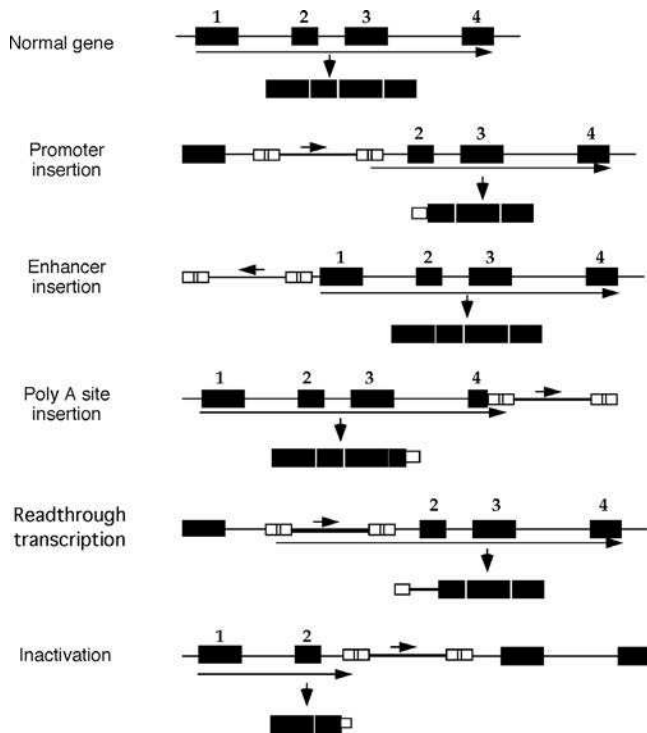


FIGURE 47.21. Genetic alterations in target gene expression induced by retroviral insertional mutagenesis. Various changes in normal gene expression that have been observed upon insertion of retroviral DNA are diagrammed. A target gene containing four exons is used in these examples (*top*). Promoter insertion: Insertion of the provirus in the same transcriptional orientation in the first intron is shown to result in the formation of a new mRNA initiated in the 3' LTR and extending into the downstream exons. Enhancer insertion: insertion upstream of the gene, in this case in reverse orientation, is shown enhancing the expression from the natural promoter. PolyA site insertion: insertion at the 3' end of the gene in the forward orientation is shown providing a polyA addition signal, increasing the levels of a prematurely truncated mRNA. Leader insertion: insertion of the provirus in the same transcriptional orientation is shown to result in the formation of an RNA initiating in the 5' LTR, extending through the provirus, and into downstream exons. Splicing results in the retention of only the viral leader on the chimeric mRNA. Inactivation: insertion is shown causing premature end formation of the mRNA, resulting in the formation of an inactive fragment.

step, and additional mutations must arise. In some tumors induced by a replication-defective transforming virus, the helper virus may provide such mutations by its own insertional activation event.

Gene inactivation, as opposed to gene activation, is also an important event in some tumors. Retrovirus insertion can frequently disrupt gene expression to effectively produce a null or hypomorphic mutation. These mutations are normally silent, since a second allele would be expected to continue to express a functional gene product. However, if the host animal is already heterozygous due to an inherited germ-line mutation in one allele, or if the insertional inactivation is coupled to a loss of the other allele by other means, the net result can be homozygous loss of function. When the target gene is a tumor suppressor, the consequence is the promotion of tumorigenesis.

Viral Determinants of Pathogenicity

Several viral genes and sequences can affect the incidence and severity of retroviral disease. The viral LTR contains the most important determinants of leukemogenicity and of the cell tropism for transformation. The enhancer and promoter elements of the LTR are responsible for proto-oncogene activation; their relative transcriptional activity thus controls the transforming ability of many viruses. If these elements are strongly tissue- or cell type-specific, the virus will be most competent for transformation of those cells in which the LTR is most active. A variety of viruses show profound tropisms for transformation that are controlled in this way.^{64,345,451} For example, the promoter of the Moloney MuLV is most active in T cells, and the virus shows strong tropism for the formation of T-cell leukemias. The Friend helper virus LTR contains an enhancer that is most active in erythroid cells; the virus is correspondingly highly tropic for erythroid cells.⁶⁵ The promoter of MMTV contains glucocorticoid response elements that provide high-level expression only in cells with high levels of the glucocorticoid receptor and only when exposed to glucocorticoids; as a result, MMTV is specific for mammary tumors.²¹⁷ Variant betaretroviruses, such as the thymotropic DMBA-LV virus, show selectivity for T cells that is probably attributable to changes in the LTR. Determinants of leukemogenicity have also been mapped to *gag*, *pol*, and *env* genes, though it is not clear what aspects of their functions are required in most cases. It may be that vigorous replication *in vivo* is the simple key feature of a highly transforming leukemia virus. There may also be *trans*-acting functions encoded by the leukemia viruses that modulate expression of specific host genes, but their roles are uncertain. The murine and feline leukemia virus LTRs encode short RNAs that can *trans* activate host genes, apparently through activation of an AP-1-like activity.

Other aspects of infection, distinct from the genetic makeup of the virus or host, can modulate the pathology associated with infection; even the route of entry of the virus can affect the disease course, presumably by determining the initial cell types infected and the route of virus spread.

Other Retroviral Diseases

A new gammaretrovirus, the xenotropic murine leukemia virus-like virus (XMRV), was first identified in familial prostate cancer cell lines, and subsequently detected in a subset of prostate tumor samples.⁶⁰⁴ The virus is similar in sequence and properties to the mouse xenotropic MLVs. XMRV was subsequently suggested as a potential cause of chronic fatigue syndrome in humans.³⁴³ Recent work has strongly suggested that the virus arose during passage of prostate tumors in nude mice by recombination events between two endogenous mouse retroviruses, and that most reported isolates are laboratory contaminants.⁴⁵² Whether humans are infected by replicating xenotropic MLVs remains controversial.

Cytopathic Viruses

Some viruses show distinctive pathogenicity mediated by specific gene products. Cas-Br-E MLV is a well-studied murine virus that induces a hind-limb paralysis with significant neuronal loss in the absence of an inflammatory response.⁴⁸⁷ Both neurons and glial cells accumulate vacuoles. The virus targets endothelial cells and microglial cells in the brain. It is likely that the infection of the microglial cells is most crucial to disease

induction. Infection may impair or block the neuronal support function of these cells, resulting in loss of neurons, though the mechanism of neuronal cell death is unclear. It is possible that the expression of the Env protein is toxic. The major determinant of pathogenicity is in the SU subunit of the Env protein.^{354,453} A number of other MuLVs, such as the ts1 mutant of the Moloney MuLV TB strain,⁶⁴⁰ can cause neurologic symptoms, including hind-limb paralysis and spongiform encephalomyelopathy.⁵³ In these cases, the SU protein is thought to be important as well. TR1.3, a Friend-related MuLV, is a neuropathogenic virus that induces fusion of capillary endothelial cells, leading to a hemorrhagic stroke syndrome. The crucial determinant in the virus is a tryptophan residue at position 102 of the SU protein. In some viruses, the LTR is also likely to play a role in disease induction,^{133,454} perhaps by determining the level of expression and the ability to spread efficiently and access the primary target cell.

A number of the ASLV group of viruses are cytopathic,⁶²⁵ and can cause an acute wasting disease characterized by poor growth, anemia, and immunosuppression associated with atrophy of the bursa and thymus.⁵⁰⁵ The disease probably reflects the ability of these viruses to lyse infected cells. The isolation of the ALV receptor for the subgroup B viruses and its identification as a member of the TNF receptor family suggests the possibility that the binding of Env to the receptor is directly triggering an apoptotic response. The cytopathic and noncytopathic viruses seem both to be able to trigger similar responses, however, so that it is not clear at this time what aspect of the interaction might be necessary and sufficient for cell killing. The vigorous replication of the virus, and an ability to mediate high-level reinfection before superinfection resistance appears, may also be significant determinants of cytopathology.

Yet another disease caused by a variant virus is the feline acquired immunodeficiency syndrome, or FAIDS. This disease was originally associated with a complex mixture of FeLV isolates. The agent responsible was shown to be an FeLV with mutations affecting the SU subunit of the *env* gene. The mutant FeLV is incapable of establishing superinfection resistance, and thus large amounts of unintegrated viral DNA accumulate during superinfection, ultimately leading to high expression of viral gene products and causing cell lysis.

The lentiviruses cause an array of important diseases in animals and humans, most notably AIDS. The major cause of disease is probably cell killing, but the most important target cells and mechanism by which infection leads to cell death are not clear. The very high level of gene expression mediated by HIV-1 infection in some cell types may be a crucial aspect of the cell killing,⁵⁶⁰ but the key viral gene products remain obscure. HIV-1 infection eventually leads to depletion of CD4-positive cells and thus to immunodeficiency, culminating in severe opportunistic infections. The lentiviruses also cause a number of other pathologies, including neurologic disease, that are poorly understood. These diseases will be discussed in Chapter 50.

Stimulation of Host Cell Proliferation

MMTVs lead to the formation of mammary tumors through the insertional activation of a number of proto-oncogenes. However, unlike other simple retroviruses, MMTVs carry an additional gene termed *sag*, for superantigen, that is important for disease induction (see 107 for review). Sag proteins bind to

MHC class II molecules in regions that are common to molecules with many different binding specificities, and thus can activate as many as 10% of all T cells.

The *sag* gene is located in the U3 region of the MMTV LTR and encodes a low-abundance glycosylated membrane protein.⁶⁷ The protein must be proteolytically processed for proper export to the cell surface. Importantly, expression of a functional Sag protein by MMTV is required to establish infection in an animal. The virus is normally transmitted in mother's milk to newborn mice, infects B cells in the Peyer's patch, and induces a vigorous Sag-mediated stimulation of T cells. There follows a B-cell response that provides a large pool of susceptible B cells for the virus¹⁶; it is these cells that then carry the virus to the mammary gland. The infection of the mammary epithelial cells ultimately leads to transformation of these cells by insertional activation. This pattern of viral spread through one intermediate cell type to ultimately lead to disease in another cell type is a paradigm for complex viral pathologies, such as that exhibited by polioviruses.

The *sag* gene of MMTV was also recognized as acting as a host gene important in disease progression when a number of mouse genes, termed Mls for minor lymphocyte-stimulating antigen, were shown to map to endogenous MMTV proviruses, and ultimately were identified as the *sag* genes. The expression of Mls results in the clonal deletion of many T cells in mice carrying the gene. Thus, mice carrying endogenous proviruses will often lose T cells needed for virus replication, and will be resistant to exogenous MMTV disease.

A number of other viruses carry variants of the normal replication genes that cause specific pathologies in the infected host. One spleen focus-forming virus, SFFV-P, causes a severe polycythemia; infection leads to a massive expansion of erythroid precursors (BFU-E and CFU-E) and a concomitant loss of mature red cells. This agent consists of a complex of a replication-defective variant and a Friend MuLV helper virus to propagate it. The defective genomes carry a mutant *env* gene encoding a shorter SU molecule, termed gp55, that no longer functions to mediate virus entry. However, gp55 can bind directly to the erythropoietin receptor (EpoR) and stimulate the mitogenic and differentiative responses normally triggered by ligand binding to the receptor. This activity allows the virus to infect these dividing pre-erythroid cells; the continued expression of the envelope protein in these cells promotes their factor-independent growth and expansion in an autocrine loop. Ultimately a frank erythroleukemia results and may be associated with proviral activation of proto-oncogenes occurring as a result of the continuing infections. It is clear that the *env* gene of the virus is sufficient to cause the disease.⁶³⁹ A very similar virus, the SFFV-A, causes a severe splenomegaly and anemia. This virus is closely related to SFFV-P, and also activates the Epo receptor to expand immature cells. Variation in the envelope between these two strains alters the target cell and the consequences of its expansion.²⁷⁵ It should be noted that this mitogenic activity of gp55 is not a completely novel property of the deleted Env. The parental F-MuLV helper Env protein has a weak ability to bind and send mitogenic signals through the Epo receptor, presumably resulting in expansion of Epo receptor-positive cells. This increase in target cell number is presumably able to enhance virus spread, and thus serves as a positively selected trait for the virus. Other Env proteins, including those of the MCF viruses, may activate the IL-2 receptor.

Another replication-defective variant, the murine acquired immune deficiency syndrome (MAIDS) virus, causes a relatively acute hyperproliferation of B-lineage cells in infected mice.^{20,249} There is a subsequent proliferation of macrophages and CD4⁺ T cells. The expansion of these cells displaces many other cell types, including T cells, and the animals eventually show a significantly defective immune response. The mechanism of the immunodeficiency is not fully clear, and there is some indication that an antigen-driven stimulation leads to an anergic state. However the immunosuppression occurs, the disease is in reality a lymphoproliferative disorder, distinct from human AIDS in its pathology. The causative agent is again a replication-defective variant carried by a replication-competent helper virus. The defective genome encodes a mutant Gag precursor in which the central portion, including the p12 region, is replaced by a foreign Gag derived from endogenous retrovirus sequences. The altered Gag has been shown to interact with the c-Abl protein, a tyrosine kinase first identified as the transduced oncogene of the Abelson MuLV, expressed in that virus as a Gag-Abl fusion protein. Thus, the MAIDS virus seems to act by forming a noncovalent interaction with the c-Abl protein as an approximate mimic of the Gag-Abl protein formed by transduction on the Abelson virus.

An ovine disease has attracted attention as a potential model for human lung cancer. Sheep pulmonary adenomatosis (SPA) is a contagious bronchiolo-alveolar carcinoma of sheep associated with an exogenous type D/B retrovirus, Jaagsiekte sheep retrovirus (JSRV). Epithelial tumor cells are sites of large amounts of viral DNA.⁴⁴⁷

Two of the epsilonretroviruses, the piscine (fish) retroviruses, cause a dermal sarcoma that shows a remarkable seasonal appearance and regression. The mechanism of transformation of cells by the virus is not totally clear, but seems to reflect the activity of a cyclin D homolog encoded by the viral genome. This gene, the *orfA* gene, may induce inappropriate entry of the cells into cycle by activation of a cyclin-dependent kinase (cdk).

Host Determinants of Retroviral Disease

A number of genes have been identified that determine sensitivity or resistance to retroviral diseases. Some of these genes act at the level of virus replication, directly controlling the ability of the virus to spread. The Fv1 locus described in the previous section is a good example of a gene that acts in a cell-autonomous way to restrict the replication of various MuLVs.²⁶⁹

A large set of those genes that affect sensitivity to viral disease modify the availability of target cells for virus growth, or the immune response to virus infection, therefore indirectly control the levels of viremia. The *Fv2* gene is an example of such a gene.³³⁵ Virus-susceptibility (*Fv2*^s) is dominant over virus-resistance (*Fv2*^r) at this locus. The virus-susceptible allele encodes a truncated form of the stem-cell kinase receptor (Stk), which promotes virus-induced erythroleukemia⁴⁷⁰; expansion of the *Fv2*^s-expressing cells may provide increased cells for virus replication. The *Fv2*^r homozygous mice are resistant due to a limited expansion of BFU-E clones, and a reduced ability of the Friend MuLV to find sensitive targets.^{60,575} Finally, mutations in certain genes can sensitize or predispose organisms to oncogenic transformation by retroviruses. Because transformation is almost always a multistep process, mutations in one of the genes in a transforming pathway can increase the frequency with which a virus-mediated loss of another gene becomes manifest as a frank

tumor. Thus, knock-out mutations of such tumor suppressors as the *p53* or *p73* genes can sensitize to transformation by a number of oncogenic retroviruses. Similarly, a preexisting transgene such as a regulated version of the *myc* oncogene, *Em-Myc*, can predispose particular cells expressing the gene to insertional activation of other proto-oncogenes in viremic animals.⁶⁰⁶ New integration sites that would not normally be detected in wild-type mice are often utilized in such mice.

ACUTE TRANSFORMING RETROVIRUSES: TRANSDUCTION OF CELLULAR PROTO-ONCOGENES

Many potent transforming retroviruses, which can initiate rapid tumor formation with a quickly fatal outcome, have been isolated and characterized. These viruses are recombinant transducing viruses, which have acquired portions of cellular genes that are responsible for the transforming activity. The prototype of these viruses is the Rous sarcoma virus, which carries a transforming version of the *c-src* gene. In the exceptional case of RSV, the viral replication functions, including the coding regions for the Gag, Pol, and Env proteins, are all intact so that the resulting transducing genome is replication competent. In nearly all other cases, the acquisition of the transforming gene from the host has occurred with a loss of one or more of the viral replication functions, so that the resulting virus is replication defective. However, these genomes retain all the *cis*-acting elements needed for their replication, and thus can be transmitted from one cell to another by a replication-competent helper virus. The concerted replication of two viral genomes in a complex—a replication-competent helper virus and a replication-defective acute transforming virus—is a common feature of most of the transforming viruses.

Transforming viral genomes exhibit a range of different structures, but have some features in common. Those segments required in *cis* for viral replication are always retained: the LTRs, the PBS, and the PPT are present because they are required for reverse transcription and forward transcription. The RNA packaging signals are retained. Much of the regions required in *trans* are often deleted, since these functions can be provided by the helper, and are replaced with the host sequences. The host gene may be expressed separately, or, more often, is fused to Gag, Pol, or Env sequences to form a fusion protein.

The formation of a transducing virus is thought to involve a complex series of events that results in the acquisition of the coding regions of a host gene by the replication-competent parental virus.⁵⁷⁷ Several models have been proposed to account for the observed structures including DNA-based events (e.g.,⁵⁴⁰), but more often, RNA-based events (summarized in 594). The most commonly accepted model includes the following steps (Fig. 47.22):

- The process begins with the insertion of a provirus upstream of the gene to be transduced. An insertion in the middle of a gene can initiate the transduction of the downstream portion of that gene.
- Next, readthrough transcription beginning in the 5' LTR generates a large RNA containing viral sequences fused to downstream sequences. This event can be enhanced by lesions in the 3' LTR that prevent normal RNA processing

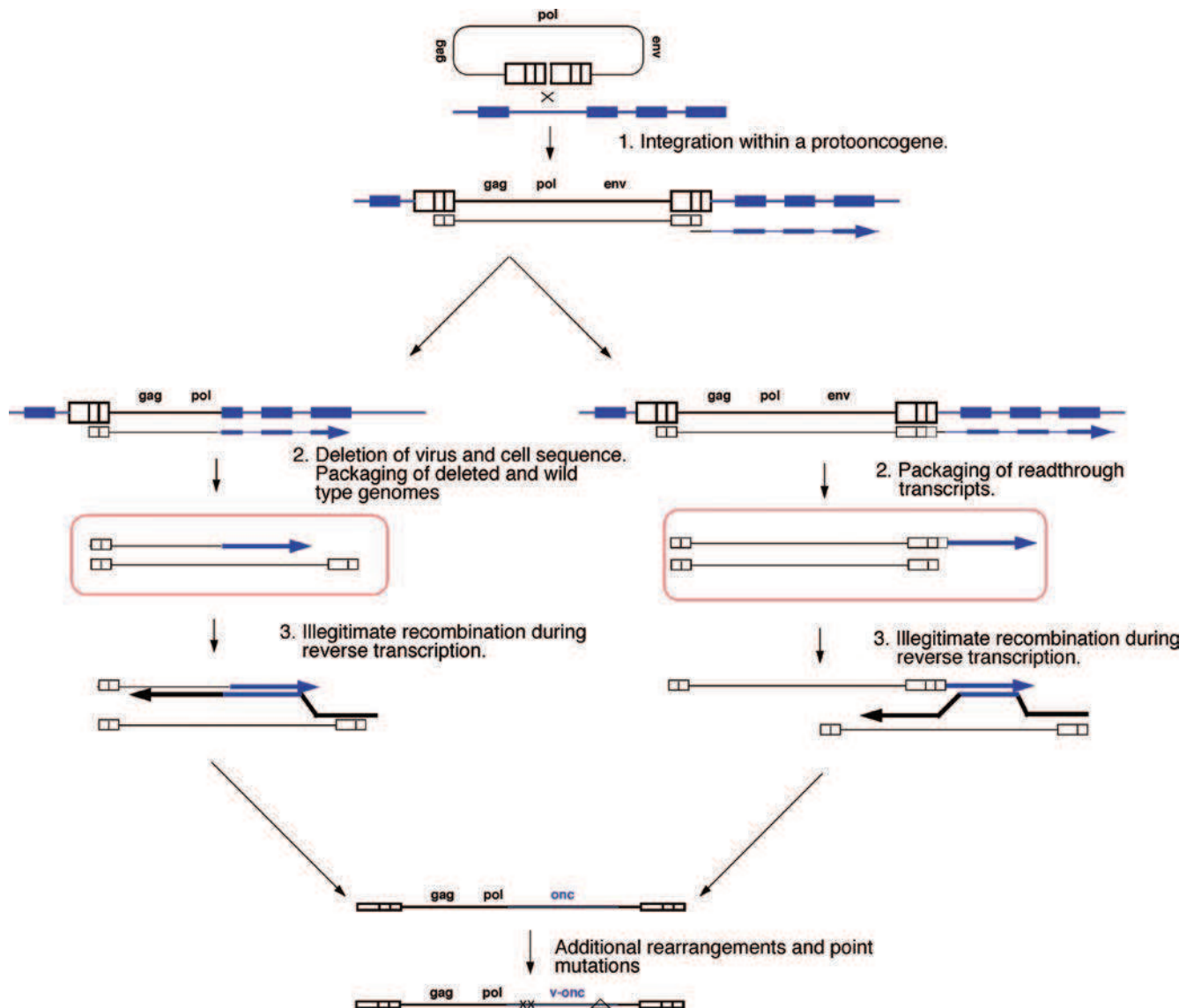


FIGURE 47.22. Two pathways for the acquisition of host oncogenes by replication-competent retroviruses in the formation of an acute transforming genome. Integration is shown establishing a provirus within a proto-oncogene in the same transcriptional orientation (*top*). Either of two processes then occurs. In one mechanism (*left*), a deletion of the chromosomal DNA fuses the 5' half of the provirus to the downstream portion of the gene. The fused DNA then encodes a fused RNA, which may be spliced and packaged into virion particles along with wild-type helper RNA. During reverse transcription, RT switches from the helper to the fusion RNA to append the 3' portion of the helper onto the hybrid RNA. The completed reverse transcribed DNA is integrated and transmitted thereafter as a replication-defective viral genome. In the other mechanism (*right*), a readthrough RNA extending from the 5' LTR through the provirus and into the downstream portion of the gene is formed. The RNA is packaged into virion particles along with wild-type helper RNA. During reverse transcription, RT switches from helper to host and back to helper RNAs to form the hybrid genome. As before, the completed reverse transcribed DNA is integrated and transmitted thereafter as a replication-defective viral genome. In either scenario, the transducing genome may undergo additional rearrangements and mutations under selective pressure for more efficient transforming activity and transmission.

and polyadenylation at this site. Alternatively, a deletion in the DNA could fuse the 5' half of the provirus to downstream sequences, again leading to the expression of a fusion RNA.

- In either mechanism, the chimeric RNA can be spliced and is then packaged into virions along with the RNA of a helper virus.^{236,578}

- Finally, nonhomologous recombination occurs during reverse transcription to append 3' viral sequences to the chimeric genome. A template switch by RT from the helper to the chimeric RNA during minus-strand synthesis can mediate such a nonhomologous event at low, but easily detected, frequencies.^{204,592,667} The completion of reverse transcription on this template would result in the generation of a provirus

with host sequences flanked by viral termini, similar to those seen in transforming retroviral genomes. Consistent with this model is the appearance of poly(A) sequences at the 3' junction between host and viral sequences in some viruses; if the translocation by RT from viral to host RNA occurs in the host poly(A) sequences, a portion will be retained in the final genome.

A key feature of the resulting genome is the presence of only the mRNA sequences—that is, only the exons and not the introns—of the host gene. Thus, very large genes can be transduced by retroviruses because they carry only the exonic coding regions of the gene. Most transforming retroviral genomes are not only a result of these relatively simple recombination events, but rather have also undergone multiple rearrangements thereafter. The RNAs encoded by these genomes often exhibit complex patterns of splicing, which can involve cryptic splice sites in both virus and host sequences. Several of the known rodent viruses carry segments of endogenous retroviral or virus-like sequences, especially the virus-like 30S (VL30) elements.

The genes that have been identified on the many acute transforming viruses are wildly diverse in their sequence and functions. These genes are among the most intensively studied of all known genes; their clear involvement in oncogenesis has focussed enormous attention on their structures and function. The genes include growth factors (v-Sis); growth factor receptors (v-erbB); intracellular tyrosine kinases (v-src, v-fps, v-fes, v-abl), members of the G protein family (H-ras, Ki-ras); transcription factors (v-myc, v-erbA); and many others. The genes are now recognized as playing major roles in mitogenic signaling pathways; in the control of the cell cycle; and in anti-apoptotic pathways that act to limit cell survival. There is no indication that all such genes have been identified, and it is likely that new transforming viruses will continue to provide new examples of genes that can be activated by transduction to initiate tumor formation.

The acquisition of these genes, as noted previously, is often associated with fusion of the coding region to Gag, Pol, or Env sequences. Thus, the expression of the oncogene results in a fusion protein that may exhibit dramatically altered biochemical activity, intracellular localization, or stability. These changes are often a key aspect of the activation of the normal function of the proto-oncogene to create the fully transforming viral oncogene. In other cases, or in addition to these alterations, there may be specific mutations that arise during or after the transduction process. These mutations, which may be as simple as a point mutation or as drastic as a frameshift or deletion mutation, can be the major cause of activation of the oncogene. Presumably, the high mutation rate of viral replication allows for the appearance of such mutations, and a selection for tumor formation in enhancing virus spread is responsible for the appearance of these mutations.

ENDOGENOUS VIRUSES AND VIRUS-LIKE SEQUENCES

Virtually all cells contain a large number of retroviral or retrovirus-like DNA elements integrated into the germ line (for comprehensive reviews, see 58,346,462,567). These endog-

enous retroviral elements can represent a substantial fraction of the total DNA in a genome; while the sequences most closely related to the exogenous retroviruses may only represent a percent or so of the total DNA in many species, the retroelements in total can occupy 10% or more of the genome.⁵⁹¹ These elements have presumably accumulated over evolutionary times, with no mechanism by which they can be removed and no strong selection against individuals that acquire them.

The retroviral provirus is closely related in structure and mode of replication to transposable elements—the retroelements—found in the genomes of all living things, from bacteria to humans.⁵⁷ Many of these elements are remarkably similar to proviruses, with LTRs that function similarly and with sequence similarity to *gag* and *pol* genes. The retroviruses have probably existed as parasites of cells from very ancient times, and evolved together with transposable elements.¹⁴⁰

Endogenous Elements in Chickens, Mice, Pigs, and Humans

Many endogenous retrovirus sequences have been characterized in chickens and other birds, and can be grouped into at least four families. The ALV-related elements were among the first to be discovered, including the replication-competent provirus RAV-0. Most of the other family members are replication defective and lack *env* sequences. The newer families of such viruses continue to be characterized.⁵⁵⁷

A vast literature describes the endogenous retrovirus sequences in inbred mice.^{39,112,255,304,568} At least eight families have been described, though only four have been studied in detail. The virus-like 30S (VL30) elements are present in the genome at a copy number of perhaps 100 to 200²; these elements encode a 30S RNA that is packaged efficiently into the virion particles of exogenous viruses and contaminates most virion RNAs. They presumably represent a parasitic RNA that spreads by exploiting exogenous viruses. The intracisternal A-type particle (IAP) elements are present at about 1,000 to 2,000 copies in the genome.^{349,423,432} These elements can express intracellular particles containing RT, but the particles are budded into the ER and not released from cells. Most lack *env* genes, and thus cannot form infectious particles. However, a few members do contain *env* sequences. Some IAPs can transpose intracellularly at low frequencies. Third, there are a small number of proviruses (0–4 per genome) closely related to MMTV that encode functional B-type viruses. Fourth, there are proviruses related to the exogenous MuLVs, present at 50 to 100 copies per genome.⁵⁶⁸ These proviruses are all very similar to one another, but can be categorized according to their similarity to exogenous viruses that utilize particular receptors into four groups: the ecotropic, xenotropic, polytropic, and modified polytropic. The distribution of these sequences among different murine species and subspecies can help reveal their evolution and spread. For example, xenotropic MLV-related proviruses are present only in *Mus musculus* subspecies, while polytropic MLV-related proviruses are found in both *M. musculus* and *M. spretus*. Replication-competent members of the family are found in many, but not all inbred mice.¹⁰⁶ (For reviews of the properties of the murine endogenous retrovirus genomes, see 180,262).

The potential to use pig organs or cells in xenotransplantation into humans has raised considerable interest in the presence of endogenous retroviral elements in the pig. Although

viruses can be rescued from porcine cells, and while these viruses can infect human cells very efficiently,^{461,635} preliminary studies suggest that they are not easily transmitted to humans in transplant settings. There remains a real possibility for their transfer to humans, however, and the consequences could be significant.

Retroviral elements are also abundant in the human genome.^{56,346,369,370} These elements are collectively termed HERVs, for human endogenous retroviruses, and subgroups are denoted by a letter indicating the amino acid specificity of the tRNA primer. Most are defective, but a very small number of these elements are still actively transcribed in somatic cells and are capable of transposition to new sites. The distribution of the HERV-K family in various primates has been surveyed to help build evolutionary trees of these species.²⁶⁸ There are also provirus families distantly related to the lentiviruses; some have the potential to encode rev-like elements that could, in principle, be pathogenic.

Properties of the Endogenous Provirus-Like Elements

The distribution of elements in a given species is relatively stable over the course of a few generations. Thus, most individuals in an inbred population show a constant, characteristic pattern of endogenous elements. The rate of loss of a given provirus is very low, and the appearance of new proviruses is rare in most animals. However, the pattern is very different in different species, and even in different strains of animals, suggesting that rearrangements happen often over longer evolutionary periods. It is known that new copies can appear at higher frequency if newborn females are viremic. Thus, early infection of germ cells can introduce new proviruses into the germ line. This route can even be used to create mutations *de novo* in laboratory mice at reasonable frequencies.

Most endogenous proviruses are transcriptionally silent; the DNA is often heavily methylated and so repressed. These may reflect the mechanisms by which the transcription of many exogenous viruses are repressed *in vivo*. Expression of many of the endogenous viral RNAs is induced by agents causing DNA damage, such as UV light and BrdU; the expression of others is stimulated by glucocorticoids. The IAPs are often induced during the differentiation of various cell types, and even more often in immortalized tumor cell lines.

The bulk of the endogenous retroviruses are fossil DNAs, grossly defective, and no longer capable of encoding proteins; the ORFs contain numerous stop codons and frameshifts that would preclude the formation of any functional viral gene products. However, these elements can often give rise to RNAs, which can be packaged efficiently by virions encoded by exogenous viruses and give rise to new proviruses. Furthermore, these co-packaged RNAs can then recombine with the exogenous viral RNAs and contribute small sequence blocks to these viruses, potentially altering the host range and replication properties of the virus. The continuous contribution of endogenous sequences to virus evolution is a fact that needs to be considered whenever genetic selections are imposed on a virus. In addition, a few of the elements are functional, and can transpos intracellularly or can even give rise to replication-competent viruses. Even when viruses are induced from the elements, however, the viruses are most often not highly pathogenic for the host in which they reside. Thus, many of the inducible ele-

ments in the mouse are xenotropic, and cannot spread in the animal; those that can do not cause an acute disease. The LTRs of the endogenous elements are often quite weak as transcriptional promoters as compared to those of exogenous viruses. This may reflect selections against highly pathogenic agents either before or after their introduction into the germ line.

The creation of a new provirus in the germ line by necessity creates a mutation, and while most such insertions probably have no significant effect, occasionally deleterious germ-line mutations occur. A number of ancient, "spontaneous" mutations upon analysis have been found to have been caused by a proviral insertion. These include such classic mutations as the rd1 allele, causing a slow retinal degeneration, and which includes an insertion affecting the beta subunit of the retinal cGMP phosphodiesterase; the hr mutation, causing a hairless phenotype; the dilute coat-color allele d; and a mutation termed Slp (for sex-limited protein) in the C4 complement gene, in which an insertion of a viral LTR renders the gene androgen responsive.

Many of the endogenous elements may be positively selected in their host species. This may be due to advantageous mutations that are created by the insertion, or to antiviral effects mediated by the gene products encoded by the endogenous proviruses. The *Fv1* and *Fv4* genes are examples of such elements. These virus-like elements confer resistance to exogenous viruses, and may serve to protect the host from leukemia induced by infection. The MMTV *sag* gene, if present on an endogenous provirus, acts to delete T cells that would respond to the superantigen; thus, subsequent infection by an exogenous MMTV cannot use *sag* to induce a proliferation of cells needed for its vigorous replication. The inherited provirus therefore protects the host from MMTV disease.

RETROVIRAL VECTORS, PACKAGING LINES, AND GENE THERAPY

The structure and mechanism of transmission of the naturally arising replication-defective transforming viral genomes provide a clear model for the directed use of retroviruses to mediate gene transfer. Retroviral vectors that mimic the structure of the transforming viruses can readily be generated, and can be engineered to carry the cDNA sequences of virtually any gene. These genomes can then be propagated with wild-type virus as helper. However, it is also possible to generate helper-free preparations of particles that transduce the vector genome via the early steps of the life cycle without delivering the helper genome, preventing subsequent spread of the vector. These helper-free particles are generated in packaging cell lines: cells engineered to express the *gag*, *pol*, and *env* genes but not expressing packageable helper viral RNAs. The first such lines simply carried a provirus lacking the Psi site, the RNA packaging signal.³⁶⁸ These cells produce virions deficient in the helper genome, and introducing a Psi+ vector construct into these cells results in the encapsidation and release of the vector RNA into those particles. These particles can then be harvested and used to deliver the vector and its gene into susceptible cells. It is also possible to generate transducing virus preparations by transiently transfecting cells with DNAs that encode the helper functions and DNAs that encode the vector. This approach is preferable in instances in which the viral

gene products are toxic and therefore difficult to express stably in a packaging cell line.

A limitation of these packaging systems is that small amounts of the Psi-minus helper RNA are encapsidated along with the vector. Endogenous retroviral genomes, such as the virus-like 30S RNAs (VL30),² are also encapsidated efficiently, and recombination events between these RNAs during reverse transcription can recreate a replication-competent virus. These events are probably similar to recombinational repair of mutations in genomes that occur during growth in cell culture. This issue has raised considerable fears that gene therapy vectors intended for therapeutic use could initiate a viremia, and perhaps a viral leukemia, in patients. More elaborate cell lines, in which the *gag*, *pol*, or *env* genes are expressed via separate RNAs, can reduce the frequency with which such recombination events occur to very low levels.

Retrovirus particles transducing a desirable gene can be directed to target cells through the use of many distinct envelope proteins. This method is possible because retrovirus particles can readily form pseudotypes; that is, they can incorporate and use the envelope proteins of a wide array of different viruses. The wide range of pseudotypes that can be formed presumably reflects the flexibility of the core-envelope interaction. The host range can be further expanded or restricted by the engineering of envelopes with new binding specificities. Chimeric envelope molecules have been particularly popular tools in targeting virions to new receptors. Another approach is to engineer animals that express a foreign receptor in a tissue-specific manner, and deliver genes with a virus envelope that only recognizes the transgenic receptor.¹⁶⁸ The envelope-receptor interaction can even be reversed: it is possible to express a particular virus receptor molecule on the virion surface, targeting the virus to those cells expressing the corresponding viral envelope.²⁴

A major limitation of early retroviral gene therapy efforts is the inability of most helper viruses to mediate the infection and transduction of nondividing cells. The major block is during the early stages of infection, when there is a strong requirement for cell division for infection by most viruses.⁵¹⁷ However, the lentiviruses have the ability to infect nondividing cells, and thus gene therapy based on lentiviral packaging systems could overcome this limitation (for review, see 9). Efforts have fully substantiated these expectations: delivery to nondividing neurons and to poorly dividing primary lymphocyte cultures has been demonstrated with vectors based on HIV-1^{55,276,408,409} as well as FIV.⁴⁸⁰

PERSPECTIVES

The study of retroviruses has led to a detailed characterization of many steps of virus replication as well as to important fundamental discoveries concerning host physiology and genetics. The viruses have served as entrees into such phenomena as cell surface receptors, cell division, DNA synthesis, the cell cycle, mechanisms of gene expression, and intracellular transport. The value of focusing on retrovirus functions in unraveling cellular functions is clear: these agents have evolved over huge periods of time to exploit key aspects of the cell, and we should make use of their success to help identify those aspects. There is every reason to believe that their continued study will reveal even more new aspects of cell physiology.

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Human T-Cell Leukemia Virus Types 1 and 2

History

Infectious Agent

HTLV-1 Gene Regulation

Tax

p12/p8

p13

HBZ

Pathogenesis and Pathology

Entry into the Host

Site of Primary Replication and Spread of the Virus

Cells and Tissue Tropism

Immune Response and Persistence

Provirus Sequences *In Vivo*

Epidemiology

Age

Morbidity/Mortality

Origin and Spread of Epidemics

Prevalence and Seroepidemiology

Genetic Diversity of Virus

Clinical Features

Adult T-Cell Leukemia

HTLV-1–Associated Myelopathy/Tropical

Spastic Paraparesis

HTLV-1 Uveitis

Infective Dermatitis

Diagnosis

Prevention and Control

Treatment

Vaccines

Prevention

Perspectives

been described in Japan. ATL is characterized by several clinical features, including skin lesions, hypercalcemia, the presence of leukemic cells with multilobulated nuclei, and an aggressive clinical course.^{354,376} Patients with ATL were clustered in a limited area of Japan, including the islands of Kyushu and Okinawa, which suggested a pathogen(s) as a causative agent. In 1981, it was reported that ATL patients had antibodies that reacted with ATL cell lines,¹³¹ suggesting that ATL was caused by an unknown virus. Thereafter, it became evident that the sequence of the provirus in ATL cells was almost identical to that of HTLV. The clinical features of ATL resemble those of mycosis fungoides. Therefore, the patient from whom the HTLV-infected cell line was established might have been misdiagnosed. The diagnosis should have been ATL.

In 1984, HTLV-1 was reported to be associated with tropical spastic paraparesis (TSP),⁹³ and the same disease was reported in Japan as HTLV-1–associated myelopathy (HAM).²⁸¹ Both diseases were subsequently classified as the same disease and are referred to as HAM/TSP. Other inflammatory diseases were found to be linked to this virus, including HTLV-1–associated uveitis, myositis, alveolitis, and infective dermatitis.^{116,204,242,243,258,341}

HTLV-2 was discovered in a cell line that was established from a patient with T-cell variant hairy cell leukemia.³¹¹ Thereafter, infection with this virus was found in the native Amerindian population of North, Central, and South America, in people in Central and West Africa, and in drug abusers in the United States and Europe.⁷⁵ HTLV-2 has been reported to cause a neurodegenerative disease resembling HAM/TSP.¹⁴⁰ However, no definite associations with lymphoproliferative diseases have been reported.

In addition to HTLV-1 and -2, HTLV-3 and -4 were isolated from bushman hunters in Cameroon.⁴⁰⁰ There are limited numbers of individuals infected with HTLV-3 and -4; thus, their pathologic roles in human diseases are unclear.

INFECTIOUS AGENT

HTLV is a delta-type retrovirus, and an HTLV virion contains two copies of single-stranded genomic RNA. Upon viral entry into a host cell, the viral RNA is reverse transcribed (the transfer RNA for proline being utilized as a primer), and the viral genome becomes integrated into the host cellular DNA as a provirus. This genome encodes the structural (Gag, Env), enzymatic (protease, reverse transcriptase, integrase), nonstructural regulatory (Tax, Rex), and accessory proteins (p12, p30, p13, HBZ).

HISTORY

The discovery of oncogenic retroviruses in animals led to a search for retroviruses in human cancer cells. In 1980, the first human retrovirus was found in a T-cell line established from a patient with mycosis fungoides, and the retrovirus was named human T-cell leukemia virus (HTLV).^{85,296} Prior to this discovery, the disease entity of adult T-cell leukemia (ATL) had

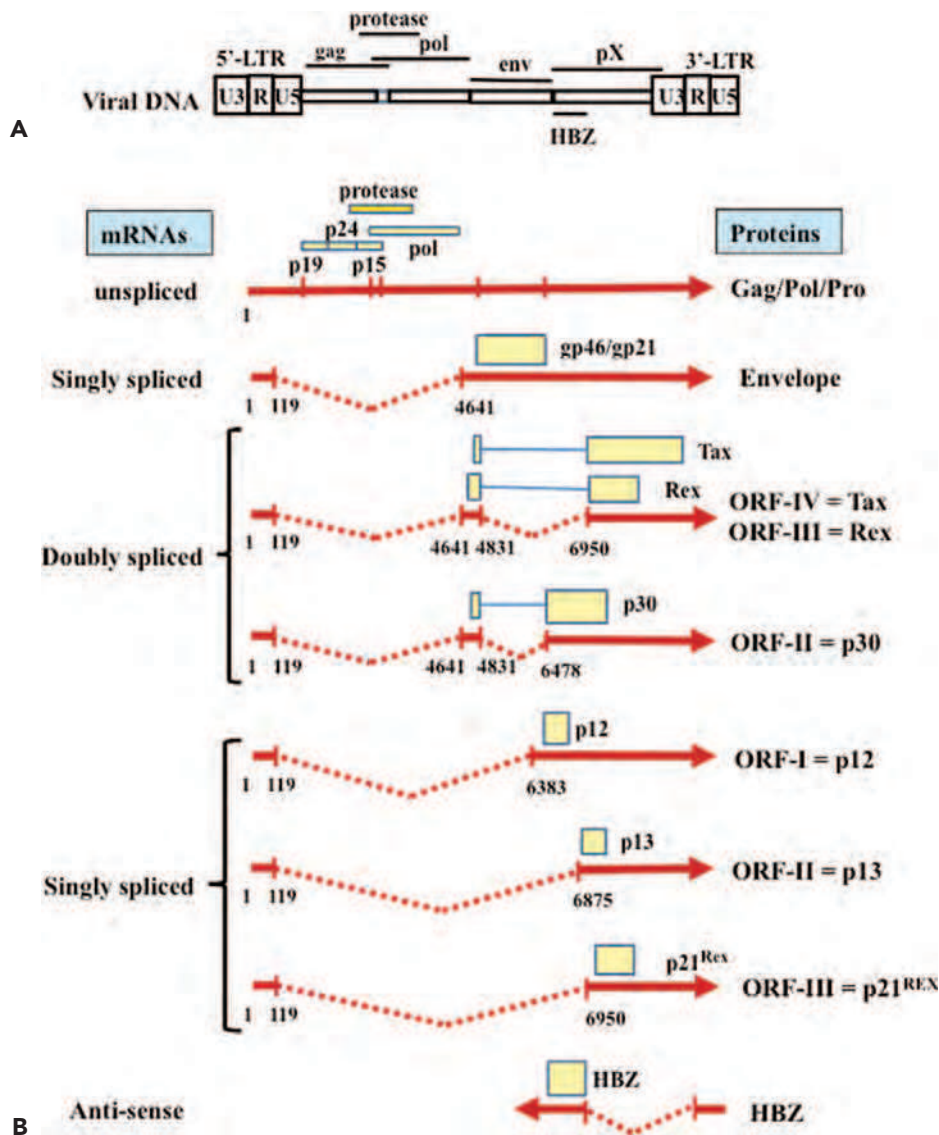


FIGURE 48.1. HTLV-1 genome organization, transcripts, and products.
A: Schematic organization of HTLV-1 proviral DNA. **B:** Structure, splicing, and orientation of HTLV-1 transcripts and their corresponding products.

Of these proteins, only HBZ is encoded by the minus strand of the HTLV-1 provirus.

HTLV-1 Gene Regulation

Overall HTLV-1 Messenger RNA Regulation

In addition to genomic unspliced messenger RNA (mRNA), HTLV-1 expresses multiple other mRNAs with distinctive splicings^{314,315} (Figs. 48.1 and 48.2). After infection of host cells, the initial dominant mRNA is the default doubly spliced *tax/rex* mRNA encoding the x-III and the x-IV open reading frame (ORF). Because the protein product Tax is a transcriptional activator of HTLV-1 transcription, Tax further amplifies the HTLV-1 transcripts, especially *tax/rex* mRNA, and their protein products. Once the other product, Rex, accumulates in a sufficient amount, it inhibits the nuclear export of *tax/rex* mRNA, instead increasing the export of singly spliced *env* mRNA and unspliced genomic RNA encoding *gag/pro-pol* (see Fig. 48.2), resulting in HTLV-1 release. Thus, Tax and Rex are essential for efficient HTLV-1 replication and production.

Tax-Dependent Transcriptional Activation

Tax is a protein of 353 amino acids, localized in both the nucleus and cytoplasm.¹⁰¹ Tax activates HTLV-1 transcription through the long terminal repeat (LTR)^{74,269,317,318,339} (see Fig. 48.2). The HTLV-1 LTR is divided into three regions: U3, R, and U5. Three repetitive 21-bp sequences in the U3 region act as Tax-responsive enhancers (TREs).³²³ Each 21-bp TRE is in turn composed of three elements—the core element and the elements 5' and the 3' to the core element—all of which are indispensable for Tax activation of HTLV-1 transcription. The core sequence of a TRE is highly similar to a cyclic adenosine monophosphate (cAMP) response element (CRE) and indeed acts as a binding site for the CRE binding protein (CREB)/activating transcription factor (ATF) family of transcription factors (CREB/ATF).^{29,421,429,430} Thus, a TRE is also referred to as a viral cAMP response element (vCRE) and is responsive to cAMP. The 5' and 3' elements within a TRE are GC-rich. Although Tax does not interact with TRE DNA by itself, it can do so when it interacts with DNA-bound CREB.^{429,430} When this happens, DNA-bound Tax interacts

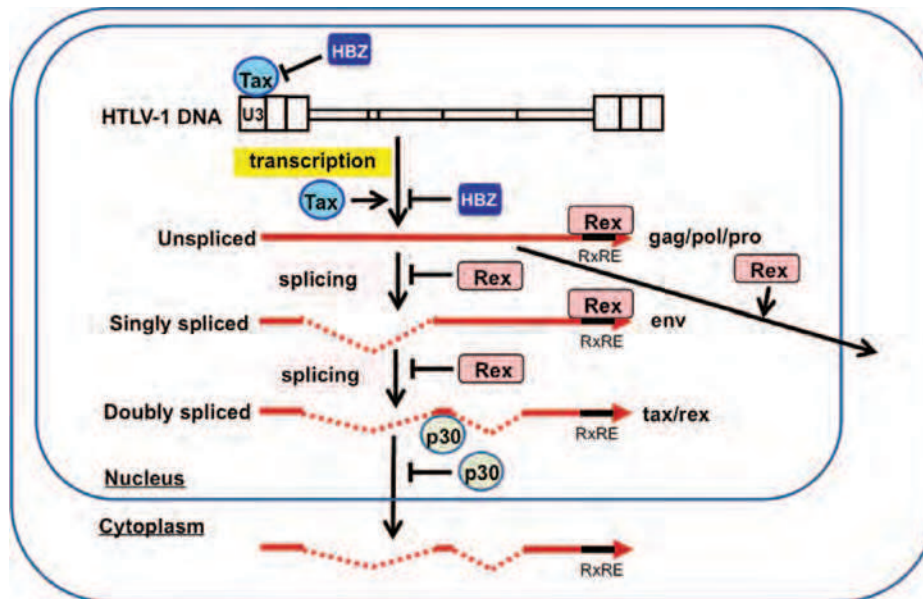


FIGURE 48.2. Overview flow chart of HTLV-1 gene regulation. HTLV-1 encodes three major transcripts: an unspliced genomic one, a singly spliced one, and a doubly spliced one, and these encode the Gag/Pro/Pol, Env, and Tax/Rex proteins, respectively. After initial infection, HTLV-1 dominantly expresses the default doubly spliced *tax/rex* transcript. Tax further augments viral transcription through the HTLV-1 long terminal repeat (LTR) promoter, and this activity is negatively regulated by HBZ. Upon accumulation of Rex, Rex inhibits the splicing of transcripts, thereby increasing the amounts of unspliced and singly spliced transcripts and their products (Gag, Pro, Pol, Env), resulting in HTLV-1 production. p30 inhibits the nuclear export of *tax/rex* transcripts to repress HTLV-1 gene expression. RxRE, Rex responsive element.

with the minor groove of the adjacent GC-rich sequences to stabilize the protein-DNA complex.^{214,215}

CREB is ubiquitously expressed and regulates several cellular genes, especially cAMP-induced genes.³⁹⁸ A cAMP-initiated signal phosphorylates CREB at serine 133, recruiting two transcriptional co-activators, CREB binding protein (CBP) and p300. An *in vitro* chromatin-based transcription study indicated that phosphorylation of CREB is essential for Tax activity through stabilizing the Tax/CREB/CBP/DNA complex. Moreover, Tax by itself induces the phosphorylation of CREB.¹⁹¹ Consistent with this observation is the fact that a drug that increases the intracellular cAMP level, and thus the phosphorylation of CREB, augments the Tax activation of HTLV-1 transcription.

CBP/p300 is a histone acetyltransferase that acetylates histone tails, thereby changing chromatin structures to activate transcription. *In vitro* and *in vivo* studies showed that p300 is crucial for Tax-dependent transcriptional activation, where p300, interacting with Tax and CREB, induces the acetylation of nucleosome histones over the TRE DNA.^{90,91,99,121,122,202,212,215,269} Interestingly, p300 recruitment by Tax to an integrated HTLV-1 promoter reduces the amount of histone H1 and H3 proteins on the promoter DNA, and stimulates the recruitment of RNA polymerase II.²¹¹ These results suggest that recruitment of CBP/p300 to the integrated promoter acetylates histone tails, thereby removing histone octamers from the HTLV-1 promoter. Histone chaperon NAP1 is a factor promoting Tax/CBP/p300-mediated histone eviction.³¹⁹

p300/CBP-associated factor (PCAF) is a co-activator for various cellular transcription factors, and it also augments Tax-induced transcriptional activation through the HTLV-1 promoter.¹⁶⁶ Like CBP/p300, PCAF is a histone acetyltransferase;

however, its activity is dispensable for co-activation of Tax. The well-known Tax mutant M47 (L319R, L320S), which is inactive for transcriptional activation of the HTLV-1 promoter but active for that of NF- κ B pathway, is defective in interacting with PCAF (Fig. 48.3).^{166,338}

Treatment of HTLV-1-infected T-cell lines with a histone deacetylase (HDAC) inhibitor (trichostatin A [TSA]) augments the expression of *tax/rex* mRNA as well as Tax protein, suggesting that HDAC(s) may inhibit HTLV-1 transcription in HTLV-1-infected cells.²²¹ Consistent with this hypothesis, HDAC1 and HDAC3 were found to inhibit Tax-induced transcriptional activation, and this inhibition was rescued by TSA treatment or expression of CBP.^{70,388} HDAC1 and HDAC3 were shown to interact with Tax, and chromatin immunoprecipitation analysis showed that HDAC1 was associated with integrated HTLV-1 promoter DNA. The amount of promoter-associated HDAC1 was reduced by expression of Tax, indicating that Tax, through interacting with HDAC1, dissociates HDAC1 from the integrated HTLV-1 promoter DNA.

TORCs and P-TEFb (CDK-9/cyclin T1) are additional positive regulators for Tax-dependent HTLV-1 promoter activation.^{53,54,167,197,336,434} Both proteins interact with Tax, and the knockdown of either by small interfering RNA (siRNA) reduces Tax-dependent activation. On the other hand, Bcl-3, by interacting with TORC3, acts as a negative transcriptional regulator of the HTLV-1 LTR.¹³⁷

Posttranscriptional Regulation of Viral RNA by Rex

HTLV-1 has two posttranscriptional regulators: Rex and p30.^{181,423} The roles of Rex and p30 in posttranscriptional mRNA regulations are described later.

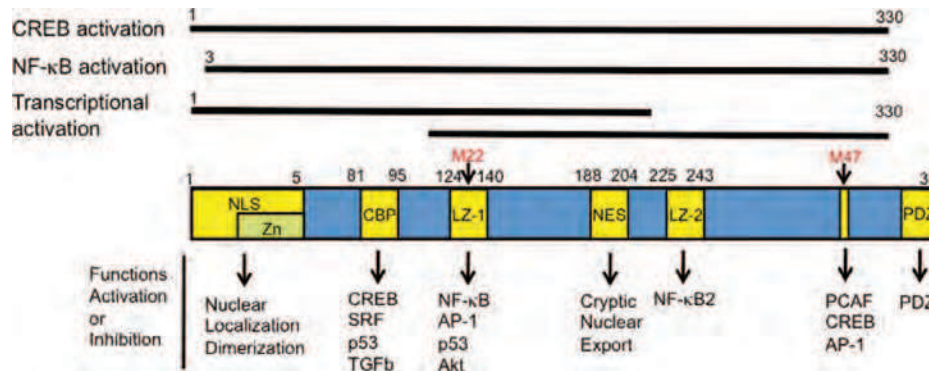


FIGURE 48.3. Functional domains of Tax. Regions of Tax required for activation of the cyclic adenosine monophosphate response element binding protein/activating transcription factor (CREB/ATF) pathway, activation of the NF- κ B pathway, and transcriptional activation function of Tax measured by a Tax fusion protein with the yeast DNA binding protein GAL4 are indicated. Positions of substitution mutations in the Tax mutants M22 and M47 are also indicated. M22 is active for the CREB pathway but inactive for the NF- κ B pathway, whereas M47 is the reverse. LZ-2 and PDZ motifs distinguish Tax1 from Tax2. NLS, nuclear localization signal; NES, cryptic nuclear export signal; Zn, zinc finger motif; LZ-1 and LZ-2, leucine zipper-like structures.

In addition to unspliced genomic mRNA encoding *gag/pro-pol*, HTLV-1 produces singly spliced *env* mRNA and doubly spliced *tax/rex* and *p30* mRNAs. RNAs with introns generally undergo splicing by the cellular RNA machinery; otherwise, they are degraded. Thus, upon initial infection of host cells, HTLV-1 dominantly expresses doubly spliced *tax/rex* and *p30* mRNA. Once the Rex protein accumulates, Rex controls the ratio of spliced forms of HTLV-1 mRNAs^{125,150} (see Fig. 48.2). Rex increases the amount of singly spliced (*env*) and unspliced (*gag/pro-pol*) mRNAs, and reduces the amount of its own doubly spliced mRNA. Rex does this by inhibiting the splicing of singly spliced (*env*) and unspliced (*gag/pro-pol*) mRNAs, stabilizing them, and promoting their transport to the cytoplasm. In the absence of Rex, unspliced HTLV-1 mRNAs are retained in the nucleus owing to two *cis*-acting repressive sequences (CRS) in the 5' and 3' LTR;^{193,316} however, Rex overcomes the inhibitory activity of the CRS and induces the translocation of the unspliced RNAs into the cytoplasm.

These Rex functions are achieved through at least three activities. The first one is sequence-specific RNA binding. Rex interacts specifically with the HTLV-1 RNA at the Rex-responsive element (RxRE) located in the U3 and R regions of the 3' LTR.^{3,40,378} RNA binding by Rex is accomplished by an arginine-rich highly basic region in Rex (aa1–19).¹¹⁴ This domain also contains a nuclear localization signal (NLS) and a nucleolus targeting signal, which allow Rex to enter the nucleolus where it can interact with the RNA.^{268,335} The RxRE RNA forms a long stem-loop structure, and one stem loop (called *stem-loop D*) is essential for Rex binding and the function.¹⁰⁶ The second Rex function (multimerization) is mediated through aa32–133.³⁹⁶ Rex mutants that cannot form multimers behave as dominant-negative proteins.^{38,395} The third Rex function is interaction with the nuclear export receptor CRM1/exportin 1, which mediates the transport of viral mRNAs from the nucleus to the cytoplasm. Interaction with CRM1 is also required for multimerization of Rex, because a Rex mutant defective for CRM1 interaction failed to form a multimer. Rex contains a typical leucine-rich nuclear export signal (NES) (aa81–94) that mediates its interaction with CRM1. HTLV-1

production is less efficient in rat cells than human cells because Rex is less active in rat cells than human cells. The rat CRM1 is impaired in promoting Rex to form multimers, which may explain the reduced activity of Rex in rat cells.^{111,426}

Rex is activated by phosphorylation.¹⁸⁷ Whereas phorbol myristate acetate (PMA), an activator of protein kinase C (PKC), transiently enhances phosphorylation of Rex, treatment of HTLV-1-infected cell lines with a PKC inhibitor reduces the amount of unspliced *gag-pol* mRNA.² Liquid chromatography tandem mass spectrometry analysis showed that Rex is phosphorylated at multiple sites, and mutation analysis indicated that phosphorylations at Ser-97 and Thr-174 are critical for its function.¹⁸⁷

Several other host factors can also positively or negatively regulate Rex activity. They include hnRNPA1, B-23, RanBP3, RIP/Rab, and eIF5A.^{1,39,110,182} hnRNPA1 binds to RxRE RNA, inhibiting the binding of Rex and thereby preventing Rex activation of the RxRE reporter.⁶⁵

Posttranscriptional Regulation of Viral RNA by p30

p30 is encoded by the doubly spliced mRNA of x-II ORF and is a protein of 241 amino acids.^{21,27} It is unusually rich in Ser (23%) and Arg (12%), and highly basic with theoretical isoelectric point of 11.71. p30 is localized in the nucleus and the nucleolus,^{26,97,98} and it interacts with the ribosomal protein RPL18a.⁹⁸

p30 inhibits the nuclear export of doubly spliced *tax/rex* mRNA through retaining the mRNA in the nucleus.²⁶² p30 binds to the splice junction region (*env* exon) of *tax/rex* mRNA. p30 has four NLS and is retained in the nucleus.⁹⁸ In addition, a fraction of p30 is localized in the nucleolus, although this localization is not essential for its activity. Studies of p30-green fluorescent protein fusion proteins identified two nucleolar retention signals, each consisting of a short Arg-rich stretch. Although p30 has a peptide with homology to an NES, heterokaryon assays indicate that p30 is not a shuttling protein.

Interplay Between Rex and p30

Although both Rex and p30 interact with HTLV-1 RNA and decrease the amount of *tax/rex* mRNA exported from the

nucleus, they generally have opposite functions in virus production. Whereas Rex stimulates virus production through augmenting the expression of structural proteins (Gag, Pol, Env), p30 has no direct effect on virus production and in fact inhibits it through reducing the expression of Tax and Rex. Interestingly, the Rex and p30 proteins interact with each other, and their interaction is augmented by the presence of the viral mRNAs.³³³ p30 has little effect on Rex activity; thus, Rex counteracts p30 activity and induces the expression of Tax/Rex proteins. Therefore, the ratio of these two proteins may be a factor controlling the transition between virus latency and virus production.

Interplay of p30 with HBZ

HBZ is another virally encoded gene (discussed later). Ectopic expression of HBZ RNA increases the expression of *tax* mRNA and Tax protein from HTLV-1–infected cells. Moreover, HBZ blocks the p30-mediated inhibition of Tax protein expression from *tax/rex* mRNA.⁵⁵ This is likely owing to an antisense mechanism, because p30 transcripts originate from a region overlapping with HBZ but in the opposite orientation.

Transcriptional and Posttranscriptional Regulation of Cellular Genes by p30

p30 can play roles in the HTLV-1 life cycle by regulating cellular genes.^{235,366,424,425} Microarray analysis using total and cytoplasmic mRNA of peripheral blood mononuclear cells (PBMCs) indicated that p30 up-regulates 15 cellular genes and down-regulates 65 at the transcriptional level. In addition, nuclear export assays indicated that p30 up-regulates 33 mRNAs and down-regulates 90 at a posttranscriptional level.³⁶⁶ Human T cells immortalized by a p30-defective HTLV-1 mutant were more sensitive to camptothecin (an anticancer drug)-induced apoptosis than those immortalized by wild-type virus, suggesting that p30 may influence sensitivity to apoptosis. In addition, p30 interacts with CBP/p300 and Tax.^{424,425} Through this interaction, p30 inhibits Tax as well as CREB-mediated transcriptional activation of the HTLV-1 LTR.

Phenotypes of Rex and p30 Knockout HTLV-1

Knockout studies indicate that Rex is essential for persistent HTLV-1 infection in rabbits but not required for immortalization of human T cells *in vitro*.⁴¹⁷ The defect in infection can be explained by the mRNA export regulatory function of Rex described earlier: Infection cannot be established if virus production is insufficient. On the other hand, studies of a p30-defective HTLV-1 (a stop codon at amino acid position 3 of p30 with an HBZ mutation of Ser-153 to Tyr) suggested that p30 is dispensable for both infectious virus production and immortalization of primary human T cells *in vitro* and persistent infection in rabbits.³³² Nevertheless, it is essential for efficient viral propagation in macaques.³⁸¹ Twelve weeks after infection of macaques, all of the p30-defective HTLV-1 had reverted to wild-type virus, indicating that p30 was crucial for maintaining HTLV-1 persistent infection. Interestingly, whereas this p30-knockout HTLV-1 produced the viral antigen equivalently to wild-type virus in a B-cell line, it does poorly in human dendritic cells (DCs),³⁸¹ suggesting that HTLV-1 infection of DCs is involved in persistent HTLV-1 infection in macaques and that p30 is necessary for this function. HTLV-1–infected DCs can transfer HTLV-1 to CD4+

T cells, and this transfer may play a crucial role in the maintenance of persistent HTLV-1 infection.

Latent HTLV-1 Infection *In Vivo*

Whereas HTLV-1–transformed T-cell lines *in vitro* express abundant amounts of HTLV-1 mRNA and proteins, fresh PBMCs of HTLV-1–infected individuals generally express only a polymerase chain reaction (PCR)-detectable level of viral mRNA and an undetectable level of viral proteins, indicating that HTLV-1 expression is suppressed in these cells. However, once such HTLV-1–infected PBMCs are cultured *in vitro*, gene expression is initiated within a few hours.

Reactivation of HTLV-1 Transcription

It should be noted that infected individuals have high titers of antibodies against HTLV-1 and high anti-HTLV-1 cytotoxic T-lymphocyte (CTL) activity. Thus, HTLV-1 replication must occur either transiently but repeatedly or constitutively in specific cells other than peripheral blood lymphocytes, which are latently infected. Indeed, there are several circumstances in which HTLV-1 is reactivated to produce viruses. First, several stress agents induce expression of HTLV-1. For instance, arsenic trioxide, an oxidative stress response inducer, enhanced virus production.¹¹ In addition, deprivation of interleukin 2 (IL-2) from IL-2–dependent HTLV-1–immortalized T cells induced expression of the HTLV-1 gag protein.³⁹¹ This result suggests that the levels of IL-2 may regulate HTLV-1 gene expression *in vivo*.

Tax

In addition to activation of viral transcription, Tax plays pivotal roles in HTLV-1 immortalization of T cells, persistent infection, inflammation, and pathogenesis. Such pleiotropic Tax functions are introduced in the following sections.

Tax Plays Crucial Roles in HTLV-1 Immortalization and Persistent Infection

Co-culture of lethally irradiated HTLV-1–infected T cells with PBMCs or cord blood mononuclear cells (CBMCs) in the presence of IL-2 for approximately 10 weeks establishes immortalized HTLV-1–infected T-cell lines (called *HTLV-1–immortalized cells*), and long-term culture of such cells occasionally confers IL-2–independent growth to some cells (called *HTLV-1–transformed cells*).^{241,409} It should be noted that such HTLV-1–immortalized/transformed cells do not produce IL-2. These results suggest that in infected individuals, HTLV-1 establishes lifelong persistent infection by immortalizing/transforming T cells, and these cells grow in an IL-2–dependent and/or an IL-2–independent manner.

Tax is indispensable for immortalization/transformation of human T cells as well as for persistent HTLV-1 infection in a host, because deletion or mutation of the *tax* gene in recombinant HTLV-1 abrogates immortalization as well as persistent infection in rabbits.³⁰⁰ Moreover, Tax by itself, without other viral products, immortalizes primary human and rat T cells in the presence of IL-2 *in vitro*.^{4,104,251} However, transduction of Tax alone into PBMC does not lead to IL-2–independent transformation of T cells, suggesting that HTLV-1 has another factor required for IL-2–independent transformation. The functions of Tax associated with immortalization of T cells and HTLV-1 pathogenesis is introduced in the following section.

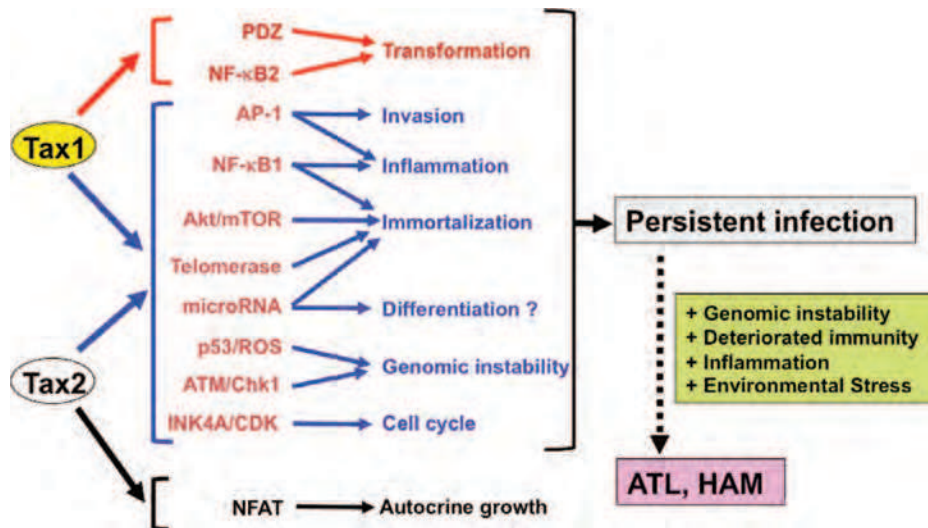


FIGURE 48.4. Functional similarities and differences between Tax1 and Tax2. The functions of Tax1 and Tax2 are classified into three groups: Tax1 specific (red), Tax2 specific (black), and common to both (blue).

ACTIVATION OF NF-κB BY TAX

Tax activates the transcription factor NF-κB, thereby inducing the expression of several cellular genes (Fig. 48.4), and this activity is crucial for multiple aspects of the HTLV-1 life cycle.^{22,41,168,216,231,301,328,342} For instance, recombinant HTLV-1 carrying a mutant Tax that cannot activate NF-κB fails to immortalize human T cells *in vitro*.³⁰⁰ Moreover, several NF-κB inhibitors induce prominent apoptosis in HTLV-1-infected T cells, indicating that NF-κB is crucial for survival of HTLV-1-infected T cells.

NF-κB is a family of transcription factors including NF-κB1 (p50), p65, c-Rel, NF-κB2 (p52), RelB, and Bcl-3, and these factors are divided into two groups belonging to the canonical (NF-κB1/p50, p65, c-Rel) and the noncanonical (NF-κB2/p52, RelB, Bcl-3) pathways. These two NF-κB pathways are both activated by Tax (see later) in T cells.⁴⁰³

To activate the canonical pathway, Tax interacts with several NF-κB regulators. IKKγ is essential for Tax activation of NF-κB, because the mutation of IKKγ in fibroblasts or T cells totally abrogates Tax activation of NF-κB.^{56,57,89,120,411} IKKγ is a scaffold component of the IκB kinase (IKK) complex, IKKα/IKKβ/IKKγ. Through interacting with IKKγ, Tax activates IKKβ to induce phosphorylation and degradation of IκBs (IκBα, IκBβ), thereby allowing nuclear translocation of p50/p65.^{56,89,102,223,344} In addition, Tax activates IKKα to phosphorylate p65 (at Ser-536), which stimulates its transcriptional activation function.²⁷⁰

Whereas NF-κB in primary T cells is activated transiently by various immune signals such as antigenic stimulation, it is constitutively active in HTLV-1-infected or Tax-expressing cells. Transient activation of NF-κB in normal cells is regulated by several negative feedback regulators. Tax interacts with one such negative regulator, Tax1BP1, via the adaptor optineurin,^{270,320} inducing constitutive NF-κB activation by Tax.

CBP/p300 appears to play a role in not only the CREB/ATF pathway but also in the NF-κB pathway. Tax and NF-κB/p65 form a ternary complex with CBP in the nucleus of HTLV-1-infected T cells, and this complex is associated with activation of the NF-κB pathway.³⁵⁰ In addition to p65, Tax has been shown to interact with other NF-κB subunits (p50, p52, c-Rel) as well as its inhibitors (IκBα, p105), although the

precise roles of these interactions in NF-κB activation by Tax have not yet been established.^{132,133,249,345} Tax also interacts with MEKK1, TAK1, TAK1-binding protein 2, and protein phosphatase 2A, which are also associated with activation of NF-κB by Tax.^{19,78,141,402,419}

Tax also activates the noncanonical NF-κB pathway.⁴⁰³ Tax1 simultaneously binds to the IKK complex (IKKγ, IKKα) and NF-κB2/p100, but not IKKβ, and thus induces IKKα-mediated phosphorylation of p100, its processing into p52, and the subsequent translocation of p52/RelB into the nucleus. The knockdown of NF-κB2/p100 by short hairpin RNA (shRNA) reduces Tax-induced IL-2-independent growth induction in CTLL-2 cells.¹²⁸ Thus, the noncanonical NF-κB pathway is also crucial for the transforming activity of Tax.

APOPTOSIS INHIBITION BY TAX

HTLV-1-infected T cells are resistant to various types of apoptosis, and this resistance is mostly mediated by Tax.^{184,248} Tax inhibits apoptosis of T cells induced by IL-2 withdrawal in an IL-2-dependent T-cell line (CTLL-2). Studies of Tax mutants (M47, M22) indicate that inhibition of apoptosis is mediated through the NF-κB pathway.¹⁵⁷ Tax activates the expression of several anti-apoptotic genes in T cells, including CTLL-2 cells through the NF-κB pathway. These genes include bcl-xl, xIAP, cIAP, and cFLIP.³⁷³ Tax also represses the expression of pro-apoptotic genes such as Bax.⁴³

ACTIVATION OF THE PI3K/AKT PATHWAY BY TAX

IL-2 activates PI3K and its downstream kinase, Akt, in normal T cells—events that are essential for apoptosis inhibition and cell growth. However, even in the absence of IL-2, HTLV-1-transformed T-cell lines show constitutive activation of the PI3K/Akt pathway. Moreover, several inhibitors of this pathway, such as LY294002 (an inhibitor for PI3K), induce growth arrest of HTLV-1-transformed T-cell lines and then induce apoptosis, suggesting that the ongoing activation of this pathway is essential for maintaining survival of HTLV-1-infected T cells.^{146,162,163} To do this, Tax directly interacts with PI3K, which consists of two subunits: the catalytic p110α subunit and the inhibitory p85α subunit. Tax directly binds to

the p85 α inhibitory subunit, causing the release of the active p110 α catalytic subunit.²⁸⁷

Activation of the PI3K/Akt pathway by Tax results in activation of the downstream target mammalian target of rapamycin (mTOR) in HTLV-1-infected T cells as well as Tax-expressing cells.⁴²² Rapamycin, an inhibitor of mTOR kinase, inhibits the growth of HTLV-1-transformed T cells and induces G1 cell cycle arrest.¹⁴⁶ This growth inhibition of HTLV-1-infected T cells is associated with reduced phosphorylations of p70S6 kinase and 4E-BP1.

G1-S PROMOTION BY TAX

In the absence of IL-2, ectopic expression of Tax in an IL-2-dependent human T-cell line (Kit225) or in PHA-activated PBMC induces cell cycle progression from G0/G1 into S phase.^{261,273,312} This activity is associated with the activation of cyclin-dependent kinase 2 (CDK2) and CDK4. CDK2 and CDK4 phosphorylate a family of retinoblastoma (Rb) proteins. The phosphorylation of Rb induces the release of the transcription factor E2F, freeing E2F to activate the expression of its target genes that control the cell cycle.

Tax has multiple distinct ways of stimulating cell cycle progression. First, Tax was found to activate CDK4 by interacting directly with p16INK4a, a CDK4 inhibitor.³⁴⁶ However, a subsequent study showed that Tax stimulates cell cycle progression even in a p16INK4a-defective T-cell line.²¹³ Indeed, Tax can also interact directly with CDK4 and its inhibitor p15INK4b, stimulating the kinase activity.^{113,220,347} Studies of Tax mutants (M22, M47) indicate that in IL-2-dependent Kit225 cells, E2F activation by Tax occurs via the NF- κ B pathway.²⁷³ E2F is a family of proteins (E2F1–E2F6). Whereas expression of E2F1 in a G0/G1-arrested fibroblast cell line induces cell cycle progression into S phase, it does not so in PHA-stimulated PBMC.²⁷³ Because Tax stimulates cell cycle progression in PHA-stimulated PBMC, in addition to E2F1 activation, Tax must have another activity that stimulates cell cycle progression in PBMC. Indeed, Tax induces the transcription of several genes associated with cell cycle regulation, including cyclin D1, cyclin D2, cyclin E, CDK2, CDK4, CDK6, and E2F1, and represses the transcription of the cell cycle inhibitor p18INK4c.^{5,155,273,305,347} In addition, knockdown experiments using short-hairpin RNA show that cyclin D2, CDK6, NF- κ B/p65, and NF- κ B2/p100 all play a role in E2F activation as well as cell cycle progression induced by Tax in Kit225 cells.¹⁵⁶ Collectively, these results suggest that Tax stimulates cell cycle progression through multiple mechanisms.

Genomic Instability in HTLV-1-Infected T Cells

HTLV-1 infected T-cells, including leukemic cells from ATL patients, have numerous genetic abnormalities such as DNA mutations, chromosomal translocations, deletions and duplications, and aneuploidy, and these genetic aberrations are linked to ATL development. For examples, homozygous deletions of p16 (CDKN2A) and p15 (CDKN2B) genes (tumor suppressor genes that regulate the cell cycle) were frequently observed in aggressive acute and lymphoma-type ATL.¹²⁴ The major viral factor that induces such genomic abnormalities is Tax, which acts via several distinct mechanisms as described later.

p53 INACTIVATION BY TAX

The tumor suppressor p53 is the “guardian of the genome” in DNA damage responses. Under normal conditions, p53 is

functionally inactive; however, upon the incidence of almost any stress, including DNA damage, p53 is accumulated, stabilized, and activated to gain competence in the transcriptional activation of genes that contribute to cell cycle arrest, apoptosis, and senescence. In HTLV-1-infected cells, such p53 functions are inactivated by Tax.^{6,248,295,412} Tax interacts with the p53 co-activator CBP/p300 and blocks its access to p53, thereby reducing the transcriptional activation function of p53.³⁴⁸ In addition, Tax induces the phosphorylation of p53 at Ser-15—a modification that triggers p53 to form an inactive ternary complex with Tax and NF- κ B/p65.^{164,295} Tax also inactivates two other p53-like proteins (p73 and p63/p51) in an HTLV-transformed T-cell line (MT-2).^{177,210} Ectopic expression of Tax in p53-negative Saos2 cells inhibits transcriptional activation of p73 and p63/p51 through the N-terminal domains. These results suggest that the inactivation of p53 and p53-related proteins is involved in the genomic instability of HTLV-1-infected cells.

ACTIVATION OF TELOMERASE BY TAX

Telomeres, the terminal sequences of chromosomes, play a crucial role in preventing chromosome abnormalities from occurring at each cell division; however, telomeres are shortened by every cell division in normal cells. In normal cells, p53-dependent replicative senescence is thus induced after a limited number of cell divisions. On the other hand, HTLV-1-immortalized cells have tricks to escape this telomere shortening, thereby avoiding replicative senescence. In HTLV-1-infected T cells, Tax activates the transcription of human telomerase reverse transcriptase (hTERT), an enzyme that synthesizes telomeric sequences and thereby extends telomeres.^{118,334} Studies using Tax mutants (G148V, M22) defective in NF- κ B activation and chromatin immunoprecipitation analysis showed that Tax induces increased binding of Sp1 and c-Myc to the hTERT promoter through the NF- κ B pathway.³³⁴

IL-2-dependent HTLV-1-immortalized T cells generally express a low amount of Tax—an amount of which may not be sufficient to maintain telomerase activity. However, IL-2 augments telomerase activity in HTLV-1-immortalized T cells. IL-2 signals inhibit the nuclear translocation of the Wilms tumor protein, an inhibitor of hTERT transcription, through a PI3K-dependent but Akt-independent pathway.³¹ The IL-2 dependence of telomerase activity in HTLV-1-infected cells suggests that continued growth of such cells in low IL-2 conditions may lead to shortened telomeres and chromosomal aberrations.

CENTROSOME AMPLIFICATION

The centrosome is a microtubule organizing center during cell division and plays a crucial role in the precise duplication and segregation of chromosomes. Whereas normal T cells have one centrosome during interphase, in HTLV-1-infected cell lines, around 30% of the cell population has more than two centrosomes, indicating that a centrosomal aberration may contribute to the chromosomal abnormalities observed in HTLV-1-infected cells.^{52,266} Such abnormal centrosomes are induced by the ectopic expression of Tax in human T cells.

This activity of Tax is mediated through its interaction with several host proteins such as TAX1BP2 and RanBP1.^{52,266} Endogenous TAX1BP2 is localized in the centrosome, and its knockdown induces centrosome amplification. Furthermore,

overexpression of TAX1BP2 reduces Tax-induced centrosome amplification. Collectively, these results indicate that Tax induces centrosome aberrations by inactivating TAX1BP2. In contrast, the knockdown of RanBP1 abrogates centrosome amplification by Tax as well as centrosome localization of Tax, indicating that RanBP1 targets Tax to centrosomes. MAD1 is another Tax-interacting protein localized in the centrosome during metaphase. Whereas the knockdown of MAD1 induces multinucleated cells, its overexpression inhibits induction of multinuclear cells by Tax.¹⁶⁹ Thus, MAD1 is also involved in the genomic instability induced by Tax in HTLV-1-infected cells.

DEFECTIVE DNA REPAIR

DNA repair activity is attenuated by Tax in HTLV-1-infected T-cell lines.^{160,179,227,238,290} Ataxia telangiectasia mutated (ATM), a member of the PI3K-like kinase family, is activated by various DNA damaging agents, and its activation is essential in initiating signaling cascades that stop DNA replication and allow the repair of damaged DNA. ATM activation by ionizing radiation (IR), and signals downstream of ATM activation, are diminished in HTLV-1-infected T-cell lines as well as cells expressing Tax alone.⁴⁹ Upon treatment with IR, both normal and Tax-expressing cells stop DNA replication; however, the interval before replication resumes is shorter in Tax-expressing cells than in the parental control cells; simultaneously, the cells expressing Tax are defective for DNA repair activity. Although the precise mechanism by which Tax blocks DNA repair has not yet been fully elucidated, Tax is known to interact with several host factors involved in DNA repair, such as DNA-dependent protein kinase, Ku, Chk1, and Chk2.^{68,284,285} In addition, Tax represses transcription of the DNA polymerase β gene, which plays a crucial role in DNA repair.¹⁶⁰

CELL CYCLE ARREST INDUCED BY TAX

Surprisingly, under certain experimental conditions, overexpression of Tax induces cell cycle arrest at G1 or G2-M as well as senescence. For instance, ectopic expression of Tax in HeLa cells or an IL-2-independent human T-cell line (supT1) induced G1 cell cycle arrest. Moreover, transient HTLV-1 infection of HeLa or SupT1 cells also induced G1 cell cycle arrest. These arrests are correlated with the Tax-dependent induction of CDK2 inhibitors (p21/waf1 and p27Kip1). In contrast, several established IL-2-independent HTLV-1-transformed T-cell lines express a high amount of p21 without cell cycle arrest, suggesting that during *in vitro* culture, HTLV-1-transformed cell lines may gain genetic change(s) that overcome cell cycle arrest. Based on these observations, it has been proposed that HTLV-1-infected cells *in vivo* are selected for their ability to escape such antigrowth effects of Tax, accumulating genetic alteration(s) during long-term HTLV-1 infection, and that this phenomenon may play a role in the numerous genetic alterations observed in HTLV-1-infected cells *in vivo*, such as chromosomal abnormalities. However, it should be noted that Tax also inactivates the growth-inhibitory functions of p21 through activating Akt: It has been reported that the activation of Akt in HTLV-1-transformed T-cell lines induces phosphorylation of p21, thereby inducing its translocation into the cytoplasm, where it is inactivated.³⁹²

REACTIVE OXYGEN SPECIES INDUCTION BY TAX

Ectopic expression of Tax in T cells stimulates the production of reactive oxygen species (ROS), which induce DNA

damage (detected by a comet assay and γ H2AX staining) and senescence (detected by β -galactosidase staining).¹⁹⁴ A ROS scavenger (N-acetylcysteine) abrogates DNA damage induced by Tax, indicating that ROS mediate the DNA damage induced by Tax. These results indicate that ROS may also contribute to the genomic abnormalities observed in Tax-expressing cells.

Inflammation Induced by Tax

INCREASED PRODUCTIONS OF CYTOKINES AND CHEMOKINES IN HTLV-1-INFECTED CELLS

One prominent feature of HTLV-1-infected T cells is the production of several cytokines and chemokines, as well as expression of their receptors. These gene inductions are mostly mediated by Tax through the NF- κ B, CREB, and AP-1 pathways^{13,20,190,250,389} and are thought to play pivotal roles in the phenotypes, activation, proliferation, differentiation, and behavior of HTLV-1-infected cells and thereby in HTLV-1 pathogenesis. For example, induction of the interleukin 2 receptor (IL-2R) complex by Tax is crucial for the immortalization of human T cells by HTLV-1.^{60,149,231} In addition, HTLV-1-infected T cells show prominent adhesion capacity, both homophilic and heterophilic, as well as cell migration activity, and they express several adhesion molecules, chemokines, and chemokine receptors involved in these functions, such as CD40, ICAM-1, OX40, and OX40L—many of which are induced by Tax in T cells.^{126,147,274,352,361} These properties would promote the resistance of HTLV-1-infected T cells to various pro-apoptotic stimuli, their migration to distant tissues through blood and lymphatic vessels, and their infiltration of tissues. Indeed, the CXCR4 antagonist AMD3100 inhibits the migration of cultured lymphoid cells from either Tax-transgenic mice or ATL patients.¹⁸³

ACTIVATION OF AP-1-DEPENDENT GENE EXPRESSION BY TAX

AP-1 activation by Tax is implicated in cell proliferation induced by Tax.²⁸⁷ The transcription factor AP-1 is transiently activated in normal T cells during antigenic stimulation, and it in turn activates several cellular genes involved in T-cell functions such as prevention of apoptosis, cytokine production, proliferation, and cell migration. In contrast, HTLV-1-infected T-cell lines have constitutive AP-1 DNA binding activity in the nucleus.⁷⁹ Such constitutive AP-1 activity is mediated by Tax. Through AP-1, Tax has been shown to activate many genes, including IL-5, IL-8, transforming growth factor beta (TGF- β), fra-1, proenkephalin, TR3/nur77, and TIMP-1.^{79,80,255,372}

AP-1 consists of protein dimers. The constituent proteins belong to two groups of transcription factors—the Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun (c-Jun, JunB, and JunD) families—and AP-1 consists of either homodimers of the Jun family or heterodimers between two groups. Among these factors, *c-fos*, *fra-1*, *c-jun*, and *junD* are all transcriptionally activated by Tax.^{79,80,255,372} Analysis of the *c-fos* promoter identified a CARG box as a Tax-inducible enhancer, where Tax interacts both with the CARG box binding protein serum response factor (SRF) and with ternary complex factor (TCF; Elk-1, SAP-1).^{81,327,343} In addition, two co-activators, CBP/p300 and P/CAF, are involved in Tax-dependent activation through the CARG box.³²⁷ The CARG box acts generally as an inducible enhancer for immediate early genes, such as *c-fos*, which are induced without *de novo* protein synthesis. Indeed, Tax, through its

action on the CARG box, induces the expression of various immediate early genes such as *c-fos*, *egr-1*, and *egr-2*.^{8,79,81}

INHIBITION OF TRANSFORMING GROWTH FACTOR BETA SIGNALING BY TAX

TGF- β is a cytokine that controls multiple cellular functions, such as cell growth inhibition, differentiation, senescence, and apoptosis, and acts as a tumor suppressor protein in solid tumors as well as leukemia/lymphoma. HTLV-1-infected T-cell lines are resistant to TGF- β -induced growth inhibition, and Tax is responsible for this resistance.^{16,208,244} TGF- β signaling is mediated through a family of transcription factors referred to as Smads. Tax, through JNK activation, induces phosphorylation of c-Jun, and phosphorylated c-Jun forms an inactive complex with Smad3.¹⁶ In addition, Tax blocks the interaction of Smads with the transcriptional co-activator p300.²⁴⁴

Deregulated microRNA in HTLV-1-Infected Cells

Although cellular microRNAs (miRNAs) are a crucial player in the host defense mechanism for clearing viral infections, many viruses have functions that counteract the antiviral activities of miRNAs. In some cases, viruses even utilize miRNAs to maintain persistent infection.³⁰² In HTLV-1-infected cells, several miRNAs are up-regulated or down-regulated relative to those in uninfected T cells.³⁰² Whereas up-regulated cellular miRNAs in HTLV-1-infected cells include miR-21, -24, -93, -130b, -146, -155, and -142-3p, down-regulated ones include miR-125a, -132, -181a, -150, and -223.^{30,291} Two miRNAs up-regulated in HTLV-1-infected T cells (miR-93 and miR-130b) target the same gene, TP53INP1, which is a p53-inducible tumor suppressor gene.⁴¹⁸ Knockdown of miR-93 and miR-130b by antagomirs in either an HTLV-1-transformed T-cell line (MT-4) or the Jurkat T-cell line increases the expression of TP53INP1 and reduces cell viability. Moreover, Tax stimulates the transcription of miR-130b. Collectively, these results suggest that Tax promotes the growth of HTLV-1-infected cells through miR-130b and TP53INP1, and that the up-regulation of certain miRNAs plays a role in the proliferation of HTLV-1-infected T cells, and thereby persistent infection and leukemogenesis.

Several miRNAs deregulated in HTLV-1-infected T cells and uncultured leukemic cells of ATL patients (miR-181a, -142, -223, and -150) are involved in hematopoiesis.³⁰ For instance, miR-181a, a gene down-regulated in HTLV-1-infected cells, is preferentially expressed in the B-lymphoid lineage in mice. Moreover, its ectopic expression in hematopoietic progenitor cells followed by *in vitro* differentiation increases the yield of the B-lymphoid lineage cells but not T-lymphoid ones. Two miRNAs (miR-150 and miR-223) are overexpressed in the PBMC of ATL patients but down-regulated in HTLV-1-transformed T-cell lines, suggesting that ATL cells and *in vitro* HTLV-1-transformed T-cell lines may originate from distinct populations. This observation may support the cancer stem cell theory for ATL development discussed later.

Distinct Activities of Tax1 Versus Tax2

HTLV-1 Tax (Tax1) and HTLV-2 Tax2 have around 80% similarity at the amino acid level and carry out many activities with equal potency. However, there are two important distinctions between the two proteins: Tax1 is associated with greater trans-

forming activity, whereas Tax2 induces IL-2 production more strongly.

THE HIGH TRANSFORMING ACTIVITY OF TAX1 RELATIVE TO TAX2

CTLL-2 is a mouse cytotoxic T-cell line, the growth of which is strictly dependent on IL-2. The lentivirus-mediated transduction of Tax1 into CTLL-2 transforms these cells, converting them from IL-2-dependent growth to IL-2-independent growth, and surprisingly, the transforming activity of Tax1 is much more potent than that of Tax2.^{128,371} These results present a simple but attractive hypothesis of the HTLV-1 pathogenesis presented later.¹²⁷ Whereas HTLV-2-infected T cells could preferentially grow and survive in conditions and/or tissues with relatively high amounts of IL-2, under low IL-2 conditions, HTLV-1-infected T cells could grow more efficiently than HTLV-2-infected cells. This distinctive characteristic of HTLV-1-infected cells would allow more clonal proliferation of infected cells *in vivo*, which is a prerequisite for ATL development. This observation may explain why HTLV-1, but not HTLV-2, is associated with ATL.

What makes Tax1 more potent than Tax2? The CTLL-2 assay identified at least two Tax1-specific functions responsible for its high transforming activity relative to Tax2: One is its interaction with selective PDZ domain proteins, and the other is its activation of the noncanonical NF- κ B2 pathway.^{128,371}

Tax1 Interaction with PDZ Domain Proteins. As mentioned earlier, studies of Tax1-Tax2 chimeras identified a PDZ domain binding motif (PBM) at the C-terminus of Tax1 (missing in Tax2) that is responsible for Tax1's high transforming activity in CTLL-2 cells. The significance of the Tax1 PBM in HTLV-1 was also indicated by the observation that deletion of the PBM from HTLV-1 (HTLV-1/ Δ PBM) abrogates its ability to persistently infect rabbits.⁴⁰⁵ Whereas the PBM is crucial for IL-2-independent growth of CTLL-2 cells, the PBM is dispensable for IL-2-dependent immortalization of primary human T cells *in vitro*, supporting the idea that the Tax1 PBM plays a role at low IL-2 conditions but not at high IL-2 conditions.

PBMs are generally located at the carboxy-terminus of cellular and viral proteins, and mediate binding with cellular PDZ (PSD-95/Discs Large/ZO-1) domain proteins. Intriguingly, PBM motifs in transforming viruses are selectively present in oncogenic viruses but not their nononcogenic relatives. For instance, human papillomaviruses (HPVs) have high-risk subtypes such as HPV16 and -18 that are associated with HPV-associated malignancies such as cervical carcinoma and low-risk subtypes that are associated only with benign hyperplastic diseases. The E6 oncoproteins of high-risk HPVs, but not low-risk ones, possess PBMs. A similar association of a PBM with a viral subtype-specific oncogenesis is also seen for human adenovirus type 9, which causes mammary tumors in rats. Interestingly, the PBMs from the oncogenic HPV E6 and the adenovirus E4-ORF1 can substitute for the Tax1 PBM in allowing Tax1 to transform CTLL-2 cells. This observation suggests that the functions of these viral PBMs and the PDZ domain proteins with which they interact are identical, and that these three oncogenic viruses utilize a common mechanism to induce malignancies. PDZ domain proteins that interact with Tax1 include Dlg1, Scribble, MAGI-3, TIP-1, and IL-16 precursor protein.^{17,271,275,399} Knockdown of Dlg1 increases the

transforming activity of Tax1 in CTLL-2 cells, suggesting that Dlg1 negatively regulates the transforming activity of Tax1.

Activation of NF- κ B2 by Tax1. Unlike Tax1, Tax2 does not activate the noncanonical NF- κ B pathway. The major defect of Tax2 here is that Tax2 cannot interact with NF- κ B2/p100.¹²⁸ Knockdown of NF- κ B2/p100 prominently reduces the transforming activity of Tax1 in CTLL-2 cells, indicating that Tax1-specific activation of the noncanonical NF- κ B pathway is crucial for the transforming activity of Tax1. In addition, transduction of p100 siRNA into large granular lymphoma cell lines derived from Tax1-transgenic mice augmented their sensitivities to pro-apoptotic stimuli and simultaneously reduced the expression of anti-apoptotic XIAP and cIAP. The leucine zipper-like region 2 (LZ-2) of Tax1 (aa 225–232) is a crucial region for Tax1-induced NF- κ B2 activation (see Fig. 48.3).³²⁶

ACTIVATION OF THE NUCLEAR FACTOR OF ACTIVATED T-CELL PATHWAY BY TAX2

Whereas Tax1 has several dominant functions over Tax2, Tax2 has one dominant function over Tax1. Ectopic expression of Tax2 in a human T-cell line (Jurkat) induces the expression of IL-2, and the activity of Tax2 is much more potent than that of Tax1 in this respect.²⁶⁴ Similarly, HTLV-2–infected T-cell lines, but not HTLV-1–infected ones, constitutively produce IL-2 protein in the culture supernatant. Importantly, anti-IL-2R antibodies inhibit the proliferation of HTLV-2–infected T-cell lines, suggesting that the IL-2/IL-2R autocrine loop is essential for this proliferation. IL-2 gene induction by Tax2 in HTLV-2–immortalized T cells is mediated through the nuclear factor of activated T cells (NFATs), especially the NFATp component. In normal T cells, NFAT is retained in the cytoplasm in an inactive, phosphorylated form, and its dephosphorylation (by the phosphatase calcineurin) induces its translocation into the nucleus and allows it to activate transcription of its target genes. Cyclosporine A, an inhibitor of calcineurin, abrogates IL-2 expression induced by Tax2, indicating that Tax2 activates IL-2 production by targeting calcineurin or elements upstream of it.

Tax3 and Tax4

HTLV-3 and HTLV-4 encode Tax3 and Tax4, respectively. Amino acid comparison of these Tax proteins indicates that Tax3, but not Tax4, has a PBM, and Tax3 has been shown to bind to the PDZ domain protein Dlg4.⁵¹ On the other hand, in the LZ-2 region (essential for NF- κ B2 activation by Tax1), Tax3 and Tax4 show more homology to Tax2 than to Tax1 (see Fig. 48.3). Therefore, these four HTLVs are classified into at least three distinct groups based on the PBM and the LZ-2 region.

In Vitro Immortalizing and Transforming Activities of Tax1

Tax immortalizes primary human or rat T cells in the presence of IL-2. Analysis of Tax mutants (M22, M47) indicates that the NF- κ B pathway is crucial for immortalization of T cells. When *tax* gene is transduced into PBMCs by a retroviral vector, Tax preferentially immortalizes CD4+ T cells. Interestingly, a Tax mutant defective in activating the CREB pathway (M47) preferentially immortalizes CD8+ cells but not CD4+ cells. These results have been confirmed by studies using whole virus: an HTLV-1 mutant carrying the same Tax mutation

(M47, M22). These results suggest that the NF- κ B pathway plays a major role in immortalization of T cells by Tax, whereas the CREB pathway is involved in the preferential immortalization of CD4+ cells.³⁰⁰

Tax Activities in Animal Models

Tax-transgenic animals (mice or rats) develop various diseases such as CD4+ T-cell leukemia/lymphoma, natural killer (NK) cell lymphoma, thymoma, and neurofibroma.^{107,123,260,272} For instance, mice transgenic for *tax* regulated by the mouse lymphocyte-specific tyrosine kinase (Lck) proximal or distal promoter both developed T-cell leukemia/lymphoma: the former, a pre-T-cell lymphoma/leukemia with a double negative (CD4–CD8–) phenotype, and the latter, a mature single-positive (CD4+ or CD8+) T-cell leukemia/lymphoma.^{123,272} Intriguingly, cancer stem cells (CSCs) were identified from the Lck (proximal)-tax-transgenic mice in a minor population of CD38–/CD71–/CD117+ cells by inoculating the splenic lymphomatous cells into immune-deficient (NOD/SCID) mice.⁴¹³ Such CSCs originated from pro-T cells or early hematopoietic progenitor cells. Inoculation of HTLV-1–infected or Tax1-transduced human CD34+ hematopoietic stem/progenitor cells (HPS/PC) into humanized SCID mice results in the development of CD4+ T-cell lymphoma.²³ These results suggest that HTLV-1 infection of CD34+ HPS/PC may play a role in ATL development.

NF- κ B activation plays a crucial role in the oncogenic activity of Tax in animals. For instance, the Tax-transgenic mice developing NK cell lymphoma show constitutive activation of NF- κ B, and the inhibition of NF- κ B by anti-NF- κ B therapeutics perturbs the progression of the disease.²³⁶

In addition to malignancies, Tax transgenic mice develop chronic inflammatory diseases such as rheumatoid arthritis, dermatitis, and exocrinopathy resembling Sjögren syndrome.^{105,153} These diseases are more frequent in HTLV-1–infected individuals than HTLV-1–negative ones. Autoimmunity through molecular mimicry of HTLV-1 proteins to host proteins is proposed to be a mechanism in the development of HTLV-1–associated chronic inflammatory diseases including HAM/TSP. HAM/TSP patients possessed antibody to hnRNP-A1 expressed in neurons, which is cross-reactive to Tax, and this antibody is associated with impaired neuronal function.²¹⁸ Intriguingly, autoimmunity against various host factors such as type II collagen is detected in Tax transgenic mice, and this activity is correlated with the development of these chronic inflammatory diseases.¹⁵² Cell surface Fas and its ligand FasL play crucial roles in the elimination of autoreactive T cells through FasL-induced apoptosis. Peripheral T cells derived from Tax-transgenic mice are more resistant to FasL-mediated apoptosis than those from nontransgenic mice. Thus, Tax, through inhibiting the elimination of autoreactive T cells by the Fas/FasL system, may play a role in inducing autoimmunity in these mice and in the development of HTLV-1–associated inflammatory diseases.¹⁹⁵ However, these transgenic mice that developed arthritis with resistance to Fas/FasL signal express both Tax and HBZ (see later discussion). It remains obscure whether this phenotype is actually caused by Tax, alternatively either by HBZ or the combination.

Tax-transgenic mice regulated by a conditional lymphocyte-specific Tet-off-*EuSR α* promoter developed lethal inflammatory

skin diseases of progressive alopecia and hyperkeratosis together with systemic lymphadenopathy and splenomegaly.²⁰³ Skin manifestations such as inflammatory dermatitis are commonly observed in HTLV-1-associated diseases; thus, these results suggest that Tax plays a role in inflammatory skin disease in HTLV-1 infection. Studies of mice carrying mutant *tax* genes (M47, M22) indicate that the NF- κ B pathway is sufficient for inducing all manifestation in these mice, whereas the CREB pathway is dispensable. Consistent with this observation, these mice highly express a number of genes associated with inflammation (such as numerous cytokines and chemokines), which are regulated by the NF- κ B pathway.

Posttranslational Modification and Stability of Tax

Tax is modified by ubiquitination, phosphorylation, and sumoylation, and these modifications regulate the stability, activities, and subcellular localization of Tax.

UBIQUITINATION

Tax has 10 lysine (K) residues, referred to as K1 through K10, throughout the protein. K4 through K10 are the main targets of both monoubiquitination and polyubiquitination.^{205,259,286} Ubiquitinated Tax is localized both in the cytoplasm and in the nucleus; however, the amount in the cytoplasm is greater than that in the nucleus. Ultraviolet radiation of cells induces massive monoubiquitination of Tax, and under these conditions, Tax is exclusively localized in the cytoplasm.⁸⁷ Tax (K4–K8) is involved in the interaction with IKKs, because mutation of these residues in a K(4-8)-R (Arg) Tax mutant abrogates the interaction with IKKs as well as translocation of NF- κ B/p65 into the nucleus. K7 and K8 are especially crucial for Tax activation of the NF- κ B pathway but not the CREB/ATF pathway.

SUMOYLATION

Tax is sumoylated by all three isoforms of SUMOs (SUMO-1, SUMO-2, SUMO-3).^{205,259} The molecular weights of Tax conjugated with HA-tagged SUMOs indicate that Tax is conjugated with one to three SUMO monomers. An NF- κ B-activation-defective Tax mutant (M22) is not sumoylated, suggesting that sumoylation of Tax plays a positive role in NF- κ B activation. Analysis of lysine mutations indicates that K7 and K8 of Tax are the major sites of sumoylation. Sumoylated Tax conjugated with HA-tagged SUMO-3 was predominantly detected in the nuclear bodies of 293T cells, whereas ubiquitinated Tax is localized diffusely in the nucleus as well as the cytoplasm—an observation suggesting that Tax sumoylation may control the nuclear localization of Tax.

PHOSPHORYLATION

Tax is a constitutively phosphorylated protein, and its phosphorylation is augmented by treatment of HTLV-1-infected T cells with PMA, an activator of PKC.⁷⁶ Tax is phosphorylated at multiple sites such as Ser-10, Ser-77, Thr-48, Thr-184, Thr-215, Ser-274, Ser-300, Ser-301, and Ser-336.⁶⁹ Of these, mutation of Thr-215 or double mutation of Ser-300 and Ser-301 abrogates the activation of both the CREB and NF- κ B pathway, whereas mutation of Thr-48 blocks activation of the NF- κ B pathway but not of CREB. Phosphorylations of Ser-300 and Ser-301 are also involved in the localization of Tax in nuclear bodies.³²

PROTEIN STABILITY

Pin1 is a peptidyl-prolyl *cis-trans* isomerase that regulates the function and stability of several proteins. Tax induces the expression of Pin1 in HTLV-1-infected T-cell lines and moreover interacts with Pin1.²⁸⁸ Pin1 interacts selectively with phosphorylated Tax at Ser-160 at the mitotic cell phase.¹⁶⁵ Modification by Pin1 results in the increased stability of Tax, thereby augmenting transcriptional activation through the NF- κ B and CREB pathways. Consistent with this observation is the finding that overexpression of Pin1 increases the transforming activities of Tax to CTLL-2 as well as NIH-3T3 cells.

p12/p8

p12 is encoded by the singly spliced mRNA of HTLV-1 ORF1.^{58,200,263} p12 is a highly hydrophobic membrane protein of 99 amino acids, and it is localized in the endoplasmic reticulum (ER) and Golgi complex.^{64,199,384} p12 has a noncanonical ER retention/retrieval motif, two putative transmembrane (TM) domains, four putative proline-rich (PXXP) Src-homology 3 (SH3)-binding domains, two putative LZ motifs, and a putative adaptin motif.^{77,83,263} (Fig. 48.5). When ectopically expressed in HeLa cells, p12 forms a homodimer through its TM domains.³⁷⁰

A p12-deficient HTLV-1 mutant (p12 mutation of Met-1 to Leu and a HBZ mutation of His-151 to Gln) had reduced infectivity of DCs, and when rhesus macaques were infected with it, viral propagation and persistent infection were poorly established.^{365,381} On the other hand, this mutant HTLV-1 produced infectious viruses comparable to wild-type HTLV-1, immortalized primary human T cells *in vitro*, and established persistent infection in rabbits. Thus, DC infection with HTLV-1 appears to be critical for persistent infection in macaques, and p12 may play a role in this process.

Effect of p12 on T-Cell Activation and Proliferation

p12 activates Ca²⁺ signaling in T cells.^{63,64} Ectopic overexpression of p12 in a T-cell line increases intracellular Ca²⁺ levels, stimulating dephosphatase calcineurin to dephosphorylate NFAT and then NFAT to activate transcription of genes such as IL-2.^{7,189} p12 increases intracellular Ca²⁺ concentration by interacting with two ER resident proteins—calnexin and calreticulin—that regulate Ca²⁺ release from the ER.⁶⁴ In addition, p12 interacts directly with calcineurin. Because HTLV-1-transformed T-cell lines generally do not exhibit constitutive NFAT binding to the IL-2 promoter,²⁶⁴ NFAT appears to be only transiently activated by p12 at a particular phase of HTLV-1 infection, such as an initial infection.

p12 in T cells can stimulate the IL-2 signaling pathway downstream of the IL-2R. p12 interacts with the beta and gamma-c chain of the IL-2R and enhances the phosphorylation of STAT5 and its DNA binding, thereby augmenting transcription.²⁴⁷ Expression of p12 in a human T-cell line transformed by a p12-defective HTLV-1 mutant augments colony formation in soft agar in low IL-2 conditions.

p12-Induced Attenuation of the Host Immune Response

p12 is implicated in reduced host immunity to HTLV-1-infected cells. HTLV-1-infected primary CD4⁺ T cells are resistant to autologous NK cell-mediated killing.³⁸⁵ Such killing is initiated by binding of NK to target cells via adhesion

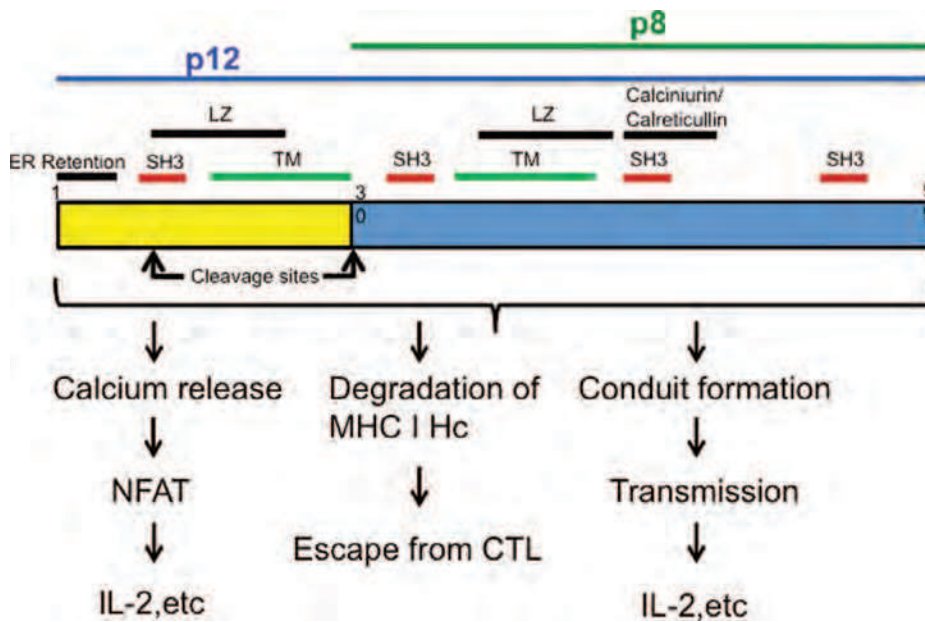


FIGURE 48.5. Schematic structure, functional domains, and functions of p12/p8. A: The protease cleavage sites are indicated. The known functions of p12/p8 are summarized. **B:** p8 induces cellular conduit formation, which is involved in HTLV-1 transmission. TM, putative transmembrane domain; LZ, leucine zipper-like structure; SH3, Src-homology 3; ER retention, endoplasmic reticulum retention signal.

molecules ICAMs. HTLV-1 infection reduces the expression of ICAM-1 and ICAM-2 in primary CD4⁺ T cells, thereby reducing NK cell binding. Lentivirus-mediated expression of p12 in primary CD4⁺ T cells down-modulates expression of ICAM-1 and ICAM-2. In addition, p12 can reduce CTL-mediated killing.¹⁷² p12, through interaction with the major histocompatibility complex (MHC) class I heavy chain, inhibits its interaction with β 2-microglobulin, thereby inducing the proteasome-dependent degradation of MHC class I.

Functions of p8

Proteolytic cleavage of p12 generates the C-terminal product p8, which may mediate HTLV-1 transmission.³⁸⁵ The cleavage of p12 removes the ER retention/retrieval motifs, and thus p8 is localized to the T-cell membrane (see Fig. 48.5). Upon T-cell receptor activation, p8 is recruited into the immunological synapse. Comparison of p8-positive and -negative HTLV-1 indicates that p8 increases cell-to-cell contact and induces lymphocyte function-associated antigen-1 (LFA-1) mediated cell clustering, augmenting the number and length of conduits (filopodia-like membrane extensions), and thereby enhancing HTLV-1 transmission.

p13

p13 is encoded by the singly spliced monocistronic mRNA of the HTLV-1 x-II ORF.^{329,331} p13 is a highly basic protein of 87 amino acids and is identical to the C-terminal 87 amino acids of p30. p13 has the following domains: an amphipathic alpha helix (residues 20–35), a mitochondrial targeting signal (MTS) (residues 21–30), a TM region (residues 30–40), and several PXXP motifs (Fig. 48.6).

A p13-defective HTLV-1 virus (with a mutation of the p13 initiation codon) showed comparable infectivity of rabbit PBMC to that of wild-type virus; however, the mutant virus could not establish infection in rabbits *in vivo*, as measured by viral loads and antibody responses, indicating that p13 plays a still-unknown role in establishing HTLV-1 infection *in vivo*.¹³⁴

p13 is localized mostly in the inner mitochondrial membrane, and its ectopic expression in isolated mitochondria induces mitochondrial swelling, depolarization, increased respiratory chain activity, and production of ROS, through activation of the inward K⁺ current.^{33,330,331} p13 also induces ROS production in primary quiescent T cells.

HBZ

The viral genes that are transcribed from the 5' LTR are encoded by the plus strand of the provirus (sense transcript). However, an antisense transcript encoded by the minus strand has also been identified in HTLV-1.^{45,88,207,252,308} The antisense transcript, originating from the 3' LTR, encodes the *HTLV-1 bZIP factor* (HBZ) gene.

Transcription of the HBZ Gene

Two major forms of the HBZ RNA have been reported: a spliced form (*sHBZ*) and an unspliced form (*usHBZ*). The first

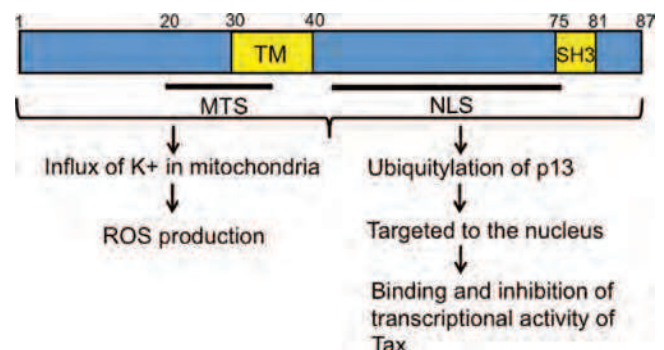


FIGURE 48.6. Schematic structure, functional domains, and functions of p13. The functions of p13 are summarized. TM, putative transmembrane domain; MTS, mitochondrial targeting signal; SH3, Src-homology 3; NLS, nuclear localization signal; LZ, leucine zipper-like structure.

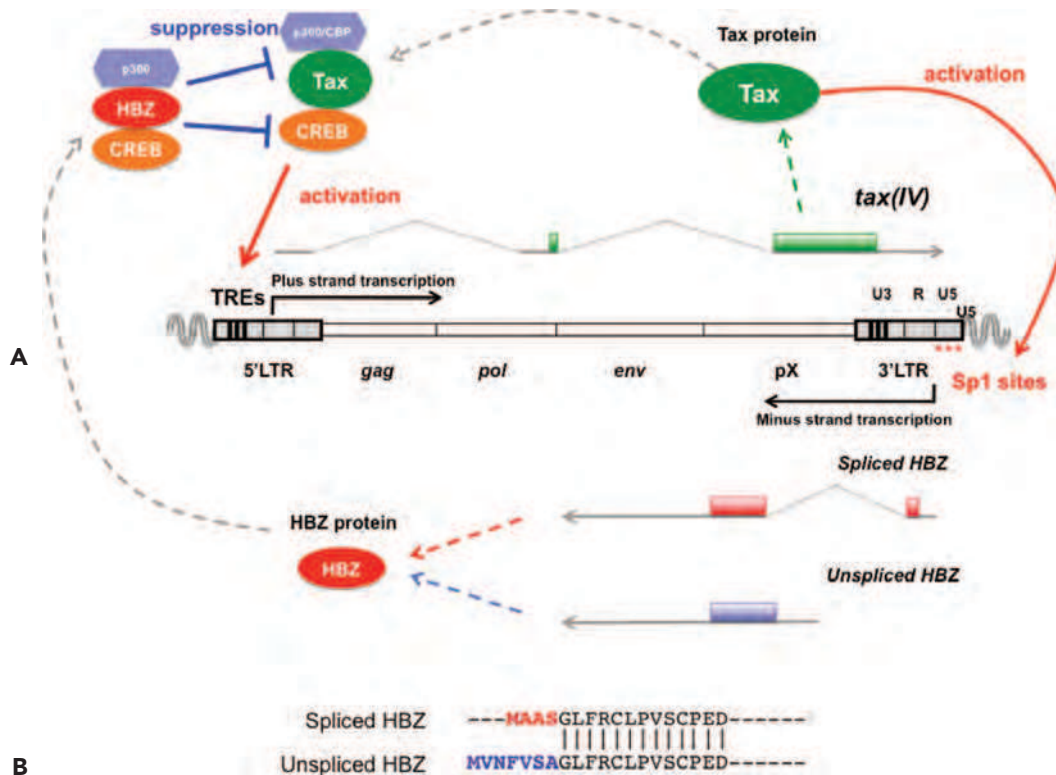


FIGURE 48.7. Transcription of the *HBZ* gene. There are two isoforms of the *HBZ* gene transcripts: spliced and unspliced forms. Transcription of the *HBZ* gene depends on Sp1, whereas the transcription from 5' LTR is highly inducible by Tax.

exon of the *sHBZ* gene transcript is located in the R region of the 3' LTR (Fig. 48.7A). Transcriptional start sites for *sHBZ* are scattered in the U5 and R regions of the 3' LTR, an observation that is consistent with the finding that the predicted promoter was TATA-less.⁴²⁰ Three Sp1 binding sites are critical for transcription of the *HBZ* gene. Because Sp1 is a well-known regulator of housekeeping genes, transcription of the *sHBZ* gene may be constitutive and relatively constant. As described later, levels of *HBZ* gene transcript are better correlated with provirus load than those of the *tax* gene transcript,³⁰³ confirming that the *HBZ* gene is constitutively expressed in HTLV-1-infected cells. Thus, transcription from the minus strand stands in contrast to that from the plus strand, which is highly inducible by Tax.

The difference between *sHBZ* and *usHBZ* is only a few amino acids, as shown in Figure 48.7B. The spliced transcript of *HBZ* is translated into a polypeptide of 206 amino acids, whereas the protein product of unspliced *HBZ* is a polypeptide of 209 amino acids. The expression level of *sHBZ* RNA is much higher than that of *usHBZ* RNA,³⁷⁹ and the half-life of *sHBZ* protein is much longer than that of *usHBZ*.⁴²⁰ Taken together, these data indicate that expression levels of *sHBZ* are much higher than those of *usHBZ*, suggesting that *sHBZ* is more important than *usHBZ* for HTLV-1-infected cells and ATL cells. TREs in the U3 region act as enhancers for sense transcription of viral genes by interaction with Tax. Similarly, Tax activates transcription of the *HBZ* gene through binding to TREs.^{206,420}

Functions of HBZ

HBZ protein is localized in the nucleus with a speckled pattern.¹³⁹ HBZ has three domains: the activation, central, and bZIP domains (Fig. 48.8). HBZ was originally reported to suppress Tax-mediated viral gene transcription from the 5' LTR via interaction with CREB2 and c-Jun.^{25,88,232} Further, HBZ interacts with various host factors with bZIP domain, including CREB, JunB, ATF-1, and ATF-3, and hinders their transcriptional activation.^{25,108,138,209} Conversely, interaction of HBZ with JunD activates transcription of target genes.³⁶⁷ Furthermore, HBZ enhances the TGF- β /Smad pathway by interaction with Smad2/3 and p300.⁴³¹ Importantly, HBZ does not interfere with expression of TGF- β /Smad target genes associated with cell cycle and proliferation. HBZ also selectively inhibits the classical NF- κ B pathway by inhibiting DNA binding of p65 and promoting the degradation of p65.⁴³²

Leukemic cells in two-thirds of ATL cases express FoxP3,¹⁸⁰ which is a master molecule of regulatory T (Treg) cells, suggesting that ATL is a malignant disease of Treg cells. Furthermore, in HTLV-1-infected individuals, the proportion of HTLV-1-infected cells is higher in Treg populations,³⁶⁹ indicating that HTLV-1 is associated with Treg cells. HBZ induces Foxp3 expression in T cells, and its induction is enhanced in the presence of TGF- β .³⁰⁹ This accounts for why HTLV-1 is associated with Treg cells. Furthermore, HBZ interacts with Foxp3 protein and impairs its functions.³⁰⁹ Thus, HBZ increases the number of functionally impaired Treg cells and may lead to the development of malignancy derived from Treg cells.

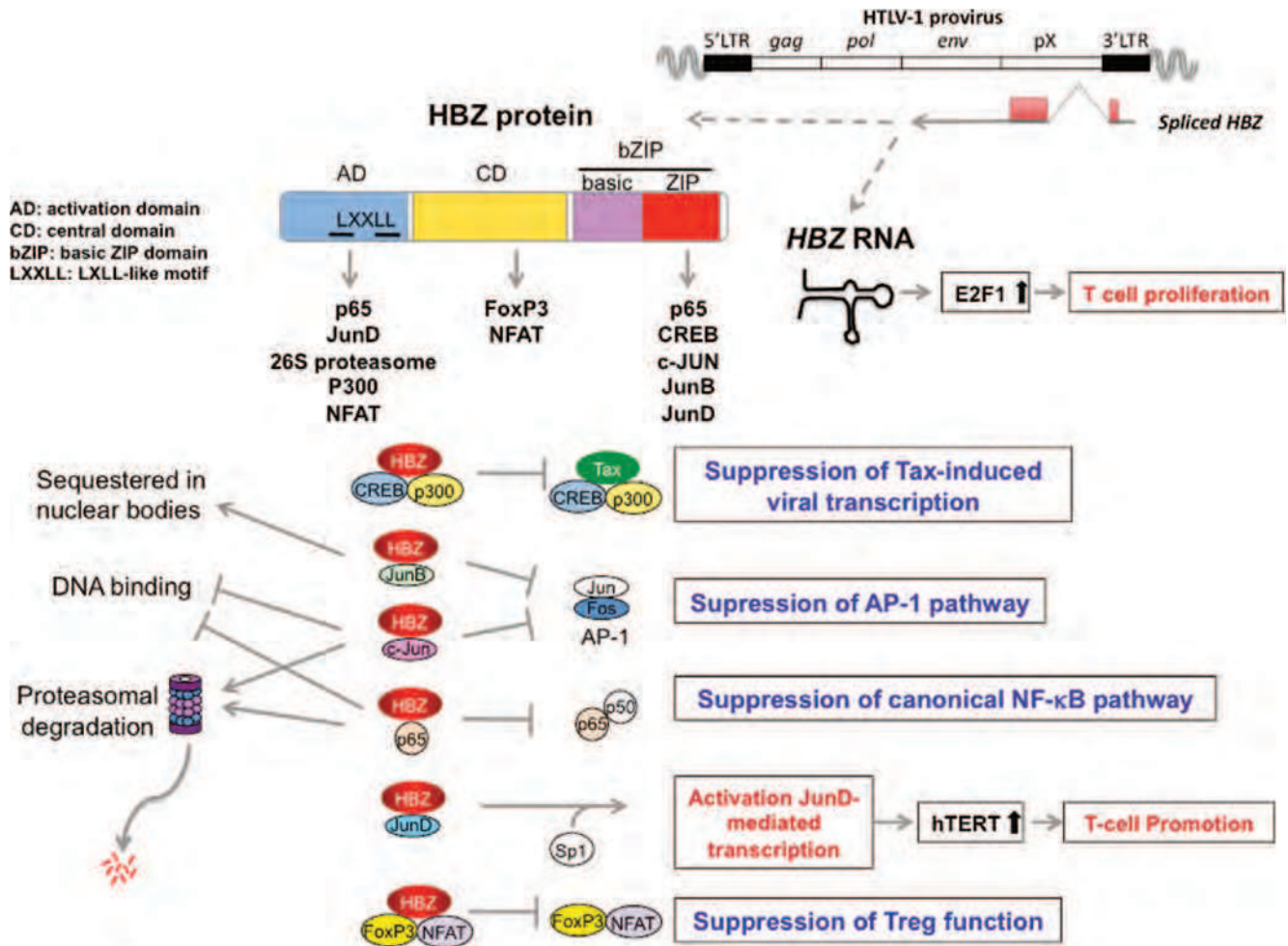


FIGURE 48.8. Functions of HBZ. HBZ has three domains: activation, central, and bZIP. The HBZ protein exerts a variety of functions by interacting with host factors. On the other hand, *HBZ* RNA promotes proliferation via up-regulation of the *E2F1* gene.

Interplay Between HBZ and Tax

For many pathways, HBZ has activities that oppose Tax (Fig. 48.9). Tax can activate both the canonical and noncanonical NF-κB pathways, whereas HBZ suppresses only the canonical pathway. These two pathways differentially control genes with anti-apoptotic functions in lymphoma cell lines. Expression of both Tax and HBZ could thus lead to predominant activation of the noncanonical pathway, a phenomenon that might be implicated in proliferation of ATL cells. Expression of Tax induces cell senescence via enhanced expression of CDK inhibitors p21 and p27.⁴³³ As a mechanism, p53 induces this senescence by stabilization of p27 protein and p21 mRNA. Co-expression of HBZ with Tax delay or prevent the Tax-induced senescence, leading to cell proliferation. Similarly, HBZ enhances TGF-β/Smad signaling, whereas Tax suppresses this pathway. Thus, it is assumed that HBZ activity is intricately interregulated with that of Tax, modulating viral gene expression, promoting cell proliferation, and leading to oncogenesis.

HBZ Promotes T-Cell Proliferation

Suppression of *HBZ* gene expression by shRNA inhibits proliferation of ATL cell lines.^{15,308} Expression of *HBZ* in transgenic

mice increases the number of T cells, whereas suppression of *HBZ* expression decreases tumor formation and infiltration of ATL cells.¹⁵ Thus, *HBZ* expression is associated with proliferation of ATL cells *in vivo* and *in vitro*. Mutation analysis of the *HBZ* gene showed that *HBZ* RNA, rather than HBZ protein, has a growth-promoting effect on T cells.³⁰⁸ Only *sHBZ* RNA, not *usHBZ* RNA, promotes the proliferation of T cells, indicating that the first exon of the *sHBZ* transcript is critical for this activity.⁴²⁰ This exon overlaps with the Rex responsive element (RxRE) in the R region of 3' LTR. Recall that the RxRE region forms a stem-loop structure that binds to Rex, and Rex promotes the export of viral RNA with an RxRE region. Kinetic analysis of viral mRNA shows strong nuclear retention of *HBZ* mRNA,²⁹⁸ which might be important for growth-promoting activity of *sHBZ* mRNA. Thus, *sHBZ* RNA promotes proliferation of T cells via a region that can form a strong stem-loop structure. Further details of how *sHBZ* RNA promotes proliferation remain to be elucidated.

APH-2

An antisense transcript similar to *HBZ* has been discovered in HTLV-2 and named antisense protein of HTLV-2 (APH-2).¹¹²

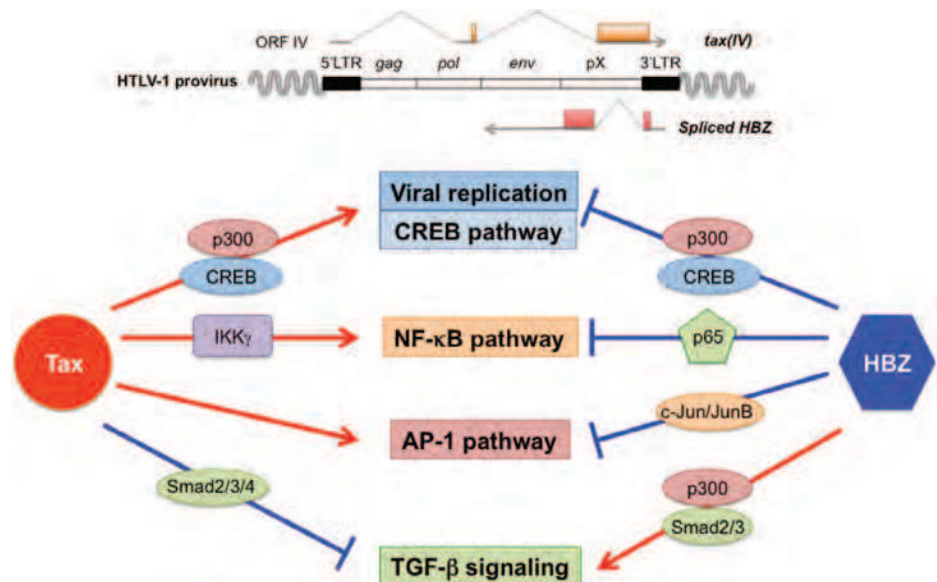


FIGURE 48.9. Interplay between Tax and HBZ. Interplays between Tax and HBZ on various pathways are shown. In most pathways, HBZ has opposite effects to Tax.

The *APH-2* gene encodes a 183 amino acid polypeptide that is localized in the nucleus. Although APH-2 does not have a bZIP domain, APH-2 interacts with CREB and represses Tax2-mediated transcription from the 5' LTR of HTLV-2. Both HBZ and APH-2 have suppressive effects on transcription of sense viral genes, although any other functions of APH-2 remain to be elucidated.

PATHOGENESIS AND PATHOLOGY

Entry into the Host

HTLV-1 transmission usually occurs through cell-to-cell contact of HTLV-1–uninfected cells with HTLV-1–infected cells, and cell-free viruses are poorly infectious. Thus, cell-free blood products do not transmit HTLV-1.²⁷⁸ HTLV-1 is primarily transmitted from infected mother to child through breast-feeding. In addition, sexual contact and blood transfusion are additional routes of transmission. Sexual transmission is mostly from male to female; transmission from female to male is far less efficient.³⁴⁰

HTLV-1 entry into susceptible cells begins with the binding of the HTLV-1 envelope proteins to a viral receptor on the membrane of the host cell, and it is followed by the fusion of viral and cell membranes.^{24,95,229} The HTLV-1 envelope protein (Env) is synthesized as a 68 kD polypeptide precursor and is cleaved to produce two subunits: the surface glycoprotein (SU) of 46 kD (gp46) and the TM glycoprotein of 21 kD (gp21). Mature envelope proteins on the virions are trimers, consisting of three SUs and three TMs linked by disulphide bonds. The cytoplasmic domain of gp21 controls envelope-mediated syncytium formation and cell-to-cell HTLV-1 entry.^{188,294} The C-terminus of gp21 contains a PDZ-domain protein binding motif, and the deletion of this motif reduces the stability of Env.³⁷ In addition, the Y-S-L-I motif in the cytoplasmic domain of gp21 is crucial for cell-to-cell HTLV-1 transmission.⁶¹

Functions of Glucose Transporter 1 in HTLV-1 Entry

Two host proteins have been identified as acting as HTLV-1 receptors: glucose transporter 1 (GLUT-1) and neuropilin-1

(NRP-1) (Fig. 48.10). Of them, the GLUT-1 glucose transporter was the first identified.^{59,174,192,230} GLUT-1 interacts with the receptor binding domain (RBD) (the first 215 aa) of HTLV-1 Env and HTLV-2 Env. Knockdown of GLUT-1 by siRNA resulted in reduced binding of the HTLV-1 and HTLV-2 RBD to the cells as well as reduced infection by HTLV-2-env pseudotype virus, and this was rescued by overexpression of GLUT-1 but not GLUT-3, another glucose transporter family member. In addition, polyclonal chicken antibody against the extracellular loop domain of GLUT-1 inhibited Env-mediated fusion as well as infection.¹⁷¹ Specific regions of GLUT-1 mediated the binding to HTLV-1 Env and virus entry into cells.²²⁸

Functions of Neuropilin-1 in HTLV-1 Entry

NRP-1 also functions as an HTLV-1 receptor.⁹⁶ Overexpression of NRP-1 in the cells expressing a low amount of endogenous NRP-1 augments HTLV-1 Env-mediated infection, and NRP-1 knockdown by siRNA in 293T cells reduces HTLV-1 Env-mediated infection. Interestingly, NRP-1 co-localizes with the Env protein at cell membranes and forms a complex with GLUT-1. Moreover, this complex formation is augmented by the expression of Env protein, suggesting that NRP-1 and GLUT-1 make a HTLV-1 receptor complex that mediates HTLV-1 binding and entry (see Fig. 48.10A), as CD4 and chemokine receptors do for human immunodeficiency virus (HIV) infection. In addition, HTLV-1 might differentially utilize GLUT-1 and NRP-1 in a cell-type dependent manner. For instance, knockdown of GLUT-1 reduced Env-mediated fusion in HeLa cells but not in U87 glioblastoma/astroglioma cells.¹⁷⁰

In addition to GLUT-1 and NRP-1, HTLV-1 infection is enhanced by several host proteins, such as ICAM-1, ICAM-3, and VCAM-1, in various settings. Heparan sulfate proteoglycans (HSPGs) have been shown to be crucial for efficient HTLV-1 binding and infection of CD4+ T cells.^{174,293} HTLV-1 virions and purified gp46 proteins bound to HSPG on CD4+ T cells, and the binding was abrogated by treatment of target cells with heparan-sulfate lyase. Moreover, soluble heparin blocked HTLV-1 infection of susceptible cells.

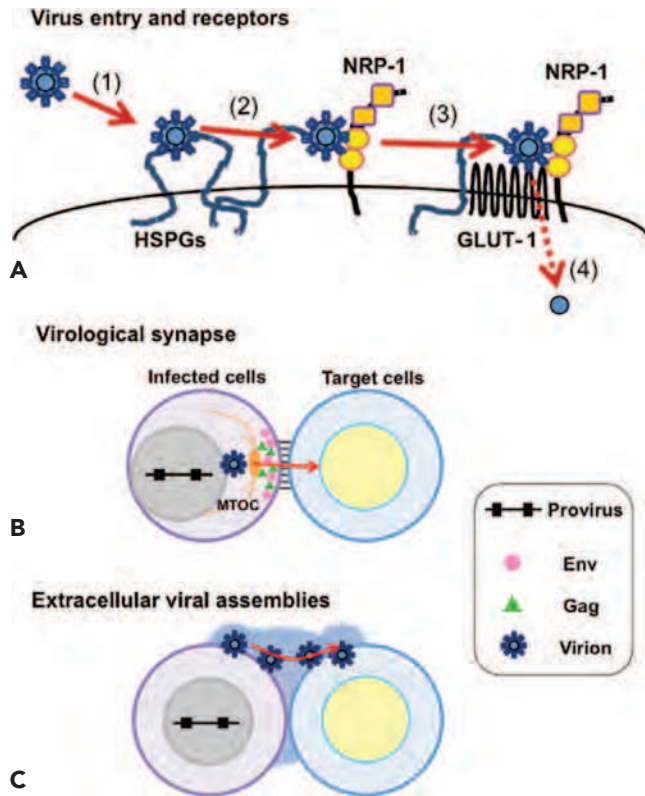


FIGURE 48.10. The HTLV-1 receptor complex and the current model of HTLV-1 entry. **A:** (1) The HTLV-1 envelope protein (Env) attaches to heparan sulfate proteoglycans (HSPGs) on the target cell, which increases the local concentration of the viruses at the cell surface. (2) HTLV-1 Env then binds to neuropilin-1 (NRP-1), and this binding induces a conformational change of Env that facilitates its interaction with glucose transporter 1 (GLUT-1). (3) The formation of a ternary complex of Env, NRP-1, and GLUT-1 induces a conformational change of Env that triggers the fusion of the viral and cell membranes. **B:** The contact site between an HTLV-1-infected cell and a target cell forms a special structure referred to as the virological synapse (VS). VS formation involves polarization of the microtubule-organizing center near the site of cell-to-cell contact in the infected cell. **C:** HTLV-1-infected cells contain infectious virions in extracellular carbohydrate-rich assemblies, and their removal reduces cell-to-cell HTLV-1 transmission. These virion-containing assemblies resemble bacterial biofilm in structure and composition.

Determinants of Tropism of HTLV-1 and HTLV-2 Entries

HTLV-1 and HTLV-2 preferentially immortalize CD4+ T cells and CD8+ T cells *in vitro*, respectively, and they are preferentially detected in CD4+ T cells and CD8+ T cells in infected individuals, respectively.^{145,299,300,390} These observations may reflect the distinctive infection tropism of HTLV-1 versus HTLV-2.¹⁷⁵ Whereas HTLV-1 infects primary CD4+ T cells much more efficiently than CD8+ T cells, HTLV-2 behaves in the reverse manner. These tropisms may be owing to distinctive viral receptor usages. Unlike GLUT-1, HSPG is not required for HTLV-2 infection, because reduction of HSPG by HS lyase has little effect on HTLV-2-Env-mediated infection. Activated primary human CD4+ T cells and CD8+ T cells express different levels of HTLV-1 receptors, and CD4+ T cells express

much more HSPG than CD8+ T cells, whereas CD8+ T cells express much more GLUT-1 than CD4+ T cells. Collectively, these results suggest that HTLV-1 and HTLV-2, through distinct usages of GLUT-1 and HSPG, preferentially infect CD4+ T cells and CD8+ T cells, respectively, thereby preferentially immortalizing selective cell types. The study of recombinant viruses has demonstrated that the envelope genes of HTLV-1 and HTLV-2 are the major determinant for immortalization tropism (CD4+ vs. CD8+) of human T cells.⁴⁰⁴ In addition, it is noteworthy that HTLV-3 utilizes GLUT-1 as a viral receptor; however, neither NRP-1 nor HSPG is required for infection, and HTLV-3 binds not only activated primary T cells but also resting ones.⁴⁰⁴ These results suggest that these three HTLVs acquired distinct entry mechanisms after diversification from their common ancestor.

DCs are implicated as key players for HTLV-1 transmission. Whereas cell-free HTLV-1 is poorly infectious for CD4+ T cells, it efficiently infects plasmacytoid and myeloid DCs.^{48,159,176} Such HTLV-1-infected DCs can immortalize CD4+ T cells, indicating that DCs can act as intermediate reservoirs for HTLV-1 infection of CD4+ T cells. Cell-free HTLV-1 infection of DCs is enhanced by DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), which interacts with ICAM-2 and ICAM-3 but not HTLV-1 Env. HSPG and NRP-1 are involved in the transfer of HTLV-1 from DCs to CD4+ T cells. These results suggest that DCs play a crucial role in the early phase of HTLV-1 transmission.

Virological Synapse and Biofilm-Like Assemblies in HTLV-1 Entry

During cell-to-cell HTLV-1 transmission, the site of contact between an HTLV-1-infected cell and a target cell forms a special structure called the *virological synapse* (VS; named thus because of its similarity to the immunological synapse)^{24,144,173,292} (see Fig. 48.10B). VS formation involves polarization of the microtubule-organizing center (MTOC) near the site of cell-to-cell contact in the infected cells. ICAM-1 and Tax appear to play a role in polarization of the MTOC during cell-to-cell transmission.

Extracellular carbohydrate-rich assemblies attached to the surfaces of HTLV-1-infected cells contain infectious virions, and their removal prominently reduces cell-to-cell HTLV-1 transmission²⁸³ (see Fig. 48.10C). These virion-containing assemblies produced by HTLV-1-infected cells resemble bacterial biofilm in structure and composition, and contain collagen, agrin, tetherin, and galectin-3. One of these proteins, tetherin, negatively controls HIV infection through inhibiting the release of HIV virions, suggesting that biofilm-like assemblies may also contain inhibitors of HTLV-1 infection.⁶⁷

Site of Primary Replication and Spread of the Virus

After transmission by breast milk, sexual contact, or blood transfusion, HTLV-1 infects DCs and/or T-cells *in vivo*, and spreads via cell-to-cell contact. HTLV-1-infected cells proliferate and spread in the body. In experimentally infected squirrel monkeys (*Saimiri sciureus*), HTLV-1 provirus was detected first in PBMCs and then in lymphoid organs including lymph node, spleen, and bone marrow.¹⁸⁶ This shows that HTLV-1 spreads in lymphoid tissues. In HTLV-1-infected individuals, bone marrow has been identified as a reservoir for HTLV-1.²¹⁷

HTLV-1 virions cannot be detected in HTLV-1-infected individuals. Instead, HTLV-1 infected cells proliferate *in vivo*: HTLV-1 copy number increases by mitosis of infected cells, whereas HTLV remains in the form of a provirus integrated in the host genome. Expression of adhesion molecules, including ICAM-1 and LFA-1, is up-regulated on HTLV-1-infected cells.⁸² In HAM/TSP patients and HTLV-1 carriers, infected cells not only circulate in blood but also infiltrate into skin, lung, and intestine. Thus, HTLV-1-infected cells persist and spread *in vivo*. If infected cells have proinflammatory properties, their proliferation and activation might induce inflammatory diseases.

Cells and Tissue Tropism

GLUT-1, a receptor for HTLV-1, is ubiquitously expressed on cells. HTLV-1 can infect various cells, including T cells, B cells, macrophages, myeloid cells, and fibroblasts *in vitro*.²⁰¹ However, the provirus is detected predominantly in T cells *in vivo*. Most infected cells are CD4+ T cells, especially effector/memory T cells and Treg cells.^{299,369,415} Approximately 10% of the provirus is detected in CD8+ T cells,^{253,415} indicating that CD8+ T cells are also a reservoir of this virus. These “preferences” may reflect either a true viral tropism or postinfection events that alter infected cells—events that are related to certain functions of HTLV-1 encoded proteins. For example, in transgenic mice expressing the *HBZ* gene, the population of effector/memory T cells and Treg cells is increased.³⁰⁹ HBZ induces Foxp3 expression, indicating that HBZ can convert infected T cells to Treg cells.³⁰⁹ Furthermore, it is thought that Tax stimulates the proliferation of CD4+ T cells. CD4+ T cells can be immortalized in *tax* gene transgenic mice by immune activation,³⁴⁹ perhaps because Tax expression synergistically augments signaling through the T-cell receptor.²³¹ Furthermore, a retroviral vector expressing Tax immortalizes CD4+ T cells *in vitro*.⁴ Thus, HBZ and Tax are considered to be responsible for the apparent cell tropism of HTLV-1.

Immune Response and Persistence

Persistence of HTLV-1-Infected Cells *In Vivo*

Free HTLV-1 virions are not detected *in vivo*. Instead, HTLV-1 spreads via cell-to-cell contact, and infected cells proliferate. Virus burdens are measured by provirus load (i.e., the number of infected cells). Compared with the highly variable sequences of HIV, HTLV-1 proviral sequences remain stable in infected individuals.^{92,382} Because proviral sequence variations are caused by error-prone reverse transcriptase during viral replication, the stability of proviral sequences indicates that HTLV-1 replicates primarily by mitosis of infected cells instead of by viral replication. Provirus load varies less than 5-fold within the same infected individuals during the clinical course. However, provirus load differs more than 1000-fold from one infected host to another, indicating that provirus load is controlled by host factors *in vivo*.⁷²

Integration sites of HTLV-1 provirus are random in the host genome.^{66,282} Therefore, clonal proliferation can be demonstrated by identifying integration sites of the HTLV-1 provirus.^{47,71} Infected T-cell clones in seroconverters were more variable than long-term carriers,³⁶⁰ indicating that over time, conditions within the host (immune response, etc.) select certain infected T-cell clones. Consistent with this finding, identifica-

tion of integration sites by high-throughput sequencing shows that negative selection is predominant in chronic infection.¹⁰⁰

Implication of HTLV-1 Provirus Load in Pathogenesis

Provirus load is an important parameter of HTLV-1 associated diseases. Higher provirus load was reported in patients with HAM/TSP and uveitis compared with asymptomatic carriers.^{254,279} High provirus load was also reported to be a possible risk factor for ATL.^{135,154} In HAM/TSP patients, an increased number of infected clones, rather than increased cell numbers in each clone, is the main contributor to increased provirus load.¹⁰⁰ In HAM/TSP and other HTLV-1-associated inflammatory diseases, HTLV-1-infected cells and lymphocytes reacting to viral antigens infiltrate into various tissues, including skin, lung, intestine, and spinal cord. Cytokines produced by infected cells and reacting lymphocytes likely induce the tissue damage that characterizes these diseases.²⁸⁰ In HAM/TSP, CD4+CD25+CCR4+ T cells produce IFN- γ , which may be implicated in the pathogenesis of this disease.⁴¹⁰

Immune Response to HTLV-1

The immune response is a major factor in determining the provirus load within an infected individual. The balance between viral gene expression and the host immune response likely determines the number of infected cells. Because Tax is a major target of CTLs,^{158,178} Tax-expressing cells are rapidly eliminated. However, transcription of the *tax* gene from the 5' LTR is suppressed *in vivo*.¹¹⁷ *In vitro* culture of HTLV-1-infected cells elicits a rapid recovery of the *tax* gene expression. Depletion of CD8+ T cells in cultured cells enhances Tax expression, indicating that CTL activity partially limits viral gene expression.¹¹⁷ This finding suggests that HTLV-1 must have a mechanism of inhibiting viral gene transcription *in vivo*. An HDAC inhibitor, valproate, induces *tax* gene transcription. Administration of valproate to patients with HAM/TSP reduces the provirus load,²¹⁹ suggesting that recovery of Tax expression induces CTLs to kill infected cells. Thus, Tax expression is suppressed by an epigenetic mechanism *in vivo*, and recovery of Tax expression induces a CTL response. Indeed, quantification of *in vivo* lymphocyte dynamics shows that CD4+CD45RO+ and CD8+CD45RO+ T-cell proliferation is elevated in HTLV-1-infected individuals. The *in vivo* proliferation of CD4+CD45RO+ T cells correlates with Tax expression,¹⁸ indicating the dynamics of HTLV-1-infected CD4+ T cells and reacting CD8+ T cells *in vivo*.

HLA-A*02 and C*08 are associated with a reduced risk of HAM/TSP and a decreased provirus load in asymptomatic carriers, whereas HLA-B*54 is linked with an increased risk of HAM/TSP and an increased provirus load in HAM/TSP patients.¹⁶¹ Furthermore, a killer cell immunoglobulin-like receptor (KIR), KIR2DL2, potentiates protective and detrimental effects of these human leukocyte antigen (HLA) class I-mediated immunity.³¹³ This study showed that the protective effect of HBZ peptides by HLA class I molecules is enhanced by KIR2DL2, suggesting that HBZ is also a critical target proteins for CTLs. The *HBZ* gene, which supports proliferation of HTLV-1-infected cells, has been found to be continuously expressed *in vivo*.³⁰⁸ Tax is highly immunogenic,^{158,178} whereas HBZ shows weak immunogenicity.¹²⁹ It has been reported that CTLs against HBZ protein determine the provirus load in HTLV-1-infected individuals,²²²

whereas CTLs against Tax are associated with inflammatory conditions.¹²⁹ Thus, the immune responses to these proteins might affect provirus load and disease status.

Viruses that cause chronic infection must evade the host immune system to enable persistent replication or proliferation. HTLV-1 appears to have evolved a strategy for allowing infected cells to evade the host immune response. One component of this strategy is the HTLV-1 accessory protein p12, which physically interacts with the free human MHC class I heavy chain and induces its degradation.¹⁷² Suppression of cell surface MHC class I expression facilitates the escape of HTLV-1-infected cells from destruction by the host immune system. A second component of this strategy is HBZ, which induces the expression of Foxp3 in infected T cells and increases the population of Treg cells *in vivo*.³⁰⁹ Indeed, in HTLV-1-infected individuals, provirus levels are disproportionately high in the Treg subpopulation.³⁶⁹ An increase in the number of Treg cells likely suppresses the host immune response, which would enable more infected cells to survive *in vivo*.

Immunodeficient State and Diseases

The host immune system controls the dynamics of HTLV-1 infection and the development of disease. This idea is supported by the clinical observations that ATL occurs fairly commonly in immunocompromised hosts. Among 24 patients with posttransplantation lymphoproliferative disorders (PT-LPD) after renal transplantation in Japan, five ATL cases have been reported.¹⁴² HTLV-1 was likely transmitted via blood transfusion during hemodialysis. It is likely that the immunodeficient state of recipients promotes the onset of ATL. Furthermore, of eight HTLV-1 carriers who received living-donor liver transplants and subsequent immunosuppressive treatment, three developed ATL.¹⁸⁵ Collectively, these findings indicate that the host immune system commonly suppresses the development of ATL, whereas an impaired immune system may allow HTLV-1-infected cells to transform into ATL cells.

HTLV-1 carriers show mild impairments of cell-mediated immunity. *Strongyloides stercoralis* infection, for example, is more severe in HTLV-1 carriers.³⁸⁷ The impairment of cell-mediated immunity is more profound in ATL patients, who frequently succumb to opportunistic infections with various pathogens, including fungus, cytomegalovirus, *Pneumocystis jirovecii*, and mycobacterium.³⁵⁵ One possible reason for this immune deficiency is that HTLV-1 infection increases the population Treg cells, because HBZ induces the expression of Foxp3.³⁰⁹

Provirus Sequences In Vivo

Analysis of viral gene transcription in ATL cells showed that in approximately 60% of cases, ATL cells did not express Tax.³⁵⁶ Three mechanisms by which Tax expression was disrupted were identified: (a) deletion of the 5' LTR,^{239,358} (b) DNA methylation of the 5' LTR,^{198,362} and (c) genetic changes of the *tax* gene (nonsense mutations, deletion, and insertion).^{73,84} In all cases, the pX region and the 3' LTR remained intact, suggesting the importance of the *HBZ* gene.

Analysis of whole HTLV-1 proviruses in ATL cells shows that all viral genes can contain nonsense mutations except the *HBZ* gene.⁷³ Most nonsense mutations were generated in a tryptophan codon (TGG) by G-to-A mutation (TGG to TGA, or TGG to TAG). The sequences of these G-to-A mutations in HTLV-1 proviruses correspond to the target sequences of

APOBEC3G.⁷³ APOBEC3G is a host factor that generates G-to-A mutations during reverse transcription. In the case of HIV, an accessory protein, Vif, promotes the degradation of APOBEC3G, which enables HIV to escape fatal nonsense mutations. HTLV-1 does not encode an accessory gene that counteracts APOBEC3G. It has been reported that the gag region of HTLV-1 suppresses the incorporation of APOBEC3G into the virion.⁶² However, this inhibitory activity is not strong enough to completely suppress all G-to-A mutations.³⁰⁶ If nonsense mutations are generated in viral genes that are not essential to proliferation, HTLV-1-infected cells can still proliferate and transform to leukemic cells. This result indicates that nonsense mutations of all viral genes except the *HBZ* gene can be generated before integration and ATL can still ensue, suggesting that *HBZ* is an unexpectedly essential gene for leukemogenesis.

EPIDEMIOLOGY

Age

HTLV-1 is transmitted via three major routes: breast-feeding, sexual transmission, and parenteral transmission. Seropositivity of infected babies was established by the age of 2 years, indicating that transmission via breast-feeding continued until that age.¹⁰ In adults, HTLV-1 seroprevalence rises according to age, and the seropositivity of females is higher than that of males.³⁶ The reason is that sexual transmission of this virus is more efficient from male to female than from female to male.^{143,340} It is of note that sexual transmission has occurred even in old HTLV-1 discordant couples, a fact that can account for the increase in seropositivity in the elder population. The average age of ATL patients was 63 years in Japan, which is 15 years older than the average age of ATL patients in the Caribbean Islands,³⁵⁵ suggesting that genetic background and/or environmental factors influence development of ATL. The mean age of onset of HAM/STP patients was 43 years.²⁸⁰ In addition, HAM/TSP rapidly develops in some cases after infection. These findings suggest that onset of HAM/TSP does not need a long latent period compared with ATL.

Morbidity/Mortality

HTLV-1 establishes persistent infection. The cumulative risks of ATL among HTLV-1 carriers in Japan is estimated to be about 6.6% for men and 2.1% for women, indicating that most HTLV-1 carriers remain asymptomatic throughout their lives.¹⁴ The incidence of HAM/TSP is estimated to be about 0.1% of these HTLV-1 carriers in Japan. There are geographical differences in the incidences of these HTLV-1-associated diseases.¹³⁶ The incidence of HAM/TSP is approximately seven times higher in Jamaican subjects compared with the Japanese population, whereas that of ATL is four times lower in Jamaicans. Differences in immune markers between Japanese and Jamaican HTLV-1-infected individuals have been reported: higher titers of antibody and more activated T-cell immunity in Jamaicans compared to Japanese individuals.³⁵ Differences in HTLV-1-related host immunity might be linked to the differences in ATL and HAM/TSP risks for these populations.

Origin and Spread of Epidemics

HTLV-1 is a member of the deltaretroviruses, which include HTLV-2, bovine leukemia virus, and simian T-cell leukemia

virus (STLV). HTLV and STLV are thought to originate from common ancestors and share molecular, virological, and epidemiological features. Therefore, they have been designated primate T-cell leukemia viruses (PTLVs). Phylogenetic analyses have revealed that HTLV-1c first diverged from STLV around $50,000 \pm 10,000$ years ago, whereas the spread of HTLV-1 in Africa is estimated to have occurred at least $27,300 \pm 8,200$ years ago. Subsequently, HTLV-1a, which is the most common subtype in Japan, diverged from the African strain $12,300 \pm 4,900$ years ago.³⁸³ Thus, these viruses have had a long history with humans after the initial interspecies transmission. In contrast, HIV-1 is thought to originate from simian immunodeficiency virus in chimpanzees (SIV_{CPZ}),⁸⁶ and the interspecies transmission to humans is estimated to have occurred about 100 years ago.⁴⁰¹ Recently, new retroviruses have been identified in bush meat hunters in Central Africa. HTLV-3 has been found in asymptomatic carriers in Cameroon.^{44,400} HTLV-3 shares high homology with its simian counterpart, STLV-3, which was detected in monkeys in Africa. The genome organization of HTLV-3 is similar to HTLV-1.²²⁵ No diseases have been reported to be associated with HTLV-3.

Prevalence and Seroepidemiology

It is estimated that 10 million to 20 million people live with HTLV-1 worldwide.²⁹⁷ Areas endemic for this virus are southwestern Japan, the Caribbean basins, Central and West Africa, and South America. In addition, epidemiological studies of HTLV-1 have revealed high seroprevalence rates in Melanesia, Papua New Guinea, and the Solomon Islands, as well as among Australian aborigines.³⁶ In Japan, approximately 1.08 million individuals were estimated to be infected with HTLV-1 in 2009, and among them, 1,000 carriers develop ATL each year.

Genetic Diversity of Virus

Unlike HIV, HTLV-1 has low sequence variability.⁹² Because reverse transcriptase is an error-prone DNA replication enzyme,

viral replication generates mutations in the proviral sequences. In the case of HTLV-1, however, the virus induces infected cells to proliferate rather than producing virions; as a result, sequence variations are rare compared with HIV. Nevertheless, several subtypes of HTLV-1 have been identified. According to previous reports, HTLV-1 strains include the six following subtypes: the cosmopolitan HTLV-1a subtype;²³⁷ the Central African subtypes HTLV-1b,^{109,386} HTLV-1d,²²⁶ HTLV-1e,³³⁷ and HTLV-1f;³⁰⁴ and the Australo-Melanesian subtype HTLV-1c.^{94,321} These subtypes are closely linked with interspecies transmission and human migration events.

CLINICAL FEATURES

After infection with HTLV-1, HTLV-1 provirus load remains relatively constant within an individual but differs among infected individuals. In other words, different individuals have different provirus load “set points.” Most individuals remain asymptomatic during their entire lives, whereas a small fraction of carriers develop HTLV-1–associated diseases (Fig. 48.11). HTLV-1 causes both a neoplastic disease, ATL, and inflammatory diseases, including HAM/TSP, uveitis, alveolitis, and infective dermatitis. In addition, HTLV-1 infection also suppresses cell-mediated immunity.³⁹⁷ Provirus load is a critical factor for pathogenesis of these disorders.

Adult T-Cell Leukemia

ATL develops predominantly in males (the male to female ratio is 1.5:1), whose age at onset ranges from 25 to 94 years, with a median age of 63 years in Japan. The predominant physical findings are peripheral lymph node enlargement (72% of cases), hepatomegaly (47%), splenomegaly (25%), and skin lesions (53%).³⁵⁵ ATL cells infiltrate into various organs/tissues, including skin, liver, lung, the gastrointestinal tract, the central nervous system, and bone. Various skin lesions, such

FIGURE 48.11. The natural course of HTLV-1 infection. After transmission of HTLV-1, infected cells proliferate owing to the actions of Tax and HBZ. The growth of HTLV-1–infected cells is suppressed by cytotoxic T lymphocytes *in vivo*. The number of HTLV-1 infected cells is thus determined by balance between viral gene expression and host immune system. About 5% of HTLV carriers develop adult T-cell leukemia after a long latent period. A smaller fraction of carriers present with inflammatory diseases, such as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis, or alveolitis. Most carriers remain asymptomatic.

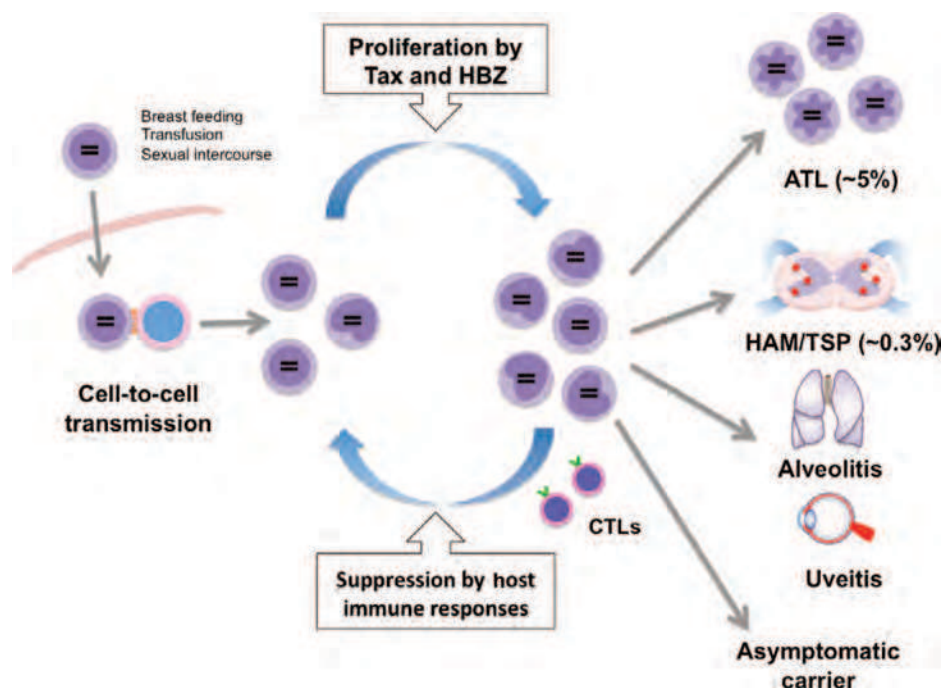




FIGURE 48.12. Skin lesion of an adult T-cell leukemia (ATL) patient. ATL cells frequently involve skin and form various lesions. In this patient, ATL cells form a tumorous lesion.

as papules, erythema, and nodules are frequently observed in ATL patients (Fig. 48.12). ATL cells densely infiltrate the dermis and epidermis, forming Pautrier's microabscesses in the epidermis (Fig. 48.13).

ATL cells in the peripheral blood have indented or lobulated nuclei (Fig. 48.14). Anemia and thrombocytopenia are rare because involvement of bone marrow is not severe. The typical surface phenotype of ATL cells is CD4+CD8–CD25+,

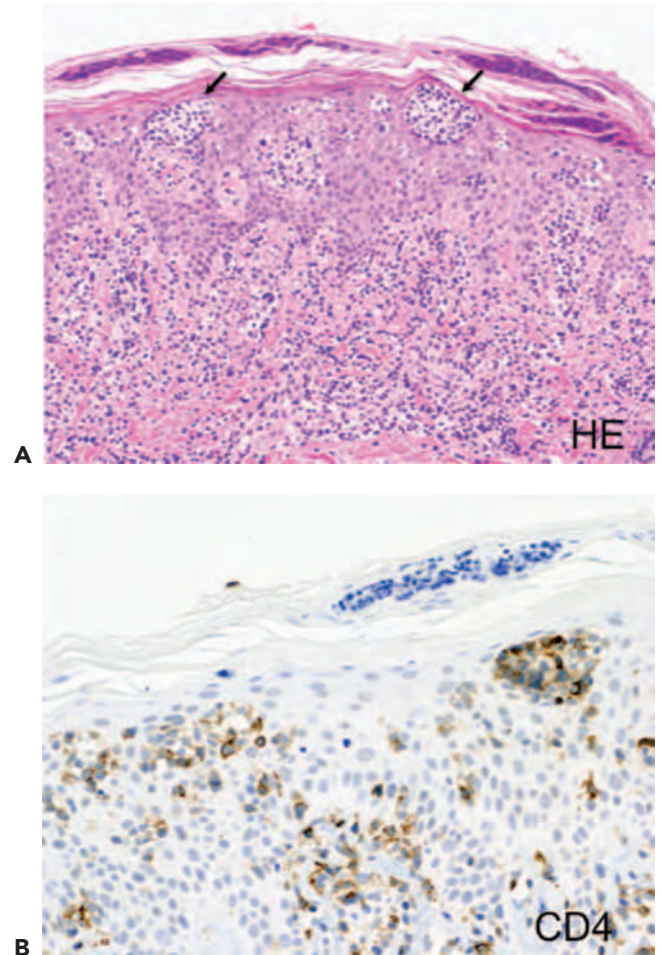


FIGURE 48.13. Skin involvement of adult T-cell leukemia (ATL). **A:** ATL cells infiltrate into epidermis and form Pautrier's microabscesses (arrows). **B:** CD4+ cells are identified by the immunohistochemical staining.

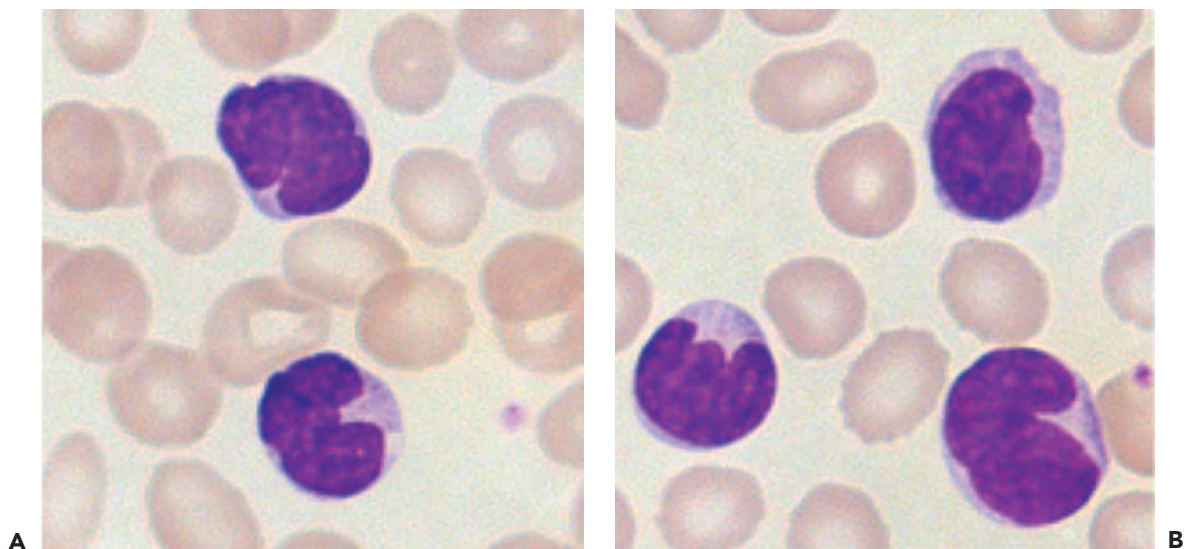


FIGURE 48.14. Adult T-cell leukemia (ATL) cells in the peripheral blood. ATL cells in an acute ATL case show specific morphology.

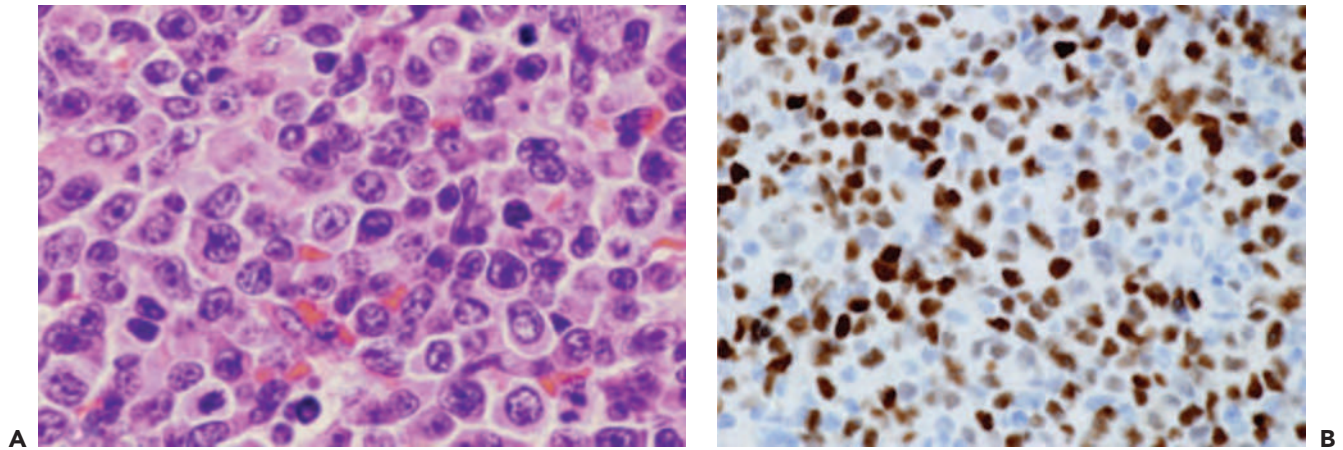


FIGURE 48.15. Lymph node lesion of an adult T-cell leukemia (ATL) patient. A: Histological analysis (hematoxylin-eosin staining) shows monotonous proliferation of ATL cells. **B:** Immunohistochemical analysis shows that ATL cells are FoxP3 positive.

which is similar to that of activated T cells and Treg cells. Foxp3 is expressed in ATL cells in two-thirds of cases (Fig. 48.15),¹⁸⁰ indicating that such ATL cells are derived from Treg cells. ATL cells express CD25 (IL-2R alpha chain) on their surfaces and secrete its soluble form. Therefore, levels of soluble IL-2Rs are elevated in the sera of patients with ATL, with the levels of soluble IL-2R being correlated with the tumor mass and clinical course.⁴¹⁴ ATL cells elaborate various cytokines that can affect the immune response and influence the pathophysiology of ATL. For example, eosinophilia, caused by elevated IL-5 levels, is frequently observed in patients with ATL.

Hypercalcemia is a complication in about 70% of ATL patients at some point during the clinical course of the disease, particularly during the aggressive stage.¹⁹⁶ Pathologic studies of ATL patients with hypercalcemia have shown that high serum Ca^{2+} levels are attributable to an increased number of osteoclasts (OCL) and accelerated bone resorption (Fig. 48.16).

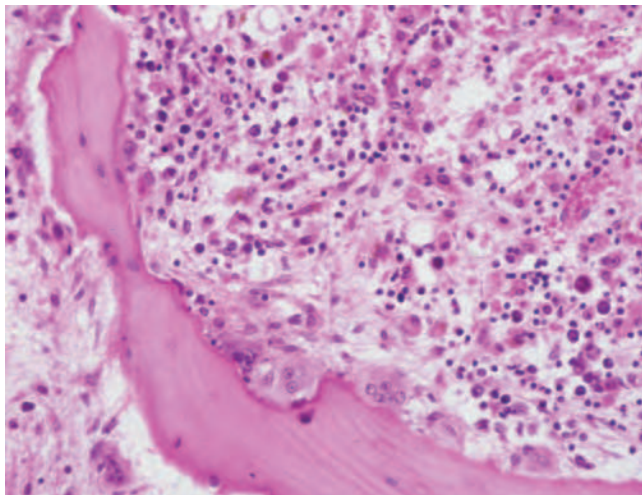


FIGURE 48.16. Increased number of osteoclasts in a hypercalcemic adult T-cell leukemia patient. In a hypercalcemic patient, the number of osteoclasts increases in the bone and accelerates bone resorption.

During differentiation of OCLs, precursor cells sequentially express c-Fms (the receptor for M-CSF) followed by receptor activator nuclear factor κB (RANK).¹² M-CSF and RANK ligand (RANKL) have been shown to be critical factors for the differentiation of OCLs and are physiologically produced by stromal cells and osteoblasts. ATL cells from patients with hypercalcemia have high RANKL transcript levels and induce the differentiation of hematopoietic stem cells into OCLs *in vitro* in the presence of M-CSF.²⁶⁷ In addition, parathyroid hormone-related protein (PTH-rP) elaborated from ATL cells activates OCLs and promotes bone resorption.³⁹⁴

HTLV-1–Associated Myelopathy/Tropical Spastic Paraparesis

HAM/TSP is characterized by a slow progressive spastic paraparesis, urinary dysfunction, and sensory disturbances.²⁸⁰ HAM/TSP patients also often have other organ disorders such as leukoencephalopathy (69%), abnormal findings on chest x-ray (50%), Sjögren syndrome (25%), and arthropathy (17%).²⁵⁶ In contrast to ATL, HAM/TSP develops predominantly in females (the male to female ratio is 1:2.9).

Pathologic studies of HAM/TSP patients demonstrated the severe involvement of the thoracic spinal cord, in which T cells cause perivascular cuffing and infiltrate into the parenchyma.²⁸⁰ In HAM/TSP, the provirus load is high compared with asymptomatic carriers,²⁵⁴ and more HTLV-1–infected CD4+ T cells infiltrate into the spinal cord.^{46,233,246} The number of HTLV-1–specific HLA class I restricted CD8+ T cells is also increased in HAM/TSP patients.¹⁵⁸ Among viral antigens, Tax has a dominant epitope that is recognized by CTLs.^{103,178} Tax-reacting CD8+ T cells also migrate into the spinal cord along with HTLV-1–infected CD4+ T cells.³⁷⁷ These HTLV-1–infected cells and reacting CTLs produce proinflammatory cytokines (tumor necrosis factor-alpha [TNF- α], IFN- γ , IL-1 β), leading to demyelination and axonal damage.

HTLV-1 Uveitis

HTLV-1 infection induces uveitis, which is an inflammatory disorder that affects the intraocular tissues. The most characteristic ocular findings of HTLV-1 uveitis were vitreous opacities

associated with mild iritis and mild retinal vasculitis. This type of uveitis is classified as intermediate uveitis, because the intermediate part of the eyeball is affected by the inflammation.²⁴² Infiltrating HTLV-1–infected cells are implicated in the pathogenesis of this uveitis. Patients with HTLV-1 uveitis are treated with topical or systemic corticosteroids.

Infective Dermatitis

Infective dermatitis, a severe, chronic, relapsing dermatitis first described in Jamaican children, was found to be associated with HTLV-1 infection.²⁰⁴ Infective dermatitis is characterized by a severe exudative dermatitis with crusting of the scalp, neck, external ears, axillae, and groin. In the skin region, *Staphylococcus aureus* or beta-hemolytic *Streptococcus* infection is detected. Oral trimethoprim-sulfamethoxazole is the treatment of choice because of low cost and effectiveness.²²⁴ Patients with infective dermatitis tend to have an elevated provirus load. Infective dermatitis has been reported prior to the development of ATL and HAM/TSP; therefore, infective dermatitis is implicated as an indicator for other HTLV-1–associated diseases.

DIAGNOSIS

HTLV-1 infection can be diagnosed by the presence of antibody to HTLV-1. The presence of serum antibodies against HTLV-1 can be demonstrated by enzyme-linked immunosorbent, particle agglutination, indirect immunofluorescence, and Western blot assays. Provirus load, which represents the number of infected cells, is measured by real-time PCR. In HTLV-1–infected individuals, provirus load ranges from less than 0.01% up to more than 50% (the percentage is shown as provirus positive cells as if a cell contains one copy of HTLV-1 provirus). High provirus load is thought to be associated with HTLV-1–associated diseases and is a risk factor for ATL.

The diagnostic criteria for ATL have been defined as follows: (a) histologically and/or cytologically proven lymphoid malignancy with T-cell surface antigens; (b) abnormal T cells present in the peripheral blood (except for lymphoma-type ATL)—these abnormal T lymphocytes can include not only typical ATL cells, the so-called flower cells, but also the small and mature T lymphocytes with incised or lobulated nuclei that are characteristic of the chronic or smoldering type of ATL; (c) antibody to HTLV-1 present in the serum at diagnosis; and (d) demonstration of monoclonal integration of HTLV-1 provirus by the Southern blot method.

PREVENTION AND CONTROL

Treatment

As expected, reverse transcriptase inhibitors (RTIs) can suppress replication of HTLV-1 *in vitro*, although the efficacies of various RTIs against HTLV-1 are different from those against HIV-1.¹³⁰ RTIs can also block HTLV-1 infection if they are administered at the time of HTLV-1 exposure.²³⁴ However, RTIs cannot suppress HTLV-1 infection if they are administered 1 week later.²⁴⁰ This finding suggests that clonal proliferation of infected cells is predominant after the initial *in vivo* spread of HTLV-1. It is consistent with the observation

that RTIs do not change provirus load in patients with HAM/TSP.³⁶⁴ Thus, the clinical use of inhibitors against HTLV-1 replication is limited to preventative administration for accidental exposure to HTLV-1–positive blood.

HTLV-1–associated diseases, ATL and HAM/TSP, must be treated. ATL is a poorly treatable neoplastic disease owing to its resistance to anticancer drugs and the complication of immunodeficiency. Patients with acute or lymphoma-type ATL are usually treated with combination chemotherapy. With the most commonly used combination chemotherapy in Japan (VCAP-AMP-VECP therapy: vincristine, cyclophosphamide, doxorubicin, and prednisone [VCAP], doxorubicin, ranimustine, and prednisone [AMP], and vindesine, etoposide, carboplatin, and prednisone [VECP]),⁴⁰⁶ 81% of the 93 eligible patients responded, with 33 patients obtaining complete response (35.5%) and 42 obtaining partial response (45.2%). The median survival time of patients was 13 months. This protocol was found to be superior to biweekly CHOP (cyclophosphamide, hydroxydaunorubicin, vincristine [Oncovin], and prednisone) therapy.³⁷⁴

The major impediments in therapy for ATL patients are the drug resistance of ATL cells to chemotherapeutic agents and the profound immunodeficient state of patients.³²⁴ The immunodeficiency of ATL patients may be caused by the immunosuppressive function of ATL cells, because they likely arise from Treg cells¹⁸⁰ and can produce immunosuppressive cytokines.²⁶⁵ The immunodeficiency of ATL patients leads to complications by various opportunistic fungal, viral, and bacterial infections, which worsens the prognosis.³⁵⁵

Successful allogeneic bone marrow transplantation was reported for an acute ATL patient in 1996.^{42,368} Furthermore, allogeneic stem cell transplantation (alloSCT) has been reported to be effective in ATL patients.³⁸⁰ However, because ATL patients are usually older (63 years in Japan), the intense conditioning (whole body irradiation, anticancer drugs) is too toxic. Patients treated with alloSCT with reduced-intensity conditioning (RIST), in which reduced dose of anticancer drugs and immunosuppressive agents are used, had overall survival at 3 years of 36%.³⁶³ These studies showed that 30% to 40% of ATL patients became long-term survivors with either conventional alloSCT or RIST.²⁷⁶ HTLV-1 provirus load decreased remarkably in many patients who received alloSCT,²⁷⁷ suggesting that cell-mediated immunity to HTLV-1 was enhanced in these patients—an observation that might account for the efficacy of this therapy. ATL cells express high levels of cell surface Fas antigen and are susceptible to Fas-mediated signaling.^{357,416} These findings suggest that ATL cells are vulnerable to CTLs, which accounts for the good therapeutic responses. In individuals who received alloSCT, CTLs to Tax peptides were activated in the recipients and provirus load became profoundly suppressed, suggesting a role of anti-HTLV-1 immune responses in the efficacious outcome.¹¹⁹ Nevertheless, further study is needed to determine whether CTLs to Tax indeed play a critical role for the efficacy of stem cell transplantation therapy.

A humanized monoclonal antibody, rituximab, dramatically improved the prognosis of B-cell lymphoma patients. Similarly, monoclonal antibodies to CD25,²⁸⁹ CD2,⁴²⁸ CD52,⁴²⁷ and CCR4¹⁵¹ have been shown to suppress ATL cells *in vivo*. KW-0761, a defucosylated humanized anti-CCR4 antibody, has shown to be effective in some ATL patients.⁴⁰⁸ Among 15

patients treated with KW-0761, 5 patients achieved positive objective responses: two complete and three partial responses. These antibody therapies are potentially beneficial for ATL patients because they avoid the adverse effects of chemotherapeutic agents on the host. In addition, it has been reported that a proteasome inhibitor, bortezomib, suppresses tumor formation by ATL cells *in vivo*.^{236,307,359} NF- κ B inhibitors also effectively induced apoptosis of ATL cells, because NF- κ B is highly activated in ATL cells.^{342,393} These findings suggest the potential efficacy of these compounds for ATL patients.

Previous studies reported that the prognosis of patients with aggressive subtypes of ATL (acute and lymphoma types) was less than 1 year, whereas that of indolent types (chronic and smoldering types) was much longer.³²⁵ It was recently reported that the mean survival time of patients with the indolent subtypes of ATL was only 4.1 years,³⁵³ indicating that the prognosis of indolent ATL is poorer than previously thought. Therapy using IFN- α combined with zidovudine has been reported to be highly effective for these indolent ATL patients.²⁸ However, viral replication has not been demonstrated in ATL cells. Instead, many nonsense mutations, deletion, and insertions of HTLV-1 provirus were reported in ATL cells.^{73,356} Taken together, these data suggest that IFN- α combined with zidovudine may suppress the growth of ATL cells without any effects on viral replication.

For treatment of patients with HAM/TSP, oral prednisone or injection of IFN- α provides a transient beneficial efficacy.²⁸⁰

Vaccines

The passive transfer of immunoglobulin derived from HTLV-1-infected individuals could block HTLV-1 infection,^{245,310} suggesting that a vaccine to HTLV-1 is capable of inhibiting its transmission. Indeed, immunization with peptide derived from envelope protein²⁵⁷ and vaccines expressing envelope protein³²² can block transmission of HTLV-1 in rabbits and monkeys. Taken together, these findings clearly show that a preventive vaccine for HTLV-1 is possible, a situation quite different from that with HIV-1.

Tax might be a target for a therapeutic vaccine owing to its high immunogenicity. In a rat model, a Tax peptide-based vaccine inhibited the growth of a Tax expressing rat T-cell line *in vivo*.¹¹⁵ However, the fact that Tax expression is frequently lost in ATL cells points to the limitation of Tax for therapeutic vaccine of ATL.

Prevention

HTLV-1 is transmitted via three routes, all of which can be preventable. Mother-to-infant transmission can be reduced by bottle-feeding or freeze-thaw processing of breast milk,⁹ which destroys the infected cells in the breast milk. However, HTLV-1 transmission still occurs in about 5% of infants who do not drink breast milk, indicating the presence of the route other than breast milk for the transmission.³⁵¹ Transmission risk by breast-feeding increased according to HLA concordance between mother and child.³⁴ This finding suggests that immunological responses influence the transmission from mother to child. Blood screening almost completely prevents viral transmission by blood transfusion.¹⁴⁸ RTIs should be useful for preventing *de novo* infection by accidental exposure to contaminated blood, although any clinical evidence has not yet been reported.

PERSPECTIVES

Since the discovery of HTLV-1, extensive studies have revealed a complex network of interactions between viral genes and host factors. This network controls the expression of viral genes and facilitates persistent infection by allowing evasion of the host immune response and promoting the proliferation of infected cells. However, many enigmas remain, including how HTLV-1 drives ATL cells and infected cells to proliferate in the absence of Tax expression, and whether the HTLV-1 provirus is necessary in ATL cells. These long-standing questions might be answered by the discovery of an antisense transcript of HTLV-1, the *HBZ* gene. Recent studies show complex interactions between HBZ and Tax, which likely regulate ATL cells and infected cells to allow their sustained proliferation in the presence of host immune pressure. The complex relationship between Tax and HBZ needs to be further clarified.

A long latent period is necessary for development of ATL, indicating that the accumulation of alterations in the host genome is needed for its onset. The detailed mechanisms of leukemogenesis should be studied further from both viral and cellular perspectives.

Another important issue is the need for better therapies for HTLV-1-associated diseases. Previously, the prognosis of ATL patients was very poor. New therapies, including stem cell transplantation and zidovudine/IFN- α , have improved the prognosis of ATL patients. However, preventive therapy and antiviral therapy are not yet available for HTLV-1. In addition, good therapeutic strategies are needed for treating HAM/TSP, which is an unrelenting debilitating disease.

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Human Immunodeficiency Viruses: Replication

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INTRODUCTION

In the late 1970s and early 1980s, previously healthy patients with symptoms of immunologic dysfunction sought advice and treatment from physicians in the United States and Europe. This new and unusual syndrome was characterized by generalized lymphadenopathy, opportunistic infections (typically *Pneumocystis carinii* pneumonia, cytomegalovirus-associated retinitis, and cryptococcal meningitis), and a variety of unusual cancers (non-Hodgkin's lymphoma and Kaposi's sarcoma). A common accompanying laboratory finding in affected individuals was marked depletion of the CD4+ T-lymphocyte subset in the peripheral blood. The disease was first brought to the attention of the general medical community in June 1981 when

the Centers for Disease Control and Prevention described five California men with severe immunodeficiency in the *Morbidity and Mortality Weekly Report*⁵²⁹ (Fig. 49.1). This notification was followed by several reports describing male homosexuals and intravenous drug users with impaired immune systems characterized by T lymphocytes that responded poorly to antigen and mitogen stimulation in functional assays. Within several months, it became clear that a similar immunodeficiency syndrome was also affecting other groups, including hemophiliacs, blood transfusion recipients, recent Haitian immigrants, and, most significantly, sexual partners or children of members of the various risk groups.

The emerging epidemiologic pattern suggested that the new disease was transmitted by a novel pathogen in contaminated blood or following sexual intercourse with an affected individual. Between late 1981 and early 1983, a variety of microorganisms were proposed as possible causes of acquired immunodeficiency syndrome, or AIDS, as the disease was soon called. In the spring of 1983, Montagnier and colleagues³² at the Pasteur Institute reported the isolation of an agent from the lymph nodes of an asymptomatic individual who presented with generalized lymphadenopathy of unknown origin. During its replication in cultured cells, the lymphadenopathy-associated virus, or LAV as it was named, released high titers of progeny virions that contained magnesium-dependent reverse transcriptase (RT) activity and exhibited electron microscopic (EM) features typical of retroviruses. However, unlike the commonly studied retroviruses such as the avian leukosis viruses (ALVs) and the murine and feline leukemia viruses (MLVs and FeLVs), LAV was highly cytopathic in human peripheral blood mononuclear cells (PBMCs), specifically killing CD4+ T lymphocytes in cell cultures.⁵³² Gallo and colleagues^{608,658} at the National Institutes of Health subsequently reported the isolation of a retrovirus from an AIDS patient, which they named human T-cell leukemia virus type III (HTLV-III) to distinguish it from the non-cytopathic HTLV-1, and obtained the first serologic evidence linking exposure to LAV-like retroviruses and immunodeficient individuals from the various groups at risk. The new retrovirus, associated with AIDS in the United States, Europe, and central Africa and exhibiting morphologic and genetic characteristics typical of the lentivirus genus, was named human immunodeficiency virus, or HIV¹⁴⁰ (and subsequently HIV-1). In 1986, a related but immunologically distinct human retrovirus (now called HIV-2) was recovered from individuals residing in several West African countries such as Senegal, the Ivory Coast, and Guinea-Bissau.¹³⁹ Prior to the discovery and use of highly active

MILESTONES IN HIV-1 RESEARCH

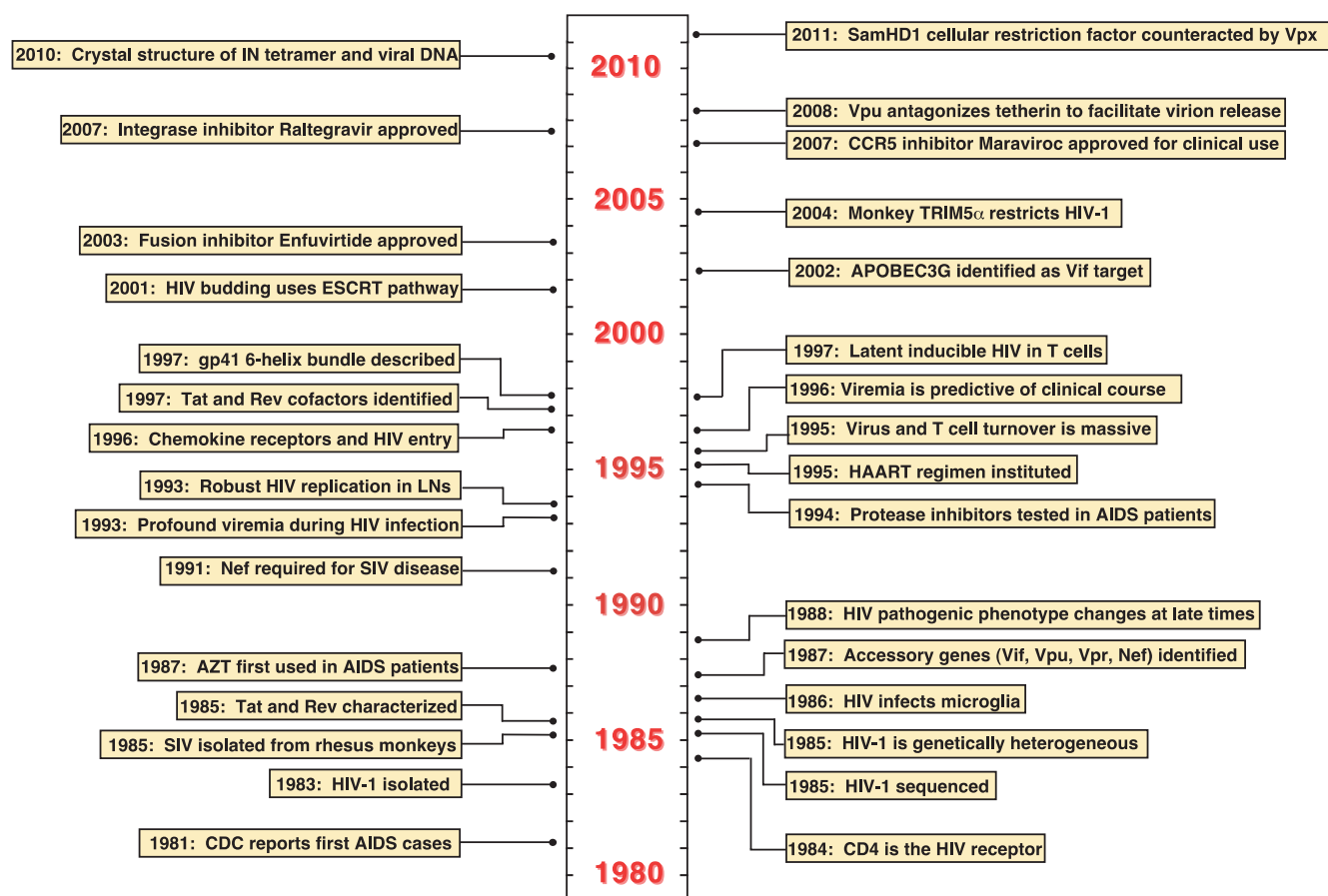


FIGURE 49.1. Landmarks in human immunodeficiency virus (HIV) research. Important discoveries and technical or conceptual advances are noted on either side of the central time line.

antiretroviral therapy (HAART), the risk of death for HIV-1–infected persons was 40-fold higher than the mortality in the general population, whereas HIV-2–infected individuals had a 2- to 5-fold higher risk.^{57,293,614}

CLASSIFICATION OF HUMAN IMMUNODEFICIENCY VIRUSES

One of the first features the Pasteur Institute group noted about HIV-1 was its particle-associated RT activity, a property that placed the new agent in the *Retroviridae* family. This feature was consistent with EM analyses of particles released from infected cell cultures, which revealed 100- to 120-nm enveloped virions, similar in size and morphology to previously studied retroviruses. The mature HIV-1 particles contained a cone-shaped cylindrical core reminiscent of that previously described for visna virus (Fig. 49.2A).²⁶⁹ The cloning and sequencing of proviral DNA, initially purified from productively infected cultures of PBMC/T-cell leukemia lines (T-cell lines), indicated that HIV-1 not only possessed a genomic organization related to other replication-competent retroviruses but also was placed taxonomically in the *Lentivirus* genus.^{620,786} This relationship

is shown diagrammatically in Figure 47.2 of Chapter 47. As their name suggests, lentiviruses were known to cause slow, unremitting disease in sheep, goats, and horses and to target various lineages of hematopoietic cells, particularly monocytes/macrophages and lymphocytes.

After the isolation, molecular cloning, and initial classification of HIV-1, several genetically distinct primate lentiviruses were subsequently discovered and their phylogenetic relationships to HIV-1 were determined. For example, viruses isolated from captive macaques or feral monkey species in Africa were shown to possess particle morphologies and genomic organizations similar to those of HIV-1 (described in Chapter 51). Because inoculation of Asian macaque species, such as rhesus monkeys, with these newly discovered agents recovered from African monkeys induced an AIDS-like illness,¹⁵⁹ these viruses were named simian immunodeficiency virus (SIV) to distinguish them from the human viruses, HIV-1 and HIV-2. The phylogenetic relationships of the two human lentiviruses are depicted in Figure 49.3; the detailed genetic interrelationships of all members of the primate lentivirus genus are presented in Figure 52.1B of Chapter 52. HIV-2 is more closely related to SIV_{sm},³²⁰ a virus indigenous to African sooty mangabey monkeys, than to HIV-1. Because of the close contact between

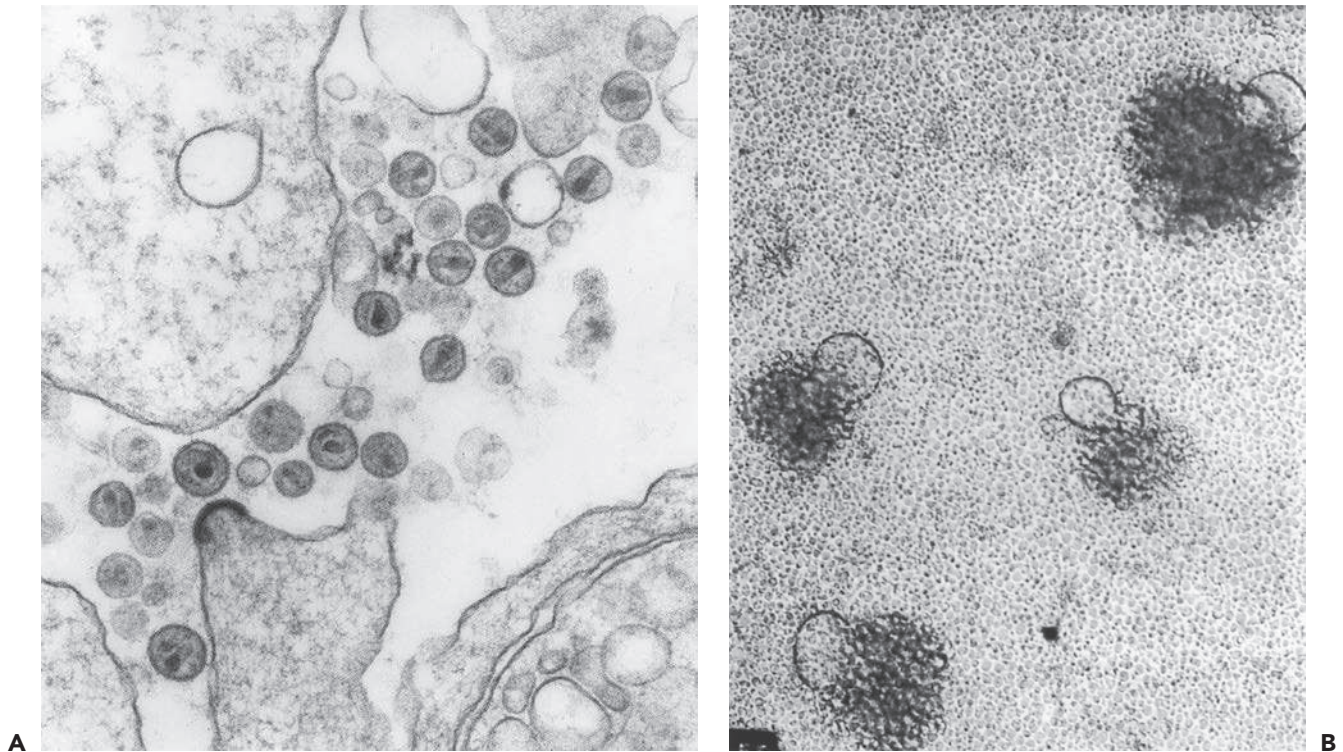


FIGURE 49.2. Primate lentiviruses have a distinct morphology and can induce syncytia during productive infections.

A: Electron micrograph showing a single human immunodeficiency virus 1 (HIV-1) particle in the process of budding from infected cultured human peripheral blood mononuclear cells (PBMCs) and several mature virions containing the characteristic conical or bullet-shaped core ($\times 100,000$). (**A** courtesy of Dr. Jan Orenstein.) **B:** Typical ballooning syncytia induced by primate lentiviruses as visualized by inverted light microscopy at the time of peak virus production.

humans and monkeys, which are hunted for food or kept as pets in West Africa, it is currently thought that HIV-2 represents a zoonotic transmission of SIV_{sm} from monkeys to humans.²⁵³

Based on previously studied replication-competent retroviruses (e.g., MLV, ALV, and HTLV), it was originally thought that HIV-1 would be genetically homogeneous. However, as proviral DNAs corresponding to HIV-1 isolates from Europe, North America, and Africa, became available and were compared to one another, their extensive genetic heterogeneity became apparent.⁴⁴ No two HIV-1 isolates were identical. When subjected to nucleotide sequence analysis, even HIV-1

samples recovered from a single individual exhibited significant heterology. Although nucleotide changes were distributed throughout the HIV-1 genome, the greatest variability occurred in the gene encoding the envelope (Env) glycoprotein, gp160. The term *quasispecies* was subsequently coined to describe the pool of diverse and changing populations of virus present in an HIV-1-infected individual.⁵²¹ Nonetheless, a highly conserved immunodominant domain, located within gp41 Env-coding sequences, was found to elicit antibodies in exposed individuals, reactive with HIV-1 samples of diverse geographic origin.²⁶³ Although not neutralizing, these antibodies could be used in enzyme-linked immunosorbent assay (ELISA) and immunoblotting assays to identify virus-infected persons.

Several factors contribute to the extraordinary genetic heterogeneity of HIV-1: (a) error-prone viral DNA synthesis during reverse transcription (1 to 2×10^{-5} mutations/nucleotide/replication cycle in tissue culture systems; 5×10^{-5} within a patient), (b) high recombination frequencies accompanying reverse transcription, (c) high levels of progeny virus production *in vivo* (10^9 particles/day; millions of replication cycles/year), and (d) large numbers of infected individuals.^{141,359,491,592,759} It has been estimated that within an HIV-1-infected person, viral genetic diversity increases by 1% per year from the founder virus strain during the early asymptomatic phase of the infection.⁶⁸⁹

The earliest phylogenetic analyses of HIV-1 isolates focused on samples from Europe, North America, and Africa;

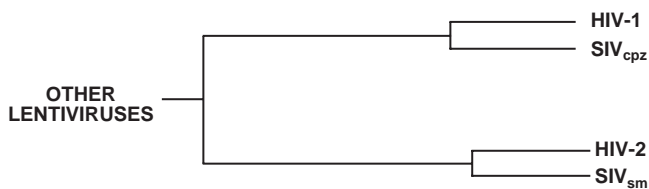


FIGURE 49.3. Phylogenetic relationships of human immunodeficiency virus 1 (HIV-1) and HIV-2 based on identity of *pol* gene sequences. Simian immunodeficiency virus (SIV)_{cpz} and SIV_{sm} are nonhuman primate lentiviruses recovered from a chimpanzee and sooty mangabey monkey, respectively.

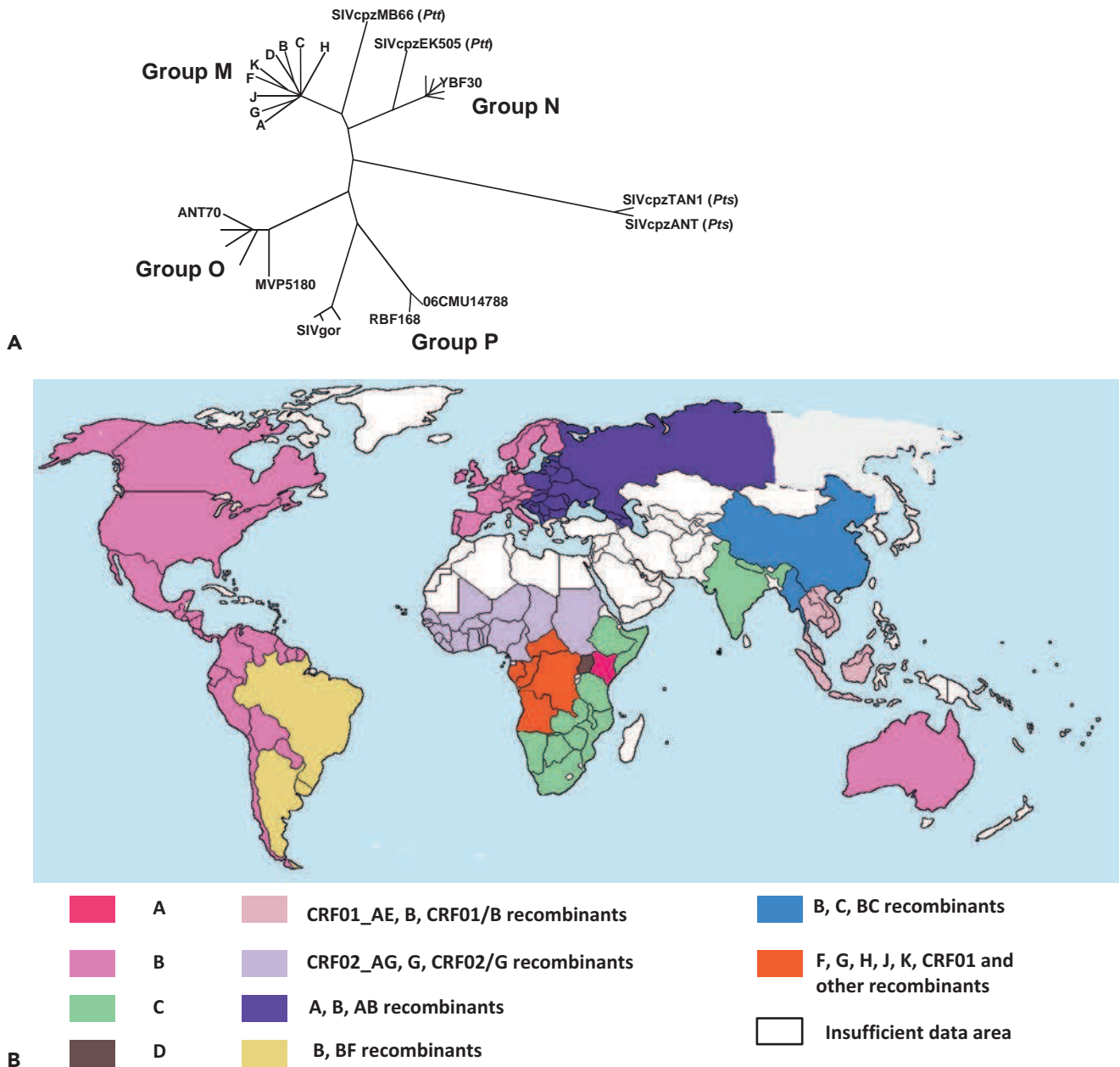


FIGURE 49.4. Human immunodeficiency virus 1 (HIV-1) genetic subtypes and their worldwide distribution. A: Phylogenetic relationships of HIV-1 groups M, N, O, and P with different simian immunodeficiency virus (SIV_{cpz} and SIV_{gor}) isolates. This maximum-likelihood tree is adapted from Foster JL, Denial SJ, Temple BR, et al. Mechanisms of HIV-1 Nef function and intracellular signaling. *J Neuroimmune Pharmacol* 2011;6:230–246 with permission. **B:** The global prevalence of HIV-1 subgroups with the predominant clades or circulating recombinant forms (CRFs) in each geographic region indicated. (Courtesy Dr. Sodsai Tovanabutra, U.S. Military HIV Research Program, Henry Jackson Foundation.)

discrete clusters of viruses were identified from these geographical regions. Distinct genetic subtypes or clades of HIV-1 were subsequently defined and classified into four groups: M (main), O (outlier), N (non-M, non-O), and P (Fig. 49.4A). The M group of HIV-1, which includes more than 98% of the global virus isolates, consists of nine clades or subgroups (A, B, C, D, F, G, H, J, and K) and 49 circulating recombinant forms (CRFs).^{462,511,588,632} HIV-1 group O isolates have been recovered from individuals living in Cameroon, Gabon, and

Equatorial Guinea; their genomes share approximately 65% identity with group M viruses.⁷⁴² Early in the epidemic, group O strains may have been responsible for more than 20% of HIV-1 infections in Cameroon but are presently associated with approximately only 1%.⁷⁷³ Group N HIV-1 strains have been identified in 13 infected Cameroonians and are detectable using currently available immunoassays. Very recently, HIV-1 isolates, identified in two Cameroonians, have been classified as members of the new HIV-1 group P.⁷⁶⁴

As shown in Figure 49.4A, the nine clades comprising group M HIV-1 isolates are phylogenetically equidistant from one another, with internucleotide distances ranging from 15% to 20% in the *gag* gene and 20% to 30% in *env*.⁶³² Within-clade distances range from 3% to 10% for *gag* and 5% to 12% for *env*; up to 10% sequence diversity in the *env* gene has been reported for a single infected person.⁴¹² All of the HIV-1 group M subtypes can be found in Africa (Fig. 49.4B). With the rapid spread of HIV-1 to southern Africa during the mid- to late 1990s, clade C viruses have become the dominant subtype and now account for nearly half of HIV-1 infections worldwide.⁵⁷⁷ Clade A viruses, the most common HIV-1 subtype in Africa early in the epidemic, currently represent about 12% of infections globally, and are present mainly in Eastern and Central African countries. Clade B viruses, the most intensively studied HIV-1 subtype, remain the most prevalent isolates in Europe, North America, and Australia.

High rates of genetic recombination, a hallmark of retroviruses, are generated as a consequence of a single cell being infected by genetically distinct viral strains and arise as a result of template switching between two different viral RNAs during the reverse transcription reaction (see section on Reverse Transcription). By 1995, it became apparent that a significant fraction of the global diversity of HIV-1 group M viruses included interclade CRFs, which arose by the intermixing of viruses co-circulating in a particular geographical locale. It is now appreciated that HIV-1 recombinants will be found in geographical areas such as Africa, South America, and Southeast Asia where multiple HIV-1 subtypes coexist and account for nearly 20% of circulating HIV-1 strains globally. Molecularly, the genomes of these CRFs resemble patchwork mosaics, with juxtaposed diverse HIV-1 subtype segments, reflecting the multiple crossover events contributing to their generation. Most HIV-1 recombinants have arisen in Africa and a majority contains segments originally derived from clade A viruses. Although an intergroup (groups M and O) recombinant virus has been described,⁵⁸⁸ no recombination between HIV-1 and HIV-2 has been reported, although mixed *in vivo* infections involving the latter are known to occur.²⁷⁹

A primate lentivirus designated SIV_{cpz}, with a genomic structure very similar to that of HIV-1, including its signature *vpu* gene, has been isolated from two chimpanzee subspecies, *Pan troglodytes troglodytes* (*ptt*) and *Pan troglodytes schweinfurthii* (*pts*). The prevalence of SIV_{cpz} recovered from *ptt* animals (SIV_{cpzPtt}) in the wild varies widely (0 to 35%) compared to the more even and higher distribution of SIVs infecting sooty mangabeys and African green monkeys. Phylogenetically, the SIV_{cpzPtt} strains are related to HIV-1 groups M and N but not to group O or SIV_{cpzPis} (Fig. 49.4A), and geographically separated chimpanzee populations carry distinct genetic lineages of SIV_{cpzPtt}.³⁸⁵ A prospective study of habituated chimpanzees in Tanzania, using noninvasive approaches to acquire fecal and urine specimens for antiviral antibody and nucleic acid assays, reported that SIV_{cpz}-infected animals experienced a 10- to 16-fold increased risk of death and virus-positive females had decreased fertility and lower infant survival than uninfected chimpanzees, indicating that these infections are pathogenic in the wild.³⁸⁴ It has been suggested that SIV_{cpzPtt} is the source of recently described SIV_{gor} lineages recovered from Western lowland gorillas.⁷⁴¹

It is now generally accepted that cross-species transmission to humans of SIV_{cpz} in Central Africa and SIV_{sm} in West

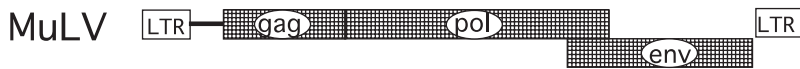
Africa gave rise to HIV-1 and HIV-2, respectively.^{252,253} Based on nucleotide substitutions over time, a common ancestor of HIV-1 group M has been proposed to have emerged in the 1920s,⁸¹³ whereas progenitors for HIV-2 groups A and B have been dated to the 1940s.⁴³⁴ The SIV_{cpz} strains giving rise to HIV-1 have been introduced into humans at least four times (as reflected in groups M, N, O, and P). It is thought that the source of HIV-1 group M, the cause of the worldwide AIDS epidemic, originated in *ptt* chimpanzees living in southeast Cameroon.³⁸⁵ Infected humans very likely migrated from there, via tributaries of the Congo River, to Kinshasa, Republic of the Congo, where HIV-1 transmission greatly accelerated. Of the numerous species-specific strains of SIV that have been identified in more than 40 different species of African monkeys (see Chapter 51), none are related to SIV_{cpz} across the entire viral genome. However, *Vpu*-coding sequences, unique to HIV-1, have been identified in the SIV isolated from the greater spot-nosed monkey (SIV_{gsn}),¹⁴⁹ and phylogenetic analyses indicate that SIV_{gsn} (in the *env* gene) and SIV_{rcm} (in the *gag* and *pol* genes) are the two SIVs most closely related to SIV_{cpz}. On the basis of these findings, it has been proposed that SIV_{cpz} arose as a result of a recombination event involving SIV_{gsn} and SIV_{rcm} lineages in a West Central African monkey species upon which chimpanzees prey.²⁸ According to this model, *ptt* chimpanzees, sharing an overlapping geographical range with greater spot-nosed and red-capped monkeys in West Central Africa, would have been the first member of the great apes to be infected with the putative recombinant progenitor of SIV_{cpz}, eventually giving rise to strains of SIV_{cpzPtt}. The subsequent four cross-species transmissions to humans, thereby generating the M, N, O, and P groups of HIV-1, would be consistent with the tree locations of these primate lentiviruses (Fig. 49.4A).

GENOMIC ORGANIZATION OF HIVs

Nucleotide sequencing of several of the original HIV-1 isolates revealed an unexpected result: in contrast to the well-characterized and intensively studied prototypical retroviruses such as ASLV and MLV, whose genomes contain only three genes (*gag*, *pol*, and *env*) encoding the structural proteins and enzymes required for productive infection, the HIV-1 genome included several additional and overlapping open reading frames (ORFs) of unknown function (Fig. 49.5). Not only were HIV-1 and HIV-2 shown to contain multiple additional ORFs, but also their genomic organizations appeared to be very similar. Further analyses revealed that HIV-1 contained the distinguishing *vpu* gene^{144,726} and HIV-2 carried a signature *vpx*³⁷⁵ gene.

Like all replication-competent retroviruses, the three primary HIV-1 translation products, all encoding structural proteins or enzymes, are initially synthesized as polyprotein precursors, which are subsequently processed by viral or cellular proteases into mature, particle-associated proteins (Fig. 49.6). The 55-kD Gag precursor Pr55^{Gag} is cleaved into the matrix (MA), capsid (CA), nucleocapsid (NC), p6, and the two spacer peptides SP1 and SP2 during or after the release of progeny virions. Autocatalysis of the 160-kD Gag-Pol polyprotein, Pr160^{Gag-Pol}, gives rise to the protease (PR), the heterodimeric RT, and integrase (IN) proteins, whereas proteolytic digestion by a cellular enzyme converts the glycosylated 160-kD Env precursor, gp160, into the gp120 surface (SU) and gp41 transmembrane (TM)

"Simple" Retrovirus



"Complex" Human Retroviruses

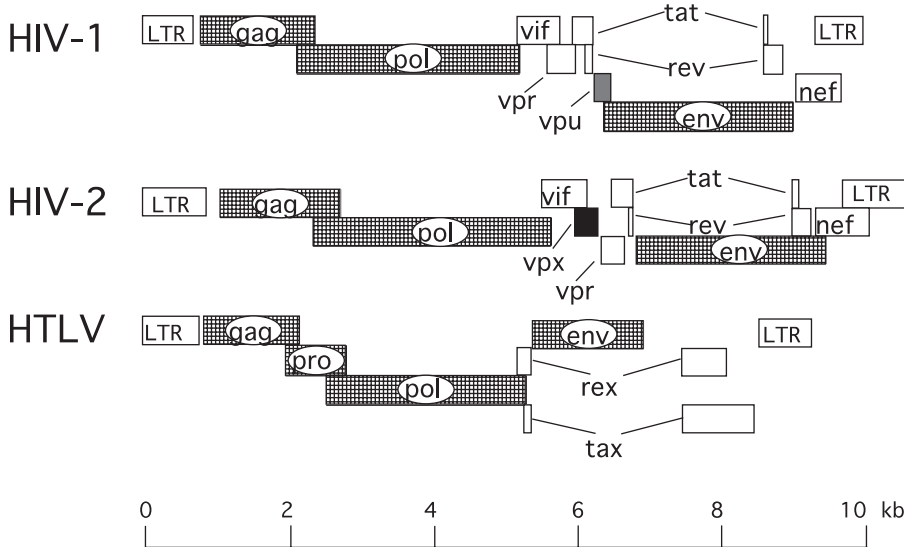


FIGURE 49.5. Genomic organization of simple and complex retroviruses. The genes of Moloney murine leukemia virus (MLV), human T-cell leukemia virus 1 (HTLV-1), human immunodeficiency virus 1 (HIV-1), and HIV-2 are depicted as they are arranged in their respective proviral DNA. The sizes of the different proviral DNA are shown in proportion to the 9.7-kb HIV provirus.

cleavage products. The remaining six HIV-1–encoded proteins (Vif, Vpr, Tat, Rev, Vpu, and Nef) are the primary translation products of spliced messenger RNAs (mRNAs).

HIV-1 and HIV-2 have incorporated multiple sequence elements into their genomic RNAs that direct the balanced and coordinated production of progeny virions. Many of these *cis*-acting RNA elements (Fig. 49.7) are present in other retroviral genomes, but a few are unique to the primate lentiviruses. The 5′ ~330 nucleotide untranslated region of HIV-1 genomic

RNA is highly structured and contains multiple elements that mediate transcriptional elongation of viral RNA transcripts, splicing, genomic RNA dimerization, packaging of full-length viral RNA, and reverse transcription. A schematic model of this 5′ leader sequence, deduced from genetic, functional, structural, and nuclease-accessibility mapping experiments, is shown in Figure 49.7, inset A.

Three regions encompassing the primer binding site (pbs) stem participate in the placement and stabilization of

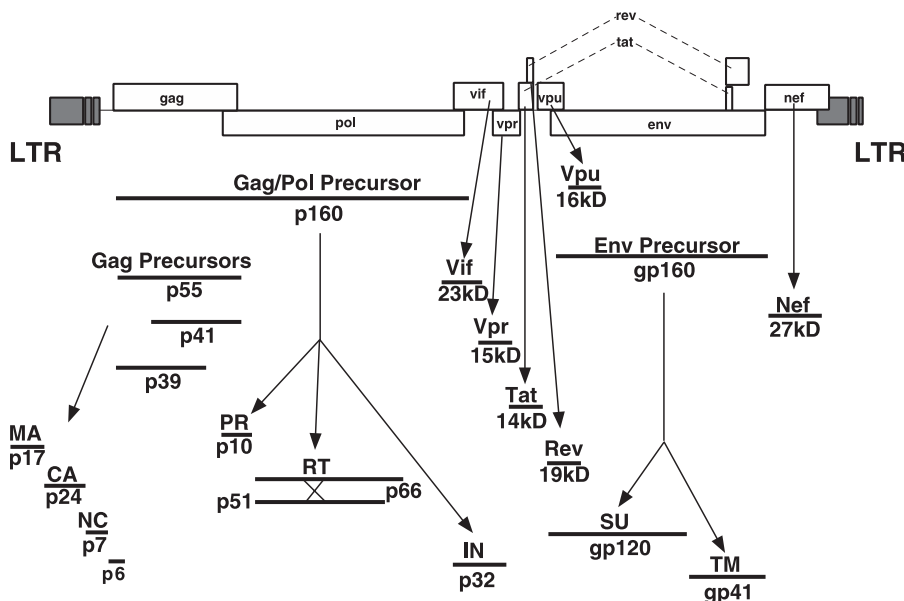


FIGURE 49.6. Human immunodeficiency virus (HIV)-encoded proteins. The location of the HIV genes, the sizes of primary translation products (in some cases, polyproteins), and the processed mature viral proteins are indicated.

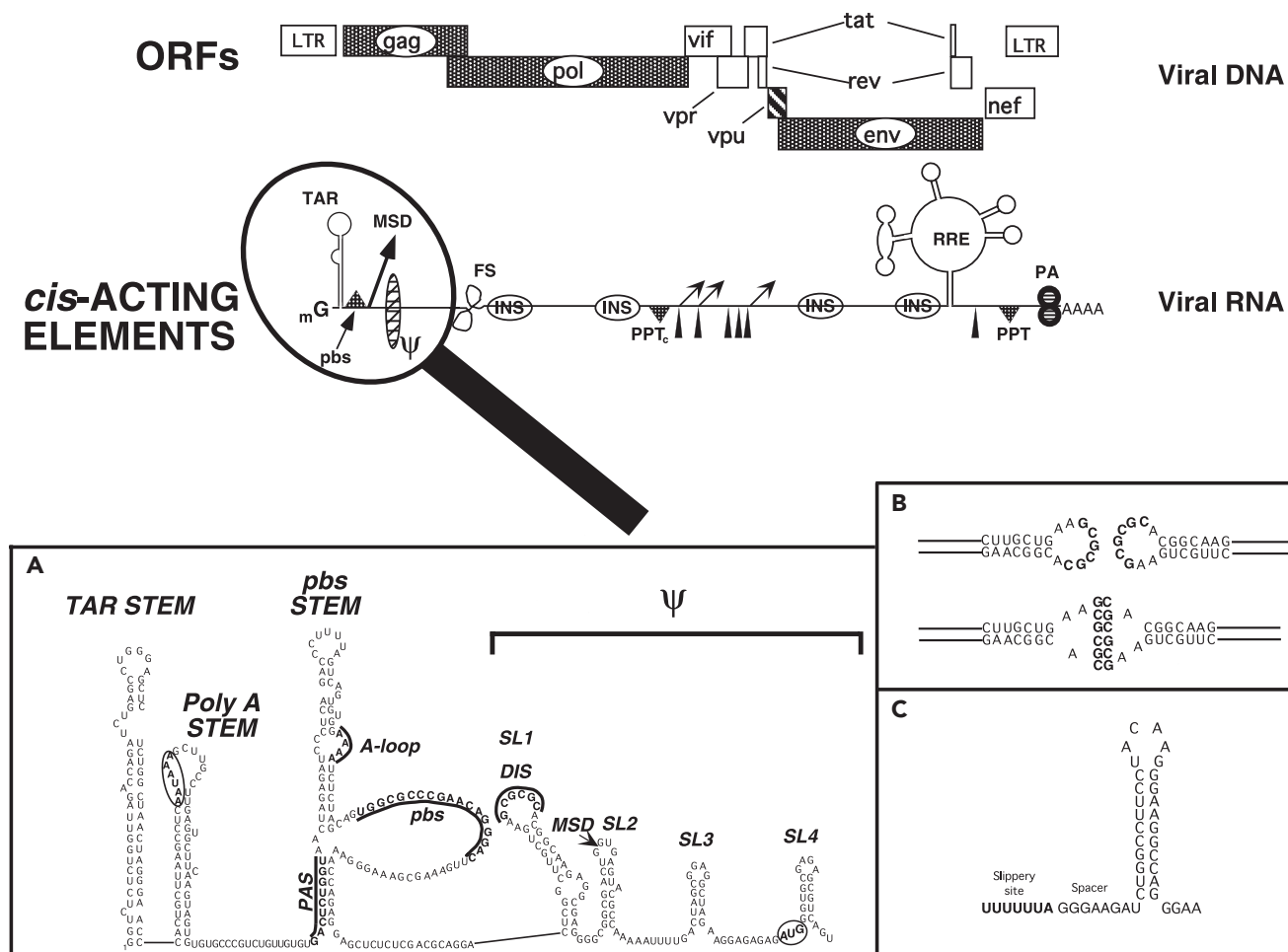


FIGURE 49.7. Cis-acting elements present in the human immunodeficiency virus 1 (HIV-1) genome. Elements associated with HIV-1 genomic RNA include the methyl-capped terminal G residue at the 5' terminus of viral RNA (mG), Tat-responsive stem-bulge-loop structure (TAR), binding site for the tRNA^{Lys} primer (pbs), major splice donor (MSD), RNA packaging site (ψ), frameshifting motif (FS), RNA instability or nuclear retention elements (INSts), central polypurine tract (PPT_c), splice acceptors (arrowheads), internal splice donors (small arrows), Rev-responsive element (RRE), canonical 3' polypurine tract (PPT), and polyadenylation signal (PA). **A:** The putative secondary structure at the 5' terminus of HIV-1 messenger RNA (mRNA). The positions of the TAR stem-loop, circled poly(A) addition signal (AAUAAA), pbs, dimerization initiation sequence (DIS), MSD, primer activation signal (PAS), ψ stem-loops 1 to 4 (SL1 to SL4), and translation initiation AUG codon for Gag are indicated. (**A** adapted from Carteau S, Gorelick RJ, Bushman FD. Coupled integration of human immunodeficiency virus type 1 cDNA ends by purified integrase in vitro: stimulation by the viral nucleocapsid protein. *J Virol* 1999;73:6670–6679.) **B:** The self-complementary DIS sequences, located at the crown of stem-loop 1, participate in the formation of “kissing loop” intermediates, an initial step in the RNA dimerization reaction. **C:** RNA stem-loop structure downstream of the UUUUUUA frameshifting sequence.

the transfer (t) RNA^{Lys3} primer, which is incorporated into HIV-1 particles and is required for the initiation of reverse transcription. These regions include the pbs itself, which can base-pair with the 3' terminal 18 nt of tRNA^{Lys3}; the primer activation signal (PAS), which interacts with the thymidine-pseudouridine-cytidine (T ψ C) arm of the tRNA to trigger the reverse transcription reaction; and the A-loop, which is complementary to the anticodon loop of tRNA^{Lys3} (reviewed in⁶⁰). The unwinding of both the tRNA primer and the pbs stem within the 5' untranslated region (UTR) of the HIV-1 genome, as well as the incorporation of the primer into nascent virions, is facilitated by the NC domain of the Gag polypeptide, which plays the role of a molecular chaperone in this process (reviewed in⁴⁴⁰).

The packaging of HIV-1 genomic RNA into progeny virus particles is also directed by the NC domain of the Gag polypeptide and is primarily dependent on its interaction with the four RNA stem-loops (SL1, SL2, SL3, and SL4) constituting the core encapsidation signal, ψ (Fig. 49.7A).¹⁵⁷ It is currently thought that a fully functional HIV ψ packaging signal may include the entire 5' UTR plus the first 300 nucleotides of the *gag* gene. In virions, the HIV-1 genome is present as an RNA dimer, noncovalently linked at the 5' end of each molecule. Particle-associated dimeric RNA provides an alternative template for reverse transcription, should the enzyme encounter a nick or break during the polymerization reaction. The dimerization initiation sequence (DIS), located at the crown of SL1, contains

a complementary, exposed, and palindromic sequence (Fig. 49.7, insets A and B).^{427,704} Most of the non-B/D HIV-1 subtypes, including the highly prevalent subtype C viruses, have a GTGCAC for their DIS, whereas clade B and D subtypes have a GCGCGC in their DIS. Dimerization is initiated via a *kissing loop* mechanism involving base-pairing between the palindromic sequences present on paired HIV-1 genomes.⁵⁷⁹ Other regions of SL1 participate in stabilizing the RNA dimer into a more extended duplex structure,²⁰⁷ a process also believed to be chaperoned by NC. The ψ packaging domain also contains the major splice donor (MSD) (near the crown of SL2) and the AUG Gag start codon (at the base of SL4).

It has been proposed that the HIV-1 leader can switch from the multiple hairpin structure shown in Figure 49.7A to an alternate, rod-like conformation, and that each conformer specifically promotes either packaging or translation of the viral RNA.³⁴⁰ In this model, the individual stem-loops are formed as intermediates during RNA synthesis, but the entire HIV-1 leader is eventually converted to the thermodynamically more stable rod structure containing an exposed AUG Gag start codon and an occluded DIS motif. The rod conformer, containing a masked DIS, may promote the translation of HIV-1 mRNAs into viral-encoded proteins. A subsequent switch to the alternative conformation, mediated by NC, would expose the DIS and ψ elements, favoring dimerization, packaging, and, ultimately, reverse transcription of the viral genomic RNA.^{160,575} Evidence for the existence of alternative RNA conformers in virions or infected cells and the hypothesized conformational switch is still forthcoming.

In HIV-1, the AAUAAA binding site for the cleavage and polyadenylation factor (CPSF) and the GU/U-rich binding site for the 3' terminal cleavage stimulation factor (CstF) are both situated within the repeat (R) regions at each end of the viral genomic RNA. An active 5' poly(A) site would obviously compromise the generation of long RNA transcripts by mediating premature cleavage and polyadenylation. A polyadenylation enhancer, situated upstream of the 3' poly(A) signals, stabilizes the binding of CPSF to AAUAAA and promotes poly(A) addition, whereas the 5' poly(A) stem or the closely positioned MSD has been reported to suppress polyadenylation activity.^{25,161,258}

Lentiviruses are unique among retroviruses in having a novel central polypurine tract (PPT) in addition to the canonical U3-proximal PPT, both of which are used for the synthesis of plus-strand viral DNA.¹¹⁴ The viral genomic RNA also contains a heptameric UUUUUUA “slippery” sequence within the *gag* gene where ribosomal frameshifting (FS) occurs. This sequence functions in conjunction with a downstream 8-nt spacer and hairpin (Fig. 49.7C) to mediate *minus 1* translational frameshifting.³⁵³ The viral RNA also folds into two additional complex structures (the transactivation response region [TAR] and Rev-responsive element [RRE]), which are involved in RNA synthesis and RNA nuclear-to-cytoplasmic transport, respectively. Multiple inhibitory sequences (INSSs), associated with the instability or nuclear retention of HIV transcripts, are scattered throughout genes encoding the *gag*, *pol*, and *env* genes.^{480,640} Finally, although not strictly considered a *cis*-acting element, HIV-1 genomic RNA contains a significantly higher adenosine deoxyribonucleotide (A) content (approximately 39%) than does mammalian DNA; this bias contributes to an unusual codon usage.⁵¹

The secondary structure of the entire HIV-1 genome has recently been determined using a technique called selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE).⁷⁹¹ SHAPE is based on the chemical modification of unpaired nucleotides in RNA molecules and the resultant termination of reverse transcription at the altered site. Regions of RNA with significant secondary structure are not readily modified, whereas looped-out RNA segments exhibit high SHAPE reactivity. This allows the determination of the folded state of RNA using a computer algorithm.⁵⁰³ Some of the tightly base-paired RNA regions identified by SHAPE correspond to previously described TAR structures at the 5' and 3' termini of the HIV-1 genome, the SP1 encapsidation site in the 5' UTR, and the RRE (Fig. 49.7). Interestingly, structured RNA segments were also present in protein-coding regions located between individual Gag, Pol, and Env subdomains, which give rise to mature protein cleavage products. In addition, base-paired regions of HIV-1 RNA are located adjacent to sequences encoding protein domains that undergo co-translational folding or unfolding.⁷⁹¹ Ribosomal pausing induced at these structured RNA segments may retard translation to allow sufficient time for a nascent protein to attain its final conformation. Nonstructured RNA regions encoding the gp120 variable loops were enclosed by stable RNA duplexes, yet another example of co-related RNA and protein structures.

BIOLOGY OF HIV INFECTIONS

The main cellular targets for HIV-1 in infected individuals are the CD4+ T lymphocytes, CD4+ cells of the macrophage lineage, and some populations of dendritic cells (DCs). A quintessential property of HIV-1 and the other primate lentiviruses is to sequentially use CD4 and a second cellular receptor during entry into susceptible cells, a subject discussed more extensively in the section dealing with virus binding and entry. The earliest studies used mitogen-activated human PBMCs for HIV-1 isolation and propagation. Some of the original HIV-1 isolates were also able to infect continuous CD4+ T leukemia cell lines such as CEM, Jurkat, Hut-78 (including its H9 derivative), and the HTLV-1-containing MT-2 and MT-4 cell lines. Human T-cell lines were obviously more logistically tractable than PBMCs and their use greatly facilitated early molecular and genetic studies of HIV-1.

Replication and Tropism

HIV-1 isolates were initially classified on the basis of their replicative, cytopathic, and tropic properties. A majority of primary virus isolates, which replicated slowly and generated small amounts of progeny particles, were labeled “slow/low” to distinguish them from “rapid/high” HIV-1 strains, which exhibit faster infection kinetics and release high titers of virions. Some HIV-1 primary isolates, particularly those recovered from symptomatic individuals, induced syncytium formation (see Fig. 49.2B). These isolates were designated syncytium-inducing (SI) to distinguish them from non-syncytium-inducing (NSI) strains. Some SI isolates are able to infect CD4+ T-cell lines but not monocyte-derived macrophages (MDMs) and have been designated T-cell-line or TCL-tropic viruses. Cell tropism was a third property used to classify HIV-1 primary isolates. All HIV-1 strains are able to productively infect activated PBMCs;

TABLE 49.1 Tropic and Biological Properties of HIV-1 Isolates

Chemokine co-receptor used	PBMC replication	Macrophage replication	T-cell line replication	Replicative phenotype	Syncytium-inducing phenotype
X4	+	—	+	Rapid/high	++
R5	+	+	—	Slow/low	—
R5/X4	+	+	+	Rapid/high	+

some can also replicate in cultures of MDMs and have been classified as macrophage- or M-tropic. Other primary HIV-1 isolates are also able to infect both MDMs and T-cell lines; such strains have been classified as dual-tropic. Although exceptions exist, M-tropic isolates are usually NSI and “slow/low,” whereas TCL-tropic strains are frequently SI and exhibit the “rapid/high” replication phenotype. Forced passage of primary isolates in continuous CD4+ human T-cell lines has given rise to T-cell-line–adapted (TCLA) HIV-1 strains, which, in contrast to their progenitors, possess increased sensitivity to both neutralizing antibodies and soluble CD4.⁷³⁰

The discovery that α and β chemokine receptors function as co-receptors (in addition to CD4) for virus entry^{127,181,216} led to a revised classification system for HIV-1 strains (Table 49.1). Isolates are now categorized on the basis of chemokine receptor usage in fusion assays, in which CD4 and an individual co-receptor protein are expressed in reporter cells, or in entry assays, in which small-molecule co-receptor–targeted inhibitors, specific for CCR5 or CXCR4, block single-cycle or spreading viral infections in T-cell lines or human PBMCs. As indicated in Table 49.1, HIV-1 isolates using the CXCR4 receptor (now designated X4 viruses⁴⁷) are frequently TCL-tropic, SI strains that exhibit enhanced cytopathicity and more vigorous replicative properties. In contrast, HIV-1 strains exclusively using the CCR5 receptor (R5 viruses) may be M-tropic and NSI. Dual-tropic R5/X4 strains exhibiting a continuum of tropic phenotypes are frequently SI. Interestingly, most T-cell leukemia cell lines express high levels of CXCR4 but are CCR5 negative, explaining in part why primary HIV-1 isolates fail to replicate in these cells.

HIV-1 Infection of CD4+ T Lymphocytes

CCR5-using viruses are the predominant HIV-1 strains detected in recently infected individuals and remain the dominant virus throughout the asymptomatic phase of HIV-1 infection. *In vivo*, CCR5 is exclusively expressed on memory CD4+ T cells, a subset present at high levels in nonlymphoid tissues (e.g., in the gastrointestinal tract and lung).¹⁹¹ Many of these tissue-infiltrating memory CD4+ T cells are also activated. In contrast, CXCR4 is expressed on both naïve and memory CD4+ T-cell populations. In peripheral blood, where a majority of the circulating lymphocytes are naïve, 80% to 95% of the CD4+ T cells may express CXCR4, whereas only 5% to 10% produce detectable CCR5.^{280,560} Because R5 HIV-1 strains predominate in recently infected individuals, marked depletion of CCR5-expressing memory CD4+ T lymphocytes in the gastrointestinal tract has been reported, whereas more modest reductions of total CD4+ T-cell numbers in the blood occur during the acute infection.⁵¹⁶ The emergence of X4 variants

during the symptomatic phase of infection, which occurs in approximately 50% of individuals infected with clade B HIV-1, is referred to as *co-receptor switching*. It is associated with accelerated CD4+ T-cell depletion and more rapid progression to AIDS. Such infected individuals have 30- to 70-fold higher frequencies of virus-infected naïve cells than persons who harbor only R5 virus, consistent with the co-receptor usage of each HIV-1 strain.⁶⁴ The accelerated and specific decline of the naïve CD4+ T cells after the emergence of X4 strains represents an additional insult to the immune system that severely impedes CD4+ T-lymphocyte renewal and heralds the beginning of a downhill clinical course.

Studies of acute HIV and SIV/SHIV infections have shown that nonactivated CD4+ T lymphocytes in the mucosal lamina propria and draining lymph nodes are the principal virus targets during this phase of the infection.^{447,562,851} Paradoxically, the innate immune responses directed against HIV following virus acquisition induce a cascade of inflammatory cytokines that greatly increases the numbers and activation status of CD4+ T cells. In contrast to these findings, multiple studies have shown that resting PBMCs in culture are highly refractory to HIV-1 infection; no viral proteins or progeny virions are produced.^{718,843} PBMCs, activated with mitogens or following incubation with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) and propagated in the presence of interleukin-2 (IL-2), are quite susceptible to HIV-1 and produce high titers of infectious virus. The block to HIV-1 replication in noncycling cultured PBMCs is not presently understood. Indeed, cell cycle studies have shown that progression into G₁b is required to achieve productive HIV-1 infections.⁴⁰⁵ *In vivo*, activated CD4+ T lymphocytes are the main source of progeny virions. Nonetheless, an inducible reservoir of resting “latently infected” memory CD4+ T cells, containing integrated HIV-1 DNA and expressing no viral proteins, is established in exposed individuals within days of transmission and is thought to be the driving factor preventing the eradication of this viral infection.^{135,137}

During infections of human T-cell lines or CD4+ T lymphocytes, HIV-1 can spread through the cultures by two different mechanisms: (a) fluid phase diffusion of cell-free particles or (b) virological synapse (VS)-mediated cell-to-cell contact. Early studies reported that cell-to-cell transmission of HIV-1 was 10²- to 10³-fold more efficient than that of cell-free virus.¹⁸⁴ The extremely low particle-to-infectivity ratio (10⁻³ to 10⁻⁵) for HIV-1 undoubtedly contributed to the inefficient spread of cell-free virions. The formation of a VS with T cells, first reported for HTLV-1,³⁴⁴ utilizes some of the components of the immunologic synapse machinery involved in the interaction between lymphocytes and antigen-presenting cells.⁷⁰⁹ The formation of the HIV-1 VS is initiated by the binding of

gp120 in the infected cell to CD4 molecules on the surface of the target cell.³⁶⁵ This conjugate is further stabilized by interactions between leukocyte function–associated antigen (LFA) and intercellular adhesion molecules (ICAMs), which promote additional recruitment of actin, viral proteins (Gag and Env), CD4, and integrins to cell–cell contact regions. Cytoskeletal remodeling drives virus assembly in the infected cell to the plasma membrane and toward the target cell. Progeny particles released into the synaptic space enter the recipient cell by fusion with the plasma membrane or following passage through endosomal compartments.⁶⁶⁰ The co-polarization of clustered viral proteins in the donor cell with receptors in multiple adjacent recipients can also lead to the formation of polysynaptic structures.³⁶⁶ Filopodial bridges and other actin-containing tubular extensions of the plasma membrane have been reported for delivering virions to distant target cells.⁶⁹³ Inhibitors of HIV-1, including neutralizing antibodies, are able to block cell-free and cell-to-cell-mediated virus infectivity, although transfer across the VS may offer some protection from these agents.¹²¹

Although HIV-1 causes the loss of CD4+ cells in culture and depletion of this T-lymphocyte subset in virus-infected individuals, the mechanisms responsible for this cytopathic effect are still incompletely understood. HIV-1–induced killing of cultured CD4+ T cells (primary T cells and T-cell lines) occurs by necrosis or cytolysis rather than by classical apoptosis, which involves the activation of cysteinyl aspartate-requiring proteases (known as caspases).⁷² Some viral-encoded proteins including Vpr and Env are cytotoxic when expressed alone or in the context of a productive HIV infection. *In vivo*, the massive depletion of CD4+ T lymphocytes in blood and tissues during acute HIV and SIV infections is now recognized to be the result of direct virus-induced cell killing.^{506,516} Nonetheless, apoptotic CD4+ T lymphocytes are present in the peripheral blood and lymph nodes of HIV-1 seropositive individuals during the chronic phase of infection. These cells are uninfected and are thought to represent the effects of bystander cell damage due to the unrelenting and generalized immune activation, a major factor contributing to HIV-1 pathogenesis.²⁸²

HIV-1 Infection of Macrophages and Dendritic Cells

All known lentiviruses have retained the capacity to replicate in nonproliferating, terminally differentiated tissue macrophage. Unlike the oncoretroviruses, which require mitosis and dissolution of the nuclear membrane to complete a cycle of replication, HIV-1 is able to productively infect G₁ (but not G₀)–arrested macrophages. This property requires that the HIV-1 preintegration complex (PIC) be translocated through intact nuclear pores, a process in which the capsid (CA) protein modulates interactions with the nuclear transport machinery in nondividing cells⁴³² (see section on Nuclear Import). In HIV-1–positive individuals, infected macrophages contribute to viral persistence and dissemination to the central nervous system (CNS). HIV-1 is thought to enter the CNS very soon following transmission as virus-free particles or by the trafficking of infected lymphocytes (and possibly infected monocytes). HIV-1 does not productively infect neurons but instead targets perivascular macrophages in the choroid plexus and parenchymal microglial cells. Multinucleated giant cells, suggestive of virus-induced fusion of microglia and/or macrophages, are a histopathologic characteristic of HIV-1–associated neuropathy.

Neurologic dysfunction affecting the CNS and the peripheral nervous system is observed in 40% to 70% of virus-positive individuals. HIV-1 replication in monocyte/macrophage lineage microglia within the CNS may persist in patients receiving HAART because of inefficient transfer of drugs across the blood–brain barrier. This viral reservoir, as well as HIV-1 present in latently infected memory CD4+ T cells, could contribute to the rebound of plasma viremia invariably observed after cessation of antiretroviral drug administration.

Virtually all information pertaining to HIV-1 replication in macrophages derives from studies of an *in vitro* surrogate: monocyte-derived macrophage (MDM). The vigorous replication by M-tropic viral strains in MDM requires that blood-derived monocytes undergo differentiation *in vitro*.⁶⁸² MDMs and tissue macrophages express lower surface levels of CD4, CCR5, and CXCR4 than CD4+ T lymphocytes.^{441,788} Nonetheless, only M-tropic R5 HIV-1 strains are able to initiate infections of MDM and only a subset of M-tropic viruses are also neurotropic.³²⁶ Most primary HIV-1 isolates from blood are weakly M-tropic; strong macrophage tropism is mainly seen with variants from tissues such as the CNS, where macrophage dependence is paramount.⁵⁹⁸ Like productive infections of T lymphocytes, HIV-1 replication in MDMs requires integration of the reverse transcript.²⁰⁶ However, in contrast to numerous reports demonstrating that assembly and release of progeny virions occur at the plasma membrane of productively infected T cells, electron microscopy of HIV-1–infected MDM revealed accumulations of particles within intracellular vacuoles of endosomal origin.^{576,589} This interpretation has been questioned by reports showing that particles within putative internal vesicles were in a compartment, distinct from multivesicular bodies, which communicates with the exterior through tubular channels continuous with the highly invaginated macrophage plasma membrane.^{45,180,798} In fact, a recent study employing electron tomography and electron microscopy with stereology has reported that MDMs release progeny virions into a pre-existing membranous compartment, contiguous with the plasma membrane, which expands in size during HIV-1 infection.⁷⁹⁷ Virus particles that assemble in this internal compartment in MDMs are rapidly translocated to the VS following contact with susceptible T cells.²⁷⁷

DCs are antigen-presenting cells that capture, transport, and present antigens to CD4+ and CD8+ T lymphocytes. Two main subtypes of DCs have been identified: myeloid DCs (MDCs) and plasmacytoid DCs (PDCs). MDCs reside in multiple tissues including the skin (where they are called Langerhans cells) and the intestinal and genital tract mucosa. The less abundant PDCs are found in the blood, T-cell zones of lymph nodes, and thymus and can be recruited to sites of inflammation. After activation, MDCs secrete IL-12, whereas PDCs produce type I interferons and inflammatory cytokines. MDCs present near mucosal surfaces may represent the first line of defense against sexually transmitted HIV-1, transporting virus particles from this portal of entry to draining lymph nodes where they are degraded into antigenic peptides for presentation to CD4+ T lymphocytes. Paradoxically, this critical initial response exacerbates the HIV-1 acute infection by delivering virus to the very T-cell subset that is susceptible to and can massively amplify progeny virus production. Because of the low abundance of DCs in tissues and the small number of these cells recoverable from clinical specimens, most studies evaluating the interaction

of DCs with HIV-1 have used monocytes differentiated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. These derivatives are known as monocyte-derived DCs (MDDCs).

Productive HIV-1 infections of MDDCs are very inefficient compared to infections of activated CD4⁺ T cells, although vigorous virus replication is observed when DCs are first pulsed with virus and then co-cultivated with CD4⁺ T lymphocytes, a process called *trans-infection*.²⁵⁹ Immature DCs (IDCs) express relatively low levels of CD4, CCR5, and CXCR4 and larger amounts of C-type lectin receptors (CLRs) such as dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and dendritic cell immunoreceptor (DCIR), which capture HIV by binding to gp120-associated high-mannose glycans. Functionally, maturation of DCs leads to (a) increased capacity to degrade and present antigen to T cells by forming immunologic synapses, (b) augmented secretion of T-cell co-stimulatory molecules, and (c) potent suppression of endolysosomal degradation. Cell surface CLR expression, including that of DC-SIGN, is markedly reduced in mature DCs; capture of HIV-1 is now considered to be CLR, gp120, and CD4 independent in mature DCs.

Early studies reported that HIV-1 was internalized in trypsin-resistant, low-pH compartments for several days in MDDCs or cells expressing DC-SIGN.⁴¹⁵ Co-cultivation with activated CD4⁺ T cells resulted in rapid *trans-infection*.²⁵⁹ EM analyses of infected MDDCs indicated that intact HIV particles were present within cytoplasmic vesicles. Subsequent imaging studies, using green fluorescent protein (GFP)-labeled HIV, revealed that DCs transferred virus to T cells via a VS, utilizing adhesion molecules similar to those used in the formation of the immunologic synapse.⁴¹⁵ Virions in the infected DC were co-polarized with CD4 and chemokine co-receptors in the T lymphocyte at the cell interfaces. More recent experiments have shown that in DCs, HIV-1 resides in a unique surface-accessible intracellular compartment, distinct from endocytic vesicles, and can rapidly traffic to the VS following contact with T cells.⁸³⁷ This unusual DC compartment communicates with the extracellular environment and, similar to structures present in HIV-1-infected macrophages, is composed of highly folded invaginations of the plasma membrane.^{45,798} DC-SIGN/gp120-mediated HIV-1 entry into IDCs by endocytosis very likely results in extensive degradation of viral proteins by an active endocytic system primed for antigen presentation. Consequently, IDCs would only transmit residual nondegraded HIV remaining on their surfaces to T lymphocytes. In contrast, mature DCs, unable to endocytose HIV in a normal fashion, sequester virions in specialized intracellular compartments and, during antigen presentation, rapidly transfer infectious particles to CD4⁺ T cells. Recent studies indicate that HIV-1 infection of DCs, and to a lesser extent mature DCs, is restricted by the interferon-induced host factor SAM domain and HD domain-containing protein 1 (SAMHD1).^{46,328,418} The ability of SAMHD1 to interfere with lentiviral infection is counteracted by Vpx (see section on Vpx).

HIV-1 ANIMAL MODELS

Chimpanzees

In the search for an HIV-1 animal model during the early phase of the AIDS epidemic, cell suspensions from virus-infected individuals were inoculated into a variety of mammalian spe-

cies including nonhuman primates, but only chimpanzees consistently became infected. It was subsequently shown that asymptomatic HIV-1 infections could readily be established in chimpanzees, but viremia was not maintained and no long-standing impairment to the immune system occurred.²⁴ This could reflect genetic changes, which occurred in the SIV_{cpz} progenitor of HIV-1 following its transmission from chimpanzees and adaptation to humans.⁶⁶³ The failure of chimpanzees to develop disease in a timely fashion following inoculation with tissue culture-adapted or patient-derived HIV-1 isolates, coupled with their endangered species status, has curtailed their use as an animal model of HIV-1-induced immunodeficiency.

Transgenic Mice

When it became apparent that HIV-1 infections could only be established in chimpanzees and humans, potentially more tractable and novel rodent systems were developed to model specific steps of *in vivo* HIV-1 infections. In one approach, transgenic mice containing the entire HIV-1 genome or individual viral genes under the control of the HIV-1 long terminal repeat (LTR) were constructed. Because mice carrying such transgenes contain HIV-1 DNA in every cell, they model the postintegration phase of the virus life cycle and provide a means for assessing possible deleterious effects of viral proteins *in vivo*. In general, these first-generation transgenic animals expressed very low levels of HIV-1 proteins and occasionally developed disease.

To overcome the intrinsically low levels of HIV-1 LTR-directed expression in rodents, transgenic mice were subsequently generated carrying viral genes under the control of cellular/tissue-specific promoters. In particular, mice carrying a complete copy of the HIV-1 genome or just the *nef* gene, directed by a chimeric (human-mouse) CD4 promoter/enhancer element, spontaneously developed an AIDS-like disease characterized by marked depletion of CD4⁺ T lymphocytes, thymic atrophy, T-cell activation, and immunodeficiency.^{292,618} Thus, targeting HIV-1 Nef synthesis to mouse CD4⁺ cells, in the absence of a spreading viral infection or the expression of other HIV-1 gene products, was sufficient to elicit a disease with some of the features of human AIDS.

Xenotransplantation Mouse Models

In addition to their use for investigations of human hematopoiesis, innate and adaptive immunity, cancer biology, and regenerative medicine, mice engrafted with human cells have been utilized as a small-animal model for studies of HIV-1 pathogenesis and vaccine development. In very early studies, severe combined immunodeficiency (SCID) mice, engrafted with human PBMC or fragments of human fetal thymus and fetal liver engrafted under the renal capsule, were shown to support HIV-1 replication and moderate to profound depletions of CD4⁺ T lymphocytes but generated no immune responses to the virus.^{543,554} Relatively low levels of human cell engraftment were achieved in these first-generation humanized mice.

Although the subsequent development of nonobese diabetic (NOD) SCID animals resulted in improved human PBMC engraftment, the relatively short life span of the mice and residual murine natural killer (NK) cell activity limited their usefulness. Targeted mutations of the mouse interleukin-2 receptor- γ chain ($\gamma_c^{-/-}$) and reconstitutions with PBMC, hematopoietic stem cells (HSCs), or human cord blood greatly increased engraftment of human tissue.³⁴⁸ Inoculation of these mice with HIV-1 by parenteral and mucosal routes

resulted in sustained disseminated infections.^{27,49} The recently described surgical implantation of fetal thymic and liver tissues in humanized mice previously engrafted with HSCs resulted in animals able to support high levels of HIV viremia, experience CD4+ T-cell depletion, sustain generalized immune activation, and generate virus-specific antibody and CD4+/CD8+ T-cell responses.⁸¹ Although great progress has been made in the development of xenotransplant mouse models over the past 20 years, they are technically demanding to make and not currently amenable for widespread use.

SIV/HIV Chimeric Viruses

When it became apparent that humans and chimpanzees were the only mammalian species that could be infected by HIV-1, attention turned to the SIV/Asian macaque model with the idea of constructing a virus containing both SIV and HIV gene segments. The earliest versions of these SIV/HIV chimeric viruses (SHIVs) consisted of the genetic backbone of SIV_{mac239}, into which the HIV-1 *tat*, *rev*, *vpr*, and *env* and, in some instances, portions of *vpr* and *nef* genes were inserted (e-Fig. 49.1). SHIVs were constructed because, unlike SIVs, they could be used as challenge viruses in vaccine experiments to monitor the effectiveness of immunogens eliciting antiviral neutralizing antibodies directed against the HIV-1 Env glycoprotein. The first pathogenic SHIVs developed all carried X4-tropic *env* genes and caused a rapid, systemic, and complete loss of CD4+ T lymphocytes within a few weeks of inoculation, high and sustained levels of viremia (>10⁷ RNA copies/mL of plasma), and death from immunodeficiency within 3 to 6 months of inoculation.^{345,361,622} This aggressive disease phenotype appeared to be ideally suited for vaccine studies and, for a few years (2000 to 2004), SHIVs rather than SIVs became the challenge virus of choice for such experiments. However, concern was raised when the results from many studies showed that although most vaccine regimens suppressed SHIV replication and disease induction, they failed to protect monkeys from acquisition of either SHIV or SIV infection. In addition, differences were demonstrated between the chemokine receptor utilization of SIV (CCR5) and most transmitted HIV-1 (CCR5) versus the early SHIVs (CXCR4) and the CD4+ T-cell subsets targeted by these viruses *in vivo* (memory [SIV and HIV-1] versus naïve [SHIV]).⁵⁶³ The development and use of R5-tropic SHIVs as challenge viruses in macaques were shown to be better surrogates for HIV-1 infections of humans.²⁹⁸

Derivatized HIVs (also known as minimal SHIVs), containing more than 90% of the HIV-1 genome and the SIV *vif* gene (to counteract macaque APOBEC3 proteins) plus a seven–amino acid segment of the SIV Gag analog of the HIV-1 cyclophilin A (CypA)-binding loop (to escape macaque TRIM5 α [for tripartite motif-containing α] restriction), have recently been reported to establish transient infections in pig-tailed macaques.³⁴⁶ When it was subsequently appreciated that pig-tailed macaques lacked TRIM5 α restriction activity against HIV-1, derivatized HIVs, containing only the SIV *vif* gene, were made and shown to transiently infect this monkey species.³⁰³

MOLECULAR BIOLOGY OF HIV-1 REPLICATION

Overview

The HIV-1 replication cycle (Fig. 49.8) begins with the binding of virus particles to CD4 molecules on the surface of susceptible

cells. In the context of tissue culture systems, susceptible cells include *activated* CD4+ T lymphocytes present in PBMC collected from uninfected individuals, T-cell leukemia cell lines, and MDMs. *In vivo*, activation of CD4+ T cells is not required for HIV-1 replication. Although binding of virions to CD4 is essential for HIV infectivity, their subsequent interaction with a co-receptor, now known to be members of the seven-membrane-spanning CC or CXC families of chemokine receptors, is required for membrane fusion and entry (reviewed in¹³). Multiple chemokine receptors exhibit activity in fusion/entry assays with HIV-1 gp120 (see later section on Env); however, the two most physiologically important co-receptors are CXCR4 and CCR5. Unlike some other enveloped viruses that enter cells by receptor-mediated endocytosis, HIV-1 and many other retroviruses fuse directly with the plasma membrane. Once inside the cell, HIV-1 encounters a cortical actin barrier, which can be remodeled by the virally encoded Nef protein. Partially uncoated virions migrate from cortical actin to the microtubule network where reverse transcription is initiated and movement to the nucleus begins (Fig. 49.8, steps 2 to 4) (reviewed in¹⁸). As with other retroelements, the partially double-stranded DNA reverse transcription product is transported through the cytoplasm and to the nucleus as a component of a PIC, which contains a subset of the Gag and Pol proteins. As noted earlier, the lentiviruses are unique among retroviruses in generating PICs that are actively transported by the nuclear import machinery into the interphase nucleus of nondividing cells arrested in the G₁ phase of the cell cycle (Fig. 49.8, step 5). After the import of the PIC into the nucleus, full-length linear copies of the reverse transcript are integrated into the chromosomal DNA of the infected cell (Fig. 49.8, step 6), a step required for efficient viral RNA synthesis and infectious particle production.

In activated T lymphocytes, integrated copies of HIV DNA serve as templates for RNA polymerase II (Pol II)-directed viral RNA synthesis (Fig. 49.8, step 7). The coordinated interaction of the HIV-encoded Tat protein and the cellular NF- κ B and Sp1 transcriptional transactivating proteins with the RNA Pol II transcriptional apparatus ensures the production of high levels of viral RNA. Unspliced or partially spliced HIV transcripts are exported from the nucleus to the cytoplasm by a unique transport mechanism mediated by the virus-encoded Rev protein, which shuttles between these compartments (Fig. 49.8, step 8). The subsequent translation of the gp160 Env precursor occurs in the endoplasmic reticulum, whereas the Gag and Gag-Pol polyproteins are synthesized on free cytoplasmic ribosomes, and each is independently transported to the plasma membrane (Fig. 49.8, steps 9 and 10). The Gag and Gag-Pol polyproteins, in association with dimers of genomic RNA, condense at the plasma membrane to form an electron-dense “bud” (see Fig. 49.2) that gives rise to a spherical immature particle containing the mature TM and SU Env glycoproteins. Proteolytic processing of the Gag and Pol proteins by HIV PR during or immediately after particle release generates the cone-shaped core characteristic of mature HIV virions (Fig. 49.8, steps 11 and 12).

Thus, HIV-1 uses the same replicative strategy as the so-called simple retroviruses, which encode only the Gag, Pol, and Env proteins. However, during its evolution, HIV-1 has acquired six additional genes to carry out functions that are either (a) performed by cellular proteins already present in the cells infected by the simple retroviruses or (b) uniquely required for virus replication, transmission, and survival in hematopoietic

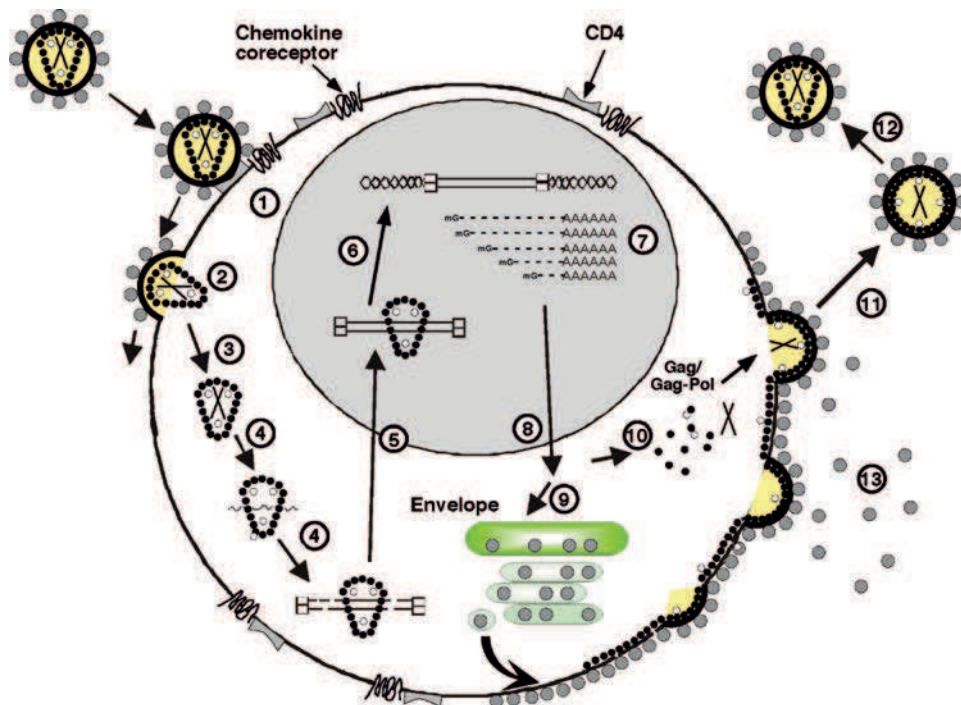


FIGURE 49.8. The human immunodeficiency virus type 1 (HIV-1) replication cycle. Productive HIV-1 infections begin with the adsorption of cell-free virions to cells and their interactions with both the CD4 and chemokine co-receptors (**step 1**). In the case of HIV, virus entry (**step 2**) is a pH-independent process that occurs after the fusion of viral and cellular membranes and results in the partial uncoating (**step 3**) of incoming virions. Reverse transcription occurs within subviral particles in the cytoplasm of infected cells (**step 4**), and the double-stranded DNA product within this preintegration complex (PIC) is transported into the nucleus (**step 5**), where integration into chromosomal DNA (**step 6**) is mediated by the virus-encoded integrase (IN) (open circles). The integrated viral DNA serves as a template for DNA-dependent RNA polymerase (Pol II) and leads to the production of messenger RNAs (mRNAs) (**step 7**), some of which are spliced and exported into the cytoplasm (**step 8**), where they are translated into viral proteins. Envelope (**step 9**) and Gag plus Gag-Pol (**step 10**) polyproteins are transported via independent pathways to the plasma membrane, where progeny virus particles begin “budding” from cells and are released as immature particles (**step 11**). Subsequent proteolysis by the virion-encoded protease (PR) generates mature particles (**step 12**) containing a characteristic condensed core. Non-virion-associated gp120 Env glycoprotein is also released from cells (**step 13**).

cells targeted by the primate lentiviruses. Some of the HIV-1 accessory proteins (Vif, Vpr, Vpu, and Nef) are not required for replication in certain human T-cell lines, although virus infectivity may be affected up to several thousandfold, depending on the accessory gene and the type of infected cell. In view of their conservation, however, these additional genes must be required for successful HIV-1 infectivity *in vivo*.

HIV-1 encodes only 15 proteins and like all other viruses must utilize a large number of cellular proteins for successful replication. Over the years, several techniques, such as immunoprecipitation/mass spectrometry and yeast two-hybrid screening, have been used to identify such host cell factors. The recent development of genome-wide small-interfering RNA (siRNA) or short hairpin (shRNA) screening has provided genetic approaches to identify potential human proteins required for HIV-1 replication, based on loss of function readouts.^{82,404,836,857} In these experiments, siRNAs are transfected into target cells, whereas shRNAs are delivered using lentiviral vectors and subsequently processed into siRNAs intracellularly.

Of the 842 genes identified as being required for HIV-1 replication in three siRNA screens, 34 were also reported in at

least two screens and three were detected in all three screens. The overlap between the three siRNAs and the single shRNA screen ranged from zero to three genes in pairwise comparisons. Eleven of the 34 genes found in two siRNA screens had also been previously reported to encode HIV-1-interacting proteins and 29 of the 34 genes are expressed in cells susceptible to HIV-1 (CD4 and chemokine coreceptor positive). The overlap between individual screens is unexpectedly small yet is statistically significant.⁹² This result is not surprising given that different challenge viruses, cell lines, siRNA libraries, periods of gene silencing prior to infection, and readouts of infection were used in the four studies. Nonetheless, all three siRNA screens identified factors associated with transport through the nuclear pores, trafficking between cellular compartments, DNA repair, RNA binding, and interaction with ubiquitin/proteasomal degradation machinery.⁹² Two of the siRNA screens identified previously reported cellular proteins known to be critical for HIV-1 infectivity including CD4, CXCR4, RelA, elongin B, and cyclin T1. Cyclophilin A, Sp1, and lens epithelium-derived growth factor (LEDGF) are among the known HIV-1 co-factors that were *not* identified in any of the screens.

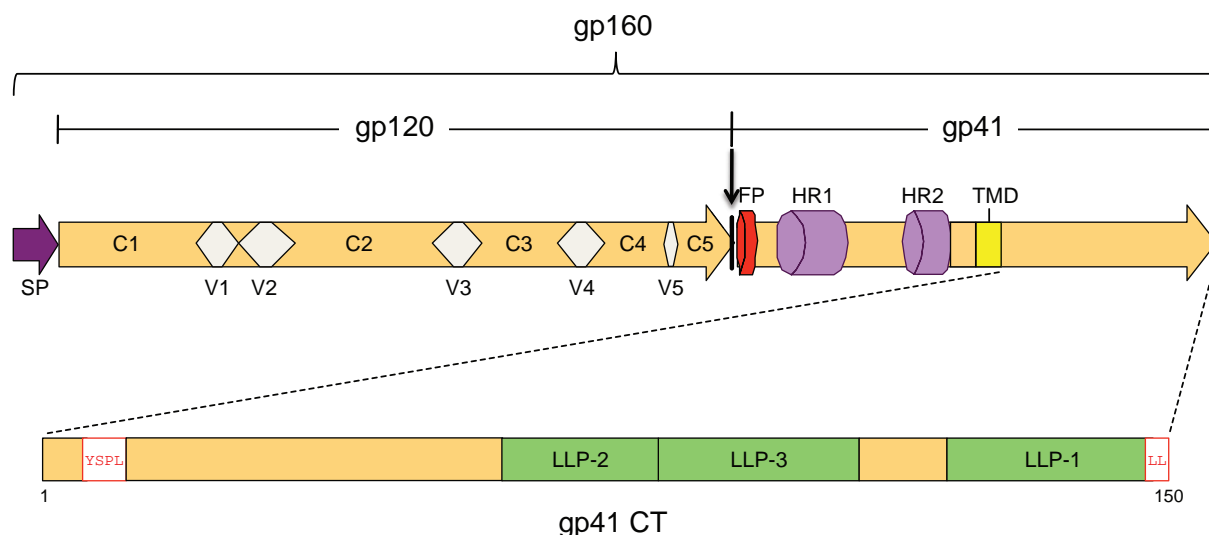


FIGURE 49.9. Linear representation of the human immunodeficiency virus 1 (HIV-1) Env glycoprotein. The vertical arrow indicates the site of gp160 cleavage to gp120 and gp41; SP denotes the signal peptide. In gp120, variable domains (V1 to V5) and conserved sequences (C1 to C5) are indicated. In the gp41 ectodomain, several domains are indicated: the N-terminal fusion peptide (FP) and the two heptad repeats (HR1 and HR2). The transmembrane domain (TMD) is represented by a yellow box. In the ~150 amino acid gp41 cytoplasmic tail (CT), the Tyr-Ser-Pro-Leu (YSPL) and Leu-Leu (LL) motifs implicated in Env trafficking and internalization are indicated. The putative helical motifs—or lentiviral lytic peptides (LLP-1, LLP-2, and LLP-3)—are shown. (Adapted from Checkley MA, Luttge BG, Freed EO. HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. *J Mol Biol* 2011;410:582–608, copyright 2011, with permission from Elsevier.)

Virus Binding and Entry: The Env Glycoproteins

The HIV Env glycoproteins are synthesized from the singly spliced 4.3-kb Vpu/Env bicistronic mRNA (see Fig. 49.6); translation occurs on ribosomes associated with the rough endoplasmic reticulum (RER). The Env glycopolyprotein precursor, gp160, is an integral membrane protein that is anchored to cell membranes by a hydrophobic stop-transfer signal in the gp41 TM domain (Fig. 49.9) (reviewed in¹¹⁷). gp160 is co-translationally glycosylated and undergoes oligomerization in the endoplasmic reticulum (ER). The predominant, biologically active oligomeric form is a trimer. gp160 is transported to the Golgi, where, like other retroviral Env precursor glycoproteins, it is proteolytically cleaved by a cellular enzyme to the mature SU and TM glycoproteins, gp120 and gp41, respectively (Fig. 49.9). The cellular enzyme primarily responsible for cleavage of retroviral Env precursors following a highly conserved Lys/Arg-X-Lys/Arg-Arg motif is furin or a furin-like protease.²⁸⁸ Cleavage of gp160 is strictly required for Env-induced fusion activity and virus infectivity.^{237,510} Subsequent to gp160 cleavage, gp120 and gp41 form a noncovalent association that is critical for transport of the Env complex from the Golgi to the cell surface. The gp120–gp41 interaction is fairly weak, and a substantial amount of gp120 is shed from the surface of Env-expressing cells.

The HIV Env glycoprotein complex, in particular the gp120 component, is very heavily glycosylated; approximately half the molecular mass of gp160 is composed of oligosaccharide side chains. During transport of Env from its site of synthesis in the ER to the plasma membrane, many of the side chains are modified by the addition of complex sugars. The numerous oligosaccharide side chains form a sugar “cloud,” obscuring much of gp120 from host immune recognition. As

shown in Figure 49.10, gp120 contains interspersed conserved (C1 to C5) and variable (V1 to V5) domains. The Cys residues present in the gp120 proteins of different isolates are highly conserved and form disulfide bonds that link the first four variable regions in large loops.⁴³⁷

After its arrival at the cell surface, the gp120–gp41 complex is rapidly internalized. Several studies have demonstrated that a Tyr-X-X-Leu sequence in the gp41 cytoplasmic tail (approximately five residues from the membrane-spanning domain) (see Fig. 49.9) is at least partially responsible for this rapid internalization.^{417,645} Analogous motifs are also present in the TM Env proteins of HIV-2, SIV, and several other retroviruses. Tyr-based motifs are known to mediate endocytosis of cellular plasma membrane proteins by binding the μ 2 chain of clathrin-associated adaptor protein-2 (AP-2) complexes, and such interactions have been observed with the HIV-1 and SIV gp41 cytoplasmic domains.^{69,568} A dileucine motif at the C-terminus of the gp41 cytoplasmic tail also participates in Env internalization and trafficking via interactions with the AP-1 complex^{52,94,820} (Fig. 49.10). Rapid Env internalization could help HIV-1 evade the host immune response and limit Env-induced cytopathicity. The cytoplasmic tail of HIV-1 and SIV gp41 has also been reported to activate NF- κ B, potentially providing a stimulus for T-cell activation, thereby enhancing viral infection.⁶¹²

CD4 Binding

The first step in HIV/SIV infection involves an interaction between gp120 and CD4, the major cell surface receptor for primate lentiviruses (for review, see⁷⁷). CD4 is a 55-kD member of the immunoglobulin (Ig) superfamily; it is composed of a highly charged cytoplasmic domain, a single hydrophobic

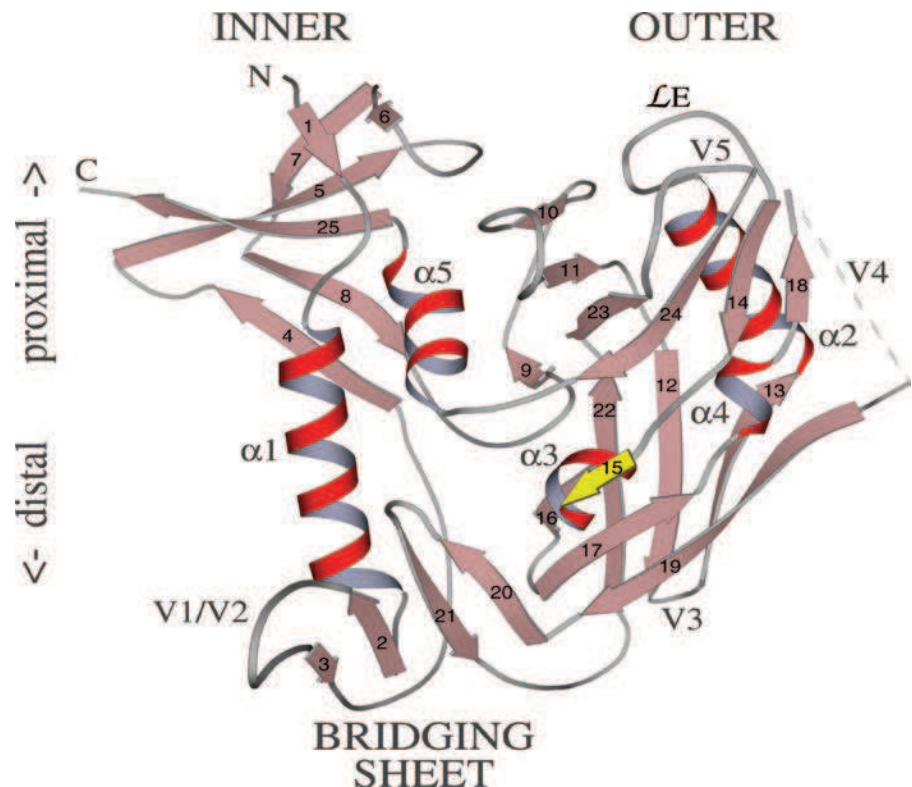


FIGURE 49.10. Ribbon diagram of the gp120 core. In this orientation, the viral membrane would be at the top, the target cell membrane at the bottom. The inner and outer domains are connected by a four-stranded β -bridging sheet. The remnants of variable loops V1/V2, V3, V4, and V5 are shown. (Reprinted from Kwong PD, Wyatt R, Robinson J, et al. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 1998;393:648–659. Copyright © 1998, *Nature*.)

membrane-spanning domain, and four distinct extracellular domains, D1 to D4.⁴⁷⁵ CD4 normally functions to stabilize the interaction between the T-cell receptor on the surface of T lymphocytes and class II major histocompatibility complex (MHC-II) molecules on antigen-presenting cells. The high-affinity CD4 binding site for gp120 has been localized to a small segment of the N-terminal extracellular domain, analogous to the second complementarity-determining region (CDR-2) loop of an Ig light chain variable domain. CD4 binding determinants in Env map to the C3 and C4 domains of gp120, although a more discontinuous, conformation-dependent domain is involved in high-affinity gp120–CD4 binding. CD4 binding serves not only to promote virion attachment to the target cell but also to induce conformational changes in gp120 and gp41 that activate Env fusogenicity.^{661,751} In most HIV-1 isolates, CD4 binding induces conformational changes in gp120 that promote gp120 binding to co-receptors.

The crystallization of a gp120 “core” domain (an unglycosylated gp120 derivative lacking the V1/V2 and V3 loops and the N- and C-termini) complexed with fragments of CD4 and a neutralizing antibody contributed greatly to our understanding of the gp120–CD4 interaction.^{416,819} The core structure reveals two major domains (referred to as the “inner” and “outer” domains) connected by a so-called bridging sheet (see Fig. 49.10). The latter is composed of a four-stranded, antiparallel β -sheet derived from sequences in the V1/V2 stem and portions of C4. Comparison of the CD4-bound HIV-1 gp120 structure with that of the non-CD4-bound (unliganded) gp120 from SIV_{mac} reveals that gp120, the inner domain in particular, undergoes a remarkably extensive conformational change upon CD4 binding.¹¹⁸ This shift in conformation leads to the formation of the bridging sheet, which is absent in the unliganded structure.

Co-receptor Interactions

Soon after the identification of CD4 as the major HIV/SIV receptor, it was recognized that this protein is not sufficient for HIV-induced membrane fusion and virus entry. In the mid-1990s, a number of studies demonstrated that members of the G protein-coupled receptor superfamily of seven-transmembrane domain proteins provided the long-sought co-receptor function.^{14,127,181} As mentioned earlier, the two major co-receptors for HIV/SIV infection are CXCR4 and CCR5. In recognition of the importance of CXCR4 and CCR5 in determining HIV-1 tropism, a nomenclature scheme was devised based on co-receptor usage: viruses (generally M-tropic) that use CCR5 are denoted R5 isolates, strains (generally TCL-tropic) that preferentially use CXCR4 are referred to as X4 viruses, and dual-tropic strains that use both CCR5 and CXCR4 are denoted R5/X4 isolates⁴⁷ (Table 49.1).

Since the discovery of HIV/SIV co-receptors, numerous additional studies refined our understanding of the activity and function of these proteins. Some of the more important findings are described here; for further information, the reader is referred to more-detailed reviews.⁴⁸

- It is clear that, depending on the virus isolate, multiple sequences in the N-terminal domain and extracellular loops of CCR5 and CXCR4 can influence co-receptor function, and that the gp120–co-receptor interaction is complex.
- Certain laboratory isolates of HIV-1, HIV-2, and SIV use co-receptors in a CD4-independent manner, that is, as primary receptors.^{205,499,613} In fact, the identification of CD4-independent HIV and SIV isolates suggests that the use of chemokine receptors in lentiviral infection may have predated the involvement of CD4.

- Direct interactions between co-receptor and gp120 have been detected. These interactions are greatly stimulated by, or are dependent on, the presence of CD4.^{425,758,816}
- In addition to CXCR4 and CCR5, a number of other chemokine receptors and related proteins can serve as co-receptors for HIV/SIV fusion and infection in cultured cells. These include CCR2b, CCR3, CCR8, APJ, Bonzo (STRL33), BOB (GPR15), and US28 (for review, see⁴⁸). What role, if any, these alternative co-receptors play in HIV/SIV infection *in vivo* is unclear.

Several domains within gp120 directly or indirectly function in Env–co-receptor interactions. Consistent with its influence on HIV-1 tropism, the V3 loop plays a major role. In addition to the involvement of variable regions in co-receptor interaction, highly conserved portions of gp120 also interact with co-receptors. Following the initial report of the gp120 core crystal structure in 1998, a number of additional HIV-1 and SIV gp120 structures have been published. Together, these structures have provided a number of key insights into the gp120 structure/function relationship. As mentioned earlier, gp120 undergoes extensive conformational changes upon binding to CD4. Particularly significant is that the co-receptor binding site is essentially created following CD4 binding.¹¹⁸ As a result, the co-receptor binding site, which would be highly susceptible to neutralizing antibodies, exists only transiently after gp120 has contacted CD4 on the target cell membrane. These structural findings are consistent with earlier reports demonstrating that binding of antibodies to the co-receptor-interacting surface of gp120 is enhanced by CD4.^{662,751} Interestingly, the co-receptor–binding surface is constitutively exposed in variants selected to replicate in a CD4-independent fashion.^{322,402} Because their co-receptor–binding surface is

constitutively exposed, CD4-independent isolates tend to be hypersensitive to neutralization.

Certain individuals, despite persistent high-risk behavior, remain HIV-1 uninfected. The discovery of CCR5 and CXCR4 as HIV-1 co-receptors raised the possibility that these individuals inherited mutant co-receptor alleles. Indeed, a mutant CCR5 allele, referred to as CCR5/Δ32, contains a 32-bp deletion and encodes a truncated protein that is not efficiently expressed at the cell surface and cannot function as an HIV-1 co-receptor.^{176,335,457,654} Homozygotes for CCR5/Δ32 are only rarely infected with HIV-1, highlighting the protective benefits of inheriting this allele. Other inherited polymorphisms in co-receptor coding regions or regulatory sequences have been reported to influence HIV-1 transmission or HIV-1-induced disease progression, though the effects are not as clear as with the CCR5/Δ32 allele.

Membrane Fusion

The primary function of viral Env glycoproteins is to promote a membrane fusion reaction between viral and target cell membranes. This membrane fusion event enables the viral core to gain entry into the host cell cytoplasm. The observation that CD4 induces conformational changes in gp120 that enhance interaction with co-receptors suggests that events leading up to membrane fusion occur sequentially (Fig. 49.11). gp120 first interacts with CD4; a ternary CD4–co-receptor–gp120 complex then forms; and finally, conformational changes take place in gp41 that trigger membrane fusion. Although fusion between the viral and cellular membrane is widely believed to occur directly at the plasma membrane after CD4 and co-receptor engagement, it has been reported that fusion can also occur in a low-pH endosomal compartment.⁵²⁷

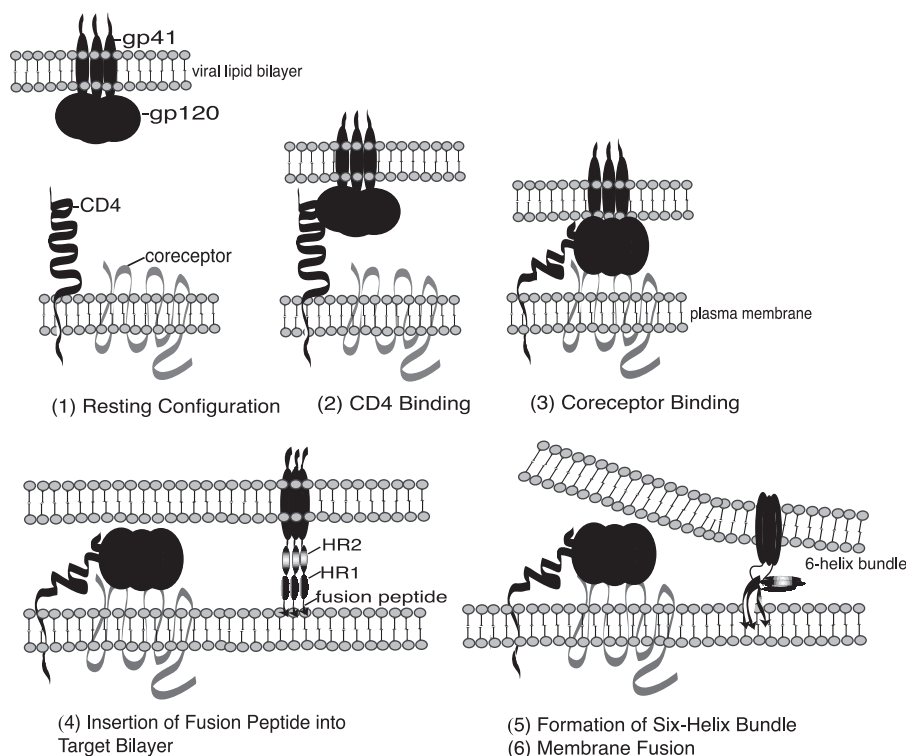


FIGURE 49.11. Schematic representation of the steps leading to membrane fusion.

In the resting configuration, the Env glycoprotein complex is in its native state (1). CD4 binding (2) induces conformational changes in Env that facilitate coreceptor binding (3). After the formation of a ternary gp120–CD4–coreceptor complex, gp41 adopts a hypothetical conformation that allows the fusion peptide to insert into the target lipid bilayer (4). The formation of the gp41 six-helix bundle, which involves antiparallel interactions between the gp41 heptad repeats (HR1 and HR2) (5), brings the viral and cellular membranes together and membrane fusion takes place (6).

Mutational analyses demonstrated that a hydrophobic region at the N-terminus of gp41, the so-called fusion peptide, plays a central role in membrane fusion mediated by HIV-1,²³⁵ HIV-2,²³⁴ and SIV⁷⁴ (see Fig. 49.9). C-terminal to the gp41 fusion peptide are two amphipathic heptad repeat (HR) domains (see Fig. 49.9) that are also required for membrane fusion. Mutations in the N-terminal HR (HR1) impair infectivity and membrane fusion activity,¹⁹⁵ and peptides derived from HR1 and HR2 exhibit potent antiviral activity in culture.⁸⁰² Structures for the ectodomain of HIV-1 and SIV gp41, including the HRs, were determined by x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy.^{95,113,743,795} These studies obtained fundamentally similar trimeric structures in which HR1 and HR2 pack in an antiparallel fashion to generate a six-helix bundle (Figs. 49.11 and 49.12). The HR1s form a coiled-coil in the center of the bundle, with the HR2s packing into hydrophobic grooves on the outside. The gp41 ectodomain structure resembles that of the fusion-competent (low-pH-induced) form of influenza HA₂, making it likely that HIV/SIV Env glycoproteins trigger membrane fusion by the same “spring-loaded” mechanism proposed for influenza virus.⁹¹ Peptides corresponding to the HRs inhibit fusion (and virus infectivity) by interacting with their

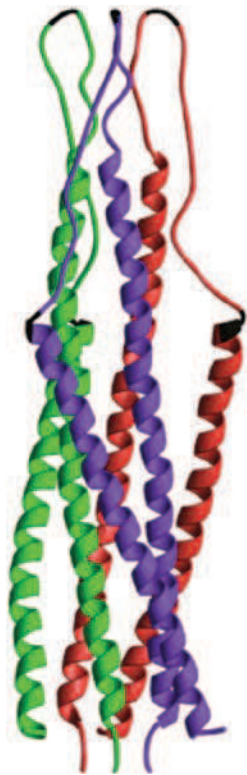


FIGURE 49.12. Trimeric structure of simian immunodeficiency virus (SIV) gp41 ectodomain residues 27 to 149. The N-terminal heptad repeats (HR1s) are located on the inside of the six-helix bundle; the C-terminal heptad repeats (HR2s) pack into hydrophobic grooves on the outside. The three subunits are shown in green, blue, and red. (Reprinted from Caffrey M, Cai M, Kaufman J, et al. Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. *EMBO J* 1998;17:4572–4584, with permission from Oxford University Press.)

complementary binding partner on gp41, thus preventing gp41 itself from forming the six-helix bundle. Binding studies using native HIV-1 Env indicate that gp41 interacts with HR2 peptide only after CD4 binding, suggesting that HR1 and HR2 undergo conformation changes after CD4 binding and that these rearrangements are required for membrane fusion.²⁴⁴ The cytoplasmic domain of gp41 also plays a role in modulating the fusion activity of Env, in part by regulating the levels of cell surface expression of the gp120–gp41 complex and by influencing the conformational of gp120 and the ectodomain of gp41 (for review see¹¹⁷).

Whereas gp41 interacts directly with the lipid bilayer to catalyze the membrane fusion reaction, a variety of domains within gp120 are involved in activating Env fusogenicity. The V3 loop of HIV-1 gp120, which elicits isolate-specific neutralizing antibodies, is an essential player in membrane fusion. Mutations throughout the V3 loop of HIV-1²³⁶ and the analogous region of HIV-2²³⁴ block syncytium formation and virus infectivity. The importance of V3 in membrane fusion appears to be in part a consequence of its role in gp120–co-receptor interactions. The structure of the V3 loop in the context of the CD4-bound gp120 core reveals that V3 protrudes out from the core, toward the target cell membrane and the co-receptor.³³¹

It has become apparent that the lipid composition of the target cell plasma membrane plays an important role in membrane fusion. Of particular interest is the concept that membrane fusion may take place in plasma membrane microdomains known as lipid rafts. These microdomains, which are enriched in glycosphingolipids and cholesterol, appear to be involved in the entry of a number of enveloped viruses, including HIV-1 (for review see⁷⁸⁴). Depletion of cholesterol from either the virus particle or the target cell membrane disrupts fusion,⁵⁷³ as does depletion of specific glycosphingolipids.^{88,336} The cholesterol-binding compound amphotericin B methyl ester (AME) is also a very potent inhibitor of HIV-1 infectivity.⁷⁸² Interestingly, HIV-1 evades AME by acquiring mutations in the cytoplasmic tail of gp41 that cause the tail to be cleaved by PR, thereby allowing infection to proceed even in the presence of the inhibitor.⁷⁸³ In addition to promoting cell-free HIV-1 infection, lipid rafts may play an important role in HIV-1 transmission between T cells³⁶⁶ and in virus uptake into DCs.⁹⁷

Progress in understanding the molecular mechanism of membrane fusion has led to the development of inhibitors that block virus fusion and entry (for review see⁷⁵²). One of these fusion inhibitors, T-20 (enfuvirtide), is now in clinical use. T-20 is a 36–amino acid, gp41 HR2-derived peptide that inhibits HIV-1 replication both *in vitro* and in infected patients.^{393,419} This peptide interacts directly with HR1 during the conformational changes that take place after CD4 binding. The interaction of T-20 with gp41 prevents six-helix-bundle formation, thereby blocking fusion. Resistance to T-20 arises both *in vitro* and in patients, primarily through mutations in HR1.⁶³⁰ Inhibitors that block gp120 binding to CD4 are being tested, and a number of CCR5 and CXCR4 antagonists are under development. Although CXCR4 knockout mice display a range of severe defects in hematopoiesis, central nervous system development, and gastrointestinal tract vascularization,^{739,864} no observable negative consequences of absent CCR5 function are evident in individuals homozygous for the CCR5/Δ32 mutation. Furthermore, in 2009, it was reported that a patient was apparently cured of HIV-1 infection following transplantation

of stem cells from a CCR5/ Δ 32 homozygous individual.³⁴¹ These observations suggest that inhibitors targeting CCR5 should be well tolerated. One CCR5-based inhibitor, maraviroc, is currently approved for clinical use in patients harboring R5-tropic strains of HIV-1 (for review see⁵⁸³). Maraviroc is thought to function by binding extracellular domains of CCR5 and inducing an allosteric change in CCR5 conformation that prevents gp120 binding.²¹¹ Resistance to maraviroc can arise in culture by acquisition of gp120 V3 loop mutations that allow HIV-1 to utilize the drug-bound co-receptor.⁸⁰⁰ In patients, CCR5 inhibitor resistance typically results from selection for CXCR4 strains that were present at low levels prior to the initiation of therapy.⁵³³ In addition to use of small-molecule co-receptor antagonists like maraviroc, strategies aimed at knocking down CCR5 expression in HIV-1-infected individuals are being contemplated. A strategy currently under development involves the use of zinc-finger nucleases that disrupt the CCR5 gene (for review see 102).

Reverse Transcription

By definition, retroviruses possess the ability to convert their single-stranded RNA genomes into double-stranded DNA during the early stages of the infection process.^{31,747} The enzyme that catalyzes this reaction is RT, in conjunction with its associated ribonuclease H (RNaseH) activity. Retroviral RTs have two enzymatic centers: (a) a DNA polymerase that can copy either RNA templates (for “minus-strand” DNA synthesis) or DNA templates (for second- or “plus-strand” DNA synthesis) and (b) RNaseH (for the degradation of the tRNA primer and genomic RNA present in DNA-RNA hybrid intermediates). RT was the first viral protein targeted by antiretroviral therapy, and RT inhibitors remain central to the treatment of HIV-infected patients (for reviews see^{337,703}).

Uncoating occurs after membrane fusion and entry of the viral nucleoprotein complex into the cytosol, giving rise to the reverse transcription complex (RTC). This multiprotein complex is composed not only of RNA and RT but also CA, NC, IN, and Vpr. While it was initially proposed that most if not all of the CA protein was lost from the RTC during uncoating, a variety of observations, including the fact that CA is the target for host restriction factors that disrupt reverse transcription (see later), support the hypothesis that at least some CA protein is retained in this complex. Recent findings suggest that uncoating and reverse transcription are linked; in the presence of an inhibitor of reverse transcription, uncoating is significantly delayed.³³⁸

The retroviral genome is packaged into the virion as two copies of single-stranded RNA. As discussed earlier, the two RNAs are held together in part by the DIS near their 5′ ends (see Fig. 49.7). Although each retroviral particle carries two strands of RNA, it appears that only one provirus is formed per virion.³³⁰ Retroviruses are therefore referred to as *pseudodiploid*. As with most other DNA polymerases, RT is dependent on the 3′-OH group of an RNA or DNA primer to initiate polymerization. Retroviruses use specific tRNAs (tRNA^{Lys3} in the case of HIV-1) to initiate DNA synthesis. The mechanism of tRNA selection and placement on the template is complex, involving interactions with RT and NC as well as with the 18-nt pbs near the 5′ end of the viral genome (see Fig. 49.7).

Reverse transcription of the retroviral RNA genome to a double-stranded DNA copy takes place after viral entry into the target cell and proceeds via a series of steps that are outlined

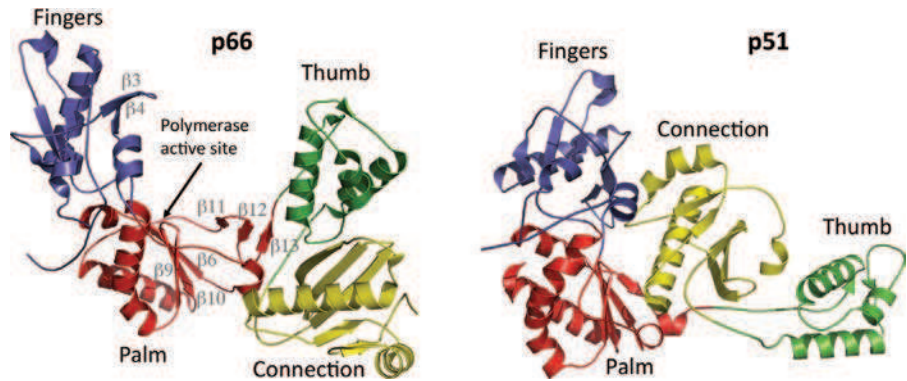
briefly here (see e-Fig. 49.2A). This is also described in Figure 47.7 of Chapter 47.

1. Minus-strand DNA synthesis is initiated from the 3′-OH of the tRNA bound to the pbs. DNA synthesis then proceeds to the 5′ end of the genome.
2. RNaseH digests the RNA portion of the newly formed RNA-DNA hybrid, freeing the resulting short, single-stranded DNA fragment (known as the minus-strand strong-stop DNA).
3. The minus-strand strong-stop DNA is transferred to the 3′ end of the genome, where it hybridizes by virtue of a short region of homology (the “repeated” or R region) present at both 5′ and 3′ ends of the RNA genome.
4. Minus-strand synthesis, accompanied by RNaseH-mediated degradation of the RNA in the resulting RNA-DNA hybrid, continues to the pbs at the 5′ end of the genome.
5. Fragments of RNA that were not removed by RNaseH serve as primers for plus-strand synthesis. The major site of plus-strand priming is the PPT (see e-Fig. 49.2A, top); however, residual RNA fragments that remain hybridized to regions outside the PPT can also be used for priming plus-strand synthesis. In the case of HIV-1, one such region, known as the central PPT, appears to be particularly important in this regard.¹¹⁴
6. After plus-strand synthesis copies a portion of the tRNA primer, RNaseH removes the tRNA. This exposes the pbs at the 3′ end of the plus-strand DNA, allowing the plus-strand DNA to hybridize with the homologous region at the 3′ end of the minus-strand DNA (*second-strand transfer*).
7. Plus- and minus-strand syntheses proceed to completion. Plus-strand synthesis terminates at the end of the minus strand and, for HIV-1, at a sequence known as the central termination signal (CTS).¹¹⁵ The position of the central PPT upstream of the CTS results in the displacement of approximately 100 nt of plus-strand DNA and the formation of a triplex DNA structure. It has been suggested that this triplex, or *DNA flap* structure, plays a role in nuclear import of the viral PIC,⁸⁴⁶ though this has been controversial. The final product of reverse transcription is a double-stranded DNA molecule capable of serving as the substrate for integration.

In addition to providing an essential function in the virus life cycle, the enzymatic activity of RT is routinely used in the laboratory to quantitatively monitor levels of progeny virions present in the supernatant of infected cultures and to elucidate the molecular details of virus replication. Also, the detection of viral DNA postinfection by real-time polymerase chain reaction (PCR) provides one of the most reliable methods to date for detecting entry and postentry steps in the virus replication cycle. Once inside the nucleus, some double-stranded viral DNA is ligated to generate circular forms; although these circular DNA products do not integrate, their formation is often used to monitor nuclear import (see later).

The mature HIV-1 RT holoenzyme is a heterodimer of 66- and 51-kD subunits. The 51-kD subunit (p51) is derived from the 66-kD (p66) subunit or some larger precursor by proteolytic removal of the C-terminal 15-kD fragment of p66 by PR (Fig. 49.6). The structure of HIV-1 RT has been determined by x-ray crystallography in a number of studies. The enzyme has been crystallized in several contexts: (a) unliganded,⁶³³ (b) bound to

FIGURE 49.13. Ribbon diagrams of the p66 (left) and p51 (right) reverse transcriptase (RT) subunits. Polymerase active site and fingers, palm, thumb, and connection subdomains are shown. (Modified by Kalyas Das and Eddy Arnold from Jacobo-Molina A, Ding J, Nanni RG, et al. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc Natl Acad Sci U S A* 1993;90:6320–6324.)



a short DNA duplex and the Fab portion of an anti-RT antibody,³⁵⁴ (c) covalently linked to a complex of primer/template and deoxyribonucleotide triphosphate (dNTP),³³² and (d) bound to an RNA-DNA duplex.⁶⁵⁵ A number of structures are also available for RT bound to nonnucleoside RT inhibitors (NNRTIs). The crystal structure of HIV-1 RT reveals that the p66 and p51 subunits are folded into similar subdomains, but these subdomains are arranged quite differently in p66 and p51. The p66 subunit can be visualized as a right hand, with the polymerase active site within the palm, and a deep template-binding cleft formed by the palm, fingers, and thumb subdomains³⁵⁵ (Fig. 49.13 and 49.14). The polymerase domain is linked to RNaseH by the connection subdomain. The active site, located in the palm, contains three critical Asp residues (Asp-110, Asp-185, and Asp-186) in close proximity, and two coordinated Mg^{2+} ions. Mutation of these Asp residues abolishes the polymerase activity of RT. The orientation of the three active-site Asp residues is similar to that observed in other DNA polymerases (e.g., the Klenow fragment of *Escherichia coli* DNA Pol I).²² The p51 subunit appears to play a structural role and does not form a polymerizing cleft; Asp-110, Asp-185, and Asp-186 of p51 are buried within the subunit. Approximately 18 bp of the primer/template duplex lie in the nucleic acid-binding cleft, stretching from the polymerase to the RNaseH active sites. In the RT–primer/template–dNTP structure,³³² the

presence of a dideoxynucleotide (ddNTP) at the 3' end of the primer allows visualization of the catalytic complex blocked just prior to attack on the incoming dNTP. Comparison with previously obtained structures suggests a model whereby the fingers close in to trap the template and dNTP prior to nucleophilic attack of the 3'-OH of the primer on the incoming dNTP. After the addition of the incoming dNTP to the growing chain, the fingers adopt a more open configuration, thereby releasing the pyrophosphate and enabling RT to bind the next dNTP. HIV-1 RNaseH, whose structure has been determined by x-ray crystallography, displays a global folding similar to that of *E. coli* RNaseH.¹⁶⁹ The structure of human RNaseH1 bound to an RNA-DNA hybrid has allowed the modeling of HIV-1 RT RNaseH complexed with its substrate.⁵⁶⁶

The high rate of variation among HIV-1 populations poses one of the fundamental challenges to effectively controlling this pathogen. Highly variable virus populations are largely a consequence of rapid virus replication rates, coupled with the error-prone nature of RT (which lacks a proofreading function) and frequent template switching during reverse transcription¹⁴¹ (for review see⁷³³). Cell-free error rates for purified RTs have been determined,^{38,616,631} as have *in vivo* retrovirus mutation rates.^{2,192,491,585,586} Errors during retrovirus replication are also likely to be introduced by the cellular DNA-dependent RNA Pol II during transcription of the viral RNA. The cell-free error

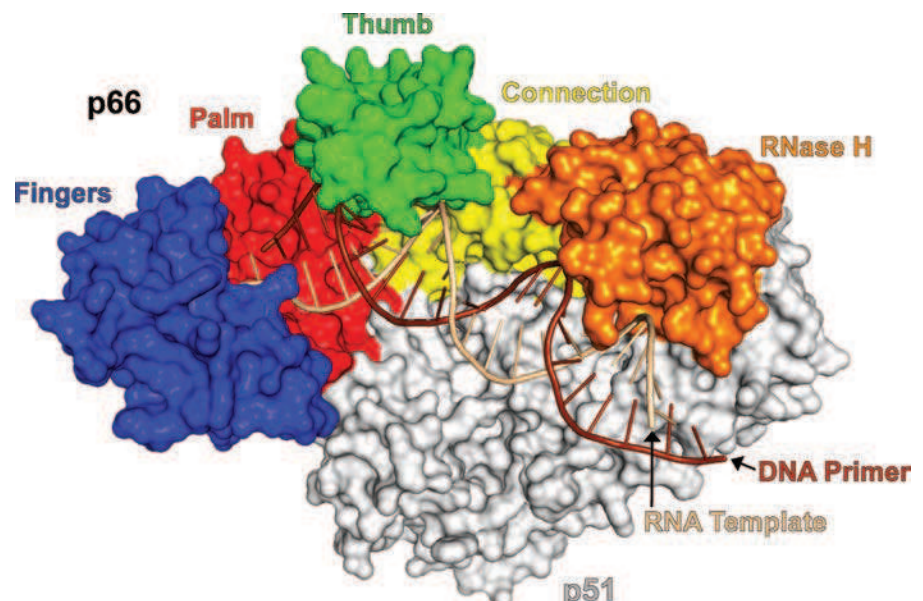


FIGURE 49.14. Human immunodeficiency virus 1 (HIV-1) reverse transcriptase (RT) bound to an RNA/DNA template/primer. (Modified by Karen Kirby and Stefan Sarafianos from Singh K, Marchand B, Kirby KA, et al. Structural aspects of drug resistance and inhibition of HIV-1 reverse transcriptase. *Viruses* 2010;2:606–638.)

rate measured for HIV-1 RT is more than 10-fold higher than the mutation rate, suggesting that the *in vitro* reaction lacks factor(s) that increase fidelity.⁴⁹¹ The total HIV-1 *in vivo* mutation rate (a composite of substitutions, frameshifts, simple deletions, and deletions with insertions) was measured at ~ 1 to 3×10^{-5} per cycle of replication.^{2,491}

As discussed earlier, retroviral particles contain two copies of single-stranded RNA. During reverse transcription, RT frequently switches from one template to the other; this occurs on the order of ~ 30 times per infection event.³⁴ RT also undergoes intramolecular jumps.⁵⁸⁴ Intramolecular jumps lead to mutations (e.g., deletions, insertions, and duplications), whereas intermolecular jumps generate recombinants if the two packaged RNAs are not genetically identical.³²⁹ The intermolecular template switches that lead to recombination can occur when RT encounters a break in the RNA during polymerization—the so-called forced copy choice model.¹⁴² Jumps during minus-strand synthesis can occur in the absence of strand breaks,³⁷¹ and the stability of RT association with template is influenced by the balance between the enzyme's RNaseH activity and polymerase activity—the “dynamic copy-choice” model.³⁴² The impact of high rates of recombination and high replication rates on HIV-1 populations is substantial. As noted earlier, a significant percentage ($\sim 20\%$)³¹² of sequenced virus isolates are intersubtype recombinants, and recombinants constitute the predominant strains currently circulating in certain parts of the world. Recombination also provides a mechanism for the rapid generation of multiple-drug-resistant HIV-1 variants.

RT has long been a target in the search for antiviral compounds. RT inhibitors are now routinely administered to AIDS patients, usually in combination with PR and, more recently, IN inhibitors. There are two major classes of drugs that block reverse transcription: nucleoside RT inhibitors (NRTIs) and NNRTIs. Examples of NRTIs include AZT (zidovudine); 2'-deoxy-3'-thiacytidine (3TC or lamivudine); 2',3' dideoxyinosine (ddI or didanosine); and 2',3' dideoxycytidine (ddC or zalcitabine). NNRTIs include nevirapine, efavirenz, and delavirdine. NRTIs are nucleotide mimics that lack the 3'-OH and thus act as chain terminators upon incorporation into DNA, whereas the NNRTIs inhibit DNA polymerization by binding a small hydrophobic pocket near the RT active site and inducing a change in the structure of RT that blocks DNA synthesis.⁴⁰¹ Resistance to RT inhibitors often develops in patients as viral variants arise encoding mutant forms of RT that are no longer blocked by the inhibitors (for review see⁶⁵⁶). Resistance to NRTIs generally takes place by one of two mechanisms: (a) resistant RTs acquire the ability to selectively incorporate natural dNTPs but exclude the NRTI, or (b) mutant RTs incorporate the NRTI but subsequently excise it from the terminated primer. Resistance to NNRTIs occurs when mutations in RT interfere with the binding of the drug to the enzyme by disrupting key drug-enzyme interactions, changing the shape of the NNRTI binding pocket, or preventing entry of the drug into the binding pocket.⁶⁵⁷ Efforts continue to develop new NRTIs that are more difficult for drug-resistant RTs to exclude, and it may ultimately be possible to develop compounds that interfere with NRTI excision. NNRTIs with increased conformational flexibility (“wiggling and jiggling”) may also be more effective and more difficult for HIV-1 to evade if the flexible inhibitor can still bind to the altered pocket in the resistant RTs.^{162,163} Finally, a significant effort has been directed toward

developing inhibitors that target the RNaseH activity of the enzyme.

Postentry Blocks to Lentivirus Infection

TRIM5 α and Family Members

While retroviruses have evolved ingenious strategies to usurp host cell machinery to facilitate their replication, they have also been forced to counter host cell defenses aimed at restricting virus infection. Decades ago, it was discovered that cells from mice of specific genetic backgrounds express dominant factors that block infection by certain subtypes of MLV. For example, the Friend virus susceptibility-1 (*Fv1*) allele^{452,601} encodes resistance to distinct strains of MLV; *Fv1ⁿ* confers resistance to B-tropic MLV, whereas *Fv1^b* cells cannot be efficiently infected by N-tropic MLV (N-MLV). The viral determinant of N- and B-tropism maps to a specific amino acid (residue 110) in the CA domain of Gag.⁴⁰⁶ The *Fv1* block occurs early postentry, after reverse transcription but before integration.³⁶³ Although the mechanism of *Fv1* restriction remains a puzzle, it was demonstrated in the mid-1990s that *Fv1* encodes an endogenous Gag-like protein.⁵⁶

Nonmurine cells do not harbor an *Fv1* gene.⁵⁶ However, seemingly analogous postentry restrictions have also been observed in a variety of mammalian cells. N-tropic MLV poorly infects cells from a number of mammalian species.⁷⁵⁶ HIV-1 infection is inefficient in cells derived from Old World (e.g., African green and rhesus) monkeys, whereas New World (e.g., owl and squirrel) monkey cells are poorly infected by SIV_{mac}.^{319,323,695} In these cases, infection appears to be blocked at or before reverse transcription.^{54,150,756} The host factor responsible for postentry restriction of HIV-1 in rhesus macaque cells was demonstrated to be the cytoplasmic body component TRIM5 α .⁷²⁷ Expression of rhesus TRIM5 α in human cells potently inhibits HIV-1 infection but has no effect on Moloney MLV infectivity. Conversely, knock-down of TRIM5 α expression using siRNA in rhesus cells markedly increases HIV-1 infectivity.⁷²⁷ Subsequent studies indicated that human TRIM5 α expression is necessary and sufficient to block N-MLV infection in human cells^{305,383,596,833} and that TRIM5 α derived from several New World monkey species restricts infection by SIV from macaques (SIV_{mac}) and African green monkeys (SIV_{agm}) in human cells.⁷¹³ Rhesus, African green monkey, and human TRIM5 α can also diminish infection by the nonprimate lentivirus equine infectious anemia virus (EIAV),³⁰⁵ and cow and rabbit cells express TRIM-family proteins that restrict diverse retroviruses.^{670,698}

As their name implies, TRIM proteins contain three major domains: a RING domain that possesses ubiquitin ligase activity, a B-box 2 domain, and a coiled-coil domain (Fig. 49.15). The α isoform of TRIM5 (TRIM5 α) also contains a C-terminal B30.2 or SPRY domain that is absent in other TRIM5 isoforms. This C-terminal B30.2/SPRY domain harbors the determinants responsible for the species specificity of TRIM5 α -mediated restriction to retroviral infection^{553,593,712,729,833}; a single amino acid change in the B30.2/SPRY domain of human TRIM5 α converts this protein from a weak inhibitor of HIV-1 infection to one that behaves like rhesus TRIM5 α in potently restricting HIV-1 infection.⁸³³ This specificity is mediated through direct binding between the B30.2/SPRY domain and the CA protein on the incoming RTC.^{423,450,686} Binding of TRIM5 α to CA requires both TRIM5 α dimerization and

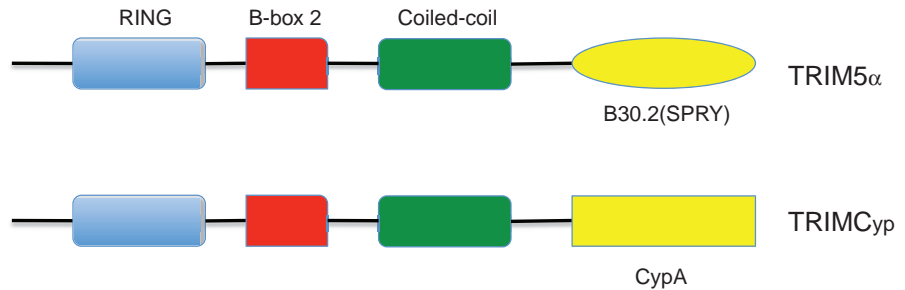


FIGURE 49.15. Organization of TRIM5 α and TRIMCyp. The major domains—RING, B-box 2, coiled-coil, B30.2 (SPRY), and cyclophilin A (CypA)—are shown. Gag-binding determinants of TRIM5 α and TRIMCyp reside in the B30.2 (SPRY) and CypA domains, respectively.

higher-order multimerization.^{423,450} The higher-order multimers have been reported to be hexameric,^{249,559} an observation that is likely relevant to the mechanism of TRIM5 restriction (see later). The importance of TRIM5 α in combating retroviral infections is supported by the finding that the CA-binding B30.2/SPRY domain in TRIM5 α has undergone positive selection during primate evolution.^{362,666,712}

In owl monkey cells, HIV-1 infection is highly inefficient but is significantly enhanced by disrupting the interaction between CA and the cellular protein cyclophilin A (CypA) (see section on Capsid). This observation suggested a link between CA–CypA binding and postentry restriction. The basis for this link was revealed when it was discovered that owl monkey cells express a form of TRIM5 α in which the C-terminal B30.2/SPRY domain has been replaced by CypA^{564,667} (Fig. 49.15). This unusual TRIM5 variant, designated TRIMCyp, restricts HIV-1 infection through a direct binding between the CypA portion of TRIMCyp and CA of the incoming particle. Thus, the ability of TRIMCyp to restrict HIV-1 infectivity in owl monkey cells is eliminated by mutations in the cyclophilin-binding domain of CA or by treating the infected cells with drugs like cyclosporine A that block the CA–cyclophilin interaction.^{564,667} Interestingly, TRIMCyp has arisen independently in several species of macaque.^{83,775,806}

TRIM family proteins assemble into cytosolic complexes often referred to as “cytoplasmic bodies.”⁶²⁴ Although pre-existing TRIM5 α or TRIMCyp cytoplasmic bodies are not required for restriction of retroviral infection,^{593,711} microscopic examination of cells overexpressing rhesus TRIM5 α early after HIV-1 infection revealed a co-localization between TRIM5 α -induced cytoplasmic bodies and viral RTCs.¹⁰⁰ Several lines of evidence support a model whereby binding of TRIM5 α or TRIMCyp to CA on the incoming RTC leads to a destabilization of the RTC and, consequently, a block to reverse transcription: (a) TRIM5 α appears to accelerate CA uncoating early postinfection in the context of either HIV-1 or N-MLV.⁷²⁸ (b) Incubation of *in vitro*-assembled core-like CA complexes with cell lysates containing TRIM5 α or TRIMCyp disrupts the assembled structures.⁶⁵ (c) Treatment of infected cells with proteasome inhibitors reverses the ability of TRIM5 α to prevent viral DNA synthesis, suggesting that TRIM5 α induces the degradation of RTCs in the proteasome.⁸¹⁷ Although proteasome inhibitors rescue the TRIM-induced defect in DNA synthesis, they do not rescue virus infectivity, suggesting a two-step mechanism of action.⁸¹⁷ (d) TRIM5 α itself undergoes proteasome-dependent degradation after exposure of cells to restriction-sensitive virus.⁶³⁶

Recent structural studies have shed light on the mechanism of action of TRIM5 α and TRIMCyp. A rhesus TRIM5 α derivative was shown to assemble *in vitro* into a two-dimensional paracrystalline hexagonal lattice.²⁴⁹ Although assembly could

occur spontaneously, it was greatly facilitated by the presence of hexagonal arrays of *in vitro*-assembled CA. These findings suggest that restricting TRIM proteins assemble on top of incoming CA hexamers, thereby leading to their disassembly and degradation. Consistent with this model, a fragment of rhesus TRIM5 α composed of the coiled-coil and B30.2/SPRY domain was shown to bind HIV-1 CA assemblies or virus-derived cores *in vitro* and induce their disassembly from an extended CA hexameric lattice to individual hexamers.⁸⁵² Binding of the CA lattice by TRIM5 α not only results in CA lattice disassembly and direct antiviral restriction but also contributes to the innate immune response against retroviral infection. Pertel and colleagues⁵⁹⁷ demonstrated that CA lattice recognition by TRIM5 α leads to the generation of free polyubiquitin chains by the ubiquitin-conjugating enzyme UBC13-UEV1A; these polyubiquitin chains in turn activate innate immune signaling through the TAK1 kinase complex.

APOBEC3G and Family Members

While lentiviral *gag* genes apparently evolved to avoid restriction by TRIM5 α and other TRIM family members, another potent suppressor of lentiviral infection drove the evolution of the lentiviral *vif* (virus infectivity factor) gene. The presence of *vif* is a highly conserved feature among lentiviruses; a *vif* product is encoded by all lentiviruses except ELAV. Soon after the discovery of the *vif* gene,^{221,706,724} it was noted that the requirement for Vif is strongly producer-cell dependent. Thus, certain cell lines (e.g., 293T, HeLa, COS, SupT1, CEM-SS, and Jurkat) are “permissive” for *vif*-defective mutants, whereas others, including primary lymphocytes, MDMs, and some T-cell lines, are “non-permissive.” This observation suggested that a host cell factor in the virus-producing cell plays a role in Vif function. Experiments performed with fusions between permissive and nonpermissive cells indicated the existence of a dominant inhibitory factor whose antiviral activity is countered by Vif.^{474,702} A major breakthrough came with the discovery that APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G) is the host factor whose antiviral activity is neutralized by Vif. This finding led to a flurry of studies that have at least partially elucidated the mechanism by which APOBEC3G inhibits HIV-1 replication and the molecular basis for the ability of Vif to overcome this inhibition¹⁵⁵ (Fig. 49.16). APOBEC3G is a member of a large family of DNA cytidine deaminases that are involved in mRNA editing and immunoglobulin gene diversification.³⁰¹ These enzymes contain one or two catalytic domains characterized by His/Cys-X-Glu-X_{23–28}-Pro-Cys-X₂-Cys motifs. In the absence of Vif, APOBEC3G is incorporated into HIV-1 virions in the producer cell. In the next round of infection, the virion-associated APOBEC3G converts cytidines to uridines during minus-strand DNA synthesis.^{300,430,487,494,692,849}

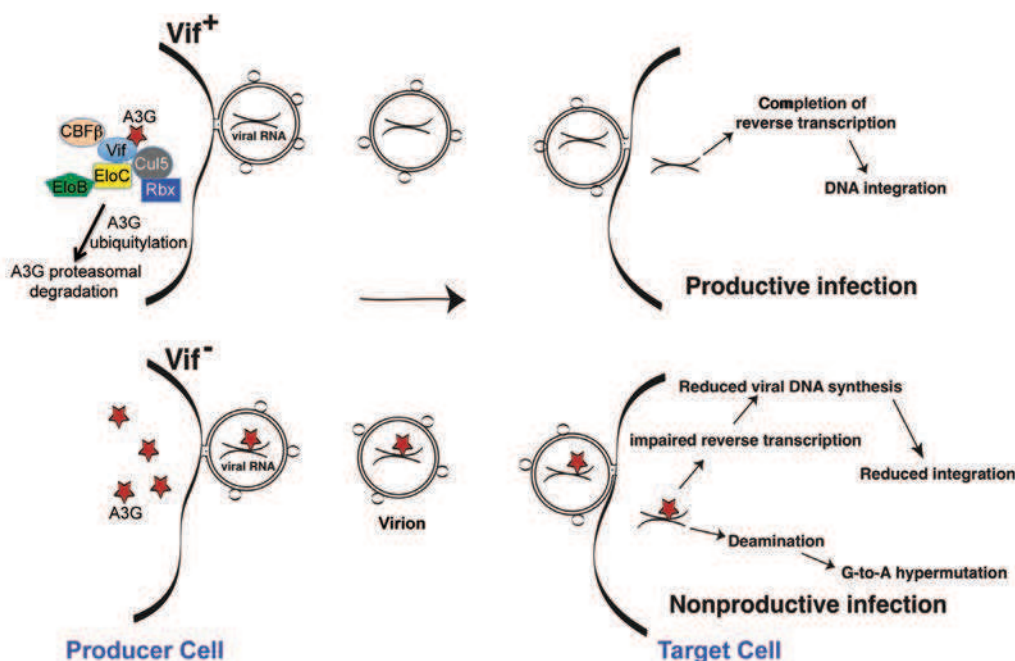


FIGURE 49.16. Proposed model for inactivation of APOBEC3G by Vif. **Top:** In nonpermissive (APOBEC3G-expressing) producer cells, Vif links APOBEC3G to the cellular proteins Cul5, elongins B and C, and ring-box-1 (Rbx1) to form an Skp1-cullin F-box (SCF)-like E3 ubiquitin ligase complex, thereby inducing the proteasomal degradation of APOBEC3G and limiting its incorporation into virions. **Bottom:** In the absence of Vif, APOBEC3G is incorporated into virions. Its presence during reverse transcription in the target cell leads to impaired reverse transcription and integration and deamination of cytidines to uridines, resulting in G-to-A hypermutation. (Adapted from Freed EO. HIV-1 and the host cell: an intimate association. *Trends Microbiol* 2004;12:170–177.)

Cytidine deamination during reverse transcription leads to G-to-A hypermutation of the viral genome, disrupting viral open reading frames. APOBEC3G-induced defects in processing of the tRNA primer, plus-strand transfer during reverse transcription, and DNA integration have also been noted.⁵⁰⁸ In nonpermissive (e.g., APOBEC3G-expressing) producer cells, Vif induces the degradation of APOBEC3G, thereby counteracting its antiviral activity⁸³⁸ (for review see¹⁵⁵). The action of cellular deaminases may contribute to the prevalence of G-to-A mutations (first noted in spleen necrosis virus⁵⁸⁶ and observed in some HIV-1 sequences *in vivo*^{357,771}). Some of the antiviral activity of APOBEC3G has been reported to be independent of deamination activity of the protein, particularly at high expression levels.⁴⁸¹ While hypermutation is clearly disadvantageous to HIV-1 replication, low levels of APOBEC-induced mutation may contribute to retroviral variation and as such could be beneficial to the virus.⁶⁵¹

Vif induces the degradation of APOBEC3G through the ubiquitin-proteasome pathway by interacting with the cellular proteins Cul5, elongins B and C, and ring-box-1 (Rbx1) to form an Skp1-cullin F-box (SCF)-like E3 ubiquitin ligase complex.^{517,838} The transcription co-factor CBF-β is also recruited to this ubiquitin ligase complex³⁵⁶ (Fig. 49.16). The formation of this complex leads to the ubiquitination of APOBEC3G and its degradation in the 26S proteasome. The ability of Vif to induce the degradation of APOBEC3G is species specific; for example, HIV-1 Vif is inactive against simian APOBEC3G, and SIV_{agm} Vif is unable to block the antiviral action of human APOBEC3G. The key difference between human and simian APOBEC3G with respect to degradation

by HIV-1 Vif maps to a single residue difference between the two APOBEC3G proteins.^{71,488,676,822} Murine APOBEC3 is also readily incorporated into HIV-1 virions even in the presence of Vif, making this rodent homolog a potent inhibitor of HIV-1 replication.^{63,494}

The ability of APOBEC3G to restrict HIV-1 replication is highly dependent on its incorporation into virions. APOBEC3G incorporation is rather promiscuous: the human protein can be packaged into, and inhibit, diverse retroviruses, including SIVs, EIAV, and MLV.^{245,300,487,494} Because these viruses are highly divergent at the protein sequence level, APOBEC3G likely gains access to virions through a highly conserved structural element or by binding to molecules present in all retroviral particles (e.g., RNA). Considering that APOBEC3G acts on the viral nucleic acid during reverse transcription, it presumably must associate with the viral core³⁹¹ and ultimately with the RTC. Finally, it is reasonable to assume that APOBEC3G is incorporated through interaction with a viral element that is essential for replication; otherwise, HIV-1 could readily escape APOBEC3G restriction simply by mutating the APOBEC3G binding site without the need for acquiring a novel protein (Vif) to counter APOBEC3G activity. Several studies have focused on the mechanism by which APOBEC3G is packaged into particles, in some cases with discordant results. Some investigators have observed a direct binding between APOBEC3G and Gag, in particular the N-terminal portion of NC.^{11,111,471,669,845} Using a highly sensitive enzymatic assay to measure APOBEC3G levels in virions, others have not observed a requirement for NC in APOBEC3G incorporation.⁷³⁴ Taken together, it appears that although genomic RNA is not absolutely required,^{111,471,734,845}

RNA (viral or cellular)—perhaps in a complex with NC—performs a central function in APOBEC3G packaging. Because RNA encapsidation plays critical roles in particle assembly and virus replication, an RNA-mediated mode of incorporation would be difficult for HIV-1 to escape. RNA-mediated incorporation would also explain the ability of APOBEC3G to gain entry into highly divergent retroviruses.

Humans encode 11 APOBEC family members, with the APOBEC3 genes (APOBEC3A to H) being the most numerous.³⁰¹ Although APOBEC3G was originally identified as the cellular restriction factor that is inactivated by Vif,⁶⁹² other APOBECs also display antiviral properties. Most notably, APOBEC3F inhibits HIV-1 replication, and, like APOBEC3G, its activity is suppressed by Vif.^{63,451,456,801,854} It thus appears that Vif evolved to counter both APOBEC3G and APOBEC3F. APOBEC3B also inhibits HIV-1 infectivity, but, unlike APOBEC3G and APOBEC3F, it is resistant to degradation by Vif.^{63,188} The biological implications of APOBEC3B's antiviral activity are unclear, because this APOBEC family member may not be expressed in most primary human tissues.^{63,188}

Some Vif-negative retroviruses have evolved distinct mechanism(s) for evading restriction by cellular cytidine deaminase. HTLV-1 appears to counter APOBEC3G restriction through a C-terminal extension of its NC domain that blocks packaging of APOBEC3G into particles.¹⁸³ Although MLV infection is inhibited by human APOBEC3G, it is insensitive to all other human APOBEC family members and is resistant to murine APOBEC3 *in vitro*.^{63,189} However, *in vivo*, disruption of the murine *APOBEC3* gene leads to enhanced MLV replication and MLV-induced tumor induction.⁴⁶³ Knockout of murine APOBEC3 also increases the susceptibility of mice to mouse mammary tumor virus infection.⁵⁷⁰ Horses encode six APOBEC3 proteins, one of which is capable of inhibiting HIV-1 replication. Despite efficient packaging into virions, this APOBEC3 does not inhibit ELAV infectivity, suggesting a mechanism of APOBEC evasion that does not rely on virion exclusion.⁷⁰

The interplay between Vif and APOBEC cytidine deaminases offers novel possibilities for antiretroviral therapy. For example, disrupting the binding between Vif and APOBECs with small-molecule inhibitors would activate the antiviral activity of the cellular enzymes. Interfering with Vif-mediated APOBEC degradation could also theoretically be achieved with proteasome inhibitors (which also block virus budding), by functional disruption of the ubiquitin-conjugating enzymes or ubiquitin ligases specifically responsible for APOBEC ubiquitination, or by blocking the interaction of APOBECs with the SCF-like E3 ubiquitin ligase complex. Some initial progress toward targeting APOBEC and Vif has been made; inhibitors that prevent Vif-mediated APOBEC3G degradation¹¹² or that induce the degradation of Vif itself⁵⁵⁵ have been reported.

SAMHD1

As mentioned previously, recent studies have demonstrated that the host protein SAMHD1 restricts an early step in lentiviral infection in myeloid cells and is counteracted by the accessory protein Vpx. This topic will be discussed in more detail later in the section on Vpx.

Nuclear Import

Most retroviral genomes gain access to host cell chromosomal DNA during progression of the target cell through the cell

cycle.^{444,634} These retroviruses are therefore unable to efficiently infect noncycling cells. In contrast, lentiviruses, including HIV-1, are able to productively infect nondividing cells (e.g., macrophages). During the events leading to its integration in the nucleus, the newly synthesized viral DNA remains associated with the PIC. This complex, which is composed of both viral and cellular proteins,^{79,87} must presumably possess the ability to cross the nuclear membrane during interphase to access the host cell genome. Because the HIV-1 PIC—estimated to be ~56 nm in diameter⁵²³—is much larger than the passive diffusion limit for nuclear pores (~9 nm),⁵⁰⁵ it has long been assumed that the PIC must harbor determinants that promote active nuclear import. Initially, it was proposed that MA contains the determinants of PIC nuclear import^{90,777}; however, this model was not validated.^{226,230,232} Subsequent findings implicated Vpr in nuclear import (see section on Vpr). Numerous studies have observed that IN localizes to the nucleus, giving rise to the hypothesis that IN regulates PIC transfer across the nuclear membrane (e.g.,⁷⁸). This model has also been controversial, because mutations that disrupt the nuclear import of IN can cause defects in multiple aspects of IN function.^{347,467} It has also been proposed that a *cis*-acting, centrally located, triple-stranded DNA structure described earlier and known as the *central DNA flap*, which is a product of lentiviral reverse transcription (see section on Reverse Transcription), promotes nuclear import of the PIC.⁸⁴⁶ Although inclusion of the central flap improves the infectivity of lentiviral vectors, there is some disagreement as to whether this sequence is required in the context of replication-competent, full-length HIV-1 molecular clones.^{19,197,454} An HIV-1 chimera-containing MLV IN and lacking all other proposed determinants of nuclear import (e.g., MA, Vpr, and the central DNA flap) retains the ability to infect nondividing cells,⁸²⁵ indicating that none of these elements is critical for HIV-1 nuclear import.

Although the viral determinants of nuclear import, and the mechanism by which they function, remain to be clarified, there is compelling evidence for a connection between CA and nuclear import. It was initially believed that HIV-1 CA is lost from the reverse transcription complex during uncoating; it now appears, however, that a significant amount of CA remains associated with this complex.^{21,187,512} Analysis of chimeras between MLV (which is unable to infect nondividing cells) and HIV-1 (which can infect nondividing cells) revealed that the ability to infect nondividing cells maps to CA.⁸²³ Furthermore, mutations in CA can impose on HIV-1 infection a requirement for cell cycle progression⁸²⁶ and can inhibit nuclear import.¹⁸⁷

As mentioned earlier, several genome-wide RNA interference (RNAi) screens have identified a large number of host factors that are putatively required for HIV-1 infection.^{82,404,857} Among these are nuclear pore proteins and karyopherins—including transportin 3 (TNPO3; also known as TRN-SR2), NUP153, and NUP358/RanBP2—that could play a role in PIC transfer across the nuclear pore. TNPO3 was shown to bind HIV-1 IN and promote PIC nuclear import.¹³¹ Subsequent work⁴¹⁰ confirmed a role for TNPO3 in HIV-1 infection and demonstrated that most lentiviruses showed a requirement for TNPO3 (feline immunodeficiency virus [FIV] being the exception) but that other retroviruses (e.g., RSV and MLV) did not. This study, however, failed to observe a role for IN–TNPO3 binding in the ability of TNPO3 to promote infection. Rather, the use of the HIV-1/MLV chimeras mentioned earlier

pointed to a role for CA in determining TNPO3 dependency.⁴¹⁰ NUP358/RanBP2 bears a cyclophilin domain that has been reported to bind directly to CA; this interaction promotes PIC nuclear import and also influences integration site preference.⁶⁷¹

In a screen designed to identify dominant factors that interfere with HIV-1 infection, a C-terminally truncated form of cleavage and polyadenylation factor 6 (CPSF6) was shown to block an early step in the HIV-1 infection process—specifically nuclear import—but had no effect on MLV infectivity.⁴³² Selection experiments gave rise to a resistant mutant that, intriguingly, contained a single amino acid change in CA (N74D). This N74D CA mutant was not only resistant to inhibition by the truncated CPSF6 but also displayed an altered requirement for karyopherins and nuclear pore factors. For example, wild-type (WT) HIV-1 infection was inhibited by depletion of TNPO3, RanBP2, and NUP153, but infectivity of the N74D mutant was relatively unaffected.^{432,504} Conversely, N74D infection was more sensitive than WT to depletion of nuclear pore proteins NUP85 and NUP155. These data again support a role for CA in PIC nuclear import and demonstrate that CA regulates HIV's utilization of nuclear transport and pore components. CPSF6 is typically localized to the nucleus and overexpression of the full-length, nuclear form has little effect on HIV-1 infection.⁴³² The truncated form that inhibits HIV-1 infection lacks the CPSF6 nuclear localization signal (NLS) and is thus localized to the cytosol. Thus, the shift in CPSF6 localization from nucleus to cytosol may play a key role in its inhibitory activity. Consistent with this model, CPSF6 becomes cytosolic in TNPO3-depleted

cells.⁴³³ Kewalramani⁴³³ showed that in TNPO3-depleted cells CPSF6 becomes cytosolic. Furthermore, CPSF6 depletion reverses the inhibitory effect of TNPO3 depletion. These data suggest that the effect of TNPO3 on HIV-1 is mediated through CPSF6. Because CPSF6 interacts with HIV-1 CA, it is conceivable that this protein plays a regulatory role in HIV-1 infection, potentially as a PIC uncoating or transport factor.

Integration

A distinguishing feature of retrovirus replication is the insertion of a DNA copy of the viral genome into the host cell chromosome after reverse transcription. The integrated viral DNA (the *provirus*) serves as the template for the synthesis of viral RNAs and is maintained as part of the host cell genome for the lifetime of the infected cell. Retroviral mutants deficient in the ability to integrate generally fail to establish a productive infection.

The integration of viral DNA is catalyzed by IN, a 32-kD protein generated by PR-mediated cleavage of the C-terminal portion of the HIV Gag-Pol polyprotein (see Fig. 49.6). The steps in the integration process were originally elucidated in studies using MLV,^{87,242} but these findings apply to HIV as well. In all retroviral systems, integration proceeds in the same series of steps (Fig. 49.17):

1. 3' processing: After its assembly with the viral DNA, IN cleaves immediately downstream from the invariant nucleotide sequence CA, typically removing two nucleotides

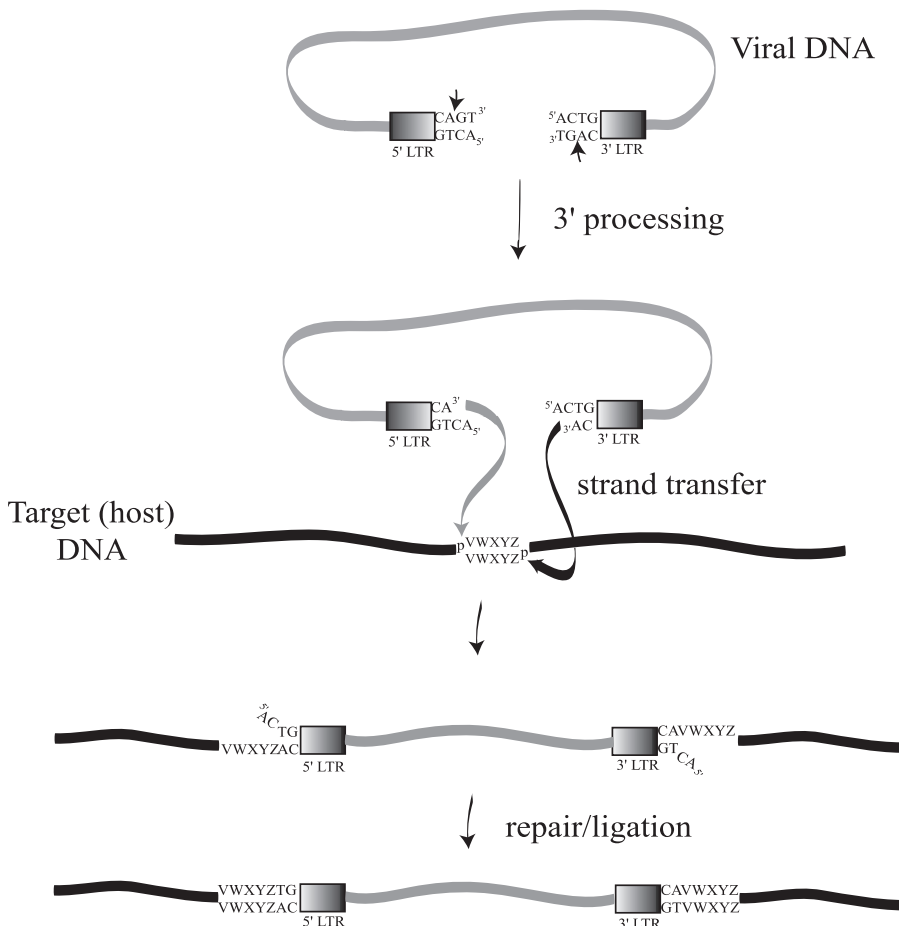


FIGURE 49.17. Schematic depiction of the integration process. Details are provided in the text.

from the 3' termini of both strands of full-length, linear viral DNA, generating a preintegration substrate with 3' recessed ends.

2. DNA strand transfer: In the nucleus, IN catalyzes a staggered cleavage in the cellular target DNA. The 3' recessed ends of viral DNA are joined to the 5' "overhanging" termini of the cleaved cellular DNA.
3. Gap repair: The cellular repair machinery fills the gap, thereby completing the integration process.^{87,242,644} All integrated proviruses terminate with dinucleotides 5'-TG and CA-3', whereas different genera yield different-sized flanking duplications based on the spacing of the staggered chromosomal DNA cut; the lentiviral duplication is 5 bp. In addition to the linear, double-stranded viral DNA that serves as the substrate for integration, two types of circular DNA are formed in the nucleus: 1-LTR circles and 2-LTR circles. Although these circular DNAs are not substrates for integration, they provide useful markers for nuclear import of the viral DNA, as their formation requires enzymatic machinery present predominantly in the nucleus.

Our understanding of the chemistry of the retroviral integration reaction was greatly assisted by the development of *in vitro* integration assays. Purified IN, expressed in *E. coli*, can carry out 3' processing and strand transfer reactions when combined with short synthetic oligonucleotides that mimic the viral DNA ends and a divalent metal ion (Mg^{2+} or Mn^{2+}).^{151,379} Using such systems, IN also catalyzes a reaction known as disintegration, which in essence is the strand transfer reaction in reverse.¹²⁸ While disintegration may not reflect a physiologically relevant activity of IN, it nevertheless has been useful in understanding the biochemistry of integration. Initial studies observed that the predominant product in *in vitro* assays using purified HIV-1 IN was a single end joined to one strand of the target, rather than the more physiologically relevant product in which both ends are integrated into the target (*concerted* or *full-site* integration).

Retroviral IN proteins are composed of three structurally and functionally distinct domains: an N-terminal zinc-binding domain (NTD), a catalytic core domain (CCD), and a C-terminal domain (CTD) (Fig. 49.18). Problems with low solubility and propensity to aggregate hindered progress in defining the structure of the IN holoenzyme in its active (tetrameric) state. However, the structures of all three domains independently and in various combinations were solved by x-ray crystallography or NMR methods. The NMR structure of the NTD⁹⁶ revealed three helical bundles with a zinc coordinated by invariant amino acids His-12, His-16, Cys-40, and Cys-43. The structure of the NTD is reminiscent of helical DNA-binding proteins that contain a helix-turn-helix motif.

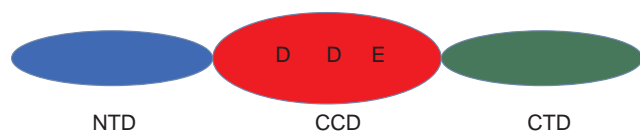


FIGURE 49.18. Schematic representation of an integrase (IN) monomer. The three major structural domains—the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD)—are shown. The catalytic triad Asp-64, Asp-116, and Glu-152 is represented as D-D-E.

Initially, poor solubility hampered efforts to solve the structure of the CCD. However, attempts at crystallography were successful when a mutation was identified that greatly increased solubility without disrupting *in vitro* catalytic activity.¹⁹⁸ Each monomer of the core domain is composed of a five-stranded β -sheet flanked by helices; this structure bears striking resemblance to that of other polynucleotidyl transferases including RNaseH and the bacteriophage MuA transposase.^{198,626} Three highly conserved residues are found in analogous positions at the catalytic center of these enzymes; in HIV-1 IN, these residues are Asp-64, Asp-116, and Glu-152—often referred to as the D,D-35-E motif (Fig. 49.18). Mutations in this catalytic triad block HIV-1 IN function both *in vivo* and *in vitro*. The IN CTD, whose structure was initially solved by NMR,^{200,461} adopts a five-stranded β -barrel folding topology reminiscent of Src homology 3 (SH3) domains. The crystal structure of an HIV-1 NTD-CCD IN fragment⁷⁸⁹ revealed a dimer of dimers, with two inner monomers and two outer monomers, with an extensive intermolecular NTD-CTD interface that provided a preview of the interface evident in the structure of the full retroviral tetramer.

A breakthrough in IN structural biology took place in 2010 with the realization that the IN of prototype foamy virus (PFV) is highly soluble and capable of concerted integration *in vitro* with short DNA substrates.⁷⁶³ Although divergent at the overall amino acid sequence level, HIV-1 and PFV IN are structurally conserved. The IN tetramer bound to DNA oligonucleotides that mimic the viral DNA ends—which together form the active nucleoprotein complex referred to as the “intasome”—was crystallized and the structure solved.²⁹⁴ This structure revealed that two monomers in the tetramer interact at a dimer interface and are responsible for all contacts with the DNA substrate; the other two monomers are on the outside of the complex and do not interact with each other or with DNA (Fig. 49.19). The residues of the catalytic triad (D,D-35-E) of the inner monomers are oriented close to the 3'-OH of the viral DNA ends. Two metal ions are coordinated at the active site by the residues of the catalytic triad. The addition of model target DNA to the intasome allowed for crystallization of a complex containing both viral and target DNAs.⁴⁷⁷ In the presence of Mg^{2+} the strand transfer reaction took place during crystallization, whereas in the absence of divalent metal ions strand transfer was blocked. This allowed capture of postcatalytic strand transfer and prestrand transfer complexes. These structures revealed that the target DNA, which binds in a cleft between the two halves of the intasome complex, adopts a highly bent conformation, allowing the well-separated intasome active sites to access the scissile phosphodiester bonds in the target DNA (Fig. 49.19). The viral (donor) DNA duplex is unpaired at the active site. Conformational changes that occur during catalysis are predicted to render the strand transfer reaction irreversible.⁴⁷⁷ Following the original publication of the structures described earlier,^{294,477} a number of additional PFV structures that contained potent inhibitors were solved (discussed later; for review see⁴⁴⁹); together these reports allowed the development of a structural model for HIV-1 integration that was validated by extensive mutational analysis.⁴⁰⁹

Although the basic mechanism by which retroviruses integrate their DNAs into the host cell genome is highly conserved, different retroviruses display clear differences in the selection of a cellular target site.⁵²⁵ For example, HIV-1 prefers to

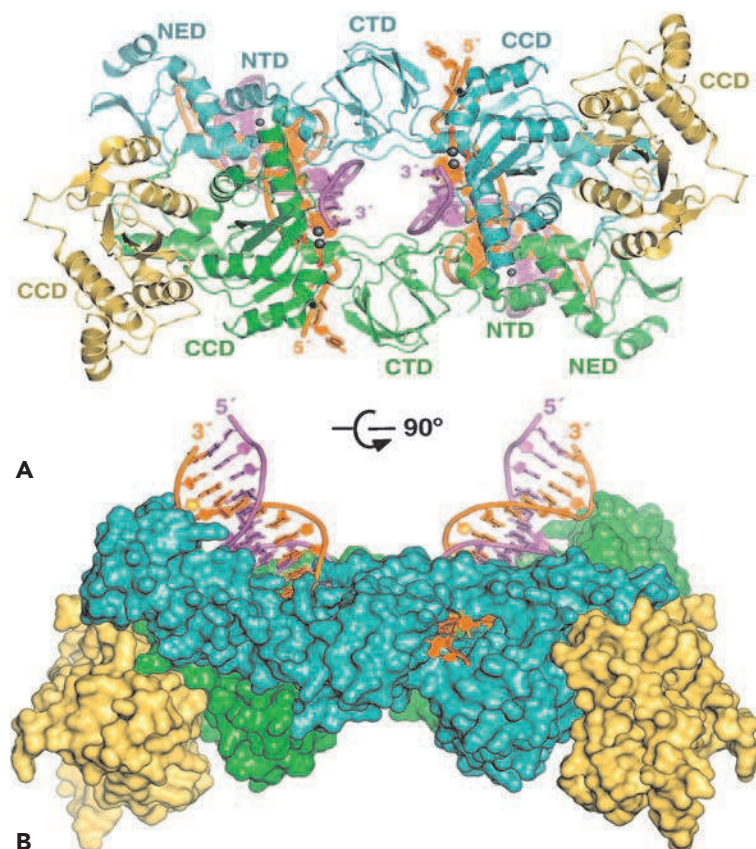


FIGURE 49.19. Structure of the prototype foamy virus integrase (IN) tetramer in complex with viral DNA ends.

The lower image is rotated 90 degrees relative to the upper image. The outer IN chains are yellow; the inner chains are green and cyan. The reactive and nontransferred strands of the viral DNA ends are magenta and orange, respectively. Active-site carboxylates are represented as red sticks. Mn^{2+} and Zn^{2+} ions are shown as large and small gray spheres, respectively. The major IN domains (see Fig. 49.18 legend) are labeled. Note that prototype foamy virus also encodes an N-terminal extension domain (NED) not present in human immunodeficiency virus 1 (HIV-1) IN. (Reprinted from Cherepanov P, Maertens GN, Hare S. Structural insights into the retroviral DNA integration apparatus. *Curr Opin Struct Biol* 2011;21:249–256, copyright 2011, with permission from Elsevier.)

integrate in actively transcribed genes. MLV favors transcription start sites but only weakly selects active genes. ASLV displays a strong preference for neither active genes nor transcription start sites; indeed, for ASLV, high-level transcription appears to be inhibitory for integration.⁵⁰⁷ Target site selectivity, which impacts upon the development of retroviruses as vehicles for gene therapy, is likely influenced by interactions between IN or other components of the PIC and chromatin-associated host factors. Several cellular proteins have been studied extensively for their contribution to integration and target site selection. The most important among these is a 75-kD, predominantly nuclear, chromatin-associated protein known as LEDGF/p75. A direct interaction between HIV-1 IN and LEDGF/p75 was reported,^{124,204} and this protein was found to stimulate the strand transfer step of the integration reaction *in vitro*¹²⁴ and increase binding of IN to DNA.⁹³ However, early studies were discordant in terms of the effect of LEDGF/p75 depletion on HIV-1 replication.^{459,769} It soon became apparent that low levels of chromatin-bound LEDGF/p75 were sufficient to promote integration in cells. Near-complete knock-down of LEDGF/p75 levels⁴⁵⁸ or use of a murine LEDGF/p75 knock-out cell line⁶⁹⁷ revealed markedly reduced (but not fully abolished) HIV-1 infectivity in the absence of detectable LEDGF/p75 expression. Infectivity was also severely compromised by overexpression of the IN-binding domain (IBD) of LEDGF/p75.^{174,458} In the case of both LEDGF/p75 depletion and LEDGF/p75-IBD overexpression, infectivity was disrupted at the integration step, and the effect was specific for lentiviruses, establishing LEDGF/p75 as a cellular co-factor for lentiviral integration. Studies in which siRNA-resistant LEDGF/p75

mutants were added back to LEDGF/p75-depleted cells identified domains of LEDGF/p75 required for its activity as an integration co-factor.^{458,697,770} The observation that the LEDGF/p75-IBD and the chromatin-binding regions (the PWWP domain and AT-hook) (Fig. 49.20) were required for function supported the hypothesis that LEDGF/p75 functions to tether the PIC to target chromosomal DNA. The crystal structure of the IN-CCD and LEDGF/p75-IBD complex provided further details of the IN-LEDGF/p75 interaction.¹²³ In addition to a clear role for LEDGF/p75 in directing HIV-1 integration to transcription units, TNPO3 and the nuclear pore protein RanBP2/Nup358 have also been implicated in the targeting of HIV-1 integration. Ocwieja and colleagues⁵⁶⁷ reported that depleting these genes shifted HIV-1 integration out of gene-dense regions. In contrast, TNPO3 depletion had no effect on the targeting of MLV integration. Use of the aforementioned HIV/MLV chimeras once again mapped the requirement for TNPO3 to Gag.⁵⁶⁷

One of the notable findings to emerge from the LEDGF/p75 depletion studies is that in the absence of LEDGF/p75 expression, HIV's preference for integrating into transcriptional units is lost.^{138,697} These results are consistent with the hypothesis that the ability of LEDGF/p75 to tether lentiviral PICs to chromatin is a major determinant for target-site selection. This hypothesis in turn suggests that the targeting of lentiviral integration could be shifted out of transcription units by substituting the chromatin-binding sequences of LEDGF/p75 with chromatin-binding domains from other proteins. Indeed, the infectivity defect conferred by LEDGF/p75 depletion can be rescued with a chimeric protein bearing the LEDGF/p75 IBD

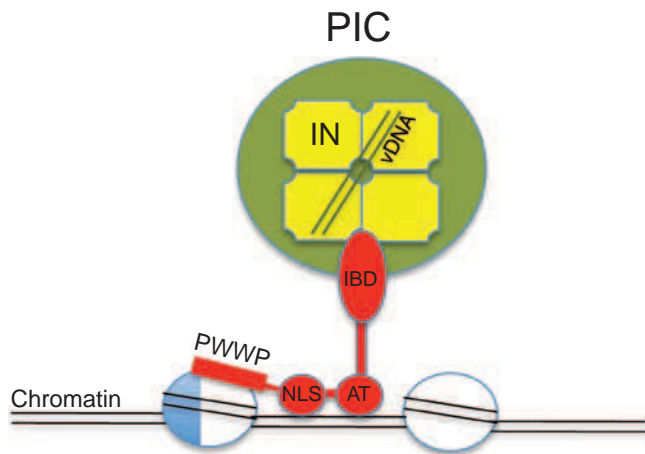


FIGURE 49.20. Model for integrase (IN)/lens epithelium-derived growth factor (LEDGF) binding to chromosomal DNA. The preintegration complex, containing an IN tetramer (yellow) and viral DNA (vDNA), is depicted in green. The IN-binding domain (IBD) of LEDGF is shown bound to the IN tetramer. The nuclear localization signal (NLS) and AT hooks (AT) are shown in complex with DNA; the PWWP domain is depicted bound to histone proteins. (Reprinted from Adamson CS, Freed EO. Novel approaches to inhibiting HIV-1 replication. *Antiviral Res* 2010;85:119–141, copyright 2010, with permission from Elsevier.)

and foreign chromatin-binding domains,⁵¹⁵ and such chimeric PIC-tethering factors are able to shift HIV-1 integration target site specificity away from actively transcribed genes.^{217,261,700} The ability to engineer such custom-designed PIC–chromatin tethering factors and control integration site specificity may have applications in lentiviral-based gene therapy. The function of LEDGF/p75 in promoting HIV-1 integration raises the possibility that the IN–LEDGF/p75 interaction could serve as a target for developing novel antiviral therapeutics. By using the IN–CCD–LEDGF/p75–IBD co-crystal structure in an *in silico* screen, Debyser and colleagues¹³² identified small-molecule “LEDGINs” (LEDGF/p75–IN inhibitors) that block the protein interaction *in vitro* and HIV-1 infection at the integration step.

Early studies showed that several classes of agents inhibit IN function *in vitro*; these included polyanions, nucleotide analogs, and DNA-binding compounds.²⁰⁹ Most of these agents are fairly nonspecific in their mode of action, and many compounds that block activity of purified IN are not inhibitory in assays using purified PICs.²¹⁰ However, di-keto acid inhibitors were found to be specific and potent inhibitors of the IN-mediated strand transfer reaction both *in vitro* and in cell-based assays.³⁰⁷ These IN strand transfer inhibitors (INSTIs) bind IN only in the presence of divalent metal and viral DNA and prevent the complex from associating with cellular (target) DNA. Although structurally diverse, INSTIs appear to have common features, including (a) a hydrophobic moiety composed of halobenzyl groups and (b) co-planar oxygen atoms presumed to chelate the metal ions in the active site.⁵⁰⁹ Work in the early 2000s showed that naphthyridine carboxamides inhibit the replication of an SHIV in rhesus macaques, setting the stage for *in vivo* use of INSTIs.³⁰⁸ Further structure-activity relationship studies led to the development of raltegravir, also known

as MK-0518 or Isentress (Merck Research Laboratories),⁷³¹ and elvitegravir (Gilead Sciences and Japan Tobacco).⁶⁵⁹ Raltegravir was approved for clinical use in HIV-infected patients by the U.S. Food and Drug Administration (FDA) in 2007 and elvitegravir was approved in 2012 (reviewed in⁵⁰⁹).

The observation that INSTIs are active against a wide variety of retroviruses,⁴⁰⁰ including PFV,⁷⁶³ allowed structures to be determined for raltegravir and elvitegravir bound to the PFV IN active site²⁹⁴; this information was subsequently extended to the HIV-1 model.⁴⁰⁹ Additional PFV structures that included highly potent second-generation INSTIs have moreover since been solved.^{295,296} These structural analyses confirmed that INSTIs chelate the metals in the enzyme active site and demonstrated that the halobenzyl moieties of the INSTI stack against the cytidine of the invariant CA dinucleotide and in doing so supplant the adenine ring and eject its associated DNA strand transfer 3'-OH nucleophile from the active site.^{294,296,409} As with other HIV inhibitors, resistance develops both *in vitro* and *in vivo*. Three major pathways of resistance have been observed for raltegravir—these involve residues N155, Q148, and Y143 (reviewed in⁵¹⁹). Secondary mutations arise at a number of additional positions. Elvitegravir treatment leads to the acquisition of mutations in IN residues T66 and S147, as well as changes associated with raltegravir resistance (at residues Q148 and N155).

Viral Gene Expression

The HIV LTR

Retroviral LTRs are generated during the process of reverse transcription and therefore exist only as “repeats” in viral DNA. During the replicative cycle, sequences mapping to the LTR serve a multitude of functions in the context of both proviral DNA and genomic RNA. First, complementary genomic RNA and minus-strand DNA sequences located in the R region mediate a critical early step of the reverse transcription reaction by forming intermolecular DNA–RNA hybrid structures (see section on Reverse Transcription) (see e-Fig. 49.2A, step 4). These hybrids link the newly synthesized DNA strand (“minus” polarity) with complementary RNA sequences at the 3' end of the same or a different genomic RNA template (“plus” polarity), thereby allowing the RT to continue its polymerizing activity and generate a full-length minus-strand DNA. Second, during the integration step of the virus life cycle, LTR elements (*att*, or attachment, sequences) located at the termini of full-length linear viral DNA molecules guide their insertion into the chromosomal DNA of host cells (see section on Integration) (e-Fig. 49.2B). Other LTR sequences, located in the context of U5 RNA sequences, have been reported to contribute, in concert with the ψ element (see Fig. 49.7), to the packaging of progeny HIV-1 RNA genomes during virus assembly (see Fig. 49.8).

In the context of integrated viral DNA, the major function of the retroviral LTR is the regulation of viral RNA synthesis. The HIV-1 transcriptional promoter and multiple regulatory elements are located within the U3 region and function in the context of the 5' LTR (Fig. 49.21). Their principal function is to recruit the RNA Pol II holoenzyme to the start site (+1) of viral RNA synthesis, also defined as the first nucleotide in the LTR R region. The recognition of the core promoter by RNA Pol II is the first step for initiating RNA synthesis. The formation of the preinitiation complex begins when the general

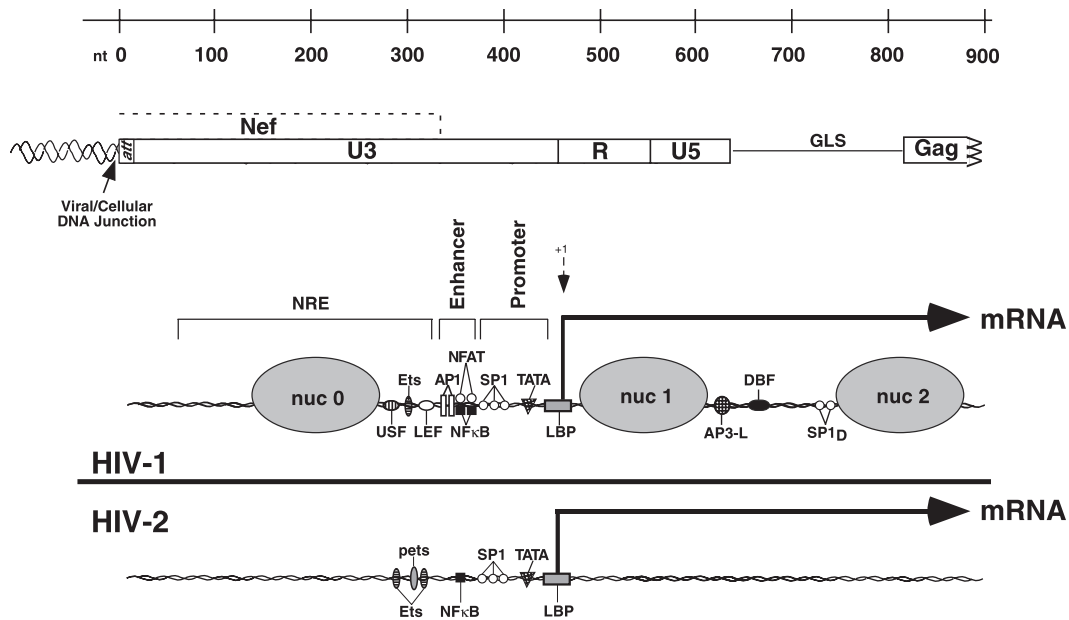


FIGURE 49.21. Fine structure of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). The HIV-1 LTR is a duplicated 630+ base-pair (bp) element located at the termini of integrated proviral DNA (as shown in Fig. 49.20A, top). A blow-up of the 5' LTR and adjacent cellular and Gag coding sequences is presented at the **top**. The HIV-1 LTR has been subdivided into three domains: the R (repeat) region is defined as a 96-nt repeat present at the 3' and 5' termini of HIV-1 genomic RNA; U5 is an 84-nt segment located immediately 3' to the R region; and U3 is a 454-nt segment situated immediately 5' to R. The portion of the *nef* gene overlapping U3 and the location of terminal attachment (*att*) sequences are shown. The 5' LTR and adjacent *gag* leader sequence (GLS) are aligned with the subdivided LTR (**middle**) to indicate functionally important binding sites for transcriptional regulatory proteins. For HIV-1, the core promoter consists of the three Sp1 binding elements and the TATA site. The two nuclear factor- κ B (NF- κ B) binding motifs, the nuclear factor of activated T cells (NFAT), and adjacent activator protein 1 (AP-1) elements compose the “enhancer” domain. The transcription start site is located at map position “+1,” which is defined as the border between U3 and R. The negative regulatory element (NRE) contains binding sites for the upstream stimulatory factor (USF) and the Ets proteins. Sequence motifs that interact with the TATA-binding protein (TBP), leader-binding protein (LBP), AP-3-like (AP3-L), DBF-1, and two downstream Sp1 elements (SP1_D) are shown. The positions of nucleosomes nuc 0, nuc 1, and nuc 2 are indicated. For HIV-2 (**bottom**), the unique binding sites for the peri-ets (pets) and E26 transforming specific (Ets) transcriptional factors are indicated.

transcription factor (GTF) TFIID binds to several core promoter elements including the TATA box, located –29 to –24 nt upstream of the transcriptional start site (Fig. 49.21). TFIID consists of the TATA box-binding protein (TBP) and 13 or 14 TBP-associated factors (TFA) that bind downstream elements including the initiator elements spanning the transcription start site. The GTF factor TFIIA then enters the complex, facilitating the binding of TFIID to the core promoter to generate a platform upon which a functional transcription complex can be assembled.

A third GTF (TFIIH), which modifies RNA Pol II allosterically and allows the transcription complex to initiate RNA polymerization and “escape” from the promoter, must also be incorporated into the RNA Pol II complex.⁴⁶⁴ TFIIH is a multicomponent transcription factor that includes a kinase activity able to phosphorylate a unique region within the largest RNA Pol II subunit, the CTD, which contains a heptad sequence (consensus YSPTSPS) that is repeated 52 times. The phosphorylated state of the CTD critically determines the functional state of Pol II. Whereas hypophosphorylated Pol II (Pol IIa) is readily recruited into the preinitiation transcription complex, the hyperphosphorylated form of the enzyme (Pol IIo) medi-

ates the elongation of nascent RNA transcripts. Three kinases, associated with the transcriptional apparatus, are known to phosphorylate the CTD. These kinases include (a) the CDK7/cyclin H subunits of the CDK-activating kinase (CAK) complex associated with TFIH, (b) the CDK8/cyclin C component of the RNA Pol II holoenzyme, and (c) the CDK9/cyclin T subunits of the positive transcription elongation factor b (P-TEFb). Hyperphosphorylation of the CTD induces conformational changes in RNA Pol II that allow the transcription complex to clear the promoter and begin elongating nascent viral RNA molecules. These cellular kinases, which participate in transcriptional elongation, will be discussed in greater detail in the section describing the function of the HIV-1 Tat protein.

In eukaryotic cells, regulatory factors, which bind to DNA elements near the promoter, modulate the basal rate of RNA synthesis in response to physiologic cues, after the recruitment of Pol II. This process results in the more frequent assembly of stable transcription complexes that are better able to escape the promoter and generate full-length primary transcripts. The human genome encodes more than 2,500 proteins with DNA-binding domains, many of which function to regulate RNA synthesis. Binding sites for distinct transcription factors

frequently flank the promoters for cellular and viral genes. The HIV promoter contains its own ensemble of DNA elements to which transcriptional regulatory factors bind (Fig. 49.21). In the context of HIV-1 replication, distinct combinations of transcription factors may be found in the different cell types (T cells and MDMs), activated states (naïve and memory CD4+ T lymphocytes), and tissues (lymph nodes, gastrointestinal tract, and CNS) targeted by the virus. The combinatorial use of subsets of these factors will determine the efficiency of progeny virus production at these various locations and activation states. The transcription of HIV-1 and cellular genes in CD4+ T lymphocytes is highly dependent on extracellular stimuli that induce the translocation of some of these regulatory factors into the nuclear compartment. Consequently, in an infected individual, the HIV-1 LTR may have to direct viral RNA production in multiple and functionally unique micro-environments.

Traditionally, the synthesis of HIV-1 RNA has been studied by (a) transfecting LTR-driven reporter gene constructs into HeLa cells or other continuous human cell lines or (b) infecting human T-cell lines or PBMCs, collected from uninfected individuals, with HIV derived from replication-competent molecular clones. In these experiments, RNA production is monitored under basal conditions or following activation with the HIV-1 Tat protein. Studies with human PBMCs have been conducted under resting or activated conditions. The LTRs of HIV and other primate lentiviruses contain three tandemly arranged binding sites for the constitutively expressed Sp1 transcription factor; these DNA elements are situated immediately upstream of a canonical RNA Pol II TATA box (Fig. 49.21). The Sp1 and TATA elements constitute the HIV-1 core promoter and must be present for basal levels of LTR-directed RNA synthesis. Functional analyses of HIV-1 LTR-driven reporter constructs have shown that mutations of individual or pairs of the three Sp1 sites have little, if any, effect on basal or Tat-transactivated expression levels.²⁹⁹ Mutation of all three HIV-1 Sp1 sites, however, markedly reduced the response to the HIV-1 Tat protein.²⁹⁹ In the context of virus infections, the role of the Sp1 elements is cell type dependent.^{438,642} Mutations that functionally inactivated all three Sp1 motifs eliminated detectable replication in Jurkat cells and delayed progeny virus production in CEM and H9 cells but had little effect on replication in activated PBMCs.

Three transcriptional factors (NF- κ B, nuclear factor of activated T cells [NFAT], and AP-1) play major roles in regulating cellular and HIV-1 RNA synthesis in human T lymphocytes after antigen-specific/MHC-restricted signaling through the T-cell receptor-CD3 complex. The two binding sites for the NF- κ B/Rel family of transcription factors constitute the principal activatable enhancer elements in the HIV-1 LTR. The NF- κ B sites (consensus sequence: GGGRNNYYCC) are located upstream and adjacent to the Sp1-binding motifs in all HIV-1 isolates.⁵⁵² In clade E strains, a single inactivating nucleotide deletion in the upstream NF- κ B element converts it to a GA-binding protein (GABP) site, and clade C isolates carry a third NF- κ B site (Table 49.2). The LTRs of HIV-2 and SIV, while retaining the triplicated Sp1 motifs, each contain only a single NF- κ B binding site (Fig. 49.21, bottom). The mammalian NF- κ B family of proteins consists of five members: p65 (Rel A), Rel B, c-Rel, p50 (derived from the p105 precursor), and p52 (derived from the p100 precursor). All NF- κ B proteins contain a highly conserved Rel homology domain (RHD),

TABLE 49.2 Binding Sites of Transcription Regulatory Factors in the Long Terminal Repeats of Different HIV-1 Clades

Clade	Ets	AP1		NF- κ B			Sp1		
		I	II	III	II	I	III	II	I
A	+	+	+	—	+	+	+	+	+
B	+	+	—	—	+	+	+	+	+
C	+	+	—	+	+	+	+	+	+
D	+	+	—	—	+	+	+	+	+
E	—	+	—	—	GABP	+	+	+	+
F	+	+	+	—	+	+	+	+	+
G	+	+	+	—	+	+	+	+	+
AG	+	+	+	—	+	+	+	+	+

AP, activator protein; NF, nuclear factor; Sp, specificity protein; GABP, GA-binding protein.

Adapted from van Opijnen T, Jeeninga RE, Boerlijst MC, et al. Human immunodeficiency virus type 1 subtypes have a distinct long terminal repeat that determines the replication rate in a host-cell-specific manner. *J Virol* 2004;78:3675–3683.

which is approximately 300 residues in size and located near the N-terminus. The RHD mediates DNA binding, dimerization with other NF- κ B partners, and interaction with the inhibitor protein, I κ B; it also contains a NLS. Some NF- κ B proteins, including p65, also carry a potent transcription activation domain located near their C-termini, which can promote the transcription of target genes by recruiting activating and repressing cellular transcription factors. The NF- κ B inhibitory proteins, I κ B α , - β , and - ϵ , all contain multiple 30- to 34-amino acid ankyrin motifs, which provide the interface for binding to various species of NF- κ B.

In vivo, NF- κ B is intimately involved in inflammation, cell proliferation, and apoptosis. It modulates the expression of cytokines (IL-6, IL-1 β , tumor necrosis factor- α [TNF α], GM-CSF), chemokines (IL-8, RANTES, macrophage inflammatory protein-1 α [MIP-1 α], monocyte chemoattractant protein-1 [MCP-1]), enzymes (inducible nitric oxide synthase [iNOS], cyclo-oxygenase-2 [COX-2]), and adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1], ICAM-1, E-selectin) in response to the stimulation of numerous receptors involved in immune responses, including T-cell receptors, B-cell receptors, TNF receptors, CD40, and the Toll/IL-1R family.⁷³ Because the various members of the NF- κ B family are present intracellularly as dimeric molecules, a heterogeneous population of homo- and heterodimers, with different functional specificities, bind to the two NF- κ B sites present in the HIV-1 LTR. For HIV-1 gene expression, the two most important forms of NF- κ B are the p50/p50 homodimer and the p50/p65 heterodimer. The functional activity of NF- κ B is controlled by its intracellular location. p50/p65 NF- κ B molecules are sequestered in the cytoplasm as a consequence of the tight binding of unphosphorylated I κ B α to the RHD of the p65 subunit of the heterodimer. It is believed that cytoplasmic retention of the NF- κ B-I κ B α complex is due to the masking of the p65 NLS, which is also located in the RHD. Following activation of the protein kinase C pathway, phosphorylation of two I κ B N-terminal serine residues (S32 and S36) occurs. Phosphorylated I κ B is rapidly degraded as a

result of ubiquitination and proteolysis via the 26S proteasome complex; the freed p50/p65 NF- κ B is then translocated into the nucleus where it can activate multiple cellular genes including I κ B. The newly synthesized (and unphosphorylated) I κ B enters the nucleus, where it captures NF- κ B and returns it to the inducible cytoplasmic pool of NF- κ B molecules. The nucleo-cytoplasmic shuttling property of I κ B and the feedback loop controlling its synthesis are responsible for the transient nature of NF- κ B activity.^{73,306}

When the NF- κ B binding sites are mutagenized in the context of HIV-1 infectivity, virus replication is affected, depending on the type of mutant constructed and the endogenous levels of NF- κ B in the cells used to generate progeny virions. Deletion of both HIV-1 NF- κ B motifs resulted in modest⁶⁴² delays in peak virus production in activated human PBMCs, whereas point mutations affecting specific nucleotides in the NF- κ B recognition sequence, but not the spatial organization of the two NF- κ B elements within the HIV-1 LTR, reduced virus production more than 10-fold.¹¹⁹ The replication of HIV-1-bearing NF- κ B mutations in several human T-cell lines was found to be inversely proportional to the basal levels of NF- κ B in the particular cell line under study. It is likely that other transcription factors present in these cells can compensate for the absence of NF- κ B in these viral mutants. The simultaneous mutation of the two NF- κ B motifs and all three Sp1 sites completely abolishes HIV-1 replication,^{438,642} consistent with the demonstration that the cooperative interaction of NF- κ B and Sp1 promotes the binding of both factors to the HIV-1 LTR and induces transcriptional activation.⁵⁹⁵ A conclusion from all of these results is that NF- κ B stimulates transcription from the HIV-1 LTR in activated human CD4+ T lymphocytes.

HIV-1 transcription can also be modulated in response to T-cell activation by other cellular transactivation proteins such as NFAT and AP-1. Like NF- κ B, members of the NFAT family of proteins are sequestered in the cytoplasm and transported into the nucleus after an increase in the levels of intracellular calcium.⁷⁵³ Changes in intracellular calcium concentration activate the calcineurin serine/threonine phosphatase and result in the phosphorylation of NFAT, exposure of the NFAT NLS, and NFAT nuclear translocation. Although, it was originally thought that NFAT binds to a site within the NRE region of the HIV-1 LTR, more recent data show that NFAT recognition sequences map to the 3' halves of the two NF- κ B motifs in the promoter-proximal region of the U3 LTR (Fig. 49.21).³⁹⁷ NFAT binds to DNA as dimers when the subclass of NF- κ B recognition sites found in HIV-1 LTR is present. Because NFAT is known to recruit the co-activator p300 and CBP to cellular promoters, it is likely to mediate nucleosomal disruption in synergy with NF- κ B to positively regulate HIV-1 LTR-directed gene expression.

The rate of HIV-1 viral RNA synthesis can also be stimulated by AP-1, which consists of Jun homodimers or Jun/Fos heterodimers. Depending on the HIV-1 subtype, AP-1 binds to one or two DNA elements located immediately upstream of the two NF- κ B sites in the HIV-1 LTR (Table 49.2). AP-1 has also been shown to cooperate with NF- κ B and activate the viral promoter via the two NF- κ B-binding motifs. AP-1 is activated by the c-Jun N-terminal kinase (JNK) and the extracellular signal-related kinase (ERK).³⁷⁶

The HIV-2 LTR also contains three Sp1 binding sites adjacent to its TATA box, but the organization of its upstream

enhancer region is different from that of HIV-1 (Fig. 49.21, bottom). This portion of the HIV-2 U3 LTR contains only a single functional NF- κ B motif as well as immediately upstream E26 transforming specific (Ets) and peri-ets (pets) binding sites.⁴⁹⁶ The two purine-rich Ets sequences are recognized by a family of proto-oncogene proteins (mainly Elf-1), and the human autoantigen DEK binds to the pets site. Thus, whereas activation of HIV-1 LTR-directed expression after T-cell activation is mediated primarily through the binding of NF- κ B to its two cognate sites, induction of HIV-2 gene expression is stimulated by a different set of cellular transactivators.

In eukaryotic cells the interactions of transcriptional regulatory proteins with their cognate *cis*-acting elements is further modulated by the packaging of DNA into chromatin. This is clearly the case for HIV-1 proviral DNA, which becomes stably integrated into host cell chromosomal DNA during productive viral infections. DNA footprinting and restriction enzyme accessibility studies of integrated viral DNA have in fact revealed the presence of nucleosomes located both upstream and downstream of the HIV-1 promoter (designated nuc-0, nuc-1, and nuc-2, respectively, in Fig. 49.21).¹⁴⁶ Nucleosome positioning defines two open chromatin regions encompassing (a) promoter/enhancer sequences (–250 to +11 [relative to the transcription start site]) and (b) a downstream segment that begins within the 3' portion of U5 and extends into the Gag leader (+150 to +250). This scheme of chromatin organization is independent of the provirus integration site. Transcription factors are thought to bind to their cognate recognition motifs to maintain these open chromatin regions. The nucleosome-free region that is promoter distal to nuc-1 and located downstream of the transcription start site contains recognition sites for three different transcription factors: AP-3-like, DBF-1, and Sp1 (Fig. 49.21). Mutagenesis of these binding motifs effectively abolishes the downstream nucleosome-free zone and markedly inhibits the transcriptional activity of the HIV-1 promoter in the context of both stably integrated LTR constructs and infections mediated by mutagenized cell-free virus.^{201,766} Taken together, these results are consistent with a model in which the positioning of nucleosome nuc-1 immediately downstream of the transcription start site sterically impedes transcription initiation and/or elongation, resulting in the characteristically low basal (uninduced) levels of HIV-1 RNA synthesis. The positioning of nuc1 is thought to reflect the binding of transcriptional regulatory factors to upstream promoter/enhancer motifs (*viz.* Sp1 and NF- κ B sites) and downstream recognition sequences situated in the U5/Gag leader region (AP-3-like, DBF-1, and Sp1), a combination that creates the two nucleosome-free regions. Transcriptional activation of cells containing the integrated HIV-1 LTR with Tat, TNF α , or inhibitors of histone deacetylation specifically results in the rapid remodeling of nucleosome nuc-1 and generates a 500-bp open chromatin region.

HIV-Encoded Regulatory Proteins

Tat

Although the mechanism underlying Tat transactivation of HIV LTR-directed expression was hotly debated for several years, it is now generally agreed upon that Tat increases the steady-state levels of viral RNA several hundredfold by directing the formation of a more processive RNA Pol II transcription complex in virus-infected cells. Tat is an indispensable viral

protein; when the HIV-1 *tat* gene is mutagenized, no detectable progeny virions are produced.^{170,222} HIV-1 Tat is a nuclear protein containing 101 amino acid residues encoded by two exons (Fig. 49.22). A shorter 72-amino acid “one-exon” HIV-1 Tat protein possesses all the transcriptional activating properties of full-length Tat, as measured in tissue culture infections or in LTR-driven reporter gene experiments. The termination codon following the first Tat exon is highly conserved among diverse HIV-1 isolates, suggesting that the “one-exon” and “two-exon” Tat proteins may mediate different functions during productive viral infections *in vivo*.

As previously noted, RNA synthesis in eukaryotic cells is usually modulated by transcription activator proteins, which bind to DNA motifs located *upstream* of their respective promoters and recruit GTFs and the RNA Pol II holoenzyme to the transcription initiation complex. Visna virus, another member of the *Lentivirus* genus, in fact encodes a DNA-binding Tat protein, which binds to AP-1 sites located in its LTR upstream of the transcription start site. In contrast, the targets of all primate lentiviral Tat transactivating proteins are sequences situated *downstream* of the start site for viral RNA synthesis.⁶³⁹ The basis for this unusual property was subsequently elucidated when it

was demonstrated that TAR does not function as a DNA recognition site but as an RNA structural element (see Fig. 49.7).⁵⁰

HIV-1 TAR encompasses the 5′ terminal 59 nt of *all* viral RNAs and folds into a stable stem-loop structure (Fig. 49.22). The minimal TAR element (mapping between bases +19 and +42) contains three critical components: a base-paired stem, a trinucleotide bulge (containing the sequence UCU at positions +23 to +25), and a hexanucleotide G-rich loop.²¹⁵ Interestingly, HIV-2 TAR forms a double stem-loop structure, each arm of which possesses a dinucleotide bulge and the hexanucleotide G-rich loop (Fig. 49.22).²⁰³ Sequences located in both the hexanucleotide loop and the bulge of HIV-1 TAR are required for Tat function. HIV-1 Tat binds to WT or “loop” mutants of TAR but *not* to TAR elements containing alterations affecting the bulge region.^{185,646} Modifications of the invariant “bulge” U₊₂₃ nucleotide and elimination of the base pairs immediately above and below the bulge (Fig. 49.22) significantly reduce the binding of Tat to TAR.⁷⁹² NMR studies have shown that the binding of a Tat peptide to TAR causes the major groove in the RNA duplex to widen, thereby generating multiple points of contact between Arg residues in the binding domain of Tat with critical nucleotides (e.g., U₊₂₃) and several phosphate groups

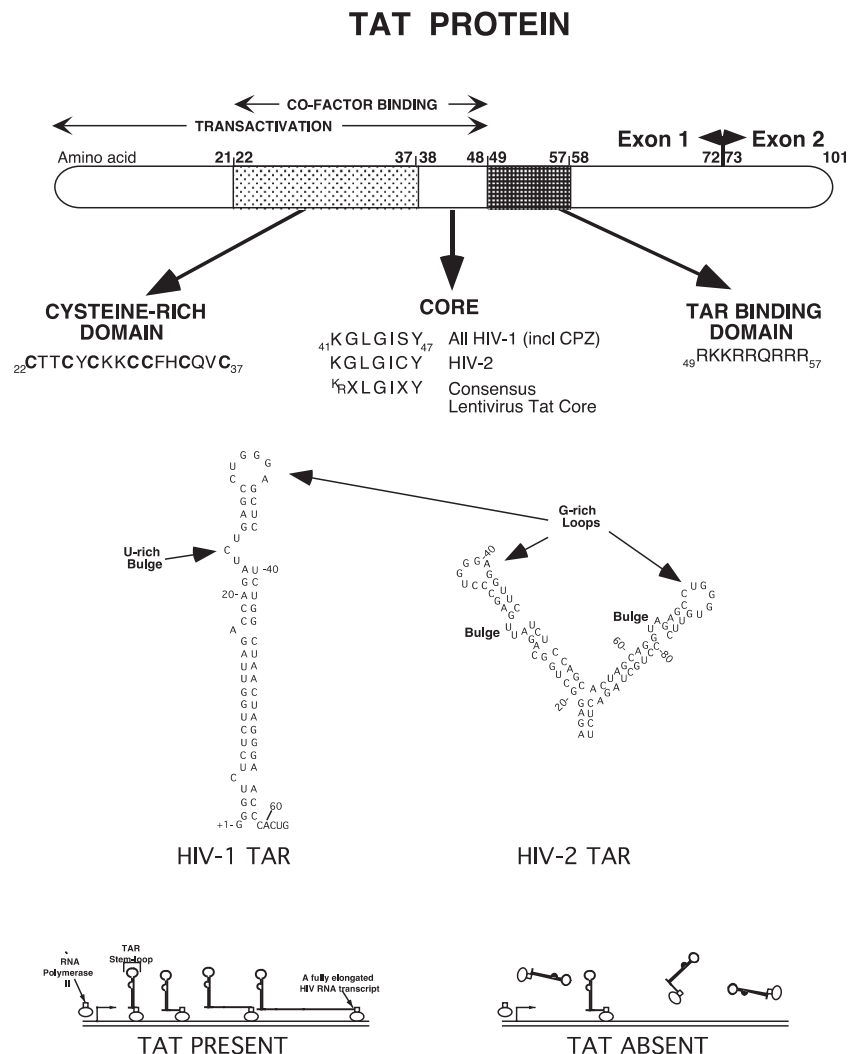


FIGURE 49.22. Tat and its transactivation response region (TAR) element. Schematic representation of the human immunodeficiency virus 1 (HIV-1) Tat protein (**top**) with the cysteine-rich activation, core, and TAR-binding domains indicated. The stem-bulge-loop configurations of HIV-1 and HIV-2 TAR elements are shown in the **middle**. The production of fully elongated HIV-1 messenger RNA (mRNA) molecules generated in the presence of Tat, compared with short HIV-1 transcripts, prematurely released from the DNA template in the absence of Tat, is depicted at the **bottom**.

comprising the TAR backbone.¹ This distortion of the TAR structure also brings the loop and bulge regions into close proximity, which could facilitate the interactions of the proteins binding to both of these TAR domains (see later).

The functional organization of the HIV-1 Tat protein has been deduced from TAR binding and transcriptional activation experiments using both WT and mutagenized derivatives of the Tat protein. The tripartite Tat activation (or effector) domain encompasses the N-terminal 48 residues, which include (a) a string of highly acidic amino acids (residues 1 to 21); (b) a Cys-rich region (seven invariant, six of which are required for function) between positions 22 and 37; and (c) a hydrophobic core segment (amino acids 38 to 48), which is highly conserved among different HIV isolates (see Fig. 49.22). The activation domain is critical for recruiting an essential cellular co-factor to TAR; mutations affecting this region of the Tat protein drastically reduce or eliminate transactivation activity.⁶⁴⁷ The TAR RNA-binding domain of Tat has been mapped to a Lys/Arg-rich region between residues 48 and 57 (Fig. 49.22); peptides from this Tat segment bind to the TAR bulge region as well as to a few base pairs surrounding the bulge, but with somewhat less affinity and specificity than do purified preparations of the full-length Tat protein.¹⁴⁸

The hallmark of HIV LTR-directed RNA synthesis in the *absence* of Tat is the accumulation of prematurely and randomly terminated transcripts, which are converted to longer RNA species when Tat is expressed (Fig. 49.22, bottom). This Tat-deficient phenotype has been observed after the transient transfection of LTR-driven reporter gene constructs in the absence of Tat and in cells harboring integrated HIV-1 proviruses with mutated Tat genes.^{212,426,755} Because Tat expression markedly stimulated transcriptional elongation in these experimental systems, attention shifted to the possible involvement of the CTD of RNA Pol II, which, as noted earlier, is hyperphosphorylated in highly processive transcription complexes. This line of reasoning was also consistent with the reported inhibition of Tat-stimulated transcription by the adenosine analog DRB, which blocks RNA Pol II-mediated elongation *in vitro* and *in vivo* by inactivating protein kinases.⁸⁰ The demonstrated requirement of the Pol II CTD for Tat-mediated transactivation of viral RNA synthesis also supported such a model.^{581,830}

Reports describing the specific interaction of HIV-2 Tat (or just the effector domain of HIV-1 Tat [residues 1 to 48]) with a cellular kinase linked many of these disparate findings and provided the first mechanistic clues about Tat function.^{316,317} The novel cellular enzymatic activity identified, initially named Tat-associated kinase or TAK, was able to phosphorylate the CTD of RNA Pol II. Contemporaneous studies evaluating Tat-stimulated Pol II processivity in cell-free systems indicated that Tat interacted with components of the TFIIF complex to enhance transcriptional elongation and raised the possibility that TAK might be a TFIIF-associated kinase.^{256,581} However, it was subsequently shown that Tat binds with high affinity to a Pol II CTD kinase complex⁸⁶³ related to the *Drosophila* multicomponent P-TEFb, which is catalytically independent of TFIIF.^{498,590,591}

The Tat-related kinase component in human P-TEFb was subsequently identified as CDK9, a 42-kD CDC2-related kinase. When antibodies were used to deplete this kinase in nuclear extracts, the residual activity for Tat-stimulated RNA chain elongation was reduced nearly 100-fold.^{590,591,863} Although

earlier studies had shown that P-TEFb interacted with the activation domain of HIV-1 Tat, the CDK9 catalytic subunit itself failed to bind to Tat in *in vitro* assays. Equally perplexing was the identification of the P-TEFb kinase activity in nuclear extracts migrating not as the 42-kD CDK9, but as a 110-kD complex.⁸³⁰ This result suggested the existence of a possible cyclin-related partner for CDK9, which might provide substrate specificity. These unresolved issues were clarified with the isolation of the “missing link”—an 87-kD protein from nuclear extracts, which bound to WT HIV-1 Tat but not to a mutant Tat protein containing a nonfunctional activation domain.⁷⁹³ The sequence of a cDNA clone encoding the 87-kD protein revealed the presence of an N-terminal cyclin box that was nearly 40% identical to human cyclin C. The new protein, initially named cyclin T (because it bound to Tat), is encoded by a gene mapping to human chromosome 12. The CDK9 family of kinases is now known to interact with cyclin regulatory subunits, many of which have been cloned and characterized.⁵⁹⁰ Several human cyclins (T1, T2a, T2b, or K) can functionally partner with CDK9, greatly increasing its kinase activity⁵⁹¹; of these, only cyclin T1 is able to directly bind to the HIV-1 Tat activation domain and is a component of P-TEFb.⁷⁹³

The binding of Tat to TAR has traditionally been the centerpiece of models for Tat-mediated transactivation. After the identification of CDK9 and cyclin T1 as cellular co-factors of Tat, attention turned to determining how they interacted with Tat, TAR, and the cellular transcriptional machinery. A large body of work revealed the following information: (a) CDK9/cyclinT1 is present in eukaryotic cells as an assembled heterodimer whose recruitment into the Pol II complex is associated with highly processive transcriptional activity; (b) recombinant cyclin T1 directly binds to the effector domain of WT Tat but not to Tat proteins containing mutations affecting this region, a result consistent with the reported interaction of Tat and purified human P-TEFb in the absence of TAR^{829,863}; (c) the binding of Tat to TAR RNA is markedly enhanced in the presence of cyclin T1,^{59,255,793} in agreement with reports showing that the interaction of Tat with purified P-TEFb greatly increased the affinity of Tat for TAR⁸⁶⁰; and (d) the Tat–cyclin T1 complex will only bind to TAR RNA in which both the loop and bulge are intact, whereas Tat alone requires only the trinucleotide bulge for stable TAR interaction. Taken together, these results suggest that multiple pair-wise interactions stabilize the binding of Tat to TAR: (a) Tat with P-TEFb, (b) Tat with the TAR bulge, (c) cyclin T1 with the TAR loop, and, (d) the TAR bulge/TAR loop with Tat/P-TEFb.

P-TEFb stimulates transcriptional elongation by phosphorylating both positive and negative elongation factors. As noted earlier, the sequential phosphorylation of the heptad repeats within the CTDs of RNA Pol II at serines 5 and 2 augments polymerase processivity. P-TEFb phosphorylation of the negative elongation factors NELF and DSIF abrogates their suppression of transcriptional elongation.²⁴⁰ Nearly half of P-TEFb is sequestered in an inactive form within ribonucleoprotein complexes containing the HEXIM protein and 7SK small nuclear RNA (snRNA).⁸⁶¹ The association of HEXIM with the cyclin T1 component of P-TEFb inhibits the kinase activity of CDK9. The remaining intracellular P-TEFb is active and associated with the Brd4 bromodomain protein, which directs P-TEFb to actively transcribed genes.⁸³¹ Expression of HIV-1 Tat during

the early phase of productive infection results in the dissociation of 7SK snRNA from P-TEFb and the formation of the Tat/P-TEFb complex.⁶⁸⁷ In this regard, the recently reported crystal structure of HIV-1 Tat complexed with human P-TEFb indicates that Tat makes extensive contacts with both the cyclin T1 subunit and the T-loop of CDK9.⁷⁴⁰ Conformational changes in CDK9 induced by its interaction with Tat may explain its reported altered enzymatic activity to phosphorylate Ser-5 of the RNA Pol II CTD.⁸⁵⁹

Tat transactivation of HIV-1 LTR-driven gene expression is quite low in rodent cells but can be greatly augmented in rodent somatic cell hybrids containing human chromosome 12.³⁰² This defect can also be corrected by overexpressing human cyclin T in rodent cells, which results in enhanced Tat transactivation of the HIV-1 LTR to levels measured in human cells.⁷⁹³ Other studies have shown that the murine homolog of human cyclin T is able to bind to HIV-1 Tat, but the resulting Tat/murine cyclin T heterodimer is not efficiently recruited to TAR.^{241,255} Substitution of Tyr-260 in murine cyclin T with the Cys residue found that that position in the human homolog also restores Tat activity in rodent cells.^{59,255} Thus, the extremely weak activity of Tat in rodent cells is not due to a failure to form the Tat/cyclin T heterodimer; rather, the cross-species heterodimer that does form is unable to bind to TAR and deliver P-TEFb to a poorly processive transcription complex.

A model of HIV Tat transactivation is presented in Figure 49.23. Tat interacts, via its activation domain, with the cyclin T1 subunit of cyclin T1/CDK9, a component of the P-TEFb elongation complex present in the nuclei of virus-infected cells. The binding of human P-TEFb to Tat induces a conformational change in Tat that alters its affinity and specificity for binding to TAR RNA. Thus, the principal function of Tat is to recruit the critical P-TEFb elongation factor to a promoter-proximal location where it can hyperphosphorylate the RNA Pol II CTD, thereby stimulating transcriptional processivity. It is worth noting that expression of both CDK9 and cyclin T1 increases after activation of resting PBMCs with phytohemagglutinin (PHA), phorbol myristic acid (PMA) plus ionomycin, anti-CD3 antibody plus IL-2, or antibodies to both anti-CD3 and the CD28 receptor.³¹⁵ Thus, T-cell activation increases steady-state levels of both NF- κ B and the two cellular co-factors of Tat, resulting in high levels of HIV-1 LTR-directed gene activity.

As noted earlier in this section, the TFIIH kinase, which phosphorylates the RNA Pol II CTD, also binds to the Tat activation domain. TFIIH is part of the preinitiation transcription complex that assembles on the promoter and is then lost from the complex during the early elongation phase of RNA synthesis.⁸⁴⁴ In contrast, P-TEFb is not a component of the preinitiation complex but functions after promoter clearance. Because P-TEFb preferentially phosphorylates a partially

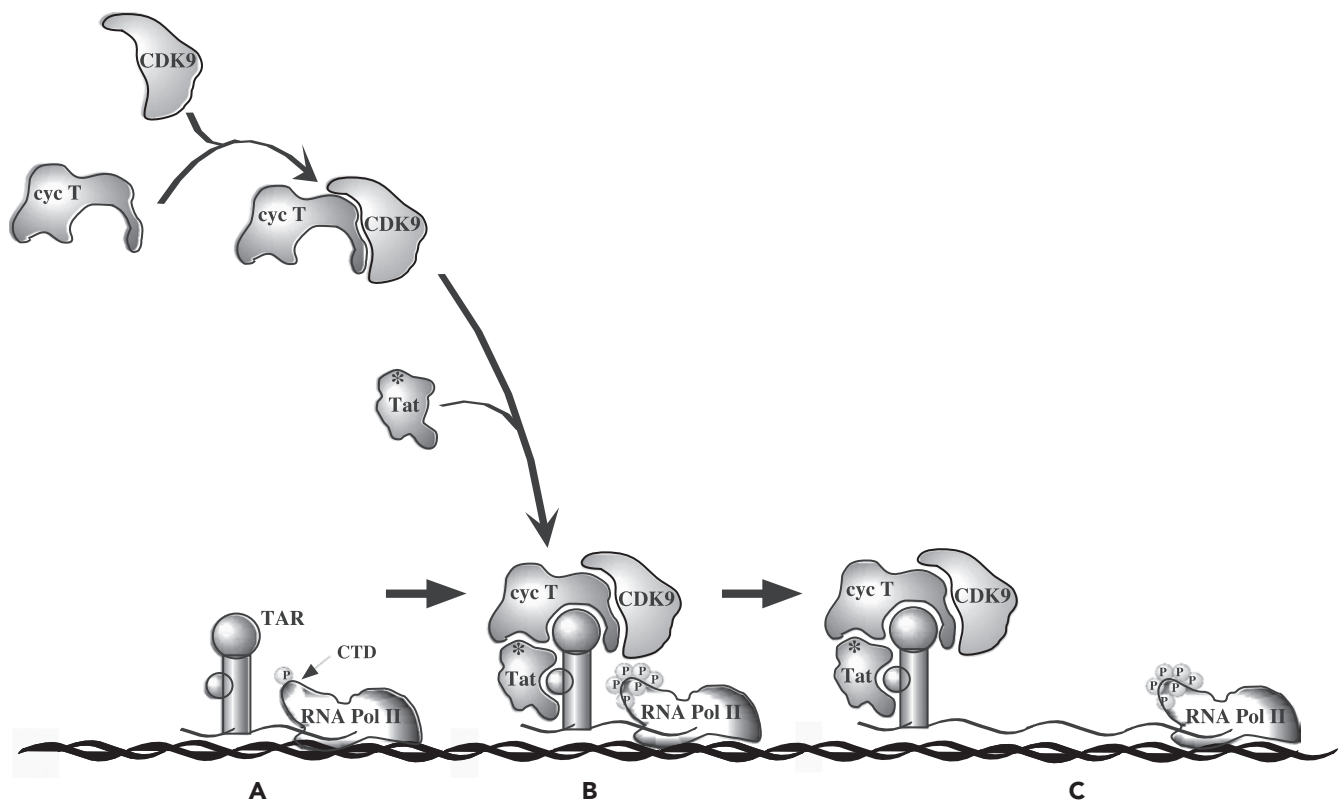


FIGURE 49.23. Tat-promoted phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (Pol II) results in processive synthesis of human immunodeficiency virus 1 (HIV-1) messenger RNA (mRNA). **A:** In the absence of Tat binding to transactivation response region (TAR), the processivity of the RNA Pol II complex is impaired. **B:** When the activation domain (*) of Tat interacts with the cyclin T (cyc T)–CDK9 complex, the conformation of Tat changes and its affinity and specificity for TAR RNA increase. **C:** By recruiting cyc T and CDK9 (the Tat-associated kinase [TAK] complex) to a promoter-proximal location, Tat mediates the hyperphosphorylation of the CTD and promoter clearance (elongation) of the transcriptional complex.

phosphorylated CTD, it has been suggested that CAK and P-TEFb act sequentially during HIV-1 RNA synthesis.⁸³² By interacting with the two kinases, Tat may be able to stimulate both phases of the elongation reaction.

In addition to initiating productive virus infections, HIV-1 is also able to establish latent infections in resting memory CD4⁺ T cells, both in untreated individuals and in patients receiving HAART.^{136,218} Latently infected lymphocytes carry a replication competent integrated provirus, which is transcriptionally silent as long as the cells remain quiescent. As a consequence, these infected T cells are invisible to the immune system. The size of this *in vivo* reservoir is quite small (approximately 10⁵ to 10⁶ cells), has an estimated half-life of 44 months, and is the primary source of viremia following cessation of HAART.⁶⁹⁹ Latently infected memory CD4⁺ T cells represent the principal barrier to HIV-1 eradication. There has been debate as to whether the very low level of viremia (less than 50 copies of viral RNA/mL of plasma) detected in patients on suppressive antiretroviral therapy^{580,812} is the result of ongoing virus replication, perhaps in tissues not efficiently accessed by the antiretrovirals, or the release of small numbers of virus particles from the latent reservoir. Evidence against the hypothesis of ongoing virus replication includes the observation that (a) treatment intensification—adding the integrase inhibitor raltegravir or other potent antiretroviral drugs on top of the suppressive HAART regimen—does not result in further reductions in viral loads^{186,513} and (b) the acquisition of drug resistance mutations is typically not detected during fully suppressive antiretroviral therapy, as would be expected if even low-level virus replication were occurring.^{186,313} However, the source of low-level viremia in patients on HAART remains to be fully defined.

The mechanisms responsible for the establishment and maintenance of latently infected CD4⁺ T cells have only been partially elucidated. It is not presently known whether they arise by the reversion of activated infected cells to a quiescent central memory T-lymphocyte phenotype^{75,495} or are the product of a direct infection of resting CD4⁺ T cells.⁹⁸ Consistent with previous studies reporting that HIV-1 preferentially integrates into active cellular genes,^{442,675} proviruses in resting CD4⁺ T cells recovered from patients on HAART have been found to be integrated into the introns of transcriptionally active cellular genes.^{291,821} This result would support an epigenetic silencing mechanism for mediating the establishment of viral latency.

Several *in vitro* cell systems have been used to investigate how postintegration latency is maintained. The earliest studies, employing cultured human T-cell lines surviving HIV-1 infections, identified the existence of two nucleosomes (nuc 0 and nuc 1 [see Fig. 49.21]) surrounding the viral promoter, which completely blocked transcription initiation. Incubation of these cells with inhibitors of histone deacetylases (HDACs) resulted in chromatin remodeling.⁷⁶⁷ Later work, using chromatin immunoprecipitation assays, reported that hyperacetylation of histone H4 at nuc 1, following HDAC treatment, correlated with activation of HIV-1 LTR-directed transcription.³⁰⁹ The subsequent development and use of clonal cell lines, derived from infected Jurkat cells, revealed that suppressed HIV-1 transcription could arise by multiple mechanisms including repressed chromatin, transcriptional interference, and the absence of transcription elongation factors or the virus-encoded Tat protein.^{367,435,794,804,805} More recently, several groups have developed primary resting human

CD4⁺ T-cell systems exhibiting HIV latency by co-cultivation, manipulation of interleukin levels, or the use of the antiapoptotic protein Bcl-2.^{381,495,652,828} The production of sufficient quantities of viable, latently infected cells remains problematic with these primary CD4⁺ T-cell systems. Recent studies have described an *in vivo* model for HIV-1 latency using the humanized bone marrow–liver–thymus mouse system.^{182,497}

Complementary biochemical and genetic analyses of tissue culture models of HIV-1 latency have shown that the recruited HDACs and histone acetyl transferases (HATs) do not directly interact with the proviral LTR. Rather, transcription regulatory proteins, binding to *cis*-acting LTR elements, recruit both positive and negative factors, which modulate chromatin structure. Some of these are indicated in Figure 49.21 and include NF- κ B, Sp1, leader-binding protein-1 (LBP-1), AP-1, NFAT, and the HIV-1–encoded Tat protein, all of which can recruit chromatin-modifying factors. For example, in latently infected cells, NF- κ B p50/p50 homodimers, lacking the transactivation domain present in the NF- κ B p50/p65 heterodimer, bind to the HIV-1 LTR and recruit HDAC-1 and HDAC-3 to the viral promoter.^{321,804} This leads to histone deacetylation and gene silencing. In contrast, in activated cells, NF- κ B p50/p65 heterodimers are rapidly imported into the nucleus, displace the NF- κ B p50/p50 homodimers, and recruit the HAT p300, thereby mediating local histone acetylation and optimizing Tat function.^{392,472} NFAT, AP-1, and Tat have all been shown to recruit activating factors to the HIV-1 LTR, while LBP-1 attracts chromatin-modifying repressors.^{43,103,445,637}

Taken together, these findings support a model of latently infected resting memory CD4⁺ T cells in which HIV-1 transcription is potently suppressed, Tat is absent, and repressed chromatin prevents the clearance of the preinitiation complex and RNA Pol II processivity.⁷⁵⁹ Under these conditions, nuc 1 is constitutively deacetylated by HDACs, NF- κ B and NFAT are sequestered in the cytoplasm, and the Tat co-factor, P-TEFb, is present in an inactive form within a large RNA-binding protein complex that includes 7SK RNA and HEXIM1. Following activation, NF- κ B p50/p65 heterodimers, NFAT, AP-1, and other positive factors are imported into the nucleus, bind to their cognate sites in the HIV-1 LTR, and recruit HATs or P-TEFb (via the Tat/TAR complex present at the 5′ terminus of all viral RNA transcripts) to the promoter.

REV

Like other primary RNA transcripts synthesized in eukaryotic cells, HIV pre-mRNAs undergo a series of modifications (capping, 3′ end cleavage, polyadenylation, and splicing) prior to their export to the cytoplasm. Retroviruses utilize the cellular posttranscriptional processing machinery to carry out these functions. With few exceptions, introns present in nascent cellular mRNAs must be removed prior to their export from the nucleus, presumably to prevent their translation into nonfunctional proteins. This requirement poses an obvious problem for retroviruses such as HIV, which must export a variety of intron-containing mRNAs into the cytoplasm (e.g., the unspliced 9.2-kb primary transcript for encapsidation into progeny virions and production of Gag and Pol proteins, as well as several other partially spliced mRNAs such as those encoding Env, Vif, and Vpr proteins). In part, retroviruses have solved the requirement for the removal of all intronic sequences from pre-mRNAs prior to nuclear export by incorporating suboptimal 3′ splice acceptors into

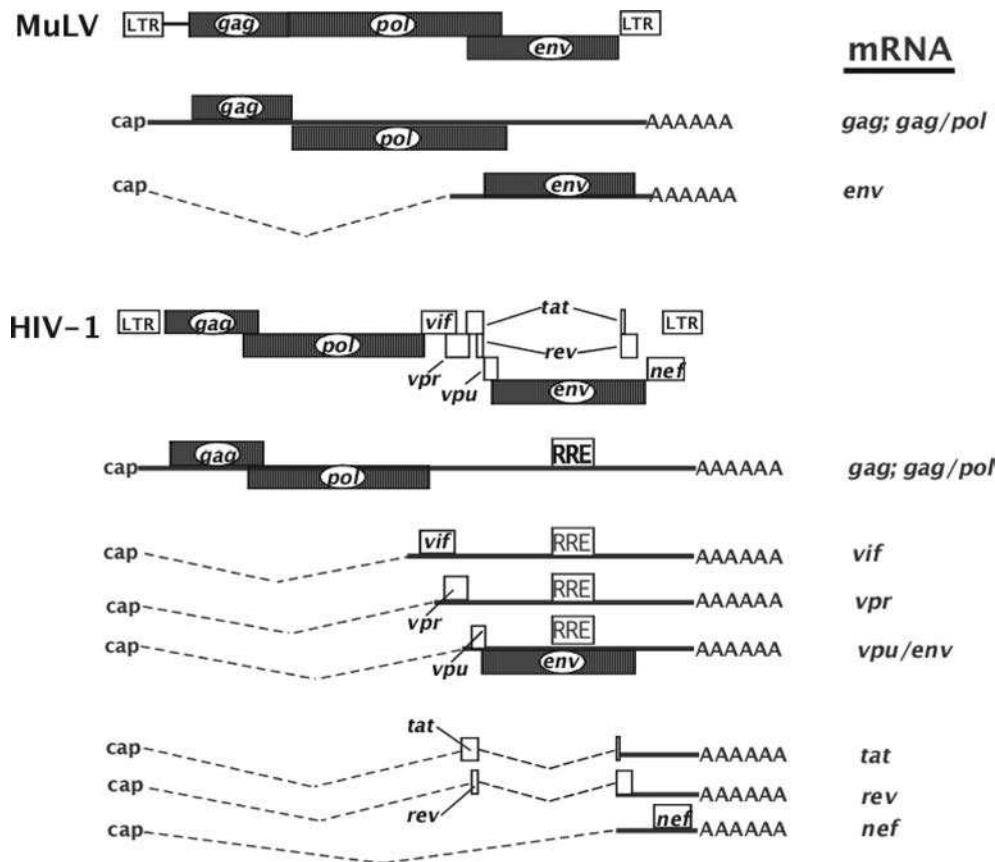


FIGURE 49.24. Retrovirus splicing patterns. In contrast to murine leukemia virus (MLV), which generates only two discrete messenger RNA (mRNA) species (the unspliced *gag/pol* and the singly spliced *env*), human immunodeficiency virus 1 (HIV-1) produces several alternatively spliced mRNAs, ranging from the unspliced *gag/pol* to the multiply spliced *tat*, *rev*, and *nef* RNA transcripts. The genomic organization of the proviral DNA, the location of protein coding sequences, and the position of the Rev-responsive element (RRE) in intron-containing mRNAs encoding viral structural proteins are indicated. The dashed lines connect the major splice donor to a downstream splice acceptor; alternative forms of *tat*, *rev*, *vpu/env*, and *nef* mRNA, some of which contain short upstream noncoding exons, are not shown.

their genomes. These acceptors are functionally impaired due to short or interrupted polypyrimidine tracts, noncanonical branch points, and the presence of *cis*-acting inhibitory sequences, which dampen splice site usage. For example, the presence of purines within the polypyrimidine tracts associated with some HIV-1 3' splice sites impairs splicing by reducing the affinity for the essential cellular splicing factor U2AF.^{268,717}

All retroviruses must splice their primary RNA transcripts to generate *env* mRNAs, eliminating upstream *gag* and *pol* sequences in the process. For simple retroviruses such as the ALVs and MLVs, this is the only splicing reaction that the viral pre-mRNA undergoes (Fig. 49.24, top). In contrast, the splicing of HIV RNA is far more complex because of the presence of both constitutive and alternatively used splice-donor and splice-acceptor motifs scattered through its genomic RNA. Three general classes of HIV-1 mRNAs have been identified in productively infected cells: (a) unspliced genomic RNA, which serves as the mRNA for synthesis of Gag and Pol proteins; (b) partially spliced RNAs, approximately 4.3 to 5.5 kb in size, which are translated into Vif, Vpr, Vpu, and Env proteins; and (c) multiply or completely spliced viral mRNAs, ranging from 1.7 to 2.0 kb in size, which encode the Tat, Rev, and Nef pro-

teins (Fig. 49.24, bottom). The first two classes of viral mRNA contain several spliceable introns yet are efficiently exported from the nucleus into the cytoplasm.

Analyses of viral mRNAs by RT-PCR have revealed the existence of more than 40 different HIV-1 transcripts in virus-producing cells.^{619,685} These mRNAs are generated as a consequence of alternative selection of the four splice donors (sd) and the eight splice acceptors (sa) embedded in the viral genome (some of which are shown at the top of Fig. 49.25). The frequency at which particular 5' and 3' splice sites are used is dependent on the relative strength of each site and the presence of adjacent *cis*-acting RNA elements. These elements include exonic and intronic splicing silencers and exonic splicing enhancers. These enhancer and silencing sequences modulate alternative splicing of the HIV-1 primary RNA transcript by binding to SR proteins and heterogeneous ribonucleoprotein particles (hnRNPs) (reviewed in⁷²²). Variable usage of HIV 5' and 3' splice sites gives rise to several sets of distinct, closely related RNAs that serve as alternative templates for translation into the same protein. For example, 12 different *rev*, 5 different *nef*, 8 different *tat*, and 16 different *env* mRNAs have been identified.⁶¹⁹ This diversity of HIV-1 mRNA is due, in part, to

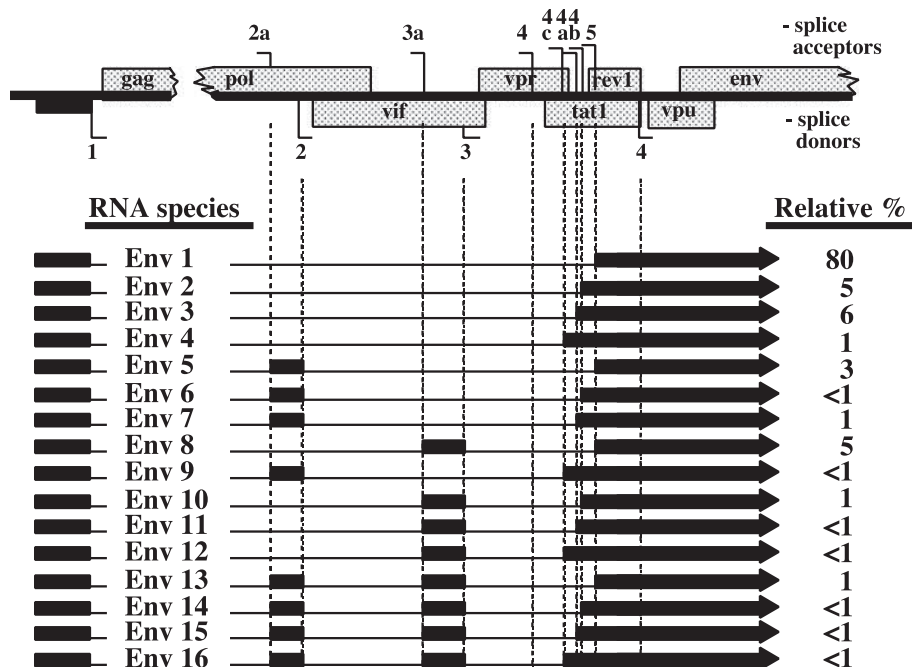


FIGURE 49.25. Structure and relative abundance of differentially spliced human immunodeficiency virus 1 (HIV-1) *env* messenger RNAs (mRNAs). For each RNA species, the dark bars and associated 3' arrows represent exons included in the spliced transcript as determined by semiquantitative polymerase chain reaction (PCR) analysis. The relative proportion of each mRNA is indicated at the right.

the variable inclusion of two upstream 50- and 74-nt noncoding exons (generated from *sa2a/sd2* and *sa3a/sd3*, respectively; Fig. 49.25) in many of the spliced RNA species. The functional significance of multiply spliced mRNAs encoding the same viral protein, their relative abundance, and the hierarchy of HIV-1 splice site usage are not presently understood.

Complex retroviruses, such as HIV, have dealt with the restriction to the nuclear export of unspliced and incompletely spliced transcripts by incorporating a novel reading frame, encoding the Rev (Regulator of expression of viral proteins) protein, into their genomes. In the absence of Rev, the unspliced *gag/pol* and the partially spliced *vif*, *vpr*, and *vpu/env* mRNAs fail to accumulate in the cytoplasm, thereby rendering the Rev-mutant viruses replication incompetent.^{213,290}

HIV-1 Rev is a 19-kD, predominantly nucleolar phosphoprotein containing 116 amino acid residues (Fig. 49.26). Like Tat, Rev is encoded by two exons and contains two functional regions: (a) an Arg-rich domain that mediates RNA binding and nuclear localization, flanked by sequences that facilitate Rev multimerization, and (b) a hydrophobic segment, located between residues 73 and 84, that contains several Leu residues within an activation domain, which also promotes nuclear export.^{33,482} Mutation of any one of three critical leucines (residues 78, 81, or 83) within this domain eliminates Rev activity. Unlike *tat*, both *rev* exons are required for virus replication.⁶⁵⁰ The HIV Rev protein regulates the expression and usage of viral transcripts by binding to a *cis*-acting target, the RRE, present in all unspliced and partially spliced viral mRNAs (see Figs. 49.7 and 49.26, bottom). The RRE, located in a 250-nt segment spanning the junction between *gp120* and *gp41* coding sequences of the *env* gene, is a complex RNA structure containing multiple stem-loops branching from a large central bubble^{213,483,640} (Fig. 49.26, bottom). The RRE must be present *within* a Rev-responsive transcript and in the *sense* orientation to confer Rev responsiveness. Nuclease protection, chemical modification, and mutagenesis studies indicate that

Rev specifically interacts with a 60+ nt portion of the RRE, designated stem-loop II (Fig. 49.26).^{171,324,484} This region of the RRE binds to Rev even when isolated from the complete RRE structure and mediates Rev responsiveness in functional assays.³³⁴ The determinants for high-affinity binding of Rev to RRE reside in the central purine-rich “bubble” of stem-loop II; this bubble contains unusual G:G and G:A base pairs that distort the duplex RNA structure and widen the major groove to accommodate the Rev protein.^{33,325,484} NMR analyses have shown that an α -helical, Arg-rich, 17-residue peptide from the RNA-binding domain of Rev burrows deep into the major groove of a stem-loop II oligonucleotide, stabilizing the non-Watson-Crick base pairs through specific interactions involving the Arg side chains.³⁵ The functionally complete RRE is approximately 350 nt in size, and an individual element may accommodate at least six Rev molecules.^{165,166,489}

It was initially believed that a dimer of Rev makes the initial contact with the RRE. A more recent study indicates that Rev binds to the stem-loop IIB region of RRE as a monomer.⁶⁰⁴ A crystal structure of truncated (N-terminal 70 aa) Rev bound to the RRE revealed that monomeric Rev folds into a helix-loop-helix structure, creating a hydrophobic core.¹⁶⁷ This core region becomes a dimerization interface for the cooperative binding of a second Rev monomer, which also interacts with a contiguous region of the RRE, thereby generating a “V”-shaped dimer structure. Further oligomerization of similarly organized dimers cooperatively assembles into a tightly bound hexameric Rev-RRE RNA ribonucleoprotein (RNP) primed for nuclear export.

Mutations affecting either the RNA-binding or the Leu-rich activation domains of the Rev protein result in loss of function. For example, activation domain mutants are able to bind to the RRE but are functionally inactive. They interfere with the transactivation mediated by WT Rev, exerting a *trans*-dominant effect in assays for Rev function.⁴⁸² Rev is also able to continuously shuttle between the nucleus and cytoplasm.⁵²⁰

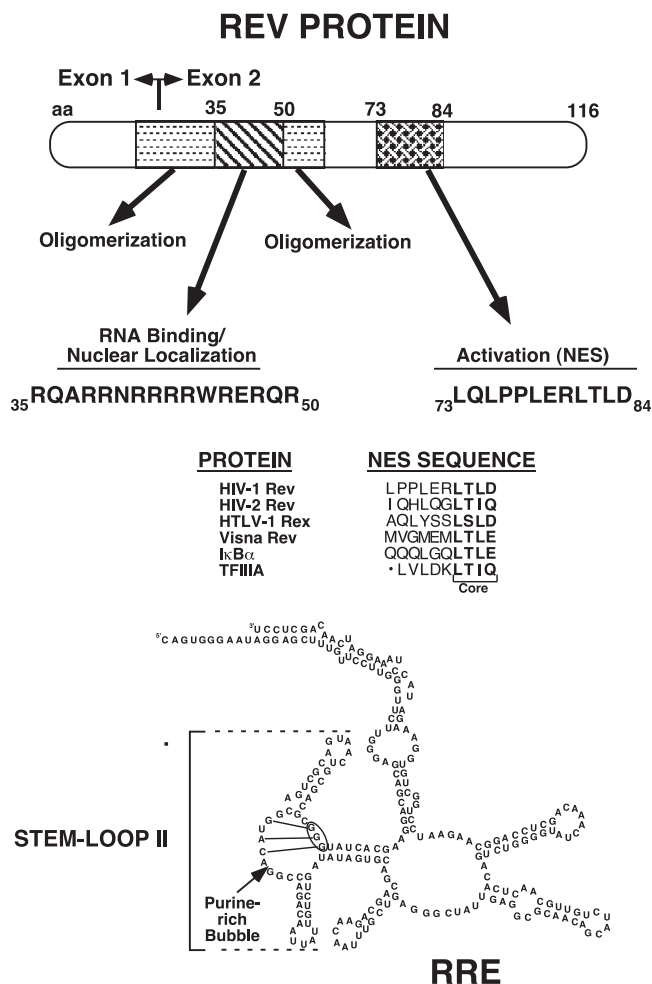


FIGURE 49.26. Rev and its response element, the RRE. A schematic representation of the human immunodeficiency virus 1 (HIV-1) Rev protein with its RNA-binding, activation, and oligomerization domains is shown at the **top**. The amino acid sequence of the leucine-rich nuclear export signal (NES) present in retroviral Rev/Rex proteins and two cellular proteins is also shown. The core tetramer motif, LxLy, where y is usually a charged residue and x is highly variable, is indicated. The structure of the RRE is presented at the **bottom** with the high-affinity target of HIV-1 Rev (stem-loop II) shown. The “bulged” G residues in stem-loop II able to form noncanonical purine-purine base pairs are circled.

Mutations affecting the Rev activation domain abolish shuttling and restrict Rev to the nucleus. Multimerization-defective Rev mutants can still bind to the high-affinity RRE site but are unable to form oligomeric complexes.⁴⁷⁶

As noted earlier, the HIV-1 genome contains multiple sequence motifs, located in the *gag*, *pol*, and *env* genes (and designated INS in Fig. 49.7), which contribute to a requirement for Rev. These *cis*-acting, AU-rich INS elements markedly impaired the expression of fused reporter genes by impeding RNA transport to the cytoplasm and/or decreasing RNA stability.^{480,640} It is quite likely that cellular proteins bind to these inhibitory sequences, contributing to the nuclear retention and the intrinsic instability of intron-containing viral RNAs. Rev is able to reverse these effects, provided that an RRE is present in such transcripts.

Although the total amount of viral RNA in cells infected with WT or Rev-deficient HIV mutants is quite similar, *gag-pol*, *env*, *vif*, and *vpr* mRNAs are either absent or markedly underrepresented in the cytoplasm of cells infected with Rev mutants compared to the levels of multiply spliced *tat*, *rev*, and *nef* transcripts in this compartment.^{213,483} Although several models were initially proposed to explain Rev function, careful analyses of HIV-1 RNA expression patterns indicated that the ratios of unspliced (or partially spliced) to completely spliced viral transcripts in the nucleus did *not* change in the presence or absence of Rev.^{213,483} Rather, the Rev⁻ phenotype was characterized by a superabundance of the 1.7- to 2.0-kb completely spliced RNAs in the cytoplasm and by greatly reduced or no detectable cytoplasmic RRE-containing unspliced or partially spliced mRNAs. Because splicing of viral RNA in the nucleus was not affected by the absence of Rev, attention shifted to the role that Rev might have on HIV-1 RNA transport.

The first unambiguous demonstration that Rev could promote the nuclear export of intron-containing viral RNA came from experiments in which purified Rev protein and RRE-containing RNA molecules were microinjected into cell nuclei.^{219,799} These studies showed that the RRE-containing unspliced RNA substrates were transported from the nucleus into the cytoplasm only in the presence of Rev. Interestingly, the excised exon (containing the RRE), derived from those transcripts that did undergo splicing, was also detected in the cytoplasm, indicating that Rev was able to mediate nuclear export *without* inhibiting RNA splicing.²²⁰ Other microinjection studies revealed that the Leu-rich Rev activation domain was indeed a nuclear export signal (NES) because, when fused to bovine serum albumin (BSA), it promoted the transport of NES/BSA molecules into the cytoplasm.^{219,799} In contrast, NES/BSA fusion proteins containing a mutated, nonfunctional Leu-rich Rev domain remained in the nucleus. The nuclear export mediated by the Rev NES was also shown to be energy dependent and was blocked by high concentrations of NES/BSA, a result that implied that the amounts of intracellular nucleus-to-cytoplasm transporting proteins were limiting. Surprisingly, the nuclear export of 5S ribosomal RNA (rRNA) and spliceosomal U (sn)RNAs, but not RNA Pol II-derived cellular mRNAs, was also inhibited when high concentrations of NES/BSA conjugates were microinjected. In this regard, a growing list of viral and cellular proteins involved in RNA metabolism and signal transduction have been shown to contain a Rev-like, Leu-rich NESs (Fig. 49.26, middle), which mediates the transport of a variety of substrates from the nucleus to the cytoplasm.^{351,505} Taken together, these results are consistent with a model in which the Rev effector domain functions as an NES, directing RRE-containing viral RNAs to the cytoplasm via a pathway used by some cellular mRNAs.

The bidirectional passage of proteins between the nucleus and cytoplasm occurs through the nuclear pore complex (NPC) and depends on the presence of NLSs and NESs (reviewed in^{283,719}). The NPC is an extremely large (greater than 100-MDa) gated structure composed of 50 to 100 different proteins, called nucleoporins. One class of nucleoporins, thought to directly participate in nuclear-cytoplasmic transport, contains Phe-Gly (FG) repeat domains and is located primarily at the periphery of the NPC. Proteins destined to be imported into the nucleus (e.g., bearing a typical basic-type NLS) initially associate with a member of a growing family of transport

“receptors” in the cytoplasm. The first receptor of this type discovered, importin- β , shares structural features and interacting partners with other family members and is considered to be the prototypical transporter. Proteins bearing the basic-type NLS usually begin the nuclear import process by binding to a related transport protein, importin- α . Importin- α serves as a bridge, linking the NLS-containing substrate to importin- β . However, in contrast to most other proteins that carry a classic NLS, Rev appears to bind directly to importin- β . Furthermore, this heterodimeric complex is transported to the NPC, where the importin- β subunit successively binds to and dissociates from the resident nucleoporins, translocating the Rev substrate through the pore and into the nucleoplasm. It is now known that the reverse process, nuclear export, uses transport receptors that are also members of the importin- β family. The best characterized of these is named Crm1 (for chromosome region maintenance 1), or exportin 1. Studies of HIV-1 Rev and related viral and cellular proteins bearing Leu-rich NES motifs (Fig. 49.26) have led to the discovery and established the functions of many proteins involved in nuclear export.

The small guanosine triphosphatase (GTPase) molecule, Ran,⁶² provides both the energy and directionality cues for nuclear import and export. Intracellularly, Ran cycles between two forms, Ran guanosine triphosphate (RanGTP) and Ran guanosine diphosphate (RanGDP), which are concentrated primarily in the nucleus and cytoplasm, respectively.³⁵² The specificity of Ran activity in nuclear transport is regulated by the nature of the bound nucleotide. Several cellular proteins are able to modulate the nucleotide-bound state of Ran and therefore contribute to the asymmetric distribution of the two Ran forms.⁶² One of these, the chromatin-associated regulator of chromosomal condensation 1 (RCC1), stimulates the replacement of GDP with GTP when Ran is in the nucleus. Conversely, two other factors, Ran GTPase activating protein 1 (Ran GAP1) and Ran binding protein 1 (Ran BP1), both augment Ran activity in the cytoplasm by promoting the hydrolysis of the Ran-associated GTP. The net effect of these interactions is the maintenance of the observed RanGTP/RanGDP intracellular gradient required for nuclear import and export functions.

The complete Rev nuclear transport cycle and its critical interacting partners are shown in Figure 49.27. During the early, postintegration phase of the virus life cycle, the HIV-1 mRNAs transported to the cytoplasm consist almost entirely of intronless, multiply spliced transcripts encoding the Tat, Rev, and Nef proteins. As noted earlier, Rev uses its NLS to bind directly to importin- β in the cytoplasm and is translocated through the nuclear pore as a Rev/importin- β heterodimer. When the complex reaches the nucleus, the binding of RanGTP triggers the Rev/importin- β heterodimer to dissociate and release the Rev subunit. In the nucleoplasm, Rev initiates its role as a nuclear exporter by binding to the high-affinity site on stem-loop II of RRE-containing HIV-1 pre-mRNAs and then multimerizes via cooperative protein–protein and protein–RNA interactions over the entire length of the RRE. When Rev oligomerizes on the RRE, its NES elements become aligned on the outside of an enlarging protein–RNA complex. Multimerized Rev/RRE then forms a functional complex with CRM1 in the presence of Ran in its GTP-bound form. The formation of this RNP is (a) sensitive to Rev NES mutations and (b) dissociated when RanGTP is hydrolyzed to its GDP-bound state.²⁶ The most

likely scenario linking all available data is that the entry of RanGTP into a pre-existing Rev/RRE/CRM1 RNP converts it into a functional export complex. Once the complex is formed, it is transported to the NPC, where the CRM1 subunit interacts with nucleoporins,²²⁴ and is then translocated into the cytoplasm. There, in the presence of Ran GAP1 and Ran BP1, the RanGTP component of the export complex is converted to the RanGDP form and the RNP disassembles.^{273,561} Rev is released along with its unspliced and partially spliced HIV-1 RNA “cargo,” allowing the latter to be incorporated into progeny virions or translated into viral proteins. Rev therefore enables lentiviral intron-containing RNAs to elude the cellular splicing machinery and be transported to the cytoplasm via an export pathway not used by most cellular mRNAs.

Virus Assembly and Release: The Gag Proteins

The Gag proteins of HIV, like those of other retroviruses, are necessary and sufficient for the formation of noninfectious, virus-like particles (VLPs). Retroviral Gag proteins are generally synthesized as polyprotein precursors, Pr55^{Gag} in the case of HIV-1. As noted previously, the mRNA for Pr55^{Gag} is the unspliced 9.2-kb transcript that requires Rev for its expression in the cytoplasm. The HIV Pr55^{Gag} precursor is targeted to the membrane rapidly after its synthesis. At the membrane, particles of sufficient size and density to be visible by EM are assembled. The formation of noninfectious VLPs does not require the participation of viral genomic RNA (although the presence of nucleic acid appears to be essential), *pol*-encoded enzymes, or Env glycoproteins. However, the production of *infectious* virions requires the encapsidation of the viral RNA genome and the incorporation of the Env glycoproteins and the Gag-Pol polyprotein precursor Pr160^{Gag-Pol}. Retroviral Gag proteins perform several major functions during virus assembly, including (a) forming the structural framework of the virion, (b) encapsidating the viral genome, (c) targeting the nascent particle for export from the cell, and (d) acquiring a lipid bilayer and associated Env glycoproteins during particle release (for review see⁸⁴). These processes require that Gag proteins participate in protein–protein, protein–RNA, and protein–lipid interactions. As described later, Gag proteins also play critical roles in the early stages of the virus replication cycle.

In the immature VLP, Gag monomers are aligned and packed radially, with the MA domain in association with the inner leaflet of the lipid bilayer and the NC domain bound to genomic viral RNA facing the center of the virion (Fig. 49.28). Gag–Gag contacts are primarily mediated through the CTD of CA and SP1. Tomographic analyses have demonstrated that in immature VLPs Gag forms a hexameric lattice^{85,173,815} (see later). The ability of the Gag lattice to curve into a sphere is afforded by a large gap in the lattice. The CA NTD is arranged in a ring around a central hole in each hexamer; the CA CTD is positioned just below the NTD and links the hexamers together to form the lattice.⁸⁵ The C-terminus of the CA CTD and adjoining SP1 are proposed to be helical,⁵³⁷ and cryoelectron tomography results suggest that the CA–SP1 junction region forms a bundle of helices that connects CA to NC.⁸¹⁵ Estimates of Gag content per virion have ranged from several thousand to ~5,000 molecules. Studies showing that the Gag lattice is continuous but contains a large gap are consistent with the lower end of this range (~2,500 molecules of Gag/particle).¹⁰⁵

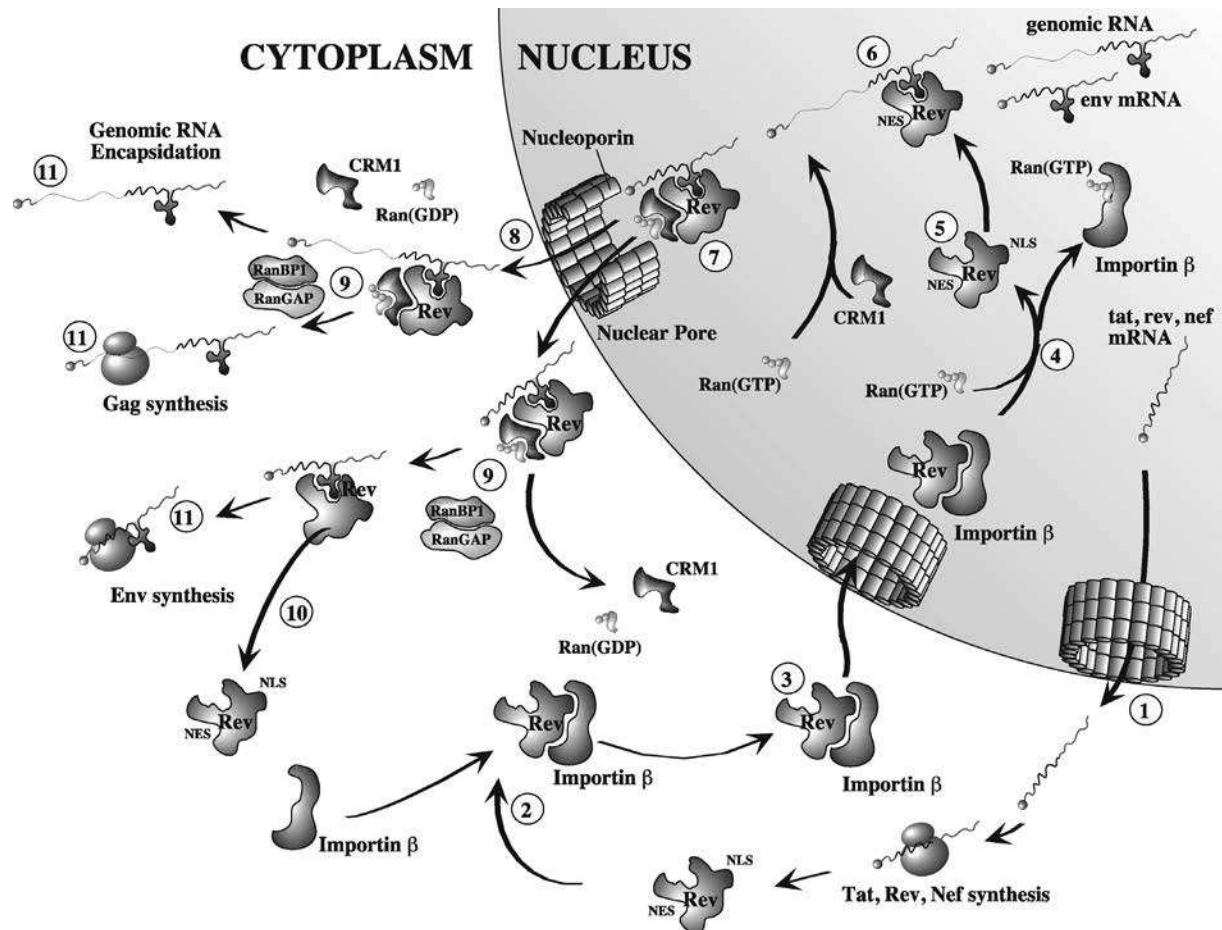


FIGURE 49.27. Rev nuclear export pathway. Intronless multiply spliced human immunodeficiency virus 1 (HIV-1) messenger RNAs (mRNAs) (*tat*, *rev*, and *nef*) use a Rev-independent pathway to exit the nucleus (1). Using its nuclear localization signal (NLS), newly synthesized Rev protein binds to importin β (2) and the resulting heterodimer (3) is translocated through the nuclear pore. In the nucleus, Rev/importin β dissociation is mediated by Ran(GTP) (4) and the free Rev protein (5) binds to Rev-responsive element (RRE)-containing viral pre-mRNA (6). The Rev-RRE RNA complex is converted to a functional export structure following the addition of CRM1 and Ran(GTP) (7) and is transported through the nuclear pore (8). In the cytoplasm, RanBP1/RanGAP mediate the conversion of Ran(GTP) in the export complex to Ran(GDP) (9), which then disassembles. After the release of Rev from intron-containing HIV-1 transcripts (10), the full-length viral RNA may be encapsidated into progeny virions or, as is the case for the partially spliced mRNA, may be translated into viral proteins (11).

When the *pol* ORF is expressed, the viral PR cleaves Pr55^{Gag} during or shortly after budding and release from the cell to generate the mature Gag proteins MA, CA, NC, and p6. Two spacer peptides are also cleaved from the Gag precursor: SP1 (originally called p2), located between CA and NC, and SP2 (originally called p1), situated between NC and p6 (Fig. 49.29A). Processing of the Gag and Gag-Pol precursor proteins by PR results in a major structural and morphologic rearrangement, referred to as maturation, during which the core condenses into the cone-shaped structure characteristic of the mature HIV particle (see section on Virus Maturation). Disruption of this maturation process abolishes virus infectivity. In the mature virion, MA is localized immediately inside the lipid bilayer of the viral envelope, CA forms the cone-shaped core structure in the center of the particle, and NC is present in the core in a ribonucleoprotein complex with the viral RNA genome (see Fig. 49.28). Detailed NMR and x-ray

crystallographic data have provided significant insights into the structure-function relationship of HIV-1 MA, CA, and NC.

Matrix (MA): Membrane Binding and Gag Targeting

The MA domain of Pr55^{Gag} (Fig. 49.29B) performs several essential functions during the viral life cycle. After Gag synthesis, MA directs Pr55^{Gag} to the membrane via a multipartite membrane-binding signal. The affinity of the MA domain for the membrane is provided in part by a myristic acid moiety covalently attached to the N-terminal Gly of MA after removal of the initiator Met by N-myristyl transferase. Mutation of the Gly myristate acceptor blocks binding of Gag to the membrane and abolishes virus assembly in most systems. Sequences in MA downstream of the myristate also contribute to membrane binding. NMR and x-ray crystallographic analyses of HIV-1 MA (as well as the MA protein of a number of other retroviruses) demonstrate that a highly basic patch of amino acids

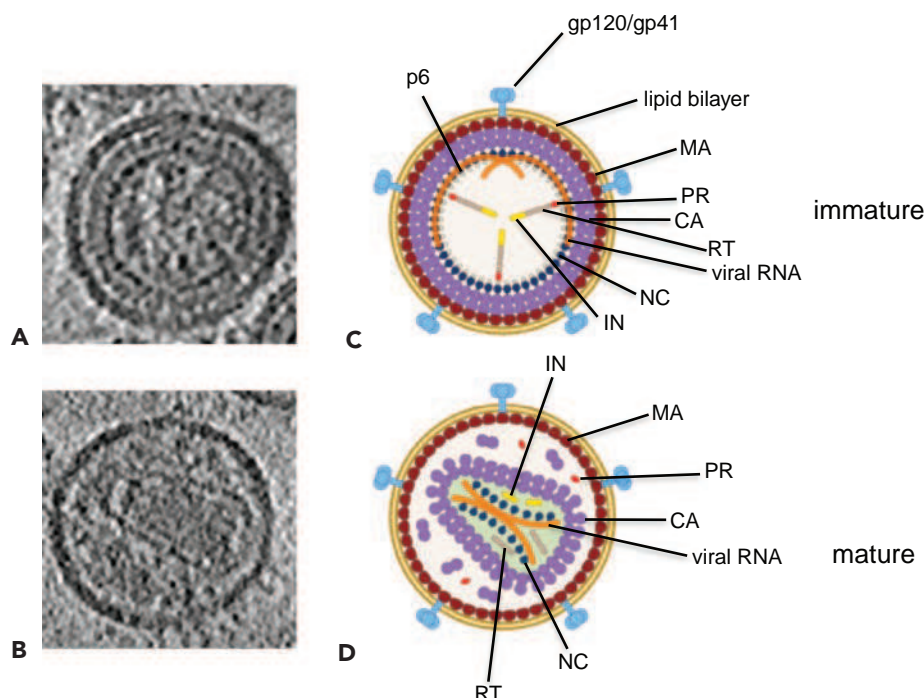


FIGURE 49.28. Structural organization of immature and mature human immunodeficiency virus 1 (HIV-1) virions. Cryoelectron micrographs of immature (A) and mature (B) virions. (A and B reprinted from Keller PW, Adamson CS, Heymann JB, et al. HIV-1 maturation inhibitor bevirimat stabilizes the immature Gag lattice. *J Virol* 2011;85:1420–1428, with permission, copyright American Society for Microbiology.) Schematic representation of immature (C) and mature (D) HIV-1 particles, with labels indicating the gp120/gp41 glycoprotein spike, the lipid bilayer, and the viral RNA. In C, the major domains in Gag (matrix [MA], capsid [CA], nucleocapsid [NC], and p6) and Gag-Pol (protease [PR], reverse transcriptase [RT], and integrase [IN]) are labeled. In D, locations of mature Gag and Pol products are indicated. (Modified from Balasubramaniam M, Freed EO. New insights into HIV assembly and trafficking. *Physiology [Bethesda]* 2011;26:236–251.)

clusters on the face of MA predicted to juxtapose the plasma membrane (Fig. 49.30).^{318,502} It has been proposed that these basic residues interact with the negatively charged acidic phospholipids on the inner leaflet of the lipid bilayer, thereby stabilizing membrane interactions.^{314,502,862} As discussed in more detail later, mutations affecting these basic residues can disrupt the targeting of Gag to the plasma membrane.^{238,842,862}

Structural analysis of myristylated MA has indicated that the myristate moiety is in equilibrium between an exposed and a sequestered conformation.⁷⁴⁶ Myristate exposure appears to be triggered both by Gag multimerization⁷⁴⁶ and by binding of MA to the phospholipid phosphatidylinositol-(4,5)-bisphosphate (PI[4,5]P₂).⁶⁴⁹ In the exposed conformation the myristate is able to insert into the lipid bilayer of the plasma membrane, indicating that both Gag multimerization and PI(4,5)P₂ binding promote Gag–membrane association.

A large body of data indicates that the assembly of electron-dense budding structures takes place predominantly at the plasma membrane. Early EM observations demonstrated that in MDMs assembly and budding take place in an internal compartment⁵⁷⁶ that was initially thought to be of late endosomal origin. Subsequent studies, however, showed that these structures are connected to the plasma membrane by a complex network of thin channels^{45,180,798} and thus represent plasma membrane invaginations rather than late endosomes. Real-time imaging of HIV-1 assembly in living MDMs

revealed that virus accumulating in this internal compartment rapidly translocates to the cell–cell junction (the VS discussed earlier) that forms between infected MDMs and uninfected T cells.²⁷⁷ Thus, assembly of HIV-1 particles internally and their movement to the MDM–T-cell VS provide a mechanism for immune evasion coupled with efficient cell–cell transfer.

Early studies indicated that MA contains sequences that regulate the site of virus assembly. Deletion of a large portion of MA redirects HIV-1 assembly to the endoplasmic reticulum,²⁰⁸ whereas single and double amino acid changes between MA residues 84 and 88 and in the highly basic domain of MA (Fig. 49.29B)^{238,572} cause virus assembly to be redirected to multivesicular bodies (MVBs).⁵⁷² Thus, MA contains sequences that not only regulate membrane binding but also determine the specificity of membrane targeting.

Although the cellular determinants that regulate the site of HIV-1 assembly remain to be fully defined, it is clear that PI(4,5)P₂ plays an important role in directing Gag to the plasma membrane. Depleting PI(4,5)P₂ from the plasma membrane leads to the retargeting of HIV-1 assembly to MVBs and severely disrupts virus particle production.⁵⁷¹ As mentioned earlier, structural data indicate that PI(4,5)P₂ binds directly to MA^{649,696} and induces the exposure of the N-terminal myristate.⁶⁴⁹ PI(4,5)P₂ also promotes the binding of Gag to liposomes.¹³³ Some of the basic residues in MA that function in targeting Gag to the plasma membrane^{572,574} appear to

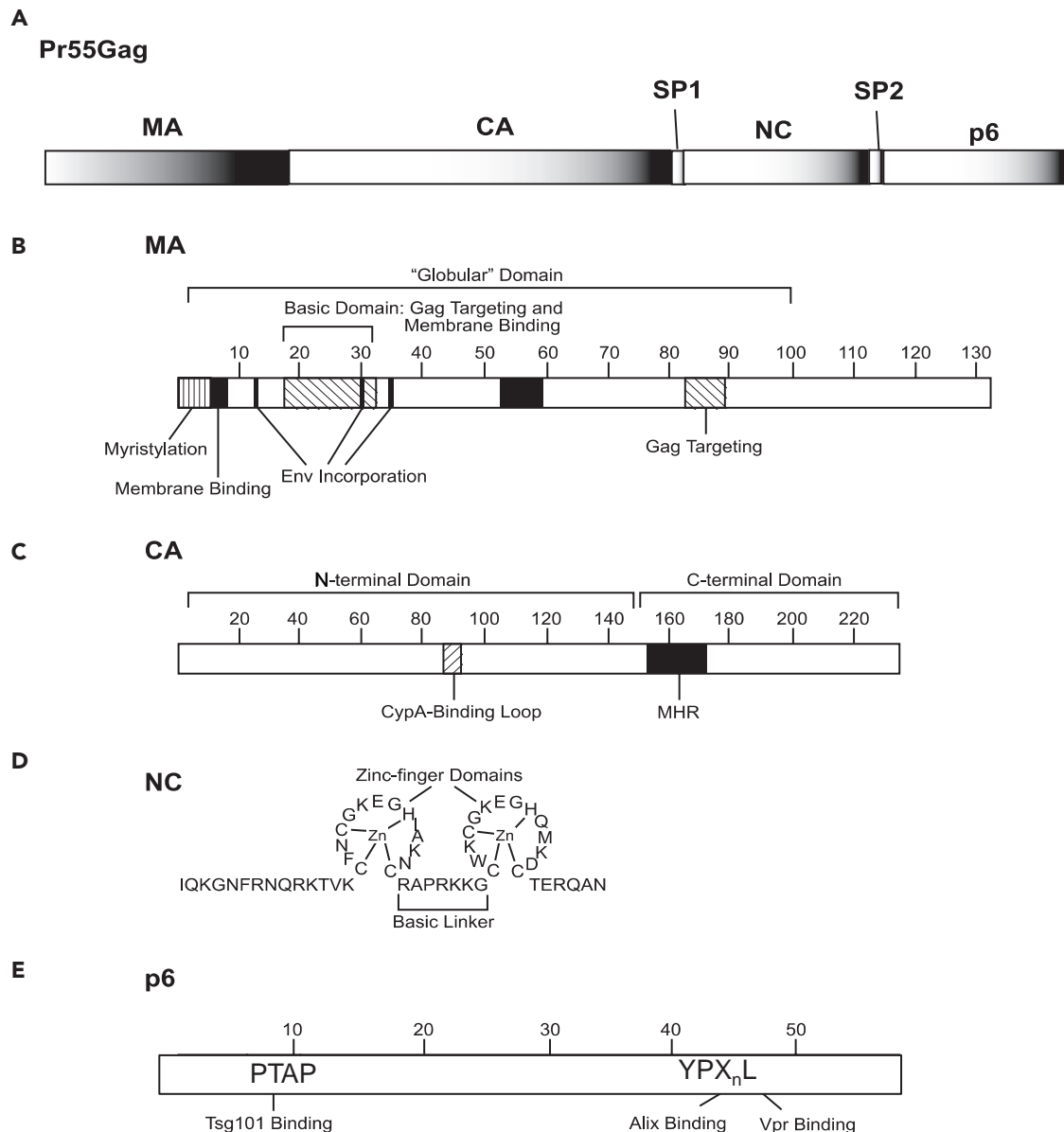


FIGURE 49.29. Linear organization of the major human immunodeficiency virus 1 (HIV-1) Gag proteins. **A:** The Gag precursor, Pr55^{Gag}, with the positions of the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 domains indicated; the spacer peptides, SP1 and SP2, are also shown. **B:** MA. **C:** CA. **D:** NC. **E:** p6. Amino acid positions are indicated, as are major functional domains (details in the text). (Adapted from Freed EO. HIV-1 Gag proteins: diverse functions in the virus life cycle. *Virology* 1998;251:1–15.)

be directly involved in PI(4,5)P₂ binding^{133,696}; mutation of these basic residues thus recapitulates the effect of PI(4,5)P₂ depletion. Interestingly, PI(4,5)P₂ also regulates the membrane binding of several other retroviral Gag proteins, for example, HIV-2⁶⁴⁸ and MLV.²⁸⁹ Although the NC domain of Gag is the primary determinant for Gag–RNA interaction, MA also binds RNA, and this RNA binding reportedly inhibits MA association with PI(4,5)P₂-deficient but not PI(4,5)P₂-containing membranes. This mode of RNA regulation likely contributes to the selectivity of Gag for PI(4,5)P₂-enriched (i.e., plasma) membranes over internal membranes that lack PI(4,5)P₂.^{12,134}

In addition to the phospholipid PI(4,5)P₂, a growing number of host cell proteins have been implicated in HIV-1 assembly and Gag trafficking.²⁹ These include a number of pro-

teins that function in cellular membrane and protein trafficking pathways, for example, the clathrin adapter protein complexes (AP-1, -2, and -3); ADP ribosylation factors (Arfs); Golgi-localized, γ -ear-containing, Arf-binding (GGA) proteins; the suppressor of cytokine signaling (SOCS1); Moloney leukemia virus 10, homolog (MOV10); and the soluble NSF attachment protein receptors (SNAREs).²⁹ Further study will be required to define the role of these proteins in HIV-1 assembly. A considerable amount of biochemical, microscopic, and pharmacologic data suggest that HIV-1 assembly takes place in lipid rafts, the cholesterol- and glycosphingolipid-enriched microdomains discussed earlier in the context of virus entry. Lipid rafts may stabilize Gag–membrane binding and facilitate assembly by serving as platforms for Gag–Gag interactions (for review see⁷⁸⁵).

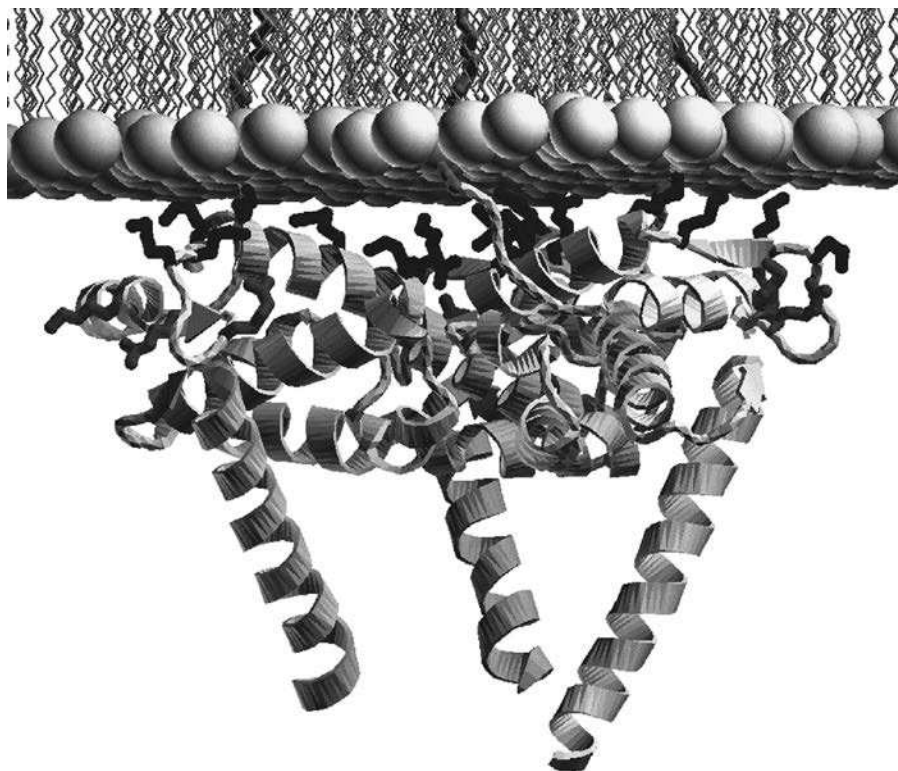


FIGURE 49.30. Structure of a matrix (MA) trimer bound to membrane. At the **top** is depicted the lipid bilayer; myristate moieties are shown embedded in the bilayer. The helical C-terminal tail of MA is shown projecting away from the globular core. (Reprinted from Hill CP, Worthylake D, Bancroft DP, et al. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proc Natl Acad Sci U S A* 1996;93:3099–3104, with permission. Copyright © 1996 National Academy of Sciences, USA.)

The MA domain not only functions in regulating Gag trafficking but also plays an important role in the incorporation of the viral Env glycoproteins into virions. This aspect of MA function will be discussed in more detail in the section on Env Glycoprotein Incorporation into Virions.

Capsid (CA): Gag Multimerization and Core Formation

The CA domain of Gag serves an important function in promoting virus assembly, and the mature CA protein forms the viral core after PR-mediated Gag processing. CA is composed of two structural and functional domains, the NTD and the CTD (Fig. 49.29C), which are connected by a short, flexible linker. This two-domain organization is a highly conserved feature among retroviral CA proteins. NMR and x-ray crystallographic data are available for both domains of HIV-1 CA^{246,262,531} (Fig. 49.31A). The NTD (residues 1 to 145) is highly helical, with an exposed, CypA-binding loop and an N-terminal β -hairpin.²⁴⁶ The CTD (residues 151 to 231) is also highly helical.²⁴⁷ The N-terminus of the CTD contains the major homology region (MHR), which, apart from the zinc-finger motifs in NC, is the only sequence in Gag that displays significant amino acid identity between divergent retroviral genera. Mutations within CA that disrupt HIV-1 replication elicit several distinct phenotypes: (a) defective immature virus particle production, (b) normal immature particle production but impaired formation of conical capsids, and (c) normal formation of immature particles and conical capsids but severely impaired infectivity.⁷⁷⁹ Mutations in the CTD often induce assembly defects, whereas changes in the NTD frequently inhibit proper virion maturation after release and severely impair infectivity.

Purified HIV-1 CA is capable of assembling into tubular or spherical particles^{199,281,778} *in vitro*. CA-NC fusion proteins form tubes *in vitro* at protein concentrations lower than those

required to assemble into similar structures with CA alone; in the case of CA-NC, tube formation appears to be dependent on the presence of nucleic acid.¹⁰¹ Interestingly, adding as few as four residues of MA to the N-terminus of CA converts *in vitro* particle assembly from tubes to spheres.⁷⁷⁸ This observation demonstrates that PR-mediated processing at the MA–CA junction causes a major refolding of the CA N-terminus and that this structural rearrangement plays a key role in promoting the morphologic changes that occur during core condensation.

In the tubes or cylinders assembled *in vitro*, CA is arranged in helical arrays in which the NTD forms hexameric rings that are linked into a continuous lattice by the CTD⁴⁴⁸ (Fig. 49.31B, C). Similar CA hexamers are visualized in cores obtained from purified HIV-1 virions,^{86,834} indicating that the CA arrays assembled *in vitro* reflect the physiologically relevant higher-order CA organization. As discussed earlier, in the immature VLP a large gap in the hexameric Gag lattice allows the lattice to curve and form a closed sphere. In the case of the mature capsid (note that the term *capsid* is used to refer to both the protein and the mature, conical core; here “CA” will be used to refer to the protein or the domain of Gag, while “capsid” will refer to the core), the formation of closed conical structures characteristic of mature lentiviral cores requires that 12 pentamers (*pentagonal defects* or *pentons*) be introduced into the hexagonal lattice, with 5 pentamers at the narrow end and 7 at the wider end (Fig. 49.31C). These cones are organized in a manner reminiscent of the fullerene structures formed by elemental carbon.²⁴⁸ Although the cores of other retroviruses are cylindrical or spherical, it has been suggested that all orthoretroviral cores are formed from similar hexagonal CA lattices and that their different shapes (cones, spheres, or cylinders) arise through differences in the spacing of the pentagonal defects.²⁴⁸ In support of this model, an HIV-1 CA mutant

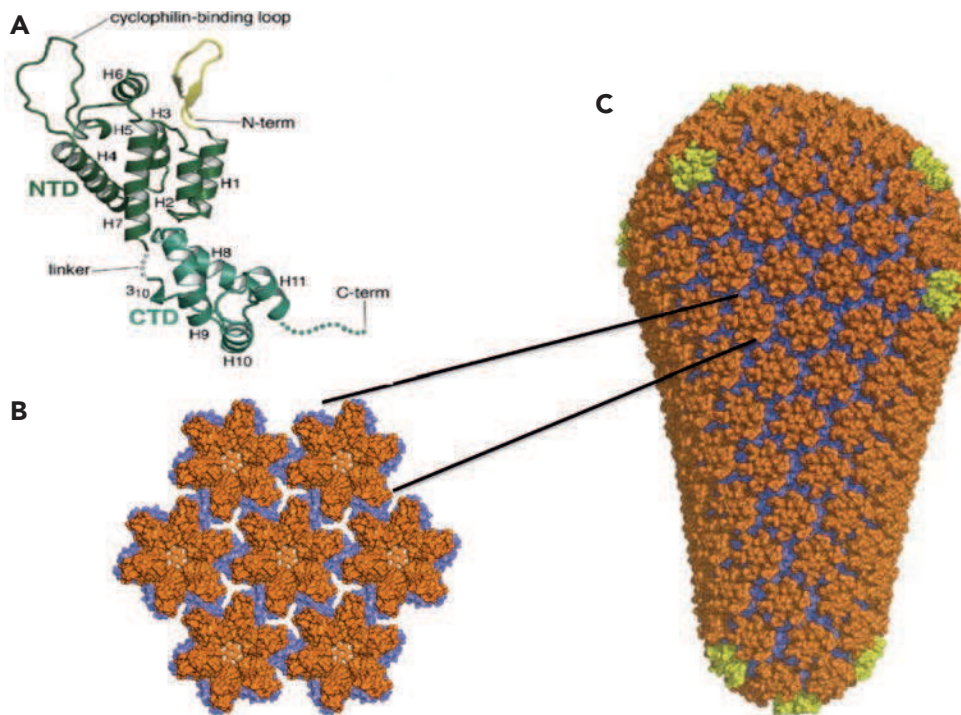


FIGURE 49.31. Capsid (CA) structure. **A:** Model of the structure of a CA monomer. N-terminal domain (NTD), C-terminal domain (CTD), linker region, and cyclophilin A-binding loop are indicated. Helices H1 to H11 are labeled. (**A** reprinted from Ganser-Pornillos BK, Yeager M, Sundquist WI. The structural biology of HIV assembly. *Curr Opin Struct Biol* 2008;18:203–217, copyright 2008, with permission from Elsevier.) **B:** Top view of a CA hexameric lattice. NTDs are highlighted in orange, CTDs in blue. Note that hexamer-hexamer contacts are mediated by the CTDs. (**B** reprinted from Pornillos O, Ganser-Pornillos BK, Kelly BN, et al. X-ray structures of the hexameric building block of the HIV capsid. *Cell* 2009;137:1282–1292, with permission.) **C:** Model of the human immunodeficiency virus 1 (HIV-1) capsid core. The color scheme for CA NTDs and CTDs are as in **B**. Pentameric defects, which close off the cone at both wide and narrow ends, are depicted in yellow. (**C** reprinted with permission from Pornillos O, Ganser-Pornillos BK, Yeager M. Atomic-level modelling of the HIV capsid. *Nature* 2011;469:424–427. Copyright © 2011, *Nature*.)

has been described that simultaneously assembles into cones, spheres, and cylinders,²⁵⁰ and an MLV CA NTD hexamer has been visualized by crystallography.⁵⁴² In addition to the CA hexamer structures mentioned earlier, structures of retroviral CA pentamers have also been determined by cryoelectron tomography¹⁰⁴ and x-ray crystallography.⁶¹⁰ These studies show that the hexamer and pentamer structures are quasiequivalent; only relatively subtle rearrangements are required for CA to shift from one to the other and analogous interaction interfaces are used in both structures. The hexameric lattice forms, and is stabilized by, three intermolecular CA–CA interfaces.⁶¹⁷

As mentioned previously, the HIV-1 CA binds members of the cyclophilin family of peptidyl-prolyl *cis-trans* isomerases.⁴⁷⁰ CypA is specifically incorporated into HIV-1 virions as the result of an interaction between CypA and a Pro-rich loop in the NTD that includes CA amino acid 90 (Figs. 49.29C and 49.31A).^{227,246,750} The relationship between CA–CypA binding and HIV-1 infectivity is complex and controversial; however, it appears that although CypA interacts with HIV-1 CA during assembly, most biologically significant interactions between CA and CypA occur postentry. At least under some circumstances, CypA binding to CA in the target cell helps to counteract restriction mediated by TRIM5 α and/or possibly other uncharacterized restriction factors.^{36,304,707}

The critical roles that CA plays both early and late in the HIV-1 replication cycle make it a promising target for the development of antiretroviral agents. Several CA-based inhibitors have been reported: one is a peptide, CAI, which disrupts HIV-1 assembly *in vitro* by binding a hydrophobic groove in the CA CTD.^{720,748} Although CAI is unable to enter cells and thus does not display antiviral activity, a technique known as hydrocarbon stapling was applied to this peptide to increase its cell permeability. The resulting circularized peptide is able to inhibit HIV-1 replication.⁸⁵⁰ A small molecule known as CAP1⁷⁴⁵ was identified in an *in silico* screen for CA-binding compounds and was found to disrupt assembly *in vitro* and in cells and to inhibit HIV-1 infectivity. CAP1 binds a hydrophobic cavity near the base of the CA NTD via an induced-fit mechanism whereby CAP1 binding displaces the aromatic ring of CA Phe-32.³⁸⁷ A different small molecule, binding a site on the CA NTD distinct from that occupied by CAP1, has been described.^{68,694} This compound inhibits both mature particle formation and virion infectivity, apparently by interfering with interactions between the CA NTD and CA CTD and by destabilizing the viral core. The identification of these CA-based inhibitors encourages efforts to develop highly specific and nontoxic small-molecule inhibitors of CA function that might be clinically active.

Nucleocapsid (NC): RNA Encapsulation, Gag Multimerization, and Nucleic Acid Chaperone Activity

The third major domain synthesized as part of the Gag precursor is NC (see Fig. 49.29D). NC is required for the specific encapsidation of full-length, unspliced (genomic) RNA into virions, and, like other Gag domains, it serves multiple roles during the virus replication cycle. All orthoretroviral NC proteins contain one or two zinc-finger motifs. Unlike most cellular zinc-finger domains, retroviral NC zinc fingers are of the CCHC type (Cys- X_2 -Cys- X_4 -His- X_4 -Cys, where X is any amino acid). HIV-1 NC contains two zinc-finger motifs that bind zinc tightly both *in vitro* and in virions^{55,714} and two clusters of basic residues flanking the first zinc finger.

NC engages in both sequence-specific and sequence non-specific interactions with nucleic acid. In general, the sequence-specific interactions involve the zinc fingers in conjunction with the basic residues, whereas nonspecific interactions are driven largely by the positive charge conferred by the basic residues. NMR data indicate that the two HIV-1 NC zinc fingers are brought together into a central globular domain by a highly basic linker domain (Fig. 49.32), which appears to be very

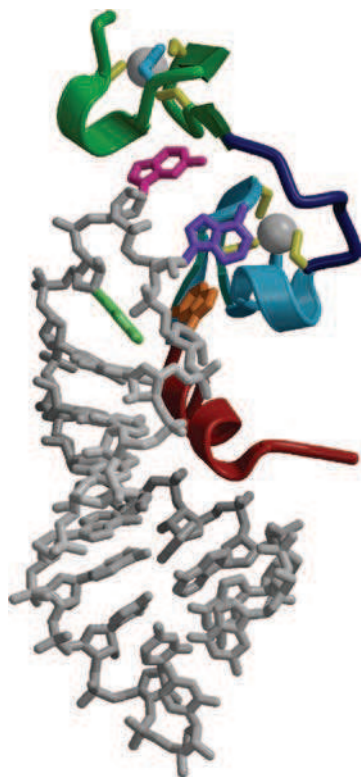


FIGURE 49.32. Complex between nucleocapsid (NC) and stem-loop 3 (SL3) of the human immunodeficiency virus 1 (HIV-1) packaging signal. The two zinc ions coordinated by the zinc-finger domains are shown as gray balls. Color scheme: N-terminal 3_{10} helix, pink; N-terminal zinc finger, cyan; linker, red; C-terminal zinc finger, green; cysteine and histidine side chains, yellow and cyan, respectively. (Reprinted from Turner BG, Summers MF. Structural biology of HIV. *J Mol Biol* 1999;285:1–32, by permission of the publisher Academic Press.)

flexible.^{538,732} Mutations that abrogate zinc binding by retroviral NC abolish genome encapsidation and virus infectivity.^{272,518} As discussed earlier, the specificity of HIV-1 genome encapsidation results in part from an interaction between NC and an approximately 120-nt sequence located between the 5' LTR and the Gag initiation codon. This sequence, variously known as the packaging signal, encapsidation element, or ψ -site, folds into a series of four stem-loops (SL1 to SL4) (see Fig. 49.7; for review on RNA encapsidation, see⁴⁶⁶). It appears that this secondary structure, rather than the primary nucleotide sequence itself, confers RNA encapsidation specificity. NC reportedly binds SL2 and SL3 with the highest affinity. Structures for SL2 and SL3 in complex with NC were solved in early studies by NMR spectroscopy.^{15,172} These structures demonstrate the involvement of both basic and zinc-finger residues in the NC/ ψ interaction. Despite some important similarities, key structural differences exist between NC–SL2 and NC–SL3 interactions, illustrating the flexibility of NC in binding to different RNA targets. Over the past decade, the structures of many small HIV-1 RNA fragments have been solved (reviewed in^{15,172}), and recently, the secondary RNA structure of the entire HIV-1 genome was determined by SHAPE technology⁷⁹¹ (see section on Genomic Organization of HIVs). It should be emphasized that the HIV-1 genomic RNA likely adopts a range of distinct conformations⁴⁶⁶; defining which conformations are required to promote specific steps in the virus replication cycle remains a challenging problem for further investigation.

Viral genomic RNA in virions is dimeric, and RNA labeling data support the presence of one RNA dimer per virion.¹²⁰ Although some studies reported that dimerization takes place after RNA encapsidation, compelling genetic evidence from studies that used recombination rates to measure RNA co-packaging supports the hypothesis that Gag selects RNA dimers for packaging.^{126,534} In the MLV system, it has been reported that RNA dimerization exposes a high-affinity binding site for NC, thus helping to explain why dimeric RNAs are packaged selectively over monomers.^{156,260,528} In the case of HIV-1, NMR analysis revealed that a stem-loop encompassing the Gag AUG start codon base pairs with a sequence upstream in U5. This interaction is proposed to expose the DIS, thereby promoting RNA dimerization and increasing NC binding.⁴⁶⁵ This structural switch provides a mechanism for regulating translation, RNA dimerization, and packaging.

Live-cell imaging demonstrates that HIV-1 genomic RNA is highly dynamic within cells but becomes stabilized at the plasma membrane through its interactions with a small number of Gag molecules.³⁶⁹ Thus, the initial Gag–RNA complex likely serves as the focal point for subsequent VLP assembly. In the absence of Gag expression or an intact ψ -signal, HIV-1 RNA is not observed at the plasma membrane.³⁸⁸ Consistent with these findings, Gag–RNA-binding assays coupled with membrane flotation analysis suggest that initial Gag–RNA contacts take place in the cytoplasm and that RNA associated with monomeric or dimeric Gag traffics to the plasma membrane where assembly proceeds.⁴¹⁴

NC–RNA interactions are critical for promoting Gag multimerization; thus, many NC mutations cause defects in virus assembly and release. The role of RNA in assembly is primarily to provide a template along which molecules of Pr55^{Gag} can align and pack. The RNA with which NC interacts need not be viral genomic RNA; expression of retroviral Gag proteins in the

absence of packageable viral RNA leads to efficient assembly, apparently through the use of cellular RNAs as a scaffold.⁵⁴⁹

In addition to its roles in RNA encapsidation and Gag assembly, other functions for HIV-1 NC have been observed. Many of these are critical for efficient reverse transcription and are attributed to the nucleic acid chaperone activity of NC.^{440,623} This property enables NC to catalyze the refolding of nucleic acid molecules into structures with the most thermodynamically favorable conformation (i.e., the greatest number of base pairs) (for reviews see^{440,623}). The nucleic acid chaperone activity of NC evidently requires that NC molecules coat nucleic acid at near-saturating levels of approximately one molecule of protein per five to eight nucleotides. NC, or the NC domain of Pr55^{Gag}, contributes to placement of the tRNA^{Lys3} primer on the pbs (see section on Reverse Transcription), unwinding of the tRNA during the initiation of reverse transcription,²⁹⁷ increased efficiency of minus-strand transfer by increasing the annealing of complementary R regions, removal of annealed RNA fragments during minus-strand strong-stop DNA synthesis, destabilization of nucleic acid secondary structure during both minus- and plus-strand DNA synthesis, and stimulation of plus-strand transfer by promoting the removal of the tRNA primer and annealing of the pbs sequence in plus-strand strong-stop DNA to its minus-strand DNA complement.⁴⁴⁰ The nucleic acid chaperone activity of NC relies on two seemingly opposing properties: the ability to weakly destabilize nucleic acid duplexes (which is central to melting secondary structures that would interfere with reverse transcription) and the ability to aggregate nucleic acids (which promotes placement of the tRNA primer on the pbs and facilitates annealing reactions during strand transfers). The duplex-destabilizing activity of NC is primarily mediated by the zinc fingers and is due to a preferential binding of the fingers to single-stranded nucleic acid.^{15,16} The ability of NC to aggregate nucleic acids appears to be at least partially due to its highly basic nature.

The nucleic acid chaperone activity of NC has been demonstrated not only in *in vitro* assays⁴⁴⁰ but also by studying the effects of NC mutation on reverse transcription and virus infectivity in cells.^{53,89,270,271,744} Mutations in HIV-1 NC impair infectivity by destabilizing newly reverse-transcribed viral DNA.⁵³ Studies performed with MLV demonstrated that changing the single CCHC zinc-finger motif to CCCC or CCHH had no effect on zinc binding, tRNA incorporation, RNA encapsidation, or RNA maturation but profoundly impaired virus infectivity.²⁷⁰ Examination of viral DNA in mutant-infected cells revealed that full-length, circular DNA products were formed inefficiently and that a variety of mutations were present at the DNA ends.²⁷⁰ Such aberrations, if present at the termini of linear DNAs, would render these DNAs unsuitable for integration. Analogous mutations in the zinc fingers of the HIV-1 NC have been reported^{89,271,744}; these changes resulted in defects in reverse transcription, leading to a lack of circular DNA production, degradation of the viral DNA ends, and impaired virus infectivity. NC has also been observed to promote integration *in vitro*^{107,254}; mutations in the HIV-1 NC zinc fingers interfere with IN-mediated end processing, leading to defects in integration in infected cells.⁸⁹

Because NC participates in multiple steps in the virus life cycle, it represents an attractive target for antiviral agents. Although many cellular proteins contain zinc fingers, the retroviral CCHC-type zinc-finger motif is relatively uncommon,

and a number of compounds have been described that appear to selectively impair NC function without causing significant cytotoxicity (for review see^{550,761}). For example, mild oxidizing agents such as 2,2'-dithiopyridine (aldrithiol-2, or AT-2) cause zinc to be "ejected" from the zinc fingers and lead to extensive protein cross-linking.^{627,628} Alkylating agents such as N-ethylmaleimide (NEM) react with zinc-bound thiols in NC and inactivate virus infectivity.⁵³⁵ Compounds that induce the ejection of zinc from NC include the S-acyl-2-mercaptobenzamide thioesters (SAMTs),³⁵⁸ which possess a novel mechanism of action in cells: the thiol generated by the reaction between NC and SAMT is acylated in the cell to regenerate an active thioester.⁵²² This recycling process likely adds to the potency and stability of the inhibitor. Although clinically effective NC inhibitors have not yet been identified, this appears to be a promising area for future research.

p6: Virus Release and Vpr/Vpx Incorporation

In addition to the MA, CA, and NC proteins described earlier, HIV-1 encodes a Pro-rich, 6-kD protein, known as p6, at the C-terminus of Pr55^{Gag} (see Fig. 49.29E). Deletion of p6 significantly disrupts HIV-1 particle production by blocking a late step in the budding process.²⁷⁵ Particles assembled from p6 mutants display a release defect characterized by a striking accumulation of tethered virions at the plasma membrane (Fig. 49.33). The virus release function of p6 maps to a highly conserved Pro-Thr-Ala-Pro (PTAP) motif near the N-terminus of p6.^{179,333}

Mutational analyses of the *gag* genes of a number of retroviruses revealed the presence of motifs that, like PTAP of p6, promote the release of virions from the plasma membrane. These short sequences have been termed *late* domains to reflect their role late in the budding process (for reviews see^{58,177,541,611}). Three general classes of retroviral late domains have been identified: PTAP (or PSAP), Pro-Pro-Pro-Tyr (PPPY), and Tyr-Pro-X_n-Leu (YPX_nL, where X is any amino acid and n = 1 to 3 residues). Specific examples of viruses in which these late domains are located include PTAP in p6 of primate lentiviral Gag, the MA domain of HTLV-1, and pp16 of Mason-Pfizer monkey virus (M-PMV); PPPY in p12 of MLV, p2b of Rous sarcoma virus, pp16 of M-PMV, and the MA domain of HTLV-1; and YPX_nL in the p9 domain of EIAV Gag and p6 of primate lentiviral Gag. In each case, mutation of these motifs disrupts virus budding and release. Interestingly, similar motifs function to promote the release of other enveloped viruses (e.g., the *Filoviridae* and *Rhabdoviridae*).

Numerous lines of evidence support the hypothesis that retroviral late domains function by interacting with components of the cellular endosomal sorting machinery.^{177,541} Cargo proteins ultimately destined for the vacuole in yeast and the lysosome in mammalian cells are recognized sequentially by four multiprotein complexes, termed ESCRT-0, -I, -II, and -III (for endosomal sorting complex required for transport-0, -I, -II, and -III).³⁸⁰ These cargo proteins are initially sorted into vesicles that bud inwardly into late endosomes to generate MVBs. ESCRT machinery also plays a key role in membrane fission during the abscission step of cytokinesis.^{106,539} It is noteworthy that retrovirus budding and release from the plasma membrane involve a membrane budding and scission event that is topologically equivalent to budding and scission steps that occur during MVB formation and cell abscission; that is, these events are oriented away from the cytosol. Thus, retroviruses appear to have evolved the ability to usurp cellular

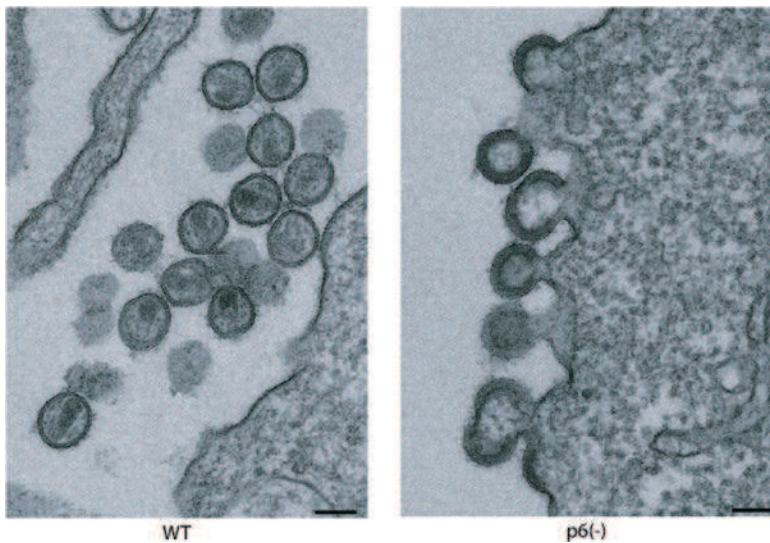


FIGURE 49.33. Morphology of wild-type (WT) versus p6-deleted human immunodeficiency virus 1 (HIV-1) visualized by thin-section transmission electron microscopy (EM). Note the presence of released, mature particles in the WT micrograph (left panel) and tethered, immature particles in the p6-mutant micrograph (right panel). (Courtesy of Kunio Nagashima.)

budding machinery to promote their release from the plasma membrane (Fig. 49.34). While the molecular details of ESCRT function are still being elucidated, it appears that ESCRT-0 first associates with endosomal membrane and cargo protein via phosphatidyl inositol-3-phosphate and ubiquitin binding, respectively, and subsequently recruits downstream ESCRTs (I, II, and III) to the membrane. *In vitro* reconstitution studies suggest that ESCRT-I and -II provide the driving force for membrane budding, whereas ESCRT-III plays a central role in the membrane scission event.^{339,810,811} The adenosine triphosphatase (ATPase) Vps4 is also implicated in membrane scission and catalyzes the recycling of ESCRT complexes back to the cytosol after membrane scission is completed. Interestingly, HIV-1 budding and release appear to require only a subset of ESCRT machinery; ESCRT-0, ESCRT-II, and some ESCRT-III components are not required^{422,540} (Fig. 49.34).

A connection between the endosomal sorting pathway and HIV-1 budding was first suggested by the observation that the ESCRT-I component Tsg101 interacts with p6, and that this interaction is dependent on the PTAP motif.^{257,774} The functional significance of this interaction was supported by the finding that siRNA-mediated depletion of Tsg101 disrupts HIV-1 release,²⁵⁷ as does overexpression of the N-terminal, Gag-binding domain of Tsg101 (TSG-5').¹⁷⁸ Inhibition of release imposed by both Tsg101 depletion and overexpression of TSG-5' is specific for PTAP-dependent budding, as the release of the PPPY-bearing MLV is unaffected.^{178,257} The significance of p6-Tsg101 binding in HIV-1 release was also supported by the finding that fusing Tsg101 directly to the C-terminus of Gag rescued a p6 late-domain defect, indicating that the primary role of p6 in virus release is to bind Tsg101.⁵⁰¹ Broad disruption of the endosomal sorting pathway by overexpressing a

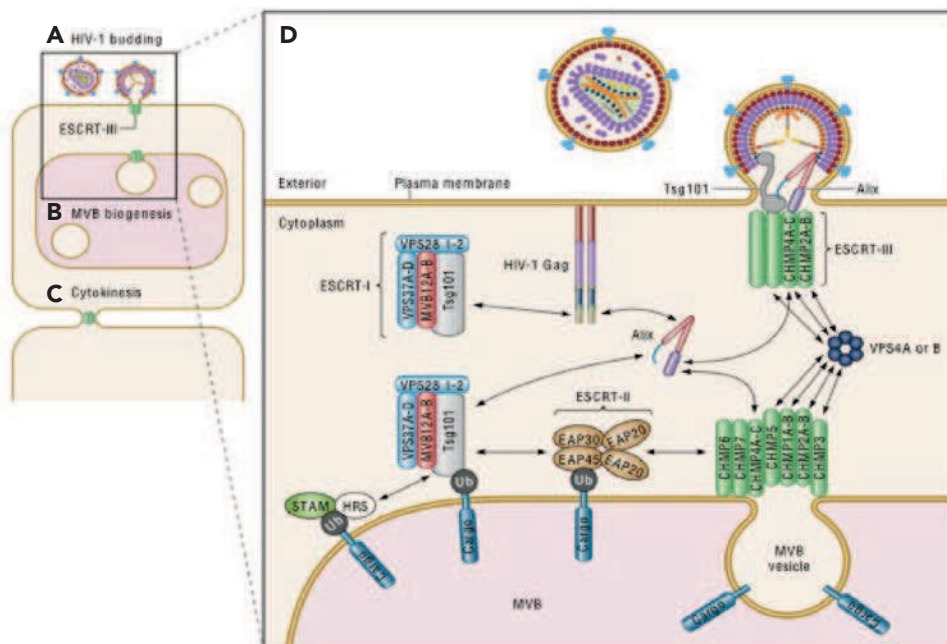


FIGURE 49.34. Role of endosomal sorting complex required for transport (ESCRT) machinery in virus budding (A), multivesicular body (MVB) biogenesis (B), and the abscission step of cytokinesis (C). ESCRT-I, -II, and -III are labeled. While not explicitly labeled as such, the STAM/HRS complex is often referred to as ESCRT-0. Note that virus budding does not appear to use ESCRT-II and requires only a subset of ESCRT-III proteins. (Reprinted from Balasubramaniam M, Freed EO. New insights into HIV assembly and trafficking. *Physiology [Bethesda]* 2011;26:236–251, with permission.)

dominant-negative mutant of the ATPase Vps4 globally inhibits the budding of a number of retroviruses, suggesting that most, if not all, retroviruses depend on the ESCRT apparatus for their release.^{177,541} Whereas PTAP late domains function by interacting with the ESCRT-I component Tsg101, the YPX_nL-type motif of EIAV interacts with the ESCRT-associated factor Alix (formerly AIP1).^{500,723,780} Alix, although not a component of an ESCRT complex, can bridge ESCRT-I and ESCRT-III by interacting with both Tsg101 and one of the ESCRT-III components^{500,723,780} (Fig. 49.34). PPPY-type late domains bind members of the Nedd4 family of E3 ubiquitin ligases.^{58,177,541}

Although under most conditions PTAP serves as the dominant HIV-1 late domain, p6 also bears an Alix-binding YPX_nL motif (Fig. 49.29E).^{500,723,780} The presence of this alternative site for interaction between HIV-1 Gag and the ESCRT machinery suggests that under some circumstances Alix plays a role in HIV-1 budding and release. Indeed, if the PTAP motif is eliminated by mutation, Alix overexpression partially rescues virus release.⁷⁶² Furthermore, mutation of the Alix binding site in p6 induces significant virus replication defects in T cells and macrophages. Simultaneous elimination of both Tsg101 and Alix binding sites in p6 abolishes virus replication.²³⁹ These findings suggest some degree of functional redundancy between PTAP and YPX_nL motifs in HIV-1 replication. In addition to binding the YPX_nL motif in HIV-1 p6, Alix has also been reported to interact with the NC domain of Gag.^{196,606,607}

Recent advances in live-cell imaging technology have provided new insights into the role of ESCRT machinery in cytokinesis and HIV-1 release. Total internal reflection fluorescence (TIRF) microscopy and fluorescently tagged Gag and ESCRT components were used to follow the recruitment of ESCRT machinery during retrovirus assembly and release.³⁷⁰ Gag assembly was typically completed within ~5 to 10 minutes of the initial appearance of an individual Gag punctum.^{349,369} For both HIV-1 and EIAV, ESCRT-III components and Vps4 were transiently recruited late in this process, concomitant with the completion of assembly. In contrast, Alix accumulated earlier in the EIAV Gag assembly process and its accumulation appeared to coincide with Gag recruitment.³⁷⁰ As expected, late domain mutation largely ablated ESCRT-III recruitment but did not affect the kinetics of assembly.^{349,370} The sequential recruitment of ESCRT machinery to the site of membrane fission is also supported by time-lapse imaging of the abscission step of cytokinesis.²⁰² ESCRT-I and -III components were observed to be recruited to ring-like structures in the intercellular bridge separating the two daughter cells undergoing cytokinesis. Subsequent recruitment of ESCRT-III and Vps4 to the constricted region at which membrane separation occurs was followed immediately by abscission. These observations are consistent with a direct role for ESCRT-III and Vps4 in membrane scission.

In addition to its role in promoting virus budding, p6 functions to direct the incorporation of Vpr and Vpx into virions.^{587,818} In the case of HIV-1, the binding site for Vpr overlaps with the sequence responsible for p6 association with Alix (Fig. 49.29E).

Env Glycoprotein Incorporation into Virions

The mechanism by which HIV-1 Env glycoproteins are incorporated into budding virus particles remains incompletely characterized. Several models for Env incorporation can be postulated¹¹⁷: (a) passive incorporation, in which the simple pres-

ence of Env glycoproteins in the plasma membrane is sufficient to allow them to be packaged into budding virus particles; (b) direct Gag–Env interaction, in which direct contacts between the cytoplasmic tail of gp41 and the membrane-proximal portion of Gag (i.e., the MA domain) are required for the active recruitment of Env into virions; (c) Gag–Env co-targeting, whereby Gag and Env traffic to a common site on the plasma membrane (e.g., a lipid raft or other type of membrane microdomain), thus concentrating Env at sites of Gag assembly; and (d) indirect Gag–Env interaction, according to which a host cell protein serves to link the gp41 cytoplasmic tail with the MA domain of Gag. Data in the literature can be invoked to support each of these models. In some cell lines, gp41 lacking the cytoplasmic tail is efficiently incorporated into virions. The Env proteins from heterologous viruses (e.g., MLV Env and vesicular stomatitis virus glycoprotein [VSV-G]) can be packaged into HIV-1 particles (a process known as pseudotyping), and host cell membrane proteins are also present within the viral envelope. However, despite this apparently nonspecific incorporation, several lines of evidence suggest that HIV-1 Env glycoproteins are actively recruited into virions via direct interactions between Env and MA: (a) mutations in the MA domain of Gag can block HIV-1 Env incorporation,^{190,233,839} and this incorporation defect can be reversed by pseudotyping virions with heterologous Env glycoproteins or by removing the gp41 cytoplasmic tail^{231,233,485}; (b) HIV-1 Env directs basolateral budding of Gag in polarized epithelial cells^{460,578}; and (c) a single amino acid change in MA reverses an Env-incorporation defect caused by a small deletion in the gp41 cytoplasmic tail.⁵⁴⁶ Although in some cell lines truncation of the gp41 cytoplasmic tail has little effect on Env incorporation, in most cell lines and in primary cell types, including PBMCs and MDMs, gp41 truncations severely disrupt Env incorporation.⁵⁴⁷ Thus, it is clear that the gp41 cytoplasmic domain plays an important role in the incorporation of Env into HIV-1 particles. The cell-type dependence of this function suggests the involvement of host cell factors or structures; these could be a common site or microdomain to which Gag and Env are co-targeted (e.g., a lipid raft) or a cellular protein that forms a bridge between MA and the gp41 cytoplasmic tail. As mentioned earlier, both Gag and Env localize to cholesterol- and sphingolipid-enriched lipid raft microdomains in the plasma membrane,⁷⁸⁵ and several host cell proteins have been proposed to bind Gag and/or Env to facilitate Env incorporation.¹¹⁷ The aforementioned observation that MA mutations can block the incorporation of full-length Env into virions and that gp41 cytoplasmic tail deletions can rescue this incorporation defect suggests that incorporation of full-length Env requires active recruitment (or at least accommodation) by MA, whereas incorporation of truncated HIV-1 Env, or heterologous glycoproteins bearing short cytoplasmic domains, is a more passive process. At this time, however, a detailed view of the host cell's role in promoting Env incorporation is lacking.

Virus Maturation: Protease (PR)

Early pulse-chase studies performed with avian retroviruses demonstrated that retroviral Gag proteins are initially synthesized as polyprotein precursors that are cleaved to generate smaller products.⁷⁷⁶ Subsequent studies revealed that the processing function is provided by the viral PR, and that proteolytic digestion of the Gag and Gag-Pol precursors is essential for virus infectivity.^{152,378} Sequence analysis of retroviral PRs

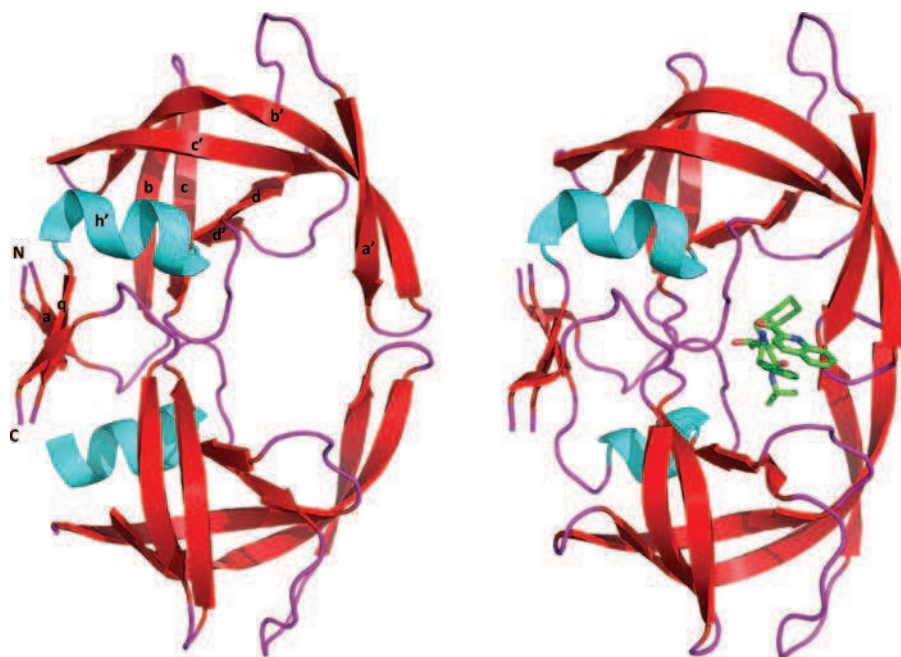


FIGURE 49.35. Structure of the human immunodeficiency virus 1 (HIV-1) protease (PR) dimer uncomplexed (A) or complexed (B) with the PR inhibitor Ro-31-8558. The following domains are indicated in the first half of the molecule: β -strands a (residues 1 to 4), b (residues 9 to 15), c, and d (residues 30 to 35). The active-site residues are at positions 25 to 27. The second half of the molecule is structurally related to the first half, with the following domains indicated: β -strands a' (residues 43 to 49), b' (residues 52 to 66), c' (residues 69 to 78), d' (residues 83 to 85), and helix h' (residues 86 to 94). The inner portion of the dimer interface is formed by β -strand q (residues 95 to 99). (Modified by Jerry Alexandratos from Wlodawer A, Erickson JW. Structure-based inhibitors of HIV-1 protease. *Annu Rev Biochem* 1993;62:543–585.)

indicated that they are related to cellular “aspartic” proteases such as pepsin and renin (for review see¹⁶⁸). Like these cellular enzymes, retroviral PRs use two apposed Asp residues in the active site to coordinate a water molecule that catalyzes the hydrolysis of a peptide bond in the target protein. However, unlike the cellular aspartic proteases, which function as pseudodimers (using two folds within the same molecule to generate the active site), retroviral PRs function as true dimers. X-ray crystallographic data from HIV-1 PR^{424,556,809} indicate that the two monomers are held together in part by a four-stranded, antiparallel β -sheet derived from both N- and C-terminal ends of each monomer (Fig. 49.35). The substrate-binding site is located within a cleft formed between the two monomers. Like their cellular homologs, the HIV-1 PR dimer contains flexible “flaps” that overhang the binding site and may stabilize the substrate within the cleft; the active-site Asp residues lie in the center of the dimer. Although some limited amino acid homology is observed surrounding active-site residues, the primary sequences of retroviral PRs are highly divergent, yet their structures are remarkably similar.

The first cleavage events catalyzed by retroviral PRs during or immediately after virion release from the cell are likely intramolecular⁵⁹⁹ and serve to liberate PR from the Gag-Pol precursor. As mentioned earlier, after the release of PR from the Gag-Pol precursor, the dimeric enzyme cleaves a number of sites in both Gag and Gag-Pol (see Fig. 49.6). The efficiency with which PR cleaves the individual target sites in Gag and Gag-Pol varies widely and is influenced by two major factors: the amino acid sequence at the site of cleavage and the context (i.e., degree of exposure or accessibility) of the cleavage site. The proteolytic processing of model proteins and substrate analogs by HIV-1 PR indicates that the binding cleft can accommodate a peptide of approximately seven residues in length, and synthetic peptides of this size are cleaved *in vitro*.⁶¹ As a consequence of the relatively divergent PR target sequences present in Gag and the varying efficiencies with which these sites serve as substrates for PR activity, Gag cleavage takes place as an ordered,

step-wise cascade. Processing at the N-terminus of NC is the most rapid *in vitro*, whereas the cleavage converting CA-SP1 (p25) to CA (p24) is the slowest. Mutations in Gag that disrupt the ordered nature of PR-mediated processing severely disrupt virus assembly or subsequent maturation. Furthermore, HIV-1 mutants engineered to overexpress PR exhibit rapid, premature processing of Gag and Gag-Pol polyproteins and a block in virus production.⁴⁰⁷ Thus, the activation of PR must be tightly controlled to prevent significant processing prior to the completion of assembly. Although the high concentration of Gag and Gag-Pol precursors achieved upon particle assembly undoubtedly facilitates activation of PR, factors that regulate retroviral PR activity remain to be fully defined.

PR cleavage of the Gag and Gag-Pol precursors leads to a dramatic change in virion morphology, a process known as maturation. Immature retroviral particles (e.g., those produced in the presence of PR inhibitors or an inactive PR) appear doughnut-shaped by thin-section EM. In contrast, mature HIV-1 virions contain conical cores. As mentioned earlier, cryoelectron tomography studies have shown that in immature virions Gag precursors are aligned in a rod-like fashion like spokes on a wheel, with the N-terminal MA domain associated with the viral membrane and the C-terminal domains projecting inward toward the center of the virion.^{243,815,835} Cleavage of Gag by PR releases CA, NC, and p6 from their attachment (via MA) to the lipid and allows CA to form a shell around the NC–RNA complex. Formation of the conical core likely involves a reassembly process rather than a concerted condensation reaction.⁴² Gag in the immature virus particle and CA in the mature core form a hexameric lattice with a unit cell spacing of ~8 nm in the case of the immature particle and ~9 nm in the case of the mature CA lattice.

Competitive inhibitors of HIV-1 PR function have proven to be highly effective antiretroviral drugs, particularly when used in combination with RT inhibitors. The development of PR inhibitors represents a classic example of structure-based drug design; detailed knowledge of the topology of the substrate-binding site, the enzyme–substrate interaction, and

the chemistry of the cleavage reaction enabled investigators to synthesize a large number of inhibitors that potently inhibit PR in culture at nanomolar concentrations. The earliest PR inhibitors were small oligopeptide derivatives that mimicked PR target sequences but contained nonhydrolyzable groups at the P1 and P1' positions flanking the scissile bond that PR would normally cleave. The early, peptide-based inhibitors were subsequently modified to improve stability and bioavailability, and the structure of the PR active site was used to design novel inhibitory compounds.⁸⁰⁹ When PR inhibitors are used to treat HIV-infected patients, virus loads decline precipitously, but drug-resistant variants soon emerge. Sequencing of the PR coding region of drug-resistant variants often reveals consistent changes. For example, saquinavir escape mutants frequently contain changes at PR residue 48 and/or 90; ritonavir resistance is often associated with mutations at PR residue 82. The initial changes that confer drug resistance generally result in only partial resistance and may be accompanied by decreased “fitness” (i.e., impaired PR function relative to WT in the absence of drug). With time, however, additional changes take place within PR that increase resistance and improve fitness. Unfortunately, mutations that arise in the presence of one inhibitor frequently induce at least partial resistance to others, a phenomenon known as cross-resistance. In addition to changes in PR, mutations that affect the natural PR targets of the enzyme (i.e., the Gag and Pol cleavage sites) have been observed.¹⁹⁴

The highly ordered nature of Gag processing and the strict dependence on complete processing for proper virion maturation make the Gag processing cascade a potential target for drug development. Indeed, the betulinic acid derivative dimethylsuccinyl betulinic acid (PA-457 or DSB, also known as bevirimat) potently inhibits HIV-1 infectivity by targeting a late Gag processing event: the cleavage of the CA-SP1 processing intermediate to mature CA.^{446,858} By specifically disrupting this step in Gag processing, treatment of virus-expressing cells with bevirimat leads to the formation of poorly infectious viral particles with aberrantly condensed cores. Selection in culture gives rise to bevirimat-resistant isolates that contain single amino acid substitutions flanking the CA/SP1 cleavage site.^{3,446,858} In patients, bevirimat can lead to significant reductions in viral loads,^{629,705} providing proof of concept that compounds capable of disrupting specific steps in Gag processing could be clinically effective against HIV-1. In the case of bevirimat, however, polymorphisms in SP1 downstream from the cleavage site reduce susceptibility to the compound in many patients.^{5,109,629} A second, structurally distinct maturation inhibitor (known as PF-46396) that blocks CA-SP1 processing has been described.⁶⁷ Interestingly, several of the mutations that confer resistance to PF-46396 map to regions of CA far upstream of the CA-SP1 cleavage site.⁷⁸⁷ These observations suggest that bevirimat and PF-46396 may occupy distinct binding sites on the assembled Gag complex, the presumed target of these compounds. In addition to compounds that block virus maturation by interfering with specific step(s) in Gag processing, condensation of a functional viral core can also be inhibited by molecules that bind the CA domain of Gag. Several compounds in this class are discussed in the CA section.

The Accessory Proteins

In addition to the viral structural proteins (Gag and Env), the *pol*-encoded enzymes (PR, RT, and IN), and the regula-

TABLE 49.3 HIV/SIV Accessory Proteins

Protein	Major proposed functions
Vif	Inactivation of antiviral activity of APOBEC family of cytidine deaminases
Vpr	Cell cycle arrest Nuclear import of viral PIC Transcriptional transactivation of viral and cellular promoters Apoptosis
Vpx	Nuclear import Inactivation of SAMHD1
Vpu	Enhancement of particle release Counteraction of tetherin Degradation of CD4
Nef	Down-regulation of surface CD4, MHC-I, CD3, and CD28 Enhancement of virus infectivity Modulation of cellular activation pathways

PIC, preintegration complex; SAMHD1, SAM domain and HD domain-containing protein 1; MHC-I, major histocompatibility complex I.

tory proteins (Rev and Tat), the HIV genome encodes several “accessory” proteins: Vif, Vpu, Vpr, Nef, and Vpx (in the case of HIV-2) (see Fig. 49.6). Early studies indicated that disruption of the ORFs for these proteins had little or no effect on virus replication in culture; indeed, laboratory-adapted virus isolates often acquire inactivating mutations in several of these genes. However, more recent tissue culture and *in vivo* experiments revealed a strong requirement for these gene products in efficient virus replication and disease induction. Remarkably, three of the four HIV-1/SIV accessory proteins (Vif, Vpu, and Vpr/Vpx) serve as adaptors to connect cellular substrates to the host cell proteasome machinery. These viral proteins function in part to counteract components of the cellular innate immune response that evolved to restrict retroviral infection. A summary of the major proposed functions of the HIV-1 accessory proteins is presented in Table 49.3.

Vif

As discussed earlier, the Vif protein derives its name (virus infectivity factor) from the observation that it plays a role in promoting virus infectivity. The finding that the requirement for Vif is producer-cell dependent suggested that Vif interacts with a host factor during virus assembly. The host factor in question was subsequently identified as the DNA cytidine deaminase APOBEC3G. The ability of Vif to counter the antiviral activity of APOBEC3G and related host enzymes is discussed in detail in the section on APOBEC3G and Family Members.

Vpr

The *vpr* gene encodes a 14-kD, 96-amino acid protein that is incorporated at high levels into virus particles.^{143,841} Virion incorporation is mediated through an interaction with a (Leu-X-X)₄ motif near the C-terminus of the p6 domain of Gag (Fig. 49.29E).^{403,468,841} The NMR structure of Vpr⁵³⁶ reveals

the presence of three α -helices folded around a hydrophobic core. The N- and C-termini of the protein are flexible. Although Vpr has little influence on HIV replication kinetics in proliferating PBMCs or T-cell lines in culture, in nondividing MDMs its effects are more substantial. Several lines of evidence indicate that Vpr is also required for efficient replication *in vivo*: Vpr mutations are observed in HIV-1 strains isolated from long-term nonprogressors,⁷¹⁰ and deletion of Vpr modestly decreases SIV pathogenicity in rhesus macaques.⁴²¹ Vpr has been reported to (a) stimulate gene expression driven by the HIV-1 LTR and enhance transcription of cellular genes, (b) promote transport of the viral PIC to the nucleus early postinfection, (c) arrest infected cells in the G₂ phase of the cell cycle, (d) induce apoptosis, and (e) modulate mutation rates during reverse transcription through interactions with uracil DNA glycosidases.

Early studies reported that Vpr weakly transactivates expression from the HIV-1 LTR,¹⁴³ potentially through Vpr interactions with Sp1,^{665,790} TFIIB,^{6,394} and/or TFIID.³⁹⁴ Vpr has also been reported to stimulate gene expression from cellular promoters, in part by interacting with the glucocorticoid receptor and enhancing its activity.³⁹⁴ Binding between Vpr and co-activators p300/CREB-binding protein may play a role in Vpr-mediated activation of both the viral LTR and cellular promoters.³⁹⁵

High levels of virion incorporation suggest a role for Vpr early in the virus life cycle prior to *de novo* gene expression. The observations that deletion of Vpr significantly reduces virus replication in terminally differentiated, nondividing MDMs,³⁰ that the HIV-1 PIC contains Vpr,³¹¹ and that Vpr localizes to the nucleus in HIV-1-infected or Vpr-expressing cells⁴⁶⁹ suggested that Vpr participates in the nuclear import of the HIV-1 PIC. However, deletion of Vpr does not block HIV-1 replication in terminally differentiated MDMs²³⁰ or other nondividing cells,⁸²⁵ and an MLV/HIV chimera lacking Vpr still infects nondividing cells.⁸²⁴

A major and well-validated activity of Vpr is the arrest of cells in the G₂ phase of the cell cycle (for review see⁶⁰³). Cell cycle arrest reportedly occurs within hours of infection and is not blocked by antiviral agents, suggesting that arrest can be initiated by virion-associated Vpr carried into the cell during infection.⁶⁰⁵ One possible scenario to explain the disruption of the cell cycle by Vpr is that it serves to link a still-unidentified cellular protein (or proteins) to the proteasomal degradation machinery. An early report identified a Vpr-binding protein (VprBP)⁸⁵³ that was later shown to bind damaged-DNA specific binding protein 1 (DDB1), which in turn associates with cullin 4 (CUL4).^{327,429,677} The interaction between Vpr and VprBP—subsequently renamed DCAF1 (DDB1- and CUL4-associated factor 1)—allows Vpr to recruit the unidentified substrate into the E3 ubiquitin ligase complex, presumably resulting in its ubiquitination and proteasome-mediated degradation. A role for Vpr–DCAF1 binding in cell cycle arrest is supported by the observations that this function of Vpr is abrogated by mutation of the DCAF1 binding site of Vpr or DCAF1 depletion.⁴²⁹ Early studies suggested that disruption of the cell cycle by Vpr involves inactivation of the p34cdc2–cyclin B kinase complex.^{310,621} This inactivation may arise through Vpr-induced modulation of host factors such as PP2A, Wee1, Myt1, and Cdc25C, which function upstream of the p34cdc2–cyclin B kinase complex (for review see⁶⁰³). A number of additional Vpr-interacting

proteins have been suggested to play a role in cell cycle arrest. These include HHR23A (the human homolog of the yeast repair protein Rad23),⁸⁰⁷ the DNA damage signaling protein ATR,⁶⁴¹ hVIP/MOV34,⁴⁷⁹ p21^{WAF154} and the family of factors known as 14-3-3 proteins, which function at several stages to regulate the cell cycle.³⁹⁶ Recent work has proposed that Vpr induces cell cycle arrest by recruiting the DDB1–CUL4 ubiquitin ligase complex to chromatin-associated nuclear foci, leading to proteasome-mediated degradation of a chromatin-bound cellular factor, and activation of ATR (ataxia telangiectasia mutated and Rad3 related), a protein involved in regulating the G₂/M checkpoint.⁴⁰ Although the precise mechanism and biological implications of Vpr-induced cell cycle arrest remain unclear, it has been proposed that increased HIV-1 LTR-driven transcription in the G₂ phase of the cell cycle could provide a rationale for the evolution of a Vpr cell cycle arrest function.²⁶⁵ The ability of Vpr to induce cell cycle arrest not only in mammalian cells but also in yeast⁴⁷³ implies the involvement of highly conserved cellular factors in this phenomenon.

Vpr was observed in yeast two-hybrid studies to interact with uracil DNA glycosylase-2 (UNG-2).⁷⁶ The ability of UNG-2 to remove uracils from DNA led to the suggestion that Vpr-mediated UNG-2 incorporation into virions could modulate viral mutation rates during reverse transcription by limiting dUTP misincorporation.^{122,490} However, subsequent studies showed that Vpr binding leads to UNG-2 degradation by linking UNG-2 to DCAF-1 and the CUL4-E3 ubiquitin ligase complex and, indeed, that Vpr(+) virions contain lower amounts of UNG-2 than Vpr(–) particles.^{7,677,678} While the biological consequences of Vpr-mediated UNG-2 degradation remain controversial, UNG-2 provides a clear example of a host cell factor that is linked by Vpr to the DCAF-1/CUL4/ubiquitin ligase complex for proteasomal degradation.⁷

Evidence from many laboratories has suggested that Vpr induces apoptosis through both caspase-dependent and caspase-independent pathways (for review see⁵⁵¹). It remains unclear whether this activity of Vpr also relates to its ability to hijack the host ubiquitination machinery. It has been postulated that Vpr-mediated apoptosis is a consequence of prolonged cell cycle arrest,¹⁷ suggesting a link between these two well-established Vpr activities.

The incorporation of Vpr into virions and its association with the reverse transcription complex and PIC postentry have provided the basis for useful tools in the study of entry and postentry events. GFP-Vpr fusion proteins have allowed investigators to track the movement of reverse transcription complexes early after infection, revealing that these complexes use dynein and the microtubule network to traffic toward the nucleus.⁵¹² The incorporation of a Vpr- β -lactamase chimera into virions and the subsequent delivery of the enzymatic activity of β -lactamase into target cells upon virion-cell fusion have provided a sensitive virus entry assay.¹¹⁰

Vpx

Members of the HIV-2/SIV_{sm}/SIV_{mac} lineage of primate lentiviruses carry an additional gene known as *vpx*. The *vpr* and *vpx* genes are highly related and it has been proposed that *vpx* likely arose by acquisition of the SIV_{agm} *vpr* gene by non-homologous⁶⁹¹ or homologous⁷⁵⁷ recombination.

Like Vpr, Vpx is incorporated at high levels into virus particles, also via an interaction with the C-terminus of Gag.^{390,818}

Interestingly, in the HIV-2/SIV_{sm}/SIV_{mac} lineage, two of the major functions of HIV-1 Vpr seem to be divided between Vpx and Vpr: Vpx is required for efficient infection of nondividing cells,⁸⁴⁰ whereas Vpr induces cell cycle arrest but has no role in nuclear targeting.²²³

The ability of Vpx to promote infection of nondividing cells (e.g., monocyte-derived macrophages and dendritic cells) is thought to result not from its karyophilic properties but rather from its ability to counteract a host cell restriction factor that blocks reverse transcription in these cell types. Vpx can elicit this function *in trans*; treating macrophages or dendritic cells with Vpx-containing virions can suppress the block to subsequent infection by Vpx-deleted SIV_{sm} or HIV-2.²⁷⁶ Likewise, as mentioned earlier, HIV-1 is known to infect dendritic cells and monocytes inefficiently. Again, this block can be reversed by prior treatment with Vpx-containing SIV_{mac}.^{276,382,486} The ability of Vpx to promote infection of dendritic cells and macrophages *in trans* extends to viruses as divergent as MLV.³⁸² This Vpx activity involves engagement of the DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex discussed earlier in the context of Vpr activity.^{690,716}

It was recently reported by several groups^{46,328,418} that SAMHD1 is a strong candidate for the host factor in myeloid cells (e.g., macrophages and dendritic cells) that is counteracted by Vpx. SAMHD1 is an interferon-induced protein that is defective in some patients with Aicardi-Goutières syndrome, an autoimmune disorder that resembles congenital viral infection and is associated with inflammation and impaired breakdown of cytosolic nucleic acids.⁶²⁵ Vpx delivered into the monocytic cell line THP-1 bound SAMHD1⁴¹⁸ and SAMHD1 was recovered in association with both Vpx and with Vpx-DCAF1/DDB1/CUL4 complexes in 293T cells but not with DCAF1/DDB1/CUL4 complexes lacking Vpx.³²⁸ Delivery of WT Vpx into primary macrophages or THP-1 cells induced the degradation of SAMHD1.^{328,418} Importantly, a Vpx mutant unable to associate with the DCAF1/DDB1/CUL4 complex did not promote SAMHD1 degradation and SAMHD1-mediated degradation was blocked by proteasomal inhibitors, consistent with Vpx serving as an adaptor to target SAMHD1 for proteasomal degradation via the DCAF1/DDB1/CUL4 complex. SAMHD1 depletion in primary MDMs and MDDCs enhanced infection by single-cycle reporter virus by increasing viral cDNA synthesis.^{46,328,418} PBMCs from patients with Aicardi-Goutières syndrome are highly susceptible to a spreading HIV-1 infection even in the absence of activation.⁴⁶

Further studies will be required to decipher the mechanism by which SAMHD1 blocks efficient reverse transcription in myeloid cells. It may be relevant that Aicardi-Goutières syndrome is associated not only with mutations in SAMHD1 but also with defective alleles for two nucleases—TREX1 and RNase H2.¹⁵³ Thus, SAMHD1 may function in regulating levels of cytosolic nucleic acids and as a consequence of this activity may reduce levels of incoming viral DNA. Alternatively, SAMHD1 has been shown to possess deoxyguanosine triphosphate (dGTP)-regulated deoxynucleotide triphosphohydrolase activity,^{267,615} leading to the suggestion that this protein might reduce dNTP pools in cells in which it is highly expressed (e.g., dendritic cells), thus limiting the efficiency of reverse transcription early postinfection.

It has been reported that the inability of HIV-1 to infect immature dendritic cells, likely a consequence of SAMHD1

expression, prevents the activation of antiviral innate immunity. Bypassing the block to HIV-1 infection in this cell type with Vpx stimulates HIV-1 infection, leading to dendritic cell maturation and induction of an antiviral type 1 interferon response.⁴⁸⁶ This response requires *de novo* synthesis of Gag and induction of the transcription factor interferon regulatory factor 3 (IRF3). The fact that HIV-1 does not trigger this dendritic cell-mediated innate antiviral response may contribute to the inability of humans to exert long-term immunologic control of HIV-1 replication.⁴⁸⁶ Thus, the absence of a *vpx* gene in HIV-1 may contribute to HIV-1 pathogenesis.

Vpu

A distinguishing feature of the HIV-1 lineage of primate lentiviruses is the presence of the *vpu* gene (see Fig. 49.6); it is absent from HIV-2 and from nearly all SIVs examined to date. Vpu is a multimeric, 81-amino acid, integral membrane phosphoprotein containing an N-terminal transmembrane anchor sequence and an approximately 50-amino acid cytoplasmic domain. Three α -helical domains have been predicted: one in the transmembrane sequence (residues 6 to 29), the others in the cytoplasmic domain (residues 32 to 51 and 57 to 72).⁸¹⁴ The sites of phosphorylation have been mapped to two Ser residues at positions 52 and 56.⁶⁸⁰ Vpu is translated from a *vpu-env* bicistronic mRNA and is synthesized at intracellular levels comparable to those of Gag in virus-infected cells. Vpu has not been detected in virus particles. Two principal functions for Vpu have been described: enhancement of virus release and degradation of CD4.

Early studies observed that HIV-1 clones lacking a functional *vpu* gene displayed a pronounced defect in virus particle production.^{681,725,726,749} The absence of Vpu had no discernible effect on the processing or transport of Gag proteins but caused apparently mature, fully budded virus particles to be retained at the plasma membrane and in intracellular vesicles.³⁹⁹ It is important to note that the Vpu-minus particle release defect is distinct from that induced by late-domain mutation; in the former case, the virions have completed the budding and maturation process but remain tethered to the cell surface; in the latter case, the particles fail to undergo membrane fission and remain morphologically immature (see Fig. 49.33). Until 2008, the mechanism by which Vpu enhances particle release remained poorly understood, though it was noted that the ability of Vpu to promote virus release is cell type dependent. Its effect on virus release is observed in so-called Vpu-restrictive cells, which include some human T-cell lines, primary CD4⁺ T lymphocytes and MDMs, and epithelial cell lines like HeLa. In contrast, particle production in Vpu-permissive cells like 293T and in simian cell lines such as COS and CV-1 occurs efficiently even in the absence of Vpu. The observation that human-simian cell heterokaryons behave like the parental simian cells led to the hypothesis that Vpu overcomes a cellular inhibitor of virus release that is expressed in some human cells.⁷⁷² It was also observed that Vpu can increase virus production of highly divergent retroviruses (e.g., MLV, visna, and HIV-2), even though these viruses do not carry a *vpu* gene,²⁷⁴ and that interferon treatment can convert a Vpu-permissive cell type to one that is Vpu restrictive.⁵⁵⁷

In 2008, two groups independently reported that the host cell factor counteracted by Vpu is an interferon-inducible protein known as CD317 or BST-2 (bone marrow stromal cell

antigen 2) and subsequently renamed tetherin.^{558,765} Vpu-permissive cells were shown to express low levels of tetherin, whereas Vpu-restrictive cells expressed high levels. Ectopic expression of tetherin in Vpu-permissive cells was demonstrated to block the release of Vpu(−) but not that of Vpu(+) HIV-1. Conversely, siRNA-mediated depletion of tetherin from Vpu-restrictive cells converted these cells to a Vpu-permissive phenotype.

Tetherin is proposed to have an unusual topology in the plasma membrane, with an N-terminal cytoplasmic domain, a transmembrane region, an extracellular coiled-coil motif, and a C-terminal glycosyl phosphatidylinositol (GPI) anchor that directs tetherin localization to lipid rafts⁴¹³ (Fig. 49.36). The extracellular domain of tetherin is glycosylated and includes three Cys residues that are involved in intermolecular disulfide cross-linking.⁵⁶⁹ X-ray crystallography studies demonstrate that the tetherin ectodomain forms an extended dimeric coiled-coil.^{679,827} Tetherin proteins from nonhuman species (e.g., rodents and nonhuman primates) retain the ability to restrict HIV-1 release but cannot be counteracted by Vpu. The key coding differences that differentiate human from nonhuman tetherins in terms of their ability to be counteracted by Vpu map primarily to the transmembrane domain, and it has been demonstrated that the transmembrane domain of human tetherin interacts directly with Vpu.^{350,638} Remarkably, an artificial tetherin composed of foreign transmembrane, external coiled-coil, and GPI anchor domains retains the ability to restrict

HIV-1 release. As expected, this artificial tetherin cannot be counteracted by Vpu.⁵⁹⁴

The mechanism by which Vpu counteracts human tetherin to stimulate virus release has not been fully elucidated. It is clear that under some circumstances Vpu expression induces tetherin removal from the cell surface and its degradation by proteasomal and/or lysosomal pathways. However, under other conditions, Vpu can disrupt the ability of tetherin to retain HIV-1 particles on the cell surface without inducing any apparent reduction in tetherin levels. Furthermore, a tetherin mutant that is refractory to Vpu-mediated degradation can still be counteracted by Vpu.^{264,526,781} These observations indicate that Vpu may counteract the antiviral activity of tetherin by removing it from the site of budding without necessarily inducing its degradation. Recent data suggest that Vpu does not alter the rate of tetherin internalization but diminishes its recycling, inhibits its transport to the cell surface, and traps it in the *trans*-Golgi network.⁶⁷⁴

As mentioned previously, early studies observed that Vpu is able to stimulate the release of retroviruses that are distantly related to HIV-1 (e.g., the gammaretrovirus MLV),²⁷⁴ suggesting that tetherin may possess a broad ability to restrict virus release. Indeed, tetherin has been shown to tether diverse enveloped viruses to the cell surface; these include many genera of *Retroviridae* (e.g., HIV-2, HTLV-1, MLV, EIAV, FIV, M-PMV, PFV) and nonretroviral enveloped viruses (e.g., filoviruses, arenaviruses, herpesviruses).^{193,368,653} Several of these viruses have, in turn, evolved mechanisms to counteract

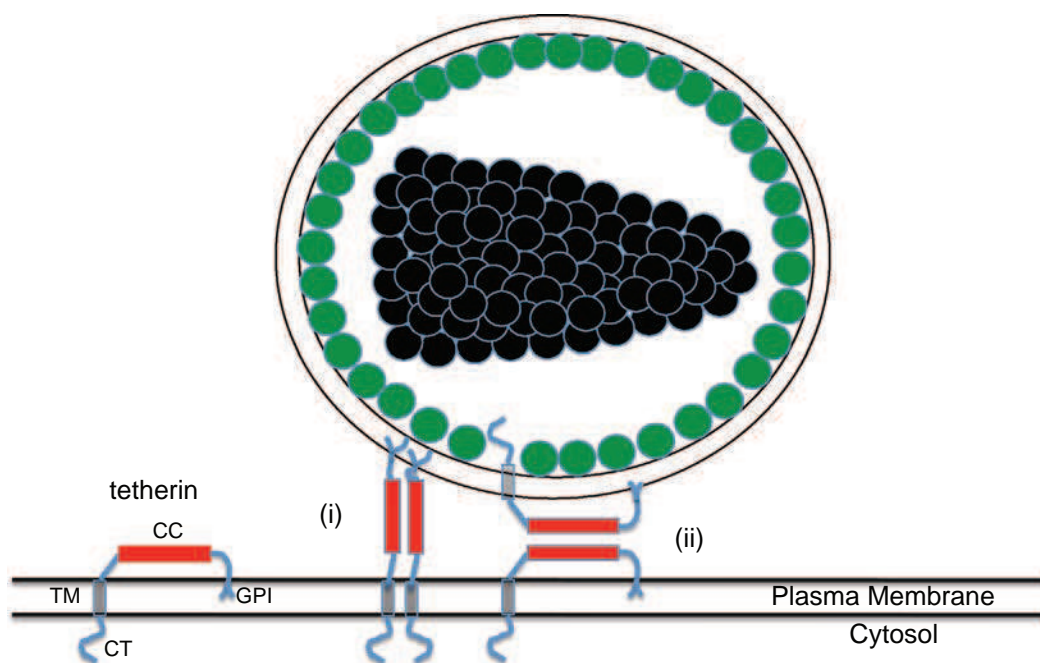


FIGURE 49.36. Hypothetical models for the tethering of human immunodeficiency virus 1 (HIV-1) virions to the cell surface by tetherin (CD317/BST-2). The tethered virion is at the **top**, with matrix (MA) in green and the capsid core in black. Tetherin is shown on the **left** anchored in the lipid bilayer of the plasma membrane, with the cytoplasmic tail (CT), transmembrane (TM), and coiled-coil (CC) domains and the putative glycosyl phosphatidylinositol (GPI) anchor indicated, with the CC domain highlighted in red. In model **(i)**, two molecules of tetherin are oriented in parallel, with the TM domains embedded in the plasma membrane and the GPI anchors in the viral membrane. In model **(ii)**, one molecule is embedded in the viral membrane, the other in the plasma membrane, with the two molecules associating via their CC domains. (Adapted from Adamson CS, Freed EO. Novel approaches to inhibiting HIV-1 replication. *Antiviral Res* 2010;85:119–141.)

tetherin restriction (for review see¹⁹³). As detailed earlier, HIV-1 uses Vpu to neutralize tetherin. Some strains of HIV-2 and SIV have evolved a mechanism whereby the Env glycoprotein induces the sequestration of tetherin in an intracellular, perinuclear compartment.²⁸⁷ The glycoprotein of Ebola virus (a filovirus) has also been reported to possess antitetherin activity.³⁷³ A number of SIVs use Nef to antagonize tetherin.^{360,848} The ability of SIV Nef to counteract tetherin is primate species specific, with critical determinants for Nef antitetherin activity mapping to the N-terminal, cytoplasmic domain of tetherin. Kaposi's sarcoma-associated herpes virus (KSHV) encodes a protein known as K5—a RING finger family ubiquitin ligase—that induces the ubiquitylation of a Lys residue in the cytoplasmic domain of tetherin and, consequently, its ESCRT-dependent lysosomal degradation.^{492,582} It is clear from the diverse strategies that enveloped viruses have evolved to counteract tetherin that this protein represents a potent and broadly acting component of the host innate immune response. Although tetherin clearly restricts the release of enveloped viruses, the role of tetherin in cell–cell viral transfer remains to be defined. Some studies have reported that tetherin restricts cell–cell HIV-1 transfer,^{108,411} whereas others concluded that tetherin actually promotes cell–cell transmission.³⁶⁴ Considering the central role of cell–cell transfer in retroviral biology, this will be an important issue to resolve.

Sequence analysis of tetherin coding regions from across a number of primate species has revealed that this gene is undergoing rapid positive selection, likely in response to lentiviral infections.⁵¹⁴ Most notably, a motif in the cytoplasmic domain of tetherin that is required for Nef's antitetherin activity is deleted in humans,⁴⁵³ rendering human tetherin resistant to antagonism by HIV-1 Nef. As discussed earlier in this chapter, HIV-1 infection in humans is thought to have originated from cross-species chimpanzee-to-human transmission of SIV_{cpz}.²⁵² Although SIV_{cpz} does encode Vpu, the SIV_{cpz} protein does not possess antitetherin activity.⁶⁶³ It therefore appears that Vpu acquired the ability to counteract tetherin after transmission of SIV_{cpz} from chimpanzees to humans.

The second main function of Vpu—CD4 degradation—has been characterized in some detail. Vpu binds directly to the cytoplasmic tail of CD4 in the ER; residues in the cytoplasmic domain of Vpu, and Vpu phosphorylation, are critical for this process. Recent findings support a model whereby Vpu mediates the degradation of CD4 by two distinct mechanisms: retention in the ER and degradation by an ER-associated degradation (ERAD)-like pathway.⁴⁷⁸ Vpu simultaneously interacts with CD4 and the human β -transducin repeat-containing protein (h- β TrCP) subunit of the SCF (Skp–Cullin 1–F-box) ubiquitin ligase complex. CD4 is then targeted for ubiquitin-mediated proteasomal degradation.⁴⁹³ The degradation of CD4 by Vpu liberates the Env glycoprotein precursor gp160 from its interaction with CD4, thereby enabling Env to continue its transport to the cell surface.⁸⁰³

In addition to counteracting tetherin and inducing the degradation of CD4, Vpu has also been reported to down-regulate CD1d and the NTB-A receptor from the cell surface.^{530,688}

Nef

The *nef* gene, present only in primate lentiviruses, overlaps approximately one-half of the 3' LTR (see Fig. 49.6) and encodes a ~25- to 34-kD membrane-associated phosphoprotein that is incorporated into virions and associates with

the viral core. Membrane binding, mediated by a covalently attached myristic acid moiety and a cluster of N-terminal basic residues,⁷⁹⁶ is critical for most Nef functions. Nef is synthesized at high levels from the first multiply spliced mRNA transcript detected postinfection. Although Nef was originally labeled a “negative factor” because of reports that it down-regulated virus replication by suppressing transcription from the HIV LTR, it is now clear that in cultured cells Nef expression either has no effect on virus spread or modestly enhances replication kinetics. Positive effects of Nef on virus replication in culture are most pronounced when target cells are not highly activated. *In vivo*, Nef appears to be a key contributor to sustained lentiviral replication and pathogenesis.

Nef is composed of several structural domains: an N-terminal “anchor” domain, a “core” domain, and a flexible C-terminal domain. The NMR structure of the core domain was determined in the mid-1990s,^{284,285} and x-ray crystal or NMR structures of the core domain were solved for the protein in a complex with Src homology 3 (SH3) domains^{23,431} or with the cytoplasmic tail of CD4.²⁸⁶ The Nef core contains an acidic cluster, a polyproline domain, and a dileucine motif important for the protein's interactions with cellular factors. Recently the structure of full-length Nef in a complex with the regulatory domain of the Src-family kinase Hck was solved by NMR.³⁷²

Deletion of the *nef* gene has a profound effect in the SIV/rhesus macaque animal model. Monkeys inoculated with *nef*-deleted mutants develop high-level antibody responses and are unable to sustain the SIV infection *in vivo*³⁸⁹; these results indicate that Nef plays a critical role in initiating and sustaining SIV infection *in vivo*. Nef has also been proposed to play a major role in pathogenesis in HIV-1-infected humans. This hypothesis stems in part from the findings of studies that show that patients infected with virus isolates harboring Nef mutations progress to disease very slowly.^{175,398} Nef-minus SIV has been used as an attenuated vaccine in the macaque model.¹⁵⁸ Nef potentiation of lentiviral replication *in vivo* is thought to result primarily from its ability to activate T cells and facilitate immune evasion.²⁰ However, because of its pleiotropic properties and multitude of interaction partners, the role of Nef in HIV/SIV pathogenesis remains incompletely understood.

A number of functions for HIV-1 and SIV Nef have been reported: (a) down-regulation of a wide array of cell surface molecules, including CD4, MHC-I, CD8, the CD3 T-cell receptor complex, and the co-stimulatory molecule CD28; (b) enhancement of virus infectivity; and (c) modulation of cellular activation and signaling pathways. Many of these functions appear to be linked to a generalized disruption of endosomal trafficking induced by Nef. The contribution of these diverse activities to the role of Nef in maintaining high viral loads *in vivo* remains to be fully understood.

Like the HIV-1 Env and Vpu proteins, Nef down-regulates cell surface expression of CD4. Nef-induced CD4 down-regulation proceeds in a step-wise fashion: (a) Nef interacts with the cytoplasmic domain of CD4⁹ and then associates with the clathrin adaptor protein complex AP-2 in clathrin-coated pits; this ternary CD4–Nef–AP-2 complex is then internalized.^{116,455} (b) Nef induces the ESCRT-dependent sorting of internalized CD4 to the MVB and, ultimately, to the lysosome for degradation.¹⁶⁴ Alternatively, it has been reported that in the endosome, Nef connects CD4 with the β -subunit of COPI coatomers (β -COP), thereby targeting CD4 for degradation in the

lysosome.^{41,600,668} In either case, Nef induces both CD4 internalization from the cell surface and sorting to the lysosome. CD4 down-regulation by Nef helps limit superinfection and may mitigate inhibitory effects of high CD4 expression on virus release and Env incorporation.^{420,643}

In addition to reducing cell surface levels of CD4, Nef down-regulates the expression of MHC-I and other surface molecules.⁶⁸⁴ The mechanism by which Nef down-regulates MHC-I is distinct from that used to reduce CD4 expression and is less well understood. Nef-induced MHC-I down-regulation reportedly involves the formation of a Nef-MHC-I complex and AP-1-dependent redirection of MHC-I from the *trans*-Golgi network to lysosomes.^{278,377,428,565,635} Redirection of MHC-I to the lysosome by Nef may also involve β -COP, but it has been suggested that Nef uses a different binding site on β -COP for MHC-I down-regulation than used for CD4 degradation.^{439,668} Some evidence supports an alternative model whereby Nef reduces surface expression of MHC-I by stimulating its internalization from the plasma membrane via an ARF6-dependent pathway.⁶⁶ MHC-I down-regulation blocks proper antigen presentation—thereby limiting the ability of cytotoxic T lymphocytes to recognize and eliminate virus-infected cells—thus providing HIV and SIV with a mechanism for partially evading at least one component of the host immune response.^{147,443,735} Removing MHC-I molecules from the cell surface exposes infected cells to attack by NK cells; HIV-1 may avoid this latter problem by not down-regulating those class I molecules (human leukocyte antigen-C [HLA-C] and HLA-E) responsible for protection from NK cell lysis.¹⁴⁵ Nef also down-regulates surface expression of CD3 and CD28, among others,^{39,736} but the mechanisms responsible for this down-regulation are less well understood than for CD4 and MHC-I. As alluded to earlier, in many cases the modulation of surface protein expression by Nef relies on its ability to serve as an adaptor between these proteins and cellular AP complexes.

In single-cycle assays, Nef modestly stimulates virus infectivity.^{130,266,524,715} This function appears to be manifested at an early postentry step in the virus life cycle, because *nef*-deleted mutants display no defect in virion-cell fusion⁷⁵⁴ yet fail to efficiently reverse transcribe their genomes after entry.^{10,129,683} At this time, the mechanism by which Nef enhances virus infectivity remains to be elucidated. Interestingly, the infectivity defect observed with *nef*⁻ virus can be partially suppressed by pseudotyping with VSV-G.⁸ The role Nef plays early postentry is therefore influenced by the route of virus entry, because VSV-G directs entry via fusion in a low-pH endosome after endocytosis, whereas HIV-1 Env is thought to primarily mediate entry via direct fusion at the plasma membrane. It has been reported that Nef stimulates cholesterol biosynthesis and increases cholesterol and ganglioside transport to rafts and virions,^{855,856} perhaps in some cell types by impairing cholesterol efflux mediated by the adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1).⁵⁴⁴ The resulting Nef-mediated increase in virion cholesterol could promote virus infection; however, this remains to be demonstrated and the relevance of these observations is unclear.^{214,708} Because Nef is a core-associated protein, it may modulate uncoating or regulate trafficking of the core early postentry, perhaps by enhancing the ability of the incoming core to penetrate the cortical actin network located beneath the plasma membrane.⁹⁹ Interestingly, HIV-1 Nef interacts with dynamin 2, and transfection

of dominant-negative dynamin 2, or dynamin 2 siRNA, in the virus-producing cell blocks the ability of Nef to enhance particle infectivity.⁶⁰² The mechanistic basis for these observations remains to be understood.

A number of studies have shown that HIV and SIV Nef influence cellular signal transduction pathways and affect the activation state of Nef-expressing cells (for review see²²⁵). The effect of Nef in potentiating T-cell activation is due in part to its interaction with the cellular Ser/Thr kinase p21-activated kinase 2 (PAK2). This interaction reportedly occurs in lipid rafts and results in the recruitment of Nef to the immunologic synapse.^{214,408} The co-localization of Nef and PAK2 at the immunologic synapse may play an important role in up-regulating the expression of the transcription factor NFAT (for nuclear factor of activated T cell),²¹⁴ which in turn leads to T-cell activation, as described earlier in the LTR section. Indeed, Nef has been reported to induce expression of a number of genes whose products positively regulate HIV replication.⁷⁰¹ Another reported consequence of the Nef-PAK2 interaction is the phosphorylation-dependent inactivation of cofilin, an actin-depolymerizing factor. Inactivation of cofilin by this route inhibits T-cell chemotaxis and motility, potentially enhancing immune evasion of HIV-1-infected cells.⁷²¹

As mentioned earlier, HIV and SIV Nef contain a highly conserved consensus binding site for the SH3 domain of Src-family Tyr kinases (Pro-X-X-Pro). Nef has been reported to bind several of these kinases (e.g., Lyn, Hck, and Lck) and modulate their catalytic activities. Because cellular kinases are intimately involved in signaling pathways, such interactions may play a critical role in Nef function. In addition to direct effects of Nef on stimulating T-cell activation, Nef expression in macrophages reportedly induces the expression and release of the β -chemokines MIP-1 α and MIP-1 β , which in turn activate resting T lymphocytes.⁷³⁸ Furthermore, Nef-expressing macrophages release factors that act on B cells to increase the sensitivity of resting T lymphocytes to HIV-1 infection.⁷³⁷ Such paracrine pathways could help explain the positive effect of Nef on HIV-1 replication *in vivo*. Nef expression also triggers the release of exosomes that contain factors including Nef itself; it has been suggested that this Nef-induced exocytosis may play an important role in Nef function.^{37,436,548}

Although it is well established that Nef is crucial to HIV and SIV pathogenesis, it remains to be determined which of the many functions attributed to Nef in culture are required for its ability to potentiate efficient replication and disease progression *in vivo*. Studies have been performed with SIV Nef mutants in an attempt to define which activities of Nef are required for the maintenance of high viral loads in infected animals. Results of these studies have suggested that mutations that interfere with the ability of Nef to down-regulate CD4, MHC-I, CD3, and CD28 attenuate virus replication *in vivo*.^{343,545,672} Mutations that disrupt the ability of Nef to enhance virus infectivity *in vitro* are also detrimental to virus replication in monkeys.³⁴³ Results are less clear for Nef-mediated activation of PAK2. This function of Nef either may not be required *in vivo* or may be required only late in disease progression.^{343,664} Attempts to establish the *in vivo* significance of the many reported activities of HIV-1 Nef are complicated by the fact that individual mutations often disrupt multiple Nef activities and by differences in the functional determinants of HIV-1 versus SIV Nef. However, understanding the differences between the Nef proteins

from SIV (which are often nonpathogenic in the natural hosts) and HIV-1 (which is highly pathogenic) may provide important clues to Nef function. For example, it has been suggested that the ability of SIV but not HIV-1 to down-regulate CD3 may correlate with the different pathogenic outcomes of infection by these viruses.⁶⁷³

As discussed earlier in the section on Vpu, the Nef proteins of many SIVs possess the ability to antagonize the host restriction factor tetherin.^{360,663,848} The capacity of Nef to counteract tetherin involves the removal of the host protein from sites of budding on the plasma membrane and requires domains of Nef that interact with AP-2.⁸⁴⁷ Thus, tetherin represents yet another host cell factor that is down-regulated by Nef through interaction with cellular clathrin adapter protein complexes.

PERSPECTIVES

Despite its relatively recent emergence as a major human pathogen, HIV-1 has become one of the most intensively studied and best-understood viruses. As this chapter illustrates, an enormous amount of information has been obtained regarding the molecular, biochemical, biological, and structural details of all aspects of the HIV-1 replication cycle. Recent breakthroughs in our understanding of how lentiviruses parasitize host cell machinery to promote virus replication and, conversely, how the host cell has evolved to specifically restrict lentivirus infection have provided remarkable insights into the dynamic nature of this complex host–pathogen interaction. Although the wealth of information available on HIV-1 and related lentiviruses has led to the development of several classes of antiretroviral drugs that effectively suppress HIV-1 replication in infected patients, controlling the AIDS epidemic remains an elusive goal, particularly in developing nations. Future progress in HIV research will undoubtedly lead to more effective approaches for controlling HIV-1 and will continue to drive advances in virology, immunology, and cellular, molecular, and structural biology.

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HIV-1: Pathogenesis, Clinical Manifestations, and Treatment

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against a variety of normally innocuous agents that become the major sources of morbidity and mortality. Over the 30 years since the original identification of a new syndrome that was ultimately shown to be caused by HIV,⁵⁵ the infection has become a grave humanitarian crisis with far ranging impact on international political and economic stability, and contributes greatly to global health inequalities in terms of access to life-saving therapies.

EPIDEMIOLOGY

The initial clues to the emerging HIV epidemic came from two linked observations in 1980–1981. One was from front-line physicians, predominantly in New York and California, who were confronted with previously healthy young men presenting with symptoms of profound unexplained immune deficiency. The other was at the Centers for Disease Control and Prevention (CDC) in Atlanta, which noted a marked increase in the number of requests for pentamidine, a drug used to treat an infection associated with severe immunodeficiency. The link between these observations was the sudden upsurge in cases of *Pneumocystis jirovecii* (formerly *P. carinii*) pneumonia being diagnosed in young gay men, an opportunistic infection previously seen in the setting of immune suppression accompanying cancer chemotherapy, but now, for the first time, appearing in persons with no obvious disposition to develop immune deficiency.

By 1984, the viral etiology of what became known as HIV type 1 (HIV-1) infection had been confirmed through the isolation of a lymphotropic retrovirus from infected persons, and the detection of antibodies in persons at risk.^{22,127,322} It was clear that certain groups were particularly at risk, including gay men, injection drug users, recipients of blood transfusions, and hemophiliacs, and that the mechanisms of transmission began to be defined.^{81,189} By April 1985, a blood test for detection of antibodies was licensed, and the extent of the epidemic began to unfold. Since the initial detection of the epidemic in the United States and Europe, it has grown to involve an estimated 60 million persons worldwide, with 5 million new infections per year, with epidemics expanding in eastern Europe, south Asia, and China³¹⁷ (Fig. 50.1). In Southern Africa, less than 1% of the population was infected in 1990, when large scale surveillance programs were initiated, whereas by 2004 antenatal screening surveys detected a prevalence well in excess of 30% in some areas.³⁰⁸

Origin of HIV

The global epidemic of HIV-1 is the result of a cross-species infection of humans by a chimpanzee lentivirus, simian

Human immunodeficiency virus (HIV) is essentially an infection of the immune system. The main clinical manifestation of infection is the progressive and ultimately profound defect in cell-mediated immune responses that are essential for protection

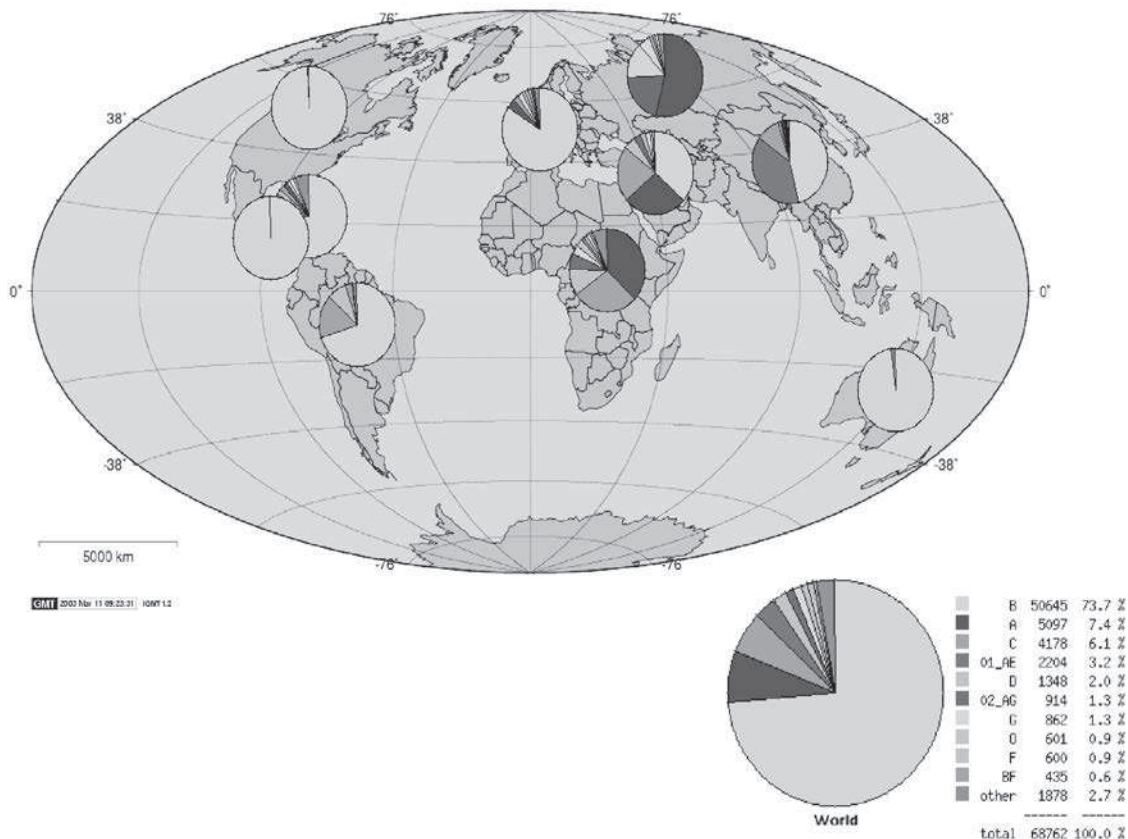


FIGURE 50.1. Global subtype distribution of human immunodeficiency virus type 1 (HIV-1) clades as reported in the Los Alamos Database.

immunodeficiency virus (SIV_{cpz}), which occurred in west central Africa.¹³¹ Chimpanzees, in turn, appear to have acquired SIV_{cpz} sometime after their divergence into multiple subspecies. *Pan troglodytes* and *P.t. schweinfurthii* are naturally infected, whereas other closely related species are not, suggesting that SIV_{cpz} is unlikely to have coevolved with its natural host.³⁶⁶ How the virus initially arose as a new virus in chimpanzees remains unclear, but it likely occurred following transmission of two other ancestral SIV species from monkeys,³⁶⁶ perhaps because chimpanzees hunt monkeys for food. Of note, SIV_{cpz} is not an asymptomatic infection in chimpanzees; the epidemiologic evidence documents decreased survival in chimpanzee troops infected by SIV_{cpz}.²⁰⁹ In contrast, other SIV infections appear to be largely asymptomatic in their natural monkey hosts (i.e., SIV_{smm} in sooty mangabeys) and do not lead to immune deficiency or decreased life span. Experimental transmission of these viruses to susceptible non-natural hosts can result in progressive and profound immunodeficiency and acquired immunodeficiency disease (AIDS).³⁵⁴ In fact, the transmission of SIV_{smm} in West Africa is the origin of the HIV-2 epidemic in humans.³⁵⁴ Although the reasons for differences in transmissibility and pathogenicity during cross-species transmission are not fully understood, it is clear that innate host defenses such as those imparted by apolipoprotein B messenger RNA (mRNA)-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), and tripartite motif 5 alpha (TRIM-5α) play a role and create barriers that viruses must overcome in order to

become established and survive within a new host species (see Chapter 49).

At least three distinct cross-species transmissions from chimpanzees to humans are thought to have occurred, giving rise to three highly divergent genetic lineages of infection, termed groups M, N, and O.^{158,302,372} Group M is by far the most widespread, accounting for most cases globally. Because the greatest genetic diversity in group M is found in Kinshasa, Zaire, the epidemic likely began in this region of Africa. The approximate date of introduction of the M group into the human population is estimated to be around 1931, based on supercomputer analysis of large numbers of sequenced HIV isolates, assuming a constant rate of evolution.²¹⁸ This approach has been validated by successfully timing the earliest historic isolate, which was sequenced from a stored plasma sample obtained in 1959 from a person who died of AIDS in Manchester, England.⁷⁷

Current Global Distribution

The most heavily affected continent at the present time is Africa, with an estimated 25 million persons currently infected with HIV, and at least 3 million with advanced disease in need of treatment. HIV infections have increased markedly over the last decade, despite knowledge of the mechanisms of transmission. Expanding epidemics in Eastern Europe, South Asia, and China may ultimately eclipse the African epidemic in terms of sheer numbers of infected persons. The viruses fueling these epidemics vary according to geographic region, with clade C

TABLE 50.1 Risk of Transmission of HIV-1**Sexual transmission**

Female-to-male transmission.....	1 in 700 to 1 in 3,000
Male-to-female transmission.....	1 in 200 to 1 in 2,000
Male-to-male transmission.....	1 in 10 to 1 in 1,600
Fellatio.....	0 (CDC) or 6% (SF)

Parenteral transmission

Transfusion of infected blood.....	95 in 100
Needle sharing.....	1 in 150
Needle stick.....	1 in 200
Needle stick /AZT PEP.....	1 in 10,000

Transmission from mother to infant

Without AZT treatment.....	1 in 4
With AZT treatment.....	Less than 1 in 10

CDC, Centers for Disease Control and Prevention; SF, San Francisco; AZT, zidovudine; PEP, post-exposure prophylaxis.

Adapted from Royce RA, Sena A, Cates W, et al. Sexual transmission of HIV. *N Engl J Med* 1997;336:1072–1078.

virus being the most prevalent worldwide and clade B being currently the most prevalent in the United States and Europe. Clades differ one from another by up to 35%; within a single clade variation between isolates can be as great as 20%. Mistakes in reverse transcription, which occur because the viral reverse transcriptase lacks an editing function, leads to variability of individual virus sequences within a single infected person that can exceed what is seen with other viruses (e.g., influenza) over the course of a global epidemic.¹³⁵

Mechanisms of Transmission

Most cases of HIV infection worldwide are the result of sexual transmission, but important modes of transmission also include parenteral transmission and transmission from mother to infant. The actual risk of transmission per exposure varies widely (Table 50.1). For sexual transmission, the risk of male to male transmission is greater than the risk of heterosexual transmission, and is highest in persons practicing receptive anal intercourse.³⁴⁸ Overall, the greatest risk of transmission is from contaminated blood transfusions, with 95 of 100 persons becoming infected, although transfusion-related infections are rare now that blood is routinely screened for this pathogen.³⁶¹ The rate of transmission depends on the inoculum size delivered, but perhaps also on local mucosal defense mechanisms that modulate infection but remain to be defined. Population studies performed in Africa suggest that there is a threshold viral load (~1,500 RNA copies/mL plasma) below which the likelihood of sexual transmission is greatly reduced.^{150,325}

Mathematical modeling suggests that the published risks of sexual transmission, which come from longitudinal studies of persons in identified risk groups, are too low to account for the huge numbers of infections worldwide and for the rapid increase in infections that occur after puberty. In South Africa, for example, 4% of 16-year-old girls are HIV seropositive, whereas by age 21 this number increases to 31%.³⁰⁸ Coital frequency estimates in these same age groups fail to account for this rapid increase in infections. More recent data suggest that transmission is amplified during periods of increased genital tract viral shedding.¹²⁸ Increased shedding certainly occurs at the time of

acute infection, which has been shown through a study of more than 15,000 persons in 56 villages in Uganda to be a particularly important period for transmission and, thus, has important public health implications.⁴¹¹ An important aspect of this study was that it followed couples who were both seronegative at the time of enrollment, so it was not potentially selecting for discordant couples who might have already demonstrated some intrinsic resistance to transmission. Other factors that may amplify the risk of transmission include other co-infections (e.g., malaria and tuberculosis, sexually transmitted diseases [STDs], or in particular co-transmission of STD and HIV).⁷¹ Estimated transmission probability likely also depends on genetic factors, such as density of the HIV co-receptor CCR5 on cervical cells.⁵⁸ In addition to using epidemiologic data, much has been learned about transmission across a mucosal barrier by studying the SIV system, where it has been shown that CD4 T cells are the first cells to be infected (as opposed to antigen-presenting cells), and that there is a small temporal window of opportunity in which antiviral therapy or immune responses can block viral dissemination.^{159,433}

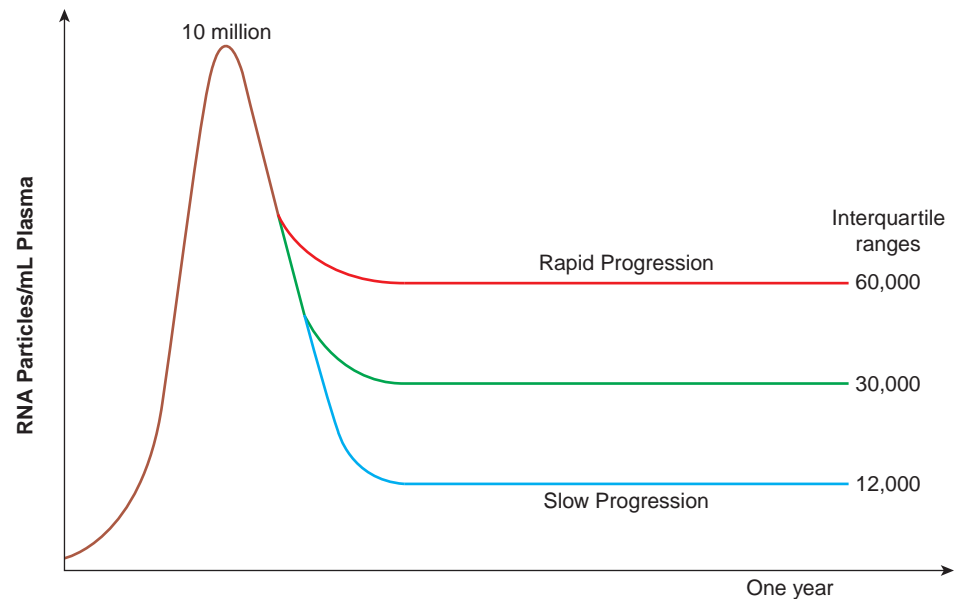
PATHOGENESIS

Human immunodeficiency virus directly infects and kills cells that are critical for effective immune responses. Through direct interaction between the viral envelope and its cellular receptor, CD4 and the chemokine coreceptor, CCR5 or CXCR4 (see Chapter 49), this virus infects key cells of the adaptive immune response, explaining the clinical manifestations of disease being profound immune suppression. The course of disease varies enormously among infected persons. The time from acute infection to the development of AIDS, defined by a CD4 cell count of less than 200 cells/ μ L or the appearance of AIDS-defining opportunistic infections or cancers, can be as rapid as 6 months.³¹⁰ Other persons have been known to be infected for more than 25 years and to maintain normal CD4 cell levels and exhibit no evidence of CD4-cell decline or immune deficiency, despite never having been treated with anti-HIV medications.²⁷¹ A precise explanation for these differences in disease development remains elusive, but increasing evidence suggests that early events at the time of acute infection, together with viral and host genetics, play a determining role in the clinical outcome of disease.

Acute Infection and CD4 Cell Depletion

Ever since the analysis of viral load in the Multicenter AIDS Cohort Study (MACS) dataset in 1996,²⁶⁷ it has been clear that the course of HIV infection can be predicted within the first 6 to 12 months based on plasma viral load, and that CD4 count at this time also independently helps to determine whose disease will progress rapidly and whose will not.²⁴⁶ The median viral load in plasma at the time of peak viremia during acute infection is approximately 10^6 to 10^7 RNA copies/mL, which drops to a mean set point of 30,000 copies/mL within the first 6 to 12 months of infection. Longitudinal studies from the MACS, which were initiated before the availability of effective antiviral therapy, revealed the interquartile ranges of viral load and the relationship to risk of disease progression (Fig. 50.2). Interestingly, at 1 year after infection, only a fivefold difference

FIGURE 50.2. Interquartile ranges of viral load within one year of acute human immunodeficiency virus (HIV) infection. (Adapted from Lyles RH, Munoz A, Yamashita TE, et al. Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. *J Infect Dis* 2000;181:872–880.)



was seen in plasma viremia between the quartile that does the best and the quartile that does the worst.²⁴⁶

Studies in both humans infected with HIV^{43,264} and the SIV macaque model^{1234,261} provide important new insights into why the period of acute infection plays such a defining role in ultimate disease progression, and why the immune system ultimately fails to control HIV in most infected persons. Infection and depletion of large numbers of both resting and activated memory CD4 cells in the gut during primary infection may be what fuels the initial peak viremia, and likely places constraints on subsequent adaptive responses^{43,264} (Fig. 50.3). Details of what is happening at a cellular level in acute infection have come from experimental infection of macaques with SIV. At the time of acute SIV infection, massive infection occurs of gut-associated lymphoid tissue, with between 30% and 60% of all gut-associated CD4 cells becoming productively infected, leading to massive depletion of these cells in only 4 days.²⁶¹ The memory cell population, including both activated and resting memory cells, seems most vulnerable.²³⁴ In the earliest stages of infection, more than half of all memory CD4+ T cells are lost, and the body is left to fight the prolonged chronic phase of infection with a severely constricted repertoire of CD4 T helper cells. These experiments in the SIV model are almost certainly representative of what is happening in acute HIV infection in humans, where the initial plasma viral load averages 1 to 10 million RNA copies/mL. Whether the subsequent decline to a steady state results from active inhibition by innate and adaptive immune responses to HIV, or a decrease in the number of cells available to support viral replication, remains an area of dispute.³¹²

Most of the CD4 T cells that are depleted from the gut during acute HIV or SIV infection are T helper cells that produce interleukin 17 (IL-17) (Th17 cells). These cells are crucial for maintaining the integrity of the mucosal barrier, and their depletion by HIV or SIV leads to loss of epithelial integrity within the gut mucosa, thereby allowing translocation of gut-associated microbial products into the systemic circulation.¹¹⁰ These products are a major driver of the systemic immune activation that is a predominant feature of all pathogenic HIV and

SIV infections.^{41,42} The loss of CD4 T cells from the gut during acute infection therefore has two effects upon the immune system: it depletes a major portion of the immune reserve in the form of memory CD4 T cells, and it allows microbial translocation, which establishes a state of chronic immune activation that further promotes systemic HIV infection and replication.

Establishment of a Latent Viral Reservoir

During the period of acute infection, a stable reservoir of HIV-infected resting memory CD4 cells is established that harbors replication-competent, integrated provirus.^{63,116} This occurs whether or not persons are started on treatment in the initial symptomatic stage of infection. Because proviruses that form this latent reservoir are not transcriptionally active, no viral proteins or enzymes are produced. These infected cells therefore are protected from the effects of antiviral drug therapy and from immune attack. This latent reservoir is present whether the viral load is high or low, and it persists even after prolonged highly active antiviral therapy.¹¹⁵ A rough estimate is that about 1 in 1×10^6 CD4 cells harbors integrated latent provirus.⁴³⁴ The half-life of infected cells is such that it would take more than 70 years to achieve viral eradication by gradual senescence of these cells even with no blips in viremia on therapy,⁴³⁴ a situation that is yet to be achieved (see below).

HOST IMMUNITY

Recovery from many human viral infections is not associated with the eradication of infection, but rather results from immune-mediated containment. For example, persons who develop herpes zoster caused by varicella zoster virus infection ultimately contain the initial infection with a potent immune response.⁹² The virus remains present for the rest of the life of the infected individual; in most persons, however, it is controlled by an effective immune response. Although HIV infection is associated with progressive and ultimately profound immune suppression, a highly variable course of disease is seen among infected persons. After more than 25 years since the humoral and cellular

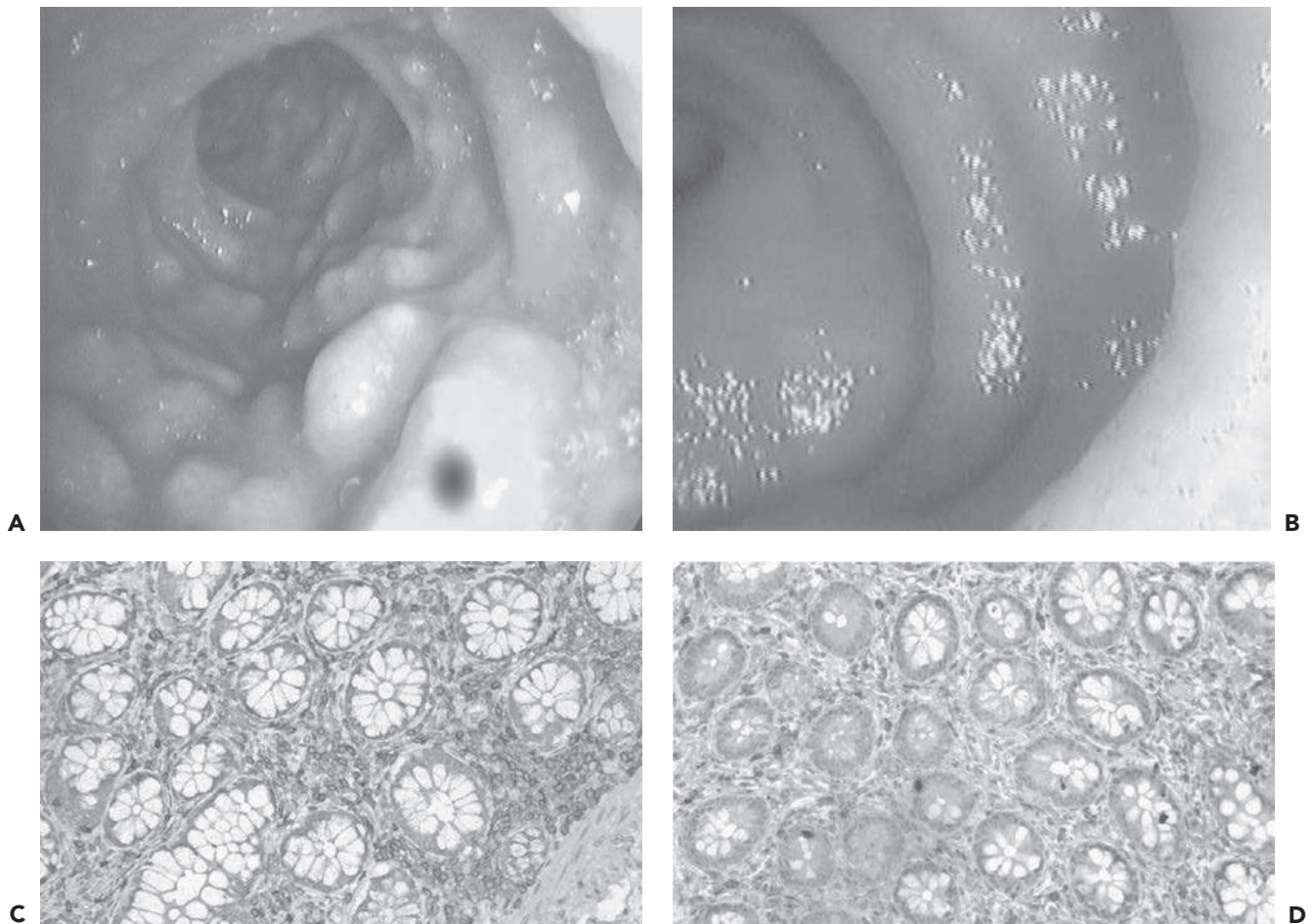


FIGURE 50.3. Appearance of the terminal ileum in human immunodeficiency virus (HIV) infection. **A:** Uninfected. **B:** HIV infected, showing dramatic loss of lymphoid tissue. **C:** Uninfected, showing numerous CD4 T cells in the lamina propria by immuno-histochemical staining. **D:** HIV infected, showing depletion of CD4 T cells in the lamina propria.

immune responses to HIV were identified, multiple correlates of immune control have been defined, but which are the cause of a better outcome, and which are the effect, remains elusive. Currently, data support contributions to disease outcome from adaptive immune responses, innate immune responses, host genetic factors, and differences in viral pathogenicity.

Innate Immune Responses

The initial immune response to HIV involves innate immune mechanisms. Toll-like receptors (TLRs) on dendritic cells (DCs) are activated by a combination of HIV-1 RNA via TLR7 and microbial products (discussed earlier) via other TLRs, resulting in high levels of interferon alpha ($\text{IFN-}\alpha$), IL-12, tumor necrosis factor alpha ($\text{TNF-}\alpha$), and IL-6, which together result in a profound activation of the immune system.^{23,42,169} These activated DCs can be infected with HIV, which may further contribute to impaired DC function in infected persons.^{245,355} Natural killer (NK) cells are also part of the initial innate immune response to HIV, and their numbers are greatly increased in acute infection. Because the increase in NK cells coincides with the initial decline in viremia after acute infection, these cells are thought to have a key role as an antiviral defense mechanism. Moreover, NK cells appear to be largely anergic in chronic infection, sug-

gesting that a pool of nonfunctional NK cells accumulates over time.⁷ The fact that expression of a killer cell inhibitory receptor, KIR3DS1, and a particular sequence in its ligand human leukocyte antigen (HLA) Bw4, is associated with slower disease progression²⁵⁷ also argues for an important role of these cells in infected persons.

Adaptive Humoral Immune Responses

HIV infection is associated with the development of antibodies that appear within weeks of initial infection. Although most of these antibodies bind, but do not neutralize,³¹⁹ a subset of these can neutralize virus, either by binding directly to the envelope glycoprotein trimer on the surface of free virions or following CD4-gp120 binding, preventing the fusion of the viral and cell membranes that is essential for viral entry.⁴⁷ The envelope is heavily glycosylated, and these sugars prevent antibody binding to the underlying peptidic structure. These neutralizing antibody responses nevertheless are sufficiently strong to influence viral evolution; indeed, the virus population *in vivo* is rapidly replaced by mutant virus that escapes recognition.^{339,412} This selection is an iterative process: new antibody responses develop to neutralize the mutant virus, which then escapes again. These responses are primarily narrowly directed

and do not appear to be able to broadly cross-neutralize many strains of HIV. During the chronic phase of infection, there is little evidence to indicate an ongoing contribution of neutralizing antibodies to immune control, as shown by depletion of antibody-generating B cells in an animal model of AIDS virus infection.³⁵⁷ Having said this, it is clear that sera from chronically infected individuals can potentially neutralize multiple strains of HIV broadly.^{27,99,235,382,409} Isolation of antibodies from these individuals has proven to be a rich source of monoclonal antibodies with broad and potent activity, and has led to the identification of potential targets of neutralizing antibodies for vaccine development.

Adaptive Cellular Immune Responses

CD8 T-Cell Responses

Adaptive immunity to viruses generally involves the rapid expansion of CD8 T cells that recognize viral proteins on the infected cell surface, presented by HLA antigen class I molecules. Recognition, via the T-cell receptor (TCR), of a foreign (nonself) protein presented as a short 8 to 10 amino acid peptide in the groove of a class I molecule can result in the directed lysis of that cell by CD8 T cells. Depending upon the kinetics of virus production, peptide presentation via class I, CD8 T-cell recognition, and the activation state of the CD8 T cell, virus-infected cells potentially can be eliminated before they are able to produce significant progeny virus.

In the early weeks following HIV infection, viral load is reduced from an average of 1×10^6 copies/mL to an average of 30,000 copies/mL.^{246,344} Coincident with this drop is the appearance of HIV-specific CD8 T cells, suggesting that these cells have an active antiviral effect^{36,221}; animal model data support this conclusion.^{194,356} When CD8 T cells are removed by administration of a CD8-specific monoclonal antibody in animals with chronic SIV infection, viral load dramatically increases, and subsequently declines again coincident with the reemergence of these cells. If CD8 T cells are depleted before acute infection in this model then viral load remains at the initially high levels.³⁵⁶ Therefore, it is clear that these cells play an ongoing role in viral containment. Because the latent viral reservoir remains invisible to these responses, CD8 T cells are unable to eradicate HIV.

The breadth and magnitude of CD8 T-cell responses can vary widely among infected persons, as measured by the ability of these cells to secrete IFN- γ following recognition of their cognate epitope. Despite animal model data showing the clear role of these cells in immune containment, other studies have failed to provide strong and consistent evidence that these responses are associated with suppression of the plasma viral load in humans. Responses have been observed to all nine HIV viral proteins and, in most persons, an average of more than a dozen viral peptides are simultaneously targeted.^{4,25} The total percent of CD8 T cells directed against HIV in the chronic phase of infection can be 25% or higher, and yet, the magnitude of these responses does not correlate with control of viremia.²⁵ One relatively consistent finding is that targeting of HIV-1 Gag over other viral proteins is associated with a lower viral load.^{326,435} Although initial animal studies suggested that targeting of proteins expressed early in the viral life cycle (e.g., Nef) might be advantageous, this finding has not been consistently borne out in larger studies in humans, although it is clear that Nef is one of the most common viral antigens targeted by the CD8 T-cell response during acute HIV infection.^{117,146} Data also indicate

that the HLA B alleles, as opposed to the A and C alleles, play the dominant role in modulating viral load,²¹³ again suggesting a key role for CD8 T cells in immune containment, but debate continues whether this is cause or effect.

Whereas these data clearly indicate that CTLs are involved in viral control, it is often difficult to determine their exact contribution in human studies. One reason for this difficulty is that both the virus and the CTL response are constantly adapting to each other over the course of the infection. Although each person has up to six different class I alleles to present viral peptides at the cell surface, only a subset of these are used in acute infection.⁶ That initial immune response is therefore narrowly directed to only a few epitopes, most of which rapidly escape, and other epitopes become targeted as the response broadens over time well after viral set point is reached.⁹ In addition, it is clear that not all CD8 T cells are the same, and based upon their different phenotypes and functions, they may have profoundly different antiviral effects.^{121,241}

Virus-Specific CD4 T Cells

The optimal production of antibodies and the function of CD8 T cells depends on the presence of virus-specific CD4 T cells.⁹² The magnitude of these responses, as measured by IFN- γ production, is consistently much lower than the CD8 T-cell responses, and a small number of peptides are recognized by most individuals.^{25,326} In contrast to the wide spectrum of responses mediated by CD8 T cells, the adaptive CD4 T-cell response is primarily directed against the Gag protein, for reasons that are unclear. In the early stage of acute infection, a robust induction of HIV-specific CD4 T cells occurs, and these cells clearly provide help to CD8 T cells, as evidenced by the ability of CD8 T cells in acute infection to proliferate *in vitro* in response to cognate epitope recognition.²³⁶ The ability of CD4 and CD8 T cells to proliferate *in vitro* is lost over the first few months of untreated infection, but the acquired defect in CD8 T-cell proliferation can be restored *in vitro* by the addition of CD4 T cells obtained at the time of acute infection or by IL-2.^{185,236} Although HIV-specific CD4 T-cell function has been augmented through therapeutic interventions (vaccination, treatment interruptions, and IL-2 therapy), none of these approaches has led to improved virologic or clinical outcomes.^{3,185,207,340,344,346}

CD4 T cells can be classified based on their major functions into multiple lineages, including T helper 1, T helper 2, T helper 17, follicular T helper, and T regulatory. Most HIV-specific CD4 T cells fall within the T helper 1 category based on their secretion of IFN- γ , but their overall function appears to be impaired.^{161,185} The fact that HIV preferentially infects and depletes HIV-specific CD4 T cells may partially explain the low frequency of HIV-specific CD4 T cells and their inability to adequately coordinate humoral and CD8 T-cell responses to HIV.¹⁰² In addition, the phenotype and function of opportunistic pathogen-specific CD4 T cells may affect their susceptibility to infection and depletion by HIV, thereby explaining some of the variation in onset of different opportunistic infections.^{52,139} Specifically, CD4 T cells that express β chemokines (which block HIV entry by binding to CCR5) are more resistant to HIV infection and depletion than those that do not.^{52,139}

Host Genetics and Viral Control

One of the strongest predictors of disease progression is the HLA type of the host. The HLA class I alleles B*27 and B*57 are associated with low viral load and prolonged asymptomatic

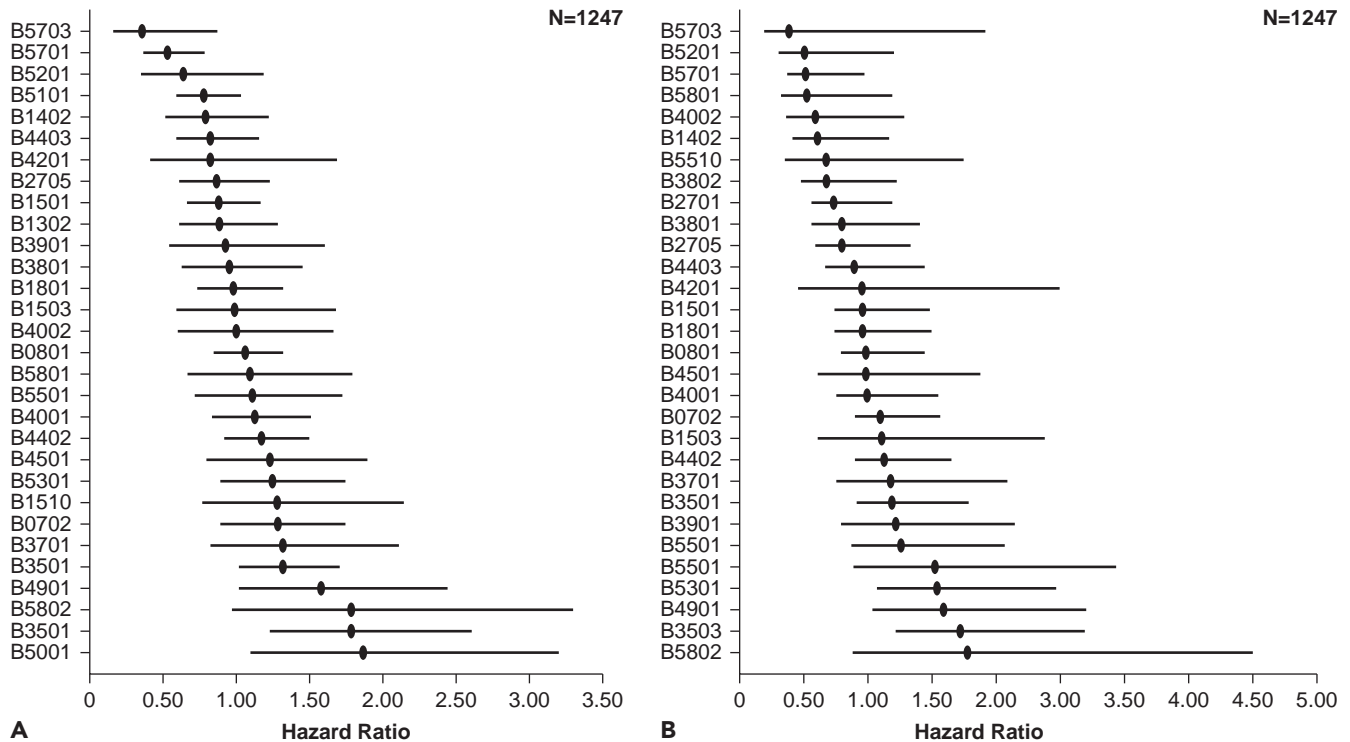


FIGURE 50.4. Relative hazard of disease progression by human leukocyte antigen (HLA) class I type. **A:** Relative hazard of progression to a CD4 count of less than 200/mm³. **B:** Relative hazard of progression to acquired immunodeficiency disease syndrome (AIDS) by the 1987 AIDS defining criteria.

infection,²⁰¹ although additional alleles have also been shown to affect viral load (Fig. 50.4). Other HLA alleles have been associated with more rapid disease progression, in particular a subtype of the HLA B35 allele referred to as *HLA B35 px*.¹³³ It is now clear that the HLA B alleles are dominant in terms of having an impact on viral load.²¹³ On a population level, clear evidence indicates viral imprinting by host CD8 T-cell responses: Persons with certain HLA alleles have a significantly increased prevalence of certain viral polymorphisms that appear to be driven by immune escape.²⁰⁸ Animal studies have shown clearly that the major histocompatibility complex (MHC) type can influence both disease progression and the relative contribution of cellular and humoral immune responses to viral control.²⁵¹ Immunization of humans with candidate HIV vaccines has also shown HLA-related differences in immunogenicity.²⁰² Remaining to be identified are the precise mechanisms whereby these alleles mediate a protective or detrimental effect, or enhance immunogenicity of candidate vaccines, or whether this represents a linkage to some other genetic factor that modulates outcome. In addition to HLA alleles, other genetic factors may also have an impact on disease progression, and are discussed below.

Reasons for Immune Failure

Functional Defects

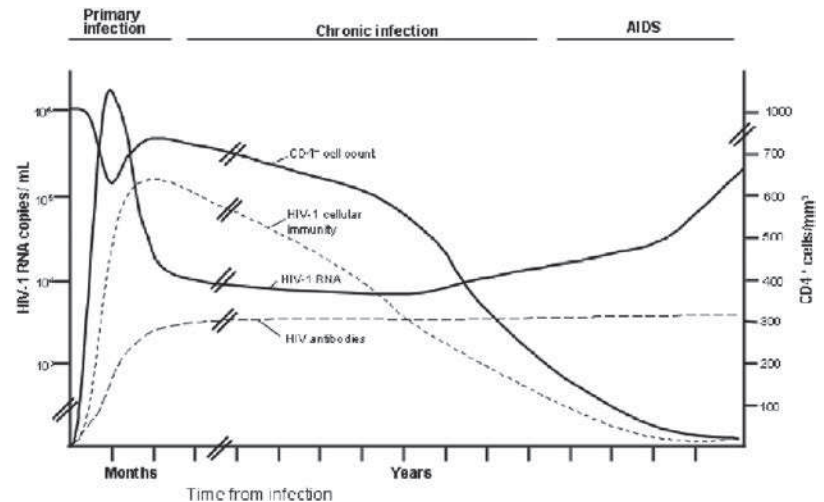
Why is it that the immune system is unable to contain HIV replication, despite detection of huge numbers of HIV-specific immune cells? An increasing amount of evidence suggests that the problem is not in the number of antiviral cells but in their function, and that most HIV-specific CD8 T cells

have some degree of functional impairment. The list of these potential defects is long, and includes lack of ability to express multiple cytokines simultaneously, to kill infected target cells, or at least to inhibit HIV replication, proliferate, and fully mature.^{26,91,170,171,272} Which of these aspects of the CD8 T-cell response in chronic HIV infection is a cause as opposed to an effect of ongoing viral replication is often unclear. What is clear, however, is that chronic antigenic stimulation by persistent viruses can lead to exhaustion of CD8 T cells by preventing them from completing the normal progression to renewable memory cells.^{416,417} In addition, studies in a murine model of chronic viral infection with many similarities to HIV infection in humans suggest that the expression, on CD8 T cells, of molecules associated with T-cell regulation (i.e., programmed death 1 [PD-1], 2B4, CD160, lymphocyte activation gene 3 [LAG-3]) may be affected by the persistent antigenic stimulation of chronic viremia, rendering those CD8 T cells ineffective in clearing virus.^{21,28} Studies of HIV-specific T cells have generally confirmed the presence of aberrant expression of co-regulatory molecules that can adversely affect HIV-specific CD8 T-cell function and longevity.^{90,307,396,429} Co-regulatory molecule expression can also be aberrant on CD4 T cells, thereby imparting indirect effects on the CD8 T-cell response.²⁰⁶

Immune Escape

The role of CD8 T cells as an antiviral mechanism is clearly implied by studies showing viral mutations arising within targeted CD8 T-cell epitopes. Studies clearly demonstrate substantial immune selection pressure as evidenced by frequent

FIGURE 50.5. Changes in plasma human immunodeficiency virus type 1 (HIV-1) RNA level, CD4⁺ cell count, HIV-1-specific antibody titers, and HIV-1-specific cellular immune responses over the course of HIV-1 infection.



polymorphisms linked to specific HLA alleles²⁰⁸ and the accumulation of HLA-associated changes during acute HIV infection. Of note, the effectiveness of CD8 T-cell responses has been linked to the fitness cost associated with viral escape, adding further evidence in support of the fact that HIV-specific CD8 T cells, although functionally impaired, still exert significant antiviral pressure on HIV, even late into infection.⁷⁹

CLINICAL FEATURES

Advancing immunodeficiency caused by the progressive loss of CD4⁺ T lymphocytes underlies the cardinal clinical manifestations of HIV-1 infection. The myriad opportunistic infections and malignancies characteristic of AIDS are a consequence of the resulting profound defect in cellular immunity. In addition,

a number of clinical syndromes are attributable directly to infection of specific organs by HIV-1. The course of HIV-1 disease can be divided into three stages: primary (or acute) infection, chronic (asymptomatic) infection, and advanced disease (AIDS) (Fig. 50.5). The duration of each stage is highly variable and can be altered by antiretroviral therapy. Several systems for classifying the different stages of HIV-1 disease have been developed, the most widely used of which include those developed by the CDC (Table 50.2) and the World Health Organization (WHO).^{56,426} Although these classification systems are valuable epidemiologic tools, their role in the assessment and clinical management of individual patients has been more limited.

Primary Infection

Acquisition of HIV-1 infection is accompanied by relatively nonspecific symptoms of an acute viral illness in approximately

TABLE 50.2 CDC Classification System for HIV Infection

CD4 cell categories	Clinical categories		
	A Asymptomatic, lymphadenopathy, or acute infection	B Symptomatic, ^a not category A or C	C Clinical AIDS ^b
>500/mm ³ (>29%)	A1	B1	C1
200–499/mm ³ (14–28%)	A2	B2	C2
<200/mm ³ (<14%)	A3	B3	C3

^aExamples of these symptoms include bacillary angiomatosis; thrush; vulvovaginal candidiasis; cervical dysplasia or carcinoma *in situ*; constitutional symptoms such as fever or diarrhea of >1 month duration; oral hairy leukoplakia; multidermatomal or recurrent herpes zoster; immune thrombocytopenic purpura; listeriosis; pelvic inflammatory disease; and peripheral neuropathy.

^bCandidiasis of the esophagus or respiratory tract; invasive cervical cancer; extrapulmonary coccidioidomycosis; cryptocryptosporidiosis; extralymphatic cytomegalovirus infection; herpes simplex with mucocutaneous ulcer >1 month duration, or bronchitis, pneumonitis, or esophagitis; extrapulmonary histoplasmosis; HIV-associated dementia; CNS lymphoma; non-Hodgkin's lymphoma; pulmonary tuberculosis; disseminated *M. tuberculosis*, *M. avium* complex, or *M. kansasii* infection; nocardiosis; *Pneumocystis jirovecii* pneumonia; recurrent bacterial pneumonia; progressive multifocal leukoencephalopathy; recurrent *Salmonella* septicemia; extraintestinal strongyloidiasis; toxoplasmosis.

Adapted from the Centers for Disease Control and Prevention. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR Morbid Mortal Wkly Rep* 1992;41 (RR-17):1–19.

50% to 70% of infected individuals.¹⁹⁸ Symptoms, which usually begin 2 weeks after exposure, frequently include fever, pharyngitis, headache, arthralgias, myalgias, malaise, and weight loss.^{85,167} A nonpruritic, maculopapular rash on the face and trunk occurs in up to 70% of cases.³⁹⁵ In addition, generalized lymphadenopathy is a frequent finding. Mucocutaneous ulceration and weight loss help distinguish primary HIV-1 infection from other viral syndromes.^{125,167} Aseptic meningoencephalitis is the most common neurologic manifestation of primary HIV-1 infection.³⁹⁵ Symptoms of primary infection resolve within 3 to 4 weeks in most patients. Persistence of symptoms beyond 8 to 12 weeks, along with a severely depressed CD4+ T-lymphocyte count and high plasma HIV-1 RNA levels may predict more rapid progression of disease.

Laboratory characteristics of primary HIV-1 infection include lymphopenia and a decrease in the absolute CD4+ T-lymphocyte count, usually accompanied by an increase in circulating activated CD8+ T cells.⁷⁵ Other hematologic abnormalities are unusual, except for mild thrombocytosis. Modest elevations in serum aspartate transaminase and alkaline phosphate may be present, but clinically significant hepatitis is uncommon.³⁹⁵ Plasma HIV-1 RNA titers generally peak 1 week following the onset of symptoms, averaging 10^6 to 10^7 copies/mL, and decline to steady-state levels (between 10^3 and 10^5 copies/mL) by 2 months after infection.^{237,246}

Chronic Infection

Following resolution of primary infection and establishment of a virologic *quasi-steady state*,¹⁷⁵ a prolonged period of asymptomatic chronic infection ensues. Although most patients remain asymptomatic during much of this phase, ongoing viral replication and CD4 lymphocyte depletion make the term *clinical latency* inappropriate. The loss of CD4 T cells proceeds at an average rate of 30 to 60 cells/mm³/year, although CD4 cell counts can remain stable for several years before a period of rapid decline.²⁶⁶ A small proportion of patients (<1%) experience progression to AIDS within 1 to 2 years.^{280,310} Rapid progression may be associated with transmission of syncytium-inducing (SI) variants of HIV-1 (i.e., viruses that use the CXCR4 coreceptor, also known as *X4 viruses*).^{64,224,287}

Fatigue and lymphadenopathy are noted by many patients during this otherwise asymptomatic phase of HIV-1 infection. Minor clinical events, such as oral hairy leukoplakia (caused by Epstein-Barr virus infection of oral epithelial cells), oral and vaginal candidiasis, herpes zoster, and a variety of other dermatologic conditions may be early signs of clinical progression. With advancing disease, night sweats and weight loss become more common.

A variety of systemic manifestations of HIV-1 infection involving nearly every organ system can occur during the chronic phase of disease. Remission in response to antiretroviral therapy suggests a direct role for HIV-1 in the pathogenesis of these disorders. Dermatologic conditions are particularly common among HIV-1-infected individuals, including seborrheic dermatitis, papular pruritic eruption, and eosinophilic folliculitis.⁷⁶ Neurologic manifestations include disorders of both the central nervous system (CNS) and peripheral nervous system, in addition to opportunistic infections and malignancies of the CNS. AIDS dementia complex and distal symmetric polyneuropathy, which occur in up to 27% and 35% of patients, respectively, are among the most frequently encountered HIV-related

neurologic complications.^{373,377} Wasting associated with HIV infection remains highly prevalent in resource-limited settings and is an independent predictor of mortality.²⁵⁰ Anorexia, malabsorption, and inappropriate nutrient utilization all contribute to the loss of lean body mass in patients with AIDS.^{154,220}

Other organ system-specific complications of HIV infection include nonspecific interstitial pneumonitis and lymphocytic interstitial pneumonitis, disorders of the gastrointestinal tract, endocrine dysfunction, anemia and neutropenia, immune-mediated thrombocytopenia, and a variety of rheumatologic syndromes. In addition, HIV-associated nephropathy leading to renal insufficiency is particularly common among African Americans and injection drug users.^{390,422}

Advanced Disease

Opportunistic infections and malignancies are rare in HIV-infected persons with CD4 counts above 500 cells/mm³, but increase as the CD4 count declines below this benchmark. Oral candidiasis, pneumococcal infections, tuberculosis, and reactivation of herpes simplex and varicella zoster viruses become more common. The risk of life-threatening complications, including *Pneumocystis jirovecii* (formerly, *P. carinii*) pneumonia, candida esophagitis, disseminated histoplasmosis and other systemic fungal infections, toxoplasma encephalitis, and cryptococcal meningitis increases substantially once the CD4 lymphocyte count drops below 200 cells/mm³.²⁵⁸ Opportunistic infections, such as disseminated *Mycobacterium avium* complex infection; reactivation of cytomegalovirus (CMV) infection, cryptosporidiosis, and microsporidiosis; and progressive multifocal leukoencephalopathy caused by JC virus reactivation, are all indicative of a profound defect in cellular immunity and usually occur at CD4 counts below 50 cells/mm³. Malignancies associated with AIDS generally are related to underlying viral infection, including Kaposi sarcoma (KS) caused by infection with human herpes virus 8; lymphomas associated with Epstein-Barr virus infection; and cervical and anal carcinoma associated with human papilloma virus infection. Although potent antiretroviral therapy has clearly reduced the risk of KS and non-Hodgkin lymphoma, the effect on Hodgkin lymphoma is less clear.^{32,33,370} Whereas the incidence of lung cancer is also increased in persons with HIV infection, age-adjusted rates of other malignancies (e.g., breast and prostate cancer) are comparable to those found in the general population.^{152,369}

Non-AIDS Complications of HIV Infection

A growing number of end-organ complications not traditionally considered “AIDS-defining” events have been recognized to occur more frequently in HIV-1–infected patients.^{253,286} These include an increased risk of cardiovascular disease, non-AIDS defining malignancies, HIV-associated neurologic dysfunction (HAND), and HIV-associated nephropathy (HIVAN), and may also include loss of bone mineral density (BMD) and other changes typically associated with increasing age, suggesting that HIV-1 infection may accelerate the aging process more generally.

With respect to cardiovascular risk, a number of studies suggest increased risk of myocardial infarction in HIV-1–infected patients as compared to control populations matched for traditional cardiovascular risk factors such as age, family history, hyperlipidemia, hypertension, diabetes, and smoking.^{177,286,398} More striking is the finding that interrupting antiretroviral

therapy significantly increases the risk of cardiovascular events, independent of CD4 cell count.^{106,313} This increased risk was correlated with an increase in plasma levels of inflammatory markers such as IL-6 and high-specificity C-reactive protein (hsCRP).^{223,347} In addition, a number of studies have shown adverse changes in surrogate markers associated with cardiovascular risk such as flow-mediated vasodilatation, carotid intima-media thickness, and coronary artery calcification in patients with HIV-1 infection.^{82,153} The extent to which these changes can be prevented or reversed by antiretroviral therapy is an active area of current research.

Patients with HIV-1 infection also show lower BMD and an increased risk of fractures compared to age-matched, uninfected controls.³⁹⁷ The causes of HIV-associated loss of BMD are poorly understood and most likely are multifactorial, including increased immune activation and inflammation, renal tubular dysfunction, low vitamin D levels, and other endocrine abnormalities.¹⁰ Whether vitamin D levels should be monitored in all HIV-infected patients, and at what level to offer supplementation, remains an area of controversy.

Determinants of Disease Progression

Numerous viral and host factors contribute to determining the rate of HIV disease progression. The plasma HIV-1 RNA level, or viral load, reflects the rate of virus replication and is a powerful independent prognostic factor for the risk of disease progression.^{267,304} Data from the MACS show that in the absence of antiretroviral therapy individuals with plasma HIV-1, RNA levels greater than 100,000 copies/mL 6 months after seroconversion are 10 times more likely to progress to AIDS within 5 years than are those with lower steady-state levels of viremia.²⁶⁵ Similarly, for patients with established HIV-1 infection, a steady-state viral load of more than 30,000 copies/mL is associated with a more than fivefold greater risk of disease progression within 3 years as compared with patients with viral loads of 3,000 to 10,000 copies/mL.²⁶⁶

According to a Poisson regression model based on data from the Concerted Action on Seroconversion to AIDS and Death in Europe (CASCADE) collaboration of 22 cohorts from Europe, Canada, and Australia, in the absence of combination antiretroviral therapy the 6-month risk of AIDS for a 25-year old patient with a CD4 cell count of 350/mm³ ranges from 0.6% at a viral load of 3,000 copies/mL to 2.5% at a viral load of 300,000 copies/mL.³¹¹ At a CD4 count of 100 cells/mm³, the AIDS risk at the same viral loads increases to 3.7% and 14.5%, respectively. The risk of disease progression has been reduced substantially since the introduction of potent combination antiretroviral therapy.¹⁰⁵ Progression to AIDS among persons infected with HIV-2 proceeds at a significantly slower rate as compared with infection with HIV-1.^{188,260} Epidemiologic and cohort studies show that HIV-2 infection generally results in lower steady-state levels of viremia (10³ copies/mL) and a more gradual decline in CD4 cell counts.^{15,24,188}

Age at the time of infection is an independent risk factor for disease progression, with older persons being at significantly greater risk, perhaps as a consequence of diminished thymic reserve.^{89,309} The role of gender in determining disease progression is less clear. Most studies show that HIV-1-infected men and women progress to AIDS at similar rates.^{134,273} Early in the course of infection, however, women tend to have significantly lower plasma HIV-1 RNA levels men.^{11,12,111} This difference dis-

appears as disease progresses. Because overall progression rates are comparable between the sexes, these observations imply that compared with men, women experience HIV-1 disease progression at lower viral loads. Whether to initiate antiretroviral therapy at lower levels of viremia in infected women than in men remains controversial (see below).

Although virus replication, and consequently viral load, is the engine that drives progression to AIDS, the CD4 cell count is the most useful marker for predicting the immediate risk of developing particular opportunistic infections.¹⁰⁵ Moreover, differences in viral load explain only a small fraction of the variability in rates of CD4 cell decline in patients not receiving antiretroviral therapy,³⁴³ suggesting that other factors such as immune activation triggered by translocation of microbial products across a damaged intestinal mucosa drive CD4 cell loss in HIV infection (see Pathogenesis). Indeed, the proportion of activated CD8+ T cells, measured as the percentage of cells expressing CD38, predicts the risk of disease progression independently of viral load and CD4 count.^{31,142,143,240} Levels of IL-6 and hsCRP are elevated in patients with HIV infection and independently predict the development of opportunistic diseases.³⁴²

The role of chemokine receptor tropism in determining the rate of disease progression remains unresolved. The prevalence of X4 variants increases with decreasing CD4 cell count, and several studies show a significantly increased risk of disease progression among patients with X4 (SI) virus.^{45,217,278,362} Macaques infected with a simian-HIV (SHIV) (SIV/HIV chimera) that expresses an X4 HIV-1 envelope show rapid depletion of CD4 cells, suggesting a causal role of X4 viruses in rapid disease progression.^{178,288} X4 variants, however, emerge in only half of patients who progress to AIDS.^{38,336} The long interval between infection and emergence of X4 viruses in most patients argues for strong selection against X4 viruses early in the course of HIV disease. Therefore, the possibility that emergence of X4 variants is a consequence, rather than a cause, of advancing immunodeficiency remains a plausible alternative explanation for the apparent association of X4 virus with disease progression. The development of chemokine receptor antagonists as a novel class of antiretroviral agents may provide new tools to address this important pathogenesis question.

The risk of disease progression is moderated also by a variety of host genetic factors. Among the most important are polymorphisms in the genes encoding the chemokine co-receptors and their ligands, and in the HLA genes. HIV-1 uses one of two chemokine receptors as co-receptors for virus entry into CD4 cells: CCR5 or CXCR4.^{97,103,113} Approximately 10% of Caucasians carry a defective allele that has a 32-base pair (bp) deletion in the CCR5 gene (*CCR5Δ32*); 1% are homozygous for this deletion and resist infection by R5 viruses.^{239,352} Infected individuals who are heterozygous for the *CCR5Δ32* allele have a slower rate of disease progression than do those who are homozygous for the wild-type allele.⁹⁴ This effect is limited to patients with R5 strains of HIV-1, presumably because syncytium-inducing viruses use the CXCR4 receptor.^{40,270} A mutation in the CCR2 gene, *CCR2-64I*, likewise reduces the rate of disease progression,^{219,376} possibly by delaying emergence of X4 virus.⁴⁰³ By contrast, mutations in the promoter region of CCR5 (*CCR5 P1*) are associated with accelerated disease progression.²⁵⁶ Other analyses have failed, however, to show a significant effect of CCR5 and CCR2 genotype after

controlling for CD4 cell count, plasma HIV-1 RNA level, and viral tropism.^{141,184}

Copy number of the gene encoding the natural ligand for CCR5 (*CCL3L1*, previously known as *MIP-1 α*) also influences the rate of disease progression. Higher *CCL3L1* copy number is associated with a lower risk of progression, which is most pronounced in patients with polymorphisms that reduce the level of functional CCR5 on the cell surface.¹⁴⁵ Variable effects on progression have been noted for the *SDF1-3'A* polymorphism, which affects the untranslated region of the mRNA encoding stromal-derived factor (SDF-1, also known as CXCL12), the natural ligand of CXCR4.^{86,145,421}

Several studies have related host HLA haplotype to the rate of HIV disease progression. For example, presence of the HLA-B27 and B57 alleles is associated with slow progression.^{8,112,305} Conversely, patients carrying class I alleles B*35 or Cw*04 progress to AIDS significantly more rapidly than do those lacking these alleles.^{51,133} Maximal heterozygosity at HLA class I loci A, B, and C is associated with delayed onset of AIDS.²⁰¹ Given that HLA antigen class I molecules play an essential role in antigen presentation to CD8+ CTL, these findings provide strong, albeit indirect, evidence for the importance of CTL in moderating the rate of HIV disease progression.

Variation in the killer immunoglobulin-like receptor (KIR) genes also affects the course of disease. These receptors are found on NK cells and regulate NK activity by recognition of certain HLA class I molecules on the surface of target cells.⁴⁰⁴ The effect of KIR alleles appears to be related to the presence or absence of specific HLA-B or HLA-C alleles.¹³⁷ For example, when present together with the HLA-B Bw4-80Ile allele, the activating KIR allele *KIR3DS1* is associated with delayed disease progression.²⁵⁷ By contrast, in the absence of HLA-B Bw4-80Ile, the presence of *KIR3DS1* is associated with more rapid disease progression. Similarly, presence of the inhibitory KIR allele *KIR3DL1* together with HLA-B*57S is highly protective against disease progression.²⁴⁴ The *KIR2DS2* allele appears to be associated with more rapid CD4 decline over time, but has no effect on viral load, whereas HLA-B*5701 and B*2705 alleles are associated with significantly lower levels of viremia.¹³⁶

Co-infecting viral pathogens have varied effects on the rate of disease progression. Patients with CMV viremia were twice as likely to experience disease progression and four times more likely to die.³⁷⁹ Asymptomatic CMV replication may also contribute to ongoing immune activation.¹⁸¹ Although the course of both hepatitis B (HBV) and hepatitis C virus (HCV) infection is worse in patients with HIV-1 infection, the effect of hepatitis co-infection on AIDS progression is less clear, with some studies suggesting accelerated progression of HIV-1 infection among co-infected patients and other studies showing no effect.^{61,350,389,392}; overall, the evidence suggests no significant effect of either HBV or HCV coinfection on the course HIV-1 infection.³⁴¹ By contrast, coinfection with the nonpathogenic GB virus C appears to have a protective effect against AIDS progression, but the mechanism of this protection is obscure.^{290,394,420}

DIAGNOSIS

The occurrence of opportunistic infections such as *P. jirovecii* pneumonia, candida esophagitis, cryptococcal meningitis, toxoplasmosis, or chronic ulcerative herpes simplex in the

absence of a known cause of immunodeficiency should raise the possibility of HIV-1 infection. Recurrent or disseminated varicella zoster infection, pneumococcal infection in a young adult, oral or recurrent vulvovaginal candidiasis, disseminated papillomavirus infection, persistent fever, night sweats, lymphadenopathy, weight loss, and chronic diarrhea all may be evidence of infection with HIV-1. A diagnosis of HIV-1 infection also should be considered in patients with unexplained lymphopenia, anemia, or neutropenia, and in cases of idiopathic thrombocytopenia.

The differential diagnosis of acute HIV-1 infection is broad, and includes other viral syndromes, such as acute Epstein-Barr virus, CMV, adenovirus, influenza, and enterovirus infection that can be associated with fever, myalgias, rash, lymphadenopathy, pharyngitis, aseptic meningitis, or lymphopenia. Acute Lyme disease, secondary syphilis, rickettsial infection, anaplasmosis, and babesiosis all have features in common with primary HIV-1 infection, but are readily excluded by appropriate laboratory tests.

Laboratory Diagnosis

The laboratory diagnosis of HIV infection usually is made by detection of HIV-1 or HIV-2 antibodies by enzyme-linked immunosorbent assay (ELISA), chemiluminescence, or rapid antibody test. The mean time to seroconversion during acute HIV infection is 25 days.⁴⁸ Antibodies to HIV become detectable within 6 to 12 weeks of infection in most infected individuals, and in virtually all patients within 6 months.^{126,176} The time to serologic detection of HIV after initial infection can be reduced by 7 days through the use of “4th generation” diagnostic tests, which combine detection of HIV antibodies and core (p24) antigen.^{108,298} In addition to the standard HIV ELISA, rapid diagnostic tests have been developed.^{166,291,374} The simplicity and wide range of operating temperature for certain of these assays make them particularly well-suited for use in point-of-care testing (e.g., a hospital emergency department or physician office) and in resource-limited settings,^{203,402} but specificity of these rapid tests may be lower than expected when applied to populations with a low prevalence of HIV infection.⁴⁰⁷

Sera that test positive are tested again to verify the initial result; repeatedly reactive sera are then tested by a confirmatory assay, usually a western blot, to ensure that reactive antibodies are directed against HIV antigens. Antibodies against the Gag proteins (p17, p24, p55) appear earliest in the course of HIV-1 infection,³²⁸ but decrease in titer with progression of HIV-1 disease, whereas antibodies to envelope (gp160 or gp120/gp41) usually persist even in advanced stages of HIV-1 disease. The sensitivity of commercial HIV-1 ELISA kits using serum from individuals with documented HIV-1 infection ranges from 98.1% to 100%,³³⁰ whereas specificity in populations with a low prevalence of HIV-1 infection ranges from 99.6% to 100.0%.^{186,293} Combined use of the HIV-1 ELISA and western blot results in a specificity approaching 100%. In settings where HIV prevalence exceeds 5%, confirmation of an initial rapid HIV antibody test by a second test that uses different antigens can serve to make a diagnosis of HIV infection.⁵⁷

The sensitivity of HIV-1 ELISA for detection of HIV-2 ranges from 50% to 93%.^{18,98} The combination HIV-1–HIV-2 antigen sandwich ELISA used by blood banks to screen simultaneously for HIV-1 and HIV-2 antibodies has a sensitivity

of 99.7% for detection of HIV-1 and 99.5% for detection of HIV-2.¹⁸

The diagnosis of primary HIV-1 infection before seroconversion depends on detection of HIV-1 capsid (p24) antigen or HIV-1 RNA in plasma. The sensitivity and specificity of the p24 antigen assay for diagnosis of acute HIV-1 infection range from 79% to 89% and 99% to 100%, respectively.^{85,167} Quantitative HIV-1 RNA assays are highly sensitive (100%), but because of occasional false-positive findings, tests have a specificity of only 95% to 97%.^{85,167} Plasma HIV-1 RNA levels generally exceed 10,000 copies/mL during acute infection, and most false-positive findings generally have values below 3,000 copies/mL.¹⁶⁷ Therefore, diagnostic accuracy of quantitative plasma HIV-1 RNA assays for diagnosing primary HIV-1 infection can be improved if only values that exceed 5,000 copies/mL are considered true positives.¹⁶⁷ Qualitative plasma HIV-1 RNA tests may prove useful in this setting, but have not been extensively evaluated for this purpose.³³¹

Serologic testing of infants born of mothers infected with HIV-1 is complicated by the presence of maternal anti-HIV antibodies that decay over 12 to 15 months. Persistence of antibody beyond 15 months of age is evidence of HIV-1 infection in the infant.⁵⁴ Two negative antibody tests obtained at least 1 month apart in infants older than 6 months of age effectively exclude HIV-1 infection. Earlier diagnosis depends on detection of HIV-1 RNA in plasma or HIV-1 proviral DNA in peripheral blood mononuclear cells. A DNA polymerase chain reaction (PCR) test is positive in approximately 40% of infected children by age 48 hours, and in 93% of infected children by age 14 days.¹⁰⁴ A qualitative HIV-1 RNA test based on

transcription-mediated amplification showed 100% sensitivity and greater than 99% specificity as compared to DNA PCR for infant diagnosis of HIV-1 infection using whole blood or dried blood spots, respectively.³⁸⁶

TREATMENT

The advent of potent combination antiretroviral therapy has had a profound influence on the course of HIV-1 infection in the developed world, where AIDS-related mortality has decreased by more than 80% since the introduction of combination therapy in the mid-1990s.^{105,179,295,323,358} (Fig. 50.6). Premature mortality among HIV-infected persons in the developed world now shows a dichotomous pattern: AIDS-related causes continue to account for deaths among those not receiving antiretroviral therapy, whereas liver-related deaths, including mortality from hepatitis C virus coinfection, account for the preponderance of premature deaths among those receiving adequate antiretroviral therapy.^{34,80,231,350} Thirty antiretroviral drugs and drug combinations in six different classes are approved for the treatment of HIV-1 infection (Table 50.3), with more in clinical development. The experience with antiretroviral therapy serves as a model for the feasibility of treating chronic viral infections, and establishes important paradigms that are being applied to the treatment of other chronic infections such as HBV and HCV.

Targets for Antiretroviral Drugs

The replication cycle of HIV-1 involves multiple steps, many of which have been successfully exploited as targets

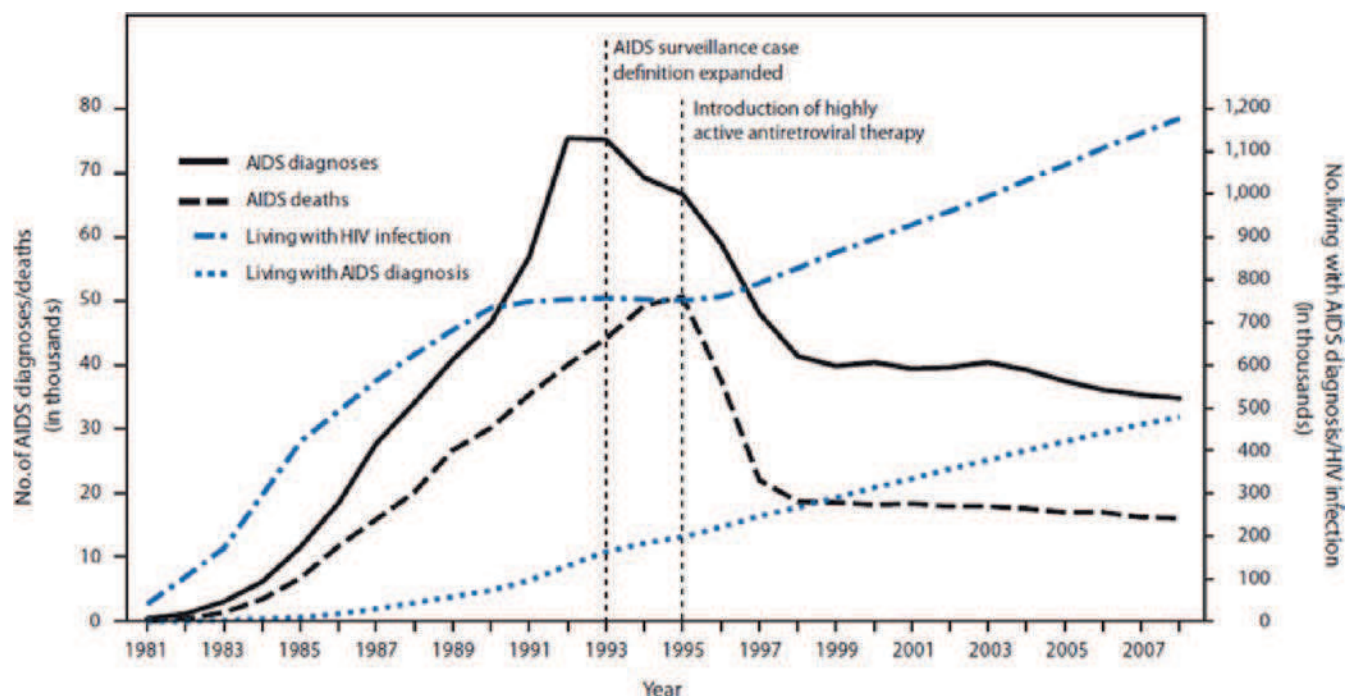


FIGURE 50.6. Estimated number of acquired immunodeficiency disease syndrome (AIDS) diagnoses and deaths and estimated number of persons living with AIDS diagnosis and living with diagnosed or undiagnosed human immunodeficiency virus (HIV) infection among persons aged 13 years or older in the United States, 1981–2008. (Centers for Disease Control and Prevention. HIV surveillance—United States. 1981–2008. *MMWR Morbid Mortal Wkly Rep* 2011;60:689–693.)

TABLE 50.3 Generic Names and Common Abbreviations for FDA-Approved Antiretrovirals (2011)

Nucleoside reverse transcriptase inhibitors (NRTIs)		Protease inhibitors (PIs)	
Abacavir	ABC	Amprenavir ^a	APV
Emtricitabine	FTC	fos-Amprenavir	fAPV
Didanosine	ddI	Atazanavir	ATV
Lamivudine	3TC	Darunavir	DRV
Stavudine	d4T	Indinavir	IDV
Zalcitabine	ddC ^a	Lopinavir ^b	LPV
Zidovudine	ZDV	Nelfinavir	NFV
		Saquinavir	SQV
		Ritonavir ^c	RTV
		Tipranavir	TPV
Nucleotide RT inhibitor (NtRTI)		Fusion inhibitor	
Tenofovir	TDF	Enfuvirtide	ENF
Nonnucleoside RT inhibitors (NNRTIs)			
Delavirdine ^d	DLV		
Efavirenz	EFV		
Etravirine	ETV		
Nevirapine	NVP		
Rilpivirine	RPV		
Integrase strand transfer inhibitor (INSTI)		CCR5 antagonist	
Raltegravir	RAL	Maraviroc	MVC

^aWithdrawn from market by manufacturer.^bAvailable only as co-formulation with ritonavir.^cUsed principally for pharmacologic enhancement of other antiretroviral agents.^dNot recommended and rarely used.

for antiretroviral drug development (Fig. 50.7). The first step in the virus life cycle is virus entry, a multistep process that involves attachment, co-receptor binding, and fusion of the cell and virus membranes. Although earlier attempts at preventing virus attachment by soluble CD4 proved unsuccessful,^{84,359,400} small molecule inhibitors that block the gp120-CD4 interaction show some promise.¹⁶⁰ Potent inhibition of HIV-1 replication has been demonstrated in randomized clinical trials by maraviroc, a small-molecule chemokine receptor antagonist that prevents binding of gp120 to CCR5.^{74,155} Similarly, a humanized monoclonal antibody (ibalizumab) that binds to domain 2 of the extracellular portion of CD4 and acts as a postattachment inhibitor of virus entry has shown potent anti-HIV-1 activity in clinical trials.^{187,225} The efficacy of the fusion inhibitor enfuvirtide (T-20), a synthetic 36-amino acid oligopeptide, was demonstrated in a series of randomized clinical trials.²²⁶ This drug blocks virus fusion by preventing the formation of a six-helix bundle by two heptad repeats (HR-1 and HR-2) in the trimeric gp41 ectodomain.^{418,419}

Inhibition of reverse transcriptase (RT) by substrate analogs (nucleoside and nucleotide RT inhibitors [NRTIs]) and by noncompetitive inhibitors (non-nucleoside RT inhibitors

[NNRTIs]) constitutes the mainstay of most antiretroviral regimens. Because the NRTIs lack a 3'-OH group, once incorporated into the growing complementary DNA (cDNA) strand, they act as chain terminators, bringing reverse transcription to a halt.³⁰⁰ Another feature of the NRTIs is their need for phosphorylation by intracellular nucleoside and nucleotide kinases to generate the active deoxynucleotide triphosphate (dNTP) forms of these drugs. Activity of these kinases can differ between cell types (lymphocyte versus monocyte or macrophage) and their activation state, accounting for variations in drug activity.¹³² Moreover, NRTIs that rely on the same phosphorylation pathway can exhibit antagonism when used in combination, as in the case of zidovudine and stavudine, which both depend on thymidine kinase for their initial phosphorylation.^{163,269,405} The NRTIs have formed the backbone of antiretroviral therapy since the introduction of zidovudine in 1987.

The NNRTIs are a chemically diverse class of drugs that occupy a potential drug-binding pocket in RT distinct from the dNTP binding site. Binding induces conformational changes that essentially inactivate RT¹⁰⁹; in this respect, the NNRTIs can be considered allosteric inhibitors of RT function.³⁸⁰ Currently approved NNRTIs generally are inactive against HIV-2, which lacks tyrosine residues at positions 181 and 188 that are essential for drug binding.³³² Drugs in this class play a central role as preferred agents for first-line regimens and for prevention of mother-to-child transmission.

Inhibition of HIV-1 protease prevents processing of the Gag and Gag-Pol polyprotein precursors into their mature constituents, which comprise the structural proteins of the virus core particle (capsid, matrix, and nucleoprotein) as well as the three virally encoded enzymes (protease, reverse transcriptase, and integrase) required for virus replication. In contrast to inhibitors of virus entry and RT, protease inhibitors do not prevent uninfected cells from becoming infected by HIV-1, but result in the release of noninfectious virions.^{119,196,301} Most protease inhibitors are peptidic or peptidomimetic compounds designed as analogs of the cleavage sites found within the Gag and Gag-Pol precursor proteins. Because of their relatively poor oral bioavailability, most protease inhibitors are coadministered with a low dose of ritonavir, an HIV-1 protease inhibitor that inhibits the 3A4 isozyme of cytochrome P450 (CYP 3A4)²¹⁰; CYP 3A4 is responsible for metabolism of most of these drugs. Protease inhibitors have proved to be highly effective in the treatment of HIV-1 infection as components of initial and subsequent treatment regimens.

Following reverse transcription, the resulting linear double-stranded DNA (dsDNA) molecule must be integrated into the host chromosome. The process of integration, which is catalyzed by the virally encoded integrase, is a multistep process that involves formation of a preintegration complex, nuclear importation, endonucleolytic processing of the 3' ends of the DNA molecule, and a strand-transfer reaction that results in covalent attachment of the viral and cellular DNA (see Chapter 49). The development of appropriate high-throughput screening assays allowed identification of specific inhibitors of the strand-transfer reaction.¹⁶⁵ The efficacy of the integrase inhibitor raltegravir has been demonstrated in phase 3 clinical trials in treatment-naïve²³⁰ and treatment-experienced patients,³⁸³ respectively.

Other potential targets for drug development include the viral accessory proteins (Nef, Rev, Tat, Vif, Vpr, and Vpu), as well as cellular proteins necessary for viral replication. A

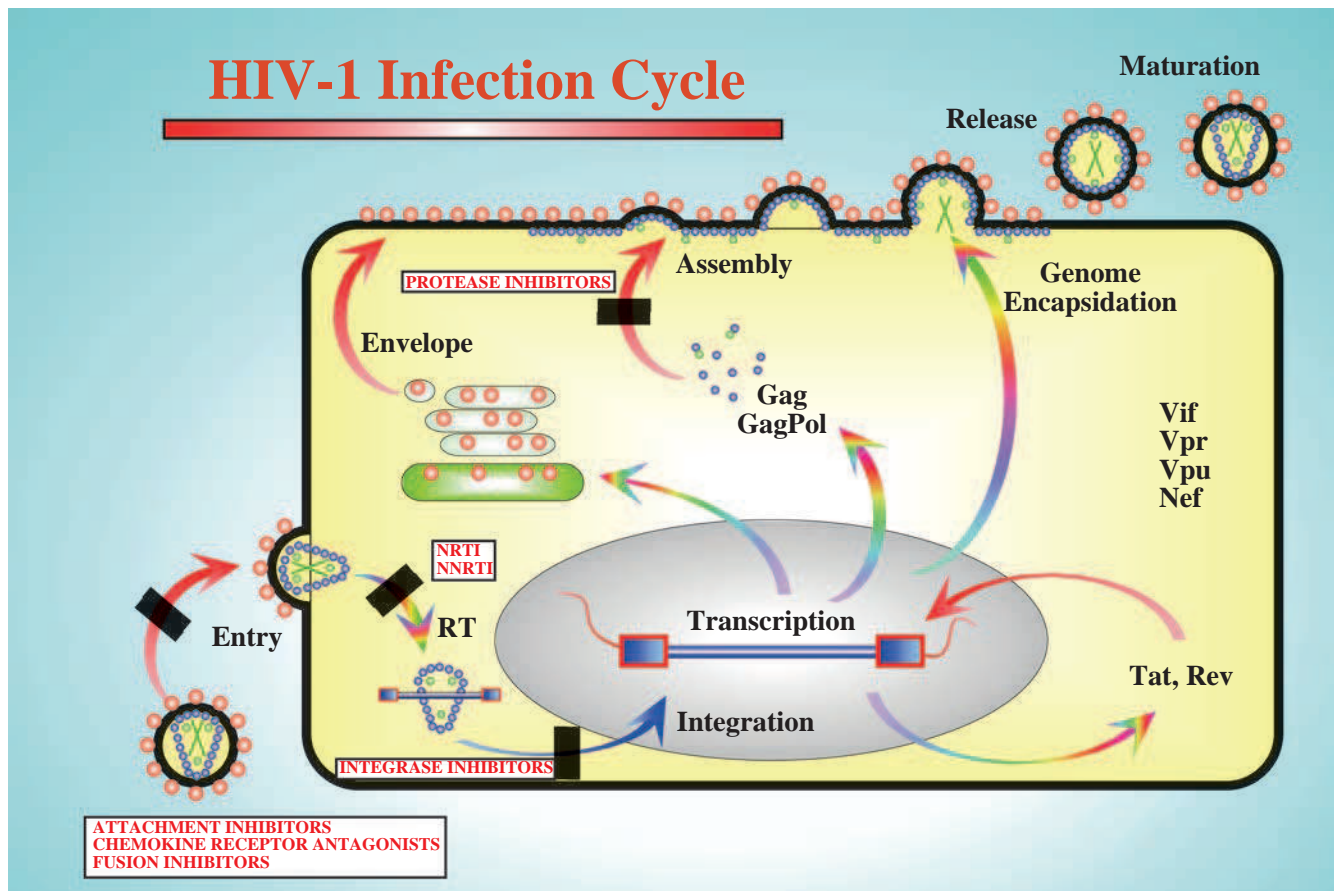


FIGURE 50.7. Points of attack in the human immunodeficiency virus type 1 (HIV-1) virus lifecycle for antiretroviral drugs.

number of cellular restriction factors that prevent HIV-1 infection have been described, including APOBEC3G and TRIM-5 α . Both factors constitute a part of the innate cellular defense against viral infection. APOBEC3G, a DNA cytosine deaminase, introduces fatal errors into the nascent viral DNA during reverse transcription, rendering the virus not infectious.²⁴⁹ The HIV-1 protein Vif counteracts the effects of APOBEC3G and could serve as a target for antiviral drug development. Likewise, Vpu counteracts the effects of tetherin, which prevents release of virus particles during the budding process, could also be a target for drug development.³⁰⁶ TRIM-5 α is a component of cytoplasmic bodies that mediate species-specific innate cellular resistance to HIV-1 by modulating early events in the virus replication cycle.³⁸⁷ Many HIV-1 variants appear to protect themselves from restriction factors through an association with the host protein cyclophilin A; derivatives of cyclosporin have demonstrated *in vitro* antiretroviral activity.³⁷⁸ Lens epithelium-derived growth factor (LEDGF), a cellular co-factor of HIV-1 integrase, offers another promising target for drug development.⁶² The identification of more than 250 HIV-dependency factors through a large-scale screen with small interfering RNA (siRNA) provides additional therapeutic targets.³⁹

A variety of gene therapy approaches have been proposed to render CD4 cells resistant to HIV-1 infection. These include the use of ribozymes,⁴²⁴ antisense RNA,¹⁸⁰ or siRNA to silence expression of viral or cellular genes.⁴¹⁵ Preliminary human studies of genetically modified cells expressing zinc-finger nucleases

that disrupt expression of host genes essential for virus replication are underway.⁴⁹ Although these approaches have shown promise in the laboratory, they face formidable challenges in demonstrating efficacy in clinical trials.

Principles of Antiretroviral Therapeutics

The demonstration in 1987 that zidovudine increases survival of individuals with advanced HIV-1 disease¹¹⁸ ushered in the era of antiretroviral therapy (ART). Subsequent development of additional NRTIs created possibilities for combination therapy.^{268,360} The NNRTIs had even greater potency as antiretroviral agents, but rapid emergence of resistance initially hindered their development.^{60,87,162} The discovery of protease inhibitors with a higher genetic barrier to resistance provided sufficiently potent regimens that could achieve durable suppression of virus replication.¹⁵⁶ At the same time, viral dynamics studies demonstrated high rates of HIV-1 replication and turnover of the HIV-1 quasi-species and suggested the inevitable emergence of drug resistance when ART does not completely inhibit replication.^{175,304,413} These observations provided the theoretic basis for use of potent drug combinations with the goal of suppressing plasma HIV-1 RNA levels to below the limits of detection of the most sensitive assays available.¹⁶⁴

Acute (Primary) HIV-1 Infection

Theoretic benefits of ART during primary HIV-1 infection include ameliorating the symptoms associated with acute

infection; reducing the steady-state level of viremia; limiting the pool of resting, latently infected CD4 T cells; preserving immune function generally and HIV-1-specific immune responses in particular; and preventing onward transmission by decreasing virus titers in blood and genital secretions. Although a number of studies have shown significant short-term improvement in virologic and immunologic markers in subjects who initiate ART during primary HIV-1 infection, most of these benefits wane once treatment is discontinued. Viral replication resumes promptly on cessation of therapy, even after prolonged periods of suppression, despite augmentation of HIV-1-specific CD4 and CD8 T-cell responses.^{207,254,344,345}

Previous approaches to the treatment of acute HIV-1 infection were based on the hypothesis that a finite course of ART initiated during acute infection or shortly after seroconversion would have virologic and immunologic benefits that persist after treatment interruption.^{168,406} However, given the risks posed by treatment interruption,¹⁰⁶ this approach no longer seems desirable, and once initiated treatment should be continued indefinitely. Moreover, the shift in treatment guidelines toward recommending initiation of ART at earlier stages of disease increasingly blurs the distinction between treatment of acute and chronic HIV infection. Nevertheless, no consensus exists at present regarding treatment of patients with acute HIV-1 infection or those who have seroconverted within the previous 6 months.²⁹⁹

Chronic HIV Infection

The benefits of ART for patients with chronic HIV infection are well established. The availability of potent, once-daily regimens that are generally well tolerated has made durable suppression of HIV-1 replication an achievable goal for most patients who require ART. A large body of data from randomized control trials and longitudinal cohort studies informs treatment guidelines developed by various groups. The general principles are clear: To be effective, treatment must be sufficiently potent to suppress plasma viremia to below the limits of detection of sensitive assays, and must be sufficiently simple and well tolerated that patients will be capable of adhering to the prescribed regimen with a minimum of missed doses. Because current therapy is incapable of eradicating HIV-1 infection, once such therapy is started most patients will require it lifelong.

There is broad consensus that ART should be offered to HIV-infected patients with CD4 counts of less than 350 cells/mm³, and for those with symptomatic HIV infection or AIDS-defining conditions regardless of CD4 cell count.^{67,138,299,393} Randomized clinical trials support the survival benefits of initiating ART at a CD4 count of 350 cells/mm³, as compared to lower CD4 counts.³⁶⁴ Less agreement exists on whether to treat patients with CD4 counts greater than 350 cells/mm³. Guidelines from the U.S. Department of Health and Human Services (DHHS) and the International AIDS Society-USA recommend initiating ART in patients with CD4 counts below 500 cells/mm³, and suggest considering treating all patients from the time of diagnosis.^{299,393} These recommendations are based on the growing appreciation of the variety of HIV-related complications and end-organ diseases that occur as a consequence of the heightened state of immune activation that accompanies uncontrolled HIV-1 replication, and that may occur independent of immunological status. In addition, analysis of data pooled from several North American cohort

studies of HIV-infected patients shows a survival advantage for those who initiated antiretroviral therapy, even at CD4 counts above 500 cells/mm³, as compared to those who deferred therapy to lower CD4 cell counts.²¹⁵ However, another large cohort study found that the survival difference of early versus deferred antiretroviral therapy is attenuated and no longer statistically significant at CD4 cell counts above 350/mm³.³⁸⁵ Guidelines from other groups such as the European AIDS Clinical Society⁶⁷ and the British HIV Association¹³⁸ generally are more conservative and recommend initiating antiretroviral therapy when the CD4 count falls below 350 cells/mm³.

Preferred regimens include the fixed-dose combination of tenofovir, emtricitabine, and efavirenz, or a fixed-dose combination of tenofovir/emtricitabine plus a ritonavir-boosted protease inhibitor (atazanavir or darunavir) (Table 50.4). Randomized trials have shown the efficacy and long-term durability of regimens that include efavirenz or a boosted protease inhibitor.^{157,173,274,292} The combination of tenofovir/emtricitabine plus the integrase inhibitor raltegravir is also considered a preferred regimen, but long-term experience with this regimen is more limited.²³⁰ Because recommendations change as new data become available, readers should consult the *Department of Health and Human Services Guidelines for Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents*, which is updated regularly and may be found at <http://aidsinfo.nih.gov>.

Pharmacologic interactions between different antiretroviral drugs, or between antiretroviral drugs and drugs used to treat common co-infections, significantly complicate the use of ART in many settings. Most troublesome are the interactions of NNRTIs and protease inhibitors (PIs) with the cytochrome P450 (CYP) system. Perhaps the greatest challenge is the difficulty of combining standard antituberculosis regimens with first-line ART because of the profound induction of CYP by rifampin, an essential component of antituberculosis regimens.²³²

Virologic and Immunologic Effects of Antiretroviral Therapy

A brisk decrease in plasma HIV-1 RNA levels should be expected following treatment initiation, with at least a 1-log₁₀ reduction within 14 days. In most patients, plasma HIV-1 RNA should fall to less than 50 copies/mL by week 16 to 24, depending on the starting viral load. Failure to achieve the expected drop in plasma viremia raises concerns regarding the degree of treatment adherence or presence of drug-resistant virus. The initial rapid decay in plasma viremia reflects the half-life of productively infected, activated cells.³⁰⁴ By contrast, the decay in latently infected resting memory (CD45RA⁻RO⁺) CD4 cells is almost imperceptible.¹¹⁵ Replication-competent HIV-1 can be recovered from these latently infected cells by the use of sensitive culture techniques.^{63,116,423} In one study, the half-life of latently infected resting memory CD4 cells was estimated to be as long as 43 months, whereas a second study documented a half-life of only 6 months.^{115,430} Decay rates of these latently infected memory cells were significantly faster in patients with consistent suppression of plasma HIV-1 RNA to less than 50 copies/mL while receiving potent ART as compared with patients who experienced transient episodes of viremia.^{327,430}

The mechanism by which the reservoir of latently infected resting memory cells persists in patients on suppressive ART remains controversial. In some studies, envelope sequences obtained at intervals show signs of evolution, which has been

TABLE 50.4 Preferred and Alternative Antiretroviral Regimens for Antiretroviral Therapy-Naïve Patients (Updated January 10, 2011)

<p>Preferred regimens^a</p> <p>NNRTI-based regimens</p> <ul style="list-style-type: none"> • EFV/TDF/FTC^b <p>PI-based regimens (in alphabetical order)</p> <ul style="list-style-type: none"> • ATV/r^c + TDF/FTC • DRV/r (once daily) + TDF/FTC <p>INSTI-based regimen</p> <ul style="list-style-type: none"> • RAL + TDF/FTC <p>Preferred regimen for pregnant women</p> <ul style="list-style-type: none"> • LPV/r (twice daily) + ZDV/3TC 	<p>Comments</p> <p>EFV should not be used during the first trimester of pregnancy or in women trying to conceive or not using consistent contraception.</p> <p>ATV/r should not be used in patients who require >20 mg omeprazole equivalent per day</p>
<p>Alternative regimens^d</p> <p>NNRTI-based regimens</p> <ul style="list-style-type: none"> • EFV + (ABC or ZDV)/3TC • NVP + ZDV/3TC <p>PI-based regimens (in alphabetical order)</p> <ul style="list-style-type: none"> • ATV/r + (ABC or ZDV)/3TC • FPV/r (once or twice daily) + either [(ABC or ZDV)/3TC] or TDF/FTC • LPV/r (once or twice daily) + either [(ABC or ZDV)/3TC] or TDF/FTC 	<p>Comments</p> <p>NVP</p> <ul style="list-style-type: none"> • NVP should not be used in patients with moderate to severe hepatic impairment • NVP should not be used in women with pre-ART CD4 count >250 cells/mm³ or men with pre-ART CD4 count >400 cells/mm³. <p>ABC</p> <ul style="list-style-type: none"> • ABC should not be used in patients who test positive for HLA-B*5701. • Use ABC with caution in patients with high risk of cardiovascular disease or with pretreatment HIV-1 RNA >100,000 copies/mL. <p>Once-daily LPV/r is not recommended in pregnant women.</p>

^aRegimens with optimal and durable efficacy, favorable tolerability and toxicity profile, and ease of use. The preferred regimens for nonpregnant patients are arranged by order of FDA approval of components other than nucleosides, thus, by duration of clinical experience.

^bFTC and 3TC may be used interchangeably in each of the regimens listed in the table.

^cATV/r, DRV/r, LPV/r and FPV/r denote dosing of the indicated protease inhibitor together with pharmacologic enhancement by low-dose ritonavir.

^dRegimens that are effective and tolerable but have potential disadvantages compared with preferred regimens. An alternative regimen may be the preferred regimen for some patients.)

The following combinations in the recommended list above are available as fixed-dose combination formulations: ABC/3TC, EFV/TDF/FTC, LPV/r, TDF/FTC, and ZDV/3TC. Abbreviations of drug names are given in Table 50.3.

Adapted from DHHS Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents (<http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>).

taken as evidence of replication.⁴³⁰ In other studies, failure of resistance mutations to emerge over time has been taken as evidence against replication in this pool of cells.^{212,349}

Although patients with plasma HIV-1 RNA levels below the limit of detection by routine clinical tests are considered to have “undetectable” viral loads, residual viremia (1–50 copies/mL) can be detected in nearly all patients when sufficiently sensitive methods are applied.²⁹⁶ Whether residual viremia reflects ongoing rounds of HIV replication, release of virus from long-lived productively infected cells or is due to activation of latently infected cells remains an area of controversy. The absence of viral evolution over time in subjects with detectable residual viremia and the inability to suppress residual viremia through intensification of ART by additional antiretroviral drugs argue against ongoing replication as a source of this virus.^{19,101,130,263} Some patients experience transient episodes of detectable plasma viremia (*blips*). In most studies, the occurrence of blips has not been associated with emergence of drug resistance or an increased risk of treatment failure.^{284,285} Mathematic modeling of the frequency of these blips suggests that they represent stochastic

bursts of replication, perhaps caused by intercurrent episodes of immune activation.^{100,303}

The rapid decline in plasma HIV-1 RNA is accompanied by a similarly brisk increase in CD4 T lymphocytes in most patients. On average, the CD4 cell count increases by 50 to 100 cells/mm³ in the first 4 weeks of therapy, followed by a more gradual, but steady, increase thereafter.²²⁸ Most of this initial increase in CD4 cell counts is thought to be the result of redistribution of memory CD4 T cells from lymphoid tissues into the peripheral blood.²⁹⁴ The subsequent slower increase is attributed, in turn, to the gradual increase in naïve CD4 T cells that is thought to represent production of naïve CD4 T cells.^{16,72,431} By the end of the first year of therapy, total CD4 cell counts increase by an average of 175 cells/mm³.^{129,242,410} Patients remaining on antiretroviral therapy over 7 years experience a CD4 count increase of 300 to 600 cells/mm³, depending on the CD4 count at the time of ART initiation.²⁴² Patients with incomplete suppression of plasma viremia also show significant increases in CD4 cell counts, but the magnitude of this increase is blunted compared with patients with complete viral suppression.^{204,205,391}

Viral suppression reduces generalized immune activation, as judged by the number or percentage of CD38+DR+ CD4 and CD8 cells.²²⁸ The proportion of activated cells remains greater, however, than that found in seronegative controls.^{193,375} Higher levels of immune activation are associated with lower levels of CD4 cell restoration on ART.¹⁹³ Reconstitution of the follicular DC network in lymphoid tissues can occur over time,⁴³² but central memory T cells (Tcm) do not appear to be reconstituted by successful ART.¹⁰⁷

The numeric increase in CD4 T cells is accompanied by laboratory and clinical evidence of reconstitution in pathogen-specific immunity. Proliferative responses to recall antigens such as *Candida albicans* and CMV can be demonstrated after 12 to 48 weeks of therapy.^{16,72,228,289,321} Prophylactic therapy to prevent new or recurrent episodes of *P. jirovecii* pneumonia,^{123,124,216} disseminated *Mycobacterium avium* complex infection,^{2,83} histoplasmosis,¹⁴⁴ and CMV retinitis^{247,399} can be discontinued safely in patients who have shown a satisfactory virologic and immunologic response to therapy. By contrast, lymphocyte proliferative responses to HIV antigens remains limited,^{16,72} although some studies have shown improvements in HIV-specific immunity by enzyme-linked immunospot (ELISPOT) assay or intracellular cytokine staining.^{35,321}

Immune reconstitution can be accompanied by a pathologic inflammatory response to previously treated or subclinical opportunistic infections. The immune reconstitution inflammatory syndrome (IRIS) occurs in up to one third of patients beginning ART, and is best described in association with CMV retinitis, disseminated *Mycobacterium tuberculosis*, or *M. avium* complex infection, and cryptococcal meningitis.^{44,329,367,368} Patients with IRIS are more likely to have started an initial ART regimen soon after diagnosis of an opportunistic infection, a low CD4 count below 50 cells/mm³, and to have a more rapid decrease in plasma HIV-1 RNA levels.^{279,368} Management includes continuation of ART, treatment of the associated opportunistic infection, and a brief course of steroids if the extent of inflammation risks significant end-organ damage or is life-threatening.¹⁷⁴

Drug Toxicity

A wide range of toxicities of varying severity has been described. In the case of the NRTIs, the more serious include anemia and neutropenia,³³⁷ peripheral neuropathy (stavudine [d4T], didanosine [ddI], zalcitabine [ddC]),^{30,114,276} pancreatitis (ddI and ddC),¹⁴⁸ and life-threatening lactic acidosis.^{59,243} Severe peripheral lipodystrophy has been associated with use of d4T, ddI, and zidovudine (ZDV), most likely caused by inhibition of mitochondrial DNA polymerase γ .^{50,199} As a result, these drugs are no longer recommended as first-line agents, although they continue to be used in resource-limited settings because of their low cost. Fatal hypersensitivity reactions can occur with abacavir in patients carrying the HLA B*5701 allele.^{172,248,255,316} Abacavir may also contribute to an increased risk of cardiovascular disease, although this finding remains controversial.^{78,335,351,427} Although tenofovir disoproxil fumarate (TDF) generally is well tolerated, uncertainty over possible long-term effects on bone metabolism limits its use in pregnancy and young children.⁵³ Similarly, concerns over potential nephrotoxicity have limited TDF use in patients with impaired renal function.^{200,214}

Use of nevirapine and efavirenz can be complicated by rash and hepatotoxicity. In the case of nevirapine, rash may be

severe (Stevens-Johnson syndrome).³²⁰ Life-threatening hypersensitivity hepatitis can occur with nevirapine, particularly in women with CD4+ T-cell counts greater than 250/mm³.^{3,353} CNS side-effects are a common cause of treatment discontinuation with efavirenz,⁶⁶ and concerns over teratogenicity restrict its use in pregnant women and women of childbearing age.⁹³

Gastrointestinal intolerance, hepatotoxicity, and hyperlipidemia are the most frequent dose-limiting toxicities associated with the protease inhibitors. The incidence of hepatotoxicity may be significantly increased among HCV co-infected patients.³⁸⁸ In addition, several drugs in this class significantly impair insulin sensitivity, possibly by an inhibitory effect on the glucose transporter GLUT4.²⁸¹ The combination of insulin resistance and hyperlipidemia can increase the risk of cardiovascular disease among patients receiving certain protease inhibitors.¹²² Hyperbilirubinemia, nephrolithiasis, and rash are additional concerns with certain members of this class. Use of the fusion inhibitor enfuvirtide (ENF), which requires subcutaneous injection, leads to development of painful subcutaneous nodules that limit long-term acceptability of this drug.²⁷⁰

When a change in therapy is prompted by drug toxicity, an alternative agent should be substituted for the offending drug without interrupting treatment. When toxicity is sufficiently severe to require suspension of dosing, all drugs in a regimen should be discontinued to avoid exposure of the virus to a partially suppressive regimen, which can result in selection of drug resistance (see below).

Treatment Failure

Numerous factors contribute to the failure of highly active antiretroviral therapy (HAART), including poor adherence (because of the complexity or poor tolerability of certain regimens)^{183,425}; pharmacologic factors, including drug-drug interactions that impair absorption or accelerate clearance²⁸²; host factors (e.g., low CD4 cell count at the start of treatment and HLA haplotype)^{5,179,315}; drug resistance acquired by transmission of a resistant isolate or selected by previous suboptimal therapy²³⁸; and preexisting drug-resistant minority variants.²³³ Each of these factors leads to incomplete suppression of viral replication, which in turn, leads to selection of drug-resistant variants.

The definition of treatment failure depends on where in the course of treatment an individual patient stands. For patients on a first or second regimen, treatment failure usually is defined as confirmed evidence of detectable viremia after an initial virologic response, or failure to achieve a response. Prompt switching to a new potent regimen is advised to reestablish virologic suppression, prevent emergence of drug resistance, and preserve future treatment options.^{192,283} Although many clinicians do not alter therapy unless the level of viremia exceeds 1,000 to 5,000 copies/mL, persistent low levels of detectable viremia can be associated with accumulation of drug resistance mutations.²⁸³

For patients with more extensive treatment histories, the situation is more complex. By this point, most patients already will have been exposed to all three classes of antiretroviral agents and may have developed resistance to at least one agent within each drug class. Therefore, the presence of detectable plasma HIV-1 RNA is less useful as a marker of treatment failure. In this situation, symptomatic evidence of treatment failure can include increasing fatigue, malaise, and night sweats; objective

evidence includes weight loss, a rising viral load, falling CD4 cell count, and occurrence of new, recurrent, or worsening AIDS-related opportunistic infections.

Even when complete virologic suppression cannot be achieved, ART continues to provide clinical benefits. Studies show that mean CD4 cell counts remain above pretherapy levels through 96 weeks of follow-up in patients experiencing virologic failure of a protease inhibitor regimen.⁹⁵ Maintenance of stable CD4 cell counts depends on continued administration of ART, even in patients with apparent virologic failure of their regimens.⁹⁶ An analysis of data pooled from 13 cohorts in Europe, North America, and Australia showed the continuing immunologic and clinical benefits of ART despite virologic failure.²²⁷ Those with plasma HIV-1 RNA levels above 10,000 copies/mL and CD4 cell counts below 200 cells/ μ L were at greatest risk of disease progression. Within each viral load and CD4 count stratum, the risk of disease progression was significantly lower for those who continued on ART despite virologic failure, confirming the persistent benefit of treatment in the setting of ongoing virus replication and presumed drug resistance.

Drug Resistance

As discussed above, the high error rate of HIV-1 RT and rapid turnover of the virus population contribute to the generation of extensive genetic variation in the HIV-1 quasi-species. These factors provide a mechanism for rapid emergence of drug resistance in the setting of partially effective ART. Resistance emerges at a rate proportionate to the frequency of preexisting variants and their relative growth advantage in the presence of drug.⁶⁸ Support for this model is provided by observations from monotherapy studies with different classes of antiretroviral drugs. For example, resistance to nevirapine and lamivudine emerges within weeks of initiating monotherapy because of point mutations that result in a thousand-fold reduction in drug activity.^{338,363} By contrast, resistance to drugs such as didanosine and tenofovir emerges slowly.^{222,252} In the case of zidovudine and most protease inhibitors, high-level resistance occurs as a consequence of the accumulation of multiple mutations over time.^{37,275} Given the extensive array of mutations implicated in resistance to various classes of antiretroviral drugs, a detailed discussion of resistance to individual drugs is beyond the scope of this chapter. The reader is referred instead to excellent reviews of this subject^{29,65,197} and to the several web sites that maintain a comprehensive listing of HIV-1 drug resistance data (www.hiv.lanl.gov; <http://hivdb.stanford.edu>; www.iasusa.org).

Several studies document the high prevalence of antiretroviral drug resistance in the developed world, and the rising incidence of transmission of drug-resistant virus.²³⁸ Data from the CDC show the prevalence of antiretroviral drug resistance to be approximately 15% among persons newly diagnosed with HIV-1 infection; resistance to drugs from two or more drug classes was found in 3% to 4%.^{140,414} A cohort study conducted in London showed the risk of accumulating at least one major drug resistance mutation was 27% by 6 years; the risk of mutations associated with resistance to drugs from at least two of the three main drug classes was 20% over the same period.³¹⁴

The clinical significance of antiretroviral drug resistance was demonstrated not long after the introduction of ZDV and ddI by studies that showed accelerated immunologic decline and disease progression in patients receiving nucleoside analogs to which their viruses had become resistant.^{88,191,222} As was

discussed, studies show the relationship between resistance to PI-based HAART regimens and clinical progression is more complex.^{95,96} Drug resistance comes at a cost to the virus in terms of replication capacity. Diminished replication capacity and residual activity of some drugs against resistant viruses may account for persisting reductions in AIDS incidence and AIDS-related mortality in the developed world despite the increasing prevalence of antiretroviral drug resistance.

Resistance to antiretroviral agents can be assessed by genotypic and phenotypic assays. These tests are an important tool for management decisions related to therapy initiation or regimen modification. Properly used, they can lead to improved virologic outcomes for patients infected with HIV-1.

Treatment as Prevention

Evidence that the risk of HIV-1 transmission correlates with viral load in the source patient leads naturally to the hypothesis that decreasing circulating levels of HIV-1 by effective antiretroviral therapy will reduce transmission.^{147,195,325,381} This hypothesis is supported by the successful use of ART to prevent perinatal and breast-milk transmission of HIV-1 from infected mothers to their infants,^{73,365} and by observational studies among HIV-1 discordant couples that show a substantially reduced risk of HIV transmission when the infected partner is receiving ART.^{13,334} These observational data have been confirmed in a randomized trial that showed a 96% reduction in the risk of sexual transmission of HIV by the group randomized to receive ART.⁷⁰ Although implementing public health policies based on these results remains an enormous challenge, the data show clearly the possibility for effective control of the HIV-1 pandemic given sufficient resources and political will.

Prospects for HIV Eradication

As discussed earlier in this chapter, the establishment of a reservoir of latently infected, resting CD4 lymphocytes results in the lifelong persistence of HIV-1 infection. Attempts to purge the reservoir by activating resting cells have thus far proved unsuccessful.³²⁴ Similarly, a report that valproic acid reduced the number of latently infected cells by inhibiting histone deacetylase (HDAC) was not confirmed by subsequent work.^{14,229,371} The effect of selective HDAC inhibitors such as members of the suberoylanilide hydroxamic acid (SAHA) class of molecules (e.g., vorinostat) on the HIV reservoir is an area of intensive study, including preliminary human trials.²⁵⁹ The report that HIV-1 is no longer detectable in an infected patient 3 years after receipt of an allogeneic bone marrow transplantation for acute myelogenous leukemia from a donor homozygous for the *ccr5Δ32* mutation has reinvigorated the search for potential cures for HIV-1 infection.^{69,182}

HIV VACCINES

Vaccines are arguably the most powerful medical intervention for preventing virus infections. Despite the obvious need, and years of intense research, the development of an effective vaccine against HIV has been elusive. Although many approaches have been tried, only four basic vaccine platforms have shown adequate promise to warrant large scale efficacy testing. Initial efforts were directed at using recombinant envelope proteins to stimulate antibodies to block infection. Although these

vaccines did stimulate envelope binding antibodies, they failed to induce antibodies capable of neutralizing strains of HIV other than easy to neutralize laboratory-grown viruses, and the trials failed to demonstrate protection against HIV infection.^{120,318} The inability to induce broadly neutralizing antibodies through vaccination has continued to be a vexing problem, and is probably related to a number of factors, including sequence variability, especially within exterior loops of the envelope that are highly immunogenic but that can easily mutate to avoid recognition, and the heavy glycosylation of the envelope.³⁸² The recent isolation of broadly neutralizing antibodies from HIV-infected subjects, and the identification of the antigenic target of these antibodies on the HIV envelope, have raised hope that an immunogen can be developed that targets conserved sites of vulnerability on the HIV envelope.^{297,408,409,428}

Because of the failure of early vaccines to protect against HIV acquisition, a subsequent approach was to stimulate broad HIV-specific CD8 T-cell responses in the hope that these responses would at least control viral replication in those who became infected with HIV, thereby limiting the impact of the infection upon the vaccinated individual, and potentially influencing the subsequent transmission of HIV to other individuals. A trial of a recombinant adenovirus-based vaccine was performed, and although it did stimulate strong CD8 T-cell responses, it failed to protect vaccinees from infection or affect their viral loads, and actually increased HIV acquisition within subjects with pre-existing immunity to the vaccine vector (adenovirus type 5).^{46,262} Although the mechanism underlying the increase in acquisition is still under investigation, the failure of this trial has severely dampened enthusiasm for T-cell-based vaccine strategies.

On a more optimistic note, vaccine that combined a pox-based vector and recombinant envelope protein did show moderate (31%) protection against HIV acquisition in a low-risk predominantly heterosexual population.³³³ This vaccine did not stimulate strong neutralizing antibody responses, and virtually no CD8 T-cell responses, so the exact mechanism of protection afforded by the vaccine is currently unknown but is certainly the subject of intense investigation. Further studies of similar vaccines are being planned. Finally, a fourth vaccine platform is being evaluated in an efficacy trial. This vaccine is based upon adenovirus type 5, but includes the HIV envelope protein and induces envelope-specific antibodies.^{190,211} The fact that an efficacy signal has been seen with a vaccine has infused optimism into the vaccine field, though it will still be years before a licensed vaccine could be available.

Other Prevention Strategies

Sexual behavioral change can clearly diminish the risk of becoming infected, as has been shown in Zimbabwe with a reduction in casual sex and a delay in sexual debut accounting for the greatest differences,¹⁵¹ and which has also been suggested for Uganda. In addition, knowledge of the mechanisms of transmission (discussed previously) has led to other nonvaccine interventions aimed at reducing transmission. It was long assumed that the mucosa of the male foreskin was a site of HIV acquisition in men, a contention supported by epidemiologic data. This was confirmed when an initial trial of more than 3,000 uncircumcised men who were randomized to be offered immediate circumcision or circumcision after a 21-month period of follow-up was stopped after an interim analysis at 18 months revealed that circumcision conferred 60% protection from HIV infection.¹⁷

These findings have been confirmed in two subsequent randomized clinical trials,^{20,149} and efforts are ongoing to deploy circumcision to high-risk male populations.

Other efforts to provide both men and women with a means of protection from infection have focused on the development of topical microbicides. Most of these efforts have proven to be failures in field trials, with at least one actually increasing the risk of HIV transmission.^{277,401} Of interest, a topical antiretroviral microbicide was effective when used intravaginally in women, but failed when given orally, potentially highlighting the importance of the local environment when designing strategies to block HIV transmission.^{1,384} The ultimate goal of prevention strategies is to have multiple options for diverse populations, including vaccines, barriers, and oral and topical prophylaxis.

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Nonhuman Lentiviruses

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Perspective

HISTORY

Use of the term “slow virus infections” and identification of the first lentivirus is generally credited to Sigurdsson et al.^{314,315–316} in Iceland. Twenty karakul sheep that were imported from Germany to Iceland in 1933 were responsible for the transmission of a chronic disease and death in massive numbers of Icelandic sheep over subsequent years. More than 100,000 Icelandic sheep died of the disease in the decades that followed. Sigurdsson et al.^{314,315–316} not only described the disease but also demonstrated that it was due to a transmissible agent. They used the term slow virus infections to refer to this disease as well as to what is now known as scrapie.³¹³ The term has since been used to describe a wide variety of nonacute, persisting viral infections. In 1960, Sigurdsson et al.³¹⁷ described the cultivation of the transmissible agent in tissue culture, and Guðnadóttir and Pálsson¹¹⁵ were able to reproduce the disease with the culture-grown virus. The diseases in the sheep

were called *maedi* (Icelandic for dyspnea, that is, a lung disease resulting in difficulty breathing) and *visna* (Icelandic for a state of progressive apathy, a “fading away,” resulting from brain disease). Both disease states result from the same virus, now referred to as maedi/visna virus (MVV). MVV and related viruses are called lentiviruses, derived from the Latin *lentus* for slow. Approximately 600,000 sheep were slaughtered in Iceland in 1965 to eradicate MVV from the island.

The maedi/visna disease in Icelandic sheep, although the first specifically shown to be caused by a defined lentivirus, is probably not the first description of a lentiviral disease in the literature. Vallée and Carré described in 1904 the infectious nature of the disease equine infectious anemia,³⁴³ which is now known to be caused by a lentivirus (EIAV). Even for MVV, a chronic, progressive interstitial pneumonia had been described in South African sheep in 1915 and in Montana sheep in 1923.²¹³ Work with EIAV was largely on a parallel track with that of MVV, and the first description of EIAV cultivation appeared in 1961.¹⁷² Only subsequently were EIAV and MVV shown to belong to the same lentivirus subfamily of retroviruses on the basis of morphologic criteria.^{111,253,363}

The scenario of disease outbreak in a susceptible population leading to the identification of a new lentivirus has repeated itself dramatically on several occasions in more recent history. In 1964, an emerging infectious disease was first detected in Bali cattle in the Jembrana district of Bali.^{272,321} Bali cattle are the domesticated form of the wild banteng (*Bos javanicus*). Within 12 months, 26,000 of the 300,000 cattle on the island died of the disease. The cause of the disease was subsequently traced to a bovine lentivirus now called Jembrana disease virus (JDV), a distinct variant of bovine immunodeficiency virus (BIV).^{36,162} Bali cattle are particularly sensitive to disease caused by this virus, whereas other species are resistant.^{322,323,366} Outbreaks of immunodeficiency disease and lymphoma in captive colonies of macaque monkeys (Asian Old World primates) were subsequently traced to the introduction of a simian lentivirus or lentiviruses from African monkeys.^{18,61,201,208} Simian immunodeficiency viruses (SIVs) naturally infect African nonhuman primates without apparent disease, but Asian macaques appear to harbor no such virus naturally. Even the origins of human immunodeficiency virus (HIV) in people have followed a similar pattern. HIV-2 in people in western Africa clearly originated from SIV of sooty mangabey monkeys.^{41,70,99,129,184,214} The natural habitat of sooty mangabey monkeys is the same geographic region in western Africa where HIV-2 is endemic, and SIV from sooty mangabey monkeys groups phylogenetically with HIV-2 distinct from other lineages of primate lentiviruses. Sooty mangabey monkeys are naturally infected with SIV at high frequency without apparent disease. The origins

TABLE 51.1 Known Lentiviruses

Species	Virus	Year that cultivation was first published and reference
Sheep/goats	Maedi/Visna virus; caprine arthritis encephalitis virus	1960 (317)
Horses	Equine infectious anemia virus	1961 (172)
Cattle	Bovine immunodeficiency virus; Jembrana disease virus	1972 (345)
Humans	Human immunodeficiency virus	1983 (13)
Monkeys	Simian immunodeficiency virus	1985 (61)
Cats	Feline immunodeficiency virus	1987 (256)

of HIV-1 in central Africa have similarly been traced to the chimpanzee *Pan troglodytes*.^{51,97,305} These examples illustrate the importance of studying animal viruses even when they are not apparently associated with any disease.

The earliest descriptions of the isolation of HIV and its association with acquired immunodeficiency syndrome (AIDS) did not appreciate that the virus was a lentivirus.^{13,270} Only subsequently, through more careful examination of electron micrographs and through sequence analysis, did this become clear.^{112,227} At the time, study of lentiviruses was an obscure discipline with which many scientists were not familiar.

From a historical perspective, MVV³¹⁷ and EIAV¹⁷² were discovered, isolated, and characterized long before the discovery of HIV; the field has retained use of the original designations for these viruses. Lentivirus groupings identified after the discovery of HIV have used a nomenclature similar to that for HIV (i.e., “immunodeficiency virus”). BIV, originally isolated from a cow with a chronic disease by Van Der Maaten et al. in 1972,³⁴⁵ received little attention until after the discovery of HIV. Subsequent to the discovery of HIV, lentiviruses were isolated from monkeys and cats (Table 51.1). Although discovery of MVV, EIAV, and BIV predates that of HIV, HIV has received such intense scrutiny that much more is known about it than all other lentiviruses in terms of the level of detailed knowledge. New information about other lentiviruses is thus usually compared with what is known for HIV.

INFECTIOUS AGENT

Overview of General Properties

Related lentiviruses have been isolated from sheep, goats, horses, cattle, cats, monkeys, and humans (Table 51.1). Genetic analysis of the virus from goats (caprine arthritis encephalitis virus; CAEV) indicates that it clusters closely with MVV,³⁴² therefore is placed in a single grouping with MVV. Based on host species and the genetic analyses described in more detail later in this chapter (Genome Organization and Composition section), five discrete evolutionary groupings of lentiviruses are generally recognized (Fig. 51.1A). It is likely that more remain to be identified. It is important to realize that even within a single grouping, discrete subgroupings based on host species, geography, and genetic distance can be defined. For example,

among the nonhuman primate lentiviruses, distinctly different subgroupings exist for the SIVs from African green monkeys, sooty mangabey monkeys, Sykes' monkeys, and L'hoesti monkeys (Fig. 51.1B). Even among African green monkeys, which inhabit virtually all of sub-Saharan Africa, discrete genetic sub-subgroupings of SIVagm can be defined that correlate with subspecies and natural geographic habitat (Table 51.2 and Fig. 51.1B).

All lentiviruses have a common morphogenesis and morphology that distinguish them from other subgroups of retroviruses (see Chapter 47). Lentiviruses bud from the plasma membrane without a preformed nucleoid, and mature particles typically have a conical or rod-shaped nucleoid (Fig. 51.2). Classification into the lentivirus subgroup by morphologic criteria alone is entirely consistent with phylogenetic analysis of *pol* gene sequences. Viruses classified as lentiviruses have *pol* gene sequences more closely related to one another than to other retroviruses, and all have the characteristic morphogenesis/morphology. All lentiviruses have a propensity to replicate in macrophages, and all are associated with a chronic progressive disease course in susceptible hosts. The primate lentiviruses have acquired use of CD4 as one of two receptors used sequentially by virus for entry into cells (Table 51.3). FIV has been shown to use an analogous two-receptor sequential mechanism for entry. However, lentiviruses from nonprimates, including FIV, do not use CD4 as a receptor for entry. The chronic disease induced by the primate lentiviruses in susceptible hosts has an immunodeficiency component presumably because of the targeting of CD4⁺ lymphocytes by use of CD4 as a receptor. All lentiviruses have a number of auxiliary genes (i.e., genes in addition to *gag*, *pol*, and *env* not found in other, simpler retroviruses). Major distinguishing properties are summarized in Table 51.3.

GENOME ORGANIZATION AND COMPOSITION

Widespread availability of DNA sequencing capabilities has greatly facilitated our understanding of phylogenetic relationships and the gene products of lentiviruses. Based on sequence analysis, five discrete groupings of lentiviruses are now recognized (see Fig. 51.1A). It is likely that other groupings remain to be discovered. Extensive diversity exists even within a grouping. For example, among the SIVs, distinct subgroups have been defined from the African green monkey, sooty mangabey monkey, L'hoesti monkey, and Sykes' monkey (see Fig. 51.1B and Table 51.2). In all, 14 discrete phylogenetic lineages of primate lentiviruses have so far been defined (Fig. 51.1B and Table 51.2). Of the 69 species known to inhabit sub-Saharan Africa, SIV infection has been demonstrated in 40 of them, partial SIV sequence information is available from 32 species, and complete SIV genome sequences are available from 20 species.¹ Since some species have not yet been surveyed, additional SIV lineages may still remain to be discovered. Extensive diversity has also been observed for FIV in wild and captive cat species.^{34,336} Even among the four subspecies of African green monkeys (vervet, grivet, tantalus, and sabeus), discrete sub-subgroupings of SIVagm have been defined (Table 51.2). These four subspecies naturally inhabit distinct or sometimes partially overlapping habitats that cover almost all of sub-Saharan Africa. JDV represents a distinct subgroup relative to the original BIV isolate, analogous to SIVagm versus SIVsm.³⁶

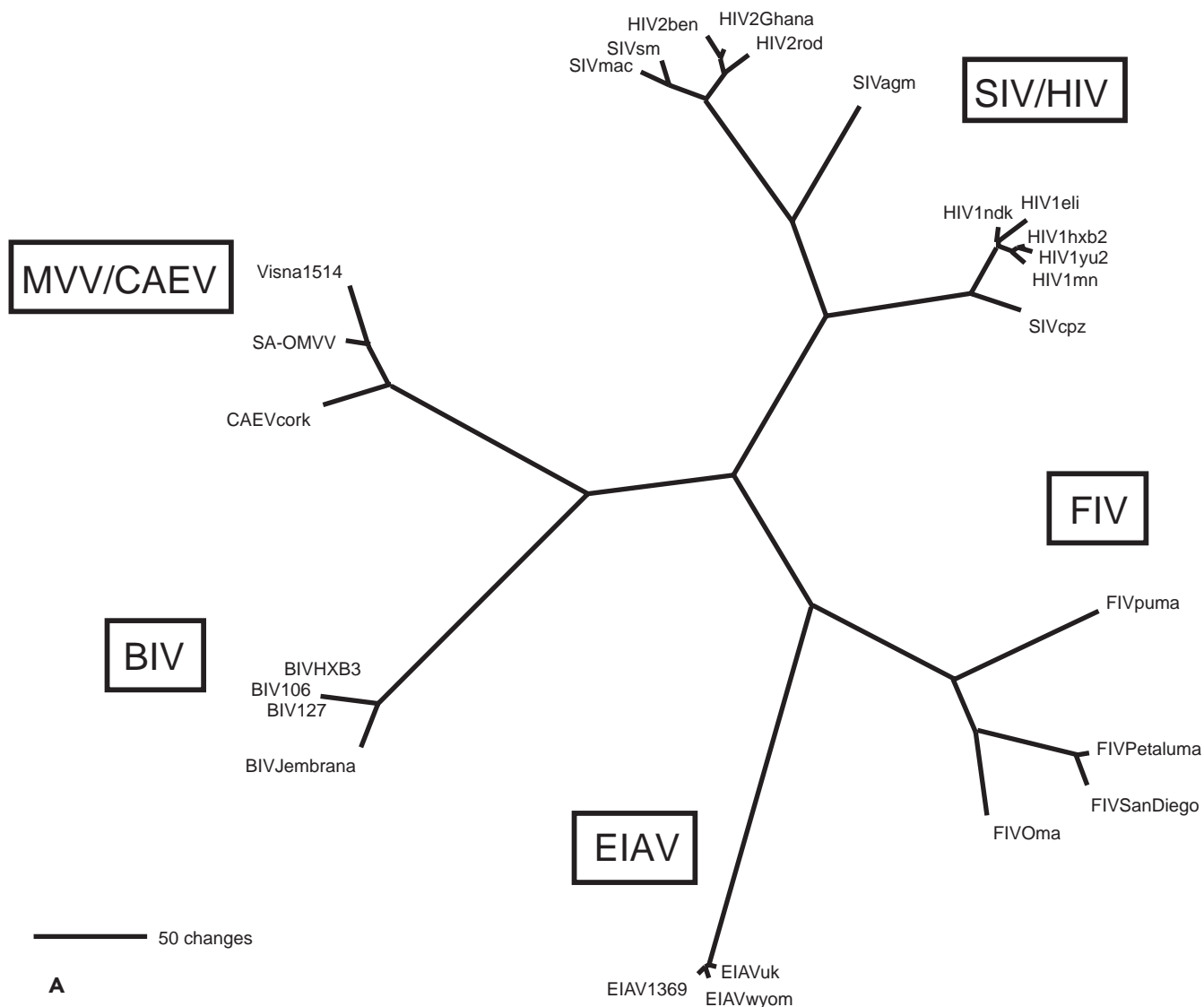


FIGURE 51.1. Phylogeny of the lentiviruses. A: Five discrete evolutionary groupings of lentiviruses. The unrooted tree depicts the phylogenetic relationships among the five recognized groups of lentiviruses. The tree is based on a 470-amino-acid alignment of reverse transcriptase sequences from representative members of each group, including the bovine (BIV, Jembrana), equine (EIAV), feline (FIV), ovine/caprine (visna, CAEV) and primate (SIV, HIV) lentiviruses. Maximum parsimony (shown) and neighbor-joining (not shown) analyses give trees of nearly identical topology. Branch lengths are proportional to the number of amino acid replacements. (*continued*)

The *pol* gene generally exhibits the greatest degree of sequence conservation. Thus, *pol* gene sequences are often used for the comparison of lentiviruses from different groups, subgroups, or sub-subgroups. Sequences from one subgroup of SIV (e.g., SIVsm) will typically exhibit only 55% to 60% identity in *pol* at the amino acid level when compared with sequences from another SIV subgroup (e.g., SIVagm). Diverse members within a subgroup may exhibit as little as 75% to 80% amino acid identity in *pol*, but the number is typically much higher. When different groups of lentiviruses are compared—for example, MVV with SIV—amino acid identity in *pol* is typically 35% or less. Relatedness in other genes is even less than that. Nonetheless, these lentiviral sequences are clearly more closely related to one another than to other retroviruses, justifying their classifica-

tion as lentiviruses on the basis of morphogenesis/morphology and biologic properties.

All known lentiviruses have at least three genes in addition to the standard *gag*, *pol*, and *env* genes that all replication-competent retroviruses possess (Tables 51.4 and 51.5, and Fig. 51.3). These extra genes likely contribute to a more complex life cycle for the lentiviruses, which includes persistent viral replication and strategies for immune evasion discussed in more detail later in this chapter (Immune Responses and Persistence section). A *rev* gene that encodes a protein responsible for controlling the relative level of full-length vs. multiply spliced viral messenger RNAs (mRNAs) is present in all lentiviruses, along with a downstream nucleotide sequence referred to as the Rev-Response Element (RRE). Interestingly, neither the sequence



FIGURE 51.1. (continued) B: Phylogeny of primate lentiviruses. The 14 groupings of primate lentiviruses are shown. (See Table 51.2 for species codes.) [Adapted from Courgnaud V, Formenty P, Akoua-Koffi C, et al. Partial molecular characterization of two simian immunodeficiency viruses (SIV) from African colobids: SIVwrc from Western red colobus (*Piliocolobus badius*) and SIVolc from olive colobus (*Procolobus verus*). *J Virol* 2003;77:744–748.]

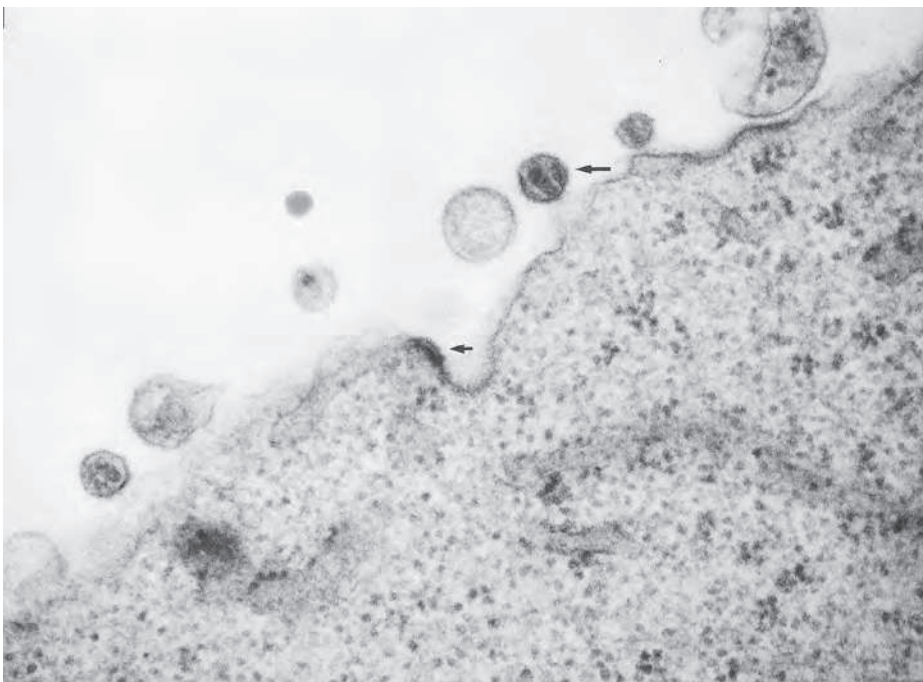


FIGURE 51.2. Lentivirus morphogenesis and morphology. This electron micrograph of a cell infected with simian immunodeficiency virus shows virus budding from the cell in the absence of preformed particles (*lower arrow*) and a mature particle with a cylindrical or rod-shaped nucleoid (*upper arrow*). (Courtesy of John MacKey.)

TABLE 51.2 Detailed Listing of Primate Lentiviruses^a

Virus designation	Primate lentivirus grouping	Species (common)	Species (formal)	Subspecies isolates	References
HIV-1	HIV-1/SIVcpz	Humans	<i>Homo sapiens</i>		(13,234)
SIVcpz	HIV-1/SIVcpz	Chimpanzees	<i>Pan troglodytes</i>	<i>P. t. troglodytes</i>	(51,97,139,258)
SIVgor	HIV-1/SIVcpz	Gorillas	<i>Gorilla gorilla</i>	<i>P. t. schweinfurthii</i> <i>G. g. gorilla</i>	(346)
SIVsm	SIVmac/SIVsm/HIV-2	Sooty mangabeys	<i>Cercocebus atys</i>		(94,129,215,233)
SIVmac	SIVmac/SIVsm/HIV-2	Macaques	<i>Macaca mulatta</i> <i>M. arctoides</i> <i>M. nemestrina</i> <i>M. fascicularis</i>		(18,61,165,234,244)
HIV-2	SIVmac/SIVsm/HIV-2	Humans	<i>Homo sapiens</i>		(45,98)
SIVagm	SIVagm	African green monkeys	<i>Chlorocebus aethiops</i>	<i>C. a. grivet</i> <i>C. a. vervet</i> <i>C. a. tantalus</i> <i>C. a. sabeus</i>	(5,6,59,91,92,130,150,153,231,249)
SIVsyk	SIVsyk	Sykes' monkeys	<i>Cercopithecus mitis</i>	<i>C. a. alboquaralis</i>	(86,128)
SIVgsn	SIVgsn/SIVmon/SIVmus	Greater spot-nosed monkey	<i>Cercopithecus mitis</i>	<i>C. a. nictitans</i>	(55)
SIVmon	SIVgsn/SIVmon/SIVmus	Mona monkey	<i>Cercopithecus mona</i>		(52)
SIVmus	SIVgsn/SIVmon/SIVmus	Mustached monkey	<i>Cercopithecus cephus</i>		(52)
SIVlhoesti	SIVsun/SIVlhoesti	L'hoesti monkey	<i>Cercopithecus lhoesti</i>	<i>C. l. lhoesti</i>	(126)
SIVsun	SIVsun/SIVlhoesti	Sun-tailed monkey	<i>Cercopithecus lhoesti</i>	<i>C. l. solatus</i>	(15)
SIVdeb	SIVdeb	DeBrazza monkey	<i>Cercopithecus neglectus</i>		(19)
SIVden	SIVdeb	Dent's mona monkey	<i>Cercopithecus denti</i>	<i>C. m. denti</i>	(62)
SIVrcm	SIVrcm	Red-capped mangabey	<i>Cercocebus torquatus</i>	<i>C. t. torquatus</i>	(16,40,105)
SIVmnd	SIVmnd 1	Mandrill	<i>Mandrillus sphinx</i>		(326,339)
SIVmnd	SIVmnd 2	Mandrill	<i>Mandrillus sphinx</i>		(257,326)
SIVdrl	SIVmnd 2	Drill	<i>Mandrillus leucophaeus</i>		(46,138)
SIVcol	SIVcol	Querza colobus	<i>Colobus querza</i>		(54)
SIVolc	SIVolc	Olive colobus	<i>Procolobus badius</i>		(53)
SIVwrc	SIVwrc	Western red colobus	<i>Ptilocolobus badius</i>		(53)
SIVtal	SIVtal	Angolia-talapoin monkey	<i>Miopithecus talapoin</i>		(202,251)
SIVtal	SIVtal	Gabon talapoin monkey	<i>Miopithecus ogouensis</i>		(257)

^aPartial *pol* sequences have also been obtained from a black mangabey (*Lophocebus aterrimus*)³³¹ and from a Schmidt's guenon (*Cercopithecus ascanius schmidtii*).³⁵³ In addition to the primate lentiviruses listed, serologic surveys for the detection of antibodies to SIV have suggested SIV infection of a variety of other species.^{202,249,334}

TABLE 51.3 Properties of Lentiviruses

Property	HIV-1	SIV	MVV, EIAV, BIV, FIV
Morphogenesis/morphology	Lenti	Lenti	Lenti
Macrophage tropism	Yes	Yes	Yes
CD4 lymphocyte tropism	Yes	Yes	No
Use of CD4 as receptor for virus entry	Yes	Yes	No
Use of chemokine receptors as receptor for virus entry	Yes	Yes	Yes (FIV)
Natural modes of transmission	Sex, blood, vertical	Sex, blood, vertical	Insects, saliva/aerosols, blood, sex, vertical
Genes in addition to <i>gag</i> , <i>pol</i> , <i>env</i>	6	5 or 6	3 or more
<i>tat</i> and <i>rev</i> activities	Yes	Yes	Yes
dUTPase	No	No	Yes (MVV, EIAV, FIV), No (BIV)
Persistent viral replication	Yes	Yes	Yes
Chronic, debilitating disease ^a	Yes	Yes	Yes
Immunodeficiency ^a	Yes	Yes	No (MVV, EIAV, BIV), Yes (FIV)

^aIn susceptible hosts. Not all hosts are susceptible to disease.

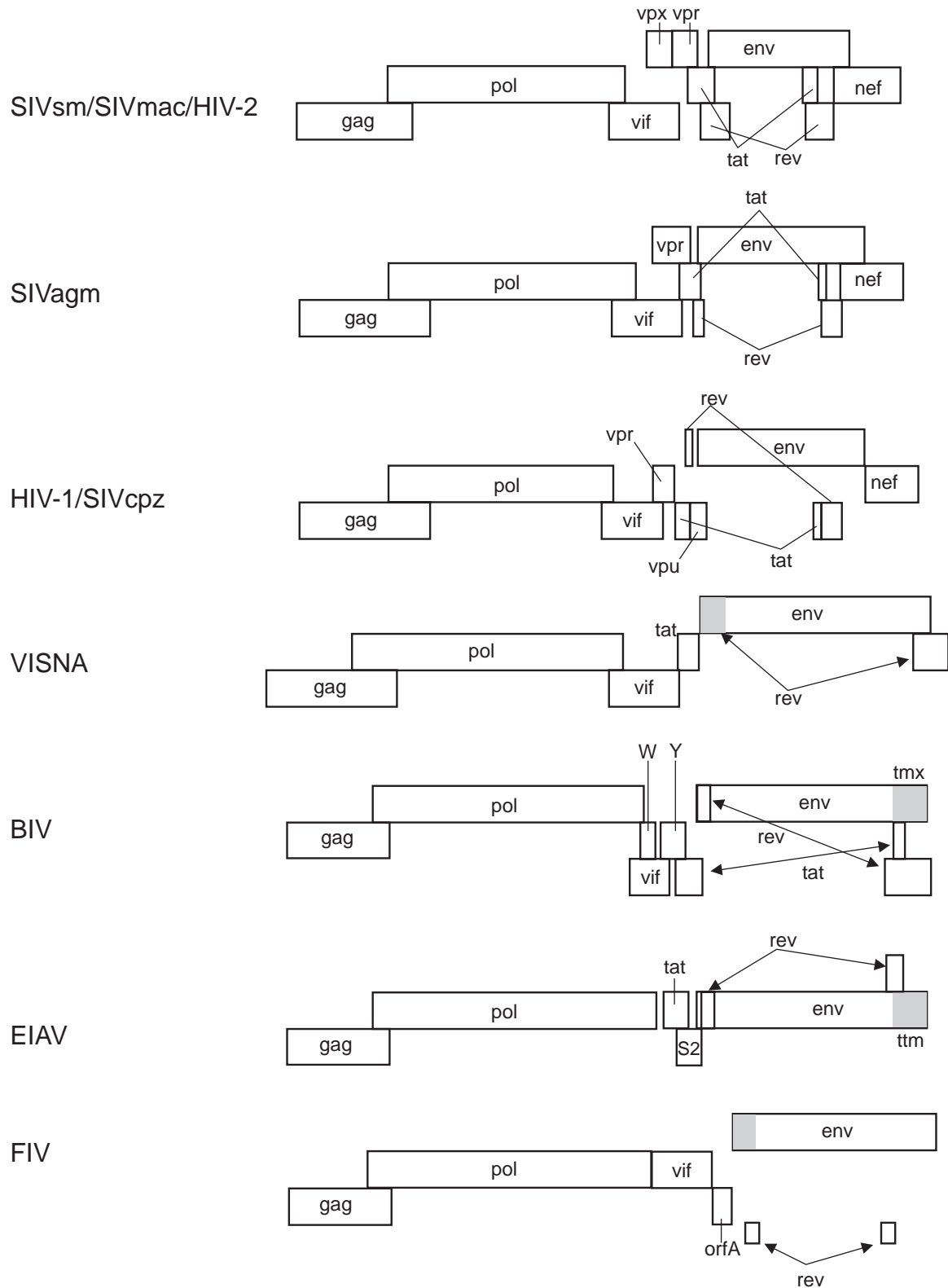


FIGURE 51.3. Genome organizations of representative lentiviruses.

TABLE 51.4 Auxiliary Genes in Nonprimate Lentiviruses

MVV ^a	EIAV ^b	BIV ^c	FIV ^d
<i>vif</i>	<i>tat</i> (S1)	<i>vif</i>	<i>vif</i>
<i>tat</i>	<i>rev</i> (S3)	<i>tat</i>	<i>rev</i>
<i>rev</i>	<i>S2</i>	<i>rev</i>	<i>tat</i> (orfA)
	<i>ttn</i>	<i>ttnx</i>	
			<i>vpw?</i>
			<i>vpy?</i>

^aReference 342.^bReference 218.^cReference 109.^dReference 250.

nor location of the RRE is conserved, but the function appears to be the same in all lentiviruses. A *vif* gene, whose main role appears to be to counteract the cellular restriction factor APOBEC, is consistently present in five of the six lentivirus groupings. EIAV, however, stands alone in apparently lacking a *vif* gene. Whether another protein may contribute *vif* function for EIAV remains to be determined. A *nef* gene is found at the 3' end of all primate lentiviruses (Table 51.5). None of the nonprimate lentiviruses contains a *nef* gene. However, cells infected with EIAV and BIV have been found to make spliced RNAs that predict protein products called Ttm and Tmx that correspond to the carboxy-terminal portion of transmembrane protein (TM).^{17,110} Whether these represent evolutionary precursors to *nef* or perform functions similar to those of *nef* is yet to be determined. All lentiviruses except FIV contain an unusually long (>120 amino acids) cytoplasmic domain of TM. In the intergenic region between *vif* and *env*, a number of other genes can be present, particularly in the primate lentiviruses. A *vpr* gene is present in this region in all primate lentiviruses that have been examined. The SIVsm/SIVmac/HIV-2, SIVrcm and SIVmnd2 subgroups of viruses contain an additional homolog of *vpr* called *vpx* (Table 51.5). The nonprimate lentiviruses also contain a gene between the *vif* and *env* genes with varied roles. BIV encodes a Tat protein from this location that acts

on a downstream Tar element similar to the primate lentiviruses.¹⁹⁷ A small protein is also encoded from this region of the ruminant lentiviruses that was originally thought to be a transcriptional transactivator but which may actually be more vpr-like in activity.³⁵⁴ Likewise, a small protein encoded in a similar region by FIV, termed OrfA, was originally thought to be a transactivator, but has recently been shown to downregulate CD134 receptor expression on the infected cell, similar to CD4 downregulation by HIV and SIV Nef.¹³³ Only two of the 14 groups of primate lentiviruses that have been analyzed to date consistently have a gene called *vpu*: HIV-1/SIVcpz and SIVgsn/SIVmon/SIVmus (Table 51.5). SIVden from a pet Dent's Mona monkey (*Cercopithecus mona denti*) was found to contain a *vpu* gene, although the virus clustered phylogenetically more closely to the SIV from DeBrazza monkeys, SIVdeb.⁶²

Except for BIV, nonprimate lentiviruses encode a deoxyuridine triphosphatase (dUTPase) activity from a distinct open reading frame located within the *pol* gene.⁸⁵ dUTPase converts dUTP to a precursor of dTTP, thus keeping the concentration of dUTP in the cell at low levels. By doing so, dUTPase indirectly prevents misincorporation of uracil into viral DNA, ultimately reducing mutagenic G>A transitions. dUTPase is particularly relevant for virus replication in nondividing cells like macrophages in which endogenous dUTPase levels are low. Curiously, these dUTPase coding sequences are absent in the primate lentiviruses but present in the type D beta retroviruses. The explanation for this variable presence of dUTPase is not entirely clear. The type D retroviruses and lentiviruses may possibly have diverged from a common ancestor that possessed such a dUTPase reading frame, and this reading frame may then have been lost in the evolution of primate lentiviruses from the more primitive ones. The type D retroviruses and lentiviruses do share a common morphology of the mature particles (i.e., a cylindrical, rod-shaped, or conical nucleoid), which also may suggest some commonality. Alternatively, the presence of a dUTPase reading frame in nonprimate lentiviruses and in type D retroviruses could be an example of convergent evolution, with two distinct lineages acquiring these sequences independently by gene capture or by sequence duplication and point mutation. Another possibility is that one viral lineage may have acquired dUTPase sequences from the other viral

TABLE 51.5 Auxiliary Genes Primate Lentiviruses

	SIVsm/ SIVmac/HIV-2	SIVagm	SIVsyk	SIVsun/ SIVhoesti	HIV- 1/SIVcpz	SIVgsn/ SIVmon/ SIVmus	SIVrcm
<i>vif</i>	+	+	+	+	+	+	+
<i>vpu</i>	—	—	—	—	+	+	—
<i>vpr</i>	+	+	+	+	+	+	+
<i>vpx</i>	+	—	—	—	—	—	+
<i>tat</i>	+	+	+	+	+	+	+
<i>rev</i>	+	+	+	+	+	+	+
<i>nef</i>	+	+	+	+	+	+	+

Note: SIVmnd2 has a *vpx* gene, but SIVmnd1 does not. SIVden from a pet Dent's Mona monkey (*Cercopithecus mona denti*) has a *vpu* gene, although it clusters more closely to the SIV from DeBrazza monkeys, SIVdeb. SIVdeb has a *vpr* gene, but no *vpx* or *vpu* genes.

lineage by a recombination or capture event.¹² The presence of a dUTPase in the endogenous SIV found in the germline of the prosimian lemur¹⁰⁷ supports the notion that dUTPase was lost in the evolution of nonprimate to primate lentiviruses. The loss of dUTPase in the evolution from nonprimate to primate lentiviruses could relate at least in part to the predominant replication of nonprimate lentiviruses in cells with little potential to divide (macrophages) where endogenous dUTPase levels are low versus the predominant replication of primate lentiviruses in cells with much greater potential to divide (lymphocytes) in which high levels of endogenous dUTPase prevail. Primate lentiviruses incorporate uracil DNA glycosylase into virions through its binding to *vpr*,²⁰⁹ thus facilitating excision and subsequent repair of misincorporated uracil in newly synthesized viral DNA. The presence of the dUTPase or uracil DNA glycosylase in virions serves to reduce the mutation frequency in reverse transcription products.

PROPAGATION

Propagation and Assay in Cell Culture

Primary isolates from all lentivirus groupings can be grown in normal macrophage cultures of the host species. Other types of cells may be more typically used for propagating the individual viruses in routine cell culture. The types of cells that can be used vary with the virus (Table 51.6) and almost certainly relate to the kinds of receptor that can be used in most cases. Isolates also can be adapted to replicate on particular cell substrates. Examples of the latter include growth of FIV on Crandell feline kidney cells and growth of SIVs and HIVs on human tumor T-cell lines (Table 51.6). It is important to note that the strong selective forces that allow replication in particular cells may make research convenient but may lead to results atypical of natural infection.

Host Range

The lentiviruses are typically restricted in their host range, but both natural and experimental cross-species infections have been documented. SIVs naturally infect a variety of African nonhuman primates, but a single example of natural infection of Asian Old World monkeys has never been documented. The SIVs from sooty mangabey monkeys and African green monkeys, when introduced into macaque monkeys (Asian Old World primates), can infect them and cause an AIDS-like disease.^{94,127,233} In fact, the SIV of sooty mangabey monkeys

was accidentally introduced into macaque monkeys in captive United States colonies on at least one occasion and disseminated unknowingly into other macaques for more than a decade before it was discovered and eliminated.^{8,59,208} At least one clear case of laboratory-acquired infection of a human with SIVmac has been documented.^{166,325}

Cross-species transmission of SIV to baboons in the wild has been documented. Baboons do not appear to harbor their own SIV naturally. Of 279 baboon sera taken from native habitats in Tanzania and Ethiopia, 277 were negative for the presence of antibodies to SIV. However, two gave strong reactivity to SIVagm antigens.¹⁷³ One of these two was subsequently shown to harbor SIVagm sequences in stored plasma. The SIVagm sequences clustered with the vervet subtype, consistent with the known cohabitation of vervet monkeys in the same region where the baboon samples were taken.¹⁵¹ An SIVagm variant has similarly been detected in a chacma baboon of southern Africa.³⁴⁷ Baboons also can be infected experimentally with HIV-2.¹⁹⁸ Evidence for cross-species transmission of SIV from West African green monkeys (*Cercopithecus aethiops sabeus*) to patas monkeys (*Erythrocebus patas*) and to white-crowned mangabeys (*Cercocebus torquatus lunulatus*) has also been presented.^{20,334} Transmission of SIV or HIV to nonprimates has not been documented.

The origins of both HIV-2 and HIV-1 in humans are now generally believed to be from cross-species transmission. HIV-2 is endemic in western Africa and has only slowly made its way to other regions of the world. The SIV from sooty mangabey monkeys is closely related to HIV-2, has the same genome organization as HIV-2, and groups phylogenetically with HIV-2, distinct from the other 13 groups of primate lentiviruses (Fig. 51.1B). The native habitat of the sooty mangabey monkey is the coastal forest regions of western African, the same region where HIV-2 is endemic. Thus, strong circumstantial evidence involving both viral sequences and geographic colocalization link the monkey and human lentiviruses in western Africa.^{42,70,99,129} Similarly, of the four subspecies of chimpanzees, at least two (*Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii*) naturally harbor lentiviruses in the HIV-1/SIVcpz group.^{51,97,305} HIV-1s from the three divergent HIV-1 groupings M, N, and O are each more closely related to the *P. t. troglodytes* SIVcpz than to the *P. t. schweinfurthii* SIVcpz. HIV-1 groups N and O are largely restricted to west equatorial Africa where *P. t. troglodytes* are found.^{51,97} Although more work needs to be done on the lentiviruses naturally harbored by the four subspecies of chimpanzees, the evidence to date provides

TABLE 51.6 Cell Substrates for Growing Lentiviruses

Lentivirus group	Growth on Macrophages	Other cell types	Adaptation to growth
HIV-1	Yes	Stimulated, primary, human CD4 ⁺ lymphocytes	Human tumor T-cell lines
SIV	Yes	Stimulated, primary, monkey CD4 ⁺ lymphocytes	Human tumor T-cell lines
MVV/CAEV	Yes	Choroid plexus cells; primary synovial cells; endothelial cells	—
FIV	Yes	Primary, stimulated feline lymphocytes	Crandell feline kidney cells
EIAV	Yes	—	Fetal equine kidney; equine dermal
BIV	Yes	—	Bovine, rabbit, canine fibroblasts



FIGURE 51.4. Chimpanzee “Sagu” eating a leg of a red colobus monkey. (Photo by Sonja Metzger, Max-Planck-Institute for Evolutionary Anthropology. See Aghokeng et al (1) and Leendertz et al.¹⁸³ (Courtesy of the Centers for Disease Control and Prevention, Atlanta, Georgia.)

strong support for the introduction of a lentivirus from *P. t. troglodytes* (or possibly from gorilla,²³⁶) into the human population on three or more occasions for the origins of groups M, N, and O of HIV-1. Use of the hypodermic syringe, deforestation, massive migrations to urban centers in Africa, and vaccination campaigns have all been suggested as possible explanations for why these cross-species transmissions and disseminations have succeeded only recently in history.

Detailed sequence analyses have revealed that SIVcpz is more closely related to SIVgsn than to other SIVs in the 3′ half of its genome and is more closely related to SIVrcm in the 5′ half of its genome.¹¹ Furthermore, until recently, HIV-1/SIVcpz and SIVgsn/SIVmon/SIVmus were the only groups of primate lentiviruses with *vpu* genes. The natural range of the west-central African chimpanzee (*P. t. troglodytes*) overlaps that of both red-capped mangabeys and greater spot-nosed monkeys. In addition, chimpanzees are known to prey on and eat monkeys for food (Fig. 51.4). Thus, the origins of SIVcpz in chimpanzees may be connected directly or indirectly to greater spot-nosed monkeys and/or red-capped mangabeys. SIVden from *C. mona denti* has now also been found to contain a *vpu* gene, although overall it clusters more closely with the SIV from DeBrazza monkey, SIVdeb.⁶² Sorting out the origins of SIVcpz, and consequently HIV-1 in humans, is complicated by the sheer numbers of species likely to harbor phylogenetically distinct SIVs, the potential for cross-species transmission, and the potential for retroviral recombination events.³⁰⁵

Attempts to transmit HIV-1 experimentally to a variety of animal species have shown a restricted host range. HIV-1 is infectious for chimpanzees, and a chimpanzee-passaged HIV-1 has shown pathogenic potential.^{246,247} An early report described HIV-1 infection of pig-tail macaques (*Macaca nemestrina*),²

but this model has not proven sufficiently robust to date for general use.

The nonprimate lentiviruses are also restricted in their host range, limited to the same or closely related genera. BIV is a notable exception in that it has been reported to infect New Zealand white rabbits.²⁶⁴ Cross-species infections do occur, however. Cross-species infection of sheep from goats by the goat lentivirus has been observed,²⁶⁵ consistent with the high degree of genetic relatedness between the caprine arthritis-encephalitis virus (CAEV) of goats and the sheep lentivirus MVV. Studies have also shown that domestic cats can be infected by puma lentivirus (FIVpco) and by lion lentivirus (FIVple).^{349,350} No overt disease symptoms have been associated with the latter infections, but long-term studies have not been performed.

Restriction

Cellular proteins have been defined in recent years that directly interfere with different stages of virus replication. These proteins, termed restriction factors, constitute an important defense against lentivirus infection at the cellular level, collectively referred to as “intrinsic immunity.” There are presently three lentiviral restriction factors that have been extensively studied: APOBEC3G/F, TRIM5 α , and tetherin/BST-2, discovered in 2002,³⁰⁷ 2004,³²⁸ and 2008,^{238,344} respectively. Although their mechanisms of antiviral activity are diverse, they are linked to innate immunity through induction by type I and type II interferons. Lentiviruses have, in turn, evolved countermeasures to each of these restriction factors to facilitate persistent replication in their respective hosts. As a consequence of this ongoing evolutionary conflict, APOBEC3G/F, TRIM5 α , and tetherin have acquired species-specific differences that represent important host range determinants of lentiviral infection.^{222,291,292}

APOBEC3G and 3F are incorporated into virus particles in the absence of Vif and result in a producer cell-dependent block to virus infectivity.^{96,357} These cytidine deaminases introduce C-to-U mutations in minus-strand DNA during the process of reverse transcription, which are then copied into G-to-A transitions upon plus-strand DNA synthesis.^{119,377} APOBEC3G and 3F preferentially deaminate the 3' dC in CC and TC dinucleotides respectively.^{14,21,38,193,365} The antiviral activity of APOBEC3G and 3F is a direct result of the accumulation of extensive mutations in the proviral genome, a phenomenon known as hypermutation.^{120,182,206,383}

HIV, SIV, and other lentiviruses are resistant to the APOBEC proteins of their respective hosts by virtue of the ability of their Vif proteins to prevent APOBEC incorporation into virions. Vif does this by serving as an adaptor to recruit the cullin-5-elongin B/C-Rbx ubiquitin ligase complex to APOBEC3G and 3F, which leads to the polyubiquitylation and subsequent proteasomal degradation of these proteins in infected cells.^{49,211,306,378} By depleting cytosolic pools of APOBEC3G and 3F, Vif significantly reduces their encapsidation into virions.

The sensitivity of APOBEC3G to Vif-mediated degradation is often species specific.³¹⁸ HIV-1 Vif can degrade the APOBEC3G proteins of humans and chimpanzees, but not African green monkeys or rhesus macaques.²¹⁰ Similarly, SIV_{agm} Vif can degrade African green monkey APOBEC3G, but not human APOBEC3G.²¹⁰ This specificity is governed by a single amino acid difference at position 128 (aspartic acid in humans versus lysine in Old World monkeys) that is critical for binding to Vif.^{22,207,298} The failure of HIV-1 Vif to counteract the APOBEC3G proteins of Old World monkeys in part accounts for the inability of HIV-1 to replicate in these species. Likewise, the inability of SIV_{agm} Vif to counteract human APOBEC3G may pose a significant barrier to the zoonotic transmission of this group of primate lentiviruses to humans.

TRIM5 α imposes a post-entry block that represents a major host range determinant for lentiviruses as well as other types of retroviruses. This block occurs before reverse transcription and susceptibility/resistance is determined by sequences in the capsid protein.^{124,132,311} Evolutionary analyses have revealed that *TRIM5* has been co-evolving with retroviral pathogens for tens of millions of years, perhaps since the radiation of eutherian mammals.²⁹⁰ Homologs of the *TRIM5* gene have been found in the genomes of primates, cows, pigs, dogs, rabbits, rats, and mice,²⁹⁰ and anti-viral activity has been demonstrated for the TRIM5 α proteins of various species of Old and New World primates^{121,287,324} as well as for the related TRIM5 proteins of rabbits and cows.^{294,376} A common theme is that these TRIM5 proteins do not block infection by retroviral pathogens of their host, but exhibit variable patterns of restriction against retroviruses of other species.¹²¹ The underlying basis for this differential restriction is sequence variation in the B30.2 domain.^{190,235,294,368,375} One of the more peculiar twists in *TRIM5* evolution is the independent emergence of a TRIM5-cyclophilin A (TRIM5Cyp) fusion protein in at least two different primate lineages. Owl monkey cells exhibit a potent post-entry block to HIV-1 infection due to a TRIM5Cyp fusion protein resulting from the LINE-1-mediated retrotransposition of an open reading frame for *CypA* into the intron between exons 7 and 8 of *TRIM5*.^{243,293} A similar TRIM5 Cyp fusion was identified in Old World primate species of the macaque genus, including rhesus macaques, pig-tailed

macaques, and cynomolgous macaques.^{27,191,241,355,369} In this case, the CypA insertion occurred in the 3' UTR of *TRIM5* and is linked to a second mutation in the splice acceptor site of exon 7 favoring splicing of the mRNA transcript to the downstream open reading frame for CypA. Macaque TRIM5Cyp poorly restricts HIV-1, but does block infection by other lentiviruses, including SIV_{agm}, HIV-2 and FIV.^{355,369} The functional blocks to lentiviral infection imposed by these TRIM5Cyp proteins, which clearly represent distinct retrotransposition events, are believed to be the result of convergent evolution.

Sequence analyses have also revealed extensive *TRIM5* polymorphism in rhesus macaques and sooty mangabeys.²⁴⁰ Clusters of synonymous and nonsynonymous nucleotide substitutions were identified in regions of the gene coding for the CC and B30.2 domains with multiple alleles present at high frequencies in both species.²⁴⁰ Moreover, a number of specific polymorphisms were found in the *TRIM5* genes of both rhesus macaques and sooty mangabeys, despite an estimated divergence time of over 8 million years.²⁴⁰ Sequence variation in the B30.2 domains of these species' TRIM5 α proteins was also shown to result in the differential restriction of a number of lentiviruses, including HIV-1, HIV-2, FIV, and ELAV.^{240,368} These observations imply that balancing selection has acted over millions of years to preserve ancient TRIM5 α polymorphisms with functional diversity in their ability to block virus infection in Old World monkeys.

TRIM5 polymorphisms have been found to account for the highly variable course of infection for SIV_{sm}E543-3 in rhesus macaques. In contrast to SIV_{mac}239, which consistently results in high viral loads with minimal animal-to-animal variation, viral loads in SIV_{sm}E543-3-infected rhesus macaques are highly variable. Kirmaier et al. found that this variation is strongly associated with differences in *TRIM5* genotype.¹⁷⁰ The resurgence of SIV_{sm}E543-3 replication in animals with restrictive TRIM5 alleles was also associated with an arginine-to-serine change at position 97 in capsid (R₉₇S) corresponding to the residue present at this position in SIV_{mac}239.¹⁷⁰ Thus, while SIV_{mac}239 has had sufficient time to adapt to rhesus macaque TRIM5 protein, SIV_{sm}E543-3 evidently has not. These observations illustrate the influence of TRIM5 polymorphisms on the differential suppression of a virus that has not fully adapted to its host.

Efforts to determine the role of the HIV-1 Vpu protein in virus release led to the identification of tetherin (BST-2 or CD317) as an interferon-inducible host-cell factor that interferes with the detachment of virus particles from infected cells.^{237,238,344} Tetherin is a type II integral membrane protein with a topology that allows both ends of the molecule to be anchored in lipid membranes.¹⁷⁴ It has an N-terminal cytoplasmic domain, a transmembrane domain, an extracellular coiled-coil domain and a C-terminal glycosyl-phosphatidylinositol anchor.¹⁷⁴ Tetherin is upregulated in response to interferon and becomes incorporated into virus particles as they attempt to bud from the surface of infected cells.^{89,116,260} Captured virions are then internalized and routed for lysosomal degradation.^{226,260}

The primate lentiviruses have evolved at least three different viral gene products to overcome restriction by tetherin. Whereas HIV-1 Vpu and HIV-2 Env antagonize human tetherin,^{180,238,344} most SIVs, including members of the phylogenetically diverse SIVcpz, SIV_{agm}, and SIV_{smm} lineages, use Nef to counteract the tetherin proteins of their nonhuman primate

hosts.^{149,289,382} An exception are the SIVs of Old World monkeys that contain a *vpu* gene (SIVgsn, SIVmon, SIVmus and SIVden), which use Vpu rather than Nef to counteract the tetherin proteins of their respective hosts.²⁸⁹ Tetherin antagonism by HIV-1 Vpu depends upon a physical interaction between the membrane-spanning domains of Vpu and tetherin^{147,281} and in part on the recruitment of the β TrCP-2 component of the Skp1-Cullin1-F-box ubiquitin ligase complex, nonlysine ubiquitylation of tetherin, and ESCRT-mediated trafficking of tetherin for lysosomal degradation.^{79,148,205,225,333} In contrast, tetherin antagonism by HIV-2 Env involves a physical interaction between the extracellular domains of Env and tetherin, and the internalization and sequestration of tetherin within the *trans*-Golgi network, without degradation, by a pathway that depends on a conserved tyrosine-based endocytosis motif in the cytoplasmic tail of gp41.^{122,180} The mechanism of antagonism by Nef remains to be defined, but it appears to involve the downregulation of tetherin from the surface of infected cells.^{149,302}

In accordance with a now familiar theme, resistance to tetherin is species dependent. HIV-1 Vpu counteracts human, chimpanzee, and gorilla tetherin, but is ineffective against the tetherin orthologs of Old World monkeys.^{149,222,289} Conversely, the Vpu proteins of SIVgsn, SIVmon and SIVmus counteract the tetherin proteins expressed by various species of Old World monkeys, but are unable to counteract great ape tetherin.²⁸⁹ Likewise, the Nef proteins of SIVcpz, SIVsmm/mac and SIVagm counteract the tetherin proteins of their respective hosts, and with varying efficiency, the tetherin proteins of other nonhuman primates.^{149,289,382} However, these Nef proteins are universally ineffective against human tetherin.^{149,289,382} The specificity of Vpu reflects amino acid variation in the transmembrane domain of tetherin and corresponding variation in the transmembrane domain of Vpu.^{149,222,289} In contrast, the specificity of Nef for nonhuman primate tetherin is dependent on a five amino acid sequence in the cytoplasmic domain (G/D₁₄DIWK₁₈ in chimpanzee, rhesus macaque, and sooty mangabey tetherin) that is missing from human tetherin.^{149,382}

The absence of sequences in human tetherin that confer susceptibility to Nef has had a profound effect on the evolution of HIV-1 and HIV-2. Since HIV-1 arose from the cross-species transmission of SIVcpz from chimpanzees to humans, and SIVcpz uses Nef to antagonize tetherin, the absence of sequences in human tetherin that confer susceptibility to Nef explains why HIV-1 Vpu acquired this activity in humans. However, this function appears only to have been acquired by the Vpu proteins of the pandemic HIV-1 group M viruses, but not by the nonpandemic HIV-1 group N or O viruses.²⁸⁹ It has therefore been suggested that antagonism of tetherin by Vpu may have contributed to the global spread of HIV-1 group M.²⁸⁹ A similar scenario may explain the role of HIV-2 Env in the antagonism of tetherin. Since HIV-2 arose from the cross-species transmission of SIVsmm from sooty mangabeys to humans, and this virus does not have a *vpu* gene, the inability of Nef to antagonize human tetherin may also account for the selective pressure for HIV-2 Env to acquire this activity.

Compensatory changes in the cytoplasmic tail of gp41 that restore resistance to tetherin were recently identified in a *nef*-deleted strain of SIV that acquired a pathogenic phenotype after serial passage in rhesus macaques. Similar to HIV-2

Env antagonism of human tetherin, these changes result in a physical interaction with rhesus tetherin and facilitate virus release by a mechanism that depends on a conserved tyrosine-based endocytosis motif in the gp41 tail.³⁰² However, unlike HIV-2 Env, these changes afford resistance to rhesus tetherin, but not to human tetherin, by stabilizing a selective physical interaction that depends on residues in the cytoplasmic domain of rhesus tetherin.³⁰² These observations are analogous to the adaptation of HIV-2 Env for antagonism of human tetherin, and imply that antagonism of tetherin is important for virus replication *in vivo* and ultimately for lentiviral pathogenesis.

Receptor Use

HIV-1 uses a sequential two-receptor system that includes both CD4 and a seven-membrane-spanning chemokine receptor for entry into target cells (see Chapter 49). The SIVs that have been examined to date are similar to HIV in their use of both CD4 and chemokine receptors for entry. Like HIV-1, the SIVsm/SIVmac/HIV-2 and SIVagm groups of viruses appear to use CCR5 as the principal second receptor. However, a variety of other chemokine receptors may be used, depending on the virus and the isolate. In addition to CCR5, assorted SIVs have been shown to use CCR2b, CCR3, STRL33 (Bonzo), GPR15 (Bob), and GPR1. The SIVsm/SIVmac/HIV-2 group of viruses in general appears to show less dependence on CD4 for a larger percentage of isolates than does HIV-1.⁸⁴ Use of CXCR4 as second receptor also appears to occur much less frequently with SIVsm/SIVmac than with HIV-1. SIV isolates from red-capped mangabeys (*Cercopithecus torquatus torquatus*) appear to be unusual in their predominant reliance on CCR2b as a second receptor.⁴⁰

Not only does FIV use a two-receptor mechanism to infect cells, it shares with HIV the use of a chemokine receptor (CXCR4) as second receptor for entry.^{267,277,367} FIV infection is modulated by soluble stromal cell-derived factor, the natural ligand for CXCR4¹³⁴ and is inhibited by the CXCR4 antagonist, AMD3100. However, the feline lentivirus uses the T-cell activation marker CD134 as a primary binding receptor rather than CD4.^{64,312} CD134 is a member of the TNF receptor superfamily and has the typical 4-domain structure. The outermost domain is responsible for receptor activity and as few as 5 amino acid changes can make the human homolog a viable receptor for FIV.⁶³ At least certain lion lentiviruses also use CD134 and CXCR4 sequentially for entry,²²⁰ but an unidentified distinct mechanism of entry may be used by the puma lentivirus.^{220,319} The two-receptor mechanism used by the primate lentiviruses and FIV allows the virus to mask critical regions of the envelope protein from the host's immune system in that binding to the primary receptor (CD4 or CD134) causes a conformational change in the envelope to expose sensitive regions involved in high-affinity binding to the chemokine entry receptor. These sensitive regions are thus only exposed transiently to the humoral immune system.

The only other nonprimate lentivirus for which information is available regarding receptor usage is ELAV. The equine virus also uses a member of the TNF receptor superfamily as a receptor, termed ELRI, that is expressed on the primary target for this virus, the macrophage.³⁸⁰ It has been suggested that ELRI may be the only receptor required for infection with the equine virus,^{380,381} with infection occurring "from within"

through vesicles in a pH-dependent manner.²⁸ However, the extent to which EIAV uses one versus a sequential two-receptor mechanism for entry remains to be determined.²⁹

Little information is currently available about the receptors used by other nonprimate lentiviruses. The immediate questions regarding receptor use by lentiviruses are obvious ones, but the answers will be instrumental in our understanding of the evolution and logic of receptor use. Do all lentiviruses use a two-receptor system for entry into target cells? Is the second receptor always a seven-membrane-spanning chemokine receptor? Is use of a chemokine receptor a theme common to all lentiviruses? What is the nature of the other primary receptors that are used? Did evolutionary changes result in a switch from a one-receptor system (chemokine receptor) to a two-receptor system (X + chemokine receptor), or a switch from one two-receptor system (X + chemokine receptor) to another (CD4 + chemokine receptor)?

Germline Integration

Many retroviruses not only infect their hosts exogenously but also are inherited in Mendelian fashion, either as highly defective genomes, single LTRs, or in the case of the murine and feline gammaretroviruses as inducible infectious agents. However, endogenous genomes of lentiviruses have not been found in the germlines of most species that have been examined, including humans and nonhuman primates; thus, such germline integration must be a much rarer event for the lentiviruses. There are, however, now two examples of germline transmission of nonreplicating lentiviral elements. The first was noted in the European rabbit (*Oryctolagus cuniculus*), with unmistakable lentivirus characteristics, yet totally unique from any known “modern” lentivirus in overall structure.¹⁵⁹ The second was identified in the genome of the grey mouse lemur (*Microcebus murinus*) of Madagascar.¹⁰⁷ Both of these primordial lentiviruses offer a plethora of information relevant to better understanding of how long lentiviruses have been around as infectious agents, the nature of lentiviral genome plasticity, and the selective forces that may have shaped lentiviral evolution in the past.

Analysis of the rabbit endogenous lentivirus termed RELIK (rabbit endogenous lentivirus type K) revealed a tRNA_{Lys} primer binding site, a hallmark of lentiviruses and, in addition to *gag*, *pol*, and *env*, evidence for both *tat* and *rev* genes. The genomic organization for RELIK is the simplest of known lentivirus structures, with greatest similarity to EIAV based on the presence of a gene encoding dUTPase in *pol*, but lack of a *vif* gene found in other nonprimate lentiviruses. The genomic data, presence of pairs of insertions that had arisen through segmental duplication of the host genome, and the number of single LTRs present in the rabbit genome, suggests an approximate age for RELIK of 5.7 to 7 million years. This is in comparison to a calculated age for more modern transmissible lentiviruses of felines and primates of 1 to 2 million years.¹⁵⁹ A later study of an ortholog of RELIK in the European hare (*Lepus europaeus*)¹⁶¹ allowed comparisons of divergence rates in the context of two lagomorph species, yielding an approximate age for RELIK of 12 million years! Thus, lentiviruses, formerly thought to be of relatively recent origin, may be much older.

The lemur endogenous element likewise showed characteristics that identified it as a lentivirus, with evidence that it represents the primordial ancestor to modern SIVs.¹⁰⁷ The virus, termed pSIVgml (grey mouse lemur prosimian immunodeficiency virus), has features more resembling the complex lentiviruses, including *vif*, *tat*, and *rev* genes but lacks evidence for *vpr* and *vpu* genes. A putative 3′ gene is present in the relative position of *nef*, but it bears no identifiable sequence relationship.

The overall genomic structure puts pSIVgml closer to FIV than to the other nonprimate lentiviruses. Of particular relevance to lentiviral evolution, pSIVgml has a gene for dUTPase between the RNase H and integrase coding regions of *pol*. This characteristic is lacking from the modern primate lentiviruses but present in the nonprimate lentiviruses. The presence of dUTPase establishes with good authority that modern primate lentiviruses lost coding capacity for this gene product as opposed to never having acquired it. As with the rabbit lentivirus, the presence of identical viral elements in segmental duplications, frequency of single LTRs, and lack of heterozygous insertion sites arising from homologous recombination is consistent with great antiquity for the virus in the lemur genome. By comparing to estimated rates of mouse and human neutral evolution, the authors calculate that pSIVgml is 1.9 to 3.8 million years old. Given the likely introduction to Madagascar via the last mammalian terrestrial incursion, the evidence suggests that lentiviruses have circulated in the population at least 14 million years. These forays into paleovirology will continue to be of value in better understanding the origins and evolutionary history of the lentiviruses.

PATHOGENESIS AND PATHOLOGY

Portals of Entry

The primary modes of natural transmission of the nonhuman lentiviruses vary considerably with the virus. EIAV may be the most interesting because it is the only lentivirus for which there is good evidence for vector-borne transmission. During disease episodes, levels of infectious virus in the plasma of horses can exceed 10⁴/mL. The horsefly appears to be more efficient than mosquitoes, fleas, or other insects for being able to transmit EIAV. Transmission has been documented by following a single horsefly that had taken a blood meal on an infected pony during an acute clinical period.¹⁴⁶ Transmission via blood can also be mediated by inappropriate veterinary practices with needles or scalpels.

Work on natural modes of infection has demonstrated the capability of both vertical and horizontal transmission for FIV, MVV/CAEV, and EIAV.³³ Vertical transmission can occur *in utero*, during parturition, and postnatally via milk. This vertical transmission by these viruses parallels that observed with HIV in humans. Contact transmission also is common, particularly for MVV/CAEV and EIAV, when animals are herded closely together in barns or stables. These viruses can be found in semen, lung excretions, and saliva. For cats, bite wounds are believed to be the most important route of transmission in adult animals.³⁷³ Transmission via grooming/licking has also been observed,⁶⁶ and consistent experimental infection of cats can be achieved via oronasal administration. FIV infection is much more prevalent in free-roaming males than in females, consistent with increased fighting and biting among male cats. Cats allowed to roam free in areas with high cat density are at greatest risk of becoming infected.

Information about natural modes of SIV transmission has been harder to come by. One study of wild grivet monkeys in Awash National Park in Ethiopia analyzed SIVagm serologic

status with age, sex, and risk.²⁶² Infection was nearly universal in females of reproductive age and nearly absent in younger females. In males, infection was observed in only those that were fully adult. The findings support a predominantly sexual mode of SIV transmission among grivets. Male-to-male transmission by aggressive contacts may also be a prominent mode by which SIV can be spread.²³⁹ Maternal–infant transmission of SIV has been documented in captive animals.¹⁷¹

Experimental infection of laboratory animals has been commonly achieved by direct needle inoculation. However, mucosal exposure is now being used more frequently, particularly for vaccine studies, as a model for the most common routes of HIV transmission.

Cell and Tissue Tropism

Despite the varied modes of transmission, all lentiviruses are disseminated to an assortment of tissue sites by the blood. A virus can be disseminated as a free virus or, more likely, by infected blood monocytes or lymphocytes. Differences in the cell tropism of primate versus nonprimate lentiviruses relate largely to receptor use discussed earlier. Infection by the primate lentiviruses SIV and HIV is seen in CD4⁺ lymphocytes and macrophages, with CD4⁺ lymphocytes vastly predominating in terms of numbers of infected cells. For the nonprimate lentiviruses, infection of macrophages typically predominates, but infection can be seen in a greater diversity of cell types. Replication in tissue macrophages is a unifying feature of all lentiviruses.

The principal anatomic sites for localization of MVV and CAEV have varied with the study and strain of virus used. Because many studies of virus localization have used experimental infection, the origin of the infecting strain and type of cell in which it was grown must be taken into account when interpreting the results of these studies. Various reports have localized MVV principally to lungs, mammary glands, joints, lymph nodes, the spleen, and the brain. The principal target cell in which MVV and CAEV sequences can be found *in vivo* is consistently the tissue macrophage. However, in keeping with the broad range of cell types that can support replication in culture, evidence has been presented for MVV/CAEV replication in other cell types in a less prominent fashion, including epithelial and fibroblast cells in choroid plexus, intestine, and kidneys. An important observation first made with MVV is that although virus may reside in the relatively undifferentiated monocytes in peripheral blood, MVV expression is greatest in the differentiated tissue macrophages.^{104,259} This led to the concept of the “Trojan horse” for MVV and other lentiviral infections, whereby undifferentiated monocytes in the peripheral blood may carry the viral genome without sustaining viral replication until transported to tissues and differentiated into their mature form, the tissue macrophage.

Anemia caused by EIAV is hemolytic and results from the formation of antigen–antibody complexes that can associate with the surface of erythrocytes. The kidney also is affected by this complex formation. A variety of studies, both old and new, have shown the tissue macrophage to be the major cell type producing EIAV.^{221,248,301} Virus production has been noted in the spleen, liver, kidney, lymph nodes, lung, heart, brain, stomach, bone marrow, thymus, adrenals, and intestine. Although EIAV can be adapted to replicate on fibroblasts, the virus that comes out of horses during viremic episodes is derived principally from macrophages.

FIV appears to be unusual among the nonprimate lentiviruses in that the virus has been more consistently found in a broader range of cell types, particularly lymphocytes, in addition to macrophages. Analyses of tissue and cellular localization of FIV have principally used experimentally infected cats and FIV grown in lectin-stimulated peripheral blood mononuclear cell (PBMC) cultures in the presence of interleukin-2. However, studies with naturally infected cats have yielded similar findings. FIV has been found in a variety of cell types including CD4⁺ T lymphocytes, B cells, CD8⁺ T cells, macrophages, bone marrow–derived cells and cells of the central nervous system (CNS). Macrophages, microglia, and astrocytes, but not neurons, in the brains of cats have been found to be targets of FIV infection.⁸⁰ The propensity of FIV to replicate in and deplete CD4⁺ T lymphocytes now makes sense in the light of use of the activated T-cell marker CD134 (OX40) as primary binding receptor and CXCR4 as second receptor for entry into cells.^{63,64,312}

The major sites of pathogenic SIV replication early after infection have been localized to gastrointestinal (GI) tract, thymus, spleen, and other lymphoid tissue.^{176,351} SIV was localized at these early time points within periarteriolar lymphoid sheaths in spleen, paracortex of lymph nodes, and medulla of the thymus. Many early studies overlooked the fact that the GI tract contains most of the lymphoid tissue in the body. Further, the T lymphocytes are activated and express CCR5 in great abundance. SIV infection of rhesus monkeys results in profound and selective depletion of CD4⁺ T cells in the intestine within days of infection, before any such changes are evident in peripheral lymphoid tissues. The loss of CD4⁺ T cells in the intestine coincides with productive infection of large numbers of mononuclear cells at this site. Thus, the GI tract appears to be a major target for SIV replication and the major site of CD4⁺ T-cell loss early in SIV infection.^{351,352} It is now clear that SIV replicates principally or exclusively in CD4⁺ CCR5⁺ cells, which represents a subset of memory T cells.¹⁸⁹ One exception to the predominant use of CCR5 is the X4-using SIV strain SIVmac155T3.²⁶³ CD4⁺ CCR5⁺ lymphocytes predominate in the gut and other mucosal sites and are present at much lower frequency in peripheral lymphoid organs. Subsequent to these pioneering studies with SIV in monkeys, the gut-associated lymphoid tissues were also shown to be a major site of HIV-1 replication during acute infection in humans.^{26,224} The predominate replication in the human gut is again associated with the marked preference for CD4⁺ CCR5⁺ memory cells.²¹⁷ Within the thymus, marked depletion of thymic progenitors has been noted by 21 days after rhesus monkey infection with pathogenic SIV; this depletion is followed temporally by increased levels of cell proliferation in the thymus and a marked rebound in thymocyte progenitors.³⁷² The distribution of virus within lymphoid organs varies with the inoculum. SIV also can be found in the CNS early after infection. The infected cells in the brain, whether early after infection or late in SIV-induced encephalitis, are primarily cells of monocyte/macrophage lineage.²⁸⁸ These may include perivascular monocyte/macrophages that have migrated from the blood or the resident microglial cells.

Immune Responses and Persistence

Monkeys infected with SIV and animals infected with other lentiviruses typically make strong antibody and cytotoxic T lymphocyte (CTL) responses to the virus. These immune responses persist at high levels for the lifetime of the infected host, whether

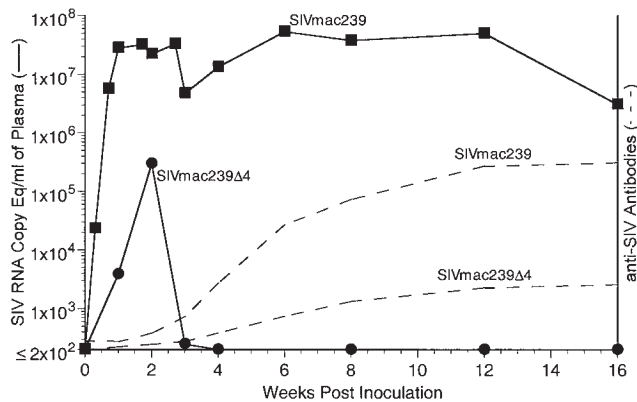


FIGURE 51.5. Attenuation by auxiliary gene deletion lowers viral load and reduces the strength of the antiviral antibody response. SIVmac239Δ4 has inactivating deletions in *vpr*, *vpx*, and two in *nef*.

infection is by natural or experimental means. Approximately 20% to 30% of macaque monkeys infected with some AIDS-causing strains of SIV develop a more rapid disease course, make few or no antibodies to the virus, and die with AIDS in 3 to 7 months. The deficiency in anti-SIV antibodies in such animals does not appear to be a matter of antigen excess because B cells from these rapidly progressing monkeys make few or no anti-SIV antibodies.⁸³ The strength of anti-SIV antibody responses also varies with the virulence of the infecting strain (Fig. 51.5). Deletion mutants of SIV that are progressively more attenuated on the basis of viral load measurements elicit progressively weaker anti-SIV antibody responses.⁶⁹ CTL responses have been shown to be major histocompatibility complex (MHC)-restricted for both SIV and animal lentiviruses. A considerable amount of new information has now emerged about MHC alleles in rhesus monkeys and the peptides that they present, which is facilitating detailed work on CTL responses.

The importance of CD8⁺ lymphocytes in limiting the extent of replication of SIV or SIV/HIV recombinant (SHIV) has been definitively demonstrated by using the approach of CD8-cell depletion.^{216,296} Extensive depletion of CD8⁺ cells was achieved by intravenous administration of large doses of specific anti-CD8 monoclonal antibodies. Depletion was not permanent because CD8⁺ lymphocytes returned in the absence of continuous monoclonal antibody. When CD8⁺ cells were depleted during primary infection, virus replication proceeded unabated after peak loads 10 to 14 days after infection; this contrasts with nondepleted animals, in which immune responses typically result in reductions in viral loads beyond 14 days.^{216,296} Elimination of CD8⁺ lymphocytes from monkeys during chronic SIV infection resulted in a rapid and marked increase in viremia that was again suppressed coincident with the reappearance of SIV-specific CD8⁺ T cells.²⁹⁶ Depletion of CD8⁺ T cells in SHIV-infected animals has facilitated the appearance of more highly pathogenic variants.¹⁴¹

In SIV-induced disease in macaque monkeys with wild-type strains of SIV, viral-specific, proliferative responses of CD4 cells are typically weak or absent. However, infection by attenuated SIV mutants deleted in *nef* results in strong, SIV-specific, CD4 helper cell proliferative responses.^{101,102} This situation appears analogous to HIV infection of humans in which

HIV-specific CD4 proliferative responses are usually weak or absent in typical progressors but are often strong in unusual nonprogressors that are controlling their infection.^{282,283} The picture that emerges with these primate CD4 receptor-using viruses is one of a battle during the early stages of infection. CD4 helper cells try to respond to sites of infection, but these activated CD4 cells at the sites of infection are the ideal targets for replicating virus. In pathogenic infections, the virus wins this early battle and eventually wins the remainder of the war. SIV-specific CD4⁺ helper cell responses have been observed in nonpathogenic infection of natural hosts, but their effectiveness is uncertain.^{82,360}

Lentiviral persistence is achieved via chronic active viral replication. The clearest demonstrations of this are with HIV in humans and SIV in macaque monkeys; during months or years of chronic infection, millions of viral particles and millions of infected cells are produced and turned over every day.^{131,362} This does not mean, however, that no cells are infected in a quiescent or latent fashion; some certainly are. The degree of chronic active replication also varies with the strain of virus and the host. The long-term persistence of antibody and CTL responses at high levels is consistent with the prolonged antigen expression of chronic active replication. Even nonpathogenic derivatives of SIV continue to replicate at low levels over prolonged periods, as evidenced by the evolution of sequence changes, persistence of antibody, and other criteria.

EIAV is unusual among the lentiviruses for the episodic nature of the persistent viral replication. Infection of horses with EIAV is associated with recurring episodes of fever, anemia, and thrombocytopenia. These episodic occurrences of clinical disease coincide with bursts of new virus replication, which may be weeks or months apart. Episodes of new virus replication yield EIAV variants that are not neutralized by the horse's own serum; however, the same serum is capable of neutralizing the EIAV variants from earlier episodes.²²⁸

The dilemma of lentiviruses is their ability to replicate persistently in the face of apparently strong immune responses. The levels of anti-HIV antibody and CTLs have been precisely measured and are enormously high. The strategies used by lentiviruses to allow persistent viral replication in the face of these apparently strong immune responses have been reviewed.^{72,87,156} These are summarized in Figure 51.6 and in points 1 through 5 below.

1. **Differentiation- or activation-dependent expression:** Like all retroviruses, the lentiviruses integrate a proviral DNA copy of their genetic information into the host cell DNA. This proviral DNA may lie dormant for prolonged periods and can be reactivated at a later date when conditions may be more suitable. Triggering events for activation of lentivirus expression can include cellular activation of lymphocytes or macrophages by natural stimuli (antigen, cytokines) and differentiation of monocytes into tissue macrophages, as originally promulgated in the Trojan horse concept.
2. **Emergence of antigenic variants:** As RNA viruses, the lentiviruses have an error-prone reverse transcriptase (RT) for replicating their genetic information. The emergence of mutant forms of virus (within a single infected individual) that are antigenic variants that escape ongoing neutralizing antibody responses was first described with EIAV, MVV, and CAEV. Subsequent work has provided exquisite detail

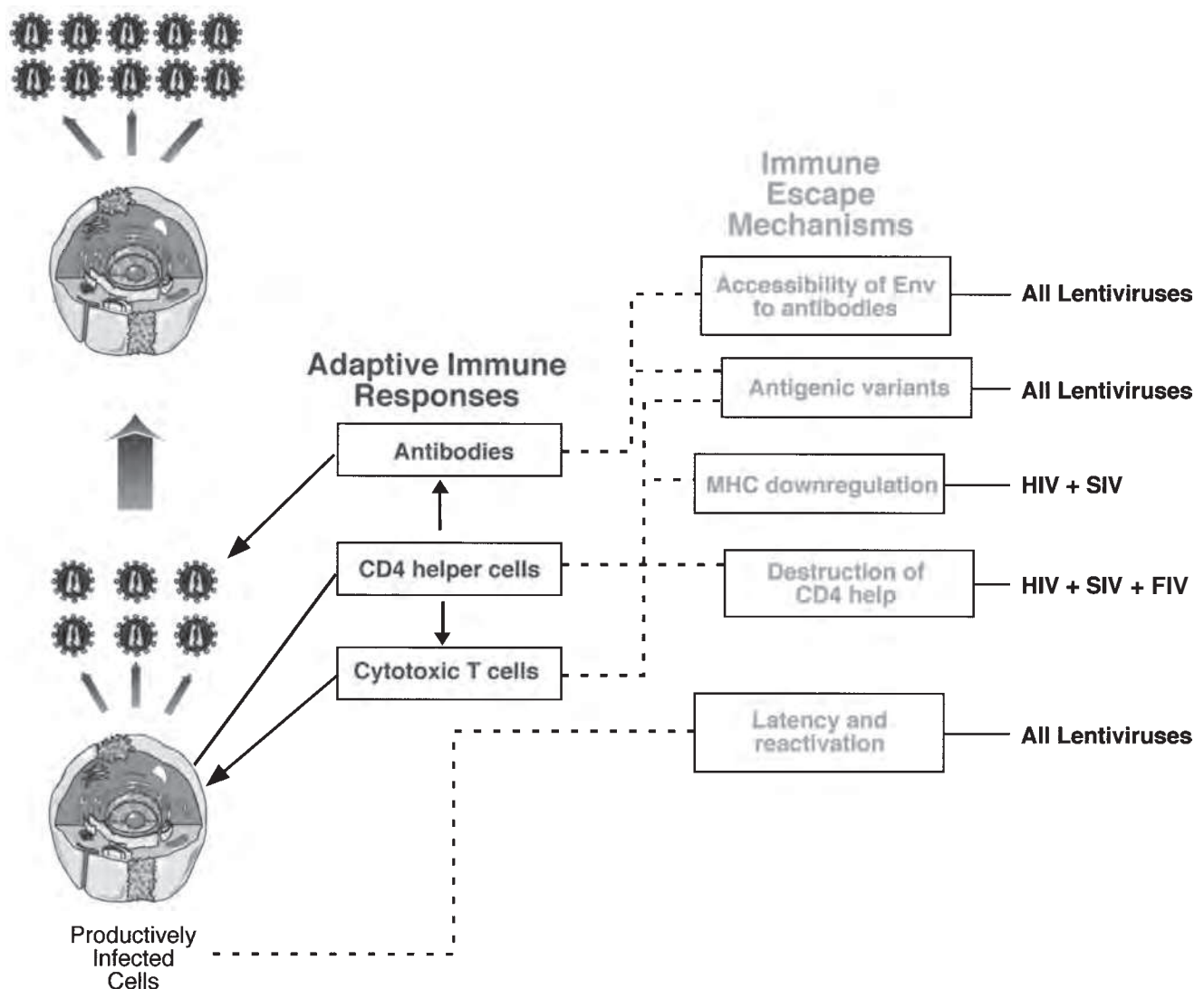


FIGURE 51.6. Human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and other lentiviruses use a variety of strategies to avoid both humoral and cellular responses.

on the emergence of SIV and HIV variants that escape both neutralizing antibody and CTL responses.^{24,31,32,88,278,361}

3. *Shielded nature of external surface glycoprotein:* The surface proteins of all lentiviruses are heavily glycosylated; in fact, the external surface glycoproteins (GPs) of lentiviruses are among the most heavily glycosylated mammalian cell proteins known. The carbohydrate groups help to serve as a barrier that limits the neutralizing antibody response to virus. Strains of SIV lacking specific carbohydrate attachment sites are more effective targets for antibody-mediated neutralization and some are able to elicit considerably higher titers of neutralizing antibodies.^{35,43,275} As mentioned previously, the two-receptor mechanism for attachment and entry also allows a certain degree of shielding of internal sites that are conserved in sequence.

The only lentiviral surface GPs for which three-dimensional data are available are HIV-1¹⁷⁵ and SIV.³⁹ Although the structures lack variable loop sequences and carbohydrate to varying degrees, a compilation of antibody mapping

and structural and functional data have revealed interesting features that reflect on the replication strategy. The CD4-binding site is contained within a difficult-to-access pocket, and the variable loops and carbohydrate appear to act largely as a shield to protect regions of the protein that would otherwise make the virus sensitive to antibody attack. The envelope proteins of HIV and SIV are packed tightly in a trimer to form the spike on the surface of virions. While monomeric gp120 is readily reactive with glycosidases and antibodies, gp120 as it exists on the surface of virions is difficult for glycosidases and antibodies to access.^{223,254} Although infected people develop high-titer antibody responses reactive with monomeric HIV-1 envelope protein, these antibodies apparently react poorly or not at all to envelope protein as it exists on the surface of virions. These antibodies in general have poor neutralizing activity.

4. *Destruction of CD4 help:* This immune evasion strategy appears to be applicable only to the primate lentiviruses, which use CD4 as a primary receptor, and FIV, which uses

CD134 as a receptor. The early destruction of virus-specific CD4 helper cell activity leaves the host without appropriate help for its B-cell and CD8-cell responses.⁷⁸

5. **MHC downregulation:** The primate lentiviruses encode an early gene product, Nef, that downregulates MHC class I molecules from the surface of cells, making them less-sensitive targets for CTL attack.^{48,300,329}

Other RNA viruses with error-prone polymerases, such as poliovirus, seem nowhere near as difficult to neutralize and nowhere near as malleable as HIV. The basis for these differences may lie in the inherent strategies used by persisting versus nonpersisting viruses. The survival of any virus depends on its ability to spread through the population. For a nonpersisting virus such as poliovirus, transmission is typically limited to the several weeks after the time of primary infection. Resistance to antibodies, once they appear, may not be so important to such a virus. Instead, it is important to replicate maximally for the brief period to maximize the likelihood for transmission. HIV, SIV, and other lentiviruses, in contrast, help to ensure their transmission by greatly lengthening the period during which transmission can occur. Thus, these viruses may sacrifice inherent replicative capacity in the short term to allow persistent viral replication over the long term.

Virulence

Not all lentiviral infections are uniformly pathogenic. The various forms of SIV are in general not associated with disease in their natural hosts. Lifelong subclinical infections also have been documented for EIAV and MVV. Breeds of horses and sheep vary in their general susceptibility to EIAV and MVV, and these may even vary in the degree of susceptibility to specific disease manifestations. For example, the classic CNS form of visna infection originally observed in Iceland is only rarely seen elsewhere. Some strains of SIV are much less pathogenic than others in susceptible macaque monkeys, and some derivatives that have been specifically attenuated by deletion mutations appear to be nonpathogenic. There are a few examples of AIDS-like disease in African monkey species naturally infected with SIV.^{196,219,252}

The diseases associated with lentiviral infections are typically chronic and manifest over a prolonged period. However, there are some prominent exceptions to the classic, prolonged course of lentiviral disease. A particular strain of SIV called SIVsmPbJ14 is acutely lethal in rhesus monkeys. Monkeys infected with SIVsmPbJ14 typically die within 14 days with very high viral loads, severe GI disease, cytokine dysregulation, lymphoproliferative disease, and organ system failure.^{74,93,95,245} The unusual properties of this strain have been attributed in large part to the unusual positioning of a tyrosine at residue 17 of the nef protein.⁸¹ The occurrence of tyrosine at this position of nef creates an immunoreceptor tyrosine-based activation motif (ITAM) of the type YXXLX₇YXXL that imparts on the virus the unusual ability to cause lymphocyte activation and to replicate to high titers in lymphocytes of PBMC cultures without any prior stimulation.⁸¹ The BIV variant that is the cause of Jembrana disease in Bali cattle also is acutely pathogenic. About 17% of Bali cattle infected either naturally or experimentally with JDV die with an acute disease within the first few weeks.^{75,76,366} During acute disease, infectious titers of virus reach 10⁸/mL of plasma. JDV has remarkable similarities to the

disease induced by the tyrosine-*nef* variant of SIV, SIVsmPbJ. In JDV, there is marked enlargement of lymph nodes and spleen, which feature proliferating lymphoblastoid cells. Proliferating lymphoid infiltrates are also found in many other tissues. The disease in horses induced by EIAV also is often considered more acute than that occurring with other lentiviruses. The first episode of EIA disease usually occurs 2 to 6 weeks after the initial infection. Subsequent disease cycles are irregular, appearing weeks to months apart, and usually last 3 to 5 days. The frequency and severity of disease episodes usually decline with time. Episodes of clinical disease usually end within the first year after an average of six to eight episodes.

Everything that we know about the pathogenesis of AIDS in macaques with SIV and in humans with HIV-1 points to the importance of viral loads. High viral loads bode poorly; low viral loads predict an improved prognosis. Because sooty mangabeys and African green monkeys do not get sick from the SIVs with which they are naturally infected, most scientists assumed that the viral loads in these species would be low. Quite unexpectedly, by and large they are not.^{30,108,276} Naturally infected sooty mangabeys and African green monkeys live a normal life with plasma viral RNA loads of 10⁵ to 10⁶, viral loads that are lethal in SIVmac-infected macaques and HIV-1-infected humans. The SIVsm and SIVagm viruses infecting these species are fully capable of inducing AIDS, since they do so when passaged in macaques. A distinguishing feature of natural hosts who are able to resist disease despite high viral loads is the absence of chronic lymphoid activation. A strong case has been made that the chronic lymphoid activation that is the hallmark of SIV- and HIV-induced AIDS results at least in part from “microbial translocation,” that is, the chronic release of bacterial antigens from the confines of the gut into the circulation.²⁵

Clinical and Pathologic Features

Good reviews are available on the clinical and pathologic features of nonhuman lentiviral infections.^{33,157} Some of the most prominent features are highlighted here.

Equine Infectious Anemia Virus

Equines are the only species susceptible to natural or experimental infection with EIAV. Clinical disease is usually divided into acute, subacute, and chronic phases. Acute disease typically results in fever as high as 108°F 1 to 4 weeks after infection. Anemia is not a prominent feature at the outset. Excessive thirst, loss of appetite, weakness, depression, and hemorrhage are seen in the acute phase. The acute form may result in death. The subacute form is characterized by relapsing fever and recurrence of other signs. Recurrence of episodes may be brought on by hard work or malnutrition. In its chronic form, animals may remain thin despite adequate availability of food, and red cell counts are typically well below normal. Clinical signs in late disease and pathology findings appear to result principally from hemolytic anemia. Erythrocytes of infected horses are coated with antibodies and complement factor 3, and destruction of red cells is immunologically mediated. Osmotic fragility, shortened half-life, and phagocytosis contribute to the destruction. Bone marrow may also be depressed, but this seems less important than immune-mediated destruction. Hemorrhage, jaundice, and edema are most commonly found at necropsy. The nature and severity of the lesions will vary with course of disease and duration of the illness.

Maedi/Visna Virus and Caprine Arthritis Encephalitis Virus

Although maedi (pneumonia) and visna (wasting, depression, paralysis) were once thought to be separate diseases, it is now clear that they are both caused by the same virus. Both systems can be affected in the same animal. Maedi disease in the Netherlands has been called *zwoegerziekte*. Polyarthritis and mastitis also are seen as a result of viral infection. Disease is usually seen only in adult sheep because of the lengthy incubation period, typically 3 to 8 years. The lungs of affected sheep may

be two to five times the normal size, and they exhibit a rubbery loss of elasticity. These abnormalities result from a gross thickening of the alveolar walls from infiltration and proliferation of reticuloendothelial or mesenchymal cells that invade the septa everywhere (Figs. 51.7A and 51.7B). Lymph nodules occur along the bronchi and bronchioles. There is progressive weight loss. Dyspnea is initially apparent only after exercise, but it progresses. Severely dyspneic sheep spend much time lying down. Lesions in the brain consist of demyelination and lymphocytic infiltration (Figs. 51.7C and 51.7D). Trembling

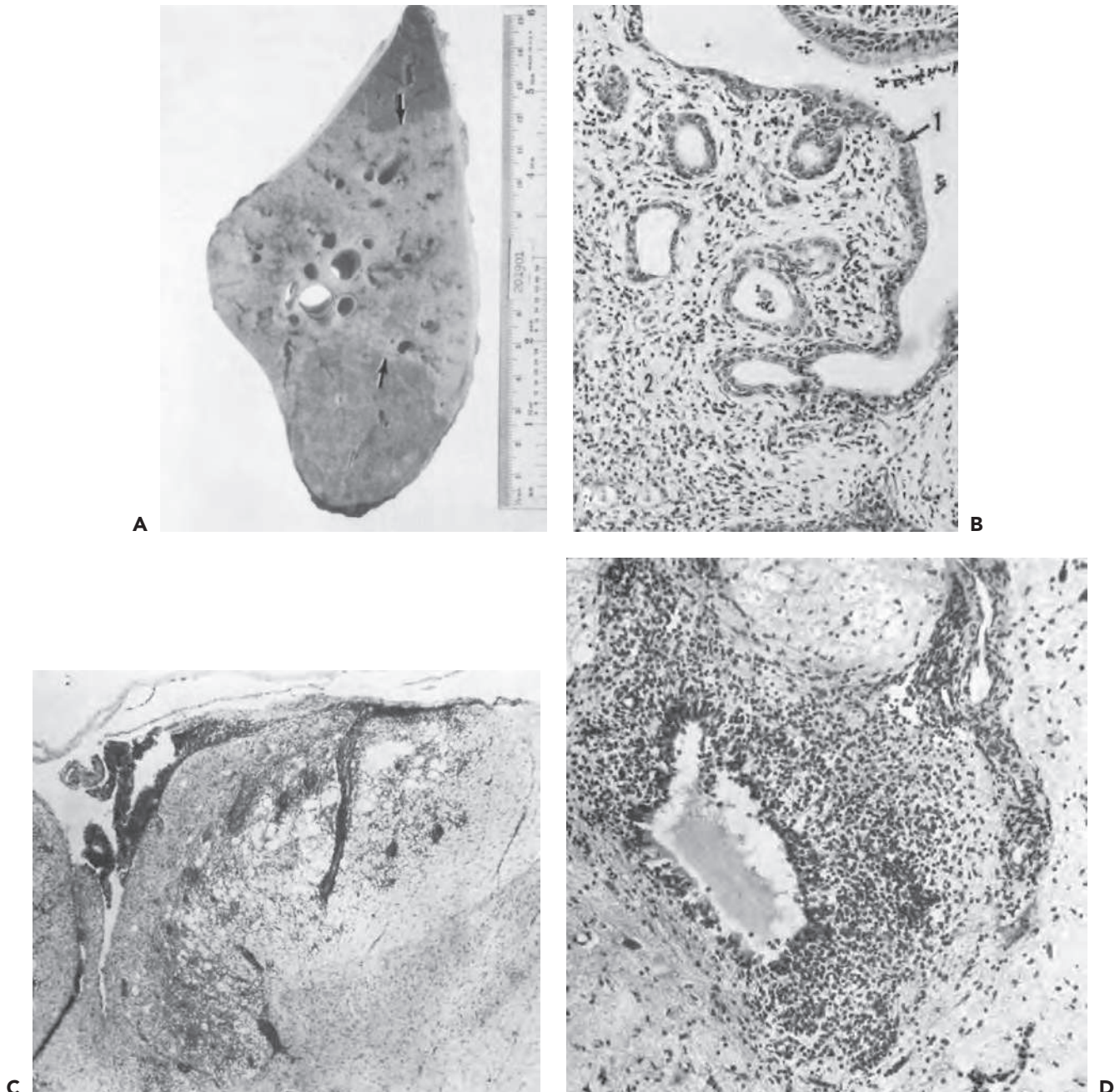


FIGURE 51.7. Pathology of maedi/visna virus (MVV) in sheep. A,B: Pneumonia. **C,D:** Encephalitis. (From Johnson W, Desrosiers RC. Viral persistence: HIV's strategies of immune system evasion. *Annu Rev Med* 2002;53:499–518.)

of facial muscles and lips may occur. The onset of clinical signs of visna usually begins with weakness of the hind legs, which eventually leads to paraplegia. Diseases caused by CAEV in goats are similar to those of MVV in sheep except that arthritis is usually most prominent and the pneumonia is usually of lesser severity. Joints are swollen and painful, exacerbated by cold weather. The basic lesion is a proliferative synovitis of joints, tendon sheaths, and bursae.

Feline Immunodeficiency Virus

Primary infection by FIV may lead to low-grade fever, generalized lymphadenopathy, and sometimes diarrhea. During the ensuing months and years, progressing disease is associated with lymphopenia, recurrent fever, lymphadenopathy, anemia, diarrhea, and weight loss of protracted duration. CD4 counts and other cell subsets may be depressed. The final stages of disease are associated with chronic secondary infections, particularly gingivitis, dermatitis, and infections of the upper respiratory tract. Opportunistic infections that have been observed include calicivirus, herpesviruses, toxoplasma, and cryptococcus. Neurologic abnormalities also have been noted, including dementia, twitching tremors, and convulsions. Pathologic lesions primarily reflect those of opportunistic infection.

Simian Immunodeficiency Virus

SIV infection of rhesus monkeys is generally considered the closest model of AIDS in humans. SIV infection of the natural host (e.g., SIVagm in green monkeys or SIVsmm in sooty mangabey monkeys) is in general not associated with any disease, although there may be occasional exceptions.^{196,219,252} When SIVsmm was inadvertently introduced into captive macaque monkey species (Asian Old World primates), AIDS-like disease and lymphomas did ensue.^{8,61,208} AIDS-like disease is generally induced experimentally in macaque monkeys with SIVmac, SIVsmm, or less frequently SIVagm. As with other lentiviruses, SIV establishes a chronic active infection with a prodromal period of months to years before clinical signs appear. Immunodeficiency is usually, but not always, associated with marked declines in CD4⁺ lymphocyte concentrations. Macrophages also are infected. Pathologically, generalized lymphadenopathy is characterized by hyperplasia and is followed by lymphoid depletion in terminal stages (Figs. 51.8A and 51.8B). The GI tract, where activated T lymphocytes predominate, appears to be the major site of early viral replication and early loss of CD4⁺ T cells (Fig. 51.8C).³⁵¹ However, CXCR4-using viruses may cause a profound loss of T cells in the periphery that is not paralleled in the intestine.²⁶³ Marked depletion of progenitor cells occurs in the thymus by 21 days; although a rebound occurs subsequently, thymic dysinvolution is typically seen at terminal stages (Figs. 51.8D and 51.8E).³⁷² Nodular lymphocytic infiltrates in a variety of tissues (Fig. 51.8C), interstitial pneumonia with syncytial cells (“giant cell pneumonia”) (Figs. 51.8H and 51.8I), and granulomatous encephalitis (Fig. 51.8F and 51.8G) are variably present. Opportunistic infections are usually present, which can influence the specific nature of the clinical signs. Common opportunistic infections include *Pneumocystis carinii* pneumonia, generalized cytomegalovirus, cryptosporidiosis, and *Mycobacterium avium*.

Many research groups have now constructed recombinant forms of SIVmac with HIV-1 *env*, *tat*, *rev*, and in some cases *vpu* genes called “SHIV” (for SIV-HIV hybrid) and selected for

pathogenic forms by passage in macaque monkeys.^{118,141,142,152,274} A uniform feature of these early, independently passaged SHIV constructions is that they rapidly and irreversibly deplete CD4⁺ T lymphocytes from the periphery.¹⁴⁰ Although most of the HIV-1 envelopes from which these SHIVs were derived are dual-tropic in that they can use either CXCR4 or CCR5 for entry in cultured human cells, in rhesus monkeys they appear to target CXCR4-expressing cells almost exclusively.²⁴² R5-using SHIVs are under development,¹¹⁸ but none have been found yet to consistently induce a chronic disease course similar to SIVmac251, SIVmac239, or SIVsmE660.

Contributions of Individual Genes and Genetic Elements

Because lentiviruses, like other retroviruses, replicate through a proviral DNA intermediate, cloned proviral DNA representing the full-length lentiviral genome can be infectious. Infectious lentiviral DNAs have been used to study the contributions to replication of open reading frames not found in other retroviruses. The unusual, persistent nature of lentiviral replication has raised speculation as to the role of auxiliary genes in the persistent replication and chronic disease of the lentiviruses. Infectious, cloned, lentiviral DNAs also have been used to gauge the relative importance and functional role of auxiliary genes in the context of experimental animal infection. The first cloned lentiviral DNA shown to be not only infectious but also pathogenic was one for SIV.¹⁶³ Infectious clones that are also pathogenic are now available for EIAV, CAEV, FIV, and other SIV isolates.^{50,66,125,255}

Deoxyuridine Triphosphatase

A dUTPase reading frame is located within the *pol* gene of the nonprimate lentiviruses and in the transition region between *gag* and *pol* in the type D beta retroviruses. dUTPase catalyzes the conversion of dUTP to dUMP and inorganic pyrophosphate. dUMP is an important precursor for dTTP, used for incorporation into DNA. dUTPase activity in the cell is believed to minimize misincorporation of dUTP into DNA, which can be mutagenic. The effects of eliminating the dUTPase gene (*DU*) have been studied in EIAV, FIV, CAEV, and visna. In all cases, *DU*⁻ virus was still replication competent in cultured cells. Loss of *DU* in EIAV and FIV had little or no effect on virus replication in permissive fibroblast cell lines that continuously divide. However, loss of *DU* in these viruses had dramatic effects on virus replication in macrophages.^{332,358} Macrophages are not dividing cells and in general have low deoxynucleotide pool sizes. *DU*⁻ EIAV exhibited five- to 10-fold lower viral loads in plasma at peak compared with the parental virus, and both EIAV and CAEV missing *DU* were attenuated for pathogenicity.^{192,358} *DU*⁻ FIV and CAEV were found to accumulate increased levels of mutations, particularly G-to-A transition mutations.^{185,340} Visna viruses lacking *DU* also showed decreased viral loads in experimentally infected sheep but still produced neuropathogenic effects upon direct intracerebral inoculation.²⁶¹

Tat and Rev

The *tat* proteins of lentiviruses can be clearly divided into two groups with respect to whether or not their transactivating activities are dependent on binding to an RNA sequence element.^{23,65,229} HIV, SIV, EIAV, and BIV all have long terminal repeats (LTRs)

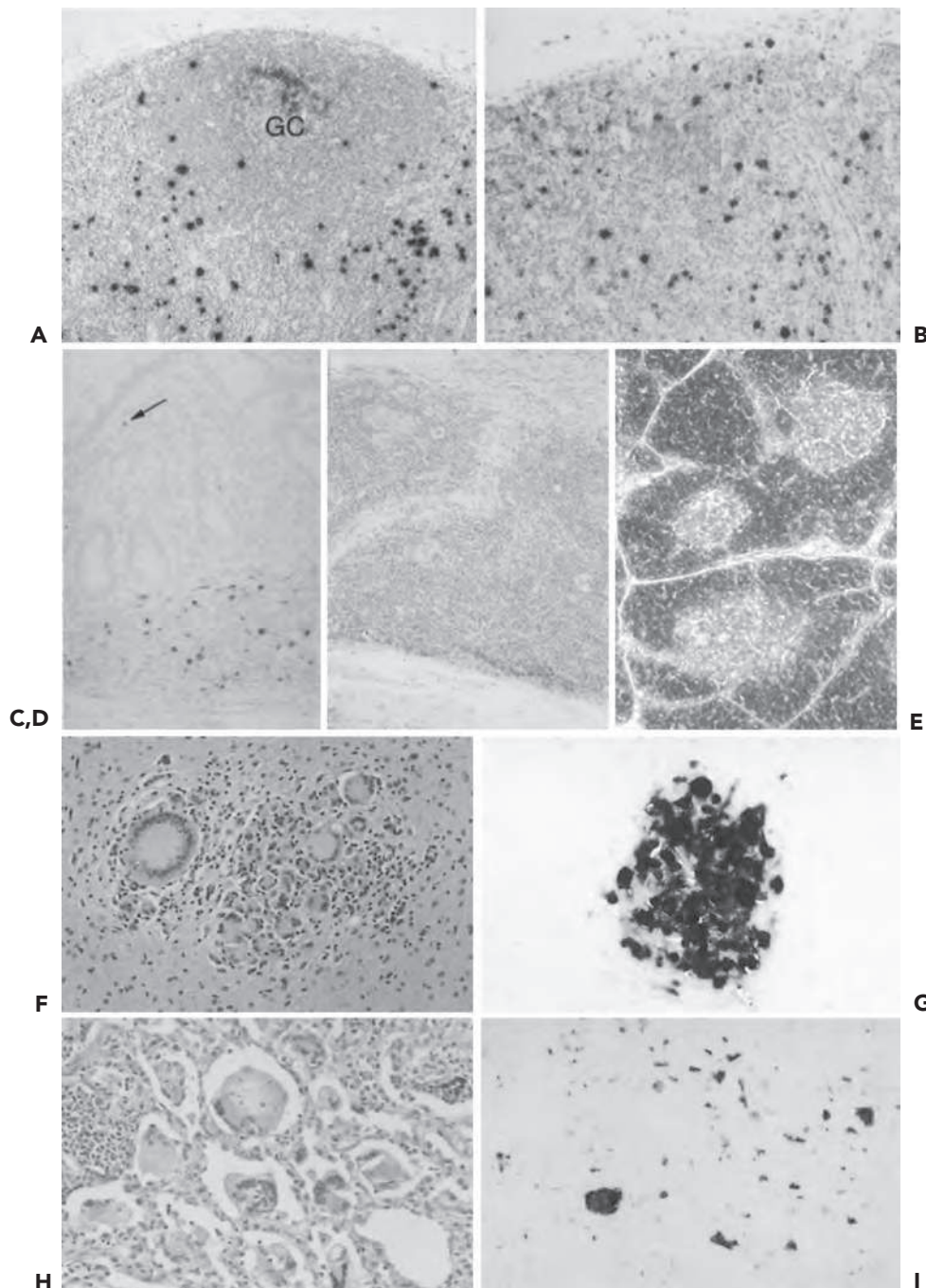


FIGURE 51.8. Acquired immunodeficiency syndrome (AIDS) in monkeys: histopathologic lesions and simian immunodeficiency virus (SIV) localization. **A–C:** *In situ* hybridization for SIV RNA in lymph nodes (**A,B**) and intestine (**C**). In **A**, numerous individual positive cells can be seen in the paracortex of the lymph node of an animal 8 weeks after infection. In addition, diffuse staining of the germinal center (**GC**) of a lymphoid follicle consistent with trapping of virus on follicular dendritic cells can be seen. Note that the lymphoid architecture is relatively intact at this early time point, in contrast to a lymph node from an animal with terminal AIDS in **B**. Although the lymph node in **B** shows severe lymphoid depletion with no evidence of lymphoid follicles, numerous SIV-infected cells can be seen. In **C**, numerous infected cells in the intestine can be seen in a submucosal lymphoid nodule with a rare positive cell (*arrow*) in the overlying lamina propria of this animal 2 months after infection. **D,E:** Thymic atrophy. Infection with SIV is associated with profound dysinvolution of the thymus (**D**), as opposed to a normal thymus (**E**) with discrete cortex and medulla. **F,G:** SIV encephalitis. Infection with SIV results in inflammation of the brain in 25% to 50% of the animals that are infected. The brain lesions are characterized by aggregates of mononuclear cells and multinucleated giant cells (**F**). Cells in the lesions contain abundant SIV nucleic acid by *in situ* hybridization (**G**). **H,I:** Giant cell pneumonia. Multinucleated giant cells in this pneumonia (**H**) as well as numerous mononuclear cells contain abundant SIV nucleic acid by *in situ* hybridization (**I**). (Courtesy of Dr. Andrew Lackner.)

with low basal activity that is strongly induced by their respective Tat proteins in a cyclin T1- and RNA sequence-dependent fashion. The LTRs of visna and FIV are strongly active in the absence of any viral proteins. Recent reports now indicate that the gene called *tat* in visna virus and OrfA in FIV may actually be more Vpr-like in their activities.^{37,103,354} FIV OrfA has now been shown to facilitate decreased CD134 receptor expression on the cell surface by sequestration in the Golgi complex,¹³³ analogous to similar activities for Vpu and Nef of primate lentiviruses. It is debatable whether there is any viral-induced transcriptional transactivation with FIV and MVV; if it exists, it is weak. BIV Tat is unusual in that it is able to bind to the RNA sequence element independent of cyclin T1, but transactivation is nonetheless dependent on the participation of cyclin T1.²³

Tat and *rev* are essential for the replication of SIV and HIV. The *rev* genes of MVV and CAEV have also been found to be essential for replication.^{297,335} *Tat* appears to be essential for replication of BIV and EIAV as well. But it is only a weak transactivator in CAEV, and it was found to be dispensable for efficient viral replication both in culture and in goats.¹¹⁷ Although *rev* is essential for SIV replication, von Gegerfelt et al.³⁵⁶ have described a strain of SIV lacking both *rev* and the *rev* response element (RRE) that becomes replication competent with the addition of a constitutive transport element (CTE). The loss of *rev* and RRE and the addition of the CTE appeared to have a significant attenuating effect.³⁵⁶ Because antibodies persisted for more than a year without decline, the *rev*⁻ RRE⁻CTE⁺ SIV appeared to have established a persistent infection but at low levels.

S2

An EIAV derivative lacking S2 replicated normally in fetal equine kidney cells, monocyte-derived macrophages, and differentiated macrophages.¹⁸⁷ The S2 deletion mutant displayed slightly decreased viral loads and thrombocytopenia-inducing potential when compared with the parental cloned EIAV.¹⁸⁶ Infected horses consistently make antibodies to S2, confirming that S2 is a bona fide virus-encoded product. Recent studies have shown that S2 causes a marked upregulation of pro-inflammatory cytokine and chemokine expression in infected macrophages, which may contribute to the rapid and early acute clinical disease unique to EIAV infection.⁵⁶

Vif

With the exception of EIAV, all lentiviruses have a *vif* gene (Tables 51.4 and 51.5). Vif is required to overcome a producer-cell block to virus infectivity imposed by members of the APOBEC family of cytidine deaminases.^{21,193,307,365,384} Accordingly, Vif is critical for virus replication *in vivo* and in cells expressing APOBEC3G and APOBEC3F.^{69,303} In the absence of Vif, APOBEC3G and 3F become incorporated into virus particles and catalyze the cytidine deamination of minus-strand DNA during the next round of reverse transcription.^{119,182,206,383} Extensive cytidine deamination of the minus strand leads to the accumulation of G-to-A transitions in the plus strand and inactivation of the viral genome as a result of catastrophic hypermutation (see previous section on Restriction). Vif counteracts this restriction by recruiting the cullin-5-elongin B/C-Rbx ubiquitin ligase complex, which mediates the polyubiquitylation and proteasomal degradation of APOBEC3G and 3F.^{49,211,306,378} By promoting the depletion

of intracellular pools of APOBEC3G and 3F, Vif prevents the incorporation of these proteins into virions, thereby subverting their antiviral activity.

Vif is important for the replication of FIV, SIV, and HIV in most cultured cells. Mutations that knock out the *vif* open reading frame of FIV drastically reduced viral replication.³⁰³ SIVmac239 with a deletion in the *vif* gene was produced in a *vif*-complementing cell line and inoculated into monkeys.⁶⁹ Inoculated animals showed no plasma antigenemia, no recoverable virus, and no SIV sequences in peripheral blood detectable by PCR. However, inoculated monkeys did develop a low-level antibody response. Thus, SIV Δ vif does appear to be infectious for rhesus monkeys, but it is the most highly attenuated strain yet studied. Similarly, knockout of the *vif* gene in FIV resulted in a high degree of attenuation even for newborn kittens.³⁰⁹

Nef

All of the primate lentiviruses, but not the nonprimate lentiviruses, have a *nef* gene (Tables 51.4 and 51.5). The 5' end of SIVmac *nef* overlaps the *env* reading frame, whereas no such overlap exists for the *nef* gene of HIV-1. Although there is correspondence in sequence for most of SIVmac Nef and HIV-1 Nef, SIVmac Nef is approximately 40 to 50 amino acids longer due to additional N-terminal sequences; these extra sequences appear to have no homologs in HIV-1 *nef*. Nef is not essential for virus replication but is important for efficient virus replication *in vivo*.¹⁶⁴ A multitude of functional activities have been attributed to Nef. These include but are not limited to: the downregulation of CD4, MHC class I and the TCR; lymphocyte activation; infectivity enhancement; and antagonism of tetherin/BST-2. HIV-1 and SIV Nef both downregulate CD4 to prevent engagement by Env on nascent virions.¹⁰⁰ Likewise, HIV-1 and SIV Nef both selectively downregulate certain MHC class I molecules to evade the recognition of infected cells by virus-specific CD8⁺ T cells, while leaving others on the cell surface to inhibit NK cell activation.^{47,68,329} However, the residues that contribute to MHC class I downregulation differ for HIV-1 and SIV Nef.³³⁰ In HIV-1 Nef, these sequences are located near the N-terminus of the molecule, whereas in SIV Nef these sequences map to the C-terminus.³³⁰ For SIVsmm/mac, HIV-2, and most other primate lentiviruses, with the possible exception of HIV-1 and SIVgsn/mon/mus, Nef downregulates the TCR through interactions with the TCR ζ chain.^{135,295} TCR downmodulation reduces the sensitivity of infected CD4⁺ T cells to activation and has been proposed to contribute to the non-pathogenic nature of SIV infection in natural hosts.²⁹⁵ An additional role for Nef in lymphocyte activation was revealed by the observation that the replication of *nef*-deleted SIV in an immortalized T-cell line was IL-2-dependent, whereas the replication of wild-type SIV, and recombinants expressing HIV-1 *nef* alleles, was not.³ Nef also enhances the infectivity of HIV-1 and SIV by a poorly understood mechanism that involves binding to Dyn2.^{44,266} In addition, the Nef proteins of most SIVs, with the exception of SIVgsn/mon/mus, counteract restriction by the tetherin proteins of their nonhuman primate hosts.^{149,289,382} This activity is dependent on a five-amino-acid sequence that is present in the cytoplasmic domain of the tetherin orthologs of apes and Old World monkeys, but is missing from human tetherin,^{149,382}

and appears to involve the downmodulation of tetherin from sites of virus release at the cell surface.¹⁴⁹

The properties of SIV strains with mutations in *nef* have been extensively studied. SIVmac239 with an in-frame stop at codon 93 of *nef* uniformly reverted to an open *nef* reading frame by 2 weeks after monkey infection.¹⁶⁴ Even a form of SIV with a four-amino-acid deletion in *nef* has reverted in about 10% of infected monkeys.³⁶⁴ So there is strong selective pressure to maintain an open *nef* reading frame. SIV with deletions in the *nef* gene are markedly attenuated compared with parental virus. Viral loads are typically reduced by two to three logs at the time of peak viremia, 2 weeks after infection and ≥ 3 to 4 logs at set point. Most monkeys infected with SIVmac239 Δ *nef* have survived without disease and with undetectable viral loads for as long as they have been studied. Despite the marked attenuation, infection with SIVmac239 Δ *nef* is clearly persistent, only at much lower levels. From 10% to 20% of monkeys infected with SIVmac239 Δ *nef* have gone on to develop moderate viral loads and to develop AIDS despite the absence of the *nef* gene. Serially passaged Δ *nef* SIV has shown a consistently pathogenic phenotype, and compensatory changes elsewhere in the genome appear to be responsible.⁴ Sequence changes in the cytoplasmic domain of gp41 of serially passaged *nef*-deleted SIV have been definitively shown to restore the virus's ability to counteract tetherin.³⁰² *Nef*⁻ SIV replicates similarly to wild-type SIV in a variety of standard cell-culture systems, including lectin-stimulated PBMC cultures supplemented with IL-2, although conflicting reports have appeared.

The attenuated properties of SIV Δ *nef* in monkeys appear to be similar to what has been observed in unusual cases of *nef*⁻ HIV-1 infection in humans. One human in the United States has been consistently infected with an HIV-1 with large deletions in *nef* since 1983.¹⁶⁸ In Australia, a blood donor with *nef*-deleted HIV-1 was responsible for infecting eight recipients; the donor and recipients have been slow or nonprogressors with low or undetectable viral loads.⁶⁷ *Nef*-deleted HIV-1 is clearly an attenuated virus with a level of attenuation similar to that of SIVmac Δ *nef*. However, as with monkeys infected with SIVmac Δ *nef*, some of the humans infected with HIV-1 Δ *nef* show signs of disease progression.^{114,181,379}

An unusual *nef* allele (YE *nef*; PBJ14 *nef*) has an ITAM that results in the ability to stimulate lymphocyte activation and to cause an unusually acute disease⁸¹; no analogous HIV-1 *nef* allele has been found.

Vpr and Vpx

Whereas all known primate lentiviruses contain a *vpr* gene, the *vpx* gene is present in only the SIVsm/mac/HIV-2, SIVrcm and SIVmnd2 lineages. *Vpr* and *vpx* are related by ancestral gene duplication and encode proteins with similar amino acid sequences. Moreover, Vpr and Vpx are both incorporated into virions through specific interactions with sequences in the p6 domain of p55 Gag. However, the activities of these accessory proteins differ. Vpr induces G₂/M cell-cycle arrest and apoptosis in infected cells,^{123,158,273,280} and has been implicated in the nuclear import of the preintegration complex in nondividing cells.⁹⁰ In contrast, Vpx is required to overcome an early block to the infection of macrophages and dendritic cells that occurs prior to the completion of reverse transcription.^{90,113,304} Both proteins associate with the Cul4^{DDB1/DCAF1} ubiquitin ligase complex by binding to VprBP1 (DCAF1) and are thought to

mediate the proteasomal degradation of specific cellular proteins.^{136,179,299,327} While the target of Vpr remains to be identified, Vpx-mediated degradation of SAMHD1 was recently shown to alleviate a block to reverse transcription in macrophages and dendritic cells.^{137,177} SAMHD1 is a deoxynucleotide triphosphohydrolase that inhibits reverse transcription in myeloid cells by depleting cellular dNTP pools. Thus, SAMHD1 imposes a block to HIV-1 infection of macrophages and dendritic cells that is overcome by Vpx.

Two lines of evidence indicate that *vpr* is not a major contributor to the replication of SIVmac in experimentally infected rhesus monkeys. SIVmac239 with a stop signal in the initiating ATG codon of *vpr* was slow to revert.¹⁷⁸ SIVmac239 with a deletion in *vpr* replicated only slightly less efficiently than did parental virus in rhesus monkeys, and all *vpr*⁻ SIV infections in animals have progressed to AIDS.¹⁰⁶ The effects of deleting *vpx* from SIVmac239 on levels of replicating virus in rhesus monkeys were more significant; nonetheless, more than half of such monkey infections progressed to AIDS.¹⁰⁶ A *vpx* deletion of SIVsmPBJ14 was strongly outcompeted when co-inoculated with parental virus,¹³⁰ consistent with these results. Deletion of both *vpr* and *vpx* markedly attenuated the virus, resulting in a virus that was about as attenuated as SIVmac239 Δ *nef*.¹⁰⁶

Env

Env is essential for virus replication. The protein products of the *env* gene are responsible for binding receptor(s) on the cell surface and mediating virus entry into cells. The products of *env* are the major or only targets of antibodies that can neutralize infectivity. Determinants of the differences in tropism of related viruses thus often map to the *env* gene. Whereas SIVmac replicates well in stimulated PBMCs from macaques, HIV-1 replicates not at all or only very poorly in macaque cells. Surprisingly, the block to replication of HIV-1 in macaque cells maps outside of the *env* gene.³¹¹ The restricting element TRIM 5 α may be overcome by changes in gag sequence.³²⁸ Derivatives of SIVmac, in which the *env* gene has been replaced by *env* of HIV-1, are replication competent in macaque cells and capable of infecting monkeys.^{142,188,203,310} Serial passage of several second-generation SHIVs has resulted in strains that are consistently pathogenic in macaques (see previous section on Clinical and Pathologic Features: Simian Immunodeficiency Virus).

U3

The U3 regions of the primate lentiviruses are unusually long compared with their counterparts in the nonprimate lentiviruses.¹⁴⁴ Most of this unusual length can be accounted for by the overlap of *nef* coding sequences with the LTR. In fact, several lines of evidence suggest that about 300 base pairs (bp) of U3 sequence in SIV and HIV may be nothing other than *nef* coding sequence.

1. In monkeys infected with SIV missing 182 bp in the region that is uniquely *nef*, the SIV progressively and consistently loses about 300 bp of sequence in the region of U3–*nef* overlap.¹⁶⁹ The 12 terminal nucleotides of U3 are consistently retained because these are needed for integration. About 50 bp at the C-terminus of *nef* coding sequence, immediately upstream of the nuclear factor- κ B NF- κ B site, are also consistently retained; this region is known to contain a poorly characterized enhancer element.^{143,212,268}

2. In a human infected with a *nef*-deleted variant of HIV-1, sequences in the U3–*nef* region also were progressively lost over time.¹⁶⁸ Again, the U3 terminal nucleotides and 50 bp upstream of the NF- κ B site were consistently retained.
3. The 300-bp region of *nef*–U3 overlap has been grossly altered in SIVmac239 by 102-point mutations without changing the predicted amino acid sequence of the encoded Nef protein.¹⁴⁴ This virus replicated like wild-type virus in rhesus monkeys without evidence of specific reversional events through at least 20 weeks of infection.

These results are most easily explained whereby these 300 bp of U3 sequence serve predominantly or exclusively as *nef* coding sequence. However, it also is possible that this region contains transcriptional control elements that respond to factors induced by *nef* or that in the absence of *nef*, any transcriptional control elements in this region provide less-selective advantage than does a shorter genome.

NF- κ B and Sp1

Sequence elements in U3 for binding NF- κ B and Sp1 transcription factors have been defined as major enhancer elements of HIV-1, HIV-2, and SIV. However, SIVmac is still able to replicate well in a variety of cell types in the complete absence of NF- κ B and Sp1 elements.¹⁴³ This is due predominantly or exclusively to an enhancer element present within the 50 bp immediately upstream of the NF- κ B element within sequences that are also *nef* coding sequences.^{143,212,268} SIVmac239 derivatives with the NF- κ B sites removed from both LTRs or with all Sp1 sites removed from both LTRs were not detectably attenuated in rhesus monkeys.¹⁴³ SIVmac239 with NF- κ B and Sp1 sites removed was detectably attenuated, but not markedly so.¹⁴⁵ HIV-1 replication appears more heavily dependent on the presence of the NF- κ B and Sp1 sites,²⁸⁵ but circumstantial evidence suggests an enhancer element within the 50 bp upstream of the NF- κ B binding sequence in HIV-1 as well.¹⁶⁸ This enhancer element remains poorly characterized. Evidence has been presented suggesting that this short stretch immediately upstream of the NF- κ B binding region may contain AP1 and C/EBP elements in HIV-1 and AP1 and PUB elements in SIVmac and HIV-2.¹⁶⁷

Multiply Deleted Derivatives

Work in the SIV system has shown that virtually any level of attenuation can be achieved simply by varying the number and locations of deletion mutations.⁶⁹ Attenuation has been scored by measure of viral load at peak, viral load at set point, strength of the antibody response, and propensity to induce declines in the numbers of CD4⁺ lymphocytes. Lack of viral persistence is a phenotype that has never been consistently associated with any mutant strain.

Genetic Resistance

Although individual examples of resistance to disease occur throughout the lentivirus subfamily, the clearest documentations of mechanisms by which it can occur come from studies with humans. In humans, decreased susceptibility to HIV-1 disease is associated with co-receptor polymorphisms, MHC class I alleles, KIR alleles and with chemokine gene dosage (see Chapter 50). In humans, disease progression is uniformly related to virus load, with high loads being of poorer prognosis and low loads being of better prognosis.

As in humans, certain MHC class I alleles have been associated with the control SIV infection in rhesus macaques. *Mamu* (*Macaca mulatta*)-A*01, a common MHC class I allele present in 22% of Indian-origin rhesus macaques, is associated with a fivefold reduction in chronic phase plasma viral loads.²³⁰ *Mamu-B*08* and *-B*17*, present in 6% and 11% of Indian-origin rhesus macaques, respectively, are significantly overrepresented among elite controllers (animals that contain plasma viremia below 1,000 copies/ml).^{199,374} The protective effect of *Mamu-B*08* is particularly strong, and is associated with more than a sevenfold reduction in chronic phase viremia.¹⁹⁹ However, the protective effect of *Mamu-B*17* is less clear, since viral loads in *Mamu-B*17*–positive animals vary considerably, possibly reflecting a role for other genetic loci.³⁷⁰ Interestingly, *Mamu-B*08* binds a very similar set of peptides as HLA-B*2705, a molecule associated with the control of HIV-1 replication in humans, suggesting that the peptides bound by these molecules are related to their ability to suppress virus replication.²⁰⁰ Although the mechanisms by which these MHC class I alleles lead to the containment of virus replication remains to be fully defined, evidence from CD8-depletion and CTL escape points to a role for CD8⁺ T cell responses.

As discussed earlier in this chapter, *TRIM5* polymorphisms are also associated with differences in the ability of rhesus macaques to control certain strains of SIV. *TRIM5Cyp* and TFP_{339–341} variants of *TRIM5 α* potentially inhibit the replication of SIV_{sm}E543-3, and probably the closely related strain SIV_{sm}E660, due to the incomplete adaptation of the capsid proteins of these viruses for resistance to rhesus macaque *TRIM5* proteins.¹⁷⁰ The differential susceptibility of SIV_{sm}E543-3 and SIV_{sm}E660 to variants of *TRIM5* is a significant factor in the interpretation of vaccine studies in rhesus macaques using these challenge viruses.

A striking example of a genetic polymorphism with analogies to the human situation has been documented in red-capped mangabeys. A 24-bp deletion (Δ 24) was noted in the *CCR5* gene of red-capped mangabeys with an allelic frequency of 86.6%.⁴⁰ Eleven of 15 red-capped mangabeys were found to be homozygous for this polymorphism. Interestingly, the SIV from red-capped mangabeys does not use *CCR5* as co-receptor but is unusual in its predominant use of *CCR2b* as co-receptor.

A prominent place to look for mechanisms of disease resistance other than in humans is in sooty mangabey monkeys and African green monkeys. These species are naturally infected with their own SIV without disease, and there is clearly no problem with the SIV that they carry because their SIVs are capable of causing AIDS when transmitted to Asian macaques. Based on experience with HIV-1 in humans, one would expect viral loads in sooty mangabeys and green monkeys to be low. Surprisingly, this is not the case. As discussed previously in the Virulence section, many sooty mangabey monkeys and African green monkeys carry SIV RNA loads in plasma in a range (10²–10⁶ copies/mL) in which disease progression uniformly occurs with HIV-1 in humans and SIV in macaque monkeys.^{30,108,160,276} The mechanisms of disease resistance in these species remain to be elucidated, but are likely to relate at least in part to an ability to resist chronic lymphoid activation (see Virulence section above) and may also relate in part to polymorphisms in co-receptor genes as suggested by the recent discovery of a high prevalence of defective *CCR5* alleles in the sooty mangabey.

An allele of *CCR5* with a 2-base pair deletion (*CCR5* Δ 2) that prevents *CCR5* expression on the cell surface was

recently identified in sooty mangabeys. This allele is present at a frequency of about 26% in sooty mangabeys; together with another allele with a 24-bp deletion (*CCR5* $\Delta 24$) present at a frequency of about 3%, it is estimated that nearly half of all sooty mangabeys are either homozygous (8%) or heterozygous (41%) for defective alleles of *CCR5*.²⁷⁹ Yet, SIVsmm is fully infectious for primary CD4⁺ lymphocytes from CCR5-null animals and is insensitive to the CCR5 antagonist maraviroc.²⁷⁹ An analysis of the ability of SIVsmm to infect cells expressing CD4 together with alternative co-receptors revealed that the virus could use GPR15, CXCR6, and to a lesser extent GPR1, but not CXCR4, CCR3, CCR8, or CCR2b.²⁷⁹ The inability of SIVsmm to use CCR2b as a co-receptor was surprising given that SIVrcm has been shown to preferentially use CCR2b as a co-receptor in red-capped mangabeys due to the high frequency of the *CCR5* $\Delta 24$ allele in this species.⁴⁰ While the co-receptor(s) used for SIVsmm infection *in vivo* remain to be defined, these results suggest that the cellular tropism of this virus is determined by a co-receptor other than CCR5. Thus, it will be important to define the cellular targets of SIVsmm infection in sooty mangabeys to determine if they are less susceptible to viral turnover or less critical for immunologic function in ways that might reconcile high levels of persistent virus replication with the lack of chronic lymphoid activation and the lack of disease progression in this species.

DIAGNOSIS

Because lentiviral infections are persistent, antiviral antibodies are present throughout the lifetime of the infected host. Detection of antiviral antibodies is thus the most widely used method for determining the presence of viral infection. A variety of methods are commonly used for antibody detection that vary with the individual virus. These include enzyme-linked immunosorbent assay (ELISA), Western blot, gel diffusion, indirect immunofluorescence, hemagglutination, complement fixation, and neutralization assays. The commonly used Coggins test for the detection of EIAV infection is a gel-diffusion assay for the detection of antiviral antibodies. Attempts are being made to develop simpler ELISAs for EIAV and other nonprimate lentiviruses. ELISA assay methods for the detection of antibodies to FIV are now routinely available. However, a partially efficacious vaccine for FIV is also now in use, which can complicate detection of infected cats. ELISA is most commonly used for the detection of antibodies to SIV; detection by ELISA is usually routine but can be complicated by the history of the monkey, whether the antibodies are to the same or a different type of SIV (i.e., cross-reactive), and, in an experimental setting, the presence of antibodies at low levels due to attenuation or intervention. Positivity can be confirmed by virus isolation or by identification of viral antigens or viral RNA in plasma or cells. The presence of specific clinical signs and clear demonstration of the presence of antilentiviral antibodies is usually sufficient for a definitive diagnosis.

PREVENTION AND CONTROL

MVV was eradicated from Iceland by a drastic slaughter policy before the availability of diagnostic tests. Test and removal pro-

grams have since been and continue to be used as an effective means of control. Test and removal programs may be voluntary or mandated. Buyers of horses have increasingly sought negative test certification for EIAV, and negative test certification is required as a condition for entry into many racetracks, salesyards, and shows. Horses imported into the United States and some other countries are required to have a negative test certificate. Testing within a state is not always compulsory, nor is it compulsory for an owner to destroy a positive horse. For FIV, testing is routinely available for cats under veterinary care and animals in shelters are also routinely screened. However, test and removal programs and certification at the point of sale have been sparingly applied to FIV.

Two vaccines are currently used in the field for the prevention of lentivirus infections. A live attenuated EIAV strain was developed by researchers by repeated passage in donkey cells.³⁰⁸ This EIAV vaccine has been extensively used in China, where it has been administered to millions of horses with apparent safety and efficacy, and in Cuba. Unfortunately, little is known in the western world about the properties of this live attenuated vaccine, its genetic composition, or details regarding its performance in the field. Sequences from Chinese EIAV vaccine strains have recently been published; an accumulation of nucleotide substitutions, not deletions, appears to be the basis for attenuation.³⁵⁹ EIAV vaccine virus derived from a single proviral clone did not fare as well in vaccine challenge experiments as the actual vaccine, which contains extensive sequence diversity.²⁰⁴ The second vaccine now in use is against FIV, initially developed using whole-infected cells as immunogen, then subsequently using killed cell-free virus preparations as a mixture of representatives from two distinct clades.²⁷¹ Efficacy in prevention of experimental FIV infections has been reported as 85%, which is less than desired but substantially better than reported for efficacy trials of HIV vaccines, at least to date.

RESEARCH ON VACCINE DEVELOPMENT

One important application of research with nonhuman lentiviruses is in the area of vaccine development. Certainly, development of a safe, effective, affordable vaccine for AIDS is one of the greatest challenges of our time. Most experimental studies have used SIV in rhesus monkeys. Use of SHIV in rhesus monkeys has also been extensively used because it allows analysis of HIV-1 *env*-containing vaccine products in a system in which challenge and meaningful viral load and disease readouts are readily possible.

Expectations were raised when inactivated whole SIV was found to provide protection against pathogenic strains of SIV.^{73,232} Hope was quickly dashed, however, when it was found that protection occurred only when the vaccine and challenge stocks were grown in human cells.^{57,58} When the vaccine was prepared in human cells and the challenge virus was grown in monkey cells, protection was not observed. Xenoantigens present in virus preparations were able to confer protection when a challenge virus was grown in the same xenogeneic cells. MHC class II was reported to be at least one of the xenoantigens capable of conferring protection.¹⁰ MHC class II antigens may actually be present in greater abundance in virus particles than the virus-encoded envelope proteins.⁹ The mechanisms by which antibodies to MHC class II present on the surface of

virus particles can block viral infection have not been defined, but it could be as simple as steric hindrance. Improved methods for purifying, inactivating, and delivering whole particles justify continued investigation of this vaccine approach.²⁸⁶ Whether the abundance of MHC class II antigens on virus particles has significance for pathogenesis remains to be determined.

Vaccine studies in animal models can provide useful information in several ways. Head-to-head comparisons of different vaccine approaches can shed light on which approaches perform more effectively, at least under the defined conditions of the experiments. In-depth analyses of individual vaccine approaches also may provide fundamental insights into immunologic control and what is needed for protective immunity. With these goals in mind, vaccine approaches that have been tried in animal models include a variety of envelope subunit approaches; poxvirus recombinants; DNA; inactivated whole particles; adenovirus recombinants; vesicular stomatitis virus recombinants; and prime and boost protocols that use combinations of these approaches. Several themes arising from these studies are worth noting.

1. The particular virus that is used for challenge is one of the single most important determinants of whether an individual vaccine challenge study will be successful. Easy-to-neutralize, nonpathogenic strains of virus have proven relatively easy to protect against. Difficult-to-neutralize pathogenic strains of virus, which could be considered representative of field strains of HIV-1, have proven very difficult to protect against. A homologous cloned virus challenge is less stringent than a closely matched uncloned virus challenge, which in turn is much less stringent than a challenge by a virus with natural, representative levels of sequence divergence. It has proven easy to prevent the acute disease course resulting from CXCR4-tropic SHIV infections using a variety of vaccine approaches.
2. Timing of the challenge is crucial. The vaccine approaches discussed earlier induce transient immune responses; they decay dramatically after the peak of vaccine-induced immune responses, which occurs within the first few weeks after the last vaccine boost. Most challenge experiments have been performed 2 to 4 weeks after the last vaccine boost.
3. It may be easier to achieve protection against a mucosal challenge than against an intravenous challenge. Most early studies used intravenous challenge because of the ability to achieve infection reproducibly in control monkeys with carefully controlled doses of challenge virus. Many studies now use vaginal or rectal mucosal challenge because these routes model the major modes of HIV transmission around the world. Repeated low-dose mucosal challenge models have also been developed.
4. Antibody responses to the envelope glycoprotein and cellular responses to core Gag–Pol antigens can contribute to protection. Which type of immune response can be most responsible for protection varies with the vaccine approach and characteristics of the challenge virus. Inclusion of SIV *gag-pol* and *env* in recombinant poxviruses resulted in improved protection in a vaccine/challenge format compared with when either *gag-pol* or *env* was used alone.²⁶⁹
5. When pathogenic, difficult-to-neutralize SIV strains have been used for challenge, protection has proven difficult to achieve with the vaccine approaches that have been tried to

date. Even when using a challenge virus identical or highly similar in sequence and challenge 2 to 4 weeks after the last vaccine boost, the levels of solid long-term protection by any criteria have seldom reached even 50%.

Live attenuated strains of SIV have performed more impressively as vaccines in experimental monkey studies.⁶⁰ Although the live attenuated vaccine approach is not under any serious consideration for practical development for use in humans, continued studies hope to identify critical components of a protective immune response and critical variables that may influence whether a protective immune response is attained. For example, it has been learned that a wide range of attenuation can be achieved by varying the number and location of deletion mutations.⁶⁹ Some strains—for example, SIVmacΔ4 (missing *vpr*, *vpx*, *nef*, and U3 sequences) and SIVΔkRXN (missing NF-κB, *vpr*, *vpx*, and *nef*)—are highly attenuated but still persist. SIVΔvif is so highly attenuated that infection is undetectable by standard criteria. In general, the ability to achieve protection varies inversely with the degree of attenuation. Thus, more highly attenuated strains are safer but less protective as vaccines.¹⁵⁵ Protection is also time dependent; a protective state can take 6 months or longer to become established. An understanding of protective immunity by live attenuated strains will require an explanation of why it takes so long to develop. Even the live attenuated vaccine strain SIVmac239Δ3 (missing *vpr*, *nef*, and U3 sequences) provides only minimal protection against heterologous challenge with SIVsmE660, which contains a level of sequence divergence that one would expect to encounter with a field strain of virus.³⁷¹ Although this difficulty could conceivably be addressed by using mixtures of sequences in the vaccine strain, the results illustrate the major hurdles that must be overcome to achieve protection against pathogenic, difficult-to-neutralize strains of SIV.⁷¹

The potential of adeno associated virus (AAV) for the delivery of antibodies with potent broadly neutralizing activity as a preventative has been nicely modeled in monkey studies.¹⁵⁴

RESEARCH ON THERAPEUTIC REGIMENS

Antiviral drugs useful against HIV-1 in humans have been developed with little or no input from animal models of lentiviral infection. This trend will likely continue. However, there is a growing realization that study of certain types of therapeutic intervention in model systems can provide valuable information. Cytokines, immunoadjuvants, immune stimulants, immune suppressants, or other highly experimental treatments that could possibly be harmful in the setting of a lentiviral infection can be examined for possible benefit in animal models. Faced with several possible lead compounds, some companies have chosen head-to-head comparisons in an animal model to help select the most promising compound to move forward. Comparisons of experimental regimens during primary infection, analysis of ways to boost virus-specific CD4⁺ T-helper cell responses during therapy, and analysis of ways to minimize maternal–infant transmission are also areas in which ongoing work in animal models may provide useful information to help guide trials of therapeutic regimens in humans.

Some of the antiviral drugs useful against HIV-1 have reduced or marginal activity against SIV. Überla et al.³⁴¹

generated an RT–SHIV in which the RT reading frame of SIVmac239 has been replaced by that of HIV-1. This RT–SHIV needs adaptive changes for optimal replication^{7,320} and is capable of inducing disease in monkeys.³⁴¹

PMPA (*R*-9-2-phosphonylmethoxypropyl adenine) is one compound with strong antiviral activity against both HIV-1 and SIV that has been used extensively in monkey studies. Tsai et al.³³⁸ were the first to show that treatment of monkeys with PMPA by 24 hours after SIV inoculation and continuing treatment for 4 weeks prevented the establishment of a persistent infection. Delaying the initiation of therapy to 48 or 72 hours or shortening the duration of treatment significantly reduced efficacy.³³⁷ Nonetheless, even when the establishment of persistent infection was not prevented, early treatment significantly reduced viral loads and prolonged AIDS-free survival.^{337,348}

In another study,¹⁹⁴ monkeys were started on PMPA therapy 24 hours after inoculation of SIVsm E660 and kept on the therapy for 28 days, similar to the studies of Tsai et al.³³⁷ and van Rompay et al.³⁴⁸ Establishment of a detectable, traditional, persistent infection was prevented, confirming the results of Tsai et al. A low-level, controlled infection was demonstrated, however, since CD8⁺ cell depletion resulted in the emergence of readily detectable virus.¹⁹⁵ Interestingly, when three such animals off therapy for 6 weeks were subsequently challenged intravenously with SIVsm E660, one animal was completely protected, and the other two showed dramatic reductions in viral load.¹⁹⁵ What is most startling and difficult to understand is that protection has been achieved in some animals in the absence of readily measurable SIV-specific antibody or cellular responses at the time of challenge. Continuation of this line of investigation promises important new insights into immune-mediated control outside the bounds of our current level of understanding.

Hematopoietic stem cell gene therapy is an attractive, futuristic approach for the prevention of HIV-induced disease, but it is clear that many developmental hurdles must be cleared. Research with SIV in monkeys is proving useful toward this goal. Transduction of CD34⁺ hematopoietic progenitor cells with an anti-*tat* gene protected T-cell and macrophage progeny from SIV infection.²⁸⁴ Infusion of lymphocytes with antisense *tat/rev* genes into macaques may have reduced SIV replication on subsequent SIV inoculation.⁷⁷

PERSPECTIVE

The worldwide crisis brought on by HIV and AIDS has focused attention on the nonhuman lentiviruses as a source for information that will shed light on the human condition. Lentiviral infections of domesticated animals are economically important in their own right and have been studied historically in this context. The lack of a reliable, usable animal model that uses HIV-1 itself and that recapitulates the features of human infection has resulted in extensive study of nonhuman lentiviruses as analog models in which susceptible hosts are infected with nonhuman lentiviruses, either naturally or experimentally. The most critical issues for future progress include better understanding of pathogenesis; improvements in therapy; improved availability of therapy; and, perhaps most important, development of a safe, effective, affordable vaccine. The most remarkable feature of these viruses, and perhaps most critical for the eventual control of HIV, is their ability to replicate continuously and unre-

lently despite apparently strong immune responses. Further advancement of therapeutic regimens and development of effective vaccines against HIV will have to deal with the strategies of immune evasion used by this group of viruses.

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Foamy Viruses

Foamy Virus Isolation and Diagnosis of Infection
Natural History and *trans*-Species Transmissions
Evolution of Foamy Viruses
Replication *In Vitro*
Replication in the Natural Host *In Vivo*
Role of Antiviral Restriction Factors

APOBEC3
 TRIM5 α
 Tetherin (CD317)
 Interferon

Virion Structure and Virion Nucleic Acid
Genome Organization
Virion-Associated Proteins

Gag
Pol
Env

Nonstructural Proteins
Tas
Bet

Regulation of Gene Expression
***cis*-Acting RNA Sequences and Packaging of RNA**
Overview of the Virus Life Cycle
 The Early Phase: Establishing the Provirus
 The Late Phase: Generation of Progeny Viruses
Apathogenicity of Foamy Viruses
Foamy Virus Vector Applications
Perspectives

The name *foamy virus* (FV) was coined in the 1950s to acknowledge the spontaneous emergence of the typical foamy cytopathic effect (CPE) produced in response to FV infection (Fig. 52.1A). This is characterized by multinucleated syncytia and vacuolation in primary monkey kidney cultures leading to a “foamy” appearance.^{47,154,186,206} Subsequently, it was found that CPE development was attributed to the fact that the monkeys were latently infected with a transmissible agent. Following the discovery of reverse transcriptase, the transmitted agent was shown to be a retrovirus.¹⁷² The first 30 years of FV research dealt mainly with the identification of infected monkeys, prior to sacrifice, as sources of primary cell cultures. Molecular cloning of the first FV—at that time believed to be a human isolate—permitted functional studies on the replication of FVs.¹⁹² FV research gained momentum following

the discovery that these viruses replicate differently from all other retroviruses.^{133,264} This culminated in the finding that the FV infectious genome appears to be DNA rather than RNA.^{160,203,269} In brief, the FV replication strategy combines those of retroviruses with some characteristics of hepadnaviruses, such as hepatitis B virus (HBV), with other properties that are unique to FVs.^{120,186,190} In virtually any aspect that has been examined, FVs replicate differently from all other retroviruses. This has led to the definition of two retroviral subfamilies, the *Orthoretrovirinae*, which encompass all retrovirus genera except FVs, and the *Spumaretrovirinae*, which constitute only the spumaviruses or FVs.¹³⁴ This chapter will summarize what is known about the biology of these viruses; for reviews on particular aspects, such as FV vectors, the reader is referred to more specialized reviews.^{132,188,246,249} We shall focus on FVs of primates and mention the nonprimate viruses only when they become relevant.

FOAMY VIRUS ISOLATION AND DIAGNOSIS OF INFECTION

FV isolation is relatively easy on primary fibroblasts from throat swabs, although any other tissue as source of virus can be used.^{94,154,166,186} Virus isolation from peripheral blood lymphocytes is greatly enhanced by the addition of anti- γ -interferon (γ -IFN) antibodies.⁵⁶ A virus isolate displaying the typical giant cells’ CPE (Fig. 52.1A) can be confirmed by the detection of reverse transcriptase activity, by immunofluorescence assay (IFA) demonstrating a predominantly nuclear antigen (Fig. 52.1B), or by nucleic acid detection methods.^{94,154,166,186,222}

The diagnosis of an FV infection can be made by demonstrating antibodies against the main structural proteins (typically the Gag doublet, see later discussion) in serologic assays,⁷¹ such as immunoblots or radioimmunoprecipitation assays (Fig. 52.2). The choice of the right antigen in these assays is important, because the reaction is, at least in part, virus type specific.^{96,102,112} To verify an FV infection, the serologic analysis should be combined with nucleic acid detection methods (typically polymerase chain reaction [PCR]). For this, the amplification of a conserved (~420 bp) fragment from the integrase (IN) domain of the *pol* gene has proven to be extremely useful.²²⁰

NATURAL HISTORY AND *TRANS*-SPECIES TRANSMISSIONS

Various vertebrate species are naturally infected with FVs. Table 52.1 and Figure 52.3A give an overview of some isolates.

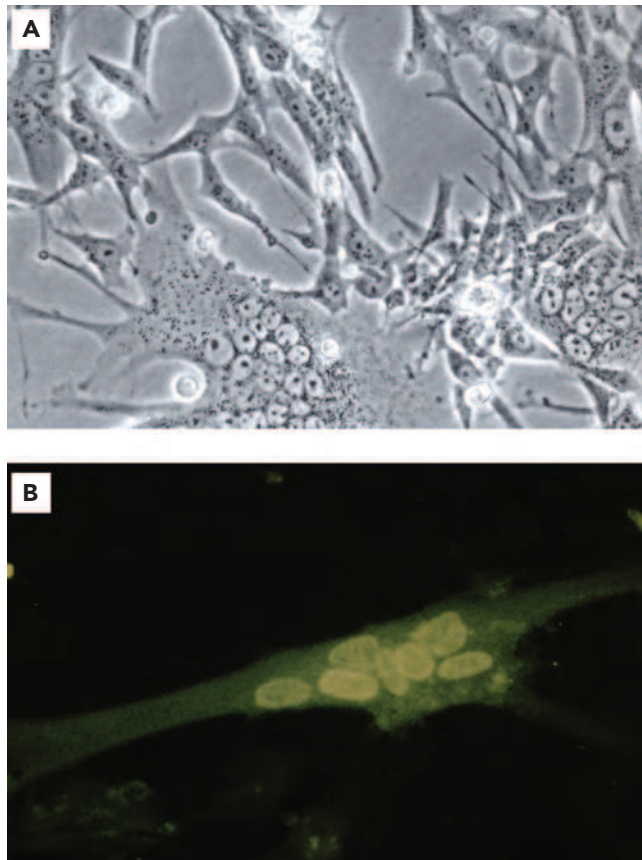


FIGURE 52.1. A: Multinucleated giant cell cytopathic effect induced by a primary monkey foamy virus (FV) isolate on baby hamster kidney 21 (BHK-21) cells. **B:** Typical nuclear fluorescence of a primary monkey FV isolate grown in human fibroblastoid cells reacted in indirect immunofluorescence assay with homologous serum. (With kind permission from Springer Science+Business Media: Neumann-Haefelin D, Rethwilm A, Bauer G, et al. Characterization of a foamy virus isolated from *Cercopithecus aethiops* lymphoblastoid cells. *Med Microbiol Immunol* 1983;172:75–86.)

In addition to FVs of Great Apes,^{94,95,154,186} Old and New World simians,^{94,154,186} and prosimians,^{99,154,186} FV infections appear to occur worldwide in bovines and other *Artiodactyla*,^{5,59,148,149a,154,186} equines,^{119,154,186,242} and felidae.^{154,186,196} Whether sea mammals are natural hosts has not been intensively investigated.^{110,154,186} Probably all monkey species harbor an FV.^{94,100,135,154,186} Prevalence in the natural host in the wild may be as high as 100% and is usually more than 30%.^{94,135,154,186} It is therefore not unlikely that FVs are, in terms of prevalence, the most successful of all retroviruses. Although host restriction factors show some species restriction (see later discussion), FVs have been reported to cross the species barrier between monkeys and apes in captivity or in the wild.^{94,124,135} In their hosts, FVs cause lifelong persistent infections of a benign nature, often in the presence of neutralizing antibodies.^{94,154,186} Laboratory animals, such as mice and rabbits, have also been infected in the absence of overt disease.^{26,93,94,208,214}

Humans are not a natural host of FVs.¹⁸⁶ Indeed, the best-studied FV was once believed to be of human origin.^{1,143} It has now been designated as the prototype foamy virus (PFV).¹⁸⁷ Ini-

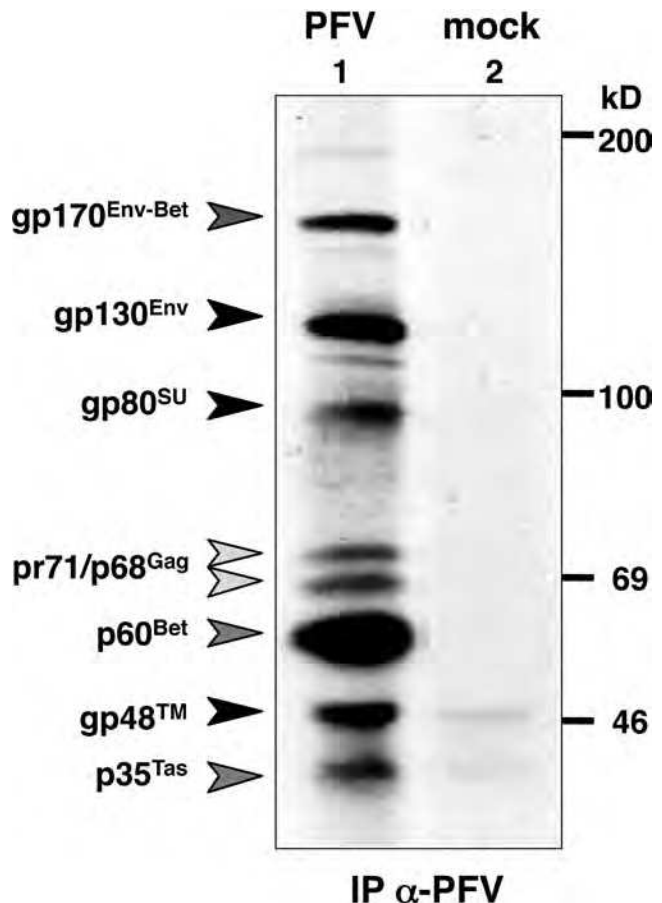


FIGURE 52.2. Radioimmunoprecipitation analysis of metabolically ³⁵S-Met/Cys labeled 293T cell lysates transfected with a prototype foamy virus (PFV) proviral expression construct (lane 1, PFV) or empty expression vector (lane 2, mock) using foamy virus–positive chimpanzee serum. The immunodominant viral proteins precipitated by this serum are indicated as well as the molecular weight markers (on the right).

tial reports on naturally occurring human infections^{144,147} were not confirmed by the large-scale screening of more than 5,000 samples using sophisticated methods such as a combination of antibody detection and PCR.^{2,222} Furthermore, postulated associations of FVs with human disease states, mainly autoimmune disorders and neurologic diseases of unknown origin could not be validated.^{40,86,201,223} Because of the close nucleotide sequence homology to FVs from the *Pan troglodytes schweinfurthii* chimpanzee *sub*-species,^{88,135} the single human isolate from a Kenyan patient is now believed to have resulted from a *trans*-species transmission of a virus from these chimpanzees that were once more prevalent in East Africa.⁵¹ Primate FVs are currently not circulating in the human population. However, humans are susceptible to zoonotic transmissions of nonhuman primate (NHP) FVs.^{218,232} Altogether, around 100 human infections with NHP FVs have been confirmed worldwide. In a survey, the Centers for Disease Control and Prevention identified approximately 2% seropositives and virus DNA positives among several hundred samples from occupationally to NHP-exposed persons and could isolate virus in several instances.^{22,87} Some of these

TABLE 52.1 Examples of Foamy Virus Isolates from Different Species of Primate and Nonprimate Origins

Virus	Host species	Accession number of full-length sequence	Reference
PFV	Chimpanzee/human	Y07725.1	213
SFVcpz	Chimpanzee	NC_001364.1	88
SFVgor	Gorilla	HM245790	217
SFVora	Orangutan	AJ544579	250
SFVbab	Baboon	n.a.	78,154
SFVmac	Macaque	X54482	117
SFVagm	African green monkey	M74895	184
SFVspm	Spider monkey	EU010385	238
SFVmar	Common marmoset	GU356395	170
SFVsqu	Squirrel monkey	GU356394	170
SFVgal	Galago	n.a.	100,154
FFV	Cat	Y08851	83,258
BFV	Cattle	U94514	92
EFV	Horse	AF201902	242
SHFV	Sheep	n.a.	59,154
SLFV	Sea lion	n.a.	110,154

PFV, prototype foamy virus; SFV, simian foamy virus; n.a., not available; FFV, feline foamy virus; BFV, bovine foamy virus; EFV, equine foamy virus; SHFV, sheep foamy virus; SLFV, sea lion foamy virus.

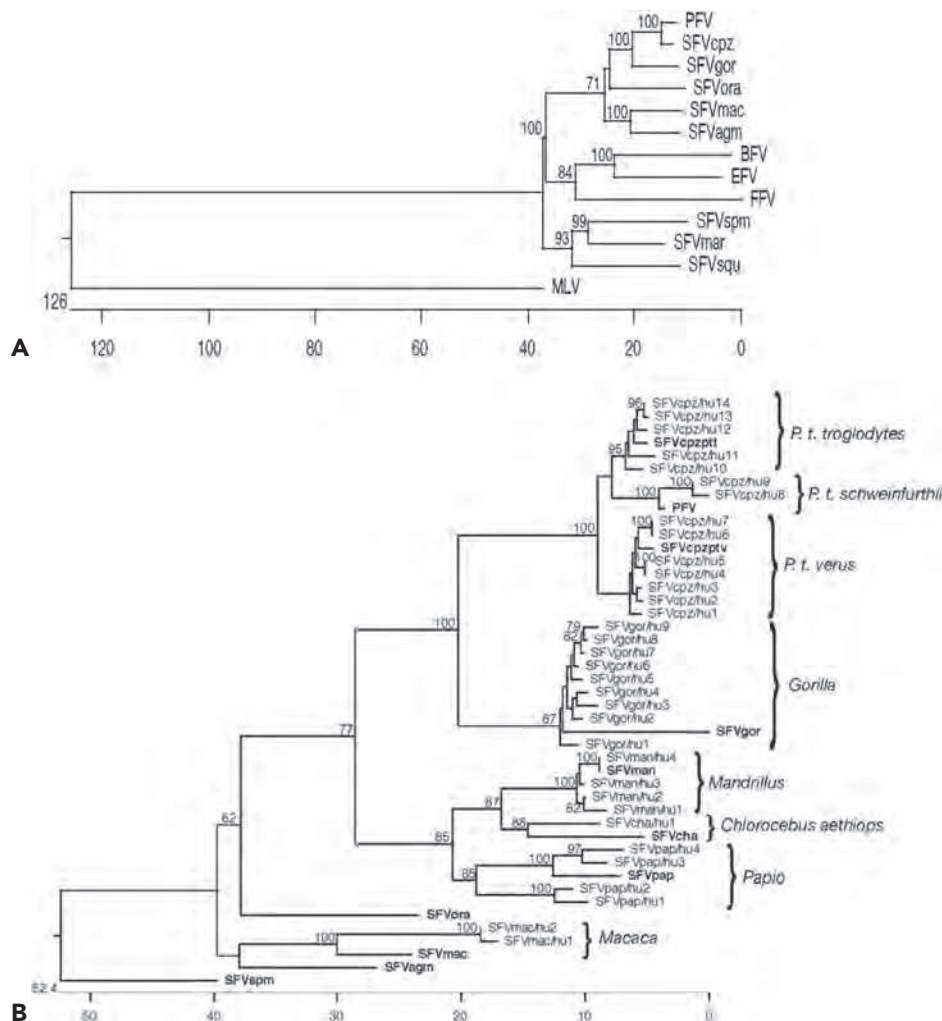


FIGURE 53.3. **A:** Phylogenetic relationship of complete foamy virus *pol* gene sequences from various isolates. **B:** Phylogenetic relationship of some human infections to the transmitted simian foamy viruses based on a conserved approximately 420-bp integrase gene fragment of the *pol* gene. To simplify matters, human infections were named consecutively after the original virus (in bold); they cluster with the viruses of the transmitting ape or monkey species. For **A** and **B**, evolutionary distances are indicated as nucleotide substitutions $\times 100$ and were calculated with 10,000 bootstrap trials. (Courtesy of J. Bodem, Würzburg, Germany.)

infections date back decades and were the result of severe monkey bites.^{22,87} FV infection has also been identified in African bushmeat hunters.³⁰ Other persons at risk are those living in close proximity to quasi-wild NHPs (e.g., at Asian temple sites) or individuals possessing an NHP pet.¹⁰¹ A very special case may be human recipients of NHP xenotransplants.⁴ Human infections are lifelong; however, none induced any disease and remained unrecognized prior to the investigation. The viral load in buffy-coat cells of infected humans revealed a very low number of FV DNA copies.³⁰ Moreover, in contrast to lentiviruses, *in vivo* adaptation to what can be called a human FV has not occurred. Even after decades of infection, FVs in humans have remained relatively unchanged at the nucleotide level, and the transmitting donor species can be readily identified^{22,87} (i.e., a baboon FV will remain a baboon FV even after decades in a human host) (Fig. 52.3B). Although the sample size is relatively small, there is no indication of human-to-human transmission even between close contacts.^{22,64a} Because FV can be transmitted by transfusion,^{25,111} infected persons have been advised not to donate blood to avoid new human retrovirus infections.⁸⁵

For reasons that are not fully understood, men appear to be a “dead-end” host for primate FVs. With respect to human infections by non-NHP FVs, it is worth mentioning that the antibody screening of more than 200 veterinarians at risk of acquiring a feline foamy virus (FFV) infection did not reveal a single positive case.²⁸ Based on this result, it appears unlikely that bovine or equine foamy virus (BFV and EFV, respectively) can infect humans. However, this has not been thoroughly investigated.

EVOLUTION OF FOAMY VIRUSES

Exogenous FVs are very ancient retroviruses. Exogenous FVs are very ancient retroviruses. There exist three examples of endogenous FVs, which suggest that exogenous FVs were around before that times: 1) Katourakis et al.¹⁰⁸ reported the detection of endogenous FVs (SloEFV) in the South American sloths genome (*Choloepus hoffmanni*), 2) Han and Worobey^{71a} described endogenous FVs (PSFVaye) in the Madagascan aye-aye, a primitive lemur species (*Daubentonia madagascarensis*), and more surprisingly, 3) in the latimera (*Coelacanth*) genome^{71b} (CoeEFV), suggesting that exogenous FVs existed more than 400 million years ago in species outside the mammals. Moreover, once an exogenous FV has adapted to its host, it mutates only slightly faster than the host mitochondrial DNA.²³⁴ The substitution rate of simian foamy viruses (SFVs) has been estimated to be around 1.7×10^{-8} per site and year.²³⁴ This makes FVs the most genetically stable of viruses, with an RNA phase in replication. For instance, the FV mutation rate is approximately ten times lower than primate T-cell lymphotropic virus type 1 (PTLV-1), a virus that replicates primarily by a proviral expansion mechanism (i.e., through DNA) (see Chapter 48).

FVs combine two favorable features: They are of extraordinary genetic stability and, if not acquired by *trans*-species transmissions, always point to the host species from which they were derived, and they are shed in feces. Owing to these features, NHP phylogeographic and conservation issues can be addressed easily without animal disturbance.^{135,220}

Curiously, the fidelity of the reverse transcriptase enzyme (RT) does not reflect this enormous genetic stability. If analyzed *in vitro*, the PFV RT was found to be of exceptional high

processivity but of low fidelity.^{23,24,197} The mutation rate of PFV RT (approximately 1.7×10^{-4} per site and replication round) is similar to that of human immunodeficiency virus (HIV).²⁴ Most mutations found were small deletions and insertions.²⁴ If analyzed in cell culture, such deletions and insertions were not found. However, a point mutation error rate of 1.1×10^{-5} per site and round of replication remained.⁶²

Thus, the genetic stability of FVs at the molecular level is not currently understood, and the involvement of as yet unidentified specific cellular factor(s) may play a role in this process.

REPLICATION *IN VITRO*

The host cell range for FVs is quite broad and includes species-independent primary cells or cell lines of fibroblastoid, epithelial, and lymphoblastoid origin, such as various B and T lymphocytes, and cells of erythroid and of myeloid lineages.^{94,154,159,186,268} Upon replication in adherent cell cultures, FVs induce massive multinucleated giant cell CPE (Fig. 52.1A), and apoptosis is thought to be the ultimate cause of cell death.¹⁵⁸ Vacuolization of cells is often only observed using primary isolates.

The paucity of cell lines resistant to FV infections has hindered the identification of the cellular receptor(s) required for entry by classical approaches. It is now appreciated that all FVs use the same cellular receptor, including those present on bird, reptile, and fish cells.^{12,89,230} Only two cell lines—Pac-2 zebrafish embryonic fibroblasts and the G1E-ER4 human erythroid precursors—have been reported to be refractory to infection.²³⁰ Thus, the means to screen complementary DNA (cDNA) libraries for FV receptor-related genes has now been established.

Whereas the characteristic CPE develops in adherent cells, this hallmark of FVs is often absent in cells of lymphoblastoid origin, in which FVs appear to become latent and intermittently reactivatable. Latently infected cells do not undergo syncytium formation and death but proliferate with normal kinetics and produce low amounts of virus.²⁶⁸ As judged from Southern blots, the viral DNA copy number oscillated in infected lymphocytes (unpublished observation). Interestingly, chemical treatment of lymphocytes (e.g., with phorbol esters) may induce the latent virus and cause cell death owing to activated viral replication.^{157,268} Although this is reminiscent of the lymphotropic herpesviruses, in FVs the molecular basis for virus reactivation has not been investigated nor have sites within the cellular or FV DNA genome responsive to the drug-mediated reactivation been mapped. Whether the methylation of FV DNA that has been observed in a cell culture model²¹⁹ contributes to *in vivo* latency remains unresolved, because there is no evidence of transcriptional down-regulation of FV vectors by methylation following their introduction *in vivo*.^{90,169,225}

REPLICATION IN THE NATURAL HOST *IN VIVO*

Recently, Liu et al.¹³⁵ used methods similar to those employed to demonstrate that human immunodeficiency virus type 1 (HIV-1) was derived from simian immunodeficiency virus from chimpanzees (SIVcpz; i.e., they collected and analyzed fecal samples from wild chimpanzees; see Chapters 49 and 50). They found that simian foamy virus from chimpanzees (SFVcpz) is widely distributed among wild chimpanzees with a

phylogeographic distribution and is transmitted horizontally, because babies younger than 2 years were free from FVs and infection rates increase with age. Moreover, they determined that superinfection by SFVs from lower primates and frequent recombination events occur. The most interesting finding by Liu et al has been the detection of viral RNA but not viral DNA in the fecal samples.¹³⁵ This finding directly relates to the FV replication pathway (see later discussion). However, Liu et al have not investigated whether the RNA-containing virus transmits the infection, and it is not known in which cell type these viruses were produced. It is possible that the DNA content in the fecal samples may have been too low to be detected even with very sensitive methods.

The tissue distribution of SFV and sites of *in vivo* replication have been investigated using another approach. As expected from the broad host cell range of FVs seen *in vitro*, viral DNA was detected at a frequency of one genome copy per 10^2 to 10^3 cells in every organ examined.⁵⁴ Because animals were perfused prior to the analysis of nucleic acids, infected lymphocytes were not detected, although these cells were the probable vehicles of virus dissemination *in vivo*.^{54,55} As judged from *in situ* hybridization experiments, viral RNA, indicative of active virus replication, was confined to superficial cells of the oral mucosa.^{54,165} Thus, it appears that only cells, which are destined to be shed, are productively infected and undergo lytic replication *in vivo*. Differential expression of yet undisclosed host factors restricting viral replication in other tissues is a likely explanation for this observation.

It is generally assumed that FVs are transmitted among NHPs through saliva via social contacts, including aggressive activities such as biting among young animals.^{31,135} These contacts and lactation are also suspected to be the main transmission route of the FFV,²⁵⁹ whereas BFV probably is mainly transmitted via milk from infected cows to offspring.¹⁹⁸

Early studies suggested that drug-induced immunosuppression of infected African green monkeys did not result in SFV-related symptoms yet enhanced the frequency of virus isolation (D. Neumann-Haefelin, Freiburg, Germany, personal communication). It was subsequently found that in dually SFV- and simian immunodeficiency virus (SIV)-infected and severely immunosuppressed macaques, the predominant site of FV replication changed from the oral mucosa to the small intestine.¹⁶⁴ However, SFV-related diseases did not occur. This was also observed in cats dually infected with feline immunodeficiency virus (FIV) and FFV.^{8,272} A case of human co-infections by HIV-1 and SFV from mandrills has also been reported, without clinical consequences that could be attributed to the SFV infection.²³³

ROLE OF ANTIVIRAL RESTRICTION FACTORS

APOBEC3

FV genomes are sensitive to editing by a variety of cellular catalytic polypeptide 3 apolipoprotein B messenger RNA (mRNA)-editing (APOBEC) proteins.^{41,141,205} To edit complementary DNA (cDNA) during reverse transcription, APOBEC3 proteins have to be encapsidated into the nascent virus particle. FVs encode the accessory Bet protein to preserve genome stability. The HIV-1 Vif protein prevents APOBEC3 particle incorporation by routing it to the proteasomal degradation pathway. In contrast, Bet prevents APOBEC3 encapsidation by binding and quantitatively trapping the deaminase.^{36,175} The PFV Bet

function was found to be broadly active against various primate APOBEC3 proteins.¹⁷⁵ However, some species specificity was also observed, as PFV Bet was found to be inactive against all or some mouse, feline, and rhesus monkey APOBEC3 proteins, as well as against human APOBEC3DE and APOBEC3H.¹⁷⁵ Because reverse transcription of FV RNA takes place to a significant degree in virus-producing cells (see later discussion), APOBEC3 restriction of FVs occurs at a different point in the replication cycle than that reported for orthoretroviruses.¹⁴¹

TRIM5 α

The tripartite interaction motif (TRIM) proteins comprise a large family of cellular proteins that are components of the innate immune defense mechanism and are active at various stages of replication against many viruses, including retroviruses.^{167,244}

TRIM5 α has been studied extensively for its activity against lentiviruses (see Chapters 8 and 49) and was found to be active against some orthoretroviruses during the very early phase of infection, prior to reverse transcription, by inducing premature disassembly of viral capsids. Lentiviral *gag* genes have evolved in such a way that their products are not neutralized by the homologous TRIM5 α but often by proteins from other species. TRIM5 α proteins have a modular organization consisting of so-called N-terminal RING domains, followed by zinc-binding B boxes, coiled-coil domains, and the C-terminal variable region B30.2/SPRY.^{167,244} The ability to recognize and bind in a species-specific manner to retroviral capsids is mediated by the B30.2 domain.²⁴⁴ FV *gag* genes are highly divergent from their orthoretroviral counterparts; thus, it was uncertain whether TRIM5 α would react against FVs and, if so, what the molecular basis would be. Nonetheless, it has been shown that primate TRIM5 α proteins restrict FVs in a species-specific manner.^{170,263} The specificity of TRIM5 α has been mapped to variable residues of the B30.2 domain, which are important for neutralization of lentiviruses, and to the N-terminal half of the FV Gag.²⁶³ The activity of TRIM5 α against divergent capsid proteins, including those of FVs, which do not mature into the canonical orthoretroviral matrix (MA), capsid (CA), and nucleocapsid (NC) subunits, implies an even wider structural recognition pattern than previously assumed.

Tetherin (CD317)

CD317/tetherin is an integral membrane protein with an N-terminal membrane-spanning domain and a C-terminal glycosyl-phosphatidylinositol anchor. CD317 interacts directly with the actin cytoskeleton and blocks the release of enveloped viruses from infected cells. In HIV-1-infected cells, it is antagonized by the accessory gene product Vpu, for HIV-2 by Env, and in some SIVs by the Nef protein (see Chapters 49 and 51). The fact that different lentiviruses developed effective strategies to neutralize CD317 argues for its *in vivo* importance. As is the case for other enveloped viruses, CD317 is also active against FVs.^{104,262} A FV protein antagonizing tetherin has not yet been identified. The activity of tetherin against PFV shows some mechanistic differences in comparison to HIV-1, because dimerization-deficient tetherin inhibits PFV replication with the same efficiency as the wild-type factor.²⁶²

Interferon

All three aforementioned restriction factors are inducible by interferon (IFN) to which FVs are vulnerable.^{56,150} Early studies indicated already that FVs are vulnerable to IFNs,^{194,195} and

it has been shown that toll-like receptor 7 (TLR7) expression in plasmacytoid dendritic cells is the likely factor in sensing FV RNA resulting in the induction of type I IFN.²⁰⁴ Replicating virus was found to be not required for this type of IFN induction. The addition of type II IFN abolishes FV replication *in vitro* almost completely, whereas the activity of type I IFN is less pronounced.^{56,150} The type I IFN-induced protein IFP35 has been demonstrated to down-regulate BFV transcription and replication by interacting with the *trans*-activator protein of BFV (Tas_{BFV}).²³⁶ Furthermore, the analysis of specific PFV *gag* arginine to lysine conversion mutants (see later discussion) revealed a likely antiviral role of IFNs that cannot be attributed solely to the three restriction factors discussed earlier.¹⁵⁰

Whether the IFN-inducible antiviral promyelocytic leukemia (PML) proteins play a role in restricting FV replication¹⁸³ needs further substantiation; PML proteins appear not to be involved in an establishing viral latency.¹⁵⁶

VIRION STRUCTURE AND VIRION NUCLEIC ACID

By ultrastructural analysis, FVs appear as immature-looking core particles surrounded by a lipid bilayer with embedded prominent Env proteins^{94,154,186,256} (Fig. 52.4). In negative-staining electron microscopy (EM), the virion has a diameter

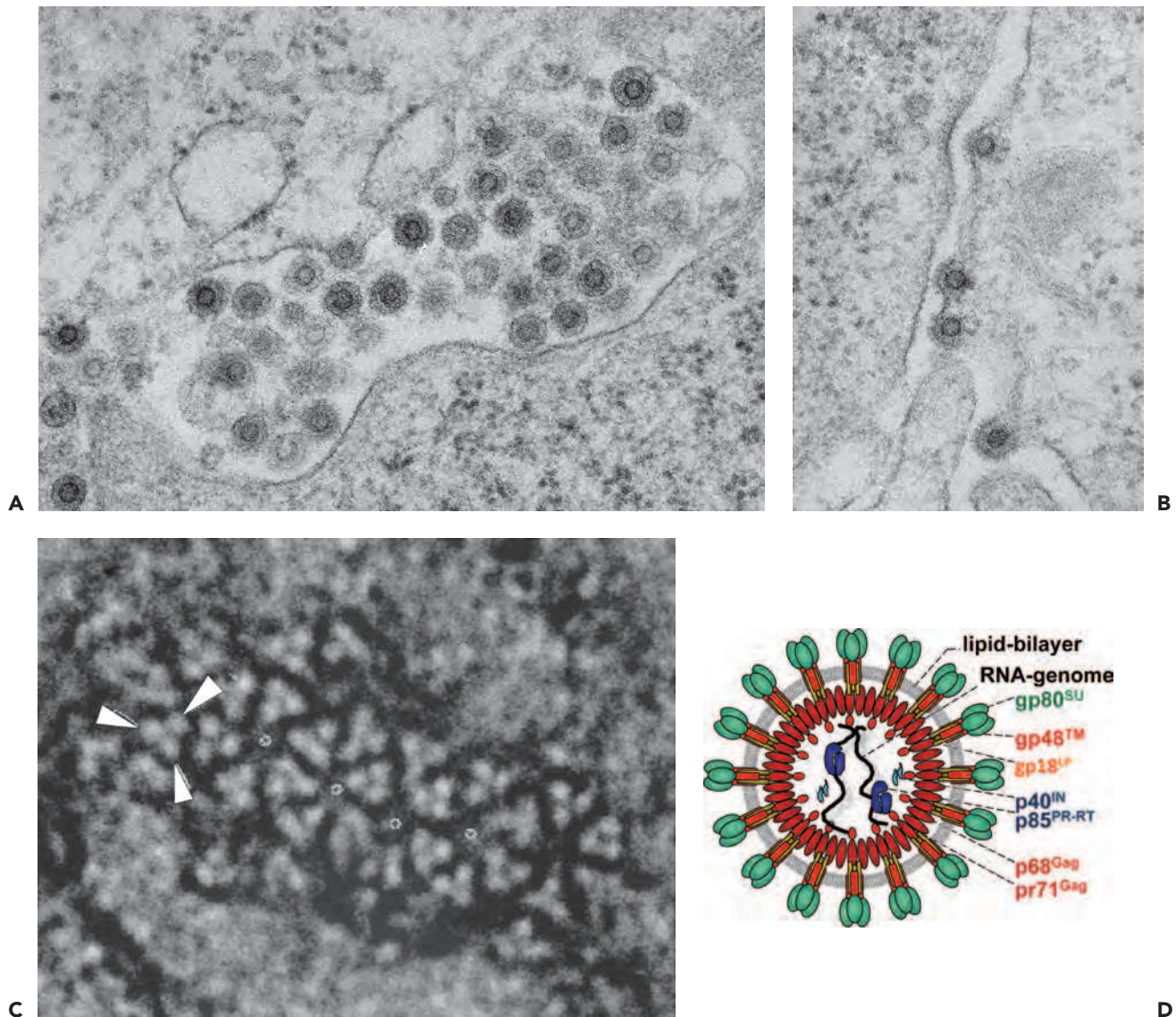


FIGURE 52.4. A–C: Electron microscopy of negative-stained prototype foamy virus. The viral Env protein is arranged in trimers (arrowheads). (Adapted from Wilk T, de Haas F, Wagner A, et al. The intact retroviral Env glycoprotein of human foamy virus is a trimer. *J Virol* 2000;74:2885–2887.) **D:** Scheme of the complete viral particle prior to reverse transcription. Glycoprotein complex: leader peptide (gp18LP), surface (gp80SU), and transmembrane (gp48TM) subunits. Capsid: Gag precursor (pr71Gag), large processing product (p68Gag). Viral enzymes: protease-reverse-transcriptase (p85PR-RT), integrase (p40IN). (Modified from Lindemann D, Rethwilm A. Foamy virus biology and its application for vector development. *Viruses* 2011;3:561–585.)

of approximately 110 nm and a core of approximately 60 nm.^{94,154,186,256} The core has an immature morphology owing to the very limited cleavage of the Gag precursor protein by the viral protease (PR) (see later discussion). The cores of infectious PFV virions are made up of the Gag precursor pr71^{Gag} and its larger processing product p68^{Gag} at a ratio of 1:1 up to 1:4.³³ This Gag doublet is seen with all FVs,⁷¹ although the nonprimate FV capsid proteins are considerably smaller than their primate relatives.^{92,119,199,242} The core is probably oriented radially with the Gag N-terminus pointing outward in the direction of the Env proteins²⁵⁶ (Fig. 52.4C). The prominent surface spikes average 15 nm in length and are organized as trimers in ring-like structures.²⁵⁶ A feature that PFV shares with HBV is the formation of subviral particles (SVPs) consisting only of membranous Env-containing vesicles and devoid of cores.^{224,228}

It appears that FV particles contain fewer Pol molecules than orthoretroviruses, which is consistent with the high processivity activity of its RT.^{23,197} The interpretation of older studies demonstrating equal amounts of Pol in foamy and orthoretroviruses³³ have been complicated by the detection of significant amounts of extraparticular Pol protein present in FV particle preparations of different origin as reported by Swiersy et al.²³¹ This study also revealed that in sharp contrast to orthoretroviruses, FV replication tolerates a great imbalance in the relative ratios of Gag and Pol molecules in virus producing cells.²³¹ This is owing to the unusual Pol encapsidation strategy of FVs (see later discussion), in which viral RNA is the limiting factor at conditions of high cellular levels of Pol.

The physical stability of FVs has not been directly compared with that of orthoretroviruses. However, owing to their immature core and a particular Env topology (see later discussion), FVs are probably quite stable. This is illustrated by the fact that vector particles can be concentrated more than 100-fold (i.e., by ultracentrifugation) without great loss of infectivity.^{65,89}

Viral nucleic acid is present within the core of extracellular FV particles, and it appears to be a mixture of RNA

and DNA. RNA dominates at a ratio of approximately 1:1 to 7:1, depending on the genomic region analyzed (long terminal repeat [LTR] vs. *gag*).^{203,269,270} Consistent with this, both forms of nucleic acid were detected in plasma and saliva of an experimentally infected cynomolgus macaque by PCR and RT-PCR.²⁵ Full-length DNA was shown to be present in roughly 5% to 20% of FV virions, and functional studies using the RT-inhibiting drug AZT indicated DNA to be the relevant genome for infection, at least at the high multiplicity of infection (MOI) studied.^{150,160} Because DNA was found in PFV and FFV virions, the idea that FVs are facultative DNA viruses may be generalized.²⁰³

However, it has not been investigated in much detail to what extent reverse transcription is taking place late in the replication cycle. For instance, it is likely that there are genomic regions, such as the gap in the plus strand cDNA (see later discussion), that still remain single stranded. The finding of more LTR than *gag* region reverse transcripts in virions is a clear indication of this.^{203,269} In addition, because more RNA than DNA is found in virions, it would be interesting to know whether purely RNA-containing viruses exist or whether most viruses contain both forms of nucleic acids.

It is with respect to reverse transcription taking place at a late step in viral replication that FVs diverge at most from orthoretroviruses, which display a RNA to DNA ratio of approximately 10⁵:1 in extracellular virions.²⁰³ The most compelling evidence for this are experiments in which the infectivity of virion-extracted FV DNA has been demonstrated.^{150,203,269} Thus, FVs functionally bridge the replication pathways of orthoretroviruses and hepadnaviruses (Fig. 52.5). It is because of this analogy to the hepadnaviral replication cycle (see Chapter 68) that the full-length FV RNA has been termed (*pre*-) *genomic*.

In addition to reverse transcription in late phases of FV particle morphogenesis, it has been shown that, similar to orthoretroviruses, reverse transcription also occurs during the early phases of FV replication upon target cell entry.^{42,270} Given

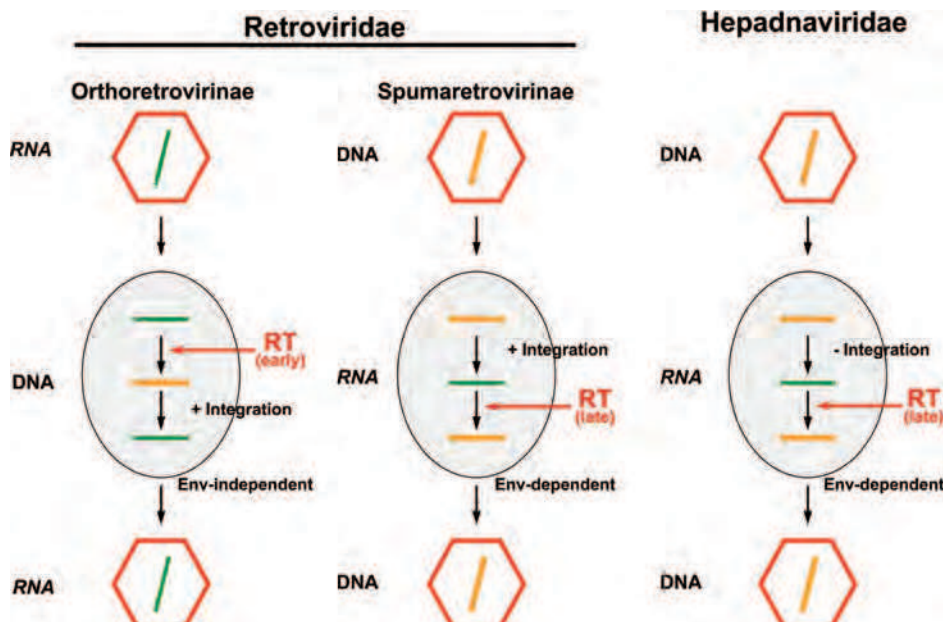


FIGURE 52.5. Principal replication strategies of reverse transcribing viruses. Whereas orthoretroviruses (**left panel**) are RNA viruses that reverse transcribe early in replication, replicate through a DNA intermediate, and exhibit obligate integration into the cellular genome, hepadnaviruses (**right panel**) essentially are DNA viruses replicating through an RNA intermediate; they must not integrate. The retroviral subfamily of spumaretroviruses (**middle panel**) functionally bridges both pathways by being DNA viruses, reverse transcribing late in replication (like hepadnaviruses), and requiring proviral integration (like orthoretroviruses).

the aforementioned ratios of RNA to DNA in extracellular viruses, reverse transcription during the early phase of FV replication may only become relevant at a very low MOI, when the amount of virion DNA may be too low to sustain a productive infection.²⁷⁰ Furthermore, the discrepant results reported about the importance of the differentially timed reverse transcription events for FV infectivity may reflect the inherent differences in the cell types used for virus production.^{42,150,160,203,269,270} In essence, the generally accepted view that virions contain either an RNA or DNA genome, but not both, may not apply to FVs.

GENOME ORGANIZATION

The schematic representation of the genome of PFV is shown in Figure 52.6. All FV genomes share common genome structures.¹⁹¹ Between the LTRs, the canonical *gag*, *pol*, and *env* genes are found; downstream of *env*, the accessory open reading frames (ORFs) are found. Proviruses are 12 to 13 kb—long

in comparison to those of other retroviruses. The large size of FVs genomes is partially attributed to the extraordinary long U3 regions of the LTR, which can be explained in part by the overlapping ORF-2 (see Fig. 52.6). However, the accessory ORF-2 reaches only to some extent (approximately 300 bp, in the case of PFV) into the more than 1.4 kb long U3 region, leaving several hundred bases without known function. In the latter, very few enhancer elements, such as those for AP-1 and Ets-1,^{152,213} and the short sequence motifs responsive to the viral transactivator (see later discussion), are present.

There are length differences in the *gag* genes in different FVs, with those in FVs from cats, bovines, and equines being shorter than those from primates.^{92,151,242,258} In sharp contrast to their orthoretroviral cousins, the FV Gag proteins are more variable in their sequence than are the Env proteins.^{186,238} For instance, primate lentiviruses have an amino acid conservation of roughly 60% in Gag and 40% in Env compared to 45% in Gag and 65% in Env among primate FVs.^{186,238} It is likely that this curiosity of FV biology is a consequence of the

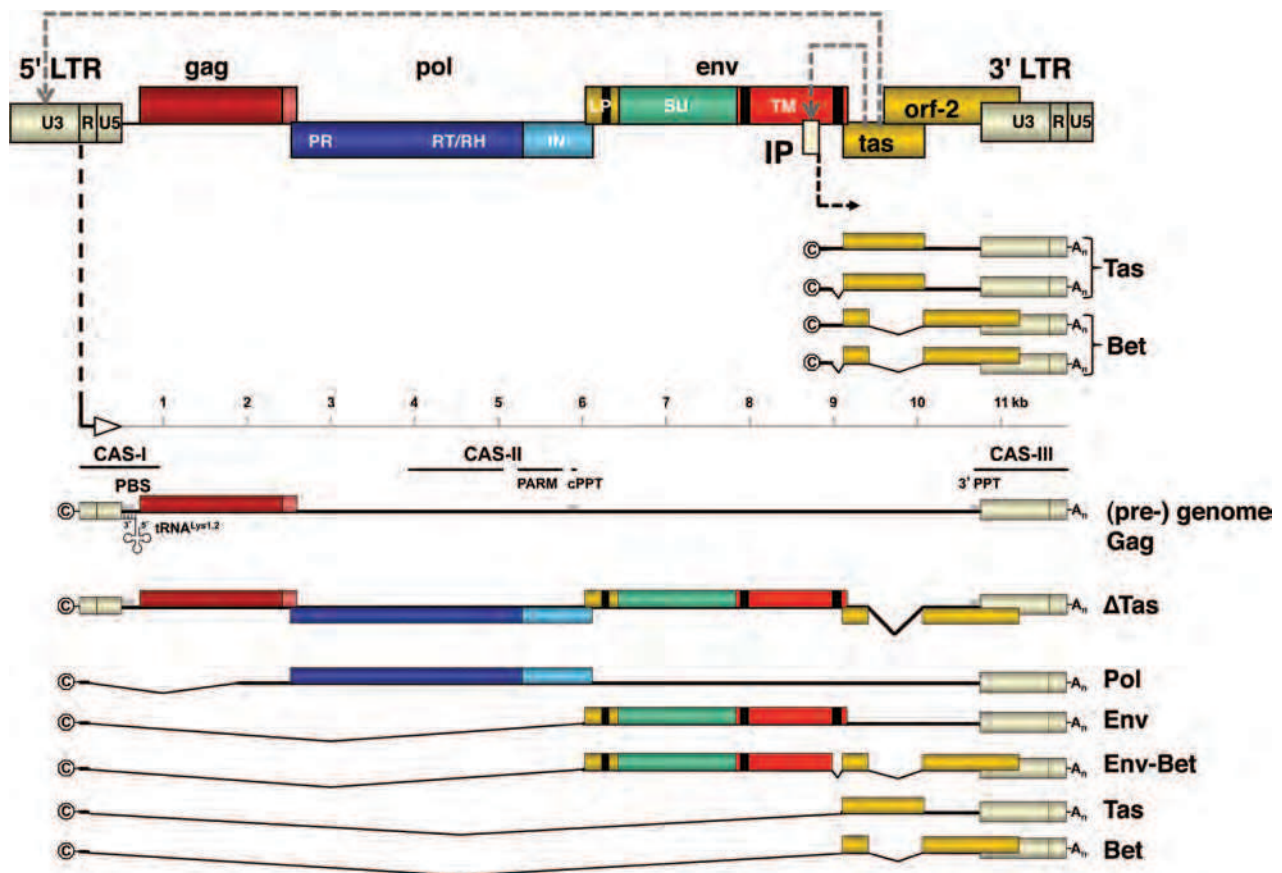


FIGURE 52.6. Prototypic foamy virus genomic structure, gene regulation, and messenger RNA (mRNA) transcripts originating at the two promoters. LTR, long terminal repeat; *gag*, *pol*, *env*, genes for virion-associated proteins; *tas* and *orf-2*, regulatory genes; IP, internal promoter giving rise to the accessory Tas and Bet proteins. If sufficient amounts of Tas have been made, the transcriptional activator switches on gene expression from the U3 promoter in the LTR. The (pre-) genomic transcript contains the primer binding site (PBS), central and 3' polypurine tracts (cPPT and 3' PPT, respectively), a motif required for activating the protease (PARM), and *cis*-acting sequences (CAS) I through III essential for packaging the RNA (pre-) genome, the Pol protein, reverse transcription, and integration. Δ Tas, resulting from reverse transcription of the depicted nearly full-length (pre-) genomic RNA from which the *tas* intron has been spliced out, is replication defective (see text for details). Δ Tas may generate some Bet protein. PR, protease; RT/RH, reverse transcriptase - RNase H; IN, integrase; LP, leader peptide; SU, surface; TM, transmembrane - subunits. ©, mRNA cap site; A_n, poly A tail. (Modified from Lindemann D, Rethwilm A. Foamy virus biology and its application for vector development. *Viruses* 2011;3:561–585.)

process of adaptation to and co-evolution with their natural hosts. Furthermore, this finding is consistent with the use of the same cellular receptor(s) by all FVs.

All FV genomes encode an internal promoter (IP) near the 3' end of their *env* genes, which drives the expression of the accessory ORFs^{137,140} (see Fig. 52.6). The primer binding site (PBS) is complementary to the cellular transfer RNA_{lys1,2} and is conserved among all FVs.¹⁵¹ Another feature of all sequenced FV genomes is the presence of a central polypurine tract (cPPT) in addition to the polypurine tract (PPT) upstream of the 3' LTR^{118,176,221,243} (see Fig. 52.6). The cPPT is believed to enhance reverse transcription.^{176,243} In contrast to HIV-1, it does not lead to a DNA *flap* region but to a single-stranded DNA *gap* without defined ends and start points of the upstream and downstream respective DNA plus strands.¹⁷⁶

VIRION-ASSOCIATED PROTEINS

Gag

The FV capsid proteins have several unusual characteristics compared to orthoretroviral Gag proteins. They are probably translated by ribosomal shunting from the (pre-) genomic RNA²¹¹ (see Fig. 52.6). This is a mechanism that is used by plant pararetroviruses to translate their *gag* homolog^{91,207} and involves the selective jumping of ribosomes to the translation initiation site.⁶¹ Even more surprisingly, FV Gag proteins are neither cleaved into the canonical MA, CA, and NC subunits, nor are several sequence motifs present, which are conserved in all orthoretroviral Gag proteins (Fig. 52.7). These include the N-terminal myristoylation signal of the MA domain, the major homology region (MHR) in the CA domain, or the cysteine-histidine (CH) boxes in the NC domain. Instead of Gag subunit processing observed with orthoretroviruses, at least half of the particle-associated FV Gag molecules are truncated approximately 3 to 4 kD C-terminally by *pol*-encoded PR processing at a singular cleavage site. In the case of PFV, this generates a large PFV p68^{Gag} and a small PFV p3^{Gag} product from the PFV pr71^{Gag} precursor molecule⁶⁰ (see Fig. 52.7). Whereas the p68^{Gag} cleavage product together with the pr71^{Gag} precursor forms the capsid of secreted PFV virions, the smaller p3^{Gag} is not thought to be particle associated and its cellular localization and fate are unclear.^{48,193a} FV Gag cleavage is required for infectivity, as FVs expressing only the pr71^{Gag} precursor are not infectious and often produce aberrantly formed capsids.^{48,114,271}

Mutants expressing only the large p68^{Gag} cleavage product are infectious albeit at low titers.^{48,229,271} Secondary protease cleavage sites, located in the central part of FV Gag, have been identified *in vitro*, using recombinant proteins and peptides¹⁷⁹ (see Fig. 52.7). They are believed to be essential and utilized for a viral disassembly process involving proteolytic processing of Gag by the FV PR and cellular proteases following entry into target cells.^{66,75,125,179} Thus, FV capsid disassembly appears to be a unique process controlled by viral and cellular proteases.

One of the most distinctive features of FVs is the failure of their capsids to spontaneously bud from cellular membranes and generate virus-like particles (VLPs). This is owing to the absence of a membrane-targeting signal in Gag.^{7,58,181} For cellular egress, FVs require the cognate envelope protein, with which FV capsids specifically interact. Co-expression of FV Gag and FV Env is required for detection of capsids secreted into the cell culture supernatant (Fig. 52.8). However, whether a direct capsid–Env interaction or an indirect cellular protein-mediated interaction exists has not been established by biochemical means. Biophysical investigations (applying surface plasmon resonance to recombinant protein fragments) with FFV and morphologic studies (with cryo-EM) of PFV^{64,257} suggest that a direct interaction between Gag and Env occurs. As a consequence of this unique feature, pseudotyping of wild-type FV capsids by anything other than FV glycoproteins cannot take place.¹⁸¹ Capsids exhibit a marked preference for virus type-specific Envs when FVs from different species are examined (unpublished own observations).

Various peptide and structural motifs have been found in FV Gag proteins, and these have been partially functionally characterized (see Fig. 52.7). Four coiled-coil domains (CC1–4) are predicted to be present in the PFV Gag, and functions have been assigned to the first three. The CC1 (aa 4–19) at the N-terminus has been suggested to interact with domain for the Env leader peptide (LP),^{129,193a} although unequivocal biochemical evidence has yet to be provided. CC2 (aa 133–146) has been reported to harbor a domain required for Gag multimerization,²⁴⁰ and CC3 (aa 161–174) is believed to be required for the incoming capsids to interact with the dynein light chain 8 for retrograde movement along the cellular microtubule network to the microtubule organizing center (MTOC).¹⁷⁸ The function of CC4 (aa 436–453) remains unknown.

Similar to Mason-Pfizer monkey virus (MPMV), a cytoplasmic targeting and retention signal (CTRS) is located at the N-terminus (aa 43–60) of PFV Gag^{32,46} (see Fig. 52.7).

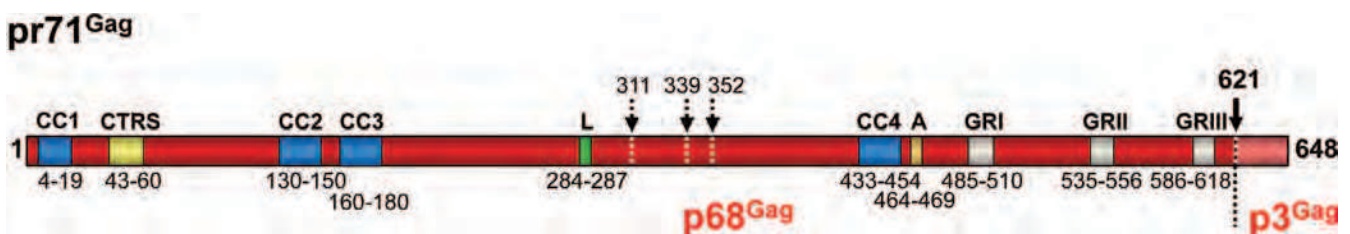
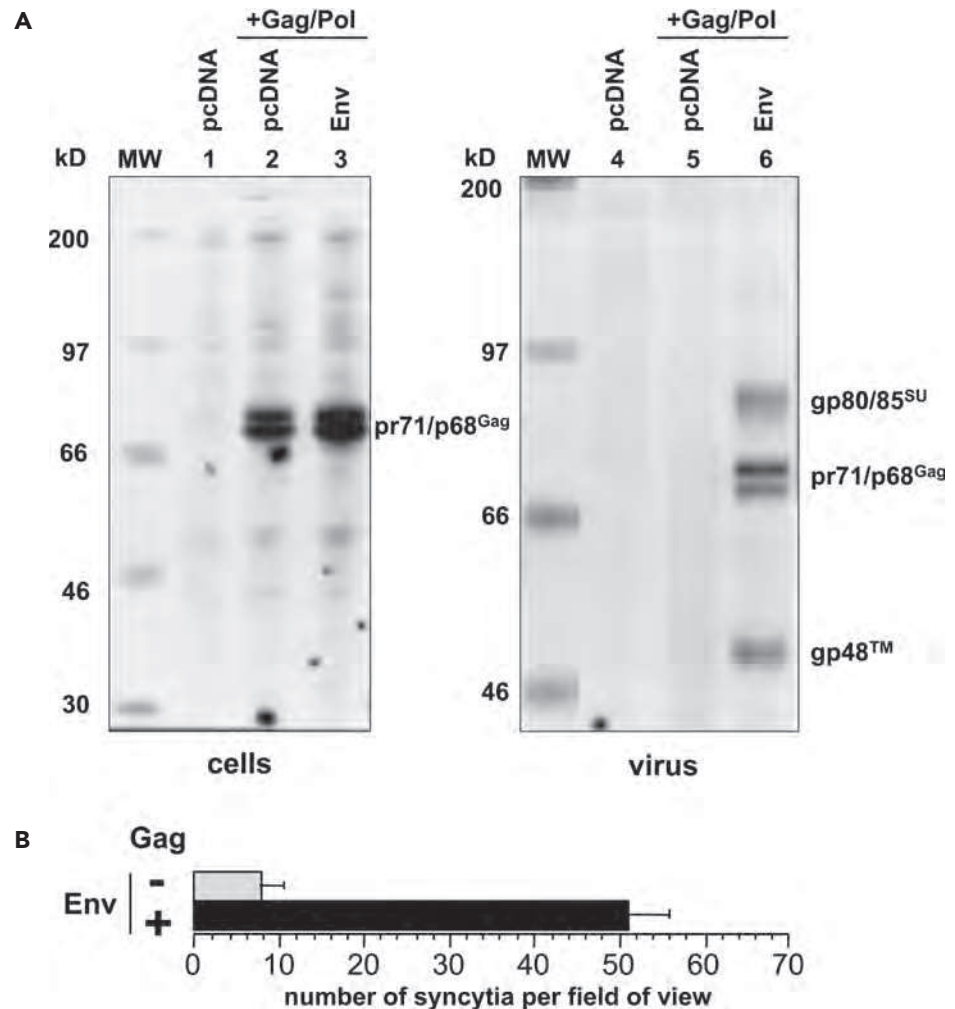


FIGURE 52.7. Primary structure of the 71-kD prototype foamy virus Gag protein. The Gag primary (solid arrows) and secondary (dashed arrows) cleavage sites by the viral PR, the four coiled-coil (CC) domains, the three glycine-arginine boxes (GR), the cytoplasmic targeting and retention signal (CTRS), the PSAP late budding motif (L), and the YXXLGL motif (A) required for correct particle assembly are indicated (see text for details). (Modified from Lindemann D, Rethwilm A. Foamy virus biology and its application for vector development. *Viruses* 2011;3:561–585.)

FIGURE 52.8. A: Autologous Env is required for FV particle (Gag) egress. Foamy virus proteins of 293T cells transfected with vector only (lanes 1 and 4), a *gag/pol* gene expression plasmid (lanes 2 and 5), or in addition with an *env* gene expression plasmid (lanes 3 and 6). Cellular lysates (cells) and particulate material from the supernatants (virus) were partially purified by ultracentrifugation and were reacted with a Gag antiserum. (Modified from Fischer N, Heinkelein M, Lindemann D, et al. Foamy virus particle formation. *J Virol* 1998;72:1610–1615, with permission.) **B:** Gag is required for surface expression of Env. As soon as env-expressing cells are co-transfected with an expression vector for the autologous *gag* gene, massive syncytium formation develop, which is indicative for the surface expression of Env. (Modified from Pietschmann T, Zentgraf H, Rethwilm A, et al. An evolutionarily conserved positively charged amino acid in the putative membrane-spanning domain of the foamy virus envelope protein controls fusion activity. *J Virol* 2000;74:4474–4482.)



However, unlike MPMV, mutation of a conserved arginine in the CTRS did not lead to a switch from a B/D to a C-type capsid assembly strategy. Instead, mutation of the analogous arginine in PFV Gag completely abolished particle release.^{46,129} Release of FV CTRS Gag mutants was rescued by the addition of a N-terminal, heterologous myristoylation signal that also permitted capsid release in the absence of Env.^{46,129,136,274} However, these altered particles were non-infectious even in the presence of the authentic Env.^{46,129} This result suggests that the CTRS of MPMV and FV Gag are functionally different.

A late assembly (L) domain, specified by the motif PSAP (AA 284–287 of PFV Gag), interacts with the cellular export machinery (vacuolar protein sorting [VPS]) via TSG101 to mediate release of virus particles from the plasma membrane^{174,227} (see Fig. 52.7). However, PSAP motifs are absent in nonprimate FV Gag proteins, and their functional L-domains remain to be identified.²²⁷ Interestingly, ubiquitination of PFV Gag—a common feature of orthoretroviral capsids upon interaction with the VPS machinery—has not been observed.^{228,276} This suggests that ubiquitin conjugation to transacting cellular factors, not the Gag protein itself, may be critical for ubiquitin-dependent particle release of enveloped viruses.^{273,274}

Three domains, rich in glycines and arginines (GR-boxes), have been identified at the C-terminal regions of PFV Gag²¹²

(see Fig. 52.7). GR-box I (AA 485–495) is believed to have nucleic acid-binding properties.²⁶⁶ PFV GR-box II (AA 547–557) was initially reported to contain a basic nuclear localization signal (NLS) in its C-terminal half,²¹² whereas the N-terminus contains a chromatin-binding site (CBS) that tethers incoming PFV to the chromatin of the host cell by interacting with H2 core histones.²⁴¹ Nuclear localization of the Gag protein is a common feature of most FVs and leads to the strong nuclear fluorescence detected in IFA using homologous serum (see Fig. 52.1B). It has been recently reported¹⁶¹ that nuclear localization of PFV Gag might be an epi-phenomenon reflecting the tethering of Gag to chromatin via its CBS during mitosis. No specific function has been assigned to GR-box III (AA 601–611). Another study has reported that the GR-boxes are important for RNA encapsidation, (pre-) genome reverse transcription, particle morphology, and virion infectivity.¹⁶² In addition, GR-box III has been reported to functionally substitute for GR-box I, indicating some functional redundancy.¹⁶² Other sequences in PFV Gag, such as a conserved YXXLGL motif (AA 464–467), have also been shown to participate in particle assembly and reverse transcription¹⁴⁹ (see Fig. 52.7).

An unusual and distinguishing feature of FV Gag proteins is their paucity of lysine residues.^{150,228,274} PFV Gag contains only a single lysine residue that is required for replication in primary cells.¹⁵⁰ In contrast, there are 64 arginine residues in

PFV Gag. The probability that this uneven distribution occurs randomly is very low (1.8×10^{-18}).¹⁵⁰ Curiously, viral replication in continuous cell lines was not altered if some of the arginine-specifying residues were changed to lysine-specifying codons. In contrast, several of these mutants replicated poorly in primary cells in the presence of type I IFN.¹⁵⁰

In summary, FV capsids share various features with the core of hepadnaviruses (i.e., glycoprotein dependence for budding, nuclear localization, and the presence of arginine-rich motifs) as well as with orthoretroviruses (i.e., presence of an L-domain and a CTRS). There are also Gag characteristics that are unique to FVs, particularly their unusual cleavage pattern. Furthermore, alterations of conserved FV Gag motifs result in morphologic defects that affect the ability of capsids to support intraparticle reverse transcription and render such mutants noninfectious.

Pol

Pol is translated separately from Gag from its own spliced mRNA, using the major splice donor in the R region of the LTR and a suboptimal splice acceptor in the PFV gag ORF, which prevents *pol* mRNA from becoming too abundant^{20,49,103,122,138,264} (see Fig. 52.6). On the other hand, FV mutants have been reported to be replication competent when *pol* was expressed in-frame with the preceding *gag* ORF or by an orthoretroviral-like frameshift mechanism.²³¹ From orthoretroviruses, it is known that expression of a Gag-Pol fusion protein alone is incompatible with viral replication owing to severe particle assembly or release defects.^{11,57,107,171,254} Furthermore, these defects often involved the orthoretroviral PR that was either found to be in- or hyperactive. The finding of FV replication tolerating expression of an in-frame Gag-Pol fusion protein indicates a mode of Pol encapsidation and regulation of PR domain activity that is unique to these viruses and different from that observed for orthoretroviruses (see later discussion). In the BFV system, equal amounts of *gag* and *pol* mRNAs have been reported.⁹² Whether this also leads to similar amounts of intracellular Gag and Pol proteins has not been investigated.

Historically, the discovery of a spliced *pol* mRNA and large amounts of virion DNA represent a landmark in retrovirus research.²⁶⁴ PFV Pol is translated as a large, approximately 127-kD polyprotein harboring enzymatic domains of PR, RT, RNase H (RH), and IN from N- to C-terminus (Fig. 52.9). Unlike orthoretroviruses, the PFV pr127^{Pol} precursor is processed by the viral PR following particle release into only two mature subunits: p85^{PR-RT} and p40^{IN}.⁶⁰ FV PR does not exist as a separate subunit. Both Pol subunits have been reported to localize to the nucleus in infected cells.⁹⁸ FV Pol precursor

processing is required for virus replication.^{114,202} For the PFV IN subunit, NLS sequence motifs have been characterized.^{6,97}

All retroviral PRs (including the FV PR) are active as dimers. So the question arises, how is FV Pol dimerization accomplished? In orthoretroviruses, this is facilitated by Gag oligomerization of the Gag-Pol fusion protein; however, this is not possible for FVs. Biochemical and biophysical evidence point to transient dimer formation of the FV Pol, as the enzyme is purified always as a monomer, except under the nonphysiologic conditions of high salt.⁷⁶ A role of RNA in PR activation was proposed by Hartl et al.⁷⁵ by identifying a *pol* ORF-located RNA protease-activating RNA motif (PARM) present on (pre-) genomic RNA. By binding to PARM, the p85^{PR-RT} PFV Pol subunit dimerizes and PR is activated. Although the entire process has not yet been elucidated, this mechanism would explain the replication competence of viral mutants expressing an in-frame Gag-Pol protein, because they also would rely on activation of PR by PARM, which would be the rate-limiting step.

Another important question deals with the mechanism of Pol protein encapsidation, because the encapsidation of Pol via protein-protein interaction of the fused Gag precursor, as it occurs with orthoretroviruses, cannot apply to FVs. Two competing views on this exist for the initial step of Pol incorporation: one favoring Gag-Pol protein-protein interactions (when proviral mutants were analyzed) and the other Gag and Pol protein-RNA interactions with viral (pre-) genomic RNA serving as a bridging molecule (when subgenomic vectors were analyzed).^{79,123,177} Probably both mechanisms are involved in encapsidating Pol; however, a definitive answer to this question remains open.

The FV RTs bear in the active center the motif YXDD and are sensitive to nucleoside analog RT inhibitors, such as AZT.^{115,121,197,200}

The solution structure of the PFV RNase H domain has been determined by Leo et al.¹²⁷ RNase H is an endonuclease covalently coupled to the RT domain of Pol, and it hydrolyzes the RNA template strand in RNA/DNA hybrids during reverse transcription. This activity is essential for virus replication.^{185,239} Like the murine leukemia virus (MLV) enzyme but unlike HIV RT, FV RNase H possesses a protruding basic loop and the so-called C-helix.²³ This structure has recently been validated for the PFV RNase H by nuclear magnetic resonance (NMR) spectroscopy,¹²⁷ and information on the function of the basic protrusion remains to be determined.

FV replication depends on integration mediated by the active IN.^{50,153} A 4-bp duplication of staggered chromosomal nucleotides occurs at the site of integration.^{50,153} Orthoretroviruses utilize 3'-end processing as the initial step of the integration

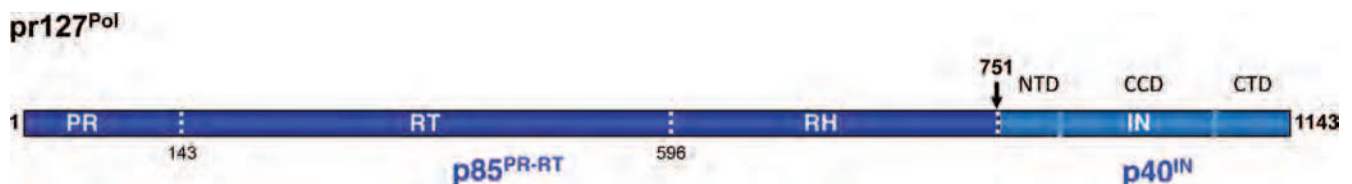


FIGURE 52.9. Primary structure of the 127-kD prototype foamy virus Pol protein. The PR domain is not separated from the RT/RH domain. The only viral PR-mediated cleavage event separates the p40^{IN} subunit from the rest of the protein. Conserved domains within the active center of RT (YVDD)^{160,197} and of IN, with the N-terminal domain (NTD) that is Zn²⁺-binding, the catalytic core domain that harbors the DD35E motif, and the C-terminal (CyD) DNA-binding domain are indicated. (Modified from Lindemann D, Rethwilm A. Foamy virus biology and its application for vector development. *Viruses* 2011;3:561–585.)

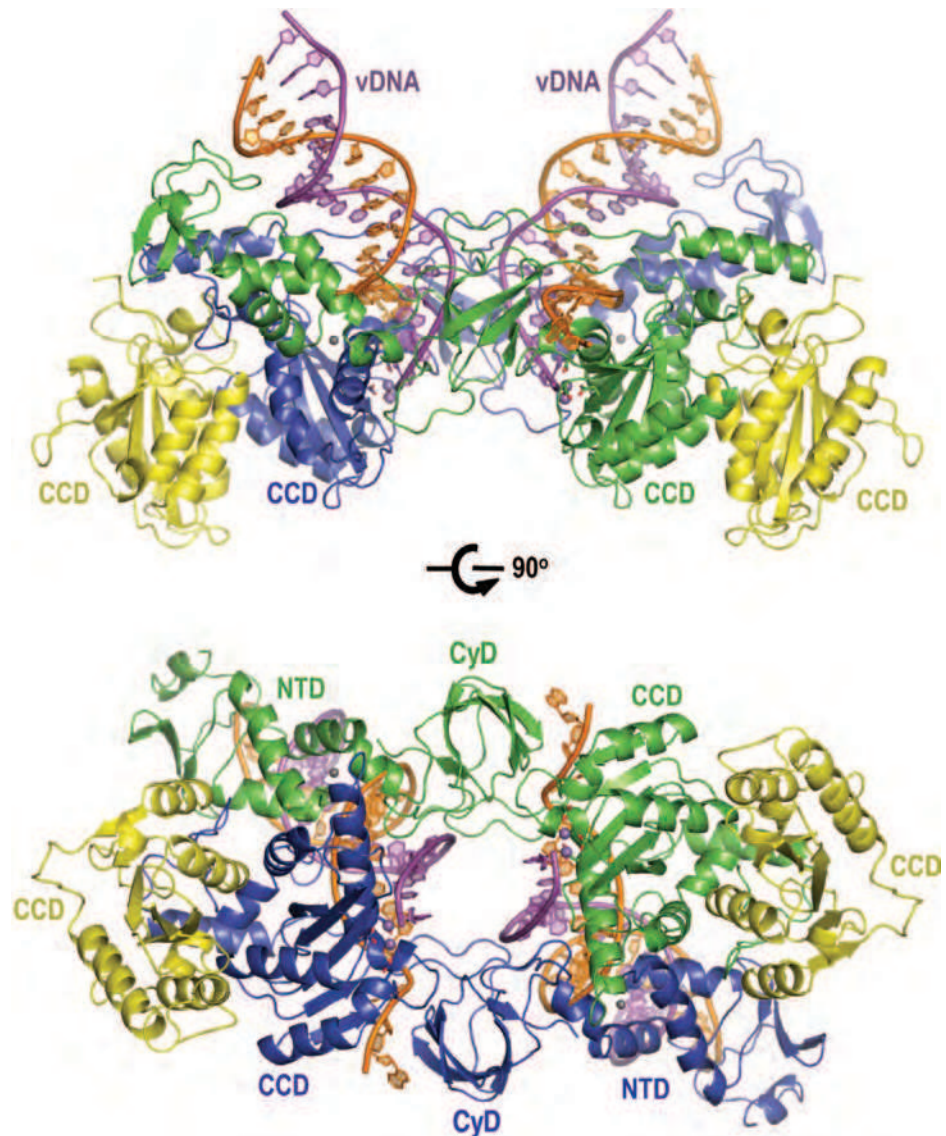


FIGURE 52.10. The architecture of the prototype foamy virus intasome. The crystal structure (PDB ID 3OY9) is shown as viewed along (**bottom panel**) or perpendicular (**top panel**) to its twofold axis. Viral DNA (vDNA) chains are shown as cartoons and colored by chain; vDNA bases and active site IN residues are shown as *sticks*. Gray spheres are metal cations. Locations of IN domains (NTD, CyD, and CCD) are indicated. (Courtesy of P. Cherepanov, London, UK.)

reaction. This involves the removal of two nucleotides from each terminus of the blunt-ended linear viral DNA. During FV integration, only the 3' terminus (within the U5 region) of the viral DNA undergoes processing, whereas the 5' end (the U3 region of the LTR) remains unprocessed, possibly because it is already suitable for integration.^{50,105}

In 2010, a seminal study was published that described the crystal structure of full-length PFV IN bound to its cognate DNA, as tight complex, termed the *intasome*⁷² (Fig. 52.10). This achievement was possible because, unlike the orthoretrovirus IN protein, the recombinant PFV IN is uniquely soluble and active *in vitro*.²⁴⁸ These properties contrast with the aggregation and poor enzymatic activity observed with other retroviral INs, regardless of the expression system used.

Classically, retroviral INs are subdivided into three domains (see Fig. 52.9): (a) an N-terminal Zn²⁺ binding domain (NTD), characterized by pairs of His and Cys residues (HHCC motif); (b) a catalytic core domain (CCD), harboring the Asp, Asp-35-Glu (DD35E) motif; and (c) a nucleic acid-binding Arg/Lys-rich C-terminal domain (CyD).²⁷ These

domains are connected by nonconserved flexible linkers. Early studies reported nonspecific DNA-binding activity by the CyD, and it was assumed that retroviral INs would adopt a dimeric or tetrameric structure when engaged with the viral DNA ends.²⁷ It was also hypothesized that multimers were highly flexible,²⁵³ and several contrasting structures of the retroviral intasome had been proposed. Crystallization of the PFV intasome has revealed the definitive answer to a long-standing puzzle. The viral integration apparatus contains a tetramer of IN, assembled on a pair of viral DNA ends, in which all three IN domains and interdomain linkers are involved in intimate protein–protein and protein–DNA interactions cross-linking the complex in a rigid structure.⁷² Further co-crystallization of the PFV intasome with target DNA (a mimic of the host cell DNA) revealed the assembly of the entire retroviral synaptic integration complex prior to and following strand transfer.¹⁴⁶ Moreover, because of the structural and functional similarity of PFV and HIV-1 INs, the mechanism of action of clinically useful HIV IN strand transfer inhibitors (raltegravir, elvitegravir, and dolutegravir) was elucidated.^{73,74,116} Using the PFV

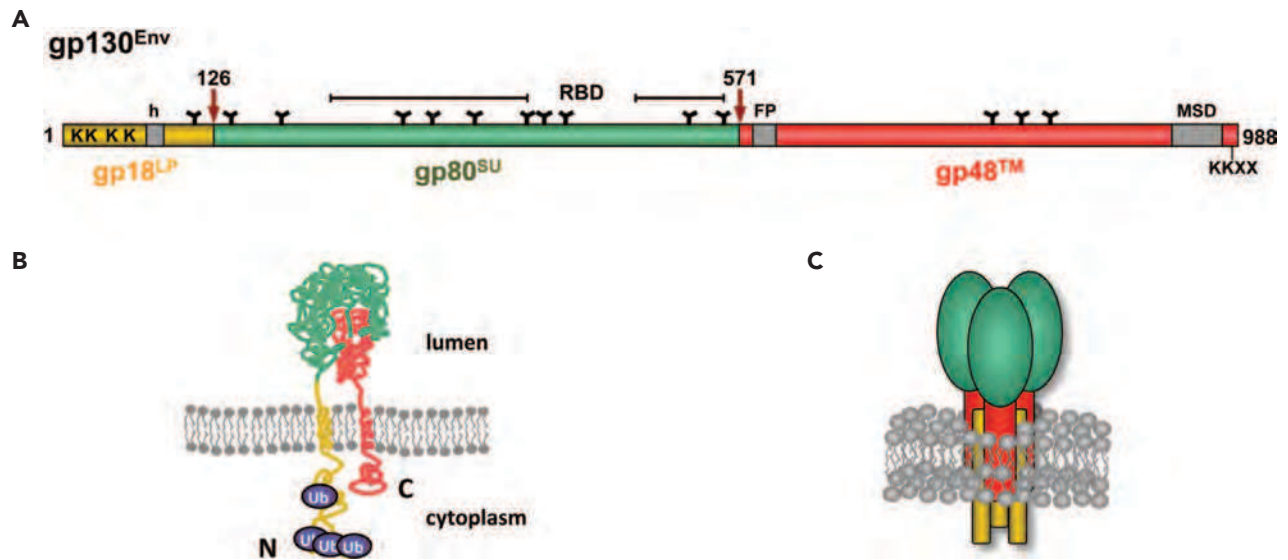


FIGURE 52.11. A: Primary structure of the 130- to 140-kD prototype foamy virus (PFV) Env protein, with cleavage sites separating gp18^{LP}, gp80/85^{SU}, and gp48TM. h, hydrophobic region of leader peptide; FP, fusion peptide; MSD, membrane-spanning region of transmembrane; RBD, receptor binding domain of SU⁴⁴; K, lysine residues in LP found to be ubiquitinated²²⁸; Y, sites of N-linked glycosylation throughout Env¹⁴⁵; KKXX, C-terminal dilysine motif in transmembrane responsible of Env retrieval to the endoplasmic reticulum.^{69,70} **B:** Schematic membrane topology of the monomeric Env protein with ubiquitination (Ub) sites in leader peptide regulating subviral particle release.²²⁸ The N- and C-terminus of the protein are indicated. **C:** Schematic view of the trimeric PFV glycoprotein. (Modified from Lindemann D, Rethwilm A. Foamy virus biology and its application for vector development. *Viruses* 2011;3:561–585.)

intasome as a surrogate for its HIV counterpart, it was shown that these small molecule inhibitors bind to the active site of IN and displace the reactive 3'-hydroxyl group of the viral DNA, thereby preventing strand transfer.³⁷ The availability of the PFV intasome co-crystal structures with target DNA may facilitate the development of “designer integrases” possessing site-specific integration properties.¹⁴⁶

Env

The Env glycoprotein of FVs has an unusual primary structure and topology (Fig. 52.11A). PFV Env is synthesized as a large precursor protein of 988 aa (gp130–140^{Env}). It is tripartite, consisting of gp18^{LP}, gp80/85^{SU} surface (SU), and gp48TM transmembrane (TM) subunits.¹³⁰ The Env precursor is not co-translationally processed by the cellular signal peptidase complex, which naturally removes N-terminal signal peptides required for targeting glycoproteins to the secretory pathway. Therefore, unlike orthoretroviruses, which have their glycoprotein membrane anchored only in the TM domain, the FV Env spans the cellular membrane twice with the N- and C-terminal regions of the precursor located in the cytoplasm¹³⁰ (see Fig. 52.11B). The peptide backbone of the PFV Env precursor protein is cleaved between LP and SU (after aa 126) and between SU and TM (after aa 571), during cell surface transport in the late Golgi complex, by furin-like cellular proteases.^{45,63} The SU-TM cleavage is required for infectivity, whereas the LP-SU cleavage is not.⁴⁵

As a consequence, the mature gp18^{LP} subunit is an integral component of the virion and transverse the viral membrane at the N-terminus (a type II *trans*-membrane protein), as does the TM subunit at the C-terminus of Env (a type I *trans*-membrane protein)^{130,257} (see Fig. 52.11B,C). All three Env subunits are heavily glycosylated. Fourteen N-linked glycosylation sites

have been mapped, only two of which (N8 and N13 located in PFV SU and PFV TM, respectively) are essential for viral infectivity¹⁴⁵ (see Fig. 52.11A).

The C-terminal cytoplasmic tail domain (CyD) of the TM subunit is rather short (comprising 16 aa), and its presence is not required for particle egress.¹⁸¹ In contrast, the N-terminal CyD of the LP subunit, comprising approximately 68 aa, is considerably longer. Alteration of conserved tryptophan residues in the LP N-terminal CyD (at aa positions 10 and 13 of the PFV LP) abolished interaction with the Gag protein.^{130,257} Not only is FV Env required for export of capsids (see Fig. 52.8A), but *gag* gene expression is also necessary for the transport of Env to the cell surface. This implies that highly specific direct or indirect interactions influence the intracellular distribution and trafficking of both proteins^{32,58,181} (see Fig. 52.8B).

Aside from its interaction with Gag, two additional factors appear to regulate Env intracellular trafficking and transport to the cell surface. First, a dilysine motif, known to be responsible for retrieval of glycoproteins to the endoplasmic reticulum (ER), is present near the C-terminus of the TM⁶⁹ (see Fig. 52.11A). Although this signal can sort Env to the ER, it is not required for efficient virus replication⁷⁰ and, in comparison to the other factors, has only a weak effect on Env intracellular distribution. Second, posttranslational ubiquitination of four of five lysine residues located within the LP subunit N-terminal CyD also appears to mediate efficient Env removal from the cell surface^{226,228} (see Fig. 52.11B,C).

The PFV Env has been shown to support not only viral particle release from cells but also release of SVP from other cellular membranes harboring the viral glycoprotein.²²⁴ This is again analogous to a similar process observed with hepadnaviruses, which secrete vast amounts of SVPs, the so-called Australia antigen (see Chapter 68). Ubiquitination appears to

suppress the intrinsic activity of the FV glycoprotein to induce SVP release, and mutants of the lysine-specifying codons in the LP CyD release large amounts of SVP.²²⁸ These PFV Env mutants appear to be particularly well suited to pseudotype orthoretroviral capsids, in contrast to the wild-type PFV Env, probably owing to its low level of cell surface expression (own unpublished observation).

Surprisingly, the gp130–140^{Env} is not the only FV glycoprotein synthesized. Using conserved splice sites within *env* ORF of the FV genome (see Fig. 52.6), alternatively spliced *env* transcripts are generated that are translated into fusion proteins consisting of Env lacking the membrane-spanning domain of TM in-frame with the Bet protein (Env-Bet)^{67,131} or, as in the case of FFV, with the ORF-2 encoded peptide sequence (Env-Bel2).¹⁹ The Env-Bet fusion protein is secreted into the supernatant of PFV-infected cells and processed into LP, SU, and ΔTM-Bet subunits that are not viral particle associated.¹³¹ This fusion protein is synthesized at 50% of the level of particle-associated gp130–140^{Env}, suggesting that it may have a useful function.¹³¹ However, replication-competent PFV mutants deficient in Env-Bet synthesis do not exhibit a distinctive phenotype, and no revertants with restored Env-Bet expression have been observed in cell culture, although this fusion protein could be functionally important *in vivo*.¹³¹

In summary, the biosynthesis and membrane topology of the FV Env are highly unusual. The Env LP subunit is an integral component of the particle-associated Env complex and harbors in its CyD the major interaction domain with FV capsid essential for viral particle budding. The interaction of Env with Gag and ubiquitination of the CyD of the Env LP subunit seem to be the main determinants of its intracellular transport and are probably dominant over the C-terminal dilysine motif. Furthermore, some properties of orthoretroviral Gag proteins, such as ubiquitination and budding functions, have been delegated to Env in FVs. In addition, the function of the unusual Env-Bet/Bel 2 fusion proteins is unknown.



FIGURE 52.12. Prototype foamy virus Tas protein with functional domains, the region shared with Bet that harbors a multimerization domain (SR/MD), the centrally located DNA-binding domain (DBD) that is followed by the region specifying the nuclear localization signal (NLS), and the C-terminal activation domain (AD).

NONSTRUCTURAL PROTEINS

Tas

Tas is the *trans*-activator of spumaviruses and is required for replication.^{142,193} PFV Tas (Tas_{PFV}) is a 35-kD nuclear protein that binds to upstream DNA elements in, and augments gene expression from, both the IP and the U3 LTR promoter^{34,109,137,139} (see Fig. 52.6). Most Tas protein is translated from a spliced mRNA initiated at the IP.^{10,19,137,163} Tas is variable in size (209 aa in FFV and 300 aa in PFV) and has a modular organization (Fig. 52.12).^{77,137,189} Its N-terminus contains a region of variable length that is shared with the Bet protein, the other accessory FV protein, and harbors a multimerization domain. Unique to Tas are a central DNA-binding domain (DBD) of approximately 100 aa, a basic NLS, and a C-terminally located acidic activation domain (AD) of around 30 aa.

Except for the AD, Tas shows no homology to known cellular proteins,¹³⁷ and there is little or no cross-*trans*-activation between different FVs.¹³⁷ The reason for this is the species specificity of the Tas DBD, which is highly variable in aa sequence among different FV Tas proteins. This is consistent with the highly divergent DNA targets that mediate Tas function among different FVs (Table 52.2). This is in contrast to the acidic Tas

TABLE 52.2 Tas Binding Sites in Different Foamy Virus Internal Promoter and U3 Promoter Elements Reveal Little Consensus

IP-TBS-Sequences (position relative to start of transcription in 5' → 3' direction)					
Virus					Reference
PFV	-163	GCCACTGGTTGCGGAAGAAAGATTG	-139		77,106
SFVmac	-69	TTGCAATCACTGGAATAGAAGTTAC	-44		275
FFV	-70	TTAAAAAGGCCAC	-58		17
BFV	-62	AGAGCTTAAAAATCAAGGTAAC	-41		235
U3-TBS-Sequences (position relative to start of transcription in 5' → 3' direction)					
Virus					Reference
PFV	-73	ATACTATAGTAATAGATTGATAGTTT	-47		77
FFV	-70	AACTTTAACTTTAGTTGC	-50		17
BFV	-368	ATACTAGAAGAATAAGGTTAAG	-346		235
	-327	ATAGCTTAGGGAGATTGTTAG	-307		
	-306	ATAGCTTAAAGAACAAGTTAAC	-285		
	-186	ATAGCTATTTTAGTAAGTTAGC	-165		

IP, internal promoter; TBS, Tas binding site; PFV, prototype foamy virus; SFVmac, simian foamy virus in the macaque; FFV, feline foamy virus; BFV, bovine foamy virus.



FIGURE 52.13. Prototype foamy virus Bet protein with the SR/MD. The domain probably involved in antagonizing APOBEC3 enzymes (AAD) is located C-terminal from the SR/MD.

AD, which shares conserved amino acids with other viral and cellular transcriptional activators and is also active in yeast.¹⁴ For transcriptional activation, the Tas protein has to multimerize—a process that is apparently facilitated by residues located within the N-terminal domain shared with the Bet protein (SR/MD). This property has been demonstrated experimentally for Tas_{BFV} and can probably be generalized to all FV Tas proteins.²³⁷

Little is known about likely cellular factors that might interact with Tas.¹¹³ Phosphorylation by DNA-PK is required for full Tas activity, and only acetylated Tas protein has full DNA-binding capacity.^{15a,18,35} In addition, the ADA2 adaptor molecule is required for Tas AD-mediated activation in yeast.¹⁴

Research carried out on cellular factors engaged with Tas_{BFV} has led to the identification of RelB, which interacts with Tas_{BFV} and activates the nuclear factor κB (NF-κB) pathway.²⁵² However, whether these results also apply to Tas proteins other than Tas_{BFV} is not known. Furthermore, the NF-κB-mediated transcriptional enhancement seen is unlikely to explain the full activity of Tas.

Tas can activate cellular genes if they—probably by chance—harbor Tas-responsive DNA elements.^{137,251} This has been investigated for various human genes activated by Tas_{PFV}.^{137,251}

Bet

Bet is the least conserved of all FV proteins (Fig. 52.13). It is translated predominantly from multiply spliced mRNAs originating at the IP and to a minor extent from mRNA variants initiating at the LTR promoter^{10,163} (see Fig. 52.6). The *tas* ORF-located splice sites utilized for generation of the *bet* mRNA are highly efficient, resulting in Bet to be always made in vast excess over Tas. Because the shared region at the N-terminus appears to harbor the multimerization domain of Tas, it is likely that Bet also multimerizes via this domain. Aside from antibodies against the Gag protein that are diagnostic, infected individuals sometimes develop antibodies against this highly expressed protein, which are of some help in confirming the serodiagnosis.⁷¹

For a long time, no clear function could be attributed to Bet. For PFV, it was shown to be dispensable for *in vitro* replication in most cell types, with only a minor decrease in viral titer.^{10,267} The first generation of FV vectors actually had the ORF-2 region encoding for most of Bet replaced by sequences of interest so that they were under Tas-dependent transcriptional control of the IP or of an inserted heterologous promoter.²¹⁵ For FFV, a more drastic reduction in viral titers was observed for Bet-deficient viruses when grown on feline CRFK cells but not on human 293T cells.³ This observation eventually led to the identification of Bet being an antagonist of APOBEC3, similar to HIV Vif.¹⁴¹ However, unlike Vif, PFV Bet does not induce degradation of APOBEC3³⁶ but instead inhibits its dimerization by directly binding to the dimerization domain.¹⁷⁵ The regions of Bet involved in this physical interaction are under

investigation. Studies with FFV Bet suggest that these regions are located outside of those shared with Tas (M. Löchelt, Heidelberg, personal communication).

The splice sites that lead to generation of the Bet protein appear to be so efficient that they are used also in (pre-) genomic RNA. An integrated FV has been described that carries the characteristic deletion leading to the generation of Bet (ΔTas)²⁰⁹ (see Fig. 52.6). As the (pre-) genomic RNA leading to ΔTas carries all features necessary for successful packaging and reverse transcription, ΔTas infects new cells where it integrates. ΔTas has been found *in vitro* and also *in vivo* in a rabbit infection model and in the monkey to a considerable extent.^{19,54,208,209} ΔTas provirus is replication incompetent because of its *tas* gene deletion; however, it is not transcriptional silent. Owing to the basal activity of the IP (see later discussion), there is still some residual *bet* gene expression. The magnitude of viral transcription depends on the number of integrated copies of ΔTas and probably also depends on the site of integration where cellular promoter/enhancer elements could augment levels of Bet mRNA.²⁰⁹ Cells expressing *bet* become resistant to superinfection by homologous virus—a feature that has so far not been further investigated.^{15,209} Furthermore, a role in promoting viral persistence has been discussed for Bet in general and ΔTas in particular.^{155,209} Functionally, ΔTas behaves like a defective interfering (DI) genome. However, whether the typical oscillating frequency of DI viruses occurs with ΔTas has not been investigated.

Because only either Bet or Tas can be made, and because FV gene expression starts with the translation of Tas protein (see later discussion), it has been speculated that Bet synthesis represents the molecular switch, which determines viral latency.¹⁵⁵

REGULATION OF GENE EXPRESSION

FV gene expression (see Fig. 52.6) begins with expression of the *tas* and *bet* genes directed by transcripts initiated at the IP located in the *env* ORF.^{139,140} This is mediated by the weak basal transcriptional activity of the IP, whereas the U3 promoter in the LTR of the provirus has no, or almost no, similar activity.^{109,139}

In FFV, enhancer elements upstream of the IP implicated in its basal transcriptional activity include sites for SP-1^{16,258}; however, their biological function has not been characterized in any depth. In BFV, AP-1 sites in this location have been partially characterized²⁶¹ and may control the basal gene expression that it directs.

As a consequence of the IP's low basal activity, some Tas protein is made, which subsequently binds with high affinity to specific DNA elements—namely, Tas binding sites (TBS) upstream of the IP—resulting in a positive feedback loop of

tas gene expression. Once sufficient amounts of Tas have been synthesized by this mechanism, Tas, with lower affinity but higher avidity, binds to upstream promoter elements in the 5' LTR U3 region. This then leads to the expression of structural genes and, to some extent, also to LTR-directed *tas* and *bet* gene expression.^{16,137}

The IP and LTR U3 TBS essential for Tas-mediated transactivation have been determined and characterized in detail (e.g., by electromobility shift assays) for PFV, SFVmac, FFV, and BFV (see Table 52.2). However, no real TBS consensus sequence has emerged. Furthermore, even within a specific FV species, the IP and U3 TBS show only very weak homology. In general, it can be noted that the finer details of Tas-mediated regulation of FV gene expression remain to be elucidated.

Similar to other complex retroviruses, FVs encode a regulatory protein acting at the transcriptional level. A *post*-transcriptional regulator, such as Rev of HIV or Rex of PTLV, has never been identified in FVs. The peculiarities of FV gene regulation—one transcriptional activator and two active promoters—allow for a biphasic mode of FV gene expression analogous to other complex retroviruses.³⁹ However, this does not circumvent a central problem of all retroviruses: regulation of nuclear export of intron-containing mRNAs containing functional splice sites.³⁸ As detailed elsewhere in this book (see Chapters 48 and 49), complex retroviruses solve this problem by interacting with the karyopherin CRM1 by using a viral regulatory protein, which binds to an RNA secondary structure embedded in viral mRNAs. In contrast, some simple retroviruses utilize the NXF1/NXT1-mediated cellular mRNA export pathway by means of a constitutive transport element (CTE) located within their genomic mRNAs.³⁸ PFV (pre-) genomic and Gag encoding mRNA appears to make use of yet another so far undisclosed pathway.²¹ Nuclear export of FV RNA is, on one hand, CRM1 dependent and, on the other, relies on the presence of additional cellular proteins. The host cell HuR protein binds to the unspliced PFV (pre-) genomic RNA, and two cellular adapter molecules, ANP32A and ANP32B, mediate the interaction between the RNA-bound complex and CRM1.^{16,21} The FV RNA elements in question have not yet been characterized. The consequences of this viral nuclear RNA export mechanism on the synthesis of early response cellular proteins, whose mRNAs normally use this export pathway, are unknown. It is tempting to speculate that FVs, by making use of a nuclear mRNA export pathway involving cellular proteins also required for the synthesis of early response proteins, may outcompete the latter.

CIS-ACTING RNA SEQUENCES AND PACKAGING OF RNA

FV packaging of (pre-) genomic RNA is quite different from that of orthoretroviruses: Three *cis*-active RNA elements (CAS I–III) essential for viral replication have been identified and partially characterized by their capacity to enable an efficient FV vector gene transfer¹³² (see Fig. 52.6).

The first region, CAS-I, is located in the 5' untranslated leader of the viral (pre-) genome and extends into the *gag* gene. It harbors the R-U5 region of the LTR, the PBS, and what is believed to be the ψ -sequence.¹³² The R region also contains the elements conferring dimerization of the (pre-) genomic RNA.^{29,53}

The second element, CAS-II, is located within the 3' half of the *pol* gene^{52,82,177,260} (see Fig. 52.6). It is mainly the presence of and requirement for CAS-II that sets FV (pre-) genomic RNA and Pol protein incorporation apart from orthoretroviral particle assembly. CAS-II is believed to harbor (in 5' to 3' direction) sequences for packaging of viral RNA¹⁷⁷; for encapsidation of the Pol polyprotein¹⁷⁷; the PARM sequence required to activate the FV PR⁷⁵; and the cPPT, which is probably engaged in efficient reverse transcription.¹⁷⁶ Furthermore, one of the RNA elements directing nuclear export via HuR appears to be located in CAS-II.²¹ These, however, are not required for efficient FV vector transfer. RNA sequences of the individual functional elements may partially overlap and mediate more than one function. Intervening sequences can be deleted without apparent loss of vector transfer function.²⁵⁵

The third element, CAS-III, is located far downstream on the viral (pre-) genome. It contains the 3' PPT and the LTR (U3-R) sequences needed for reverse transcription and integration as well as transcription initiation and polyadenylation upon proviral integration¹³² (see Fig. 52.6).

OVERVIEW OF THE VIRUS LIFE CYCLE

The Early Phase: Establishing the Provirus

FVs bind to yet unknown, probably ubiquitously expressed and evolutionary conserved, cellular receptor molecules (Fig. 52.14). Proteoglycans contribute to FV entry but do not appear to be the major cellular receptor.^{210a,230} Uptake of most FVs predominantly seems to involve endocytosis and a pH-dependent FV Env-mediated fusion process, although PFV Env displays a significant fusion activity at neutral pH.¹⁸⁰ After release into the cytoplasm, FV capsids migrate along the microtubular network to the centrosome, where they accumulate.²¹⁰ At the centrosome, intact FV capsids can remain functional active for weeks in G₀-resting cells, allowing productive infection to proceed upon re-entry of targets cells into the cell cycle.¹²⁶ It is generally agreed upon that mitosis is required for FV replication and that the latency period from cell entry to integration and gene expression, owing to the facultative viral DNA genome, can be very long.^{13,43,126,173,245} Most productive infections are attributed to FV particles that have reverse transcribed their viral RNA (pre-) genome prior to target cell infection.^{150,160} However, some genome reverse transcription takes place upon FV host cell entry and seems to add to viral infectivity predominantly under conditions of low MOI.^{42,270} Disassembly of FV capsids accumulated at the centrosome is reported to involve Gag cleavage by viral and cellular proteases in a cell-cycle-dependent manner.^{66,75,125} Upon mitotic breakdown of the nuclear membrane, the viral DNA and fragments of Gag then gain access to the chromosome, whereas active nuclear import of Gag into interphase nuclei is not observed.¹⁶¹ Productive viral replication requires insertion of the provirus into the host genome mediated by the viral integrase,^{50,153} although nonintegrated viral DNA appears to be transcriptionally active, similar to reports of orthoretroviruses.⁴³ Like other retroviruses, there are no preferred sites of FV integration. Analyses of FV integration patterns have revealed no preference for actively transcribed genes and a much lower frequency of integration into the proximity of cellular promoters than MLV.^{168,247}

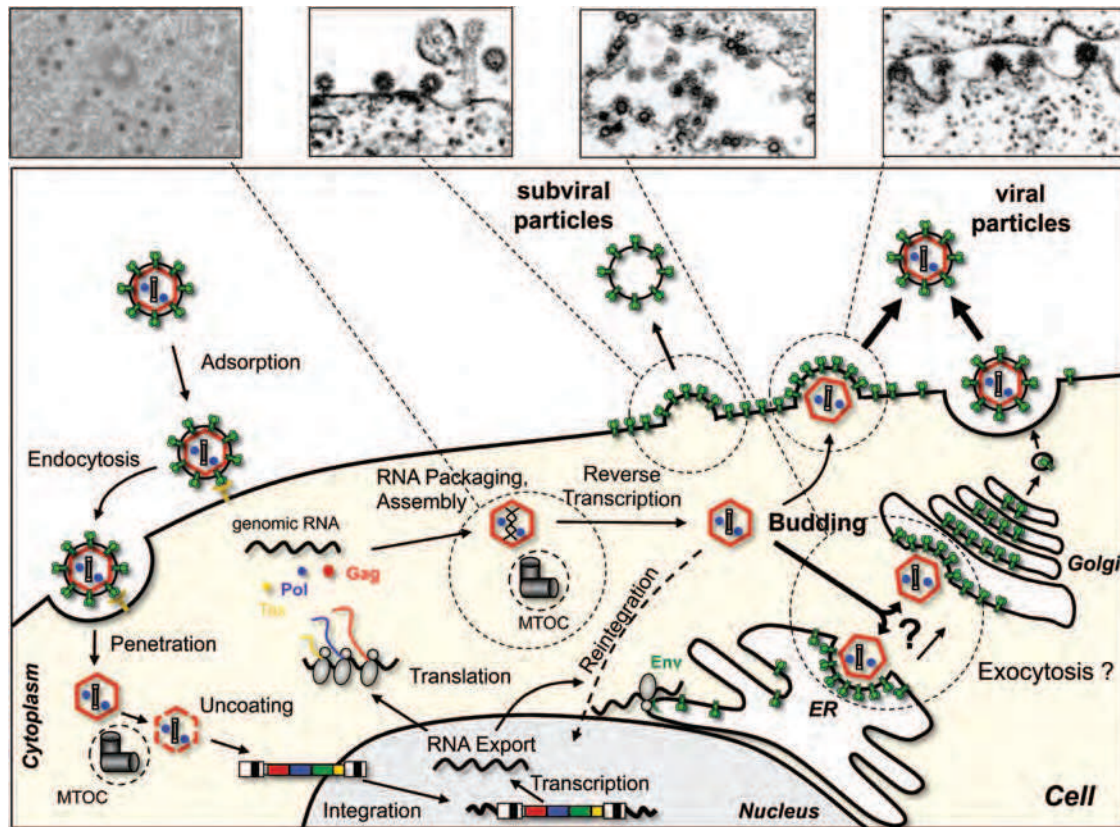


FIGURE 52.14. Overview of the prototype foamy virus replication cycle (see text for details). (Modified from Lindemann D, Rethwilm A. Foamy virus biology and its application for vector development. *Viruses* 2011;3:561–585.)

The Late Phase: Generation of Progeny Viruses

Like orthoretroviruses, FVs exploit the cellular transcription machinery for viral transcription. FV transcription is special, as a cascade of events is launched by the action of two viral promoters and one transcriptional transactivator. FV nuclear RNA export also appears to be unique.

The Gag and Pol proteins, as well as the accessory gene products Tas and Bet, are translated on free ribosomes in the cytoplasm. The Env protein is targeted to the secretory pathway by ribosomal translation in the rough endoplasmic reticulum and displays a highly unusual biosynthesis and membrane topology.^{130,257}

FV capsid assembly follows a retrovirus type B/D morphotype strategy. This includes the microtubule-dependent transport of Gag proteins to the centrosome involving a CTRS within Gag.^{46,265} A Gag CC domain mediates FV capsid preassembly at the centrosome.²⁴⁰ Unlike orthoretroviruses, FV capsids lack membrane-targeting signals. Virion budding and egress requires co-expression of the viral Env protein. This process involves a highly specific interaction between the viral glycoprotein complex and capsid proteins. This interaction, mediated by an N-terminal region in the capsid and the CyD of the envelope LP subunit,^{32,130,257} presumably takes place at the *trans*-Golgi network (TGN).²⁶⁵ Together with the involvement of the cellular VPS machinery in FV particle release, this is suggestive for an exocytotic budding pathway.^{130,265} However, there are also clear indications for budding of FVs at the plasma membrane.¹¹⁹

Although the exact time point of reverse transcription with respect to capsid assembly and maturation is unknown, preassembled intracellular FV capsids contain significant amounts of reverse transcribed, infectious viral genomic DNA.^{150,160,203,221,269} In this respect, FVs show a similarity to HBV replication (see Chapter 68). Gag, but not Pol precursor protein cleavage, is a prerequisite for intraparticle reverse transcription that occurs late in the FV replication cycle.^{48,202}

Orthoretroviruses can occasionally behave like retrotransposons and reshuttle their genome to the nucleus without an extracellular phase. The frequency of such intracellular retrotransposition (IRT) has been estimated to be 1 per 10⁶ proviruses and is thought to reflect the frequency at which the RNA genome is prematurely reverse transcribed.^{80,203} For PFV, this frequency is much higher (approximately 5%)—a finding concordant with late reverse transcription of the RNA (pre-) genome.⁸⁰ It was subsequently found that IRT strongly depended on the cell type used for analysis and on the particular FV isolate.^{81,203} Thus, IRT is not a general phenomenon of FVs. However, the late phase of reverse transcription of the RNA (pre-) genome is required for this process.

APATHOGENICITY OF FOAMY VIRUSES

Apart from very rare reports of subclinical laboratory findings, *in vivo* infections by FVs follow a benign course. Asking the

reason for this is probably posing the wrong question. The pathogenicity of an infectious agent requires an explanation, not the apathogenicity of what has been called a “perfect parasite”²⁴⁹ whose only “interest” is to multiply its genome without doing harm. It appears that FVs evolved over millions of years to do exactly this.

Certainly, some arguments can be made in favor of this hypothesis. The site of active replication *in vivo* determines to a large extent the pathogenicity of an infectious agent. FVs appear to replicate only in cells that are destined to be eliminated (e.g., the oral mucosa and occasionally the intestine). However, during the establishment of persistence, it is likely that lymphocytes become infected and produce low amounts of virus that disseminate throughout the body. Once persistence is established, FV genes may no longer be expressed in lymphocytes. A second factor contributing to the benign character of FV infections is that the Tas transactivator appears to be specific for its autologous cognate viral promoters. The off-target activation of gene expression is probably a rare event. Pleiotropic effects, as they occur with PTLV-I Tax (see Chapter 48), does not occur. Furthermore, the integration of FVs does not induce the activation of cellular oncogenes because they lack strong enhancer elements. In addition, a strong polyadenylation signal in the LTR seems to prevent a read-through of viral transcripts into cellular genes for activation.^{84,216}

FOAMY VIRUS VECTOR APPLICATIONS

There are several advantages that FV vectors have over the more common orthoretroviral vectors (summarized in Table 52.3). FV vectors have been shown to be extremely useful in transducing stem cells of hematopoietic or mesenchymal origin.^{128,255} The same targets are transduced with at least the efficiency of HIV-1 vectors pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV-G). However, even better transduction and engraftment rates of hematopoietic stem cells have been observed when a mutant of the FV *env* gene, deficient in LP ubiquitination and optimized for expression in human cells, is used to pseudotype HIV-1 vectors (H. Hanenberg, Indianapolis, Indiana, personal communication).

TABLE 52.3	Advantages of Foamy Virus Vectors Compared with Ortho-/gammaretroviral Vectors
<ol style="list-style-type: none">1. Derived from nonpathogenic parental viruses2. Transduction of stem cells with exceptional high efficiency3. Favorable integration profile4. No evidence of oncogene activation in relevant animal models5. Possibility of particle concentration6. No gene silencing7. Relatively stable DNA genome8. Large capacity to accommodate foreign genes9. Natural of self-inactivating type, use of tissue-specific promoters permitted10. Efficient pseudotyping of orthoretroviral vectors by modified foamy virus Env	

Basically, FV vector applications follow the field of gammaretroviral and lentiviral vectors using the same models of single gene defects that can be corrected by gene transfer into hematopoietic or mesenchymal stem cells. However, two models are well advanced and may lead to human clinical trials in the near future. One is the replacement of a defective *cd18* gene. CD18 is essential for leukocytes to adhere to endothelial cells and to migrate from the blood stream to sites of infection. The CD18 defect results in a disease known as leukocyte adhesion deficiency (LAD). Canine LAD has been successfully treated in a clinically relevant large animal dog model⁹; the recipient animals have been followed for more than 4 years of age and are clinically completely healthy.¹⁶⁹

The other disease is Fanconi anemia (FA). FA results from a defect in double-strand DNA breakage repair. There are 16 so-called complementation groups known, because at least 16 genes can be defective. Probably some more very rare variants remain to be discovered. *Fanca* and *fancc* defects account for approximately two-thirds of the cases. Large animal models of the disease are not available. However, knockout mice for some of the genes have been successfully treated on a NOD/SCID background by transfer of gene-corrected human hematopoietic stem cells.²²⁵ The repopulating cells have been serially transferred documenting their stem cell character.²²⁵ A forthcoming clinical trial to combat FA will rely on HIV-derived vectors pseudotyped with a PFV-derived envelope.⁶⁸

These rare life-threatening diseases serve as model systems for more common conditions. Therefore, one may speculate about tackling more widespread diseases with these apparently benign vectors in the future.

PERSPECTIVES

In the past few years, FV research has resulted in several new findings that impact on fundamental aspects of retrovirus

TABLE 52.4	Some Future Foamy Virus Research Topics
<ol style="list-style-type: none">1. What is the nature of the vacuoles (foam) induced upon FV replication?2. What (cellular) factors are responsible for replication in certain epithelia only?3. What factors induce and control latency?4. What is the cellular immune response like?5. What factors are responsible for the enormous genetic stability of FVs?6. What is the nature of the FV receptor?7. What roles do the antiviral restriction factors actually play in FV replication?8. What is the physical stability of FV virions?9. What is the actual FV budding site?10. What are the cellular Tas and integrase interaction factors?11. What is the role of the central polypurine tract in FV replication?12. How is the virion in terms of proteins and nucleic acids composed, and how are RNA and Pol encapsidated?	

FV, foamy virus.

replication. It is likely that future studies of FVs will unravel new aspects of these pathways. Some topics worth investigating have already been alluded to in this text and are summarized in Table 52.4. Aside from basic research into FV replication and necessary *in vivo* and *ex vivo* studies (e.g., cellular immune responses to FVs), increasing interest will focus on the use of FV-derived vectors. To cover this aspect, the reader is referred to more specialized reviews.^{132,188,246}

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Polyomaviruses

History

Classification

Virion Structure

Genome Structure and Organization

Structure of T Antigens

Stages of Replication

Mechanism of Attachment

Entry, Intracellular Trafficking, and Uncoating

Transcription

Translation

Replication of Viral Genomic DNA

Assembly

Release

Pathogenesis and Pathology

Immune Response

Persistence

Transformation

Epidemiology

Clinical Features

BKPyV-Associated Diseases

JCPyV and Progressive Multifocal

Leukoencephalopathy

Merkel Cell Polyomavirus and Merkel

Cell Carcinoma

Trichodysplasia Spinulosa Polyomavirus

Infections

WUPyV and KIPyV and Respiratory Illness

SV40 and Humans

Association of BKPyV and JCPyV with

Human Tumors

Avian Polyomavirus and Disease

Perspective

Polyomaviruses are composed of nonenveloped capsids with a simple, double-stranded DNA (dsDNA) genome of approximately 5,000 base pairs containing a single origin of replication and a bidirectional promoter that drives expression of messenger RNA (mRNA) transcripts encoding five to nine proteins. The small size of the polyomavirus genome, comparable to a simple plasmid, and its limited number of genetic elements have enabled research that continues to be at the forefront of biology including DNA replication, gene expression, signal transduction, and oncogenesis.

The history of polyomavirus research began in the 1950s when Ludwig Gross¹⁰³ noted that when he passaged a mouse leukemia virus in mice, the recipients occasionally developed salivary and parotid gland tumors rather than leukemia. He isolated this specific activity and demonstrated that the parotid agent differed from murine leukemia virus (MLV) in its sedimentation, filtration, and heat stability properties. Stewart et al²⁴⁷ observed the formation of multiple tumor types in newborn mice inoculated with this agent and coined the name *polyomavirus*, derived from the Greek word *poly*, meaning “many,” and *oma*, denoting “tumor.” The mouse polyomavirus is often called simply “polyomavirus” but will be referred to as MPyV in this chapter, consistent with the most recent taxonomic classification (Table 53.1).¹²⁸ Initially discovered at the same time as MPyV, murine pneumotropic virus (MPtV), also known as Kilham virus or K virus, causes severe interstitial pneumonia in newborn mice.^{101,144}

The next member of the family to be isolated was simian virus 40 (SV40) by Sweet and Hilleman²⁵⁶ in 1960. They were screening samples from poliovirus vaccine lots produced in rhesus monkey kidney cells for the presence of contaminating viruses. SV40 was the 40th virus isolated in this screen and caused cytopathic or vacuolating effects in African green monkey kidney cells but not in the rhesus monkey cells used for vaccine production. It soon became clear that the early production batches of poliovirus vaccine were contaminated with SV40. Although the Salk poliovirus vaccine was inactivated by formalin treatment, SV40 was relatively resistant to this treatment and survived. The presence of SV40 in the vaccine quickly became a public health concern when the oncogenic potential of purified virus was demonstrated in newborn hamsters.

Since that time, many polyomaviruses have been isolated from a variety of mammalian and bird species. The first two human polyomaviruses, JC (JCPyV) and BK (BKPyV), were isolated in 1971 from immunocompromised patients. JCPyV was isolated by the transfer of brain tissue from a patient with the demyelinating disease progressive multifocal leukoencephalopathy (PML) into cultures of human fetal brain tissue.¹⁹⁹ BKPyV was isolated from the urine of a renal

HISTORY

Polyomaviruses are found ubiquitously in a broad range of avian and mammalian species. Polyomaviruses can cause severe illness and death on an epidemic scale in birds. In contrast, polyomaviruses typically cause lifelong and asymptomatic infections in healthy humans but severe illness in immunocompromised patients. Recognition of the ubiquitous presence of polyomaviruses in their natural hosts as well as their disease-causing ability has prompted a variety of research efforts.

TABLE 53.1 Taxonomic Classification of Polyomaviruses

#	Full name	Abbreviation	Alternative names	Nucleotides	Entrez genome	Large T antigen	Small T antigen	VP1	VP2	VP3	Additional proteins
1	Avian polyomavirus	APyV	Budgerigar fledgling disease polyomavirus	4981	NC_004764	599	145	343	341	235	Agno 1a 176 1b 112 2a 109 2b 79
2	B-lymphotropic polyomavirus	LPyV	African green monkey polyomavirus	5270	NC_004763	697	189	368	356	237	
3	Baboon polyomavirus	SA12	Simian agent 12	5230	NC_007611	699	172	364	352	234	Agno 68
4	Bat polyomavirus	BatPyV	Myotis polyomavirus VM-2008	5081	NC_011310	670	162	357	353	237	Agno 30
5	BK polyomavirus	BKPyV	BK, BKV	5153	NC_001538	695	172	362	351	232	Agno 66
6	Bornean orangutan polyomavirus	OraPyV1		5168	NC_013439	693	197	366	311	190	
7	Bovine polyomavirus	BPyV		4697	NC_001442	619	124	365	353	232	Agno 118
8	California sea lion polyomavirus 1	SLPyV		5112	NC_013796	662	186	495	340	223	Agno 50
9	Canary polyomavirus	CaPyV		5421	NC_017085.1	625	167	356	369	245	
10	Chimpanzee polyomavirus	ChPyV		5086	NC_014743	615	194	497	309	190	
11	Crow polyomavirus	CPyV		5079	NC_007922	636	166	353	333	227	ORF-X 150
12	Finch polyomavirus	FPyV		5278	NC_007923	612	166	358	354	244	ORF-X 205
13	Goose hemorrhagic polyomavirus	GHPyV		5256	NC_004800	636	160	353	326	217	ORF-X 169
14	Gorilla polyomavirus	GggPyV			HQ385752.1	801	186	393	242	197	
15	Hamster polyomavirus	HaPyV		5366	NC_001663	751	194	372	345	221	Middle T antigen 401
16	Human polyomavirus 6	HPyV6		4926	NC_014406	669	190	387	336	215	
17	Human polyomavirus 7	HPyV7		4952	NC_014407	671	193	380	329	209	
18	Human polyomavirus 9	HPyV9		5026	NC_015150	680	189	371	352	233	
19	JC polyomavirus	JCPyV	JC, JCV	5130	NC_001699	688	172	354	344	225	Agno 71
20	KI polyomavirus	KIPyV	KI	5040	NC_009238	641	191	378	400	257	
21	Merkel cell polyomavirus	MCPyV	MCV	5387	NC_010277		186	423	241	196	
22	Murine pneumotropic polyomavirus	MPtV	Kilham	4754	NC_001505	648	158	373	341	222	
23	Murine polyomavirus	MPyV		5297	NC_001515	785	195	383	319	204	Middle T antigen 421
24	Pan troglodytes verus polyomavirus 1a	PtvPyV1a		5303	HQ385746	802	186	412	240	195	

25	Pan troglodytes verus polyomavirus 2a	PtvPyV2a	5309	HQ385748	802	186	396	242	197	
26	Simian virus 40	SV40	5243	NC_001669	708	174	364	352	234	Agno 62 VP4 125
27	Squirrel monkey polyomavirus	SqPyV	5075	NC_009951	655	163	357	332	207	Agno 70
28	Sumatran orangutan polyomavirus	OraPyV2	5358	FN356901.1	735	194	380	317	202	
29	Trichodysplasia spinulosa-associated polyomavirus	TSPyV	5232	NC_014361	697	198	375	312	194	
30	WU polyomavirus	WUPyV	5299	NC_009539	648	194	369	415	272	
Additional polyomaviruses										
31	Bandicoot papillomatosis carcinomatosis virus type 1	BPCV1	7295	NC_010107		224				L1 505 L2 469
32	Bandicoot papillomatosis carcinomatosis virus type 2	BPCV2	7277	NC_010817		225				L1 502 L2 469
33	Japanese eel endothelial cells-infection virus		15131	NC_015123	698					
Additional polyomaviruses with incomplete genomic sequence										
A	Athymic rat polyomavirus	RatPyV								Papovaviral sialoadenitis
B	Rabbit kidney vacuolating virus	RKV								
C	Cynomolgus polyomavirus	CyPyV								
D	Baboon polyomavirus 2	BPyV2								

transplant patient after inoculation into African green monkey kidney cells.⁸⁹

The advent of advanced molecular biology techniques including polymerase chain reaction (PCR), rolling circle amplification, and deep DNA sequencing led to the identification of seven additional human polyomaviruses. WUPyV and KIPyV were cloned from respiratory secretions of young children by investigators at Washington University and the Karolinska Institute, respectively. The discovery of Merkel cell polyomavirus (MCPyV) reflected recognition that the incidence of Merkel cell carcinoma, a rare form of skin cancer, was more frequent in severely immunocompromised patients, suggesting an infectious cause.⁷¹ MCPyV was recovered from Merkel cell carcinomas by digital transcriptome subtraction, a method that used high-throughput sequencing of cellular transcripts to identify sequences that did not match the human genome but were distantly related to BKPyV.⁷⁸ Two additional polyomaviruses, HPyV6 and HPyV7, were cloned from the skin or hair follicles of healthy adults using a technique called rolling circle amplification that takes advantage of the small circular nature of the polyomavirus dsDNA genome.²²⁶ Using the same technique, trichodysplasia spinulosa-associated polyomavirus (TSPyV) was found in a patient with a rare skin disease named trichodysplasia spinulosa.²⁶⁸ HPyV9 was identified in the serum of a renal transplant patient and on the skin of a patient with Merkel cell carcinoma.^{222,227} Undoubtedly, additional human polyomaviruses will be discovered. Although identification of novel polyomaviruses by DNA sequencing has become easier, isolation of virion particles remains a technical challenge.

Primate polyomaviruses from Old World monkeys include the simian agent 12 (SA12), isolated from a South African vervet monkey kidney culture in 1963, and B-lymphotropic polyomavirus (LPyV), isolated from an African green monkey lymphoblast cell line, as well as polyomaviruses directly isolated from animals including Bornean (OraPyV1) and Sumatran orangutan polyomavirus (OraPyV2), gorilla polyomavirus (GggPyV), and chimpanzee polyomavirus (CHPyV).¹⁶⁰ The first New World monkey polyomavirus was isolated from squirrel monkey (SqPyV2).²⁷²

In addition to MPyV and MtPyV, a number of nonprimate mammalian polyomaviruses have been identified. Hamster polyomavirus (HaPyV) was discovered in a spontaneously occurring hair follicle epithelioma from a Syrian hamster. This is an interesting member of the polyomavirus family because the behavior of the virus in the tumors most closely resembles that of the papillomaviruses in that viral particles can be found in the highly differentiated layers of the tissue. However, analysis of the HaPyV DNA sequence and its genome organization revealed that it is indeed a polyomavirus and most closely related to MPyV.⁶¹ Rabbit kidney vacuolating virus (RKV) was originally isolated from a rabbit papilloma but was shown to be involved in subclinical infections in rabbits. Bovine polyomavirus (BPyV) contains the smallest of the polyomavirus genomes with fewer than 4,700 base pairs. Sea lion polyomavirus (SLPyV) was isolated from a sick animal with kidney swelling, interstitial nephritis, and an intestinal lymphoma.⁴⁶

Polyomaviruses have been isolated from several bird species. The first of these, budgerigar fledgling disease virus (BFDV), was isolated from a parakeet in 1986.¹⁹⁰ Unlike the strict host range restriction of mammalian polyomaviruses, BFDV can infect and cause disease in a wide variety of bird species and is now referred

to as avian polyomavirus (APyV). Recently identified bird polyomaviruses include canary (CaPyV), crow (CPyV), finch (FPyV), and goose hemorrhagic polyomavirus (GHPyV).^{105,129,131} In general, the avian polyomaviruses cause a severe inflammatory illness that often results in death. For example, infection with GHPyV causes hemorrhagic nephritis and enteritis.¹³⁰

For many years, the polyomaviruses were studied principally as model systems for understanding basic eukaryotic cell processes including DNA replication, RNA transcription, splicing and processing, and oncogenic transformation. The cloning and sequencing of the SV40 genome ushered in the era of recombinant DNA research. Indeed, the SV40 genome may be the most intensively studied DNA molecule per base pair. Several genetic elements from the SV40 genome are used in nearly every molecular biology laboratory in the world today.²⁰⁸

Interest in the polyomaviruses as human pathogens lagged behind these more basic biological studies because for many years the incidence of polyomavirus-associated diseases was rare and not well recognized. The onset of the human immunodeficiency virus type 1 (HIV-1)/acquired immunodeficiency syndrome (AIDS) epidemic, however, led to a dramatic rise in the incidence of PML, a JCPyV-induced disease. In addition, recent advances in immunosuppressive regimens for bone marrow and solid organ transplant recipients and biological therapies for autoimmune diseases led to increases in JCPyV-, BKPyV-, and TSPyV-associated diseases. More recently, the apparent transforming activity of the MCPyV and its contribution to Merkel cell carcinoma has generated widespread interest in the polyomaviruses. Studies have led to the emerging view that while many polyomavirus features are shared, there are many fundamental differences that distinguish each virus.

CLASSIFICATION

Polyomaviruses were originally classified within the *Papovavirus* family that included *papillomavirus*, *polyomavirus*, and *vacuolating virus*.¹⁸¹ The vacuolating or cytopathic effect of SV40 virus on host cells during lytic infection led to this distinction. Polyomaviruses were split from the papillomaviruses in 2000. Recently, a proposed classification organized polyomaviruses into three genera based on the DNA sequence of the viral genome and its normal host. The genera *Orthopolyomavirus* and *Wukipolyomavirus* contain polyomaviruses isolated from mammalian species, while *Avipolyomavirus* contains all avian species (Fig. 53.1).¹²⁸ The *Wukipolyomavirus* genus contains WUPyV, KIPyV, HPyV6, and HPyV7, and the *Orthopolyomavirus* genus contains all other polyomaviruses including the bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1) and type 2 (BPCV2).

Several polyomaviruses have not been officially classified within the family because the complete viral genomic sequence is not available. These include the baboon polyomavirus 2 (BPyV2), RKV, athymic rat polyomavirus (RatPyV), and cynomolgus polyomavirus (CyPV).

VIRION STRUCTURE

Polyomavirus virions are nonenveloped, 45- to 50-nm particles consisting of three virally encoded capsid proteins, VP1, VP2,

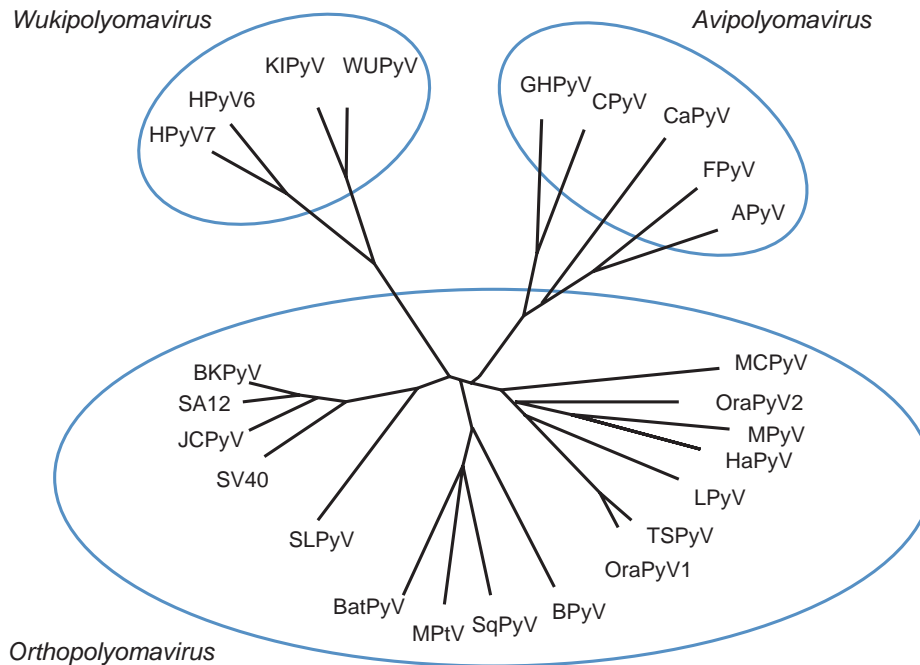


FIGURE 53.1. Phylogenetic relationships among the *Polyomaviridae* family based on whole genomic nucleotide sequences. The mammalian polyomaviruses are contained in the *Orthopolyomavirus* and *Wukipolyomavirus* genera, while the bird polyomaviruses are contained in the *Avipolyomavirus* genus. See Table 53.1 for full names and Genbank accession numbers. (Reproduced from John R. Buck CB, Allander T, et al. Taxonomical developments in the family Polyomaviridae. *Arch Virol* 2011;156(9):1627–1634, with kind permission from Springer Science+Business Media.)

and VP3, containing a circular dsDNA genome wrapped with cellular histones H2A, H2B, H3, and H4.²⁶² The virion minichromosome exhibits the same nucleosome structure as cellular chromatin except for the absence of histone H1 that becomes associated with the viral genome only when in the infected cell. The particles have a $T = 7$ icosahedral symmetry and sediment at 240S in sucrose density gradients (Fig. 53.2). The density of mature virions is 1.34 g/mL and of empty capsids is 1.29 g/mL, as determined by cesium chloride equilibrium gradient centrifugation. The polyomaviruses are relatively resistant to heat

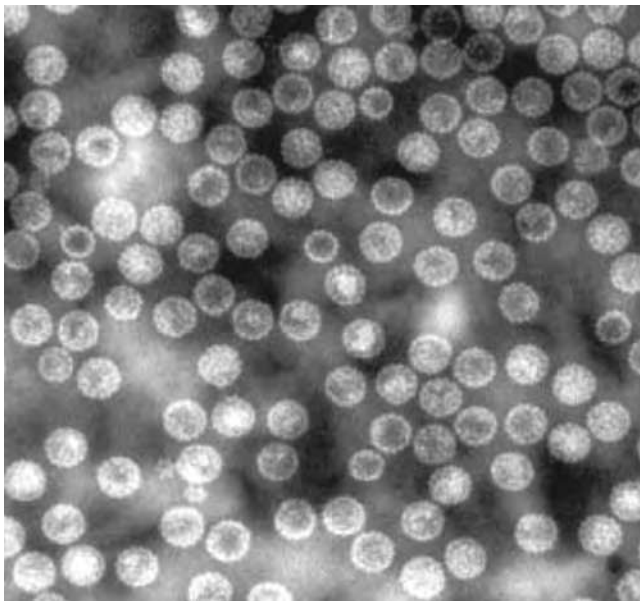


FIGURE 53.2. Composite electron micrograph of a 40-nm polyomavirus icosahedral structure derived from assembly of VP1, negatively stained with 2% phosphotungstic acid (50,000 \times).

and formalin inactivation, demonstrated by the isolation of viable SV40 from the Salk poliovirus vaccine.⁵¹ Because polyomaviruses are nonenveloped, they are resistant to lipid solvents. Similar to most viruses, preparations of polyomavirus virions contain many different types of particles. For example, in addition to mature virions, one can find empty capsids and capsids that contain cellular, rather than viral, DNA.

The polyomavirus capsid contains 360 molecules of VP1 arranged in 72 pentamers or capsomeres each containing 5 molecules of VP1 and 1 molecule of VP2 or VP3. Only the VP1 molecule is exposed on the surface of the capsid. The icosahedral capsid has both five- and sixfold axes of symmetry, with 12 pentamers surrounded by 5 other pentamers and 60 pentamers surrounded by 6 pentamers (Fig. 53.2). Capsomeres with fivefold symmetry are unusual but supported by the high-resolution structure of SV40.^{166,246} The C-terminus of each VP1 molecule extends out of the pentamer and contacts the neighboring capsomere. This structure is flexible and thereby provides the means to form an icosahedron. Capsomere contacts are stabilized by the presence of calcium ions, and mutations in residues that bind calcium result in premature disassembly.¹⁶⁵ Treatment of virus with EGTA under reducing conditions results in the dissociation of the capsid into VP1 pentamers. In addition to VP1, VP2, and VP3, APyV expresses VP4 (agnoprotein 1a) that interacts with the C-terminus of VP1 and may be incorporated into viral capsids.²³⁴ The capsid contains posttranslational modifications including disulfide bridges that form between the pentameric capsomeres. In addition, VP2 undergoes myristoylation at its N-terminus. A recent report found a large number of posttranslational modifications on the BKPvV VP1 protein, although the role of these modifications during infection is not known.⁷⁴

GENOME STRUCTURE AND ORGANIZATION

The polyomavirus dsDNA circular genome contains approximately 5,000 base pairs and can be divided into three parts: the

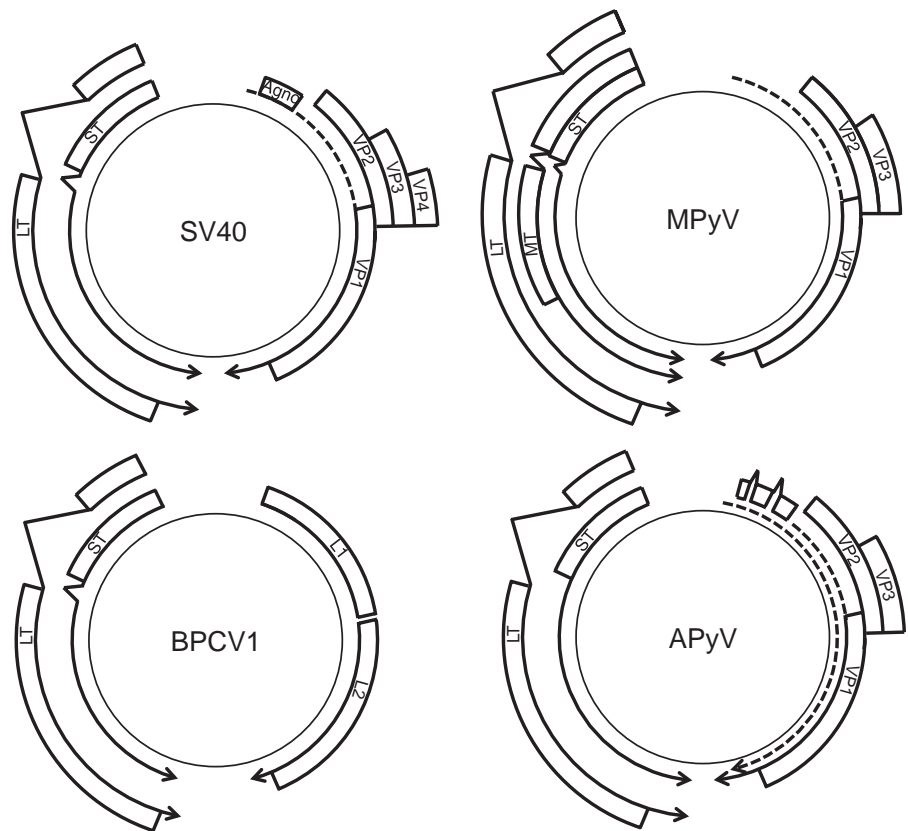


FIGURE 53.3. Genome organization for simian virus 40 (SV40), mouse polyomavirus (MPyV), bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1), and avian polyomavirus (APyV). All polyomaviruses express large T (LT) and small T (ST) antigen from the early region (counterclockwise arrows). MPyV also expresses middle T (MT) antigen. The late region (clockwise arrows) expresses the viral coat proteins VP1, VP2, and VP3 for SV40, MPyV, and APyV. SV40 also expresses a variably spliced small protein called VP4. BPCV1 expresses L1 and L2 coat proteins. At the 5' end of the late transcript, SV40 expresses agnoprotein, while APyV expresses VP4. APyV late region expresses two transcripts that encode all late proteins.

early region encoding genes that are expressed prior to the onset of DNA replication; the late region encoding genes expressed after viral DNA replication commences; and the regulatory region, containing the origin of DNA replication as well as the promoters for early and late viral genes (Fig. 53.3). The early and late promoters give rise to primary transcripts from opposite strands of the DNA. The regulatory region including the origin is often referred to as the noncoding control region (NCCR). The numbering system for the polyomavirus genome differs from virus to virus with nucleotide position 1 defined in different ways.²⁶² There has been precedent in recent years, however, to call the nucleotide preceding the A in the large T antigen ATG nucleotide 1, with numbering proceeding in the late direction, that is, away from the large T antigen open reading frame.

The small size of the polyomavirus genome made it amenable to classical genetic approaches.²⁶² Infection with temperature-sensitive mutants of SV40 led to the identification of five complementation groups, A, B, BC, C, and D. Mutations in group A mapped to the large T antigen gene; groups B, BC, and C to the *VP1* gene; and group D to the *VP3* gene. MPyV mutants have been classified into similar complementation groups, although no standard nomenclature was developed for this virus. Other mutants of MPyV known as host range (hr-t) mutants were selected for their inability to grow in established cell lines but retained the ability to replicate in primary cells or transformed cells. These mutations were later mapped to the middle T and small T antigen genes.²⁴⁰ The analysis of these early mutants set the stage for detailed and directed mutational studies enabled by recombinant DNA technology.

Another early genetic approach to the study of polyomaviruses was the selection for so-called evolutionary variants. In these experiments, viruses were passaged at high multiplicities of infection and variants were isolated. Many of these variants had alterations in the regulatory region that imparted a growth advantage to the virus. Passage of polyomavirus in culture often leads to duplications, deletions, and other rearrangements in the regulatory region.¹⁸⁵ Although high-multiplicity passage of JCPyV in human glial cell cultures does not result in alterations in the regulatory region, the regulatory regions of BKPyV and JCPyV are frequently found to be rearranged when isolated from diseased tissues or patients' blood.

The polyomavirus early and late promoters are contained within the regulatory region and overlap each other as well as the origin of replication. Early transcription progresses from the early promoter around the genome in one direction. Late transcription proceeds from the late promoter around the genome in the opposite direction. The early mRNAs are produced by posttranscriptional processing at a polyadenylic acid or poly(A) tail site that is located about halfway around the circular genome from the start site and by removal of introns by the cellular splicing machinery. SV40 was one of the first experimental systems where it was demonstrated that RNA polymerase II transcribes past the 3' end of the mature mRNA molecule, implying that the 3' end, including the poly(A) tail, was generated posttranscriptionally.⁸²

Each of the polyomaviruses encodes at least two early mRNAs by alternative splicing that are translated into the large T and small T antigens (Fig. 53.4). MPyV and HaPyV produce a third alternatively spliced mRNA that codes for middle

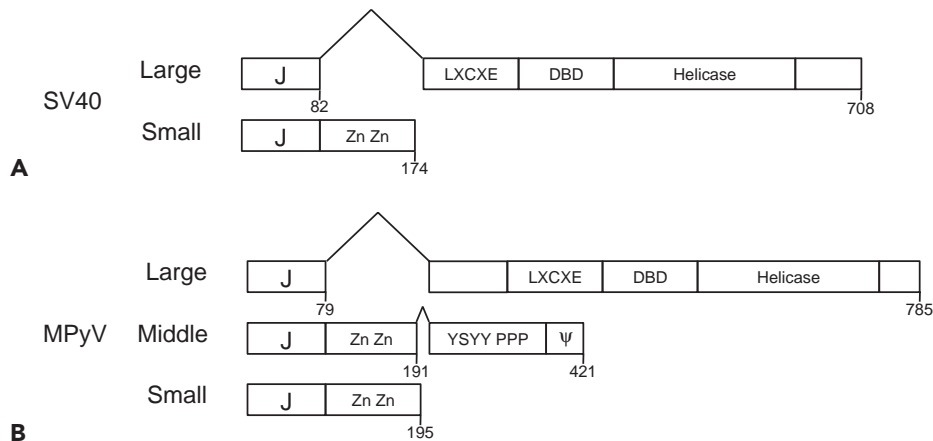


FIGURE 53.4. Simian virus 40 (SV40) (A) and mouse polyomavirus (MPyV) (B) T antigens. The N-terminal J domain is shared with all T antigens. SV40 expresses large and small T antigens and MPyV expresses large, middle, and small T antigens. The large T antigens contain the LXCXE motif that binds directly to RB1, a DNA-binding domain (DBD) that binds to the origin of replication, and a helicase domain. The small T antigens contain a unique domain not shared with large T antigen that binds to two Zn molecules. The MPyV middle T antigen shares the J domain and Zn-binding domain with small T antigen and also contains tyrosine and serine residues (YSY) that become phosphorylated, a proline-rich region, and a C-terminal hydrophobic domain.

T antigen. The “T” in T antigen derives from the initial identification of these proteins as tumor antigens that were recognized by antisera from tumor-bearing animals inoculated with SV40.^{21,107} For many years, it was believed that large, middle, and small T antigens were the only early transcripts, but more recently it has been demonstrated that these viruses encode additional early mRNAs that differ in their splicing patterns, particularly for large T antigen. In SV40 and MPyV, an additional mRNA encodes a protein called 17KT and tiny T, respectively. Similarly, JCPyV produces a series of alternatively spliced mRNAs that encode proteins referred to as T’135, T’136, and T’165, and BKPyV encodes a molecule called truncated TAg. MCPyV encodes a full-length, 816-residue large T antigen as well as an alternatively spliced 57-kD T antigen that corresponds to the first 440 and last 100 residues of the full-length large T antigen.²³⁷

The late mRNA is transcribed in the opposite direction from the early mRNAs. As with the early transcripts, the late transcript has a single poly(A) site approximately halfway around the genome and is alternatively spliced (Fig. 53.3). The polyomavirus late transcript encodes three capsid proteins, VP1, VP2, and VP3. Notably, VP3 is translated in the same open frame as VP2 but uses an alternate AUG start codon downstream of the VP2 start codon and thereby shares all residues with VP2. In addition, SV40 encodes a VP4 protein that uses an internal AUG start codon even further downstream from VP3 and functions as a viroporin that promotes virus release from the infected cell.²¹² The late region transcript from SV40, JCPyV, and BKPyV as well as SA12, BatPyV, BPvV, SLPyV, and SqPyV encode an additional protein called agnoprotein.

Avian polyomaviruses express early transcripts encoding large and small T antigen, but the late transcripts have several distinct features (Fig. 53.3). There are two late transcription start sites, PL1 and PL2, that give rise to at least eight different transcripts due to alternative splicing.¹⁶⁴ PL1 encodes two forms of VP4 (agnoprotein 1a) and VP4d (VPΔ4, agnoprotein 1b), while PL2 gives rise to two forms of agnoprotein 2a and

2b that use the same splice sites as VP4 and VP4d but are translated in a different reading frame. Avian agnoprotein 2a and 2b bears some similarity to the SV40 agnoprotein. All of the late avian transcripts also encode for VP1 or VP2 and VP3.

The late strand of several polyomaviruses encodes a microRNA (miRNA).^{39,253} The SV40 miRNA maps just 3' of the late poly(A) site and appears to correspond to SAS (SV40-associated small RNA), a small RNA molecule identified 25 years earlier, albeit of then-unknown function. These miRNAs are complementary to the early mRNAs, target the early mRNAs for degradation, and may serve to limit the expression levels of the T antigens. MiRNA have been identified in SV40, BKPyV, JCPyV, MCPyV, SA12, MPyV, BPCV1, and BPCV2.³⁹

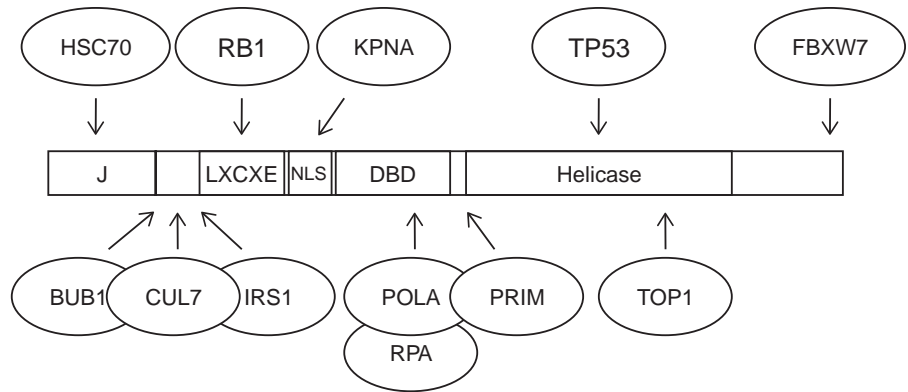
Structure of T Antigens

Much of what is known about the structure of polyomavirus T antigens comes from studies of SV40. All polyomavirus T antigens share an N-terminal region of approximately 80 residues that shows structural and sequence homology to the DnaJ or J domain found in host cell HSP40 homologs (Fig. 53.4). Full-length large T antigen is a nuclear phosphoprotein of approximately 700 residues. The molecule’s atomic coordinates have been assembled from crystallography of isolated domains, including the DnaJ and the retinoblastoma protein-binding domains; the DNA-binding domain, also known as the origin-binding domain; and a central domain consisting of residues 251 to 627 that forms a hexamer and contains the adenosine triphosphatase (ATPase) and helicase activities required for viral replication.¹⁶³ Studies using scanning transmission electron microscopy, negative staining with atomic force microscopy, and single-particle reconstruction of cryoelectron microscopy (cryo-EM) images revealed that large T antigen forms a double hexamer in a head-to-head arrangement when bound to the origin of DNA.⁵⁰

A number of functional domains contained within large T antigen are required for viral replication. Functions intrinsic to SV40 large T antigen include the ATPase/helicase domain and

FIGURE 53.5. Simian virus 40 (SV40) large T antigen binds to cellular host proteins.

The J domain binds to HSC70, the LXCXE motif binds to RB1, the helicase domain binds to TP53, and the phosphorylated threonine residue 701 binds to FBXW7. The cellular proteins BUB1, CUL7, and IRS1 bind to a region between the J domain and the LXCXE motif. Karyopherin (KPNA) binds to the nuclear localization sequence (NLS). Large T antigen recruits replication protein A (RPA), topoisomerase 1 (TOP1), DNA polymerase α (POLA), and primase (PRIM) to promote viral DNA replication.



the DNA-binding domain that mediates direct interactions with specific DNA sequences at the origin of replication. In addition to these intrinsic functions, the SV40 large T antigen domains serve to recruit host factors important for viral replication (Fig. 53.5). For example, the N-terminal J domain, containing the canonical residues HPDK, binds and activates the ATPase activity of host cell HSC70.³¹ The DNA-binding domain binds to replication protein A, while the helicase domain binds to the DNA polymerase α /primase complex.¹¹⁸ In addition, the helicase domains of many but not all polyoma large T antigens bind to p53. The outer surface of each SV40 large T antigen hexamer subunit can bind directly to the DNA-binding domain of p53.¹⁶⁷ The large T antigen from MPyV is a notable exception among polyomaviruses because it does not bind to p53, although the ability of all the other polyomavirus large T antigens to bind to p53 has not yet been reported.

Smaller functional motifs within large T antigen include the nuclear localization signal (NLS).^{132,154} Mutations that disrupt the NLS result in the cytoplasmic localization of SV40 large T antigen and inability to support the viral lytic life cycle. All mammalian polyomavirus large T antigens contain the conserved residues LXCXE (where X is any residue) that bind directly to the retinoblastoma family of tumor suppressor proteins including pRb (RB1), p107 (RBL1), and p130 (RBL2).^{60,68} The large and small T antigens from GHPyV, CPyV, and FPyV each contain the LXCXE motif, while those from APyV contain a related sequence, LXAXE. It is not known if any of the bird polyomavirus T antigens can bind to pRb or p53.

SV40 large T antigen contains a series of posttranslational modifications, including phosphorylation, O-glycosylation, acylation, poly(ADP)-ribosylation, and acetylation. In addition, phosphorylation of C-terminally located threonine residues in SV40 large T antigen creates a phospho-degron motif that binds directly to FBXW7, an F-box substrate adapter. Large T antigen binding to FBXW7 blocks binding to cyclin E and prevents its degradation by the CUL1^{FBXW7} RING ubiquitin ligase (Fig. 53.5).²⁷⁷ Phosphorylation regulates some of the functions of the molecule, including its subcellular localization and its ability to participate in the initiation of viral DNA synthesis. These regulatory events will be discussed later in the context of the life cycle.

Polyomavirus small T antigen is found in both the nucleus and the cytoplasm. Small T antigen is a cysteine-rich protein ranging in size from 124 to 198 residues and shares its N-terminus with large T antigen (i.e., those residues encoded

up to the 5' large T antigen splice site) but contains a unique C-terminal region. Small T antigen contains the same N-terminal J domain as large T antigen and a unique C-terminal domain (Fig. 53.4). The unique domain of the mammalian polyomavirus small T antigens contains a highly conserved set of cysteine and histidine residues that bind to two zinc molecules.^{43,44} These zinc-binding domains serve an important role in binding to the cellular protein phosphatase 2A (PP2A).²⁰⁰ PP2A is a trimeric complex consisting of two regulatory subunits A and B that bind to the catalytic C subunit. Small T antigen binds directly to the PP2A A subunit where the B subunit normally binds and thereby displaces or replaces the B subunit. SV40 small T antigen binds specifically to the A α (PPP2R1A) subunit, while MPyV small T antigen binds to both the A α and A β (PPP2R1B) subunits.⁴ The small T antigen–PP2A complex contains also the C α (PPP2CA) or C β (PPP2CB) catalytic subunit. There are at least 18 different B PP2A subunits identified in mammalian cells. At the very least, SV40 small T antigen can displace the B56 α (PPP2R5A), B56 γ (PPP2R5C), and PR72/PR130 (PPP2R3A).²¹⁹ It is likely that the polyomavirus small T antigen–PP2A complex not only serves to disrupt the cellular PP2A complexes but also is likely to retain specific phosphatase activity directed toward substrates. Notably, the bird polyomavirus small T antigens do not contain the conserved cysteine/histidine residues that serve to bind zinc, and it is not known if they are capable of PP2A binding.

The middle T antigen of MPyV shares its N-terminal J domain and PP2A-binding domain with small T antigen (Figs. 53.4 and 53.6). The MPyV middle and small T antigens are identical for the N-terminal 191 residues until a splice junction that removes four nucleotides resulting in an additional 230 residues at the C-terminus of middle T antigen. In contrast, small T antigen contains only four unique amino acids after this intron. The unique middle T antigen C-terminus is encoded by an alternate reading frame used for coding the second exon of large T antigen. Notably, middle T antigen contains an N-terminal J domain in common with both large and small T antigen and, like small T antigen, binds to the A and C subunits of PP2A. The additional MPyV middle T residues mediate binding to several proteins involved in signal transduction, including the SRC tyrosine kinase, the SHC1 phosphotyrosine docking protein, phospholipase C (PLCG1), and phosphatidylinositol 3-kinase (PIK3CA and PIK3R1).²²³ MPyV middle T antigen can also bind to the SRC-related tyrosine kinases YES1 and FYN. The C-terminus of middle T antigen contains

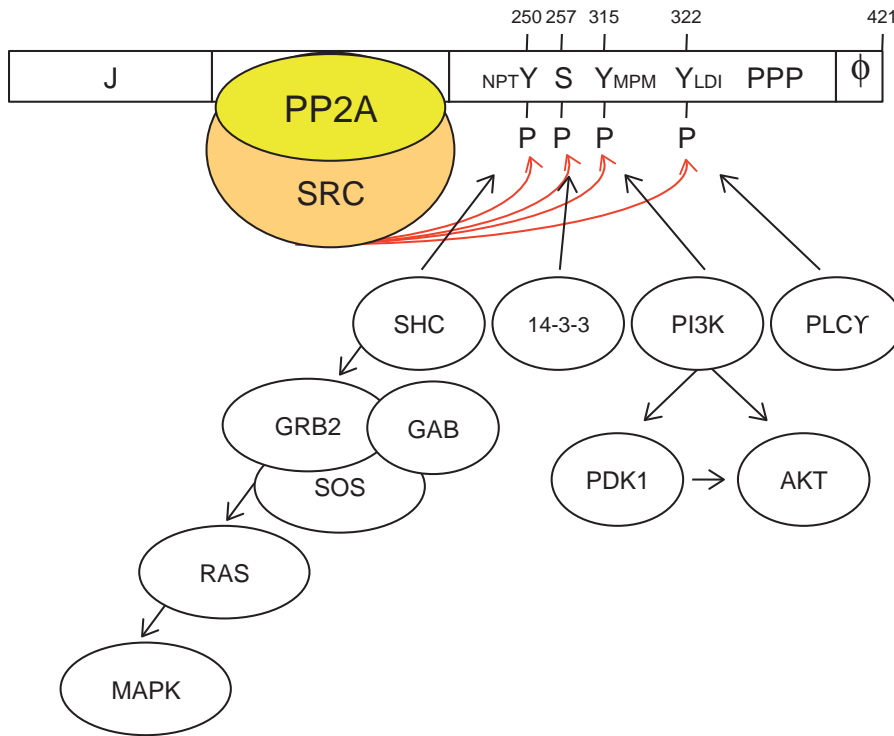


FIGURE 53.6. Mouse polyomavirus (MPyV) middle T antigen assembles an activated signaling complex. Tyrosine residues 250, 315, and 322 are phosphorylated by associated SRC kinase. Residues surrounding the phosphorylated tyrosine residues are required for specific association with SHC, 14-3-3, phosphoinositide 3-kinase (PI3K), and phospholipase C. PPP represents the proline-rich sequence. The C-terminus contains the hydrophobic membrane attachment site. (Adapted from Schaffhausen BS, Roberts TM. Lessons from polyoma middle T antigen on signaling and transformation: a DNA tumor virus contribution to the war on cancer. *Virology* 2009;384[2]:304–316.)

a 22-residue hydrophobic domain that moves the newly translated middle T antigen from the cytoplasm through the endoplasmic reticulum (ER) to the inner plasma membrane.²⁸⁵ The combination of membrane localization with recruitment and activation of several enzymes enables middle T antigen to function as a constitutively activated tyrosine kinase that triggers downstream signaling in the RAS and MEK pathways.

STAGES OF REPLICATION

Mechanism of Attachment

Prior to the 21st century, the identity of the cell surface receptors for polyomaviruses was poorly understood. Early studies on SV40 indicated that it used the major histocompatibility complex (MHC) class I antigens to bind to cells.²⁹ Supporting evidence for this model included observations that antibodies against MHC class I blocked virus binding to rhesus monkey kidney cells and the inability of virus to bind well to human cells that do not express MHC class I antigens. Experimentally induced expression of MHC class I in nonexpressing cells restored binding. Although the MHC class I antigens were implicated as the SV40 receptor, they were not sufficient to account for all binding. For example, virus binding occurred only on the apical surface of polarized monkey epithelial cells, while MHC class I antigens were expressed on both the apical and basolateral surfaces. In addition, expression of MHC class I on human kidney epithelial cells was not sufficient for SV40 infection.

Subsequent results challenged the notion that SV40 uses a protein molecule as its receptor, indicating instead that it uses the branched ganglioside, GM1 (Fig. 53.7).²⁶⁵ This finding is more consistent with the route of entry of the virus through endosomes. In these studies, a rat cell line that did not express gangliosides and was unable to be infected by SV40 was ren-

dered susceptible by preincubation with GM1. Gangliosides are glycosphingolipids that combine a sialylated oligosaccharide with ceramide consisting of sphingosine and a fatty acid. The sialic acid is critical for viral binding to the cell. In addition to providing a binding site for the virus, the gangliosides direct the virus to the correct endocytic pathway.²⁰⁹ The efficiency of SV40 infection is dependent on the relative concentration of GM1 on the cell surface as well as its ability to activate focal adhesion kinase (PTK2).²⁴⁴

Other polyomaviruses use different forms of gangliosides as receptors (Fig. 53.7). For example, MPyV uses GT1b or GD1b.^{92,265} After initial binding to the ganglioside, MPyV interacts with $\alpha 4\beta 1$ integrin that may serve as a secondary or cell type-specific receptor for viral entry.³⁵ BKPyV also uses gangliosides to enter the cell, as judged by restoration of infectivity to otherwise resistant cells upon preincubation with GT1a and GD1a.¹⁷¹ These branched gangliosides are found on the renal tubular epithelial cells that BKPyV normally infects. Another report indicated that an N-linked glycoprotein containing sialylated oligosaccharides can also mediate BKPyV binding.⁶⁶ Both the glycolipid and glycoprotein contain sialic acid, consistent with early reports that BKPyV can hemagglutinate human red blood cells and that this activity was neuraminidase sensitive. There are conflicting reports regarding the receptor for MCPyV. Although it has been shown that MCPyV VP1 capsomeres can bind the ganglioside GT1b,⁷² pseudovirions can bind heparin moieties.²²⁵

JCPyV binds to lactoseries tetrasaccharide c(LTSc), a linear sialylated oligosaccharide that differs from the branched forms reported for other polyomaviruses (Fig. 53.7). LTSc is a pentasaccharide with the terminal sialic acid linked by an $\alpha 2,6$ bond to the penultimate galactose.^{194,266} In addition, JCPyV uses the 5HT_{2A} serotonin receptor, perhaps as a cell type-specific receptor, for viral entry.⁶⁹ This receptor is expressed on

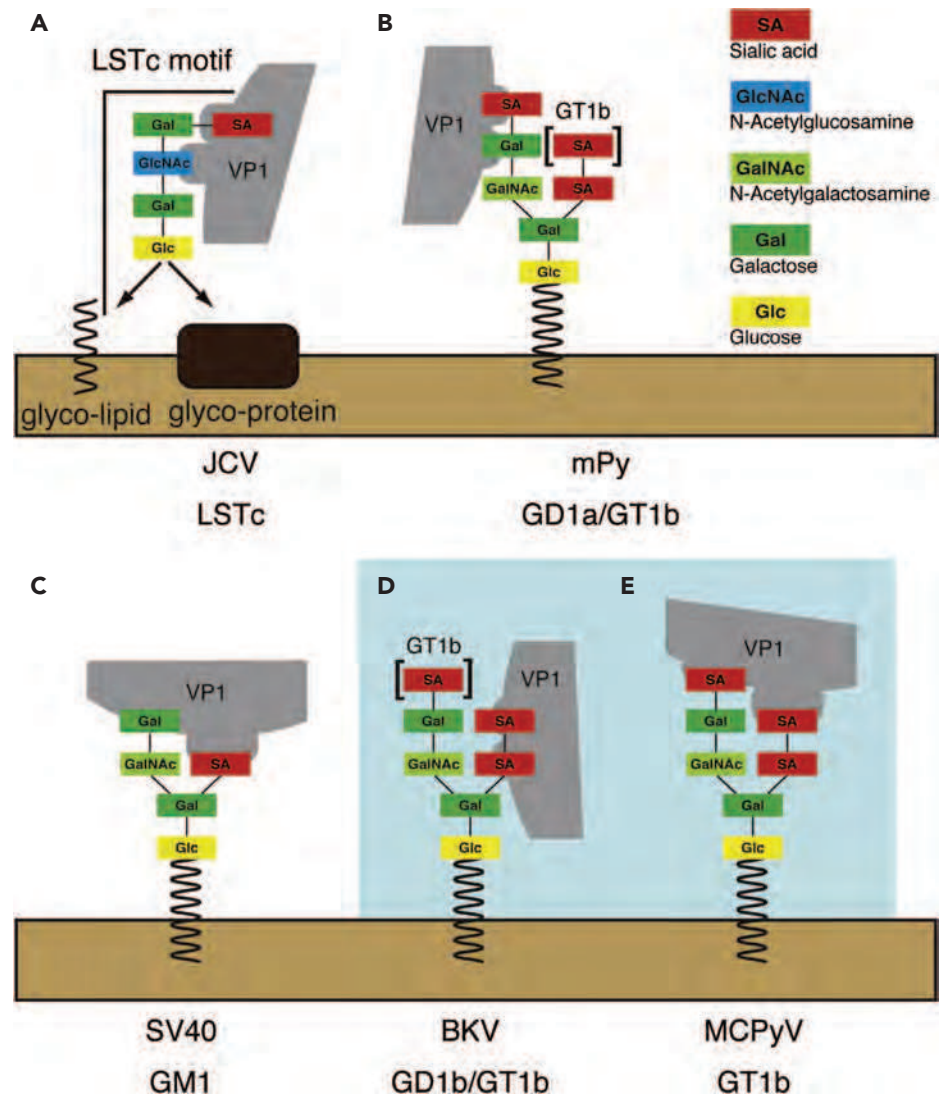


FIGURE 53.7. Polyomavirus VP1 interactions with gangliosides. **A:** JC polyomavirus (JCPyV) VP1 binds the branched LSTc attached to a lipid or protein. **B:** Mouse polyomavirus (MPyV) VP1 binds to the sialic acid galactose on GD1a or GT1b. **C:** Simian virus 40 (SV40) VP1 binds galactose and sialic acid residues on both branches of GM1. **D:** BK polyomavirus (BKPv) VP1 binds the disialic acid motif of GD1b or GT1b. **E:** Merkel cell polyomavirus (MCPyV) may interact with the sialic acid on both branches of ganglioside GT1b. (Reproduced from Tsai B, Inoue T. A virus takes an “L” turn to find its receptor. *Cell Host Microbe* 2010;8[4]:301–302, copyright © 2010, with permission from Elsevier.)

glial cells, the major target cell for JCPyV. Antibodies to the 5HT_{2A} receptor and receptor antagonists block JCPyV infection, while expressing the receptor in otherwise noninfectible cells renders them susceptible to infection.

Entry, Intracellular Trafficking, and Uncoating

The polyomaviruses use several pathways to enter into the cell and pass through the endosomes to the ER (Fig. 53.8). The pathway from the endosomal compartment to the ER and from the ER to the nucleus is not well understood. After binding to gangliosides on the cell surface, SV40 and BKPv enter the cell using a pathway involving caveolin. The virus is delivered to a neutral pH organelle called the caveosome by endocytosis and then to the ER, where it could be detected by electron microscopy. Recent work has indicated that SV40 may also traffic through a more traditional endocytic pathway to the ER that does not involve a caveosome.⁷⁰ Evidence also indicates that entry of SV40 into the cell requires engagement of a signal transduction cascade through interactions at the cell surface.⁷⁰

Studies with various inhibitors of intracellular structures and processes have shed some light on how the virus travels

within the cytosol. Molecules that interfere with microtubules and prevent movement of vesicles from the endosome to the ER interfere with SV40 infection.²³⁵ Infection can be blocked by the drug, brefeldin A, which inhibits trafficking between the ER and the Golgi apparatus,¹⁹⁷ and a number of other drugs that interfere with endosome maturation and trafficking to the ER.⁷⁰

The polyomavirus capsid begins to disassemble in the ER. Evidence for this includes the appearance of epitopes on VP2 and VP3 that become accessible for immunostaining.¹⁹⁷ In addition, structural changes to the capsid, including disulfide bond reduction and isomerization mediated by ER-resident protein disulfide isomerases, can be detected biochemically at the point when the virus enters the ER.^{93,172,224,274} Furthermore, transmission EM experiments have detected changes in the morphology of virions that were isolated from the ER.¹²¹ It is thought that this change leads to exposure of hydrophobic surfaces of the VP1 molecule on the capsid that facilitates transport across the ER membrane. This step in trafficking also involves ER chaperone proteins. Similar to SV40, it has been shown that the conformation of the MPyV capsid begins to change in the ER due to the action of ERp29, a member of

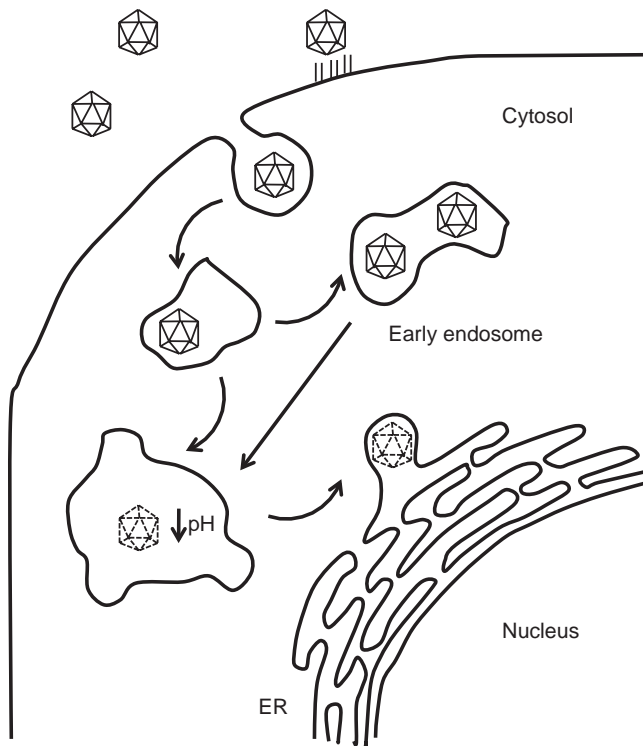


FIGURE 53.8. Simian virus 40 (SV40) binds to its receptor (GM1) and induces internalization from the plasma membrane by caveolin-dependent or -independent endocytosis. The virus is transported to early endosomes that mature to form endolysosomes. Acidification is required for SV40 internalization and subsequent transport steps. Virus transport to the endoplasmic reticulum (ER) occurs from the late compartments of the endocytic pathway. (Adapted from Jiang M, Abend JR, Tsai B, et al. Early events during BK virus entry and disassembly. *J Virol* 2009;83[3]:1350–1358.)

the protein disulfide isomerase family.^{172,265} Capsid disassembly in the ER or the cytosol may be required because the viral particle is bigger than the functional capacity of the nuclear pore. Evidence for a multistep disassembly process for SV40 includes the exposure of certain VP2 and VP3 epitopes in the ER, while further disassembly occurs in the cytoplasm at a later time point as evidenced by immunoassay detection of the viral genome.¹⁵⁰ The ER-associated degradation pathway, which functions to target misfolded cellular ER proteins for proteasome-mediated degradation, has also been implicated in polyomavirus disassembly.^{91,96,125}

The question of how the genome gets carried to the nucleus remains to be answered. For SV40, it has been speculated that release from the vesicular compartment might involve VP1, as noted earlier, or VP2, by virtue of its myristoylated N-terminus inserting into the lipid bilayer.¹⁹⁷ An NLS in VP3 may mediate entry through the nuclear pore complex, because mutations in this NLS inhibit entry of the virion into the nucleus but do not affect capsid assembly or the production of new virions.¹⁹¹

JCPyV enters the cell through clathrin-coated pits, as indicated by use of inhibitors of this pathway as well as the demonstration that JCPyV co-localizes with transferrin, which is known to use clathrin-mediated entry into the cell.²⁰⁶ Both microtubules and microfilaments play a role in trafficking of

JCPyV to the nucleus.⁷ As with SV40, binding of JCPyV to its receptor induces a signal transduction cascade required for efficient entry that can be inhibited by genistein, a tyrosine kinase inhibitor.²¹¹ JCPyV appears to signal through the extracellular signal-regulated kinase (ERK) or mitogen-activated protein kinase (MAPK) pathway. Using fluorescently labeled virus-like particles (VLPs), it has been shown that JCPyV particles do not disassemble before they reach the nuclear pore and that the nuclear localization signal of VP1 is required for entry into the nucleus.²¹⁰

Transcription

After the polyomavirus genome enters the nucleus, it serves as a template for transcription by the cellular RNA polymerase II. Once inside the nucleus, the viral genome becomes wrapped in nucleosomes containing histone H1 in addition to the four core histones that are present in the virus particle.²⁷⁰ Within the cell, the SV40 viral genome contains 24 nucleosomes with a nucleosome-free region of 400 base pairs encompassing the NCCR of early and late promoters and origin of replication. This is in contrast to the virion, where all regions of the SV40 genome are covered with nucleosomes devoid of H1. Chromatin immunoprecipitation can detect transcriptionally active chromatin, defined by the presence of RNA polymerase II, as early as 30 minutes postinfection with SV40.¹¹ The chromatin at this time also contains hyperacetylated histone H3 and H4,^{10,182} which have been associated with chromatin undergoing transcription initiation and elongation.

Transcription of polyomavirus genes is governed by *cis*-acting sequences in the regulatory region. The SV40 regulatory region has been the most intensively studied and serves as a paradigm for the other polyomaviruses. Seminal studies involving SV40 include the demonstration that AT-rich sequences designated TATA boxes act to direct RNA polymerase II to the proper initiation site for transcription.¹⁵ The SV40 early promoter also contains a series of GC-rich sequences within the 21–base-pair repeat region.^{73,84} The protein SP1, one of the first eukaryotic transcription factors to be cloned, binds to these sequences.⁶⁷ The SV40 early promoter also contains a duplicated element called the 72–base-pair repeat, which was the first *cis*-acting DNA sequence to be deemed a transcriptional enhancer because it could activate transcription when placed several thousand base pairs distant from the transcription start site.^{104,187} Interestingly, most clinical isolates of SV40 carry only a single copy of the 72–base-pair repeat, and it appears that duplication can be selected during passage in culture.¹⁵⁷

In MPyV, the enhancer element consists of two neighboring enhancers called A and B, alternatively α and β , that can act independently. The activity of the MPyV enhancers is dependent on the cell environment, as viral variants that are selected for growth on differentiated or undifferentiated embryonal carcinoma cells have mutations that map to the enhancers.^{54,180} The MPyV early promoter is regulated by the cellular factors characterized as polyomavirus enhancer A binding proteins (PEA) or RUNX1, CBFB, and ETV4 that are expressed when cells are growth stimulated with serum.^{122,176}

The JCPyV promoter shows a distinct tissue-specific activity that correlates with the sites of acute infection. While normal JCPyV virions can attach and enter many types of cells, its host range is restricted to those cells that have the appropriate DNA-binding proteins.²⁶⁷ The JCPyV early promoter

contains sequences that act as transcriptional promoters. These sequences include the TATA box as well as binding sites for the transcription factors SP1, YB1 (YBX1), Pur α (PURA), AP-1, a heterodimer of JUN and FOS, nuclear factor 1 (NFAT), and NF1X. A nuclear factor- κ B (NF- κ B) binding site that includes the NFAT site has been shown to be active for late transcription and possible positioning of other DNA-binding proteins to initiate efficient transcription.⁸⁶ NFAT consists of a family of proteins that are expressed in many cells but have multiple classes with tissue-specific expression. The NF-1 class X (NF1X) is highly expressed in human glial cells, stromal cells, B lymphocytes, and CD34+ stem cells that have all been reported to support JCPyV transcription and replication.⁷⁹ Interactions between YB1 and PURA may also provide cell-specific regulation.⁸⁶

The transcription factors that govern kidney tissue-specific activity of the BKPyV early promoter are not known. While numerous candidate transcription factor binding sites have been identified,¹⁸⁵ it remains unclear which of those factors bind the BKPyV regulatory region in an infected kidney cell.

Rearrangements in the NCCR have also been found in BKPyV and JCPyV.^{30,124,214} Viral regulatory regions referred to as archetype are thought to be associated with naturally circulating polyomaviruses, while rearranged regulatory regions arise when high levels of viral replication occur in culture or during disease. For example, JCPyV early promoter undergoes duplication in virus isolated from patients with PML. These rearrangements may serve to allow higher levels of large T antigen expression and viral replication that are tolerated by the patient's immunocompromised state.⁹⁸ Notably, the δ SV40/JCV hybrid virus contains JCPyV T' antigen and VP1, VP2, and VP3 coding sequences but a hybrid regulatory region with elements of the SV40 enhancer. This laboratory-generated hybrid virus grows with faster kinetics and to higher titers than wild-type JCPyV and has an expanded host range to human and monkey kidney cells as well as monkey glial cells.²⁶⁷

The SV40 early promoter undergoes negative feedback regulation by large T antigen.²⁵⁹ When cells are infected with a temperature-sensitive large T antigen mutant of SV40 at the nonpermissive or restrictive temperature, early mRNAs are overproduced. The ability to repress early transcription is dependent on the binding of large T antigen to the promoter. Mutations that disrupt the DNA-binding domain of large T antigen also typically lead to higher levels of large T antigen.¹³³ Three binding sites, referred to as sites I, II, and III, in the regulatory region are all involved in repression by large T antigen. Because site III overlaps the early promoter elements, it is possible that large T antigen binding acts to prevent binding or displace other transcription factors to that region. Early transcription of MPyV is also regulated by large T antigen, but to a lesser degree and in a DNA binding site-independent manner.

Early gene expression in SV40 is down-regulated by miRNAs encoded by the late transcript.²⁵³ The other primate polyomaviruses have similar sequences that are predicted to be able to form the characteristic hairpin structure of miRNA.²²⁸ Somewhat surprisingly, an SV40 mutant virus lacking the miRNA does not produce more virus than wild type in cultured monkey kidney cells. However, cells infected with the mutant virus are more sensitive to killing by cytotoxic T lymphocytes *in vitro*. This observation led to the proposal that the miRNA serves to limit production of antigens recognized by

the immune system and thereby protecting the infected cell.²⁵³ A mutant MPyV that cannot express its miRNA shows no difference in pathogenesis in animals.²⁵⁴ Therefore, the role of the miRNAs during infection remains to be fully elucidated.

Similar to the early promoter, the late promoter elements span the regulatory region and have been defined in a variety of *in vivo* and *in vitro* systems. In SV40, maximal late transcription requires sequences in the 21-base-pair GC repeats and the 72-base-pair enhancers. Late gene transcription occurs concomitantly with the onset of DNA replication, although replication is not required for activation of late transcription. Large T antigen can promote viral replication as well as late gene expression. Large T antigen can stimulate late transcription, although large T antigen binding the origin of replication is not required for this activity. Rather, transcription activation is accomplished through large T antigen interactions with components of the basal transcription machinery such as TATA-binding protein, a component of TFIID and TBP-associated factor 1 (TAF1), as well as transcription factors late SV40 factor (LSF, TFPCP2), TEF-1 (TEAD1), and SP1. These interactions increase the binding of TBP and another basal transcription factor, TFIIA, to the TATA element.⁵³

The control of late gene expression in MPyV is more complicated. Host range or hr-t mutants that do not express functional small or middle T antigen produce equivalent amounts of late proteins, as does wild-type virus.⁸⁷ However, infection with a virus containing similar mutations in cells with a different genetic background demonstrated a stimulatory role of the two T antigens during the late phase. This does not appear to be solely due to an indirect effect because of amplification of the genome, but also involves a direct stimulation of transcription because the RNA/DNA ratio increases.⁴⁰ These same studies also indicated a role for middle and small T antigens in the induction of early gene expression. As these two T antigens induce signaling pathways that lead to activation of transcription factors known to bind the polyomavirus enhancer, this effect is not surprising. The murine virus also differs from its primate counterparts in how its late primary RNA transcript is processed. The MPyV late poly(A) site is a relatively weak site, resulting in the RNA polymerase circling the viral genome multiple times.^{1,161} The first exon of the late transcript can therefore be spliced to itself multiple times, although the protein-coding sequence is only present once on each mature mRNA.

Translation

Limited studies have been performed to address perturbations in translation by the polyomaviruses. One important question in the polyomavirus life cycle is, how does translation of VP3 occur when the same reading frame on the late transcript encodes both VP2 and VP3? VP3 uses an AUG sequence downstream of the VP2 start codon. Mutagenesis of the SV40 late region between the VP2 and VP3 start codons revealed evidence for two internal ribosome entry sites (IRESs) that could potentially promote cap-independent translation of VP3.²⁷⁹ This observation may be particularly important because SV40 infection leads to a decrease in cap-dependent translation.²⁸⁰ SV40 small T antigen leads to decreased phosphorylation of 4E-BP1 that in turn binds to and represses eIF4E, reducing cap-dependent translation. This inhibitory effect on phosphorylation 4E-BP1 required the SV40 small T antigen PP2A binding domain and was most evident at late times after infection.

The ability of SV40 small T antigen to reduce levels of 4E-BP1 phosphorylation during infection contrasts with the effects of MCPyV small T antigen constitutively expressed in Merkel cell carcinoma. As described later, part of the MCPyV genome is randomly integrated into the host cell chromosomes in a manner that permits expression of small T antigen and the N-terminal half of large T antigen.⁷⁸ In cell lines derived from Merkel cell carcinoma that express MCPyV small T antigen, downstream targets of AKT and mTOR including 4E-BP1 were hyperphosphorylated, leading to increased cap-dependent translation.²³⁸ This activity appeared to contribute to the transforming activity of MCPyV small T antigen because a phosphorylation-resistant form of 4E-BP1 reduced cell growth in the Merkel cell carcinoma cell lines. Notably, this activity was independent of its PP2A-binding domain, suggesting the possibility that this represents a function unique to MCPyV small T antigen.

Replication of Viral Genomic DNA

To a great extent, polyomavirus large T antigen orchestrates the viral life cycle. Studies of SV40 large T antigen led to groundbreaking insights into eukaryotic DNA replication including the role of its DNA-binding and helicase activities, culminating in the establishment of the first *in vitro* system for eukaryotic DNA replication. The history of these seminal discoveries and the scientists who made them is well described in a recent review.⁷⁵ While many of the insights into the molecular functions of polyomavirus large T antigen have come from studies on SV40, investigations into other polyomaviruses have also made substantial contributions to our understanding of viral replication.

The large T antigen binds directly to the viral origin of replication and functions as a helicase to unwind the viral dsDNA to enable replication. Large T antigen also serves to recruit cellular proteins including replication protein A (RPA), DNA polymerase/primase, and topoisomerases I and II to facilitate viral genome replication. In addition, large T antigen induces changes in the cell to facilitate viral replication including the formation of viral replication centers or foci within the nucleus.

Polyomaviruses normally infect quiescent or growth-arrested cells that are not actively proliferating, yet they require the host cell's DNA synthetic machinery that is fully active during the S phase of the cell cycle. Large T antigen plays a role in inducing exit from the quiescent state and entry into S phase. Large T antigen also functions to counter the cell's apoptotic responses induced by viral DNA replication. The cellular DNA damage response to replicating viral genomes is also triggered by large T antigen and appears to be required for efficient viral replication.

The SV40 origin of replication contains three regions, a core origin of 64 base pairs with four pentanucleotide (GAGGC) sequences organized as a pair of inverted repeats flanked by an early palindrome (EP) on the early side and an A/T-rich region on the late side (Fig. 53.9).²⁴² The large T antigen DNA-binding domain binds directly to the pentanucleotide sequence.¹⁷⁹ After two molecules of large T antigen bind to opposing inverted pentanucleotide sequences, they each recruit five additional molecules of large T antigen to form a hexamer. The two hexamers face each other in a head-to-head orientation that surrounds the origin. After double-hexamer formation, large T antigen initiates melting of the DNA in the EP region and untwisting of the A/T region. Viral DNA is opened to form single-stranded DNA (ssDNA) followed by large T antigen–recruiting RPA and topoisomerase I. The helicase domain of large T antigen recruits the DNA polymerase α /primase complex (POLA1, POLA2, PRIM1, PRIM2) that contributes to RNA Okazaki primer formation on the lagging strand (Fig. 53.5).

Besides its role in initiation of DNA synthesis, large T antigen is also required for elongation of the growing chains. Elongation is carried out by DNA polymerase δ (POLD1, POLD2, POLD3, POLD4) in conjunction with its accessory factors, proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C). Large T antigen has an ATPase-dependent 3' to 5' helicase that advances each hexamer in opposite orientations along the viral DNA. Replication occurs in a bidirectional manner and ends with the de-catenation of the two linked circular molecules by topoisomerase II.

The bidirectional nature of SV40 DNA synthesis was demonstrated in two ways. First, Danna and Nathans⁵⁶ performed an elegant experiment in which they pulse-labeled infected cells with ³H-thymidine for 5 to 15 minutes, isolated fully replicated DNA molecules, and determined what parts of the genome were labeled. The theory behind this approach is that the labeled thymidine would appear farthest from the origin of replication in cells that were labeled for the shortest time periods, because those were the closest to completion, and extend back toward the origin as the labeling time was extended. This allowed the mapping of the origin as well as the determination that replication was bidirectional. The second approach used shadowing of DNA with electron microscopy imaging of replicating SV40 and MPyV.²⁷¹ Here, replication forks were visualized and mapped in relation to the ends of the DNA molecules, which had been digested with a restriction endonuclease at a unique site on the chromosome.^{48,76,102}

Examination of the crystal structure of the large T antigen helicase domain when bound to adenosine triphosphate (ATP) or adenosine diphosphate (ADP) led to the proposal that ATP

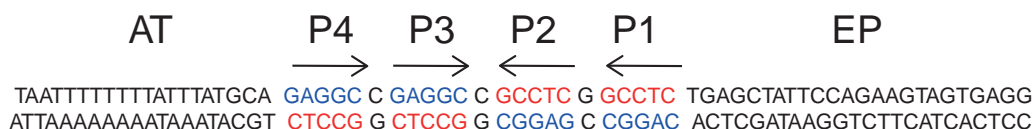


FIGURE 53.9. The minimal simian virus 40 (SV40) origin of 64 base pairs contains a central perfect palindrome with four pentanucleotides (P) GAGGC, the early palindrome (EP), and AT-rich region. The large T antigen DNA-binding domain binds to the pentanucleotide sequence. A second large T antigen binds to another pentanucleotide sequence on the opposite strand. Each large T antigen forms a hexamer leading to a double hexamer in a head-to-head orientation surrounding the origin. Adenosine triphosphate (ATP) hydrolysis by the hexamer leads to melting of the AT-rich and unwinding of the EP regions.

hydrolysis induces conformational changes that cause twisting of the hexamers resulting in expansion and constriction of the central channel that resembles the movement of an iris within an eye.¹⁶³ This could explain how large T antigen generates the motive force that is necessary to move DNA for replication. It should be emphasized that although many of the steps involved in polyomavirus DNA replication and many cellular factors that contribute to this process have been identified, there are many important questions to be resolved. In particular, it is not known where the formation of the ssDNA intermediates occurs relative to large T antigen, how it passes through the large T antigen hexamer, and how the DNA moves through the double hexamers to enable bidirectional DNA synthesis.

Phosphorylation of large T antigen regulates its ability to drive DNA synthesis.²⁴² In SV40, phosphorylation of large T antigen threonine residue 124 by cyclin-dependent kinases enhances binding of the protein to the origin of replication and subsequent unwinding.^{178,184} In MPyV, the corresponding large T antigen threonine residue 278 is also phosphorylated, and mutation of this residue abolishes replication function. Conversely, phosphorylation of SV40 large T antigen serine residues 120 and 123 inhibits replication.³⁷ The serine residues can be phosphorylated by casein kinase I and dephosphorylated by PP2A.

The N-terminal J domain of large T antigen contributes to viral DNA replication, functioning through binding to HSC70. While the J domain is not required for viral DNA replication *in vitro*, it plays a significant role *in vivo*.³¹ Notably, the J domain from the human HSP40 homolog HSP70-1 protein can support SV40 DNA replication when it was substituted for the homologous domain of large T antigen, suggesting that interaction of large T antigen with HSC70 and the host cell heat shock system facilitates viral replication. The mechanism by which the large T antigen J domain stimulates DNA replication is not known, but may involve chaperone-mediated rearrangements of the initiation complex on the DNA.

An essential contribution of large T antigen to viral replication is to induce the cell to enter the cell cycle. Although polyomaviruses normally infect cells that are not actively dividing, they require the host cell's DNA synthetic machinery, produced and activated during S phase, for replication of the viral genome. Large T antigen drives the resting cell into S phase by binding to pRb (RB1), the product of the retinoblastoma susceptibility gene, and the Rb-related proteins p107 (RBL1) and p130 (RBL2).⁵⁹ The Rb tumor suppressor proteins, sometimes referred to as pocket proteins, prevent entry of the cell into S phase through binding and repression of the E2F family of transcription factors.⁶² During the quiescent (G0) and G1 phase of the cell cycle, pRb and p130 form complexes with E2F on the promoters of genes required for DNA synthesis. Notably, p130 binds to the repressor E2F4/DP1 heterodimer and recruits a five-protein complex to the promoters of more than 800 E2F-dependent, cell cycle-regulated genes.¹⁶⁹ The DREAM (DP1, RBL2, E2F4, and MuvB) complex is disrupted when large T antigen binds to p130, thereby releasing the MuvB core complex and enabling activation of E2F-dependent genes. When a cell is induced to divide physiologically, cyclin-dependent kinases serve to phosphorylate the Rb family proteins, enabling their release from E2F and thereby permitting gene expression. Large T antigen subverts this pathway by binding to Rb family proteins and disrupting their binding to E2F. The LXCXE

motif in large T antigen binds directly to the Rb proteins.¹⁴⁵ In addition, the large T antigen N-terminal J domain cooperates with the LXCXE motif binding of Rb family proteins to facilitate their release from E2F. The SV40 large T antigen J domain also perturbs the phosphorylation status and stability of Rb-related proteins, thereby contributing to loss of their E2F repression functions.^{251,252}

In addition to causing the cell to enter S phase, SV40 large T antigen carries out another important role in establishing the proper cellular environment for replication. The inappropriate entry into S phase induced by large T antigen causes the cell to activate a p53-dependent growth arrest and proapoptotic response. The p53 tumor suppressor protein was first discovered by virtue of its being co-immunoprecipitated with SV40 large T antigen from cellular protein extracts.^{153,168} Large T antigen binds directly to the DNA-binding domain of p53, thereby disrupting p53's ability to transactivate its target genes such as *p21* (*CDKN1A*), *PUMA*, and *MDM2* involved in cell cycle arrest, apoptosis, and autoregulation, respectively.

The ability of large T antigen to activate p53 is due to expression of ARF (alternate reading frame), a product of the *CDKN2A* gene that also expresses the CDK4 inhibitor p16-INK4A. ARF functions to stabilize p53 by binding to and inhibiting the E3 ubiquitin ligase activity of MDM2 directed toward p53. Although normal cell cycle entry does not induce ARF expression, SV40 large T and MPyV middle T antigens induce expression of ARF. Although many polyomavirus large T antigens can bind to p53 and thereby inactivate its growth inhibitory and apoptotic response, MPyV large T antigen is notable for its inability to bind directly to p53. Nonetheless, MPyV is able to counter the p53-mediated growth arrest by large T antigen inactivation of the pRb family proteins⁶³ and by small T antigen inhibition of ARF.¹⁸⁸

Recent evidence indicates that the cellular DNA damage response (DDR) is required for SV40 replication.²⁸² SV40 infection induces a cellular DDR response that includes activation of the ATM kinase, resulting in phosphorylation of the specialized histone γ -H2AX and the checkpoint kinase CHK2. Large T antigen co-localizes with phosphorylated ATM and γ -H2AX on DNA to form nuclear foci that include the MRN complex (Mre11, Rad50, and Nbs1) and 53BP1. Large T antigen alone without viral DNA can induce the DDR, and this may be dependent on large T antigen interaction with BUB1.¹¹¹ In addition to the DDR, much attention has been focused recently on the role of nuclear structures called promyelocytic leukemia nuclear bodies (PML-NB) during replication and infection.²⁷ In BKPyV infection, large T antigen co-localizes with PML-NB at very early times and, concomitant with the onset of viral DNA replication, induces a dramatic reorganization of PML-NB.¹²⁶ It remains unclear if the DDR and changes in the PML-NB are a consequence or a requirement of viral replication.

Assembly

Assembly of polyomaviruses begins with the translocation of VP1, VP2, and VP3 into the nucleus and the formation of capsomeres. The VP1, VP2, and VP3 from SV40 and MPyV each contain nuclear localization sequences. VP2 and VP3, in the absence of VP1, do not accumulate in the nucleus but rather require VP1 for nuclear transport, indicating that these

proteins may be imported as a complex. The VP2 or VP3 NLS may be more important for trafficking of incoming virus from the ER to the nucleus than for import of the newly synthesized proteins during the late phase of the infection. This model fits with the data indicating that VP2 and VP3 become exposed as the virus transits through the ER.

Capsomeres assemble into viral particles and DNA is incorporated into the virion. It is unclear whether empty capsids are precursors to mature, DNA-containing virions or dead-end products. While it has been reported that a *cis*-acting sequence in the origin region of SV40, *ses*, enhances DNA packaging,⁵² DNA molecules that lack this sequence element can still be encapsidated.

Purified recombinant VP1, expressed in bacteria, yeast, or baculovirus-infected insect cells, can self-assemble into capsomeres. Under appropriate conditions, these capsomeres form capsid structures referred to as VLPs. Therefore, viral DNA, VP2, and VP3 are not required for capsid formation. Changes in ionic strength and pH of the buffer can lead to formation of other structures including tubular moieties and T = 1 icosahedrons. It has been shown that chaperones such as HSP70 are required for efficient capsid assembly. VLPs are very similar to authentic virions and have been used for basic studies on receptor binding, virus entry, intracellular trafficking, and the immune response. VLPs have also been investigated as vehicles for delivery of genes and small molecules^{38,88,258} and have been extensively used for serologic assessment of polyomavirus infection.^{34,273}

Release

The manner by which polyomaviruses leave the cell is poorly understood. While some studies indicate that the virus causes lysis of the cell, others indicate that virus can be shed from intact cells. For example, SV40 infection can cause a cytopathic effect (CPE) or vacuolating effect with destruction of the cell. Alternatively, SV40 can leave polarized epithelial cells from the apical surface and can exit nonpolarized epithelial cells without killing them.⁴⁵ A regulated virus exit process is suggested by the observation that SV40 can be detected in cytoplasmic membrane vesicles using electron microscopy, and release of virions is inhibited by monensin, which blocks vesicular transport. Alternatively, SV40 VP4, translated from the VP1 mRNA using an internal methionine codon in the VP1 open reading frame, may function as a viroporin that disrupts membranes to promote virus release.²¹²

JCPyV causes cell death through lytic infection of glial cells. Infection of astrocytes cultured *in vitro* supports a progressive JCPyV infection that leads to necrotic cell death and virion release.²²⁹ Notably, this model system did not yield any evidence for apoptosis by JCPyV infection.

The agnoprotein may also contribute to assembly and egress. SV40 isolates containing mutations in agnoprotein replicate with wild-type kinetics but produce plaques that are smaller than wild type due to inefficient release of mature virions from the cell. Other phenotypes of agnoprotein mutants that could account for inefficient viral replication include improper localization of VP1. At the nuclear envelope, agnoprotein has been shown to dissociate heterochromatin protein 1 (HP1) from the lamin B receptor, resulting in destabilization of the envelope, perhaps explaining how agnoprotein could facilitate egress.¹⁹⁸ While some of the large T antigen host range

mutations affect agnoprotein expression, the assembly defect in these mutants cannot be complemented by expression of agnoprotein *in trans*.²⁴⁵ Additional functions have been proposed for the JCPyV agnoprotein, including as a viroporin on the nuclear membrane.¹⁴²

PATHOGENESIS AND PATHOLOGY

The principal target cell for initial entry of polyomaviruses has been difficult to identify. It appears that once exposed to a polyomavirus, an individual maintains a lifelong persistent infection present in a variety of tissues. Therefore, it can be difficult to distinguish the site of initial entry from a site that acts as a reservoir for lifelong infection. While many studies have found the presence of polyomaviruses in a variety of tissues from immunocompromised patients, there is scattered evidence that these tissues may represent reservoirs for virus in normal immune competent individuals as well. For example, many polyomaviruses including SV40, BKPyV, and JCPyV are present in the kidney and excreted in the urine. Exposure to infected urine could be a source of infection for these polyomaviruses. There have been reports of JCPyV infection in human colonic epithelial cells and isolation of BKPyV and JCPyV from stool specimens.²³ Notably, BKPyV, JCPyV, MCPyV, KIPyV, and WUPyV have been frequently detected in sewage samples,²⁵ while BPyV has been detected in farm wastewater samples.¹¹⁹

JCPyV is able to productively infect tonsillar stromal cells in culture with efficiency nearly comparable to human glial cells. JCPyV DNA has also been found in lymphoid tissues including bone marrow and spleen.¹¹⁷ JCPyV DNA has been identified in tonsil stromal cells and in B cells isolated from tonsils.¹⁸⁶ The ability of JCPyV to infect tonsillar stromal cells in culture and the presence of JCPyV DNA in tonsil tissue suggest the possibility that the initial infection can occur in this tissue.

BKPyV has been detected in lung tissue from one case of interstitial pneumonia in a hematopoietic stem cell transplant patient and in the respiratory tract and tonsil tissue in another,^{100,221} as well as in salivary glands.¹²³ This, together with the observation that the majority of the population in both developed and underdeveloped regions of the world seroconverts in early to midchildhood, suggests a respiratory route of transmission.

WUPyV and KIPyV can be detected in respiratory secretions that represent either the site of acute infection or latency. For example, WUPyV was frequently detected in tonsils and nasopharyngeal lymphoid tissue from immunocompetent children.⁴⁷ WUPyV and KIPyV were also found in plasma, urine, and respiratory samples of renal transplant patients.⁴⁹

For some polyomaviruses, there is evidence for maternal-fetal transmission. MPyV can be acquired vertically through transplacental transmission or direct contact as neonates.²⁸¹ BKPyV has also been reported to undergo *in utero* and transplacental transmission.²⁸ In contrast, a recent study determined that the human polyomaviruses WUPyV, KIPyV, and MCPyV were not present in more than 500 fetal tissues, although serologic assessment indicated that nearly half of the mothers had been infected with MCPyV.²²⁰

Polyomavirus infection in birds spreads quickly in susceptible flocks. Avian polyomaviruses can be found in the urine and

fecal matter in flocks, suggesting an oral route of transmission. A recent report demonstrated upward vertical transmission for APyV by blowfly larvae present in nests.²⁰⁷ Bird fledglings become viremic after being bitten by the larvae and shed virus from their cloaca, thereby infecting the breeding adults. In parrots, viremia was followed by cloacal shedding within 1 week of experimental infection.²⁰⁵ Infected birds may harbor virus persistently throughout life, but acute infection can cause neurologic damage and death in younger birds.²⁴⁸

Immune Response

Humoral immune responses that are immunoglobulin G (IgG) or IgM occur in all animal species infected with polyomaviruses and show some cross-reactivity within rodent, primate, or avian families but not across those barriers unless immunized with laboratory-made virions or proteins. Antibody against polyomavirus is IgG, although a number of reports identified an IgM response with onset of clinical symptoms of infection.^{80,81} IgM and IgA antibodies have been detected against BKPyV in bone marrow and renal transplant patients.⁶⁴ The Multicenter AIDS Cohort Study found that approximately 80% of all participants had immunoglobulin titers specific for MCPyV VP1. Within the remaining group of seronegative participants, approximately 26% seroconverted in the following 4 years. Notably, no clinical signs or symptoms associated with acute MCPyV infection and subsequent seroconversion were appreciated.²⁶¹

Measurements of cellular immune responses to JCPyV and BKPyV in infected individuals have identified CD8+ cytotoxic T lymphocytes (CTLs) that recognize epitopes on VP1 and large T antigen. The epitope on BKPyV VP1 amino acid residues 108 to 116 elicits a T-lymphocyte response that also recognizes JCPyV epitope VP1 p100.¹⁴⁹ Using tetramer assays or CTL cell lysing assays, CD8+ T cells can be found in the peripheral circulation that are human leukocyte antigen (HLA) restricted to genotype A*02, present in a large proportion of the population.⁶⁵ Viral-specific CD8+ T lymphocytes show a good correlation with time of survival in PML patients and could be useful as a prognostic indicator of disease progression.¹⁴⁷

A CD8+ cytotoxic T-cell response was noted in an analysis of mRNA expression profiling of Merkel cell carcinoma.²⁰⁴ Notably, patients with a better prognosis had a statistically significant increase in genes related to the CD8+ response. The response was most likely due to infiltrating CD8+ cells that were captured when the mRNA was prepared from the tumor.

There is also a T-cell-mediated immune response in SV40-infected monkeys and MPyV-infected mice that confers protection from infection and is thought to play a role in establishing viral persistence in SV40- and MPyV-infected animals. Stimulation of CD8+ CTLs in mice, however, affects different pathways involving CD28 and CD40 ligands during the course of either acute or persistent infection.¹⁴⁰

Persistence

One of the interesting biological characteristics of all the polyomaviruses is the maintenance of a chronic and lifelong viral infection in their hosts. Usually viral persistence is established in the kidney as judged by viral excretion in the urine that can show remarkably high levels of virus release ranging from 10^3 to 10^8 viral copies per milliliter. Virus can be found in uroepithelial cells and in ductal pathways. However, there is no apparent pathology associated with such high levels of urinary excretion

unless there is an underlying immune-compromised condition. There is no information, however, on the mechanisms of viral persistence, what triggers viral synthesis and multiplication, and the consequences of lifelong virus shedding in the urine. There is no evidence that polyomaviruses enter a true latent state in which there is little or no viral gene expression or genome replication as with the herpes family of viruses.

For the human polyomaviruses, the kidney or urothelium is the primary location for BKPyV, whereas the kidney and lymphoid organs are locations for JCPyV with differing consequences of infection. The sites of persistence for WUPyV and KIPyV have not been determined, although viral DNA has been recovered from respiratory secretions. MCPyV, HPyV6, and HPyV7 can be isolated from normal skin.²²⁶ MCPyV was shed as virions from the skin. Given the rarity of Merkel cells in skin, this raises the possibility that MCPyV is produced by the stratified epidermal keratinocytes rather than less abundant Merkel cells.

Transformation

Since their initial discovery, polyomaviruses have been used to study the transformation of normal cells. The initial observations of the tumor-causing ability by MPyV followed by the observation that injection of SV40 into newborn hamsters caused tumors heralded the potent transforming activities of polyomaviruses. Since then, many more polyomaviruses have been identified and their transforming activity has been compared to MPyV and SV40. The transforming activity of the polyomaviruses is limited to the T antigens. The VP coat proteins or agnoprotein does not have transforming activity. The large and small T antigens of most mammalian polyomaviruses as well as middle T antigen of MPyV and HaPyV have transforming activities. Large T antigen transforms cells in part by binding to and inactivating the Rb and p53 tumor suppressor proteins. The contribution of small T antigen to transformation is dependent in part on binding to the protein phosphatase PP2A. Middle T antigen has unique transforming activities related to its ability to assemble an activated, tyrosine kinase signaling complex.

The study of cellular transformation and its relationship to tumorigenesis was led by seminal studies of the polyomaviruses. Early studies on transformation included colony or focus formation studies of primary mouse embryo fibroblasts or established 3T3 cells. Using SV40, Risser and Pollack²¹⁵ described a hierarchy of the transformed phenotype that began with immortalization of primary cells, followed by their growth in reduced serum conditions, anchorage-independent growth, and ultimately formation of xenograft tumors in mice. The degree of transformation was dependent in part on the level of large T antigen expression; the higher level of large T antigen expressed enabled the complete transformed phenotype.

The polyomaviruses have been used to study transformation and oncogenesis in a variety of ways. In addition to using the whole virus genome, expression vectors for the T antigens has been used to study transformation. Mammalian expression vectors that drive the expression of the early region containing large and small T antigen or complementary DNAs (cDNAs) specific for large T antigen have been used in cells as well as in transgenic mice.

A seminal breakthrough in the study of human carcinogenesis was made when it was demonstrated that the combination

of only four genetic elements could fully transform normal human cells. Expression of the SV40 large T and small T antigens, an activated form of HRAS, and the catalytic subunit of human telomerase reverse transcriptase (TERT) could completely transform normal human cells, including the ability to form tumors as a xenograft in an animal.^{108,109} This strategy has been used to transform a wide variety of human cell types including astrocytes, myoblasts, and epithelial cells from lung, breast, and prostate tissues.¹⁴¹ In addition, substitution of SV40 large and small T antigen with human oncogenes and tumor suppressor genes led to the establishment of a model system of human oncogenesis composed of completely human genetic elements.⁴¹ This approach has been expanded in high-throughput screens of cDNAs for gain-of-function and short hairpin RNA interference (shRNAi) molecules for loss of function to identify novel oncogenes and tumor suppressor genes, respectively.

The transforming activity of polyomaviruses is balanced by its capacity to lyse cells during infection. Polyomaviruses can transform cells and form tumors under circumstances when the T antigens are expressed but the virus does not undergo replication at sufficiently high levels to cause lysis of the cell. If the infected cell is permissive for virus replication, then it will undergo lysis and not undergo transformation. Conversely, if a cell is restrictive for replication but enables T antigen expression, then it may undergo transformation. Typically, a restrictive cell undergoes transformation when the polyomavirus DNA becomes integrated into the host chromosomal DNA in a way that sustains expression of the T antigens. This situation occurs when MCPyV DNA becomes integrated into the host genome and contributes to the development of Merkel cell carcinoma. Alternatively, a cell can be semipermissive and sustain low levels of viral replication while expressing sufficient levels of T antigens to change the cellular growth characteristics.

The nonpermissive or restrictive environment is generally caused by species-specific differences in DNA replication factors. The best understood of these is the interaction of large T antigen with the DNA replication machinery. Large T antigen binds to the DNA polymerase α /primase complex and recruits it to the viral origin of replication. SV40 cannot replicate its DNA in rodent cells, at least in part due to its inability to bind the DNA polymerase α /primase in these cells.

Another level of permissiveness involves the interaction between large T antigen and p53. Just as the large T antigen can affect p53 function, p53 has reciprocal effects on the ability of large T antigen to function as a replication factor.⁸³ A tissue-specific example of this restriction is found in the differential ability of SV40 to replicate in human fibroblasts and mesothelial cells.²² In fibroblasts, the virus replicates quite efficiently, whereas replication is severely limited in mesothelial cells. The latter cells have higher steady-state levels of p53 than the former, and decreasing p53 expression in mesothelial cells with antisense strategies increases viral replication. Changes in the regulatory region of the virus can also change the outcome of the infection from lytic to nonlytic. Monkey kidney cells transduced with a viral genome containing a deletion of the SV40 origin of replication that disables replication are readily transformed.⁹⁵ Rearrangements in the BKPyV NCCR that cause a decrease in DNA replication also enhance the transforming ability of the virus.²⁷⁵

A wide variety of transgenic mice lines have been generated that express polyomavirus early region or individual T antigens. Many of these animals develop tumors dependent on the specific promoter used. For example, transgenic mice expressing the SV40 early region have led to the TRAMP mouse model of prostate cancer and the rat insulin promoter (RIP)-Tag model for pancreatic neuroendocrine tumors. MPyV middle T antigen transgenic mice have been developed using a variety of promoters. Mice with middle T antigen expression driven by the mouse mammary tumor virus long terminal repeat (MMTV-LTR) develop multifocal mammary adenocarcinomas that can become metastatic to the lung in 3 months.¹⁰⁶ Several different strains of mice expressing MPyV middle T antigen in the prostate tissues have been developed. Mice with middle T antigen driven by the C3 promoter develop mouse prostatic intraepithelial neoplasia (mPIN).²⁶⁰ Another strain using the (ARR)2-Probasin promoter develop mPIN as early as 8 weeks of age that develops over time to become invasive cancer.¹⁵⁹

BKPyV induces different types of tumors dependent upon the rodent species and route of inoculation. Intracerebral inoculation of BKPyV in hamsters results in ependymomas, neuroblastomas, and pineal gland tumors. Intravenous inoculation causes pancreatic islet cell tumors, fibrosarcomas, and osteosarcomas. Mouse inoculations frequently result in fibrosarcomas, liposarcomas, nephroblastomas, gliomas, and choroid plexus tumors that are also common in SV40 T protein-expressing transgenic mice.¹⁹² BKPyV tumor induction shows some viral strain variation, with the prototype isolate, BKPyV Gardner, possessing the greatest oncogenic potential. It is unclear whether *trans*- or *cis*-acting factors influence tumor formation frequency, but it is assumed that the regulatory sequence variations have a dominant role.

JCPyV inoculation into hamsters also results in multiple tumor types including medulloblastomas, glioblastomas, and pineocytomas. However, unlike BKPyV or SV40, JCPyV induces a grade 4 glioblastoma multiforme or malignant astrocytoma upon intracranial inoculation into owl and squirrel New World monkeys. In contrast, JCPyV does not induce tumors when inoculated into Old World macaques or rhesus or African green monkeys. Malignant brain tumors induced by JCPyV inoculation revealed that the viral genome was integrated into the cellular DNA.¹⁸³ The astrocytoma cells can be explanted and grown in culture. In some tumor cell explants, JCPyV large T antigen expression gradually diminishes upon continuous culture and it enters into senescence. One owl monkey tumor, however, demonstrated unique properties, not only surviving in culture but also becoming transplantable back into other owl monkeys. JCPyV DNA in these tumor cells, termed 586, was both integrated in the cell chromosome and episomal in high copy number. JCPyV infectious virus was produced continually in culture, resulting in a persistent viral or carrier culture.¹⁷⁵

EPIDEMIOLOGY

Members of the family *Polyomaviridae* are found throughout the world in bird, rodent, nonhuman primate, and human populations. The presence of polyomaviruses in the human population has been extensively studied. In one approach, evidence for prior infection by individual polyomaviruses has been performed with serum collected from individuals. For the most

part, sera testing with viral particles or more recently using VLPs prepared from recombinant VP1 corresponding to the different polyomaviruses enables determination of prior exposure. These serologic studies have revealed that infection with polyomavirus occurs as early as several months of age and increases in frequency until, by adulthood, most types of polyomaviruses have infected nearly all individuals. In the second approach, sequencing of polyomavirus DNA collected from the urine or the skin enabled determination of the presence of the unique strain of polyomaviruses. This approach has revealed that for the most part, an individual maintains a lifelong infection with polyomavirus. Although protective immunity against individual polyomaviruses may prevent reinfection, it may not be sufficient to prevent infection with a different strain of the same polyomavirus. In addition, these studies have revealed that polyomaviruses have co-evolved along with the human population.

Evidence for prior infection with specific polyomaviruses can be determined in a sensitive enzyme-linked immunosorbent assay (ELISA). Recombinant forms of VP1 from the corresponding polyomavirus are produced in bacteria or insect cells and spontaneously form VLPs. The purified VLPs are adhered to a plate and used to capture specific immunoglobulin molecules from sera. The relative amount of antibody captured is detected using anti-IgG- or anti-IgM-labeled secondary antibodies. In a useful variation of this assay, the recombinant VLP from a polyomavirus can be used to deplete the sera of specific antibodies prior to exposure to the VLP attached to the plate. Depletion of immune sera with a polyomavirus-specific VLP permits determination of cross-reactivity of antibodies to different polyomaviruses. Using the VLP capture ELISA together with prior immunodepletion, evidence for infection with specific polyomaviruses can be obtained in a large collection of sera samples.

VLP-based assays indicate that infection with human polyomaviruses can occur in the first few years of life. Serologic surveys of populations for the detection of antibodies specific for JCPyV and BKPyV indicate that seroconversion takes place early in life.¹⁴⁶ Detection of maternal antibody in newborns diminishes within the first 3 months and is followed by evidence for newly acquired infection as early as the first year of life. Evidence for exposure continues to increase into adulthood, with titers corresponding to prior infection observed in approximately 50% to 80% of the sera tested.^{139,264,269} As evidenced by antibody titers, WUPyV and KIPyV infections can occur very early in life and then progressively become more common with age, with one report indicating 100% for KIPyV in healthy blood donors older than 50 years of age.^{193,195}

The VLP assay was initially used to detect evidence for prior infection with BKPyV and JCPyV in human sera and then expanded to search for evidence of prior infection with SV40. A high degree of cross-reactivity in human sera has been observed between VLPs from BKPyV and SV40 and to a smaller degree between JCPyV and SV40.^{34,139,273} Immune depletion of sera with BKPyV VLPs significantly reduces the titer of IgG-recognizing SV40 VLPs. Notably, reactivity of human sera against the African green monkey-derived lymphotropic polyomavirus (LPV) has been recognized for many years that was not appreciably depleted by preincubation with the VLP corresponding to SV40 or other known polyomaviruses. The discovery of human HPyV9, highly homologous to LPV, led to a direct comparison of sera reactivity for the

two corresponding VP1 proteins. Depletion with HPyV9 VLP removes most of the reactivity against monkey LPV.²⁶⁴

Antibodies to both BKPyV and JCPyV are quite prevalent in populated countries but less prevalent in remote populations. In a study analyzing sera from Amerindian tribes from remote regions of South America, a low percentage of samples were positive, demonstrating low titers compared to a collection of Japanese samples with the majority positive with high antibody titers, particularly to JCPyV.¹⁷⁴ It also seemed that introduction of the human polyomaviruses into the Amerindian population occurred at the time of human contact from other countries.

In general, detection of viral DNA by PCR is less sensitive than evidence for infection by serology-based testing. A direct comparison of evidence for infection using VLP assays, neutralization assays, and PCR-based detection of viral DNA isolated from the skin of more than 400 patients was performed. In this study, 65% had evidence for prior infection with MCPyV based on serology, but only 18% were positive by PCR amplification of DNA isolated from skin. This may be due to the inability of certain PCR primers to amplify all possible genomes of a given virus. Notably, nearly all patients with detectable amounts of MCPyV DNA on the skin had evidence for infection by serology against the virus.⁷⁷

Because excretion of the human polyomaviruses in the urine is relatively common, a number of studies have tested urine by PCR for the prevalence of BKPyV and JCPyV. Sequencing of the polyomaviruses has revealed variation in all regions of the viral genome, especially the NCCR and the sequences encoding the C-termini of large T antigen and VP1. Sequencing of BKPyV has identified four major subgroups with evidence for co-evolution with known migration patterns of human hosts.²⁷⁸

This approach demonstrated that excretion of the identical JCPyV virus occurred over 5 to 7 years in 19 different individuals and indicated persistent shedding rather than reinfection by different strains of JCPyV.^{136a} In addition, sequencing of JCPyV genomes revealed that the identical sequence was present in four of five families including parents and children, indicating that the virus spread horizontally within close quarters.²⁸³

Given that infection with JCPyV and BKPyV frequently occurs early in childhood among individuals living together, studies have been performed that reveal that polyomaviruses have co-evolved with humans.^{148,284} For example, a study collected urine samples from young and older individuals in Okinawa who had contact with American military and their families. The samples were analyzed using PCR to identify JCPyV DNA and nucleotide sequencing that could "type" variants of the DNA. One type was specific to Japan, while others were prominent in the United States. The results suggested that none of the JCPyV DNA types found commonly in the American population were present in any age group in Japan, suggesting that virus is not easily transmitted between populations.¹³⁶ Additionally, urine samples collected from second- and third-generation Japanese Americans living in Los Angeles revealed that strains of JCPyV more commonly detected in Japan were present in the second- and third-generation Japanese American families.²⁵⁵

Infection with MCPyV can also be traced back to childhood location by geographic areas based on sequences unique to specific polyomaviruses. Comparing the NCCR regions from different isolates revealed substitutions, small deletions, insertions, and duplications. Notably, strains detected in individuals from North

America or Europe could be distinguished from those from Asia, supporting co-evolution of viruses along with the host.²²⁶

CLINICAL FEATURES

Human diseases caused by polyomaviruses result from lytic infection of target cells including JCpV in the brain; BKpV in the kidney, bladder, and ureter; and TSPyV in the hair follicle. There is no evidence that the initial or primary infection by these polyomaviruses directly results in disease. Instead, it appears that the low levels of virus that are maintained by a healthy immune system can reach very high levels when an individual's immune system is affected by HIV-1/AIDS, organ transplantation, or immunosuppressive drugs. High levels of JCpV, BKpV, and TSPyV lead to destruction of infected tissues. Furthermore, high levels of MCPyV that can occur in immunocompromised patients may increase the chances for accidental integration of the viral genome into Merkel cells. The immunocompromised state of the patient may also disable mechanisms for effective clearance of the T antigen expressing Merkel cells, enabling it to advance to a cancer.

BKpV-Associated Diseases

Reactivation of BKpV infection is an important cause of illness in immunocompromised patients. BKpV has been most consistently identified with kidney failure after renal allograft and hemorrhagic cystitis and ureteric stenosis in hematopoietic stem cell transplant (HSCT) recipients.^{5,6} BKpV has also been implicated in ureteric stenosis and associated with kidney failure in HSCT patients.¹⁴³ In both patient groups, immunosuppressive drugs for graft protection allow reactivation of latent BKpV in the urinary tract. BKpV has also been associated with a limited number of cases of retinitis, nephritis, pneumonia, and encephalitis.

Approximately 40% of renal allograft recipients shed BKpV in the urine either transiently or continually over weeks to months, with interstitial nephritis occurring in 10% of renal allograft recipients and graft loss caused by BKpV infection in 1% to 2%. This syndrome is known as polyomavirus-associated nephropathy (PVAN) (Fig. 53.10). The definitive diagnosis of PVAN requires a kidney biopsy, although this test may lack sensitivity. Generally, viral cytopathic effects show nuclear inclusions in the tubular epithelial cells, with chromatin damage and enlarged nuclei indicating viral multiplication (Fig. 53.10). Such extensive virus growth causes damage and leakage to the tubules, which results in fluid in the interstitial spaces and gradually atrophy of the tubules and fibrosis.¹¹² PVAN is suggested by the presence of decoy cells, epithelial cells with viral inclusions, in the urine reflective of BKpV replication and the presence of virus that can reach 10^7 virion particles per milliliter of urine. The presence of urine casts composed of polyomavirus aggregates or *Haufen* (German for “cluster” or “stack”) may also suggest the presence of productive BKpV infection in the renal tubules.²⁴³

In the setting of failing kidney function, evidence for increasing levels of BKpV in the blood and urine should prompt suspicion of PVAN. Quantitative PCR assays are used clinically to detect BKpV viral load.¹³ The diagnosis of PVAN is suggested by BKpV DNA in the blood (greater than 10,000 copies/mL) or VP1 mRNA (greater than 6.5×10^5 copies/ng total RNA) in the urine.¹¹³ In renal transplant patients, the incidence of BKpV disease appears to correlate with high antibody titers in the donor. Evidence also suggests that there is greater vulnerability for graft loss if the recipient is seronegative for BKpV and conversely that high levels of interferon- γ (IFN- γ)-secreting cells in the recipient are protective. It should be noted that both JCpV and MCPyV could be detected in the urine of patients with PVAN. However, it appears unlikely that JCpV and MCPyV are a significant cause of PVAN in the absence of BKpV.^{120,170}

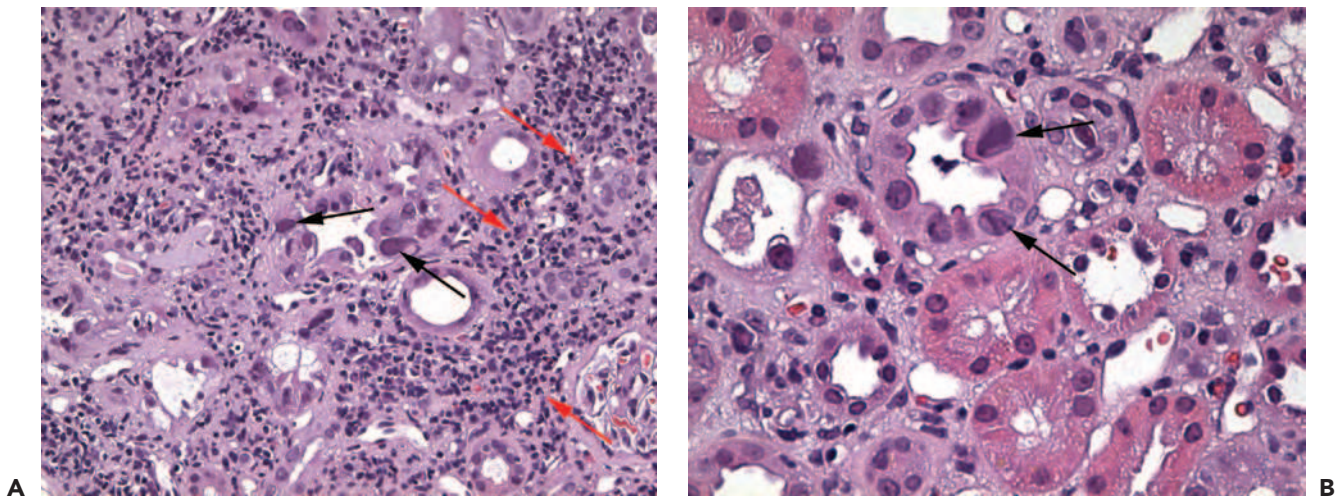


FIGURE 53.10. Polyomavirus-associated nephropathy. Photomicrographs illustrating BK polyomavirus (BKpV) viral inclusions in the kidney of a transplant patient. **A:** Red arrows point to the intense inflammation associated with the viral infection that resembles acute cellular rejection with lymphocytes and eosinophils. The black arrows show the basophilic viral inclusions in the nuclei of tubular cells. **B:** Higher-power view of viral inclusions in the nuclei of tubular epithelial cells (arrows). (Courtesy of Kent Johnson, University of Michigan, Ann Arbor, MI.)

Active BKPyV infection in renal allograft patients at times mimics graft rejection, complicating the use of immune-suppressive drugs. Antirejection therapy can lead to graft loss due to an increase in viral multiplication.²¹³ Consequently, viral infection leading to interstitial nephritis and graft dysfunction may be sufficient reason to decrease immunosuppressive therapy and closely monitor acute rejection. A number of strategies for decreasing immunosuppressive drugs have been reported. A number of antiviral therapeutics have been tried without much success to reduce the severity of PVAN. There is a critical need for case-controlled studies.

HSCT patients undergo thorough immunosuppressive therapy before transplantation. The frequency of BKPyV disease is higher in adult allogeneic than autologous transplant patients. Both groups have BKPyV in their peripheral circulation.²⁶ In a comprehensive study of children between 7 and 17 years of age followed for nearly 1 year, all the BMT patients who developed hemorrhagic cystitis had antibodies to BKPyV prior to transplant, as did the marrow donors. However, there was a significant increase in IgM antibodies in cystitis patients compared with those with no signs of cystitis, indicating a new or reactivated infection. There was a correlation of the immune status to BKPyV of the donor and the risk of cystitis in the recipient. Donors of HSCT patients without occurrence of cystitis had higher antibody levels compared with donors of HSCT patients who did develop hemorrhagic cystitis.²⁶ However, as in the adult patients, there was not a direct correlation between BKPyV infection and hemorrhagic cystitis.

JCPyV and Progressive Multifocal Leukoencephalopathy

Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of immune-compromised individuals caused by a lytic infection of JCPyV in oligodendrocytes, the myelin-producing cells in the white matter of the human brain. Clinically, PML patients demonstrate a triad of neurologic

symptoms including muscle weakness and gait disturbance leading to hemiparesis, cognitive abnormalities consistent with a subcortical dementia, and sensory and visual deficits.⁷⁹ PML can occur at any time during life, even in childhood,¹⁹ although it usually is found in adults with severe immunodeficiencies. Life expectancy ranges from several months to 1 year, depending on the severity of the immunocompromised state and the viral burden in the brain.¹⁸ There is a strong correlation between longer-term survival defined as greater than 12 months and the levels of CD4/CD8 cells at the time of diagnosis.¹⁷ In patients with higher levels of CD4/CD8 cells, enhancement of lesions on gadolinium magnetic resonance imaging (MRI) scans may reflect inflammation in the brain due to immune cell recognition of viral antigens (Fig. 53.11). PML lesions are most frequently present in the cerebral hemispheres including the temporal, frontal, occipital, and parietal regions but also in the posterior fossa in the cerebellum and brainstem. PML lesions are typically located at the gray–white matter junction in areas of highest cerebral blood flow, implicating virus seeding by a hematogenous route. PML plaque lesions are asymmetrical, unlike the demyelinating lesions in multiple sclerosis (MS), due to gradual virus release from lytic infection that infects new oligodendrocytes by cell–cell contact.

The characteristic features of the histopathology of PML were first described in 1958.⁹ The demyelinated areas form multifocal plaque lesions that contain enlarged, hyperchromatic oligodendrocytes, macrophages, and bizarre astrocytes. Perivascular infiltrates, usually T lymphocytes, have also been described in some PML lesions. In heavily infiltrated regions, few infected oligodendrocytes can be detected. In these lesions, MHC class I has been identified on bizarre astrocytes while MHC class II has been found on macrophages, microglia, and endothelial cells.² Intrathecal antibody to JCPyV VP1 has been detected in the cerebrospinal fluid (CSF) of PML patients.²⁷⁶ These observations suggest immune clearance of infected cells with JCPyV antigen presentation in the infected brain.

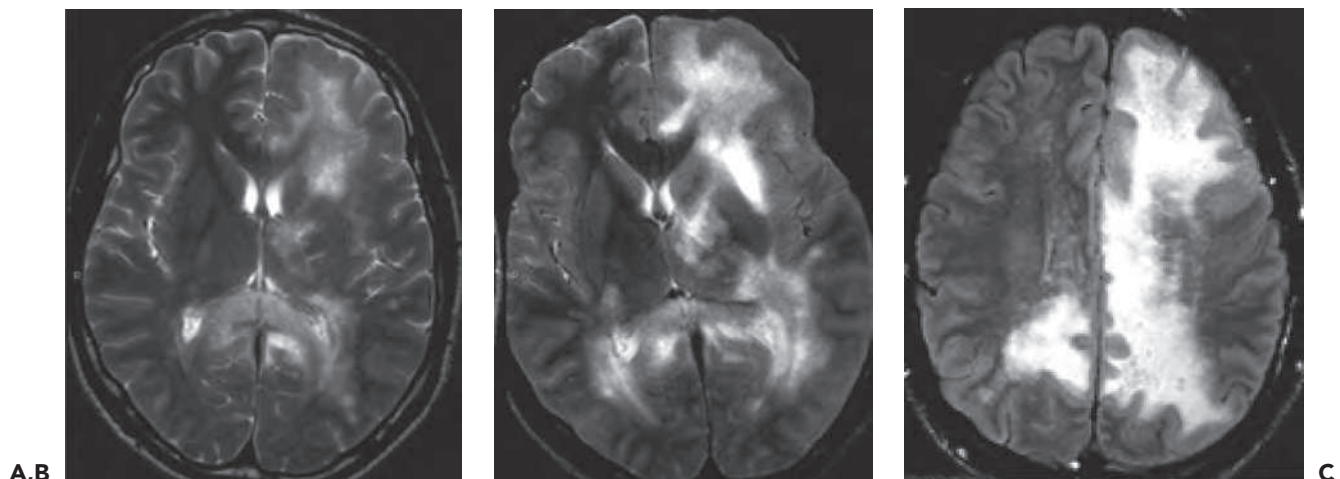


FIGURE 53.11. Magnetic resonance imaging of progressive multifocal leukoencephalopathy (PML). A, B: T2-weighted images show a progressive increase in high-signal-intensity lesions over a period of 1 month in a patient with human immunodeficiency virus 1 (HIV-1) infection. Lesions are seen in the frontal lobe, the internal capsule, and the splenium of the corpus callosum with spread to the opposite hemisphere. C: Section from the frontal lobe of the same patient shows effacement of the cortical sulci and some midline shift suggestive of inflammation due to immune reconstitution inflammatory syndrome (IRIS).

Before the HIV-1/AIDS epidemic, PML was considered a rare disease in the United States with only several hundred cases reported. Up to the mid-1980s, the most common predisposing immune disorders in patients with PML were lymphoproliferative disorders, cancer, granulomatous and inflammatory diseases (e.g., sarcoidosis), and immune-suppressive drugs used in allograft recipients. With the advent of an increased population with severe immune deficiencies, particularly HIV-1/AIDS, the incidence of PML has risen dramatically, not only in the United States but also throughout the world. PML has been recognized in an increasing number of immunocompromised patients due to systemic lupus erythematosus, autoimmune vasculitis, non-Hodgkin Lymphoma, chronic lymphocytic leukemia, and bone marrow transplantation.^{3,137} More recently, PML has become a significant adverse event in autoimmune patients treated with immune-modulatory, biological therapies.^{79,173}

The highest number of cases of PML occur in patients infected with HIV-1, reaching as high as 5% of all AIDS cases and accounting for nearly 80% of all PML cases. PML has been the presenting AIDS-defining illness in 1% to 3% of HIV-1-infected individuals. Although the use of highly active or combined antiretroviral therapy (HAART or cART) has reduced the number of opportunistic infections, PML remains a substantial neurologic complication in HIV-1/AIDS patients. With the increased incidence of PML in AIDS patients, there has been the opportunity to evaluate the potential effect of HAART. Several studies have demonstrated that HAART can lead to improvement in clinical status, radiologic features, and a clearance of JCPyV DNA in the CSF. These observations suggest that eliminating HIV-1 burden may reduce the multiplication and spread of JCPyV into the brain either by direct viral interactions or an increase in immune reactivity to JCV.

There is a spectrum of JCPyV-induced brain diseases in HIV-1 patients treated with HAART, which include PML as well as a variety of focal lesions in the brain.³⁶ A surprising number of new PML cases have been reported within weeks to months after HAART was initiated, including some patients exhibiting immune reconstitution inflammatory syndrome (IRIS).²⁵⁷ The mechanism that underlies this observation is not known but may be associated with influx of latently infected immune cells into the brain upon immune reconstitution.²³⁹ It should be noted that BKPyV, WUPyV, and KIPyV have been detected in brain tissue of patients with HIV-1/AIDS, but it is unlikely that they contribute to the development of PML.¹²

Progression of neurologic symptoms including cognitive decline and motor dysfunctions with neuroradiologic evidence of white matter lesions is necessary but not sufficient to make a diagnosis of PML. Because white matter lesions identified in patients with AIDS are caused by HIV-1 and not by JCV-induced demyelination, evidence for JCPyV is critical. JCPyV viral DNA or protein must be demonstrated in the patient to make a definitive diagnosis. The mechanisms that account for the high incidence of PML in patients with HIV-1/AIDS are unknown. Whether a specific immune control of JCPyV latency that is deficient exists in patients with AIDS or a direct interaction occurs between the two viruses remains an open question.

Natalizumab, efalizumab, and rituximab are therapeutic monoclonal antibodies that can induce specific immune defects that can predispose the patient to PML.³³ Natalizumab is a humanized monoclonal antibody to the cellular adhesion α -4

(ITGA4, CD49D) integrin molecule. ITGA4 binds with β -1 (ITGB1, CD29) or β -7 (ITGB7) to form the very late antigen-4 (VLA-4) receptor. The antibody prevents T cells from binding to the VLA-4 receptors on endothelial cells and blocks extravasation into tissues such as the brain and colon. Natalizumab was approved by the U.S. Food and Drug Administration for relapsing, remitting multiple sclerosis and Crohn disease, an inflammatory bowel disorder; both are considered autoimmune-mediated diseases. During a phase III global trial, two patients with multiple sclerosis and one patient with Crohn disease developed PML.^{16,155} These patients had JCPyV detectable by PCR in the brain and peripheral blood. Prior use of immunosuppressive therapy with mitoxantrone, methotrexate, and azathioprine increases the risk of developing PML when treated with natalizumab.¹³⁴ Notably, prolonged treatment of multiple sclerosis with natalizumab can cause specific reactivation of JCPyV with increased frequency of detection in the urine.⁴²

While JCPyV isolated from kidney and urine contains the archetype NCCR without repeat elements, viruses obtained from PML brain tissue and blood from the same patient often contain direct tandem repeats. Interestingly, no two PML patients show identical nucleotide sequence arrangement, but each patient shows consistent nucleotide sequences.²¹⁴ There is also evidence for variations in the gene that codes for the VP1 capsid protein, with the suggestion that such alterations may lead to a more neurovirulent form of JCPyV.⁹⁷ In HIV-1/AIDS patients with PML, variations occur in the VP1 coding sequences but not the T antigen sequences that remain highly conserved.⁹⁴ Similar results were observed in natalizumab-treated patients.²¹⁴

Many laboratories and clinical centers have adopted PCR detection of JCPyV DNA in CSF samples in place of biopsy as the laboratory tool for confirmation of PML. Several recent studies have further shown that quantitative measurement of JCPyV DNA can also be used as a prognostic indicator of the progression of disease. Reduced viral genome copy number predicts long-term survival of PML patients. Viral DNA can be detected in longitudinal CSF samples months to even years after PML diagnosis, indicating that JCPyV can persist in the brain causing continued neurologic deficits regardless of immune responses.²¹⁸

There has been little progress in the treatment of JCPyV diseases. Antiviral treatment of PML patients has typically used nucleoside analogs in attempts to block JCPyV DNA replication. Cytarabine (cytosine arabinoside, ARA-C) has been used most frequently but without consistent results. Several reports had described remission of PML symptoms and brain lesions upon intrathecal administration of ARA-C. However, other patients treated with combined intravenous and intrathecal ARA-C did not show neurologic improvement, although in some cases there was a reduction of viral DNA in the CSF. Several other drugs have been used in patients with PML, including cidofovir. Recently, an esterified analog of cidofovir, 1-0-hexadecyloxypropyl (CMX001), has shown efficacy in reducing JCPyV replication in a human brain-derived cell line, SVG, and in primary human astrocytes at concentrations that were not toxic to the host cells.^{99,127} However, clinical use of CMX in PML patients has gut and hepatic toxicity, although it does reduce viral load in the CSF.

Reactivation of SV40 in monkeys with simian/human immunodeficiency virus (SHIV)-immunosuppressed rhesus monkeys can result in a PML-like disease. Monkeys that were

seropositive for SV40 prior to SHIV inoculation as well as a monkey that seroconverted for SV40 after inoculation with SHIV SV40 disease developed a PML-like disease.¹¹⁵ Infection with SV40 can also cause meningoencephalitis in SHIV rhesus monkeys.⁵⁵

Merkel Cell Polyomavirus and Merkel Cell Carcinoma

Merkel cell carcinoma (MCC) is a relatively rare skin cancer. Merkel cell carcinoma typically presents on sun-exposed areas of elderly patients. Intense and lifelong UV exposure from the sun seems to be a particular risk for Caucasian patients especially from Australia. Merkel cell carcinoma can occur in non-sun-exposed areas in young adults as well. Risk for developing Merkel cell carcinoma is increased in patients with severe immunocompromise including HIV-1/AIDS, solid organ transplantation, cancer, and chemotherapy. In particular, there is an increased risk for developing Merkel cell carcinoma in patients with chronic lymphocytic leukemia and vice versa.

The increased risk for developing MCC in HIV-1/AIDS patients prompted an intense search for a pathogen etiology. High-throughput sequencing of transcripts from four MCC specimens led to the identification of an open reading frame with weak homology to polyomavirus large T antigen. Complete sequencing of the viral genome revealed that it contained typical features of a polyomavirus with a large and small T antigen VP1, VP2, and VP3 and an NCCR containing the origin of replication.⁷⁸ In the original report, Southern blot detection of MCPyV DNA revealed random integration of viral DNA into the tumor host cellular DNA in 8 of 10 unique MCC tumor specimens. Significantly, a metastatic lesion had the same restriction fragment length pattern as the primary tumor, indicating

clonal integration of the polyomavirus DNA into the tumor cell DNA prior to metastasis. In addition, the integrated viral DNA incurred mutations in the large T antigen coding region that led to truncations of the large T antigen that retained the N-terminal J domain and LXCXE Rb-binding motif but eliminated the DNA-binding and helicase domains (Fig. 53.12A). The small T antigen sequence remained intact.

Since the original report, several labs have confirmed the presence of MCPyV in most if not all Merkel cell carcinomas studied.^{14,135,215a} Furthermore, expression of the truncated large T antigen and small T antigen is noted in a similar percentage of tumors.^{236,238} Frequently, the copy number of viral genomes can vary from as low as a single copy to several hundred copies or more when comparing one tumor to another. Furthermore, the expression levels of large T antigen vary from barely detectable to intense staining by immunohistochemistry (Fig. 53.12B). A few studies have reported that the presence of MCPyV in the Merkel cell carcinomas predicts a better prognosis. Of note, a recent expression profiling study noted that infiltration of the tumor with activated CD8 T lymphocytes predicted a better prognosis.²⁰⁴

A neutralization assay based on a pseudovirion generated by expression of the MCPyV VP1 and VP2 molecules that encapsulate a reporter plasmid capable of expressing luciferase²⁰² found that patients with Merkel cell carcinoma tended to have higher titers of serum antibodies against MCPyV than healthy adults. This suggests the possibility that Merkel cell carcinoma may have developed from a period of high MCPyV production. Serologies corresponding to prior exposure to MCPyV have been determined for healthy children and adults and for patients with Merkel cell carcinoma. Patients with Merkel cell carcinoma had the highest titers.²⁶³

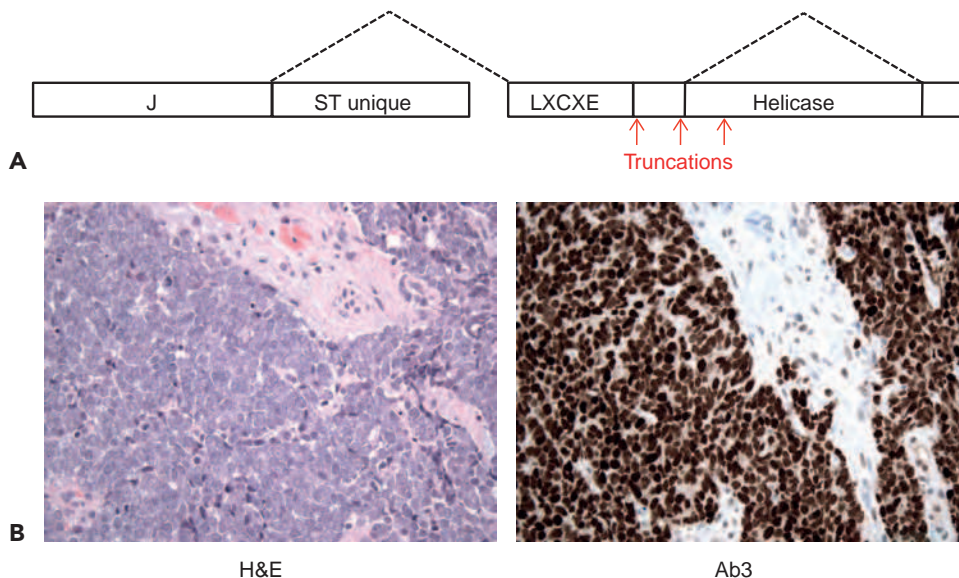


FIGURE 53.12. Merkel cell carcinoma. A: The Merkel cell polyomavirus (MCPyV) viral DNA early region containing large and small T antigen is clonally integrated into the tumor cell DNA. Small T antigen remains intact while large T antigen undergoes mutations resulting in truncations that occur after the LXCXE RB1-binding motif. The alternative spliced form (57 kD) of large T antigen is shown. **B:** Merkel cell carcinoma with hematoxylin and eosin (H&E) (left panel) and immunohistochemistry stain (right) with a monoclonal antibody (Ab3) specific for MCPyV large T antigen showing expression in tumor cells. (Photomicrographs courtesy of Scott Rodig, Harvard Medical School, Boston, MA.)

Antibodies specific for MCPyV large and small T antigens were evaluated in patients with Merkel cell carcinoma and healthy controls. Antibodies specific for the T antigens were present in less than 1% of more than 500 control subjects, while 40% of 205 patients with Merkel cell carcinoma had antibodies specific for the shared residues in the N-terminus of large and small T antigen. Notably, antibody titers against the T antigens decreased in patients who remained disease free after treatment and rose in titers for patients with recurrent disease.²⁰³

Several Merkel cell carcinoma cell lines containing integrated copies of the MCPyV genome have been established. Expression of the small and large T antigen is required for the continued growth of these cell lines as RNAi depletion of either large or small T antigen caused growth arrest.^{116,238} Typical for all polyomaviruses, the MCPyV large T antigen contains an LXCXE Rb-binding motif that is required for binding to pRb. Expression of large T antigen mutant in the LXCXE motif disrupts binding to pRb and is no longer able to promote cell growth. As noted earlier, MCPyV small T antigen is capable of promoting phosphorylation of 4E-BP1 and increasing cap-dependent translation. This activity appears to be critical for MCC cell growth.

Treatment for localized Merkel cell carcinoma includes surgical excision and sentinel lymph node biopsy.¹⁶² Adjuvant radiation therapy may be used for larger lesions. Merkel cell carcinoma tends to be sensitive to radiation therapy and certain forms of chemotherapy including cisplatin and etoposide. However, after the initial responses to radiation or chemotherapy therapy, Merkel cell carcinoma tends to recur and become resistant to further treatment.

Trichodysplasia Spinulosa Polyomavirus Infections

Trichodysplasia spinulosa is an exceedingly rare skin disease observed in immunocompromised patients. The first case of trichodysplasia spinulosa was reported in 1999.¹¹⁰ Since then, less than 25 cases have been reported.¹⁷⁷ Trichodysplasia spinu-

losa occurs in adult and pediatric patients after solid organ transplantation on immunosuppressive treatment or with hematologic malignancy and chemotherapy.

The trichodysplasia spinulosa syndrome typically presents with alopecia especially of the eyebrows but can extend over the entire body. The hair follicles themselves can be inflamed. Instead of hair, fragile white spinous processes extend from the hair follicles (Fig. 53.13). Histology reveals changes within the hair follicle including plugging with keratotic debris and dystrophic inner root sheath cells that contain hyaline or glassy appearing granules. The hyaline material gives rise to the spinous processes.

The trichodysplasia spinulosa polyomavirus (TSPyV) was cloned by using rolling circle amplification of the spinous processes obtained from a newly described pediatric patient. TSPyV is a unique human polyomavirus most closely related to OraPyV1 and MCPyV.²⁶⁸ TSPyV DNA was detected by PCR from the skin of three kidney transplant patients. Importantly, the amount of TSPyV genomes detected was significantly higher in lesions (10^6 copies/cell) than in the occasionally positive samples from normal skin (less than 10^2 copies/cell).¹³⁸ Furthermore, detection of TSPyV VP1 protein was only observed in lesions. Electron micrograph imaging of hair follicles revealed small viral particles less than 45 nm suggestive of a polyomavirus rather than the larger papillomavirus particle size of 50 to 55 nm. The virus particles have been observed within the inner root sheath cells in the extracellular debris.¹⁷⁷

The frequency of TSPyV infection is probably similar to most other human polyomaviruses as determined by serology testing of more than 500 healthy subjects. The presence of IgG antibodies specific to TSPyV VP1 range from 10% in infants to 80% in adults,²⁶⁹ indicating the increasing incidence of a lifelong persistent infection. Notably, in 80 kidney transplant patients, 89% were serologically positive for TSPyV.

WUPyV and KIPyV and Respiratory Illness

WUPyV was originally discovered by high-throughput sequencing of respiratory secretions of patients with symptoms of acute

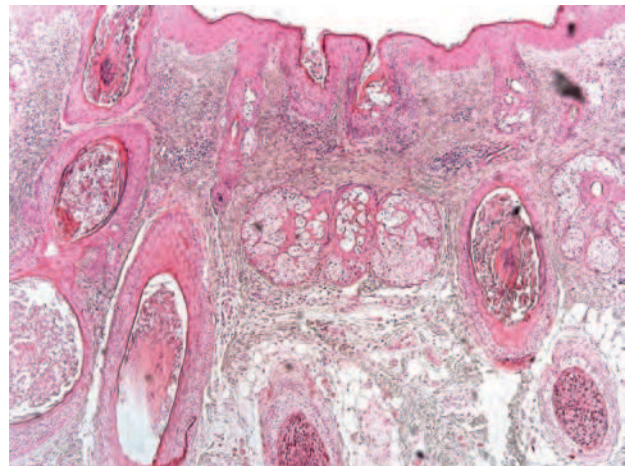


FIGURE 53.13. Trichodysplasia spinulosa. Affected nose with papules and spicules (**left panel**). Biopsy of a hyperkeratotic follicular papule from the forehead. The epidermis reveals enlarged, hyperplastic hair bulbs and hypercornification within a distended follicular infundibulum (hematoxylin and eosin [H&E] stain, 10×) (**right panel**). (Reproduced from van der Meijden E, Janssens RW, Lauber C, et al. Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromised patient. *PLoS Pathog* 2010;6[7]:e1001024, under Creative Commons Attribution License.)

respiratory illness.⁹⁰ The initial survey found evidence for WUPyV DNA in 2% to 3% of more than 4,000 patients with acute respiratory tract infections.¹⁵⁶ However, these patients typically were infected with additional viruses including adenovirus and respiratory syncytial virus well known to cause respiratory illness.^{114,196} The detection of WUPyV and KIPyV in respiratory secretions from patients with respiratory illness was higher in patients immunocompromised by stem cell transplant.^{8,189} WUPyV and KIPyV were also more frequently detected in lymphoid tissue in autopsy specimens from patients with HIV-1/AIDS compared to controls without immunodeficiency.²³³ To date, it does not appear that infection with WUPyV or KIPyV leads to significant respiratory illness.

SV40 and Humans

From the mid-1950s to 1963, the simian polyomavirus SV40 was unknowingly introduced into 100 million people that had received contaminated poliovirus vaccines.²³⁰ Because SV40 is tumorigenic in rodents and can transform human cells in culture, there has been concern about whether SV40 can contribute to cancer development in humans vaccinated with the contaminated polio vaccine. There has also been controversy regarding whether SV40 can replicate and establish a productive infection in humans.

Follow-up studies from the initial polio vaccination efforts have concluded that there has not been an increase in neoplasias over three decades in the population that was directly exposed to SV40 compared with matched age, gender, and geographical control populations not exposed to SV40.²³¹ A retrospective cohort study of data collected by programs designed to track tumor incidence in specific populations did not find any association between SV40 exposure and increased risk.²⁵⁰ The FDA Office of Vaccine Research and Review convened a meeting of international authorities to discuss the topic of SV40 in the human population and the technical approaches needed to evaluate its presence as an infectious agent and role, if any, in human tumors.²⁴¹ The Institute of Medicine also published a study that concluded that the epidemiologic evidence regarding SV40 infections in humans is inconclusive and recommended additional research.²⁴⁹ There have been sporadic reports that SV40 DNA and large T antigen protein can be identified in human tumors such as ependymomas, osteosarcomas, mesotheliomas, and choroid plexus papillomas.^{20,32,58,158} However, unlike the frequent presence of integrated MCPyV viral genome in Merkel cell carcinoma, there have been no reports of integrated SV40 viral genomes in human tumors.

It is not certain if SV40 can establish a persistent infection in humans. While SV40 can replicate at very low levels in human fibroblasts and mesothelial cells in culture, it does not appear to be capable of replicating in lymphocytes.^{22,232} Notably, while BKPyV and JCPyV are frequently detected in human sewage, the presence of SV40 has not been reported.²⁴ In addition, while some human sera are reactive against SV40 VP1, suggesting a sustained infection, most of this activity can be depleted by preincubation with BKPyV or JCPyV VP1.^{139,216}

Association of BKPyV and JCPyV with Human Tumors

Reports have identified BKPyV DNA in primary brain tumors, osteogenic sarcomas, colorectal carcinomas, urinary tract neoplasms, and meningiomas. However, BKPyV DNA has also

been described in normal brain, bone, peripheral blood, and gastrointestinal and urinary tracts, so it is difficult to draw conclusions on the importance of its presence in tumor tissues. BKPyV has also been detected in preneoplastic prostatic tissues.⁵⁷ It is not clear if BKPyV contributes to development of the preneoplastic state or whether these cells represent an idealized host cell that supports BKPyV replication.

Because experimental infection with JCPyV induces glial tumors in nonhuman primates, the major focus of interest in the oncogenic potential for JCPyV in humans has been the brain. There have been several reports of PML patients with multiple astrocytomas or oligodendroglioma, along with evidence of anaplastic astrocytes.⁸⁵ However, this occurrence is not common in PML, nor has there been evidence of JCPyV in these tumors or in bizarre or anaplastic astrocytes. A study examined 225 human brain tumors, predominantly gliomas, for nucleotide sequences for JCPyV, BKPyV, and SV40. All specimens were evaluated in a blinded manner by two independent laboratories. Although one lab detected polyomavirus in nine tumors, the other lab could not confirm these results. No tumor had polyomavirus DNA identified by both laboratories.²¹⁷

In addition to brain tumors, the presence of JCPyV DNA was found in high copy number in colorectal tumor tissue compared with normal, noninvolved colon tissue from the same patient.¹⁵² Similar to the presence of BKPyV in preneoplastic prostate lesions, the presence of JCPyV in colon carcinoma cells may represent a passenger and not a driver during oncogenesis.

Avian Polyomavirus and Disease

Hemorrhagic nephritis and enteritis of geese develops rapidly after infection with GHPyV. Typical symptoms of infected birds include generalized edema, gout, hemorrhagic enteritis, interstitial nephritis of the kidney tubular epithelium, and disruption of lung, feather follicle, and endothelial cells. Lymphoid tissues can also be affected with depletion of most lymphocytes. These features have been observed in naturally and experimentally infected birds.^{151,201} In addition to GHPyV, infection with APyV, FPyV, and CPyV are associated with fatal disease often including hepatitis, ascites, enteritis, and nephritis.¹³¹

PERSPECTIVE

Since Ludwig Gross first described the parotid agent or MPyV in 1953, the polyomaviruses have proven to be a rich model system for understanding fundamental biological processes in eukaryotic cells. In addition to the insights that have contributed to our understanding of transcription factors and enhancers, RNA splicing and polyadenylation, and DNA replication, the study of polyomaviruses has enriched our understanding of tumor suppressors, oncogenes, tyrosine kinase signaling, immunity, and tumorigenesis. The intensive study of polyomavirus cell receptors, entry, and trafficking through the cell has brought remarkable insights that have challenged previously held models. Importantly, the information gained by study of polyomaviruses has provided critical insights of human disease. As the population of immunosuppressed individuals increases due to HIV-1/AIDS and developments in transplant medicine, the incidence of clinical polyomavirus disease continues to climb. A clear example of this has been the occurrence of

JCPyV-induced PML in patients with multiple sclerosis or Crohn disease who were treated with an antibody that blocked T lymphocytes from binding the VLA4 receptor through the $\alpha 4$ integrin molecule.

Continued investigation into the interaction between polyomaviruses and their hosts will be crucial in framing our ability to intervene with patients in the future. Given new technology and model systems, it should be possible to develop high-throughput screens to identify more effective compounds that inhibit the viral life cycle. It is imperative that evidence-based clinical studies, guided by impeccable cellular- and molecular-based studies, are implemented to benefit immunocompromised patients suffering with polyomavirus-related diseases.

The contribution of MCPyV to Merkel cell carcinoma deserves special mention. To date, all studies indicate that expression of the MCPyV small T antigen and a truncated form of large T antigen is present in at least 80% of all Merkel cell carcinomas. Consistent with the large body of literature on polyomavirus-mediated transformation, MCPyV is clonally integrated into the Merkel cell carcinoma cells in a manner that enables persistent expression of the T antigens. There are many questions that remain unanswered regarding the role of MCPyV T antigens in Merkel cell carcinoma. In addition, it is not understood what the contributions of age, UV exposure, and immunosuppression are to the risk for developing this cancer. Furthermore, it is not known what is the normal host cell or tissue type for MCPyV and what is the special relationship with Merkel cells. Also, the factors in the host and virus that sustain lifelong infection but permit reactivation of polyomavirus infection remain to be defined not only for MCPyV but also for all polyomaviruses.

The recent discoveries of new polyomaviruses in humans, primates, marsupials, and birds have brought renewed vigor to the field. It is likely that more polyomaviruses will be discovered. More importantly, it can be expected that investigation of the new and previously discovered polyomaviruses will continue to bring important new and fundamental insights into biology and disease. It can certainly be concluded that the polyomaviruses have paid huge dividends in the amount of knowledge they have yielded. Consequently, the future seems to hold great promise that study of polyomaviruses will continue to help us demystify fundamental cellular processes and give direct benefits to patients whose active infection becomes life threatening.

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Papillomaviruses

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The papillomaviruses (PVs) comprise a group of nonenveloped epitheliotropic DNA viruses that induce benign lesions of the skin (warts) and mucous membranes (condylomas). Some PVs have also been implicated in the development of epithelial malignancies, especially cancer of the uterine cervix, other tumors of the urogenital tract, and upper airway cancers. The recognition that PVs are an important cause of human cancer has led to the development of a preventive virus-like particle (VLP)-based vaccine targeted to the human papillomavirus (HPV) types most often found in the cancers. This chapter focuses primarily on the HPVs.

HISTORY

Warts were known to the ancient Greeks and Romans. Their infectious nature was recognized, but until the nineteenth century, genital warts were usually considered to be a form of syphilis or gonorrhea. The viral nature of human warts was demonstrated in the early 1900s when cell-free filtrates from lesions were shown to transmit the disease.¹¹⁰ PVs were subsequently identified in a variety of vertebrate species in addition to humans.^{346,592} Because PVs are species-specific in their host range, it has not been possible to study the biology of HPVs in animals.

The first animal PV was identified in the 1930s by Richard Shope, who characterized the transmissible nature of cutaneous papillomas arising in wild cottontail rabbits.⁵⁵³ The Shope papillomavirus, now designated the cottontail rabbit papillomavirus (CRPV), was the first DNA tumor virus identified. Shope's research also showed that although systemic injection with papilloma suspensions did not produce detectable infection, it could induce serum neutralizing antibodies and protect rabbits against high-dose cutaneous viral challenge.⁵⁵² These findings laid the groundwork for believing that a preventive vaccine against a PV could be based on the induction of humoral immunity. In addition to causing benign papillomas, some warts induced by CRPV were observed to undergo malignant progression,^{513,599} and for the next two decades, CRPV was an important model for the fundamental study of viral tumorigenesis.^{328,598} However, its use as a model tumor virus was largely supplanted by the discovery in the late 1950s of the polyomaviruses, which could replicate in cultured cells and induce morphologic transformation *in vitro*, in contrast to CRPV, and were tumorigenic for experimental animals.⁴¹⁷

In addition to CRPV, the rabbit oral papillomavirus (ROPV) was also identified in the 1930s as a distinct virus of domestic rabbits.⁴⁵⁹ ROPV infected the oral mucosa of rabbits but not their skin, whereas CRPV had the opposite host range, and neither virus was infectious for heterologous hosts.

Furthermore, ROPV was not oncogenic, in contrast to CRPV, and neutralizing antibodies against one virus were shown not to be neutralizing for the other. The research on CRPV and ROPV thus established the multiplicity of PVs, the narrow host range of PVs to sites with stratified squamous epithelia that were cornified (skin) or noncornified (mucosa), and the notion that protection against one PV may not confer protection against another PV.

Although the PVs were studied less intensively in the 1950s and 1960s, that period was associated with some important advances, including the physicochemical analysis of PV virions and the demonstration that PV replication was associated with the differentiation process of the infected epithelium.^{379,515} However, it was the advent of molecular cloning in the 1970s that initiated more extensive studies of PVs. This technical advance enabled investigators to partially circumvent the inability to culture PVs, as the cloning of PV genomes greatly enhanced the ability to study their biologic and biochemical properties. The sequencing of the cloned PV genomes led to the identification of open reading frames (ORFs) as putative viral genes and permitted investigators to determine the function of viral genes by reverse genetics, resulting in a much wider interest in PV research.^{97,125,126} The bovine papillomavirus type 1 (BPV1) represented the standard PV for initiating these studies because the virus induced focal transformation of established rodent cell lines.^{45,163} The molecular cloning of the HPV genomes also led to the recognition that there were multiple HPV genotypes, and that a subset of these types was closely associated with human cancers, including cervical cancer.^{56,162,446} The appreciation of their medical importance, combined with improved tools for analyzing PVs, further enhanced the utility of PVs as a model of viral tumorigenesis. Although the study of animal PVs continues to bring new information to the field, the medical importance of HPVs has shifted emphasis toward the analysis of HPV, especially when it was established that the biochemical properties of some nonstructural viral proteins differed from those of their BPV-1 counterparts.^{165,525}

CLASSIFICATION

Historically, the PVs were classified together with the polyomaviruses as a single family, the *Papovaviridae*. This grouping arose because although PV genomes and virions are larger than those of polyomaviruses, the viruses share many features, including a double-stranded circular DNA genome, an icosahedral capsid composed of 72 pentamers, a nonenveloped virion, and the nucleus as the site of viral replication and virion assembly. However, sequencing of PV genomes indicated that although PVs share a common genetic organization, they differ from that of polyomaviruses and have no major sequence homology to polyomaviruses, and PV transcription is unidirectional, in contrast to the bidirectional transcription of polyomaviruses. Recognition of these differences, and others, have led to PVs being designated as a distinct family, the *Papillomaviridae*, by the International Committee on the Taxonomy of Viruses (ICTV) in 2000.⁶²⁸

PVs have been isolated from many mammalian host species, birds, and reptiles, but thus far have not been identified in nonvertebrates. There are hundreds of PV types (as defined in

the next paragraph). PVs are species-specific and many different PV types can infect a given host species. HPVs have been analyzed most intensively; by 2010, 120 different HPV types had been identified, and there are likely to be more.³⁹

PVs are classified primarily according to the host species they infect and have been traditionally referred to as types based on their DNA sequence. A distinct type is one whose L1 DNA sequence is at least 10% different from that of other HPV types. The PV genomes have been organized phylogenetically based on their DNA sequence (Fig. 54.1; 44), according to the comparative homology of the L1 ORF. Similar phylogenetic relationships are also seen when homologies between other regions of the genome are compared, as PVs appear to have arisen primarily via point mutations scattered throughout the genome, rather than via recombination between PVs.⁹¹ These similarities are consistent with the conclusion that PVs have accompanied their host species during evolution and have evolved with them.⁴⁰ Although all PVs share a similar genetic organization, the L1 DNA sequence identity is just over 40% between the most divergent genomes. On the other hand, two very closely related isolates may differ by only a single nucleotide. The current classification attempts to provide logical designations to cover this wide range of homology: genus, species, type, subtype, and variant. The broadest category is a *genus*. PVs are divided into 29 genera, each of which is designated by a letter of the Greek alphabet (Fig. 54.1). Within a given genus, the L1 DNAs of all members share more than 60% identity; conversely, they have less than 60% identity with members of other genera. A *species* is designated for those PVs within a given genus that share 60% to 70% identity. A viral *type* within a species has 71% to 89% identity with other types within the species. Within a type, there can be *subtypes*, which share 90% to 98% identity, and *variants*, which have more than 98% identity. Although there are relatively few subtypes,⁴⁰ many variants have been identified for HPV-16, the type that has been examined in greatest detail because of its medical importance.¹⁰⁰

Using this classification, HPVs are clustered among 5 of the genera: alpha, beta, gamma, mu, and nu, with the other 24 genera being occupied exclusively by animal PVs.³⁹ The host species associated with each PV genus tend to be closely related evolutionarily. Therefore, PVs that infect nonhuman primates are found within the genera that include HPVs, and some HPVs are more closely related to nonhuman primate viruses than to some of the other HPVs in the genus. The HPVs of greatest medical importance, that is, those that are associated with genital and mucosal cancers, are members of the alpha genus. Most alpha PVs primarily infect genital and nongenital mucosal surfaces and the external genitalia. This group of PVs is often referred to collectively as the “genital-mucosal” types. The types that are associated with cervical cancer, often designated as “high-risk” types, are found in species 5, 6, 7, 9, and 11.^{423,529} HPV16, the type found most frequently in cervical cancer, is a member of species 9, whereas the next most common cancer-associated type, HPV18, is a member of species 7. HPV-6, which causes most cutaneous genital warts, is a species 10 member.

In contrast to most species of the alpha genus, members of alpha species 4 (HPV2, -HPV27, and HPV57) are primarily infectious for nongenital skin. The beta, gamma, mu, and nu viruses also infect nongenital skin. The beta HPVs

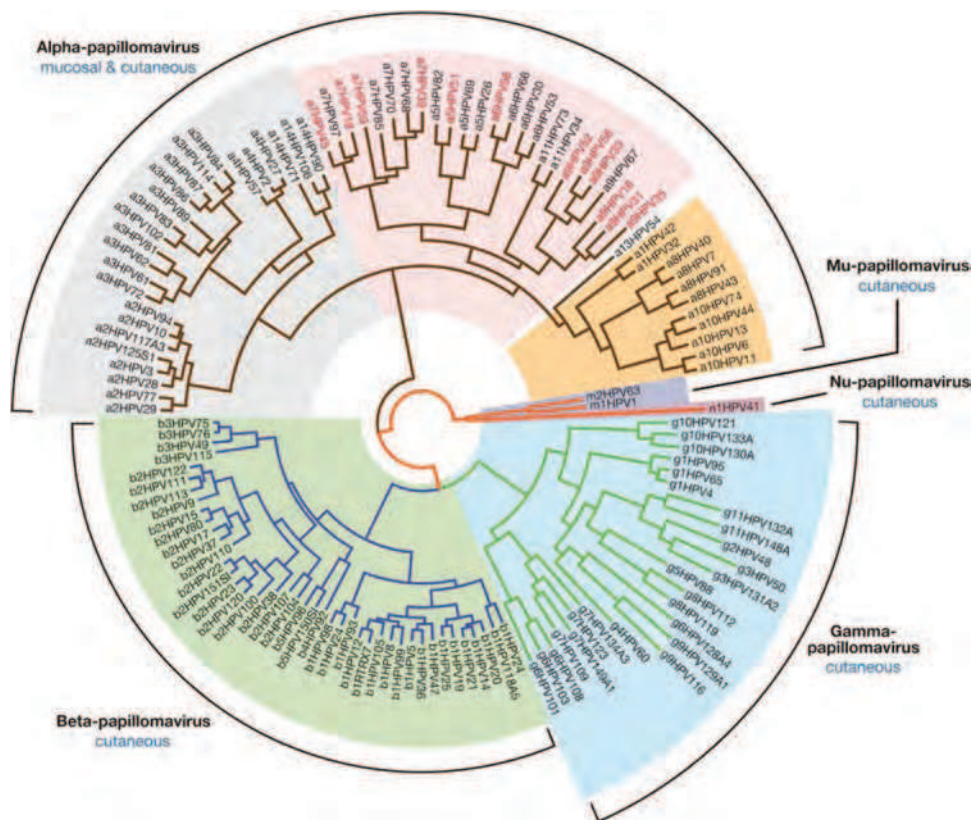


FIGURE 54.1. Phylogenetic tree demonstrating the evolutionary relationship among human papillomaviruses (HPVs).

HPVs comprise five groups with different epithelial tropisms and disease associations. The alpha papillomaviruses include the low-risk mucosal types (many of which are within the orange-shaded branch) that cause genital warts, and the high-risk mucosal types (contained within the branch highlighted with pink shading) associated with anogenital preneoplasias and cancers. Although the cutaneous HPV types—most of which are contained within the gray (alpha), green (beta), and blue (gamma) shaded branches—are not generally associated with cancers, certain beta types have been implicated in the development of nonmelanoma skin cancers (NMSC) in immunosuppressed individuals and in epidermodysplasia verruciformis (EV) patients. The lower case letter and number preceding the HPV type refer to its genus and species. (Generated by John Doorbar; reprinted from Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses. *Vaccine* 2012;30(Suppl 5):F55–F70, with permission.)

include those that are often designated epidermodysplasia verruciformis (EV) specific, because they cause lesions mainly in patients with EV, a genetic susceptibility to widespread non-genital HPV lesions. Some PVs, including many members of the beta and gamma species, may behave as commensal agents, as they are frequently isolated from normal skin or plucked hair from humans and animals.^{12,59}

The PVs in the delta genus, which include BPV1 and some other PVs of ungulates, cause fibropapillomas, rather than papillomas. This distinct pathology results from a proliferative dermal fibroblastic component under the epithelial portion of the lesion, because members of this genus induce nonproductive transformation of the fibroblasts, in addition to the productive infection of the overlying epithelium. The ability to transform nonepithelial cells is not species-specific. It can lead to the induction of nonproductive fibroblastic tumors in heterologous hosts under natural conditions, as in equine sarcoid of horses (from BPV1 or BPV2), or experimental hosts, such as hamsters. It also endows viruses such as BPV1 and BPV2 with the ability to induce focal transformation of cultured rodent cells.

VIRION STRUCTURE

Papillomaviruses are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. The PV particles are approximately 60 nm in diameter (Fig. 54.2). The virion particles consist of a single molecule of double-stranded circular DNA about 8,000 base pairs (bp) in size, contained within a spherical protein coat, or capsid, and composed two viral proteins L1 and L2. The DNA constitutes approximately 12% of the virion by weight, accounting for their density in cesium chloride of 1.34 g/mL.¹¹⁶

Fine structural analysis by cryoelectron microscopy (cryo-EM) and three-dimensional image reconstruction techniques has revealed that the viruses consist of 72 pentameric capsomers arranged on a $T = 7$ surface lattice.^{22,621} The capsomers comprise five L1 molecules with an L2 molecule occupying the axial lumen.⁷³ As with the polyomavirus capsids, the capsomers exist in two environments, one capable of making contact with six neighbors as observed in the 60 hexavalent capsomers and the other with five neighbors in the 12 pentavalent vertex capsomers (Fig. 54.3). Analysis of proteins in the virus particle

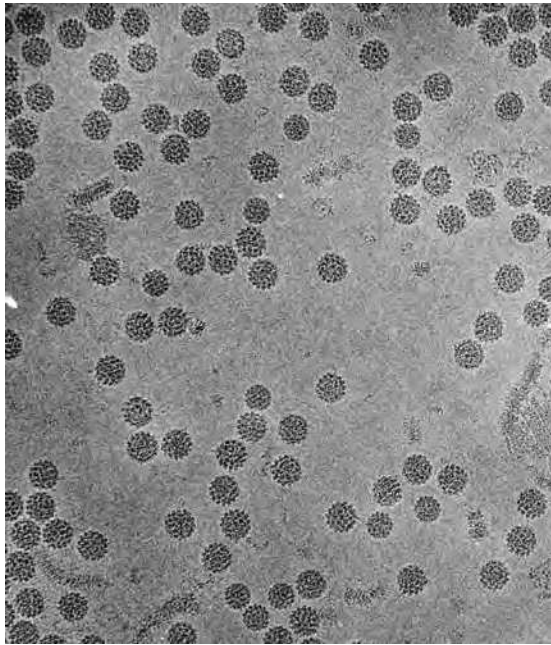


FIGURE 54.2. Electron micrograph of bovine papillomavirus 1 (BPV1) virion particles (55 nm in diameter). (Reprinted from Baker TS, Newcomb WW, Olson NH, et al. Structures of bovine and human papillomaviruses—analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys J* 1991;60:1445–1456, with permission.)

showed that the viral DNA is associated with cellular histones to form a chromatin-like complex.^{172,469}

VLPs can be produced from different PVs by expressing L1 alone using mammalian or nonmammalian expression systems.^{234,311,511} The morphology of VLPs containing only L1 appears identical to intact virus particles in low-resolution cryo-EM reconstructions.²³³ The structure of a truncated T = 1 HPV16 L1 VLP containing 12 pentamers has been solved by x-ray crystallography to 3.5 Å resolution.⁹⁹ The structure of

full-size BPV1 virions was recently solved to 3.6 Å resolution using cryo-EM.⁶⁶⁸

GENOME STRUCTURE AND ORGANIZATION

The genomes of many of the human and animal papillomaviruses have been sequenced in their entirety, and the genomic organization of each of the papillomaviruses is similar. One characteristic of the genomic organization of all papillomaviruses is that all of the ORFs are located on one strand of the viral DNA, thus indicating that all of the viral genes are located on one strand. Transcriptional studies indicate that only one strand serves as a template for transcription.

The coding strand contains approximately 10 designated translational ORFs that are classified as either early (E) or late (L) ORFs, based on their location in the genome. The early region of the papillomavirus genomes encodes viral regulatory proteins including those viral proteins that are necessary for initiating viral DNA replication. The L1 and L2 ORFs encode the viral capsid proteins and are expressed only in productively infected cells.²¹ The position, size, and function of many of the ORFs are well conserved among the various PVs that have been sequenced and studied in detail thus far. The functions of the individual ORFs, the functions of which have been well characterized, are described in more detail in the appropriate sections of this chapter.

There is a region, of approximately one kilobase, in each of the papillomavirus genomes that contains no ORFs. This region has been referred to by several terms including the long control region (LCR), the upstream regulatory region (the URR), and the noncoding region. This region contains the origin of DNA replication as well as important transcription control elements. The genomic organization of HPV16 is shown in Figure 54.4.

VIRUS REPLICATION

The PVs are highly species-specific and have a specific tropism for squamous epithelial cells. The productive infection of cells

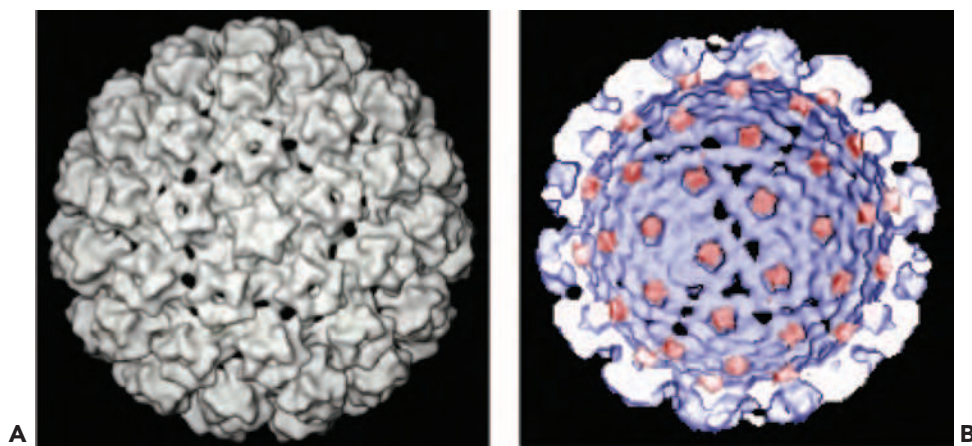


FIGURE 54.3. **A:** Three-dimensional (3D) reconstruction of a BPV virion viewed down a fivefold axis.⁶²¹ **B:** 3D reconstruction of an interior/cutaway view of an human papillomavirus (HPV) L1/L2 virus-like particle (VLP) with the L2 specific density shown in red. (Adapted from Buck CB, Cheng N, Thompson CD, et al. Arrangement of L2 within the papillomavirus capsid. *J Virol* 2008;82:5190–5197.)

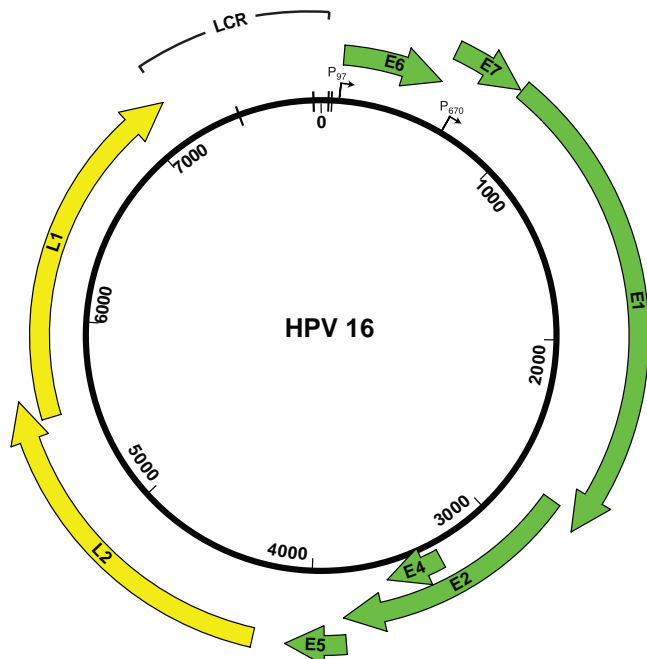


FIGURE 54.4. HPV16 genomic map. The numbers inside the circle indicate the nucleotide positions. The individual open-reading frames (ORFs) of the early (E) and late (L) regions are depicted as areas outside the double-stranded circular genome. Only one strand is transcribed, and transcription occurs in the clockwise direction. The early promoter (P_{97}) is indicated by an arrow at the approximate nucleotide position of the RNA initiation site. P_{670} is the late promoter whose initiation sites map within the E7 ORF. The long control region (LCR) designates the long control region that contains the origin of DNA replication. The hatch marks indicate the four E2 binding sites within the LCR.

by the PVs can be divided into early and late stages. These stages are linked to the differentiation state of the epithelial cell. The specific tropism of the PVs for squamous epithelial cells is evidenced by the restriction of the viral replication functions, such as vegetative viral DNA synthesis, the production of viral capsid proteins, and the assembly of virions to differentiated epithelial cells. The close link of the papillomavirus life cycle with the differentiation program of the squamous epithelium is depicted in Figure 54.5.

The basal cell is the only cell in the squamous epithelium capable of undergoing cell division. Therefore, the virus must infect the basal cell in order to establish a persistent lesion. By *in situ* hybridization, it has been demonstrated that the viral DNA is indeed present within the basal cells and the parabasal cells of a papilloma.⁵³⁶ Furthermore, using probes to the early gene regions of the PVs, viral transcripts have been detected in the basal cells of the epidermis,⁵⁸⁴ and at least some early viral protein is found in basal cells.⁷⁹ Late gene expression, synthesis of capsid proteins, vegetative viral DNA synthesis, and assembly of virions occur only in terminally differentiating squamous epithelial cells.

Virion Attachment, Entry, and Trafficking

Papillomaviruses have a unique infectious process that is intimately linked with their life cycle in stratified squamous epithelia. As noted above, productive papillomavirus infection

is thought to require infection of the basal layer cells of the epithelium.¹⁵² To achieve selective infection of basal keratinocytes, the virions have evolved to preferentially bind initially to heparan sulfate proteoglycans (HSPGs) on the basement membrane exposed at sites of epithelial trauma or permeabilization, rather than to cells (Fig. 54.6).^{285,500} *In vitro*, the capsids bind directly to most epithelial cell lines in an HSPG-dependent manner.²¹² This difference probably reflects the adaption of *in vitro* propagated epithelial cells to the expression of HSPGs with modifications that resemble those that are normally found only on basement membrane *in vivo*.¹³⁰ Basement membrane binding induces a conformational change in the capsid that exposes a highly conserved N-terminal L2 peptide motif to cleavage by furin or the closely related proprotein convertase 5/6.³¹⁰ According to one model, cleavage induces a conformational change that exposes a capsid-binding site (probably on an L1 surface) for an as yet unidentified cell-surface receptor on keratinocytes and other cell types.³¹⁰ Alternatively, a recently proposed model suggests that the virions may interact with cell surfaces as high molecular weight complexes also containing cleaved HSPGs and bound growth factors through an interaction with a variety of growth factor receptors.⁵⁹⁴ There is a remarkably long delay between cell surface binding and viral genome transcription of 1 to 3 days, both *in vivo* and *in vitro*.^{129,500} Internalization of the capsids from the cell surface takes at least 2 to 4 hours, and is very asynchronous, with some capsids remaining on the surface for a much longer time.¹²³ *In vitro* studies indicate that the capsids are transported on the cell surface from filopodia at the leading edge of migrating cells to the central cell body via linkage to actin retrograde flow.⁵²⁶

The endocytic pathway involved in internalization and intracellular trafficking by PV capsids is controversial. Most studies have observed trafficking to acidified late endosomes either via clathrin-dependent or clathrin-independent uptake (Fig. 54.7).^{57,129,561} However, one study has implicated caveolin-dependent uptake with eventual trafficking to the endoplasmic reticulum,³⁴⁷ whereas another has suggested that tetraspanin-enriched microdomains may be involved in endocytosis.⁵⁷⁵ Several cell factors have been implicated in internalization and trafficking. These include cyclophilin B, a peptidyl-prolyl *cis/trans* isomerase, being required for exposure of the furin cleavage site in L2⁴²; sorting nexin 17, an adaptor protein involved in endosome cycling that inhibits routing of the capsids to lysosomes³⁵; and FAK, a focal adhesion kinase.¹ At least partial uncoating occurs in Lamp-2–positive late endosomes, but not until at least 8 to 12 hours after cell surface binding.¹²⁷ L2–genome complexes escape the late endosomes, whereas the genomes packaged in L1-only capsids do not.²⁹³ A conserved C-terminal L2 peptide with strong membrane penetrating and disrupting activity *in vitro* may be directly involved in endosomolysis. In addition, the activity of the transmembrane protease γ -secretase is required for infection, perhaps functioning in endosome escape.²⁹⁶ Movement through the cytoplasm to the nucleus likely occurs along microtubules in association with the motor protein dynein.¹⁸³ The fact that infection, at least *in vitro*, requires cell division has led to the conjecture that entry of the L2–genome complex into the nucleus may require nuclear envelope breakdown during mitosis, rather than active transport through nuclear pores.⁴⁸⁵ After cell division, the L2–genome complexes predominantly localize to specific nuclear structures, N10 bodies (also designated promyelocytic leukemia protein (PML) oncogenic domains, or

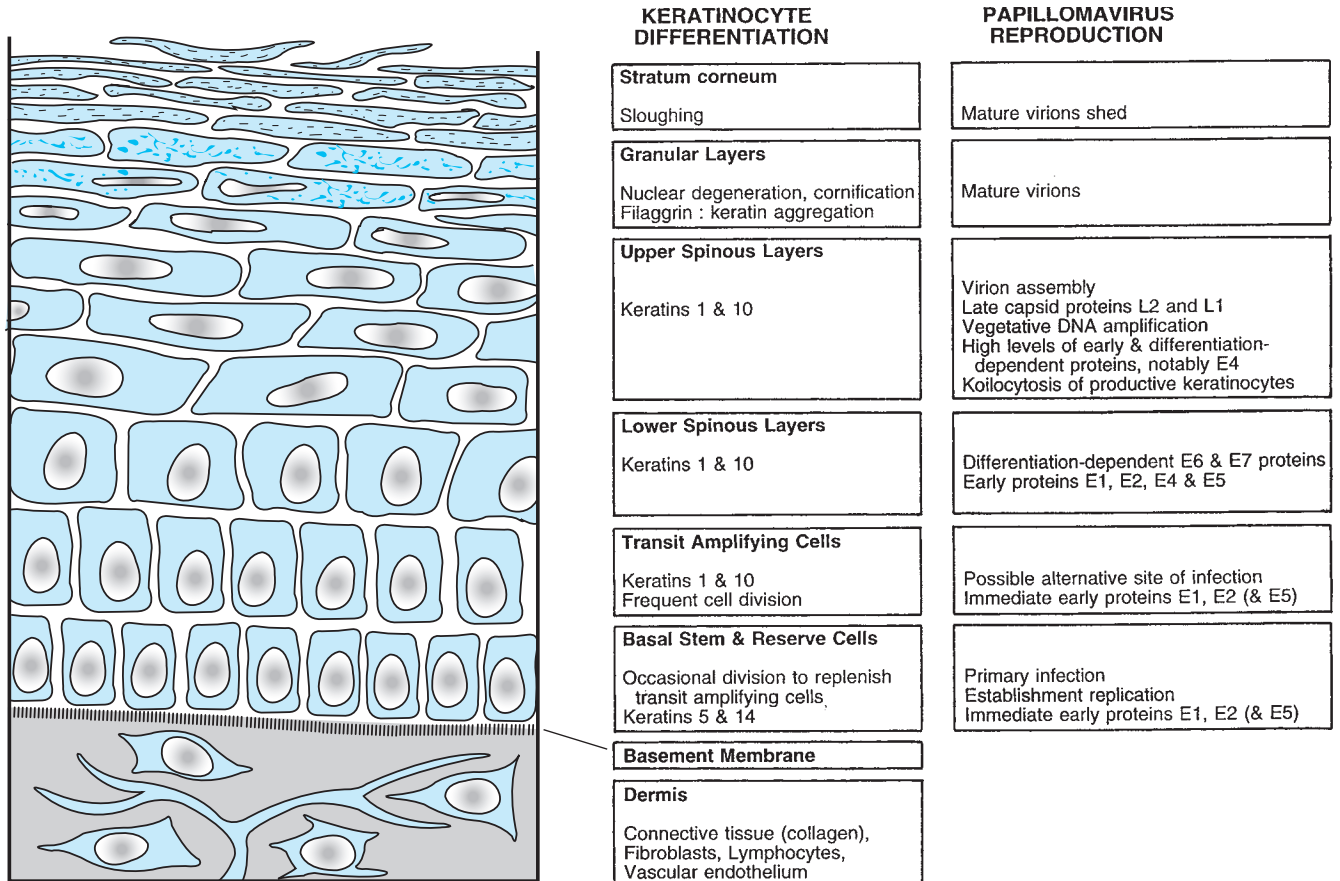


FIGURE 54.5. Differentiation of normal cutaneous squamous epithelium and papillomaviral activities in productively infected benign lesions. The various epithelial strata and the host differentiation, stage-specific, gene-expression profile are indicated in the left and center panels. In nonkeratinized squamous epithelia, such as cervical or laryngeal, keratins 4 and 13 are expressed in the place of keratins 1 and 10 in the differentiated cells. Although profilaggrin is expressed, there is no granular layer or stratum corneum in nonkeratinized squamous epithelia. The viral activities in the corresponding strata during productive infection shown on the right have been determined or inferred from *in situ* hybridization studies. (Reproduced from Chow LT, Broker TR. Small DNA tumor viruses. In Nathanson N, ed., *Viral Pathogenesis*. Philadelphia: Lippincott-Raven, 1997:267–301.)

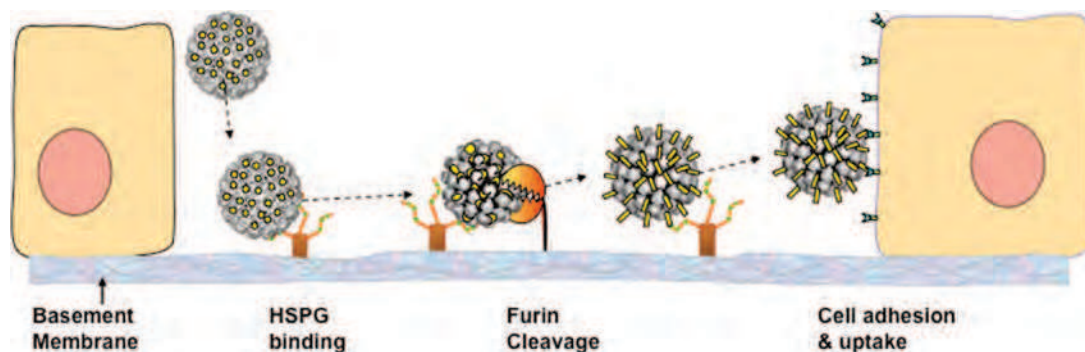
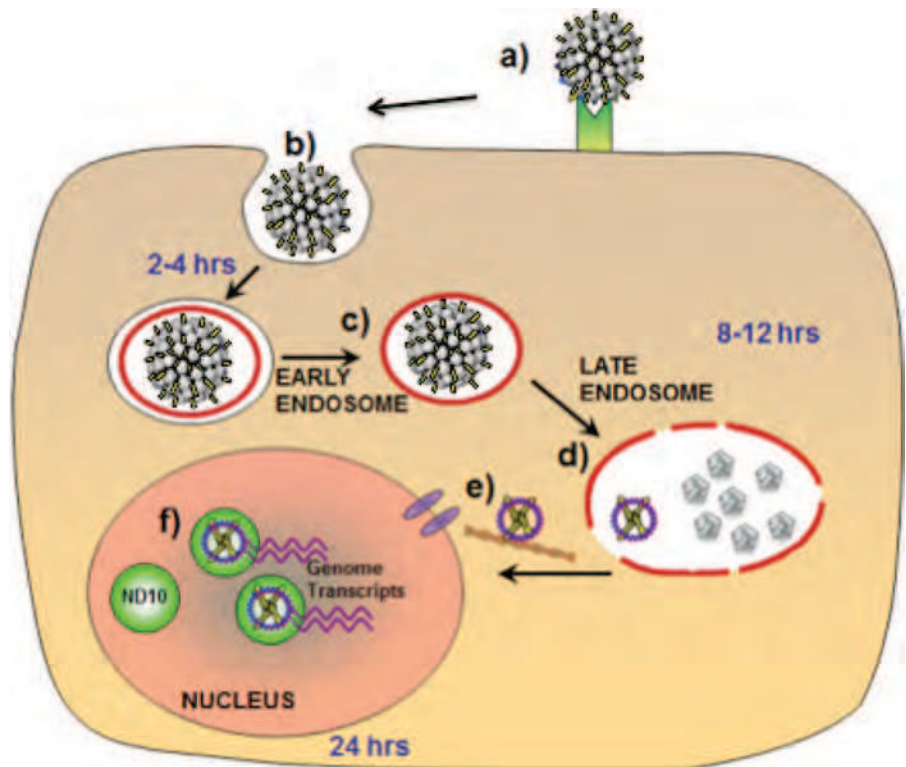


FIGURE 54.6. Model of in vivo papillomavirus infection. The virion first binds to heparan sulphate proteoglycans (HSPGs) on the basement membrane exposed after disruption. This induces a conformational change exposing a site on L2 (depicted in yellow) susceptible to proprotein convertase (furin or PC5/6) cleavage. After L2 cleavage, an L2 neutralizing epitope is exposed and a previously unexposed region of L1 binds to an unidentified secondary receptor on the invading edge of the epithelial cells.

FIGURE 54.7. Infectious process after cell binding. After binding to a cell surface receptor (a), the virus enters the cell via an endocytic pathway (b) and within 4 hours localizes in the early endosome (c). By 12 hours the virus uncoats within the late endosome and the viral genome complexed with L2 is released (d). The L2–genome complex traffics through the cytoplasm, perhaps via microtubules, and enters the nucleus by 24 hours (e). After nuclear entry, the complex co-localizes with ND10 and viral genome transcription begins (f).



PODs).¹²⁷ This localization promotes transcription of the viral genome. Potentiation of PV infection by association with ND10 bodies contrasts with the inhibitory activity of these structures in infections by herpes viruses, which target PMLs for degradation early in infection.¹⁷¹

Viral Transcription

The replicative phase of the papillomavirus life cycle is tightly linked to the differentiation program of the squamous epithelium. Historically, BPV1 served as the prototype for analyzing the papillomavirus transcription program. The studies have been carried out in a variety of systems, including viral RNAs from rodent cells transformed by BPV1 as well as those from infected wart tissues. More recently viral transcription studies have been extended to some of the HPVs associated with genital tract lesions—such as HPV11, HPV16, HPV18, and HPV31—by using HPV-positive clinical lesions, xenograft tissue in nude mice, cervical carcinoma cell lines, as well as organotypic culture systems. This portion of the chapter on transcription focuses largely on HPV16 and HPV31, both of which have been extensively analyzed by *in vitro* culture techniques.⁴¹¹

Viral RNAs and Promoters

Papillomavirus transcription is complex due to the presence of multiple promoters, to alternate and multiple splice patterns, and to the differential production of messenger RNA (mRNA) species in different cells.

A transcription map of HPV31 is shown in Figure 54.8. As with BPV1, multiple promoters are involved in generating the various mRNA species for the genital tract HPVs. For HPV31, P₉₇ is the major promoter active in nonterminally differentiated cells. This promoter, which directs the expression of E6 and E7 as well as several other early gene products, is analogous to P₉₇ of

HPV16 and P₁₀₅ of HPV18. Upon differentiation of immortalized keratinocytes harboring episomal HPV-31 DNA, there is activation of the differentiation-dependent, late promoters, P₆₇₀ for HPV16 and P₇₄₂ for HPV31, that direct the expression of the late gene products, including E4, L1, and L2, as well as an increase in the level of the E1 mRNA.^{321,517}

An important difference in the structures of the E6 and E7 mRNAs and in the manner by which they are expressed distinguishes the “high risk” and “low risk” HPV types. For the “high risk” HPVs such as HPV16 and HPV18, a single promoter (P₉₇ for HPV16 and HPV31, or P₁₀₅ for HPV18) directs the synthesis of mRNAs with E6 and E7 intact or with splices in the E6 gene (Fig. 54.8). The species with E6 intact could be translated into E6 but not E7 since there is insufficient spacing for translation reinitiation. The mRNAs with the spliced E6 splice the 5′ end of the E6 ORF (referred to as E6*) to a translation frame with stop codons that provide sufficient spacing for translation reinitiation of the E7 ORF and are therefore likely to represent the E7 mRNAs. In contrast, the E6 and E7 genes of the “low risk” HPVs such as HPV6 and HPV11 are expressed from two independent promoters.¹⁰⁵

Regulation of Transcription (Cis Elements)

Papillomavirus transcription is tightly regulated by the differentiation state of the infected squamous epithelial cell. This is evident from the analysis of the differential expression of viral RNAs in cells from the different levels of the epithelium in warts.¹⁰⁷ It is also evident from studies of infected keratinocytes using organotypic and suspension tissue culture systems that permit epithelial cell differentiation.^{149,321,410} Proper transcription of the viral genome is dependent upon the differentiation state of the epithelial cells and on cellular transcription, splicing, and other RNA processing factors.³⁷³

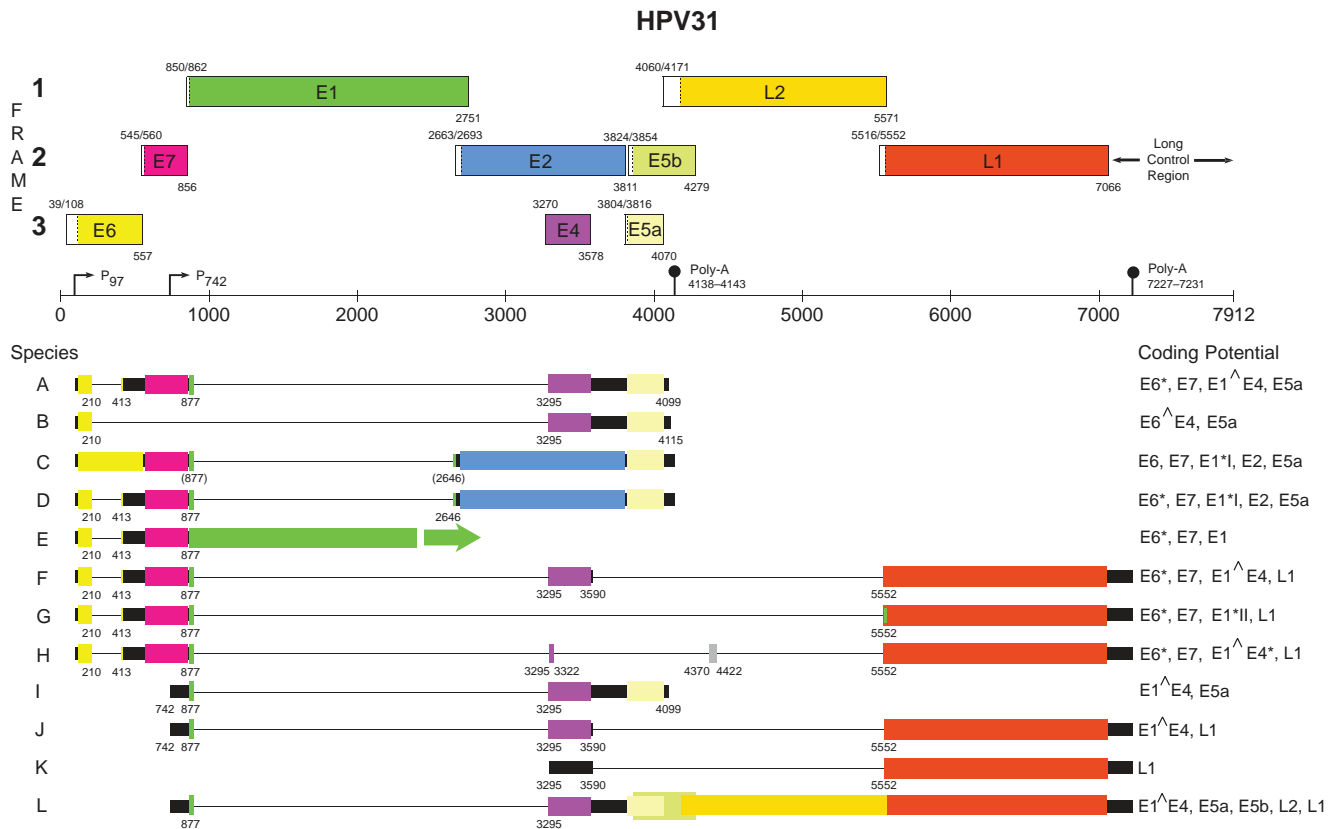


FIGURE 54.8. Transcription map of HPV31.^{266,267,321,448} A linearized version of the genomic map is shown at the top. Transcripts initiated at the early viral promoter, designated P₉₇, are expressed in the nonterminally differentiated cells in the lower portion of the epithelium, whereas those initiating from the late promoter (P₇₄₂) are expressed upon differentiation in the cells committed to the replication of the progeny virions. (Reproduced from the PAVE transcription maps, <http://pave.niaid.nih.gov>.)

The LCR (also referred to as the URR) region of papillomavirus contains enhancer elements that are responsive to cellular factors as well as to virally encoded transcriptional regulatory factors. Each of the viral LCRs that have been studied in detail have been found to contain constitutive enhancer elements that have some tissue or cell-type specificity. These constitutive enhancer elements play an important role for the initial expression of the viral genes after virus infection and may also be important in the maintenance of viral latency. A number of transcription factor binding sites have been identified in the LCRs of the various papillomaviruses that have been carefully studied. Included among them are sites that bind AP1, SP1, Oct-1, and YY1, among others.^{38,373} The HPV16 LCR has also been shown to contain nuclear matrix attachment sites that may be important for controlling viral gene expression.⁶⁰⁴ In addition to the binding sites for cellular transcription factors, the LCR contains binding sites for the virally encoded E2 regulatory proteins and the origin of DNA replication that binds the E1 replication factor.

E2 Regulatory Proteins

The papillomavirus E2 proteins have well-characterized regulatory functions affecting viral transcription, viral DNA replication, and long-term plasmid maintenance. E2 was first described as a transcriptional activator⁵⁷² capable of activating viral transcription through E2 responsive elements located within the

viral genome.⁵⁷¹ The E2 proteins are relatively well conserved among the papillomaviruses in two domains: a sequence-specific DNA-binding and dimerization domain located in the carboxy terminal region of the protein and a transactivating domain that is located within the amino terminal half of the protein.^{211,398} These two domains are separated by an internal hinge region that is not well conserved in size or in amino acid composition among different papillomaviruses. The E2 proteins bind the consensus sequence, ACCN₆GGT,^{9,362} and can regulate transcription from promoters containing E2 binding sites.^{242,251,570} E2 binds ACCN₆GGT motifs as a dimer; the DNA binding dimerization domain localizes to the carboxy terminus of E2.³⁹⁹

The E2 proteins have been best studied in the BPV system, where three species have been identified (Fig. 54.9). The full-length protein (E2TA) can function as a transactivator or a repressor depending on the location of the E2-binding sites within the enhancer/promoter region. The two shorter forms of E2 called E2TR and E8/E2 have been described as repressors because they can inhibit the transactivation function of the full-length E2TA.^{344,345} The shorter E2 proteins contain the DNA binding and dimerization domains of the C terminus but lack the transactivation domain. E2TR and E8/E2 can inhibit the transcriptional transactivating function of the full-length polypeptide by competing for its cognate DNA binding sites and by forming inactive heterodimers with the full-length transactivator protein. The crystal structure of the

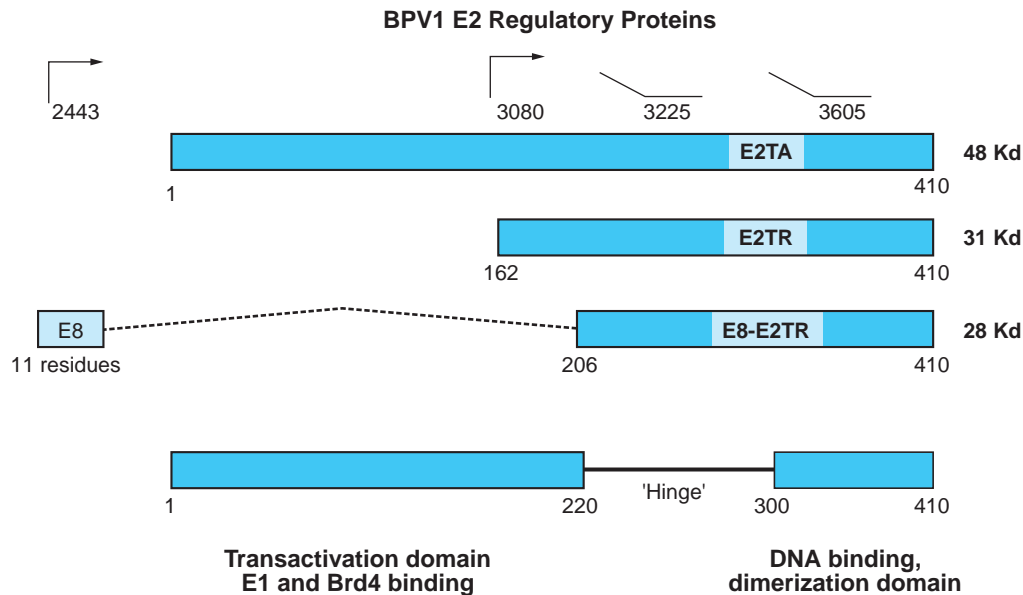


FIGURE 54.9. Structure of the BPV1 E2 gene products. The structures of the three known proteins encoded by the BPV1 E2 ORF are indicated. The 48-kD full-length E2 transactivator can be expressed from an unspliced message from P₂₄₄₃ or from a spliced messenger RNA (mRNA) from upstream promoters by utilizing a splice acceptor at nt 2558. The 31-kD and 28-kD forms of the repressor are expressed from P₃₀₈₀ and as an E8/E2 fusion by a spliced mRNA as shown, respectively. The transactivation domain consists of a region of approximately 200 amino acids at the N-terminal region of the full-length E2 protein that is relatively well conserved among papillomaviruses. This region, which is acidic and is predicted to contain amphipathic helices, is only present in the full length form of E2. The N-terminus of E2 contains the site for binding E1. The 110 C-terminal amino acids are also conserved and comprise the DNA-binding and dimerization domain. The basic region and the hydrophobic repeats are indicated.⁴⁰⁰

dimeric DNA-binding domain of BPV1 E2 revealed a previously unobserved structure for a DNA-binding protein, which is a dimeric antiparallel β barrel.²⁴⁶ The crystal structure of the N-terminal transactivation domain has also been resolved for HPV16 and HPV18.^{14,240} The structural studies of the E2 N-terminal domain indicated that it could form a dimer both in the crystal and in solution. Because amino acids that are necessary for transactivation are located at the dimer interface, the dimer structure may be important in the interactions of E2 with viral and cellular transcription factors. The dimer formation may contribute to the stabilization of DNA loops, which may serve to relocate distal DNA-binding transcription factors to the site of human papillomavirus transcription initiation.¹⁴

Mutations in the BPV1 E2 ORF have pleiotropic effects, disrupting transformation, replication, and transcriptional functions. Studies have shown that expression of the early region viral genes is under the control of the viral E2 gene product through E2 responsive elements located within the viral LCR. The actual role of the E2 transactivation function in the PV life cycle remains to be elucidated, however. Studies with an HPV31 genome that carried a mutant E2 gene that was defective for transactivation but competent for DNA replication competent could still be established as a stable episome and could induce differentiation-dependent late functions.⁵⁸⁹

E2 transcriptional regulation has also been well studied for the genital tract-associated HPVs. The binding of E2 to its cognate sites within the LCR of the HPV genomes results in the modulation of viral promoter activity. The E6 and E7 transforming genes of HPV16 and HPV18 are transcribed from the major early promoter (P₉₇ and P₁₀₅, respectively) contained

within the LCR of their respective genomes. In human epithelial cells, the HPV16 P₉₇ promoter and the HPV18 P₁₀₅ promoter display basal activities that can be repressed by full-length E2.^{37,506,608,609} There are four E2-binding sites upstream of the P₉₇ and P₁₀₅ promoters that mediate this repression. In addition to binding at its cognate sites, the E2 transcriptional activation function is required for E2-mediated promoter repression. Specific conservative point mutations within the bovine or human E2 transactivation domain that eliminate E2-mediated transcriptional activation also eliminate E6/E7 promoter repression.^{219,436} The bromodomain containing protein 4 (Brd4) is involved in this transcriptional repression.⁶⁷⁷ An unbiased whole genome small interfering RNA (siRNA) screen validated the involvement of Brd4 in this transcriptional repression and also revealed independent roles for the histone demethylase known as SMCX as well as components of the TIP60 histone acetylase complex including EP400.⁵⁶⁰

E2 can suppress the growth of HPV-positive cervical cancer cell lines through the transcriptional repression of the viral E6 and E7 genes.^{156,188,269,436,609} This repression results in the reactivation of the Rb and p53 tumor suppressor pathways that are inhibited by E6 and E7 and induce a cell cycle arrest and cellular senescence.^{217,218,649}

E2 is a multifunctional protein. Its functions as a transcriptional activator and repressor are likely mediated by interactions with specific cellular factors, some of which have now been identified (Table 54.1). In addition to its role as a transcriptional regulator, E2 has critical role in viral DNA replication. The full-length E2 proteins are critical auxiliary factors for viral DNA replication.^{103,137,623} This aspect has been

TABLE 54.1 Cellular Targets and Functions of the Papillomavirus E2 Regulatory Proteins

Associated cellular proteins	Functional consequences	References
Bromodomain protein 4	Tethers E2 and DNA to mitotic chromosomes	(688)
	Mediates transcriptional activation function	(539)
	Mediates transcriptional repression	(560,677)
TATA binding protein (TBP)	Transcription activation	(494)
G-protein pathway suppressor 2 (AMF-1/Gps2)	Enhance transcriptional activation	(67)
	Enhance E2 interaction with p300	(464)
Yin and Yang protein 1 (YY1)	Transcription regulation	(355)
Nucleosome assembly protein 1	Ternary complex with p300	(496)
Tumor suppressor protein 53 (p53)	Apoptosis (high risk HPV types)	(454)
CREB binding protein (CBP)	Transcriptional activation	(353)
Papillomavirus E1 protein	Viral DNA replication	(416)
Topoisomerase II binding protein 1 (TopBP1)	Enhance transcription and replication	(51)
HTLV1 TAX1 binding protein (TAXBP1)	Regulates transcription and stability	(645)
Chromosome loss-related protein 1 (ChlR1)	Loads E2 onto chromatin	(453)
Cullen 3 complex	E2 ubiquitylation	(692)
Skp2/SCF complex	E2 ubiquitylation	(34)

Abbreviations: Histone acetyltransferase CBP/p300 is a transcriptional coactivator. Skp2 is the S-phase associated kinase 2 that functions as an adaptor in the cullen based SCF E3 ligase complex.

best studied in the BPV system, where E2 was first shown to complex with E1 and to strengthen the affinity of E1 for binding to the origin of DNA replication.^{416,623}

E2 is also required for long-term episomal maintenance of viral genomes within replicating cells.^{27,273,357,475,556} The critical role of E2 in viral DNA replication is not sufficient to support long-term maintenance of the viral genomes. Genome maintenance requires, in addition, *cis* minichromosome maintenance elements consisting of multiple E2 binding sites.⁴⁷⁵ In the presence of E2, plasmids containing viral E2 binding sites associate with mitotic chromosomes. Furthermore, the chromatin attachment function correlates perfectly with the stable episomal maintenance of the viral plasmids.²⁷³ E2 facilitates viral genome segregation by interacting simultaneously with condensed mitotic chromatin and viral genomes, linking the viral plasmids to the cellular mitotic chromosomes, and thereby ensuring the viral genomes are contained within the nuclear envelope when it reforms during telophase. The TA domain is required for the association of E2 with mitotic chromosomes and for the genome maintenance function in dividing cells. Specific mutations in the TA domain have been shown to disrupt the tethering of viral genomes to mitotic chromosomes.^{2,28,693} The cellular protein Brd4 mediates the association of BPV-1 E2 to mitotic chromosomes and may play an important role for some other PVs.⁶⁸⁸ Furthermore the binding of E2 to Brd4 is conserved among all the papillomaviruses.^{274,339,688}

Additional cellular factors are likely to be involved in E2-mediated tethering to mitotic chromosomes and PV genome maintenance. For instance ChlR1 has been shown to be important for loading E2 onto mitotic chromosome.⁴⁵³ The E2 proteins of some of the alpha genus HPV types (HPV11, HPV16, and HPV18) have been shown to associate with the mitotic spindle rather than the chromosomes.⁶²⁹ It has been proposed that an association between mitotic spindles and these HPV E2 proteins might provide a mechanism for HPV viral genome persistence in host cells. Finally HPV8 E2 binds as large speckles at the ribosomal DNA loci at the pericentro-

meric regions of chromosomes.⁴⁸⁰ Therefore, different papillomavirus E2 proteins use different mechanisms to ensure the stable maintenance of their genomes in host cells and evaluation of these differences is likely to be fruitful.

The interaction of E2 with Brd4 is required for the transcriptional activation function of E2.⁵³⁹ Amino acid residues in the E2 TA domain required for Brd4 binding are also required for transcriptional activation, and siRNA knockdown of Brd4 protein levels reveals a role for Brd4 in E2 transcriptional activation.⁵³⁹ Brd4 is a member of the bromo and extra terminal (BET) family, a group of structurally related proteins characterized by the presence of two bromodomains and one extraterminal (ET) domain of unknown function.¹⁴¹ Bromodomains in general have been shown to interact with acetylated lysines in histones and are involved in chromatin targeting and remodeling.⁶⁷⁶ Unlike other bromodomain proteins, which are released from chromatin during mitosis, BET family members remain bound to chromatin during mitosis. Brd4 has been shown to influence the general RNA polymerase II-dependent transcription machinery by interacting with the core factors of the positive transcription elongation factor b (P-TEFb).^{281,684}

Late Gene Expression

The viral late functions, such as vegetative viral DNA synthesis, capsid protein synthesis, and virion assembly, occur exclusively in differentiated keratinocytes. Transcriptional regulation of the late genes is directed from a specific promoter that becomes active only in terminally differentiated keratinocytes. The late genes include the capsid genes L1 and L2, as well as E4, which is located in the early region of the viral genomes. Of interest the late promoters for the human papillomaviruses that have been analyzed do not map to the LCR. Instead, a differentiation-specific promoter (referred to as P₇₄₂ in HPV31 and P₆₇₀ in HPV16) has been identified within the E7 coding region which gives rise to mRNAs that map heterogeneously over a 100-bp region in the E7 gene.^{221,266} Keratinocyte differentiation by itself is able to activate low levels of late transcription, and genome

amplification also increases the level of late gene expression.^{48,574} Recent studies have also established differentiation changes in the levels of the CCAAT/enhancer binding protein (C/EBP), β repressors and activators in the regulation of the HPV late promoter.²³⁰

Papillomavirus L1 and L2 gene expression is also regulated at a posttranscriptional level. Cis elements have been described for BPV1 as well as several HPV types that regulate late gene expression at a posttranscriptional level. In the 3' untranslated regions (UTRs) of the late RNAs of each of these viruses there are negative regulatory elements that can inhibit the stability of late messenger RNAs. A negative regulatory element in the HPV16 3' UTR contains multiple 5' splice-like sequences, as well as an inhibitory GU-rich region that reduces mRNA stability and binds to specific cellular factors.^{142,305,322} Three different cellular factors (the U2 auxiliary splicing factor 65-kD subunit, the cleavage stimulation factor 64-kD subunit, and the Elav-like HuR protein) interact with the RNA and regulate its levels at a post-transcriptional level.³²² In HPV1, an AU-rich inhibitory region has been identified in the 3' UTR that also binds the Elav-like HuR protein.⁵⁶⁸ The model emerges that these, and perhaps additional cellular factors, are responsible for the nuclear retention or cytoplasmic instability of the nuclear retention element (NRE)-containing late transcripts. Keratinocyte differentiation would then lead to changes in these cell-encoded factors, thereby relieving the inhibition of late mRNA processing.

Virion Assembly and Release

Virion assembly takes place in the nuclei of terminally differentiated keratinocytes in which vegetative viral genome replication and expression of the virion proteins has occurred.¹⁵² Nuclear entry of L1 and L2 is mediated by cellular karyopherins, particularly the Kap alpha2/beta1 heterodimer.^{174,408} In addition to nuclear transport, karyopherin binding may prevent premature L1 assembly in the cytoplasm.⁴³ As noted above, L1 can assemble into VLPs. However, L2 may increase the efficiency of the assembly reaction.^{313,694} Hsc70 may also participate in the assembly reaction, since it is found in association with nuclear L2 but is displaced in L1/L2 capsids that have packaged DNA.¹⁸⁴ Packaging of the viral genome by the capsid proteins does not appear to require a sequence-specific packaging signal because many bacterial plasmids with no PV sequences can be efficiently packaged, at least in cultured cells, provided they are less than 8 kb in length.⁷⁴ Preferential encapsidation of the viral genome may involve a size discrimination mechanism. Nascent capsids might randomly coalesce around any nuclear DNA but would generally form unstable open structures. A stable closed structure could assemble only if the DNA molecule is approximately the size of the 8-kb viral genome. Consistent with this hypothesis, linear fragments of cellular DNA less than 8 kb in length are efficiently encapsidated if the nuclei of L1/L2 expressing cells are gently lysed and exposed to a double-stranded DNA endonuclease.⁷⁵ In cell culture systems, the L2 dependence for DNA encapsidation varies by PV type. For example, almost no DNA is encapsidated when BPV1 L1 is expressed alone, whereas HPV16 L1 alone rather efficiently encapsidates pseudoviral genomes or linear cellular DNA fragments, although the resulting DNA-containing L1-only capsids are essentially noninfectious.⁷⁵

Upon exposure to an oxidizing environment, as occurs in the upper layers of a terminally differentiated squamous epi-

thelium,¹¹⁴ the capsids are further stabilized by the formation of disulfide bonds between conserved cysteines on adjacent L1 monomers. This maturation process condenses the capsid and increases its regularity and resistance to proteolytic digestion.⁷⁵ Formation of disulfide-linked L1 dimers and trimers was observed after capsid production in replicating cultured cells or *in vitro* raft cultures.^{75,115} Neither L2 nor encapsidated DNA appreciably influences the formation of L1 disulfide bonds. However, the extent of cross-linking varies by PV type, for example, being much greater for BPV1 than HPV16.^{75,668} PVs are not believed to be cytolytic, and release of the virions is thought to occur as a result of the normal loss of nuclear and cytoplasmic membrane integrity during terminal differentiation of the infected keratinocyte. E4-mediated collapse of cytokeratin filaments might assist in virion release.¹⁵³

Viral DNA Replication

Little is known about the initial stages of viral DNA replication and amplification that occur following infection of a basal keratinocyte, when there is an amplification of the viral genome to approximately 50 to 100 copies. In cells in which the viral DNA has been established, the viral DNA is maintained as a stable multicopy plasmid. The viral genomes replicate an average of once per cell cycle during S-phase in synchrony with the host cell chromosome.²⁰⁷ As a multicopy plasmid, this type of DNA replication ensures a persistent infection in the basal cells of the epidermis. Vegetative DNA replication occurs in the more differentiated epithelial cells of the papilloma. Such differentiated cells have exited the cell cycle and are no longer capable of supporting cellular DNA synthesis. Through E6 and E7, however, the HPVs activate the DNA replication machinery to support vegetative viral DNA synthesis producing the genomes to be packaged into progeny virions.

Origin of DNA Replication

Papillomavirus DNA replication requires the origin of DNA replication in *cis* and the viral E1 and E2 proteins in *trans*. The minimal origin of DNA replication contains an A+T rich region (ATR), the E1 binding site that includes a region of dyad symmetry (DSR), and an E2 binding site.⁶²⁴ Origin-dependent DNA replication can be achieved *in vitro* with cell extracts containing high levels of E1 alone in the absence of E2.^{543,681} E1 is the essential virus-encoded replication factor and functions as an ATP-dependent helicase. The role of E2 is as an auxiliary factor in viral DNA replication; the binding of E1 to the origin of replication is stabilized through its interaction with E2 and the binding of E2 to its cognate sites adjacent to the origin.^{416,624}

The E1 Protein

The E1 protein is highly conserved among the PVs, and the BPV1 E1 protein is a 68-kD nuclear phosphoprotein that binds specifically to the origin of replication.^{616,624,664} By itself, E1 binds the origin with weak affinity; however, this binding is stabilized through its interaction with E2. E1 has DNA-dependent ATPase and DNA helicase activities.^{65,543,682} E1 is required for both the initiation and elongation of viral DNA synthesis.³⁶⁸ In addition to its interaction with E2, E1 has been shown to bind a number of cellular proteins. E1 interacts with the p180 subunit of the cellular DNA polymerase α -primase and thereby recruits the cellular DNA replication initiation machinery to the viral replication origin.^{52,457} Several additional host

cellular proteins have been found to bind E1, including histone H1,⁵⁹⁶ SW1/SNF5,³⁵⁴ cyclin E/Cdk2,^{121,385} Hsp40/Hsp70,³⁶⁹ and Ubc9.^{492,685} Although the physiologic significance of some of these interactions remains to be determined, several appear to be quite interesting. In particular, the efficient cell cycle-regulated replication of papillomavirus genomes is dependent upon the association of E1 with the S-phase specific cyclin E-Cdk2 complex.¹²¹ In addition, the interaction of E1 with Ubc9 is required for efficient origin-dependent replication.⁶⁸⁵ E1 is small ubiquitin-like modifier 1 (SUMO-1) modified by Ubc-9, and this modification is required for the intranuclear accumulation of E1.⁴⁹³

E2 Protein Replication Functions

Papillomavirus DNA replication requires E2 as an auxiliary factor.^{623,681} Although not essential for origin-dependent DNA replication *in vitro*, E2 greatly stimulates the ability of E1 to initiate DNA replication.⁶⁸¹ E2 interacts with E1^{46,383,416} and greatly enhances the ability of E1 to bind the replication origin.^{416,541,544} E2 can relieve nucleosome mediated repression of papillomavirus DNA replication *in vitro*.³⁶¹ The E1–E2 complex is a precursor to a larger multimeric E1 complex, which after the removal of E2 can distort the replication origin and ultimately unwind the DNA (Fig. 54.10).³⁸⁴ E2 serves as an auxiliary factor that fosters the assembly of the preinitiation complex at the origin, but E2 itself plays no intrinsic role in viral DNA replication. A hexameric form of E1 protein is associated with the ATPase and DNA helicase activities intrinsic to its initiator function in DNA replication.^{186,542}

Vegetative Viral DNA Replication

Vegetative replication of papillomavirus DNA is necessary to generate the genomes to be packaged in virions, a process that normally occurs only in the terminally differentiated epithelial cells of a papilloma. The mechanisms regulating the switch from plasmid maintenance to vegetative viral DNA replication are not known. The switch may involve the presence or absence of controlling cellular factors in differentiating keratinocytes. In addition, or alternatively, the relative levels of viral factors such as E1 or E2 (or their modification) may change in terminally differentiating keratinocytes. There have been few studies that have examined the mode of vegetative viral DNA replication in differentiated cells. One might anticipate that, as with the polyomaviruses, vegetative DNA replication occurs bidirectionally through theta structure intermediates, as it does in the maintenance replication phase. One intriguing study, however, suggests that there may be a switch from a bidirectional mode of replication to what could be a rolling circle mode.¹⁸² Additional studies on the mechanism of vegetative replication would appear to be warranted.

VIRAL TRANSFORMATION

BPV-1 Transformation

Certain papillomaviruses are capable of inducing cellular transformation in tissue culture. The best studied of the transforming papillomaviruses is BPV1. Morphologic transformation in tissue culture was first described for BPV in the early 1960s.^{45,50,612} In the late 1970s, a focus assay was developed using established cell lines to study BPV1 transformation.¹⁶³ In general, investigators have relied upon mouse C127 cells and NIH 3T3 cells for these transformation studies, although a

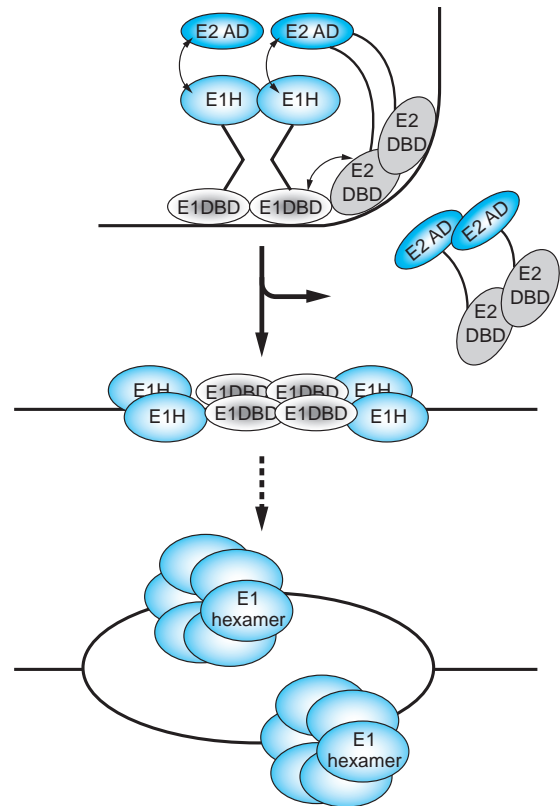


FIGURE 54.10. Proposed pathway for the assembly of an initiation-competent complex at the bovine papillomavirus (BPV) origin of DNA replication. The E1 initiator binds cooperatively with E2 to the ori forming a specific E1₂E2₂–DNA complex. As a consequence of the interaction between the E1 and E2 DNA binding domains (DBDs), a sharp bend is induced in the ori DNA. The bend promotes the interaction between the E1 helicase domain (E1H) and the E2 transactivation domain (E2AD). The resulting highly sequence-specific complex serves to recognize the ori. In a reaction requiring ATP hydrolysis, E2 is displaced and additional E1 molecules are added to the complex, resulting in the formation of a complex where four molecules of E1 are bound to the ori. This complex can distort the DNA duplex to and give rise to partially single-stranded regions. Subsequently, additional molecules are added. In a final step, E1 is assembled onto the exposed single strands forming a hexameric ring-like structure that constitutes the replicative helicase. (Courtesy of Arne Stenlund; modified from Enemark E, Chen G, Vaughn DE, et al. Crystal structure of the DNA binding domain of the replication initiation protein E1 from papillomavirus. *Molec Cell* 2000;6:149–158.)

variety of other rodent cells, including hamster and rat cells, are susceptible to BPV1-mediated transformation. Transformation of mouse C127 cells by BPV1 causes alterations in morphology, loss of contact inhibition, anchorage independence, and tumorigenicity in nude mice.¹⁶³

One notable characteristic of BPV1 transformed rodent cells is that the viral DNA is maintained as a stable multicopy plasmid.³⁴⁹ Integration of the viral genome is not required for either the initiation or maintenance of the transformed state. However, transformation is dependent upon the continued expression of viral genes as evidenced by the loss of the transformed phenotype in mouse cells that have been “cured” of the viral DNA by treatment with interferon.⁶²²

Genetic studies mapped the BPV1 transforming genes to the E5, E6, and E7 ORFs. The E5 gene is the major transforming gene of BPV1 in transformed cells. E5 encodes a small (44 amino acid) integral membrane protein that is sufficient for the transformation of certain established rodent cells in culture, and does so by activating the platelet derived growth factor (PDGF) β receptor to transform cells in a ligand-independent manner.^{467,468} The molecular biology of BPV1 E5 and its mechanism of transformation are described in more detail in Chapter 7. E5 is highly conserved among the group of papillomaviruses that induce fibropapillomas in their natural host and have the capacity to induce fibroblastic tumors in hamsters. The E5 gene is believed to be responsible for the proliferation of dermal fibroblasts in fibropapillomas.

The E6 and E7 genes of all the papillomaviruses encode proteins with conserved structural motifs. They contain domains of almost identically spaced CYS-X-X-CYS motifs (four in E6 and two in the carboxy-terminal portion of E7). It has been postulated that the E6 and E7 genes may have arisen from duplication events involving a 39-codon core sequence containing one of these motifs.¹¹² The CYS-X-X-CYS motifs found in a number of nucleic acid binding proteins are characteristic of zinc-binding proteins. The papillomavirus E6 and E7 proteins bind zinc through these cysteine residues.^{24,225} BPV1 E6 and E7 have not been studied as extensively as their HPV counterparts, and they appear to transform through p53- and pRB1-independent mechanisms. As such, studies on the mechanisms by which BPV1 E6 and E7 transform cells could provide insights into the p53 and pRB independent activities of the HPV E6 and E7 oncoproteins, discussed in detail below.

HPV Immortalization and Transformation

The HPV16 and HPV18 genomes are not as efficient as BPV1 DNA at inducing transformation of established rodent cells; however, transformation can be achieved when the HPV DNA is transfected along with a second selective marker, such as the neomycin resistance gene.⁶⁸⁶ Immortalization assays employing primary rodent cells, primary human fibroblasts, and/or primary human keratinocytes have proven more informative. In such assays, the high-risk HPVs, such as HPV16 and HPV18, are positive for immortalization or transformation, whereas the low-risk viruses, such as HPV6 and HPV11, are not.^{535,585} These assays permitted the mapping of the E6 and E7 as oncogenes for the high-risk HPV types.

In established rodent cells, such as the NIH3T3 cells, the E7 ORF scores as the major HPV transforming gene.^{471,605,640,647,687} HPV16 and HPV18 by themselves are not able to transform primary rat fibroblasts or baby rat kidney cells.^{343,471} However, the E7 gene can cooperate with an activated *ras* oncogene to fully transform primary rat cells.^{30,362,440,471,585}

The DNAs of the high-risk HPVs can also be distinguished from the DNAs of the low-risk HPVs by their abilities to immortalize primary human fibroblasts, human foreskin keratinocytes, or human cervical epithelial cells.^{161,476,535,646,673} The resulting cell lines are neither anchorage-dependent nor tumorigenic in nude mice, but they do display altered growth properties and are resistant in the response to signals for terminal differentiation.^{161,302,476,535}

HPV E6

The HPV E6 proteins are approximately 150 amino acids in size and contain four Cys-X-X-Cys motifs that are involved

in binding zinc.^{24,225,226} The first transforming activity identified for the high-risk alpha genus HPV E6 proteins (such as HPV16 and HPV18) was the ability complement E7 in the immortalization of human keratinocytes.^{245,421} This activity was soon explained by its ability to complex p53,⁶⁵⁵ a property not possessed by the low-risk HPV E6 proteins. Through its interaction with p53, E6 blocks the transcriptional function of p53 to activate p53-responsive promoters.⁴¹² The protein levels of p53 are generally quite low in HPV-positive carcinoma cell lines and in cells immortalized by the HPV oncoproteins,⁵²³ due to the ability of the E6 proteins of high-risk HPV types to promote the ubiquitin-dependent degradation of p53.⁵²⁵ Expression of the high-risk E7 proteins and their engagement of the pRB family of proteins results in an increase in the levels of p53 within cells, which in turn transcriptionally activates the expression of cell cycle arrest genes or proapoptotic genes.²⁸⁷ Indeed the half-life of p53 is dramatically decreased in E6-expressing cells, and E6 prevents the increase in p53 levels when cells are challenged with genotoxic agents.^{260,523} In targeting p53, the high-risk HPV E6 proteins inhibit DNA damage and oncogene-mediated cell death signals.^{167,287,308} Therefore, like SV40 Tag and Ad E1B that also target p53, HPV E6 has antiapoptotic activities and can interfere with the cell cycle regulatory functions of p53. E6 also can induce genomic instability, as evidenced by the development of translocations and aneuploidy in culture,^{498,657} as well as maintenance of stable episomal replication during the viral life cycle,⁴⁵⁸ and immortalization of human mammary epithelial cells.^{23,549}

HPV16 E6 induces p53 degradation by forming a complex with the cellular ubiquitin-protein ligase E6AP,^{263,264} which is then able to bind and ubiquitylate p53.⁵²² E6AP is the founding member of a class of ubiquitin-protein ligases called HECT E3 proteins, which directly transfer ubiquitin to their substrates.⁵²⁴ The catalytic domain of HECT proteins is a conserved 350 amino acid region defined by its homology to the E6AP carboxy terminus (HECT).²⁶² The HECT domain binds to specific E2 enzymes and contains an active site cysteine residue that forms a thioester bond with ubiquitin.^{262,524} (Fig. 54.11). Structure studies have determined that the HECT domain is a bilobed structure, with a larger N-terminal lobe that interacts with the ubiquitin-conjugating enzyme, and a smaller C-terminal lobe containing the catalytic cysteine residue.²⁵⁷

Levels of p53 in E6-immortalized cells or in HPV-positive cervical carcinoma cells are, on average, two- to threefold lower compared to primary cells.⁵²³ In uninfected cells, intracellular p53 levels increase significantly in response to DNA damage or genotoxic stress.²⁹⁹ The higher levels of p53 can result in a G₁ growth arrest or apoptosis, as part of a cell defense mechanism that allows for either the DNA damage to be repaired prior to the initiation of a new round of DNA replication or the removal of the cell. E6-expressing cells, however, do not manifest a p53-mediated cellular response to DNA damage,³⁰⁸ indicating the ability of E6 to promote the degradation of p53 and prevent the steady level of p53 to rise above a certain threshold level (Fig. 54.12). Under DNA-damaging conditions, the E6-stimulated degradation of p53 abrogates the negative growth regulatory effect of p53, and as such contributes to genomic instability. E6AP does not normally regulate p53 ubiquitylation in the absence of E6. In binding E6AP, E6 directs E6AP to p53 allowing it to form a ternary complex. It should be noted that E6AP does not regulate p53 protein

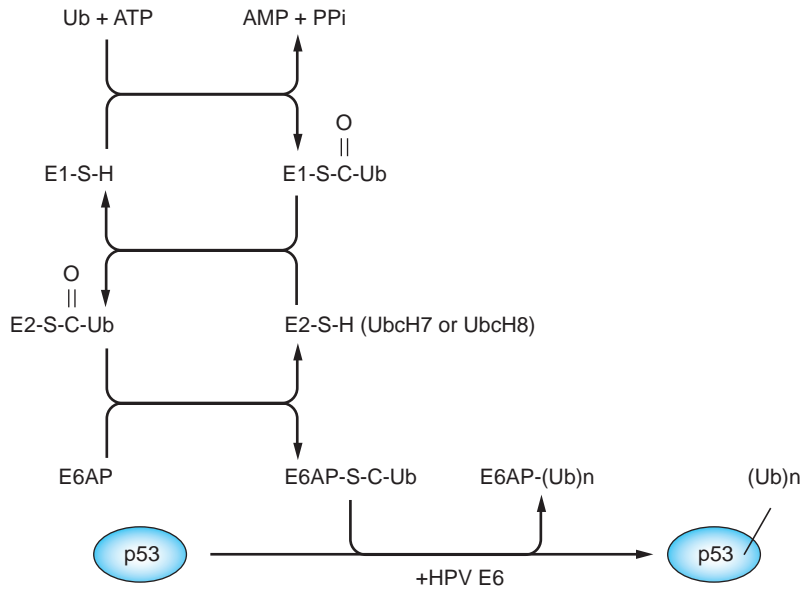


FIGURE 54.11. A ubiquitin thioester cascade model for the human papillomavirus (HPV) E6 dependent ubiquitination of p53. The E6 protein binds to the cellular protein E6AP, and the complex together functions as an E3 (ubiquitin protein ligase) in facilitating the ubiquitination of p53.⁵²² The ubiquitination of a protein involves three cellular activities: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin protein ligase). Ubiquitin is activated in an ATP-dependent manner and forms a high energy thioester with E1, which can then be transferred to the E2 through a thioester linkage. Ubiquitin can then be transferred to a cysteine within the Hect domain of E6AP, again as a thioester linkage⁵²⁴ through the direct binding of E6AP with UbcH7 or UbcH8.³³⁷ In conjunction with HPV-16 E6, E6AP then recognizes p53 and catalyzes the formation of an isopeptide bond between the carboxy-terminal glycine of ubiquitin and a lysine side chain of p53. In catalyzing the ubiquitination of p53, HPV-16 E6 also induces the self-ubiquitination and proteolysis of E6AP.²⁹⁵

stability in non-E6-expressing cells^{31,602}; the ubiquitin ligase MDM2 is the major E3 ubiquitin ligase responsible for p53 degradation in the absence of E6.^{244,334}

The HPV16 E6 protein binds to E6AP within the N-terminal substrate recognition domain, directing E6AP to ubiquitylate p53.²⁶⁵ The E6AP protein is encoded by the UBE3A gene that is located in an imprinted region on chromosome 15q11-q13, and it has been linked to Angelman's syndrome, a neurogenetic disorder characterized by severe mental retardation, ataxia, loss of speech, seizures, and other abnormalities.^{314,396} Several potential E6-independent substrates of E6AP have now been identified, including the human homolog of the yeast RAD23 protein involved in nucleotide excision repair (HHR23A), the src-family kinase Blk, and the MCM7 subunit of replication licensing factor.^{335,338,442} In addition, E6 induces self-ubiquitylation of E6AP.²⁹⁵ It is conceivable that the redirection of E6AP activity toward p53 by E6 might affect (either enhance or inhibit)

the targeting of the normal substrates of E6AP, and that such an alteration of E6AP activity could account for some of the transforming activity of E6. E6AP is a component of a number of cellular complexes, including the proteasome and a 2MDa complex that contains Herc2 and Neurl4.³⁹³ E6 is recruited to each of these complexes by E6AP. E6 encodes a number of p53-independent functions that are relevant to cellular transformation and immortalization, and there are HPV16 E6 mutations that separate p53 degradation from cellular immortalization.^{315,372} It is possible that some of the p53-independent activities of E6 are still mediated by E6AP through the activities of these other cellular complexes.

A number of additional cellular targets have been identified for the high-risk alpha genus HPV E6 proteins (Table 54.2). Of note, the high-risk E6 oncoproteins contain an X-(S/T)-X-(V/I/L)-COOH motif at the extreme C-terminus that mediates binding with cellular PDZ domain-containing proteins. This motif

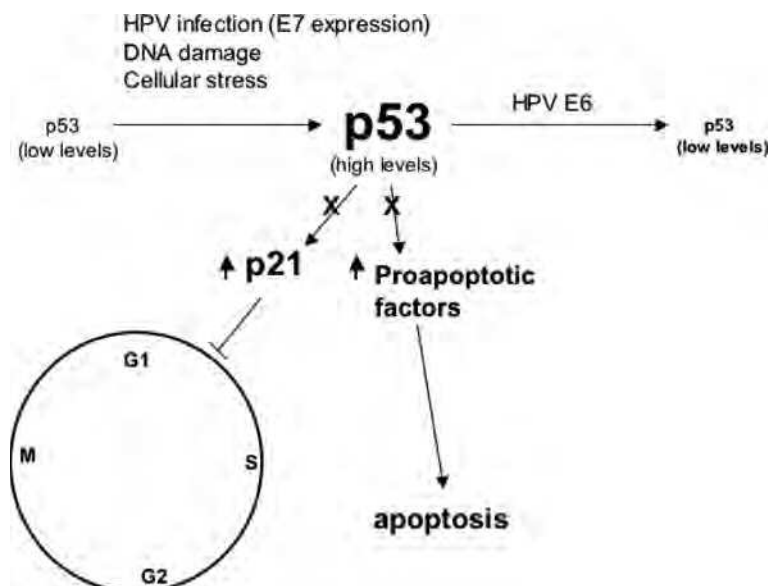


FIGURE 54.12. The level of p53 in primary cells is generally low. DNA damaging agents, viral infection, and expression of E7 increase the level of p53. Elevated levels of p53 can lead to either apoptosis or a cell-cycle checkpoint arrest in G1 through the transcriptional activation of proapoptotic genes or p21^{cip1}. Viral oncoproteins may interfere with this negative growth regulatory function of p53, either by sequestering p53 into a stable, but nonfunctional complex (such as with SV40 TAg or the Ad5 55 kD E1B protein), or by ubiquitylation and enhanced proteolysis as observed with the high risk human papillomavirus (HPV) E6 proteins.

TABLE 54.2 Cellular Targets of the Papillomavirus E6 Oncoproteins

Associated cellular proteins	Functional consequences	References
E6-associated protein (E6AP) (Alpha genus HPVs)	Ubiquitylation and proteolysis of associated proteins	(522)
Mastermind-like 1 (MAML1) (Cutaneous PVs)	Ubiquitylation of E6	(295)
p53	Repress Notch transcription and signaling	(70,603)
Reticulocalbin 2 (Erc55 or E6BP)	E6AP dependent ubiquitylation and proteolysis	(525,655)
Paxillin	Unknown	(98)
PDZ domain-containing proteins: HDlg, MUPP1 and hScrib (HR HPVs only)	Disruption of the actin cytoskeleton	(619,631)
Interferon regulatory factor 3 (IRF-3); Clathrin adaptor complex AP-1	E6AP dependent ubiquitination and proteolysis	(197,356,427)
Bak	Inhibition of β -interferon induction	(510)
CBP/p300	Unknown	(618)
Myc	Inhibition of Bak induced apoptosis	(611)
NFX1-91	Inhibition of p53 transcriptional activity	(462,697)
E6 oncoprotein targeted protein 1 (E6TP-1)	Activation of cellular telomerase	(371)
ADA3	Activation of cellular telomerase	(205,206)
FADD/Caspase 8	E6 induced degradation	(196)
	Transcriptional regulation	(336)
	Block apoptosis	(180,198)

Gene abbreviations: Tumor suppressor protein 53 (p53); CREB-binding protein (CBP); Bcl2 antagonist killer (Bak); myelocytomatosis viral oncogene homolog (Myc); Nuclear factor, X-box binding (NFX1-91); Homolog of yeast putative transcriptional adaptor (ADA3); Fas-associated protein with death domain (FADD).

is unique in the high-risk HPV E6 proteins and is not present in the E6 proteins of the low-risk alpha genus HPV types. E6 serves as a molecular bridge between these PDZ domain proteins and E6AP, facilitating their ubiquitylation and mediating their proteolysis. Among the PDZ domain proteins implicated as E6 targets are hDlg, the human homolog of the *Drosophila melanogaster* discs large tumor suppressor, and hScrib, the human homolog of the *Drosophila* scribble tumor suppressor.^{197,427} Additional PDZ domain proteins shown to be capable of binding to E6 are the membrane associated guanylate kinase protein 1, 2 and 3 (MAGI 1, MAGI 2 and MAGI 3), the multi-PDZ domain protein (MUPP1) and the a cytoplasmic interacting protein containing a PDZ domain (TIP 2/GIPC).^{173,215,356,613} Several of the PDZ-containing proteins have been shown to be involved in negatively regulating cellular proliferation. Therefore some of the p53-independent transforming activities of the high-risk E6 oncoproteins may be linked to their ability to bind and degrade some of these PDZ motif-containing proteins.

An important p53-independent activity of HPV16 E6 is its ability to activate telomerase in keratinocytes,³²⁰ through the transcriptional upregulation of the rate-limiting catalytic subunit of human telomerase (hTERT).^{315,443,632} Maintenance of telomere length is an important step in cellular immortalization and transformation, which occurs either through transcriptional activation of hTERT expression or through the activation of the ALT recombination pathway. Activation of hTERT is observed in most human cancers, including HPV-positive cervical cancers. The mechanism of hTERT promoter activation by E6 is complex. It involves the c-Myc transcription factor that binds to the hTert promoter, and it involves the E6AP-dependent degradation of a transcriptional repressor of the hTERT promoter, NFX1-91.^{205,206} Interactions of E6 with E6AP as well as c-Myc have been shown to be important in the transcriptional activation of the hTERT promoter.^{370,371,633,679}

HPV16 E6 has also been reported to bind the transcriptional co-activator p300/CBP, a target also of Ad E1A and

SV40 large T antigen.^{462,697} This interaction is limited to E6 proteins of high-risk HPVs associated with cervical cancer that have the capacity to repress p53-dependent transcription. The repression of p53 transcriptional activity by targeting the p53 co-activator CBP/p300 provides a second mechanism which can inhibit p53. A subsequent study has shown that *in vitro*, E6 can inhibit p300-mediated acetylation on p53 and nucleosomal core histones.⁶¹⁴ A variety of other E6 cellular targets have been identified (Table 54.2); however, the physiologic relevance to transformation or immortalization has not yet been elucidated. It is possible that the binding of E6 to some of these targets might contribute to the virus-host cell functions unrelated to cellular transformation.

Other p53-independent activities for the alpha genus HPV E6 proteins have been described in the literature, including the activation of cap-dependent translation.⁵⁷³ Recent reviews that provide more detail on these activities have been published.^{256,403,418}

Most studies thus far on the molecular biology of the HPV E6 proteins have been on the alpha genus high-risk HPV types. Studies of the cutaneous HPVs are more limited, and less is known about the cellular activities of the cutaneous HPVs of the beta genus. Recently, the beta HPV E6 types as well as BPV1 E6 have been shown to bind Mastermind-like 1 (MAML1) and other members of the Notch transcription complex.^{70,603} MAML1 is a core component of the transcriptional activation complex that mediates the effects of the canonical Notch signaling pathway.⁶⁷⁵ BPV1 and beta-HPV E6 repress Notch transcriptional activation, and this repression is dependent on an interaction with MAML1. Furthermore, the expression levels of endogenous Notch target genes are repressed by beta-HPV E6 proteins.⁶⁰³

Notch-dependent transcriptional programs are critical in the differentiation and cell cycle arrest of keratinocytes.^{378,491} In addition, inactivating Notch pathway mutations have been recently reported in squamous cell carcinomas of the head and

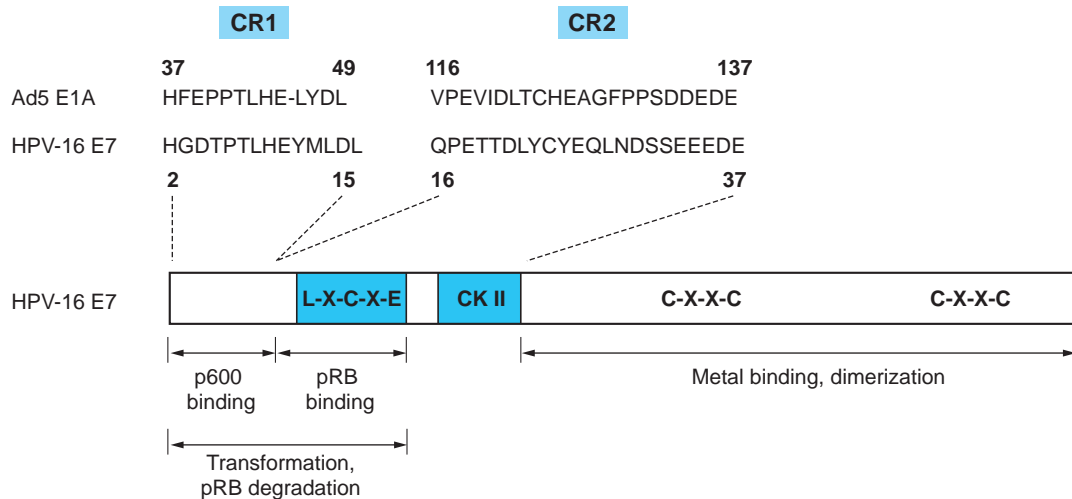


FIGURE 54.13. Amino acid sequence similarity between portions of conserved regions 1 and 2 (CR1 and CR2) of the Ad5 E1A proteins and the amino terminal 38 amino acids of HPV16 E7. CR2 contains the pRB binding site and the casein kinase II (CKII) phosphorylation site of HPV16 E7.

neck,^{3,588} and the skin,⁶⁴³ consistent with the notion that Notch signaling is a tumor suppressor pathway in squamous epithelial cells.¹⁵⁵ E6 binding to MAML1 provides a novel mechanism of viral antagonism of HPV16 Notch signaling, and suggests that Notch signaling is an important epithelial cell pathway target for the beta-HPVs. Of interest, papillomavirus E6 proteins appear capable of binding to E6AP or to MAML1.

HPV E7

The E7 protein encoded by the “high risk” HPVs is a small protein of about 100 amino acids, has been shown to bind zinc, and is phosphorylated by casein kinase II (CK II).⁴⁰² E7 is a multifunctional protein that shares some functional similarities with adenovirus (Ad) 12S E1A.⁴⁷¹ The HPV proteins also share important amino acid sequence similarity with portions of the AdE1A proteins and the SV40 large tumor antigen (TAG) (Fig. 54.13). These conserved regions are critical for the transforming activities in all three viral oncoproteins, and have been shown to participate in the binding to a number

of important cellular regulatory proteins, including the product of the retinoblastoma tumor suppressor gene pRB, and the related pocket proteins, p107 and p130.^{135,165,659} Complex formation with pRB involves conserved region 2 of the Ad E1A protein and the corresponding region in the E7 protein and in SV40 large Tag.^{135,660}

The retinoblastoma protein is a member of a family of cellular proteins that also includes p107 and p130, which are homologous in their binding “pockets” for E7, AdE1A, and SV40 TAG. Its phosphorylation state is regulated through the cell cycle, being hypophosphorylated in G₀ and G₁ and phosphorylated during S, G₂, and M. pRB becomes phosphorylated at multiple serine residues by cyclin-dependent kinases at the G₁/S boundary and remains phosphorylated until late M, when it becomes hypophosphorylated again through the action of a specific phosphatase (Fig. 54.14). The hypophosphorylated form represents the active form with respect to its ability to inhibit cell cycle progression. HPV16 E7, like SV40 TAG, binds preferentially to the hypophosphorylated form of pRB, resulting

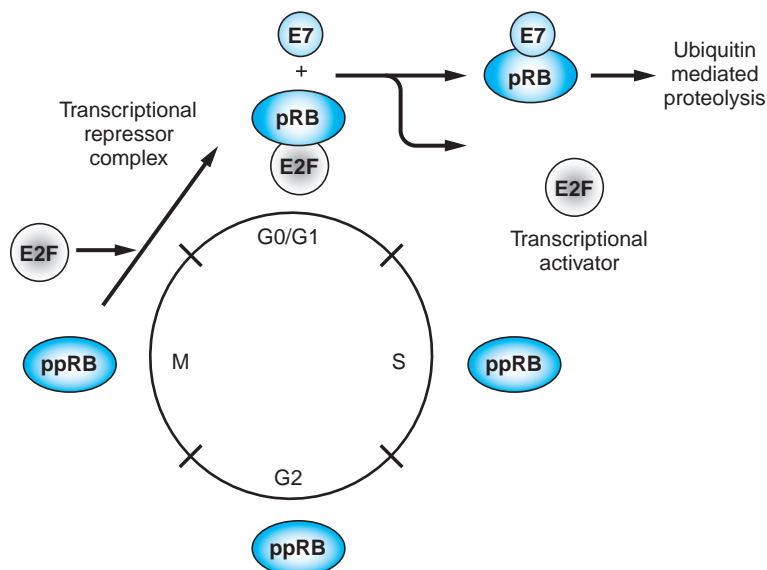


FIGURE 54.14. E7 abrogates the cell cycle regulation mediated by pRB (as well as the related proteins p107 and p130) by complex formation. During the cell cycle, pRB is differentially phosphorylated, and the underphosphorylated form is detected only in the G₀/G₁ phase. This underphosphorylated form is the active form of pRB, acting as a negative regulator of the cell cycle. During the transition to the S-phase, pRB is phosphorylated by cyclin-dependent kinases (cdk), resulting in the inactivation of its cell cycle regulatory functions. Members of the E2F family of cellular transcription factors are preferentially bound to the under-phosphorylated form of pRB, and in complex with pRB cannot activate transcription. Phosphorylation of pRB or complex formation with E7 results in the release of the E2F factors, allowing them to function as transcriptional activators of cellular genes involved in cellular DNA synthesis and progression into the S phase of the cell cycle.

TABLE 54.3 Cellular Targets of the Human Papillomavirus E7 Oncoproteins

Associated cellular proteins	Functional consequences	References
Retinoblastoma protein and related pocket proteins pRB, p107, p130	Disruption of E2F transcription factor complexes Degradation	(164,165) (61,287)
UBR4 (p600) and KCMF1	Inhibit anoikis	(139,261,658)
Cyclin dependent kinase inhibitors (p21 and p27)	Inactivation of cdk inhibitory activity	(194,286,689)
Zer1 (HPV-16 specific)	pRB ubiquitylation	(658)
TATA binding protein (TBP)	Transcription activation	(395)
ATM, ATR		
Activator protein 1 (AP-1)	Activation of c-jun transcriptional activation function	(11)
Centrosome components (Gamma tubulin)	Aneuploidy	(430)
IGFBP-3	Inhibition of IGFBP-3 mediated apoptosis	(389)
E2F transcription factor 6 (E2F6) (cdk)	Prevent repression by E2F6 polycomb group complexes	(401,402)
Histone deacetylase (HDAC)	Activate transcription	(66,374,375)
IRF1 and p48	Block IFN response	(25,456)
Forkhead transcription factor MPP2	Activation of MPP2 transcriptional activity	(382)
Cullen 3	Not yet known	(658)

Gene abbreviations: Ubiquitin protein ligase component n-recogin 4 (UBR4); Potassium channel modulatory factor 1 (KCMF1); Target recruitment subunit in a cullen 2 E3 ubiquitin ligase (Zer1); Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 related (ATR); Insulin-like growth factor-binding protein 3 (IGFBP-3); Interferon regulatory factor 1 (IRF1) and Interferon stimulated gene 3 gamma (p48); Mitosis phase phosphoprotein 2 (MPP2)

in the functional inactivation of pRB through the release of E2F transcription factors, thus permitting progression of the cell into S phase of the cell cycle. This property of the viral oncoproteins to complex pRB accounts, at least in part, for their ability to induce DNA synthesis and cellular proliferation. The high-risk HPV E7 proteins associate with the pocket proteins and induce their proteasomal degradation.^{61,287} The LXCXE motif within the CR2 homology domain of E7 is sufficient for pocket protein binding,^{166,422} but additional sequences located in the immediate amino terminal CR1 homology domain of E7 are required for pocket protein degradation,²⁸⁷ and these sequences are also necessary for the transforming activities of E7.⁴⁷⁰

E7 targeting the pocket proteins, including pRB, is not sufficient to account for its immortalization and transforming functions, indicating that there are likely to be additional cellular targets of E7 that are relevant to cellular transformation.²⁸³ Table 54.3 provides a list of various cellular targets with which E7 has been shown to bind. The physiologic relevance of many of these interactions is unclear.

Of particular interest are the reports that E7 can interact with cyclin-dependent kinase (cdk) inhibitors. As with E1A, HPV16 E7 interacts with and abrogates the inhibitory activity of p27^{kip1}.⁶⁸⁹ Because p27^{kip1} is involved in mediating cellular growth inhibition by transforming growth factor (TGF)- β in keratinocytes, this activity may contribute to the ability of E7 to override TGF- β -associated growth arrest.⁴⁷³ HPV16 E7 can also associate with p21^{cip1} and abrogate its inhibition of cdks as well as its inhibition of proliferating cell nuclear antigen (PCNA)-dependent DNA replication.^{194,286} p21^{cip1} is normally induced during keratinocyte differentiation,⁴¹⁵ and presumably its inhibition by E7 may be critical to allowing the replication of papillomavirus DNA in differentiated squamous epithelial cells.¹⁰¹

E7 is necessary for the stable maintenance of HPV episomes in epithelial cells,^{181,610} and sequences in E7 that contribute to cellular transformation are also important for the functions in the viral life cycle.^{373,610} Hence, the ability of E7 proteins to induce DNA replication through the release of E2F transcription factor

complexes and the inactivation of p21^{CIP1}^{194,286} and p27^{KIP1}⁶⁸⁹ is an essential component of the HPV replication strategy.

The high-risk HPV E7 proteins cause genomic instability in normal human cells.⁶⁵⁷ HPV16 E7 induces G1/S and mitotic cell cycle checkpoint defects and uncouples synthesis of centrosomes from the cell division cycle.⁴²⁰ This causes formation of abnormal multipolar mitoses, leading to chromosome mis-segregation and aneuploidy.¹⁵⁷ Moreover, there is an increased incidence of double-stranded DNA breaks and anaphase bridges, suggesting that in addition to numerical abnormalities, high-risk E7 proteins also induce structural chromosome aberrations.¹⁵⁸ Abnormal centrosome duplication rapidly results in genomic instability and aneuploidy, one of the hallmarks of a cancer cell. This activity is therefore likely to be functionally relevant to the contribution of high-risk HPV to malignant progression.

The HPV and BPV E7 proteins also bind to UBR4 (also known as p600).^{139,261} This binding appears to be conserved among all HPV E7 proteins (alpha as well as beta genera).⁶⁵⁸ The binding to p600 might therefore be important in mediating some of the pRB-independent functions of E7 such as modulating anoikis.¹³⁸ UBR4 may be involved in N-end rule ubiquitylation, but its cellular function remains to be determined.

The high-risk HPV E7 proteins have also been shown to reprogram cellular transcriptional programs. This has been reviewed recently.⁴⁰³ E7 binds to both canonical and noncanonical E2F family members^{270,402} and affects their transcriptional activities. E7 binds E2F6, a component of the polycomb repressive complex, and the detection of E2F6/polycomb repressive complexes is decreased in E7-expressing cells.⁴⁰²

The repressive H3K27 marks, which are necessary for binding of polycomb repressive complexes, are decreased in HPV16 E7-expressing cells due to the transcriptional induction of the KDM6A and KDM6B H3K27-specific demethylases.⁴⁰¹ HPV16 E7-mediated KDM6B induction accounts for expression of p16(INK4A).⁴⁰¹ Moreover, KDM6A- and KDM6B-responsive Homeobox genes are expressed at significantly

higher levels, indicating that HPV16 E7 results in reprogramming of host epithelial cells. These effects are independent of the ability of E7 to inhibit the retinoblastoma tumor suppressor protein.

What are the roles of the E6 and E7 oncoproteins in the normal life cycle of an HPV infection? It is likely that they function to allow the replication of the viral DNA. The viral E1 and E2 proteins are necessary for the initiation of viral DNA replication, but the virus is otherwise totally dependent on host cell factors, including DNA polymerase α , thymidine kinase, PCNA, and so on, for the replication of its DNA. These are proteins that are normally only expressed in S-phase during cellular DNA replication in cycling cells. Vegetative DNA replication for the papillomaviruses, however, occurs only in the more differentiated cells of the epithelium that are no longer cycling (see Fig. 54.5). Therefore, the papillomaviruses have evolved a mechanism similar to that of the polyomaviruses and the adenoviruses, to activate the cellular genes necessary for the replication of their own DNA in otherwise quiescent cells. These viruses may do so through the E7 proteins and their ability to release the E2F transcription factors by binding the pocket proteins including pRB (Fig. 54.13). In addition, E7 binds and inhibits the cdk inhibitor p21^{cip1} that is normally induced during keratinocyte differentiation, again presumably for the purpose of permitting viral DNA replication in a differentiated cell. The high-risk HPV E7 proteins, when expressed in the absence of E6, result in increased levels of p53 and in either a G1-mediated cell cycle arrest or apoptosis, depending upon the cell type. The mechanistic link between the pRB and p53 pathways is discussed in Chapter 7. E7 creates a signal that increases p53 levels. E6, by promoting the degradation of p53, counters this activity of E7 and permits the E7-dependent activation of the cellular DNA replication genes required for viral DNA replication.

HPV E5

Many of the papillomaviruses that induce purely epithelial papillomas (such as CRPV and the HPVs) contain E5 genes with the potential to encode short hydrophobic peptides. The structural similarity of these peptides to the BPV1 E5 protein has prompted studies of the potential transforming activities of the HPV E5 genes. The E5 proteins of the HPVs are required for optimal growth.^{175,201} In tissue culture, various HPV E5 genes have been shown to have some modest transforming activities, and in transgenic mice HPV16 E5 expressed in basal keratinocytes can alter the growth and differentiation of stratified epithelia and induce epithelial tumors at a high frequency.²⁰²

Although the biochemical mechanisms by which the E5 genes of the epitheliotropic papillomaviruses exert their growth stimulatory effects have not yet been fully elaborated, they may involve interactions with the epidermal growth factor receptor (EGFR) or the 16-kD subunit of the vacuolar ATPase, each of which has been shown to bind HPV E5 proteins.^{113,202,268,586,587,691} As with the BPV1 E5 protein, HPV16 E5 can bind the 16-kD subunit of the vacuolar ATPase and can inhibit the acidification of endosomes.^{113,502,586} It should be noted, however, that the E5 gene is not expressed in most HPV-positive cancers, suggesting that if the E5 gene does stimulate cell proliferation *in vivo*, it presumably functions in benign papillomas and not in the cancers. It might also par-

ticipate in the initiation of the carcinogenic process or in some other aspects of the viral–host cell interaction relevant to the pathogenesis of the HPV infection. Indeed there are some data that would implicate E5 in the down-regulation of major histocompatibility complex (MHC) class II antigen expression.⁶⁹⁰

Propagation and Assay in Cell Culture

PVs have proved difficult to propagate *in vitro* because these viruses replicate in stratified squamous epithelium, which is not mimicked in monolayer cultures. Most clinical identifications of PV infection, therefore, rely on a technique that identifies the viral DNA, such as polymerase chain reaction (PCR) or a molecular hybridization technique, rather than on virus replication in culture.

Although the species specificity of PV has also limited the utility of animals for the study of HPV, an early approach for HPV propagation was to first expose primary cultured epithelial cells to virus and then to place the cells under the renal capsule of nude mice.²⁵⁵ This immunologically protected site can support the growth of heterologous cells and foster formation of a multilayer epithelium that resembles a stratified squamous epithelium. The xenograft approach led to the successful propagation of several HPV types, although it is too cumbersome for routine virus isolation or for the molecular or biochemical analysis of virus replication.

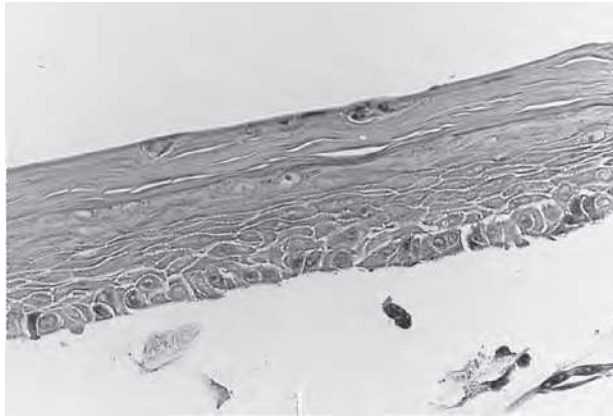
The complete virus life cycle can be recapitulated in cultured cells by using an organotypic “raft” culture system composed of stratified squamous epithelial cells placed at an air–water interface. This technique is more complex than growing cells in monolayers, and complete replication usually occurs in a small minority of the cells.^{106,410} It can be used, however, to analyze aspects of PV biology, genetics, and biochemistry (Fig. 54.15). Late gene expression can also be studied by suspending HPV-containing epithelial cells in semisolid medium.⁵¹⁶

Efficient methods for generating papillomavirus gene transfer vectors, designated pseudovirions, in monolayer cell culture have been developed.⁷⁴ The most widely employed procedure involves transient expression of codon modified L1 and L2 genes in SV40 T-antigen expressing cells in which high numbers of copies of a target pseudogenome containing the SV40 origin of replication are generated. Pseudovirions containing plasmids expressing marker genes such as GFP or secreted alkaline phosphatase are used in studies of virus infection and *in vitro* neutralization assays.⁴⁶¹ Transfection of authentic recombinant papillomavirus genomes together with an L1 and L2 expressing plasmid that is too large to be packaged in the capsids results in the production of “quasivirus” that can be used as substitute for lesion-derived virions in basic virologic studies.⁴⁸⁴

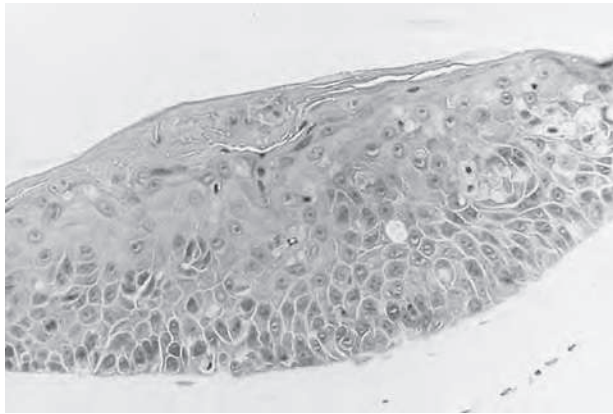
PAPILLOMAVIRUS INFECTION OF EXPERIMENTAL ANIMALS

The species-specific nature of PV has thus far prevented adaptation of authentic HPV infection to experimental animals. Despite these limitations, several useful animal PV models have been described.⁸⁰

Wild cottontail rabbits (*Sylvilagus floridanus*) represent a natural host for cottontail rabbit papillomavirus.⁵⁵³ Experimental studies of CRPV can be carried out in the natural host, but difficulties in maintaining cottontail rabbits under typical



A



B

FIGURE 54.15. Raft cultures showing normal keratinocytes (**A**) and keratinocytes transfected with the full-length HPV16 genome (**B**). The normal keratinocytes stratify and differentiate with an increase in the cytoplasmic-nuclear ratio in cells of the upper half of the epithelium and a prominent granular layer. In keratinocytes transfected with HPV16, while stratification takes place, differentiation is abnormal. There is hyperplasia of cells in the parabasal layer, and mitotic figures are observed in the upper half of the epithelium. No granular layer is seen, and the cytoplasmic-nuclear ratio does not change throughout the stratified epithelium. This morphology is similar to cervical intraepithelial neoplasia (CIN). (Courtesy of D. McCance.)

cage conditions have led to most CRPV studies being carried out with domestic rabbits (*Oryctolagus cuniculus*).⁶⁹ Cottontail and domestic rabbits are closely related species, which probably accounts for the ability of CRPV to infect domestic rabbits. CRPV can readily induce papillomas in domestic rabbits, where their persistence and progression to cutaneous cancer occurs more frequently than in cottontails. Although papillomas in cottontails usually contain large amounts of infectious CRPV, the lesions in domestic rabbits contain little or no infectious virus. Papillomas can also be induced by application of naked CRPV genomic DNA, permitting mutational analyses of the viral life cycle in this model.³²⁹ CRPV genomes have also been incorporated into the L1/L2 capsids of other papil-

lomavirus types and the resulting pseudovirions used to assess the protective capacity of HPV prophylactic vaccines.⁴⁰⁵

Rabbit oral papillomavirus was isolated from domestic rabbits.^{459,662} It is a model for oral mucosal infection without oncogenic potential. Although CRPV and ROPV are closely related phylogenetically, they are sufficiently distinct antigenically that resistance to one virus does not confer resistance to the other.

The canine oral papillomavirus (COPV) causes oral mucosal lesions.⁴³² The papillomas it induces usually regress 1 to 2 months after infection, which makes this model well suited to studying aspects of host defense mechanisms against PV infection.²⁸⁰

BPV1 is the prototype of a group of animal PVs, found in ungulates, that cause fibropapillomas.³⁴⁵ The ability of these PVs to induce nonproductive transformation of dermal fibroblasts leads to their having a wider host range than other PVs, although the increased host range may be limited to fibroblasts. Benign dermal tumors known as *equine sarcoid* appear to arise following accidental dermal infection of horses with BPV1 or BPV2.⁹⁰ An experimental counterpart is the ability to induce nonneuronal tumors in hamsters inoculated intracerebrally with BPV1.

BPV4 can induce oral mucosal lesions in cattle, as well as esophageal papillomas.⁸⁰ When cattle are fed bracken fern that contains chemical carcinogens, it can lead to esophageal cancers. One interesting feature of these cancers is that, although the BPV4 infection plays a role in their induction, the tumors themselves do not contain detectable BPV4 DNA.⁸² This represents a rare instance of virally induced tumors that arise via a "hit-and-run" mechanism.

A rhesus papillomavirus (RhPV) has been described that appears to be sexually transmitted between monkeys and to be associated with the development of cervical cancer.⁶⁷¹ RhPV and HPV16 are highly homologous, which suggests this animal model might have many similarities with human cervical infection with HPV. Infectious RhPV has not yet been isolated or propagated, however, severely limiting experimental analysis of this potentially useful model. The ability to generate RhPV quasivirions in cultured cells might increase the utility of this model.

Cervicovaginal infection by mucosatropic HPVs can be assessed using a pseudovirus-based mouse intravaginal challenge model.⁵⁰⁰ Infection is generally monitored by whole animal luminescence imaging after inoculation with luciferase-expressing pseudovirions. Similar models have been developed to examine cutaneous infection.⁶

The first and only laboratory mouse PV, designated MusPV1, was recently identified and its genomic sequence published.²⁸⁴ The virus was isolated from a colony of immunodeficient inbred mice that spontaneously developed papillomas at cutaneous surfaces near the mucocutaneous junctions of the nose and mouth. Although not yet extensively characterized, this domestic mouse papillomavirus represents a major new tool for applying the power of mouse genetics and immunology to questions of papillomavirus biology.

PAPILLOMAVIRUSES AND CANCER

Whereas some PVs do not appear to have oncogenic potential, a subset of PVs is clearly implicated in the development of

TABLE 54.4 Cancers Associated with Papillomaviruses

Species	Cancer	Predominant virus	Co-factors
Humans	Anogenital tract cancers	HPV16, -18	Tobacco
	Oropharyngeal cancers	HPV16	
	Nonmelanoma skin cancers in patients with EV	HPV5, -8	Sunlight
	Malignant progression of RRP	HPV6, -11	X-Irradiation
Cattle	Alimentary tract cancers	BPV4	Bracken fern
	Eye and skin cancers	Not characterized	Sunlight
Rabbit	Cutaneous cancers	CRPV (Shope virus)	Methylcholanthrene and coal tar (experimentally)

HPV, human papillomavirus; EV, epidermodyplastic verruciformis; RRP, recurrent respiratory papillomatosis; BPV, bovine papillomavirus; CRPV, cottontail rabbit papillomavirus.

malignancy in humans and animals^{69,403,429,699} (Table 54.4). In humans, these include several mucosal epithelial cancers, with cervical cancer being the most important from a public health perspective. HPVs are also implicated in other anogenital cancers, including anal cancer, vulvar cancer, and penile cancer, as well as in oropharyngeal and laryngeal cancers (Fig. 54.16). Although virtually all cases of cervical cancer are attributable to HPV infection, HPV accounts for only a portion of the other cancers. There may be concordant HPV infection at multiple mucosal sites,^{216,607} and patients who have had one HPV-associated cancer may have an increased risk of a second HPV-associated cancer.⁹⁶

In the developing world, cervical cancer accounts for more than 90% of the HPV-associated cancers,¹³² whereas the non-cervical cancers may represent close to one-half of the HPV-associated cancers in the United States and other industrialized countries (Fig. 54.17).⁸⁸ This difference is attributable to substantial reductions in the incidence of cervical cancer brought about by Pap smear screening, as well as an increasing incidence of HPV-positive anal cancer⁴³³ and, especially, HPV-positive oropharyngeal cancer.⁹⁵ In the developing world, less than 5% of HPV-associated cancers occur in males, whereas in the United States approximately 30% of these cancers arise in males.

HPV may also be involved in the cutaneous squamous cell cancers that develop in association with epidermodyplasia verruciformis (EV).⁴⁴⁴ HPVs have also been implicated in some cutaneous squamous cell cancers in the general popula-

tion or in immunosuppressed individuals, but the frequency is unclear.⁴³⁵ Some claims have been made of an association of HPV with several other common cancers, including those of the lung, breast, esophagus, colon and rectum, and prostate, but a consistent causal relationship has not been demonstrated.^{210,325} Except for esophageal cancer, the latter tumor types do not arise in stratified squamous epithelia.

In the well-studied examples of human cancers attributable to HPV, the tumors predominantly develop in a stratified squamous epithelium, they do not occur until many years after the initial infection, persistent infection is required for progression to invasive cancer, and maintenance of the transformed phenotype depends on the continued expression of at least some viral genes, especially E6 and E7. On the other hand, most infections, even those caused by HPV types implicated in cancer, have a benign outcome, either because they are self-limited or do not progress to cancer, even when persistent.

The long interval between the initial infection and the development of cancer implies that, in addition to persistent infection by an appropriate HPV type, additional environmental factors and/or host factors contribute to malignant progression. Immune status is one important host parameter, with impaired cellular immune function being associated with a greater risk of persistent infection and cancer.⁵⁷⁸ Adult patients with Fanconi anemia, an inherited disease with defective DNA repair, have a greater than 100-fold increased risk of developing solid HPV-associated tumors.^{8,340,380} Exogenous exposure

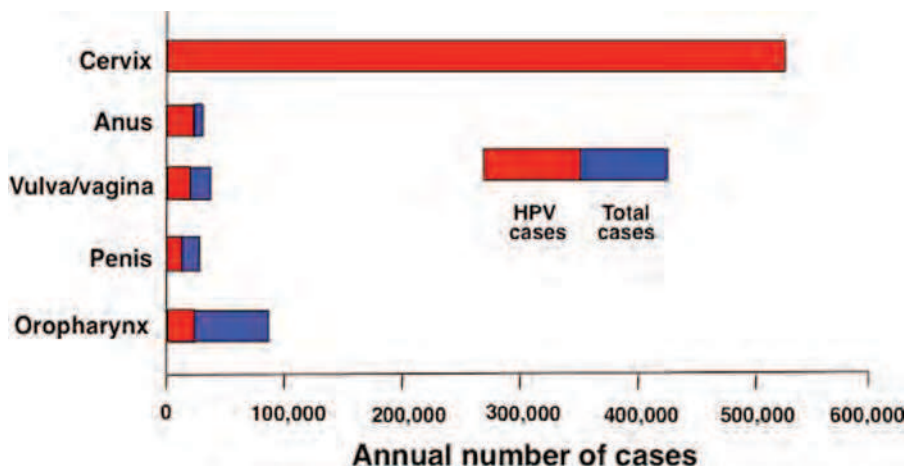
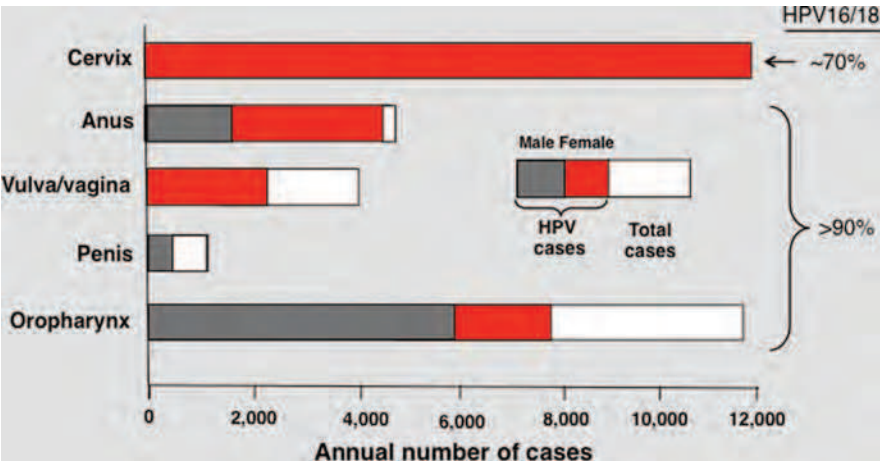


FIGURE 54.16. Worldwide incidence and distribution of cancers attributable to human papillomavirus (HPV). (Adapted from de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol* 2012;13:607–615.)

FIGURE 54.17. Annual incidence and distribution of cancers attributable to HPV in the United States (2004–2008). (Adapted from Human papillomavirus-associated cancers—United States, 2004–2008. *MMWR Morb Mortal Wkly Rep* 2012;61:258–261.)



of PV-induced lesions to cocarcinogens may represent an environmental mechanism. For example, most cutaneous cancers in EV occur on sun-exposed skin, which implies that UV light is serving as a co-carcinogen.

Most HPV-associated cancers appear to depend on the continuous expression of viral genes (e.g., E6 and E7). However, it remains possible that viral gene expression may not be obligatory at all stages for some HPV-induced cancers. In a small minority of human tumors that contain HPV DNA, viral gene expression seems to have been selectively suppressed.²²² Development of esophageal cancer in cattle by BPV4 and the carcinogen in bracken fern is associated with complete loss of the viral genome in the malignant tumor.⁴²⁹ This hit-and-run phenomenon suggests that in these instances the virus is required for tumor induction, but that its continued presence is selected against in progressed lesions.

HUMAN PAPILLOMAVIRUS INFECTIONS OF THE GENITAL TRACT

HPV infection of the anogenital tract represents the most important medical burden from infection with this group of agents (Table 54.5). Infection affects the genital skin and mucosa, including the vaginal tract, cervix, and anal canal. Most of these infections are sexually transmitted, which means that their prevalence is usually correlated with measures of sexual promiscuity, such as number of lifetime sexual partners, a recent change in sexual partner(s), and a history of other sexually transmitted infections.

HPV infection of the anogenital tract is extremely common, with a wide range of clinical manifestations and outcomes, varying from asymptomatic and self-limited, to persistent and associated with malignant progression. Viral factors and host factors each contribute to determining the outcome. The alpha-HPVs cause most anogenital infections, and the majority of oncogenic HPV types are from the alpha-7 and alpha-9 species (see Fig. 54.1).

Cervical Cancer

Cervical cancer is the third most common malignancy among women worldwide, with approximately 530,000 newly diagnosed cases each year and about 275,000 deaths annually.^{17,132}

Despite its worldwide distribution, the frequency of cervical cancer varies considerably, being about 10 times more common in some developing countries than in some industrialized ones. More than 85% of cervical cancer occurs in developing countries, where it is frequently the most common cancer of women, accounting for as many as one-fourth of female cancers. Pap smear screening has decreased the frequency of cervical cancer in industrialized countries. In the United States, approximately 12,000 new cases are diagnosed annually,⁸⁸ and about one-third of these women will die of their malignant disease. The

TABLE 54.5 Major Clinical Association of Genital Tract and Other Mucosal Human Papillomavirus (HPV)

Clinical association	Viral type(s)
Genital tract	
Subclinical infection	All genital HPV
Exophytic condyloma (any site)	HPV6, -11
Flat condyloma (especially cervix)	HPV6, -11, -16, -18, -31, others
Bowenoid papulosis	HPV16, -18
Giant condyloma	HPV6, -11
Cervical cancer	
Strong association	HPV16, -18, -31, -45
Moderate association	HPV33, -35, -39, -51, -52, -56, -58, -59, -68
Weak or no association	HPV6, -11, -26, -42, -43, -44, -53, -54, -55, -62, -66
Vulvar and vaginal cancer	HPV16
Penile cancer	HPV16
Anal cancer	HPV16
Recurrent respiratory papillomas	HPV6, -11
Conjunctival papillomas	HPV6, -11
Oral cavity	
Focal epithelial hyperplasia	HPV13, -32
Infection with genital HPV	HPV6, -11, -16
Lesions on lip	HPV2
Oropharyngeal cancer	HPV16

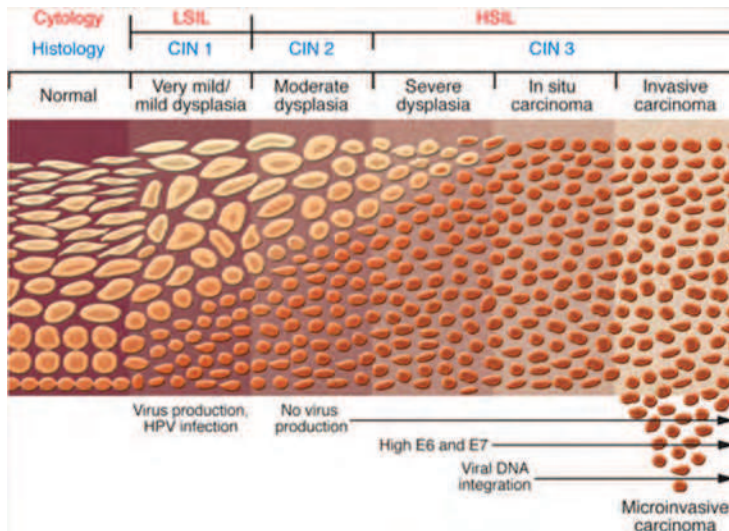


FIGURE 54.18. Progression from a benign cervical condylomatous lesion to invasive carcinoma. Infection by oncogenic human papillomavirus (HPV) types, especially HPV-16, may directly cause a benign condylomatous lesion, low-grade dysplasia, or sometimes even an early high-grade lesion. Carcinoma *in situ* rarely occurs until several years after infection. It results from the combined effects of HPV genes, particularly those encoding E6 and E7, usually after integration of the viral DNA into the host DNA and a series of genetic and epigenetic changes in cellular genes. HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia. (Modified from Lowy DR, Schiller JT. Prophylactic human papillomavirus vaccines. *J Clin Invest* 2006;116:1167–1173.)

incidence of cervical cancer in the United States varies considerably between ethnic and, especially, socioeconomic groups.¹⁹¹

Most cancers occur in the transformation zone of the cervix, where the columnar cells of the endocervix form a junction with the stratified squamous epithelium of the exocervix.^{439,527} About 85% of cervical cancers are squamous cell cancers. Most of the other cases are adenocarcinomas, with a small number being small cell neuroendocrine tumors.

Lesions that are destined to become malignant squamous cell carcinomas typically undergo a series of dysplastic changes over many years. The severity of the lesion is determined by the degree to which the squamous epithelium is replaced by basaloid cells, with the entire thickness being replaced in the most severe dysplasias. In the histologic classification of cervical intraepithelial neoplasia (CIN), grades 1, 2, and 3 correspond, respectively, to mild dysplasia, moderate dysplasia, and severe dysplasia or carcinoma *in situ* (Fig. 54.18). The cervical dysplasias have their counterpart in the exfoliated cells present in the Papanicolaou (Pap) smear, by the presence of basaloid cells and koilocytosis. In the cytologic Bethesda System,³³⁹ abnormalities are classified as low-grade and high-grade squamous

intraepithelial lesions (SIL) with low-grade corresponding to mild cytologic abnormalities, and high-grade encompassing the more severe abnormalities (Fig. 54.19). Atypical squamous cytology of undetermined significance (ASCUS) is the cytologic designation for equivocal lesions.

Most dysplasias do not progress and, in fact, resolve spontaneously, with the likelihood of resolution decreasing with the severity of the dysplasia (Fig. 54.20). More severe dysplasias, however, generally arise from less dysplastic lesions after several years,^{289,364} although some high-grade dysplasias can develop rapidly without passing through a low-grade stage.³²⁶ Because of the long interval between the development of cervical dysplasia and invasive cancer, Pap smear screening programs can identify most premalignant lesions. Appropriate follow-up of women with these abnormalities, together with appropriate treatment, can thereby prevent the development of most cases of cervical cancer. In some countries with screening programs, however, the incidence of adenocarcinoma and adenosquamous cell carcinoma has been increasing, suggesting that Pap smear screening is less effective in identifying the precursors to these tumor types.^{77,551,638}

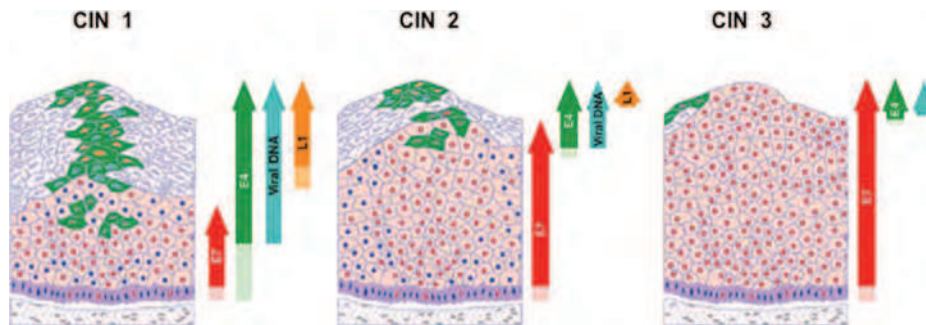
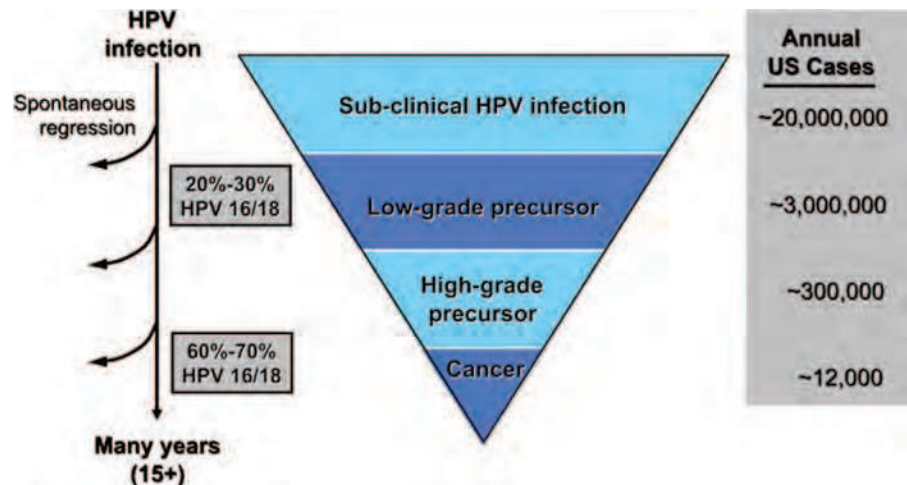


FIGURE 54.19. Changes in the human papillomavirus (HPV)-16 life cycle from cervical intraepithelial neoplasia (CIN)1 to CIN3. During progression, normal regulation of the papillomavirus life cycle is lost. CIN1 lesions generally resemble productive lesions caused by other supergroup A HPV types, and express virus coat proteins at the epithelial surface. In CIN2 and CIN3, the order of life cycle events is unchanged, but the extent of E7 expression is increased. (Reproduced from Doorbar J. The papillomavirus life cycle. *J Clin Virol* 2005;32(Suppl 1):S7–15, with permission.)

FIGURE 54.20. Natural history of cervical human papillomavirus (HPV) infection. Approximate number of U.S. cases of the different categories of infection. Most subclinical infections and low-grade dysplasias regress spontaneously. Even high-grade dysplasia has some potential to regress spontaneously. Infection with HPV-16 or HPV-18 represent a minority of subclinical infections and low-grade dysplasias, while they represent a majority of the high-grade dysplasias and invasive cancers.



Role of HPV in Cervical Cancer

Cervical cancer was recognized for decades to behave as a sexually transmitted disease, long before sexually transmitted HPV infection was implicated in its pathogenesis. In the mid-1970s, Meisels and Fortin⁴⁰⁴ recognized, on morphologic grounds, that HPV infection of the cervix occurred frequently, often with the histologic characteristics of mild CIN. These observations coincided with the proposal by zur Hausen⁶⁹⁸ that HPV infection might be the putative sexually transmitted agent responsible for cervical cancer. The CRPV system had provided the initial experimental evidence that animal PV could induce malignant tumors, whereas ROPV lacked oncogenic activity. The development of skin cancer in patients with EV had demonstrated that some HPV might have malignant potential.^{383,444} As with ROPV, only a subset of the HPV types isolated from patients with EV was found in the skin cancers, implying that HPV types might vary in their oncogenicity. The identification in the early 1980s of HPV16 and HPV18 by zur Hausen and colleagues^{56,162} provided the field with HPV types that could be shown to be present in most cervical cancers and could, by lower stringency hybridization techniques, identify HPV DNA in an even greater proportion of these tumors. Subsequent studies, carried out by many investigators, have sought to understand the natural history of HPV infection of the genital tract, determine the biologic properties of different HPV types, elucidate the role of the virus in the pathogenesis of cervical disease, and identify nonviral factors that may influence the outcome of HPV infection.

About 40 HPV types, mostly from the alpha genus, can infect the genital tract. Only a subset of these types, however, is found regularly in cervical cancers, and in a higher proportion than in controls, leading these types to be designated as *high-risk*. On the other hand, HPV types that are found less frequently in tumors than in controls are designated *low-risk*. A worldwide study of almost 1,000 cervical cancers, from paraffin-embedded sections, indicated that more than 90% contain HPV DNA.⁵⁵ The results confirmed and extended observations made in more restricted studies of cervical cancer.²⁷¹ Although more than 20 HPV types were found in the tumors, four types (HPV16, HPV18, HPV31, and HPV45), from the alpha-7 (types 18 and 45), and alpha-9 (types 16 and 31) species, accounted for close to 80% of the HPV-positive cancers. Conversely, low-risk HPV

(e.g., HPV6 and HPV11) was found in only two of these cancers. In almost all areas of the world, HPV16 is the most common type found in the cancers. Some regional variation exists in the specific proportion of HPV types.⁵⁶⁴ A follow-up evaluation of the cancers in the international study that were initially believed to be HPV-negative indicated either that they were false-negative results, or that the DNA in the specimens was too degraded for the negative results to be deemed reliable.⁶⁴² The conclusion from this study was that at least 99.9% of cervical cancers contain HPV DNA. Subsequent systematic assessment of a group of CIN3 and invasive cancers also supports the conclusion that virtually all bona fide cases are associated with HPV DNA.⁴⁹ Likely, cases reported as being HPV DNA-negative represent false-negative findings.

An international analysis of 1,918 cases of squamous cell cancers and 1,928 controls pooled from 11 case-control studies identified 15 HPV types as high-risk, three additional ones as probably high-risk, and 11 types as low-risk (Table 54.6).⁴²³ Of the cases, 90.7% were HPV DNA-positive. Eight of the HPV types (16, 18, 31, 33, 45, 52, 58, and 35) accounted for 95% of the positive cases. With two exceptions (HPV70 and HPV73), the phylogenetic relationship between the HPV types correlated perfectly with the high-risk or low-risk potential of the types. The odds ratios for developing cervical cancer associated with HPV16 and HPV18 were 434 (95% confidence interval [CI] 278–678) and 248 (95% CI 138–445), respectively. Although other high-risk types also had high odds ratios, HPV16 and HPV18 were the only HPV types whose lower limit 95% CI was greater than 100. These odds ratios are among the highest for a human carcinogen. In a worldwide meta-analysis of 14,595 cervical cancers, of which 89.7% were HPV-positive, HPV16 accounted for 55.2%, followed by -18 (12.8%), -45 (4.6%), -31 (3.8%), and -33 (3.7%).⁵⁶⁴ Prospective population-based studies in the United States and Europe that used progression from asymptomatic infection to CIN3 or worse (CIN3+) as the end point confirmed that HPV16 is the most oncogenic type, with the next most virulent types appearing to be HPV18, -31, and -33 (Fig. 54.21).^{316,528}

Whereas most studies have focused on the more common squamous cell cancers, most adenocarcinomas, adenosquamous carcinomas, and those carcinomas with neuroendocrine differentiation also contain HPV DNA.^{7,87} In an international

TABLE 54.6 Phylogenetic and Epidemiologic Classification of Human Papillomavirus (HPV) Types, Based on Presence or Absence in Cervical Cancer

Phylogenetic classification	Epidemiologic classification		
	High-risk	Probable High-risk	Low-risk
High-risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 82	26, 53, 66	70
Low-risk	73		6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, cand-89

analysis that pooled eight case-control studies, 81% of the tumors were HPV DNA-positive, and HPV16 and HPV18 accounted for 81% of these tumors.⁸⁷ More of these tumors are associated with HPV18, compared with squamous cell carcinomas, and in some studies the number of HPV18-associated tumors is greater than those associated with HPV16. Some evidence suggests that HPV18 infection may progress more rapidly and carry a poorer prognosis than HPV16.

The distinction between high-risk and low-risk HPV types has considerable utility, but should not be overinterpreted. Even low-risk HPV types may rarely be associated with cervical cancer.²²² HPV6 and HPV11 isolated from cervical cancers may have alterations in their genome that could have increased their oncogenic potential. Alternatively, an HPV type, such as HPV26, might be more oncogenic in an immunocompromised host.²³⁸ It should also be recognized that this classification may not apply to HPV infections in all sites. For example, the Buschke-Lowenstein tumor, which is a low-grade squamous cell carcinoma of the external genitalia, is usually associated with the low-risk HPV6 or HPV11.²²⁸ Laryngeal cancer complicating recurrent respiratory papillomatosis (RRP) may also involve these low-risk types,³⁶⁶ although most HPV-positive laryngeal cancers arise in patients without RRP and contain a high-risk HPV, especially HPV16.¹⁵⁹

Natural History of Genital Human Papillomavirus Infection

Genital HPV infection is considered the most common sexually transmitted viral infection, with an estimated life-time risk of at least 75%.^{26,239,530} The estimated prevalence of infection varies with the age of the population and depends on the sensitivity of the HPV assay employed. A recent population-based study of cervical infection in the United States, which used a very sensitive DNA-based test, found 42.5% of women age 14 to 59 were HPV-positive, with those age 20 to 24 having the highest prevalence, 53.8%, decreasing to 38.8% in women age 50 to 59 (Fig. 54.22). The infections were divided approximately equally between high-risk and low-risk types. Because sexually active women 19 or younger acquire genital HPV infection at a high rate,^{252,672} the comparatively low prevalence of 32.9% among those 14 to 19 probably arises because many in this age group are not yet sexually active. The decreasing prevalence of HPV infection beyond age 24 results from a combination of the self-limited nature of most infections, decreasing HPV exposure with age, and resistance to reinfection. It is known that HPV can establish latency,^{151,178,391} but it is not clear whether to attribute increased detection at this age to a decrease in immune surveillance, a cohort effect, or some other explanation.

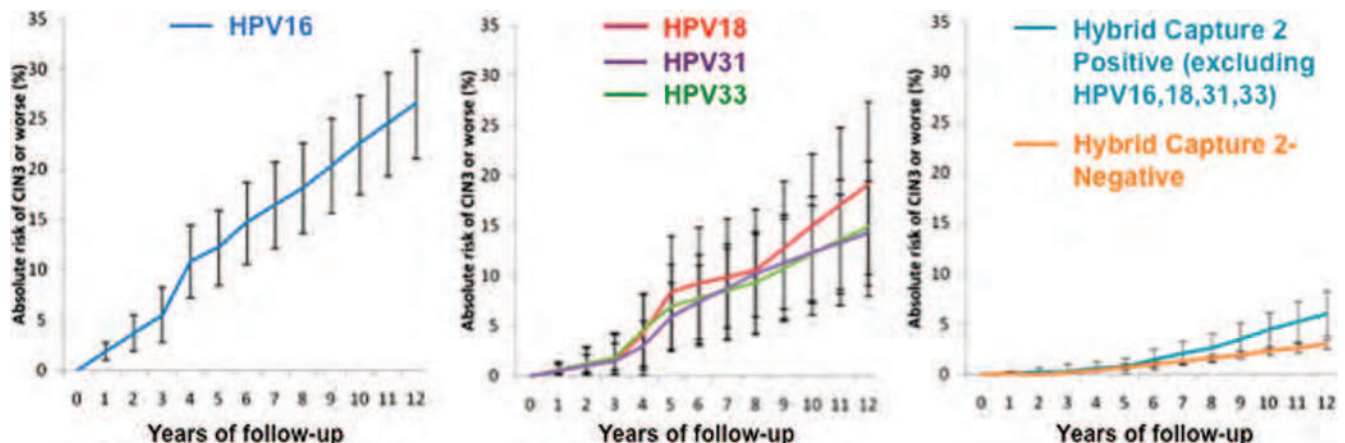


FIGURE 54.21. Data from the Danish Cohort Study shows that the human papillomavirus (HPV) type affects the rate of development of cervical intraepithelial neoplasia (CIN)3 or worse in women with normal cytologic findings at baseline.³¹⁶

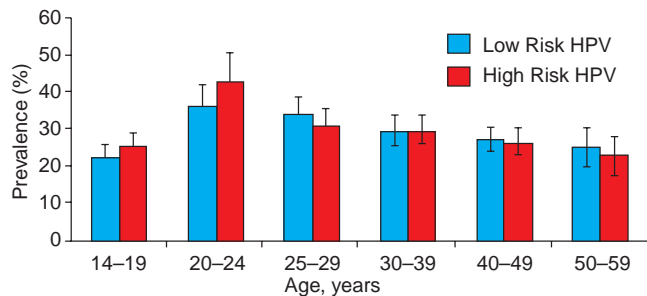


FIGURE 54.22. Distribution of prevalent cervical infection by high-risk and low-risk human papillomavirus (HPV) types in U.S. women 14 to 59 years old. (Adapted from Hariri S, Unger ER, Sternberg M, et al. Prevalence of genital human papillomavirus among females in the United States, the National Health and Nutrition Examination Survey, 2003–2006. *J Infect Dis* 2011;204:566–573.)

The natural history of genital HPV infection in men has been studied less systematically than in women.^{213,460} As with women, male infections are very common, and most are self-limited. In males, however, prevalence does not tend to decrease with age.²¹⁴ When controlled for lifetime number of sexual partners, male circumcision has been identified as an important negative risk factor for the prevalent infection in the men and for cervical cancer in their current sexual partners.^{4,86}

Although most women with HPV infection of the genital tract do not have detectable cytologic abnormalities, the epidemiology of genital HPV infection appears to account for the epidemiology of cervical neoplasia, including the dysplasias that precede cervical carcinoma.⁵⁴ Following the development of validated HPV DNA assays,^{497,566} many studies have consistently shown that infection with high-risk HPV represents the major risk factor for high-grade cervical dysplasias and invasive cancer.^{88,54,316,528} In cytologically normal women, being positive for a high-risk HPV places them at much higher risk

for developing cytologic abnormalities compared with women who are HPV-negative.^{316,326,528} In addition, results of a validated serologic assay that can identify currently and previously infected individuals implicated HPV16-related viruses prospectively in the development of cervical cancer.³⁵⁸

As noted above, most genital infections are self-limited, and the majority clear within 12 months.^{26,252,667,672} Clearance of HPV infection appears to return an individual to the same low risk of CIN3 or invasive cancer as an individual in whom HPV has not been detected previously. Low-grade dysplasia may be caused by infection with either low-risk or high-risk HPV. Persistent (i.e., long-term) infection with a high-risk HPV type, which occurs in a minority of infected women, is the single most important risk factor for developing CIN3 or invasive cancer (CIN3+). However, the magnitude of the risk depends on the HPV type (Fig. 54.21), and even on the variant within a given type.⁵³¹ In practical terms, persistence is usually defined as meaning that the same HPV type has been identified in two or more genital samples taken over a certain period, with the interval usually being 4 to 12 months. Persistent infections may clear spontaneously, but are less likely to do so the longer they persist. Conversely, only some persistent infections progress to CIN3, and only some CIN3 progress to invasive cancer (Fig. 54.23). HPV16 infections are more likely to persist than infection by other HPV types.³⁶⁵ However, persistence *per se* is not sufficient for progression to high-grade dysplasia, because low-risk HPV types that persist are much less likely to progress than high-risk types.⁵²⁹ The distinct biologic activities of high-risk E6 and E7 are likely to represent at least partial explanations of the differences in the likelihood of progression. Despite some enthusiasm for quantitative measurement of viral loads, their presence has not been consistently associated with progression to high-grade disease.^{377,678}

As persistent infection with a high-risk HPV constitutes the main risk factor for progression to CIN3 or invasive cancer and high-risk HPV DNA is found in virtually all cases of cervical cancer,⁶⁴² it has been concluded that HPV infection is

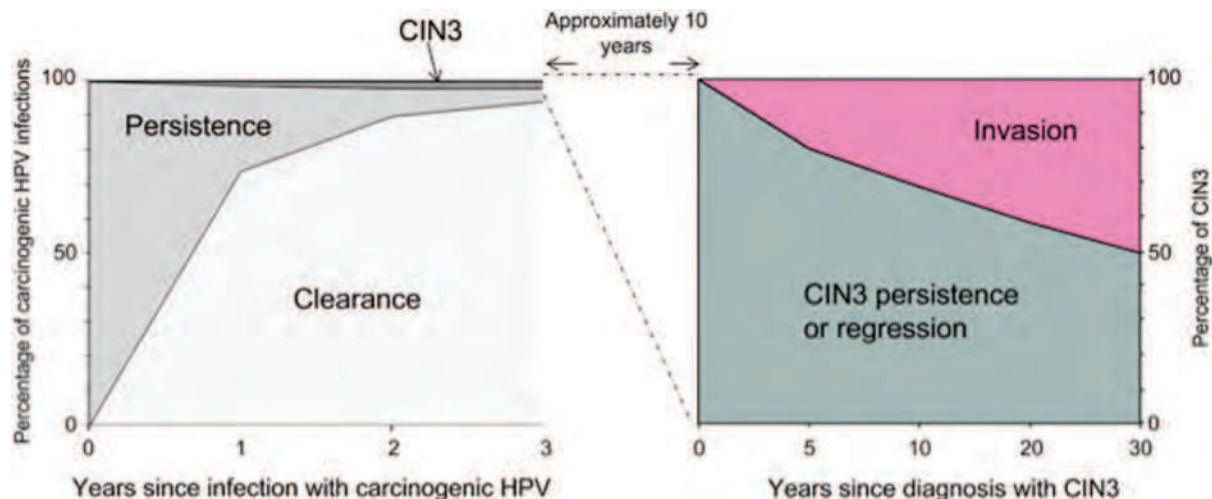


FIGURE 54.23. Risk of persistent human papillomavirus (HPV) infection and progression to cervical intraepithelial neoplasia (CIN)3 and invasive cervical cancer. (Reprinted from Schiffman M, Wentzensen N, Wacholder S, et al. Human papillomavirus testing in the prevention of cervical cancer. *J Natl Cancer Inst* 2011;103:368–383, with permission.)

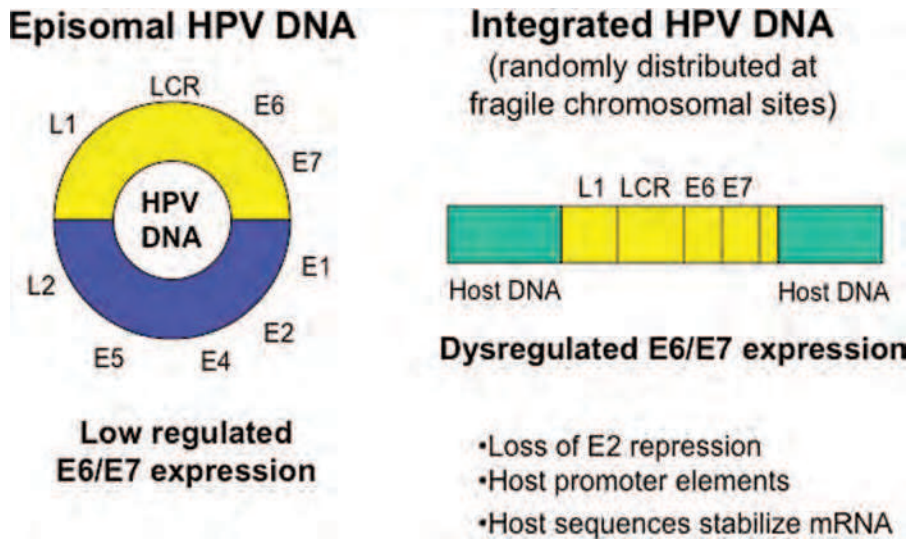


FIGURE 54.24. Integration of human papillomavirus (HPV) DNA results in high-level expression of E6 and E7. In low-grade lesions, the viral genome is maintained as an episome, which is associated with low level expression of E6 and E7. Viral DNA integration into the host DNA, which occurs in high-grade dysplasia or cancer, is usually associated with deletion of portions of the viral genome, with preferential retention of the long control region (LCR) E6-E7 region and the higher levels of expression of E6 and E7, attributable to multiple factors.

a necessary cause of cervical cancer. Persistent HPV infection, however, is not sufficient to cause cancer, because some individuals who have persistent infection with a high-risk HPV do not develop serious lesions.

Molecular Pathogenesis of Cervical Cancer

High-risk HPV types can infect the genital skin, the vaginal tract, or the cervix. If the cervix is not infected initially, the virus must spread locally, by autoinoculation, to the cervix for the individual to be at risk of developing cervical lesions. These can be single or multiple, and only develop in cells that have been infected. A recently identified subset of cervical cells in the transformation zone that expresses specific markers may be particularly susceptible to tumor development when they become infected.²⁴⁷ The production of progeny virions is usually limited to asymptomatic or low-grade lesions, as the full viral replication cycle is tied to the differentiation process.¹⁵¹

In high-grade dysplasias, a more restricted number of viral genes are expressed, primarily E6 and E7, and their expression is now found in the basal, proliferating layer of the epithelium, in contrast to most of their expression being in suprabasal cells during productive infection. E6 protein is expressed at substantially lower levels than E7, and in early lesions, the level of E7 may be more limiting than E6.⁵¹² Both genes are expressed from a single promoter, with alternate splicing determining their relative level of expression,⁶⁰⁶ and progression to high-grade disease may be associated with a splicing pattern that favors E7 production. E6 and E7, which have many activities, appear to be the main drivers for progression to high-grade dysplasia and cancer, by orchestrating a series of pathogenetic changes. Methylation of the HPV16 viral genome appears to change the risk of progression to moderate or high-grade disease, with methylation of CpG sites in L1, L2, and E2/E4 being associated with an increased risk of precancer,⁴¹⁴ whereas methylation of LCR sites is associated with a decreased risk.⁵⁹⁰

Integration of HPV DNA, via nonhomologous recombination, represents a key change that appears to stabilize the high expression of E6/E7 and is frequently associated with more severe lesions. In one study of 155 samples, integration was not detected in any normal or CIN1 lesions, but was present in 5%

of CIN2 lesions, in 16% of CIN3 lesions, and in 87% of invasive cancer.³¹⁹ Other studies suggest that integration occurs in a higher proportion of CIN3 lesions.²⁵⁴ The frequency of viral DNA integration may vary with the HPV type, being integrated in most tumors with HPV18 and -45, more than one-half of those with HPV16, and less than 40% of those with HPV31 and -33, using an assay that determines whether the viral RNA originates from integrated or unintegrated DNA.⁶³⁵ Viral DNA integration can occur at many sites throughout the genome, but is found preferentially at genomic fragile sites.^{615,653} In a given lesion, it usually involves only one locus or a few loci.⁶⁹⁵

Viral DNA integration is characteristically associated with deletion of large segments of the viral genome, and with transcription of sequences downstream from the integrated LCR (Fig. 54.24).⁵³⁷ In this integrated form, the E6 and E7 ORFs remain intact in the integrated viral DNA, and can be transcribed from the LCR, which lies upstream in the integration site. Experimental data suggest that disruption of the viral E1 and E2 genes, as well as of downstream viral sequences, may permit higher levels of E6 and E7 transcription,^{282,505} whose RNA may be stabilized following fusion to downstream cellular sequences, but integration is not invariably associated with higher levels of expression.²³² Cellular promoter elements near the integration site may also contribute to increased viral gene expression. When more than one viral DNA integration site exists, viral gene expression from a single site predominates via methylation of the other sites.^{292,630}

Viral DNA integration occurs in almost all tumors and tumor-derived cell lines that harbor HPV18, and in most with HPV16, although the DNA remains episomal in some HPV16 tumors and cell lines.^{122,635} Cell lines derived from cervical cancer continue to express E6 and E7, which appear to be necessary for their continued viability and their ability to proliferate.^{136,140} It is noteworthy that HPV16- and HPV18-positive cell lines derived from cervical and anal cancers harbor wild-type versions of p53 and pRb, in contrast to HPV-negative tumor-derived cell lines, many of which have mutant p53 and pRb.^{119,120,523,674} This observation implies that there is sufficient functional inactivation of p53 and pRb by HPV E6 and E7 and that there is virtually no selective pressure for the

genetic inactivation of the *p53* and *pRb* genes. Another consistent feature of dysplasias and cancer is that most cells express p16, as a cellular response to the E7-dependent inactivation of pRb,^{520,639,651} again in contrast to many tumors not associated with HPV, in which p16 tends to be silenced.

High-risk HPV induce abnormal centrosome duplication, which can result in genomic instability and aneuploidy.^{157,474} The deregulation of this mitotic event appears to depend on both E6 and E7, with the latter protein being more responsible for the effect.³²⁴ Aneuploidy may result in part from an association between E7 and NuMA (nuclear mitotic apparatus protein 1).⁴³¹ In a study that compared the incidence of aneuploidy and viral DNA integration, both were associated with increasing dysplasia.⁴⁰⁷ Although 95% (19 of 20) of lesions with integrated viral DNA were aneuploid, only 59% (19 of 32) of aneuploid lesions had integrated viral genomes. Because aneuploidy was found significantly more frequently than integration, it was concluded that deregulated viral oncogene expression results first in chromosomal instability and aneuploidization, which are subsequently followed by viral DNA integration.

The activation of telomerase (hTERT) represents another important HPV-dependent effect relevant to cancer.^{315,320} In human keratinocytes, this activity is induced primarily by E6 through a mechanism that is largely independent of p53 and probably includes an interaction with hTERT complexes.³⁷⁰ Efficient immortalization of keratinocytes by E6 and E7 probably depends on the ability of E6 to inactivate p53 and to activate telomerase, although E7 also contributes to this process, and keratinocyte immortalization can be induced by E7 plus either a mutant p53 or the catalytic component of telomerase. In the context of the viral genome, factors in addition to E6 may also contribute to hTERT activation.^{576,580} In cervical lesions, hTERT activation is associated with progression to severe dysplasia and cervical cancer, and cell lines derived from cervical cancers are uniformly positive for hTERT.^{303,567} A gene may exist on chromosome 6 that antagonizes HPV-induced activation of telomerase, as suggested by *in vitro* studies⁵⁸⁰ and by finding increased allelic imbalances at 6q14-q22 in CIN3 and cervical cancer with elevated telomerase activity.⁶²⁷

Although the studies mentioned above imply a central role for high-risk E6 and E7 genes in cervical cancer pathogenesis since tumors that contain only integrated HPV DNA have usually lost the E5 gene, evidence suggests that, in HPV16-associated tumors that retain extrachromosomal copies of the viral genome, E5 may also contribute to their pathogenesis. In one study, HPV16 E5 protein was detected in 12 (60%) of 20 of invasive cancers.⁹² Of the 12 E5-positive cases, 3 cases contained only episomal forms, whereas the other 9 had both episomal and integrated HPV DNA. The presence of E5 protein was significantly associated with the high-level expression of EGF-R and of the related ErbB4 protein. Consistent with the inference that E5 expression in the tumors has pathogenetic relevance, E5 in mice can stimulate hyperplasia that depends on EGFR,²⁰² and induce cervical tumors whose efficiency is increased by E6 or E7.³⁹⁷

Cellular Events in Cervical Cancer

Although infection with high-risk HPV may be necessary for the development of cervical cancer, or other cancers attributable to HPV infection, it is clearly not sufficient. Cancer must arise only after other factors have collaborated with the infection. As

discussed above, some changes (e.g., integration) may be virus-specific. Other changes associated with progression include genetic and epigenetic alteration of cellular genes. These alterations, which reflect the consequence of long-term viral gene expression, especially E6/E7, include downregulation of tumor suppressor genes and proapoptotic genes, or upregulation of protooncogenes and antiapoptotic genes.^{301,352,420,565} The specific changes are heterogeneous, although some occur relatively frequently. Epigenetic silencing of numerous genes has been reported in high-grade dysplasias and cancer.^{150,177,600,652} Tumors typically have several cytogenetic alterations, which implies loss or gain of function of several cellular genes. In most instances, the specific gene that has been affected by a given cytogenetic alteration has not been unequivocally identified. Whereas most of these cytogenetic changes are probably not a direct consequence of viral DNA integration, cellular genes can be directly affected by the viral integration. For example, viral DNA integration near the c-Myc protooncogene occurs in about 10% of cervical cancer and has been associated with its increased expression.^{160,653}

Consistent with the multistep nature of tumorigenesis, cervical cancers may show additional cytogenetic alterations when compared with adjacent dysplastic lesions.³⁰⁷ Some cytogenetic changes have been found in a relatively high proportion of tumors.³⁵² Those found in one-fourth to one-half of tumors include loss of heterozygosity in chromosome regions 3p14, 4p16, 4q21-35, 6p21-22, 11p15, 11q23, 17p13.3, and 18q12-22. Loss of heterozygosity in 3p has been identified in cervical dysplasias adjacent to cancers that also have this alteration.²³¹ This observation implies that inactivation of a putative tumor suppressor gene in this region may occur as an early event that could predispose to further progression. The *FHIT* (fragile histidine triad) gene, which is a putative tumor suppressor gene, is a candidate for the 3p locus. It is located at 3p14.2, and its expression is inversely correlated with the severity of dysplasia or cancer and with prognosis.^{44,258} The tumor suppressor of lung cancer 1 (TSLC1) gene, which is implicated in mediated cell adhesion, may be the tumor suppressor gene associated with loss of 11q23.^{579,683}

In contrast to the multiple loci with loss of heterozygosity, a gain of chromosome 3q24-28 has been reported to be present in almost all cervical cancers, but to be absent from most cases of severe dysplasia.²⁴⁹ The gene encoding the catalytic subunit of the class 1A phosphatidylinositol 3-kinase (PIK3CA), which is located in 3q26.3 and is part of the PI3 kinase/AKT signaling pathway, has been proposed as a candidate oncogene for this amplified locus.³⁸⁵ The PI3 kinase/AKT pathway, which promotes growth and is antiapoptotic, is active in most cervical tumors. This activity is associated with inactivation of the phosphatase and tensin homolog (PTEN) tumor suppressor gene and frequent PIK3CA amplification.^{41,102} EGF-R overexpression may also have prognostic significance.^{306,438}

Evidence suggests that cervical cancers have undergone changes that may help them to evade the immune system,^{190,294,582} whereas the presence of T regulatory cells in the tumor microenvironment may interfere with the ability of the immune system to mount a strong response against the tumor.^{472,625} The most frequent changes in the tumor cell involves the downregulation of HLA antigen class I alleles, which are normally expressed on keratinocytes, in contrast to class II.^{118,583} Consistent with a role in progression, downregulation

of one or more class I alleles occurs in most tumors, and is reported to be greater in metastases compared with the primary tumor. Patients who are HLA-B7 positive whose tumors downregulate this allele have a poorer prognosis, and selective loss of HLA-B7/40 allelic products is associated with lymph node metastases. Several mechanisms may account for the downregulation of class I expression.^{63,118,323} Some affect class I alleles directly by mutation or deletion, whereas others affect the class I processing system, primarily by downregulation of the TapI transporter, which is required for class I processing. The HLA antigen region is located on the short arm of chromosome 6, at 6p21.3, which is within the 6p21-22 region that, as noted above, has been reported to be a frequent site of cytogenetic abnormalities in cervical cancer. Therefore, it is likely that many of the cytogenetic rearrangements in this region reflect alterations in class I alleles. In addition to these class I changes, most primary tumors aberrantly express HLA antigen class II molecules, perhaps in response to inflammatory cytokines produced by infiltrating lymphocytes. Class II expression is not a feature of metastatic lesions.

Other Co-factors in Cervical Cancer

Because only a few women infected with high-risk HPV are destined to have a malignant outcome, host and environmental factors have been examined for their possible influence on the incidence of cervical cancer and its precursor lesions.^{85,93} When controlled for the presence of HPV infection, smoking, oral contraceptives, early age of pregnancy, and multiparity have consistently been found to be associated with increased risk,^{16,15,86} with nutrition, micronutrients, and other sexually transmitted diseases⁵⁶³ to be associated with increased risk in some studies. In most instances, it is not clear how the co-factor contributes to dysplasia and carcinogenesis. Possibilities include increasing the risk of establishing infection, decreasing local or

systemic immunity, stimulating the growth of HPV-infected tissue, and inducing mutations in infected tissue. It may be relevant that higher levels of a tobacco-specific carcinogen have been identified in the cervical mucus of cigarette smokers.⁴⁸³ Although not all studies have found positive results, specific alleles of some immune response genes appear to be associated with susceptibility to, or protection from, serious HPV infection. These include HLA antigen class I and II genes and killer immunoglobulin-like receptor (KIR) genes, which recognize specific HLA antigen class I allotypes.^{83,93}

Human Papillomavirus in Other Genital Sites Anogenital Warts (Condylomas)

As with cervical HPV infection, condylomas are most prevalent among young, sexually active adults, and their frequency usually parallels that of other sexually transmitted conditions (Fig. 54.25).^{26,318,460} The estimated prevalence in the United States is 1% of the sexually active population aged 15 to 49, with about 7% of women and 4% of men giving history of at least one episode.¹⁴⁶ The increase in sexual promiscuity that began in the mid-1960s was associated with at least a four-fold increase in the incidence of condylomas, which decreased by about one-third in the 1990s, and increased about twofold between 2000 and 2010. Infection may be subclinical.⁶⁰

Condylomas are usually exophytic lesions that are frequently multiple. They can arise anywhere on the external genitalia and can be found simultaneously in multiple sites. In men, they occur most commonly on the penis and anus, and in women on the perineum and anus. The anus can develop multiple lesions that coalesce to surround the anal canal. Condylomas are usually self-limited, regressing spontaneously or after local treatment, but some lesions can persist for years. As with other HPV infections, genital warts, when they arise in patients with impaired cellular immunity, can be extremely

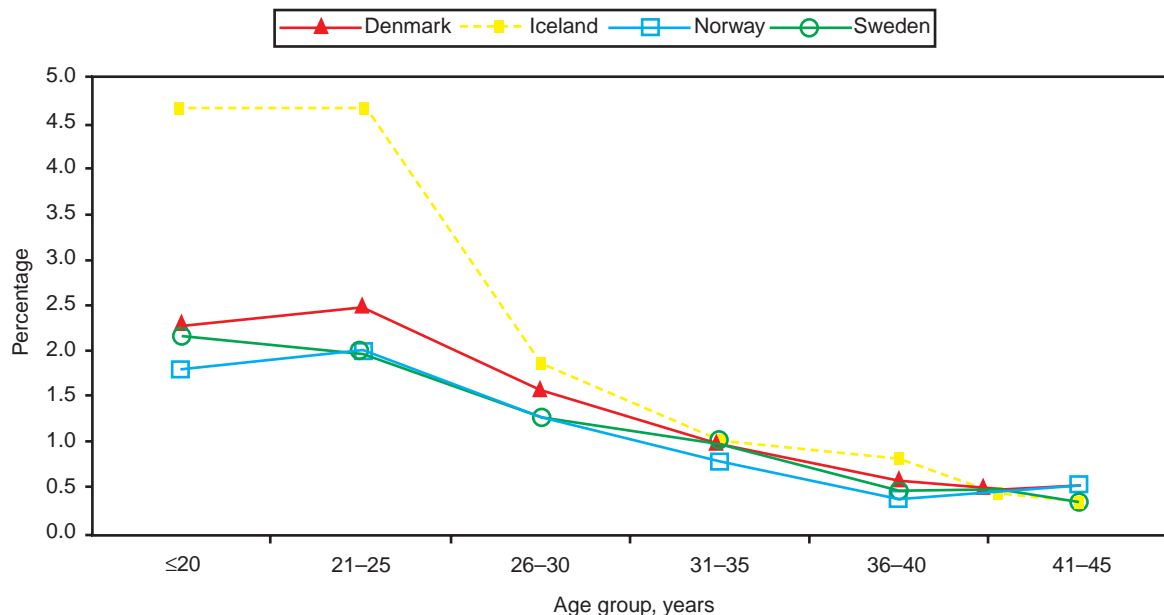


FIGURE 54.25. Proportion of self-reported clinically diagnosed genital warts during the last 12 months by age and country. (Adapted from Kjaer SK, Tran TN, Sørensen P, et al. The burden of genital warts: a study of nearly 70,000 women from the general female population in the 4 Nordic countries. *J Infect Dis* 2007;196:1447–1454.)

refractory to treatment. They can also increase in size and number during pregnancy, and regress following delivery. This sequence of events may also be a reflection of the immune suppression associated with pregnancy.

About 90% of genital warts are caused by HPV6 or HPV11, which are closely related to each other, with HPV6 predominating.²²³ Other HPV types, including HPV16, may also be found in these lesions. Bowenoid papulosis is a related entity.²²⁴ The lesions usually consist of multiple small papules that histologically resemble Bowen's disease or squamous cell carcinoma *in situ*. Most of these lesions contain HPV16, but the rate of transition to frank malignancy appears to be much lower for the external genitalia than for the cervix. Some genital warts in children may be sexually transmitted, secondary to sexual abuse.^{111,441} Genital lesions in children, however, can commonly result from virus inoculation at birth or from incidental spread from cutaneous warts. In contrast to anogenital lesions in adults, a significant proportion of genital warts in children contain HPV types that are usually isolated from nongenital warts, perhaps because, compared with adults, the genital skin in children is more susceptible to infection with these nongenital HPV types.

Vulvar, Vaginal, and Penile Malignancy

Genital HPV can routinely infect other genital areas that contain stratified squamous epithelium. The risks associated with HPV infection at these sites appear to be similar to that of cervical infection. HPV DNA, usually HPV16, is found in a subset of cancers of the vulva, vagina, and penis.^{20,562} Giant condyloma acuminata, also called the Buschke-Lowenstein tumor, is a low-grade, locally invasive squamous cell carcinoma that involves the external genitalia, most frequently the penis. It is associated with low-grade HPV types, such as HPV6 and HPV11.^{228,394}

In squamous cell carcinoma of the vulva, most invasive tumors designated morphologically as being warty or basaloid arise in younger women and contain HPV DNA, especially HPV16.²¹⁰ Smoking and infection with herpes simplex virus type 2 have been identified as possible co-factors for vulvar cancer. Vulvar and vaginal dysplasia occur more frequently in women with a previous history of cervical dysplasia,¹⁴⁸ and some evidence, based on virus-host DNA junction analysis, suggests that, in some instances, dysplastic cells may actually have been self-transplanted from the cervix to these sites.⁶³⁴ In contrast to basaloid vulvar cancers, HPV DNA is found in only a few keratinizing squamous cell carcinomas of the vulva, which is associated with older women and with mutation of p53.

Anal Cancer

Anal cancer shares important similarities with cervical cancer, as well as some differences. The disease is somewhat more frequent in women than in men, with about 3,100 female cases and 1,700 male cases per year in the United States.⁸⁸ The incidence has been gradually increasing since the mid-1980s, with a similar rate of increase in men and women between 1992 and 2004.²⁹⁰ Anal HPV infection appears to be sexually transmitted in most instances, and a history of receptive anal intercourse in women and of homosexual activity in men is associated with an increased risk for this cancer.¹⁹³ Such a history is often lacking, however, which makes it likely that some anal infections have spread from other genital areas. Consistent with this interpretation, simultaneous cervical and anal infection, often with the same HPV type, is common. The rate of anal infection by HPV appears to be

similar to that of cervical infection, although anal HPV infection has been studied less systematically than cervical infection.⁶⁶³ As with cervical cancer, high-risk HPV has been found in most anal cancers, with an even greater preponderance of HPV16 than in cervical cancer, and most anal cancers arise in the transition zone between columnar and squamous epithelium. In one large study from Scandinavia, 83% of HPV-positive tumors contained HPV16, and 93% of tumors from women were HPV positive, whereas 69% of the tumors from men were HPV positive.¹⁹³ A meta-analysis of anal cancer drew qualitatively similar conclusions: 86.5% of female cases were HPV positive, compared with 76.7% of male cases.²⁵³ It would appear, therefore, that most anal cancers in women are attributable to HPV infection, whereas some proportion of anal cancers in men may not be.

One important difference from cervical cancer is that the risk of anal cancer in the general population appears to be much lower than for cervical cancer, although it is not known what may account for these differences in rates of progression. In countries with effective cervical cancer screening programs, comparisons between the incidence of cervical cancer and anal cancer underestimate the difference in risk of progression because these programs prevent many potential cases of cervical cancer. In developing countries, however, where almost no impact exists of Pap smear screening on the incidence of cervical cancer, the incidence of anal cancer in women is less than one-tenth that of cervical cancer.

The risk of anal cancer among individuals who are human immunodeficiency virus (HIV) positive is much greater than in the general population, with especially high rates for HIV-positive male homosexuals.⁴⁵⁵ According to the AIDS-Cancer Match Registry Study in the United States, HIV infection was associated with a relative risk, for invasive cancer, of 6.8 for women and 37.9 for men.¹⁹² The difference in risk between women and men was attributable to the group of HIV-positive homosexual men, whose relative risk was 59.5, compared with a relative risk of 5.9 for male HIV-positive male intravenous drug users. As discussed in more detail below, a high proportion of men and women with HIV infection also have anal HPV infection and associated dysplasia.¹³³ Studies from San Francisco report anal HPV infection in 93% of HIV-positive male homosexuals (versus 61% of HIV-negative homosexual males), compared with 76% of HIV-positive women. These modest differences in gender-dependent infection rates suggest that the heightened risk of male homosexuals to anal cancer may result from a feature specific to receptive anal intercourse (e.g., trauma and/or the size of the viral inoculum) that collaborates with HIV and HPV infection.¹⁹² Consistent with this interpretation, at least some of the increased risk of male homosexuals to anal cancer seems to have predated the HIV era.⁴⁰⁶ The high risk of anal cancer among homosexual and bisexual men who are HIV positive has led some investigators to suggest routine screening of this population for anal squamous epithelial lesions.⁴⁵⁵

HPV INFECTIONS OF THE AERODIGESTIVE TRACT

Infections of the Oral Cavity

Benign HPV infections of the oral cavity occur commonly.^{209,481,597} They may be asymptomatic or associated with single or multiple lesions in any part of the oral cavity.

Genital-mucosal HPV types, especially HPV6, HPV11, and HPV16, have frequently been recovered from oral tissue. HPV is usually identified in at least one-half of papillomatous lesions in the oral cavity, but some lesions appear to be HPV-negative. HPV6 and HPV11 seem to be responsible for most benign HPV oral lesions, although HPV16 may also be found. Focal epithelial hyperplasia (FEH) is a well-defined clinical entity that occurs only in the oral mucosa. Its distribution is worldwide, but it is most prevalent in the indigenous populations of Central and South America and of Alaska and Greenland. In Greenlandic Eskimos, the prevalence in different localities varied between 7% and 36%. In contrast, the prevalence among Caucasian residents in the same localities was less than 1 in 300. Most infection is attributed to HPV13 or HPV32, two types that appear predominantly to infect the oral cavity. Maternal transmission of genital-mucosal types to newborns occurs, but its relevance to disease outside the oral cavity seems to be limited to recurrent respiratory papillomatosis, as discussed below.¹⁴⁴

Head and Neck Cancers

Cigarette smoking and alcohol consumption are two well-known risk factors for head and neck cancer. Although HPV does not appear to be involved in most cancers in the mouth (oral cavity),³⁶⁷ consistent data from several industrialized countries indicate that a subset of head and neck cancers are attributable to HPV infection.^{32,62,208,330,558} HPV16 accounts for about 90% of the HPV-positive tumors. Most of these HPV-associated cancers are located in the oropharynx, which includes the tonsils, tonsillar fossa, base of the tongue, and soft palate. It is not understood why the HPV-positive tumors preferentially develop in the oropharynx. In the United States, the incidence of these oropharyngeal cancers, which usually develop at a younger age than the HPV-negative cancers, increased more than threefold between 1988 and 2004 (Fig. 54.26).⁹⁵ If this trend continues, these tumors are predicted to account for more

HPV-positive cancers than cervical cancer, although the incidence of oral HPV infection is an order of magnitude lower than that of anogenital.^{209,239,333} About three-fourths of the patients with HPV-positive tumors are male, and most of the increased incidence is confined to white males. The decrease in tobacco use has been associated with a decreased incidence of HPV-negative oropharyngeal tumors. In the United States, HPV-positive tumors now account for more than two-thirds of the oropharyngeal cancers.

The behavioral risk factors for developing HPV-positive oropharyngeal cancer are similar to those for anogenital HPV-positive cancer.¹²⁴ A history of oral-genital sex is also an important risk factor. Some studies suggest that tobacco and alcohol do not increase the risk of HPV-positive tumors,¹²⁴ whereas others have reported they are associated with an increased risk for both HPV-positive and HPV-negative tumors.⁵⁵⁹ The HPV-positive tumors share many molecular features with those of HPV-positive anogenital tumors. Specifically, the oropharyngeal tumors usually have integrated HPV DNA that preferentially expresses E6 and E7, and their p53 and pRb genes are wild-type, and most of them express p16,^{10,288,341} in contrast to the HPV-negative tumors, which tend to have mutant p53 and to be p16-negative. Despite the similarities with HPV-positive anogenital cancers, there is thus far no clearly identifiable premalignant oropharyngeal lesion for HPV-positive tumors.

The HPV-positive oropharyngeal cancers carry a better prognosis than the HPV-negative ones, but cigarette smokers with an HPV-positive cancer have a poorer prognosis than nonsmokers.^{10,490} Blacks who develop oropharyngeal cancer tend to have a poor prognosis, which appears to be attributable to their very low incidence of HPV-positive tumors.⁵⁴⁶ The basis for this low incidence is unclear, as blacks are not resistant to HPV-positive anogenital tumors.

Recurrent Respiratory Papillomatosis (Laryngeal Papillomatosis)

Laryngeal papillomatosis is a rare condition the papillomas of which can severely compromise the airway, particularly in young children.^{204,298,348,554} Most lesions are caused by genital HPV types that are also associated with external genital warts, especially HPV types 6 and 11. The vocal cords of the larynx is the site most commonly affected, but papillomas can arise at other sites, such as the trachea, lungs, nose, and oral cavity, even without laryngeal involvement. Lesions can be single or multiple.

Symptomatic papillomas are generally treated surgically, but the recurrent nature of the disease means that periodic surgical treatment may be required. It is important to avoid the necessity of performing a tracheostomy in patients with RRP, because papillomas often grow along the tissues involved in the tracheostomy, resulting in severe morbidity.

Epidemiologic studies suggest at least two modes exist by which RRP develops.³⁴⁸ One primarily affects young children and the other when RRP is first diagnosed in older individuals (Fig. 54.27). RRP may occur at any age, but the risk of developing it decreases after the age of 5 years. In children younger than this age, a maternal history of genital warts is a risk factor for RRP. In a population-based study in Denmark, close to 1% of children whose mothers had a history of genital warts developed RRP, corresponding to a 231 times higher risk of RRP relative to women without a history of genital warts.⁵⁵⁵ First pregnancy and young age of the mother are associated with an increased

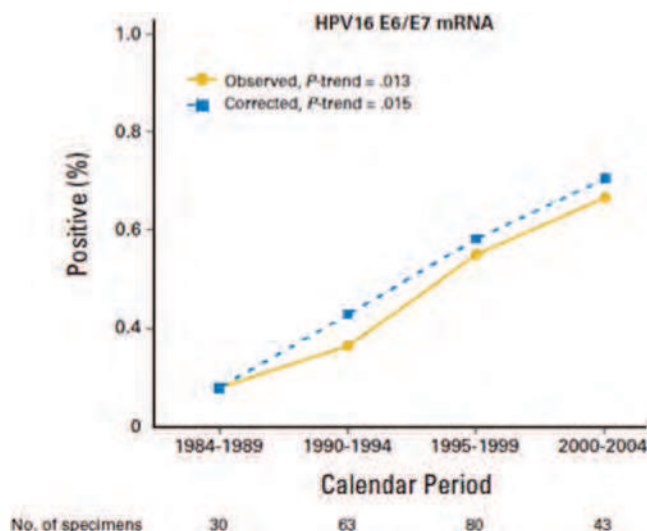


FIGURE 54.26. Increasing prevalence of human papillomavirus (HPV)-positive oropharyngeal cancers over time in the United States. (Adapted from Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol* 2011;29:4294–4301.)

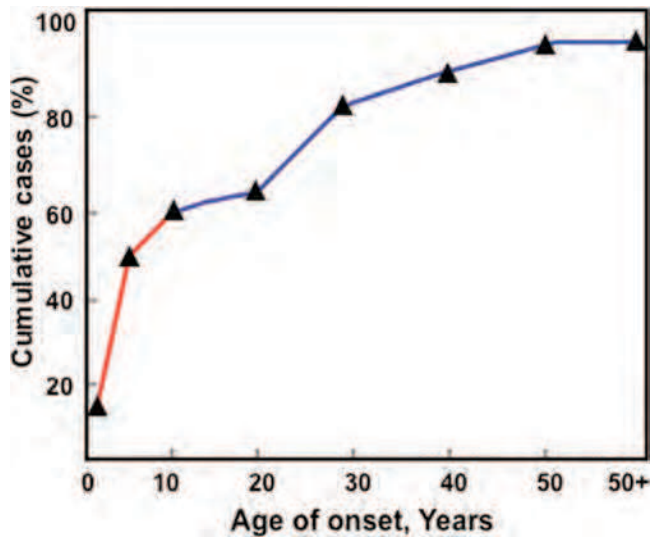


FIGURE 54.27. Cumulative percentage of cases of recurrent respiratory papillomatosis (RRP) in the United States. RRP in patients up to 5 years of age has a strong maternal inheritance component. (Adapted from Mounts P, Shah KV. Respiratory papillomatosis: etiological relation to genital tract papillomaviruses. *Prog Med Virol* 1984;29:90–114.)

risk, whereas cesarean section carries a lower risk.⁵⁴⁷ HLA antigen class II polymorphism may also be a contributing factor.²⁰⁰ Most cases of RRP that arise in early childhood are probably attributable to intrapartum transmission of HPV. *In utero* transmission also occurs, but it is rare. A history of oral-genital sex may be a risk factor in adult RRP. Despite the low incidence of juvenile-onset RRP, it has been estimated that annual U.S. medical costs for managing this condition is \$150 million.³⁴⁸

Although HPV6 causes genital warts more frequently than HPV11, the reverse is true of RRP, and cases with HPV11 tend to be more severe than those associated with HPV6.^{298,487,661} Given the presumed single exposure to HPV during vaginal delivery, the long and variable latent period of up to 5 years for childhood RRP remains to be explained. One possible explanation could be that viral latency is established in a proportion of exposed neonates before they develop symptomatic infection; latency has been shown in the larynx of patients with RRP who are in remission.³⁹¹

RRP is associated with a low risk of spread to the bronchi and lungs, progression to severe dysplasia, and even to cancer.²⁰⁴ The risk of such progression is increased if the papillomas have been subjected to X-irradiation, which was a common treatment of RRP in the 1940s. In contrast to cervical cancer, HPV6 and HPV11 in RRP are clearly associated with severe dysplasia and cancer, with HPV11 predominating, although high-risk types such as HPV16 may sometimes be found in laryngeal cancer not associated with RRP.⁶⁴

HUMAN PAPILLOMAVIRUS AND THE NONGENITAL SKIN

Nongenital Skin Warts

Nongenital skin warts are benign papillomas that occur most commonly on the hands and feet, although they can arise in

almost any location.^{78,277} They occur frequently in older children and young adults,²³⁵ with some surveys of adolescents indicating a prevalence of at least 20%.³⁰⁹ PV can remain infectious in the environment for extended periods, and transmission via fomites can occur relatively commonly.²⁰³ Because of the apparent need for PV to infect basal cells at the time of inoculation, maceration of the skin may predispose to cutaneous infection. Multiple lesions are most common, and they can be distributed symmetrically or be unilateral. Lesions involving apposing areas of skin or mucous membrane, as occur frequently, probably develop by autoinoculation. Most infections are present for several months and regress spontaneously within 2 years, although some persist indefinitely.

Although not absolute, a reasonably good correlation is seen between the clinical lesion and the HPV type. HPV1, HPV2, HPV4, HPV27, HPV57, and HPV65 are usually associated with common warts and plantar warts, including mosaic warts, whereas HPV3, HPV10, HPV28, and HPV41 are found in flat warts. Butchers and meat handlers may have an unusually high prevalence of hand warts; HPV7 is found in most lesions. It is believed that the combination of maceration and trauma predispose these workers to the development of clinical lesions when they are exposed to this HPV type.⁴⁷⁹

Regression of lesions is thought to be mediated immunologically. When most lesions regress spontaneously, there may be a mild mononuclear cell infiltrate, although regression of flat warts can be associated with an erythematous reaction around the lesions in association with an intense inflammatory infiltrate. The lower incidence of warts in older individuals⁴⁷⁹ may imply that immune mechanisms have rendered them relatively resistant to infection.

Epidermodysplasia Verruciformis

Epidermodysplasia verruciformis is a rare disorder in which affected individuals have a unique susceptibility to cutaneous HPV infection.^{387,444} The warts usually develop in childhood, become widespread, do not tend to regress, and, in some instances, may progress to squamous cell cancers. Two predominant types of lesions are seen, which can occur in the same patient. Some lesions have the appearance of flat warts, whereas others are flat, scaly, red-brown macules. The flat warts are caused by the same HPV types that induce flat warts in the general population, usually HPV3 or HPV10. The scaly lesions are associated with a range of beta-HPV types, especially HPV5 and HPV8. Patients with EV are frequently infected with several HPV types.

About one-third of patients with EV will develop skin cancers in association with their lesions. Most of the malignant tumors remain local, but regional and distant metastases may occur. The risk of malignant progression is limited to the pityriasis-like lesions, which are the lesions that contain the EV types. HPV5 and HPV8 seem to be the most oncogenic, because most of the skin cancers contain one of these two types. The skin cancers usually develop on sun-exposed areas, implying that carcinoma develops in EV by a combination of infection by an oncogenic EV HPV type plus the cocarcinogenic effects of UV light. p53 mutations are common in EV-associated cancer,⁴⁴⁹ in contrast to the mucosal cancers associated with HPV.

EV occurs as an inherited disorder in approximately one-half of affected patients, and many families with EV have a

history of parental consanguinity. Inheritance has an autosomal recessive pattern in most affected families, although one family with apparent X-linked recessive inheritance has been reported, as has another with apparent genetic dominance (no history of parental consanguinity, and EV in father and son).⁴⁰⁰ The cases with autosomal recessive inheritance are genetically heterogeneous, as the condition in different families have been mapped to two distinct chromosomal loci, at 2p21-p24 and 17q25.⁴⁸⁹ Two adjacent novel genes (EVER1 and EVER2) with nonsense mutations in some EV families have been molecularly identified at the 17q25 locus.⁴⁸⁸

Patients with classic EV do not ordinarily display an increased susceptibility to clinical infection with other microbial agents, including the mucosal HPV types or those HPV types associated with common warts. Because many healthy individuals have been found to harbor EV-specific HPV types in normal skin, patients with EV are unusual in that these HPV types produce clinically apparent lesions. The lesions in patients with EV are resistant to usual treatment modalities. Global defects in cellular immune function can be demonstrated in most, but not all, patients with EV. In addition, acquired EV has been described in association with HIV infection,^{36,279} and clinical lesions associated with EV-specific HPV types have been described in some other immunosuppressed individuals. These include patients with a genetically defined subset of severe combined immunodeficiency disease³⁴²; some with common variable immunodeficiency syndrome^{342,641}; and a family with a T-cell defect attributable to a premature stop codon in the RHOH gene, a GTPase expressed predominantly in hematopoietic cells, which renders the patients susceptible to multiple infections, including clinical lesions attributable to beta-HPV.¹¹⁷

In familial classic EV, the genetic lesion is present in the cutaneous keratinocytes as well as the cells of the immune system. It is therefore difficult to infer unambiguously whether the susceptibility to clinical lesions in classic EV is attributable mainly to an epithelial cell defect, immune deficiency, or both.⁴⁴⁴ With acquired EV, by contrast, the defects have been limited to various types of immunodeficiency. This observation implies the development of acquired EV is attributable to immune defects that render the cutaneous keratinocytes permissible for the development of clinical lesions. Molecular analyses of the EVER genes, which interact with the zinc transporter ZnT-1,³⁵¹ do not yet permit a clear answer for classic EV. On the one hand, keratinocytes that are null for EVER2 grew faster than wild-type keratinocytes and had higher nuclear zinc levels.³⁵¹ On the other, compared with their expression in keratinocytes, the EVER genes are expressed at substantially higher levels in T cells, and T cells from EVER2 patients have elevated zinc levels and impaired T-cell activation.³⁵⁰

Nonmelanoma Skin Cancer

Nonmelanoma skin cancers (NMSCs), which are extremely common, are generally subdivided into basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). They generally arise on exposed areas of skin, most commonly in light-skinned individuals. Sunlight exposure is a predominant risk factor, and tumors associated with sun exposure are usually locally invasive, but only rarely metastasize. The skin of immunosuppressed individuals is at high risk of developing warts as well as premalignant lesions and NMSCs, especially SCCs, on exposed areas.^{227,445}

The known oncogenic potential of at least some HPV types and the consistent finding of certain beta genus HPV types in SCCs associated with EV make HPV infection an attractive etiologic agent for at least some NMSCs in individuals who do not have EV.¹⁷⁶ Beta HPVs are known to encode candidate oncoproteins that can interfere with UV-induced apoptosis,²⁷⁸ which might allow keratinocytes with UV-induced mutations to survive and progress to carcinomas. Beta HPV DNA is frequently detected in SCCs using sensitive PCR-based detection methods, but it is also frequently detected in normal skin.^{58,482,548} Furthermore, the genome copy number is usually much less than one copy per tumor cell,⁶⁴⁸ in contrast to HPV-associated anogenital and oropharyngeal cancers. A recent study employing an unbiased analysis involving high-throughput sequencing of randomly primed mRNAs detected virtually no HPV transcripts in SCC specimens.¹⁹ Weak or no association was also observed in L1-based serologic assays for individual cutaneous HPV types,^{58,477} although an association with seropositivity to beta HPVs in aggregate was detected in some analyses.

Overall, the association between HPV infection and NMSCs must be considered weak at present, since continuous expression of predominant HPV types has not been as clearly identified in NMSCs as in EV-associated cancers or mucosal cancers associated with HPV. In addition, the HPV-positive tumors are not clinicopathologically distinct from the HPV-negative ones, in contrast to vulvar or oropharyngeal cancer. It remains formally possible that cutaneous HPVs induce SCCs but are not required for their maintenance,⁶⁴⁸ and there could be strong biological or immunological selection for their loss during progression. Alternatively, the detection of HPV DNA may be an epiphenomenon. This latter possibility needs to be seriously considered, because healthy skin often contains HPV DNA, especially of beta types,¹² and removal of the superficial layers of NMSC lesions has been reported to result in a drastic reduction in the proportion of HPV-positive lesions.¹⁸⁵

HUMAN IMMUNODEFICIENCY VIRUS AND HUMAN PAPILLOMAVIRUS INFECTIONS

The immune suppression resulting from HIV infection is associated with an increased susceptibility to infection by many other microbial agents. The risk of genital HPV infection may be particularly high for HIV-infected individuals because infection by both viruses is often acquired via sexual transmission, and it is well known that exposure to one sexually transmitted agent places an individual at higher risk for exposure to other sexually transmitted agents. Therefore, individuals at high risk for sexual exposure to HIV would tend to be at high risk for exposure to genital HPV infection.

It is well documented that cervical HPV infection is detected more frequently in HIV-positive women than in those who are HIV-negative; the infection is even more likely to persist, it is more likely to result in cytologic abnormalities, and a much greater risk is seen for high-grade dysplasia.^{451,591} In one prospective study of high-risk women in New York, cervical swabs taken at the initial examination detected HPV DNA approximately twice as frequently in HIV-positive women compared with HIV-negative women (56% versus 31%), and persistent infection was six times as frequent in the HIV-positive women (24% versus 4%).⁵⁹¹ Low-grade dysplasia

(CIN1) was about three times as frequent in HIV-positive women (13% versus 4%), whereas high-grade dysplasia was seven times more frequent (7% versus 1%). Low CD4 counts in HIV-positive women represent an independent risk factor. The rate of oral infection is also higher in HIV-positive individuals.²⁹

Consistent with the cervical findings, retrospective studies from New York reported that an unexpectedly high proportion of cervical cancers diagnosed in women younger 50 years of age were in HIV-positive women,³⁸⁶ leading the Centers for Disease Control and Prevention (CDC) in 1993 to designate cervical cancer as an acquired immunodeficiency syndrome (AIDS) case-defining illness. Analysis of large cohorts has confirmed that HIV infection places women at increased risk of developing cervical cancer.^{96,192,545} HIV infection represents a risk factor for other malignancies associated with HPV infection, including cancers of the vulva, penis, and anus.¹⁹² As noted, the relative risk for anal cancer is especially high for HIV-infected male homosexuals. HPV types with limited malignant potential in the HIV-negative population may be associated with aggressive tumors in HIV-positive patients.^{237,238}

In contrast to the inverse correlation between CD4 levels and the risk of HPV infection and dysplasia, the risk of invasive cancer may not be associated with low CD4 levels.²⁷⁵ In addition, highly active antiretroviral therapy (HAART) has been associated with only a modest reduction in HPV-associated dysplasia, and no reduction in HPV-associated cancers in most studies.^{104,134,275,413,450} EV lesions also appear to be resistant to HAART.²⁷⁹ These observations are in contrast to the substantial HAART-associated reduction in the incidence of Kaposi's sarcoma-associated herpesvirus (KSHV)-associated tumors and with Epstein-Barr virus (EBV)-associated non-Hodgkin's lymphoma.

Immunity

PVs typically induce persistent epithelial infection with little or no pathologic consequence to their host. This generally peaceful co-existence reflects a basic viral strategy of minimizing host cell damage and thereby the immune responses to its presence.¹⁸⁹ Although some infections, particularly with some species of the HPV beta genus, may persist lifelong in an occult fashion, most infections are cleared, or controlled to undetectable levels, in a span of months to years. Incident infection rates tend to decline with age after they peak, consistent with the acquisition of effective immunity. Both humoral and cellular immunity likely play a role in preventing, controlling, and/or clearing PV infection, so the virus has evolved mechanisms to evade both arms of the immune system.

The fact that PVs have evolved into hundreds of genotypes that are essentially distinct serotypes that cannot be effectively cross-neutralized by antibodies induced by natural infection strongly implies that such antibodies play an important role in virus-host interactions.⁶²⁶ It is unlikely that virion antibodies play a role in clearing infections because the virion proteins are not expressed on the surface of living cells, and so are not susceptible to known antibody-mediated mechanism of immune regression. However, neutralizing antibodies induced by infection likely play a role in preventing successive rounds of autoinoculation, reinfection from an external source, and perhaps spread to another host. The latter can be readily envisioned for genital mucosal infections, where the virions are shed into mucus that can also contain virion-neutralizing antibodies.⁴²⁸

Virion antibody responses have been best characterized for genital infections in women. They are generally not detected until months after initial infection, and the titers induced are modest.¹⁴³ Response may be somewhat more robust for genital wart types than high-risk types.⁸⁴ Many infections clear without the induction of a measurable antibody response. There is some disagreement in the literature over whether virion antibodies measured after natural infection can protect from reinfection.^{518,636} However, these studies are confounded by difficulties in distinguishing reinfection from reactivation of latent infection and in distinguishing low level of virion-specific from non-specific antibodies. It seems quite likely that women with higher than average virion antibody titers to mucosal types in their serum are afforded at least partial protection from type-specific reinfection.⁵¹⁸ Most adults are seropositive for L1 antibodies to one or more cutaneous HPVs, and the antibody responses tend to persist.¹³ The role of these antibodies in the natural history of cutaneous HPV infection is not well understood.

Substantial antibody responses to the early viral proteins are seldom detected, except in the case of invasive cancers. In cervical cancers, responses to E6 and/or E7 are detected in approximately half of the patients.⁶³⁷ Contributing factors in the weak humoral responses to viral infection include the absence of viremia, the low level or absence of the viral proteins in the basal layers of the epithelium, the release of the virions onto external skin or mucosal surfaces, and the limited inflammation induced by the superficial nonlytic infections. Low intrinsic immunogenicity of the viral proteins does not seem to play a role. For instance, the capsids can be highly immunogenic when exposed to the systemic immune system, as exemplified by the VLP vaccine studies discussed below.

Adaptive cell-mediated immunity likely plays a key role in inducing the regression of PV-induced lesions and preventing reactivation of latent infection. When multiple warts are present, it is not uncommon for removal of one wart to be followed by the regression of others, suggesting a systemic immunologic response triggered by release of antigen from the treated wart is responsible for regression. Patients with conditions that affect T-cell immunity, such as pregnancy, immunosuppressive chemotherapy, or HIV infection, are at increased risk of HPV infection that persists, as well as an increased risk of cervical cancer.^{18,133,179,192} Likely, at least some of these infections represent reactivation of latent infection, rather than being secondary to new exogenous exposure. Even benign HPV infections in immunosuppressed patients are notoriously difficult to treat, and reduction of the immune suppression may be associated with the spontaneous improvement of the HPV-induced lesions.

The immune effectors mechanisms and viral antigens involved in lesion regression and prevention of reactivation are not well defined. Descriptive studies of cell-mediated immune responses have mostly focused on genital infections in women. Until recently, most studies have evaluated systemic responses, whereas local mucosal responses are likely to be the critical determinants. Modest systemic CD4+ and CD8+ cytotoxic T-cell (CTL) responses to E6 and E7 are often detected in women with previous or current cervical HPV16 infections.^{170,425,426} Interestingly, failure to clear infection was associated with lack of induction of CTL to E6, but not E7.⁴²⁵ In women with HPV16-associated CIN1, CD4 T-cell responses to E2 and E6 were most frequently detected, and Th1-type CD4 responses to

these viral proteins are also most frequently detected in women without infection.^{131,650,670} In a prospective study, the presence of HPV16 E2–specific responses in an interferon-gamma enzyme-linked immunosorbent spot (ELISPOT) assay correlated with lack of progression of HPV16–associated lesions.⁶⁷⁰

The local tissue responses in regressing cervical lesions were recently characterized. Granzyme B–expressing CD8 T cells predominated, and a higher ratio of CD8 and Foxp3–negative CD4 cells over CD4 Foxp3–positive (regulatory) CD4 T cells was detected.^{447,669} In contrast, the infiltrates in persistent and progressing lesions were associated with a suppressive environment, including granzyme B–negative and PD-1–expressing T cells (a sign of exhaustion) and high numbers of Foxp3+ Tregs, and macrophages.^{236,276,297} Studies in E7 transgenic mice suggest that persistent epithelial expression of the viral proteins in a non-inflammatory setting can actively tolerize CTL precursors.¹⁴⁷

In a prospective study of high-grade HPV16–associated CIN lesions, infiltration of CD8+ T cells into the epithelium was observed in lesions destined to regress.⁶²⁰ They were excluded from the epithelium in lesions that persisted, although infiltrates in the submucosa adjacent to the lesions were frequently observed. The results suggest that dysregulation of T-cell trafficking and/or adhesion plays a role in viral immune evasion. Finally, loss of epidermal Langerhans cells (the primary professional antigen presenting cell in the epidermis) has been documented in mucosal and cutaneous lesions caused by HPVs of the alpha, gamma, mu, and nu genera, which may help to create an environment permissive for viral persistence.³⁶⁰

The induction of cancers by certain PV types is an aberration to both the host and the virus, since cancers are too dedifferentiated to produce virions. Therefore, carcinogenesis plays essentially no role in the evolution of papillomavirus immunobiology. However, immune evasion during carcinogenic progression is of obvious clinical interest. HPV-induced cancer and high-grade dysplasias are genetically unstable and therefore readily able to evolve to evade immune recognition. The majority of cervical cancer and CIN3 lesions have defects in MHC class I presentation, generally through downregulation of specific class I alleles or defects in antigen processing for class I presentation.^{53,323} Therefore, it appears that most tumors have undergone strong selection for escape from CD8+–mediated immune responses.

Given that PVs have evolved to persistently infect their host, it is not surprising that their early proteins have specific activities involved in inhibiting immune recognition and/or clearance. The E5 of several PV types, including BPV1, BPV4, HPV2, HPV6, and HPV16, has been shown to downregulate the expression of cell surface MHC class I molecules, which can reduce recognition of E5–expressing cells by HPV-specific CD8+ T cells.⁸¹ In addition, HPV16 E5 was recently been shown to increase cell surface GM1 ganglioside.⁵⁹³ Because gangliosides are known to inhibit killing by CTLs, their upregulation by E5 may be another mechanism of preventing immune clearance.

Type I interferons are involved in control of most viral infections, by direct inhibition of virus replication and by aiding in the induction of adaptive immune responses.³⁷⁶ PV E6 and E7 genes blunt type I interferon responses by several specific mechanisms. HPV16 E6 binds interferon regulatory factor-3 (IRF-3) and in so doing inhibits its ability to act as a transcriptional transactivator for interferon gene expression in response to viral infection.⁵¹⁰ Viral genes also disrupt inter-

feron signaling. HPV16 E7 inhibits induction of interferon alpha inducible genes by specifically disrupting the interferon-stimulated gene factor 3 complex (ISGF3).²⁵ HPV18 E6, but not HPV11 E6, was also shown to interfere with interferon alpha activity by binding Tyk2, a molecule involved in interferon alpha receptor 1 signaling.³⁶³ Expression of E6 and E7 of high-risk types can also downregulate expression of TLR9, the pattern recognition receptor of the innate immune system that recognizes CpG oligonucleotides, and IL-8, a potent chemoattractant of T cells and neutrophils.^{241,259} The E6 and E7 of both low- and high-risk types inhibit transcription of MIP3 alpha, a potent chemoattractant of Langerhan's cells.²²⁹

DIAGNOSIS AND TREATMENT

Human Papillomavirus Diagnosis

The approach used for the diagnosis of HPV infection may depend to a considerable degree on the underlying goal for making the diagnosis. These goals can include a determination of whether HPV is present, whether an active infection is present, which HPV type(s) is associated with the infection, and the degree of cellular atypia associated with the infection. If routine *in vitro* propagation of HPV from clinical samples were available, its isolation from productive infections, theoretically, would be possible. No such assays exist, however, and their utility would be limited by the fact that high-grade dysplasias and cancers do not produce infectious virus. The potential use of virus-specific antisera in tissue sections or extracts is limited by analogous considerations. L1 and E4 proteins can be expressed at high levels in productive lesions, but not in high-grade dysplasias and cancers.¹⁵¹ The expression of E6 or E7 proteins should be higher in advanced lesions, but clinical utility has not yet been validated.⁵⁴⁰

Serologic assays in an enzyme-linked immunosorbent assay (ELISA) format that monitor the antibody response to L1 in VLPs may have utility for population-based research studies,^{84,143,272} and a high-throughput neutralization assay with comparable sensitivity has also been developed.⁴⁶¹ However, these assays, which measure both current and past infection, are not sufficiently sensitive or specific to be used for routine clinical diagnosis. Invasive cervical cancers, but not the premalignant lesions, are associated with serum antibodies against E6 and E7.⁴⁰⁹ Most patients with HPV16–positive oropharyngeal cancer have detectable anti-E6 and/or E7 serum antibodies.⁵⁵⁷

On the other hand, sensitive, reproducible, and robust molecular assays have been developed to detect HPV DNA and RNA in cervical swabs and biopsy samples, several of which have been approved by the FDA.^{272,291,437,532,601} Approaches for such assays include PCR consensus primers (or alternative amplification systems) that can be used in conjunction with a reverse line blot for specific hybridization, synthetic RNA probes that capture viral DNA, real time PCR, and microarrays. Some assays detect L1 DNA sequences, whereas others detect E6 or E7 DNA or RNA. The most sensitive tests may be preferred if the goal is to detect as many infections as possible.

For cervical cancer screening, the goal is to maximize identification of those infections associated with high-grade abnormalities (true positives), which usually should be treated, while excluding as many infections with low-grade abnormalities (false positives) as possible, which should not be treated

because most are destined to clear spontaneously.^{300,499,509,528} Achieving this balance means that the signal used for screening must exceed a certain threshold, in order to reduce the number of false positives, while still identifying most true positives. Some assays use a cocktail that can detect more than 10 high-risk HPV types, whereas other assays are type-specific. The greater oncogenicity of HPV16, and HPV18 to a lesser extent, may also argue for the importance of knowing whether a patient is infected with either of these HPV types.

Other biomarkers may also have the potential, as stand-alone or ancillary tests, to contribute to distinguishing between those HPV infections associated with precancer and those that are not. The most advanced is p16, which is elevated in response to the inactivation of pRb by E7 from high-risk viruses.^{503,520,644} Methylated viral DNA or methylation of specific cellular genes,^{168,414} as well as other markers, are also being evaluated.^{569,654} Although population-wide screening efforts are directed toward cervical cancer, the high risk of anal cancer among men who have sex with men has led some investigators to consider screening high-risk populations for this cancer.⁴⁵⁰

Since its introduction in the 1950s, cytologic screening with Pap smears, the main goal of which is to prevent cancer by identifying premalignant lesions that can then be treated, has been the basis for the substantial reduction in the incidence of and mortality from cervical cancer. The greater sensitivity of HPV-based assays gives them several advantages over cytology. These include the greater negative predictive value of HPV testing, which can permit longer intervals between screens, and the greater sensitivity of HPV testing in detecting cervical adenocarcinoma precursors, which cytologic screening tends to miss.^{300,499,509,532} In the United States, cervical cancer screening guidelines now recommend cytology-based screening every 3 years for women 21 to 65 years of age or co-testing with cytology and a U.S. Food and Drug Administration (FDA)-approved HPV test every 5 years for women 30–65.⁵²¹ HPV testing is also recommended for women with equivocal (ASCUS) Pap smears. Screening only by cytology is recommended for women younger than 30 because of their high prevalence of HPV infection without severe dysplasia, together with the likelihood that most infections in these young women will clear spontaneously. Primary HPV-based screening has been initiated elsewhere,⁴⁹⁹ and it seems likely that the United States will also make this shift in the future, especially if there is high uptake of the preventive HPV vaccine.⁵

In the developing world, the cost of cytologic screening has been too high for implementation of population-wide cervical cancer screening. The development of inexpensive HPV tests that are suitable for screening may soon make it cost-effective for population-wide screening in developing nations.⁴⁸⁶ The limited resources in developing countries make it likely that women there could not be screened more than three times in a lifetime. Under those circumstances, HPV testing can reduce cervical cancer mortality in women older than 30 years of age, whereas the lower sensitivity of cytology (or visualization of cervical lesions with acetic acid) limit their effectiveness in such settings.⁵¹⁹

Treatment

In cervical HPV infection, treatment of low-grade dysplasia is not usually warranted, given that most of these lesions will clear spontaneously. High-grade dysplasias represent precancerous lesions that are unlikely to resolve spontaneously, and their

treatment is recommended to prevent cervical cancer. Depending on the setting, treatment of cervical dysplasia can be surgical, with cryotherapy, via loop electrosurgical excision repair, or by laser. In most instances, this approach prevents cervical cancer. HPV testing can be used in this setting, because most successfully treated cases become negative for HPV DNA, whereas incompletely treated cases may remain positive.⁶⁹⁶ Cervical cancer is treated by surgery, radiotherapy, and chemotherapy, with early stage tumors having a better prognosis than more advanced tumors.^{392,504}

Given what is now known about key molecular events in HPV infection, considerable potential exists for developing antiviral therapies against HPV in addition to immunotherapy.^{187,577,581,665} An antiviral that targeted a molecular activity common to all HPV types, or at least to a large number of them, such as the interaction between high-risk E6 and E6AP, might have the theoretic advantage of being active against multiple types, in contrast to the predominantly type-specificity of most viral antigens. Antisense and ribozyme approaches may also have some potential, but their activity is likely to be type-specific.

Two immunomodulatory agents, interferon and imiquimod, are approved for use against genital warts, although destructive therapy is often used to treat these lesions. In placebo-controlled trials, intralesional and parenteral interferon therapy was active against refractory genital warts,¹⁰⁹ whereas topical imiquimod, which activates TLR7 and induces interferon and other cytokines, was also effective.^{199,220,680} Neither agent, however, cures more than two-thirds of treated patients.

Interferon therapy of recurrent respiratory papillomatosis has been less successful than that of genital warts, and surgery remains the principal therapeutic modality, with only a few patients appearing to derive therapeutic benefit. Controlled trial results with antivirals have been disappointing.^{89,424,550}

As with other HPV infections, no specific antiviral therapy is available for nongenital warts. Most treatments are aimed at destroying the lesional tissue while causing as little long-term damage as possible to the surrounding normal tissue. No treatment will cure all warts, which has led to the wide range of therapies. At least partial regression can be obtained with many therapies, but even complete clearance that is then followed by recurrence is usually of limited clinical value. Traditional therapies include topical application of caustic agents (e.g., salicylic acid, podophyllin), cryotherapy, inhibitors of DNA synthesis (5-fluorouracil), and surgical therapy or laser treatment.

PREVENTION

Interruption of Transmission

As noted above, the epidemiology of various forms of HPV infection can differ drastically. Therefore, approaches to control genital HPV infection would be distinct from those to control nongenital infections. Pap smear screening can prevent most cervical cancers, which represent the most serious public health consequence of HPV infection but presumably do not have appreciable impact on transmission.

Public health efforts that can prevent other sexually transmitted infections should, in principle, also be effective in preventing genital HPV infection. Indeed, decreased sexual promiscuity on the part of both sexual partners should reduce the likelihood of exposure to genital HPV. In the absence of

specific treatment that would eradicate genital infections, investigation of sexual partners of infected individuals would not be expected to have much impact on HPV transmission, although it may help identify infected individuals who otherwise might not realize that they are infected.

Although HPV can infect genital areas that are not covered by condoms, condom use may reduce the incidence of genital HPV infection. Consistent with this possibility, several studies in men have reported that condom use is associated with fewer genital HPV infections and a shorter duration of infection in those men who were infected.^{434,460} For women, a meta-analysis found no consistent evidence that condom use in their sexual partners reduced the risk of acquisition of cervical HPV infection, but it was associated with some protection against genital warts, CIN2, CIN3, and cervical cancer.³⁸⁸ However, in a prospective study, consistent condom use by their partners reduced cervicovaginal HPV infection in young women by two-thirds.⁶⁶⁶ As another possible intervention measure, circumcision of adult men was shown to reduce their acquisition of high-risk HPV infection by almost one-half after 2 years.⁶¹⁷

HPV topical microbicides are under active evaluation. Carrageenan, a complex polysaccharide derived from red algae, is a potent inhibitor of HPV infection of cultured cells. In vivo, it mitigates the potentiating effects on cervicovaginal HPV pseudovirus infection induced by nonoxynol 9—containing over-the-counter spermicides and Pap smear collection in mouse and macaque models.^{76,500,501} Carrageenans are widely used in food and cosmetic products and are the main gelling agent in some over-the-counter sexual lubricants. In a trial designed to test the efficacy of a carrageenan gel in prevention of HIV infection, some protection against HPV infection at the exit visit was observed, but only in the subgroup that was most compliant with use instructions.³⁹⁰ Clinical trials designed to formally evaluate carrageenan gels as HPV microbicides will soon commence.⁴⁶⁵ Epidemiologic findings, discussed earlier, suggest that most cases of juvenile-onset RRP that arise in infants and young children have been transmitted from exposure to the mother's HPV during vaginal delivery. This hypothesis raises the possibility that the use of cesarean section for the delivery of infants of mothers with known genital HPV infection would reduce the risk of HPV exposure. The incidence of genital HPV infection is high, however, whereas that of RRP is low, which means that many cesarean sections would be needed to prevent each case of RRP. These considerations suggest that the morbidity and mortality risk to the mother associated with cesarean section may, in most situations, be greater than the risk of RRP to the baby, which has led some investigators to argue against the widespread use of this procedure to prevent RRP. However, because the risk of RRP is greater for younger women and for first pregnancies, a subset of HPV-positive women may exist for whom the cost-to-benefit analysis might favor cesarean section.⁵⁴⁷ HPV vaccination with Gardasil (discussed below) or use of an HPV topical microbicide might be effective interventions to reduce the rate of RRP, although it would be difficult to demonstrate their effectiveness in a clinical trial because of its low incidence.

Prophylactic Vaccination

The important public health consequences of genital HPV infection make it highly desirable to develop an effective vac-

cine against those HPV infections associated with cancer, especially cervical cancer. Vaccines to prevent oncogenic HPV infections could potentially prevent the full spectrum of HPV-associated cancers, in contrast to current screening programs that are only effective at preventing squamous carcinoma of the cervix. Although it would be ideal if a vaccine could both treat established infection and induce long-term protection against incident infection, efforts to develop therapeutic vaccines, have thus far had limited success in human clinical trials. Most HPV studies have focused on prophylactic vaccine strategies, because of the success of preventive vaccines against other viral diseases.⁴⁷⁸

Early studies with CRPV indicated that systemic injection with papilloma suspensions that did not produce detectable infection could induce serum neutralizing antibodies and protect rabbits against high-dose cutaneous viral challenge.⁵⁵² Intradermal injection of a vaccine composed of formalin-inactivated COPV virions was shown to protect against COPV-induced oral lesions under field conditions.³³ This result demonstrated that systemic immunization can induce protective immunity against natural transmission of a mucosal PV infection. However, the viral determinant(s) that conferred protection were not definitively identified in these studies.

The inability to produce preparative amounts of HPV virions, together with the presence of oncogenes in the viral genomes, suggested that a subunit vaccine would be a preferred approach. A key observation in the development of a prophylactic vaccine was finding that L1 can self-assemble into VLPs, which are empty capsids that closely resemble those of authentic virions morphologically and immunologically.^{234,313,511} As with authentic virions, L1 VLPs were highly immunogenic, inducing high titers of neutralizing antibodies that were conformation-dependent and type-restricted.³⁸¹ In several animal PV models, systemic immunization with L1 VLPs induced strong protection against high-dose experimental challenge by the homologous virus.^{68,108,312,595} Protection was type-specific, of long duration, and could be passively transferred with immune immunoglobulin G (IgG), implying that neutralizing antibodies were sufficient for protection. L2 also contains neutralization epitopes, but they are not exposed in the context of the mature virion.⁵³⁴ Therefore, although co-expression of L1 and L2 in cells results in L1/L2 VLPs, these VLPs were neither more immunogenic nor more protective than L1 VLP.⁶⁸ Human trials, therefore, went forward with L1 VLPs, with the main focus on HPV16 and HPV18, the two most oncogenic HPV types. Despite the excellent results with the preclinical models, none represented a genital infection, and it was unclear how relevant these models would be to genital infection under natural conditions. In addition, most vaccines protect against systemic infection that has a viremic phase, which exposes the virus in contact with antibodies in the blood, whereas HPV induces an infection that spreads locally.

Two commercial prophylactic HPV vaccines have been developed. GlaxoSmithKline's Cervarix is a bivalent vaccine composed of L1 VLPs of HPV16 and HPV18, whereas Merck's Gardasil is a quadrivalent vaccine composed of L1 VLPs of HPV6, -11, -16, and -18. In addition to their valency, the two vaccines differ in production system, adjuvant, and recommended injection schedule (Table 54.7). Both vaccines were generally safe and able to consistently induce high titers

TABLE 54.7 Characteristics of HPV VLP Vaccines

	Cervarix	Gardasil
Manufacturer	GlaxoSmithKline	Merck
VLP Types	HPV16/18	HPV6/11/16/18
Dose of L1 Protein	20/20 µg	20/40/40/20 µg
Producer Cells	<i>Trichopulsia ni</i> (Hi 5) insect cell line infected with L1 recombinant baculovirus	<i>Saccharomyces cerevisiae</i> (Baker's yeast) expressing L1
Adjuvant	500 µg aluminum hydroxide, 50 µg 3-O-deacylated-4'-monophosphoryl lipid A	225 µg aluminum hydroxyphosphate sulfate
Injection Schedule	0, 1, 6 months	0, 2, 6 months

Gardasil® (Merck & Co., Whitehouse Station, NJ USA)

Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium)

HPV, human papillomavirus; VLP, virus-like particle.

of capsid-reactive antibodies in early phase trials.⁵³³ In phase III clinical trials of young women, both vaccines were also highly effective at preventing acquisition of cervical infection and low- and high-grade CIN caused by the types targeted by the vaccine (Table 54.8). These results have led to worldwide licensure of the two vaccines for prevention of CIN and cervical cancer caused by the vaccine-targeted types. Remarkably, Cervarix was recently shown to induce a high level of protection against persistent cervical infection 4 years postvaccination, even after administration of only one or two doses of the vaccine.³³² Both vaccines also induced a modest degree of protection against cervical infection caused by specific nonvaccine types closely related to HPV16 or -18. For instance, both vaccines induced partial protection against persistent infection by HPV31. Cervarix, but not Gardasil, induced significant protection against HPV45, and neither vaccine protected significantly against HPV35 or -58.^{72,248,656}

Gardasil was also highly effective at preventing genital warts, and vulvar and vaginal intraepithelial neoplasia, and it has been licensed for these indications^{145,317} (Table 54.8).

Cervarix was not evaluated for these end-points, probably because it does not target HPV6 and HPV11, the types that cause most genital warts. Neither vaccine induced significant clearance of established infections or regression of established lesions, and so they are not licensed for treatment of HPV infection or disease.^{195,250}

Prophylactic protection by the two vaccines against vaccine-targeted types does not appear to be waning in the more than 5 years since initiation of the VLP vaccine trials. Neutralizing antibody titers induced by the vaccines have remained essentially stable, and above the levels induced by natural infection, since the second year postvaccination period, supporting optimistic projections for long-term, perhaps lifelong, protection without the need for booster immunizations.^{507,514} However, cross-neutralizing titers against related types, when they are detected, are much lower than the titers against the vaccine-targeted types,³⁰⁴ and there are indications that protection against these types may wane over time.⁵⁰⁷

Gardasil was also tested for efficacy in men. Strong protection from genital warts was documented²¹⁴ (Table 54.8).

TABLE 54.8 Four-Year Efficacy of HPV VLP Vaccines Against Vaccine Targeted Types in According to Protocol Analyses of Phase III Clinical Trial

Study	Vaccine	Sex/age	End point	Efficacy	95% CI	Reference
PATRICA	Cervarix	Females 15–25	CIN2+	94.9%	87.7–98.4	(359)
FUTURE I/II	Gardasil	Females 15–26	CIN2+	100%	94.7–100	(317)
FUTURE I/II	Gardasil	Females 15–26	VIN2+/ValN2+	100%	82.6–100	(317)
FUTURE I/II	Gardasil	Females 15–26	Genital Warts	99.0%	96.2–99.9	(145)
CVT	Cervarix	Female 18–25	6-Month Persist. Infect.	90.9%	82.0–95.9	(248)
CVT	Cervarix	Females 18–25	Anal Infection at Exit	83.6% ^a	66.7–92.8	(332)
Merck 020	Gardasil	Males 16–26	Genital Warts	89.4%	65.5–97.9	(214)
Merck 020	Gardasil	Males 16–26	6-Month Persist. Infect.	85.6%	73.4–92.9	(214)
Merck 020	Gardasil	Males 16–26	AIN2+	74.9%	8.8–95.4	(452)

95% CI, Ninety-five percent confidence interval; PATRICIA, Papilloma TRial against Cancer In young Adults; FUTURE, Females United To Unilaterally Reduce Endo/Ectocervical Disease; CVT, Costa Rica Vaccine Trial; CIN2+, Cervical Intraepithelial Neoplasia grade 2 or worse; VIN2+, Vulvar Intraepithelial Neoplasia grade 2 or worse; ValN2+, Vaginal Intraepithelial Neoplasia grade 2 or worse.

^aSubjects were cervical HPV-16/-18 DNA negative at entry but anal HPV not evaluated at entry.

Relatively few penile intraepithelial lesions were detected in the trials, so the trial was unable to critically evaluate this end-point. The subset of the men who had sex with men was concurrently enrolled in a study of anal infection and anal intraepithelial neoplasia (AIN).⁴⁵² Excellent protection against vaccine type-related anal infection and AIN was observed (Table 54.8). The findings have led to licensure for these indications and for prevention of AIN and anal cancer in the United States, but not in Europe. U.S. licensure was extended to anal neoplasia in women based on the argument that AIN is indistinguishable in the two sexes. Although Cervarix has not been evaluated for prevention of AIN in either sex, it was shown to significantly reduce the prevalence of anal HPV infection at the end of a 4-year trial in young women.³³¹

The above trials were conducted in young women and men ages 15 to 26 years. Immunobridging studies were used to extend licensure to 9 to 14 year olds. Antibody responses to both vaccines were significantly higher (two- to threefold) in younger than in the older age groups.^{47,463} The higher responses in children led to a comparison of two- and three-dose vaccination protocols. For both vaccines, the antibody responses of children after two doses given 6 months apart was not inferior to the responses of young adults to three doses given according to the recommended schedules (Table 54.7).^{327,508} These findings have led some jurisdictions to initiate two-dose vaccination programs in children, with ongoing assessment of the need for administering a third dose.

Although highly immunogenic, both vaccines were well tolerated in both sexes. The primary side effects attributable to the vaccines were short-term pain, swelling, and erythema at injection site and low-grade fever and/or headache.^{214,243,466,538} In post-licensure studies, no pattern of serious adverse events has been associated with vaccination, except for rare anaphylactic reactions at rates comparable to that of other vaccines.^{199a}

Neutralizing antibodies are believed to be the primary, if not exclusive, immune effectors for the VLP vaccines, although a formal immune correlate of protection has not been determined. In part, this shortcoming is due to the remarkably small numbers of vaccine failures to date and the inability to definitively distinguish incident infection from emergence or reactivation of infections present at the time of vaccination. Antibodies induced by systemic VLP vaccination could reach the anogenital sites of infection by two mechanisms. One mechanism is transudation of serum IgG, which is especially pronounced at the cervix.⁴²⁸ Alternatively, systemic antibodies could be directly exudated at the site of trauma required for initiating infection.⁵⁰⁰ The second mechanism appears to be fully able to prevent infection, since strong protection was observed against genital warts on external genitalia sites, not normally exposed to transudated antibodies in mucus. In a mouse cervicovaginal challenge model, VLP-induced antibodies were shown to protect from keratinocyte infection by two distinct mechanisms.¹²⁸ At high concentrations, the antibodies prevented binding of the capsids to the basement membrane of the cervicovaginal epithelium. At lower concentrations that were nonetheless effective at preventing infection, the capsids were able to bind the basement membrane and undergo the conformational change that exposes L2 N-terminal epitopes. However, they were subsequently unable to stably associate with the

cervicovaginal keratinocytes, suggesting that lower antibody occupancy is needed to prevent capsid binding to the keratinocyte surface receptor than binding to the heparan sulfate proteoglycans of the basement membrane.

Since their initial approval in 2006, Cervarix and Gardasil have been licensed in more than 100 countries. However, they have been introduced into the national vaccination programs of only about 30 countries, mostly in the most developed ones because of the high cost of the vaccines and the implementation programs. National programs are mostly centered on vaccination of preadolescent or adolescent girls, ages 9 to 15 years, since most HPV-associated cancers occur in women,⁹⁴ genital HPV infections are often acquired soon after initiating sexual activity, and children responded better than adults to the vaccines. However, the recent evidence that Gardasil protects young men from genital warts and anal cancer precursors has provided additional support for considering male vaccination programs.

Australia was one of the first countries to achieve at least 70% coverage of adolescent girls, in this case with Gardasil, and is the first country to report public health impact of an HPV vaccination program. Remarkably, in a large sexual health center between 2007/2008 and 2010/2011, the number of genital wart cases in women and heterosexual men under 21 years old decreased by 90%, despite low coverage rates in boys.⁴⁹⁵ No decline was observed in women or men older than 29 years old. In another study involving a cervical cytology registry, the incidence rates of high-grade CIN in women younger than 18 years, but not older age groups, decreased by almost half within 3 years of implementation of the vaccination program.⁷¹

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All cited references are available in the e-book.

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Adenoviridae

Classification**Virion Structure****Genome Structure and Organization****Stages of Replication**

Mechanism of Attachment

Mechanism of Entry, Intracellular Trafficking,
and Uncoating

Transcription

Viral DNA Replication

Virion Assembly

Virus Release

Perspectives

Adenoviruses were first isolated and characterized in 1953 by two groups who were searching for the etiologic agents of acute respiratory infections.^{134,283} The two isolated viruses were related and named *adenoviruses*, after the original tissue (adenoids) in which they were discovered. Human adenoviruses are responsible for only a small portion of acute respiratory morbidity in the general population and for about 5% to 10% of respiratory illness in children. But they can be responsible for epidemic outbreaks in nursing homes and among military recruits.¹⁷⁴ Oral administration of enteric-coated live human adenovirus (HAdV) species B types 4 and 7, the serotypes responsible for most outbreaks among military recruits, was introduced in the 1970s and was an effective vaccine for this population. But cessation of vaccine production in 1998 led to a resumption of adenovirus-associated respiratory illness at US military training centers,^{153,221} resulting in severe pneumonia and one death from HAdV species B type 14.³³⁰ Consequently, there is a renewed effort to produce adenovirus vaccines.^{166,204}

More than 100 members of the adenovirus group have been identified that infect a wide range of vertebrate hosts. All of these viruses contain a linear, double-stranded DNA (dsDNA) genome encapsidated in an icosahedral protein shell with fiber proteins of varying lengths extending from the vertices of the icosahedron that bind to receptors on host-cell surfaces through a terminal globular domain.

In humans, besides respiratory disease, adenoviruses cause conjunctivitis²⁴² and infantile gastroenteritis.⁹ In immunocompetent patients, adenoviruses usually cause a mild, self-limiting acute infection. However, in neonates and immunosuppressed patients, including patients with AIDS, adenoviruses can cause fulminant fatal pneumonia, hepatitis, and/or encephalitis.^{192,331}

Laboratory diagnosis is most rapidly done by polymerase chain reaction (PCR)-based assays.^{161,191}

In 1962, Trentin and colleagues³⁴² made a seminal discovery: human adenovirus type 12 induces malignant tumors after inoculation into newborn hamsters. This was the first time that a human virus was discovered to be oncogenic. No epidemiologic evidence has been reported linking adenoviruses with malignant disease in humans; extensive searches have generally failed to find adenovirus nucleic acids in human tumors.²⁰⁸ While in one recent study HAdV DNA was detected in >50% of pediatric central nervous system (CNS) tumors by PCR, it was also detected in normal CNS tissue from the same patients.¹⁷¹ Thus, the CNS may be a common site of latent, persistent adenovirus infection in children. While there is as yet little evidence that adenoviruses contribute to the etiology of human tumors, the ability to induce tumors in animals and to transform cultured primary cells established adenovirus as an important model system for probing the mechanisms of oncogenesis.

As the interest in adenoviruses as tumor viruses intensified, their virtues as an experimental system became evident. The prototype human adenoviruses are easily propagated to produce high-titer stocks, and they initiate synchronous infections of established cell lines. Further, the viral genome is readily manipulated, facilitating the study of adenovirus gene functions by directed mutational analysis. Studies of adenovirus-infected cells have made numerous contributions to our understanding of viral and cellular gene expression and regulation, DNA replication, cell-cycle control, and cellular growth regulation. Perhaps the most recognized contribution of the adenovirus system to modern biology was the discovery of messenger RNA (mRNA) splicing.^{24,53} Today, the utility of adenovirus as a vector for gene therapy is the subject of intense exploration. This chapter overviews the structure of the adenovirus particle, the adenovirus replication cycle in human cells, and its interactions with host cells and host organisms.

CLASSIFICATION

Adenoviridae have been isolated only from vertebrates—however from every major class from fish to mammals. Bioinformatic analysis of genome sequences⁷¹ indicate four major lineages corresponding to four genera: *Mastadenovirus*, isolated from mammals, including all human adenoviruses; *Aviadenovirus*, isolated from birds; *Atadenovirus*, so named because of their unusually high A+T content, isolated from reptiles, birds, a marsupial and mammals; and *Siadenovirus*, isolated from a reptile and birds. Fifty-six human adenovirus types have been recognized and are classified into seven species (A–G) on the basis of

TABLE 55.1 Classification Schemes for Human Adenoviruses (HAdVs, Genus *Mastadenovirus*)

Species	Hemagglutination Groups	Types	Oncogenic potential		% GC	Associated disease
			Tumors in animals	Transformation in cell culture		
HAdV-A	IV (little or none)	12, 18, 31	High	Positive	46–47	Cryptic enteric infection
HAdV-B	I (complete for monkey erythrocytes)	3, 7, 11, 14, 16, 21, 34, 35, 50	Moderate	Positive	49–51	Conjunctivitis Acute respiratory disease Hemorrhagic cystitis Central nervous system Endemic infection Respiratory symptoms
HAdV-C	II (partial for rat erythrocytes)	1, 2, 5, 6	Low or none	Positive	55	Keratoconjunctivitis in immunocompromised and AIDS patients
HAdV-D	III (complete for rat erythrocytes)	8, 9, 10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, 53, 54	Low or none (mammary tumors)	Positive	55–57	
HAdV-E	III	4	Low or none	Positive	58	Conjunctivitis Acute respiratory disease
HAdV-F	III	40, 41	Unknown	Negative	51	Infantile diarrhea
HAdV-G	Unknown	52	Unknown	Unknown	55	Gastroenteritis

Adapted from Benko M. Adenoviruses: Pathogenesis. In: Mahy BWJ, Van Regenmortel MHV, eds. *Encyclopedia of Virology*. 3rd ed. Oxford, UK: Elsevier, 2008: 24–29.

serology, hemagglutination, oncogenicity in rodents, transformation of cultured primary cells, and genome sequencing^{152,280} (Table 55.1). A proposal has been made recently for systematically naming human adenovirus types (superseding the term “serotype”) based on genome DNA sequences.¹² Types are to be defined by the amino acid sequence of the major capsid protein hexon, which contains the major neutralizing epitopes. These same authors have proposed that intertypic human adenovirus recombinants be designated by the identity of the hexon gene (*H*) and that of the fiber gene (*F*) encoding the second most important neutralizing epitope. For example, HAdV-H7/F3 will designate a virus with a type 7 hexon and a type 3 fiber.¹² This parsimonious nomenclature makes it unnecessary to assign a new adenovirus type number to each new recombinant between previously established types that have been isolated from patients apparently infected simultaneously with two or more types in military recruiting centers and nursing homes.

VIRION STRUCTURE

Adenoviruses are nonenveloped icosahedral particles ~90 nm in diameter (Fig. 55.1) with fibers projecting from the vertices of the icosahedron (Fig. 55.2). Most structural studies of adenoviruses have focused on the closely related human adenoviruses types 2 and 5. The particles (virions) have a mass of $\sim 150 \times 10^6$ D³⁵¹ and contain DNA (13% of mass), protein (87% of mass), no membrane or lipid, and trace amounts of carbohydrate because the virion fiber protein is modified by addition of glucosamine.¹⁴⁸ Virions consist of a protein shell (capsid) surrounding a DNA-containing core. Virion polypeptides were initially characterized by disruption of iso-

lated virions with sodium dodecylsulfate and gel electrophoresis.^{209,351} Comparison of electrophoretic results with genomic open reading frames (ORFs) suggests there are 12 virion proteins numbered by convention II–IX, IIIa, IVa, μ , terminal protein, and the p23 viral protease, with no polypeptide I because the moiety originally designated I proved to be a mixture of aggregated molecules (Fig. 55.3, Table 55.2).

The current highest resolution structure of the Ad5 virion is based on 3.6 Å resolution cryo-electron microscopic (cryo-EM)¹⁹⁴ and 3.5 Å resolution x-ray crystallographic²⁷⁴ structures of the entire virion (Fig. 55.1), an ~3 Å resolution x-ray crystal structure of the isolated hexon trimer^{285,286} (Fig. 55.4), and ~1.5 Å resolution structures of the penton base⁴⁰³ (Fig. 55.5A), fiber shaft,³⁵³ and fiber knob³⁵² (Fig. 55.5B). The icosahedral shell is composed primarily of 240 capsomeres of hexon trimers (12 per triangular facet of the icosahedron), 12 pentameric penton capsomeres at each vertex of the icosahedron, and 12 fibers extending from the pentons, each a trimer of the fiber polypeptide. Loops with hypervariable sequence on the external surface of hexons are important for type-specific immunogenicity and neutralization²⁸⁶ (Figs. 55.4C and 55.4D).

Only the bases of the flexible fibers were visualized in the cryoEM structure that depends on methods that average density signals from multiple virions. However, the full lengths of fibers are evident in negatively stained transmission electron micrographs of single virions (Fig. 55.2). Most human adenoviruses encode a single type of fiber. But types 40, 41,¹⁶² and 52¹⁵² encode two fiber proteins, with one or the other bound to each penton base. In contrast, avian adenoviruses also encode two fiber proteins, but one of each binds to each penton base.¹³² Because the fiber knob (Fig. 55.5B) interacts with a cellular receptor protein, the incorporation of two fiber proteins

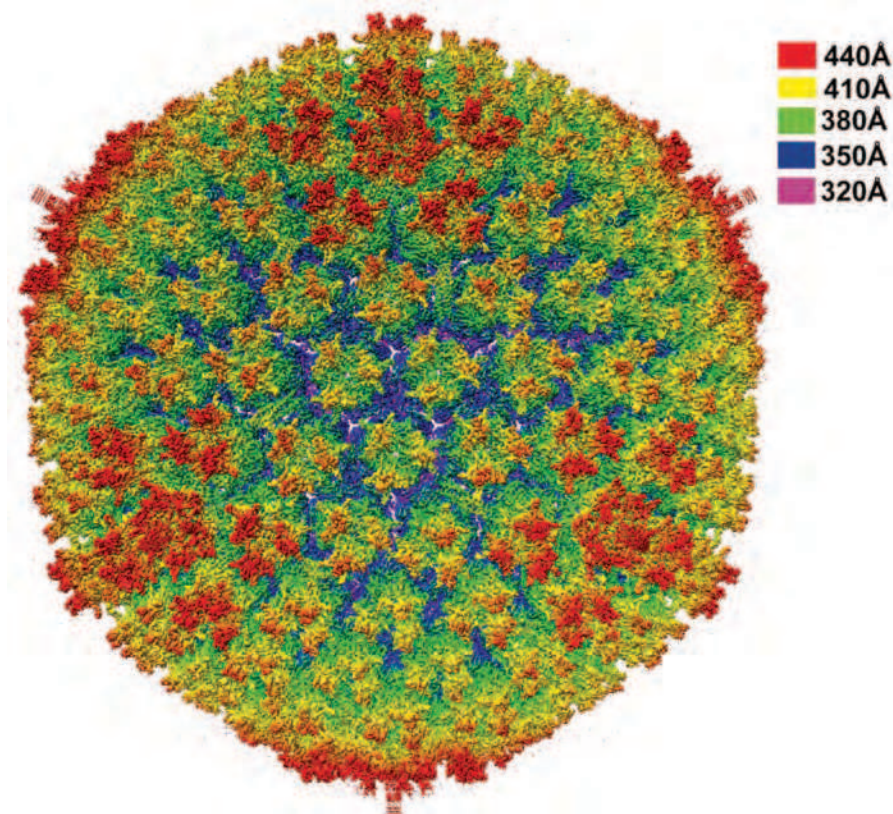


FIGURE 55.1. Ad5 virion. Color coding (*upper right*) represents distance from the center of the virion in angstroms. (Courtesy of Z. Hong Zhou. Adapted from Liu H, Jin L, Koh SB, et al. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* 2010;329:1038–1043.)

might extend the range of cell types to which these viruses bind. The fiber shaft of the HAdVs is composed of repeats of an ~15-residue structural motif,³⁵³ and the length of the shaft varies among types from six repeating units in Ad3 to 21 in Ad2 and Ad5. An unusual “symmetry mismatch” occurs in the

interaction between the fivefold symmetric penton base and the threefold symmetric trimeric fiber. The interaction is mediated by a hydrophobic ring around a central pore on the top surface of the penton base, hydrophobic residues on the bottom of the fiber shaft, and flexible N-terminal tails (aa 10–19) of the fiber monomers (Figs. 55.5B and 55.5C) that insert into three of five available grooves formed by neighboring subunits of the penton base.^{196,403} The N-terminal residues of the fiber monomers (aa 1–9) extend to the bases of penton loops¹⁹⁶ containing RGD sequences that bind to integrins on the target cell plasma

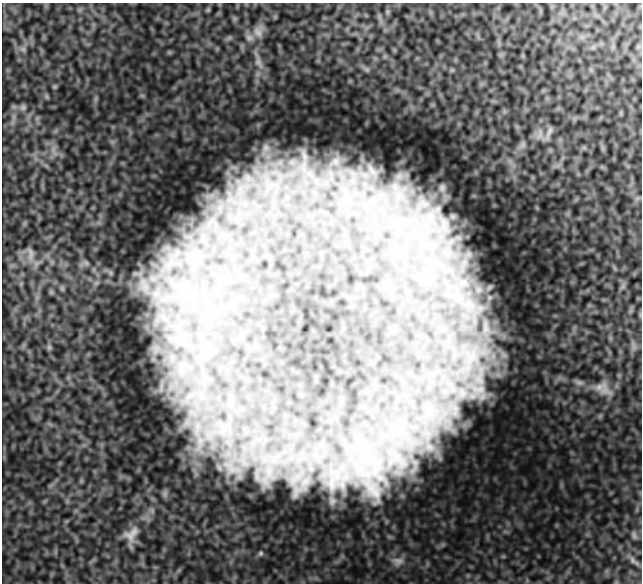


FIGURE 55.2. Visualization of adenovirus fibers in a negatively stained transmission electron micrograph. (Courtesy of Robley C. Williams.)

TABLE 55.2 Adenovirus 5 Structural Proteins		
Polypeptide	aa residues in monomer	Copies in virion
II (hexon)	952	240 trimers
III (penton base)	571	12 pentamers
IIIa	585	60 monomers
IV (fiber)	581	12 trimers
IVa2	449	<10
V (core; pI = 10.3)	368	157 ± 1
VI	250	60 hexamers
VII (core; pI = 12.3)	174	830 ± 20
VIII	227	120
IX	140	240
μ	36	~100

Adapted from Liu H, Jin L, Koh SB, et al. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* 2010;329:1038–1043.

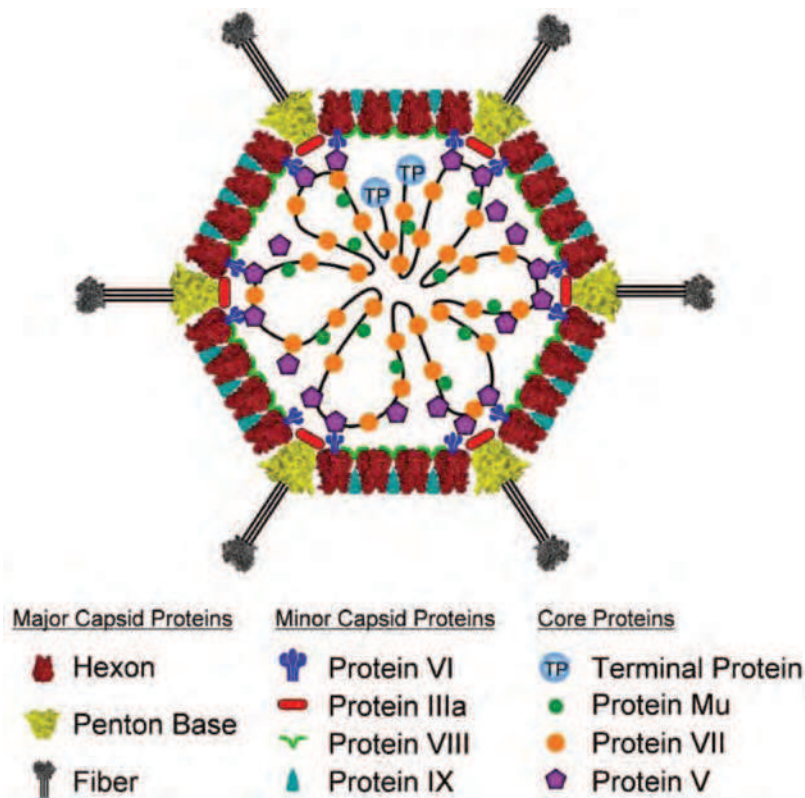


FIGURE 55.3. Diagram of the location of virion proteins in the human adenoviruses. (From Nemerow GR, Pache L, Reddy V, Stewart PL. Insights into adenovirus host cell interactions from structural studies. *Virology* 2009;384:380–388, with permission.)

membrane, triggering endocytosis of the virion (see Mechanism of Entry section).

Minor capsid proteins IIIa, VIII, and IX stabilize nonequivalent interactions between hexon trimers, allowing the same hexon trimer to be used in four different chemical environments on the surface of the capsid^{194,274} (Figs. 55.6 and 55.7). Networks of interactions between the minor capsid proteins stabilize two groups of capsomeres and hold them together: a group-of-nine hexon trimers on each triangular facet of the icosahedron,²⁶⁸ and a group-of-six capsomeres at each vertex composed of five hexon trimers with a central penton-base pentamer (Fig. 55.7).

The core of the virion contains seven known viral proteins and the viral genome. Polypeptides VII (174 amino acids), the major core protein with over 800 copies per virion,³⁵¹ and polypeptides V (368 amino acids) and μ (36 amino acids) are basic, arginine-rich proteins^{141,284} that contact the viral DNA^{8,45} and likely condense the viral DNA within the core. Protein VI associates with a cavity on the inner surface of hexon trimers^{261,274,287} (Fig. 55.7B) and with protein V,²¹⁷ tethering the highly ordered capsid to the less ordered DNA-protein core. A basic 115-residue disordered region in the middle of each polypeptide VI is proposed to contribute to an interaction with the core.²⁸⁸ The N-terminus of each penton-base monomer in the pentamer also interacts with the core, and the C-terminus of protein IIIa may as well,¹⁹⁴ consistent with reports that these proteins also interact with protein V^{33,87,300} and that IIIa is responsible for type-specific packaging of viral DNA into virions.²⁰⁶ Protein IVa2 is present in only a few copies at one vertex of the icosahedron,⁵⁵ binds to the packaging sequence at the left end of the genome, and is required for packaging viral DNA into the capsid (see Virion Assembly). The sixth protein in the core is the *terminal protein*, which is covalently

attached to the 5' ends of the viral DNA and therefore present in only two copies per virion. As discussed in Viral DNA Replication below, the terminal protein serves as a primer for DNA replication. The core also contains about 10 molecules of the p23 viral cysteine protease that functions to cleave precursors of several virion proteins during assembly and maturation of the virus particle and in virus disassembly and escape from endosomes during the infection process.³⁶⁷

GENOME STRUCTURE AND ORGANIZATION

Adenovirus genomes are linear dsDNAs ranging in size from 26 to 45 kb.⁷¹ A protein primer of DNA replication called terminal protein is covalently bound to the 5' phosphate of each strand (see Viral DNA Replication). Homologous genes shared by all adenoviruses encode the three viral proteins required for viral DNA replication, the terminal protein, viral DNA polymerase, and viral single-stranded DNA (ssDNA) binding protein, and major structural components of the virion described (see Virion Structure), except that genes encoding the core protein V are found only in the mastadenoviruses isolated from mammals.⁷¹ These common genes are encoded in the central portion of the genome. Additional genes specific to adenoviruses from different vertebrates are encoded primarily at the termini of the genome. Also, the genomes of all adenoviruses have inverted terminal repeat sequences ranging in size from 36 to over 200 bp.⁷¹ As discussed in Viral DNA Replication below, the inverted repeats function as DNA replication origins at each end of the viral genome and enable single strands of viral DNA displaced during asymmetric strand synthesis to circularize by base-pairing of their terminal sequences. The resulting

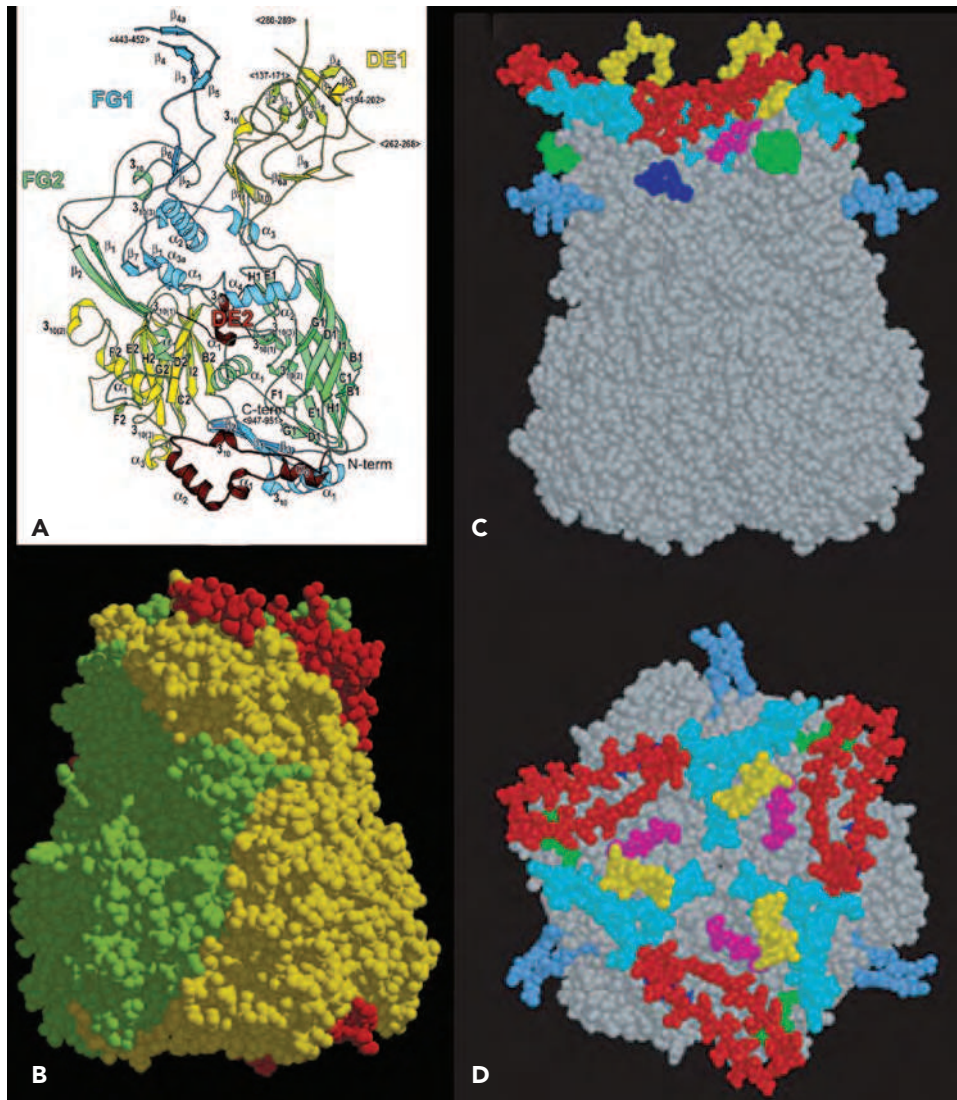


FIGURE 55.4. Ad2 hexon structure. **A:** Ribbon diagram of the Ad2 hexon. View from the inside of the trimer. Loops DE1, FG1, and FG2 comprise the outer surface of hexon in the virion. DE2 is a small loop in the hexon monomer interface. **B:** Space filling model of the hexon trimer. Subunits of the trimer are colored *red, green, and yellow*. **C,D:** Regions that are hypervariable in sequence between different human adenovirus types are modeled in different colors. These hypervariable regions are important for type-specific immunogenicity. (**A** reprinted from Rux J.J, Kuser PR, Burnett RM. 2003. Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. *J Virol* 2003;77:9553–9566, with permission. **B** from Rux J.J, Burnett RM. Type-specific epitope locations revealed by X-ray crystallographic study of adenovirus type 5 hexon. *Mol Ther* 2000;1:18–30, with permission. **C,D** from Roberts, DM, Nanda A, Havenga MJ, et al. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* 2006;441:239–243, with permission.)

base-paired “panhandles” in the otherwise ssDNA molecules function as origins for replication of displaced single strands.

Most human adenovirus genomes encode homologous genes represented by the extensively studied, closely related human species C adenoviruses types 2 and 5 (Fig. 55.8). HAd2 DNA was the first adenovirus genome to be completely sequenced,²⁷⁹ and it includes a total of 35,937 base pairs (bp). This chapter will concentrate on the human adenoviruses, all having the same general genome organization.

The human adenovirus genomes include several repeats of a *cis*-acting packaging sequence between the left terminal

repeat and the first protein-coding region (E1A).¹²⁹ This packaging sequence must be located within several hundred bp of an end of the chromosome to direct the proper packaging of viral DNA into infectious virions.¹²⁹

The human adenovirus genomes contain five early transcription units (E1A, E1B, E2, E3, and E4), four intermediate transcription units transcribed at the onset of viral DNA replication (IX, IVa2, L4 intermediate, and E2 late), and one late transcription unit (major late) that is processed to generate five families of late mRNAs (L1–L5), all of which are transcribed by RNA polymerase II (Fig. 55.8). As discussed in

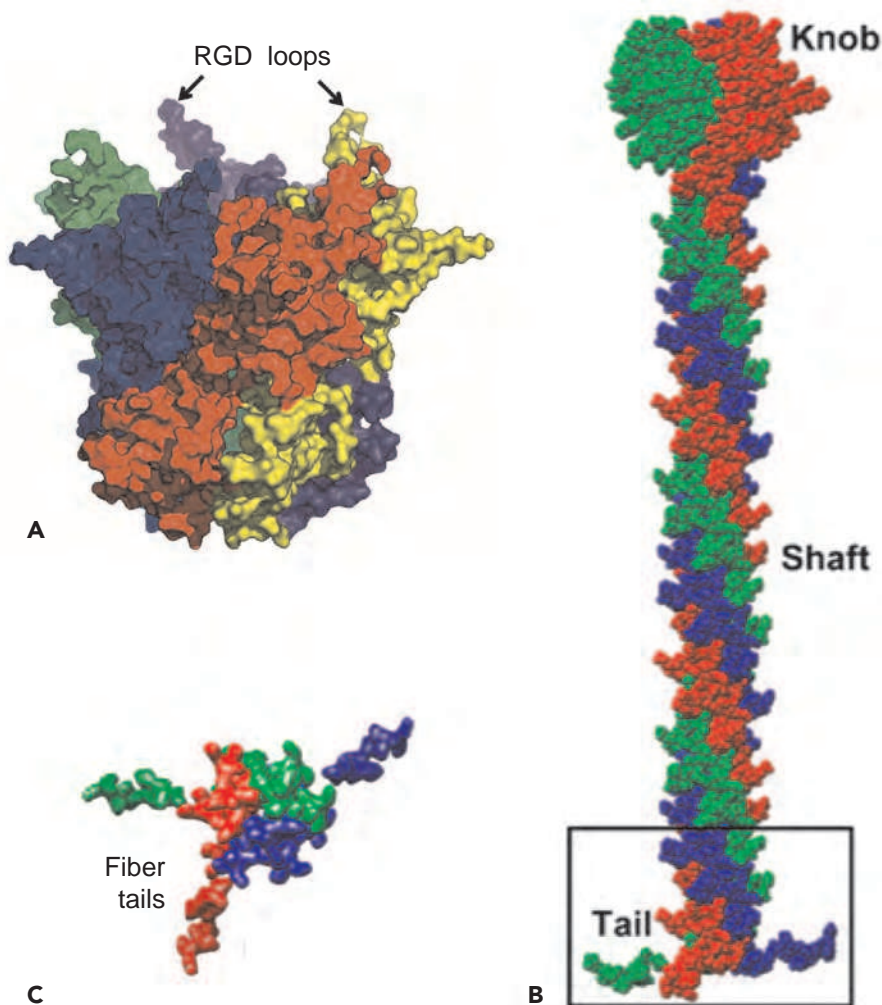


FIGURE 55.5. Structures of Ad2 penton base and fiber. **A:** Space filling model of a penton base. Each of the five penton base subunits is shown in a separate color. **B:** Model of fiber shaft and knob. Each of the three fiber subunits is shown in a separate color. **C:** View of the fiber shaft from the bottom with three fiber N-terminal tails that fit into three of five grooves between the subunits at the top of the penton base. (**A** from Zubieta C, Schoehn G, Chroboczek J, et al. The structure of the human adenovirus 2 penton. *Mol Cell* 2005;17:121–135. **B, C** from Liu H, L. Wu L, Zhou ZH. Model of the trimeric fiber and its interactions with the pentameric penton base of human adenovirus by cryo-electron microscopy. *J Mol Biol* 2011;406:764–774.)

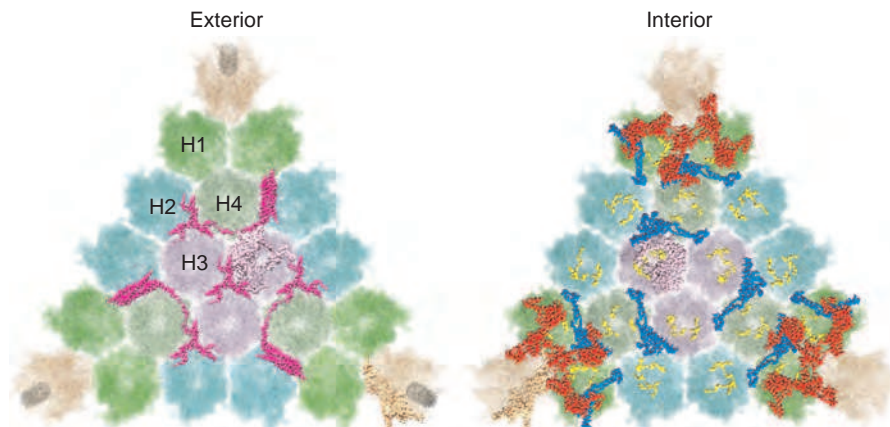


FIGURE 55.6. Four different chemical environments of hexon trimers and locations of minor cement proteins. Models of one of the twenty facets of an Ad5 icosahedron viewed from outside (*exterior*) and inside (*interior*) the virion. Penton bases with the base of a fiber trimer are at the three corners of the triangular facet. Hexon trimers are in four distinct chemical environments, each shown in a different color shade: (H1) two hexon trimers neighboring each penton base; (H2) two hexon trimers on the edges of each facet between the penton base-associated hexon trimmers; (H3) three hexon trimers at the center of each facet; and (H4) three hexon trimers between the central hexon trimers and the penton-associated hexon trimers. These nonequivalent interactions are stabilized by protein IX (purple) between hexon trimers, visible from the exterior surface, and proteins IIIa (red) and VIII (blue) on the inner surface. (Courtesy of Z. Hong Zhou. Adapted from Liu H, Jin L, Koh SB, et al. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* 2010;329:1038–1043.)

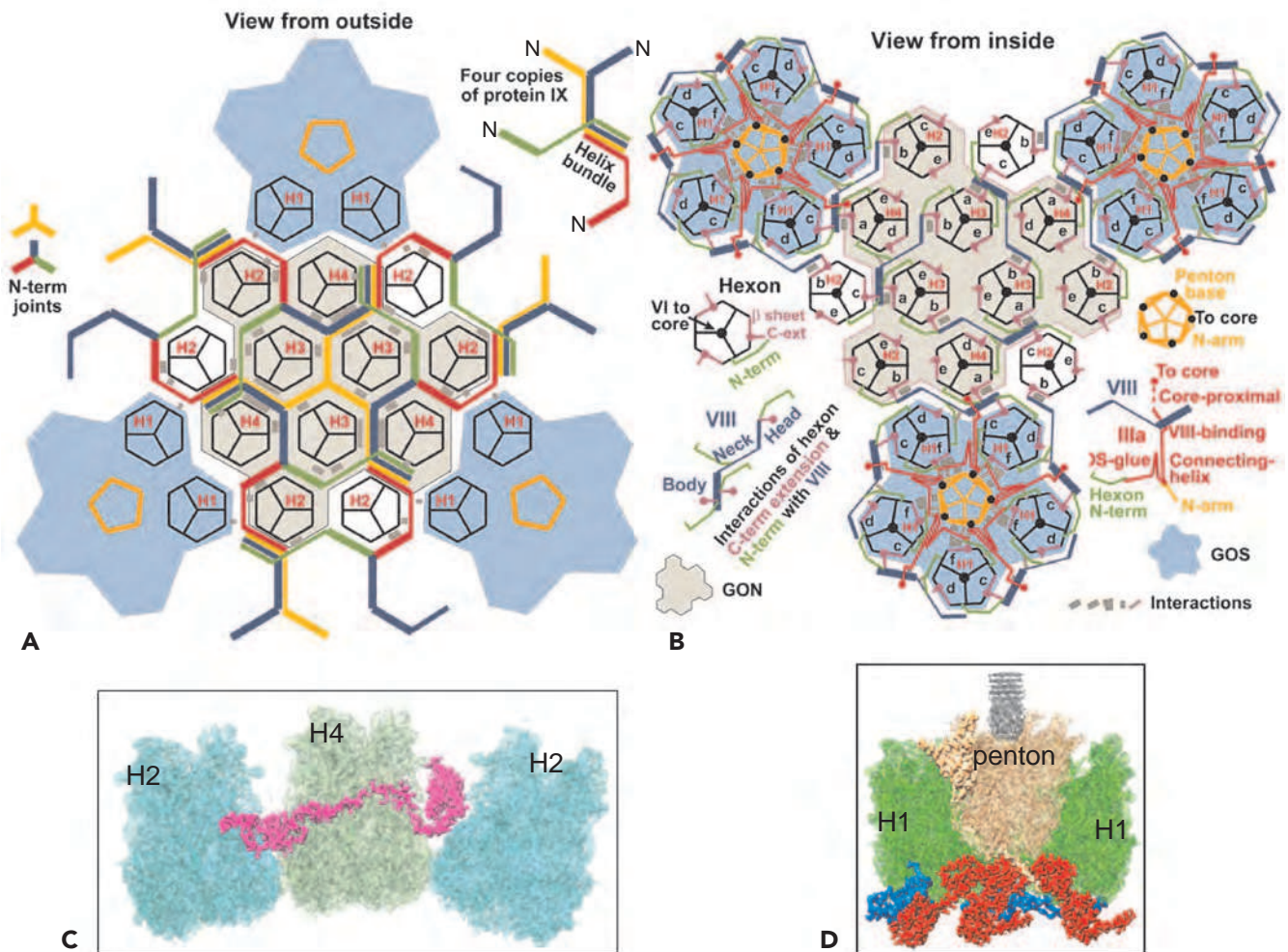


FIGURE 55.7. Diagram of the interactions of minor proteins IIIa, VIII, and IX that stabilize nonequivalent hexon trimer interactions. **A:** Hexon trimers are labeled H1 to H4 as described in Figure 55.6. The diagram at the upper right represents four different conformations of protein IX shown in different colors. N indicates their amino termini. These link hexon trimers in a group of nine (gray), and link groups of nine from neighboring facets. Groups-of-nine hexon trimers are released when virions are disrupted under mild conditions.²⁶⁸ **B:** Letters a through f indicate hexon monomers with distinct conformations of their extended N- and C-termini. As diagrammed at the lower left, three hexon C-termini and four hexon N-termini interact with one protein VIII. At each vertex of the icosahedron, five copies of protein IIIa link five hexon trimers to the central penton base, forming a group-of-six capsomeres (GOS, shaded in blue). Protein VIII links groups-of-nine hexon trimers (GON) to groups-of-nine hexon trimers in neighboring facets and groups-of-nine hexon trimers to groups-of-six capsomeres at the vertices. Protein VI binds in a central cavity on the inner surface of hexon trimers, represented as a black circle. **C:** Side view of protein IX (purple) inlaid into the canyons between hexon trimers. **D:** Side view of proteins IIIa (red) interacting with penton base and H1 hexons and protein VIII (blue) interacting with H1 hexons on the inner surface of the capsid shell. (Courtesy of Z. Hong Zhou. Adapted from Liu H, Jin L, Koh SB, et al. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* 2010;329:1038–1043.)

the Late Transcription section, the major late promoter is also transcribed at a low level early during infection, generating one of the L1 family mRNAs encoding the p52/55 kD protein (a 48-kD polypeptide that runs as an apparent doublet of 52 and 55 kD on SDS polyacrylamide gels because of variation in the degree of phosphorylation). Also, after the onset of viral DNA replication, the E2 transcription unit is transcribed from an alternative promoter called the E2 late promoter as well as the early E2 promoter active before and after the onset of viral DNA replication. The chromosome also carries one or two (depending on the type) virus-associated (VA) genes transcribed by

RNA polymerase III. By convention, the map is drawn with the E1A gene at the left end. Both strands of the viral DNA are transcribed with the rightward reading strand as the template for the E1A, E1B, IX, major late, VA RNA, and E3 transcription units, and the leftward reading strand as the template for the E4, E2, and IVa2 units.

Except for the IVa2 and pIX transcription units, each of the adenovirus genes transcribed by RNA polymerase II gives rise to multiple mRNAs that are differentiated by alternative splicing, and in the case of the major late, E2, and E3 transcription units by the use of alternative poly(A) sites as well. The L4

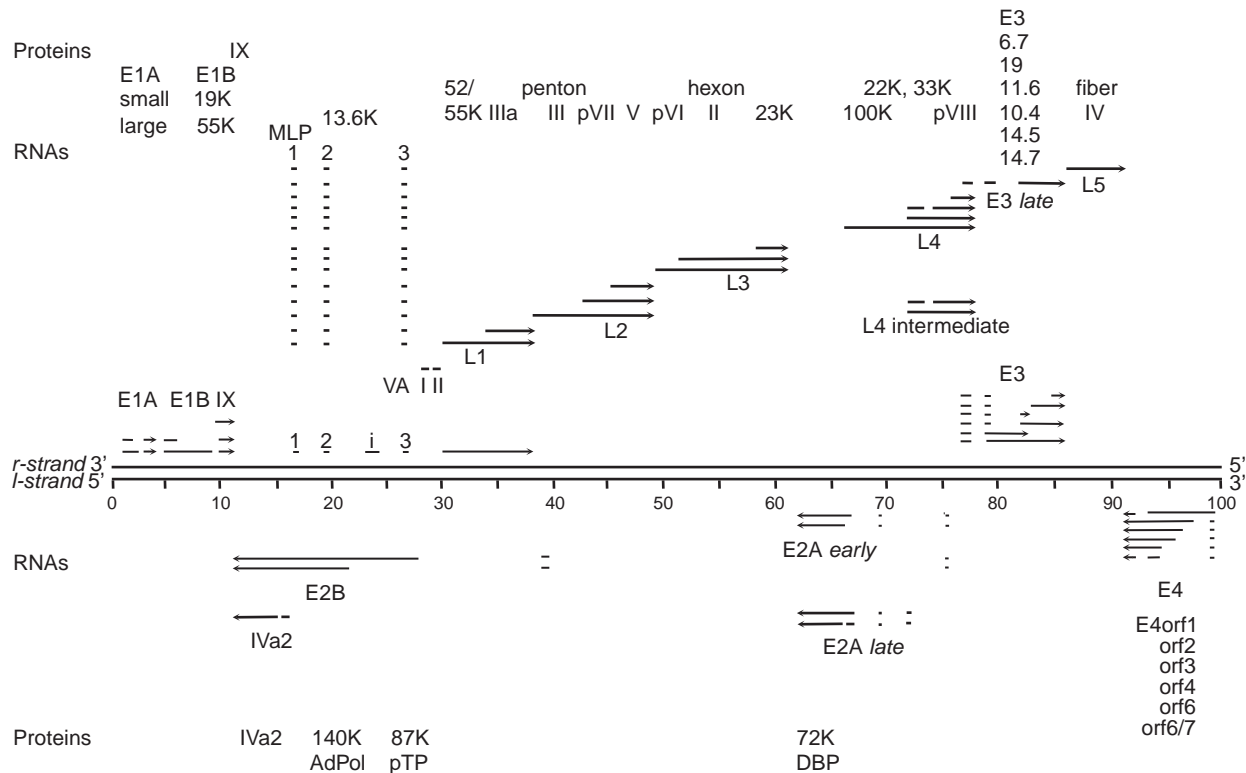


FIGURE 55.8. Genomes of adenoviruses type 2 and type 5. The genome of 35,937 base pairs (type 2) is divided into 100 map units. Early mRNAs are diagrammed with their exons represented by *thin lines*, late mRNAs with *heavy lines*, and intermediate RNAs (IX, IVa2, L4 intermediate, and E2A late) by *lines of intermediate thickness*. Arrowheads represent polyadenylated 3' ends. Most late mRNAs originate at the major late promoter (MLP) at 16.8 map units and contain the tripartite leader whose exons are labeled 1, 2, and 3. E2B mRNAs encoding the adenovirus DNA polymerase (AdPol, 140K) and terminal protein precursor (pTP, 87K) are also expressed from the E2 late promoter and contain the same noncoding 5' exon as the E2A late mRNAs. The L4 intermediate mRNAs encoding proteins L4 22K and L4 33K (from the spliced mRNA) are inferred from the recent discovery of a promoter at ~72 map units and expression of these proteins at intermediate times postinfection.²²⁶ Proteins translated from the mRNAs transcribed to the right and left are named at the top and bottom, respectively. Penton represents the penton base, which is also designated virion protein III. Hexon is also designated virion protein II, and fiber, virion protein IV. pVI, pVII, and pVIII refer to precursor polypeptides that are cleaved during virion maturation. 52/55K represents a single protein that migrates as a doublet on SDS-polyacrylamide gels because of heterogeneous phosphorylation.

intermediate mRNAs shown in Figure 55.8 encoding proteins L4 22K and L4 33K (the spliced mRNA) are inferred from the recent discovery of a promoter at ~72 map units and expression of these proteins at intermediate times postinfection.²²⁶ As mentioned earlier, the analysis of adenovirus mRNA structure led to the discovery of RNA splicing^{24,53} (Fig. 55.9).

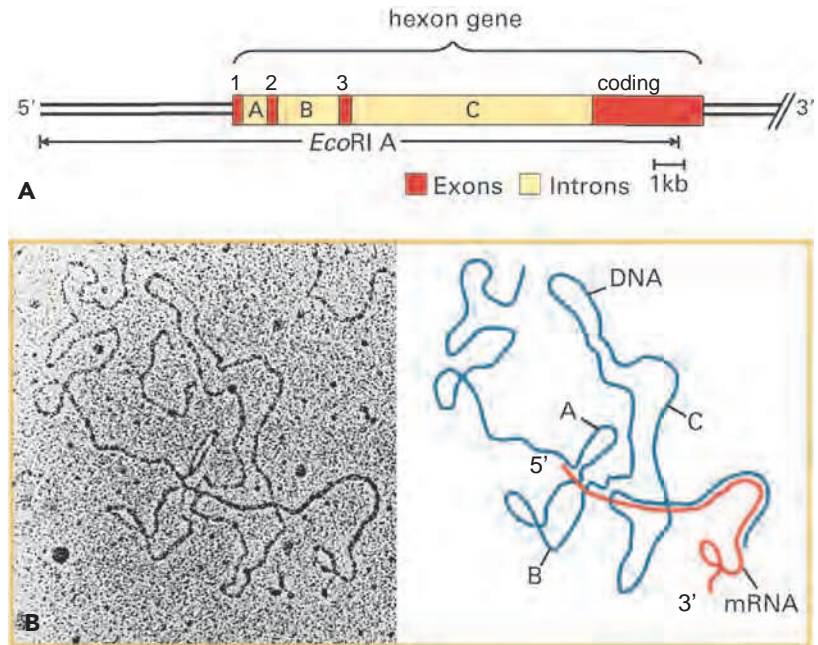
Some of the protein products generated from the same transcription unit share amino acid sequence, such as the two major polypeptides encoded by the E1A region (Fig. 55.10) and the 22K and 33K proteins of the L4 region (Fig. 55.11). Others have no sequence in common, such as the two major E1B-coded proteins (Fig. 55.10) and the 100K, 22K, and pVIII proteins encoded in the L4 region (Fig. 55.11). No consistent terminology has been adopted for naming viral proteins: the early E1A proteins are named large and small E1A proteins and are often referred to by the sedimentation coefficient of the mRNAs that encode them (13S and 12S); E1B and E3 proteins are designated by their apparent molecular mass estimated from SDS gel electrophoresis; E2 proteins are named for

their functions; E4 proteins are named for ORFs; and the proteins encoded by the major late transcription unit are named for the virion proteins discussed above in Virion Structure and the molecular mass for nonvirion proteins. The various historical names of viral polypeptides are used in this chapter, generally preceded by the name of the transcription unit or family of late mRNAs that encodes them (e.g., E4orf6, L4-100K).

Many of the individual adenovirus transcription units encode a series of polypeptides with related functions. As will be discussed, the E1A unit encodes two principle proteins that activate transcription and induce the host cell to enter the S phase of the cell cycle; E1B encodes two proteins that block apoptosis; E2 encodes three proteins that function directly in DNA replication; E3 encodes products that modulate the response of the host to infection; and the late family of mRNAs is concerned with the production and assembly of capsid components. Only the E4 unit encodes an apparently disparate set of functions. E4 products mediate transcriptional, RNA splicing, and translational regulation; mRNA nuclear export;

FIGURE 55.9. Discovery of spliced Ad2 hexon mRNA by electron microscopy of an RNA-DNA hybrid.

A: Diagram of the Ad2 EcoRI A fragment and the hexon gene with exons 1, 2, and 3 comprising the 5' tripartite untranslated leader region and the 3' coding exon shown in red, and introns labeled A, B, and C. **B:** Electron micrograph of a hybrid between hexon mRNA and the EcoRI A coding strand visualized by formamide spreading. In this method, RNA-DNA hybrid appear as a smooth filament whereas single-stranded DNA and RNA appear slightly thinner and less smooth.⁷⁰ A diagram of this RNA-DNA hybrid is shown at the right with hexon mRNA in red and single-stranded DNA of the hybridized EcoRI A fragment in blue. Regions where the red RNA parallels the blue DNA represent base-paired regions of the RNA-DNA hybrid. (**B, left**, from Berget SM, Moore C, Sharp PA. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc Natl Acad Sci U S A* 1977;74:3171–3175, with permission.)



and modulate DNA replication and apoptosis. The grouping might be driven in part by the advantage of using a single transcriptional control element to regulate the expression of multiple polypeptides that are needed simultaneously to execute a function such as DNA replication. As for bacteriophage, the grouping of coding regions for products that interact physically or functionally may have been selected during virus evolution so that recombination between different viral genomes in co-infected cells did not separate genes for interacting proteins.

STAGES OF REPLICATION

The replication cycle is divided by convention into two phases that are separated by the onset of viral DNA replication. Early events commence as soon as the infecting virus interacts with the host cell. These include adsorption, penetration, movement of partially uncoated virus particles to a nuclear pore complex (NPC), transport of viral DNA through the NPC into the nucleus, and expression of an early set of genes. Early viral gene products mediate further viral gene expression and viral DNA

replication, induce cell cycle progression (presumably to activate host cell genes for nucleotide and protein synthesis), block apoptosis, and antagonize a variety of host antiviral measures (see Chapter 56, Adenoviruses by W.S.M. Wold and M. Ison). In HeLa cells infected at a multiplicity of 10 plaque-forming units per cell, the early phase lasts ~ 6 hours, after which viral DNA replication is first detected. Concomitant with the onset of viral DNA replication, the late phase of the cycle begins with expression of late viral genes and assembly of progeny virions. The infectious cycle is completed after 24 to 36 hours in HeLa cells. At the end of the cycle, about 10^5 progeny virus particles per cell have been produced, along with the synthesis of a substantial excess of virion proteins and DNA that are not assembled into virions.¹¹³ Cells infected at high multiplicity seldom divide; hence, at the completion of the replication cycle, the DNA and protein content of the infected cell has increased by a factor of about two.

Studies of the human adenovirus replication cycle have focused primarily on the closely related HAdV-2 and HAdV-5 (Ad2, Ad5) viruses. These closely related types have been favored because they are easily grown in the laboratory, and

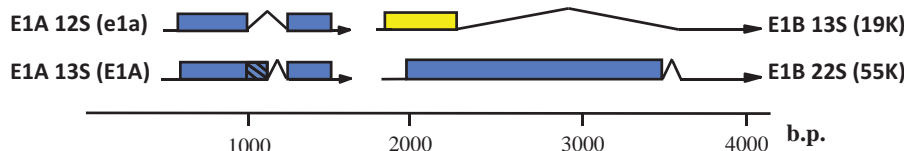


FIGURE 55.10. Ad2 and Ad5 major E1A and E1B mRNAs and proteins expressed during the early phase of infection and in transformed cells. The large and small E1A proteins are translated from spliced mRNAs with single introns having alternative 5' splice sites and the same 3' splice site (12S and 13S E1A mRNAs). Translation starts at the first AUG of both mRNAs, and the second exon of both mRNAs is translated in the same open reading frame. As a consequence, the large E1A protein (289 aa residues) contains 46 amino acids near the middle of the protein that are absent from the small E1A protein.²⁶³ Two E1B proteins of 21K (often called 19K because of its apparent molecular weight on SDS-polyacrylamide gels) and 55K are translated from the first and second AUG in alternative reading frames of the large, 22S E1B mRNA. The 21K protein is also expressed from the first AUG of the smaller 13S E1B mRNA.³⁰

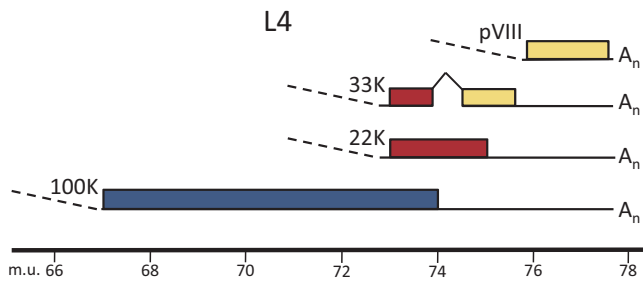


FIGURE 55.11. Ad5 mRNAs and proteins from the L4 region. The three alternative reading frames of the genome sequence are shown in blue, red, and yellow. Dashed lines at the left represent splicing to the third exon of the tripartite leader.

an extensive collection of mutant viruses have been developed. When other human types have been studied, their growth strategies usually have proved similar to the paradigm established for these prototypes. Most studies of adenovirus growth have been performed by infection of HeLa or KB cells at fairly high

multiplicities of infection (more than 10 plaque-forming units per cell). High multiplicities of infection have been used so that all cells in the culture are synchronously infected, allowing the ordered series of biochemical events during the infectious cycle to be observed in a time-wise fashion. HeLa and KB cells have been favored as hosts because they are easily propagated in large quantities, and because Ad2 and Ad5 grow in them rapidly and to high yield. These tumor cells support more rapid viral growth than human diploid fibroblasts, in which the replication cycle is prolonged to about twice as long due to extension of the early phase of infection.¹⁰⁷

Mechanism of Attachment

Adenovirus uses distinct cellular receptors for attachment and internalization (Fig. 55.12). Initial attachment of HAdVs in species A, C, D, E, and F (but not species B) is mediated by high-affinity binding of the fiber knob domain⁷⁵ to the host-cell transmembrane CAR protein (coxsackie B, adenovirus receptor).^{23,281,339} The CAR protein (345 residues in human) is a component of epithelial cell tight junctions with extracellular domains in the immunoglobulin (Ig) superfamily.⁵⁸ It is

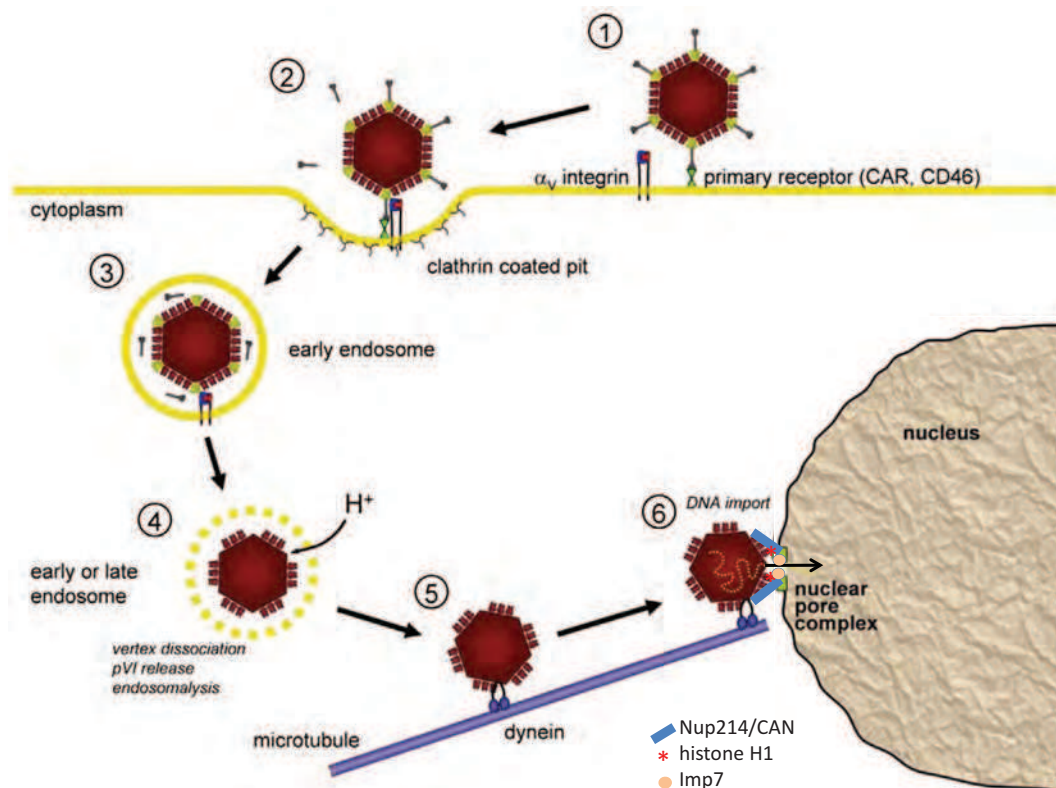


FIGURE 55.12. HAdV Attachment, internalization, intracellular transport and uncoating. (1) The fiber knob binds to the primary cellular receptor, CAR (HAdV species A, C, D, E, F) or CD46 (HAdV species B). (2) The RGD region of the penton monomers (Fig. 55.4A) bind α_v integrins, stimulating endocytosis in clathrin coated vesicles for species C. (3) Fibers begin to dissociate from pentons at the plasma membrane and completely dissociate in early endosomes. (4) The vertices of the particle composed of penton base and peripentoneal hexon trimers dissociate in endosomes, probably as a result of acidification, and the internal capsid proteins IIIa, VI, and VIII are released. Protein VI lyses the endosome, releasing the partially uncoated particle into the cytosol (5) where it is transported on microtubules by dynein motor proteins to the microtubule organizing center, and then associates with the cytoplasmic filament nucleoporin NUP214/CAN (6). The viral DNA bound by protein VII is then imported through the nuclear pore complex by the abundant importins Transportin and Imp7. (Adapted from Nemerow GR, Pache L, Reddy V, Stewart PL. Insights into adenovirus host cell interactions from structural studies. *Virology* 2009;384:380–388; and Greber UF. Signalling in viral entry. *Cell Mol Life Sci* 2002;59:608–626.)

abundantly expressed in heart, pancreas, the central and peripheral nervous systems, prostate, testis, lung, liver, and intestine, but little or no CAR is expressed on hematopoietic cells or adult muscle.²²⁰ Infection does not require the CAR cytoplasmic or transmembrane domains,³⁶³ implying that CAR function in infection is primarily as an anchor for high-affinity binding. The finding that most CAR is localized below the apical surface of polarized epithelial cells comprising the respiratory epithelium at tight junctions on the basolateral surface raises the question of how species C respiratory HAdVs like types 2 and 5 initially infect such tissues from the luminal side. It has been suggested that infections may initiate in specialized nonpolar cells that express CAR on their apical surface, or that lesions of the epithelium are required to expose basolateral surfaces of epithelial cells during the initial phase of infection.²²⁰

The x-ray crystal structure of a complex between the HAdV-12 fiber knob and the extracellular N-terminal Ig-like domain (D1) of CAR showed that three CAR molecules bind to the three interfaces between fiber knob monomers²⁶ through the same surface used by CAR for homotypic interactions.⁹⁹ The avidity generated from the three independent interactions generates high-affinity binding with a $K_D \approx 1$ nM.²⁰⁰ Free fiber proteins produced during the late phase of infection in excess over fiber proteins incorporated into virions are released with progeny virions from the basolateral surfaces of infected airway epithelial cells and interfere with CAR oligomerization at tight junctions. This probably promotes release of progeny virions to the airway lumen.³⁵⁹ In this way, the fiber-CAR interaction serves two functions: initial attachment of virions to host cells during infection, and dissemination of progeny virions to new host cells and host organisms.

In contrast to other HAdV species, fiber knobs of HAdVs in the B species and species D HAdV-37 bind CD46, a regulator of the complement cascade present on the plasma membrane of most cell types including hematopoietic cells.^{102,295,301,382} As in the case of CAR binding, three CD46 extracellular domains are bound by the three fiber monomer interfaces in the fiber knob.²⁶⁴

As mentioned earlier, HAd-40 and -41 incorporate one of two different fibers at each vertex of the virion. The longer of these binds to CAR. The receptor for the second, shorter fiber protein of HAdV-40 and -41 is not known, but it is postulated to contribute to the tropism of these viruses for intestinal epithelium and hence the enteritis with which they are associated.⁶⁰ The stability of HAdV-40 and -41 at low pH no doubt also contributes to this tropism.⁸⁹

The fiber knob of HAdV species D type 37 and probably other species D types can bind three molecules of sialic acid near its top³⁹ as opposed to the interaction of CAR and CD46 with the lateral surface of the fiber knob. Consequently, this terminal saccharide present on many glycoproteins may also function as an initial attachment site for HAdV species D types. Infection by HAdVs elicits a potent, long-term humoral immune response. While most neutralizing antibodies bind to the major virion protein hexon and are primarily responsible for the classification of HAdVs into serotypes,^{104,266,278,323,340} neutralizing antibodies to fiber are also generated⁹⁰ and neutralize synergistically with antibodies to penton base, which interacts with the secondary HAdV receptor.¹⁰³

In addition to these well-studied host receptors, experiments with HAdV-5-based gene delivery vectors led to the

discovery that most vectors infect hepatocytes following intravenous administration. This was found to result from binding of coagulation factor X through its negatively charged Gla-domain to the central depression of the Ad5 hexon and subsequent uptake into hepatocytes through an interaction with heparan sulfate proteoglycans.³⁵⁷ A review of other potential receptors for human adenoviruses in specific tissues is presented in.¹⁴

Mechanism of Entry, Intracellular Trafficking, and Uncoating

Following high-affinity binding of the HAdV-5 fiber knob to CAR, the RGD loop domains of penton base subunits (Fig. 55.5) associate with $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins on the host-cell surface with 50-fold lower affinity than the interaction of fiber with CAR^{234,235,373} (Fig. 55.12). Integrins are abundant heterodimeric transmembrane proteins involved in cell adhesion to the extracellular matrix and neighboring cells via RGD peptide motifs in the bound, extracellular protein and are exploited by many viruses for cell entry.³¹⁶ At saturation, ~ 4 integrins are bound to the pentameric penton base.⁵² Neutralizing antibodies that bind loops on the outer surface of penton are generated that block association with integrins.¹³⁷ However, a monoclonal antibody directed against the RGD loop cannot neutralize the virus, whereas Fab fragments from the same antibody can.³¹⁵ This is presumably because steric hindrance due to the central fiber and the short distance (~ 60 Å) between the RGD loops prevents binding of the intact antibody to all five penton base subunits.

Interaction of the penton base with integrins is presumably facilitated by their high local concentration resulting from high-affinity binding of the fiber knob to transmembrane CAR. Binding of the penton base to integrins requires the flexibility of the fiber shaft.³⁸¹ Clustering of integrins through the binding of several integrins to the subunits of one pentameric penton base triggers integrin transmembrane signaling, leading to activation of phosphoinositide-3-OH kinase (PI3K), p130CAS, and Rho family GTPases.¹⁸⁶ This results in the localized actin polymerization required for endocytosis of the virion via clathrin-coated pits in the classic receptor-mediated endocytosis that involves clathrin adapters and the large GTPase dynamin²²⁰ (Fig. 55.12). Dynamin multimerizes into a spiral-shaped structure that promotes the membrane fusion required for separating an endosome from the plasma membrane.^{105,201,362}

In contrast to the other HAdV species, species B HAdVs, with fiber knobs that bind CD46 as the primary receptor, enter cells through a subsequent penton base interaction with αv integrins that stimulates an alternative, clathrin-independent mechanism of endocytosis called macropinocytosis.^{6,154} Macropinocytosis generates large vesicles near the plasma membrane and requires activation of the PAK1 protein kinase, protein kinase C (PKC), the small GTPase Rac1, Na^+/H^+ exchangers, and the C-terminal binding protein 1 (CtBP1). CtBP1 (C-terminal binding protein) is so named because it binds to the C-terminal conserved region 4 of HAdV E1A proteins.⁵¹ Remarkably, CtBP1 and 2 also function in the nucleus to regulate transcriptional activation by E1A (see Regulation by E1A). Species C HAdVs also stimulate macropinocytosis, but do not use this process as a primary route of infection.²¹⁹ However, this phenomenon probably accounts for the observation that infection of cells with untreated or inactivated HAdV-2 or -5 can stimulate uptake of macromolecules from the medium.^{59,95}

Uncoating of the virion begins at the cell surface. The interaction of penton base with integrins at the cell surface and during endocytosis leads to detachment of fibers, so that a fiberless virion is endocytosed.²³² Structural studies revealed substantial conformational flexibility in the central region of the penton base pentamer such that an ~ 15 Å diameter pore in the cryoEM structure of wild-type HAdV-5¹⁹⁶ becomes an ~ 28 Å pore in the x-ray crystal structure of the isolated penton base⁴⁰³ and can expand to a diameter of ~ 50 Å in the crystal structure of an HAdV-5 engineered with a short fiber derived from HAdV-35 to facilitate crystallization.²⁷⁴ Such an expansion of the penton base central pore may facilitate release of the fiber. Studies of virion disassembly using fluorescently labeled virions and fluorescence resonance energy transfer (FRET), fluorescence anisotropy measurements of the segmental motions of fluorophores, and fluorescence lifetime imaging microscopy using a pH-sensitive probe indicated that the half-time for detachment of the fiber is 3 minutes following adsorption at 4° followed by warming to 37°.²¹⁴

Following endocytosis of clathrin-coated vesicles containing HAdV-2, the vesicles mature into endosomes, and subviral particles lacking fibers escape from endosomes into the cytosol ~ 15 minutes after binding to the cell surface.¹¹⁰ Release from endosomes may require further signaling from α_v integrins following endocytosis since release is blocked by inhibitors of protein kinase C, which prevent transport of virion containing vesicles through the actin cortex under the plasma membrane into the cytosol.^{109,232} Release of penton base, peripentoneal hexon trimers and the internal capsid proteins IIIa, VI, and VIII occurs in the endosome, thought to be triggered in part by acidification during endosomal maturation¹¹² (Fig. 55.12). Exposure to a reducing environment in the maturing endosome also results in re-activation of the virion-associated viral protease, which functions during virion assembly (see Virion Assembly), and cleavage of protein VI. This is required for final disassembly of subviral particles at nuclear pore complexes and DNA import into the nucleus.¹¹¹ Lysis of the endosome and release of partially uncoated virions into the cytosol is mediated by an amphipathic helical, membrane lytic domain of protein VI³⁷⁵ that appears to be sequestered in the intact virion in cavities on the inner surface of hexon trimers.²⁷⁴ Thus, the virion, which is very stable outside the cell, is dismantled by an ordered elimination of structural proteins on entry to the cell so that it can deliver its DNA to the nucleus.

For HAdV species A, B, C, and D, release of penton base and protein VI and lysis of endosomes are blocked by α -defensins, antimicrobial cysteine-rich peptides released from host neutrophils.^{181,239,305} CryoEM and other studies indicate that thousands of defensin molecules bind to HAdV species A, B, C, and D (but not E or F) virions. Defensins bound at the interface between fiber and penton base are particularly important for inhibiting infection by preventing the loss of the vertex region and therefore preventing escape of protein VI and endosome lysis.³⁰⁶ As a consequence, virion-containing endosomes mature into late endosomes and fuse with lysosomes where virion proteins and DNA are degraded.³⁰⁶ Anti-hexon neutralizing antibodies cross-link hexons and also may prevent the release of peripentoneal hexons as well as the subsequent release of protein VI and endosome lysis so that virions are delivered to lysosomes and degraded.

In the absence of these host defenses, endosomal lysis occurs. The subviral particles released into the cytosol are

transported to the nucleus on microtubules^{66,183,207} (Fig. 55.12). Fluorescently labeled virion particles in live cell imaging are observed to switch rapidly between minus-ended microtubule motility toward the juxtannuclear microtubule organizing center (MTOC) and plus-ended motility away from the nucleus. But transient activation of protein kinase A (PKA) by integrin signaling and activation of the p38 MAP kinase and its target MAPKAP kinase 2 (MK2) by an unknown mechanism increase the frequency and velocity of minus-end directed movement of subviral particles by the dynein motor protein complex³²⁴ until they reach the MTOC.¹⁸ One neutralizing monoclonal antibody to hexon does not interfere with endosome lysis, but rather blocks infection by inhibiting the association of virions with microtubules, suggesting that it is hexon that binds to microtubule motors.³⁰⁴

From the juxtannuclear MTOC, subviral particles associate with nuclear pore complexes (NPCs)¹¹⁰ via an interaction between hexon trimers and the FG-repeat-containing domain of NPC cytoplasmic filament protein CAN/Nup214.³⁴⁴ Surprisingly, the activity of exportin 1 (CRM1), a nuclear export factor, is required for this association with the cytoplasmic side of NPCs since association of subviral particles with NPCs is blocked by leptomycin B and ratjadone A, highly specific inhibitors of exportin 1, and by small interfering RNA (siRNA) to exportin 1 mRNA.³²¹ But how exportin 1 functions in this process is unclear.

The diameter of subviral particles bound to the NPC (~ 90 nm) is too large to pass through diffusional channels in the pore, ~ 5 to 9 nm.⁴ Viral uncoating occurs at the nuclear pore as shown by exposure of core polypeptide VII and hexon epitopes that are hidden in intact virions.¹¹⁰ Association of intact subviral particles with NPCs occurs by ~ 60 minutes postinfection, and particle disassembly assayed by epitope exposure occurs by ~ 120 minutes.³⁴⁴ FRET experiments with fluorescently labeled virions indicate that the half-time for disassembly of the icosahedral capsid, probably coincident with unpackaging at the NPC, is 60 minutes.²¹⁴ Blocking NPC binding by microinjection of wheat germ agglutinin or specific antibodies that bind to central domains of the NPC blocked exposure of the epitopes, indicating that an interaction with the NPC is required to trigger final capsid disassembly.¹¹⁰ This mechanism assures that viral DNA is released only at the NPC in preparation for transport into the nucleus.

Viral DNA bound by the major basic core protein VII^{110,310,358,386} enters the nucleus by ~ 120 minutes postinfection, while most hexon and protein IX remain associated with NPCs, indicating that the viral DNA–protein VII complex separates from the partially dissociated capsid during the nuclear import process.¹¹⁰ Protein VII and the viral DNA to which it is bound are imported into the nucleus by a Ran-dependent mechanism involving protein VII binding by the abundant importin Transportin.^{135,379} In addition, free histone H1, which is known to be in rapid dynamic equilibrium with chromatin-bound H1,^{184,223} binds to a cluster of 16 acidic residues on one of the hexon surface loops conserved among type C HAdVs.³⁴⁴ Subsequent import of a small fraction of the hexon complexed to histone H1 by an Importin 7–importin β heterodimer is proposed to contribute to the nuclear import of the viral DNA–protein VII complex.³⁴⁴ Experiments with HAdV-2 containing a green fluorescent protein–protein V fusion in its core indicate that the core protein V remains on the cytoplasmic side of the

nuclear pore and does not enter the nucleus with the protein VII–viral DNA complex.²⁷⁰ Protein VII remains associated with viral DNA after its import into the nucleus until it is dissociated by transcription of the viral genome.^{47,358,386}

The process of HAdV infection activates several components of the innate immune response that recognize pathogen-associated molecular patterns (PAMPs). Binding of purified recombinant HAdV-5 fiber knob to the CAR receptor of human A549 cells derived from alveolar type II epithelial cells caused a sharp peak in activity of MAP kinases ERK1/2 and JNK 5 to 20 minutes after addition to cells, and NF- κ B translocation to nuclei by 4 hours.³²⁷ Although the mechanism of signaling between fiber knob bound CAR and the MAP kinase and NF- κ B activation pathways remains unclear, this was associated with expression of proinflammatory chemokines interleukin-8, CXCL-1 (GRO- α), CXCL-3 (GRO- γ), CCL5 (RANTES), and CXCL-10 (interferon-inducible protein 10). No activation was observed with purified penton base or hexon.³²⁷ Toll-like receptor 9 (TLR9) is activated by viral DNA released into endosomes in plasmacytoid dendritic cells, resulting in secretion of type 1 interferon α .^{21,42,146,399} In macrophage, following endosomal lysis by protein VI, the cytosolic NLRP3 inflammasome²⁶⁵ is activated by released cytosolic viral DNA. This triggers activation of caspase 1, which processes pro interleukins 1 β and 18, leading to their secretion.²³⁰ In mouse models of intravenous administration of defective HAdV vectors, infection of splenic macrophage led to secretion of the potent interleukin 1 α .⁷⁶ These and potentially other innate proinflammatory immune responses likely contributed to the well-publicized fatal systemic inflammatory syndrome in an ornithine transcarbamylase (OTC)–deficient patient following infusion into a hepatic artery of 4×10^{13} particles of an HAdV-5 gene therapy vector with a substitution of an OTC expression cassette for the viral E1A and E1B regions and deletion of E4.²⁷²

The robust humoral adaptive immune response elicited by HAdV infections in immunocompetent patients results in production of neutralizing antibodies primarily to hexon, but also to fiber and penton base as discussed previously. In addition, a cellular adaptive immune response is induced. Both cytotoxic CD8⁺ T-cell^{179,180,328,329} and memory CD4⁺ T-cell^{179,245,328} responses occur to epitopes in hexon that are highly conserved between different HAdV species. Consequently, it has been suggested that adoptive transfer of adenovirus-specific T cells from healthy adults may be useful in preventing or treating HAdV infections of multiple different types in immunocompromised patients.¹⁷⁹

Transcription

As mentioned previously, during the infection process adenovirus DNA is transported into nuclei bound by the most abundant virion core protein, the 20-kD, highly basic protein VII.^{110,310,358,386} Protein VII serves the crucial role of suppressing activation of the cellular dsDNA break response (DSBR) when the viral DNA associated with protein VII first enters the nucleus.¹⁵⁸ In the absence of such protection, the cellular MRE11–RAD50–NBS1 complex is activated by the termini of the viral DNA and degrades the ends of the genome, inhibiting viral DNA replication.¹⁵⁸

Release of core protein VII to facilitate transcription of the infecting viral genome is stimulated by its interaction with several abundant acidic nuclear proteins, including template

activating factor I.¹²⁰ The normal function of this protein is to remove protamine from sperm chromatin following oocyte fertilization,²¹⁶ a similar process in that small basic proteins are removed from DNA. Chromatin immunoprecipitation assays¹⁶⁸ indicate that cellular histones replace much of protein VII early in infection by a mechanism that requires viral transcription,⁴⁷ generating viral genomes bound by both protein VII and acetylated nucleosomes. The DNA of an HAdV-5–based gene therapy vector was also shown to be assembled into physiologically spaced nucleosomes containing histone H3.3, the isotype associated with transcribed cellular genes, dependent on the activity of the histone chaperone HIRA.²⁸² Further convincing evidence for the association of host histones with adenoviral DNA early during infection came from immunofluorescence microscopy showing co-localization of phosphorylated histone H2AX with infecting viral genomes of an E4orf3-minus/E4orf6-minus double mutant that induces a dsDNA break response (see E4).¹⁵⁸

Although early and late are convenient terms for description of events that occur during the replication cycle, the functional distinction between early and late events is often blurred. Early genes continue to be expressed at late times after infection, and the promoter controlling expression of the major late transcription unit directs a low level of transcription early after infection. Also, the viral genes encoding proteins IVa2, IX,²⁸ L4-22K and L4-33K²²⁶ begin to be expressed at an intermediate time, thus forming a delayed-early category.

Regulation of Viral and Host-Cell Transcription by E1A Proteins

There are three main goals of early adenovirus gene expression. The first is to induce the host cell to enter the S phase of the cell cycle, providing an optimal environment for viral replication. E1A and E4 gene products play roles in this process. The second is to set up viral systems that protect the infected cell from various antiviral defenses of the host organism. The E1A, E1B, E3, and VA RNA genes contribute to these defenses, which are also discussed. The third is to synthesize viral gene products needed for viral DNA replication. All three of these goals depend on transcriptional activation of the viral genome and the induction of S-phase in noncycling cells in order to provide the precursor dNTPs required for viral DNA synthesis, NTPs and RNA processing proteins required for viral mRNA synthesis, and to activate pathways required for protein synthesis.

The E1A region is the first region to be transcribed following infection²³⁷ due to strong enhancer activity of the ~500 bp upstream of the E1A promoter.¹³⁰ Two major E1A mRNAs and encoded proteins are expressed early after infection from alternatively spliced mRNAs that use one of two 5′-splice sites separated by 138 bp and the same 3′ splice site²⁶³ (Fig. 55.10). Large E1A protein auto activates transcription of the E1A region about fivefold and stimulates transcription from the E1B, E2 early, E3, and E4 start sites more than 10- (E1B) to about 100-fold (E2early, E3, and E4).^{224,225,378} Activation requires a stable, highly specific interaction of the unique region of the large E1A protein, conserved region 3 (CR3), with the MED23 subunit of the human Mediator of transcription complex.^{35,314} Activation domain interactions with the Mediator MED23 subunit both increase the assembly of preinitiation complexes on promoters and stimulate transcription elongation.^{40,360} An interaction between CR3 and the major cellular nuclear acetyl transferase

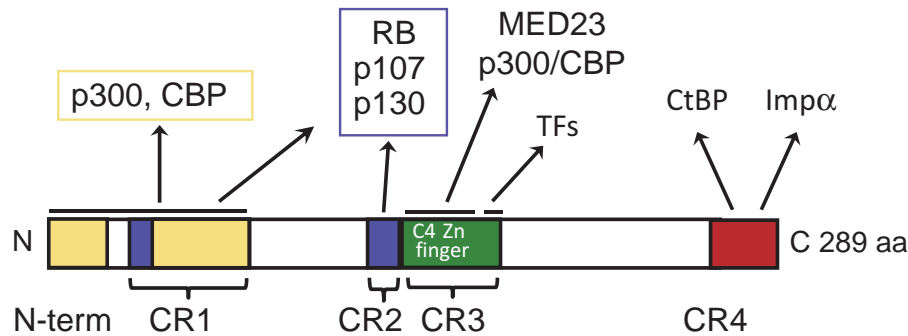


FIGURE 55.13. Protein–protein interaction domains of the large E1A protein. Extensively conserved amino acid sequences in the primate adenoviruses are designated CR1, 2, 3, and 4, although the N-terminal region is also extensively conserved.²⁷³ The two regions of both large and small E1A that interact with the closely related nuclear lysine amino transferases p300 and CBP are shown in yellow. The two regions that associate with the pocket domain of RB-family proteins are shown in blue. The N-terminal C4-Zn-finger region in CR3 unique to the large E1A protein that binds the MED23 subunit of the mediator of transcription complex and the C-terminal region of CR3 proposed to interact with the DNA-binding domains of host-cell transcription factors that bind the early viral promoters¹⁹³ are diagrammed in green. CR4 is shown in red. It includes a C-terminal nuclear localization signal and a PXDLS sequence bound by CtBP 1 and 2 N-terminal to the nuclear localization signal.^{51,205}

p300 also contributes to early gene activation by large E1A.^{259a} In addition, a glutamic acid-proline repeat, (EP)₆, immediately C-terminal to CR3 in Ad2 and Ad5, is essential for large E1A activation of the early adenovirus promoters.³²⁰ Finally, the CR3 region of Ad5 also interacts with the lysine acetyl transferase PCAF, which may also contribute to transcriptional activation by the large E1A protein.²⁵⁹

The small and large E1A proteins through their common sequences can each stimulate G₀- and G₁-arrested cells to enter S-phase in the absence of other mitogenic signals such as serum growth factors.^{106,139,142} Presumably, this reflects the situation when Ad2 and Ad5 initiate an infection of a human host by infecting end-differentiated, G₀-arrested upper respiratory tract epithelial cells. Induction of cellular DNA synthesis requires E1A protein interactions with the abundant, closely related nuclear lysine acetyl transferases p300 and CBP (KAT3B, KAT3A)¹⁴² and the retinoblastoma family proteins RB1, RBL1 (p107), and RBL2 (p130)^{17,20,82,83,142,187,371,372} (Fig. 55.13). E1A proteins must form a trimeric complex between an RB-family protein, E1A, and p300 or CBP to oncogenically transform primary baby rat kidney cells.³⁶¹ A model of the trimeric structure has been presented⁹³ based on the structures of the complexes between Ad2/5 E1A residues 54 to 82 with the TAZ2 domain of CBP,⁹³ residues 37 to 49 with the cleft between cyclin folds in the RB1 pocket domain¹⁹⁷ and the LxCxE-containing peptide of HPV E7 protein, homologous to E1A CR2, with a complementary groove on the side of the C-terminal cyclin fold in the RB1 pocket domain¹⁷⁸ (Fig. 55.14). The two interactions with RB-family proteins remove RB-family proteins from the E2F family of transcription factors that control genes required for entry into S-phase.^{17,92,197,348} The interactions of e1a with CBP and p300 and the RB-family members induce a global change in the localization of these transcriptional regulators throughout the host-cell genome.⁹²

In NIH-3T3 cells arrested in G₀ by culturing in media with 0.05% serum for 60 hours, the promoter regions of the cell cycle-regulated genes *Cdc6* and cyclin A controlled by E2F transcription factors are bound by the repressing E2F4 tran-

scription factor associated with RBL2 (p130), which is in turn associated with the histone deacetylation complex HDAC1/2-mSin3B.¹⁰⁶ The promoter regions are also associated with the Suv39H1 histone methylase transferase that methylates histone H3 lysine 9 (H3K9), generating binding sites for the repressive heterochromatin protein 1 (HP1).^{106,297} Expression of E1A led to loss of p130, replacement of the repressing E2F4 with activating E2Fs 1, 2, and 3 and acetylation of H3K9.¹⁰⁶ This was not observed with a mutant in the cysteine of the CR2 LxCxE-motif that does not bind RB-family proteins, demonstrating the importance of this interaction for displacing p130 from E2F4 and the association of the activating E2Fs 1, 2, and 3.¹⁰⁶ Furthermore, an E1A mutant (*dl26-35*) that cannot bind the histone acetyltransferase/chromatin remodeling TRRAP-PCAF-p400 complex¹⁰¹ eliminated the E2F4-p130 co-repressor complex and H3K9me₂, but did not induce acetylation of H3K9/14 or transcription of *Cdc6* and cyclin A and entry into S-phase.²⁹⁷ These results demonstrate that E1A induces cells to enter S-phase by reconfiguring chromatin structure both by displacing repressing E2F4 and its co-repressor p130 associated with HDAC1/2-mSin3B and Suv39H1, dependent on the interaction of CR2 with RB-family proteins, and by inducing H3 acetylation by the TRRAP-PCAF-p400 complex.^{106,297}

Genomic studies with an Ad5 mutant that expresses only small e1a revealed that, remarkably, small e1a associates with the promoters of most cellular genes in a specific temporal order either activating or inhibiting their expression so as to favor viral replication.⁹² In contact-inhibited, G₁-arrested primary human fibroblasts, between 2 and 6 hours postinfection small e1a associated with promoter regions of genes involved in inflammatory responses and responses to pathogenic organisms, amounting to ~20% of all cellular genes. At these early times postinfection small e1a also became associated with the promoter regions of genes involved in passage through the cell cycle, DNA, RNA, and protein synthesis, amounting to ~30% of all cellular genes. By 12 hours postinfection, association with these first two classes of genes diminished greatly and small e1a

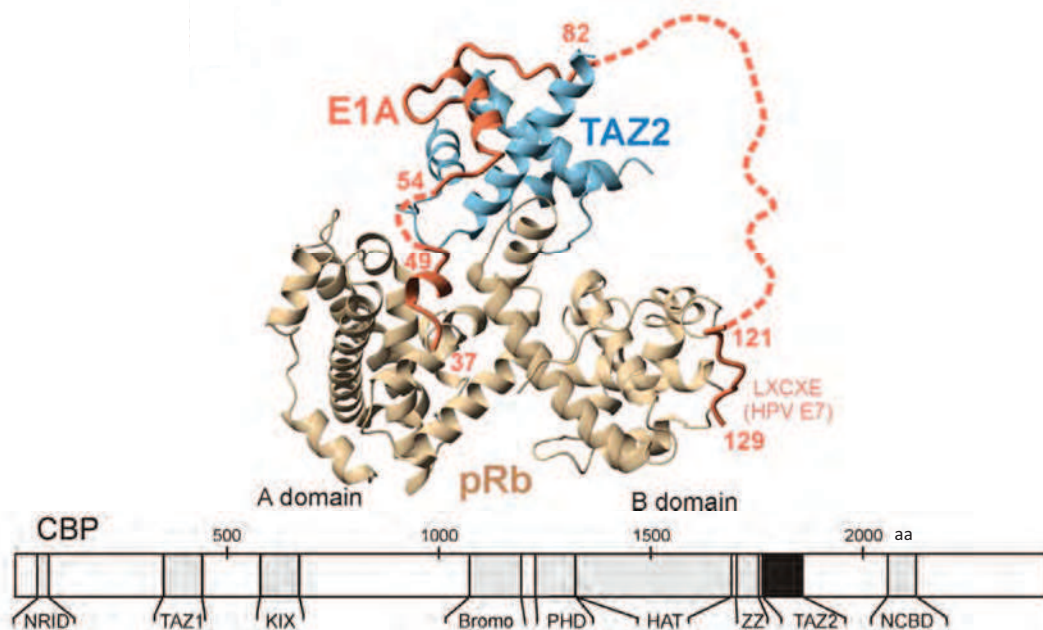


FIGURE 55.14. Model for the trimolecular complex between E1A residues 37 to 129 with the RB1 pocket domain and the CBP TAZ2 domain. Ribbon diagrams of the RB1 pocket domain (*brown*), the TAZ2 domain of CBP (*blue*) and regions of E1A (*orange*) for which the structure has been determined in complex with the RB pocket domain or the CBP TAZ2 domain are shown. Linker regions of E1A that may be unstructured are shown as a *dashed orange line*. The structure of the complex between E1A residues 37 to 49 and the RB1 A domain was reported in,¹⁹⁷ between E1A residues 54 to 82 and the CBP TAZ2 domain in.⁹³ The structure of the LxCxE containing region in CR2 is based on the structure of the homologous LxCxE region of HPV E7 bound to the RB pocket domain reported in.¹⁷⁸ The sequence of CBP is diagrammed at the bottom with HAT representing the lysine acetyl transferase catalytic domain, TAZ2 protein interaction domain shown in *black*. (From Ferreón JC, Martinez-Yamout MA, Dyson HJ, et al. Structural basis for subversion of cellular control mechanisms by the adenoviral E1A oncoprotein. *Proc Natl Acad Sci U S A* 2009;106:13260–13265, with permission.)

became associated with the remaining ~50% of genes involved in differentiated cell functions.⁹²

Small e1 activated transcription of the ~30% of genes involved in cell cycling and macromolecular synthesis. Induction of these genes was associated with the removal of all three RB-family proteins from their promoter regions and acetylation of histone H3K18 at and downstream from their promoter regions.⁹² CBP and p300, which are primarily responsible for acetylating H3K18 *in vivo*,¹³⁹ became associated with these promoter regions. Induction of many of these genes was also observed in contact-inhibited human fibroblasts infected by wt Ad5,²²² indicating that their induction is primarily due to activities of the common regions of the E1A proteins. The ~20% of genes involved in inflammation and other defenses against pathogenic organisms bound by small e1a at early times were repressed, and became associated with both the p300 and CBP acetyl lysine transferases and the RB-family transcriptional repressors RB1 and RBL2 (p130).⁹² Repression of genes involved in innate immune responses and inhibition of cell cycling as well as induction of genes involved in metabolic pathways contributing to cell growth were also observed after infection of HeLa cells with wt Ad2.^{108,397} The observation that promoter regions of host genes involved in antiviral defenses became associated with RB, p130, p300, and CBP⁹² suggests that the trimeric complex of RB or p130, e1a, and p300 or CBP³⁶¹ associate with the promoters of host genes involved in cellular antiviral defenses, repressing their transcription. By

24 hours postinfection, RBL1 (p107) became associated with repressed host genes involved in differentiated fibroblast functions.⁹² Consequently, it appears that after e1a dissociates RB-family proteins from E2F4 and other transcription factors to induce S-phase and cell growth, it exploits the same RB-family proteins to repress antiviral response genes and differentiated fibroblast functions that would otherwise inhibit virus replication and spread to new host cells. Three additional, alternatively spliced E1A mRNA species designated 9S, 10S, and 11S accumulate later in the infectious cycle that contains the same 3'-exon, but have additional sequences spliced out of the 5'-exons.^{313,325} However, as yet no distinct functions have been described for their translation products.

The conserved C-terminal region of E1A, CR4 (Fig. 55.14), contains both a five-residue nuclear localization signal (NLS) at the very C-terminus²⁰⁵ and a transcription repression domain.³⁰⁷ Transcriptional repression results from binding of either of two closely related 48-kD cellular proteins named for this activity (CtBP1/2 for C-terminal binding protein 1/2) that bind to the highly conserved sequence PXDLS just N-terminal to the NLS in all primate adenoviruses.^{34,291} The *Drosophila* ortholog of CtBP was subsequently discovered to be a co-repressor that binds to the same PXDLS sequence in several *Drosophila* repressors that function during embryogenesis.²⁴⁰ Thus far, the significance of this interaction for adenovirus replication remains unclear, but the interaction is required for oncogenic transformation of primary rodent cells by Ad2 E1A and E1B.^{50,51}

Transcription of the Other Early Regions and Functions of the Encoded Proteins

E3

Once the large E1A protein is expressed, it activates transcription of early regions E1B, E2 early, E3, and E4. The E3 promoter consists of a TATA-box and binding sites extending to ~200 bp from the transcription start site for transcription factors NF1, AP1, and the family of transcription factors called ATFs, comprised of homo- and heterodimers of basic-leucine zipper proteins.¹⁴⁵ In lymphoid cells, an NF- κ B site at ~145 is critical to E3 expression.³⁷⁶ E3 expresses several alternatively spliced mRNAs encoding proteins that inhibit innate and acquired immune responses to viral infection. Consequently, activation of E3 by NF- κ B, which activates multiple cellular genes involved in innate and acquired immune responses, likely functions in a regulatory feedback loop that protects the virus-infected cells from these host antiviral mechanisms. The functions of the E3 proteins are discussed in Chapter 56, Adenoviruses.

E1B

The E1B transcription unit maps immediately to the right of the E1A region and encodes two abundant spliced mRNAs called 13S and 22S encoding proteins, commonly referred to as E1B-19K and E1B-55K (Figs. 55.8 and 55.10). The E1B promoter is composed primarily of an Sp1 transcription factor binding site closely juxtaposed to a canonical TATA-box.³⁸³

Additional transcription factor binding sites in the 3'-end of the E1A transcription unit stimulate E1B transcription further,²⁵⁷ as does "transcriptional read-through" from the E1A region.²¹⁸

The two major E1B proteins inhibit p53-dependent induction of apoptosis by two different mechanisms. The level and activity of the tumor suppressor p53 are induced by the abnormal stimulation of cell cycling induced by the E1A proteins.^{73,202} Apoptosis is induced following infection with an E1B-19K mutant because of proteasomal degradation of the critical cellular antiapoptotic BCL-2 family member MCL-1.⁶¹ E1B-19K is homologous to cellular BCL-2 family proteins⁶² and functions as an antiapoptotic MCL-1 viral mimic. It binds to the proapoptotic BCL-2 family members BAK and BAX, preventing them from co-oligomerizing and forming pores in the outer mitochondrial membrane. In the absence of E1B-19K, formation of such BAK-BAX pores in the outer mitochondrial membranes of infected cells releases apoptogenic proteins such as cytochrome c and Smac/DIABLO,⁶² leading to the activation of caspase-9 and -3 and the ensuing apoptosis program.³¹¹ This pathway is blocked in wild-type virus-infected cells by the sequestration of activated BAK and BAX by E1B-19K binding (Fig. 55.15).

p53 function is directly inhibited by the E1B-55K protein through multiple mechanisms. Like SV40 T-antigen, E1B-55K binds p53 directly,²⁸⁹ an observation that led to the discovery that p53 is a major tumor suppressor.¹⁸⁵ E1B-55K binds to the amino-terminus of p53, which also contains the p53 activation domain and the region bound by MDM2, the major

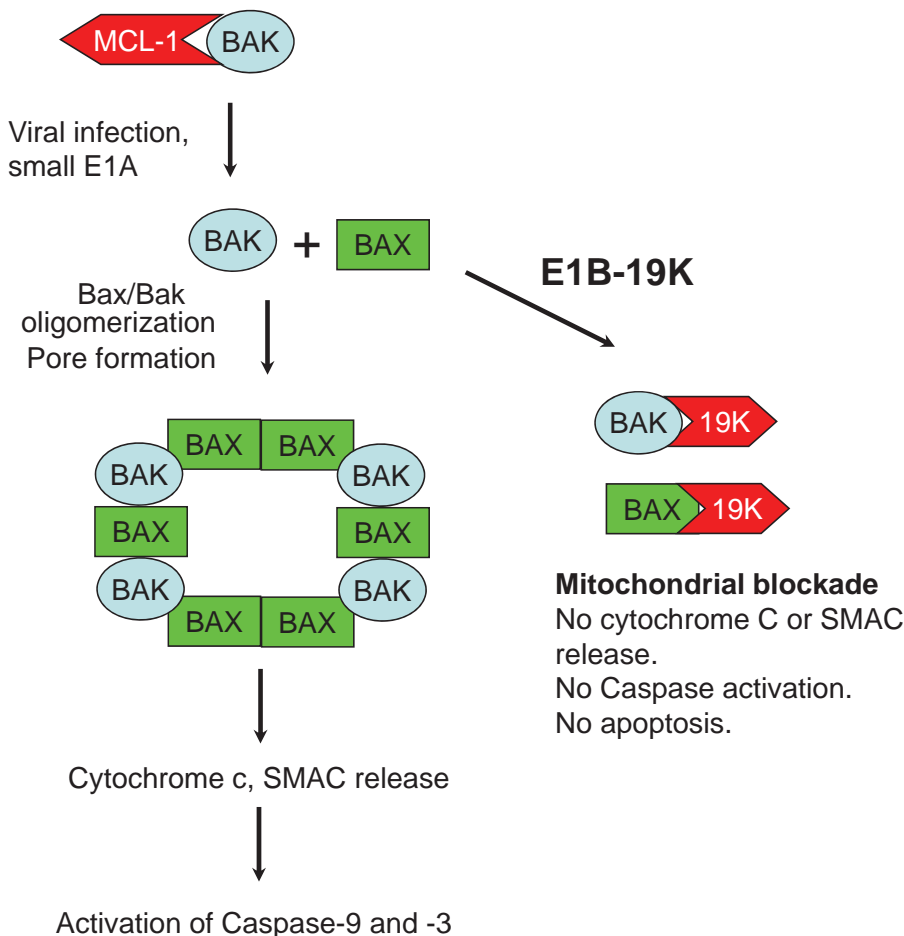


FIGURE 55.15. E1B-19K inhibition of pro-apoptotic BCL2 family members BAK and BAX. Infection of cells with an E1B-19K mutant, or expression of E1A proteins in the absence of other viral proteins results in the proteasomal degradation of MCL-1. This releases and activates BAK, which oligomerizes with BAX to form pores in the outer mitochondrial membrane. These pores release apoptogenic molecules such as cytochrome c and Smac. E1B-19K blocks this proapoptotic BCL-2 pathway by binding and sequestering BAK and BAX, preventing the formation of pores and the release of apoptogenic factors from mitochondria.³⁷⁰

ubiquitin ligase controlling p53 proteasomal degradation.^{157,190} As a result, p53 is stabilized and transcriptional activation by p53 is inhibited.^{387,388} The E1B-55K protein functions as a SUMO1 E3-ligase for p53²⁶⁰ resulting in the sumoylation of ~1% of p53 and association of the complex with PML-nuclear bodies.^{80,198,229,260} Sumoylation of a small fraction of a nuclear protein can result in the association of nearly all of the nuclear protein with PML-nuclear bodies.¹²⁷ In the case of the E1B-55K-p53 complex, this can be explained by the formation of a large network of p53 tetramers cross-linked by the binding of E1B-55K dimers²¹³ to the amino termini of p53 monomers in the p53 tetramers, and the binding of the small fraction of sumoylated E1B-55K^{84,377} and p53 by SUMO-interacting motifs in PML proteins, the major component of PML-nuclear bodies.^{260,377} Association of E1B-55K-SUMO1 complexes with PML-nuclear bodies promotes their nuclear export by exportin 1 (CRM1).^{163,173,260} In cells transformed by the integration of the E1A and E1B regions into cellular chromosomal DNA and when E1B-55K is expressed in the absence of other viral proteins, E1B-55K-p53 complexes exported to the cytoplasm are transported on microtubules by dynein motor proteins to the microtubule organizing center (MTOC) where they accumulate, forming large cytoplasmic inclusion bodies³⁹² called aggresomes.^{198,260}

E1B-55K protein also forms a complex with the E4orf6 protein that associates with cellular proteins Elongin B and C, cullin 5, and RBX1 to form an E3 ubiquitin ligase that poly-ubiquitinates p53, leading to its proteasomal degradation^{124,271} (Fig. 55.16). E1B-55K also binds the host cell MRE11-RAD50-NBS1 (MRN) complex through a distinct region from the E1B-55K region that binds p53.²⁹⁴ E1B-55K-MRN complexes also associate with PML-nuclear bodies and subsequently are exported to aggresomes at the MTOC.¹⁹⁸ The E1B-55K-E4orf6-Elongin B/C-cullin 5-Rbx1 ubiquitin ligase also poly-ubiquitinates subunits of the MRN complex, inducing their proteasomal degradation.³¹⁹ During infection, MRN complex and p53 are first inhibited by being sequestered in PML-nuclear bodies and then by their concomitant nuclear export and proteasomal degradation. Sequestration in PML-nuclear bodies and their subsequent nuclear export prevents them from interacting with nuclear substrates, and transport to the MTOC accelerates the rate of their degradation, probably because proteasomes and components of the ubiquitination pathway are concentrated at the MTOC.¹⁹⁸ The E1B-55K-E4orf6-containing ubiquitin ligase complex also targets other proteins involved in the dsDNA break response, including DNA ligase IV¹⁹ and the Bloom helicase,²⁴⁷ probably because the interaction of these proteins with the termini of adenovirus genomes would interfere with viral DNA replication. Another substrate of this viral ubiquitin ligase is integrin $\alpha 3$.⁶⁷ The degradation of this substrate may function to promote release and spread of progeny virions. E1B-55K and E4orf6 are also required for the inhibition of host mRNA nuclear export during the late phase of infection^{22,122,267} and a high rate of nuclear export of viral late mRNAs.^{122,267} The activity of the E1B-55K-E4orf6-containing ubiquitin ligase is also required for these activities,^{29,380} but the relevant substrate(s) remains to be identified.

E4

The E4 promoter extends at least 180 bp upstream of the major transcription start site and contains binding sites for at least

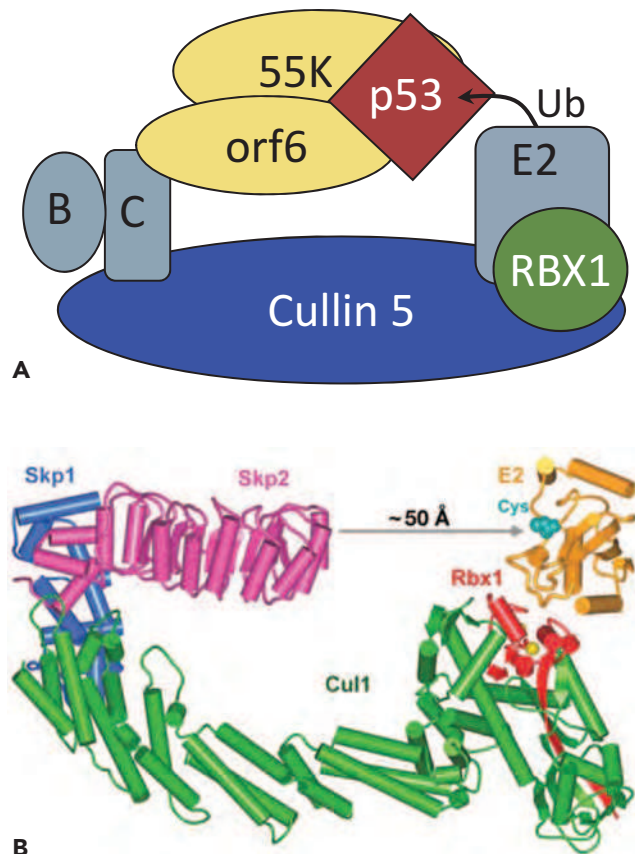


FIGURE 55.16. Diagram of the E1B-55K-E4orf6-Elongin B/C-RBX1 ubiquitin ligase complex. **A:** The Elongin B/C complex is bound to the N-terminus of Cullin 5, which interacts with the RBX1 protein through its C-terminus. E4orf6 binds to the Elongin C and E1B-55K interacts with E4orf6. An unknown E2(s) interacts with RBX1 and transfers ubiquitin to p53. The complex also induces degradation of the MRE11 subunit of the MRN complex,³¹⁹ DNA ligase IV,¹⁹ and the Bloom helicase.²⁴⁷ **B:** Model for the SCF cullin-based ubiquitin ligase complex³⁹⁸ on which the model in **A** is based. Skp2 is the substrate-binding subunit. (**A** adapted from Querido E, Blanchette P, Yan Q, et al. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev* 2001;15:3104–3117. **B** from Zheng N, Schulman BA, Song L, et al. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* 2002;416:703–709, with permission.)

seven sequence-specific binding proteins in an HeLa nuclear extract.³⁶⁴ These include E4F,⁹¹ E4TF1,³⁶⁴ and the several basic-leucine zipper proteins collectively known as ATFs.^{121,356} Several alternatively spliced mRNAs are expressed from E4 that encode proteins named for the E4 ORFs in their order from 5' to 3'³⁵⁵ (Fig. 55.17).

E4orf1 and E4orf4 function to activate the protein kinase mTOR in the absence of nutrient and mitogenic signaling.²⁴⁸ Activated mTOR stimulates protein synthesis by phosphorylating and inhibiting 4EBP, which otherwise inhibits the eIF4E cap-binding translation initiation factor. Activated mTOR also phosphorylates and activates the protein kinase S6K, which stimulates translation by phosphorylating ribosomal protein S6, and additional targets that lead to increased synthesis of

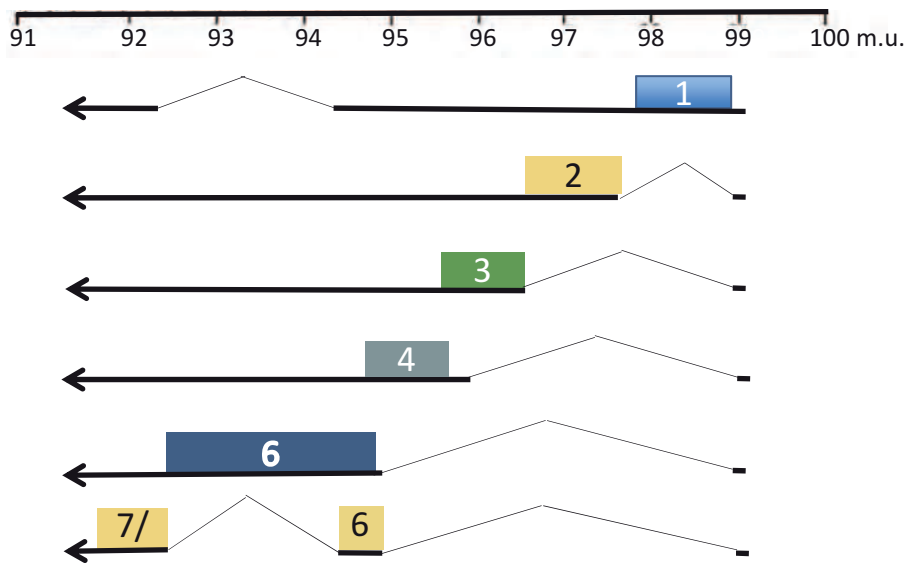


FIGURE 55.17. E4 mRNAs and proteins.

Alternative splicing locates the first AUG of the leftwardly transcribed E4 open reading frames to the 5' end of the E4 mRNAs. E4orf7 is translated as a fusion with the 5' portion of orf6, generating the E4orf6/7 protein. Additional mRNAs not shown encoding E4orfs 1, 3, and 4 are also spliced to remove the intron that fuses orfs 6 and 7. But since this splice is 3' of the first open reading frames in these mRNAs, they do not alter the translated protein.³⁵⁵

ribosomes and translation factors.⁴⁰² Mitogens activate mTOR by activating phosphatidylinositol 3-kinase (PI3K) through a pathway involving Akt/protein kinase B, the tumor suppressor complex TSC1–TSC2, and the Ras-family GTPase Rheb that ultimately activates mTOR.⁴⁰² E4orf1 activates mTOR by activating PI3K through interactions with several host-cell PDZ-domain-containing proteins at the plasma membrane.^{56,100,248} Recently, it has been discovered that proteins encoded by many viruses interact with PDZ-domain-containing members of the membrane-associated guanylate kinase protein family, disrupting tight junctions between epithelial cells and, consequently, probably aiding in progeny virus dissemination.^{151,176} But the molecular mechanism by which E4orf1 binding to PDZ-domain proteins leads to potent PI3K activation is as yet not understood.

E4orf4 also activates mTOR by another mechanism that is independent of PI3K and Akt.²⁴⁸ E4orf4 binds to substrate-binding β subunits of the trimeric protein phosphatase PP2A family.^{167,211} This interaction is required for mTOR activation.²⁴⁸ How this interaction leads to mTOR activation is not understood, but it seems likely that alterations in the substrate specificity or activity of PP2A are involved.³⁹⁶

E4orf3, a small (11-kD) hydrophobic protein, inhibits induction of the cellular DNA double-stranded break response (DSBR), protecting the termini of the viral genome. It does so by sequestering the cellular MRN complex and p53 in nuclear bodies with PML protein that have altered morphology, often described as track-like, compared to PML nuclear bodies in uninfected cells.^{86,309,319} Recall that E1B-55K also inactivates the MRN complex and p53 by itself and as the substrate binding subunit of the E1B-55K–E4orf6 ubiquitin ligase complex that induces the degradation of MRN subunits. Consequently, adenovirus employs several independent mechanisms to inactivate p53 and the MRN complex that otherwise interfere with viral DNA replication. E4orf3, like E1B-55K, also induces nuclear export of MRN complex subunits to cytoplasmic aggresomes.¹³ These processes inhibit MRN complex activation of the ATR protein kinase that otherwise activates the DSBR.⁴¹ E3orf3 also induces histone H3 lysine 9 methylation of p53 target promoters, inhibiting their binding by p53.³⁰⁹

E4orf3 is particularly important for counteracting the antiviral interferon response by inhibiting innate antiviral functions of PML nuclear bodies mediated by PML and the associated host cell Daxx protein.³⁴⁷ E4orf3 also localizes components of cytoplasmic P-bodies, sites of translation inhibition and mRNA degradation, to aggresomes, potentially accounting for E4orf3's stimulation of late viral mRNA accumulation and late protein synthesis.¹¹⁴

The fifth short ORF originally identified in the Ad2 E4 sequence¹³¹ is apparently not translated. E4orf6 was discussed earlier in the context of the E1B-55K–E4orf6–Elongin B/C–Cul5–Rbx1 ubiquitin ligase complex. E4orf6 also interacts with and inhibits p53 independently of E1B-55K.⁷⁸ E4orf6,^{133,312} but not E1B-55K,^{212,312,374} also can bind to p73 and inhibits its function. The p73 protein is a member of the p53 family that is able to activate transcription through p53 response elements. It is transcriptionally induced by small E1A, probably through E2F binding sites associated with one of its two alternative promoters.⁹⁶ Thus, E4orf6 antagonizes the function of both p53 and p73, blocking their potential inhibitory effects on viral replication.

E4orf7 is expressed as a fusion with the N-terminal region of E4orf6 generated by RNA splicing and translation of a protein called E4orf6/7 (Fig. 55.17). E4orf6/7 forms a dimer that binds to and effectively dimerizes cellular E2F transcription factors, greatly increasing their affinity for the E2 early promoter by cooperative binding to the appropriately spaced inverted E2F binding sites.^{215,233,245}

E2

Transcription of the E2 region peaks after the other early regions,²³⁷ and while the other early regions continue to be transcribed throughout the late phase of infection, E2 transcription reaches much higher levels than the other early regions, in part because of the activation of a second E2 promoter, the E2 late promoter⁵⁴ (Fig. 55.8). The E2 early promoter is composed of two binding sites for the E2F family of transcription factors in inverted orientation upstream of a noncanonical TATA-box as well as a binding site for the E1A-EF transcription factor just upstream of these.^{172,302} The E2 late promoter extends

~160 bp upstream from the transcription start site and includes a noncanonical TATA-box and binding sites for two Sp1 transcription factors and two CCAAT boxes that can be bound by several CAAT-box binding factors.²⁷

E2 encodes the three viral proteins required for adenovirus DNA replication, the preterminal protein (pTP) which primes DNA synthesis at the 5' end of each daughter strand, the Ad DNA polymerase (Ad Pol), and the Ad ssDNA binding protein (DBP). DBP, encoded in the E2A region, is required in much larger amounts than the Ad Pol or the pTP encoded in the E2B region (Fig. 55.8). Much more of the E2A mRNAs, which both encode DBP, are expressed than the E2B mRNAs as the result of alternative cleavage and polyadenylation of the E2 primary transcript most frequently at the E2A polyA site near 62 map units. However, some of the E2 primary transcripts are spliced from the second E2 exon at 68 map units to the 5' end of a third exon at 39 map units and then to a fourth pTP mRNA exon at 29 map units or the fourth Ad Pol 3' exon at 23 map units, splicing the E2A polyA site out of the pre-mRNAs (Fig. 55.8). Translation of these mRNAs begins at an AUG in the third exon at 39 map units and continues into the open reading frame that encodes most of the pTP in the longer E2B mRNA or the open reading frame that encodes most of the Ad Pol in the shorter of the E2B mRNAs.²⁹⁹ The E2B mRNAs utilize the same polyA site as the IVa2 mRNA expressed at intermediate times postinfection.³¹⁷

It is remarkable that adenovirus regulates the expression of the viral proteins required for viral DNA replication in part by activating the same E2F family of transcription factors that regulate cellular genes required for entry into S phase. This economical arrangement by which E1A activates both viral and cellular genes probably evolved to coordinate the timing of viral DNA synthesis with the activation of cellular genes required for synthesis of deoxyribonucleotide triphosphates and other aspects of cell physiology conducive to DNA replication.

LATE TRANSCRIPTION

During the early phase of infection, the major late promoter (MLP) at 17 map units is active, but only the longest L1 mRNA encoding the 52/55K protein is expressed.²⁹⁸ Adenovirus late genes encoding virion polypeptides, and the L4-100K protein (Fig. 55.11) involved in late-phase translational regulation and virion assembly (see Regulation of Translation and Virion Assembly) begin to be expressed efficiently at the onset of viral DNA replication. The adenovirus late coding regions are organized into a single large transcription unit with the MLP at 17 map units and a length of ~28 kb.^{236,401} This transcript is processed by differential poly(A) site utilization and splicing to generate at least 14 distinct mRNAs (Fig. 55.8). These mRNAs are grouped into five families, termed L1 to L5, based on the use of common poly(A) addition sites.^{54,236,400} Each family consists of alternatively spliced mRNAs that all contain a 201-nucleotide untranslated tripartite leader generated from the splicing of three short exons (1, 2, and 3, Fig. 55.8)^{3,24,53} to a longer exon containing an open reading frame that generally is not interrupted by introns, except in the case of the L4-33K mRNA (Fig. 55.11). Different open reading frames in each late mRNA family are accessed by alternative RNA splicing of the 3' end of the third tripartite leader exon to different splice sites that define the 5' end of the longer, protein-coding 3' exon (Figs. 55.8 and 55.11). The 5' leader in the L1-53/55K mRNAs expressed at early times also

contains an additional 440 nucleotide exon called the i-leader (Fig. 55.8) that encodes a 135 amino acid protein. That protein is not required for viral replication in cultured cells, but the presence of the i-leader dramatically reduces the half-life of L1 mRNAs from ~26 hours for L1 mRNAs lacking the i-leader to ~4 hours for L1 mRNAs containing the i-leader.³⁰⁸

Expression of this large family of late mRNAs is controlled by the major late promoter (MLP). This promoter exhibits a low level of activity early after infection, but becomes several hundred-fold more active on a per DNA molecule basis at late times.²⁹⁸ There appear to be at least two distinct components that contribute to the delayed activation of the major late promoter: a *cis*-acting change in the viral chromosome dependent on viral DNA replication,³³⁴ and expression of two virus-coded transacting factors at intermediate times postinfection. As yet, there is no molecular explanation for the requirement for viral DNA replication. The IVa2^{256,343} and L4-22K^{16,226,250} proteins greatly stimulate transcription from the MLP by binding to sites in the first intron between leader exons 1 and 2.^{16,182,250,343} These two proteins also bind cooperatively to repeated sequences at the left end of the genome, promoting packaging of viral DNA into subviral particles (see Virion Assembly). IVa2 is transcribed at intermediate times postinfection dependent on initiation of viral DNA replication²⁸ from a TATA-less promoter with a strong initiator sequence that determines the transcription initiation site¹⁵⁹ and additional control elements downstream within the IVa2 coding region.⁴⁶ IVa2 then activates a TATA-less promoter upstream of the L4-22K and L4-33K coding region, within the 100K coding region, stimulating expression of the L4-22K and L4-33K proteins and transcription from the MLP.²²⁶ L4-22K also stabilizes the IVa2 protein, resulting in a positive feedback loop that increases the expression of both proteins and results in a rapid increase in MLP transcription at the initiation of viral DNA synthesis.²²⁶ The requirement for viral DNA replication for expression of IVa2 and L4-22K delays activation of the MLP and expression of the virion structural proteins until they are needed for packaging of replicated viral DNA. L4-22K also stimulates transcription from the IX promoter (Fig. 55.8), contributing to activation of this promoter at intermediate times postinfection.¹⁶

REGULATION OF TRANSCRIPTION ELONGATION AND RNA SPLICING DURING THE LATE PHASE

Two viral proteins—E4orf4 which, as discussed previously, binds to protein phosphatase 2A complexes, likely altering their substrate specificity,^{36,396} and L4-33K³⁴¹—remodel the host cell splicing machinery to modify the splicing of viral pre-mRNAs and probably host-cell pre-mRNAs during the late phase. During the early phase, transcription from the MLP generates only mRNA with the L1 polyA site encoding the 52K/55K protein.^{2,238} During the late phase, mRNAs are synthesized from all five late families using one of five alternative polyA sites (Fig. 55.8), and splicing of L1 pre-mRNA to the downstream alternative 3' splice site generates the protein IIIa mRNA.^{2,175,238}

The early 52/55K 3' splice site is a much better fit to the mammalian consensus 3' splice site sequence than the IIIa splice site. It contains a long polypyrimidine tract, which is a binding site for the essential U2AF splicing factor that recruits the U2snRNP to the pre-mRNA splicing branch point. The L4-33K protein functions through the sequence immediately upstream of the IIIa 3' splice site, functioning as a splicing

enhancer that induces splicing to this site.³⁴¹ Early in infection, host-cell SR proteins bind to a sequence just upstream from the IIIa branch point A, blocking binding of the U2snRNP to the IIIa splice site.^{69,155} Dephosphorylation of host SR proteins dependent on Eforf4 relieves this inhibition of IIIa splicing.¹⁵⁶ Since the phosphorylation of SR proteins is required for their association with consensus splicing signals, the dephosphorylation and inactivation of SR proteins probably contributes to the inhibition of host-cell gene expression during the late phase.¹⁵⁶ Several other 3' splice sites in the MLP pre-mRNA with short polypyrimidine tracts also are activated during the late phase of infection, probably through similar mechanisms to the control of the IIIa 3' splice site.¹ At present, the molecular mechanism(s) that prevents RNA polymerase II elongation to the end of the genome during the early phase is poorly understood, as is the mechanism that relieves this inhibition of elongation during the late phase. However, together with the activation of the MLP by proteins IVa2 and L4-22K and the regulation of 3' splice site selection by proteins E4orf4 and L4-33K, prodigious amounts of the late mRNAs are expressed at appropriate high concentrations to serve as mRNAs for translation of the large amounts of virion structural proteins required for the generation of ~100,000 virion particles per infected HeLa cell.

REGULATION OF TRANSLATION

During the early phase of infection, viral early mRNAs are translated along with the continued translation of host-cell mRNAs. However, during the late phase of infection, translation of host-cell mRNAs is inhibited and virtually all of the host-cell translation apparatus becomes dedicated to translating the late viral mRNAs, especially mRNAs processed from the MLP pre-mRNA with the 5' untranslated sequence generated by the splicing of exons 1, 2, and 3 to generate the 201-bp tripartite leader sequence that precedes the initiation codon. Translation initiates at the first AUG downstream from the tripartite leader, so that splicing of exon 3 to the alternative 3' splice sites in the L1 to L5 mRNA families accesses the coding regions for each of the virion structural proteins and the L4 proteins not incorporated into the virion (L4-100K, -33K and -22K).

In addition to their facilitated transport from the nucleus to the cytoplasm discussed earlier,²² late after infection viral mRNAs are preferentially translated when they reach the cytoplasm.⁶⁴ During this period, when viral mRNAs constitute about 20% of the total cytoplasmic pool,³²⁶ they are translated to the exclusion of host mRNAs.¹⁵ The inhibition of host mRNA translation results from dephosphorylation of the translation initiation factor that binds the 5'-m7GpppN cap structure on most cellular mRNAs, eIF4E.¹⁴³ eIF4E is a subunit of the multimeric initiation factor eIF4F that includes a large scaffold subunit, eIF4G, to which are bound the cap-binding subunit eIF4E and eIF4A, an RNA helicase, as well as other proteins. The entire complex functions as a cap-dependent RNA helicase that unwinds secondary structure at the 5' ends of mRNAs and promotes binding of the 40S ribosomal subunit and scanning of the 40S ribosomal subunit to the translation initiation codon.¹⁵⁰ Dephosphorylation of eIF4E reduces cap-dependent translation by reducing the interaction of eIF4F complexes with capped mRNA.

In uninfected cells, eIF4E is phosphorylated by the protein kinase Mnk1 when both are bound to the same molecule of eIF4G. During the late phase, the N-terminus of the abundantly

expressed L4-100K protein (Figs. 55.8 and 55.11)²⁴⁷ binds to eIF4G, displacing the Mnk1 kinase, resulting in dephosphorylation of eIF4E by an unopposed phosphatase.^{63,65} Late viral mRNAs transcribed from the major late promoter continue to be translated because of the 201-base 5' tripartite leader sequence generated from the three short exons spliced to their coding exons (Figs. 55.8 and 55.9).^{25,79,199} The tripartite leader overcomes the inhibition due to hypophosphorylation of eIF4E by two mechanisms. First, the tripartite leader promotes an alternative form of translation initiation, referred to as ribosome shunting.³⁹¹ Rather than scanning from the 5' cap through the 5' untranslated region to the initiation codon, the 40S ribosomal subunit initially binds to the 5' cap, then is directly transferred to the initiation codon without scanning through the tripartite leader 5' untranslated region. Ribosome shunting is largely dependent on three regions of the tripartite leader that are complementary to the 3' end of 18S rRNA, evoking a mechanism of translation initiation similar to that in bacteria.³⁹¹ Similar complementarity between the 5' untranslated region of the IVa2 mRNA and 18S ribosomal RNA also promotes ribosome shunting and maintains IVa2 translation during the late phase.³⁹¹

In addition to the primary (and probably also the tertiary) structure of the tripartite leader, L4-100K is required for the stimulation of translation by the tripartite leader during the late phase.¹²⁸ 100K binds both the tripartite leader^{384,385} and the eIF4G scaffold subunit of eIF4F, thereby directly stimulating ribosome shunting.³⁸⁵ The tripartite leader also stimulates late mRNA export from the nucleus during the late phase, possibly through an interaction with 100K.¹⁴⁴ The multifunctional 100K protein also functions as a chaperone in the folding of the trimeric hexon protein, encoded by the most abundant late mRNA. Interaction with the cap structure puts it at high local concentration during the synthesis of hexon.

VAI AND VAII TRANSCRIPTION BY RNA POLYMERASE III

The small, abundant VA RNAs were named when their viral origin was still uncertain.²⁷⁶ Different adenovirus types encode one or two VA RNAs; Ad2 and Ad5 encode two species, termed VAI and VAII. The VA RNAs are each about 160 nucleotides in length, are GC rich, adopt stable secondary structures that are important for their function, and are transcribed by RNA polymerase III³⁶⁹ from an internal promoter with A- and B-boxes similar to those of transfer RNA (tRNA) genes.^{97,115} VA RNA synthesis begins during the early phase of the infectious cycle and dramatically accelerates during the late phase as the viral DNA template replicates. VAI accumulates to about 10⁸ molecules per infected HeLa cell, roughly the abundance of ribosomes, and VAII reaches about 10⁷ molecules per cell.

The initial indication of the function of VA RNAs came from analysis of a mutant Ad5 virus in which the VAI gene was deleted.³³³ The virus grew poorly, and its defect was traced to inefficient protein synthesis during the late phase of infection. Additional work identified a defect in polypeptide chain initiation^{277,293} resulting from phosphorylation of the eukaryotic initiation factor-2 (eIF-2) α -subunit.^{277,292} eIF-2 binds to guanosine triphosphate (GTP) and the initiator tRNA to form a ternary complex, which then interacts with the 40S ribosomal subunit. When the 40S ribosomal subunit binds the initiating AUG, eIF-2 hydrolyzes the GTP to GDP and leaves the 40S subunit with the initiator tRNA in the ribosomal P site.

Subsequent steps in the initiation process involving additional factors result in the binding of the 60S ribosomal subunit and chain elongation.¹⁵⁰ For eIF-2 to participate in another round of initiation, its GDP must be replaced with GTP in a reaction catalyzed by eIF-2B, a guanosine nucleotide exchange factor. The exchange reaction is inhibited by phosphorylation of the α subunit of eIF-2. The eIF-2- α (P) forms a tight complex with eIF-2B, preventing it from cycling and catalyzing the exchange reaction. As a result, initiation is brought to a halt when about one-third to one-half of eIF-2 is phosphorylated, trapping all available eIF-2B.

Phosphorylation of eIF-2 is mediated by the cellular protein kinase PKR. Synthesis of an inactive form of PKR is induced by interferon, and the latent enzyme that can phosphorylate eIF-2 α is activated by dsRNA. PKR is activated by autophosphorylation, which appears to require that two molecules of the enzyme interact with one molecule of dsRNA.¹⁷⁰ VAI folds into a structure with extensive base pairing that binds PKR, blocking rather than stimulating its activation.^{160,165,242} As a result, VAI RNA blocks the antiviral effect of dsRNA generated from transcription of both strands of the viral genome during the late phase and blocks the antiviral effect of interferon.¹⁶⁵

In contrast to VAI, VAIL exhibits limited ability to block PKR activation. However, both VAI and VAIL inhibit the processing of cellular micro RNAs (miRNAs). miRNAs are ~22 nucleotide RNAs incorporated into RNA silencing complexes (RISC) that inhibit translation of mRNAs to which they base pair with a small number of base-pair mismatches.⁵ They are transcribed as long precursors called pri-miRNAs and processed by the nuclear enzyme Drosha into ~65 nucleotide stem-loop RNAs called pre-miRNAs. pre-miRNAs are exported from the nucleus to the cytoplasm, where they are further processed by the ribonuclease Dicer into the ~22 nucleotide miRNAs incorporated into RISC complexes. RISC complexes can also be programmed by transfected siRNA with the characteristics of products of Dicer digestion: ~22 bp dsRNAs with two base 3' single-stranded regions. When the siRNA in an RISC complex hybridizes to perfectly complementary regions of an mRNA in vertebrate cells, the RISC complex cleaves the mRNA, initiating its rapid degradation by exonucleases.⁸¹

VA RNAs are synthesized in the nucleus and transported into the cytoplasm by exportin-5.^{119,389} This same exportin transports pre-miRNAs from the nucleus to the cytoplasm, where they are processed into miRNAs by Dicer. During the late phase of infection, the abundantly expressed VA RNAs interfere with the nuclear export of pre-miRNAs by competing with them for binding to exportin-5.²⁰³ VA RNAs in the cytoplasm also bind to Dicer and competitively inhibit pre-miRNA processing.^{10,203} Specific ~22 nucleotide fragments of the VA RNAs are incorporated into RISC complexes that are functional, but do not target viral mRNAs because the VA RNAs are transcribed from the region of the genome that encodes introns from the major late and E2B transcription units (Fig. 55.8).¹⁰ The competitive inhibition of pre-miRNA nuclear export and cytoplasmic processing by VA RNAs interferes with the expression of cellular miRNAs. Since Dicer is a critical enzyme in processing dsRNA into miRNAs and siRNAs, the VA RNAs are expected to interfere with endogenous miRNA interference, and any siRNA interference that might otherwise result from dsRNAs generated by hybridization of

viral pre-mRNAs in the nucleus. However, as of now, it is not clear to what extent the inhibition of the miRNA and siRNA processing pathway contributes to the success of an adenovirus infection.

Viral DNA Replication

As E2 gene products accumulate in response to transcriptional activation of the E2 early promoter by large E1A and stimulation of E2F transcription factor binding by E4orf6/7 as described previously, the stage is set for viral DNA replication. Ad2 and Ad5 DNA replication begins ~6 hours after infection of HeLa cells at a multiplicity of 10 plaque-forming units per cell, and it continues until the host cell dies.

Adenovirus DNA replication occurs by a mechanism requiring a minimal set of replication proteins: the pTP terminal protein primer of DNA synthesis, the viral DNA polymerase, Ad Pol, and the ssDNA binding protein, DBP. The inverted terminal repeats of the viral chromosome serve as replication origins. Viral DNA replication takes place in two stages¹⁷⁷ (Fig. 55.18). First, synthesis is initiated at either terminus of the linear DNA and proceeds in a continuous fashion to the other end of the genome. Only one of the two DNA strands serves as a template for the synthesis; thus, the products of this stage of replication are a duplex consisting of a daughter and parental strand plus a displaced single strand of parental DNA. In the second stage of the replication process, a complement to the displaced single strand is synthesized. The single-stranded template circularizes through annealing of its self-complementary termini that result from the inverted terminal repeat at the ends of the genome. The resulting duplex "panhandle" in the otherwise single-stranded circle has the same structure as the termini of the duplex viral genome. This structure allows it to be recognized by the same initiation machinery that operates in the first stage of replication, and complementary strand synthesis generates a second completed duplex consisting of one parental and one daughter strand. Adenovirus DNA was the first eukaryotic DNA to be replicated *in vitro*,⁴⁴ and increasingly defined *in vitro* replication systems allowed analysis of replication in considerable detail.

The *cis*-acting sequences composing the replication origins are located within the inverted terminal repeats of the viral chromosome. Three functional domains have been defined within the terminal 51 bp of the repeats. Domain A consists of the first 18 bp of the viral DNA, and it functions as a minimal origin of replication. This domain is required for replication but supports only limited replication on its own. The sequence between base pairs 9 to 18 (5'-ATAATATACC-3') is conserved among different human adenovirus types, and a complex of two viral proteins binds here: the preterminal protein (pTP) and the DNA polymerase.^{48,227,332} The E2B-coded terminal protein (TP) is synthesized as an 80-kD polypeptide (pTP) that is active in initiation of DNA replication^{43,85,147,317} and, as discussed previously, is found covalently attached to the 5' ends of the viral chromosome. It is subsequently processed by proteolysis at three sites during assembly of virions to generate a 55-kD fragment (TP) that is covalently attached to the genome, but it appears that the entire protein with cleaved peptide bonds remains associated with the genomic termini.²⁹⁰ The E2B-encoded DNA polymerase (AdPol) is a 140-kD protein^{94,318} in the eukaryotic Pol α family.^{149,195} Like other Pol α s, AdPol contains both 5' to 3' DNA polymerase activity and 3'

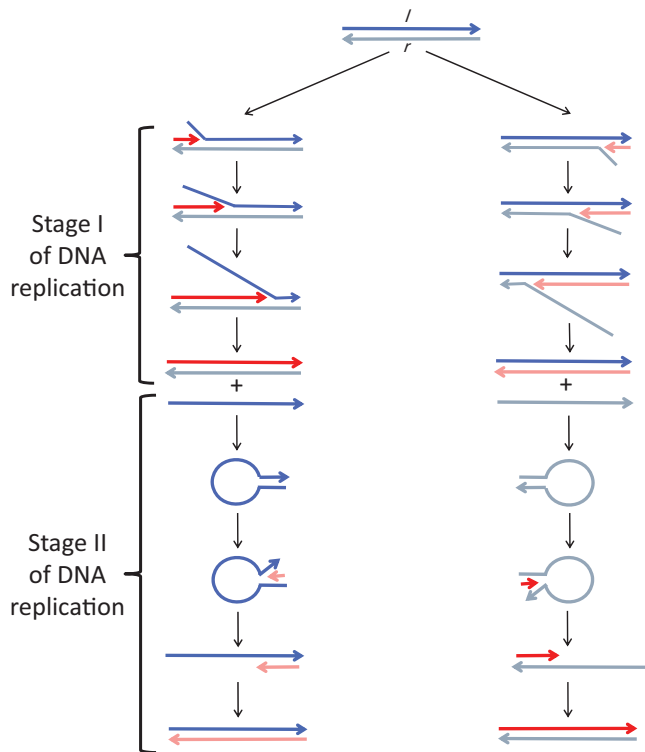


FIGURE 55.18. Simple mechanism of adenovirus DNA replication.

The parental strands are at the top. The strand transcribed to the left is the l-strand (blue); to the right, the r-strand (gray). The ends of the genome are identical because of the genome inverted terminal repeat (102 base pairs in Ad2). Replication beginning on the left end is shown on the left; beginning at the right end on the right. Arrowheads represent 3' ends. Replication occurs in two stages: synthesizing the entire length of one strand first (Stage 1), and the entire length of the complementary strand is synthesized second (Stage 2). With replication beginning at the left, daughter l-strand (red) remains base-paired to the r-strand template, while the parental l-strand is displaced as a single-stranded DNA (bound by the viral DBP, which is not shown). Replication is primed by the pTP protein (not shown) covalently bound to dCMP whose 3'-OH group is extended by the Ad Pol. The entire l-strand is displaced as a single stranded DNA when l-daughter strand synthesis proceeds to the end of the genome. Because of the inverted terminal repeat, the left and right end of the displaced, single-stranded parental l-strand base pair over the length of the repeat, generating a double-stranded region (a "panhandle") identical to the end of the parental genome. Priming of DNA replication and extension of the r-daughter strand reaches the end of the duplex region of the panhandle, breaking the interaction between the ends of the parental l-strand and generating a linear DNA that is partially single stranded with a growing r-daughter strand. Extension of the r-daughter strand completes the synthesis of two double-stranded DNA daughters of the parental DNA with one newly synthesized daughter strand. Replication from the right end (right) proceeds by the same mechanism for the complementary strands. Note that unlike the mechanism for replication of most other DNA molecules, lagging strand synthesis does not occur as short (Okazaki) fragments that are ligated together. The adenovirus mechanism requires only three proteins: pTP, Ad Pol, and DBP. (Adapted from Lechner RL, Kelly Jr. T.J. The structure of replicating adenovirus 2 DNA molecules. *Cell* 1977;12:1007–1020.)

to 5' exonuclease activity, which serves a proofreading function during polymerization.^{94,164} The pTP and the polymerase form a heterodimeric complex in solution that binds to the conserved base pair 9-18 sequence in origin domain A.^{85,188,318,332}

Two more binding sites in the left end of the terminal repeat that are bound by host-cell transcription factors NF1^{31,48,228} and OCT1^{228,243,269} are not absolutely required for adenovirus DNA replication, but they substantially enhance the efficiency of the initiation. The DNA-binding domain of NF1 interacts with AdPol^{48,228} and the DNA-binding domain of Oct-1 binds to the pTP.^{32,350} The binding of NF1 at domain B is stimulated by the third viral protein that functions in DNA replication, DBP.^{57,322} The resulting protein–DNA and protein–protein interactions stabilize binding and properly position the pTP–AdPol complex at the origin. Consequently, the preinitiation complex is composed of the terminus of the viral DNA bound by three viral proteins, pTP, AdPol, and DBP, and two host-cell transcription factors, NF1 and OCT1.

As a protein primer, pTP preserves the integrity of the viral chromosome's terminal sequence during multiple rounds of DNA replication. The priming reaction begins with the formation of an ester bond catalyzed by the AdPol between the β -OH of a serine residue in pTP (serine 580)³⁰³ and the α -phosphoryl group of deoxycytidine monophosphate (dCMP), the first residue at the 5' end of the DNA chain.^{43,189} pTP melts the end of the DNA duplex, allowing the template strand to enter the active site of the polymerase.⁷² The 3'-OH group of the pTP–dCMP complex then serves to prime synthesis of the nascent strand by AdPol. Replication and late transcription take place in large nuclear domains called replication centers.³⁷ pTP-binding to the CAD enzyme, which catalyzes three steps in pyrimidine synthesis,¹¹ and association with the poorly soluble nuclear matrix fraction,^{98,290} may localize the replicating DNA to microenvironments in the nucleus where dNTPs are synthesized²⁵⁵ and may help to localize the viral replication centers.

Chain elongation requires the virus-encoded AdPol and DBP, as well as cellular topoisomerase I.²³¹ The abundantly expressed virus-coded DNA-binding protein is a 59-kD phosphoprotein that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 72 kD. It binds tightly and cooperatively in a sequence-independent fashion to ssDNA.³⁴⁹ The three-dimensional structure of the larger C-terminal DBP DNA-binding domain sufficient for *in vitro* replication was determined by x-ray crystallography.³⁴⁵ The DBP C-terminal extension has a flexible hinge that hooks onto an adjacent DNA-binding protein monomer. This drives the formation of long, multimeric protein chains bound to ssDNA. Polymerization of the DNA-binding protein on ssDNA drives strand separation, consistent with the lack of a requirement for a DNA helicase to unwind the double-stranded template at the growing fork in reconstituted adenovirus DNA replication reactions.⁷⁴ In the presence of DBP, AdPol is highly processive and can travel the entire length of the chromosome after it has separated from pTP.⁹⁴ Topoisomerase I enhances the synthesis of nascent chains >9 kb long; hence, it must be needed to overcome a DNA structural problem that arises only after extensive replication.

In summary, a set of proteins has been identified that mediates the initiation of adenovirus DNA replication (pTP, AdPol, DBP, NF1, and OCT1) and chain elongation (AdPol, DBP, and topoisomerase I). These polypeptides, together

with a template containing an adenovirus replication origin, are sufficient to reconstitute the complete viral DNA replication reaction *in vitro*. The simplicity of this DNA replication mechanism and the location of the viral DNA packaging sequences at the left end of the genome allowed the development of helper-dependent adenovirus vectors in which nearly all of the viral DNA is substituted with other DNA sequences of interest, while the proteins required for DNA replication and the packaging of vector DNA are supplied by a helper virus genome with its packaging sequence removed.^{136,258}

Virion Assembly

The replication of viral DNA, coupled with the production of large quantities of the adenovirus structural polypeptides, sets the stage for virus assembly. In addition to suppressing the translation of host-cell mRNAs and stimulating the translation of viral late mRNAs with the tripartite leader, L4-100K acts both as a chaperone to facilitate the folding of hexon and as a scaffold to facilitate assembly of trimers.¹³⁸ Penton capsomeres consisting of a pentameric penton base and trimeric fiber assemble in the cytoplasm, subsequently joining to form a complete penton capsomere.^{140,354} After their production, hexon and penton capsomeres are imported into the nucleus, where assembly of the virion occurs.

Ad DNA is packaged into capsids in a polarized fashion starting at the left end of the genome.^{68,123,129,335} Extensive mutational analysis showed that seven AT-rich sequences with the consensus sequence 5'-TTTG-N₈-CG-3' located between Ad5 bp 200 to 400 function in viral DNA encapsidation.²⁵³ This ~200-bp packaging sequence also functions in virion assembly when placed at the right end of the genome, but must be within ~600 bp of an end.¹²⁹ Results from mutational studies of the packaging sequence, *in vitro* DNA-protein binding studies, and chromatin immunoprecipitation assays of infected cells indicate that the viral IVa2, L4-22K, and L1 52/55K bind the packaging sequence and promote viral DNA packaging into pro-capsids.^{88,250,252,262,395} Mutants of both IVa2³⁹⁵ and 52/55K^{117,126} are defective for virion packaging. IVa2 appears to provide the sequence specificity for binding to the packaging motifs,^{88,254,262,346,394} while 52/55K makes specific protein-protein interactions with IVa2.^{88,118} Protein IIIa also associates with empty capsids, interacts with the 52/55K protein, and participates in the packaging process.²⁰⁶ IVa2 and L4-22K also function in late-phase activation of the MLP (see Late Transcription) where they bind sequences similar to the repeated AT-rich repeated packaging sequence in transcription control elements in the first intron of the late mRNAs.

Analysis of the IVa2 sequence from human and nonhuman adenoviruses showed that they contain conserved Walker A- and B-box consensus sequences found in ATPases.¹⁶⁹ Consistent with this, IVa2 binds ATP²⁵¹ and mutation of the conserved lysine in the Walker A-box required for activity of ATPases resulted in loss of viability.²⁵⁶ Significantly, IVa2 is associated with one vertex of the mature virion particle.⁵⁵ Thus, IVa2 functions as an ATP-driven motor for inserting viral DNA into a pro-capsid, as observed for large bacteriophage.¹⁶⁹ 52/55K mutants assemble empty capsids,^{117,125,395} while a IVa2 null mutant assembles unstable empty particles.^{95,395} Similarly, deletion of the packaging sequence to which IVa2 binds prevents the appearance of empty capsids.¹²⁹ A temperature-sensitive 52/55K mutant accumulates the mutant protein in

particles containing ~1 kb of viral DNA from the left end of the genome,¹²⁵ but the wild-type protein is not found in mature virions. These results indicate that 52/55K assists a IVa2 packaging motor in stuffing viral DNA into the capsid.

The major histone-like virion core protein VII is synthesized as a precursor, pVII,⁷ that associates with viral DNA during the late phase.^{38,366} pVII interacts with IVa2 and 52/55K.³⁹³ Consequently, viral DNA—with its associated preterminal proteins—is packaged as a complex with pVII and the other virion core proteins V and a precursor of μ .^{366,393}

Finally, cleavage of the precursors of proteins VI, VII, VIII, μ , and terminal protein by a viral protease included in the particles completes assembly of infectious virions. The L3-coded 23-kD viral protease,³⁶⁷ a cysteine protease that requires DNA and a C-terminal fragment of pVI as cofactors,^{77,116,210,336,368} functions late in the assembly process. Its activity is required for removal of 52/55K protein from maturing capsids¹²⁵ as well as maturation of the virion proteins. These cleavages stabilize the particle and render it infectious. A mutant virus with a defective L3-coded protease accumulates noninfectious, virion-like particles with a set of unprocessed polypeptides.³⁶⁵ The requirement of DNA and a pVI fragment for activity of the protease prevents it from processing precursors until both the protease and the precursors are incorporated into maturing virion particles. As discussed in the section on Adsorption and Entry, virion-associated L3 protease activity is also required for uncoating during the process of infection.¹¹¹

Virus Release

There are several processes that facilitate the release of progeny virions from infected cells and the spread of progeny virus in infected tissues. Late in the infectious cycle, the L3-23K viral protease cleaves the cellular cytokeratin K18.⁴⁹ This cleavage event occurs at amino acid 74 of the cytokeratin, creating a “headless” protein that is not able to polymerize and form filaments; rather, it accumulates in cytoplasmic clumps. A normal intermediate filament system helps to maintain the mechanical integrity of cells, and perturbations to the network would be expected to make the infected cell more susceptible to lysis.

The second system that facilitates the release of progeny virions involves the E3 11.6-kD protein. This protein, which is also referred to as the adenovirus death protein, kills cells as it accumulates during the late stage of infection and promotes their lysis.³³⁷ While it is expressed at very low levels from rare mRNAs transcribed from the E3 promoter during the early phase of infection, it is expressed at very high levels from an mRNA with a tripartite leader transcribed from the MLP late in infection.³³⁸ This 101 amino acid protein is an integral membrane N-linked, O-linked palmitoylated glycoprotein that localizes to the nuclear membrane, endoplasmic reticulum (ER), and Golgi, where it interacts with the MAD2B protein.³⁹⁰ MAD2B is related to MAD2, which regulates the activity of the anaphase promoting complex ubiquitin ligase in response to the spindle assembly cell-cycle checkpoint during mitosis. But the function of MAD2B and how its interaction with the E3 protein leads to cell death are not known.

As discussed in the E1B section, the virus-induced ubiquitin ligase complex of E1B-55K-E4orf6-Elongins B/C-Cul5-RBX1 (Fig. 55.16) induces degradation of integrin $\alpha 3$, which appears to decrease attachment of infected cells to their

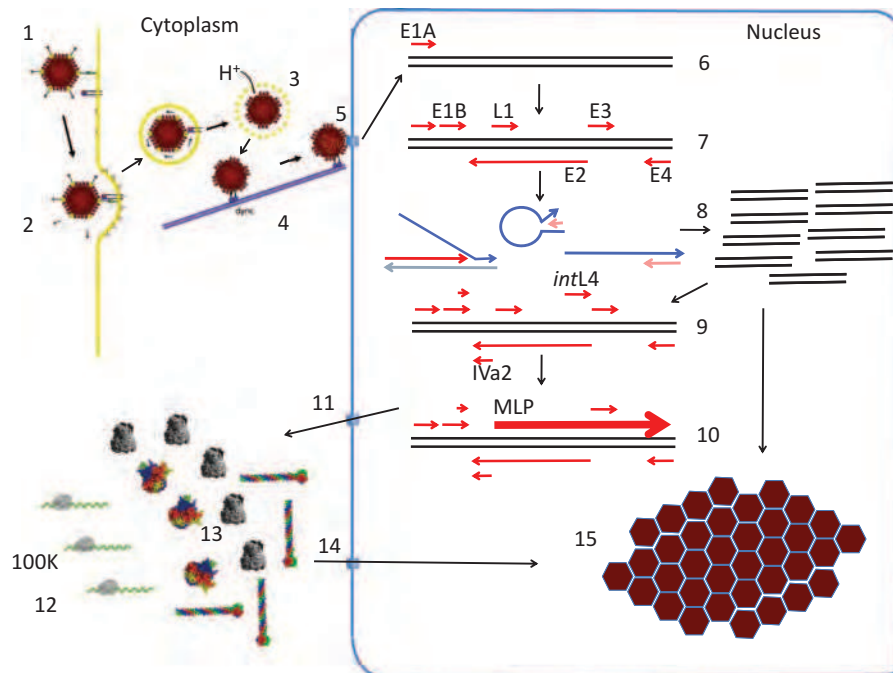


FIGURE 55.19. Summary of adenovirus infection cycle: (1) The globular fiber knob domains of human adenoviruses in species A, C, D, and E make high-affinity interactions with the extracellular domain of CAR (the coxsackie and adenovirus receptor). The fiber knob of HAdV-Bs interact with the extracellular domain of CD46. (2) Penton bases, now at high local concentration relative to the extracellular domains of α_v -integrins on the cell surface, make a lower affinity interaction that stimulates endocytosis of the particle via clathrin-coated pits as fibers dissociate from the pentons. (3) As the endosome matures and the interior becomes more acidic, conformational changes in virion proteins result in the release of pentons and peri-pentonial hexons. Multiple copies of protein VI pass through the open-particle vertices and lyse the endosomal membrane, releasing the partially uncoated virion into the cytosol. (4) The particle associates with dynein motor proteins that carry it along microtubules to the microtubule organizing center adjacent to the nucleus. (5) The partially uncoated particles associate with nuclear pore complexes (NPCs) and the viral DNA bound by protein VII is transported into the nucleus. (6) In a pre-early phase of infection, a transcriptional enhancer in the left 500 base pairs of the genome activates the transcription of early region 1A (E1A). E1A proteins are translated in the cytoplasm and transported back into the nucleus, where they stimulate the host cell to enter the S phase of the cell cycle and (7) activate transcription from E1B, L1, E2, E3, and E4 during the early phase. E1A, E1B, E3, and E4 proteins counter cellular antiviral defenses and stimulate cellular protein synthesis. (8) E2-encoded proteins replicate the viral DNA by the strand displacement mechanism. (9) DNA replication promotes an intermediate phase, during which additional regions—including IVa2 and intermediate L4—are transcribed. These encode viral transcription factors that bind to sites in the first intron of the transcript from the major late promoter, MLP, (10) greatly stimulating MLP transcription. L4-33K regulates RNA splicing. Multiple mRNAs are processed from the MLP transcript—encoding virion structural proteins and the 100K protein. (11) These late mRNAs are preferentially transported through NPCs to the cytoplasm. (12) 100K causes hypophosphorylation of the cap-binding translation initiation factor eIF4E, resulting in inhibition of host mRNA translation. However, the 201 base tripartite leader on all transcripts from the MLP permit initiation by hypophosphorylated eIF4E, resulting in exclusive translation of viral mRNAs into prodigious amounts of the virion structural proteins (13). These are transported into the nucleus through NPCs, where they assemble with replicated viral DNA into progeny virions. After assembly of ~100,000 progeny virions, the cell lyses, releasing them to carry out another cycle of infection.

substratum. This likely increases virus spread in the respiratory tract. As discussed previously, the interaction of E4orf1 protein with PDZ-domain-containing members of the membrane-associated guanylate kinase protein family disrupts tight junctions between epithelial cells and, consequently, probably aids in progeny virus dissemination.^{151,176} Finally, as discussed in the section Adsorption and Entry, tight junctions are also disrupted by free fiber trimers released from infected cells that interfere with CAR oligomerization.³⁵⁹ This also probably promotes the release of progeny virions to the airway lumen in the respiratory tract. Figure 55.19 presents an overview of the adenovirus infection cycle discussed previously.

PERSPECTIVES

As fundamental research on all aspects of adenovirus biology continues, the virus maintains its status as fertile ground for uncovering new knowledge, understanding, and insight regarding multiple aspects of animal cell biology and human antiviral defenses. Successively deeper understanding of the structure and assembly of the virion provides exquisite examples of macromolecular interactions and assembly. It also allows the design of adenovirus-based gene transduction vectors with engineered receptor-binding domains that promote infection of specific cell types. Replication over literally an astronomical number of

generations has led to the evolution of multiple viral proteins that interact with several cellular proteins, every one of which has profound functions in the biology of the cell. Through these interactions, the adenoviruses literally point a finger at cellular proteins and protein complexes that are nodes in the regulation of cellular processes. The viral proteins provide us with subtly engineered molecular tools for exploring these cellular processes. The large number of new references to fundamental advances in all aspects of adenovirus biology demonstrates that adenovirus research continues to be a productive and rewarding area of discovery. Adenovirus continues to have much to teach us.

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Adenoviruses

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Perspectives

HISTORY

During attempts to establish tissue culture lines from tonsils and adenoidal tissue surgically removed from children, Rowe and colleagues⁶¹³ recognized that a transmissible agent was causing degeneration of the epithelial-like cells. Thus, adeno-

viruses (Ads) were first cultured and reported as distinct viral agents in 1953.⁷⁸⁷

A nomenclature for Ads was adopted in 1956, and then Ads were reclassified in 1999.⁷⁴³ The family name is *Adenoviridae*, and there are four accepted genera: *Mastadenovirus*, from mammals; *Aviadenovirus*, from birds; and *Atadenovirus* and *Sia-**adenovirus*, from a broad range of hosts.^{50,141,743} A fish adenovirus falls into a fifth clade.¹⁴¹ Human Ads are divided into seven species, A, B, C, D, E, F, and G, based on serum neutralizing and hemagglutination epitopes, genome sequence and function, oncogenic properties in newborn hamsters, and pathology in humans. These species were previously referred to as groups or subgroups.

Historically, the human Ad isolates were designated as serotypes, based on neutralization of productive infection by homologous sera.⁵⁰ Recently, with the advent of high-throughput Ad genome sequencing and bioinformatics analysis, new insights have been obtained into Ad genome structure and taxonomy.^{141,262} Evolution of Ads seems to have been driven by not only sequence divergence but also frequent recombination between different serotypes.^{444,450,593,594,643,644,756,764,763} Researchers have taken the view that Ad isolates should be designated as *types* rather than *serotypes*, per definitions of the International Committee on Taxonomy of Viruses. Two similar but not exact proposals have been advanced to characterize Ads.^{17,642} With one proposal, for example, human Ad serotype 1 will be designated *type HuAdV-1*, with the “1” referring to hexon (the major capsid protein) identity. The rationale is that hexon should remain the major identifier “because it contains the major neutralizing epitope, which is targeted in molecular diagnosis”.¹⁷ With the other proposal, human Ad serotype 1 will become *type HAdV-C1*, with the “C” referring to species C.⁶⁴² In the current article we will use *serotype* to indicate *type* because serotype has been used in nearly all the literature. According to current standard nomenclature, “H” should precede the serotype number, for example, HAdV-5 for human serotype 5. For brevity, we will use “Ad5.”

There are 57 serotypes in the seven species of human Ads: species A (Ad12, 18, 31), species B (Ad3, 7, 11, 14, 16, 34, 35, 50, 55), species C (Ad1, 2, 5, 6), species D (Ad8 to 10, 13, 15, 17, 19, 20, 22 to 30, 32, 33, 36 to 39, 42 to 49, 51, 53, 54, 56), species E (Ad4), species F (Ad40 and 41), and species G (Ad52) (Table 56.1). Species B can be further divided into species B1 (Ad3, 7, 11, 16, 21, 50) and B2 (Ad11, 14, 34, 35). Ad52 to 57 are recent isolates. Ad52 (species G) was isolated from a patient with gastroenteritis.³⁴³ Ad53^{357,763} and Ad54³²⁶ are in species D and associated with epidemic keratoconjunctivitis (EKC). Ad56, species D, which caused a rare neonatal fatality and keratoconjunctivitis, seems to be a complex recombinant

TABLE 56.1 Infections Associated with Adenovirus Subgroup and Serotype

Subgroup	Serotype	Major site of infection
A	12, 18, 31	Respiratory, urinary, GI
B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55	Respiratory, eye, urinary, GI
C	1, 2, 5, 6, 57	Respiratory, urinary, GI
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, 53, 54, 56	Eye, GI
E	4	Eye, respiratory
F	40, 41	GI
G	52	GI

GI, gastrointestinal.

of Ad9, 15, 26, and 29.⁵⁹⁴ Ad55, species B, isolated from a respiratory outbreak in China, is a recombinant between Ad14 (97% of genome) and Ad11.⁷⁶⁶ An isolate from the feces of a 4-year-old patient⁴⁴⁴ was recently proposed, based on computational analysis, to be Ad57 in species C: it has a fiber similar to Ad6 but a unique hexon.⁷⁶⁵ The genome of many human Ad serotypes has been sequenced.^{450,643,765,766,776} Also, the genome sequence of the Ad5 reference material (ARM) was published as a reference strain for Ad5-based vectors.⁶⁹¹ The genomes of serotypes within a species are highly related and are modestly diverged from species to species. This chapter will tend to concentrate on the relatively recent literature on Ad pathogenesis in humans. Some of the earlier literature can be found in the fourth and fifth Editions of *Fields Virology*.^{308,787}

INFECTIOUS AGENT

Propagation and Assay in Cell Culture

Primary human embryonic kidney (HEK) cells are probably the best host for the entire range of human Ads, but such cells are expensive and may be contaminated with adeno-associated virus. Continuous epithelial lines, such as HEp-2, HeLa, KB, HEK 293, and A549, are also highly sensitive. Ads in monolayer cell culture have a characteristic cytopathic effect (CPE). The cells round up, swell, and detach from the culture surface into grape-like clusters, and the nuclei become enlarged³⁶³ (Fig. 56.1). Eventually the cells lyse, leaving cell debris. This CPE is the result of the infection passing into the “late” stage of infection, when Ad DNA, messenger RNA (mRNA), and proteins are being made in large quantities and virions are assembling in the cell nucleus.

Biological Characteristics

Ads have a double-stranded DNA genome of ~36,000 base pairs (bp) enclosed by a protein capsid and with no membrane (see Chapter 55). The virion binds to specific receptors on the cell surface and enters the cell by endocytosis, and the genome is transported to the cell nucleus. “Early” genes are expressed, viral DNA replicates, “late” genes are expressed, virions assemble in the cell nucleus, and the cells lyse to release progeny

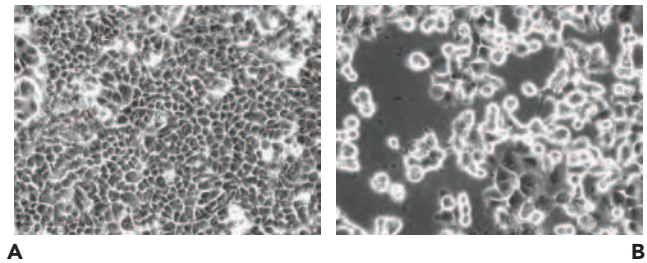


FIGURE 56.1. Cytopathic effect induced by adenovirus type 5 (Ad5) in human A549 lung adenocarcinoma cells. **A:** Mock-infected cells. **B:** Ad5-infected culture showing rounding, ballooning, and clustering of cells.

virus (see Chapter 55 and the legend to Fig. 56.10). There are about 20 early genes and 15 late genes. Ads increase glycolysis in continuous cell lines and thereby induce the cells to produce large quantities of acid. Rapid cytopathology can be induced within several hours of inoculating concentrated crude virus preparations and is not related to viral replication; rather, it is caused by the penton base component of the free viral penton capsomere.

Nuclear morphologic changes in infected tissue cells can be used for diagnostic purposes. The nuclear changes include overall enlargement and intranuclear inclusions that initially are Feulgen negative and eosinophilic but become Feulgen positive and basophilic as the infection progresses.⁷⁰ The Feulgen-positive inclusions contain Ads by electron microscopy (EM) (see Fig. 56.2), and it is the DNA within the viruses that

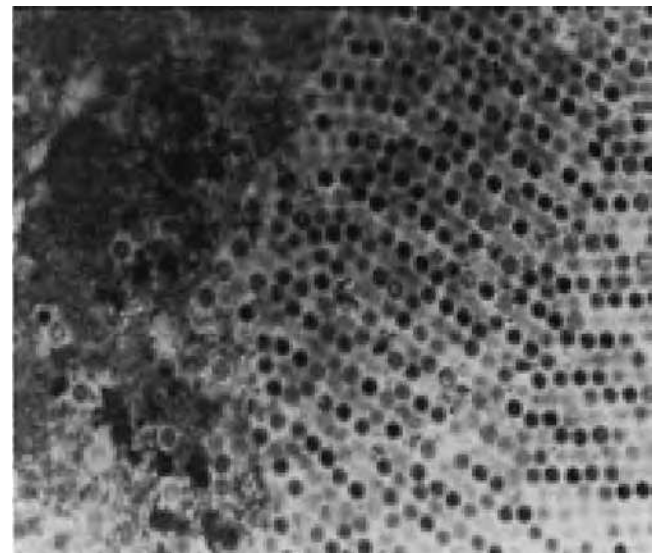


FIGURE 56.2. Electron microscopy (EM) of a nuclear inclusion body in a lung tissue cell from a fatal case of adenovirus pneumonia. The inclusion is full of virions, of which there may be as many as 10,000 particles per cell (EM, $\times 237,200$). (From Myerowitz RL, Stalder H, Oxman MN, et al. Fatal disseminated adenovirus infection in a renal transplant recipient. *Am J Med* 1975;59:591–598. Copyright 1975, with permission from Elsevier.)

contributes to the Feulgen stain. In addition, there are paracrystalline aggregates that contain viral protein without nucleic acid.⁴⁸⁴

Description of Key Antigens

The clinically predominant Ad antigens are the three capsid proteins: hexon, penton base, and fiber. Most early studies indicated that the hexon and to a lesser extent the fiber proteins contain most of the epitopes recognized by neutralizing antibodies, but there are neutralizing epitopes on penton base as well (reviewed in^{109,218,302,448,569,591,693,786}). The neutralization properties of polyclonal antibodies are often concordant with the inhibition of Ad-induced hemagglutination (HA) of selected red blood cells. However, the HA functions are the property of fiber (reviewed in⁵²¹), which usually must be linked to the penton base for complete HA to occur. Because of recombination within species in clinical isolates, it is not uncommon to isolate a virus that demonstrates discordant reactions in the neutralization and HA inhibition reactions.

One recent study of naturally infected humans found that the majority of neutralizing antibodies were against the hypervariable regions of hexon, but some were also against fiber and possibly penton base.^{591,693} In contrast, another recent study concluded that Ad5 neutralizing antibodies to fiber are more common than to hexon in the naturally infected population, but that immunization with a replication-defective Ad5-based vector raised more neutralizing antibodies to capsid proteins other than fiber.¹⁰⁹ Most studies in animal models infected with Ad5-based vectors have found that the predominant neutralizing antibodies are directed at hypervariable regions in hexon.^{404,591,810} Mechanisms by which neutralizing antibodies function include virus aggregation, virus destabilization, and blocking virus receptor interactions and integrin-mediated internalization (reviewed in⁶⁷⁰). One study with the Ad5 neutralizing hexon monoclonal antibody 9C12 reported that following infection and endosome penetration of the virus–9C12 complex, microtubule-dependent translocation to the microtubule-organizing center was inhibited.⁶⁷⁰ Another study with the same monoclonal antibody as well as polyclonal Ad5 neutralizing antibodies concluded that the cellular cytosolic protein TRIM21 binds to the antibody in the internalized antibody–virus complex and targets the virus to the proteasome for degradation.⁴⁵⁴

There are group- and type-specific epitopes on both hexon and fiber. Type-specific domains have been mapped to unique sequences in loop 1 (amino acids 281 to 292) and loop 2 (amino acids 441 to 455) of hexon by generating neutralizing antibodies to peptides from each of these regions. This epitope is referred to as the E determinant.⁴⁴⁸ Loops 1 and 2 had previously been shown to be on the surface of the virion by crystallography. Differences in the HA properties of rhesus and vervet erythrocytes for two important subtypes of Ad11 (Ad11p and Ad11a) have been related to nucleotide sequence differences in the shaft and knob region of fiber. The knob region of fiber, which has HA properties that are used for HI (hemagglutination inhibition) tests, includes the γ determinant.⁴⁴⁸ Ad11p and Ad11a have some differences in tissue tropism in that Ad11p can be persistent in the urinary tract and Ad11a causes acute respiratory tract infections. These properties of the whole virus may be related to the changes in fiber polypeptides that are otherwise identical for 92.3% of their amino acids.⁴⁶⁸

There are group-reactive antibodies that react with conserved domains of hexon from all human serotypes.⁵²⁹ These interactions were classically measured by the complement fixation (CF) test and were useful in identifying an agent as an Ad. Subsequent serologic techniques, such as immunofluorescent (IF) antibody and enzyme-linked immunosorbent assay (ELISA) determinations, were also capable of detecting group reactivity shared by most of the human Ads.

Ad-specific CD4+ T lymphocytes have been detected in peripheral blood monocytes (PBMCs) in nearly all naturally infected humans of all ages (reviewed by⁴⁰⁹).^{84,102,278,321,414,536,541,542,750} When PBMCs were stimulated in bulk culture (e.g., by incubation with intact Ad particles, Ad-infected cell extracts, or purified Ad proteins or with pools of peptides that span various Ad proteins), CD4+ T lymphocytes specific to hexon were identified from healthy donors.^{199,540,542,636,750,814} Other studies using peptides corresponding to parts of several Ad proteins identified a number of CD4+ T-cell epitopes, including the dominant human leukocyte antigen (HLA) DP4-restricted H910-924 epitope, located in the base of the hexon protein, conserved among Ad serotypes, and detectable in PBMCs from 78% of healthy adults analyzed.^{540,700} Many other CD4+ T-cell epitopes located in the conserved regions of Ad5 hexon and that are conserved across Ad serotypes have been identified that are recognized by PBMCs.^{410,411,636,750,813} In one study in which PBMCs from 44 healthy donors were analyzed, 10 CD4+ T-cell immunodominant hexon epitopes were detected in more than 50% of subjects examined.⁶³⁶ The HLA restriction element for some of these peptides is known.^{636,813} Although not as frequently found as are Ad- and hexon-specific CD4+ T cells, hexon-specific CD8+ T cells have also been detected in PBMCs from healthy donors; the cells are cross-reactive against various Ad serotypes, they secrete interferon- γ , and they have cytolytic activity in culture.^{199,320,321,345,410,414,669,700,813} CD4+ and CD8+ T cells specific to Ad proteins other than hexon, including penton base⁴¹¹ and the DNA polymerase,^{344,345} have also been identified.¹⁹⁹ There is increasing evidence that cytotoxic T lymphocytes (CTLs) specific to hexon are protective in humans.⁴¹⁰ Indeed, T-cell “lines” (mixture of CD4+ and CD8+ cells) specific to hexon (and other Ad proteins) have been shown to be effective by adoptive cell transfer in treating infection by various Ad serotypes in allogeneic hematopoietic stem cell transplant patients.^{198,411,413,813} The therapeutic effect is believed to be through the coordinated action of the adoptively transferred CD4+ and CD8+ T cells.⁸¹³

Infection of Experimental Animals

Human Ads are mostly species specific in their replication cycle, as are most Ads of other mammals or avian species. Even nonhuman primates are poor hosts for human Ads. Human species C Ads can replicate in the lung of cotton rats,²²⁷ and the pathogenesis of Ad pneumonia has been studied in these animals.^{54,227,574} An ocular model of infection with either Ad5 or Ad8 has been described in cotton rats, and the clinical manifestations, including subepithelial corneal opacities, were similar to epidemic EKC.^{359,730} The animals shed virus, developed specific antibodies to the infecting virus, and were able to spread the infection to control cotton rats. A similar ocular model was reported in New Zealand White rabbits after topical or intrastromal inoculations of Ad5.^{602,729} The virus appeared to replicate, and most of the animals developed a humoral immune

response. There were findings of blepharitis, conjunctivitis, iritis, corneal edema, and subepithelial corneal infiltrates that were consistent with immune-mediated clinical disease. The Ad5 New Zealand White rabbit model has been used to evaluate the anti-Ad activity of several compounds⁶⁰¹ including cidofovir and 2'-3' dideoxycytidine,⁶⁰³ *N*-chlorotaurine,⁶⁰⁴ and dexamethasone povidone-iodine.¹²³ Ad5 can also replicate in porcine tissues.⁷²³

A number of groups have explored the mouse as a model for pathogenesis of human Ads or as a model to evaluate oncolytic (replication-competent) Ad vectors for cancer gene therapy. The results from these studies have been mixed. Human Ads can infect cells of virtually all mammalian species including the mouse, especially if high multiplicities of infection are used (~100 plaque forming units [pfu] per cell). The early proteins (synthesized prior to Ad DNA replication) are expressed at good levels, but most workers have found that Ad DNA does not replicate (or barely replicates), and therefore that late genes are not expressed (or are expressed at very low levels). Nevertheless, there are reports that Ad5 or Ad2 can replicate to low levels in several mouse carcinoma cell lines^{221,258,503,772}; however, this is not a universal finding (e.g.,³⁴¹). When high doses of Ad5 were inoculated intravenously into CBA mice, there appeared to be several orders of magnitude of replication in the liver.¹⁶⁹ On the other hand, no replication was seen in the lungs of C57BL/6 mice following intranasal administration of Ad5.²²⁸ Also, little or no replication was observed in any organs following intravenous administration of an Ad5-based oncolytic vector in C57BL/6 mice⁸⁰¹ (this vector retains all Ad genes except some in the E3 region).

Ad5 replicates modestly in cotton rat cells,^{726,682} canine cells,⁷⁰³ porcine cells,³⁴¹ and Syrian hamster cells.⁷⁰⁵ In three Syrian hamster cancer cell lines, the burst size (virus yield per cell) was about 1,000, only 10-fold less than in A549 cells.⁷⁰⁵ In Syrian hamsters, about four orders of magnitude of Ad5 replication were seen in the lung following intranasal or intratracheal administration.^{292,487,705} Ad5 also replicates in the liver of Syrian hamsters following intravenous administration.⁸⁰¹

Because Syrian hamster tissues are quite permissive for Ad5 and there are numerous Syrian hamster cancer cell lines, Syrian hamsters have been used as a model to investigate the toxicology and antitumor efficacy of oncolytic Ad5-based vectors.^{157,369,705,707} Immunocompetent and immunosuppressed (by treatment with cyclophosphamide) hamsters bearing subcutaneous tumors formed by injection with various Syrian hamster cancer cell lines were treated by intratumoral injection with a variety of oncolytic Ad vectors.^{63,64,66,94,95,155,156,361,384,648,677,705,706}

In general, these studies show that oncolytic Ad5-based vectors suppress the growth of tumors, and that there is a rapid adaptive immune response to the vector that appears to eliminate the vector from the tumor. Syrian hamsters have also been used to study the biodistribution and toxicity of oncolytic Ad5-based vectors in advance of clinical trials.^{374,429,460,675,801}

Immunocompetent,^{159,727} newborn,⁸¹⁵ and immunosuppressed⁷²⁷ Syrian hamsters have been used to evaluate compounds to inhibit Ad5 replication. A mouse model was used similarly to test cidofovir against disseminated mouse Ad type 1 infection.⁴¹⁷

Human Ads inoculated into a variety of rodent species cause tumors. Ad12, 18, and 31 are highly oncogenic in newborn Syrian hamsters, and much has been learned about the

mechanism of action of the genes in the E1A and E1B regions from these viral models (see Chapter 55). The integrated Ad12 sequences in these hamster tumors are a model to understand epigenetic consequences of foreign DNA integrations.¹⁶⁰ Ad9 causes fibroadenomas and mammary sarcomas in rats, and an early region 4 gene seems to be important in this model.⁴⁰⁵

Functions of Adenovirus E3 Proteins

The proteins coded by the Ad E3 transcription unit are believed to provide protection of infected cells from the host antiviral response (Fig. 56.3) (reviewed in^{57,196,259,309,430,445,465,646,785}). The Ad2 or Ad5 E3-gp19K protein is a type I glycoprotein localized in the endoplasmic reticulum (ER) (reviewed in²⁵⁹). E3-gp19K binds to major histocompatibility complex class I (MHC-I) heavy chain in the ER, prevents transport of MHC-I to the cell surface by virtue of an ER retrieval signal on E3-gp19K, and prevents killing of Ad-infected cells by CTLs.^{14,82,131,582} E3-gp19K and its function are conserved among serotypes in all human Ad species²¹² except species A in which the E1A proteins cause down-regulation of MHC-I.³⁴⁰ E3-gp19K binds with higher affinity to HLA-A than to HLA-B, and it binds poorly if at all to HLA-C.^{212,259} E3-gp19K binds via a conserved domain (among Ad serotypes)^{213,641} to the outer surface of the peptide-binding groove on MHC-I molecules.^{259,437} E3-gp19K also binds to *transporter associated with processing* (TAP), prevents formation of the TAP–tapasin complex, and limits the inclusion of TAP into the peptide-loading complex of antigenic peptide, MHC-I, and chaperones.⁵² This property of E3-gp19K could possibly retard cell surface expression of MHC-I in individuals with HLA-B and HLA-C MHC-I molecules.²⁵⁹

Reduced expression of MHC-I could render Ad-infected cells susceptible to killing by natural killer (NK) cells. To potentially enhance this possibility, the Ad E1A proteins up-regulate ligands recognized by the NKG2D receptor on NK cells and sensitize the E1A-expressing cells to NK cell–mediated cell lysis.^{467,611} Multiple ligands for NKG2D are known, including the MHC-I chain-related A (MICA) and B (MICB) proteins. As is the case with MHC-I, E3-gp19K causes retention of MICA and MICB in the ER, prevents their transport to the cell surface, and reduces killing of E3-gp19K–expressing cells by NK cells.^{467,641}

The Ad E3-14.7K protein inhibits tumor necrosis factor (TNF)-induced cytotoxicity of Ad-infected cultured cells.^{232,232,234,305,306,579} E3-14.7K binds to cellular IKK γ /NEMO^{373,426} and is reported to modulate NF- κ B activity and inhibit TNF-induced apoptosis.⁴²⁶ E3-14.7K also is reported to bind to caspase 8.^{107,373} In addition, E3-14.7K inhibits internalization of TNF receptor 1 as well as formation of the death-signaling complex (DISC) that is required for TNF-induced apoptosis.⁶²⁵ Two studies report that E3-14.7K inhibits TNF-induced signaling through NF- κ B,^{90,426} whereas another study did not observe such activity for E3-14.7K.⁶²⁵ In a recent report, E3-14.7K stably expressed in mouse cells inhibited induction by TNF of the chemokine CCL2 (monocyte chemoattractant protein-1 [MCP-1]) by preventing phosphorylation of glycogen synthase kinase-3 β and recruitment of NF- κ B to the CCL2 promoter.⁶⁷⁸ Some researchers^{107,794} but not others⁷¹⁴ report that E3-14.7K inhibits apoptosis by Fas ligand. E3-14.7K also inhibits TNF-induced release of arachidonic acid,^{390,709,822} a property that might be important in reducing inflammation.

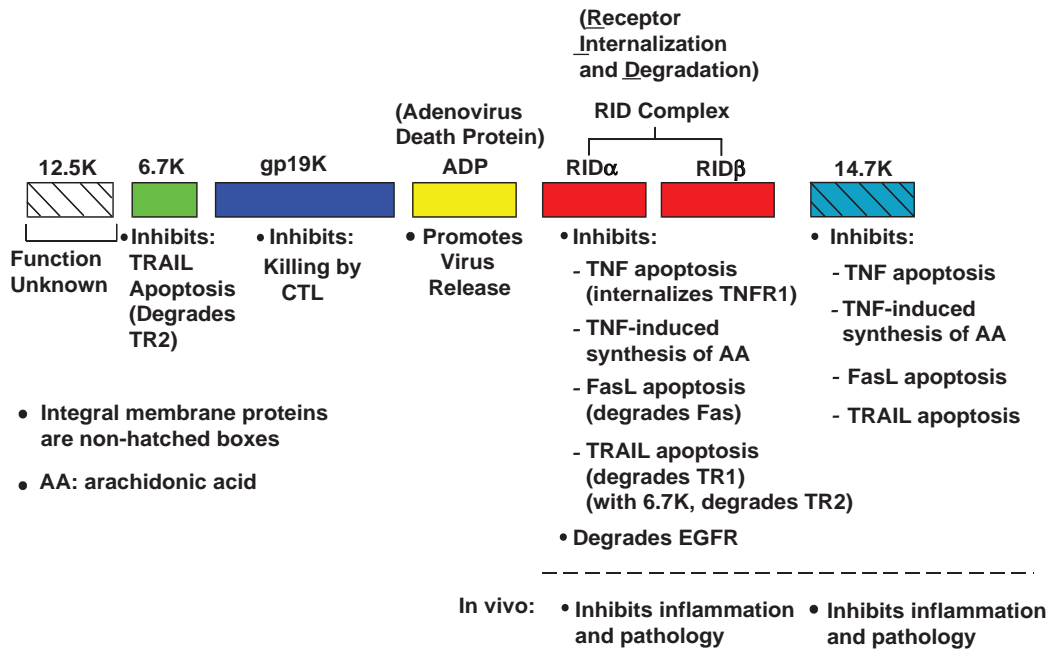
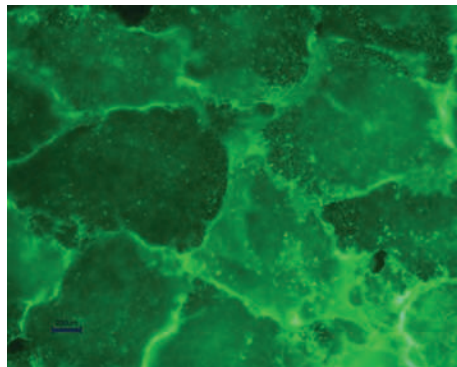


FIGURE 56.3. Schematic illustrating the E3 proteins and their functions. Each bar represents a protein, with the name of the protein above the bar. Colored bars without hatches are integral membrane proteins. The adenovirus death protein (ADP) was originally named the 11.6K protein.^{717,718} The receptor internalization and degradation (RID) protein was originally named E3-10.4K/14.5K, and RID α and RID β were named E3-10.4K and E3-14.5K, respectively.⁷¹⁴

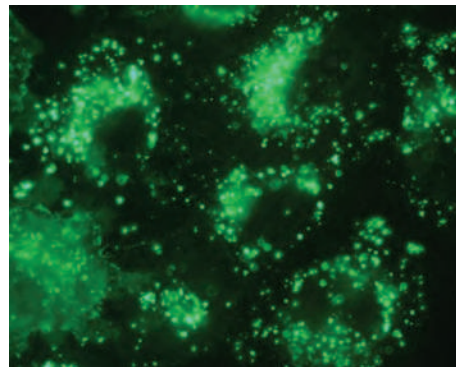
Two other E3 proteins, of 10,400 daltons (10.4K) and 14,500 daltons (14.5K), which were originally named E3-10.4K⁷¹⁶ and E3-14.5K,⁷¹⁵ respectively, form a molecular complex originally named E3-10.4/14.5K.⁷¹⁹ The E3-10.4K protein⁸⁸ and then later the E3-10.4K/14.5K complex⁷¹⁹ were reported to be required to clear the epidermal growth factor receptor (EGFR) from the cell surface (Fig. 56.4). The E3-10.4K and E3-14.5K proteins were later renamed *receptor internalization and degradation* (RID) α and RID β , respectively, when it was shown that these proteins also are able to cause the internalization and lysosomal degradation of not only EGFR but also cell surface Fas,^{180,464,662,714} TRAIL receptor 1,^{49,720} and TNF

receptor 1.^{110,111} RID inhibits cytolysis induced by Fas ligand (i.e., by an agonist antibody)^{428,662,714} and TNF.^{233,390,812} RID, functioning in concert with the E3-6.7K (6,700 daltons) protein, also down-regulates TRAIL receptor 2 from the cell surface.^{49,427} One laboratory⁴⁹ but not another⁴²⁷ reported that E3-6.7K is required together with RID to down-regulate TRAIL receptor 1. A cytosolic tyrosine sorting motif (YXX ϕ) in RID β ^{110,428} and a cytosolic dileucine motif²⁸⁶ and tyrosine-containing motifs¹¹⁸ in RID α are required for these proteins to function.

RID also has been shown to inhibit TNF-induced translocation of cytosolic phospholipase A₂ to membranes (from where arachidonic acid is generated)³⁹⁰ and to inhibit



A



B

FIGURE 56.4. Clearance of the epidermal growth factor receptor from the surface of human A549 cells by Ad5. In mock-infected cells (A), the receptor is abundant on the cell surface, whereas in Ad5-infected cells (B), the receptor has been internalized into endosomes. The internalization of the receptor is mediated by the E3 receptor internalization and degradation (RID) complex of proteins.

lipopolysaccharide- and interleukin-1 β -mediated signaling responses.¹⁵⁰ Thus, both RID and E3-14.7K might function *in vivo* to inhibit inflammatory responses associated with infection.

In contrast to most laboratories who have studied RID, one laboratory has reported that RID α can function in the absence of RID β to down-regulate EGFR.^{118,293,645} This laboratory also reported that RID α alone is able to regulate endosome maturation by mimicking guanosine triphosphate (GTP)-Rab7⁶⁴⁵ and to activate an autonomous cholesterol regulatory mechanism.¹¹⁷ Possible explanations for how RID α , RID β , and the RID α /RID β complex (i.e., RID) could function independently have been discussed.⁶⁴⁵

As mentioned, the E3-6.7K protein is required together with RID to down-regulate TRAIL receptor 2 from the cell surface in Ad-infected cultured cells.^{49,427} In transiently or stably transfected Jurkat cells, E3-6.7K alone inhibited apoptosis induced through Fas, TNF receptor, and TRAIL receptors and prevented TNF-induced release of arachidonic acid.⁴⁸⁰ Further, E3-6.7K was shown to interact with calcium modulator and cyclophilin ligand (CAML), a calcium-modulating protein,²⁴¹ to prevent calcium efflux from the ER, maintain calcium homeostasis, and inhibit apoptosis induced by thapsigargin (which inhibits calcium uptake by the ER).^{241,480}

The functions for the E3 proteins as described previously have been determined by studies in cell culture, and the question arises as to whether the E3 proteins have these same functions or other unknown functions *in vivo* (animal models and humans). Some and perhaps all of the E3 proteins are not essential for Ad5-based oncolytic vectors to replicate in Syrian hamsters⁷⁰⁵ or in cancer patients, but there is good evidence that E3 proteins have functions *in vivo*. In one study, expression of all the E3 proteins from a replication-defective Ad vector prolonged transplants of human cells in immunocompetent mice.⁷²⁴ In another study, the entire Ad2 E3 region stably expressed in an immortalized Gunn rat hepatocyte cell line protected against allograft rejection following transplantation into rats.⁴⁵⁸ This protection correlated with reduced Fas expression and inhibition of Fas-mediated apoptosis in the transplanted cells (which could be mediated by RID). Expression of E3-gp19K plus RID by lentivirus vectors in an insulin-producing β -cell line corrected diabetes in allogeneic mice, whereas the cell line without E3-gp19K plus RID did not protect.³⁸³ In transgenic mice, the entire Ad2 E3 region that was placed under the control of the rat insulin promoter (RIP) for targeted expression in murine pancreatic islet cells prevented autoimmune diabetes in a virus-induced (lymphocytic choriomeningitis virus) murine model of type I diabetes or in non-obese diabetic (NOD) mice.^{179,755} In further studies in which all E3 genes except E3-gp19K or except the E3B region genes (E3-RID plus E3-14.7K) were expressed from the RIP in transgenic mice, one study found that E3-gp19K but not the E3B region genes prevented autoimmune diabetes,³¹⁰ but another study obtained the opposite result.⁵⁶⁶ In studies addressing E3-14.7K, the gene for E3-14.7K, which was cloned into vaccinia virus together with the *tnf* gene, increased the virulence of the vaccinia virus carrying the *tnf* gene alone by reversing the antiviral effects of TNF.⁷³¹ In other studies, a transgenic mouse was constructed in which E3-14.7K is expressed selectively in the distal respiratory epithelium (alveolar and bronchial) from the surfactant protein B promoter.²⁶⁴ E3-14.7K protein suppressed pulmonary epithelial cytotoxicity and lung

inflammation in response to respiratory infection with a replication-defective Ad vector or to intratracheal administration of lipopolysaccharide.²⁶⁴ In an influenza virus study, E3-14.7K reduced expression in the lung of CCL2, a chemokine that functions in recruitment of inflammatory monocytes and lymphocytes.⁶⁷⁸ E3 genes were required to reduce inflammation in an Ad5 pneumonia model in cotton rats²²⁷ and mice.⁶⁷⁶ The E3-6.7K plus E3-gp19K proteins were required to prolong persistence of an oncolytic Ad in Syrian hamsters.⁶⁵ These various studies suggest that E3 proteins function *in vivo* in a manner consistent with their functions seen *in vitro*.

PATHOGENESIS AND PATHOLOGY

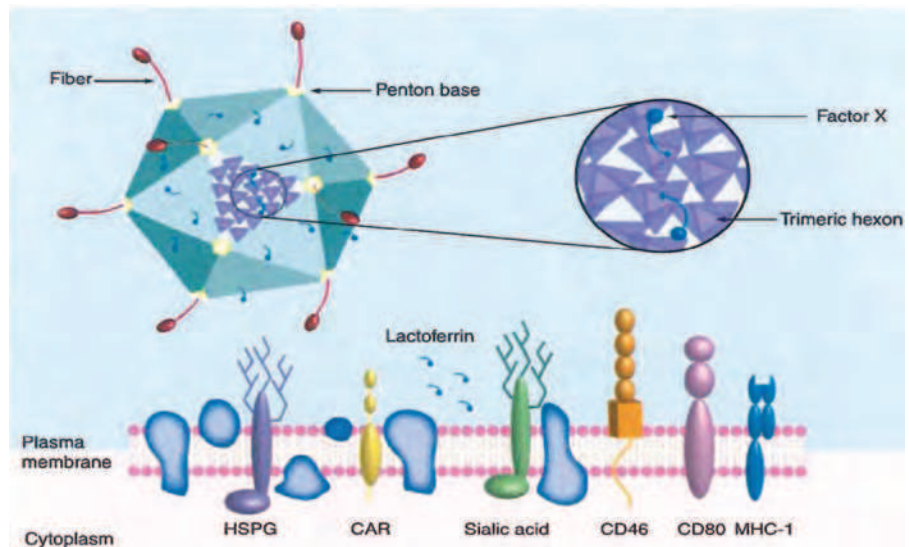
Ad infection causes inhibition of cellular DNA, mRNA, and protein synthesis. The infected cell degenerates in specific ways that help the pathologist diagnose Ad infection on biopsy or autopsy tissue.⁵⁰⁰ The respiratory epithelial cells that are affected during Ad pneumonitis have enlarged nuclei containing amphophilic or basophilic inclusion bodies surrounded by thin rims of cytoplasm. Some of these cells are referred to as *smudge cells* and contrast with those infected cells with nuclear inclusions but intact nuclear membranes. As in tissue culture, the epithelial cells are the primary target for Ad cytopathology *in vivo*.⁷⁰

One of the Ad structural proteins, the penton base, made in much larger excess than needed for the assembly of Ad, binds to the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins via an arginine-glycine-aspartate (RGD) motif in penton base²⁸ and causes cells to detach rapidly from monolayer cell culture dishes.⁷⁸⁰ This effect can be demonstrated even in cells that are not directly infected with Ads; it has been postulated that this protein might be involved in pathogenesis. It had been thought that the penton cell-rounding effect led to cell death, but it now appears that the cells are viable upon removal of the viral protein.⁵⁰⁸ Although the importance of penton in human disease has not been determined, it has been found in the blood of several fatal cases of Ad pneumonia.⁴⁰⁰

Ad-induced changes at the level of single cells result in considerable organ toxicity during serious tissue invasion by Ads. Necrotizing bronchitis, bronchiolitis, and interstitial pneumonia, as well as fibrin and hyaline membranes within the alveoli, characterize Ad pulmonary syndromes.¹⁶⁸ Comparable lesions occur within the conjunctiva, in which exudative and mononuclear infiltrates are found. However, the lesions occur beneath the epithelium, so that ulceration or neovascularization of the cornea usually does not occur.^{335,478}

Proliferative responses similar to transformation have not been recognized in acute Ad infections in humans. However, lymphatic tissue is often hypertrophied, and active germinal centers are found. For example, the appendices of children undergoing surgery to correct bowel obstruction found in intussusception may be associated with enlarged mesenteric lymph nodes and Ad isolation from stool specimens.⁸⁰⁷ These lymphocyte changes are often in close proximity to areas of desquamated epithelial cells in which typical viral inclusions may be found. The inclusions contain viral particles in crystalline arrays that are visualized by electron microscopy (EM). Some of the lymphocytes are probably CD8+ CTLs that recognize proteolytic peptide products of viral proteins.

FIGURE 56.5. Some of the receptors used by human adenoviruses (Ads). CAR, sialic acid, CD86/CD80, CD46, and MHC-1 are membrane proteins. Other ligands are present in a soluble form, such as lactoferrin and coagulation factor X, which are used as a bridge between the virus and the cell to target the liver. The receptor used depends on the Ad type and the targeted tissue. CAR, coxsackievirus B and adenovirus receptor; HSPG, heparan sulfate proteoglycans. (From Enaff D, Salinas S, Kremer EJ. An adenovirus traffic update: from receptor engagement to the nuclear pore. *Future Microbiol* 2011;6:179–192, with permission.)



Entry into the Host

Ads enter susceptible hosts by the mouth, nasopharynx, or ocular conjunctiva. Recent experiments have defined the Ad receptors (Fig. 56.5) (reviewed in ^{406,469,521,770,817}). The cell protein named CAR (coxsackie-adenovirus receptor) is a receptor shared by these two groups of unrelated viruses and is the target of fiber in many if not all serotypes in species A, C, D, E, and F.^{55,55,597} CAR is present on tight junctions of polarized cells where it mediates cell–cell adhesion. Species B Ads can be differentiated based on their receptor usage.⁷³² Species B, group 1 (Ad16, 21, 35, 50) viruses nearly exclusively utilize CD46 as a receptor.^{253,457} Initially, Ad37, a species D Ad, was also thought to use CD46,⁷⁹¹ but recent data suggest that GD1a glycan is the more likely major receptor for this virus.⁵²⁵ CD46 (also known as membrane co-factor protein) is expressed in virtually all cells where it acts as a co-factor for inactivation of the complement components C3b and C46. Species B, group 2 (Ad3, Ad7, 14) viruses share desmoglein 2 as the high-affinity receptor.^{769,770,771} Desmoglein 2 is a calcium-binding transmembrane glycoprotein belonging to the cadherin protein family. When the Ad fiber binds to desmoglein 2, there is opening of intercellular junctions that results in increased access to receptors trapped deep within the junction (i.e., CD46 and Her2/*neu*).⁷⁷⁰ Further, such dissociation of the intercellular junctions may facilitate the lateral viral spread in epithelial cells and, potentially, the penetration of Ad into subepithelial cell layers and the bloodstream. Lastly, species B, group 3 (Ad11) viruses preferentially interact with CD46 but also utilize desmoglein 2 if CD46 is blocked.⁷⁷⁰

Heparan sulfate glycosaminoglycans, which are long heterogeneous, heavily sulfated carbohydrates that are abundant within the extracellular matrix, can mediate the CAR-independent binding of Ad2 and Ad5 to cells, but it is not known if these are *bona fide* receptors.⁸¹⁷ The class I MHC molecule has been proposed as a second receptor for Ad5³⁰⁴ but has not been confirmed by other groups.^{142,463} Three species D Ads, Ad8, Ad19, and Ad37, all major causes of EKC, appear to bind to α 2,3-linked sialic acid present in the GD1a ganglioside on the corneal cell surface.⁵²⁵ Several integrins participate in Ad uptake into cells by interacting with the RGD motif on the Ad penton base protein.⁸¹⁷

Most of the studies described previously were conducted *in vitro*. Recent studies of systemic administration of Ad vectors in mice raise the possibility that infection of tissues *in vivo* may be different (see section on Adenoviruses as Vectors for Vaccination and Gene Therapy). These studies propose a CAR-independent model in which Ad infection of murine hepatic cells occurs through binding of Ad to blood factors, especially factor X, directing the complexes to hepatocellular receptors including heparan sulfate proteoglycans (Fig. 56.5). Whether this pathway operates in humans is a key²⁸¹ question. Recent studies clearly demonstrate that all of the tested species of Ads, except for the species D viruses, efficiently bind human factor X.⁷⁵⁷ The high-affinity interaction of Ads with factor X may facilitate “bridging” the hexon protein in the Ad capsid to heparan sulfate proteoglycans expressed on the surface of hepatocytes.^{352,757} The Ad–factor X complex binds to the cell surface through the serine protease domain of factor X and not through a direct interaction of the virus with the cell surface.⁷⁵⁷ Once bound to the cell surface, efficient and rapid intracellular transport of the Ad, though, remains dependent upon engagement of α_v integrins via the penton base protein.⁷¹

The lower serotypes, Ad1, 2, 5, and 6, are ubiquitous, particularly in young children, and may be shed for months, especially in the stool, which is probably responsible for the endemic spread of these agents by the fecal–oral route to new pools of susceptible infants and children.²⁰⁵ An epidemic form of Ad keratoconjunctivitis (EKC) has been spread in swimming pools²⁰⁷ from contaminated water and in medical settings³³⁶ from Ad-infected ophthalmologic instruments. A third pattern of spread was unique in the military setting, occurring during the early period after induction into service. Ad4 and Ad7 caused acute respiratory distress (ARD), including pneumonia, and were the result of air-borne inoculation of the respiratory tract¹⁶⁸ as well as acquisition after contact with contaminated surfaces in living quarters.⁶¹⁵

Site of Primary Replication

It is clear from the original findings of Ads in tonsils and adenoids that these tissues of the oropharynx are a major initial site of replication for the entering Ads. For serotypes causing

respiratory disease, the initial replication most likely occurs in the nonciliated respiratory epithelium, although some limited replication and persistence can also occur within lymphocytes (reviewed in⁴⁴⁵). These conclusions are based not on careful observations of cells *in situ* but on the cells in which Ad replicates in tissue culture. Ciliated respiratory epithelium of the lower airway is difficult to infect with Ads through the apical surfaces, which do not contain the CAR receptor; however, disruption of the integrity of cell–cell contact allows basolateral infection of such polarized epithelial cells using CAR.^{565,768}

Spread of Virus and Tissue Tropism in the Host

Most of the manifestations of Ad infection are locally in the eyes and pharynx, but contiguous extension into the lungs results in some cases. One possible molecular mechanism facilitating the spread of the virus over respiratory epithelia is that the fiber protein, which is synthesized in great excess in the infected cell and is released when the cell is lysed, binds CAR on the basolateral surface. This binding disrupts the CAR homodimers in tight junctions, thus increasing paracellular permeability. This in turn allows the virus to escape onto the apical surface of the respiratory epithelium, thereby making it possible for the virus to infect other areas of the respiratory tract.⁷⁶⁷ Likewise, for the species B viruses that bind to desmoglein 2, binding to desmoglein 2 resulting in dissociation of the intercellular junctions may facilitate spread of the virus locally and into deeper tissue layers, allowing access to the bloodstream.⁷⁷⁰ Even in the nonreplicating mouse model of human Ad infection, the virus can cause inflammation in the lungs, which is strong evidence for direct extension into these target organs.^{228,676} Ads have been cultured from the blood during fatal Ad respiratory disease, suggesting viremic spread in some situations (see the section later on immunocompromised patients). As CAR is present on endothelial cells, viremia might be promoted by the fiber protein through the mechanism described earlier. The successful use of oral, live, microencapsulated Ad vaccines by the military to prevent ARD suggests that if the respiratory tract can be physically bypassed by Ad4 and Ad7, intestinal replication of the virus causes an immunizing rather than a virulent infection.¹²⁹ Although most Ads replicate in the intestine without causing gastroenteritis, Ad40 and 41 are responsible for intestinal disease. Ad disease in the urinary bladder, primarily by species B Ads in immunocompetent hosts, suggests that the virus probably is viremic at some stage in order to reach this organ. There is no evidence for ascending infection for these species B serotypes, which are less commonly found in the intestine and are more common in young males than in females. The route of infection of the liver, especially in immunosuppressed liver transplant recipients, is unknown, but some patients might be infected by latent Ads that are present in cells such as lymphocytes in the transplant.

Immune Response

Various aspects of the immune response to Ads have been described in other sections, as have mechanisms that the virus uses to evade the immune response (see Fig. 56.3 and section on Functions of Adenovirus E3 Proteins). The innate response to Ad also has been reviewed^{439,504} (see section on Adenoviruses as Vectors for Vaccination and Gene Therapy). In the airways, the virus must penetrate the surface fluid, and sialic acid present in the mucus may bind and inhibit species D Ads that use sialic acid as a receptor.^{342,525} The virus must also survive chemical defenses of

the host. These include a large variety of antimicrobial peptides that are able to neutralize microbes directly. Among these peptides are the defensins, a family of small cationic amphipathic peptides divided into two classes, α -defensins and β -defensins (reviewed in²⁷³). The α -defensins HNP1 and HD5 were shown to neutralize Ad serotypes in species A, B1, B2, C, and E, but not in species D and F.^{270,671,672} The defensins bind Ad particles outside the cell, block uncoating of the virion, and restrict the release of virions from endocytic vesicles.⁶⁷¹ Thousands of molecules of α -defensins bind to a sensitive serotype; neutralization of the virus depends on binding to critical determinants in a region spanning the fiber and penton base proteins.⁶⁷² Binding to these determinants is proposed to prevent the release of fiber from the virion, the first step in the virion uncoating process within the endosome.⁶⁷² HNP1 is expressed primarily in neutrophils, monocytes, lymphocytes, and natural killer cells, while HD5 is expressed mainly by Paneth cells in the intestine.^{273,671} It is not known if these or other defensins play a role in Ad infections in humans.

With respect to cellular defenses, alveolar macrophages and Kupffer cells play an important role in elimination of Ad vectors from the lung and liver, respectively, in murine models^{439,504} (Fig. 56.6) (see section on Adenoviruses as Vectors for Vaccination and Gene Therapy). These cells take up the vectors rapidly and secrete inflammatory cytokines such as TNF, interleukin-6 (IL-6), and IL-8. A robust inflammatory response characterized by early cytokine (IL-1 β , IL-6, interferon- γ [IFN- γ], IL-12, and TNF) and chemokine release as well as neutrophilic and monocytic infiltration have been described in the murine pneumonia model after nonpermissive infections with species C Ads.^{228,349} Increased levels of IL-6, IL-8, and TNF have also been associated with Ad infection in children.⁴⁷⁶ A recent study suggested that there may be specific cytokine signatures in pediatric stem cell transplant recipients with localized and invasive Ad infection. Patients with invasive infections had increased levels of the pro-inflammatory cytokines IL-1 β , IL-6, IL-8, IL-12, IFN- γ , and TNF- α , as well as IL-17, macrophage inflammatory protein-1 α (MIP1 α), oncostatin M (OSM), and IFN- γ -inducible protein 10 (IP10).²⁷² Invasive Ad infection was also associated with the simultaneous release of the cytokines IL-1 β , IL-17, IL-18, OSM, MIP-1 α , and IP10.²⁷²

Ad infection of cultured cancer cells, respiratory epithelial cells, hepatocytes, and antigen-presenting cells activates the NF- κ B and MAP kinase pathways.⁵⁴⁷ Induction of IL-6 and IL-8 does not require Ad gene expression and likely occurs via interaction of the virion with integrins on the cell surface. A biphasic mononuclear cell response in the cotton rat suggested an early appearance of monocytes and NK cells followed by specific T and B lymphocytes. The cell infiltrate was changed from a mononuclear to a polymorphonuclear leukocyte response by deletion of the Ad E3 anti-TNF genes (*14.7k*, *rida*, *ridb*). Deletion of the anti-MHC Ad E3-gp19K protein increased the intensity of the inflammatory response in the cotton rat but not in the mouse.^{227,676} These observations suggest that immunopathology plays a role in the host response to Ad infection, and this process is ameliorated by the E3 inhibitors of the host inflammatory response.^{227,574} Another Ad protein, the L4-100K protein, which is expressed at late stages of infection, inhibits granzyme B-mediated death by CTL.¹⁶ Thus, it is not clear at this point whether the cell-damaging effects of Ad infection or the host immune responses to the

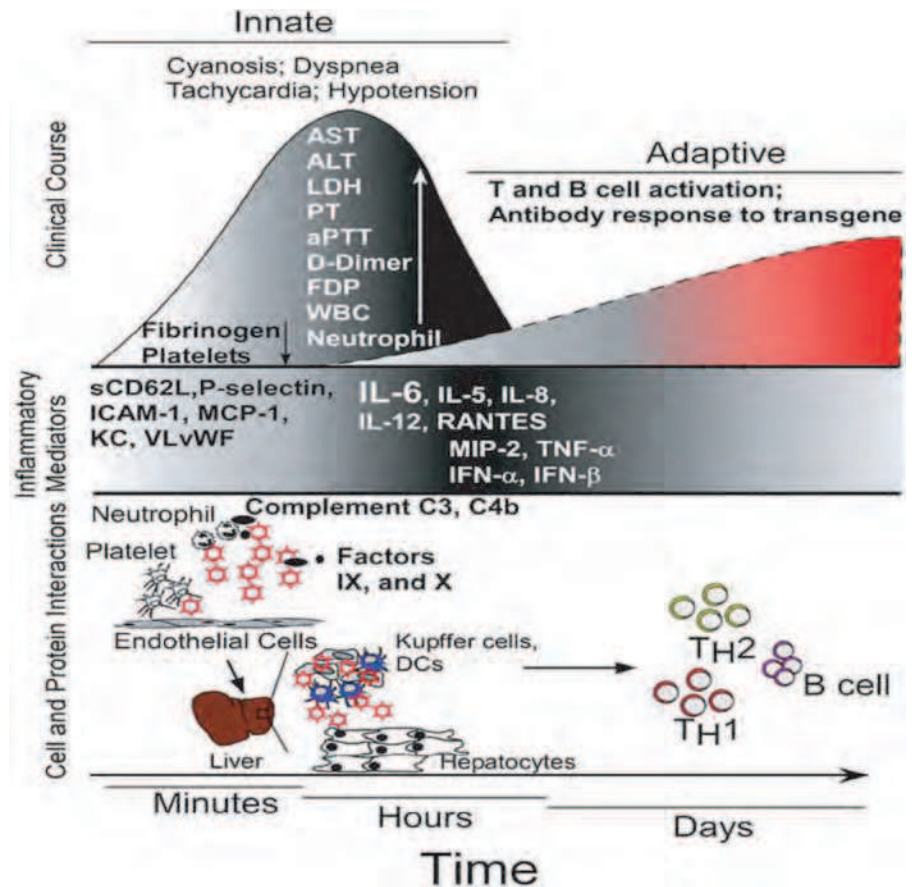


FIGURE 56.6. Complex blood-borne protein and cellular factors interact with helper-dependent adenovirus (HDAd) vector within minutes of systemic administration (bottom). The cascade of inflammatory mediators initiated by the immediate cellular interactions (**middle**) exacerbates the clinical manifestations shown (**top**) over several hours. The induction of innate immune responses initiated days earlier leads to activation of antigen-presenting cells and subsequent T helper (Th) 1/2 cell expansion and an antitransgene humoral immune response to the virus and transgene. (From Seiler MP, Cerullo V, Lee B. Immune response to helper dependent adenoviral mediated liver gene therapy: challenges and prospects. *Curr Gene Ther* 2007;7:297–305.)

pathogen are responsible for the tissue pathology and clinical manifestations.

Induction of type I interferons (α and β) is part of the innate response to Ad infection. Ads have at least two mechanisms to counteract these interferons (see Chapter 55). First, the E1A proteins block the assembly of interferon-induced transcription factors, and second, VA-RNA₁, a 159-nucleotide RNA, binds to the protein kinase named PKR, prevents its activation by double-stranded RNA, and thereby prevents PKR from phosphorylating eukaryotic initiation factor eIF2 and shutting down protein synthesis.

Neutralizing and nonneutralizing antibodies are discussed in the section, Description of Key Antigens. After infection, most patients develop both group- and type-specific antibodies to the infecting Ad strain. Group-specific antibodies are not neutralizing of viral infectivity but are useful in measuring patient response when infection with an Ad is suspected but no isolate was obtained. In this case, acute and convalescent sera taken several weeks apart may demonstrate rising antibodies to a group-specific Ad antibody such as that measured by CF, IF, or ELISA, using a viral-infected tissue culture extract or purified hexon from a single Ad type. Type-specific antibodies are measured by either the neutralization test or inhibition of HA, as described in the section Viral Serology. It is clear that patients may continue to shed Ads intermittently, especially in their stools, for many months after a successful humoral immune response has occurred.²⁰⁵ Neutralizing antibodies may be protective against disease manifestation in the previously infected host or against reinfection with the same serotype, but they do not eliminate the carrier state.

Recent studies have begun to define the T-cell response to Ad infection in humans and Ad (see the section on Description of Key Antigens) (reviewed in^{382,409,762}). Although most of the early work in humans described Ad-specific CD4⁺ T cells, recent studies have detected and characterized Ad-specific CD8⁺ T cells. These CD4⁺ and CD8⁺ T cells are frequently cross-reactive among Ad serotypes, and many of the epitopes have been mapped to the conserved regions of hexon.

Although it is not known if these T-cell epitopes are protective against Ad in humans, most likely T cells in general are protective because the absolute lymphocyte level and the CD4⁺ T cell level correlate inversely with Ad infection and disseminated adenoviremia in immunosuppressed transplant patients (see section on Diseases Associated with Immunocompromised Patients). For example, in adult stem cell transplant recipients, clearance of human Ad viremia coincides with emergence of a coordinated CD8⁺ and CD4⁺ T-cell response against Ad hexon epitopes.^{199,277,813}

Although donor lymphocyte infusions can be used in hematopoietic stem cell transplant recipients with Ad infections to improve infectious diseases outcomes, potentially life-threatening graft-versus-host disease (GVHD) may develop.¹²⁵ Adoptive transfer of CTL against Ad can rapidly reconstitute anti-Ad immunity after stem cell transplantation without causing GVHD.^{199,277,410,813} Methods are being developed to activate and expand T cells *in vitro*, and these interventions appear to be a promising potential therapy for immunocompromised patients with serious Ad infections (reviewed in^{5,367,409,412,474,813}).

Virulence

It is not known why certain serotypes characteristically cause disease in some organs and not in others. Tissue culture experiments and the existing animal models do not explain the mechanisms of such tissue tropism or organ-specific pathogenicity. There is nothing obvious about the bioinformatics analysis of multiple Ad genomes that explains differences in pathogenicity, although most differences among species are in the E3 region.¹⁴¹ Because most Ads can attach to the CAR receptor, it is unlikely that this receptor can explain differences in the *in vivo* tropism of the various serotypes. Even the oral vaccine strains used to prevent respiratory disease in military personnel seem to depend more on physical formulation (i.e., enteric coating of a live virus so that it bypasses the oropharynx and first exposes the intestinal epithelium) than on mutation of virulence genes.⁴⁴⁶

Epidemiology

Human Ad serotypes are generally not pathogenic to animals, and animal Ads are pathogenic only within the species of origin.⁷⁰² However, simian species occasionally have been shown to have antibodies to human Ad12,³⁵³ and antibodies to simian Ads have been detected in human sera.¹⁸⁷ Recently, a novel Ad named titi monkey Ad, which caused a deadly outbreak of pneumonia and hepatitis in a closed colony of titi monkeys in California, caused a respiratory infection in a researcher in close contact with the monkeys, and this infection spread to a family member.¹⁰⁵ A screen of 81 random adult blood samples revealed neutralizing antibodies to this virus in 2 individuals. The native species for this virus is unknown. Of added interest, Ads isolated from great apes are phylogenetically related to human Ads in species B, C, and E, consistent with the possibility of interspecies spread at some point in time; however, frequent interspecies infections are unlikely.⁶¹⁴

In humans, transmission of Ad infection and disease varies from sporadic to epidemic. The pattern often correlates with the viral serotype and the age (children or adults) of the susceptible population. A virus watch study was undertaken in New York and Seattle,^{205,206} and much of the Ad epidemiology in civilian populations was learned from these studies, including that there was a high incidence of recurrent shedding, especially of the lower numbered serotypes, in fecal specimens. A large number of asymptomatic Ad infections were documented. Fecal–oral transmission accounts for most infections in young children.⁴³ Initial spread may occur by the respiratory route, but the prolonged carriage in the intestine makes the feces a more common source during both the acute illness and intermittent recurrences of shedding.²⁰⁵ The epidemiologic importance of the long latency in tonsil tissue is not known.

Ads are estimated to cause 8% of clinically relevant viral disease globally. They probably account for 3% of the infections in civilian populations and for about 5% to 10% if only febrile illnesses are calculated.^{205,206} The corresponding figures for young children are about 5% and 10%, respectively. In a recent PCR-based multicenter study, 4.4% of pediatric patients with diarrhea in Asia were shown to have Ad-positive stool samples, with Ad40 and 41 being the most prevalent serotypes.⁴²⁴ According to a survey carried out in Manchester, United King-

dom, 61.3% of patients with Ad infection were younger than 5 years of age, 24.2% were adults, and 5.6% were children between 5 and 18 years old.¹²⁶ The most prevalent serotypes were Ad2 (18.6%), Ad3 (14.9%), Ad1 (12.1%), and Ad41 (10.9%). While Ad2, 1, and 41 were isolated mostly from infants, Ad3 and, less frequently, Ad4 (8.3%) was recovered from adults.

Serologic surveys have furnished estimates of the prevalence of Ad infections. Early surveys indicated that antibodies to Ad1, 2, and 5 are most common and are present in 40% to 60% of children.^{27,73,317} The incidence of antibodies to Ad3, 4, and 7 is low at the same ages. These antibody results probably explain why adults are uncommonly infected with Ad1, 2, and 5 but are more susceptible to infections with Ad3, 4, and 7. During the surveillance for the virus watch studies, only about 75% of the Ad isolates were accompanied by an antibody response, as measured by the CF test.²⁰⁵

More recent surveys have provided additional epidemiologic information. Ad5 neutralizing antibodies have been found in ~80% to 90% of the population in Sub-Saharan Africa.^{1,36,106,167,301,332,459,533,693,710} In one study of sera from Africa, the seroprevalence of Ad5 was very high in neonates (93%, with 48% having neutralizing titers of greater than 1,000), and it correlated with maternal titers.⁷¹⁰ Interestingly, the seroprevalence was 13% in subjects aged 6 months to 1 year, was 28% at age 1 to 2 years, and then reached high adult levels at age 7. These data suggest that neonates acquired maternal antibody, which then declines following birth, but the children acquire new infections as they age.⁷¹⁰ Seroprevalence for Ad5 is 40% to 70% in Japan,^{301,754} ~85% in China (54% with neutralizing titers greater than 1,000),⁶⁹⁴ 70% to 80% in Brazil (with 14% greater than 1,000 neutralizing titers),^{187,459} nearly 100% in northern India with 25% of individuals having high neutralizing titers (greater than 1,000) and 31% having very high titers (greater than 10,000),⁵⁶⁷ and 82% to 94% in Thailand.^{36,459} In the United States, Ad5 seroprevalence ranges from 30% to 70% in various studies.^{25,36,108,301,459,533,627,754} In Europe, the seroprevalence of Ad5 is 50% to 60%.^{301,385,459,754} Ad6 (species C) is being evaluated as a vaccine vector^{263,776}; overall in the world, about half of subjects have neutralizing antibodies to Ad6, but mostly of low titer.^{138,459,624} For Ad2, which is closely related to Ad5 and Ad6 in structure and clinical properties, the seroprevalence was found to be 83% in one study of subjects from Philadelphia.²⁵

Seroprevalence studies have also been conducted for more rare serotypes, motivated in part because of considerations to use these serotypes as vectors. For Ad35 (species B), the seroprevalence was estimated to be between 0²⁵ and 10%^{36,533,754} in the United States, less than 10% in Europe,^{385,754} ~15% in Japan⁷⁵⁴ and Thailand,³⁶ and ~3% to 20% in Sub-Saharan Africa.^{36,385,533} With Ad11 (species B), seroprevalence is 10%⁷¹⁰ to 30%^{1,301} in Sub-Saharan Africa and Japan. Ad26 (species D) seroprevalence is 10% (with low titers) in the United States^{106,459}; 44% in Brazil¹⁸⁷; 10%¹⁰⁶ and 50% in Thailand⁴⁵⁹; and ~20%,¹ ~50%,^{36,459} and 60% to 80%¹⁰⁶ in Sub-Saharan Africa. Thus, Ad26 is not a rare serotype. Ad49 and Ad50 are rare in most areas of Sub-Saharan Africa, but Ad48 is more common in East Africa.^{1,36,710} With Ad36 (species D), which has been linked to increased obesity, neutralizing antibodies are rare in the United States and Thailand but were found in about half the population in Brazil and parts of Sub-Saharan Africa.⁴⁵⁹ The Ad28 seroprevalence is less than 10% in the United States.³⁴⁷

It should be noted that the serologic studies described previously refer primarily to neutralizing antibodies. Generally, the prevalence of total antibodies against the Ad is higher. Further, nearly all adult humans contain T lymphocytes, primarily CD4+ but also CD8+ specific for Ads.^{84,106,320,322} These T-lymphocyte analyses do not identify the serotype (by definition) to which they are specific, and the T lymphocytes appear to be broadly reactive because of cross-reactive epitopes (often on hexon).

The epidemic forms of Ad disease were studied in different ways from the sporadic endemic occurrences. The epidemics of ARD were well known during World War II, and this awareness preceded the isolation and the characterization of the first Ad by about one decade. This ARD, which occurred almost exclusively in recently assembled military recruits, was most common in winter. It did not occur in senior personnel in close contact with the recruits and was later identified as an Ad4 or Ad7 infection in most outbreaks (reviewed in^{287,575}). This disease rarely occurred in similarly congregated college students, suggesting that additional factors, such as more crowded sleeping conditions or the fatigue associated with basic training, contributed. The observation that ARD-causing Ads did not spread to civilian personnel in contact with the military supports these conditions as co-factors. In recruits congregated during the summer months, ARD often did not occur until the onset of colder weather in the fall. Influenza A could be distinguished because it affected both experienced and new recruits. Ad-induced ARD often affected 80% of the recruits, with 20% to 40% hospitalized. The duration of infectivity was rather short; virus was not demonstrable after 4 days of illness.⁶¹² Controlled studies of routes of infectivity for the ARD-causing Ads have demonstrated that aerosolized virus inhaled into the lungs of volunteers produced the disease, whereas application to the mouth, the nasal mucosa, or the intestine in enteric-coated capsules failed to produce the lower respiratory disease.¹²⁹ ARD outbreaks were effectively controlled by vaccination of recruits in the first few days of military service; however, recent interruption in the supplies of Ad vaccine has resulted in the reappearance of the epidemic form of ARD. This is discussed further in the section on Adenoviruses as Vectors for Vaccination and Gene Therapy.

The epidemiology of pharyngoconjunctival fever and keratoconjunctivitis, both of which may occur in epidemic proportions, is described later with the individual disease entities. Infection resulting in several of the Ad syndromes can be acquired in hospitals and can be spread as nosocomial infections.

CLINICAL FEATURES

Ads can commonly infect and replicate at various sites of the respiratory tract as well as in the eye and gastrointestinal tract. Less frequently, Ads can infect the urinary bladder and liver. On occasion, these viruses may also cause disease in other organs, such as the pancreas,⁵²⁴ myocardium, or central nervous system, which may be involved in meningoencephalitis. Although there are at least 57 distinct human Ad serotypes, most human disease is associated with only one-third of these types.⁵⁹⁵ Many Ad infections are subclinical and result in antibody formation that probably is protective against exogenous reintroduction of the same Ad serotype. However, the virus itself may be grown, especially from the gastrointestinal tract²⁰⁵ and respiratory tract,¹⁸⁹

for months after the initial infection and immune response. The more common illnesses associated with various Ads are described in Table 56.1 and in the following paragraphs. The association of Ad with the disease in question is often attributed to the detection of the virus or antibodies to the virus in the blood, or to the detection of the virus in specific tissue. Often very sensitive techniques are used such as nested PCR. It is important to bear in mind that the presence of the virus or viral DNA in clinical specimens does not necessarily imply a cause-and-effect relationship between the virus and the disease, especially when PCR or nested PCR has been used. We do not understand whether and how Ad persists at very low levels in humans, so it is possible that the detection of Ad is merely a coincidence. On the other hand, the detection of the virus should not be disregarded.

Respiratory Diseases

Endemic Adenovirus Infections of Young Children

About 7% of upper respiratory tract infection cases in children younger than 5 years of age are due to an Ad.⁵⁵⁷ The usual symptoms include nasal congestion, coryza, and cough. Other patients may have an exudative tonsillitis that may be clinically indistinguishable from disease caused by the group A streptococcus. The respiratory symptoms are often accompanied by systemic manifestations, such as generalized malaise, fever, chills, myalgia, and headache. The common serotypes are Ad1, 2, 5, and 6 (species C or HA group III) and occasionally Ad3 and Ad7 (species B or HA group I), which are endemic in most populations. Sporadic cases may be indistinguishable from other viral respiratory infections, such as influenza, parainfluenza, and respiratory syncytial virus.⁵⁵⁷ If conjunctivitis accompanies the signs and symptoms already described, the disease is designated as *pharyngoconjunctival fever*. The Ad serotype most commonly involved is Ad3, but Ad7 and Ad14 within the same HA group have been isolated from such patients.³⁰⁸

Ads also cause lower respiratory tract infections in children and are probably responsible for about 10% of the pneumonias of childhood.^{455,557} Most patients recover, but some epidemics have resulted in considerable mortality. Sequelae in those who recover may include bronchiectasis that can clinically manifest years after the primary infection. Ad7, in particular strain Ad7h in recent years, has been a problem in South America for pediatric lower respiratory tract infections.⁸⁷ In one retrospective study in Buenos Aires, Argentina, Ad7h was associated with 29 (2.4%) of 1,233 cases (mean age 8.8 months) with a mortality rate of 34.5% between 1984 and 1988.⁴⁹⁷ In another study of 22 cases in 1991–1992 in Buenos Aires, Ad7 was found in 82% of patients (12 of 14 were Ad7h) and species C in 18% of patients.⁸⁷ Four patients died, three of whom had Ad7h. In a similar study in Uruguay in 1994–1998, 32% of pediatric patients (mean age 8.8 months) had species B (all but one Ad7h) and 61% had species C.²⁰⁸ In a Taiwan study in 1999–2000, nine children (mean age 22 months) had Ad3, one had Ad2, and one had Ad11.¹¹⁵ In a U.S. study, there was a 6-month outbreak of Ad30 (species D) in a neonatal intensive care unit that involved 21 of 333 patients (6.3%) and that had pneumonia in 8 patients.¹⁹⁰ Six infants died, and death was associated with pneumonia.

Acute Respiratory Disease in Adults

In adults, the serotype subspecies B1 Ads (Ad3, Ad7, Ad16, Ad21, and Ad50) and species E (Ad4) are commonly associated with acute respiratory disease, whereas subspecies B2 viruses

(Ad11, Ad14, Ad34, Ad35) are more frequently associated with urinary tract and opportunistic infections in immunocompromised patients. Without significant circulation in North America, Ad14 emerged as a significant cause of acute and sometimes severe acute respiratory disease in 2006. Initially, the infection was recognized in three military bases under continuous systematic surveillance,⁴⁷¹ but widespread outbreaks were demonstrated shortly thereafter in Washington, Oregon, Alaska, Wisconsin, and Pennsylvania, and to a lesser extent in other states.^{92,93,188,313,350,421,441,701} The DNA sequence of the prototype Ad14 (de Wit strain, isolated in 1955) was published recently.⁶⁴³ Sequencing of the outbreak strain *elA*, *hexon*, and *fiber* genes suggests that Ad14p1 arose from recombination among similar Ad11 and Ad14 ancestral strains. A deletion of two amino acids in the knob region of the fiber protein is the only identified unique characteristic of Ad14p1.³⁵⁰ Low antibody titers against Ad14 in recruits sampled at admission to training camp and initial detection of Ad14p1 in cities on the U.S. West Coast that represent major ports of entry into the country from Asia suggest that travel and commerce played a major role in the introduction of this rare Ad subspecies B2 virus into the United States.^{350,701} Current studies are ongoing to better understand why the virus resulted in significant morbidity and mortality in a small subset of infected individuals; in one of the earliest outbreaks, in Oregon, there were 29 hospitalizations with 7 associated deaths.^{350,421} During the investigation of this initial civilian outbreak, 67 cases of Ad infection were detected during the study period and 40 (60%) involved Ad14.⁴²¹ Of those with medical records available for review, most presented with fever and cough; 29 (76%) required hospitalization, 23 (61%) required supplemental oxygen, 18 (47%) required critical care, 9 (24%) required vasopressors, and 7 (18%) died. Older age, chronic underlying condition, low absolute lymphocyte counts, and elevated creatinine levels were associated with severe illness. In most instances, no epidemiologic link between cases could be established.^{188,421}

Acute Respiratory Disease of Military Recruits

In many respects, ARD is similar to the description furnished earlier of the respiratory infection of children. The syndrome is predominantly caused by Ad4 (92.8% in one study conducted in 2004–2006),²⁴⁴ and less commonly Ad7. After 2005, coincident with similar findings in the civilian populations, there was the simultaneous emergence of increased diversity of infections due to subspecies B1 serotype 3, 7, and 21 and subspecies B2 serotype 14.⁴⁷¹ ARD is a syndrome that frequently occurs under the special conditions of fatigue and crowding created soon after the induction of young military recruits (reviewed in^{308,575}). Some cases have had a fatal outcome from the pneumonitis that may accompany and complicate the other, milder respiratory symptoms. A significant increase in severe and fatal cases was seen recently with the emergence of Ad14 infections in two U.S. military facilities.^{471,518} On the other hand, recent retrospective studies of Air Force recruits with pneumonia at Lackland Air Force Base did not find evidence that Ad14 was associated with excess overall morbidity as compared to pneumonia due to other Ad serotypes.⁷⁵² Further aspects of this disease are discussed in the sections entitled Epidemiology and Vaccines.

Pertussis-Like Syndrome

The association of Ad infection with a pertussis-like syndrome has led to speculation that Ads can cause clinical whooping

cough.⁶⁸⁹ Ad5 was isolated from multiple organs of a patient with severe whooping cough with lymphocytosis that ended fatally.¹²⁴ Later data from a controlled study of 134 children with a pertussis-like illness and 101 healthy controls reported the common association of Ads with whooping cough symptoms.⁵⁰⁶ Ads have been isolated from other studies of respiratory tract infections including pertussis-like syndrome.^{195,586} However, there was no evidence that the Ads alone were responsible for the syndrome. The large number of Ad isolates may be due to conditions favorable for reactivation of latent viruses from tonsillar tissue during concurrent *B. pertussis* infection.⁵¹⁶

Infections of the Eye

An acute follicular conjunctivitis may occur as part of a respiratory-pharyngeal syndrome or as a separate entity.^{53,496} Both bulbar and palpebral conjunctival involvement may occur and affect both eyes. The disease is often accompanied by significant preauricular lymphadenopathy.⁴⁴ Complete recovery without sequelae is the most common result of this rather mild illness. The incubation period is usually 6 to 9 days, but it was as short as 2 days in experimental infections in volunteers.^{46,348} Epidemiologically, these infections can occur sporadically or cause disease in large groups of contacts. Family members may be affected. When the source is a swimming pool or small lake, large numbers of children and young adults may develop symptoms. Swimming pool conjunctivitis is probably most commonly due to Ads.²⁰⁷ Although the virus is isolated from the conjunctiva of affected individuals, it has not been isolated from water samples from putatively infected sources. The common-source water-borne outbreaks usually occur in summer and are caused by Ad3 and Ad7; however, other types, such as Ad1, 2, 4, 6, 9 to 11, 15 to 17, 20, and 22 (species B, C, D, and E and HA groups I, II, and III), have been associated with this syndrome.^{53,496}

In contrast to the milder form of ocular disease described previously and limited to the conjunctiva, EKC is a highly contagious and more serious disease. The clinical entity was first described before the isolation of Ads and occurred among German workers in the late 19th century.^{299,334} It was subsequently observed in shipyard workers in Hawaii and in the continental United States. “Shipyard eye” was probably transmitted in the medical facilities that cared for chemical and physical trauma to the eyes of the workers. After an 8- to 10-day incubation period, a follicular conjunctivitis with edema of the eyelids, pain, lacrimation, and photophobia began. Corneal subepithelial infiltrates often followed the initial conjunctival involvements.¹⁴³ The disease is often unilateral with preauricular lymph node hypertrophy. Occasionally, other lymph nodes were involved, and constitutional symptoms occurred, especially in children. However, most often the disease was limited to the involved eye and its draining lymph nodes. Corneal opacities in some cases lasted for several years and, uncommonly, would remain for longer periods. In some patients, the involvement slowly progressed to a hemorrhagic conjunctivitis that should be distinguished from the rapidly evolving acute hemorrhagic conjunctivitis associated with enterovirus 70.¹⁴³ In addition to viral inoculation during an ophthalmologic procedure such as tonometry, EKC has occurred as a late summer and fall epidemic in certain parts of Japan, Taiwan, and Vietnam. Ad8 was the original cause of EKC, but outbreaks of Ad3, Ad19, Ad34, Ad37, Ad53, and Ad54 (a novel hexon-chimeric intermediate Ad22,37/H8)

have also been described.^{18,23,47,97,103,327,360,365,561,699,704} Recent outbreaks of EKC between 1995 and 1997 were caused by a unique isolate, designated Ad8I, which has Ad9 sequences in the hypervariable region of the *hexon* gene that may have allowed the virus to escape pre-existing neutralizing antibodies to Ad8 in the population.²² Treatment of severe infections caused by Ad8 with *N*-chlorotaurine, an antimicrobial agent, shortened the duration of illness and was well tolerated.⁷⁰⁴

Ad4 has been recognized to cause a range of symptoms, from EKC to pharyngoconjunctival fever.¹⁹ Ocular infections by Ad4 were rare up to the 1960s, but beginning in the latter half of the 1970s Ad4 caused many worldwide outbreaks of conjunctivitis.²²

Acute Hemorrhagic Cystitis

Acute hemorrhagic cystitis, an illness occurring almost exclusively in boys and associated with Ad11, is characterized by gross hematuria.⁵³⁰ Its significance lies in the potential confusion with other, more serious diseases of the kidney (such as glomerulonephritis). This self-limited disease is usually not accompanied by fever or hypertension, and tests of renal excretory and concentrating functions have been essentially normal. Ad21, like Ad11, is a species B HA type I and can also cause hemorrhagic cystitis.⁴⁹³ In Japan, when acute hemorrhagic cystitis occurs in a boy between the ages of 6 and 15 years, an Ad isolation from urine or a rise in neutralizing antibody occurs in about 70% of patients.⁵³⁰ In comparable studies of hemorrhagic cystitis in the United States, only 20% of cases can be linked to an acute Ad infection, and for 60% of the total, the etiology remains unexplained.⁴⁹³

The other population that develops hemorrhagic cystitis is immunosuppressed transplant recipients. According to one study, Ad infections account for 3.9% of hemorrhagic cystitis among pediatric hematopoietic stem cell transplant (HSCT) recipients.²³⁵ Another study of mostly adult HSCT recipients found that Ad was associated with 9.8% of cases of hemorrhagic cystitis.⁴⁸⁶ Ad hemorrhagic cystitis in HSCT patients is more frequently associated with T-cell purging and was less common in patients with acute GVHD than other causes of hemorrhagic cystitis.⁴⁸⁶ In the kidney transplant population, Ad can also cause hemorrhagic cystitis with or without concomitant nephritis; such patients typically present with fever and often feel poorly.^{12,261,282,380,544} In some patients, it appears that the infection was introduced with the transplanted kidney.^{389,746} In addition to Ad11, two other species B serotypes, Ad34 and Ad35, were isolated first from renal transplant recipients. Ad34 was isolated from urine, whereas type 35 was isolated from kidney and lung tissue at autopsy.^{283,500} Although neither was accompanied by the symptoms of hemorrhagic cystitis, the Ad35 clearly contributed to the patient's demise from pneumonia. Ad34, 2, and 31 were isolated from allogeneic bone marrow, hematopoietic stem cell, and liver transplant recipients with hemorrhagic cystitis, respectively.^{485,632}

Meningoencephalitis

It is rare to isolate any of the Ads from either the cerebrospinal fluid (CSF) or the brain. However, several reports have directly demonstrated Ads in CSF (Ad3, 5, 6, 7, 7A, 12, and 26).^{113,166,364} One patient with malignant lymphoma, immunosuppressed by chemotherapy, had an Ad32 isolated from the

brain at autopsy.⁶⁰⁶ A patient with large B-cell lymphoma developed meningoencephalitis due to Ad7.²⁰⁰ Ad5 was cultivated from CSF in two immunocompetent patients, one with meningoencephalitis and the other with meningitis.⁶⁷⁴ A bone marrow transplant developed fatal subacute Ad meningoencephalitis.¹⁴⁰ A 12-year-old immunocompetent girl developed tubulointerstitial nephritis with acute renal failure, hepatitis, and meningoencephalitis following systemic Ad infection; she recovered with supportive care.¹⁸⁶ There are other cases of meningoencephalitis in which viral isolation from extraneural sites or antibody titer increases have been used to make a diagnosis, especially associated with epidemic Ad7 pneumonia in children.⁶⁶⁴ A case of sudden unilateral deafness was associated with an Ad3 infection of the nasopharynx.³³⁰

Gastrointestinal Diseases

Gastroenteritis, or inflammation of the stomach and small and large intestines, is characterized by fever, vomiting, and diarrhea. It is frequently caused by viruses, bacteria, or parasites. Viruses that cause gastroenteritis include rotaviruses, Ads, noroviruses, calciviruses, astroviruses, and Norwalk virus. Rotaviruses are the leading cause of diarrhea in the world, and calciviruses cause the most gastroenteritis outbreaks in industrial nations.²²⁹ The role of Ads is discussed in the next few sections.

Diarrhea

The relationship between Ads and diarrhea has had a long and complicated history but has now been clarified.^{308,787} Because many Ads replicate efficiently in the intestine and are excreted in the stool, it was assumed that they would be strong candidates for causing diarrhea. However, most earlier epidemiologic studies generally found as many Ad isolates in the stools of controls as in those with diarrhea.⁵⁷⁸ The failure to correlate Ad growth from stool with clinical illness was a good example that Ads should not be designated as the cause of a whole spectrum of medical illnesses just because they can be cultured from the stool of an individual with a disease. Asymptomatic children can clearly shed Ads in stool and often develop antibodies to the particular type grown.^{205,290} These subclinical infections probably result in lifelong immunity.

The whole issue of Ads as a cause of infantile gastroenteritis was re-examined based on the observation that initially "noncultivable" Ads were seen on EM examination of stool smears of affected children.²⁰¹ Serologic detection methods such as ELISA and alternate tissue culture host-cell systems, such as the HEK 293 line, have identified several of these "noncultivable" viruses.^{147,781} Two different enteric Ads, Ad40 and 41, have been associated with diarrhea.^{147,226,781}

Epidemiologic studies to assess the importance of these agents have been completed for several population groups. A report of 14 enteric Ad-related cases of diarrhea in 27 hospitalized patients studied during a 12-week period suggested that these viruses may be an important cause of acute gastrointestinal disease in hospitalized young children and may be nosocomially transmitted; this report also suggested that respiratory symptoms may be a prominent part of the clinical manifestations.⁸⁰² The incidence of Ad-related gastroenteritis differs considerably in the various studies and locations reported by many authors. In general, it is not as prevalent as rotavirus diarrhea, occurs most often in children younger than 4 years of age, and is not easily distinguished on clinical grounds from rotavirus

infection.⁵⁸⁷ In Bangladesh, it was responsible for 2.8% of the cases of diarrhea but reached 12.3% in some months as diagnosed by a monoclonal antibody specific for Ad40 and Ad41.³³³ In day care centers in Houston, 38% of 249 children present during 10 separate outbreaks had diarrhea associated with enteric Ads. Of these patients, 46% were asymptomatic, demonstrating that even during epidemics of enteric Ads, many infected children do not develop gastroenteritis.⁷⁴⁵ In another study, the prevalence of all Ads in stool was 8%, and Ad40 and Ad41 were 2% in both 565 patients with diarrhea and 129 controls.⁴²⁰ In a recent study of 44 infants in a day care center in Tokyo, Japan, Ad was found in 12.5% of fecal specimens from symptomatic and 11.5% from asymptomatic infants; one outbreak of acute gastroenteritis in these infants was linked to Ad12.⁶ In another recent study using ELISA analysis of 3,577 fecal specimens from infants and children with acute gastroenteritis in Japan, Korea, and Vietnam during 1998 and 2001, 4.4% were positive for Ad.⁴²⁴ More than half of these were Ad41, but Ad40, 2, 3, 8, and 31 were also detected. Enteric Ads were detected in a fraction of stool samples of children with gastroenteritis in a number of countries, as follows: 6.9% in the United States⁷²; 8.0% in Sweden⁷³⁵; 8.3% in Germany⁵³⁸; 7.9% in the East Anglia, United Kingdom⁶⁶⁵; 9% in Belfast, United Kingdom⁵³⁴; 1.4% in Blantyre, Malawi¹³⁵; 1.5% in Brazil⁶⁷³; 6.7% in Iran⁶¹⁹; 4% in Jakarta, Indonesia⁶⁹⁰; 10.8% in Shenzhen, China²⁷⁴; and 4% in Jakarta, Indonesia.⁶⁹⁰ In many of these studies, nonenteric Ads were also detected but at lower frequencies. In Japan and Iran, antibodies to enteric Ad were detected in sera from about half of healthy children analyzed.^{618,659} Thus, it is clear that the role of enteric Ads as a cause of diarrhea is roughly the same in different areas of the world.

Intussusception

Another intestinal syndrome, intussusception, has been linked in some patients to Ad infection.^{45,687} The telescoping bowel characteristic of intussusception may be caused by mesenteric adenitis acting as a lead point to the mechanical obstruction. Ads (species C, HA group III, Ad1, 2, 5, and 6) have been isolated from both stool cultures and the involved lymph nodes removed at surgery.^{122,571} The percentage of children with intussusception showing evidence of Ad is high as evidenced by shedding into stool, detection in throat swabs, anti-Ad antibodies, Ad inclusion bodies in tissue samples, and identification of Ad by electron microscopy. The percentages found in a number of studies range from 22% to 61% (see references in³⁰⁸). Most of the studies examined control patients, and in all cases the presence of Ad was statistically more significant than in the controls. These studies were from several parts of the world (United States, United Kingdom, Spain, France, Taiwan, Nigeria). In an analysis using immunohistochemistry and PCR of formalin-fixed intestinal specimens from 12 Mexican pediatric patients, 4 patients (33%) were positive for species C Ad.²⁵¹ There is no evidence that Ad40 and Ad41 are involved in this syndrome.⁵⁹ A proposed alternative explanation is that some hyperirritability of the small intestine might be caused by Ad infection and lead to the intussusception.⁸⁰⁸ However, many patients with intussusception have no evidence of Ad infection, and the disease is probably multifactorial. Ad inclusions have been seen in about one-third to one-half of appendices removed at surgery.³⁰⁸

Celiac Disease

Celiac disease is a common autoimmune disease thought to be caused by ingesting the proteins of the gliadin, hordein, and secalin classes found in wheat, barley, and rye. Structural homology between the Ad12 E1B-55K protein and A-gliadin, a major component of gluten proteins known to activate celiac disease, was noted. In addition, most patients with celiac disease had evidence of prior Ad12 infection, in contrast to matched controls. These observations raise the possibility that the E1B-55K protein from an Ad found in the intestine may play a role in the pathogenesis of celiac disease, perhaps by inducing cross-reacting antibodies to A-gliadin.³⁴⁶ In support of this possibility, T lymphocytes from celiac patients recognize a synthetic dodecapeptide shared by the Ad12 E1B-55K protein and A-gliadin.⁴⁵⁶ On the other hand, in three studies using PCR to determine whether there was persistent Ad12 infection in the intestinal mucosa of patients with celiac disease, 4 of 18 patients had detectable Ad12 DNA, as did 2 of 24 controls, leading the authors to conclude that Ad12 persistence is not a major element in celiac disease.⁴⁵¹ Similar conclusions were reached by others but do not preclude that prior Ad12 infection might have been involved in the evolution of celiac disease.^{407,753}

Myocarditis

Myocarditis is an inflammatory disorder of the myocardium characterized by necrosis of myocytes and infiltration of inflammatory cells. The most common form of this disease, viral myocarditis, is generally thought to be caused by enterovirus infection (e.g., coxsackievirus), although there is evidence that Ad may be a significant cause as well (reviewed in^{69,356,453}). Ads do not normally cause symptomatic infection of the heart, and the reason that it is associated with myocarditis and dilated cardiopathy is unknown. Both enteroviruses and Ads can infect cardiac myocytes in culture. It is of interest that coxsackieviruses and Ads both use CAR as the receptor to enter cells. CAR expression in healthy and diseased hearts is reported to be variable, and it has been suggested that this variability plays a role in enterovirus and Ad myocarditis.⁵⁷⁰

A number of reports have diagnosed Ad in myocarditis and dilated myocardiopathy. In a study of 126 conscripts diagnosed on the basis of serial electrocardiograph changes during an acute infection, Ad-specific antibodies increased in 19 patients, although most did not have myocarditis.³⁶² Ad DNA has been detected by PCR in myocardial biopsies primarily from children with acute myocarditis in three studies (see³⁰⁸). Although there was clinical and electrocardiogram evidence of myocarditis, most of the Ad-positive biopsies did not show classic inflammation, in contrast to those that appeared to be associated with enterovirus infection. In children with myocarditis or dilated cardiomyopathy, Ad was detected in anywhere from 8.1% to 36% of patients.^{67,68,83,397,621,626,657} Sequencing of the PCR products from one of these studies established that 80% of the Ad isolates were Ad2; most of the remainder were Ad5 and one was Ad6; nonfatal cases caused by Ad1 have also been described.⁶²¹ In another study of 142 Ad-positive patients with clinical diagnosis of myocarditis, 57 (40%) had histopathology that was consistent with myocarditis; none of the control patients were positive for Ad.⁶⁷ Some patients with detected virus remain positive for a prolonged period of time (mean of 6.8 months); clearance of the virus was associated with improved symptoms.³⁹⁷ Myocardial involvement with Ad may

be patchy, suggesting that biopsies may miss affected regions and thereby underestimate the incidence of Ad-associated disease.²⁶⁸ These studies suggest that Ad may be a cause of or a contributing factor for cardiomyopathy.

Ads may also be associated with idiopathic left ventricle dysfunction in adults. Ad DNA (mostly Ad2) was found by nested PCR analysis of myocardial tissue without evidence of myocarditis from 12 of 94 patients with this disease.⁵⁵⁶ This same group has conducted a phase II clinical study of interferon- β treatment of 22 patients with long-term cardiac dysfunction and with Ad (7 patients) and enterovirus (15 patients) persistence as detected by nested PCR analysis of myocardial biopsy specimens.³⁹⁶ Twenty-four weeks of interferon- β treatment was associated with viral clearance and improved left ventricle function in most patients.

Among heart transplant patients, the presence of Ad genome within biopsy specimens has been correlated with adverse outcomes in two independent studies.^{491,661} In the first study of 553 consecutive biopsy samples from 149 pediatric heart transplant recipients, 30 samples were positive for Ad.⁶⁶¹ Eighty-five percent of patients with positive PCR results had an adverse cardiac outcome within 3 months of the biopsy and just over a quarter had graft loss due to coronary vasculopathy, chronic graft failure, or acute rejection; the odds of graft loss were 6.5 times higher in those with a detectable virus by PCR.⁶⁶¹ In the second study, of 928 serial endomyocardial biopsies from 94 pediatric heart transplant patients, Ad genome was detected in 9.6% of patients. Presence of detectable viral genome by PCR was associated with decreased graft survival (2.4 years vs. 8.7 years in the PCR-negative group) and earlier development of transplant coronary artery disease.⁴⁹¹ For 2 years of the study, patients with detectable virus were treated with intravenous immunoglobulin (IVIG) and this intervention was associated with a trend toward improved graft survival ($p = .06$).⁴⁹¹

Adenovirus Infection During Pregnancy

A number of studies have established that the presence of Ad in amniotic fluid is more frequently associated with abnormal fetuses than normal ones. In one prospective observational study of second-trimester women undergoing either genetic amniocentesis or evaluation of fetal abnormalities, amniotic fluid was analyzed for Ad and other viruses by multiplex PCR.⁴¹ The prevalence of Ad was similar in women with normal (39/652) and anomalous (23/364) fetuses, but echogenic liver lesions with or without hydrops and neural defects in fetuses were more common with Ad.

Diseases Associated with Immunocompromised Patients

Although Ads are not nearly as prevalent as the various herpesviruses during immunosuppression, Ads have been isolated from immunocompromised hosts, in particular transplant patients, and have contributed to their morbidity and mortality (reviewed in^{98,197,328,434}). The precise incidence of Ad illness in transplant recipients remains poorly defined because available studies have incorporated different definitions of disease, have used different diagnostic techniques (culture vs. PCR vs. histopathology), and have evaluated diverse patient populations; large prospective studies have not been performed.³²⁸

In the stem cell transplantation (SCT) population, the incidence of disease ranges from 3% to 47%.^{30,80,100,202,256,295,399,435,655} Available data suggest that Ad infections are more frequent in allogeneic SCT recipients compared to those receiving autologous grafts (8.5% to 30% vs. 2% to 12%); children compared to adults (20% to 47% vs. 9% to 13.6%); patients who receive T-cell-depleted grafts (45% vs. 11%); and patients with acute GVHD.^{30,80,99,100,184,202,256,295,399,434,435,436,655} Severe lymphopenia (less than 300 cells/mm³) is associated with a progression to disseminated and often fatal disease.^{100,101,184,434,436} Likewise, T-cell depletion, through either the use of alemtuzumab or *ex vivo* T-cell depletion of grafts (i.e., CD34+ selection) or the use of cord blood, are associated with enhanced risk of Ad disease.^{355,501,592} In the SCT patient Ad can cause severe respiratory disease, hepatitis, colitis, hemorrhagic cystitis, adenoviral keratoconjunctivitis, and disseminated disease. Stem cell patients can also be divided into patients with subclinical viremia, viremia with disease symptoms, and disseminated disease. The incidence of disseminated disease is 1% to 7% with a reported mortality of 8% to 26%.⁴³⁴

Development of quantitative PCR (qPCR) assays in the past several years that are broadly reactive against many or all Ad serotypes have allowed important new insights into Ad infections and Ad-associated disease in SCT patients (reviewed in^{98,197,328,434}). These studies have led to suggestions on prediction and perhaps management of Ad-associated disease. In a retrospective study of 303 pediatric and adult patients who underwent allogeneic SCT, 35 (11.5%) were found using non-PCR procedures to have Ad infection (serotypes 1 [$n = 6$], 2 [$n = 7$], and 31 [$n = 4$] were the most common).⁶² In an analysis of 132 consecutive pediatric patients undergoing SCT, 36 (27%) were positive by qPCR for Ad in species A, B, C (78% of all positive cases), D, and F.⁴³⁵ Except for gastroenteritis in some patients with Ad in stool, the isolation of Ad from other sites (throat, urine) was not linked to disease and mortality was not different from patients that were negative for Ad. Ad was detected in the peripheral blood of 11 children, 9 with species C and 2 with species A, and 7 (82%) of these children died.⁴³⁵ With most of the children that died, repeated PCR testing revealed a 10-fold rising load of Ad in the peripheral blood with a median of 3 weeks prior to the onset of fatal outcome. The maximum virus load in the blood for those patients who died ranged from about 10⁴ to 10⁷ or more genome copies/mL. T-cell depletion *in vivo* by immunosuppression and T-cell depletion *in vitro* by CD34+ selection were risk factors for Ad infection.⁴³⁵

In a study of 86 consecutive bone marrow patients who received alemtuzumab (Campath; a monoclonal antibody against CD52) to deplete T cells, Ad was detected in 11 (18%) patients.²⁶ Five patients died of progressive Ad disease. The authors concluded that the severity of lymphocytopenia and the continuation of immunosuppression were risk factors for Ad-associated death. In a study of 155 consecutive pediatric allogeneic SCTs, 126 of whom underwent T-cell depletion, Ad (species A [43%], B [5%], C [21%], and F [43%]) was detected by qPCR and other methods in 26 (17%) patients.³⁵⁴ Death from Ad was closely associated with lack of T-cell recovery (to greater than 300 cells/ μ L) following immunosuppression.³⁵⁴ This link between T-cell levels and adenoviremia and death is in accord with work by Chakrabarti et al¹⁰⁰ and other researchers (see later). The importance of T cells was also found in a retrospective study of 328 consecutive pediatric patients with allogeneic

SCTs in which Ad was found in 37 (12%) patients, mostly in patients with greater than a 2-log depletion of T cells.⁷⁴⁴ Death occurred in seven of seven patients with Ad DNA in serum and decreasing lymphocyte counts. In another study, Ad infection occurred in 21 (44%) pediatric SCT patients and progressed to viremia in 6 patients who had low lymphocyte counts.²⁷⁶ Survival of these patients correlated with an increase in lymphocyte counts soon after the viremia, including Ad-specific CD4+ T cells, as well as neutralizing antibodies.

The SCT studies described previously were conducted on pediatric patients only. In an SCT study that included both pediatric patients and adults, Ad was detected in the whole blood by real-time PCR in 4 of 27 (14.8%) children and 8 of 96 (8.6%) adults.²⁷⁹ Ad was found in 5 of 306 (1.6%) blood samples from healthy donors. The Ad loads were higher in children (median of 1.7×10^5 Ad genomic DNA copies/mL; range of 4.2×10^2 to 1.1×10^{10} copies/mL) than adults (2.3×10^3 copies/mL; range 3×10^2 to 5.8×10^5 copies/mL). The highest load in the blood of the healthy control group was 1.7×10^3 copies/mL. In the transplant patients, symptoms suggesting disseminated Ad infection were seen in only two of six patients, both children, with high virus loads in the blood. One child with greater than 1×10^{10} copies/mL died.²⁷⁹ In a different study of SCT, a child died following a progressive increase in Ad DNA to about 10^{10} copies/mL in peripheral blood.⁴⁰³

The link between a high Ad load in the peripheral blood and fatal disseminated Ad disease was also established using qPCR in a retrospective analysis of 328 allogeneic SCT pediatric patients, 36 of which (11%) were positive for Ad.¹²¹ Seventeen patients had positive Ad cultures but no symptoms, 12 had symptoms but survived (10 had enteritis, 1 had hemorrhagic cystitis), and 7 had fatal disseminated Ad. In four of six fatal cases, a steep increase in Ad DNA in the serum was observed in the month preceding death, ranging from about 10^8 to 10^{12} copies/mL. However, some of the asymptomatic patients had Ad DNA loads in the serum of up to about 10^7 copies/mL, so quite high loads do not necessarily cause symptoms. In a prospective study of Ad DNA loads conducted by the same workers in pediatric transplants, Ad was found in the plasma of 12 of 48 patients (25%).¹²¹ Six patients developed disseminated Ad and three died; two of the three that were thought to die from adenoviremia had plasma loads of about 10^7 and 10^{11} copies/mL. The authors concluded that serum or plasma Ad DNA loads of greater than 10^6 copies/mL have an increased risk for fatal complications.¹²¹ In a retrospective qPCR study of 26 pediatric bone marrow transplant patients, 11 (42%) had Ad in the whole blood.⁷⁶¹ Of the children who had symptoms of Ad infection, Ad was isolated a mean of 17 days following transplant. Ad infection occurred more frequently in patients who had received T-cell depletion, and the two patients who died had very low absolute lymphocyte counts (less than $330/\mu\text{L}$) at the time when Ad was detected. These two patients developed an Ad DNA load of greater than 10^5 copies/mL in the blood.

One study in pediatric SCT patients also attempted to assess the link between Ad in the stool and blood with active disease. In 153 consecutive allogeneic transplantations involving 138 pediatric patients, Ad was detectable in serial stool specimens in all cases of Ad viremia during the posttransplant course. Peak virus levels of Ad in stool specimens above 1×10^6 copies per gram were associated with adenoviremia with a median of 11 days (range 0 to 192) between detection in the

stool and blood. Further, serial measurement of Ad levels in stool specimens permitted early diagnosis of impending invasive infection with a sensitivity and specificity of 100% and 83%, respectively.⁴³⁶

Several studies on Ad and transplantation have been conducted in adults. In an analysis of 2,889 adult bone marrow transplants using culture and histopathology methods to detect Ads, 85 (3%) of patients were positive.³⁹⁹ The mortality rate for Ad-positive patients was 26%. Perhaps the incidence of Ad would have been higher had PCR methods been used in this study. Consistent with this suggestion, in a prospective study of 76 adult SCT patients, 15 (19.7%) were positive for Ad by standard PCR assay.¹⁰⁰ Interestingly, the virus was found exclusively in patients whose T cells had been depleted, and 6 of the 15 patients (40%) developed Ad disease. Severe lymphopenia (less than 300 absolute lymphocyte count/ μL) when Ad was first detected was a major risk factor for development of Ad disease. Other key risks were positive Ad PCR in the blood and failure to reduce immunosuppression.

In another study of 62 adult T-cell-replete SCT recipients, serial blood samples were assessed retrospectively for detection of Ad by PCR.¹⁸⁴ Ad was detected in 21 (87.5%) of 24 patients with proven Ad disease, in 4 (21%) of 19 patients who shed Ad, and in 1 (10.5%) of 19 uninfected control patients. In those who progressed to proven Ad disease, viral loads were significantly higher (median maximum viral load of 6.3×10^6 copies/mL, range of 0 to 1.0×10^9 copies/mL), while all patients who shed Ad had symptoms compatible with Ad disease, suggesting possible infection. The minimal plasma viral load detected among patients with proven or possible infection was 10^3 copies/mL. Adenoviremia was detectable at a median of 19.5 days (range, 8 to 48 days) and 24 days (range, 9 to 41 days) before death for patients with proven and possible Ad disease, respectively.

Summarizing these data, weekly qPCRs to monitor the Ad DNA load and immune-reconstitution monitoring (CD3 counts) after HSCT are now widely used methods in many pediatric bone marrow transplantation units. Weekly monitoring is required because of the kinetics of Ad replication, which can be rapid. Such close monitoring can be useful in determining which patients are at risk for progressive infection (stable or rising DNA loads in patients with significant lymphopenia). Interventions have only been demonstrated prospectively to be effective in children, although suggestive data are emerging in adults.^{98,184,434,436} Reduction of immune suppression, reconstitution of Ad-specific immunity via adoptive transfer, and/or the use of antiviral therapy in such patients may prevent progressive disease. In some studies, there is anecdotal evidence that treatment with cidofovir and its lipid ester (CMX001) to suppress Ad may be beneficial (see section on antiviral drugs).^{328,434} Furthermore, Ad PCR-based monitoring can assess the impact of the intervention and determine if additional interventions are needed.⁴³⁴

Incidence data for adenoviral disease in solid-organ transplant recipients are even more limited than for SCT recipients. Ad infections appear most commonly in liver transplant recipients, pediatric transplant recipients, patients who receive antilymphocyte antibodies, and patients with donor-positive/recipient-negative Ad status.³²⁹ The problem of Ad hepatitis in 5 of 224 pediatric hepatic transplant recipients has been demonstrated.⁸⁶ The serotypes involved were common types, such as Ad1, Ad2, and Ad5. The clinical manifestations were

often the direct result of infection of the transplanted liver, and viral intranuclear inclusions were readily identified. The disease was acquired *de novo* or by reactivation of latent virus from an endogenous source. Forty-nine of 484 pediatric liver transplant recipients had an Ad isolated from either the liver, lung, or gastrointestinal tract. Twenty children had invasive Ad infection, which culminated in the death of nine patients.⁴⁷³ Some immunocompromised patients recovered from their Ad infections after discontinuation of immunosuppressive drugs. Enterocolitis occurs more commonly in small bowel transplant recipients and may mimic rejection.^{56,568} Adenoviral pneumonia is associated with graft loss, death, or progression to obliterative bronchiolitis for lung transplant recipients.⁷⁵ Ad genome detection in heart biopsy specimens has also been associated with worse outcomes among pediatric heart transplant patients (see Myocarditis section for more details).^{491,661}

Two studies have documented the natural history of asymptomatic adenoviremia in solid-organ transplant recipients. The incidence of Ad viremia was 6.5%, 6.7%, 8.3%, and 22.5% for adult kidney, heart, liver, and lung recipients, respectively.^{318,319} Few patients were symptomatic at the time that viremia was detected, viral loads in these patients were low, and none developed end-organ disease. There was no compromise of pulmonary function among the studied lung transplant recipients.³¹⁸ These data suggest that, unlike in stem cell transplant patients, monitoring patients for adenoviremia by PCR is not predictive of disease and is therefore not recommended.³²⁹

Another situation in which Ad is linked to immunosuppression is acquired immunodeficiency syndrome (AIDS). Twelve percent of patients with AIDS were reported to have species B Ads in their urine.^{148,311} Restriction endonuclease analyses of viral DNA of 24 urinary isolates have shown that all were related to Ad34 and Ad35. However, some of the AIDS urine isolates had the Ad7 hemagglutinin by serologic classification, suggesting recombination between the Ad35 and Ad7 species B (HA group I) agents. In addition to the HAs of Ad7 and Ad35, HAs from Ad3 and Ad11 have been found in other Ad35-like isolates from AIDS patients.³¹¹ In AIDS patients, the seroprevalence of Ad5 compared to Ad35 was 60% versus 7% in the Netherlands and 90% versus 20% in Sub-Saharan Africa.³⁸⁵ Occasionally, Ad35 has caused fatalities from pneumonia or hepatitis, as observed in three pediatric patients.³⁹⁴

Ads were observed by EM in colonic tissue of 5 of 67 (7.5%) and 30 of 377 (8.0%) homosexual men infected with human immunodeficiency virus type 1 (HIV-1) and studied because of persistent diarrhea.⁷⁰⁸ However, it is not clear whether species D Ads are associated with the chronic diarrhea that is common in these immunocompromised patients. The tissue biopsy samples had foci of mucosal necrosis, chronic inflammatory cells, and amphophilic nuclear inclusions observed by light microscopy. Thus, Ads, primarily newer isolates of species D, are present in the colonic lesions of some immunocompromised patients and may cause diarrhea but do not appear to be the etiology of the large number of intestinal dysfunctions that affect AIDS patients.^{285,331}

Persistence and Latency

Ads were discovered because of their propensity for persistence in adenoidal tissue.² These and other observations suggest that Ads may be able to establish long-term low-level persistent or perhaps even latent infections. We use the term *latent* to

imply that, as is the case with herpesviruses, the Ad genome is maintained in the cell in an unintegrated episomal state, and that few if any Ad genes are expressed. Little is known about the mechanism of persistence or latency for Ads in humans or animals (reviewed by^{466,787}).

In earlier work, Ad DNA was detected in tonsils²⁴⁸ and adult human lymphocytes.^{2,307} An Epstein-Barr–positive lymphocyte line and Ad-infected tissue culture cells have also documented persistence of Ad infection.^{15,114,203,204,401,663} In one study in primary peripheral blood mononuclear cells, unstimulated lymphocytes were mostly refractory to Ad productive infection, but replication occurred, especially with Ad11p and Ad35, in stimulated T lymphocytes.⁶³⁰ Not all cell lines support lytic infection, and latently infected cells apparently underwent phenotypic changes including decreased CAR expression that rendered the cells resistant to further infection by Ad5.⁸¹⁸ In another study, the majority of subjects analyzed had Ad in the gut tissue and gut-associated lymphocytes, as detected by PCR.⁸⁴ qPCR-based studies also suggest the possibility that Ads, at least those in species C, form latent infections in lymphocytes.^{224,452,465,466} In these studies, T cells appear more likely to have persistence than B cells with significant DNA-copy-per-cell variations (10 to greater than 2×10^6).^{224,225} Persistence has been most clearly demonstrated for species C Ad. The fraction of children with Ad DNA increased from about 60% at age 2 to higher at age 4 and then declined. Stimulation of DNA-positive cells with phorbol myristate acetate and ionomycin resulted in productive replication in 85% of the samples, suggesting the latent infection may be activated in life.²²⁵

Persistent or latent Ads could explain the clinical illnesses observed in immunocompromised hosts following tissue transplantation. One recent study after allogeneic stem cell transplantation proposed that the Ad complications seen were due to reactivation of persistent Ad in the patient rather than *de novo* infection.⁷⁵¹

The mechanism that maintains lymphoid cells in humans and in cell culture in an Ad latently infected state is unknown and is of considerable interest. All Ad promoters contain binding sites for transcription factors, and the delayed early promoters (E1B, E2, B3, E4) are transactivated by the E1A proteins. However, the E3 promoter is unique in that it contains two binding sites for NF- κ B, a transcription factor that functions to activate genes involved in inflammation and immunity.⁷⁸³ The E3 promoter is activated through these NF- κ B sites in an E1A-independent manner by TNF and other activators of NF- κ B.^{153,452} The E3 proteins protect Ad-infected cultured cells from destruction by CTL and the death ligands TNF, Fas ligand, and TRAIL, and cultured T and B lymphocytes are protected by the E3 RID protein from Fas ligand-induced apoptosis⁴⁶⁵ (see Fig. 56.3 and the section Function of Adenovirus E3 Proteins). Therefore, the E3 proteins may prevent the immune system from eliminating the infected lymphocytes.

Is There a Link Between Adenoviruses and Human Cancer?

Species A and to a lesser extent species B Ads can induce tumors in newborn Syrian hamsters, and Ad9 in species D can induce mammary tumors in rats (see Chapter 55), so the question arises as to whether Ads cause cancer in humans. The Ad E1A and E1B region sequences are sufficient for transformation of rodent cells by various Ad serotypes, and when Ads induce

tumors in newborn hamsters, the E1A and E1B regions, and often other regions as well, are integrated into cellular DNA. These integrated genes are expressed as proteins that mediate the transformed state. In most studies addressing the Ad etiology of human cancer, no Ad macromolecules (DNA, RNA, or proteins) have been found that are suggestive of an Ad infection.^{247,248,386,470} One group of investigators found Ad nucleic acid in neurogenic tumors,³²³ but these results await confirmation by others.

Other researchers have considered the possibility that Ads may play a role in acute lymphoblastic leukemia (ALL).^{252,303,748} Epidemiology studies suggest a link between *in utero* infection with viruses and ALL,²⁴⁶ and it has been reported that approximately 5% of amniotic fluid samples from sonographically sound pregnancies contain Ad DNA as detected by PCR, suggesting that Ad infections *in utero* are fairly common.⁴⁷⁵ In one study in which DNA extracted from Guthrie cards was analyzed by PCR for species C Ad DNA (the PCR primers were targeted Ad2 hexon DNA), such DNA was detected in 13 of 49 neonatal blood spots from ALL patients but only in 3 of 47 controls.²⁵² However, this intriguing association was not confirmed in a follow-up study by the same group in an analysis of DNA from Guthrie cards from 243 children who developed ALL and 486 matched controls,³⁰³ nor was such an association found by another research group using PCR primers specific for hexon DNA from species A, C, and F.⁷⁴⁸

Therefore, at this point there is no clear evidence that Ads cause human cancer, given the assumption based on rodent models that human cancer cells would retain and express *e1a* and *e1b* genes. For unknown reasons, human cells are resistant to transformation by the genes in E1A and E1B, although there are exceptions (reviewed by⁷⁸⁴). However, it is important to emphasize that the studies discussed previously do not rule out a “hit and run” mechanism for Ad-induced cancer in humans in which the virus causes changes in cells that lead eventually to cancer but the viral genome is not retained by the cells. In fact, a hit-and-run mechanism of transformation has been reported in which *e1a* plus either *e4orf6* or *e4orf3* cause transformation of primary rat cells without retention of these Ad sequences, possibly by inducing mutations in cellular DNA.⁵¹⁷

DIAGNOSIS

Differential Diagnosis

Many Ad infections of the respiratory tract are difficult to distinguish from other respiratory viruses and even some bacterial infections. In children younger than 3 years of age, small discrete white spots of exudate can appear on the tonsils and are consistent with Ad infection. They have to be distinguished from streptococcal infections and the manifestations of the Epstein-Barr virus mononucleosis syndrome or even some cases of herpes stomatitis, which in older children or young adults might start as exudate on the faucial tonsils. The most characteristic manifestation of Ad infection is the conjunctivitis as part of the keratoconjunctivitis syndrome, which usually begins unilaterally, causes moderate enlargement of the preauricular lymph node, and may subsequently involve the contralateral eye. The Ad-induced upper respiratory syndromes or pneumonias are impossible to distinguish clinically from other viral infections of these organs and must employ laboratory

diagnosis for clarification of the etiologic agent. Hemorrhagic manifestations of the urinary tract can be caused by bacterial infections as well as by Ad infections. In patients immunosuppressed with cyclophosphamide for bone marrow transplantation, manifestations of hemorrhagic cystitis can be due to drug toxicity in the bladder soon after transplantation. However, the isolation of Ads later in the course as a cause of the cystitis was reported in 13% of cases.³⁸¹ Ad as a cause of hemorrhagic cystitis is more common in Japan than in the United States, for reasons that have not been explained.

Laboratory Diagnosis

Collection and Preparation of Specimens

Collection of specimens from affected sites early in the illness is necessary to optimize viral isolation and to detect Ad antigens or nucleic acid directly in clinical samples. The duration of excretion of Ads at the time of acute infection is about 1 to 3 days from the throat in adults with the common cold; 3 to 5 days from the nose, throat, stool, and eye from patients with pharyngoconjunctival fever; 2 weeks from eye cultures from EKC; 3 to 6 weeks from the throat or stool of children with respiratory or generalized illnesses; and 2 to 12 months or longer from urine, stool, throat, or organ biopsies of immunocompromised patients.²⁸⁴ However, after the acute period, Ads may be latent in some tissues, such as the tonsils, or may be reisolated intermittently from throat or stool cultures for periods of months to years after primary infection in some patients.^{205,290} Ads obtained from throat swabs, nasal washes, conjunctival swabs or scrapings, and anal swabs are generally stable agents and withstand freezing and storage at -70°C , if immediate inoculation is not possible. Because some of the urine may be toxic to the tissue culture lines employed, it is best to remove the input inoculum after 2 to 4 hours of incubation. This time allows the virus to be absorbed before the cultures are re-fed with fresh medium. When cultures of blood mononuclear cells are desired, heparinized blood is fractionated on Ficoll-Hypaque gradients, and the washed cells are cultured in RPMI medium with fetal calf serum.³⁶³ Portions of these cells should also be co-cultivated on such cells as HEK cells, which are susceptible to Ad infection.

A common method for identifying Ad in the clinical virology laboratory is cell culture. For respiratory infections, specimens include nasopharyngeal swabs, washings, or aspirates, bronchoalveolar lavage fluid, or bronchial washings. For ocular infections, a swab of the infected conjunctiva is used. The inoculum is often placed into tubes of cell monolayers that are rotated. Cell types that are highly permissive for all human Ads except Ad40 and Ad41 are human neonatal kidney cells (these are semipermanent cell lines at very low passage) and primary HEK cells. Highly permissive permanent human cell lines that are used are A549 (lung adenocarcinoma), HeLa (cervical carcinoma), KB (nasopharyngeal carcinoma), and HEP-2 (hepatocellular carcinoma). Fibroblast cell lines are less permissive for Ad than the aforementioned epithelial cells. The highly fastidious enteric Ads, Ad40 and Ad41, can be grown on HEK293 cells. The ability of Ad40 to grow on HEK293 cells is a function of the Ad E1B-55K protein.⁴⁶¹

In current practice in many clinical virology laboratories, Ad detection in epithelial cells has been greatly accelerated by centrifugation of viral specimens directly onto the tissue culture cells (HEP-2), using the shell vial technique. This approach has

yielded positive cultures in 1 to 2 days for most serotypes or in 3 days for ocular Ads.³⁸⁸ In a clinical setting, initial testing is often done to distinguish between multiple viruses (influenza A and B, parainfluenza virus types 1 to 3, respiratory syncytial virus, and Ad).^{33,170,325,679,778} Nasopharyngeal swab, nasopharyngeal aspirate, and nasopharyngeal wash specimens are collected. A modified shell vial technique is used that combines a mixture of mink lung cells (Mv1Lu) and human A549 cells³¹⁶ or a mixture of A549 and primary rhesus monkey kidney cells.¹⁸³ The A549 cell line is a good host for most human Ads. After growing for 1 to 2 days, the cells are tested with monoclonal antibodies to identify the virus; Ads are identified by antibody recognition of the group-reactive epitope on hexon. Ad detection is best after at least 2 days of culture.^{170,183}

Respiratory infection or ocular samples are sometimes assayed directly for Ad without culturing the virus. Similarly, Ad can be diagnosed rapidly from the urine of patients with suspected Ad hemorrhagic cystitis.⁴⁸ The epithelial cells in the sample are washed by centrifugation, pelleted, and spotted onto microscope slides. The cells are then examined by IF using antibodies specific to Ad and visualized in a fluorescent microscope. Only 50 to 100 cells are required for this procedure. The method is rapid but is only 50% as effective as cell culture.

A commercial enzyme-linked immunoassay kit that has been approved by the Food and Drug Administration (FDA), named Adenoclone (Meridian Biosciences, Inc.), can be used to detect Ad directly in respiratory ocular or stool specimens.^{236,782} The assay detects most if not all human Ad serotypes, takes about 1 hour, and can be performed in the doctor's office. However, a high concentration of virus is needed such as is found within 1 to 3 days of clinical onset. Therefore, the assay is less sensitive than cell culture or IF staining.³⁰⁸ Thus, negative results need to be confirmed by cell culture methods.

In most instances in the routine clinical virology laboratory, once a positive Ad diagnosis is made, further work is not conducted to identify the serotype. However, as more is being learned about the pathogenesis of particular Ad serotypes, and for research purposes, PCR and qPCR, as well as restriction enzyme digestion of viral DNA, have become highly developed.

Identification and Classification of Viral Isolates

VIRAL SEROLOGY

Agents growing in tissue culture and demonstrating typical Ad CPE can be further processed to identify the isolate definitively. The methods used traditionally have been serology but now include restriction enzyme digestion of viral DNA and especially PCR analysis. Serologic confirmation in Ad typing can be separated into two categories. The IF and CF tests, using tissue culture material as antigens and reference antibodies, measure group characteristics of the Ads and are therefore usually shared by all human isolates.^{149,308} The hemagglutination inhibition (HAI) and serum neutralization (SN) tests measure type-specific parameters that can distinguish members of each group.³⁶³ See reference⁷⁸⁷ for details.

VIRAL DNA

In recent years, analysis of Ad DNA has gained prominence for detection of Ad in clinical samples and for subgroup typing. Characterization of the viral DNA by both hybridization and restriction endonuclease digestion patterns has been used for clinical isolates (see³⁰⁸).^{23,148,185,375} Because of small differences

in the DNA from various isolates, multiple restriction enzymes are often needed for a definitive identification. Although these small DNA differences can make interpretation of results difficult, they do lead to identification of strains that are of interest in the general epidemiology and pathogenesis of Ad.

Currently, PCR assays are being used for diagnosis, typing, and quantitation of Ad infections in various tissue specimens and body fluids.^{3,10,32,121,175,177,216,250,408,558,737,759,774,795,796} One study used PCR to amplify a portion of the *hexon* gene from all 51 serotypes followed by restriction fragment length polymorphism analysis and DNA sequencing.⁶⁵⁶ This method was especially useful for diagnosis of serotypes in species D (Ad8, 19, 37) and E (Ad4) that cause the majority of EKC in Japan. Most of the reports describe standard PCR but with the reactions set up in a multiplex format in which mixtures of primers and probes are used so that multiple serotypes can be detected simultaneously. The primers and probes are slightly degenerate in sequence to take into account small differences in DNA sequences among the different serotypes. One approach that could detect all 56 serotypes as well as many genome variants of Ads 1, 3, 4, 5, 7, 11, 19, 40, and 41 was to conduct PCR using primers specific to the conserved region of the *hexon* gene followed by restriction enzyme digestion of the PCR amplification products.¹⁰ This PCR assay could detect Ad40 and Ad41 on sodium dodecylsulfate/ethylenediaminetetraacetic acid (EDTA)-pretreated paper strips that have been proposed as a method to collect, store, and transport inactivated Ad from stool.⁸²³ Other PCR-only multiplex assays have been used for species C³ and D⁴ Ads using fiber-specific primers. Multiplex assays based on hexon have been used for species B Ad.^{216,408,795} Other research groups have developed multiplex assays based on fiber that can detect serotypes in all species, A through F.^{177,558,796} One assay, termed *PCR adenovirus consensus*, uses two degenerate primers specific to the Ad VA-RNA gene to amplify DNA from 51 serotypes.⁷³⁷ The amplified DNA is hybridized to a biotin-conjugated probe and is detected with a streptavidin peroxidase conjugate. Positive samples can be diagnosed further using species-specific hexon-specific primers. The assay is available commercially (Argene). Multiplex assays that can detect Ads, rotaviruses, and Norwalk-like viruses in a single reaction have been described.^{534,598}

Some workers have employed qPCR to detect and quantitate Ad DNA sequences (reviewed by⁷⁷³). This method was used for Ad4 in clinical samples^{191,314} and is versatile enough to be used in field situations to diagnose Ad4-induced ARD in military recruits.¹⁹¹ Nested qPCR has been combined with an antigen ELISA for broad detection of Ads.⁴⁷² Two groups have developed multiplex qPCR methods with primers and probes specific to hexon that can detect all serotypes in species A, B, and C.^{121,250} Both these assays had a sensitivity of about 50 to 250 copies/mL of Ad DNA. In another study, 51 serotypes in species A to F could be detected in one PCR reaction using a consensus primer and probe specific to a conserved region of hexon, but low stringency conditions were necessary.²⁷⁹ In a series of studies that took advantage of the DNA sequence of the *hexon*¹⁷³ and *fiber* genes, Ebner et al¹⁷⁵ developed a stringent qPCR assay that can detect all Ad serotypes. The DNA sequence differences in the conserved regions of the *hexon* and *fiber* genes precluded the design of a single PCR specific to all serotypes with optimal specificity, so a two-reaction PCR assay was developed. One reaction

covered serotypes in species A, C, and F, and the other was specific to species B, D, and E.¹⁷⁵

Quantitative PCR assays can be used to assess the antiviral efficacy of antivirals in cell culture⁶⁸⁵ and are useful to predict progression to disseminated disease in pediatric and, to a lesser extent, adult SCT recipients.^{98,184,197,222,337,434,436,351,539} Lastly, such qPCR methods have been useful in predicting response to interventions among immunosuppressed patients treated for Ad infections.^{13,181,419,632} In such situations, a decrease in the viral load of greater than or equal to 1 log in the 7 to 10 days after the first dose was administered was predictive of a successful outcome.⁴¹⁹

Lastly, a range of molecular methods have been developed that allow simultaneous identification and typing of strains of Ad from clinical specimens.^{11,60,174,182,244,443,449,462,573,599} Such assays have been used to identify the prevalent strains of Ad and risk factors for disease in a number of populations. Given the ease of such systems, compared to traditional serologic methods, most labs have moved to using such molecular systems to type viruses. Such assays have also been advocated as a major component of contemporary Ad designation systems.¹⁷

These various PCR and qPCR assays have been used to diagnose and sometimes quantitate Ad in respiratory and ocular specimens and in the blood, serum, and peripheral blood cells from immunosuppressed patients. A number of commercial laboratories offer analysis of clinical specimens for 56 serotypes using PCR or qPCR or will sell kits for these analyses. Further, there are a number of multiplex systems, particularly focused on diagnosing respiratory and gastrointestinal viral infections, that are able to detect Ad as part of a panel of other viruses that cause a similar clinical syndrome.^{29,217,395,438}

Direct Microscopy of Clinical Specimens

EXFOLIATED CELLS

Rapid diagnosis by direct examination of the cytopathology of exfoliated cells has shown variable degrees of success; reports indicate that IF techniques detected only one-third of the nasopharyngeal secretions from which Ads subsequently grew in tissue culture²²³ or that IF is as sensitive as tissue culture growth.³⁰⁸

Nasopharyngeal secretions and swab specimens have been used for direct fluorescent antibody testing (DFA).³⁰⁸ DFA can be a rapid initial test for seven respiratory viruses (influenza A and B, parainfluenza virus types 1 to 3, respiratory syncytial virus, and Ad). Cells are pelleted, transferred to slides, fixed, and stained with monoclonal antibodies specific for the seven virus groups.^{652,697,816} This assay is often run in parallel to shell vial culture for improved sensitivity.

The IF technique has not been useful in early diagnosis of conjunctival Ad lesions but may be more useful in studies of exfoliated bladder cells from patients with acute hemorrhagic cystitis.⁴⁸ Latex agglutination tests for detecting enteric Ad proteins in stool are available. They depend on specific Ad antibody to agglutinate latex beads in the presence of Ad proteins. Although evaluation of their sensitivity and specificity has indicated that they are useful in diagnosing enteric Ad infection, they are less sensitive than culture, they may give positive results with nonenteric Ads, and a significant number of positive reactions of stool extract with the latex controls can invalidate the test. Development of latex agglutination tests using monoclonal antibodies has increased the use of this assay.²⁴⁰

Electron Microscopy

EM of clinical specimens has been used in special situations to identify viral agents rapidly and specifically. The discovery of the “noncultivable” enteric Ads was made by EM examinations of stool extracts.²⁰² The EM morphology of the Ads is unique. The Ads in stool are primarily the enteric Ads, types 40 and 41. The other usual method to detect Ad in stool is an enzyme-linked immunoassay such as Adenoclone. These fastidious enteric Ads can also be grown on HEK293 cells and characterized further. On specimens from other body sites, immune EM for rapid diagnosis of respiratory infections was successful in detecting 19 of 25 throat swabs from which Ads were grown.¹⁷⁸ Although less sensitive than tissue culture growth, the EM technique was rapid. However, such approaches are not generally used to diagnose Ad infection of the respiratory tract.

PREVENTION AND TREATMENT

Therapy

With the emerging appreciation that Ads are a serious problem in EKC and in immunocompromised patients, there is recent interest in developing anti-Ad drugs (reviewed in^{176,197,294,328,378,409,416,418,434,762}). A class of drugs known as acyclic nucleoside phosphonates is effective against many DNA viruses including Ad¹⁴⁶ (reviewed in^{144,312}). One member of this class, (S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl] cytosine, known as cidofovir, has been studied extensively off label in Ad infections. Cidofovir is an analog of deoxycytosine monophosphate in which the ribose has been replaced with an acyclic moiety. Because cidofovir is already a monophosphate, it does not require a viral enzyme for its phosphorylation (e.g., thymidine kinase [TK], which is required to phosphorylate acyclovir), and cidofovir can be converted to the di- and triphosphate forms by cellular enzymes. These triphosphorylated acyclic nucleoside phosphonates in general have higher affinity for viral DNA polymerases than for cellular DNA polymerases, thereby providing specificity for virus-infected cells. They act as inhibitors of the viral DNA polymerase, the triphosphate is a substrate for the polymerase, and, acting through a variety of mechanisms, they function as a DNA synthesis chain terminator.^{144,312}

Cidofovir is a potent inhibitor of Ad replication in cell culture.^{378,502} Cidofovir-resistant Ad5 mutants have been isolated that have sequence changes in the Ad5 DNA polymerase near the nucleotide binding site, implying that the target of cidofovir is the Ad5 DNA polymerase.³⁷⁷ In one study, cidofovir inhibited replication in HEp-2 cells of serotypes from species A, B, D, E, and F.^{482,483} Cidofovir has shown some efficacy in the rabbit^{123,378,601,602,603} and cotton rat³⁵⁹ models of ocular models of Ad5 infection.

A large multicenter trial was initiated in the United States to evaluate cidofovir against Ad ocular infections in humans (discussed in³⁷⁸). Significant efficacy was observed, but the trial was discontinued because of a narrow efficacy/toxicity ratio. Two other clinical trials have been conducted for cidofovir treatment of EKC: no efficacy was seen in one trial using 0.2% cidofovir plus 1% cyclosporine,²⁸⁸ whereas in the other trial, 1% cidofovir plus 1% cyclosporine lowered the frequency of severe corneal opacities but caused local toxicity.²⁸⁹

Cidofovir was examined in a number of retrospective studies as well as in case reports, alone or sometimes

in combination with ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), to treat systemic Ad infections in immunosuppressed patients.^{378,382,409} However, there have not been controlled clinical trials for systemic use of these drugs to treat Ad, and the drugs have not been licensed for this use. In early studies, cidofovir showed some efficacy against Ad in immunosuppressed HSCT and bone marrow transplant patients.^{62,295,415,440,589} Cidofovir was effective against Ad-associated hemorrhagic cystitis in bone marrow transplant patients.^{193,271} With eight pediatric SCT patients, cidofovir seemed to provide long-term suppression of Ad without dose-limiting nephrotoxicity.²⁹⁵ In a study in which Ad was detected in 26 of 155 pediatric HSCT patients, ribavirin was used when Ad was first detected and cidofovir was used in patients with persistent viremia.³⁵⁴ The authors concluded that, although not curative, the antiviral therapy appeared to control the Ad infection. In a study in which 57 of 177 pediatric HSCT patients were diagnosed with Ad, with eight having disseminated disease, cidofovir treatment led to clinical symptom resolution in 56 patients.⁸⁰⁹ In a study in which 71 pediatric STC transplants were monitored, there were eight cases of Ad infection, three of whom died, but cidofovir seemed to provide benefit.⁶⁶⁷ In a recent study, eight immuno-

suppressed patients (three HSCT recipients, two liver–small bowel transplant recipients, one liver transplant recipient, one recipient with severe combined immune deficiency, and one with a T-cell deficiency) with adenoviremia and invasive Ad disease were monitored by qPCR analysis to determine their response to cidofovir (5 mg/kg every week for 2 weeks, then every other week).⁴¹⁹ Five patients clearly responded virologically and clinically to cidofovir; the remaining three patients had stable viral replication and eventually died. Of note, there was a significant delay between onset of symptoms and institution of therapy in three fatal cases (median time to death, 18 days). A decrease in the viral load of greater than or equal to 1 log in the 7 to 10 days after the first dose was administered was predictive of a successful outcome.⁴¹⁹ Other groups have documented a similar correlation of poor early response to cidofovir and progression to fatal disease.^{13,515} In another study, seven pediatric HSCT developed high-risk Ad infections and were preemptively treated with intravenous cidofovir (1 mg/kg three times weekly for nine doses).¹³ High-risk Ad infections resolved in all seven patients without renal toxicity. Cytomegalovirus (CMV) viremia occurred in two of seven patients during or shortly after therapy with cidofovir, suggesting benefit of early antiviral therapy.

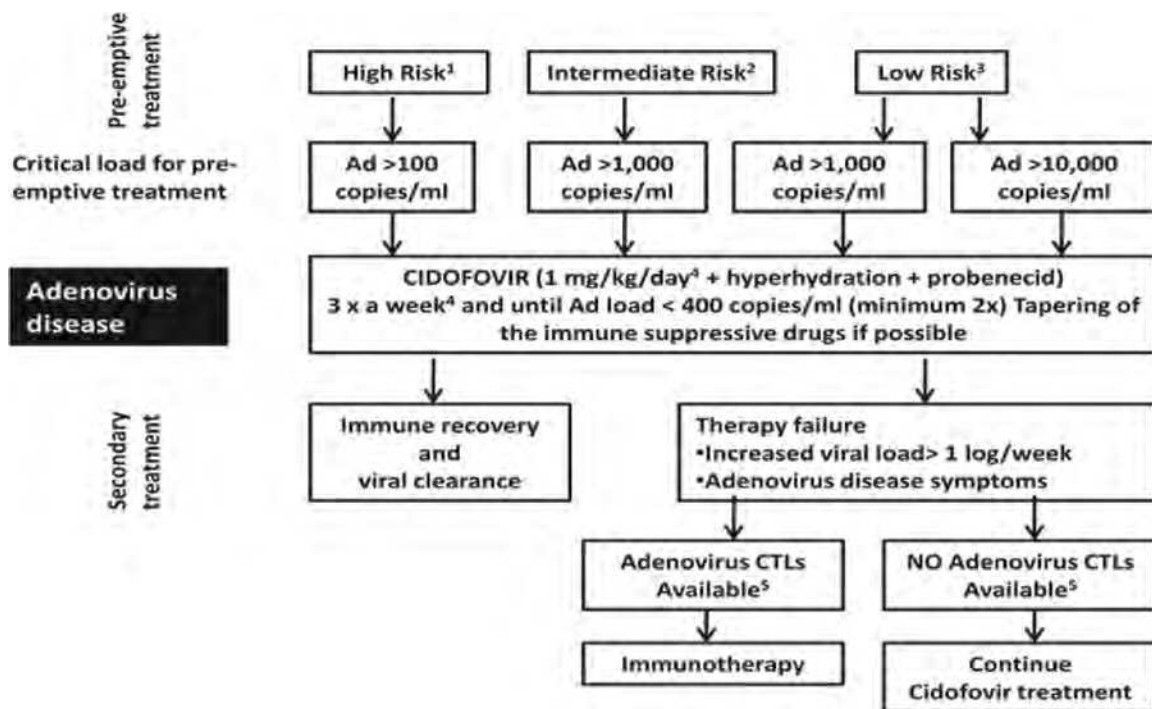


FIGURE 56.7. How Lindemans et al⁴³⁴ treat adenovirus (Ad) infections in pediatric hemopoietic stem cell transplant (HSCT) recipients.¹ Cord blood donor/T-cell–depleted graft recipient less than 1 month post-SCT AND/OR immune suppression: prednisone greater than or equal to 1 mg/kg/day and greater than or equal to one lymphocyte proliferation inhibitor (e.g., cyclosporine A).² Cord blood donor/T-cell–depleted graft recipient 1 to 4 months post-SCT AND/OR immune suppression: prednisone 0.5 to 1 mg/kg/day and greater than one lymphocyte proliferation inhibitor.³ Donor source other than cord blood or T-cell–depleted graft, or 4 or more months post-SCT for any donor source. Immune suppression maximum: one lymphocyte proliferation inhibitor and/or prednisone less than or equal to 0.5 mg/kg/day. CD3 monitoring when Ad greater than 100 genome copies/mL: CD3 less than 25/ μ L at detection OR less than 300/ μ L within 2 weeks; CD3 greater than 25/ μ L at detection AND greater than 300/ μ L within 2 weeks.⁴ An alternative is cidofovir 5 mg/kg intravenously weekly.⁵ For centers that have Ad-specific cytotoxic T lymphocytes (CTLs) readily available, CTLs are immediately initiated for all high-risk patients and for all patients with Ad symptoms before awaiting cidofovir effect. (Adapted from Lindemans CA, Leen AM, Boelens JJ. How I treat adenovirus in hematopoietic stem cell transplant recipients. *Blood* 2010;116:5476–5485.)

In case reports, cidofovir appeared to have anti-Ad activity in pediatric liver^{120,366} and renal¹⁷⁶⁰ transplants and in HSC,^{515,523} heart,⁵⁸³ and renal⁶⁹² transplants. Cidofovir seemed to resolve disseminated Ad in a pediatric liver transplant patient⁹¹ and Ad7 in a B-cell lymphoma adult patient with meningoencephalitis.²⁰⁰ Two adult renal transplant patients with disseminated Ad11 infection were successfully treated with cidofovir, intravenous immunoglobulin, and reduction in immunosuppression.⁶²⁰

In summary, these data and reports support the view that cidofovir can be employed against Ad infections with significant anti-Ad activity, but toxicity of cidofovir may limit its use. In fact, one group of authors have described a general approach in which they strictly monitor pediatric HSCT patients for Ad by qPCR and then for Ad-positive patients treat with cidofovir preemptively⁴³⁴ (Fig. 56.7); such approaches have not been studied in adult patients. Nevertheless, it still must be noted that a major concern with the systemic use of cidofovir is nephrotoxicity caused by accumulation of the drug in renal proximal tubules and cytopenias.^{144,145}

Because of the presence of the phosphate group on cidofovir, the drug shows poor oral bioavailability.^{144,312} However, a new series of ether lipid-ester prodrugs of cidofovir and other acyclic nucleoside phosphonates has been developed that increase oral absorption in the small intestine and facilitate cellular uptake

and metabolism.³¹² The lipid–nucleotide conjugate was designed to mimic lysophosphatidylcholine (LPC) and thus use the LPC uptake pathway in the small intestine. These prodrugs spontaneously insert into cellular membranes, the lipid moiety is hydrolyzed away by phospholipase C, and the compound is converted to the di- and triphosphate form by cellular enzymes. These di- and triphosphate forms tend to stay inside the cell because they are negatively charged and cannot easily pass through the plasma membrane. Alkoxyalkyl ester derivatives of cidofovir or (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine [(S)-HPMPA] were reported to be 2 to 3 logs more effective than cidofovir or (S)-HPMPA in inhibiting five serotypes of Ad (Ad3, 5, 7, 8, 31) in cell culture.²⁶⁶ These compounds also have broad-spectrum activity against many double-stranded DNA viruses as compared with the unmodified nucleobases. Hexadecyloxypropyl-cidofovir (HDP-CDV; developed as CMX001 by Chimerix, Inc.) was the first of these compounds synthesized, it has been characterized extensively *in vitro* and in animal models, and it is being studied in clinical trials.³¹² As compared to cidofovir, CMX001 is not toxic to the kidney, although there is some gastrointestinal toxicity. In a Syrian hamster model for Ad5 infection, CMX001 was highly effective in eliminating Ad5 infection in the liver (Fig. 56.8), salivary gland, and pancreas when administered orally and daily beginning 1 day before or as long as 2 days

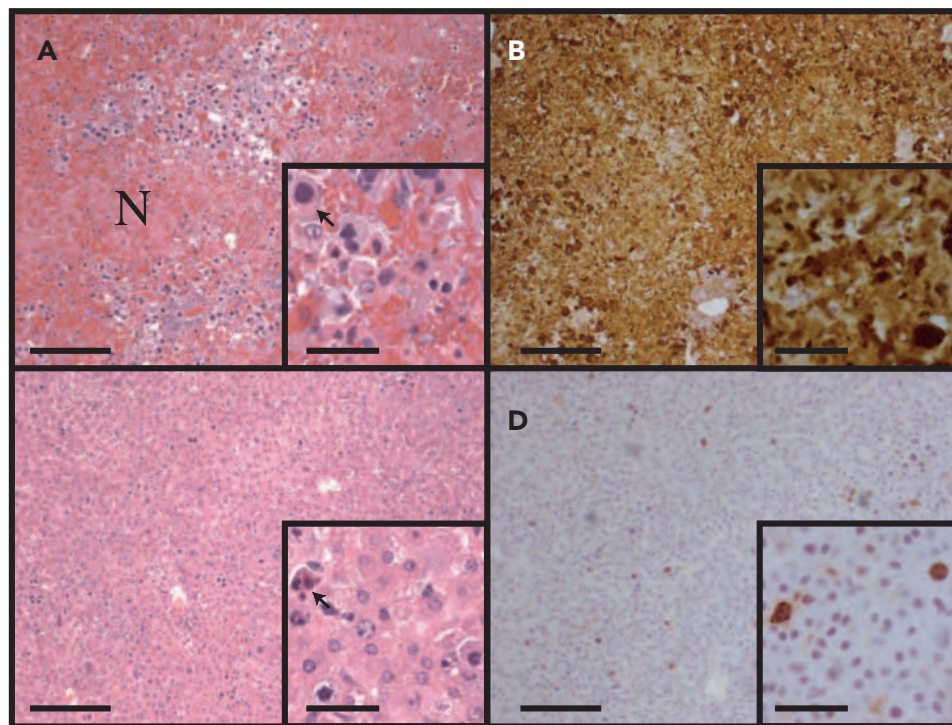


FIGURE 56.8. CMX001 decreases adenovirus type 5 (Ad5)-induced lesions in the liver. Syrian hamsters were immunosuppressed using cyclophosphamide, then infected intravenously with 1.9×10^{12} virus particles/kg of Ad5. Livers of hamsters sacrificed at day 6 were subjected to histopathologic and immunohistochemistry (IHC) evaluation. Animals infected with Ad5 and not treated with CMX001 exhibited extensive coagulation necrosis throughout the liver (**A**) and widespread replication of Ad5, demonstrated by staining for fiber (**B**). Treatment of Ad5-infected hamsters with CMX001 resulted in a significant reduction in hepatocellular injury (**C**) and greatly reduced IHC staining for fiber (**D**). The arrows indicate intranuclear inclusion bodies. The scale bars represent 200 μm for the larger pictures and 50 μm for the insets. N, necrosis. (From Toth K, Spencer JF, Dhar D, et al. Hexadecyloxypropyl-cidofovir, CMX001, prevents adenovirus-induced mortality in a permissive, immunosuppressed animal model. *Proc Natl Acad Sci U S A* 2008;105:7293–7297. Copyright 2007 National Academy of Sciences U.S.A.)

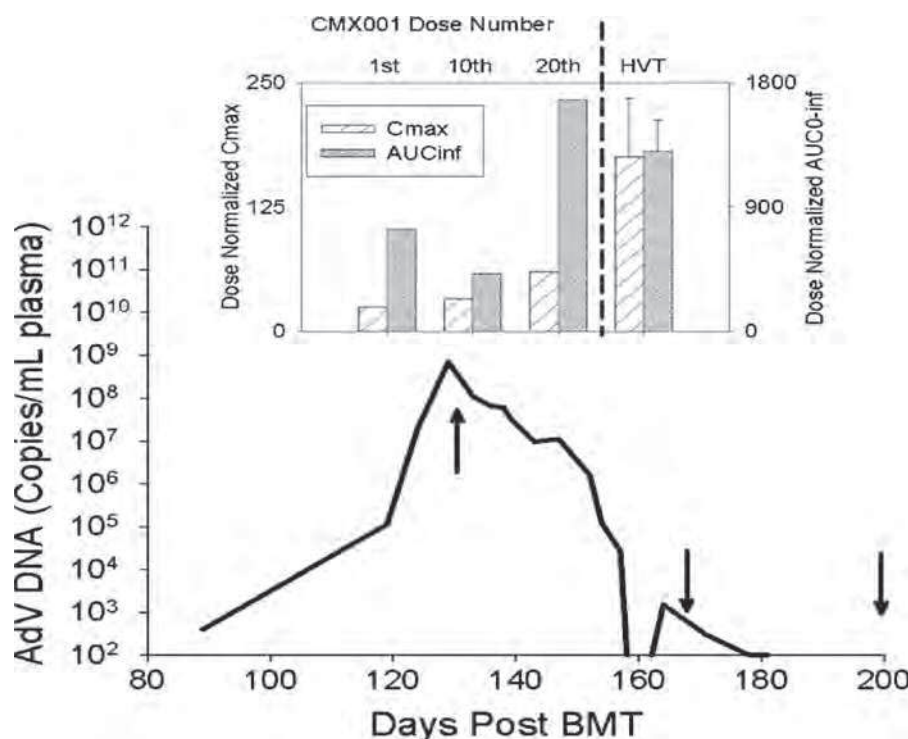


FIGURE 56.9. Plasma adenovirus (Ad) levels prior to (day +132 posttransplant) and during (day +133) treatment with CMX001.

Ad was first detected day +89 posttransplant, with a continued rise in Ad load despite administration of intravenous cidofovir days +92 to +132 posttransplant and intravenous immunoglobulin (IVIG) (day +129 posttransplant). Treatment with CMX001 was initiated on day +133 posttransplant at 2 mg/kg administered twice weekly increasing to 3 mg/kg after the sixth dose. After the virus became undetectable (less than 10² copies/mL) (day +159 posttransplant), administration of CMX001 continued at 3 mg/kg but the schedule was reduced to once weekly for maintenance. The inset shows the patient's dose normalized maximum plasma concentrations (C_{max} , ng/mL/dose [mg/kg]) and systemic exposure (AUC0-inf, h*ng/mL/dose [mg/kg]) of CMX001 after the 1st, 10th, and 20th doses (first three bars) in comparison to adult healthy volunteers (HVTs) administered a single dose (fourth bar). Arrows indicate timing of 1st, 10th, and 20th CMX001 doses. (From Paolino K, Sande J, Perez E, et al. Eradication of disseminated adenovirus infection in a pediatric hematopoietic stem cell transplantation recipient using the novel antiviral agent CMX001. *J Clin Virol* 2011;50:167–170. Copyright 2011, with permission from Elsevier.)

after an intravenous administration of approximately the lethal dose 50 of Ad5.⁷²⁷ CMX001 also displayed a remarkable ability to eradicate a disseminated Ad2 infection in a pediatric HSCT patient.⁵⁵⁰ In this patient, Ad DNA levels in the plasma increased to ca. 10⁹ copies/mL despite cidofovir therapy, and the patient experienced renal deterioration caused by cidofovir; shortly following CMX001 administration, Ad DNA levels in the plasma decreased dramatically and the patient recovered (Fig. 56.9).⁵⁵⁰ In July 2011, Chimerix announced the beginning of the AdV HALT Trial, which is a randomized, placebo-controlled, multisite phase II clinical trial to evaluate the safety and efficacy of pretreatment with CMX001 for prevention of Ad disease following HSCT. In the trial, CMX001 will be administered orally to immunocompromised pediatric patients who have Ad infections but not evidence of disease. CMX001 (and similar lipid-linked prodrugs of acyclic nucleoside phosphonates) appears to hold great promise.

Ribavirin is a broad-spectrum antiviral drug with activity *in vitro* against a variety of viruses, especially RNA viruses, that is used to treat hepatitis C infections of the liver in combination with interferon- α and also respiratory tract infections in children by respiratory syncytial virus. The activity of ribavirin against

Ad replication in cell culture is controversial.⁴¹⁶ In general, ribavirin was considered to be modestly effective against only species C Ads,⁴⁸³ but in a more recent study ribavirin was reported to inhibit replication *in vitro* of clinical isolates of species A, B, and D as well as species C.⁴⁸² Mixed results have been obtained in about a dozen studies in the clinic with immunosuppressed patients (reviewed in^{378,409,416}). Successful use of ribavirin in combination with cidofovir has been reported.^{354,623} In a case series of four patients serially monitored with Ad DNA loads, ribavirin failed to have a meaningful clinical or virological impact, even though three of the patients clearly had species C Ads.⁴⁰²

Digoxin and furosemide are commercially available and show *in vitro* activity against Ad but have not been studied for the treatment of adenoviral infections.²⁶⁵ Likewise, ganciclovir has modest activity against Ad,⁴¹⁶ but there are limited data for the efficacy of ganciclovir in the treatment of adenoviral infections.³²⁸ Two studies revealed that patients who did not receive ganciclovir for prophylaxis were at greater risk of developing adenoviral infection and disease (odds ratio [OR], 3.4; 95% confidence interval [CI], 2.1 to 5.6).^{26,80}

A quite large number of other compounds have been identified that inhibit Ad replication in cell culture and in

animal models (reviewed in^{378,416}). Olomoucine II, that is, 6-(2-Hydroxybenzylamino)-2(*R*)-[[1-hydroxymethyl]propyl]amino]-9-isopropylpurine³⁰⁰; zalcitabine; alovudine; stavudine^{219,358,502,733}; halo-substituted stavudine phenyl phosphoramidate derivatives⁷³⁴; sanilbudine⁷³³; interferon- β ³⁹⁶; an antioestrogen peptide⁷³³; the sulfated sialyl lipid NMS03; the microbicide *N*-chlorotaurine^{378,416}; the cobalt chelate CTC-96 (Doxovir)^{378,416}; and aribidol⁶⁵⁴ have shown activity *in vitro* against Ad but have not been studied clinically for the treatment of Ad infections and are not undergoing active clinical development for this indication. Further, dehydroepiandrosterone, epiandrosterone, and some of their analogs inhibited Ad5 replication *in vitro*.⁶⁰⁵ Human α -defensins HNP1 and HD5 inhibit infection by a variety of Ad serotypes with a half-maximal inhibitory concentration (IC_{50}) of 3 to 4 μ M.⁶⁷² The defensins or their analogs could, therefore, be used for treatment of Ad infections. Likewise, the soy isoflavone, genistein, inhibits phosphorylation of p130^{CAS} and inhibits Ad late mRNA translation and therefore could also be investigated as an anti-Ad antiviral.^{422,792} Additionally, A3, which interferes with *de novo* pyrimidine biosynthesis, and GSK983, a novel tetrahydrocarbazole, have been demonstrated to have activity *in vitro* against Ad5.^{269,296}

An interesting and alternative approach to anti-Ad drugs is to exploit biological aspects of the host that the virus must employ in order to replicate efficiently. Ad5 enhances signaling through the Ras pathway; this activity is required for efficient replication inasmuch as pharmacologic inhibitors of MEK1/2 or ERK1/2 inhibited replication greater than 100-fold in cell culture.⁶²⁸ Perhaps such inhibitors could be used for Ad infections.

Ad-specific T cells are being explored as a therapy against Ad infection, with great promise (see the sections on Description of Key Antigens and Immune Responses).^{5,199,277,367,410,474,813}

Vaccine

As a result of the significant impact of Ad infection on the U.S. military, particularly new recruits in the 1960s, the U.S. Department of Defense and the National Institutes of Health (NIH) contracted with Wyeth Laboratories to develop and produce oral, live, enteric-coated vaccine tablets against Ad serotypes 4 and 7.⁴⁴⁶ These vaccines were administered to several million recruits over 25 years with excellent efficacy and safety. The manufacturer discontinued production of the vaccine in 1996, and Ad-associated acute respiratory illness returned to prevaccine levels.^{243,616} To address the re-emergence of Ad, the Department of Defense contracted with Barr Laboratories to develop and produce a new live, attenuated Ad4 and Ad7 vaccine.⁴⁴⁶ The new vaccine utilized the same Ad4 and Ad7 strains that were utilized in the original vaccine.⁴⁴⁶ The vaccine virus strains have been sequenced, annotated, and compared to prototype Ad4 and Ad7 strains. In doing so, it was determined that the Ad4 strains are recombinant: the inverted terminal repeat (ITR) of the Ad4 vaccine strain is identical to species C Ad, whereas the ITR of the prototype Ad4 strain is identical to species B1 Ad.⁵⁷⁵ Interestingly, neither the Ad4 nor the Ad7 vaccine strain appears to be attenuated based on bioinformatics analysis.⁵⁷⁵

In March 2011, the oral, enteric-coated vaccine was again approved for use in military populations 17 to 50 years of age, but it is not licensed for use in civilians. It is delivered as a single Ad4 and a single Ad7 tablet. The vaccine serotypes are packaged together in enteric capsules so that they bypass the respiratory epithelium and only replicate once they reach the intestine.¹³⁰

The enteric infection is typically asymptomatic, and it results in good neutralizing antibody responses. Circulating antibody is a reliable standard of immunity after administration of Ad vaccines. Hematuria, gastroenteritis, febrile gastroenteritis, gastritis, pneumonia, and hematochezia were recognized serious adverse events in vaccine recipients, but the incidence of any adverse effect was similar to those who received placebo. The vaccine efficacy was 99.3% (95% CI: 96.0% to 99.9%) for Ad4, while seroconversion was documented to be 94.5% (95% CI: 93.4% to 95.5%) and 93.8% (95% CI: 92.4% to 95.2%) for Ad4 and Ad7, respectively. Seroconversion is more likely among individuals who shed virus in the stool. No vaccine-type viruses were detected in throat swabs or blood of individuals who received the vaccine as part of a clinical study.⁴⁴⁶

The Ad vaccine has not been extended for use in children or civilians because of the risk of clinically symptomatic transmission between contacts. In a clinical trial of Ad4 vaccine in a small number of children, the infection spread to household contacts and resulted in a few clinically manifest illnesses.⁴⁹² Similar vaccine administration to adults commonly spread to sexual partners but infrequently to other adults housed together.⁶⁸¹ This transmissibility is likely the result of inadequate attenuation, as has been shown with sequencing data.⁵⁷⁵ Similarly, a small oral vaccine study in adults with Ad types 1, 2, and 5 resulted in virus shedding in the stool, some replication in the pharynx, and seroconversion, but no clinical illness.⁶²⁹

Adenoviruses as Vectors for Vaccination and Gene Therapy

As of 2011, ~415 gene therapy trials have been conducted or are ongoing with Ad vectors, accounting for ~24% of all clinical trials (<http://www.wiley.co.uk/genetherapy/clinical/>). Some trials have employed Ad vectors for gene therapy; for example, to deliver the correct version of a gene (typically as a complementary DNA [cDNA]) to a patient with a mutation in that gene, some vectors have been used as vaccines, but the vast majority of clinical trials with Ad vectors have been for cancer treatment. These various trials have indicated that Ad vectors are very well tolerated. Of further note regarding safety, millions of soldiers have been immunized safely with live Ad4 and Ad7 vaccines. The advantages of Ad vectors are multiple: the biology of the virus is well studied, they can be grown to produce stable and high-titer stocks, and they can infect both dividing and nondividing cells and cells and tissues of many different types (reviewed in^{76,77,257,725,803}). Ads rarely integrate into the host chromosome; thus, the vector DNA is maintained episomally.

Ads as vectors (Fig. 56.10) can be classified as replication defective (RD) or replication competent (RC). RD vectors serve to deliver the transgene into the target cell, whereas virus replication in the target cell is the intended mechanism of action for RC vectors.

RD Ad vectors have one or more essential viral genes deleted. With the “first generation” vectors these deletions encompass the essential E1 region (i.e., E1A and E1B), where the transgene is inserted, and the nonessential (in cultured cells) E3 region, which serves to increase cloning capacity (see Fig. 56.10B). These vectors can infect most cells and transcribe the inserted transgene. The constructs usually are made with plasmids into which the transgene is inserted, flanked by Ad genome packaging sequences (into virions), the origin of genome replication at the physical left end of the DNA (the

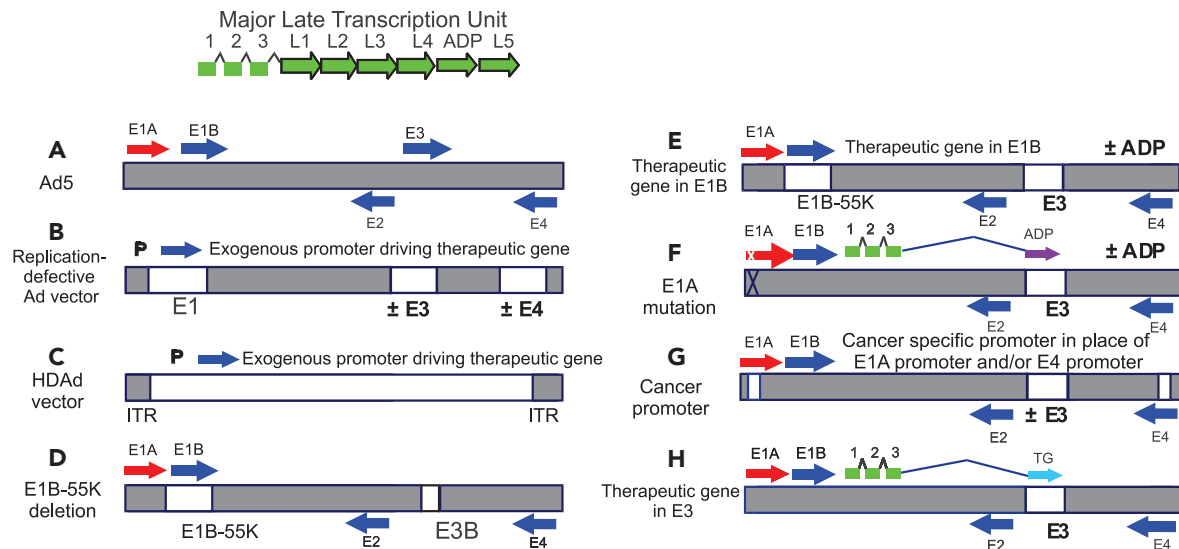


FIGURE 56.10. Schematic of the genome of adenovirus type 5 (Ad5) and Ad5-based vectors. **A:** Ad5. The horizontal bar indicates the duplex DNA genome of 36 kbp encoding ca. 35 genes. The arrows indicate transcription units. The “immediate early” E1A proteins derived from the E1A transcription unit (red arrow) induce expression of the “delayed early” proteins coded by the E1B, E2, E3, and E4 transcription units (blue arrows). Viral DNA begins to replicate at about 7 hours postinfection (p.i.), and then “late” proteins derived from the major late transcription unit (green arrows) are synthesized. The major late messenger RNAs (mRNAs) are formed by alternative splicing and polyadenylation of a large pre-mRNA initiated at the single major late promoter and extending to the right end of the genome. All late mRNAs have a tripartite leader (leaders 1, 2, and 3) at their 5' termini that facilitates translation. Beginning at 20 to 24 hours p.i., virions begin to assemble in the cell nucleus, and then after 2 to 3 days the cells begin to lyse and release virions, with lysis complete by about 5 to 6 days. Efficient cell lysis is mediated by adenovirus death protein (ADP). ADP is a late protein derived from the major late transcription unit. The vectors depicted are based on the Ad5 backbone. However, vectors based on serotypes other than Ad5 have also been developed. The reason for this is that Ad5 uses the coxsackie-adenovirus receptor (CAR) receptor, whereas some other serotypes use different receptors (e.g., CD46 and sialic acid as receptors), and sometimes this is an advantage. Also, pre-existing antibody levels in humans are more prevalent for Ad5 than for certain other serotypes. **B:** Replication-defective vectors. The E1A and E1B regions (transcription units) (i.e., the E1 region) are deleted and replaced with an expression cassette with an exogenous promoter driving expression of the therapeutic gene. In gene therapy vectors, this gene can be one to correct a genetic defect. In vaccine vectors, the gene is the antigen. In cancer gene therapy vectors, the gene (e.g., *p53*) induces cell death. Usually the E3 region is deleted. Deletion of E3 does not affect the growth of the vector because the E3 genes are not required for Ad growth in cell culture. Also, deletion of E3 allows for larger inserts into the E1 region because only up to 105% of the genome can be packaged into virions. E1-deleted vectors are defective for replication because the E1A proteins, and in some cells the E1B proteins, are required for virus growth. The vectors are propagated in cell lines such as 293 or PER.C6, which retain and express the E1A and E1B proteins. Although the E1A proteins are required for expression of other Ad genes, these vectors can be leaky and express other Ad genes in an E1A-independent manner, especially at long periods following infection and if high multiplicities of infection are used. In animal model studies, this leakiness has led to elimination of vector-transduced cells by cells of the immune system. For this reason, second-generation vectors also lack the E4 region. E4 regions are essential for Ad replication, including late gene expression, so deletions of E4 eliminate the leakiness. Some vectors also lack the E2 region. These E4- and E2-deleted vectors must be grown on cell lines that complement the E1, E4, and E2 deletions. **C:** Helper-dependent adenovirus vectors. These vectors lack all or most Ad genes but retain the *cis*-acting sequences such as the inverted terminal repeats (ITRs) at each end of the genome as well as the packaging sequence at the left end that are required for the genome to replicate and be packaged. These vectors are propagated in the presence of a helper Ad, which must be eliminated from the large-scale vector stocks. It is difficult to completely eliminate the vector. **D–H:** Replication-competent vectors. These vectors are mostly used for cancer gene therapy. The schematic in **D** depicts the vector ONYX-015, the first replication-competent (oncolytic) Ad vector to be described. It lacks the *E1B-55K* gene as well as the *rid* and *14.7k* genes in the E3B region. Because of the *E1B-55K* deletion, this vector grows better in cancer cells than in noncancerous cells. However, in many cancer cells, the vector does not grow as well as Ad5. Many other oncolytic Ad vectors have a similar design (e.g., H101), and as shown in **E**, by incorporating therapeutic genes into the *E1B-55K* gene deletion, and in some cases expressing the ADP from the E3 region (purple arrow). ADP enhances the spread of the vector from cell to cell. As shown in **F**, some vectors are targeted to cancer cells by virtue of deletions in the *e1a* gene that knock out binding of the E1A proteins to the pRB family of tumor suppressors, and also the p300/CBP proteins. Some vectors also express ADP from an alternatively spliced mRNA derived from the major late promoter. As shown in **G**, many vectors are targeted to cancer cells versus noncancerous cells by replacement of the E1A promoter/enhancer by a cancer-specific promoter. These vectors grow in cancer cells in which the promoter is active. Many different promoters have been used. In some vectors, the E4 promoter is replaced by a cancer-specific promoter. In **H**, a therapeutic foreign gene is expressed from the E3 region (TG, light blue arrow). The foreign gene can have anticancer properties (e.g., granulocyte-macrophage colony-stimulating factor [GM-CSF]); in some vaccine vectors, the foreign gene codes for an antigen.

ITR), as well as sites for recombination with the rest of the viral DNA to reconstitute adenovirions.^{58,291} These vectors are grown on cell lines that stably express the necessary E1A and E1B proteins. The best known such cell line is HEK293, which was generated by immortalizing primary human kidney cells with sheared Ad5 DNA, has the E1A and E1B regions integrated into cellular DNA,²³⁹ and is now known to have a neuronal lineage.⁶⁴⁹ Unfortunately, this cell line contains Ad DNA extending beyond the 3' end of the *E1* genes; at a very low frequency homologous recombination occurs between the Ad DNA in HEK293 cells and the DNA in E1-deleted vectors, thus generating contaminating RC Ads in RD vector stocks. Therefore, other complementing cell lines that do not carry any Ad sequences that overlap with the vector DNA were constructed, for example, PER.C6¹⁹² and N52.E6.⁶²²

The *in vivo* use of first-generation Ad vectors for gene replacement therapy is limited because of issues with immunity.^{76,77,631,633} Ads induce both humoral and cell-mediated immune responses^{137,505} (see Fig. 56.6) that result in toxicity, decrease the time that the transgene is expressed, and interfere with the effective readministration of the vector.⁵⁰⁴ The immunity seen with Ad vectors, which typically are applied in very high doses ($\sim 10^9$ to 10^{12} virus particles [vp] per kg), is probably different from what occurs in natural infections with much less virus.⁶⁵⁰ One problem with first-generation Ad vectors is the leakiness of expression of other Ad genes,⁶⁵⁸ especially those for the immunogenic virion proteins.⁸⁰⁰ Although E1A proteins are required to induce efficient transcription of other Ad genes, these genes can be expressed in an E1A-independent manner if high multiplicities of infection are used and if the infection is extended for several days. Thus, the first-generation

Ad vectors were altered further by deleting the E4 region and/or the E2 regions and then growing the vector on complementing cell lines that provide the E2 and/or E4 proteins.²³⁸ Such second-generation vectors have considerable promise as vaccine vectors.^{587,545}

A promising type of vector is the so-called helper-dependent Ad vectors (HDAds) (also referred to as gutless, gutted, or high capacity), which contain only the necessary *cis*-acting elements, namely, the two origins of DNA replication at each end of the genome and the packaging sequences from within the first 500 base pairs of the left-hand end (Fig. 56.10C)^{490,554} (reviewed in^{76,77,631}). These vectors, besides lacking any immunogenic Ad genes expressed by the vector, have a high cloning capacity (~ 37 kb), making it possible to insert whole loci, minigenes, and multiple transgenes. The HDAd vector genome is maintained as an episome in the nucleus in transduced cells; shortly after transduction, the Ad DNA packaging protein VIII is replaced by cellular histones and the genome assumes a transcriptionally active chromatin-like configuration.^{608,609} HDAd vectors cannot efficiently package genomes smaller than about 75% of the wild-type Ad genome, so “stuffer” DNA (eukaryotic DNA is superior to prokaryotic DNA) is used to make up for the required genome size.

HDAd vectors require a complementing helper Ad that provides all the required early and late gene products for the assembly of progeny virus. In a commonly employed method for HDAd production, the producing cell line for HDAd vectors conditionally expresses Cre recombinase, which, when activated, excises the loxP site–flanked packaging signal from the helper Ad's genome, thus ensuring preferential packaging of the HDAd DNA.^{260,553} (Fig. 56.11). Using this method and

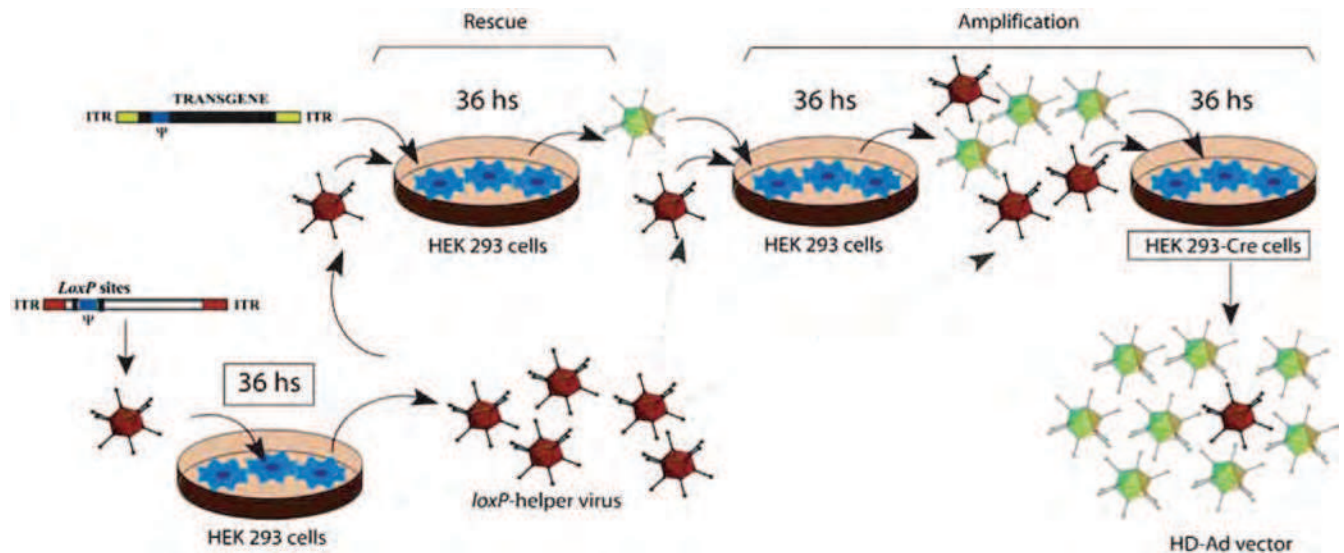


FIGURE 56.11. Helper-dependent vector production and helper virus removal strategies. Generation of helper-dependent adenovirus (HDAd) vectors starts with the rescue of the HDAd DNA construct to its viral form. This is most commonly achieved by transfection of a complementing producer cell line (i.e., HEK293) with linear HDAd DNA followed by infection with a previously prepared helper virus stock (red virions). After cytopathic effect is evident, typically 36 to 48 hours postinfection, HDAd virions (green) are collected from the crude lysate and used along with helper virus supplementation (dashed gray arrows) for vector amplification. Using the Cre/loxP recombination-based approach, helper virus removal is achieved by using a Cre recombinase–expressing cell line capable of cutting out the helper genome packaging domain (Ψ) flanked by loxP sites, making it unpackageable. (From Segura MM, Alba R, Bosch A, et al. Advances in helper-dependent adenoviral vector research. *Curr Gene Ther* 2008;8:222–235, with permission.)

novel Cre-expressing 293 cells adapted for growth in suspension and a helper virus resistant to mutation, high yields of HDAd could be produced and helper virus contamination reduced to low levels (0.01% to ~1%).⁵⁴⁶ Another method for HDAd production has flt sites flanking the Ad packaging signal; excision of the packaging signal is mediated by FLP recombinase stably expressed in a complementing cell line.^{519,736}

For laboratory purposes, the HDAd is separated from the helper Ad based on their different buoyant densities in cesium chloride density gradients. Amplification of the HDAd vector and a gradual decrease in helper contamination requires multiple serial rounds of co-infection and purification of the HDAd away from the helper. For industrial or academic scale-up, infected cell lysates are concentrated by ultrafiltration and the virions are purified by anion exchange (typically) chromatography followed by polishing using size exclusion chromatography.^{161,549,631} Two phase I/II clinical trials have been conducted with HDAd vectors.^{684,779}

HDAd vectors are less toxic and transgene expression is longer than seen with first- or second-generation vectors.^{77,78,499,548,631,633,777} Transgene expression continued for more than 2 years in one study with HDAd vectors in baboons⁷⁶ and up to 964 days in another study in baboons when a balloon occlusion catheter was used to deliver the vector to the liver.⁷⁹

The innate immune response to Ad capsid proteins (see Fig. 56.6) begins within minutes of intravenous administration, peaks at about 6 hours, is lethal when a high dose is used (e.g., $>10^{13}$ virus particles/kg in large animal models^{489,531}), and varies with the human Ad vector species.²⁰ Ad vectors induce biphasic production of pro-inflammatory mediators, including the cytokines TNF- α , IL-6, IL-1 β , IFN- γ , and IL-12 and the chemokines MIP-2, IP-10, RANTES, MIP-1 α , MIP-1 β , and MCP-1.⁴⁹⁹ These molecules are produced mainly by dendritic cells and macrophages including Kupffer cells. This ability of the Ad capsid to induce the innate immune system rapidly was illustrated by the tragic death of a patient who, after intravenous treatment with a large dose of first-generation Ad gene therapy vector expressing ornithine transcarbamylase, had a massive elevation of inflammatory cytokines and disseminated intravascular coagulation resulting in multiple organ failure.^{580,581} This death is a singular event; one review states that 16,000 patients have been treated with Ad vectors of various types with a good safety profile.⁵⁶³

The infection events and the innate response to systemically administered Ad5-based vectors in rodents (mostly mice) are very complex and include multiple components (reviewed by⁶⁵⁰) (see Fig. 56.6). The vectors interact with red blood cells,^{89,522,634} platelets,⁶⁸⁶ neutrophils,¹²⁸ macrophages in the spleen,¹⁵⁸ and Kupffer cells in the liver (see later). Liver is the main organ infected by intravenously administered Ad5 and Ad5-based vectors, even though the Ad5 receptor CAR is in tight junctions in the liver and presumably unavailable to adenovirions.¹³² Interestingly, Ad delivered into mice systemically does not infect hepatocytes directly using CAR, but rather by binding to blood coagulation factors VII, IX, and X and protein C, all of which could mediate liver infection through heparin sulfate proteoglycans on hepatocytes.⁵⁵² More recent studies indicate that factor X is the most important factor in hepatocyte transduction (see Fig. 56.5).^{551,757,758} Factor X binds directly with high affinity to Ad5 trimeric hexon through hypervariable region 5^{7,9,352,757} and more importantly

hypervariable region 7.⁹ The Gla domain in factor X binds to those hypervariable regions in hexon, and a cluster of basic amino acids in the serine protease domain provides a “bridge” to heparin sulfate proteoglycans on hepatocytes^{127,757} through the sulfated side chains on the proteoglycans.⁷¹ Many but not all serotypes in species A, B, C, and D bind factor X; the serotypes that do not bind are in species D.⁷⁵⁷ Factor X-mediated liver transduction occurs independently of Kupffer cells,⁷ and it occurs not only in rodents but also in nonhuman primates (*Microcebus murinus*).⁸

In addition to blood coagulation factors, adenovirions interact with proteins (C4, C4BP) in the classical and alternative complement pathways, leading to complement activation, infiltration of leukocytes, platelet aggregation, and induction of pro-inflammatory chemokines and cytokines.^{119,339,371,637,638,639,640,713} Antibodies to Ad can opsonize the virions, resulting in Fc-mediated uptake and secretion of cytokines by activated macrophages.^{747,811} Further, platelet-activating factor generated from the reticuloendothelial system helps generate a syndrome resembling septic toxic shock.⁷⁹⁷

In Ad vector-transduced lymphoid cells, the pattern recognition receptors are sensors activated by virion components leading to activation of NF- κ B and interferon regulatory factors and transcription of type I interferon and cytokine and chemokine genes.⁶⁵⁰ Toll-like receptor 2 (TLR2) is activated by the vector capsid at the plasma membrane, and TLR9 senses the genome in endosomes.^{21,96,819} MyD88 (myeloid differentiation primary response gene 88) is required for signaling from all TLRs except TLR3.²⁶⁷ The sensing of intracellular Ad DNA is important in the innate response, and both TLR-dependent and TLR-independent pathways are involved. One study in murine plasmacytoid dendritic cells concluded that Ad DNA is recognized by TLR9 in endosomes.^{42,819} Other studies in murine conventional dendritic cells and primary macrophages found that Ad DNA is sensed through TLR-independent cytosolic components.^{194,526,527,819} In a study in both murine and human macrophages, internalized Ad DNA induced caspase-1 processing of pro-IL-1 β to IL-1 β via NLRP3/NALP3 and ASC; the latter are components of the innate cytosolic complex termed the *inflammasome*, which functions to detect “dangerous DNA”.⁴⁹⁸ This study⁴⁹⁸ did not reveal a role for TLR9, but another study in human primary macrophages concluded that TLR9 sensing of endosomal membranes containing Ad DNA led to activation of the NLRP3/NALP3 inflammasome and IL-1 β release; these results were not observed in murine macrophages.³⁵ Another study concluded, based on intravenous injection of RD Ad into mice as well as *in vitro* studies, that the vector, interacting with macrophage β 3 integrins via the RGD motif on the penton protein, and acting independently of TLR9 or the NLRP3/NALP3 inflammasome, triggers a predominantly IL-1 α -IL-1 receptor 1 inflammatory response (Fig. 56.12).^{158,507} In other studies in murine cells, the TLR/MyD88 pathway appears to cooperate independently but additively with the NOD2 (nucleotide-binding and oligomerization domain) signaling pathway in the innate response to HDAd vectors.⁶⁹⁵ The responses are speculated to occur in activated macrophages or hepatocytes and may involve sensing of vector DNA.⁶⁹⁵ Type I interferon induced by these signaling pathways in dendritic cells and macrophages activates NK cells to eliminate vector-transduced cells in the liver.^{820,821} In mice injected intramuscularly with HDAd vectors, multiple TLR/MyD88

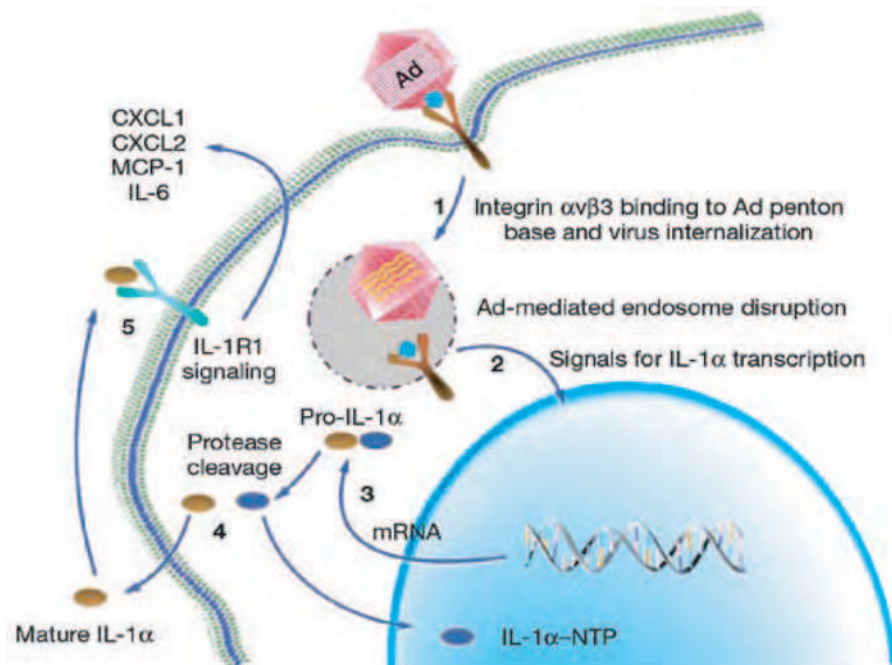


FIGURE 56.12. Schematic diagram of adenovirus (Ad)-induced cytokine production in splenic macrophages as proposed by Di Paolo et al.¹⁵⁸ (1) Ad penton base interaction with integrin $\alpha v \beta 3$ promotes virus internalization into early endosomes. (2) Partial disassembly of the virion in the low-pH environment of the endosome allows release of the viral membrane lytic protein that disrupts the lipid bilayer. (3) Membrane disruption acts as a signal for increased transcription of pro-interleukin-1 α messenger RNA (pro-IL-1 α mRNA) as well as increased cytokine protein production. (4) Proteolytic cleavage of pro-IL-1 α by neutral proteases results in nuclear localization of the N-terminal (NTP) fragment and secretion of the mature IL-1 α protein. (5) Binding of IL-1 α to its receptor (IL-1R1) induces further signaling that produces a defined set of cytokines and chemokines. MCP-1, monocyte chemoattractant protein-1; mRNA, messenger RNA. (From Nemerow GR. A new link between virus cell entry and inflammation: adenovirus interaction with integrins induces specific proinflammatory responses. *Mol Ther* 2009;17:1490–1491. Copyright 2011, with permission from Elsevier.)

signaling pathways contribute to the development of vector-specific CD8⁺ T cells.⁵⁸⁸ Certain differences among these various reports will need further resolution.

After intravenous injection of an Ad vector, Kupffer cells take up ~90% of the vector, dying in the process, but playing a major role in the inflammatory response.^{431,788} Kupffer cells take up Ad vectors via scavenger receptors^{255,798}; the hypervariable regions of hexon appear to be involved in this vector uptake, independently of the binding of these regions to factor X.^{7,368} Although both Ad5 and Ad6 are closely related in species C, Ad6 has lower affinity for Kupffer cells (and hepatocytes) than Ad5, and swapping the Ad5 hexon hypervariable region of Ad5 with that of Ad6 reduced Kupffer cell uptake and liver infection.³⁶⁸ Serotypes in species B and D also have low affinity for Kupffer cells and liver infection.⁶⁴⁷ To increase the specificity of Ad delivery to select cell types (to avoid getting the vector into the Kupffer cells) and to address the difficulties of transducing some cells that lack CAR, some investigators have swapped the fiber of Ad5 to fiber of other serotypes.^{391,479,680,780}

The other aspect of antiviral immunity that limits the *in vivo* use of Ad5 as a gene therapy vector is the high prevalence of neutralizing antibodies against Ad5 in the population. To circumvent this obstacle, researchers have developed vectors based on other serotypes^{37,301} and nonhuman Ads.³¹ In one study, all seven hypervariable regions of the Ad5 hexon were exchanged with the corresponding regions of Ad48, a rare

serotype.⁵⁹¹ The vectors were mostly resistant to neutralizing antibodies against Ad5.

Other workers are developing chemical shielding techniques for Ad vectors. A common method is to covalently link the uncharged hydrophilic polymer polyethylene glycol (PEG) to virus particles via free amine groups on the capsid proteins.^{39,393,481,537,775,789} Although PEGylation can reduce infection/transduction efficiency, it can have significant benefits. PEGylation protects the virus against pre-existing neutralizing antibodies and Ad-specific cellular immunity; it reduces (a) the development of neutralizing antibodies,^{133,543} (b) innate immune responses^{134,481} including the activation of complement,^{139,713} (c) uptake of vector by Kupffer cells,⁴⁸¹ and (d) binding of the vector to erythrocytes and platelets.²⁹⁷ The size of the PEG moiety attached to the virion is important. Large PEG moieties (e.g., of 20,000 [20K] molecular weight) detarget the liver in mice as compared to PEG molecules of ~5K.^{162,298,790} Interestingly, an oncolytic Ad conjugated with 20K PEG retained its ability to suppress tumor growth in mouse models, and with reduced liver transduction.¹⁶²

Regarding the clinical trial use of first- or second-generation RD Ad vectors for gene therapy, among the first examples are delivery of the cystic fibrosis transmembrane transporter⁶⁰⁷ and dystrophin.⁵⁷⁶ Other examples are ornithine transcarbamylase; blood clotting factors VII, VIII, and IX; aquaporin; and bilirubin uridine 5'-diphospho (UDP) glucuronosyltransferase

(reviewed in⁸⁰³). Growth factors have been expressed in Ad vectors to promote the growth of new vessels to replace occluded ones.²⁴⁹ Ad vectors have been used to prevent the overgrowth of the arterial wall during the healing phase after angioplasty is performed to open blocked cardiac arteries.^{651,721} The goal of some ongoing trials is to transduce angiogenesis-promoting genes (vascular endothelial growth factor, fibroblast growth factor, hypoxia-induced factor α , platelet-derived growth factor) into vascular epithelia at sites of limited blood perfusion such as damaged heart muscle or the limbs of diabetic patients.

First- and second-generation Ad vectors are excellent for use as vaccines because they generate both a humoral and strong T-cell response to the transgene, tending to a T helper cell 1 (Th1) response (reviewed in⁴⁰⁴). The most extensive use of an Ad vector for vaccine purposes in humans has been in a series of clinical trials for immunization against HIV-1 (reviewed in^{81,165,214,242}).^{24,116,136,245,263,332,372,520,559,572} In these various trials, the vaccine was well tolerated, with repeat injections (intramuscular) as high as 10^{11} vp per injection. Among the first reports was the so-called STEP study, a phase IIb, double-blind, randomized, placebo-controlled, test-of-concept trial, using a mixture of three Ad5-based E1-deleted vectors expressing HIV-1 *gag*, *pol*, or *nef*, manufactured by Merck Research Laboratories.⁸¹ The trial was stopped early (in September 2007) because the vaccine did not reduce risk of HIV-1 acquisition or viral load after infection, and there appeared to be a higher number of HIV-1 infections in the vaccine group than in the placebo group.

This unexpected result raised concern in the HIV vaccination field.^{165,242} One hypothesis is that the Ad5 vector activated and expanded memory Ad5-specific CD4+ (and CD8+) T cells that became hosts for HIV-1 infection. Such cells could contact HIV-1, especially if they homed to mucosal surfaces.⁵¹ Arguing against this hypothesis, individuals without detectable Ad5-specific neutralizing antibodies at baseline nevertheless have Ad5-specific CD4+ (and CD8+) T cells, as do Ad5 seropositive individuals, and the CD4+ T cells expand similarly following vaccination with the vector.¹⁶⁵ Another hypothesis to explain the STEP trial results, supported by *in vitro* data,^{562,572} speculates that pre-existing Ad5 neutralizing antibodies can form complexes with the vaccine vector to promote infection of CD4+ T cells with HIV.¹⁶⁵ Whatever the explanation for the STEP trial results, the issues raised are of great interest in the use of Ad vectors for vaccine purposes and for gene therapy in general.

With Ad vectors to combat cancer, the vector nearly always has been injected directly into tumors, as reviewed in.⁸⁰³ Some vectors express immunomodulators to augment antitumor immunity (e.g., IFN- α , IFN- β , IFN- γ , IL-2, IL-7, IL-12, granulocyte-macrophage colony-stimulating factor [GM-CSF], CD40 ligand, and B7.1). Other vectors express tumor antigens (e.g., MART-1, gp100, L523s, pp65, α -fetoprotein, GA733-2, MUC-1, and prostate-specific antigen). Still other vectors express endostatin to inhibit tumor angiogenesis, a dominant-negative version of the epidermal growth factor receptor, somatotropin, the sodium iodide symporter, cell cycle inhibitors (pRB, p21, and p16), or pro-apoptotic proteins such as TNF- α , Melanoma differentiation-associated-7 (MDA-7)/IL-24, TRAIL, or p53.

Another approach is to have the vector express pro-drug-converting enzymes such as herpes simplex virus (HSV) TK, which converts ganciclovir to the monophosphate form; CD, which converts 5-fluorocytosine to 5-fluorouracil; or bacterial

nitroreductase, which converts the pro-drug CB1954 into a strong alkylating agent. One TK-expressing vector, named sitimagene caradenovec, has been evaluated in phase I/II and III clinical trials for operable primary high-grade malignant glioma.⁷⁴² The European Medicines Agency (EMA) concluded that the data did not provide sufficient evidence of clinical benefit and therefore they rejected marketing authorization.^{477,742} Other Ad TK-expressing vectors have been tested in clinical trials for hepatocellular carcinoma⁴²⁵ and prostate cancer.⁶⁶⁰

Another Ad5-based RD vector, named CTL-102, which expresses bacterial nitroreductase, was examined in a phase I/II clinical trial for localized prostate cancer.⁵⁵⁵ Prodrug CB1954 plus vector doses of 5×10^{10} to 1×10^{12} vp showed minimal toxicity and suggestive evidence for antitumor efficacy.

MDA-7/IL-24, a member of the IL-10 family, is a secreted cytokine that is considered to be a tumor suppressor that, when expressed intracellularly, can cause cell cycle arrest or death of a variety of cancer cells.¹⁵² In a phase I clinical trial conducted on patients with advanced melanomas and carcinomas, an RD Ad5-based vector expressing MDA-7/IL-24 was considered to be safe and to demonstrate a significant clinical response rate.^{152,171} Ad5-based vectors expressing interferon- β have been tested in clinical trials for mesothelioma⁶⁸³ and malignant glioma.¹¹²

The most clinical data, by far, has been obtained with two Ad5-based RD vectors, named Advexin and Gendicine, that express the transcription factor and tumor suppressor protein p53 from the E1 region of the vector (see Fig. 56.10B). Most cancers have nonfunctional mutations in the *p53* gene or are defective in the mechanisms that control p53 levels or p53-mediated functions. Expression of p53 causes death or inhibition of proliferation of vector-infected cells, most likely due to induction by p53 of genes involved in apoptosis, cell cycle arrest, and DNA repair and direct interaction of p53 with antiapoptotic proteins in the mitochondria.

More than a dozen clinical trials have been conducted with Advexin for cancers including head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer, colorectal cancer, hepatocellular carcinoma, glioma, prostate cancer, breast cancer, ovary cancer, and bladder cancer.^{324,513,610,635} Several phase I and II trials were conducted on patient groups with recurrent or refractory HNSCC, with Advexin intratumoral monotherapy, in perioperative or postoperative settings, and in various dosing regimes up to 2.5×10^{11} vp/dose, directly into tumors (reviewed in^{315,513}). These studies indicated that Advexin is safe and had minor durable antitumor activity. Two phase III trials were conducted, one that compared Advexin monotherapy to methotrexate in advanced recurrent HNSCC.^{509,513} Patients with a favorable p53 biomarker profile (no p53 or with wild-type p53) had an increase in survival of 7.2 months versus 2.7 months for patients with an unfavorable p53 profile (mutant p53 that could be a dominant-negative inhibitor of p53 expressed by Advexin) ($p < .0001$).⁵⁰⁹

Advexin is similar to Gendicine (reviewed by^{447,560,653}), which was approved in 2003 by the State Food and Drug Administration of China (SFDA) for the intratumoral treatment of HNSCC in combination with chemotherapy. Gendicine is the first gene therapy product to be approved anywhere for commercial use. Publications describing some of the HNSCC clinical trial results with Gendicine have appeared in the Chinese literature. One review describes that Gendicine is

quite safe (the side effects mentioned are similar to those with Advexin discussed earlier) and has significant anticancer activity including synergistic effects with radiotherapy and chemotherapy.⁵⁶⁰ More recent reviews state that more than 7,000 patients have been treated with Gendicine⁴⁴⁷ and describe additional clinical studies with Gendicine for a variety of cancers.^{315,447,653} Typically, 1×10^{12} vp have been administered intratumorally, with repeated administration.

Replication-competent Ad vectors are also being explored as drugs to treat cancer (reviewed in^{38,172,230,393,398,563,577,653,687,688,728,799,805}). Oncolytic Ad vectors kill cancer cells as part of the natural virus life cycle, so following replication the virions are released from the lysed tumor cell to infect other cells in the tumor. Because Ads rely in part on the host machinery for DNA replication, cells with a deregulated cell cycle (such as cancers) are more permissive than quiescent cells⁷³⁸ or normal human cells.²³¹ Nevertheless, many oncolytic Ad vectors incorporate additional features that restrict their replication, such as tumor-specific promoters driving expression of the E1A or E4 regions of Ad (see Fig. 56.10G). Vectors of this type have been evaluated in phase I clinical trials for prostate cancer, with encouraging results.^{154,596,668,804}

The first oncolytic Ad vector to be proposed for the treatment of cancer in humans was ONYX-015.⁶¹ This vector is actually the Ad5 E1B-55K deletion mutant *dl* 1520 (see Fig. 56.10D).³⁴ The E1B-55K protein binds to and neutralizes p53, a step postulated to be required for Ad to replicate. Because normal cells express p53, it was thought in theory that ONYX-015 should not replicate in these cells. Most cancer cells have defects in the p53 signal transduction pathway, so it was proposed that ONYX-015 should replicate in those cells. However, E1B-55K is a multifunctional protein and is required for Ad mRNA export from the nucleus. It now appears that the lack of E1B-55K-mediated export of Ad mRNA is the reason that ONYX-015 does not grow well in noncancerous cells.⁵³⁵ Cancer cells complement this defect in mRNA export, allowing the vector to replicate better in cancer cells than normal cells,⁵³⁵ although replication is reduced in many cancer cells compared to Ad5.

ONYX-015 has been evaluated in more than 15 phase I and II clinical trials for several different types of cancer,^{151,220,254,275,315,370,488,495,510,511,512,514,584,585,698,749} and more than 300 patients have been treated.⁴³² The initial focus of these trials was HNSCC.^{220,370,510,512} In these and later trials, up to 2×10^{11} vp were repeatedly administered intratumorally, either as monotherapy or in combination with chemotherapy. There was evidence of vector replication in tumors but not normal tissue. Tumor response rates were modest, and toxicity was limited and included injection site pain and transient fever.

Interesting results were obtained when ONYX-015 was evaluated in trials for refractory colorectal metastases to the liver. In a phase I/II clinical study of 35 metastatic colorectal cancer patients that had failed prior treatment with 5-fluorouracil/leucovorin,^{585,698} the vector was administered by hepatic artery infusion over multiple cycles in most patients in combination with 5-fluorouracil/leucovorin. No dose-limiting toxicities were observed, and the median survival of patients with stable disease or partial responses was 19 months versus 5 months in other patients. An acute enlargement of tumor masses was observed after infusion with vector followed by regression of these masses in 7 of the 11 patients.^{585,698} This tumor enlargement suggests that there was an inflammatory response to the vector and that

that response, perhaps a “cytokine storm,” may have contributed to the regression of the tumors.⁵⁸⁵

A number of other vectors similar to ONYX-015 that lack the *E1B-55K* gene are in evaluation in clinical trials. One such vector, named H101 (Oncorine), was evaluated in phase I, II, and III clinical trials in China.^{315,442,447,563,653,793,805,806} Significant antitumor responses were observed with relatively little toxicity, and no serious adverse events were reported. The H101 vector was approved in China in 2005 for treatment of HNSCC in combination with chemotherapy.³³⁸ This is the first oncolytic vector of any kind approved for commercial use, including viruses other than Ad. A report published in 2009 stated that more than 600 patients had received intratumoral injection with H101 in clinical trials.⁴²³

Another type of *E1B-55K*-deleted vector has reached clinical trials for prostate cancer (reviewed by²¹⁰). One vector, named Ad5-CD/TKrep, which expresses a CD-TK fusion protein from the deleted E1B region (see Fig. 56.10E), has proved to be safe and effective for the treatment of prostate cancer (injection into the prostate tumor, 10^{10} to 10^{12} vp per injection) when used in combination with radiation therapy and 5-fluorocytosine plus valganciclovir²¹¹ or 5-fluorocytosine plus ganciclovir.²⁰⁹ These compounds are converted by the CD-TK enzyme into metabolites that inhibit DNA synthesis in the infected and neighboring cells, and they sensitize cells to radiotherapy. A new version of this vector expresses an improved yeast CD-TK fusion protein from the E1B region and an Ad protein named adenovirus death protein (ADP) from the E3 region.⁴⁰ ADP, an 11.6-kD integral membrane glycoprotein, mediates the efficient lysis of cells at the culmination of infection, allowing Ad to be released from cells.^{717,718} High expression of ADP from oncolytic Ad vectors causes the vector to spread more rapidly from cell to cell^{163,164} and to spread in tumors.⁴⁰ Altogether, 79 prostate cancer patients have been treated with these vectors without serious side effects and with an improvement in survival relative to historical controls.²¹⁰

A commonly employed group of vectors has a deletion in *conserved region 2* (CR2) of the E1A protein, the region that binds to and inactivates members of the pRB family.^{215,280} This deletion permits efficient replication of the vector in cancer cells with a disrupted pRB pathway, and it reduces replication in normal cells. One such CR2 deletion, so-called delta24 (Δ or D24), has been built into a variety of *infectivity-enhanced* vectors.^{563,799} The issue addressed with such enhanced vectors is that many tumors (e.g., of the ovary and bladder) have low levels of CAR, the primary receptor for Ad5 and Ad5-based vectors. Therefore, researchers have incorporated an RGD-containing peptide into the HI loop of the Ad5 fiber knob; this allows the vector to infect cells via the $\alpha_v\beta$ integrins that are expressed abundantly on many cancer cells.^{391,696} Another approach is to construct vectors with a chimeric fiber consisting of the shaft of Ad5 fiber but the knob of Ad3 fiber.³⁹² The receptor for Ad3, desmoglein 2,⁷⁷⁰ appears to be expressed well on many cancer cells.⁷³² Several clinical trials and clinical studies have been conducted recently with the infectivity-enhanced and/or D24 vectors. The various vectors are named Ad5- Δ 24-RGD,³⁷⁶ Ad5/3-Cox2L-D24,⁵⁶⁴ and ICOVIR-7.⁵²⁸ In all these trials, the authors concluded that the vector was well tolerated and that evidence was seen for vector replication and anticancer activity.

Certain oncolytic Ad vectors as well as this class of infectivity-enhanced and/or D24 vectors have been “armed” by expression of GM-CSF from the E3 region of the vector.

GM-CSF is a secreted cytokine that stimulates growth and differentiation of myelomonocytic lineage cells, especially dendritic cells (DCs). One concept is that the GM-CSF will attract DC precursors to the vector-infected tumor and stimulate them to differentiate into DCs that will take up and process tumor antigens and migrate to local lymph nodes and induce antitumor responses. This enhanced antitumor response might be sufficient to overcome the generally immunosuppressive environment of tumors. Oncolytic vaccinia virus and herpesvirus vectors expressing GM-CSF have been evaluated in advanced clinical trials with excellent results.^{74,722} Clinical studies with oncolytic Ad vectors expressing GM-CSF are at an earlier stage^{94,95,104,384}; in general, the vectors appear to be well tolerated, with evidence of induction of antitumor immunity and of clinical benefit.

Stability and Inactivation of Adenoviruses

Being nonenveloped viruses with a well-organized capsid and a double-stranded DNA genome, Ads are expected to be stable. Ads survive for long periods in liquid or on surfaces in a desiccated state.²³⁷ Ads in simulated conjunctival samples can be shipped cross-country at ambient temperatures without losing titer.⁶⁰⁰ There are recent studies addressing methods to inactivate Ads. One study examined 21 germicides for their ability to inactivate Ad8.⁶¹⁷ Based on their results, these authors recommend disinfecting ophthalmologic equipment with 70% ethyl alcohol or ~5,000 parts per million chlorine. High-level disinfectants such as 0.55% *ortho*-phthalaldehyde, 2.4% or higher glutaraldehyde, or 0.2% peracetic acid may also be used, but equipment must be compatible and rinsed thoroughly. Environmental surfaces may be rinsed with ~1,900 parts per million chlorine, 65% ethanol with 0.63% quaternary ammonium compound, or 79% ethanol with 0.1% quaternary ammonium compound. Two disinfectants recommended by the Centers for Disease Control and Prevention and Association for Professionals in Infection Control and Epidemiology for elimination of Ad8 from ophthalmic instruments, 70% isopropyl alcohol and 3% hydrogen peroxide, were ineffective in this study.⁶¹⁷

A number of studies have employed ultraviolet light to inactivate Ad. One report described cross-linking Ad DNA with amotosalen-HCl and long-wavelength ultraviolet light,⁴³³ especially for enteric Ads that may contaminate water supplies. Enteric Ads can be inactivated, but they are more resistant to ultraviolet light than most other water-borne viruses.^{379,532,712} Ad40 can also be inactivated by ozone.⁷¹¹

There is very little information on the levels of Ads in the environment. It is well recognized, though, that Ads are stable in surface and ground water for a prolonged period of time.⁵⁹⁰ In one novel study, van Heerden et al⁷⁴⁰ used PCR, DNA sequencing of the PCR product, and real-time PCR to detect, type, and quantitate Ads in drinking water and river water in South Africa. Ads were detected in 5.5% (10 of 188) and 22.2% (10 of 45) of different samples of drinking and river water, respectively, at a level of less than one copy per liter of water. These are minimum numbers because the Ads were isolated from the water by adsorption onto glass wool, a process that is unlikely to be 100% efficient. Species D predominated in the drinking water and, perhaps not surprisingly, enteric Ads were found most frequently (70%) in the river water. In an earlier study by these authors using culture methods to detect Ad, Ad was detected in 30% (59/198) of drinking water samples, 16% (8/50) of dam water,

and 44% (22/50) of river water samples.⁷⁴¹ In swimming pool water that conformed to accepted specifications for treatment, Ad was found in 11% to 21% of samples.⁷³⁹

Several new techniques are currently under development or approved outside the United States to inactivate a wide range of pathogens in blood and blood products. Most of these systems appear to successfully inactivate Ad if present in the blood. The INTERCEPT Blood System utilizes amotosalen and 3 J/cm² long-wavelength ultraviolet light and has been shown to result in a 5-log reduction in titers in plasma.⁶⁶⁶ Another system using the frangible anchor linker effector (FRALE) compound S-303 also appeared to result in a 5-log reduction of Ad titers in whole blood.⁴⁹⁴ Other systems in development combine riboflavin (vitamin B₂) and long-wavelength ultraviolet light and appear to significantly reduce the titer of adenovirus present in blood.⁸⁵

PERSPECTIVES

Much has been learned from studying Ad replication and pathogenesis for the past ~60 years. Recently, we have gained a greater appreciation of the serious complications that arise from Ads in immunosuppressed patients. The future should see continued development of drugs as well as T-cell therapy to treat these infections. We have come to understand much about the innate and adaptive response to Ads in mice, and the challenge is to learn more about Ad-induced immunity in humans.

Another key challenge is to understand the molecular basis for Ad pathogenesis including differences that exist among the serotypes and species. No doubt further work on the molecular biology of Ads and the interaction of Ads with their receptor on cells in culture and especially in tissues will be instructive. Research on Ad vectors in animal models and in clinical trials will be necessary.

HDA vectors will probably be employed more frequently in gene therapy clinical trials. HDA and first- and second-generation vectors will continue to be developed as vaccines, in particular applying lessons learned from the Ad5-vector HIV-1 vaccine clinical trials.

Both RD (Gendicine) and RC (H101) vectors have been approved as drugs to treat cancer in China. Other vectors are making their way through trials elsewhere in the world, and it seems likely that one or more of these will reach commercialization. These vectors hold much promise, especially because they can be effective against tumors that are resistant to other treatments. Hopefully, information gleaned from the widespread use of Gendicine and H101 in China will facilitate the development of vectors elsewhere in the world. These and other studies hopefully will lead to an understanding of exactly which cells in which tissues are initially infected with the different serotypes, whether the lytic infection as described in cell culture studies occurs in these cells, how the virus spreads from cell to cell, and how the innate and adaptive immune response limits the infection.

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INTRODUCTION AND HISTORY

Parvoviruses are small, nonenveloped viruses with a linear, single-strand DNA (ssDNA) genome of about 5,000 bases. The family *Parvoviridae* (Table 57.1) contains two subfamilies: *Parvovirinae* and *Densovirinae*. The latter infect invertebrates and will not be described in detail in this chapter. (The interested reader is referred to the latest report of the International Committee on Taxonomy of Viruses [234]). The *Parvovirinae* are divided into five genera: *Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus*, and *Parvovirus*. The adeno-associated viruses (AAVs) among the dependoviruses require co-infection with a

helper virus for productive infection, whereas the others are all autonomous, although they require the host cell to go through S phase for viral DNA replication but do not induce cell division.²³⁴ The AAV helper virus is most commonly an adenovirus, or less frequently a herpesvirus.

In the first half of the 20th century, feline diseases characterized by enteritis, panleukopenia, and cerebellar ataxia were recognized as being of viral origin. In the late 1940s, a similar disease was noted in mink; in 1952, it was determined that those diseases were caused by similar small, DNA-containing viruses.^{399,431} Parvoviruses were isolated from rats (Kilham rat virus),²³³ and the H-1 virus isolated from human tumor tissue infected hamsters.⁴³⁰ Between 1960 and 1962, small particles were observed within Adenovirus preparations by electron microscopy, and the smaller virus (AAV) depended on co-infection of the cells with adenovirus for replication^{24,196,335} or on herpesviruses.⁶⁸ The human parvovirus B19 was identified in 1974 during screening of blood, and the name derives from the code for that sample. The B19 virus is clearly associated with transient aplastic crisis (TAC) in patients with sickle cell disease, as well as with the childhood fifth disease and with postinfection arthralgia. In 1978, a new virus of dogs (canine parvovirus [CPV]) caused myocarditis of neonatal puppies and enteritis in older dogs, and CPV proved to be a variant of the long-known feline panleukopenia virus (FPV).³⁴⁰ Over the years, many different parvoviruses have been isolated from animals and cell cultures, and by discovery of viral DNA using direct cloning and sequencing approaches.^{223,224,395} Many of the latter viruses grow poorly or not at all in cultured cells, or are not clearly associated with disease in animals.

BIOLOGY OF THE PARVOVIRUSES

The parvoviruses have small (~25 nm diameter) and structurally stable capsids that bind host cell receptors, small ssDNA genomes with few genes that control their interactions with their hosts, and they replicate only in dividing cells or in the presence of a helper virus. Parvoviruses are widespread, and the presence of integrated viral DNA in the genomes of many different vertebrates and invertebrates indicates that related viruses have likely been infecting animals for millions of years.^{41,222,226,267} The capsids transmit between hosts by routes that include fecal–oral, urine, and respiratory spread, and they are stable in the environment and are readily transmitted by contaminated fomites. The routes of entry into the body likely involve infection of dividing epithelial or lymphoid cells of the upper respiratory tract, oropharynx, or intestine. The viruses are relatively simple and do not induce the host cell to proliferate or manipulate the immune responses directly through viral gene products. Where

TABLE 57.1 Classification of *Parvoviridae*

Subfamily: <i>Parvovirinae</i>	Genus: <i>Bocavirus</i>
Genus: <i>Parvovirus</i>	Members:
Members:	<i>Bovine parvovirus</i>
<i>Minute Virus of Mice</i>	<i>Canine minute virus</i>
<i>Chicken parvovirus</i>	
<i>Feline panleukopenia virus</i>	Genus: <i>Amdovirus</i>
<i>H-1 parvovirus</i>	Member:
<i>HB parvovirus</i>	<i>Aleutian mink disease virus</i>
<i>Kilham rat virus</i>	
<i>Lapine parvovirus</i>	Subfamily: <i>Densovirinae</i>
<i>Lull virus</i>	Genus: <i>Densovirus</i>
<i>Mouse parvovirus 1</i>	Members:
<i>Porcine parvovirus</i>	<i>Junonia coenia densovirus</i>
<i>RT parvovirus</i>	<i>Galleria mellonella densovirus</i>
<i>Tumor virus X</i>	
Genus: <i>Erythrovirus</i>	Genus: <i>Iteravirus</i>
Members:	Member:
<i>Human parvovirus B19</i>	<i>Bombyx mori densovirus</i>
<i>Pig-tailed macaque parvovirus</i>	
<i>Rhesus macaque parvovirus</i>	
<i>Simian parvovirus</i>	
Genus: <i>Dependovirus</i>	Genus: <i>Brevidensovirus</i>
Members:	Members:
<i>Adeno-associated virus-2</i>	<i>Aedes aegypti densovirus</i>
<i>Adeno-associated virus-1</i>	<i>Aedes albopictus densovirus</i>
<i>Adeno-associated virus-3</i>	
<i>Adeno-associated virus-4</i>	Genus: <i>Pefudenovirus</i>
<i>Adeno-associated virus-5</i>	Member:
<i>Avian adeno-associated virus</i>	<i>Periplaneta fuliginosa</i>
<i>Bovine adeno-associated virus</i>	<i>densovirus</i>
<i>Canine adeno-associated virus</i>	
<i>Duck parvovirus</i>	
<i>Equine adeno-associated virus</i>	
<i>Goose parvovirus</i>	
<i>Ovine adeno-associated virus</i>	

the mechanisms have been defined, the host and tissue tropisms are determined by the requirement for dividing cells of the autonomous parvoviruses, by host receptor binding, or by the cell-specific control of viral gene expression.

AAVs are cryptic viruses and are not clearly associated with any pathology. Natural hosts likely include most vertebrates, and the viruses are widespread and have been isolated from humans and other primates, horses, birds, cows, and sheep (see Table 57.1). Viral persistence is likely related to the ability of the DNA to integrate and establish a latent infection, and AAV sequences are found in many tissues.^{163,365} If AAV infects a healthy cell in culture in the absence of a helper virus, the viral genome does not replicate but may establish a persistent infection by chromosomal integration, and stable concatemers of AAV genomes in an extrachromosomal state are frequently found. In cell culture, the integrated AAV genome can be rescued by superinfection of the cells with a helper virus.¹⁹⁷ Extrachromosomal or integrated latency are likely functionally equivalent if the infected cell does not divide; however, in dividing cells, the nonreplicating extrachromosomal AAV DNA

would likely be diluted out. Stressing host cells allows some limited AAV replication. In cell culture, this can be achieved by exposure of the cell to genotoxic conditions that include ultraviolet irradiation, ionizing radiation, and cycloheximide.^{386,470}

There are complex two-way relationships between AAVs and their helper viruses. AAVs can inhibit the replication of adenovirus in a process that depends on the relative amounts of the two viruses and on the temporal relationship of the co-infection.^{81,82} Adenovirus inhibition likely results from AAV Rep protein inhibition of heterologous promoters, and that inhibition is also seen in co-infections of AAV and SV40, papillomavirus, and some herpesviruses.^{192,238,254,473}

TAXONOMY AND CLASSIFICATION

The Family *Parvoviridae* has unenveloped capsids of about 25 nm diameter with icosahedral symmetry composed of 60 copies of the capsid proteins. The nucleic acid is single-stranded linear DNA between 4 and 6 kb in length. The *Parvovirinae* infect vertebrates, and the *Densovirinae* infect insects and other invertebrates. The *Parvovirinae* are subdivided into five genera: *Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus*, and *Parvovirus*. The *Densovirinae* are subdivided into four genera: *Brevidensovirus*, *Densovirus*, *Iteravirus*, and *Pefudenovirus* (see Table 57.1). Genera of the *Parvovirinae* are defined by their genomic and biological properties, whereas species are defined by their DNA sequence relatedness, biological host ranges, and/or capsid antigenicity.

There is significant variation in the details of the viral genomes and gene expression. Parvoviruses may package mostly negative-strand DNA, or both DNA strands in variable proportions, and the ssDNA genome has different hairpin structures at each end. There are generally two messenger RNA (mRNA) promoters and a single polyadenylation site near the 3' end. The erythroviruses are related to the B19 human parvovirus, and mature virions contain equivalent proportions of positive and negative sense ssDNA, approximately 5 kb in size. The DNA molecules contain inverted terminal repeats (ITRs), and there is a single transcriptional promoter as well as two polyadenylation signals. Amdoviruses are related to the Aleutian mink disease virus (AMDV), whereas the bocaviruses are related to bovine parvovirus and the canine minute virus and include many viruses of humans and other hosts. Dependovirus virions package equivalent numbers of positive- or negative-strand ssDNA genomes, with ITRs of approximately 145 nucleotides—of which the first nearly 125 nucleotides form a palindromic sequence.

Structure of Capsid and General Properties

The particle has a molecular weight (MW) of 5.5 to 6.2×10^6 daltons. The buoyant density of the intact virion in cesium chloride (CsCl) is 1.39 to 1.42 g/cm³,⁴²⁴ and the sedimentation coefficient of the virion in neutral sucrose gradients is 110 to 122 . The virions are resistant to inactivation, being largely stable for 60 minutes between pH 3 and 9 and at 56°C. The viruses can be inactivated by formalin, β -propiolactone, hydroxylamine, and oxidizing agents.

The capsids have T = 1 icosahedral structures assembled from 60 copies of between 2 and 4 forms of a single structural protein (virus protein [VP]: VP1, VP2, and VP3), which encapsidate the linear, ssDNA genome⁴³⁵ (Fig. 57.1). The atomic

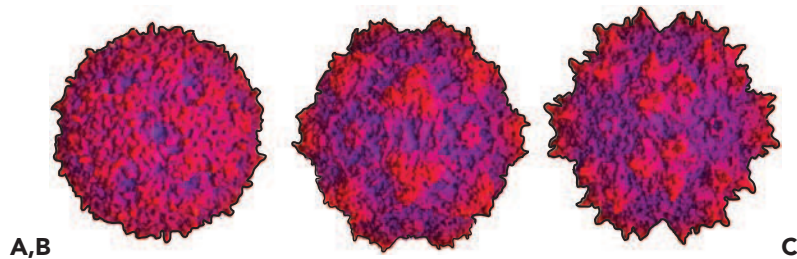


FIGURE 57.1. Surface topologies of the capsids of various members of the *Parvoviridae*, calculated from the atomic structures of the viruses. **A:** The insect-infecting densovirus *Galleria mellonella* densovirus (GmDNV). **B:** The autonomous parvovirus minute virus of mice. **C:** The dependovirus adeno-associated virus type 2. (Courtesy of Agbandje-McKenna, University of Florida.)

structures of the capsids of many parvoviruses and AAVs share a similar structure^{5,229,402,435,447,469} (see Fig. 57.1). An eight-stranded, antiparallel β -barrel makes up one-third of the sequence of the major structural protein, and large loops connecting the strands of the β -barrel make up the capsid surface and determine host and tissue interactions, bind receptors, and form the epitopes recognized by antibodies. Antiparallel β -ribbons form a cylinder about the icosahedral fivefold axes and make a channel through the capsid in some of the viruses.^{118,150,229} N-terminal sequences of some VP2 molecules are externalized through that channel, allowing them to be cleaved to VP3, and DNA packaging occurs through that channel, leaving sequences from the 5' end of the genome outside the capsid.^{118,123,350} Other features of the capsid may include one large or three smaller spike-like protrusions surrounding the icosahedral threefold axes of symmetry, depressions about the fivefold axes, and dimple-like depressions at the icosahedral twofold axes^{87,435,469} (Fig. 57.1).

Conformation-dependent neutralizing epitopes are important targets of neutralizing antibodies on the exposed surface of CPV, minute virus of mice (MVM), AAV, and B19 capsids, and the raised regions around the threefold axes are favored binding sites.^{272,381,415,460,477} Linear epitopes include the exposed N-terminus of VP2 of parvovirus full capsids and the N-terminus of VP1 for the B19 parvovirus.^{113,141,257,307,381}

Functions of the Capsid Proteins

Most autonomous parvovirus and AAV capsids assemble from combinations of three overlapping proteins—MWs of 80,000 to 86,000 (VP1), 64,000 to 75,000 (VP2), and 60,000 to 62,000 (VP3)—although AMDV, B19, and simian parvovirus assemble from only VP1 and VP2.⁴²⁴ For the autonomous viruses, VP1 and VP2 are derived from alternatively spliced viral messages, and VP3 is generated in DNA-containing capsids by proteolytic cleavage of VP2. AAVs have three coat proteins: VP1 (~87,000), VP2 (~73,000), and VP3 (~62,000). The capsid proteins are not glycosylated but are variably phosphorylated.

The unique regions of VP1 of most or all parvoviruses contain a calcium-dependent phospholipase A₂ (PLA₂) enzymatic activity that is buried within the capsid but becomes exposed during cell entry.^{151,418,480} The VP1 unique sequence is exposed on the surface of B19 capsids, which may reflect a difference in the biology of that virus.¹⁴² The PLA₂ activities of the different viruses differ significantly, with the highest activity seen for porcine parvovirus (PPV).⁷¹ The N-terminus of VP1 contains basic

amino acid motifs that likely function as nuclear localization motifs during cell entry and capsid assembly.^{199,270,444,459}

Genome Structures and Organizations

All parvoviruses of vertebrates have similar genome structures, with terminal repeats required for DNA replication, non-structural protein genes on the left half of the genome (the 3' end of the -ve strand DNA), and capsid protein genes on the right half of the genome (Fig. 57.2). Smaller proteins are produced by alternative splicing of some viruses and include the NS2 protein in the viruses similar to MVM and the NP1 protein in the bocaviruses (see later discussion). The functions of the noncoding sequences between the capsid protein and the 5' terminal hairpin of the autonomous parvoviruses are less well understood; however, in the B19 viruses, there is a short open reading frame (ORF) encoding a protein (p11), and that region may also be involved in regulating the packaging of the

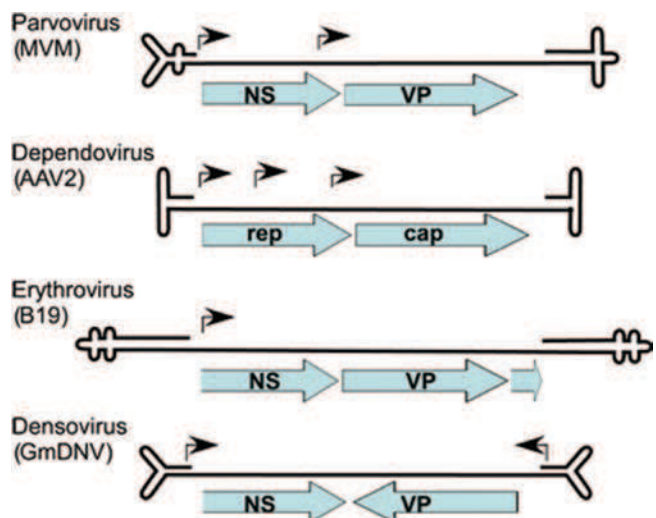


FIGURE 57.2. The genomes structures of different members of the *Parvoviridae*, showing genomes of three of the nine genera, the parvoviruses, the dependoviruses, and the erythroviruses, indicating the promoters and open reading frames of the major genes, non-structural and viral protein. The terminal hairpins are magnified approximately 20-fold relative to the intervening single-stranded sequences.

viral DNA.^{118,119} The smaller ORFs include the NS2 protein in several viruses, the 11KDa protein in the B19 human parvovirus, and a small ORF (SAT) that overlaps the VP1 and VP2 N-terminal sequence in an alternative reading frame.⁴⁸¹ Various alternative splice products have been identified for many viruses, although the predicted proteins produced have generally not been characterized.

Epidemiology and Antiviral Immunity

Many autonomous parvoviruses, including CPV, FPV, porcine parvovirus (PPV), and B19 human parvovirus, cause acute infections of their hosts that last for fewer than 10 days. The virus is cleared by the host immunity, and infectious virus is not subsequently shed.³³⁶ However, some parvoviruses show prolonged replication and persistence: Rodent parvoviruses may persist in the kidneys and be shed in the urine, whereas AMDV persists in mink and continues to replicate for the life of the animal.^{10,52,354} For B19 in humans, rare chronic persistent infections occur in individuals who are immunosuppressed, or who do not develop effective immunity.^{63,158,478}

The mechanisms of transmission among the parvoviruses vary and in many cases are not well defined. Carnivore parvoviruses such as CPV and FPV are spread by fecal–oral transmission.^{283,284,336} Some rodent parvoviruses also replicate in the intestine but may also be transmitted through urine after replication in the kidney.^{28,214} The human B19 virus replicates primarily in the bone marrow and is thought to be transmitted by respiratory routes.⁶¹

Cell-mediated immunity likely assists in recovery from infection; however, humoral immunity alone can protect animals against infection and is also important for recovery from infection, as immune immunoglobulin G (IgG) can arrest CPV replication in dogs and can terminate chronic human infections by the B19 parvovirus.^{61,63} Antibodies produced in older mink reduce titers of AMDV but do not eliminate the virus, thus a persistent infection continues.^{9,12,52}

Any role for antigenic variation in the epidemiology of the parvoviruses is not well understood. Viruses of different genera or species are distinguished by polyclonal sera; however, little antigenic variation is present within virus species, and any epidemiologic significance is not known, and the same changes often alter host range or other properties of the virus.^{85,287,310,333,338} For MVM, antigenic variants were readily selected with neutralizing monoclonal antibodies in tissue culture or in persistent infections of SCID mice,²⁷² although it is not known whether similar variation occurs in nature.

Genetic Variation and Evolution

These DNA viruses are replicated by host cell DNA polymerases; however, the single-stranded nature of the genome may make it susceptible to modification, and the mode of DNA replication may lead to significant sequence variation. The temporal rate of sequence variation is high ($>4 \times 10^{-4}$ variations per site per year) when measured over defined periods or during the growth of CPV through serial passage in tissue culture.^{25,276,393,394} MVM mutant strains grown persistently in mice or under monoclonal antibody selection showed variation at several sites owing to host or immune selection.^{272,275}

All parvoviruses are related through a distant common ancestor, and the sequences can be subdivided into several clades that show some correlations with the hosts of origin,²⁸⁰

although there are many variants. Parvovirus-related sequences are integrated into the genomes of several animals.^{41,222,226,267} Most were only partial or degenerate sequences, which would not generate infectious virus or recombine with infectious virus sequences. The finding of some syntenic sequences in related mammals shows that the integration occurred prior to the host evolutionary divergence, thus at least some of the integrations occurred millions of years ago.

Various different viruses have been seen to infect single host species. For example, within cows, three parvovirus sequences have been discovered by searching for nonhost DNAs.¹⁴ The several erythroviruses from primates (B19 and related human and simian parvoviruses) are most closely related to each other and to the chipmunk parvovirus; however, those are distinct from other human parvoviruses.^{109,198,391,475} Most viruses from rats, mice, and hamsters were found to be within the same clade along with CPV and the related viruses of carnivores and PPV,^{27,448} whereas AMDV appears to infect mink and grey foxes and is only distantly related to other parvoviruses of vertebrates. Recombination is quite common among the parvoviruses.^{280,392}

The human B19-like parvoviruses include three distinct clades that differ by 5% to 20% in sequence; within each clade, the viruses differ by less than 1% to 4%.^{72,162,198,391} Viruses from patients with persistent infections may show more variation than viruses from patients with acute infections. Many parvovirus strains have worldwide distributions, although some geographic clustering occurs.

The emergence of CPV illustrates specific features of the parvoviral evolution. The most recent common ancestor to all CPVs likely arose during the early 1970s.^{394,434} The CPV lineage included two major variants, and related viruses recovered from raccoons and other hosts suggested extensive exchange among the related hosts.¹⁶ Virus strains differed in various combinations of the two neutralizing epitopes, which were in many cases associated with capsid changes that influence receptor binding and host range.^{16,69,338,339}

Significant variation (of up to 16%) was seen in the genomes of AMDV isolates, most likely owing to the co-circulation and co-infection of animals with variant virus strains. Because that virus can form persistent infections with continuously circulating virus, mixed infections likely occur.³²² A hypervariable sequence within the capsid protein gene alters residues on the surface of the VP2 protein structure.^{51,291,321}

Infection Pathways

Parvoviruses bind to one or more cell surface receptors followed by endosomal trafficking within the cell within vesicles, release into the cytoplasm, and active or passive nuclear entry. There appear to be no substantial differences between the infections by the autonomous viruses and AAV (Fig. 57.3).

Capsid Structures and Cell Infection

The parvovirus capsid is a stable sphere of about 26 nm in diameter (see Fig. 57.1). Flexibility is required during infection to allow genome release and to expose protein structures from within the capsid. The VP1 unique region of 40 to 230 residues is not required for capsid formation or DNA packaging,^{439,444} and that sequence includes a basic amino acid motif, which likely acts as a nuclear localization sequence, and a PLA₂, which is active when released from the capsid.^{71,270,418,444,480} The

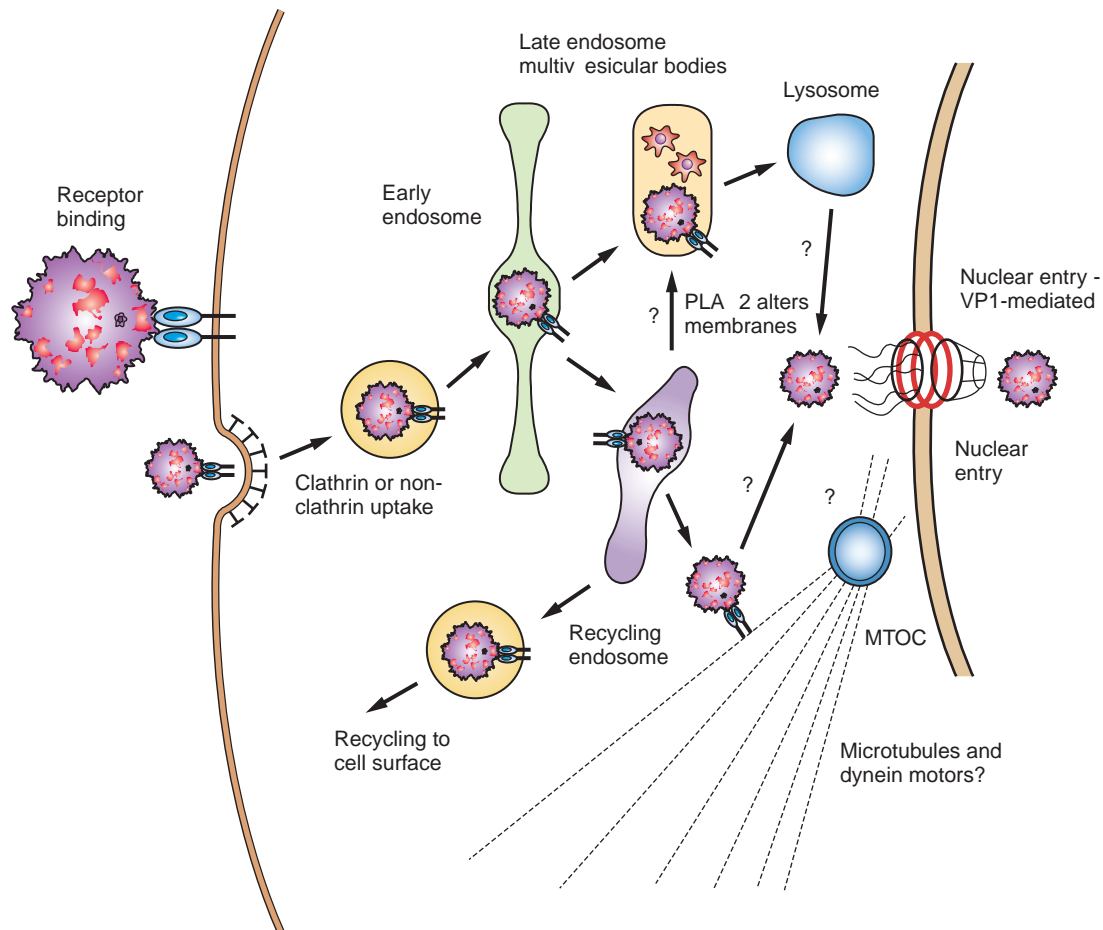


FIGURE 57.3. A general summary of the known or proposed infection pathways of viral capsids from the cell surface to the nucleus, as exemplified by the canine parvovirus binding to the transferrin receptor and being taken up by clathrin-mediated endocytosis. The later steps of the pathway, including sorting the endosomal system of the cell, and trafficking within the cytoplasm and into the nucleus may vary between viruses and are still not completely defined.

unique region of PPV showed approximately 100-fold higher PLA₂ activity than those of AAV2 or the human B19 parvovirus.⁷¹ The PLA₂ modification of the lipid components of the endosome may allow more efficient viral release.¹⁵¹

The viruses of vertebrates show various surface features, including raised regions around the threefold axis of symmetry, a depressed region spanning the twofold axis, and in many cases a pore at the fivefold axis of symmetry that runs through the capsid^{16,402,403,435} (see Fig. 57.1). The insect parvoviruses show a relatively smooth surface.^{228,402} The host ranges and tissue tropisms of CPV and FPV, and of MVM variants, are controlled by a small number of residues on the capsid surface.^{4,6,29,85,436,469}

Variability and flexibility in the capsid structure are important for viral infectivity and capsid functions—in particular, the externalization of VP1 and VP2 N-terminal sequences. Flexibility of surface loops was seen in CPV, FPV, and MVM capsids at different pH, and removal of Ca⁺⁺ or other cations from capsids altered their structures.^{114,401} For many parvoviruses and AAVs, the VP1 unique region can be exposed to the exterior after certain mild treatments, and that exposure was increased by changes of residues surrounding the fivefold axis of icosahedral symmetry.^{113,150,247,350,444}

Cell Receptor Binding

Various molecules mediate parvovirus cell binding and infection. A cellular receptor for parvovirus B19 is globoside or erythrocyte P antigen (glycosphingolipid tetrahexoseceramide), and people who genetically lack P antigen (p phenotype) are not susceptible to parvovirus B19 infection.^{57,59} The role of globoside binding is not well understood, and in some studies the capsids did not bind the globoside *in vitro*.²²⁷ Globoside is expressed on mature erythrocytes and erythroid precursors; in tissue culture, excess soluble globoside or anti-P monoclonal antibody can protect erythroid progenitors from infection. However, on other cells, it is uncertain whether the globoside is displayed on the cell surface or functions as a B19 receptor. The $\alpha 5 \beta 1$ integrin is a co-receptor for infection of erythroid progenitor cells.^{454,455}

Transferrin receptor type 1 (TfR) is used by CPV and FPV for cell infection, and receptor binding controls differences in the host ranges of those viruses.^{202,332} Capsids bind through contacts between the TfR apical domain and the capsid threefold spike.^{201,331} CPV and FPV also bind to sialic acids on some erythrocytes and host cells, although that binding does not mediate infection.^{34,37} MVM capsids bind sialic acids and

hemagglutinate mouse erythrocytes, and cell infection can be blocked by neuraminidase treatment of the cells prior to virus binding.¹¹⁶ Differences in affinity of binding result in differences in plaque size and pathogenicity in mice.^{6,273}

The AAVs can have broad cellular tropisms and infect various hosts. Infection may involve participation of more than one cell surface molecule. Receptors or co-receptors for AAV2 include heparan sulfate proteoglycan,^{231,421} human fibroblast growth factor receptor 1,³⁵⁹ and $\alpha V\beta 5$ integrin.⁴²⁰ The differences in tropisms of AAV serotypes allow matching receptors on desired target cells with specific forms of transducing virions. Retargeting AAV capsids to alternative receptors using a variety of approaches has allowed transduction of cells that are normally not susceptible.^{189,308,344,353,371,467}

Endocytosis and Endosomal Release

Parvoviruses require receptor-mediated endocytosis for cell infection. Capsids of CPV and MVM are taken up rapidly into cells by clathrin-mediated endocytosis, and the capsids become associated with clathrin-coated pits and vesicles during uptake.^{266,334} Infection is inhibited by treatment of cells with lysosomotropic agents, including ammonium chloride (NH_4Cl), chloroquine, or bafilomycin A1, indicating that low endosomal pH is required for infection.^{36,334,373,418} After uptake, capsids are detected in endosomes by antibody staining for several hours^{187,334,419,453}; by *in situ* hybridization, viral DNA was localized with the capsids in perinuclear compartments for at least several hours.⁴¹⁹ The infectious process appears to be slow, and CPV infection could be blocked by anticapsid antibodies injected into the cytoplasm of cells 4 or more hours after virus uptake.^{444,445}

Uptake and endocytosis of the AAV capsids follow similar pathways, and infection may require signaling after receptor binding.^{137,384} Infection can be affected by the activity of the proteasome system, as protease inhibitors can enhance the infection of AAV2 and AAV5 vectors.^{373,472} The capsids may be retained for long periods in the endosomal system before escaping into the cytoplasm and being transported to the nucleus.^{35,138,186}

The mechanisms of escape from endocytic vesicles into the cytosol are not fully understood, although wholesale lysis of the endosomal vesicles does not occur.^{334,418} The PLA_2 activity of the VP1 unique region is essential for infection, and that enhances the release of the capsids into the cytoplasm.^{71,142,151,171,418,480}

Transport Within the Cytoplasm

Capsids are likely released into the cytoplasm from a vesicle in a perinuclear location, and further processing and trafficking events in the cytoplasm likely occur before transport to the nucleus. Infection of cells by MVM is affected by the activity of the proteasome, because infection can be reduced by some proteasome inhibitors, including those of the chymotrypsin-like activity (N-tosyl-L-phenylalanine chloromethyl ketone and aclarubin) but not by inhibitors of the trypsin-like activity.³⁷³ Mechanisms involved in that activity are not clear; however, there is no clear evidence for ubiquitination of the capsid within the cells or direct proteolytic digestion of the capsids.³⁷⁴

Active mechanisms likely transport capsids to the vicinity of the nucleus. Transport to the perinuclear region and nuclear transport are blocked by treatment of cells with nocodazole to depolymerize microtubules and by injection into the cells of an antibody against the intermediate chain of dynein.^{417,419,445} By

electron microscopy, capsids were seen associated with tubulin and dynein structures *in vitro*, and viral capsids were also precipitated from infected cells along with intermediate chain of dynein.

Nuclear Transport

Nuclear transport is thought to involve the nuclear pore complex, although this has not been strictly proven, and injection of MVM capsids into the cytoplasm of *Xenopus* oocytes was shown to affect the structure of the nuclear envelope.¹⁰⁶ However, other studies suggest that parvovirus capsids pass through the nuclear pore intact.^{417,445} Nuclear entry may require modification of the capsid to expose nuclear localization sequences (NLS) in the VP1 unique region. A VP1 sequence can function for nuclear transport when conjugated to bovine serum albumin (BSA),⁴⁴³ which appears to be exposed on incoming virions, and antibodies to the VP1 unique region blocked infection when injected into cells before virus inoculation.⁴⁴⁴ MVM capsids have NLS in both VP1 and VP2, with two NLS mapped near the VP1 N-terminus (Fig. 57.4). A VP1 unique region that could mediate nuclear transport is required for efficient cell infection by MVM capsids, and infection was reduced significantly by mutations in the basic sequences in the VP1 N-terminal sequence.^{270,438} An internal basic sequence acts in a conformation-dependent manner when present in a trimer of VP2 proteins.^{269,270,372}

Viral DNA Release From the Capsid and Initiation of Replication

It is not clear how the viral DNA genome is released from the capsid for replication. Full capsids of MVM and CPV, and likely other viruses including AAV2, have 20 to 30 nucleotides of the 5' end of the viral genome exposed on the outside of the capsid, and the NS1 or Rep protein is covalently attached to the 5' end of that DNA in newly produced capsids.^{123,449} The 3' end of the viral DNA becomes exposed outside the capsid without capsid disintegration.^{113,114,444} In the nucleus, that extra-capsid DNA could be used to initiate DNA replication by the host cell DNA polymerase, where the DNA could be removed without disassembly of the stable capsid.

Autonomous Virus DNA Replication

The autonomous parvovirus genome is a linear ssDNA with terminal palindromes (Fig. 57.5), and many viruses have different palindromes at each end^{22,368} and primarily package the minus DNA strand.⁴²⁴ DNA replication has been most extensively studied for the rodent parvoviruses.^{23,425} Only two non-structural (NS) proteins are encoded by most parvoviruses. Replication depends on the DNA replication machinery of the cell and various cellular proteins, and the cell must pass through S phase.^{369,423,461}

The current model for autonomous rodent parvovirus DNA replication is shown in Figures 57.6 and 57.7.^{119,121} A hairpin formed by the palindrome at the 3' end of the viral genome serves as a primer for complementary strand synthesis (see step 1, Fig. 57.6). The elongating strand becomes covalently linked to the hairpin form of the 5' end of the template to form a linear duplex model covalently cross-linked at both ends by DNA hairpins (see step 2, Fig. 57.6). The hairpin formed at the right end is nicked on the newly synthesized strand by NS1

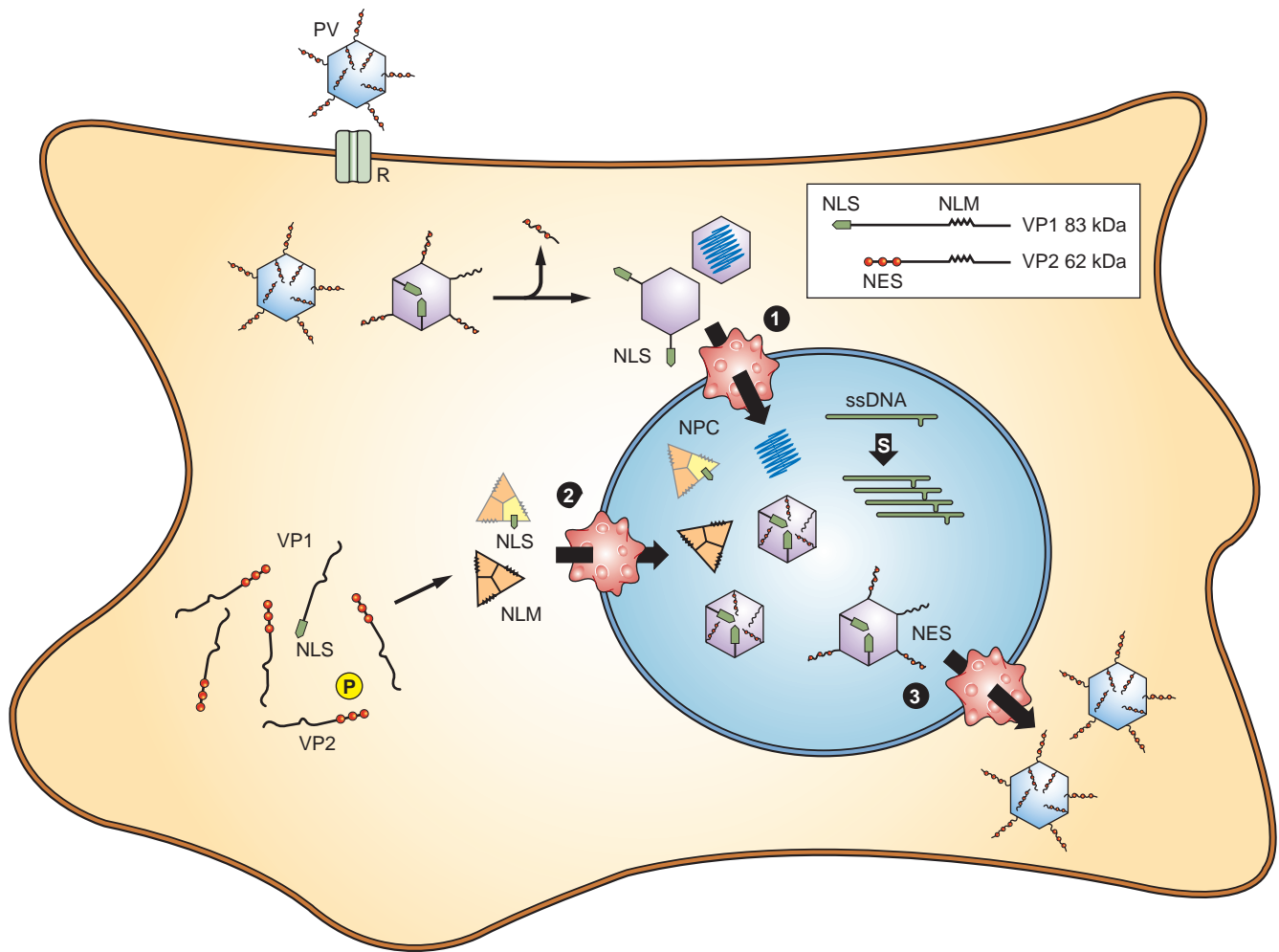
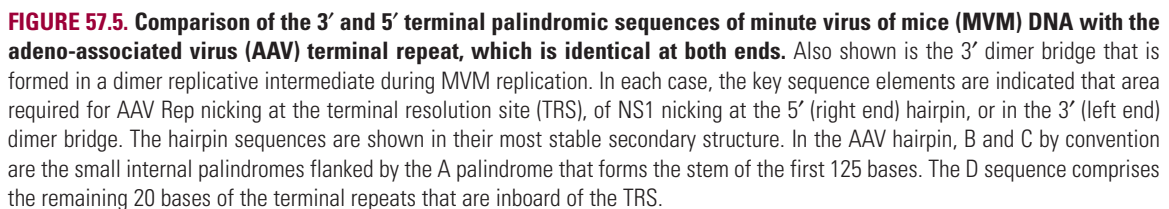


FIGURE 57.4. The nuclear transport of the parvoviral capsid proteins and capsids in the life cycle. Part (1) shows the processes include the cleavage of the VP2 N-terminal sequences containing a nuclear export sequence (NES) from the incoming particle and exposure of the N-terminal sequences of the VP1 protein containing a nuclear localization sequence (NLS). Part (2) shows association between the VP1 and phosphorylated VP2 results in translocation into the nucleus as dimers, where the particles assemble. Part (3) shows how the newly assembled particles are transported out of the nucleus and into the cytoplasm using the phosphorylated N-terminus of the VP2 protein as a NES. (Adapted from Valle N, Rioloobos L, Almendral JM. Synthesis, post-translational modification and trafficking of the parvovirus structural polypeptides. In: Kerr JR, Cotmore SF, Bloom ME, et al, eds. *Parvoviruses*. London: Hodder Arnold; 2006:291–304. Reproduced with permission of Taylor and Francis Books UK, and the authors.)

at a site 18 nucleotides downstream from the original 5' end, and NS1 is covalently linked to the 5' end formed by the nick (see step 3, Fig. 57.6). The 3' OH allows repair synthesis to fill the gap at the 3' end of the newly synthesized strand so that the right end of the intermediate is now a double-stranded form of the original 5' palindrome with NS1 covalently linked to the 5' end of the parental strand. The restored 5' end is 18 nucleotides longer than the 5' end of virion DNA (step 4, Fig. 57.6). The extended form of the right end palindromic sequence is denatured so that both strands can form hairpins (see step 5, Fig. 57.6), and the 3' OH at the end of the hairpin on the progeny strand primes DNA synthesis that extends through both strands of the linear duplex monomer so that a linear duplex dimeric structure is formed (see step 7, Fig. 57.6). The left end palindromic sequence is now in a linear double-stranded form at the center of the dimer intermediate (the dimer bridge), with

the GAA sequence from the bubble present in the left end palindrome hairpin on the left side of the dimer bridge and the GA sequence to the right (see Figs. 57.5 and 57.7), and those sequences determine the generation of virion DNA strands as outlined later.

Three sites in the dimeric intermediate are potentially susceptible to single-strand nicking by NS1: two in the covalently closed right end hairpins at each end of the intermediate and a third on the right side of the dimer bridge at GA/TC (nicked at TC; the GAA/TCC sequence on the left side of the dimer bridge is not a site for cleavage)¹²¹ (see Figs. 57.6 and 57.7). The resolution process is shown in Figure 57.7.¹²¹ Nicking at TC leaves NS1 covalently linked to the 5' side of the nick and a free 3' OH that can serve as a primer for DNA synthesis. The helicase activity of the bound NS1 unwinds the helix in the 3' to 5' direction, allowing the palindromic sequence of the dimer



cleaved by NS1 and converted to the extended duplex form, then the strands separated to provide a 3' OH to prime synthesis, which would displace a single strand containing both the plus and minus strands linked by the extended left end palindrome (Fig. 57.7). The original nick in the dimer bridge leaves NS1 covalently linked at the 5' end of the template strand for the last reaction and thus provides a stopping point for synthesis. The duplex molecule created is cross-linked at one end by the right palindrome and could go through multiple rounds of displacement synthesis to produce minus strands with the original flip orientation of the left palindrome for packaging into the virion, as seen in MVM.^{23,17} This model conforms to the observation that NS1 cannot cleave oriL when the left end palindrome is in the hairpin conformation but only when the left palindrome is in the extended duplex form of the dimer

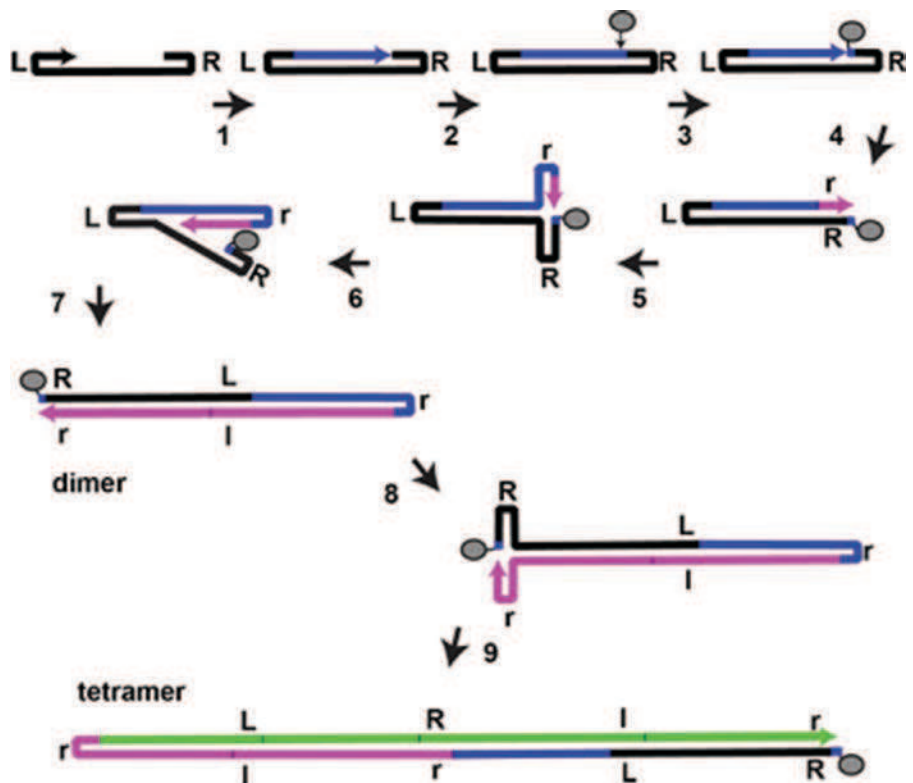


FIGURE 57.6. The replication cycle of the autonomous parvovirus DNA, based on the rolling hairpin model for minute virus of mice (MVM). The viral genome is represented by a continuous line (shaded black for the original genome and different colors for the newly synthesized DNAs); the 3' end is indicated by an arrowhead. The letters L and R represent the left-end and right-end palindromic sequences, respectively. Upper- and lowercase represent the “flip” and “flop” versions of the sequences, which are inverted complements of each other. (From Cotmore SF, Tattersall P. Parvovirus DNA replication. In: *DNA Replication in Eukaryotic Cells*. New York: Cold Spring Laboratory Press, 1996:799–813, with permission of Cold Spring Harbor Laboratory Press and the authors.)

bridge in the RF. However, NS1 can nick oriR when the right end palindrome is hairpinned.

The genomic ssDNA is generated and packaged when capsids are present. The RF structures giving ssDNA production for encapsidation are undetermined but do not appear to be simple linear duplex monomers; they are likely complex NS1/DNA complexes that are associated with capsid structures.^{119,121}

NS1 is the major parvovirus regulatory protein and is a helicase, adenosine triphosphatase (ATPase), and site-specific nickase that binds to the sequence 5'ACCA3'.^{101,111,458} When NS1 nicks, it becomes covalently attached to the 5' side of the nick through residue Y210.^{26,123,317} This site in the protein is in a motif characteristic of proteins involved in rolling circle replication (RCR motif).²⁰⁶ NS1 cleaves at a site within the extended duplex form of oriL in the dimer bridge and at a site within the hairpinned form of the right end palindrome, but not the hairpinned form of the left terminal palindrome (see Figs. 57.5–57.7). NS1 can presumably cleave the extended duplex form of oriR that would exist in a concatemer bridge with a tail-to-tail junction of monomeric units. Whether the type of asymmetry observed in the cleavage of the oriL bridge would occur has not been reported. The left end palindrome contains a short sequence that is not complementary; when extended in the dimer bridge, there is a 5'GA/TC to the right of the axis of symmetry and a 5'GAA/CTT to the left. Cleavage only occurs at the 5'GA/TC site, owing at least in part to a requirement for a cellular protein (parvovirus initiation factor [PIF]) to be part of the cleavage reaction.^{97,99} The sequence of the nucleotides in the “bubble” mismatch is not critical; however, the number of nucleotides is, owing to the need for interaction between NS1 and the cellular protein so that adenosine triphosphate (ATP) hydrolysis can occur,

allowing the double helix to be unwound and NS1 cleavage of its single-stranded substrate. Both NS1 and PIF can bind to both sides of the dimer bridge, although only the dinucleotide bubble sequence allows formation of the necessary ternary complex (see Fig. 57.5). PIF is a cellular transcriptional factor of the KDWK motif family, which functions as a heterodimer with subunits of 96 and 79 kda.¹⁰⁰

Three sequences are required for NS1 cleavage of the covalently closed hairpinned form of oriR found in the dimer RF. An NS1 binding site is near the axis of symmetry in the right end palindrome and another just outboard of the nicking site, and NS1 binding to the two sites leads to looping of the double helix of the hairpin and positions NS1 relative to the nicking site to allow the helicase to expose the site and NS1 to cleave.^{112,120} A cellular protein, either HMG1 or 2,¹²⁰ acts in concert with NS1. HMG1/2 preferentially binds to bent DNA, heterocruciforms, and Holliday junctions, and likely assists NS1 in creating the bend in the double helical hairpin to allow oriR cleavage.

Two additional sequences near the right end of the MVM genome affect DNA replication. There is a 65-nucleotide repeat from 4760 to 4850; deletion of one copy of the repeat reduces replication by 100-fold.³⁸² There is also a so-called internal replication sequence from 4489 to 4695.⁶⁷ Deletion of this sequence reduces the extent of DNA replication by 5-fold. Whether these regions function as internal oris is unknown.

Additional cellular proteins are required for MVM DNA replication, including phosphokinase C (PKC), where PKC ϵ is required for NS1 to function in RCR,^{107,316,319} and PKC λ phosphorylates NS1 at T435 and S473 so that NS1 can function as a helicase.²⁵⁵ G1 phase cell extracts do not support MVM DNA replication *in vitro*; however, the addition of cyclin A to the G1 phase extract allows DNA replication.

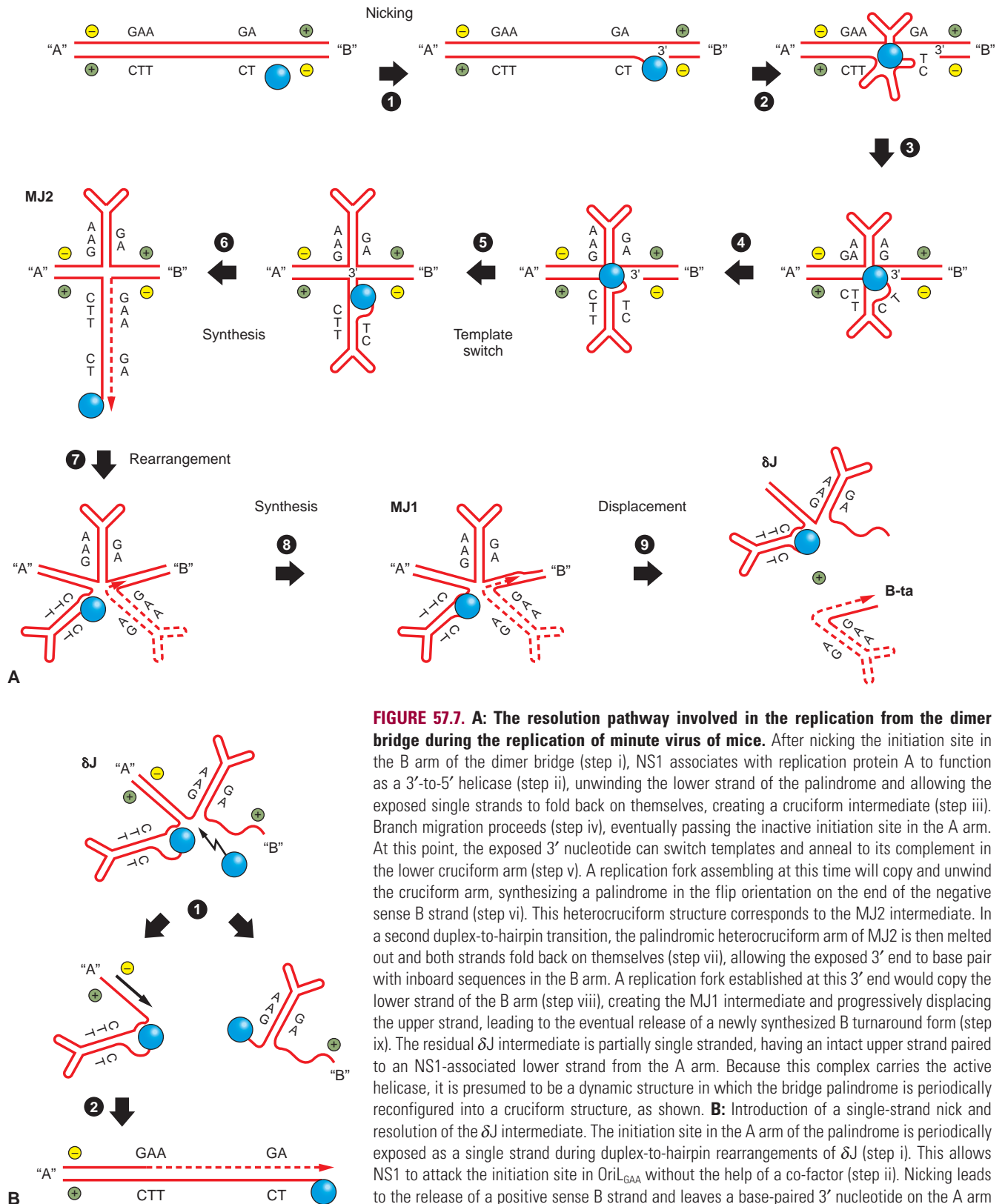
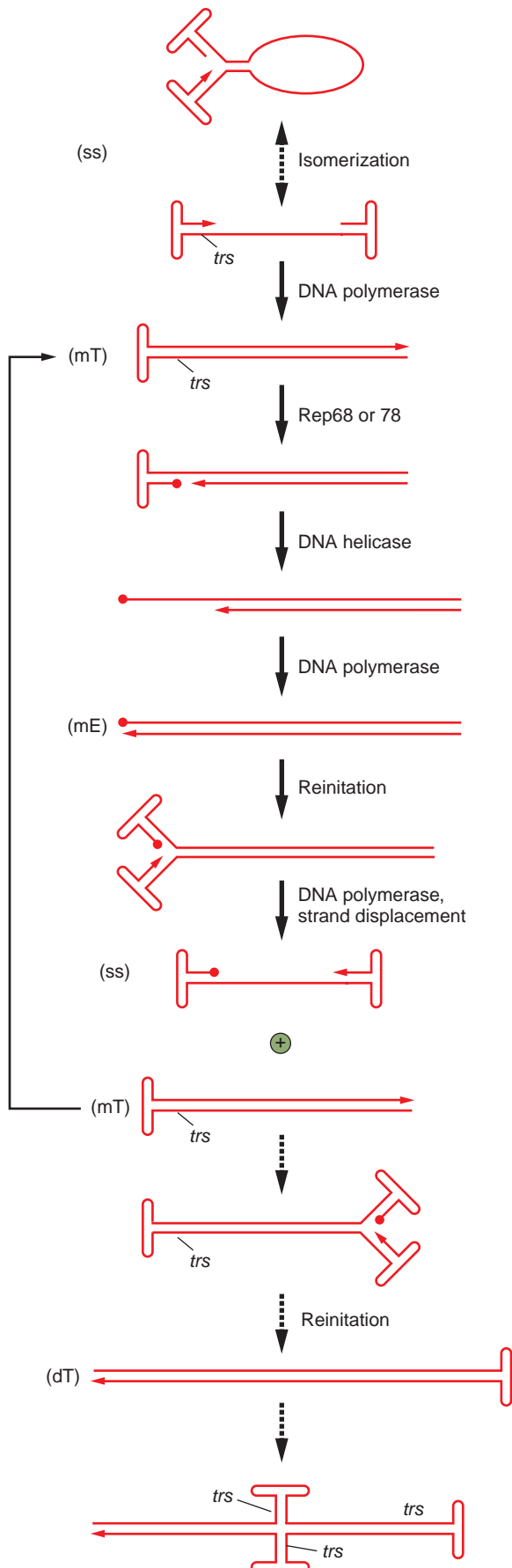


FIGURE 57.7. A: The resolution pathway involved in the replication from the dimer bridge during the replication of minute virus of mice. After nicking the initiation site in the B arm of the dimer bridge (step i), NS1 associates with replication protein A to function as a 3'-to-5' helicase (step ii), unwinding the lower strand of the palindrome and allowing the exposed single strands to fold back on themselves, creating a cruciform intermediate (step iii). Branch migration proceeds (step iv), eventually passing the inactive initiation site in the A arm. At this point, the exposed 3' nucleotide can switch templates and anneal to its complement in the lower cruciform arm (step v). A replication fork assembling at this time will copy and unwind the cruciform arm, synthesizing a palindrome in the flip orientation on the end of the negative sense B strand (step vi). This heterocruciform structure corresponds to the MJ2 intermediate. In a second duplex-to-hairpin transition, the palindromic heterocruciform arm of MJ2 is then melted out and both strands fold back on themselves (step vii), allowing the exposed 3' end to base pair with inboard sequences in the B arm. A replication fork established at this 3' end would copy the lower strand of the B arm (step viii), creating the MJ1 intermediate and progressively displacing the upper strand, leading to the eventual release of a newly synthesized B turnaround form (step ix). The residual δJ intermediate is partially single stranded, having an intact upper strand paired to an NS1-associated lower strand from the A arm. Because this complex carries the active helicase, it is presumed to be a dynamic structure in which the bridge palindrome is periodically reconfigured into a cruciform structure, as shown. **B:** Introduction of a single-strand nick and resolution of the δJ intermediate. The initiation site in the A arm of the palindrome is periodically exposed as a single strand during duplex-to-hairpin rearrangements of δJ (step i). This allows NS1 to attack the initiation site in OriL_{GAA} without the help of a co-factor (step ii). Nicking leads to the release of a positive sense B strand and leaves a base-paired 3' nucleotide on the A arm (step iii) to prime assembly of a fork that will copy the hairpin, creating an extended form of the A arm (step iv). (Adapted from Cotmore SF, Tattersall P. Resolution of parvovirus dimer junctions proceeds through a novel heterocruciform intermediate. *J Virol* 2003;77:6245–6254, with permission from the American Society of Microbiology, and the authors.)



In vivo MVM replication has been found to evoke a DNA damage response (similar to AAV); H2AX, Nbs1, RPA32, Chk2, and p53 are phosphorylated and are recruited to MVM replication centers, where they co-localize with NS1.^{3,378} Parvovirus DNA replication takes place in discrete subnuclear structures sometimes known as PARs, which contain DNA polymerases α and δ .^{38,128,205}

Adeno-Associated Virus DNA Replication

The AAV genome is 4,500 to 5,000 nucleotides long and has palindromic terminal sequences that serve as the primers for DNA replication of AAV (see Fig. 57.5). AAVs have several forms of Rep with multiple functions in DNA replication, but no DNA polymerase or ssDNA binding protein, and additional factors are supplied by either the helper virus and/or the cell. Because of the AAV ITR and the evidence for inversion of the palindromic sequences at both termini, replication can occur by single-strand displacement^{190,416} (Fig. 57.8). The 3' terminal repeats fold over to form hairpin structures²⁸¹ that serve as primers for DNA synthesis⁴¹⁶ (see Fig. 57.8). Complementary strand synthesis continues either to the end of the parental template strand, forming a linear duplex molecule cross-linked at one end by the terminal hairpin, or the growing end of the complementary strand may become covalently linked to the hairpinned form of the ITR at the 5' end of the parental strand template to form a linear duplex cross-linked at both ends. Resolution is achieved by Rep (68 or 78) binding to the hairpin (which has a Rep binding sequence [RBS], as well as a secondary binding site on one of the cross-arms [see Fig. 57.5]) and nicking the original parental strand at the terminal resolution site (TRS), which is opposite the phosphodiester bond between the 3'OH of the primer and the 5'PO₄ of the first nucleotide inserted into the progeny strand.^{54,92,290,380} Rep covalently binds to the 5'PO₄ created at the nick,^{133,207,405} and the 3'OH serves as a primer for "repair" synthesis, filling in the resultant gap at the 5' end of the parental strand using the displaced hairpin sequence as the template.²⁰⁸ Thus, the original 3' end of the parental strand is transferred to become the 5' end of the new strand, and the terminal palindromic sequence is inverted. The 3' end of the newly synthesized strand may fold over to prime synthesis of another new strand, which can use both complementary strands of the original unit length duplex as a template to produce a linear, duplex dimer-length

FIGURE 57.8. The replication cycle of the adeno-associated viral DNA. The single-stranded DNA released from the virion after uncoating (top two possible forms) is extended from the 3' end of the hairpin to form a full-length hairpin. The hairpin is nicked at the terminal recognition site by Rep68 or rep78, leaving the Rep protein attached to the end of the DNA. The hairpinned end is unwound, and the 3' end formed by Rep cleavage is extended to the end of the template strand. The ends of each strand refold into their alternative self-base pairing hairpin structures, and full-length DNA synthesis from the 3' primer on the left end of the genome produces one single-stranded genome and one duplex structure, which can each serve as a substrate for additional round of replication. mT, monomer turnaround; dT, dimer turnaround replicative form; ss, single-strand viral DNA; mE, monomer extended replicative form, TRS, terminal recognition site.

molecule.^{313,416} The structure of the initial primer strand may potentially be either a linear single strand with a hairpinned 3' end or a single-stranded circle held together by H-bonding between the ITRs (see Fig. 57.8).

The large Rep proteins introduce site-specific nicks at the TRS, and their helicase activities are necessary for the hairpin unwinding during resolution of the hairpinned termini.^{208,408,462} The endonuclease activity selectively works on the hairpin structure, but poorly, if at all, on the linear duplex form of the same sequence.^{92,290} The Rep protein binds ATP, and its ATPase activity is required to unwind the double helix, but not for the endonucleolytic activity.^{208,408,462} The site-specific DNA binding and endonuclease activities have been mapped to the N-terminal domain of the larger Rep 68/78 proteins.⁴⁰⁶ The RBS is a tandem repeat of four copies of GAGC.^{92,290,380,407,457} Only two copies of the GAGC sequence are required to bind Rep, and there is apparently wide latitude in the number of copies of the tetranucleotide that are required for Rep to function.^{93,290,462} Rep 68/78 functions as a multimeric complex that binds to the RBS in the stem of the T-shaped structure of the folded ITR and also to a second site (RBS', GTTTC) that is at the tip of the cross arm farthest removed from the TRS^{54,380} (see Fig. 57.5). The TRS consists of a seven base sequence (3'CCGGT/TG5') that is recognized only on the correct strand.⁵⁵ The functional complex of Rep is a pentamer.¹⁹³ Once Rep is bound to the hairpin, Rep unwinds the stem of the ITR in a reaction that requires ATP hydrolysis to generate a single-stranded TRS,^{55,133,407} then the endonuclease active site carries out a transesterification to link tyrosine 156 of Rep to the 5' phosphate end of the nick.^{133,208,405} Rep remains covalently bound until packaging occurs and can be detected on the surface of the viral particle.³⁵⁷

The two smaller Rep 52/40 proteins can bind ATP and have helicase activity⁴⁰⁶ and may be involved in packaging of newly synthesized DNA strands into preformed capsids.⁸⁸ Although Rep 40 does not bind specifically to RBS, it does bind to ssDNA, dependent on two lysine residues at positions 404 and 406.⁴⁷⁶ The functional distinctions between the two isoforms of both Rep 78/68 and Rep 52/40 are not well delineated.

AAV DNA replication requires both cellular and helper virus components. Adenovirus encodes a DNA polymerase; however, AAV replication appears to use the cellular DNA polymerase δ . Data with regard to use of other cellular DNA polymerases is not conclusive. Recently, Nash et al³¹² have been able to reconstitute AAV DNA replication *in vitro* by fractionating extracts from adenovirus-infected cells. Cellular factors included replication factor 3, polymerase δ , proliferating cell nuclear antigen (PCNA), and the so-called minichromosome maintenance complex, as well as the AAV rep. Herpes simplex virus (HSV) encodes a DNA polymerase, and AAV replication will use that polymerase.^{300,456} Genetic studies of the helper virus gene products required for AAV DNA replication have identified several functions. Adenovirus ssDNA binding protein greatly enhances AAV DNA replication but is not an absolute requirement.^{80,217} HSV gene products include the UL30/UL42 HSV DNA polymerase; the helicase-primase complex of UL5, UL8, and UL52; and the UL29 product ICP8, which is the HSV ssDNA binding protein.^{300,456} Cells co-infected with AAV and helper viruses show co-localization of AAV DNA replication with both the viral ssDNA binding proteins and replication protein A (RPA), the cellular ssDNA

binding protein, as well as various proteins associated with the DNA damage response.³⁹⁰ In adenovirus co-infected cells, AAV replication can be associated with PML bodies; however, this is not seen with the HSV helper. Indeed, the PML bodies are dissociated as the consequence of HSV infection. Yet even when certain HSV ICP0 mutants were used as the helper and the PML bodies were maintained, AAV replication was not associated.¹⁵⁵ Recent cytochemical studies of HSV/AAV-infected cells have shown the association of various cellular proteins with AAV DNA replication; these include not only replication factor C (RFC), PCNA, and RPA but also many factors normally associated with cellular DNA repair, including the Mre 11/rad50/Nbs1 complex, Ku70 and -86, and other mismatch repair proteins.^{313,314} Nuclear high mobility group protein 1 (HMG1) binds to cruciform DNA¹¹⁰ and hence binds to AAV hairpins and Rep and stimulates Rep nicking, ATPase activity, and repression of AAV gene expression from the P5 promoter. The 52 kDa FK 506 binding protein FKBP52 binds to the single-stranded form of the D region of the ITR, the 20 nucleotide of the ITR beyond the palindrome.^{286,360} The phosphorylated form of the protein binds and inhibits DNA replication, whereas when the nonphosphorylated form binds, synthesis can occur. Phosphorylation is controlled by the epidermal growth factor receptor tyrosine kinase and correlates with the ability of AAV vectors to transduce cells in the absence of a helper virus.

Transcription

The AAV genome contains three transcription units with promoters at map positions 5, 19, and 40^{174,282,363} (Fig. 57.9). A single, functional polyadenylation signal is at map position 96,⁴¹² thus all three transcripts cover the 3' half of the plus strand. A single intron lies just beyond mp 40; four of six AAV mRNAs have this sequence excised.²⁶⁰ The ratio of spliced to unspliced mRNA species depends on binding of Rep 78/68 to the transcription template.^{363,364,432} Four mRNAs cover all or part of the ORF in the left half of the genome. Two initiate at the P5 (mp5) promoter, one is spliced and produces Rep 68, and the other is not spliced and forms Rep 78. The P19 promoter also produces two mRNAs producing Rep 52 and Rep 40. The P40 produces two major mRNAs splice variants; the one utilizing the upstream splice acceptor encodes VP1. In the variant, the VP1 initiator codon is spliced out, an ACG initiator codon is used to translate VP2,³⁰⁶ and the first AUG in phase is used as the initiator codon for VP3. An additional protein is encoded by an alternative ORF (nucleotides 2717–3340) within the *cap* gene; an unusual initiator codon CTG is used. The 23-kd protein has been shown to promote AAV2 capsid formation within the nucleolus.⁴¹⁰ In AAV5, most transcripts originating from P7 and P19 are polyadenylated at a site in the intron.^{362,474} Polyadenylation of P7 and P19 transcripts at the proximal site is governed by an element 5' to P41.

AAV transcription depends on the intracellular milieu. In a healthy cell, AAV represses its own transcription and DNA replication; however, limited Rep expression following second-strand DNA synthesis permits the establishment of latent infection. Cell stress by heat (39°C), ultraviolet irradiation, gamma irradiation, inhibition of protein synthesis by cycloheximide, and exposure to cytosine arabinoside or hydroxyurea can also allow AAV expression.⁴⁷⁰ Adenovirus helper factors include early regions (E) 1A, 1B, 2A, and 4 (ORF 6).^{8,370} The ICP0 gene

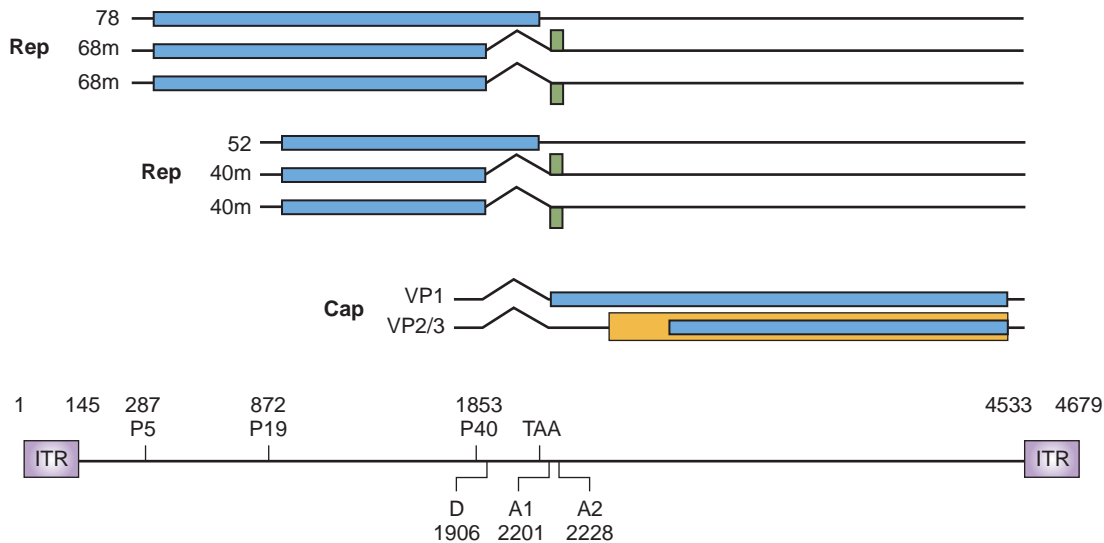


FIGURE 57.9. Transcriptional map of the adeno-associated virus type 2 (AAV2) genome, which includes three promoters (P5, P19, P40), the small intron donor (D), and acceptors (A1 and A2), the termination sites of the Rep and Cap messages. The major transcripts and the proteins they encode are shown, and the different open reading frames used are shown with different shading. (Adapted from Qiu J, Yoto Y, Tullis G, et al. Parvovirus RNA processing strategies. In: Kerr JR, Cotmore SF, Bloom ME, et al, eds. *Parvoviruses*. London: Hodder Arnold, 2006:252–273. Reproduced with permission of Taylor and Francis Books UK, and the authors.)

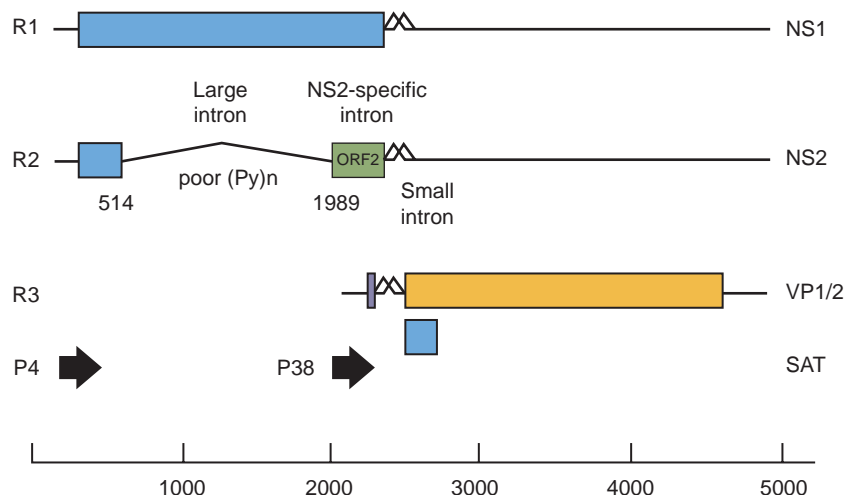
product of HSV-1 turns on AAV transcription and can activate transcription from an integrated latent viral genome.¹⁶⁷ Cellular transcriptional regulatory elements turn AAV RNA synthesis on and off, and the P5 promoter sequence contains binding sites for cellular, helper, and AAV transcriptional factors.^{253,345} Transcription is regulated during the replication cycle, and the final ratio of stable transcripts from P5, P19, and P40 are 1:3:18, respectively.

Autonomous Parvovirus Transcription

The transcriptional schemes of the autonomous parvoviruses are highly variable (Figs. 57.10 and 57.11). Many use promoters around map positions 4 and 38 to generate two transcripts, along with one polyadenylation signal near the right end of the genome.^{219,304,348} A small intron is between map positions 44

and 46, and a large intron is between map positions 10 and 39. For MVM, all messengers have the small intron spliced, although alternative splice donor and acceptor sites are used. Half of the P4 transcripts also have the larger intron spliced at alternative splice donor and splice acceptor sites, producing a total of nine mRNAs.^{115,219,304,368} Where the P38 transcript uses the upstream splice donor site, the mRNA is translated to VP1, whereas use of the downstream donor site gives VP2. The erythroviruses have one promoter at map position 6 but two polyadenylation signals: one near the middle of the genome and the other at the right end^{53,90,179,328} (see Fig. 57.11). Read-through of the internal polyA site occurs only with genome replication, and the large transcript is translated into the capsid protein.¹⁸⁰ The amdoviruses have promoters at map positions 3 and 36, and the mRNAs have complex splicing patterns.^{361,413,424} The

FIGURE 57.10. Transcriptional maps of the autonomous parvovirus minute virus of mice. The three major transcript classes (R1, R2, and R3) are shown relative to the viral genome, and the P4 and P38 promoters are indicated. The proteins encoded are shown as NS1, NS2, VP1/2, and the SAT proteins. The alternative open reading frames used are shown as different shading patterns. (Adapted from Pintel DJ, Gersappe A, Haut D, et al. Determinants that govern alternative splicing of parvovirus pre-mRNAs. *Semin Virol* 1995;6:283–290. With permission of author and Elsevier Press.)



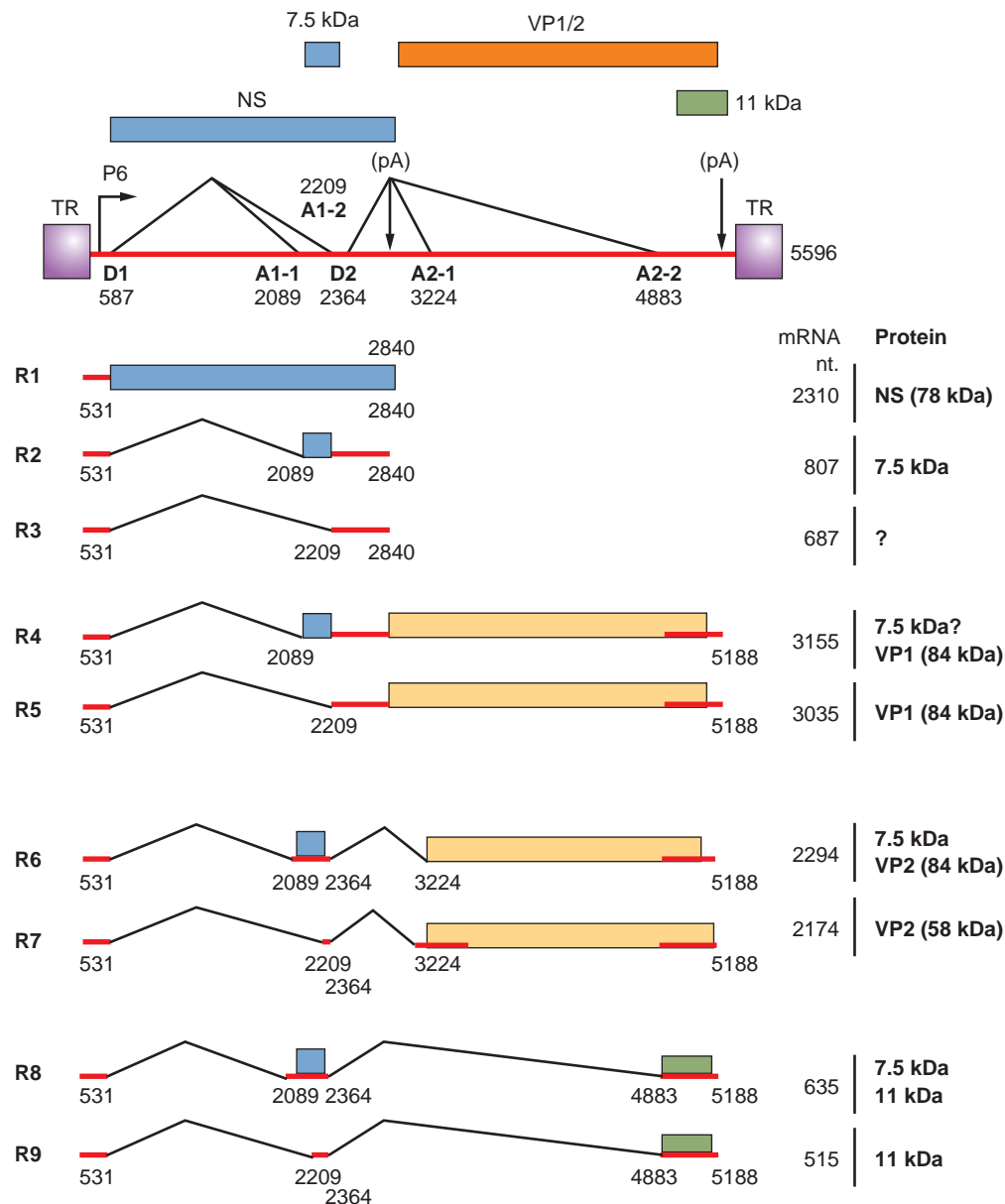


FIGURE 57.11. Transcription map of the B19 genome, showing the level of complexity of the RNAs produced by alternative splicing and polyadenylation. The main features shown include the viral promoter (P6), the various splice donors (D1 and D2) and acceptors (A1-1, A1-2, A2-1, A2-2), and the polyadenylation sites (pA). Other features shown are the open reading frames (*open* or *closed* boxes) and the products likely generated from each transcript. (Adapted from Guan W, Cheng F, Huang Q, et al. Inclusion of the central exon of parvovirus B19 precursor mRNA is determined by multiple splicing enhancers in both the exon and the downstream intron. *J Virol* 2011;85:2463–2468, with permission from the American Society of Microbiology and the authors.)

betaparvoviruses have promoters at map positions 4.5, 13, and 39; NS proteins and VPs are encoded in large ORFs in the left and right sides of the genome, and a small ORF that overlaps the 3' terminus of the NS ORF encodes NP1.^{89,424}

Regulation of transcription and posttranscriptional modification is complex. For MVM, splicing of the large intron (10–39) depends on splicing of the small intron (44–46),⁴⁸³ because the small intron assists entry of the large intron into the spliceosome. Modification of the polypyrimidine tract in the large intron relieves the requirement for initial splicing

of the small intron; however, nonsense mutations in the NS2 exon inhibit splicing of the large intron in a *cis*-active manner. NS1 transactivates P39 transcription; this activity has been localized to the C-terminal 129 amino acids.^{140,367} The three *cis*-active genomic sequences near P39 required for transactivation are a tar (transactivation response element at –139) to which NS1 binds in a reaction that is ATP dependent,^{98,277,278} an Sp1 site (–50), and the TATA box.^{7,278} NS1 can also interact with CBP, Sp1, TBP (TATA binding protein), and TFIIA in the absence of DNA.^{245,278} The promoter contains binding sites

for Sp1, CREB/ATF, E2F, and NF- κ B.^{134,135,160,346} Several regulatory mechanisms link MVM expression and replication to the cell cycle.^{134,135,346} The host range and tropism of MVM may be controlled in part at the level of P4 activation. Transgenic mice with β -galactosidase under the control of P4 showed expression in specific tissues and dependence on differentiation.¹³¹ Two MVM subtypes also differ in tissue specificity, as MVMi replicates in T lymphocytes, but not in fibroblasts, whereas MVMp has the reverse phenotype. Two amino acid sequence changes in the capsids control the phenotype and involve the control of the ratio of NS2 to NS1.⁹⁴ Mutation of a single base at the 3' splice site of the large intron enhanced the ability of MVMi to replicate in fibroblasts. NS2 interacts with the nuclear export protein Crm-1, and mutations enhancing MVMi NS2 binding to Crm-1 influenced MVMi replication in fibroblasts.^{94,275}

Protein Synthesis

Protein synthesis depends on the transcription. NS protein transcripts appear earliest in the course of infection, and one or both of NS proteins regulate gene expression.^{103,139,264} Both NS1 and NS2 are phosphorylated after translation.^{108,136,318,319} NS1 dimerizes prior to nuclear localization,^{319,320} and one interaction domain maps between amino acids 261 and 280.³⁵⁸ MVM NS2 is required for efficient virus growth in a host-dependent manner and is essential for growth in mouse cell lines and in animals.^{129,275,309,378}

Adeno-Associated Virus Proteins

The four NS proteins—Rep78, 68, 52, and 40—are named according to their apparent MWs, and protein amounts correspond to the levels of their transcripts. Rep78 and 68 are predominantly nuclear proteins, whereas Rep52 and 40 are also found in the cytoplasm.²⁹⁵ Rep52 retains helicase activity,⁴⁰⁶ whereas the Rep78/68 unique region plus a few amino acids shared with Rep52/40 are sufficient for nicking a single-stranded TRS substrate,¹³³ and a slightly smaller N-terminal region is sufficient for RBE binding.³²⁷ Rep sequences involved in terminal repeat binding have been mapped.^{132,165,288,289,440} Repression of the p5 promoter was most pronounced with Rep78, followed by Rep68 and Rep52.²⁰⁰ Transactivation of the p40 promoter was caused by either Rep78 or 68.⁴⁵²

VP1 to VP3 are translated from two alternatively spliced p40 mRNAs.³⁰⁶ The most abundant is translated to form VP2 from a nonconventional ACG codon and VP3 from a downstream AUG. VP1 is translated from the low-abundance spliced p40 message (see Fig. 57.9). All three proteins share the same ORF, and protein sequences begin with amino acid 1 of VP1, whereas VP2 begins at amino acid 138 and VP3 at 203.

Expression of the structural proteins alone leads to capsid assembly. VP2 is required for self-assembly; either VP1 or VP3 could be omitted, although VP1 and VP3 are required for infectivity.³⁷⁷ VP3, however, lacks an NLS⁴⁵⁹; if a heterologous NLS is fused to VP3, that protein can self-assemble into empty particles.⁴⁵⁹ At least one NLS has been mapped to the N-terminus of VP2¹⁹⁹; however, there appear to be redundant NLS.⁴⁶⁷ The heparan sulfate binding region of the AAV2 capsid protein has been mapped to two clusters of basic amino acids: residues 509 to 522 and 561 to 591.^{396,468} The N-terminus of VP2 and a portion of the N-terminus of VP1 are also on the capsid sur-

face. The N-terminus of VP3 and the C-terminus are internal to the capsid and essential for capsid structural integrity, and one mutant (R432A) makes only empty capsids.

Virion Assembly

Capsid proteins translated in the cytoplasm are transported to the nucleus, where they assemble³⁷² (see Fig. 57.4). The major capsid proteins can assemble alone into capsids that are structurally very similar to normal capsids,^{240,377,479} and those package viral DNA when they are expressed with replicating genome.⁴³⁸ However, capsids are only infectious when they contain the largest of the minor capsid proteins (VP1), likely because of their PLA₂ activity and nuclear localization functions.^{151,171,247,480}

DNA incorporation occurs by insertion of viral ssDNA into the preassembled capsid and requires the activity of viral larger nonstructural proteins, and the helicase activity is most likely involved in translocating the ssDNA into the capsid^{118,144,350,484} (Fig. 57.12). As described earlier, the polarity of DNA packaging of some viruses results from differences in the use of the sequences at each end of the genome for initiating replication, as when one sequence is used preferentially, and then one DNA strand is packaged more frequently. When replication initiates at both ends, the viruses package both DNA strands.¹¹⁹ After assembly, the capsids may be retained within the nucleus, translocated into the cytoplasm, or transported out of the cell in a process regulated at least in part by the phosphorylation state of the capsid proteins and the activation of gelsolin in the cytoplasm.^{33,147,270,479} (see Fig. 57.4).

Adeno-Associated Virus Latent Infection

AAV can establish a latent infection, allowing the genome to persist until conditions are favorable for the production of progeny. A cell culture model of AAV latent infection required inoculation of a fairly high multiplicity of infection in the absence of helper³⁴⁷ and then the genomes persisted for as many as hundreds of passages. The AAV genome can integrate into the cell genome; wild-type AAV2 integrated at a specific site (known as AAVS1) on the q arm of human chromosome 19 (q13.4), which contained a Rep binding site (RBS) and a terminal resolution sequence (TRS)^{203,242–244,383,441} (Fig. 57.13). Analysis of the sequences in the target showed that it included a 33-nucleotide sequence containing the RBS and the TRS separated by an 11-nucleotide spacer.^{265,296} Virus functions included the expression of Rep 78/68, which was required *in trans*, and the presence of the RBS.^{215,230,347} Viral DNA is generally integrated as a concatemer,^{91,203,293} and slightly more than a genome equivalent is required for successful rescue.¹⁷⁰ In the absence of Rep expression, integration occurs approximately randomly.²⁹³ The AAV genome can also survive for prolonged periods as an extrachromosomal element and be expressed in the absence of a Rep gene. The unusual ends of the DNA appear to allow it to resist intracellular exonucleases and may prevent DNA modification, which would turn off expression. The extrachromosomal forms of AAV DNA are concatemers that are thought to be circular.⁴⁰⁹ Many people likely harbor viral DNA sequences that can be reactivated, and the female genitourinary tract is a frequent site for AAV sequences.^{70,159} It is not clear whether the viral sequences are maintained in tissues as extrachromosomal elements, or as integrants, either at AAVS1 or at random locations. No negative

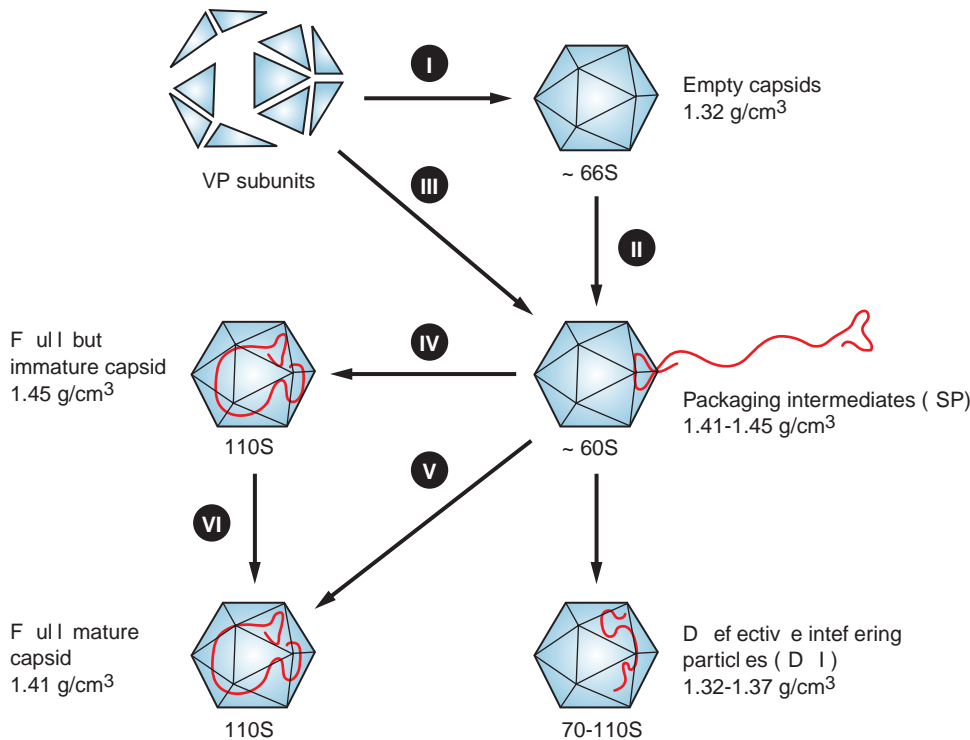


FIGURE 57.12. Possible pathways for parvovirus assembly and DNA incorporation, based on various studies of adeno-associated and autonomous viruses. Several mechanisms are possible; however, the current data proposes the assembly of capsids from viral protein subunits, possibly trimers (see Fig. 57.4), some of which remain as empty particles. The single-strand DNA is packaged during replication into the more or less intact capsids through the activity of the nonstructural or Rep protein. A final step involves the “maturation” of the capsids, which is seen as a small change in the buoyant density of the particle from 1.45 g/cm³ to 1.41 g/cm³. (Adapted from Kleinschmidt JA, King JA. Molecular interactions involved in assembling the viral particle and packaging the genome. In: Kerr JR, Cotmore SF, Bloom ME, et al, eds. *Parvoviruses*. London: Hodder Arnold, 2006:305–319. Reproduced with permission of Taylor and Francis Books UK, and the authors.)

consequences of AAV infection have been demonstrated in people in controlled studies.

PATHOGENESIS AND CONTROL OF TISSUE TROPISM

Parvoviruses appear to infect most species of vertebrates, as well as many arthropods and crustacea, and most are not associated

with disease. The major vertebrate pathogens are listed in Table 57.2. Autonomous parvoviruses require S-phase of the cell cycle for their replication, so there is an age dependence to many of the diseases seen. However, in general, only some dividing cells are targeted for infection, and the cell and tissue tropism controls the precise pathogenesis.³³⁶ Viral genes, including the capsid and NS2 sequences, govern the host range or pathogenicity of parvoviruses.^{29,50,274,337,434} There are likely many parvoviruses that cause only asymptomatic or very mild infections.^{14,15,218}

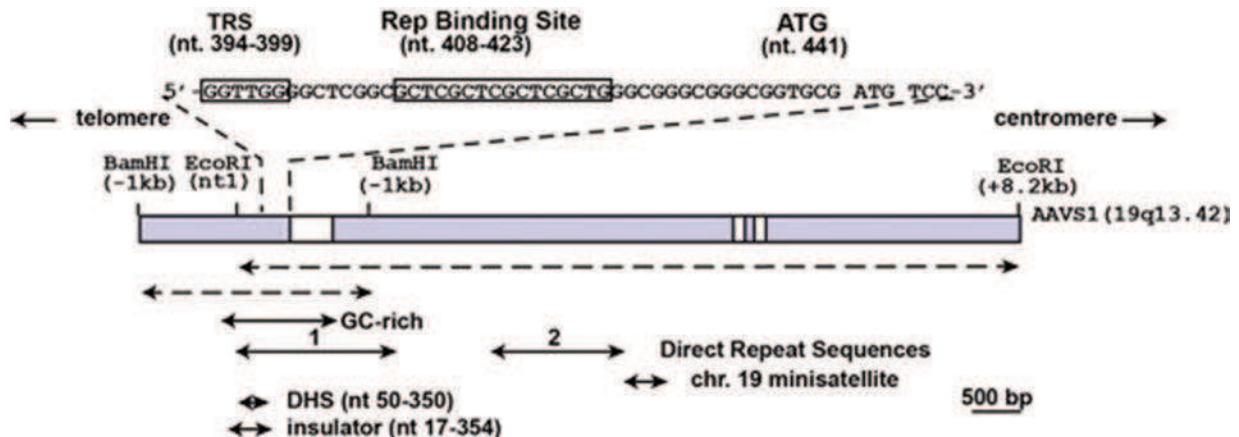


FIGURE 57.13. Schematic representation of the adeno-associated virus S1 region, the favored site of integration into the human chromosome position 19q13.42 chromosome, within the BamHI and EcoRI fragments as mapped by (REF and REF), where the EcoRI site is indicated as position 1. The sequence and position of the Rep binding site (RBS), terminal resolution site (TRS), and the MBS85 gene translation start sequence (ATG) are indicated, which allow Rep recognition and nicking. Locations of the GC-rich region, direct repeat sequence, chromosome 19 minisatellite sequence, DNase I hypersensitive site (DHS), and insulator element as indicated by arrows. (Adapted from Dutheil N, Linden RM. Site-specific integration by adeno-associated virus. In: Kerr JR, Cotmore SF, Bloom ME, et al, eds. *Parvoviruses*. London: Hodder Arnold, 2006:213–236. Reproduced with permission of Taylor and Francis Books UK, and the authors.)

TABLE 57.2 Major Parvovirus Pathogens of Vertebrates

Virus	Disease
Human parvovirus B19 and primate erythroviruses	Erythema in children, polyarthritides and arthralgia, transient aplastic crisis, fetal hydrops
Feline parvovirus (canine parvovirus and feline panleukopenia virus, mink enteritis virus)	Enteritis; generalized neonatal disease, myocarditis, ataxia following cerebellar hypoplasia
Porcine parvovirus	Fetal infection and death, abortion, infertility
Aleutian mink disease virus	Chronic immune complexes
Canine minute virus	Mild diarrhea, fetal or neonatal death
Minute virus of mice, rat virus, H1 virus of rats	Congenital fetal malformations, persistent subclinical infections
Goose parvovirus and duck parvovirus	Hepatitis and myocarditis

Tissue Specificity, Viral Entry, and Cryptic Infection

Viral tropisms may be determined by various factors. Certain MVM strains differ in cell tropism, and MVM(p) is restricted in T lymphocytes at the level of gene expression, whereas the related variant MVM(i) is permissive in those cells.⁴¹¹ The host range restriction is caused by mutations in the capsid.^{29,149,311} In other cells, transcription can occur, although DNA replication is inhibited. The narrow cell tropism of B19 virus for erythrocyte precursors is partly owing to the distribution of its receptor and to controls of gene expression.^{59,180} Some viruses show tissue-specific gene expression,²⁴⁸ and NS and capsid genes have been implicated in the PPV tissue tropism, whereas in MVM, NS2 has been implicated.^{129,147,309,376} No evidence of functional latency or genome integration of infectious autonomous parvoviral DNA has been detected during either active or abortive infections.

Fetal and Neonatal Infections

Fetal and newborn animals are highly susceptible to infection owing to the large number of dividing cells, and several parvoviruses cause fetal death or abortion. Maternal immunity completely protects the fetus, and maternal antibodies protect the newborn for some weeks after birth. Where immunity is lacking, fetal or neonatal infections can be caused by PPV,^{126,220,297} bovine parvovirus,⁴¹⁴ H-1 parvovirus,⁴²⁹ goose and duck parvovirus,⁵⁸ parvovirus B19,^{83,428} and AMDV.⁵⁶ Some infections are fatal, whereas in other cases the surviving fetus suffers severe sequelae such as congenital cerebellar ataxia by infecting cells in the developing cerebellum.^{232,430} Neonatal dogs infected with CPV may develop myocarditis.^{261,262} MVM infects renal vascular endothelial cells and lymphocytes in baby mice.^{65,214} Newborn mink infected with AMDV develop a severe respiratory distress syndrome subsequent to infection of type 2 alveolar pneumocytes, which proliferate in the neonatal period.^{12,446}

Older Animals

Parvoviral diseases in older animals result from the direct effects of virus infection on target tissues, as well as from the subsequent immune response. Direct infection of the target organ includes enteritis in CPV-infected dogs or FPV-infected cats, hepatitis in hamsters and geese, and erythrocyte aplasia in humans (B19 and related viruses) (reviewed in 52,61,122,336,478). Several disorders result from the host response to infection,^{102,109} the most dramatic being the chronic immune complex disease caused by persistent infection with AMDV, which can be worsened by vaccination.^{1,52} Certain PPV isolates induce immunologically mediated vesicular skin lesions in late-gestation swine fetuses,^{42,246} whereas the immune response likely causes the erythema seen in fifth disease and the polyarthropathy caused by B19 infection.^{464,478}

Parvovirus and AAV may show significant differences in host range or tissue tropism among closely related strains or isolates.^{50,84,86,87,130,323,337,340,434,468} Many sequences controlling these aspects of viral biology map to the viral capsids, mostly on the surface exposed regions.^{4,6,149,173,202,240,268,273,274,403} Although the capsid genes appear to be a major viral determinant of host range and pathogenicity, nonstructural gene products also play a role in some systems.^{66,129,275,309}

Human Parvoviruses

There are several human parvoviruses. The B19 and the related erythroviruses are associated with primary diseases, whereas the human parvovirus 4 (Parv4) and human bocavirus are of lower pathogenicity and are associated with diseases primarily in mixed infections with other pathogens. Parvovirus B19 infection may be subclinical. When present, disease is generally an acute, biphasic illness with a high viremia that peaks at days 8 and 9.^{19,96,351,356,464} (Fig. 57.14). Viremia is accompanied by nonspecific, influenza-like symptoms, including fever, malaise, and myalgia. Red cell aplasia coincides with viremia, leading to a drop in hemoglobin, reticulocytopenia, and modest lymphopenia and neutropenia. Appearance of specific immunoglobulin M (IgM) and IgG antibodies 10 to 14 days after inoculation is accompanied by classic fifth disease symptoms and with circulating immune complex formation.^{19,164} Acute infection with parvovirus B19 causes the childhood exanthem fifth disease (erythema infectiosum),^{21,63,96} which occurs after the viremic phase of infection and likely results from the host immune response to infection. Adults with acute B19 infection may suffer a symmetric polyarthropathy that can persist for weeks or months,^{463,464} likely resulting from the host immune response.

Parvovirus B19 is found in the nasopharynx, and transmission is probably through the upper airway,¹⁹ as well as by the transfer of contaminated blood products or tissues.^{221,349,466} The major sites of viral replication are the adult bone marrow and the fetal liver.^{251,252} Pathologic changes found in the hematopoietic tissues include giant pronormoblasts in bone marrow aspirates and fetal liver tissue sections with depletion of later erythroid precursor cells.^{17,83,249,252,329} Temporary depression of erythropoiesis results from B19 infection but is not significant in people without underlying hemolytic disorders. In people with a shortened red cell life span and reduced erythropoiesis, acute B19 infection causes TAC and a precipitous worsening of anemia.^{19,20,61,478} Predisposing conditions include sickle cell anemia, hereditary spherocytosis, enzymopathies, thalassemias,

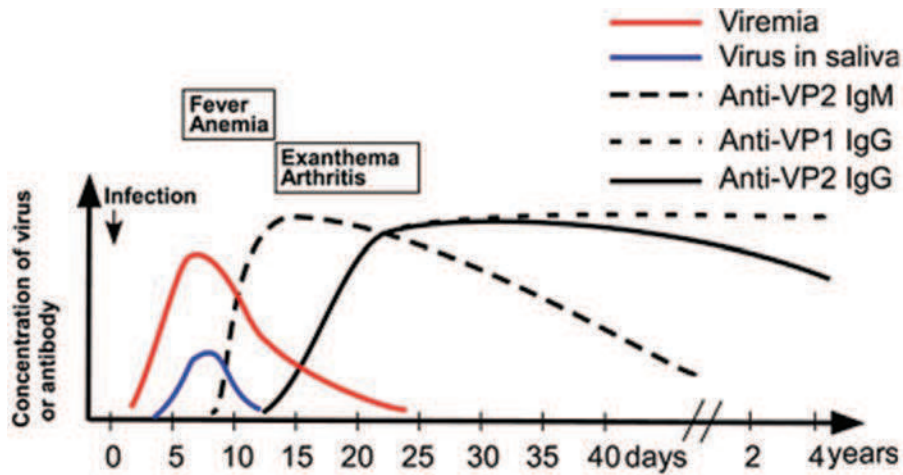


FIGURE 57.14. Pathogenesis of B19 in humans. The graph shows the changes in the amounts of the virus or the immune response to the various viral capsid components at different times in an acute human infection. (From Kerr JR, Modrow S. Human and primate erythrovirus infections and associated disease. In: Kerr JR, Cotmore SF, Bloom ME, et al, eds. *Parvoviruses*. London: Hodder Arnold, 2006:385–416, with permission. Reproduced with permission of Taylor and Francis Books UK, and the authors.)

and acquired hemolytic anemias. Resolution of the crisis begins about 7 to 10 days after onset, coincident with the appearance of antiviral antibody and viral clearance. Viral replication occurs at high levels only in erythroid progenitor cells,³³⁰ and erythroid colony formation by the erythroid colony forming unit and the erythroid burst-forming unit are strongly inhibited by infection; susceptibility of marrow cells increases with erythroid differentiation.⁴²² Productive virus propagation depends on the presence of erythropoietin,^{397,398} and susceptibility is related to the effects of erythropoietin on erythroid differentiation.^{302,303}

Fetal infection may cause nonimmune hydrops fetalis characterized by severe anemia, high-output cardiac failure, and often fetal death.^{18,148,428,465} Erythroblasts in the fetal liver are infected, and fetal myocarditis may contribute to the pathogenesis.^{17,83,355} The risk for a fatal outcome is greatest during the first two trimesters, and fetal infection may persist after birth as pure red cell aplasia (PRCA).^{40,153,465} About 30% of maternal infections are vertically transmitted, and fetal death occurs in 2% to 10% of maternal infections; there is no evidence of congenital abnormalities after fetal B19 infection.^{60,428}

Simian parvoviruses are associated with severe chronic anemia and persistent simian parvovirus infection in cynomolgus monkeys immunosuppressed by infection with the type D simian retrovirus.^{62,324,325} Related parvoviruses are found in rhesus monkeys, pig-tailed macaques, chimpanzees, and gorillas.^{175,395} Acute diseases are similar to those seen in humans, with symptoms ranging from inapparent to very mild, although without the arthropathy seen in humans.^{62,325,326} An erythrovirus-like parvovirus has been found in Manchurian chipmunks.⁴⁷⁵

Canine and Feline Parvoviruses

CPV and FPV are very closely related and cause disease primarily in young animals, with a lessening of severity as the animals age.³³⁶ The relationship between the viruses is complex; FPV is found in cats, raccoons and related hosts, and CPV is a dog-specific variant that emerged in 1978.^{340,394} CPV has since undergone variation in antigenicity, as well as host range for cats and other carnivores.^{16,433} The viruses infect through the oral-nasal route, replicate in the pharyngeal lymphoid tissues, then spread as a free or cell-associated viremia to the other lymphoid tissues.^{74,75,124,125,283,284,299} There is a marked loss of lymphocytes from many tissues (thymus, lymph nodes, spleen, Peyer's

patches). The virus spreads to the small intestine, infecting the rapidly dividing cells in the crypts of Lieberkühn, resulting in a loss of epithelial cells from the small intestine and shortened, nonabsorptive villi and subsequent diarrhea.^{73,285} Animals that survive recover normally and are protected against reinfection by the immunity they develop.

Porcine Parvovirus

Infection of older pigs mostly is mild or subclinical. However, in pregnant pigs, infection can lead to fetal infection; those infected prior to reaching immune competence (55–70 days gestation) have an extensive infection and die.^{127,220,297} After infection of the sow, the virus takes approximately 15 days to reach the fetus, and early infection can result in fetal death and resorption or mummification.^{126,127,297} After approximately 70 days of gestation, the fetuses are generally less severely infected, and the virus replicates in the lymphoid tissues.⁶⁴ PPV has also been associated more rarely with poorly understood skin vesicular disease.²⁴⁶ Combined infections of PPV and porcine circovirus type 2 may cause a severe disease termed *postweaning multisystemic wasting syndrome*, which appears to result from the immune stimulation resulting from the PPV infection.^{13,181,188,259}

Aleutian Mink Disease Virus

AMDV mostly causes disease in mink of certain susceptible genotypes but commonly infects mink, and the same or related viruses can also infect skunks, ferrets, and foxes.^{52,154,211,263} Disease is age dependent; in neonatal mink, an acute disease results from infection of type II pneumocytes and pneumonitis owing to loss of surfactant production.¹¹ In older animals, or in young animals treated with antiviral antibodies, the disease is chronic and characterized by plasmocytosis, hypergammaglobulinemia, glomerulonephritis, arteritis, focal hepatitis, and death.^{10,12} Lesions in chronically infected mink result from viral antigen/antibody complexes that are not efficiently cleared.¹⁹¹ The (Aleutian) coat color mutation is linked to a lysosomal storage disease similar to the human Chediak-Higashi disease, in which the phagocytosed immune complexes are not degraded.¹⁸² Vaccination is not effective and may enhance the disease; however, control can be achieved by testing and culling infected animals, as transmission is relatively inefficient.

Rodent Parvovirus Diseases

Many different parvoviruses infect rodents, including several that infect experimental mice and rats, as well as wild mice and other rodents.^{44,45,214,216,471} Rodent parvoviruses can develop persistent infections but are not usually associated with overt clinical disease.^{161,213} They can also infect rodent and other cells in tissue culture and be introduced into animals by transfer of those cells.^{44,292,471} The viruses may cause fetal abnormalities or death owing to their tropism for dividing cells. Control is most effectively by physical isolation and barriers, by serologic or DNA testing, and culling of the infected animals or populations, with cesarean derivation of the young into a clean environment.^{216,451}

Other Pathogenic Parvoviruses

Goose parvoviruses can infect goslings of 8 to 30 days of age can result in focal or diffuse hepatitis and widespread degeneration of striated, cardiac, and smooth muscles.^{172,236} Duck parvovirus causes a similar disease in ducks. Bovine and rabbit parvoviruses are not commonly associated with clinical disease. Minute virus of canines (canine minute virus; canine bocavirus) is widespread in dogs.^{46,389} The infection is generally subclinical; in fetuses or neonatal puppies, the main target organs are the lung and small intestine.⁷⁸ The virus has been isolated from puppies with diarrhea, although mostly in conjunction with another enteric pathogen.³⁰¹

Dependoviruses

Natural AAV infections are assumed to occur via the respiratory or gastrointestinal route. It is not clear which tissues are the preferred sites of latency or infection in humans, although some evidence suggests that muscles may be involved. The virus has not been detected in human lung samples but is recovered from a small percentage of hematopoietic cells and from the female genital tract.^{159,178} AAV has been found at higher frequency (20%) in muscle biopsies.⁴²⁶ The chromosome 19 integration site is associated with the *MBS85* gene, and integration alters that gene expression.^{145,146,152} Skeletal muscle resists adenovirus and herpes infection, thus this could be a reservoir protected from rescue by AAV helper viruses.

IMMUNE RESPONSE, KEY ANTIGENS, AND VACCINATIONS

Immunity to parvovirus infection includes both antibody and T-cell responses. Antibodies protect animals against infection by most parvoviruses and aid in recovery, as antibody therapy can resolve chronic human infections with B19 and clear canine infections by CPV.^{157,250,298,315} The immune responses in hosts that recover protect for many years. The major antigens recognized by antibodies are the conformational epitopes of the capsids.^{272,385, 2009 #15684,415,460} Antibodies to the capsids of AAV can interfere with gene therapy.^{442,450} Other structures recognized by neutralizing antibodies include the VP1 unique region of the B19 virus^{307,375} and an exposed peptide of the VP2 N-terminus of CPV.²⁵⁸ Antibody may also induce the immune complex-mediated manifestations of some infections. Relatively little is known about the T-cell responses to parvoviruses; however, these are likely important in recovery from infection.^{156,209,225,400}

Vaccines

Modified live and inactivated or subunit vaccines have been developed against parvoviruses. In many cases, they can protect against infection. A candidate recombinant vaccine has been developed for B19.^{30,31,43} Modified live and recombinant vaccines protect dogs and cats against CPV and FPV,^{77,271} and vaccines against PPV are also successfully used.^{341,342}

EPIDEMIOLOGY

Prevalence and Incidence

Many parvoviruses appear to be widespread, and most circulate readily among the susceptible individuals. Parvovirus B19 infects almost all humans, and IgG antibody generally persists for life; 50% of children have anti-B19 antibodies by 15 years of age, as do more than 90% of elderly people.^{60,104,166} Viremia is relatively short lived, and although virus may be transmitted by blood, few donated blood units contain high titers of B19.^{105,221,241,294,404,437} B19 infections are seasonal, peaking in late winter, spring, and summer,⁶⁰ and they cycle in 3- to 4-year periods. Between 10% and 60% of susceptible schoolchildren and 20% to 30% of susceptible or adult school and daycare personnel can develop fifth disease in school outbreaks,^{60,96,169} and sibling-to-sibling transfer is probably a major path of transmission.²³⁹ Nosocomial transmission likely occurs but is difficult to distinguish from community-acquired infections.³⁹ Because of the resistance of parvoviruses to heat and solvents, virus may survive the inactivation treatments employed for blood products and pass through filters; thus, the virus can be spread throughout pooled preparations, although infections appear uncommon.^{237,466}

In normal CPV or FPV infections, virus is shed at very high titers in feces and can be found in places where infected animals are housed.³³⁶ Maternal antibodies protect animals against infection by CPV and FPV, and pups or kittens between 6 and 16 weeks of age with waning maternal immunity are highly susceptible.³⁵² Vaccination with modified live vaccines provides strong protection; however, that is blocked when maternal antibody is present.^{76,77}

Adeno-Associated Virus Infections

AAV infections most likely occur in the presence of an adenovirus or herpesvirus infection, and respiratory, fecal-oral, direct conjunctival, and sexual transmission have all been suggested.^{47,168,185,388} AAVs infect a wide variety of mammals; however, cross-species transmission is not known. AAV2, AAV3, and AAV5 have been obtained from humans in the presence of numerous adenovirus serotypes.^{32,48,49} AAV3 was isolated during an outbreak of adenovirus type 3 conjunctivitis among children and adults; it was found in conjunctival, throat, and fecal specimens.³⁸⁸ AAV5 was isolated from a male with a flat condylomatous genital lesion.³² AAV DNA (primarily AAV2) has been detected in peripheral blood cells, cervical biopsy samples, and tissues from spontaneous abortions, as well as tissue culture cells and adenovirus isolates.^{163,178,185,387,427} Antibodies to AAV can reduce the efficiency of gene transduction, and gene therapy vectors derived from a variety of serotypes are being examined to attempt to avoid antibody neutralization.^{183,184,379,482}

Treatments

A few specific treatments are used for most parvovirus infections. Chronic or persistent B19 infections can be treated with commercial immunoglobulin preparations.^{210,249,250}

ADENO-ASSOCIATED VIRUS AS A VECTOR FOR GENE THERAPY

AAV is being developed as a vector for human gene therapy owing to its persistent infection and lack of pathogenicity. Different AAV serotypes have different cell receptors and tissue tropisms or host ranges; as well, they offer the possibility of overcoming humoral immunity to a specific serotype.^{95,177,308} Current AAV vector genomes contain the transgene(s) and regulatory sequences between two copies of the AAV ITR, and no viral genes are expressed and little of the unique sequence of the AAV genome remains. The capacity of a vector based upon the AAV genome is approximately 4.5 kb,¹⁷⁶ although it is possible to include different parts of a transgene in two vectors and have them expressed as a spliced, functional protein product.^{143,256,343}

Production of AAV vectors involves the vector genome (transgene between two ITRs) in a plasmid; the AAV internal, unique sequences (minus the ITRs) in a plasmid; and required helper functions, such as the adenovirus or the HSV genes required for transcription and DNA replication. AAV vectors have been used to transduce cells *ex vivo*, as well as *in vivo* in intact animals and in people.⁷⁹ The vector is primarily maintained as an extrachromosomal element, although it may integrate into the genome.⁴⁰⁹ Transgene expression may be at high levels and maintained for more than a year in rodents and for several years in dogs.² It may be sufficient to functionally correct diseases, including hemophilia,¹⁹⁴ acute macular degeneration,³⁰⁵ diabetes,²⁰⁴ parkinsonism,²³⁵ and $\alpha 1$ antitrypsin deficiency.²⁷⁹ Animal models have included rodents, dogs, and subhuman primates. Vector has been administered *ex vivo* and *in vivo* intramuscularly, intravenously, bronchially, by the upper respiratory route, and by injection into the eye, and minimal toxicity has been observed.

Many clinical trials using various AAV vectors had been completed or are ongoing.⁷⁹ Among the diseases being targeted are cystic fibrosis, hemophilia B, Leber congenital amaurosis, arthritis, human immunodeficiency virus (HIV; vaccine), muscular dystrophy, hereditary emphysema, Parkinson, Canavan, Batten, Alzheimer, and malignant melanoma. Different routes of administration have been used, including intramuscular, hepatic artery, intracranial, intradermal, and *ex vivo*. In all of these trials, the vector has shown minimal or no evidence of toxicity. Clinical efficacy has been observed in patients with Leber congenital amaurosis²¹² and hemophilia B.¹⁹⁵ Other promising trials have involved Duchenne muscular dystrophy and $\alpha 1$ antitrypsin deficiency. Different AAV serotypes can show dramatically variant rates of transduction in various tissues; thus, clinical trials may involve different serotypes and alternative routes of administration.

Current vectors lack Rep proteins or Rep binding sites and therefore do not specifically integrate into the human genome.^{242,243,383} The transduced DNA remains within the cell primarily as extrachromosomal elements, and loss by dilution

may occur if the cells undergo cell division. Targeting site-specific integration may overcome the challenges of cell division, and vectors in which the Rep function is provided in *trans* may allow site-specific integration under conditions where the Rep gene remains extrachromosomal; and such vectors have been developed and appear to work in animal models.³⁶⁶

SUMMARY AND CONCLUSIONS

Parvoviruses include a wide variety of viruses that infect most animals, from mammals to crustaceans. Although many parvoviruses are associated with various acute and chronic diseases, there are many others that cause no or very mild disease, which are often only uncovered by DNA detection or sequencing methods. The small genomes encode two large genes that are expressed in various spliced versions, as well as several small transcripts and proteins that are often less well characterized. The viral capsids assemble from 60 copies of two or three forms of the same protein, and each variant protein has important functions for receptor binding and cell infection. The small size and limited genetic capacity of the viruses means that they highly depend on host cell functions for their replication and only complete their replication cycles in cells undergoing mitosis or those co-infected with a helper virus. The lack of pathogenicity and efficient transduction capabilities of the AAVs has made them a favored candidate for gene therapy in humans.

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Circoviridae

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HISTORY

The prototype circovirus, porcine circovirus (PCV), was first reported in 1974 as papovavirus-like and picornavirus-like particles in a contaminated porcine kidney cell line PK-15.¹⁰⁶ The name circovirus was proposed in 1982 when the viral genome was determined to be a circular single-stranded DNA molecule.¹⁰⁴ PCV was not known to be pathogenic¹⁰⁵ until 1997 when a variant strain, PCV type 2 (PCV2), was isolated from pigs with a wasting disease.² The PK-15 cell-derived virus was designated PCV1 to distinguish it from the pathogenic PCV2. The first circoviruses identified in avian species are chicken anemia virus (CAV) and subsequently psittacine beak and feather disease virus (BFDV), both of which are associated with diseases in birds. CAV was isolated in 1979,¹¹⁸ although its complete genomic sequence was not determined until 1991.⁷⁹ BFDV was first isolated as a novel virus with a single-stranded circular DNA genome in 1989 from cockatoos with beak and feather disease.⁹²

Definitive evidence of human infections by known porcine or avian circoviruses is lacking. Antibodies to PCV1 were reportedly detected in humans,¹⁰⁵ although subsequent studies could not confirm the initial report.^{3,28,39} Recently, novel circovirus-like DNA sequences were detected in stool samples from humans,⁵¹ although the biological and clinical significances of these novel circovirus sequences in humans remain unknown. The recent discovery of PCV1 and PCV2 DNA in

live-attenuated human rotavirus vaccines prompted the U.S. Food and Drug Administration (FDA) to temporarily suspend the use of rotavirus vaccines.^{4,111}

CLASSIFICATION

Viruses in the family *Circoviridae* infect mammalian and avian species. The genomic organization and replication strategy of circoviruses are similar to those of plant geminiviruses and nanoviruses. In fact, animal circoviruses may have evolved from a plant nanovirus through host-switch followed by a recombination event with a picorna-like virus in a mammalian host.³⁷ Two genera of circoviruses, *Circovirus* and *Gyrovirus*, have been recognized by the International Committee on Taxonomy of Viruses (ICTV). The genus *Circovirus* consists of at least 11 species including BFDV, PCV1, PCV2, canary, duck, finch, goose, gull, pigeon, starling, and swan circoviruses.¹¹⁵ CAV, which has larger virion and genome size as well as a different genomic organization, is the sole member of the genus *Gyrovirus*. The Torque teno virus (TTV) and mini-TT virus, which were once classified in the *Circoviridae*, have now been reclassified in a new family *Anelloviridae*.⁸ The “*Cyclovirus*” recently discovered from the stool samples of humans and chimpanzees may represent a previously unrecognized genus in the family *Circoviridae*.⁵¹

VIRION STRUCTURE

Circoviruses contain a single-stranded circular DNA genome enclosed within a capsid, which is presumably the only structural protein in the virion. Morphologically, members of the genus *Circovirus* appear as small, nonenveloped, icosahedral particles of approximately 15 to 20 nm in size (23,70; e-Fig. 58.1). The virion particles of CAV in the genus *Gyrovirus* are larger, with a diameter of 25 to 26.5 nm (23,36,64; Fig. 58.1). Members of *Circoviridae* all have a T=1 structure containing 60 copies of the capsid protein.²³ However, the capsids of genus *Circovirus* consist of 12 flat pentameric morphological units, whereas the capsid of CAV in the genus *Gyrovirus* consists of 12 pentagonal trumpet-shaped units. Therefore, the virions of the genus *Circovirus* have a smoother and more featureless surface than that of CAV of the *Gyrovirus* (23; Fig. 58.1).

GENOME STRUCTURE AND ORGANIZATION

The genome of *Circoviridae* is a single-stranded circular DNA molecule of 1.7 to 2.0 kb for genus *Circovirus*, and 2.3 kb for

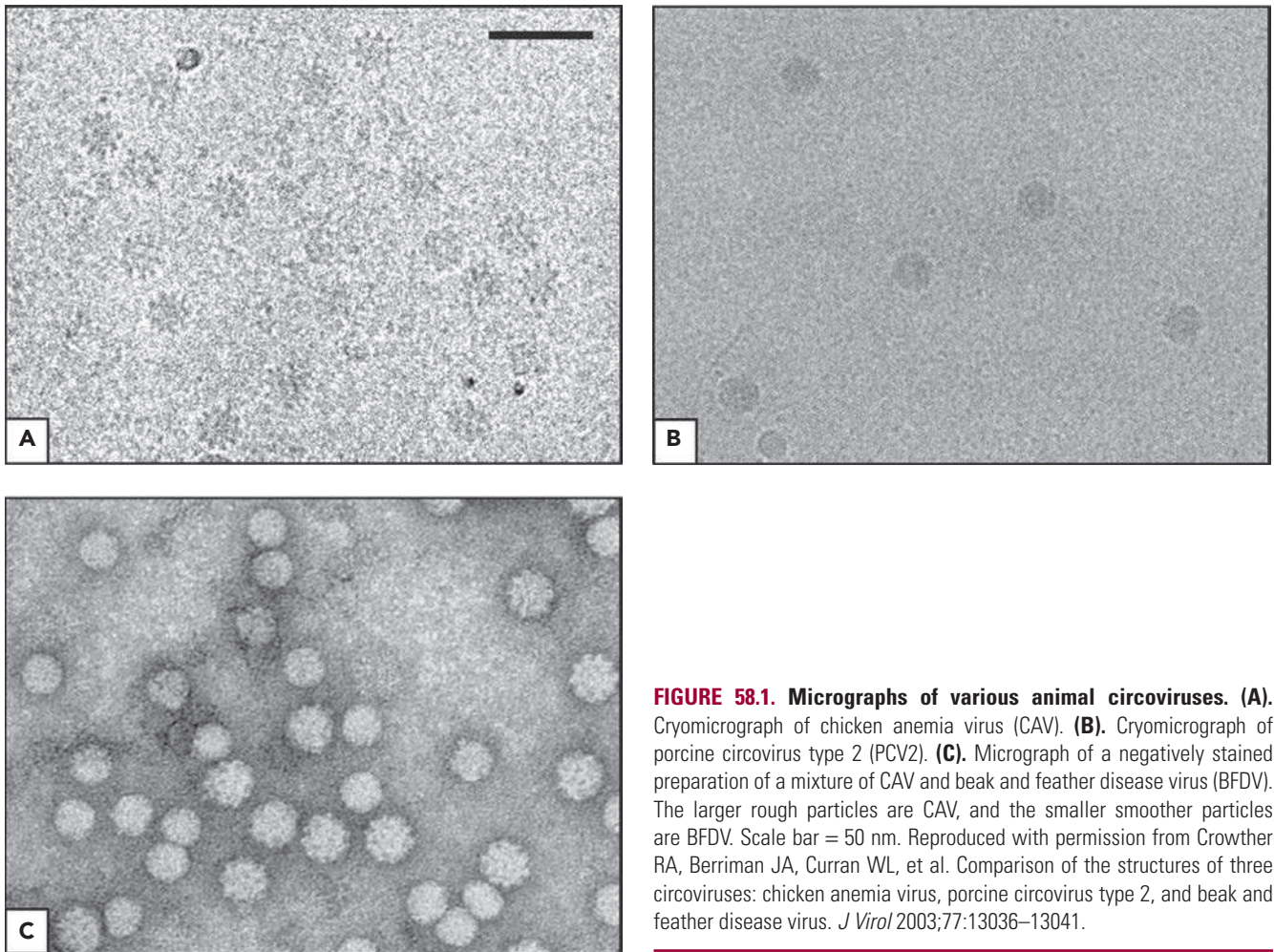


FIGURE 58.1. Micrographs of various animal circoviruses. (A). Cryomicrograph of chicken anemia virus (CAV). **(B).** Cryomicrograph of porcine circovirus type 2 (PCV2). **(C).** Micrograph of a negatively stained preparation of a mixture of CAV and beak and feather disease virus (BFDV). The larger rough particles are CAV, and the smaller smoother particles are BFDV. Scale bar = 50 nm. Reproduced with permission from Crowther RA, Berriman JA, Curran WL, et al. Comparison of the structures of three circoviruses: chicken anemia virus, porcine circovirus type 2, and beak and feather disease virus. *J Virol* 2003;77:13036–13041.

Gyrovirus CAV.^{96,115} These are the smallest DNA viruses known to infect mammals and birds, and the genome size is reduced to the absolute necessities for the two basic functions of a virus: copying and packaging of viral genome.

For the genus *Circovirus*, the genome contains two major open-reading frames (ORFs) coding for the replicase protein (Rep) and capsid protein (Cap), respectively. The *rep* and *cap* genes are oriented in the opposite direction resulting in an ambisense genome organization. The Rep and Rep' are produced from alternatively spliced RNA transcripts. An intergenic region between the 5' ends of *rep* and *cap* genes contains the origin of viral genome replication (*Ori*), which is characterized by a stem-loop structure with a nonamer motif in its apex (e-Fig. 58.2). Three or four hexamer repeat motifs adjacent to the stem-loop serve as the binding sites for Rep and Rep' to initiate rolling-circle replication of viral genome.^{33,61} The *rep* or *Ori* between PCV1 and PCV2 are fully exchangeable,^{5–7,29–31} indicating conserved functionality of these regions among members of the genus *Circovirus*.

The structure and organization of the CAV genome in the genus *Gyrovirus* is different from that of the genus *Circovirus*.^{22,79} The CAV genome is negative sense with approximately 2.3 kb, and contains three partially overlapping ORFs, a promoter-enhancer region, and a polyadenylation signal.^{74,87} The ORF1 codes for the VP1 capsid protein, and the ORF2 and ORF3

code for VP2 and VP3 nonstructural proteins. The ORF3 completely overlaps ORF2, whereas ORF2 partially overlaps ORF1. The promoter-enhancer region in the 5' NCR of CAV genome contains four or five 21-bp direct repeats (DRs) and a 12-bp insert between the second and third DRs.^{65,74} Host cell transcription factors bind to the DRs and the 12-bp insert, and at least two DRs and the 12-bp insert are required for efficient transcription and replication.^{73,74}

STAGES OF REPLICATION

Among the 11 species in the genus *Circovirus*, only PCV1 and PCV2 can be propagated *in vitro*. Although the CAV of the genus *Gyrovirus* can be propagated in cell cultures, very little is known regarding its replication. Therefore, the knowledge of circovirus replication is derived mostly from the studies of PCV1 and PCV2, and to a lesser extent, of CAV.

Attachment, Entry, and Uncoating

Glycosaminoglycans (GAG) heparin, heparan sulfate, and chondroitin sulfate B are attachment receptors for PCV2.⁶⁸ It is not surprising that PCV2 utilizes GAG as the general attachment receptors (33, e-Fig. 58.3), since the virus targets

multiple organs and tissues in infected pigs, even though cells in the monocyte and macrophage lineage are the preferential targets for PCV2 *in vivo*.⁹⁹ A yet-to-be identified specific receptor may be needed for more efficient binding and entry of PCV2 into cells.³³

PCV2 is internalized by dendritic cells (DCs), and the internalization was observed with both mature and immature cells including blood DCs, plasmacytoid DCs, and DC precursors, and thus suggestive of a nonmacropinocytic uptake of the virus.^{112,113} PCV2 virus-like particles (VLPs) quickly bind to porcine monocytic cells 3D4/31, and enter cells predominantly via clathrin-mediated endocytosis and require an acidic environment for infection.⁶⁹ The epithelial cells are also major targets for PCV2 *in vivo*. Although PCV2 quickly attaches to epithelial cells, virus entry was slow.⁶⁷ It appears that a dynamin- and cholesterol-independent, but actin- and small GTPase-dependent pathway, allows PCV2 entry and internalization leading to full replication in epithelial cells.⁶⁷ After entry, PCV2 is localized in the endosomes (33, e-Fig. 58.3). As the endosomal vesicles move toward the nuclear membrane and become acidic, a serine protease appears to be required for PCV2 release from the endosome, suggesting that a proteolytic cleavage of Cap may be a part of the uncoating process.^{66,68,69} PCV2 infection of untreated and chloroquine diphosphate-treated PK-15 cells was blocked by a serine protease inhibitor, suggesting that serine protease-mediated PCV2 disassembly is enhanced in porcine epithelial cells but inhibited in monocytic cells after inhibition of endosome-lysosome system acidification.⁶⁶

Transcription

A total of nine RNA transcripts were synthesized during productive PCV2 infection in PK-15 cells: Cap protein RNA (CR), five Rep-associated RNAs (Rep, Rep', Rep3a, Rep3b, and Rep3c), and three NS-associated RNAs (NS515, NS672, and NS0) (21; Fig. 58.2). Rep', Rep3a, Rep3b, and Rep3c are produced from Rep by alternate splicing. The three NS-associated RNAs are transcribed from three different promoters inside ORF1, and share only the 3' sequence with Rep.²¹ A stop codon introduced at the 5'-end of CR did not affect Rep-associated viral antigen or DNA synthesis.¹⁴ Altering the consensus dinucleotides at the splice junctions of the minor Rep- and NS-associated RNAs or introducing a stop codon in the abundant NS0 RNA also had no effect on viral protein or DNA synthesis. However, mutations resulting in truncated Rep or Rep' reduced viral protein synthesis by more than 99% and abolished viral DNA replication, indicating that both Rep and Rep' are essential for PCV2 replication.²⁰ In contrast to the pathogenic PCV2, a total of 12 RNAs were synthesized in PCV1-infected PK15 cells¹⁴ including the viral CR RNA, eight Rep-associated RNAs, and three NS-associated RNAs.¹⁴ The promoter for *cap* is mapped within the ORF1 (nt 1328-1252), and the promoter for *rep* is located in the intergenic region (nt 640-796) and overlaps the *Ori* of PCV1.⁶²

Several cellular gene transcripts were upregulated in both PCV2-infected PK-15 cells and affected tissues including two transcripts with homology to an RNA splicing factor (SPF30) and a hyaluronan-mediated motility receptor (RHAMM).¹⁰ Microarray analyses of the genes in lymph nodes of PCV2-infected pigs revealed altered expression levels in genes that are involved in innate immune defense (TLR1, CD14, and

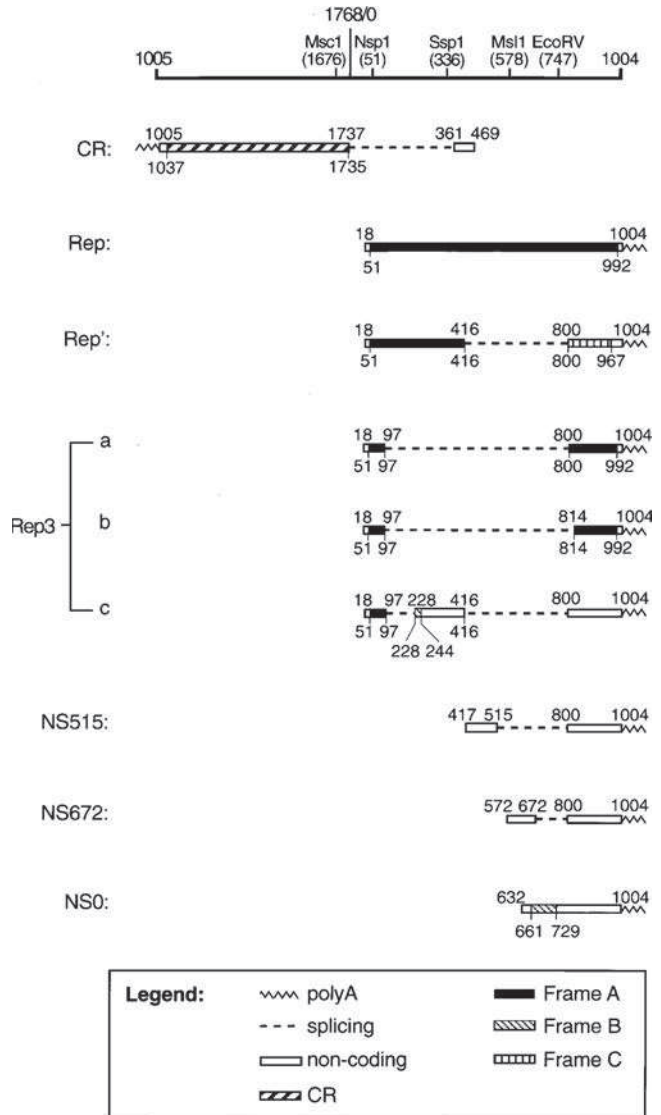


FIGURE 58.2. Summary of the porcine circovirus type 2 (PCV2) RNA transcripts. The schematic representation of the PCV2 genome with relevant restriction enzyme sites is shown on top. The RNA is annotated on top with nt coordinates that indicates the last nucleotide of each respective exon. The coding sequence of each transcript was shaded and their nt coordinates are indicated below each RNA. Reproduced with permission from Cheung AK. Transcriptional analysis of porcine circovirus type 2. *Virology* 2003;305:168–180.

CD180), immunosuppressed responses (FGL2 and GPNMB), pro-inflammatory signals (galectin-3) and fasting processes (Angiopoietin-like 4, ANGPTL-4), suggesting that PCV2 has developed an intricate mechanism to induce immunosuppression, inflammatory cell infiltration and weight loss in pigs.⁴⁹ PCV2, but not PCV1, induces interleukin 10 (IL-10) secretion by monocytic cells, which led to repression of IL-12 in peripheral blood mononuclear cells (PBMCs).⁴⁵ The PCV2 ORF3 protein binds to a regulator of G protein signaling (RGS), and co-localized with poRGS16 in lipopolysaccharide (LPS)-activated porcine PBMC. The poRGS16 appeared to participate in the translocation of ORF3 protein into the nucleus.¹⁰³

NF- κ B was activated concomitantly with PCV2 replication, and treatment of cells with an NF- κ B inhibitor reduced virus protein expression and virion production, suggesting that NF- κ B activation is important for PCV2 replication.¹¹⁴

Three viral proteins of CAV in the genus *Gyrovirus* are derived from a single 2.0-kb mRNA species.^{76,85} Several minor mRNA species of 1.6, 1.3, and 1.2 kb in size are also identified (42, e-Fig. 58.4). The 1.3-kb RNA had a splice site joining nt 1222 to nt 1814 and encoded head-to-tail VP1. The 1.2 kb RNA possessed a splice site joining nt 994 to nt 1095 and encoded several putative proteins with frameshift mutations. CAV contains a single promoter–enhancer region with four consensus cyclic AMP response element sequences that are similar to the estrogen response element consensus half-sites. These sequences are arranged as direct repeats, an arrangement that can be recognized by members of the nuclear receptor superfamily, and may provide a mechanism to regulate CAV activity in situations of low virus copy number.⁶⁵

Translation

The genome of genus *Circovirus* consists of two major ORFs: ORF1 encodes the Rep and Rep', and the ORF2 encodes Cap. Rep is translated from the full-length *rep* transcript, whereas Rep' is produced from a spliced transcript.³³ Both Rep and Rep' are essential for the initiation of virus replication.^{19,59,60} Mutation within motifs I to III and the putative dNTP-binding (GKS) box of the Rep and Rep' interfered with viral replication. Motifs I to III are essential for PCV1 *Ori* cleavage.¹⁰¹ The repression of *rep* promoter is mediated by binding of Rep to H1 and H2 hexamers in the *Ori* of PCV1; however, transcription of *cap* promoter is not influenced by viral proteins.^{33,60} Both Rep and Rep' co-localize in the nucleus of infected cells and form homomeric and heteromeric complexes.^{34,59} Three putative nuclear localization signals (NLSs) are present in the N-termini of Rep/Rep': NLS1 and NLS2 mediate nuclear accumulation, whereas NLS3 enhances the nuclear transport of Rep and Rep'.³⁴ PCV2 Rep interacted with an intermediate filament protein, similar to human syncoilin, and with the transcriptional regulator c-myc.¹⁰² The PCV Rep also binds three porcine cellular proteins^{1,35}: ZNF265 is an alternative component of the spliceosome, whereas VG5Q and TDG were linked to transcriptional regulation.

The Cap of the genus *Circovirus* can self-assemble into VLPs,^{11,70,117} and it elicits neutralizing antibodies in vaccinated animals.^{9,97} At least five different but overlapping conformational epitopes were identified within residues 47 to 63 and 165 to 200 and the last four amino acids at the C terminus of PCV2 Cap.^{50,57} Two amino acid mutations in the Cap, P110A and R191S, enhance the growth ability of PCV2 *in vitro* but attenuate PCV2 *in vivo*.³² The Cap localized in the nucleoli of PCV2-infected cells⁵⁴ and in the nucleoli of cells at an early stage of PCV1 infection.³⁴ The PCV Cap interacts with numerous cellular proteins^{35,102} including complement factor C1qB, E3 ubiquitin ligase family member MKRN1, and proapoptotic gene product Par-4.³³ The exact function of these cellular factors in PCV replication remains to be elucidated. An ORF3 has been identified in PCV1 but is truncated in PCV2. The *in vitro*–expressed ORF3 protein of PCV2-induced apoptosis through the activation of caspase 8 and caspase 3 pathways.⁵² The ORF3 protein interacts with pPirh2 and competes with p53 in binding to Pirh2 and mediates the deregulation of p53

homeostasis, leading to increased p53 levels and apoptosis of the infected cells.^{44,53} It was reported that abrogation of the ORF3 function attenuated PCV2 in pigs,⁴³ although other studies showed that PCV2 pathogenicity is not solely determined by ORF3.^{13,41} In fact, whether or not PCV2 infection causes apoptosis remains controversial.^{48,58,91,113}

The CAV genome encodes three proteins: the VP1 Cap and the VP2 and VP3 nonstructural proteins.^{94,108} The amino acid residue 394 of VP1 is a determinant of virus pathogenicity.¹¹⁶ The VP2 is a multifunctional protein that also serves as a scaffold protein during virion assembly.^{47,76,94} The VP2 has dual serine and tyrosine protein phosphatase activities,^{85,86} and mutations in VP2 attenuated CAV.⁸⁴ Both VP1 and VP2 are required to elicit neutralizing antibodies against CAV.^{27,47,75,77} The VP3 of CAV, also known as apoptin, induces apoptosis in chicken thymocytes and lymphoblastoid cells.⁷⁸ VP3 triggers the intrinsic mitochondrial death pathway, resulting in loss of mitochondrial membrane and release of cytochrome c and apoptosis-inducing factors in mitochondria.^{12,25,55} The VP3-induced apoptosis is independent of tumor suppressor p53, and Bcl-2 does not inhibit the VP3-induced apoptosis in tumor cells.^{24,26,95} Synthesis of VP3 alone induces apoptosis in human tumor cell lines, but not in normal human diploid cells,²⁴ and thus VP3 is considered as an anticancer agent.^{71,72}

Replication of Genomic DNA, Assembly and Release

After uncoating, the single-stranded circular viral DNA genome is converted to double-stranded DNA (dsDNA) DNA intermediate by host enzymes in the nucleus. Binding of Rep and Rep' to *Ori* unwinds the dsDNA intermediate to initiate genome replication.^{15–19,33} Viral genomes with mutations on either or both arms of the inverted repeats (IRs, palindrome) were still capable of synthesizing viral proteins and producing infectious viruses with restored or new palindromes, indicating that a flanking palindrome at *Ori* is not essential for initiation of viral DNA replication. A rolling-circle “melting-pot” model for circovirus DNA replication was proposed (15,18, Fig. 58.3). The Rep and Rep' complex binds, destabilizes, and nicks the *Ori* sequence to initiate leading-strand DNA synthesis.¹⁵ The four strands of the destabilized IRs exist in a “melted” configuration, and the minus-strand viral DNA and a palindromic strand serve as templates simultaneously during initiation or termination of viral DNA replication. The palindromic sequences flanking the *Ori* can potentially form single-stranded stem-loop cruciform structures that are essential for circovirus replication.¹⁸ Three conserved rolling-circle replication motifs (RCR-I, RCR-II, and RCR-III) and a deoxyribonucleotide triphosphates (dNTP)-binding motif were identified within PCV Rep and Rep'.^{33,40,100,110} Mutations of the conserved motifs negatively affect PCV replication.^{60,101} The RCR-II motif is involved in the nicking of the viral DNA, and the Tyr-93 residue within the RCR-III motif cleaves the phosphodiester bond to produce a 3'-hydroxyl group that serves as a primer for viral genome replication, and a 5'-phosphate of the cleavage product that covalently attaches to Rep and Rep'.^{100,110} After a round of replication, the newly synthesized viral genome DNA is cleaved again, and the 5'-phosphate is ligated to the 3'-hydroxyl group, resulting in the release of unit-length single-stranded monomer genomes.³³

The genome replication of the *Gyrovirus* CAV is also thought to be via a rolling-circle replication mechanism.^{94,109}

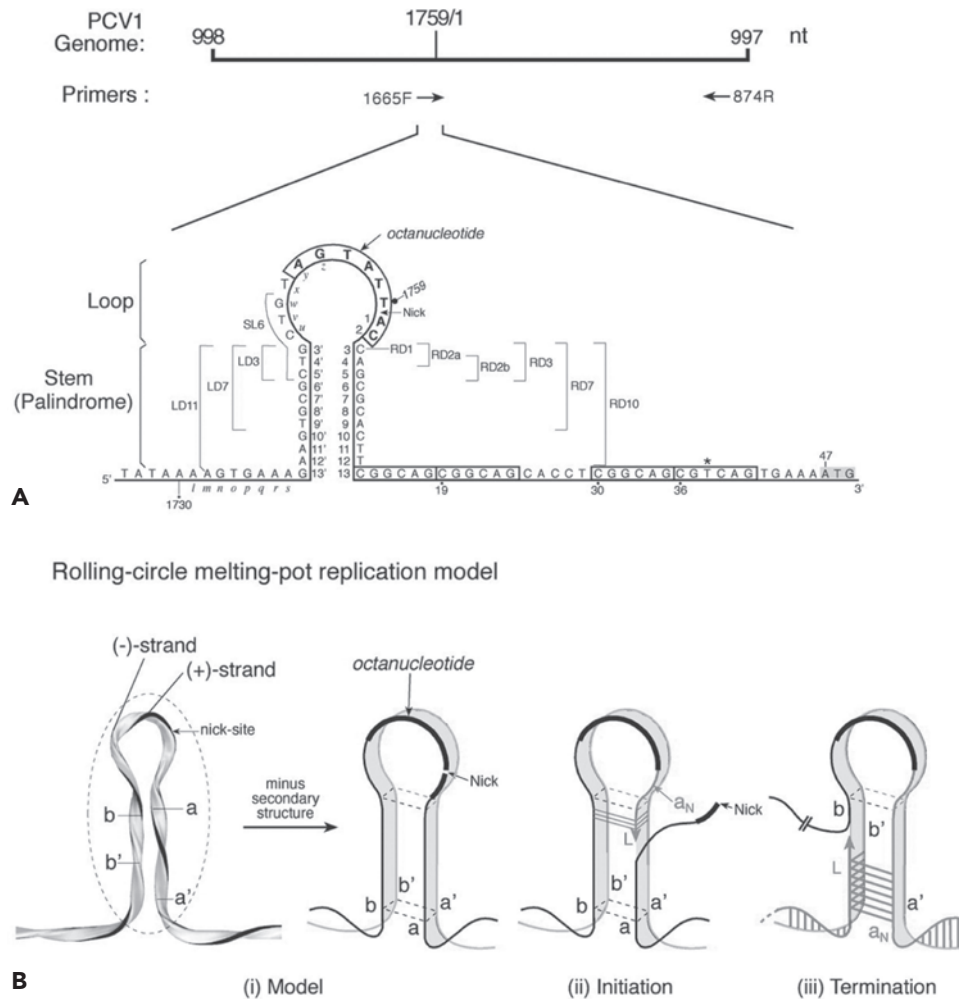


FIGURE 58.3. A: Schematic representation of the porcine circovirus type 1 (PCV1) *Ori*, indicating potential base pairing of the flanking inverted repeats. The genome sequence (1,759 nucleotides) and coordinates (1, 2, 3, etc.) are based on GenBank accession number AY184287. The nucleotide coordinates (3', 4', 5', etc.) are arbitrarily assigned to show the nucleotide complementarity of the palindromic sequences. The octanucleotide containing the presumed nick site (AGTATT↓AC) is boxed and indicated in bold. The palindrome is divided into six regions (right arm, RD3, RD7, and RD10; left arm, LD3, LD7, and LD11). The six-nucleotide tandem repeats located at nucleotides 13, 19, 30, and 36 (not perfect at nucleotide 38 and indicated by an asterisk) are in boxes. Relevant nucleotide sequences are assigned arbitrary positions (l-m-n-o-p-q-r-s and u-v-w-x-y-z) to assist in retracing the templates used during replication. **B:** The rolling-circle melting-pot replication model. (i) PCV1 *Ori* after Rep binding to the octanucleotide (prior to nicking) with the plus- and minus-strand genomes in proximity to each other. The destabilized environment (i.e., the melting pot) is enclosed by a dotted circle. (ii) Schematic representation of the DNA templates available during initiation of DNA replication after removal of the secondary structure in the model. The leading strand (L) displaces strand a, and uses strand a' or strand b as the template. (iii) Schematic representation of the DNA templates available during termination of DNA replication after removal of the secondary structure in the model. The leading strand (L) displaces strand b and uses the newly synthesized strand a_N or strand b' as the template. The plus-strand genome is indicated in black, the minus-strand genome is indicated in blue, and the potential base-pairing opportunities available for the current round of DNA replication are indicated in red. Reproduced with permission from Cheung AK. Palindrome regeneration by template strand-switching mechanism at the origin of DNA replication of porcine circovirus via the rolling-circle melting-pot replication model. *J Virol* 2004;78:9016–9029.

Homologous recombination occurs in cloned head-to-tail repeat replicative form of CAV genomes.¹⁰⁹ The VP1 contains a three-amino acid motif associated with rolling-circle replication.¹⁰⁷ The phosphatase activity of VP2 is important but not required for CAV replication.^{85,86} The VP3 is essential for completion of CAV life cycle as truncated VP3 lacking the C-terminal 11 amino acid residues failed to induce apoptosis.^{74,78}

PATHOGENESIS AND PATHOLOGY

Porcine Circovirus-Associated Diseases

PCV2 is associated with various disease conditions in pigs including postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS), collectively known as porcine circovirus-associated

diseases (PCVADs).^{38,80,89} The oronasal exposure is likely the natural route of PCV2 transmission,⁹⁶ although pigs can be experimentally infected via intramuscular, oral, oronasal, and intrauterine routes of inoculation.^{56,81–83} Lymphoid depletion and histiocytic replacement are characteristic lesions in lymphoid tissues (80, e-Fig. 58.5). PCV2 is likely shed through respiratory and oral secretions, urine, and feces of infected pigs.⁸⁰ The interaction of PCV2 with the host immune system is a critical factor in PCV2 pathogenesis. Immunostimulations by co-infecting agents such as porcine reproductive and respiratory syndrome virus (PRRSV) or by adjuvants such as keyhole limpet hemocyanin exacerbate PCVADs.^{80,89} An increase in IL-10 and proinflammatory cytokines such as IL-1 and tumor necrosis factor α (TNF- α) and a decrease in IL-2 and IL-4 expression were observed in PCV2-infected pigs with PCVAD.⁸⁹ An interferon-stimulated response element (ISRE) sequence was identified in the Ori of PCV2 genome. When present in the context of intact virus but not in isolation, the ISRE influences the interferon-mediated enhancement of PCV2 replication *in vitro* and plays a potential role in viral pathogenesis *in vivo*.^{88,90}

Psittacine Beak and Feather Disease (PBFD)

PBFD, caused by BFDV of genus *Circovirus*, is one of the most frequently diagnosed viral diseases in psittacine birds.^{92,115} BFDV is now known to infect more than 60 different species of psittacine birds, and circovirus infections are common in many other avian species. The natural routes of exposures are thought to be by way of aerosolized virus particles or direct ingestion of contaminated materials. BFDV has tropism for rapidly dividing cells in mitosis stage such as basal follicular epithelium, lymphoid tissues, and intestinal epithelium. Virus transmission is through virus shedding in feather dander followed by fecal shedding and feeding of chicks with regurgitated crop contents. Gross pathological lesions include feather loss and dystrophy, and beak deformities. Microscopically, necrosis and inflammation are seen in dystrophic feathers. Lymphofol-

licular hyperplasia with necrosis and lymphoid depletion are commonly seen in lymphoid tissues of BFDV-infected birds.¹¹⁵

Chicken Infectious Anemia (CIA)

The natural route of exposure for CAV is likely oral.⁹³ Feces from infected chickens are the main source of virus for horizontal transmission. Vertical transmission in commercial flocks through hatching eggs is an important means of virus dissemination. Under field condition, vertical transmission occurs for a period of 3 to 9 weeks after exposure to CAV. Gross lesions include thymic and bone marrow atrophy, and less commonly bursal atrophy. Hemorrhagic-aplastic anemia syndrome characterized by intracutaneous, subcutaneous, and intramuscular hemorrhages is associated with severe anemia in CAV-infected chickens. Microscopically, CAV-infected chickens are characterized by generalized lymphoid atrophy and depletion, and panmyelophthisis. Infection of hemocytoblasts in the bone marrow and lymphoblasts in the thymus cortex in the early infection stage (6 to 8 days post-infection) cause a rapid depletion of these cells by apoptosis with drastically reduced numbers of erythrocytes, white blood cells, and thrombocytes.⁹⁸

EPIDEMIOLOGY AND CLINICAL FEATURES

PCVAD affects grower pigs of 5 of 18 weeks of age, and rarely occurs in 1- to 3-week-old pigs, presumably due to maternal antibody protection.⁶³ PCV2 infection is widespread worldwide, with 100% seropositivity in some herds; however, the morbidity is low and only a small proportion of infected animals (5% to 30%) actually develop clinical PCVAD.^{38,80,89} PCV2a and PCV2b genotypes, which differ by as much as 10% at nucleotide sequence level, are most prevalent worldwide.⁶ The main clinical signs of PCVAD include progressive weight loss or decreased rate of weight gain, paleness or icterus, and gauntness and ill thrift (80, Fig. 58.4A). The infected pigs may



FIGURE 58.4. A: An 8-week-old pig experimentally co-infected with porcine circovirus type 2 (PCV2) and porcine parvovirus (PPV) showing icterus and a poor body condition typical of systemic porcine circovirus-associated disease (PCVAD). **(B):** A 12-week-old pig with from porcine dermatitis and nephropathy syndrome (PDNS). The perineal region, ventral abdomen, and legs are covered with raised coalescing red-purple lesions. Reproduced with permission from Opriessnig T, Meng XJ, Halbur PG. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest* 2007;19:591–615.

also experience labored respiration with coughing and diarrhea (e-Fig. 58.6). PCV2 is associated with a number of diseases including pneumonia, enteritis, reproductive failure, and PDNS (Fig. 58.4B).^{80,89}

BFDV infection is usually seen in young psittacine birds younger than 3 years of age and young pigeons of less than 1 year of age, although older birds can also be infected. In general, circovirus infection in birds is associated with high morbidity but low mortality. The prevalence of BFDV infection varied in wild and captive bird populations. For example, approximately 41% to 95% of the free-ranging psittacine birds in New South Wales are seropositive, whereas 5% of the captive birds in the United States are positive for BFDV.¹¹⁵ Mortality and clinical signs in BFDV-infected birds varied dependent on the age, species, and concurrent infection status. The most common signs in circovirus-infected pigeons include poor performance, diarrhea, and ill-thrift, whereas loss of flight and tail feathers are the main clinical signs in turtle doves.¹¹⁵ The majority of the infections are subclinical.

CAV infection is ubiquitous in chicken flocks worldwide. CAV infection of chickens of 2 to 4 weeks of age induced anemia, which is characterized by hematocrit values ranging from 6% to 27%. The mortality is about 10% to 20%, but generally does not exceed 30%. Surviving chickens completely recover from anemia by 20 to 28 days postinfection, although secondary bacterial or viral infection may slow down the recovery process. Chickens develop age resistance to CAV-induced anemia by approximately 3 weeks of age.⁹³

DIAGNOSIS, PREVENTION, AND CONTROL

The diagnosis of clinical PCVAD requires the demonstration of characteristic pathological lesions associated with the detection of PCV2 antigen or DNA in the affected tissues.⁸⁰ Prior to the availability of vaccines, good herd management practices, co-infection control, and disinfection of animal facilities can minimize the impact of PCV2 infection. At least four commercial vaccines are available against PCV2 infection and PCVAD,^{6,7,30,31,46} and these vaccines are very effective. Definitive diagnosis of circovirus infections in birds requires the detection of viral antigen or DNA in affected birds. Virus isolation is not possible for most circoviruses. Currently, there is no commercial vaccine against BFDV, and thus control of circovirus infection in free-ranging birds is difficult. CAV is the only avian circovirus that can be propagated in lymphoblastoid T-cell lines (MDCC-MSB1 and MDCC-JP2) and B-cell line (LSCC-1104B1). Virus isolation in susceptible cells and detection of CAV DNA and antigen in tissues are means of diagnosis of CAV infection. Commercial live-attenuated vaccines against CAV are used to immunize chickens of 9 of 15 weeks of ages. Complete elimination of CAV from chicken flocks is not realistic, and thus good management and hygiene procedures are important to minimize the impact caused by CAV infection.

PERSPECTIVE

Members of the *Circoviridae* are associated with several important animal diseases including PCVAD in pigs and PBFD and CIA in avian species. Although circovirus-like DNA sequences

have recently been detected in stool samples from humans, their biological significances remain unclear. The discovery of porcine circoviruses in live-attenuated human rotavirus vaccines raised a concern of vaccine safety, although definitive evidence of zoonotic human infections by animal circoviruses is still lacking. Only porcine circoviruses and CAV can be propagated *in vitro*, and a specific cellular receptor(s) for circoviruses has not yet been identified. Many steps in the circovirus life cycle including uncoating, replication, assembly, and release are poorly understood. Future studies are warranted to elucidate the molecular mechanism of circovirus pathogenesis, identify cellular and viral factors that determine species and tissue tropisms, assess the zoonotic potential of animal circoviruses, and determine the clinical significances of novel circovirus-like sequences in humans and farm animals.

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Herpesviridae

Definition

Inclusion in the Family *Herpesviridae*

Distribution in Nature

Biological Properties

Nomenclature and Classification

Herpesvirus Species

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with a hole running down their long axis, plus one capsomeric structure that serves as the portal for packaging and release of the viral genome (the complex of the core and capsid is the nucleocapsid); an amorphous-appearing, sometimes asymmetric material that surrounds the nucleocapsid and is designated the tegument; and an envelope containing viral glycoprotein spikes on its surface. Based on the morphologic criteria, highly divergent viruses with hosts that range from bivalves to humans have been identified as herpesviruses (Fig. 59.2). Originally classified into a single family, the availability of extensive nucleotide sequence data led to establishment of a new taxonomic order, the *Herpesvirales*,^{28,102} that encompasses three virus families: the herpesviruses of mammals, birds, and reptiles (the *Herpesviridae*),¹⁰⁰ herpesviruses of fish and amphibians (the *Alloherpesviridae*),⁹⁹ and herpesviruses of bivalves (the *Mala-coherpesviridae*).¹⁰¹ This and subsequent chapters in this book are concerned primarily with viruses that have long been recognized as the family *Herpesviridae*.

The objectives of this chapter are to provide definitions and examples of many of the terms and concepts that are relevant across the diverse collection of herpesviruses (a glossary is provided in e-Table 59.1). Briefly summarizing such a broad area of active research can be accomplished only at the cost of oversimplification and overgeneralization. Subsequent chapters will delve into most of these areas in much greater depth. Students, especially, should understand that the paradigms presented here represent significant opportunities for conceptual and experimental challenge.

DISTRIBUTION IN NATURE

Herpesviruses are highly disseminated in nature. Most animal species have yielded at least one herpesvirus and frequently several distinct herpesviruses on examination. Inasmuch as few herpesviruses naturally infect more than one species, the number of herpesviruses in nature is likely to exceed the more than 200 identified to date. Thus far, nine herpesviruses have been identified that have humans as their primary host: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and *Human herpesviruses 6A, 6B, and 7* (HHV-6A, HHV-6B, HHV-7), and Kaposi's sarcoma-associated herpesvirus (also known as HHV-8). Some key properties of many of the known herpesviruses are listed in Table 59.1, and a more comprehensive list of viruses is provided in e-Table 59.2. We have not tabulated the many herpesviruses identified primarily on the basis of small segments of nucleotide sequence (e.g., 35,36 and references therein).

*Who made the world I cannot tell;
'Tis made, and here I am in hell.
My hand, though now my knuckles bleed,
I never soiled with such a deed.*

A. E. Housman, No. XIX in *More Poems*

DEFINITION

Inclusion in the Family *Herpesviridae*

Herpesviruses have historically been defined based on the architecture of the virion (Fig. 59.1). A typical herpesvirion consists of a core containing a linear double-stranded DNA (dsDNA, ranging from 124–295 kb in length); an icosahedral capsid approximately 125 nm in diameter containing 161 capsomeres

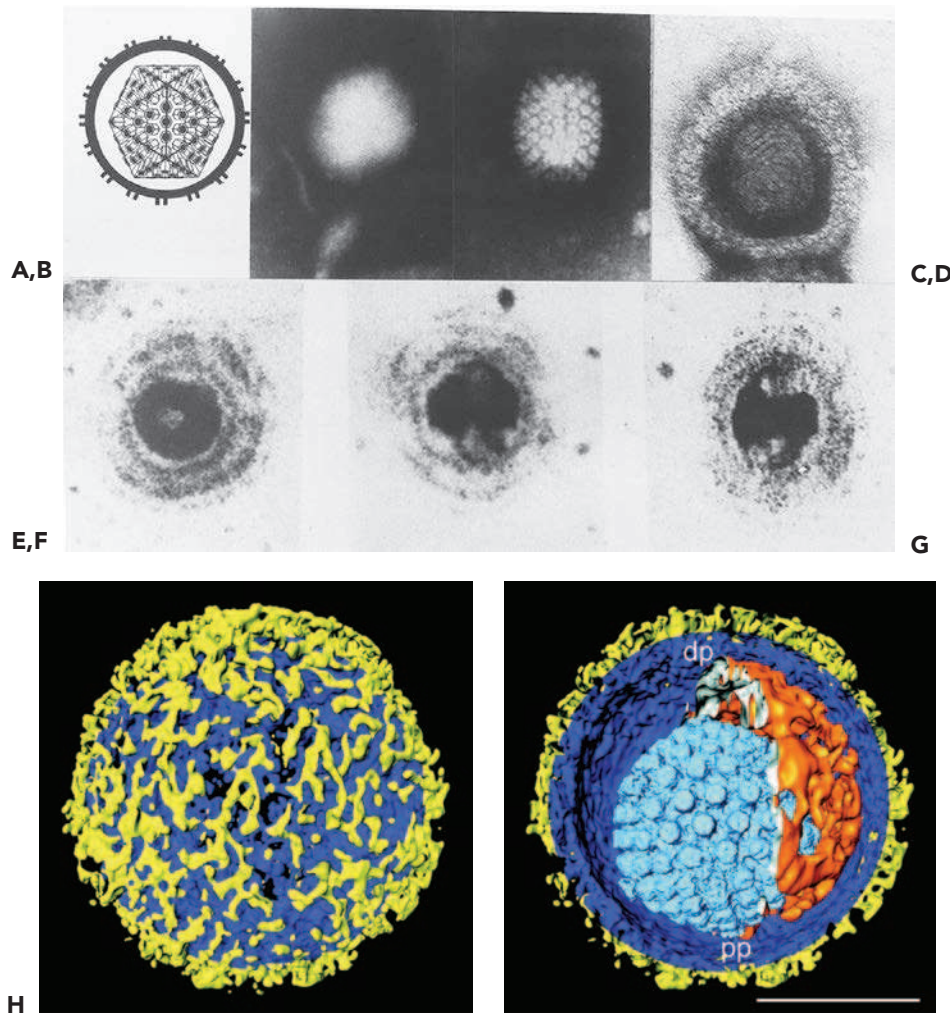


FIGURE 59.1. Herpesvirus morphology as visualized by transmission electron microscopy. **A:** Schematic representation of the herpesvirion seen through a cross-section of the envelope with spikes projecting from its surface. The sides of the icosahedron forming the capsid show twofold symmetry. The irregular inner perimeter of the envelope is meant to represent the occasional asymmetric arrangement of the tegument. **B:** An intact negatively stained HSV-1 virion. The intact envelope is not permeable to negative stain. The diameter of the virion is approximately 120 nm. **C:** A HSV-1 capsid exposed to negative stain and showing twofold symmetry matching the diagrammatic representation of the capsid in **A**. **D:** HSV-1 nucleocapsid containing DNA permeated with uranyl acetate. The electron micrograph shows the presence of thread-like structures 4 to 5 nm wide on the surface of the core. **E–G:** Electron micrographs of thin sections of HSV-1 virions showing the core cut at different angles. The preparation was stained with uranyl acetate and counterstained with lead citrate. The DNA core preferentially takes up the stain and appears as a toroid with an outer diameter of 70 nm and an inner diameter of 18 nm. The toroid appears to be suspended by a fibrous cylindrical structure. The micrographs show the toroid seen looking down the hole (**E**), in cross-section (**F**), or from the side (**G**). The electron micrographs shown in **D** to **G** are from Furlong et al.⁴¹ **H, I:** Segmented surface rendering of a single virion tomogram after denoising. Outer surface showing the distribution of glycoprotein spikes (yellow) protruding from the membrane (blue) (**H**). Cutaway view of the virion interior, showing the capsid (light blue) and the tegument “cap” (orange) inside the envelope (blue and yellow) (**I**). pp, proximal pole; dp, distal pole. Bar = 100 nm. (**A, B, C, H, I** from Grunewald K, Desai P, Winkler DC, et al. Three-dimensional structure of herpes simplex virus from cryo-electron tomography. *Science* 2003;302:1396–98, with permission. **D** through **G** from Furlong D, Swift H, Roizman B. Arrangement of herpesvirus deoxyribonucleic acid in the core. *J Virol* 1972;10:1071–1074, with permission.)

BIOLOGICAL PROPERTIES

Members of the family *Herpesviridae* share four significant biological properties:

1. They specify a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis (e.g., DNA polymerase, helicase, primase), and processing of proteins (e.g., protein kinases), although the exact array of enzymes may vary from one herpesvirus to another (Table 59.2).
2. Virus gene transcription, synthesis of viral DNA, and nucleocapsid assembly occur in the nucleus. Most virions

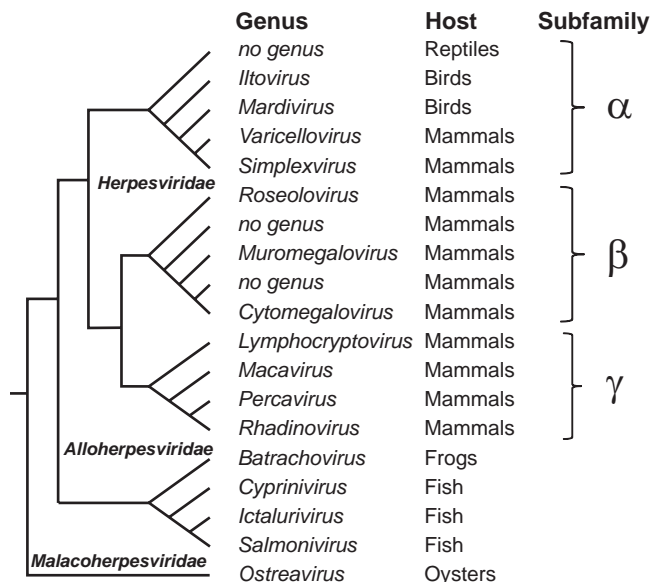


FIGURE 59.2. Major phylogenetic relationships and taxonomic subunits within the order Herpesvirales.¹⁰² The schematic shows branching patterns, not evolutionary distances.

acquire at least part of their tegument and are enveloped in the cytoplasm.

3. Production of infectious progeny virus (lytic infection) is generally accompanied by the destruction of the infected cell.
4. The herpesviruses examined to date employ cellular latency as a mechanism for lifelong persistence in their hosts (Fig. 59.3).

Herpesviruses also differ with respect to many of their biological properties. Some have a wide host cell range, multiply rapidly, and quickly destroy the cells they infect (e.g., HSV-1, HSV-2). Others have a narrow host cell range (EBV, HHV-6) or a long replicative cycle (HCMV). Herpesviruses can differ substantially with respect to the details of the mechanisms they use to manage host responses to infection and in the pathogenic mechanisms and clinical manifestations of diseases they cause.

NOMENCLATURE AND CLASSIFICATION

Herpesvirus Species

The definition of viral species accepted by the International Committee on Taxonomy of Viruses (ICTV) is “A virus species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche”.¹²⁵ Consistent with this, “A herpesvirus may be classified as a species if it has distinct epidemiologic or biological characteristics and a distinct genome that represents an independent replicating lineage”.¹⁰² Several circumstances have arisen that might seem to challenge these definitions, but as illustrated here, the definition allows resolution of the issues.

The EBV variants, EBV-1 and EBV-2, differ markedly in several EBNA gene sequences. The differences lead to differ-

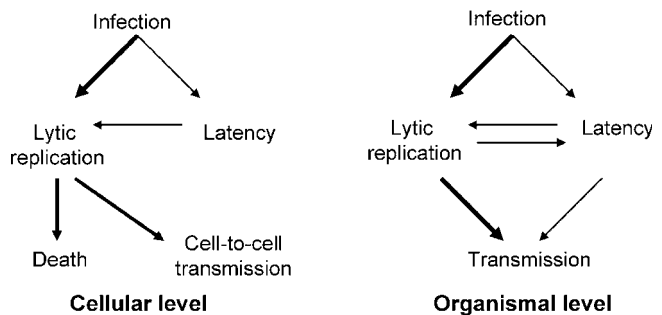


FIGURE 59.3. Outcomes of lytic and latent infections at the cellular and organismal levels. Thicker arrows represent more common events.

ences in some biological properties, including transforming potential. However, the variants do not occupy distinct ecological niches, the differences map to a small number of genes, and intermediates carrying one variant allele at one locus and the other variant allele at another locus have been detected. Thus, the EBV variants are recognized as allelic variants of the same species. At least two alleles have been identified of the KSHV K15 gene, which maps at the right end of the unique segment of the genome.¹⁰⁵ As for the EBV variants, recombinants have been detected between viruses encoding the different K15 alleles; thus, this represents another example of intraspecies allelic variation. It is possible that the EBV and KSHV allelic variants represent early steps in speciation. The process of adaptation to a specialized niche would likely result in the gradual emergence of a new virus lineage characterized by additional mutations across its genome, a unique epidemiology, and reduced biological fitness of recombinants with the parental lineage. The net product would be a new virus species.

Species Nomenclature

Two forms of nomenclature are employed for herpesviruses: an informal (also known as vernacular or colloquial) nomenclature that often traces to the early days of virology, and a formal nomenclature that is sanctioned by the ICTV. For example, the virus informally known as Epstein-Barr virus (EBV) is formally known as *Human herpesvirus 4*. Table 59.1 and e-Table 59.2 include formal and informal names for each virus. In the ICTV-endorsed formal nomenclature, herpesvirus names consist of the family, subfamily, or genus of the natural host of the virus, the word “herpesvirus,” and a serial Arabic number (e.g., *Cercopithecine herpesvirus 1*; Table 59.1). Human herpesviruses are an exception to the host name rule (e.g., *Human herpesvirus 7*). ICTV-recognized virus species names are italicized, and the first letter of the first word of the name is capitalized.¹²⁴ It has been proposed to rename herpesvirus species according to their subfamily, for example, *Human herpesvirus 1* would be renamed as *Human alphaherpesvirus 1*, but this has not been formally approved.

The host name–serial number nomenclature system was adopted in 1973^{106a} in an attempt to rectify problems associated with earlier systems in which viruses were named based on their disease associations, discoverer, geographic source, or whatever inspired the discoverer. Some viruses were given multiple names, and some names were applied to multiple viruses. Naming viruses after their associated diseases caused problems because

TABLE 59.1 Herpesviruses of Humans and of Veterinary or Scientific Importance^a

Formal name ^b	Abbreviation	Vernacular names and synonyms ^c	Subfamily and genus ^d	G+C (%)	Group	Genome size (Kbp) ^e	Genome accession no. ^f
Family HERPESVIRIDAE							
Viruses of humans							
<i>Human HV 1</i>	HHV-1	Herpes simplex virus [type] 1	α S	68.3	E	152	NC_001806
<i>Human HV 2</i>	HHV-2	Herpes simplex virus [type] 2	α S	70	E	155	NC_001798
<i>Human HV 3</i>	HHV-3	Varicella-zoster virus	α V	46	D	125	NC_001348
<i>Human HV 4</i>	HHV-4	Epstein-Barr virus	γ L	60	C	172	NC_007605 (type 1) NC_009334 (type 2)
<i>Human HV 5</i>	HHV-5	Cytomegalovirus (CMV)	β C	57	E	236 230	NC_006273 ^f NC_001347 ^f
<i>Human HV6A</i>	HHV-6A	HHV-6 variant A	β R	43	A	159/170 ^g	NC_001664
<i>Human HV6B</i>	HHV-6B	HHV-6 variant B	β R	43	A	162/168 ^g	NC_000898
<i>Human HV 7</i>	HHV-7		β R	36	A	145	NC_001716
<i>Human HV 8</i>	HHV-8	Kaposi's sarcoma–associated HV (KSHV)	γ R	59	C	170/210 ^g	NC_003409 NC_009333
Viruses of nonhuman primates (order <i>Primates</i>)							
<i>Cercopithecine HV 9</i>	CeHV-9	Medical Lake macaque, Liverpool vervet HV, Patas monkey HV, simian varicella HV	α V	40	D	124	NC_002686
<i>Macacine HV 1</i>	McHV-1	B virus, HV simiae, [<i>Cercopithecine HV 1</i>]	α S	75	E	157	NC_004812
<i>Macacine HV 3</i>	McHV-3	Rhesus monkey CMV, [<i>Cercopithecine HV 8</i>]	β C	49	F	221	NC_006150
<i>Macacine HV 4</i>	McHV-4	Rhesus EBV-like HV, rhesus lymphocrypt HV, [<i>Cercopithecine HV 15</i>]	γ L	62	C	171	NC_006146
<i>Macacine HV 5</i>	McHV-5	Rhesus Rhadinovirus, [<i>Cercopithecine HV 17</i>]	γ R	52	B	131 ^h	NC_003401
<i>Saimiriine HV 2</i>	SaHV-2	Squirrel monkey HV, HV saimiri	γ L	46	B	155	NC_001350
<i>Saimiriine HV 3</i>	SaHV-3	Squirrel monkey CMV 1	β C	46		190	FJ483967
Undesignated		Macaca fuscata Rhadinovirus	γ R u	51		131	NC_007016
Viruses of other mammals							
Order Artiodactyla							
<i>Alcelaphine HV 1</i>	AIHV-1	Wilbebeest HV, malignant catarrhal fever HV of European cattle	γ M	61	B	160	NC_002531
<i>Bovine HV 1</i>	BoHV-1	Infectious bovine rhinotracheitis HV	α V	72	D	140	NC_001847
<i>Bovine HV 2</i>	BoHV-2	Bovine mammillitis	α S	64	E	133	
<i>Bovine HV 4</i>	BoHV-4	Movar HV	γ L	41	B	109 ^g	NC_002665
<i>Bovine HV 5</i>	BoHV-5	Bovine encephalitis HV	α V	75	D	138	NC_005261
<i>Suid HV 1</i>	SuHV-1	Pseudorabies virus, Aujeszky disease	α V	74	D	143	NC_006151
Order Carnivora							
<i>Felid HV 1</i>	FeHV-1	Feline HV 1, feline rhinotracheitis HV	α V	46	D	136	FJ478159
Order Rodentia							
<i>Murid HV 1</i>	MuHV-1	Mouse CMV	β M	59	A	235	NC_004065
<i>Murid HV 2</i>	MuHV-2	Rat CMV, Maastricht strain	β M	61	A	230	NC_002512
<i>Murid HV 4</i>	MuHV-4	Mouse HV strain 68, murine gammaherpesvirus 68	γ L	47	B	135	NC_001826

(continued)

TABLE 59.1 Herpesviruses of Humans and of Veterinary or Scientific Importance^a (Continued)

Formal name ^b	Abbreviation	Vernacular names and synonyms ^c	Subfamily and genus ^d	G+C (%)	Group	Genome size (Kbp) ^e	Genome accession no. ^f
Order							
<i>Perissodactyla</i>							
<i>Equid HV 1</i>	EHV-1	Equine HV 1, equine abortion HV	α V	57	D	150	NC_001491
<i>Equid HV 2</i>	EHV-2	Equine HV 2; equine CMV	γ P	58	A	184	NC_001650
<i>Equid HV 4</i>	EHV-4	Equine HV 4; equine rhinopneumonitis virus	α V	51	D	146	NC_001844
Order Proboscidea							
<i>Elephantid HV 1</i>	EIHV-1	Endotheliotropic elephant HV 1	β P				
Viruses of birds							
<i>Anatid HV 1</i>	AnHV-1	Duck plague HV, duck enteritis virus	α M	45	D	161	JF999965
<i>Gallid HV 1</i>	GaHV-1	Infectious laryngotracheitis virus	α I	46	D	165	NC_06623
<i>Gallid HV 2</i>	GaHV-2	Marek disease HV 1	α M	47	E	180	NC_002229
<i>Gallid HV 3</i>	GaHV-3	Marek disease HV 2	α M		E		NC_002577
<i>Meleagrid HV 1</i>	MeHV-1	Turkey HV 1	α M	47	E	161	NC_002641
<i>Psittacid HV 1</i>	PsHV-1	Parrot HV; Pacheco disease virus	α I	60		163	NC_005264
Family ALLOHERPESVIRIDAE							
<i>Cyprinid HV 3</i>	CyHV-3	Koi herpesvirus	Cy	59	A	295	NC_009127
<i>Ictalurid HV 1</i>	IcHV-1	Channel catfish HV	Ic	56	A	130	NC_001493
Family MALACOHERPESVIRIDAE							
<i>Ostreid HV 1</i>	OsHV-1	Pacific oyster HV	Os	39	E	207	NC_005881

^aThe table was extracted and extended from information compiled by the *Herpesvirales* Study Group of the International Committee on Taxonomy of Viruses (ICTV). For details, see refs. 24,56,100,102,107,108.

^bFormally recognized HV species are italicized. Information about hosts is in bold.

^cRetired formal names are in brackets.

^dGenus designations: *Alphaherpesvirinae*: S, *Simplexvirus*; V, *Varicellovirus*; M, *Mardivirus*; I, *Iltovirus*. *Betaherpesvirinae*: C, *Cytomegalovirus*; M, *Muromegalovirus*; R, *Roseolovirus*; P, *Proboscivirus*. *Gammaherpesvirinae*: L, *Lymphocryptovirus*; R, *Rhadinovirus*; M, *Macavirus*; P, *Percavirus*. *Alloherpesviridae*: Ba, *Batrachovirus*; Cy, *Cyprinivirus*; Ic, *Ictalurivirus*; Sa, *Salmonivirus*. *Malacoherpessviridae*: Os, *Ostreavirus*.

Viruses that have not been approved as species are not italicized. Formally unassigned species and viruses are indicated by U and the subfamily (if known). Unofficial provisional assignments made by the authors are indicated by u.

^eGenome lengths are fuzzy for some viruses; unique segment lengths are sometimes known, and often the unit length of TR, but not TR copy number.

^fAccession numbers are provided for a clinical isolate of HCMV (Merlin) and widely studied laboratory strain (AD169), respectively.

^gValues obtained in different laboratories may reflect differences in strains.

^hComposition and length data are for the long unique segment, not including terminal repeats.

some viruses do not cause a specific disease, some viruses cause multiple and quite different diseases, and other viruses cause diseases whose etiologies are comprised of multiple agents. The formal nomenclature scheme was not meant to supplant the informal names that have been grandfathered by time and usage. The intent was to create an orderly system in which each virus would be named unambiguously and independently of classification or properties, which at that time were largely unknown. An important, and sometimes misunderstood, extension of this is that the species number is not intended to imply anything about the relationship between a virus and other herpesviruses that infect the same host species (e.g., HHV-7 and HHV-8 are members of different subfamilies) or between similarly num-

bered viruses that infect different host species (e.g., EHV-2 and BoHV-2 are members of different subfamilies).

Classification

In the late 1970s, before viral DNA and amino acid sequences were known, herpesviruses were initially classified into one family (the *Herpesviridae*) and three subfamilies (the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae*) on the basis of biological properties.¹⁰⁷ Remarkably, this framework remains useful and is supported by a deep and growing body of information extracted from nucleotide sequences. The viruses have been further classified into genera based on DNA sequence similarity, similarities in genome sequence

TABLE 59.2 Genes Conserved among the Alpha-, Beta-, and Gammaherpesviruses

Function ^a	HSV Homolog	Gene Block ^b	Function ^a	HSV Homolog	Gene Block ^b
Gene Regulation			<i>Capsid</i>		
Multifunctional regulator of expression (MRE)	UL54	3	Major capsid protein (pentons and hexons; MCP)	UL19	5
Nucleotide Metabolism			Portal protein (PORT)	UL6	6
Ribonucleotide reductase, large subunit (RR1)	UL39	1	Portal capping protein (PCP)	UL25	4
Uracil-DNA glycosylase (UNG)	UL2	7	Capsid triplex monomer (TRI1)	UL38	1
Deoxyuridine triphosphatase (dUTPase)	UL50	3	dimer (TRI2)	UL18	5
DNA Replication			Small capsid protein (SCP) at hexon tips	UL35	1
Helicase/primase complex			<i>Tegument</i>		
ATPase subunit (HP1)	UL5	6	Encapsidation and egress protein (EEP)	UL7	6
RNA pol subunit (HP2)	UL52	3	Myristoylated/palmitoylated cytoplasmic egress tegument protein (CETP)	UL11	6
Subunit C (HP3)	UL8	6	Virion protein kinase (VPK)	UL13	6
DNA polymerase (POL)	UL30	2	Encapsidation chaperone protein (ECP)	UL14	6
ssDNA binding (SSB)	UL29	2	CETP binding protein (CETPbp)	UL16	6
DNA polymerase processivity subunit (PPS)	UL42	1	Capsid transport tegument protein (CTTP)	UL17	6
Virion			Cytoplasmic egress facilitator 2 (CEF2)	UL21	5
<i>Nonstructural; roles in virion maturation</i>			Cell-to-cell fusion inhibitor	UL24	4
Alkaline exonuclease (NUC)	UL12	6	Large tegument protein (LTP)	UL36	1
Capsid transport nuclear protein (CTNP)	UL32	1	LTP binding protein (LTPbp)	UL37	1
Terminase binding protein (TERbp)	UL33	1	Cytoplasmic egress facilitator 1 (CEF1)	UL51	3
Terminase (TER)			<i>Envelope</i>		
TER ATPase subunit (TER1)	UL15	6	Glycoprotein B (gB)	UL27	2
TER DNA recognition subunit (TER2)	UL28	2	Glycoprotein H (gH)	UL22	4
Assembly protease (PR)	UL26	4	Glycoprotein L (gL)	UL1	7
Assembly protein precursor (pAP)	UL26.5	4	Glycoprotein M (gM)	UL10	6
Capsid nuclear egress complex			Glycoprotein N (gN)	UL49.5	3
Nuclear egress membrane protein (NEMP)	UL34	1			
Nuclear egress lamina protein (NELP)	UL31	1			

^aNomenclature and abbreviations are as described and proposed in.⁸⁴

^bGene blocks are as illustrated in Figure 59.5.

arrangement, and immunologic relatedness of important viral proteins.

There has been an explosion of herpesviruses discovered through the use of degenerate polymerase chain reaction (PCR) primers that target highly conserved regions in core genes, such as the DNA polymerase.¹¹⁰ Most of this work has been done by assaying blood specimens, thus the list of known lymphotropic gammaherpesviruses has expanded greatly and additional beta-herpesviruses have been identified. Expansion of the hunt to somewhat more difficult to obtain materials such as saliva or throat swabs and ganglia will likely yield a new array of novel viruses, including alphaherpesviruses. These new observations are important in that they thus far affirm the long-held hypothesis that individual animal species, or groups of closely related species, are hosts to particular herpesvirus species, with mammalian hosts being inhabited by viruses that represent each of the major mammalian herpesvirus lineages. Although the PCR

amplimers sequenced in many of these studies are less than 400 base pairs (bp) long, the information is usually sufficient for reliable assignment of the novel virus to an established subfamily; genus-level assignments are less reliable in the absence of more extensive information.

Alphaherpesvirinae

This subfamily is defined on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily—but not exclusively—in sensory ganglia. This subfamily contains the genera *Mardivirus* (GaHV-2), *Iltovirus* (GaHV-1), *Simplexvirus* (HSV-1), and *Varicellovirus* (VZV). *Simplexviruses* and *Varicelloviruses* have mammalian hosts, while *Mardiviruses* and *Iltoviruses* have avian hosts. Reptilian herpesviruses belong to the alphaherpesvirus lineage, but do not belong to any of the currently designated genera.⁷⁶

Betaherpesvirinae

A nonexclusive characteristic of the members of this subfamily is a restricted host range. The reproductive cycle can be long (over 7 days), and the infection progresses slowly in cultured cells. Infected cells frequently become enlarged (cytomegalia), and carrier cultures are readily established. Betaherpesviruses can establish latency in secretory glands, lymphoreticular cells, kidneys, and other tissues. This subfamily contains the genera *Cytomegalovirus* (HCMV), *Muromegalovirus* (MCMV), *Proboscivirus* (EIHV-1), and *Roseolovirus* (HHV-6).

Gammaherpesvirinae

The host range of the members of this subfamily is restricted to the family or order of the natural host. *In vitro* all members replicate in lymphoblastoid cells, and some can lytically infect particular types of epithelioid and fibroblastic cells. Viruses in this group are usually specific for either T or B lymphocytes. Latency is ordinarily established in lymphoid tissue. This subfamily currently contains four genera: *Lymphocryptovirus* (EBV), *Macavirus* (AIHV-1), *Percavirus* (EHV-2), and *Rhadinovirus* (KSHV). Rhadinoviruses are mainly hosted by primates, Macaviruses are related to the malignant catarrhal fever viruses of ruminants, and the identified Percaviruses are hosted by perissodactyl and carnivore species. The lymphocryptoviruses consist of two major lineages that appear to have co-evolved with their hosts: viruses of Old World (humans, chimpanzees) and New World (marmosets) primates.^{43,77}

VIRION ARCHITECTURE

The Virion

Mature herpesvirus virions vary in size from 120 to as much as 260 nm (reviewed in 109). The variation is in part due to variability in the thickness of tegument. Another major source of observed variability is the state of the envelope. Intact envelopes are impermeable and generally retain the quasispherical shape of the virion during preparation for electron microscopy (Fig. 59.1). Damaged envelopes are permeable to negative stains and the virions lose their quasispherical shape; permeated virions spread out on solid surfaces, resulting in a sunny-side-up egg appearance with a diameter much larger than for intact virions. The precise number of protein species contained in the virions is not known and may vary from one virus to another. Estimates based on analysis of purified virions in protein gels have generally been in the range of 35 to 45 major species. Proteomic analyses have identified from 24 to 71 virally encoded proteins in virions (4–7 in the nucleocapsid, 9–>20 in the tegument, and 4–19 in the envelope, plus a number of proteins whose location within virions is unknown).^{56,59,82,98,126} These and other studies also identified a number of host proteins in virion preparations, including moderately abundant quantities of cellular structural proteins, enzymes, and chaperones; actins and annexins have been consistently identified. The roles and necessity of the host-derived proteins in infection are not known. The abundance of individual protein species varies widely, from less than one copy per virion to over 1,000. Individual virions harbor on the order of 10,000 individual protein molecules. In addition to proteins, virions can include viral- and cellular-encoded messenger RNAs (mRNAs) that can be translated immediately after infection.^{11,114}

While a lot is known about the average composition of populations of virions, the molecular definition of what constitutes an infectious virion remains to be defined. For example, gB is present in virion preparations at levels that correspond to an average of approximately 800 copies per virion. It is not known how widely the number of copies per virion ranges, or how many copies of gB are required to be present on a virion for it to be infectious. Importantly, most of the virus particles released from infected cells are not competent for virus replication but are bioactive in various ways. These include particles that appear by electron microscopy to be intact and complete virions of the sort just described. In addition, herpesviruses can produce large numbers of nonvirion particles of unknown biological significance, such as the dense bodies of HCMV, which are capsid-free, enveloped collections of tegument proteins.

Virion Components

The Core

The core of the mature virion contains a single molecule of the viral genome, in the form of nonchromatinized dsDNA that is packed in an orderly manner in the form of a torus.^{40,41,64,93,138} In some herpesvirions, the torus appears to be suspended by a proteinaceous spindle consisting of fibrils embedded in the underside of the capsid and passing through the hole of the torus. The precise arrangement of the DNA in the torus is not known, but the DNA is packed tightly, such that the internal volume of the capsid is approximately equal to the cylindrical volume of the genome. Because of the repulsive forces generated by the negatively charged phosphates that make up the backbone of the genome, such compact packing necessitates the abundant presence in the core of the anion spermine.⁴⁵ Packaging of the viral genome into the capsid core requires ATP and results in a pressurized system that appears to be important for injection of virus genomes through the nuclear pore complex into nuclei of newly infected cells.⁶³

The Capsid

The structural features of the capsid—that is, its 100-nm diameter, 161 capsomeres (150 hexons and 11 pentons), portal complex, and capsid triangulation number ($T = 16$)—are characteristic of all herpesviruses, including the distantly related fish and oyster viruses.^{9,31,64} Nonenveloped capsids are present in infected cells in three main forms: A-, B-, and C-capsids.⁴⁴ A-capsids have no core structure, B-capsids contain the assembly scaffold but no genome, and C-capsids are DNA-containing species that no longer house the scaffold. The four conserved capsid proteins that comprise the major structural features of the capsid include the major capsid protein (MCP), the monomer and dimer proteins of the triplex (TRI1 and TRI2, respectively), and the small capsomere-interacting protein (SCP; HSV homologs are listed in Table 59.2). MCP is present in six copies per hexon ($6 \times 150 = 900$ copies per capsid) and five per penton ($5 \times 11 = 55$ copies per capsid), for a total of 955 copies per capsid. The triplex proteins interact with $\alpha\beta$ stoichiometry and form complexes that are present at the 320 sites of threefold symmetry. Hexameric capsomeres are 9.5×12.5 nm in longitudinal section; a channel of 4 nm in diameter runs from the surface along their long axis.¹³³ Penton channels are generally somewhat narrower and are nearly closed at their midpoint in B-capsids.¹³⁵ The twelfth pentonal position is the portal for transit of genomic

DNA into and out of the capsid; it is composed of 12 copies of the capsid portal protein (PORT). The portal capping protein (PCP) is associated with mature, DNA-containing nucleocapsids. Cryoelectron microscopic image analysis has enabled reconstruction of capsid structures to greater than 10 Å resolution.¹³⁹ Among other things, this has revealed a unique protein fold in the herpesvirus major capsid protein that is shared with the capsid proteins of tailed DNA bacteriophages, in support of a primordial linkage between these viruses.⁷ Beautiful animated representations of herpesvirus capsids and capsid components are available at <http://www.eicn.ucla.edu/animations>.

The Tegument

The tegument, a term introduced by Roizman and Furlong¹⁰⁹ to describe the proteinaceous structure between the nucleocapsid and the envelope, has no distinctive features in thin sections but may appear to be fibrous on negative staining.^{90,91,133} The tegument is sometimes distributed asymmetrically, and its thickness may vary, depending on the location of the virion within the infected cell. When the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space.⁴⁰ Some evidence suggests that the amount of tegument is more determined by the virus than by the host.⁷¹ Teguments can contain more than 20 different virally encoded proteins, some of which are present at hundreds of copies per virion. Structural polarity across the tegument has been visualized by immunoelectron microscopy,¹²⁰ indicating that it is an ordered structure, an observation supported by cryoelectron microscopic observations of tegument–nucleocapsid interactions.^{123,134} This is further evidenced by tegument proteins that are closely associated with the nucleocapsid (inner tegument) being acquired in the nucleus and by interactions between envelope glycoproteins and tegument proteins at the tegument periphery. Following nuclear egress, subsequent components of the tegument are likely added in a somewhat ordered manner as the virus particle matures during its trek through the cytoplasm.⁸⁰ One purpose of the tegument is to carry into newly infected cells an assortment, or toolbox, of already synthesized proteins that can immediately begin to manage the host environment to meet the needs of the virus, such as by shutting down host protein synthesis, inhibiting infection-triggered cell defenses, and stimulating viral gene expression.

The Envelope

Electron microscopic studies on thin sections have shown that the outer covering of the virion, the envelope, has a typical trilaminar appearance.³⁸ The presence of lipids was demonstrated by analyses of virions⁵ and by the sensitivity of the virions to lipid solvents and detergents.¹¹⁸ Virion envelopes are derived from patches of altered membrane that trace to the organelle where envelopment occurs.^{3,40,66,91} A major constituent of virion envelopes is a collection of virally encoded glycoproteins. The number and relative amounts of viral glycoproteins vary among herpesviruses. HSV specifies at least 11 different virion-associated glycoproteins, and the copy number of individual glycoproteins can exceed 1,000 per virion. The glycoproteins form numerous protrusions on virion envelopes that are more numerous and shorter than those present on the surface of many other enveloped viruses.¹³³

GENOMIC AND GENETIC ARCHITECTURE

Genome Size and Base Composition

Herpesvirus DNAs extracted from virions and characterized to date are linear and double stranded, but they circularize immediately on release from nucleocapsids into the nuclei of infected cells.

Distinguishing features of herpesvirus DNAs include their length and base composition. The length of herpesvirus DNAs varies from approximately 124 to 295 kbp (Table 59.1 and e-Table 59.2). The variability in genome lengths of different herpesviruses is distinct from the generally less extensive polymorphism in the size of DNAs of individual viruses. Thus, herpesvirus genomes contain terminal and internal reiterated sequences that can vary in copy number, as well as sequences that can be lost or duplicated during passage in cell culture, leading to intraspecies variation in genome lengths that can exceed 10 kbp.

The base composition of herpesvirus DNAs varies from 31% to 77% total G+C content (Table 59.1 and e-Table 59.2). Furthermore, herpesvirus DNAs vary with respect to the extent of homogeneity of G+C content across the length of the genome (generally higher G+C composition in terminal repeats). The extent of inhomogeneity in the base composition varies from minimal (e.g., HSV) to very extensive. For example, the genome of MuHV-4 has a G+C content of 46% in its unique region and 78% in its terminal repeats.¹³⁰

The genetic requirements for efficient replication in cultured cells can differ from the *in vivo* requirements. Spontaneous deletions have been noted in HSV and EBV strains passaged outside the human host (e.g., EBV strain P3HR1, HSV-1 strain HFEM). Highly passaged strains of HCMV lack a segment encoding at least 19 genes that are present in wild-type isolates.¹⁷ A progression of changes in a small number of genes enables clinical isolates of HCMV to begin replicating efficiently in cultured cells.²³

Sequence Arrangements

An interesting feature of herpesvirus genomes is their sequence arrangement. The sequence arrangements shown in Figure 59.4 emphasize the presence and location of reiterations of terminal sequences greater than 100 bp. According to this scheme, herpesviruses genomes can be divided into six groups designated by the letters A to F. In the genomes of viruses comprising group A and exemplified by HHV-6, a large sequence from one terminus is directly repeated at the other terminus. In the group B genomes, exemplified by herpesvirus saimiri (SaHV-2), the terminal sequence is directly repeated numerous times at both termini; furthermore, the number of reiterations at the termini may vary. In the group C genomes exemplified by EBV, the number of direct terminal reiterations is smaller. In addition, group C genomes can harbor other direct sequence arrays that subdivide the unique (or quasiunique) sequences of the genome into several well-delineated stretches. In group D genomes, exemplified by VZV, the sequence from one terminus is repeated in an inverted orientation internally. In these genomes, the domain consisting of the stretch of unique sequences flanked by inverted repeats (Small or S component) can invert relative to the remaining sequences (Large or L component) such that the DNA extracted from virions or infected cells consists of two equimolar populations differing solely in the orientation of the S component

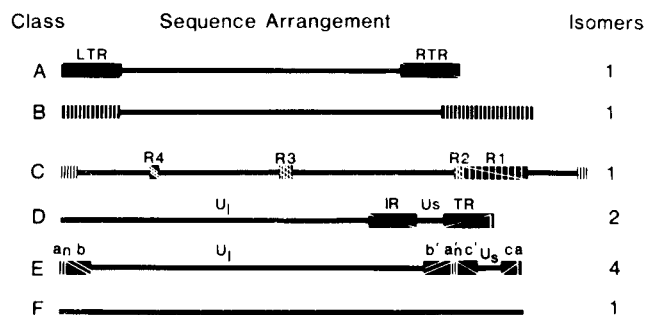


FIGURE 59.4. Schematic diagram of the sequence arrangements in the six classes of genomes of the viruses comprising the family *Herpesviridae*. Group A, B, C, D, E, and F genomes are exemplified by the human herpesvirus 6, herpesvirus saimiri, EBV, varicella-zoster virus, herpes simplex viruses, and tupaia herpesvirus, respectively. In the schematic diagram, *horizontal lines* represent unique or quasiunique regions. The reiterated domains are shown as *rectangles* and are designated as left and right terminal repeats (LTR and RTR) for group A, repeats R1 to R4 for internal repeats of group C, and internal and terminal (IR and TR) repeats of group D. The termini of group E (e.g., HSV) consist of two elements. One terminus contains *n* copies of sequence *a* next to a larger sequence designated as *b*. The other terminus has one directly repeated *a* sequence next to a sequence designated as *c*. The terminal *ab* and *ca* sequences are inserted in an inverted orientation (denoted by primes) separating the unique sequences into long (*U_L*) and short (*U_S*) domains. Terminal reiterations in the genomes of group F have not been described. In group B, the terminal sequences are reiterated numerous times at both termini. The number of reiterations at each terminus may vary. The components of the genomes in groups D and E invert. In group D, the short component inverts relative to the long. Although the long component may also rarely invert, most of the DNA forms two populations differing in the orientation of the short component. In the group E genomes, both the short and long components can invert, and viral DNA consists of four equimolar isomers.

relative to the L component. In group E viral genomes, exemplified by HSV and HCMV, sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genomes into two components, each of which consists of unique sequences flanked by unrelated pairs of inverted repeats. In this instance, both components can invert relative to each other, and DNA extracted from virions or infected cells consists of four equimolar populations of isomers that differ in the relative orientation of the two components. The genomes comprising group F are exemplified by tupaia herpesvirus 1 (TuHV-1); the terminal sequences are not identical and are not repeated either directly or in an inverted orientation. Although the potential of inverted repeat sequences to cause inversions and gene expansion is well appreciated, their role and the advantage they confer to viral replication are not understood.

All herpesvirus genomes examined to date contain at their termini conserved *cis*-acting signals for packaging of the DNA into capsids and cleavage of concatemeric genomes to unit length (cleavage/packaging, or *pac* sequences).³²

Genetic Architecture Herpesvirus Genes

Most herpesvirus genes contain a collection of promoter/regulatory sequence elements that are located 50 to 200 bp

upstream of a TATA box, a transcription initiation site 20 to 25 bp downstream of the TATA box, a 5' nontranslated leader sequence of 30 to 300 bp, a single major open reading frame with a translation initiation codon that meets the host requirements for efficient initiation, 10 to 30 bp of 3' nontranslated sequence, and a canonical polyadenylation signal with standard flanking sequences (e-Fig. 59.1A). As illustrated, variation and exceptions exist and are important. Genes without a TATA box (e.g., HSV-1 γ 134.5 gene)²⁰ and initiation from a second in-frame methionine⁶⁹ have been reported. In the case of HSV late genes, the promoter-regulatory sequences may be located 3' to the TATA box.⁷⁰

Gene overlaps are common. For head-to-tail gene arrangements, the upstream gene can overlap with the promoter-regulatory sequences of the downstream gene (e-Fig. 59.1B). In several instances, a second transcriptional unit is embedded in a protein coding sequence to yield a shorter polypeptide that is initiated at an internal methionine within the same open reading frame as the larger protein (e.g., HSV UL26 and UL26.5; e-Fig. 59.1C).⁶⁵ The resulting proteins thus share a domain of identical amino acid sequence, yet can differ markedly in their function. Protein-encoding open reading frames can be expressed from mRNAs that are antisense to each other (e.g., HSV-1 γ 134.5 and ORF P and O; e-Fig. 59.1D).⁶² Common features of herpesvirus genomes are clusters of 3' co-terminal transcripts, each designed to express a different open reading frame (e-Fig. 59.1B). Within these clusters, coding domains seldom overlap by more than a few codons. Although most genes are transcribed by RNA polymerase II; some small transcripts are transcribed by RNA polymerase III, for example, the EBV EBER transcripts.⁵²

The predominant form of transcript for most herpesvirus genes is unspliced; that is, the primary transcription product only needs to be polyadenylated to form the mature mRNA. The number of genes for which the predominant transcript is spliced varies from very few for some herpesviruses to about 10%. One strategy is to use splicing to enable differential regulation of a gene at different parts of the virus life cycle. In some cases, this means the same open reading frame can be accessed from different promoters. Differential splice-site selection can be used to express different open reading frames from the same primary transcript (e-Fig. 59.1E). Recent application of deep sequencing to transcript analysis demonstrated that RNA splicing (including alternative splicing) is more common than previously recognized.⁴² Herpesviruses also encode noncoding RNAs, some of which are highly abundant, e.g., the Ori_S RNA and latency-associated transcripts of HSV, the EBERs of EBV, and microRNAs.^{104,115,121}

Genomes of members of the *Herpesviridae* encode between 70 (the smallest genome) and 200 (the largest genome) protein-coding genes. These estimates are rooted in studies of viral mutants, transcriptional mapping, analysis of the translation products of particular transcripts, and computational analyses of protein-coding potential. Current estimates probably understate the number of gene products. Noncoding RNAs are not included in these counts, nor are very small open reading frames that have not been identified as functional. Functions that arise as products of alternative splicing, translational frame shifting, internal translation initiation sites, antisense open reading frames, and tissue-specific transcription are only beginning to be fully appreciated. Elucidation of the true genetic

and corresponding functional complexity encoded in herpesvirus genomes remains a major objective of herpesvirus research.

Naming of Genes and Their Products

ICTV has established a framework for naming of herpesviruses, but there is no formal system for naming viral genes or their products. This has led to several problems that can be particularly daunting to newcomers to the field: the nomenclature applied to viral genes and their products is a hodgepodge, difficult to follow, and potentially misleading. Historical precedents, self-interests, and common usage maintain nomenclatures whose time has long passed. The nature of the problems facing us can be summarized with a few examples.

The HSV-1 virion transactivator was first identified as a component of the virion and designated by its migration in denaturing gels relative to that of other virion proteins (VP16).¹¹⁷ Subsequently, it was designated by its migration in denaturing gels relative to other infected cell proteins (ICP25).⁵¹ Still later it was designated by its apparent molecular weight in denaturing polyacrylamide gels (vmw65),¹⁶ its best known function (α -trans-inducing factor or α -TIF),¹⁰³ or by the position of the gene encoding it (UL48).⁷⁴ Another example is the major regulatory protein of HSV-1, which was designated initially as ICP4 and subsequently as vmw175 and α 4.

HSV is not the only herpesvirus beset by problems in nomenclature of its genes and proteins. For example, with the exceptions of the proteins expressed during latent infection, EBV genes and their products are named after a restriction endonuclease-generated DNA fragment in which the gene maps. This nomenclature precludes all but the most dedicated from visualizing the position of the gene.

The least precise current designations are based on apparent molecular weight. For example, ICP4 (vmw175) is expressed in several forms that differ in electrophoretic mobility in denaturing polyacrylamide gels. In addition, the apparent molecular weights relative to standards may vary, depending on the composition of the gel. Furthermore, although the HSV-2 homolog has a higher apparent molecular weight, it is sometimes designated by the apparent molecular weight of its HSV-1 counterpart.

The naming of homologous genes based on their HSV-1 counterparts enables a relational database but leads to a loss of information and may be misleading. For example, glycoprotein B is encoded as HSV UL28, HHV-6B U39, HCMV UL55, KSHV orf8, and so on. A designation such as gB (HHV-6B) addresses sequence homology but not map position. An additional hazard is the assumption that all functions of orthologous gene products are fully conserved from virus to virus. Most herpesvirus proteins studied in detail have multiple functions; proteins sharing a conserved block of amino acids may diverge in the functions encoded in their other domains; future nomenclatures may need to account for the multifunctionality of viral proteins. Ideally, protein names would convey key information useful to the field, such as major function, kinetic class, and genome map location.

What is being done? Gene name cross-references are often available in papers that describe complete herpesvirus genome sequences (e.g., 2,6,46,95), as well as in freely available compendia.^{67,83,84,106b} The unified nomenclature and abbreviation system proposed for genes conserved across the *Herpesviridae*⁸⁴ is used in this chapter. In addition, a more highly curated set of reference herpesvirus genome sequences has been compiled at

Genbank (<http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html>). These sequences have an accession number with an "NC_" prefix and are listed in Table 59.1 and e-Table 59.2. Reference sequences are derived from the original sequence submissions, but are maintained and curated separately to ensure a degree of uniformity in describing gene boundaries and names.

Gene Functions

The viral functome is the net functional manifestation of the viral genome, transcriptome, and proteome. A challenge in fully defining the viral functome is the fact that no experimental system can fully mimic the biology of the natural host. A variety of methods have been developed to enable genetic studies of herpesviruses. A widely used approach involves cloning complete herpesvirus genomes into plasmids that can replicate in *E. coli* as bacterial artificial chromosomes (BAC).^{131,137} This allows the use of powerful and specific mutagenesis methods, followed by transfection of the mutagenized genome into susceptible mammalian cells where the virus can be regenerated.

Two key characteristics define the functionality of herpesvirus gene products. First, they frequently have multiple functions that may or may not be related. For example, HSV-infected cell protein 0 (ICP 0 or α 0) is a promiscuous transactivator, but at a molecular level it first localizes sequentially in the nucleus and later in the cytoplasm, and interacts with several host proteins located in both compartments.^{39,60} Even some of the smaller proteins (e.g., HSV-1 US11 and γ 134.5) have been ascribed more than one function. The decision as to which function is performed by a particular protein may be determined by the nature of posttranslational modifications to which many herpesvirus proteins are subjected. Full assessment of the multifunctional nature of viral proteins and their dependence on interactions with other viral proteins requires that they be studied in the context of infection.

The second key characteristic relates to the role of virus-encoded proteins in viral replication. For HSV, more than 50% of the open reading frames are dispensable for growth in cells in culture. For the most part, however, these genes are not dispensable for viral replication or for reactivation from the latent state in experimental animal systems. In contrast, in assays involving establishment of latency, no virus gene has been found to be indispensable. Genes that are necessary for virus replication in cultured cells are sometimes referred to as "essential" or "fundamental," and dispensable genes as "nonessential" or "accessory."

Virally encoded proteins are required for entry into the cell, regulation of viral gene expression, nucleotide metabolism, synthesis of viral DNA, structural proteins, and virion assembly, as well as for management of host defenses, metabolism, and macromolecular biosynthesis and transport. Regulatory genes are specifically adapted to the requirements of the cell type inhabited by the virus *in vivo*. Some subsets of herpesviruses (e.g., the alphaherpesviruses) share clear homologs of regulatory genes such as α 4, α 0, and α 27. Other groups of herpesviruses have important regulatory genes that have little obvious sequence similarity, although they may occupy similar genomic locations and even share splicing patterns (e.g., the immediately early genes of the betaherpesviruses).

Every herpesvirus studied to date establishes latent infection in a specific population of cells. The mechanisms differ from one virus to the next. There appears to be no general strategy or shared genetic component for establishment, maintenance, or

termination of the latent state, even though latency is the cornerstone of herpesvirus biology. Although members of some genera of herpesviruses encode several proteins that are expressed during maintenance of the latent state (e.g., EBV), others (e.g., HSV) have no absolutely required expression of the viral genome during this phase of latency.

The cellular and organismal host ranges of herpesviruses differ enormously, from very wide (e.g., HSV) to very narrow (e.g., EBV, HHV-6B). The host range is determined only in part by the availability of receptors. Little is known about the virus- and cell-specific factors that enable herpesviruses to replicate in specific hosts or cell types.

Herpesviruses are able to alter the cellular environment to suit their needs. These alterations include shutting off or stimulating host-cell macromolecular synthesis, inducing or inhibiting host-cell DNA replication, or immortalizing the host cell. Many viral genes have been identified as specific regulators of these processes; this remains an area of active research.

The environment in which herpesviruses replicate is decidedly unfriendly to the virus. An armamentarium of viral functions is encoded by herpesviruses to fend off cellular and organismal reactions to the presence and expression of viral genes (reviewed in 8,27,85,86). At the cellular level, the virus blocks the induction of programmed cell death and the activation of the interferon pathway (e.g., by activation of protein kinase R). At the organismal level, the response is highly varied and depends in part on the cells in which the virus replicates. Here the responses range from blocking the presentation of antigenic peptides on the surfaces of antigenic cells to molecular mimicry, in which herpesvirus-encoded proteins perform some, but not all, functions of immunomodulators. A common consequence is a delay in the elimination of the infected cell—long enough to enable the virus to replicate, colonize the host, and become available for transmission to another nonimmune individual. The range of genes evolved to this end varies from one genus to another, with limited conservation among members of subfamilies. Alphaherpesviruses have adopted a different general strategy for accomplishing this than the beta- and gammaherpesviruses, in that the former sequesters cellular proteins and modifies them to perform novel functions (e.g., HSV-1 $\gamma_{134.5}$ and the cellular protein phosphatase 1 α). Beta- and gammaherpesviruses encode numerous orthologs of cellular proteins designed to mislead the cell or directly perform the desired function (often with altered regulatory properties). Herpesviruses have evolved multiple ways in which to attain the same objectives: for example, the many ways they block the action of interferon or block the attempt of infected cells to commit suicide (apoptosis). The diversity of approaches is a remarkable testimony both to the depth and complexity of host defenses and to the adaptive prowess of the virus. That the virus wins in the infected cell but normally allows the host to survive attests to an evolutionary necessity.

Regulation of Gene Expression

Herpesvirus genes belong to one of several regulatory classes; the more they are studied, the more complex the systems that regulate their expression appear to be. Standing apart are genes expressed during latency, and even here, as in the case of EBV, there are multiple forms of latency differing with respect to the genes that are expressed. Herpesvirus gene expression during productive infection represents a classic regulatory cascade,

the basic features of which are conserved across the family. In productively infected cells, viral genes form at least four groups differing with respect to the order of gene expression and the mechanisms that drive it. These are the α (immediate-early) genes that require no new (cellular or viral) protein synthesis for their expression, β (early) genes whose transcription is totally independent of viral DNA synthesis, γ_1 (leaky-late) genes whose expression is augmented by the onset of viral DNA synthesis, and γ_2 (true late) genes whose expression is totally dependent on viral DNA synthesis. The immediate-early, early, and late nomenclature is rooted in early phage studies; it has the virtue of instant recognition but does not fully describe the events occurring in the infected cell. Both systems are used widely and interchangeably.

As mentioned, most herpesvirus genes are transcribed by the host RNA polymerase II, as regulated by numerous viral and cellular proteins. An important aspect of transcriptional regulation relates to the state of chromatinization of the viral genome, which is dynamically regulated in a virus- and cell-specific manner from the time the viral genome is injected into the nucleus at the initiation of infection, and then throughout the processes of lytic replication and establishment, maintenance, and reactivation of latent infections. Transcriptional expression of herpesvirus genes during lytic infection thus appears to be regulated by sequential derepression of viral genes that were repressed by the host at the moment of viral DNA entry into the nucleus. At least for HSV-1, the initial stages of virus gene transcription during reactivation from neuronal latency can be disordered with respect to viral gene classes. This suggests that reactivation can involve a general, rather than targeted, derepression of viral gene expression.³³

Gene Relatedness and Arrangement

Complete genomic sequences have been determined for more than 50 herpesviruses (Table 59.1 and e-Table 59.2); smaller segments have been sequenced from many others. From this information, several fundamental principles of herpesvirus genetic architecture have been deduced.

Herpesvirus gene sequences are related to each other in ways that reflect the long-standing, biology-based classification of herpesviruses into subfamilies and their respective genera, as described here. These relationships are substantial and easy to detect with any of the widely available tools for sequence analysis. As mentioned, for viruses of the *Herpesviridae*, the nucleotide sequence of a 400-bp PCR product amplified from the viral DNA polymerase gene by the use of degenerate consensus primers is sufficient to establish the identity of a herpesvirus and place it into the appropriate subfamily. Higher resolution and more robust classification can be obtained by expanding the size of the comparison sequence and by analyzing more than one gene. Phylogenetic trees topologically similar to those obtained by comparing amino acid sequences have been obtained from comparisons based on which members of conserved sets of genes are represented in a given lineage or virus species.^{1,87} A representation of herpesvirus taxonomic and phylogenetic relationships is shown in Figure 59.2.

Forty-one genes, referred to as the herpesvirus core genes, have been identified as conserved across the alpha-, beta-, and gammaherpesviruses (Table 59.2). Each gene belongs to one of six core gene blocks. Within each gene block, gene order and polarity are conserved (Fig. 59.5). Across the family, the

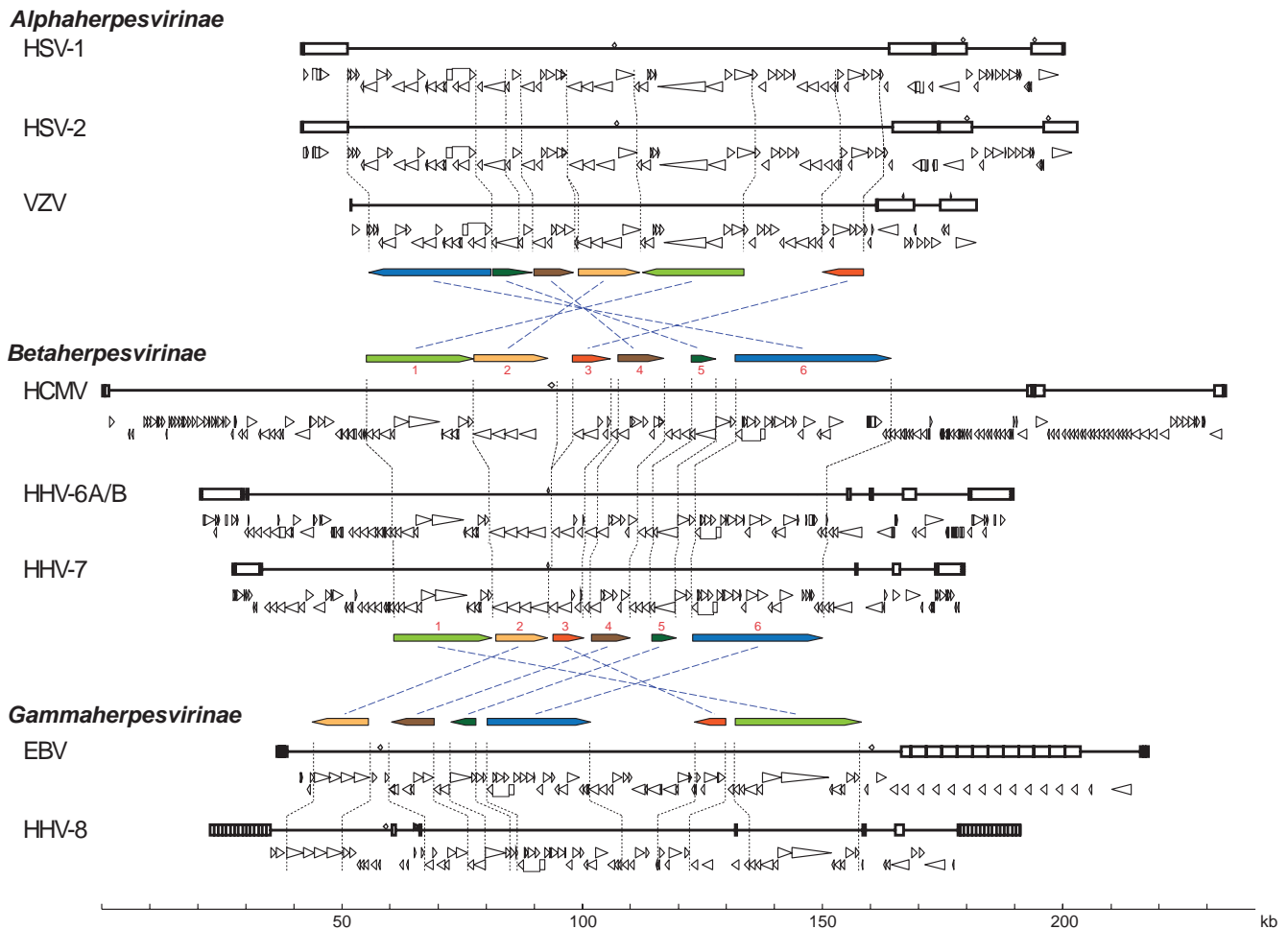


FIGURE 59.5. Genomic and genetic architectures of the human herpesviruses. Major repeat elements are indicated on each genomic schematic as *boxes*. Beneath each genome, open reading frames considered likely to encode expressed proteins are indicated as *triangles* that are oriented to show their direction of transcription. 5' exons of spliced genes are indicated as *boxes* that are connected by *bars* to 3' exons. The seven conserved herpesvirus sequence blocks (block 1 through block 7) are diagrammed to show their relative locations and orientations in the three major lineages. Diagrams are based on annotations and coordinates in Genbank accession numbers X14112 (HSV-1 strain 17), Z86099 (HSV-2 strain HG52), X04370 (VZV strain Dumas), NC_006273 (HCMV strain Merlin), X83413 (HHV-6A strain U1102), AF157706 (HHV-6B strain Z29), U43400 and AF037218 (HHV-7 strains JI and RK, respectively), NC_007605 (EBV strain B95-8), and U75698 (HHV-8 BC-1). Detailed descriptions are available in virus-specific chapters that follow. TRL, long component terminal repeat; IRL, long component internal repeat (inverted relative to TRL); IRS, short component internal repeat; TRS, short component terminal repeat (inverted relative to IRS); DRL and DRR, left and right direct repeats, respectively; TR, terminal direct repeat; IR, internal direct repeat. Abbreviations for conserved proteins are as listed in Table 59.2. A large format version of this diagram is available as [e-Fig 59.2](#).

core gene blocks are found in various permutations of order and orientation.^{18,30} Block arrangement, and thus gene order, is conserved at the subfamily level. As illustrated in Figure 59.6, conserved genes do not share identical sequences. In some instances, homologs share low-level sequence identity or similarity across their full lengths, while in other instances only a small portion is identifiably conserved. The implication is that the resulting proteins are an amalgam of conserved and diverged functions.

Some genes are conserved at the subfamily level. Thus, genes unique to alphaherpesviruses include their latency-associated genes, glycoprotein D, a tegument-associated protein that induces transcription from α -genes, and a transcriptional

regulatory protein related to HSV $\alpha 4$ (Fig. 59.6). Betaherpesviruses encode a block of 14 genes that have no counterparts elsewhere in the family, sets of diversified genes that are related to HCMV US22, and highly divergent immediate-early genes that nonetheless have shared evolutionary history. Gammaherpesviruses uniquely encode conserved proteins needed to maintain latent genomes in dividing cells.

An interesting variation on the linkage of gene sets to subfamilies is offered by the origin-binding protein (OBP) and its binding site. OBP binds to its cognate sites in origins of lytic replication and serves to nucleate the DNA replication machinery and play a role in initiating DNA replication. Only alphaherpesviruses and members of the *Roseolovirus* genus of

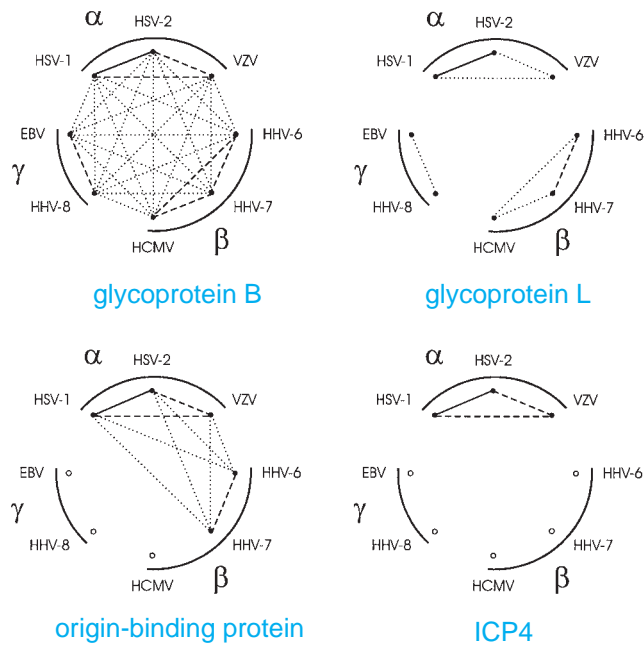


FIGURE 59.6. Patterns of gene conservation among the human herpesviruses. Viruses encoding the protein are indicated by the filled dots; empty dots indicate viruses that do not encode the protein. Degrees of similarity are indicated by the lines connecting the viruses; solid, dashed, and dotted lines represent >70%, 40% to 70%, and <40% identity, respectively. Glycoprotein B is highly conserved and is represented in all of the viruses; glycoprotein L is conserved within subfamilies; the origin-binding protein is conserved among the alpha herpesviruses and a subset of betaherpesviruses; ICP4 is found only in the alpha herpesviruses. The HHV-6A and HHV-6B counterparts of these proteins are highly related to each other and have similar distances to the other viruses, thus they are represented here as a single entity.

the betaherpesviruses encode OBP homologs and OBP binding sites⁵⁴ (Fig. 59.6). Other betaherpesviruses and the gamma herpesviruses do not encode OBP homologs or binding sites, and initiate DNA replication by another mechanism.

Herpesviruses generally encode at least one gene of obvious host origin (reviewed in 53,106); KSHV has acquired at least 12.¹¹¹ Examples include thymidylate synthase and *bcl-2*. These genes appear to have been acquired by retrotransposition events from cDNAs, given that they are most often encoded on the viral genome as continuous open reading frames that do not require splicing. Some of these genes appear to have been acquired independently by different herpesvirus lineages (again, *bcl-2* and thymidylate synthetase). Host-acquired genes tend to be located toward the viral genomic termini and between core gene blocks. In some cases, the host-acquired genes retain a function similar to its cellular counterpart; expression from the viral genome allows the desired product to be produced when needed by the virus. In other cases, the host-acquired gene has been modified to alter its function. For example, the KSHV homolog of the cellular D-type cyclin does not respond to signals that inhibit the cellular version, rendering the viral version constitutively active and capable of transforming cells.⁴

BIOLOGICAL CYCLE OF HERPESVIRUSES

The herpesvirus biological cycle can be divided into three major components: initiation of infection, lytic replication, and latency (Fig. 59.7). These components are linked by a biological decision in the infected cell to follow either the lytic or latent pathway, as well as by the ability of latently infected cells to be reactivated to a lytic state. Here we provide a general outline of the herpesvirus biological cycle; detailed descriptions of the diverse mechanisms employed by the various viruses to accomplish this are provided in subsequent chapters. It is important to

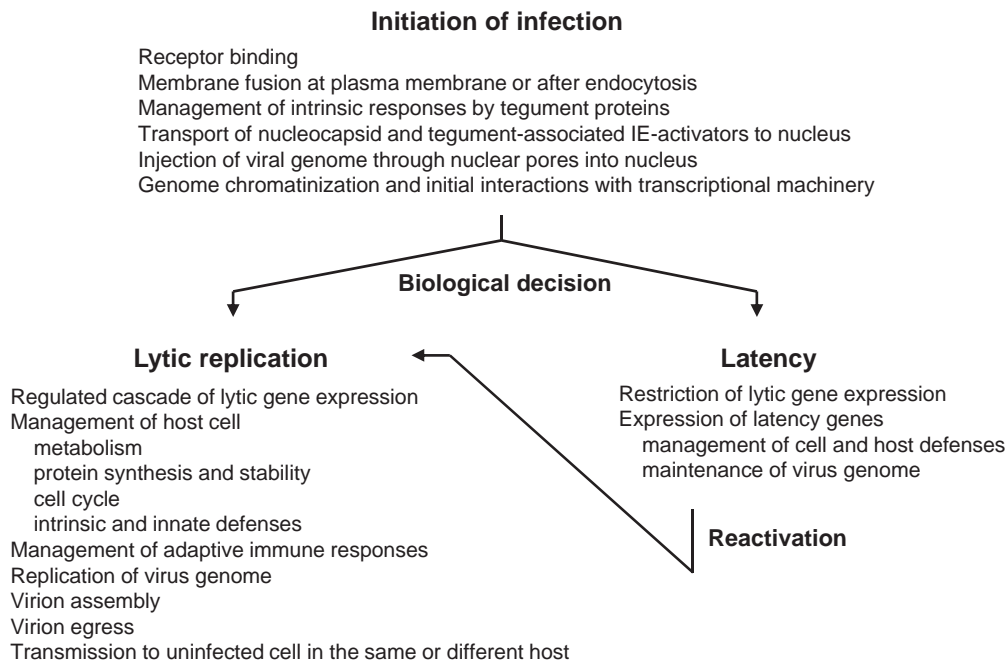


FIGURE 59.7. Herpesvirus biological cycle.

bear in mind that essentially every step and stage of the herpesvirus biological cycle is dependent on the virus making use of or adapting for its purposes cellular systems, structures, and materials. Host interactions include modification of cellular systems for regulating gene expression (transcription, mRNA processing and transport, RNA stability, translation, posttranslational processing, management of protein lifetimes), modulation of the cell cycle (e.g., to drive it to particular stages to ensure that necessary replicative precursors are available as needed), reprogramming metabolic pathways for energy production and generation of biosynthetic precursors, remodeling of the secretory apparatus, using intracellular transportation systems throughout the infectious cycle, and managing host defenses.

Initiation of Infection

Initiation of infection spans the events that begin with receptor binding through the initial interactions of the virus genome with the host transcriptional machinery in the nucleus (e-Fig. 59.3A). The general features of this stage occur in cells destined for immediate entry into the lytic state, as well as in cells that will become latently infected.

Because they infect wide varieties of cell types, individual herpesviruses employ multiple cell surface receptors for virion entry. These interactions are mediated by individual, or combinations of, virion surface virus glycoproteins. For most, if not all herpesviruses, the initial binding of the virion to the host cell involves interactions of gB with cell surface heparan sulfate proteoglycans. After receptors are fully engaged, herpesviruses enter cells by two major pathways: fusion of the virion envelope with the plasma membrane at the cell surface, or membrane fusion after virion uptake by endocytosis. Fusion of virion and cellular membranes is mediated by gB and the gH/gL complex. gB is a Class III fusion protein that has structural features in common with membrane fusion proteins encoded by viruses as diverse as vesicular stomatitis virus and baculoviruses.⁵⁰ In addition to their roles in virion entry, virion–receptor interactions activate cellular signaling pathways, some of which the virus takes advantage of, while others need to be redirected or blunted.

As part of entry, virus particles must interact with and ultimately penetrate the cytoskeleton. Because of the macromolecular density and highly structured nature of the cytoplasm, objects the size of nucleocapsids are unable to migrate from the cell surface to the nucleus by diffusion alone. By way of interactions that ultimately link to microtubule motors, nucleocapsids hitch rides on microtubules for active transport to nuclear pores. By unknown mechanisms, nucleocapsids dock with the nuclear pore complex and then release their DNA so it is translocated into the nucleus.⁶³ Injection of the genome into the nucleus is powered in part by pressurization of the capsid during virion assembly plus active consumption of ATP during translocation of the genome through the nuclear pore complex. Almost immediately upon entry into the nucleus, the virus genome is partially or unstably chromatinized. The nature of the chromatinization and the availability and state of particular viral and cellular transcription regulators are major determinants of the biological decision to either enter the lytic replication cycle or establish a latent infection.⁹⁴ Tegument proteins that are released into the cytoplasm during entry can have effector activities in the cytoplasm or nucleus. These include management of intrinsic defense responses and transcriptional activation of α genes.

Lytic Replication

The purpose of lytic replication is to manufacture infectious virions that can infect other cells and hosts. As outlined in Fig. 59.7 and e-Fig. 59.3, lytic replication includes regulated expression of virus genes, genome replication, virion assembly, egress, and transmission, all the while managing a wide range of host activities and defenses. As with any assembly line manufacturing process, virion assembly is dependent on production of appropriate levels of the various parts, plus mechanisms to ensure their presence in the proper locations when they are needed. Coordination of events during lytic infection is regulated as a function of state of infection rather than by synchronization to a molecular clock. Lytic replication involves sophisticated use of preexisting cellular compartments such as the nucleus, cytoplasm, and cytoplasmic organelles, as well as their modification and sometimes subcompartmentalization. As studied in detail for HSV, at early times after infection, gene transcription, genome replication, and nucleocapsid assembly take place in distinct but adjacent subnuclear compartments. Later in infection these appear to merge into a single large nuclear compartment, but the underlying functional organization is likely to remain.

Expression and Activities of Lytic Genes

Regulatory properties of herpesvirus gene classes were described earlier. Regardless of whether the lytic pathway is entered directly as part of a new infection, or via reactivation from latency, a key event is triggering α gene transcription in a manner that drives the coordinated cascade of lytic gene infection. As detailed in subsequent chapters, mechanisms for regulating initiation of the lytic cascade differ from virus to virus, in different cell types, and for new versus reactivated infections.

Herpesviruses typically encode approximately five α genes, whose products perform a number of regulatory functions, including activation of β gene transcription, modulation of the cell cycle, chromatin structure, RNA transport and splicing, and blunting innate, intrinsic, and adaptive immune responses. Most β gene products are involved in virus genome replication. This includes enzymatic activities that ensure adequate pools of nucleotides for viral DNA synthesis, as well as the proteins and enzymes necessary for DNA synthesis at the replication fork. Most γ gene products are virion proteins.

Genome Replication

Replication of the virus genome is dependent on the presence of sufficient biosynthetic precursors, *cis*-acting signals in the virus genome that guide assembly of the replication complex and initiation of DNA replication, and *trans*-acting factors that replicate the virus genome in a manner that is sufficiently orderly to generate several thousand genomic copies from as few as one starting template, then efficiently encapsidate the nascent genomes. In addition to encoding enzymes involved in nucleotide metabolism, some herpesviruses (e.g., HSV) alter the flow through metabolic pathways to result in enhanced pyrimidine biosynthesis,¹²⁷ while others (e.g., HCMV) appear to manipulate the tricarboxylic acid cycle to help ensure adequate supplies of lipids that are used for other stages of replication.^{92,127,136}

Herpesvirus genomes have 1 to 3 *cis*-acting sites that can act as origins of lytic DNA replication (*oriLyt*). For alphaherpesviruses and a subset of betaherpesviruses (members of the *Roseolovirus* genus), assembly of the replication complex at

these sites is dependent on the virally encoded origin-binding protein (OBP), which binds to specific oriLyf sequences and then triggers conformational changes in the virus genome that enable assembly of the replication complex and initiation of replication. Other herpesviruses employ virally encoded DNA-binding transcription factors for oriLyf activation. Regardless of how oriLyf is activated, a set of six proteins that are conserved across the *Herpesviridae* interact to form the enzymatic complex that replicates the genome. These proteins include the viral DNA polymerase (POL) and its associated processivity subunit (PPS), a single-stranded DNA (ssDNA) binding protein (SSB), and the three components of the helicase-primase complex (HP1, HP2, and HP3). Some host proteins are involved, but their roles have not been fully defined. Other forms of replication might be involved, but rolling-circle replication appears to be the major source of new genomes. Because packaged genomes are not chromatinized, thus far undefined mechanisms are needed to either prevent chromatinization during and after viral DNA replication or to remove nucleosomes from newly replicated viral DNA prior to packaging.⁹⁴

Virion Assembly and Egress

BIOSYNTHESIS AND INTRACELLULAR DELIVERY OF VIRION COMPONENTS

Dissection of the process of virion assembly needs to account for the structure of mature infectious virions and the topological constraints imposed by cellular compartmentalization (e-Fig. 59.3C). The different stages of virion assembly occur first in the nucleus and then in the cytoplasm, with extensive use made of cellular systems for synthesis, processing, and trafficking of membrane proteins, plus nucleus-cytoplasm, intra-cytoplasmic, and vesicular transport systems. The process is dependent on coordinated delivery of virion subcomponents to appropriate subcellular addresses. We can consider virion assembly to begin with translation of mRNAs for structural genes. There is no evidence to suggest it, but it is conceivable that structural gene mRNAs are delivered to ribosomes situated to facilitate transport of their products to the intended destination. Thus, mRNAs for virion glycoproteins need to be translated by ribosomes associated with the endoplasmic reticulum (ER), to begin the trafficking through the ER, Golgi apparatus, and trans-Golgi network required for sequential maturation of nascent glycoproteins and their ultimate delivery to the organelle(s) where virions are enveloped. Proteins that form structures assembled in the nucleus must be routed to systems that enable transport through the nuclear pore complex. Tegument proteins that are assembled onto virions in the cytoplasm need to be routed to areas nucleocapsids will traverse on their way to acquiring their envelope at a cytoplasmic organelle that connects to a path of cellular egress.

ASSEMBLY AND NUCLEAR EGRESS OF THE NUCLEOCAPSID

In the nucleus, nascent capsids are assembled on temporary scaffolds, then filled with newly replicated DNA in an energy-dependent manner.^{64,81,84} In addition to the structural proteins, capsid assembly is dependent on several other viral proteins, including the assembly protein precursor (pAP), the mature assembly protein (AP), and the assembly protease (PR) that is self-excised from a precursor polypeptide (prePR) that contains PR, pAP, and AP. As DNA is being spooled into the capsid through the portal, a head-full sensing mechanism

operates, triggering cleavage of the genome at the conserved *pac* sequences that define the genomic termini. The cleavage is mediated via interactions among PORT, the heterodimeric terminase (TER1 and TER2), TER binding protein (TERbp), and the portal capping protein (PCP). Filled and capped nucleocapsids are pressurized and very rigid.

The mechanisms employed for transport of filled nucleocapsids from the nucleus are not fully understood. Three paths of nuclear egress have been considered in depth^{14,37,57,79–81}: envelopment at the inner nuclear membrane followed by subsequent cytoplasmic transport on the interior (within the lumen) of membrane-bounded tubes and vesicles such as cisternae of the rough ER and Golgi apparatus; envelopment at the inner nuclear membrane, then de-envelopment during entry to the cytoplasm; and direct transport of nonenveloped particles to the cytoplasm through enlarged nuclear pores or a disintegrated nuclear membrane (e-Fig. 59.3C).

The luminal transport model predicts that the envelope acquired at the inner nuclear membrane is the same membrane present on mature extracellular virions. Two of the major lines of evidence against this model are: some tegument and envelope proteins acquired during envelopment at the inner nuclear membrane are absent from mature virions; and most tegument proteins are acquired in the cytoplasm, not in the nucleus, and no mechanism has been postulated for addition of tegument proteins to enveloped virions.

In the envelopment/de-envelopment model, an initial layer of nucleocapsid-associated tegument is acquired in the nucleus. A nuclear envelopment complex containing the conserved viral proteins, nuclear egress membrane protein (NEMP) and nuclear egress lamina protein (NELP), mediates a viral and cellular kinase-driven disassembly and rearrangement of the nuclear lamina that enables filled nucleocapsids to access the nuclear face of the inner nuclear membrane. In parallel, a subset of the population of viral glycoproteins is routed to the inner nuclear membrane, where they become part of the primary virion envelope as nascent virions bud into the lumen of the nuclear membrane. In comparison to mature infectious virions, primary enveloped virions present in the lumen of the nuclear membrane do not contain the full complement of virion glycoproteins and only a small subset of tegument proteins, but contain NEMP, which is not present in mature virions. Primary enveloped virions “infect” the cytoplasm from the lumen of the nuclear membrane by a membrane fusion process that for many herpesviruses is dependent on the same viral fusion proteins (gB and gH/gL) responsible for membrane fusion during initial infection at plasma or endocytic vesicle membranes. The envelopment/de-envelopment model is supported by ultrastructural, genetic, and biochemical data for members of all three *Herpesviridae* subfamilies. Nonetheless, as discussed further in Chapter 60, key elements of its proof are lacking, including identification of a fusion receptor on the luminal side of the outer nuclear membrane.

The third model is the simplest mechanistically. It is supported by limited ultrastructural and genetic data,⁶¹ and appears to apply in limited circumstances.

CYTOPLASMIC TEGUMENTATION, SECONDARY ENVELOPMENT, AND EGRESS

As delivered into the cytoplasm, nonenveloped nascent virions consist of nucleocapsids and a set of nucleocapsid-associated

tegument proteins (the inner tegument) that were acquired in the nucleus. The next assembly steps involve addition of the final complement of tegument proteins and some RNAs, and then acquisition of the mature envelope while budding into a cytoplasmic organelle from which the virion can be transported to the cell surface for release.^{48,57,84} Few mechanistic details are known of the tegumentation process. Virion membrane proteins must possess trafficking signals appropriate for being routed to exit organelles, where they form concentrated patches. Nucleocapsids are transported from the site of nuclear egress to their exit vesicle through associations with microtubule motors. Some tegument proteins interact with each other to form subassemblies that associate with the developing virion during transport to the exit organelle, and some tegument proteins associate with cytoplasmic projections of virion membrane proteins. When the necessary associations and structures have formed, tegumented nucleocapsids bud into the exit organelle, forming mature virions.

Herpesviruses can differ with respect to the organelle where they acquire their mature envelope. The unifying concept is that the exit organelle is adapted from an organelle that is normally involved in vesicular transport and delivery of cargo for release at the plasma membrane. Thus, HSV uses the trans-Golgi network, HCMV uses recycling endosomes, and HHV-6A uses multivesicular bodies. Vesicles containing enveloped virions are transported to the plasma membrane, where vesicle membranes fuse with the plasma membrane; mature virions are then released to the extracellular space where they can infect other cells in the same or different individuals.

Latency

Persistence in the host organism via cellular (or microbiologic) latency is a defining property of herpesviruses. Cellular latency is different from clinical (or organismal) latency, which is the period between infection and disease, and during which extensive lytic replication might be occurring at the cellular level (Figs. 59.3 and 59.7). In cells harboring latent virus, viral genomes generally take the form of closed circular molecules from which only a small subset of viral genes is expressed. Latent genomes retain the capacity to reactivate to a lytic state during which new infectious virions are produced. Latently infected cells differ from chronically or persistently infected cells in that infectious progeny are not produced during latency; the capacity to reactivate differentiates latent from abortive infections. Reactivation from cellular latency can result in disease due to a combination of the cellular and tissue damage directly caused by lytic virus replication, plus the immunologic responses to the reactivated lytic replication.

The precise molecular mechanisms that lead to establishment of and reactivation from the latent state are not fully understood and can differ from one herpesvirus to another. A common feature is epigenetic regulation of transcription during latency to ensure transcription of latency-specific transcription, as well as readily reversed suppression of lytic gene transcription.⁹⁴ Each herpesvirus establishes latency in a specific set of cells, with the cellular site of latency differing from one virus to another. For example, whereas HSV establishes latency in neurons of dorsal root ganglia, latent EBV is present primarily in B lymphocytes. Within an organism, a virus can be simultaneously latent in some cells and actively replicating in others. Thus, there are circumstances when virus is latent in essentially

all infected cells (as seems to be the case after clearance of primary VZV infection, and to a lesser extent, for HSV); virus is lytically active in a subset of infected cells, but there are no associated symptoms (perhaps the most common state for all of the human herpesviruses except for VZV); and lytic virus activity is of a nature that results in illness (with some cells remaining in a latent state) (Fig. 59.3).

Oncogenicity

Although many herpesviruses encode genes that can transform cells in experimental systems outside the context of viral infection, few herpesviruses are oncogenic (EBV and KSHV of the human herpesviruses). In only a few circumstances, such as the immortalization of B cells by EBV in the process of establishing its latent repository, is transformation or immortalization an integral part of the *in vivo* life cycle.⁸⁸ There is some irony in the development of recombinant herpesviruses for clinical application as oncolytic agents.¹⁵

HERPESVIRUS EVOLUTION

A number of reviews explore various aspects of herpesvirus evolution in depth.^{25,27,75,78}

Virus Origins

Although their precise origins remain open to speculation, the similarity of mechanisms and pathways of replication of herpesviruses with DNA phages cannot be explained totally on the basis of convergent evolution, and places the ancestry of herpesviruses at the early stages of the evolution of parasitism. Many genetic elements of herpesviruses trace to deep branches of evolutionary trees. Homologs of the terminase gene encoded by HSV-1 UL15 are found in all herpesviruses, including the fish and mollusk viruses²⁵; a related function is encoded by some DNA bacteriophages of the *Caudovirales*. Further evidence of a deep evolutionary relationship between herpesviruses and bacteriophages is the identification in their capsids of a shared unique fold that consists of three α -helices and two β -sheets.⁷

Numerous shared features suggest that herpesviruses trace to a common ancestor. The ancestral virus diverged into at least three highly diverged lineages, one represented by the alpha-, beta-, and gammaherpesvirus subfamilies that infect birds and mammals (the *Herpesviridae*), the others represented by the herpesviruses of oysters and of fish and amphibians (Fig. 59.2). Although there is little obvious genetic similarity between the latter herpesviruses and the herpesviruses of birds and mammals, their capsid structures are well conserved.^{9,31}

The ancestral herpesvirus of birds and mammals must have encoded homologs of most, if not all, of the core genes that are shared by the contemporary progeny. The three major lineages of these viruses emerged prior to the mammalian radiation.⁷³ According to one analysis, the alphaherpesviruses emerged prior to the beta- or gammaherpesviruses⁷³; a different method suggested that HHV-6 is closest to being a progenitor herpesvirus and that the alphaherpesviruses emerged relatively recently.⁵⁸ The evolutionary distances between closely related herpesviruses that infect different species—such as the rhadinoviruses KSHV (humans) and herpesvirus saimiri (squirrel monkeys)—suggest that in many instances viral speciation occurred at approximately the same time as did host speciation.^{73,89} The

situation is somewhat more complex in some closely related and more recently diverged lineages.⁷⁷ Thus, herpesviruses have an intimate shared evolutionary history with their hosts.

Gene Acquisition

Primordial Gene Acquisition

Although many eukaryotic genes have bacterial homologs, bacterial DNA polymerases are not related to eukaryotic replicative DNA polymerases. Rather, the eukaryotic DNA polymerases are related to a group of DNA polymerases encoded by some bacteriophages (e.g., T4); various archaea; and pox-, baculo-, and herpesviruses. This has led to the hypothesis that eukaryotic replication proteins had their origins in DNA viruses.¹²⁸

Other genes with primordial roots are also involved in the fundamental process of viral genome replication. Herpesvirus alkaline exonucleases have features in common with a superfamily of deoxyribonucleases that includes restriction endonucleases, exonucleases, and nicking enzymes, with members of the group being found in several virus families, including the phage lambda exonuclease.¹³ Herpesvirus primases (homologs of HSV-1 UL52) are members of the archaeoeukaryotic primase superfamily, a deeply rooted group whose origins trace to proteins of bacteriophages or bacteria.⁵⁵ Herpesviruses interact with many intracellular regulatory pathways, exemplified by the deubiquinating activity embedded in the conserved large tegument protein (homologs of HSV-1 UL36).¹¹²

In addition to connections to bacteriophages, homology has also been detected between the glycoproteins encoded by members of the HCMV RL11 gene family and human adenovirus E3 genes.²⁶

Evolutionarily Recent Gene Acquisition

Many of the more recently acquired genes are involved in modulation of host immune responses (reviewed in 27,96,106) and are of obvious host origin. Most of these genes are encoded in the viral genomes as single open reading frames that lack the intron/exon structure of their host homologs, indicating that their acquisition by the virus involved a reverse transcription step, possibly the product of a co-infecting retrovirus. An example of an immunomodulatory gene that was acquired independently in different herpesvirus lineages is the IL-10 homologs encoded by gammaherpesviruses, which branch with IL-10 homologs from primate species, and the IL-10 homolog of equine herpesvirus 2, which branches with the horse IL-10.⁵³ There are examples of recent introductions into limited lineages, such as a glycosyltransferase (core β -1,6-*N*-acetylglucosaminyltransferase-mucin) acquired from an ancestor of the African buffalo that is present only in viral lineages closely related to the gammaherpesvirus, bovine herpesvirus 4.⁶⁸ In addition to the primordial connections to DNA bacteriophages and other viruses, a more recent intervirus gene transfer is represented by the presence of homologs of the parvovirus *rep* gene in the genomes of some betaherpesviruses.^{122,129}

Mechanisms of Evolution

A number of rational mechanisms have been used to explain the development and divergence of herpesviruses; because of their time scale and net complexity, they are essentially impossible to prove directly. These mechanisms would have been used to assemble the current genomes from diverse sources such as those described previously, and include recombination-

based acquisition and subsequent evolution of chunks of cellular DNA, as well as transposition of viral or cellular DNA or RNA.^{12,119} Shifts in base and dinucleotide composition can occur as a function of viral DNA polymerase mutation rates or in response to concentrations of intracellular nucleotide precursors and pools. Intraspecies recombination enables relatively rapid exchange of information within a virus lineage. It requires simultaneous infection of the same cell by different viral genotypes, a recombination event, then selection and propagation of the recombinant. Despite the expected rarity of the simultaneous infection step, there is abundant evidence for intraspecies recombination occurring in nature,^{21,22,97,140} with recombination contributing to HSV-1 strain variability at a level comparable to mutation.¹⁰

The strategy of gene duplication and divergence has been used across the herpesvirus family, as exemplified by the evolution of the dUTPase gene of the mammalian/avian/reptilian herpesviruses, which appears to have undergone a duplication and subsequent fusion that involved permuting the order of its subdomain.^{29,72} Further duplication and divergence events led to the current family of genes that encode proteins with dUTPase function, as well as proteins that lack residues that are essential for dUTPase activity, and have diverse or unidentified functions. Some of these proteins are essential for viral replication, whereas others are not. The betaherpesviruses have exploited this duplication and divergence strategy extensively, with HCMV encoding five gene additional families that each have from 2 to 12 members.¹⁸ Modifying a protein that is already able to be incorporated into the virion to execute a new function is a much smaller evolutionary challenge than a *de novo* strategy that requires simultaneous or successive development of function and its incorporation into virions. Thus, the strategy of adapting functionally flexible structural scaffolds to new purposes can confer a significant selective advantage.

Functional Convergence

An important regulatory circuit for T-cell activation operates through a member of that tumor necrosis factor receptor superfamily, the herpesvirus entry mediator (HVEM). This pathway is modulated by HSV-1 by binding of gD to HVEM, which blocks the binding of two regulatory ligands of HVEM, LIGHT, and BTLA. HCMV UL144 encodes a protein that mimics the BTLA binding site on HVEM, blocking the BTLA–HVEM interaction.¹⁹ Thus, these divergent herpesviruses have evolved independent mechanisms for modulating this immune regulator. There are numerous parallel examples, including the previously mentioned intersections with paths that regulate interferon and apoptosis.

Herpesvirus Phylogeny

The virus lineage that resulted in the present day alpha-, beta-, and gammaherpesvirus subfamilies is rooted at approximately 400 Ma (million years before the present).⁷⁶ As mentioned, to a first approximation, relationships among virus species within the major subdivisions of the alpha-, beta-, and gammaherpesvirus subfamilies mirror host phylogeny.

In contrast to the beta- and gammaherpesviruses, the alphaherpesvirus lineage includes viruses of reptiles and birds. Analysis of limited sequence data from several reptilian herpesviruses suggests that the avian and mammalian alphaherpesviruses descended from a lineage that included the reptilian

viruses. Current information cannot discriminate between the possibilities that the reptilian and mammalian viruses co-evolved with their hosts, and the avian lineages arose via transfer from reptiles or mammals; or the reptilian and avian viruses co-evolved with their hosts, with the mammalian viruses arising via a transfer event.⁷⁶ It remains to be determined whether there are single or multiple nodes through which the reptilian viruses connect to the alphaherpesvirus lineage.^{76,132}

Phylogenetic analyses can lead to testable hypotheses. Fleshing out of the rhadinovirus phylogenetic tree by amplifying novel viruses from a host of nonhuman primates has led to identification of two sublineages of Old World rhadinoviruses, each of which has identified homologs in chimpanzees and gorillas or gibbons. KSHV belongs to one of these lineages, but a human virus has not been identified for the other, leading to an ongoing directed search for a potential novel human gammaherpesvirus.^{35,113}

Host Interactions and Herpesvirus Evolution

Some viruses have evolved to rapidly spread new genotypes through the host population, such as can occur via the rapid global distribution by birds of newly reassorted influenza viruses. This is in marked contrast to viruses such as KSHV that have been relatively stable in their hosts over a time course that corresponds to the global radiation of humans from our African origins.⁴⁹ Other herpesviruses also show marked geographically defined genotype distributions.

A concept of some relevance is that of the “genetic budget” of viruses and its effect on virus–host interactions.^{17a} According to this theory, the error-prone replication of the generally smaller RNA viruses is a more primitive/crude escape mechanism that works over the short term and can be associated with significant host damage. With the greater genetic budget afforded by their larger genomes, viruses such as herpesviruses have acquired an array of genes and genetic functions that allow a more finely regulated virus–host interaction that functions over the course of a long-term relationship between the virus and an infected individual.

In summary, herpesviruses are well adapted to their hosts. In the natural immunocompetent host, fatal infections are rare; from the point of view of viral survival, fatal infections are counterproductive because they do not lead to transmission of virus. This is not always the case when herpesviruses infect a heterologous host, such as the frequently fatal outcomes of infection of cattle with pseudorabies virus, of humans with simian B virus, and the severe malignant catarrhal fever in cattle and bison that is caused by infection with *Ovine herpesvirus 2*, a virus that is a relatively benign inhabitant of sheep.³⁴ Thus, in addition to adaptation to specific cellular niches, many of the dispensable genes must also function to strike the optimal balance between virus growth in the host and a combination of avoidance and stimulation of host responses so that the virus can persist in populations of organisms over an evolutionary time scale.

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Herpes Simplex Viruses

Introduction and History

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- Tegument
- Capsid
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B Virus

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Disease Pathogenesis and Clinical Presentation
Control of B Virus Infection

But of all such cases, the most formidable were those which took place about the pubes and genital organs. Such was the nature of these cases when attended with sores, and proceeding from an external cause; but the same things occurred in fevers, before fevers, and after fevers.

Hippocrates, "Of the Epidemics," 400 BCE

INTRODUCTION AND HISTORY

The publication of the sixth edition of this book approaches the 100th anniversary of the formal recognition of herpes simplex virus (HSV) as an infectious agent,⁹⁶¹ having met the requirements of Koch's postulates. A revisionist historian would point to the facts that the lesions caused by HSV may have been described in the ancient literature millenia ago, and that the infectious nature of the disease was well described more than two centuries ago, as was the experimental transmission of the virus from person to person. Thus, descriptions of genital lesions that resemble those caused by HSV were described in a Sumerian tablet from the third millenium BC and in the Ebers Papyrus (c. 1500 BC). Hippocrates used the Greek word *herpes* to describe lesions that seem to creep or crawl along the skin. In all instances, the lesions described in these early accounts could have been attributed to viral, fungal, or parasitic infections virtually unknown today, to concurrent infections with several pathogens, or even to cutaneous malignancies. Celsus may have been the first to describe an actual herpetic lesion as initially round but then diffused as a serpent to form a belt—a description that would fit varicella-zoster virus better than HSV. Galen elaborated on Herodotus' description and indicated that the lesions recurred at the same anatomic site. In the intervening centuries between Galen's initial description of lesions similar to those caused by HSV and the 17th century, the term *herpes* was applied to many different skin ailments.⁹⁶¹ Probably the most relevant to our age are the writings of John Astruc, physician to King Louis XIV.

At that time, French prostitutes (*puellae publicae*) were under medical surveillance, Astruc and others studied their afflictions, and he was the first to describe *herpes genitalis*.³⁹ A most enlightening description of recurrences of genital lesions was published by Unna in 1883.¹¹⁷² He wrote that herpes was "*so to say a vocational disease*," recognized as being "*one of the most benign of affections both to the patient and her public*." Shortly afterward, the first book dedicated to herpes—*Les Herpes Genitaux*—was published.²⁷⁶ In 1896, Fournier wrote about the diagnosis and treatment of genital herpes.³⁴³ His advice was "*For recurrent herpes the general treatment of arthritis* [by which he may have meant inflammation and pain] *may be necessary. As far as hygiene, forbid alcohol, tobacco, also wine fatigue, and sexual excesses*".³⁴³ But it was Vidal¹¹⁹⁰ who had the dubious distinction of passing infection from one human to another and thus proving beyond a doubt that HSV was infectious. While these human studies were being performed, Gruter initiated a series of animal studies that unequivocally demonstrated the infectious nature of HSV.³⁹⁸ He showed how HSV could be transmitted serially from rabbit to rabbit, and although Gruter published his data years after they were completed and after Lowenstein,⁶⁸³ it is to him that the virology community gives credit for the isolation of HSV.

Although HSV was recognized as an infectious agent in the last quarter of the 19th century, many more years passed before the connection between HSV and recurrent infections was finally established. In the 1930s, one of the milestones of HSV biology was the observation made by Andrews and Carmichael that recurrent infections occurred only in adults who carried neutralizing antibodies, an occurrence in sharp contrast to the behavior of other known infectious agents at that time. Their article stimulated an international debate that ultimately shaped the perception of HSV as a disease agent. On one extreme, Doerr, a doyen of European virology of the time, stated that HSV infections in humans resulted from the endogenous production of a virus-like agent by the cell, under the influence of certain stimuli, and were not caused by exogenous infection.²⁸⁴ Once the agent had been produced, it would act on the cells of susceptible animals (not man) as a true virus. One year later, Burnet and Williams articulated the modern view that "*Herpes simplex infections, however, once contracted, seem to persist for life. The virus remains for the most part latent; but under the stimulus of trauma, fever, as so forth it may at any time be called into activity and provoke a visible herpetic lesion*".¹⁴¹ In retrospect, however, Doerr had come perilously close to understanding the true nature of latency, namely that endogenous virus production by the cell is based on information supplied by the viral DNA retained within the cell. The modern age of HSV research had arrived.

HSVs were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. Their attractions are their biological properties, particularly their abilities to cause various infections, to remain latent in their host for life, and to reactivate to cause lesions at or near the site of initial infection. They serve as models and tools for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, gene therapy, cancer therapy, and myriad other biological problems, both general to viruses and specific to HSV. For years, their size and complexity served as formidable obstacles to intensive research. More than 40 years passed from the time

of their isolation until Schneweis¹⁰¹⁶ demonstrated that there were in fact two serotypes of HSV—HSV-1 and HSV-2—whose formal designations under the International Committee on Taxonomy of Viruses (ICTV) rules are now the two species human herpesviruses 1 and 2.⁹⁵¹ Not until 1961 were practical plaque assays published,⁹⁵⁷ and only much later were the genome sizes and the extent of homology between these two viruses reported. This chapter recounts well-established facts; however, its main emphasis is on burning issues—the problems whose time has come.

To the prefaces of earlier editions, which we affirm but are not repeated here, we add two additional comments. Foremost, the field has grown enormously since the dawn of the molecular biology of HSV more than 45 years ago. Studies on HSV are entering, at last, a most exciting period for two reasons. First, the words *structure and function* are beginning to have an operational meaning. Second, HSV gene products have become powerful probes for the study of cellular metabolic pathways. Host factors crucial to virus multiplication and, potentially, to latency are being identified. The armamentarium for a major assault on the mysteries underlying the biology of these viruses is in place and reflects the contributions of many laboratories over many, many years. However, we are also confronted with the fact that only a fraction of viral gene products are extensively studied and that for many products their reason for existence has not been established.

The literature has grown *pari passu* with the field and tasks our capacity to place myriad collected data into a comprehensible total. The task before us was far greater for not having followed Ludwig Wittgenstein's dictum that "*Whereof one cannot speak, thereof one must be silent,*" but chapters of citations bereft of commentary are dull. The decision to avoid assembling an annotated bibliography was in large part dictated by the space available to us. As a result of these space limitations, the references from 2008 to the present are listed in the book, whereas all of the cited references can be found in the eBook form of this publication.

VIRION STRUCTURE

The HSV virion consists of four elements: (a) an electron-opaque core containing the viral DNA, (b) an icosahedral capsid surrounding the core, (c) a largely unstructured proteinaceous layer called the *tegument* that surrounds the capsid, and (d) an outer lipid bilayer envelope exhibiting spikes on its surface⁹⁵² (Fig. 60.1A). Early studies defined the structure of the HSV virion through electron microscopy and biochemical analysis of the virion components. More recent electron cryo-microscopy studies have defined the nucleocapsid structure to 8.5 Å resolution.¹³¹⁹ The most detailed analysis of the whole virion used cryo-electron tomography to define its structure to 7-nm resolution³⁹⁷ (see Fig. 60.1B,C). This study defined the virion as a spherical particle with an average diameter of 186 nm, which extended to 225 nm with spikes included. The nucleocapsid was located in an eccentric position with one edge of the nucleocapsid close to the envelope and the other, the distal pole, 30 to 35 nm from the envelope. The tegument showed a particulate structure with some 7-nm filaments apposed to the membrane. The tegument cap on the distal pole was connected to the envelope by 4 nm wide "linkers."

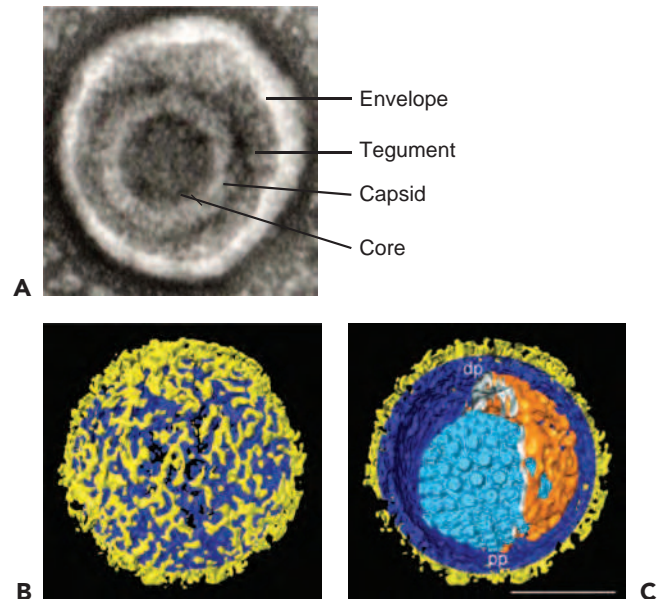


FIGURE 60.1. Structure of the herpes simplex virus (HSV) virion.

A: Electron micrograph of a negative-stained HSV-1 virion. The envelope, tegument, capsid, and core are indicated. (Micrograph provided by Travis Taylor.) **B:** Surface image of HSV derived from cryo-electron tomography.³⁹⁷ Glycoprotein spikes are shown in yellow, and the membrane is blue. **C:** Cutaway view of the virion interior derived from cryo-electron tomography. Capsid is shown in light blue, the tegument cap is shown in orange, the envelope is dark blue, and the glycoprotein spikes are yellow. (Parts B and C courtesy of Alasdair C. Steven.)

Virion Proteins

Early studies on purified HSV-1 virions suggested that they contain more than 30 distinct proteins that were designated as virion polypeptides (VP) and given serial numbers.^{437,1079} HSV proteins have also been named based on serial numbering of the virion proteins on a gel (e.g., VP1/2), on the open reading frame (ORF) encoding them (e.g., U₁8), or as infected cell proteins (ICPs; e.g., ICP5). Mass spectrometry analysis of purified extracellular virions demonstrated several host proteins,⁶⁸² although the critical issue will be to determine if any host proteins are essential for virion structure. Of the approximately 30 known and 10 suspected virion proteins, at least 11 are on the surface of the virion (accessible to antibody) and at least 10 are glycosylated.

Core

The core contains the double-strand (ds) DNA genome wrapped as a toroid³⁵⁶ or spool¹³¹⁸ in a liquid crystalline state.¹¹¹ A small fraction of the virion DNA may be circular.^{878,1103} The viral DNA genome is described in detail later.

The core does not contain highly basic proteins, including histones, that would neutralize the negative charges on viral DNA to allow proper folding within the capsid; however, highly purified virions do contain the polyamines spermidine and spermine in a nearly constant ratio of $1.6 \pm 0.2:1$, or approximately 70,000 molecules of spermidine and 40,000 molecules of spermine per virion.^{374,376} The polyamines appear

to be tightly bound and cannot be exchanged with exogenously added, labeled polyamines. Disruption of the envelope with nonionic detergents and urea removed the spermidine but not the spermine. The spermine contained in the virion is sufficient to neutralize approximately 40% of the DNA phosphate.³⁷⁶

Tegument

The space between the undersurface of the envelope and the surface of the capsid, designated as the tegument,⁹⁵² is largely unstructured, except for the fibrils described previously and some apparent icosahedral structure around the pentons¹³¹⁸ and is comprised of at least 18 viral proteins (see the following gene list). The most notable of the proteins associated with the tegument are the VP16 virion transactivator protein (also known as α -trans-inducing factor or α TIF, encoded by the *U_L48* ORF); the virion host shutoff (VHS) protein (*U_L41*) VP22 (*U_L49*), which was reported to have the ability to spread cell to cell³¹²; and a very large protein (VP1-2; *U_L36*), which plays a role in DNA release at the nuclear pore during viral entry.⁷³ Other tegument proteins include *U_L14*, *U_L17*, VP11/12 (*U_L46* gene), VP13/14 (*U_L47*), *U_S9*, *U_S10*, and *U_S11*. Recent studies are starting to define the protein-protein interactions both within the tegument and between the tegument and capsid and envelope proteins.¹¹⁹³

Highly purified HSV virions contain cellular and selected viral gene transcripts.¹⁰²¹ These are likely packaged in the tegument because three RNA binding proteins (*U_S11*, *U_L47*, and *U_L49*) were identified in the tegument.¹⁰²³

Capsid

The capsid is comprised of 162 capsomers, including 140 hexons, 11 pentons, and one portal, arranged in a T = 16 icosahedral symmetry. Cryo-electron microscopy studies have shown that the HSV nucleocapsid is composed of an outer layer arranged in a T = 16 symmetry and an intermediate layer organized in a T = 4 lattice.¹⁰¹⁸ The same study concluded that the outer and intermediate layers are organized so that channels along their icosahedral twofold axes coincide, forming a direct pathway and potential channel between the DNA layer and the exterior of the virion.¹⁰¹⁸ Another study of intact virions has shown that the channel is plugged, possibly with tegument proteins.¹³¹⁸

The outer shell of the capsid is composed of four viral proteins, VP5 (*U_L19*), VP26 (*U_L35*), VP23 (*U_L18*), and VP19C (*U_L38*). VP5, the major capsid protein, is present in five copies in each penton capsomere and six copies in each hexon capsomere in this icosahedral shell. VP26 is present in six copies as a ring on top of the VP5 subunits on each hexon.¹³²⁰ Triplexes made up of one VP19C molecule and two VP23 molecules link adjacent capsomeres as a result of the triplex forming a pseudotrimer with each subunit interacting in a roughly similar manner with two capsomeres.¹³¹⁹ The capsid also contains the *U_L6* protein, which forms a dodecamer thought to form a portal through which viral DNA is packaged¹¹⁶³ and VP24 (*U_L26*), the protease that processes the scaffolding during DNA encapsidation. The *U_L16* protein may be a capsid-associated protein because it plays a role in DNA encapsidation.

Envelope

The envelope consists of a lipid bilayer with as many as 13 distinct viral glycoproteins embedded in it. The virion enve-

lope glycoproteins are gB (VP7 and VP8.5, encoded by the *U_L27* gene), gC (VP8, *U_L44*), gD (VP17 and VP18, *U_S6*), gE (VP12.3 and VP12.6, *U_S8*), gG (*U_S4*), gH (*U_L22*), gI (*U_S7*), gK (*U_L53*), gL (*U_L1*), and gM (*U_L11*). The presence of gJ (*U_S5*) and gN (*U_L49.5*) in virions has not been demonstrated. Virion envelopes also contain at least two (*U_L20* and *U_S9*) and possibly more (*U_L24*, *U_L43*, and *U_L34*) nonglycosylated intrinsic membrane proteins.

Lipids

It has been assumed that HSV acquires the envelope lipids from its host. The hypothesis that the lipid composition of the viral envelope is determined by the host was supported by the observation that the buoyant density of the virus was host cell dependent on serial passage of HSV-1 alternately in HEp-2 and chick embryo cells.¹⁰⁷⁸ More recent studies¹¹⁷⁹ suggest that the virion lipids are similar to those of cytoplasmic membranes and different from those of nuclear membranes of uninfected cells.

STRUCTURE AND SEQUENCE ARRANGEMENT OF VIRAL DNA

The bulk of packaged HSV DNA is linear and double stranded.⁵⁴⁶ The ends of the genome are probably held together or are in close proximity inasmuch as a small fraction of the packaged DNA appears to be circular and the bulk of the linear DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells.⁸⁷⁸ DNA extracted from virions contains ribonucleotides, nicks, and gaps.^{94,351} Based on physical characterization studies, the HSV genome was originally estimated to be approximately 150 kilo base pair (kbp), with a G + C content of 68% for HSV-1 and 69% for HSV-2.⁵⁴⁶ Complete sequencing of the HSV-1 strain 17 genome described the genome as 152,260 bp and 68.3% G + C (GenBank sequence accession number NC_001806),⁷²⁷ and minor updates altered this sequence to 152,261 bp.²⁸⁵ Complete sequencing of the HSV-2 strain HG52 genome defined it as 154,746 bp and 70.4% G + C (GenBank sequence accession number NC_001798).²⁸⁵ However, these numbers include only single copies of the *a* sequence at the ends of the L component and do not take into account the variation in the size of the *a* sequences (200–500 bp each) or the variable number of direct repeats present throughout the genome but especially in the inverted repeats flanking the L and S components.

Illumina high throughput sequencing further defined the genomic sequence of two HSV-1 viruses: the F laboratory strain and the H129 clinical isolate.¹¹¹⁴ This analysis defined the unique and single copies of the repeated sequences of the genomes and based the numbers of repeats on the strain 17 prototype genome. Comparison of the three sequences showed a limited amount of sequence diversity, less than 0.7%, between any pair of the viruses.¹¹¹⁴

The HSV genome can be viewed as consisting of two covalently linked components, designated as L (long) and S (short) (Fig. 60.2). Each component consists of unique sequences bracketed by inverted repeats.¹¹⁹⁸ The repeats of the L component are designated *ab* and *b'a'*, whereas those of the S component are *a'c'* and *ca* (see Fig. 60.2). The number of *a* sequence repeats

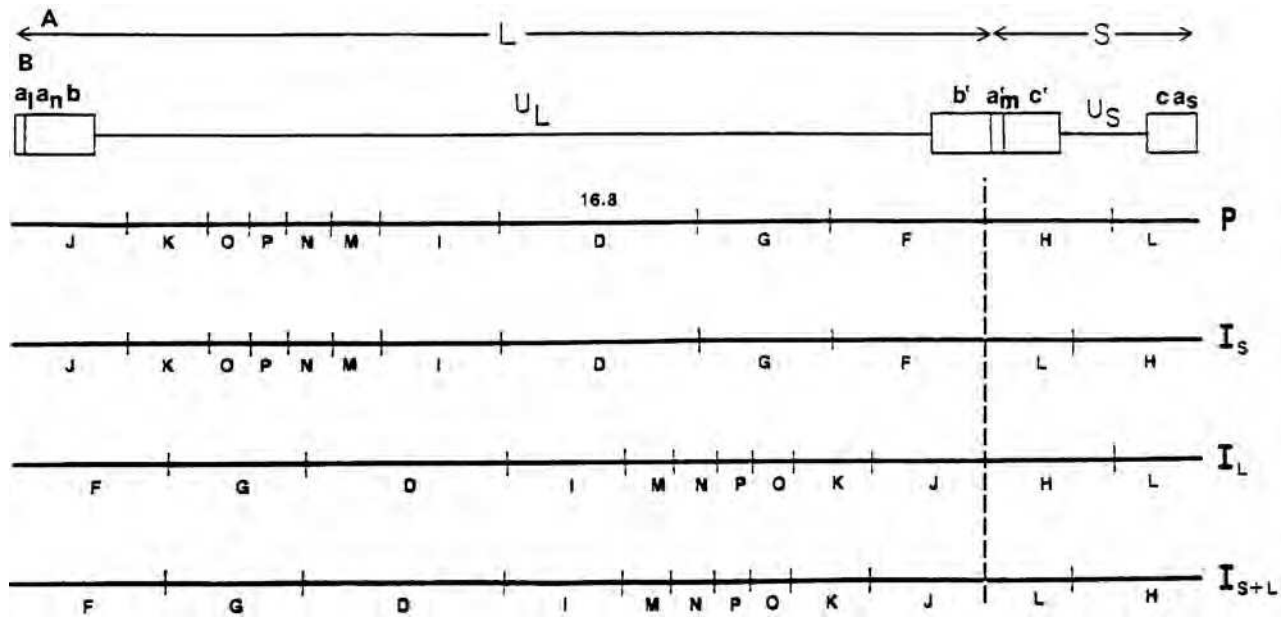


FIGURE 60.2. Schematic representation of the arrangement of DNA sequences in the herpes simplex virus (HSV) genome. **A:** The domains of the L and S components are denoted by the arrows. The second line shows the unique sequences (thin lines) flanked by the inverted repeats (boxes). The letters above the second line designate the terminal a sequence of the L component (a_L), a variable (n) number of additional a sequences, the b sequence, the unique sequence of the L component (U_L), the repetitions of the b sequence and of a variable (m) number of sequences (a_m), the inverted c sequence, the unique sequence of the S component (U_S), and finally the terminal a sequence (a_S) of the S component. **B:** The HindIII restriction endonuclease map of HSV-1 (F) strain for the P, I_S , I_L , and I_{S+L} isomers of the DNA. Note that because HindIII does not cleave within the inverted repeat sequences, there are four terminal fragments and four fragments spanning the internal inverted repeats in concentrations of 0.5 and 0.25 M, respectively, relative to the concentration of the viral DNA.

at the L–S junction and at the L terminus is variable; thus, the HSV genome can then be represented as

$$a_L a_n b - U_L - b' a'_m c' - U_S - ca_S,$$

where a_L and a_S are terminal sequences with unique properties described below, and a_n and a_m are terminal a sequences directly repeated 0 or more times (n) or present in one to many copies (m).^{666,948,949,1198,1202} The overall structure of the a sequence is highly conserved; however, it consists of a variable number of repeat elements. In the HSV-1 F strain, the structure of the a sequence can be represented as

$$DR1 - U_b - DR2_n - DR4_m - U_c - DR1,$$

with DR1 being a 20-bp direct repeat, U_b being a 65-bp unique sequence, DR2 being a 12-bp direct repeat present in 19 to 23 copies per a sequence, DR4 being a 37-bp direct repeat present in 2 to 3 copies, U_c being a 58-bp unique sequence, and a final copy of DR1.^{759,760} Adjacent a sequences share an intervening DR1. The size of the a sequence varies from strain to strain, reflecting in part the number of copies of DR2 and DR4. Linear virion DNA contains asymmetric terminal a sequence ends. The terminal a sequence of the L component (a_L) contains a truncated DR1 with 18 bp and one 3' nucleotide extension, whereas the terminal a sequence of the S component (a_S) ends with a DR1 containing only 1 bp and one 3' overhanging nucleotide.⁷⁶¹ The two truncated DR1 sequences form a complete DR1 upon circularization.

The L and S components of HSV are found inverted relative to one another, to yield four linear isomers (see Fig.

60.2B). Populations of unit-length DNA from wild-type virus-infected cells consist of equimolar concentrations of the four predicted isomers.^{263,430} The isomers have been designated as P (prototype), I_L (inversion of the L component), I_S (inversion of the S component), and I_{S+L} (inversion of both S and L components).⁴³⁰ The first evidence for the existence of repetition of terminal sequences in an inverted orientation was from electron microscopic studies of denatured HSV-1 DNA allowed to self-anneal¹⁰³¹ and partial denaturation profiles of HSV DNA, which revealed that the terminal repeats are also repeated internally and that the repeats flanking the L component differ from those of the S component in size and sequence arrangement.¹¹⁹⁸ The demonstration that restriction endonucleases that cleave outside the inverted repeats yield four terminal fragments, each present in one-half of the molecules (also called *half molar* or *0.5-M fragments*), and four L–S component junction fragments that are each present in one-fourth of the molecules (also called *quarter molar* or *0.25M fragments*)⁴³⁰ (see Fig. 60.2B), as well as analyses of the partial denaturation profiles of Wadsworth et al,¹¹⁹⁸ supported the conclusion that the L and S components can invert relative to each other.

The internal inverted repeat sequences are not essential for growth of the virus in cell culture; thus, mutants from which portions of unique sequences and most of the internal inverted repeats have been deleted have been obtained in all four arrangements of HSV DNA.^{498,879} The genomes of these mutants do not invert and are frozen in one arrangement of the L and S components, although all retain their viability in cell culture.

Herpes Simplex Virus Genetic Variation

Herpes Simplex Virus 1 Versus Herpes

Simplex Virus 2 Variation

Comparison of the complete DNA sequences of an HSV-1 and HSV-2 genome confirmed the colinearity of the two genetic maps and showed an overall identity of 83%.²⁸⁵ Nevertheless the two genomes differ with respect to the location of endonuclease cleavage sites in their genomic DNAs and in the apparent sizes of the encoded proteins. The variations with respect to restriction endonuclease cleavage sites and apparent size of the proteins were used to map the approximate genomic location of genes on the basis of analyses of HSV-1 X HSV-2 recombinants.^{716,768,896}

Herpes Simplex Virus 1 Strain Variation

The first evidence of HSV-1 strain polymorphisms emerged from studies of virion structural proteins and indicated that nonglycosylated proteins vary sufficiently in electrophoretic mobility to be used as strain markers.⁸⁶⁴ Although specimens from epidemiologically related individuals appeared to yield similar electrophoretic profiles, the usefulness of virion proteins as markers for molecular epidemiologic studies was limited by the effort required to purify virions for such analyses.

At the DNA level, differences between HSV-1 strains appear to result from (a) base substitutions that may add or eliminate a restriction endonuclease cleavage site, and on occasion, change an amino acid and (b) variability in the number of repeated sequences present in a number of regions of the genome (e.g., $\gamma_134.5$, U_511 , etc.).^{195,941} The restriction endonuclease cleavage patterns of a given strain are relatively stable, whereas the numbers of repeats are not.^{135,429,950,1151} Thus, no changes in restriction endonuclease patterns were noted in isolates from the same individual over an interval of 13 years or in genomes of an HSV-1 strain passaged serially numerous times in cell culture. However, restriction endonuclease site polymorphisms were readily noted in isolates from epidemiologically unrelated individuals.^{416,960} On the basis of these properties, restriction endonuclease site polymorphism was used in several epidemiologic studies of HSV transmission in the human population.^{135,950,960} In addition, restriction endonuclease analysis of virus isolates has been used to trace the spread of infection from patients to hospital personnel,¹³³ from patient to patient,⁶⁵³ and from hospital personnel to patient.^{4,134} One paper⁹⁸² reported clustering of divergent sites along geographically and racially distinct areas. Although these authors concluded that the evolution of HSV-1 may be host dependent, it seems more likely that random mutations were conserved and dispersed in different populations.

The limited amount of phylogenetic analysis of HSV genomes conducted thus far has revealed limited sequence diversity among HSV isolates. Sequence comparison and phylogenetic analysis of the U_54 , U_57 , and U_58 genes of 28 HSV-1 clinical isolates showed at most 2% divergence between isolates but revealed three major clades.⁸¹⁵ Sequence analysis of the U_54 , U_57 , and U_58 genes of 27 HSV-2 clinical isolates from Tanzania, Norway, and Sweden showed only 0.4% divergence; nevertheless, at least two genotypes were identified.⁸¹⁶ Thus far, no HSV-2 low-passage clinical isolate genomes have been sequenced, and it will be interesting to determine how these differ in DNA sequence from the highly attenuated HSV-2 strain HG-52 viral genome. Sequence analysis of HSV genomes has

shown that HSV-1 and HSV-2 diverged 8 million years ago²⁸⁵ and the HSV-1 clades diverged about 700,000 years ago.⁸¹⁷ There is evidence of high-level recombination in the evolution of HSV genomes^{119,815,816,817}; therefore, this could help to explain the limited amount of genetic diversity found in current HSV clinical isolates.

Viral Genes: Pattern of Organization and Expression

Distribution and Organization of Transcriptional Units

HSV DNA encodes, by current count, at least 90 unique transcriptional units. (The HSV-1 and HSV-2 genomic sequences and gene maps can be found at the following websites: <http://stdgen.northwestern.edu> and <http://www.viprbrc.org>.) At least 84 of these transcriptional units encode proteins. As described in more detail later, these genes are classified into at least three general kinetic classes: α or immediate-early, β or early, and γ or late genes. The key elements of genome organization are as follows:

1. With three known exceptions, each viral transcript encodes a single protein. In the first exception, a single transcript serves as the template for both ORF P and ORF O proteins. Protein synthesis is initiated from a single methionine but diverges between the 1st and 35th codons to yield different polypeptides.⁹¹⁶ Second, the U_L26 gene encodes a polypeptide that cleaves itself to form two proteins. The N-terminal product functions as a protease. The C-terminal product, ICP35a,b, is a component of the capsid scaffolding. The third exception is the messenger RNA (mRNA) that encodes the U_L3 protein. This RNA, 2.9 kb in size, contains the ORFs of U_L1 , U_L2 , and U_L3 ; however, U_L1 and U_L2 proteins are encoded by distinct mRNAs.¹⁰⁵² The mechanism of expression of the U_L3 protein is unknown.
2. Many clusters of transcriptional units are 3' co-terminal. In addition, these clusters may be arranged either head to head, head to tail or tail to tail. The precise transcriptional initiation sites are not known for all transcriptional units. In many instances, but especially late in infection, transcriptional termination sites are ignored with the consequence that run-on transcripts of giant size are present along with the properly terminated transcripts.
3. There are several instances of transcriptional units wholly embedded in the coding sequence contained in the larger transcript. In each instance, the transcripts are 3' co-terminal, and the coding sequences of the smaller mRNA are identical to a C-terminal portion of the protein encoded by the larger mRNA. For example, the transcript encoding $U_51.5$ is 3' co-terminal with that of the $\alpha 22$ gene. The protein encoded by $U_51.5$ contains the 250 C-terminal residues of the 420-residue product of the $\alpha 22$ gene.¹⁶⁰ A similar relationship exists between U_L26 and $U_L26.5$ ^{657,658} and between U_53 and $U_53.5$.⁸⁸⁶ The two products may differ in function. For example, both U_53 and $U_53.5$ are protein kinases that phosphorylate similar substrates. However, whereas U_53 protects cells from proapoptotic viral or cellular proteins, $U_53.5$ protein kinase does not.⁸⁸⁶
4. Some of the expressed ORFs are antisense to each other. These include $\gamma_134.5$ and ORF P, U_L43 and $U_L43.5$, and gB and $U_L27.5$. The existence of additional antisense ORFs cannot be excluded.

5. Few of the transcripts accumulating in infected cells arise as a consequence of splicing of RNA. In only a few cases ($\alpha 0$ and U_L15 of HSV-1 and both genes plus $\gamma_134.5$ of HSV-2) are there introns within coding domains. The intron of U_L15 is transcribed antisense to U_L15 and encodes two ORFs, U_L16 and U_L17 .⁷²⁷ In all other cases (genes encoding ICP22, ICP47), the introns are in the 5' noncoding domains. The major intron of ICP0 is more stable than the mRNA encoding the protein and is translocated into the cytoplasm. The differences between viruses carrying wild-type and intronless $\alpha 0$ genes add urgency to the investigation of the role of this intron on the course of viral infection.
6. Several transcripts appear to not encode proteins. Those best known are the latency-associated transcripts (LATs), described in the Latent Infection section and the *Ori_s* mRNA that is expressed late in infection and is 3' co-terminal with the mRNA encoding ICP4. The functions of these RNAs in productive infection are not known.

Functional Organization of Herpes Simplex Virus Genomes

α Genes Map Near the Termini of the L and S Components

$\alpha 0$ and $\alpha 4$ map within the inverted repeats of the L and S components, respectively, and are therefore each present in two copies per wild-type genome. However, a single copy of each is sufficient inasmuch as mutants lacking the internal inverted repeats are viable but are attenuated for growth in animals.⁸⁷⁹ With few exceptions, β and γ genes are scattered in the unique sequences of both the L and S components. The exceptions are the $\gamma_134.5$ and *ORF P* genes located in the reiterated sequences flanking the L component between the terminal *a* sequence and the $\alpha 0$ gene. At present, only two functional gene clusters are strikingly apparent, although their significance is uncertain: The β genes specifying the DNA polymerase and the ICP8 single-stranded DNA binding protein flank the L component origin of DNA synthesis (*Ori_L*), and the γ genes specifying membrane glycoproteins D, E, G, I, and J map next to each other within the unique sequences of the S component.

Genetic Techniques for the Identification of Gene Product Function

The various genetic techniques used for analysis of HSV have been described in depth previously⁹⁵⁶; therefore, we will review this area only briefly. Key to the identification of viral functions and mapping of viral genes encoding these functions are conditional lethal, such as temperature-sensitive (ts) and null mutants. Earlier studies of ts mutants identified approximately 30 complementation groups,¹²³⁰ which was enormously helpful in identifying and mapping some genes and the functions of their gene products. Nevertheless, ts mutant phenotypes placed constraints on the range and types of mutants that could be isolated. Thus, several methods have been developed for localized mutagenesis of the viral genome. Genetic alterations have been introduced into the viral genome by a co-transfection procedure involving the introduction of infectious viral DNA with a viral DNA fragment that contains a marker or mutation to be introduced into the viral genome by a double cross-over event.^{561,976,1102} This has been used for marker rescue mapping of viral ts mutants, introduction of markers into a viral genome

(marker transfer,^{561,976} and for introduction of mutations into a viral genome.^{199,221} Alternatively, the second major technique is based on the use of bacterial artificial chromosomes to propagate the viral genome in bacterial cells. Mutations can be introduced into the HSV sequences, and introduction of the recombinant bacterial artificial chromosome into mammalian cells yields infectious virus.⁴⁶⁷ The genomes cloned as bacterial accessory chromosomes are also amenable to random insertional mutagenesis by transposons.

A protocol for site-specific insertion/deletion of viral genes was based on selection of recombinants generated by double recombination through homologous flanking sequences between an intact viral DNA molecule and a DNA fragment containing an insertion or deletion and a selectable marker.⁸⁹³ The selectable marker used in these studies was the viral thymidine kinase (*tk*) gene because (a) it can be deleted from the HSV genome without affecting the growth of virus in cell culture; (b) a plasmid-borne *tk* gene can be altered so that it cannot recombine by double cross-over to repair the deletion in the genomic *tk* gene; (c) viruses carrying a functional *tk* gene can be selected against by plating viral progeny in the presence of nucleoside analogs phosphorylated by the viral thymidine kinase (TK) e.g., Ara T; and (d) viruses expressing the *tk* gene can be selected for by plating the virus in TK⁻ cells in medium containing methotrexate or aminopterin, which blocks the conversion of thymidine monophosphate (TMP) from 2'-deoxyuridine 5'-monophosphate (dUMP) by thymidylate synthetase and precludes the *de novo* pathway of TMP synthesis. This procedure permits the selection of viable mutants with deletions or insertions in genes that appear to be nonessential for growth in cells in culture.

A second popular approach for mutagenesis of a specific gene is to replace the gene with an expression cassette containing *Escherichia coli lacZ* coding sequences so that the β -galactosidase enzyme is expressed by the recombinant virus. The recombinant virus can be identified by the formation of blue plaques in the presence of chromogenic substrates such as X-GAL.³⁸⁴ If the recombinant virus can be isolated and propagated on normal cells, such as Vero cells, the mutated gene is considered to be nonessential. Insertion of a green fluorescent protein (GFP) expression cassette into a gene results in a virus that gives a green fluorescent plaque,⁹⁸⁹ and recombination of other sequences into the genome to replace the GFP cassette to give a virus that forms a nonfluorescent plaque also provides a screen for introduction of new sequences into a viral genome.¹²²²

If the viral gene is essential for growth in that cell line, stable complementing cell lines have often been used to propagate these null mutant viruses.²⁶⁵ In this protocol, the gene to be mutated is transfected into and stably maintained in a cell line that serves as the complementing cell line. The complementing cell line is then transfected with intact viral DNA and the mutated DNA fragment. The progeny viruses are screened for mutants that form blue plaques or multiply only in the complementing cells.²⁶⁵ It should be emphasized that the procedures for generating site-specific mutations can introduce unintended mutations at distant sites. The obvious and necessary control is to restore the original sequence and then compare parent, mutant, and rescued virus for the predicted phenotype of the mutation.

The genes known to be dispensable for viral replication in cells in culture are listed in the Gene Functions List. It should be

stressed that most of the dispensable genes are required for replication in experimental animal systems and that in no instance has a virus lacking a dispensable gene been isolated from human lesions (although viruses that fail to react with a specific monoclonal antibody are readily isolated).

Genes dispensable for viral replication in cells in culture fall into several groups whose products are involved in entry of HSV into cells, regulation of gene expression, posttranslational modification of proteins, exocytosis, inhibition of host response to infection, and spread of virus from cell to cell. It could be predicted that viral genes that specify products whose functions are identical and interchangeable with those of cellular genes would be dispensable, at least in cells that express these functions. In this category are the *tk* gene and the genes specifying ribonucleotide reductase. The observation that some virion proteins are dispensable for infection and replication of virus at least in cell culture was very puzzling for many years. Although we cannot exclude the possibility that cells express proteins with similar functions that complement the deletion mutants, a more likely scenario is that cells in culture express many more genes than cells *in situ* in animal organs. Moreover, recent studies have demonstrated that many of the dispensable genes block host responses to infection. These responses may be effective in some cells but not others. Obvious examples of functional mimicry are the TK, ribonucleotide reductase, and so forth. The less obvious homologs are the cellular protein kinases (e.g., protein kinase A) that substitute for some of the viral enzymes (e.g., U_53). The complexity involved in defining the function of the numerous genes whose products are dispensable for viral replication in at least some cells in culture is offset by the fact that these genes are excellent probes of cellular functions. Once the riddle is solved, the answers can provide an unexpected wealth of information about both the virus and the host cell.

HERPES SIMPLEX VIRUS GENE FUNCTIONS LIST

This section summarizes the known gene products and their functions as an introduction to the sections dealing with viral replication and virus–host cell interactions. Although it is useful, the list has numerous limitations:

1. The list understates the number of the gene products. The HSV genome encodes a large number of ORFs with 50 or fewer codons, and not all of the ORFs have been probed to determine whether they are expressed. In addition, standard annotations exclude ORFs that are antisense to known ORFs or that do not have TATA boxes or other motifs that indicate that they encode proteins. HSV encodes several proteins whose ORFs are antisense to each other.
2. We have assigned a gene product designation to every transcriptional unit defined as giving rise to a specific RNA. Three points should be noted. First, HSV encodes numerous long noncoding RNAs and stable introns whose functions have not been thoroughly investigated. Examples are the large intron of ICP0, OriS RNA, and the recently reported U_55-1 RNA.⁵¹⁹ Second, several mRNAs are embedded inside and are 3' co-terminal with a large mRNA and encode a protein identical to the carboxyl terminal domain of the protein encoded by the larger mRNA. Examples include the $\alpha 22$

and $U_51.5$ transcripts, the U_53 and $U_53.5$ transcripts, and the U_{L26} and $U_{L26.5}$ transcripts. Lastly, transcripts arising late in infection frequently do not terminate at predicted termination signals.

3. There is an irresistible urge to classify proteins as essential or dispensable for replication, at least in some cells in culture. There are two limitations to this classification. The first is the definition of the cutoff value for essentiality. Is the protein essential if, in its absence, the yield is reduced by 10-fold, 100-fold, or 1,000-fold? A second limitation is that a specific protein may be essential in some cell lines but not others, or at high multiplicity but not at low multiplicity. For example, ICP0 is essential at low multiplicities of infection in most cells but not in U2OS cells. Notations of cell type restrictions are important in that they point to potentially important cellular functions that enhance or block viral functions.
4. Virtually all viral proteins studied in detail appear to perform multiple functions. Not all of the functions are known. In many instances, we do not fully understand the contribution of each function to the biology of the virus.
5. The descriptions of the ORFs and noncoding RNAs include their size as defined in the current reference sequence for HSV-1 strain 17. It should be noted that strain differences are common; thus, they should serve as a guide rather than as an absolute value.

Open Reading Frames

$\gamma_134.5$ (248 codons, γ_1 , nonessential). $\gamma_134.5$ encodes ICP34.5, which consists of two unequal domains linked by a variable number of alanine-proline-threonine repeats. The C-terminal domain is homologous to the C-terminal domain of GADD34 and functions as a phosphatase accessory factor, which binds phosphatase 1 α and redirects it to dephosphorylate the α subunit of the translation initiation factor 2 (eIF-2). In the absence of $\gamma_134.5$, protein kinase R is activated, eIF-2 α is phosphorylated, and all protein synthesis ceases. Loss of this function correlates with avirulence in animal models and humans. Another domain of $\gamma_134.5$ protein interacts with Beclin and blocks autophagy. The $\gamma_134.5$ protein plays a dual role in blocking both innate and adaptive immunity, the latter through evasion of major histocompatibility complex (MHC) class 2 responses.^{433,434,630,1157}

ORF-P (233 codons, pre- α , nonessential). ORF-P encodes the ORF-P protein, which binds p32 and localizes in spliceosomes. ORF-P is encoded on the strand complementary to $\gamma_134.5$. Transcription of ORF-P is blocked by ICP4 bound to a high-affinity site at the transcriptional initiation site of ORF-P. Derepression of ORF-P results in decreased expression of $\gamma_134.5$. Overexpression of ORF-P results in decreased expression of viral products of spliced RNAs.^{612,614}

ORF-O (117 codons, pre- α , nonessential). ORF-O encodes the ORF-O protein, which blocks the binding of ICP4 to its cognate high-affinity DNA binding sites *in vitro*. The N-terminus is identical to that of ORF-P. The amino acid sequence beyond residue 35 is in an alternate reading frame. Expression of ORF-O is repressed by ICP4.⁹¹⁶

$\alpha 0$ (775 codons, α , nonessential at high multiplicities). $\alpha 0$ encodes ICP0, an E3 ubiquitin ligase that promotes viral gene expression and inhibits host cell responses. The three exons encode a 775-residue protein containing a RING finger

domain. In transfected cells, it acts as a promiscuous transactivator of genes introduced by transfection or infection. Early in infection, it localizes near ND10 bodies. During this phase, it turns over with a half-life of approximately 1 hour. At late times, it accumulates in the cytoplasm. Several major interactions with cellular proteins have been reported. Thus, (a) it acts as a ubiquitin ligase, and in conjunction with UbcH5A ubiquitin conjugating enzyme, it degrades promyelocytic leukemia (PML) and SP100 proteins, resulting in dispersal of ND10 bodies. A consequence of the dispersal of ND10 bodies is loss of activation of antiviral genes by exogenous interferon (IFN). (b) ICP0 binds to CoREST (corepressor of the RE1 silencing transcript factor) and dissociates histone deacetylases (HDACs) 1 and 2 from the HDAC CoREST/LSD1/REST repressor complex. As a consequence, ICP0 blocks repression of viral genes by the repressor complex. (c) ICP0 also interacts with cyclin D3 to recruit CDK4 and with BMAL1 to recruit the CLOCK histone acetyl transferase (HAT) to the viral replication compartments. Additional functions have been described.^{181,400,401,402,409,526,527,644}

U_L1 (224 codons, γ_1 , essential). *U_L1* encodes glycoprotein L (gL), which chaperones gH (U_L22) through the Golgi to the plasma membrane. gL is not anchored to the plasma membrane and appears to regulate fusogenic activity of gH. It is conserved among herpesvirus subfamilies. gL is a target for neutralizing antibody.^{479,968}

U_L2 (334 codons, β or γ_1 , nonessential). *U_L2* encodes a uracil-DNA glycosylase enzyme, a highly conserved enzyme associated with the base excision repair pathway. Uracil-DNA glycosylase replaces uracil in G:U base pairs resulting from deamination of cytosine residues in DNA.^{156,1266}

U_L3 (235 codons, γ_2 , nonessential). *U_L3* encodes a nuclear non-structural protein of unknown function that is phosphorylated by the U_L13 protein kinase. U_L3 co-localizes with ICP22-U_S1.5 proteins in small dense nuclear structures. In the absence of ICP22 or U_S1.5, it is diffuse throughout the nucleus. The protein is translated predominantly from the second methionine of its reading frame. It interacts with ICP22 possibly through formation of complexes that include other proteins.^{51,714,1274,1277}

U_L4 (199 codons, γ_2 , nonessential). *U_L4* encodes a nuclear non-structural protein of unknown function. U_L4 co-localizes with ICP22/U_S1.5 in small dense nuclear structures formed prior to the onset of viral DNA synthesis. In the absence of other viral proteins, it remains in the cytoplasm. U_L4 forms complexes with other viral proteins.^{494,520,1277,1283}

U_L5 (882 codons, β , essential). *U_L5* encodes a component of the heterotrimeric helicase-primase complex that contains sequence motifs shared by members of the superfamily of RNA and DNA helicases. The U_L5-U_L52 complex has DNA-dependent adenosine triphosphatase (ATPase) and guanosine triphosphatase (GTPase), DNA primase, and DNA helicase activities. U_L5 function depends on its interactions with ICP8.^{282,414,1323}

U_L6 (676 codons, γ , essential). *U_L6* encodes the virion portal protein. U_L6, a putative leucine zipper protein, forms a dodecameric ring located at one vertex of the HSV capsid. U_L6 interacts with U_L15 and U_L28 (the putative terminase) proteins. Resistance to a class of thiourea drugs that block cleavage and packaging of DNA was mapped to the *U_L6* ORF.^{803,1236,1290}

U_L7 (296 codons, γ_1 , nonessential). *U_L7* encodes a virion tegument protein reported to interact with mitochondrial adenine nucleotide translocator 2.^{818,1125}

U_L8 (750 codons, β , essential). *U_L8* encodes the putative primase subunit of the heterotrimeric helicase primase complex and is required for the transport of U_L52 and U_L5 into the nucleus. It interacts with ICP8, U_L9, U_L30, and U_L42. It is required for unwinding of ICP8-coated DNA by ICP8.^{326,715,1134}

U_L8.5 (487 codons, γ_1 , unknown). *U_L8.5* encodes a protein corresponding to the C-terminal domain of U_L9 protein. It was reported to be synthesized both early and late in infection and to bind to the origin of DNA synthesis. In transient assays, the U_L8.5 protein inhibited DNA synthesis and may play a role in shifting the pattern of synthesis for *de novo* initiation at origins to a rolling circle model of viral DNA synthesis.^{60,61}

U_L9 (851 codons, β , essential). *U_L9* encodes the viral origin binding protein. U_L9 binds to specific sites flanking the origins of viral DNA synthesis. It acts as an ATPase and helicase and is required for initiation but not for continued DNA synthesis. hTid-1 enhances the binding of a multimer of U_L9 to the origin of DNA synthesis. NFB42, an F box component of SCF E3 enzyme binds phosphorylated U_L9 and targets it for ubiquitin-proteasomal degradation. The phosphorylation and degradation of U_L9 may enable the switch from *de novo* initiation of DNA synthesis at origins to a rolling circle type of DNA replication.^{314,569,628}

U_L9.5 (477 codons, γ_2 , unknown). *U_L9.5* encodes a protein of unknown function but may be the same as the U_L8.5 protein because the transcript of *U_L9.5* is co-terminal with those of *U_L8*, *U_L8.5*, and *U_L9*.⁶¹

U_L10 (473 codons, γ_2 , nonessential). *U_L10* encodes glycoprotein M (apparent M_r 53,000–63,000), predicted to contain six to eight transmembrane segments. It forms a complex with U_L49. Although a $\Delta gM\Delta gE$ HSV mutant showed no major impairment of growth, gM/U_L49.5 altered the localization and decreased the fusogenic activity of HSV glycoproteins.^{52,238}

U_L11 (96 codons, γ , nonessential). *U_L11* encodes a myristoylated, palmitoylated tegument protein of unknown function. ΔU_{L11} mutants exhibit reduced levels of envelopment and egress of virus from infected cells. The protein associates with lipid rafts, binds the cytoplasmic face of cellular membranes, and recycles to the Golgi. It is particularly abundant in the Golgi apparatus. U_L11 interacts with and directs U_L16 to the Golgi.^{53,581,677,698}

U_L12 (626 codons, β , nonessential). *U_L12* encodes an alkaline exonuclease that potentially functions as a resolvase, an enzyme required for processing of replication intermediates that would interfere with the packaging of viral DNA into capsids. It forms a complex with ICP8 that has recombinase activity.^{56,385,717,932}

U_L12.5 (~500 codons, nonessential). *U_L12.5* encodes a protein that corresponds to the C-terminal portion of the U_L12 protein. The protein accumulates in the cytoplasm, contains a mitochondrial localization signal, and degrades mitochondrial DNA.^{228,717,981}

U_L13 (518 codons, γ , nonessential). *U_L13* encodes a serine/tyrosine protein kinase enzyme that phosphorylates many viral and cellular proteins. It is present in the virion tegument;

- however, its function as a tegument protein is unknown. *U_L13* appears to regulate multifunctional proteins and disrupt nuclear lamins. The kinase specificity of *U_L13* is similar to that of *cdc2*.^{36,247,906,1064}
- U_L14*** (219 codons, γ_2 , nonessential). *U_L14* encodes a virion tegument protein. ΔU_{L14} mutants replicate well in cell culture but are highly attenuated in the mouse. The only function attributed to *U_L14* is enhancement of nuclear localization of *U_L17* and *U_L26*. Co-expression of *U_L14* enhances nuclear import of the *U_L35* (VP26) and *U_L33* proteins and increases luciferase expression, suggesting that it may facilitate folding of various proteins. *U_L14* has been implicated in cell-to-cell trafficking of virus.^{242,1284,1286}
- U_L15*** (735 codons, γ , essential). *U_L15* encodes a protein that binds *U_L28* and *U_L6* and is required for cleavage and packaging of viral DNA (terminase). The ORF consists of two exons. The intron is antisense to the *U_L16* and *U_L17* ORFs. The protein is cleaved at its N-terminus—a reaction coupled with maturation of viral DNA into unit-length molecules.^{49,235,988}
- U_L15.5*** (293 codons, γ , unknown). *U_L15.5* encodes a capsid-associated protein of unknown function from exon 2 of *U_L15*.⁴⁹
- U_L16*** (373 codons, γ_1 , essential). *U_L16* encodes a tegument protein that interacts with the *U_L11* and *U_L21* proteins, and is encoded by a sequence antisense to the *U_L15* intron. *U_L16* dissociates from capsids on interaction with receptors.^{425,791,988}
- U_L17*** (703 codons, γ , not essential). *U_L17* encodes a putative tegument protein that plays a role in the terminal stages of DNA cleavage and packaging. This ORF is encoded in sequences antisense to exon 2 of *U_L15*. In productive infection, *U_L17* may localize preformed capsids to the replication compartment.^{986,987,1130}
- U_L18*** (318 codons, γ_1 , essential). *U_L18* encodes VP23 (*M_r* 34,000), a capsid protein that together with the VP19C (*U_L38*) forms triplexes consisting of two copies of VP23 and one copy of VP19C. The 320 triplexes connect adjacent hexons⁷⁰⁶ and pentons.^{53,1162}
- U_L19*** (1,311 codons, γ_1 , essential). *U_L19* encodes VP5 (*M_r* 149,000), the major capsid protein present in hexons that appear as tower-like complexes on the capsid in hexons and pentons.^{120,804,1265}
- U_L20*** (222 codons, γ_1 , nonessential). *U_L20* encodes a protein that is predicted to be polytopic with four predicted transmembrane segments. It localizes to virions, nuclear membranes, and Golgi but is absent from plasma membranes. When expressed singly, it localizes mainly to endoplasmic reticulum. Co-expressed with gK, it localizes to the Golgi and inhibits cell–cell fusion. A deletion mutant in the *U_L20* gene is defective for transport of the virions out of the perinuclear space, particularly in cells with fragmented Golgi. The ΔU_{L20} mutant is syncytial. Its postulated role is to prevent fusion of the infected cells with adjacent cells. *U_L20* interacts with gK and gB.^{45,54,197,341}
- U_L20.5*** (160 codons, γ_2 , nonessential). *U_L20.5* encodes a protein of unknown function that localizes to small dense nuclear structures containing ICP22, *U_S1.5*, *U_L3*, and *U_L4*. The *U_L20.5* ORF is located upstream of the *U_L20* ORF.¹²¹⁸
- U_L21*** (535 codons, γ , nonessential). *U_L21* encodes a nucleotidylated tegument protein weakly associated with capsids. It binds to microtubules but not to purified tubulin. It binds *U_L6*.^{48,98,1121}
- U_L22*** (838 codons, γ , essential). *U_L22* encodes glycoprotein H (gH), which is essential for virion infectivity and cell–cell fusion. In cells infected with a gH deletion mutant or a ts mutant at the nonpermissive temperature, viruses lacking gH egress from the cell but are not infectious. Transport of gH from the endoplasmic reticulum to the Golgi and plasma membranes requires the interaction with soluble gL. gH is an essential component of the complex enabling cell–cell fusion and induces neutralizing antibodies.^{271,340}
- U_L23*** (376 codons, β , nonessential). *U_L23* encodes the TK. Although known primarily as a TK, it is a wide-spectrum nucleoside kinase capable of phosphorylating both purine and pyrimidine nucleosides and their analogs. TK plays a crucial role in activating nucleoside analogs used for treatment of HSV infections.^{226,557,732}
- U_L24*** (269 codons, γ_1 , nonessential). *U_L24* encodes a membrane-associated nuclear protein (*M_r* 30,000). Mutant viruses defective for *U_L24* form small polykaryocytes in cell culture, yield less virus, and exhibit decreased virulence in mice. *U_L24* is associated with dispersal of nucleolin. Other functions remain to be determined. The *U_L24* coding sequences are contained in six different mRNAs originating from three different initiation sites and terminating at two different 3' termination sites, and the synthesis and cytoplasmic accumulation of these transcripts is regulated by ICP27. The *U_L24* ORF partly overlaps the *TK* gene on the opposite strand.^{89,419,692,857}
- U_L25*** (580 codons, γ_2 , essential). *U_L25* encodes a capsid protein involved in release of viral DNA after penetration and in capsid assembly after packaging of DNA and possibly for retention of DNA in the capsid.^{735,798,825}
- U_L26*** (635 codons, γ , essential). *U_L26* encodes a protease and scaffolding protein involved in capsid assembly. The *U_L26* product is cleaved in *cis*- or *trans*- by itself at two sites: the N-terminal polypeptide being a serine protease required for assembly of a scaffolding within capsids for DNA packaging, and the larger, middle cleavage product a component of the scaffolding.^{657,658}
- U_L26.5*** (326 codons, γ , essential). *U_L26.5* encodes a protein that is identical to the C-terminal sequence of the *U_L26* protein. The promoter and coding domain of the *U_L26.5* gene is contained in its entirety within the *U_L26* ORF. *U_L26.5* is cleaved by the protease at its C-terminus at the same site as the *U_L26* protein.⁶⁵⁸
- U_L27*** (904 codons, γ_1 , essential). *U_L27* encodes glycoprotein B (gB), which interacts with gH/gL and with gD is an essential step for entry by fusion of the envelope with the plasma membrane. Virions lacking gB egress the cell but are non-infectious. The protein binds heparan sulfate. Mutations in the cytoplasmic tail cause formation of syncytia. The tail also contains endocytosis motifs, which mediate gB endocytosis into large vacuoles. Down-modulation of gB by gK at cell surfaces may be responsible for negative control of cell–cell fusion. A ts mutation in the ectodomain (tsB5) affects virus entry into the cell. gB induces neutralizing antibodies. gB decorated by O-linked glycans interacts with the paired immunoglobulin (Ig)-like type 2 receptor α .^{29,144,196,441,859,998}
- U_L27.5*** (undefined but not >575 codons, γ_2 , unknown). *U_L27.5* encodes a cytoplasmic protein of unknown function with an

- apparent M_r of 43,000; its size is smaller than the predicted capacity of the ORF. The ORF maps antisense to *U_L27*.¹⁷⁷
- U_L28*** (785 codons, γ , essential). *U_L28* encodes a putative subunit of the terminase, a component of the DNA cleavage and packaging complex. It interacts with *U_L15*, *U_L6*, *U_L14*, and *U_L33*. *U_L28* binds *pac1* sequences and is required for correct generation of the L-terminus.^{76,492,1288}
- U_L29*** (1,196 codons, β , essential). *U_L29* encodes ICP8, the viral major DNA-binding protein, which unwinds and binds single-stranded DNA. ICP8 plays a key role in viral gene expression and an essential role in synthesis of viral DNA. ICP8 enables the unwinding of the DNA at the origin by *U_L9* and allows the entry of the machinery that replicates the DNA. ICP8 may also serve as a scaffold for organization of the replication complex. ICP8 in combination with *U_L12* or another nuclease has recombinase activity.^{188,221,932}
- U_L30*** (1,235 codons, β , essential), *U_L30* encodes the catalytic subunit of the viral DNA polymerase. The coding sequence is contained in several transcripts differing in size and temporal patterns of accumulation.^{104,1228,1229}
- U_L31*** (306 codons, γ , essential in a cell-type dependent fashion). *U_L31* encodes a nuclear protein required for viral disruption of the nuclear lamina and promotes primary envelopment at the inner nuclear membrane. *U_L31* requires *U_L34* protein for localization at the inner nuclear membrane and for its stability.^{98,179,935,1051,1295}
- U_L32*** (596 codons, γ_2 , essential). *U_L32* encodes a cysteine-rich, zinc-binding protein that is highly conserved among different herpesviruses and plays an essential role in the cleavage of concatemeric viral DNA into monomeric genomes and their packaging into preformed capsids. Localization of *U_L32* to the nucleus requires the presence of *U_L6*, *U_L25*, *U_L17*, and *U_L28*.^{178,619}
- U_L33*** (130 codons, γ_2 , essential). *U_L33* encodes a nuclear protein that along with *U_L6*, *U_L15*, *U_L17*, *U_L28*, and *U_L32* is required for packaging of viral DNA into preformed capsids. *U_L33* is reported to be translocated into the nucleus along with VP26 by *U_L14*.^{75,933,1286}
- U_L34*** (275 aa, γ_1 , essential). *U_L34* encodes a type 2 membrane protein phosphorylated by the *U_S3* kinase that localizes in nuclear membranes and is required for nuclear envelopment. *U_L34* is present in perinuclear virions but not in extracellular virions. Exclusive localization of *U_L34* protein at the nuclear membrane requires *U_L31* in certain cells.^{907,935,1035,1285}
- U_L35*** (112 codons, γ_2 , nonessential). *U_L35* encodes the VP26 capsid protein, which forms a hexameric structure located on the outer surface of each hexon. It interacts with dynein light chains RP3 and Tetex1 and is involved in intracellular transport.^{215,267,289}
- U_L36*** (3,165 codons, γ_2 , essential). *U_L36* encodes a tegument protein designated VP1-2 ($M_r > 270,000$), the largest protein encoded by HSV. It is required for egress of virions through the cytoplasm. A ts mutation in *U_L36* (*tsB7*) blocks release of viral DNA into the nucleus at the nonpermissive temperature. Release of DNA requires proteolytic cleavage of the protein. Late in infection, cells contain a truncated protein consisting of the amino-terminal domain that exhibits a cysteine protease deubiquitinating activity. *U_L36* and *U_L37* form tufts of elongated strands at the vertices of capsids.^{73,270,518,534,796,1008}
- U_L37*** (1,123 codons, γ_1 , essential). *U_L37* encodes a tegument phosphoprotein that binds to DNA in the presence of ICP8. In the absence of *U_L37*, nucleocapsids accumulate aberrantly in the nucleus and unenveloped capsids accumulate in the cytoplasm. The protein interacts with VP1-2 (see *U_L36*).¹⁰¹³
- U_L38*** (465 codons, γ_2 , essential). *U_L38* encodes the capsid protein VP19C. This protein forms triplexes consisting of two copies of VP23 (*U_L18*) and one copy of VP19C. The 320 triplexes connect adjacent hexons and pentons.^{1082,1162}
- U_L39*** (1,137 codons, β , nonessential). *U_L39* encodes ICP6, which is the large subunit of ribonucleotide reductase. ICP6 is anchored in membranes and has protein kinase activity mapping to the N-terminus; however, this is not required for ribonucleotide kinase activity. *U_L39* may play a role in maintaining dTTP pools in infected cells.^{202,248,862}
- U_L40*** (340 codons, β , unknown). *U_L40* encodes the small subunit of ribonucleotide kinase.⁴⁸⁶
- U_L41*** (489 codons, γ_1 , nonessential). *U_L41* encodes a tegument protein with an endoribonuclease activity with a substrate specificity similar to that of ribonuclease A (RNase A). This is termed the VHS function and protein. VHS introduced into cells during infection degrades (a) stable mRNAs in polyribosomes from 5' to 3' and (b) mRNAs containing A-U-rich elements by deadenylation and endonucleolytic cleavage in the 3' untranslated region (UTR). Other features suggest that the degradation of mRNA is selective. Newly made VHS is neutralized by VP16 (*U_L48*) and VP22 (*U_L49*). VHS also interacts with ICP27.^{317,1022,1105,1116,1118,1120}
- U_L42*** (488 codons, β , essential). *U_L42* encodes the DNA polymerase processivity factor, which binds double-strand DNA and tethers the polymerase to its template and increases its processivity. *U_L42* also associates with cdc2 and topoisomerase II α .^{11,389}
- U_L43*** (434 codons, γ , nonessential). *U_L43* encodes a hydrophobic, myristoylated integral membrane protein of unknown function.^{161,699}
- U_L43.5*** (91 codons, γ_2 , nonessential). *U_L43.5* encodes a hydrophilic protein localizing in nuclear structures containing capsid proteins. The ORF is encoded antisense to *U_L43*.¹²¹⁷
- U_L44*** (511 codons, γ_2 , nonessential). *U_L44* encodes glycoprotein C (gC), which mediates attachment of virions to cells by binding to glycosaminoglycans of heparan sulphate or to chondroitin sulfate to a site near its N-terminus. Δ gC mutants attach to cells with reduced efficiency. gC contains two domains involved in modulating complement activation: one binds C3, and the other is required for blocking C5 and properdin binding to C3. Each region contributes to virulence, as viruses lacking these domains are less virulent than wild-type virus. gC is heavily N- and O-glycosylated.^{354,443,685}
- U_L45*** (172 codons, γ_2 , nonessential). *U_L45* encodes a type II membrane protein of unknown function.^{286,1192}
- U_L46*** (718 codons, γ_1 , nonessential). *U_L46* encodes tegument proteins VP11 and VP12, differentiated solely by their migration in denaturing gels. VP11-12 associates with capsids and membranes, binds VP13-14 (*U_L47*) in stoichiometric amounts with VP16 (*U_L48*), and enhances the latter's activity. In the absence of *U_L48* protein, *U_L46* inhibits activation of α promoters. VP11-12 is stabilized by *U_S3* kinase and activates Lck kinase.^{66,720,731,778,1200,1201,1311}

U_L47 (693 codons, γ_1 , nonessential). *U_L47* encodes the VP13 and VP14 tegument proteins, which along with *U_L46* enhance the activity of VP16 (*U_L48*). *U_L47* in conjunction with ICP27 binds and displaces PABC1 from the poly(A)-binding protein (PABP) binding complex on the cap structure. It is reported to shuttle between the cytoplasm and nucleus.^{66,98,279,287,744,1023}

U_L48 (491 codons, γ , essential). *U_L48* encodes VP16 or (α -trans-inducing factor (α -TIF), a multifunctional tegument protein. It induces α genes by interacting with two cellular proteins, HCF and Oct-1. The complex binds to specific sequences with the consensus GyATGnTAAT-GArATTCy-NC. It is also required for virion assembly. Together with VP22 (*U_L49*), it interacts with and blocks the ribonuclease (RNase) activity of *U_L41*.^{665,695,860,1158}

U_L49 (301 codons, γ_2 , nonessential). *U_L49* encodes the VP22 tegument protein, which binds RNA and translocates it from infected to adjacent, noninfected cells. It has been shown to interact with chromatin remodeling proteins and to block nucleosome assembly in transfected cells. It binds to microtubules and after cell division to chromatin. It has also been reported to bind membranes and to induce the stabilization and hyperacetylation of microtubules (putative microtubule-associated protein). It is not required for tegument assembly; however, it plays a critical role in neutralization of VHS. Mutants lacking VP22 express a defective VHS.^{98,123,882,1022,1023,1180}

U_L49.5 (91 codons, γ_2 , essential). *U_L49.5* encodes the gN membrane-associated protein with apparent *M_r* of 6700 that is abundant in virions. It interacts with gM (*U_L10*), and gM may be required for packaging of *U_L49.5* in virions. Reported to block endogenous antigen presentation.^{6,67,831}

U_L50 (371 codons, β , nonessential). *U_L50* encodes a monomeric deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase).^{57,66}

U_L51 (244 codons, γ_2 , nonessential). *U_L51* encodes a tegument protein that localizes to the Golgi apparatus and is involved in virus egress.^{66,246,819}

U_L52 (1,058 codons, β , essential). *U_L52* encodes the primase subunit of the HSV helicase-primase complex. Its strongest affinity appears to be for ICP8; however, both *U_L8* and *U_L5* proteins are required for DNA binding.^{95,291,558}

U_L53 (338 codons, γ , nonessential). *U_L53* encodes glycoprotein K (gK), whose sequence predicts three transmembrane segments. Epitope mapping predicts an extracellular N-terminus and intracellular C-terminus. In infected cells, gK localizes to the Golgi apparatus. In transduced cells, gK localizes mainly in the endoplasmic reticulum (ER). Colocalization with Golgi markers requires the *U_L20* protein. Plasma membrane localization is less clearly defined. gK is the most frequent locus of syncytial (syn) mutations. A Δ gK mutant virus is defective in virion exocytosis and is syncytial. gK inhibits fusion in the cell-cell fusion assay. Inhibition is augmented by co-expression with *U_L20* protein. One function of gK may be to prevent infected cells from fusing with adjacent cells.^{45,342,480,496,880}

U_L54 (512 codons, α , essential). *U_L54* encodes the ICP27 multifunctional regulatory protein. Early in infection, its major role is to block pre-mRNA splicing and thus contributes to the shutoff of host protein synthesis. At the same time, and particularly at late times after infection, ICP27 is required

for transcription of some β and γ_1 , and perhaps all γ_2 genes. Thus, ICP27 recruits cellular RNA polymerase II (pol II) to the viral genome to promote efficient viral transcription. It shuttles between nucleus and cytoplasm, promoting the cytoplasmic accumulation of some mRNAs. It is intimately involved in translation. It binds RNA and interacts with numerous cellular and viral proteins.^{98,245,313,339,497,995,1314}

U_L55 (186 codons, γ_2 , nonessential). *U_L55* encodes a nonstructural protein of unknown function that is associated with sites of virion assembly.¹²⁸²

U_L56 (234 codons, γ_2 , nonessential). *U_L56* encodes a phosphorylated type II virion membrane protein. It was reported to interact with and relocalize a member of the Nedd4 family of ubiquitin ligases and to bind kinesin motor protein KIF1A. It was reported to be required for pathogenesis in mice.^{579,580,970,1177}

$\alpha 4$ (1,298 codons, α , essential). $\alpha 4$ encodes ICP4, a protein that acts as a repressor and as a transactivator of viral transcription. As a repressor, it binds to high-affinity sites overlapping the transcription initiation sites of its own gene and that of ORF-P and ORF-O. It also interacts with cellular transcription factors (e.g., TFIID) and is present in transcriptional complexes containing ICP22, ICP27 and the CLOCK HAT, and with DNA at low-affinity sites whose sequences are significantly different from that of high-affinity sites. The interaction of ICP4 together with transcription factors at low-affinity sites may account for the transactivating function of ICP4. The role of low-affinity sites remains unclear.^{264,394,526,843}

$\alpha 22/U_S1$ (420 codons, α , nonessential). $\alpha 22/U_S1$ encodes the ICP22 regulatory protein, which is required in experimental animal systems and some nonhuman cell lines but not in Vero or HEp-2 cells. The properties encoded in its C-terminal domain are similar to those of *U_S1.5* described later and are principally manifested by reduced accumulation of a subset of γ_2 genes. Deletion of the entire ORF exacerbates the attenuation phenotype. The protein is extensively phosphorylated by viral and cellular kinases and nucleotidylated by casein kinase II. Thus, in the absence of ICP22 or *U_L13*, a set of late proteins (*U_L41*, *U_S11*, and *U_L38*) accumulates in smaller amounts. Both ICP22 and *U_L13* are required for the activation of *cdc2* and degradation of the partners, cyclin A and B. *cdc2* partners with *U_L42* and together bind and mediate postranscriptional modification of topoisomerase II α . These steps are essential for optimal expression of *U_L41*, *U_S11*, and *U_L38*. *U_L13* and ICP22 mediate the phosphorylation of the C-terminal domain of RNAi pol II. In addition, ICP22 interacts with *cdk9* in a *U_S3* kinase-dependent fashion to phosphorylate RNAi pol II. ICP22 also affects the accumulation of $\alpha 0$ mRNA. ICP22 and the *U_L13* protein kinase mediate the degradation of cyclin B. *cdc2* acquires a new partner—the *U_L42* polymerase accessory factor—and together they recruit topoisomerase II to enable the expression of the subset of γ_2 genes exemplified by *U_L41*, *U_S11*, and *U_L38*. It has been detected in complexes containing ICP4, ICP27, TFIID, and CLOCK HAT.^{11,304,526,673,757}

U_S1.5 (250 codons, α , nonessential). *U_S1.5* is expressed from a promoter and coding domain contained within the $\alpha 22$ ORF, and the $\alpha 22$ methionine 171 codon acts as the initiator codon of the *U_S1.5* ORF. Most of the known functions of ICP22 map to the *U_S1.5* ORF.^{11,159,675}

U_S2 (291 codons, γ_2 , nonessential). *U_S2* encodes a tegument protein reported to interact with cytokeratin 18.^{388,506}

U_S3 (481 codons, γ_1 , nonessential). *U_S3* encodes a multifunctional serine-threonine protein kinase that phosphorylates HDAC1 and 2, CoREST, U_L34, and a host of other viral and cellular proteins. Its substrate specificity is similar to that of cellular protein kinase A. It promotes egress of capsids from nuclei and is essential for blocking apoptosis induced by viral or exogenous inducers. A key function of the *U_S3* protein kinase is to alter the structure of nuclear membranes so as to enable budding of capsids into the lumen between the inner and outer nuclear membranes.^{82,639,907,977}

U_S3.5 (405 codons, γ_1 , nonessential). *U_S3.5* encodes a truncated *U_S3* protein kinase enzyme initiated at the *U_S3* methionine 71 codon on an mRNA that is smaller than that encoding *U_S3*. *U_S3.5* mediates the phosphorylation of HDAC1 and does not block apoptosis.⁸⁸⁶

U_S4 (283 codons, γ_1 , nonessential). *U_S4* encodes glycoprotein G (gG), whose precise function is not known; it has been shown to bind to chemokines.¹¹⁹¹ The HSV-2 gG is larger than HSV-1 gG; thus, gG can be used to differentiate HSV-1 from HSV-2 antibody responses.^{3,626}

U_S5 (92 codons, γ , nonessential). *U_S5* encodes glycoprotein J (gJ), which blocks apoptosis induced by granzyme B, Fas, or by infection of cells with glycoprotein D (gD; *U_S6*) minus viruses and other agents.^{501,1316}

U_S6 (394 codons, γ , essential). *U_S6* encodes gD, a multifunctional protein with the following properties: (a) it interacts with three cellular receptors for entry—HvE, nectin1, and a modified heparin sulfate; hence, it defines viral tropism. (b) Upon receptor binding, an ensuing change in conformation exposes fusion domains that enable the fusogenic glycoproteins gB, gH, and gL to complete fusion of the envelope with the plasma membrane. (c) The N-terminal domain may be replaced with ligands for entry via other receptors. (d) gD protects the cell from apoptosis induced by Δ gD mutants. Antiapoptotic activity is mediated by mannose-phosphate receptor. (e) Δ gD mutants grown in cells ectopically expressing gD produce virions that exit the cell but are noninfectious.^{157,209,368,1131,1316}

U_S7 (390 codons, γ_1 , nonessential). *U_S7* encodes glycoprotein I (gI), which forms a heterodimer with gE. The gE-gI complex constitutes a viral Fc receptor for monomeric immunoglobulin G (IgG). The gE-gI complex has basolateral localization in epithelial cells and facilitates basolateral spread of progeny virus in polarized cells. *U_S7* is dispensable in transformed cells but critical in nontransformed cells.^{277,508,674}

U_S8 (550 codons, γ_1 , nonessential). *U_S8* encodes glycoprotein E (gE), the major component of the viral Fc receptor. It is phosphorylated by the U_L13 kinase and forms a heterodimer with gI (*U_S7*). *U_S8* has been implicated in cell-to-cell spread and in virion transport in neurons and antihist defenses.^{74,277,686,1212}

U_S8.5 (159 codons, γ_1 , nonessential). *U_S8.5* encodes a nuclear protein of unknown function that localizes to nucleoli. Its mRNA is among the most abundant species packaged in virions.^{367,675,1023}

U_S9 (90 codons, γ , nonessential). *U_S9* encodes a type II membrane-associated protein. *U_S9* may play a role in axonal spread.^{675,728,814,1066}

U_S10 (312 codons, γ_1 , nonessential). *U_S10* encodes a tegument phosphoprotein of unknown function that co-purifies with the nuclear matrix.^{675,814,1281}

U_S11 (161 codons, γ_2 , nonessential). *U_S11* encodes an RNA-binding protein with multiple functions: (a) it binds RNA in sequence- and conformation-dependent fashion and is in part responsible for the packaging of RNA in virions. Virion-associated *U_S11* binds to ribosomes and localizes to polyribosomes. (b) When expressed from an early promoter, it blocks activation of protein kinase R. (c) Late in infection, *U_S11* is also found in nucleoli. (d) *U_S11* interacts with and blocks homeodomain-interacting protein kinase 2 (HIPK2).^{165,379,675,887,963,1023}

α 47/*U_S12* (88 codons, α , nonessential). α 47/*U_S12* encodes ICP47, which selectively binds to human TAP1/TAP2 and blocks the transport of antigenic peptides into the ER for presentation at the cell surface.^{446,723,1152,1302}

Noncoding RNAs

LAT. The primary LAT is a low-abundance transcript of 8.5 to 9 kb in size and extends through the length of the *ab* and *b'a'* sequences flanking the unique long sequences. The smaller LATs are stable introns and terminate antisense to and within the coding sequences of the α 0 gene. Deletion of the sequences encoding LAT increases the mortality and morbidity in experimental animal systems and decreases the number of neurons harboring latent virus. The decrease in the number of neurons in LAT minus mutants has been linked to increased viral gene expression and pro-apoptotic manifestations of latent virus. The LAT promoter has neuron-specific elements. Accumulation of LAT correlates with increased assembly of viral DNA into heterochromatin.^{206,329,360,866,1101,1214}

ORI_S RNAs. These 5' co-terminal RNAs originate in the *c* sequences flanking the unique short sequences at or near the transcription initiation sites of the α 22 or α 47 genes. Ori_{S1} is synthesized early in infection in the absence of protein synthesis and runs antisense to α 22 or α 47 genes, terminating at the transcription initiation sites of these genes. Ori_{S2} extends across the ORI_S sequence and co-terminates with the transcript encoding ICP4. The HSV-1 Ori_{S2} contains an ORF, whereas the HSV-2 Ori_{S2} does not. Ori_{S2} RNA is detected late in infection. The function of these RNAs is not known.⁴⁷⁶

α X and β X RNAs. These overlapping RNAs are 0.9 and 4.9 Kb in size, originate upstream of ORF P, and extend across the L-S junction. Their function is not known.^{108,644}

AL-RNA. Reported to be antisense to the 5' sequences of LAT.⁸⁶⁸

α 0 Intron 1 RNA. This is a family of four RNAs with a common 5' end but differing 3' sequences. The RNAs are stable, nonpolyadenylated, and accumulate in the cytoplasm. Their functions are not known.¹⁶⁰

***U_S5–1* RNA.** The RNA initiates in *U_S5* and terminates in the α 22 ORF. It is expressed antisense to *U_S5*, *U_S4*, *U_S3*, and ICP22 mRNAs. This transcript is expressed with γ 2 kinetics and has a half-life of 80 minutes. There is no evidence that it encodes a protein.⁵¹⁹

MicroRNAs. MicroRNAs have been detected in trigeminal and sacral ganglia of mice latently infected with HSV-1

and HSV-2, respectively. The microRNAs are largely derived from the genome domains encoding the LAT precursor RNA. To date, no evidence has emerged that the microRNAs play a defined role in the establishment or maintenance of latency.^{240,521,1126,1169,1170}

VIRAL REPLICATION

Overview of Herpes Simplex Virus Replication

It is convenient to begin this section on viral replication with a bird's eye view of the major events of HSV replication (Fig. 60.3).

To initiate infection, the virus must attach to cell surface receptors. Fusion of the envelope with the plasma membrane or an internal membrane rapidly follows the initial attachment.

The de-enveloped tegument-capsid structure is then transported to the nuclear pores, where DNA is released into the nucleus.

Transcription of the viral genome, replication of viral DNA, and assembly of new capsids take place in the nucleus. Viral DNA is transcribed throughout productive infection by host RNA pol II, but with the participation of viral factors at all stages of infection. The synthesis of viral gene products is tightly regulated: Viral gene expression is coordinately regulated and sequentially ordered in a cascade fashion. The gene products studied to date form at least five groups (α , β_1 , β_2 , γ_1 , and γ_2) as a result of both transcriptional and posttranscriptional regulation. The α or immediate-early genes are expressed first and are defined as the genes that are transcribed in the absence of *de novo* viral protein synthesis. The α gene products are involved in activating expression of the β or delayed-early genes. Several of the β gene products are enzymes and

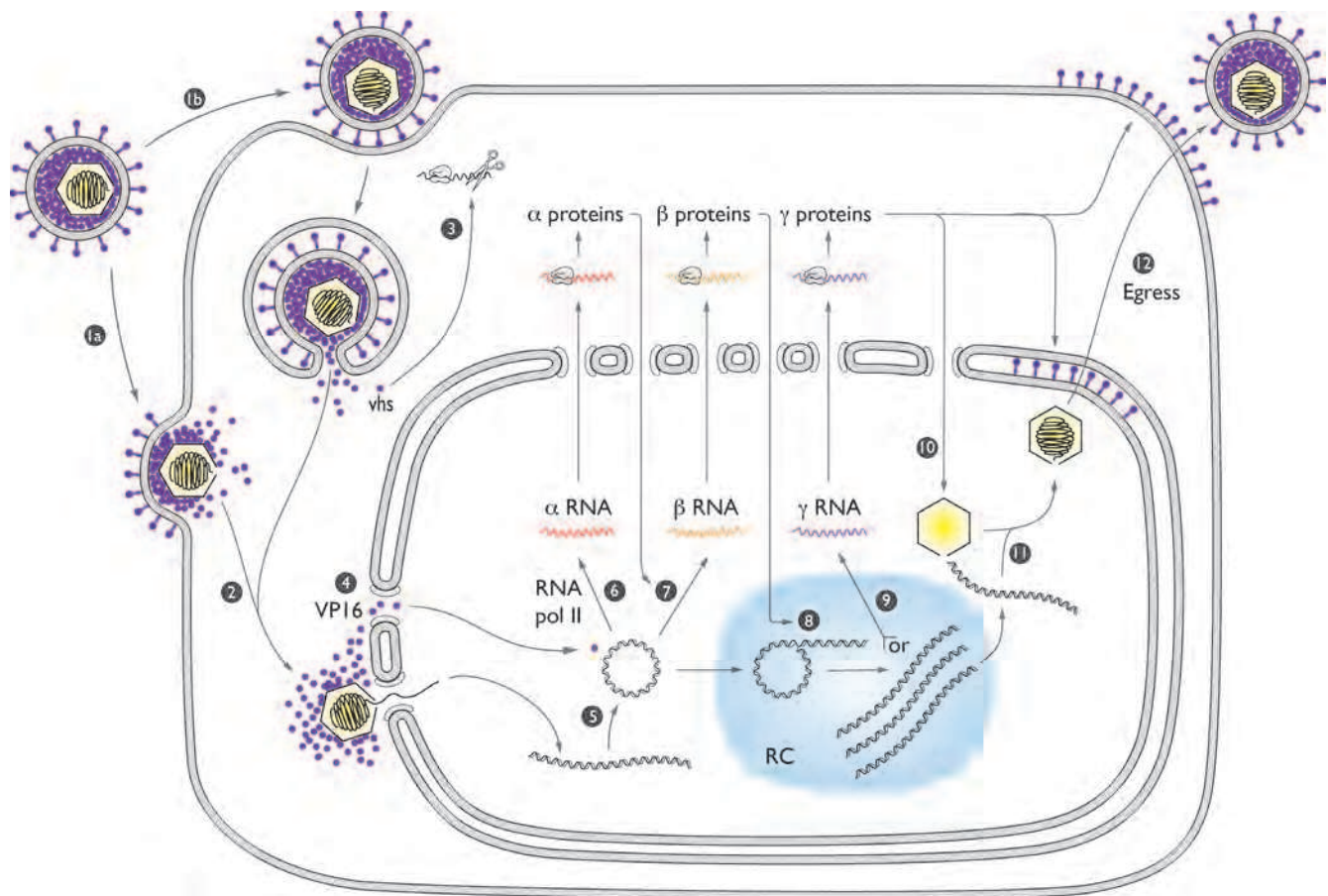


FIGURE 60.3. Diagram of the replication cycle of herpes simplex virus. 1: The virus binds to the cell plasma membrane and the virion envelope fuses with the plasma membrane (1a) or the virus enters by endocytosis (1b), releasing the capsid and tegument proteins into the cytoplasm. 2: The capsid is transported to the nuclear pore, where the viral DNA is released into the nucleus. 3: The vhs protein causes degradation of host messenger RNAs (mRNAs). 4: VP16 localizes into the nucleus. 5: The viral DNA circularizes. 6: It is then transcribed by host RNA polymerase II to give first the α mRNAs. α gene transcription is stimulated by the VP16 tegument protein. Five of the six immediate-early proteins act to regulate viral gene expression in the nucleus. 7: α proteins transactivate β gene transcription. 8: The β proteins are involved in replicating the viral DNA molecule. 9: Viral DNA synthesis stimulates γ gene expression. 10: The γ proteins are involved in assembling the capsid in the nucleus and modifying the membranes for virion formation. 11: DNA is encapsidated in the capsid. 12: The filled capsid buds through the inner membrane to form an enveloped virion, and the virion exits from the cell by mechanisms described in the text. (Copyright Lynne Chang and David Knipe.)

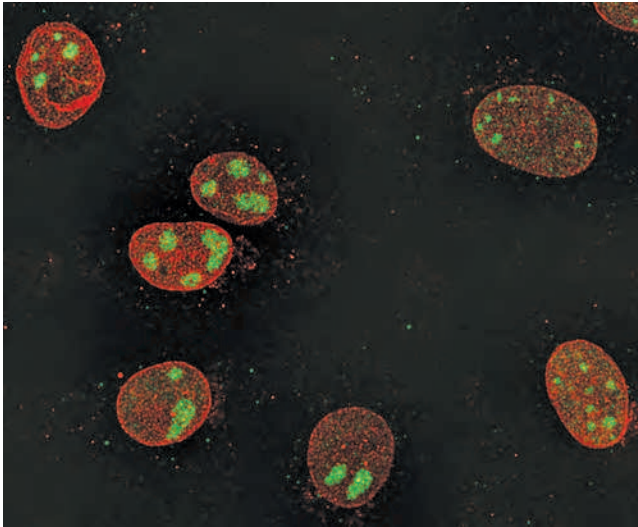


FIGURE 60.4. Replication compartments in the nuclei of herpes simplex virus (HSV)-infected cells. Shown are Vero cells infected with HSV-1 strain KOS virus and fixed at 7 hours postinfection and stained with anti-ICP8 antibody (green) to mark replication compartments and anti-lamin B1 antibody (red) to mark the nuclear lamina and define the boundaries of the nucleus. The punctate structures containing ICP8 within the larger globular replication compartments co-localize with sites of viral DNA synthesis. (Copyright Lynne Chang and David Knipe.)

DNA-binding proteins involved in viral DNA replication in nuclear replication compartments (Fig. 60.4). The bulk of viral DNA is synthesized by a rolling circle mechanism, yielding concatemers that are cleaved into monomers during the process of nucleocapsid assembly. The γ or late genes are then transcribed efficiently following viral DNA replication, and these gene products are largely involved in assembly of the progeny virions.

Assembly occurs in several stages. After packaging of DNA into preassembled capsids, the filled capsid or nucleocapsid matures into a virion and acquires infectivity by budding through the inner lamella of the nuclear membrane. The transit of virions from the space between the inner and outer nuclear membranes to the subcellular space is less well defined. It has been suggested that the virion envelope is processed by transit through Golgi stacks, by being de-enveloped and then re-enveloped at the trans Golgi network, or by the nucleocapsid exiting the nucleus through nuclear pores and then budding into the Golgi apparatus. In fully permissive tissue culture cells, the entire process takes approximately 18 to 20 hours.

HSV infection reorganizes the host cell in many ways, some of which have only recently been discovered. HSV causes extensive reorganization of cell structure, with nuclear changes including margination of chromatin, enlargement of the cell nucleus, formation of replication compartments, disruption of the nuclear lamina and nucleoli, and cytoplasmic changes including disruption of the Golgi apparatus and microtubules.⁹⁵⁶ It was recently discovered that HSV infection alters cellular metabolism, in part by diverting the central carbon metabolism toward the production of pyrimidine nucleotide components.¹¹⁸⁸ The study of the metabolomics of

HSV-infected cells will be an important new field for the next few years.

Entry of Herpes Simplex Virus

Pathways of Herpes Simplex Virus Entry Into Cells

Early electron microscope studies by Epstein led to the conclusion that entry of virus into cells occurs by fusion of the envelope with the plasma membrane. Fusion at the plasma membrane has been well documented for Vero and HEp-2 cells^{808,1270} and primary neurons.⁶⁹⁰ In contrast, although the presence of enveloped virus particles in the cytoplasm after exposure to the virus was reported approximately 40 years ago by Hummeler et al.⁴⁷⁷ and others,⁹⁵² it is only recently that entry by endocytosis became accepted as a significant pathway of entry for productive infection. HSV enters via endocytosis in other cell types such as Chinese hamster ovary (CHO) and HeLa cells.^{752,807,808} In turn, the endocytic pathway may be through acidic endosomes in some cells and through neutral endosomes in other cells,^{752,807,809} although the same viral glycoproteins are required for both pathways.⁸⁰⁹ Thus, whether HSV enters by fusion at the plasma membrane or following endocytosis is a cell line- or cell-type-dependent phenomenon. The cellular molecules that can serve as determinants of the pathway of entry into cells have come to light only recently. One such determinant appears to be $\alpha_3\beta_3$ -integrin. Thus, HSV-1 enters CHO cells by an endocytic pathway. In the absence of $\alpha_3\beta_3$ -integrin, the entry is by an endocytic pathway independent of lipid rafts and dynamin 2. Expression of $\alpha_3\beta_3$ -integrin in CHO cells routes HSV-1 to entry by an endocytic pathway dependent on lipid rafts and dynamin 2.³⁷² The properties of the gD receptor additionally contribute to determine the route of entry. Thus, entry into CHO cells via nectin is by lipid-raft-independent endocytosis. When nectin is modified and redirected to lipid rafts, entry is through lipid-raft-dependent endocytosis.

The endocytic pathway may well be the default entry pathway in certain situations. For example, in the absence of gD-receptor interactions, virions are taken up by endocytosis but are then degraded by lysosomal enzymes. Specifically, cells expressing gD take up wild-type virus in endocytic vesicles. Electron micrographs showed that the virions were rapidly degraded, and viral DNA could no longer be recovered after several hours.¹⁴⁸ gD^{-/-} mutants (i.e., virus stocks lacking both the gD gene and gD in their envelopes) are taken up by endocytosis and degraded by lysosomal enzymes. At high multiplicities gD^{-/-} virions induce apoptosis as defined by laddering of cellular DNA and other assays.¹³¹⁶

Attachment to the Cell Surface

Entry of HSV into cells involves the interaction of at least five virion surface glycoproteins with several molecules (receptors) on the cell surface and is the consequence of fusion of the envelope with a cellular membrane. The first step in the process of entry is the binding of virions to glycosaminoglycans (GAGs) on the cell surface. The interaction of virions with GAGs is mediated by two glycoproteins: gC and gB. Removal of GAGs from the cell surface or site-specific mutagenesis of gC and gB to disable the interaction with GAGs reduces virus yields by 10- to 20-fold. The available data suggest that GAGs serve as anchors or platforms from which virions interact with entry receptors. The hypothesis that the interaction of GAGs with virions is fully reversible has not been tested adequately.

The second step varies depending on the nature of the surface of the cells to which the virions are attached. Irrespective of the pathway of entry—endocytosis or fusion at plasma membrane—entry requires at the very least the interaction of virion glycoprotein gD with its receptors.

Interaction of Glycoprotein D With Its Receptors

The next series of steps in virus entry consists of the interaction of gD with its receptors and execution of fusion between the envelope and the cellular membrane by the heterodimer gH/gL and gB. The interaction of gD with its receptors has been extensively studied in many laboratories. The consensus is that, for entry, gD interacts with one of three natural receptors. The receptors are nectins, a protein designated as herpesvirus entry mediator (HVEM), and a selected form of 3-O-sulfated heparan sulfate (3-OS HS).^{150,209,368,765,1044,1077,1220} Nectin and HVEM are found on cells of numerous types and tissues, and this readily explains the ability of HSV to infect many seemingly diverse cells.

Nectins are intercellular adhesion molecules expressed on epithelial and neuronal cells, and are members of the extended Ig family. The N-terminal domains of a nectin dimer on the surface of one cell forms *trans*-dimers with the corresponding domains of nectins on adjacent cells. The C-terminal domains embedded in the cytoplasm interact with afadin, which in turn anchors the nectins to cytoskeleton.⁹⁸³ Earlier studies showed that gD of HSV-1 interacts with the amino-terminal V1 domain of nectin 1. This domain includes two loops—C-C' and C'-C"—and both loops play a role in the interaction with gD essential for virus entry.²⁰⁸ Recently, the crystal structure of gD bound to nectin 1 has extended earlier studies. Phenylalanine 129, at the tip of the loop connecting β -strands F and G of nectin 1 binds to the gD core.²⁷⁵ HSV-1 strains with specific mutations in gD and wild-type HSV-2 can also enter cells via nectin 2. The binding site on gD for with nectin 1 is complex and includes amino acid residues 34, 38, and 215 through 218.^{222,705}

HVEM is a member of the extended tumor necrosis factor (TNF) receptor family, expressed mainly by T lymphocytes,⁷⁷⁶ which in turn binds the lymphotoxin β receptor. Its natural ligand is LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV gD for HVEM, a receptor expressed by T lymphocytes), which is constitutively expressed on T and natural killer (NK) cells and appears to be a regulator of mucosal immune system.²¹⁴ The functional and structural interactions of HVEM to gD have been mapped to

the N-terminal residues 7 through 31 of gD,¹⁵⁷ a region of gD that can be deleted.¹³⁰¹

The third receptor, 3-OS HS, results from modification of HS by the HS 3-O-sulfotransferase. This enzyme is present in the brain and led to speculation that this receptor may play a role in that organ.¹⁰⁴⁴ However, little is known of the role played by this receptor in the natural history of HSV infection and of the mechanism of entry into cells via this receptor.

The sequence of events subsequent to the interaction of gD with its receptors has been postulated as follows. The conformation of gD is altered in a specific manner by its interaction with any of its receptors. The consequence of the conformational change is changes to gH and gL, and ultimately activation of gB, which enables the fusion of the envelope with the cellular membranes.

There are several issues regarding the interaction of gD with its receptors that need to be resolved. The role of each of these receptors in human infection remains to be defined. In genital infection of mice by HSV-2, knockout of HVEM has little effect on infection, whereas knockout of nectin 1 attenuates infection but does not entirely prevent infection of the central nervous system (CNS).¹¹³¹ By the intracranial route of infection, nectin 1 is required for infection and the ensuing encephalitis.⁵⁷⁷

Perhaps the key issue is the exact function of gD in fusion. Crystallographic and functional studies have defined several domains in gD.^{157,275,1308} These are an N-terminal domain containing the bulk of the binding sites for HVEM, an Ig-folded core (residues 56–184) and a profusion domain (residues 260–316) required for fusion with the cellular membranes (Fig. 60.5). The crystallographic studies on truncated soluble forms of HSV-1 gD suggest that the N-terminal domain and the core fold over the C-terminal portion of the soluble form, N terminal to residue 260, and that the unliganded gD adopts a closed conformation. Interaction of gD with its receptor changes the conformation of the gD. This process presumably activates gH/gL and gB, which will be addressed later.

A linear schematic representation of the monomeric form of gD is shown in Figure 60.5. Residues 1 through 38 are dispensable and can be replaced by a heterologous ligand for a novel receptor (e.g., interleukin [IL]-13 for IL-13 α 2, urokinase plasminogen activator for its receptor, or a single-chain antibody), and residues 61 through 218 can also be replaced. In this instance, the 158 residues comprising the Ig-folded domain were replaced with a single-chain antibody against HER-2 containing 268 residues. In each instance, the modified virions

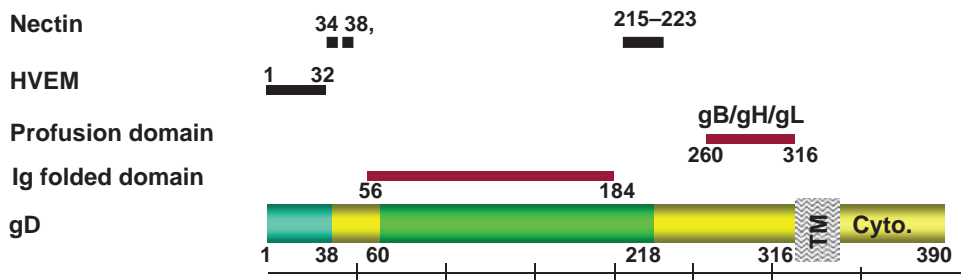


FIGURE 60.5. Diagram of the domain structure of glycoprotein D. Shown are the residues required for nectin binding, the HVEM binding site, the profusion domain, and the immunoglobulin folded domain.

entered cells through the novel receptor. To further confound the issues, a recombinant virus (R5322), in which the wild-type gD was replaced with two independently expressed polypeptides, enters cells via the urokinase plasminogen activator. In this instance, the first polypeptide consisting of the signal peptide of gD, the amino terminus of the urokinase plasminogen activator fused to the peptide comprising the residues 32 to 60, formed a heterodimer with the independently expressed polypeptide containing residues 219 to the C terminus of gD. The expectation that each of the novel ligands on interaction with their cognate receptors provokes an identical conformational change in gD makes little sense.

The fundamental problem stems from two assumptions. The first is that the transmembrane and cytoplasmic domains pose no restrictions on the conformation adopted by gD. The crystallographic studies by necessity were restricted to the ectodomain, and simulation of the gD homodimer was done by synthetically engineering a homodimer consisting of ectodomains. The notion that the transmembrane and cytoplasmic domains are merely anchors and that higher order structures are not functionally required is belied by the observation that they do in fact play a role in the life cycle of the virus. In studies designed to define the regions of gD necessary to block apoptosis induced by endocytosed virus lacking the gD gene, the minimally effective gD construct is a complex consisting of two components: the ectodomain and transmembrane domain of gD, and the transmembrane and cytoplasmic domain of the same protein. Substitutions of the cysteine residue in the transmembrane domain of either component of the complex abolish the antiapoptotic activity of gD. The observation that such heterodimers arise spontaneously in transduced cells is a strong indication that homodimers could also arise by a similar mechanism. Parenthetically, a low-resolution, three-dimensional structure obtained with the aid of electron microscopy predicts that gD has a homotetrameric structure.⁸⁷⁶

The second assumption is more insidious and independent of the first. Regardless of the interaction of gD with its receptor, the fusion assays are done on intact cells. If the interaction of virions with GAGs were neutral (i.e., do not result in modification of the virion surface), the rate of random collisions between virions and gD receptors should not be affected by presence of or absence of GAGs. One possible explanation for the reduced infectivity of viruses lacking ability to bind to GAGs or on exposure of wild-type virus to cells from which GAGs have been removed is that to interact with GAGs, gB and gC must project from the surface of the virion. The interaction of gB and gH/gL may introduce conformational changes in these proteins as well as gD. In essence, it is conceivable that the postulated conformational changes following exposure to receptor are in fact effected by GAGs. The role of gD with respect to gB and gH/gL is to recruit them and position them properly to enable interaction with GAGs and the lipid bilayers of the cellular membranes and summarized elsewhere.^{560a}

Roles of Glycoprotein B and Glycoprotein H/Glycoprotein L

Studies on the roles of gB and gH/gL remain the focus of numerous laboratories; however, the roles they play in the entry process are less contentious. Crystallographic studies of the ectodomain of gB showed that it was structurally homologous to the vesicular stomatitis virus (VSV) glycoprotein (G).⁴⁴¹ The

solved structure for gB shows a trimer with an α -helical coiled-coil core, which related gB to class I viral membrane fusion glycoproteins, and a domain carrying two extended β hairpins with hydrophobic tips, homologous to fusion peptides in VSV G, which related gB to class II fusion proteins. Because of this new structure, gB was defined as a class III fusion protein (see Chapter 3). Both class I and class II fusion proteins undergo conformational changes to carry out fusion of the virion envelope bilayer with that of the cellular membrane; therefore, it is likely that gB also promotes fusion through this same process. A recent study showed that the pH-dependent change in gB was a local change.¹⁰⁸⁸ gB has been shown to bind to the paired immunoglobulin-like type 2 receptor- α (PILR α) to trigger viral fusion in certain cell types⁹⁹⁸; however, the precise role of this interaction in viral entry remains to be determined.

gH and gL appear to form a tight complex, and in HSV, neither protein is stable without the other. In contrast to gB, the structural studies of gH/gL showed no homology with fusion domains of other viral glycoproteins.¹⁹⁸ Both gB and gH/gL bind, independently of the other, to the profusion domain of gD,^{44,371} and one function of gD may well be to enable formation of complexes with gB and gH/gL assembled in the correct configuration. This interaction does require initiation of fusion.⁴⁰ The exact role of gH/gL has not been determined; however, one hypothesis is that the interaction of gD with its receptors changes the conformation of gH/gL, which in turn induces gB to adopt its fusion active conformation and thus to carry out fusion of the membrane bilayers. The caveat is that any structure of gD interacting with any of the receptors listed previously must be able to activate gH/gL.

Entry and Signaling

Viral entry into Vero cells by fusion at the cell surface leads to increases in intracellular calcium concentration, and blocking this response inhibits viral entry.¹⁹⁰ Binding of virus did not activate calcium release; however, entry triggered local calcium release followed by a more global release of calcium in the cell.¹⁹¹ Binding to heparan sulfate proteoglycans and engagement of a gD receptor was required for the calcium signaling.¹⁹¹ Therefore, calcium seems to play a critical role in HSV entry, although the exact mechanism during entry has not been defined. Infection with ultraviolet (UV)-inactivated virus and binding of gD to HVEM also lead to activation of NF- κ B,^{737,1020} and this could be another signaling pathway activated by infectious virus during productive infection.

Translocation of Capsids to the Nucleus From the Site of De-Envelopment

Upon release from the envelope, capsids are transported to the nuclear pores. Evidence obtained from several labs indicates that the capsids are transported from the site of de-envelopment to the nuclear pore along the microtubular network^{592,1069,1154} by the microtubule motor dynein.¹⁰⁶⁹ Capsids covered with inner tegument proteins recruit microtubule motors and move along microtubules *in vitro*.^{912,1272}

The accumulation of empty capsids at the nuclear membrane was reported by Morgan et al.⁷⁶⁶ and Miyamoto and Morgan,⁷⁵⁸ suggesting that the release of DNA takes place through some port in capsids and does not require its dissolution. Evidence that viral DNA is released at nuclear pores emerged from studies of the HSV-1 Δ B7 mutant. At the nonpermissive

temperature, cells infected with the mutant virus accumulate capsids containing DNA at nuclear membranes. The DNA is released from the capsids on temperature shift-down, and viral gene expression ensues. The *tsB7* mutation maps in the *UL36* gene encoding a tegument protein (VP1-2) that is tightly bound to capsids.^{72,559} More recent studies have shown that the *UL36* protein must be cleaved to allow release of the viral DNA.⁵¹⁸ Host nuclear factor importin β as well as the nuclear pore complex proteins Nup358/RanBP2 and Nup214/CAN are involved in docking of capsids to nuclear pores.^{227,828,849} Furthermore, *in vitro* reconstruction studies showed that capsids bind to nuclear pores and release viral DNA in the presence of cytosol and an energy source. Purified importin β can substitute for cytosol.⁸²⁸ Antibody to importin β blocked the release. The capsid proteins interacting with importin β have not been identified; however, *UL25* has been shown to be involved in uncoating.^{849,897} After docking, viral DNA genomes are likely released through the *UL6* portal, by a pressure-driven ejection mechanism, similar to bacteriophages, in a polarized process with the S terminus first.⁷⁹⁷ It is not known whether histones, the host factor transportin,⁶⁰⁹ or other host or viral factors are then required for targeting of the input genome into the nucleoplasm or the nuclear lamina. A recent study showed that ectopic expression of *UL25*, an inner tegument protein associated with *UL36*, did not block uncoating of the viral DNA but did block viral gene expression⁹⁴⁶; therefore, *UL25* could play a role in movement of the viral genome from the nuclear pore to sites of transcription.

As emphasized elsewhere in this chapter, tegument proteins are released into the cytoplasm concurrently with the capsid. Their destination within the cells varies. As noted earlier, the products of *UL36*, VP1-2, migrate with the capsid to the nuclear pore. The product of *UL41* localizes in the cytoplasm,¹¹¹⁹ whereas VP16, the product of *UL48*, makes its way into the nucleus independently of the capsid.^{73,892}

Viral Gene Expression

Classification of Viral Gene Products: General Summary

The total coding capacity of HSV includes at least 84 transcripts that encode a diversity of proteins, several long noncoding RNAs, and as many as 16 to 17 microRNAs as described previously. In addition, the virions contain and bring into the infected cells a diverse set of mRNAs that do not fully reflect the concentration or composition of the mRNAs present in the infected cells during the assembly of the virions. Viral gene products form several groups expressed coordinately in a cascade fashion^{463,464} (Fig. 60.6). The α (immediate-early) genes are expressed first, followed by β_1 (early-early), β_2 (late-early), γ_1 (leaky-late), and γ_2 (true late). α genes do not require prior viral protein synthesis or their expression. A common property of the six α genes encoding the proteins ICP4, ICP0, ICP22, ICP27, *US1.5*, and ICP47 is the presence of a response element in their upstream sequences. β_1 and β_2 genes exemplified by the *UL29* gene encoding ICP8 and the *UL23* gene encoding the viral TK require for their synthesis at least two of the six α proteins, ICP4 and ICP0. The designations reflect the order of their accumulation in infected cells, although their accumulation is not affected by the absence of viral DNA synthesis. The accumulation of the products of γ_2 genes, as exemplified by

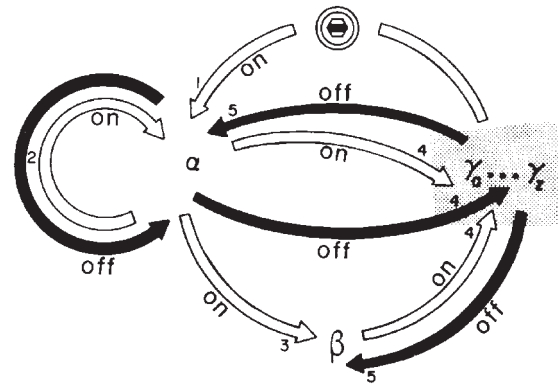


FIGURE 60.6. Schematic representation of the regulation of herpes simplex virus gene expression. Open and filled arrows represent events in the reproductive cycle that turn gene expression “on” and “off,” respectively. **1:** Turning on of α gene transcription by VP16, a γ protein packaged in the virion. **2:** Autoregulation of α gene expression. **3:** Turning on of β gene transcription. **4:** Turning on of γ gene transcription by α and β gene products through transactivation of γ genes, release of γ genes from repression, and replication of viral DNA. Note that γ genes differ with respect to the stringency of the requirement for DNA synthesis. The heterogeneity is shown as a continuum in which inhibitors of viral DNA synthesis are shown to have minimal effect on γ_1 gene expression but totally preclude the expression of γ_2 genes. **5:** Turn off of α and β gene expression by the products of the γ genes late in infection.

the *US11*, *UL38*, and *UL44* gene products, require viral DNA synthesis and the viral proteins ICP4, ICP0, ICP22, ICP27, and *US1.5*. A characteristic of at least some γ_2 genes is that the requirement for viral DNA synthesis is encoded within the 5′ transcribed noncoding domains.

In contrast to the β gene products, the accumulation of γ_1 gene products exemplified by VP5, the major capsid protein, and so forth is affected to various degrees by inhibitors of viral DNA synthesis. Neither β nor γ_1 promoters share common response elements, and most likely, the order and quantity of the gene product that accumulates in the infected cells reflects the embedded cellular response elements and the abundance of transcriptional factors present in the infected cells.

In permissive infected cells, progeny virus accumulates at an exponential rate between 6 and 18 hours after infection and at a reduced, linear rate for at least 24 more hours. In cells exposed to 10 plaque-forming units (PFU) of virus per cell, α gene products reach peak rates of synthesis between 2 and 4 hours after infection, although the actual synthesis of the proteins continues for many hours. The β_1 gene products are expressed very soon thereafter. Overall, β gene products are readily detected by 4 hours after infection but reach highest rates of accumulation between 6 and 12 hours after infection. Viral DNA synthesis can be detected as early as 3 hours after infection and continues for at least 10 to 12 more hours.

State of the Viral DNA and Proteins Prior to the Initiation of Transcription of Viral DNA

CIRCULARIZATION OF VIRAL DNA

Upon entry into the nucleus, viral DNA circularizes quickly at very early times after infection and in the absence of viral

protein synthesis, as first shown using assays that detected (a) viral DNA end joining in cells infected with recombinant viruses lacking internal repeated sequences⁸⁷⁸ or (b) viral DNA that remained in the well in pulsed-field gels.³⁵⁹ These results also led to the development of viral DNA replication models involving theta molecule intermediates and rolling circle mechanisms,¹⁰⁶ as described later. Furthermore, a later study of an HSV recombinant strain containing an insertion of the cleavage-packaging sequence within the genome confirmed the circularization of the HSV-1 genome during productive infection.¹¹⁰³ This study also showed that the end joining reported earlier⁸⁷⁸ was not attributable to concatemer formation or recombination between terminal repeats but rather to circularization.¹¹⁰³ Circularization of viral DNA, formation of endless genomes, and efficient viral replication require the cellular DNA ligase IV/XRCC4, as shown by studies of mutant cell lines and small interfering RNA (siRNA)-mediated knockdown.⁷⁸⁰

ASSOCIATION OF VIRAL DNA WITH HISTONES

Two lines of evidence support the conclusion that the host attempts to silence viral DNA immediately upon entry into the cells.⁵⁶⁰

The first centers on the evidence that the host cell attempts to assemble free incoming DNA molecules into chromatin.¹⁷¹ Following assembly of the incoming DNA with nucleosomes, posttranslational modifications of the histone tails lead to compaction of the chromatin into a form called *heterochromatin*. Reversal of the heterochromatin marks and addition of other histone modifications are needed to form the more open, active chromatin known as *euchromatin*. Similar to transfected DNA, HSV gene promoters introduced on the infecting viral genome are quickly associated with chromatin lacking euchromatin marks at early times postinfection.^{207,824} At later times, the amount of chromatin associated with viral promoters is reduced, and euchromatin marks are associated with the histones associated with the viral genome (H3).^{444,541} Furthermore, nuclease digestion studies have shown that there are few nucleosomes on HSV DNA during productive infection⁶³³ or that they are not arranged in a regular repeating pattern.^{541,635} Other studies have reported that the nucleosomes associated with HSV DNA are less tightly associated than host chromatin.⁶⁰⁸

Although chromatin immunoprecipitation (ChIP) results assume that the fraction of DNA in the immunoprecipitates is representative of the total and should be interpreted with caution, the ChIP studies are supported by other biochemical and genetic results. Thus, a key second support of the hypothesis that the host attempts to silence viral DNA is based on analyses of the requirements for initiation of transcription, which show that the virus mobilizes both host and viral factors to alter the structure of histones and remove the repressors bound to viral DNA as described later and summarized elsewhere.^{560a}

INTRANUCLEAR TARGETING OF HERPES SIMPLEX VIRUS DNA

The input viral genomic DNA is targeted to a location near the nuclear lamina early after entry into the nucleus. Because HSV DNA cannot be detected by *in situ* hybridization until DNA replication occurs, input viral genomes must be detected indirectly. Several lines of evidence argue for the localization of input genomes to the nuclear periphery. First, ICP4 foci were observed at the periphery of the nucleus within minutes of infection.³²⁴ Second, fluorescently tagged repressors to detect

viral genomes with an operator sequence showed genome foci at the nuclear periphery.¹⁰⁷⁶ Third, in cells infected with a virus expressing GFP-VP16 in the presence of an inhibitor of protein synthesis, GFP-VP16 targets to foci at the nuclear lamina.¹⁰⁴⁷ Fourth, early replication compartments, as detected by ICP8 immunofluorescence,^{255,1046} are located near the inner nuclear membrane. Fifth, knockout of the lamin A/C gene in mouse embryonic fibroblasts (MEFs) causes the release of replication compartments from the nuclear periphery.¹⁰⁴⁶ Assembly of the VP16 activator complex likely occurs on the nuclear lamina,¹⁰⁴⁷ possibly because Oct-1 is believed to be associated with nuclear lamins.⁷⁰²

ASSOCIATION OF VIRAL DNA WITH NUCLEAR DOMAIN 10 STRUCTURES

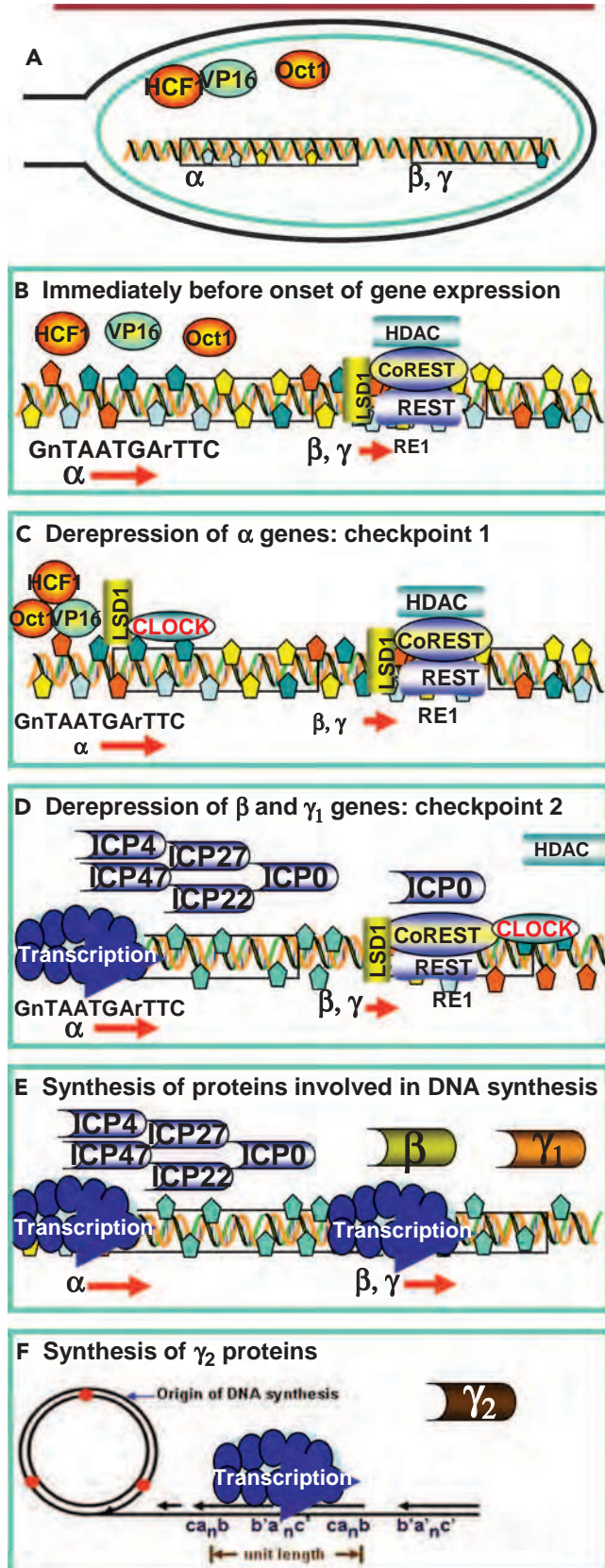
Concurrent with the association of viral DNA with histones, histone and chromatin-modifying enzymes, and repressors, nuclear domain 10 (ND10) or PML bodies are organized adjacent to the newly entered DNA.^{322,489} ND10 bodies have been associated with many functions, including transcriptional activation and epigenetic regulation of gene expression.¹¹⁵⁵ They are dynamic structures regulated by PML. They may contain upward of 20 proteins. They increase in number in cells transfected with DNA upon infection, treatment with IFN, or incubation with histone deacetylase (HDAC) inhibitors. The precise function of ND10 bodies vis-a-vis infection is unknown and remains a controversial issue. Thus, Chee et al¹⁸¹ reported that wild-type virus replicated equally well in murine PML^{-/-} and sibling PML^{+/+} cells. The key finding is that IFN- α or - γ had a major effect in PML^{+/+} cells and minimal effect in PML^{-/-} cells. In contrast, Everett et al³²³ reported that the yields were lower in PML^{-/-} cells than in PML^{+/+} cells. In fact, Chee et al¹⁸¹ observed that replication of an ICP0 mutant virus was threefold higher in PML^{-/-} as compared with PML^{+/+} cells, whereas Everett et al³²³ found that the plaquing efficiency of an ICP0 mutant virus was increased by three- to fivefold by short hairpin RNA (shRNA) knockdown of PML. Therefore, there appears to be agreement that there is a slight increase in ICP0 mutant viral replication when PML is not present in cells during the initial infection of cells but that a larger effect is exerted via IFN- α/β in surrounding cells.

Regardless of their effect on viral replication, two facets of the ND10 functions have become prominent. Foremost, they are the centers of sumoylation that affects the longevity and function of key components of the cell transcriptional apparatus. Second, the dissolution of the ND10 bodies presages the rise of the replication compartments (see Fig. 60.4). Replication compartments form near ND10 bodies, although PML protein and ND10 bodies are not required for their formation.

REQUIREMENTS FOR THE SYNTHESIS AND CHARACTERISTICS OF VIRAL GENE PRODUCTS

Transcription of viral DNA takes place in the nucleus, and as would be expected, all viral proteins are synthesized in the cytoplasm. The key constituent of the viral transcriptional apparatus is host RNA polymerase II,^{16,236} and several viral proteins either become components of the transcriptional apparatus or recruit specific cellular proteins, as discussed in detail later. RNA pol II is responsible for transcription of all viral genes during infection, although viral gene products may modify its activity and structure.

Productive infection



The studies reported largely during the past 5 to 10 years have altered our perception of viral gene expression from serial activation of transcription to serial derepression and transcriptional activation of the viral genome. It is convenient to describe the key events in viral gene expression in terms of three checkpoints (Fig. 60.7). The first involves the specific derepression of α gene promoters and activation of transcription of α genes. In brief, the tegument protein VP16 serves both of these functions by becoming part of a tripartite complex comprised of octamer-binding protein 1 (Oct-1), which binds to the α promoter response element described below, and host cell factor 1 (HCF-1). The complex performs two functions. First, it recruits lysine-specific demethylase 1 (LSD1) to demethylate H3K9me2 and at least one HAT—CLOCK—and most likely other factors to derepress α promoters. Second, it recruits transcriptional factors to initiate the expression of α genes.

The expression of β and γ_1 genes, the second checkpoint, requires ICP4 and, at low multiplicities of infection, ICP0. A major role of ICP0 is the inactivation of the HDAC1 or 2/CoREST/LSD1/REST repressor complex. The CoREST/LSD1/REST complex is best known for its role in repressing genes in nonneuronal cells. The complex through REST binds RE1, a large and somewhat degenerate response element present at more than 5,000 sites in the human genome. ICP0 also enhances the recruitment of several other factors (e.g., cyclin D3, CDK4, BMAL1, and CLOCK) to the nuclear domain that ultimately becomes the viral replication compartment. ICP4 is a component of the transcriptional complex involved in the expression of all post- α genes. The role of ICP4 is in promoting the initiation of transcription of the post- α genes.

As noted earlier, the expression of γ_2 genes, the third checkpoint, requires ongoing DNA synthesis, ICP4, and ICP0. Except for the apparent involvement of response elements in the 5' transcribed noncoding domain and the important roles of ICP27 and ICP22, little is known of the mechanisms underlying expression of γ_2 genes.

DEREPRESSION AND TRANSCRIPTION OF α GENES

The key players in the derepression and transcriptional activation of α genes are the protein complex involving the viral VP16 protein; the unique α gene response element; the host factors recruited by the VP16 complex, including Set1, LSD1, and CLOCK; and a large number of transcriptional factors, discussed later.

VP16 encoded by U_L48 is one of 18 virion tegument proteins. In productively infected cells, it forms complexes with VHS encoded by U_L41 and VP22 encoded by U_L49 . It is also an essential structural component of the virion. Upon entry into the host cell in the virion, it is a key component of complex of proteins that derepresses and activates α gene expression. Upon

FIGURE 60.7. Molecular checkpoints regulating viral gene expression during productive infection. α , β , and γ designate genes of those kinetic classes. **A:** Viral DNA entering the nucleus is free from histones and nucleosomes. **B:** Histones associate with viral DNA immediately upon entry of viral DNA in nucleus. **C:** Checkpoint 1. Derepression of α genes. **D:** Checkpoint 2. Derepression of β and γ_1 genes by effects of ICP0 and associated proteins. **E:** Synthesis of viral DNA replication proteins. **F:** Checkpoint 3. Derepression of γ_2 genes following viral DNA replication.

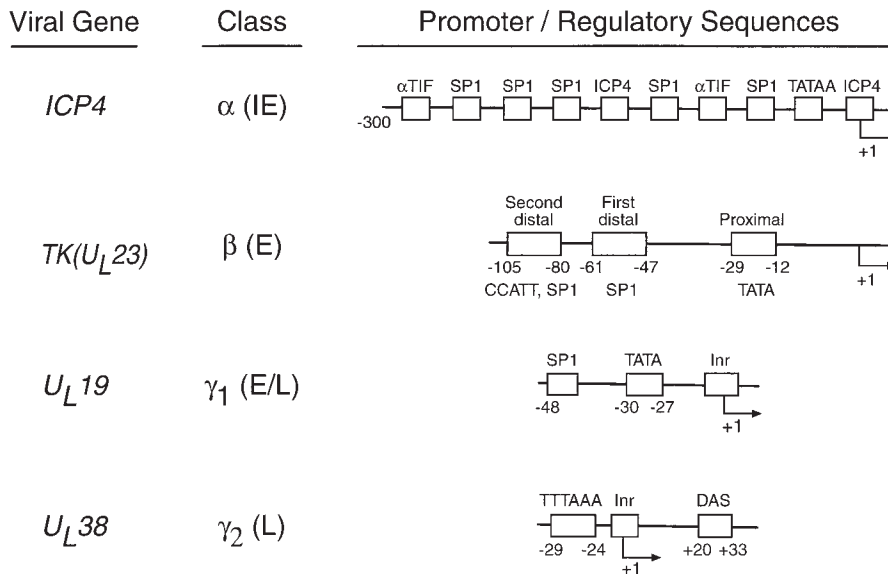


FIGURE 60.8. Diagram of the structure of prototypic herpes simplex virus gene promoters. The promoter/regulatory sequences of prototypic genes of α , β , γ_1 , and γ_2 kinetic classes are diagrammed.

release from the tegument, VP16 binds to the cellular protein HCF-1.^{369,594} It has been reported that HCF-1 carries VP16 into the nucleus in transfected cells⁶⁰⁶; however, VP16 localizes normally to the cell nucleus at late times in infected cells that express a ts mutant HCF-1⁶⁰⁶ or in cells in which HCF-1 is knocked down.⁷⁹² Thus, at late times and conceivably at early times in infected cells, other factors, possibly viral tegument proteins, assist in nuclear localization of VP16. Within the infected cell nucleus, the VP16-HCF complex binds to Oct-1 bound to viral DNA, forming the activator complex. Oct-1 binds to the octamer core sequence, and VP16 binds to Oct-1 and the adjacent HSV sequences.

The α gene promoters contain numerous cellular response elements including SP1 (Fig. 60.8); however, the sequence 5'GyATGnTAATGArATTCTTGGG3' is the key response element. It is present in one or several copies within several hundred base pairs upstream of the cap site.^{694,695} Oct-1 is an abundant ubiquitous transcriptional factor that, as its name indicates, binds to the ATGnTAAT octamer.

A key step in the expression of α genes is the recruitment of LSD1 to the viral α gene promoters by the VP16/HCF-1/Oct-1 complex. LSD1 demethylates H3K9me2 bound to α promoters to enable α gene transcription.⁶⁴⁵ Depletion of LSD1 or pharmacologic inhibition of LSD1 increased H3K9me3 modification of histones associated with HSV α gene promoters and decreased α gene transcription.⁶⁴⁵ It is noteworthy that LSD1 is normally stable in association with CoREST and is rapidly degraded in the absence of its partner. Indeed, depletion of CoREST or REST prior to infection results in the depletion of LSD1 and a decreased accumulation of α gene products.²⁹⁴ Because the CoREST/REST complex is not associated with the α gene promoters, it may be inferred that LSD1 is detached from the CoREST/REST complex by the HCF-1–VP16 complex or that HCF-1 in the VP16 complex recruits LSD1 that is not associated with CoREST/REST.⁶⁴⁵ Furthermore, HCF-1 is part of the Set1 and MLL1 histone H3 Lys4 methyltransferase complexes; therefore, the transactivation complex recruits the HCF-1–Set1 or HCF-1–MLL1 histone methyltransferase (HMT) complex to α promoters resulting in the euchromatic histone modification; trimethylation of the histone H3 lysine

4 residue (H3K4me3),^{475,793} which provides a euchromatic modification; and binding of various factors that stimulate α gene transcription.

The second step in α gene expression is the activation of transcription. Part of this process involves the recruitment by the VP16 acidic C-terminal domain of transcription factors IIB (TFIIB), TFIIF, TFIID, TATA-binding protein (TBP), and TBP-associated factors, which promote formation of the pol II preinitiation complex on α promoters.⁵⁹³ VP16 has also been reported to recruit chromatin-modifying co-activator proteins CBP, p300, BRG-1, or BRM onto viral promoters.⁴⁴⁴ CBP and p300 have HAT activities associated with them and promote active chromatin or euchromatin. BRM and BRG-1 are the mammalian homologs of the ATPase subunit of the yeast SWI/SNF complex, which remodels host chromatin. Interestingly, knockdown of these co-activators did not reduce HSV α gene transcription, suggesting that their presence is gratuitous or their function is redundant with unknown host factors.⁶⁰³ CLOCK knockdown reduces α gene expression, although the mechanism(s) of its role in α gene expression have yet to be determined.

Whereas the results of biochemical studies leave no doubt as to its importance in viral gene expression, the role of VP16 in rapidly dividing cells in culture is minimal. As illustrated in the case of the gene encoding ICP4 (see Fig. 60.8), α gene promoters also contain binding sites for other cellular transcriptional activators (e.g., SP1) in addition to Oct-1–VP16 response element. The precise mix of cellular and viral response elements varies between different α gene promoter/regulatory sequences, and their relative contribution to the abundance and duration of transcription has been investigated in only a few instances. In rapidly dividing cells, VP16 increases the accumulation of α transcripts by approximately fourfold and is not a critical factor in viral gene expression.¹⁰⁸⁰ Presumably, other cellular transactivating factors account for the basal level of α gene expression. The requirement for VP16 may be different in resting cells or differentiated cells in which these transcriptional factors are unavailable or tightly regulated.

The cellular hSNF2H chromatin remodeling complex subunit has also been linked to efficient ICP0 gene expression.¹²⁹

This host factor is associated with the *ICP0* gene promoter at early times postinfection¹²⁹ but has not been linked to VP16.

SHUT OFF OF α GENE EXPRESSION OR FUNCTION

There is significant evidence that HSV controls the accumulation or function of at least two α proteins: ICP4 and ICP0.

1. ICP4 can repress its own expression. ICP4 binds to two sets of DNA sequences. The first, reported by Faber and Wilcox,³²⁵ was ATCGTCNNNNYCGRC and is defined as the consensus binding site. The second set of sequences bears no relationship to the consensus binding sites or, for that matter, to each other.⁷⁴⁹ Consensus binding sites that span transcription initiation sites serve as sites for repression of transcription by ICP4, as illustrated by the *ICP4* gene in Figure 60.8. Another consensus binding site spans the transcription initiation site of *ORF P/ORF O*. The effectiveness of the binding site in repressing transcription in the context of the viral genome varies depending on the distance from the transcriptional initiation site. The farther downstream it is, the less the repression. Also, displacement by half of a helical turn is more effective in minimizing repression than a full helical turn.⁶³⁸ It should be noted that whereas mutagenesis of the ICP4 binding site across the transcription initiation site resulted in a tenfold increase in accumulation of transcripts, the added mutagenesis of the upstream and more distant binding site had barely a twofold effect on transcript accumulation.⁶³⁸

It is less clear whether ICP4 represses the expression of the other α genes. ICP4 binds upstream of the *ICP0* gene transcriptional initiation site,⁵⁹⁵ and ICP4 represses the *ICP0* promoter in transfected cells.³⁶⁵ Nevertheless, the specific effect of ICP4 on *ICP0* gene expression in infected cells has not been defined by construction of a mutant virus with the binding site in the *ICP0* gene mutated. Consensus ICP4 binding sites are not evident in or near the promoters of the other α genes.

2. As noted previously, ICP4 blocks the expression of ORF O/ORF P by binding to the consensus binding site or at the transcription initiation site of these genes. Studies of the function of these genes revealed that the ORF O protein blocks the binding of ICP4 to its consensus binding sites.⁹¹⁶ The precise role of ORF O remains to be defined.
3. During productive infection, β gene products down-regulate α gene expression.⁴⁶³ One β protein identified as a candidate is ICP8, the single-stranded DNA binding protein encoded by the *U_L29* gene. ICP8 decreases the expression of all genes from the parental viral DNA molecules^{381,382}—in particular, transcription of the gene encoding ICP4.³⁸³ The mechanism of this effect of ICP8 is not known but may involve the sequestration of viral DNA molecules in replication complexes, changes in the conformation of viral DNA, or displacement of DNA to sites of DNA synthesis away from those required for transcription.
4. During the first 6 hours after infection, ICP0 is localized in the nucleus. Thereafter, it accumulates in the cytoplasm. The half-life of ICP0 at very early times during the nuclear phase is less than 1 hour but increases with time after infection. Analyses of the cleavage products suggest that ICP0 is degraded by at least two different proteases.⁴⁰¹ Therefore, ICP0 nuclear function is limited by its transport to

the cytoplasm and/or rapid turnover in the nucleus as the infection progresses.

5. At early times, VHS has been shown to degrade both viral and cellular mRNAs. Observation of cells infected with *vhs* mutants that the transition from α to β and subsequently to γ protein synthesis lags behind that observed in wild-type virus-infected cells led to the conclusion that one function of *vhs* is to synchronize sequential viral gene expression.⁶⁰⁵ The proposed model is that the rate of transcription of viral genes exceeds the ability of VHS to degrade the mRNAs, and that unless destabilized, α mRNAs would outcompete β mRNAs, and so on.

EXPRESSION OF β AND γ_1 GENES

Because the temporal order of expression of β and γ_1 genes overlap, it is convenient to lump them together. Expression of these genes requires ICP4 and is increased by ICP0, in particular at low multiplicities of infection. Expression of these genes requires, to a variable extent, also ICP27, ICP22, and U_S1.5, and, in the case of γ_1 genes, the onset of viral DNA synthesis. It is convenient to consider the contribution of each gene product separately.

ICP0

ICP0 is a multifunctional protein that plays the major role in the derepression of the post- α genes by countering host chromatin silencing mechanisms. ICP0 is not essential for viral replication in cell culture at high multiplicity of infection (MOI) or in experimental animal systems but does increase viral replication in animals and at low MOI infection of cultured cells.⁴⁰⁹ At low MOI, cells infected with *ICP0* deletion mutants yield approximately 10- to 1,000-fold less virus than cells infected with wild-type virus in certain cell types. At more than 5 PFU per cell, the yields of Δ ICP0 mutant virus may be similar to those of wild-type virus. The cell line most permissive for Δ ICP0 mutant viruses is U2OS. Thus, U2OS cells serve as the standard for infectivity titrations of ICP0 mutant virus stocks for experimental studies. Even using this cell line for titrations, the particle:PFU ratio for ICP0 mutants may be higher than the wild-type or rescued viruses; thus, this may be a factor in certain experiments. During the first 6 hours after infection, ICP0 accumulates in nuclei, initially near ND10 structures^{681,721}; later, it expands to occupy most of the nucleus. In wild-type virus-infected cells, ICP0 then accumulates in the cytoplasm between 6 and 9 hours after infection. ICP0 turns over very rapidly at early times (half-life <1 hour) and at much slower rates late in infection.

The major phenotypic properties of ICP0 are threefold. Foremost, ICP0 promotes but is not essential for viral replication in experimental animal systems⁶³² and in cell culture at very low MOI. In these cells, Δ ICP0 mutants express α gene products; however, the transition to β and γ gene expression does not ensue. A second key phenotypic property of ICP0 is that it acts as an indiscriminate promiscuous transactivator of both viral and nonviral genes introduced into cells by transfection or infection. Lastly, ICP0 plays very prominent roles in viral defenses against cellular innate immune responses. It is convenient to look at the molecular mechanisms underlying each of the phenotypic properties separately.

Several observations led to our current models by which ICP0 is believed to enable the expression of β genes by countering the epigenetic silencing of these genes. First, HDAC inhibitors

such as trichostatin A⁴⁵³ and sodium butyrate⁸⁸⁴ could rescue at least part of an ICP0 mutant defect. This argued that one of the functions of ICP0 was to inhibit HDAC activity. Second, ICP0 was reported to interact with class II HDACs and to reverse repressive effects of these HDACs in transfected cells.⁶⁷² Third, ICP0 binds to CoREST *in vitro* and dislodges HDAC1 from the HDAC1/CoREST/LSD1/REST (HCLR) complex in wild-type virus-infected cells. Over time, a fraction of CoREST, HDAC1, and LSD1 are exported from the nucleus to the cytoplasm of wild-type virus-infected cells but not from Δ ICP0 virus-infected cells. This step requires the onset of viral DNA synthesis and may involve other viral gene products.⁴⁰⁰ Fourth, replacement of both copies of the *ICP0* gene with a gene encoding a dominant negative CoREST capable of binding REST but unable to bind HDAC1 led to a 100-fold increase in the yield of virus in Vero cells and at least a 10-fold increase in HEP-2 cells following low PFU/cell infection.⁴⁰² The involvement of HDACs was reinforced by the observation that HDAC1 and 2 are phosphorylated by U₃ protein kinase and that expression of reporter genes is augmented by co-transfection with U₃.⁸⁸³ Lastly, a virus encoding an ICP0 unable to bind CoREST showed reduced ability to reactivate quiescent HSV-1, consistent with a role for REST/CoREST/HDAC1/2/LSD1 in repression of quiescent genomes.³³² Despite the weight of this evidence, knockdown of CoREST did not affect replication of an *ICP0* mutant in human HepaRG cells, terminally differentiated human hepatic cells.³¹⁸ The differences here may be owing to the differences in cell types used or to the fact that CoREST both inhibits viral gene expression and stabilizes LSD1, which is needed for viral α gene expression.¹³¹⁷

The hypothesis that ICP0 counters silencing by reversing host chromatin silencing mechanisms is confirmed by the observation that ICP0 promotes decreased total histone H3 association with viral promoters and increases the acetylation of histone H3 that is associated with viral promoters, a histone modification associated with euchromatin or active chromatin.²⁰⁷ Furthermore, ICP0 promoted the removal of heterochromatin markers from viral chromatin during reactivation from quiescent infection in cell culture.³³² An unresolved issue is whether ICP0 also affects host chromatin. ICP0 increases the expression of host gene products, including p21, gadd45, and mdm2, potentially owing to chromatin effects.⁴⁵³ HSV infection reduces histone H3 levels on the *GAPDH* gene promoter⁴¹⁸ but not on a *GAPDH* pseudogene.²⁰⁷ Further studies are warranted to determine the effects of HSV infection and ICP0 specifically on host gene chromatin.

ICP0 has at least three other functions that contribute to the transactivation of viral genes. The first two relate to the binding of ICP0 to BMAL1 and cyclin D3.⁵³⁷ BMAL1 is a partner and stabilizer of the CLOCK HAT. First, recent studies have shown that CLOCK and its partner BMAL1 localize at ND10 structures and the replication compartments¹¹⁷⁴ that evolve near them. BMAL1 binds to ICP0, and the data suggest that ICP0 enhances the recruitment of CLOCK and stabilizes it. Overexpression of functional CLOCK enhances the replication of Δ ICP0 mutants, whereas overexpression of CLOCK lacking HAT activity did not. Depletion of CLOCK drastically reduced expression of α genes. It is noteworthy that cells depleted of CLOCK produced only small amounts of ICP0 but no other α proteins.⁵²⁶ These results reinforced the hypothesis generated in other studies in cells infected at low multiplicities that ICP0

is made first, that ICP0 helps recruit other factors, and that other α proteins and more ICP0 are then produced. A corollary of this hypothesis is that the CLOCK HAT is essential for the expression of the full complement of α proteins but not for the initial transcription of the gene encoding ICP0. CLOCK is a component of the transcriptional complex throughout viral replication.⁵³⁰

Second, the interaction of cyclin D3 with ICP0 is abolished by the D199A substitution. The mutated ICP0 is also retained in the nucleus throughout the infection. The Δ ICP0 mutants were complemented by replacement of ICP0 with cyclin D3; thus, a key function of ICP0 appears to be the recruitment of cyclin D3 to replication compartments to activate cdk4.⁵²⁷

Third, the interaction of ICP0 with ND10 and, in particular, the degradation of PML and of other components of ND10 play a role in the activation of expression of β genes; however, the mechanism of degradation of PML is more difficult to define in molecular terms. The fundamental conclusion reported elsewhere is that the degradation of ND10 and its components and transition from α to β gene expression are interdependent.

The functions of ICP0 as a promiscuous transactivator were recently re-examined in detail. In brief, transfection of cells with DNA containing expressible reporter genes, and lacking known promoter or ORFs, results in an increase in the size and number of ND10 structures. Infection of cells transfected with extraneous DNA results in retention of ICP0 in the nucleus that depends on the size and amount of DNA but not on the content or composition of the DNA. The hypothesis that ICP0 is retained in the nucleus until all DNA at ND10 bodies is “processed” is supported by the concurrent expression of reporter gene and viral genes in cells infected with wild-type virus but not in cells infected with Δ ICP0 mutant virus.⁵²⁹ This finding suggests that the tasks of degrading ND10 and its components and the derepression of viral genes occur concurrently until both are completed.

The domains on ICP0 (Fig. 60.9) that are relevant to gene activation include the RING domain in exon 2, which is a C₃HC₄ zinc binding domain⁶⁸ from residues 106 through 149 containing a ubiquitin E3 ligase activity,^{116,410,1184} the CoREST binding site from residues 669 through 718,⁴⁰² and the BMAL1 binding site from residues 20 through 241.⁵³⁷ Two studies have shown that the RING domain and CoREST binding site are functionally related, in that both are involved in IFN sensitivity⁴⁰⁴ and viral gene activation.^{331,404}

FUNCTION OF THE RING DOMAIN LIGASE ACTIVITY

The RING domain E3 ligase activity is essential for ICP0's functions in transactivation of post- α gene expression and for promotion of viral replication at low MOI. However, the molecular mechanism of these effects has not been elucidated. ICP0 has been linked to the degradation of numerous proteins in that ICP0 mutant viruses do not show the normal degradation associated with HSV infection. However, there is little evidence that ICP0 directly ubiquitinates several of these proteins. ICP0 has been linked to degradation of PML^{183,321}; the SUMO-modified form of Sp100^{183,772,846}; centromere proteins CENP-A, -B, and -C^{320,670,671}; the catalytic subunit of DNA-protein kinase^{629,847}; the ubiquitin-specific protease 7 (USP7)¹¹⁵; the RNF8 cellular RING finger ubiquitin ligase⁶⁴⁹; ubiquitinated histones⁶⁴⁹; and the RNF168 cellular E3 ligase.⁶⁴⁹ However,

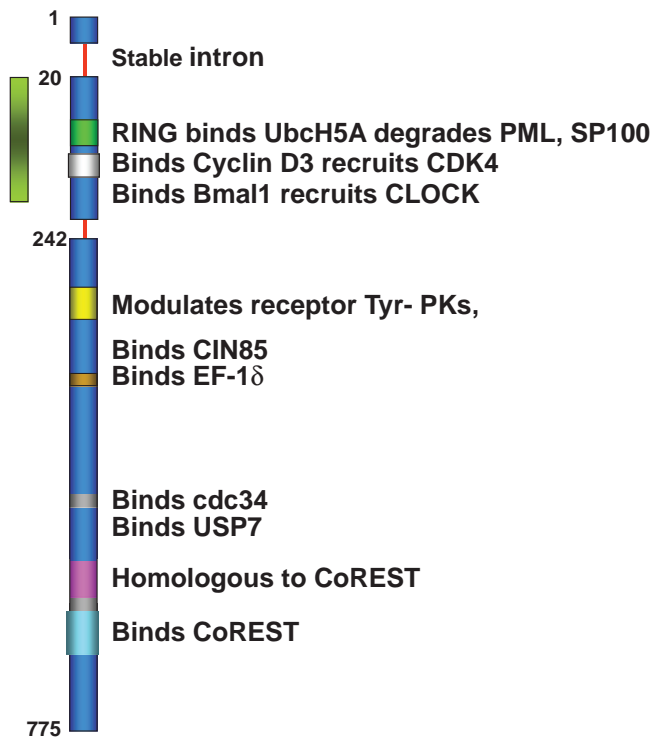


FIGURE 60.9. Diagram of the domain structure of ICP0. Shown are functional domains and protein-binding sites. Green bar at left indicates the BMAL1 binding region.

ICP0 has been shown to interact directly with only a few of these proteins, such as USP7.¹¹⁵ Therefore, it remains a paradox how ICP0 promotes the degradation of the large number of proteins. ICP0 binds to USP7 and promotes its ubiquitination *in vitro*.¹¹⁵ ICP0 binds to the DNA repair protein RNF8, and purified ICP0 ubiquitinates RNF8 *in vitro*.⁶⁴⁹ These are the only known direct substrates of the ICP0 E3 ligase activity. The correlation of ICP0 with degradation of PML and Sp100 and disruption of ND10 bodies raises this as a potential important activity of ICP0; however, there is no evidence of direct interaction of ICP0 with these proteins. The only direct evidence that ICP0 degrades PML and SP100 is the fact that dominant negative UbCh5A blocks the degradation.⁴⁰³ The question of the precise molecular interactions that lead to the effects of ICP0 is likely to be an important one for the field.

ICP4

ICP4 fulfills the second function needed for post- α gene transcription: recruitment of RNA polymerase II to these gene promoters. ICP4 is required for maximal levels of all post- α gene expression,^{204,561,894,1223} and the effect of ICP4 is exerted at the transcriptional level in infected cells, as demonstrated by nuclear run-off assays.³⁸³ ICP4 has been shown to also activate gene expression in transfected cell assays^{319,366,827,910} and in *in vitro* transcription systems.³⁹⁹

Two properties of ICP4 are relevant to its roles in regulation of viral gene expression: its ability to bind to DNA and its ability to interact with transcription factors. ICP4 has both sequence-specific and nonspecific DNA-binding activities.^{595,750} Binding of ICP4 to specific DNA sites seems unlikely to explain

its transactivation activity, because the strongest affinity of ICP4 is for sites known to repress rather than activate transcription (e.g., at the transcription initiation sites of the *ICP4* or *ORF P* genes), and destruction of the sole sequence-specific binding site of ICP4 upstream of the transcription initiation site of the *ICP4* gene attenuated repression in the context of the viral genome.⁶³⁸ With regard to the role of DNA binding in the transactivation function of ICP4, early mutational analysis showed that the sequence-specific binding activity of ICP4 correlated extensively with its ability to activate β gene expression in infected cells.¹⁰³³ In addition, insertion of ICP4 binding sites was reported to render reporter genes responsive to ICP4, albeit in transfected cells.¹¹³³ However, one viral mutant has been obtained in which the DNA-binding activity of ICP4 was greatly reduced but the ability to activate β genes in infected cells was nearly unchanged.¹⁰³² Furthermore, most post- α genes do not have recognizable consensus binding sites for ICP4, and mutation of consensus sites has not affected the level of expression of the surrounding genes in the viral genome.¹⁰⁶⁰ Thus, the bulk of the data from studies of infected cells has failed to correlate ICP4 DNA binding with transactivation of β genes. Nevertheless, there may be a role for the DNA-binding ability of ICP4 in stabilizing the binding of cellular transcription factors to β and γ gene promoters.^{841,842}

Second, ICP4 has been shown to interact with basal transcription factors to form complexes with TBP, TFII-B, and TAF250^{158,641,991,1061} as well as ICP27, ICP22, and the cellular CLOCK HAT.⁵³⁰ ICP4 promotes the formation of transcription preinitiation complexes *in vitro* on the *gC* gene promoter, at least in part by stimulating the binding of TFIID to the promoter to form preinitiation complexes.³⁹⁴ ICP4 also interacts with the mediator complex⁶⁴¹—a large protein complex that bridges upstream activators and the preinitiation complex. Thus, most current models for ICP4 transactivation of viral β and γ genes involve interactions with cellular transcription factors to stimulate transcription. It is still not clear how this stimulation is focused specifically on viral genes. Again, given the ability of ICP4 to both activate and repress gene expression, it is important to distinguish in which cases the binding of cellular transcriptional factors represents the transactivation or the repressive function of ICP4.

ICP27

ICP27 is an α protein that is reported to have multiple regulatory functions, including promoting expression of certain β proteins, late gene transcription, RNA export from the nucleus to cytoplasm, and translation of viral mRNAs. Early studies of viral gene expression concluded that ICP27 is required only for viral DNA replication and late gene expression^{936,980}; however, further studies showed that ICP27 is also needed for efficient expression of certain β genes^{729,990,1175}—in particular, the less abundant viral DNA replication proteins.¹¹⁷⁵ Thus, ICP27 is required at least in part indirectly for viral DNA replication by increasing the mRNA levels of β gene products essential for viral DNA synthesis. These studies have shown that ICP27 increases the mRNA levels for these β genes, although it has not been determined if this effect is transcriptional or posttranscriptional. ICP27 affects the cytoplasmic levels of only a subset of β mRNAs because northern blot studies have shown that an ICP27 null mutant virus expresses levels of ICP8 mRNA at 6 hours postinfection (hpi) that are equivalent to wild-type virus.¹¹⁷⁵

HSV ICP27 mutant viruses are defective for late *gC* gene transcription, as assayed by intracellular pulse labeling.⁴⁹⁷ Consistent with this, ICP27 associates with RNA polymerase II holoenzyme¹³¹⁴ through its interaction with the C-terminal domain of the large Pol II subunit,²⁴⁵ consistent with an ability to enhance initiation or termination of transcription. ICP27 is also associated with the CLOCK HAT.⁵³⁰ ICP27 has been shown to bind to ICP4,⁸³⁸ to alter the phosphorylation of ICP4,^{936,1107,1276} and to localize to replication compartments.²⁵⁷ ICP27 interacts directly with ICP8,⁸²⁹ which associates with viral progeny DNA.⁶²⁷ Therefore, ICP27, through its interactions with ICP8 and RNA pol II, is proposed to link pol II to progeny viral DNA to promote late viral gene transcription.⁸²⁹ Consistent with this, immunofluorescence studies have shown that certain ICP27 mutant viruses are defective for recruitment of pol II to replication compartments in infected cells.²⁴⁵ These properties are all consistent with effects on transcription of late HSV genes. The ability of ICP27 to promote γ viral gene expression is at least partly distinct from its ability to stimulate viral DNA synthesis, because at least one mutant virus, *n504*, separates these two functions. This mutant is defective for γ gene expression, in particular γ_2 proteins, while maintaining normal levels of viral DNA synthesis.⁹³⁷ This mutant virus has further been used to demonstrate that ICP27 stimulates transcription of γ genes independently of its role in stimulating β gene expression and viral DNA replication.⁴⁹⁷ This study found that ICP27 promotes transcription of at least two γ_2 genes, *U_L44* and *U_L47*, in infected cells, as assayed by *in vivo* pulse labeling of RNA, without any apparent effects on transport or stability of these transcripts. ICP27 has also been reported to affect the elongation, termination, and/or polyadenylation site selection during transcription.^{419,729,734}

ICP27 has been shown to shuttle between the nucleus and cytoplasm^{736,874,994,1070} and is reported to promote RNA export in several studies.^{184,994,1070,1071} However, in some cases, the viral mutants used may have an additional defect in late gene transcription.¹⁰⁷⁰ There are differing results on the role of ICP27 in viral mRNA export. One study found that the β ICP8 mRNA is present in the cytoplasm of an ICP27 null mutant virus-infected cell at nearly normal levels (82%) compared to that in wild-type virus-infected cells.¹¹⁷⁵ Another study found substantially lower amounts of ICP8 as well as other E and L mRNAs in the cytoplasm of ICP27 mutant-infected cells.⁹⁹⁴ Furthermore, measurement of viral transcript levels by microarray technology shows that a broad spectrum of viral poly (A)⁺ transcripts are retained in the nucleus in cells infected with ICP27 mutant viruses,⁵¹² whereas studies looking at specific viral mRNAs using northern blot hybridization found that (a) the long *U_L24* gene transcript was increased in the nucleus of ICP27 mutant-infected cells but the short *U_L24* gene transcript was unaffected⁸⁵⁸; (b) VP16 mRNA export was not altered in ICP27 mutant infected cells,³¹³ and (c) ICP27 is not required for cytoplasmic accumulation of *gD* and ICP5 mRNAs.³³⁸ Therefore, there are only a limited number of specific transcripts that show a dependence on ICP27 for nuclear export and cytoplasmic accumulation. In total, ICP27 binds to pol II, promotes late gene transcription, promotes association of RNA-binding proteins to late transcripts, may escort mRNAs to the cytoplasm, and enhances the binding of translation factors to mRNA. Further studies should define how ICP27

participates in all of these processes, from synthesis of the mRNAs to their translation.¹¹¹⁸

β GENES AND THEIR REGULATION

Unlike α genes, β or γ genes do not have unique promoter elements that instantly predict the timing, duration, or abundance of gene expression. Rather, the overall impression is that they appear to be made up of a diversity of elements, and both the organization and the context in which these elements are placed determine the expression of the gene. Nevertheless, the β gene promoters thus far studied contain binding sites for two to three cellular transcription factors upstream of the transcriptional start site (see Fig. 60.8—*tk* gene). The mechanism of activation of β genes has also been approached in studies attempting to map the *cis*-acting sequences needed for activation by virus infection or ICP4. The most extensively studied β gene promoter is that of the *U_L23* or *tk* gene. Extensive mutagenic analyses of the promoter showed that the sequences needed for basal level transcription⁷³³ or activated transcription²¹³ were the same (see Fig. 60.8): a proximal signal from bp -12 to -29 containing a TATA box and two distal signals from bp -47 to -61 and -80 to -105 containing an SP1 transcription factor binding site and an SP1 site and a CCAAT transcription factor (CTF) site, respectively. These studies also supported the hypothesis that ICP4 transactivates β gene promoters through interactions with cellular basal transcription factors.

EXPRESSION OF γ GENES

Once viral DNA replication has initiated, expression of γ genes is increased,⁴⁶⁵ largely as a result of increased transcription.³⁸³ Late transcription takes place in replication compartments within the infected cell nucleus, as evidenced by (a) the localization of ICP4^{562,917} and pol II⁹³⁹ to replication compartments at late times and (b) RNA-pulse labeling of replication compartments at late times.⁸⁷⁵

It is known that transcription of late genes increases upon replication of the viral genome³⁸³; that the alteration in the viral DNA template is *cis*-acting⁷²⁴; and that the ICP4, ICP22, ICP27, and ICP8 proteins are required. The *cis*-acting effect on the template could be owing to changes in the viral DNA molecules themselves by exposure of single-stranded regions or by conversion of the viral genome from a circular form to a linear form. Alternatively, the *cis*-acting effect could be owing to proteins tightly bound to the viral parental DNA that do not exchange to other DNA molecules in the infected cell. ICP4 promotes γ gene transcription by promoting the assembly of preinitiation complexes on the γ_2 *gC* gene promoter.¹³⁰⁵ ICP27 and ICP8 have been hypothesized to stimulate late gene transcription by recruiting pol II onto progeny DNA through a direct interaction that bridges ICP27 bound to Pol II with ICP8 bound to viral progeny DNA.⁸²⁹ Additional interactions of pol II holoenzyme with ICP4 would lead to preinitiation complexes on late gene promoters.

As a general rule, whereas sequences upstream of transcriptional initiation sites are sufficient to endow a reporter gene with the kinetics of β gene expression both in transfected cells and in the context of the viral genome,^{213,1225} this is not generally true of γ genes.^{724,1048} In the context of the viral genome, expression of reporter genes as γ_2 genes also requires regulatory elements present in the 5' transcribed noncoding domains.⁴⁶² Even then, such chimeras are expressed as β genes

in cells transfected with the chimera and then superinfected with the virus.¹⁰⁴⁸

The HSV γ_1 promoters have not been studied as extensively as some of the other viral promoters; however, the *ICP5* (*U_L19*) gene promoter elements have been defined (see Fig. 60.8). The essential elements of the minimal *ICP5* gene promoter are an SP1 binding site at -48, a TATA box at -30, and an essential *cis*-acting element between -2 and +10 whose sequence resembles the HIV initiator element.⁴⁷⁴ A cellular factor that binds at the cap site has been identified.⁴⁷³ The γ_1 gene promoters are heterogeneous in that the minimal *VP16* gene promoter from bp -90 to +6 contains an E box (CACGTG) at -85, a CAAT box at -77, an SP1 site at -48, and a different initiator element.⁶⁴⁶

Analyses of γ_2 viral gene promoters have shown that the upstream sequences consist of a TATA box with few other upstream transcription factor binding sites^{334,461,462,513} and with additional sequences needed for activation within the 5' UTR.^{408,462,724,1095,1226} For example, the γ_2 *U_L38* gene promoter (see Fig. 60.8) contains three elements: (a) an unusual TATA element with the sequence TTAAAA at -31, (b) a consensus initiator element at the transcriptional start site, and (c) a downstream activation sequence (DAS) from bp +20 to +33 that is required for normal levels of gene expression.⁴⁰⁸ The DAS seems to increase transcriptional initiation,⁴⁰⁷ and several other HSV γ genes, including *U_S11*, *gC* (*U_L44*), *gB* (*U_L27*), *L/ST*, and *U_L49.5*, have similar downstream control elements in their promoter.⁴⁰⁷ In addition, the *gC* gene DAS can partially substitute for the *U_L38* DAS,⁴⁰⁷ suggesting that common mechanisms may act on the different DAS sequences. The Ku70 or DNA-binding subunit of the DNA-dependent protein kinase⁸⁷³ and hTAF(II)70⁸⁷² have been identified as factors binding to the DAS. Given that HSV infection leads to the degradation of the catalytic subunit of DNA-protein kinase and loss of kinase activity,⁶²⁹ this effect may free up the Ku70 subunit for interaction with viral DNA.

ROLE OF ICP22/U_S1.5 IN LATE GENE EXPRESSION

ICP22 is required in certain cell types for optimal accumulation of a subset of late (γ_2) proteins exemplified by the *U_S11*, *U_L38*, and *U_L41* proteins.^{823,885,905} This function maps in the C-terminal domain of ICP22 and can be contributed by the smaller *U_S1.5* gene product.⁸²³ Two distinct functions mapping to that site may account for this activity. It is not known whether the effect is transcriptional or posttranscriptional; however, several interactions and effects of ICP22 could explain this function. First, ICP22, in conjunction with the *U_L13* protein kinase, mediates the phosphorylation of RNA polymerase II.^{673,938} ICP22, in conjunction with the *U_L13* protein kinase, mediates the degradation of cyclins A and B and the acquisition of a new partner, the *U_L42* DNA polymerase processivity factor. The cdc2-*U_L42* complex recruits topoisomerase II α in an ICP22-dependent manner to promote γ gene expression.⁸⁻¹⁰ It has been proposed that the ICP22/*U_L42*/topoisomerase complex enables transcription of newly synthesized concatemeric DNA. This DNA accumulates in tangles, which may block access of the transcriptional machinery in the absence of ICP22. Second, ICP22 also causes the loss of serine 2 phosphorylated pol II,³⁴⁸ which could affect activity levels of pol II. Third, ICP22 binds cdk9, and this complex phosphorylates the C-terminus of pol II.³⁰⁵ Finally, ICP22 promotes the formation of nuclear

foci near replication compartments that contain chaperone proteins, proteasomal components⁷¹ that could affect late viral transcription in replication compartments.

EXPORT OF VIRAL RNA FROM THE NUCLEUS

Export of cellular mRNA from the nucleus is coupled to RNA splicing in the nucleus. Because HSV infection inhibits RNA splicing, this raises the question of the mechanism for transport of nascent transcripts from the site of transcription to the cytoplasm. In addition to the potential role of ICP27 in nuclear export of viral RNAs as described previously, there is accumulating evidence that a new export system is assembled in infected cells. Recent studies have shown that HSV replication compartments coalesce at nuclear speckles and that this movement involving active transport using nuclear actin and myosin and ongoing transcription promotes export of late viral mRNAs.¹⁷⁵

TRANSLATION

Like all viruses, HSV must utilize the host protein synthesis apparatus for synthesis of its own proteins. As a result, HSV has evolved several strategies to commandeer the host ribosomes and translation factors for its own protein synthesis. First, HSV infection inhibits host transcription and RNA splicing, thereby interrupting the supply of host mRNA to the cytoplasm, as described elsewhere in this chapter. Furthermore, HSV induces the degradation of many pre-existing host mRNAs through the *vhs* function.⁹²⁵ In addition, HSV blunts the host innate responses such as IFN and protein kinase R (PKR) responses, both of which are described in more detail later. HSV infection has also been shown to exert some specific effects on the host translation apparatus to enhance translation of its mRNA. In addition to decreasing the host mRNA levels, HSV infection stimulates the activity of the cellular cap-dependent translational machinery by increasing the formation of the eIF4F cap-binding complex. This is accomplished by inducing phosphorylation and proteasomal degradation of the eIF4E binding protein 1 (4E-BP1), which releases eIF4E to facilitate eIF4F complex formation.¹²¹⁰ HSV infection also causes phosphorylation of eIF4E, which enhances viral protein synthesis, particularly in quiescent cells.¹²¹⁰ ICP0 is required for both of these effects; however, this is at least in part an indirect effect because ICP0 stimulates the expression of the viral ICP6,²⁶⁸ which binds to the eIF4G protein, a component of the eIF4F complex, and promotes the formation of the complex.¹²⁰⁹ HSV also activates eIF4E via *U_S3* phosphorylation of tuberous sclerosis complex 2 (TSC2), allowing constitutive activation of mTORC1.²⁰⁰

HSV ICP27 has also been shown to interact with host translation factors^{339,622} and to enhance the translation of late mRNAs in infected cells,^{313,338} or an mRNA to which ICP27 is tethered.⁶²² The mechanism of this effect of ICP27 on viral translation has not been elucidated; however, ICP27 has been recently reported to act with *U_L47* to promote the nuclear accumulation of the cellular PABP²⁷⁹; however, others have not observed this effect.⁹⁸⁵

ICP0 has several potential functions during its cytoplasmic sojourn. ICP0 has been also shown to interact with elongation factor 1 δ .⁵³⁶ The interaction of ICP0 with eF-1 δ , an elongation factor responsible for adenosine biphosphate-adenosine triphosphate (ADP-ATP) exchange, appears to be significant inasmuch as a truncated ICP0 polypeptide containing the

binding site interfered with *in vitro* synthesis of a reporter protein. The significance that HSV places on eF-1 δ is underscored by the observation that U_L13 phosphorylates eF-1 δ and that this protein is also phosphorylated in cells infected with representative β - and γ -herpesviruses. ICP0 also forms a complex with CIN85 and Cbl—a ubiquitin ligase that also carries cargo from the cells surface.⁶⁴⁴ Furthermore, ICP0 was shown to bind the SH3 domains of the adaptor protein CIN85 and members of the *src* family.^{642,644} The function of ICP0 as a ubiquitin ligase is firmly established. ICP0 has been reported to interact with at least two additional ubiquitin-conjugating enzymes, UbCH6 and UbCH9; however, the targets of these E2 enzymes have not been firmly established.^{116,410} On the other hand, several proteins turn over rapidly in infected cells, and some have been specifically linked to expression of ICP0. Yet not all the cellular proteins that disappear during productive infection are likely to be the targets of ICP0. For example, the receptor of TNF α turns over very rapidly; in infected cells, it is not replenished because of the degradation of its mRNA.⁶⁴³ A similar case has been made for the disappearance of IFN- γ receptor.⁶⁴² Accelerated degradation of cellular proteins by posttranslational modifications initiated by phosphorylation of cellular proteins by the viral protein kinases has also been described.⁶⁴² As late proteins, the accumulation of viral protein kinases would depend on the synthesis of ICP0.

MICRORNAS

A total of 16 and 17 microRNAs have been discovered for HSV-1 and HSV-2, respectively, many of which are encoded proximal to or within the *LAT* locus; however, some arise from the S component inverted repeated region.^{240,521,1126,1128} The first of these discovered—HSV-1 miR-H1—is expressed relatively abundantly in productively infected cells as a late gene.²⁴⁰ HSV-1 microRNAs miRH2 through H8 and miRH11 through H17, as well as HSV-2 miRH2 through H7, H9, H11 through H13, and H19 through H25, are also expressed during productive infection.⁵²¹ There is limited information on targets of HSV microRNAs and even less on their biological functions because a limited number of mutant viruses have thus far been constructed and characterized. HSV miR-H2, -H3, and -H4 are perfectly complementary to HSV mRNAs encoded on the opposite strands and can down-regulate the expression of proteins encoded by these mRNAs when expressed in transfected cells. HSV-1 miR-H2 inhibits ICP0 expression of the immediate-early protein ICP0,¹¹⁶⁸ and HSV-2 miRH3 inhibits ICP34.5 expression in transfected cells¹¹²⁶ and in infected cells.¹¹²⁸ The seed region of HSV-1 miR-H6 has complementarity with the mRNA encoding the immediate-early protein ICP4 and can down-regulate its expression in transfected cells.¹¹⁶⁸ In contrast, the HSV-2 miR-H6 does not down-regulate ICP4 expression in transfected or infected cells.¹¹²⁷ Repression of viral transcriptional activators ICP0 and ICP4 by microRNAs may be important for repression of HSV-1 productive-cycle gene expression, although it remains to be shown that these effects are exerted in virus-infected cells.

Viral DNA Replication

Production of progeny virus requires the amplification of the input viral DNA genomes for assembly into progeny viral particles. Many of the viral β proteins prepare the infected cell for or are directly involved in viral DNA replication through targeting and

assembly of viral proteins into intranuclear structures, where viral DNA replication initiates (prereplicative sites) and where viral DNA replication proceeds (replication compartments; see Fig. 60.4), and for enhancement of levels of nucleotide precursors for viral DNA synthesis in resting cells. As described earlier, the input viral genomes target to the nuclear periphery soon after entry into the nucleus. Following α and β gene transcription from the viral DNA molecules, likely at these sites, the viral DNA replication proteins then localize to these genomes on the nuclear periphery to form structures called *prereplicative sites*.^{255,909} Prereplicative sites near ND10 sites are the sites of initial viral DNA replication,¹¹⁷⁴ and these structures grow into replication compartments as DNA replication proceeds.²⁵⁵ The replication compartments enlarge, merge, and fill the nucleus, concomitant with peripheral margination of the host chromatin,^{763,1051} suggesting that the HSV replication compartments displace the cellular chromatin from internal sites in the nucleus.

The mechanism of HSV DNA replication has not been fully elucidated, although the origins of viral DNA replication and essential viral gene products have been defined. The major DNA replicative products inside infected cells are long “head-to-tail” concatemers containing the four sequence isomers described earlier,⁴⁹¹ and the mechanisms of viral DNA synthesis need to accommodate this observation.

It is generally accepted that HSV DNA replication is a two-stage process involving initiation by binding of the HSV U_L9 origin binding protein to an origin and formation of the replication complex, which replicates the molecule through an intermediate such as a Cairns circle or theta molecule (Fig. 60.10) in a process that yields circular progeny DNA molecules. At a later time, there is a switch to an origin-independent replication mechanism. Both rolling circle¹⁰⁶ and recombinational^{812,1262} mechanisms have been proposed for the second stage of HSV DNA synthesis. Rolling circle mechanisms have been the favored mechanism because (a) rolling circle replication on preformed templates has been achieved *in vitro*,^{327,911} and (b) it is simpler to see how this mechanism could generate the tandem repeats of viral DNA in defective viral genomes.^{77,1194} These two types of mechanisms are not mutually exclusive, however, because models involving both rolling circle replication and recombination have also been proposed.⁹⁵³

Seven viral proteins are required for viral DNA replication. The seven gene products are the viral DNA polymerase catalytic subunit (U_L30) and its processivity factor (U_L42); an origin-binding protein (U_L9); the ICP8 single-stranded DNA-binding protein (U_L29); and the helicase-primase complex of three proteins—U_L5, U_L8, and U_L52.^{173,221,904,1275} Host cell factors (HCFs) are presumably also involved in viral DNA synthesis, although these have not been identified, in large part because HSV DNA replication involving origin-dependent initiation and synthesis has not been achieved *in vitro*. Nevertheless, host enzymes, including the DNA polymerase α -primase, DNA ligase, and topoisomerase II, are probably also required. Early immunofluorescence studies showed that cellular RPA single-stranded DNA binding protein, PCNA, Rb, p53, DNA ligase 1, and pol α co-localized with ICP8.¹²⁵⁷ Inhibition of topoisomerase II prevents efficient replication of HSV-1 DNA,⁴¹⁵ possibly by preventing decatenation of newly synthesized daughter molecules. Proteomic studies showed that numerous cellular DNA repair, DNA damage response, and

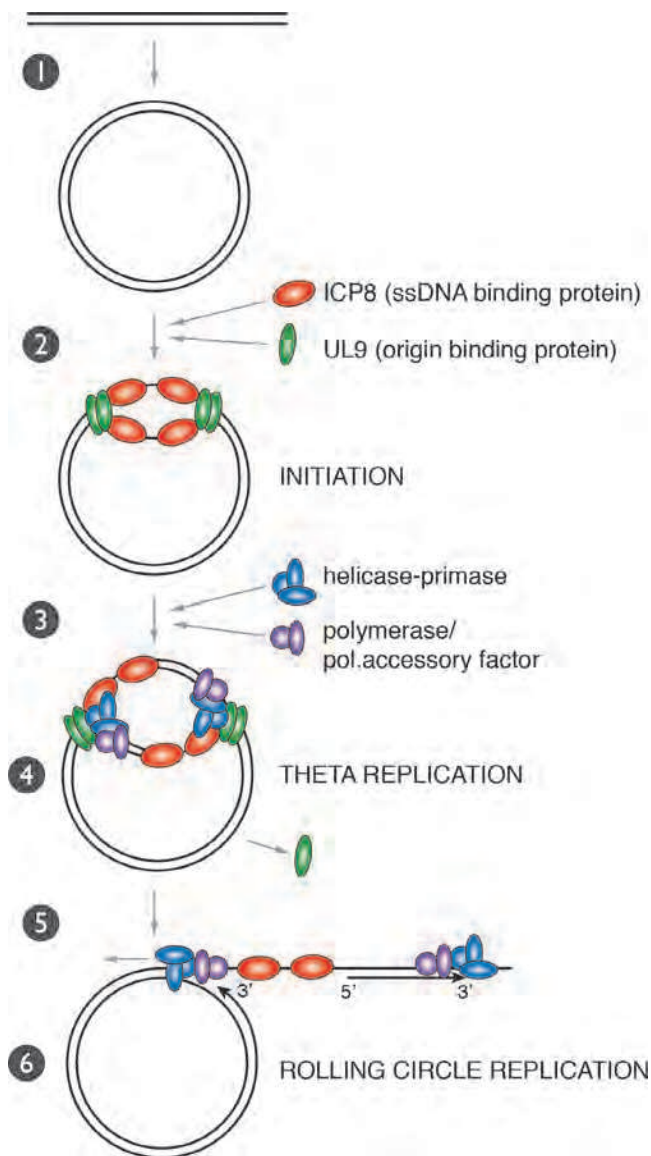


FIGURE 60.10. Diagram of a model of herpes simplex virus DNA replication. **1:** Input DNA is circularized upon entry into the nucleus. **2:** U_L9 (the origin binding protein) initially binds to specific elements in the origin (either *oriL* or *oriS*) and begins to unwind the DNA. U_L9 then recruits ICP8 (the single-stranded DNA binding protein) to the unwound single-stranded DNA. **3:** U_L9 and ICP8 recruit the five remaining viral DNA replication proteins to the replication forks. **4:** The helicase-primase proteins and the viral polymerase complex assemble at each replication fork for initial rounds of theta form replication. **5:** Replication switches from theta to rolling circle mode by an unknown mechanism. U_L9 is not necessary for rolling circle replication, as it is not origin dependent. **6:** Rolling circle DNA replication produces long head-to-tail concatamers of viral DNA, which are cleaved into monomeric molecules during packaging. (Copyright Lynne Chang and David Knipe.)

recombination proteins are associated with ICP8¹¹³² and localize to replication compartments.^{1132,1261} Mre11, Rad50, Nbs1, RPA, MSH2, BRCA1, BLM, WRN, DNA ligase 1, RAD51, and Ku86 are located in prereplicative sites and replication compartments.^{393,648,1043,1132,1257,1261} Cells defective for WRN

helicase¹¹³² or Mre11 or ataxia telangiectasia mutant (ATM)⁶⁴⁸ show reduced HSV replication; thus, these proteins promote viral replication in some way. It is not known if these host proteins are involved in viral DNA replication at this time.

The origins of viral DNA synthesis were identified through mapping of sequences found in defective viral genomes^{350,667} and sequences needed for plasmid DNA amplification in transfection studies.^{761,1195,1231} The origins include *oriS*, a sequence located in the *c* sequences bounding the S component and therefore present in two copies in the viral genome, and *oriL*, a sequence located between the divergent transcription units of the genes for two viral DNA replication proteins: ICP8 and DNA polymerase. Both *oriL* and *oriS* are palindromic structures, with *oriL* being a 144-bp palindrome and *oriS* being a shorter palindrome of 45 bps that center around AT-rich regions of 20 and 18 bps, respectively. Inverted repeats that contain binding sites for the U_L9 protein, called box I, are located on either side of the AT-rich region of *oriL*. *oriS* contains a box I sequence to the 5' side of the AT-rich region and a similar sequence with a tenfold lower binding affinity for U_L9, called box II, located 3' to the AT-rich region. In *oriL*, the box I sequences are flanked by another homologous sequence, box III, which has greatly reduced affinity for U_L9. *oriS* contains one copy of box III flanking the 5' box I. *oriL* is notoriously unstable when cloned into plasmids, thus little genetic analysis has been performed on this sequence. Box I is required for *oriS* function, and mutations in box II greatly reduce DNA replication. Although box III shows weak binding to U_L9 *in vitro*, mutations in box III reduce replication by approximately fivefold in transfection assays.

The reason(s) for three potential origins of replication in the viral genome are not apparent at this time. Neither origin is specifically required for viral replication. A mutant virus with a deletion in *oriL* is viable and showed normal burst size and latent infection.⁸⁸¹ In addition, mutant viruses with both *oriS* sequences deleted showed at most a fourfold reduction in viral yields and only slightly delayed viral DNA synthesis.⁴⁸³ It has been suggested that one of these origins may represent vestigial origins from the L and S components of the viral genome,⁹⁵⁹ and that one of these origins, *oriL*, may function in reactivation from latent infection.⁴²⁰ One study found that an *oriL* mutant virus shows reduced replication in mouse tissues and reduced reactivation from latent infection.⁵⁹ Thus, *oriL* may be required for DNA replication in certain tissues; alternatively, the expression of the bounding *U_L29* and *U_L30* genes may be affected in these tissues.

Viral DNA Replication Proteins

The seven essential HSV DNA replication proteins are reviewed in detail elsewhere.^{106,955,1219}

DNA POLYMERASE

The HSV DNA polymerase has been studied extensively, partly as a result of its potential as a target for antiviral drugs. A new DNA polymerase activity was detected in HSV-infected cells in the 1960s; however, formal genetic proof that it was virus encoded was not provided until the late 1970s, when ts and drug-resistant mutations affecting the properties of the polymerase were isolated and mapped to the viral genome. The HSV DNA polymerase holoenzyme is a heterodimer of the 136 kDa U_L30 protein complexed with the 65 kDa U_L42 protein.

The U_L30 protein contains the polymerase activity, and it contains three sequence motifs that are homologous and align with sequence motifs I, II, and III of other DNA polymerases. The U_L30 protein also has an intrinsic 3′-5′-exonuclease activity, which can serve as a proofreading activity. The crystal structure of U_L30 defined the pre-NH2 domain, NH2-terminal domain, 3′-5′-exonuclease domain, polymerase palm subdomain, finger subdomain, and thumb subdomain.⁶⁶⁰

U_L42 increases the processivity of the U_L30 DNA polymerase activity. The structure of U_L42 protein bound to the C-terminal fragment of U_L30 has been determined,¹³²⁴ and U_L42 is unusual in binding DNA directly as a monomer rather than as a toroid wrapping around the DNA as other processivity factors do. U_L42 is capable of linear diffusion along DNA despite its high-affinity binding to DNA.⁹¹⁸ The interaction between U_L30 and U_L42 is essential for viral DNA replication *in vivo*, and these interaction sites are being investigated as possible targets for antiviral compounds. U_L42 also interacts with cdc2 and topoisomerase II α , as noted previously.

The HSV DNA polymerase interacts with a broader range of deoxynucleoside triphosphates than cellular polymerases, and this has allowed the development of compounds that specifically inhibit the viral DNA polymerase. These compounds are discussed later and in Chapter 13.

ORIGIN BINDING PROTEIN U_L9

The U_L9 origin binding protein forms a homodimer and binds specifically to the sequence CGTTCGCACTT. U_L9 also has ATP-binding and DNA helicase motifs that are essential for viral replication. Binding of U_L9 to origin sequences induces a bend in the DNA and formation of a single-stranded stem-loop structure. The addition of the HSV single-strand DNA binding protein, ICP8, allows U_L9 to unwind Box I of *oriS* if an 18-nucleotide single-strand tail is present 3′ to Box I. Thus, ICP8 may provide a single stranded DNA region in the AT-rich region, from which U_L9 can separate the strands. As an origin binding protein, U_L9 would play a role in origin-dependent synthesis originating from the circular molecules, but once synthesis has converted to a rolling circle or recombination-based mode, U_L9 would presumably not be needed as an origin binding protein.

SINGLE-STRANDED DNA BINDING PROTEIN, ICP8, U_L29

ICP8 is an abundant protein in HSV-infected cells and was first identified as the major DNA-binding protein in HSV-infected cells. ICP8 binds preferentially to single-stranded DNA, and this single-stranded DNA binding function is essential for viral DNA replication. ICP8 also exhibits a helix-destabilizing activity but can also catalyze the renaturation of complementary single strands. Probably as a consequence of the latter two activities, ICP8 can promote strand transfer, which may contribute to the high frequency of homologous recombination observed in infected cells. In addition to its single-stranded DNA binding function, ICP8 interacts with several other viral DNA replication proteins, as evidenced by its physical interaction and stimulation of U_L9 helicase activity; stimulation of the U_L5/8/52 helicase activity; functional interaction with DNA polymerase; and co-immunoprecipitation with U_L9, U_L8, and pol proteins. Consistent with these numerous interactions, ICP8 is required for localization of other viral proteins and cellular proteins to prereplicative sites in infected cell nuclei. As a result of its size and numerous protein interactions,

ICP8 is likely to play a scaffold role in assembly of HSV DNA replication complexes. The crystal structure of a 60-residue C-terminal deletion form of ICP8 showed a novel fold consisting of a large N-terminal domain (residues 9–1,038) and a small C-terminal domain (residues 1,049–1,129).⁷⁰⁷ The overall structure provided a potential groove containing aromatic and basic amino acid residues that could serve as the single-stranded DNA binding site. ICP8 possesses strand-melting¹⁰⁷ and strand-annealing activities³⁰⁶ and has been reported to mediate limited strand exchange.^{112,811} A recent study has also shown that ICP8 can promote strand invasion.⁸¹²

DNA HELICASE–PRIMASE COMPLEX

The DNA helicase–primase complex, which contains the protein products of the *U_L5*, *U_L8*, and *U_L52* genes, was first identified as a helicase activity from infected cells. The complex unwinds short oligonucleotides annealed to single-stranded M13 DNA in the 5′ to 3′ direction. A complex of U_L5 and U_L52 has DNA-dependent ATPase, helicase, and primase activities, thus this constitutes the core enzyme. U_L8 promotes the nuclear localization of this complex and, in concert with ICP8, stimulates optimal activities of the core enzyme. Therefore, the holoenzyme can unwind a 2.3-kbp nicked plasmid in the presence of ICP8. The primase activity produces oligoribonucleotides 6 to 13 bases in length, and the preferred template sequence is 3′AGCCCTCCCA, with synthesis initiating at the first C.

Model for Herpes Simplex Virus DNA Replication

Based on the available information, a model for HSV DNA replication (see Fig. 60.10) has been formulated.¹⁰⁶ The first steps in HSV DNA replication involve the binding of U_L9 protein and/or ICP8 to the origin sequences and the looping and distortion of the origin sequences by U_L9. ICP8 then binds to U_L9 and/or single-stranded DNA regions, and the U_L9 helicase activity unwinds the DNA. The helicase–primase complex is then recruited to the origin by interactions with U_L9 and/or ICP8. Leading strand synthesis involves the unwinding of the DNA and synthesis of a primer by the HSV helicase–primase complex, from which leading strand synthesis can be accomplished by the HSV pol-U_L42 holoenzyme. Alternatively, primers may be synthesized by the cellular polymerase α -primase. Lagging-strand synthesis is then accomplished by primer synthesis and pol-U_L42 extension of the DNA strand. Because the progeny DNA is largely in the form of head-to-tail concatemers,⁴⁹¹ it is believed that although synthesis initiates from the theta form, it is rapidly converted to a rolling circle replicative mechanism, by which concatemeric molecules can be synthesized.

Accessory Viral Gene Products Involved in Viral DNA Synthesis and Postsynthesis Modification

Several other HSV gene products, including the TK, deoxyuridine triphosphatase (dUTPase), ribonucleotide reductase, and uracil DNA glycosylase, are not essential for viral replication in cultured cells but are likely to be essential for nucleotide metabolism and viral DNA synthesis and repair in resting cells, such as neurons. The corresponding host cell enzymes are not expressed in resting cells, and it is likely that the virus has evolved to encode these enzymes to optimize its own DNA synthesis in these cells.

THYMIDINE KINASE

The TK enzyme was first identified as a deoxythymidine kinase, giving rise to its name. In fact, it phosphorylates pyrimidines and even purine nucleosides. In addition, it has a thymidylate activity. Its broad substrate specificity allows it to phosphorylate nucleoside analog molecules, which then can serve as antiviral compounds. The HSV TK consists of a homodimeric complex of the U_L23 protein, the structure of which has been solved. HSV presumably encodes a TK activity to provide nucleoside triphosphate precursors for DNA synthesis in resting cells such as neurons, where the cellular enzyme is not expressed (see later discussion). The viral enzyme leads to an increase in dTTP pools relative to that seen in uninfected cells or early in infection. This complicates the use of radioactive thymidine for labeling of viral DNA synthesis in infected cells, because the specific activity of the labeled DNA will be lower at later times of infection.

DEOXYURIDINE TRIPHOSPHATASE

HSV dUTPase, a monomer of U_L50 protein, hydrolyzes dUTP to dUMP and pyrophosphate, preventing the incorporation of uracil into DNA. HSV-1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency.⁹⁰⁸

RIBONUCLEOTIDE REDUCTASE

Ribonucleotide reductase catalyzes the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphate. The HSV enzyme consists of a complex of the U_L39 and U_L40 proteins as an $\alpha_2\beta_2$ tetramer.⁴⁶ The HSV ribonucleotide reductase is not subject to the same allosteric controls as the cellular enzyme; thus, the HSV enzyme is not inhibited by the increased dTTP pools in HSV-infected cells. The HSV ribonucleotide reductase is required for viral replication in nondividing cells. The large subunit has an intrinsic autophosphorylating kinase activity that is separable from the ribonucleotide reductase activity.

URACIL DNA-GLYCOSYLASE

The enzyme uracil DNA-glycosylase removes uracil bases from DNA—bases that arise in DNA by the deamination of cytosine to form uracil by the cleavage of the N-glycosidic bond linking uracil to the deoxyribose sugar. The site is then repaired so that a mutagenic event converting a G-C base pair to an A-T base pair does not occur. The HSV enzyme is encoded by the U_L2 gene.¹⁵⁶

ALKALINE NUCLEASE

The enzyme alkaline nuclease is a phosphoprotein encoded by the U_L12 gene.⁸⁹⁵ It has endo- and exonuclease activities and is active at pH 9 to 10. As described later, this nuclease plays a role in DNA maturation and encapsidation. The HSV nuclease may be required to resolve concatenated viral DNA for packaging. U_L12 interacts with ICP8,¹¹⁴⁰ possibly to allow the nuclease to act directly on viral replication intermediates or products. U_L12 and ICP8 facilitate strand exchange *in vitro*,⁹³⁰ and ICP8 stimulates nuclease activity by increasing processivity of the enzyme.⁹³¹

Viral DNA Recombination

Homologous recombination is very efficient in HSV-infected cells, and multiple cross-over events between co-infecting viral genomes are apparent in progeny viruses, even between HSV-1

and HSV-2 genomes. Viral DNA replication is required for this high level of homologous recombination, and the time courses of DNA replication and recombination are parallel in cells transfected with viral *oriS* plasmids. The mechanism by which replication of the DNA promotes recombination is not known; however, the single-stranded regions or the concatemeric molecules might be targets for cellular or viral recombination machinery. In addition, ICP8 is known to promote strand transfer, thus it might play a role in recombination in infected cells.

Inversion of the genomic L and S segments also involves recombination between the terminal repeats and the internal inverted repeats. As described earlier, HSV virion DNA contains four populations of molecules bearing the four orientations of the L and S components of the genome. This novel feature of the genome of HSV and certain other, but not all, herpesviruses (see Chapter 59) has long intrigued the field, and interest has been further piqued by the observation that viruses without internal repeats replicate well in continuous cell lines but poorly in primary human cells.^{498,879} These mutant viruses are nonpathogenic, suggesting that the internal inverted repeats play a key role in primary human cells and *in vivo*. The L–S junction sequences—in particular, the *a* sequences—promote high-efficiency inversion in that insertion of a copy of these sequences at other sites in the genome leads to inversion of these sequences. Duplication of certain other viral sequences at a second site in the viral genome can lead to additional inversion events; however, these events appear to be less efficient than *a* sequence-mediated inversion. The 95bp Uc-DR1 sequence is specifically required for inversion at the *a* sequence, although it remains to be determined if this event involves site-specific recombination or a hot spot for recombination.

ACTIVATION OF HOST DNA REPAIR PATHWAYS

The interactions of HSV with DNA repair pathways are complex. The presence of nicks and gaps in the incoming viral DNA is likely to result in activation of DNA repair pathways, as discussed above. Consistent with this, HSV infection has been shown to activate the host DNA damage repair pathway.^{648,1261} Some of this host response may be due to parental viral DNA, but the peak response requires and/or coincides with viral DNA replication.^{648,1261} Phosphorylation of ATM is observed as early as 0.5 hpi but increases until 8 hpi during the time of viral DNA synthesis.⁶⁴⁸ Thus, DNA damage sensors may recognize the input viral DNA, yet the optimal response involves newly replicated DNA. HSV infection activates the ATM-dependent signaling pathway while it inhibits the DNA-PKcs and ATR (ATM and Red 3-related)-dependent pathways.^{7,28–30,648,762,847,1261} To counter the inhibitory effects of DNA repair mechanisms on viral replication, ICP0 promotes the degradation of RNF8 and RNF168, which are mediators in the ATM pathway.^{649,650} The effects of the DNA repair components on HSV replication are complex in that some components are inhibitory to viral replication and others stimulate viral replication.^{648,1132} It is conceivable that whereas HSV inhibits the DNA repair pathways, it has co-opted DNA repair mechanisms for its own genome replication, much as the host cell uses DNA repair mechanisms as part of its genomic synthesis.⁷¹⁹

Viral Nucleocapsid Assembly

Following their synthesis, the γ capsid proteins localize into the infected cell nucleus, where capsid assembly occurs. Empty

shells containing an internal scaffolding are assembled first, then viral DNA is inserted or encapsidated into the capsid concomitant with loss of the scaffolding. Our knowledge of the mechanisms of assembly of the HSV capsid has come from several lines of experimentation: (a) study of infected cell complexes and structures, (b) assembly of capsid structures from extracts of insect cells infected with baculoviruses expressing HSV capsid and scaffolding proteins, (c) study of protein localization using immunofluorescence, and (d) genetic analysis of the functions of capsid and scaffolding proteins. Each of these approaches has contributed important information to our understanding of this process.²²⁴

Immunofluorescence studies have shown that the initial stages of assembly of at least some capsid proteins occur in the cytoplasm. VP5, the major capsid protein that makes up the hexons and pentons, VP26 located on the outer tips of hexons, and triplex protein VP23 are incapable of nuclear localization on their own. Thus, complexes involving these proteins may form in the cytoplasm and localize into the nucleus. VP5 may be carried into the nucleus by triplexes or by pre-VP22a, a scaffolding protein. VP26 localizes into the nucleus only when it is expressed with both VP5 and VP19C or pre-VP22.

Electron microscopic studies have shown that the final assembly of capsids occurs in the nucleus. Three types of capsids—A, B, and C—have been identified in infected cell nuclear extracts using sucrose density gradient ultracentrifugation.³⁷⁵ All three types of capsids are approximately 120 nm in diameter with an outer shell consisting of hexons and pentons made up of VP5, the major capsid protein. The capsomers are linked by triplex structures comprised of the two minor capsid proteins VP19C (*U_L38*) and VP23 (*U_L18*), each complex on average consisting of one molecule of VP19C and two molecules of VP23. C capsids contain viral DNA and a heterodimer of viral proteins, *U_L25* and *U_L17*, that form the capsid vertex-specific component (CVSC).^{210,1156} This was originally thought to be specific for C capsids¹¹⁶⁴; however, this complex was also seen in reconstructions of all three forms of capsids,²¹⁰ albeit at perhaps at lower amounts in A- and B capsids,²²⁴ thus the more general CVSC name has been used. C capsids mature into infectious virions by budding through the nuclear membrane. B-capsid cavities are filled with VP22a and VP21, the cleaved forms of the scaffolding protein, and a viral protease, VP24. The scaffolding protein, but not the protease, is removed upon encapsidation of DNA to form C capsids. A capsids are not filled with DNA or scaffolding protein and are believed to be abortive forms that result from failed attempts to package DNA.

Scaffolding Proteins and Maturation Protease

Although the other capsid proteins are encoded in separate ORFs and transcriptional units (see Gene List.), the scaffolding proteins and maturational protease are encoded by the overlapping *U_L26* and *U_L26.5* genes, which encode a complex set of gene products involved in formation of a core for capsid assembly and for capsid maturation. The *U_L26* gene encodes a 635 amino acid residue precursor protein with an intrinsic protease activity that cleaves either autoproteolytically or *in trans* after residues 247, the R site, and 610, the M site, in the precursor molecule. The amino terminal 247-residue fragment is VP24, which retains protease activity. The fragment from residues 248 through 610 is VP21, which can serve as a scaffolding for capsid assembly. The *U_L26.5* gene mRNA initiates

within the *U_L26* gene and encodes a protein that is read in the same reading frame as *U_L26*. The *U_L26.5* protein, pre-VP22a, is equivalent to the C-terminal 329 residues of *U_L26* and is also subject to cleavage by the *U_L26* protease at the M site near its C-terminus to give a 304-residue protein, which is known as VP22a. Of the *U_L26* and *U_L26.5* gene products, the pre-VP22a protein functions as the major scaffolding protein for capsid assembly. The carboxy-terminal 14 residues of pre-VP22a (and VP21) are recognized by VP5 during capsid assembly.

The protease activity encoded by the *U_L26* gene is a serine protease⁶⁵⁶ whose activity is required for virus assembly.³⁵⁸ Cleavage at the R site is required for viral infectivity,⁹⁴² and cleavage at the M site is believed to be involved in release of the scaffolding protein from the capsid interior. Mutagenesis studies have identified two histidines and two glutamic acid residues essential for proteolytic activity; however, a conserved cysteine was dispensable in the 247 amino acid polypeptide.^{655,656} Proteases have been targets for drug development in many biological systems, and considerable effort has been devoted to identifying specific inhibitors of the HSV protease as possible antiviral compounds. The crystal structures of the HSV-1 and HSV-2 proteases have been solved.⁴⁶⁶

Capsid Assembly

In the nucleus, the VP5–pre-VP22a complexes come together as a result of self-interactions of pre-VP22a molecules. The triplex proteins are then added to form a partial capsid. As hexons and pentons are added, the partial capsid assembles into a round procapsid.⁸⁰⁰ Although it was originally proposed that the portal complex of 12 *U_L6* protein molecules is assembled onto the procapsid through interactions of *U_L6* with *U_L26.5* protein,⁸⁰⁴ the portal may be the nidus upon which the rest of the capsid is built into a sphere,⁷⁹⁹ because that would ensure one portal per capsid. The procapsid structures undergo a structural transformation and become angular and polyhedral.^{203,1147,1162} It is likely that DNA is encapsidated into procapsids as in the model of Newcomb et al.⁸⁰⁰

Much of our knowledge of the capsid assembly process has come from analyses of structures formed in insect cells infected with baculoviruses expressing HSV capsid or scaffolding proteins^{1129,1148} and in extracts from these infected cells.⁸⁰² In these studies, a series of baculovirus recombinants were constructed that each express one capsid or scaffolding protein, and B capsids with normal structure were formed when insect cells were co-infected with viruses encoding the VP5, VP19C, VP23, and *U_L26* or *U_L26.5* gene products. When the *U_L26* and *U_L26.5* genes were left out, no intact capsids were formed, indicating that these gene products are needed to form a scaffold structure around which the shell proteins could form a closed capsid. Similar phenotypes were observed with HSV strains in which the *U_L26* and *U_L26.5* genes were mutated.²⁶⁹ In the baculovirus studies, when the *U_L26* gene was omitted, large-cored B capsids were observed because in the absence of the maturational protease, the *U_L26.5* protein is not cleaved. Thus, cleavage of the *U_L26.5* scaffolding protein leads to condensation of the core. When the *U_L26.5* gene was omitted, intact capsids were observed, but no core structure was apparent. Viruses mutated for *U_L26.5* can still produce infectious virus; however, progeny virus yields are reduced by 10²- to 10³-fold relative to wild-type virus.³⁵⁸ These data indicate that the *U_L26* gene products can serve as scaffolding proteins, although they form a different

core and one that is not as effective as cores containing *U_L26.5* gene products.

In vitro assembly of B capsids can occur when extracts from insect cells individually infected with baculoviruses expressing VP5, VP19, VP23, *U_L26*, and *U_L26.5* gene products are mixed and incubated.⁸⁰⁰ *In vitro* assembly of procapsids requires only the purified viral components VP5, VP19C, VP23, and a scaffolding protein, thus assembly of procapsids does not require any cellular proteins.⁸⁰¹ It has been hypothesized that procapsids may be involved in the early stages of viral DNA packaging, much like the prohead structures of bacteriophages.⁴⁶⁰

Assembly of capsids takes place at early times of infection within replication compartments at sites near those of viral DNA replication^{203,256} and at later times in certain cell types also in nuclear structures called *assemblons*.^{791,1216} It is likely that the next step in viral replication—encapsidation—occurs in replication compartments.

Encapsidation of Viral DNA

Encapsidation of HSV DNA involves a process in which the terminal L components of HSV progeny concatemeric DNA molecules¹³¹⁰ are fed into capsids in an energy-dependent process,²⁵⁰ and the concatemers are cleaved into unit-length monomers by a linked process, as originally shown for pseudorabies virus. Insertion of the L component first would lead to packaging of the S component last, consistent with it being the first to exit the capsid.⁷⁹⁷ This process is not well defined; however, the concatemeric progeny molecules are likely fed into the capsid concomitant with displacement of scaffolding molecules VP21 and VP22a from the capsid. The viral DNA concatemer is thought to be cleaved upon encapsidation of a length of DNA that fills the capsid or when a *headful* (a term that originated with bacteriophage head assembly) of DNA has been inserted. Varmuza and Smiley¹¹⁸⁷ first mapped signals for cleavage and packaging within the *U_b* and *U_c* domains of the *a* sequences, and Deiss et al²⁶¹ mapped these precisely and designated the two DNA packaging elements as *pac1* and *pac2*, respectively.

Concatemers are cleaved into unit-length molecules during packaging. Cleavage occurs site specifically within the DR1 sequences of the *a* sequences⁷⁵⁹ and involves two site-specific breaks at defined distances from the *pac1* and *pac2* packaging signals.^{261,1059,1187} The maturational process duplicates the sequences between these two cleavage sites so that cleavage at sites bearing only one *a* sequence leads to two molecules with terminal *a* sequences.^{262,1059,1187} Several models have been proposed to explain the cleavage and metabolism of viral DNA during encapsidation.^{261,1187} In general, these models propose that a packaging complex binds to the DNA and scans for a *U_c* sequence. Cleavage occurs at a DR1 element proximal to the *U_c* sequence, then the packaging complex will scan the DNA as it is packaged until a directly repeated junction is encountered. Alternatively, two cleavages at the L and S termini will produce a monomer molecule. The amplification of the *a* sequence may occur by staggered nick-repair or gene conversion. The terminal *a* sequence generated by the cleavage may be recombinogenic in either a single- or double-stranded form and promote inversions and/or *a* sequence amplification. In any event, the process must yield the virion form of the genome with an L terminus containing one or more *a* sequences and a 3′ single base overhang, a unit-length genome with an L and S compo-

nent, and an S terminus containing a single *a* sequence and a 3′ single base overhang.

Encapsidation of viral DNA requires at least seven viral gene products, including *U_L6*, *U_L15*, *U_L17*, *U_L25*, *U_L28*, *U_L32*, and *U_L33*,²²⁴ although the mechanism(s) of this process remain to be defined. As described earlier, a complex of 12 *U_L6* proteins serves as the portal at one capsid vertex as the portal for entry of viral DNA into the capsid.⁸⁰³ A complex of *U_L15*, *U_L28*, and *U_L33* proteins is proposed to serve as the terminase, which cleaves the concatemeric DNA into monomers when a monomer is packaged into the capsid. The *U_L28* protein binds to the *pac1* cleavage-packaging sequence,⁷ a sequence needed for termination of DNA packaging.⁴⁵⁴ The *U_L15* protein has homology to the bacteriophage T4 terminase large subunit, including a Walker A motif, which is present in a wide range of ATPases, including bacteriophage terminases.²⁵³ The structure of a cytomegalovirus (CMV) homolog suggests that the *U_L15* homologs are a family of proteins with nuclease activity mediated by an RNase H-like domain.⁷⁸¹ *U_L33* is assembled in the complex with the two proteins⁷⁶ and may reinforce the *U_L28/U_L15* interaction.¹²⁸⁸ Therefore, the complex of the *U_L28* and *U_L15* proteins has the specific DNA-binding and nuclease activities needed for a terminase.^{492,1288,1289} *U_L15* may also have a role in translocation of the DNA into the capsid.¹²⁹¹ *U_L25* seems to be involved in retaining DNA in the capsid,⁷³⁵ possibly by acting as a plug in the C capsid⁸²² or by adding structural rigidity to the capsid. *U_L17* interacts with *U_L25* and is an important part of the CVSC, and its role is probably to make the capsid competent for DNA packaging.^{1017,1156}

Viral Egress

Once the mature nucleocapsid is formed in the nucleus, its route to the extracellular space is a complicated one in that it must traverse the nuclear membranes, cytoplasm, and plasma membrane.⁵⁰⁷ Cell lysis is a very late aspect of HSV infection, thus viral egress must occur through a specialized pathway. At least three general models have been proposed for HSV egress from the infected cell,⁹⁵⁶ including envelopment–de-envelopment–re-envelopment,¹⁰⁵⁴ the luminal pathway,^{510,956} and the nuclear pore egress pathway¹²⁶⁰ (Fig. 60.11).

Although this has been a controversial area, the first model—envelopment–de-envelopment–re-envelopment—is supported by substantial data⁵⁰⁷ and is considered to be the “most prominent model of virion egress”.⁵⁰ The presence in the cytoplasm of filled capsids juxtaposed to curved, invaginated vesicles was first interpreted as evidence of envelopment at cytoplasmic membranes.¹⁰⁸⁷ We will consider this model and then consider the alternative models.

Primary or nuclear envelopment involves the budding of the mature nucleocapsid through the inner nuclear envelope. In the HSV-infected cell nucleus, the marginated host chromatin and the nuclear lamina constitute barriers to the budding of filled capsids through the inner nuclear membrane. To deal with these barriers, HSV infection causes both dispersal of the marginated host chromatin¹⁰⁵¹ and disruption of the nuclear lamina.^{934,1051} Primary envelopment is promoted by the viral *U_L31* and *U_L34* proteins, a complex referred to as the nuclear egress complex (NEC), in that primary envelopment and viral replication are decreased by 10³- to 10⁴-fold with *U_L31* null mutant viruses¹⁷⁹ and 10³- to 10⁵-fold with *U_L34* null mutant viruses in some but not all cell lines.⁹⁶⁵ The *U_L31* null mutant

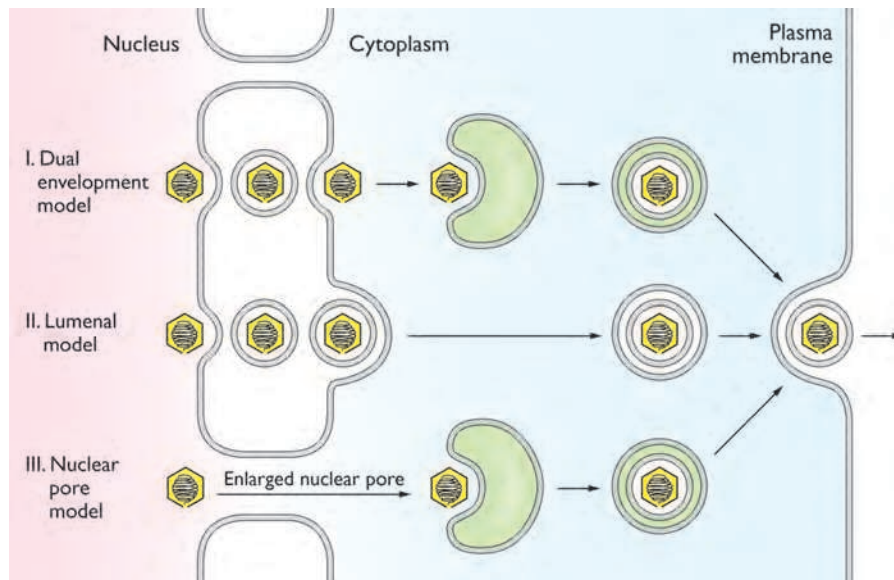


FIGURE 60.11. Models for egress of herpes simplex virus (HSV) from the host cell. Shown are the proposed pathways for maturation of filled capsids from the nucleus to the exterior of the infected cell. **I:** In the dual envelopment pathway,^{748,1054,1087} nucleocapsids undergo primary envelopment at the inner nuclear membrane, become de-enveloped at the outer nuclear membrane, undergo secondary envelopment at cytoplasmic membranes, and then are transported in vesicles to the plasma membrane where fusion of the vesicle with the plasma membrane releases the virion to the extracellular space. **II:** In the luminal model,³¹⁵ the nucleocapsid is enveloped at the inner nuclear membrane, enters a vesicle at the outer nuclear membrane, and is then transported to the plasma membrane where the virion is released. **III:** In the nuclear pore model,¹²⁶⁰ filled capsids exit the nucleus through enlarged nuclear pores and become enveloped by budding into cytoplasmic vesicles, and the virion is transported to the plasma membrane where the virion is released. Wild et al¹²⁶⁰ have proposed a dual pathway for egress, in which HSV uses the luminal pathway at early times of infection but the bulk of extracellular virus is formed by the nuclear pore pathway at late times of infection. (Copyright Lynne Chang and David Knipe.)

also showed a defect in viral DNA replication¹⁷⁹; therefore, U_L31 may play a role in that process as well as envelopment. U_L34 is a type II integral membrane embedded in the inner nuclear membrane, and U_L31 is a nucleoplasmic phosphoprotein that is retained at the inner nuclear membrane through its interaction with U_L34.

U_L31 and U_L34 may promote the disruption of the nuclear lamina in several ways: (a) U_L31 and U_L34 bind directly to lamin A/C and modify the conformation of these lamins,⁹³⁴ (b) recruitment of protein kinase C to the nuclear lamina and phosphorylation of lamin B,⁸⁴⁴ and (c) recruitment of the viral U_S3 protein kinase to the nuclear lamina and phosphorylation of lamin A/C.⁷⁶⁹ U_L31 and U_L34 are also required for the dispersal of the marginated cellular chromatin,¹⁰⁵¹ which may be an indirect effect of disruption of the nuclear lamina, to which cellular chromatin is tethered.³⁹⁶ The contribution of these two proteins differ in different cells because there are differing genetic requirements for U_L31, U_L34, and U_S3 for primary envelopment in different cell types.

Budding of mature nucleocapsids involves binding of the capsid to the inner tegument proteins—VP1/2, U_L37, VHS, VP22 and VP16—which are associated with the inner tails of viral glycoproteins. Full capsids or C capsids are preferentially enveloped and move into the cytoplasm.¹¹⁹⁵ As discussed earlier, C capsids contain a complex of U_L17 and U_L25 at the vertices that lock the filled capsid structure in place. A recent study has shown that the U_L17/U_L25 complex binds to U_L31¹²⁸⁷; there-

fore, an attractive mechanism for the selective envelopment of C capsids is that the U_L17/U_L25 marked capsids bind to U_L31 at the inner nuclear membrane to initiate primary envelopment. The NEC may also recruit HSV glycoproteins to the inner nuclear membrane for primary envelopment.¹²⁶⁴ HSV glycoproteins gM, gB, gH/gL, and gD are found in the inner nuclear membrane and in perinuclear virions, yet their role in primary envelopment has not been defined.

De-envelopment, the next step in egress in this pathway, involves the fusion of the envelope of the virion in the perinuclear space with the outer nuclear membrane, releasing the capsids and tegument into the cytoplasm. The mechanism of this fusion process is not known; however, the viral gB and gH/gL glycoproteins are required for de-envelopment, as shown by a gB/gH double mutant virus.³²⁸ It is interesting that the single mutants affected egress to a much lesser extent. U_S3, which encodes a viral kinase, promotes de-envelopment through phosphorylation of gB and U_L31.^{770,1269} Although the gB⁻ gH⁻ gL⁻ mutant virus shows increased numbers of enveloped particles in the perinuclear space, extracellular virions are reduced by much less; thus, there may be other mechanisms for egress that are independent of these viral glycoproteins. Definition of the de-envelopment mechanism requires identification of receptors in the inner surface of the outer nuclear membranes that would enable membrane fusion, and hence de-envelopment, to take place.

Secondary envelopment involves the budding of tegument-coated capsids into cytoplasmic membranes, possibly including

the Golgi apparatus, the *trans*-Golgi network (TGN), and endosomes, producing infectious virions within a vesicle. HSV has been reported to undergo secondary envelopment at sites coincident with markers of the TGN¹¹⁰⁸; however, HSV infection leads to reorganization of cytoplasmic membranes so that markers of Golgi, TGN, and endosome membranes are spread throughout the cytoplasm and this effect varies among different cell lines.^{149,1267} Budding may occur into multivesicular bodies (MVBs), as shown by the requirement for MVB machinery such as Vps4 and Vps24 for viral assembly or egress.^{145,239}

Transport of virions to the surface involves normal exocytosis mechanisms carrying vesicles containing HSV particles to the plasma membrane, and fusion of the vesicle membrane with the plasma membrane leads to release of the virions to the exterior of the host cell. HSV infection may inhibit TGN recycling loops so that vesicle flow is largely from the TGN to the plasma membrane, thereby actively promoting its own release from cells.

The envelopment–de-envelopment–re-envelopment egress pathway seems to be a very complex process, and many aspects such as the energetics of the process and the de-envelopment receptor remain to be defined. However, evidence supporting the dual envelopment egress pathway includes the following: (a) the U_L31 and U_L34 proteins are present in perinuclear virions but not in extracellular virions^{682,935}; (b) the lipid composition of extracellular virions resembles that of cytoplasmic rather than nuclear membranes¹¹⁷⁹; and (c) mutant forms of gD that are retained in the endoplasmic reticulum are present in virions accumulating at the nuclear membrane but not in extracellular virions, consistent with this model.¹⁰⁵⁴

The second model—luminal secretion—was originally based on the observations that capsids are enveloped at the inner nuclear membrane, that enveloped capsids are frequently contained in perinuclear vesicles, and that these transport vesicles fuse with the plasma membrane and release enveloped capsids into the extracellular space.³¹⁵ Although this pathway is attractive in being simpler than the dual envelopment model, as discussed earlier, the lipid content of extracellular virions is similar to cytoplasmic membranes rather than nuclear membranes, arguing against this model.

As the basis for the third model of egress—nuclear pore egress—Wild et al¹²⁶⁰ reported that the nuclear pores of HSV-infected cells become enlarged to a size readily capable of accommodating the egress of unenveloped capsids from the nucleus. They proposed that HSV then exits the cell by budding through cytoplasmic membranes (see Fig. 60.11). According to this model, a fraction of the capsids become enveloped at the inner nuclear membrane and egress the cell through the single nuclear envelopment model. A larger fraction exits the nucleus via the nuclear pore and is enveloped at cytoplasmic membranes. However, other studies have not observed enlargement or other changes in nuclear pores that would allow movement of nucleocapsids to the cytoplasm.⁴⁵⁵ Acceptance of the nuclear pore model hinges on either demonstration of nuclear pore size changes or that capsids interact specifically with nuclear export proteins and that their depletion blocks cytoplasmic envelopment.

Regulators of Membrane Fusion

Certain mutations in specific viral membrane proteins, including gB, U_L20, and gK, lead to aberrant fusion of infected cell

membranes, leading to the syncytial phenotype.^{507,976} U_L20 and gK interact with gB, the fusion protein,¹⁹⁷ consistent with their regulation of membrane fusion. They may also prevent cell-to-cell fusion by interfering with the trafficking of the fusion glycoproteins to and from the plasma membrane, such that their steady-state amounts at the plasma membrane are reduced.⁴⁵ However, the functions of U_L20 and gK are more complex in that they also seem to promote de-envelopment. A U_L20 mutant virus accumulated enveloped particles in the perinuclear space.⁵⁴ gK mutant viruses also showed defects in nuclear and cytoplasmic egress.^{481,496}

Targeting of Virus During Egress

In certain specialized cell types, HSV egress can be targeted. Pathways for release of HSV from neuronal axons will be discussed later. In polarized epithelial cells, for viruses that bud through the plasma membrane, targeting of viral glycoprotein(s) to the apical or basolateral surface defines the site of viral release from the cell. HSV glycoproteins target to the basolateral membranes of polarized epithelial cells.¹⁰⁸⁶ For HSV, with the complex pathways of release described earlier, targeting of viral proteins to specific plasma membrane sites does not direct virus budding to those sites. In fact, HSV egress is targeted to lateral membranes, in particular to cell–cell junctions in polarized epithelial cells, and this process requires the viral gE/gI glycoprotein complex.⁵¹¹ It is believed that the gE/gI complex accumulates in one domain of the TGN and promotes secondary envelopment there and that this domain targets the sorting of virion vesicles to the cell–cell junctions and the basolateral membrane.⁵⁰⁹ The ability of HSV to spread cell to cell is likely to occur via tight junctions in polarized epithelial cells; however, the virus can also spread to other cell types in the presence of antibodies, forming the basis of plaque assays conducted in the presence of antiviral antibodies.^{311,456} This allows HSV to spread *in vivo* from one epithelial cell to another without being neutralized by antiviral antibody. Similar spread to and between neurons may also be possible to a limited extent.

INTRINSIC IMMUNITY, INNATE IMMUNITY, AND ANTI ANTIVIRAL DEFENSES

The host defense mechanisms against HSV are best described in terms of sophisticated arrays of restriction factors, sensors, and preemptive responses arranged all along the pathway of viral replication, from the moment of contact with the cell surface to the point of late viral gene expression. Certain host factors are expressed constitutively and block essential viral replication processes; this restriction has been called *intrinsic immunity*.⁹¹ Other host mechanisms are induced in response to viral infection and are called *innate immune responses* (see Chapter 8). Some of these responses and restrictions act to limit viral replication within the primary infected cell, whereas other responses act to protect surrounding cells from infection. The overall objective is to shut off the invader or, in extremis, to commit suicide for the greater good. Although it is convenient to illustrate the specific defense mechanisms as if they are present and fully operational in every cell likely to be exposed to the virus, this is not the case. For heuristic purposes, it is convenient to segregate host defenses on the basis of the compartment in

which they are located and whether they are triggered before or after the synthesis of viral gene products.

Cytoplasmic Compartment Prior to Synthesis of Viral Gene Products

The cytoplasmic pattern recognition receptors (PRRs) fall into at least three groups: the Toll-like receptors (TLRs), the RIG-I-like receptors, and the AIM-2-like receptors.^{27,922} At the cell surface, the major PRR relevant to defense against HSV infection is Toll-like receptor 2 (TLR2). Furthermore, current studies indicate that cell surface integrins may also affect TLR2 signaling.¹⁴⁷ In cells lacking $\alpha_3\beta_1$ integrins, HSV enters through a neutral compartment, whereas in cells expressing $\alpha_3\beta_1$ integrins, viral entry is effected through an acidic compartment, likely endocytosis of the virion followed by fusion of the envelope with the endocytic vesicle.³⁷² Targeting of viral entry to lipid rafts could increase viral exposure to TLRs, which are reported to be enriched at lipid rafts after microbial infection.¹⁰⁷⁴ Furthermore, targeting of viral entry through an acidic endosome may increase degradation of the virion and increase the efficiency by which virion components are detected by PRRs in the endosome. The significance of the interaction of virions with the $\alpha_3\beta_1$ integrins in terms of overall defenses of the cell remains a promising area of future investigation.

TLR2 has emerged as a major plasma membrane sentinel of viral interaction on the cell surface.⁹²² HSV infection of cells expressing TLR2 leads to induction of proinflammatory cytokines through the NF- κ B pathway.^{601,602} Sensitization appears to lead to heterodimerization of TLR2-TLR1 through binding of MyD88 and MAL, the activation of NF κ B, and MAP kinases and a whole range of proinflammatory cytokines.⁹²² Murine CMV induces type 1 IFNs via TLR2,⁶² presumably by translocation of TLR2 to endosomes in which a TLR2-TLR1 heterodimer in a complex with MAL and MyD88 activates the Traf3-IRF3/IRF7 signaling pathway; however, there is no evidence of this for HSV. There is evidence that the HSV virion components that trigger TLR2 signaling are surface glycoproteins gB and gH/gC.⁶³⁷ A role for TLR2 in human infections was revealed in a study revealing that two polymorphisms in the *TLR2* gene are associated with increased shedding of HSV-2 and higher recurrent disease rates.¹⁰⁵ In animal studies, TLR2 played a role in protection against HSV-2 disease following intravaginal inoculation but not following intraperitoneal infection.¹⁰⁷⁵ In contrast, TLR2 sensing of HSV-1 infection by the intraperitoneal or corneal routes can lead to increased encephalitis and lethal infection.^{602,996} The ability of different HSV-1 and HSV-2 strains and preparations to induce TLR2 signaling differs,^{997,1181} which may provide some of the basis of virulence of different isolates.

A major endosomal pattern recognition sensor of DNA viruses is TLR9, although TLR7 and a MyD88 dependent pathway are also involved but perhaps to a lesser extent. TLR9 activation requires acidic endosomes, at least in part for viral entry.⁶⁸⁸ In principle, TLR9 recognizes hypomethylated DNA; exposure to HSV DNA in endosomes would occur only if virions were degraded in the endosomes. TLR9 and TLR7 in conjunction with MyD88 induce the phosphorylation of IRF3 and IRF7. Phospho-IRF3 or IRF7 form homo- or heterodimers as part of transcriptional complexes that include NF- κ B and ATF-c-Jun. These transcriptional complexes are powerful acti-

vators of proinflammatory genes that include IFN- α/β , IL-6, IL-12, and TNF- α . The importance of the endosomal TLRs in HSV infection is supported by observations of severe HSV encephalitis in patients with mutations in UNC93B1,¹⁶⁴ which is required for trafficking of TLR7 and TLR9 to the endosome.

This discussion would be remiss without mention of other IFN-inducing cytosolic receptors whose primary function is the detection of DNA. These include DNA-dependent activators of interferon regulatory factors (DAI); leucine-rich repeat flightless-interacting protein 1 (LRRFIP1), which recognizes the AT-rich B form of DNA and GC-rich Z form of double-stranded DNAs; the IFN-inducible protein IFI16; DHX36 and DHX9 shown to bind CpG-A and CpG-B DNA; RNA polymerase III; and inflammasomes activated by DNA viruses that include NLRP3 and AIM2. DAI mediates TBK1-IRF3-dependent induction of IFN in cells exposed to synthetic DNA or HSV.¹¹²³ IFI16 recruits STING to activate the TBK1-IRF3 pathway; it appears to play an important role in innate immunity against HSV.¹¹⁷³ Although IFI16 has been described as a cytosolic DNA sensor,¹¹⁷³ one recent paper reported that IFI16 acts in the nucleus to sense infection by another herpesvirus—Kaposi's sarcoma-associated herpesvirus.⁵⁴² Similarly, in the absence of viral gene expression, HSV can activate IRF3 signaling through nuclear IFI16 in human fibroblasts.⁸³³ DHX36 and DHX9 DEAD box helicases activate IFN via MyD88.⁵⁴⁸ Perhaps the most novel of this group of signaling molecules is RNA polymerase III. The product of this polymerase is uncapped 5' triphosphate RNAs, which induce IFN through activation of RIG-I.^{2,193}

Nuclear Compartment Before Synthesis of Viral Gene Products

Cells recognize as potentially inimical the entry of free DNA into the nucleus, whether it is synthetic and totally devoid of coding sequences, a transfected plasmid, or a viral genome brought into nucleus as a consequence of infection by a virus. In essence, on entry into the nucleus, viral DNA becomes the focus of recruitment of histones,^{207,444,824} histone-modifying enzymes,⁴⁴⁴ repressors of gene expression,⁴⁰⁰ and the assembly of nuclear structures designated PML bodies or ND10s.⁴⁸⁹ The ND10 organizing factor is PML. ND10 bodies are dynamic structures that vary in size and composition. They are present normally in cells but are amplified by IFN, by introduction of DNAs by infection or transfection, and by HDAC inhibitors.⁵²⁸ In infected cells, viral genomes are localized directly adjacent to ND10 structures.⁷²² Some or all of these effects may be related; thus, future experiments to understand the relationship between these different effects are of major importance. The full scope of the modifications imposed on the entering HSV DNA is best described and appreciated from the point of view of the events that lead to its expression, as described earlier.

Activation of Proteolytic and Nucleolytic Enzymes During Entry

In productive entry, endocytosed virus fuses its envelope with the membrane of the endocytic vesicle. The capsid is released into the cytoplasm and is then transported to the nuclear pore, where it releases the viral DNA into the nucleus. Fusion depends on the presence of the viral fusogenic machinery. There is likely always a population of input virions that do not enter, or are incapable of entering, by fusion and could enter and induce signaling through

other pathways. The effects of lack of fusion are shown by the example of gD-negative mutants in which fusion of the envelope with the plasma membrane will not ensue and the virion is degraded. At high multiplicities of infection, massive release of lysosomal enzymes destroys both the virus and the cell.⁷⁷⁴

Summary

The fundamental strategies of the host defenses are three-fold. First, the cytoplasmic organelles and cytosol contain an abundance of pattern recognition sensors capable of detecting virion components prior to *de novo* synthesis of viral gene products. The principal sentinels are PRRs. The signal transduction pathways converge to activate in a cell type-dependent manner the antiviral gene products that include IFN- α/β and proinflammatory cytokines. Second, there are attempts to shut off the synthesis of viral genes both at the point of entry of viral DNA into the virus nucleus and immediately after the onset of synthesis of viral DNA. Third, there is an attempt to commit suicide to preclude the synthesis and dissemination of the virus in the body.

Second Host Defense Perimeter: Activation of Cellular Defense Mechanisms After Onset of Synthesis of Viral Gene Products

Current studies point to three host defense mechanisms that become effective after the onset of accumulation of viral gene products. Of these two, activation of PKR and programmed cell death (apoptosis), if they run their course, commit the infected cell to death.

PKR is activated by IFN and also by double-stranded RNA. In brief, numerous viral transcripts made after the onset of viral DNA synthesis are complementary to each other. Analyses of double-stranded RNA formed by annealing RNA extracted from cells late after infection showed that it had the predicted T_m of and represented transcripts from approximately 50% of the genome.^{493,585} Self-annealing of this RNA could form double-stranded RNA to activate PKR resident in the cytoplasm. Activated autophosphorylated PKR forms dimers that phosphorylate the α subunit of translation elongation factor eIF-2 and I κ B α . The consequences are complete shutoff of protein synthesis by eIF2 α -P and activation of NF- κ B.⁴³⁴

Second, toll-like receptor 3 (TLR3) is an endosomal PRR that is also activated by double-stranded RNA. The importance of TLR3 in host defenses to HSV infection emerged from studies on patients with HSV encephalitis who exhibit mutations in TLR3^{405,1309}; TRIF, one of its adaptor molecules,⁹⁹³ or TRAF3, which interacts with TRIF.⁸⁶⁵ TLR3 is presumably sensing the viral double-stranded RNA molecules described earlier; however, it remains to be defined how these molecules are sensed by TLR3 in the endosome.

Cell death as a preemptive response to infection is triggered by at least three events. First, as described previously, high multiplicity infection with a virus that is unable to enter the cell by fusion of the envelope with the membrane of the endocytic vesicle triggers a massive influx of lysosomal enzymes, resulting in the lysis of the virus and the killing of the cell.⁷⁷⁴ Second, activation of PKR may lead to cessation of all protein synthesis. Third, infection with α gene mutants (e.g., $\Delta\alpha 4$, $\Delta\alpha 27$) that are unable to express post- α or - β genes may trigger apoptosis. Cell death results from activation of caspases.⁷⁷⁴ Overexpression of ICP0 can cause apoptosis; however, the full range of

viral products that trigger apoptosis under normal infections is not known.

Finally, it would be remiss not to mention activation of autophagy. In principle, shortage of nutrients could force the cell to resort to proteolysis of its own proteins to meet its current shortage of amino acids. Autophagy typically initiates with the appearance of cup-shaped membrane structures in the cytoplasm that ultimately evolve into an autophagosome through the intervention of lipid kinase Vps34. This enzyme phosphorylates lipids in association with an array of Atg proteins that include Beclin (Atg6) and LC3 (Atg8).^{14,1053} Protein degradation is the consequence of an influx of lysosomal enzymes into the autophagosome. The role of autophagosomes as a host defense mechanism is at this stage speculative, although evidence is mounting. Autophagosomes could hypothetically furnish viral components to class II antigen presentation pathways or expose viral DNA and RNA to PRRs.

Strategy of Viral Conquest of the Host Cell

The strategy employed by the virus is to preempt any response on the part of the cell to the presence of the virus or its components. Some preemptive strikes are carried out by virion components brought into the cell during infection or made after infection. A key component of the strategy is to block any potential response by multiple mechanisms expressed by different gene products. On the basis of the number of functions directed to suppress a specific host response, the synthesis of IFN and of IFN-dependent host gene products emerge as the major targets of the viral strategy of conquest. It is convenient to consider the preemptive acts in terms of their objective.

Shutoff of Host Responses Prior to Onset of Viral Expression of Viral Gene Products

There are two fundamental mechanisms by which the virus preempts the host responses to infection prior to *de novo* synthesis of viral gene products: suppression of degradation of virions by lysosomal enzymes and degradation of cellular stress response mRNAs.

SUPPRESSION OF DEGRADATION OF VIRIONS BY LYOSOMAL ENZYMES

As noted previously, virions lacking gD are endocytosed and degraded by an influx of lysosomal enzymes. The escape of wild-type virions from degradation is attributed to the presence of gD. This conclusion is based on the observation that apoptosis does not take place in cells transfected with a plasmid encoding gD prior to exposure of the cells to a Δ gD mutant virus. gD appears to block fusion of lysosomes with the endocytic vesicles.¹³¹⁶ This argues that normal virions unable to fuse could also undergo degradation and induce TLR signaling and apoptosis.

DEGRADATION OF CELLULAR STRESS RESPONSE MESSENGER RNAs

Within a very brief interval after exposure to HSV, the cell responds by activation of NF- κ B and the synthesis of a wide array of stress response mRNAs with the ultimate objective to block the synthesis of viral gene products. After a brief burst of activation of NF- κ B by gD binding to HVEM⁷³⁷ and the U_L37 tegument protein binding to TRAF6,⁶⁶⁴ HSV reduces this

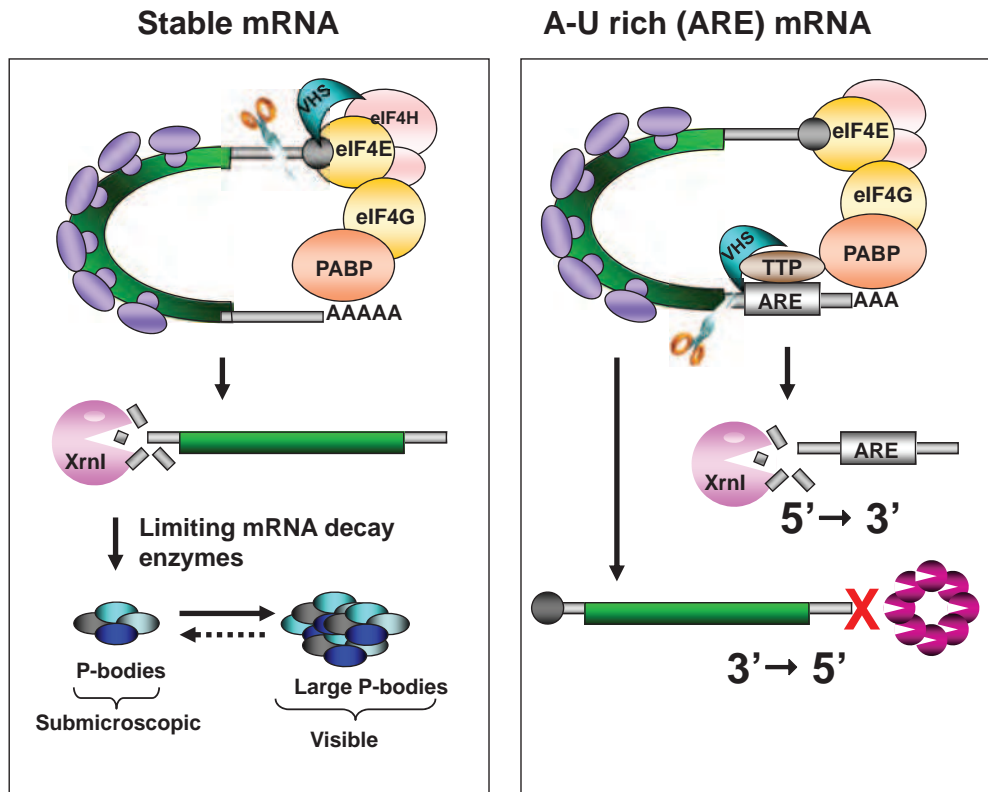


FIGURE 60.12. Model of messenger RNA (mRNA) degradation by VHS-RNase. **Left:** Degradation of Stable mRNAs. VHS binds to polyribosome-associated translation factors and cleaves the mRNA in the 3' untranslated region, leading to 3' to 5' degradation. **Right:** Degradation of A-U-rich (ARE) mRNAs. VHS binds to polyribosome-associated tristetraprolin (TTP), leading to cleavage at or near the ARE elements.

response by degradation of the stress response mRNAs by the VHS RNase, a tegument protein encoded by the *UL41* gene.³¹⁷

VHS is an endoribonuclease with the specificity of RNase A.¹¹¹⁶ It has been shown to bind to both cap- and poly(A)-binding proteins. A model of its activity is illustrated in Figure 60.12. Briefly, VHS can degrade mRNAs in polyribosomes, implying that it binds to mRNAs in the course of translation.¹¹²⁰ VHS rapidly degrades stable mRNAs exemplified by GAPDH or actin mRNAs by binding to cap structures and cleaving the mRNAs 3' to the cap.⁸³⁶ The mRNAs are then degraded 5' to 3'. The high amounts of RNAs targeted for degradation precipitate an increase in size and number of processing bodies.¹¹²⁰ The process of degradation of A-U-rich (ARE) mRNAs is different. ARE RNAs contain stretches of A-U-rich sequences in the 3' UTRs and in uninfected cells have characteristically a short half-life. The A-U-rich elements bind to members of the HuR protein family and to tristetraprolin, which sequesters the ARE mRNAs for degradation. Tristetraprolin is induced in infected cells, and it has been shown to bind VHS.³¹⁶ In wild-type virus-infected cells, ARE mRNAs are cleaved at or near the A-U-rich elements, presumably by VHS bound to tristetraprolin.¹¹¹⁵ The portion of the mRNA 5' to the cleavage site tends to linger in infected cells for many hours,³¹⁷ presumably because of a dearth of enzymes that degrade mRNA from 3' to 5'.

Whereas the VHS RNase functions efficiently in the absence of viral proteins made after infection, in practice the

pre- and postviral protein synthesis activities of VHS cannot be readily differentiated. In synchronously infected cells, VHS is active for at least 5 to 7 hours. To be ectopically expressed in transfected cells, the RNase activity of VHS must be neutralized by concomitant production of VP22 (*UL49*) and V16 (*UL48*).¹¹¹⁷ Viral replication does not ensue in the absence of a neutralizing partner. For example, replication-competent Δ VP22 mutants exhibit mutations in the VHS genes.¹⁰²²

VHS is highly effective in degrading stable mRNAs. A key unresolved question is the mechanism by which HSV transcripts escape total degradation. In transfected cells or *in vitro*, VHS is highly effective in degrading viral mRNAs. The prevailing doctrine is that so much viral mRNA is produced that a sufficient amount escapes degradation. This view is difficult to reconcile with the evidence that in Δ *UL41* mutant virus-infected cells, the transition from α to β and γ protein synthesis is delayed. The hypothesis that the RNase activity is selective or defined by interacting partners is sustained by the observation that some mRNAs appear to be stable even while other mRNAs synthesized concurrently are degraded. ICP27 is one potential partner of VHS that is identified on the basis of its interactions with VHS and potential co-localization in mRNA undergoing translation.^{530,1118}

Other Tegument Proteins

The dominant role of VHS in shaping the response of the infected cells has overshadowed investigation of the role of

other tegument proteins in blocking host responses to infection. The functions of known tegument proteins are shown in the Herpes Simplex Virus Gene Functions List section. A smaller set comprising three proteins is considered next.

VP22 has been reported to be secreted and taken up by adjacent cells. The protein also binds and packages mRNA, both viral and cellular, in virions. The viral mRNAs packaged in virions, although highly diverse, are not random. The RNAs transported by VP22 are expressed in recipient cells; however, the contributions of these mRNAs to the preparation of the infected cell to reproduce the infecting virus is unknown.¹⁰²³

The *U_L11* gene product is an abundant tegument protein. It binds both single- and double-stranded RNA.⁹⁶² It is known to block PKR late in infection and to be associated with polyribosomes at early times.^{165,964} Its role, if any, in blocking host responses to infection at early times is not readily apparent because deletion of the gene has a negligible effect on the pathogenicity of the virus.

ICP0 is defined as a tegument protein by its presence in virions.¹²⁹³ Its function as a tegument protein is not readily apparent and remains to be elucidated.

Shutoff of Host Responses After Onset of Synthesis of Viral Gene Products

The fundamental mechanisms by which the virus shuts off the host after the onset of synthesis of viral gene products are as follows: (a) inhibition of host gene transcription, (b) continuing degradation of cellular mRNAs by VHS described earlier, (c) suppression of silencing of viral DNA by cellular proteins, (d) degradation of specific cellular proteins, (e) disruption of splicing of cellular mRNA accompanied by enhancement of cytoplasmic transport of unspliced mRNA, (f) induced disappearance of short-lived cellular proteins owing to degradation of the mRNAs, (g) inhibition of apoptosis and of autophagy by viral gene products, (h) inhibition of presentation of the antigenic peptides to the adaptive immune system, and (i) inhibition of cytotoxic effects of the cellular immune system.

INHIBITION OF HOST GENE TRANSCRIPTION

HSV infection reduces host gene transcription as measured by nuclear runoff transcription assays.¹⁰⁸¹ However, the quantitative effects of HSV infection and the viral gene products required vary with the cellular gene being studied, although ICP27 was required for inhibition of many of the cellular genes studied.¹⁰⁸¹

SUPPRESSION OF SPLICING OF CELLULAR PRE-MESSENGER RNAs

ICP27 plays a key role in blocking host responses to infection.⁹⁹⁵ In addition to inhibiting host gene transcription, ICP27 performs two key functions. First, early in infection, it interacts with SR proteins, which are components of the pre-mRNA splicing machinery, and recruits the SR protein kinase 1 into the nucleus where it phosphorylates the SR proteins so that spliceosome assembly is inhibited.^{652,1019} This function is executed for several hours and ceases on onset of expression of late viral genes. The net effect is that most cellular pre-mRNAs are not processed correctly and are not translated. A second function of ICP27 is to chaperone viral mRNAs virtually from the time of synthesis to their translation in the cytoplasm

inasmuch as ICP27 has been pulled down in complexes with RNA polymerase II^{245,1314}; the ICP4 transactivator⁸³⁸; CLOCK HAT⁵³⁰; RNA-binding proteins RNA helicase A and hnRNP-F¹³¹⁵; RNA export protein Aly/REF¹⁸⁴; and translation factors including PABP, eukaryotic initiation factor 3 (eIF3), and eukaryotic initiation factor 4G (eIF4G).³³⁹ Still unresolved is the significance of the interaction of VHS with ICP27.¹¹¹⁸ It is conceivable but as yet unproven that ICP27 binds to VHS to block the degradation of the mRNA that it chaperones.

SUPPRESSION OF SILENCING OF VIRAL DNA BY CELLULAR PROTEINS

Immediately upon entry into cells, viral DNA becomes the focus of assembly of histones, histone and DNA modifying enzymes, and repressors. HSV gene products combat this host cell silencing. In addition, ND10 nuclear bodies assemble near the DNA. Viral gene expression depends on remodeling of the viral chromatin. Thus, at least as a preemptive move, HSV degrades components of ND10. These topics have been discussed in detail in the Viral Gene Expression section and in recent reviews.^{560a,947}

Degradation of Cellular Proteins

A key event early in infection associated with the localization of ICP0 at ND10 bodies is the degradation of two key components of ND10: PML and Sp100. In this instance, ICP0 acts as an E3 ubiquitin ligase.^{116,410} It recruits the UbcH5A ubiquitin conjugating enzyme to degrade both proteins.⁴⁰³ The E3 activity maps to the RING domain encoded by exon 2 of ICP0. As a consequence, the components of ND10 bodies become dispersed and are replaced by viral and cellular proteins that ultimately form the viral replication compartments.¹¹⁷⁵ As described earlier, ICP0 promotes, either directly or indirectly, the degradation or reduction in the levels of several cellular proteins. One key function of ICP0 is to block host intrinsic immunity factors via degradation of important cellular proteins such as PML and Sp100. A second key function of ICP0 is to block innate immune responses through reduction of the levels of important signaling molecules. For example, ICP0 causes reduced induction of IFN- β by Sendai virus co-infection, potentially by sequestering IRF3 in the nucleus away from the IFN promoter and by accelerating its degradation.^{742,743} Nuclear ICP0 expressed by HSV-1 *d*106 virus in the absence of expression of other viral proteins can block IFN- β induction by Sendai virus or IFN- β induction by HSV infection,⁷⁴³ thus this seems to be a direct effect of ICP0 and not downstream effects of later events in the infection cycle. In human fibroblasts, IFI16 is required for IFN- β and IFN-stimulated gene responses to HSV infection, and HSV ICP0 causes intranuclear relocalization and degradation of IFI16.⁸³³

To counter the production of proinflammatory cytokines, ICP0 inhibits the TLR2 signaling pathway and, at least under certain circumstances, causes a reduction in levels of the adaptor molecules in this signaling pathway.¹¹⁸³ Although the expectation would be that ICP0 causes this effect during its cytoplasmic phase late in infection, a virus expressing a mutant ICP0 that remains nuclear can still inhibit TLR2 signaling.¹¹⁸³ This would seem to be an example of an indirect effect exerted by ICP0. There is also evidence that cytoplasmic ICP0 can block activation of IRF3⁸³⁷ and that cytoplasmic ICP0 can block TLR signaling via promotion of the nuclear export of

the USP7 deubiquitinase,²⁵¹ although the latter results have not been corroborated.

Overcoming the Shutoff of Protein Synthesis by Activated Protein Kinase R

Virtually every virus that synthesizes complementary RNA must find a way to block the activation or function of PKR. HSV encodes two proteins that thwart the function of PKR.

As the name indicates, ICP34.5, the product of the γ_1 34.5 gene, is a late protein whose accumulation is augmented by the onset of viral DNA synthesis. ICP34.5 has at least two key functions. First, the terminal domain of ICP34.5 contains the motif of a phosphatase accessory factor: It binds protein phosphatase 1a and redirects it to dephosphorylate eIF-2 α -P, thereby enabling uninterrupted protein synthesis.⁴³⁴ Curiously, ICP35.5 does not block activation of NF- κ B. One explanation is that HSV requires one or more NF- κ B-dependent proteins inasmuch as HSV replicates to a lower level in NF- κ B-minus cells.^{20,853} The second known function of ICP34.5 maps in the N-terminal domain of the protein. It interacts with Beclin/Agt6 and blocks autophagosome formation.^{14,1053}

U_s11 blocks activation of PKR by binding to it prior to its activation.¹⁶⁵ U_s11 is a late protein, and its function as an inhibitor of PKR is most apparent when the gene is driven by an α rather than a γ_2 promoter.⁴³²

Blocking Apoptosis

HSV expresses several gene products that preclude the self-destruction of the infected cell. These include, in addition to gD and gJ noted previously, the U_s3 protein kinase.⁷⁷⁴ Cells infected with mutants unable to express ICP4, ICP27, or ribonucleotide reductase undergo apoptosis in a cell-type-dependent fashion. Implicit in these findings is that (a) the cell senses the presence of noxious viral gene products that trigger apoptosis, and (b) a gene function that is expressed later in infection and whose expression requires the missing gene functions blocks apoptosis. Consistent with this view, wild-type virus blocks apoptosis in cells induced by high temperatures or by sorbitol, a potent osmotic shock inducer. Indeed, ectopic expression of U_s3 protein kinase blocks apoptosis induced by sorbitol, or overexpression of caspase 3 or the BAD proapoptotic inducer. To block apoptosis, the U_s3 protein kinase requires the protein kinase activity mapping in its C-terminal domain and a sequence mapping close to the N-terminus. Recent studies led to the identification of a protein—the cellular programmed cell death protein 4 (PDCD4)—that binds to the U_s3 protein kinase. Depletion of this protein precludes apoptosis following infection with Δ ICP4 mutant.¹²¹⁵ Nevertheless, the U_s3 protein kinase is unlikely to express the only antiapoptotic function of HSV inasmuch as Δ U_s3 mutants do not induce apoptosis.

Inhibition of Adaptive Immunity

In addition to their effects on innate immunity, HSV gene products also inhibit adaptive immune responses in several ways.

1. *ICP47 and MHC class I presentation.* The small ICP47 protein blocks presentation of MHC class I peptides by binding in the pore of the transporter of antigen presentation (TAP) and blocking transport of peptides from the cytoplasm into the lumen of the ER for loading on MHC class I molecules.¹³⁰² The net result of this effect would be to reduce or totally block CD8⁺ T-cell induction. The significance of this viral gene product in viral infection has not been determined experimentally because ICP47 does not bind to TAP of small animals commonly used for HSV infection models, but only to TAP of larger domestic animals, nonhuman primates, and humans.⁴⁴⁶ This function of ICP47 might be especially relevant in neuronal cells that express low levels of TAP and MHC class I molecules.
2. *gE and Fc receptor activity.* HSV gE and gI form an Fc receptor that binds the Fc region of IgG and blocks antibody neutralization of virions, antibody-dependent cytotoxicity, and phagocytosis.^{296,345,1185}
3. *gC and complement.* HSV-1 gC binds complement component C3b and inhibits the interaction of the next components of the pathway, C5 and properdin, thereby preventing activation of the classical and alternative pathways.^{353,582}
4. *HSV and T-cell receptor signaling.* HSV-infected cells cause a loss of T-cell functions in co-incubated T cells, including cytotoxicity, and production of cytokines.⁵⁰⁰ The functional defect is owing to a failure of the T-cell receptor signal to propagate beyond the adaptor molecule, the linker for activation of T cells, owing to the lack of recruitment of PLC- γ 1, Grb2, and GADS to the adaptor complex. The viral genes involved in this block have not been identified. For this effect, HSV has to directly infect the T cells; however, HSV can spread from other cell types to T cells via virological synapses.⁴¹
5. *HSV and cytotoxic T-cell (CTL) function.* HSV-1 infection renders cells resistant to CTL-induced apoptosis.⁵⁰³ HSV-1 infection blocks apoptosis induced by anti-Fas antibody or UV irradiation, whereas HSV-2 clinical isolates do not block these effects.⁵⁰² Deletion of the U_s3 gene reduced inhibition of UV-induced apoptosis and partially inhibited Fas antibody-induced apoptosis, whereas deletion of the U_s5 gene had the opposite effect.⁵⁰² HSV-1 gJ (U_s5) inhibits caspase activation and apoptosis induced by granzyme B or Fas.⁵⁰¹ In contrast, U_s3 may block TCR signaling.¹⁰⁵⁷ These effects show at least part of the reasons why the host immune system cannot completely control HSV infection prior to spread to neuronal cells for establishment of latent infection.
6. *HSV and CD4⁺ T-cell function.* HSV infection of B-lymphoblastoid cells reduces their ability to induce CD4⁺ T-cell clone proliferation and cytokine expression.⁶³ The effect was not associated with down-regulation of MHC class II molecules or co-stimulatory molecules. HSV-1 U_s1 expression is necessary and sufficient for this effect and also rendered cells less susceptible to CD4⁺ T-cell-mediated lysis.⁶³ These effects provide further means by which HSV evades host immune responses.
7. *gB, U_s3, and NKT cells.* HSV-1 infection rapidly down-regulates CD1d expression and inhibits NK T-cell function. HSV-1 does this primarily by suppressing CD1d recycling from the ER to the cell surface.¹³⁰⁴ A recent study identified gB and U_s3 as two viral factors functioning in suppressing CD1d antigen presentation by retaining CD1d in the *trans*-Golgi network.⁹¹⁹

Interferon: Herpes Simplex Virus Enemy Number 1

The main objective of the previous sections was to relate suppression of cellular responses to infection by specific viral gene functions. Shutting down IFN-dependent host responses to infection readily emerges as the main objective of the virus on the basis of the number of viral functions aimed at shutting down the cell. Degradation of mRNAs, inhibition of cellular protein synthesis by inhibiting splicing of pre-mRNAs, degradation of PML and IRF3 signaling molecules, and dispersal of ND10 bodies and alteration of functions of cellular proteins by the viral protein kinases are prime examples of the scope and range of functions deployed to suppress the IFN response.^{182,742}

LATENT INFECTION

The ability of HSV to establish a lifelong latent infection in its human host is one of the most intellectually challenging aspects of HSV biology. Owing to space constraints, we are not able to discuss and cite all of the extensive studies in this area; nonetheless, there are several recent, more detailed reviews.^{102,810,867,947} The available data on HSV latent infection support the following sequence of events (Fig. 60.13). Following spread in the tissue at the primary site of infection, the virus enters sensory neurons by fusion at the axonal termini, and the nucleocapsid is carried by retrograde axonal transport to the nucleus in the cell body of the neuron (see Fig. 60.13A). Viral DNA persists in the nucleus in a circular episomal form associated with nucleosomes (see Fig. 60.13B). Lytic gene expression is repressed; however, LAT is expressed at high abundance, which yields several RNA species upon processing and splicing. No replicating virus can be detected in the sensory ganglia during latent infection. In a fraction of neurons harboring latent HSV, the virus is periodically reactivated (see Fig. 60.13C). Infectious virus is carried by anterograde axonal transport to peripheral tissues, usually to cells at or near the site of initial infection. Depending on several factors, including the host immune status, the reactivation may be asymptomatic or lead to a recurrent lesion, which may vary considerably in severity from punctate lesions that are invisible to the naked eye to severe, debilitating lesions in immunosuppressed individuals.

The ability of sensitive PCR tests to measure HSV DNA in genital secretions has led to the detection of frequent viral DNA shedding in the genital tract of both asymptomatic and symptomatic individuals.¹²⁰⁷ This frequent shedding has led to questions about whether HSV latent infection is actually a low level chronic infection producing constant shedding of virus rather than a true latent infection.¹⁰⁰⁶ However, the amount of virus shed in many of these individuals is very low, and this could represent viral DNA molecules that are shed in epithelial cells rather than virions. The number of DNA molecules detected, even if it does represent recent release, would only require a limited number of neurons to be reactivating at any one time. Therefore, it seems likely that the bulk of the infected neurons are undergoing a latent infection, as described previously, and only a small number are reactivating at most times. Nevertheless, the possibility of low-level chronic infection, especially with HSV-2, remains.

Latent infection was first detected experimentally by co-cultivation of sensory ganglia tissue with permissive cells and

detection of cytopathic effect owing to reactivated virus.¹⁰⁹⁹ Infectious virus was not detected in homogenized ganglia; therefore, the virus was latent in the tissue at the time of explant. This led to the original definition of latency—virus must be able to reactivate from the latent state under appropriate experimental conditions. Operationally, this meant that virus could be detected after incubation of intact ganglionic tissue with suitable susceptible cells, but not by inoculation of the susceptible cells with homogenized ganglia. The definition of latency was later extended to include viruses that can be detected in sensory ganglia several weeks after infection by *in situ* hybridization with probes for the LATs or by assays to detect viral DNA in ganglia.^{212,535,632,634} At the present time, the only technique for proving that a virus is incapable of establishing latent infections is the inability to detect viral DNA by polymerase chain reaction (PCR) assays of DNA extracted from whole ganglia. This extension of the definition of latent infection enables an

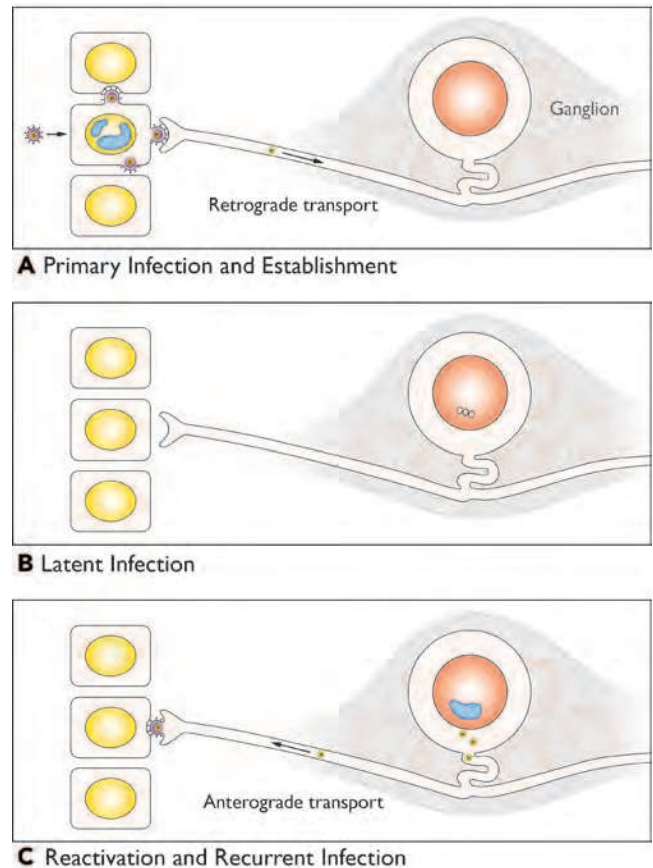


FIGURE 60.13. Stages of herpes simplex virus (HSV) infection of the host. A: HSV is introduced onto a mucosal surface or a break in the skin, and it replicates productively in epithelial cells at the site of inoculation and spreads through the tissue. Virus enters sensory neuron axons and is transported to the cell body in a ganglion. **B:** HSV establishes a latent infection in the neuronal cell nucleus. Viral DNA is circular and assembled in chromatin. **C:** Upon neuronal damage or activation, the virus reactivates and undergoes at least a limited productive cycle. Capsids are transported by anterograde transport to the axonal termini, and virions are released. Reactivated virus causes a recurrent infection of the mucosal tissue, causing the shedding of virus. (Copyright Lynne Chang and David Knipe.)

assessment of the roles of specific genes in the establishment and maintenance of latency, separate from their roles in reactivation. The downside of the revised definition is that it equates the presence of viral DNA with a bona fide latent state defined biologically as at least capable of reactivation. Because viral DNA has been detected in the CNS and even at peripheral sites, the redefinition arguably extends the sites of viral latency beyond currently proven sites.

The burning question of HSV latency remains: How can HSV undergo a productive infection in epithelial and other nonneuronal cells but undergo a latent infection in sensory neurons? Numerous corollary questions derive from this: Are there intrinsic nonpermissive features of neurons that allow the latent infection? Does the virus play an active or passive role in the latent infection? Does the host immune response control the latent infection or just the lytic infection? Some of these questions are addressed next.

Herpes Simplex Virus Latency in Experimental Systems

Latent infection by HSV has been achieved in cell culture systems only under special conditions that usually involve HSV inhibitors.^{32,146,1258} As a result, the most meaningful models have been animal infection systems. The most useful animal model systems are mice and rabbits for HSV-1 infection and guinea pigs for genital infection by HSV-2. In all three species, inoculation of wild-type virus results in viral replication at the peripheral site and retrograde transport of virus to the nucleus of dorsal root neuron, followed ultimately by establishment of latency. In all three systems, a fraction of sensory neurons replicate the virus following entry into the neuron.

There are several key differences in the animal models. In rabbits, both HSV-1 and HSV-2 reactivate spontaneously. The rabbit eye model is particularly useful for studies of spontaneous reactivation because the virus is readily detected in lacrimal secretions. The guinea pig is a useful model of HSV-2 genital infections. The virus reactivates spontaneously, causing lesions containing small amounts of virus, although the acute and recurrent phases are somewhat artificially defined. The virtues of the mouse model are twofold. First, highly inbred strains differ with respect to their susceptibility to infection, and knockout mice have been particularly useful in studies of immune responses to infection.⁶⁷⁸ Second, detection of spontaneous reactivation of virus resulting in the appearance of infectious virus either in ganglia or at peripheral sites is very rare in mice. Latent virus in mice can be induced to reactivate, leading to appearance of virus in sensory ganglia or at peripheral sites by peripheral tissue trauma, UV light exposure, hyperthermia, or administration of drugs that stimulate prostaglandin synthesis. Reactivation has been studied in murine systems following explant of ganglia in culture¹⁰⁹⁸ or *in vivo* owing to tissue trauma,⁴⁴⁸ transient hyperthermia,¹⁰⁰² UV irradiation,⁶²⁴ or epinephrine iontophoresis in the eye.¹²⁶³

Viral DNA During Latent Infection

In latently infected neurons, viral genomes acquire the characteristics of endless or circular DNA^{347,741,943,944} assembled in nucleosomal chromatin.²⁷² Histone modifications, particularly methylation and acetylation, play an important role in defining the type of regulation imposed on eukaryotic genes by nucleo-

somal chromatin, with particular modifications resulting in chromatin-mediated gene activation (active or euchromatin) or repression (inactive or heterochromatin).⁴⁹⁹ During latent infection, the LAT promoter and 5' exon are associated with acetylated histones or active chromatin, whereas lytic gene promoters are not associated with acetylated histone H3.⁵⁹⁷ In contrast, viral lytic gene promoters are associated with heterochromatin forms of histones during latent infection.¹²¹⁴ The heterochromatin modifications include histone H3 lysine 27 trimethylation,^{206,604} and histone H3 lysine 9 trimethylation²⁰⁶ but little of the constitutive heterochromatin marker H3K20 me3.²⁰⁶ HSV DNA is not methylated during latent infection.^{292,598} Current models for maintenance of HSV latent infection include the silencing of all viral lytic genes except for the *LAT* gene by heterochromatin and microRNAs.

Viral RNAs Expressed During Latency: Herpes Simplex Virus Latency-associated Transcripts and microRNAs

During latent infection, the only viral gene products expressed at abundant levels are the LATs, first detected in latently infected murine ganglia,¹¹⁰¹ and microRNAs, first detected in latently infected human¹¹²⁶ and murine ganglia.¹¹⁶⁸ The LATs were subsequently found in latently infected rabbit⁹⁴⁵ and human^{590,1100} ganglia. The *LAT* transcriptional unit (Fig. 60.14) yields several RNA species by splicing, and these are collectively referred to as LATs. The LATs constitute a family of transcripts mapping to the inverted repeats flanking the U_L sequence. The full-length 8.3- to 9-kb transcript accumulates at low levels in latently infected neurons, whereas 2- and 1.5-kb introns processed from a full-length transcript are abundant and accumulate in the nucleus.¹¹⁹⁹ These introns are highly stable as a result of unusual lariat structures.³²⁹ The 2-kb LAT is the major species detected in neuronal nuclei by *in situ* hybridization¹¹⁰¹ (Fig. 60.15). LATs are expressed from a neuron-specific promoter/enhancer.¹³²⁶ Although the 2-kb LAT is detected in productively infected cells at late times postinfection, this is likely to be the result of splicing of read-through transcripts that are common at late times postinfection^{383,493} or another promoter and not transcription from the true LAT promoter.¹⁸⁷ Despite reports of proteins encoded by LATs,^{283,1139} most workers in the field have found no evidence for expression of proteins by the LATs.²⁹³

Organization of the *LAT* Gene

The primary LAT initiates at bp 118,802 on the HSV-1 genome based on RNase mapping of the primary transcript²⁷⁴ or at 118,803 using *in vitro* transcription¹⁰⁶⁸ and extends for 8.3 to 9 kbp, possibly reading through the L-S junction and overlapping the *ICP4* gene at its 3' end (see Fig. 60.14). The 5' exon extends from bp 118,803 to 119,465, and the major intron extends from bp 119,465 to 121,417. The *LAT* promoter is from bp 118,003 to 118,878. A second promoter, sometimes referred to as the LAP2 promoter, has been identified in transient assays only¹⁸⁷ and extends from bp 118,867 to 119,461. An enhancer allowing long-term expression of LAT (long-term enhancer element, or LTE) extends from bp 118,077 to 119,461 and is located downstream from the *LAT* gene transcriptional start site.^{87,668,669}

There are several other transcripts near or overlapping the *LAT* gene or its promoter that could conceivably play a

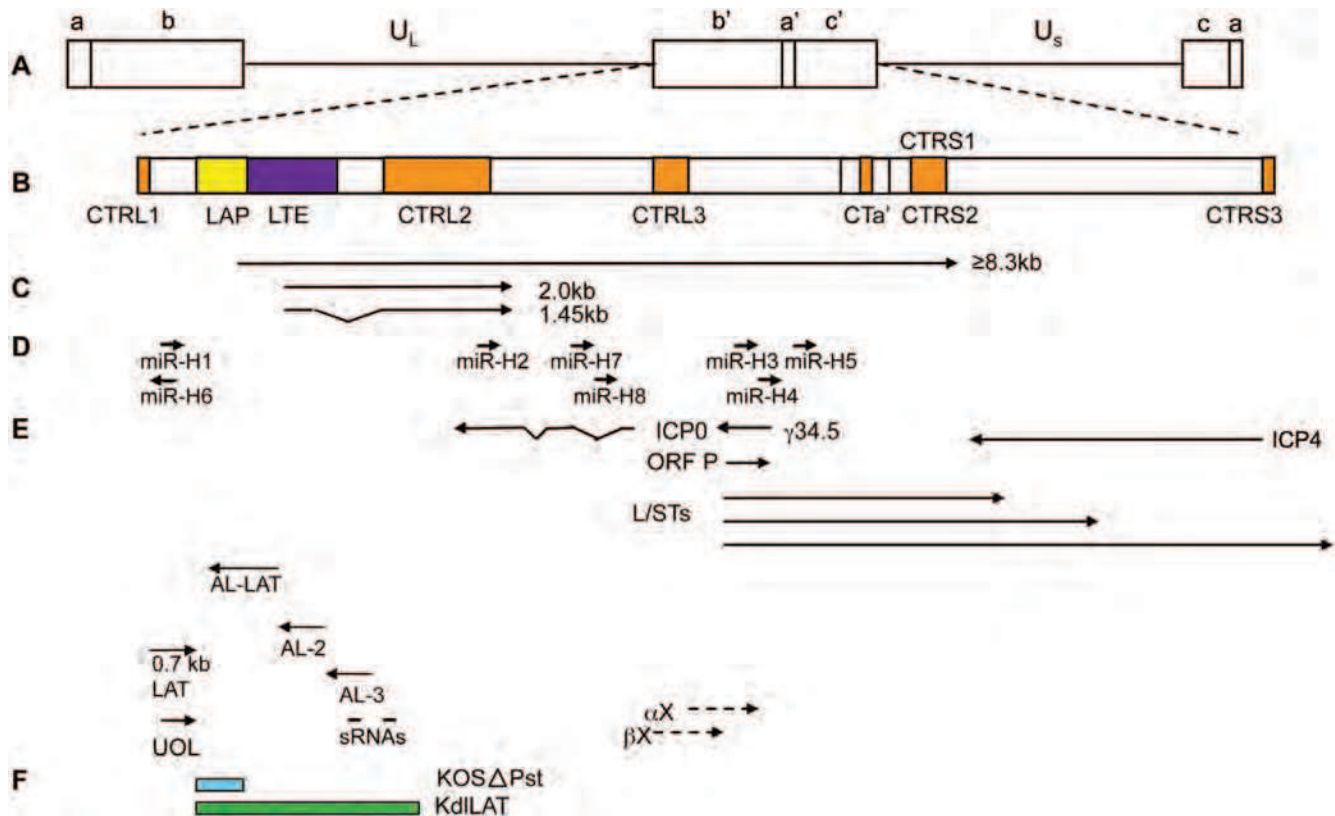


FIGURE 60.14. Map of the *LAT* transcriptional unit. **A:** HSV-1 genome in the prototype orientation; U_L and U_S denote the unique sequences of the long (L) and short (S) components of the genome, respectively. Boxes denote repeated sequences. **B:** Expanded view of the L–S junction of the HSV-1 genome showing the regulatory elements, including the *LAT* promoter (LAP),^{280,1325} long-term enhancer element (LTE),^{86,668} CTCF (CCCTC-binding factor) motifs and putative insulator sites labeled as CTRL1, CTRL2, CTRL3, CTa' and CTRS1, CTRS2, and CTRS3.¹⁸ **C:** LATs including the ≥ 8.3 -kb *LAT* primary transcript; 2-kb and 1.5-kb *LAT* stable introns. **D:** MicroRNAs encoded by the *LAT* region: miR-H1, miR-H2, miR-H7, miR-H3, miR-H5 and miR-H4, miR-H6, and miR-H8. **E:** Locations and orientations of transcripts including infected cell protein (ICP) 0, ICP4, and $\gamma 34.5$ ^{194,195}, open reading frame (ORF) P⁶¹³, L/STs¹²⁹⁸, upstream of *LAT* (UOL)⁷⁹⁰, 0.7-kb *LAT* (only in strain 17 and McKrae, not KOS) antisense to *LAT*s AL-*LAT*, AL-2, and AL-3^{490,869}; small RNAs⁸⁶³; and partially characterized transcripts αX and βX .^{108,109} **F:** *LAT* mutations in the KOS strain: KOS Δ Pst is an *LAT* promoter deletion between two PstI sites, and KdILAT is a 1.8-kb deletion of PstI–HpaI that eliminates the *LAT* promoter and 1.6 kb from the 5' end of the *LAT*.

role in *LAT* function or contribute to the phenotypes of *LAT* gene mutant viruses (see Fig. 60.14). First, for example, a 0.7-kb transcript encoded on the same DNA strand as *LAT* but upstream of *LAT* is expressed by HSV-1 strains 17 and McKrae but not by strains KOS and F.¹³²¹ Second, transcripts that are complementary to the *ICP4* gene transcripts, called *anti-4 transcripts*, were detected by reverse transcriptase (RT)-PCR.¹⁸⁵ The *anti-4* transcript(s) may be expressed from a new transcriptional unit downstream of the primary *LAT* transcriptional unit or expressed as a read-through transcript from *LAT*; however, in any case, *anti-4* transcription is promoted by *LAT* expression.¹⁸⁵ Third, a transcript that is antisense to the 5' end of *LAT* has been identified as encoded by HSV-1 strain McKrae and reported to express a protein in infected rabbits.⁸⁶⁹

Two ORFs encoded with the domain transcribed by the 8.3-kb *LAT* are ORF P and ORF O.^{613,614} Because their mRNAs are tightly down-regulated by ICP4, these were candidates for gene products that might affect latency. However, inactivation of the ORFs had no effect on the ability of the virus to replicate

in experimental animal systems, and only a slight effect on the amount of virus recovered from latently infected ganglia.⁹¹⁵

The literature on LATs is enormous but does lend itself to a brief summation. Thus, some studies reported that viruses deleted in various domains of *LAT* establish latency at normal levels,^{495,631,1096} whereas others reported that the number of neurons harboring *LAT*-negative viruses were decreased by three- to fivefold.^{1002,1146} It is conceivable that assaying numbers of latently infected cells is a more quantitative assay of latent infection than measurement of total viral DNA molecules. Still others reported reduced explant reactivation of virus from ganglia latently infected with *LAT*-negative mutant viruses,⁶³¹ although in some cases *LAT*-negative mutant viruses show reduced replicative ability.¹⁰⁰ The region of the LATs associated with decreased reactivation has been mapped to a 348-bp sequence in the 5' end of *LAT*.^{103,447}

Several studies have shown effects of *LAT* on viral gene expression or replication. First, *LAT*-negative mutant viruses show elevated productive viral gene expression in sensory neurons during acute infection³⁶⁰ and during latent infection.¹⁸⁶

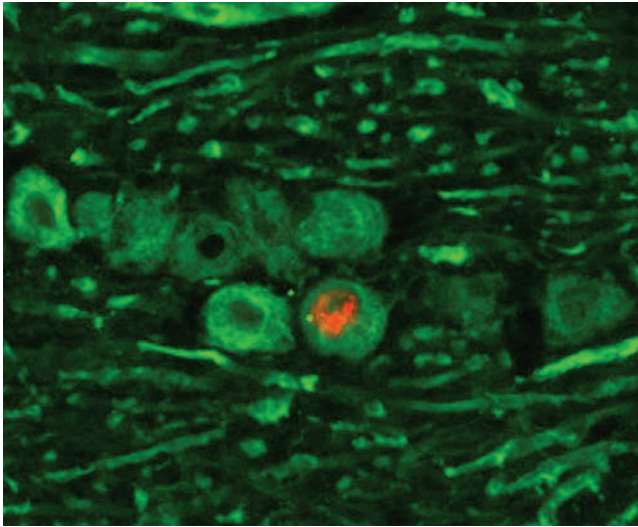


FIGURE 60.15. Detection of herpes simplex virus stable latency-associated transcript (LAT) RNA in latently infected murine trigeminal ganglion neurons by fluorescence *in situ* hybridization (FISH). Mice were infected with HSV-1 following corneal scarification. At 30 days postinfection, the mice were sacrificed, and trigeminal ganglia were frozen and sectioned. The sections were hybridized with a labeled DNA probe detecting the LAT RNA (shown as *red*). Neurons were stained with antibodies detecting neurofilament proteins (*green*). (Copyright Lynne Chang and David Knipe.)

Consistent with this, expression of LAT has been shown to reduce viral gene expression and replication in cultured cells.⁷⁰⁰ LAT-negative mutant viruses have been shown to give higher pathology in trigeminal tissue,⁶³¹ to cause neuronal death,¹¹⁴³ and to cause higher mortality in infected mice.^{1143,1214} This silencing effect of LAT has been attributed in part to the assembly of heterochromatin on viral lytic gene promoters.¹²¹⁴ Recent studies using viruses that mark infected cells⁹⁰⁰ have shown that fewer neurons survive infection with an LAT-negative mutant virus.³⁰⁹ A further function attributed to LATs is that they protect neurons from apoptosis. Thus, a virus deleted for LAT was reported to induce apoptosis in rabbit trigeminal ganglia at slightly higher levels than the wild-type virus.⁸⁶⁶ The effect of LAT on apoptosis in mice is less clear because some did not observe the same effect in mice,^{866,1144,1145} whereas others have observed this effect of LAT in mice.¹² LAT expressed from a plasmid vector can protect cells from cell death induced by etoposide, ceramide, or other agents.^{12,487,870} Although the original hypothesis was that the LATs protected sensory neurons from apoptosis during acute infection,⁸⁷⁰ others have argued that the effect of LATs on establishment of latent infection may be minimal and that it is more likely that the LATs play a role in blocking apoptosis during reactivation.¹⁰¹ LATs can also protect neuronal-derived cells from CD8⁺ T-cell killing.⁵⁰⁵ Thus, a common theme of LAT studies has been that LATs protect neurons from death,¹⁰¹ either by reducing viral gene expression, by protecting against apoptosis, or by other mechanisms. Nevertheless, a few studies have shown that LAT increases lytic gene expression³⁷⁸ and reduces heterochromatin.⁶⁰⁴

LAT expression has also been reported to promote CD8⁺ T-cell exhaustion in trigeminal ganglia through increased expression of PD-1 and Tim-3.^{15,189} LAT directly or indirectly up-regulates both PD-L1 and MHC class I on mouse neuroblastoma cells. Further studies are needed to define the relationships between this new function and the other LAT functions.

microRNAs

A large number of microRNAs have now been shown to be expressed during latent infection. In latently infected murine trigeminal ganglia, these include miR-H1,^{240,589} miR-H2, miR-H3, miR-H4, miR-H5, and miR-H6,¹¹⁶⁸ as well as miR-H7, miR-H8, miR-H17, and miR-H18.⁵²¹ In guinea pig ganglia latently infected with HSV-2, microRNAs shown to be expressed are miR-I (miR-H3), miR-II (miR-H4), and miR-III (miR-H2). In human trigeminal ganglia, these include miR-H1-8¹¹⁶⁹; in human sacral ganglia, they include miR-H3, 4, 7, 9, and 10.^{1126,1170} Many of these microRNAs are likely to be processed from the primary LAT because their expression depends on the LAT promoter.^{589,1127} Furthermore, miR-H6 expression is reduced in a LAT promoter mutant virus,⁵⁸⁹ suggesting that the LAT promoter is bidirectional.

Expression of HSV-2 miR-H3 by transfection reduced ICP34.5 expression in transfected or infected cells,¹¹²⁶ whereas expression of HSV-1 miR-H2 reduced ICP0 expression and miR-H6 reduced ICP4 expression.¹¹⁶⁸ Thus, these microRNAs could contribute to latent infection by down-regulating critical viral gene products needed for lytic infection. A few studies have looked at the function of microRNAs when expressed from the virus; however, much remains to be done in this area. Tang et al¹¹²⁷ found that reduction in expression of HSV-2 miR-H6 did not result in any detectable phenotype with regard to acute or latent infection of guinea pigs.

Events of Latent Infection and Reactivation

We will now examine the mechanisms of latent infection in chronological order in more detail.

Establishment of Latency: Initial Stages

Electron microscopic studies indicate that in neurons infected in cell culture, the viral particle transported by retrograde transport along microtubules in the axon is the unenveloped nucleocapsid or nucleocapsid-tegument structure.^{691,861} Viral gene expression, α , β , γ , and LAT RNAs, are observed in ganglia of infected animals over the first 24 to 72 hours.^{583,586,1178} Several host immune response effectors are postulated to play a role in control of early viral replication, including IFN- γ , macrophages, $\gamma\delta$ T cells, and CD8⁺ T cells. CD8⁺ T cells infiltrate the ganglia starting around 5 to 7 days postinfection, coincident with the time at which virus starts to decrease around day 7.^{663,1037} CD8⁺ T cells have been shown to play an essential role in control of HSV replication in the nervous system¹⁰⁵⁰ and in controlling viral replication in the ganglia during acute times of infection.⁶⁶² Cytokine and chemokine expression increases in the acutely infected sensory ganglia.

The silencing of viral genes in sensory neurons appears to be a slow and multistep process. Although the events occurring during the first few hours after entry of viral DNA into dorsal root ganglia have not been defined (Fig. 60.16), a key factor is likely to be the reduced expression of α genes. The reduced α

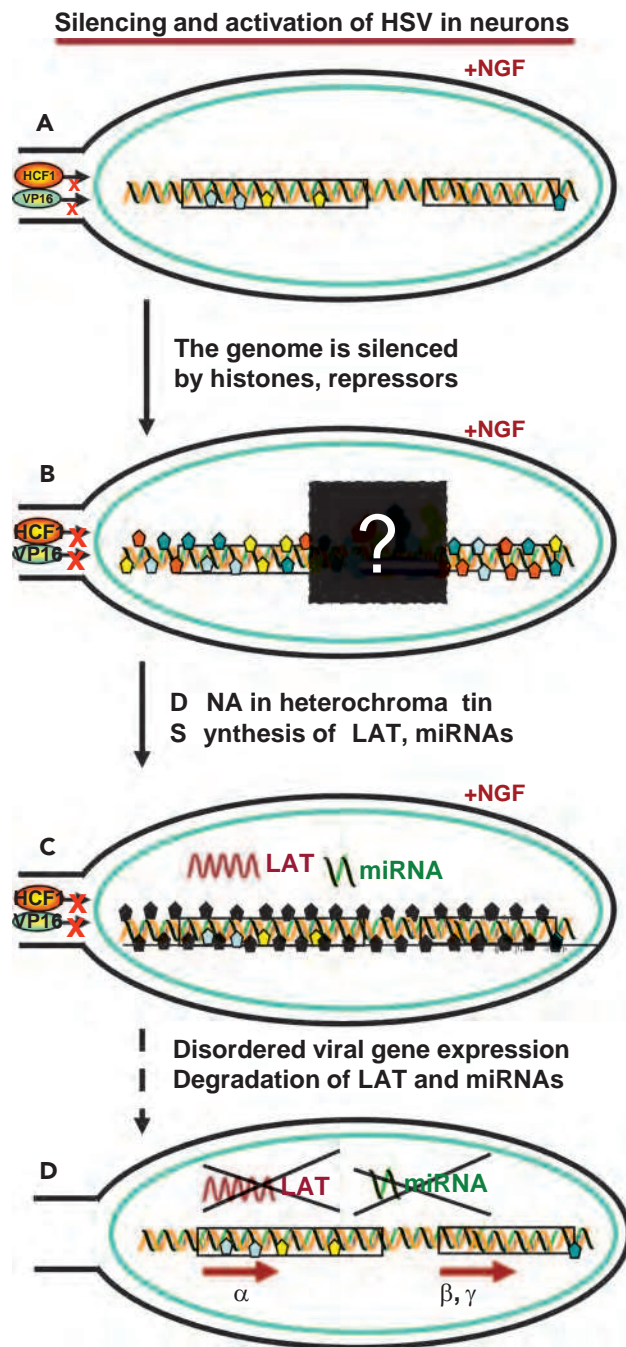


FIGURE 60.16. Molecular events during establishment of and reactivation from latent infection. **A:** Viral DNA is bound by host proteins upon entry into the neuronal nucleus. **B:** Viral DNA is silenced by histone and repressor association. The question mark indicates that we know little about the precise mechanisms of this process. **C:** Latency-associated transcripts (LATs) and possibly microRNAs play roles in maintenance of viral chromatin silencing. **D:** Removal of nerve growth factor or explant leads to disordered viral gene expression and turnover of LAT and microRNAs.

gene expression is likely explained by several mechanisms acting in combination (see Fig. 60.16):

1. *Neuronal repressors.* The host Oct-2 factor was postulated to repress α gene expression through its interaction with

TAATGARATT sites blocking Oct-1 activation.^{540,651} However, other investigators could detect no Oct-2 expression in sensory neurons, and even when Oct-2 was overexpressed in cells, it did not inhibit expression from a complete α -promoter/enhancer-bearing reporter gene,⁴¹¹ reducing the potential for α gene repression by Oct-2. The neuron-specific Zhangfei factor has also been postulated to block activation of α gene promoters through interaction at Oct-1 binding sites.¹³ Similarly, expression of a dominant-negative form of the host repressor REST reduced viral replication in neurons,²⁹⁵ indicating that REST is part of the mechanism of silencing of the viral genome in neurons.

2. *Hormonally regulated repression of viral gene expression.* Nerve growth factor (NGF) treatment of sensory neurons has been shown to restrict viral lytic gene expression,¹²⁵⁹ and anti-NGF treatment of latently infected neurons increases viral lytic gene expression.²⁹⁵
3. *Lack of viral and cellular factors necessary to overcome host silencing and allow α gene transcription.* Several studies have raised the idea that sensory neurons lack essential factors for expression of HSV α genes, thereby explaining the latent infection in these cells. In studies using *in situ* hybridization to detect Oct-1 mRNA, it was found that Oct-1 is not expressed in cells of the sensory ganglia.^{435,1178} More sensitive assays showed Oct-1 and GABP α/β are present but at low levels in sensory neurons.⁴¹¹ Kristie et al.⁵⁹⁶ showed that HCF, or C1, the cellular co-factor required for enhancement of α gene expression along with Oct-1 and VP16, resides in the cytoplasm in trigeminal ganglion neurons and is translocated into the nucleus only under conditions of reactivation such as explantation of ganglia in culture; thus, HCF may not be capable of localizing to the nucleus when HSV infects and cannot transactivate α gene transcription. In addition, HCF has been reported to transport VP16 into the cell nucleus.⁶⁰⁷ Therefore, in sensory neurons, even if the VP16 in the incoming virion were translocated to the neuronal cell body, it would not be localized into the neuronal nucleus, and it could not participate in transactivation of α genes. Further, it has been proposed that VP16 from input virus particles is not translocated to the neuronal nucleus in the cell body to enter the nucleus or cannot function in some way to activate α gene expression in neurons.¹⁰²⁶ In support of this, the limited α gene expression in neuronal cells is reported to be independent of VP16.⁹⁰¹
4. *Viral RNA-mediated repression of α gene expression.* LAT-negative mutant viruses show elevated lytic gene expression in trigeminal ganglion neurons during acute infection at 3 to 5 days postinfection.³⁶⁰ Therefore, LAT or one of its products seems to exert some of its repressive effects at early times following infection of neurons.

It is informative to look at the kinetics of LAT and microRNA expression and accumulation. Expression of LAT is detected by 26 hpi in murine trigeminal ganglia⁵⁸⁶ and continues to increase for at least 3⁵⁸⁶ to 9 days postinfection.²⁹⁵ Similar levels and kinetics of LAT expression are observed for a TK-negative mutant virus, which does not replicate in TG neurons, indicating that LAT expression at these times largely occurs from input viral genomes.⁵⁸⁶ In one study, microRNAs were detected at 3 dpi in murine trigeminal ganglia at levels of 30 molecules/cell in one study and increased significantly

by 30 (dpi).⁵⁸⁹ Levels of LAT were much higher, approximately 1,000 molecules/cell, at 3 days post infection (dpi).⁵⁸⁹ In another study, microRNAs were detected by 1 dpi and continue to increase until approximately 9 dpi.²⁹⁵ In that study, levels of LAT and microRNAs were similar. Therefore, LATs and microRNAs could be expressed early enough to add to the repressive effects of pre-existing host repressors.

CHROMATIN SILENCING OF VIRAL LYTIC GENES

Chromatin assembly with incoming viral DNA is very rapid in cells in culture.²⁰⁷ In trigeminal ganglia, histone H3 association with HSV DNA is apparent by 7 dpi, and the H3K9me2 modification is apparent by 15 dpi.¹²¹⁴ Further studies have shown that whereas the viral lytic gene promoters are associated with H3 histone by 7 dpi, the H3K27me3 heterochromatin modification was not detected on HSV lytic gene chromatin until later, 14 dpi,²⁰⁵ arguing that the earliest chromatin effect is association of unmodified chromatin followed by the H3K27me3 modification. In this infection model, acute viral replication in the trigeminal ganglia has largely declined by 7 dpi⁶³¹; therefore, H3K27 methylation appears to occur after resolution of the infection. This would argue that events earlier than heterochromatin modifications of histones are responsible for the initial silencing of the viral genome.

Genetic analysis of viral mutant virus strains has attempted to define the role of viral gene products in establishment of latent infection. HSV must have access to the nerve endings in order to establish latency; therefore, it could be expected that the greater the number of peripheral cells that become infected and support virus multiplication, the larger the number of neurons that will harbor latent virus. Indeed, viruses that are replication defective show reduced levels of latent infection, although they can establish and maintain latent infections but not reactivate.^{211,535,1027} In essence, no deletion mutant totally failed to establish latent infection.

Studies on the neuronal subtype in which latent infection is established in murine model systems have shed light on the mechanisms of establishment of latent infection. In mice infected by the ocular route with HSV-1 strain KOS virus, latent infection is preferentially established in a subset of neurons identified with the A5 antibody recognizing a specific lactoseries glycoconjugate.¹²⁹² Further studies showed that HSV-2 preferentially established latent infection in KH10 MAB-reactive sensory neurons in either trigeminal or dorsal root ganglia.⁷⁰⁸ A recombinant HSV-2 virus that expresses the HSV-1 LAT showed an HSV-1 phenotype (i.e., preferential establishment of latency in A5-positive neurons),⁷⁰⁸ and a recombinant HSV-1 virus expressing the HSV-2 LAT showed an HSV-2 phenotype (i.e., preferential establishment of latency in KH10-positive neurons).⁴⁸⁵ These studies showed that LAT influenced establishment of latent infection in specific neuronal subtypes. Furthermore, studies of infection of murine adult sensory ganglion neurons *in vitro* have shown that A5-positive sensory neurons are nonpermissive for HSV-1 strains KOS and 17 *in vitro* but are permissive for HSV-2.⁸⁸ Although the LAT recombinant viruses were not examined in these studies, these studies, in total, raise the general model of LAT controlling productive infection in specific neuronal subtypes. Therefore, these studies support the idea that both viral and host factors contribute to establishment of latent infection.

The question of whether viral infection leads directly to latent infection versus lytic infection and/or whether lytic infection can convert to latent infection has been debated extensively. Studies using viruses that express CRE recombinase from viral promoters found that approximately one-third of the latently infected neurons have experienced immediate-early promoter activation prior to establishment of latent infection but have not experienced TK or VP16 promoter activation.^{900,901} This argues that at least α gene expression can precede latent infection and raises that possibility that these gene products could affect the host responses or viral chromatin and affect the type of latent infection that ensues.

Maintenance Phase of Latency

As described previously, extensive studies on ganglia harboring latent HSV have been rewarded by an extreme paucity of evidence for viral gene expression, except for LATs and microRNAs. Nevertheless, low-level expression of α ICP4 and β TK gene transcripts have been observed in murine trigeminal ganglia.⁵⁸⁷ ICP4 protein has been observed in rabbit trigeminal ganglia³⁹²; however, owing to the significant rate of spontaneous reactivation in rabbit TGs, the possibility that this was attributable to reactivation cannot be eliminated. Kramer and Coen⁵⁸⁷ hypothesized that the low level of lytic transcripts was likely owing to a low level of lytic gene breakthrough during latent infection, whereas others have found rare neurons in murine ganglia that express high levels of lytic transcripts, and they have hypothesized that the low levels of lytic transcripts observed in latently infected ganglia are attributable to a few cells undergoing reactivation rather than numerous cells showing low-level expression.³³⁰ Further studies looking at HSV transcript levels in individual neurons¹⁰⁰⁰ should determine the fraction of neurons that show expression of lytic transcripts.

There is prolonged expression of cytokines and chemokines^{151,185,225,413,1038} and CD8⁺ T-cell infiltration^{543,662} in the latently infected murine ganglia and prolonged expression of cytokines and chemokines in human latently infected ganglia.¹¹³⁸ This indicates that there is continued antigenic stimulation of dendritic cells (DCs) and macrophages and continued T-cell activation in the ganglionic tissue. As described elsewhere in this chapter, the ongoing immune surveillance is likely in response to the presence of viral antigens in the ganglion owing to low-level reactivation.

Heterochromatin likely plays an important role in maintaining the HSV lytic genes in a silenced state during latent infection,^{206,560,1214} whereas the LAT promoter and enhancer have euchromatin associated with them.⁵⁹⁷ How are these separate chromatin domains maintained? Amelio et al¹⁸ have identified candidate insulator elements containing CCTC factor (CTCF) binding sites at the U_L/c boundary (bp 120,503–120,635), and in the LAT intron (bp 117,158–117,342), and they propose that these insulators keep the LAT enhancer activity within that boundary and heterochromatin outside of those boundaries.

The latent HSV infection, in addition to inducing the recruitment of immune cells into the ganglia, leads to perturbation of neuronal host cell gene expression. Using microarray studies, Kramer et al⁵⁸⁸ showed that numerous cellular genes were up-regulated or down-regulated in latently infected murine trigeminal ganglia. Little is known about the effects of these gene expression changes on the host neuron.

COPY NUMBER OF VIRAL DNA IN LATENTLY INFECTED NEURONS

Early studies determined that there are between 0.1 and 1 viral genome equivalents per cell genome during latent infection.^{142,943,944} In calculations based on neurons accounting for only about 10% of the total cells in a sensory ganglion, it was obvious that each latently infected neuron must contain more than one viral genome.⁹⁵⁸ This was shown more directly in studies showing that approximately 20% of single purified murine trigeminal ganglion neurons contain viral DNA with an average of 20 to 30 HSV DNA molecules per cell and a range from less than 10 to greater than 1,000 molecules per cell.^{1000,1145} In studies of human neurons obtained by laser capture microdissection of trigeminal tissue, 2% to 10% of the neurons contain viral DNA with a median value of 11 genomes per cell and a range of 5 to 3,955 copies per cell.¹²¹³ The range of DNA copy numbers raises the issue of heterogeneity of latent infection in different cells. Furthermore, only 0.2% to 1.5% of the human TG neurons express LAT, as detected by *in situ* hybridization.¹²¹³ Thus, there are many more neurons containing DNA than expressing LAT, at least as detected by *in situ* hybridization. This extends the issue of heterogeneity of latent infection by demonstrating that some latently infected neurons have all viral genes but LAT silenced, whereas other neurons have the entire viral genome silenced. This raises another important issue: What contributes to the high levels of viral DNA molecules per neuron?

In terms of the events and mechanisms leading to the high copy number of viral DNA in latently infected cells, this could be owing to multiple viruses infecting each neuron. Alternatively, the viral DNA could be replicated by viral or cellular mechanisms. Studies using HSV mutant strains that do not replicate viral DNA in neurons, such as TK-negative viruses, do show lower levels of viral DNA per cell,^{1058,1145} supporting the idea that viral DNA replication contributes to the levels of DNA in the latently infected neuron. Nevertheless, even the ganglia infected with TK-negative mutant viruses contain multiple genomes per cell, so there may be some leaky viral DNA replication in these cells or there is still the potential for infection by multiple viruses or that nonviral replication mechanisms contribute to the levels of viral DNA.⁹⁵⁸

Reactivation of Virus From the Latent State

As described earlier, there are several experimental systems for studying *in vitro* and *in vivo* reactivation. Upon induction of reactivation by *in vitro* explant, LAT and microRNA transcript levels decrease and lytic gene transcripts increase,^{295,584,1083} and the histones associated with the *LAT* gene become deacetylated.¹⁷ Similarly, lytic genes such as the *ICP0* gene become associated with acetylated histones,¹⁷ and lytic gene transcripts accumulate.^{273,584} The assumption has been that viral gene expression during reactivation follows the α - β - γ paradigm, and some evidence has supported this.^{273,584} However, Tal-Singer et al¹¹²⁴ reported that they could detect β gene expression prior to α gene expression during explant reactivation. In this study, they used RT-PCR to detect viral transcripts but did not show that the assays for each of the transcripts was equally sensitive; thus, it is conceivable that the differences in kinetics of appearance reflected sensitivity of the detection assay rather than the true order of appearance. Some *in vivo*^{583,584} and cell culture⁸⁰⁶ studies have suggested that optimal viral gene expression in neurons depends on viral DNA replication, although

viral spread was not ruled out in the *in vivo* and *ex vivo* studies. However, as first suggested by Sawtell and Thompson¹⁰⁰¹ and then shown by Pesola et al⁸⁷¹ and Sawtell et al,¹⁰⁰³ when viral spread is prevented, there is no apparent role for viral DNA synthesis in stimulation of α and β gene expression in the explant situation. Nevertheless, one study of infection of sensory neurons in culture does support the idea that viral gene expression in neurons is affected by viral DNA synthesis, in that Nichol et al⁸⁰⁶ observed that in HSV-infected sensory neurons in culture, viral DNA synthesis stimulates α and β gene expression.

The events leading to viral gene expression during reactivation continues to be debated. Thompson and Sawtell¹¹⁴² have defined the exit from latency as the expression of viral lytic proteins as detected by immunohistochemistry using anti-HSV antiserum, and they defined reactivation as the production of infectious virus. In the transient hyperthermia reactivation model, they observe an average of one cell per ganglion in which virus has escaped latency and approximately 70% of the ganglia reactivate with an average yield of 10 pfu of virus per ganglion.¹¹⁴² An ICP0 mutant showed similar escape from latency but no reactivation. Thompson and Sawtell¹¹⁴² concluded that ICP0 does not initiate reactivation from latency *in vivo*; however, the process of exit of latency in this system is of low efficiency and could have minimized the differences between wild-type and mutant viruses.

A second study in this *in vivo* reactivation system tested the ability of a VP16 mutant virus—*in1814*—to reactivate and found that the *in1814* virus reactivated in *in vitro* explant but not in the *in vivo* reactivation system.¹¹⁴¹ They concluded that VP16 is needed for *in vivo* exit from latency but not in *in vitro* explant. They argued that VP16 is expressed prior to the immediate-early proteins; however, VP16 promoter activity was measured by β -galactosidase staining, whereas viral proteins were measured by immunohistochemistry, and β -gal staining may be more sensitive. This interesting hypothesis merits further investigation.

A recent study found that in explanted ganglia incubated with anti-NGF antibody with or without cycloheximide, viral genes of α , β , and γ kinetic classes, including VP16, are expressed simultaneously and not sequentially ordered.²⁹⁵ The RT real-time PCR assays used in this study provide more sensitive assays than protein detection, and this may explain the differences between this study and the two previous ones. Similar results were obtained in an *in vitro* neuronal latent infection system that showed an initial widespread derepression of viral genes prior to VP16-dependent transcription.⁵⁴⁷ These results argue that reactivation from a state of heterochromatin over the entire genome occurs by cellular mechanisms affecting the entire genome uniformly and emphasize the differences in the mechanisms of reactivation versus initial infection of neurons and other cell types.

In humans, latent virus is reactivated after local stimuli such as injury to tissues innervated by neurons harboring latent virus, or by systemic stimuli such as physical or emotional stress, hyperthermia, exposure to UV light, menstruation, and hormonal imbalance, which may reactivate virus simultaneously in neurons of various ganglia (e.g., trigeminal and sacral). A modest amount of evidence and the most plausible common denominator is that injury or stimulation of cells innervated by dorsal root neurons harboring latent virus is a common trigger of recrudescence of lesions caused by reactivated virus.

The important question of the nature of the transduced signal remains to be answered.

The fate of the neurons after viral reactivation is a hotly debated topic. Proponents of the notion that neurons survive to reactivate again and again base their arguments on two observations. The first is that local anesthesia is not a sequelae in patients suffering from frequent recurrences at the same site. A formal rebuttal of this argument is that nerve endings from adjacent unaffected neurons grow into the healed area and re-establish a network.

The second and more significant observation is that women with recurrent genital lesions shed virus in the interim between clinically manifest lesions. The shed virus appears to originate from punctate, microscopic lesions that arise frequently and for long intervals. If each microscopic lesion arises from a reactivated neuron, there would not be enough neurons to support viral reactivation over many years if the neurons perish as a consequence of this process. The question that has not been resolved is whether each lesion is the consequence of reactivation and whether chronic infections of mucosal tissues characterized by the observed microscopic lesions can be ruled out.

ASSEMBLY OF PROGENY VIRUS IN NEURONS

As described earlier, reactivation leads to expression of sufficient viral proteins and viral DNA replication to produce some progeny virions. These are ultimately released at the axonal termini to initiate the recurrent infection at the site of innervation of the nerve. The mechanisms of assembly of HSV progeny virions in neurons has been controversial, as there has been evidence of intact viral particles undergoing anterograde transport as well as evidence of independent transport of capsids and vesicles containing the viral glycoproteins.^{26,1268} New components of the virion move by anterograde transport to both peripheral and central branches of the neuron.⁶²³ Virion assembly and/or release have been reported along the axon shaft and at the axon tip.^{826,984,1153} The question is how virions or virion components are transported by anterograde transport to these sites of virion release. Early studies of HSV infection of human fetal neurons followed by electron microscopy, immuno-electron microscopy, and immunofluorescence analyses concluded that capsids are transported independently from vesicles containing viral glycoproteins.^{457,861} Immunofluorescence and live cell imaging of human neuroblastoma cells infected with HSV also showed separate sites of staining or localization for glycoproteins relative to capsid proteins.^{1065–1067} A further study using high-resolution electron microscopy has shown that 75% of the capsids in the axons of rat neurons were in virion particles.⁷⁹⁵ Recent studies of “two-color” HSV recombinants expressing fluorescent capsid proteins and a fluorescent glycoprotein have continued to find different results, in that one study found that 64% to 70% of the capsids were associated with glycoproteins while undergoing anterograde transport,²⁶ although the other found that 80% and 67% of the capsids in two neuronal systems were independent of glycoproteins.¹²⁶⁸ Thus, there is the potential that either of two mechanisms of transport could occur to different extents in different neuronal cells under different conditions. Genetic studies have provided some insight in this area. One study has shown a defect in transport of envelope proteins, VP22 tegument, and VP5 capsid proteins into the optic nerve in mice infected with a glycoprotein E-negative virus,¹²¹²

implying that axonal localization of certain glycoproteins and capsid and teguments may be linked.

ROLE OF THE HOST IMMUNE RESPONSE IN LATENCY

The role of the host immune response in controlling acute lytic infection is clear; however, its role in latent infection is less clear. It has been reported that viral CD8⁺ T cells and/or IFN- γ can block viral gene expression and replication, leading to reactivation in explanted ganglia.^{259,661,662} There were sufficient HSV-specific T cells in acutely infected ganglia to control reactivation in explanted ganglia but not in latently infected ganglia.⁶⁶² Despite the latter results, the prolonged CD8⁺ T-cell infiltrate in latently infected ganglia has led to the proposal that CD8⁺ T cells “control” latency.⁵⁴⁴ Although some studies have attempted to correlate T-cell infiltrates with LAT-positive neurons,^{1138,1189} a recent study found that most LAT-positive neurons do not have CD3⁺ T cells around them. This study concluded that “the majority of LAT⁺ neurons is not directly controlled by T cells”.⁴⁴⁰ Therefore, we favor the idea that latent infection is maintained by neuronal and viral factors such as LAT and microRNAs, and CD8⁺ T cells are activated and retained in the ganglia in response to low-level expression of viral antigens. Supporting the idea that immune responses do not control latency, Gesser et al³⁷⁰ showed that HSV can establish latent infection in immunodeficient animals. Furthermore, van Lint et al¹¹⁸² observed that CD8⁺ T cells nonspecifically traffic into latently infected ganglia, although only the HSV-specific CD8⁺ T cells are activated and specifically retained in the ganglia. It seems likely that the host immune mechanisms respond to and control lytic infection and that latent infection of neurons involves intrinsic nonpermissive properties of sensory neurons and viral functions.

PATHOGENESIS OF HUMAN INFECTION

Transmission of HSV depends on intimate, personal contact between a seronegative (susceptible) individual and someone excreting HSV. Virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. Following oropharyngeal infection, usually caused by HSV-1, the trigeminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. Virus replicates in the genital, perigenital, or anal skin sites with seeding of the sacral ganglia.

Accumulated clinical experience suggests that after primary infection, replication of virus at the portal of entry, usually oral or genital mucosa, results in infection of sensory nerve endings; virus is transported to dorsal root ganglia.^{65,70,1097} The more severe the primary infection, as reflected by the size, number, and extent of lesions, the more likely it is that recurrences will ensue. This notion is attributed to the large quantity of virus present when primary disease is extensive as compared with asymptomatic infections.

Fundamental to disease pathogenesis is the propensity of virus to replicate at mucosal surfaces, to be transported to dorsal root ganglia, and to become latent. Although replication can sometimes lead to disease and can infrequently result in life-threatening CNS infection, the host–virus interaction leading to latency predominates. With reactivation, virus is detected at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers, or can simply be excreted in the absence of symptoms.

Primary infection can spread beyond the dorsal root ganglia, thereby becoming systemic. For example, studies of children with primary gingivostomatitis demonstrated HSV DNA in the blood by PCR in about one-third of patients.⁴⁷² Similarly, 24% of adults with primary genital herpes had viral DNA detected by PCR in the blood.⁵¹⁶ Multiorgan disease is encountered in disseminated neonatal HSV infection, visceral organ disease of pregnancy, and, rarely, in immunosuppressed patients.

For individuals susceptible to HSV infections (namely, those without pre-existing antibodies), the first exposure to either HSV-1 or HSV-2 results in primary infection. The epidemiology and clinical characteristics of primary infection are distinctly different from those associated with recurrent infection. Reactivation of HSV is known as recurrent infection and clinically results in a limited number of vesicular lesions, as occurs with HSV labialis or recurrent HSV genitalis. An individual with pre-existing antibodies to one type of HSV (i.e., HSV-1 or HSV-2) can experience a first infection with the opposite virus type (i.e., HSV-2 or HSV-1, respectively) at a different site. Under such circumstances, the infection is known as an initial infection rather than as a primary infection. For example, an initial infection occurs in those individuals who have pre-existing HSV-1 antibodies who then acquire a genital HSV-2 infection. Because many individuals are infected with HSV and do not experience a primary infection, the first recurrence has been identified as first episode infection. Reinfection with a different strain of HSV occurs, although it is uncommon in the normal host.

UNIQUE BIOLOGICAL PROPERTIES OF HERPES SIMPLEX VIRUS

HSV has two unique biological properties: neurovirulence and latency. The propensity of HSV to invade and replicate in the CNS can result in profound disease with severe neurologic devastation, as occurs with HSV encephalitis. Alternatively, virus simply establishes latency, providing a reservoir for virus transmission to susceptible persons if reactivated.

Neurovirulence

Neurovirulence encompasses both neuroinvasiveness from peripheral sites and the ability of HSV to replicate in neuronal cells. Whereas this property may be the function of numerous genes, the deletion of one gene in particular, $\gamma_134.5$, removes the ability of HSV to both invade and replicate in the CNS, as defined earlier.

Predictably, mutations affecting neuroinvasiveness have also been mapped in genes encoding glycoproteins. Access to neuronal cells from usual portals of entry into the body requires postsynaptic transmission of virus and, therefore, a particularly vigorous capacity to multiply, being dependent on glycoprotein expression. In addition, because neuronal cells do not make cellular DNA, they lack the precursors for viral DNA synthesis that are also encoded by those viral genes that are dispensable for replication in cell culture.

Latent Infection in Humans

Latent virus has been retrieved from the trigeminal, thoracic, sacral, and vagal ganglia of humans.^{65,70,1221} As but one example, virus isolation was attempted from the trigeminal ganglia of 90

cadavers and was documented in 44 individual situations. In 26 of these cases, HSV was isolated bilaterally from both ganglia. Similarly, when 68 sacral ganglia were co-cultivated, only 9 were positive and all for HSV-2.

Patients treated for trigeminal neuralgia by sectioning a branch of the trigeminal nerve can develop herpetic lesions along the innervated areas of the sectioned branch.^{162,243,387,855,856} Microvascular surgery of the trigeminal nerve, performed to alleviate the pain associated with tic douloureux, resulted in recurrent herpetic lesions in more than 90% of seropositive individuals.

Reactivation of latent virus depends on an intact anterior nerve route and peripheral nerve pathways. Induction of viral replication by axonal injury¹²¹¹ follows sectioning of a peripheral nerve and results in the appearance of virus within the ganglia 3 to 5 days after the surgical manipulation. Not surprisingly, if an attempt is made to excise the lesions induced by HSV, vesicles will recur adjacent to the site of excision. Recurrences occur in the presence of both cell-mediated and humoral immune responses. Recurrent herpes labialis is three times more frequent in febrile patients than in nonfebrile controls.

PATHOLOGY

The pathologic changes induced by HSV replication are similar for both primary and recurrent infection but vary in the extent. The histopathologic characteristics of HSV skin lesions are shown in Figure 60.17,⁹⁵⁶ representing a combination of virus-mediated cell death and associated inflammation. Viral infection induces ballooning of cells and the appearance of condensed chromatin within the nuclei of these cells, followed by subsequent degeneration of the cellular nuclei, generally within parabasal and intermediate cells of the epithelium. Cells lose intact plasma membranes and form multinucleated giant cells. With cell lysis, clear (referred to as vesicular) fluid containing virus appears between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells, and, often, multinucleated giant cells. In dermal substructures, there is an intense inflammatory response, usually in the corium of the skin. With healing, the vesicular fluid becomes pustular with the recruitment of inflammatory cells and then it scabs. Scarring is uncommon but has been noted in some

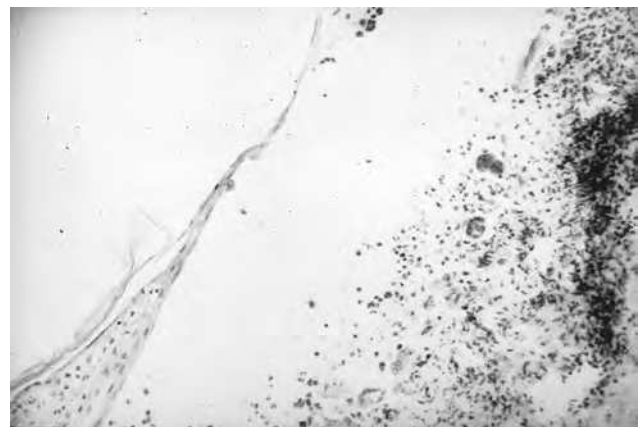


FIGURE 60.17. Histopathology of herpes simplex virus infection.

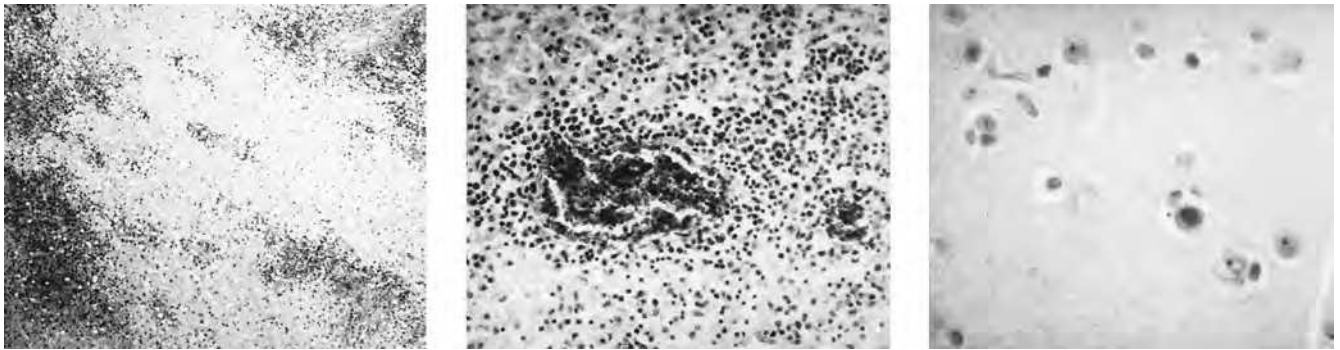


FIGURE 60.18. Perivascular cuffing with areas of hemorrhagic necrosis of brain.

patients with frequent recurrences. When mucous membranes are involved, vesicles are less likely to be prominent. Instead, shallow ulcers are more common because the vesicles rapidly rupture as a result of the very thin cornified epithelium.

Vascular changes in infected tissue include perivascular cuffing and areas of hemorrhagic necrosis⁹⁵⁶ (Fig. 60.18). These histopathologic findings become particularly prominent when organs other than skin are involved, such as HSV encephalitis or disseminated neonatal HSV infection. In these cases, widespread areas of hemorrhagic necrosis, mirroring the area of infection, are prominent. When the brain is involved, oligodendrocytic involvement and gliosis are common, as is astrocytosis; however, these changes develop late in the disease course. Local lymphatics can show evidence of infection with intrusion of inflammatory cells, draining infected secretions from the area of viral replication. The intensity of the inflammatory response is significantly less with recurrent disease. As host defenses develop, an influx of mononuclear cells is detected in infected tissue.

MODELS OF DISEASE PATHOGENESIS

Animal models allow study of the pathogenesis of infection outside the human; however, herein lies a series of fundamental problems. The animal species (indicative of susceptibility), virus species, route of infection, state of immune competence, and specific viral strain all influence disease pathogenesis. Both HSV-1 encephalitis (virulence) and latency models have been established following inoculation by the eye route. However, the endpoints of virulence and latency do not routinely correlate with human disease. After ocular inoculation by corneal scarification, replication of virus in the eye peaks within 48 hours and declines over the next 6 days. Virus appears in the trigeminal ganglia approximately 1 day after inoculation, with peak replication occurring 4 to 6 days later. If sufficient quantities of virus and/or a virulent strain of virus are inoculated onto the eye, encephalitis follows virus replication within the trigeminal ganglia.⁵⁶³

For HSV-1 cutaneous infections, mice (particularly the hairless mouse)¹¹⁷¹ and rabbits or guinea pigs with abraded or punctured skin are used to study replication and disease pathogenesis as well as to evaluate antiviral therapies. The hairless mouse, guinea pig, and rabbit are all artificial models of human cutaneous infections, particularly for recurrent infections of humans.

HSV-2 mucosal infections have been studied most extensively in an intravaginal guinea pig model. Both HSV-1 and

HSV-2 have been used in this model. These animals tend to experience continual recurrences of lesions; however, retrieval of HSV from these lesions is variable. The guinea pig genital infection model has been used extensively in the assessment of vaccines and antiviral drugs, although infection of cotton rats with HSV-2 has also been shown to cause recurrent genital disease, albeit less well characterized.¹³⁰⁰

Models of life-threatening disease include the intranasal inoculation of HSV-1 or HSV-2 in young (3-week-old) Balb/C mice, resulting in CNS and visceral (usually lung) disease (mimicking neonatal HSV infection). However, inoculation of older mice with similar quantities of either virus may fail to cause disease, or, if it does lead to disease, it might be encephalitis but not multiorgan disease. Intracerebral inoculation of virus provides an unnatural route of infection even for the study of antiviral therapeutics; however, it is used to screen new drugs.

Accurate and predictive models of human HSV encephalitis have been described using rabbit^{1009,1010} and mouse models. The resultant disease leads to focal localization of infection in the rabbit brain, as compared with diffuse pancortical infection of the mouse brain. Virus is inoculated directly into the olfactory bulb or upon abraded nasal epithelium of the rabbit over the nerves from the olfactory bulb.¹¹⁰⁶

Human models of both labial and genital herpes have been reported. Induced reactivation of HSV labialis occurs by exposure of the vermillion border of the lip of seropositive individuals to UV light.⁹⁶⁶ The resulting lesions and course of infection are similar to those that occur naturally. Lesions develop in approximately 70% of UV-exposed volunteers after approximately 3 days. This model has been exploited for the evaluation of antiviral therapies.^{966,967} Similarly, UV exposure of the buttocks and thigh in HSV-2-infected individuals results in reactivation in approximately 35% and a longer incubation period of about 5 days.⁹⁶⁷

EPIDEMIOLOGY

Orolabial Herpes Simplex Virus Infections: Primary and Recurrent

HSV is distributed worldwide and have been reported in both developed and underdeveloped countries, including remote Brazilian tribes.^{96,676} Humans are the sole reservoir for transmission of HSV to other humans. Virus is transmitted from

infected to susceptible individuals during close personal contact. There is no seasonal variation in the incidence of infection. Because infection is both rarely fatal and these viruses become latent, over half of the world's population has been infected by HSV and, therefore, is capable of transmitting HSV during episodes of productive infection.

Geographic location, socioeconomic status, and age are the primary factors that influence the acquisition of HSV infection, regardless of the mode of assessment.^{281,1025} In developing countries and lower socioeconomic populations, infection occurs early in life. By 5 years of age, approximately one-third of children seroconverted, increasing to 70% to 80% by early adolescence.^{97,784} Predictably, middle-class individuals of industrialized societies acquire antibodies at a later age in life. Seroconversion occurred over the first 5 years of life in 20% of children, followed by no significant increase until the second and third decades of life, at which time the prevalence of antibodies increased to 40% and 60%, respectively.^{380,726,999,1063,1232} Seroconversion of susceptible university students occurs at an annual frequency of approximately 5% to 10%.^{373,380}

Examples of early lessons on the epidemiology and pathogenesis of infection are in order. Following the association of HSV and gingivostomatitis,^{141,281} virological screening was used to study the epidemiology and natural history of infection. Primary infection led to the shedding of virus in mouth and stool and from the former site for as long as 23 days (average, 7 to 10 days).^{21,281} Neutralizing antibodies appeared between 4 and 7 days after the onset of disease and peaked in approximately 3 weeks. Virus was isolated from the saliva of asymptomatic children, occurring in approximately 20% of the children in the age range of 7 months to 2 years.¹³⁶ Despite no overall race or sex differences, young African American children had higher rates of both neutralizing antibodies and virus isolation than did their Caucasian counterparts. Virus shedding in children younger than 6 months was uncommon, as has been noted in more recent studies.⁵²² In older children, 3 to 14 years of age, asymptomatic shedding was documented in 18%. Retrieval of infectious virus decreased with advancing age, being only 2.7% in individuals more than 15 years of age. These frequencies of shedding are similar to contemporary cross-sectional surveys, ranging from 2% to 5%.⁹⁵⁶ Application of more contemporary technology, namely PCR, demonstrates higher frequencies of shedding and for intermittent periods.⁷⁰⁹

The influence of age is underscored by other studies. Evidence of oral herpetic infection was found in approximately 13% of children over a 10-year period. Children 1 to 2 years of age were most commonly infected, accounting for more than half of all cases. No differences in sex or seasonal variation were detected.^{260,956} Most children experienced asymptomatic infection, accounting for more than 65% of all cases.^{23,172}

With the development and licensure of species-specific serologic assays, the seroprevalence of HSV-1 and HSV-2 infections was redefined using sera obtained from two randomized National Health and Nutrition Examination Surveys (NHANES).^{335,787} The seroprevalence of HSV-1 is more common than HSV-2. By 5 years of age, more than 35% of African American children versus 18% of Caucasian children are infected with HSV-1. No significant differences in seroprevalence exist between the three studies: one performed on sera collected in 1978 and the other between 1988 and 1994. Subsequently, through adolescence, African Americans had approximately a twofold higher prevalence of antibodies to

HSV-1 than did Caucasians, and females had a slightly higher prevalence than did males. Furthermore, the prevalence of HSV-1 was slightly higher for females than males. By 40 years of age, both African Americans and Caucasians had similarly high prevalence of antibodies, in excess of 60%.^{1279,1280}

When similar analyses were performed on populations from various countries around the world, notable differences have been detected. For example, only 5 of 18 countries surveyed had an HSV-1 antibody prevalence of less than 70%. Antibody prevalence in excess of 95% was detected for adults 20 to 40 years of age in Spain, Italy, Rwanda, Zaire, Senegal, China, Taiwan, Haiti, Jamaica, Uganda, and Costa Rica. Thus, a high prevalence of antibodies to HSV-1 exists worldwide; however, there is significant country-to-country variation.^{484,676}

In summary, these studies demonstrate a significantly lower prevalence of antibodies during childhood, adolescence, and even later in life among relatively middle and upper socioeconomic classes. Primary infection occurs much earlier in life, generally very early, in children of the developing world as well as in those of lower socioeconomic classes, whereas in developed countries and more affluent classes, primary infection may be delayed until adolescence or perhaps even adulthood. The frequency of direct person-to-person contact, indicative of crowding encountered with lower socioeconomic status, appears to be the major mediator of infection.

The largest human reservoir of HSV-1 is latent infection in the trigeminal ganglia, which reactivates leading to recurrent herpes labialis. Studies performed to assess the frequency and severity of recurrent infection in the immunocompetent host are limited. A positive history of recurrent herpes labialis was noted in 38% of 1,800 graduate students.^{1041,1042} New lesions occurred at a frequency of one per month in 5% of the afflicted students and at intervals of 2 to 11 months in 34% of the afflicted students. Recurrences of one per year or less often were found in 61%. The predictable frequency of recurrence is relatively constant—approximately 33% in several studies.^{352,1039,1040} Lower frequencies of recurrences have been reported (16%); however, under vastly different socioeconomic conditions (upper vs. lower socioeconomic conditions) than previous studies.^{290,1303} Ironically, one other study found that recurrences occur more frequently among the more socially privileged,¹⁰⁴⁰ suggesting more attention to relatively trivial medical illnesses.

Recurrent infection occurs in the absence of clinical symptoms. Asymptomatic excretion of HSV in healthy children is about 1% to 5%.^{380,1238} Nearly 1% of pregnant women and nursery personnel excrete HSV at any time, providing a source of virus for transmission to the newborn. Asymptomatic excretion of virus is not limited to the healthy adult, occurring in nearly one-third of seropositive transplant recipients.^{172,851,852}

Unusual Epidemiologic Infections

Clustered outbreaks of human HSV infections have been reported; however, there is no indication from either clinical or molecular epidemiologic studies that HSV causes epidemics. Most studies involved families in which several individuals had HSV infection at approximately the same time. No index case could be identified, although recurrent labial lesions of one family member were frequently incriminated.

Outbreaks within hospitals have been identified, yet here also, no clear epidemic nature of the disease could be determined.^{412,903} Herpetic stomatitis outbreaks have been

reported within orphanages,^{412,522} where the attack rate for apparent primary infections was approximately 75% of the susceptible patients. Similarly, an outbreak of HSV encephalitis was recognized in Boston; however, no common virus was identified.⁴¹⁶ Interestingly, increased transmission of HSV in daycare centers has not yet been reported to be problematic.¹⁰¹²

Genital Herpes Simplex Virus Infections: Primary and Recurrent

Genital herpes can be caused by either HSV-1 or HSV-2. Because infections with HSV-2 are usually acquired through sexual contact, antibodies to this virus are rarely found before the onset of sexual activity.¹²⁷⁸ Importantly, an ever-increasing proportion is attributable to HSV-1.^{531,1273} Indeed, more than 50% of primary episodes of genital herpes at some sexually transmitted disease (STD) clinics are caused by HSV-1.¹²⁷⁸ The distinction in virus species is not insignificant; genital HSV-1 infections are usually both less severe clinically and less prone to recur.^{231,928} Sexual transmission of both viruses is the consequence of intimate contact, namely oral–genital or genital–genital.^{552,1238}

The most accurate assessment of the prevalence of HSV-2 infections is derived from species-specific serologic assessments of targeted populations. From the most recent NHANES study, the overall seroprevalence of HSV-2 in the United States is about 17%, as illustrated in Tables 60.1 and 60.2.^{335,1280} A rate of more than 20% was reported for middle and upper socioeconomic classes as well.⁶³⁶ This rate of infections was similar to that reported in the 1997 NHANES study.³³⁵ As many as 50% to 60% of lower socioeconomic women in the United States and elsewhere develop antibodies to HSV-2 by adulthood.^{335,788} Antibodies to HSV-2 are virtually nonexistent in nuns.⁹²⁴ This compares with similar data from Birmingham, Alabama, whereby seroprevalence was 11.4% in 1985 and 28% in 1990. Sera collected from pregnant women in the mid-1980s from Padova, Italy, Orebro, Sweden, and Stockholm, Sweden showed seroprevalence

rates varying between 8% and 28%. In contrast, 10% to 20% of women in higher socioeconomic groups are seropositive. Seroprevalence continues to increase over time, indicating widespread acquisition of HSV-2 infection. Factors found to influence acquisition of HSV-2 include gender (greater for women than for men), race (more frequent among African Americans than for Caucasians), marital status (more for divorced individuals than for single or married individuals), crowded personal living conditions, and place of residence (more in the city than in the suburbs). Importantly, the number of sexual partners correlates directly with probability of acquiring HSV-2 infection.⁵⁵²

The highest prevalence of antibodies to HSV-2 in the United States was identified in female prostitutes (75%)—a rate almost identical to that of prostitutes in Tokyo.⁷⁸⁷ Seropositivity among female prostitutes in Dakar, Senegal, was even higher, at 95.7% in 1985. Homosexual men have seroprevalence rates to HSV-2 varying from a high of 83.1% in San Francisco (1985–1986) to lows of 21.6% in Seville, Spain (1985–1986); 24.2% in Tokyo (1988); and 50.0% in Amsterdam (1986). Seroprevalence is higher in STD clinics, as would be expected.³⁹¹ The implications for HIV and STD risk reduction have also been addressed.³⁹⁰ Numerous seroprevalence studies have been reported.^{787,1007,1278} Some countries have surprisingly low HSV-2 seroprevalence rates. For example, in Italy, whereas the seroprevalence was high in STD clinics (26%), it was significantly lower in obstetric populations of Modena, being only 5.3%.²⁴⁴ An excellent review summarizes HSV-2 trends.⁷⁰³

As noted, the number of different sexual partners correlates directly with acquisition of HSV-2.⁷⁸⁷ For heterosexual women living in the United States having one partner, the probability of acquisition of HSV-2 is less than 10%. The probability increases to 40%, 62%, and greater than 80% as the number of lifetime sexual partners increases from 2 to 10, from 11 to 50, or greater than 50, respectively. For heterosexual men, similar data are 0% for one lifetime sexual partner and 20%, 35%, and

TABLE 60.1 Herpes Simplex Virus 2 Seroprevalence in NHANES III (1988–1994) According to Sex, Age, and Race or Ethnic Group

Variable	Overall ^a		Whites		Blacks		Mexican Americans	
	Sample size	Percent prevalence (95% CI)	Sample size	Percent prevalence (95% CI)	Sample size	Percent prevalence (95% CI)	Sample size	Percent prevalence (95% CI)
<i>Sex</i>								
Both sexes	13,094	21.9 (20.2–23.6)	4727	17.6 (15.7–19.8)	3,884	45.9 (43.9–47.9)	3,991	22.3 (21.2–23.5)
Male	6,407	17.8 (15.6–20.2)	2383	14.9 (12.3–18.1)	1,798	34.7 (32.5–37.0)	1,992	19.2 (17.9–20.6)
Female	6,687	25.6 (24.0–27.3)	2344	20.2 (18.3–22.2)	2,086	55.1 (52.7–57.5)	1,999	25.7 (24.2–27.2)
<i>Age (y)</i>								
12–19	2,396	5.6 (4.3–7.2)	605	4.5 (3.0–6.8)	806	8.7 (6.4–11.9)	870	5.4 (4.0–7.3)
20–29	2,750	17.2 (15.0–19.7)	675	14.7 (12.0–18.1)	891	33.6 (30.3–37.2)	1,072	14.8 (12.6–17.5)
30–39	2,567	27.8 (24.8–31.2)	792	21.9 (18.7–25.6)	884	54.4 (50.5–58.6)	793	28.7 (26.3–31.4)
40–49	2,061	26.6 (23.5–30.0)	724	19.9 (16.4–24.1)	634	58.9 (55.7–62.4)	612	33.0 (28.4–38.3)
50–59	884	25.1 (21.5–29.4)	456	19.4 (15.6–24.1)	211	62.7 (54.4–72.3)	185	42.5 (31.6–57.1)
60–69	1,069	24.3 (20.2–29.1)	480	18.2 (13.7–24.0)	258	76.8 (72.3–81.5)	310	38.7 (32.0–46.8)
≥70	1,367	27.7 (24.6–31.1)	995	23.3 (20.0–27.0)	200	74.3 (67.6–81.8)	149	44.6 (38.8–51.2)

NHANES, National Health and Nutrition Examination Survey; CI, confidence interval.

^aTotals differ from the sums for whites, blacks, and Mexican Americans because other races and ethnic groups are included in the overall totals.

TABLE 60.2 Changes in Age-adjusted Herpes Simplex Virus 2 Seroprevalence Between NHANES II (1976–1980) and NHANES III (1988–1994)

Category of subjects	NHANES II		NHANES III		Percent relative increase
	Sample size	Age-adjusted percent seroprevalence (95% CI)	Sample size	Age-adjusted percent seroprevalence (95% CI)	
<i>All races and ethnic groups^a</i>					
Both sexes	3,597	16.0 (14.7–17.4)	13,094	20.8 (19.2–22.5)	30
Men	1,681	13.4 (11.7–15.4)	6,407	17.1 (15.0–19.5)	27
Women	1,916	18.4 (16.5–20.5)	6,687	24.2 (22.7–25.7)	32
<i>Whites</i>					
Both sexes	2,153	12.7 (11.2–14.3)	4,727	16.5 (14.7–18.5)	30
Men	1,021	10.7 (8.7–13.0)	2,383	14.1 (11.6–17.2)	32
Women	1,132	14.5 (12.4–17.0)	2,344	18.7 (17.0–20.5)	29
<i>Blacks</i>					
Both sexes	1,130	43.6 (40.0–47.6)	3,884	47.6 (45.4–49.9)	9
Men	495	34.1 (30.0–38.7)	1,798	37.5 (34.8–40.3)	10
Women	635	51.4 (47.6–55.6)	2,086	55.7 (53.3–58.2)	8

NHANES, National Health and Nutrition Examination Survey; CI confidence interval.

Note: Seroprevalence has been age adjusted to the 1980 census. The age range is ≥12 years.

^aTotals differ from the numbers for whites and blacks because other races and ethnic groups are included in the category of all races and ethnic groups.

70% for each of the subsequent three risk groups. In contrast, for homosexual men, seroprevalence increases from greater than 60% to 90% for those with 11 to 50 and more than 50 partners. Thus, multiple sexual partners (*polypartnerism*), irrespective of sexual preference, correlate directly with acquisition of HSV-2 infection.⁷⁸⁷ In addition, male circumcision has been shown to decrease the risk of acquisition of both HSV-2 and HIV.¹²²⁷

Overall, the number of new cases of genital HSV infections has been conservatively estimated to be approximately 500,000 individuals per year.⁷⁸⁸ Estimates of individuals with genital herpetic infection in the United States are 40 to 60 million individuals.^{488,552,701}

Vaccine and recent antiviral studies have defined the risk of transmission.^{234,621} Several observations are relevant. First, women remain more susceptible to HSV infection than men. Second, transmission usually occurs from an asymptomatic individual and does so in more than 70% of cases. Third, overall, the annual rate of transmission is approximately 4% to 5% per annum. The rate of acquisition by women is about 8% annually, whereas it is lower for men at about 2%.

As with HSV-1 infections of the mouth, HSV-2 can be excreted in the absence of symptoms at the time of primary, initial, or recurrent infection.^{923,1161,1207} Among women evaluated prospectively after their first episode of genital herpes, asymptomatic shedding was detected in approximately 12%, 18%, and 23% of women with primary HSV-1, primary HSV-2, and first-episode HSV-2 infection, respectively.⁵⁶⁵ Among women with established genital HSV-2 infection, asymptomatic shedding was detected on 1% to 4% of all days that cultures were obtained.¹²⁴ Shedding of virus occurs more frequently in the first year following genital infection than in subsequent years.¹²⁰⁶ Seropositive individuals with no history of lesions shed virus as frequently as those who are symptomatic.¹²⁰⁷ Asymptomatic shedding is the most significant source for virus transmission.

The use of PCR to detect virus shedding markedly increases these rates, as is discussed later.

The incidence of HSV-2 infection has been assessed prospectively in selected populations. Among low-risk individuals, namely college students, the rate of acquisition was approximately 2% per year over 4 years, compared with 4% per year for homosexual men.⁷⁸⁷ The incidence of HSV-2 infection during pregnancy is about 2.5% per gestation but was reported by other investigators to be as low as 0.58%.¹¹³ As noted earlier, acquisition of HSV-2 infection between monogamous sexual partners with discordant infection status is about 5% yearly.⁷⁴⁵

Latent infection of sacral ganglia is the largest reservoir of HSV-2, and reactivation of this virus leads to recurrent HSV-2 infection. Recurrent HSV-2 infection, like HSV-1, can be either symptomatic or asymptomatic. A recurrence is associated with a shorter duration of viral shedding and fewer lesions.²³¹ The frequency of recurrences varies somewhat between males and females, with calculations of 2.7 and 1.9 per 100 patient-days, respectively.²³¹ Detecting HSV by both culture and PCR, viral excretion was documented at 6% annually; however, PCR detection of viral DNA occurred on 18% to 25% of days—a three- to fourfold increase over viral culture. Notably, there was an association between detection of virus by PCR (but not by culture) and person-to-person transmission.^{1161,1207}

Overall, several studies have reported a frequency of recurrence as high as 60%.¹⁷⁶ Broadly, nearly 90% of HSV-2-infected patients will have 1 or more recurrences per year, 38% will have 6 recurrences, and 20% more than 10 recurrences.⁸⁰ The type of genital infection (HSV-1 or HSV-2) is predictive of the frequency of recurrence.⁹²⁸ HSV-1 infection recurs less frequently than does HSV-2 infection.^{80,610} The changing prevalence of HSV-2 infections is illustrated by surveys of representative populations (Table 60.3).

TABLE 60.3 Prevalence of Herpes Simplex Virus 2 Antibodies in Different Populations

Site of study	Year(s) of study	Percent prevalence of HSV-2 antibodies
<i>Different individuals</i>		
Reykjavik, Iceland	1979	4.1
(pregnant women)	1985	23.4
Lyon, France (pregnant women)	1977–1978	10.7
	1985	17.3
Orebro, Sweden (pregnant women)	1982	11.8
	1985	16.1
Zaire	1959	21
Kinshasa	1985	60
Rural areas	1959	6
	1985	32
<i>Same individuals</i>		
Alabama (pregnant women)	1985	6-mo acquisition rate = 2%
South Carolina (university students)	1983	0.4 (acquisition rate = 2%/y)
San Francisco (homosexual men)	1987	7
	1978	44 (acquisition rate = 4%/y)
	1985	72

HSV-2, herpes simplex virus 2.

Herpes Simplex Virus and Human Immunodeficiency Virus Interactions

HSV-2 infection, by the nature of being an ulcerative disease, is associated with increased risk of acquisition of both human immunodeficiency virus type 1 (HIV-1) and human T-cell lymphotropic virus type 1 (HTLV-1) by one-and-one-half- to more than threefold,^{349,1205} in particular in regions where HIV and HSV-2 are both epidemic (Fig. 60.19). The association between acquisition of HIV-1 and HSV-2 has been documented in heterosexual populations, as well.¹⁶⁷ Although treatment with HSV

drugs reduces HIV blood levels,⁷⁸² a clinical trial of acyclovir failed to prevent HIV acquisition,¹⁶⁷ and a clinical trial of acyclovir in HSV seropositive individuals exposed to HIV-infected partners failed to prevent transmission.¹⁶⁸ Arguably, there is evidence of submucosal replication of virus and inflammation that led to the acyclovir failure.^{349,783,1322} Furthermore, a recent study showed that acyclovir blood levels are lower in African subjects than non-African subjects.⁶⁸⁴ Therefore, further studies are needed to determine the optimal drug concentrations needed for international use and to define an efficacious genital herpes vaccine for use in studies to prevent genital herpes as well as to prevent HIV acquisition in high-risk areas for HIV infection.

Molecular Epidemiology

Studies on isolates based on restriction enzyme polymorphism showed that viruses isolated from epidemiologically related individuals (parents and child, sexual contacts, hospital contacts, etc.) cannot be differentiated from each other. In contrast, isolates from epidemiologically unrelated individuals can be readily distinguished on the basis of the restriction endonuclease patterns of their DNA. In a small number of cases, it has been shown that the isolates consist of a mixture of viruses with different restriction enzyme patterns.^{133,134} These rare cases suggest that under some circumstances an individual may become infected with multiple strains of HSV.^{617,1011}

Maternal Genital Herpes Simplex Virus Infections

Genital HSV infection in the pregnant woman is as common as in age-matched, nonpregnant women. However, it must be considered separately because of the risk to fetus and newborn.⁴⁰⁶ An uncommon problem encountered with HSV infections during pregnancy is that of widely disseminated maternal disease.¹²³⁸ In a limited number of cases, dissemination after primary oropharyngeal or genital infection has led to life-threatening disease, such as necrotizing hepatitis with or without thrombocytopenia, leukopenia, disseminated intravascular coagulopathy, and encephalitis. The mortality rate among these pregnant women is reported to be greater than 50%. Fetal deaths also have occurred in more than 50% of cases, although mortality did not necessarily correlate with the death of the mother. Surviving fetuses

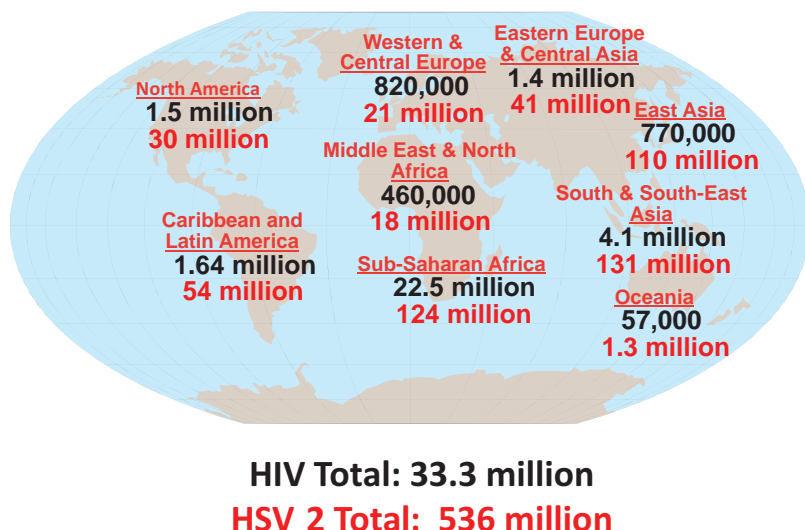


FIGURE 60.19. Numbers of cases of herpes simplex virus 2 infection in 2003 (red)⁶⁷⁶ and persons living with human immunodeficiency virus in 2009 (black) (UNAIDS) globally. (Copyright David Knipe.)

were delivered by cesarean section either during the acute illness or at term, and none had evidence of neonatal HSV infection. Fortunately, with the advent of safe antiviral therapy, specifically acyclovir, HSV disease is treated in the pregnant woman.

From several prospective trials, the major risk to the fetus is maternal primary or initial genital HSV infection.^{127,599} Thus, identification of the woman at risk for primary infection (seronegative for HSV-2 or both HSV-1 and HSV-2) is important. Studies of the discordant serologic status of sexual partners and rates of acquisition of genital HSV-2 infection define this problem.^{127,898} Primary or initial genital infection occurs at rates from 5% to 10% per year.^{130,898} If a pregnant woman experiences initial or primary genital infection in the last trimester of gestation, the likelihood of transmission to the fetus is between 30% and 50%. On the other hand, recurrent maternal infection is associated with a rate of transmission of 3% or less.¹²⁶ These different rates of transmission have direct implications on strategies to prevent neonatal disease or at least ameliorate it if the child is inadvertently delivered through an infected birth canal. In the United States, approximately 1 in 2,000 live births (~2,000 per year) are infected with HSV.

Maternal primary infection before 20 weeks gestation in some women has been associated with spontaneous abortion.⁷⁸⁵ Precise data, indicating the true risk of spontaneous abortion after primary infection during gestation, are not known. However, the contribution of primary maternal genital infection to spontaneous abortion must be weighed above that of a routine rate of fetal loss of approximately 20%. Infection that develops later in gestation has not been associated with the termination of pregnancy.^{395,406,423}

Recurrent infection is the most common form of infection during gestation. Transmission of infection to the fetus is usually related to shedding of virus at the time of delivery. The actual prevalence of viral excretion at delivery is about 0.5% for all women, irrespective of past history¹¹⁹⁷ and perhaps as high as 0.6%.¹²⁵

Several prospective studies have evaluated the frequency and nature of viral shedding in pregnant women with a known history of genital herpes. In a predominantly white, middle-class population, documented recurrent infection occurred in 84% of pregnant women with a history of herpes.¹¹⁹⁷ Asymptomatic viral shedding occurred in at least 12% of the recurrent episodes. Viral shedding from the cervix occurred in 0.56% of symptomatic infections versus 0.66% of asymptomatic infections and higher in some populations (3%),⁴²⁸ as reported in other populations.^{33,110,923} The frequency of recurrences has not been shown to be different from one pregnancy to the next for a given woman.⁴²⁴ The frequency of shedding does not appear to vary by trimester during gestation.^{424,1197} Most infants who develop neonatal disease are born to women who are completely asymptomatic for genital HSV infection and have neither a past history of genital herpes nor a sexual partner reporting a genital vesicular rash.^{1247,1252,1256} These women account for 60% to 80% of all women whose infected children become clinically ill.

animal models provided most data that address the relationship between host defense and disease pathogenesis. Host genetic background, innate immune responses, macrophages, NK cells, specific T-cell subpopulations, specific antibodies, and cytokine responses are important host defenses against HSV infections. However, more recently, human studies have been far more insightful.¹⁰⁸⁹ Local control mechanisms of viral spread aim to neutralize the infectious agent and lead to viral clearance. Following primary HSV infection, the initial, local immunological responses involve both nonspecific defense mechanisms, namely IFN- α/β , activated NK cells, and macrophages, as well as HSV-specific responses, such as CTLs.⁸⁸⁸ The role of T cells has attracted increasing attention, particularly as it relates to vaccine development.^{470,566,615,616,890}

In response to a viral infection, the initial cellular response is synthesis and secretion of type I IFNs (α and β). IFNs induce an antiviral state in infected and surrounding cells. The antiviral activity is modulated in part by IFN-mediated activation of cellular enzymes such as 2'-5' oligoadenylate synthetase (2'-5' AS) and double-stranded RNA-dependent protein kinase, as well as intracellular signaling molecules through the activation of the JAK/STAT kinase pathway. More specific to HSV infection, IFN- α appears to inhibit immediate-early gene expression.²⁵⁸ Thus, the antiviral mechanism directly affects transactivation of the immediate-early responsive element necessary for synthesis of viral proteins.

In addition to antiviral activity, IFNs are potent immunomodulators. As such, they mediate macrophage and NK cell activation, activate CTLs, induce MHC class I and II antigens, stimulate cytokine secretion, and induce local inflammation. IFN- γ may aid in the control of HSV infection. Evidence that $\gamma\delta$ T cells, NK cells, CD4⁺ T cells, and possibly neurons produce IFN- γ and TNF in response to HSV infection in the nervous system has been reported. IFN- γ down-regulates priming of CD4⁺ Th2 cells, which are responsible for inducing Ig isotype B-cell switching from immunoglobulin A (IgA) to IgG, thereby exerting a major effect on humoral immune responses.⁴⁵⁹

NK cells lyse pathogen-infected cells before virus-specific T-cell immunity is generated and constitute first-line defense against infection. *In vitro* and *in vivo* experiments have demonstrated that NK cells protect from HSV challenge in a murine model.⁷⁴⁷ Severe herpetic disease has been correlated with low *in vitro* NK activity in newborns, as well as in a patient lacking NK cells.⁹³ Other mononuclear cells, such as macrophages, are recruited to the site of infection and, upon activation, release immune cell mediators such as TNF and ILs. Macrophages play a major role in mediating antibody-dependent cellular toxicity for viral clearance and antigen presentation.⁶⁷⁹

An important aspect of immune responses to HSV infections is the maturation of DCs at the site of infection. Mobile DCs travel from mucosal or skin areas of infection and prime antigen-specific, naive T cells in draining lymph nodes (DLNs). Studies using HSV-1 footpad infection and fractionating DC subpopulations in the DLN show that classic, CD8 α^+ dermal DCs, rather than specialized epidermal Langerhan's cells (LCs), are able to prime naive CD8⁺ T cells.⁷⁹ In the case of vaginal HSV-2 infection, dermal DCs rather than LCs again seem to be the physiologically active cell population in a similar DLN investigation.¹³¹² LCs are certainly able to present HSV antigens to memory HSV-specific T cells and may participate in primary or recurrent immune reactions. Plasmacytoid dendritic

HOST IMMUNOLOGICAL RESPONSES

Primary Immune Responses

The natural history of HSV infections is influenced by both innate and induced host defense mechanisms. Historically,

cells (pDCs) react to HSV by producing copious amounts of IFN- α .¹⁰⁴⁵ pDCs are recruited to sites of infection, participate in viral clearance, and express relevant TLRs, including TLR 7, 8, and 9—the latter having been shown to recognize HSV.^{689,997} Of interest, a low pDC number or poor pDC reactivity with HSV is associated with severe HSV infection in humans.^{1,249}

As infection progresses, virus-specific immune responses are detected. On days 4 and 5 post infection, HSV-specific CD4⁺ Th1 lymphocytes are detected in genital lymph nodes and in smaller numbers in peripheral blood; they can subsequently be found in the genital mucosa.⁷⁵¹ CD8⁺ responses also occur quite quickly in the mouse⁷⁷¹; relevant human studies have not yet been reported.

Humoral immune responses rapidly follow initial HSV infection. The predominant mucosal antibodies are of the IgA isotype, being secreted by plasma cells. These antibodies can be detected as early as day 3 following infection, peaking within the first 6 weeks after disease onset, and are followed by appearance of IgG1 and IgG3 subclasses of antibodies, which are typically found following viral infections. HSV-specific IgA antibodies are present for at least 6 weeks, gradually decreasing to undetectable levels. Immunoglobulin M (IgM)—secreting B cells have also been detected in secretions of the female genital mucosa.⁷⁷⁷ Shorter periods of viral shedding in women with primary genital herpes have been positively correlated with the presence of secretory IgA in vaginal secretions.⁷⁴⁷ From a systemic perspective, analyses of antibody responses by immunoblot and immunoprecipitation to infected cell polypeptides have been correlated with the development of neutralizing antibodies.^{34,83,84,308,1327} Soon after the onset of infection, antibodies to structural proteins are followed by those directed against gD, gB, ICP4, gE, gG-1 or gG-2, and gC.⁵²³ Both IgM and IgG antibodies can be detected, depending on the time of assessment after infection.

Recurrent Herpes Simplex Virus Infection

Early on, cytokine production was incriminated in the pathogenesis of frequently recurrent genital and labial HSV infection. A decrease in both IFN- γ production and NK cells was reported during the disease prodrome.²⁴¹ With more knowledge, it was learned that HSV-specific CD8⁺ and CD4⁺ T cells persistently infiltrate latently infected trigeminal ganglia in mice and humans.^{543,1189} These cells appear to act via IFN- γ .⁶⁶¹ They have cytolytic activity, but neuron loss is not seen clinically, and inhibitory receptor-ligand pairings can be documented in the ganglia that may modulate their cytotoxic activity.¹¹¹³ In the periphery, HSV-2-specific CD4⁺ and CD8⁺ T cells localize to sites of recurrent HSV-2 infection and to the cervix.^{567,568} Using *in situ* staining, HSV-2-specific CD8 CTL has been shown to persist at the epidermal/dermal junction adjacent to sensory nerve endings.¹³²² Damage to these cell populations in immune-compromised persons may lead to increased HSV replication either centrally or peripherally. In fact, this finding has been incriminated as the basis of failed acyclovir trials on the prevention of person-to-person transmission of infection in individuals with¹³²² HIV-acquired immunodeficiency virus (AIDS) (Medical Research Council [MRC], HIV Prevention Trials Network [HPTN]). Repeated subclinical episodes of HSV excretion may be a source of antigenic stimulation leading to long-term HSV-specific immune memory.⁶⁷⁹ In recurrent HSV-2 infections, NK and HSV-specific CD4⁺ cells are detected earlier than CD8⁺ cells in genital lesions.⁴⁵⁹ CD4⁺

T cells and, more recently, CD8⁺ T cells have been highlighted as major mediators of viral clearance from mucocutaneous lesions in recurrent episodes.^{567,888,889} Low IFN- γ titers in vesicle fluid have been associated with a shorter time to the next recurrence in patients with frequent recurrences. T-cell proliferation is decreased in these patients in comparison to patients with less frequent recurrences.⁶⁷⁹ In as much as the involvement of cytokines has been studied, IFN- γ has been reported to have a role in viral clearance from mucocutaneous sites, whereas altered cytokine production appears to correlate with recurrence.¹⁰⁹²

As with primary HSV infection, a shorter duration of viral shedding occurs in women with recurrent genital herpes who have detectable secretory IgA in vaginal secretions.⁷⁴⁷ IgA, IgG1, and IgG3 antibodies have been found in the sera of all patients with recurrent HSV-2 episodes, whereas IgM and IgG4 antibodies were detected in 70% to 80% of these patients. However, there does not appear to be clear correlation between humoral immune responses and disease prognosis.⁴²⁷

Persistence of Immune Responses

The host's immune responses persist and partially control HSV disease; recurrent episodes are generally less severe and of shorter duration over the years, perhaps owing to progressive enhancement of long-term immunity.⁸¹ Furthermore, some degree of cross-protection exists between HSV-1 and HSV-2, as best evidenced by partial protection of newborns by maternal antibodies.¹²⁵⁰ Finally, HSV-specific T-cell infiltrates are detected in herpetic lesions during early disease resolution.⁸⁸⁸

Studies indicate that persistent cell-mediated immune responses are more important than humoral immune responses in the resolution of HSV disease.⁸⁵ NK cells, macrophages, and T lymphocytes, as well as cytokines such as IFN- α and - γ , IL-2, and IL-12, all have central roles in resolving HSV disease.⁹⁴⁰ HSV-specific CD4⁺ and CD8⁺ cells are detected in lesions from recurrent episodes, indicating their potential role in controlling HSV disease.^{567,888} By contrast, agammaglobulinemic patients do not experience more severe or more frequent herpetic recurrences than the general population.⁵⁹¹ Importantly, several vaccine trials have demonstrated that the presence of neutralizing antibodies to HSV glycoproteins does not provide protection against HSV infection or disease. Although a recent vaccine study suggested benefit for genital HSV-1 infection,⁷⁸ the contribution of HSV-1 to recurrences and acquisition of HSV-2 is significantly less medically important.

Relationship Between Immune Response and Disease

Efforts to precisely incriminate that arm of the host response responsible for disease have not been established. Humoral immunity to HSV infection has been evaluated exhaustively in disease pathogenesis. From a clinical perspective, prior HSV-1 infection has a somewhat protective effect on the acquisition of HSV-2 infection,⁷⁴⁵ albeit controversial. In animal models, polyclonal antibodies have been used to alter disease lethality, particularly in the newborn mouse or to limit progression of both neurologic and ocular disease.^{252,278,533,574,600,730,820,821,927,1049} Monoclonal antibodies to selected specific infected cell polypeptides, especially the envelope glycoproteins gB and gD, confer protection from lethality.^{55,278,927} Importantly, gD2 is a known target of neutralizing antibodies, antibody-dependent

cell-mediated cytotoxicity, and CD4 and CD8 T-cell-mediated responses.^{436,514,756,1307} As a consequence, this antigen has been a prime component of subunit vaccines.

Efforts to correlate the frequency recurrences with immune responses have failed to identify any specific humoral response to specific polypeptides.^{83,85,524} Thus, further efforts have focused, in large part, on cell-mediated immune responses. As noted, lymphocyte blastogenic responses are demonstrable within 4 to 6 weeks after the onset of infection and sometimes as early as 2 weeks.^{233,850,921,974,975,1036,1094,1109} These responses are typically mediated by CD4⁺ T cells. With recurrences, boosts in blastogenic responses occur; however, these responses, as after primary infection, decrease with time. Nonspecific blastogenic responses do not correlate with a history of recurrences. HSV-1 and HSV-2 are cross-reactive in these assays at the whole virus levels, although individual T-cell clonotypic responses can be either type common or type specific.⁵⁶⁴

Lymphokine production has been incriminated in the pathogenesis of frequently recurrent genital and labial HSV infection. Notably, several investigators have recognized a decrease in both IFN- γ production and NK cells during disease prodrome.^{241,835,1034} Nevertheless, there are no reproducible data from selected populations to confirm these observations. The relevance of lymphokine expression in vaccine development can be assessed only in prospective field trials.

Host response of the newborn to HSV must be defined separately from that of older individuals. Immaturity of host defense mechanisms is a cause of the increased severity of some infectious agents in the fetus and the newborn. Factors that must be considered in defining host response of the newborn include the mode of transmission of the agent (viremia vs. mucocutaneous infection without blood-borne spread), time of acquisition of the infection, and the potential of increased virulence of certain strains, although this last point remains purely speculative. Two broad issues are of relevance: protection of the fetus by transplacental antibodies and definition of host responses of the newborn. Transplacentally acquired neutralizing antibodies either prevent or ameliorate infection in exposed newborns, as does antibody-dependent cell-mediated cytotoxicity.^{575,899,1297} Importantly, pre-existing antibodies, indicative of prior infection, significantly decrease the transmission of infection from pregnant women to their offspring,¹²⁵ contributing to the rationale for the development of a HSV vaccine.

Humoral IgG and IgM responses have been well characterized. Infected newborns produce IgM antibodies specific for HSV within the first 3 weeks of infection. These antibodies increase rapidly in titer during the first 2 to 3 months, and they may be detectable for as long as 1 year after infection. The most reactive immunodeterminants are the surface viral glycoproteins, particularly gD. Humoral antibody responses have been studied using contemporary immunoblot technology, and the patterns of response are similar to those encountered in adults with primary infection.^{525,1109} The quantity of neutralizing antibodies is lower in babies with disseminated infection.^{1109,1297}

Cellular immunity has been considered to be important in the host response of the newborn. The T-lymphocyte proliferative response to HSV infections is delayed in newborns compared to older individuals.¹¹⁰⁹ Most infants studied in a recent evaluation had no detectable T-lymphocyte responses to HSV 2 to 4 weeks after the onset of clinical symptoms.^{850,920,1109} The correlation between these delayed responses may be of signifi-

cance in evaluating outcome to neonatal HSV infection. Specifically, if the response to T-lymphocyte antigens in children who have disease localized to the skin, eye, or mouth at the onset of disease is significantly delayed, disease progression may occur at a much higher frequency than babies with a more appropriate response.^{192,1109}

Infected newborns have decreased production of IFN- α in response to HSV when compared to adults with primary HSV infection.¹¹⁰⁹ The importance of the IFN generation on the maturation of host responses, particularly the elicitation of NK-cell responses, remains to be defined.^{131,571} Lymphocytes from infected babies have decreased responses to IFN- γ during the first month of life.^{137,571,1109} In general, the newborn has poorer immune responses than older children and adults. Antibodies plus complement and antibodies mixed with killer lymphocytes, monocytes, macrophages, or polymorphonuclear leukocytes will lyse HSV infected cells *in vitro*.⁹⁷¹ Antibody-dependent cell-mediated cytotoxicity has been demonstrated to be an important component of the development of host immunity to infection.⁵⁷⁰ However, the total population of killer lymphocytes of the newborn seems to be lower than that found in older individuals, and monocytes and macrophages of newborns are not as active as those of adults.^{30,451,572,573,680,1160} These findings are supported by animal model data.

Host Susceptibility

Recent data indicate that a select group of children are prone to develop encephalitis with primary infection and are at risk for recurrent life-threatening HSV infections. These children have been found to have inborn errors of *TLR3*, *TRIF*, *UNC93B1*, or *TRAF3*. These unique studies warrant further elaboration in defining genetic susceptibility to recurrent HSV infections.^{164,993,1309}

CLINICAL MANIFESTATIONS OF DISEASE

HSV disease ranges from the usual case of mild illness, nondiscernible in most individuals, to sporadic, severe, and life-threatening disease in a few infants, children, and adults. Although HSV-1 and HSV-2 are usually transmitted by different routes and involve different areas of the body, there is a great deal of overlap between the epidemiology and clinical manifestations of these two viruses. Historically, primary HSV-1 oropharyngeal infections occurred in the young child (<5 years) and were most often asymptomatic. More recently, HSV-1 infections have become a significant cause of genital herpes as the first manifestation of disease, as is discussed later.¹²⁸⁰ When the oropharynx is involved, the mouth and lips are the most common sites of HSV-1 infections, causing gingivostomatitis; however, any organ can become infected with this virus.

Primary/Recurrent Oropharyngeal Disease

The clinical symptoms of primary HSV-1 infections vary greatly. Infection can be totally asymptomatic or result in combinations of fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, edema, localized lymphadenopathy, anorexia, and/or malaise. Asymptomatic infection is the rule rather than the exception. The incubation period ranges from 2 to 12 days, with a mean of approximately 4 days.



FIGURE 60.20. Herpes simplex gingivostomatitis.

Symptomatic disease in children is characterized by involvement of the buccal and gingival mucosa¹⁰⁸⁴ (Fig. 60.20). The duration of clinical illness may be from 2 to 3 weeks with fever of 101°F to 104°F. Children with symptomatic primary infection often are unable to swallow liquids because of the significant edema and ulcerative lesions of the mucosal membranes and the associated pain. Lesions within the mouth evolve from vesicles to shallow ulcerations on an erythematous base and then heal slowly. In contrast to cutaneous lesions, intraoral lesions are not associated with the development of scabs. Submandibular lymphadenopathy is common with primary HSV gingivostomatitis but rare with recurrent infections. A clinical distinction should be drawn between intraoral gingival lesions and lip lesions indicative of presumed primary and recurrent infections, respectively. Utilizing PCR detection of viral DNA, evidence of viremia can be documented in approximately one-third of symptomatic patients.⁴²²

With primary HSV infections during adulthood, HSV pharyngitis in association with a mononucleosis syndrome is common. Under such circumstances, ulcerative tonsillar lesions on an erythematous base with associated submandibular



FIGURE 60.22. Recurrent herpes simplex labialis.

lymphadenopathy are common.³⁸⁰ The differential diagnosis of both primary HSV gingivostomatitis and pharyngitis focuses on other mucosal lesions of the oropharynx. These would include herpangina (caused by coxsackieviruses), candidal infections of the mouth, Epstein-Barr virus–induced mononucleosis, lesions induced by chemotherapy or radiation therapy, and Stevens-Johnson syndrome.

The onset of recurrent orolabial lesions is heralded by a prodrome of pain, burning, tingling, or itching, which generally lasts for less than 6 hours and is followed by vesicle formation.¹⁰⁸⁵ The natural history of recurrent herpes labialis is illustrated in Figure 60.21.¹⁰⁸⁴ Vesicles appear most commonly at the vermillion border of the lip and persist in most patients only 48 hours at the longest¹²³⁸ (Fig. 60.22). Vesicles generally number from three to five. The total area of involvement usually is less than 100 mm², and lesions progress to the pustular or ulcerative and crusting stage within 72 to 96 hours. Pain is most severe at the outset and resolves quickly over 96 to 120 hours. Similarly, the loss of virus from lesions decreases

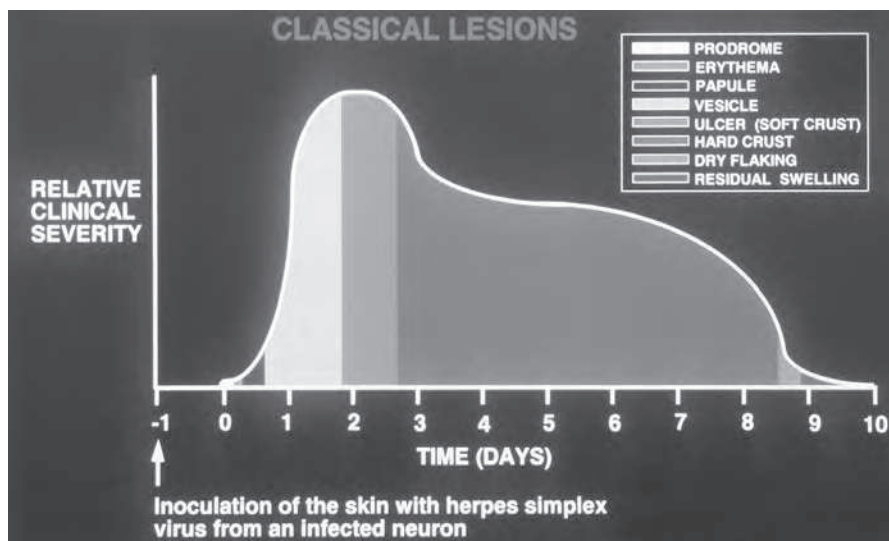


FIGURE 60.21. Herpes simplex labialis natural history.

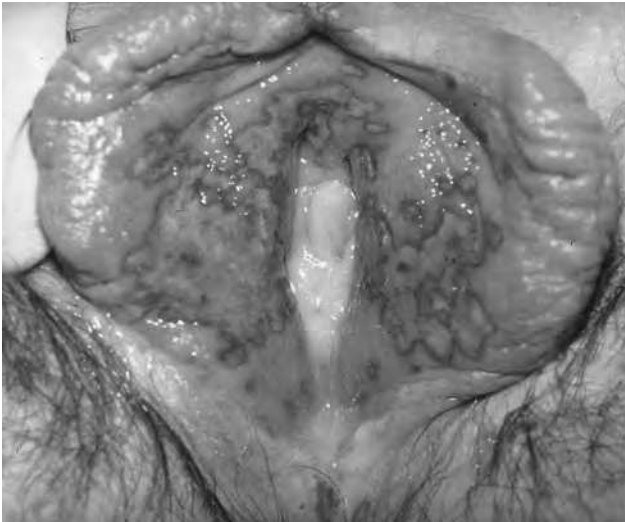


FIGURE 60.23. Genital herpes simplex virus infection (female).

with progressive healing over 2 to 3 days.^{47,1085} Healing is rapid, generally being complete in 8 to 10 days. The frequency of recurrence varies among individuals.¹⁰⁸⁵ The factors responsible for recurrence in humans are both highly variable and poorly defined but include fever, stress, immunosuppression, and exposure to UV light.^{1028,1040}

Primary/Initial/Recurrent Genital Disease

Symptomatic primary genital infection is usually the most severe. After acquisition of HSV infection at a mucocutaneous site, macules and papules appear, followed by vesicles, pustules, and ulcers. The duration of lesions averages 3 weeks. There are both similarities and differences in the clinical symptomatology between men and women with infection.^{229,231} Furthermore, there appears to be no difference in clinical symptoms associated with primary genital HSV infection attributed to either virus species.

Primary infection is associated with (a) larger quantities of virus replicating in the genital tract ($>10^6$ viral particles per 0.2 mL of inoculum) and (b) a period of viral excretion that may persist for an average of 3 weeks. Systemic complications in men are relatively uncommon; however, aseptic meningitis can develop. Paresthesias and dysesthesias that involve the lower extremities and perineum can result from genital herpetic infection. Primary infections can be associated with fever, dysuria, localized inguinal adenopathy, and malaise in both men and women. The severity of primary infection and its association with complications are statistically higher in women than in men, for unknown reasons.²³¹ Systemic complaints are common in both sexes, approaching 70% of all cases. Extragenital lesions are common.

In women with primary infection, lesions appear on the vulva and are usually bilateral,⁹⁵⁶ as shown in Figure 60.23, with the cervix being invariably involved. The actual frequency of primary cervical infection in the absence of vulvar infection is unknown. Lesions usually are excruciatingly painful, associated with inguinal adenopathy and dysuria, and may involve the vulva, perineum, buttocks, cervix, and/or vagina. A urinary

retention syndrome occurs in 10% to 15% of females, and as many as 25% will develop aseptic meningitis.

In men, primary genital HSV infections are most often associated with vesicular lesions superimposed on an erythematous base, usually appearing on the glans penis or the penile shaft,⁹⁵⁶ as shown in Figure 60.24. The total number of lesions can vary significantly. Extragenital lesions of the thigh, buttocks, and perineum can occur.

Complications of primary genital herpetic infection have included sacral radiculomyelitis, which can lead to urinary retention, neuralgias, and meningoencephalitis.^{152,445,450,1056,1136} Primary perianal and anal HSV-2 infections, as well as associated proctitis, are common in male homosexuals.³⁸⁶ HSV-2 can be isolated from the rectum of heterosexual males and females as well. As with HSV-1 infections, many primary HSV-2 illnesses are subclinical, involving the mouth or the uterine cervix.¹²⁹⁹

Nonprimary but initial genital infection (i.e., occurring in an individual with pre-existing antibody) is less severe



FIGURE 60.24. Genital herpes simplex virus infection (male).

symptomatically and heals more quickly than primary infection. The duration of disease is approximately 2 weeks. The number of lesions, severity of pain, and likelihood of complications are significantly decreased. Pre-existing antibodies to HSV-1 appear to have an ameliorative effect on disease severity of HSV-2^{611,1011,1207} but do not appear to prevent infection.

Recurrent genital herpes is the mildest form of disease. With recurrent genital herpetic infection, a limited number of vesicles, usually three to five, appear on the shaft of the penis of the male or as simply a vulvar irritation in the female.⁵ The duration of disease is approximately 7 to 10 days and parallels that encountered with recurrent HSV labialis. Neurologic or systemic complications are uncommon with recurrent disease; however, paresthesias and dysesthesias can occur.

Some individuals experience their first-episode genital infection years after an asymptomatic or atypical primary infection. In such circumstances, disease can be as severe as true primary infection.

The extent of viral replication is different for recurrent infection as compared with primary infection. Virus is shed for an average of only 2 to 5 days and at lower concentrations ($\sim 10^2$ to 10^3 per 0.2 mL of inoculum in tissue culture systems). Recurrent genital infection in both men and women is characterized by prodrome (which is a useful marker for therapeutic trials)⁶⁸⁷ and localized irritation.

The major problem with recurrent genital HSV infection is the frequency of recurrences, which varies from one individual to the next. Anecdotally, the severity of primary infection correlates directly with the frequency of recurrences—that is, the more severe the primary infection, the more likely and frequent the recurrences. Notably, virtually all patients will suffer from recurrences, whether symptomatic or asymptomatic; however, it does vary by virus type. For those individuals with genital HSV caused by HSV-1, the probability of recurrences is significantly less and, in some patients, virtually not at all. On the other hand, HSV-2 genital infections are prone to recur. One-third of patients are estimated to have recurrences in excess of eight or nine times per year, one-third will have two to three per year, and the remaining one-third will have four to seven.²³¹ Obviously, with recurrences, either symptomatic or asymptomatic, transmission of infection to sexual partners can occur with intimate contact. HSV DNA can be detected by PCR^{218,1203} three to five times more frequently than the isolation of virus. Recent data suggest much more frequent intermittent shedding of reactivation with episodes lasting only a few hours. These data provide a rationale for ongoing transmission of infection.

Neonatal Herpes Simplex Virus Infection

Incidence and Transmission of Newborn Infection

The estimated incidence of neonatal HSV infection is approximately 1 in 3,000 to 1 in 5,000 deliveries per year.⁵⁵² Neonatal HSV infection occurs far less frequently than genital HSV infections in the adult childbearing population. At least four factors influence transmission of infection from mother to fetus. First, the risk of transmission with maternal primary or first-episode genital infection during the third trimester is 30% to 50% as compared with 3% or less with recurrent infection.^{125,127} In part, this reflects prolonged viral excretion during the third trimester of gestation as well as an inadequate

transplacental antibody transfer. Second, transplacental maternal neutralizing and antibody-dependent cell-mediated cytotoxic antibodies appear to have at least an ameliorative effect on acquisition of infection and disease presentation, as noted earlier.^{38,786,899,1296,1297} Third, prolonged rupture of membranes (>6 hours) increases the risk of acquisition of virus. Fourth, certain forms of medical intervention in the labor and delivery suites may increase the risk of neonatal HSV infection, including the use of fetal scalp monitors.^{538,848}

Acquisition and Presentation of Infection

Infection of the newborn can be acquired *in utero* (congenital), intrapartum, or postnatally. The mother is the most common source of infection for the first two of these routes of transmission. The reader is referred to a more detailed treatise on this disease.⁴⁰⁶

CONGENITAL INFECTION

In utero infection can occur as a consequence of either transplacental or ascending infection.^{58,482} The incidence has been estimated to be 1 in 200,000 deliveries.⁵⁸

Placental necrosis and inclusions in the trophoblast can occur.^{357,361,1271} Intrauterine infection is characterized by the triad of skin vesicles or skin scarring, eye disease (chorioretinitis or microphthalmia), and the far more severe manifestations of microcephaly or hydranencephaly.

INTRAPARTUM INFECTION

The most common route of infection is that of intrapartum contact of the fetus with infected maternal genital secretions. Likely, 75% to 80% of infected babies acquire HSV infection by this route. Babies who are infected intrapartum or postnatally with HSV infection are classified as having (a) disease localized to the skin, eye, and mouth; (b) encephalitis with or without skin involvement; and (c) disseminated infection that involves multiple organs, including the CNS, lung, liver, adrenals, skin, eye, and/or mouth.^{551,1254}

Babies with the worst prognosis both for mortality and morbidity have disseminated infection.¹²⁴⁷ These children are usually referred to tertiary care centers for therapy at 9 to 11 days of age; however, signs of infection usually begin an average of 4 to 5 days earlier. The principal afflicted organs are the liver and adrenals; however, there can be involvement with multiple other organs, including the larynx, trachea, lungs, esophagus, stomach, lower gastrointestinal tract, spleen, kidneys, pancreas, and heart. Constitutional signs and symptoms include irritability, seizures, respiratory distress, jaundice, bleeding diatheses, shock, and, frequently, the characteristic vesicular exanthem, which is often considered pathognomonic for infection. Encephalitis appears to be a common component of this form of infection, occurring in about 60% to 75% of children with disseminated infection. More than 20% of these children do not develop skin vesicles during the course of their illness.^{35,1247} Mortality in the absence of therapy exceeds 80%; all but a few survivors are impaired. The most common cause of death in babies with disseminated disease is either HSV pneumonitis or disseminated intravascular coagulopathy.

Babies with infection of the CNS alone or in combination with disseminated disease present with the findings indicative of encephalitis. Overall, nearly 75% of babies with disseminated disease have evidence of acute brain infection. Clinical

manifestations of either encephalitis (alone or in association with disseminated disease) include seizures (both focal and generalized), lethargy, irritability, tremors, poor feeding, temperature instability, bulging fontanelles, and pyramidal tract signs. Whereas babies with disseminated infection often have skin vesicles in association with brain infection, the same is not true for the baby with encephalitis alone. This latter group of children may only have skin vesicles in approximately 60% of cases at any time in the disease course.^{35,406,1110,1247} Death occurs in 50% of babies with localized CNS disease who are not treated and is usually related to brain system involvement. With rare exceptions, survivors are left with severe neurologic impairment.^{1239,1248}

The long-term prognosis, after either disseminated infection or encephalitis, is poor in the absence of antiviral therapy. As many as 50% of surviving children have some degree of psychomotor retardation, often in association with microcephaly, hydranencephaly, porencephalic cysts, spasticity, blindness, chorioretinitis, or learning disabilities. Recent data suggest that CNS damage can be progressive after initial clearance of the viral infection, as predicated upon long-term antiviral suppressive data administered to babies with encephalitis.⁵⁵⁴

Subclinical infection of the CNS has been detected in babies with disease apparently localized to the skin, eye, and mouth. These children had normal cerebrospinal fluid assessments but PCR-detectable HSV DNA at the onset of disease.⁵⁵⁰ Subsequently, a subset of these children developed neurologic impairment.

Importantly, in as many as 40% of children with disease localized to the CNS (e.g., the 2- to 3-week-old baby with cells and protein in the cerebrospinal fluid), skin vesicles (the classic sign of disease) are not present. For the neonate with cerebrospinal fluid findings indicative of infection, HSV must be

considered along with other bacterial pathogens (e.g., group B *streptococcus*, *E. coli*). A reasonable diagnostic approach—if all antigen and Gram stain studies and PCR evaluations are negative—would be serial cerebrospinal fluid examinations to document progression in protein and mononuclear cell counts.

Infection localized to the skin, eye, and/or mouth is associated with lower mortality; however, significant morbidity can occur. When infection is localized to the skin, the presence of discrete vesicles remains the hallmark of disease. With time, the rash can progress to involve other areas of the body as well, particularly if viremia occurs. Vesicles occur in 90% of children with skin, eye, or mouth infection. These children present at about 10 to 11 days of life. Babies with skin lesions invariably will experience recurrences over the first 6 months (and longer) of life, regardless of whether therapy was administered. Although death is not associated with disease localized to the skin, eye, and/or mouth, approximately 30% of these children eventually develop evidence of neurologic impairment.^{1239,1248,1252} The skin vesicles, as shown in Figure 60.25, usually erupt from an erythematous base and are usually 1 to 2 mm in diameter. Other manifestations of skin lesions have included a zosteriform eruption.⁷⁷⁹

Infections involving the eye may manifest as keratoconjunctivitis or, later, as chorioretinitis. The eye can be the only site of HSV involvement in the newborn.¹²⁴⁸ These children present with keratoconjunctivitis or, surprisingly, evidence of microphthalmia and retinal dysplasia.

POSTNATAL ACQUISITION

Lastly, postnatal acquisition of HSV-1 accounts for about 10% of cases.^{288,303,344,417,545,647,653,786,1111} The presentation is similar to that described previously and usually is the consequence of family contact with the newborn.

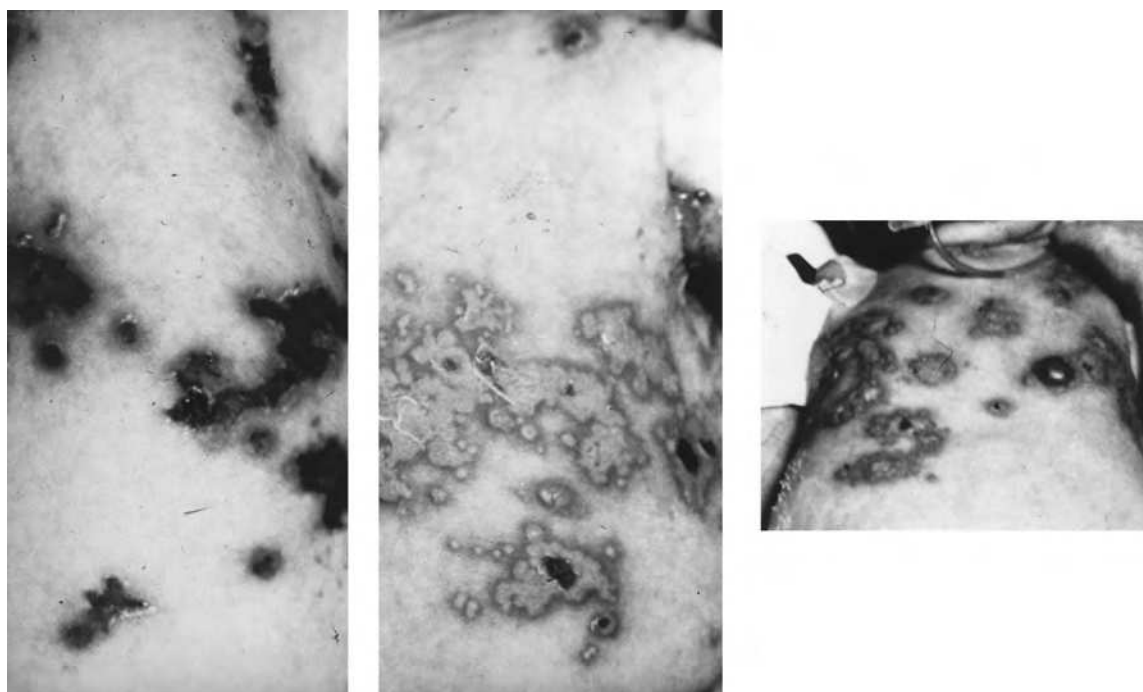


FIGURE 60.25. Vesicular rash of neonatal herpes simplex virus infection.

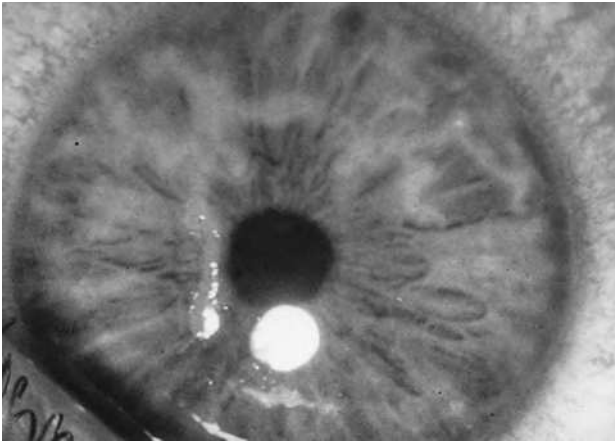


FIGURE 60.26. Dendritic lesions of herpes simplex keratoconjunctivitis.

Herpes Simplex Keratoconjunctivitis

Viral infections of the eye are usually caused by HSV-1 beyond the newborn age.^{92,834,1024} Approximately 300,000 cases of HSV infections of the eye are diagnosed yearly in the United States. These infections are second only to trauma as the cause of corneal blindness in the United States. Primary herpetic keratoconjunctivitis is associated with either unilateral or bilateral conjunctivitis, which can be follicular in nature, followed soon thereafter by preauricular adenopathy. HSV infection of the eye is also associated with photophobia, tearing, eyelid edema, and chemosis, accompanied by the pathognomonic findings of branching dendritic lesions⁹⁵⁶ (Fig. 60.26). Less commonly, with progressive disease, the infection is associated with a geographic ulcer of the cornea⁹⁵⁶ (Fig. 60.27). Healing of the cornea can take as long as 1 month, even with appropriate antiviral therapy.

Recurrent HSV infections of the eye are common. Recurrences parallel that which was described for herpes labialis. Most frequently, recurrences are unilateral in involvement,

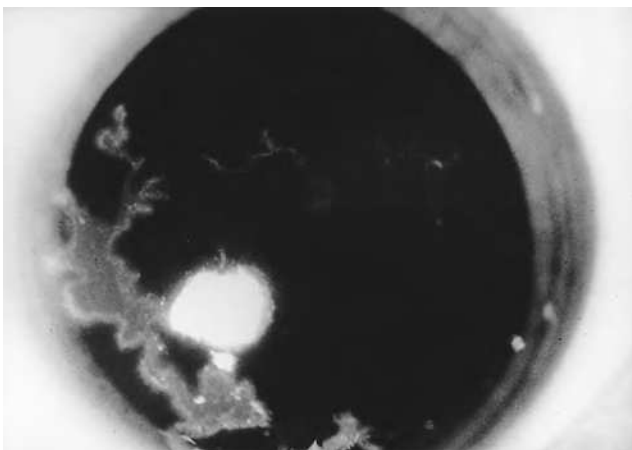


FIGURE 60.27. Geographic corneal ulcer caused by herpes simplex virus infection.

although a small percentage of cases involve both eyes. Characteristically, either dendritic ulceration or stromal involvement occurs. Visual acuity is decreased in the presence of the ulcers; with progressive stromal involvement, opacification of the cornea may occur. Repeated recurrences may last for weeks or even months after appropriate antiviral therapy. The route and pathogenesis of infection remain unknown. Progressive disease results in visual loss and even rupture of the globe.

Skin Infections

Skin infections caused by HSV generally manifest as eczema herpeticum in patients with underlying atopic dermatitis, occurring in 1% to 2%.^{310,903,1135,1234} The lesions can either be localized, resembling herpes zoster with a dermatomal distribution, or disseminated. The latter occurs commonly in Kaposi's varicella-like eruption.⁹⁷³ HSV infections of the digits, known as herpetic whitlow, have been reported and are particularly common among medical and dental personnel.⁹⁶⁹ The estimated incidence is 2.4 cases per 100,000 individuals per year, and the cause may be HSV-1 or HSV-2.³⁷⁷

In addition to individuals with atopic disease, patients with skin abrasions or burns appear particularly susceptible to HSV-1 or HSV-2 infections, and some may develop disseminated infection.³³⁶ Disseminated HSV infections have been also reported among wrestlers (herpes gladiatorum).¹²³⁵ Other skin disorders associated with extensive cutaneous lesions include Darier disease and Sezary syndrome.^{431,452} Localized recurrences followed by a second episode of dissemination have been reported.⁸³² HSV infections of either type can trigger erythema multiforme.¹²³³ The presence of HSV DNA in skin lesions of erythema multiforme is as high as 80%.

Infections of the Immunocompromised Host

Patients compromised by immunosuppressive therapy, underlying disease, or malnutrition are at increased risk for severe HSV infections. Renal, hepatic, bone marrow, and cardiac recipients are all at particular risk for increased severity of transplant HSV infection.^{773,851,1251} An example of cutaneous dissemination after shaving, in a renal transplant recipient, is shown in Figure 60.28.⁹⁵⁶ In organ transplant recipients, the presence or quantity of HSV antibodies before treatment predicts the individual at greatest risk for recurrence.⁸⁵² Acquisition of HSV infection from a transplanted organ (kidney) has been reported.³⁰¹ These patients may develop progressive disease involving the respiratory tract, esophagus, or even the gastrointestinal tract.^{578,764} The severe nature of progressive disease in these patients appears to be directly related to the type of immunosuppressive therapy.⁹¹⁴ Esophagitis is a common occurrence in the immunocompromised host and can be caused by HSV, CMV, or *Candida albicans*. Notably, acyclovir-resistant HSV disease can occur in the immunocompromised host and can be progressive. Reactivation of latent HSV infections in these patients can occur at multiple sites, and healing in these patients with severe progressive disease occurs over an average of 6 weeks.¹²⁵⁵

Since the first reports of AIDS, the severity of HSV clinical disease in these severely immunocompromised hosts was noted.^{704,1208} Herpetic disease, even more exaggerated than in the cancer patient (e.g., proctitis), has been reported and is an essential target for antiviral therapy. Disease severity correlates with CD4 count and the probability of developing resistance to antiviral therapy. As noted earlier, genital ulcerative disease,



FIGURE 60.28. Cutaneous dissemination of herpes simplex virus infection in an immunosuppressed host.

including HSV infection, increases the risk for acquisition of HIV infection.^{69,1203} Furthermore, despite highly active antiretroviral therapy, HSV recurrences are more common than in the normal host.⁸⁹¹ Asymptomatic excretion of HSV can occur even in the immunocompromised host, as is best exemplified in HIV-infected individuals.^{1004,1005,1207}

Infections of the Central Nervous System

Herpes simplex encephalitis is one of the most devastating of all HSV infections⁹⁵⁶ (Fig. 60.29), being the most common

cause of sporadic, fatal encephalitis in this country.^{830,1244} The incidence of severe hemorrhagic focal encephalitis is approximately 1 in 250,000 individuals per year, for a national annualized rate of approximately 1,250 cases in the United States.

HSV encephalitis is primarily a focal encephalitis associated with fever, altered consciousness, bizarre behavior, disordered mentation, and localized neurologic findings. These clinical signs and symptoms generally are associated with evidence of localized temporal lobe disease, as demonstrated by neurodiagnostic procedures.^{1253,1254} There are no pathognomonic findings

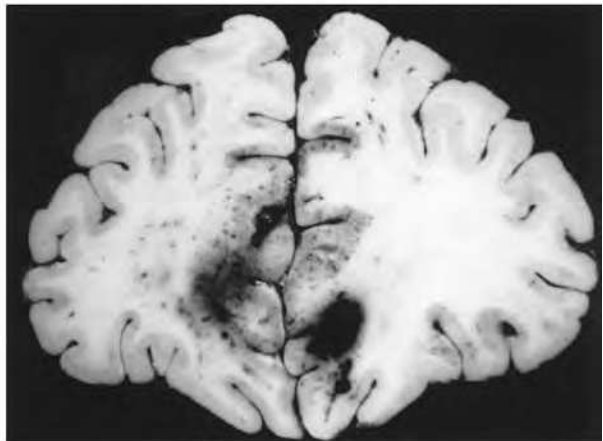


FIGURE 60.29. Coronal section of brain from a patient with herpes simplex encephalitis.

for herpes simplex encephalitis; however, a progressively deteriorating level of consciousness, fever, an abnormal cerebrospinal fluid formula, and focal neurologic findings in the absence of other causes should make this disease highly suspect. Other diagnostic evaluations should be initiated immediately because other treatable diseases mimic HSV encephalitis.¹²⁴⁵ Mortality in untreated patients is in excess of 70%, and only 2.5% of all patients return to normal neurologic function.

Neurodiagnostic procedures used in the evaluation of patients with suspect HSV encephalitis include cerebrospinal fluid examination; electroencephalogram; and one or more scanning procedures such as technetium, computed tomography, or a magnetic resonance image scan. Characteristic abnormalities of the cerebrospinal fluid include elevated levels of cells (usually mononuclear) and protein. Red blood cells are found in most (but not all) cerebrospinal fluids obtained from patients with HSV encephalitis. The electroencephalogram generally localizes spike and slow-wave activity to the temporal lobe. A burst suppression pattern is considered characteristic of HSV encephalitis. Imaging will allow for localization of disease to the temporal lobe. Early after onset, only evidence of edema is detectable, if at all. This finding is followed by evidence of hemorrhage and a midline shift in the cortical structures.

PCR detection of HSV DNA in the cerebrospinal fluid has become the diagnostic of choice; however, it must be performed by a qualified laboratory.⁶¹⁸ Serologic assessments of serum or cerebrospinal fluid are not helpful early in the disease course when therapeutic decisions are mandatory.⁷⁸⁹

Other Neurologic Syndromes

In addition to encephalitis, HSV can involve nearly all anatomic areas of the nervous system, including meningitis, myelitis, and radiculitis, among others.²³⁷ Aseptic meningitis is a common occurrence in individuals with primary genital HSV infections. Recurrent aseptic meningitis (Mollaret syndrome) will occur in patients with genital herpes.¹¹⁶⁶ The relationship between HSV infections of the brain and chronic degenerative disease, psychiatric disorders, or Bell palsy requires further definition.^{64,223}

Other Forms of Infection

Other forms of infection are noted briefly. HSV was isolated from the respiratory tract of adults with adult respiratory distress syndrome and acute onset bronchospasm.¹¹⁶⁵ Both were associated with increased mortality and morbidity. In addition, HSV has been associated with Bell palsy.⁷⁷⁵

DIAGNOSIS

The appropriate use of laboratory tools is essential if a diagnosis of HSV infection is to be achieved.²³⁰ Virus isolation is a definitive diagnostic method; however, PCR detection of viral DNA has gained increased acceptance even for routine skin infections, replacing culture in most laboratories. If a culture is obtained, skin vesicles/lesions should be swabbed/scraped and transferred in appropriate virus transport media to a diagnostic virology laboratory. In addition to skin vesicles, other sites from which virus may be isolated include the cerebrospinal fluid,

stool, urine, throat, nasopharynx, and conjunctivae. Clinical specimens should be shipped on ice for inoculation into cell culture systems (e.g., foreskin fibroblasts, Vero cells) that are appropriate for the demonstration of the cytopathic effects characteristic of HSV replication.¹⁰¹⁵ Cytopathic effect tends to develop within 24 to 48 hours after inoculation of specimens containing infectious virus. The shipping and processing of specimens should be expedited.

The advantage of cell culture is the ability to obtain a virus isolate for further testing (e.g., resistance or typing). Typing of an HSV isolate may be accomplished by one of several techniques, which may not be routinely available. Because outcome with treatment does not appear to be related to the virus type, identification is only of epidemiologic and pathogenetic importance and, therefore, not usually necessary.

Every effort should be made to confirm infection by viral isolation or PCR detection of viral DNA. Today, such efforts are both reasonable and the expected standard of care. Cytopathologic detection of evidence of infection is less rewarding. These methods have a sensitivity of only approximately 60% to 70% in babies, and slightly higher in adults, and therefore should not be the sole diagnostic determinant of infection.^{611,788} Cellular material obtained by scraping the periphery of the base of lesions should be smeared on a glass slide and promptly fixed in cold ethanol. The slide can be stained according to the methods of Papanicolaou, Giemsa, or Wright before examination by a trained cytologist. The demonstration of intranuclear inclusions and multinucleated giant cells are indicative, but not diagnostic, of HSV infection. Electron microscopic assays have limited use.⁶²⁷

Serologic diagnosis of HSV infection is clinically valuable in the counseling of patients regarding genital herpes provided that the assays are type specific. This is especially the case with the development of very sensitive and specific assays that distinguish HSV-1 gG-specific antibodies from those of HSV-2 gG-specific antibodies.¹²⁰⁵ Therapeutic decisions cannot await the results of serologic studies. Several commercial and Food and Drug Administration (FDA)-approved assays are available.

The development of PCR has revolutionized the diagnosis of HSV infections and also has allowed for detailed studies of disease pathogenesis. The experience with PCR indicates that it is the most useful tool for diagnosis of HSV encephalitis^{22,42,43,266,472,555,556,902,972,1159} as well as skin lesions—either orolabial or genital. Using primers from an HSV DNA sequence that was common to both HSV-1 and HSV-2 (either the gB or DNA polymerase genes), HSV DNA can be detected in the cerebrospinal fluid of patients undergoing brain biopsies for HSV encephalitis with a sensitivity of more than 95% and a specificity that approaches 100%.⁶¹⁸ False-negative assessments can be found when there is contamination of hemoglobin in the cerebrospinal fluid, when inhibitors are present, or very early in the course of disease.⁶¹⁸ PCR evaluation of cerebrospinal fluid can be used to follow therapeutic outcome in patients with HSV encephalitis. Persistence of HSV DNA in the cerebrospinal fluid of newborns with HSV encephalitis at the completion of antiviral therapy predicts poor neurologic outcome.⁵⁵⁶ PCR is being used to detect HSV DNA in lesions because it has improved sensitivity and specificity.^{219,220,421,1203} As the cost of PCR determinations decreases, an added benefit will be realized.

PREVENTION AND CONTROL OF HERPES SIMPLEX VIRUS INFECTIONS

Genital Herpes Simplex Virus Infections

Because of the increased awareness of genital herpes and neonatal herpes and its association with increased risk of acquisition of HIV, every effort should be made to prevent HSV-2 infections. Until a vaccine is proven efficacious, educational efforts must be developed for adolescents and those at greatest risk. The use of condoms should be promoted, as use significantly decreases the probability of acquiring genital HSV infection. Condoms are more effective in the prevention of infection of females than males.^{163,620,1204} Valacyclovir has been demonstrated to decrease but not totally prevent person-to-person transmission of genital herpes in discordant couples.²³⁴

Prevention of Neonatal Herpes Simplex Virus Infection

Surgical abdominal delivery is associated with the decreased transmission of infection when membranes are ruptured less than 4 hours; however, cesarean section has not been proven efficacious when membranes are ruptured for longer periods of time.¹²⁷

For women with a past history of genital HSV infection, some obstetricians prescribe an antiviral medication during the last 4 weeks of gestation, recognizing a decreased probability of virus excretion.²⁵ However, recent data indicate that despite maternal compliance, maternal transmission can still occur, resulting in severe neonatal HSV disease.⁸⁷⁷

For babies born to mothers with known primary infection during the third trimester of gestation, the American Academy of Pediatrics Red Book Committee recommends 10 days of high-dose intravenous acyclovir therapy followed by careful serial medical assessment.¹⁹ If a child is born through a known infected birth canal, surface cultures and careful follow-up are in order. Should the cultures become positive or the child ill, further evaluation (i.e., cerebrospinal fluid and blood assessment by PCR) are indicated with therapy initiated until a diagnosis is either confirmed or excluded.

At the time of presentation to the labor and delivery suite, a careful vaginal examination is of paramount importance. Although visualization of the cervix is often difficult, speculum examination for documentation of recurrent lesions is extremely important and should be attempted in all women, because it will guide management of the newborn (see later discussion).

Hospital Staff

At many institutions, a policy that requires transfer or medical leave for nursery personnel who have a labial HSV infection is impractical and causes an excessive burden in those attempting to provide adequate care. Temporary removal of personnel who have cold sores is advocated for some clinical services. Education regarding the risk of transmission of virus and the importance of hand washing when lesions are present should be repeatedly emphasized to healthcare workers. In addition, hospital personnel should wear masks when active lesions are present.

Prevention of Other Herpes Simplex Virus Infections

At the present time, the only mechanism for prevention of mucocutaneous HSV infection is to avoid contact with

infected secretions. There are no proven effective postexposure antiviral therapies or vaccines.

VACCINE DEVELOPMENT

Vaccination remains the ideal method for prevention of viral disease; however, prevention of HSV disease introduces unique problems because of recurrences in the presence of humoral and cell-mediated immune responses. Nevertheless, protection from life-threatening disease can be achieved in animal models with avirulent, inactivated, or subunit glycoprotein vaccines, each of which has unique differences. Excellent reviews summarize HSV vaccine development.^{298,515,1089,1090,1246}

Human HSV Vaccine Development

An important consideration for human HSV vaccines is the definition of expectations, warranting a distinction between infection and disease. From animal data, disease can be ameliorated if not prevented; however, infection still occurs. Similarly, the potential of altering the immune response to change the pattern of recurrences in previously infected individuals is unlikely. Thus, in human studies, a goal is to prevent disease, although it is highly unlikely that infection will be altered. Early investigations provide important insights into these expectations according to candidate vaccine: (a) wild-type virus, (b) inactivated or killed virus, (c) subunit vaccines, and (d) live vaccines.

Wild-type Virus Vaccines

Numerous investigators attempted to either prevent or alter the pattern of recurrences by inoculation of (a) autologous virus, (b) virus from another infected individual, or (c) virus recovered from an experimentally infected rabbit. In some cases, inoculation led to recurrences of latent infection.^{99,625} These live viruses were subsequently abandoned because many patients failed to develop lesions at the site of inoculation; therefore, it was concluded that the virus did not produce an “adequate take”,^{90,121,346} as compared to smallpox immunization.

Inactivated (or Killed) Virus Vaccines

Killed viruses were used as vaccines in various animal model systems, often with good results, as reviewed elsewhere.⁷³⁸ The initial inactivated vaccines were made from phenol-treated tissues obtained from infected animals.^{90,121,346} Because of the potential for demyelination after the administration of animal proteins, these vaccines attracted little biomedical attention. UV light inactivation of purified virus derived from tissue culture experiments provided greater impetus for the vaccine field. As reviewed,⁷³⁸ viral antigens obtained from various cell cultures were inactivated either by formalin, UV light, or heat and used administered to thousands of patients.¹²⁴⁰ Although decreased recurrences were reported in as many as 60% to 80% of patients,^{302,794} no controls were used.

Several important observations were made from these studies. First, despite repeated inoculations, antibody titers (as measured by neutralization or complement fixation) remained unchanged in most patients^{132,442,929} or demonstrated only slight increases.^{1072,1196} Second, although few side effects were reported, some authors noted concern that in patients with keratitis, autoimmune phenomena might make the herpetic

disease worse.^{640,1014,1073} Lastly, the need for placebo-controlled observations is imperative.

Subunit Vaccines

Subunit vaccines evolved from attempts to remove viral and cellular DNA, to enhance antigenic concentration and induce stronger immune responses, and, finally, to exclude any possibility of contamination with residual live virus. The immunogenicity of envelope glycoproteins was demonstrated in animals.^{154,155,746,1055} Generally, the subunit vaccines elicited some protection, as evidenced by reduction of morbidity and mortality in the immunized animals. However, the predictability of these models for human studies is disappointing. Furthermore, several injections are required to induce protection, and they must also include adjuvant. Protection in the rodent is significantly easier than in nonhuman primates. Vaccination of primates, specifically rhesus monkeys,¹⁵³ chimpanzees,^{153,154} and *Cebus* monkeys,⁴⁴⁹ induces neutralizing antibodies, which can lead to an amnestic response after subsequent injection months later.

Subunit vaccines have failed in human experiments despite initial promising results. Both HSV-1 and HSV-2 antigens have been prepared in human diploid cells and chicken embryo fibroblasts. The first extensive and thoughtful vaccine experience was reported for individuals receiving the Merck⁴⁴⁹ envelope subunit vaccine.^{37,1306} In uninfected sexual partners of patients known to have genital herpes, the number of individuals developing HSV infection was nearly equal for both placebo and vaccine recipients, indicating that the vaccine was not effective.

Further extensive studies have been completed with two gB and/or gD recombinant vaccines in humans. Initially, a gD-2 construct (30 μ g or 100 μ g with alum) was administered to 24 human volunteers to determine reactogenicity and immunogenicity. These studies demonstrated an increase in the geometric mean neutralizing antibody titer to gD-2; however, lymphocyte blastogenic responses were inconsistent.¹¹⁰⁴ Subsequently, gD-2 combined with a lipophilic muramyl tripeptide was shown to afford a high level of protection from HSV disease in animal models,^{138,992} although this adjuvant was abandoned because of reactogenicity. An important observation from these studies is that the quantity of neutralizing antibodies elicited by immunization and the total HSV antibody titer (as measured by enzyme-linked immunosorbent assay [ELISA]) were higher after vaccination than after natural infection.¹⁰⁹¹

The next series of experiments used alum and gD-2, demonstrating immune responses similar to natural infection¹¹⁰⁴ with the suggestion of decreased recurrent genital herpes.¹²⁴² A controlled clinical trial using gD-2 and gB-2 in a squalene oil and water emulsion (Chiron) failed to demonstrate efficacy for prevention of either infection or disease.²³²

A second candidate vaccine consisting of gD-2 with an alum and monophosphoryl lipid A adjuvant (GlaxoSmith-Kline) was shown to induce titers of HSV gD-specific antibody that were higher than those observed in patients who had sexually acquired genital HSV-2 infection.¹⁰⁹³ In these studies, women who were seronegative for both HSV-1 and HSV-2 were significantly protected from disease (72% efficacy; $p = 0.01$ – 0.02), and there was a trend toward protection against infection (43% efficacy; $p = 0.06$ – 0.07). However, in individuals seropositive for HSV-1, irrespective of sex, and seronegative

men, no significant clinical benefit could be demonstrated. Thus, this genital herpes vaccine (gD-Alum/MPL) was effective in preventing HSV-1 or HSV-2 genital herpes disease in a subset of volunteers—that is, women who were HSV-1 and HSV-2 seronegative (HSV1–/2–) prior to vaccination. However, these studies were neither designed nor powered to assess efficacy exclusively in HSV1–/2– women and therefore did not meet their primary endpoints of overall efficacy. The vaccine had no effect in women who had pre-existing antibodies to HSV-1 or men regardless of their serologic status. A large placebo-controlled study to verify these results has just been completed. The vaccine showed only 20% efficacy in preventing genital herpes disease overall (the primary endpoint) but did show 58% efficacy for preventing HSV-1 genital disease.⁷⁸ The vaccine showed only 22% protection from HSV infection overall but 35% efficacy for preventing HSV-1 infection. Notably, rates of HSV shedding were higher in the vaccine than the placebo group. Both of these subunit vaccines required administration on three occasions.

The difference in response to these two vaccines warrants comment. The Chiron gB-2 and gD-2 vaccine had an adjuvant that induced Th2 responses, whereas the later GlaxoSmith-Kline vaccine induced Th1 responses.

Live Virus Vaccines

For decades, live virus vaccines have been considered most likely to provide a high level of protection as occurs with measles, mumps, and rubella. Live viral vaccines replicate in the recipient, and the resulting immunity should both mimic natural host responses and be longer lasting. Moreover, these vaccines usually require smaller quantities of antigen and, therefore, should be more economical. Several approaches to live virus vaccines have been attempted, including HSV mutants, heterologous herpesviruses, antigens expressed in non-HSV viral vectors, and genetically engineered viruses.

GENETICALLY ENGINEERED HERPES SIMPLEX VIRUS VACCINES

Current technology allows the construction of recombinant HSV as prototype vaccines.^{739,954} These vaccines were engineered with the objectives that they should be attenuated so as not to cause primary disease or to reactivate; able to protect against HSV-1 or HSV-2 disease; provide biomarkers that distinguish vaccine from wild-type infection; and, potentially, serve as vectors to express immunogens of other human pathogens. Notably, there is overlap between engineered vaccine candidates and HSV constructs that are used for gene therapy (see later discussion).

The initial construction of candidate vaccines was based on the use of HSV-1. An engineered HSV was constructed that deleted the viral *TK* gene and the junction region of the U_L and U_S segments in order to excise some of the genetic loci responsible for neurovirulence as well as to create convenient sites and space within the genome for insertion of other genes. An HSV-2 DNA fragment encoding the HSV-2 glycoproteins D, G, and I was inserted in place of the internal inverted repeat. The purpose of the HSV-2 genes was to broaden the spectrum of the immune response and to create a chimeric pattern of antibody specificities. The resulting recombinant, designated as R7017, was further altered by insertion of the *TK* gene next to the HSV-2 DNA fragment (R7020). Because this virus expresses TK, it is susceptible to antiviral chemotherapy with

acyclovir. When analyzed by restriction enzyme digestion, the DNA of the recombinants has a unique pattern that facilitates their unambiguous identification.

When evaluated in rodent models, the two constructs were considerably attenuated in pathogenicity and ability to establish latency and were capable of inducing protective immune responses. The recombinants did not regain virulence on passage, nor did they change DNA restriction enzyme cleavage patterns when subjected to serial passages in the mouse brain.⁷⁴⁰ These results were corroborated by studies in owl monkeys (*Aotus trivirgatus*). Although 100 pfu of wild-type virus administered by peripheral routes was fatal to the monkeys, recombinants given by various routes in amounts at least 10⁵-fold greater were innocuous or produced mild infections, even in the presence of immunosuppression by total lymphoid irradiation.⁷⁴⁰ When R7020 was administered to humans to determine reactogenicity, immune responses were elicited¹⁴³; however, R7020 was not effective at the relatively low doses tested at that time.

The application of these principles to HSV-2 constructs was undertaken and assessed in animal models. The $\gamma_134.5$ gene of HSV-1 was proven to be a mediator of CNS replication, providing a potential vaccine construct upon deletion.¹⁹⁴ Furthermore, deletions of $\gamma_134.5$ significantly decrease the ability of this construct to establish latency.

One promising approach to an HSV vaccine is using replication-defective viruses.²⁹⁷ Although first studied over a decade ago,^{767,805} a current construct deletes U_L5 and U_L29 in HSV-2 to generate the dl5-29 vaccine candidate. Importantly, this vaccine candidate was compared to a gD-2 subunit vaccine in Freund's adjuvant. Significant protection was induced along with high titers of neutralizing antibodies and CD8⁺ T cells.^{299,468,469} This vaccine candidate is targeted for human trials in the near future.

A vaccine deleted for the *gH* gene was studied in a phase II trial in the United Kingdom. This vaccine, known as the HSV-DISC vaccine, undergoes a single cycle of replication. Because gH is deleted, no further replication is possible. The results of this phase II study failed to demonstrate effectiveness in reducing reactivation and disease in persons with recurrent HSV-2.²⁵⁴ An HSV-2 vaccine deleted for several genes involved in immune evasion (ImmunoVEX HSV-2) is in phase I clinical trials.

VECTORED GENE EXPRESSION AND OTHER APPROACHES

Vaccinia virus has been used as a vector for delivering antigens to animals or humans.¹⁰⁶² The principle of inserting foreign genes into a vaccinia vector was exploited for the expression of the gD and gB genes of HSV.⁸³⁹ Because of concern for the development of adverse effects to the vaccine (e.g., vaccinia gangrenosum and disseminated vaccinia), interest in this approach is virtually nonexistent. Furthermore, immune memory in individuals who have previously received vaccinia may prevent recognition of any foreign gene insert.

Adenoviruses have also been proposed as expression vectors on the grounds that they might be safer than vaccinia.⁷²⁵ Animal models show good efficacy when adenovirus expressing gD are administered.¹³¹³ Newer adenovirus platforms have been found in the HIV research field to stimulate strong CD8 responses to the inserted foreign gene,³⁰⁰ rendering this platform attractive for HSV. The varicella zoster virus vaccine strain has been engineered to express HSV glycoproteins. Two

of these constructs have been shown to afford animals protection against experimental HSV-2 genital herpes.^{438,439}

Other approaches being tested include plasmid DNAs encoding HSV genes and HSV-2 peptides complexed with heat shock proteins.

Summary

HSV vaccine development remains a high priority. Current approaches have relied upon prevention of infection as an endpoint of efficacy. Future studies will need to consider the impact of vaccines on amelioration of disease and rely on novel new constructs. As noted earlier, the most advanced at this time is a construct that deletes U_L5 and U_L29 in HSV-2 to generate the dl5-29 vaccine candidate. One such construct will consider the utilization of immunologically dominant T-cell epitopes as a means to improve immune responses.

ONCOLYTIC HERPES SIMPLEX VIRUS THERAPY

Genetically engineered HSVs have mainly been assessed for the treatment of human glioblastoma multiforme. These constructs have included mutations in the viral genes for TK, DNA polymerase, ribonucleotide reductase, and $\gamma_134.5$ ^{118,174,504,532,712,718,754} and have been identified as HF10, HSV1716, NV1020, G207, G47 Δ , OncoVEX^{GM-CSF}, rRp450, M032, and C134.^{166,654,697,739,755,813} Although virtually any alteration of HSV ameliorates neurovirulence, only the deletions in the $\gamma_134.5$ gene consistently demonstrate safety and efficacy in animal models. Tumorcidal effects *in vitro* and *in vivo* in multiple glioma models (mouse, rat, and human glioma cell lines; human glioma explants) are demonstrable. *In vivo* models include tumor reduction in subrenal capsule and flank subcutaneous implants but, more importantly, increased survival and some tumor cures in intracranial implant models. These effects are reproducible *in vivo* for both immune-deficient animals (nude, *scid* mice)^{174,712,718,754} as well as immune-competent models (rats and mice).^{117,504,532,753}

Animal Model Studies

Studies in animal models of gliomas of various constructs of HSV (engineered viruses deleted in $\gamma_134.5$) have been performed. These studies demonstrate the following principles: (a) the time course of infection (quantitative virology and PCR) represents impaired replication with limited spread of virus across the brain using marker genes (*lacZ* under an ICP6 promoter) with HSV antibody staining,^{504,532} (b) two selected mutations appear to avoid second-site mutations with reversion to wild-type phenotype ($\gamma_134.5$ and ribonucleotide reductase deletions),¹²⁹⁴ (c) the retention of the native HSV TK allows for acyclovir susceptibility,¹⁷⁴ (d) the safety of these constructs was established in susceptible primates (*Aotus*),¹²⁹⁴ and (e) HSV could be used to successful vector genes.^{24,201,362,845}

Indeed, one construct, namely G207⁷¹⁸ demonstrated an adequate safety profile in both cell culture and animal models^{478,1112} and was efficacious in several tumor models *in vivo*.^{118,174,504,532,712,718,754} This candidate therapeutic is deleted in both copies of the $\gamma_134.5$ gene as well as ribonucleotide reductase. Sufficient quantities of virus were produced under

good manufacturing practice (GMP) conditions for a phase I study in humans with recurrent glioblastomas, as described later.

Numerous other constructs have been developed, including cytokine/chemokine genes, enzymes, and receptors.⁷¹⁰ However, other investigators have taken the approach of altering host recognition of HSV by deleting the $\alpha 47$ gene and thereby allowing host MHC-I processing. The status of these viruses is summarized in the next eight construct.

HF10

HF10 is a nonengineered virus, derived following serial passage of the wild-type HSV-1 strain HF.⁸¹³ This virus is not neuroinvasive and was determined to have stable duplications in the *UL53*, *UL54*, and *UL55* genes, as well as deletion of the *UL56* gene. Notably, HF10 contains both native copies of the $\gamma_1 34.5$ neurovirulence gene; however, its lack of neuroinvasiveness is presumably attributable to the absence of *UL56*, or to *syn* alterations that may also have an effect.¹¹⁷⁶ HF10 has been evaluated in murine models of peritoneal tumor distribution as well as breast and bladder cancer.^{576,1122,1137} Preliminary clinical studies of HF10 demonstrated its safety following intratumoral injection into metastatic squamous cell carcinoma of the head and neck, as well as metastatic breast cancer.^{355,549} A phase I clinical trial investigating the safety of HF10 in patients with refractory head and neck cancer is recruiting patients. This study will directly inject 1×10^5 to 1×10^7 pfu into squamous cell carcinoma of the head and neck that failed standard of care chemotherapy and radiation (Clinical Trial ID NCT01017185).

HSV1716

HSV1716 is one of two conditionally replication-competent, engineered HSVs directly injected into malignant glioma with demonstrated safety and the absence of neurovirulence. HSV1716 has been clinically studied for the treatment of malignant glioma in the United Kingdom. A series of phase I studies of direct intratumoral and surrounding normal parenchyma administration of HSV1716 at doses ranging from 1×10^3 to 1×10^5 pfu proved safety and, subsequently, viral replication in the tumor.^{426,840,913}

HSV1716 has also been assessed as a therapy for oral squamous cell carcinoma (1×10^5 pfu of virus intratumorally) and melanoma with evidence of safety but no documentation of viral replication.^{693,696}

NV1020

First engineered as a vaccine to prevent HSV-1 and HSV-2 infections, NV1020 is notable in that it is deleted in only one of the two $\gamma_1 34.5$ genes.⁷³⁹ NV1020 has been studied as an oncolytic HSV for hepatic colorectal adenocarcinoma metastases at doses ranging from 3×10^6 to 1×10^8 pfu followed by chemotherapy 2 to 3 days later.⁵³⁹ Serious adverse events were minimal. Concomitant sampling of the hepatic vein for viral DNA by PCR detected little evidence of systemic exposure, a perceived risk of administering virus intra-arterially. Additional phase I studies have been performed that support the safety of this approach, including multiple infusions at doses of 1×10^8 pfu.^{357,364} Efficacy cannot be determined from these studies; however, the investigators concluded that NV1020 may render infected malignant cells moderately susceptible to chemotherapy regimens against which they are resistant.

G207

G207, derived from the HSV-1 (F) strain, is deleted for both $\gamma_1 34.5$ genes and is further attenuated by insertion of the *lacZ* gene into the *UL39* locus, preventing expression of ribonucleotide reductase. G207 was demonstrated to be safe following direct intracranial injection in both mice and in highly susceptible *Aotus nancymae* primates.^{478,1112}

The first phase I safety trial with G207 evaluated direct intratumoral injection of virus in escalating doses ranging from 1×10^6 to 3×10^9 pfu. A total of 21 patients with recurrent grade III to IV malignant glioma were enrolled in the trial.⁷¹³ Even at the highest dose (3×10^9 pfu), no significant adverse events related to G207 administration were reported. Importantly, no evidence of encephalitis was apparent in any biopsy samples taken to monitor tumor progression or at postmortem.

Additional phase Ib studies in patients with recurrent glioblastoma multiforme have utilized various designs to define safety upon injection into a resected tumor bed, the ability of the virus to replicate in tumors, the effect of multiple administrations, and the definition of the contribution of radiation to improved outcome.⁷¹¹ At total doses of 1.15×10^9 pfu of G207, the results continue to define safety, evidence of virus replication at the highest doses, radiographic improvement, and some long-term survivors. Further studies are in progress.

G47 Δ

G47 Δ , directly derived from G207 and thus lacking both $\gamma_1 34.5$ gene copies as well as containing a *lacZ* gene insertion within *UL39*, is additionally deleted for the $\alpha 47$ gene. This latter modification prevents G47 Δ from down-modulating MHC-I presentation and additionally alters the kinetics of *UL11* gene expression from late to early in infection.¹¹⁵⁰ These modifications increase the immunogenicity and replicative ability of G47 Δ without affecting neurovirulence.¹¹⁵⁰ G47 Δ reduces tumor mass in multiple models.¹¹⁴⁹ The *in vivo* efficacy of G207 and G47 Δ have been directly compared, with G47 Δ providing an equal or improved survival advantage over G207.^{659,1150,1186} A phase I/II clinical trial investigating the safety and efficacy of G47 Δ in recurrent glioblastoma multiforme is recruiting patients in Japan.

OncoVEX^{GM-CSF}

OncoVEX^{GM-CSF} is derived from the pathogenic clinical isolate JS-1.⁶⁵⁴ Attenuations in this vector include replacement of both $\gamma_1 34.5$ genes with the gene encoding human granulocyte-macrophage stimulating factor (GM-CSF) and deletion of the $\alpha 47$ coding sequence such that the *UL11* gene is expressed from the immediate-early $\alpha 47$ promoter. OncoVEX^{GM-CSF} is “armed,” meaning that it expresses a transgene hypothesized to enhance antitumor effects by complementing the lytic mechanism of tumor clearance. In the case of OncoVEX^{GM-CSF}, this additional mechanism is immune stimulation through the cytokine GM-CSF—a hematopoietic growth factor that promotes the maturation and development of DCs as well as elicits the formation of Th1 skewed immune responses. OncoVEX^{GM-CSF} is the first armed oncolytic HSV vector to be evaluated in clinical trials.

OncoVEX^{GM-CSF} has been evaluated in several phase I clinical trials enrolling patients with refractory cutaneous or subcutaneous metastases of breast, gastrointestinal adenocarcinoma, malignant melanoma, or epithelial cancer of the head

and neck.⁴⁷¹ Direct intratumoral injection of one or three doses (1×10^6 – 1×10^8 pfu) established safety. OncoVEX^{GM-CSF} is being evaluated in malignant melanoma.¹⁰²⁹ No severe events have been attributable to virus administration. In addition, 13 patients have achieved no evidence of disease (NED) status, 10 of which achieved this status through the effects of the vector alone, with no additional therapy. A randomized, controlled phase III trial has been under way since April 2009 to investigate OncoVEX^{GM-CSF} for the treatment of patients with nonresectable stage III and IV malignant melanoma.

The safety of OncoVEX^{GM-CSF} has also been demonstrated in stage III and IV head and neck squamous cell carcinoma (HNSCC) in a phase I/II trial. A randomized controlled phase III study is planned to establish efficacy.

rRp450

rRp450 is derived from the first-generation vector hrR3. hrR3 was engineered by replacing the *U_L39* gene encoding RR of the wild-type strain KOS with *lacZ*. rRp450 followed with the replacement of the *lacZ* genes with the *CYP2B1*, the gene encoding the rat cytochrome p450 enzyme responsible for prodrug metabolism.¹⁸⁰ Cytochrome p450 2B1 expression is hypothesized to serve as a method of concentrating active metabolites of chemotherapeutics in malignant cells. It is notable that rRp450 retains both native copies of $\gamma_{134.5}$. Preclinical studies with rRp450 demonstrated its selective replication in tumor cells with increased expression of mammalian ribonucleotide reductase, demonstrating a level of targeting afforded by the ICP6 deletion, as well as increased cytotoxicity of these cells when infection was combined with nitrogen mustard alkylating agents *in vitro*.¹⁸⁰ *In vivo* studies demonstrated synergistic efficacy of rRp450 and cyclophosphamide against a model of hepatic metastases of colon adenocarcinoma in mice.⁸⁵⁴

M032 and C134

The engineered HSV recombinants M032 and C134 are similar in that both were originally derived from the wild-type HSV-1 (F) strain. They share identical deletions of the $\gamma_{134.5}$ gene but differ with regard to the inserted genes replacing $\gamma_{134.5}$. M032 expresses the human immunostimulatory cytokine IL-12, which promotes the activation of IFN- γ secretion and Th1 polarization of CD8⁺ T lymphocytes and also has antiangiogenic effects. Preliminary studies of a murine IL-12 expressing virus M002 demonstrate increased survival in intracranial tumor-bearing mice treated with M002 and increased infiltration of innate and adaptive immune cells into tumors.⁸⁴⁵ An Investigational New Drug (IND) application has been submitted to evaluate the safety of M032 in patients with recurrent glioblastoma and is awaiting FDA approval. C134 expresses the CMV PKR evasion and IFN- γ resistance molecule IRS1.¹⁰³⁰ IRS1 has equivalent functions as the $\gamma_{134.5}$ gene product ICP34.5 in terms of PKR evasion; however, unlike ICP34.5, it does not contribute to neurovirulence. Cells infected with C134 do not undergo PKR-mediated host protein shutoff. This has been found to occur in neuroblastoma cell lines, prompting the submission of an IND application for a phase I clinical trial of C134 in pediatric patients with neuroblastoma.¹⁶⁶

Summary

Engineered HSV constructs have been proven consistently safe when administered intracranially to humans at high doses. With

some constructs, suggestions of efficacy are apparent. Likely, no one construct or approach will cure all tumors. Future work will need to improve replication competence without sacrificing safety, improved delivery, and a better understanding of host gene expression to optimize the oncolytic approach.

ANTIVIRAL THERAPY

Advances in the treatment of HSV infections have led the way in the development of antiviral therapeutics. In the 1970s, vidarabine (adenine arabinoside) became the first licensed antiviral therapeutic for the treatment for herpes simplex encephalitis and neonatal HSV infections, as well as varicella zoster virus infection. Quickly, however, vidarabine was replaced by acyclovir in the physicians' armamentarium for the treatment of all HSV infections. Today, acyclovir and its prodrug valacyclovir, as well as the prodrug of penciclovir, famciclovir, are the most useful and widely used therapeutics for the treatment of HSV infections. Both valacyclovir and famciclovir have a distinct advantage over acyclovir. Specifically, the oral bioavailability of acyclovir following valacyclovir administration allows for improved pharmacokinetics. The same case is also made of plasma levels of penciclovir following famciclovir administration. The mechanism of action of acyclovir and penciclovir are summarized in Chapter 13. Brief reference will be made to the use of these medications, as well as foscarnet and cidofovir, in the management of HSV infections.

Table 60.4 summarizes the use of the antiviral therapeutics in the treatment of HSV infections. A brief summary of the status of therapy for these entities is provided.

Life-threatening HSV infections, including herpes simplex encephalitis, neonatal HSV infection, and progressive or visceral disease in the immunocompromised host, are routinely treated with intravenous acyclovir. For infections involving the CNS, most notably herpes simplex encephalitis, mortality is decreased from 70% in placebo recipients to 25% in those who receive intravenous acyclovir therapy. Of the survivors, only one out of three returns to normal function. For neonatal HSV infection, multiorgan disseminated involvement, as defined earlier, can be treated with acyclovir with a resultant mortality of 25%. Neonatal HSV encephalitis is associated with a mortality of 5% following therapy. For both of these entities, survivors are left with significant neurologic impairment in most cases, namely 60%. Factors that predict a poor outcome include disseminated intravascular coagulopathy and prematurity for those babies with disseminated neonatal herpes, whereas for those with encephalitis seizures and prematurity predict a poor neurologic outcome.^{551,1241,1243,1249} These data indicate the requirement for improved therapies of HSV infections of the CNS.

Recently, 6 months of suppressive acyclovir therapy, following 3 weeks of intravenous therapy, has been shown to improve neurologic outcome for newborns with brain disease.⁵⁵³ In a placebo-controlled clinical trial, the treated babies had Bayley Developmental scores that were 30 points higher on average than the controls. These data suggest that low-grade continuing viral replication in the brain can be suppressed.

The concomitant use of monoclonal antibodies, as has been demonstrated in animal models, may prove effective adjunctive therapy. Similarly, the evaluation of anti-inflammatory

TABLE 60.4 Drugs for Herpes Simplex Virus

Infection	Drugs	Adult dosage ^a
Orolabial	<i>Topical</i>	
	Acyclovir:	5% cream 5×/d × 4 d
	Ointment	
	Docosanol	10% cream 5×/d until healing
	Penciclovir	1% cream applied q2h while awake × 4
	<i>Oral</i>	
Genital	Acyclovir	400 mg PO 5×/d while awake × 4
	Famciclovir	400 mg PO bid × 7 d
	Valacyclovir	2 g PO q12h × 1 d
	First episode	
	Acyclovir	400 mg PO tid or 200 mg PO 5×/d × 7–10 d ^b
	Famciclovir	250 mg PO tid × 7–10 d
	Valacyclovir	1 g PO bid × 7–10 d
	Episodic treatment of recurrences	
	Acyclovir	800 mg tid or 400 mg PO tid × 3–5 d
	Famciclovir	125 mg PO bid × 5 d
Suppression of recurrences	Valacyclovir	500 mg PO bid × 3 d
	Acyclovir	400 mg PO bid
	Famciclovir	250 mg PO bid
Mucocutaneous (immunocompromised)	Valacyclovir	500 mg or 1 g PO 1×/d
	Acyclovir	5 mg/kg IV q8h × 7–14 d or 400 mg PO 5×/d × 7–10 d
	Famciclovir	500 mg bid × 7–10 d
Encephalitis	Valacyclovir	500 mg or 1 g bid × 7–10 d
	Acyclovir	10–15 mg/kg IV q8h × 14–21 d
Neonatal	Acyclovir	20 mg/kg IV q8h × 14–21 d
Keratoconjunctivitis	Trifluridine	1% solution 1 drop q2h (max 9 drops/d)
Acyclovir resistant (severe infection, immunocompromised)	Foscarnet	40 mg/kg IV q8h × 14–21 d

^aDosage adjustment may be required for renal insufficiency.

^bFor severe initial genital herpes, IV acyclovir (5–10 mg/kg q8h for 5–7 d) can be used.

Note: Generic and trade names of drugs are as follows: acyclovir (Zovirax), docosanol (Abreva), penciclovir topical (Denavir), famciclovir (Famvir), valacyclovir (Valtrex), trifluridine (Viroptic), and foscarnet (Foscavir).

drugs, namely corticosteroids, early in the course of neurologic disease, might be beneficial. A lipophilic derivative of cidofovir, CMX001, may help to improve outcome, because it penetrates the CNS to a greater extent than acyclovir and does not have nephrotoxicity. Regardless, alternative therapeutic strategies for both herpes simplex encephalitis and neonatal HSV infections need to be developed.

Ocular HSV infections are routinely treated with topical trifluorothymidine with or without concomitant oral acyclovir, valacyclovir, or famciclovir therapy. Topical vidarabine and idoxuridine have also been used for ocular infections. Acyclovir, and likely valacyclovir and famciclovir, can be used to suppress recurrences of HSV keratoconjunctivitis. However, it should be noted that no clinical trials of valacyclovir or famciclovir have been performed for this entity.

The most significant advances in antiviral therapy have been achieved in the management of mucocutaneous HSV infections. The prototypic treatment and suppressive regimens have been developed for the management of genital HSV infec-

tions. Acyclovir, valacyclovir, and famciclovir are efficacious in the treatment of both primary and recurrent genital HSV infections. Valacyclovir and famciclovir⁹⁷⁹ can be administered to patients on a suppressive basis to prevent reactivation of infection.⁵⁵² Although these medications significantly reduce the frequency of recurrences, and therefore the probability of reactivation, periodic excretion of virus still occurs during suppressive therapy and can result in person-to-person transmission of infection. Indeed, even valacyclovir at approved doses does not prevent intermittent shedding of HSV.²³⁴ In a seminal clinical trial, valacyclovir has been shown to decrease person-to-person transmission by approximately 48% when drug is administered once daily at 500 mg.²³⁴ Suppressive therapy can be successfully achieved with valacyclovir administered once daily, whereas famciclovir requires twice-daily dosing for suppression. Both valacyclovir and famciclovir^{28,979} can be administered for short periods of therapy (3 days and 1 day, respectively) to treat recurrences of genital herpes with efficacy compared to standard treatment regimens.^{517,554}

Acyclovir, valacyclovir, and famciclovir are approved for the treatment of herpes labialis. As with genital HSV infection, short-course therapy provides excellent therapeutic results. At the present time, although acyclovir has been studied for the management of HSV gingivostomatitis, registrational trials have not been submitted to the FDA.

HSV infections in the immunocompromised host pose unique challenges. Intravenous acyclovir is used routinely for progressive and/or life-threatening disease. Valacyclovir and famciclovir are useful in the management of HSV infections in individuals with HIV/AIDS. However, in some patient populations, progressive disease occurs in the presence of intravenous acyclovir therapy, implying the development of resistance. The propensity to develop resistance to these medications is greatest in hematopoietic stem cell transplant recipients and in individuals who have advanced HIV/AIDS with low CD4 counts. When HSV isolates are mapped for resistance, the most common mutations are found in TK. Alternatively, mutations have been detected in HSV DNA polymerase, rendering acyclovir triphosphate and penciclovir triphosphate ineffective. Under such circumstances, foscarnet or, rarely, cidofovir, drugs with alternative mechanisms of action have been employed.

Future Needs

The development of new antiviral agents for the management of HSV infection has been stymied by the overwhelming success of drugs such as acyclovir, valacyclovir, and famciclovir. In large part, the pharmaceutical industry does not identify a niche for the development of future antiviral drugs in this area. Regardless, the persistent mortality and elevated morbidity in patients with HSV infections of the CNS defines a medical need for which improved therapy should be developed. Recently, a new helicase/primase inhibitor has been shown to be safe and effective for the treatment of genital HSV infection.¹¹⁶⁷

B VIRUS

B virus, endemic in macaque monkeys, has the unique distinction of being the only one of nearly 35 identified nonhuman primate herpesviruses that is highly pathogenic for humans.¹²³⁷ The unique biology of B virus includes its neurotropism and neurovirulence. Because untreated B virus infections are associated with high mortality in humans, individuals handling macaques or macaque cells and tissues are at risk for infection. Human infection is associated with a breach of skin or mucosa and subsequent infection with virus. Fomites and contaminated particulates or surfaces can serve as a source of virus infection. Human-to-human transmission has been documented. Prior to the advent of antiviral therapy, the mortality was 80%. However, since the early 1980s, 80% of infected individuals have survived.

History

In 1932, a young physician (WB) was bitten by a monkey and later developed localized erythema at the site of the animal bite that was followed by lymphangitis, lymphadenitis, and, ultimately, a transverse myelitis with demise ascribed to respiratory failure. Autopsy tissue specimens revealed an ultrafilterable agent recovered from neurologic tissues that caused a cytopathic effect in tissue culture similar to HSV.³⁶³

Within a year of this first report, Sabin independently also found an ultrafilterable agent in the tissue of this index patient. He identified this as “B” virus,⁹⁷⁸ naming the virus by the initial of the patient’s last name. Thus, the virus subsequently has been called *herpes B virus*, *herpesvirus simiae*, *Cercopithecine herpesvirus 1*, or more simply, *B virus*. The virus was also noted to share similarity with pseudorabies, among other viruses, including SA8 and two additional nonhuman primate alphaherpesviruses: HPV-2 and Langur herpesvirus.^{139,140,307,926} Because of neurovirulence in a foreign host, B virus is considered by some to be one of the most dangerous occupational hazards for those who work directly with macaques or their tissues or with monkeys who have been in contact with macaques.

Disease Pathogenesis and Clinical Presentation

The most striking characteristic of human B virus infection is the involvement of the CNS, specifically the upper spinal cord and brain stem. These areas are the principal sites for virus replication. For the most part, human disease reflects the sites of viral replication; however, there is great variability in presentation. Most often, illness is apparent within days to weeks; however, in some cases, there appears to be a delay in development of acute disease. The reasons for this delay are unknown, and although rare, delays may even range from months to years, making diagnosis difficult. Once symptoms appear, the clinical progression is associated with relatively consistent symptoms, including influenza-like illness, lymphadenitis, fever, headache, vomiting, myalgia, cramping, meningeal irritation, stiff neck, limb paresthesias, and urinary retention with an ascending paralysis culminating in respiratory failure, requiring ventilatory support. Cranial nerve signs, such as nystagmus and diplopia, are common in most published cases. Sinusitis and conjunctivitis have been observed in some cases.¹²³⁷ The array of symptoms may be related to the dose of virus with which the individual was infected or the route of inoculation. Ophthalmic zoster-like symptoms have been reported as well,³³³ and in one particular case, reactivation of latent infection was postulated. A summary of descriptions of human cases can be found in a comprehensive review.¹²³⁷

Virus can be recovered at skin sites of inoculation for extended periods of time, and viral DNA can be detected in cerebrospinal fluid by the time neurologic symptoms are experienced. Virus has also been recovered from throat, buccal, and conjunctival sites as well as from lesions, vesicles, or injury sites as late as weeks to months after infection. Most clinical cases are associated with bites (50%), fomites, (8%) saliva (<5%), and aerosols (10%). Histopathologic findings included edema and degeneration of motor neurons. Even with advanced disease, Cowdry type A eosinophilic intranuclear inclusions are uncommon. Gliosis and astrogliosis are late histopathologic findings; thus, there can be evidence of myelitis, encephalomyelitis, or encephalitis, or combinations of each of these conditions. Histopathologic examination of the eye has revealed a multifocal necrotizing retinitis associated with a vitritis, optic neuritis, and prominent panuveitis. B virus can infect and destroy retinal tissue similar to that of other herpesviruses.

The highest percentage of deaths occurs within a few weeks after onset of disease. In some cases, however, life was prolonged artificially for months or years. Surviving cases have varying degrees of morbidity, ranging from little or no neurologic impairment to more extensive CNS involvement.^{31,122,128}

Some survivors experience slow neurologic decline, whereas others report few, if any, long-term effects. In several reports, ocular disease has been reported.³³³

Control of B Virus Infection

Multiple levels of prevention are used to prevent B virus infection in both humans and nonhuman primates, ranging from attempts to eliminate virus from colonies to designing methods to work safely in environments where there is increased risk for contracting this agent. The Centers for Disease Control and Prevention (CDC) has published detailed guidelines for maximizing protection of individuals working with macaque monkeys.^{169,170,216,458} Further, the National Institutes of Health's (NIH) National Center for Research Resources has funded the development of B virus-free colonies for NIH-funded research involving these animals in an attempt to eliminate this virus from colonies used for biomedical research. Nonetheless, B virus-infected monkeys are plentiful and can be handled safely if strict guidelines are followed, including barrier precautions.

When B virus is detected, inactivation is accomplished with either heat or formaldehyde.¹²³⁷ Other virus inactivators include detergents and bleach; however, individuals who are working in a decontaminated area should still be alert for injury prevention. Minimizing fomites decreases worker risk and reduces virus spread among animals. One B virus infection in a human was acquired from a cage after sustaining a scratch, underscoring that surface decontamination can play an important role in infection control.

No vaccine is available; thus, only antiviral therapy is recognized as effective in prevention when administered early after exposure.^{114,217,458,1224} For B virus disease, acyclovir and the related family of nucleoside analogs were noted to be effective when given in high doses—for example, acyclovir at 10 mg/kg intravenously three times daily for 14 to 21 days.⁴⁵⁸ Some physicians follow intravenous therapy with long-term oral suppression (acyclovir, valaciclovir, or famciclovir). Ganciclovir, which has a greater efficacy *in vitro* and thus was used in a few cases since 1989 with success, has been recommended by some experts as the treatment of choice for CNS disease associated with B virus. Antivirals are used by an increasing number of facilities for postinjury prophylaxis or after laboratory results indicate that an animal may have been actively infected around the time of the exposure. Postinjury prophylaxis has been performed with acyclovir, famciclovir, or valaciclovir, and these have demonstrated efficacy *in vitro*. Recommendations and guidelines have been published by the CDC and can be readily accessed.²¹⁶ Only a handful of physicians have had experience in the treatment of B virus zoonosis, and their participation and expertise has been important in the development of the CDC guidelines.

Finally, with respect to prevention, the value of immediate wound cleansing and first aid after a potential exposure owing to a bite, scratch, splash, or other suspicious injury is very important. Guidelines for wound cleaning are described in detail by the CDC (<http://www.cdc.gov/herpesvirus/firstaid-treatment.html>). Every institution working with macaques should have an injury protocol with immediate availability of first aid, a secondary care plan, and an infectious disease specialist who is a member of the institution's prevention and care response team.

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Epstein-Barr Virus

History

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Virus Structure

Genome Structure

Steps in Replication

Attachment and Entry

Transcription: Transformation of Primary B Lymphocytes by EBV

Translation: EBV Gene Products Associated With Latency

EBV Gene Products Associated With Lytic Replication

Entry into Host: Portals of Entry, Site of Primary Replication, Cell Types Infected

Immune Response

Innate Immune Response

Antibody Response

Cellular Immune Response

Immune Escape

Epidemiology

Primary Infection

Healthy Carriers

Infectious Mononucleosis

EBV-Associated Diseases

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Chronic Active EBV and Related Disorders

EBV Lymphoproliferative Disease: Congenital, Iatrogenic, and Posttransplant

Burkitt Lymphoma

Hodgkin Lymphoma

Nasopharyngeal Carcinoma

Gastric Carcinoma

Non-Hodgkin Lymphomas and Other Malignancies in Nonimmunocompromised Persons

EBV and HIV

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Hodgkin Lymphoma

Nasopharyngeal Carcinoma

Other Therapies

Prevention and Vaccines

Perspective

Acknowledgments

HISTORY

While descriptions of patients with symptoms resembling infectious mononucleosis were reported in the medical literature in the late 19th century, infectious mononucleosis was first named in 1920.¹³⁸⁷ The initial description of the use of heterophile antibodies to diagnose infectious mononucleosis was in 1932.¹¹⁴³ In 1958, Denis Burkitt¹⁴⁴ described a tumor involving the jaws in Africans in areas where malaria was holoendemic, and this tumor is now referred to as Burkitt lymphoma. Epstein-Barr virus (EBV) was discovered using electron microscopy to detect viral particles in cultured Burkitt lymphoma cells in 1964 by Epstein et al.³⁶³ Gertrude and Werner Henle⁵⁶⁰ described an immunofluorescent antibody test for the virus in 1966 and found that patients with Burkitt lymphoma as well as most Americans had antibody to EBV. In 1968, EBV was shown to be a cause of infectious mononucleosis.⁵⁶² Three years later, in 1971, EBV was found to transform human B lymphocytes in cell culture,¹⁰⁹⁴ and in 1984, the virus was shown to replicate in human epithelial cells.¹³⁵⁴ Strong epidemiologic evidence that EBV was a cause for Burkitt lymphoma came from pioneering studies of de-The and colleagues.³⁰³ Since its initial discovery in Burkitt lymphoma, the virus was found in nasopharyngeal carcinoma in 1970,¹⁶⁴⁵ in non-Hodgkin lymphoma in patients with acquired immunodeficiency syndrome (AIDS) in 1982,¹⁶³⁵ in T-cell lymphoma in 1988,⁶⁹⁸ and in Hodgkin lymphoma in 1989.¹⁵³⁸ The virus has been associated with other malignancies. Its relationship to other diseases such as multiple sclerosis and systemic lupus erythematosus is uncertain.

CLASSIFICATION

The gammaherpesvirus subfamily consists of the gamma 1 or *Lymphocryptovirus* (LCV) genus, which includes EBV and other primate lymphocryptoviruses, and the gamma 2 or *Rhadinovirus* (RDV) genus, which includes herpesvirus saimiri (HVS) and Kaposi's sarcoma-associated herpes virus (KSHV) as well as related viruses of primate and non-primate species.^{15,1256} Many Old

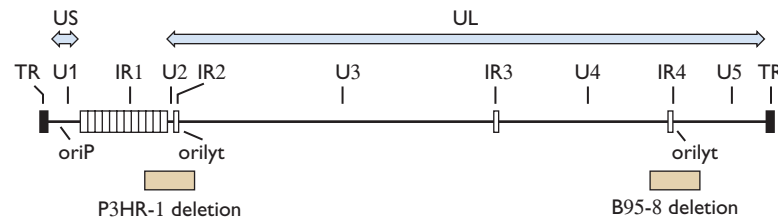


FIGURE 61.1. Schematic depiction of the linear Epstein-Barr virus (EBV) genome. A: Linear representation of the overall genome arrangement with the unique short (US) and unique long (UL) regions shown. Within the genome the terminal repeats (TR), internal repeats (IR1 to 4), and unique sequence domains (U1 to U5) of the EBV genome are depicted in proportion to their overall size. The position of the *cis*-acting element for episome maintenance and replication in latent infection, oriP, is indicated. The origins for EBV DNA replication in lytic infection (oriLyt) are in U3 and U5 just to the right of IR2 and IR4, respectively. The deletions in B95-8 and P3HR-1 genomes are shown.^{540,681,700}

World and some New World primate species have endemic LCVs that are collinearly homologous to EBV.^{114,229,230,375,376,451,452,551–553,1226,1227} In contrast, RDVs are endemic not only in most primate species but also in many subprimate mammalian species.^{14,16,288,361,1005,1433} Overall, the nucleotide sequences of gammaherpesvirus genomes have substantial collinear homology and much less co-linearity and homology with alpha- and betaherpesvirus genomes.^{288,1005}

Amongst the gammaherpesvirus genomes, LCVs are likely to have evolved from RDVs. RDVs have more diverse genomes than LCVs, are endemic in more diverse mammalian species, and characteristically have multiple cell complementary DNAs (cDNAs) in their genomes.^{288,1005} Old World LCVs have collinear homologous genomes with a unique group of recently evolved nuclear protein genes, which lack homology to RDV or cell genes and are only partially present in New World LCV genomes.¹²²⁶ New World LCVs may be intermediates in the evolution of Old World primate LCVs.

Aside from the absence of two nuclear protein 3 genes in the marmoset LCV genome, LCV genomes are very similar to each other in structure, gene organization, and collinear homology. The LCV genome shared features include 0.5–kilobase pair (kbp) tandem terminal direct repeats (TR), 3-kbp tandem internal direct repeats (IR1), and short tandem internal direct repeats (IR2, IR4)^{280,281,468,1198} (Fig. 61.1). RDVs have longer and more highly reiterated TRs and lack long internal direct repeats. Old World primate LCV genes encode latency 3–associated nuclear antigen proteins EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-1, which are expressed when EBV growth transforms primary B lymphocytes to lymphoblastoid cell lines (LCLs). LCVs also include genes that encode integral membrane proteins important for efficient latency 3 infection—associated B-lymphocyte growth transformation and survival,^{388,730,921} genes that encode two small nonpolyadenylated EBV-encoded small RNAs (EBERs),^{615,859} genes that encode differentially spliced Bam A rightward transcripts, genes that encode an interleukin-10 (IL-10) and a bcl-2 homolog,^{1043,1144} genes that encode distinctive microRNAs (miRNAs),^{151,1162,1163} and a gene that encodes a glycoprotein that binds to the B-lymphocyte surface protein, CD21.^{1080,1427,1428}

The EBV genome and gene organization is shown in Figures 61.1 and 61.2.¹²⁷⁹ EBV was the first herpesvirus genome to be completely cloned in sets of overlapping fragments,^{280,1198} based in part on previously derived restriction endonuclease

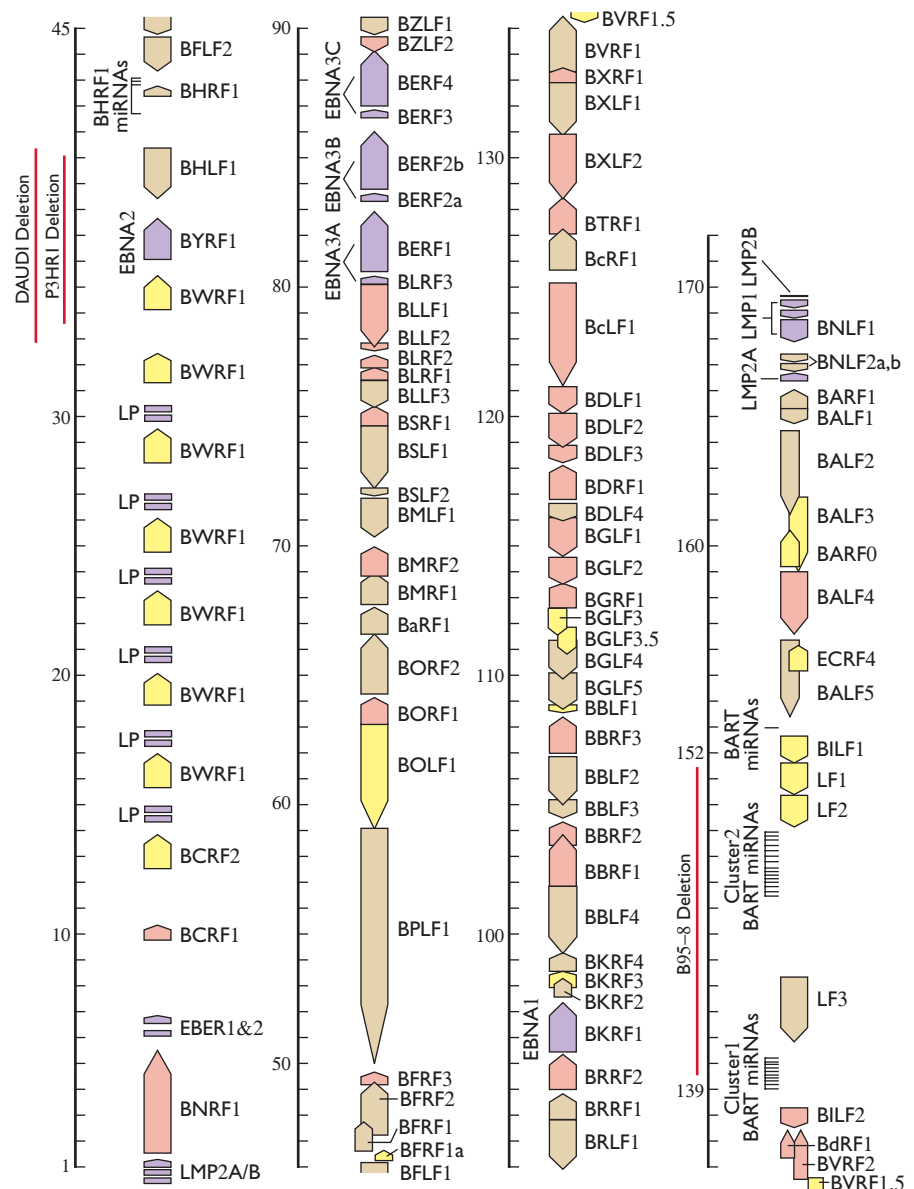
maps.⁴⁶⁶ The EBV genome was subsequently sequenced from BamHI-digested cloned DNA fragments⁶⁵ and open reading frames (ORFs) were designated by their order in a rightward or leftward direction within each BamHI fragment (Fig. 61.2). The majority of KSHV-predicted ORFs are collinearly homologous to ORFs of EBV.¹²⁵⁶ The mean amino acid identity to EBV among the 54 homologous KSHV ORFs is 35%, with only two ORFs less than 21%.

Herpesvirus DNAs have conserved distantly homologous gene blocks with conserved gene order and collinear homology at the predicted protein level.^{288,1005} Gammaherpesvirus genes conserved with alpha- and betaherpesviruses are primarily genes that encode proteins involved in nucleotide metabolism, proteins that replicate and process viral DNA, and proteins that make up the structural components of the virion capsid, tegument, and envelope. Genes that are at least partially shared among the gammaherpesviruses and have more limited representation in the genomes of other herpesviruses include BZLF1 and BRLF1, which encode the immediate early regulators of viral gene expression; BALF1, which encodes a bcl-2 homolog; and LMP1 and LMP2, which encode integral membrane proteins with 6 and 12 membrane-spanning domains, respectively. LCVs and RDVs also have analogous but nonhomologous DNA sequences and nuclear proteins that are necessary and sufficient for persistence of the genomes as episomes in dividing cells.^{72,266,1588}

The gammaherpesvirus subfamily classification was initially established on the basis of biologic properties including oncogenic associations, restricted host range for infection in cell culture, and episome persistence in dividing cells. Gammaherpesviruses also establish latent infection in lymphocytes but are not unique in this regard. Betaherpesviruses such as human herpesvirus 6 (HHV-6), HHV-7, and even cytomegalovirus (CMV) can also latently infect lymphocytes and other hematopoietic cells. Segregation into the gammaherpesvirus subfamily based on nucleotide and protein sequence homology and genome collinearity provides a more enduring basis for phylogenetic classification.^{288,1005} Taxonomists have named EBV as HHV-4. Although most authors still use the name EBV, some indexing services have adopted HHV-4.

Two EBV types circulate in most populations. EBV types 1 and 2 (also named types A and B) differ largely in nuclear protein genes that encode leader protein (EBNA-LP), EBNA-2, EBNA-3A, EBNA-3B, and EBNA-3C, which are expressed in latency 3 growth transformation of B cells to lymphoblastoid

FIGURE 61.2. Schematic depiction of the linear Epstein-Barr virus (EBV) genome with encoded open reading frames (ORFs). EBV ORFs are named based on their location (first ORF, second ORF, etc.) and direction (rightward or leftward) within the Bam H1 fragment (A, B, C, and so on, in decreasing size).⁶⁵ The prototypic EBV type 1 genome DNA sequence (171,820 bp) found in the National Center for Biotechnology Information (NCBI) is actually a composite of two viral genomes (B95-8 and Raji) due to deletions within the viral genome during passage in tissue culture.^{65,1136,1187} Size and direction of the ORFs are indicated, as are the noncoding RNAs EBER1 and EBER2. The latency-associated ORFs or exons that make up these ORFs are colored purple. The early lytic ORFs are beige and late lytic ORFs are shaded with red. Yellow arrows depict ORFs of unknown gene class or are hypothetical. LMP2A and LMP2B are encoded across the circularized genome. The locations of microRNA (miRNAs) encoded by the genome are shown. Numbers refer to kilobase pairs. (From Sample JT, Marendy E, Hughes DJ, et al. The Epstein-Barr virus genome. In: Damania B, Pipas JM, eds. *DNA Tumor Viruses*. New York: Springer, 2009, with kind permission from Springer Science+Business Media B.V.)



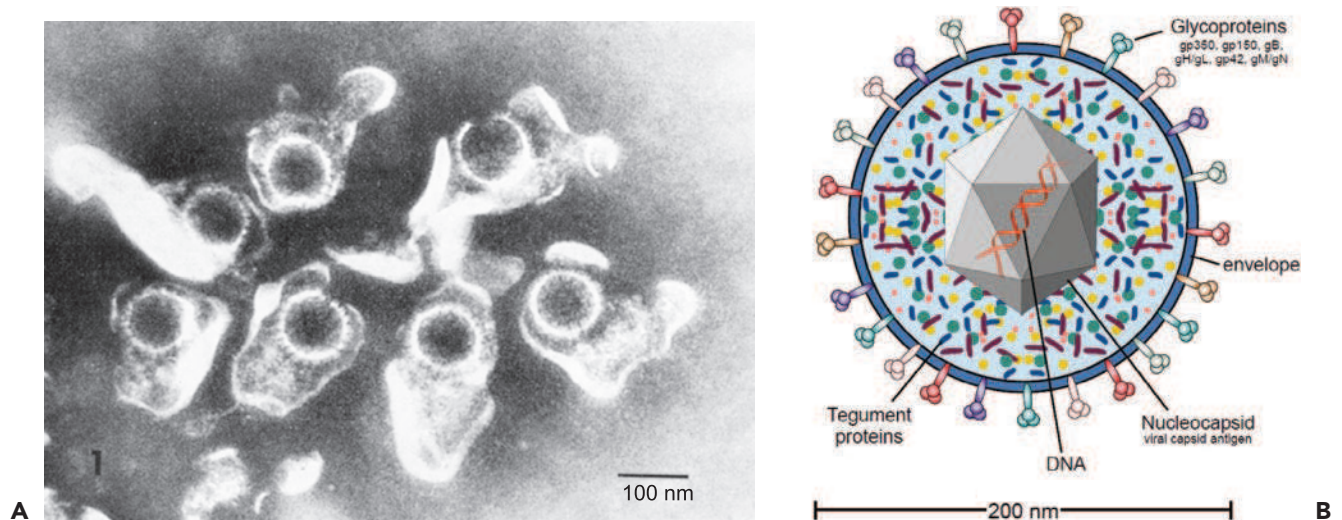


FIGURE 61.3. Electron micrograph and schematic diagram of Epstein-Barr virus (EBV) virions. A: EBV particles purified from the supernatant of B95-8 marmoset cells. Bar represents 100 nm. (A from Miller G, Lipman M. Comparison of the yield of infectious virus from clones of human and simian lymphoblastoid lines transformed by Epstein-Barr virus. *J Exp Med* 1973;138[6]:1398–1412, © 1973, Rockefeller University Press.) B: Diagram of EBV virion. The major structural components of the virion are shown including the virion envelope, which is studded with glycoproteins important for binding and subsequent fusion of the virion envelope with cellular membranes; the tegument, which contains both virus-encoded proteins and cellular proteins; the capsid; and the viral genome.

protein (BOLF1), 15-kD myristylated protein (BBLF1), 32-kD myristylated protein-binding protein (BGLF2), 58-kD capsid-associated protein (BVRF1), 58-kD packaging protein (BGLF1), 27-kD palmitylated protein (BSRF1), and 47-kD serine-threonine protein kinase (BGLF4), which are common components of herpesvirus teguments.⁶⁹⁵ In addition, EBV has a 140-kD major tegument protein (BNRF1), 19-kD BLRF2, 72-kD BRRF2, 54-kD BDLF2, and 42-kD BKRF4, which are gammaherpesvirus specific.⁶⁹⁵ Cell proteins actin, HSP70, cofillin, β -tubulin, enolase, and Hsp90 are also significant components of the EBV tegument, potentially related to cytoplasmic re-envelopment. The major glycoprotein components of the envelope are gp350 (BLLF1), gH (BXLF2), gB (BALF4), gp42 (BZLF2), gM (BBRF3), gp78 (BILF2), gN (BLRF1), gp150 (BDLF3), and gL (BKRF2).⁶⁹⁵ EBNA-1, LMP1, LMP2, gp64 (BILF1), BMRF2, and herpes simplex virus (HSV) homologs UL7 (BBRF2), UL14 (BGLF3), UL31 (BFLF2), and UL34 (BFRF1) are not detected in EBV virions.

GENOME STRUCTURE

The EBV genome is a linear, double-stranded DNA composed of 60 mole percent guanine and cytosine.^{65,758,1185} The prototypic EBV type 1 genome DNA sequence (171,820 bp) found in the National Center for Biotechnology Information (NCBI) is actually a composite of two viral genomes B95-8 and Raji due to deletions within the viral genome during passage in tissue culture (Fig. 61.1).^{65,1136,1187} Deletion during extensive passage in culture is a common feature of EBV because three other commonly used viruses, Daudi, Raji, and P3HR-1, also contain deletions.^{540,681,700} Recently, type 2 strains have been sequenced. The prototypic type 2 strain deposited in NCBI is 172,760 bp and as described earlier is highly homologous to type 1 DNA.³¹⁸

The characteristic features of EBV and most other lymphocryptovirus genomes include (a) a single overall format and gene arrangement^{280,466–468,1198}; (b) 2 to 5 tandem, 0.5-kbp, direct repeats of the same sequence at both termini (TR)⁴⁶⁸; (c) 6 to 12 tandem reiterations of a 3-kbp, internal direct repeat (IR1)²¹⁹; (d) short and long largely unique sequence domains (US and UL) that have almost all of the genome coding capacity; (e) perfect and imperfect tandem DNA repeats, most of which are within ORFs; and (f) a duplicated region, IR2, near the left end of UL, which consists of multiple, highly conserved, G-C-rich, tandem 125-bp repeats and 2 kbp of adjacent unique DNA, all of which have extensive homology to a region, IR4, near the right end of UL. IR4 is composed of G-C-rich, tandem 102-bp repeats and 1 kbp of nearby unique DNA.²⁸⁴ The IR2 and IR4 repeats are within ORFs. Because the IR2 repeat is 125 kbp and not divisible by three, sequential iterations are translated in different reading frames, resulting in an unusual protein. IR2 and IR4 also include the origins for initiation of viral DNA replication.^{524,1260}

The reiteration frequency of the EBV tandem perfect repeats becomes variable during viral DNA replication, with the average number of repeats being identical to the parent genome and most of the progeny having similar numbers of repeats as the parental genome. When EBV infects a cell, the genome becomes an episome with a characteristic number of TRs, dependent on the number of TRs in the parental genome and the unique cleavage and joining events of the single infecting viral genome. If infection is nonpermissive for viral replication and permissive for latent infection and continued cell growth, each EBV episome in progeny-infected cells will usually have the same number of TRs as the parent cell. Homogeneity or heterogeneity in the number of TRs is therefore useful in determining whether a group of latently infected cells arose from a single common progenitor infected cell or from multiple

progenitors.¹¹⁹⁹ The number of 3-kbp IR1 repeats also varies among EBVs and among progeny of EBV replication but is more difficult to assess as a marker of heterogeneity because of the 18- to 30-kbp total size. Other, smaller, imperfect, or less highly reiterated repeats within ORFs are more stable during replication but differ sufficiently among different virus isolates that the size of the encoded proteins can be used to uniquely identify each isolate.^{554,563-568,1014,1633}

STEPS IN REPLICATION

Attachment and Entry

EBV uses different glycoprotein combinations to infect B cells and epithelial cells (Fig. 61.4), but similar to other human herpesviruses, the core fusion complex consists of gB and gH/gL. gB and gH/gL were originally designated gp110 and gp85/gp25, respectively, but because of homologous proteins found

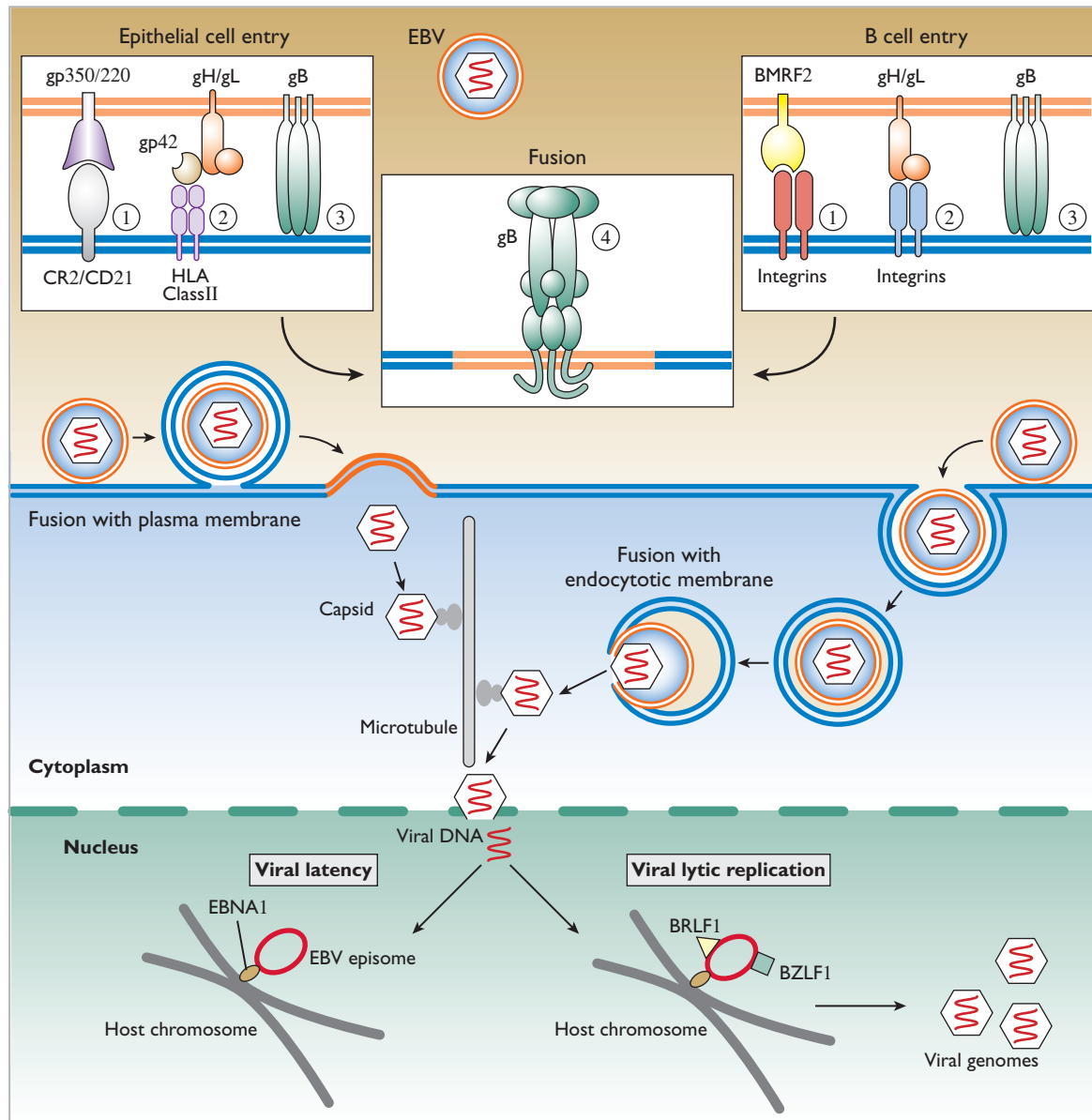


FIGURE 61.4. Schematic representation of Epstein-Barr virus (EBV) entry into epithelial and B cells. EBV enters epithelial cells by fusion at the plasma membrane and B cells by fusion with an endocytic membrane after endocytosis. For B-cell entry, EBV virions first bind to CR2/CD21 via gp350/220 (1). Following this interaction, gp42 binds to human leukocyte antigen (HLA) class II (2), triggering fusion mediated by gH/gL and gB (3). For epithelial cell entry, EBV virions may first bind via an interaction of integrins with BMRF2 (1). Following this binding, gH/gL binds to integrins (2) and triggers fusion mediated by gB (3). Following fusion, the capsid is released into the cytoplasm and is transported to the nuclear membrane by microtubule-mediated transport. The EBV genome is released into the nucleus through a nuclear pore. Latency can result, in which the genome is tethered to host chromosomes via an interaction with Epstein-Barr virus nuclear antigen 1 (EBNA-1). Lytic replication resulting in the production of viral genomes may also result, induced by the BRLF1 and BZLF1 EBV gene products.

in all human herpesviruses, these proteins are now generally referred to as gB and gH/gL. EBV gB is the fusogen for viral entry. The gB structure is composed of trimers nearly identical to the structure of HSV gB.^{64,548} The solved gB structure likely represents a postfusion conformation because the structure is very similar to the low-pH or postfusion form of the G protein of vesicular stomatitis virus.¹²³³ The mechanism of entry into two major target cells of EBV follows.

B Lymphocytes

B lymphocytes and epithelial cells are the major sites for EBV infection in the human host. EBV binding to B cells is mediated by CD21, also known as complement receptor 2 (CR2). Reflecting the importance of CD21 for B-cell infection, CD21 is abundantly expressed on B cells and binds the virus-encoded glycoproteins gp350/220.^{390,416,1081,1082,1427} gp350/220 has homologs in primate gammaherpesviruses but not in other human herpesviruses.¹⁵²² gp350 and gp220 are encoded by the same messenger RNA (mRNA), but a single splice generates the gp220 reading frame.^{88,639} No known distinct function of either gp350 or gp220 has been determined because they both contain the domain that binds CD21.¹⁴²⁸

CD21 is the receptor for the C3d component of complement and is part of a large family of complement regulatory proteins. Family members have one or more extracellular structural motifs known as short consensus repeats (SCRs). As well as being expressed on B cells, CD21 is expressed on other cell types including tonsillar epithelial cells.⁶⁸⁸ The extracellular domain of CD21 contains multiple SCRs, but only SCR-1 and SCR-2 are required for EBV and C3d binding.^{175,929,981} Soluble CD21 binds to the EBV virion and can block B-cell infection.¹⁰⁸³ The structure of the key gp350 binding domain on CD21 was solved¹¹⁸⁶ and the region of gp350 required for binding to CD21 is contained within a portion of gp350 that is not glycosylated.¹⁴¹⁶ Compatible with this finding, deglycosylated gp350 binds to CD21, as does the fully glycosylated gp350.¹⁴¹⁶

Binding of EBV virions or gp350 coated beads to CD21 results in capping of CD21 followed by endocytosis,^{1079,1427} consistent with a role of gp350/220 initiation of CD21 intracellular signal transduction in EBV B-lymphocyte entry.^{124,277,1348,1402,1427} The binding of gp350/220 to CD21 parallels the interaction of HSV gC with cell surface proteoglycans in tethering virions to target cells. This initial step is not absolutely required for infection because EBV deleted for gp350/220 or HSV-1 deleted for gC have only modest defects in virus entry.^{571,678} After binding to B cells, EBV virions are endocytosed¹⁰²⁵ and fusion of the viral membrane is thought to occur via the interaction of gp42 with human leukocyte antigen (HLA) class II followed by fusion mediated by gH/gL and gB.

HLA-DR was found to bind gp42 in an expression library screen for proteins that bound a soluble form of gp42.¹³⁸⁶ Both structural and functional studies have shown that gp42 exhibits features of the C-type lectin family^{1065,1386} and has no known sequence homologs in other human herpesviruses but is found in the EBV-related primate viruses.¹⁵²² The gp42/HLA-DR interaction is essential for EBV infection of B cells and antibodies to gp42 and HLA-DR block infection.^{876,877,1024} In addition, virus deleted for gp42 does not infect B cells unless soluble gp42 is provided *in trans* or polyethylene glycol is added to promote fusion.^{1530,1531} Also indicative of the important role

of gp42 in B-cell infection, virosomes lacking gp42 bind B cells but are unable to fuse.⁵¹⁵ The binding site of gp42 on HLA class II has been extensively studied by mutagenesis^{511,513} and by protein crystallography.¹⁰⁶⁵ Because of the key role of HLA class II in antigen presentation, studies have suggested that gp42 may block HLA class II antigen presentation, preventing CD4 T-cell activation.^{1220,1219,1386}

gp42 stably interacts with gH/gL and links receptor binding with fusion mediated by gH/gL and gB. The structure of gp42 unbound to a receptor indicates that a conformational change occurs upon binding of gp42 to HLA class II, widening a hydrophobic pocket on the surface of gp42, which suggests a conformational change is important in triggering fusion by engaging other components of the EBV fusion complex or a cellular ligand.⁷⁸⁰ The EBV gH/gL structure has also been solved,⁹⁹⁵ revealing an elongated rod-like shape with the middle being the widest and gL forming intimate contacts with the N-terminal residues of gH, compatible with the known chaperone function of gL for gH expression, processing, and transport.^{547,877,1039,1111,1586} Of particular interest, a KGD sequence on the gH/gL surface has been implicated in integrin binding and epithelial cell entry²¹⁷ and is near a potential gp42 binding domain.⁹⁹⁵ Competitive binding between gp42 and integrin to gH/gL is consistent with the observation that gp42 and gp42-derived peptides block fusion and entry of epithelial cells.^{121,778,779,908} Gp42 interacts with gH/gL via a domain contained within the amino terminus of gp42 located outside the gp42 C-type lectin domain.^{778,877,908}

EBV virions produced by B lymphocytes are deficient in gp42 as a consequence of gp42 binding to HLA class II instead of gH/gL during EBV lytic replication.¹²¹ EBV deficient in gp42 is defective in B-cell infectivity but is enhanced in epithelial cell infectivity.¹²¹ The absence of gp42 enables the gH/gL complex to engage an epithelial cell receptor,¹²² which is otherwise occluded by gp42. During virus egress from epithelial cells, the absence of HLA class II results in virions containing gp42 bound to gH/gL that efficiently infect B lymphocytes.

Epithelial Cells

In contrast to B-cell entry, EBV entry into epithelial cells occurs at the cell surface in the absence of endocytosis.¹⁰²⁵ In addition to CD21, which is expressed on tonsillar epithelial cells, suggesting the gp350/220 interaction with CD21 may be important for epithelial cell infection,⁶⁸⁸ BMRF2 may also have a role in epithelial entry. BMRF2 is a multispan membrane protein that contains an extracellular RGD motif that is a ligand for $\alpha 1$, $\alpha 5$, $\alpha 3$, and αv integrins.¹⁵⁶⁸ This interaction is important for infection of polarized epithelial cells,¹⁴⁷⁷ and antibodies to BMRF2 or $\alpha 5 \beta 1$ integrin block EBV infection of polarized epithelial cells through the basolateral cell surface. Interestingly, BMRF2 is not abundant in the EBV virion⁶⁹⁵ and appears not to have a role in fusion,⁵¹² so BMRF2 may function similar to gp350/220 to tether virions to target cells and induce signal transduction pathways. Integrin signaling has been shown to be important in HHV8 infection.¹³²⁹ Most recently, $\alpha v \beta 6$ and $\alpha v \beta 8$ integrins have been identified as epithelial cell receptors for gH,²¹⁷ and this binding triggers fusion in a two-step reaction.²¹⁶ Earlier studies had shown that a soluble form of gH/gL bound specifically to epithelial cells but not B cells.¹²² In

TABLE 61.1 EBV-Encoded Latent and Lytic Genes

EBV gene	HSV gene	KSHV gene	EBV name	Known or proposed function
BNRF1		ORF75		Major Tegument Protein
BNLF2b				
BNLF2a				Interacts With Tap, Immune Evasion
<i>EBER1,2</i>				<i>Small RNAs Cell Survival Factor</i>
BCRF1				IL-10 Homolog—Host Immune Modulator
BCRF2				
BCLT1				Nuclear Noncoding RNA—Lytic Replication
BCLT2				Nuclear Noncoding RNA—Lytic Replication
<i>BWRF1</i>			<i>EBNALP</i>	<i>Regulator of Latent Viral Gene Transcription (EBNA5)</i>
<i>BYRF1</i>			<i>EBNA2</i>	<i>Major Regulator of Viral Gene Transcription</i>
BHRF1		ORF16		Bcl-2 Homolog
BHLF1				
BFLF2	UL31	ORF69		Required for Nuclear Egress—Binds BFRF1
BFLF1	UL32	ORF68		Virion Protein—DNA Cleavage/Packaging
BFRF1	UL34	ORF67		Nuclear Egress—Binds BFLF2
BFRF2		ORF66		
BFRF3	UL35	ORF65		Capsid Protein
BPLF1	UL36	ORF64		Deneddylase
BOLF1	UL37	ORF63		Cytoplasmic Phosphoprotein
BORF1	UL38	ORF62		Capsid Assembly
BORF2	UL39	ORF61		Ribonucleotide Reductase (Large Subunit)
BaRF1	UL40	ORF60		Ribonucleotide Reductase (Small Subunit)
BMRF1	UL42	ORF59		Polymerase-Associated Processivity Factor
BMRF2	<i>UL43</i>	ORF58		Transmembrane Glycoprotein, 53/55 kD, Binds Integrins
BSLF2/BMLF1	UL54	ORF57		Posttranscriptional Regulator of Viral Gene Expression
BSLF1	UL52	ORF56		DNA Replication—Helicase/Primase Complex
BSRF1	<i>UL51</i>	ORF55		
BLRF1		ORF53		gN, Virion Protein
BLRF2		ORF52		p23 Capsid Antigen
BLLF1a			gp350	Virion Binding to CR2 (CD21), Virion Protein
BLLF1b			gp220	Virion Binding to CR2 (CD21), Virion Protein
BLLF3	UL50	ORF54		dUTPase
<i>BLRF3-BERF1</i>		<i>EBNA3C</i>		<i>Regulator of Latent Viral Gene Transcription (EBNA6)</i>
<i>BERF2a,b</i>		<i>EBNA3B</i>		<i>Regulator of Latent Viral Gene Transcription (EBNA4)</i>
<i>BERF3-BERF4</i>		<i>EBNA3A</i>		<i>Regulator of Latent Viral Gene Transcription (EBNA3)</i>
BZLF2			gp42	Complexes with gp25 and gp85—Binds HLA Class II, Virion Protein
BZLF1			Zebra, ZTA, EB1	Lytic Gene Transactivator
RAZ				Z Regulator
BRLF1		ORF50	Rta, EB2	Lytic Gene Transactivator
BRRF1		ORF49		Transcription Factor
BRRF2		ORF48	Na	Lytic Gene Transactivator, Virion Protein
<i>BKRF1</i>			<i>EBNA1</i>	<i>Maintenance of Viral Episome</i>
BRKF2	UL1	ORF47	gp25	gL—Complexes with gp42 and gp85, Virion Protein
BKRF3	UL2	ORF46		Uracil-DNA Glycosylase
BKRF4	<i>UL3</i>	ORF45		Nuclear Phosphoprotein
BBLF4	UL5	ORF44		DNA Replication—Helicase/Primase Complex
BBRF1	UL6	ORF43		Capsid Protein—Portal
BBRF2	UL7	ORF42		
BBLF3	UL8	ORF41		DNA Replication—Helicase/Primase Complex
BBLF2	UL9	ORF40		DNA Replication—Helicase
BBRF3	UL10	ORF39		gM, Virion Protein
BBLF1	UL11	ORF38		Myristylated Virion Protein
BGLF5	UL12	ORF37		Exonuclease, Host Shut-off, Immune Evasion
BGLF4	UL13	ORF36		Virion Protein Kinase
BGLF3	UL14	ORF34		Putative Virion Tegument Protein, Antiapoptotic
BGLF3.5		ORF35		

TABLE 61.1 EBV-Encoded Latent and Lytic Genes (Continued)

EBV gene	HSV gene	KSHV gene	EBV name	Known or proposed function
BGRF1	UL15	ORF29a		DNA Packaging Protein
BGLF2	UL16	ORF33		Capsid Maturation/Assembly Protein
BGLF1	UL17	ORF32		Capsid Maturation/DNA Packaging, Virion Protein
BDLF4		ORF31		
BDRF1	UL15	ORF29b		DNA Packaging Protein
BDLF3		<i>ORF28</i>	gp150	Enhances Epithelial Infection, Virion Protein
BDLF3.5		<i>ORF30</i>		
BDLF2		ORF27		Glycoprotein—Complexes with BMRF2
BDLF1	UL18	ORF26		Minor Capsid Protein
BcLF1	UL19	ORF25		Major Capsid Protein
BcRF1		ORF24		
BTRF1	<i>UL21</i>	ORF23		
BXLF2	UL22	ORF22	gp85	gH—Complexes with gp25 and gp42, Virion Protein
BXLF1	UL23	ORF21		Thymidine Kinase, Virion Protein
BXRF1	UL24	ORF20		
BVRF1	UL25	ORF19		Virion Protein
BVRF2	UL26	ORF17		Protease
BVLF1.5				
BdRF1				Capsid Scaffolding
BILF2			gp78/55	Virion Protein
BILF1				GPCR
BALF5	UL30	ORF9		DNA Polymerase
BALF4	UL27	ORF8	gp110	gB—Fusion Protein, Virion Protein
BALF3	UL28	ORF7		DNA Cleavage/Packaging
BALF2	UL29	ORF6		ssDNA Binding Protein
BALF1				Bcl-2 Homolog
<i>BARF0/RK-BARF0</i>				<i>Regulator of Notch Pathway</i>
<i>BARTS</i>				<i>MicroRNAs</i>
BARF1				Binds CSF-1
<i>BNLF1a,b,c</i>			<i>LMP1</i>	<i>Constitutive CD40 Mimic—Oncoprotein</i>
			<i>LMP2A</i>	<i>Constitutive B-Cell Receptor Mimic (TP1)</i>
			<i>LMP2B</i>	<i>Regulator of LMP2A and LMP1 Function? (TP2)</i>
Raji LF3				
Raji LF2		ORF11		Binds BRLF2 and IRF7
Raji LF1				Part of BILF1

Genes in “EBV” column in italics are expressed in latent infection.

In the HSV and KSV columns, the homology with the genes in italics with EBV is not significant, but they may be positional homologs.

EBV, Epstein-Barr virus; HSV, herpes simplex virus; KSHV, Kaposi’s sarcoma–associated herpes virus; IL, interleukin; HLA, human leukocyte antigen; CSF-1, colony-stimulating factor-1.

addition, gH-null virus does not bind to epithelial cells,^{1039,1112} and a monoclonal antibody to gH/gL can reduce both gH/gL and virus binding.^{122,1039} These observations all point to an essential role of gH/gL in the infection of epithelial cells.

Other modes of EBV infection may also exist besides virus-induced membrane fusion. Virus coated with immunoglobulin A can bind to the polymeric IgA receptor.¹³⁵⁶ This form of infection may be relevant in nasopharyngeal carcinoma, where high levels of EBV-specific IgA antibodies are found in the blood.⁵⁶¹ However, EBV is transcytosed in polarized epithelial cells without evidence of replication.⁴⁴⁴ Interestingly, antibodies directed against gp350/220 can enhance EBV epithelial infection independent of Fc receptor interactions, and cell contact also enhances epithelial infection.^{653,1328,1479} Finally, cell-associated virus is very efficient in infecting epithelial cells or B cells.^{653,1326,1327,1328}

Transcription: Transformation of Primary B Lymphocytes by EBV

One of the most interesting aspects of infection by EBV is the ability of the virus to immortalize B lymphocytes in cell culture and establish a largely latent infection in which few viral genes are expressed. B lymphocytes infected with EBV become immortalized and proliferate indefinitely as lymphoblastoid cell lines (LCLs). It is this characteristic of EBV that likely contributes to the association of EBV with human cancers. This section will focus on viral genes expressed in LCLs because these genes have been extensively studied. Only a subset of the approximately 80 genes encoded by EBV are abundantly expressed in latently infected B lymphocytes (Table 61.1). In addition to protein products, a number of RNAs are expressed, including the EBERs and microRNAs. The major proteins expressed are either nuclear (EBNAs—Epstein-Barr

nuclear antigens) or found in cellular membranes (LMPs—latent membrane proteins). Their known or proposed functions are shown in Table 61.1. These genes are not conserved in any other human herpesvirus, but homologs to these genes have been identified in the related LCVs that infect non-human primates.

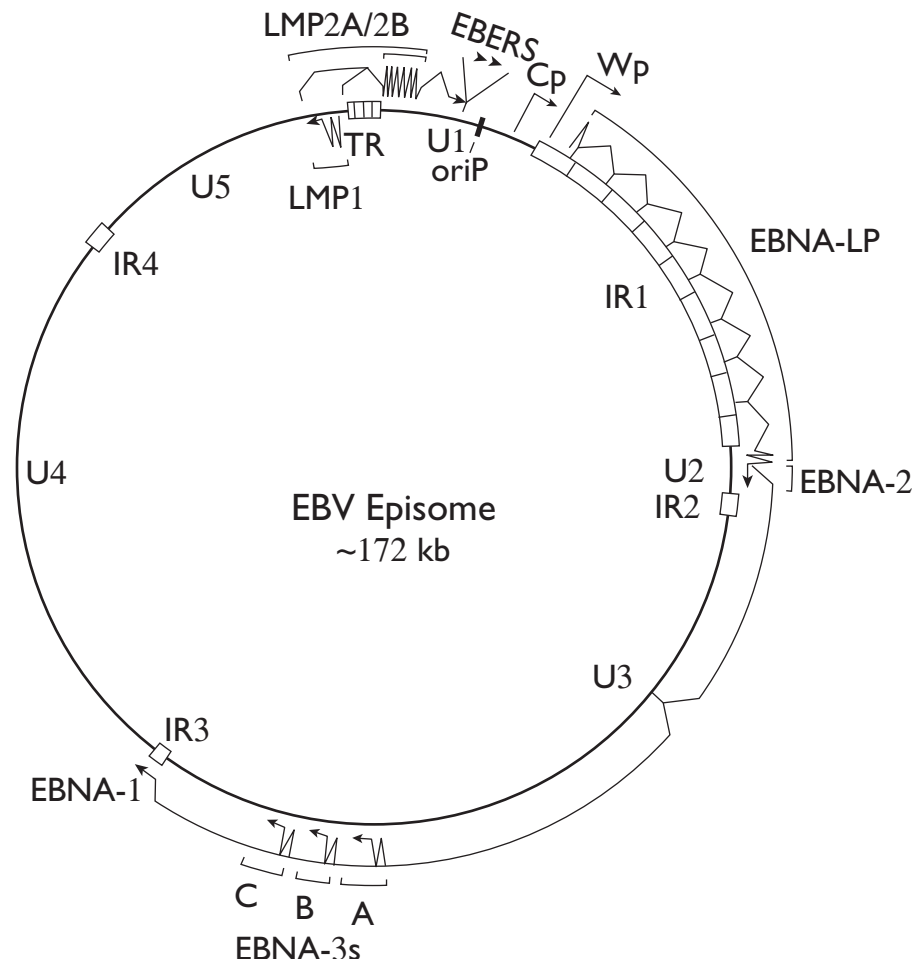
In addition to the pattern of viral gene expression observed in LCLs, EBV establishes other patterns of gene expression in EBV-positive tumors and in the blood of infected persons. In latency 1, defined by Burkitt lymphoma, only EBNA-1 protein is expressed, although recent studies have shown other latency-associated proteins are sometimes expressed (described later). In latency 2, characterized by nasopharyngeal carcinoma, EBV-positive Hodgkin lymphoma, and T-cell lymphoma, EBNA-1, LMP1, and LMP2 are expressed. In latency 3, defined by LCLs grown *in vitro*, all the known latent genes are expressed. More recent studies analyzing expression of viral genes in immunocompetent individuals latently infected with EBV have shown that few if any EBV genes are expressed in virus-infected B lymphocytes in the blood, resulting in EBV latency 0.

Following infection of primary B cells, host cell RNA polymerase II transcribes viral mRNAs with the first transcripts resulting from Wp promoter encoding EBNA-LP and EBNA-2 (Fig. 61.5).^{17,1189,1274,1303,1381,1382,1491,1521,1548,1600,1601} The Wp upstream regulatory element extends from -1168 to -87

and has components that bind YY1, RFX1, RFX3, MIBP1, CREB /ATF-1, and BSAP/Pax5 that provide B-cell specificity.^{90,776,1223,1449} Early in infection of primary B cells, Wp is used exclusively to drive transcription of the EBNA genes, followed by an EBNA-2 and EBNA-1 induced switch to use of the Cp promoter.^{1550,1552,1566} Interestingly, recent data suggest a novel posttranscriptional mechanism for *trans*-repression of Wp-dependent gene expression upon activation of Cp that may guard against overexpression of the EBNAs prior to the switch in promoter usage.⁶³⁴

EBNA-LP and EBNA-2 reach the level that is maintained in transformed B cells (LCLs) by 24 to 32 hours postinfection.¹⁷ By 36 hours the Wp and the upstream Cp EBNA promoters are both functional, resulting in rightward transcripts extending through at least 70% of the genome.^{17,379,494,534,898,902,1148,1150,1274,1275,1381,1383} LCLs transformed by a virus lacking the EBNA-2-responsive Cp element are biased toward Wp usage but not substantially impaired in transformation efficiency or different from wild-type virus infected cells in EBNA expression.¹⁵⁹⁹ Indeed, Cp-mediated transcription suppresses Wp.¹¹⁹⁰ Interestingly, deletion of the Wp -169- to -369-bp upstream control region, which affects both Wp and Cp activity, resulted in compensatory use of downstream copies of Wp and decreased transformation efficiency.^{1189,1601} The Wp promoter appears to be active in many cell types and has a region that is required for B-cell transformation that binds the

FIGURE 61.5. Schematic depiction of the Epstein-Barr virus (EBV) episome. The EBV episome is circularized through terminal repeats (TR). Shown are the transcripts, messenger RNAs (mRNAs), and major proteins in type III latent B-lymphocyte infection. Largely unique (U1 to U5) and internal (IR1 to 4) or terminal repeat DNA segments are indicated. The origin for latent infection EBV episome replication, oriP, is also indicated. Exons encoding EBV nuclear proteins (EBNA-1, -2, -3A, -3B, -3C, and -LP) or EBV integral latent membrane proteins (LMP 1, 2A, or 2B) are indicated by vertical lines radiating from the circular episome map. EBNA transcription is shown as it is first initiated from the IR1 or Wp promoter. In many infected lymphocytes, after expression of EBNA-LP and EBNA-2, the Cp promoter in U1 dominates EBNA transcription. Alternative polyadenylation and splicing result in expression of the various EBNA mRNAs. EBNA-LP is encoded by repeating exons from IR1 and two short unique exons from U2. The EBERs are two small, nonpolyadenylated RNAs. LMP1 is transcribed in direction opposite to the EBNAs, LMP2A and LMP2B, and EBERs. LMP2A has an upstream EBNA-2 response element, whereas LMP1 and LMP2B share the same EBNA-2 response element.



B-cell-specific BSAP/Pax5 activator protein.¹⁴⁵⁰ The Cp promoter also appears to be B-cell specific.²⁵⁹ Mutational analysis of the Wp and Cp promoters has suggested that transcription from Cp interferes with transcription initiation at the downstream Wp promoter.¹¹⁸⁹ Interestingly, deletion analysis has demonstrated that the Cp promoter is not essential for *in vitro* transformation of primary B lymphocytes by EBV.¹⁴¹¹

EBNA-2 up-regulates specific EBV and cell promoters and EBNA-LP potentiates EBNA-2 effects.^{2,534,1098,1349,1473,1518–1520,1524,1548–1550} Major viral targets of EBNA-2 include Cp, LMP1, LMP2B, and LMP2A promoters through a bidirectional enhancer element shared by the LMP1 and LMP2B promoters and an enhancer element in the LMP2A promoter.^{368,458,694,834,835,1357,1358,1473,1524,1578,1636} Interestingly, EBNA-LP has less co-activation effects with EBNA-2 on the EBV LMP2A promoter, the cell CD21, and HES-1 promoters.¹¹⁵³ The relative role of EBNA-LP in the EBNA-2-responsive CD23, c-fgr, and c-myc promoters has not been characterized.^{17,263,527,534,792,1098,1153,1518–1520,1598} EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-1 do not reach levels seen in LCLs until 46 to 72 hours after infection, consistent with dependence on EBNA-2 and EBNA-LP up-regulation of the Cp promoter. Although EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-1 mRNAs accumulate at very low levels, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-1 proteins are remarkably stable and expressed at high levels in LCLs. EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-1 protein levels are positively affected by an internal ribosome entry site (IRES) in the U exon that is common to their mRNAs and by their intrinsic stability.^{99,359,658} Full steady-state LCL levels of EBNA-1 at 72 hours after infection ensure EBV episome transmission to progeny cells and further enhance transcription from promoters around oriP; oriP is an EBV DNA fragment that includes an EBNA-1-dependent enhancer and an EBNA-1-dependent replication origin.^{442,1215,1216,1404,1588} To regulate the promoter activities of Cp and Wp by EBNA-2; EBNA-3A, EBNA-3B, and EBNA-3C compete with EBNA-2 for binding to RBP-Jκ/CBF1.^{696,979,1228,1229} RBP-Jκ/CBF1 is a cellular protein that by binding to EBNA-2 is able to recruit the transcriptional activation function of EBNA-2 to DNA through its DNA sequence-specific binding function on the Cp enhancer.^{494,694,899,902}

Following initial EBV infection, EBV up-regulates c-myc and CD21 RNA and c-myc protein expression are increased at 16 to 24 hours after B-lymphocyte infection, soon after EBNA-2 and EBNA-LP are expressed.¹⁷ CD23 expression is delayed relative to CD21. Patches of LMP1 are detectable in B cells by 48 hours and LMP1 protein is at levels observed in LCLs by 72 hours.¹⁷ By 46 hours after infection 15% of cells are in S phase, and at 72 hours a substantial fraction of infected cells have divided.

By 72 hours EBERs are expressed in the cell nucleus. EBER 1 and EBER 2 RNAs are transcribed by cell RNA polymerase (pol) III, which is enhanced by EBNA-1.^{49,615,616,680,859,1124} The EBERs have intragenic control regions common to pol III transcripts and upstream Sp1, ATF, and TATA elements characteristic of pol II transcriptional sites.^{615,616} At steady-state levels in latently infected B lymphocytes, the EBERs are the most heavily transcribed and most abundant EBV RNAs. EBERs are estimated to accumulate at about 50,000 copies per cell nucleus.^{49,615–617,773,774,826}

In forms of latency other than latency III, where there is reduction in latent gene expression, this change is at least in part due to epigenetic mechanisms. In persistent infections, there is a down-regulation of latency 3 promoters and a switch to latency 1, 2, or 0. During latent infection, the EBV genome can undergo progressive methylation and condenses with nucleosomes.^{95,339,372,373,624,677,775,1323} Regulatory domains and genes involved in maintaining latent infection such as oriP tend to be undermethylated in latently infected cells relative to DNA that is expressed only in lytic infection.^{372,1032–1034,1104,1429} Extensive methylation of lytic genes and their regulatory elements probably helps to maintain latency by inhibiting lytic gene expression.¹⁴¹⁹ Treatment of latently infected cells with drugs that reduce DNA methylation increases the frequency of spontaneous activation to lytic infection.^{991,1230} Methylation of the EBNA-2 response element may be important for down-regulation of Cp promoter activity *in vivo* in some EBV-related malignancies.^{642,691,1231,1409} In latency 1, in which no other EBNA proteins are expressed, Cp/Wp promoter activity is inactivated and EBNA-1 is driven by an EBNA-1-specific promoter termed Qp that is located more than 50 kbp from Cp and Wp.^{1103,1272,1294,1295} Qp is a TATA-less promoter that is negatively regulated by two EBNA-1 binding sites downstream of the transcriptional initiation site.^{1272,1273} In addition, the transcription factor E2F can reverse EBNA-1-mediated repression of the Qp promoter by displacement of EBNA-1 from Qp by binding to two E2F-like sites that overlap the EBNA-1 binding sites.¹⁴⁰⁹ The Qp promoter is functional when introduced into a variety of cell types,^{1101,1274,1295} suggesting that Qp promoter activity may be constitutive. EBNA-1 is the only known negative regulator of Qp activity. In the absence of Cp- or Wp-mediated expression of EBNA-1, the Qp promoter is functional, thus ensuring expression of EBNA-1 to maintain the viral episome.¹¹⁰² Histone acetylation and methylation at essential promoter elements also contributes to the regulation of latent viral gene expression.^{13,197,293} EBV genomes are not randomly distributed in the nucleus but localize to perichromatic regions that are enriched for specific preferentially methylated and acetylated histones indicative of transcriptionally active regions.³⁰⁴ CTCF binding sites have also been identified in the EBV genome. CTCF is an 11-zinc finger nuclear protein that binds to diverse and long DNA sequences, thereby mediating long-range chromatin interactions. CTCF can function as an insulator of transcriptional enhancers and a regulator of genomic imprinting. Genome-wide studies have identified CTCF binding sites at several key regulatory elements in the EBV genome.^{197,198,1267} Studies analyzing CTCF binding at Qp found that viral mutants containing mutations in the CTCF sites near Qp were reduced in recovery of stable episomes from viral bacmids, as well as a decrease in Qp transcription initiation and an increase of histone and DNA methylation at the Qp initiation site.¹⁴³⁹ More recent studies have shown that differential expression of CTCF and DNMT1 and DNMT-3B are not critical for restricted latency. EBV mutants lacking the Cp CTCF binding site had sustained Cp activity relative to wild-type virus, but transition to latency 1 was observed, indicating that CTCF contributes to the establishment of restricted latency but is not absolutely required.⁶³⁵ As well as acting as an insulator to silence EBV gene expression, CTCF binding to the EBV genome may anchor the genome to the nuclear matrix and

direct specific chromatin architectures that can mediate alternative promoter targeting by the OriP enhancer.¹⁴³⁸

More recent studies have shown that cells latently infected with EBV encode additional proteins as well as miRNAs compared with the limited repertoire of genes originally identified. In particular, weak Wp activity can drive the expression of BHRF1. In 15% of endemic Burkitt lymphomas (BLs), a transformation-defective EBNA-2–deleted virus was selected⁷³⁷ and infection was associated with activation of the Wp latent promoter with broadening of latent protein expression beyond that typically seen in latency I.^{737,739,740} This finding led to the discovery that BHRF1 and BALF1 are expressed in B cells immediately following infection²³ and BHRF1 transcripts can be detected at low levels in LCLs long term.⁷³⁸ Overall, these findings indicate that the four patterns of latent gene expression that have been described should be used only as a guide in subdividing types of EBV latency, because other viral genes may also be expressed. EBV genes newly identified as expressed in EBV latent infections will be discussed more fully after the functions of well-recognized latency-associated genes are described.

Translation: EBV Gene Products Associated With Latency

EBNA-1

EBNA-1 (Fig. 61.6A) associates with chromosomes during mitosis^{469,492,544,1117,1160,1174} and supports replication and stable maintenance of plasmids in human cells via a latent origin termed *oriL*.^{1587,1588} *OriP* consists of two functional elements separated by 1 kbp, the dyad symmetry (DS) element and the family of repeats (FR)^{1209,1216} (Fig. 61.6B). Interaction of EBNA-1 with the specific sequence *oriP* within the viral genome enables the viral genome to persist as an episome in latently infected LCLs.^{27,211,227,947} EBV *oriP* contains multiple copies of an 18-bp EBNA-1 recognition sequence, which is clustered in two noncontiguous functional elements, the family of repeats and the DS element⁹⁴⁷ (Fig. 61.6B). The DS element contains four EBNA-1 binding sites, a 65-bp region of dyad symmetry, and is the site for initiation of DNA replication.^{310,947,1105,1106,1171,1209} Within *oriP*, FR is an EBNA-1–dependent enhancer; six to seven copies are essential for enhancer function.^{1216,1566} The FR, but not DS, is important for the localization of the EBV genome in perichromatic regions that are enriched for histone methylation marks that are hallmarks of transcriptionally active regions.³⁰⁴ The separate DS has four copies and is the origin for episome DNA synthesis.^{441,1566} Telomere repeat binding factor 2 (TRF2), TRF2-interacting protein hRap1, and the telomere-associated poly(adenosine diphosphate [ADP]-ribose) polymerase bind to nonamers in DS and the binding is EBNA-1 dependent.²⁹⁹ A minor component, termed Rep*, is a nearby EBNA-1–independent origin for DNA synthesis.⁷⁷⁷ Three copies of Rep* can complement deletion of DS.⁷⁷⁷ Cell DNA origins can also replace the DS element.^{807,992} The function of EBNA-1 and *oriP* plasmid maintenance in rodent cells requires replacing DS with a rodent DNA replication origin.⁸⁰⁶

EBNA-1 binds as a dimer, and both the DNA binding and dimerization domains map near the carboxyl terminus of the protein^{27,79,112,211} (Fig. 61.6B). The repeat unit of *oriP* acts as an enhancer in the presence of EBNA-1, which is important for latent gene transcription from the Cp promoter.^{66,442,1157,1171}

In addition, lower-affinity EBNA-1 binding sites near the Qp promoter are important for the expression of EBNA-1 in the non-latency 3 programs.^{3,26,59,570,1101,1295} The transcriptional activation of EBV latent genes by EBNA-1 is essential for EBV transformation of primary B cells.²⁴

The structure of EBNA-1 bound, and not bound, to DNA has been solved^{79,112,113} (Fig. 61.6C,D). The EBNA-1 dimer is composed of multiple domains. The dimerization domain extends from amino acid 504 to 604.^{79,112,113} Amino acids 459 to 503 include a helix that projects into the major groove and an extended chain that moves along the minor groove and makes all the sequence-specific contacts with the DNA of the binding site. Amino acids 477 to 489 form a helix, which is oriented perpendicularly to the axis of the DNA, and amino acid 477 contacts the DNA. Amino acids 490 to 504 form an extended loop that connects the helix to the first β strand of the core β barrel. Amino acids 461 to 469 form an extended chain that tunnels along the minor groove near the outside of the binding site, wraps around the DNA toward the center of the binding site, and exits between the second and third bases away from the middle of the binding site. These amino acids appear to provide most of the sequence-specific DNA contacts. Amino acid 469 is connected to amino acid 477 by a loop that traverses along and forms multiple hydrogen bonds with the DNA phosphate backbone.

EBNA-1 binding to its cognate sequence appears to be cooperative,^{1030,1209,1406} suggesting that either EBNA-1 molecules interact cooperatively in binding to DNA or that the DNA template is modified as a consequence of initial EBNA-1 binding. Crystallography, electron microscopy, and biochemical data indicate that EBNA-1 binding to the family of repeats and the DS element results in high-order structures that lead to bending and distortion of the DNA duplex.^{421–424,1013,1121} Early findings suggested that cellular proteins were required for episome maintenance and not required for enhancer activity, because EBNA-1 binding to the family of repeats enhances transcription in mouse or human cells, whereas EBNA-1 promotes episome replication only in primate cells.⁸⁰⁶ Recent studies have identified a number of cellular proteins that likely participate in EBV genome replication. Subunits from the cellular origin recognition complex (ORC) and the minichromosome maintenance (MCM) complex associate with the DS element of *oriP*, suggesting a role for these factors in the initiation and licensing of EBV genome replication.^{199,309,701,1300} It is not known how ORC is recruited to the DS element. EBNA-1 may facilitate ORC recruitment to DS by disrupting nucleosomes formed at the DS element, allowing ORC access to DNA.^{56,1631} EBNA-1 binding to its recognition sites *in vitro* is greatly stimulated by binding to the cellular deubiquitinating enzyme, USP7. USP7 can form a ternary complex with DNA-bound EBNA-1¹²⁸⁴ and is part of a histone deubiquitinating complex. Other proteins that may have roles in EBNA-1 function include Cdt1,³⁰⁹ TAF-1 (β isoform),¹⁵²⁸ TRF2,^{298,299,897} TRF2-interacting protein hRap1,^{298,299} and the telomere-associated poly(ADP-ribose) polymerase (Tankyrase).^{298,299} EBNA-1–binding protein 2 (EBP2) is important for interactions between EBNA-1 and chromosomes in metaphase and association with metaphase chromosomes.^{712,713,1078,1339} Poly(ADP-ribose) polymerase (PARP) is an abundant, chromatin-associated, nicotinamide adenine dinucleotide (NAD)-dependent enzyme that functions in multiple

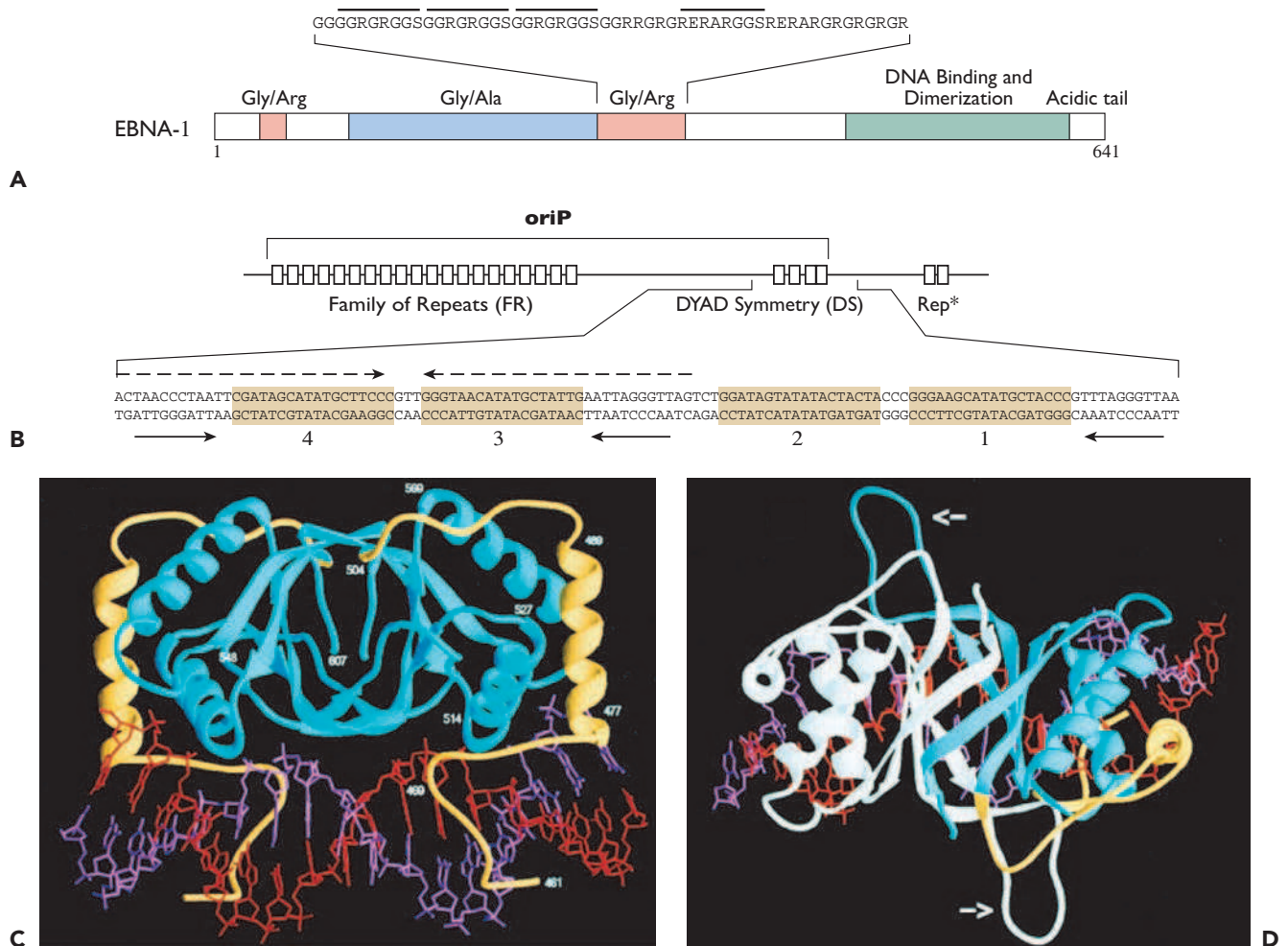


FIGURE 61.6. Structure of Epstein-Barr virus nuclear antigen 1 (EBNA-1) bound to DNA, schematic of the oriP DNA arrangement, and EBNA-1 functional domains. **A:** Important EBNA-1 functional domains. The amino terminal Gly/Arg domain is important for replication, segregation, and transcription and has been shown to bind P32/TAP. The central Gly/Arg sequence is also important for replication, segregation, and transcription. This domain has been shown to bind a variety of cell proteins including EBP2, Nap1, p32/TAP, MDF1, PRMT1, and PRMT5. The DNA binding and dimerization domain at the C-terminus of EBNA-1 is essential for all known functions of EBNA-1 including replication, segregation, and transcription. Regions outside these domains of EBNA-1 are generally thought to be nonessential but have been shown to bind cellular proteins including Tankyrase and USP7. **B:** The oriP family of repeats (FR) and dyad symmetry (DS) elements are shown with an expanded view of the nucleotide sequence of DS with the EBNA-1 binding sites 1 to 4. The nonamer repeats (*solid arrows*) and the 65-base-pair dyad symmetry (*broken arrows*) are also shown. EBNA-1 binding sites are shown as boxes with the dyad present in DS denoted by the head-to-head arrows. The Rep* element is located 240 bp downstream of DS. **C:** EBNA-1 bound to DNA with a ribbon diagram showing the core domain, which contains residues 504 to 607 from each monomer, in blue. The flanking domains from each monomer are shown in yellow. The amino acids are numbered according to their position in the full-length EBNA-1 protein derived from B95-8. **D:** A view down the noncrystallographic axis that shows one monomer in white and the other monomer in the same color scheme as used in C. The proline loops are indicated by the arrows. (From Bochkarev A, Barwell JA, Pfuetzner RA, et al. Crystal structure of the DNA-binding domain of the Epstein-Barr virus origin-binding protein, EBNA1, bound to DNA. *Cell* 1996;84[5]:791–800, copyright © 1986 Elsevier.)

chromosomal processes, including DNA replication and chromatin remodeling, and overall negatively affects EBNA-1 function.¹⁴³⁷

An important feature of EBNA-1 is its ability to act as a bridge coupling the EBV genome to host chromosomes. Multiple RG rich EBNA-1 residues are required for EBNA-1 binding to host chromosomes.^{641,974,1078,1339,1560,1561} This

interaction is also mediated by proteins that associate with EBNA-1 such as EBP2^{713,1339} or through direct interactions of EBNA-1 with cellular DNA as suggested by EBNA-1 glycine- and arginine-rich regions that resemble AT hooks.¹³¹³ More recently, these regions have been shown to have a strong preference for G-quadruplex RNA; G-quadruplex RNA-interacting drugs block EBNA-1 functions, suggesting a role of RNA

binding for the function of EBNA-1.^{1107,1108} Further studies are clearly needed to fully understand the mechanism of EBNA-1 binding to cellular DNA. Cellular chromatin and chromosomal proteins such as high mobility group I (HMG-I) amino acids 1 to 90 or histone H1 can functionally substitute for the EBNA-1 regions responsible for host chromosome binding in enabling chromosome binding and long-term episome persistence.^{641,1313} EBNA-1 is a serine phosphoprotein.^{544,1160,1174} Functionally, serine phosphorylation is important for genome segregation,^{329,825,1340} and interestingly, roscovitine, a selective inhibitor of CDK1, blocks EBNA-1 serine phosphorylation, nuclear localization, transcription, and episome maintenance.⁷⁰⁸

EBNA-1 has been proposed to have a role in cell growth and survival. Two studies using EBNA-1 transgenic mice indicate a greater incidence of lymphoma in these mice alone^{1543,1544} and in cooperation with Myc.³²⁷ However, in two more recent studies, there was no increased incidence of lymphoma. The EBNA-1 transgenic mice did not display any gross phenotypic differences when compared to littermate controls.^{709,710} Other studies indicate that EBNA-1 induces genomic instability and a DNA damage response.^{499,500} However, EBNA-1 is not a dominant transforming gene of EBV because EBNA-1 deletion does not compromise immortalization of B cells by EBV or outgrowth in severe combined immunodeficiency (SCID) mice, but only the efficiency of transformation because the EBV genome must integrate into the host genome to allow genome persistence in the absence of EBNA-1 expression.⁶³⁷ The contribution of EBNA-1 to B-cell immortalization, outside of activation of EBV latent genes²⁴ and maintaining the EBV genome in infected cells, appears to be limited in comparison with other latent EBV proteins such as LMP1, EBNA-2, EBNA-LP, or EBNA-3C that when mutated have more dramatic effects on EBV transformation of B cells (see relevant sections within this chapter).

Despite having a limited role in EBV-mediated transformation of B cells, EBNA-1 may have importance in the development of EBV-associated cancers. EBNA-1 may alter cell growth by enhancing expression from EBNA-1-binding sequences in cell DNA or by interaction with cell proteins (see earlier). Expression of a dominant negative form of EBNA-1, which blocks EBNA-1 interaction with cognate DNA, in an LCL with integrated EBV DNA resulted in no change in cell growth or virus or cell gene expression,⁷⁰⁷ consistent with observations that EBNA-1-negative LCLs do not differ in growth from wild-type LCLs.⁶³⁷ Furthermore, EBNA-1 strongly enhances transcription from an episome with an oriP FR near a promoter but has much less effect on the same elements integrated into cell DNA, unless their endogenous expression level is minimal.^{707,743} Additionally, expression of EBNA-1 has little effect, or a negative effect, on the growth of EBV-negative BL cells.⁷⁴² On the other hand, EBNA-1 partially protects EBV-negative BL cells from the apoptotic effects of p53 overexpression,⁷⁴² and inhibition of EBNA-1 in EBV-positive BL cells can induce apoptosis,⁷⁴² consistent with EBNA-1 or another EBV genome product affecting EBV-positive BL cell survival. Also, EBNA-1 can associate with Mdm2 and the p53 ubiquitin specific protease USP7 and could affect p53 stability, although the affinity of EBNA-1 for USP7 is less than that of p53.¹²⁸³

High-affinity EBNA-1-binding sequences have not been identified in cell DNA.⁶⁰⁹ More recently, a number of studies

using transcription profiling, chromatin immunoprecipitation followed by microarray hybridization (ChIP-Chip), and ChIP combined with deep sequencing (ChIP-Seq) have identified a large number of potential cellular targets of EBNA-1.^{166,326,932,1371}

Many studies have been done analyzing the potential of EBNA-1 to alter the expression of cellular genes that may provide a growth advantage to cells expressing EBNA-1. Survivin, an apoptosis suppressor protein, is up-regulated by EBNA-1.⁹³⁶ EBNA-1 modulation of host cell gene transcription affects the STAT1 and transforming growth factor- β (TGF- β) signaling pathways.¹⁵⁵³ ChIP-Seq has been used to identify cellular sites bound by EBNA-1. A cluster of binding sites were identified on chromosome 11 and EBNA-1 bound close to the transcriptional start site of a large number of cellular genes.⁹³² The significance of these findings needs to be determined.

EBNA-2

Early studies identified a transformation-incompetent but replication-competent laboratory strain of EBV, P3HR-1, in BL cells from a culture of Jijoye BL tumor cells.^{583,584,1023} The search for the biochemical basis of the nontransforming phenotype led to the discovery and characterization of EBNA-2.^{253,282,283,566,758,772,773,1185,1200} Transfection of cells infected with the P3HR-1 with cloned wild-type EBV DNA that spanned the deletion resulted in homologous recombination and restoration of transformation competence.^{252,253,523} Transfection with cloned EBV DNA fragments deleted for part of EBNA-2 ORF or containing a stop codon one-third of the way into the EBNA-2 ORF failed to restore transforming ability. Recombinant EBVs with a nontransforming EBNA-2 mutation could also be recovered by infection of EBV-negative BL cells.⁹⁷² EBNA-2 differs extensively between EBV types 1 and 2, and this results in a differential ability to cause LCL outgrowth.^{253,282,1222} Restoration of the P3HR-1 deletion with type 1 or type 2 EBNA-2 indicates that EBNA-2 is the principal determinant of efficient transformation of primary B lymphocytes.²⁵³ Primary B lymphocytes infected with type 2 P3HR-1 EBV harboring type 1 EBNA-2 DNA proliferate into LCLs as rapidly as lymphocytes transformed by type 1 EBV strains. In contrast, recombination of cloned type 2 EBNA-2 DNA in place of the P3HR-1 deletion results in deficient transformation, characteristic of type 2 EBV. Interestingly, studies have shown that type 1 EBNA-2 induces more cell genes including CXCR7, suggesting that this may be in part why type 1 EBNA-2 causes more robust LCL outgrowth.^{168,938}

EBNA-2 is a specific transactivator of cell CD23, CD21, c-fgr, and c-myc and EBV LMP1, LMP2A, LMP2B, and Cp promoters. EBNA-2 is a transactivator that has no DNA-binding activity, but rather is brought to promoters that contain EBNA-2 response elements by cellular DNA-binding proteins. EBNA-2 stably associates with the cell sequence-specific DNA-binding protein RBP-J κ /CBF1.^{494,694,899,902} EBNA-2 can transactivate through a multimerized RBP-J κ site^{899,902} and is at least partially dependent on RBPJ κ sites for transactivation of LMP1 and LMP2B,^{694,834} LMP2A,¹⁶³⁷ Cp,⁶⁹¹ CD21,⁹⁶³ and CD23⁸⁹⁹ promoters. Other elements important for EBNA-2 responsiveness include the PU.1^{694,834,1578} and CRE binding site in the LMP1 promoter¹³⁵⁹ and the AUF1 binding site in the Cp and CD21 promoters.^{433,1459} Based on the similarity to EBNA-2 with an activated Notch, the cellular binding partner

of RBP-J κ /CBF1, cellular genes that are activated by EBNA-2 have been globally studied.^{958,1305,1384,1628} Studies using CHIP-seq and an analysis of long-range chromosomal interactions (HiC) indicate that EBV utilizes intrinsic B-cell transcriptional programs mediated by EBNA-2 and RBP-J κ /CBF1 to achieve B-cell immortalization by targeting H3K4me1-modified, nucleosome-depleted, nonpromoter sites to drive proliferation of resting B cells by activation of cell growth genes.¹⁶³⁰ In EBV LCLs, constitutive Notch signaling does not replace EBNA-2 function to maintain B-cell proliferation.^{475,598} Partial rescue of proliferation could only be achieved by co-expressing LMP1 independently of EBNA-2 or by expressing activated Notch at high levels.^{475,598} Further studies based on this observation have shown that EBNA-2 and activated Notch induce pro-apoptotic cellular genes, whereas EBNA-2 only up-regulates antiapoptotic genes.⁷⁹³

Recombinant EBV genetic analysis of EBNA-2 using marker rescue of primary B-lymphocyte transforming activity in the background of EBNA-2–deleted P3HR-1 EBV identified three essential EBNA-2 domains (Fig. 61.7), which are also critical for LMP1 and Cp promoter up-regulation. The essential domains correspond to a requirement for N-terminal amino acids 1 to 58 or 97 to 210,^{252,253,1577} which mediate homotypic association⁵³⁵; amino acids 280 to 337,^{252,536,1467,1578} which mediate interaction with RBP-J κ /CBF1 through amino acids PWWP^{494,559,1578} and SKIP¹⁶³²; and amino acids 420 to 464,^{244,252,1467} which are an acidic activation domain.^{244,250,252}

In activating transcription through RBP-J κ , EBNA-2 mimics a constitutive Notch receptor. The WWP sequence in EBNA-2 is similar to the WFP sequence in the Notch receptor intracellular domain that normally mediates tight association with RBP-J κ in up-regulating HES-1 in tissue development.¹⁴²³ An EBNA-2 WW to SS mutation ablates EBNA-2 up-regulation of the Cp promoter, reduces LMP1 promoter activation by 50%, and ablates EBV transformation of primary B lymphocytes

into LCLs.¹⁵⁷⁸ A subtle EBNA-2 WW to FF mutation also substantially reduces EBNA-2 transactivation of the Cp and LMP1 promoters.¹⁵⁷⁸

Once near promoter sites, the EBNA-2 acidic activating domain recruits basal and activation-associated transcription factors including TFIIB, TAF40, TBP, RPA70, TFIIF, and CBP/P300 (Fig. 61.7).^{1464–1466,1527} In B lymphocytes, but not CHO cells, the EBNA-2 acidic domain is nearly as strong in activation of basal reporter constructs with multiple upstream Gal-4 binding sites as the core VP16 14 amino acids, while the corresponding EBNA-2 14 amino acids have only 25% of the VP16 activity.²⁵⁰ Despite their greater activity in transient assays, the VP16 14 amino acids can substitute for the corresponding EBNA-2 amino acids in primary B-lymphocyte conversion to LCLs, when expressed in an EBV recombinant.²⁴⁴ Much of the EBNA-2 acidic domain is complexed with a cell nuclear protein, p100, that has co-activating effects with EBNA-2.¹⁴⁶⁵ p100 is a scaffolding protein for the acidic domain, for TFIIE, for c-myc, and for PIM-1.^{1205,1465} Efficient assembly of multiple transcription factors at promoter sites appears to require multiple EBNA-2 acidic domains, because two separate EBNA-2 domains mediate homotypic association.⁵³⁵

EBNA-2 is associated with nucleoplasm, chromatin, and nuclear matrix fractions and localizes to large nuclear granules.^{566,1160} EBNA-2 is phosphorylated on serine and threonine residues and may undergo significant posttranslational modification other than phosphorylation, because the size of the nascent protein is 10 kD smaller than stable intranuclear EBNA-2.^{484,1521} Numerous studies have suggested a role of phosphorylation in EBNA-2 function. Serine 243 is a target of both BGLF4 protein kinase and cdc2/cyclin B1 kinase and phosphorylation results in suppression of EBNA-2 activity.^{1614,1615} Although not essential for transformation or transcriptional activation, the domain that is carboxyl-terminal to the polyprolines has also been implicated in

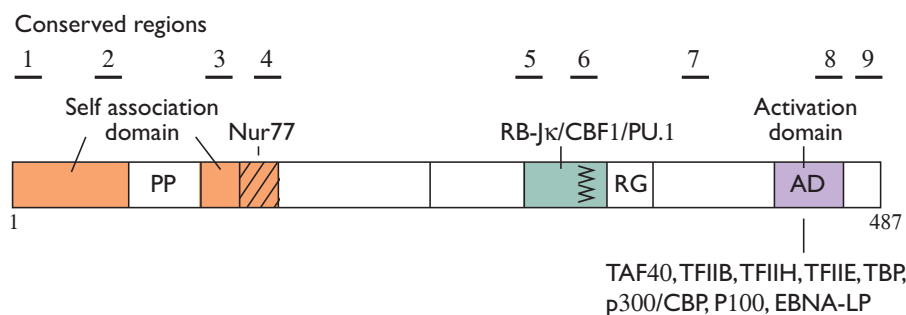


FIGURE 61.7. Schematic depiction of Epstein-Barr virus nuclear antigen (EBNA)-2 domains. EBNA-2 contains a domain that interacts with the cellular sequence-specific DNA binding proteins RBP-J κ and PU.1 to bring it to specific promoters, including the latent membrane protein 1 (LMP1) promoter. The EBNA-2 N-terminus has two domains that can mediate homotypic association with other EBNA-2 molecules. Within this region there is an interactive domain for Nur77. The poly-proline (PP) and poly-arginine-glycine (RG) as well as the activation domain (AD) are shown. The tryptophan-tryptophan-proline (WW) sequence in EBNA-2, which is similar to a sequence in the Notch receptor intracellular domain that normally mediates tight association with RBP-J κ in up-regulating HES-1 in tissue development, is shown. Mutation of this sequence ablates EBNA-2 transactivation.¹⁵⁷⁸ The EBNA-2 acidic domain (AD) interacts with TAF40, TFIIB, TFIIF, TFIIE, TBP, P300/CBP, p100, and EBNA-LP in stimulating transcription. The EBNA-2 acidic domain is extensively associated with p100, which is a scaffolding protein for c-myc and PIM-1. Nine conserved regions of EBNA-2 (bars above the protein) have been identified by comparing type 1 and type 2 EBNA-2 with those from related lymphocryptoviruses (LCVs) that infect nonhuman primates.⁹⁰³

interactions with human SNF5/INI1 and the DEAD box protein DP103.^{502,1557,1558} DP103 is a DEAD box protein with RNA helicase activity that appears to have transcriptional repressive effects. SNF5/INI1 and DP103 interact with EBNA-2 amino acids 112 to 344 in yeast two-hybrid screens,^{1557,1558} and the interaction of EBNA-2 with SNF5/INI1 has been mapped to several sites within EBNA-2 including a casein kinase II (CKII) phosphorylation site.⁸²¹ Finally, Nur77 associates with EBNA-2 via conserved region 2 (CR2) and the resulting interaction can block apoptosis by etoposide and 5-fluorouracil (Fig. 61.7).^{847,848}

The EBNA-2 RG repeat domain has an unusual, partly understood role in EBNA-2 activation and EBNA-LP co-activation (Fig. 61.7).^{1148,1150,1467} EBV recombinants deleted for the RG domain are deficient in transforming primary B cells to LCLs and hyperactivate the LMP1 promoter in transient transfection assays. The RG repeat binds histone H1, EBNA-1, and nucleic acids, especially poly(G). The strong interaction of the EBNA-2 RG domain with histone H1 is consistent with the effect of the RG domain deletion in releasing EBNA-2 from the chromatin to the nucleoplasm.¹⁴⁶⁷ These data are most compatible with a model in which the RG domain modulates EBNA-2 interactions with chromatin in transcriptional regulation. Studies with the EBNA-2 acidic domain provided the key link between acidic domains and p300/CBP recruitment to promoters. More recently, genome wide studies have found EBNA-2 to have evolved to enter approximately 5,000 enhancer elements throughout the human genome in B lymphocytes to up regulate myc and other cell genes necessary for B cell growth and survival.¹⁶³⁰

EBNA-LP

EBNA-LP is composed of several 22- and 44-amino acid segments encoded by IR1 W1 and W2 exons and 11- and 34-amino acid segments encoded by Y1 and Y2 exons (Fig. 61.8A). Because the number of IR1 repeats varies among EBV isolates, EBNA-LP frequently varies in size in different EBV isolates or even different progeny of the same virus.^{313,391,1521}

The 44-amino acid domain encoded by W2 exons has two runs of basic amino acids, RRHR and RRVRRR, which enable EBNA-LP nuclear localization (Fig. 61.7B).^{1154,1425} EBNA-LP is phosphorylated at multiple sites, probably at least once within each 44-amino acid repeat, because the number of iso-electric forms varies with the W1W2 exon repeat number.¹¹⁶⁰ The number of repeats has a direct effect on the transforming ability, with optimum transforming function observed with viruses containing five repeats.¹⁴⁵² EBNA-LP is phosphorylated on serine residues and *in vitro* is a substrate for both casein kinase II and the cyclin-dependent p34cdc2^{783,1598} and the EBV-encoded kinase BGLF4.⁷²¹ This phosphorylation occurs in a cell cycle stage-specific manner, with the protein being hyperphosphorylated in late G2 consistent with a role for p34cdc2 in phosphorylation of EBNA-LP.^{783,1160}

EBNA-LP is transcribed along with EBNA-2 at the initiation of EBV infection in primary B cells and cooperates with EBNA-2 in the activation of virus and cell gene expression.^{17,534,1098,1154,1349} Co-activation is evident in transient expression assays with artificial LMP1 promoter constructs and with endogenous LMP1 expression in EBV-infected BL cells that are otherwise in latency 1 and fail to express LMP1 until EBNA-2 and EBNA-LP are co-expressed by gene transfer. Association of EBNA-LP with EBNA-2 is evident when the two proteins are expressed and purified from bacteria and incubated under physiologic conditions or when EBNA-LP deleted for its C-terminal 11 amino acids is expressed with EBNA-2 in human B cells.¹¹⁴⁸ EBNA-LP does not significantly associate with EBNA-2 in LCLs.¹¹⁴⁸ However, EBNA-LP can bind to and co-activate through the EBNA-2 acidic activating domain or through an EBNA-2 N-terminal 58-amino acid domain.¹¹⁵⁰

EBV recombinant genetic analyses of EBNA-LP have focused on the C-terminal two exons, because mutations in the repeating exons of EBNA-LP are more difficult to construct. Specific deletion of DNA encoding the C-terminal two exons or placement of a nonsense codon at the beginning of the last two exons resulted in EBV recombinants that are deficient in

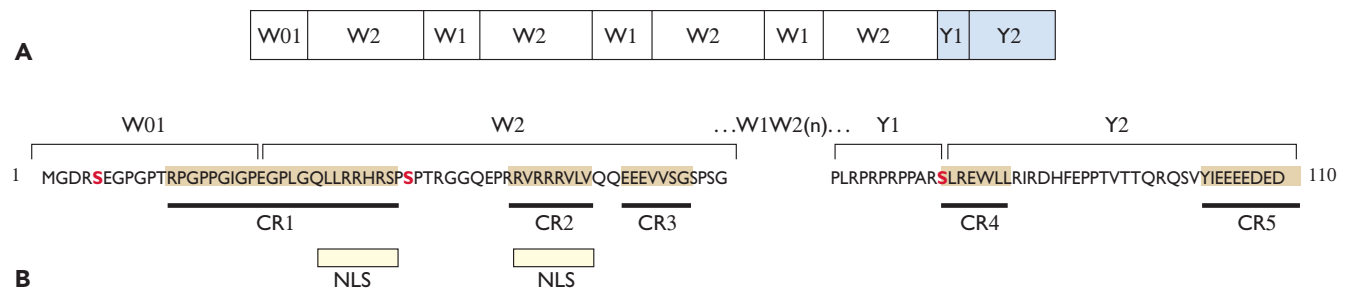


FIGURE 61.8. Schematic diagram of the repeat and unique exon and domain structure of Epstein-Barr virus nuclear antigen leader protein (EBNA-LP). The exons of EBNA-LP are derived from the BamHI W and Y fragments with initiation of transcription from Wp or Cp. **A:** The EBNA-LP AUG is generated by a unique alternative 5' splicing event. The W1 and W2 regions are repeats of 22 and 44 amino acids, respectively. This EBNA-LP repeat domain co-activates transcription mediated by the EBNA-2 acidic domain. The unique C-terminus of EBNA-LP consists of Y1 and Y2, which is dispensable for EBNA-2 co-activation and modulates EBNA-LP function. **B:** Amino acid sequence of the unique W01 exon and the W2, Y1, and Y2 exons. A single W1/W2 repeat is shown. Also shown are serines that are phosphorylated and conserved regions (CRs) in EBNA-LP proteins encoded in EBV and the nonhuman primate lymphocryptoviruses (LCVs).

primary lymphocyte transformation into LCLs.^{523,970} In two series of experiments that resulted in many wild-type EBV recombinant LCLs, only two LCLs were obtained that were infected with recombinants that were deleted for the C-terminal two exons, and these LCLs were deficient for growth. Transfection of one of these LCLs with an expression vector for wild-type EBNA-LP enabled the LCLs to grow out more rapidly under selection than control LCLs.¹⁸ Despite growth of infected primary B cells on fibroblast feeder layers, the frequency of derivation of EBNA-LP nonsense recombinant transformed LCLs was less than 5% of wild-type recombinants, and cell growth was slower than with wild-type recombinants. One mutant recombinant-infected LCL was obtained that had wild-type growth characteristics, and that LCL proved to be infected with a revertant.⁹⁷⁰ Passage of the deletion or nonsense EBNA-LP recombinants into other primary B lymphocytes further confirmed the reduced transformation phenotype.⁹⁷⁰ Infection of multiple primary B cells with higher-titer mutant recombinant virus enabled better outgrowth of LCLs infected with the mutant EBNA-LP EBV recombinants, presumably because of cross-feeding among the multiple mutant EBV-infected cells.⁹⁷⁰ There was no difference in latent EBV gene expression other than the absence of wild-type EBNA-LP. The mutant virus-infected LCLs were more highly differentiated toward immunoglobulin secretion, compatible with the possibility that wild-type EBNA-LP inhibits differentiation. In other experiments, transient expression of EBNA-LP and EBNA-2 in primary B cells co-stimulated with gp350 resulted in induction of cyclin D2.¹³⁴⁹ As described earlier, EBNA-LP potentiates EBNA-2-mediated transcription from the Cp and LMP1 promoters in transient transfection assays with reporter constructs and also co-activates EBNA-2 up-regulation of LMP1 expression in type 1 EBV-infected BL cells. Although EBNA-LP did not further co-activate CD21, LMP2A, and HES-1 promoters with EBNA-2—indicating promoter specificity in EBNA-LP co-activation,¹¹⁵³ EBNA-LP co-activation with Gal4-EBNA-2 in BL cells indicates that promoter specificity is somewhat relaxed.¹¹⁵⁰ Overall, EBNA-LP has a key role in up-regulating viral and cellular gene expression critical for LCL outgrowth, which is likely to be its primary role in EBV conversion of primary B cells to LCLs.

The unique 3' exons of EBNA-LP encode 11- and 34-amino acid C-terminal domains. While the repeating exons are efficient co-activators, addition of 11 or even 35 of the C-terminal residues results in nearly complete loss of co-activating effects where as, addition of the entire 45 C-terminal amino acids restores wild-type co-activation. These data indicate that the first 35 C-terminal amino acids have a strong negative regulatory effect on co-activation and the last 10 amino acids effectively silence the negative regulatory effect.^{534,1148} The negative effect of the stop codon mutation that prevented expression of the last 45 amino acids on primary B-lymphocyte transformation⁹⁷⁰ is evidence of the importance of this complex regulatory domain for efficient lymphocyte growth transformation. Differential phosphorylation of EBNA-LP by p34 cdc2 or casein kinase II may be important in the natural regulatory effects of the C-terminal domain.

An effect of EBNA-LP on retinoblastoma protein (Rb) and p53 has been suggested based on *in vitro* biochemical interactions and co-localization with Rb.¹⁴¹⁸ However, EBNA-LP is not associated with Rb family proteins or p53 in cell lysates

and does alter Rb- or p53-mediated transcription.⁶⁵⁷ Expression of EBNA-LP in transgenic mice had no discernible effect on development or tumor incidence.⁶³² The EBNA-LP transgenic mice died of heart failure, which is likely to be due to a toxic effect of EBNA-LP expression or an insertion site effect.⁶³² Immunoprecipitations of EBNA-LP from B lymphoblasts have resulted in the identification of the DNA-PK catalytic subunit, an AKAP 95 homolog, HA95, HSP72, and HSP27 as EBNA-LP-associated proteins.^{526,630,784,971} EBNA-LP associates at a very high level with HA95 in B cells and is also associated with DNA-PK, probably through HA95.⁵²⁶ HA95 appears to mediate EBNA-LP co-activation by heterodimerizing with EBNA-LP and removing NCoR and HDACs from matrix associated deacetylase bodies and freeing chromatin from repressors.⁵²⁷ EBNA-LP is partially localized in the cytoplasm and more often associated with the nuclear matrix fraction than other EBNAs.¹¹⁶⁰ HSP72 up-regulates EBNA-LP co-activation with EBNA-2.¹¹⁴⁹ In immune microscopy of LCLs or of non-EBV-infected BL lymphoblasts in which EBNA-LP is expressed alone by gene transfer, EBNA-LP localizes to promyelocytic leukemia (PML) nuclear bodies.^{1417,1521} Sp100A, a PML nuclear body-associated protein, is another EBNA-LP-interacting protein.⁹⁰¹ EBNA-LP interacts with and displaces Sp100 from PML nuclear bodies.⁹⁰¹ Sp100A mutants deleted for the PML targeting domain, which is also the domain that interacts with EBNA-LP, can co-activate EBNA-2 independent of EBNA-LP, suggesting that displacement of Sp100A from PML nuclear bodies is an important EBNA-LP function.⁹⁰¹ Deletion of the HP1 α interaction domain in Sp100A prevents Sp100A co-activation, suggesting that EBNA-LP function may in part involve chromatin modification.⁹⁰¹ Studies in support of this have shown that EBNA-LP interacts with HDAC4 and 14-3-3 and sequesters HDAC4 in the cytoplasm. More recent studies have shown that isoforms of EBNA-LP smaller than 42 kD shuttle between the nucleus and cytoplasm.¹¹⁷⁸ Isoforms larger than 62 kD have no detectable nuclear-cytoplasmic shuttling activity and retain co-activation function. Moreover, the ability to interact with HDAC4 and shuttle between the nuclear and cytoplasmic compartments is not sufficient to mediate the co-activation function.⁹⁰⁴ Finally, EBNA-LP associates with NCoR and dismisses NCoR, NCoR and RBPJ, or NCoR, RBPJ, and EBNA-2 from matrix-associated deacetylase (MAD) bodies, which increases gene expression.¹¹⁷⁹

EBNA-3A, EBNA-3B, and EBNA-3C

The EBNA-3 genes are tandemly placed in the genome (Fig. 61.5), are collinear distant homologues, are transcribed under control of the far upstream latency 3 Cp or Wp EBNA promoters, share a U exon splice with EBNA-1, have individual splice acceptor sites that compete with EBNA-1, and have short and long exons, and have individual downstream polyA sites.^{65,115,116,564,692,748,1159,1161,1274,1275} LCLs have very few copies of each EBNA-3 mRNA. EBNA-3A, EBNA-3B, and EBNA-3C have repeating oligopeptides near their C-termini, which serve as unusual examples of domain amplifications within ORFs that are amplified from a common progenitor. The type 1 EBNA-3A, EBNA-3B, and EBNA-3C are 944, 938, and 992 amino acids, respectively (Fig. 61.9A).¹³⁴⁶ The type 2 EBNA-3A, EBNA-3B, and EBNA-3C proteins are 925, 946, and 1,069 amino acids and are 84%, 80%, and 72% identical to their type 1 counterparts, respectively.¹²⁷⁸

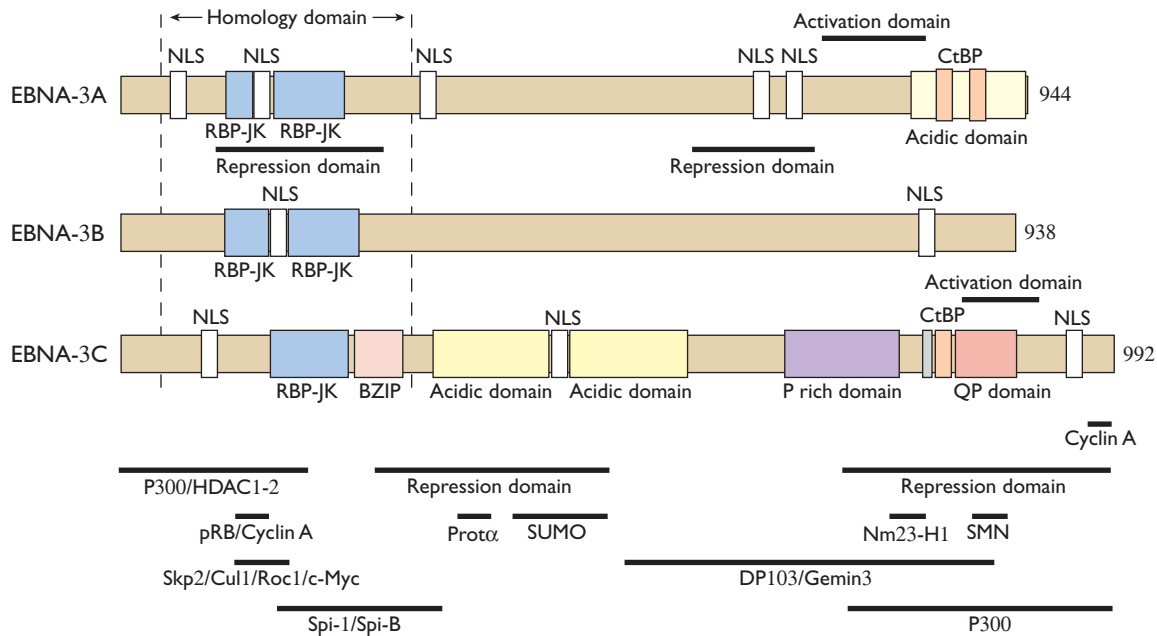


FIGURE 61.9. Schematic diagram of the Epstein-Barr virus nuclear antigen (EBNA)-3A, EBNA-3B, and EBNA-3C and their functional domains. EBNA-3A, EBNA-3B, and EBNA-3C proteins are shown with the number of their amino acids and with a common domain that contains the RBP-J κ interaction domain. Also indicated are domains that have activating or repressive effects in addition to a variety of domains that associate with cellular proteins including CtBP, p300/HDAC1-2, pRB/cyclin A, prothymosin- α (Prot α), Skp2/Cul1/Roc1/c-Myc, Spi-1/Spi-B, SUMO, Nm23-H1, SMN, DP103/Gemin3, and p300. Nuclear localization signals (NLSs), a leucine zipper (BZIP), acidic domains, a proline (P)-rich region, and a glutamine (Q) and proline region are shown. (From Sims K, Saha A, Robertson ES. Regulation of cellular processes by the Epstein-Barr Virus nuclear antigen 3 family of proteins. In: Robertson ES, ed. *Epstein-Barr Virus: Latency and Transformation*. Philadelphia: Caister Academic Press, 2010:81–100, with permission.)

Although significantly divergent in nucleotide and amino acid sequence, type 1 and type 2 EBNA-3A, EBNA-3B, and EBNA-3C have similar effects in B-cell growth transformation.¹⁴⁶¹ EBV recombinants made in a type 2 EBV P3HR-1 background with type 1 EBNA-LP and EBNA-2 have the robust primary B-cell transformation phenotype characteristic of type 1 EBV strains. Replacement of the P3HR-1 type 2 EBNA-3s with type 1 EBNA-3s does not alter infected B-cell outgrowth to LCLs or the ability of the recombinant virus to replicate in response to induction of permissive infection. Thus, EBNA-3 type specificity does not affect latent or lytic virus infection of B lymphocytes *in vitro*.

EBNA-3B is not critical for latent infection of primary B cells, for LCL outgrowth, for cell survival, or for lytic virus replication.^{201,773,1462} Finding that EBNA-3B is not essential for any aspect of lymphocyte infection *in vitro* is surprising because EBNA-3B epitopes are frequently recognized by human immune cytotoxic T lymphocytes. Immune recognition would be expected to provide strong *in vivo* selection against a nonessential gene. Consistent with the lack of effect of the putative null EBNA-3B mutation on lymphocyte infection and LCL outgrowth *in vitro* and the expectation that EBV strains with altered or absent EBNA-3B genes would be expected to emerge under immune selection, an EBV mutant with a truncated EBNA-3B ORF was discovered in a transplant patient with EBV-associated lymphoproliferative disease who was undergoing therapy with anti-EBNA-3B cytotoxic T lymphocytes.⁴⁷⁹

In contrast to the lack of phenotype associated with truncation of the EBNA-3B ORF, nonsense codons after codon 302 of EBNA-3A or codon 365 of EBNA-3C result in EBV recombinants that are unable to transform primary B cells.^{741,1463} These experiments were the first to indicate that EBNA-3A and EBNA-3C are critical for B-cell growth transformation or survival and differ significantly from EBNA-3B in their roles in EBV infection. Interestingly, despite collinear homology, rhesus LCV EBNA-3A, EBNA-3B, and EBNA-3C cannot substitute for EBV EBNA3s in transformation of human B cells into LCLs, consistent with species-specific roles for EBNA-3A, EBNA-3B, or EBNA-3C in LCL outgrowth.⁶⁸⁶

Studies with conditional mutant forms of EBNA-3A or EBNA-3C confirm the importance of these proteins for LCL growth and have allowed extensive studies of domains of EBNA-3A or EBNA-3C required for LCL growth.^{985,986,988–990,1360} This genetic analysis used viral genomes in which the EBNA-3A or EBNA-3C terminus is fused in frame to a 4OH-tamoxifen (4HT)-dependent mutant estrogen receptor hormone binding domain.⁹⁸⁵ The resultant EBV EBNA-3AHT or EBNA-3CHT transformed LCLs conditionally express EBNA-3AHT and EBNA-3CHT. In 4HT, EBNA-3AHT or EBNA-3CHT LCLs grow similarly to wild-type LCLs. In the absence of 4HT, the LCLs slowly stop growing and gradually die. Addition of 4HT restores exponential cell growth.

Wild-type or mutant EBNA-3A or EBNA-3C complementation in LCLs transformed by the conditional EBV

EBNA-3AHT or EBNA-3CHT has identified regions within EBNA-3A and EBNA-3C that are not required for LCL growth.^{985,989} These studies have also identified domains of EBNA-3A and EBNA-3C that are required for continued LCL growth^{986,989,990,1360} (Fig. 61.9A). EBNA-3A and EBNA-3C mutants in the RBP-J κ binding site are deficient in repression of EBNA-2 activation of the EBV Cp enhancer and in LCL growth.^{986,989} Thus, EBNA-3A and EBNA-3C regulation of transcription through RBP-J κ /CBF1 is critical for LCL growth. More recent studies have been particularly illuminating in regard to EBNA-3A and EBNA-3C function. Initially, it was shown that EBNA-3C inactivation resulted in the accumulation of p16 (INK4A) and decreases in the hyperphosphorylated form of Rb, resulting in a smaller number of cells in S or G2/M phase.⁹⁸⁸ In more recent studies, it was demonstrated that epigenetic repression of p16 (INK4A) and p14 (ARF) expression mediated by EBNA-3A and EBNA-3C are required for LCL growth.^{990,1360} Moreover, human papillomavirus type 16 E7 and E6 expression was able to sustain LCL growth after inactivation of EBNA-3C or EBNA-3A.⁹⁹⁰ Interestingly, binding to the C-terminal-binding protein (CtBP) by both EBNA-3A and EBNA-3C is required for LCL growth.¹³⁶⁰ Thus, the interaction of EBNA-3A with CtBP is only now beginning to be understood⁵⁷⁷ as well as the significance of the finding that the cellular protein BIM is epigenetically regulated by EBNA-3A and EBNA-3C.^{36,1140}

Despite the absence of homology, EBNA-3A, EBNA-3B, and EBNA-3C are similar to EBNA-2 in stably associating with RBP-J κ .^{696,802,979,1228,1229,1512,1627} The EBNA-3 family can significantly alter cell gene transcription, and in many cases there is a remarkable similarity in this regulation consistent with coordinated effects on cell gene transcription.^{202,572,958,1384,1540,1626} In associating with RBP-J κ , the EBNA-3s compete for RBP-J κ with EBNA-2 and Notch and limit EBNA-2 up-regulation through RBP-J κ . An RBP-J κ domain as small as amino acids 125 to 181 can bind to EBNA-3C, and amino acids 172 to 223 of EBNA-3C, which are relatively well conserved in EBNA-3A and EBNA-3B, can interact with RBP-J κ (Fig. 61.9A).^{696,802,979,1228,1229,1512,1627} EBNA-3A amino acids 224 to 556 can also bind to RBP-J κ .²³⁹ EBNA-3A, EBNA-3B, and EBNA-3C bind more tightly to RBP-J κ than EBNA-2 and can displace EBNA-2 from RBP-J κ . Most of the RBP-J κ in LCLs is associated with EBNA-2, EBNA-3A, EBNA-3B, and EBNA-3C.⁶⁹⁶ Two- to threefold overexpression of EBNA-3A in LCLs is sufficient to dissociate RBP-J κ from the strong up-regulatory effects of EBNA-2, decrease c-myc expression, and stop LCL growth.²⁶¹

Other studies have also highlighted the importance of EBNA-3C in mediating gene transcription. EBNA-3C interaction with RBP-J κ is important for up-regulating TCL1.⁸⁵⁰ EBNA-3C has also been implicated in suppressing the ATM/Chk2-mediated DNA damage-responsive signaling pathway that is important in EBV transformation.^{235,1093}

Several lines of evidence indicate that EBNA-3C can have transcriptional up-regulatory effects alone or in concert with EBNA-2. When expressed alone in B-lymphoma cells, EBNA-3C can up-regulate CD21 expression and does not affect CD23, CD10, CD30, CD39, CD40, CD44, or cellular adhesion molecule expression.¹⁵¹⁸ Raji latency 3 BL cells do not express EBNA-3C as a consequence of a deletion in the Raji EBV genome. When Raji cells reach saturation, they undergo

growth arrest in G1 and LMP1 levels fall. EBNA-3C expression in Raji cells sustains LMP1 levels when the cells grow to saturation,²⁰ consistent with the notion that EBNA-LP co-activation of the LMP1 promoter with EBNA-2 ceases at saturation and is replaced by EBNA-3C co-activation. In support of this hypothesis, EBNA-3C is unique among EBNA-3s in co-activating the LMP1 promoter with EBNA-2.^{690,892,1629} The EBNA-3C leucine zipper interacts *in vitro* with the PU.1-Ets domain and appears to up-regulate transcription through the LMP1 promoter PU.1 binding site.¹⁶²⁹ The EBNA-3C effect is independent of the RBP-J κ binding site in the LMP1 promoter and of the RBP-J κ interaction domain in EBNA-3C.¹⁶²⁹ Furthermore, EBNA-3C fully co-activates with WW to SS mutant EBNA-2, which cannot interact with RBP-J κ .⁸⁹² EBNA-3C amino acids 365 to 545, which have repressive activity in the context of fusions to the Gal4 DNA binding domain,⁷⁰ are essential for co-activation with EBNA-2.^{892,1240} Point mutations of residues within that domain abrogate EBNA-3C co-activation with EBNA-2. SUMO1, 2, and 3 bind to this site in EBNA-3C. Point mutations that disrupt EBNA-3C co-activation also disrupt SUMO binding. Furthermore, overexpression of SUMO or UBC9 co-activates the LMP1 promoter with EBNA-2 in the absence of EBNA-3C.¹²⁴⁰ Moreover, EBNA-2-mediated promoter activation is dependent on p300, and EBNA-3C potentiation of p300 transcriptional effects is abrogated by the same point mutations. These data are consistent with a model in which SUMO association with EBNA-3C at the LMP1 promoter activates p300 and potentiates EBNA-2-enhanced transcription.^{892,1240} Other data identify EBNA-3C glutamine- and proline-rich amino acids 724 to 826 as activators of transcription from a GAL4-responsive promoter when fused to the GAL4 DNA binding domain.⁹⁷⁹ The EBNA-3C C-terminal domain can interact with Nm23-H1 in transcriptional activation¹⁴⁰¹ and modulate necdin activity.⁷²⁴ EBNA-3C can also recruit the p300 histone acetylase through an interaction with prothymosin α .^{265,1399,1400} EBNA-3B binds Spi-1/Spi-B, which is important for binding an EBNA-3C-responsive element in the LMP1 promoter.^{834,1629} EBNA-3C can directly bind to the tumor suppressor protein p53 and repress its functions, in part by blocking its transcriptional activity as well as facilitating its degradation through stabilization of its negative regulator, Mdm2.¹²⁶⁵ More recent studies have demonstrated that EBNA-3C can negatively regulate p53-mediated functions by interacting with the inhibitor of growth family proteins ING4 and ING5.¹²⁶³ Both ING4 and ING5 bind to N-terminal domain residues 129 to 200 of EBNA-3C,¹⁵⁹² which associates with p53 and is also essential for LCL growth. EBNA-3A activity as well as Mdm2-dependent mechanisms may be important in preventing apoptosis of LCLs in which the p53 pathway is active.⁴¹¹ EBNA-3C has repressive effects on the EBV Cp promoter, which is dependent on RBP-J κ sites in the promoter plasmid and partially on HDAC1 binding.^{1201,1202} Full-length EBNA-3C fused to the GAL4 DNA binding domain also represses transcription and EBNA-3C amino acids 280 to 525 and 580 to 992 have repressive effects in the context of Gal4 fusions.^{70,125}

EBNA-3C has been reported to cooperate with Ras in transforming rat embryo fibroblasts and to interact at a low level with Rb in activation of an E2F site in the B-myc promoter.^{1137,1138} In NIH3T3 fibroblasts or human U2OS cells compelled to arrest by serum withdrawal, EBNA-3C

expression can inhibit p27 (K1P1) accumulation and cause bi- or multinucleated cells by abrogating the mitotic spindle checkpoint.¹¹³⁸ Because bi- or multinucleated cells are not a characteristic of EBNA-3C expression levels in latency 3 EBV-infected B cells, the relevance of these observations is uncertain. EBNA-3C has also been shown to interact with and modulate cyclin activity including the cyclins A, D1, and E. This modulation may effect progression through the cell cycle in EBV-transformed cells.^{787,788,1264} EBNA-3C recruits the ubiquitin ligase Skp1/Cul1/F-box complex, resulting in ubiquitination of the cell cycle regulators Rb and p27.^{789,790} Recently, it was shown that EBNA-3C stabilizes Gemin3 to block p53-mediated apoptosis.¹⁵⁰

EBNA-3A also represses Cp promoter activity in B cells and in epithelial cells, and this activity is largely dependent on the domain through which EBNA-3A interacts with RBP- κ .²³⁹ Using GAL4 EBNA-3A fusion proteins and GAL4-responsive promoters, the EBNA-3A-repressive domain has been mapped to 143 amino acids that do not interact with RBP- κ and can mediate down-regulation of transcription.^{125,986} EBNA-3A also has a weak, B-cell-biased, activation domain that is evident when EBNA-3A deleted for the amino acids 100 to 364 repressive domain is fused to the GAL4 DNA binding domain and expressed in B lymphoblasts along with a GAL4-responsive promoter.²³⁹ An EBNA-3A bait in a yeast two-hybrid search for interacting proteins retrieved the carboxyl part of the epsilon subunit of the chaperonin T-complex protein 1 and the p38 subunit of the aryl hydrocarbon receptor complex.^{717,718} EBNA-3A associates with the p38 subunit and partially relocates it to the nucleus.

Single gene transfer of EBNA-3B into DG75 BL cells results in up-regulation of vimentin, CD40, and Bcl-2 expression and down-regulation of CD77, compatible with these being EBNA-3B-regulated genes.^{1343,1344} A more comprehensive analysis identified 550 genes regulated by EBNA-3C. As expected, EBNA-3C-regulated genes overlapped significantly with genes regulated by EBNA-2 and EBNA-3A, consistent with coordinated effects on cell gene transcription.¹⁶²⁶

LMP1

LMP1 is a constitutively active tumor necrosis factor (TNF) receptor mimic (Fig. 61.10),^{667,669,670,1060} essential for primary B-lymphocyte conversion to LCLs.^{314,315,730-732,762} LMP1 supports LCL survival and growth by inducing cell activation pathways, such as mitogen-activated protein kinase (MAPK), JNK, phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor- κ B (NF- κ B), and interferon response factor 7 (IRF7).^{348,351,354,522,761,960,1035,1095,1373,1623} Depending on cell type, LMP1 is transcribed from two different promoters separated by about 600 bp. In B cells, LMP1 is encoded by a 2.8-kb mRNA, which initiates from a site (ED-L1 promoter) that is dependent on EBNA-2.⁶⁹⁴ ED-L1 can also be activated independently of EBNA-2 by cellular factors, such as STATs, IRF7, and activating transcription factor 4 (ATF4).^{206,844,1096,1359} The p38 signaling pathway up-regulates LMP1 expression.⁶⁹⁷ Enhancement of LMP1 expression up to 200-fold can be mediated by the EBNA-1-dependent transcriptional enhancer FR in *oriP* acting across the fused TRs.⁴⁴² In epithelial cells, LMP1 transcription is mediated by an upstream promoter (L1-TR) that initiates transcription from multiple TATA-less sites in the nearest copy of TR resulting in a

3.5-kb transcript with a long untranslated 5' exon^{461,1262} and is in part dependent on the cellular factors Sp1 and STAT3.^{461,1262} Interestingly, LMP1 expression was observed to be greater with fewer TRs¹²¹⁷ and LMP2A can negatively regulate the expression of LMP1 from this promoter.¹³⁹³ The LMP1 ORF has two short introns.^{74,388} While EBNA proteins are remarkably stable, LMP1 has a short half-life. Nevertheless, LMP1 accumulates at high levels and localizes to a large plasma membrane patch in LCLs.^{563,888,890} LMP1 can have opposing effects on cell growth, enhancing proliferation at physiologic doses but being cytostatic at high levels.^{275,406,525,733} This in part is likely related to the observation that LMP1 can induce autophagy.⁸⁴³

In LCLs, LMP1 constitutively forms discrete patches or a large cap in the plasma membrane.⁵⁶³ LMP1 has six transmembrane domains (Fig. 61.10). Live cell protease cleavage studies of LCLs indicate that the LMP1 N- and C-termini are on the cytoplasmic side of the plasma membrane. Only three short reverse turn domains are on the extracellular side of the plasma membrane, and almost half of LMP1 is in the plasma membranes.⁸⁹⁰ In epithelial cells or fibroblasts, LMP1 is more broadly distributed in surface and internal cytoplasmic membrane patches.¹⁵¹⁵⁻¹⁵¹⁷ LMP1 aggregation is an intrinsic effect of the multiple hydrophobic transmembrane domain and does not change in the absence of exogenous growth factors.^{888,890} Extraction of cells with nonionic detergents reveals more than half of LMP1 to be associated with the cell cytoskeleton.^{67,888,969,1049,1051} Although vimentin is not ordinarily found in a plasma membrane patch, vimentin co-localizes with LMP1 in latently infected B-cell plasma membranes and is required for LMP1 association with the lymphocyte cytoskeleton.^{887,888} Vimentin can also further direct LMP1's localization. When vimentin relocates into perinuclear rings and coils in response to Colcemid, LMP1 relocates to vimentin rings. However, LMP1 also forms patches and caps in plasma membranes of cells that lack vimentin and does not require vimentin for signal transduction.⁸⁸⁷ The association of LMP1 with plasma membranes is at least in part mediated by cholesterol-rich membrane microdomains termed *lipid rafts*.^{45,46,581}

LMP1 is phosphorylated on serine and threonine residues at a ratio of 6:1 and is not tyrosine phosphorylated.^{888,969,1049,1051} The half-life of LMP1 in LCLs as measured by time to reach stable levels of ³⁵S-methionine in LMP1 and by pulse chase is 3 to 5 hours.^{67,888,969} Nonionic detergent-soluble LMP1 has a half-life of much less than 5 hours and is converted to more stable cytoskeletal-associated and phosphorylated LMP1.^{888,969,1049,1051} LMP1 is also cleaved near the beginning of the C-terminal cytoplasmic domain, resulting in a transiently stable soluble 25-kD C-terminal cytoplasmic domain.^{1049,1051} The principal phosphorylation sites are in the 25-kD cleavage product.¹⁰⁵⁰

In single gene transfer experiments using heterologous promoters, LMP1 has transforming or "oncogene" effects in continuous rodent fibroblast cell lines.¹⁵¹⁵ LMP1 alters Rat-1 or NIH 3T3 cell morphology, enables growth in medium supplemented with low serum, causes loss of contact inhibition, and enhances growth as tumors in nude mice. Rat-1 or Balb/c 3T3 cells also lose anchorage dependence and grow in soft agar after LMP1 expression.^{1515,1516} The growth of Balb/c 3T3 cells in soft agar correlates quantitatively with the extent

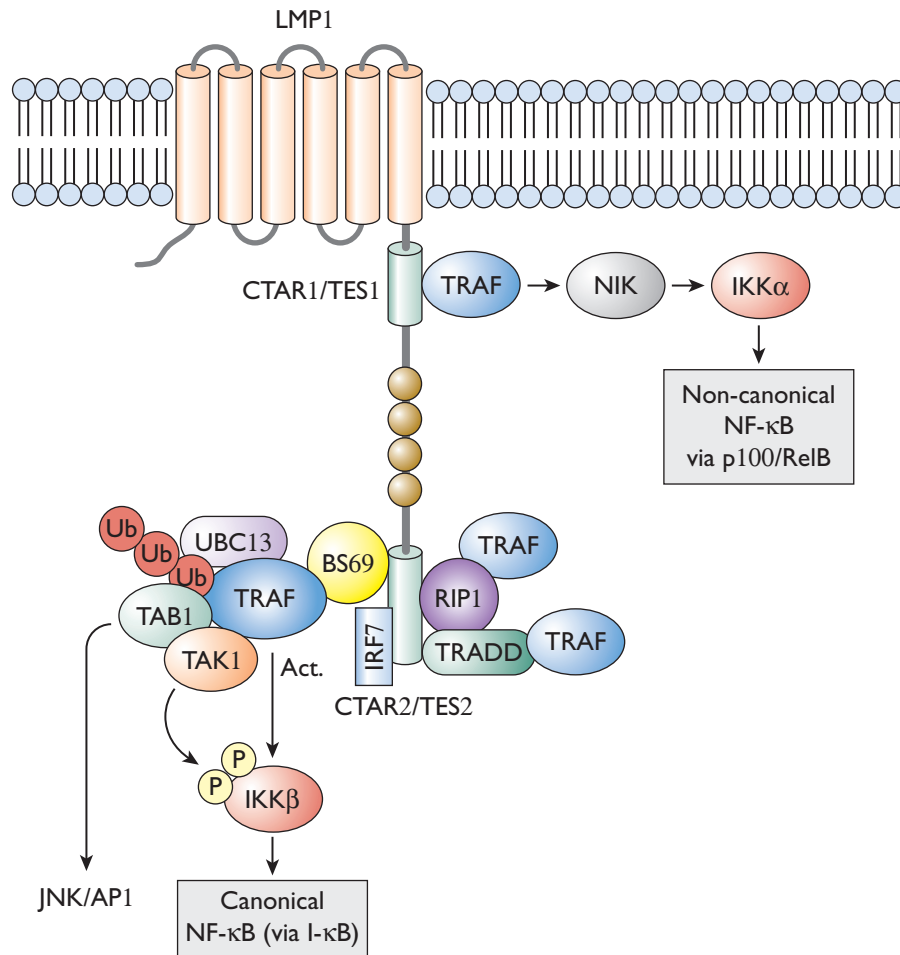


FIGURE 61.10. Schematic diagram of biochemical mechanisms by which latent membrane protein 1 (LMP1) affects cell growth and survival pathways by constitutively mimicking the activated tumor necrosis factor (TNF) receptor CD40. The six hydrophobic transmembrane domains of LMP1 enable it to aggregate in lipid rafts within the plasma membrane. The amino terminal cytoplasmic domain is fully dispensable for primary B-lymphocyte growth transformation and its primary function is in tethering the amino terminus of the first transmembrane domain to the cytoplasmic side of the plasma membrane. The transmembrane domains and the C-terminal cytoplasmic domain are essential for primary B-lymphocyte growth transformation. The C-terminal cytoplasmic domain is 200 amino acids and has two essential components (CTAR1/TES1 and CTAR2/TES2). CTAR1/TES1 follows the transmembrane domains and the first 44 amino acids associate with TNF receptor-associated factors (TRAFs), which activate noncanonical nuclear factor- κ B (NF- κ B) signaling through NF- κ B-inducing kinase (NIK) and inhibitor of κ B kinase α (IKK α). The second, CTAR2/TES2, is at the C-terminus of LMP1 and associates with the TNFR-associated death domain proteins including TNFR1-associated death domain (TRADD) and receptor-interacting protein (RIP) without propagating a death signal. BS69 and IRF7 also bind to CTAR2/TES2. Canonical NF- κ B signaling through IKK β is mediated through a TAB1/TAK1-dependent mechanism. TRAF activation involves UBC13-dependent Lys 63-linked polyubiquitination. The JNK/AP1 pathway is TRADD independent. The linker region between CTAR1/TES1 and CTAR2/TES2 can be deleted without loss of B-lymphocyte-transforming activity.

of LMP1 expression up to the levels ordinarily expressed in LCLs.^{69,1051,1516} LMP1 mutants that have transforming activity when expressed at levels similar to LCLs can be toxic when expressed at higher levels.⁹⁸³ Comparison of the transforming properties of LMP1 with those of D1LMP1, which has only transmembrane domains 4 and 5 and the C-terminus and is expressed in lytic infection, indicates that D1LMP1 lacks these effects^{68,1050,1516} and can inhibit the transforming function of LMP1.¹¹³⁰ Because D1LMP1 lacks the N-terminal cytoplasmic domain and the first four transmembrane domains, inserts into cell membranes, and does not

patch, these data indicate that the amino terminus and the first four transmembrane domains are essential for LMP1 aggregation in membranes and transforming effects in rodent cells. Addition of the 25-amino acid N-terminal cytoplasmic domain to the amino terminus of D1LMP does not restore the ability to cause anchorage-independent growth of Balb/c 3T3 cells.^{68,1050} C-terminal cytoplasmic domain truncations have variable effects but are consistently unable to cause loss of contact inhibition in Rat-1 cells.¹⁰⁵⁰ These data suggest that the C-terminal cytoplasmic domain is important for loss of contact inhibition in Rat-1 cells.

LMP1 dramatically alters the growth of EBV-negative BL lymphoblasts, primary B lymphocytes, or epithelial cells when expressed stably or transiently at the appropriate level following gene transfer.^{110,887,1151,1152,1155,1248,1517,1518} LMP1 induces many of the changes usually associated with EBV infection of primary B cells or with antigen activation of primary B cells, including cell clumping; increased villous projections; increased vimentin expression; increased cell surface expression of CD23, CD39, CD40, CD44, and class II major histocompatibility complex (MHC); increased IL-10 expression; decreased expression of CD10; and increased expression of the cell adhesion molecules leukocyte function-associated antigen-1 (LFA-1), intracellular adhesion molecule-1 (ICAM-1), and LFA-3.¹⁵¹⁷ LMP1 not only increases plasma membrane expression of adhesion molecules but also activates adhesion and induces higher levels of LFA-1 mRNA. LMP1 effects on cell adhesion are important because homotypic B-cell adhesion or adhesion to other cells can facilitate autocrine or paracrine growth factor effects. D1LMP1 has none of these effects, confirming the importance of the amino terminus and multiple transmembrane domains. LMP1 also protects B lymphocytes from apoptosis, in part through induction of bcl-2 expression.^{490,558,1248} Despite the robust and pleiotropic effects of LMP1 expression in human B-lymphoma cells, the effects of deletion mutants that partially affect the membrane patching properties of LMP1 on each of the phenotypes is intermediate, without a clear loss of some effects and the retention of others.⁸⁸⁹ LMP1 expression also alters cell growth and gene expression in a variety of cell types with resulting phenotypes and changes in cell gene expression compatible with the broad effects LMP1 has on cell survival, gene expression, and signaling.^{234,289,291,292,337,369–371,427,558,623,798,812,813,913,1500,1604,1605}

LMP1 also affects epithelial and B-lymphocyte cell growth, survival, and differentiation in transgenic mouse models. LMP1 expression in the skin and esophagus of three lineages of mice under control of the polyomavirus enhancer and promoter results in induction of the expression of a hyperproliferative keratin, K6; epidermal hyperplasia; and thickening of both parabasal and keratinizing layers.¹⁵⁴⁵ Further, LMP1 expression under the control of the immunoglobulin heavy chain promoter and enhancer in three lineages of transgenic C57/bl6 mice backcrossed into Balb/c mice results in markedly increased B-cell hyperplasia and lymphomas.⁸¹¹ Massive spleen enlargement and lymphoma increases significantly with age. Lymphomas are unusual before 12 months and develop in 42% of transgenic mice over 18 months of age. The lymphomas are monoclonal or oligoclonal by immunoglobulin H (IgH) rearrangement and frequently invade and metastasize, and LMP1 is expressed at LCL levels. These data indicate that LMP1 can be oncogenic in B cells *in vivo*. B cells of transgenic mice expressing LMP1 under the control of the immunoglobulin promoter and enhancer also have enhanced expression of activation antigens, spontaneously proliferate, and produce antibody.¹⁴⁸¹ In CD40-deficient or normal backgrounds, LMP1 expression mimics CD40 signals and induces extrafollicular B-cell differentiation, blocking germinal center formation. Transgenic mice expressing LMP1 under the control of an immunoglobulin heavy chain promoter and enhancer develop lymphoma at a threefold higher incidence than LMP1-negative mice. LMP1 immunoprecipitates with TNF receptor-associated factors (TRAFs) 1, 2, and 3, and NF- κ B, c-Rel, Akt, JNK, STAT, and p38 are activated in LMP1-associated lymphomas.^{1325,1447}

Illustrative of the similar pathways that LMP1 and CD40 activate, mice expressing a constitutively active CD40 receptor, in the form of an LMP1/CD40 chimeric protein, have B cells with an activated phenotype, prolonged survival, and increased proliferation, which results in B-cell lymphomas.^{601,1208} EBV conversion of primary B cells to LCLs is stringently dependent on LMP1 expression and therefore is a suitable system to identify the important LMP1 components.^{730,732} The LMP1 first transmembrane domain (TM1) and cytoplasmic C-terminus (CT) are essential for LCL outgrowth.^{730,732} LMP1 deleted for the N-terminal cytoplasmic domain and part of TM1 is a stable protein, which localizes to the plasma membrane and does not aggregate or enable LCL outgrowth.^{730,889} Similarly, LMP1 containing only TM3-6 and the CT is a null mutation, which localizes to all cytoplasmic membranes and does not accumulate or aggregate in the plasma membrane or enable LCL outgrowth. These mutant effects are unlikely to be due to specific interactions mediated by the N-terminal cytoplasmic domain, because deletion of any part of the N-terminal cytoplasmic domain results in at least 10% residual transforming efficiency and the resulting LCLs grow normally.⁶⁶⁸ Further, at least part of the CT is critical because expression of the N-terminus and TM1-6 without the 200-amino acid CT is null for LCL outgrowth.⁷³² Moreover, an EBV recombinant that expresses the LMP1 N-terminus, TM1-6, and CT first 44 amino acids is dependent on fibroblast feeder layers or cross-feeding from multiple transforming events for LCL outgrowth, whereas an EBV recombinant that expresses the LMP1 N-terminus and TM1-6 and is deleted for only the CT first 24 amino acids does not result in LCLs.⁶⁶⁹ These data indicate that the CT first 24 amino acids are essential for LCL outgrowth and that efficient LCL outgrowth also requires the CT last 155 amino acids.^{315,669} Surprisingly, within the CT last 155 amino acids, mutation of the most C-terminal YYD to ID identifies this site as critical for LCL outgrowth, whereas deletion of amino acids 232 to 351 has no effect on LCL outgrowth, LCL growth characteristics, or lytic EBV replication.^{666,670} Thus, the non-transforming phenotype with a deletion of the N-terminus and TM1 or a deletion of the CT first 24 amino acids, and the near-null transforming effect of mutation of the CT last three amino acids from YYD to ID, identify TM1 and the CT first 24 and last 3 amino acids as critical for LCL outgrowth, whereas amino acids 232 to 351 are not important. The two critical C-terminal transformation sites are therefore referred to as CT transformation effector sites 1 and 2, or TES1 and TES2.

In parallel with the initial genetic analyses of the role of the LMP1 CT first 44 amino acids in LCL outgrowth,⁷³² investigations of the biochemical basis of LMP1 effects in up-regulating B-cell activation markers, adhesion molecules, the HIV promoter, and A20 expression identified LMP1 to activate NF- κ B.^{522,822} Based on the role of the LMP1 CT first 44 amino acids in enabling transformation, yeast two-hybrid, biochemical pull-down, and in-cell-association experiments were undertaken, which identified TES1 interactions with a human protein TRAF3,¹⁰⁶⁰ which is related to mouse TNF receptor II (TNFRII) factors.¹²⁴² A related human protein, TRAF1 also associated with LMP1.¹⁰⁶⁰ This was of interest because CD40 is the principal pro-survival TNFR for B cells and is similar to the LMP1 CT TES1 amino acid sequence. CD40 engages TRAF3 and has effects in B cells that are similar to LMP1.^{73,306,729,1060}

This allowed the realization that LMP1 is a constitutively activated TNFR. In support of this model, TES1 correlated with a principal LMP1 CT NF- κ B activation domain, C-terminal activating region (CTAR) 1, and TES2 correlated with a second LMP1 NF- κ B activation domain, CTAR2.^{633,1035} The LMP1 CT/TES2/CTAR2 terminal 11 amino acids interact with and associate with TNFR1-associated death domain (TRADD) and receptor interacting protein (RIP) death domains but do not initiate a death signal.^{667,670,671} LMP1 also associates with a number of other cellular proteins including A20 and Ubc9.^{97,428} A20 regulates LMP1 activation of IRF7.¹⁰⁹⁷ The interaction with Ubc9 was localized to a region between CTAR1/TES1 and CTAR2/TES2 (see later) termed CTAR3. This region was previously reported to bind and activate DNA binding of STAT1⁴⁶⁴; however, more recent studies indicate that JAK3 only weakly associates with both LMP1 and LMP1 lacking the putative JAK3 binding domain.⁵⁸² More importantly, this region was shown not to induce STAT1 transcriptional activity or be important for the functions of LMP1 described later. Although essential for LMP1 signaling, the LMP1 TMs can be replaced by CD2-CTAR1 or CD2-CTAR2 expression, using anti-CD2 antibody to induce aggregation.^{405,465} The two LMP1 CT activation and transformation domains activate NF- κ B, JNK, p38, IRF and cdc42 pathways.^{133,305,350–354,404,405,420,541,760–762,1026–1029,1191,1280,1415,1481,1589,1590}

Within the LMP1 CTAR1/TES1 region is a PXQXTDD motif that approximates CD40 interactions with a shallow canyon on the TRAF3 surface, stabilizing trimer formation.^{1563,1589} Extensive studies have shown that LMP1 utilizes TRAFs and proteins essential for the NF- κ B and IRF7 pathway to signal.^{44,117,133,305,306,353,404,482,541,569,643,805,961,1027,1060,1095,1266,1280,1373,1482,1563,1569–1571} Through the interactions with TRAF1-3 and TRAF5-6 LMP1 CTAR1/TES1 activates both the non-canonical pathway and the canonical NF- κ B pathways by stabilizing NF- κ B-inducing kinase (NIK), which phosphorylates inhibitor of κ B kinase α (IKK α)-activating NF- κ B.^{53,349,353,1029,1127,1266,1415} TRADD, RIP, and IRF7 binding within CTAR2/TES2 appears to primarily activate TRAF6 and IKK β .^{53,939–941,1306,1310,1373} BS69, a cellular MYND domain-containing protein, also binds CTAR2/TES2, and knock-down of BS69 interferes with JNK activation.^{1513,1514} LMP1 also induces Lys 63-linked polyubiquitination of TRAF6, which is likely mediated by UBC13.^{939,1374} A unique aspect of LMP1-mediated canonical signaling is incomplete dependence on IKK γ .⁹⁴⁰ Furthermore, interleukin- α receptor-associated kinase 1 (IRAK1) is required for LMP1 noncanonical and canonical signaling, due to a surprisingly central role for IRAK1 in p65 S536 phosphorylation.¹³⁷⁴

LMP1 provides both growth and survival signals for LCL growth. In an LCL with conditional LMP1 expression, cells exit the cell cycle in the absence of LMP1 and survive for 5 days.⁷⁶² LMP1 protects BL cells from the pro-apoptotic effects of growth factor withdrawal^{558,1248} and is the principal activator of NF- κ B in LCLs.^{146–149} NF- κ B inactivation results in rapid apoptosis, which proceeds despite high bcl-2 and bcl-XL levels. Mitochondrial membrane potential decreases rapidly, cytochrome C is not released, and bax is activated. Caspases are also activated. Caspase inhibitors can prevent activation but not apoptosis. Bfl-1, c-Flip, A20, and c-IAP mRNAs decrease rapidly, implicating these proteins as candidate triggers of cell death.

Because LMP1 activation of NF- κ B is critical for cell survival and is dependent on effective LMP1 aggregation, the basis for TM aggregation has been investigated. Surprisingly, LMP1 containing TM1-2 and CT can induce 30% to 40% of wild-type LMP1 CT signaling, whereas LMP1 with TM3-4 and CT or TM5-6 and CT lack activity.^{243,1585} Further, LMP1 with TM1-4 and CT has 70% to 80% of wild-type signaling.¹⁵⁸⁵ A point mutation analysis of the role of TM1-2 specific amino acids in effective aggregation identified FWLY41 at the external surface of TM1 as critical for LMP1 signaling.¹³⁷⁵ LMP1 with FWLY mutated to AALA is markedly deficient in raft association and has less than 20% of wild-type LMP1 NF- κ B activation. TM1-2 with the AALA mutation is also very deficient in intermolecular association with TM3-6 expressed as another protein. These data are consistent with a model in which TM1 FWLY is critical for raft association and intermolecular interactions necessary for signaling.

LMP2A and LMP2B

LMP2A constitutively mimics signaling from the B-cell receptor (BCR) (Fig. 61.11). As a result, EBV latent membrane proteins mimic two signals that are essential for normal B-cell development and function: LMP1 mimics a CD40 signal, while LMP2A mimics a BCR signal. LMP2B is related to LMP2A but lacks the domain required for LMP2A tyrosine kinase signaling (Fig. 61.11). LMP2A and LMP2B in latency 3 are expressed from unique EBNA-2-responsive promoters that have RBP-J κ and PU.1 sites, which LMP2A has and LMP2B shares with the LMP1 promoter.^{834–836,838,1008,1277,1636} In latency 2, in which EBNA-2 is not expressed and LMP1 and LMP2A are expressed, LMP2A has been shown to autoactivate its expression through Notch, providing a mechanism for expression independent of EBNA-2.³³ Interestingly, LMP1 is not as consistently expressed in latency 2 as LMP1.^{136,576} A recent study has shown that LMP2A in epithelial cells can block LMP1 expression by LMP2A, inhibiting IL-6 secretion and preventing autocrine secretion and activation of the L1-TR LMP1 promoter by STAT3 activation.¹³⁹³ The LMP2A first exon encodes 119 N-terminal cytosolic amino acids that precede a methionine, which is the translational start site for the LMP2B mRNA. LMP2A and 2B mRNAs share remaining exons, which encode 12 membrane-spanning domains separated by short reverse turns and a 27-amino acid C-terminal cytosolic domain.^{836,1277} (Fig. 61.11). LMP2B consists primarily of 12 membrane-spanning domains and lacks the LMP2A N-terminal domain. Recently, using RNA-seq, alternative LMP2 splicing events have been identified.²⁵⁸ Several studies have suggested that LMP2B can augment LMP1 signaling and negatively regulate the function of LMP2A.^{1212,1213,1245} No antibody reagents specific to LMP2B have been developed to date, and as a result, expression patterns for LMP2A are what have been most characterized. Similar to LMP1, LMP2A localizes to patches in membranes of latently infected B lymphocytes, where it co-localizes with LMP1.^{920,921} A related LMP2A transcript has been identified in NK- and T-cell lymphoproliferative disease that initiates from within the EBV terminal repeats and containing downstream, epitope-encoding exons that may encode targets for cellular immunotherapy.⁴¹⁴

LMP2A is palmitoylated, although the function is uncertain.^{581,723,993} LMP2A also associates with lipid rafts, which

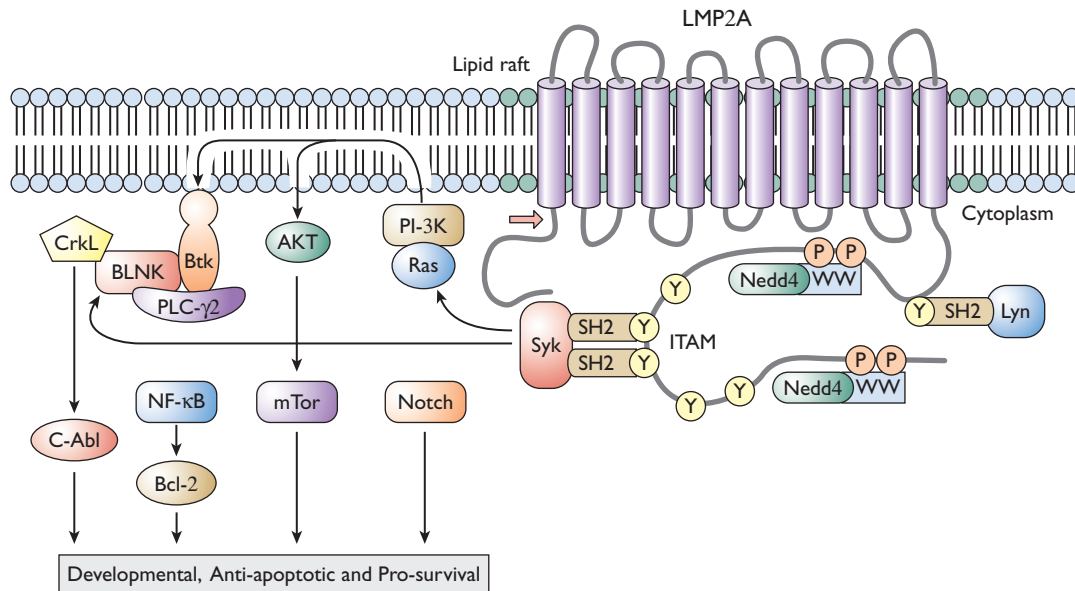


FIGURE 61.11. Schematic diagram of biochemical mechanisms by which latent membrane protein 2 (LMP2) activates signaling pathways by constitutively mimicking an activated B-cell receptor. LMP2A is constitutively localized to lipid rafts and binds Src family tyrosine kinase such as Lyn, leading to constitutive phosphorylation of LMP2A at multiple tyrosine residues. Syk is recruited by the phosphorylated intracellular adhesion molecule (ITAM), where it becomes activated. LMP2A also binds E3 ubiquitin ligases in the Nedd4 family and may also bind mitogen-activated protein kinase (MAPK). LMP2A blocks the ability of the B-cell receptor (BCR) to enter lipid rafts. Phosphatidylinositol 3-kinase (PI3K) is constitutively phosphorylated in cells expressing LMP2A, as is SLP-65 and Btk. The antiapoptotic serine/threonine kinase Akt is constitutively phosphorylated in cells expressing LMP2A, representing one possible way in which LMP2A inhibits apoptosis. MAPK and Ca^{2+} -dependent signal transduction may result from activation of PI3K, SLP-65, and Btk. E3 ubiquitin ligases mediate the degradation of LMP2A and associated factors, including Lyn. LMP2A can block BCR signal transduction in certain B-cell lines; however, it is unclear whether this is solely due to denying entry of BCR into rafts, sequestration of Syk and Lyn, desensitization of cells, or a combination of these effects.

is likely important for initial contact with src family tyrosine kinases.^{338,581} LMP2A lacking TM3-12 and the C-terminal cytoplasmic domain is diffusely localized in the plasma membrane.⁹²⁰ Indeed, the C-terminal cytoplasmic domain can have a direct role in self-association,^{993,1460} although LMP2A deleted for TM 6–12 and the C-terminal cytosolic domain can still partially block BCR signaling.¹⁰¹⁹ Even with deletion of TM3-12, the LMP2A C-terminus is still tyrosine phosphorylated.⁹²⁰

LMP2A is a substrate for, is associated with, and is constitutively phosphorylated by B-lymphocyte src family tyrosine kinases, especially Lyn.^{143,1017,1246} Lyn is likely recruited to LMP2A by the amino acids DQSL in LMP2A, which are similar to the amino acids DCSM in Ig- α .¹¹⁷² Lyn likely binds to Y112 of LMP2A through its SH2 domain and phosphorylates all LMP2A tyrosines (Y), resulting in recruitment of Syk to Y74 and Y85 and activation of PI3K, Btk, BLNK, Akt, and mTor.^{360,1011,1012,1017,1042,1413} LMP2A Y23, Y31, Y60, Y64, and Y101 phosphorylations are unnecessary for blocking BCR signaling.¹⁴¹³ LMP2A causes constitutive phosphorylation of PI3K and CrkL in B cells.^{360,1017} Antiphosphotyrosine antibodies localize LMP2A sites of constitutive tyrosine phosphorylation in the infected cell plasma membrane.⁹²⁰ LMP2A is one of the few stably tyrosine-phosphorylated proteins in BL cells or LCLs and as a result recruits cellular proteins that contain SH2 domains like Shb.⁹⁹⁴ LMP2A may also undergo serine/threonine phosphorylation. LMP2A S15 and S102 bind MAPK *in vitro* and are substrates for MAPK phosphorylation.^{920,1131}

The LMP2A N-terminus has two PPPPY motifs, which bind WW domains of Nedd4 family ubiquitin ligases, such as AIP4/Itchy, WWP2, Nedd4, or Nedd4-2.^{645,647–649,1546} This interaction causes the ubiquitination and degradation of LMP2A and associated proteins.⁶⁵¹

LMP2A and 2B have been extensively mutated in EBV recombinants.^{764,922–925,1234,1380} Except for one anomalous result,¹³¹ the specifically mutated recombinants transform primary B lymphocytes with wild-type efficiency. The resultant LCLs grow the same as wild-type EBV-transformed LCLs under standard culture conditions, in reduced serum, in soft agarose, and in SCID mice. Virus replication is also similar following treatment of latently infected lymphocytes with chemical inducers of lytic infection.^{764,922–925} LMP2A is required for outgrowth of LCLs that lack expression of a functional BCR.^{964,965}

LMP2A expression in BL lymphoblasts constitutively blocks the signal transduction events that would follow the cross-linking of surface IgM (sIgM), CD19, or class II MHC on cells that do not express LMP2A.^{1018,1019} This blockade is similar to the desensitization that occurs after sIg signal transduction.^{139,162} LMP2A mutants that are deficient in association with Lyn and Syk do not block signal transduction.^{430–432} LMP2A may deplete the cell of Lyn and Syk by the ubiquitin ligases, induce desensitization, or deplete lipid rafts of Lyn, thereby depriving the BCR of Lyn.³³⁸ Consistent with a desensitization mechanism, the basal tyrosine phosphorylation of many cell proteins, including Lyn, Syk, and the p85 subunit of

PI3K, are higher in LCLs transformed by EBV recombinants that express wild-type LMP2A versus EBV recombinants that do not express LMP2A. Furthermore, cross-linking of sIg on wild-type EBV-transformed LCLs results in abrogation of tyrosine kinase signaling at the first steps of src family and syk phosphorylation, whereas cross-linking of sIg on LMP2A null mutant recombinant-transformed LCLs results in normal sIg protein tyrosine kinase signal transduction, including transient phosphorylation of Lyn, Fyn, Syk, p85, Vav, PLC- γ , SHC, and Grb2.¹⁰¹⁷ Consistent with the desensitization model, CD8 fusion to the LMP2A N-terminus and cross-linking of CD8 result in an increase in intracellular Ca^{2+} and then refractoriness.⁸⁴

Cross-linking of sIg on primary B lymphocytes transformed by LMP2 null mutant recombinants results in normal sIg-mediated signal transduction and activation of EBV replication.¹⁰¹⁸ Further, the LMP2A block of lytic replication can be bypassed by activating protein kinase C and raising intracellular free calcium with phorbol ester and calcium ionophore treatment. Indicative of the ability of LMP2A to activate cellular pathways, expression of LMP2A can in some cases activate lytic replication.¹²⁹²

A second role for LMP2A may be to provide survival and differentiation signals to B cells. LMP2A expression in transgenic mice under control of the IgH chain promoter and enhancer enables sIg-negative B cells to escape from the bone marrow and colonize peripheral lymphoid organs, bypassing normal B-cell developmental checkpoints by constitutive activation of the Erk/MAPK pathway.^{34,155,156} Thus, LMP2A can mediate sufficient constitutive forward signaling to affect normal B-cell survival. The LMP2A effect is also evident in RAG 1(−/−) mice; requires Syk, Btk, and BLNK; and is dependent on the LMP2A immunoreceptor tyrosine-based activation motif (ITAM).^{360,1011,1012} Thus, LMP2A expression could have a role in latency type 2–infected lymphocytes in enhancing B-cell survival through activation of PI3K, Akt, and Ras.^{435,1183} Interestingly, in transgenic mice high-level LMP2A expression results in B-1 cells in bone marrow, spleen, and periphery, whereas low-level expression results in spontaneous germinal centers.^{179,646} Also, the level of LMP2 expression is partially regulated by N-terminal monoubiquitination.^{645,648} In murine models of Burkitt lymphoma, LMP2A has been shown to dramatically accelerate disease, and this is in part due to bypassing the requirement of mutation of the p53 pathway.^{106,142} Remarkably, tumors derived from LMP2A/Myc mice share a similar pattern of gene expression with Myc tumors derived from transgenic mice despite the absence of p53 pathway mutations in the LMP2A/Myc mice.¹⁰⁷

LMP2A is also expressed in type 2 latency in Hodgkin lymphoma and nasopharyngeal carcinoma. Constitutive forward signaling from LMP2 could also be important in the pathogenesis of these malignancies. LMP2A expression during B-cell development in mice results in decreased expression of E2A, EBF, and Pax-5, which are important for normal B-cell development^{1180–1183} and furthermore activates Notch, which may alter B-cell identity.³⁵ Transgenic LMP2A expression in normal mouse epithelium under control of the keratin 14 promoter resulted in no apparent effect.⁹¹⁹ LMP2A expression in epithelial cell lines results in hyperproliferation in raft cultures, alterations in differentiation, increased cloning efficiency in soft agar, and promotes epithelial cell spreading

and migration.^{21,413,436,799,935,1307–1309} AKT activation increases β -catenin and cell growth through the LMP2A ITAM and PY motifs.^{1055–1057,1147} LMP2A can also increase LMP1 signaling and inhibit TGF- β -associated apoptosis.^{290,437} LMP2A has also been shown to activate the human endogenous retrovirus HERV-K18 in infected B cells, which encodes a superantigen that strongly stimulates T cells.^{618,619}

In summary, LMP2A, like LMP1, delivers a potent cell survival signal to B lymphocytes and may provide a therapeutic target in EBV-associated cancers.

EBERs

The EBER RNAs are by far the most abundant EBV RNAs in latently infected cells. Estimates place the EBERs at 10^7 copies per cell.^{49,615,617} EBER1 is about 10-fold more abundant than EBER2 and is transcribed by RNA polymerase II and III, whereas EBER2 is solely transcribed by RNA polymerase III.^{615,616} The EBERs localize to the cell nucleus where they are complexed with the cellular proteins La and L22 proteins.^{409,859,1455–1457} EBER RNAs have stable secondary structures that persist in RNA La protein complexes.⁴⁷² La protein associates with the 3' terminus of the EBER RNAs,⁴⁷² while L22 associates with three sites in EBER1.^{316,410,1457} Upward of 50% of the cellular pool of L22 is found in complex with EBER1, and most EBER1 is precipitable with antibody to L22.¹⁴⁵⁵ Recent studies have shown that the EBERs can be secreted from cells in complex with La resulting in Toll-like receptor 3 (TLR3) signaling.⁶⁶³ Confocal microscopy has detected a small fraction of EBERs in the cytoplasm of interphase cells, although EBERs are tightly associated with chromosomes in metaphase cells, consistent with nuclear entry after mitosis.¹³¹² Although L22 shuttles from the nucleus to the cytoplasm, EBERs remain in the nucleus.⁴⁰⁹ Furthermore, a substantial fraction of L22 relocates from nucleoli to the nucleoplasm in the presence of EBER1.¹⁴⁵⁵ EBERs are not expressed in lytic infection.⁴⁶⁰

EBER expression in uninfected Akata cells partially duplicates the effect of EBV reinfection of Akata cells in restoring growth in soft agar, tumorigenicity in SCID mice, resistance to apoptosis, resistance to interferon- α (IFN- α)-induced apoptosis, and induction of bcl-2 or IL-10.^{796,797,987,1076,1251–1254,1335} Also, EBV latency 2 infection or EBER expression in MT-2 T cells results in a shorter cell doubling time, higher saturation density, and IL-9 secretion.^{1579,1603} In addition, EBV reinfection or EBER expression in gastric carcinoma cell lines increases insulin-like growth factor 1 (IGF1) expression.⁶⁶² The effects of EBER expression can be opposed by concomitant L22 expression.³⁴⁷ Studies have suggested that the interaction of the EBERs with L22 is functionally significant because Burkitt lymphoma cells expressing mutant forms of EBER1 are incapable of binding to and relocating L22 to the nucleoplasm from the nucleoli and have reduced capacity to enhance cell growth of Burkitt lymphoma cells compared to Burkitt lymphoma cells expressing wild-type EBERs.⁶¹⁴

EBERs are similar to adenovirus VA1 RNA, which blocks interferon-induced PKR phosphorylation and inhibition of translational initiation factor eIF-2 α .⁷⁸² EBER1 and EBER2 can partially substitute for VA1 in adenovirus replication¹⁰² and can inhibit PKR *in vitro* at levels that are similar to VA1 RNA.^{238,1076,1330} In contrast, PKR activation *in vivo* is unaffected by EBER expression.^{1251,1533} This may reflect the distinct

subcellular compartmentalization of the EBERs being exclusively nuclear, while PKR is predominately found in the cytoplasm. Both EBERs have also been shown to activate retinoic acid-inducible gene I protein (RIG-I) and stimulate type I interferon signaling,¹²⁷⁰ but like PKR, RIG-I is generally thought to be a cytosolic protein and the observed interaction was observed via overexpression.

EBV recombinants specifically deleted for the EBER genes have been compared with wild-type recombinants in LCL outgrowth.^{489,1412,1564,1576} In the first experiments, EBER-deleted EBV was equivalent to wild-type EBV in efficiency of primary B-cell conversion to LCLs, in time to LCL outgrowth, in LCL morphology, in LCL regrowth after dilution in fresh medium, or in transition to EBV replication.¹⁴¹² In the later experiments, the efficiency of primary B-cell conversion to LCLs with EBER-restored virus was 20-fold more than that with EBER-deleted virus, LCLs infected with EBER-restored virus grew twice as rapidly as EBER-deleted virus, and LCLs infected with EBER-restored virus grew back after higher dilution than EBER-deleted virus, indicating that EBERs are important in LCL outgrowth.¹⁵⁷⁶ The discrepancy between the two data sets may be due to a complementation effect of the EBER-deleted infected cells by irradiated LCL, which were the source of virus for the LCL outgrowth assays used in the first study. Further studies indicated that deletion of EBER2 is all that is required for the growth defect, suggesting that the EBERs may have different functions.¹⁵⁶⁴ In the most recent studies, the authors observed no significant change in transformation efficiency or growth of LCLs in response to the deletion of the EBERs.⁴⁸⁹ Interestingly, the authors observed changes in the expression of cellular genes in LCLs transformed with EBER deletion viruses. At present, there is not an obvious explanation for the differences observed in transformation efficiency for EBER-deleted virus. It may be related to the methodology used in the various studies or related to the different virus strains utilized in the various studies.⁴⁸⁹

The exact role of the EBV-encoded EBERs is still enigmatic and remains to be determined. The consistent expression of the EBERs in EBV latently infected cells provides a convenient means to identify EBV-infected cells and likely highlights important roles of the EBERs in the biology of EBV infections.

BHRF1, BALF1, BART RNAs, and miRNAs

Early studies had indicated that BHRF1, a virally encoded bcl-2 homolog, is nonessential for *in vitro* transformation of B lymphocytes,^{849,973} can protect cells from apoptosis,^{557,998} and is expressed early in EBV latently infected lymphocytes.^{233,1144} The BHRF1 NMR structure indicates similarity to bcl-2, but BHRF1 differs from bcl-2 in predicted interactions with bax, bak, bid, and bad.⁶²⁵ BHRF1 exerts antiapoptotic effects by binding to Bim,³⁰⁰ VRK2 (human vaccinia virus B1R kinase-related kinase 2),⁸⁷⁴ hPRA1 (human prenylated rab acceptor 1),⁸⁷⁵ Bid, Puma, and Bak.⁸²⁰ Although controversial in function,^{94,408,980} BALF1 has also been shown to block apoptosis,^{408,980} and recent studies have shown that BHRF1 and BALF1 have redundant functions and at least one is required for transformation of newly infected primary B cells *in vitro*.²³ Studies have now shown that BHRF1 is expressed as a latent protein by weak Wp promoter activation in growth-transformed cells *in vitro* using an assay in which BHRF1 is recognized by CD4 T cells⁷³⁸ and functions as a survival factor in the

Wp-restricted Burkitt lymphoma cell line P3HR-1.¹⁵³⁵ Studies have also shown that *cis*-acting effects on RNA processing and Drosha cleavage result in the selective expression of BHRF1 miRNAs, limiting BHRF1 expression in latency 3.¹⁵⁷²

Bam A rightward transcripts, or BARTs, are mRNAs expressed at low levels in all forms of latent and lytic EBV infection *in vitro* and *in vivo*.^{137,207,208,461,462,591,716,868,1163,1261,1405,1621} The BART mRNAs encode BARF0 and RK-BARF0,^{429,759,818} A73,¹³⁶⁷ and RPMS1¹³⁶⁷ proteins, whereas the introns encode microRNAs.^{151,1163} Two TATA-less promoter regions, P1 at nucleotide (nt) 150641 and P2 at nt 150357 in the B95 sequence, likely transcribe the BARTs.^{65,205,294,1367} P1 is active in epithelial cells, is the initial B-cell promoter, and is down-regulated by IRF7 and 5, whereas P2 is more active in epithelial cells and is up-regulated by c-myc and C/EBP.²⁰⁵ BARF0 or RK-BARF0 can interact with Notch receptor cytosolic domains, but the proteins have not been detected in EBV-infected human cells and humans have little detectable antibody to BARF0 or RK-BARF0.^{1448,1487} Similarly, interesting interactions have been described for A73 and RPMS1,¹³⁶⁷ but these proteins have not been detected in EBV-infected cells.

More recent studies, which may be more indicative of the function of the BARTs, have identified a distinct group of about 40 miRNAs encoded by EBV that likely play a critical role in the establishment and/or maintenance of EBV latency and/or contribute to pathogenesis in the human host.^{151,501,1163,1634} Two clusters of miRNAs have been found corresponding to transcripts from the regions of the EBV genome encoding the BHRF1 and BART genes (Fig. 61.2). In general, the BHRF1 miRNAs exhibit expression that is restricted to latency 3 in which Cp/Wp drive EBNA transcription, whereas the BART miRNAs are expressed in all latency types.^{29,151,264,501,763,1163,1573,1634} Induction of lytic replication increases the expression of both the BHRF1 miRNAs^{340,1573} and the BART miRNAs.⁷⁷

Specific EBV miRNA targets identified include the viral DNA polymerase,⁷⁷ LMP1, and LMP2A.^{946,1207} Cellular target proteins include PUMA,²³⁶ CXCL-11,¹⁵⁶⁷ BRCA-1,¹⁶³⁴ stress-induced natural killer (NK) cell ligand MICB,¹⁰⁷² and Bim.⁹⁷⁶ Using immunoprecipitation of RNA-induced silencing complexes (RISCs), the number of potential targets for EBV microRNAs has greatly expanded, with a comprehensive study indicating 44 cellular transcripts may be targeted by EBV miRNAs and 2,337 cellular transcripts are targeted in infected cells.³²⁰ A more recent study used photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) to comprehensively examine the viral and cellular miRNA targetome in B95-8-transformed LCLs¹³⁶¹; 7,827 miRNA interaction sites in 3,492 cellular 3' untranslated regions (UTRs) were identified, of which 531 of these sites contained seed matches to viral miRNAs. A number of the interactions were confirmed by reporter assays. Interestingly, this study also showed that myc-regulated host miRNAs from the miR-17/92 cluster regulate latent viral gene expression.¹³⁶¹

A comprehensive analysis of the function of EBV-encoded miRNAs in B-cell transformation by EBV in which mutations were systematically introduced in the precursor miRNA transcripts encoded by EBV has been done.^{383,1320} In the two studies in which mutations were introduced into the BHRF1 locus to inhibit production of BHRF1 miRNAs, it was found that these miRNAs inhibit apoptosis and favor cell cycle progression and proliferation during the early phase of infected human

primary B cells. This may reduce viral antigenic load at early stages of growth proliferation when other latent genes are not highly expressed.^{383,1320} Studies with BART locus miRNAs did not uncover any gross defect in regard to EBV transformation as monitored in cell culture.¹³²⁰ Compatible with this observation, no defects were found in the regulation of the EBV lytic phase in both studies.^{383,1320} The BART9 miRNA alters LMP1 levels and affects the growth rate of NK T-cell lymphomas.¹²⁰⁷ Interestingly, editing of the BART6 miRNA results in suppression of EBNA-2.⁶⁴⁴ Other studies have suggested that the EBV-encoded miRNA profile in infected cells may differ when normal infected tissues are compared to neoplastic tissues.¹¹⁹³

Finally, EBV-encoded miRNAs and viral-encoded proteins such as LMP1 and gp350 have been found in exosomes released from EBV-infected cells, including nasopharyngeal carcinoma and B cells.^{330,397,481,613,650,750,1006,1146,1485,1498} Interestingly, in peripheral blood mononuclear cells from patients with increased EBV loads, EBV BART miRNAs are present in both B-cell and non-B-cell fractions, suggestive of miRNA transfer.²⁹ Exosomes may manipulate the tumor microenvironment to influence the growth of neighboring cells through the intercellular transfer of virally encoded proteins such as LMP1, signaling molecules, and viral or cellular miRNA.

In addition to miRNAs encoded by EBV, virus infection activates the expression of a number of cellular miRNAs. Most well studied is the enhanced expression of miR-155 by EBV infection or expression of LMP1 that activates NF- κ B and p38/MAPK signal transduction.^{164,328,449,687,786,931,1061,1063,1204,1596} The expression of a number of cellular targets has been studied in the context of EBV up-regulation of miR-155 including c-Myb,⁶⁵⁶ c-Ski,⁶⁵⁶ and components of the BMP signaling pathway.^{1595,1596} Evidence for a direct role in EBV pathogenesis is scant, although some studies indicate direct effects on normal EBV processes such as the expression of EBNA-1 mRNA and the EBV copy number in latently infected cells,⁹³¹ regulation EBV lytic activation,¹⁵⁹⁶ and growth of EBV-transformed LCLs.⁹⁰⁵ This suggests that miR-155 contributes to EBV immortalization by modulation of NF- κ B signaling and the suppression of host innate immunity to latent viral infection. Another cellular miRNA, miRNA200, is decreased in EBV-infected epithelial cells and by transfection of EBV latent genes. Down-regulation of miRNA-200 resulted in down-regulation of E-cadherin expression.¹³³⁷ Other studies with the miR-200 family suggest possible roles in regulating lytic EBV lytic replication in epithelial cells as well in B cells⁸⁹⁴ and in regulating cell growth in nasopharyngeal carcinoma.¹⁶⁰⁹ In regard to regulating EBV lytic replication, studies have shown that miR-200 family members can down-regulate ZEB1 and ZEB2,³⁵⁷ two cellular proteins that repress BZLF1 expression⁸⁰⁴ (see EBV Gene Products Associated With Lytic Replication).

EBV Gene Products Associated With Lytic Replication

BZLF1 and BRLF1

Early studies had shown that various induction strategies could induce lytic replication in a variety of latently infected cells grown in tissue culture. Phorbol esters are among the most reproducible and most broadly applicable inducers of replication.^{37,279,380,402,837,1432,1644} Other agents that can induce lytic replication include calcium ionophore,³⁶⁷ histone deacetylase

inhibitors,⁹⁴² and DNA demethylating agents.⁹⁵ Recent studies have begun to elucidate the signals that result in replication *in vivo*. Early studies suggested that induction of lytic replication may be linked to differentiation of cells harboring latent EBV. Studies of oral hairy leukoplakia found that EBV lytic replication occurred in the outermost differentiated layers of the epithelium.^{1090,1607} More recent studies using B cells have suggested that when B cells differentiate into plasma cells, EBV lytic replication is induced,⁸²⁴ and XBP-1, a plasma cell differentiation factor, is essential for this induction.^{103,1407} Interestingly, down-regulation of BLIMP1- α by LMP1 disrupts plasma cell differentiation, which may be important both in regulating EBV lytic replication and in EBV B-cell lymphomas.¹⁵⁰²

BZLF1 and BRLF1 were identified as transactivators of virus replication as the result of the observation that P3HR-1 Burkitt lymphoma cells replicate and package highly defective EBV that induces lytic infection in Raji cells.^{1020,1022} These viruses express BZLF1 or BRLF1 using the Wp promoter.²⁶⁸ BZLF1 expression in latently infected cells activates EBV replication.^{268,683,684,1020} Compatible with the important role of BZLF1 and BRLF1 expression in inducing lytic replication, both proteins were shown to be expressed after induction of lytic replication following activation of B-cell receptor signaling¹⁴²¹ and found to be essential for activation of lytic replication³⁸² but not important for transformation by EBV of primary B cells.⁷²² Recent studies have shown that cellular expression of early growth response genes are required prior to the expression of BZLF1 and BRLF1 and subsequent activation of lytic replication.¹⁵⁹¹ BRRF1 (also known as Na), which is adjacent to BZLF1 and BRLF1 in the genome, also can induce lytic replication through a TRAF2/JNK-dependent manner in cooperation with p53.^{517,602}

Studies analyzing viral gene expression following induction of lytic replication also established the roles of BZLF1 and BRLF1 as immediate-early viral genes.^{108,1421} More recent studies indicate that BZLF1 is a key inducer of lytic replication as an immediate-early protein^{28,109} but maximal expression of BZLF1 is a result of a BZLF1 autoactivation loop requiring protein synthesis.^{109,399,403,1484,1594} Early promoter regions are coordinately up-regulated by BZLF1 and BRLF1 activating the expression of viral genes involved in replication.^{220,267,269,380,399,425,463,491,496-498,524,600,638,640,745,884,885,1109,1110,1196,1236,1484,1620} Promoter activation has a strong positive effect on DNA synthesis from the lytic origin.¹²⁹⁹ The *cis*-acting domains of early promoters have many similarities including multiple BZLF1- and BRLF1-responsive elements.^{200,220,221,538,880,884,885}

BZLF1 is encoded by a three-exon transcript initiating from the BZLF1 promoter (Zp).⁹⁶⁷ BRLF1 is encoded upstream of Zp and is transcribed in the same orientation as BZLF1 under the control of BLRF1 promoter (Rp).⁹⁶⁷ Rp produces three alternately spliced mRNAs. Two larger transcripts encode BRLF1, whereas the third generates a BZLF1/BLRF1 fusion protein known as RAZ, which is a fusion of the amino terminus of BLRF1 and the BZLF1 DNA binding and dimerization domains.⁹⁶⁷ RAZ is an inhibitor of BZLF1 transactivation and may suppress BZLF1 targets in EBV lytic replication.^{440,1316} BZLF1 and BRLF1 are typically not expressed in EBV latently infected cells.^{1421,1617} The three BZLF1 exons encode a separate functional domain.^{380,882} The first exon encodes amino acids 1 to 167, which transactivate when fused to a Gal4 DNA binding domain.²²² The activating domain can recruit TAFs and

stabilize TFIID association with TATA elements.^{881,883,886} The second exon encodes amino acids 168 to 202, which include a strongly basic domain with homology to a conserved region of the c-fos and c-jun family of transcriptional modulators. This domain confers the ability to interact with AP1-related sites in DNA and targets BZLF1 to the nucleus.¹⁰¹⁶ The third exon encodes amino acids 203 to 245, which include a near-perfect leucine and isoleucine heptad repeat capable of coiled-coil dimer formation.⁴⁰¹ Like c-fos, BZLF1 dimerizes and binds directly to AP1-related sequences. Dimerization may facilitate high-affinity interaction with templates having two or more BZLF1 binding sites.¹⁷⁶ BRLF1 is also a DNA sequence-specific acidic transactivator and has distant homology to c-myc; c-myc can interact synergistically with BZLF1 in transactivating the BMRF1 promoter.^{747,968} Two domains of BRLF1 when fused to the Gal 4 DNA binding domain have transactivating activity for Gal 4 response elements upstream of basal promoters.^{538,539,968} Amino acids 416 to 519 are weakly activating in B cells, while the C-terminal amino acids, 520 to 605, are a potent acidic activator similar to VP16. Similar to BZLF1, the BRLF1 transactivation domain interacts with a number of cellular transcription factors including TBP, TFIIB, and CBP to facilitate promoter activation.^{498,966,968,1414}

BZLF1 contains leucine zipper dimerization motifs.^{401,579,800,882} X-ray crystallography has shown that BZLF1 dimerizes through the leucine zipper, but this dimerization is stabilized by the folding back of amino acids extending beyond the dimerization motif.^{578,1053,1158,1297,1347} This observation may in part explain the inability of BZLF1 to heterodimerize with members of the leucine zipper family of transcription factors.

BZLF1 and BRLF1 are both targets of posttranslational modification. BZLF1 is multiply phosphorylated by casein kinase II, protein kinase A (PKA), and PKC on serines 173, 186, and 336.^{80,278,794} Phosphorylation regulates DNA binding and transcriptional activation. Phosphorylation of S186 in response to PKC activation stimulates transcription but decreases affinity for cognate DNA.⁸⁰

BZLF1 is highly phosphorylated following activation of lytic replication.²⁷⁸ PKC phosphorylates BZLF1 at T159 and S186 and phosphorylation at S186 increases BZLF1 transactivation and DNA replication.^{80,81} T159 is near the activation domain, whereas S186 is in the BZLF1 DNA binding domain. CKII can phosphorylate BZLF1 at S173,⁷⁹⁴ and mutation of this site impairs the ability of BZLF1 to activate lytic replication.^{342,344,345} Other domains within the activation domain of BZLF1 are also phosphorylated, but their function is currently

not known.³⁴⁶ The viral tegument protein BGLF4 phosphorylates BZLF1 and regulates the BZLF1 transactivation function.^{50,51} Both BZLF1 and BRLF1 are modified by sumoylation, which regulates their activities.^{5,7,192,517,1067} BRLF1 sumoylation can be induced by the EBV LF2 protein, which binds BRLF1 and relocalizes BRLF1 to the extranuclear cytoskeleton, resulting in repression of BRLF1 activity.^{153,546}

Regulation of Zp has been studied extensively and contains many *cis*-acting regulatory elements that have been shown to be important in different cellular contexts (Fig. 61.12). Initial studies identified four sequence-related elements termed ZIA, ZIB, ZIC, and ZID and a unique element designated ZII.^{399,402} Two additional sites, ZIIIA and ZIIIB, were also identified that bind BZLF1.³⁹⁹ Further studies found that ZIIIB binds BZLF1 with higher affinity.^{109,399} The ubiquitous transcription factors Sp1/Sp3 bind to the AT-rich ZIA, ZIC, and ZID elements,⁹¹⁰ while the myocyte enhancer factor 2D (MEF2D) binds to the ZIA, ZIB, and ZID elements.⁹¹¹ ZII is a cyclic adenosine monophosphate (cAMP)-response element (CRE)-like motif that binds CREB, ATF family members, C/EBPs, and the AP-1 family of transcription factors.^{6,402,909,1534} Although the ZIA-ZID and ZII elements have distinct DNA sequences and are bound by different regulatory factors, they mediate the induction of lytic virus replication by chemical inducers and BCR cross-linking. Treatment of cells with calcium ionophore and phorbol ester such as TPA induces a rise in intracellular free calcium and activates protein kinase C and is a partial surrogate for Ig cross-linking.²⁷⁹ Ionomycin activates lytic replication through the Ca²⁺-dependent signaling pathway with MEF2D bound to ZIA, ZIB, and ZID and ATF bound to ZII.¹⁹⁶ BCR cross-linking activates phospholipase C- γ . Phospholipase C- γ releases IP₃ and diacylglycerol, which mobilize calcium from intracellular stores and activate protein kinase C, respectively. Elements upstream of the BZLF1 promoter that mediate the Ig cross-linking response include Sp1, Sp3, MEF2D, ATF-1, and ATF-2 sites.^{911,1334} The ATF-1 and ATF-2 binding site is regulated by CAM kinase IV.¹⁹⁶ Inhibition of calcineurin blocks Ig cross-linking-induced EBV activation.¹³⁸

Because the BZLF1 functions as a key switch between latent and lytic replication of EBV, Zp must be tightly repressed to maintain latency. Silencing is achieved by multiple negative regulatory elements^{803,804,909,1612,1625} including elements within ZIIR⁹⁰⁹ and ZV/ZV'^{803,804,1612} Depending on cell type, the cellular proteins ZEB1 or ZEB2 bind to ZV/ZV' to suppress induction of lytic replication^{356,803,804} and a 2-bp mutation in the ZV element results in spontaneously lytic infection.¹⁶¹² The

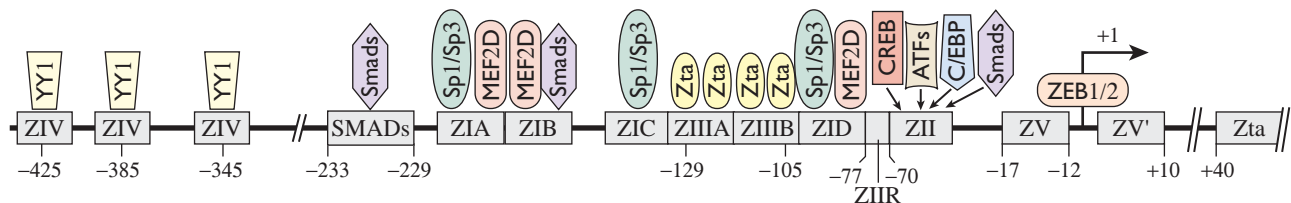


FIGURE 61.12. Schematic representation of Epstein-Barr virus (EBV) BZLF1 promoter elements. The BZLF1 promoter has a large number of binding sites for regulators of gene expression. Shown in the diagram are the *cis*-acting regulatory elements present within nucleotides -221 through +40 of the BZLF1 promoter relative to the transcription initiation site. Shaded rectangles along the BZLF1 promoter indicate approximate locations of identified regulatory elements with the relevant *trans*-acting factors indicated previously.

cellular microRNAs 200b and 429 can induce EBV latently infected cells to undergo lytic replication by reducing expression of ZEB1/ZEB2.³⁵⁷ The ZRII element of Zp has also been shown to be important for repressing lytic replication by possibly binding a cellular factor.¹⁶¹¹ EBV with mutations within this element is impaired for production of latently infected cell lines and enhanced for lytic replication.¹⁶¹¹ More distal sites from Zp between nt -551 and -222 have also been shown to regulate Zp including a reversible YY1 silencing element.^{909,1040,1041,1311,1442,1616}

A phosphorylated form of MEF2D bound to ZIA, ZIB, and ZID can also repress Zp by recruiting histone deacetylases (HDACs) to maintain chromatin in a repressed state.^{138,1003} A site within ZIIR is a potent silencing element,⁹⁰⁹ and mutations within this element are defective in establishing proliferating latently infected cells and are spontaneous for lytic replication.¹⁶¹¹ The ZIIR is likely targeted by an unknown cellular protein. Studies have identified variants in Zp between malignant and nonmalignant samples from AIDS patients.⁹⁸⁴ Histone acetylation is critical for BZLF1 promoter activation.⁶⁸²

Very recent studies have been particularly informative in understanding how the activation of EBV lytic replication and the establishment of EBV latency are regulated. Methylation of sites within the BRLF1 promoter (Rp) represses the expression of BRLF1, and BZLF1 preferentially binds to these sites.¹⁰⁵ A BZLF1 serine 186 mutant that fully interacts with Zp but is unable to induce lytic replication does not activate Rp and is unable to interact with a methylated Zp.^{8,104,417,418} Structural studies have shown that this serine, along with cysteine 189, interacts with the methyl motif of the CpG dinucleotide.^{715,1298} Studies comparing BZLF1 and BRLF1 deletion viruses²²⁸ and analyzing the temporal order of BZLF1 and BRLF1 expression following induction of EBV lytic replication indicate that BZLF1 is critical in the initial stages of induction of lytic replication.^{228,1539} The importance of DNA methylation in regulating EBV lytic replication is demonstrated in studies indicating that binding of BZLF1 to viral and certain cellular promoters is methylation dependent, allowing BZLF1 to overcome epigenetic silencing.^{98,311,545,1293} Recent studies have suggested that BZLF1 expression early in infection is essential for driving initial proliferation and lytic replication, but initially BZLF1 does not induce lytic replication because the incoming viral genome is not methylated and as a result is not activated by BZLF1.⁷⁰³ Only genomes that are methylated efficiently undergo lytic replication.⁷⁰² Studies using mice reconstituted with human immune systems or SCID mice implanted with LCLs transformed with BZLF1 or BRLF1 mutant viruses demonstrate that expression of BZLF1 or BRLF1 may be important for development of EBV lymphoma.^{603,950,1397}

BZLF1 also interacts with NF- κ B, p53, virion proteins, the human IL-10 promoter and PML bodies and inhibits MHC class I expression.^{92,509,734,957,1319,1624} BZLF1 interaction with PML bodies results in BZLF1 sumoylation and disruption of PML bodies. NF- κ B inhibits BZLF1 transactivation, whereas the interaction of the BZLF1 coiled-coil dimerization domain with the p53 C-terminus transcriptionally inactivates both proteins. BZLF1 inhibition of p53 may be important in preventing p53-induced apoptosis in response to lytic EBV DNA replication. Both BZLF1 and BRLF1 have been found to alter the expression of a variety of cellular genes. This is in part due to high-affinity transcriptional binding sites that

are able to bind BZLF1 and BRLF1. Genes shown to be up-regulated include IL-10,⁹⁵⁷ IL-6, TGF- β ,¹⁸³ EGR-1,¹⁹⁴ c-Fos,⁴⁰⁰ VEGF,⁶⁰⁴ tyrosine kinase TKT,⁹³³ Myc,⁵¹⁰ and matrix metalloproteinases.⁹³⁴ In addition, BZLF1 has been shown to inhibit MHC class I⁷³⁴ and MHC class II expression.⁸⁷⁰ BZLF1 interacts with and directs the degradation of p53 by an interaction with the ECS E3 ubiquitin ligase complex. BZLF1^{1286,1287,1624} also interacts with NF- κ B.^{92,509} Interestingly, it has been reported that BZLF1 can be taken up by cells and reach the nucleus, raising the possibility that BZLF1 may affect transcription in uninfected cells.⁹⁵⁶ BZLF1 and BRLF1 can inhibit induction of IFN- β and expression of IRF3 and IRF7.^{96,518} BZLF1 protein inhibits TNFR1 expression through its effects on cellular C/EBP proteins.¹³² BZLF1 has also been shown to interact with Ku80, which enhances BZLF1 transcriptional activation.²⁰³ Finally, BZLF1 has been shown to induce growth arrest^{182,184,1559} and repress Myc, while Myc expression repressed BZLF1 transactivation.^{896,1235} BRLF1 interacts with Oct-1, which can promote disruption of viral latency¹²³²; this does not require Oct-1 binding to DNA.¹²³²

Other Genes Associated With Early Lytic Replication

Following induction, cells that are permissive for virus replication undergo cytopathic changes characteristic of herpesviruses, including margination of nuclear chromatin,⁷²⁷ inhibition of host macromolecular synthesis,⁴⁵³ replication of viral DNA at the center of the nucleus, assembly of nucleocapsids at the nuclear periphery, nucleation of nucleocapsids, envelopment by budding through the nuclear membrane, and final envelopment at cytoplasmic membranes.^{473,1134,1288} Virus gene expression follows a temporal and sequential order as with other herpesviruses.^{378,1421,1613} The vast majority of genes that are encoded by EBV that are required for lytic replication are conserved within the herpesvirus family and are contained in blocks of genes that are found in different herpesvirus families. Table 61.1 lists the EBV-encoded genes and their HSV and KSHV gene homologs. As can be readily seen, the vast majority of genes that are conserved across these three herpesvirus family members are related to replication, cleavage, assembly, and packaging of the viral genome.

EBV early replicative cycle genes are operationally differentiated from late genes by their persistent transcription in the presence of inhibitors of viral DNA synthesis. By a combination of Northern blot,^{108,379,459,629-631,638,1421} which considers the sensitivity to inhibition of viral DNA synthesis, and array data, which consider the peak time and sensitivity to inhibition of viral DNA synthesis following Ig cross-linking in Akata BL cells, 38 EBV mRNAs are early mRNAs and 40 EBV mRNAs are late mRNAs.^{930,1613} Early and late mRNAs are intermingled through most of the EBV genome. Frequently, different promoters initiate nested transcripts, which begin with different ORFs and terminate at the same polyadenylation site. The longer mRNAs are co-terminal with the shorter mRNAs. Some early and late genes are spliced, while others are not.

BSMLF1

BSMLF1 (SM), also designated EB2 and Mta, is the most rapidly activated early RNA following Ig cross-linking of Akata cells^{1421,1613} and is essential for lytic replication.^{495,528,1258} SM

is the functional homolog of HSV US11 or ICP27. SM is similar to US11 in that it increases the export and levels of most intronless RNAs,^{141,260,746,1221,1257,1259,1317} rather than increasing transcription.^{1085,1259} SM also increases the efficiency of 3' processing and the stability of the EBV DNA polymerase RNA,^{751,1259} which is intrinsically deficient in 3' processing.⁴³⁹ SM amino acids 60 to 140 and 218 to 237 are nuclear export signals,^{126,209} 152 to 172 form an RXP RNA binding domain,^{140,585,1257} and 470 to 474 are core hydrophobic folding residues; the hydrophobic residues and an HX3CX4C motif are conserved in ICP27 and are essential for SM function in replication.¹²⁵⁸ Although SM has broad RNA-binding activity, SM has different affinities for EBV mRNAs⁵²⁹ and different effects on specific EBV messages.¹⁴⁹⁶ A second arginine-rich RNA-binding activity maps to 33 residues C-terminal to the RXP motif and is also essential for SM function.¹²⁵⁸ SM acts as an alternative splicing factor directing donor splice site usage of a cellular gene to a novel alternate 5' site.¹⁴⁹⁷ SM interacts with the splicing factor SRp20, which modulates the activity of SM-directed alternative splicing.¹⁴⁹⁵ Similar to HSV US11, SM binds to and inhibits PKR activation.¹¹⁷⁷ Much of the effect of SM is in rescuing intronless RNA from components of the exon junction complex, in stabilizing the RNAs, and in exporting the RNAs to the cytoplasm. SM interacts with a variety of proteins including cellular RNA-binding proteins, CRM-1, and Sp100.^{126,154,585} SM can also interact with the PML nuclear body component Sp110b and synergize with SM in increasing intronless gene expression.¹⁰⁸⁵

Virus-Encoded Kinases (BXLf1 and BGLf4)

The BGLf4 serine threonine protein kinase, which is homologous to the HSV UL13 and KSHV ORF36, is expressed during lytic replication^{210,454} and is a nuclear protein.^{454,457,1526} It dissociates from the virion in a phosphorylation-dependent manner⁵⁰ and phosphorylates several viral proteins including BMRf1,^{210,455} BZLF1,⁵¹ and EBNA-LP,⁷²¹ as well as cellular proteins including EF-1 γ ,⁷²¹ TIP60,⁸⁷⁸ CKII β ,⁷²⁶ MCM4,⁸⁰⁸ lamin A/C,⁸⁴² stathmin,²¹² and condensin.⁸⁴¹ BGLf4 activates topoisomerase II,⁸⁴¹ and along with its induction of changes in nuclear morphology⁸⁴¹ and modification of the DNA damage response,⁸⁷⁸ these findings suggest that BGLf4 modifies the cell to facilitate virus replication. BGLf4 has an essential role in nuclear egress of capsids from infected cells.⁴⁵⁶ Finally, using EBV mutants in BGLf4 and BXLf1 (the viral thymidine kinase), BGLf4, but not BXLf1, was shown to be required for ganciclovir or acyclovir inhibition of lytic replication.¹⁰¹⁰ Earlier studies reported that EBV BXLf1 phosphorylates ganciclovir and acyclovir *in vitro*⁹⁰⁶ and enhances cytotoxicity in cells treated with these antivirals,¹⁰⁴⁵ whereas other studies found that BXLf1 poorly phosphorylates ganciclovir and acyclovir^{508,1478} and does not increase cellular cytotoxicity in cells treated with ganciclovir.⁵⁰⁸ These studies highlight the importance of performing studies in the context of virus infection.

DNA Replicative Proteins

EBV early proteins important for DNA replication include the viral DNA polymerase (BALF5), major DNA-binding protein (BALF2), ribonucleotide reductase (BORF2 and BaRF1), thymidine kinase (BXLf1), and alkaline exonuclease (BGLf5) (Fig. 61.2). In addition to its role in DNA replication, BGLf5 mediates host shutoff during productive EBV infection¹²⁴⁷ and

facilitates immune evasion.¹⁶⁴² For further details, see the section on Immune Escape, which describes this and other EBV lytic proteins important for modulating the immune response. The EBV DNA polymerase⁷⁰⁴ is associated with several other EBV nuclear proteins, including the 50-kD processivity factor. The catalytic subunit expressed in insect cells has 3' to 5' proof-reading exonuclease activity in addition to DNA polymerase activity.¹⁴⁷⁵ Unlike cellular polymerases, the EBV polymerase is active in 100-mM ammonium sulfate or in KCl and is sensitive to inhibition by phosphonoacetic acid, phosphonoformic acid, arabinofuranosylthymine, and acyclovir triphosphate.^{19,215,226,285,286,493,1119} The EBV DNA polymerase also differs from HSV DNA polymerase in its salt sensitivity and its relative resistance to aphidicolin. The EBV DNA polymerase also weakly distinguishes between 2-amino-purine nucleoside triphosphate and dATP, so that 2-amino-purine is preferentially incorporated into viral as opposed to cell DNA.⁴⁹³

Considerable progress has been made in reconstituting an *in vivo* minimal DNA replication system similar to the Challenge HSV system.^{395,396} The components include BALF5, which encodes the core DNA polymerase; BALF2, the single-strand DNA-binding protein; BMRf1, the processivity factor; BSLF1 and BBLF4, the primase and helicase complex; BBLF2/3, a spliced primase helicase complex component; and BKRF3, the uracil DNA glycosylase. A surprising aspect of EBV DNA replication is its dependence on expression of the BZLF1 and BRLF1 transactivators.^{395,396} The interaction of the C-terminal tail of BZLF1 with its core leucine zipper is required for DNA replication.¹⁰⁰⁴ In addition, mutations in the BZLF serine 173 phosphorylation site and DNA recognition domain are competent for the induction of early lytic genes but have a reduced capacity to bind DNA and activate lytic DNA replication; however, overexpression of BSLF1, BMRf1, and BALF2 partially restores DNA replication and binding to oriLyt.^{342,343} EBV DNA replication requires topoisomerase 1 and 2 activity.^{728,1004} EBV DNA replication induces growth arrest indirectly by activation of a DNA damage response.^{809,810}

DNA Packaging and Virion Egress

The EBV TR has several similarities to the HSV "a" sequence.^{1038,1379} Both EBV TR and the HSV "a" sequence are directly repeated at both ends of the DNA. Both sequences are high in G+C composition, with runs of three to five Cs. In both HSV and EBV, there is a short extra oligonucleotide segment (DR1) where the repeat joins the unique DNA. The EBV DR1 oligonucleotide GCATGGGGGG brackets the multiple copies of TR. In HSV-1, DR1 is the site of cleavage of headful length DNA for packaging into virions. There is little sequence specificity for DR1 because the nonhomologous oligonucleotides of HSV-1, HSV-2, and CMV are interchangeable. HSV or CMV cleavage and packaging occur 33 base pairs 3' to a conserved sequence of C(G)₆₋₈ TGT G/T (T)₃ NG C/G (G)₆ G/C T/C. A similar sequence C (G)₅ TGT(T)₂ CCT(G)₅ CC occurs 25 base pairs before the EBV DR1 and is also the key cleavage packaging recognition sequence for EBV. The EBV rightmost TR acquires a short unique sequence from the leftmost TR during cleavage and packaging, and that sequence becomes the left end of the DNA.¹⁶³⁸ Plasmids containing the EBV latent infection episome origin, the EBV lytic origin, and the EBV TR are stably maintained as an episome in latently infected cells, are replicated as linear concatemers by the EBV DNA polymerase when

lytic infection is induced, and are cleaved and packaged into infectious virions.¹⁵²³

Early electron microscope observations of lymphocytes lytically infected with EBV report the presence of nucleocapsids in the cytoplasm. While this could occur as a consequence of nuclear disruption late in infection or as a consequence of reinfection of the infected cell by released virus, an alternative model is that virus acquires an initial envelope as it buds through the nuclear membrane, that de-envelopment occurs within cytoplasmic vesicles resulting in release of nucleocapsids into the cytoplasm, and that re-envelopment occurs prior to release of infectious virus as postulated for other herpesviruses. BFLF2 and BFRF1 are positional homologs of UL31 and UL34 of HSV and are essential for the egress of newly assembled capsids across the nuclear membrane in HSV. Deletion of BFLF2 from EBV impairs virus egress but does not affect lytic DNA replication or B-cell transformation.^{474,483} Interestingly, the BFLF2 deletion virus has a further defect in DNA packaging, suggesting there may be some divergence in the function of this protein in herpesviruses.^{474,483} As in other herpesviruses, EBV gM and gN are important for egress of EBV virions from infected cells. These two proteins form a stable complex in infected cells, and EBV that lacks gN is deficient in envelopment and de-envelopment.^{827,828}

ENTRY INTO HOST: PORTALS OF ENTRY, SITE OF PRIMARY REPLICATION, CELL TYPES INFECTED

Most EBV infections occur through contact with infected saliva from persons infected in the past. Saliva contains virus that is high in EBV gp42, indicating that the virus is derived predominantly from MHC class II–negative cells (i.e., epithelial cells rather than B cells).⁶⁸⁹ Virus in saliva is more efficient in binding B cells than epithelial cells. During primary infection, EBV infects epithelial cells in Waldeyer's ring in the oropharynx where it replicates¹³⁵⁴ and then can infect resting naïve B cells that traffic through the oropharynx, or may infect resting, naïve B cells in the tonsillar crypts directly (Fig. 61.13).^{246,714} Virus replication has been found in rare tonsillar epithelial cells⁶²⁸ and in epithelial cells during infectious mononucleosis (IM)⁸⁵⁸; in contrast, other studies have reported replication only in B cells in the tonsils.³² While some B cells undergo lytic infection,

most become latently infected. Initially the cells are lymphoblasts with a type 3 pattern of latency and express each of the EBV latency genes^{32,817} (Table 61.2). Up to 10% of B cells in the blood early in infection are EBV positive. Most of these cells are thought to be killed by NK cells, antibody-dependent cell-mediated cytotoxicity (ADCC), or cytotoxic T cells.

A controversy exists as to whether EBV-infected B cells mimic the pathway in which naïve B cells differentiate to become resting memory B cells. Thorley-Lawson and colleagues^{912,1445} postulate that EBV-infected naïve B cells enter germinal centers and differentiate to resting memory B cells, similar to the process in which naïve B cells are activated by antigen to become B-cell blasts; enter the germinal center of lymphoid follicles; undergo class switch recombination and somatic hypermutation; and subsequently differentiate into resting memory B cells (Fig. 61.14). Only B cells whose surface immunoglobulin (B-cell receptor) has a high affinity for antigen avoid apoptosis and become CD27+ memory B cells. Similarly, naïve B cells infected with EBV express EBV type 3 latency genes resulting in B-cell proliferation and blast formation. These cells then migrate to a lymphoid follicle to begin the germinal center reaction. EBV-infected cells then express EBV latency 2 genes. EBV LMP1 up-regulates activation-induced cytidine deaminase (AID) to induce somatic hypermutation or immunoglobulin class switching.⁵⁴² EBV LMP2A stimulates antigen receptor signaling.¹⁵⁶ This allows the cell to survive its transit through the germinal center.

Upon exiting the germinal center into the blood, the virus-infected cells differentiate into memory B cells and express the EBV latency 0 pattern of gene expression with no viral proteins expressed.⁵⁹⁶ Some of the cells may undergo differentiation to plasma cells with EBV lytic replication, and the resulting virus can infect naïve B cells. Other EBV-infected B cells are thought to traffic back to the oropharynx, where they can undergo differentiation into plasma cells and either shed virus into the saliva directly or infect epithelial cells, which shed EBV into the saliva.

Several lines of evidence suggest that EBV-infected B cells may not always follow this pathway of infecting naïve B cells, transiting germinal centers, and trafficking to the oropharynx. First, EBV can infect both naïve and memory B cells.^{341,817} Second, EBV is present in both non-isotype-switched and isotype-switched memory B cells¹⁸⁹; the latter are thought to be independent of the germinal center. Third, EBV-infected B cells are usually in extrafollicular areas rather than the germinal

TABLE 61.2 EBV Latency Gene Expression Patterns

Latency	EBERs	EBNA-1	EBNA-2	EBNA-3	EBNA-LP	LMP1	LMP2	BARTs	Disease
0	+	— ^a	—	—	—	—	—	?	Blood in healthy carriers
1	+	+ ^b	—	—	—	—	—	+	BL, gastric carcinoma
2	+	+ ^b	—	—	—	+	+	+	NPC, HL, TCL
3	+	+ ^c	+	+	+	+	+	+	PTLD

^aEBNA-1 is expressed only when cells divide.

^bEBNA-1 is driven by the BamH1 Qp promoter.

^cEBNA-1 is driven by the BamH1 Cp or BamH1 Wp promoter.

EBER, Epstein-Barr virus–encoded RNA; EBNA, Epstein-Barr virus nuclear antigen; LMP, latent membrane protein; BL, Burkitt lymphoma; NPC, nasopharyngeal carcinoma; HL, Hodgkin lymphoma; TCL, T-cell lymphoma; PTLD, posttransplant lymphoproliferative disease.

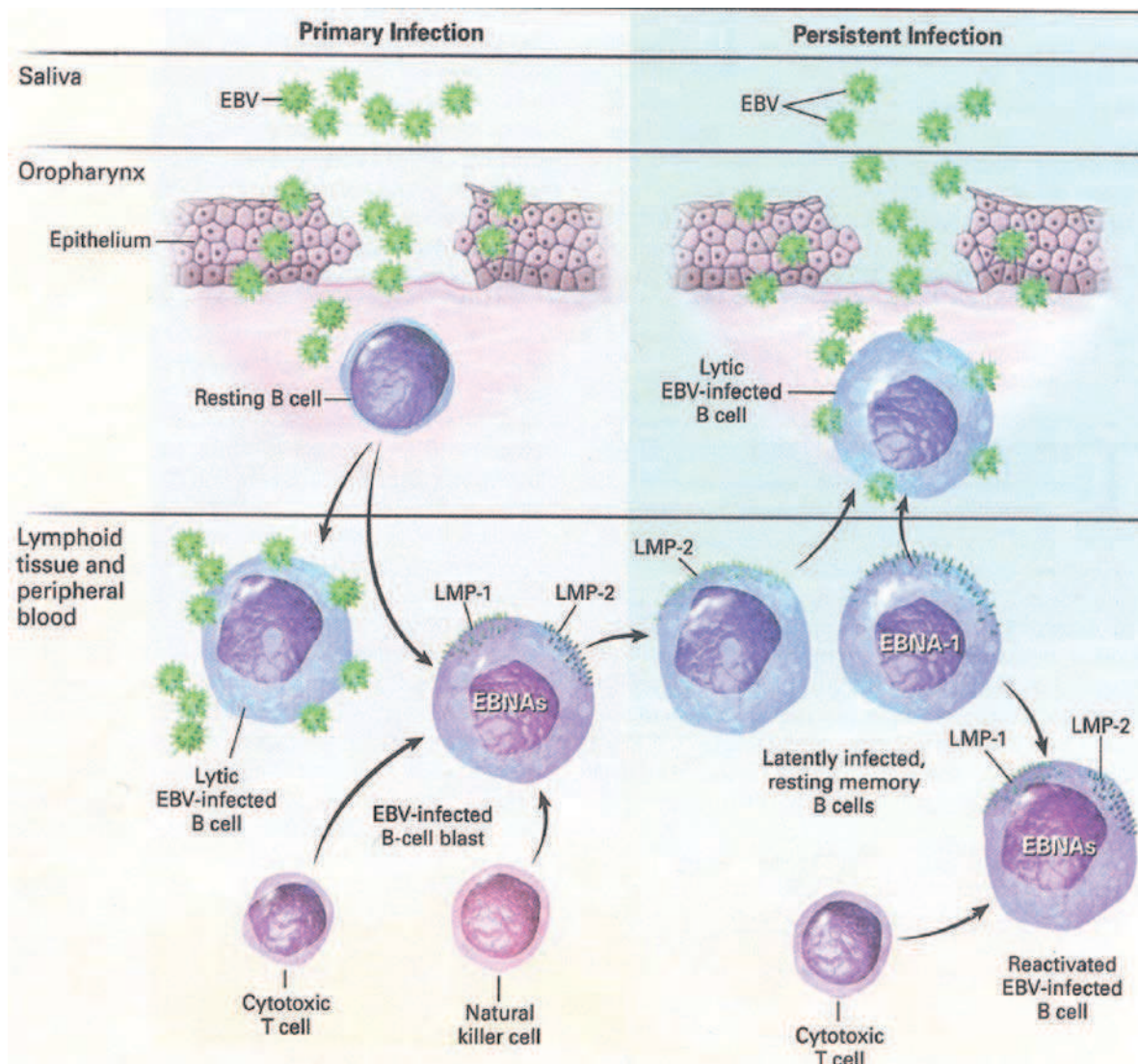
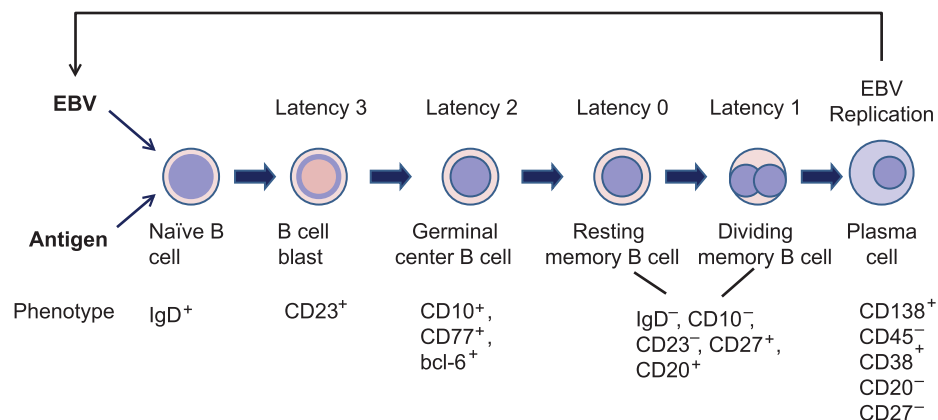


FIGURE 61.13. Epstein-Barr virus (EBV) life cycle. EBV infects resting B cells either directly or by virus released from epithelial cells in the oropharynx. EBV-infected B cells in the lymphoid tissues and blood undergo lytic infection or become B-cell blasts, which are controlled by NK cells and cytotoxic T cells. Latently infected resting memory B cells express no viral proteins unless they divide; if they reactivate, they are controlled by cytotoxic T cells. Some latently infected cells traffic to the oropharynx where they undergo lytic replication and release virus. (Adapted from Cohen JI. Epstein-Barr virus infection. *N Engl J Med* 2000;343[7]:481–492.)

FIGURE 61.14. Epstein-Barr virus (EBV) infection of B cells parallels the response of B cells to antigenic stimulation. EBV infects naïve B cells, which become B cell blasts with type 3 EBV latency. The cells undergo a germinal center reaction with type 2 EBV latency and exit the germinal center with type 0 EBV latency. EBV-infected resting memory B cells can differentiate in plasma cells whereby EBV undergoes lytic replication and can infect new naïve B cells.



centers.^{817,1089} Fourth, patients with X-linked lymphoproliferative disorder (XLPD) have absent or poorly formed germinal centers,⁹⁴⁹ but they are infected with EBV (see later). Patients with X-linked hyperimmunoglobulin M syndrome lack classical memory B cells and their B cells cannot undergo a germinal center reaction and therefore are not class switched (IgD[−], CD27⁺); however, these patients can be infected with EBV.²⁵⁷ These patients have EBV in nonclassical memory B cells (IgD⁺, CD27⁺). Therefore, germinal center reactions and classical memory B cells are not necessary for EBV infection. Fifth, EBV-infected B cells in the tonsils express the CD10 and CD77 germinal center markers in some¹²⁴³ but not other¹⁸⁹ studies. Thus, EBV-infected cells may not require transit through the germinal center. Sixth, EBV-infected B cells in the germinal center may not participate in germinal center reactions during IM.⁸¹⁶ Seventh, EBV-infected B cells in tissues have a wide variety of expression of EBV latency proteins with patterns other than latency patterns 0 to 3.⁸¹⁷ Thus, another model posits that EBV infects and proliferates in memory B cells and that the germinal center reaction is not essential.⁸¹⁴ Finally, virus shedding still occurs in the absence of detectable B cells in the peripheral blood,⁶⁰⁵ which indicates either that other cells can also transport EBV to the oropharynx or that EBV is maintained in B cells in the oropharynx in the absence of B cells in the periphery.

While EBV infects naïve and memory B cells from tonsils *ex vivo*, EBV is less efficient in infecting memory B cells from the blood.³²⁵ IgA-positive or IgG-positive memory B cells from the tonsils are infected more efficiently with EBV than IgM memory B cells from the tonsils. EBV infects memory B cells in nasal-associated lymphoid tissue more efficiently than memory B cells in other lymphatic tissues; β 1 integrin on the memory B cells in nasal-associated lymphoid tissue increases the susceptibility of these cells to EBV infection.³²⁵ EBV has been detected in plasmacytoid B cells.³²

B cells are essential for EBV latency. Persons with X-linked agammaglobulinemia who lack mature B cells are not infected with EBV and have no cellular immunity to EBV.³⁸¹ Hematopoietic stem cell transplantation, which eliminates host B cells but not epithelial cells, can eliminate EBV from the body.⁴⁸⁵ In addition to B cells, EBV infects epithelial cells. EBV replication is detected in epithelial cells of the tongue but not in epithelial cells of the salivary glands.^{419,1508} EBV also infects human umbilical cord–derived endothelial cells.⁶⁹⁹

While EBV is present in B cells in the blood of healthy individuals, in some patients with elevated EBV levels in the blood (e.g., patients with HIV, transplant recipients), EBV has been detected in other cell types, usually at a lower frequency than in B cells. EBV has been detected in the blood in cells other than B cells including CD4 and CD8 T cells in patients with HIV,⁸⁹ γ/δ T cells, and NK cells in patients with chronic active EBV disease⁷⁷⁰; plasmablasts/plasma cells and monocytes in patients with lymphoproliferative diseases and HIV^{152,1476}; and pre-Langerhans cells in the blood of persons with HIV as well as healthy individuals.¹⁵¹¹

IMMUNE RESPONSE

Innate Immune Response

The innate immune response includes interferon responses and NK cells. Herpesvirus nucleic acids are detected by TLRs and

RIG-I, which in turn activate a number of adapter molecules, including IRFs, which lead to expression of IFN- α and IFN- β . These bind to IFN receptors and activate expression of interferon-stimulated genes (ISGs).

Treatment of monocytes with EBV DNA or virus stimulates release of IL-8 through TLR2 and TLR9, while release of MCP-1 and IL-10 was mediated primarily by TLR2.³⁹² EBV BLLF3 (dUTPase) activates NF- κ B through TLR-2 and induces expression of proinflammatory cytokines.⁴⁷

Expression of EBERs activates RIG-I and IRF-3 and induces expression of IL-10.¹²⁷¹ Expression of EBERs in cells expressing RIG-I induces expression of type I IFN and ISGs; this requires NF- κ B and IRF-3.¹²⁷⁰ EBERs are transcribed by RNA polymerase 3, which then triggers RIG-I activity.⁴ EBER1 is released from virus-infected cells in a complex with the cellular protein La and activates TLR3 to induce expression of IFN- β , IFN- γ , and TNF- α .⁶⁶³

EBV up-regulates expression of TLR7; treatment of B cells with UV-irradiated virus and a TLR7 ligand enhances B-cell proliferation.⁹⁸² In contrast, EBV lytic infection down-regulates TLR9. CpG increases proliferation and transformation of EBV-transformed B cells and increases T-cell co-stimulatory molecules, up-regulation of IL-6, and release of virus from lymphoblastoid cell lines.⁶⁵⁹ TLR2 or TLR7/8 ligands also enhance EBV-induced B-cell transformation.

Incubation of plasmacytoid dendritic cells with EBV or viral DNA results in release of IFN- α through TLR9.³⁹² LMP1 up-regulates expression of ISGs, which induces an antiviral effect.¹⁶²²

NK cells are important for early control of EBV. Patients with X-linked lymphoproliferative disease have impaired NK-cell killing, which contributes to fulminant primary EBV infection observed in many patients. NK cells from the tonsils produce high levels of IFN- γ and inhibit EBV transformation.¹³⁹⁶ IFN- γ from NK cells also delays latent EBV antigen expression in virus-infected cells and reduces B-cell proliferation during the first 7 days of infection *in vitro*.

NK-cell numbers are increased at the onset of IM and have enhanced ability to kill virus-infected cells.¹⁵⁴¹ During IM, higher NK-cell counts are associated with a lower viral load in the peripheral blood. Cytokines that regulate NK-cell activation (IFN- α , IFN- β , IFN- γ , and IL-12) are significantly increased in persons with IM compared with healthy controls.

Antibody Response

Antibody is likely important for protection against IM but may not be sufficient to prevent infection; vaccine trials using gp350 as an immunogen showed reduction of IM but no difference in infection.¹³⁶⁹ Initial control of EBV infection is due to NK cells and non-HLA-specific cytotoxic T cells, while later during convalescence HLA-specific CD8 and CD4 T cells are important for control of infection (see Cellular Immunity below).

Acute EBV infection induces polyclonal B-cell activation with elevated levels of IgG, IgM, and IgA. Heterophile antibodies are produced, which are not EBV specific. One type of heterophile antibody, used for diagnosis of acute infection, measures the dilution of serum that agglutinates sheep, horse, or cow erythrocytes after absorption with guinea pig kidney. Heterophile antibodies persist for up to 1 year after IM and are often absent in children younger than 5 years old or in the elderly.

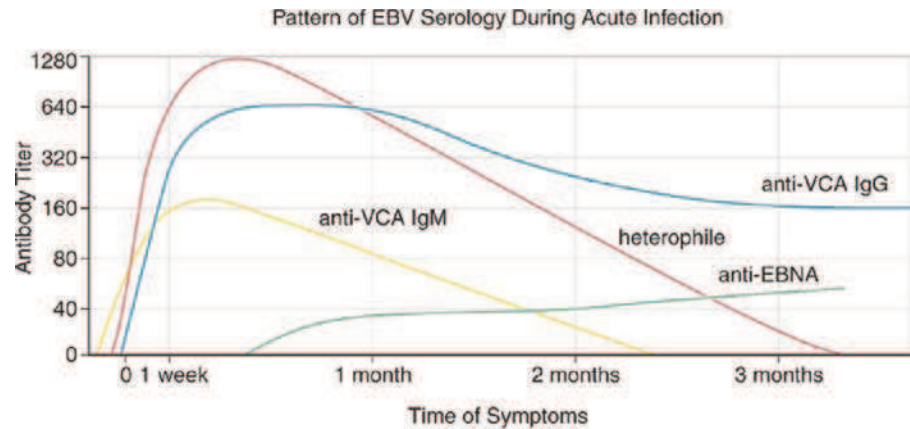


FIGURE 61.15. Pattern of antibodies to Epstein-Barr virus (EBV) during acute infection. (From Cohen JL. Epstein-Barr virus. In: Young NS, Gerson SL, High KA, eds. *Clinical Hematology*. Philadelphia: Elsevier, 2006:956–966, copyright © 2006 Elsevier.)

In addition to heterophile antibody, IgM and IgG antibodies to the viral capsid antigen (VCA) are usually present at the onset of symptoms of IM (Fig. 61.15).²⁴⁷ Later in infection, antibody to EBNA is detected and IgM antibody to VCA disappears. IgG antibody to VCA²⁵ and EBNA persists for life. Antibodies to EBV early antigens (EAs) are classified as either diffuse (EA-D, diffusely in the nucleus and cytoplasm, methanol resistant) or restricted (EA-R, restricted to the cytoplasm, methanol sensitive). EA-D antibodies are often present 3 to 4 weeks after the onset of IM, especially in patients with severe illness, and are frequently detected in patients with nasopharyngeal carcinoma (NPC) or chronic active EBV (CAEBV). EA-R antibodies are often detected in CAEBV or African BL. IgA antibody to EBV is often elevated in patients with NPC, and IgA antibody to EBV EA is present in patients with IM.¹⁰¹

Cellular Immune Response

Acute Infection

During IM there is a massive expansion of CD8, but not CD4, T cells.⁹⁵⁹ Monoclonal or oligoclonal populations of CD8 T cells (based on T-cell receptors) are present, which react with EBV latent antigens,^{160,959} but there are not clonal populations of CD4 T cells. Oligoclonal populations of CD8 T cells may also be present that react with EBV lytic epitopes.³⁸

Most EBV-specific CD8 T cells have an activated memory phenotype and express HLA-DR and CD45RO with down-regulation of CD63L and CD45RA.¹⁶¹ Cytotoxic T cells to lytic and latent antigens during IM display a CD45RO+, CCR7– effector phenotype; later lytic antigen responses have either a CD45RA+, CCR7–, or a CD45RO+, CCR7– effector memory phenotype, while latent antigen responses have either a CD45RO+, CCR7+ central memory phenotype or a CD45RO+, CCR7– effector memory phenotype.⁵⁸⁶ CD8 T cells during IM have cytotoxic activity and contain perforin^{159,1389,1390}; most of the cells have a very short lifespan and undergo apoptosis when the level of EBV antigen declines.^{159,331,1422} During convalescence the frequency of EBV-specific T cells declines and in long-term carriers up to 5% of cytotoxic T lymphocytes (CTLs) are directed against EBV antigens.⁵⁸⁶ Expression of programmed death receptor 1 (PD1, a marker of lymphocyte exhaustion) is elevated on EBV-specific CD8 T cells during IM, correlates with the EBV viral load, and returns to low levels during convalescence.⁴⁸⁷

Initially CD8 T cells are directed predominantly against EBV lytic antigens; later in infection cytotoxic T cells are

directed to latent antigens⁵⁸⁶ (Fig. 61.16). CD8 T cells to additional latent antigens may arise 3 to 4 months after acute infection.^{586,1554} Up to 44% of CD8 T cells can recognize a single lytic epitope during IM in some persons.^{161,1188,1389,1390} While the level of CTLs to EBV immediate-early (IE) antigens declines rapidly after infection, the level of CTLs to latency proteins declines much more slowly. Of the lytic antigens, CD8 T cells recognize IE proteins (BZLF1, BRLF1) more often than early proteins (BMRF1 and BMLF1), and late proteins least frequently.^{1188,1389} (Fig. 61.17). This hierarchy of IE over E proteins may be due to expression of immune evasion genes that block CD8 T-cell responses later in the lytic replication cycle. There is a direct correlation between CD8 T-cell immunodominance among the lytic antigens and the efficiency with which these antigens are processed and presented to CD8 T cells in lytically infected cells.¹¹⁸⁸ Up to 5% of CD8 T cells are directed to latent antigens, especially EBNA-3A, -3B, and -3C.^{161,1389}

CD4 T-cell responses to EBV proteins have been less well studied than CD8 T-cell responses, especially in IM. CD4 T cells from patients with IM recognize EBNA-3C to a greater extent than EBNA-1 or EBNA-2, while EBNA-1 is predominant in long-term carriers.¹⁵⁵⁴ During IM, CD4 T-cell responses to BZLF1 are higher than CD8 T-cell responses.¹³⁰¹ CD4 T cells recognize BZLF1, BMLF1, and EBNA-3A in patients with IM and the responses rapidly decline after acute infection to less than 1% at 1 year.¹¹⁸⁴ While the CD8 T-cell response favors IE over early EBV proteins, the CD4 T-cell response is distributed more equally to IE, early, and late viral proteins.⁹¹⁶ These findings are consistent with CD8 T-cell expansions being induced by direct contact with IE antigens on lytically infected cells before MHC class I immune evasion proteins encoded by EBV are expressed, while CD4 T-cell expansions are due to multiple antigens released from infected cells and presented to CD4 T cells by dendritic cells.

While there are large numbers of EBV-specific T cells in the blood during IM, relatively few of these T cells traffic to the tonsils.⁵⁸⁷ CD8 T cells that recognize lytic antigens are fourfold more prevalent in the tonsils than in the blood, and CD8 T cells that recognize latent antigens are 10-fold more prevalent in the tonsils.⁵⁸⁷ CD8 T cells that recognize EBV lytic and latent epitopes in the blood of patients with IM have low levels of the lymphoid homing receptors CCR7 and CD62L.^{161,331,586,587} While CD8 T cells in the blood that recognize latent antigens may be CCR7+ and CD62L+ and accumulate in the tonsil

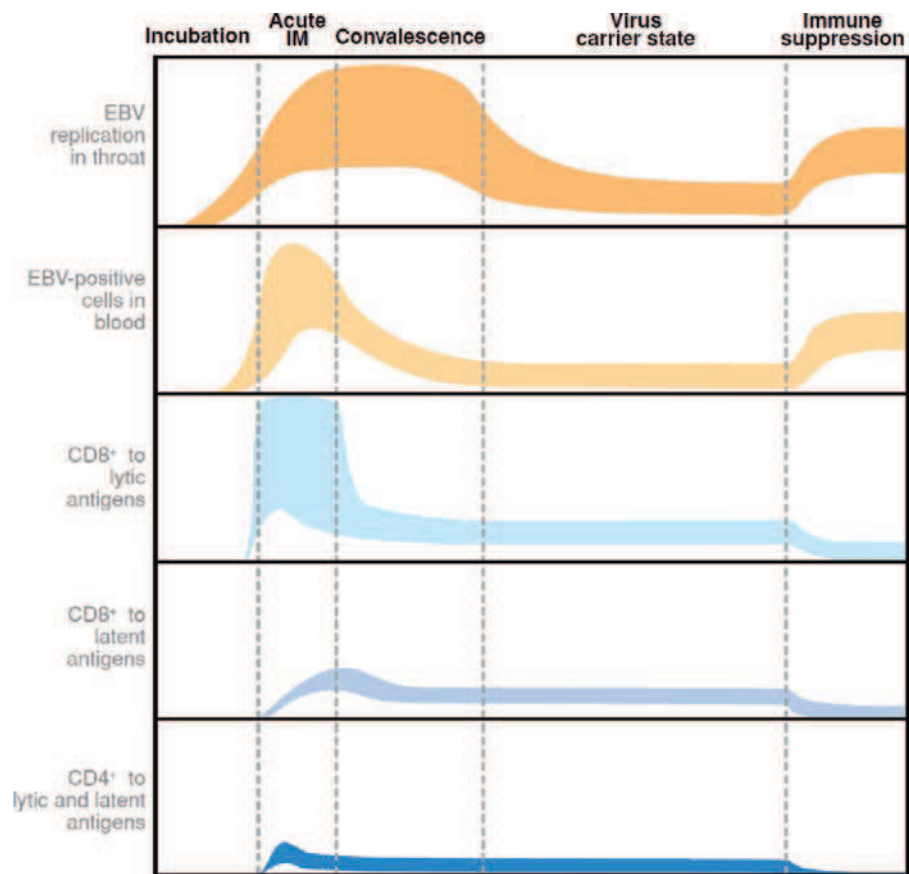


FIGURE 61.16. Levels of Epstein-Barr virus (EBV) in the throat and blood and cellular immunity during the course of infection. (From Hislop AD, Taylor GS, Sauce D, et al. Cellular immunity responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* 2007;25:587–617, with permission.)

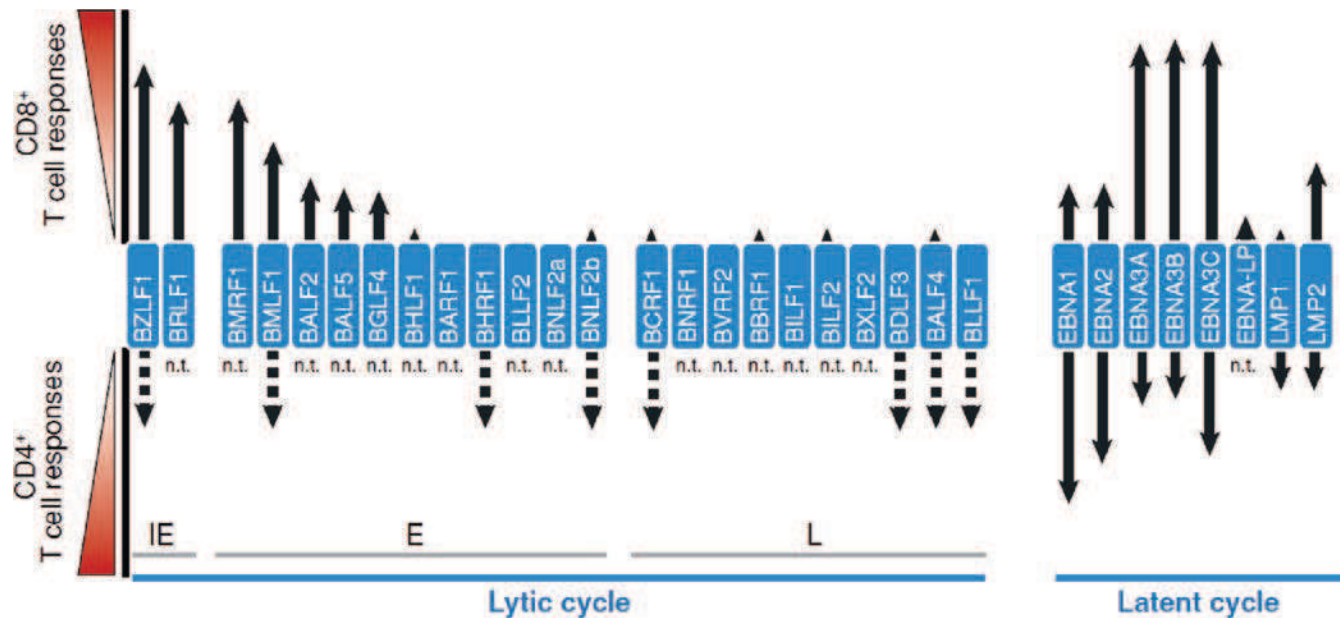


FIGURE 61.17. CD8+ and CD4+ T-cell responses to Epstein-Barr virus (EBV) immediate-early (IE), early (E), late (L), and latent proteins. (From Hislop AD, Taylor GS, Sauce D, et al. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* 2007;25:587–617, with permission.)

after IM, CD8 T cells that recognize lytic antigens remain CCR7[−] and CD62L[−] much longer after IM. This might allow virus-infected cells in the tonsil to undergo persistent lytic replication. In long-term carriers, the tonsils show a 10- to 20-fold enrichment of T cells that recognize latent epitopes and a 2- to 5-fold enrichment of T cells that recognize lytic epitopes compared with the peripheral blood.⁵⁸⁷ The CD38 B-cell activation marker is present on CD8 T cells that recognize lytic or latent EBV epitopes in the blood during IM but is down-regulated during convalescence.^{181,586}

Healthy Carriers

In healthy carriers, the frequency of EBV-specific CD8 T cells is about 0.2% to 2% for viral lytic proteins and 0.05% to 1% for latent proteins and remains stable over time.⁵⁹⁰ In healthy carriers CD8 T cells predominantly recognize the EBNA-3A, -3B, and -3C latency proteins.^{754,1070} IE BZLF1 and BRLF1, and early BMRF1 and BMLF1 proteins are also prominent targets of CD8 T cells.⁵⁸⁶ Other EBV early (BALF2, BALF5, BGLF4) and latency (LMP2 and to a lesser extent EBNA-1 and EBNA-2) proteins are also targets for CD8 T cells; late proteins are less frequently targeted. Clonal expansions of EBV-specific CD8 T cells contract over time but do persist and have cytotoxic activity.^{867,1341,1342} EBV-specific cytotoxic CD8 T cells are perforin(−/+), granzyme (Grm) B(+), GrmA(+), and GrmK(+); perforin and GrmB, but not GrmA and GrmK, correlated with cytotoxic activity.⁵³⁷

The frequency of CD8 T cells that recognize EBV proteins is 10-fold higher in healthy EBV carriers than for CD4 T cells.^{855,915} CD4 T cells predominantly recognize EBNA-1 (especially the carboxyl terminus) in healthy EBV carriers, and to a lesser extent EBNA-2 and EBNA-3C.^{550,855,1472} EBNA-1 can be recognized by endogenous processing in the proteasome.^{1434,1501} EBNA-1 accumulates in cytoplasmic autophagosomes where lysosomal processing enhances EBNA-1 recognition by CD4 T cells.¹¹²⁹ Studies using B cells with inducible expression of EBNA-1 and EBNA-3B showed that CD8 T cells recognized the latency proteins rapidly but that recognition declined quickly when protein expression was suppressed; in contrast, CD4 T-cell recognition was delayed when latency protein expression was induced but persisted for days after protein expression was suppressed.⁹⁵³ These results imply that *de novo* EBV-infected cells are recognized predominantly by CD8 T cells, while latently infected cells are better recognized by CD4 T cells.

Other latency (EBNA-3A and -3B, LMP1, and LMP2) as well as multiple IE, early, and late lytic proteins (BZLF1, BMLF1, BHRF1, BNLF2b, BCRF1, gp110 [BALF4], gp350 [BLLF1]) are recognized by CD4 T cells from healthy carriers,⁹ although the relative frequencies of individual IE, early, and late proteins are not known. The broad array of latency proteins recognized by CD4 T cells is presumably due to CD4 cells recognizing antigens released from infected cells, rather than by direct contact with cells expressing latency proteins. *In vitro* studies show that gp110 and gp350 antigen is transferred to B cells by uptake of virions, resulting in MHC class II presentation of the glycoprotein antigens to CD4 T cells that are cytolytic and inhibit proliferation of virus-transformed B cells. EBNA-2 and EBNA-3C are thought to be recognized by CD4 T cells upon transfer of antigen of these EBV proteins between cells, rather than processing by autophagy.¹⁴³¹

EBV-specific CD4 T cells produce IFN- γ and TNF- α but less frequently produce IL-2 in response to antigen.³⁰ Some EBV-specific CD4 T-cell clones are cytotoxic or inhibit outgrowth of virus-transformed cells, while others do not.⁹¹⁵ Most EBV-specific CD4 T cells express CD27 and CD28.

Immune Escape

EBV uses a number of strategies to avoid detection by the immune system. Activation of EBV replication, which allows the immune system to detect viral proteins, results in down-regulation of MHC class I, class II, and β_2 -microglobulin with impaired antigen presentation by virus-infected cells.^{504,734} Activation of virus replication increases the susceptibility of virus-infected cells to killing by NK cells, which corresponds to down-regulation of HLA-A, B, and C (which bind to KIR inhibitory receptors on NK cells) and down-regulation of HLA-E (which binds to the NKG2D inhibitory receptor).¹¹³³ EBV inhibits dendritic cell development by inducing apoptosis of monocyte precursors.⁸⁷³ EBV encodes a number of proteins that help the virus to avoid killing of infected cells by the immune system.¹²¹⁸

The EBNA-1 Gly-Ala repeats were initially reported to inhibit processing of EBNA-1 by the proteasome and presentation of EBNA-1 peptides to CD8 T cells.^{865,866} (Table 61.3). More recent studies indicate that the dominant role of the EBNA-1 Gly-Ala repeats is to reduce the translation efficiency of EBNA-1, resulting in fewer EBNA-1 peptides expressed on the surface of cells, which results in less efficient recognition by EBV-specific CD8 T cells.^{1435,1597} Newly synthesized EBNA-1, rather than long-lived stable EBNA-1, is the primary source of the protein's epitopes that are presented to CD8 T cells.^{1434,1501}; this suggests that defective ribosome EBNA-1 products may be the major source of EBNA-1 epitopes. The mRNA that encodes the EBNA-1 Gly-Ala repeats is enriched for purines, which results in a lack of secondary structure, inhibits the efficiency of protein translation, and reduces antigen presentation.¹⁴³⁶ The EBNA-1 Gly-Ala repeats also inhibit translation initiation of EBNA-1 mRNA,⁴² and the rate of inhibition of translation correlates with reduced antigen presentation of EBNA-1.⁴¹ EBNA-1 inhibits pre-mRNA processing of its transcripts, resulting in autorepression of EBNA-1 in latently infected cells, which may reduce the level of protein available for detection by the immune system.¹⁶⁰² While presentation of some CD4 epitopes from EBNA-1 requires autophagy,¹¹²⁹ localization of EBNA-1 to the nucleus limits its processing by autophagy and its presentation to CD4-specific T cells.⁸⁶⁰ Regardless of these findings, EBNA-1 is recognized by CD8 T cells.^{851,1434,1501}

LMP1 inhibits its presentation to MHC class I by self-aggregation and possible destruction of LMP1 epitopes in proteasomes.¹³⁶⁶ LMP1 levels in EBV-infected cells vary over time, and this may allow cells expressing low levels of the protein to avoid killing by CD8 T cells.¹³⁵ LMP1 peptides suppress T-cell activation, inhibit T-cell proliferation, and reduce NK-cell cytotoxicity; however, it is not clear if such peptides are present and at levels required to have immunosuppressive activity.³³⁰ LMP1, secreted in exosomes from virus-infected cells, inhibits T-cell proliferation.^{397,750} LMP2A and LMP2B increase the degradation of IFN receptors in epithelial cells, which may limit the antiviral response in virus-infected cells.¹³²⁴ LMP1 and LMP2A up-regulate the galectin-1 promoter; galectin-1 triggers apoptosis of EBV-specific cytotoxic T cells.¹¹²³

TABLE 61.3 EBV Proteins and RNAs that Inhibit the Immune System

EBV protein	Effect
EBNA-1	Inhibits its own proteasome processing Reduces its own translational efficiency and initiation of translation
LMP1	Up-regulates galectin promoter to induce apoptosis of CTLs Up-regulates IL-10, up-regulates A20 and bcl-2 to inhibit apoptosis
LMP2	Increases degradation of IFN receptors Up-regulates galectin promoter to induce apoptosis of CTLs
BARF1	Soluble colony-stimulating factor receptor, inhibits IFN- α secretion
BCRF1	IL-10 homolog; inhibits synthesis of IFN- γ by T cells; reduces MHC class II expression; reduces IL-1 α , IL-1 β , TNF- α , and IL-6 production in monocytes; reduces levels of MHC class I, ICAM1, CD80, and CD86 on monocytes; reduces mRNA levels of TAP1 and proteasome protein LMP2
BGLF5	Blocks synthesis of MHC class I and II molecules Down-regulates TLR9
BHRF1	Bcl-2 homolog, blocks apoptosis
BILF1	Binds MHC class I molecules and removes MHC class I from cell surface Reduces rate of newly synthesized class I molecules on the cell surface by diverting them from the exocytic pathway Forms heterodimers with CXCR4 and impairs binding to CXCL12
BLLF3 (dUTPase)	Up-regulates IL-10, TNF- α , IL-1 β , IL-8, and IL-6
BNLF2a	Interacts with TAP, inhibits its ATP and peptide-binding functions, reduces MHC class I at the cell surface
BZLF1	Up-regulates IL-10; inhibits IRF7 (and type I IFN); inhibits IFN- γ -induced MHC class II expression; inhibits activation of IRF1, CIITA, and p48 and nuclear translocation of STAT1; decreases levels of IFN- γ receptor- α mRNA and level of TNF receptor 1
BZLF2 (gp42)	Binds the HLA-DR β chain of class II and inhibits antigen presentation, inhibits generation of CTLs in mixed lymphocyte reactions
LF2	Inhibits dimerization of IRF7, inhibits IFN- α production
EBV RNA	
EBER	Up-regulates IL-10, induces resistance to IFN- α -mediated apoptosis
miR-BART2-5P	Reduces expression of MICB, a stress-induced NK-cell ligand, to reduce killing by NK cells
miR-146a	Inhibits expression of IFN-responsive genes

EBV, Epstein-Barr virus; EBNA, Epstein-Barr virus nuclear antigen; LMP, latent membrane protein; CTL, cytotoxic T lymphocyte; IL, interleukin; IFN, interferon; MHC, major histocompatibility complex; TNF, tumor necrosis factor; ATP, adenosine triphosphate; mRNA, messenger RNA; HLA, human leukocyte antigen; NK, natural killer.

EBV BNLF2a co-localizes with the transporter associated with antigen processing (TAP) in the endoplasmic reticulum and is expressed early in infection.⁶¹⁰ BNLF2a interacts with TAP and inhibits its adenosine triphosphate (ATP) and peptide-binding functions and reduces MHC class I at the cell surface, resulting in inhibition of CD8 T-cell recognition of antigens on target cells.^{589,1565} BNLF2a is expressed early in the lytic cycle of EBV replication and it affects presentation of IE and early EBV proteins, but not late proteins.²⁷² Nonetheless, CD8 T-cell clones can lyse lymphoblastoid cells expressing BNLF2a.⁸²⁹

The EBV host shutoff function of BGLF5 down-regulates MHC class I expression and inhibits virus-specific CD8 T-cell recognition of endogenous antigens by MHC class I.¹⁶⁴² BGLF5 blocks synthesis of both MHC I and II molecules¹²⁴⁷ and down-regulates TLR9.¹⁴⁹⁰

BILF1, a constitutive G protein-coupled receptor, binds to MHC class I molecules, resulting in increased turnover of MHC class I from the cell surface and increased degradation by lysosomes.¹⁶⁴⁰ The G protein signaling activity of BILF1 is independent of its effect on MHC class I. BILF1 also reduces the rate of newly synthesized class I molecules on the surface by diverting them from the exocytic pathway. The effect of BILF1

on the exocytic pathway accounts for about 70% of its immune evasion activity.¹⁶⁴¹ Deletion of BILF1 from the virus increases recognition of virus-infected cells by CD8 T cells. BILF1 also forms heterodimers with various chemokine receptors,¹⁴⁹⁹ and binding of BILF1 to CXCR4 impairs the latter's binding to CXCL12 and reduces CXCL12 signaling.¹⁰⁹²

EBV BCRF1 shares 70% amino acid identity with human IL-10 and, like its human homolog, BCRF1 inhibits synthesis of IFN- γ by T cells.^{621,1044} BCRF1 reduces MHC class II expression on monocytes, resulting in both reduced antigen presentation and antigen-specific T-cell proliferation.²⁹⁵ BCRF1, like human IL-10, reduces IL-1 α , IL-1 β , TNF- α , and IL-6 production in monocytes. BCRF1 also reduces levels of MHC class I, ICAM1, CD80 (B7.1), and CD86 (B7.2) on monocytes.¹²⁶⁸ BCRF1 reduces mRNA levels of TAP1, and LMP2 (low molecular mass polypeptide 2), a subunit of the proteasome, reduces the amount of peptide translocated into the endoplasmic reticulum and reduces levels of MHC class I on the surface of B cells.¹⁶¹⁸ Other EBV proteins and RNA including LMP1,¹⁰⁷⁴ BZLF1,^{840,957} and EBERs⁷⁸¹ up-regulate IL-10. EBV BLLF3 (dUTPase) up-regulates IL-10, as well as TNF- α , IL-1 β , IL-8, and IL-6, in resting peripheral blood mononuclear cells (PBMCs).⁴⁷⁰

EBV BARF1 is secreted from infected cells and acts as a soluble colony-stimulating factor 1 (CSF-1) receptor that blocks the activity of CSF-1.¹³⁹⁵ BARF1 inhibits IFN- α secretion from mononuclear cells.²⁵¹ EBV LF2 tegument protein interacts with IRF7, inhibiting its dimerization and reducing production of IFN- α .¹⁵⁶²

EBV BZLF1 interacts with IRF7 and inhibits its activity, which is essential for production of type I IFN.⁵¹⁸ BZLF1 also inhibits the activation of IRF7 by double-stranded RNA. BZLF1 inhibits a number of activities of IFN- γ including IFN- γ -induced MHC class II expression; activation of IRF1, CIITA, and p48; and nuclear translocation of STAT1.¹⁰⁵⁹ BZLF1 also decreases levels of IFN- γ receptor- α mRNA and levels of TNF receptor 1, which impairs TNF- α signaling.¹⁰⁵⁸

gp42 (BZLF2) binds the HLA-DR β chain and soluble gp42 inhibits antigen presentation.¹³⁸⁶ Soluble gp42 inhibits the proliferative response of PBMCs to antigen and inhibits generation of cytotoxic T cells in mixed lymphocyte cultures. gp42 binds to class II peptide complexes and sterically hinders T-cell receptor domains and inhibits MHC class II antigen presentation to T cells.¹²²⁰ gp42 is present as a type II membrane protein and as a soluble protein secreted from cells; both forms bind MHC class II and inhibit class II-restricted antigen presentation to T cells.¹²¹⁹

An EBV miRNA (miR-BART2-5P) reduces expression of MICB, a stress-induced NK-cell ligand; cells expressing miR-BART3-5P showed reduced killing by NK cells.¹⁰⁷² EBV LMP1 up-regulates expression of miR-146a, which is expressed at high levels in EBV type 3 but not type 1 latency.¹⁶⁵ miR-146a inhibits expression of IFN-responsive genes.

Several EBV gene products inhibit apoptosis. EBV BHRF1 is a homolog of bcl-2 and blocks apoptosis.⁵⁵⁷ BHRF1 blocks apoptosis by binding to Bim, a proapoptotic bcl-2 protein,³⁰⁰ as well as to Bid and PUMA.⁸²⁰ LMP1 up-regulates expression of bcl-2, which blocks apoptosis, and A20, a negative regulator of NF- κ B.⁸¹¹ The EBERs induce resistance to IFN- α -mediated apoptosis, although the mechanism by which this occurs is controversial.^{1076,1251}

EPIDEMIOLOGY

Over 90% of adults are infected with EBV. Infection is most common between the ages of 2 and 4 years and at around age 15 years.¹⁰⁸⁸ Primary infection with EBV in infants and young children is usually symptomatic or results in nonspecific symptoms. In developing countries infants and young children are infected with EBV and infectious mononucleosis is much less common. For example, in areas of Africa where Burkitt lymphoma is common, 50% of children are infected with EBV before 1 year of age. In contrast, in developed countries, about 50% of adolescents and young adults are EBV seronegative, and about 25% of these seronegative persons who become infected with EBV develop infectious mononucleosis. In studies performed at five English colleges, Yale University, and the United States Military Academy at West Point, infectious mononucleosis occurred in 28% to 74% of seronegative students.

EBV is usually spread by infected saliva. EBV infection is increased in sexually active young adults. While this may be due to kissing with sharing of oral secretions, some observations suggest that the virus may be spread by sexual intercourse. The

risk of IM was lower in students who always used condoms, suggesting that the virus may also be transmitted by sexual intercourse,⁵⁸⁰ and the risk was higher in women than men, in sexually active persons, and in those with a higher number of sexual partners.^{270,271} In addition, detection of EBV in both male and female genital secretions^{660,1353} provides a mechanism for sexual transmission of EBV; however, the level of EBV in genital secretions is lower than in saliva.¹⁴⁴³ EBV has also been transmitted by latently infected B cells through blood transfusion and by bone marrow transplant.

EBV is classified into two types, 1 and 2, based on sequence polymorphisms in EBNA-2, -3A, -3B, and -3C. Type 1 EBV isolates are more efficient in B-cell transformation *in vitro* than type 2 isolates, although differences in EBV types have not correlated with human diseases and both types co-exist in all populations that have been studied. While type 1 strains are believed to be more common in most populations, many of these studies used outgrowth assays, which may be skewed by the higher transforming efficiency of EBV type 1 strains. Type 2 isolates are nearly equal in prevalence to type 1 isolates in New Guinea and equatorial Africa. Intertypic recombinants containing portions of type I and type II EBV strains have been reported.^{1015,1584}

EBV is further classified into strains based on sequence differences. Strain differences are generally segregated with different ethnicities (African, Southeast Asia, and Caucasian). Asymptomatic carriers have multiple strains of EBV that are often different in the blood and in the throat and change over time.^{1350,1505} Patients with IM have multiple strains of EBV in the oropharynx, plasma, and PBMCs; multiple strains have been shown to be transmitted from a carrier resulting in primary infection.¹³⁵¹ Most patients with IM showed two or more EBV strains in the blood and throat, although only one strain is usually detected using *in vitro* transformation assays.¹⁴⁵¹ Multiple EBV strains persist over time; there is a trend toward restriction of the number of EBV strains in the saliva, but not in the blood.³⁶⁶

Some persons have CTL responses to polymorphic EBV T-cell epitopes and carry two distinct strains of virus with different T-cell epitope sequences.¹³⁴ While a deletion in the carboxyl terminus of LMP1 was reported to be associated with EBV-associated malignancies, this may be due instead to geographic differences.²¹³ Six variants of LMP1 that differ in sequence have been identified; however, these proteins do not differ in their transforming or signaling properties *in vitro*.⁹⁶² The majority of LMP1-specific T-cell epitopes are highly conserved in viruses from around the world.³³⁴ Differences in EBNA-1^{514,954} and LMP1 sequences¹⁴⁵ are more likely related to differences in geographic areas than to differences in biological activities of the proteins.

PRIMARY INFECTION

Healthy Carriers

In healthy carriers in developed countries, EBV is present in about 1 to 50 per million B cells in the peripheral blood.¹⁰³⁶ These cells are classic antigen-selected resting memory B cells (IgD-, CD27+, sIg+, CD20+) and are isotype switched and somatically mutated.^{61,62,1377} In Africa, particularly in areas where malaria is endemic, EBV is present in the blood of children at higher levels, approaching those seen with infectious mononucleosis.^{1046,1099}

While transcripts for LMP2 and EBNA-1 have been detected in the blood of carriers,¹⁴⁵³ more recent studies suggest that virus-infected cells in the blood have a latency 0 phenotype and express no viral genes with the exception of the EBERs.⁶⁰ The EBV viral load varies among individuals but is relatively constant at a set point within a single individual, both in healthy persons and in those with HIV.^{1036,1168} All EBV-infected persons shed virus into the saliva, although the levels vary considerably.⁹⁰⁰

In the tonsils EBV-infected CD10+ B cells are present at about three to four cells per germinal center.¹²⁴⁴ EBV-infected memory B cells in the tonsils are IgD[−] and express a type 2 latency phenotype.^{61,62} EBV-infected B cells are present at similar levels in the tonsils, adenoids, and peripheral blood, but at 20-fold lower levels in the spleen and the mesenteric lymph nodes, indicating that the subtype of memory B cells or their environment influences infection.⁸²³ EBV gene expression is similar in the spleen and tonsil (latency 2), unlike that of the peripheral blood (latency 0). When B cells in the tonsils differentiate into plasma cells, the EBV BZLF1 promoter is activated and viral replication initiates.⁸²⁴

While the EBV viral load is similar in the peripheral blood and the bone marrow, there is a threefold higher number of EBV lytic antigen-specific T cells in the bone marrow compared with the blood, although the numbers of latent antigen-specific T cells are similar at both sites.¹¹²⁸

Infectious Mononucleosis

The incubation period for IM is about 4 to 6 weeks. IM presents with fever, lymphadenopathy, and pharyngitis in more than half of patients.²⁴⁵ Posterior cervical lymphadenopathy and a tonsillar exudate are common. Other signs and symptoms include splenomegaly, hepatomegaly, rash, periorbital edema, and fatigue. Rarely EBV-positive genital ulcers can occur. Serious complications include upper airway obstruction due to enlarged tonsils, aplastic anemia, severe thrombocytopenia, granulocytopenia, autoimmune hemolytic anemia, hemophagocytic syndrome, hepatitis, jaundice, myocarditis, splenic rupture, encephalitis, meningitis, and Guillain-Barre syndrome. Most patients with encephalitis do not present with IM. While most patients' symptoms resolve in 2 to 4 weeks, up to 10% will have fatigue persisting for 6 months or more. Patients with symptoms that persist for 6 or more months do not have higher EBV DNA levels or differences in immune responses to the virus.¹⁶³

The peripheral blood shows leukocytosis, an increase in T cells but not B cells, and atypical lymphocytes. These latter cells have large amounts of cytoplasm often with vacuoles (Fig. 61.18) and are predominantly activated T cells (HLA-DR⁺), but also include NK cells. There is an increase in CD8 T cells and a decrease in CD4 T cells. T cells from patients with IM have reduced responses to mitogens *in vitro*. Pathology of lymph nodes and tonsils shows reactive follicles with proliferation of immunoblasts with reactive T cells; binuclear immunoblasts that resemble Reed-Sternberg cells are often present, which can confuse the diagnosis of IM with B-cell lymphoma. Polyclonal B-cell activation results in elevated levels of immunoglobulins, production of heterophile antibody (see Diagnosis later), and in some cases antinuclear antibodies, cold agglutinins, rheumatoid factor, or antiplatelet antibodies. Tissues have elevated levels of IL-1 β , IL-6, IL-10, TNF- α , lymphotoxin, and IFN- γ -induced chemokines (MIG, IP10).^{412,1322} The serum shows elevated levels of liver enzymes including serum transaminases and alkaline

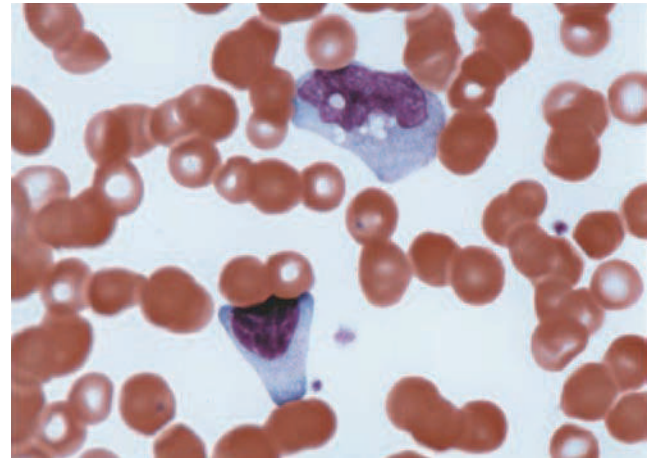


FIGURE 61.18. Atypical lymphocytes with prominent cytoplasm and vacuoles. (From Cohen JL. Epstein-Barr virus. In: Young NS, Gerson SL, High KA, eds. *Clinical Hematology* Philadelphia: Elsevier, 2006:956–966, copyright © 2006 Elsevier.)

phosphatase. Symptoms of IM are due to the large expansion of CD8 T cells and the cytokines they produce (IL-6, IL-10, IL-12, IFN- γ , TNF- α , TGF- β ^{1113,1556}) rather than to lytic infection of B cells. In contrast, asymptomatic EBV infection is associated with a high level of EBV in the blood but not the large expansion of CD8 T cells, which occurs in IM.¹³⁴⁵ Therefore, the large expansion of CD8 T cells in IM that are associated with symptoms might not be required for control of virus infection. High-level persistent shedding of virus occurs for weeks to months after IM despite the high levels of CD8 T cells that recognize EBV lytic antigens.

It is uncertain why IM is a disease of adolescents and young adults. Some of the CD8 T cells that recognize EBV lytic proteins can cross-react with other proteins from pathogens previously seen by the immune system and might contribute to lymphoproliferation. Two patients with IM had CD8 T cells that recognized EBV BMLF1 and cross-reacted with the influenza M1 protein and accounted for one-third of the cells that recognized BMLF1.²⁴⁰ HLA MHC class I polymorphisms in HLA-A01 correlate with development of IM, severity of symptoms, and viral load in persons with primary EBV infection.⁹⁹⁷ HLA-DR polymorphisms also correlated with development of IM.¹²⁰⁶ Lymphoblastoid cell lines obtained from HLA-A01 individuals also fail to present latent EBV peptides.¹³⁰ Persons with certain polymorphisms in IL-10 have more severe disease with acute infection.^{555,556}

Patients with IM have high levels of EBV in the peripheral blood and shed high titers of virus from the throat (Fig. 61.16). Virus may be produced in lytically infected B cells⁷¹⁴ as well as in epithelial cells. The level of EBV-infected B cells in the blood drops rapidly ($t_{1/2}$ = 7.5 days) after infection, followed by a slow decline during the first year.⁵¹⁶ During the latter slow decline, the half-life of the viral load is 39 days.⁶¹² EBV levels in the peripheral blood decline during the first 6 weeks of infection and rapidly fall in the plasma during the first 2 weeks.^{71,366} High levels of virus shedding persist in the throat for prolonged periods (greater than 180 days) of time after IM. The number of infected B cells is maintained at about 1 to 50 B cells per

million after recovery.⁶¹ The half-life of EBV-specific T cells during the first 2 weeks of IM is 2.9 days.⁶¹²

From 1% to 20% of B cells in the peripheral blood are infected with EBV during IM. While up to 50% of memory B cells are latently infected with EBV, few naïve B cells are infected.⁵⁹⁷ The virus-infected cells are resting, not proliferating, memory cells (IgD⁺, sIg⁺, CD27⁺, CD5⁺, and CD20⁺), and while some cells have detectable transcripts for all of the latency genes,¹⁴⁵³ most do not express viral latency genes except for EBNA-1 when the cells divide.⁵⁹⁶ Viral replication in the blood is rare during IM, and nearly all the cells are latently infected. EBV-positive cells in the blood have more somatic hypermutation than EBV-negative cells.¹³⁷⁶

The frequency of regulatory T cells (CD4⁺, CD25^{hi}) in the blood is significantly lower in persons with IM than in seropositive blood bank donors; however, a similar frequency of regulatory T cells was observed in the tonsils of patients with IM and controls.¹⁵⁴⁷

B cells in tonsils from persons with IM show EBV latency 3 gene expression along with other forms of latency including type 1 and type 2 with variable expression of LMP1.⁸¹⁷ EBV-infected memory B cells and B cells in the germinal center of lymph nodes expand in the absence of somatic hypermutation.⁸¹⁶

EBV-ASSOCIATED DISEASES

X-linked Lymphoproliferative Disease

Patients with X-linked lymphoproliferative disease (XLP) type 1 (XLP-1) present with hemophagocytic syndrome (32% of cases), dysgammaglobulinemia (22%), a family history of XLP-1 (17%), lymphoma (14%), fulminant IM (8%), or other symptoms.¹²⁰ Acute infection with EBV usually results in a fulminant infection with organs infiltrated by proliferating B and T cells; hemophagocytic lymphohistiocytosis (HLH), bone

marrow failure with lymphocytopenia, and hepatic failure often occur.¹³¹⁵ Other patients have aplastic anemia, lymphomatoid granulomatosis, or vasculitis. Patients have an excessive T helper cell 1 (Th1) response with elevation of IFN- γ levels and large numbers of activated T cells and NK cells in the blood. Survivors of acute EBV infection often have low levels of IgG and high levels of IgM and IgA; about 20% subsequently develop B-cell lymphomas. Some patients with mutations in SAP that have not been infected with EBV develop B-cell lymphomas, vasculitis, or hypogammaglobulinemia. These patients do not have more severe infections with other viruses. Patients with XLP-1 have reduced IgG responses to vaccines, reduced IL-10 production in T cells,⁹⁴⁸ and absent NK T cells.^{1087,1141} SAP knockout mice have impaired B-cell memory.²⁷³

XLP-1 is due to a mutation in SAP (SLAM-associated protein) also known as SH2D1A^{242,1086,1291} (Fig. 61.19). SAP is an adapter protein containing a single SH2 domain. SAP is expressed in T, NK, NK T, and some B cells and interacts with SLAM on T, B, and dendritic cells.¹⁷⁰ This interaction inhibits production of IFN- γ , which is normally induced by T-cell activation. The interaction of SAP with SLAM may result in reduced IFN- γ production either by (a) impairment of SLAM binding to SHIP¹²⁹¹ or (b) recruitment of FynT, SHIP, Dok1, and Dok2 with activation of Ras-guanosine triphosphatase (GTPase)-activating protein.⁸³² SAP also interacts with other members of the SLAM family.¹⁷⁰ These include 2B4 and NTB-A, which mediate NK-cell killing; and interaction with Ly9 and CD84. The interaction of SAP with 2B4 results in recruitment of FynT, SHIP, and Vav-1, which results in increased NK-cell cytotoxicity. Impaired SAP interactions with SLAM, 2B4, and NTB-A may reduce CD8³³² and NK-cell^{123,1139} killing of EBV-infected B cells and result in excessive production of IFN- γ , which can contribute to uncontrolled T-cell proliferation and HLH. SAP also has pro-apoptotic activity and promotes reactivation-induced cell death, which is important for T-cell homeostasis.^{869,1073,1368}

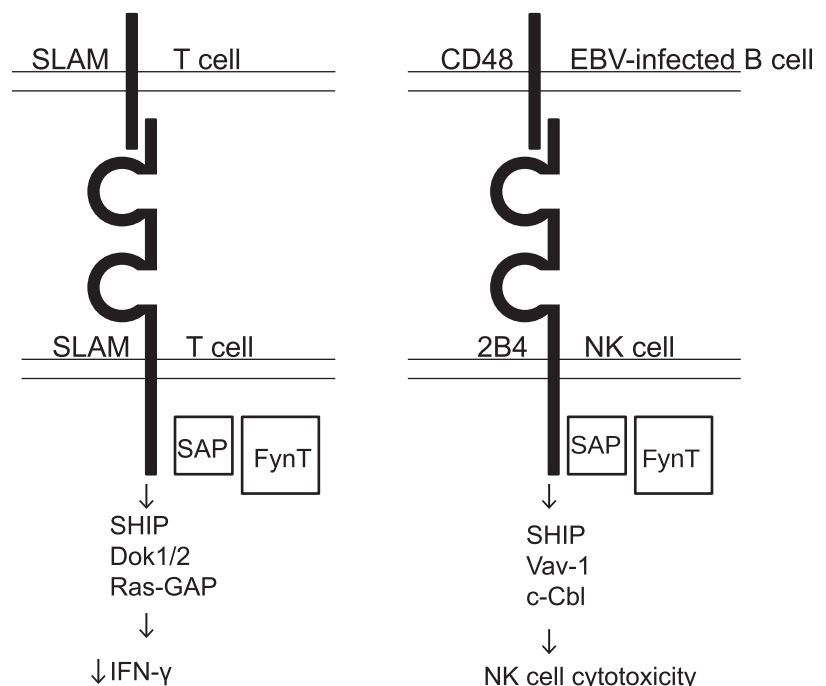


FIGURE 61.19. Interaction of SAP with SLAM and 2B4. SAP binds to SLAM on T cells and initiates a cascade resulting in decreased interferon- γ (IFN- γ). SAP binds to 2B4 on natural killer (NK) cells, which results in increased NK-cell cytotoxicity.

T cells from patients with XLP-1 are resistant to apoptosis mediated by T-cell receptor stimulation, which may contribute to the massive lymphoproliferative disease with the disease.

Patients with XLP-1 who survive infection with EBV have quantitatively normal EBV-specific CD8 T-cell responses, but their T cells are impaired for recognition of SLAM ligand-positive (but not SLAM-negative) EBV-infected B cells.⁵⁸⁸ Patients with XLP-1 lack IgD[−] CD27⁺ class-switched memory B cells, and those who survive carry EBV in nonswitched memory cells (IgM⁺, IgD⁺, CD27⁺).^{190,949} Patients with other family members with XLP-1 nearly always have mutations in SAP, whereas sporadic cases are less likely to have mutations. CD4 T cells from patients with XLP-1 do not provide optimal B-cell help, efficiently differentiate into IL-10–positive effector cells, or efficiently up-regulate inducible co-stimulator (ICOS), a potent inducer of IL-10. Adoptive transfer experiments in mice lacking SAP show that the animals have normal B cells but defective CD4 T cells.²⁷³ This results in markedly reduced numbers of virus-specific long-lived plasma cells and memory B cells with impaired long-term antibody responses, but normal or increased levels of virus-specific memory CD4 T cells. Mice²⁷⁴ and humans⁹⁴⁹ lacking SAP have markedly reduced germinal center B cells; germinal center responses require early and sustained interactions between B and T cells and SAP is essential for these interactions.¹⁶⁹ SAP expression in T cells, but not B cells, is required for normal antibody production and formation of germinal centers.¹⁴⁹³

Intravenous immunoglobulin has been used to prevent EBV infection in boys with mutations in SAP; fatal breakthroughs have occurred. While antiviral therapy and cytotoxic chemotherapy have not been successful, rituximab (anti-CD20 monoclonal antibody) has been reported to be life-saving in several patients with XLP-1 and fulminant IM.¹⁰³¹ Hematopoietic stem cell transplantation (HSCT) cures the disease and survival rates of 80% have been reported with good immune reconstitution.¹²⁰ Patients with active HLH have a lower rate (50%) of survival. Patients who are not transplanted are usually given intravenous immunoglobulin and the survival rate is 63%.

Patients with mutations in X-linked inhibitor of apoptosis (XIAP, also termed BIRC4) have many features of XLP, and this disease is referred to as XLP-2.¹²²⁵ These patients often present with HLH, recurrent splenomegaly, fever, and cytopenia. The disease is usually triggered by primary infection with EBV. XLP-2 is usually less severe than XLP-1. HLH is more common with XLP-2 than XLP-1, while hypogammaglobulinemia and low NK T-cell numbers are more common in XLP-1.^{389,1126} The high incidence of HLH and the lack of lymphomas in patients with XLP-2 have caused some authors to reconsider its classification as a form of X-linked familial HLH rather than XLP-2.⁹⁷⁸ Lymphocytes from patients deficient in XIAP have increased apoptosis in response to Fas and the TRAIL receptor.¹²²⁵ HSCT is recommended for XLP-2.³⁸⁹

Two sisters with mutations in IL-2–inducible T-cell kinase (ITK) died with uncontrolled lymphoproliferative disease after infection with EBV.⁶²⁷ NK T cells were absent from the one patient tested and high levels of eomesodermin in CD8 T cells were present in both children.

Chronic Active EBV and Related Disorders

CAEBV disease, also referred to as EBV-positive lymphoproliferative disorder of childhood, is a rare disease more common

in Asia and natives of Mexico and Central and South America. Patients present with IM-like symptoms with fever, lymphadenopathy, and hepatosplenomegaly and develop lymphoproliferative disease involving the liver, lungs, nervous system, or other organs.^{249,767,768,1118} HLH is common with fever and pancytopenia. Asian patients may have hypersensitivity to mosquito bites. The disease often presents after primary EBV infection, and patients have high viral loads and/or extremely high antibody titers to EBV VCA and EA, and infiltration of tissues with EBV-positive T, NK, or much less commonly B cells.⁷⁶⁵ Many patients have absent antibody to EBNA-1. In Japanese patients with CAEBV, EBV is often clonal in T or NK cells, while in the United States the virus is often in B cells.²⁴⁹ Patients with CAEBV have dysregulation of cytokines and often have elevations in both Th1 and Th2 cytokines.¹¹¹⁶ Patients often have low levels of EBV-specific CD8 T cells, implying a failure to control infection.¹⁴⁰³ One patient has been described who had mutations in both alleles of perforin⁷¹⁹ and another patient had a mutation in a magnesium transporter protein (MagT1),⁸⁷¹ but the etiology of the disease in other patients is not known. Patients with EBV in T cells have a poorer prognosis and often have high fever and higher EBV antibody titers to VCA and EA than those with virus in NK cells, while patients with EBV in NK cells often have hypersensitivity to mosquito bites, high IgE, and large granular lymphocytes in the blood.⁷⁶⁶ If untreated, most patients die from hepatic failure, refractory T- or NK-cell lymphomas, or opportunistic infections due to a progressive loss of immune cell function. EBV latency 2 gene expression is present in the peripheral blood of patients with CAEBV.⁶⁶⁴ While a number of therapies, including antiviral agents, immunosuppressive agents, cytotoxic chemotherapy, and autologous EBV-specific CTLs have been tried, only hematopoietic stem cell transplantation (HSCT) has been curative.⁴⁷⁸

Hydroa vacciniforme-like lymphoma is associated with CAEBV and is more common in Asians and natives of Central and South America. EBV-positive T-cell hydroa vacciniforme presents on sun-exposed areas of the skin with papules and vesicles on the face.⁶⁶⁵ EBV-positive NK-cell disease often presents with hypersensitivity to mosquito bites and patients may develop HLH. EBV-positive cells are usually clonal and have EBV latency 1 gene expression. The disease is indolent and can regress spontaneously; some cases progress with systemic symptoms with lymphadenopathy and hepatosplenomegaly. High levels of EBV DNA are in the blood. A milder form of hydroa vacciniforme presents in children or young adults with persistent papules and vesicles on sun-exposed areas, especially the face, which ulcerate. EBV is present in T cells in the lesions and the prognosis is good.

Hemophagocytic lymphohistiocytosis (HLH) is associated with CAEBV as well as with certain infections, including primary infection with EBV.⁶⁵⁴ Patients present with HLH and fever and often develop bone marrow and liver failure. The condition is frequently, but not always, seen in patients with CAEBV disease and is more common in Asians. Patients have elevated serum levels of TNF- α , IFN- γ , IL-1, sTNF-R1, and cytochrome c.⁷²⁵ Infection of T cells *in vitro* with EBV results in up-regulation of TNF- α and IFN- γ , which activates macrophages and may drive HLH.⁸³⁹ Elevated levels of IFN- γ , macrophage inflammatory protein-1 α (MIP-1 α), MIG, and IP10 are present in tissues.¹⁴⁴¹ EBV infects circulating T cells, CD5 is down-regulated on CD8 T cells, and increased HLA-DR-positive cells are observed.¹⁴⁵⁸

Adults with EBV-associated HLH have high numbers of EBV genomes in circulating NK cells.⁴¹⁵ While an elevated EBV DNA load correlates with a poorer prognosis, IgH or TCR rearrangements are not associated with a worse outcome.¹¹ In patients who do not respond to conservative measures, EBV-associated HLH is often treated using the HLH 2004 protocol with corticosteroids or cyclosporine and etoposide to control HLH.⁶⁵⁵ In most cases etoposide is not required.¹³³⁸ Patients with persistent disease or elevated viral loads are usually treated with HSCT.¹¹¹⁵

EBV Lymphoproliferative Disease: Congenital, Iatrogenic, and Posttransplant

Patients with congenital (e.g., severe combined immunodeficiency, Wiskott-Aldrich syndrome, ataxia telangiectasia), acquired (HIV), or iatrogenic (methotrexate therapy) T-cell immunodeficiencies often have elevated EBV DNA levels in the peripheral blood and throat and are at risk for EBV lymphoproliferative disease and lymphoma (Table 61.4). The underlying defect in these patients is impaired T-cell function and inability to control EBV-infected B cells. Pathology ranges

from polymorphic lymphoproliferative disease to diffuse large B-cell lymphoma.

Patients with rheumatoid arthritis or polymyositis who receive methotrexate may develop EBV lymphoproliferative disease that often resolves when methotrexate is discontinued.^{1269,1037} In this case methotrexate may increase the risk of virus reactivation as well as impair T-cell function.³⁸⁴ Patients receiving TNF- α inhibitors for rheumatoid arthritis have also developed EBV lymphoproliferative disease⁹⁷⁵; some cases resolved after stopping the drug.

EBV posttransplant lymphoproliferative disease (PTLD) occurs in 1% of kidney transplant recipients, 1% to 2% of liver transplant recipients, 1% to 3% of heart transplant patients, 2% to 5% of lung transplant recipients, and 20% of small intestine transplant patients. Bone marrow transplant recipients have a 1% incidence of PTLD, which increases in those who receive T-cell-depleted marrow or HLA-mismatched marrow. The risk of EBV PTLD is increased in persons who have a primary EBV infection, graft-versus-host disease, cytomegalovirus infection, or who receive second transplants or anti-T-cell antibodies.^{831,1504} Certain immunosuppressive

TABLE 61.4 EBV-Associated Malignancies in Immunocompromised Persons

Disease	EBV frequency	EBV gene expression	EBV latency	Genetic mutations	Cell of origin	Latency period after EBV infection	Comments
Lymphoproliferative disease in immune deficiency	100%	EBNA-1, -2, -3, -LP; LMP1, 2	3		B cell, GC, or post-GC	<3 months	Reduced T-cell immunity
Posttransplant lymphoproliferative disease	Early: <1 year after transplant >90%; late: EBV less common	EBNA-1, -2, -3, -LP; LMP1, 2	3	Early: none; late: bcl-6 +/- ras, p53, c-myc	B cell, GC, or post-GC	Early: <1 year; late: >1 year	Reduced T-cell immunity
HIV malignancies							
Burkitt lymphoma	30%–70%	EBNA-1	1	c-myc, bcl-6, +/- p53	B cell, GC	Early in HIV	35% of HIV lymphomas
Hodgkin lymphoma	>95%	EBNA-1, LMP1, LMP2	2		B cell, post-GC	Early in HIV	5% of HIV lymphomas
DLBCL—centroblast	30%	EBNA-1	1	+/- bcl-6	B cell, GC	Early in HIV	25% of HIV lymphomas
DLBCL—immunoblastic	>90%	EBNA-1, -2, -3, -LP; LMP1, 2	3		B cell, GC, or post-GC	Late in HIV	15% of HIV lymphomas, low CD4 cell numbers
Primary CNS lymphoma	>95%	EBNA-1, -2, -3, -LP; LMP1, 2	3		B cell, GC, or post-GC	Late in HIV	Low CD4 cell numbers
Primary effusion lymphoma	>90%	EBNA-1, +/- LMP1, LMP2	1 or 2	bcl-6	B cell, GC, or post-GC	Late in HIV	HIV lymphomas HHV-8+
Plasmablastic lymphoma	50%–80%	EBNA-1	1		B cell, post-GC	Late in HIV	Very rare; CD20-
Smooth muscle tumors	>95%	EBNA-2, +/- LMP1	?		Smooth muscle cell	Late in HIV	Rare

EBV, Epstein-Barr virus; EBNA, Epstein-Barr virus nuclear antigen; GC, germinal center; LMP, latent membrane protein; DLBCL, diffuse large B-cell lymphoma; CNS, central nervous system.

drugs, including tacrolimus and IL-2 receptor antagonists, are associated with an increased risk of disease; other immunosuppressive drugs such as alemtuzumab are associated with a high viral load but a low risk of PTLD.¹⁷⁷ The rate of EBV PTLD varies depending on the transplant, with higher rates associated with increasing immunosuppression.

Patients with PTLD often have elevated levels of IL-6, a B-cell growth factor, in the peripheral blood.¹⁴⁶⁹ Transplant recipients with a polymorphism in the TNF- α promoter and the TNF receptor I promoter are at increased risk for PTLD.⁹⁹⁶ Increased levels of TNF- α are also seen in the plasma of patients with PTLD. Polymorphisms in Nramp⁷⁶ and in IFN- γ ⁸⁵³ are predictive for development of PTLD in liver transplant recipients. Polymorphisms in IL-10 and TGF- β are more frequent in solid organ transplant patients with PTLD.⁶³ Transplant recipients with elevated antibody to EBV early antigens were at increased risk of developing EBV PTLD after transplant¹⁰ in one study, but not another.¹⁷⁸ Patients with EBV PTLD had a significantly higher frequency of an HLA B51 allele than transplant patients without the allele.⁴⁷⁶ HLA-B18 and HLA-B21 were associated with a higher risk of developing PTLD, while HLA-A03 and HLA-DR7 were associated with a lower risk.¹³⁹⁸

About 55% of patients (especially early after transplant) present with an IM-like illness with fever, enlargement of tonsils and adenoids, and lymphadenopathy; biopsies show plasmacytic hyperplasia and lesions are polyclonal or oligoclonal. About 30% of patients have polymorphic PTLD with monoclonal or oligoclonal lesions (Fig. 61.20). About 15% of patients have monomorphic PTLD with monoclonal disease, which includes diffuse large cell lymphoma, Burkitt lymphoma, classical

Hodgkin lymphoma-like PTLD, and plasma cell myeloma. Distinct EBV-positive clones can arise at one or more sites. In contrast, T-cell lymphomas after transplant are rarely EBV positive. The polyclonal and polymorphic PTLD lesions that occur during the first year after transplant are almost always EBV positive and have a type 3 pattern of latency. In contrast, the later monomorphic lesions are often, but not always, EBV positive and may have different patterns of latency with variable expression of EBNA-2 and LMP1. Early plasmacytic hyperplastic or polymorphic PTLD lesions usually do not have chromosomal abnormalities,¹⁷² providing further evidence that these tumors are driven by EBV. In contrast, monomorphic PTLD lesions that occur later after transplant that are centroblastic diffuse large B-cell lymphomas may have mutations in *bcl-6*.^{172,186} Monomorphic lesions may also have mutations in *p53*, *N-ras*, and *c-myc*.⁷⁹¹ Unlike many Burkitt lymphoma cell lines, which have *c-myc* translocations and often do not require EBV for survival, cells from PTLD patients require EBV to inhibit apoptosis and to drive the cells out of the G1/G0 phase of the cell cycle.¹⁴⁹⁴

PTLD may involve the lymph nodes, but lesions are often extranodal and present in the gastrointestinal tract, liver, lungs, kidney, or central nervous system (CNS). In organ transplant recipients the lesions are often in the transplanted organ, probably due to antigenic stimulation with production of cytokines and B-cell growth factors. Tumors are usually of donor origin in bone marrow transplant recipients and of recipient origin in solid organ transplant recipients. EBV-positive Hodgkin lymphoma can also occur after transplant, and in a large series all of these tumors occurred in persons who were EBV seronegative prior to transplant.¹¹⁹⁵

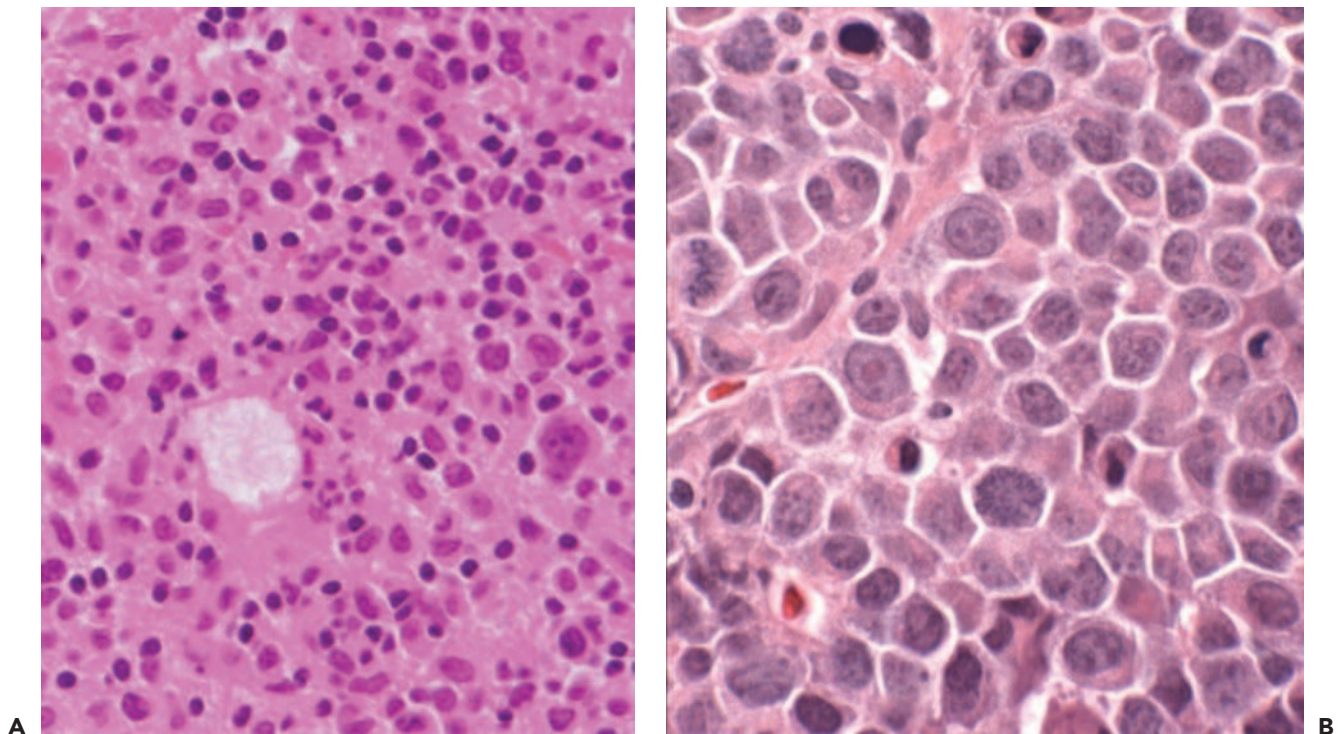


FIGURE 61.20. Biopsy from a patient with polymorphic (A) or monomorphic (B) posttransplant lymphoproliferative disease. (Courtesy of Dr. Elaine Jaffe.)

Sequencing of immunoglobulin heavy chain (IgH) genes from PTLD lesions show that some (especially early after transplant) have germline IgH sequences indicative of naïve B cells, while others have IgH somatic mutations that arise from antigen-selected memory B cells; in contrast, others (usually late after transplant) have mutated IgH genes (lacking a functional B-cell receptor) that are often not compatible with B-cell survival but more commonly seen with Hodgkin lymphoma.^{128,172,1454}

In immunosuppressed solid organ transplant recipients, elevated viral loads in half of the patients are due to increased numbers of cells with type 0 EBV latency in the blood, while the other patients had both type 0 latency and lytically infected cells in the blood.⁶⁰ Peripheral blood cells from solid organ transplant recipients expressed EBERs, BARTs, and LMP2, but not other latency genes.⁴⁷⁷ In a larger study of heart transplant patients with high EBV loads,⁶⁰⁶ blood from 53% of patients expressed LMP2, 25% expressed gp350, 17% expressed EBNA-3C, and 11% expressed LMP1. In another study of organ transplant recipients, 85% of those with high viral loads had LMP1 and LMP2 expression in the peripheral blood, and 20% had EBNA-1, EBNA-2, or BZLF1 expression in the blood.¹¹⁹⁴ In other studies, solid organ transplant recipients with elevated viral loads had high EBV copy numbers in cells¹²³⁹ and EBV in cells lacking surface immunoglobulin.¹²⁹⁶ PTLD biopsies

overexpress galectin-1, which tolerizes dendritic cells and induces apoptosis of cytotoxic T cells; neutralizing antibody to galectin-1 inhibited galectin-1–induced apoptosis of virus-specific CD8 T cells.¹¹²³

Detection of elevated levels of viral DNA in the blood or cerebrospinal fluid may provide a clue to the diagnosis of PTLD and also may be useful for follow-up after treatment.^{480,505} In general, very high levels of EBV DNA in the blood are predictive of PTLD, while low levels are common and often resolve without therapy.¹ Detection of EBV DNA in the plasma is more predictive of PTLD than detection in whole blood.⁷⁷¹ Patients at risk for EBV PTLD have lower levels of EBV-specific CD8 T cells in the blood,¹⁰⁰⁷ and combined viral load measurements and EBV-specific T cells may be more predictive of PTLD than either alone.^{39,1007,1363} Staging of the extent of disease is performed with computed tomography and positron emission tomography (PET) scans. Diagnosis of EBV PTLD requires demonstration of EBV and appropriate histopathology in tissue biopsies. Biopsies of PTLD lesions usually show type 3 EBV latency.¹⁶⁰⁶

Burkitt Lymphoma

Endemic BL is usually an EBV-positive tumor that occurs in equatorial Africa and New Guinea, where *Plasmodium falciparum* malaria is also endemic (Table 61.5). The disease occurs

TABLE 61.5 EBV-Associated Malignancies in Nonimmunocompromised Persons

Disease	EBV frequency	EBV gene expression	EBV latency	Genetic mutations	Cell of origin	Latency period after EBV infection
Burkitt lymphoma	Endemic 95%–100%; sporadic 15%–85%	EBNA-1	1	<i>c-myc</i> , +/- <i>p53</i> , <i>RB2</i>	B-cell GC centroblast	3–8 years
Hodgkin lymphoma	MC, LD 60%–80%; NS 20%–40%	EBNA-1, LMP1, LMP2	2		B-cell post-GC	1 year or more
Nasopharyngeal carcinoma	Anaplastic 100%; keratinizing 30%–100%	EBNA-1, LMP2, +/-LMP1	2	Various tumor suppressor genes	Undifferentiated epithelial cell	>30 years
Gastric carcinoma	Lymphoepithelioma 100%; adenocarcinoma 5%–15%	EBNA-1	1		Gastric epithelial cell	>30 years
Diffuse large B-cell lymphoma of the elderly	Up to 25%	EBNA-1, LMP1,2 +/-EBNA-2	2 or 3		B cell, GC, or post-GC	>30 years
Peripheral T-cell lymphoma associated with CAEBV or acute EBV infection	100%	EBNA-1, LMP2, +/-LMP1	2		Mature CD4+ T cell	None or several months
Angioimmunoblastic T-cell lymphoma	>90%	EBNA-1, LMP1,2	2	Trisomy 3 or 5	Lymphoma in CD4 T cells; EBV in B cells, GC	>30 years
Pyothorax lymphoma	100%	EBNA-1, 2, +/-LMP1	3	p53	B cell, GC, or post-GC	>30 years
Extranodal NK/T nasal	100%	EBNA-1, LMP2, +/-LMP1	2		CD3–, CD56+	>30 years

EBV, Epstein-Barr virus; EBNA, Epstein-Barr virus nuclear antigen; RB2, retinoblastoma-like 2; GC, germinal center; MC, mixed cellularity; LD, lymphocyte depleted; NS, nodular sclerosis; LMP, latent membrane protein.

at 5 to 10 cases per 100,000 children and is the most common childhood tumor in these areas.³⁶⁵ Endemic BL usually presents as a tumor in the jaw. The B-cell proliferation and reduction in virus-specific cytotoxic T cells associated with malaria are thought to increase the EBV viral load and enhance the risk of chromosomal *c-myc* translocations present in BL cells.

Sporadic BL occurs in the United States and Europe at a rate of 0.2 to 0.3 cases per 100,000 persons and only 20% of tumors are EBV-positive. The disease is more common in South America and North Africa, with rates of 1 to 2 per 100,000 persons, where up to 85% of BL is EBV positive. Sporadic BL usually presents in children with abdominal tumors. About 40% of BLs in patients with AIDS are EBV positive. The disease occurs early in the course of HIV when immune function is relatively intact. HIV, like malaria, also induces B-cell proliferation and impairs EBV-specific CTL activity. Pathology of BL shows atypical B cells with basophilic, vacuolated cytoplasm and irregular nuclei with prominent nucleoli in a background of phagocytic histiocytes resulting in a “starry sky” pattern (Fig. 61.21). The lymphoid tumor cells often show mitotic figures and evidence of apoptosis.

BL cells shows dysregulation of the *c-myc* oncogene due to an 8:14, 8:2, or 8:22 chromosomal translocation in which a portion of the immunoglobulin heavy chain (on chromosome 14) or light chain (on chromosome 2 or 22) is fused to *c-myc* (on chromosome 8). Analysis of the structure of the chromosome breakpoints suggest that the *c-myc*/immunoglobulin gene translocations occur during the germinal center reaction either due to somatic hypermutation or to an error during class-

switch recombination.⁸¹⁴ Increased expression of *c-myc*, due to its proximity to the immunoglobulin enhancer sequence, affects expression of greater than 15% of genes in BL cells.⁸⁷⁹ *c-myc* reduces the immunogenicity of EBV-positive cells by inhibiting expression of TAPs, reducing expression of MHC class I, and inhibiting expression of proteasome subunits that are important for antigen presentation.¹³⁸⁸ However, impairment of proteasome function by *c-myc* may be compensated by the ability of *c-myc* to activate another pathway involving tripeptidylpeptidase II for proteolysis.⁴⁵⁰ *c-myc* can substitute for many of the growth-transforming functions of LMP1 and EBNA-2.¹¹⁷³ Expression of *c-myc* impairs NF- κ B and interferon responses in BL cells.¹³⁰⁴ Transgenic mice expressing *c-myc* driven by immunoglobulin light chain sequences develop tumors resembling BL.⁸⁰¹ Expression of *c-myc* is not compatible with expression of EBNA-2, which, if transcriptionally active, is functionally mutated.⁷³⁷ In addition, EBNA-2 down-regulates expression of *c-myc*.⁶⁹³ Therefore, high levels of *c-myc* expression limit EBV latency gene expression in BL.

Analysis of immunoglobulin gene rearrangements shows that EBV-positive endemic BLs and AIDS-related BLs have high IgH mutation rates with signs of antigen selection, while EBV-negative sporadic BLs have low mutation rates without signs of antigen selection.⁹³ This indicates that endemic and AIDS-associated EBV-positive BLs evolve from late germinal center B cells (or memory B cells), while sporadic EBV-negative BLs evolve from early centroblasts. Sequencing of immunoglobulin genes from endemic BL cells showed that multiple mutations evolve *in vivo*.¹⁹⁵ BL cells express cell surface markers present on germinal center cells with CD77 (BL antigen), CD10, *bcl-6*, CD38, and PAX5.¹⁴⁴⁴ Unlike lymphoblastoid cells, BL cells do not express adhesion molecules (e.g., LFA-1, ICAM1), B-cell activation antigens (e.g., CD23, CD70), co-stimulatory molecules (e.g., CD80, CD86), or *bcl-2*. Gene expression profiling confirms that BL cells have high-level expression of *c-myc*, expression of a subset of germinal center genes, and low-level expression of MHC class I genes.²⁸⁷ Low-level expression of MHC class I and TAPs in BL cells reduces recognition by class I-restricted CTLs; however, BL cells do express MHC class II.⁷⁵⁵

EBV endemic BL contains clonal viral genomes and usually has a type I EBV latency pattern in tissues and *in vitro*. The cells express EBNA-1 driven by the Q μ promoter and grow as single cells *in vitro*. EBNA-1 expression is critical for survival of BL cells; inhibition of EBNA-1 induces apoptosis and reduces survival of EBV-positive BL cells.⁷⁴² More sensitive assays indicate that some BL cells express LMP2.⁹¹ Some endemic BL cells express EBNA-1, EBNA-3A, EBNA-3B, EBNA-3C, a truncated EBNA-LP, and low-level LMP2, but not LMP1, and there is a deletion in EBNA-2.⁷³⁷ These grow as single cells *in vitro* and use the W μ promoter for expression of EBNAs. Rare BLs have been reported with cells containing different patterns of EBV latency that may enhance the ability of the tumor to escape apoptosis.⁷³⁹ Other BL cells, especially those resistant to apoptosis, express EBV BHRF1, a *bcl-2* homolog.⁷³⁸ Transcriptional profiling of type I EBV latency BL cell lines showed expression of lytic genes, especially BHRF1 and LF3 at high levels.⁸⁹⁵ While some EBV BL cells lose their EBV genome during passage *in vitro*,¹³³⁵ this has not been observed *in vivo*, indicating that the EBV genome has an important role in maintaining the tumor even with limited viral gene expression.

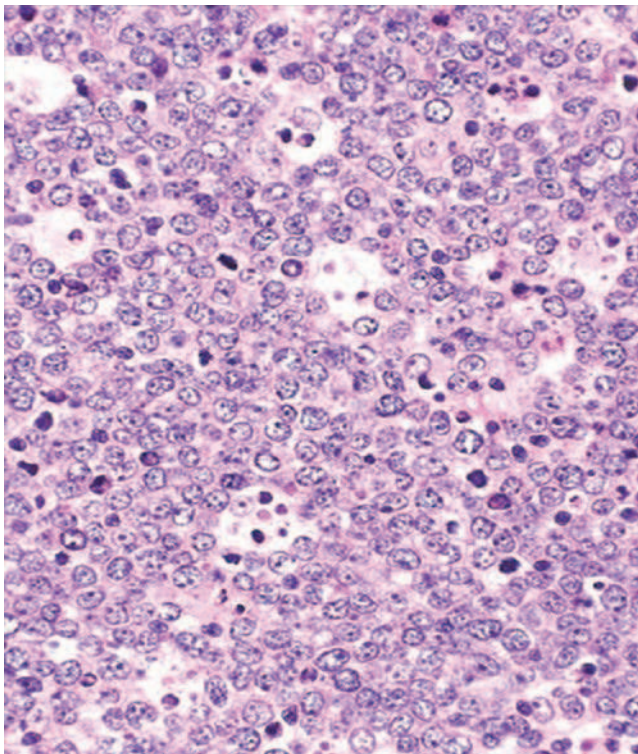


FIGURE 61.21. Biopsy from a patient with Burkitt lymphoma. The intensely stained B cells in a background of lightly stained histiocytes give a “starry sky” appearance. (Courtesy of Dr. Elaine Jaffe.)

In addition, BL cells that lost their EBV genomes *in vitro* had a less malignant phenotype with reduced tumorigenicity in mice. While some studies have postulated that the EBERs are primarily responsible for EBV's contribution to the malignant phenotype in some BL cells,⁷⁹⁵ this has not been observed in other BL cells.⁹⁸⁷

With increasing passage in cell culture, some BL cell lines switch from a latency 1 to a latency 3 pattern of EBV expression and the EBNAs are driven by the Cp or Wp promoters.^{1249,1272} These cells grow in clumps and express adhesion molecules (ICAM1, LFA3), B-cell activation markers (CD23, CD70), co-stimulatory molecules (e.g., CD80, CD86), and bcl-2. These latter cells express lower levels of c-myc. Cells that use the EBV Wp promoter require EBV to inhibit apoptosis induced by Bim and to proliferate efficiently.¹⁴⁹⁴

EBV-positive endemic BL has fewer secondary genomic alterations than EBV-negative sporadic BLs.⁷⁸ However, EBV infection of virus-negative BL cells results in chromosome instability; while this effect is seen in BL cells with EBV latency 1 gene expression, it is more prominent in BL cells with latency 3 gene expression.⁷⁰⁵

Children in Africa with increased antibody titers to EBV VCA are at increased risk of BL.³⁰³ Patients with endemic BL often have elevated antibody titers to EBV VCA as well as EA-R. Elevated serum antibody titers to EBV lytic and latent proteins were observed in children in areas where malaria was holoendemic and the levels remained elevated, while antibody titers were lower and declined in persons from areas where malaria was sporadic.¹¹⁶⁶ Children with BL in Malawi had higher levels of antibodies to EBV and malaria and were more likely to be HIV positive.¹⁰⁷¹ Children in Gambia with malaria had higher EBV viral loads than those without malaria and had evidence of impaired EBV-specific CD8 T cells during onset of malaria.¹⁰⁹⁹ Children with BL in Africa where malaria is holoendemic were less likely to have EBNA-1-specific IFN- γ responses compared with healthy children in the same area, while CD8 T-cell responses to other latent and lytic antigens were similar in children with endemic BL and controls.¹⁰⁴⁸ Children aged 5 to 9 years old who lived in an area holoendemic for malaria had lower EBV-specific IFN- γ responses than those in a non-holoendemic area; this coincided with the peak age incidence of endemic BL in this area.¹⁰⁴⁷ This effect was not seen in children younger than 5 or older than 9 years old. Children living in areas of Africa where malaria is holoendemic had higher EBV DNA loads than those in areas where the disease was sporadic, and viral loads were highest in very young children.¹⁰⁴⁶ Children in South America, where Burkitt lymphoma is sporadic, had high levels of EBV DNA in the blood at the time of diagnosis.⁹⁵¹ Antimalarial therapy resulted in a decline in the EBV viral load in the blood 2 weeks after treatment, but not in the saliva.³²³ A domain in the *P. falciparum* membrane protein 1 is a polyclonal B-cell activator, and incubation of this protein domain with PBMCs increases the EBV DNA copy number.²¹⁴ This suggests that chronic exposure to malaria may promote B-cell proliferation and BL.

Hodgkin Lymphoma

Classical Hodgkin lymphoma (HL) consists of four histologic subtypes: in the United States and Europe 75% to 80% of classical HL is the nodular sclerosis subtype, 15% to 20% is the mixed cellularity subtype, 5% is the lymphocyte-rich subtype,

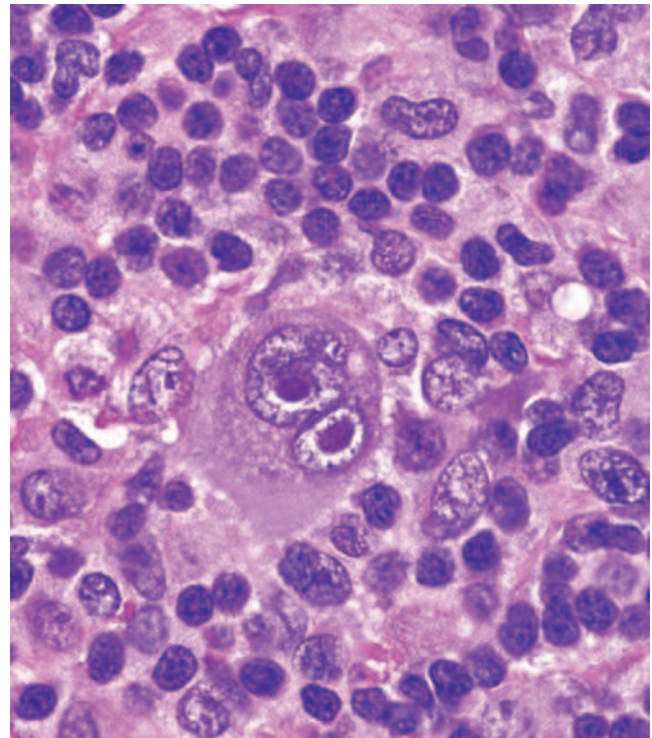


FIGURE 61.22. Biopsy from a patient with Hodgkin lymphoma, mixed cellularity. The large binucleate cell in the center is a Reed-Sternberg cell. (Courtesy of Dr. Elaine Jaffe.)

and less than 1% is lymphocyte depleted. The frequency of EBV positivity is 80% to 90% for lymphocyte depleted, 60% to 75% for mixed cellularity, 20% to 40% for nodular sclerosis, and less than 10% for lymphocyte rich. EBV proteins and clonal viral DNA are present in Reed-Sternberg cells (large multinucleated cells of B-cell lineage) in about 40% of cases in the United States and Europe. Reed-Sternberg cells have clonal immunoglobulin gene rearrangements and express B-cell activation antigens CD25 and CD70, although they usually do not express CD19 or CD20. Patients present with lymphadenopathy and often have involvement of the bone marrow, liver, and spleen. Pathology shows rare (less than 1%) Reed-Sternberg cells in a background of lymphocytes, granulocytes, histiocytes, and plasma cells (Fig. 61.22).

EBV-positive HL is more common than EBV-negative disease in patients in developing countries including Central and South America (80% to 90% EBV-positive) compared with affluent countries (30% to 40% EBV-positive) in some⁴⁷¹ but not all studies.¹²¹⁰ HL is more commonly EBV positive in children and older adults than in young adults.⁴⁸ HL is nearly always EBV positive in immunosuppressed patients.

Elevations in IgG antibody to EBV VCA, EBNA-2, and EA-D are present at a mean of 4 years before diagnosis of HL.¹⁰⁶⁴ Increases in each of these antibodies carries a three- to fourfold relative risk of subsequent development of HL. Patients with EBV-positive HL have detectable EBV DNA in the plasma before therapy.⁴⁴⁵ EBV DNA in the plasma declines in response to therapy and increases before disease recurs.¹³⁷⁸ The level of EBV is higher in the blood of persons with EBV-positive HL

compared with EBV-negative disease⁷⁵² and higher in patients with advanced-stage disease.⁵⁹⁹

The risk of EBV-positive HL is higher within 1 year in persons with IM and decreases to normal after 10 years.⁵⁹² There is a 2.6–⁵⁹³ to 3.4-fold^{594,595} increased risk of HL after IM. The risk is fourfold in younger adults. In these studies, HL occurred a median of 2.9 years after IM. In contrast, there was no increase in other lymphoid tumors after EBV IM,⁵⁹³ including EBV-negative HL or non-Hodgkin lymphoma.^{100,595}

Sequencing of immunoglobulin genes from HL indicates that the tumor cells have mutated immunoglobulin genes and are derived from crippled germinal center B cells.⁷¹¹ Of the reported cases of HL with crippling immunoglobulin gene mutations, about 90% of EBV-positive HLs had these mutations, while only about 40% of EBV-negative HLs had these mutations.¹²⁷ These immunoglobulin gene mutations, which result in loss of functional immunoglobulin, would be expected to be lethal for the cells; however, the presence of EBV LMP1 and LMP2A, which inhibit apoptosis by stimulating NF- κ B¹⁴⁸ and providing a B-cell growth stimulus mimicking the Ig receptor,¹⁵⁶ respectively, may allow these cells to survive. In this way EBV gene expression may substitute for host cell gene mutations in I κ B α or amplification of *REL* that also activates NF- κ B in EBV-negative HL.⁸¹⁵ EBV-infected B cells lacking surface immunoglobulin (and therefore B-cell receptor deficient) with or without inactivating mutations in IgH sequences have also been generated *in vitro* by infecting germinal center cells from tonsils with EBV.^{85,188} Therefore, EBV can rescue B-cell-receptor-deficient germinal center B cells from apoptosis so that they no longer are dependent on survival signals that uninfected B cells require.

Biopsies from EBV-positive HL have type 2 EBV latency with expression of LMP1, LMP2A, and LMP2B; EBNA-1 driven by the Qp promoter; EBERs; and BARTs.²⁹⁶ Expression of LMP1 in germinal center B cells results in reduced expression of genes that are commonly down-regulated in HL such as CD79A, CD19, CD20, and CD22.¹⁵⁰⁰ EBNA-1 down-regulates expression of Smad2, which in turn down-regulates expression of the protein tyrosine phosphatase receptor κ , which enhances proliferation and viability of HL cells.³⁹⁸ LMP1 up-regulates Bmi-1 in HL cells, which may increase survival of these cells.³³⁷ EBV infection of HL cells induces expression of autotaxin, which increases lysophosphatidic acid and increases growth and survival of HL cells.⁸³ Autotaxin is also increased in EBV-positive HL tissue. EBV-positive HL shows higher levels of CCL20, which can induce trafficking of regulatory T cells into the tumors.⁸²

Polymorphisms in the HLA class I region associate with EBV-positive HL, while those in HLA class III correlate with EBV-negative disease.³¹² This suggests that presentation of EBV peptides to CTLs is related to the pathogenesis of HL and that ethnic variations in the disease may be related to HLA sequences. Further studies showed that patients with an HLA-A02 allele have a reduced risk of developing EBV-positive HL; conversely, those with an HLA-A*01 allele have an increased risk.^{594,1091} A polymorphism in the IL-10 promoter was more common in persons with EBV-positive HL than in EBV-negative disease.²⁷⁶ Biopsies from patients with EBV-positive, but not EBV-negative, classical HL show down-regulation of miR-96, miR-128a, and miR-128b.¹⁰⁷⁷

EBV-specific T-cell function is impaired in HL. Galectin-1, an immunomodulatory protein, was expressed in the majority of

classical HL tissues examined (and in Reed-Sternberg cell lines from HL) and expression was associated with reduced LMP1- and LMP2-specific CD8 T-cell responses and reduced CD8 T-cell infiltrates in tumor tissues.⁴⁴⁷ CD8 T cells specific for LMP1 and LMP2, but not EBNA or lytic proteins, from patients with HL have impaired function. Galectin-1 reduces degranulation and recognition of epitopes by LMP-specific T cells.¹³⁶⁴ Regulatory T cells are increased in the blood of patients with HL with active disease, compared with those in remission.⁴⁴⁶ Expression of LAG-3, which is a marker of regulatory T cells, is increased in infiltrating lymphocytes in EBV-positive HL tissue and is associated with impaired EBV LMP1- and LMP2-specific CD8 T-cell responses. Many patients with EBV-associated HL lack detectable CD4-specific T-cell responses to EBNA-1.⁵⁴⁹

EBV positivity in HL improves prognosis in children younger than 15 years of age but has a poorer prognosis in adults 45 years or older.⁷³⁵ Overall survival is higher in patients with EBV-positive HL than EBV-negative disease, independent of gender, stage of disease, and B symptoms.⁶⁷⁹

Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is classified as keratinizing or nonkeratinizing with differentiated or anaplastic subtypes. In the United States, 63% of NPC is anaplastic, 25% keratinizing, and 12% differentiated, while in Southern China 95% of NPC is anaplastic. Anaplastic NPC is more prevalent in certain areas including Southeast Asia (especially Southern China), Northern Africa, and Eskimos in the Arctic.³⁰² In Southern China the rate of disease is 50 per 100,000 in men over 50 years old. The disease has a two- to threefold higher rate in men than women. The incidence of disease peaks at ages 50 to 59, with a smaller peak in adolescents and young adults. The rates are also elevated in Chinese persons who migrate from China. Environmental factors, especially dietary habits (use of salted fish and preserved foods), as well as genetic factors, may play a role in development of the disease. The rate of NPC has been declining in developed areas of Southeast Asia, perhaps due to changes in dietary habits. NPC occurs less commonly in the United States and Europe, with rates of 0.5 to 2 per 100,000.

Genome-wide association studies have identified multiple NPC-associated genes including γ -aminobutyric acid type B receptor 1, TNFRSF19, integrin- α 9, and loci within HLA-A.^{87,1474} A polymorphism in TLR3 was also associated with a higher risk of NPC.⁵⁴³

Virtually all anaplastic NPC is EBV positive. EBV is also present, although at a lower frequency, in more differentiated (keratinizing) forms of NPC, especially in areas where anaplastic NPC is endemic. EBV is clonal in high-grade preinvasive lesions (carcinoma *in situ* or dysplasia) as well as in NPC,¹¹⁴² but not in low-grade preinvasive lesions. Thus, the virus likely infects epithelial cells that already have a genetic abnormality. Pathology of anaplastic NPC shows uniform malignant epithelial cells with prominent nuclei in a dense background of lymphocytes (Fig. 61.23). EBV DNA is present in the transformed epithelial cells but not in the infiltrating lymphocytes. The tumors are sometimes referred to as lymphoepitheliomas. However, in contrast to many other EBV malignancies, NPC tumors have normal antigen processing and are efficiently recognized by virus-specific CTLs.⁷⁵⁶

NPC is believed to be a multistep process initially with overexpression of *p53* and loss of expression of the tumor

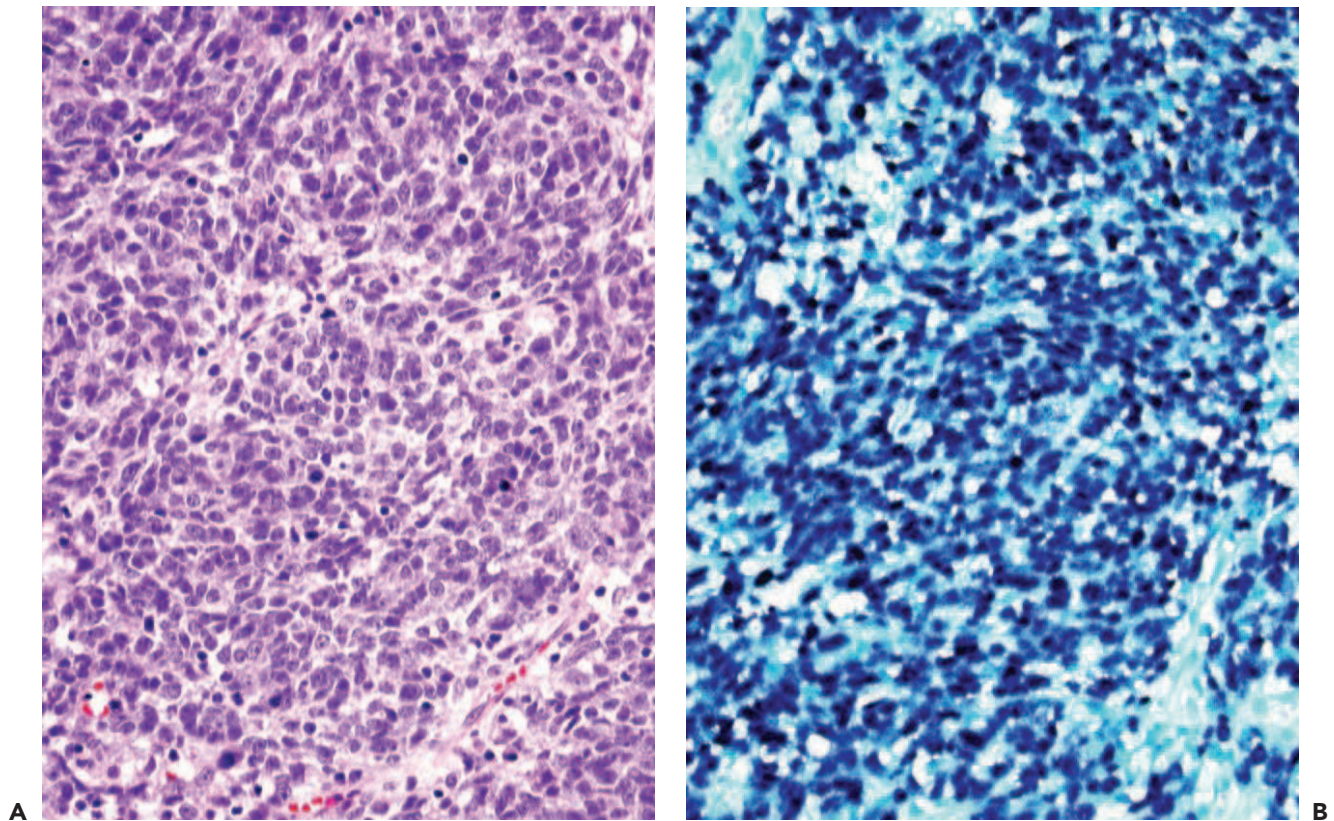


FIGURE 61.23. Biopsy from a patient with nasopharyngeal carcinoma. A: Undifferentiated anaplastic carcinoma. **B:** *In situ* hybridization shows Epstein-Barr virus (EBV)-encoded RNA (EBER)-positive carcinoma cells. (Courtesy of Dr. Paul A. VanderLaan.)

suppressor genes *p16* and *p27* (and in some cases increased *p21Ras*) resulting in a hyperplastic lesion. This is followed by EBV infection and increased expression of *bcl-2* resulting in a dysplastic lesion, and then overexpression of *p53* and invasive NPC (Fig. 61.24).³⁷⁷ A large number of tumor suppressor genes are hypermethylated resulting in reduced expression in NPC including *TFPI-2*,¹⁵²⁹ *DLEC1*, *BLU*, and *E-cadherin*.⁵⁸ Twist and Snail, which are repressors of E-cadherin and regulators of epithelial–mesenchymal transition, are induced by LMP1 and correlate with the metastatic behavior of NPC.⁶⁰⁸

The risk for NPC is much higher (hazard ratio 6.8) for family members (when one member has the disease) compared with others in the same community.⁶²² The risk of NPC is higher for those with elevated anti-EBV DNAase antibody or anti-EBV VCA IgA (hazard ratio 2.8) and even higher when both antibodies are elevated (adjusted hazard ratio 15.1). Persons with a family history of NPC and elevated EBV serologies had a higher risk of NPC (hazard ratio was 31) than those without a family history and negative serology for DNAase and VCA IgA.⁴²⁶ A prospective study found a 21-fold increased risk of NPC in persons with rising titers of IgA antibody to EBV VCA.¹⁷¹ Elevated levels of anti-EBV EBNA1 IgA were associated with a fivefold risk of NPC.¹⁶¹⁰

Patients with NPC usually have high titers of antibody to EBV VCA, EBNA, and EA-D IgA; unlike other EBV tumors, elevated antibodies to EBV occur in NPC regardless of the geographical location. IgA to EBV VCA or EBV DNAase in serum

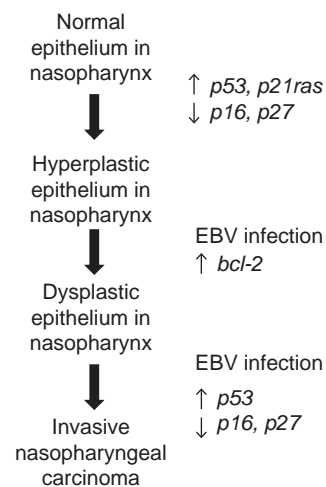


FIGURE 61.24. Steps involved in the development of nasopharyngeal carcinoma. Epstein-Barr virus (EBV) infection occurs after the cells have undergone initial genetic changes and have a hyperplastic or dysplastic phenotype. (Adapted from Fan SQ, Ma J, Zhou J, et al. Differential expression of Epstein-Barr virus-encoded RNA and several tumor-related genes in various types of nasopharyngeal epithelial lesions and nasopharyngeal carcinoma using tissue microarray analysis. *Hum Pathol* 2006;37[5]:593–605.)

or saliva has been used for screening in endemic areas.²²⁵ Persons with a fourfold increase in IgA VCA have an 18% risk of developing NPC within 3 years in China; some patients have loss of antibody and loss of risk. EBV IgA antibody levels are elevated up to 10 years before the diagnosis of NPC with a mean duration of 37 months before diagnosis.⁶⁸⁵

NPC patients often have elevated levels of EBV DNA in the plasma.⁸⁹³ The sensitivity of EBV DNA, VCA IgA antibody, and combined EBV DNA and VCA IgA for diagnosis of NPC was 95%, 81%, and 99%, respectively.⁸⁶¹ The specificity of EBV DNA and VCA IgA was 98% and 96%, respectively. Higher EBV plasma levels at the time of diagnosis and rising levels of EBV VCA or EA IgA antibody, or EBV DNA after therapy is associated with a poorer prognosis. After therapy the serum level of EBV DNA and IL-6 often declines.^{31,1424} Another noninvasive test for NPC involves brushing suspicious areas of the nasopharynx and performing polymerase chain reaction (PCR) for EBV DNA and detecting EBV DNA and RNA.¹³⁹² High levels of EBV DNA in brushings had a specificity of 98% and sensitivity of 90% for NPC. EBNA-1 and BARF1 mRNA were found in 86% and 74% of brushings, respectively, from NPC-positive patients.

Patients with NPC have lower levels of EBNA-1-specific CD8 T cells⁴⁰⁷ and lower levels of LMP1- and LMP2-specific T cells⁸⁷² than controls. Patients with NPC have lower levels of naïve CD3+CD45RA+ and CD4+CD25- cells in the blood and higher levels of activated CD4+CD25+ T cells and CD3-CD16+ NK cells than controls. EBV-specific CTL activity could be reactivated in peripheral blood mononuclear cells by incubation with autologous lymphoblastoid cell lines, while tumor-infiltrating lymphocytes from NPC biopsies lacked cytotoxic activity and did not produce IFN- γ when incubated with autologous lymphoblastoid cell lines. NPC tumors have high levels of regulatory T cells (CD4+CD25^{hi}, Foxp3).⁸³³ These cells may inhibit immune surveillance of the tumors.

NPC usually has type 2 EBV latency with EBNA-1 driven by the Qp promoter, expression of EBER and BARTs, and variable expression of LMP1; in many cases LMP1 is undetectable.⁹¹ In contrast, LMP1 is routinely expressed in preinvasive lesions.¹¹⁴² LMP2A is usually expressed in NPC biopsies, and its expression may be important for NPC, because LMP2A expression in epithelial cells increases expression of integrin- $\alpha 6$ proteins, which increases their migration and invasiveness.¹¹⁴⁷ In addition, LMP2A activates PI3K and Akt, resulting in activation of β -catenin and inhibition of differentiation of epithelial cells.¹⁰⁵⁷ Higher mutation rates in the sequence of LMP2 were seen in NPC tumors than in isolates from the oropharynx of healthy persons.¹⁵³² While BARF1¹³⁹² and BZLF1 were present in some tumor cells,²⁴¹ other lytic proteins were generally not expressed. microRNAs from BART, but not BHRF1, are expressed in NPC.^{264,914,1634} BART miRNAs interact with the 3' nonterminal region (NTR) of LMP1 and reduce its expression. This may explain the variable expression of LMP1 protein in NPC despite frequent detection of LMP1 transcripts.⁹¹⁴ BART miRNAs may also inhibit apoptosis.⁹⁷⁷ miR-218 suppresses NPC progression by down-regulating survivin.¹²

NPC cells release exosomes that contain galectin-9, which can induce apoptosis of EBV-specific CD4 T cells; galectin-9 is also present in exosomes from plasma of patients with NPC.⁷⁸⁵ Exosomes from NPC cells also contain LMP1, viral miRNAs, and signal transduction molecules, which activate signaling

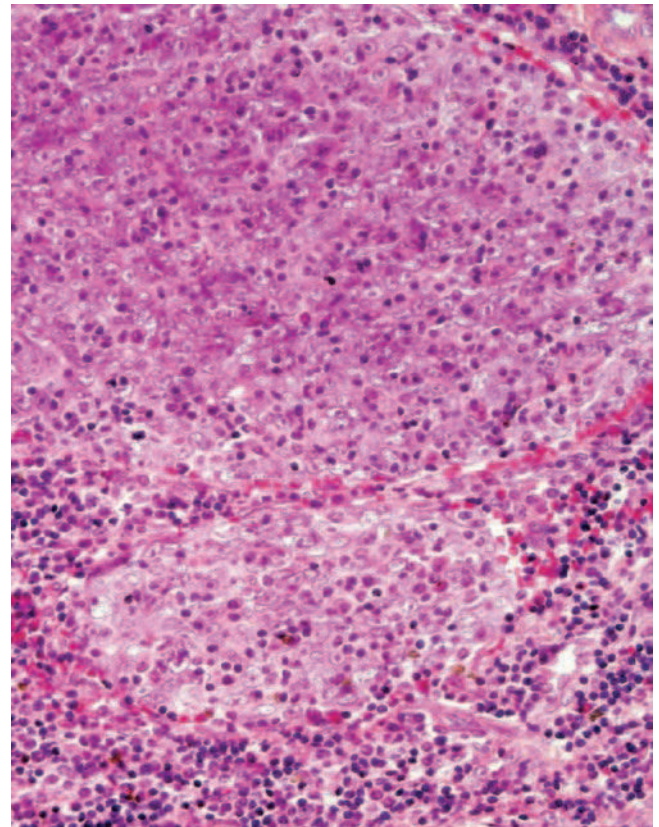


FIGURE 61.25. Biopsy from a patient with lymphoepithelioma involving tonsil. The biopsy shows nodular infiltrates of undifferentiated tumor cells with macronucleoli. Lymphocytes infiltrate the tumor nodules. The tumor cells were positive for Epstein-Barr virus (EBV) by *in situ* hybridization. (Courtesy of David Kleiner.)

pathways in recipient cells.¹⁰⁰⁶ Lesions from patients with NPC often show down-regulation of TAP1, tapasin, and MHC class I, and up-regulation of IL-10.¹¹¹⁴

EBV is also present in other epithelial cell tumors (known as lymphoepitheliomas or undifferentiated carcinomas of the nasopharyngeal type) including the tonsils (Fig. 61.25), salivary glands, thymus, larynx, lungs, skin, and cervix. Like NPC, these tumors are more common in certain areas of the world, especially Southeast Asia and Eskimos in the Arctic. Also similar to NPC, pathology shows malignant EBV-positive epithelial cells with a dense infiltrate of lymphocytes. When a family member was diagnosed with NPC, the risk to other members for carcinoma of the salivary gland was 8.4 and for carcinoma of the uterine cervix was 2.2.⁴²⁶

Gastric Carcinoma

EBV is present in about 9% to 10% of gastric carcinomas including over 90% of gastric lymphoepithelioma-like carcinomas, 7% of moderately to well-differentiated gastric adenocarcinomas, and 6% of poorly differentiated gastric adenocarcinomas.^{434,652} Worldwide, gastric carcinomas may be the most common EBV-associated malignancy. The prevalence of EBV-positive gastric carcinoma is similar in the United States, Europe, and Asia.¹⁰⁶⁸ Lymphoepithelioma-like carcinomas have

a similar pathology to NPC with EBV DNA in malignant epithelial cells and a dense infiltrate of EBV-negative lymphocytes and macrophages surrounding the tumor cells.¹³³¹ EBV-positive gastric carcinomas often involve the upper third of the stomach. Meta-analyses found that the prevalence of EBV positivity in gastric carcinoma was twofold higher in men than in women, more common in younger persons, more frequent in the gastric cardia or corpus than the antrum, and more common in post-surgical gastric remnants.^{846,1068}

Gastric carcinoma cells are clonal or oligoclonal for EBV DNA. EBV-positive gastric carcinomas have an EBV type 1 latency pattern, with EBNA-1 driven by the Qp promoter, expression of EBERs and BARTs, and variable expression of LMP2A.^{434,1405} EBNA-1 expression on gastric carcinoma cells is associated with loss of PML bodies, impaired response to DNA damage, and reduced apoptosis.¹³⁵²

BARF1 is also expressed in the tumors, in the absence of other lytic genes.¹³²¹ Expression of p16, p27, E-cadherin, and p73 is reduced in EBV-positive gastric cancer, while expression of cyclin D1 and NF- κ B are increased compared with EBV-negative cancer.^{86,193} p53 and bcl-2 are overexpressed in some but not all EBV-positive gastric cancer. The tumor cells do not express CD21, the B-cell EBV receptor. DNA methylation of CpG sequences in the promoters of tumor suppressor genes such as *PTEN* is induced by LMP2 by up-regulation of *de novo* methyltransferase 1.⁴³⁴ MHC class I is down-regulated in EBV-positive gastric carcinoma cells.³³⁶ Like NPC, BART miRNAs, but not BHRF1 miRNAs, are expressed in tissues from patients with EBV-positive gastric carcinoma.⁷⁶³

EBV was not detected in preneoplastic (dysplasia and intestinal metaplasia) lesions in patients with EBV-positive gastric carcinomas, but only in the carcinomas.¹⁶⁴³ This suggests that like NPC, EBV infection is a late event in the development of gastric carcinoma. EBV gastric carcinoma is associated with atrophic gastritis and lymphocytic infiltration.

Like NPC, levels of anti-EBV VCA IgA are higher in persons who subsequently develop EBV-positive gastric carcinoma than controls.⁸⁶⁴ Levels of anti-EBV VCA IgG and anti-EA IgG antibodies are higher in patients with EBV-positive gastric carcinoma than those with EBV-negative cancer.⁶⁵² EBV anti-VCA and anti-EBNA antibodies titers are higher in persons with dysplasia on gastric biopsy, suggesting that EBV reactivation could be related to an early phase of gastric carcinoma.¹³⁰²

EBV-positive gastric carcinomas with a lymphoepithelial-like histology and large numbers of tumor-infiltrating lymphocytes generally have a better prognosis than EBV-positive carcinomas with an adenocarcinoma histology and a patchy lymphocytic infiltrate.¹³⁷² EBV-positive gastric carcinomas are less likely to spread to lymph nodes than EBV-negative tumors.

Non-Hodgkin Lymphomas and Other Malignancies in Nonimmunocompromised Persons

EBV is present in about 6% of cases of non-Hodgkin lymphoma (NHL)⁵²⁰ in Europe. This includes EBV-positive diffuse large B-cell lymphoma in the elderly, diffuse large B-cell lymphoma, peripheral T-cell lymphoma, angioimmunoblastic T-cell lymphoma, lymphomatoid granulomatosis, and pyothorax-associated lymphoma. In Asia, EBV is associated with NK/T-cell lymphoma and aggressive NK-cell leukemia/lymphoma.

miRNA expression is altered in EBV-positive versus EBV negative NHL.⁶⁵⁶

EBV positive diffuse large B-cell lymphoma of the elderly usually presents in persons older than age 60 with fever and weight loss. The disease is often extranodal and can involve the skin, stomach, and lung. Disease is thought to be due to senescence of EBV-specific T-cell responses and the proportion of EBV-positive cases increases with age.¹¹²⁵ Tumor cells are monoclonal and EBER positive, express LMP1, and have variable expression of EBNA-2. The disease has a poorer prognosis than diffuse large B-cell lymphoma.¹⁵⁵¹ About one-third of cases have an HL-like morphology with Reed-Sternberg-like cells. The disease has a poorer prognosis than classical HL in elderly persons.^{52,1336}

EBV-positive mucocutaneous ulcers are also more common in the elderly and they respond to reduction in immunosuppression or may spontaneously regress.³¹⁷

Diffuse large B-cell lymphomas are EBV positive in 10% of nonimmunosuppressed persons; the rate of EBV positivity is higher in immunocompromised persons. EBV-positive diffuse large B-cell lymphomas have a poorer prognosis than EBV-negative lymphomas and are associated with increased age, extranodal disease, fever, and a poorer response to therapy.¹¹³⁵ About 40% of CD30+ anaplastic large B-cell lymphomas are EBV positive and express LMP1 with variable expression of EBNA-2.⁸¹⁹ About 15% of large B-cell lymphomas in the oral cavity are EBV positive,¹³⁷⁰ while approximately 20% of T-cell-rich B-cell NHL is EBV positive.⁵⁷

Peripheral T-cell lymphomas usually present with fever and lymphadenopathy and are often associated with HLH.⁷⁰⁶ The disease is more common in Asians and persons from South America. Macrophage activation is often present with elevated serum levels of TNF- α , IFN- γ , and other cytokines.⁸³⁹ Elevated levels of TNF- α in the serum are associated with a poorer prognosis.¹⁰⁵⁴ Patients present either during primary EBV infection or during the course of chronic active EBV.^{698,1197} T-cell lymphomas have clonal EBV DNA and usually have type 2 EBV latency, although expression of LMP1 can be variable.²⁰⁴ The lymphoma can involve α/β or γ/δ T cells.

Angioimmunoblastic T-cell lymphoma is a peripheral T-cell lymphoma that presents with fever, night sweats, weight loss, hepatosplenomegaly, lymphadenopathy, and hypergammaglobulinemia. The malignant T cells are usually clonal and patients may develop EBV-positive diffuse large B-cell lymphoma. Autoantibodies including immune complexes, cold agglutinins, rheumatoid factor, and anti-smooth muscle antibodies may be present. EBV is present in over 90% of cases and the virus is present in the proliferating B cells that accompany the malignant T cells.¹⁵³⁷ EBV-infected B cells can be polyclonal, oligoclonal, or clonal and are deficient in immunoglobulin receptor.¹²⁹ EBV DNA is elevated in the serum and parallels the clinical course.⁵⁵

Lymphomatoid granulomatosis usually presents with fever and masses in the lungs but also can involve the kidneys, skin, liver, and central nervous system. Many patients with lymphomatoid granulomatosis have reduced numbers of CD8 and CD4 T cells; some patients have HIV or XLP1 or have received organ transplants.⁶⁷⁴ Pathology shows EBV in rare B cells, an exuberant T-cell response, and destruction of small blood vessels. Separate clonal lesions can arise at different sites. Lesions express EBV EBNA-2 and LMP1,¹⁴²⁶ and EBV DNA levels in the blood are usually not elevated. IFN- α is often effective in early-stage disease, while late-stage disease requires cytotoxic chemotherapy.

Pyothorax-associated lymphoma is an EBV-positive diffuse large B-cell lymphoma that originates in the pleural cavity after a history of pyothorax. Tumors usually express EBNA-1, EBNA-2, and/or LMP1.⁴⁰ The disease is probably due to chronic inflammation, persistent cytokine production, and reduced host immunosurveillance. Similar localized EBV-positive lymphomas, termed *diffuse large B-cell lymphoma associated with chronic inflammation*, can occur at sites of chronic osteomyelitis, cardiac myxomas, splenic cysts, and skin ulcers and near metallic prostheses.⁹²⁶

Extranodal NK/T-cell lymphoma—nasal type, previously referred to as midline granuloma, usually presents in the nose but can also involve the sinuses and palate. Other portions of the upper airway may be involved; much less commonly the gastrointestinal tract and skin are infected.^{672,673} Pathology shows invasion of blood vessels with necrosis. Most tumor cells are CD56+ and surface CD3– and express granzyme and perforin; however, in some cases cells are CD56–, CD3+. These tumors are more common in Southeast Asia and Central and South America, especially in men. The disease may be associated with HLH. The tumors are nearly always EBV positive, are clonal for EBV, and have type 2 EBV latency with variable expression of LMP1.^{224,414,1575} EBV LMP1 expression is associated with up-regulation of integrin α M, which may be important for angiogenesis of these tumors.⁹⁰⁷ Elevated levels of interferon-induced cytokines MIG and IP10 are present in areas of tissue necrosis.¹⁴⁴⁰ Tumor cells often show NF- κ B and Akt activation⁶²⁶ and express TNF- α with dysregulation of STATs and Rel A. Some patients have disease involving the peripheral blood, termed NK-cell leukemia, and the malignant cells are EBV positive. High levels of EBV DNA in the blood correlate with a poorer prognosis.^{620,1410} Treatment involves chemotherapy and radiation therapy; early-stage disease is usually curable, while disseminated disease has a poor prognosis.⁵⁴

Aggressive NK-cell leukemia/lymphoma presents with fever, night sweats, weight loss, pancytopenia, hepatosplenomegaly, and large granular lymphocytes in the blood and bone marrow. About 50% of cases are EBV positive and the disease is more common in Asians; some patients initially present with CAEBV. The level of EBV DNA in the plasma is elevated at onset of disease and parallels the course of the disease.⁵⁵

Inflammatory pseudotumor-like follicular dendritic cell sarcoma presents with fever and usually involves the spleen and liver.²¹⁸ The tumors contain clonal EBV DNA with a type 2 pattern of EBV latency.

Breast cancer has been associated with EBV based on earlier reports using PCR and antibodies to EBV proteins that detected EBV in a small fraction of the tumor cells; however, more recent studies using *in situ* hybridization and immunohistochemistry have generally not supported an association of EBV with the disease.^{301,1069}

EBV and HIV

Patients with late-stage HIV often have higher levels of EBV-specific antibodies and lower levels of virus-specific CTLs, shed higher levels of virus from the throat, and have 10-fold higher levels of EBV in the peripheral blood than healthy persons.¹¹¹ In contrast to transplant recipients, the level of EBV in the blood alone is not predictive of EBV-associated lymphoma and may not correlate with the CD4 count in patients with HIV.¹¹⁶⁹ The level of EBV DNA and the BZLF1-specific T-cell response

in the blood may rise after acute HIV infection and reach a new EBV DNA set point. EBV viral loads rise in HIV patients during the late stage of AIDS. The CD4 T-cell response to EBNA-1 and BZLF1 was lower in untreated patients with HIV than in healthy persons or in those on highly active antiretroviral therapy (HAART).¹¹⁷⁰ The EBV viral load of children with untreated HIV is often similar to that seen in healthy children with acute EBV infection.⁸⁹ EBV is predominantly in B cells, and EBV transcripts for LMP2, but not EBNA-1, LMP1, or lytic genes, are present in the cells.¹³⁹¹

In patients begun on HAART, the EBV DNA load did not change, and there was a decline in BZLF1-specific CD4 and CD8 T cells suggestive of less EBV reactivation. In contrast, there was an increase in EBNA-1-specific CD4 and CD8 T cells to levels similar to those seen in healthy persons.¹¹⁶⁵

A longitudinal study of EBV DNA loads and cellular immunity in HIV patients showed that patients who did not develop NHL had stable levels of EBV DNA and EBV-specific CTL precursors, while patients who developed NHL had increasing EBV loads and decreasing EBV-specific CTLs before the onset of lymphoma.⁷⁴⁹ In another study, HIV patients who developed NHL had increasing EBV DNA levels in the blood, and although they did not have a decline in the number of EBV-specific T cells, these cells lost their ability to produce IFN- γ in response to EBV peptides.¹⁴⁸⁶ These studies indicate that loss of immunity to EBV and rising viral loads correlate with development of lymphomas. Primary infection with EBV does not alter the HIV viral load or progression to AIDS.¹¹⁴⁵

The overall rate of EBV-positive lymphomas has declined with HAART. Lymphomas that occur early in the course of HIV infection—BL and HL—have not declined in incidence with HAART, while lymphomas that occur later when patients are very immunocompromised—immunoblastic and primary CNS lymphomas—have decreased in incidence.¹⁶⁷

Oral hairy leukoplakia is a nonmalignant disease that occurs in patients with late-stage AIDS and in some transplant recipients.^{488,1471} The disease presents with white corrugated “hairy” lesions on the lateral aspect of the tongue or occasionally on the palate or buccal mucosa and is due to lytic EBV replication in the outer layer of epithelial cells, but not the basal layers. Pathology shows hyperkeratosis of the squamous epithelium with acanthosis and intranuclear inclusions, but no inflammatory cells. Biopsies show linear (replicating) EBV DNA and herpesvirus particles on EM. EBV lytic antigens BZLF1, EA, and VCA, as well as EBNA-1, EBNA-2, and LMP1 are expressed in epithelial cells.^{1509,1536} Multiple EBV strains have been detected in single lesions with EBV variants due to recombination in and adjacent to the EBNA-2 gene between viruses.¹⁵⁰⁷ The disease responds to acyclovir, which inhibits EBV replication; however, relapses are common when acyclovir is stopped; HAART can resolve lesions.

Lymphoid interstitial pneumonitis is characterized by diffuse interstitial pulmonary infiltrates. The alveoli are infiltrated with EBV-positive B cells, immunoblasts, and plasma cells.³⁹³ The disease is more common in children with HIV, although it can occur in adults with autoimmune disease.

Burkitt lymphomas account for about 35% of cases of AIDS-associated lymphoma (Table 61.4). About 30% of BLs in HIV are EBV positive. The disease occurs in patients with HIV usually before they develop severe immunodeficiency. These

tumors usually have a type 1 EBV latency pattern, or less commonly a type 2 pattern, and have c-myc translocations.

Hodgkin lymphoma occurs early in the course of HIV, and like patients without HIV, EBV is more often present in tumors of the lymphocyte-depleted or mixed cellularity subtypes.

Non-Hodgkin lymphomas in patients with HIV are EBV positive in 50% of cases and are clonal for the viral genome.^{173,174,1332} EBV was detected more often in benign lymph nodes of HIV patients who subsequently or concurrently had NHL than in those without lymphoma.¹³³³ Patients with HIV progressing to NHL had loss of EBV-specific CD8 T and CD4 T cells, including EBNA-1-specific CD4 and CD8 T-cell responses.¹¹⁶⁷

Diffuse large B-cell lymphomas include centroblastic and immunoblastic lymphomas. Centroblastic lymphoma involves the lymph nodes and accounts for 25% to 30% of HIV lymphomas, and 30% of the tumors are EBV positive. Immunoblastic lymphomas account for 10% of HIV lymphomas, 90% are EBV positive, and the tumors are extranodal. Diffuse large B-cell lymphomas often have type 2 or type 3 EBV latency, although variable expression of latent EBV genes has been reported.^{521,522,1211}

CNS lymphomas in patients with AIDS are nearly always EBV positive, are immunoblastic lymphomas with type 3 EBV latency, and occur late in the course of AIDS when CD4 T-cell counts are low⁹⁵⁵ (Fig. 61.26). The cerebrospinal fluid is usually EBV positive. While studies before the era of HAART found that detection of EBV in the cerebrospinal fluid of patients with mass lesions was highly predictive of CNS lymphoma,²³⁷

more recent studies suggest that the positive predictive value may be 30% or less.^{262,661} Increased metabolic activity on a PET scan along with EBV DNA in the cerebrospinal fluid increases the likelihood of CNS lymphoma in these patients. HIV patients who subsequently developed CNS lymphomas lacked EBV-specific CD4 T cells regardless of their T-cell counts and whether they had undergone immune reconstitution.⁴⁴⁸ Other immunoblastic lymphomas in patients with AIDS occur in the late stages of the disease and are usually EBV positive.

Plasmablastic lymphomas are usually EBV positive and occur in the mouth or jaw of patients with HIV; less often they present in the sinus, gastrointestinal tract, or soft tissues.^{324,1224} These tumors are EBER positive, have a type 1 latency pattern, and do not express CD20. They generally occur late in the course of HIV and have a poor prognosis.

Primary effusion lymphomas are located in the pleural, pericardial, or peritoneal cavity. The cells are positive for HHV-8 DNA and 90% or more of the tumors contain EBV DNA. When positive for EBV, the EBV genome is required for cell proliferation.⁹⁵² EBV inhibits HHV-8 lytic replication in primary effusion lymphoma cells.¹⁵⁷⁴ The tumor cells express EBNA-1 and may express LMP1 and LMP2.^{157,607}

EBV-positive smooth muscle tumors, leiomyosarcomas, and leiomyomas occur in patients with AIDS,^{999,1192} in transplant recipients,^{845,1385} and in patients with congenital immunodeficiencies. These tumors usually present in the lungs or gastrointestinal tract but also can involve the liver, kidney, lymph nodes, central nervous system, and adrenal glands.⁴⁴³ The smooth muscle cells contain clonal or oligoclonal EBV DNA. Multiple tumors in the same patient may be from distinct EBV clones, indicating that they can arise independently.³⁰⁸ The disease usually occurs in the late stage of AIDS or at a mean of 3 years after organ transplant. Tumors can be either donor or recipient in origin in transplant patients. EBV gene expression varies; some tumors show type 3 EBV latency, while others express EBNA-2 but not LMP1. In contrast, leiomyomas and leiomyosarcomas from immunocompetent persons do not contain EBV DNA.

Model for EBV Oncogenesis

While a large number of malignancies are associated with EBV and new associations continue to be made, certain criteria have proved important for linking EBV to tumors. First, EBV should be in virtually every tumor cell. Second, the viral DNA should be clonal (or oligoclonal), indicating that the malignancy arose from an EBV-infected cell. Third, in the case of epithelial cell malignancies, viral DNA should be in dysplastic lesions, indicating that EBV infection occurred during oncogenesis. Fourth, at least one EBV-associated latency gene should be expressed, indicating that the virus has an active role in maintaining the tumor.

Some EBV-associated malignancies are strongly linked with the virus, which clearly is required for development of the tumor (Fig. 61.27). These malignancies have a short latency period between EBV infection or virus reactivation and development of malignancy, and occur in highly immunosuppressed persons with little or no EBV-specific T-cell immunity. The tumor cells express most or all of the EBV-associated latency proteins and have few if any host gene mutations. Examples include EBV lymphoproliferative disease occurring early after transplantation or in patients with congenital immunodeficiencies. The low level of cellular immunity to EBV allows

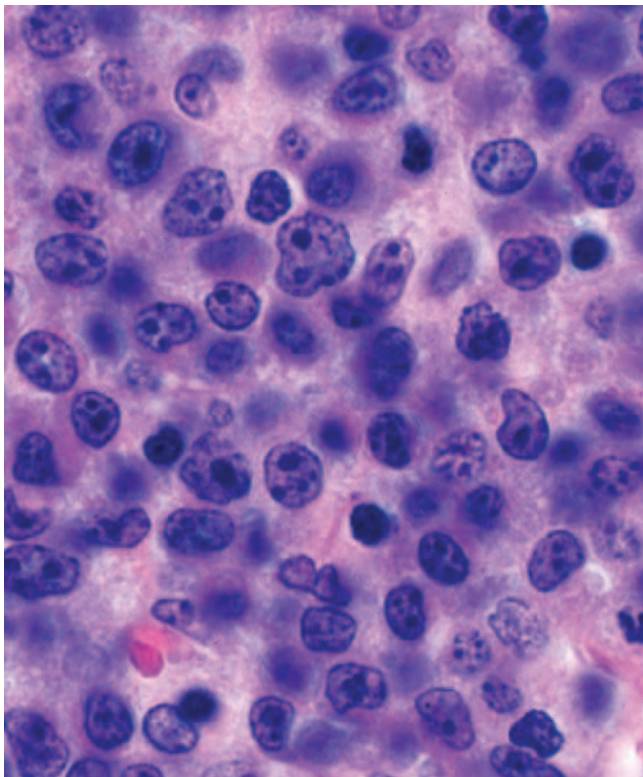


FIGURE 61.26. Biopsy from a patient with immunoblastic lymphoma of the central nervous system. (Courtesy of Dr. Elaine Jaffe.)

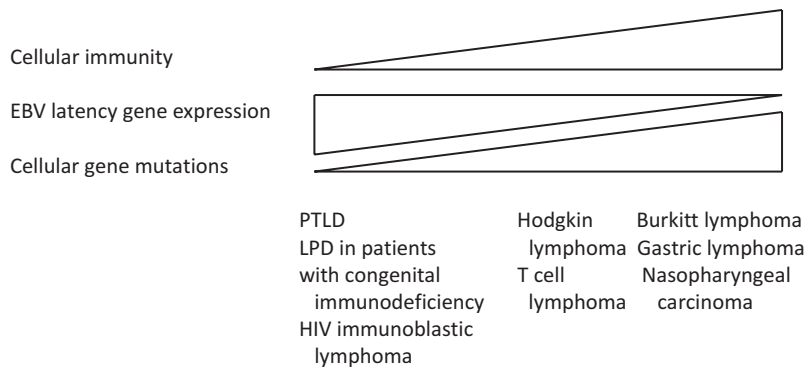


FIGURE 61.27. Model of Epstein-Barr virus (EBV) oncogenesis. EBV-associated malignancies in immunocompromised patients show full expression of EBV latency genes and few or no mutations or changes in cellular gene expression (**left**), while EBV-associated malignancies in persons with otherwise normal immunity have limited expression of EBV latency genes and more mutations or changes in cellular gene expression (**right**).

expression of the EBV-transforming proteins, which rapidly results in transformation without the need for secondary cellular genetic changes.

In contrast, other malignancies (e.g., HL) occur in otherwise healthy persons, have longer latency periods, and express an intermediate number of EBV latency genes. These patients have impaired T-cell immunity,³⁹⁴ but to a much lesser extent than those with EBV PTLD. Viral gene products can substitute for mutations in host cell genes that occur in the analogous EBV-negative tumors (e.g., crippled germinal center cells in HL).

A third class of EBV-associated malignancies (e.g., BL, NPC, gastric carcinoma) has mutations in cellular genes and more limited EBV latent gene expression. In EBV-associated epithelial cell malignancies (NPC, gastric carcinoma), genetic mutations are associated with inactivation of tumor suppressor genes and hyperplastic changes occur before EBV infection of the cells. In BL, EBV may initially drive proliferation of B cells, resulting in chromosomal translocation and oncogene activation (*c-myc* in BL). In these diseases, EBV is clearly a cofactor and virus infection is one of several steps in development of the fully malignant phenotype. However, the presence of clonal EBV in every tumor cell, without loss of viral DNA after tumors are established, indicates that the fully malignant phenotype is dependent on EBV.

Other Diseases Associated With EBV

Chronic lymphocytic leukemia may have a subpopulation of cells infected with EBV, and these cells express LMP1 and EBERs. EBV has been associated with transformation of chronic lymphocytic leukemia to diffuse large B-cell lymphoma.³¹⁹ EBV has also been detected in T-cell prolymphocytic leukemia cells.⁸³⁰

Rheumatoid arthritis patients often have elevated EBV DNA loads in the blood.¹⁵⁸ These patients have an increased risk of lymphomas (HL and diffuse large B-cell lymphoma), even in the absence of immunosuppressive therapy, and 24% of the lymphomas are EBV positive.

Multiple sclerosis (MS) has been associated with EBV in several studies. Primary infection with EBV is associated with an increased risk of MS⁸⁶²; in contrast, persons who are EBV seronegative have a very low risk of MS. A meta-analysis showed a 2.2-fold increased risk of MS after IM.⁵³⁰ A case-control study showed that 83% of children with MS had evidence of EBV infection, compared with 43% of controls.²² A study of the prevalence of EBV-specific antibodies prior to the onset of MS found that serum antibodies to the EBNA complex in persons 25 years old or older were two- to threefold higher in those who

subsequently developed MS compared to those who did not.⁸⁶³

The risk of MS was 36-fold higher in persons with high titers of anti-EBNA complex IgG antibodies and 8-fold higher in those with high EBNA-1 titers than those with low titers.¹⁰⁶⁶ A meta-analysis of several studies found an association between MS and anti-EBV VCA IgG, anti-EBNA IgG, and anti-EBNA-1 antibody, but not anti-EBV EA IgG, viral DNA in the serum, or DNA in cerebrospinal fluid or brain.¹²⁸¹ Patients with MS have increased numbers and broadened specificity of CD4 T cells to EBNA-1.⁹⁴³ Elevated levels of both antibodies and T cells to EBNA-1 were associated with development of MS.⁹⁴⁵ EBNA-1-specific T cells cross-reacted with myelin more frequently than autoantigens not associated with MS, and the myelin cross-reactive T cells produced IFN- γ and IL-2.⁹⁴⁴

While most studies have not found EBV DNA or RNA in the brain, one study reported that nearly all postmortem brains from patients with MS showed EBV RNA (EBER) and BFRF1 protein in B-cell follicles, and LMP1 and EBNA-2 in perivascular areas in white matter lesions; EBV gene expression was not detected in brains from persons without MS.¹³¹⁸ CD8 T cells were present at the site of EBV-infected B cells in the brain. In contrast, EBV RNA was generally not detected in active plaques or in B cells in the cerebrospinal fluid in another study of patients with MS.¹²⁸² One study reported higher levels of antibodies to peptide sequences from EBV BRRF2 and EBNA-1 in the serum and cerebrospinal fluid from patients with MS compared with controls; antibodies to the viral proteins were present in oligoclonal cerebrospinal fluid IgG.¹⁸⁵ Increased levels of EBV antibody were detected in the serum and cerebrospinal fluid of patients with MS¹¹⁰⁰; however, intrathecal synthesis of EBV antibody was uncommon, absent, or nonspecific in other studies.^{180,1122,1282} Further studies will be needed to confirm many of these findings, and the role EBV in the pathogenesis of MS is still unclear.

Systemic lupus erythematosus (SLE) is associated with elevated antibody titers to EBV; however, a prior history of IM was not associated with increased risk of SLE.¹⁴⁸³ In one study, 99% of children with SLE had evidence of prior infection with EBV compared with 70% of age-matched controls.⁶⁷⁶ EBV DNA was detected in the blood of 100% of patients with SLE, compared with 72% of controls. Patients with SLE often have higher EBV loads, increased numbers of latently infected B cells, and certain EBV-specific antibodies that cross-react with SLE autoantigens.⁶⁷⁵ Patients with SLE more often have antibody to EBNA-1 and produce antibody to more regions of EBNA-1 than healthy persons.¹⁰⁰¹ Antibody to 60-kD Ro,

which cross-reacts with EBNA-1, was detected in some patients with SLE prior to the onset of disease; rabbits immunized with either a 60-kD Ro or a cross-reacting EBNA-1 peptide developed autoantibodies and SLE-like autoimmune disease.¹⁰⁰⁰ Immunization of rabbits and mice with an EBNA-1 peptide homologous to part of the Sm antigen resulted in autoantibodies and SLE-like autoimmune disease.¹¹⁷⁵ While there is vigorous B-cell activation in SLE and impaired control of EBV, EBV-associated malignancies are not increased in these patients. At present, there is not a strong causal relationship between EBV and SLE.

DIAGNOSIS

The diagnosis of IM in patients with typical symptoms is made by a positive heterophile antibody. In patients with atypical symptoms, or in those with typical symptoms and a negative

heterophile test, titers of EBV-specific antibodies are measured. Antibody to EBV VCA IgM antibody is the most useful test because the antibody is present only during the first 2 to 3 months of the illness (see Fig. 61.15). EBV VCA IgG antibody is often elevated at the time of diagnosis, but because it persists for life, it is not useful for diagnosis of acute infection. Seroconversion to EBV EBNA positivity can be useful because antibodies take 3 to 6 weeks after the onset of illness to become positive and they persist for life. Antibody to EBNA-2 is present early in IM, while antibody to EBNA-1 is present later during convalescence.

Detection of EBV DNA in the blood can be useful for diagnosis of IM if serologic results are equivocal. Measurement of EBV DNA in the blood is also used to follow patients after transplant to predict the development of EBV PTLD or to follow patients after therapy.^{505,769} Detection of EBV DNA in the cerebrospinal fluid of AIDS patients with lesions in the brain is useful for prediction of lymphoma. Culture of throat

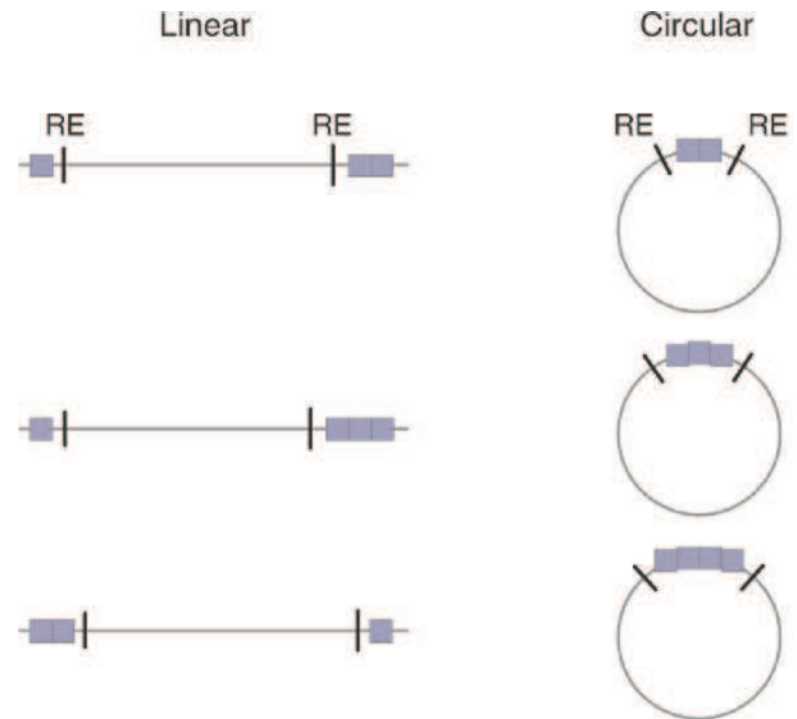


FIGURE 61.28. Identification of replicating, latent clonal, or latent oligoclonal Epstein-Barr virus (EBV) in biopsies. Linear (upper left) and circular (upper right) EBV genomes have differing numbers of terminal repeats (gray bars) bounded by restriction endonuclease sites (RE). Southern blotting with a probe to the terminal repeats shows different patterns in cells with replicating (linear EBV) or latently infected clonal (single form of terminal repeats) or oligoclonal (different forms of terminal repeats) EBV DNA (bottom). (Adapted from Katz B, Raab-Traub N, Miller G. Latent and replicating forms of Epstein-Barr virus DNA in lymphomas and lymphoproliferative diseases. *J Infect Dis* 1989;160:589–598, by permission of the Infectious Diseases Society of America.)

	Replicating	Latent clonal	Latent oligoclonal
Circular	≡	—	≡
Linear	≡		

washings for EBV is not useful for diagnosis of IM because the virus persists for life in the throat of infected persons. PCR for the terminal repeats is used to determine if EBV is clonal in tissues, because individual clones have a fixed number of terminal repeats (Fig. 61.28).

EBV is usually detected in tissue by *in situ* hybridization using a probe for EBER (Fig. 61.29) because this RNA is present at thousands of copies per cell. EBV latency proteins, including EBNA-1, EBNA-2, or LMP1, can also be detected in tissues.

INHIBITORS OF REPLICATION

Acyclovir and ganciclovir inhibit the viral DNA polymerase²⁸⁵ but do not inhibit latent infection. Phosphorylation of acyclovir and ganciclovir is mediated by the EBV BGLF4 protein kinase, not the viral thymidine kinase (encoded by BXLFI).¹⁰¹⁰ While treatment of chronic EBV carriers with acyclovir stops EBV shedding from the oropharynx, 1 month of therapy has no effect on the EBV viral load in the blood.¹⁵⁸¹ However, 1 year of antiviral therapy resulted in a modest reduction in the EBV viral load in the blood but did not affect the EBV DNA

copy number per B cell.⁶¹¹ CMX-001, a hexadecyloxypropyl ester of cidofovir, is greater than 100 times more potent as an inhibitor of EBV replication than acyclovir.¹⁵⁴² Maribavir, an l-ribofuranoside benzimidazole, blocks phosphorylation of viral proteins by the EBV protein kinase and inhibits viral transcription, which results in reduced EBV replication.¹⁵²⁵

TREATMENT

Infectious Mononucleosis

Treatment of IM is supportive. Contact sports should be avoided during the acute phase of the disease due to the risk of splenic rupture. While antiviral therapy reduces or eliminates virus replication and shedding from the oropharynx, it has no effect on symptoms.^{1468,1488} Corticosteroids reduce the duration of fever and sore throat; however, they may inhibit the development of cellular immunity to the virus and are not recommended for most cases of IM. The combination of corticosteroids and antiviral therapy did not reduce time off from work or school or the duration of the disease.¹⁴⁸⁰ Corticosteroids are used for life-threatening complications such as upper airway obstruction due to tonsil enlargement, severe hemolytic anemia, and some cases of cardiac or central nervous system EBV disease.

EBV Posttransplant Lymphoproliferative Disease

Reduction in immunosuppression can allow an increase in EBV-specific T-cell activity⁵⁰⁶ against virus-infected B cells and in some cases, especially disease that occurs early after organ transplant, can cause tumor regression.^{480,573} Rituximab, which depletes CD20-positive B cells, can be effective,²³¹ especially in combination with chemotherapy. Recent studies show that rituximab and reduced-dose chemotherapy have been successful in children.⁵⁰⁷ Surgery can be effective when PTLD lesions are confined to a single site. Cytotoxic chemotherapy (e.g., CHOP) is often effective early after transplant²³² or in cases of rituximab failure.¹⁴⁷⁰ Radiation as well as high-dose methotrexate is often used for disease involving the central nervous system.¹⁴²⁰ Antiviral therapy has no effect once PTLD is established, because the EBV genome replicates in latently infected cells using the host, not viral, DNA polymerase.

Most tumors are of donor origin in hematopoietic stem transplant (HSCT) recipients. Thus, in these patients infusion of donor lymphocytes has been successful, although graft-versus-host disease can occur.^{481,1120,1132} (Table 61.6). Repeated infusions of cells may be required, and graft-versus-host disease may occur when large numbers of cells are infused. Treatment of EBV PTLD with EBV-specific CTLs after HSCT resulted in complete remissions in 11 of 13 patients.⁵⁷⁵ Partially HLA-matched EBV-specific CTLs from third parties have been successful for treatment of PTLD in HSCT recipients.^{75,1009} Functional gene-marked CTLs persisted for up to 9 years. Immune escape from EBV-specific CTLs was reported in one bone marrow transplant recipient whose tumor cells mutated, deleting two EBNA-3B epitopes, which resulted in a fatal outcome.⁴⁷⁹

Most EBV PTLD in organ transplant patients is recipient in origin. In these patients reduction of immunosuppressive therapy can increase the level of EBV-specific T cells and may cure the disease. In some cases EBV-specific CTLs have been expanded from organ transplant recipients and successfully

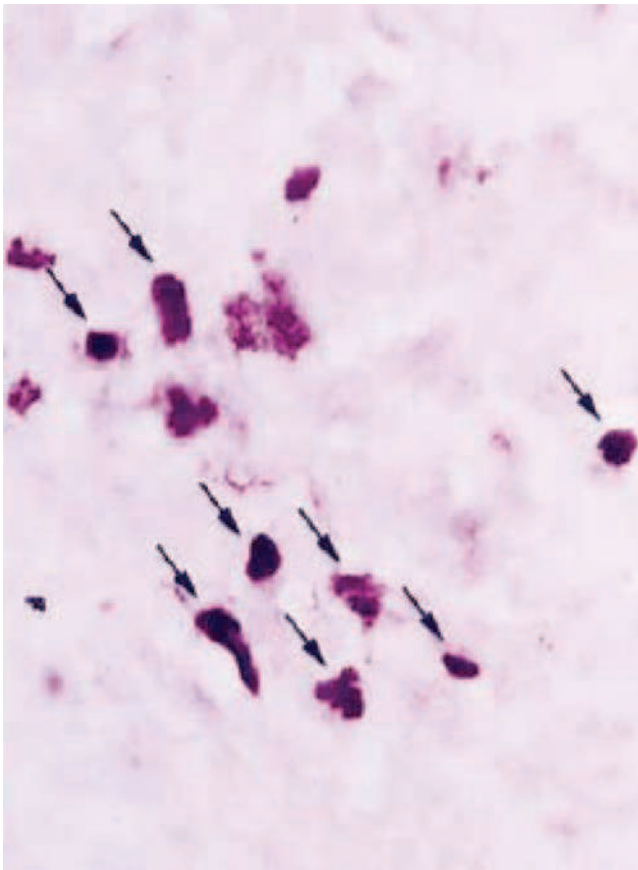


FIGURE 61.29. Epstein-Barr virus–encoded RNA (EBER) staining from a biopsy showing Epstein-Barr virus (EBV) lymphoma. Arrows show cells that hybridize with the EBER probe. (Adapted from Cohen JI. Epstein-Barr virus infection. *N Engl J Med* 2000;343[7]:481–492.)

TABLE 61.6 EBV-Specific Approaches to Treatment of EBV Malignancies

Approach	Method	Results	References
Infusion of EBV-specific CTLs	CTLs directed against EBV latent proteins (LCLs)	Effective in PTLD	118,256,533,1120,
	CTLs directed against LMP2	Some responses in HL, NPC	1237,1394
Therapeutic vaccination	DC with LMP2 peptides	CR for HL and NHL	119
Induction of EBV replication	Arginine butyrate and ganciclovir	Tumor regression in NPC	891
	HDAC inhibitor and cytotoxic chemotherapy	Tumor responses in PTLD and NHL	1156
	Azacytidine	Tumor responses in mice	386
	Gemcitabine or doxorubicin and ganciclovir	Demethylation of EBV promoter, activation of BZLF1	191
	Adenovirus expressing BZLF1 or BRLF1	Tumor response in mice	385
Reduction of EBV episome copy number	Low-dose hydroxyurea	Tumor response in mice	387
Cell cycle arrest of EBV-transformed B cells	Rapamycin	Responses in EBV CNS lymphoma	1362
Inhibition of NF- κ B	Ritonavir, BAY 11-7082, bortezomib	Tumor responses in mice	1492
Reduction of EBNA-1 expression	Hsp90 inhibitor	Tumor responses in mice	307, 736,720,1639
	miR-155 inhibitor	Tumor responses in mice	1408
	siRNA	Reduced EBV copy number <i>in vitro</i>	931
Reduction of LMP1 expression	Antisense RNA	Inhibited growth of NPC cell line <i>in vitro</i>	1593
		Induced apoptosis of EBV-transformed B cells	744

EBV, Epstein-Barr virus; CTL, cytotoxic T lymphocytes; LCL, lymphoblastoid cell line; PTLD, posttransplant lymphoproliferative disease; NPC, nasopharyngeal carcinoma; LMP, latent membrane protein; CR, complete remission; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; DC, dendritic cell; HDAC, histone deacetylase; NF, nuclear factor; miR, microRNA; siRNA, small interfering RNA.

treated the disease.^{757,1290} In 2 organ transplant recipients with active PTLD and in 10 at risk for the disease who received autologous EBV-specific CTLs, one patient with active disease had a complete response and one had a partial response, while none of the patients at risk developed PTLD.¹²⁹⁰ Partially HLA-matched allogeneic T cells from a donor bank that covered 95% of common HLA haplotypes resulted in a complete remission in 42% of patients with PTLD (most of whom had undergone organ transplant) who had progressive disease despite conventional therapy⁵³³; 86% of these patients in complete remission at 6 months were still in remission at 4 to 9 years.⁵³² Better responses were observed in recipients who received higher numbers of CD4 T cells.

EBV-specific T cells have also been derived from the blood of EBV-seronegative persons.^{1176,1289} CTLs that recognize adenovirus, CMV, and EBV have been developed by transducing EBV lymphoblastoid cell lines with a vector expressing adenovirus and CMV genes; infusions of the CTLs into transplant recipients and their subsequent expansion in the blood were associated with lower levels of EBV DNA in the blood and resolution of EBV PTLD in one recipient.⁸⁵⁷ A rapid method of producing EBV-specific T cells involves stimulation of donor cells with EBV-specific peptides and antibody-mediated capture of cells expressing surface IFN- γ ; this technique allowed isolation of EBV-specific T cells from donor blood in 36 hours and resulted in remissions in patients with early-stage PTLD.¹⁰⁵²

While most EBV-specific T-cell therapies have focused on CD8 T cells that recognize EBV antigens, some CD4 T cells recognize EBV-transformed B cells not through viral antigen, but likely by cellular antigens that are induced by the virus.⁹¹⁸ The latter may provide additional targets for T-cell therapy. Lymphokine-activated autologous killer cells have been effective in small numbers of organ transplant recipients with disease.¹⁰⁷⁵

Hodgkin Lymphoma

HL cell lines can process and present EBV proteins to HLA class I–restricted EBV-specific CTL clones; thus, they should be amenable to CTL therapies.⁸⁵² Adenovirus expressing a portion of EBNA-1 linked with epitopes from LMP1 and LMP2 was used to stimulate CD8 T cells from patients with HL; this reversed the functional impairment of the T cells and the cells responded to tumor cells expressing EBNA-1 and LMP1 and LMP2 *in vitro*.¹³⁶⁵ Infusion of allogeneic EBV-specific CTLs into patients with refractory HL resulted in reduction in disease in some patients; however, the donor cells could not be detected in the recipients.⁹³⁷

CTLs were made to EBV-transformed B cells and infused into patients with EBV-positive HL; of 11 patients with measurable tumors, 2 had a complete response, 5 had stable disease, 1 had a partial response, and 3 had no response.^{118,1241} However, CTLs produced using dendritic cells transduced with LMP2 resulted in a higher frequency of LMP2-specific CTLs

and induced a complete response in 4 of 6 patients with HL or NHL and relapsed disease and maintained remissions in 9 of 10 patients.¹¹⁹

Nasopharyngeal Carcinoma

A poxvirus (MVA) vector expressing the carboxyl terminus of EBNA-1 fused to LMP2 reactivated LMP2-specific CD8 T-cell responses from EBV-infected persons *in vitro*.¹⁴³⁰ A phase I trial showed that CD4 and CD8 T-cell responses were induced *in vivo*.⁹¹⁷ Dendritic cells from patients with NPC were pulsed with LMP2 peptides *in vitro* and when injected into patients they resulted in epitope-specific T-cell responses in the blood coincident with tumor regression in some patients.⁸⁹¹ Dendritic cells infected with adenovirus expressing LMP2 and a portion of LMP1 did not increase the frequency of LMP1/LMP2-specific T cells in the blood and had limited efficacy.²²³ Poxvirus³³⁵ and adenovirus³³³ vectors expressing multiple HLA-A2–restricted LMP1 and/or LMP2 epitopes induced LMP-specific CTL responses in mice expressing HLA-A2 and inhibited growth of tumor cells expressing LMP1.

Infusion of autologous EBV-specific T cells in patients with late-stage NPC resulted in control of disease in 6 of 10 patients; 3 of 4 patients with LMP2-specific immune responses after infusion had a clinical benefit.²⁵⁶ A second study by the same authors used higher doses of EBV-specific T cells after lymphodepletion by chemotherapy and showed control of disease in 6 of 11 patients.¹³¹⁴ In another trial, 10 patients with advanced NPC were treated with autologous EBV-specific CTLs; 4 patients who had been in remission remained disease free, and of 6 with refractory disease before treatment, 2 had complete responses, 1 had a partial remission, 1 had stable disease, and 2 had no response.¹³⁹⁴ A phase I/II study of 23 patients (including 10 patients reported previously¹³⁹⁴ with recurrent or refractory NPC) showed that infusion of autologous EBV-specific T cells resulted in disease-free outcomes in 62% of patients who had been in remission at the time of infusions, while 49% who had active disease at the time of the infusions had a complete or partial response.⁹²⁷ Patients with metastatic disease had a worse outcome than those with locoregional disease. Infusions of anti-CD45 monoclonal antibodies to deplete lymphocytes prior to EBV-specific CTLs for NPC enhanced the expansion of the cells *in vivo*.⁹²⁸

Other Therapies

Other therapies to treat EBV diseases include the use of combined cytotoxic chemotherapeutic agents and ganciclovir to induce virus replication, express the viral protein kinase to phosphorylate ganciclovir (which is toxic to cells), and kill EBV-infected cells.³⁸⁵ Combined administration of ganciclovir and arginine butyrate (a histone deacetylase inhibitor) resulted in EBV-positive tumor responses in 10 of 15 patients.¹¹⁵⁶ Histone deacetylase inhibitors also increase the effectiveness of chemotherapeutic agents by inducing lytic replication in animal models of EBV lymphoma.³⁸⁶ Adenovirus-expressing EBV IE genes reduced EBV tumor growth in mice.³⁸⁷ Demethylating agents such as 5-azacytidine also activate viral gene expression.¹⁹¹ Other approaches include low-dose hydroxyurea to reduce EBV episome copy number,¹³⁶² and rapamycin, which induces cell cycle arrest at the G1 phase in EBV-transformed B cells and inhibits growth of EBV lymphomas in SCID mice.^{1084,1492} Ritonavir, an HIV protease inhibitor, down-regulates survivin

and cyclin D2, inhibits activation of NF- κ B, induces apoptosis in EBV-transformed B cells, and inhibits growth of lymphoma cells in immunocompromised mice.³⁰⁷ Hsp90 inhibitors reduce EBNA-1 expression and inhibit the growth EBV-transformed B cells in SCID mice.¹⁴⁰⁸ Inhibition of cellular microRNAs might also inhibit EBV disease. An miR-155 inhibitor reduced EBNA-1 mRNA and EBV copy number in latently infected cells.⁹³¹

A variety of compounds that block the activity of EBNA-1 or LMP1, including antisense molecules to LMP1,⁷⁴⁴ small interfering RNA (siRNA) to EBNA-1,¹⁵⁹³ and intracellular¹¹⁶⁴ and extracellular antibody to LMP1,²⁹⁷ are active *in vitro* but have not been used in clinical studies. Bortezomib, a proteasome inhibitor, and Bay 11-7082 block NF- κ B, induce apoptosis of EBV-transformed B cells, and enhance survival of mice injected with the cells.^{736,1639}

PREVENTION AND VACCINES

Preemptive therapy for EBV lymphoproliferative disease has included antiviral therapy with acyclovir or ganciclovir. Results have been mixed, with more recent studies reporting a reduction in disease^{438,1002} and other studies⁴⁸⁶ showing no effect. Depletion of B cells from bone marrow used for transplantation reduces the risk of EBV PTLT, presumably due to removal of virus-infected cells.⁵¹⁹ A single dose of rituximab (anti-CD20 antibody) given to allogeneic HSCT patients at high risk for EBV PTLT with rising viral loads reduced the rate of disease from 49% based on a historical cohort to 18% in the treated group and prevented death at 6 months.¹⁴⁸⁹ A second study showed similar results.¹⁵⁵⁵ However, relapses due to CD20-negative EBV-infected B cells have occurred that require other therapy.²⁵⁴ An alternative approach is to monitor patients with rising viral loads and to give rituximab or EBV-specific CTLs only when systemic symptoms develop.¹⁵⁰³ Infusion of EBV-specific T cells (directed against EBV latency-associated proteins) has been effective in reducing EBV load in the blood and preventing EBV-associated PTLT in HSCT recipients.^{480,574,1238} A study of EBV-specific CTLs for prevention of EBV PTLT found that none of 101 patients who received CTLs developed the disease.⁵⁷⁵ Donor-derived EBV-specific CTLs have prevented disease in matched unrelated and haploidentical stem cell transplants.⁸⁵⁶ Intravenous immunoglobulin did not provide any additional effect when added to antiviral therapy.⁶³⁶

Reduction of immunosuppression is often effective early after transplantation when viral loads increase.¹⁸⁷ In a study of 73 liver transplant recipients in which immunosuppression was tapered when EBV viral loads were elevated, one patient developed allograft rejection, but the incidence of PTLT declined from 16% to 2%.⁸⁵⁴ EBV-specific T cells prevented development of EBV-positive lymphomas when they were given as prophylaxis to T-cell-depleted bone marrow transplant recipients with high EBV loads after transplant and at high risk of the disease¹²³⁷; the cells can persist for at least 18 months.⁵⁷⁴ Autologous EBV-specific T cells have also been used to prevent EBV lymphomas in solid organ transplants with high viral loads.²⁵⁵

Vaccination to prevent EBV infection or disease will be important in certain high-risk groups including transplant recipients, boys with mutations in SAP or XIAP, and areas

where the frequency of NPC and BL are high.²⁴⁸ Vaccination that reduces the rate of IM might reduce the frequency of HL, which is increased during the first 5 years after IM.

gp350 has been the major immunogen in vaccine studies; it is the major target for neutralizing antibody¹⁴⁶ and it is a target of CTLs.⁷⁵⁷ Infusion of immunodeficient mice, reconstituted with PBMCs from EBV-negative persons, with monoclonal antibody to gp350 prevents development of EBV lymphomas after challenge with virus.⁵³¹ Soluble gp350 protected cotton-top tamarins that had been challenged intravenously with high doses of EBV from lymphoma.³⁶⁴ After immunization of cotton-top tamarins or common marmosets with gp350, protection from lymphoma¹²⁰³ or infection,³⁵⁸ respectively, did not always correlate with development of neutralizing antibody. Rhesus lymphocryptovirus is the homolog of EBV in rhesus macaques and contains an ortholog of each EBV gene. Immunization of rhesus macaques with soluble rhesus lymphocryptovirus gp350 resulted in better protection from infection and reduced viral loads 2 years after infection in animals that became infected after challenge when compared with virus-like replicon particles expressing rhesus lymphocryptovirus gp350, EBNA-3A, and EBNA-3B.¹²⁸⁵

A study of 19 seronegative children (1 to 3 years old) in China randomized to vaccination with vaccinia virus expressing gp350 or control showed that the vaccine induced neutralizing antibody to EBV; at 16 months all 10 unvaccinated children were infected with EBV, while only 3 of 9 vaccinated children were infected.⁵⁰³ Soluble gp350 vaccine induced neutralizing antibody in volunteers.¹⁰⁶² A study of 181 EBV-seronegative adults randomized to receive three doses of soluble gp350 in monophosphoryl lipid A/alum adjuvant or placebo showed that the vaccine reduced the rate of EBV IM by 78% but did not affect the rate of virus infection.¹³⁶⁹ A phase I trial of children with chronic kidney disease using three injections of a lower dose of gp350 in alum showed that the vaccine induced neutralizing antibody in 33% of subjects receiving the highest dose of gp350 and immune responses declined rapidly; the authors concluded that a more prolonged vaccine schedule or an improved adjuvant was needed for these patients.¹²¹⁴

An alternative or complementary strategy is to induce potent T-cell responses to control primary infection so as to reduce the viral load after infection and potentially reduce the risk of EBV-associated malignancy. A study of 14 HLA B*0801 EBV-seronegative adults randomized to receive an HLA B*0801 EBV EBNA-3A peptide (FLRGRAYGL) and tetanus toxoid in a water-in-oil adjuvant showed that 8 of the 9 volunteers developed epitope-specific T-cell responses.³⁵⁵ At 2 to 12 years after follow-up, one of two placebo recipients developed IM, while four of four vaccinees that became infected with EBV did not develop IM. Virus-like particles expressing nearly all the EBV genes except for BZLF1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and LMP1 and the terminal repeats induced neutralizing antibody and T-cell responses in mice.¹²⁵⁵

PERSPECTIVE

The true importance of the innate immune response, NK cells, and CD4 cells in controlling EBV is unclear. It is not apparent why EBV IM is primarily a disease of adolescents and young adults. Treatment of prolonged fatigue that occurs sometimes

after IM is unsatisfactory. The role of EBV in multiple sclerosis and SLE is still unclear.

While small-scale trials have shown that a vaccine can reduce the incidence of IM, a larger trial is needed to confirm these findings. Similarly, it is unknown if such a vaccine would reduce the rate of PTLD in seronegative transplant recipients or prevent or reduce the rates of EBV-associated malignancies, including HL, BL, NPC, and gastric carcinoma. The immune correlates of protein against EBV infection, IM, and EBV-associated malignancies are not well understood.

EBV continues to be associated with additional malignancies, and the mechanisms involved in viral oncogenesis continue to be unraveled. Treatment of EBV-associated malignancies is usually not directed against viral proteins. Additional drugs that can target viral or cellular proteins involved in oncogenesis are needed. While preliminary studies suggest that EBV-specific CTLs might be used to control NPC and HL, more effective therapies are needed. Additional therapies to induce EBV replication and kill virus-infected cells, infusions of EBV-specific CTLs followed by vaccination to boost CTL numbers, and treatments that block EBV immune evasion mechanisms might be useful for treating EBV-associated malignancies.

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Cytomegaloviruses

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INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous virus infection with worldwide distribution. The virus is the most significant infectious cause of congenital disease, an important opportunist in the immunocompromised host and an occasional cause of febrile illness as well as infectious mononucleosis in the general population. HCMV infects an overwhelming majority of the population, spreading efficiently throughout life and all over the world through direct contact with bodily secretions. Like other human herpesviruses, HCMV is never completely cleared and remains latent for the life of the host. Persistently and sporadically shed virus is an important recurrent source of virus for transmission. Susceptibility to HCMV disease is associated with a compromised immune system, particularly related to defects in cell-mediated CD4 and CD8 T-cell function. During pregnancy, intrauterine transmission to the fetus results in sensorineural damage. In immunocompromised

individuals (following solid organ transplantation and hematopoietic cell allografting, immunosuppressive therapies, and genetic or acquired immunodeficiency), this virus remains clinically important despite available antiviral therapies aimed at reducing the overall disease burden. More effective and safe, orally bioavailable antiviral drugs are needed.

Transplacental transmission during pregnancy, rare with other human herpesviruses, underscores the medical importance of HCMV as well as the motivation for universal vaccination.⁴⁸¹ Virus transmits during primary maternal infection (in HCMV-seronegative women) in a pattern reminiscent of rubella; however, HCMV causes recurrent maternal infection (in HCMV-seropositive women) following either reinfection with additional viral strain or reemergence of persistent/latent infection. It is now recognized that this virus is transmitted in at least 1% of pregnancies worldwide regardless of population serostatus. Sensorineural damage (hearing loss, eye sight compromise, and learning disabilities) impacts roughly 12% to 25% of infected newborns,¹⁶² with half developing disease over the first few years of life. A small proportion of congenital infections cause severe systemic, life-threatening cytomegalic inclusion disease (CID). Overall awareness of disease risk is poor, both in the general population and among physicians, possibly owing to the limited options that are available to treat congenital HCMV disease. Because person-to-person transmission depends on direct contact with infected bodily secretions, viral shedding patterns in urine, saliva, breast milk, and genital secretions mediate exposure. Good hygiene practices (e.g., handwashing) can reduce rates, although widespread shedding and transmission results in universal transmission in populations worldwide. Transmission is most frequent in childhood and is mostly asymptomatic. A varying proportion of the population, ranging as high as 50% to 60% in areas of North America and Europe, escapes infection early in life and remains susceptible as adults. Primary infection during pregnancy in HCMV-naïve women is associated with an average 33% risk of transplacental transmission,²⁸⁹ whereas roughly 1% of recurrent infections result in transmission.⁹⁵ Therefore, HCMV universal vaccination is highly desirable²⁹ and includes the challenge of preventing primary as well as recurrent intrauterine maternal transmission.⁵⁶²

Space limitations dictate emphasis on HCMV, with some reference to key observations in animal models, in this chapter. A growing number of publications and reviews are available. The reader is strongly advised to consult earlier reviews and chapters focusing on cytomegaloviruses to obtain a more complete understanding of this field. In particular, *Cytomegalovirus: Biology and Infection*,²⁴⁷ the last version of this chapter⁴⁰⁴ as well as earlier versions^{18,397,402,404,446,627} and the reference book, *Human Herpesviruses: Biology, Therapy and Immunopathology*,²⁸ provide more extensive information. Given the limited number of references that can be included in the print version of this chapter, we generally reference a key recent report or review, expecting that this will lead the reader to relevant earlier work. The online materials provide more extensive references.

History

HCMV has been recognized as an opportunistic pathogen in immunocompromised hosts as well as an important infectious cause of birth defects for half a century.^{704,705} Observations of cytopathology associated with HCMV disease in the immunocompromised host and newborns extends back nearly 100 years

before cell culture or virus isolation methods were available. Severe HCMV-associated CID was first recognized by its association with “owl’s eye” cytopathology in autopsy specimens prior to any knowledge of viral etiology. Surrogate animal models employing natural cytomegaloviruses of mice, rats, and guinea pigs played important roles in early appreciation of pathogenesis.²⁴⁷ More recent studies using rhesus macaque cytomegalovirus (rhCMV) have been possible due to isolation of specific pathogen-free monkeys.^{41,490} By the early 1950s, diagnosis of HCMV disease was facilitated by the identification of inclusion-bearing cells in urine along with the demonstration of virus-like particles by low-resolution electron microscopy (EM). One investigator in particular, Margaret Smith, pursued studies on salivary gland virus of mice, which today is called murine CMV (MCMV), for at least two decades before the mid-1950s, when human salivary gland virus was isolated from urine of congenitally damaged newborns by her, at Washington University, as well as by Thomas Weller at Harvard and Wallace Rowe at National Institutes of Health (NIH). MCMV and HCMV replicated and produced a similar cytopathology, but only on fibroblasts from the homologous species. Species-specificity is now recognized as a key characteristic of all cytomegaloviruses. In the period from 1956 through 1970, the viral etiology of HCMV disease in newborns and immunocompromised transplant recipients was elucidated.^{704,705} Biologically similar viruses associated with a characteristic tissue and cell type distribution were isolated from many mammals. By the early 1970s, diagnosis of HCMV by virus isolation was established as a gold standard. By the turn of the 21st century, quantitative polymerase chain reaction (PCR) detection of viral DNA in blood and secretions became the standard for diagnosis of infection.¹⁶⁴

As the health economic and societal consequences of HCMV damage, including lifelong sensorineural hearing damage and neurological impairment, became fully appreciated, this earned a high priority status as a target for universal vaccination.^{29,643} Primary maternal infection leads to frequent transplacental transmission and is tied to severe disease in newborns.⁶⁵ Viral transmission from primary and recurrent maternal infection both contribute to the overall congenital disease burden in the United States⁶⁹⁵ and other developed countries, with recurrent infection predominating in other areas of the world. In the overall population, transplacental transmission is infrequent, and ranges broadly from roughly 0.3% to 2% of newborns, depending on many factors. Transmission is more frequent in younger women as well as during the third trimester, although transmission during the first or second trimester is associated with greater risk of congenital disease. Placental infection^{122,469} may be much more frequent and may be a barrier to intrauterine transmission, although this area needs more systematic study. Perinatal and postnatal infection of full-term newborns, often acquired from breastfeeding,²³² is of little disease consequence. Premature or immunodeficient infants risk HCMV disease during delivery, from blood transfusion, or from breast milk,²³² and sometimes results in a systemic disease constellation.⁵² Lactating HCMV seropositive mothers commonly shed virus in milk along with HCMV-specific antibody that may neutralize virus, thereby dampening transmission.¹⁷³ Once infected, infants and children shed virus in saliva and urine for months to years and remain an important source of virus infecting parents as well as other childcare providers.⁹⁵ Remarkably low awareness of HCMV in the general population

as well as among physicians⁵³⁶ poses a challenge to efforts in dealing with congenital disease.

HCMV exhibits remarkable genome variation and heterogeneity during natural infection,^{139,216,217,520} a characteristic that has been documented in virus shed by congenitally infected infants.⁵²⁰ The consequences of this variation are presently unknown. Because transmission occurs commonly through contact with bodily secretions and fomites, handwashing is an effective intervention to prevent primary infection in pregnant women.¹¹ Development of a vaccine to prevent congenital HCMV remains an area of unmet medical need and potential promise.⁵⁰

HCMV remains an important etiologic agent of opportunistic infections and disease in immunocompromised hosts, generally emerging in the face of poor T-cell function. Infection in immunocompetent adults is clinically benign, although febrile illness and heterophile-negative infectious mononucleosis may be common.¹³⁶ The T-cell response to HCMV is remarkable. High frequencies of antigen-specific major histocompatibility complex (MHC) class II-restricted CD4 and MHC class I-restricted CD8 T-cell responses develop over the course of life,^{290,644} increasing in frequency with age.⁶⁹¹ T-cell immunity is important for the lifelong suppression of virus replication, as well as for maintaining latency. Helper CD4 and, in particular, cytotoxic CD8 T-cell response levels are recognized as central to the effective host control of viral infection and disease.^{489,530,672,691} HCMV infection and disease predominate during immunosuppressive therapy, particularly in settings of allograft rejection following solid organ transplant (SOT) and allogeneic hematopoietic cell transplant (HCT) recipients, as well as in acquired immunodeficiency disease syndrome (AIDS). Given that settings where this virus causes disease can sometimes be predicted because they result from medical interventions, preemptive therapy and prophylaxis strategies are commonly employed to suppress infection before disease appears.^{190,302} The currently licensed antiviral drugs, ganciclovir/valganciclovir, are used to treat clinically evident disease as well as for prophylaxis and preemptive therapy strategies, with foscarnet and cidofovir as back-up drugs.⁵⁰⁵ Although highly beneficial, these antiviral drugs have been recognized as inadequate in many settings that affect outcomes in transplantation as well as in congenital disease.

General Characteristics

All beta herpesviruses share common characteristics, including appearance in electron micrographs (Fig. 62.1) a prolonged replication cycle in cell culture, species specificity, and a tropism for differentiated hematopoietic and epithelial cell types. Cytomegaloviruses isolated from a mammalian species are most readily propagated in cultured fibroblasts from the homologous host species. During natural infection, HCMV engages epithelial and myeloid (monocyte/macrophage and dendritic) cells, as well as fibroblasts and endothelial cells.⁵²³ HCMV-infected cells develop characteristic cytopathology, exhibiting both nuclear and cytoplasmic inclusions. The latter is associated with a distinct cytoplasmic viral assembly compartment (AC) composed of cellular membranes and organelles that support viral final steps in maturation and release.¹⁴⁴ When propagated in human fibroblasts, HCMV clinical isolates acquire mutations¹⁴¹ in a manner that suggests a process of adaptation.^{139,619} A comparison of laboratory-propagated strain AD169 and wild-type strain Merlin is depicted in Figure 62.1. Mutation of RL13 and UL128 (Table 62.1) occurs rapidly, even after few passages,

suggesting that these membrane impede viral replication in fibroblasts.⁶¹⁹ Although the negative impact of either gene product in fibroblasts remains elusive, pUL128, along with pUL130 and pUL131A (any of which mutates during virus passage in fibroblasts), forms a pentameric envelope complex that contains glycoprotein (g)H and gL and facilitates entry into epithelial and endothelial cells.^{523,539,699} TB40/E is a low passage endothelial tropic strain used for experimental investigations.⁵⁸⁴

Human fibroblasts (e.g., MRC5) are commonly employed for isolation as well as propagation of HCMV, and these cells have been key to understanding how viral gene functions control the various steps in replication (Fig. 62.2). Fibroblasts retain full permissiveness when immortalized with either telomerase or human papillomavirus E6/E7 oncoproteins. Retinal pigment epithelial (e.g., ARPE-19) and astrocytoma (e.g., U373-MG) cancer cell lines are susceptible to HCMV, but other transformed cell lines are typically nonpermissive. Although replication stalls, cytomegaloviruses can enter and proceed through the early stages of the viral replication cycle in many cell types, including cells from other animal species. When exposed to HCMV fibroblasts from nonhomologous animal species such as mouse, attachment, entry, uncoating, and viral IE gene expression proceed (see Fig. 62.2), but viral DNA replication fails to occur. Studies on HCMV infection of human fibroblasts suggest roles for virus-encoded cell death suppressors³⁷⁷ as well as other viral gene controlling early steps⁵⁶⁸ as critical determinants to overcome the species barrier.

HCMV DNA or antigens in peripheral blood (PB) are important and specific markers of infection and disease,²⁰⁷ particularly in at-risk immunocompromised transplant recipients.^{190,302} Two common methods, quantitative DNA PCR amplification, which can be applied to whole blood, tissue, and body fluids, and detection of viral antigen, applied to PB cells as an antigenemia assay, are complementary methods for diagnosis of infection.²⁰⁷ Viral antigens and nucleic acids may also be assessed directly in affected tissues. These indicators of active HCMV infection are used in combination with clinical diagnosis to guide antiviral therapy.⁵⁰⁵ Primary infection in immunologically normal children or adults, including pregnant women and newborns, is readily diagnosed by detection of virus or viral DNA in urine or saliva. These body fluids may contain virus continuously for months to years, and virus reappears in these fluids sporadically during life. Serologic assessment of HCMV-specific antibodies is a more common means of diagnosing previous infection, and is a highly specific and accurate indicator of long-term infection. Natural latency of HCMV occurs in blood marrow (BM)-derived hematopoietic cells⁵⁸⁰ where viral DNA is present at very low frequencies (10^{-5}) and low copy number (2 to 10 genome equivalents per cell)^{581,589} and is associated with limited viral transcription.^{47,515} The precise status and regulation of HCMV during life-long infection remains a topic of active investigation.⁵⁸¹

Viral Strains and Cell Tropism

HCMV strains accumulate deletion and point mutations when propagated in cell culture.^{139,141,619} Replication and release of progeny virus improves with adaptation through repeated passage on fibroblasts, while, at the same time, ability to infect endothelial and epithelial cells is compromised.^{139,523,619} As a result, laboratory strains such as AD169¹¹² and Towne^{167,417} carry substantive mutations and rearrangements compared to

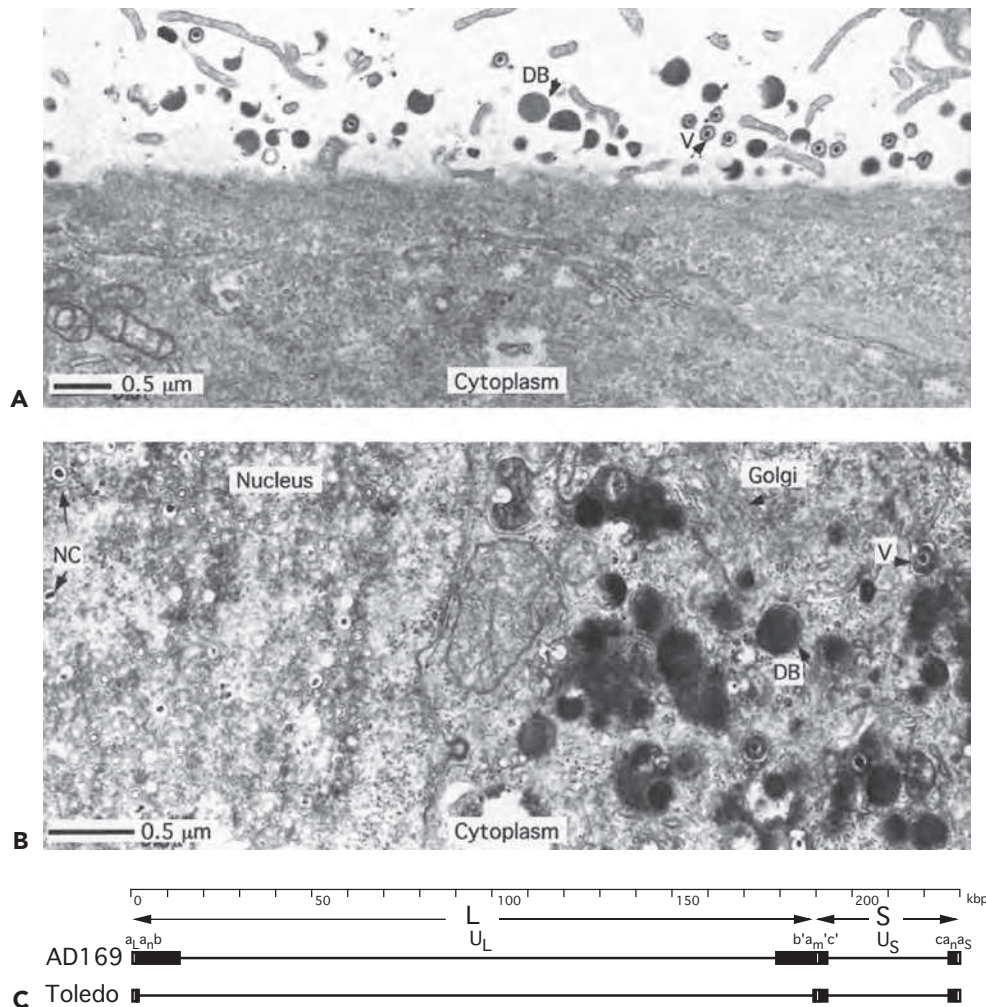


FIGURE 62.1. Virus particles, replication, and genome structure. **A:** Surface of a cell onto which virions (V) and dense bodies (DB) of human cytomegalovirus (HCMV) (Towne strain) have attached. This illustrates the size and approximate ratio of these two types of particle in a virus preparation and the ability of both to attach to the cell surface. **B:** Productively infected cell with nucleocapsids (NC) in the nucleus and maturing virus (V) and dense bodies (DB) in the enlarged Golgi region of the cytoplasm, which forms the characteristic cytoplasmic inclusion of HCMV. Many capsids lacking a dense core of nucleic acid can be observed in the nucleus where, along with nucleocapsids, they form a characteristic inclusion within a kidney-shaped nucleus that is displaced by a cytoplasmic inclusion, a compartment where final steps in maturation occur (giving an “owl’s eye” appearance to cells).²⁴⁷ Envelopment of nucleocapsids (and capsids) occurs at the inner nuclear membrane, where thickened patches of modified membrane develop, followed by final envelopment in the cytoplasm. Dense body envelopment occurs exclusively at cytoplasmic membranes. The bar represents 0.5 μm . **C:** HCMV genome structure. The top line is a size scale in kbp (kilobase pairs = 1000 bp). The second line depicts the L and S components of the genome by arrows. The complete sequenced AD169varUK strain genome structure^{112,159} is shown on the third line with unique sequences (thin lines) flanked by inverted repeats (boxed areas). The genome structure of a low passage strain Merlin as an example of a wild strain is depicted on the fourth line.^{106,159} Note that the region shown as *b* sequence repeat on the left end of the L component in the AD169 strain is retained in wild strains as unique sequence; the *b* sequence repeat on the right is replaced by additional unique (U_L) sequence. The lettering above the genome depicts the following features. The L-terminal *a* sequence repeat (a_L), zero to several (*n*) additional copies of the *a* sequence (a_n), L-terminal *b* sequence repeat of the L component, the unique sequences of the L component (U_L), the L-S junction *b* sequence inverted repeat (b'), one to several (*m*) additional copies of the *a* sequence inverted repeats at the L-S junction (a'_m), the L-S junction *c* sequence inverted repeat (c'), the unique sequences of the S component (U_S), S-terminal *c* sequence repeat, and the S-terminal *a* sequence repeat (a_S) with a variable number of additional copies of the *a* sequence (a_n). The *b* and *b'* repeats are also called TRL and IRL, respectively; and *c'* and *c* repeats are also called IRS and TRS, respectively. In different strains of CMV, including clinical isolates, the *a* sequence ranges in size from 700 to 900 bp.

TABLE 62.1 Summary Information on HCMV Gene Productsⁱ

HCMV gene ^{a,b}	HHV-6/T HHV-7 ^a	HCMV gene family/expression ^c	Function/comments ^d
RL1		RL1 family, DE	
β 2.7 (RNA2.7)		Most abundant noncoding RNA, DE	Metabolic regulator targeting mitochondrial complex I ^{513,737}
RL5A		RL11 family	¹⁴⁸
RL6		RL11 family, LL	¹⁴⁸
RNA1.2		abundant noncoding RNA, DE	
RL8A		L	Multiple spliced variants ²⁰⁴
RL9A		L	²⁰⁴
RL10		Virion envelope gp, LL	⁶⁰⁴
RL11		RL11 family; virion envelope gp, L	gp34, binds IgG Fc ^{35,337,609}
RL12		RL11 family; membrane gp, LL	gp95, binds IgG Fc (Hengel, pers comm)
RL13		RL11 family; virion envelope gp, LL	Impairs replication in fibroblasts and epithelial cells ⁶¹⁹
UL1		RL11 family, LL	
UL2		Putative membrane protein, L	
UL4		RL11 family; virion envelope gp, DE	gp48; Translation stalling by upstream uORF2 ⁵⁴
UL5		RL11 family; virion envelope gp, DE	
UL6		RL11 family; putative membrane gp	
UL7		RL11 family; shed membrane gp, L	CD229/SLAM family homolog inhibits inflammatory cytokine production ¹⁷⁷
UL8		RL11 family; putative membrane gp	
UL9		RL11 family; putative membrane gp, L	Impairs replication in fibroblasts ¹⁶⁶
UL10		RL11 family; putative membrane gp	Impairs replication in retinal pigment epithelial (RPE) cells ¹⁶⁶
UL11		RL11 family; membrane gp, DE	Interacts with CD45 to inhibit T-cell function ¹⁹⁹
UL13		Putative secreted protein, DE	
UL14		UL14 family; putative membrane gp, L	
UL15A		Putative membrane protein, L	
UL16		Membrane gp, DE	Impairs NK cell activity by blocking NKG2D ligands MICB, ULBP1, and ULBP2; impairs replication in RPE cells ^{411,709}
UL17		DE	
UL18		MHC family, L	Peptide-binding MHC-I homolog; LIR-1 ligand; NK decoy ⁷²²
UL19			
UL20		Putative T cell receptor γ chain homolog	Rapidly degraded in lysosomes ²⁷⁰
UL21A		DE (also called UL20A)	Enhances replication in fibroblasts at early times ^{184,185}
UL22A		Virion-associated, secreted gp, L (also called UL21.5)	RANTES chemokine decoy receptor ⁶⁹⁶
UL23	U2	US22 family; tegument	Impairs replication in fibroblasts ¹⁶⁶
UL24	U3	US22 family; tegument	Enhances replication in microvascular endothelial cells (HMVECs) ¹⁶⁶
UL25		UL25 family; tegument, L	
UL26		US22 family; tegument, DE	Transcriptional activator of MIEP; influences phosphorylation of tegument proteins and virion stability ^{349,413}
UL27	U4	Maribavir resistance, DE	Inhibits histone acetyltransferase KAT5/Tip60, inducing CDK inhibitor p21/Cip1 to arrest cell cycle ⁵¹⁸
UL29-UL28	U7 x 2/U5 U7 x 1/U7 U8	US22 family; tegument, L	Stimulate IE gene expression by modifying NuRD complex. ^{396,656}
UL30	U9 ^d	UL30 family	Alters PML morphology ⁵⁴⁶
UL30A		UL30 family	Potential ACG initiation codon ¹⁴⁷
UL31	U10	DURP family, L	¹⁵¹
UL32	U11	Tegument, DE	pp150, Nucleocapsid-proximal stabilization protein (NSP) ^{43,651,729}
UL33x1	U12 x 1	GPCR family, virion envelope	Constitutive signaling GPCR
UL33x2	U12 x 2		
UL34	U13 ^e	DE	Represses US3 IE transcription ³²⁸

TABLE 62.1 Summary Information on HCMV Gene Productsⁱ (Continued)

HCMV gene ^{a,b}	HHV-6/T HHV-7 ^a	HCMV gene family/expression ^c	Function/comments ^d
UL35	U14	UL25 family; tegument, DE	DNA damage response modifier ⁵⁴⁵ ; virus particle formation ⁵⁵⁹ ; alters PML morphology ⁵⁴⁶
UL36x1	U17 x 1	US22 family; tegument, IE	vICA, Inhibitor of caspase-8-induced apoptosis; blocks apoptosis in macrophages ^{123,379,585}
UL36x2	U17 x 2	Localizes to ER and mitochondria, IE	vMIA, Mitochondrial inhibitor of apoptosis; sequesters Bax and Bak and drives fission; blocks serine protease-dependent death during; release of Ca ²⁺ from the ER ^{286,377,380,435,574}
UL37x1			Multiple spliced variants with vMIA domain at amino terminus ⁷⁵
UL37x3	U18	Membrane gp, IE	HDAC1 interacting protein, endoplasmic reticulum stress-induced cell death suppressor, antagonizes tuberous sclerosis complex ^{407,501,656}
UL38	U19	Tegument, DE	Enhances HLA-E-mediated inhibition of NK cells ⁷⁰⁹
UL40		Membrane gp, LL	
UL41A		Virion envelope (also called UL41.5), L	
UL42		Putative membrane protein	
UL43	U25	US22 family; tegument, L	
UL44	U27	(core) DNA synthesis, DE	PPS, DNA polymerase processivity subunit ^{19,21,301,443}
UL45	U28	(core) Tegument, DE	RR1, Large subunit of ribonucleotide reductase homolog (enzymatically inactive) ⁴⁵⁷
UL46	U29	(core) Capsid, LL	TRI1, Capsid triplex component 1; interacts with pUL85; minor capsid binding protein ^{80,113,669}
UL47	U30	(core) Tegument, LL	LTPbp, Associates with largest tegument protein, pUL48, supporting intracellular capsid transport; ⁴⁴
UL48	U31	(core) Tegument, DE	LTP, Largest tegument protein; associates with pUL47; intracellular capsid transport; deubiquitinase ^{80,293,701}
UL48A	U32	(core) Capsid, L	SCP, Smallest capsid protein (also called UL48.5 and UL48/49) located on tips of hexons in capsids ^{37,72,80,315,728}
UL49	U33	(betagamma), LL	
UL50	U34	(core) Nuclear egress, tegument, DE	NEC1, Nuclear egress complex membrane anchoring component 1; interacts with pUL53 at inner nuclear membrane to control capsid egress ^{92,392,547,565}
UL51	U35	(core) DNA encapsidation	Necessary for formation of nucleocapsids; interacts with terminase ²¹² (Messerle, pers comm; McVoy, pers comm)
UL52	U36	(core) DNA encapsidation	Necessary for formation of nucleocapsids ⁷³
UL53	U37	(core) Nuclear egress, tegument, DE	NEC2, Nuclear egress complex component 2; interacts with UL50 membrane protein and nuclear lamina ^{92,392,486,547,565}
UL54	U38	(core) DNA synthesis, DE	POL, DNA polymerase catalytic subunit; target of ganciclovir and foscarnet antivirals ^{21,443}
UL55	U39	(core) Virion envelope gp, DE	gB, heparan sulfate-binding fusion mediator of viral entry; forms homomultimers ¹³¹
UL56	U40	(core) DNA encapsidation, DE	TER2, Terminase subunit 2; binds to DNA packaging motif, exhibits nuclease activity; target of Ietermovir antiviral ^{215,659}
UL57	U41	(core) DNA synthesis, DE	SSB, Single-stranded DNA-binding protein ^{21,443}
oriLyt	oriLyt	DNA synthesis initiation site	DNA synthesis origin for productive infection; position conserved in roseoloviruses ^{21,277,443}
vRNA-1		oriLyt-associated RNA, DE	Virion RNA ²¹
vRNA-2			
(RNA4.9)			
UL69	U42	Noncoding RNA (core) Tegument, LL	MRP, Multiple regulatory protein; cell cycle block; nucleocytoplasmic shuttling; enhances nuclear export and translation of unspliced mRNA ^{22,304}
UL70	U43	(core) DE, DNA synthesis, LL	HP2, DNA helicase-primase subunit; primase homology ^{21,443}
UL71	U44	(core) Tegument	Secondary envelopment; forms oligomers ^{388,557,716}

(continued)

TABLE 62.1 Summary Information on HCMV Gene Productsⁱ (Continued)

HCMV gene ^{a,b}	HHV-6/T HHV-7 ^a	HCMV gene family/expression ^c	Function/comments ^d
UL72	U45	(core) DURP family; tegument, LL	DURP, Deoxyuridine triphosphatase homolog (enzymatically inactive) ⁹⁸
UL73	U46	(core) Virion envelope gp, L	gN, complexes with gM to support envelopment; high sequence variability ^{358,478,479}
UL74	U47	Virion envelope gp,	gO, enhances gH:gL delivery and release of virions ^{271,540,679}
UL74A	UL47A	Putative virion gp (also called UL73.5)	Multiple spliced variants ^{204,556}
UL75	U48	(core) Virion envelope gp, TL	gH, complexed with gL; gH-gL associates with gO or UL128-UL131A; role in entry ^{131,680}
UL76	U49	(core) Virion-associated protein	Translational impact on UL77 ²⁶³
UL77	U50	(core) DNA encapsidation, DE	CVC1, putative capsid vertex-specific component 1
UL78	U51	GPCR family; virion envelope gp, DE	Putative chemokine receptor ³⁹¹
UL79	U52	(betagamma)	L gene transactivator ^{264,471}
UL80	U53	(core), L	PR-AP precursor, maturational protease (N terminus) and capsid assembly (scaffold) protein (C terminus) ^{80,730}
UL80.5	U53.5	(core), L	AP precursor, capsid assembly (scaffold) protein ^{80,730}
LUNA			Latency-associated ^{46,511}
UL82	U54	DURP family; tegument pp, L	pp71, VTA, upper matrix protein; ND10 localized; degrades Daxx and Rb, relieving repression ^{258,430}
UL83		DURP family; major tegument pp, LL	pp65, lower matrix protein; suppresses interferon response ³⁷¹
UL84	U55	DURP family; DNA synthesis, DE	Initiation of DNA synthesis; binds IE2, UL112-113 and PPS ^{21,277,295,443}
UL85	U56	(core) Capsid, LL	TRI2, capsid triplex component 2; interacts with TRI1/UL46; minor capsid protein ^{80,113,669}
UL86	U57	(core) Capsid, L	MCP, major capsid protein; component of hexons and pentons ^{80,113,669}
UL87	U58	(betagamma)	L gene transactivator ²⁶⁴
UL88	U59	(betagamma) Tegument	Putative cytoplasmic egress
UL89x1	U66 x 1	(core) DNA encapsidation, LL	TER1, terminase subunit 1 ATPase; inhibition by antiviral compounds ^{425,658}
UL89x2	U66 x 2		
UL90			
UL91	U62	(betagamma), L	
UL92	U63	(betagamma), L	
UL93	U64	(core) DNA encapsidation, L	CVC2, putative capsid vertex-specific component 2
UL94	U65	(core) Tegument, TL	Secondary envelopment in association with pp28 ^{344,475}
UL95	U67	(betagamma), DE	L gene transactivator ²⁶⁴
UL96	U68	Tegument, DE	Stabilization of nucleocapsids during nuclear-cytoplasmic translocation ⁶⁵²
UL97	U69	(core) Viral serine-threonine protein kinase; tegument, DE	VPK, viral protein kinase, phosphorylates ganciclovir; mimics cdc2/CDK1; target of maribavir antiviral; phosphorylates many viral and cellular proteins ^{254,330,494}
UL98	U70	(core), DE	NUC, deoxyribonuclease ³⁰⁹
UL99	U71	(core) Myristylated tegument pp, TL	pp28, secondary envelopment; multimerization ^{570,571}
UL100	U72	(core) 8TM virion envelope gp	gM, complexes with gN; role in envelopment ^{80,307,308}
UL102	U74	(core) DNA synthesis, DE	HP3, DNA helicase-primase subunit ^{21,443}
UL103	U75	(core) Tegument, L	VEP, Virion and dense body egress protein; Enhances release ¹⁶
UL104	U76	(core) DNA encapsidation, DE	PORT, portal protein; DNA encapsidation ¹⁵⁷
UL105	U77	(core) DNA synthesis, DE	HP1, DNA helicase-primase subunit; helicase homology ^{21,443}
(RNA5.0)		Noncoding RNA, IE	Stable intron ³¹¹
UL111A		L	vIL-10, viral interleukin 10 and latency-associated (LA) vIL-10 ^{36,587}
UL112–113	U79	DNA synthesis accessory, DE	Multiple spliced variants; transcriptional activation, orchestration of DNA synthesis ²⁹⁵
UL114	U81	(core) DNA synthesis accessory, DE	UNG, Uracil-DNA glycosylase; role in excision of uracil from DNA and control of DNA synthesis ^{496,503,633}
UL115	U82	(core) Virion envelope gp, TL	gL, complexed with gH; gH-gL associates with gO or UL128-UL131A in cell tropism; role in entry ^{74,131,680}

TABLE 62.1 Summary Information on HCMV Gene Productsⁱ (Continued)

HCMV gene ^{a,b}	HHV-6/T HHV-7 ^a	HCMV gene family/expression ^c	Function/comments ^d
UL116 UL117	<i>U84</i>	Putative membrane gp, LL L	Enhances replication compartment formation; impairs cell DNA synthesis ⁵⁰⁰
UL118-UL119 UL120 UL121	<i>U85^d</i>	IgG Fc-binding virion envelope gp, DE UL120 family; putative membrane gp, L UL120 family; putative membrane gp, L	gp68, binds IgG Fc, related to OX-2, ^{35,609}
UL122	<i>U86</i>	IE (alternatively spliced with UL123) (L60, LL, and L40, TL)	IE2, principle transactivator of host cell transcriptional machinery; sequence-specific DNA-binding repressor; cell cycle impact ^{552,631}
UL123	<i>U90^e</i>	IE (major, alternatively spliced with UL122)	IE1, Major IE protein; enhances activation by IE2; indirect enhancement of transcription; binds HDACs; disrupts PML bodies/ND10 ^{576,654}
ORF94 UL124 UL128	<i>U91^e</i>	Latent, DE Membrane gp, DE Virion envelope gp, DE	Inhibits 2',5' oligoadenylate synthetase ⁶⁴⁸
UL130		Virion envelope gp, L	Pentameric gH:gL:pUL128:pUL130:pUL131A complex enhances monocyte, endothelial and epithelial cell tropism ^{523,539,636,699}
UL131A		Virion envelope gp, L	Pentameric gH:gL:pUL128:pUL130:pUL131A complex enhances endothelial and epithelial cell tropism ^{523,539,699}
UL132 UL148		Virion envelope gp, LL Putative membrane gp, L	Pentameric gH:gL:pUL128:pUL130:pUL131A complex enhances endothelial and epithelial cell tropism ^{523,539,699}
UL147A UL147		Putative membrane protein, L UL146 CXC family; putative chemokine, L	Enhances replication in fibroblasts ³⁰⁵
UL146		UL146 CXC family, L	vCXCL1; hCXCR1/hCXCR2-specific chemokine; high sequence variability ³⁵⁵
UL145 UL144		RL1 family, membrane gp, L	TNF receptor homolog; regulates lymphocytes via BTLA; high sequence variability ⁴⁸⁴
UL142		MHC family; membrane gp	Impairs NK cells by blocking NKG2D ligands MICA and ULBP3 ^{31,48,107}
UL141		UL14 family; membrane gp	Inhibits NK cell cytotoxicity by downregulating CD155 and CD112 ^{497,664}
UL140 UL139 UL138		Putative membrane protein Putative membrane gp Membrane protein, DE and latent	Sensitizes to TNFR1 killing; impairs replication in CD34+ and enhances replication in endothelial cells ^{321,406,675}
UL136 UL135 UL133		Membrane protein Membrane protein Membrane protein	⁶⁷⁵ ⁶⁷⁵ ⁶⁷⁵
UL148A UL148B UL148C UL148D UL150 UL150A		Putative membrane protein Putative membrane protein Putative membrane protein Putative membrane protein Putative secreted protein	
<i>IRS1</i> US1 US2 US3		US22 family; tegument, IE US1 family US2 family; membrane gp, DE US2 family; membrane gp, IE	PKR inhibitor; transcriptional activator ³⁷¹ Dislocation of MHC-I from ER ^{350,438} Inhibits processing and transport of MHC-I and, possibly, MHC-II ³⁵⁰
US6 US7		US6 family; putative membrane gp, LL US6 family; membrane gp, LL	Inhibits TAP-mediated ER peptide transport ³⁵⁰

(continued)

TABLE 62.1 Summary Information on HCMV Gene Productsⁱ (*Continued*)

HCMV gene ^{a,b}	HHV-6/T HHV-7 ^a	HCMV gene family/expression ^c	Function/comments ^d
US8		US6 family; membrane gp, DE	Binds MHC-I ³⁵⁰
US9		US6 family; membrane gp, DE	³⁶⁰
US10		US6 family; membrane gp, DE	Degrades HLA-G ⁴⁴⁴
US11		US6 family; membrane gp, DE	Dislocation of MHC-I from ER ^{350,438}
US12		US12 family; putative 7TM protein, DE	
US13		US12 family; putative 7TM protein, DE	
US14		US12 family; putative 7TM protein, DE	¹⁴²
US15		US12 family; putative 7TM protein, LL	
<u>US16</u>		US12 family; putative 7TM protein, DE	Enhances replication in HMVECs ¹⁶⁶ Bronzini et al (in press)
US17		US12 family; putative 7TM protein, DE	Nuclear, fragmented ¹⁴²
cORF29		DE	Nuclear rim-associated cytomegaloviral protein (RASCAL) interacts with pUL50 ³⁹³
US18		US12 family; putative 7TM protein, DE	¹⁴²
<u>US19</u>		US12 family; putative 7TM protein, DE	Enhances replication in HMVECs ¹⁶⁶
<u>US20</u>		US12 family; putative 7TM protein, DE	
US21		US12 family; putative 7TM protein	
<u>US22</u>	<i>DR1x1</i> <i>DR1x2</i>	US22 family; tegument, DE	
US23		US22 family; tegument, DE	
US24		US22 family; tegument, DE	Enhances early infection ¹⁸⁷
US26	<i>DR6x1</i> <i>DR6x2</i>	US22 family; tegument, DE	Enhances release ³⁴⁹
US27		GPCR family; 7TM virion envelope gp, DE	Enhances release ⁴³⁶
US28		GPCR family; 7TM virion envelope gp, DE	Broad spectrum CC and CX3C chemokine receptor; cellular activation and migration ^{528,637}
US29		Putative membrane gp, LL	Enhances replication in RPE cells ¹⁶⁶
<u>US30</u>		Putative membrane gp, DE	Impairs replication in fibroblasts ¹⁶⁶
US31		US1 family	
US32		US1 family, L	Alters PML morphology ⁵⁴⁶
US33A		DE	²⁰⁴
US34		Putative secreted protein, DE	
US34A		Putative membrane protein, DE	
<i>TRS1</i>		US22 family; tegument, IE	PKR inhibitor, transcriptional activator; capsid assembly ³⁷¹

ⁱCoordinated with HCMV genome map (Figure 62.3).

^aCommonly annotated open-reading frames (ORFs) of human cytomegalovirus (HCMV), together with selected ORFs conserved in roseoloviruses, human herpesvirus (HHV)-6 (A or B), and HHV-7. Additional references⁴⁰⁰ and functional dissection by mutagenesis^{167,727} showing replication better than wild-type (*underlined normal type*), little difference from wild-type (*normal type*), or one of two broad growth deficient phenotypes: failure to replicate (*bold black²*), or poor replication (*bold gray³*), when viruses are assayed in fibroblasts. ORFs that impact levels of replication in a cell type-specific manner are underlined.

^bBeta herpesvirus-common genes are in italics, and include 40 herpesvirus core functions, labeled "core" as well as genes conserved in beta- and gamma herpesviruses, labeled "betagamma." Gene family, characteristics, and functional information are provided for HCMV, where a blank indicates the absence of information.

^cGene family (see Fig. 62.2) and expression class based on transcript accumulation (IE, immediate early; DE, delayed early; LL, leaky late; TL, true late; L, late, not further studied).

^dFunctional information is based on information from studies on HCMV, murine cytomegalovirus (MCMV), and other herpesviruses. References limited to recent publications focused on gene function in HCMV.

^ePositional homolog lacking sequence similarity.

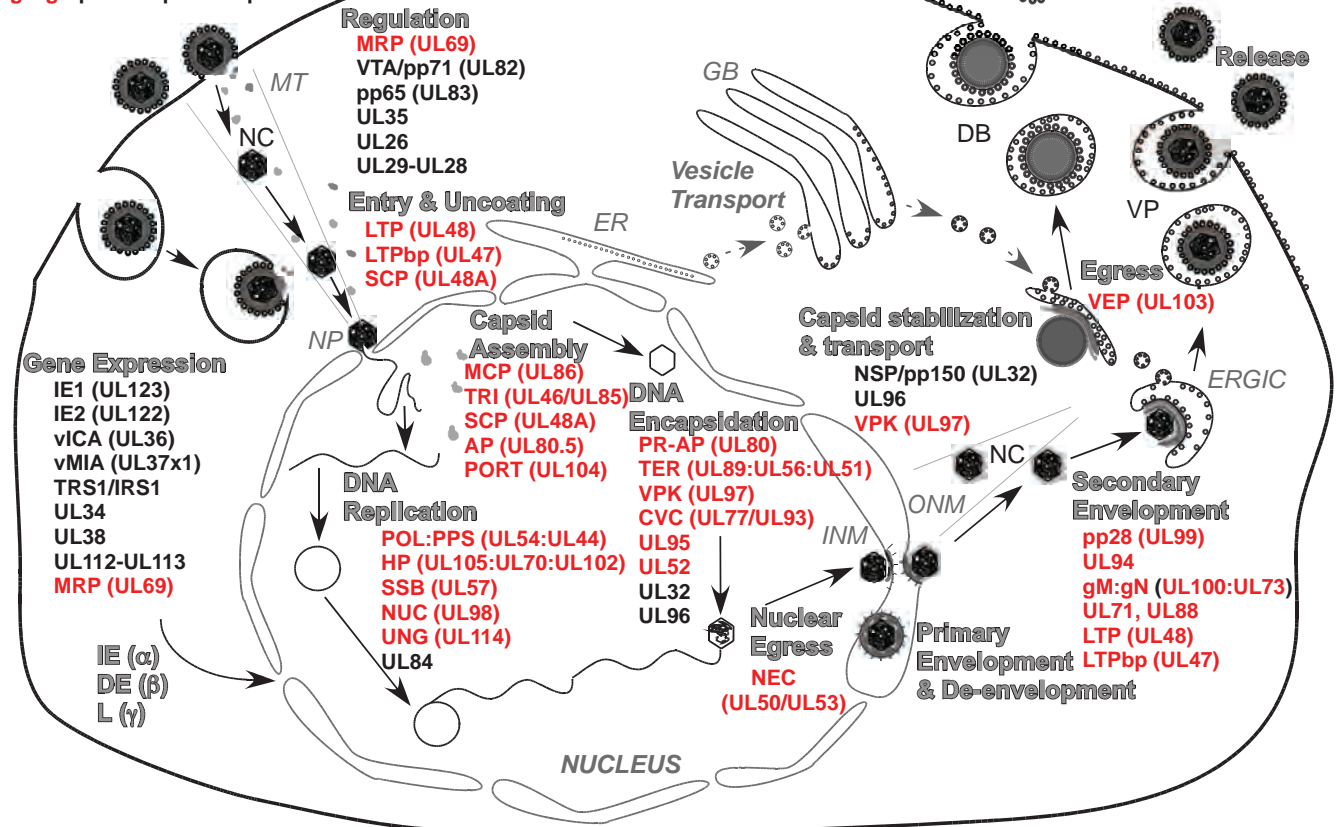
Attachment & Penetration**gB (UL55)****gH:gL (UL75:UL115)****gH:gL:gO (UL75:UL115:UL74)****gH:gL:pUL128:pUL130:pUL131A**

FIGURE 62.2. Summary of the human cytomegalovirus (HCMV) replication pathway. Major steps in productive replication are indicated in *large gray font with outline*, black arrows indicate the progression of steps and viral functions (see Table 62.1 for summary descriptions). Individual gene products listed under each step are identified by provisional abbreviated names^{399,400} as either herpesvirus core (red text) or beta herpesvirus conserved (black text). Viral attachment and penetration occur either via direct fusion at the cell surface (fibroblasts), dependent on gB, gH:gL and gH:gL:gO, or via endocytosis into other cell types (endothelial and epithelial cells) where the pentameric complex, gH:gL:p128:p130:p131A, also facilitates entry (see text). In addition to the interferon (IFN)-like activation of cells by the process of attachment and penetration, input virion tegument proteins (UL69/MRP, pp71/VTA, pp65, UL35, UL26, and UL29-UL28) regulate cellular pathways. NC-associated UL47, UL48, and smallest capsid protein (SCP) are predicted to facilitate the final steps in entry and uncoating that deliver input nucleocapsid (NC) via microtubules (MT) to nuclear pore (NP) complexes where the viral genome is released into the nucleus. Transcriptional regulation of viral and host cell gene expression is mediated by IE genes (IE1, IE2) or DE genes (UL34, UL35, UL112-UL113, and UL69); cell death suppression is mediated by IE gene products vICA and vMIA, and other regulatory processes are facilitated by UL34, UL38, and UL112-UL113 proteins. DNA replication depends on core proteins (POL:PPS, HP, SSB, NUC, and UNG) as well as one beta herpesvirus-specific protein (UL84 gene product) that facilitates initiation of DNA synthesis. Capsids assemble from MCP, TRI, SCP, PORT, and AP. Preformed capsids process PR-AP (UL80) and AP as viral DNA is encapsidated by the TER complex (UL89, UL56, UL51) through a PORT (UL104) pentamer, followed by predicted addition of the CVC complex (UL77, UL93) onto NC pentamers, with UL95, UL52, UL32, and UL96 added for NC stabilization. Nuclear egress of the NC is mediated by the NEC (UL50, UL53). Following primary envelopment at the inner nuclear membrane (INM), and de-envelopment at the outer nuclear membrane (ONM), capsid stabilization is ensured by the function of NSP/pp150 (UL32) and UL96, with nuclear egress and transport facilitated by VPK (UL97). Glycoproteins incorporated into the envelope are synthesized in the endoplasmic reticulum (ER), glycosylated in the Golgi body (GB), and delivered by vesicle transport (dashed gray arrow) to join NC at sites of secondary envelopment on ER Golgi intermediate compartment (ERGIC). Secondary envelopment requires UL99, UL94, gM/UL100:gN/UL73, UL71, UL88, UL47, and UL48, acting together with VPK. Following the acquisition of an envelope, virus particle (VP) as well as capsidless dense body (DB) egress is facilitated by VEP/UL103 for release into the extracellular space.

a wild-type HCMV prototype^{106,139,141,149,150} (see Fig. 62.1). Adapted strains replicate very well in fibroblasts but poorly on cultured macrophages, dendritic cells, endothelial cells, or epithelial cells. In addition to adaptive mutations, it has become clear that viral stocks often consist of multiple strain variants and that laboratory adapted viral strains in common use around the world differ markedly.^{77,138} Serial propagation of common laboratory strains results in stable strain variants^{77,138} that exhibit unique biological properties such as upregulation of cell cyclin-dependent kinases resulting in pseudomitosis²⁴⁶ as well as other alterations in host cell response to infection.³⁹⁹ Comparison of viral genomes from low passage or even non-propagated wild-type strains in clinical samples facilitated current estimate of HCMV genome coding capacity^{106,139,141,149,150} and led to the derivation of bacmid clones of low-passage strains for experimental use.^{584,619} Recent deep DNA sequencing has revealed considerable heterogeneity as well as the presence of multiple viral strains within clinical samples.^{139,216,217,520} Deep sequencing approaches have also facilitated assembly of a high-resolution transcriptome²⁰⁴ and unveiled a level of messenger RNA (mRNA) greater than previously appreciated for this virus, two features that still need to be integrated into the growing understanding of HCMV biology.

The AD169 and Towne stocks distributed by the American Type Culture Collection (ATCC) include a mixture of genomes. Replication-competent variants with substantive genome rearrangements and deletions have been independently propagated from various AD169 and Towne preparations.^{77,138} Cell tropism factors have come from such studies⁵³⁸ and have opened the way to a more complete understanding of HCMV biology. Two examples include the role that virion envelope glycoprotein gpRL13 plays in suppressing replication in fibroblasts⁶¹⁹ and the role that the pentameric complex composed of gH:gL:pUL128:pUL130:pUL131A plays in facilitating entry into epithelial and endothelial cells.^{523,539,699}

CLASSIFICATION

The starting point for classification of cytomegaloviruses infecting humans and other animals is comparative biology, physico-chemical characteristics, and virion morphology. Over the last 20 years, genome sequence analysis has eclipsed other approaches in viral taxonomy.^{146,465} Cytomegaloviruses have been isolated from a wide variety of mammalian species, including dogs, horses, bats, cows and pigs, although many have not been fully characterized.²⁴⁷ Beta herpesviruses, in general, are associated with universal infection in their natural host species. Four groups of beta herpesviruses have been officially recognized⁴⁶⁵: (1) known human and primate cytomegaloviruses; (2) muromegaloviruses, including MCMV and rat CMV; (3) beta herpesvirus causing roseola (human herpesvirus type 6B [HHV-6B]) as well as two close relatives (HHV-6A and HHV-7); and, (4) proboscivirus, endotheliotropic elephant herpesvirus. Other beta herpesviruses, such as guinea pig CMV and porcine CMV, as well as Tupaia herpesvirus, remain unclassified.⁴⁶⁵ The beta herpesvirus subfamily exhibits a greater level of evolutionarily and genetic divergence than either alpha herpesviruses or gamma herpesviruses. This situation poses a challenge to investigators trying to understand virus biology by studying these surrogate animal models. MCMV and HCMV exhibit many common biological

attributes in pathogenesis, immunomodulation, and latency, but appear to achieve these ends via evolutionarily divergent mechanisms and gene products.^{348,394,398,401,405} RhCMV has been championed as more HCMV-like^{41,490} and has raised interest in cytomegaloviruses as vaccine vectors in the face of preexisting immunity.²³⁷ Importantly, nonhuman primate surrogates have obvious limitations in that the host animals are limited, plus RhCMV is evolutionarily divergent. There remains a need for intensive investigation of natural HCMV infection and immunity, as well as for further optimization of humanized small animal models^{593,675} to facilitate direct study of HCMV.

The HCMV genome is the largest of any characterized herpesvirus, at 236,000 bp (HCMV), with an annotated capacity to encode at least 167 protein-coding gene products¹³⁹ (Table 62.1 and Fig. 62.3), with extensive alternate mRNA splicing in certain regions²⁰⁴ (Table 62.2), plus noncoding RNAs as well as micro (mi)RNAs^{156,182,223,623} (Fig. 62.3). Other cytomegaloviruses exhibit a level of genome complexity similar to HCMV, whereas roseoloviruses have smaller genomes encoding approximately 85 genes. Alternative annotation methods^{82,473} and sensitive experimental detection^{156,182,204,223,623} have complemented each other in the annotation of HCMV genome coding potential.

Neither human roseoloviruses nor nonhuman cytomegaloviruses share significant DNA sequence identity with HCMV. Evolutionary relationships between beta herpesviruses emerge from comparisons of predicted protein coding sequences and appear to follow relationships of host animal species consistent with longstanding pathogen–host co-evolution.⁴⁰⁵ Independent coevolution belies the biological similarity of cytomegaloviruses infecting diverse mammalian hosts. Only 75 of the estimated 167 HCMV protein-coding genes are conserved across this group, including 40 core herpesvirus and seven (UL49, UL79, UL87, UL88, UL91, UL92, and UL95) betagamma conserved genes (Table 62.1). On one end of this spectrum, 163 of the predicted 168 chimpanzee CMV proteins are homologous and colinear with HCMV.¹⁴⁹ Only about 111 RhCMV genes are homologous to HCMV genes⁵³¹; in guinea pig CMV, relatedness falls off to 84 homologs,²⁸² and in MCMV or rat CMV genomes 75 sequence homologs are present.²⁸² These comparisons have consistently shown that beta herpesviruses are remarkably diverse in genetic composition. As information accumulates, it appears that the immune modulators in these viruses evolved to target a common set of host immune control pathways from evolutionarily distinct origins and using distinct mechanisms. Because of the smaller number of total genes, a high proportion of human roseoloviruses genes have homologs in HCMV,¹⁴⁹ and these overlap with the genes retained in all animal cytomegaloviruses. With few exceptions, both beta herpesvirus-conserved genes and herpesvirus core genes are concentrated between UL23 to UL123, with additional US22 family members flanking this region. Many genes within as well as outside this central region are involved in modulation of the host response to virus infection. Genes near the ends of viral genomes represent the most recent evolutionary acquisitions and provide evidence of evolutionary adaptation to host defense pathways.⁴⁰⁵

Like all herpesviruses, beta herpesviruses have linear DNA genomes with direct terminal repeats containing the *cis*-acting signals (*pac1* and *pac2*) recognized by the encapsidation machinery to initiate packaging and direct genome cleavage.⁷⁰² HCMV and guinea pig CMV have large (approaching 1,000 bp) terminal repeats, whereas MCMVs has a small (<50 bp)

TABLE 62.2 Summary Information on HCMV miRNAs

miRNA ^{a,b}	Sequence ^c	Abundance ^d	Function/comments ^e
miR-UL22A-1-5p	TAAC TAGCCTTCCCGTGAGA	0.041	Detected in cells infected with HCMV AD169 ²²¹ and clinical isolates AF2380 U8795, ⁶²⁶ but not Towne ⁶²³
miR-UL22A-1-3p	TCACCAGAATGCTAGTTTGTAG	0.094	
miR-UL36-1-5p	TCGTTGAAGACACCTGGAAAGA	0.284	
miR-UL36-1-3p	TTTCCAGGTGTTTTCAACGTG	0.004	
miR-UL70-1-5p	TGCGTCTCGGCCTCGTCCAGA	ND	
miR-UL112-1-5p	CCTCCGGATCACATGGTTACTCA	<0.001	Downregulates pUL123 (IE1), ^{222,419} pUL112/113, ²²² pUL120/121, ²²² pUL114, ⁶²⁶ MHC I-related chain B (MICB), ⁶²⁵ and interferon regulatory factor 1 (IRF-1) ³³¹ ; elevated expression in plasma of hypertensive patients versus normals (p < 0.0001) ³³¹
miR-UL112-1-3p	AAGTGACGGTGAGATCCAGGCT	0.012	
miR-UL148D-1	TCGTCCTCCCCTTCTTACCCT	0.001	
miR-US4-1-5p	TGGACGTGCAGGGGATGTCTG	0.002	
miR-US4-1-3p	TGACAGCCGCTACACCTCTCT	<0.001	
miR-US5-1	TGACAAGCCTGACGAGAGCGT	0.001	Synergizes with miR-US5-2-3p to downregulate pUS7 ⁶⁶¹
miR-US5-2-5p	CTTTCGCCACACCTATCCTGAAA	0.001	Synergizes with miR-US5-1 to downregulate pUS7 ⁶⁶¹
miR-US5-2-3p	TATGATAGGTGTGACGATGTCT	0.382	
miR-US22-1-5p	TGTTTCAGCGTGTGTCCGCGGG	0.007	
miR-US22-1-3p	TCGCCGGCCGCGCTGTAACCAGG	0.001	
miR-US25-1-5p	AACCGCTCAGTGCTCGGACC	0.063	
miR-US25-1-3p	GTCCGAACGCTAGGTGCGTTCT	<0.001	Down regulates cyclin E2 and other cell cycle genes by targeting 5' UTRs ²²⁴ ; downregulates viral DNA replication but not specific to HCMV ⁶²⁶
miR-US25-2-5p	AGCGGTCTGTTCAGGTGGATGA	0.028	
miR-US25-2-3p	ATCCACTTGGAGAGCTCCCGCGG	0.037	
miR-US33-1-5p	GATTGTGCCCGGACCGTGGGCG	0.015	
miR-US33-1-3p	TCACGGTCCGAGCACATCAA	0.001	
miR-US33as-1-5p	TGGATGTGCTCGGACCGTGACG	0.022	Downregulates viral DNA replication but not specific to HCMV ⁶²⁶
miR-US33as-1-3p	CCCACGGTCCGGGCACAATCA	0.002	

^amiRNAs were identified by a combination of small RNA cloning and sequencing,^{168,473} comparison of HCMV and chimpanzee genomes,²²¹ and deep sequencing of small RNAs⁶²³ from infected fibroblasts. miR-UL23-5p, miR-UL23-3p, and miR-US24¹⁶⁸ were renamed miR-UL22A-5p, miR-UL22A-3p, and miR-US25-1, respectively.¹⁶¹

^bmiRNAs are named for the viral open-reading frame (ORF) with which they overlap; or, if they reside between ORFs, they are named for the nearest upstream annotated ORF encoded on the same strand of the viral genome. ORF designations are followed by a number to distinguish multiple miRNAs in the same ORF location, and the two arms processed from a common pri-miR hairpin are distinguished by 5p versus 3p.

^cMost abundant form in fibroblasts at 72 hpi with the HCMV Towne strain.⁶²³

^dRelative abundance at 72 hpi is based on a calculation from deep sequencing results reported in⁶²³; that is, the number of sequence reads for each miRNA divided by the combined sequence reads for all miRNAs (except miR-UL70-1–5p, which was not detected, ND).

^eTarget identification/validation methods included: Luciferase reporter assay or ectopic expression; Protein assay in infected cells; Virus mutated for miRNA; Virus mutated for target site; RISC immunoprecipitation.

terminal repeat that suffices for genome cleavage and packaging.³⁸⁵ Similar to human alpha herpesviruses, primate cytomegaloviruses have an internal inverted copy of the *a* sequence terminal repeat that supports homologous recombination-driven genome rearrangement during replication.⁴²⁴ As a result, HCMV and chimpanzee CMV have class E genomes (Fig. 62.1; see Chapter 59, Herpesviridae) generating four isomers that appear to package with equal efficiency, whereas RhCMV, roseoloviruses, muromegalovirus, and guinea pig CMV have linear DNA genomes that do not rearrange at all (referred to as class A genomes). Genome isomerization is dispensable for HCMV replication⁵⁵⁵ as it is in other herpesviruses. The importance of genome isomerization in the biology of any herpesviruses remains a complete mystery.

Consistent with the conserved set of proteins and despite variation in genome structure and existence of genome isomers, all beta herpesviruses, follow a replication pathway (Fig. 62.2) from DNA replication^{21,424} through capsid formation and DNA encapsidation,^{84,99} nuclear egress, and final virion maturation steps^{80,653} common to all herpesviruses.

VIRION STRUCTURE

HCMV has a structure characteristic of all herpesviruses,^{80,84,99} with a DNA core inside of a highly stable icosahedral capsid made up of 162 capsomeres surrounded by an envelope derived from host cell membrane containing viral glycoproteins to

control attachment and entry into cells. Herpesviruses have a particularly thick tegument (or matrix) layer of virus-encoded proteins between the capsid and envelope. Cryoelectron microscopy in combination with computer-assisted tomographic image reconstruction (cryoEM or cryoET)^{84,342} has provided accurate nucleocapsid dimensions as well as other structural details of the 200 to 230 nm virion particle. HCMV has a 130-nm icosahedral nucleocapsid, somewhat larger than other herpesviruses, that accommodates a large genome. Overall, the virion is the most structurally complex of the characterized herpesviruses. Whether observed in thin sections of infected cells (Fig. 62.1) or as purified virion and dense body preparations resolved either by transmission EM or cryoEM, virus particles have a pleomorphic appearance with a voluminous tegument layer that is asymmetric.⁷²⁹

HCMV capsids are composed of four herpesvirus core proteins, major capsid protein (MCP, the UL86 gene product) comprising hexons and most pentons, triplexes composed of two subunits, triplex subunit 1 (TRI1, the UL46 gene product or minor capsid protein) and triplex subunit 2 (TRI2, the UL85 gene product or minor capsid protein binding protein), and the smallest capsid protein (SCP, the UL48A gene product). Unlike the situation in HSV-1 where SCP is dispensable, all capsid proteins are essential for HCMV replication.^{72,80,315} By analogy with HSV-1,^{84,99} one specialized penton composed of the portal protein (PORT, the UL104 gene product) acts as a channel for both encapsidation and release of viral DNA together with two principal subunits of the terminase, subunit 1 (TER1, the UL89 gene product) and subunit 2 (TER2, the UL56 gene product). A capsid vertex-capping (CVC) complex composed of UL77 and UL93 proteins decorates all pentons¹³³ and the proteins encoded by UL51 and UL52 likely provide stability. Inside the HCMV nucleocapsid is a ~236-kb linear DNA genome together with two virion (v) RNAs, ~300 nucleotide vRNA-1, and ~500 nucleotide vRNA-2, embedded as an RNA–DNA hybrid in an essential region the lytic origin of DNA synthesis (*oriLyt*).^{21,443} The nucleocapsid is enclosed in a tegument (or matrix) composed of at least 32 virus-encoded proteins, many of which are phosphorylated. Small amounts of cytoplasmic proteins⁶⁸¹ as well as RNAs⁶⁵⁷ are captured into mature virus particles, likely passively. The tegument is surrounded by a lipid bilayer envelope that is derived from the ER–Golgi intermediate compartment (ERGIC) or recycling endosomal compartment modified by insertion of approximately 23 virus-encoded glycoproteins. Five envelope glycoproteins (gB, gH:gL, gM:gN) provide essential replication functions and are targets of neutralizing antibody. In total, the virion is composed of at least 66 virus-encoded proteins that play diverse roles during infection (Table 62.1 and Fig. 62.2). Many virion proteins mediate and modulate entry and egress, influence cell tropism, and interface with the host response to infection. Although additional minor tegument and envelope components are likely to be recognized given the large coding capacity and truly extensive mRNA splicing,²⁰⁴ HCMV has the most complex of herpesvirus virions.

Purified HCMV preparations contain an abundance of noninfectious defective particles in addition to virions. Infected cells release both capsidless and capsid-containing particles such that defective particles constitute roughly 99 percent of material in highly purified virus preparations. The most carefully purified HCMV preparations exhibit particle-to-PFU (particle forming unit) ratios exceeding 100, and high

multiplicity propagation can result in particle-to-PFU ratios approaching 10,000 due to enrichment of defective interfering particles.⁶³⁰ Capsidless dense bodies constitute approximately 50% of particles collected from culture supernatants (Fig. 62.1). Dense body formation depends upon pp65 tegument protein and takes place in the same cytoplasmic AC using the same cytoplasmic membranes as virions (Fig. 62.2). Noninfectious enveloped particles (NIEPs) form when genomeless capsids mature through the cytoplasm and are released.²¹⁰ Even carefully executed studies seeking to identify virion and dense body structural components^{657,681} have encountered heterogeneity, cross-contamination, and difficulty in removing host contaminants even though these virus particles have been enriched by differential sedimentation. Furthermore, virus strain-to-strain variability may also influence protein composition of virus particles (Plachter, personal communication). An abundance of DNA-containing defective particles are produced during infection but cannot be distinguished or separated from infectious virions by physicochemical properties.

Capsid

CryoEM analyses³⁴² revealed unique characteristics of the inner capsid surface of HCMV and simian CMV nucleocapsids, in addition to the larger volume to accommodate these large genomes. The icosahedral nucleocapsid exhibits icosahedral T = 16 symmetry assembled from 162 capsomeres like all herpesviruses and the HK97 group of tailed bacteriophages.^{84,99} One hundred fifty hexons, each consisting of six molecules of MCP, make up the triangular faces of the capsid. Eleven pentons, each consisting of five molecules of MCP together, plus one specialized penton made of PORT complete the capsid wall. Hexons and pentons together form the bulk of the 15-nm-thick capsid walls. Therefore, like all herpesviruses,^{84,99} the HCMV capsid comprises 955 molecules of MCP, 12 molecules of PORT, 320 copies of a 2:1 complex consisting of TR2 and TR1 making contact with three capsomers just above the capsid floor, and 900 molecules of SCP, forming six member rings on MCP at hexon tips. The importance of UL77 and UL93 as a CVC complex or the precise role of UL51 and UL52 in nucleocapsid stabilization, have not yet been resolved in structural studies. The organized layer of material on the outer surface of the capsid has been ascribed to one of the major tegument proteins, pp150,⁷²⁹ a structural detail that is unique to HCMV.

Based on studies with HSV-1, an HCMV procapsid shell is likely formed when MCP is translated and imported into the nucleus together with scaffold subunits comprised of assembly protein (AP, UL80.5 gene product) and maturational protease (PR), a PR–AP fusion protein (the UL80 gene product).²¹⁰ After chaperoning subunits to the nucleus where maturation proceeds, the scaffold is replaced as viral DNA is packaged by encapsidation machinery. The capsid assembly process is also common to other herpesviruses^{84,99} and yields three distinct nuclear capsid forms, termed A, B, and C capsids. C capsids represent DNA-containing nucleocapsids that appear to be in the process of maturing; whereas, A and B capsids represent aberrant particles that appear to have failed to complete encapsidation. Viral DNA is arranged in three-dimensional hexagonally packed arrays within the interior of the nucleocapsid together with polyamines and *oriLyt* RNA but without additional virus or host proteins. Some HCMV B capsids complete maturation to become NIEPs. In addition, B capsids accumulate when

maturation is blocked, either using HCMV-specific encapsidation inhibitors,^{158,215} by inhibiting expression of the PR-AP⁷³⁰ or by employing other viral mutants that fail to encapsidate viral DNA.^{73,80} Therefore, there is strong evidence that viral DNA encapsidation drives the generation of C capsids (nucleocapsids) and that these translocate to the cytoplasmic AC where maturation continues and final envelopment takes place.

Tegument

The 32 known tegument proteins (Table 62.1) carry out diverse activities, from conditioning the host cell at the beginning of infection to orchestrating the final stages of virion assembly. They are added to the maturing nucleocapsid in sequential layers, beginning in the nucleus and continuing in the cytoplasmic AC. At the start of infection, these proteins, located entirely within the virion between the nucleocapsid and the lipid bilayer envelope, direct nucleocapsid translocation on microtubules to nuclear pore complexes, delivering the viral genome to the nucleus while also overtaking the host cell machinery (Fig. 62.2). During the final stages of maturation, tegument proteins control nucleocapsid stability, trafficking, and envelopment from the nucleus through to final steps in egress (Fig. 62.2). Many tegument proteins are conserved across herpesviruses. The small amounts of viral and cellular RNAs as well as host proteins that appear to be passively acquired during envelopment reside in the tegument. Most tegument proteins are phosphorylated and many are highly immunogenic.^{80,262}

The most abundant tegument protein in virions is pp65 (lower matrix protein, UL83 gene product). This protein is the major constituent of capsidless dense bodies and is acquired during envelopment in the AC. Despite its abundance and potential importance during natural infection in humans, UL83 is dispensable for replication in cultured cells.⁵⁶⁴ pp65 is highly immunogenic and has proven very useful for monitoring virus-specific immunity in the population because it is a target of MHC class I-restricted CD8 and MHC class II CD4 T-cell responses.⁶⁴⁴ This tegument protein is also the most abundant viral protein in virus-infected cells and may be transferred to neutrophils that come into contact with virus-infected cells during natural infection. Detection of pp65 in PB neutrophils has been the basis of the antigenemia diagnostic assay.³³⁶ Immediately following viral entry, pp65 localizes to the nucleus of infected cells where it has an immunomodulatory role dampening the interferon-like cellular response to infection.²⁶² Late in infection, pp65 is associated with the AC where virion and dense body envelopment occur.

The HCMV UL32, UL48, and UL82 genes encode abundant tegument proteins that play crucial roles during infection. The virion transactivator (VTA) pp71 (upper matrix protein, UL82 gene product), a sequence homolog of UL83, localizes to the nucleus following entry and recruits cellular transcription machinery to activate immediate early (IE) gene transcription.^{472,631} The pp150 (large matrix phosphoprotein, UL32 gene product) is capsid-proximal in virions⁷²⁹ and plays an essential role sustaining stability of maturing nucleocapsids during translocation from the nucleus to the cytoplasmic AC.^{651,653} This phosphoprotein is recognized by more than 80% of HCMV-seropositive sera and is also an important target of cellular immunity. The largest tegument protein (LTP; UL48 gene product) is capsid proximal,⁷²⁹ and is expected to stabilize nucleocapsids, like its HSV-1 homolog.^{560,645} An important enzyme,

the viral protein kinase (VPK, the UL97 gene product),⁴⁹⁴ is incorporated into virions as a tegument constituent and may impact early stages of infection,³⁶⁶ even though its major role is later in facilitating a number of steps during maturation.⁴⁹⁴

Many protein-protein contacts are involved in the organization of the tegument layer. A number of approaches have been employed to recapitulate these interactions, most recently focused on building a systems-level framework of binary relationships between structural proteins.^{474,662} Interactions between pp150 tegument protein and MCP, pUL47/pUL48, and MCP as well as pUL48 and pp28 may help direct envelopment in the AC.⁸⁰ It is likely that many additional contacts serve to establish and maintain the structure of the tegument and provide continuity between the nucleocapsid and the envelope.

The remaining tegument proteins account for a small percentage of virion or dense body proteins,⁶⁸¹ but contribute to entry and maturation (Table 62.1 and Fig. 62.2). One class is involved in replication steps, including disassembly of virions and release of the viral genome into the nucleus, transcriptional regulation, or virion assembly. Other tegument proteins modulate or modify the host cell response to infection, inactivating host cell transcriptional repression mechanisms, blocking intrinsic cell defenses, altering the host cell cycle, and optimizing the intracellular environment for virus replication. US22 family members (UL23, UL24, UL25, UL26, UL29-28, UL36, UL43, IRS1, US22, US23, US24, US26, and TRS1 gene products) modulate host cell signaling and cell death pathways.

Envelope

Virions, dense bodies and other noninfectious virus particles are enclosed in a lipid bilayer envelope derived from cytoplasmic ERGIC or endosomal membranes^{80,653} as depicted in Figure 62.2. Remarkably, as many as 23 different viral glycoproteins have been associated with purified virion and dense body preparations.⁶⁸¹ Some of these contribute to attachment and entry, but most are more likely involved in modulation of the host cell response to infection. In contrast to the alpha- and gamma herpesviruses where a subfamily-specific envelope glycoprotein such as gD (herpes simplex virus type 1 [HSV-1]) and gp350 (Epstein-Barr virus [EBV]) dictates attachment and entry, HCMV relies on homologs of the three major conserved glycoprotein complexes (gC1, gCII, and gCIII, comprised of gB, gM:gN, and gH:gL, respectively). gB and gH:gL are key to attachment and entry, whereas gM:gN is involved in maturation (Table 62.1, Figs. 62.2 and 62.3). These glycoproteins accumulate on internal infected cell membranes as well as on the plasma membrane during infection, and they are the principal targets of antibodies that neutralize virus.⁸⁰

UL55-coded HCMV gB forms a trimer (gC1) on the envelope to mediate membrane fusion in attachment and entry. This is a major target of neutralizing antibody and, like other viral fusion proteins, undergoes a conformational change to fuse the virion envelope and target cell membranes during entry. Based largely on structural comparisons to truncated gB from HSV-1 or EBV,¹³² which are closely related sequence homologs, HCMV gB is a class III fusion protein related to rhabdovirus G and baculovirus gp64. gB mediates binding to heparan sulfate proteoglycan, an initial step in attachment, as well as either pH-independent entry directly at the plasma membrane, as occurs in fibroblasts, or pH-independent entry via the endocytic route, as occurs in endothelial and epithelial

cells.²⁶² gB is not involved in maturation or release of progeny virions, but is important for both cell-to-cell spread and cell–cell fusion leading to syncytia, both of which involve membrane fusion. Cellular receptor(s) for gB are still being investigated. Candidates include cell surface integrins $\alpha 2\beta 1$, $\alpha 6\beta 1$, and $\alpha V\beta 3$ on all cells,²⁶² and particularly $\beta 1$ integrin via a disintegrin-like domain,¹⁸⁶ epidermal growth factor receptor (EGFR) on monocytes,¹⁰⁸ and platelet-derived growth factor receptor α (PDGFR) on endothelial, epithelial, and fibroblast cells.⁶⁰² HCMV receptors (also called entry mediators) may enhance early viral gene expression through signaling pathways, but the role of signaling in entry remains unclear.^{262,434}

Late in replication, as virion maturation proceeds, gB is cleaved by a cellular furin-like protease to generate a 116-kD surface component linked by disulfide bonds to a 55-kD transmembrane component. Unlike the situation with many RNA viruses, proteolytic cleavage is not a requisite for gB function under any conditions that have been studied.²⁶² HCMV neutralizing antibody in convalescent sera recognizes gB as well as other envelope glycoproteins. A soluble form of gB has been shown to elicit protective immunity as an oil-in-water (MF59) adjuvanted subunit vaccine,^{228,456} suggesting that this strategy may lead to a safe vaccine to prevent infection and disease. When administered prophylactically, pooled human gammaglobulin with high gB-specific binding antibody titer has been reported to benefit SOT recipients⁵⁹⁵ and congenitally infected infants.^{359,431} Antibodies that recognize different antigenic domains can inhibit viral attachment or prevent fusion,⁴⁸⁸ reinforcing both aspects of gB function during entry.²⁶²

A second envelope glycoprotein complex (gcII) includes UL100-coded gM and UL73-coded gN. gM may be the most abundant envelope glycoprotein.⁶⁸¹ gM:gN is clearly important during maturation^{307,308} and has not been implicated in entry. gM is an eight-membrane spanning protein that binds heparan sulfate. Experimentally, only a small portion of gM is required for complex formation with gN.³⁵⁷ gN is one of the most highly variable gene products and has been used to track viral strains in disease settings. One particular variant (gN4) has been associated with an increased congenital disease risk.⁴⁷⁹

The third envelope glycoprotein complex is composed of gH (UL75 gene product) and gL (UL115 gene product) that may be modified with additional proteins to control infection in different cell types. The main function of gH:gL is to influence attachment and gB-mediated fusion. HCMV gH:gL and gB together mediate cell fusion more efficiently than gB alone,²⁶² similar to the situation in other herpesviruses.¹³² Therefore, most evidence indicates gH, in particular, controls postattachment enhancement of fusion.²⁶² Detailed crystallographic structural studies completed on HSV-1 and EBV gH:gL¹³² have not provided insight into the gH:gL control of gB activity. gH is a transmembrane protein that requires gL as a chaperone to properly mature.⁸⁰ gH alone, as a component of gH:gL or in other higher order complexes, behaves in ways that suggest it controls the interaction with a cellular receptor,⁶⁷⁹ and although integrin $\alpha v\beta 3$ may be engaged,⁷⁰³ this area remains elusive.

As first shown with EBV, subpopulations of envelope gH:gL associate with additional proteins that act as tropism determinants and dictate entry efficiency for particular cell types. A majority of gH:gL exists as the unmodified heterodimer glycoprotein complex. One additional HCMV component, the UL74-encoded gO, may either be a chaperone^{540,710} or a structural component,⁶⁷⁹

and apparently facilitates entry.^{262,569} In addition to the trimeric gH:gL:gO complex, a pentameric gH:gL complex containing UL128, UL130, and UL131A gene products⁵⁴¹ enhances infection in epithelial and endothelial cells^{539,699} as well as virus interactions with neutrophils, dendritic cells, and many other cell types.⁵²³ This complex may recognize novel receptors to initiate an endocytic entry pathway or facilitate fusion at a postattachment step that follows endocytosis.^{523,539} Viruses lacking components of the pentameric gH:gL:pUL128:pUL130:pUL131A complex enter and replicate without compromise in fibroblasts. Attachment and endocytosis into endothelial and epithelial cells is also independent of the pentameric complex, but the final step of entry into the cytosol is inefficient in its absence.^{539,541} Evidence is accumulating around the pentameric complex that virus particles produced in one cell type differ in biological characteristics from particles produced in another cell type.⁵⁶⁹ For reasons that remain largely unexplained, mutations in UL128, UL130, or UL131A accumulate rapidly as HCMV is propagated in fibroblasts, where entry occurs directly at the cell surface, have given the impression that the pentameric complex expression is deleterious to replication in this cell type.^{262,539,569}

Many of the HCMV envelope glycoproteins that are not directly involved in entry or egress have immunomodulatory potential. The virion contains glycoproteins that bind to IgG encoded by RL11 and UL119-118⁶⁰⁹ as well as secreted glycoproteins, such as the UL22A gene product, the RANTES (Regulated and Normal T Cell Expressed and Secreted) chemokine decoy receptor (Table 62.1) that impact the host response to infection. G protein-coupled receptor (GPCR) homologs encoded by UL33, UL78, US27, and, particularly, US28, have attracted the most attention as immunomodulators. US28 encodes a constitutively strong and ligand-inducible CC/CX3C chemokine receptor,^{202,298,426} long suggesting a role in infection or behavior of virus-infected cells as well as pathogenesis. US28 has been associated with signaling via phosphorylation-, beta-arrestin-, G $\alpha 12$ - and RhoA-dependent activity, and induction of apoptosis and cell migration.^{575,686,690} US27 does not signal but influences viral spread,⁴³⁶ and virion-associated UL33 and UL78 gene products have biological roles that remain unclear.^{575,686,690} Receptor heterodimerization may contribute to the activities of viral GPCRs.⁶⁷⁰

VIRAL GENOME

The genomes of HCMV and closely related chimpanzee CMV align closely, retaining a class E genome arrangement with unique long (U_L) region and unique short (U_S) regions flanked by terminal and internal repeats in an arrangement first described for HSV-1 ($ab-U_L-b'a'c'-U_S-ca$) (see Chapter 59, Herpesviridae) and shown in Figure 62.1. This arrangement of terminal and internal inverted a sequences, which contain the genome cleavage/packaging signals, promotes genome isomerization during replication. Such genome inversion generates four equimolar and independently infectious isomers with regard to the orientation of the short (S) and long (L) genome components. This process is dispensable for replication⁵⁵⁵ and the artificial creation of an inverted copy of the terminal repeat in a viral genome that naturally lacks such repeats, triggers isomerization.³⁸⁶ The c' and c repeats (also called IRS and TRS) flanking the S genome component have a partially duplicated

IE gene set (IRS1 and TRS1) that are conserved in HCMV strains. In contrast, the large *b* and *b'* repeats (also called TRL and IRL) flanking the L genome component originally characterized in the AD169 strain, are apparently the result of extensive cell culture propagation.¹⁴⁹ The RhCMV genome^{239,531} has direct terminal repeats but lacks internal repeats or genome isomerization. Directly repeated *a* sequences of variable length and copy number are found at genome termini and include two herpesvirus-conserved *cis*-acting cleavage/packaging signals, *pac1* and *pac2*. These elements are recognized by the encapsidation machinery to initiate genome packaging and feed the DNA into a capsid as well as to signal cleavage once a genome-length is reached. The cleavage event produces ends with single overhanging 3'-nucleotides, with the location determined by the location of *pac1* and *pac2* motifs.

The viral genome contains an origin of DNA synthesis (*oriLyt*), a second *cis*-acting function, located between the divergent UL57 and UL69 genes in the middle of the U_L region. This position is conserved in all characterized beta herpesviruses. *oriLyt* is required to support viral DNA replication. The *oriLyt* in cytomegaloviruses is large (~1,500 bp) and structurally complex, as assayed in a transient replication assay or within the context of the viral genome.^{21,443} In HCMV, it includes a pyrimidine-rich sequence, reiterated elements, direct and inverted repeat sequences, transcription factor-binding sites, and RNA–DNA hybrids.^{21,443,495}

The HCMV genome has two transcription enhancers controlling IE promoters, a third type of *cis*-acting element, as well as numerous⁶²⁹ transcriptional promoters that become active at different times during infection through a regulatory cascade that controls the behavior of host cell RNA polymerase (RNA pol) II machinery. The best studied is the major IE promoter (MIEP) enhancer, a large genomic region that activates transcription of IE1 (p72) and IE2 (p86). A second complex promoter–enhancer controls US3 IE gene expression. When isolated from HCMV and linked to heterologous genes, the MIEP-enhancer directs efficient transcription in a wide-range of mammalian expression vectors and cell lines. In the context of the viral genome, expression is influenced by cell type and, importantly, is repressed in undifferentiated cells and at late times during productive replication.^{628,629} Therefore, the MIEP enhancer plays a pivotal role in activating viral gene expression immediately following entry into permissive cells, in repressing viral gene expression during latency and in activation of viral gene expression during reactivation.^{510,512,515,628} The enhancer may also function as a latent origin of DNA replication to maintain the viral genome in dividing hematopoietic progenitor cells.⁴⁰³ The MIEP enhancer of HCMV, as well as the analogous enhancer found in other cytomegalovirus genomes, is composed of a dense assembly of transcription factor binding sites that extend over several hundred base pairs of DNA.^{628,629} A *cis*-acting repression signal (*crs*) located between the MIEP TATA box and transcription initiation site interacts with IE2 gene products and shuts down MIEP expression as productive infection proceeds. The different *cis*-acting elements act on host RNA pol II transcription initiation^{628,629} to control the cascade of both protein-coding and noncoding gene products that are made during replication.

Genome Organization and Expression

The conventional depiction of the organization of the HCMV genome (Fig. 62.3), as established in initial published work,¹¹²

depicts one of the four natural isomers with the L component to the left and S component in a multiline layout. Additional considerations and modifications have emerged from genome comparisons,¹⁴⁵ including the current arrangement of protein coding genes RL1-13-UL1-150-IRS1-US1-US34-TRS1 (Table 62.1). The numbering of genes has resulted in the UL148 to UL133 segment being inverted due to the unusual organization of the Toledo strain genome where this region was first characterized.¹⁰⁶ The current consensus genome map omits some potential ORFs and emphasizes a uniform nomenclature.³⁹⁹ A few genes have been added as a result of strain comparisons and empirical mapping, resulting in the current estimate of a minimum of 167 protein-coding genes, plus genes for four large noncoding RNAs, two *oriLyt* RNAs, and at least 23 miRNAs (Table 62.2). The genome includes *cis*-acting signals for replicative DNA synthesis (*oriLyt*), cleavage/packaging (*pac1* and *pac2* within terminal *a* sequences) and the RNA transcription, including the MIEP-enhancer within the U_L (located between UL124 and UL128) and the US3 promoter-enhancer within the U_S region (located between US3 and US6). As in all herpesviruses, genes encoding different HCMV temporal classes of protein-coding and noncoding RNAs are interspersed across the viral genome. For example, the first set of genes to be expressed during infection, IE genes, include the IE1-IE2 locus and the UL36-UL37 locus within U_L, US3 within U_S and IRS1/TRS1 within the S component inverted repeats (Table 62.1). These features are conserved in chimpanzee CMV but diverge in other primate and nonprimate cytomegaloviruses.^{41,369,490,531}

During passage in fibroblasts,^{141,619} both HCMV (AD169, Towne) and RhCMV (68.1) strains accumulate point mutations, deletions, and duplications such that common laboratory strains lack as many as 20 genes while retaining a consistent genome size through sequence duplication.¹⁰⁶ A common duplication affects a large segment of the RL region,¹⁰⁶ which is naturally part of U_L (Figs. 62.1 and 62.3). It should be noted that HCMV strains do not spontaneously lose any of the many genes that are dispensable for replication. Despite spontaneous deletions, HCMV maintains a consistent, 236 kbp genome size.^{145,166,399,727}

HCMV genome annotation remains provisional. Protein-coding regions shorter than about 80 codons or regions overlapping larger ORFs have typically not been included unless verified as biologically functional.^{145,399} The refinement of HCMV annotation has included many methods, including comparisons of strains to unpropagated HCMV, chimpanzee CMV, alternative algorithms, and relating protein composition to coding capacity. Liberal estimates of coding capacity,⁴¹⁸ improved annotation methods,⁸² transcriptome analysis,²⁰⁴ and ribosomal profiling⁷⁴¹ all contribute to the current estimate. The functional analysis of HCMV genes has generally followed one of two strategies. Many viral genes have yielded biological activity when studied in isolation or when mutated in the virus. Many have revealed their function through a specific phenotype in viral replication, pathogenesis, immune modulation, or latency. Alternatively, directed or random mutagenesis of viral genomes has yielded phenotypes that define biological function during replication or pathogenesis. The virus strain, host cell type, precision of the mutagenesis, and approaches to evaluation may all impact the physiological relevance of the data that is generated by either approach. A wider

variety of viral gene products have been studied in isolation than have been studied in the context of viral infection, which leaves the physiological function of many gene products in need of further study. The genome complexity of HCMV, together with the range of settings where this virus interfaces with host cell and host defense pathways for replication, persistence, pathogenesis, and latency provides opportunity for additional insights to emerge.

Although different strains of HCMV exhibit >95% DNA sequence homology, certain regions exhibit a high interstrain variability. UL73, UL74, UL144, and UL146 glycoprotein genes, in addition to the terminal repeat α sequence, are the most polymorphic (Table 62.1), variability that has been very useful in tracking viral genome transmission in clinical settings. These polymorphic genes have been used in attempts to correlate particular genotypes with disease as well as for assessing geographic distribution of strains and to establish the level of recombination between strains in reinfected individuals. Recent deep sequence evaluation of viral genomes in clinical samples has revealed a truly immense level of variation that occurs within infected individuals¹³⁹ and has suggested that HCMV undergoes error-prone replication with sequence variation levels as high as some RNA viruses.⁵²⁰

HCMV encodes 23 miRNAs (Table 62.2) during productive viral replication.^{168,221,473,623} In contrast to alpha herpesviruses and gamma herpesviruses, where miRNAs are clustered, HCMV miRNA genes are dispersed across the genome. All but miR-UL70-1-5p, which has not been tested, associate with Argonaute protein silencing complexes⁶²³ and initiate a diversity of biological consequences.^{156,674} Most mature miRNAs that have been assayed²²¹ show DE kinetics, except miR-UL70-1, which shows an IE pattern. Many of the miRNAs are in regions of the genome known to be dispensable for replication in fibroblasts (e.g., miRNAs mapping to US4, US5, and US22), such that no single virus-encoded miRNA is crucial for replication. Several play accessory roles modulating gene expression that influences cell behavior or host response, and may be important in pathogenesis. The sequences of miRNAs are not conserved between herpesvirus subfamilies or even between human beta herpesviruses HCMV and HHV-6B.⁶⁷³ Although conservation between HCMV and chimpanzee CMV²²¹ limited (miR-US5-2) conservation with RhCMV have been noted,²³⁵ MCMV carries a completely unique set of miRNAs.^{88,160} More work will be needed to determine whether the lack of sequence conservation reflects a type of evolutionary divergence that immunomodulatory proteins are already known to exhibit. HCMV encoded miRNAs have not yet been demonstrated during the latent phase of infection, although cellular miRNA has been implicated in a CD34+ cell model of latency.⁴⁸⁵

REPLICATION

HCMV pathogenesis involves productive replication in a variety of cell types,^{81,523} including epithelial, endothelial, neuronal, myeloid, and fibroblast cells. Most studies aimed at understanding viral replication properties have been carried out in fibroblasts, a stromal cell present in all tissues of the host. The ease of studying HCMV replication in these cells, together with significantly greater yields of virus and gene products,⁶⁶⁸ has made it possible to establish the main themes

of viral gene expression and regulation, DNA synthesis and maturation (Fig. 62.2). Viral tropism differences for various cell types were initially recognized through studies employing clinical strains propagated on either cultured endothelial or myeloid cells, including vascular endothelial cells, macrophages, and dendritic cells.⁵⁸³ Viral strains propagated in this manner retained tropism characteristics that are lost during propagation on fibroblasts, but still accumulate mutations.¹⁴¹ Cell lines such as ARPE-19 epithelial cells proved useful in studies of viral tropism determinants. Clinical isolates must undergo adaptation to replicate efficiently in fibroblasts, overcoming poorly understood negative impacts of RL13 and UL128-UL131A¹⁴¹ that drive genome mutations in fibroblasts.⁶¹⁹ Evidence clearly shows that loss of any one of three components (pUL128, pUL130, and pUL131A) of the gH:gL pentameric complex compromises replication efficiency on epithelial and endothelial cells.^{523,539,541,698}

Following entry, three kinetic classes of genes, IE (or α), delayed early (DE or β) and late (L or γ), are expressed sequentially in a coordinately regulated manner over the course of a 48 to 72 hour productive replication cycle. Expression of major IE gene transcription is controlled by upstream enhancers on the viral genome as well as a tegument protein (pp71) that acts as the virion transactivator to increase expression. IE gene expression depends upon preformed host and viral machinery and is independent of *de novo* viral or host expression. Host cell RNA pol II and related transcription machinery controls transcription of the viral genome throughout infection, although transcription is regulated via virus-encoded transactivators. Translation depends entirely on host cell ribosomes. Expression of DE genes is required for viral DNA synthesis and requires expression of functional IE gene products. The DE class of β genes may be further divided into early-late subclasses, β_1 and β_2 , which differ in the pattern of expression.³⁹⁹ Expression of L genes is dependent on expression of DE genes and includes gene products that form virions and control maturation. Two distinct categories of L genes are recognized as leaky late, or γ_1 and true late, or γ_2 , based on the pattern of expression and dependence on viral DNA synthesis.³⁹⁹ Similar to other herpesviruses, the expression of most HCMV L genes is leaky, and occurs independent of viral DNA synthesis though dependent on DE gene expression. Typically, viral DNA synthesis inhibitors such as foscarnet (phosphonoacetic acid) have been used to block viral DNA synthesis to assist in dissecting DE and L patterns of gene expression. The leakiness of late gene expression and the recognition of kinetic subclasses spawned the alternate terminology— α , β , and γ —first used to describe HSV-1 patterns. Although the replication cycle of HCMV is slow, requiring 48 to 72 hours to reach final stages of maturation and release of progeny, the expression of IE gene products starts within minutes of infection. The switch from early phase to late phase is prolonged, from 24 to 36 hours postinfection (hpi) and may be even longer dependent on cell type. Initiation of IE gene expression is sensitive to cell cycle status, linked to regulation of p53,⁷³⁹ such that cells in S, G2, or M phases do not produce IE proteins until cells return to G1. Maximum levels of virus progeny are released from fibroblasts starting around 5 days postinfection and continue for several days before cells die, succumbing to a serine protease-dependent programmed death pathway the timing of which is controlled by the viral mitochondrial inhibitor of apoptosis (vMIA).³⁷⁷

Attachment and Entry

Entry occurs in distinct steps³⁹⁹: (a) binding to specific cell surface receptors, (b) viral envelope fusion with cellular membranes to release nucleocapsids into the cytoplasm, either directly at the plasma membrane (as occurs in fibroblasts) or after endocytosis into cells (as occurs in endothelial and epithelial cells), (c) nucleocapsid translocation toward the nucleus on cytoskeletal filaments, (d) nucleocapsid interaction with nuclear pores, and (e) release of the viral genome into the nucleus (see Fig. 62.2). At the same time, independent of nucleocapsid translocation to the nucleus, tegument proteins are released into the cytosol and traffic to sites where they function in diverse ways, modulating the initial host response to infection and orchestrating the transcription of IE genes. This process has common characteristics among all herpesviruses, so understanding of how these steps are controlled comes from direct study of HCMV as well as from surrogates such as MCMV, guinea pig CMV, and simian CMV, as well as comparisons to other herpesviruses. Initial contact with cells is via cell surface heparan sulfate, a feature common to many herpesviruses. Investigations have shown that envelope glycoproteins gB and gM bind heparan sulfate, and may overlap to make initial contact with cells.²⁶² HCMV attachment does not require dedicated subgroup-specific receptor-recognizing envelope glycoprotein, such as gD (HSV-1/HSV-2) or gp350 (EBV).^{132,285} Instead, HCMV completes attachment as well as fusion steps with herpesvirus-conserved gB and gH:gL. Initial binding of virions to cells leads to a cascade where gB trimers and gH:gL heterodimers orchestrate events through sequential cellular receptors that ultimately results in fusion between the viral envelope and cell membrane,²⁶² and release of the nucleocapsid and tegument proteins into the cytoplasm. This entry step is disrupted when cholesterol is reduced.²⁷⁵ In addition to the major envelope glycoproteins controlling entry into any cell type, a trimeric complex consisting of gH:gL:gO and a pentameric complex comprised of gH:gL:pUL128:pUL130:pUL131A facilitate entry in certain settings.^{523,582} The pentameric complex facilitates attachment and entry into epithelial and endothelial cell types,^{523,541,698} suggesting that this complex may play an important role in HCMV pathogenesis. Additional entry options have come to light, such as the requirement for pUS16 to mediate efficient viral infection of endothelial and epithelial cells when the pentameric gH:gL complex is absent.⁸³

Considerable attention has been focused on gB because of its common role in attachment and fusion of all herpesviruses, mediating delivery nucleocapsids into the cytosol. Several receptor and entry mediator candidates have been shown to interact with gB. Some of these have turned out to be dispensable for infection of susceptible cells.¹³¹ In this regard, β_2 microglobulin, annexin II, and aminopeptidase N (CD13) are unlikely receptors for viral entry. More recent identification of cell surface integrins $\alpha 2\beta 1$, $\alpha 6\beta 1$, and $\alpha V\beta 3$,²⁶² as well as EGFR on monocytes¹⁰⁸ and PDGFR on endothelial, epithelial, and fibroblast cells,⁶⁰² remain under evaluation. Furthermore, host membrane characteristics conferred by tetherin expression enhance entry.⁶⁸⁷ In addition to mediating the delivery of the nucleocapsid to the cytoplasm, the interaction of HCMV envelope glycoproteins with cell surface proteins may trigger cellular signaling pathways to enhance viral and cellular gene expression to facilitate infection.²⁶² Toll-like receptor 2 (TLR2) is one such candidate on monocytes. Candidate receptors have also been found to

interact with gH, including $\alpha v\beta 3$ integrin.¹³¹ Although there is little question that HCMV enters cells through specific interactions between major envelope glycoproteins and cellular plasma membrane receptors, much remains to be learned about the pathways that are involved.^{131,262,399,523}

Intracellular Trafficking and Uncoating

Once the HCMV nucleocapsid is deposited into the cytoplasm, cytoplasmic microtubules are predicted to facilitate translocation to the nucleus where viral DNA is released.³⁹⁹ In HCMV, the large tegument protein (LTP; UL48), as well as a binding protein (LTPbp; UL47) play essential roles in replication,^{167,727} potentially analogous to the HSV-1 UL36 gene product, controlling uncoating and release of viral DNA from nucleocapsids. Both HCMV UL47 and UL48 are likely to be important for replication,^{80,400} but so far only viral mutants in a dispensable deubiquitinating function of UL48 have been studied.²⁹³ Greater focus on uncoating and nucleocapsid translocation to nuclear pores is needed.

IE Gene Regulation and Function

Once the HCMV genome is delivered to the nucleus, IE gene expression ensues. RNA pol II transcription machinery transcribes IE as well as all other protein-coding and noncoding RNAs made from the HCMV genome. Regulation of viral gene expression occurs via two broad strategies: (1) viral as well as cellular factors that directly influence the transcription machinery by binding to promoter/enhancer elements directly (transcription factors) or through interactions with other proteins (adaptors) and (2) viral factors that alter chromatin remodeling by regulating the opposing activities of histone acetyl transferases (HATs) acting together with demethylases and histone deacetylases (HDACs) and methylases. HDAC-dependent repression of viral IE gene expression, in particular, is a cell-intrinsic host defense mechanism that must be defused before productive replication can ensue. Epigenetic regulation is important in permissive cells, even though the viral genome does not take on a recognizable chromatin structure, and also during latency, where viral genomes take on an organized chromatin arrangement and viral HDAC inhibitors can drive reactivation.^{510,512,579}

The regulation and activities of IE gene products have been a focus of considerable review.^{399,510,512,579,628,629,631,707} Five loci distributed across the viral genome give rise to IE gene transcription (Table 62.1), UL36 and UL37, IE1 and IE2 (UL122 and UL123), TRS1, IRS1, and US3. IE1/IE2 and US3 gene expression is under control of independent transcriptional enhancers. The major IE (MIE or IE1/IE2) gene is transcribed from the MIE promoter (MIEP) into alternatively spliced and polyadenylated mRNAs encoding two predominant nuclear phosphoproteins, IE1-p72 and IE2-p86, along with minor IE proteins. Although IE2-p86 is recognized as the most important viral transactivator and key to viral gene expression during productive replication, IE1-p72 and IE2-p86 together defuse epigenetic repression, activate DE and L classes of viral genes during productive replication, autoregulate IE gene expression, and establish nuclear sites where lytic viral DNA synthesis proceeds. In addition, these major IE proteins contribute in crucial ways to the switch between latency and reactivation.

MIEP expression is dependent on a transcriptional enhancer (also called the HCMV enhancer) that is subject to positive- and negative-acting viral and cellular chromatin

remodeling pathways controlled by HATs and HDACs acting together with methyltransferases and demethylases.^{510,512,579} This locus is the only region of any cytomegalovirus genome known to exhibit CpG suppression, consistent with a need for the region to remain unmethylated during latency and reactivation.⁶²⁹ No matter the cell type, epigenetic repression of the MIEP must be relieved by the concerted and sequential activity of tegument proteins such as pp71 and ppUL69 and the major IE proteins IE1-p72 and IE2-p86, acting with other viral proteins and host transcription machinery to sustain viral gene expression over the very long HCMV replication cycle.^{323,374,654} The failure of HCMV to replicate in cells that have entered S phase is due to the inability of these viral functions to overcome epigenetic repression⁷⁴⁰ and is associated with the failure to encode IE proteins.⁵⁵⁰

Individual MIE proteins counteract cell-intrinsic resistance mechanisms triggered by viral infection.^{427,512} MIE proteins IE2-p86 and IE1-p72 induce cell cycle arrest with prodeath or pro-survival impact depending on the setting.^{377,427,631} MIE protein IE1-p72 binds chromatin,^{516,576} orchestrates STAT (Signal Transducer and Activator of Transcription) signaling,⁴²⁷ disrupts nuclear domain 10 (ND10; also called promyelocytic leukemia protein [PML] bodies or PML oncogenic domains [PODs]),^{323,374,654} in addition to binding HDACs⁵⁷⁶ and Daxx^{511,512} and contributing to chromatin remodeling and direct regulation of gene expression.

Three additional IE regions (UL36/UL37, TRS1, and IRS1) encode functions that modulate other aspects of the cell-intrinsic response to viral infection in addition to their direct impact on gene expression, and one IE gene (US3) is involved in posttranslational down modulation of MHC class I gene levels on the host cell to evade cytotoxic T-cell surveillance. UL36, UL37 × 1, UL37 × 3, TRS1, and IRS1 are each individually dispensable for viral replication in fibroblasts; each carries out modulatory activities that contribute to viral pathogenesis. The US3 protein is the earliest viral protein disrupting MHC class I antigen presentation to make infected cells less attractive targets for cytotoxic T-cell immune surveillance⁴⁸⁰ and will be discussed together with other US2 and US6 family members.

UL37 × 1 encodes the very potent mitochondrial cell death suppressor, vMIA. This small protein localizes to the outer mitochondrial membrane and sequesters Bax and Bak, but does not prevent oligomerization of these Bcl2 family members or mitochondrial fission that results from this association.^{286,435} Through interactions with Bax, Bak, Bcl_L, and GADD45 family members, vMIA prevents cytochrome *c* release, the key step in the activation of executioner caspases driving apoptosis.³⁷⁷ vMIA associates with endoplasmic reticulum (ER)-derived mitochondrial-associated membranes,^{75,711,713} increases ER release of calcium stores, and enhances cell rounding⁵⁷⁴ and, as a consequence, vMIA has been associated with HCMV cytopathic effects mediated through viperin.⁵⁷³ Although vMIA is a very potent inhibitor of apoptosis,³⁷⁷ its role in HCMV infection is to prevent a novel mitochondrial HtrA2 serine protease-dependent death pathway that terminates the viral replication cycle in fibroblasts.³⁸⁰ Because of its role in titrating out mitochondrial proteins that antagonize infection, vMIA may appear essential for replication (e.g., AD169) or dispensable for replication (e.g., Towne). Small mitochondrial Bax and Bak inhibitors with sequence characteristics of UL37 × 1 are evolutionarily conserved in beta herpesviruses.³⁷⁷ MCMV

encodes two mitochondrial-localized cell death suppressors with related function, m38.5/vMIA and m41.1/vIBO, that independently target Bax^{26,435} and Bak,⁹¹ respectively.

UL36 is a US22 family member that encodes the viral inhibitor of caspase 8 activation (vICA).³⁷⁷ vICA suppression of extrinsic apoptosis is crucial for viral infection of macrophages,^{123,379} but apparently dispensable for replication in other cell types. HCMV UL36, as well as the RhCMV homolog are mutated in commonly used strains.⁵⁸⁵ UL36-encoded vICA binds procaspase 8 and prevents cleavage/activation, behaving in a manner that is mechanistically similar to the long form of cellular FLICE (another name for caspase 8) inhibitory protein (cFLIP_L). vICA is highly conserved, with a homolog encoded by all beta herpesviruses. The contribution of vICA-associated caspase 8 suppression during infection and pathogenesis comes from studies using MCMV mutant viruses in mice.¹²³ Viruses that lack vICA replicate fine in fibroblasts but are attenuated due to susceptibility to extrinsic apoptosis in macrophages.^{123,377,405} The evolutionary acquisition of multiple cell death suppressors in HCMV and MCMV has facilitated identification of programmed necrosis as a bona fide host defense pathway that evolved in mammals to defend against viruses.⁴⁰⁵ Necrotic death is triggered by vICA suppression of caspase 8, causing release of receptor interacting protein (RIP) kinase 3 to form a complex with DNA-dependent activator of interferon signaling (DAI)⁶⁷⁷ and is inhibited by MCMV M45-encoded viral inhibitor of RIP activation (vIRA).⁶⁷⁸ An HCMV RIP inhibitor has not been identified.

IRS1 and TRS1 are highly homologous US22 family members that inhibit interferon-inducible protein kinase R (PKR).³⁷¹ Either must be retained in order for viral replication to proceed.³⁷⁰ Independent of PKR, TRS1 inhibits macroautophagy,¹¹¹ and TRS1, more than IRS1, may influence DE gene expression, DNA synthesis,^{278,634} and capsid assembly,³⁷¹ all of which may ultimately be related to inhibition of PKR.³⁷⁰

Regulation of MIEP Enhancer

The MIEP-enhancer is a common tool for eukaryotic expression vectors in experiments and commercial production of biologics because it is constitutively active and strong.⁵⁶¹ In the context of the viral genome, the MIEP-enhancer is epigenetically silenced and requires the activity of input viral tegument proteins pp71 and ppUL69 as well as IE and DE proteins that are made during infection to remain active.^{579,628,629} Regulation focused on cellular chromatin remodeling machinery bears on models of viral latency and reactivation where MIEP expression is key to this switch.⁵¹² Mutations within the MIEP enhancer result in viruses that exhibit MOI-dependent growth properties and that are compromised in models of latency and reactivation.^{512,628,629} Once released from nucleocapsids following entry, HCMV genomes delivered to the nucleus associate with ND10, in a manner that has been observed in many herpesviruses and other DNA viruses.³⁷⁴ Initiation of IE gene transcription, modulation of cell intrinsic signaling, and inhibition of the DNA damage response all take place in association with ND10,¹⁷⁹ which becomes disrupted by IE1-p72, even though this disruption is dispensable for viral replication.⁵⁷⁶ Relief from HDAC-mediated repression is initially carried out predominantly by the tegument protein pp71, encoded by UL82, is the principal virion transactivator (VTA) of HCMV. VTA relieves HDAC repression^{511,512,628} by localizing to ND10, degrading Daxx to

prevent Daxx/ATR-X-mediated repression of the MIEP, which is mediated via HDACs.^{258,352,430,492} These properties of pp71 make it useful in experimental settings to increase production of infectious virus following transfection of viral DNA or bacmids. pp71 associates with tegument protein ppUL35,⁵⁵⁹ an HCMV DNA damage response modifier.⁵⁴⁵ Upon entry, the pp71:ppUL35 tegument complex more efficiently stimulates MIEP expression than pp71 alone, whether surveyed in reporter assays or in the context of virus infection.⁵⁵⁹ Viral mutants lacking UL82 or UL35 exhibit MOI-dependent replication similar to that observed with IE1-p72, MIEP enhancer, and *crs* mutant viruses.^{628,629} Following the initiation of IE gene expression, HDAC suppression is sustained by IE1-p72.

Other tegument proteins contribute to optimal MIEP expression. Virion ppUL69 enhances the initial stages of viral infection, although it has its major impact later in infection. ppUL26 is a transcriptional activator of the MIEP that probably regulates phosphorylation levels of other tegument proteins,⁴¹³ and pp65 activates the MIEP possibly by recruiting the cellular IFI16 protein to the control region.¹³⁷ ppUL29/28, encoded from a spliced mRNA, stimulates MIE gene expression by modifying the HDAC1-containing nucleosome remodeling and deacetylase protein (NuRD) complex, handing off to pUL38 in association with the MTA2 component of NuRD at early times in infection.^{396,656} As infection proceeds, MIE gene products impose additional regulation on the MIEP enhancer, as described in the next section. In addition to the viral proteins, cellular signal transduction pathways induced by virus particles or other factors such as serum growth factors as well as cell type and cell cycle state, impact MIEP expression. This is particularly evident at low MOIs as well as in particular cell types.⁵⁷⁹

Mutation and substitution of enhancer components have reinforced the role of the MIEP enhancer in the initiation of viral gene expression during replication. The MIEP enhancer is composed of an upstream region (−580 to −300 relative to the transcription start site) that is less important in most assays than the proximal region (−300 to −39 relative to the transcription start site).^{628,629} The multitude of transcription factor binding sites contributing to enhancer activity during infection^{628,629} includes multiple cyclic AMP response element binding protein ATF/CREB and NF-κB binding sites. Investigations have shown that many can be disrupted without compromising replication, consistent with a highly redundant role for this complex transcriptional control region. Substitution experiments replacing MIEP enhancers have shown that MCMV carrying a HCMV enhancer replicates normally in mouse fibroblasts, but not in mice. The MCMV enhancer does not work in HCMV, but the simian CMV enhancer confers normal growth levels and plaque efficiency on HCMV.^{628,629} These experiments reinforce the role of the enhancer as a critical *cis*-acting signal for MIE expression as well as some level of evolutionary adaptation to the homologous host species within the context of the viral genome.

IE1 and IE2

The principal products of the MIE gene, IE1-p72 and IE2-p86, result from alternative mRNA splicing that places 85 amino terminal amino acids together with carboxyl terminus derived from UL123 and UL122, respectively. Both of these proteins are primarily involved in regulating viral transcription to initiate productive replication as well as the latency-reactivation axis, although expression from this locus is not sufficient to drive reac-

tivation.^{512,628,629,707} These proteins modulate the host cell response to infection,^{367,654} acting in concert with virion tegument as well as other IE gene products to establish a cellular environment to support replication. IE2-p86 is both a crucial activator of later kinetic classes (DE and L) of viral genes as well as a repressor of MIE expression,⁶³¹ whereas IE1-p72 functions primarily as a coactivator together with IE2-p86.²⁰⁵ IE1-p72 disrupts ND10^{374,629} and sustains an open chromatin configuration initiated by the VTA, and probably acts independent of VTA in reactivation from latency.⁵¹¹ In addition, less abundant IE proteins IE1-p38, IE2-p55, and IE2-p18, have no known function.⁶²⁹ Later during infection, abundant leaky L (p60) and true L (p40) proteins are made from independent promoters embedded within UL122, and these are colinear with the carboxyl terminus of IE2-p86. Although both of these have the ability to repress MIE expression by binding to the *crs*, neither plays a crucial role in replication.^{629,706}

IE2 proteins that share the carboxyl-terminus of IE2-p86 all negatively autoregulate expression from the MIEP by binding to the minor groove of the *crs* between −13 and −1 relative to the transcription start site (CGTTTAGTGAACC^{−1}), thereby blocking transcription by inducing a repressive chromatin state^{509,510} rather than by disrupting transcription by RNA pol II more directly. This was the first IE2-p86 binding site identified in the HCMV genome, but there are many others within the promoters of DE and L genes that enhance transcription. A sequence related to the *crs* in the US3 gene (CGT^G/T-CAGTCCACACG⁺¹) is repressed by ppUL34 rather than IE2-p86.^{55,629} The highly abundant leaky L 60-kD and very abundant L 40-kD (L40) IE2 proteins, once thought to maintain repression throughout infection, are largely accessory. Only IE2-p86 appears crucial for the activation and repression necessary to complete viral replication.⁷⁰⁶

The major function of IE1-p72 and IE2-p86 is to mediate activation of viral genes expressed through the remainder of infection.⁶²⁹ IE1-p72 and IE2-p86 associate with ND10. IE1-p72 disrupts these domains and undermines ND10-dependent activation of interferon (IFN), but does not alter their organizational role in viral gene expression or in initiation of viral DNA synthesis. Although an independent role of IE1-p72 in counteracting IFN activation is established,⁴⁵⁹ infection with IE1-p72-deficient virus at an MOI greater than 1 leaves ND10 intact and replication at levels similar to that of wild-type virus. The theme established by pp71 continues with the MIE genes, and IE1-p72 inactivates HDACs associated with ND10 to facilitate efficient, IE2-p86-dependent DE and L gene expression.^{511,512,631} Although normally disrupted by IE1-p72, the ND10 areas become sites of DNA synthesis independent of IE1-p72 function, starting with ppUL84 associating with IE2-p86, attracting UL112-113 gene products and viral replication complex proteins to initiate viral DNA replication.^{21,278,295,443} The replication defect of IE1-p72-deficient virus at low MOI likely results from failure to inactivate HDACs and allow for adequate activation of DE gene expression rather than either a failure to disrupt ND10, suppress the IFN response, or initiate DNA replication.^{511,512} It should be noted that the UL97 kinase⁶⁶⁷ and other gene products expressed later in infection (UL30, UL35, and US32) alter PML morphology or disrupt ND10.⁵⁴⁶

HCMV IE2-p86, a major nuclear phosphoprotein, is crucial for replication, although exact mechanisms have proven difficult to establish because of technical difficulties with conditional

mutant viruses that have been made. This gene has been subjected to extensive mutagenesis and evaluation by transient assay and many mutants have been built into virus constructs that fail to replicate. Most attention has been focused on the full-length MIE product, IE2-p86, the only UL122 gene product that is absolutely necessary for replication. The additional IE, DE, and L gene products encoded in the UL122 region are completely dispensable.^{90,551–553,631,706,707} The MCMV homolog (called ie3) seems to function analogously to HCMV IE2-p86,^{314,372} although the MCMV homolog of HCMV IE1 (called ie1) and a novel MCMV ie2 gene are dispensable for all aspects of viral infection⁷⁰⁸ and fail to contribute to transcriptional patterns during infection.³¹⁴ HCMV IE2-p86 transactivates promoters through direct interaction with DNA (the MIEP *crs* and many other *crs*-like sites distributed throughout the genome), influencing RNA pol II transcription machinery directly by interacting with p53, cdk9 and cdk7, cycinT1, Bed4, HDAC1 and HDAC2 in a viral transcriptosome^{283,284} as well as via epigenetic regulation, and employing functional domains that are located in both amino-terminal and carboxyl-terminal regions of the protein.^{631,707} In the absence of IE2 mutant viruses to reveal core aspects of IE2-p86 function, transient assays defined IE2-p86 domains that are important for positive or negative regulatory impact, and these have proven useful. The regions contributing to transactivation include two domains located between aa 25 and aa 85 as well as between aa 544 and aa 579, at the extreme carboxyl terminus, as well as dimerization (aa 388 to 542) and helix-loop-helix aa (463 to 513) regions that overlap a core region (aa 450 and 544) important for most associated activities.^{631,707} The 450 to 544 region directs the interaction with a number of cellular transcription factors that have been implicated in regulation by IE2-p86,^{283,284} as well as for interaction with the viral UL84 protein, which counteracts IE2-p86 transactivation function but is crucial for initiation of DNA replication. IE2-p86 also interacts with a broad number cell cycle regulated proteins, sustaining the unique cell cycle block instituted during infection. Transactivation involves an adapter-like protein stabilizing RNA pol II transcription factor D TFIID-associated factors on the promoters, interacting with the carboxyl terminus of TATA element binding protein TBP and TFIIB.^{631,707}

DE Gene Regulation and Function

Following peak expression of MIE regulatory proteins, between 8 and 12 hpi, the second class of early genes, DE, become transcriptionally active independent of the host cell type.⁶⁶⁸ Although some DE gene products are produced in abundance from the start, most of the 65 proteins (Table 62.1) and assorted miRNAs (Table 62.2) accumulate gradually. The DE period of viral replication continues through 18 to 24 hpi when viral DNA synthesis initiates. DE genes are crucial for viral DNA synthesis and include several functions that become important later in infection for maturation and egress. Many DE genes have a substantial impact on replication when disrupted.^{167,727} DE gene products dispensable for replication in fibroblasts may contribute to modulation of the host cell and host animal response to infection. Several HCMV DE genes switch or add transcriptional start sites later in infection, which means that transcript levels represent the combined products of different kinetic classes of gene expression.

The principles of DE gene regulation⁷⁰⁷ are intertwined with subversion of host cell cycle regulated pathways⁵⁵⁰ and

both depend on the key regulatory protein IE2-p86 functioning together with a number of collaborating factors.^{629,631} The promoter regions of three DE genes (UL112-UL113, UL54, and UL4) have been studied in detail. Although transient cotransfection assays first revealed candidate transcriptional control elements in the promoters for these genes, it was only after mutations were introduced into the viral genome and evaluated in the context of infection that concrete information emerged. The activation of these three genes illustrates the subpatterns of early gene expression that is dependent on IE gene expression as well as the differential regulation by host and viral functions as infection proceeds. Expression of the UL112-113 transcripts⁷⁰⁷ that are differentially spliced to yield four unique proteins (pp34, pp43, pp50, pp84), starts by 8 hpi and remains constant through L times (β_1 pattern). Transcription shifts to a different start site at L times but the same mRNA splicing pattern and a single polyadenylation site is used throughout infection. Promoter-proximal ATF/CREB and IE2-p86 binding sites are important for sustained transcription throughout infection, with the ATF/CREB site more important early and the IE2-p86 binding site more important at L times. Expression of UL54,⁷⁰⁷ encoding DNA POL, starts by 8 hpi and increases steadily through L times (β_2 pattern), relying on a single transcription start site but two polyadenylation sites. Unlike L genes discussed in the next section, expression of β_2 genes is not reduced in the presence of viral DNA synthesis inhibitors. Promoter-proximal SP1 and IE2-p86 binding sites are more important early, and an ATF/CREB site is crucial for the increase at L times during infection, although the transcription start site remains unchanged. Finally, UL4,⁷⁰⁷ which encodes a minor virion envelope glycoprotein (gp48), shows a transcription pattern during infection that is similar to UL54 (β_2 pattern), but this gene is predominantly regulated at the translational level by ribosome stalling.^{54,543} Transcriptional activation is dependent on Elk-1 and IE2-p86 binding sites in the promoter plus some consequence of MAPK signaling that acts via the TATA element. Translational stalling is dependent on a carboxyl-terminal Pro within a 22 aa ORF (uORF2) within the transcript upstream UL4, thereby preventing full expression of gp48. Translation stops because uORF2 fails to release peptidyl tRNA dependent on the release factor eRF1,⁵⁴³ and this blocks the large ribosomal subunit exit tunnel and halts the ribosome.⁵⁴ Although elegant studies have deduced that this form of regulation is common across eukaryotes and prokaryotes, the natural context for this exquisite level of regulation as well as a mechanism to relieve inhibition in HCMV remain to be elaborated.

A number of DE gene products regulate gene expression. pUL34 is a specific repressor of US3 expression, acting on a novel *crs*.³²⁸ Four UL112-113 gene products (pp34, pp43, pp50, pp84), function together with IE1-p72, IE2-p86, and TRS/IRS to modulate DE gene expression, UL21A enhances replication at early times,^{184,185} and DE proteins contribute to continued suppression of epigenetic repression by HDACs. UL26 activates the MIEP,^{349,413} pUL38 antagonizes the tuberosclerosis complex to prevent ER stress (407 Qian, 2011 #7341), and pUL29-28 stimulates gene expression by interfering with HDACs, modifying the nucleosome remodeling and deacetylase (NuRD) complex^{396,656} to sustain derepression of the MIE gene throughout infection. In addition, UL97 promotes KAT5/Tip60 HAT phosphorylation to increase expression³³⁰ and UL27 inhibits KAT5/Tip60 HAT and suppresses

expression of cyclin-dependent kinases.⁵¹⁸ Finally several abundant tegument proteins, including pp65, pp71, pp150, and LTP/ppUL48 are transcriptionally active as DE genes, although their activities support virion maturation or steps during infection of subsequent cells.

Cellular gene expression is activated during HCMV infection. Global transcription^{204,246,434,623,668} and, most recently, global proteomic/metabolic analyses,^{412,549} has shown the extensive stimulation of cellular lipid metabolism and energy pathways to favor HCMV replication, contrasting with the extensive shut-off of the host cell by HSV.⁶⁸² Viral replication and maturation follow stimulation and parallel accumulation of viral DNA synthesis functions, giving the impression that the prolonged replication cycle of this virus may require sustained cellular functions and proceeds only as they become available.⁵⁵⁰ HCMV infection stimulates cellular RNA as well as protein synthesis while completely dysregulating the cell cycle⁵⁵⁰ such that infected cells appear to be at G₁/S, G₂, or even M. Some of this comes as a result of dysregulation of cellular cyclin-dependent kinases²⁴⁶ and mislocalization of proteins normally associated with checkpoint control to sites of viral maturation in the cytoplasm.²⁰³ In fibroblasts, cellular DNA synthesis is usually held in check as a pseudo-G1 block,²⁴⁶ with contributions from tegument proteins, pp71 and ppUL69, as well as IE2-p86 in maintaining this cellular environment.⁵⁵⁰ Systems level analyses have repeatedly confirmed more focused earlier assessments showing that glucose metabolism,^{731,732} mitochondrial energy production,^{377,711} and cell cycle-regulated expression⁵⁵⁰ are all broadly activated, whereas cytoskeletal, extracellular matrix, adhesion functions, and cellular DNA synthesis⁵⁵⁰ are broadly down modulated.

One abundant noncoding RNA, the β 2.7/RNA2.7, accumulates to represent more than 60% of total viral polyadenylated transcripts at L times of infection.^{204,668} This most abundant DE gene product is dispensable for replication³⁸⁴ but associates with mitochondrial complex I (nicotinamide adenine dinucleotide-ubiquinone oxidoreductase) to stabilize ATP production and prevents apoptotic cell death when respiration is blocked by rotenone treatment⁵¹³ as well as necrotic death from ischemia/reperfusion injury.⁷³⁷ Although naturally a noncoding RNA, the β 2.7/RNA2.7 promoter allows for high-level expression of heterologous genes, regulated with DE kinetics, within the HCMV genome.⁶⁰⁵

DNA Synthesis and Nucleotide Metabolism

Lytic HCMV DNA synthesis occurs within the nucleus of infected fibroblasts, starting as early as 14 to 16 hpi, increasing by 24 hpi, and reaching greater than 10,000 viral genome copies per cell⁶⁶⁸ at the time progeny virions start to form.^{21,443} In fibroblasts, viral DNA labeled for 1 h at either 19 or 23 hpi quantitatively chases into maturing virions and leaves the cell via the cytoplasm over the next 24 to 48 hours.⁴⁶⁷ In epithelial and astrocytoma cells, viral replication is more subdued and maximal viral DNA levels do not exceed 1,000 viral genomes per cell.⁶⁶⁸ DNA synthesis initiates from the complex oriLyt site between the UL57 and UL69 genes (Fig. 62.3), although the mechanism is still unclear. The HCMV, MCMV, or GPCMV oriLyt region is large, almost 3,000 bp, making this replication origin considerably more complex than others, including those from other human beta herpesviruses. This region can be divided into Essential Region I, with a bidirectional

ppUL84:IE2-p86-responsive oriLyt promoter and a pyridine-rich Y-block,⁴⁴³ and Essential Region II, which includes an RNA–DNA hybrid structure⁴⁹⁵ and an adjacent RNA stem-loop structure capable of binding ppUL84.¹³⁰ This region is also rich in other direct and inverted repeat sequences and transcription factor-binding sites. Viral DNA destined for replication circularizes after being delivered to the nucleus, associating with ND10, sites of early transcription where DNA synthesis initiates following the successive localization of IE1-p72, IE2-p86, and ppUL84, then UL112-113 proteins and, finally, ppUL44, which brings in other replisome components. A distinct subregion containing viral DNA and replisome components eventually dominates the nucleus as a distinct replication compartment. Six herpesvirus core replication fork proteins compose the replisome that synthesizes DNA.⁴²⁴ In HCMV, this replisome composed of the UL54-encoded DNA polymerase catalytic subunit (POL) together with the UL44-encoded polymerase processivity subunit (POL:PPS complex), the UL57-encoded single-strand DNA binding protein (SSB), and the heterotrimeric helicase-primase (HP) consisting of UL105-encoded HP1, UL70-encoded HP2, and UL102-encoded HP3.

Initiation of DNA synthesis requires oriLyt promoter activity, which is dependent on a ppUL84:IE2-p86 complex. ppUL84, a DE gene long known to play a key role in this process,⁴⁴³ forms homomultimers and binds to IE2-p86, thereby antagonizing transactivation by this protein. Its role in viral DNA synthesis is highlighted by the phenotype of mutant viruses.⁴⁴³ In complex with all UL112-113 proteins,²⁹⁵ ppUL84, a putative DExH/D box protein⁴⁴³ recruits ppUL44/PPS.⁶⁵⁵ Interactions between these components must form to support viral replication.²⁹⁵ As such, oriLyt recognition by ppUL84 bridges to the replisome via PPS. In addition, cellular functions such as C-EBP²⁷⁷ and hnRNP-K²⁷⁸ associate with these complexes and are necessary for viral DNA synthesis. Genetic evidence strongly supports the role of a ppUL84:IE2-p86 complex in initiation of oriLyt-dependent viral DNA synthesis,⁴⁴³ although the lack of a cell-free system leaves other details to be investigated. Other HCMV IE genes pUL36/vICA, pUL37 \times 1/vMIA, ppIRS1, and ppTRS1 make the host cell environment more conducive to the process without being actively involved in the replication compartment.⁴⁴³

HCMV DNA synthesis may start as theta form before switching to a rolling circle mode of replication. The architecture of replicating DNA is consistent with concatemeric structures generated by rolling circle form of replication, and this is the likely template for the genome encapsidation. IE2-p86, ppUL84, and four UL112-113-encoded phosphoproteins (pp34, pp43, pp50, pp84) produced via alternative mRNA splicing⁷⁰⁷ all associate with prereplicative sites near ND10 sites in the nucleus to coordinate assembly of the viral replisome. Based on studies of viral mutants, all of these proteins must be simultaneously coexpressed to form a complex, colocalize with ND10, and recruit ppUL44/PPS to prereplication foci.²⁹⁵ It has long been appreciated that ppUL44/PPS and ppUL57/SSB show distinct nuclear localization patterns prior to the initiation of viral DNA synthesis, as the replication compartment forms around 19 hpi.⁴⁶⁷ Although IE2-p86 is believed to facilitate prereplication complex formation, it is the UL112-113 proteins that independently associate with ND10 and recruit the replisome to the viral genome, apparently via ppUL44/PPS.²⁹⁵ A shortage of UL112-113 gene products may underlie

the MOI-dependent replication defect in IE1-p72 mutant virus.²⁰⁵ The four UL112-113 gene products, ppUL84, IE2-p86, and six herpesvirus-conserved replisome proteins remain associated with the replication compartment throughout the remainder of infection.

Viral DNA synthesis is the target of antiviral drugs (foscarnet, ganciclovir/valganciclovir, and cidofovir) in current widespread use. Acyclovir/valacyclovir, although sometimes employed for HCMV prophylaxis, has no basis of action against HCMV due to the absence of a virus-encoded thymidine kinase. All of the effective anti-HCMV drugs act on or through the viral DNA POL. Intravenous ganciclovir and oral valganciclovir represent gold standards for prevention and treatment of HCMV infection and disease.^{188,596} Although active against other herpesviruses, these are rarely used outside of HCMV due to toxicity. Foscarnet inhibits viral DNA synthesis by binding the pyrophosphate release site similar to phosphonoacetate. Structure–function analysis has shown that foscarnet, like phosphonoacetate, inhibits POL enzymatic activity by maintaining a closed, untranslocated state on the viral DNA template.⁷³⁵ In cell culture, ganciclovir or foscarnet have proven to be excellent HCMV DNA synthesis inhibitors and are superior to phosphonoacetate, due to lower toxicity during the extended treatment periods necessary with HCMV. Ganciclovir (as well as valganciclovir, a pro-drug that is converted to ganciclovir in the body) is monophosphorylated by UL97 kinase, a viral protein kinase able to recognize this nucleoside.^{341,640} Phosphorylation of ganciclovir monophosphate is completed by host nucleoside kinases, and the triphosphate form is incorporated by POL and chain terminates viral DNA synthesis. UL97-independent phosphorylation and modest selectivity for POL over host cell DNA polymerases underlie ganciclovir/valganciclovir toxicity. Cidofovir, a nucleotide triphosphate analog that does not require UL97 phosphorylation, also causes chain termination, but POL is positioned differently on the template. Cidofovir is, like foscarnet, a second-line choice. As would be expected, clinical isolates exhibiting resistance to ganciclovir arise with mutations either in the protein kinase gene (UL97) or the DNA POL gene (UL54).

Additional anti-HCMV drugs are under development, and these will be mentioned here, although their targets lie outside DNA synthesis machinery. A terminase inhibitor AIC246 exhibits exquisite specificity for HCMV.³⁶⁸ The protein kinase, ppUL97 is a target of maribavir, for which difficulty was experienced in clinical development.³⁷³ Poor therapeutic effectiveness of maribavir may result from overlap in UL97 function with cyclin-dependent kinase (CDK) activity.^{246,494} The activity of maribavir varies dramatically with cell culture conditions.¹²⁰ The synergy observed between maribavir and CDK inhibition directly implicates overlapping function to sustain viral infection.²⁴⁶ Resistance to maribavir may be conferred by mutations in UL97 itself, as well as in UL27. Somewhat surprisingly, mutations in UL27 also develop spontaneously, as a UL97 mutant virus is propagated in the absence of drug,¹¹⁷ implicating UL27 influence on CDK activities during infection. UL27 directs the proteasomal degradation of histone acetyltransferase KAT5/Tip60,⁵¹⁸ leading to increased CDK inhibitor p21/Cip1. UL97 phosphorylates many targets in infected cells,⁴⁹⁴ including KAT5/Tip60, a modification proposed to increase viral gene expression.³³⁰ Compromised UL27 function may result in higher CDK activity that supplants the need for ppUL97 kinase.

Other HCMV DE gene products contribute to DNA synthesis as well as host DNA damage and ER stress responses. UL114-encoded uracil DNA glycosidase (UNG) and UL98-encoded nuclease (NUC) are core proteins that play poorly understood roles in viral DNA replication.³⁹⁹ UNG sponsors excision of uracil from DNA and control of DNA synthesis.^{496,503,633} The need for UNG, like UL97 kinase, is dependent on the cell culture conditions and cell cycle state. HCMV has a strong effect on DNA repair and the DNA damage response,^{203,354,502} and viral replication is compromised in ataxia telangiectasia-mutated (ATM) protein kinase-deficient cells due to the failure to upregulate H2AX-mediated stabilization of the MRE11-NBS1-RAD50 complex.⁷¹⁸ HCMV pUL35 associates with USP7 deubiquitinase as well as with components of the Cullen 4-DCAF1 E3 ubiquitin ligase complex and induces H2AX levels, thereby activating the DNA damage response.⁵⁴⁵ Therefore, pUL35, which is both a DE protein and a component of the tegument where it associates with pp71, is implicated in the modulation of the DNA damage response during infection. ER stress is also dramatically enhanced in HCMV-infected cells.^{20,87} UL38 modulates the ER stress response⁵⁰¹ and contributes to resistance of HCMV-infected cells to accumulating reactive oxygen species^{408,660} that can arise from activation of Mammalian Target of Rapamycin (mTORC)1⁴⁰⁷ as well as PKR-like ER kinase (PERK) pathways.⁷¹⁹ Therefore, the resistance of infected cells to ER stress,^{125,126} likely relies on pUL38, as well as pUL69, that together modulate the mRNA cap-binding complex so that it continues to function through the pUL38-imposed block.²² UL69 is a homolog of HSV-1 ICP27, which also exhibits translational regulatory activity. The most extensively studied aspect of pUL69, shared with ICP27, is its role in the transport of unspliced mRNA from nucleus to cytoplasm.³⁴⁰ UL69 binds RNA and interacts with the DExD/H-box RNA helicase UAP56 or the related URH49 protein, cell proteins involved in RNA transport.

Late Gene Regulation

Expression of HCMV L (γ) genes is maximal after viral DNA replication has begun. In fibroblasts, 24 hpi is typically designated as the dividing point between DE and L phase of replication. Leaky L (γ_1) genes are expressed even in the presence of viral DNA synthesis inhibitors, whereas true L (γ_2) expression is blocked by DNA synthesis inhibition. Observations that most L gene expression can continue even when viral DNA replication is inhibited and has kinetic subclasses were first made on HSV-1, and resulted in the use of γ instead of L. Leaky L (γ_1) and true L (γ_2) genes have very simple promoters defined by the region surrounding the TATA motif, although some have multiple transcription start sites. HCMV transcription at L times involves a considerable level of mRNA splicing and is symmetric off both DNA strands.²⁰⁴ Furthermore, the pattern of transcription and timing varies with cell type.⁶⁶⁸ In general, expression of DE genes continues during the L phase as L genes become active. Three HCMV true L genes have been studied in some detail: UL94, which encodes a tegument protein utilizing a single start site; UL99, which encodes tegument protein pp28, utilizes two separate start sites; and, UL75, encoding envelope gH. L40, a true L gene product encoded from a promoter element within the UL122 region, has been useful as a marker of true late gene expression. The promoter region required for authentic regulation of UL99, UL94, or UL75 is small and

poorly responsive to viral transactivators (IE1-p72, IE2-p86, TRS1) in transient assays. In the context of the viral genome, a UL99 promoter region containing little more than a TATA element and start site (−40 to +6 relative to the start site) is sufficient for regulated transcription, and a G:C-rich hairpin structure naturally present within the first 25 nt of the transcript regulates translation efficiency.²¹ Recently, the importance of specific late transactivators has come to light because “beta-gamma” genes in murine gamma herpesvirus 68 were shown to transactivate L genes promoters from that viral genome. Incisive studies of UL79, UL87, and UL95 control of HCMV true L gene expression^{264,471} revealed these essential genes to be necessary for UL99 and UL75 expression, as well as for the activation of a true late promoter element within UL44.²⁶⁴ An orchestrated L gene-specific complex may form through the association of UL79, UL87, and UL95 proteins with ppUL44/PPS in prereplication foci prior to the initiation of DNA synthesis.²⁶⁴

L gene products control capsid maturation, DNA encapsidation, virion and dense body maturation, and egress from the cell. By the L phase of infection, host cells have been subjected to substantial modification by viral gene products, with a large nuclear inclusion that contains replicating viral DNA and capsids as well as a cytoplasmic inclusion where final envelopment and egress from the cell take place. The balance of transcriptional and translational controls appears important throughout infection, as does the need to balance cellular metabolism and stress.

Capsid Assembly and DNA Encapsidation

The basic features of HCMV capsid assembly and DNA encapsidation^{80,653} are shared across the herpesviruses^{84,273} and evolved from those employed by the HK97 DNA bacteriophages,⁹⁹ including T4.⁵⁸ The process of virus maturation is orchestrated by protein–protein interactions employing common herpesvirus core functions and modified by viral and host enzymes, including protein kinases, proteases, and terminase (Table 62.1). Assembly begins in the nucleus where newly synthesized viral DNA is packaged into preformed capsids and concludes when nucleocapsids obtain an envelope at cytoplasmic membranes. The HCMV maturation machinery produces infectious virions as well as an abundance of noninfectious particles, all of which egress from infected cells by vesicular transport. Three capsid forms accumulate in the nucleus of herpesvirus-infected cells.²¹⁰ Two of these appear to be dead-ends: A capsids that lack both scaffold and packaged viral DNA; and B capsids that contain scaffold but lack viral DNA.^{84,99} The third form, C capsids, contains viral DNA in place of scaffold and probably represents nucleocapsids in the process of maturation. Tegument proteins are added to nucleocapsids sequentially starting in the nucleus and continuing in the cytoplasm, providing stability during nucleocapsid translocation from the nucleus to the cytoplasm and directing nucleocapsid trafficking to sites of envelopment in the cytoplasm.

The process of capsid formation begins when newly translated MCP associates with the maturational protease precursor complex (pPR-AP:pAP) and is transported into the nucleus.²¹⁰ Both pPR-AP, the UL80 gene product, and the assembly protein precursor (pAP), the UL80.5 gene product, are produced independently. The MCP:pPR-AP:pAP complexes are transported into the nucleus where proteolytic processing results in assembly into procapsid shells (Fig. 62.2).^{84,99,210} The maturational protease (PR) processes both pPR-AP and pPR in a

pathway that releases PR, pAP, and AP.²¹⁰ Although pAP is sufficient for procapsid assembly, self-cleavage of pPR-AP to PR and release of a number of pAP and pPR-AP products are required for proper DNA encapsidation and production of nucleocapsids. Precise protease cleavage steps lead to the release of MCP, inactivate the protease, and orchestrate the replacement of the scaffold in procapsids with viral DNA. PR and AP, as well as pAP forms, are completely removed from nucleocapsids into which DNA has been packaged. Properly formed nucleocapsids (C capsids) mature more efficiently than defective nucleocapsids such as scaffold-containing noninfectious enveloped particles (NIEPs).

The procapsid shell is made up of MCP-containing capsomeres, with six copies of MCP per hexon and five copies of MCP per penton. One of the 12 pentons in each capsid is composed entirely of PORT, the UL104 protein, a self-assembling homododecamer.²⁴⁸ The PORT penton provides a channel for viral DNA encapsidation. Triplex (TRI) complex TRI1:TRI2 is added to stabilize hexons and pentons,^{84,99} and SCP decorates the outer capsid surface, interacting with MCP at hexon tips. Based solely on predictions from HSV-1, a putative capsid vertex capping complex (CVC), composed of UL77 (CVC1) and UL93 (CVC2) proteins, decorates penton tips,¹³³ and the UL51 and UL52 proteins would be added to provide capsid stability. Available data on UL77, UL93, UL51, and UL52 mutant viruses^{73,212} predict such roles, although the order of addition of capsid vertex and stabilizing proteins remains to be established. In addition, it is not yet resolved whether these proteins are to be considered components of the capsid or tegument.^{84,99}

The preformed procapsids are made in the nucleus proximal to DNA replication compartments. Encapsidation of unit-length viral DNA genomes depends on a terminase (TER1:TER2) complex^{658,659} interacting with a specialized PORT penton. Terminase machinery recognizes free genomic ends and threads a single genome length of DNA through the PORT channel into each capsid. This process begins and ends at *pac* elements within terminal repeated *a* sequences, proceeding in a directional manner (S component first) on concatemeric DNA. A 129-bp region contains both *cis*-acting *pac* elements (*pac1* and *pac2*) and is sufficient to direct cleavage and packaging,⁷⁰² leaving single-base 3' extensions at both genomic ends. The packaging machinery of herpesviruses is analogous to distant evolutionary relatives, such as T4 bacteriophage.⁵⁸

Specific small molecule terminase inhibitors such as 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (BDCRB) have been exceptionally useful to synchronize viral infection to study maturation and egress steps.¹⁶ BDCRB, which interacts with the ppUL56:ppUL89 terminase, is chemically related to maribavir, the UL97 kinase inhibitor, although the two drugs act on different viral replication steps.¹⁸⁰ B capsids accumulate during BDCRB treatment of HCMV-infected cells but fail to encapsidate viral DNA after release of drug block, suggesting that DNA packaging machinery operates as capsids are assembled. The ATP binding site within ppUL56 and the nuclease site within ppUL89 contribute to activity. The nuclease site has a structure reminiscent of retroviral RNaseH-integrases and can be bound by integrase inhibitor.⁴²⁵ The antiviral drug candidate AIC246 apparently targets the ppUL56 terminase subunit.^{215,493}

Capsid localization, packaging, and cleavage of viral DNA are all regulated through phosphorylation. UL97 kinase (VPK) and cell cycle-regulated kinases overlap in function to support

viral replication.^{246,366,494} Once formed in the nucleus, nucleocapsids translocate to the cytoplasm.^{80,653} The most capsid-proximal major tegument protein in HCMV is the UL32 protein, pp150.⁷²⁹ pp150 is added to nucleocapsids in the nucleus^{548,651} and accompanies maturing particles to the cytoplasm. UL32 or UL96 mutant viruses accumulate nuclear pp150.^{651,652} pp150 and ppUL96 stabilize nucleocapsids in a common pathway of translocation to cytoplasmic sites of envelopment.⁶⁵²

Translocation, Envelopment, Egress, and Release

Virion maturation follows a two-stage envelopment and egress process that begins in the nucleus and finishes with virion release by exocytosis from the cell,^{80,653} depicted in Figure 62.2.

Nucleocapsid translocation from the nucleus to the cytoplasm is carried out by a herpesvirus-conserved nuclear egress complex (NEC), which carries out nucleocapsid translocation from the nucleus to the cytoplasm. The NEC is located at the inner nuclear membrane. This is probably one quality control step where preference is afforded to DNA-containing C capsids (nucleocapsids) over A or B capsids, possibly mediated by tegument proteins that associate with C capsids.^{80,653} The NEC is composed of a type II membrane-spanning component (NEC1, UL50 gene product) together with a nuclear lamina-interacting component (NEC2, UL53 gene product).³⁶⁶ These together facilitate egress from the nucleus by recruiting cellular and viral protein kinases to phosphorylate and disrupt the nuclear lamina cage and allow nucleocapsid passage. Consistent with this, NEC1 or NEC2 mutant viruses in many herpesviruses including MCMV and HCMV accumulate nucleocapsids (C capsids) in the nucleus. In HCMV, host protein kinase C functions interchangeably with viral ppUL97/VPK, cellular p32, and the lamin B receptor in the NEC to phosphorylate lamins.^{366,392} An HCMV-encoded nuclear rim-associated cytomegaloviral protein (RASCAL), the product of cORF-29, also interacts with ppUL50³⁹³ and may also contribute to NEC-dependent disruption of nuclear lamina. Following primary envelopment/de-envelopment, nucleocapsids are delivered to the cytoplasm with pp53/NEC2 remaining attached. ppUL53 accompanies the nucleocapsid as it is transported through the cytoplasm and becomes part of the tegument in mature virions. ppUL50/NEC1 remains in the nucleus and apparently cycles ppUL53. Together with MIE and replisome components, ppUL50 makes a sensitive indicator of nuclear contamination within purified virus particle preparations. Other cellular proteins, including BiP/GRP78, interact with ppUL50, and, together with dynein, contribute to nucleocapsid translocation⁸⁶ as well as the organization of the AC, although these pieces have not been entirely fit into a pathway.

Nucleocapsids most likely reach the cytoplasm by obtaining a temporary envelope at the inner nuclear membrane passage through the perinuclear space and de-envelopment at the outer nuclear membrane (Fig. 62.2). Once delivered to the cytoplasm, transport is directed to sites where tegumented nucleocapsids obtain a “second,” final envelope to become virions. This final envelopment occurs at ERGIC/endosomal/exosomal membranes based on lipid composition^{343,666} and results in formation of mature virus that is released from cells. Once formed, virions are translocated to the cell surface within small vesicles using cellular exocytic transport machinery. All of these important cytoplasmic steps in maturation, from tegumentation through release, are closely associated with the specialized AC. The AC consisting of a remodeled cellular

secretory apparatus that distorts the cell nucleus, resulting in a characteristic “kidney” shape while the cell takes on a characteristic “owl’s eye” appearance.²⁴⁷ In electron micrographs, virions and dense bodies can be seen concentrated within this region, some undergoing envelopment. Within the AC, the *cis*- and *trans*-Golgi network, ER, ERGIC, and endosomes are arranged into a compact nested and cylindrical array adjacent to the nucleus.^{143,144} Cellular markers for endosomal sorting and transport (ESCRT) and Rab GTPases localize to the AC together with viral structural proteins.^{80,653} Rab6, in particular, appears to facilitate maturation events in the AC.²⁶¹ The AC is a prominent feature of infected cells, the cytoplasmic inclusion nestled into a kidney-shaped nucleus. Although replication of all herpesviruses is thought to follow a similar pattern, the AC is a unique feature of beta herpesvirus-infected cells. In addition to virions, the AC supports maturation of many noninfectious particles, the most numerous of which are capsidless dense bodies that constitute about half of the mature particles produced. These form in the cytoplasm and follow envelopment and release steps similar to virions.^{80,653}

Tegument proteins associate with nucleocapsids in the cytoplasmic AC and facilitate envelopment. The AC is the principal location where final tegument components assemble on nucleocapsids and virions form. Envelopment produces mature, infectious virions within vesicles that are transported to the cell surface for release. Partially tegumented and partially enveloped nucleocapsids (often considered as being caught in the act) can also be observed in the AC. Infection with replication defective mutant viruses affecting UL71,^{557,716} UL94,⁴⁷⁵ UL96,⁶⁵² and UL99⁵⁷⁰ reveal greater proportions of tegumented nucleocapsids and partially enveloped particles. The UL99 gene product, tegument protein pp28 (a myristoylated and phosphorylated tegument protein), performs a key function in virion envelopment^{572,570} and acts through multimerization.⁵⁷¹ ppUL94, a binding partner of pp28,^{474,662} is involved at the same level as pp28 based on mutant virus phenotype.⁴⁷⁵ ppUL71 also controls the acquisition of the envelope,^{557,716} oligomerizing through a leucine zipper motif.³⁸⁸ Therefore, the emerging theme in directing acquisition of an envelope is protein–protein interactions, possibly in an oligomerization-driven process.

Other tegument proteins and envelope glycoproteins that localize to the AC have their major impact upstream or downstream of final envelopment. Virus lacking pp150, encoded by UL32, produces nuclear nucleocapsids that appear normal and deliver viral DNA to the cytoplasm but fall apart during this translocation, producing an AC that lacks recognizable capsids.⁶⁵¹ Therefore, pp150 is absolutely crucial for the maintenance of nucleocapsid stability during its translocation to the AC. pp150 may control further steps based on its interaction with bicaudal D1-Rab6 because this complex controls dynein-dependent vesicular trafficking.²⁶⁰ ppUL96 appears to cooperate with pp150 to stabilize nucleocapsids during translocation. A UL96 mutant also produces nucleocapsids that appear normal in the nucleus but become unstable in the cytoplasm.⁶⁵² Envelope glycoprotein gM, most likely together with gN, either contributes directly to secondary envelopment or helps organize the AC. The cytoplasmic domain of the multiple membrane-spanning gM interacts with FIP4-Rab11 complex to harness endosomal recycling and provide a platform for final envelopment to occur.³⁰⁸ UL100 mutants disrupting this domain are slow to form an AC and produce less infectious

virus due to defective final envelopment.³⁰⁷ The gN cytoplasmic domain is also crucial for this process,³⁵⁸ consistent with the need to assemble a gM:gN complex and in agreement with the colocalization of gM:gN with ESCRT components that are also involved in endosomal recycling. Most important, both VPS4A and CHMP1A components localize within the AC, with a striking colocalization of CHMP1A and assembled gM:gN.⁶⁵⁰ Using a series of dominant negative inhibitors, this study implicated CHMP1A ESCRT component and the ATPase VPS4A directly in final envelopment.

Once virions are enveloped, they are carried in vesicles to be released through exocytosis. This step carries virus particles as well as dense bodies to the extracellular space and is carried out by the cellular exocytic pathway. Surprisingly, HCMV is the first herpesvirus with evidence of a viral function that facilitates exocytosis of viral particles. UL103 mutant virus is defective in releasing virus to the extracellular space.¹⁶ Therefore, ppUL103 influences vesicle trafficking after envelopment to facilitate more efficient egress of fully enveloped virions and dense bodies out of the cell. These studies employed the DNA encapsidation inhibitor BDCRB to synchronize infection at late times, and showed that maturation and release is complete within about a day. Progeny virus is efficiently released from cells, reaching steady state levels where half of the infectivity at very late times postinfection is found in the culture fluid and half remains cytoplasm associated.

Effects on the Host Cell

Above and beyond the cellular changes that facilitate replication, presented in the rest of this section on replication, HCMV infection modulates cell-intrinsic host defense in three important ways: (1) altering pathogen recognition receptor signaling; (2) subverting cytokine and interferon activation to reduce the impact of cytokines and interferons; and (3) suppressing cell death to prevent or delay the premature demise of infected cells. As with other aspects of viral modulation of host defense, cell intrinsic pathways are dampened but not necessarily eliminated. Furthermore, modulation of intrinsic host defense develops along with the stages of the replication cycle, so it is sometimes difficult to distinguish a role in replication from a role in immunomodulation. Furthermore, HCMV dramatically stimulates cells through virus particle attachment-dependent^{131,262} as well as early gene expression-dependent pathways such that virus-infected cells to be cycling without proceeding into a true S phase as viral DNA synthesis and maturation take place, based on a variety of gene expression pattern analyses.^{371,550,707} Therefore, infection suppresses some cell signaling pathways, such as interferon activation, that limit viral replication, while stimulating others, such as cell cycle kinases, that benefit replication. As infection proceeds from the early phase to the late phase, a wide range of changes occur in conjunction with development of a dramatic cytopathology. Both a cytoplasmic AC and a nuclear replication compartment form as infection progresses along with a striking metabolic signature^{376,549,682,731} and increasing resistance to stress.^{377,378} A wide variety of viral gene products have been implicated in the control of cellular changes as well as in blocking pathways that become triggered (Table 62.1).

The impact of HCMV infection varies with both viral strain and cell type. Experiments with viral mutants together with either knock-down or knock-out cells or animal models facilitate the identification of viral functions and the cellular

pathways being targeted; such experiments have also revealed viral strain and strain variant differences in behavior.^{246,377,378} Significant effort has gone into the identification of HCMV gene functions that contribute to replication in a cell type-specific manner, distinguishing behavior in fibroblasts from behavior in epithelial, endothelial, macrophage, dendritic cell, and myeloid progenitors that are available as continuously propagated cell lines or cultured directly from human tissues. Even the most commonly studied fibroblast host cell type sometimes give diametrically opposed responses to viral infection depending on host cell variation, as revealed when UL97 function¹²⁰ or M45 function⁶⁷⁸ was assessed. Therefore, CMV infection sometimes unveils crucial host cell differences where none were suspected, such as the way RIP3 and DAI levels are known to control the susceptibility to virus-induced programmed necrosis.^{677,678} Viral strain variability is more complex, because CMV is known to undergo sequence drift during propagation in cell culture. To circumvent viral strain variability, efforts to employ low passage strains as well as to evaluate multiple viral strains for common properties have been paired with study of multiple cell types. This situation has become a particular concern where the impact of a viral gene product is subtle as well as in situations where no underlying mechanism is demonstrated. An eventual integrated understanding of the impact HCMV has on host cells will clearly depend on the generation of complementary data from different settings.

The orchestrated NF- κ B-dependent cytokine and IRF3 plus NF- κ B-dependent interferon responses that follow HCMV particle attachment and penetration leads to diverse host cell gene expression that includes features of growth factor activation and second messenger-type responses associated with calcium mobilization.^{131,262,734} This IFN-like response pattern predominates independent of virus strain or cell type, with NF- κ B activation following a two-phase pattern starting as early as 5 minutes after exposure to virus particles, which is followed up with a subsequent activation of this pathway around 24 hpi.^{131,262,734} This activation pattern is reminiscent of pathogen recognition receptor responses,⁵³⁷ where there is a primary response as well as a secondary response to factors like TNF and IFN that result from the primary response, and, in agreement with this, antiviral gene products are produced.^{131,262,573} DNA-dependent activator of interferon (DAI, also called ZBP1 and DLM-1) is a cytosolic DNA sensor that activates IRF3⁶⁴⁷ and NF- κ B²⁸¹ and has been implicated as the main pathogen recognition receptor in postattachment events that trigger virus particle-induced IFN response in fibroblasts.¹⁵³

Although dramatic, the IFN-like impact of HCMV on cells is dampened by input virion proteins as well as by additional viral gene products made during infection. Input pp65, the major tegument protein, localizes to the nucleus where it reduces activation of cytokines and IFN by input particles,³⁷¹ while itself being dispensable for replication. Input virion ppUL69, ppUL97, and pp71/ppUL82, all known for impacting viral gene expression, also modulate the host cell response to infection. In monocyte cell lines, a further damper on cytokine production is the UL7 gene product, a CD229-SLAM homolog expressed late in infection.¹⁷⁷ Viral proteins encoded shortly after infection, including the MIE gene products, IE1-p72 and IE2-p86, IE gene products from the UL36-UL37 locus, and a wide array of DE and L gene products modulate the host cell response to infection by sustaining cell viability as well as energy

production and metabolism. Host cell modulation by IE1-p72 involves formation of a complex with STAT1 and STAT2 to prevent association with IRF9, thereby dampening activation of IFN-responsive promoters.⁴⁵⁸ IE2-p86 contributes to down modulation of this response in ways that have not yet been distinguished from its overall repressive effect on cell viability.⁶⁵⁵ The pathogen recognition receptor DAI senses incoming viral DNA and triggers either an IFN-like response in HCMV infection¹⁵³ or, in MCMV, a RIP3-dependent cell necrotic death naturally suppressed by the virus.^{677,678} M45-encoded vIRA, a RIP homotypic interaction motif (RHIM) inhibitor, is an important suppressor expressed by MCMV^{508,676,677,678} that can also deflect NEMO-dependent NF- κ B signaling during infection.¹⁹¹ Whether HCMV encodes functions that modulate DAI-RIP3 death remains to be investigated. Therefore, initial contact of HCMV with cells triggers DAI activation of NF- κ B and IFN β ,¹⁵⁴ whereas MCMV completely suppresses this signaling in infected cells. In this way, necrotic cell death, apoptotic cell death, and the cytokine activation pathways are all tightly regulated as they have evolved to carry out cell-intrinsic host defense^{279,677,678} to eliminate virus-infected cells.⁴⁰⁵ As a result of studies on cytomegaloviruses, host defense pathways under the control of caspase 8 and RIP3 are becoming recognized as evolutionary adaptations to virus-encoded cell death suppressors.²⁸⁰

The cell cycle state at the time virus enters a cell is a crucial determinant that dictates whether infection is impeded or proceeds. Cell cycle is a factor in reactivation from latency as well. Permissive fibroblasts in G0 or G1 at the time of viral entry support IE gene expression and proceed into viral replication, but cells in S or G2 suppress IE gene expression, and, surprisingly independent of the ND10/PML-repression pathway,⁷⁴⁰ quite distinct from epigenetic control of viral gene expression in G0/G1 cells.⁷³⁹ Curiously, treatment with either cdk inhibitor or DNA damaging agents reverses this suppression, suggesting that there is a yet-to-be unveiled cdk/DNA repair-dependent pathway of regulation that can shut down virus at a pre-IE phase following entry.^{283,284,739,740} To support viral replication, cells must complete cell division and cycle back to G1 when IE gene expression occurs and replication proceeds.⁷⁰⁷

Once replication initiates in G0/G1 cells, input viral tegument proteins together with newly expressed IE proteins dysregulate normal cell cycle progression such that many cellular gene products associated with cell division are available to the virus, although cellular DNA synthesis is blocked.^{371,550,707} There is overlap in the impact of viral proteins on viral and host gene expression, as described in the section on replication. Virion VTA/pp71 induces resting cells into G1 phase by interacting with cellular Daxx and ATRX, thereby dampening PML-dependent repression of gene regulation.^{352,544,579} analogous to derepression of viral IE gene expression.^{579,628,629} Cellular genes responsive to pp71 may also respond to pathogen sensor signaling, stress, and/or autophagy.^{382,491} Virion-associated ppUL69, a herpesvirus-conserved multifunctional regulatory protein (MRP) that is similar to HSV-1 ICP27, is required to regulate gene expression as well as to inhibit cell cycle progression during infection, although the mechanisms remain to be established. Like HSV-1 ICP27, ppUL69 can bind RNA and shuttles between the cytoplasm and nucleus, thereby contributing to differential viral and cellular mRNA transport. Export of unspliced RNA in HCMV-infected cells involves the cellular DEXD/H-box (DEAD-box-related) RNA helicase UAP56,³⁴⁰

an aspect that appears distinct from HSV-1. Once infection begins, both IE1-p72 and IE2-p86 may alter cell cycle progression, although studies do not distinguish indirect impacts via regulation of other viral genes. IE2-p86, in particular, binds to p53, as well as other cell cycle-regulated proteins, and may influence cell cycle progression or orchestrate some activity of p53 and Cdks that drive the infected cell into a state that appears similar to actively cycling cells.^{257,628,629}

To successfully replicate in the host, HCMV must counteract cell death triggered by diverse insults arising from cell-intrinsic stress, pathogen sensors, TNF-family death receptors, and cytotoxic NK and CD8 T cells. The HCMV IE gene UL37 \times 1 encodes a mitochondrial inhibitor of apoptosis (vMIA)^{201,214,377,378} that is the most potent inhibitor of mitochondrial cytochrome *c* release known. vMIA naturally prolongs HCMV replication by defusing a programmed cell death pathway dependent on a mitochondrial resident serine protease, HtrA2.³⁸⁰ vMIA also dampens release of ER Ca⁺² stores.⁵⁷⁴ vMIA has two critical domains: an amino terminal hydrophobic membrane anchor that associates with lipid rafts in the ER^{712,713} to target the protein to the outer mitochondrial membrane, as well as an internal alpha helical antiapoptotic domain that binds and sequesters the Bcl2 homologs Bax and Bak as an oligomer.^{286,694} While blocking cytochrome *c* release, the impact of vMIA is to induce mitochondrial fission. In addition to Bax and/or Bak, this cell death suppressor must also engage growth arrest and DNA damage 45 (GADD45) and Bclx_L proteins via its antiapoptotic domain to prevent apoptosis.⁵⁹¹ This vMIA-dependent change blocks release of cytochrome *c* following induction of intrinsic or extrinsic apoptosis and prevents activation of executioner caspase 3.^{377,378} MCMV encodes two viral proteins, one (UL38.5-encoded vMIA) inhibits Bax activation^{377,378} and another (UL41.1-encoded viral inhibitor of Bak oligomerization [vIBO]) inhibits Bak oligomerization.^{91,363} Viruses with mutations in UL37x1 fail to encode a diverse family of differentially spliced UL37 gene products—most of which are glycosylated—that can all suppress cell death. Cells infected with UL37 \times 1 mutant viruses become more sensitive to inducers of caspase-dependent apoptosis³⁸⁰ and succumb to an HtrA2-dependent serine protease programmed death pathway that naturally terminates infection in fibroblasts.³⁸⁰ The amino terminal mitochondrial targeting domain (aa 5–34) and a carboxyl terminal antiapoptotic domain (aa 118–147) are together necessary and sufficient to block cell death induced in a wide variety of ways.²⁰¹ In addition to vMIA, a noncoding RNA, β 2.7 accumulates to very high levels during infection,²⁰⁴ and interacts with mitochondrial complex I to suppress apoptosis resulting from exposure to the respiration toxin rotenone as well as from cell stress.⁵¹³ β 2.7 RNA also prevents metabolic changes that are controlled at the mitochondrion.⁷³⁷ Neither β 2.7 nor vMIA is essential for viral replication in fibroblasts, although some viral strains are more or less dependent on these functions, possibly due to overlap in sustaining mitochondrial respiration through the very long HCMV replication cycle. Other viral gene products, including UL38, IE1, and IE2, contribute to cell death suppression³⁷⁸ in ways that may be related to their impact on gene expression.

Viral IE protein vICA inhibits caspase 8 activation in a manner analogous to cellular FLICE inhibitory protein (cFLIP) without exhibiting any obvious sequence similarity to cFLIP or any vFLIP. Like cFLIP_L, vICA prevents activation by

binding to the prodomain of procaspase-8, blocking both basal as well as TNF receptor-induced activity of this caspase.⁵⁸⁵ Therefore, vICA blocks extrinsic apoptosis induced by TNF family death receptors, but fails to block intrinsic cell death. vICA is one of the genes that becomes mutated in HCMV, as well as RhCMV, upon laboratory propagation, and this change increases susceptible to death receptor-induced apoptosis.⁵⁸⁵ In macrophages, vICA-deficient viruses induce premature apoptosis that cuts short replication even in the absence of inducers of extrinsic apoptosis.^{377,405} The other potent inhibitors of apoptosis such as vMIA and $\beta 2.7$ cannot rescue cells in these settings. The function of vICA is likely to counteract the consequences of death receptor TNFR1 upregulation mediated by UL138, an early gene product that is also latency-associated.^{321,406} In RhCMV, the nonfunctional UL36 homolog in the commonly used 68.1 strain³⁸¹ has little apparent effect on infection in macaques,^{237,238,240} suggesting that additional cell death suppressors are active. However, mutation of MCMV M36-encoded vICA, like HCMV UL36, results in premature apoptosis in mouse macrophages and attenuates virus replication in mice.¹²³ In MCMV, where viruses with a mutated M45 gene are severely attenuated,^{677,678} vICA inhibition of caspase 8 sensitizes cells to DAI-RIP3-mediated programmed necrotic host defense pathway.⁴⁰⁵ The predicted consequence of expression of vICA (together with vIRA) in MCMV assures that death receptor signaling is shunted toward cytokine activation and away from apoptosis or programmed necrosis. Therefore, evolution has left this cell death suppressor with different roles despite high conservation across primate and rodent CMVs.

Infection with HCMV and other herpesviruses results in production of double-stranded RNA (dsRNA), reaching high levels at late times during replication.²⁰⁴ Viral dsRNA, like dsDNA, triggers IFN activation via pathogen recognition receptors such as TLR3, which scores as an important receptor in MCMV infection.⁶⁴⁶ dsRNA also contributes to activation of proteins that mediate IFN-induced antiviral resistance, including protein kinase R (PKR) and 2'-5' oligoadenylate synthetase (OAS). If not suppressed by viral modulators, the pathogen recognition response and IFN effector pathways shut down protein translation in infected cells, prematurely terminating viral replication. HCMV encodes proteins that bind dsRNA to prevent activation of the PKR and OAS pathways. HCMV latency-associated gene product ORF94, a nucleolar protein, blocks OAS activation.⁶⁴⁸ In HCMV, either of the US22 family members, pIRS1 or pTRS1, modulates the interferon response within infected cells by blocking dsRNA-dependent activation of PKR or OAS as soon as these IE proteins accumulate during replication.^{116,370,371} Similarly, in MCMV, two US22 family members, m142 and m143, block PKR activation, although these function together as a complex.^{89,371}

PATHOGENESIS

HCMV infection in humans initiates when exposure to virus-infected body fluids overcomes innate immune barriers and sustains replication and dissemination. These events occur most frequently at mucosal sites. A systemic infection takes place where virus can be detected in PB leukocytes that are responsible for dissemination to salivary glands and kidneys. Depending on the age of the host, persistent shedding in saliva and urine

may proceed for months to years. HCMV is readily detected in body fluids during primary infection regardless of the immune status of the host or any disease pathogenesis. In immunocompromised individuals, HCMV may infect endothelial cells in addition to myelomonocytic cells at levels that may contribute to disease pathogenesis in the immunocompromised host. By far the most clinically useful virus-positive cell is the neutrophil, which forms the basis of the antigenemia test.²⁰⁸ Although viral antigen-positive neutrophils accumulate in relation to the intensity of the infection, the interaction represents an abortive infection, possibly due to the phagocytic properties of this leukocyte. Currently, virus is most commonly detected in body fluids and bloodstream by DNA PCR infection rather than by virus culture or antigenemia. Therefore, infection by any route culminates in a leukocyte-associated viremia that deposits virus in sites from which shedding can infect new hosts. When resolved through an effective cellular immune response, a latently infected myeloid cell population remains in the bone marrow precursors of monocytes, macrophages, and dendritic cells. This source of latently infected cells allows distribution throughout the body and contributes to the risk of transfer of HCMV with any tissue or organ during transplantation.

In contrast to the mucosal route that characterizes transmission in most of the human population, transmission during pregnancy involves the placenta,⁴⁶⁹ although the precise steps that gain access to the fetus are not yet established.³⁵⁹ Persistent and recurrent shedding in urine and saliva is more common in children than in adults.⁹³ Persistent shedding in urine contributes to transmission among children as well as from children to adults. Saliva is an important source in all age groups. In addition, breast milk transmission from mothers to children is important, but is only associated with a disease risk in low-birthweight newborns^{173,313} where viral inactivation methods are effective.²¹³ Finally, cervical or seminal secretions may contribute to transmission patterns in adults.⁶²²

HCMV is a classic opportunist such that disease pathogenesis follows either primary infection or reactivation from latency primarily when the immune response is compromised. The virus has long been known to reactivate and replicate to levels that cause pathology in the inflammatory environment set up by allogeneic tissue rejection; however, viral factors and mechanisms that underlie these viral properties remain to be characterized. Less well-appreciated is the fact that viral reactivation occurs frequently following trauma, surgery, and autografting, but disease appears to be rare in these settings,¹³⁴ suggesting that additional host factors contribute to disease pathogenesis in allogeneic transplant settings. Through their impact on cell intrinsic, innate and adaptive host defense pathways, virus-encoded immune modulatory gene products may represent important virulence determinants in disease pathogenesis. HCMV RhCMV, or MCMV each encode at least 100 gene products contributing to the host-pathogen interaction.

Due to strict species specificity, HCMV immunomodulatory gene products have been studied primarily in cell culture models. These have been complemented by some studies in animal models of infection, immunity, and disease pathogenesis. Many phenomena have been catalogued in both arenas but few concrete principles have emerged regarding the role of specific viral gene products or host pathways to disease. On the one hand, are differences in cultured cell systems that have been the focus of studies on HCMV mutant viruses, making

comparisons and generalization difficult. On the other hand, strain variability and adventitious mutations discussed above have complicated phenotypic studies with HCMV mutants. It is worth recounting that, despite the remarkable potency of unique immunostimulatory proteins encoded by HCMV US28 chemokine receptor,⁴²⁶ UL146/vCXCL1 chemokine,⁴⁶⁶ and UL111A/cmvIL-10 cytokine⁶⁰⁸ there is still remarkably little concrete understanding of how these properties contribute to outcomes during natural infection.

During infection, viral IL-10 is expressed in different forms, one (cmvIL-10) dominating productive replication and another (LAcmvIL-10) dominates latency in myeloid progenitors.⁵⁸⁸ These forms have subtle differences in their impact on T and B cells.⁶⁰⁷ Related cytomegaloviruses, such as MCMV or RhCMV, modulate host immunity and inflammation in ways that appear analogous to HCMV, but each of these surrogate viruses employs different mechanisms to achieve a common set of ends. Primate CMVs encode viral IL-10 and most closely related follow HCMV. All cytomegaloviruses engage myeloid lineage cells in the host as a component of dissemination during acute infection and as a site of latency during lifelong association with the host, although this may not be an exclusive relationship. The presence of latent virus in CD34+ and myeloid lineage progenitor cells^{403,510,515,580} likely gives rise to viral genome positive cells that distribute the latent viral reservoir throughout the host. Progenitors give rise to macrophages and myeloid dendritic cell lineages that take up residence in all tissues. A distribution of latent virus in monocyte-derived macrophages and dendritic cells remains the most likely explanation for virus transfer in high-risk SOT settings (seropositive donor organ into seronegative recipient), as predicted more than 40 years ago.^{704,705} Therefore, these specialized antigen-presenting cells very likely facilitate continued dissemination and distribution of latent virus throughout life while also being subjected to immunomodulation by viral gene products.^{403,510,515,580}

The potent immune modulators expressed by HCMV are likely to allow the virus to escape the full impact of immune clearance and to facilitate persistence.^{378,398,401,403} Modulatory functions are predicted to facilitate primary infection, contribute to efficient dissemination, and prolong replication. The adaptive T-cell response, which is less developed in children,⁶⁷¹ effectively controls active HCMV replication only after a prolonged period of time. This response must also contribute to maintenance of viral latency, either through suppression of reactivation, or, more likely, by preventing viral amplification following reactivation. T-cell immune deficit, such as occurs during AIDS, allogeneic HCT, following SOT, or during treatment with strongly T-cell suppressive lymphoma therapies leave patients open to HCMV infection and disease. Disease pathogenesis in these settings is widely believed to follow as a result of three collaborating events: (a) a proinflammatory cytokine-driven process that stimulates the latently infected cell to differentiate and reactivate latent virus to replicate; (b) immunosuppression that allows amplification via productive replication, either locally or systemically; and (c) direct or indirect viral or host immune-mediated damage that manifests as acute or chronic disease.²⁴⁷

Because of species specificity, study of HCMV in the context of a naturally infected host has remained largely unexplored. Animal models using available surrogate viruses such as MCMV or RhCMV, and cell culture models using HCMV, have been slow to dissect the basis of the virus–cell interactions

that underlie viral persistence in myeloid lineage cells of the bone marrow. An immature cell type where host epigenetic regulation of viral gene expression dominates has long been considered key to establishment and maintenance of latency, as a change in this repressive environment is key to reactivation and amplification as myeloid cells differentiate, particularly in an inflammatory environment.⁵¹² Current models of that focus on macrophages and dendritic cells have not yielded much evidence of viral control, save gene products such as UL138, LAcmvIL-10, and ORF94 that all have a primary effect as immunomodulators. Latency is crucial to the survival of HCMV in the immunocompetent host, yet, even the most scrupulous of models have only given rudimentary insights.⁵⁸⁸ Many preexisting expectations have been reinforced by these studies, but few of the biological details have emerged such that we still do not understand how HCMV remains for life in everyone who becomes infected.

Entry, Dissemination, Shedding, and Control in the Host

HCMV transmission patterns occur by direct contact, mainly as children acquire infection from parents and other children and then gradually throughout life.^{42,620} Age dependence of acquisition is linked to shedding patterns as well as hygiene.^{95,566} HCMV intrauterine transmission results in infection of approximately 1% of newborns, and is a worldwide concern.⁷²⁰ Child-to-child and adult-to-child transmission patterns are dependent upon virus shed in saliva, urine, and breast milk. Adult-to-adult transmission relies on sexual transmission. Virus is transferred by direct contact with secretions.^{93,621} Co-infection with multiple strains, reinfection with additional strains, changes in viral persistence, and reactivation of latent infection all likely contribute to transmission patterns. Adaptive T-cell immunity, in particular, maintains low viral levels, but this protection is not absolute, and, as discussed in the section on Immunity, the modulation by viral functions enables the virus to escape control. Consistent with this view, any other compromise to cellular immunity, in particular, contributes to the importance of this virus as a classic opportunist, whether it is congenital disease where the virus crosses the placenta to an environment where the immune system is not fully developed, or severe, life-threatening disease that follows immunodeficiency, immunotherapy, or immunosuppression.

Primary infection typically initiates with replication in mucosal epithelium at a portal of entry that follows direct contact with infectious secretions from another individual who either has a primary or recurrent infection. A high incidence of infection in certain populations and accumulating evidence that hand washing among caregivers prevents acquisition of HCMV from infants provide excellent evidence that this virus reaches a large proportion of the population through a direct route of transmission.⁹⁴ A systemic phase of infection depends upon a leukocyte-associated viremia that may be prolonged. Immature monocytes recruited from bone marrow (BM) seem to function as vehicles for viral dissemination, ferrying the salivary glands and kidneys, as well as other secretory organs, where the virus infects ductal epithelial cells, is shed and transmitted to new hosts. MCMV has provided key evidence for such a mode of dissemination as well as for the control of infection.¹⁴⁰ Dendritic cells together with NK cells produce cytokines that regulate development of adaptive immunity that is crucial for lifelong

host control of the virus. Neither HCMV nor MCMV can be detected cell-free in PB, although viral DNA in plasma is used to monitor the clinical status of immunocompromised individuals for active infection. Plasma viral DNA likely represents either viral genome fragments or encapsidated DNA that has been released from infected cells and tissues. This method of viral DNA detection is important in the assessment of immunocompromised hosts, along with assays for viral DNA, RNA, or antigen in PB neutrophils, mononuclear cells, and endothelial cells. This virus exhibits a relatively broad range of tropism for differentiated cells of various lineages when immune surveillance is compromised. Viral DNA is detected in PBMCs of long-term infected immunocompetent hosts, and has been used to identify latent reservoirs, but virus is not detected in plasma, neutrophils, or endothelial cells in immunocompetent individuals. The systemic phase of primary infection in adults is accompanied by the highest levels of persistent viral shedding in urine, saliva, breast milk, and genital secretions, and, as such may be the most important sources for transmission between hosts. The distribution of virus during acute infection has only been studied in the immunocompromised host, where a range of endothelial, epithelial, fibroblast, and hematopoietic cells in PB, BM, and tissues become infected. The involvement of these cell types in immunocompetent individuals is less well understood. Hematopoietic cells are likely to control dissemination⁵²⁴ and ductal epithelial cells are sites of viral production and shedding into bodily secretions. There is no direct evidence supporting a role for endothelial cells in primary infection or latency, although these cells are likely to play an important role in systemic infection that leads to a well-documented seeding of salivary gland and kidney tubule ductal epithelia. Viremia continues for a long period of time an adaptive immune response is first detected, typically lasting months in adults and years in young children. This prolonged period of primary infection is surprising given the intensity and quality of the host immune response, although this pattern reinforces the balance that characterizes the general picture of HCMV pathogenesis. An initial persistent infection is often observed, with gradual clearance of acute infection in immunocompetent individuals that correlates with an effective cell-mediated immune response rather than antibody levels. The relatively prolonged period of persistent shedding observed in infants correlates with their poor cellular immune response.⁶⁹¹

Immune Response and Modulation

Primary HCMV infection is brought under control by a combination of a coordinated innate and adaptive immune response. Much of what is considered to be important in the initiation of the immune response against HCMV comes from decades of immunological investigation of MCMV-infected mice.⁶⁹¹ Innate interferon and NK cell responses are important in the immediate control of viral infection, and, although monocyte-derived macrophages act as vehicles for dissemination and also regulate T-cell responses, they are not critical in innate clearance of MCMV.¹⁴⁰ Protective adaptive responses appear to be T-cell mediated, with antibody playing a secondary role in most settings, although important exceptions exist. Seropositive recipients of a HCMV-infected SOT are better able to control active viral infection and disease than seronegative recipients, and, concomitantly, seropositive donors of HCT provide better control of active viral infection than do seronegative donors in seropositive recipients. The severity of

disease due to primary HCMV infection in renal transplant patients is controlled through pretransplant immunization with the live-attenuated Towne strain of HCMV,⁴⁸³ which stimulates both antibody and cellular immunity.²⁶⁹

The natural immune response to HCMV has been characterized as very broad, strong, and durable, particularly with regard to the antiviral CD4 and CD8 T-cell frequency in the peripheral blood.^{57,268,644} The robust response and ease of detecting HCMV-specific CD4 and CD8 T cells has facilitated study of virus-specific immunity in humans.²⁶⁸ Despite robust T-cell and neutralizing antiviral antibody immunity, immune control of this virus is incomplete. Recurrence, reinfection, and shedding occur sporadically throughout life, particularly in pregnancy.^{96,566} The contrast between a robust immune response and incomplete control is often ascribed to the diversity of immune modulators encoded by HCMV and their effect on host defense.²⁶⁸ A high level of immune responsiveness is present years beyond primary infection at levels that exceed those of other herpesviruses or persistent viruses,⁶⁹¹ and the pattern of response to certain epitopes increases in an inflationary pattern that has been characterized in MCMV, RhCMV, and HCMV infection. Somewhat surprisingly, the high level of HCMV specific T cells in the periphery is not reflected in the lymph nodes,⁵¹⁹ which has been taken to indicate that this virus does not consume immunological attention to undermine responses to other pathogens. Although seropositive women occasionally transmit HCMV, the incidence of congenital disease in their offspring is less frequent and less severe than occurs in offspring of seronegative women with primary HCMV infection, indicating the value of immunity. Seropositive women who transmit virus transplacentally exhibit reduced antigen-specific T-cell proliferation,⁵²⁵ particularly in the quality of CD4 T-cell responses,³³⁵ as well as lower avidity IgG,³¹⁹ consistent with a role for adaptive immunity in protection from transmission. Transmission among children also results in a skewed CD4 T-cell response profile^{310,671} that may underlie prolonged shedding that contributes to both child-to-child and child-to-adult transmission patterns. Placenta contains virus in relatively high frequency and is associated with stillbirth.²⁶⁶ Transmission through the placenta⁴⁶⁹ and subsequent infection of the fetus may be blocked by neutralizing viral antibodies.^{359,431} Pooled human gammaglobulin, containing HCMV neutralizing antibodies, has been employed for prophylaxis as well as therapy in congenital disease settings.^{432,433} Lifelong HCMV infection has been associated with immunosenescence in the elderly.⁴⁶⁰ Despite susceptibility to reinfection, immunocompetent individuals can better control reinfection and avoid disease correlates following subcutaneous inoculation.⁴⁸³ Antiviral CD4 and CD8 T-cell immunity is crucial for lifelong control of infection and resistance to reinfection.^{268,714} In AIDS, successful anti-HIV therapy has reduced HCMV disease primarily as a result of increased level of HCMV-specific T cells.³³⁴

A characteristic feature of HCMV infection in the immunocompetent host is the persistence of active primary infection viremia, which is accompanied by virus excretion for months (adults) or even years (children) despite a fully functional immune response. The ability of this virus to avoid clearance by host immune mechanisms may be due in part to the many gene products that exhibit potential to interfere with the cellular or host response to infection. In excess of 40 HCMV gene products have been ascribed some role in modulating host systemic immune responses to viral infection, disrupting innate

immunity controlled by IFNs, chemokines, other cytokines or NK cells, and deflecting adaptive antibody or T cell-mediated immunity. Escape functions provide a balance to deflect host clearance and foster viral persistence and latency in immunocompetent hosts; however, the contribution of HCMV genes to pathogenesis and latency has remained elusive given the difficulty in identifying appropriate model systems.

Escape from cytotoxic T-lymphocyte responses is due to a nonrelated set of functions that prevent MHC class I antigen presentation with ER retention, proteasomal degradation, and peptide transport blockade by US2, US3, US6, and US11 gene products important in HCMV, and the generation of nonfunctional complexes, lysosomal degradation, and Golgi body retention by m04, m06, and m152 in MCMV. In addition to these mechanisms for interfering with MHC class I presentation, US2 gene product can cause degradation of MHC class II proteins, preventing their presentation of antigen, and the host CD4⁺ T lymphocyte response to HCMV MCMV m152 downregulates NK cell ligands in the RAE-1 family in addition to MHC class I. The extent to which some putative mechanisms of immune evasion are responsible for the persistence or virulence of HCMV infection is not yet clear. In some cases, the apparent mechanism of action of candidate immune escape functions remains unresolved, as with the HCMV-encoded MHC I homolog (UL18 gene product) initially suggested to play a role blocking NK cell activity through binding with killer inhibitory receptors but later shown to enhance NK cell killing of infected fibroblasts in a pattern that correlated with expression levels. More relevant contexts in which to test such potential immune escape functions are needed.

Natural Killer Cells

Extensive evidence from mice as well as supportive evidence from human infections shows that NK cells are the earliest immune cells to control levels of infection.^{268,685} This area of innate host defense, together with important consequences of viral modulation of NK cells, has developed to a remarkable level over the last several years, continuing to be led by incisive studies in mice.^{439,499,526} Remarkably, the antiviral NK cell response includes immune memory somewhat akin to T cells.^{439,641,642} The link between resistance to viral infection and NK cell function was first established in genetically deficient mouse strains, as well as in mice that were depleted of NK cells, infected with MCMV. Remarkably, certain strains of mice have a more effective NK response due to direct activation by a viral protein, encoded by m157,^{23,592} although an array of other MCMV and HCMV functions focus on impeding the NK response.^{268,439,526,641} NK-mediated strain differences in susceptibility mediated via MCMV m157 are dependent on direct binding to the activating NK cell receptor Ly49H, driving more efficient killing, associated with approximately 10-fold reduction in levels of virus early after infection. Despite the fact that m157 readily mutates under experimental selection and the activating receptor Ly49H is rare in wild mice, additional mouse and viral determinants with similar relationships have emerged and it now appears that a similarly remarkable phenomenon may accompany HCMV infection of humans.¹⁹² It is very clear that NK cells play an important role in host defense against HCMV, but in a complex pathogen-host relationship that is just becoming clarified.

Evidence supporting the importance of NK cells in defense against HCMV infection comes from the growing

numbers of viral gene products that block NK cell-mediated recognition as well as evidence that MHC class I down modulation by this virus makes cells more susceptible to NK lysis.²⁶⁸ A variety of host MHC interactions by viral MHC mimics and other interacting proteins have come to the fore as inhibitors of NK activity. In HCMV, the viral UL16, UL18, UL40, UL140, UL141, and UL142 proteins all play roles in NK cell down modulation, by downregulating activating ligands for NKG2D (UL16), by mimicking HLA class I (UL18) and interfacing with UL40,^{498,722} by binding to HLA-E (UL40) to retain NK repression via CD94:NKG2A,⁴⁹⁸ by downregulating the activating ligand CD155 (UL141) that is recognized by the activating receptor CD226,⁴⁹⁷ by retaining activating ligands in the Golgi (UL142),³¹ as well as by unknown mechanisms (UL140).^{268,709} MCMV encodes an evolutionarily distinct arsenal of NK-modulating functions.^{439,499,526}

Antibody

Experimental and clinical evidence indicate that humoral immunity to HCMV plays a role in protecting the host from disease, particularly in the setting of congenital disease.^{431,433} In mice or guinea pigs, passive or active immunization against a single envelope glycoprotein (gB) protects from challenge and disease. In humans, a subunit vaccine composed of gB in an adjuvant (MF59) was able to convey protection from infection in phase 2 studies,^{448,456} and certainly, maternally acquired antibody is an important determinant of protection from disease among very low birth weight newborns who acquire HCMV from mother's milk or blood products.¹⁷³

Many HCMV proteins are immunogenic such that nearly all immune human sera have both binding and neutralizing antibody to envelope glycoproteins gB (UL55) and gH (UL75), as well as binding antibody to a number of charged proteins such as the tegument phosphoprotein pp150 (UL32) as well as to a nonstructural DNA binding phosphoprotein, pp52 (UL44). Neutralizing antibody has been detected against principal envelope glycoproteins gB, gM:gN, gH:gL, and complexes formed between gH/gL and pUL128, pUL130, and pUL131A.³⁵⁶ Any of these antibodies may be associated with protection from infection and disease. It has become clear that assays for neutralization on fibroblasts underestimate the titers in human sera, such that low passage clinical isolates such as VR1814 infection of epithelial cells such as ARPE-19 cells shows significantly higher titers.^{356,542,697} Given that virus is largely cell associated in the host, but cell free in secretions, neutralizing antibody is likely to play an important role in controlling transmission. The extent to which humoral immunity from naturally acquired infection or from active immunization contributes to prevention of disease or reinfection remains to be fully elaborated.

HCMV encodes potent IgG Fc receptors from RL11, RL12, and UL118-UL119,⁶⁰⁹ and also incorporates bound antibody that facilitates entry into cells via Fc.³⁶¹ Few studies have been performed in animal models to provide guidance as to the significance of these activities during viral pathogenesis, although MCMV Fc receptor m138 is known to interact with B7-1 (CD80) and impact endosomal trafficking.⁶⁶³

Cell-Mediated Immunity

There is ample evidence supporting the crucial role of T-cell immunity in control of HCMV infection, perhaps none is more persuasive than the observation that severe HCMV disease is a

universal problem in patients with normal antibody levels who have profoundly impaired cell-mediated immunity, such as in recipients of allogeneic HCT or in AIDS. The severity of disease due to HCMV infection parallels the degree of T-cell impairment in AIDS as well as in solid organ transplantation and HCT, where adoptive reconstitution of T-cell immunity is effective.^{176,504} Understanding the adaptive T-cell immune response to HCMV infection, both in terms of the role of specific components of the immune response and specific viral proteins that are recognized, has been facilitated by the frequency of viral antigen-specific CD4 and CD8 T cells together with assays that assess responses to HCMV proteins and peptides. Investigations using murine models showed the central role of cytotoxic T-cell immunity in control of active viral disease as well as reactivation from latency and amplification. In humans, the T-cell response is broad and persistent and, in adoptive transfer experiments, CD8+ T cells with specificity for HCMV tegument proteins appear protective, although dependent upon the presence of CD4+ T cells in the transfer. Although a full description of adoptive immunotherapy to control of HCMV disease is beyond the scope here, evidence supporting a critical role for CD8+ T cells as cytotoxic effectors recognizing HCMV-infected targets as a component of immune clearance is incontrovertible.

The very convincing body of evidence that supports the role of cytotoxic T cells in host defense against HCMV infection is accompanied by a similarly extensive list of viral gene products that block T-cell recognition in either the MCMV model or in HCMV cell culture assays. Three MCMV gene products (m04, m06, and m152) modulate MHC class I. In HCMV, four glycoproteins (gpUS2, gpUS3, gpUS6, and gpUS11) down modulate cell surface expression of MHC class I proteins, all acting via posttranslational mechanisms²⁶⁸ that result in reduced immune surveillance rather than any impact on dendritic cell-dependent antigen presentation.⁵⁵⁸ US2 and US3 also effect MHC class II expression. The role of this class of immune modulator in surveillance is reflected in classic studies carried out in MCMV as well as more recent RhCMV studies.^{163,238} The viral UL18 MHC class I homolog is not subjected to down modulation due to an interaction with UL40.⁴⁹⁸ The novel MHC class I proteins expressed by cytотrophoblasts are not affected by these modulators, although US10 controls the levels of the novel HLA G, so may be important in modulation of fetal infection.⁴⁴⁴ All US2 and US6 family members are related to single immunoglobulin domain proteins that probably arose by duplication of an ancestral gene. US2 is most similar to US3, and US6 is most similar to US11. Mutants disrupting US2 to US11 completely fail to downregulate MHC class I expression in a number of cell types, including endothelial cells, epithelial cells, and dendritic cells.²⁶⁸ Any one of the four is sufficient to prevent translocation of MHC class I proteins to the cell surface, consistent with nonredundant roles. Although the activity of these proteins predicts a powerful role in escape from killing by cytotoxic T lymphocytes, thus far only limited evidence has shown that fibroblasts infected with a US2-US11 mutants are recognized and killed by a greater number of T-cell clones than are cells infected with wild-type virus. Upregulation of nonclassical MHC proteins such as MIC-A, which engages NKG2D on T cells as well as NK cells, may enable cytotoxic T cells to kill even though MHC class I levels are depressed by viral infection. In addition, HCMV retains the ability to down modulate MHC class I

expression even in the face of strong upregulation. Despite incisive mechanistic investigation in HCMV, a more complete evaluation of the physiological consequence of eliminating viral immunomodulatory gene products that block MHC class I expression has been possible with MCMV. Down modulation of MHC class I levels on MCMV-infected cells helps the virus avoid CD8 T-cell immune surveillance, but is not involved in altering T-cell memory set points.^{268,594}

A significant body of information has been generated studying US2, US3, US6, and US11. US3, an IE gene product, localizes to ER, possibly through interaction with calnexin, and down modulates tapasin-dependent peptide loading onto MHC class I proteins, thereby preventing egress to the Golgi. US2 and US11 encode DE gene products that degrade unassembled MHC class I via a Sec61 complex-dependent process and ubiquitin-dependent proteasome-mediated degradation. An immunoglobulin-like ER-luminal domain of gpUS2 is essential for binding to the $\alpha 3$ domain of MHC class I heavy chain, but requires the carboxyl terminus to direct degradation. Although gpUS2 and gpUS11 exhibit many of the same activities, gpUS2 binds more selectively to MHC class I haplotypes and MHC class II, whereas US11 gene product interacts with Derlin-1 and counteracts the misfolded protein response. US6 is expressed at late times and binds to the transporter of antigen processing (TAP) in the lumen of the ER and blocks TAP conformation in a manner that prevents ATP-dependent peptide transport. Although the US2-US11 appear to block effectively, the block can be overcome by certain T cells as well as in dendritic cells.

HCMV infection also down modulates constitutive MHC class II. gpUS2 and gpUS3, which modulate MHC class I levels, may also affect class II levels. Although the viral function remains to be identified, the principal MHC class II transcription factor, CT_{IIA}, is inhibited at early times of infection via enhanced degradation of Janus-activated kinase (JAK) with a resultant impact on JAK/STAT signaling that leads to CT_{IIA} expression. HCMV-encoded vIL-10 also exhibits an ability to reduce levels of MHC class II expression.

Interference with antigen presentation likely provides substantial benefit to the virus in the escape from clearance by CTL, but these functions do not abrogate the development of an effective CTL response. Viral functions that interfere with the level of T-cell responses are only beginning to be identified. In MCMV, one role of the viral chemokine is to recruit inflammatory monocytes from bone marrow that reduce the effectiveness of T-cell priming.¹⁴⁰ The balance of host and viral factors likely contributes to the level of viral persistence and the maintenance of latency in the host.

Persistence and Latency

As with all herpesviruses, HCMV latency is maintained in everyone who experiences primary infection. The universal risk of transplanting an organ or tissue from a CMV-seropositive donor to a CMV-seronegative recipient provides the most dramatic evidence of life-long infection, and propensity of virus to reactivate following immunosuppression or immunodeficiency is a central factor in HCMV-associated diseases. Most settings where HCMV disease occurs in immunocompromised hosts, such as SOT and allogeneic HCT recipients as well as in AIDS, are derived from reactivation. The earliest evidence of HCMV latency was the demonstration that leukocytes transmit HCMV infection despite the absence of infectious virus, resulting in the

current practice of leukodepletion that has dramatically reduced transfusion-acquired infection. Although evidence supporting HCMV latent infection has accumulated, the role of low-level persistent replication remains a topic of debate. Persistent replication occurs in epithelial cells of salivary glands and kidneys, with sporadic shedding in body fluids throughout life; whereas, hematopoietic progenitors harbor latent virus. Various myeloid progenitor cell types as well as the more differentiated cell lineages that arise from progenitors have been implicated in latency.^{510,515} Natural latency has been difficult to quantify beyond the identification of HCMV DNA-positive cells, along with occasional success at experimental reactivation from the natural latent reservoir. The balance between natural latency and reactivation is apparently controlled by cell type or cellular differentiation-specific factors that act at both genetic and epigenetic levels to restrict viral regulatory gene expression and replication.⁵¹² Considerable evidence suggests that viral genes fall under cellular control during latency. The MIE genes, IE1 and IE2, are repressed via the transcriptional enhancer and activated possibly as a result of cytokine or viral activation of transcription factors such as NF- κ B. Therefore, HCMV latency and reactivation seem to be controlled by host cell gene repression and activation that may be tied to differentiation signals that may be influenced by the cytokine and growth factor environment. Differentiation and activation of myeloid cells by proinflammatory cytokines that drive cell maturation continue to be considered important signals for viral reactivation.²⁵¹ These have also been observed using MCMV. This type of environment results from allograft rejection where HCMV has long been a prominent pathogen causing both acute and chronic disease. The maintenance of the viral genome appears to be limited to mononuclear myeloid progenitors that reside in BM,⁵⁸⁹ in cells that are as primitive as CD34+, lineage marker negative stem cells and as mature as monocyte-derived macrophages⁶⁰⁰ and DCs.⁵¹⁴ Virus reactivation occurs in mature cells. Myeloid lineages distribute widely as tissue resident macrophages and dendritic cells, suggesting they are the source of virus transmission in organ transplantation as well as dissemination within the infected individual. Neither granulocytes nor lymphocytes retain the HCMV genome and are unlikely to contribute as vehicles of virus dissemination.

The differentiation state of macrophages or dendritic cells, influenced by the proinflammatory cytokine environment and the level to which viral replication functions are encoded, has been associated with different outcomes, including restriction of viral replication with viral genome maintenance (latency), failure to support viral genome maintenance, and support of productive replication associated with reactivation.^{251,512,515} Latency in hematopoietic progenitor cells is considered an important biological reservoir, where reactivation is viewed as the first step in viral disease pathogenesis during transplantation or AIDS and may also underlie recurrent transplacental transmission as well as reseeding of epithelial cells that support sporadic shedding throughout life.

As a species-specific virus, HCMV does not infect any laboratory animal model. The species restriction results in a postpenetration failure in replication following IE gene expression before viral DNA replication and may be related to induction of cell death.⁸⁵ Experimental work on HCMV latency and reactivation employs both primary and continuous human cell culture models, with investigators selecting cell types suspected

of playing an important role in the naturally infected host. Considerable effort over the last 15 years has been focused on PB monocyte-macrophages,⁶⁰⁰ myeloid progenitors,⁵⁸⁸ and dendritic cells,^{251,514} all pointing to an important contribution of progenitors derived from BM in latency. Initially, focus on PB monocytes followed the demonstration that these cells harbor viral DNA but not infectious virus in healthy, HCMV seropositive individuals. Experimental models of latency have consistently shown the maintenance of the viral genome in a nonproductive state, with evidence of circular forms, and the ability to drive reactivation and replication through cellular differentiation pathways.^{512,515} One important study showed detection of viral DNA in naturally infected monocyte preparations that reactivated when differentiated into monocyte-derived macrophages under conditions that recapitulated allogeneic rejection,⁶⁰⁰ although triggering reactivation has proven to be one of the most elusive and poorly reproducible assays. Attempts to further investigate quantitative or qualitative properties of this natural latent reservoir have not succeeded and have been further complicated by the very low frequency of HCMV DNA positive monocytes in PBMC from healthy seropositive individuals. Therefore, even though there is little question that seropositive individuals are latently infected and sometimes have DNA-positive PB monocytes, variability apparently comes into play in determining the frequency of HCMV DNA positive cells in the PB of seropositive individuals at any particular time. Despite this, BM-derived or G-CSF mobilized PB mononuclear cells from seropositive donors contain viral genome positive cells at frequencies ranging from 10^{-4} to 10^{-5} with 2 to 15 HCMV genome equivalents per cell.⁵⁸⁹ These natural levels are consistent with cell culture models and implicate these cells as a reservoir of latency. Recent observations on reactivation using naturally infected progenitors further support the importance of primitive cells as a reservoir.^{46,510,515,580,588} Experimental infection of total progenitors, including CD34+-enriched cell populations, has been carried out with fetal liver, cord blood, adult BM, or G-CSF mobilized adult PB and each of these results in a quiescent infection appearing to reflect latency. Naturally infected BM-derived progenitors express unique transcripts, and these have been characterized as candidate HCMV latency-associated transcripts when also detected in natural latency.⁴⁶ Experimental infection of cultured CD34+CD38- progenitors is associated with a gradual shutdown in viral gene expression along with a sustained presence of a range of viral transcripts, including candidate latent transcripts. In addition to LUNA,⁴⁶ whose function remains unknown, three functional latent gene products have been elucidated: LAcvIL-10, expressed in latently infected myelomonocytic progenitors⁵⁸⁸; UL138, an inducer of TNFR1 expression^{321,406}; and ORF94, a 2'-5' OAS inhibitor.⁶⁴⁸

In addition to cell culture models of HCMV tissue tropism and latency, surrogate animal models of latency have been investigated to varying levels. Surrogates such as MCMV have continued to play a crucial role in understanding the overriding principles of viral pathogenesis and latency both because of the close biological relatedness and because of the array of mutant strains of mice that facilitate mechanistic studies affecting cellular and immune pathways common to rodents and humans. Therefore, MCMV recapitulates the general processes of latency and reactivation, providing insights into the distribution and type of cells that carry viral DNA, express viral

RNA, and reactivate as well as evidence that the cytokine environment necessary to observe reactivation is highly inflammatory and is influenced directly by TNF levels. The regulation of the HCMV major IE gene by TNF is well-established, and, together with UL138 function, suggests that TNFR1 may be crucial to natural latency and reactivation. Finally, interest in immune control of MCMV latency has suggested a direct role of CD8+ T lymphocytes independent of CD4+ T lymphocytes, B lymphocytes, or antibody, consistent with what is known about HCMV latency and reactivation following allogeneic HCT.

Although immunocompetent individuals eventually control primary infection through a strong and broad T lymphocyte response, persistent shedding of free virus in saliva, urine, and other bodily secretions continues for months or years and this is followed by intermittent asymptomatic reactivation and shedding in a proportion of healthy individuals. HCMV DNA is detected in a small proportion of the general population in saliva or urine and therefore virus must either reside in these sites, or be sporadically reseeded from a latent reservoir, possibly reseeded from a myeloid reservoir. Even in settings where disease occurs: (a) immunocompromised individuals where disease may result from primary as well as recurrent/reactivated infection, and (b) transplacental transmission during pregnancy where primary infection of the fetus arises from either primary or reactivated infection in pregnant women, the contribution of latently infected myeloid cell lineage cells is apparently critical to HCMV pathogenesis.

A full understanding of HCMV functions involved in the control of latency and reactivation will require the continued pursuit of appropriate cell-based and reconstituted animal-based models. In such settings, viral mutants that show little effect on productive replication can be evaluated for behavior and phenotype during latency, and host cell functions that contribute to either repression or activation of viral gene expression can be identified. Surrogate systems, including MCMV infection of the natural mouse host and RhCMV in monkeys will continue to provide complementary information.

EPIDEMIOLOGY OF HCMV INFECTION

Prevalence and Demographics

HCMV is universally distributed among human populations.⁹⁶ Table 62.3 lists data on prevalence of infection in women of childbearing age from Europe, North and South America, Africa, and Asia based on selected studies. HCMV epidemiology has focused on this population because of the public health importance of primary infection during pregnancy. Studies including both sexes consistently report prevalence rates that are slightly higher in women than in men.^{42,242,620} Although prevalence of infection increases with age in every group that has been studied, the overall prevalence of infection and the age at initial acquisition of virus vary greatly according to living circumstances. In general, prevalence of infection is greater and virus is acquired earlier in life in developing countries compared to the United States and Europe, where prevalence is greater in the lower socioeconomic strata, among nonwhites, and in immigrant populations from developing countries.^{96,220,225,620} Although prevalence is higher in nonwhite compared to white populations, these differences reflect exposure frequency related to living circumstances rather than to

TABLE 62.3 Prevalence of CMV Infection Among Women of Childbearing Age

Study location, date	Population	N	Prevalence (%)
Ankara, Turkey	Women 15–49 years	745	99
Cotonou, Benin	Pregnant women	211	97
Seoul, South Korea	Prenatal clinic	575	96
Sendai, Japan	Prenatal clinic	10,218	95
Sao Paulo, Brazil	Pregnant women, middle SES	427	67
	Pregnant women, lower SES	179	84
Northern Italy	Women, pregnant or hospital patients	12,568	77
Helsinki, Finland	Women, prenatal clinics	1,088	71
Birmingham, USA	Prenatal, middle SES	12,140	54
	Prenatal, lower SES	4,078	77
Grenoble, France	Women, prenatal clinic	1,018	52

racial factors. Infection reaches a majority of the adult population worldwide, although neither seasonal variation nor epidemics have been observed. Very high rates of transmission that occur in settings such as care of young children can be reduced through hygienic measures, including handwashing.

Seroepidemiologic studies of HCMV infection have largely relied on convenience samples, including patients at a particular health care institution, healthy blood donors, or hospital employees. Population-based estimates of HCMV prevalence have been derived from the National Health and Nutrition Examination Survey (NHANES), starting with a 1988 to 1994 set and continuing with 1999 to 2004 set (6 years of age or older).^{42,620} Based on these large samplings, the overall age-adjusted prevalence of HCMV infection in the United States remains about 59%, with about one-half of the U.S. population aged 6 to 49 infected.^{42,620} The age-related prevalence of HCMV infection derived from NHANES 1988 to 1994 is shown in Figure 62.4. More female patients (63.5%) than male (54.1%), and more non-Hispanic blacks (75.8%) as well as Mexican Americans (81.7%) than non-Hispanic whites (51.2%) were infected. Other than ethnic and racial correlates, multivariate analysis revealed that fewer years of education, residence in the South, larger family size, birth outside the United States, and government-provided health insurance (Medicaid) were also associated with higher seroprevalence, all factors related to socioeconomic status as well as living circumstances. Overall, the 1988 to 1994 results were similar to data from a more recent, 1999 to 2004 study period, with the important recognition of household income as an independent inverse risk factor for HCMV infection.⁴² The precise activities of lower socioeconomic groups that contribute to transmission have not been identified, but it is notable that long-term longitudinal studies in The Netherlands and Denmark have revealed that the greatest risk of HCMV infection in developed countries is associated with the childhood years,⁴¹⁰ and studies seeking to understand

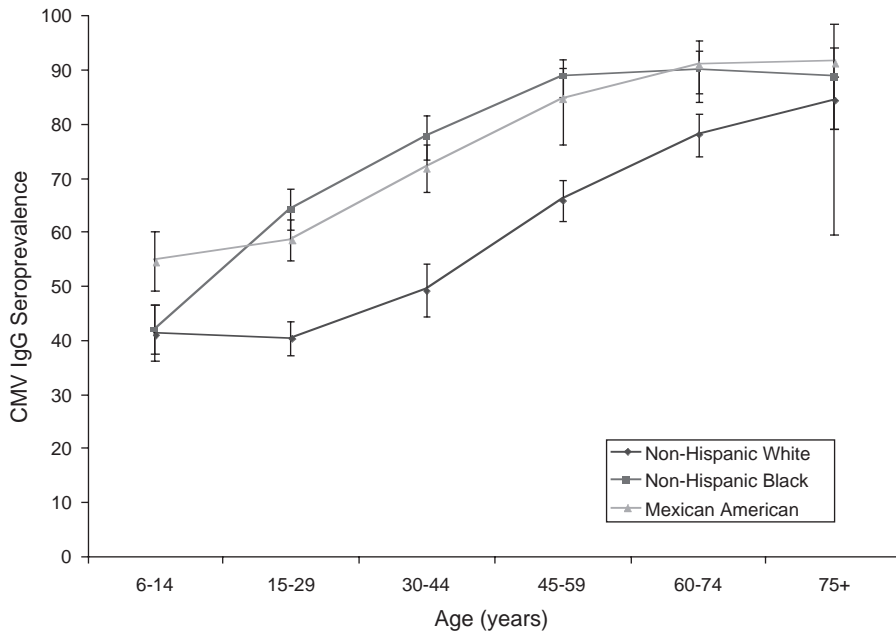


FIGURE 62.4. CMV seroprevalence in the United States by age and race. (Reprinted from Staras SA, Dollard SC, Radford KW, et al. Seroprevalence of cytomegalovirus infection in the United States, 1988–1994. *Clin Infect Dis.* 2006;43(9):1143–1151, with permission from Oxford University Press.)

risk of primary infection during pregnancy consistently reveal that exposure to the urine and saliva of young children is a highly significant risk.^{95,259} Table 62.4 lists rates of HCMV seroconversion for various groups, including childcare providers, parents of young children, and patients attending a sexually transmitted disease clinic. Two types of exposures have consistently been linked with horizontal transmission of HCMV: contact with saliva and urine from young children and sexual activity in adults. The extended, multi-year pattern of shedding in children results from slow development of a virus-specific CD4 T-cell response,^{114,209,671} providing an ample source for transmission, with exposure to children the most dominant risk for seroconversion in susceptible adults.^{196,259}

TABLE 62.4 Incidence of CMV Infection in Various Groups

Group	Rate
Blood donors	1.57
Hospital employees	
Richmond, VA	2
Birmingham, AL	2.2
Pregnant women	
Middle income	2.5
Low income	6.8
Women in STD clinic	37
Adolescent females	13.8
Parents of CMV shedding child	
0–12 months old	47
≤18 months old	32
19 months–6 years	13
Daycare workers	7.920

Rate is percent per year that seroconverted from antibody negative to antibody positive.

Maternal Transmission Patterns

HCMV stands out among the human herpesviruses in patterns of intrauterine transmission across the placenta that result in infection of the fetus during pregnancy.⁴⁶⁸ Overall, maternal transmission occurs during pregnancy, where infection is associated with congenital disease, as well as at birth (intrapartum) or following birth, where disease is uncommon. Breastfeeding is the most frequent route of maternal transmission, but transplacental transmission is the most medically important. Intrauterine transmission of HCMV occurs in women who have already been infected before conception as well as in women who experience primary infection during pregnancy.⁶¹¹ Transplacental transmission in seropositive women that results in congenital infection is termed *recurrent infection* to acknowledge a likely contribution of reinfection, persistent infection, and reactivation of latent virus in this setting. Although pre-existing natural immunity reduces the risk of intrauterine transmission below the frequencies observed in primary maternal infection, estimates of this benefit¹⁹⁴ have been complicated by the difficulty of assessing recurrent infection directly in seropositive populations.^{70,720} The role of recurrent maternal infection as a source of fetal infection was initially suggested by the occurrence of congenital infection in consecutive offspring of the same mother and was confirmed by a cohort study where the rate of congenital infection in offspring of seropositive women was relatively high.⁶¹⁵ In addition, high rates of congenital infection occur in populations with near universal acquisition of virus before sexual maturity, including Brazil, where these rates are higher in seropositive populations than in HCMV-naïve populations.^{423,567,614} A proportion of congenital disease following recurrent infection results from reinfection with different strains based on serologic response characteristics.^{70,720} Although the most severe CNS damage follows primary gestational infection in naïve women, particularly early in pregnancy, maternal infection is a worldwide problem^{15,66,100,195,612,613} given high seroprevalence rates in

women of childbearing potential.^{720,721} Transplacental transmission is less frequent earlier in gestation and more frequent later, similar to other causes of congenital infection. Higher rates of congenital disease are associated with earlier transmission. A small cohort study of primary infections during the third trimester of pregnancy reported a transmission rate of 75%, without observing congenital disease in any of the 20 infected newborns.²¹¹

Newborns with congenital infection shed virus in urine and saliva at birth. Between 0.3% and 1.3% of newborns shed HCMV at birth based on a review of studies of 800 or more children in industrialized countries.¹⁶² Higher rates of congenital infection, from 2% to around 4% have been reported in developing countries.^{288,423,567} In the United States, the overall rate of primary maternal infection during pregnancy varies from less than 0.5% to around 4%, with higher rates occurring in young, unmarried women of lower socioeconomic status.⁶¹⁴ Intrauterine transmission reaches high frequency in confirmed primary maternal infection, with overall rates of approximately 33% congenital infection,²⁸⁹ based on a review of studies from many highly developed countries around the world. Importantly, newborns with HCMV in urine or saliva up to 3 weeks of age acquired the virus during gestation, whereas newborns remaining negative for this period that start to shed virus at around 6 weeks of age acquired the virus during or following birth.

Intrapartum and postnatal mother-to-child transmission follow maternal shedding patterns. Approximately 10% of women shed HCMV from the vagina or cervix; near the time of delivery, rates of 2% to 28% have been reported,⁶¹⁴ with the higher rates in younger women.³⁰⁰ If the newborn comes into contact with virus in the genital tract at the time of delivery, the rate of transmission is approximately 50%.⁵²⁷ Although intrapartum exposure contributes to the proportion of infected children, the most common route of mother-to-infant transmission is via breastfeeding. Studies detecting viral DNA by PCR found evidence of shedding in more than 95% of milk samples from seropositive mothers,^{30,249,272,688} extending earlier studies that employed virus isolation. Shedding follows primary or recurrent maternal infection, with no evidence of ongoing systemic infection because blood remains virus negative.²³² Transmission to infants of seropositive mothers is related to duration of nursing such that infection occurs in infants nursed for more than one month but not in infants nursed for less than one month.¹⁷⁰ A large proportion (69%) of infants acquire HCMV when nursed by shedding mothers, compared with only 10% of infants nursed by nonshedding seropositive mothers. Shedding peaks within the first few months and then declines. When studied, 70% of milk samples between 9 days and 3 months postpartum were virus positive, whereas samples were negative at other times.¹⁴ HCMV DNA PCR appears more sensitive than virus culture for detecting virus in milk,²³² and presence of either indicator correlates with transmission.

Neither intrapartum nor breast milk transmission of HCMV is associated with morbidity, except in very low birth weight premature newborn infants. Both routes are important in the epidemiology of infection. The proportion of infants who acquire virus during the first year of life is directly related to the prevalence of maternal infection and the proportion of mothers who nurse their infants.^{395,616} In countries where HCMV infection is universal and breastfeeding is common, around 50% of infants acquire virus before one year of age. In the United States,

this level is much lower. Young infants who acquire virus from a maternal source usually shed for years and become important sources for virus transmission to other children.

Horizontal Transmission

HCMV generally transmits most efficiently in settings where susceptible individuals are exposed to body fluids from individuals shedding virus, either by direct contact or contact with objects that have been recently contaminated with infected body fluids such as saliva and urine. Transmission on dry surfaces, by the airborne route or by aerosols, is not known to occur.⁶³² Following initial acquisition of HCMV, virus replicates and causes a systemic infection, sometimes detected as a leukocyte-associated viremia, and disseminates to infect tubular epithelial cells of secretory organs such as salivary glands and kidney where replication produces virus found in secretions. Not surprisingly, rates of infection are increased in any setting with a risk of exposure to infected body fluids. Child-to-child and child-to-adult transmission occurs in home and daycare, patterns that are reduced by frequent handwashing.⁹⁵ Virus may be shed in any body fluid, including urine, saliva, tears, semen, and cervical secretions, and persistent shedding may continue for months to years, depending on age and immune status of the host. The shedding period is longer in children than in adults, but clinically apparent disease rarely accompanies infection in immunocompetent populations. After resolution of primary infection, virus is shed sporadically for life.

Spread occurs frequently where young children congregate and interact.⁴⁴⁹ Cohort studies indicate that children in certain daycare centers frequently transmit virus to each other, whereas other centers have very low transmission rates.^{6,414,452} Young children shed virus in saliva and urine for long periods of time, providing ample opportunity for continued spread to classmates, parents, and daycare providers. Transmission in this setting is enhanced by frequent mouthing of hands and toys that become contaminated with virus,^{97,256} contributing to the exchange of body fluids with other children and adults. Childcare providers have markedly higher risk of acquisition directly related to the proportion of children shedding virus. This has been reported to be 7% to 20% per year in selected sites,^{7,193,415,451} although in the general population, acquisition varies dramatically such that family size emerges as the only correlate of seropositivity.⁶²¹ Rates are higher overall in parents of children than in similar aged adults not exposed to children.¹⁹⁶ Therefore, adults caring for young children in groups at increased risk for infection in families with multiple siblings,⁶²¹ home daycare,⁴⁰ or large daycare centers may all be at greater risk of infection proportional to the levels of shedding by children. Consistent with these patterns, parents of premature babies who acquire this virus nosocomially while in the hospital nursery are at risk of becoming infected.^{8,453,724} Overall, risk of infection is highly correlated with the presence of children who shed; a seroconversion rate of 45% per year was found in parents of toddlers who shed virus compared with 0% among parents of nonshedders.⁴⁵³ Restriction enzyme analysis of viral DNA has confirmed viral strain transmission from child-to-child, child-to-parent, and child-to-daycare provider.^{8,9,454}

Transmission also occurs between adults, and this mode predominates in sexually active populations.²⁵⁹ In adults, where sporadic shedding in saliva, tears, cervicovaginal secretions, and semen occurs, intimate contact is important for transmission.²³¹ Shedding patterns in saliva range from about 7% in healthy

seropositive adults to over 20%⁹⁵ in adult populations with greater numbers of sexual partners and those with other sexually transmitted diseases.^{110,135,259,470,601} A high incidence of infection has been reported in sexually active adolescents,⁶¹⁸ a population where contact with young children is a confounder.⁶²² Importantly, sexual transmission predominates in married adults without children where one partner is HCMV seropositive.¹⁹⁶ The similar DNA restriction enzyme digest patterns observed in viral strains from partners confirmed person-to-person transmission.²³⁶ Studies of hospitalized patients have shown virus may be frequently shed in urine or saliva,⁴⁵⁵ related to both immune status and trauma,¹³⁴ although hospital workers are not at increased risk of infection. The lack of evidence for increased risk of HCMV transmission to hospital workers provided the first evidence that good hygiene and infection control procedures (e.g., handwashing and avoiding contact with body fluids) can effectively interrupt HCMV horizontal transmission.

Horizontal transmission accompanies blood transfusion as well as transplantation of cells, tissues, or solid organs from seropositive donors. Universal screening has reduced the risk of acquisition from the blood supply in many areas of the world, including the United States and Europe. Transmission continues to be associated with transplantation and is clinically important as discussed below.

Molecular Epidemiology

The large size of the HCMV genome and the presence of genes with substantial interstrain variability have facilitated the characterization of viral isolates based on genetic differences by restriction fragment length polymorphism (RFLP) of entire genome as well as by comparing amplified viral DNA segments representing variable regions or genes such as the gB/UL55, and, more recently, gN/UL73 and UL144.^{24,479} All of these approaches are based on the general premise that epidemiologically linked isolates share nucleotide sequence identity and, conversely, isolates that are not epidemiologically related exhibit sequence diversity. Molecular genotyping of viral isolates has provided evidence for the following: vertical transmission of reactivated endogenous virus,²⁵⁰ transmission from child-to-child and child-to-parent,⁸ as well as to childcare providers,⁷ from child-to-mother-to-fetus,^{416,454} between newborns in a hospital nursery,⁶⁰⁶ and from organs in transplant recipients.¹²¹ In some cases, these genotyping tools have also discounted spread from a suspected source.^{10,726} Viral strains can be distinguished (by RFLP, sequencing hypervariable regions or other methods) such that multiple strains shed from different body sites as well as sequential detection of a different strain in the same person supports multiple infection even in immunocompetent individuals. The assumption that primary HCMV infection involves a single, genetically homogeneous strain has been drawn into question. Although studies based on genotyping or specific genes sequences obtained shortly after primary infection support the prevalence of a single strain in most settings,^{218,420,521} recent studies that employ deep DNA sequencing have revealed a high degree of genome wide variability within the host. These new methods raise questions about strain definition and heterogeneity^{216,217,520} and predict a bottleneck in transmission that is reminiscent of RNA viruses. Nevertheless, multiple infection occurs, based on detection of more than one genetically distinct strains in the same person has been reported in immunocompromised patients,^{165,322} healthy children,³⁹ sexually active women,¹⁰⁹ and mothers of newborns with congenital infection.⁷⁰

Although few studies have been carried out, one viral genotype has not predominated in a particular geographic location. A comparison of 223 strains from around the world did not associate gN genotype with geographic origin,⁴⁷⁸ and, studies of gB genotypes from various locations in Europe, North America, and Asia have not revealed associations.^{25,38,420,476,477,649}

CLINICAL FEATURES

Acquired Infection in the Immunocompetent Host

As a rule, infection with HCMV in the immunologically normal host is clinically silent.⁴⁴⁷ HCMV symptomatic infection occasionally results in a febrile, mononucleosis-like illness. More serious disease or systemic illness with complications involving specific organ systems is rare. During primary maternal infection most (>90%) pregnant women remain asymptomatic.^{226,612} In addition, intrapartum or postnatal transmission from mother to infant during birth or through breast milk is typically silent. Young and premature infants may exhibit hepatomegaly, elevation of liver enzymes, and pneumonitis,^{312,610} although these signs and symptoms have not been observed in studies of term infants.^{170,462,527,617} Asymptomatic infection predominates in all age groups, from preschool-aged children^{6,452} to immunocompetent adults.¹²⁹

Mononucleosis

Clinical signs and symptoms in patients with HCMV mononucleosis are listed in Table 62.5.^{274,299,442} The picture that emerges from these older reports is illness characterized by fever for more than 10 days, malaise, myalgias, headache, and fatigue. Body temperature elevation to 39°C to 40°C is common. Splenomegaly, hepatomegaly, adenopathy, and rash are less common. Several hallmark signs and symptoms of EBV-associated mononucleosis, including pharyngitis, adenopathy, and splenomegaly, are less common with HCMV.

Diagnostic laboratory findings associated with HCMV mononucleosis include lymphocytosis with activated or atypical lymphocytes and elevated hepatic transaminases. Increased

TABLE 62.5 Clinical Findings in CMV Mononucleosis, Compiled from Three Published Reports Covering a Total of 45 Adult Patients^{274,299,442}

Feature	Frequency (%)
Fever >10 days	94–95
>18 days	41–53
Cervical adenopathy	6–56
Sore throat	56
Exudative pharyngitis	0
Splenomegaly	33–53
Hepatomegaly	0–53
Rash	0–44
Elevated serum ALT or AST	78–100
Lymphocytosis (>5,000/ μ l)	78–95
Atypical lymphocytes (>1,000/ μ l)	71–95%

Reported patients typically had malaise, fatigue and myalgia as well as features listed in the table.

serum bilirubin and jaundice, signs of hepatitis, are less common. A 1998 review of 116 immunocompetent hospitalized patients with symptomatic HCMV infection reported similar signs and symptoms (compared with Table 62.5), noting sweats in 35 (35%) of 99 and chills in 40 (39%) of 103 of cases. In addition, 20 (17%) of 115 had abdominal pain, 7 (6%) of 112 had diarrhea, and 5 (5%) of 103 experienced 10% or greater weight loss.¹⁸³ A laboratory-based study reviewed clinical findings in 124 immunocompetent adults with serologic evidence of primary HCMV infection (patients whose illnesses had led their general practitioners to test them for HCMV infection)⁷¹⁷ found similar symptoms: 32% of subjects experiencing a constellation of findings that included abnormal liver function tests, malaise, jaundice, sweats, and fever, including 46% with fever, 24% with jaundice, 17% with arthralgia, and 3% with thrombocytopenia. The average duration of symptoms was about 8 weeks (range 1 to 20 weeks) with 12% of patients experiencing relapse. Mononucleosis-like illness can also occur in children. A comparison of adults and children with HCMV mononucleosis found that children were less likely to be febrile (43% versus 94%), but were more likely to have hepatomegaly (100% versus 53%) and splenomegaly (86% versus 53%).⁴⁴²

Severe Infection in the Immunocompetent Host

Rarely, HCMV infection in apparently immunocompetent individuals has been associated with complications involving specific organ systems, and can sometimes even be life-threatening. Reported cases describe a prolonged course of infection that develops into severe illness with multiorgan system involvement.^{171,316} In these rare cases, abnormalities are similar to those encountered in immunocompromised patients, including enteritis, thrombotic disease, hemolytic anemia, thrombocytopenia, encephalitis, neuropathies, myocarditis, and ocular disease, and raises questions about the true immune status of the host. Severe disseminated or focal disease due to HCMV is sufficiently rare that even when suspected, other possible causes should be carefully sought and the immunocompetence of the patient should be assessed.

Because most adults harbor latent HCMV and the virus can actively replicate in critically ill patients for reasons unrelated to illness,¹³⁴ distinguishing HCMV-specific disease can be difficult. Enteritis in association with HCMV infection illustrates this point. A meta-analysis of 41 cases of HCMV colitis reported in immunocompetent individuals between 1980 and 2003 found only 10 cases without comorbidity and 5 cases that were subsequently diagnosed as inflammatory bowel disease.²⁰⁰ Although reports of improvement in following antiviral treatment suggest an etiologic role, a lack of controlled studies, occurrence of spontaneous resolution, and variable outcome of reported cases make it impossible to draw firm conclusions about the relationship of HCMV to enteritis in immunocompetent patients.^{297,533} However, cases are attributed to HCMV when no co-morbidities or evidence of immune impairment are found, and no other potential causes can be identified.

Rare complications in immunocompetent hosts have been linked to primary or ongoing infection, and include venous thromboembolism and pulmonary embolus and recurrent anterior uveitis with iris atrophy.^{3,365} Transverse myelitis, persistent thrombocytopenia, hemolytic anemia, and encephalitis have each been associated with symptomatic HCMV infection in previously healthy persons.^{198,554,683} In a review of HCMV encephalitis in

immunocompetent hosts, evidence of HCMV infection included the detection of virus in the CNS, intrathecal production of antibody to HCMV, or temporal association of symptoms with either a virus culture-positive body fluid or serologic evidence of infection.²⁷ Signs of encephalitis in these patients include confusion, seizures, coma, aphasia, dysphasia, and cranial nerve abnormalities. Deaths during the acute illness have been reported, although encephalitis attributed to HCMV typically resolves spontaneously; however, recovery may take months and leave permanent memory or speech impediment.^{27,638}

Among critically ill intensive care unit patients, active HCMV infection, defined by detection of HCMV DNA in plasma, leukocytes, or tracheal secretions, was unrelated to illness severity.^{134,244} HCMV appeared to cause pneumonia and encephalitis in two patients who died from multiorgan dysfunction. HCMV illness in critically ill patients who are not immunosuppressed is uncommon.

Congenital Infection and Disease

Congenital HCMV infection associated with signs and symptoms of disease, ranging from subtle sensorineural damage such as hearing and eyesight compromise to severe CNS damage and multiorgan involvement, is termed *symptomatic*. A review of 15 relatively large studies (published 1970 to 2004) that screened newborns for HCMV virologically reported that 12.7% of 117,986 newborns with congenital CMV infection were symptomatic at birth.¹⁶² The principal clinical and laboratory findings are shown in Table 62.6, summarizing findings in

TABLE 62.6 Clinical Features of 285 Infants with Symptomatic Congenital CMV Infection Reported to the National Congenital CMV Disease Registry in the U.S. from January 1, 1990 through December 31, 1993

Characteristic	% of infants
Nonneurological abnormalities	
Petechiae or purpura	54
Small for gestational age	47
Hepatosplenomegaly	40
Jaundice at birth	38
Hemolytic anemia	11
Pneumonia	8
Neurologic abnormalities	
One or more of the following	68
Intracranial calcifications	37
Microcephaly	36
Unexplained abnormality	27
Hearing impairment	25
Chorioretinitis	11
Seizures	11
Neonatal death	9
Laboratory abnormalities	
Platelet count $\leq 75,000/\text{mm}^3$	48
Direct bilirubin $\geq 3 \text{ mg/dL}$	36
Alanine aminotransferase level $>100 \text{ U/L}$	23

Adapted from Istaş et al.²⁶⁵



FIGURE 62.5. Computed tomographic scan of the brain of an infant with symptomatic congenital CMV infection demonstrating periventricular calcification.

285 newborns with symptomatic congenital HCMV infection reported to a national registry from 1990 through 1993.²⁶⁵ In an analysis of 106 newborns with disease from Birmingham, AL, two-thirds had clinical evidence of CNS involvement, such as microcephaly, poor feeding, lethargy, generalized hypotonia, or seizures.⁶⁹ Cerebrospinal fluid exhibited elevated protein to greater than 120 mg/dL in approximately 50% of cases. Cranial computed tomographic scans were abnormal in 75% of symptomatic newborns, and periventricular calcification, a hallmark of congenital disease, was the most common abnormality (Fig. 62.5).⁶⁸ Other reported brain abnormalities include cortical atrophy, lissencephaly, and ventriculomegaly. Hearing loss is common in newborns with symptomatic infection; it was present in 56% of tested newborns in the Birmingham study and in 25% in the national registry report, and chorioretinitis was found in 10% and 11% of patients, respectively.^{69,265} Although jaundice, hepatitis, hepatosplenomegaly, petechiae, and thrombocytopenia usually clear spontaneously within a few weeks to a few months following birth, disease in infected newborns can be severe, leading to prolonged hospitalization for supportive care. Neonatal death occurs in approximately 10% of symptomatic newborns. Considering both the frequency and outcome of congenital HCMV infection, it has been estimated to be a leading cause both of sensorineural deafness and of infectious brain damage in children in the United States. Most symptomatic newborns will have some combination of deficits, including mental retardation, cerebral palsy, sensorineural hearing loss, and impaired vision. A literature review estimated a 13.5% rate of CNS sequelae at birth, mainly sensorineural hearing loss.¹⁶² An approximately equal proportion of infants who are asymptomatic at birth have risk may develop CNS sequela over the first months to years of life,⁵³⁵ which has been a motivating factor for universal screening.³¹⁸

The outcome of congenital HCMV infection is influenced by many factors, including gestational age at time of maternal infection, type of maternal infection (primary versus recurrent), specific newborn clinical findings, and viral load in newborn body fluids. Primary maternal infection during the first trimester of pregnancy is more likely to lead to clinical sequelae than infection later during pregnancy. Clinical abnormalities were detected by prenatal ultrasound, autopsy of abortus, or newborn examination in 10 (26%) of 38 congenital cases when primary infection occurred before 20 weeks of gestation, compared with 1 (6.2%) of 16 later in gestation.³³² Newborns with congenital infection exhibited CNS sequelae in 11 (32%) of 34 when primary infection occurred in the first trimester, compared with 6 (15%) of 40 when infection occurred later.⁴⁵⁰ Primary maternal infection has long been associated with more significant CNS sequelae and symptomatic congenital infection than recurrent maternal infection,^{195,612} although severe disease occurs after recurrent maternal infection, leading an impression that maternal infection may not always be predictive of outcome.^{15,66,206} Signs and symptoms of congenital HCMV disease include chorioretinitis (Fig. 62.6), microcephaly, an abnormal neurologic examination, and an abnormal computed tomographic scan of the brain. Congenitally infected newborns shed HCMV in urine and other body fluids at levels, but these levels are not related to risk of disease. In contrast, like other settings of HCMV disease, the level of viremia (typically assessed by quantitative DNA PCR) at birth or early in infancy correlates with congenital disease.^{67,76,317} Comparison of genotype of envelope gN (UL73) from affected and unaffected infants with congenital infection showed a consistent association between gN4 genotype and disease, whereas gN1 and gN3 were associated with less risk of sequelae.⁴⁷⁹ Previous attempts to link outcome to gB or UL144 genotype produced conflicting results. If the gN results can be confirmed, genotyping of strains could provide clinically relevant information and the knowledge of gN genotype associated virulence would likely influence evaluation of candidate vaccines.

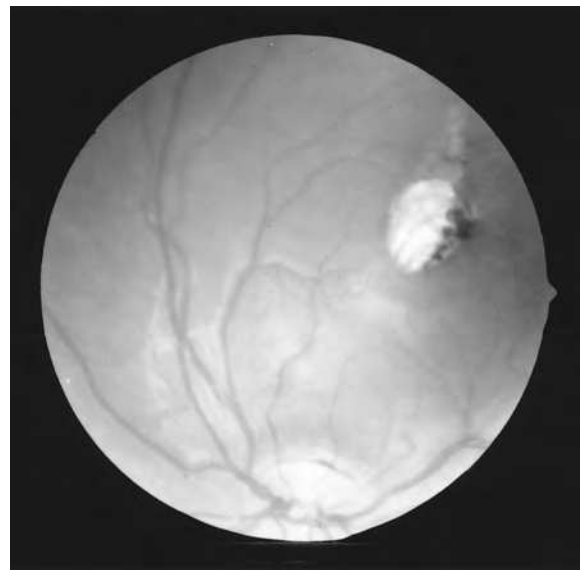


FIGURE 62.6. Retinal lesions due to congenital CMV infection. (Courtesy of Dr. Catherine Amos, Birmingham, AL.)

Infection in Premature Infants

Among very low birth weight premature infants, acquired HCMV infection is a potential cause of morbidity as shown by studies of transfusion-acquired infection. Exposure to multiple units of blood has been associated with HCMV infection and a clinical syndrome of hepatosplenomegaly, atypical lymphocytosis, thrombocytopenia, worsening of respiratory status, and a septic appearance. Risk for infection was related to very low birth weight and birth to a seronegative mother (correlated with a lack of passively acquired maternal antibody). Transfusion-acquired HCMV infection may be limited by using blood products from seronegative donors or by filtering seropositive blood to remove leukocytes that may transfer virus. Even with current standards that control transfusion-transmitted infections, HCMV infections continue to occur in premature newborns as a result of breastfeeding. Premature infants who acquired virus from a maternal source developed neutropenia, lymphocytosis, thrombocytopenia, hepatosplenomegaly, and worsening of respiratory status.⁷²⁵ Hospitalized very low birth weight premature infants (less than 32 weeks of gestation or less than 1,500 g at birth) in Tübingen, Germany were followed with serial PCR and virus isolation.⁶⁸⁸ Postnatal infection occurred in 59% of premature newborns of seropositive mothers who were breastfed on HCMV-infected milk. Infection was accompanied by symptoms in approximately half of those infected. A sepsis-like syndrome (apnea, bradycardia, gray pallor, bowel distention) was seen in the most severely affected infants who were typically from the lowest birthweight category and had onset of infection within the first 2 months after birth.^{232,234,688} Additional reports identified a similar source of virus, but lower rates or even lack of symptomatic infection.^{387,421,723}

Compared with term infants, premature newborns have lower levels of HCMV-specific IgG, especially when delivered before 28 weeks of gestation. In addition, premature and very low birth weight newborns often rapidly lose maternal IgG because of repeated blood sampling (for supportive care) and transfusion with HCMV-negative blood products. Therefore, the postnatal infection in these infants could be viewed as being similar to congenital infection caused by a primary maternal infection, raising the question of whether small, premature newborns with postnatal HCMV infection might be subject to the same types of sequelae seen in congenital infection.⁴⁴⁵ A number of studies failed to find evidence of neurologic sequelae of postnatal HCMV infection in this population, including sensorineural hearing loss, the most frequent hallmark of congenital HCMV disease.^{272,689} Given the variable study results regarding morbidity of HCMV infection acquired from breastfeeding in small, premature newborns, no consensus exists on what steps should be taken to prevent or treat these infections.⁵⁶³

Opportunistic Infection in the Immunocompromised Host

HCMV is one of the most common, significant, and difficult opportunistic pathogens affecting immunocompromised patients. Infection is common due to reactivation of latent virus, reinfection of patients who have had past infection, and initiation of primary infection through infected tissues and organs. Procedures that are components of patient care (e.g., transfusions or organ transplants) may transmit this virus at a time when the patient is maximally immunosuppressed. HCMV infections in these patients are significant in that severe,

life-threatening disease occurs in a number of settings. Finally, HCMV infections are difficult because diagnosis requires the detection of virus as well as the demonstration that HCMV is associated with disease. HCMV shedding and viremia are common in patients with impaired cellular immunity, even in the absence of disease caused by this virus. The disease risk is proportional to levels of viral markers in the bloodstream, and these roughly parallel the degree to which the patient is immunocompromised.¹⁶⁴ The highest levels of viremia and most consistent severe disease outcomes are seen in recipients of allogeneic HCT as well as in other settings where T-cell immunity is compromised, such as in patients with AIDS with very low CD4 counts. Disease is also seen in SOT recipients, in patients receiving immunosuppressive chemotherapy for cancer or collagen vascular disease, and in congenital immunodeficiencies. Infection may be clinically silent even in the immunocompromised host, and, when it occurs, disease may range from a brief, self-limited febrile illness to multisystem disease with severe consequences.

In addition to the clinical importance of HCMV in the management of immunocompromised patients, infections in various settings have improved understanding of the viral pathogenesis. For example, evidence that reinfection and reactivation can cause disease was first observed in immunocompromised patients. The importance of T cell-mediated immunity in host defense against HCMV, with virus-specific cytotoxic CD8+ T lymphocytes controlling infection, was established in HCT recipients.⁵²⁹ The suggestion that prophylaxis with passive antibody may prevent disease risk was derived from SOT recipients. The first evidence for clinical efficacy of a HCMV vaccine came from studies of live-attenuated Towne vaccine in renal transplant recipients. The development of HCMV-specific antiviral therapies resulted primarily from the need to manage infections in immunocompromised patients, and studies that led to definition of a molecular basis for antiviral resistance were performed in these populations. Finally, the widespread use of automated quantitation of HCMV DNA in blood and body fluids results from the need to manage infection in immunocompromised patients.

Opportunistic Infection Following Solid Organ Transplantation

Managing HCMV infection remains a major problem in transplant recipients and a significant component of the overall cost of an SOT. HCMV disease in this population is most risky when a seronegative recipient is transplanted with a seropositive organ (D+R-) and primary infection occurs in the recipient while receiving immunosuppressive therapy. Studies from the United States, United Kingdom, and France each reported that patients with HCMV disease require more days of hospital care and substantially higher cost of care.^{181,324,375} A 2001 review of the impact of HCMV infection on the cost of solid organ transplantation concluded that HCMV infection in liver transplant recipients was one of the most important variables affecting cost; HCMV disease resulted in approximately \$35,000 increased cost and 18 days longer hospital stay per affected patient during the first year after transplant.⁴⁶¹ A review of hospital readmissions within two years of transplantation among adult renal and heart transplant recipients from five U.S. centers, 1998–1999, found that the average length of stay for care due to HCMV disease was 10 to 11 days at an average cost of \$22,598 for renal transplant recipients and \$42,111 for heart transplant recipients.²⁴⁵

Given the widespread presence of latent virus infection in the population, the potential for HCMV infection must be considered in all solid organ transplant (SOT) recipients. The risk of HCMV disease in this population follows the likelihood of systemic infection: D+R- > D+R+ > D-R+, with D-R- at no risk. Therefore, clinical manifestations of infection in this population can arise from virus acquired with the transplant or from latent virus reactivation within the recipient and may be caused by (a) an acute, systemic, febrile illness (sometimes called *CMV syndrome*), (b) effects on specific organs, or (c) indirect effects.^{188,189,346} Principal signs and symptoms of CMV syndrome include fever and malaise; with occasional arthralgia and rash. Common laboratory manifestations include neutropenia, thrombocytopenia, and elevated hepatic transaminases. Although these nonspecific abnormalities could result from other causes, the presence of HCMV above a certain threshold in blood (usually determined by pp65 antigenemia or PCR detection of DNA as markers of viremia). The role of HCMV in end-organ disease is reinforced by the concurrent onset of abnormalities and rising presence of viral markers in blood. End-organ disease attributed to HCMV on SOT recipients includes pneumonitis, gastrointestinal lesions, hepatitis, retinitis, pancreatitis, myocarditis, and rarely, encephalitis or peripheral neuropathy. Evidence of an etiologic role for HCMV is strengthened by biopsy-proven detection of virus in tissue, assessed histopathologically. Even with current antiviral approaches to prevent severe disease, indirect effects of HCMV infection are seen in this population, including graft rejection, accelerated coronary artery vasculopathy (heart transplant), and increased risk of opportunistic fungal and bacterial infections. Primary HCMV infection in this population has been consistently linked with dysfunction of the transplanted organ, such as renal impairment in kidney recipients, hepatitis after liver transplantation, vasculopathy and chronic graft rejection in heart transplant, and pneumonitis as well as bronchiolitis obliterans following lung or heart-lung transplant. The association between infection (especially primary infection) and increased risk of opportunistic infections, recognized more than 30 years ago, continues to be observed despite available antiviral drugs.^{188,189}

The onset of HCMV infection, as indicated by detection of virus in blood or shedding, is approximately 4 to 8 weeks after SOT, and corresponds to the occurrence of clinical signs and symptoms.¹⁸⁹ The onset of infection and disease is influenced by both the type and level of immunosuppression as well as by the timing of antiviral prophylaxis after transplantation. The sources of infection include reactivation of the latent virus, either within the recipient or from the transplanted organ. Given widespread practice of leukodepletion, blood products are rarely the source of virus, which is why D-R- rarely become infected even when they receive blood from HCMV-seropositive sources. Primary infection acquired by an HCMV-naïve recipient from an organ is recognized as the highest risk setting, although this varies with the specific organ as well as the degree of immunosuppression. Studies performed before the widespread use of prophylactic or preemptive antiviral treatment showed that most patients with primary infection after transplant experienced a febrile illness related to the onset of HCMV excretion, with at least half resolving the infection spontaneously with clinical support. A more recent multicenter study showed that, in the absence of prophylactic antiviral therapy, HCMV disease occurred in 45% of D+/R- renal transplants

and in 6% of patients who were seropositive prior to transplant.³⁵¹ A study of D+/R- liver transplants from a multicenter trial reported that HCMV disease occurred in 17.6% of patients within 12 months of transplant, despite antiviral prophylaxis, up to 100 days after transplant.¹⁹⁷ Although antiviral prophylaxis has proved effective in preventing disease while the antiviral drug is being given, there is a risk of late-onset disease at the time prophylaxis is stopped.^{17,339,506} HCMV disease was reduced to 16% in a randomized, controlled trial comparing 200 days with 100 days of valganciclovir prophylaxis, where most cases were diagnosed as CMV syndrome.²⁵² Alternatively, patients may be monitored for viral markers by quantitative PCR and treated only when viral load exceeds a defined value in whole blood.¹⁶⁴ Such preemptive therapy has a similar efficacy to that of prophylaxis in preventing HCMV end organ disease,^{276,292,517} as recently demonstrated in a large cohort.³⁴

Opportunistic Infection Following Hematopoietic Stem Cell Transplantation

HCMV infection following allogeneic HCT poses the greatest risk when a seropositive recipient receives an allograft from a seronegative donor (D-R+). This setting leaves the transplant recipient without the benefit of immunity, in particular memory CD8+ T cells, while greater histoincompatibility increases graft versus host disease (GvHD), allowing viral reactivation and amplification in the posttransplantation period. Although the incidence of this disease after allogeneic HCT has been dramatically reduced over the last 30 years, antiviral prophylaxis is generally not applied in the immediate posttransplantation period due to concerns that ganciclovir and valganciclovir would delay engraftment. Infection remains a significant problem in this setting because of breakthrough disease in high-risk patients, late onset disease, and indirect effects that adversely affect outcome. Considering that 25 to 30 years ago approximately 25% of at-risk allogeneic HCT recipients developed HCMV disease (usually, pneumonitis) associated with a mortality rate of approximately 85%, remarkable progress has been made. From 1975–1985 to 1992–1994, rates of HCMV disease decreased from 13% to 2.2% and deaths attributed to this virus decreased from 13% to 0% at a transplant center in Sweden.³⁴⁵ Similar results have been reported from a major U.S. transplant center where the incidence of viral disease in allogeneic HCT recipients during the first 100 days posttransplantation dropped from 38% to 5% over an approximate 10-year period.⁵⁹ Reasons for the decline in disease due to this virus include prevention of transfusion-acquired HCMV infection in seronegative patients, decreased frequency of GvHD, use of preemptive antiviral therapy and improved methods for rapid diagnosis and prediction of patients at risk, the availability of effective antiviral agents, and improved understanding of the use of antiviral drugs to prevent disease.

The most common clinical manifestations of HCMV disease in allogeneic HCT recipients are pneumonitis and gastrointestinal disease. Pneumonitis produces an interstitial pattern on radiographs, respiratory distress, and hypoxemia. The diagnosis is supported by clinical signs in temporal association with detection of viral markers in blood, either pp65 antigenemia or quantitative PCR, or by PCR in bronchoalveolar lavage fluid.^{346,665} Gastrointestinal disease usually involves mucosal inflammation or erosion and can occur throughout the tract from esophagus to distal colon. In a review of HCMV

infection and disease in allogeneic HCT recipients in a Swedish hospital, 384 transplants were performed in which donor, recipient, or both were seropositive for HCMV over a 20-year period, and 33 (8.6%) developed HCMV disease.³⁴⁵ Among patients with HCMV disease, 21 (63.6%) had pneumonitis; 3 (9.1%) had pneumonia plus gastrointestinal disease; 6 (18.2%) gastrointestinal disease alone; and there was one case each of hepatitis, encephalitis, and retinitis. The widespread use of preemptive antiviral treatment has reduced the incidence of HCMV disease during the first 3 to 4 months after HCT transplant to approximately 5% or less. In current practice, late-onset disease, usually defined as HCMV disease more than 100 days after transplantation, remains a significant problem in transplant recipients who have had incomplete immune reconstitution.⁶¹

In addition to pneumonitis, gastrointestinal disease, and other clinical abnormalities attributed to this virus, evidence indicates that infection has indirect effects on the outcome in allogeneic HCT recipients just as it does in solid organ transplantation. Although the use of preemptive therapy has benefited the highest risk group for HCMV disease (D–R+), the D+R– setting can experience greater opportunistic bacterial and fungal infections.⁴²⁸ In addition, D+R– HCT experience the most infectious disease complications and highest overall mortality, suggesting a contribution of HCMV reactivation in susceptibility to other pathogens.⁴²⁸ Among patients with myelodysplastic syndromes undergoing HCT, probability of disease-free survival was greater for those who were HCMV seronegative and treatment-related mortality was higher for those who were HCMV seropositive.¹⁰¹ The effect of donor serostatus when donor and recipient are HCMV-seropositive remains controversial.⁶³ In addition to increasing the risk of other opportunistic infections, immunomodulatory effects of HCMV infection enhance GvHD, although mechanisms remain undefined.

In addition to the donor and recipient serostatus, the clinical variables associated with high risk for HCMV disease include donor–recipient histocompatibility mismatch, the conditioning regimen used to ablate the recipient's bone marrow, and the type of immunosuppression used after transplantation.⁶³ HCMV disease risk in HCT is as follows: D–/R+ > D+R+ > D+R–, and is enhanced when the donor and recipient histocompatibility match is less than ideal. Therefore, a matched unrelated donor source is typically more risky than a related donor, and, risk of disease is low following autologous or twin-to-twin HCT. Pretransplantation conditioning regimens that have a greater impact on recipient T cells (e.g., myeloablation) are associated with increased risk for HCMV disease. Similarly, posttransplantation immunosuppression associated with lymphopenia or more severe impairment of T cells (high-dose steroids, mycophenolate mofetil, T-cell depletion, anti-CD52, or anti-thymocyte globulin) result in higher risk of HCMV disease. Graft-versus-host disease is also associated with HCMV disease. Many of these risk factors are interrelated.

Although the approach to diagnosis, prevention, and treatment of HCMV infection varies considerably among stem cell transplant centers, the general consensus is that successful management of HCMV infection requires pretransplant donor and recipient screening, prevention of blood product transmission of HCMV, virologic and clinical screening for HCMV infection and disease posttransplantation, and effective use of antivirals. Because peripheral shedding of HCMV is not predictive of disease in HCT recipients, laboratories have focused

on measuring the quantity of HCMV in blood to determine when antiviral treatment should be given.

Opportunistic Infection in Human Immunodeficiency Virus/Acquired Immunodeficiency Disease Syndrome

Before the use of highly active antiretroviral therapy (HAART), approximately 20% to 40% of adults with AIDS experienced HCMV disease. Among persons with HIV infection, the risk of HCMV disease is linked closely with the loss in cellular associated with a low CD4 T-cell count. Conversely, the use of HAART has resulted in reconstitution or preservation of CD4 T-cell immune function in patients with HIV infection and has dramatically reduced the occurrence of HCMV viremia and disease.⁶²⁴ In the pre-HAART era, the most prominent AIDS-associated HCMV disease was retinitis followed by esophagitis and colitis and less common clinical presentations such as encephalitis, peripheral neuropathy, polyradiculoneuritis, pneumonitis, gastritis, and hepatitis.¹¹⁵ HCMV disease has always been less common in HIV-1–infected children than in adults, a consequence of the lower prevalence of HCMV infection in younger populations. HCMV remains a significant opportunistic infection in patients with HIV infection with significant immune impairment, and guidelines for preventing HCMV disease are focused on patients with fewer than 50 CD4 cells per μL of blood.^{102,103}

Co-infection with HCMV occurs in approximately 90% of HIV-infected women of childbearing age, and women with HIV are more likely to shed HCMV than are HIV-negative women,¹²⁴ probably due to the effect of HIV on immune function. One study in Brazil did not detect a difference in the rates of congenital HCMV infection in newborns of HIV-infected and HIV-negative women⁴²²; however, large cohort studies in the U.S., France, and Spain reported congenital HCMV infection in 2.3% to 4.6% of babies born to HIV-infected mothers, significantly higher than the rate of around 0.7% expected for the general population.^{169,229,364} In addition, decreased rates of HCMV transmission from mother to fetus have been reported in the HAART era compared with pre-HAART data from the same centers.^{229,364} The rate of acquisition of HCMV during birth and early infancy is higher in HIV-infected infants compared to HIV-negative infants.^{229,303,422}

HCMV has been suspected of being a co-factor in the development of AIDS because clinical studies link HCMV and progression of HIV-1 infection, and laboratory studies provide a biologic framework for HCMV enhancement of HIV-1 infection. Although some clinical studies in adults have linked markers of active HCMV infection (e.g., viremia or quantitative plasma HCMV load determined by PCR) with progression of HIV infection, others have not found HCMV to be a risk factor for progression of HIV-1 disease. A study in patients with CD4 counts less than 100/ μL , who were receiving HAART, reported that detection of HCMV DNA in blood by PCR was associated with HCMV disease, a new AIDS-defining condition and death; effects that remained significant in multivariable analyses.¹⁵² During HAART, asymptomatic HCMV infection contributes directly to T-cell activation.²⁵⁵ Among children with HIV-1 studied in the pre-HAART era, HCMV infection was associated with progression to AIDS, encephalopathy, and death.³⁰³ Studies have not been extended to children during the post-HAART era.

Association with Chronic Diseases

In view of the considerable experimental evidence for a key role of vascular endothelial cells in the pathogenesis of HCMV infection and the numerous viral gene products that appear to modulate the host inflammatory response, it is not surprising that this virus has been suspected of playing a role in vascular disease. HCMV contributes to cardiac allograft vasculopathy in heart transplantation⁴⁸⁷ and has been implicated in chronic diseases afflicting the general population, including immune senescence and frailty⁷⁰⁰ as well as cancer,^{128,389,603} although these remain controversial.³²⁵ In addition, the suggestion that HCMV may contribute to autoimmune vascular disorders (systemic sclerosis, polyarteritis nodosa, systemic necrotizing vasculitis, systemic lupus erythematosus, and Sjögren's syndrome). Evidence for a causative role for CMV based on antibody prevalence or serum level of antibody to CMV (or specific viral proteins) is not persuasive; it is difficult to control for the impact of the autoimmune disorder or its treatment on activation of CMV. A report associating antibody to HCMV UL94 with induction of endothelial cell activation, apoptosis, and proliferation of fibroblasts suggests a mechanism through which HCMV could influence the pathogenesis of autoimmune vascular disease.³⁵³

The role of HCMV in atherosclerosis has received considerable attention, but remains controversial. A number of studies link the virus with atherosclerosis, whereas others find no association.⁵³⁴ Studies in elderly women⁷⁰⁰ and Latinos⁵³² reported a greater risk of all-cause mortality and with cardiovascular disease mortality associated with HCMV infection. A large U.S. population-based study showed an association between HCMV seropositivity and all-cause mortality, an association that remained significant in multivariate analyses but did not show any association with cardiovascular disease mortality.⁵⁷⁸ The effect on all-cause mortality was independent of elevated C-reactive protein levels, so further studies may deconvolute the complex relationship between this virus, inflammation, and mortality.

There is growing interest in changes in the relationship with HCMV in aging populations. The elderly, and in particular, frail elderly, have fewer naïve T cells, and more immunocommitted T cells with shorter telomeres, combined with heightened cytokine levels, which has been called “immunosenescence.” Biomarkers of immunosenescence have been found in HCMV-seropositive adults between 42 and 81 years of age but not in an HSV-

seropositive/HCMV seronegative comparison group.¹⁵⁵ The clinical correlates are speculative, but may include excess mortality and impaired immune responses, including to vaccines.⁴⁶⁰

DIAGNOSIS

Differential Diagnosis

When acquired HCMV infection is symptomatic in the normal host, it must be distinguished from other viral and nonviral causes of acute infectious illness characterized by relatively prolonged fever (often, >10 days) and malaise. Other infectious agents that can cause mononucleosis or similar illness (e.g., such as Epstein-Barr Virus, *Toxoplasma gondii*, HIV, and hepatitis viruses A, B, and C) should be considered. Many viral and bacterial infections as well as some autoimmune diseases can cause prolonged fever and malaise, however; when fever persists for more than 14 days, the differential diagnosis is similar to that for fever of unknown origin. Serologic evaluation can be of value in assessing primary HCMV infection.

Congenital HCMV infection cannot be reliably distinguished on clinical grounds from congenital infection caused by other agents such as *Toxoplasma gondii*, syphilis, and rubella virus. Sometimes newborns with postnatal bacterial sepsis have hepatic or splenic enlargement, petechiae, direct hyperbilirubinemia, thrombocytopenia, and neurologic signs similar to those seen in newborns with symptomatic congenital HCMV infection. Newborns with noninfectious diseases such as erythroblastosis fetalis, osteopetrosis, and metabolic diseases can present with jaundice, petechiae, and organomegaly reminiscent of congenital infection. Specific laboratory tests to isolate or detect HCMV resolve the diagnosis in newborns quickly and reliably so long as they are applied within the first 3 weeks of age.

In transplant recipients, many signs of HCMV infection are nonspecific (Table 62.7). A variety of viral (notably, EBV and HHV-6) and other pathogens as well as rejection episodes or GvHD can result in fever, leukopenia, malaise, hepatitis, pneumonitis, enteritis, or impaired graft function. Disease caused by HCMV in patients with AIDS typically presents as retinitis, enteritis, or esophagitis. Although retinitis caused by this virus can be identified with reasonable accuracy by ophthalmologic examination, myriad other possible viral, bacterial,

TABLE 62.7 Common Clinical Effects of HCMV Infection in Immunocompromised Patients

	Solid organ transplantation	Hematopoietic cell transplantation	HIV/AIDS
DIRECT EFFECTS	Acute febrile illness with leukopenia Pneumonitis Esophagitis, gastritis, or enterocolitis Hepatitis Retinitis or other organ involvement	Pneumonitis Esophagitis, gastritis or enterocolitis Less common: encephalitis, retinitis, hepatitis	Retinitis Esophagitis, gastritis, or enterocolitis Peripheral neuropathy, encephalitis Pneumonitis Hepatitis
INDIRECT EFFECTS	Impaired graft function Accelerated coronary stenosis (heart transplant) Opportunistic fungal and bacterial infections Graft failure (controversial)	Opportunistic bacterial and fungal infections Increased mortality (controversial)	Progression to AIDS (controversial)

and fungal causes exist for the other signs and symptoms of HCMV disease in patients with AIDS. Sorting out the role of HCMV infection in immunocompromised patients is facilitated by quantitation of virus in blood by detection of HCMV in affected tissue (lung, eye, CSF, or gastrointestinal mucosa; see *Laboratory Diagnosis* section) and assessment of response to antiviral therapy aimed at HCMV.

Laboratory Diagnosis

Serology

Serologic tests for antibody to HCMV are highly accurate in establishing prior HCMV infection. This assessment is of great clinical importance for organ, HCT, and blood donors, as well as in the pretransplant evaluation of prospective transplant recipients. In addition, serologic tests for seroconversion and for IgM antibody to HCMV are commonly used to establish whether an infection in the immunocompetent host occurred recently. Antibody tests are not useful in the diagnosis of HCMV disease in the immunocompromised host. Although detection of virus-specific IgM antibody has been used to diagnose congenital HCMV infection, direct detection of viral markers is more accurate and is preferred.

Virus Detection

Virus culture detection of HCMV requires inoculation of human embryonic lung or foreskin fibroblast monolayers with clinical material and identification of the slowly developing, focal cytopathic effect (CPE) that is characteristic of HCMV, an approach that is labor intensive and time-consuming. Rapid HCMV isolation in tissue culture based on enhancement of infection of the monolayer by low speed centrifugation and detection of immediate early antigens using monoclonal antibodies allows detection of virus within 24 to 48 hours, and shows similar sensitivity to traditional virus isolation. Real time DNA PCR has become the preferred method for HCMV detection in many clinical virology laboratories because results may be available in a matter of hours, it is more sensitive than culture, and provides a quantification of this viral marker in the sample.

Laboratory Diagnosis of HCMV Infection in Specific Clinical Settings

Primary Infection in the Normal

Host-Maternal Infection

The question of primary maternal infection usually arises as a result of maternal febrile illness, known risk of exposure to HCMV through childcare or sexual activity, or because of a prenatal serologic screening with a positive HCMV-IgM result. The routine testing of pregnant women for antibody to HCMV is controversial; the American College of Obstetricians and Gynecologists and the British Royal College of Obstetricians and Gynecologists state in their respective practice guidelines that screening of pregnant women for HCMV is not recommended and should not be offered as part of routine prenatal care.^{4,507} Primary maternal infection is typically demonstrated by seroconversion from virus-specific IgG negative to positive; however, the initial serum available is often HCMV-IgG positive and other approaches are required. The presence of HCMV-IgM antibody is strongly suggestive of primary infection in symptomatic patients, although antibody assays can vary significantly in sensitivity and specificity and the positive predictive value of a

HCMV-IgM test is likely to be less than 50% when used for prenatal screening. The accuracy of diagnosis of primary maternal HCMV infection has been improved by use of HCMV-IgG antibody avidity assays.^{219,320} Commercial kits for HCMV-IgG antibody avidity determination may vary widely in cut-off values that define low avidity as well as in kinetics of transition from low to high avidity.⁵²² It is important to note that viral shedding in urine, saliva, or genital tract, or even detection of viremia, establishes a primary infection within the preceding 1 to 4 months, which is often the key question when a pregnant woman is first evaluated for possible primary HCMV infection.

Congenital Infection

Congenital HCMV infection is easily diagnosed by the isolation of virus from urine or saliva or detection of viral DNA by PCR within the first 3 weeks after birth. Because HCMV can be transmitted from mother to newborn during birth or in breast milk, virus shedding in newborns first detected after 3 weeks of age cannot be used as an indicator of congenital infection. Congenital infection has been diagnosed retrospectively by PCR detection of HCMV DNA in dried blood spots collected from newborns at birth for metabolic screening. Some investigators report PCR detection of HCMV DNA in blood of all newborns with congenital infection,^{56,327} whereas others report lower rates of viremia using real-time PCR on blood of symptomatic newborns.⁷⁶ A study that screened more than 20,000 newborns reported a low sensitivity of 34% for real-time PCR detection of HCMV in dried blood spots compared with rapid HCMV isolation (detection of early antigen) from saliva,⁷¹ results that argue for refinement of DBS methods to detect congenital infection unless DBS-positive newborns turn out to be more likely to have hearing loss or other sequelae. Results of quantitative DNA PCR in newborns with congenital HCMV infection may have value for prognosis; newborns with higher levels of viral DNA in blood are more likely to have sensorineural hearing loss or other sequelae.^{67,317} There is a nonlinear relationship between viral load and hearing loss, with most damage occurring in those with high viral loads.⁶⁹³ This could explain why short-course ganciclovir therapy in newborns with symptomatic infection protected hearing without an impact on chronic low-level infection.²⁹⁶

In pregnancies complicated by primary maternal infection, the prenatal diagnosis of congenital HCMV infection may be made by detection of virus in amniotic fluid, using virus culture or DNA PCR. Testing amniotic fluid collected at 20 or more weeks of gestation and 6 to 8 weeks from the onset of maternal infection is recommended.³³²

Opportunistic Infection in the Immunocompromised Host

Serologic methods are not useful for identifying HCMV disease episodes in these populations. Viral shedding in urine and saliva is very common in patients with impaired cell-mediated immunity even when they are not experiencing disease. Viral shedding is of limited value except in seronegative populations where shedding can provide evidence of primary infection. The most valuable laboratory diagnostic tests in immunocompromised patients are those that quantitate viral antigens or DNA in blood. In general, higher levels of virus markers in the blood correlate with greater risk of disease. Although commercial quantitative DNA PCR methods for use on whole blood or plasma have been in place at major medical centers,¹⁶⁴ recommendations

regarding threshold quantities of viral DNA that predict disease remain dependent on the clinical setting and other factors. Other approaches are in use—including pp65 antigenemia, quantitative competitive PCR, NASBA for pp67 mRNA and hybrid capture—to complement real-time PCR. Commercial kits are available for some of these, and a number of institutions use in-house assays. Among HCT recipients, lower levels of pp65 antigen–positive cells or of viral DNA in whole blood are predictive of disease than would be considered predictive in solid organ transplantation. Studies that have compared real-time PCR with antigenemia consistently report that more transplant recipients are positive by the former and that infection is detected earlier after transplant with real-time PCR than with the antigenemia assay.^{60,230,326,390} Until a standard approach to quantitation of HCMV in blood is widely accepted, clinicians who manage HCMV infections in immunocompromised patients will need to work closely with their clinical laboratories to determine locally the levels of antigenemia or HCMV DNAemia that are correlated with disease occurrence, recognizing that the threshold levels may vary in different immunocompromised populations.

ANTIVIRAL TREATMENT

The four antiviral agents currently approved by the FDA for treatment of HCMV disease are listed along with dosage information in Table 62.8; their pharmacology is reviewed in Chapter 13. U.S. Food and Drug Administration (FDA)–approved indications for each of these agents are limited to treatment of HCMV infection in immunocompromised patients. Each of these agents has been shown to reduce or eliminate viremia or viral shedding and to prevent or control viral disease in specific settings in immunocompromised patients. Each agent, however, also has the potential for significant toxicity and, therefore, their use is limited to patients who are at risk for serious (disabling or life-threatening) disease. Because HCMV infections in the normal host resolve spontaneously, antiviral treatment is not indicated. Antiviral treatment of

the extremely rare patient with a presumably normal immune system and severe HCMV infection is probably justified if the disease is life-threatening or likely to lead to disability, but will not be discussed further here.¹⁷¹

Treatment and Prevention of HCMV Disease in Patients with HIV Infection

With progressive decline in immune function, active HCMV infection (viral shedding, viremia) is common among HIV/AIDS patients. Before the use of highly active, antiretroviral therapy, HCMV disease accompanied advanced AIDS in adults approximately 40% of the time in the absence of HAART, and a number of clinical trials evaluated antiviral therapy for prevention and treatment of HCMV disease, as reviewed elsewhere.¹⁰² The risk of HCMV disease is closely associated with the level of immune system damage; with a CD4+ T-lymphocyte count of less than 50 cells/ μ L. The ability of HAART antiretroviral therapy to prevent or reverse profound immune impairment in patients with AIDS has resulted in reconstitution of immune responses to HCMV and a dramatic reduction in the frequency of HCMV disease.

Detailed recommendations for preventing and treating HCMV disease in patients with HIV or AIDS have been published and are updated from time to time, and these are posted at the following URL: www.cdc.gov.^{102,103} Consideration of ganciclovir prophylaxis is recommended for HIV-infected adults, adolescents, and children who are HCMV seropositive and who have CD4+ T-lymphocyte counts less than 50 cells/ μ L. Prophylaxis with intravenous ganciclovir is recommended for all patients with HIV or AIDS to prevent recurrences of previous HCMV end-organ disease; valganciclovir orally, cidofovir intravenously, and intravitreal foscarnet are listed as alternatives for adults and adolescents, but not for children. Usual dosages for ganciclovir, valganciclovir, foscarnet, and cidofovir for induction and maintenance therapy are listed in Table 62.8. Discontinuing of prophylaxis for HCMV should be considered for patients with sustained (≥ 6 months) increases in CD4+ T-lymphocyte counts in response to HAART therapy. Ganciclovir or valganciclovir is preferred for antiviral therapy for HCMV

TABLE 62.8 Antiviral Agents Approved for Treatment or Prevention of HCMV Infection in Immunocompromised Adults

Generic (trade name)	More information	Route	Usual adult dose for induction treatment ^a	Maintenance dosage	Major toxicity
Ganciclovir (Cytovene®)	www.rocheusa.com/products/cytovene/pi.pdf	IV oral	5 mg/kg, q12 hour, 14–21 days Not applicable ¹	5 mg/kg/ q24 hour 1,000 mg tid	Hematologic
Valganciclovir (Valcyte®)	www.rocheusa.com/products/valcyte/pi.pdf	oral	900 mg bid, 21 days	900 mg once per day	Hematologic
Foscarnet (foscavir®)	www.rxlist.com/cgi/generic2/foscarnet_cp.htm	IV	90 mg/kg, q12 hr, 14–21 days	90–120 mg/kg once per day	Renal
Cidofovir (Vistide®)	www.gilead.com/pdf/vistide.pdf	IV	5 mg/kg once per week ² , for 2 weeks	5 mg/kg once every 2 weeks	Renal neutropenia

^aRecommended dosages vary considerably depending on renal function, other organ dysfunction, concomitant medications, and indication for treatment; consult the indicated source for prescribing information.

¹Oral ganciclovir is not used for induction treatment.

²Dosage must be individualized based on patient's renal function and probenecid is given prior to dose to minimize renal secretion of drug.

end-organ disease among adults and adolescents with AIDS; cidofovir and foscarnet are listed as alternatives.¹⁰² Following initial induction treatment of 14 to 21 days or until symptomatic improvement has been achieved, chronic maintenance therapy is recommended for patients with HCMV retinitis or neurologic disease but not for patients who have had HCMV pneumonitis, esophagitis, or colitis.¹⁰² Treatment recommendations for children are similar, although there is a lack of data on cidofovir and foscarnet in children mentioned.¹⁰³ For both adults and children, optimized therapy of HIV with antiretroviral drugs remains a very important strategy for prevention and control of HCMV disease in this patient population.

Antiviral Strategies to Prevent Disease in Transplant Recipients

Antiviral agents are commonly used in transplant medicine to prevent HCMV disease, either as a daily prophylactic regimen or in a preemptive approach in which laboratory surveillance for evidence of infection in blood (commonly viral DNA levels by PCR is used as a surrogate for viremia) is used to identify patients at risk of developing HCMV disease who are provided with antiviral therapy until the indicator of infection is eliminated. If the preemptive strategy is employed, monitoring frequency (once or twice weekly or biweekly) and threshold level of viremia at which antiviral treatment is initiated vary according to the type of transplant and other risk factors. An assessment of risk for HCMV disease and potential side effects of antiviral therapy influences the choice of approach. Transplant recipients who are HCMV seronegative and receive a graft from a seronegative donor or an autologous hematopoietic cell graft have little or no risk of HCMV disease, and, assuming transfusion-transmitted infection is avoided, these populations never require preventive antiviral treatment. The highest risk group for HCMV disease in SOT recipients (D+R-) as well as the next highest risk group (D+R+) benefit from prophylaxis or surveillance with preemptive therapy. Additional factors influence approaches to prevention, including type of organ transplanted, donor-recipient histocompatibility, immunosuppressive regimen, and other underlying disease issues.

Consensus panel reports based on critical review of published studies of prophylactic and preemptive approaches to prevention of HCMV disease in SOT recipients provide recommendations based on the available evidence. In general, prophylactic antiviral treatment is recommended for all D+/R- kidney, liver, pancreas, heart, lung, and heart-lung transplants, as well as for all R+ (D-R+ and D+R+) lung and heart-lung transplants and for all patients treated with anti-lymphocyte or antithymocyte globulins or anti-OKT3 monoclonal antibody.^{287,302} Among R+ kidney, liver, pancreas, and heart transplant recipients, either universal prophylaxis or preemptive therapy are considered acceptable approaches; some centers choose to monitor low risk R+ patients and not use a preventive strategy. Antiviral agents used for prevention of HCMV disease in SOT patients include intravenous ganciclovir or oral valganciclovir or valganciclovir; CMV immune globulin is used in some centers. A meta-analysis of studies of prophylactic and preemptive strategies in SOT patients reported no difference between the two in occurrence of HCMV disease.⁵⁹⁰ The choice of preventive approach and duration of preventive medication varies based on specific organ being transplanted, donor and recipient HCMV serostatus, immunosuppressive regimen,

and local custom. Recent reviews should be consulted for details of specific preventive regimens as well as the evidence basis for them.^{174,253,440}

A consensus panel of experts issued guidelines for prevention of opportunistic infections, including HCMV infection in HCT recipients.⁶⁶⁵ The guidelines recommend use of an HCMV disease prevention program for all R+ and all D+/R- HCT recipients from the time of engraftment until 100 days after transplantation. Either prophylactic or preemptive treatment with intravenous ganciclovir was recommended for recipients of allogeneic hematopoietic cells; foscarnet was listed as an alternative antiviral for prophylaxis. If high-dose acyclovir or valganciclovir are used for prophylaxis, virological monitoring is recommended, and treatment with intravenous ganciclovir or foscarnet is recommended if there is virological evidence of HCMV infection. The choice between these two strategies (prophylactic versus preemptive) should be based on an assessment of risks and benefits of each approach, patient factors, and the availability of a clinical laboratory that can provide the rapid turn-around time for quantitation of HCMV in blood that is required for a successful preemptive approach. Virological monitoring should be performed weekly from 10 days to at least 100 days posttransplantation. It was also noted that HCMV-seropositive autologous HCT recipients who have underlying hematologic malignancies or receive specific intense immunosuppressive regimens are at risk for HCMV disease and should be monitored weekly using pp65 antigenemia or quantitative PCR for viremia for the first 60 days after transplantation. Specific recommendations on dose and duration of both prophylactic and preemptive therapy were included in these recommendations.⁶⁶⁵

Although a prophylactic rather than preemptive preventive strategy is usually favored for SOT patients at greatest risk of HCMV disease, this is not necessarily the case for allogeneic HCT recipients. The use of prophylactic antiviral treatment means that all patients are subject to the disadvantages of this approach (notably marrow suppression and delayed engraftment) to prevent HCMV disease that occurs in about one-third of allogeneic HCT recipients without any preventive strategy. Guidelines favor use of the preemptive over the prophylactic approach for D+/R- HCT patients.⁶⁶⁵

Antiviral Treatment for Transplant Patients with Disease

Although preventive strategies have been highly effective in reducing morbidity and mortality from HCMV disease in both SOT and HCT recipients, HCMV disease remains a concern in patients who are either not on a preventive regimen or present with late-onset disease more than 100 days after transplant. In addition, HCMV disease is seen in some patients who were judged to be at low risk and not placed on any preventive program or as breakthrough disease in a small percentage of those on prophylaxis or preemptive regimens. Evidence-based guidelines for treatment of HCMV disease are not available, and no randomized clinical trials have been conducted of antiviral treatment of HCMV disease in transplant recipients. Recommendations for treatment of HCMV have been made in recent reviews.^{174,253} Intravenous ganciclovir or oral valganciclovir are the preferred antiviral agents for treatment, usually given for 2 weeks or until clinical symptoms resolve and viremia falls below a threshold value. Failure to clear viremia has been associated with recurrence of HCMV disease. Maintenance

therapy is used for patients who are at substantial risk for HCMV disease recurrence because of continued immunocompromise; among HCT recipients, maintenance therapy is often continued through the first 100 days after transplantation. Foscarnet is an alternative for treatment of HCMV disease in patients whose virus is resistant to ganciclovir, and in those who fail to respond to ganciclovir, those with marrow failure, or those who cannot tolerate ganciclovir. Recent reviews of antiviral treatment of HCMV disease in SOT and HCT patients provide details of treatment regimens and their effectiveness^{62,174,227,253,347,665}

Antiviral Treatment of Congenital Infection

Antiviral treatment of congenital HCMV infection is currently limited to newborns with signs of congenital HCMV infection at birth. A randomized trial of ganciclovir was limited to newborns that had clear evidence of central nervous system involvement at birth.²⁹⁶ Six weeks of intravenous ganciclovir (6 mg/kg/dose every 12 hours) was compared with no treatment. The proportion of patients with worsening of hearing at 1 or more years of age was 21% in the ganciclovir treated infants, compared with 68% in those who were untreated, $P < 0.01$. In addition follow-up developmental testing of participants showed that a higher proportion of the untreated group failed to attain multiple developmental milestones at the expected ages.⁴³⁷ The American Academy of Pediatrics endorsed treatment of newborns with symptomatic congenital HCMV infection with evidence of CNS damage, but pointed out that whether newborns with less severe disease would benefit from treatment was unknown.¹ Treatment of symptomatic congenital HCMV infection in HIV-exposed or HIV-infected infants with intravenous ganciclovir, 6 mg/kg of body weight every 12 hours for 6 weeks, is recommended.¹⁰³ Newborns with congenital HCMV infection who have no abnormalities at birth should not be treated with intravenous ganciclovir, as the majority of them will have no sequelae of congenital infection and there is currently no means of identifying the approximately 10% to 15% who will subsequently develop hearing loss. Neutropenia was common in newborns treated with ganciclovir and resolved with decreasing dose or suspending treatment.²⁹⁶ Carcinogenicity and reproductive toxicity occur in experimental animals at ganciclovir levels similar to those achieved in treating HCMV infection in humans, another reason to limit ganciclovir in newborns as recommended by the American Academy of Pediatrics. The pharmacokinetics and pharmacodynamics of valganciclovir, a ganciclovir prodrug with good oral bioavailability, suggest it could replace intravenous ganciclovir for treatment of symptomatic congenital HCMV infection. Six months of oral valganciclovir is currently being compared to shorter-course intravenous ganciclovir.⁵⁷⁷

Antiviral Drug Resistance

Chronic administration of antiviral drugs in immunocompromised patients is associated with appearance of mutations in HCMV genes for the UL97 phosphotransferase and UL54 DNA polymerase.¹¹⁸ Specific mutations have been associated with phenotypic resistance to antiviral drugs both *in vitro* and *in vivo* (see Chapter 13 for discussion of the mechanisms and genetics of antiviral drug resistance). Clinically, development of antiviral drug resistance is associated with treatment failure, although it is important to recognize that breakthrough disease or treatment failure occurs in severely immunocompromised

patients with HCMV disease, independent of the appearance of mutations associated with resistance. Genetic mutations associated with resistance are uncommon in patients before antiviral treatment, but are quite common after three or more months of antiviral therapy.²⁶⁷ Patients are often infected with multiple strains of HCMV that may differ with regard to antiviral susceptibility, and resistant strains can persist for months after antiviral therapy has been discontinued.^{119,178} Recognizing and managing antiviral drug resistance in the clinical setting is a complex problem because *in vitro* susceptibility testing is not widely available and the relationship between *in vitro* susceptibility or genotypic changes and clinical effectiveness of ganciclovir, foscarnet, or cidofovir have not been well-defined. The problem is further compounded because one patient isolate selected for evaluation may not accurately reflect the strain or strain mix causing disease. In addition, cross-resistance to cidofovir often occurs in patients with high-level ganciclovir resistance, and patients with multiresistant viruses have been reported.^{119,241}

Clinically significant antiviral drug resistance should be considered a possibility for patients who are responding poorly to treatment. Patients with more significant degrees of immunocompromise, continued HCMV replication while on treatment, or with longer exposure to antiviral therapy are at greater risk for drug resistance. Ganciclovir resistance of HCMV isolates from R+ SOT recipients is rare; among recipients of kidney or pancreas or lung transplants resistance rates range from 1% to 4.4%.³³⁸ Resistance rates are higher among HCMV isolates from D+/R- transplant recipients, ranging from 0 to 5% in kidney, heart, and liver transplant recipients and from 9% to 27% in kidney or pancreas and lung transplant recipients. Among HCT recipients, ganciclovir resistance rates range from 0 to 8.3%.⁶³ If phenotypic or genotypic testing of viral isolates is available, this can be used to guide selection of alternatives, although in practice it may be necessary to make an empiric change in therapy before such results are available. Selecting drugs based on patient isolate susceptibility, optimizing the dose of antiviral medication based on serum levels within the limits of toxicity, and use of combination therapy may all prove beneficial.

PREVENTION OF INFECTION AND DISEASE

Community Acquired Infection

Preventing horizontal transmission of HCMV poses challenges: infected individuals shed virus for months to years; infection in normal hosts is almost always clinically silent and the exposures most likely to transmit HCMV are difficult to avoid. These features of HCMV infection make it difficult to plan strategies for prevention based on limiting exposure to the virus. Exposure to urine and saliva from young children together with adult sexual activity remains the most important sources of virus. However, such exposures are a normal part of life. Two studies provide evidence that the incidence of infection may be reduced in pregnant women by increased hygiene. Women who knew they were seronegative benefited from instruction in techniques for limiting exposure to body fluids, particularly from handling children.^{11,684} The Centers for Disease Control and Prevention (CDC) recommends informing caregivers of infants and young children about HCMV—how it is spread and hygienic measures (e.g., hand-washing) to reduce transmission; in addition, it

is recommended that pregnant women who work with young children should be informed about their risk of HCMV infection as well as the risk of infection to their offspring.^{104,105}

Health Care Workers

Hospital workers do not appear to have an increased risk of HCMV infection, which is consistent with control via infection-control procedures (e.g., handwashing and avoiding contact with body fluids) that are a routine in hospitals and other health care settings. The Hospital Infection Control Practices Advisory Committee of the CDC recommends only standard precautions (the same precautions used to prevent contact with blood and body fluids of all patients) for HCMV and does not recommend screening of hospital workers for HCMV antibody.⁶⁴

Prevention of Infection using Medical Procedures

Blood Transfusion

Transmission of HCMV through blood products to patients who have significant risk of HCMV disease should be avoided. Patient groups at risk for such disease from transfusion include seronegative newborns (especially premature newborns), seronegative pregnant women, patients with AIDS, and organ transplant recipients; however, any patient with impaired cell-mediated immunity may be at risk. Transfusion-transmitted HCMV infections have generally been brought under control by using either leukoreduced or seronegative blood products to prevent exposure of the most at-risk patients. Serologic screening for HCMV and the allocation of blood products according to donor HCMV status adds cost and is of limited value in communities where a high proportion of donors are seropositive. Seronegative blood products have been administered to certain populations (e.g., premature newborns) without prior screening and without a complete understanding of risk, further increasing the demand for seronegative blood products. Given that leukocytes are the main reservoir of HCMV in blood, procedures that remove white blood cells from whole blood by filtration have reduced risk of transmission. Both methods—provision of blood products from seronegative donors and leukoreduction—are effective in preventing transfusion transmitted HCMV, but these two methods may not be equivalent. A study in donor and recipient HCMV-seronegative allogeneic and seronegative autologous HCT recipients ($N = 807$) found a significant difference in the rate of transfusion-transmitted HCMV (1.7%) when only seronegative blood products were used compared to a period when leukoreduction was used (4%),⁴²⁹ and shows that exposure filtered blood from seropositive donors was the key risk factor for HCMV transmission. Similar studies with weekly screening of recipients for HCMV infection and sample sizes sufficiently large to detect low infection rates have not been performed in other at risk populations. Customs vary from institution to institution as to the use of seronegative or filtered blood to prevent HCMV infection, and some differences exist among regulatory authorities and professional associations as to recommendations.⁴⁴¹

Solid Organ Transplantation

The most common source of HCMV infection in organ transplant recipients is reactivation of the transplant recipient's latent virus. Although selection of seronegative donors for seronegative recipients would prevent primary HCMV infection in this

setting, this approach is impractical due to the limited availability of donors. Prophylactic and preemptive antiviral regimens have been used to prevent disease from primary and reactivated HCMV infection as described in the section on antiviral treatment. It is common practice to provide only irradiated, HCMV-seronegative or filtered blood products for transplant recipients. In addition, seronegative transplant recipients and others with profoundly impaired immunity should be cautioned about the risk of HCMV infection through contact with saliva and urine from young children or as well as through sexual contact with adults.

Hospital Cross Infection

Standard precautions are recommended for control of this virus in the hospital.⁶⁴ Human milk is a potential vehicle for nosocomial transmission of HCMV where banking of milk is employed for hospitalized newborns. Milk from seropositive donors should not be given to seronegative newborns, especially those who are premature or hospitalized. A possible exception could be provision of a seropositive mother's milk to her own hospitalized, premature newborn who is seronegative because of prematurity and postnatal decline of passively acquired maternal antibody. Even this situation carries a risk for HCMV disease. Methods for processing milk that reduce HCMV infectivity have been evaluated²³³; a review of HCMV transmission to premature newborns by this route concluded that there was insufficient evidence to support either avoidance or inactivation methods.³¹³

Assisted Reproductive Technology

HCMV has been detected in semen from 2.9% to 33.5% of seropositive donors based on virus culture, DNA hybridization, or PCR.^{53,78,329,362,715} HCMV is transmitted during artificial insemination, resulting in maternal as well as fetal infection. A number of years ago, the American Fertility Society recommended serologic screening of semen donors for antibody to HCMV.¹³ The British Andrology Society has gone further and recommended limiting semen donors to seronegative men with the goal of eliminating the chance of HCMV transmission, a position that elicited dissenting views.^{79,333,383} The American Society for Reproductive Medicine guidelines recommend testing sperm donors for IgG and IgM antibody to HCMV initially and every 6 months, with the donor excluded if the HCMV-IgM antibody test is positive. The serologic tests are repeated before a semen sample is released from quarantine.³³ Donors who are HCMV-IgG and HCMV-IgM negative and those who are positive only for HCMV-IgG (including those who were previously HCMV-IgM positive) are acceptable. Data on HCMV in donated oocytes are very limited; a study of 71 women having infertility evaluations (62% seropositive) reported no detection of HCMV by PCR in any donated oocytes.⁷¹⁵ The American Society for Reproductive Medicine, however, has cautiously recommended the same approach to screening oocyte donors as is recommended for sperm donors.³²

Passive Immunization

CMV-IG is used by some transplant centers in combination with antiviral drugs in SOT patients as part of a prophylactic regimen to reduce HCMV disease. A multicenter, randomized, placebo-controlled clinical trial in D+/R- renal transplant recipients performed more than 25 years ago showed a statistically significant reduction in HCMV disease and opportunistic

fungal and parasitic infections among CMV-IG recipients compared with placebo recipients,⁵⁹⁸ although the rate of HCMV infection was similar in CMV-IG and placebo subgroups. Another multicenter trial performed in liver transplant recipients failed to show efficacy for prevention of HCMV disease in high risk D+/R- recipients, although an overall reduction was seen in severe disease and opportunistic fungal infections.⁵⁹⁷ In the interval since these randomized, placebo-controlled clinical trials of CMV-IG were performed, significant advances in the use of antiviral drugs to prevent HCMV disease in organ transplantation and changes in immunosuppressive regimens have made the role of CMV-IG less clear. A randomized, open-label comparison of CMV-IG with no treatment in HCMV-seropositive lung transplant recipients found no evidence of efficacy for prevention of HCMV disease, viremia, acute rejection, or bronchiolitis obliterans.³⁰⁶ Results of studies of prophylactic regimens comparing combinations of CMV-IG and ganciclovir with single agent therapy differ as to whether the addition of CMV-IG was superior to ganciclovir alone. In an American Society of Transplantation review of diagnosis and management of HCMV infection in SOT patients, there was no recommendation for use of CMV-IG for prevention, but a number of centers employ CMV-IG in conjunction with antiviral agents for prophylaxis of high risk (D+/R-) heart, lung or heart-lung transplant recipients.²⁵³

CMV-IG is not recommended for prophylaxis of HCMV infection in HCT⁶⁶⁵ and is no better than IV-IG for reducing GvHD. Passive immunization with donor-derived HCMV specific T cells has been shown to decrease the risk of HCMV disease in allogeneic HCT recipients; however, this approach is difficult to adapt to clinical use.^{127,175,463,464,692}

A randomized, double-blind, placebo-controlled clinical trial evaluated the use of CMV-IG in premature newborns that were transfused with blood from donors not screened for HCMV antibody.⁵⁹⁹ No difference was noted in overall HCMV disease rates between CMV-IG and placebo recipients. Use of blood from seronegative donors or from units that have had white blood cells removed by filtration has been so successful in preventing transfusion-acquired HCMV infection in premature newborns that it has eliminated the need to consider CMV-IG for this indication. Whether CMV-IG could prevent HCMV disease in small, premature newborns infected from a maternal source (intrapartum or milk) is not known.

CMV-IG treatment of pregnant women with primary HCMV infection to prevent fetal infection as well as to treat fetuses with prenatal infection reduced the rate of transmission to the fetus and improved the clinical outcome.⁴³² This study has attracted considerable attention, even though it was not randomized, lacked a control group, study participants self-selected for treatment or no treatment, and investigators who evaluated clinical outcomes were not blinded to treatment used. Although no conclusions regarding efficacy of CMV-IG for preventing transplacental transmission of HCMV infection can be drawn, the possibility of a benefit provided a rationale for further study of CMV-IG for HCMV-infected pregnant women; two randomized, placebo-controlled clinical trials are now addressing this question.^{2,172}

Vaccines

Although an HCMV vaccine has not been licensed, there is a significant body of evidence from clinical trials indicating that

HCMV vaccine candidates can decrease the occurrence of HCMV disease and even prevent infection. Encouraging results were recently reported from clinical trials of a HCMV-subunit glycoprotein B vaccine and from a bivalent plasmid DNA vaccine. More than 20 years ago an efficacy trial in renal transplant recipients using a live virus vaccine (Towne HCMV) showed that pretransplant immunization of seronegative patients with Towne vaccine protected them from severe disease due to primary HCMV infection in the immediate posttransplantation period⁴⁸² as well as protection from a challenge with HCMV strain Toledo,⁴⁸³ although vaccine-induced protection was overcome by a lower dose of challenge virus than the protection afforded by natural infection. Immunization with Towne vaccine did not prevent infection, it did not reactivate, and vaccine virus was never recovered from immunocompromised vaccine recipients. Towne HCMV vaccine was also evaluated in a placebo-controlled trial in seronegative parents of preschool age, HCMV-infected children¹²; however, no evidence of vaccine efficacy was observed and the infection rate remained identical to that of the placebo control group. A subunit vaccine composed of a secreted gB antigen in an oil-in-water emulsion adjuvant (MF59) was evaluated in a phase 2 randomized, placebo-controlled, double-blind clinical trial in young mothers.⁴⁵⁶ The HCMV infection rate was reduced by 50% in vaccinees compared with placebo recipients. Furthermore in a phase 2 randomized, placebo-controlled clinical trial in SOT recipients, HCMVgB/MF59 vaccine was immunogenic and appeared to benefit recipients.²²⁸ Higher antibody titers to gB were associated with shorter duration of viremia; among D+/R- transplants, HCMVgB/MF59 recipients had reduced duration of viremia and reduced need for antiviral treatment compared with placebo recipients. A phase 2 double-blind, placebo-controlled clinical trial of a bivalent (HCMV gB and pp65) plasmid DNA vaccine in HCMV-seropositive HCT patients reported that vaccine recipients had fewer episodes of viremia and were less likely to have repeat episodes compared with placebo recipients.²⁹¹

A number of other vaccines have reached clinical trials evaluating immunogenicity and safety including: vaccines based on a canarypox virus vector,⁴⁹ a chimeric live virus with portions of Towne and Toledo genomes,²⁴³ a modified alphavirus vector (Venezuelan equine encephalitis virus) expressing immunogenic HCMV proteins,⁵¹ and a recombinant HCMV gB antigen with a proprietary adjuvant.⁶³⁹ Additional approaches to vaccine development have been pursued using animal models including subviral particles,⁴⁵ priming with plasmid DNA expressing multiple HCMV genes followed by boost with formalin inactivated virus,⁴⁰⁹ vaccinia vectored gB, pp65 and IE1,⁷³³ replication-deficient adenovirus vectored HCMV antigens,⁷³⁸ and an attenuated HCMV expressing the ligand for an NK-activating receptor.⁵⁸⁶

The relative merits of specific combinations of antigens, adjuvants, and vaccine platforms will be revealed by additional clinical trials. Continued preclinical studies will likely reveal new and possibly superior approaches to any that have been in clinical trials. In addition, there are unanswered questions regarding the optimal target populations for HCMV vaccine clinical trials as well as the prevention of HCMV disease. Although it is unclear when an HCMV vaccine will be licensed, there is continued optimism that vaccines will eventually be able to play a major role in control of HCMV disease.

PERSPECTIVES

As with previous editions of this textbook, we have sought to update the basics of HCMV replication and latency together with a rapidly growing body of information on the interaction with the human host. Our understanding of pathogenesis, immune response, clinical disease settings, and both prophylactic and therapeutic interventions has seen remarkable strides. The breadth of cell intrinsic, innate, and adaptive immune processes that control viral replication is balanced by an armory of virus-encoded modulatory gene products aimed at deflecting those same pathways. It has become clear that HCMV immune modulators that support such a complicated lifestyle, together with those elaborated by other viruses and microbial pathogens, have contributed to the evolutionary adaptation resulting in host defense pathways found in mammals.

In the last few years, a complete sequence of a prototypic HCMV genome has been assembled, and combined with analysis of viral strain variants that arise under different culture conditions as well as during natural replication in patients, an unexpected plasticity has come to light. This plasticity contributes to three broad areas that continue to challenge experimental consensus: (1) seemingly variable behavior of viral strains in cells of a defined cell type, distracting from the goal of mechanistic understanding of key viral properties of persistence, latency and reactivation; (2) common features that engender this virus with a dual capacity for transplacental transmission during pregnancy and opportunistic infection in immunocompromised hosts; and (3) adaptive immune control mechanisms that limit viral transmission as well as disease and would suffice for vaccination to prevent the range of clinically significant infections associated with HCMV. As was true when we prepared the last perspective, we still do not have a definitive picture of the array of cell types that can harbor latent virus, and we certainly do not comprehend the molecular principles that govern latency and reactivation. Despite challenges posed, greater emphasis must be placed on determining whether principles that emerge from cell culture or animal models apply in the natural human host, and these must include observational studies in settings where disease is the outcome as well as clinical trials focused on defined interventions. This virus remains the only human herpesviruses where a subunit-adjuvant vaccine may prevent virus acquisition, which must be pursued. At the same time, no issue remains as glaring as the question of how host immune control is compromised or overcome to allow transplacental transmission during pregnancy.

Bacmid-based molecular genetics and methods for complementation of defective mutants have contributed to a remarkable understanding of HCMV gene function. HCMV studies in cell culture, paired with studies in animal models, have shown the benefit of viral genes that modulate host response to infection, predicting where cell intrinsic, innate NK cell, and adaptive T cell-mediated immune surveillance are most crucial. Endothelial, epithelial, and myeloid cells have begun to yield up requirements for differences in the way viral entry takes place as well as differences in the cellular response to infection manifest by pathogen sensors. The challenge viral strain variants that arise in cultured fibroblasts as well as in all other cultured cell types must be overcome. The macromolecular interactions crucial to viral replication, pathogenesis, latency, and host defense have just started to emerge, and these must to

be extended to natural infection settings where the information will improve clinical management of disease.

Finally, the possible involvement of HCMV in chronic diseases such as vascular disease, immunosenescence, glioblastoma, and salivary gland cancer continues to attract evidence. The path forward is not entirely clear: current antiviral drugs are not always effective in situations where viral etiology is established, and large epidemiological studies needed to develop a definitive correlation are costly and must include settings not confounded by the prevalence of HCMV infection in the population. Immunization might ultimately reduce the universal risk of congenital disease while establishing the contribution this virus makes to any chronic disease.

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Varicella-Zoster Virus

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INTRODUCTION AND HISTORY

Varicella-zoster virus (VZV) belongs to the alphaherpesvirus subfamily of the *Herpesviridae*. Primary VZV infection usually occurs in childhood and causes varicella (chickenpox), characterized by fever and a generalized, pruritic vesicular rash. VZV has tropism for T lymphocytes, which allows dissemination of the virus from mucosal sites of inoculation to skin during primary infection (Fig. 63.1). VZV then becomes latent in ganglionic neurons and may reactivate to cause zoster (shingles), characterized by pain and a dermatomal rash. Declining cell-mediated immunity to VZV, associated with advancing age or immunosuppression, increases the risk of zoster. Zoster may be followed by chronic pain (postherpetic neuralgia) and other neurologic and ocular disorders. Chronic radicular pain and these other disorders also occur in the absence of rash (zoster sine herpete). VZV persists in the human population by transmission to susceptible individuals from contacts with varicella or zoster.

Once thought to be the same disorder, varicella and smallpox were shown to be distinct entities in 1768 by William Herberden. Inflammation of ganglia corresponding to the dermatomal distribution of zoster rash was described by Fredrich Von Baresprung in 1863. In 1900, Head and Campbell mapped the cutaneous dermatomes through an extensive pathologic analysis of ganglionic lesions associated with zoster.²⁵³ In 1892, Von Bokay proposed an infectious etiology for varicella and zoster, postulating that they were related,⁶⁴¹ a concept later supported by the occurrence of varicella in children inoculated with zoster lesion fluid.⁵⁹¹ Garland¹⁸⁵ and Hope-Simpson^{268,269} suggested that zoster was caused by reactivation of latent virus acquired during varicella. In 1953, Weller propagated VZV in tissue culture and found that cytopathic effects, virion morphology, and antigenic profiles were indistinguishable when cells were inoculated with lesion fluid from patients with varicella or zoster.^{672,673} Proof of VZV latency in ganglia came in 1983 from Gilden et al., who showed that radiolabeled VZV DNA probes hybridized to DNA extracted from trigeminal ganglia²⁰⁵ and from Hyman et al., who found

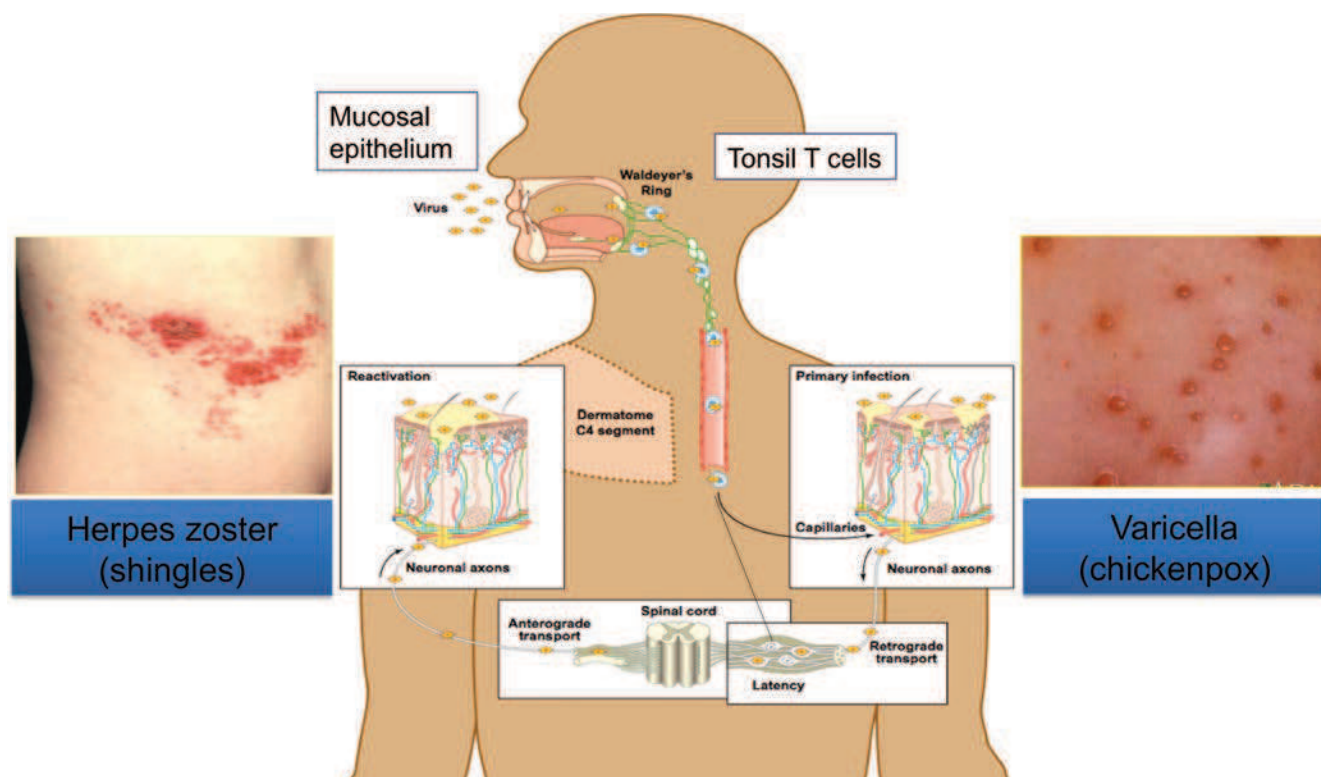


FIGURE 63.1. Model of the pathogenesis of varicella-zoster virus (VZV) infection. Primary VZV infection is acquired by inoculation of mucosal epithelial cells of the upper respiratory tract. Replication at the site of entry allows VZV transfer into tonsils and other local lymphoid tissues, where T cells become infected. Infection of dendritic cells in the mucosal epithelium may support virus transfer to regional lymph nodes. VZV-infected T cells transport the virus to skin sites of replication via a cell-associated viremia. After a 10- to 21-day interval, skin infection produces the vesicular rash associated with varicella (chickenpox). In the course of primary infection, VZ virions gain access to the sensory nerve cell body by retrograde axonal transport or via T cell viremia to establish latent infection within neurons of the sensory ganglia. Episodes of reactivation, during which VZV gains access to skin via anterograde axonal transport, can cause symptoms of zoster (shingles).

that radiolabeled VZV RNA hybridized *in situ* to trigeminal ganglionic neurons.²⁷⁶ In 1984, Straus et al. used restriction endonuclease analysis of VZV DNA from viruses isolated from an immunocompromised patient with varicella and a later episode of zoster to show that reactivation was caused by the original infecting virus.⁶⁰⁷

In 1986, Davison and Scott¹¹⁹ determined the complete nucleotide sequence of the VZV genome (strain Dumas), making VZV the first herpesvirus to be fully sequenced. Since then, sequencing of more than 30 VZV isolates has revealed the remarkable stability of the VZV genome.^{56,407,562} Mutagenesis of the VZV genome using cosmid or bacterial artificial chromosome (BAC) methods has permitted functional analyses of many VZV genes in recombinant viruses.^{100,395,621,715} VZV gene products and promoter elements involved in pathogenesis and modulation of host cellular defenses *in vivo* have been identified by Arvin et al.^{23,24,427,428,707,709} using VZV recombinants containing defined mutations to infect human skin, T cell, and dorsal root ganglia xenografts in a SCID mouse model.^{23,24,427,428,707,709} Major clinical advances were achieved with the development of the live attenuated varicella vaccine from the Oka strain by Takahashi in 1974, making varicella^{610,611} and zoster⁴⁸² the only vaccine-preventable human herpesvirus infections and the

identification of antiviral agents effective against varicella and zoster in the 1980s.^{36,142,677,678}

CLASSIFICATION

The genus *Varicellovirus* of the alphaherpesvirus subfamily includes VZV, simian varicella virus (SVV) (cercopithecine herpesvirus 9), pseudorabiesvirus (PRV) (suid herpesvirus 1), and equine herpesvirus 1 (equid herpesvirus 1). VZV is most closely related to SVV.^{215,216,217} These viruses disseminate through their natural hosts to infect the skin, mucous membranes, and visceral and nervous system tissues. They also share a genomic arrangement in which isomeric forms are generated by inversion of the shorter unique DNA segment and its flanking repeat elements. VZV is the species name of the only varicellovirus known to infect humans. The three human alphaherpesviruses are VZV, which has a very narrow host range restricted almost exclusively to cells of human or simian origin, and the herpes simplex viruses HSV-1 and HSV-2, which replicate in cells and tissues of many mammalian species. All three viruses become latent in ganglia along the entire neuraxis, from which they can reactivate to cause recurrent viral shedding or disease.

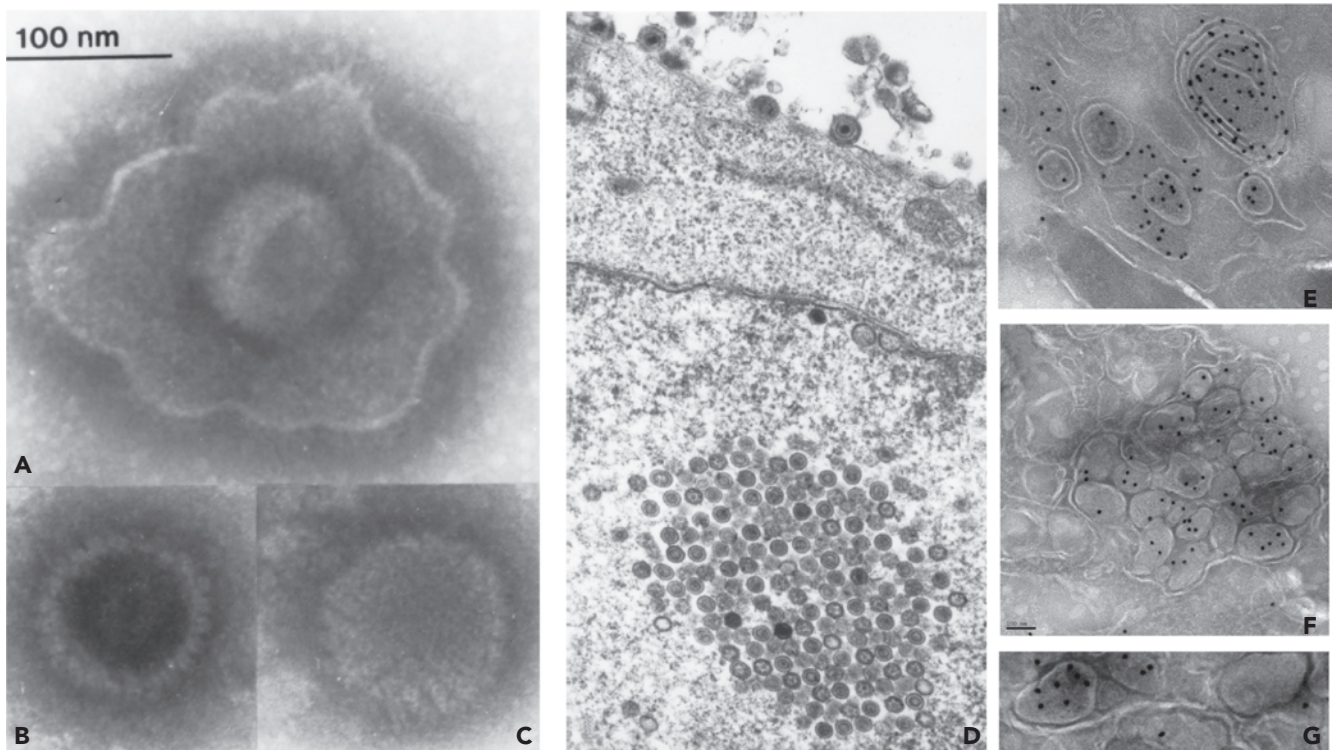


FIGURE 63.2. Electron microscopic appearance of the varicella-zoster virus particle, infected cells and a representative tegument protein. **A:** Enveloped particle. **B:** Purified nucleocapsid. **C:** Negatively stained nucleocapsid preparation. **D:** Infected melanoma cell. **E–G:** Localization of the ORF9 tegument protein in Golgi (**E**), intracellular vacuole (**F**), and extracellular virions (**G**) shown in 100-nm Tokuyasu cryosections with immunogold staining using rabbit anti-ORF9 (**A–D** adapted from Straus SE, Ostrove JM, Inchauspé G, et al. Varicella-zoster virus infections: biology, natural history, treatment, and prevention. *Ann Intern Med* 1988;108:221–237. **E–G** courtesy of M. Reichelt, X. Che and A. Arvin).

VIRION STRUCTURE

Morphology

The VZ virion structure has not been investigated completely, in part because cell-free progeny virions cannot be produced efficiently from cultured cells. Nonetheless, the four major components of VZV particles, like those of the other herpesviruses, are the core, nucleocapsid, tegument, and envelope (Fig. 63.2).

The Core

One copy of the linear, double-stranded DNA (dsDNA) VZV genome is presumed to be incorporated into each virion. Particles fixed with nondehydrating agents and imaged by electron microscopy (EM) reveal a loose fibrillar cage of strands surrounding a dense cylindrical core of DNA fibers.⁵¹² The packaging of the HSV genome in regularly spaced layers within the nucleocapsid may be relevant to VZV structure.

The Nucleocapsid

The VZV nucleocapsid is indistinguishable from those of other herpesviruses. It consists of 162 capsomere proteins with a 5:3:2 axial symmetry, in which pentameric proteins form the vertices of an 80-nm to 120-nm icosahedron, while hexameric elements comprise its facets. VZV nucleocapsids recovered from cell culture often lack dense cores associated with genome

packaging, consistent with the low infectivity of VZV particles produced *in vitro*.²⁴⁵ The VZV gene products that comprise the assembled nucleocapsid have not all been verified, but are expected to include the products of VZV open reading frames (ORFs) 20, 21, 23, 33, 40, and 41, by analogy with their herpesvirus homologs.

The Tegument and the Envelope

The VZV tegument includes three immediate early (IE) proteins encoded by ORFs 4, 62, and 63.^{321,324} In addition, the ORF9 to ORF12 gene cluster, conserved in alphaherpesviruses, encodes three tegument proteins, ORF9 to ORF11, and the putative tegument protein, ORF12.⁷⁶ The viral kinases, encoded by ORF47 and ORF66, are also tegument proteins.⁵⁹⁶ ORFs 22, 38, 53, 57 and 64/69 are putative tegument proteins.⁷¹⁵ Tegument proteins are acquired by the nucleocapsid, which is then enveloped within patches of host cellular membranes that have been modified to display virally encoded glycoproteins. The particle is further modified as it is transported along membranous networks that extend from the nuclear membrane to the cell surface. Virions accumulate at the plasma membrane, transit across this membrane and infect cells in close proximity. Since particles can capture membranous elements of the endoplasmic reticulum (ER), cytoplasmic vesicles, and the cell surface, the VZV envelope does not appear to have a single source.¹¹³ The mature VZV particle, consisting

of the nucleocapsid, tegument, and lipid membrane envelope, is pleomorphic-to-spherical and 180 to 200 nm in diameter.

Physical and Chemical Properties

The VZV lipid-rich envelope renders it susceptible to degradation and loss of infectivity by brief treatments with organic solvents, detergents or proteases, heating to 60°C, prolonged storage at temperatures of –70°C or above, extremes of pH, or ultrasonic disruption.^{69,141,210} In cesium chloride gradients, enveloped VZV particles band at a buoyant density of 1.274 g/cm³ while VZV DNA exhibits a buoyant density in cesium chloride of 1.705 g/cm³.

GENOMIC STRUCTURE AND ORGANIZATION

The dsDNA genomes of the alphaherpesviruses range in size from 124,784 to 164,270 base pairs (bp); VZV and SVV are the smallest at ~125,000 bp.^{91,217} The VZV genome consists of a unique long region (UL) of about 105,000 bp flanked by inverted repeat regions, the terminal repeat long (TRL) and internal repeat long (IRL) sequences, each 88.5 bp, and a unique short region (US) of 5,232 bp that is flanked by internal repeat regions, the terminal repeat short (TRS) and inverted repeat short (IRS), each 7,319.5 bp (Fig. 63.3). Overall, 46% of VZV bases are G + C, whereas the G + C content of its repeat elements is 59% for the TRS and IRS regions and 68% for the TRL and IRL regions. Unlike SVV and other varicelloviruses, VZV lacks a terminal region with flanking inverted repeats at the left end of the genome.²¹⁷

VZ virions usually contain the VZV genome as a linear molecule; a circular, supercoiled conformation is detected as a rare alternate structure.^{327,603} The genome has four potential isoforms but two predominate,^{148,603} with 50% of VZV genomes extracted from nucleocapsids showing the US directed in a single orientation and 95% showing a single orientation of

the UL region.^{117,327} The linear VZV genome has an unpaired cytosine at its left end and a guanine at the right end, which can pair to form a circular molecule.¹¹⁷ In latency, the termini are covalently joined, circularizing the genome.⁸⁸

ORFS/L (or ORF 0), located at the end of the UL segment, encodes a protein essential for replication.^{302,308,714} However, mutagenesis of the extreme 5' terminus of ORFS/L also results in defective DNA cleavage.³⁰² This UL sequence is not duplicated at the opposite end of the UL, which may explain why UL is found in a single orientation in 95% of genomic DNA extracted from nucleocapsids.

The VZV genome encodes at least 70 ORFs, three of which are present in two copies in the IRS and TRS regions (Fig. 63.3; Table 63.1). Mutagenesis studies indicate that about 44 ORFs are essential for replication *in vitro*, with some variations depending on experimental conditions. Twelve of 26 genes dispensable for replication in cell culture are in the genome region from *ORFS/L* to *15*.^{91,715} Gene duplication in the IRS and TRS regions is highly preserved since removal of one copy of the gene pairs *ORF62/71*, *ORF63/70* or *ORF64/69* is corrected after a few replication cycles.^{472,580} Many VZV ORFs are arranged in unidirectional groups of up to four genes with a single putative polyadenylation signal at the 3' end of the group. Eleven VZV ORFs have overlapping coding sequences. *ORFs 42* and *45* are thought to be spliced segments of the same gene.¹¹⁹ *ORFS/L* is spliced in the 3' noncoding region³⁰⁸ in VZV strains including parent OKA, but it is not spliced in vaccine Oka and produces two protein products.³³⁷

The VZV genome has five small regions, R1 through R5, which vary in length and contain different numbers of identically oriented short sequence repeats; VZV has relatively few repeats compared to PRV and HSV-1.¹⁵⁶ These repeat regions result in distinct electrophoretic profiles by restriction enzyme analysis.^{119,604} The R1, R2, and R3 regions are GC rich and located in the UL segment of the genome. The R1 region, located within *ORF11*, consists of four separate repeats of 15 or

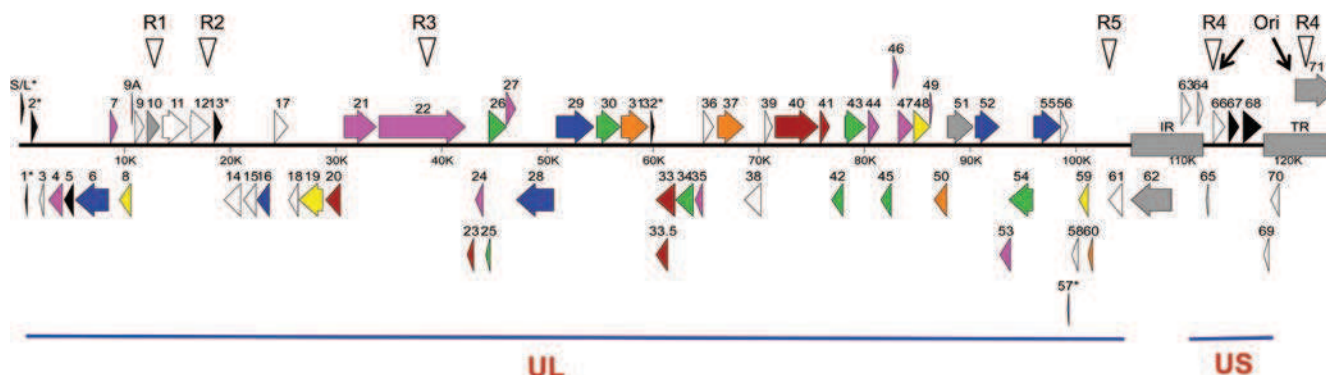


FIGURE 63.3. Organization of the varicella-zoster virus (VZV) genome and known or predicted open reading frames encoding viral proteins. The VZV genome has unique long (UL) and unique short (US) regions flanked by internal repeat (IR) and terminal repeat (TR) regions. VZV has 40 core genes that are conserved among α -, β - and γ -herpesviruses; these gene products participate in DNA replication (blue), DNA cleavage and packaging (green), nucleic acid metabolism (yellow), capsid protein assembly (red), or are envelope glycoproteins (orange) or have other functions (pink). The genes that are not conserved among the other herpesviruses may be nonessential for VZV replication in cultured cells (white), or may be essential (gray), three of which are envelope glycoproteins (black). Six VZV genes have no HSV-1 homolog (*). Open arrows and dark arrows indicate repeats (R1–R5) and the origins of DNA replication (*ori*), respectively. When the genome is circularized, ORF S/L (ORF 0) begins at the right end and extends across the terminal repeat into the UL region at the left end.

TABLE 63.1 VZV Genes and Proteins with Known or Predicted Functions

ORF	Virion location	Protein characteristics and/or functions ^b	Requirement for growth in cell culture ^c	Infection of skin and T-cell xenografts in the SCID mouse model		HSV- 1 homolog
				Skin	T cells	
0 (S/L)		RNA transport	Essential/impaired ^c			None
1		Membrane protein	Dispensable			None
2			Dispensable			None
3			Impaired			UL55
4	Tegument	Transactivator	Essential			UL54 (ICP 27)
5		gK ^b	Essential			UL53
6		Helicase/primase complex ^b	Essential			UL52
7			Dispensable			UL51
8		Deoxyuridine triphosphatase	Impaired			UL50
9	Tegument	Complex with gE	Essential			UL49
9A		gN ^b ; syncytia formation	Essential ^c			UL49A
10	Tegument	Transactivator; efficient virion assembly	Dispensable	Impaired	NL	UL4 (VP16)
11	Tegument	RNA binding	Dispensable	Δ10: Impaired (–) RNA binding: NL		UL47 (VP13–14)
12		Predicted tegument protein	Dispensable	NL		UL46 (VP11/12)
13	Envelope	Thymidylate synthetase	Dispensable			None
14		gC	Impaired			UL44
15		Integral membrane protein	Dispensable			UL43
16		DNA polymerase processivity factor ^b	Essential			UL42
17		RNA cleavage	Essential ^c			UL41 (VHS)
18		Ribonucleotide reductase, small subunit	Impaired			UL40
19		Ribonucleotide reductase, large subunit	Impaired			UL39
20	Capsid ^a	Intercapsomeric triplex component ^b	Essential			UL38 (VP19C)
21	Capsid ^a	Nucleocapsid protein	Essential			UL37
22	Tegument ^a		Essential			UL36
23	Capsid	Small capsid surface protein	Impaired	Required		UL35 (VP26)
24			Essential			UL34
25		DNA packaging ^b	Essential			UL33
26		DNA packaging ^b	Essential			UL32
27		Nuclear matrix protein ^b	Essential			UL31
28		DNA polymerase	Essential			UL30
29		ssDNA binding protein	Essential			UL29 (ICP8)
30		DNA packaging ^b	Essential			UL28
31	Envelope	gB	Essential	(–) furin cleavage: Impaired		UL27
32		ORF47 kinase substrate	Dispensable			None
33		Major capsid scaffold protein; protease ^b	Essential			UL26 (VP24)
33.5		Minor capsid scaffold protein ^b	Essential			UL26.5 (VP22)
34		DNA packaging ^b	Essential			UL25
35		Cell–cell fusion	Impaired	Impaired	Impaired	UL24
36		Deoxypyrimidine (thymidine) kinase	Dispensable			UL23
37	Envelope	gH; three domains DI–DIII	Essential	(–) DI extreme N-terminus: Impaired (–) DIII fusion loop residues: Impaired		UL22

(continued)

TABLE 63.1 VZV Genes and Proteins with Known or Predicted Functions (Continued)

ORF	Virion location	Protein characteristics and/or functions ^b	Requirement for growth in cell culture ^c	Infection of skin and T-cell xenografts in the SCID mouse model		HSV- 1 homolog
				Skin	T cells	
38	Tegument ^a		Essential			UL21
39		Integral membrane protein ^b	Essential			UL20
40	Capsid	Major nucleocapsid protein; hexon/penton component ^b	Essential			UL19 (VP5)
41		Minor capsid protein ^b	Essential			UL18 (VP23)
42/45		DNA packaging ^b	Essential			UL15
43		DNA packaging ^b	Essential			UL17
44	Tegument ^a		Essential			UL16
46	Tegument ^a		Essential			UL14
47	Tegument	Serine/threonine kinase (conserved)	Dispensable (defective virion assembly)	Δ47: No growth (–) kinase: Impaired	No growth No growth	UL13
48		Deoxyribonuclease ^b	Essential			UL12
49			Impaired			UL1
50		gM	Impaired ^c			UL10
51		Origin binding protein	Essential			UL9
52		Helicase/primase complex (presumed)	Essential			UL8
53			Essential			UL7
54			Essential			UL6
55		Helicase/primase complex ^b	Essential			UL5
56			Essential			UL4
57	Tegument ^a	Virion egress ^b	Dispensable			None
58			Dispensable			UL3
59		Uracil-DNA glycosylase	Dispensable			UL2
60	Envelope	gL, chaperone for gH	Essential			UL1
61		Transactivator/repressor; PML dispersal by SUMO binding motifs (SIMs); E3 ligase	Essential ^c	ΔSIM: Impaired Δ250–320 hydrophobic domain: Impaired		ICP0
62/71	Tegument	Transactivator; IFN inhibition	Essential	Single copy: No growth		ICP4
63/70	Tegument	Phosphoprotein	Essential ^c	Single copy: NL (–) S/T residues: Impaired	NL NL	US1.5 (co-linear with ICP22)
64/69			Dispensable (large plaques)	NL	NL	US10
65		Virion protein	Dispensable	NL	NL	US9
66		Serine/threonine kinase (α herpesviruses only)	Dispensable	Δ66: SI. impaired (–) kinase: SI. impaired	Impaired Impaired	US3
67	Envelope	gL; binds gE	Impaired	ΔgL: No growth (–) gE binding: Impaired	No growth	US7
68	Envelope	gE; large unique N-terminus (aa 1–187); binds gL, IDE; natural variant: MSP-gE	Essential	Δ105–125: No growth (–) gL binding: Impaired (–) IDE binding: Impaired Δ51–187: No growth (–) TGN targeting: Impaired MSP gE: Enhanced	T cell entry NL NL No growth Impaired	US8

NL: normal as compared to wild type VZV.

^aIndicates predicted function based on conserved sequence.^bDefined by mutagenesis of the VZV genome (pOka or vaccine Oka) using cosmids or BACs to delete, insert stop codon, or introduce targeted changes in the coding sequence.^cIndicates differences among published observations about whether the gene is essential or dispensable, including variations in mutations tested and/or experimental conditions used to assess growth requirement.

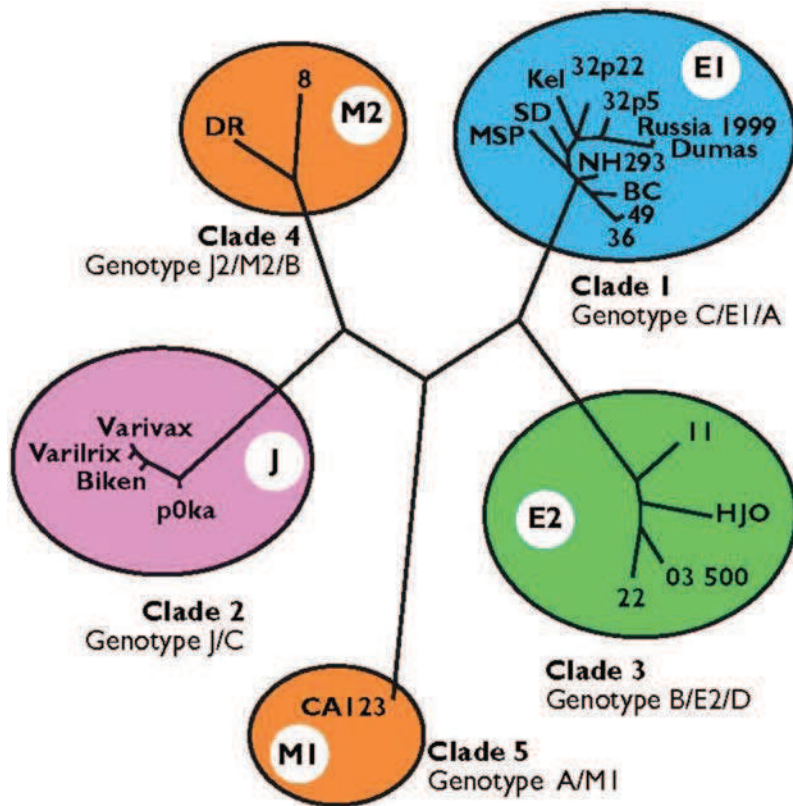


FIGURE 63.4. Phylogenetic tree of varicella-zoster virus (VZV) genogroups and their nomenclatures. Clades 1, 3, and 5 include isolates of European origin; clade 2 has Asian isolates, including the parent Oka virus used to derive VZV vaccines, and clade 4 has isolates from tropical Africa. (Adapted from Breuer J. VZV molecular epidemiology. *Curr Top Microbiol Immunol* 2010;342:15–42).

18 bp. The R2 region is located within *ORF14*, which encodes glycoprotein C (gC), and consists of 42-bp repeats in multiples that differ among VZV isolates.³²⁸ The largest repeat region, R3, is about 1,000 bp in length and located in *ORF22*.¹¹⁹ The GC-rich R4 region is located in both IRS and TRS regions between *ORF62* and *ORF63* (and *ORF70* and *ORF71*), consisting of multiple 27-bp elements.⁶⁸ The A-T rich R5 region, located between *ORF60* and *ORF61*, consists of both 88-bp and 24-bp repeats.¹⁹⁷ The Dumas strain of VZV has only one copy of the R5 repeat, while other strains of VZV have multiple R5 repeats.

Full-length genomic sequencing of more than 30 VZV isolates has revealed that the VZV genome is very stable and exhibits little geographic diversity.^{56,251,451,562} The few consistent differences among geographically distinct VZV isolates allow their classification into five distinct phylogenetic clades (Fig. 63.4). Clades 1 and 2 are the most divergent but are still 99.83% identical. The parent clinical isolate used to derive the vaccine Oka stock virus is clade 2. Comparison of the sequence of the Oka vaccine virus with that of the parent Oka virus revealed 63 sites that differ.¹⁰⁹ The nucleotide sequence could not be unambiguously determined in 27 sites because the Oka vaccine stock is a mixture of genomes with nucleotide polymorphisms.^{211,298a} Of interest, 15 of the 42 nucleotide differences in Oka vaccine are in *ORF62* and account for 8 of the 20 predicted amino acid changes. VZV has been calculated to undergo about 20 replicative cycles in the human host before latency is established, suggesting limited opportunity for the accumulation of genetic mutations.²²³ Mutations in the VZV genome were also minimal after more than 1,200 serial passages in tissue culture.²⁴⁶

Comparison of the VZV genome with those of the seven other human herpesviruses indicates that VZV shares at least 40 conserved genes, all of which are among the 62 genes located in the UL region. These conserved genes encode an IE protein (*ORF4*); the DNA polymerase; helicase-primase complex proteins; other viral enzymes; the *ORF47* kinase and structural proteins, including the major nucleocapsid and related proteins for assembly; and the glycoproteins gB, gH, gL, gM, and gN.

Large portions of the VZV genome are co-linear with the HSV-1 genome, and the predicted amino acid sequences of many VZV and HSV-1 gene products have identical or similar regions (Table 63.1). Of the 62 genes in the VZV UL region, 56 have HSV homologs, as do all four of the genes encoded in the VZV US region.⁹⁰ Nevertheless, while functions may be conserved even with limited sequence similarities,¹⁷² predictions must be confirmed empirically, as illustrated by differences in the roles of VZV *ORF61* and HSV-1 *ICP0*,⁶⁵⁴ and of *ORF10* and HSV-1 *VP16* (UL48).⁷⁷

Six VZV ORFs (*ORFs 1, 2, 13, 32, 57, and S/L*) have no HSV-1 homologs and each is dispensable for VZV replication in cell culture.^{100,103,114,308,338,521,553} *ORFs 1, 32, and 57* have homologs in SVV and the equine herpesviruses.²¹⁷ *ORF13* has no related gene in the other alphaherpesviruses, but has a homolog in a gammaherpesvirus, the human herpesvirus 8.

STAGES OF REPLICATION

Overall Pattern of Replication

As with other herpesviruses, VZV is presumed to attach to cells by fusion of the viral envelope with the cytoplasmic membrane

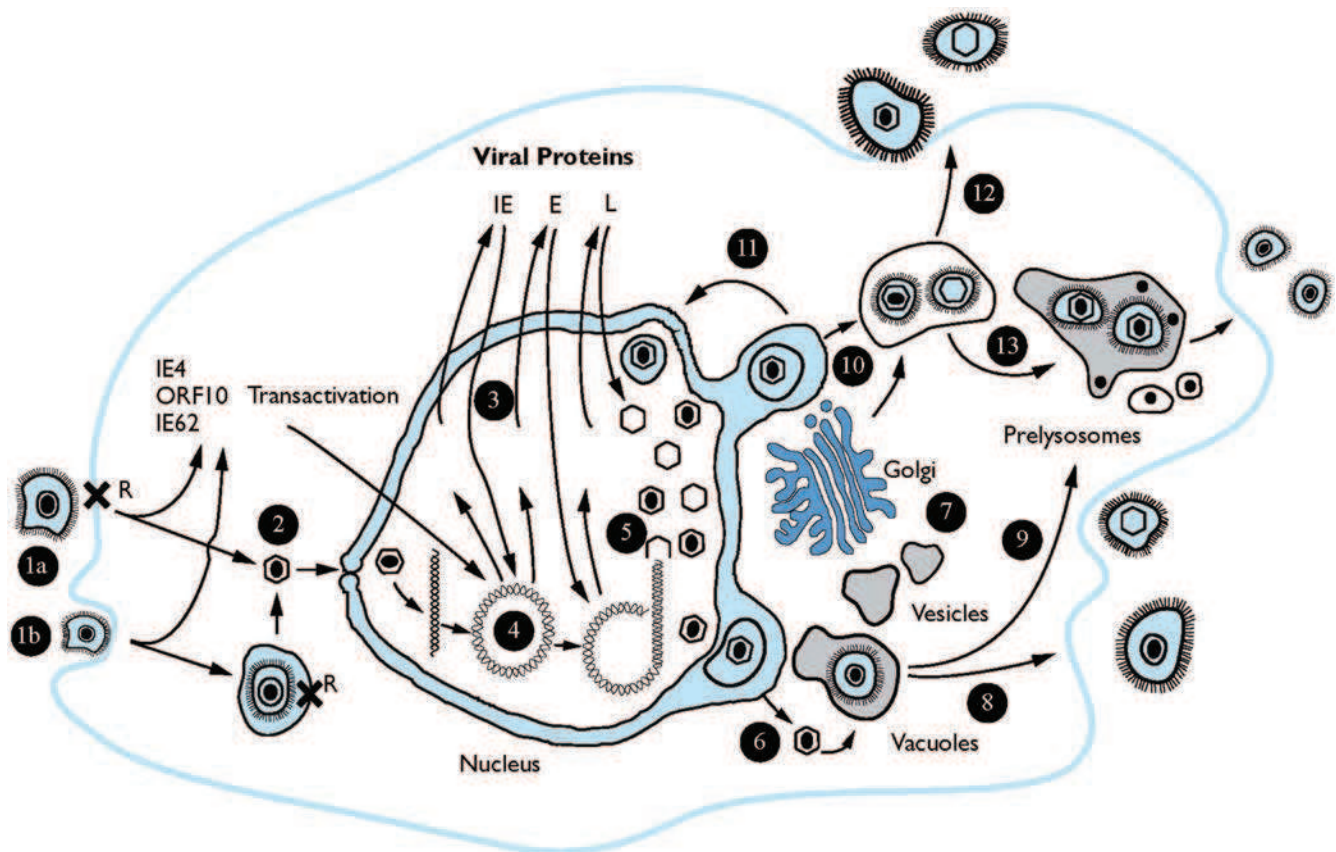


FIGURE 63.5. Replication of varicella-zoster virus (VZV). The schema shown accommodates observations from multiple sources and relies on analogies drawn from studies of herpes simplex virus (HSV) replication. VZV may enter cells by attaching to cell-surface glycosaminoglycans followed by binding to receptor(s) (*R*) on the plasma membrane (1a), or by endocytosis followed by interaction with a receptor(s) in the endosome (1b). The nucleocapsids penetrate the cell (2) and enter the nucleus where, with the aid of regulatory tegument proteins encoded by IE4, ORF10, and IE62, a cascade of viral immediate-early (*IE*), early (*E*), and late (*L*) transcripts and proteins is synthesized (3). Viral DNA replication follows a rolling-circle model (4). Nucleocapsids are assembled and package newly synthesized DNA (5). Nucleocapsids appear to be transiently enveloped as they bud through the inner nuclear lamellae (6). These nucleocapsids lose their envelopes upon fusion with the rough endoplasmic reticulum, resulting in the release of naked nucleocapsids into the cytoplasm (7). Virion glycoproteins are synthesized in the rough endoplasmic reticulum, mature in the Golgi apparatus, and line the inner surfaces of cytoplasmic vesicles. Viral tegument proteins are synthesized by free ribosomes and line the outer surfaces of the cytoplasmic vesicles. Nascent particles are drawn into these vesicles, where intact, enveloped virions coalesce (8).¹⁹³ These vacuoles may fuse with the cell membrane to release infectious progeny (9), although some fuse with prelysosomes in which the particle is partially degraded before release. Alternatively, nucleocapsids may retain their envelopes as they egress the nucleus (10).²⁴⁵ Their envelopes are glycosylated by fusion with Golgi-derived vesicles. Late in infection, some particles may reenter the nucleus (11), although most are transported to the cell surface for release (12) or to prelysosomes (13).

followed by endocytosis (Fig. 63.5). On entry, VZV tegument proteins encoded by *ORFs* 4, 10, and 62^{321,324} are likely transported with the nucleocapsid to the nucleus, where they may initiate viral gene transcription. ORF61 is expressed along with IE62 shortly after virus entry⁵²⁵ but is not a tegument protein. Other tegument proteins, including IE63 and the viral kinases ORF 47 and 66, are also likely to be important early in infection. VZV IE messenger RNAs (mRNAs) are transcribed, transported to the cytoplasm and translated, and the encoded proteins are transported back to the nucleus. VZV early mRNAs may then be transcribed, translated, and shuttled to the nucleus to facilitate viral DNA replication. Replication compartments containing IE62, the single-stranded DNA (ssDNA) binding protein, ORF29, and nascent VZV DNA

are formed within 4 hours after infection.⁵²⁵ Thereafter, VZV late mRNAs are transcribed and translated and the proteins enter the nucleus for assembly into nascent capsids. Newly replicated VZV DNA is packaged into capsids that egress from the nucleus and are enveloped in the cytoplasm. The life cycle from entry to formation of mature enveloped VZ virions is completed in about 9 hours *in vitro*. By 12 hours, enveloped viruses are abundant in the cytoplasm and extracellularly along plasma membranes. The externalized enveloped virus particles are presumed to bind surface receptors, fuse with membranes of adjacent cells and begin synthesis of progeny virions. While syncytia formation is a hallmark of VZV infection, cell–cell fusion is not required to initiate the infectious process; however, the uninfected cell must be in close proximity to an

infected cell, suggesting that virions attached to cell surfaces are transferred into neighboring cells.⁵²⁵

The basic events in VZV replication have been defined in cell culture using intact virus and VZV recombinants with targeted mutations in viral genes and promoter regions to map protein and promoter functions (Table 63.1). However, many important characteristics of the regulation of gene expression and the essential or important functions of viral gene products during the viral life cycle are not identifiable *in vitro*, but are evident when specialized human cells are infected *in vitro* or in their tissue microenvironments in human xenografts in the SCID mouse model *in vivo* (Table 63.1).

Attachment, Entry, and Uncoating

The initial attachment of alphaherpesvirus particles to the cell surface appears to entail nonspecific electrostatic interactions between viral envelope glycoproteins and cellular surface glycosaminoglycans such as heparan sulfate. In HSV, glycoproteins B and C (gB, gC) participate in electrostatic attachment to cells. VZV gB binds to heparan sulfate,²⁸⁸ but it is uncertain whether gC does so. Whereas HSV gD and its homologs in other alphaherpesviruses bind to cell surface receptor molecules known as herpesvirus entry mediators, VZV lacks a gD homolog and may not use any of these receptors. The cation-independent mannose 6-phosphate receptor may facilitate VZV entry by interacting with viral glycoproteins that contain phosphorylated N-linked complex oligosaccharides.^{82,716}

After attachment, studies of other herpesviruses indicate that the highly conserved glycoproteins gB, gH, and gL are required for fusion of the viral envelope with the cell membrane. The VZV gB structure, including the putative fusion loops, is similar to that of HSV-1 gB by homology modeling, and gB residues in the primary loop are essential for VZV replication.⁴⁷³ VZV gH appears to facilitate fusion of the VZV envelope with the cell membrane since antibodies to gH block VZV entry in cultured cells; however, anti-gH antibody is also internalized by infected cells,⁶³⁹ inhibiting syncytia formation and virion egress.⁵³⁴ The gH C-terminus has a loop structure essential for gB/gH/gL-mediated fusion.⁶⁴⁰ The extreme N-terminus of VZV gH is important for replication *in vitro* and in skin and may interact with a cell-surface protein since this region of gH in other herpesviruses interacts with $\alpha\beta$ integrins.⁶⁴⁰ Interactions of gB with the myelin-associated glycoprotein (MAG) contributes to cell–cell fusion mediated by gB and gH/gL in neural cells.⁶⁰⁹ VZV gE is an abundant glycoprotein that forms heterodimers with gI and binds to insulin-degrading enzyme (IDE) in VZV-infected cells.³⁶⁴ Inhibition of IDE reduces VZV infectivity while IDE expression enhances infection of nonhuman cell lines, suggesting a contribution to entry. However, IDE binds to immature gE in the cytoplasm, which is not a receptor–ligand interaction,⁶⁶ and gE mutants with disrupted IDE binding can infect cells in culture and are not impaired in their ability to enter T cells *in vitro* or *in vivo*.⁴²

Once virus entry has occurred, herpesvirus nucleocapsids are transported to the nuclear membrane and the viral genome is delivered into the nucleus. Neither the role of specific viral proteins and cellular cytoskeletal elements in facilitating the centripetal movement of VZ virions to the nucleus nor the composition of the virion reaching the nuclear membrane has been determined.

Transcription and Translation

The transcription and translation of VZV genes in infected cells depends on viral transactivating proteins that act in conjunction with host-cell RNA polymerase II and the cellular transcription and translation machinery. VZV proteins with transactivating capacity are encoded by *ORFs* 4, 10, 61, 62, and 63; IE62 is the predominant viral transcription factor. While VZV gene transcription is likely to follow a coordinated temporal program analogous to that of HSV, the transcriptional cascade has remained undefined because synchronous infection cannot be established with low titers of cell-free VZV recoverable from cultured cells. However, the kinetics of expression of six representative VZV proteins, ORF61, IE62, IE63, ORF29 (ssDNA binding protein), ORF23 (small capsid protein) and gE was established using markers differentiating inoculum from newly infected cells⁵²⁵ (Fig. 63.6). IE62 and ORF61 are synthesized first (within 1 hour); IE63 is expressed at 4 hours. The localization of IE62 to small nuclear punctae is a marker of the earliest stage of replication. Infection in the presence of cycloheximide followed by actinomycin-D indicates that VZV *ORF4*,¹²² as well as *ORF62* and *ORF63*,¹²⁰ encode IE proteins. Although each of the HSV-1 homologs of *ORF* 4, 62, and 63 contains an upstream TAATGARAT sequence element, only the VZV *ORF62* promoter region has TAATGARAT-like promoter elements.^{329,437}

Transcriptional arrays of VZV-infected monkey kidney cells show that *ORFs* 9, 33, 49, 57, 63, and 64 are the most abundant viral transcripts,¹⁰⁷ whereas arrays from virus-infected melanoma cells showed highest expression of *ORFs* 9, 48, 49, 57, 58, and 64.³¹² Transcripts not known to encode proteins are detected from sequences between *ORFs* 60 and 61, 61 and 62, and 62 and 63.¹⁰⁷ VZV transcription mapping revealed 78 abundant transcripts of 6.8 kb or smaller,³⁹⁵ many of which were located in regions corresponding to the *ORFs* predicted by Davison and Scott,¹¹⁹ but most could not be assigned with certainty to a given gene.

As is observed in other herpesviruses, multiple co-linear and co-terminal transcripts can encode a single VZV protein. VZV uses TATA elements and polyadenylation signals whose sequences may differ from consensus sequences.^{119,370} VZV, like HSV-1, contains GU-rich sequences GTCTGTGT and TGGTGGTA downstream of polyadenylation sites that may be important for termination of transcription.³⁷⁰ VZV genes homologous to HSV-1 genes may have different patterns of transcription. For example, *ORF14* (gC)³⁷⁰ and *ORF61*⁵⁹⁵ appear to be expressed from unspliced transcripts, in contrast to their HSV-1 homologs. Different strains of VZV may also vary in the level of transcription of a given gene. For example, VZV strain Scott expresses about 20 times more gC mRNA than does VZV strain Oka.³⁷⁰ Another feature of VZV transcription is the regulation of VZV polymerase (*ORF28*) and the major DNA binding protein (*ORF29*) by a single bidirectional promoter with shared promoter elements. Both this *ORF28–29* bidirectional promoter^{411,412,519,541} and the *ORF4* promoter are activated by the interaction of IE62 with the transcription factor upstream stimulatory factor (USF). A binding site for the nuclear regulatory protein Sp1 modulates the activity of both of these promoters, whereas one TATA box controls expression from the *ORF28* promoter and two other TATA elements regulate expression from the *ORF29* promoter.⁶⁹⁵ Transcription of *ORFs* 62 and 63 is also mediated from a bidirectional promoter.²⁹⁵

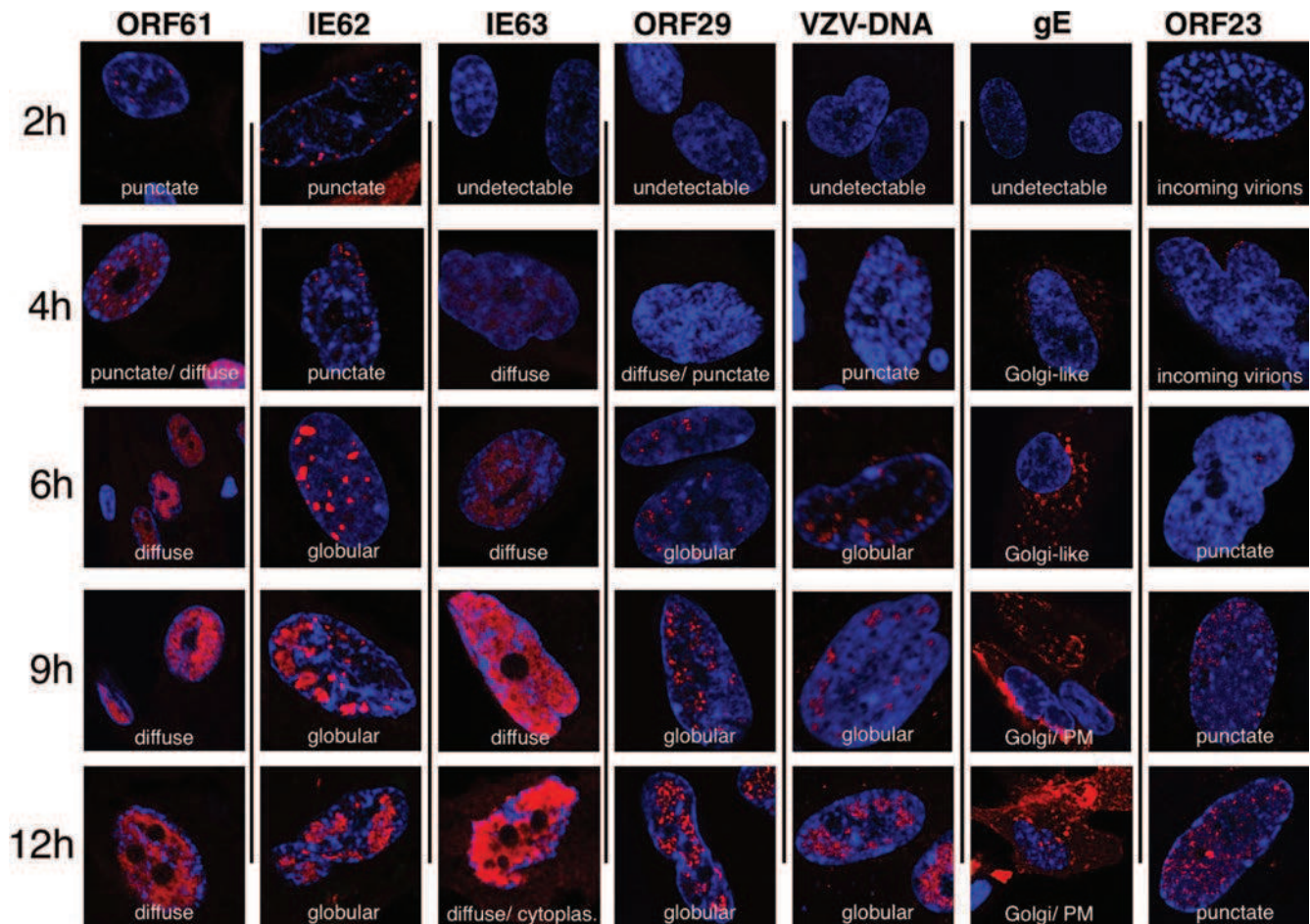


FIGURE 63.6. The spatio-temporal pattern of expression of selected varicella-zoster virus (VZV) immediate-early, early, and late proteins and viral genomic DNA. Cells were fixed and imaged at 0 (not shown), 2, 4, 6, 9, and 12 hours after inoculation of unlabeled human fibroblasts with input cells labeled with green CellTracker. Cells were then stained with specific antibodies for VZV proteins ORF61, IE62, IE63, ORF29, gE, and ORF23 (from left to right) followed by Texas red-conjugated secondary antibodies to detect proteins (red). VZV DNA was detected by *in situ* hybridization (red). Cell nuclei were counterstained with Hoechst 22358. Ten fields of 30 to 50 output cells were scanned to determine the representative staining patterns in newly infected cells at each time point. Single representative cells or nuclei are shown. PM, plasma membrane; cytoplasm, cytoplasm. (From Reichelt M, Brady J, Arvin AM. The replication cycle of varicella-zoster virus: analysis of the kinetics of viral protein expression, genome synthesis, and virion assembly at the single-cell level. *J Virol* 2009;83(8):3904–3918, with permission.)

VZV Proteins that Regulate Viral Gene Transcription

Five VZV proteins, encoded by *ORFs* 4, 10, 61, 62, and 63, can regulate viral gene expression. Most promoters of VZV genes also have binding motifs for cellular transcription factors, such as Sp1, USF and activating transcription factor (ATF), that contribute to viral gene expression. These cellular transactivators can be critical for VZV replication *in vitro* and in human skin and T cell xenografts *in vivo*.⁵⁴¹ All of these proteins except ORF61 are known tegument components.³²¹ ORF61 and IE62 also have important functions in counteracting intrinsic antiviral cellular responses (see Effects on the Host Cell).

IE62

ORF62 (at least one copy) is essential for VZV replication.⁵⁴⁹ This gene encodes IE62, a 1310-amino acid protein,^{167,324} which is the most potent and promiscuous viral transactivat-

ing factor. IE62 has an acidic transcriptional activation domain and a DNA binding domain. IE62 binds directly to the cellular transcription factors Sp1 and USF and to the cellular TATA binding protein and general transcription factor (TFIIB).⁵⁴¹ IE62 binding to the viral proteins IE4 and IE63 modulates its transactivating functions. IE62 has a nuclear localization signal³⁹ and is localized primarily in the nuclei of infected cells, with some cytoplasmic expression at later stages of infection (Fig. 63.6). IE62 is phosphorylated and requires the kinase activity of ORF47 protein for cytoplasmic versus nuclear localization.⁴⁵ Co-expression of the ORF66 kinase with IE62 results in cytoplasmic accumulation of IE62³²³ (although not in T cells),⁵⁶⁰ and ORF66 is required for IE62 incorporation into virions in cultured cells.³²²

A single amino acid change in the DNA binding domain of IE62 results in loss of its transactivating function.⁶²³ IE62 is the homolog of HSV-1 infected cell protein (ICP)4 and can complement ICP4 mutants.^{135,167} However, the DNA binding

domain of IE62 is less sequence specific and, unlike ICP4, does not induce DNA bending on binding its own promoter.⁶²⁴ The transcriptional activation domain (TAD) at the IE62 amino terminus is not conserved in HSV-1 ICP4 or HSV-1 viral protein (VP)16.^{93,496,541} This IE62 TAD interacts with the mammalian Mediator complex involved in RNA polymerase II-mediated transcription,⁶⁹⁶ an interaction that appears to be required for IE62 TAD-mediated activation, specifically through the MED25 subunit (eFig. 63.1).

IE62 transactivates expression of putative IE (*ORF4*, *ORF61*), early, and late gene promoters in transient expression assays^{278,495} and activates its own promoter in a lymphocyte-derived cell line but not in simian cells.^{136,167,497} The differential activation of promoters by IE62 reflects its capacity to interact with cellular transcription factors and depends on the sequence of the TATA motif in the targeted promoter.⁴⁹³ IE62 also increases the infectivity of transfected VZV DNA.⁴⁴⁵

Recruitment of IE62 is enhanced by the presence of an Sp1 site within the gI promoter.⁴⁹² Interaction with Sp1 and possibly other Sp1 family members appears to be an important aspect of IE62 activation. Several other Sp1 sites within VZV promoters have been validated as important for IE62-mediated activation, including the essential proteins gE⁴⁰ and ORF61.⁶⁵³ Bioinformatics analysis of the VZV genome has identified Sp1 sites within almost all predicted promoter elements.⁵⁴¹

The mechanism of activation of the *ORF62* promoter is important because of the central role of IE62 in VZV gene transcription. The upstream region of *ORF62* contains three TAATGARAT-like elements that contribute to the ability of VZV ORF10 protein to transactivate expression of IE62.⁴³⁷ The *ORF62* promoter also contains cAMP-responsive and GA-rich elements that are important for its transactivation. The ORF10 protein forms a complex with two of the TAATGARAT-like elements in the *ORF62* promoter that lack an overlapping octamer-binding motif, but not with the TAATGARAT-like element that possesses an octamer motif. Two cellular proteins, Oct1 and host cell factor (HCF), form a complex with ORF10 and at least one of the TAATGARAT-like elements on the *ORF62* promoter. HCF-1 is critical for activation of immediate-early gene expression by ORF10.⁴⁶¹ The Oct2 cellular protein binds to the TAATGARAT-like elements on the *ORF62* promoter and inhibits ORF10-mediated transactivation. However, ORF10 is dispensable for VZV replication *in vitro* and *in vivo*. Thus, self-regulation of the *ORF62* promoter by IE62 that enters the cell with the infecting virion may be the mechanism that initiates expression of additional IE62 and other IE regulatory proteins.^{541,676} The NF-Y CCAAT box-binding protein and the activating transcription factor/adaptor protein 1 (ATF/AP-1) family also bind to the *ORF62* promoter.³³⁴

ORF61 Protein

ORF61 encodes an essential 467-amino acid protein that is expressed shortly after VZV entry and localizes to the nuclei of infected cells^{595,597} (Fig. 63.6); ORF61 is not detected in the virion tegument. ORF61 protein transactivates putative IE, early and late promoters in transient expression assays,⁴⁴⁴ although not in a T-cell line.⁴⁹⁵ When ORF61 is truncated or mutations of *ORF61* promoter elements restrict its expression, VZV replication is severely compromised.^{653,715} Limiting ORF61 expression by promoter mutations also shows that ORF61 is most important for gE expression. ORF61 dimerization through a domain located at residues 250 to 320 is required for ORF61

regulatory functions and normal replication *in vitro* and in skin xenografts *in vivo*.⁶⁵⁵

ORF61 can either repress or enhance activation of other VZV genes by IE4 and IE62, depending on the cell line and transfection conditions.^{444,460,495,653} The ORF61 N-terminus contains a RING-finger domain that is required for the transactivating activity of the full-length protein.^{163,436} Carboxyl-terminal truncation mutants of ORF61 that retain the RING-finger domain act as dominant-negative transrepressors and reduce the infectivity of viral DNA. Transient expression of ORF61 enhances the infectivity of VZV DNA,⁴⁴⁴ which can be explained by its role in dispersing promyelocytic leukemia (PML) nuclear bodies.⁶⁵⁴ ORF61 is related to HSV ICP0 and can functionally complement an HSV-1 ICP0 deletion mutant.⁴⁴²

IE4

ORF4 encodes the 52-kd essential immediate-early protein IE4.^{95,550} which requires co-expression with *ORF62* for its nuclear localization.¹²¹ IE4 must dimerize for gene activation, and several regions of the protein are required for its transactivation function.^{38,439} Mutation of the KYFKC residues that mediate IE4 dimerization is lethal.⁵⁵⁰ In transient expression assays, IE4 transactivates some, but not all, putative IE, early or late promoters, and also synergizes with IE62 to transactivate promoters of genes from all three kinetic classes. IE4 activation of target genes occurs at both transcriptional⁴⁹⁴ and posttranscriptional levels.¹²² IE4 interacts directly with TFIIB and the nuclear factor- κ B (NF- κ B) p50 and p65 subunits,⁹⁶ and also binds to an underphosphorylated form of IE62.⁵⁸⁵ IE4 may function as a viral mRNA export factor through its RNA binding capacity.⁴⁸¹

IE4 has homology to HSV-1 ICP27 but does not complement an ICP27 mutant.⁴⁴¹ The carboxyl portions of IE4 and ICP27 share the most homology and contain putative zinc-finger domains; however, mutagenesis indicates that several cysteine residues in IE4 are not required for its regulatory activity.⁴⁹⁴ ICP27 cannot complement the growth of an *ORF4* deletion mutant⁹⁵ and, unlike ICP27, IE4 does not appear to have transrepressing activity.^{124,278,441,495}

IE63

ORF63 encodes a 45-kd protein and additional smaller products of 38 and 28 kd.^{120,321,463} IE63 is phosphorylated predominantly in the carboxy-terminal region⁵⁹⁸ by casein kinases I and II, CDK1, and CDK5.^{52,235} Deletion of >90% of both *ORF63* and its duplicate *ORF70* was compatible with very limited VZV replication in melanoma cells; providing IE62 allows an *ORF63* deletion mutant to replicate in U2OS cells, which are also permissive for HSV-1 ICP0 mutants.^{95,580,715} The carboxy-terminal 70 amino acids of IE63 are dispensable, but further deletion of the carboxy-terminus or replacement of some of the serine or threonine phosphorylation sites of the protein with alanines impairs VZV replication^{33,94} and skin infection.³³ IE63 is located primarily in nuclei of infected cells (Fig. 63.6). Phosphorylation of certain amino acids of IE63 is important for its cellular localization when expressed alone, but not when expressed in the context of the whole virus.^{52,94} When expressed alone in neurons, IE63 nuclear import requires ORF61.⁶⁴⁸ IE63 binds to IE62 and the two proteins exhibit some colocalization in infected cells; IE63 also binds to the cellular RNA polymerase II³⁸² and to antisilencing protein 1, interfering with its histone binding capacity.⁸ IE63 is reported to repress expression of VZV promoters in some transient transfection

assays,^{52,285,339,382} but IE62 is the only viral gene product that is significantly reduced in cells infected with an IE63-null mutant.²⁶⁷ IE63 also transactivates the EF-1 α promoter in the absence of other viral proteins.⁷²⁰ IE63 is the homolog of the HSV-1 U_S1.5 protein, which is expressed co-linearly with HSV-1 ICP22 (U_S1).³³

Replication of Viral DNA

Replication Proteins

VZV encodes homologs of each of the seven HSV-1 genes required for origin-of-replication (ori)-dependent replication of viral DNA (Table 63.1); however, the minimal repertoire of genes needed for VZV DNA replication is not known.

DNA Polymerase

ORF28 encodes the large subunit of the VZV DNA polymerase.¹¹⁹ The VZV DNA polymerase has been partially purified from infected cells and requires magnesium for maximal activity. Unlike the cellular polymerase, the VZV enzyme is sensitive to phosphonoacetic acid, although less so than HSV-1 DNA polymerase,³⁰⁵ and its activity is enhanced in the presence of ammonium sulfate.^{399,420} *ORF16* is predicted to encode the small subunit processivity factor of the VZV polymerase.

DNA Binding Proteins

ORF29 encodes a 130-kd protein that localizes to nuclei of infected cells via a noncanonical nuclear localization signal^{91,249,586} (Fig. 63.6). Both *ORF29* and its HSV-1 homolog, ICP8, bind ssDNA, although the VZV protein binds with lower efficiency²⁴⁹ and cannot substitute for ICP8.⁶⁶⁵ *ORF29* binds to the *gI* promoter *in vitro*.⁵³ Furthermore, *ORF29* protein increases or decreases transactivation of a reporter gene by IE62, depending on the cell line.⁵³ *ORF29* protein is secreted from VZV-infected cells and can be endocytosed by neurons.¹⁵ *ORF29* is also part of a complex that binds to a sequence in the Ori_s, which functions as a negative regulator of VZV DNA replication.³¹⁸

ORF51 encodes the ori binding protein.⁶⁰¹ The carboxyl portion of *ORF51* protein binds *in vitro* to three DNA sequence motifs in the VZV ori with similar affinities.⁷⁹ As in the HSV-1 homolog, UL9, a CGC triplet in the VZV ori is required for *ORF51* binding. *ORF51* can substitute for HSV-1 UL9 in an origin-dependent HSV DNA replication assay⁶⁶⁵ and can partially complement growth of an HSV-1 UL9-null mutant.⁸⁰ VZV DNA binds five other proteins of unknown identity or function, ranging from 21 to 175 kd in size.⁵³²

Genome Replication

By analogy with HSV, VZV DNA replication is presumed to occur in four stages.¹¹⁷ First, the linear DNA molecule circularizes shortly after virion entry. This event is facilitated by the complementary unpaired nucleotides at each 3' end of the VZV genome. The presence of a fused UL-US joint, as demonstrated by cloning of VZV genomic DNA, supports this concept. The second stage involves replication of the circular genomic template. Isomerization of progeny genomes is thought to occur by homologous recombination between the inverted repeats. Third, replication of the circular molecule generates head-to-tail concatemers, probably by a rolling-circle mechanism (Fig. 63.3).⁶⁰⁰ Fourth, these concatemers are

cleaved by sequence-specific nucleases to generate linear DNA for packaging into virions.

Both the VZV and HSV-1 genomes contain AT-rich and GC-rich sequences near their leftward and rightward termini, respectively. Since these sequences are implicated in cleavage and packaging of HSV-1 DNA, the equivalent VZV sequences may serve similar roles. Because VZV exists predominantly as two isomers, 95% of cleavage appears to occur at one U_L-U_S junction, but occasionally cleavage at the other UL-US joint yields the two minor isomers.³²⁷

The VZV genome contains duplicate ori_s between ORFs 62 and 63 in the IRS and between ORFs 70 and 71 in the TRS^{119,600} (Fig. 63.3). Transfection of cells with plasmids containing the VZV ori_s followed by VZV infection results in replication of these plasmids. The VZV genome does not have a homolog of the HSV-1 ori_L sequence.

The VZV ori_s contains a near-perfect 45-bp palindrome with the sequence (TA)₁₇ at its center. Mutations of the palindrome impair replicative activity. The VZV ori_s also includes a GA-rich region and three binding sites for the VZV origin-binding protein, ORF51. These sequences are CGTTCGCACTT (also found in both the ori_s and the ori_L of HSV-1)⁶⁰⁰ and CATTCGCACTT. Unlike the HSV ori_s and the ori_L, all three VZV ori-binding protein sites are upstream of the palindrome on the same strand and are oriented in the same direction.⁷⁹ DpnI DNA replication assays indicate that the 45-bp palindrome and the sequence CGTTCGCACTT closest to the palindrome are essential for replication, whereas the sequence CATTCGCACTT contributes additional activity but is not essential.⁶⁰¹

Viral Enzymes

Thymidine Kinase

ORF36 encodes a viral thymidine kinase (TK)⁵⁵⁷ dispensable for VZV replication. Since the VZV enzyme has more deoxycytidine than TK activity, it is more accurately a deoxypyrimidine kinase.⁴⁷¹ The enzyme is a 70-kd homodimer composed of two 35-kd subunits. It is most active *in vitro* at a higher pH and has broader substrate specificity than its cellular homolog.⁸³ VZV TK is more thermostable and more susceptible to inhibition by deoxythymidine triphosphate than is HSV-1 TK.⁴⁷¹ TK phosphorylates and thereby activates acyclovir and its analogs. Most acyclovir-resistant VZV isolates have point mutations in the TK gene near the adenosine triphosphate (ATP) or thymidine-binding sites or have mutations that result in a truncated protein.^{435,556} Differences between the structures of VZV and HSV TK may explain some differences in their substrate activities.⁴⁸

Ribonucleotide Reductase

ORFs 18 and 19 encode proteins homologous to the small and large subunits of HSV-1 ribonucleotide reductase, respectively.¹¹⁹ VZV ribonucleotide reductase has enzymatic activities similar to those of its HSV-1 homolog. Unlike cellular ribonucleotide reductase, the VZV and HSV-1 enzymes are not inhibited by increased intracellular levels of deoxyribonucleotides.⁵⁸³ Inhibition of VZV ribonucleotide reductase with small molecules enhances the antiviral activity of acyclovir, presumably by decreasing the intracellular pool of deoxyribonucleoside triphosphates and increasing the concentration of acyclovir triphosphate. Deletion of *ORF19* from the viral genome abolishes viral ribonucleotide reductase activity, impairs the growth of the virus *in vitro*, and potentiates inhibition of replication by acyclovir.²⁵⁴

ORF47 and ORF66 Protein Kinases

As components of the virion tegument, ORF47 and ORF66 are likely to be important for activation of regulatory proteins in the newly infected cell. *ORF47* encodes a 54-kd serine-threonine protein kinase and is a tegument component. ORF47 phosphorylates both acidic and basic proteins, but with a marked preference for acidic amino acids, using both ATP and guanosine triphosphate (GTP) as phosphate donors; it also phosphorylates casein kinase II.^{316,462} The HSV homolog, UL13, has a similar catalytic domain.⁵⁹⁶ ORF47 protein autophosphorylates and also phosphorylates viral proteins ORF32,⁵²¹ IE62,^{317,463} IE63,³¹⁷ gI,³¹⁶ and gE³¹⁵ but is not required for phosphorylation of gE, IE62, or IE63 in VZV-infected cells.^{255,317} ORF47 binds to IE62 through an N-terminal domain.⁴⁴ The N-terminal DYS motif is required for ORF47 kinase activity which, if disrupted, results in nuclear retention of ORF47 and IE62. Deletion of ORF47 does not alter plaque formation²⁵⁵ but does lead to accelerated gE expression on membranes and cell–cell spread.³¹⁶ However, even though plaques appear normal in the absence of ORF47, the protein and its kinase activity are required for efficient VZV virion assembly and transport to cell surfaces.⁴⁴ ORF47 is important for T cell and dendritic cell infection *in vitro*, is important for skin infection, and essential for T-cell tropism *in vivo*.^{44,271,582}

ORF66 encodes a 393-amino acid phosphoprotein present in the virion tegument and expressed in the nucleus, in puncta surrounding nucleoli, and in cytoplasm of infected cells.^{153,560} ORF66 is a serine-threonine protein kinase that autophosphorylates⁵⁵⁹ and phosphorylates IE62. The ORF66 catalytic domain (residues 93–378) is homologous to the HSV-1 US3 and other members of this protein kinase superfamily.¹⁵² IE62 phosphorylation by ORF66 determines its late accumulation in the cytoplasm of infected cells, where packaging into virions occurs.³²² Mutagenesis of the glycine 102 residue in a highly conserved, glycine-rich ATP-binding motif in this domain blocks ORF66 kinase function.⁵⁶⁰ ORF66 also phosphorylates the cellular nuclear matrix protein, matrin 3, which may modulate RNA processing^{157,158} and targets histone deacetylases to promote transcription.⁶⁴⁷ ORF66 is dispensable for replication *in vitro* and in human skin xenografts in the SCID mouse model; however, the absence of ORF66 or disruption of its kinase activity leads to impaired growth and production of virions in human T cells in xenografts.^{428,559,560} VZV infection of primary corneal fibroblasts also requires ORF66 functions.¹⁵⁹

Other Viral Enzymes

ORF8 encodes a deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase), which hydrolyzes deoxyuridine 5′-triphosphate (dUTP) to deoxyuridine 5′-monophosphate (dUMP) and pyrophosphate.⁵³⁷ *ORF8* is not required for growth *in vitro* nor is *ORF13*, which encodes the viral thymidylate synthetase.¹⁰⁰ *ORF59* encodes the VZV uracil DNA glycosylase that removes uracil residues incorporated into newly synthesized DNA strands.⁵²² This protein is not necessary for replication in cell culture. VZV-infected cells express a DNase activity⁸³ thought to be encoded by ORF48, the homolog of HSV-1 UL12.

ORF33 encodes the precursor of the VZV serine protease, which is cleaved autocatalytically at two sites.^{184,408} The mature 31-kd protease is the amino-terminal cleavage product. The active form of the protease is predicted to be a homodimer. The VZV protease crystal structure is similar to that of the

cytomegalovirus (CMV) protease.⁵¹³ A serine and two histidines comprise the catalytic site of the VZV protease; mutation of either one of these histidines in the protease precursor protein prevents self-cleavage.

ORF17 encodes the homolog of the viral host shut-off protein encoded by HSV-1 UL41. *ORF17* is dispensable for replication, but deletion of this gene yields a temperature-sensitive mutant. ORF17 protein induces RNA cleavage much less effectively than does its HSV-1 homolog,⁵⁵¹ and the host shut-off function in VZV-infected cells is correspondingly less efficient.

The ORF9-12 Gene Cluster

ORF9 encodes an essential protein present in the virion tegument that interacts with IE62 and is phosphorylated by ORF47.^{76,584,621} *ORF9A* encodes the putative 7-kd gN protein located in the membranes of infected cells.⁵³⁷ Although this protein is not required for replication, blocking ORF9A expression impairs syncytia formation.

ORF10 encodes a 50-kd protein that is incorporated into the viral tegument³²⁴ and localizes to the *trans*-Golgi network in infected cells. ORF10 is the homolog of HSV-1 VP16, but unlike its HSV-1 counterpart, it is dispensable for replication.^{77,102} ORF10 complements an HSV VP16 mutant and enhances the infectivity of transfected VZV virion DNA.⁴⁴³ ORF10 transactivates the *ORF62* promoter, but not the *ORF4* or *ORF61* promoter. ORF10 forms a complex with TAATGARAT-like sequences and Oct1 and HCF to transactivate the ORF62 promoter.⁴³⁷ ORF10 is important for replication in human skin, but not in T-cell xenografts in SCID mice.⁷⁷

ORF11 is expressed as a 118-kd polypeptide present in the virion tegument and in the nucleus and cytoplasm of infected cells. ORF11 alone has little effect on VZV gene transcription, but IE4, IE62, IE63, and gE proteins are reduced if ORF11 is deleted from the viral genome.^{75,76} ORF11 functions as an RNA binding protein with the binding region in the first 22 residues of the protein. RNA binding is conserved among the UL47 alphaherpesviruses homologs. ORF11 is critical for VZV infection of human skin xenografts, but its RNA binding function is not required.⁷⁵

ORF12 encodes a predicted tegument protein *ORF12* has effects on all cycle progression by activation of the P13 kinase/Akt pathway.^{371a} It is dispensable for replication *in vitro* and in skin xenografts *in vivo*.⁷⁶

Nucleocapsid Proteins

Three forms of nucleocapsids are found in VZV-infected cells.²⁴³ Immature B capsids lack viral DNA and contain 32- and 36-kd proteins, presumed to be the mature viral protease and assembly proteins, respectively, present in the nuclear matrix of VZV-infected cells.¹⁷⁷ Mature C capsids contain viral DNA but lack the 32- and 36-kd proteins. Intermediate B/C capsids are thought to contain the VZV genome and the 32- and 36-kd proteins.

ORF40

ORF40 encodes the 155-kd major nucleocapsid protein that localizes to the nuclei of infected cells. A monoclonal antibody to this protein cross-reacts with the HSV-1 UL19 homolog.⁶³³

ORF33

ORF33.5, located in the 3' portion of the *ORF33* gene, encodes the 40-kD precursor of the VZV assembly protein. The mature 37-kD assembly protein is released after cleavage from its precursor by the VZV protease.^{408,506} The assembly protein is presumed to provide a scaffold for nucleocapsid assembly. When expressed *in vitro*, ORF33.5 forms long, hollow rods that accumulate predominantly in the nuclei of cells.⁵⁰⁶

ORF23

ORF23 encodes a conserved capsid protein, called VP26 (UL35) in other alphaherpesviruses, which is expressed as a late protein^{74,525} (Fig. 63.6). Unlike VP26, ORF23 localizes to nuclei through an SRSRVV motif in the carboxy-terminus and mediates nuclear import of ORF40, the major capsid protein. ORF40 is also translocated by ORF33.5. ORF23 is not essential for VZV replication, but deletion disrupts capsid assembly *in vitro*. ORF23-mediated nuclear transport of the major capsid protein is necessary in skin xenografts *in vivo*, and the ORF33.5 interaction with ORF40 does not provide a redundant capacity for this function.

ORF21

ORF21 encodes a 115-kD protein located in the nucleocapsid.³⁸³ The protein localizes to both the nucleus and the cytoplasm of infected cells. ORF21 is essential for VZV replication, and its HSV-1 homolog, UL37, cannot complement its absence *in vitro*.⁶⁹⁰

Other Putative Late Proteins

ORF35 encodes a protein that localizes to the nuclei of infected cells. A mutant deleted for this gene is impaired for cell-to-cell spread and for infection of human skin and T-cell xenografts in SCID mice.²⁸¹ Deletion of *ORF64* results in virions that induce extensive cell fusion with large syncytia.⁵⁸⁰ *ORF65* encodes a 16-kD virion protein that localizes to the Golgi apparatus of virus-infected cells⁹⁹; it is dispensable for VZV infection of human T cells and skin xenografts in SCID mice.⁴⁶⁶

Glycoproteins

VZV glycoproteins are major constituents of the viral envelope that are also expressed on intracellular membranes and have critical interactions with other viral and cellular proteins during VZV entry, replication, and cell–cell spread.

Glycoprotein B

gB (formerly gpII), encoded by *ORF31*, is the most highly conserved of the herpesvirus glycoproteins. In addition to its presumed role in attachment and entry, gB expression along with gH/gL is sufficient to induce cell fusion.^{609,640} Unlike HSV gB, VZV gB has a furin recognition motif at residues 491 to 494 and is cleaved into two polypeptides that are bound together by disulfide residues. Thus, under nonreducing conditions, gB migrates in gels as a 140-kD protein, whereas under reducing conditions, gB resolves as two proteins of 60 and 70 kD. Virion morphology, protein localization, plaque size, and replication kinetics are not altered by mutations eliminating gB furin cleavage, but furin cleavage is necessary for normal VZV replication in human skin xenografts *in vivo*.⁴⁷³ gB contains two tyrosine-based motifs in its cytoplasmic domain that are required for endocytosis to the Golgi network^{257,259}; gB asso-

ciates with clathrin during endocytosis. The carboxy-terminal 36 amino acids of gB are required for normal trafficking to the cell surface.²⁵⁸ gB has both N- and O-linked sugars and is sialylated, sulfated, and palmitylated.^{150,221,234,429} An immunoreceptor tyrosine-based inhibition motif has recently been identified in the gB cytoplasmic domain that regulates cell fusion and skin pathogenesis.^{474a}

Glycoprotein C

gC (formerly gpV), encoded by *ORF14*, is dispensable for growth in cell culture. Because the number of R2 repeats in the gC coding sequence differs, gC varies from 80 to 170 kD. The gC endodomain consists of only six amino acids. gC is heavily glycosylated, with about one-third of its molecular weight resulting from N- and O-linked glycosylation.²²² gC transcription and translation is delayed compared to other VZV glycoproteins, with little VZV gC detected until 72 hours after infection. The quantity of gC transcripts and protein produced also varies depending on the VZV isolate.^{326,328,370} In contrast to cultured cells, skin lesions show abundant gC in a pattern consistent with virion envelope expression⁵⁹⁹ (eFig. 63.2). This difference may be related to the limited production of infectious virus particles *in vitro*.

Glycoprotein E

gE (formerly gpI) encoded by *ORF68* is an 85- to 100-kD protein. Unlike its homologs, VZV gE is an essential protein^{421,431} and has a unique N-terminus of 188 residues necessary for VZV replication.⁴² Although gE has been considered to be a late gene product, it is detectable in a Golgi-like cytoplasmic distribution by 4 hours and is expressed extensively on plasma membranes by 9 hours⁵²⁵ (Fig. 63.6). While gE homologs are encoded within the US, *ORF68* extends into the repeat regions. gE contains both N- and O-linked sugars, is heavily sialylated^{221,431} and is modified by myristylation, palmitylation, and sulfation.¹⁵⁰ Its monomeric form is phosphorylated at serine and threonine residues by casein kinase II, whereas the homodimeric form of gE is phosphorylated by a tyrosine kinase.^{475,699} gE binds the Fc receptor of human immunoglobulin.³⁷² Expression of gE in epithelial cells induces formation of tight junctions.⁴²²

After synthesis and maturation in the Golgi apparatus, gE is transported to the cell membrane. The short gE cytoplasmic domain (62 amino acids) has an endocytosis motif, YAGL, a TGN-targeting AYRV tyrosine motif and an acidic amino acid-rich phosphorylation motif.^{221,315,426} The AYRV motif and the acid patch are required for gE transport.^{717,718} Endocytosis to the TGN requires the YAGL motif, which allows recycling of gE through endosomes back to the cell membrane.⁴⁷⁶ Phosphorylation of gE by the ORF47 protein kinase is necessary for endocytosed gE to traffic to the TGN. In cells infected with a mutant unable to express ORF47, gE accumulates on cell membranes.^{315,316} gE endocytosis from the plasma membrane is necessary for incorporation into virions.⁴⁰² The YAGL endocytosis motif is required for virus replication; the AYRV motif is important for VZV replication in human skin and T-cell xenografts in SCID mice.⁴²⁶

Residues in the unique gE N-terminus are important for normal gE expression, secondary envelopment and cell-to-cell spread, and are essential for skin infection.⁴² Residues within amino acids 27 to 90 are necessary for gE binding to insulin-degrading enzyme (IDE), normal cell-to-cell spread

in vitro^{1,7,41,42} and for virulence in skin xenografts, but not for virus entry into T cells.⁴² Nonglycosylated immature gE binds to IDE in the ER.⁶⁶ The unique gE residues 51 to 187 are necessary for VZV infection of skin as well as T cells. VZV-MSP is a naturally occurring mutation in the unique gE N-terminus, changing the amino acid of codon 150 from aspartic acid to asparagine. The mutant shows altered cellular egress and enhanced cell–cell spread *in vitro* and in skin xenografts.⁵⁴⁷

Correct gE trafficking and maturation requires heterodimerization of gE with gI.^{320,372,698} gE/gI heterodimer formation depends on the cysteine-rich region (amino acids 208–236) also present in gE encoded by other alphaherpesviruses. The capacity of gE to bind to gI is a major determinant of VZV virulence in skin xenografts, indicating that cell–cell fusion and polykaryocyte formation depends on this interaction.⁴¹ Disrupting gE/gI heterodimer formation also results in aberrant VZV infection of dorsal root ganglion (DRG) xenografts, causing severe tissue damage despite impaired cell-to-cell spread. In contrast, mutations of the gE AYRV trans-Golgi localization motif and blocking gE/IDE binding did not alter VZV neurovirulence in DRG xenografts.⁷¹¹

Glycoprotein L

gI (formerly gpIV), encoded by *ORF67*, is a 58- to 62-kd glycoprotein that contains N- and O-linked sugars.^{221,431,697} gI heterodimer formation with gE requires residues 105 to 125 and four highly conserved cysteine residues that are also necessary for gI structure and virion incorporation.⁴⁷⁴ gI is not required for VZV replication in cultured human cells but is required in Vero cells. Deleting gI impairs efficient cell-to-cell spread of virus, adsorption to cells, and syncytia formation *in vitro*. gI is essential for infection of human skin and T-cell xenografts in SCID mice.^{97,395,425} Mutations that alter Sp1 or USF binding sites in the gI promoter reduce gI expression and impair infection of human T-cell and skin xenografts in SCID mice, but not in cultured cells.²⁸⁰

The gI cytoplasmic tail is phosphorylated by a cyclin-dependent kinase⁷⁰²; the phosphorylation sites are not required for VZV skin infection. A threonine residue in the gI endo-domain targets gI to the TGN when expressed alone in cultured cells, although gI is more rapidly localized to the TGN in the presence of gE.^{6,657} The carboxy-terminal domain of gI is required for envelopment of VZ virions in the TGN.⁶⁵⁶ While gI is also endocytosed from the cell surface and has a methionine-leucine internalization motif in its cytoplasmic tail, the gE-gI complex undergoes endocytosis more efficiently than does gE or gI alone.^{6,477} VZV gI is required for normal maturation of gE and for efficient distribution of gE to the cell surface.

Glycoprotein H

gH (formerly gpIII), the product of *ORF37*, is a 118-kd glycoprotein that contains N-linked but not O-linked sugars and is sialylated and sulfated.^{150,221,222,306,430} gH forms a heterodimer with gL. gH is secreted into the medium of cultured cells.⁵⁷⁴ gH requires gL for processing in the Golgi network and transport to the cell surface.^{144,171,400} The short cytoplasmic tail of gH contains a tyrosine-based motif (YNKI) that mediates clathrin-dependent endocytosis of gH in infected cells.⁴⁸⁸ Endocytosis of gH results in reduced cell surface expression and decreased cell–cell fusion; gE enhances fusion mediated by gH.⁴⁰¹

Co-expression of gH with either gE or gI allows transport of an immature form of gH to the cell surface, but this form is not fusogenic.¹⁴³ gH forms a complex with gE in virus-infected cells and during incorporation into virions.^{402,366}

The gH ectodomain has three predicted subdomains—DI, DII, and DIII, based on homology with HSV gH—that have specific functions in cell fusion and VZV pathogenesis as demonstrated by site-directed mutagenesis.⁶⁴⁰ The extreme N-terminal residues in DI are important for skin tropism, T-cell entry, and fusion. DII helices and a conserved disulfide bond are essential for gH structure and VZV replication. Disulfide bonds and a bridging strand in DIII are required for the structural stability of this domain and are critical for membrane fusion. The biologic importance of gH DIII function during VZV replication is evident since a naturally occurring compensatory mutation was acquired during VZV replication when the ⁷²⁴CXXC⁷²⁷ motif was mutated; this DIII mutation stabilized the bridging strand and rescued both a replication-deficient virus and a lethal cysteine mutation that disrupted the fusion function of gH.⁶⁴⁰

Glycoprotein L

gL (formerly gpVI), encoded by *ORF60*, is a 20-kd glycoprotein that forms a complex with gH, acting as a chaperone that associates with the immature but not the mature form of gH.¹⁴⁴ gL is thought to be glycosylated with an N-linked oligosaccharide.¹⁴⁴ Whereas gH requires gL for its maturation, gL is fully processed in the absence of gH. When expressed alone or with gH, gL accumulates only in the cytoplasm.¹⁴³ gL contains an ER-targeting sequence and is recycled between the ER and the Golgi network.¹⁴³ Co-expression of gH and gL supports syncytia formation, whereas expression of either protein alone does not.^{222,144}

Glycoprotein K

gK, encoded by *ORF5*, is a 40-kd glycoprotein containing N-linked sugars and is required for VZV replication.⁴²³ Overexpression of gK in a cell line inhibited syncytia formation by wild-type virus. HSV gK cannot complement growth of gK-deleted VZV.

Glycoprotein M and Glycoprotein N

gM, encoded by *ORF50*, is a 435-amino acid membrane glycoprotein. *ORF50* has one full-length transcript that produces gM and three alternatively spliced transcripts that are rarely translated *in vitro*.^{434,694} gM is predicted to contain eight transmembrane domains, an N-glycosylation site, and a cysteine residue in the first ectodomain. gM is 42 to 48 kd in infected cells and virus particles; a 37-kd precursor, not yet modified by N-linked oligosaccharides, is present in infected cells. gM localizes to the Golgi and endosomes, where it may be incorporated into the virion envelope. The functions of gM are not defined, although its presence in virus particles suggests a role in viral entry and virion maturation.⁴³⁴ As observed in other herpesviruses, gM forms a complex with gN through a disulfide bond; the interaction with gN is necessary for gM maturation, transport and virion incorporation. *ORF50* is dispensable, but gM deletion mutants have impaired plaque formation and spread, with vacuoles containing electron-dense material appearing in the cytoplasm.⁶⁹⁴ An *ORF50* deletion mutant did not replicate in melanoma cells.⁷¹⁵

Assembly and Release

Infectious VZV progeny are synthesized and released efficiently from cells infected *in vivo* (see Pathogenesis and Pathology). Fluid aspirated from varicella or zoster vesicles contains large numbers of infectious cell-free virions.^{69,615} In contrast, VZV is highly cell associated in cultured cells.²⁴⁵ Low titers of infectious cell-free virus can be recovered by sonicating VZV-infected cells,²²⁴ but yields are only 1 to 2×10^9 particles per milliliter and the particle-to-infectivity ratio is 40,000:1⁶⁴ compared to 60:1 for HSV-2. Moreover, virions remain attached to cell membrane fragments. VZV virions are usually enclosed in membrane-lined vacuoles within the cytoplasm. Viewed by transmission electron microscopy (TEM), the virions appear to be degraded during transit through the cytoplasm, while analysis by three-dimensional electron microscope (EM) tomography shows that most particles are intact.⁵²⁸

Because VZV replication in cultured cells is expected to differ from that in differentiated cells in the human host, any model of assembly and egress derived from *in vitro* observations will be incomplete. However, the current model is that nucleocapsids transit the inner and outer nuclear membranes for delivery into the cytoplasm in a process that involves envelopment, fusion and de-envelopment.¹⁹³ VZV ORF24 and ORF27 are likely to have functions in nuclear egress similar to those of their respective HSV homologs, UL31 and UL34. In the cytosol, the naked nucleocapsids are enveloped by cisternae of the TGN. The cisternae are decorated with VZV-encoded glycoproteins with their cytoplasmic tails extending into the cytosol. Tegument proteins adhere to the cytosolic faces of TGN membranes (Fig. 63.2). The naked nucleocapsids attach to the tegument and invaginate into the cisternae. In cultured cells, these vesicles are usually diverted to prelysosomes, where they are subjected to degrading enzymes (Fig. 63.5). Many nascent particles enter late endosomes, probably because some of their envelope proteins are glycosylated with mannose-6-phosphate residues.^{82,180} Mannose-6-phosphate side chains render the evolving particles susceptible to binding and uptake by lysosomes bearing the cation-independent mannose 6-phosphate receptor (MPR^{ci}).⁸² Cell lines deficient in MPR^{ci} release cell-free VZV more efficiently than other cultured cells. This latter feature of the model of VZV assembly and release implies that an alternative secretory pathway is operative in some cell types *in vivo* to allow virus egress without lysosomal degradation (Fig. 63.5). Superficial epidermal skin cells, which lack MPR^{ci}, show a pattern of VZV maturation resembling that seen in cells deficient in this receptor.⁸²

Syncytia Formation

In cultured cells, VZV induces foci of rounded, refractile cells in human diploid cells. Cell-cell fusion with formation of large syncytia is a hallmark of VZV replication in cell culture (Fig. 63.7), becoming detectable at about 9 hours after infection of melanoma cells.⁵²⁵ Syncytia formation involving about 75% of the monolayer is observed by 48 hours, and the fused cells detach from the surface at about 60 hours. These multinucleated giant cells have enlarged nuclei, abnormal nucleoli and marginated chromatin. In addition to its importance in cultured cells, syncytia formation is important for VZV pathogenesis in skin, where polykaryocytes are prominent. VZV also induces the fusion of neurons and satellite

cells in DRG.³²⁷ In contrast, VZV does not cause fusion of T cells; instead, spread in T-cell xenografts is associated with the release of infectious cell-free virions.^{427,428} VZV also induces the formation of “viral highways” in which viral particles decorate the surface of infected cells, especially within the syncytia and on filopodia^{65,221,245} (Fig. 63.7), and an increase in cell surface virions is associated with more rapid cell-to-cell spread.⁵⁴⁷

The gB/gH/gL complex, which is presumed to function in fusion of the viral envelope with the cell-surface membrane during entry, is essential for cell fusion and syncytia formation.⁶⁴⁰ Residues in gH domain III are necessary for cell-to-cell fusion and virulence in skin xenografts. Formation of typical VZV syncytia also requires gE/gI heterodimerization and functions that depend on the unique gE N-terminus.^{41,42} Deleting gI (ORF67) or reducing its expression by promoter mutations results in a small-plaque phenotype with distorted polykaryocyte formation.^{97,395} Syncytial formation is also reduced if the products of both ORF8 and ORF9A are not expressed.⁵³⁷ Conversely, typical syncytial formation can be retained *in vitro* even with mutations that severely disrupt VZV virion formation, such as those blocking ORF47 expression or eliminating its kinase activity; the deficiency is evident only by markedly impaired skin infection.^{47,48}

Replication in Specialized Cell Types in Culture

VZV replication and gene requirements have been studied *in vitro* using primary human cells, cell lines, and organ cultures that reflect the tropisms of the virus *in vivo* (Fig. 63.8). Human tonsil T cells are highly permissive for VZV replication,³⁴³ whereas VZV infects T cells at low frequencies in peripheral blood mononuclear cell (PMBC) cultures from adult peripheral blood or cord blood⁵⁸²; infection can be increased by T cell activation.³³⁶ VZV has only limited capacity to replicate in transformed human B cells.³³⁶ VZV replicates in immature but not in mature dendritic cells.^{2,273}

Skin organ cultures are permissive for VZV. Deleting ORF 7, 10, 14, and 47 disrupts VZV infection of skin organ cultures but not of cultured cells, whereas deletions of ORF 0, 18, 19, 23, 32, 35, 49, and 68 affect both.⁷¹⁵ Infected keratinocytes release cell-free virus *in vitro* but infectious virus yields are low, whereas *in vivo* infection yields high titers.¹⁹⁰

VZV infects dissociated cells cultured from nervous system tissues, including both neuronal and nonneuronal cells (Schwann cells and astrocytes).²⁶⁶ VZV also infects neurons and supporting cells in explanted intact ganglia⁵⁸⁹ and neurons derived from human embryonic stem cells.⁴⁰⁴ Infection of human fetal neurons with a clinical VZV isolate resulted in sevenfold more virus replication compared to infection with the Oka vaccine.⁵⁷⁹ Infection of differentiated neurons in culture with cell-free VZV did not produce cytopathic effects or infectious virus, although VZV DNA, virus-specific transcripts and protein, including gE, were found several weeks later.⁵¹⁰

VZV Effects on the Host Cell

Cell Protein Synthesis, Cell Cycle Changes, and Other Effects

Although ORF17 encodes a homolog of the HSV virion host shut-off (VHS) protein, ORF17 is not present in the tegument and has little impact on host protein synthesis.⁵⁵¹

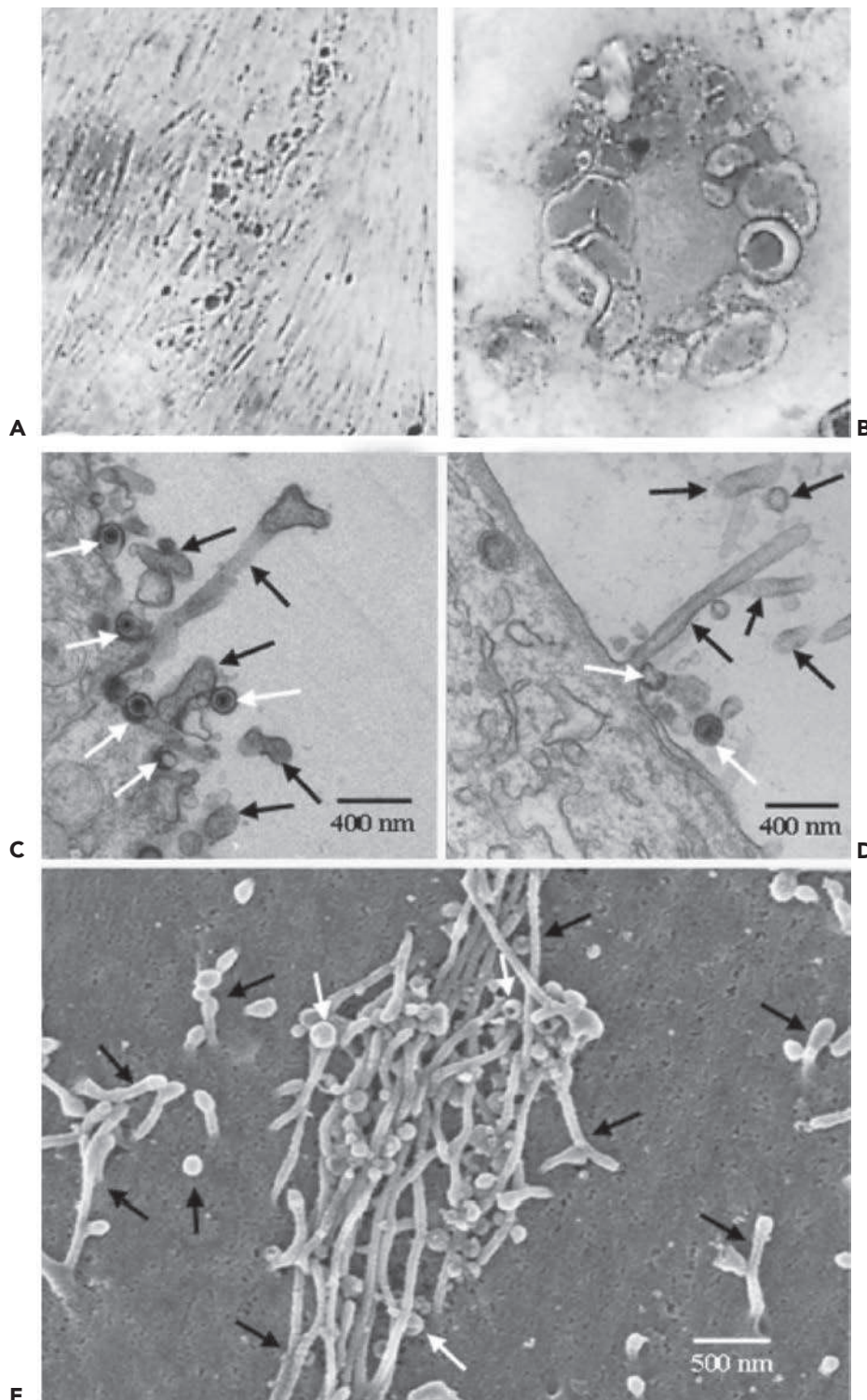


FIGURE 63.7. Varicella-zoster virus (VZV) infection of human cells *in vitro*.

A: An early focus of VZV infection in a monolayer of human fibroblasts stained with hematoxylin and eosin (H&E, $\times 240$) **B:** A multinucleated cell containing intranuclear inclusion bodies from a monolayer of VZV-infected human fibroblasts (H&E, $\times 2,600$). **C–E.** Microstructural composition of “viral highways,” the distinctive linear patterns from which VZV emerges as complete and aberrant viral particles (white arrows) and cellular projections (black arrows) of 70- to 100-nm diameter (by transmission electron microscopy): **C:** five viral particles and several projections; **D:** one elongated projection surrounded by portions of other projections; **E:** viral highway covered by cellular projections with viral particles in between (by scanning electron microscopy). (Adapted from Breuer J. VZV molecular epidemiology. *Curr Top Microbiol Immunol* 2010;342:15–42.)

However, VZV infection dysregulates and arrests the cell cycle in fibroblasts (eFig. 63.3), an effect characterized by high levels of expression of cyclins A, B1, and D3 without extensive synthesis of cellular DNA.^{356,424} Cyclin-dependent kinase (CDK) activity is essential for VZV replication; CDK1 phosphorylates IE62 and is present in virions.³⁵⁵ VZV replication may be associated with activation of the DNA damage response, leading

to cell-cycle arrest. VZV may also activate transcription factors and increase cell-cycle regulatory proteins through the mitogen-activated protein kinase pathways, extracellular-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). p27, p53, and ATM kinase are other examples of host regulatory proteins induced in infected cells. VZV infection also alters the organization of actin in the cell.³⁴⁵

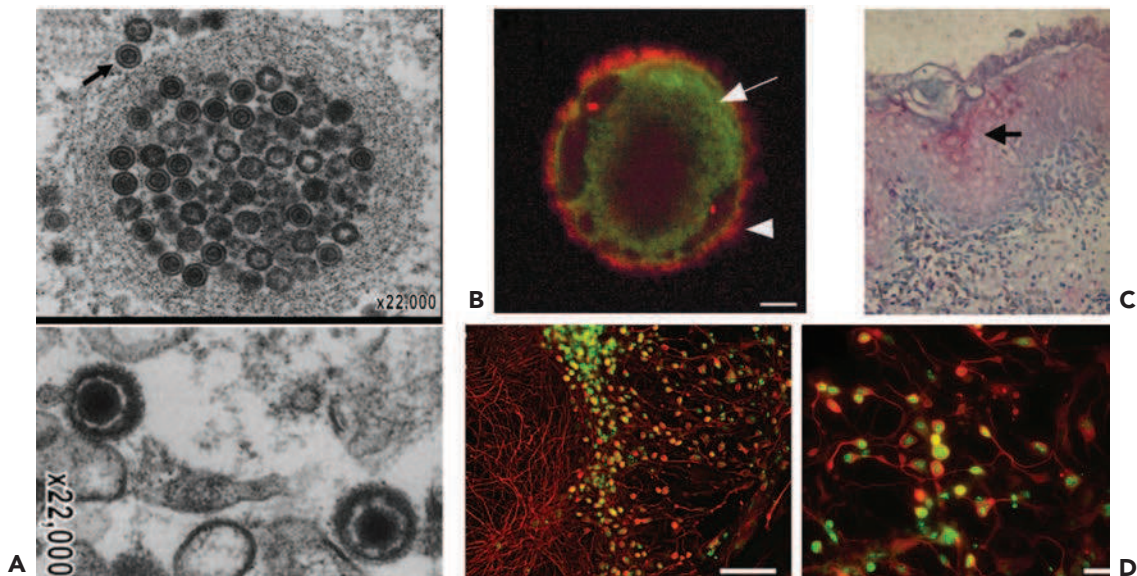


FIGURE 63.8. Varicella-zoster virus (VZV) infection of specialized cell types. **A:** VZ virions in T cells infected with pOkα at 14 days postinoculation, showing intranuclear capsids (*upper*) and complete virions within a large cytoplasmic vesicle (*lower*). **B:** Dendritic cells two days after infection and stained with monoclonal antibody to CD1a (*arrowheads*) and rabbit antibody to IE4 (*arrows*). **C:** Immunohistochemistry of VZV-infected skin organ cultures on day 6 after inoculation, stained with human anti-VZV serum and visualized with Vector Red and hematoxylin counterstain. **D:** Human embryonic stem cell–derived neurons infected with recombinant VZV expressing green fluorescent protein fused to ORF23. Immunostaining of infected neurons with bIII-tubulin (*red*) shows a dense plexus of neuronal processes at the left and many infected neurons (*green, left panel*); higher magnification reveals a large number of infected neurons (*right panel*). (**A** adapted from Schaap A, Fortin J-F, Sommer M, et al. T-cell tropism and the role of ORF66 protein in pathogenesis of varicella-zoster virus infection. *J Virol* 2005;79:12921–12933. **B** adapted from Abendroth A, Morrow G, Cunningham AL, et al. Varicella-zoster virus infection of human dendritic cells and transmission to T cells: implications for virus dissemination in the host. *J Virol* 2001;75:6183–6192. **C** adapted from Taylor S, Kinchington PR, Brooks A, et al. Roscovitin, a cyclin-dependent kinase inhibitor, prevents replication of varicella-zoster virus. *J Virol* 2004;78:2853–2862. **D** adapted from Markus A, Grigoryan S, Sloutskin A, et al. Varicella zoster virus infection of neurons derived from human embryonic stem cells: direct demonstration of axonal infection, transport of VZV and productive neuronal infection. *J Virol* 2011;85(13):6220–6233).

Inflammasome Formation

In response to VZV infection, host cells mount an innate response through pattern recognition and the formation of the nucleotide-binding domain and leucine-rich repeat-containing/pyrin domain–containing 3 (NLRP3) inflammasome. NLRP3 protein recruits the adaptor apoptosis-associated speck-like protein (ASC) and caspase-1, resulting in processing of the proinflammatory cytokine interleukin (IL)-1β by activated caspase-1.⁴⁷⁰ NLRP3 is also upregulated in VZV-infected skin xenografts *in vivo*.⁴⁷⁰

Interference with Intrinsic Antiviral Responses

CELL SIGNALING.

Activation of several cellular pathways that support antiviral responses is targeted for inhibition by VZV. ORF66 interferes with the induction of phosphorylated signal transducer and activator of transcription 1 (STAT1) after exposure to interferon gamma (IFN-γ) in VZV-infected T cells and fibroblasts.⁵⁵⁹ VZV also interferes with nuclear factor (NF)-κB activation in cultured fibroblasts and in epidermal cells in skin xenografts *in vivo* by rapidly sequestering p50 and p65 in the cytoplasm and by inhibiting degradation of the NF-κB inhibitor, IκBα.²⁹³ However, VZV infection of human monocytes induces NF-κB and IL-6 through the toll-like receptor 2 pathway.⁶⁵¹ VZV also

inhibits the expression of STAT 1-α and Janus kinase 2 (JAK2) proteins as well as the transcription of IFN regulatory factor 1 and the major histocompatibility (MHC) class II transactivator, mediating IFN-γ signal transduction.

In contrast to its inhibition of STAT1, VZV induces STAT3 activation in T cells and fibroblasts, as is also observed in cells infected with the oncogenic herpesviruses KSHV and EBV.⁵⁶⁸ STAT3 phosphorylation requires infectious VZV and is a direct rather than a cytokine-mediated effect. STAT3 phosphorylation leads to the induction of the cell protein survivin *in vitro* and in infected skin cells (Fig. 63.9). Inhibition of STAT3 phosphorylation diminishes VZV virulence in skin xenografts and survivin inhibition reduces VZV replication. Since STAT1 and STAT3 activation are inversely activated signaling events, VZV-mediated STAT3 activation may enhance replication indirectly by blocking STAT1-mediated innate responses and directly by promoting survival of infected cells through enhanced survivin expression (Fig. 63.10).

INTERFERON AND INTERFERON-REGULATED RESPONSES.

As is true of other viruses, IFNs significantly inhibit VZV. The induction of the type I IFN pathway is initiated by IFN-β primarily through activation of IFN response factor (IRF)-3, leading to IFN-α production. VZV blocks IFN-β production efficiently and downregulates transcription of IRF3-dependent

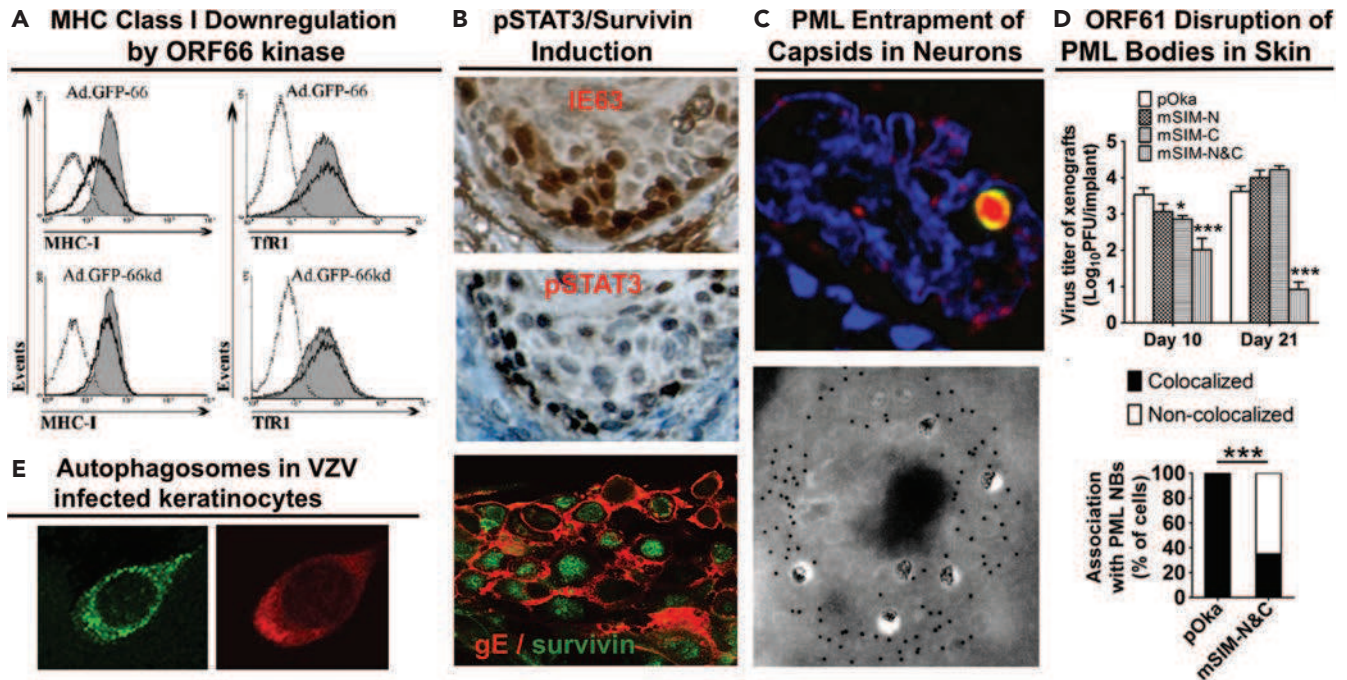


FIGURE 63.9. Effects of varicella-zoster virus (VZV) on the host cell. **A:** Kinase-dependent and specific downregulation of surface MHC-I by ORF66. MRC5 cells were infected with an adenovirus vector expressing ORF66-GFP (Ad.GFP-66) or a kinase mutant (Ad.GFP-66kd) with a tet-off construct (Ad.Tet-Off) with or without doxycycline; at 36 hours, cells were stained with fluorophore-conjugated antibodies to MHC-I and transferrin receptor (TfR1) or isotype controls and analyzed by flow cytometry. **B:** STAT3 activation and induction of survivin in VZV-infected skin sections obtained 21 days after infection and analyzed by immunohistochemistry. *Upper two panels:* Sections were probed with antibodies to VZV IE63 to identify foci of infected cells and pSTAT3; expression was detected with DAB (brown) chromogen and hematoxylin counterstain. *Lower panel:* VZV-infected skin sections were stained with antibodies to gE (red) and survivin (green) and examined by confocal microscopy. **C:** PML entrapment of nucleocapsids in neurons in human DRG xenografts in SCID mice. *Upper panel:* Neural cell with large ring-like PML cage (green) that sequesters ORF23 protein (red) in infected human DRG xenograft. *Lower panel:* a large, ring-like PML cage that sequesters numerous VZV NCs in infected neural cells shown by PML-specific immunogold-labeling of ultrathin (80-nm) cryosection. **D:** ORF61 disruption of PML nuclear bodies in skin. *Upper panel:* Replication of pOka and ORF61 SIM mutant viruses in human skin xenografts in SCID mice; mean titer \pm SEM at 10 and 21 days postinfection. *, $p < 0.05$; ***, $p < 0.001$; vs pOka (two-way ANOVA). *Lower panel:* ORF61/PML NB association in pOka- or pOka-mSIM-N&C-infected cells shown as percentage of cells that did/did not have ORF61 co-localization with PML NBs; ***, $p < 0.0001$. **E.** Autophagosomes in a varicella lesion. A keratinocyte exhibited LC3B puncta characteristic of LC3B-II in autophagosomes in a VZV-infected cell identified by IE62 immunoreactivity. (A adapted from Eisfeld AJ, Yee MB, Erazo A, et al. Downregulation of class I major histocompatibility complex surface expression by varicella-zoster virus involves open reading frame 66 protein kinase-dependent and -independent mechanisms. *J Virol* 2007;81(17):9034–9049. B adapted from Sen N, Che X, Rajamani J, et al. STAT3 Activation and survivin induction by varicella zoster virus promotes viral replication and skin pathogenesis. *PNAS* 2012;109:600–605. C adapted from Reichelt M, Wang L, Sommer M, et al. Entrapment of viral capsids in nuclear PML cages is an intrinsic antiviral host defense against varicella-zoster virus. *PLoS Pathog* 2011;7(2):e1001266. D adapted from Schoeb TR, Eberle R, Black DH, et al. Diagnostic exercise: papulovesicular dermatitis in rhesus macaques (*Macaca mulatta*). *Vet Pathol* 2008;45:592–594. E adapted from Carpenter JE, Jackson W, Benetti L, et al. Autophagosome formation during varicella-zoster virus infection following endoplasmic reticulum stress and the unfolded protein response. *J Virol* 2011;85(18):9414–9424).

genes, as shown by comparing VZV-infected and uninfected cells from the same culture.⁵⁶⁷ IE62 alone prevents TANK-binding kinase (TBK1)-mediated IRF3 function and IFN- β secretion through a novel mechanism in which IRF3 phosphorylation is blocked. This antiviral activity of IE62 is independent of its function as a transactivating protein. IFN- α triggers production of cell proteins that phosphorylate the alpha subunit of eukaryotic initiation factor 2 (eIF-2 α), which blocks translation. VZV can block eIF-2 α phosphorylation through an IE63-dependent process, preventing this IFN- α effect,^{7a} possibly as a redundant mechanism if IE62 interference with

IFN- β is incomplete. ORF47 kinase contributes to inhibition of IRF3 activation by altering its phosphorylation and blocking homodimer formation,⁶³⁴ and ORF61 degrades phosphorylated IRF3⁷¹⁹ in HEK293 cells; these cells do not support productive infection, however.

PROMYELOCYTIC LEUKEMIA PROTEIN NUCLEAR BODIES (PML-NBs).

PML-NBs, also known as ND10 bodies, restrict herpesvirus gene expression in newly infected cells and are upregulated by IFNs. Whereas HSV ICP0 degrades PML protein and

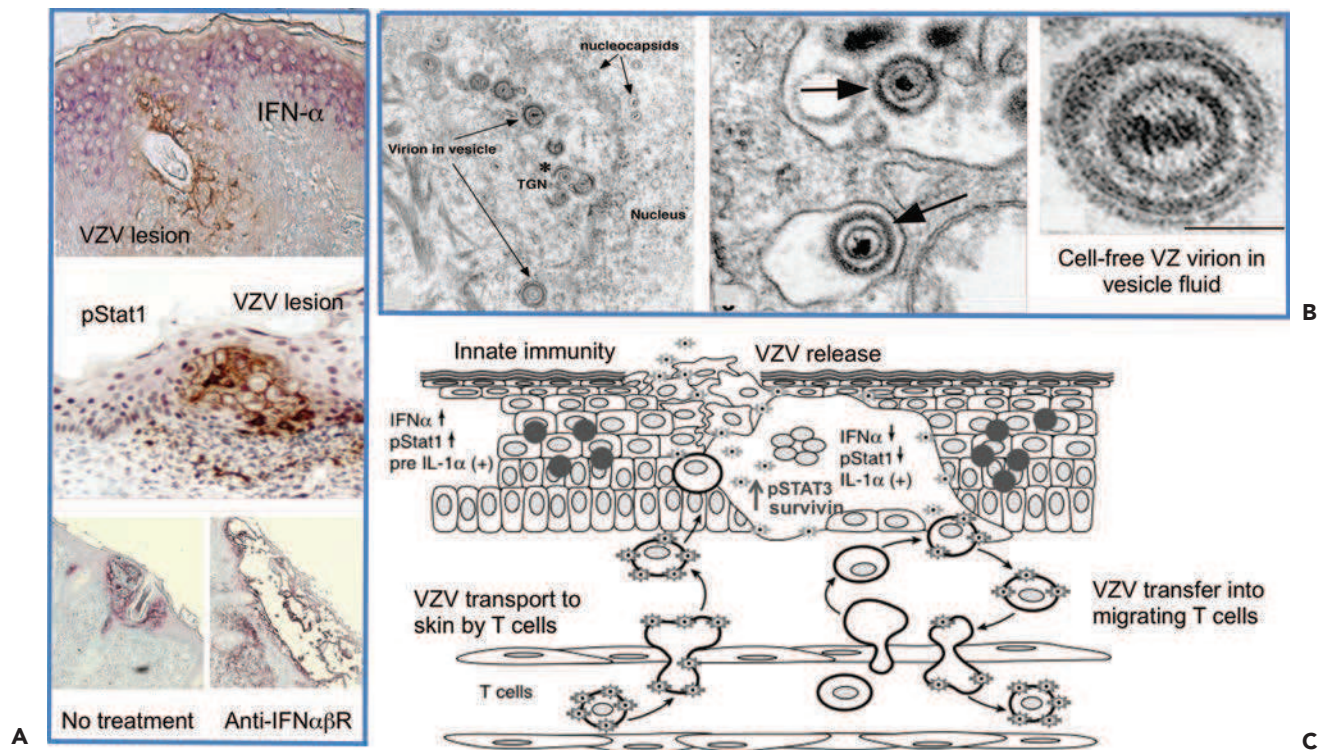


FIGURE 63.10. Varicella-zoster virus (VZV) infection of skin. Formation of epidermal lesions that penetrate the skin surfaces requires 10 to 21 days because of potent innate antiviral responses of epidermal cells. **A:** VZV lesions are formed by cell-to-cell spread overcoming the interferon barrier (IFN- α and pSTAT) in surrounding uninfected cells; interference with the innate IFN response by treatment of SCID mice with antibody against the IFN α/β receptor results in formation of large skin lesions. **B:** Electron micrograph of the suprabasal epidermis of a varicella skin lesion showing all stages of viral envelopment in infected keratinocytes. Unenveloped nucleocapsids are seen in the nucleus while nucleocapsids are being enveloped (*) by specialized cisternae of the TGN/transport vesicles (arrows), each containing a single enveloped virion (left panel). Accumulating cytokeratin filaments are visible in the cytoplasm of the keratinocytes (lower left of center panel). Keratinocytes of the suprabasal layers of the VZV-infected epidermis secrete intact virions to the extracellular space (arrows in right panel indicate two extracellular virions). A virion from a varicella vesicle is illustrated at high magnification and imaged in bright-field; note the spikes on the viral envelope, the complexity of the underlying tegument, and the apparent connections between the nucleocapsid and the tegument (scale bar = 100 nm) (right panel). **C:** Schema of skin infection during primary VZV infection. Activation of innate responses (IFN, pStat1, NF κ B) in uninfected cells is triggered by VZV replication. Within infected cells, VZV activates STAT3 and the antiapoptotic protein survivin. Uninfected T cells that traffic through sites of VZV lesion formation may amplify VZV viremia, and infected dendritic cells may transfer the virus to regional lymph nodes, enhancing T cell-mediated viremia. (A adapted from Ku CC, Zerboni L, Ito H, et al. Varicella-zoster virus transfer to skin by T cells and modulation of viral replication by epidermal cell interferon- α . *J Exp Med* 2004;200:917–925. B adapted from Gershon MD, Gershon AA. VZV infection of keratinocytes: production of cell-free infectious virions in vivo. *Curr Top Microbiol Immunol* 2010;342:173–188. C adapted from Ku CC, Zerboni L, Ito H, et al. Varicella-zoster virus transfer to skin by T cells and modulation of viral replication by epidermal cell interferon- α . *J Exp Med* 2004;200:917–925; and Sen N, Che X, Rajamani J, et al. STAT3 Activation and survivin induction by varicella zoster virus promotes viral replication and skin pathogenesis. *PNAS* 2012;109:600–605).

PML-NBs, VZV-infected cells do not exhibit PML degradation,³⁴⁷ although the architecture of PML-NBs is disrupted in VZV-infected cells *in vitro* and in human skin xenografts through an ORF61-mediated effect. Dispersal of PML-NBs depends on small ubiquitin-like modifier (SUMO)-interacting motifs (SIMs) in ORF61 (Fig. 63.9).⁶⁵⁴ In the absence of SIMs, ORF61 association with PML-NBs is reduced, most PML-NBs remain intact, and VZV skin infection is severely impaired. The IFN response in VZV-infected skin is associated with even higher numbers of PML-NBs in dermal and epidermal cells, and the ORF61 SUMO-binding capacity is necessary to counteract this intrinsic barrier. Furthermore, incomplete dispersal

of PML-NBs by VZV inhibits egress of VZ capsids from the infected-cell nucleus, impairing the synthesis of infectious virus progeny. This antiviral mechanism has biologic significance, since neurons and satellite cells in human DRG and skin cells infected with VZV *in vivo* contain large PML-NBs that sequester newly assembled nucleocapsids (Fig. 63.9).⁵²⁶ This function is mediated by the unique carboxy-terminus of PML IV, which interacts with the ORF23 capsid surface protein to trap capsids and inhibit VZV replication. PML-dependent sequestration of nucleocapsids appears to be a common cytoprotective function of these PML-NBs, in which aberrant proteins are sensed and aggregated in neuronal cell nuclei.

Modulation of Surface Expression of Cellular Proteins Involved in the Host Response

VZV downregulates expression of MHC class I proteins on the surface of infected fibroblasts and T cells, with these proteins retained in the Golgi compartment in virus-infected cells,¹ although the total amount and rate of synthesis of class I molecules is unchanged in VZV-infected cells. Most of this effect is mediated by ORF66 (Fig. 63.9).^{1,157} VZV also inhibits IFN- γ induction of MHC class II molecules on the infected cell surface,⁴ preventing recognition of infected cells by VZV-specific CD4 or CD8 T cells. Infection of mature dendritic cells with VZV results in downregulation of surface MHC class I, CD80, CD83, and CD86, and inhibits their ability to stimulate proliferation of allogeneic T cells.⁴⁴⁶ VZV also downregulates Fas expression on the surface of dendritic cells.²

Regulation of Apoptosis and Autophagy

VZV induces apoptosis with chromatin condensation and DNA fragmentation in Vero cells, B cells, T cells, and human fibroblasts, but not in primary human DRG neurons.⁵⁴⁴ Conversely, expression of IE63 in sensory neurons inhibits apoptosis induced by withdrawal of nerve growth factor.²⁶⁶ Caspase-8 transcripts are reduced, whereas two IRF gene transcripts (ADAR, MxA) are increased in VZV-infected T cells. In contrast, caspase-8 transcripts are not reduced in virus-infected fibroblasts or skin cells.²⁹⁴ VZV ORF66 expression inhibits apoptosis induced by VZV in T cells⁵⁵⁹ and is required for efficient growth in corneal epithelial cells, although interference with apoptosis is not the only ORF66 function required in these cells.¹⁵⁹ STAT3 activation by VZV results in upregulation of survivin, exerting a proviral effect by inhibiting apoptosis.⁵⁶⁸

Whereas HSV-1 ICP34.5 inhibits autophagy, VZV lacks an ICP34.5 homolog. VZV infection induces autophagy-associated proteins and formation of autophagosomes in cultured cells and in keratinocytes that express IE62 within varicella and zoster skin lesions (Fig. 63.9).^{66a,612} Autophagy appears to be preceded by ER stress and the unfolded protein response triggered by excessive glycoprotein synthesis, such as the abundant synthesis of gE in VZV-infected cells. These processes are associated with the inhibition of apoptosis.

PATHOGENESIS AND PATHOLOGY

In the usual course of VZV infection, the virus exhibits tropism for T cells, cutaneous epithelial cells, and cells of sensory nerve ganglia (Fig. 63.1). VZV can also infect other tissues when cell-associated viremia and viral replication are not controlled by intrinsic or adaptive host immune responses. Under these circumstances, disseminated infection may involve the lungs, liver, central nervous system (CNS), and many other organs.⁴⁵² Pathologic changes in these tissues may be caused by replicating virus and, in some instances, by inflammatory or immune-mediated injury. VZV and simian varicella virus (SVV) pathogenesis have similarities consistent with the genetic relatedness of these two primate *Varicelloviruses*.^{148,215,216,217,275,314,375,384,285,386,387,388,389} The clinical manifestations of varicella and zoster that are associated with events in VZV pathogenesis and the pathologic changes associated with VZV syndromes are described in the Clinical Features section.

Entry into the Host

Primary VZV infection (varicella) is presumed to be initiated by inhalation of respiratory droplets containing virus particles or by inoculation of mucosal epithelium with infectious vesicular fluid from an individual with varicella or zoster. The potential for aerosol transmission is suggested by epidemiologic reports, and VZV DNA is detected in air samples from rooms occupied by patients with varicella or disseminated zoster.⁵⁵⁵

Site of Primary Replication and Viral Spread

Initial VZV replication is likely to occur in respiratory mucosal epithelial cells (Fig. 63.1). Following an incubation period of 10 to 21 days, primary VZV infection results in varicella. Although T-cell tropism appears to be a necessary component of primary VZV infection, the site and mechanisms whereby T cells become infected cannot be defined in the human host. Tonsillar T cells—especially those that are activated and express skin homing markers, predominantly memory CD4 T cells—are most permissive for VZV infection, suggesting that spread of VZV from infected mucosal epithelial cells to these T cells initiates cell-associated viremia.³⁴³ Respiratory mucosal epithelial cells line tonsil crypts, penetrating deep into tonsillar tissues that contain many T cells, and migrating T cells traffic across this epithelial cell layer into tonsils. Dendritic cells are also present in mucosal epithelium and may transfer the virus to T cells in regional lymph nodes.³ Viremia may be amplified during infection as T cells traffic through infected skin and visceral organs, such as the liver. VZV is detected in PBMCs from individuals just before or after the onset of the rash.^{28,483,558,642} Infected cells are present at low frequency (0.01% to 0.001% of PBMC)³³⁶ and are usually cleared within 24 to 72 hours after the appearance of the rash. SVV also causes a robust cell-associated viremia that precedes the cutaneous exanthem,^{90,215,416,683} whereas VZV produces a limited viremic phase in guinea pigs.^{378,453}

VZV proteins that are required for or contribute to T-cell tropism *in vivo* as identified in T cell-containing xenografts in the SCID mouse model include gI, ORFs 66, 35, and 47 as well as residues 51 to 187 in the unique gE N-terminus, the residues required for gE binding to gI and, to a lesser extent, the gE AYRV TGN targeting motif (Table 63.1). VZV T cell-associated viremia is likely to be facilitated by downregulation of MHC class I protein, inhibition of apoptosis by ORF66, and by STAT3 activation with survivin induction.

Experiments in the SCID mouse model indicate that VZV is transported by infected human T cells to skin xenografts soon after they enter the circulation. However, the spread of VZV in skin is countered by a potent innate immune barrier resulting from constitutive IFN expression and upregulation of the cellular transactivators STAT1 and NF- κ B in epidermal cells, along with the high frequency of PML nuclear bodies in basement membrane cells of the dermis.^{293,342,344,654} (Fig. 63.10). Blocking IFN responses accelerates VZV infection in skin dramatically. The long interval between VZV exposure and the appearance of varicella skin lesions appears to reflect the time required for VZV to overcome these robust intrinsic obstacles.

Skin Tropism

During varicella, skin cells become major sites of virus replication (Fig. 63.10). The virus may gain access to cutaneous

epithelial cells by migration of intact, infected T cells out of the capillaries into cutaneous tissue, followed by release of virions. Alternatively, infectious virus may move from T cells into capillary endothelial cells. Inclusions and VZV proteins have been observed in capillary endothelial cells and adjacent epithelial cells in VZV lesions.⁴⁶⁷ Cells lining the lymphatics of the superficial dermis become dilated and contain intranuclear inclusions, suggesting VZV infection. Cutaneous dendritic cells and Langerhans cells are susceptible to VZV and may contribute to local replication and spread.³

Classic studies of varicella lesions by Tyzzer in 1906⁶²⁸ showed that the earliest changes consist of vasculitis involving the endothelium of small blood vessels, whereas the emergence of enlarged, multinucleated epithelial cells with intranuclear eosinophilic inclusions is typical of the second, maculopapular stage of the lesions. Progressive degeneration of epithelial cells, coalescence of fluid-filled vacuoles between cells, and destruction of the basement membrane occur as maculopapular lesions evolve into vesicles (Fig. 63.1).

VZ virions are abundant in keratinocytes and in vesicular fluid (Fig. 63.10). In skin biopsy specimens, early lesions show IE63 in keratinocytes; later, gE, gB, and IE63 are detected in keratinocytes, sebocytes, Langerhans cells, dermal dendrocytes, monocyte-macrophages and endothelial cells. Viral proteins are also expressed in dermal nerves and in perineural type I dendrocytes.¹⁵ Infiltration of the involved skin sites by inflammatory cells is minimal in the early vesicular phase, whereas later, upregulation of adhesion molecules on capillary endothelial cells is associated with extensive mononuclear cell migration into the skin lesions.²⁶³ In the final phase, cutaneous lesions progress to ulceration and necrosis.

VZV proteins that are required for or contribute to skin tropism, as assessed in skin xenografts in the SCID mouse model, include ORFs 10, 11, 31, 35, 47, 61, and 66; gI and particular residues in gI; and specific motifs within gE (Table 63.1). The passage of the Oka virus in tissue culture alters its capacity to infect skin, and analysis using vaccine/parental Oka chimeric viruses indicates that multiple VZV genes are involved in skin tropism.⁷⁰⁶

Like VZV, SVV produces a cutaneous rash within 10 to 12 days after close contact with infected animals or following intratracheal inoculation.^{140,384,386,416}

Release from the Host

Many cell-free VZV virions are released into vesicular fluid of skin lesions, which facilitates transmission by direct contact. VZV differs from other human herpesviruses in that infectious virus can be released into respiratory secretions and spread to susceptible persons by airborne transmission.²³⁵ Viral DNA can be detected by PCR in specimens obtained from respiratory sites in the late stage of the incubation period and during the first few days of rash.^{27,558} VZV DNA can also be detected in saliva during and after zoster, but it is not known whether infectious particles are present.^{409,459}

Virulence

Naturally circulating strains of VZV do not exhibit intrinsic differences in virulence, as judged by the clinical consequences of primary VZV infection. Nonetheless, circulating strains of VZV do differ in their genome sequences, allowing assignment to clades (Fig. 63.4). The enhanced growth of the naturally

occurring VZV-MSP variant in skin xenografts in SCID mice suggests that a single amino acid change in gE can alter pathogenesis.⁵⁴⁷ VZV TK-negative viruses have been isolated from patients given prolonged acyclovir therapy, but altered pathogenicity has not been described.⁶⁷⁷ VZV isolates from three patients with encephalitis did not have distinctive genetic characteristics.⁵⁶ A study of Oka vaccine viruses isolated from skin revealed the same DNA sequence (a clonal isolate), whereas virus isolated from lungs contained mixed DNA sequences but with no identifiable changes related to tropism.⁵¹⁵ In addition, viruses isolated from skin lesions after vaccination have been found to have *ORF62* genes that encode amino acids characteristic of the parent strain rather than the substitutions identified in the vaccine, suggesting that a natural selection for these residues occurs during replication in the human host.^{513a} As demonstrated in the SCID mouse model, many VZV genes and promoter regulatory elements contribute to VZV cell tropism and virulence (Table 63.1).

Latency

VZV gains access to sensory ganglia, either hematogenously or by centripetal neural transport from mucocutaneous lesions during primary infection, where it establishes latency.^{155,593} The importance of cell-associated viremia in the delivery of the varicelloviruses to sensory ganglia is supported by clinical reports of VZV detection at these sites before rash has appeared and in nonhuman primates infected with SVV.^{387,388,389} Alphaherpesvirus latency is characterized by the presence of viral DNA in ganglionic neurons (Fig. 63.11), limited viral gene transcription, and the potential for reactivation. VZV becomes latent in neurons in cranial nerve, dorsal root, and autonomic ganglia along the human neuraxis.^{179,203,204,276,311,348,392,393,617,652} Based on autopsy studies, the prevalence of VZV latency in the population varies from 63% to 100%^{111,274,367,392,502}; in the largest study, VZV DNA was found in 94% of 414 trigeminal ganglia.²⁷⁹ Use of precise laser capture microdissection and PCR revealed VZV DNA in 4.1% of neurons and in less than 0.1% of satellite cells.⁶⁵²

Latent VZV DNA assumes a circular or concatameric form⁸⁸ and is present at a frequency of 2 to 9 genome copies in 1% to 7% of neurons, which corresponds to a virus burden of 30 to 3,500 copies per 100 ng of ganglionic DNA.^{111,267,300,311,348,360,502,652} The wide range of genome copy number in latently infected ganglia may reflect the extent of primary VZV infection, since both the extent of cell-associated viremia and the number of cutaneous lesions is highly variable.^{126,394,536} Episodes of VZV reactivation or possibly reinfection may also increase the virus burden in latently infected ganglia.

VZV gene expression is highly restricted during latency. *In situ* hybridization detected transcripts mapping to *ORFs* 4, 18, 21, 29, 40, 62, and 63 but not to *ORFs* 28 or 61 in human trigeminal ganglia.³⁰⁹ Transcripts mapping to *ORFs* 21, 29, 62, 63, and 66, but not *ORFs* 4, 10, 40, 51, or 61, were detected in latently infected trigeminal ganglia by PCR amplification of cDNA libraries^{104,112} and confirmed by sequence analysis of the 3'-polyadenylated termini.^{104,105,106,111,112,410} Most recently, multiplex PCR technology (GeXP) was applied to mRNA extracted from 26 latently infected trigeminal ganglia and one control trigeminal ganglion negative for VZV DNA from 14 individuals, 16 to 84 years of age, within 24 hours after death; the

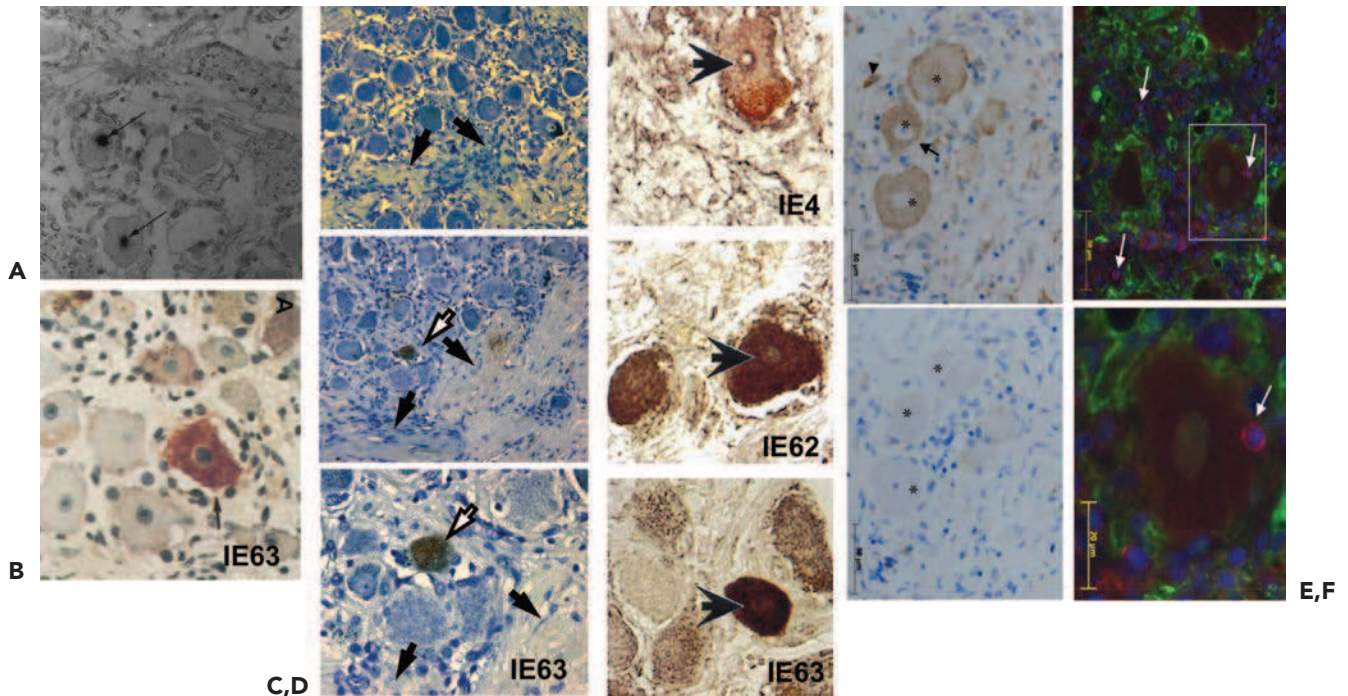


FIGURE 63.11. Varicella-zoster virus infection of human sensory ganglia. **A:** Detection of VZV DNA by *in situ* hybridization in the nucleus of a neuron in sensory ganglion section obtained postmortem from a patient without signs of zoster. **B:** Immunohistochemical detection of IE63 protein in neuron cytoplasm (arrow) in a thoracic ganglion section using rabbit anti-VZV ORF63. **C:** Immunohistochemical detection of IE63 protein in dorsal root ganglion sequential sections using rabbit preimmune IgG antibody (upper panel) and matched high-titer rabbit anti-IE63 IgG at magnifications $\times 100$ (center panel) and $\times 200$ (lower panel); white arrows indicate DAB-positive cells; black arrows indicate fibrous bands as a reference point. **D:** Immunohistochemical detection of IE4, IE62, and IE63 in sensory ganglion sections from a patient with zoster at time of death using purified anti-VZV protein rabbit antibodies and AP-conjugated goat anti-rabbit Ig secondary antibody. **E:** Immunohistochemical detection of CXCL10 in dorsal root ganglia from a patient with zoster rash at time of death. The ganglion innervating the site of the rash contained CXCL10-positive neurons (arrow) and infiltrating cells (arrowhead, upper panel). Lower panel shows isotype control staining of the consecutive section. **F:** Immunofluorescence detection of CXCR3 (red) and S100b (green) and counterstained with DAPI (4',6-diamidino-2-phenylindole) (blue) in the same ganglia as in **E**. Nonneuronal CXCR3-positive cells were observed throughout the reactivated lumbar DRG (arrows), with some CXCR3-positive cells juxtaposed to neurons (boxed region, upper panel); higher-power magnification of the inset box (lower panel). (**A** adapted from Mahalingam R, Kennedy PGE, Gilden D. The problems of latent varicella zoster virus in human ganglia: precise cell location and viral content. *J Neurovirol* 1999;5:445–448. **C** adapted from Zerboni L, Sobel RA, Ramachandran V, et al. Expression of varicella-zoster virus immediate-early regulatory protein IE63 in neurons of latently infected human sensory ganglia. *J Virol* 2010;84(7):3421–3430. **D** from Lungu O, Panagiotidis CA, Annunziato PW, et al. Aberrant intracellular localization of varicella-zoster virus regulatory proteins during latency. *Proc Natl Acad Sci U S A* 1998;95:7080–7085, with permission. **F** adapted from Steain M, Gowrishankar K, Rodriguez M, et al. Upregulation of CXCL10 in human dorsal root ganglia during experimental and natural varicella-zoster virus infection. *J Virol* 2011;85(1):626–631).

presence of *ORF 4*, *29*, *40*, *62*, and *63* transcripts was confirmed and *ORF 11*, *41*, *43*, *57*, and *68* transcripts were detected.⁴⁵⁵ The 10 VZV ORFs transcribed during latency represented putative IE, early, and late genes. The detection of transcripts corresponding to all stages of the VZV life cycle contrasts with findings in HSV latency, in which only a single HSV latency-associated transcript (LAT) has been identified thus far. Further information on the extent of HSV gene transcription awaits the use of GeXP to study ganglia latently infected with HSV.

By quantitative analysis, *ORF63* is the most abundant and prevalent VZV transcript.^{111,455} *ORF63* transcripts in individual ganglia vary more than 2,000-fold, ranging from 1 to 2,785 copies per 10,000 copies of GAPdH transcript,¹¹¹ and *ORF63* transcripts were detected in 17 of 28 ganglia, with as many as

29,000 copies per 1 μ g of input mRNA. The consistent detection of *ORF63* transcripts in high abundance is not understood.

Deep sequencing of RNA extracted from human trigeminal ganglia positive for VZV and HSV-1 DNA revealed microRNAs (miRNAs), the small noncoding RNA molecules that can alter transcript stability and translation, mapping to the HSV-1 genome but not the VZV genome.⁶³⁰ Since HSV-1 miRNAs map to the LAT locus and VZV has no similar region, the lack of VZV miRNA may reflect a basic difference in the mechanism by which these neurotropic alphaherpesviruses maintain latency.

Chromatin immunoprecipitation (ChIP) assays followed by PCR have been used to elucidate the epigenetic state of VZV genomes.¹⁸⁸ Like HSV-1 DNA, histones are associated with VZV DNA at all stages of the viral life cycle, but the histone

composition differs during productive infection and latency. Acetylated histone H3K9(Ac), indicative of a euchromatic (transcriptionally active) state, is associated with the *ORF62* and *ORF63* promoters during both latent infection in human ganglia and lytic infection of human melanoma (MeWo) cells. Neither *ORF14* (glycoprotein C) nor *ORF36* (thymidine kinase) is transcribed or associated with H3K9(Ac) during latent infection. Chromatin remodeling appears to contribute to the restricted pattern of latent VZV gene transcription. An IE63 interaction with human anti-silencing function 1 protein interferes with its capacity to bind histones h3.1 and h3.3.⁸

VZV protein expression in latently infected ganglia has been assessed using antibodies that detect viral proteins in cultured cells to stain ganglion tissue sections. IE63 was first identified in rare ganglion neurons by Mahalingam et al.³⁹¹ (Fig. 63.11). Detection of IE62^{274,617} IE63,³⁸¹ ORF66¹⁰⁶ and proteins encoded by *ORFs* 4, 21, and 29²¹⁹ during latency has also been described. Some of these reports identify IE62 and IE63 as restricted to the cytoplasm of neurons, in contrast to their prominent nuclear localization during lytic infection. This pattern has been proposed to indicate a mechanism by which latency is maintained; IE63 and other VZV proteins have been referred to as VZV latency-associated proteins. However, not only does the frequency of neurons reported to express VZV proteins vary substantially among these studies, ranging from very few to more than 30%, but also the frequency of neurons expressing VZV proteins in some reports exceeds the frequency of neurons harboring VZV genomes. Furthermore, viral proteins, such as IE4, are detected but their transcripts are not. These discrepancies suggest technical problems with analysis of autopsy specimens using antibody reagents that are specific for VZV proteins in cultured cells.

It is now recognized that ascites-derived monoclonal antibodies and rabbit antisera may contain endogenous antibodies to human blood group A determinants. Ascites-derived monoclonal antibodies to VZV IE62, gE, and ORF40 capsid protein produce a cytoplasmic Golgi-like pattern of staining in neurons of blood group A subjects (~30% to 40% prevalence)⁷¹²; isotype reagents do not serve as a control for this reactivity. The apparent expression of VZV proteins in neurons at high frequency is no longer detected if endogenous anti-A antibodies are eliminated by deriving monoclonal antibodies in tissue culture or adsorbing antisera with erythrocytes from blood group A individuals.⁷¹² IE63 expression was rare when ganglion sections were stained with anti-IE63 rabbit antiserum and with preimmune serum to control for blood group A reactivity,⁷¹⁰ which confirmed two earlier reports^{309,391} (Fig. 63.11). The presence of neuronal cell pigments is another confounding factor in evaluating viral protein expression by immunohistochemistry, and IE62 has an epitope that cross-reacts with brain-derived neurotropic protein found in the neuron cytoplasm.²³⁸ Together, the evidence points to the presence of VZV proteins in only a few neurons in sensory ganglia from latently infected individuals with no signs of zoster at the time of death if blood group A and other variables are eliminated in the staining conditions. Thus, VZV protein expression is not a characteristic of VZV latency, with detection in rare neurons probably reflecting early or abortive reactivation.

Investigations of VZV neurotropism *in vivo* have used the SCID mouse model, in which human DRG are xenografted under the mouse kidney capsule and infected with VZV. In this

model, a limited lytic infection of DRG occurs in the first 14 days (Fig. 63.12). VZV virions are detected in neurons and satellite cells, and infectious virus is produced.^{527,707,709} By 1 month, no infectious virus is released from cells, virion assembly ceases, and VZV genome copy numbers are reduced. ORF63, and less consistently ORF62, continue to be transcribed but gB expression is silenced. Viral genomes remain in DRG for at least 8 months. This transition from lytic to persistent infection indicates that VZV latency can be established in human neurons in the absence of adaptive immunity, since SCID mice do not generate antigen-specific T cell or humoral immunity.

Like VZV, SVV establishes latent infection in ganglionic neurons at multiple levels of the neuraxis, but not in lung or liver.^{314,386} VZV neurotropism has also been investigated in rodent and guinea pig models.^{81,86,310,378,543} Inoculation of cotton rats by injections adjacent to the spine and sensory ganglia with VZV mutants showed that *ORFs* 1, 2, 10, 13, 14, 17, 21, 32, 47, 57, 61, 66, and 67 were dispensable for VZV DNA persistence in ganglion tissues whereas *ORF* 4, 63, and 66 mutants were impaired.

Reactivation

The factors that trigger VZV reactivation from latency in infected neurons are unknown. Three possible outcomes of reactivation are: abortive infection restricted to the ganglia; transport of virus particles to skin but with lesion formation blocked by innate and adaptive immunity; and cutaneous lesion formation, manifest as zoster (see Clinical Features). Zoster is a vesicular rash typically confined to a dermatomal distribution²⁶⁹ and differs from the patchy distribution of cutaneous HSV vesicles (Fig. 63.13). The histopathology and viral protein expression in zoster and varicella lesions are similar^{449,467} (Fig. 63.11); viral proteins and inclusion bodies are observed within the small nerves in the dermis.¹⁵ Unlike HSV, infectious VZV has only been recovered in human ganglia from patients with active zoster.^{429,505}

The pathology of VZV reactivation in ganglia is characterized by inflammation and hemorrhagic necrosis with associated neuritis, localized leptomeningitis, unilateral segmental myelitis, and degeneration of related motor and sensory roots.^{127,253} Demyelination is seen in areas with mononuclear cell infiltration and microglial proliferation. Intranuclear inclusions, viral DNA and proteins (Fig. 63.11), and viral particles are found in acutely infected ganglia.^{78,160,197,205,380} VZV-infected cells have been detected in subependymal microvessels in fatal zoster cases. VZV reactivation may be followed by vasculopathy affecting large and small vessels with resultant cortical and subcortical infarction and ovoid mixed necrotic and demyelinating lesions, particularly in deep white matter and periventricular ependyma.³³³ VZV reactivation has been demonstrated in ganglia in the absence of cutaneous rash.¹³⁹

The lytic phase of VZV infection of DRG xenografts in SCID mice can be considered a model of events that occur during VZV reactivation⁵²⁷ (Fig. 63.14). Both neurons and satellite cells are productively infected.^{707,709} Importantly, VZV induces cell-cell fusion between neurons and satellite cells and spread from satellite cells to adjacent uninfected satellite cells. Infection of these cells surrounding neuronal cell bodies facilitates access of the virus to other neuronal cell bodies. Similar cell-cell fusion has been observed in ganglia obtained at autopsy from patients with zoster.¹⁶⁰ During VZV

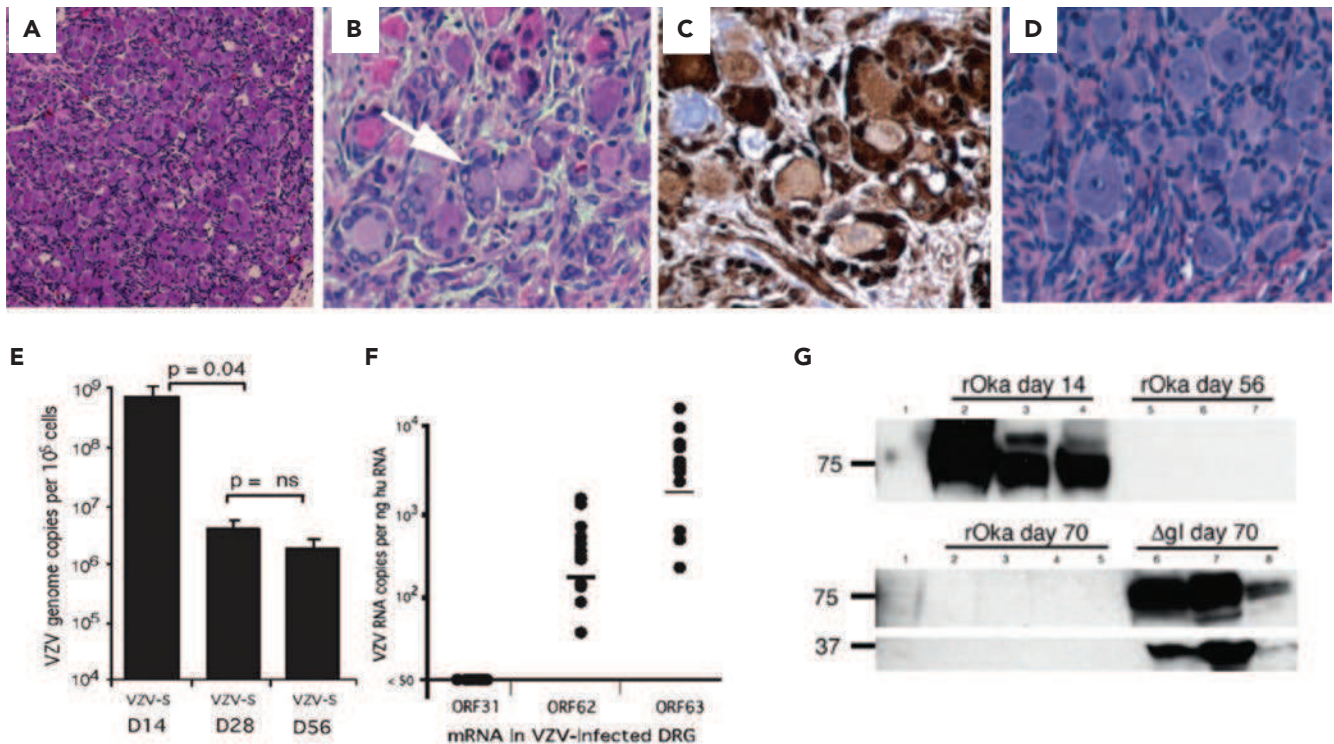


FIGURE 63.12. Varicella-zoster virus (VZV) infection of dorsal root ganglia xenografts in the SCID mouse model. **A:** DRG xenografts retain organotypic features of normal human ganglia (H&E stain; $\times 100$). **B:** DRG xenograft 14 days after infection; *arrow* indicates cytopathology in neurons (H&E stain; $\times 200$). **C:** DRG xenograft 14 days after infection stained with rabbit polyclonal antibody to VZV IE63 (brown, DAB signal) and counterstained with hematoxylin ($\times 200$). **D:** DRG xenograft 56 days after VZV infection, showing no evidence of productive infection (H&E stain; $\times 200$). **E:** VZV genome copy numbers in VZV-infected DRG xenografts evaluated by quantitative real-time DNA PCR at 14, 28, and 56 days postinfection using a probe to ORF62. **F:** VZV gene transcripts evaluated by quantitative real-time RNA PCR at 56 days postinfection using probes to ORF31 (gB), ORF62, and ORF63 mRNAs. **G:** Immunoblot (*upper panel*) of DRG extracts using human polyclonal anti-VZV antibody, primarily reactive against VZV gE in VZV-infected DRG at days 14 and 56 postinfection; immunoblot (*lower panel*) of DRG extracts using human polyclonal anti-VZV antibody and rabbit polyclonal antibody to IE63 in DRG at 70 days after infection with VZV or the VZV gI deletion mutant Δ gI, indicating cessation of protein expression in VZV- compared to Δ gI-infected DRG, which exhibit prolonged active infection. (Adapted from Zerboni L, Ku C-C, Jones CD, et al. Varicella-zoster virus infection of human dorsal root ganglia in vivo. *Proc Natl Acad Sci U S A* 2005;102:6490–6495; and Zerboni L, Berarducci B, Rajamani J, et al. Varicella-zoster virus glycoprotein E is a critical determinant of virulence in the SCID mouse-human model of neuropathogenesis. *J Virol* 2011;85(1):98–111.)

reactivation, amplification by spread to other neuronal cell bodies within the ganglia presumably allows delivery of virus particles from many neurons along axons to the skin in the affected dermatome. VZV damage to both satellite cells and neurons in the ganglia helps to account for the neurologic consequences that are rare during HSV reactivation.

SVV also reactivates in a dermatomal distribution either spontaneously or after drug- or radiation-induced immunosuppression.^{387,388,389} SVV glycoproteins are detected in zoster skin lesions, lungs, and multiple ganglia of most immunosuppressed monkeys, but not in control animals.³⁸⁸ Of interest, subclinical SVV reactivation after irradiation of rhesus macaques latently infected with SVV resulted in disseminated varicella in another irradiated SVV-seronegative monkey in the same colony.³³⁵

Postherpetic Neuralgia (PHN)

PHN is common after VZV reactivation (see Clinical Features). Two nonmutually exclusive theories to explain this pain are: an

altered excitability of ganglionic or even spinal cord neurons; and the presence of persistent or low-grade productive virus infection in ganglia. Analysis of ganglia from a case of PHN of 2.5 months' duration revealed diffuse and focal infiltration by chronic inflammatory cells⁵⁷⁷; collections of lymphocytes were prominent in ganglia from a patient with PHN of two years' duration⁶⁵⁹ (Fig. 63.13). Evidence that PHN may be due to low-level ganglionitis associated with prolonged viral infection has come from the detection of VZV DNA and proteins in PBMCs of patients with PHN.^{132,390,632} While antiviral therapy is not recommended for PHN, the favorable response of some patients to antiviral treatment supports the possibility that chronic ganglionitis may contribute to PHN.^{200,616}

Reinfection

Reinfection with VZV appears to be rare based on clinical criteria. Second episodes of varicella have been documented,¹⁹⁵ but confirmation of true reinfection with VZV as opposed to incorrect diagnosis of a previous vesicular rash is difficult. In

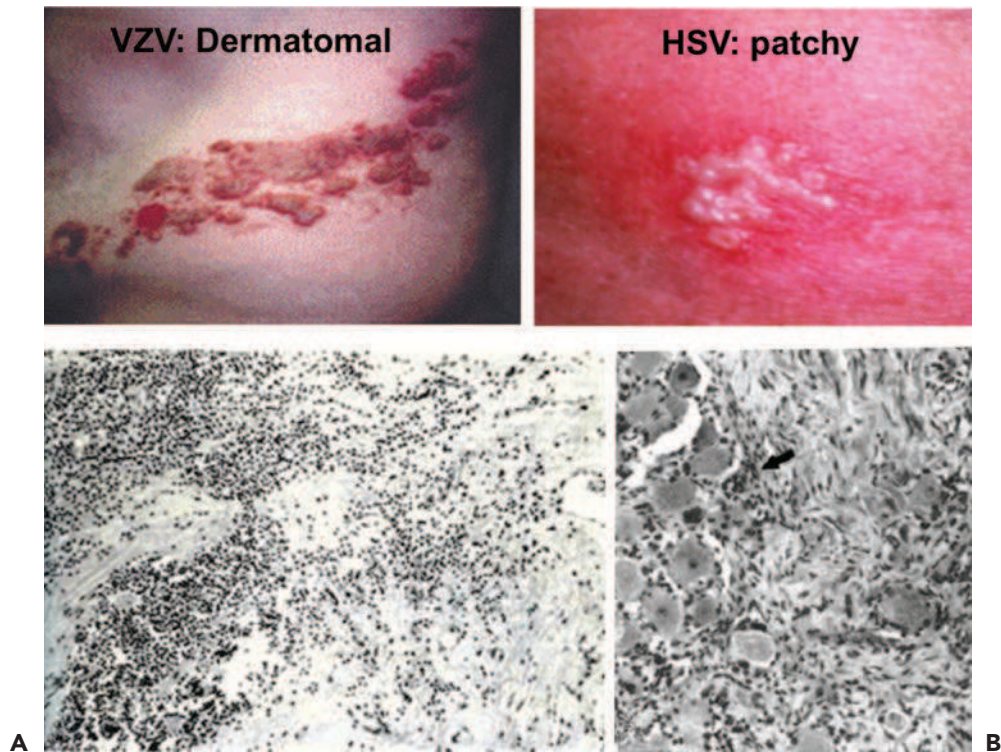


FIGURE 63.13. Characteristics of vesicular rash produced by varicella-zoster virus (VZV) versus herpes simplex virus reactivation and of ganglionitis associated with VZV reactivation and postherpetic neuralgia. **A:** Extensive dermatomal lesions characteristically produced by VZV compared to the patchy skin lesions produced by HSV. **B:** Sections of dorsal root ganglia (H&E staining) from two patients with PHN lasting 2.5 months (*right panel*) and 2 years (*left panel*) reveals diffuse and focal infiltration by chronic inflammatory cells; *arrow in right panel* points to a prominent collection of lymphocytes. (Adapted from Watson CPN, Deck JH, Morshead C, et al. Postherpetic neuralgia: further post-mortem studies of cases with and without pain. *Pain* 1991;44:105–117; and Smith FP. Pathological studies of spinal nerve ganglia in relation to intractable intercostal pain. *Surg Neurol* 1978;10:50–53.)

a cohort of adults who developed varicella despite a previous episode, none had VZV antibodies in sera obtained before the acute illness, suggesting that the earlier exanthem was not caused by VZV.⁶⁴⁵ Most immunocompromised patients who have had prior VZV infection and who develop signs suggestive of a second episode of varicella likely have generalized, atypical reactivation without dermatomal involvement rather than reinfection. Immunologic evidence of reinfection in healthy and immunocompromised patients is indirect; infection may not be necessary to trigger the increases in antibody and cellular immunity to VZV that occur when immune subjects have close contact with an infected person. Antigen presentation with enhancement of adaptive immunity can occur without any viral replication, as observed when immune individuals receive inactivated varicella vaccine. Since vaccinated individuals can develop zoster due to wild-type VZV, subclinical reinfection can occur despite vaccine-induced immunity.¹⁸⁹

Immune Response

IMMUNE CONTROL OF PRIMARY VZV INFECTION.

In addition to local antiviral responses mediated by epidermal cells (Fig. 63.10), innate cell-mediated responses are likely to be important in the initial control of VZV infection. Natural

killer (NK) cells from nonimmune individuals lyse VZV-infected fibroblasts²⁸² and also produce granulysin, which has antiviral activity.²⁴⁹ IFN- α is detected in serum at the onset of varicella²⁰ and may limit early viral replication, as suggested by its clinical efficacy in modifying the severity of varicella in immunocompromised children and by the association of more severe varicella in adults with reduced serum IFN- α .^{22,646} The innate response to primary VZV infection also appears to involve invariant natural killer T cells (iNKT) and antigen presentation to these unusual T cells by CD1, a nonclassical MHC class I molecule, as suggested by the disseminated infection after administration of varicella vaccine virus in a child with deficient iNKT cells and CD1 expression but no other evidence of a primary immunodeficiency disorder.³⁷

The initial stages of primary VZV infection before and in the first few days of rash evoke little or no adaptive immunity, as measured by antibody production or assessment of T cells that recognize VZV antigens. Nevertheless, although the virus can evade induction of and recognition by CD4 and CD8 T cells transiently, replication is well contained when VZV-specific immunity is induced¹⁷ (Fig. 63.15). Robust cellular immunity is elicited even when the varicella rash consists of fewer than 10 lesions. Unless the host is compromised, this pattern of

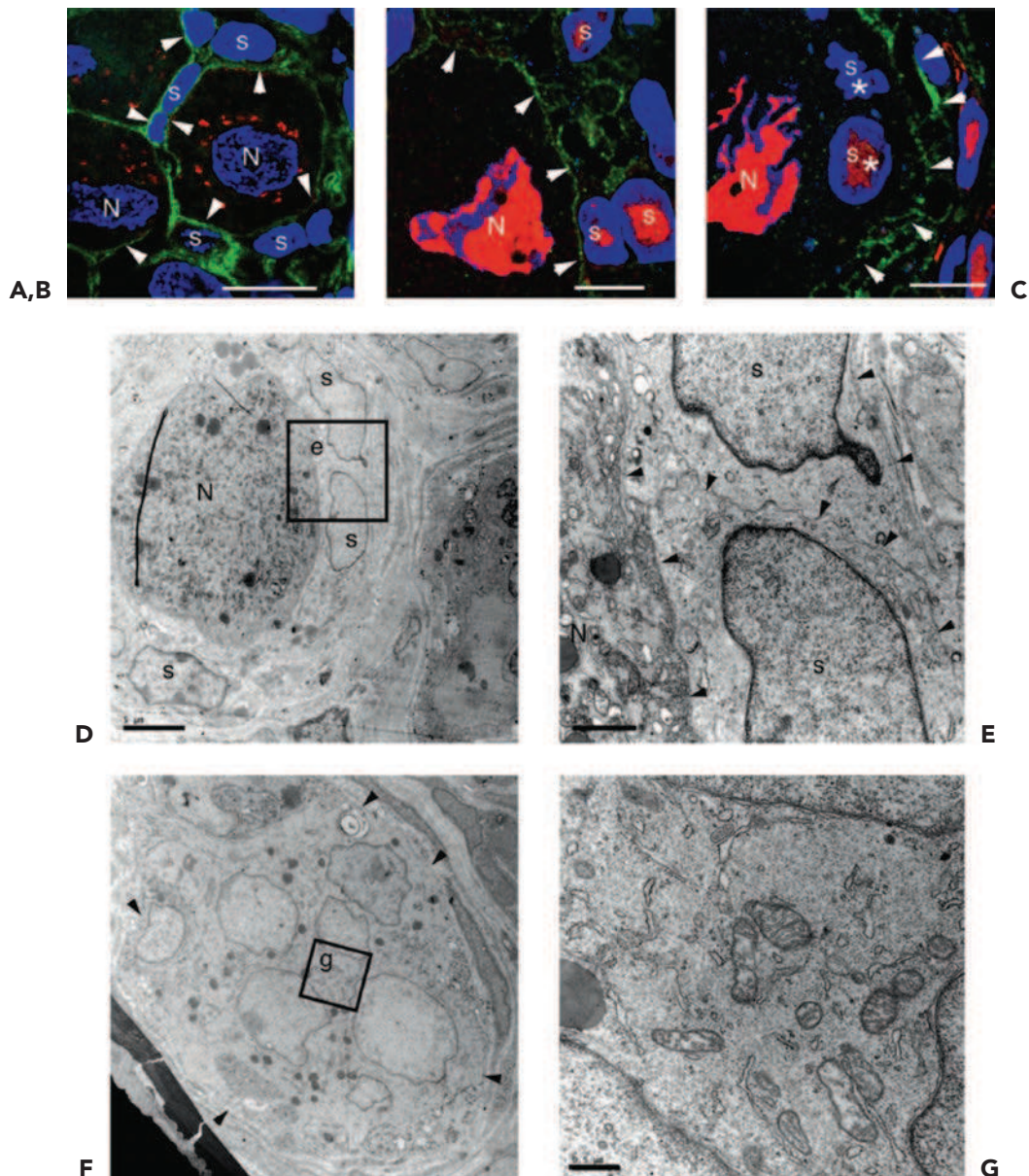


FIGURE 63.14. Polykaryon formation in acutely infected DRG. A–C. Immunofluorescence analysis of acutely infected DRG. Cryosections of VZV-infected DRG were stained with mouse monoclonal anti-NCAM antibody (**A–C**; green), rabbit polyclonal anti-synaptophysin antibody (**A**; red), rabbit polyclonal anti-IE62 antibody (**B, C**; red) and Hoechst stain (blue). Texas red-labeled goat anti-rabbit or FITC-labeled goat anti-mouse antibodies were used for secondary detection. Satellite cells (s) and neurons (N) are marked with letters; *asterisks* (*) indicate nuclei of satellite cells located within a putative polykaryon. *Arrowheads* point to cell boundaries detected by NCAM staining (green). Scale bars = 10 μm. **D–G.** EM analysis of acutely infected DRG. *Arrowheads* point to the cell membrane surrounding satellite cells (s) or the neuron cell body (N) or a polykaryon (**F, G**). Black boxes in **D** and **F** indicate the area shown at higher magnification in **E** and **G**, respectively. Note that despite the normal morphology of the mitochondria, the ER and the nuclear envelope in the polykaryon (**F, G**), no cell membranes between the nuclei of the polykaryon are detected. (Adapted from Reichelt M, Zerboni L, Arvin AM. Mechanisms of varicella-zoster virus neuropathogenesis in human dorsal root ganglia. *J Virol* 2008;82(8):3971–3983).

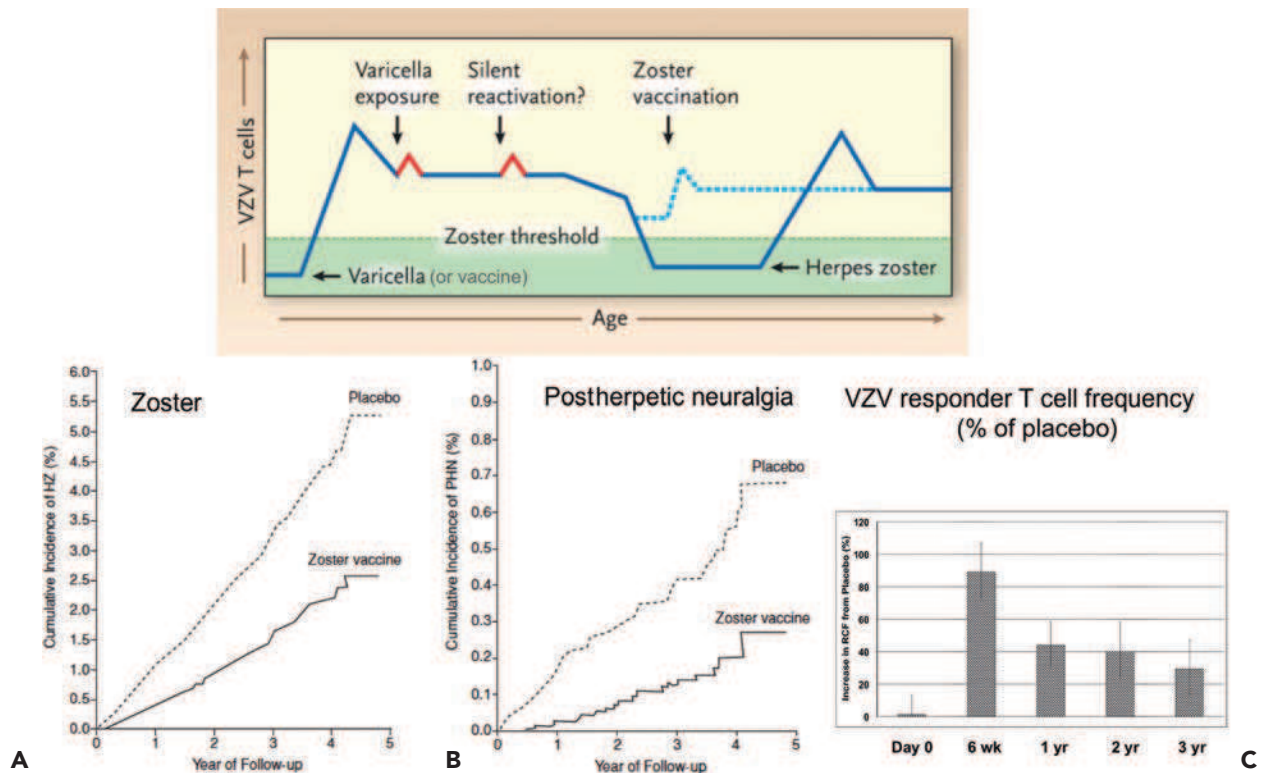


FIGURE 63.15. The immune response to varicella-zoster virus (VZV) infection. Upper panel: Schematic of VZV T cell immunity. Varicella is the primary infection caused by VZV, and its resolution is associated with the induction of VZV-specific memory T cells (blue line); varicella vaccine also induces memory T cells. Memory immunity to VZV may be boosted periodically by exposure to varicella or silent reactivation from latency (red peaks). VZV-specific memory T cells decline with age. The decline below a threshold (dashed green line) correlates with an increased risk of zoster. The occurrence of zoster, in turn, is associated with an increase in VZV-specific T cells. Administration of zoster vaccine to older persons may prevent VZV-specific T cells from dropping below the threshold for zoster occurrence (dashed blue line). (From Arvin A. Aging, immunity, and the varicella-zoster virus. *N Engl J Med* 2005;352:2266–2267, with permission.) **Lower panel:** Effects of zoster vaccination. **A:** Reduction of zoster. **B:** Reduction of postherpetic neuralgia. **C:** VZV-specific memory T cells in healthy adults given high-potency live attenuated VZV vaccine or placebo. (Adapted from Oxman MN, Levin MJ, Johnson GR, et al. Shingles Prevention Study Group. A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. *N Engl J Med* 2005;352:2271–2284; and Weinberg A, Levin MJ. VZV T cell-mediated immunity. *Curr Top Microbiol Immunol* 2010;342:341–357).

virus–host interaction initially favors the virus because cutaneous viral replication allows VZV transmission to other susceptible individuals, while the host is favored thereafter through the protection from illness on subsequent exposures afforded by memory B and T cells that recognize VZV antigens. Disease that could be initiated by VZV reactivation is also blocked as long as VZV-specific memory T cells are maintained in sufficient numbers.⁶⁶⁹

Primary VZV infection induces the production of IgG, IgM and IgA antibodies directed against many viral proteins.^{17,137,196} Antibodies can be detected in low concentrations in some individuals at the onset of the varicella rash and are usually present within 3 days. Antibodies to VZV proteins neutralize VZV replication, either directly or with complement, and also function in antibody-mediated cellular cytotoxicity.^{250,305} However, the humoral immune response appears to have little role in controlling primary VZV infection, since children with agammaglobulinemia have uncomplicated varicella. Among healthy children, the early detection of

high levels of circulating VZV-specific IgG or IgM antibodies does not predict disease severity. Moreover, children with T-cell immunodeficiencies may develop fulminant varicella, despite VZV antibody responses.²⁰

VZV antibodies administered passively after the appearance of varicella rash have no effect on disease progression. If present at or shortly after an exposure to VZV, passively acquired virus-specific antibodies can modulate or even prevent VZV infection of a susceptible individual.^{536,704} Transplacentally acquired IgG antibodies to VZV also protect infants from varicella or modify its severity. VZV-specific antibodies interfere with the initial phases of VZV replication *in vivo*, as shown by the reduced varicella attack rate in individuals given VZV immunoglobulin prophylaxis shortly after exposure.⁷⁰⁴ Passive antibodies may limit replication at initial sites, e.g., mucosal epithelium or lymphoid tissue, or may block or diminish cell-associated viremia or the early phase of VZV infection of epidermal cells. Anti-gH antibodies have neutralizing activity and may be important for this effect, as suggested by the restricted

skin infection in SCID mice given anti-gH antibody at the time of VZV inoculation.⁶³⁹

The critical role of VZV-specific cell-mediated immunity in controlling primary VZV infection is supported by correlations between the induction of virus-specific T cells and disease outcome.¹⁷ Cellular immunity appears to be important for terminating viremia and limiting virus replication at localized cutaneous sites. Healthy children who had T cells that recognized VZV antigens within 24 to 72 hours experienced milder varicella.²⁰ T-cell proliferation in response to VZV antigen is accompanied by the production of many cytokines, including IL-2 and IFN- γ , which amplify the initial antiviral response.²⁹⁰ Conversely, lymphopenia and failure to develop VZV-specific T-cell proliferation in immunocompromised children correlates with persistent viremia and risk of visceral dissemination.²⁰ Children with immunosuppression due to underlying diseases or interventions or those with primary cellular immunodeficiency diseases experienced progressive and often fatal varicella before the availability of antiviral drugs.⁴⁵² A similar pattern of acquisition of cell-mediated immunity and resolution of viremia and rash is observed in SVV infection; SVV-specific CD4 and CD8 T cells reach peak levels by 14 days.^{384,417}

MEMORY IMMUNITY.

Memory immunity to VZV is required to prevent symptomatic reinfection with exogenous virus and to prevent symptomatic VZV reactivations. The extent to which memory immunity contributes to the preservation of viral latency in neurons is not known. IgG antibodies that bind to viral glycoproteins and many other VZV polypeptides¹³⁷ and VZV-specific IgA antibodies are maintained, likely functioning as the first line of defense against reinfection.

Cell-mediated immunity to VZV proteins persists for years in the immunocompetent host. VZV-specific T cells respond to gB, gC, gE, gH, gI and the immediate early proteins IE62 and IE63.^{17,175,252} Healthy VZV-immune adults have circulating T cells that recognize an average of 7 of 10 IE62 peptides and 6 of 10 gE peptides.⁴³ Such individuals also have T cells that exhibit class I and class II MHC-restricted lysis of target cells expressing VZV antigens.^{134,570} Cytotoxic T lymphocytes (CTL) that recognize IE62 protein or gE are present at equivalent precursor frequencies in both the CD4⁺ and CD8⁺ memory T-cell populations.²⁶ VZV gC, gI, ORF4 protein, and ORF10 protein are also CTL targets.^{17,25} VZV-specific memory T cells circulate at low frequencies of about 5 per 100,000 PBMCs in immune adults,⁶⁶⁹ and memory CD4⁺ and CD8⁺ CTLs specific for individual VZV proteins circulate at frequencies of about 1 per 150,000 PBMCs. Intracellular cytokine detection assays showed that the mean percentage of VZV-specific CD4⁺ T cells based on IFN- γ or TNF- α production was about 0.12%.³⁰ Immune subjects also have persistent delayed-type hypersensitivity (DTH) responses to VZV skin test antigens.

Mechanisms by which memory immunity to VZV is maintained may include exposure to VZV through close contact with an infected person, since enhanced VZV T-cell proliferation, DTH, and VZV IgG antibodies often follow such contacts,²¹ and endogenous re-exposure to VZV antigens by subclinical reactivations. That endogenous re-exposures help to maintain VZV-specific T-cell responses is consistent with VZV-specific T-cell enhancement in older individuals given VZV vaccine.³⁶¹ Repeated antigenic stimulation may also result

if subclinical VZV reactivations lead to viral transfer to skin or mucosal sites of replication without progressing to zoster. This possibility is supported by the recovery of cell-mediated immunity to VZV in bone marrow transplant recipients who had no clinical episodes of zoster but had subclinical reactivations detectable by PCR.⁶⁸⁰ It is not known whether conditions necessary for CD4 and CD8 T-cell recognition of neurons harboring VZV are present during latency; if so, VZV immunity may be maintained by intermittent local expression of viral proteins during abortive reactivations. However, viral peptides can only be detected by T cells when present in MHC complexes, and class I MHC requires IFN- γ exposure for upregulation on neurons. Attempts to analyze local T-cell responses during latency have been hampered by the lack of a marker for cells harboring VZV genomes because VZV has no LAT region and viral proteins are rarely expressed.^{391,710,712}

IMMUNITY AND REACTIVATION.

T cells are present in ganglia obtained postmortem from latently infected individuals, but the extent to which VZV-specific T cells provide surveillance for early stages of reactivation in ganglionic neurons is unknown. Diminished T-cell responses to VZV antigens in older and immunocompromised individuals correlate with enhanced susceptibility to zoster,^{17,359,361} indicating the significant impact of waning immunity on the probability of symptomatic disease upon virus reactivation. The restoration of VZV T-cell immunity and the reduced risk of zoster in immunocompromised and elderly individuals given VZV vaccines has confirmed this hypothesis⁴⁸² (Fig. 63.15). Age-related immunosenescence is also associated with a decrease in DTH responses to VZV antigens. Conversely, susceptibility to VZV reactivation is not associated with decreasing titers of VZV IgG antibodies. Zoster in young children after intrauterine or early postnatal varicella and the short interval between primary and recurrent VZV infections in HIV-infected children probably reflects suboptimal induction of VZV cell-mediated immunity.^{32,128} Severe and prolonged suppression of VZV-specific cellular immunity creates the highest risk for zoster, viremia, and dissemination.

When reactivation occurs, VZV T-cell responses increase promptly, peaking at a responder cell frequency of about 10 per 100,000 PBMC at 6 weeks.⁶⁶⁹ The resolution of rash is also accompanied by local production of IFN- α in lesions.⁵⁹⁴ While information is limited, autopsy studies indicate that an extensive inflammatory response, including CD4, CD8, and NK cells, persists in ganglia for several months after zoster even without PHN.²¹⁴ Upregulation of CXCL10 occurs in ganglia during reactivation, and lymphocytes that express the CXCR3 receptor for CXCL10 are prominent among infiltrating cells⁵⁸⁸ (Fig. 63.11). Enhanced VZV T-cell immunity persists for at least 3 years after zoster⁶⁶⁹ and may explain why second episodes of zoster are rare.

EPIDEMIOLOGY

Incidence and Prevalence

VZV is a ubiquitous human pathogen with a worldwide geographic distribution. The annual incidence of varicella in the United States was equivalent to the annual birth rate (~4 million) before varicella vaccine was introduced in 1995,⁶⁷⁴ and the

prevalence of anti-VZV IgG antibodies indicates that up to 99% of adults are infected.³¹⁹ By 20 to 29 years of age, only about 5% of individuals remain susceptible in geographic areas with temperate climates. In tropical regions, only about half of individuals younger than 24 years of age have had varicella.^{186,396,478} The varicella attack rate is very high among susceptible adults who emigrate from tropical areas to temperate zones.

Given the high incidence of varicella, most adults are at risk for VZV reactivation and one-third will develop zoster. In the United States, approximately one million cases of zoster are diagnosed annually, two-thirds of which occur in individuals older than 50 years and nine in 10 are immunocompetent.⁷⁰¹ Hope-Simpson²⁶⁹ observed that the incidence rose to 7.8 cases per 1,000 people older than 60 years of age, compared with 2.5 per 1,000 people 20 to 50 years of age. The risk is as high as 50% among those who reach 85 years of age. Since the risk of zoster increases with age, and the population over age 65 is expected to be 72 million in 2030, zoster and its attendant serious neurologic complications will continue to be a significant health care burden.

Zoster incidence rates are equivalent between men and women but are lower in black than in white Americans.⁵⁶¹ Zoster is particularly frequent among patients with leukemia; Hodgkin's and non-Hodgkin's lymphoma; small-cell carcinoma of the lung; and after bone marrow transplantation (>25%) and renal transplantation (15%). Patients with systemic lupus erythematosus have an estimated incidence of 22 cases per 1,000 person years.^{398,487} HIV infection increases the risk for zoster by 15- to 25-fold regardless of age.^{60,192,208} Although cancer is correlated with a higher risk of zoster, zoster in healthy individuals is not associated with an increased risk for malignancy.⁵¹⁶ Zoster is rare in childhood, but varicella during the first year of life and infection *in utero* are associated with a higher risk.^{32,227,501}

Epidemic Patterns

Episodes of zoster provide a continuous source for VZV transmission to susceptible individuals, who then develop varicella. Varicella epidemics probably arise from sporadic cases in children after exposures to recurrent VZV in adults. Before varicella vaccination was introduced, most susceptible children became infected during annual epidemics due to efficient VZV transmission by the respiratory route and contact with high titers of infectious virus in skin lesions. The attack rate for previously uninfected household or day-care center contacts exposed to varicella is about 90%, compared to 12% to 33% for more casual exposures such as school classrooms.⁵⁹ Zoster is not seasonal because it originates from the reactivation of endogenous latent virus. Some reports have suggested that exposures to varicella can precipitate episodes of zoster, but there is no known mechanism by which reactivation would be triggered by re-exposure to the virus. While a zoster vaccine is available, only 6.7% of individuals over age 60 years have been immunized in the United States,³⁷⁹ leaving more than 900,000 of every one million individuals in this age group at risk for zoster.

Morbidity and Mortality

The high prevalence of VZV infection means that almost all individuals experience some VZV-related morbidity.^{72,674} The

risk for severe morbidity or mortality during primary or recurrent VZV infection depends on host factors rather than virulence characteristics of the virus.

Varicella complications resulted in hospitalization rates of 11,000 per year in the United States (2.2 to 4.3/1,000 cases) before varicella vaccine was introduced.⁷¹ Serious complications are least common among children 1 to 9 years of age and are 6 to 15 times higher among adults. Case fatality rates are 10 and 24 times higher in infants (6.7/100,000 cases) and adults (17.1/100,000 cases), respectively, than in children 1 to 4 years of age (0.7/100,000).⁴⁶⁴ Serious complications are usually secondary bacterial infections, varicella pneumonia in adults, or neurologic syndromes.^{169,228} CNS morbidity is higher among patients younger than 5 years and older than 20 years of age.²⁹²

Before effective antiviral drugs were available, 32% to 50% of children with malignancy developed visceral dissemination of the virus; varicella pneumonia occurred in 20% of cases, and the mortality rate was 7% to 17%.¹⁶⁵ Varicella occurs in 0.7 to 2 per 1,000 pregnancies, with a predicted maternal mortality rate of 0.5 per 1,000 cases¹⁵⁴ and a risk of congenital varicella syndrome. Rarely, the pregnancy may terminate by spontaneous abortion, fetal demise, or premature delivery.³⁵ The incidence of congenital varicella syndrome was 0.4% when maternal varicella occurred from 0 to 12 weeks' gestation, compared with 2% for infection from 13 to 20 weeks' gestation.¹⁵⁴ The virus appears to be transmitted to the fetus during the second and third trimester of maternal varicella with about the same frequency as in the first trimester, but a study found no infants with congenital varicella syndrome or other morbidity at birth if the mother had varicella between 25 and 36 weeks of gestation.¹⁵⁴ Infants are at risk for neonatal varicella when maternal varicella occurs at the end of gestation, with an attack rate of about 20% unless antibody prophylaxis is given, and a 30% mortality rate without antiviral therapy.

One in 10 individuals with zoster experiences at least one zoster-related nonpain complication, while one in four experiences zoster-related pain that persists 30 days or more.⁷⁰¹ The frequency of PHN is higher in older patients, increasing from 3% to 4% in those between 30 to 49 years of age to 21%, 29%, and 34% in patients 60 to 69, 70 to 79, and more than 80 years of age, respectively.^{18,679} Recent data from the United States indicate a risk of PHN of 1.38 cases per 1,000 person-years in healthy adults older than 60 years.⁴⁸² The annual medical care cost of treating zoster is estimated at \$1.1 billion, most of which is spent to treat immunocompetent adults 50 years and older.⁷⁰⁰ The incidence of zoster-associated CNS disease, most of which are cases of VZV vasculopathy, ranges from 0.2% to 0.5%, with risk factors including older age and cranial nerve involvement during the acute zoster episode. Cerebral angitis following zoster has a case-fatality rate as high as 20%. Immunosuppressive therapy for malignancy increases the morbidity and mortality associated with zoster, although the risk for fatal infection is less than 1%. Zoster is not unusual in pregnancy, but it does not cause significant maternal or fetal morbidity.¹⁵⁴

CLINICAL FEATURES

Varicella

Varicella follows an incubation period that ranges from 10 to 21 days. Prodromal symptoms of fever, malaise, headache, and

abdominal pain often precede the appearance of the rash by 24 to 48 hours. Fever, irritability, lethargy, and anorexia are prominent during the 24 to 72 hours after the first cutaneous lesions are noted; respiratory symptoms and vomiting are unusual. Body temperature usually rises to less than 101.5°F but can be as high as 106°F. The varicella exanthem begins on the scalp, face, or trunk. The cutaneous lesions consist of erythematous macules that evolve rapidly (within several hours) to form a clear, fluid-filled vesicle surrounded by an irregular erythema margin, the classic “dew drop on a rose petal.” (Fig. 63.1). Lesions in the maculopapular or vesicular stages are usually intensely pruritic. After 24 to 48 hours, the vesicular fluid becomes turbid, the lesions umbilicate, and crusting begins. Later crops of lesions form on the trunk and subsequently the extremities, and can resolve without progressing to form vesicles. Lesions of the mucous membranes of the oropharynx, conjunctivae, and vagina are common. In the final phase, the crusted lesions are sloughed as new epithelial cells are generated at the lesion site. Varicella vesicles are common on eyelids and conjunctivae, but eye disease is unusual.¹⁵¹

New varicella lesions emerge for 3 to 6 days, with the total number of lesions ranging from less than 10 to 2,000; most children have fewer than 300 lesions. True subclinical varicella is rare.⁵³⁶ Children who develop varicella after household exposure to an index case are more likely to have more severe varicella.^{142,536} Skin damage such as eczema or sunburn exacerbates the severity of the exanthem. Hypopigmentation of the skin at sites of varicella lesions often persists for several weeks, but extensive scarring is unusual. Low polymorphonuclear leukocyte and lymphocyte counts during the first 72 hours of rash are followed by lymphocytosis⁶¹; slight elevations of liver function tests are common.

Staphylococcus aureus and *Streptococcus pyogenes* (group A β -hemolytic streptococcus) cause secondary bacterial infection of skin lesions, cellulitis, bacterial lymphadenitis, or subcutaneous abscesses.^{61,116,169,226,286,490,500,681,682} (eFig. 63.4). Varicella gangrenosa, a form of necrotizing fasciitis, is a life-threatening infection usually caused by *S. pyogenes*.^{57,713} Bacteremia, usually caused by *S. aureus* or *S. pyogenes*, can lead to acute bacterial sepsis or focal infection such as pneumonia, arthritis, or osteomyelitis.

Varicella pneumonia in otherwise healthy adults^{468,511,644} presents with fever, cough, tachypnea, and dyspnea beginning about 3 days (range 1 to 6 days) after the onset of rash and may be associated with cyanosis, pleuritic chest pain, or hemoptysis (eFig. 63.5). Varicella pneumonia is often transient, resolving completely within 24 to 72 hours, but interstitial pneumonitis with severe hypoxemia progresses rapidly to cause respiratory failure in severe cases. Varicella pneumonia is associated with active infection of the epithelial cells of the pulmonary alveoli. Infection induces mononuclear cell infiltration of the alveolar septa and edema of alveolar septal cells, along with the accumulation of exudate, formation of hyaline membranes, and cellular desquamation into the alveolar spaces, which inhibit oxygen transfer. Desquamated septal cells in the alveoli and bronchiolar epithelial cells contain eosinophilic intranuclear inclusions and virions. Focal necrosis is characteristic of fatal varicella pneumonia.

Varicella hepatitis is associated with extensive replication in the liver, lysis of hepatocytes, and inflammation. Varicella hepatitis can occur subclinically, although it may be associated

with severe vomiting. Aspirin is contraindicated in children with varicella because it predisposes to liver damage (Reye's syndrome).

Varicella encephalitis or cerebellar ataxia usually occurs within 2 to 8 days.^{147,292,333,499,643} Patients with encephalitis typically have sudden onset of seizures and altered sensorium, whereas those with cerebellar disease show irritability, nystagmus, and gait and speech disturbances. Some patients have fever, headache, and meningismus only, without altered consciousness or seizures. The cerebrospinal fluid (CSF) usually shows a mild lymphocytic pleocytosis (<100 cells/mm³) and a slight to moderate protein level (<200 mg) with normal glucose. Encephalitic symptoms usually resolve rapidly (within 24 to 72 hours); ataxia can persist for days to weeks. A few cases of optic neuritis, transverse myelitis, and Guillain-Barré syndrome have been associated with varicella.^{198,292,374} Varicella encephalitis is observed most often in immunocompromised patients who have persistent viremia, suggesting a role for direct viral invasion of CNS tissue in some cases. The detection of herpesvirus particles and viral DNA in cerebrospinal fluid supports this possibility. The histopathology of brain tissue from rare cases of fatal varicella encephalitis demonstrates vasculitis of large and small vessels, demyelination, axonal damage, perivascular infiltration with mononuclear cells, microglial proliferation, and neuronal degeneration; however, histologic abnormalities are often minimal.^{11,333} In other cases, varicella-related neurologic syndromes resolve rapidly and completely, which may indicate a predominantly inflammatory or immune-mediated pathogenesis. The observation that cerebellar ataxia and encephalitis can occur during the pre-eruptive phase of varicella may indicate early transfer of VZV to neural tissues, triggering local inflammatory responses.^{373,643} CNS disease may also result from ischemic arteriopathies; one-third of these cases in children are associated with varicella.^{11,54,86}

Acute thrombocytopenia is a complication of varicella that causes petechiae and purpuric skin lesions, hemorrhage into the varicella vesicles, epistaxis, hematuria, and gastrointestinal bleeding. Hemorrhagic complications are usually transient. Purpura fulminans, caused by arterial thrombosis and hemorrhagic gangrene, is a rare but life-threatening complication. Thrombocytopenia may occur from 1 to 2 weeks or longer after varicella, as a result of direct infection of bone marrow megakaryocytes or immune-mediated processes. Platelet survival may also be reduced as a result of endothelial cell damage caused by vasculitis or transient hypersplenism, or as part of the syndrome of intravascular coagulopathy. As a postinfectious complication, thrombocytopenia may be associated with increased production but shortened platelet survival due to antiplatelet antibodies.

Varicella nephritis causes hematuria, proteinuria, diffuse edema, and decreased renal function, with or without hypertension. A few cases of nephrotic syndrome and hemolytic-uremic syndrome have been reported in children with varicella. Varicella arthritis is rare; it resolves spontaneously within 3 to 5 days with no residual joint disease. Other unusual complications of varicella include myocarditis, pericarditis, pancreatitis, and orchitis. Adrenal infection, characterized by focal necrosis, is common in fatal disseminated varicella. Renal pathology can occur, with hypercellularity of the glomeruli, epithelial proliferation, focal necrosis and interstitial inflammation.

Varicella in High-Risk Populations

Varicella vesicles in immunocompromised children are usually larger and more numerous, and new lesion formation often continues for at least 7 days, with an average time to lesion crusting of 14 days.⁶⁷⁷ In one series, all of the deaths from varicella in children with leukemia occurred within 3 days after the diagnosis of varicella pneumonia.¹⁶⁵ Varicella hepatitis and thrombocytopenia lead to coagulopathy; hemorrhage into lesions is a sign of severe varicella, as is severe abdominal or back pain. Encephalitis is rarely the immediate cause of death. Disseminated VZV infection in children with cancer has also produced myocarditis, nephritis, pancreatitis, necrotizing splenitis, esophagitis, and enterocolitis. Neutropenia enhances susceptibility to secondary bacterial infections. Children who acquire varicella after organ transplantation are also at risk for progressive VZV infection.^{178,263} Children on long-term, low-dose steroid therapy for asthma are not usually at risk for serious varicella,⁵⁸⁷ but fatal varicella has been described in patients receiving higher doses of prednisone during the incubation period.

Untreated varicella can be fatal in children with severe combined immunodeficiency or T-cell disorders, including adenosine deaminase or nucleoside phosphorylase deficiency and cartilage hair hypoplasia and NK-cell deficiencies.¹⁶¹ Varicella can also be severe in children with Wiskott-Aldrich syndrome or ataxia telangiectasia. Children with HIV infection may have unusual hyperkeratotic lesions and new lesion formation for weeks or months.^{128,192,225,297,307,353,608}

Placental infection with chronic villitis has been described after maternal varicella in pregnancy.⁵³³ VZV can be transmitted to the fetus, and in rare instances, causes congenital varicella syndrome (eFig. 63.6). Infants with congenital varicella syndrome have unusual cutaneous defects, with cicatricial skin scars, atrophy of an extremity, microcephaly, seizures, mental retardation, intrauterine growth retardation, and other sequelae, including chorioretinitis, micro-ophthalmia, and cataracts.^{35,154,507} Varicella embryopathy exemplifies the consequences of VZV infection of spinal ganglion cells and damage to the developing autonomic nervous system. Severe microcephaly with cortical atrophy and calcification follows intrauterine encephalitis and necrosis of brain parenchyma.

Varicella in the newborn period can be progressive, exhibiting the pathologic changes observed in other immunocompromised patients. Infants with neonatal varicella whose lesions are present at birth or within the first 5 days of life are not at risk for severe disease, probably because of transplacental transfer of maternal IgG antibodies to VZV. Those who are born within 4 days before, or 2 days after, the onset of maternal varicella may develop progressive varicella with hepatitis, pneumonia, and coagulopathy.

Zoster

Zoster is characterized by pain and a vesicular eruption on an erythematous base in one or several dermatomes (Fig. 63.1). Rash and pain usually develop within a few days of each other, although pain may precede rash by 7 to more than 100 days. The initial cutaneous lesions often appear in groups at one or several sites anteriorly and posteriorly within each affected dermatome; discrete vesicles resembling varicella lesions may merge to form larger, fluid-filled lesions as the exanthem evolves. Skin lesions resolve within 1 to 2 weeks, while

complete cessation of pain usually takes 4 to 6 weeks. Disseminated cutaneous lesions occasionally accompany the localized dermatomal infection even in the normal host. Many patients with uncomplicated zoster have a CSF pleocytosis and elevated protein. VZV can be isolated from CSF and detected by PCR.^{86,233,509} VZV reactivation in the immunocompromised host often causes a more extensive dermatomal rash and cell-associated viremia,¹⁶⁴ which allows VZV dissemination to lungs, liver, CNS, and other organs. Dissemination can occur without rash, suggesting that T cells can become infected while trafficking through the infected ganglia. Patients with HIV infection may have chronic hyperkeratotic skin lesions (eFig. 63.7).

Because VZV becomes latent in any ganglion, zoster can develop in any dermatome. The trunk from T3 to L2, which is innervated by twelve pairs of ganglia, is the most frequently involved (Table 63.2), followed by the face and the extremities. The face, which is the second most common site, is supplied by

TABLE 63.2 Segmental Localization of 180 Cases of Zoster

Right	Ganglion	Left
.....	V
.	VII	.
	C1	
.	2
...	3	...
..	4	..
..	5	.
..	6	
.	7	...
.	8	
.	T1	.
..	2	.
....	3
....	4	.
.....	5
....	6
....	7
..	8
.....	9
....	10
.	11	
...	12
....	L1
....	2
..	3	..
	4	
.	5	..
.	S1	.
.	2	.
	3	...
	4	
	5	

Each dot represents a single case of zoster.
(From Hope-Simpson RE. The nature of herpes zoster: a long-term study and a new hypothesis. *Proc R Soc Med* 1965;58:9–20, with permission.)

afferent fibers only from the two trigeminal ganglia; these are the most frequently latently infected ganglia,⁴⁵⁵ as is the case for HSV-1. In the classic study by Hope-Simpson,²⁶⁹ about half of the cases involve the thoracic dermatomes, particularly T5 to T12. Lumbosacral dermatome disease was observed in 16% of these patients, predominantly in the L1 to L2 distribution. HSV reactivations in this region are patchy rather than dermatomal (Fig. 63.13).

Zoster affects the head in approximately 19% of cases, with nearly all rashes (97%) in the trigeminal distribution.^{62,269} In patients with trigeminal zoster, the ophthalmic division of the fifth nerve is most frequently affected (eFig. 63.8). There have been 14 reported cases of trigeminal zoster accompanied by alveolar bone necrosis and tooth loss.³⁹⁷ The seventh cranial nerve ganglion is the next most commonly affected. Weakness of facial muscles is usually associated with lesions in the ear (Ramsay Hunt syndrome; geniculate zoster). Vesicles may also be observed on the anterior two-thirds of the tongue.⁴⁹¹ Commonly associated with geniculate zoster are eighth-nerve deficit,¹¹⁵ fifth-nerve zoster or zoster of the occiput and neck,³³⁴ ophthalmoplegia,⁶¹⁹ and multiple lower cranial nerve palsies.^{116,590} Importantly, multiple forms of VZV reactivation in a trigeminal^{146,264,691} and facial^{433,448,617} distribution as well as polyneuropathy^{450,480} may occur in the absence of rash.

The mechanism by which the cranial nerves are involved in zoster is not clear. Examination of one case with oculomotor nerve involvement and death 4 days after cutaneous signs revealed neuronal degeneration, with microglial and lymphocytic infiltration in the oculomotor nuclear complex, and a leptomeningeal infiltrate most marked over the lower medulla and at the point of entry of the trigeminal and oculomotor nerve roots.²¹² Orbital changes consisted of perineural and intraneural lymphocytic infiltrates of the retrobulbar, episcleral, and intrascleral segments of the long posterior ciliary nerves, with minimal lymphocytic infiltrates and sarcolemmal proliferation in extraocular muscles. Thus, cranial neuropathy may be due to a circumscribed myositis or lymphocytic mononeuropathy. PHN, the most common neurologic complication of zoster, is often defined clinically as pain that persists for at least 3 months after resolution of zoster rash. Age is the most important factor in predicting the development of PHN. Pain is usually constant, severe, stabbing or burning, and frequently associated with allodynia (increased sensitivity to light touch). The incidence of PHN appears to be slightly higher in women²⁷⁰ and after zoster involving the trigeminal ganglia.^{125,535} VZV reactivation can also produce chronic radicular pain without rash (zoster sine herpete) as well as other zoster-related neurologic and ocular disorders in the absence of rash.

Zoster paresis (weakness) is manifest by arm weakness or diaphragmatic paralysis^{58,602} after cervical distribution zoster, leg weakness after lumbar or sacral distribution zoster, and by urinary retention after sacral distribution zoster.^{283,289} Magnetic resonance imaging (MRI) reveals involvement not only of the posterior horn and posterior roots, but also of the anterior roots and anterior horn at the spinal level corresponding to the clinical deficit.^{240,630} Rarely, clinical deficit in cervical zoster paresis extends to the brachial plexus.⁸⁴ The prognosis varies. In one series of 45 patients with zoster paresis, 67% had near-complete recovery,²³¹ and in another 61 cases, 55% had complete functional recovery.⁶¹⁹

VZV reactivation may also present as meningitis or meningoencephalitis. Many reported cases of VZV encephalitis

may actually be VZV vasculopathy.¹⁹⁹ Recent reports of VZV meningitis,^{234,331} meningoencephalitis,²³⁰ and cerebellitis (gait ataxia and tremor predominated)^{447,520}—all in the absence of rash and confirmed by the detection of VZV DNA or anti-VZV antibody in CSF—reveal that VZV is not an uncommon cause of aseptic meningitis.

VZV produces multiple ocular disorders, including both acute retinal necrosis (ARN) and progressive outer retinal necrosis (PORN) (Fig. 63.16).^{301,663,679} ARN develops in both immunocompetent and immunocompromised hosts, with patients exhibiting periorbital pain and floaters with hazy vision and loss of peripheral vision. PORN presents with sudden painless loss of vision, floaters, and constricted visual fields with resultant retinal detachment. Multifocal, discrete opacified lesions begin in the outer retinal layers peripherally and/or posterior pole; only late in disease are inner retinal layers involved. Diffuse retinal hemorrhages and whitening with macular involvement bilaterally are characteristic findings (Fig. 63.16). Although PORN can also be caused by HSV and cytomegalovirus, most cases are produced by VZV in AIDS patients with CD4+ counts less than 10 cells/mm³²²⁹ as well as in other immunosuppressed individuals.³⁶³ PORN may be preceded by retrobulbar optic neuritis and aseptic meningitis,¹⁷⁴ central retinal artery occlusion or ophthalmic-distribution zoster,⁴¹³ and may occur together with multifocal vasculopathy or myelitis. Optic neuritis after zoster may be bilateral.^{418,566} Reports of visual loss in association with pain on eye movement and clinical findings of papillitis and central scotomata or optic atrophy weeks after VZV infection suggest an immunologic pathogenesis in some cases.⁶⁷ The third nerve is affected more frequently than the sixth as a cause of zoster ophthalmoplegia. Least affected is the fourth, although isolated VZV-associated trochlear nerve palsies have been described.²¹⁸ Combinations of third, fourth, and sixth nerve palsies are not unusual. Like optic neuritis, ophthalmoplegia often appears weeks to months after cutaneous signs.^{149,304}

VZV infection of cerebral arteries (VZV vasculopathy) is a serious complication of VZV reactivation, which causes both ischemic and hemorrhagic stroke. In adults with zoster, the risk of stroke was increased by 30% within the following year²⁹⁹ and 4.5-fold when zoster was in the ophthalmic distribution of the trigeminal nerve.³⁶⁹ VZV vasculopathy affects both immunocompromised and immunocompetent individuals and can present as headache, mental status changes, and focal neurologic deficits. Both large and small vessels are involved.⁴⁵⁶ Lesions at gray-white matter junctions are frequently seen on brain imaging (Fig. 63.16). In more than two-thirds of patients, angiography reveals focal arterial stenosis and occlusion, aneurysm, or hemorrhage. In 30 virologically verified cases of VZV vasculopathy, both large and small arteries were involved in 50%, small arteries in 37%, and large arteries alone in only 13%.

VZV vasculopathy produces bland or hemorrhagic infarctions. Deep white-matter lesions often predominate and are ischemic or demyelinating, depending on the size of blood vessels involved (Fig. 63.16). Case reports have revealed a wide range of vascular pathology, ranging from neointimal proliferation to necrosis with and without inflammation.³³² Infected cerebral arteries contain multinucleated giant cells, Cowdry A inclusion bodies, herpesvirus particles detected by electron microscopy, as well as VZV DNA and viral proteins. VZV

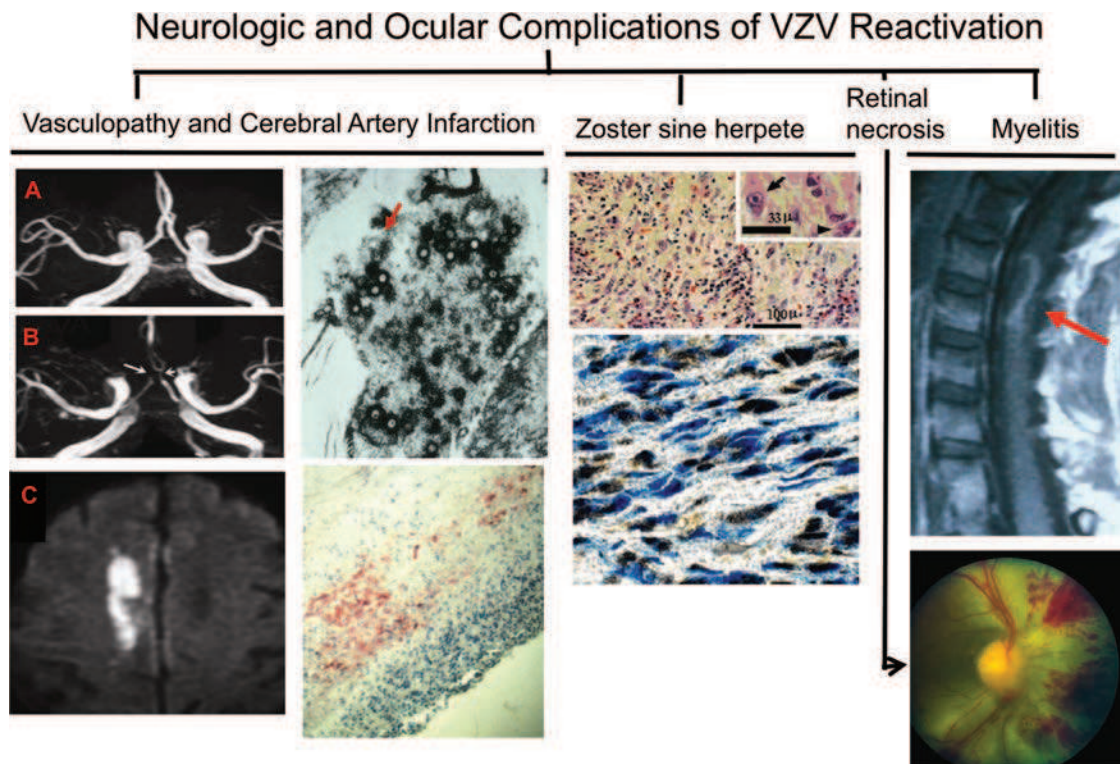


FIGURE 63.16. Neurologic and ocular complications of varicella-zoster virus reactivation. **A:** VZV-induced vasculopathy and infarction. Three-dimensional time-of-flight magnetic resonance angiography of the Circle of Willis 5 months before infarction shows normal anterior cerebral arteries (**a**), while the same analysis at the time of infarction (**b**) shows marked narrowing of anterior cerebral arteries, with a new flow gap at the junction of A1 and A2 segments of the right anterior cerebral artery, indicating occlusion on the right (*long arrow*) and marked stenosis on the left (*short arrow*). (**c**) Diffusion-weighted image (B value 1,000) shows restricted diffusion in the right anterior cerebral artery territory, indicating acute infarction. *Upper right:* Multiple herpes virions within a cerebral artery. *Lower right:* VZV antigen (red) in the media of a cerebral artery of a patient with VZV vasculopathy. **B:** Ganglionitis and intranuclear inclusions in zoster sine herpette. H&E staining of the trigeminal ganglion (*upper panel*) shows widespread chronic inflammation with fibrosis and loss of neurons. Cells in some foci contain Cowdry type A intranuclear inclusions (*arrow, inset*) indicative of virus infection; the inflammatory cells are mainly lymphocytes, with some plasma cells (*arrowhead, inset*). Immunohistochemical staining of the same ganglion (*lower panel*) with mouse monoclonal antibody against VZV IE63 indicates VZV antigen (*brown staining*) in multiple cells throughout the ganglion. Adjacent sections stained with antibody against HSV or with normal rabbit serum were negative (not shown). **C:** Fundus photograph of a patient with VZV vasculopathy and progressive outer retinal necrosis. Note the diffuse retinal hemorrhages and whitening, with macular involvement. **D:** MRI abnormality in a patient with VZV myelitis; note the longitudinal, serpiginous enhancing lesion.

vasculopathy predominantly affects intracranial rather than systemic arteries, possibly due to inherent cellular and structural differences. For example, intracranial cerebral arteries do not contain the external elastic lamina that may affect transmural migration of virus and cells.³⁵⁷ Like horseradish peroxidase, which migrates from the external surface of cerebral arteries to trigeminal ganglia,^{405,406} reactivated VZV may also travel along ganglionic afferent fibers to the adventitia of cerebral arteries, a concept supported by the presence of viral antigen predominantly in the adventitia of cerebral arteries in early VZV vasculopathy.⁵⁴⁵ Elements of VZV vasculopathy that may contribute to stroke include a thickened arterial intima composed of myofibroblasts and cells of medial smooth muscle origin, a disrupted internal elastic lamina, and decreased numbers of smooth muscle cells.⁴⁵⁸

VZV myelopathy, also called postinfectious myelitis, usually presents days to weeks after zoster as a self-limiting, monophasic spastic paraparesis, with or without sensory fea-

tures and loss of sphincter function. The CSF usually contains a mild mononuclear pleocytosis, with a normal or slightly elevated protein. Rarely, VZV myelitis recurs.¹⁹⁸ VZV myelopathy may also be an insidious, progressive and sometimes fatal myelitis, usually in immunocompromised individuals, especially those with AIDS. MRI reveals longitudinal serpiginous enhancing lesions (Fig. 63.16). Pathologic and virologic analyses of the spinal cord from fatal cases have shown frank invasion of VZV in the parenchyma³³² and in some instances, spread of virus to adjacent nerve roots.¹³⁰ Importantly, VZV myelitis may develop without rash. VZV-induced vasculopathy can also produce spinal cord infarction.⁴⁷⁹

Zoster sine herpette is chronic radicular pain without rash. The first two virologically confirmed cases were verified by detection of VZV DNA in CSF.^{198,201} A third case of thoracic-distribution zoster sine herpette, in which electromyography of paraspinal muscles demonstrated frequent fibrillation potentials restricted to chronically painful thoracic root segments,

was confirmed by detection of VZV DNA in blood MNCs and anti-VZV IgG antibody in CSF.¹² In a recent report of a patient with zoster sine herpete, the CSF did not contain amplifiable VZV DNA but did contain anti-VZV IgG, with reduced serum/CSF ratios of anti-VZV IgG indicative of intrathecal synthesis.⁴⁹ The most compelling evidence that persistent radicular pain without rash can be caused by a chronic active VZV ganglionitis came from analysis of a trigeminal ganglionic mass removed from an immunocompetent adult who had experienced relentless trigeminal distribution pain for more than 1 year; pathologic and virologic analyses (Fig. 63.16) revealed that the patient's zoster sine herpete was caused by an active VZV ganglionitis.²⁶⁴ Ocular disorders caused by VZV can also occur in the absence of rash. PORN,^{176,183} severe unremitting eye pain,²¹³ third cranial nerve palsies,²⁶⁵ retinal periphlebitis,⁴⁶⁹ uveitis,^{5,265} iridocyclitis,⁶⁹² and disciform keratitis⁵⁷⁶ have been confirmed to be caused by VZV without rash.

The detection of VZV DNA and anti-VZV IgG antibody with reduced serum/CSF ratios in the CSF of patients with meningoencephalitis, vasculopathy, myelitis, cerebellar ataxia, and polyneuritis cranialis, all without rash, has expanded the spectrum of neurologic disease produced by VZV in the absence of rash. An illustrative example is provided by the report of an immunocompetent 45-year-old woman with multiple episodes of neurologic disease (multifocal vasculopathy, meningoencephalitis, recurrent myelitis and inflammatory brain-stem disease) over an 11-month period produced by VZV in which the CSF contained anti-VZV IgG antibody, but not VZV DNA, throughout her illness.²⁴⁷

DIAGNOSIS

Differential Diagnosis

Varicella must be differentiated from vesicular rashes caused by enteroviruses or *S. aureus*, scabies, drug reactions, contact dermatitis or, rarely, rickettsial pox or disseminated HSV infection. The clinical diagnosis of zoster can be difficult when acute pain and paresthesias precede the cutaneous eruption; the pain can be sufficiently severe to mimic myocardial infarction, cholecystitis, appendicitis, and other conditions. Localized contact dermatitis is the most common alternative etiology. A recurrent vesicular rash in lumbosacral dermatomes is usually HSV infection.

Laboratory Diagnosis

Laboratory techniques are most useful to guide decisions about antiviral treatment for VZV infection, especially for high-risk patients and for the differential diagnosis of neurologic disorders.³⁵⁸ Methods for diagnosing varicella and zoster infections are described in Chapter 17. Briefly, the most sensitive method to identify VZV in any tissue is detection of viral DNA by PCR^{86,196}; VZV proteins can be identified by immunohistochemical analysis of cells from cutaneous lesions prior to crusting. Infectious virus can be isolated from lesion specimens in tissue culture but cultures are often negative, especially if the specimen is not processed rapidly or the lesions are later stage. Assays for VZV IgG antibodies are most useful for determining the immune status of individuals whose clinical history of varicella is equivocal.³⁴⁰ The fluorescent-antibody membrane antigen assay (FAMA) is the most sensitive and specific method and the only method documented to predict susceptibility.¹⁹⁶

Most commercial enzyme immunoassay methods have high specificity, generating few false-positive results, but 10% to 15% of immune individuals may be misidentified as susceptible. Commercial VZV IgM antibody assays often lack specificity and should not be used as the only evidence of VZV-related disease.

If VZV is considered a potential cause of a neurologic disorder without rash—for example, meningoencephalitis with or without cerebellitis, chronic radicular pain, vasculopathy, myelitis, or retinal necrosis—then CSF and blood MNCs should be tested for VZV DNA by PCR and CSF for anti-VZV antibody.^{12,206} VZV DNA is most likely to be found in patients with acute meningoencephalitis and cerebellitis and in some patients with VZV vasculopathy and myelitis. VZV vasculopathy is difficult to diagnose because many patients do not have a preceding zoster rash or CSF pleocytosis; VZV DNA PCR analysis of CSF is only 30% sensitive and symptoms often occur months after zoster.⁴⁵⁷ The best criterion to confirm VZV vasculopathy is detection of anti-VZV IgG and/or anti-VZV IgM in CSF or anti-VZV IgM in serum. Demonstration of the intrathecal synthesis of anti-VZV IgG antibodies indicates chronic active infection⁵²⁴ and is superior to detection of VZV DNA in CSF for diagnosis of VZV vasculopathy, recurrent VZV myelopathy, and zoster sine herpete.^{49,247,457}

TREATMENT

While human leukocyte interferon and vidarabine were the first antiviral treatments with clinical benefit,^{22,414} acyclovir and related drugs (see Chapter 14) are more effective and safer and are now used to treat both varicella and zoster.^{36,145,414,678} Two nucleoside analog prodrugs have been approved in the United States.^{46,626} Valacyclovir is a six-valine ester derivative of acyclovir that is de-esterified by hepatic enzymes to yield acyclovir. Famciclovir is a diacetyl derivative of penciclovir, a guanosine analog, which is enzymatically converted to penciclovir by intestinal and hepatic enzymes. These prodrugs are better absorbed from the gastrointestinal tract than are their parent compounds acyclovir and penciclovir, allowing higher serum levels of drug after oral administration.

Molecular proof of the pivotal role of the VZV TK in the action of acyclovir and penciclovir has come from both the cloning and stable expression of the gene in TK-deficient cells and from sequencing of the VZV gene in acyclovir-resistant viral mutants.^{556,613} Missense mutations in the TK ATP- or nucleoside-binding sites, and mutations clustered between these two sites confer acyclovir resistance. Nonsense mutations leading to expression of grossly truncated VZV TK protein also result in drug-resistant viruses. Rarely, acyclovir resistance can be conferred by mutations in the VZV DNA polymerase. VZV strains that are resistant to both acyclovir and penciclovir retain sensitivity to the pyrophosphate analog, foscarnet.⁵⁵ Cidofovir, an acyclic nucleoside phosphonate, is also active against acyclovir-resistant VZV strains and is an alternative to foscarnet.⁶³⁸

Sorivudine (1-β-D-arabinofuranosyl-E-[2-bromo-vinyl]uracil) is an oral drug with potent *in vitro* activity against VZV and is effective *in vivo* in healthy adults with varicella and HIV-infected patients with zoster, but it is not licensed in the United States because of toxicity resulting from interaction with 5-fluorouracil.^{50,209} Sorivudine also limits SVV infection.⁵⁷⁸ However, the marginal benefit of this drug over acyclovir in HIV-infected

patients with zoster suggests that early administration is more important than potency of the antiviral drug. Whereas all of the licensed antiviral drugs that inhibit VZV replication act on the viral DNA polymerase, other as yet unapproved compounds have different targets. A thiourea compound inhibits VZV by interfering with VZV ORF54 protein, the putative portal protein, and encapsidation of viral DNA.⁶³⁶ Roscovitine, an inhibitor of cyclin-dependent kinases, also inhibits VZV replication in cultured cells.⁶¹⁴ New agents that target host-cell proteins and restrict VZV replication *in vitro* and in human tissue xenografts have also been identified.⁵⁴⁰

Varicella

Acyclovir therapy diminishes the clinical severity of varicella in immunocompromised children by ensuring that cell-associated viremia is terminated, despite an impaired host response. Early therapy prevents progressive varicella and visceral dissemination.⁵⁰⁸ In addition to preventing life-threatening dissemination, early acyclovir therapy minimizes cutaneous disease and may therefore reduce the risk of secondary bacterial infections. Immunocompromised patients who have pneumonia, hepatitis, thrombocytopenia, or encephalitis require immediate treatment with intravenous acyclovir.⁶⁷⁷

Oral acyclovir ameliorates varicella symptoms in healthy children, adolescents, and adults when administered within 24 hours after the appearance of the initial cutaneous lesions.^{10,142,644} Adults with varicella pneumonia, including pregnant women, require immediate treatment with intravenous acyclovir. Acyclovir therapy does not prevent the host from mounting an effective immune response to VZV, consistent with the concept that limited viral replication provides sufficient antigenic stimulation to induce VZV memory immunity.

Zoster

Acyclovir and the related drugs are beneficial therapies for zoster in healthy and immunocompromised patients. Among healthy individuals, treatment initiated within 72 hours reduces the period of continued new lesions in the involved dermatome and the time to complete healing.^{677,685} Antiviral treatment is especially important in immunocompromised patients because of their risk of disseminated disease, and for adults with ophthalmic zoster because of their risk for developing acute uveitis and chronic keratitis. All patients with ocular disease caused by VZV should have an immediate evaluation by an ophthalmologist. Acute neuropathic pain is reduced by early treatment with acyclovir, valacyclovir, or famciclovir; effects on chronic PHN are less obvious. The benefits of antiviral therapy for limiting acute rather than chronic pain suggest that different mechanisms may contribute to these two components of zoster-related pain.⁶⁷⁹

Acyclovir is usually given intravenously to immunocompromised patients who are at high risk for disseminated zoster; less severely immunocompromised patients are given the oral prodrug valacyclovir or famciclovir. Treatment reduces the duration of new lesion formation, acute pain and time to complete healing; some benefit is observed even when therapy is delayed for more than 72 hours because the pathologic process is more extensive in these patients.^{36,303,678} Although acyclovir therapy usually eliminates the risk for visceral dissemination, relapse of zoster occurs in some immunocompromised patients.²⁸⁷ Most

patients respond to treatment with a second course of acyclovir, indicating that relapse reflects an inadequate host response rather than antiviral resistance.^{287,371}

While not life-threatening, PHN is difficult to treat. Neuroleptic drugs and various analgesics, including opiates, are used to alleviate pain, but no universally accepted treatment exists. Drugs include pregabalin, oxycodone, morphine sulfate, tricyclic antidepressants such as amitriptyline, nortriptyline, maprotiline and desipramine, and levorphanol.^{138,538,539} Morphine and gabapentin in combination decreases pain more than either drug alone or placebo.²⁰⁷

Patients with VZV vasculopathy can be treated with intravenous acyclovir and prednisone. Steroids are also used to treat patients with myelopathy,⁵⁰⁴ although some improve spontaneously.⁷⁰ Some patients with myelitis respond to antiviral therapy^{85,129,565}; the benefit of steroids in addition to antiviral agents is unknown. PORN is treated with intravenous acyclovir, steroids, and aspirin followed by oral acyclovir.⁵¹ Intravitreal injections of foscarnet and oral acyclovir have been used in early milder cases, although retinopathy may persist or recur despite acyclovir treatment.²⁹¹ PORN patients given ganciclovir alone or combined with foscarnet had a better final visual acuity than those treated with acyclovir or foscarnet.⁴³² AIDS patients given highly active antiretroviral therapy (HAART) appear to have less risk of PORN.¹⁶

PREVENTION

In most circumstances, preventing VZV transmission to susceptible individuals is difficult because patients with varicella may be contagious for 24 to 48 hours before the onset of their rash. Infection control practices, including caring for infected patients in isolation rooms with filtered air systems, are essential in hospitals that treat patients for whom varicella might be life-threatening.^{354,555}

Passive Antibody Prophylaxis

Varicella zoster immunoglobulin (VZIG) is no longer available; the related product, VariZIG,⁷³ is prepared from high-titer immune human serum. High-dose intravenous immunoglobulin may also be used for passive immunization. VZV antibody prophylaxis is recommended to reduce the occurrence of infection or disease severity in individuals with risk factors for serious varicella and recently exposed to a person with acute varicella or zoster.^{10,73} To be effective, prophylaxis must be given within 96 hours and preferably within 48 hours after exposure.¹⁰ VZV antibody prophylaxis does not eliminate the possibility that primary VZV infection will occur.^{419,704} Even with prophylaxis, the incidence of varicella is higher after household exposures and is related to the VZV IgG titer of the preparation. Passive antibody prophylaxis does not reduce the risk for VZV reactivation in high-risk populations, and passive antibody administration after the onset of illness does not alter the severity of varicella or zoster.⁵⁹⁴

Antiviral Prophylaxis

Administration of acyclovir during the incubation period following exposure of susceptible individuals to VZV can prevent disease if given early; however, the benefit of using acyclovir for prophylaxis has not been compared directly with acyclovir

treatment of individuals in whom the varicella rash has just erupted.^{204,460} Daily administration of oral acyclovir for 12 to 24 months can diminish the risk of zoster in bone marrow transplant recipients,^{620,677} but zoster occurs when acyclovir is discontinued and drug-resistant VZV mutants may emerge. The alternative is prompt initiation of acyclovir for VZV reactivation.⁴⁹⁸

Vaccines

Varicella

The live attenuated varicella vaccine is the first and remains the only human herpesvirus vaccine licensed for clinical use in several countries.^{236,341} Its development and initial clinical evaluation were first reported by Takahashi in 1974.^{610,611} The vaccine virus was derived from a clinical isolate of VZV, the Oka strain, which was propagated in guinea pig embryo fibroblasts and then in WI38 human fibroblasts. In contrast to the outcome in susceptible children inoculated with infectious VZV from vesicular fluid,⁶⁴¹ tissue-culture propagation appears to attenuate the virus so that vaccine containing as much as 17,000 plaque-forming units (pfu) per dose of infectious virus induces immunity to VZV but rarely produces clinical symptoms.⁶⁶⁷ Attenuation is maintained, although varicella vaccine stocks contain mixtures of VZV genomes with varying mutations.⁵⁶² Experiments comparing the parent Oka and vaccine Oka viruses in the SCID mouse model indicate that passage in fibroblasts reduces the ability of the virus to replicate efficiently in human skin, although T-cell tropism and neurotropism are not affected.^{427,707} Less than 5% of children develop skin lesions after vaccination with Oka-derived vaccines; however, the vaccine Oka strain of VZV can be transmitted from these individuals.^{19,145,350,351,427,428} (eFig. 63.9). Whether the virus remains attenuated is not known because of the low frequency of secondary cases. When the vaccine virus was transmitted from a healthy child to a pregnant contact, no laboratory evidence indicated transplacental transmission,⁷¹ consistent with the low risk for wild-type VZV spread across the placenta.

In prelicensure clinical trials, the live attenuated (Oka-Merck strain) vaccine was administered to more than 7,000 children and more than 1,600 healthy, susceptible adults.^{246,366,675} It is now recommended for routine vaccination of infants and susceptible older children and adults in the United States.¹⁰ In the clinical trials, a single dose of vaccine containing 1,000 to 3,000 pfu of attenuated Oka virus induced seroconversion rates of more than 90% in children 12 years of age and younger, and afforded complete protection against disease in about 85% of exposures. On exposure to varicella, some previously vaccinated children experience breakthrough infection, but the resulting disease usually remains mild, with fewer than 50 cutaneous lesions and no associated fever.^{181,284,662} Modified disease severity is consistent with priming of the host response by immunization. The varicella vaccine also induces VZV-specific cellular immunity,^{454,661} which is likely to be essential for its long-term protective efficacy.^{134,570} Varicella immunization elicits T cells that proliferate and produce lymphokines in response to stimulation with the IE62 protein and the viral glycoproteins^{30,43} and induces cytotoxic T cells that can lyse cells expressing VZV proteins.^{290,571} The VZV-specific T-cell response occurs shortly after vaccination, with VZV-specific T cells detectable within 10 to 14 days in most indi-

viduals.²⁹ In contrast, IgG antibodies to VZV were detected in only 40% of children tested at 2 weeks. This early T-cell response may account for the efficacy of vaccination when given immediately after exposure.²⁹ Varicella vaccine is also immunogenic when administered concurrently with measles-mumps-rubella (MMR) vaccines or as a quadrivalent MMR-V vaccine, although the risk of febrile seizures is increased after the first dose of the quadrivalent vaccine.^{155,572}

Achieving high VZV IgG seroconversion rates in susceptible adolescents and adults requires two doses of the vaccine administered at an interval of at least 4 weeks,^{194,454} consistent with the evidence of a diminished host response to primary VZV infection and more severe varicella in adults. However, cellular immunity to VZV in adults given two doses of vaccine was significantly lower compared with children who received a single dose of vaccine, and VZV-specific T-cell proliferation correlated with lower IFN- γ responses.^{290,454} Adults are also more likely than children to have adverse effects to the vaccine, particularly transient local reactions and rash, and to have waning VZV antibody titers. Despite their less robust immune responses, susceptible adults are at risk of developing serious varicella, and protection from or modified severity of the illness by vaccination is documented.¹⁸⁹

The persistence of VZV memory immunity after immunization has been difficult to assess because the continued circulation of wild-type VZV provides opportunities for boosting vaccine-acquired immunity through exogenous exposure. However, VZV IgG and T-cell responses have been documented in 94% to 100% of varicella vaccine recipients for 7 to 10 years after vaccination, and protection has been sustained in most vaccinees.^{13,194,346,464,571,635} In postlicensure surveillance done before vaccine coverage rates were high, no change in the frequency of breakthrough varicella or severity of symptoms with time after vaccination was found; however, those with low or undetectable VZV IgG titers 6 weeks after immunization were four times more likely to experience varicella. Prelicensure studies showed that a two-dose regimen induces higher humoral and cellular immune responses in children evaluated just after vaccination and after 1 year.^{454,495}

Although the incidence of varicella is dramatically lower in all age groups in the United States, the widespread use of the varicella vaccine has meant that as many as half of the cases of varicella in well-vaccinated populations are in immunized children. Because the wild-type virus is transmissible from cases of breakthrough varicella, community outbreaks can be sustained. The observations of the enhanced host response to two doses, along with the surveillance data on breakthrough infections, has led to the use of a two-dose regimen in children as well as in adolescents and adults. Varicella immunization using a two-dose regimen of MMR-V also enhances the primary immune response to the varicella component.⁵⁷²

The Oka-Merck varicella vaccine has also been given to children with acute leukemia in remission, reducing the attack rate after household exposure to 13%.^{192,194} Seroconversion in these patients is associated with a high degree of protection after two doses of vaccine. Cell-mediated immunity is elicited less reliably in leukemic children given varicella vaccine, consistent with the often diminished T-cell proliferation to VZV antigens after natural varicella in immunocompromised children.¹⁹² Because it contains infectious virus, varicella vaccine must be given to these children with careful attention

to the status of their underlying disease and immunosuppressive therapy regimens.²⁷⁷ Vaccine-related rashes occurred at about 1 month after immunization in ~50% of children with leukemia in remission. Rashes occurred at the site of inoculation but also as widely scattered vesicles, indicating that the vaccine virus remains capable of causing cell-associated viremia. This experience is consistent with the finding that the vaccine retains tropism for T cells in the SCID mouse model. In healthy children, the limited replication of the vaccine virus at the site of subcutaneous inoculation likely allows time for induction of adaptive VZV-specific immunity before viremia can occur, whereas vaccine virus may escape from infected epithelial cells into T cells, causing viremia and varicella-like symptoms in some immunocompromised children.¹³ When children with leukemia developed rash, the vaccine virus was transmitted to their healthy, susceptible siblings, but the contacts had mild illness or asymptomatic seroconversion.¹⁹² That vaccine virus was not transmitted unless the immunocompromised child had rash indicates that no significant replication of the vaccine virus occurred at respiratory sites.⁶²² Immunizing susceptible, healthy household contacts of high-risk children may also reduce the risk of household exposure.^{134,546} VZV was detected by PCR in oropharyngeal secretions from 7% of children given varicella vaccine in Japan, but no infectious virus was recovered.⁴⁸⁴

Reactivation of the vaccine strain of VZV has been described in a few healthy vaccine recipients; however, analysis of the incidence of zoster in leukemic children who had vaccine-induced immunity and in those with past natural infection showed that the vaccine virus reactivated significantly less often than wild-type VZV.²⁴² Whether this observation indicates that the vaccine virus is less likely to establish latency or has less potential for reactivation is not known. VZV-specific T-cell responses were higher in leukemic children who had been vaccinated but did not experience recurrent VZV compared with children who developed zoster.³⁵² If symptomatic reactivation does occur, the vaccine virus can be inhibited by acyclovir. Vaccinated individuals can also develop zoster through subclinical infection with wild-type VZV despite vaccine-induced immunity.²³⁷ Information about the prevalence of vaccine or wild-type VZV in neurons after immunization is limited, but VZV was detected by PCR in ganglia from children who died from trauma or nonimmunocompromising conditions.¹⁸⁹ If use of the varicella vaccine in early childhood continues to greatly diminish the annual varicella epidemics, as has occurred in the United States, exposures should be reduced significantly and opportunities for superinfection with wild-type VZV will be minimal. If exogenous exposures are important for maintaining memory immunity in individuals with naturally acquired infection, then zoster might become more common in highly vaccinated populations, although current evidence argues against this possibility.^{357a}

Zoster

The development of the varicella vaccine raised the possibility that vaccination might also be effective for reducing the risk of zoster associated with waning immunity in the elderly.⁴⁸² When high-potency VZV vaccine was given to healthy older adults, VZV-specific responder cell frequencies increased from 1 per 68,000 to 1 per 40,000 in healthy adults over 55 years of age, which was equivalent to the VZV responder cell numbers in naturally immune individuals aged 35 to 40 years.³⁶¹

Administration of the vaccine to elderly individuals was associated with increased production of IFN- γ by T cells stimulated with VZV antigen and recovery of skin test reactivity to VZV antigen in most vaccinees.³⁶ To avoid risks associated with infectious virus, a heat-inactivated preparation of varicella vaccine was given to patients receiving autologous bone marrow transplantation; recovery of VZV T-cell immunity was accelerated and zoster was less common in vaccinated transplant recipients as compared with unvaccinated controls.²⁴⁸

A high-potency preparation of the live attenuated VZV vaccine has now been shown to reduce the morbidity of zoster significantly in healthy adults aged 60 years and older.⁴⁸² This vaccine contains about 14-fold more attenuated Oka virus than the pediatric vaccine. Comparison of vaccinees and placebo recipients in a large randomized trial of almost 40,000 subjects showed a reduction in the burden of zoster illness, considering both incidence and severity of disease, by 61.1% ($P < 0.001$) over a median follow-up period of 3 years. The incidence of PHN was reduced by 66.5% ($P < 0.001$) and zoster incidence decreased by 51.3% ($P < 0.001$) in zoster vaccinees (Fig. 63.15). Mild injection-site reactions were more common in vaccinees compared with placebo recipients, but no significant adverse events attributable to the vaccine were observed. Although the impact of vaccination on zoster occurrence was more substantial in participants aged 60 to 69 years compared with those 70 years and older, both groups benefitted clinically because the older subjects who are more likely to develop PHN experienced less PHN with the vaccine. This study demonstrates that VZV-specific immunity can be boosted in older adults and can reduce the risk of VZV reactivation or prevent its clinical consequences. The vaccine is also approved for zoster prevention in those aged 50 to 59 years based on an efficacy study of 22,000 adults although routine use in this age group is not recommended at this time.^{73a}

PERSPECTIVE

Many new insights about VZV genomics, replication, specific gene functions and virus–host interactions have been gained over the past decade. Infectious VZV genomes can be generated using cosmids and BACs and can be readily mutated to assess gene functions and their regulation. Because the VZV genome can express foreign genes and because the varicella vaccine has proved to be safe, VZV is now being considered for use as a vaccine platform and has been engineered to express Epstein-Barr virus gp350, hepatitis B surface antigen, and HSV-2 gD.^{256,377,573} Recent biochemical and proteomics studies have advanced the understanding of interactions between viral proteins and with host cell proteins^{592,629} and will help to guide new analyses of the molecular mechanisms of VZV replication. Many aspects of VZV–host interactions require further study, such as how the virus takes over host T cells to cause viremia and how VZV reactivates from latency in neurons. While the introduction of antiviral drugs and vaccines to treat and prevent varicella and zoster represent major clinical achievements in VZV control, further insight into VZV replication, the functions of specific viral genes in subtypes of host cells, and viral immune evasion strategies using new tools promises to provide further progress in preventing VZV

morbidity and mortality in healthy populations and safer vaccines for high-risk patients.

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Human Herpesviruses 6 and 7

History

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Perspectives

HISTORY

Human herpesvirus 6 (HHV-6) was discovered in 1986 in patients with lymphoproliferative disorders.²³³ Initially named human B-lymphotropic virus, it was later found mainly to infect and replicate in lymphocytes of the T-cell lineage. HHV-6 isolates are classified into two closely related groups that have been termed variants A (HHV-6A) and B (HHV-6B), and are now recognized as distinct herpesvirus species. HHV-6B is the major causative agent of exanthem subitum (ES),³⁰⁶ but no disease has been clearly associated with HHV-6A.

Human herpesvirus 7 (HHV-7) was isolated in 1990 from a healthy individual whose cells were stimulated with antibody against CD3 and then incubated with interleukin-2 (IL-2).⁹³ The virus is one of the causative agents of ES²⁶⁸ and has been associated with febrile convulsions in young children.²⁹⁸ HHV-6 and HHV-7 are ubiquitous, with more than 85% of adults having antibody to both viruses.

INFECTIOUS AGENT

Classification

Human herpesviruses 6A, 6B, and 7 share many properties with other herpesviruses, including virion structure, many features of genomic and genetic architecture, high prevalence in their natural host, and the ability to establish latent infections in the host. The lytic replication cycle (Fig. 64.1) is generally similar to that for other herpesviruses. These viruses belong to the *Roseolovirus* genus of the betaherpesvirus subfamily, and are characterized by growth in T lymphocytes (although they can also infect other cell types), high prevalence, and association with febrile rash illness. As betaherpesviruses, roseoloviruses are related to cytomegaloviruses (CMV), including having genetically colinear genomes, encoding several subfamily-specific genes, and having protracted replication cycles. In addition to the human roseoloviruses, a virus closely related to HHV-6A and HHV-6B has been identified in chimpanzees¹⁵⁶; a somewhat more distantly related virus has been identified in mandrills.¹⁵⁵

HHV-6A and HHV-6B are closely related, yet molecularly and biologically distinct, viruses (eFig. 64.1) that differ with respect to their cell tropisms, interactions with cells and signaling pathways of the immune system, DNA sequences, and epidemiology.^{1,34,78,126}

Propagation and *In Vitro* Cell Tropism

Human herpesviruses 6A and 6B infect a variety of cultured human cells, such as T cells (preferentially mature CD4+), natural killer (NK) cells, $\gamma\delta$ T lymphocytes, primary fetal astrocytes and dendritic cells, olfactory-ensheathing cells (HHV-6A but not HHV-6B), and peripheral blood mononuclear cells (PBMC). Fresh isolates can be adapted to grow in continuous T-cell lines, such as HSB-2 and J JHAN for HHV-6A, and Molt-3 and MT-4 for HHV-6B. Cell lines derived from megakaryocytes and glioblastomas—as well as neural, epithelial, and fibroblastic cells—can also support limited HHV-6 replication.^{34,69}

HHV-6A has a greater capacity than HHV-6B for replication in cultured cells of neuronal origin.⁷⁰ In primary astrocytes, HHV-6A establishes a productive lytic infection, whereas HHV-6B produces little cytopathic effect and only small amounts of viral nucleic acids, proteins, or infectious progeny, and establishes a long-term, low-level persistent infection.⁷⁹ In an oligodendrocyte cell line, over the course of 30 days HHV-6A transitioned from a lytic state to a quiescent infection that may represent latency (reactivation was not demonstrated), whereas HHV-6B infection was nonproductive and

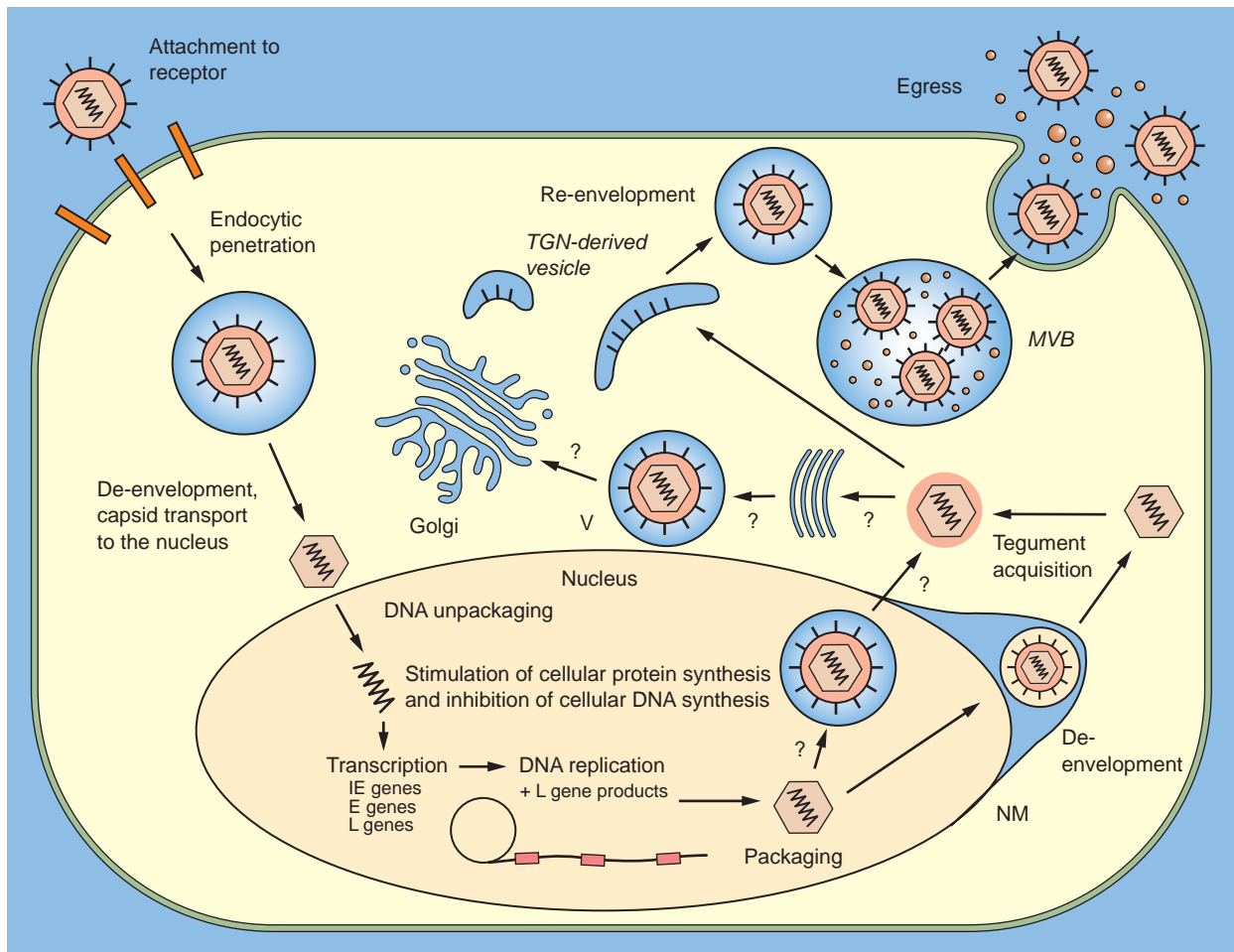


FIGURE 64.1. Stages of replication. The replication scheme is based on published data from refs.^{27,80,170,250,257} A single receptor is shown, although it is likely that the virus employs multiple receptors to enable entry into a wide range of cell types. AL, annulate lamellae; G, Golgi complex; HS, heparan sulfate; Nu, nucleus; NM, nuclear membrane; R, receptor (CD46 for HHV-6A and HHV-6B, CD4 for HHV-7); V, vacuole, MVB (multivesicular body).

did not persist.⁷ In some cell types, the HHV-6 genome integrates into a host-cell telomere (chromosomally integrated, or ciHHV-6), establishing a quiescent form of persistence that may represent a form of latency.¹⁶

In contrast, HHV-7 has a narrow *in vitro* cell tropism, thus far being restricted to primary phytohemagglutinin (PHA)-stimulated CD4⁺ T cells obtained from umbilical cords or PBMC, and an immature continuous T-lymphoblastoid cell line, SupT-1.²⁸ HHV-6 and HHV-7 induce similar cytopathic effects, characterized by ballooning refractile cells (Fig. 64.2).

Virion Structure

Roseoloviruses share the standard features of herpesvirus virions: an icosahedral capsid 90 to 110 nm in diameter that contains the 145- (HHV-7) and up to 170-kb (HHV-6) double-stranded DNA (dsDNA) genome, a tegument that surrounds the capsid, and a lipid bilayer envelope studded with virus-specified membrane proteins and glycoproteins. Enveloped extracellular virions are 160 to 200 nm in diameter^{24,25,144,312} (Fig. 64.3). By thin-section electron microscopy (EM), HHV-6 teguments are smooth and fill the space between the capsid and envelope,

whereas the HHV-7 tegument has an electron lucent space between the tegument and envelope.^{25,144} Immunoelectron microscopy with a monoclonal antibody (mAb) against a tegument protein encoded by the HHV-7 *UI4* gene showed reactivity primarily at the outer periphery of the tegument.²⁵³ Virions consist of at least 29 polypeptides, including 6 in the envelope fraction and cellular p53.^{34,265} There are at least three distinct sets of glycoproteins: gp105k and gp82k; gp116k, gp64k, and gp54k; and gp102k.

Genome Structure and Organization

Genomic Architecture

As packaged in nucleocapsids, roseolovirus genomes are composed of a central unique segment (U) bracketed by a pair of direct repeat structures, DRL and DRR (Fig. 64.4; eFig. 64.2; Table 64.1).^{78,163,176} Some forms of HHV-6 intracellular replicative intermediates do not have duplicated DR segments; the unit form of these concatemeric or circular genomes consists of one copy of the U segment and a single DR.³¹ From left to right, HHV-6 DRs consist of 25 to 29 base pairs (bp) of unique sequence, a conserved *pac1* (cleavage and packaging) element,

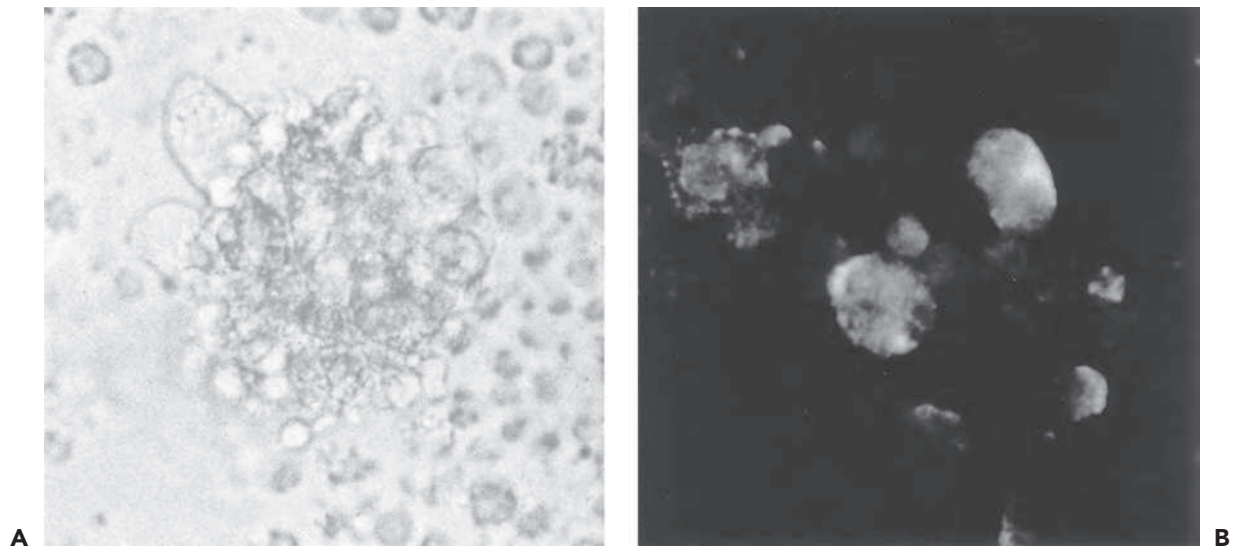


FIGURE 64.2. Cytopathic effect by HHV-6B. **A:** Balloon-like cells surround clumps of lymphocytes with positive staining by immunofluorescent antibody (IFA). **B:** Balloon-like cells stained by IFA using convalescent serum from a patient with exanthem subitum. Similar cytopathic effects and staining are seen for HHV-6A, HHV-6B, and HHV-7.

an array of heterogeneous long terminal repeats (LTR) based on the sequence TA(A/G)(C/G)(C/T)C that span about 300 bp in laboratory-passaged strains and up to 3 kb in low-passage viruses, about 7 kb of unique sequence that encodes a handful of genes, an array of sequences similar to those present at the telomeres of mammalian chromosomes (R-TRS, [TAACCC]_n), a

conserved pac2 element, and 16 to 18 bp of unique sequence (eFig. 64.3). The length of the telomeric sequence can vary from isolate to isolate and is stable during serial passage and over time in humans.³ The HHV-7 R-TRS is more heterogeneous than for HHV-6 (eFig. 64.3).²²⁷ Despite the differences, HHV-7 cleavage and packaging signals are recognized and

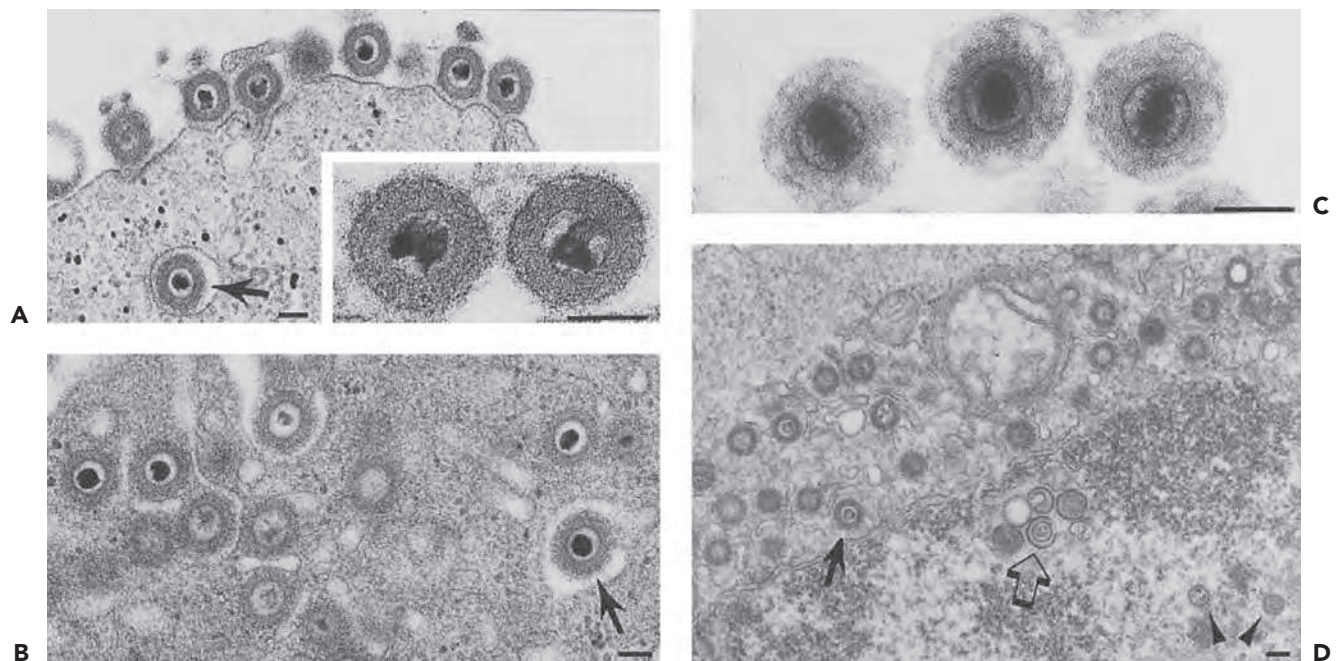
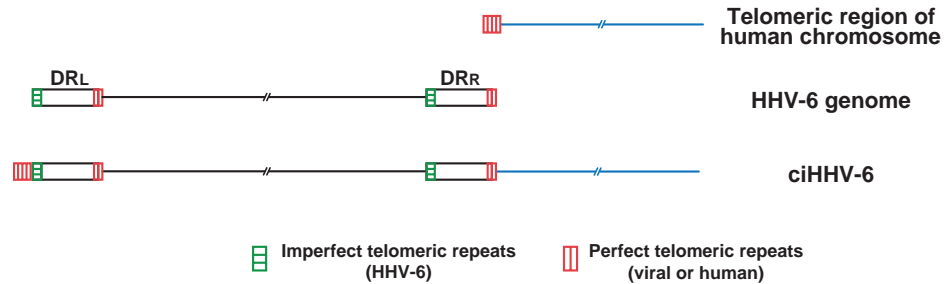


FIGURE 64.3. Ultrastructural characteristics of HHV-6B(Z29) and HHV-7(SB) grown in human umbilical cord blood lymphocytes. **A:** HHV-6B virus particles accumulated along the plasma membrane and within a vesicle (*arrow*) of an infected cell. **Inset:** Detail of HHV-6B virions. **B:** Tegument-coated nucleocapsids of HHV-6 within the cytoplasm and apparently budding into the cisternae of the Golgi apparatus (*arrow*). **C:** Extracellular HHV-7 virions. **D:** HHV-7 nucleocapsids within the nucleus (*arrowheads*), accumulated within the perinuclear space (*open arrow*), and surrounded by tegument within the cytoplasm (*arrow*). Bars, 100 nm. (Courtesy of C. S. Goldsmith, C. Lopez, P.E.P., and J. B. Black, Centers for Disease Control and Prevention.)

FIGURE 64.5. Structure of ciHHV-6

genomes. (Adapted from Arbuckle JH, Medveczky MM, Luka J, et al. The latent human herpesvirus-6A genome specifically integrates in telomeres of human chromosomes in vivo and in vitro. *Proc Natl Acad Sci U S A* 2010;107:5563–5568.)



ORF (Fig. 64.4 and eFig. 64.2). The HHV-7 genome is more compactly arranged than that of HHV-6, with a similar genetic complement being encoded in a U segment that is 12 kb shorter than for HHV-6 (Table 64.1). In addition to the repeat arrays, two genomic regions have no obvious protein coding capacity: the region between U41 and U42 that harbors the origin of lytic DNA replication (oriLyt), and the region between U77 and U79.

Many of the genes encoded in the central portion of roseolovirus genomes are conserved among the alpha-, beta-, and gammaherpesviruses^{78,101,180,198} (Table 64.2; Fig. 64.4; eFig. 64.2; and Chapter 59). A betaherpesvirus-specific gene cluster is located to the left of the core genes. Clusters of genes unique to roseoloviruses flank the core genes.

Over the conserved domains, roseoloviruses are genetically colinear with cytomegaloviruses, although the cytomegaloviruses encode many genes with no roseolovirus counterpart. Between HHV-6 and HHV-7, amino acid sequence identities range from 22% to 75%, most being approximately 50%. HHV-6A and HHV-6B have overall nucleotide sequence identity of 90%; the portions of the genomes that span U32 to U77 are highly conserved (95% identity), whereas the segment spanning U86 to U100 is only 72% identical.⁷⁸

Intraspecies sequence variation is very low. Across segments spanning several kilobases, HHV-6A strains differ by less than 0.5%, although there are short segments of greater variability.³⁴ HHV-6B strains HST and Z29 differ by less than 1% across their entire genomes. Length differences in the telomere-like sequence can be used for strain tracking.³ Other than repeat copy number differences, HHV-7 strains JI and RK differ by only 179 bp (0.1%) over their genomes. Conservation can be even higher at the amino acid sequence level.^{90,221} Subgroups of HHV-6B and HHV-7 isolates have been identified at some genomic loci.^{54,90}

Roseoloviruses differ somewhat from each other in their genetic complement. Nine HHV-6A ORFs do not have HHV-6B counterparts, and nine HHV-6B ORFs have no HHV-6A counterpart.⁷⁸ Interpretation of these differences is difficult because somewhat different criteria have been employed to predict functional ORF of the sequenced viruses. In addition, in the absence of clear similarity with genes from other herpesviruses or in the sequence repositories, such predictions must be considered provisional pending experimental study. Of six B variant-specific genes analyzed, transcripts were detected across all six, with three (B3, B6, and B7) being expressed as immediate early (IE) genes.²¹⁰ Thus, at least some of the variant-specific genes are functional.

DR3, U6, U22, U83, and U94 are encoded by HHV-6A and HHV-6B, but not HHV-7. U94 is a homolog of the

parvovirus ns1/rep gene. A related protein is encoded adjacent to a U95 homolog in rat CMV; however, in addition to not being encoded by HHV-7, neither human nor murine CMV encode a U94 homolog.²⁹¹ HHV-7 encodes a duplicated gene (U55A and U55B) that is encoded as a single copy by HHV-6A and HHV-6B. Genes unique to roseoloviruses include U20, U21, U23, U24, U24A, U26, and U100.

Although no amino acid sequence similarity has been identified, the general layout and splicing patterns of two human cytomegalovirus (HCMV) immediate-early (IE) regions are conserved in the roseoloviruses. Thus the roseolovirus IE-B domain that spans U17 to U19 corresponds to the HCMV UL36 to U38 IE locus, and the IE-A domain spanning U86 to U90 corresponds to the HCMV major IE locus.^{175,198} Every betaherpesvirus encodes several members of a family of duplicated and diverged genes that are related to HCMV US22 and are referred to as the US22 gene family.¹⁹⁸ For HCMV, some US22 family members are transcribed as IE genes and encode transactivating functions. In the roseoloviruses, DR2, DR6/7, U2, U3, U5, U7 (U5/7 in HHV-7), U8, U17/16, U25, and U95 are all members of this gene family. At least four of the HHV-6A US22 homologs encode transcriptional transactivators (DR6, U3, U16, and U25).

HHV-6 has been subject to little direct genetic experimentation. Kondo et al.¹⁵⁰ were able to replace the HHV-6B U3-U7 gene cluster with a selection marker, demonstrating that these genes are not essential for virus replication or latency in cultured cells. HHV-6A genomes have been cloned as bacterial artificial chromosomes (BACs) in three laboratories. One of the BACs is capable of regenerating infectious virus after transfection into susceptible human cells, and has been used to identify glycoprotein Q1 (gQ1) as an essential protein.²⁷³ The other two BACs expressed some viral genes and could be maintained in transfected cells under antibiotic selection, but did not produce infectious virions.^{16,31}

Stages of Replication

Entry

A cellular receptor for HHV-6,²³⁵ CD46, is also a member of the regulator of complement fixation family and is expressed on the surface of all nucleated human cells; it is also a cellular receptor for measles virus. Evidence for the CD46 receptor activity includes (a) downmodulation of CD46 expression in activated primary human CD4+ lymphocytes infected with HHV-6A or HHV-6B; (b) inhibition of HHV-6 infection and associated cell fusion by mAb against CD46 and by soluble CD46; and (c) nonhuman cells being rendered susceptible to HHV-6-mediated membrane fusion and HHV-6 entry by expression of CD46 (reviewed in 274). The short consensus repeats 2 and

TABLE 64.2 HHV-6 and HHV-7 Genes of Known or Implied Functions and Their HCMV Homologs

HHV-6 ORF	HHV-7 ORF	HCMV ORF	Gene block	Kinetic class	Properties or implied functions
DR1	DR1				HCMV US22 gene family
DR6	DR6				HCMV US22 gene family, transactivator
U2	U2	UL23	Beta	U	HCMV US22 gene family
U3	U3	UL24	Beta	E	HCMV US22 gene family, transactivator
U7	U7	UL27/UL28	Beta	E	HCMV US22 gene family
U11	U11	UL32	Beta	L	Antigenic tegument protein; HHV-6A p100, HHV-6B 101K, HHV-7 89K 8989K89K
U12	U12	UL33	Beta	E	G-protein coupled receptor
U14	U14	UL25	Beta	E	Antigenic tegument protein; HHV-7 pp85, HCMV UL25/UL35 gene family
U15	U15		Beta	E	HCMV UL25/UL35 gene family
U17	U17	UL36	Beta	E	HCMV US22 gene family, IE-B
U18	U18	UL37EX3	Beta	E	IE-B
U19	U19	UL38	Beta	E	IE-B
U20	U20			E	Glycoprotein
U21	U21			Bi	Glycoprotein
U22				L	Glycoprotein
U23	U23			L	Glycoprotein
U25	U25	UL43		E	HCMV US22 gene family
U27	U27	UL44	1	E	Polymerase processivity factor (PA)
U28	U28	UL45	1	E	Ribonucleotide reductase large subunit (RR)
U29	U29	UL46	1	L	Capsid assembly and DNA maturation
U30	U30	UL47	1	L	Tegument protein
U31	U31	UL48	1	L	Large tegument protein (Teg)
U32	U32			L	Capsid protein
U33	U33	UL49	1	E	Virion protein
U34	U34	UL50	1	E	Membrane-associated phosphoprotein
U35	U35	UL51		E	DNA packaging
U36	U36	UL52	1	E	DNA packaging
U37	U37	UL53	1	E	Putative phosphoprotein
U38	U38	UL54	2	U	DNA polymerase (Pol)
U39	U39	UL55	2	E	Glycoprotein B (gB)
U40	U40	UL56	2	E	Transport/capsid assembly (TP)
U41	U41	UL57	2	E	Major DNA binding protein (MDBP)
U42	U42	UL69	3	E	Transactivator
U43	U43	UL70	3	L	Helicase/primase complex (HP)
U46	U46	UL73	3	L	Membrane protein
U47	U47	UL74		L	Glycoprotein O (gO)
U48	U48	UL75		L	Glycoprotein H (gH)
U49	U49	UL76	4	L	Putative fusion protein
U50	U50	UL77	4	L	DNA packaging
U51	U51	UL78		E	G-protein coupled receptor
U53	U53	UL80	4	L	Proteinase
U54	U54	UL82/UL83		L	Virion transactivator
U56	U56	UL85	5	L	Capsid protein
U57	U57	UL86	5	L	Major capsid protein (MCP)
U64	U64	UL93	6	E	DNA packaging; tegument protein
U65	U65	UL94	6	L	Tegument protein
U66	U66	UL89	6	L	Putative terminase
U69	U69	UL97	6	E	Phosphotransferase; ganciclovir kinase
U70	U79	UL98	6	E	Alkaline exonuclease (Exo)
U71	U71			E	Myristylated virion protein
U72	U72	UL100	6	L	Glycoprotein M (gM)
U73	U73			E	Origin binding protein (OBP)
U74	U74	UL102	6	E	Helicase/primase complex (HP)
U76	U76	UL104	6	L	DNA packaging, virion protein

(continued)

TABLE 64.2 HHV-6 and HHV-7 Genes of Known or Implied Functions and Their HCMV Homologs (*Continued*)

HHV-6 ORF	HHV-7 ORF	HCMV ORF	Gene block	Kinetic class	Properties or implied functions
U77	U77	UL105	6	U	Helicase/primase complex (HP)
U79	U79	UL112/ UL113		IE	DNA replication
U81	U81	UL114	7	E	Uracil-DNA glycosylase
U82	U82	UL115	7	E	Glycoprotein L (gL)
U83				Bi	Interocrine cytokine
U85	U85			IE	OX-2 homology, glycoprotein
U86	U86	UL122		U	IE-A
U90	U90			IE	IE-A (IE1), transactivator
U91	U91			IE	IE-A
U94				E	Parvovirus <i>rep</i> homolog (Rep)
U95	U95			IE	HCMV US22 gene family
U100	U100			L	Spliced envelope glycoprotein (HHV-6 gp82-gp105, HHV-7 gp65)

Implied functions of homologous genes are based on previous analyses.^{53,78,101,126,180,198,286}
HHV, human herpesvirus; HCMV, human cytomegalovirus; ORF, open reading frame; IE, immediate early.

3 of the CD46 ectodomain are required for HHV-6 receptor activity.¹⁰⁵ HHV-6A can mediate fusion from without (FFWO; fusion that does not require viral protein synthesis) in a variety of human cells,¹⁹¹ an activity that is dependent on CD46 expression. HHV-6B strain HST does not mediate FFWO but strain PL-1 can.²¹³ mAb against gB and gH inhibit HHV-6 infection and FFWO,¹⁹¹ demonstrating that gH and gB are critical for virus entry and probably have fusogenic activity. HHV-7, but not HHV-6, uses CD4 as a receptor, although the virus can infect cells that do not express detectable CD4.²⁸

The human immunodeficiency virus type 1 (HIV-1) co-receptors, CXCR4 and CCR5, have been studied to determine whether they serve similar functions for the roseoloviruses. Cells from individuals lacking CCR5 were able to support growth of all three viruses, and these individuals were seropositive for the viruses, indicating that this molecule is not essential for viral replication.³⁰⁸ Data for CXCR4 are conflicting. An mAb against CXCR4 did not inhibit infection by any of the viruses,^{244,308,325} nor did a specific CXCR4 antagonist that can inhibit HIV-1 infectivity.³²⁵ Nonetheless, the natural CXCR4 ligand, SDF-1 α , inhibited HHV-7 infection at 2.5 mg/mL²⁴⁴ but not at 1.0 mg/mL.³²⁵

On the virus side, two HHV-7 glycoproteins have been identified as being able to bind cell surface proteoglycans heparan and heparan sulfate: gB, and the spliced glycoprotein encoded by U100.^{218,243,248} Other virion proteins are likely involved in receptor interactions or entry, such as the gH-gL complex, which along with products of U100, are targets for complement-independent neutralization.^{34,69} gQ1, a spliced product of the U97, U98, U99 and U100 ORFs, is unique to roseoloviruses.²⁷⁴ The gene is expressed via complex splicing, producing a number of envelope-associated polypeptides. The HHV-6 gH-gL complex associates with the 80-kd virion envelope form of gQ1 (gQ-80K; gQ1). A different small transcript from *U97* gene encodes an unrelated protein of 37 kd (gQ-37K; gQ2) that interacts with the gH/gL/gQ1 complex in infected cells and virions.⁸ Interaction of the virion with the CD46 receptor is via the gH/gL/gQ1/gQ2 complex (Fig. 64.6).^{8,193,234}

HHV-6A strains GA and U1102, and HHV-6B strain PL-1, bind CD46; HHV-6B strain HST does not. HHV-6 U47 is a positional homolog of the HCMV glycoprotein O (gO) gene. U47 encodes a component of a different envelope complex that also includes gH/gL, but is not a CD46 ligand and likely plays a different role from gH/gL/gQ1/gQ2.¹⁸⁸ gQ and gO have much greater sequence divergence between HHV-6A and HHV-6B than do other glycoproteins (76.8% and 72.1% identity, respectively), suggesting that they contribute to the biologic differences between these viruses. Lipid rafts are critical for HHV-6 entry. Depletion of cholesterol from virion and cell membranes inhibits virus infection,¹¹⁸ and HHV-6A binding to cells triggers rapid redistribution of CD46 to lipid rafts.^{190,272} Envelope glycoproteins are enriched in raft fractions and raft components are incorporated into virions.¹³⁶

Transcription

Herpesvirus genes generally belong to one of two main categories: latent or lytic. There are three general kinetic classes of

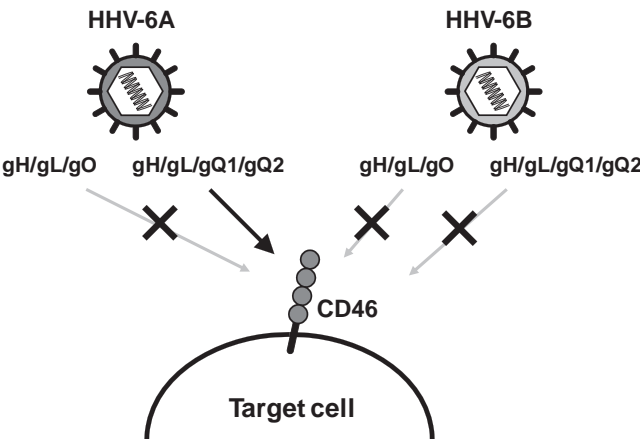


FIGURE 64.6. Virion-receptor interactions.

lytic genes: immediate early (IE) genes that provide functions for regulation of gene expression, early (E) genes that encode proteins needed for DNA replication, and late (L) genes that encode virion proteins.

Roseolovirus gene expression follows the general program of other herpesviruses. For HHV-6B, 8 genes are expressed as IE, 44 as early, 44 as late, and 4 were biphasic²⁸⁶ (Table 64.2). For HHV-6A and HHV-6B, U83 and U89/90 encode experimentally proven IE genes (expression not dependent on *de novo* protein synthesis).^{91,127,221,236} For both loci, multiple differently regulated transcripts are expressed (eFig. 64.4). The IE transcript of U83 is spliced and expresses a truncated version of the chemokine encoded by U83, whereas the full-length transcript is expressed as a late gene.^{91,328} U42 encodes a homolog of the HSV IE gene $\alpha 27$ and HCMV UL69, which is regulated as an E gene; HHV-6B U42 is not transcribed in the absence of *de novo* protein synthesis; thus, its regulation is similar to that of its HCMV counterpart.²²¹ Expression of the U90 IE gene rose to more than 10 copies per cell within 8 hours, whereas a structural gene transcript (U11) took 16 hours to reach comparable levels²²¹. At 72 hours after infection, U11 was expressed at several hundred copies per cell. Transcripts of the gene needed for initiation of DNA replication (U73) and the parvovirus ns1/rep homolog (U94) were expressed at much lower levels (<10 copies/cell).

Most transcripts expressed by these viruses are not likely to be spliced, although several are, some more than once.^{219,248} In addition, the use of some splice sites is kinetically regulated^{181,186,236,328}; others use noncanonical donor and acceptor sequences.^{91,328} Megaw et al.¹⁸⁰ provided detailed predictions of splicing patterns.

The HHV-6 R3 repeat element (R2 in HHV-7) is a potential IE enhancer. R3 is located about 300 bp upstream of the oppositely oriented U89/90 and U95 genes; it is present in more than 25 copies per genome (Table 64.1) and contains potential transcription factor-binding sites (nuclear factor- κ B [NF- κ B] and AP2 in HHV-6A; PEA3 and AP2 in HHV-6B; and PEA3 in HHV-7). U95 has amino acid sequence similarity with the murine CMV (MCMV) IE2 gene and is a member of the HCMV US22 gene family. Deletion of three R3 units decreases promoter activity 15-fold, and NF- κ B family members p50 and c-Rel bind to NF- κ B sites in R3.²⁶⁷ The HHV-6 major immediate early gene (U90–89) promoter (MIEp) is highly active in T cells; its activity is reduced in the absence of intron 1. The NF- κ B-binding site in the the R3 repeat is critical for this activity.¹⁷⁷

Promoters of the genes encoding the HHV-6A DNA polymerase (U38) and its processivity factor (U27) were active in infected, but not in uninfected, cells; thus, they have virally responsive elements. The U38 promoter is TATA-less, and its activity is dependent on a palindromic activating transcription factor (ATF)/CREB-binding site.⁵

Four latency-associated transcripts from the IE1/IE2 locus are oriented in the same direction as the IE1/IE2 genes and share their protein-coding regions with IE1/IE2, but have latency-specific transcription initiation sites and exons.¹⁵¹ In addition, transcripts from the U94 gene have been detected in primary PBMC that were polymerase chain reaction (PCR)-positive for HHV-6 DNA, and negative for other lytic gene transcripts.²²⁹ If a role for U94 in HHV-6 latency is identified, this would constitute a significant biologic difference from HHV-7 because

HHV-7 does not encode a U94 homolog. Hints to U94 function may come from studies of rat CMV, which encodes a U94 homolog that binds single- and dsDNA and is not essential for virus replication.²⁸⁹

Translation and Transport

HHV-6 U94 is expressed as a low copy number-spliced transcript that encodes several small AUG-initiated ORF upstream of the U94 ORF.²²¹ None of the initiation codons, including that for U94, has the hallmarks of being efficiently translated. This suggests that in addition to tight control of its transcription, U94 is translationally regulated to prevent high expression.

HHV-6 U51 encodes a G protein-coupled receptor homolog that is expressed on the surfaces of transfected T-cell lines but in other cell lines accumulates intracellularly without being transported to the cell surface,¹⁸² suggesting the presence of a cell-specific transport or processing function.

Virally Encoded Transcription Regulators

Roseoloviruses encode genes that regulate transcription of viral and cellular genes. The regulators that have been studied are encoded by the HHV-6A IE-A and IE-B loci, DR6, U3, U27, and U94. Positive regulatory effects have been seen by transfection or infection in a variety of cell types on heterologous viral promoters, including the HIV-1 and human T-cell lymphoma 1 (HTLV-1) LTR, Epstein-Barr virus latent membrane protein 1 (LMP-1) and Epstein-Barr virus nuclear antigen 2 (EBNA-2), HSV gD, human papillomaviruses 16 and 18, and the adenovirus E3 and E4 genes.^{34,178,192} In addition, negative regulatory effects have been observed on cellular promoters for p53, H-ras, and CXCR4,^{14,133,308} along with mixed effects on the CD4 promoter (positive by HHV-6A and negative by HHV-7).^{87,241} Upregulation of the HIV-1 LTR is variously dependent on the presence of the NF- κ B, AP-1, trans-acting response (TAR), and SP1 sites, whereas its suppression by U94 is independent of NF- κ B and SP1 but dependent on the TAR element.

Functions of Immediate Early Gene Products

HHV-6 IE-A consists of two genetic units, IE1 and IE2, corresponding to U90 to U89 and U90 to U86/87, respectively.²¹² IE2 proteins with molecular masses of 100, 85, and 55 kd are detectable 3 days after infection, whereas IE1 proteins of >170 kd are detectable within 8 hours.²¹² IE2 is expressed via differential splicing and alternative translation initiation. IE2 proteins show a mixed cytoplasmic and nuclear localization pattern. At earlier times (8 to 48 hours after infection), it is present as intranuclear granules, whereas at later time points (72 to 120 hours after infection), IE2 coalesces into a few large immunoreactive patches. IE2 can induce the transcription of a complex promoter, such as the HIV-LTR, as well as simpler promoters, whose expression is driven by a unique set of responsive elements (*cis*-acting replication element [CRE], nuclear factor of activated T cells [NFAT], NF- κ B).¹⁰⁴ Moreover, minimal promoters having a single TATA box or no defined eukaryotic regulatory elements can be activated by IE2, suggesting that it plays an important role in initiating the expression of several HHV-6 genes as a promiscuous transactivator.²⁸² HHV-6 IE2 interacts with the heterogeneous nuclear ribonucleoprotein K (hnRNP K) and the beta subunit of casein kinase 2 (CK2 β).¹⁸⁹ Although IE2 interacts with Ubc9, the protein responsible for conjugation of Sumo to target protein, IE2 does not undergo sumoylation,

and the interaction negatively affects its transactivator function.²⁸³ U95 interacts with the mitochondrial GRIM-19 protein that promotes cell death induced by IFN- β and retinoic acid.³¹¹ Short-term HHV-6B infection of MT-4 T-lymphocytic cells induced syncytial formation, resulted in decreased mitochondrial membrane potential, and led to progressively pronounced ultrastructural changes, such as mitochondrial swelling, myelin-like figures, and a loss of cristae. The high affinity between U95 and GRIM-19 may be closely linked to the detrimental effect of HHV-6B infection on mitochondria.

HHV-6 IE1 stably interacts with PML-bodies [also known as ND10 or nuclear promyelocytic leukemia protein (PML) oncogenic domains (POD)].²⁵¹ Remarkably, PML bodies remain structurally intact and associate with the IE1 protein throughout lytic HHV-6 infection, unlike other herpesviruses, which trigger PML-body dispersal. IE1 is covalently modified by conjugation to the small ubiquitin-like protein, SUMO-1,²⁵¹ which does not influence POD targeting.¹⁰³

HHV-6 U16 is in the IE-B region of the genome. The protein-coding region of one transcript is generated by an in-frame splicing of ORF U17 and U16, and another includes ORFs U16 and U15. A third differentially spliced complementary DNA (cDNA) (U16+) was identified by 5' rapid amplification of cDNA ends. These transcripts arise from at least two transcription initiation sites; the U17/U16 spliced transcript is expressed under IE conditions, and a multiply spliced gene product encoded by U16 is expressed as a late gene. The U17/U16 and the U16+ gene products can transactivate the HIV LTR.⁸⁹

Functions of Other Virally Encoded Gene Products

The U3 to U7 gene cluster is not essential for virus replication or latency in cultured cells.¹⁵⁰

ORF U11 encodes p100 and 101K, the HHV-6A and HHV-6B homologs of HCMV pp150.^{197,216} The protein is a tegument protein and is the dominant antigen recognized by HHV-6-specific IgG in immunoblots. The HHV-7 U11-encoded counterpart, p86, is one of the major HHV-7 antigens.²⁵² HHV-7 U14 encodes pp85, an 85-kD, tegument-associated phosphoprotein that is also a major antigen.²⁵²

gB (encoded by U39) is a conserved herpesvirus glycoprotein that is found on virions of most herpesviruses, including roseoloviruses. It has roles in attachment and penetration and is a target for complement-independent neutralization. The primary HHV-6A gB translation product of about 112 kD is proteolytically cleaved to form disulfide-bond-linked subunits of 64 kD and 58 kD; for HHV-6B and HHV-7, the corresponding products were approximately 102, 59, and 50 kD; and 112, 63, and 51 kD, respectively. As with the gB of other herpesviruses, the extracellular domain of HHV-7 gB binds to cell-surface heparan sulfate proteoglycans, indicating a role in adsorption of the virion to cells.²⁴³

HHV-6 U42 is a homolog of HCMV UL69. Like pUL69, pU42 homodimerizes and shuttles from between the nucleus and cytoplasm, but pU42 does not interact with the cellular DExD/H-box helicases UAP56 and URH49, and thus does not export mRNA.³²⁷

As for other herpesviruses, the conserved virion glycoproteins gH and gL (encoded by ORFs U48 and U82, respectively), heterodimerize and together have roles in membrane fusion associated with both virus entry and cell-to-cell spread.²⁷⁴

As mentioned, the HHV-6 gH–gL complex forms independent envelope complexes with either gQ1/gQ2 or gO.^{42,188} gQ1 is essential for HHV-6A replication.²⁷³

The proteinase required for capsid maturation, DNA packaging, and virus assembly is encoded by U53. The HHV-6 proteinase, like its homologs in other herpesviruses, is translated as a precursor molecule that is autolytically processed at two sites (the release and maturation sites) to produce the mature proteinase and assembly proteins. As in the other herpesviruses, the processing sites consist of an Ala-Ser sequence. In contrast to the other herpesviruses, roseoloviruses lack an internal autocatalytic cleavage site (Ala-Ala instead of Ala-Ser), which results in their proteinase being a single-chain enzyme.²⁷⁹

The HHV-6 U69 gene product (pU69) is the presumed functional homolog of HCMV UL97-encoded kinase (pUL97), which converts ganciclovir to its monophosphate metabolite in HCMV-infected cells.^{12,68,174,231} The efficiency of ganciclovir phosphorylation in HHV-6-infected cells, and by vaccinia virus-expressed pU69, however, is relatively poor.

Viral DNA replication was reduced 50-fold, and cytopathic effects were also inhibited in T cells stably expressing short interfering RNA (siRNA) specific for the HHV-6 G-protein-coupled receptor (GPCR) homolog encoded by U51, suggesting that HHV-6 U51 is a positive regulator of virus replication.³²⁶

The U94 genes of HHV-6A and HHV-6B encode homologs of the parvovirus ns1 or rep gene, which is essential for parvovirus DNA replication (see Chapter 57). The Rep68 protein of adeno-associated virus type 2 (AAV-2) is a site-specific adenosine triphosphate-dependent endonuclease and helicase, involved in site-specific integration of the viral genome into the host genome. HHV-6A U94 can serve as a helper for AAV-2 replication, and U94 can complement a point mutant of AAV-2 rep.²⁷⁸ As mentioned, U94 transcripts have been detected in PBMC during latency,²²⁹ and HHV-7 does not encode a U94 homolog, although rat CMV does. Activities associated with U94 include suppression of H-ras and bovine papillomavirus 1 (BPV-1) transformation and transcription from the H-ras and HIV-1 LTR promoters¹⁵; the ability to bind to single-strand DNA (ssDNA)⁷⁷ and to human TATA-binding protein¹⁸⁹; and the ability to inhibit the replication of HHV-6 and other betaherpesviruses (HHV-7 and HCMV), but not HSV.^{42,229} This confirms the activity of U94 in the regulation of HHV-6 replication and has implications for co-reactivations and latency of human betaherpesviruses.⁴² In addition, expression of U94 in cultured endothelial cells inhibits the formation of capillary-like structures, reduces cell migration, and blocks angiogenesis.⁴⁰

Direct repeat 6 (DR6) is a nuclear protein that interacts with the U27-encoded viral DNA polymerase processivity factor, p41.²³⁷ HHV-6A lineages that spontaneously lost the genomic segment spanning DR1 through the first exon of DR6 appear to have a growth advantage in at least some cell lines.³³

Replication of Genome

NUCLEOTIDE METABOLISM

Roseoloviruses encode several enzymes that have roles in nucleotide metabolism and provide the precursors needed for nucleic acid synthesis: ribonucleotide reductase, a phosphotransferase, alkaline exonuclease, and uracil-DNA glycosylase, but do not encode a thymidylate synthase or thymidine kinase.

Roseolovirus ribonucleotide reductases consist of a single subunit homologous to the large subunit of two-subunit enzymes.

INITIATION AND ELONGATION

For HHV-6 and HHV-7, lytic DNA replication is initiated at an oriLyt that is similar to alphaherpesvirus oriLyts and markedly different from the oriLyts of CMV and gammaherpesviruses.^{123,154,227,290} The oriLyts are located between the 5' end of U41 and the 3' end of U42. The minimal oriLyt segment is about 300 bp long, and a fully functional oriLyt is about 800 bp long. Multiple tandem iterations of the origin sequence enable more efficient replication of plasmids in transient replication assays, and confer a replication advantage on virus genomes.⁷⁵ A critical element near the center of oriLyt consists of two palindromically arranged sequences, OBP-1 and OBP-2, that are recognized by the origin-binding protein (OBP) encoded in roseoloviruses by U73 (a homolog of HSV-1 UL9). The core OBP sites flank an AT-rich sequence of about 30 bp (eFig. 64.5). Several positions within the OBP binding sites are conserved among the viruses that encode OBP homologs, whereas others differ in a manner that provides viral specificity. Replication efficiency is also affected by the sequence and composition of the AT-rich spacer. HHV-6A and HHV-6B are able to support replication of each other's oriLyt. Plasmids containing the HHV-6 oriLyt, however, were replicated in HHV-7-infected cells, but not vice versa, suggesting that oriLyt sequences and binding specificities differ between the viruses. It is thus surprising that the HHV-6 OBP binds strongly to the HHV-7 OBP binding sites, but the HHV-7 OBP binds strongly to only one of the HHV-6 OBP sites.¹⁵³

In addition to OBP, roseoloviruses encode homologs of the six other virally encoded proteins needed for replication of plasmids containing an HSV oriLyt. They include the major DNA-binding protein, the DNA polymerase and its processivity factor, and three gene products that comprise the helicase–primase complex. In an *in vitro* DNA synthesis system that employed the single-stranded M13 genome as a template, the HHV-6A DNA polymerase alone synthesized molecules shorter than 100 nucleotides.¹⁶² Addition of the polymerase processivity factor homolog encoded by U27 (p41), led to synthesis of molecules >7,200 nucleotides in length, similar to the processivity increase produced by its homologs in other herpesviruses. The N-terminal portion of the processivity factor was required for this activity.

GENOME PACKAGING AND CLEAVAGE

About 5% of genomes contained in nucleocapsids are circularized, creating a juxtaposition of direct repeat right (DRR) and direct repeat left (DRL) that results in assembly of a complete cleavage and packaging signal. The structure of such circular genomes is as follows: DRR-(terminal resolution site [TRS])n-pac2-N16–18-junction-N25–29-pac1-het(TRS)n-DRL.^{73,78,100,227,242,277} Some heterogeneity is seen in the sequences of concatemeric junctions, with 1 to 8 bp of additional, possibly untemplated, DNA being inserted into some molecules.^{78,242} Circularized molecules provide the template for rolling-circle replication, which leads to production of concatemers of nascent DNA suitable for packaging into capsids.²²⁷ In the model of Frenkel and Roffman,⁹² concatemers are cleaved between properly juxtaposed pac1 and pac2 sequences after a headful threshold has been reached during packaging, leading to

unit-length packaged genomes. Relatively few multimeric concatemers are generated during replication, and compared with HSV, HHV-6 replication intermediates are not highly branched, suggesting less frequent homologous recombination among replicating molecules.²⁴⁵ The genome of an HHV-6A BAC that is unable to replicate as an infectious virus has a single DR and is hypothesized to represent a replication intermediate,³¹ but it has not been determined how a mature genome flanked by DR elements could be generated from such a structure.

Identification of the oriLyt and cleavage and packaging signals allowed development of amplicon systems based on HHV-6 and HHV-7^{32,73} that feature T-cell replication, oriLyt, and cleavage-packaging signals, and can be used for expression of foreign genes. They have potential use as gene therapy vectors.

ASSEMBLY AND RELEASE

Roseolovirus virion maturation and egress appear to involve a process of successive envelopment, deenvelopment, and reenvelopment steps as the virion moves from one membrane-bounded compartment to another (Fig. 1). DNA replication and capsid assembly take place in the nucleus; nascent capsids that contain DNA appear about 3 days after infection.^{25,29} A feature that is likely to be a result of the envelopment-development process is the presence of abundant nonenveloped, tegumented nucleocapsids in the cytoplasm. In some studies, particles with neither tegument nor envelope were observed in the cytoplasm^{25,285}; teguments appeared to be acquired cytoplasmically in pools of electron-dense material. Membrane-bound nuclear structures (tegusomes) have been proposed as a site of tegument acquisition,²²⁶ but given the relatively low frequency with which they are observed, this is not the major pathway. Because viral glycoproteins gB, gH-gL, and gQ1 are absent from the plasma membrane, envelopment or egress by budding directly from the cytoplasm are not likely. These glycoproteins are concentrated in cytoplasmic structures known as annulate lamellae,^{37,55,285} which may correspond to the endosome- and TGN-related intracellular membranes where final envelopment of virions occurs.¹⁹⁰ HHV-6 virions collect in multivesicular bodies (MVB) that contain numerous mature virions and small exosomal vesicles.¹⁹⁰ MVB membranes can fuse with the plasma membrane, resulting in release of mature virions and the exosomal particles. In addition to exocytosis, mature virions can be released by cell lysis.^{55,285}

Fate of Host Cell

Roseolovirus infection has profound effects on host cells, including margination of chromatin, shut-off of host-cell DNA synthesis, generalized stimulation of host-cell protein synthesis, and development of the classic cytopathic effect of ballooning, refractile, multinucleated giant cells, as has been reviewed elsewhere.^{34,69} For at least HHV-7, the multinucleated cells develop, not by fusion of cells into syncytia, but by polyploidization that is linked to dysregulation of cdc2 and cyclin B. This leads to an accumulation of cells in the G2 to M phase of the cell cycle, with nuclei continuing to reproduce in the absence of cell division.²³⁸ Cytokines can be induced, including interferon- α (IFN- α), interleukin-1 β (IL- β by HHV-6A but not HHV-6B), and tumor necrosis factor (TNF), as well as the G-protein-coupled peptide receptor, EB1. In addition, HHV-6A and HHV-6B can inhibit the proliferative responses of PBMC to antigens and mitogens.³⁴

HHV-6A infection induces cell-surface expression of CD4, which then renders NK cells susceptible to HIV-1 infection. HHV-6A, but neither HHV-6B nor HHV-7, downregulates cell-surface expression of CD3; HHV-7 sharply downregulates CD4.^{28,34}

Downregulation of expression of the HIV-1 co-receptor CXCR4 and a coupled reduction in Ca^{2+} flux in response to SDF-1 α was observed in two studies^{244,308}; in contrast, no effect of HHV-7 infection on CXCR4 expression was seen in another study.³²⁵ HHV-6 affects HIV-1 infection in a co-receptor-dependent manner, suppressing CCR5-tropic but not CXCR4-tropic HIV-1 replication in human lymphoid tissue *ex vivo*, apparently via induction of the CCR5 ligand regulated on activation normal T cells expressed and secreted (RANTES), the most potent HIV-inhibitory CC chemokine. Exogenous RANTES mimics the effects of HHV-6 on HIV-1.¹⁰⁷ HHV-6 infection induces *de novo* synthesis of RANTES in primary human endothelial cell cultures,⁴¹ the highest induction being in microvascular endothelial cells that have very low susceptibility to HHV-6 infection.

HHV-6A infection induces a p53-independent G2 cell cycle arrest in human cord blood mononuclear cells²¹¹; newly synthesized viral proteins are necessary for this.⁶⁷ HHV-6B infections of T-cell lines led to G1/S and G2/M arrests concomitant with an increased level and enhanced DNA-binding activity of p53. HHV-6B induced phosphorylation of p53 at Ser15 and Ser20 by casein kinase 1,¹⁷² suggesting that viral suppression of T-cell proliferation links to p53 phosphorylation and accumulation.²⁰⁹ p53 protein levels increase in HHV-6-infected cells, and p53 ubiquitination decreases, indicating that the deubiquitination increases p53 stability. p53 mainly localizes in the cytoplasm of HHV-6-infected cells, but it translocates into the nucleus of mock-infected cells. Infected cells are resistant to UV-induced apoptosis, suggesting that HHV-6 has a mechanism for retaining p53 within the cytoplasm, providing protection from apoptosis.²⁶⁶ Since HHV-6 gene expression is enhanced in the absence of active p53,²¹¹ p53 induction may be a cellular response to the invading pathogen. p53 interacts with the HHV-6B U14 tegument protein, and is incorporated into viral particles.²⁶⁵

Some parallels have emerged in studies of apoptosis induction during infections with HHV-6 and HHV-7. In fresh umbilical cord blood lymphocytes, infected cells are apoptotic,^{25,120} whereas in cell lines, infected cells die by necrotic lysis and apoptosis is triggered in apparently uninfected or nonproductively infected cells.^{124,240} The virus may be able to inhibit apoptosis in at least some cells and its replication might be enhanced by suppression of apoptosis. This was borne out experimentally for HHV-7 in cells expressing high levels of the apoptosis suppressor, Bcl-2.²³⁹ During *in vivo* primary HHV-6B infections, apoptosis was detected in 15% to 20% of circulating PBMC.³⁰⁹

HHV-6 attachment and/or entry in the absence of *de novo* protein synthesis can induce expression of the human endogenous retrovirus K18-encoded superantigen.^{260,288}

In HHV-6-infected immature monocyte-derived dendritic cells, viral early and late antigens are expressed, and nucleocapsids containing a DNA core are observed, but few virions are detected in the cytoplasm. In co-cultures, HHV-6 can be transmitted from these cells to stimulated CD4⁺ T cells, suggesting that DCs may be one of the first cell populations targeted by the virus and may transmit the virus to T cells.²⁶⁴

PATHOGENESIS AND PATHOLOGY

Detailed referencing of historical literature in this area is available in several comprehensive and recent reviews.^{34,86,217,297}

Transmission

HHV-6B and HHV-7 are ubiquitous viruses, with horizontal infection commonly occurring during infancy. HHV-6 DNA has been detected in saliva, throat swabs, and nasal mucous of children and their mothers, as well as other healthy adults, suggesting horizontal infection through saliva and nasal secretions. HHV-6 is also detectable in the female genital tract and on the cervix of pregnant women in the late stages of their pregnancies, suggesting the possibility of transmission to neonates during vaginal delivery. No difference was found in the prevalence rates of HHV-6 infection between breast- and bottle-fed infants or between babies born by cesarean section and vaginal delivery. Local or seasonal exanthem subitum outbreaks are rare, but have been observed in pediatric institutions.

Finally, other routes of horizontal transmission have been reported, such as via organ transplantation. HHV-6B was isolated from recipients about 2 weeks after liver transplantation, accompanied by significant rises in HHV-6 antibody titers,³¹⁷ suggestive of primary infection.

While most HHV-6 infections occur via horizontal routes, approximately 1% of the population acquires the HHV-6 genome via germline cells that trace to either or both of their parents.^{64,194,214,271} In such individuals, an intact HHV-6 genome is integrated at the telomere of a chromosome, with copies of that chromosome being present in every cell in the body. While HHV-6 DNA has been detected in fetuses and in blood of neonates, indicating that intrauterine transmission is possible, most cases of congenital transmission of ciHHV-6 can be attributed to either genetic transmission of ciHHV-6 from either or both parents or to transplacental transmission from mothers with ciHHV-6.^{108,111} In addition, HHV-6-positive dried umbilical cord blood spots were more common for babies born to HIV-positive mothers.⁶³

Infectious HHV-7 can be readily isolated from saliva of healthy adults.³⁰¹ Probable transmission of HHV-7 from grandparents to parents and to children through close contact within a household was detected by using molecular epidemiologic techniques.²⁶³ It has been speculated that breast-feeding may be a route for HHV-7 transmission, but this is not the main route.⁹⁴ Thus, HHV-7 transmission may follow pathways similar to that for HHV-6B, except that congenital HHV-7 infections have not been detected.^{44,109}

Primary Infection

Site of Primary Replication

HHV-6 and HHV-7 possibly initiate infection through respiratory pathways, including lymphocyte-rich tonsils and olfactory-ensheathing cells present in the nasal cavity.

Cell and Tissue Tropism

The *in vivo* host-tissue range of HHV-6 includes lymphoid and endothelial cells, as well as cells present in the liver, CNS tissues, and salivary glands.^{34,36,69}

In vivo, HHV-7 infects CD4⁺ T lymphocytes (believed to be the site of latent infection), and salivary gland epithelial cells (a site of productive infection and viral shedding).²⁸ In

addition, cells expressing an HHV-7 structural antigen were detectable in lungs, skin, and mammary glands, and at reduced frequencies in liver, kidney, and tonsils. This antigen was not detected in the large intestine, spleen, or brain.¹³⁹

Persistence

HHV-6 DNA and antigens can be detected in saliva, nasal secretions, brain, lung, bone marrow progenitors, and PBMC. HHV-6 DNA was detected mainly in the monocyte fraction of PBMC from patients convalescing from ES and from healthy adults, and appears to establish latency in monocytes or macrophages and CD34-positive progenitor cells.^{10,148,167} As described previously, latency-associated transcripts have been detected from the IE region and U94. In a prospective study, 89% of children from 1 to 12 years of age were PCR positive for HHV-6 at least at one time point, with 1.2% of specimens being positive for reverse transcription PCR (RT-PCR), indicating active infection.⁴⁷ This activity was not associated with illness.

The HHV-6 variants differ with respect to their absolute and relative frequencies of detection in PBMC and cerebrospinal fluid (CSF). Of over 1,000 HHV-6 PCR-positive PBMC specimens, 2.5% were positive for HHV-6A and 99% for HHV-6B (some were positive for both). Of 84 HHV-6 PCR-positive CSF specimens, 17% were positive for HHV-6A and 86% for HHV-6B (some dual positives). Thus, HHV-6B was detected much more frequently in CSF than was HHV-6A, but the ratio of 6A to 6B was higher in CSF than in PBMCs, indicating a greater relative neurotropism for HHV-6A.¹¹⁰

Germ line ciHHV-6 enables a form of persistence in which the virus genome is present in every cell in the body. Under most circumstances, the virus is apparently quiescent. It has been hypothesized that the default latency pathway for HHV-6 in individuals without germline ciHHV-6 involves chromosomal integration,¹⁷ but this has not been demonstrated.

HHV-7 establishes latent infections in CD4+ cells,⁹³ and persistent lytic infections in salivary glands.³⁰¹ As described previously, HHV-7 lytic antigens are present in a variety of tissues, suggesting that persistent infection occurs in many tissues *in vivo*.¹³⁹ None of three HHV-7 lytic cycle transcripts studied were detected in HHV-7 DNA-positive PBMC,¹⁸¹ evidence that the virus is truly latent in these cells.

Immune Response

Immune responses to HHV-6 and HHV-7, and their immunomodulatory activities, have been reviewed in detail.^{69,296,297}

Antigens

Responses are generated to many of the virally encoded proteins. The major HHV-6 antigen in immunoblots is a protein encoded by U11 (101K or p100). Responses to HHV-7 are directed predominantly to pp85 and pp89 (encoded by U14 and U11),²⁵² as well as to a 52-kd species.¹⁹⁵ Consistent with their sequence similarities, sufficient antigenic cross-reactivity exists between HHV-6A and HHV-6B to preclude the use of conventional serologic assays for the study of variant-specific immune responses. A recently described serologic assay appears to enable accurate variant-specific serology.²⁷⁵ Although a degree of antigenic cross-reactivity is seen, serologic assays can distinguish between responses to the HHV-6 species and HHV-7. These levels of cross-reactivity are reflected in the frequencies of cross-reactive mAb and T-cell clones.^{18,28,310}

Primary Infection

Serologic studies on the association between ES and HHV-6 infection have mainly come from Japan. In patients with ES, IgM antibodies were detected on day 5, and persisted for 3 weeks, but were not detectable in most sera 1 month after the onset of disease. IgG antibodies are first detected about 7 days after onset of illness, increase for 3 weeks, and persist thereafter (eFig. 64.6). The viremia during primary infection subsides coincident with the appearance of neutralizing antibodies. HHV-6 antibody titers are boosted during infection with HHV-7.^{134,270}

Cellular immunity appears to be important for control of primary HHV-6 infection. A T-cell immune response against HHV-6 is generally present in healthy adults.^{295,304} Some T-cell clones with activity against HHV-6 also responded to HHV-7 and HCMV, indicating the presence of shared T-cell epitopes.³¹⁰

Plasma IFN- α levels are elevated during the acute febrile phase of ES compared with convalescence.²⁶² NK-cell activity is also elevated in the exanthem phase of ES, probably induced by IL-15.^{88,262} These early responses likely play pivotal roles in control of the infection.

In *in vitro* studies, IFN- α activity was detected in PBMC from healthy seropositive adults as early as 12 hours after exposure to HHV-6; they plateaued at days 2 to 5, and gradually decreased thereafter. The response of cord blood mononuclear cells was lower.¹⁴² HHV-6 also induces IL-1 β and TNF- α , but not IL-6 in PBMC.⁸⁴ Thus, HHV-6 can exert a strong immunomodulatory effect by stimulating cytokine production in cells of myeloid lineage. IFN- γ regulates the production of IL-10 and IL-12 at a transcriptional level mainly through inhibiting endogenous IL-10 production in HHV-6-infected monocyte-macrophage cells.¹⁵⁹

Reports on the immune response during HHV-7 primary infection are scarce because HHV-7 primary infection is seldom apparent. During a documented infection, 16 days after onset, HHV-7 antibody titer rose from less than 1:10 to 1:320 by an immunofluorescence antibody (IFA) test, whereas the HHV-6 titer remained less than 1:10.²⁶⁸ In a patient with two independent ES episodes within 2 months, HHV-6 and HHV-7 were sequentially isolated; seroconversion to HHV-7 during the second episode was observed 4 days after disease onset. HHV-7 infection induces IL-15 production, which then results in an enhancement of NK-cell activity that may play a major role in control of the infection.²²

Immunomodulation

HHV-6 infects a variety of immune cells, as described previously, and has numerous immunomodulatory effects *in vivo* and *in vitro*.

Infection of PBMC or T-cell-enriched cultures by HHV-6A suppressed IL-2 synthesis after mitogen stimulation, and levels of IL-2 transcripts were diminished in experiments with enriched T-cell cultures or Jurkat cells.⁸⁵

Suppression of bone marrow function may be one of the most serious effects of HHV-6 infection in bone marrow transplant recipients. *In vitro*, HHV-6 infection of normal bone marrow mononuclear cells reduced the outgrowth of granulocyte-macrophage colony-forming units (GM-CFU), granulocyte-erythrocyte-macrophage-megakaryocyte colony-forming units (GEMM-CFU), erythroid burst-forming units, and marrow stromal cells by 40% to 74%.³⁹ Although HHV-6A was generally a more potent inhibitor than HHV-6B, it did

not exhibit as significant a suppressive effect on the formation of GM colonies. In hemopoietic progenitor cells derived from human umbilical cord blood lymphocytes, HHV-6A suppressed erythroid colonies, HHV-6B suppressed erythroid and GM colonies, and HHV-7 had no effect on either lineage.¹²⁸ These results could be the consequence of the virus either encoding or inducing a soluble mediator or mediators that interfere with the responses of bone marrow to growth factors and block differentiation of macrophages from marrow precursors.³⁹

An *in vivo* model for immune suppression by HHV-6 was developed in severe combined immunodeficient (SCID-hu) Thy/Liv mice.⁹⁹ HHV-6A and HHV-6B efficiently infected the human thymic tissue implanted in these mice, leading to graft destruction. Virus replication was associated with severe, progressive thymocyte depletion involving all major cellular subsets. Intrathymic T progenitor cells were more severely depleted than the other subpopulations, and a preferred tropism of HHV-6 for these cells was demonstrated. Thus, thymocyte depletion by HHV-6 may be caused by infection and destruction of their immature precursors.

HHV-6A and HHV-6B, but not HHV-7, modestly (two-fold) induced several T-cell adhesion markers, including CD2, CD4, CD11a, CD44, CD49d, and HLA antigen-DR. HHV-6B infection had little effect on CD4+ cytotoxic lymphocytes, but HHV-6A and HHV-7 decreased their activity; the inhibition by HHV-7 was overcome by lectin stimulation.⁹⁵ Thus, the three viruses differ in their effects on T cells.

Human lymphocyte antigen (HLA) class I expression on dendritic cells (DC) was downregulated after infection with HHV-6A but not HHV-6B, whereas no significant change was seen in the expression of CD1a, CD83, and MHC class II; IE or E gene expression was required for this.¹¹⁷ Preexposure to HHV-6 markedly impaired IFN γ - and lipopolysaccharide-induced DC maturation. HHV-6, but not HHV-7, dramatically suppressed the secretion of IL-12 p70 by DC, whereas the production of other cytokines that influence DC maturation (i.e., IL-10 and TNF- α) was not affected.²⁴⁹ Children who acquired HHV-6 after 18 months of age were more likely to express IgE to common allergens, suggesting that early life exposure to HHV-6 might reduce allergic responses, possibly due to the virus downregulating Th2 responses.²⁰² While HHV-6 infection led to reduced IL-5 expression in DC, systemic IL-5 levels are elevated during convalescence from primary infection.³¹⁸

HHV-6 infection of monocyte-derived immature DC induced CD80, CD83, CD86, and HLA antigen class I and class II molecules, but decreased their capacity to present alloantigens and exogenous virus antigens to T lymphocytes.¹³² Levels of DC-specific intracellular adhesion molecule-grabbing non-integrin (SIGN) messenger RNA (mRNA) and intracellular protein decreased following HHV-6 infection.²⁰⁰

The cytokine encoded by HHV-6A U83 is an agonist for CCR1, CCR4, CCR5, CCR6, and CCR8 present on immune effector and antigen presenting cells.^{48,76} HHV-6B U83 can induce transient calcium mobilization through the CCR2 receptor and serve as a chemoattractant for mononuclear cells.^{171,328}

HHV-6 and HHV-7 U12 and U51 encode GPCR homologs. HHV-6 pU12 is a functional β -chemokine receptor, with selectivity that differs somewhat from its cellular homologs in its higher affinity for RANTES relative to macrophage inhibitory proteins 1 α and 1 β or monocyte chemoattractant protein 1.¹²⁷ When stably expressed in cell lines,

HHV-6 U51 specifically binds the CC chemokine RANTES and competes for binding with other β -chemokines, such as eotaxin; and monocyte chemoattractant proteins 1, 3, and 4. In epithelial cells already secreting RANTES, U51 expression induces specific transcriptional downregulation of RANTES.¹⁸⁴ U51 constitutively activates phospholipase C. It also binds several chemokines and promotes chemotaxis and internalization of chemokines, suggesting roles in virus dissemination or host transmission by chemotaxis of infected cells to sites of chemokine secretion specific for U51.^{49,83} Suppression of U51 expression by siRNA reduces the replication of HHV-6, demonstrating that U51 is important for virus growth.³²⁶

HHV-7 U12 and U51 also encode functional calcium-mobilizing receptors for β -chemokines, which include thymus and activation-regulated chemokine, macrophage-derived chemokine, EBI1-ligand chemokine, and secondary lymphoid-tissue chemokine.^{196,259} Furthermore, EBI1-ligand chemokine and secondary lymphoid-tissue chemokine induce migration in Jurkat cells stably expressing U12, but thymus and activation-regulated chemokine, and macrophage-derived chemokine, do not.²⁵⁹

HHV-6A had little direct effect on cytokine production in primary adult astrocytes, but profoundly affected the response of these cells to a mixture of proinflammatory cytokines (TNF- α , IL-1 β , and IFN- γ), inducing anti-inflammatory molecules (IL10 and IL-11), chemotactic and growth factors, and regulators of type 1 IFN production.¹⁷⁹

HHV-6B IE1 is resistant to IFN- α/β antiviral activity as a result of improper IFN-stimulated gene (ISG) expression.^{129,130} HHV-6B IE1 interacts with STAT2 and sequesters it in the nucleus, preventing ISG3 from binding to IFN-responsive promoters. This results in silencing of ISG, suggesting that IE1 has a role in regulating cellular antiviral responses.

HHV-6 and HHV-7 U21 proteins bind to HLA class I molecules and divert them to endolysosomal compartment, thereby removing them from the cell surface, leading to reduced or delayed cytotoxic T-lymphocyte recognition of infected cells.^{98,119}

HHV-6 and HHV-7 U24 proteins cause internalization of the T-cell receptor/CD3 complex at the cell surface, resulting in improper T-cell activation by antigen-presenting cells.²⁵⁵ U24 also downregulates the transferrin receptor, suggesting that U24 acts by blocking early endosome recycling.²⁵⁶

Epidemiology

HHV-6 and HHV-7 epidemiology has been reviewed in depth elsewhere.^{28,34,69,297}

Serologic assays used to date do not enable discrimination of prior infections with HHV-6A from HHV-6B, but recently described assay appears to enable variant-specific serologic testing, which will hopefully enable many aspects of HHV-6A and HHV-6B epidemiology to be untangled. Infants appear to be protected against HHV-6 infection by maternal antibody. HHV-6 seroprevalence decreases from 0 to 5 months of age, as maternal antibody wanes. Beginning at about 6 months, seroprevalence increases rapidly, with almost all children becoming positive by 2 years of age.^{205,324} Internationally, HHV-6 seroprevalence is high in almost all areas, but ranges from approximately 39% to nearly 100% among some ethnically diverse adult populations. In the United States, Europe, and Japan, most early childhood infections are with HHV-6B,

rather than HHV-6A. The situation seems to be reversed in sub-Saharan Africa,²³ suggesting significant international differences in the epidemiology of HHV-6 variants. In areas where early childhood infections with HHV-6B predominate, HHV-6A has been detected by PCR in blood or tissues from as many as 50% of adults. Little is known of the course of such infections that occur in the face of preexisting immunity against the closely related species.

HHV-7 infection generally occurs somewhat later than for HHV-6.³⁰² As for HHV-6, seroprevalence declines over the first 5 to 6 months, as maternal antibody wanes, then increases fairly rapidly through about 4 years of age. It reaches its highest level from the teens until the end of the third decade, decreasing thereafter.^{269,315}

Clinical Features

Primary Infection

Exanthem subitum (ES) was first described in 1910 by Zahorsky³²² and is a common disease of infants internationally. In the classic presentation, an infant develops sudden fever, which lasts for a few days, followed immediately by a rash that appears on the trunk and face and spreads to lower extremities as the fever subsides. A virus was hypothesized as the cause. In experiments now considered unambiguously unethical, human-to-human transmission of the infection was observed following experimental inoculation of blood of patients with ES into other infants.^{114,138} Efforts to isolate the causative agent of ES were unsuccessful until the first report linking HHV-6 infection and ES by Yamanishi et al. in 1988.³⁰⁶ HHV-6 was isolated at high frequency from patients with ES during the acute phase of the disease. About 10 days after cultivation, a cytopathic effect (CPE) could be observed, which showed balloon-like cells. When cells were fixed and stained with sera during the acute and convalescent phases of ES, positive staining was observed only using convalescent sera (Fig. 64.2). Subsequent studies found that HHV-6 primary infection can occur without clinical symptoms of rash or fever, or even without any clinical symptoms. ES is caused by HHV-6B, and the magnitude of the virus replication in infants with ES is proportional to the severity of disease.¹⁹ In most cases, ES is self-limiting, and is often associated with other symptoms, including diarrhea, cough, lymph node swelling, and bulging fontanels. In adults,

primary infections can cause mononucleosis-like disease and hemophagocytic syndrome.

Although the frequency of clinical illness is lower, primary infection with HHV-7 causes illnesses similar to those caused by HHV-6, including ES, high fever, and neurologic symptoms (e.g., febrile convulsions).^{45,268,298} An example of successive bouts of ES caused by HHV-6 and then HHV-7 is illustrated in eFigure 64.7.

Symptoms Associated with Primary Infection

The pathogenesis of HHV-6B primary infection is illustrated in Figure 64.7.

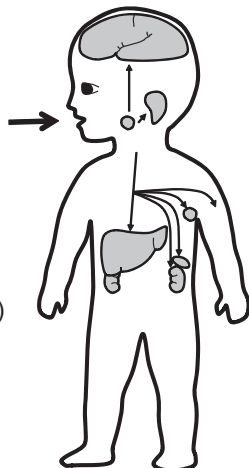
The most commonly reported severe symptom associated with ES is febrile convulsion, which generally has a benign resolution. In a large study, Hall et al.¹¹² found that of the 160 infants and young children with acute HHV-6 infection, 21 (13%) were hospitalized, and 21 had seizures. The seizures often appeared late and were prolonged or recurrent. HHV-6 infections accounted for one third of all febrile seizures in children up to the age of 2 years. In this study, 7 (24%) of 29 CSF specimens were positive for HHV-6 by PCR, including 2 of 7 from children with convulsions. HHV-6 DNA was also detected in CSF from patients with neurologic manifestations at the time of primary infection of HHV-6.^{46,254,319} In contrast with the frequency of HHV-6 in CSF during clinically apparent acute episodes, in a prospective study, no seizures were reported among children who had a well-defined time of primary infection.³²⁴ HHV-6 persists in the CNS and has been reported to increase the risk for recurrence of febrile convulsions.¹⁴⁹ A recently reported prospective study, however, suggests that a first febrile seizure associated with an acute HHV-6 infection may be associated with a reduced risk for recurrent seizures.¹³¹

Encephalitis/meningoencephalitis/encephalopathy can occur during primary HHV-6 infection. Of 89 cases identified in a Japanese national survey, half of the patients had neurologic sequelae and 2 died.³²⁰ In comparison to stem-cell recipients, HHV-6 encephalitis during primary infection is characterized by low viral DNA levels in the CSF and differences in cytokine responses.¹³⁷

Liver dysfunction is also associated with HHV-6 infection; although usually mild, fatal hepatitis and chronic hepatitis have been also reported.^{21,261} Primary HHV-6 infection has

Transmission

- Close contact through saliva (major route)
 - Mother to children
 - Siblings (at home)
 - Among infants (at day care center)
- Congenital infection
 - Transplacental
 - ciHHV-6



Major clinical symptoms of ES

- | | | |
|---------------|---|---------------|
| Fever | → | Convulsions |
| CNS infection | → | Encephalitis |
| Lymph node | → | Lymphadenitis |
| Skin | → | Rash |
| Liver | → | Hepatitis |

FIGURE 64.7. Pathogenesis of HHV-6B primary infection.

also been associated with cases of idiopathic thrombocytopenic purpura.^{143,247}

Primary HHV-7 infection can cause febrile illness, sometimes complicated by seizures and acute hemiplegia.²⁹⁸

Neurologic symptoms accompanying primary HHV-6 and HHV-7 infections sometimes occur in temporal coincidence with childhood vaccinations, leading to the likely incorrect conclusion that the vaccine caused the illness.²⁹⁸

Disease in Immunocompromised Patients

Because herpesviruses persist in their hosts after primary infection and can be reactivated, they are important pathogens in immunocompromised hosts. Exogenous reinfections are also possible and may contribute to disease in such patients.⁶

BONE MARROW TRANSPLANT RECIPIENTS

Asymptomatic HHV-6 reactivation is common after allogeneic bone marrow or hematopoietic cell transplantation, but reactivation has also been linked to bone marrow suppression, encephalitis, delirium and neurocognitive decline, gastroenteritis, colitis, pneumonitis, rash, and acute graft-versus-host disease (GVHD).^{34,60,69,115,292} In skin and rectal biopsy specimens from 34 allogeneic bone marrow transplant (BMT) recipients and 23 autologous recipients, HHV-6 DNA was detected in PBMC of 74% of autologous and 76% of allogeneic recipients, and in biopsy tissue of 48% of autologous and 71% of allogeneic recipients.¹³ HHV-6 DNA was detected more frequently in allogeneic recipients with severe GVHD (92%) than in those with either moderate (55%) or mild GVHD (22%), suggesting that the presence of HHV-6 DNA in the skin or rectum may be a factor in determining GVHD severity.

HHV-6 infection has also been associated with pneumonitis, with the infected cells being primarily intra-alveolar macrophages, plus some lymphocytes.³⁸ Concentrations of HHV-6 genomes in lung tissue and their relation to changes in serologic titers support an association between HHV-6 infection and idiopathic pneumonitis in immunocompromised hosts.⁵⁹

Prospective monitoring and prophylactic antiviral strategies for prevention of roseolovirus-associated disease have not been recommended in formal practice guidelines.^{164,323}

SOLID ORGAN TRANSPLANT RECIPIENTS

Children seronegative for HHV-6 before transplantation rejected their kidneys early after transplantation,²⁹⁴ and HHV-6 has been associated with kidney rejection.^{4,204,321} About 30% of renal transplant recipients showed a significant increase of serum antibody titer against HHV-6 after transplantation, all of whom suffered severe kidney rejection. HHV-6 antigens were detected in the tubular epithelium as well as in infiltrating histiocytes and lymphocytes. The frequency of HHV-6 and/or HHV-7 plasma DNA was significantly higher in chronic allograft nephropathy (CAN) patients (25/28, 89.3%) compared to control patients (15/50, 30.0%, $p = 0.0001$).⁵² CAN patients also had more frequent dual active infections (20/25, 80% and 2/15, 13.3%, $p = 0.007$, respectively). HHV-6B was identified in all 34 HHV-6 positive cases. The presence of HHV-7 DNA in plasma preceded the presence of HHV-6 DNA. Early development of CAN and graft loss was detected only in patients with simultaneous HHV-6 and HHV-7 plasma DNA.

The relationship between clinical features and HHV-6 infection has been investigated in liver transplant recipients.^{246,247}

HHV-6 infection occurred in four liver transplant recipients a median of 50 days after transplantation, accompanied by severe cytopenia. One of the four patients had interstitial pneumonitis caused by HHV-6. These authors suggest that idiopathic bone marrow suppression is the predominant clinical sequelae of HHV-6 in these patients. One liver transplant recipient experienced an acute febrile illness with life-threatening thrombocytopenia, progressive encephalopathy, and skin rash in association with invasive HHV-6B infection. Liver transplant recipients with ciHHV-6 experienced more bacterial infections and allograft rejection than those without ciHHV-6.¹⁵⁷

HHV-6, HHV-7, and HCMV may cause some symptoms by reactivating doubly and triply.^{74,106,208,222}

The AST Infectious Diseases Community of Practice has made the following statements: solid organ transplant recipients should not be routinely monitored for roseoloviruses, asymptomatic viral activity does not warrant antiviral treatment, antiviral therapy (intravenous ganciclovir or foscarnet) should be initiated for HHV-6 encephalitis and other more serious syndromes associated with the virus, treatment should include reduction of immunosuppression, and prophylactic and preemptive antiviral therapy are not recommended.^{223–225}

ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

The possible role of HHV-6 as a co-factor in HIV infection has been considered because both viruses can infect CD4⁺ cells, and HHV-6 can transactivate the HIV LTR.³⁴ Furthermore, HHV-6 can induce CD4 gene transcription and expression in CD4-negative cells, which renders them susceptible to HIV infection. It has been hypothesized that the most important issue with respect to HHV-6–related disease in HIV-positive individuals may not be a direct effect on specific target organs, but by HHV-6 contributing to immunodeficiency.¹⁶⁹

Comparisons between patients infected with HIV and uninfected controls have shown either no difference in seroprevalence and titer or higher or lower prevalence among those infected with HIV. The detection rate of HHV-6 DNA in PBMC and saliva has also been variable. Two studies found no correlation of HHV-6 seroprevalence with progression to AIDS, whereas another found that patients whose disease progressed slowly had decreased HHV-6 seroprevalence compared with those whose disease advanced rapidly. A marked association was seen between HHV-6 infection and progression of HIV disease in Thai children.¹⁵²

Lung infection with HHV-6 was sufficiently extensive in one patient to account for fatal pneumonitis. In other tissues, increased numbers of HHV-6–infected cells were related to the presence of lymphocyte infiltrates or residual lymphoid tissue, with lymphocytes being predominantly infected.¹⁴⁵ HHV-6–associated retinitis has been seen in patients with AIDS.²²⁰ HHV-6 was more extensively disseminated in neural cells of brains of pediatric patients with HIV, suggesting a contribution to the pathogenesis of AIDS encephalopathy.²³² Adult patients with AIDS had areas of demyelination in their CNS tissue at the time of death.¹⁴⁶ The presence of HHV-6 in an organ was significantly associated with an elevated HIV-1 proviral load.⁸¹

Co-infections of macaques with HHV-6A and simian immunodeficiency virus (SIV) resulted in a plasma viremia that was associated with clinical manifestations that progressed to AIDS more rapidly than in monkeys infected only with SIV,

providing *in vivo* evidence that HHV-6A may promote AIDS progression.¹⁶⁸

POSSIBLE DISEASE ASSOCIATIONS

Neoplasia. HHV-6 DNA has been detected in non-Hodgkin's lymphoma, Hodgkin's disease, S100-positive, T-cell chronic lymphoproliferative disease, oral salivary gland carcinoma tissues, pediatric and adult glial tumors, and other neoplasias.^{34,61,62,303} Although there has been no conclusive demonstration that either HHV-6A or HHV-6B is involved in a causative way with any malignancy, the frequency of detecting HHV-6 in certain tumors suggests a possible co-factorial role in multistep carcinogenesis.

Chronic Fatigue Syndrome. Conflicting reports have appeared of a connection between chronic fatigue syndrome (CFS) and HHV-6 infection. We do not consider the evidence to be supportive of an etiologic association.

Multiple Sclerosis and Other Neurologic Disorders.

Numerous studies have found that HHV-6B is a commensal virus of the human brain. The high prevalence of the virus in the olfactory bulb suggests that a route of CNS entry might be via olfactory ensheathing glial cells present in the nasal cavity.¹¹³

Multiple sclerosis (MS) is characterized clinically by neurologic signs and symptoms that wax and wane over time and involve multiple nerve pathways. Several studies have suggested an association between HHV-6 and MS, but the association remains uncertain.²⁹³ Staining of neurons and oligodendrocytes in plaque regions was observed in MS patients using mAb against HHV-6 virion protein 101K and DNA-binding protein p41.⁵¹ Elevated IgM serum antibody responses to HHV-6 early antigen (p41/38) were found in patients with relapsing-remitting MS, compared with patients with chronic progressive MS, other neurologic disease, other autoimmune disease, and with controls.²⁵⁰ HHV-6 DNA was detected with high frequency in MS plaques isolated by laser microdissection⁵⁰ and in neurologic and inflammatory cells in acute-phase lesions tissue of MS.¹⁰² Viral DNA and infectious virus was detected in CSF collected early in the course of MS,²²⁸ and viral transcripts were detected at higher levels in brains of patients with MS,²⁰⁷ with the highest loads being in lesional tissues. HHV-6 activity in MS patients has also been associated with poor response to interferon β 1b treatment⁹⁶ and a particular allele of an MHC regulatory gene.⁹

Negative results, however, were obtained in other studies.²⁹³ No significant difference was found in detection of HHV-6 DNA in sera and CSF between groups of patients with MS, patients with other neurologic diseases, and controls.⁸² Furthermore, no significant difference was seen between patients with MS and patients not having MS by immunohistochemical staining of brains or by PCR of defined brain regions.^{57,287} Further experiments are needed, including multicenter evaluation of blinded, carefully defined specimens.

HHV-6 has been detected in CSF from patients with encephalitis. In some patients, HHV-6 antibody and CSF DNA levels increased during the disease.³⁰⁷ In addition, herpes simplex encephalitis and HHV-6-associated encephalopathy give markedly different patterns as visualized by computerized tomography or magnetic resonance imaging.²⁰¹ In some instances, the HHV-6/encephalitis association was probably a nonspecific finding that resulted from the affected individual harboring ciHHV-6.

HHV-6 DNA has been detected in resected brain tissues from patients with mesial temporal lobe epilepsy.^{161,276} In one study, this association was restricted to patients with history of encephalitis.¹⁹⁹

Progressive multifocal leukoencephalopathy (PML) is a primary demyelinating disease of the CNS occurring almost exclusively in individuals with impaired cell-mediated immunity. The JC polyoma virus has been accepted as the etiologic agent of PML. Using a two-step *in situ* PCR procedure to amplify and detect genomic DNA of HHV-6 in formalin-fixed paraffin-embedded archival brain tissues, a high frequency of infected cells was consistently detected in PML white matter, both within and surrounding demyelinating lesions, the HHV-6 genome being found mainly within oligodendrocytes. Lesser amounts of HHV-6 genome were detected in tissues of patients with AIDS, patients with other neurologic disease, and controls. Immunocytochemistry for HHV-6 antigens showed actively infected nuclei of swollen oligodendrocytic morphology within the demyelinating lesions of PML but not in adjacent uninvolved tissue. In addition, HHV-6 antigens were not detectable in control tissues, including brains of patients with HIV-1 encephalopathy but without PML. Double immunohistochemical staining for JC virus large T antigen and HHV-6 antigens demonstrated co-labeling of many swollen intralosomal oligodendrocytes in the PML cases. Thus, HHV-6 activation in conjunction with JC virus infection is associated with the demyelinating lesions of PML.¹⁸⁷

Myocarditis. Laboratories in Europe and Japan have reported detection of HHV-6 DNA in affected heart tissues from children and adults with acute myocarditis.^{11,58,173}

Drug Hypersensitivity. Drug-induced hypersensitivity syndrome is characterized by a severe, potentially fatal, multiorgan reaction that usually appears after prolonged exposure to certain drugs.²⁸⁰ Clinical features overlap those for Stevens-Johnson syndrome and toxic epidermal necrolysis. Clinical signs include a maculopapular rash progressing to exfoliate erythroderma, fever, and lymphadenopathy. Reactivation of herpesviruses—including HHV-6B, HHV-7, HCMV, and EBV—have been detected during severe drug-induced hypersensitivity syndrome and the related illnesses,^{258,281} but it is uncertain whether the virus reactivation causes the disease, is triggered by the disease, or contributes to the disease.

Pityriasis Rosea. Pityriasis rosea was reported to be associated with HHV-7 infection,⁸⁰ but the association was not seen in other investigations.^{140,300}

Animal Model

Animal models for roseolovirus infections have been developing slowly. HHV-6 antibodies have been found in several species of monkeys, suggesting the presence of HHV-6 or an HHV-6-related virus.¹¹⁶ Related viruses were subsequently detected by PCR in mandrills and chimpanzees.^{155,156} PBMC from chimpanzees are permissive to productive HHV-6 infection *in vitro*, indicating that the host range of HHV-6 may not be restricted to humans.¹²⁹ In addition, cynomolgus and African green monkeys inoculated with HHV-6 developed antibody responses and HHV-6 DNA was detected in lymph nodes and spleens.³⁰⁵ The infection was asymptomatic, except for one monkey with rash.

Diagnosis

Diagnosis of HHV-6 and HHV-7 infections has been reviewed comprehensively elsewhere.^{34,215,217,297}

Differential Diagnosis

Because ES and the other symptoms associated with primary HHV-6 and HHV-7 infections are generally mild and self-limiting, specific laboratory diagnosis is seldom warranted in these children. Nonetheless, these viruses warrant inclusion in panels of agents to be investigated in cases of neurologic complications, particularly if the child was recently vaccinated.^{297,298} In some parts of the world primary HHV-6 and HHV-7 infections are occasionally misdiagnosed as measles or rubella virus infections.^{26,206} Roseoloviruses may also be considered in immunocompromised patients experiencing any of the wide variety of symptoms described earlier, including pneumonitis, fever, rash, GVHD, and CMV disease.

After early childhood, most individuals harbor latent infections with these viruses; latent infections have not been clearly associated with any disease. The diagnostic challenge is in distinguishing virus activity against the latent background. This is often facilitated by comparing paired acute and convalescent specimens, or consecutive specimens in patients who are being monitored closely, such as organ transplant recipients. Viral activity can be detected as changes in status, as measured by quantitative PCR or serologic titers. Direct evidence of active infection includes detection of infectious virus in cultured cells, lytic antigens in circulating lymphocytes or in affected tissues, transcripts of lytic cycle genes by RT-PCR, or viral DNA in acellular fluids such as plasma or serum.^{217,297} Recent infections can be identified through the use of antibody avidity assays, which differentiate the high avidity antibody that develops over 2 or so months following primary infection, from the sometimes high-titered but low-avidity antibody that develops in the days and weeks immediately following primary infection.²⁹⁷

VIRUS DETECTION

Culture. HHV-6 is easily recovered from PBMC of patients with ES during the acute phase. Lymphocyte activation with either PHA or antibody to CD3 and maintenance in IL-2 is required.³⁰⁶ CPE develops within 7 to 10 days, as shown in Figure 64.2. The refractile giant cells usually contain one or two nuclei; after CPE develops, the cells degenerate. Mononuclear cell-associated viremia caused by HHV-6 was detected in 66% of blood samples from children with ES between days 0 and 7 of the disease. The rate of virus isolation from mononuclear cells was 100% on days 0 to 2 (just before appearance of skin rash), 82% on day 3, 20% on day 4, 7% on days 5 to 7, and 0% on day 8 and thereafter. Cell-free virus was also detected in blood samples during the same period.²⁰ HHV-7 is sometimes isolated from peripheral blood of patients with ES, and can be readily isolated from saliva by using methods as described earlier for HHV-6. Isolation of HHV-6 from saliva is uncommon, although its DNA is often detectable there.

Detection of Viral Genome, Transcripts, and Proteins.

HHV-6 and HHV-7 DNA are commonly detected by quantitative polymerase chain reaction assays (qPCR). Numerous primer sets have been described, including rapid quantitative assays that enable identification and discrimination of HHV-6A and HHV-6B, simultaneous detection of HHV-6 and

HHV-7, and multiplex assays for all of the HHV.^{141,160,185,230} Loop-mediated isothermal amplification can also be used to detect HHV-6 and HHV-7 DNA.^{121,316} A monoclonal antibody-based quantitative antigenemia assay enables enumeration of HHV-6B-infected cells and has been used to monitor HHV-6 activity in liver transplant recipients.¹⁶⁶

A multicenter external quality assessment among 51 laboratories that employ PCR for HHV-6 found that most labs use in-house quantitative assays, there was good qualitative agreement on specimens containing at least 200 copies/mL, and quantitative reporting was much more variable.⁷¹

Latent and replicating virus can be discriminated by assays that combine reverse transcription and PCR (RT-PCR).^{203,314} Such assays target spliced mRNAs, enabling easy discrimination between mRNA (short amplicon because of intron removal) and residual DNA contamination (long amplicon). Because HHV-6 DNA is usually cell associated,² it can be more useful to describe qPCR results in terms of a ratio between virus and cell genomes, rather than in terms of virus genome copies per volume of fluid (e.g., virus genomes per mL of blood) that can contain variable concentrations of cells.⁹⁷

HHV-6 DNA is easily detectable by PCR in peripheral blood of patients with ES during the acute phase. Although detection of cell-free virus in serum or plasma by PCR offers the possibility of diagnosing active HHV-6 infections, PCR-based assays of plasma were not reliable for discriminating primary HHV-6 from ciHHV-6.⁴³ qPCR was less sensitive than culture for detecting primary infection, while RT-PCR was approximately as specific as culture for detection of primary infection.

ciHHV-6 presents several challenges to PCR-based methods for HHV-6 diagnosis.^{56,214,299} Because at least one copy of the virus genome is present in every cell, uncommonly high copy numbers will be detected in most clinical specimens, even if the virus is not actively replicating or otherwise contributing to the illness. Sufficient viral DNA can leak from lysed cells to cause false-positives in bodily fluids that are normally relatively acellular. Cell lysis during serum clotting and during the period between blood collection and plasma separation can also lead to false positives in these acellular fluids. ciHHV-6 should be suspected when uncommonly high levels of HHV-6 DNA are found. Persistent loads $>5.5 \log_{10}$ copies/mL are consistent with ciHHV-6.²¹⁴

Serology

Numerous HHV-6 and HHV-7 serologic assays have been described,^{34,215,217} including IFA, anticomplement IFA, enzyme-linked immunosorbent assay (ELISA), neutralization (NT), radioimmunoprecipitation, and immunoblot; IFA is the most commonly applied method, with HHV-6-infected cells being used as the antigens. For detection of IgM, separation of serum IgM from IgG and IgA significantly increases the specificity, but IgM testing is not useful for clinical diagnosis.⁷² The products of U11 (86 kD) and U14 (85 kD) were identified as sensitive and specific markers for HHV-7 serology.²⁵² In a comparison of immunoblot, IFA, and ELISA assays for HHV-7 antibodies, the ELISA was the most sensitive, whereas the immunoblot was the most specific.³⁰ As mentioned, antibody avidity assays enable identification of recent primary infections.²⁹⁷ Serologic responses to HHV-6A and HHV-6B cannot be discriminated by the assays described earlier. A recently described assay appears to enable variant-specific serologic testing.²⁷⁵ Materials

for HHV-6 and HHV-7 IFA are available commercially, and testing is also available from commercial reference laboratories. Sources are listed in.²¹⁷

Prevention and Control

In immunocompromised patients, reduction of immunosuppression can improve immune control of roseolovirus infections.²²⁵ The European Conference on Infections in Leukaemia provided evidence-based guidelines for management of roseolovirus infections in patients with hematologic malignancies and after hematopoietic stem cell transplantation (HSCT).¹⁶⁵

Inhibitors of Replication

Ganciclovir, phosphonoformate (foscarnet) and cidofovir are potent inhibitors of roseolovirus replication *in vitro*, and are used clinically to treat these viruses. Acyclovir (ACV) and other thymidine kinase-dependent drugs are only marginally effective.^{34,69} The inhibitory effects of IFN- α and IFN- β on HHV-6 replication were also demonstrated *in vitro*, as described earlier. Ganciclovir is activated by the phosphotransferase encoded by gene U69, which can confer ganciclovir susceptibility on recombinant baculoviruses.¹² Methods have been described for rapid identification of U69 mutations in instances of clinical resistance to ganciclovir.¹²⁵ Other compounds with *in vitro* activity against HHV-6 include CMV423, a protein tyrosine kinase inhibitor,⁶⁶ and artesunate.¹⁸³ The sensitivity of HHV-7 to the guanine analogs was different from HHV-6, suggesting a difference in selectivity of specific viral enzymes.³¹³

PERSPECTIVES

Experimental study of the roseoloviruses has been hampered by several factors, including the difficulty of preparing high-titered, cell-free virus; the availability of relatively few monoclonal antibodies; lack of a useful animal model system; and no convenient system for genetic manipulation of the viruses. Fortunately, progress has been made on these fronts. Several interesting features of these viruses have been identified, including the ability of HHV-6 to be transmitted in a germline chromosomally integrated form; the presence near the viral genomic termini of arrays of sequences (terminal resolution site [TRS]) that are similar to sequences found at mammalian telomeres; the presence in HHV-6A and HHV-6B genomes of a homolog of a parvovirus replication or/regulatory protein; the presence of an OBP homolog and cognate-binding sequences that are similar to those previously found only in the alphaherpesviruses; effects on host-cell metabolism different from those described for other herpesviruses; presence of the key cell regulatory protein p53 in HHV-6 virions; and the expression of virally encoded functional GPCR. We anticipate that the implications of these observations will stimulate further study, and that other novel aspects of the biology and molecular biology of these viruses will be identified in future studies. Although the genomic sequences of these viruses are known, the functions of most viral gene products of HHV-6 and HHV-7 have not yet been elucidated. Exploitation of the recently described recombinant virus system for HHV-6A and its extension to HHV-6B and HHV-7 are needed to enable detailed examination of functions of the viral genes in the context of infection.

Much of the pathogenesis associated with roseolovirus infections is still undefined. Although the main routes of transmission of HHV-6 and HHV-7 appear to be via close contacts between infants and parents or siblings through saliva, alternative paths (e.g., vertical transmission) require additional study, and an explanation is still needed for primary HHV-7 infection generally following HHV-6, although infectious HHV-7 is present in abundance in saliva. Numerous questions remain about the role of integrated genomes in HHV-6 biology and their effect on the host. Widespread application of a recently described serologic assay will enable delineation of aspects of HHV-6A and HHV-6B epidemiology that have been hidden.

The mechanisms by which HHV-6 and HHV-7 regulate latency are not known. With man as the only host for HHV-6 and HHV-7, the identification of relatives of these viruses in nonhuman primates opens the window to developing animal models suitable for learning the *in vivo* functions of viral genes and for closer examination of pathogenic processes.

The high prevalence of the Roseoloviruses, coupled with their ability to establish life-long infections and their relatively low associated morbidity and mortality, suggest that these highly evolved viruses have much left to teach us about host-pathogen interactions.

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Kaposi's Sarcoma–Associated Herpesvirus

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Acknowledgements

Kaposi's sarcoma–associated herpesvirus (KSHV) is the eighth human herpesvirus and is formally known as human herpesvirus 8 (HHV8). KSHV is the most recently identified herpesvirus among the human herpesviruses. Chang et al¹²⁵ first identified the virus from acquired immunodeficiency syndrome (AIDS)-associated Kaposi's sarcoma (KS) tissues. KSHV has been classified as a member of the lymphotropic gammaherpesvirus subfamily. In this chapter, we review the discovery of KSHV, epidemiologic evidence linking the virus to multiple human cancers, and the clinical description of these viral cancers. We will also discuss the detailed latent and lytic life cycles of the virus and the contribution of viral proteins to disease pathogenesis.

KAPOSI'S SARCOMA–ASSOCIATED HERPESVIRUS: DISCOVERY AND CLASSIFICATION

KS was first described by a Hungarian dermatologist named Moritz Kaposi in the late 19th century as *Idiopathisches multiples Pigmentsarkom der Haut*.³²⁰ Classically considered an indolent disease of older men, the disease was later found to be

more common in the Mediterranean region as well as in parts of Africa.^{18,284} In several regions of Africa, KS afflicts children as well as adults. Prior to the emergence of human immunodeficiency virus (HIV) infection in the human population, KS was considered a rare neoplasm in the United States and Western Europe. However, with the advent of pandemic HIV in the early 1980s, KS emerged as the most common AIDS-defining cancer. The linkage between KS and HIV infection first prompted speculation that HIV was the etiologic agent of KS; however, this was disregarded in light of many epidemiologic studies. First, classical KS occurred before the AIDS epidemic and was originally seen in HIV-negative individuals. Second, even among HIV-positive subjects, certain groups are distinctly different in their risk for KS development. KS was more widespread among patients who acquired HIV via sexual routes (e.g., men who have sex with men), compared to individuals who acquired HIV parenterally (e.g., children and hemophiliacs).⁴⁶ These data suggested that KS might be acquired by a sexually transmitted pathogen other than HIV, which initiated a hunt for the etiological agent in question. In 1994, Chang et al¹²⁵ identified KSHV genomic DNA in KS lesions but not in normal tissue using representational difference analysis. By 1996, the Chang and Moore group (and others) had sequenced and cloned the entire viral genome.^{496,497,501,571}

It is now generally accepted that there are four classes of KS, only one of which is strongly linked to HIV infection: classic KS, endemic or African KS, iatrogenic KS associated with immunosuppressive therapies in transplant patients, and epidemic or AIDS-related KS. These classes of KS are described in further detail later in the chapter.

Although KSHV was first identified in an endothelial tumor, it was later found to be associated with lymphoproliferative disorders, which were greatly increased in AIDS patients. The KSHV genome is consistently present in two B-cell lymphoproliferative diseases.^{107,625} One such disease is primary effusion lymphoma (PEL), which is an expansion of B cells predominantly in serosal cavities such as the pericardium, pleura, and peritoneum, although infiltration of solid organs does occur. B cells in PEL are clonal in origin, as discovered by analysis of immunoglobulin rearrangements.^{247,338} Every PEL cell harbors KSHV DNA in the form of a circular episome, usually in multiple copies. Most PEL also harbor Epstein-Barr virus (EBV); however, KSHV is the defining pathogen for a diagnosis of PEL.

PEL can be established in culture relatively easily^{58,110,575} and form tumors in immunodeficient mice.^{58,99,533,631} PEL cell lines were the first system where KSHV replication could be induced *in vitro*,⁵⁶⁴ and they have yielded latent and lytic antigens for serologic test development.

Another B-cell disorder associated with KSHV infection is multicentric Castleman's disease (MCD). MCD is a rare, polyclonal,¹⁸⁸ lymphoproliferative disease that can develop in both HIV-negative and HIV-positive individuals. There are two histologic forms of Castleman's disease: a plasmablastic variant of MCD that is highly associated with KSHV and a hyaline variant that is more common in the unicentric form and not associated with KSHV infection. MCD is a systemic disease characterized by fever, weight loss, sweats, splenomegaly, and lymphadenopathy. MCD is an aggressive disorder that has a higher incidence in AIDS patients, where it is always associated with KSHV infection.⁶²⁵ The KSHV viral genome is present in the B cells of the mantle zones surrounding the germinal centers

(GCs). In a given germinal center, approximately 30% of B cells appear infected with virus.^{191,322,521} All KSHV-positive cells in MCD bear lambda light chains together with immunoglobulin M (IgM) heavy chains.¹⁸⁸ In HIV-negative individuals, only 50% of all MCD cases are linked to KSHV infection.^{522,625} Both PEL and MCD are described in greater detail later in the chapter.

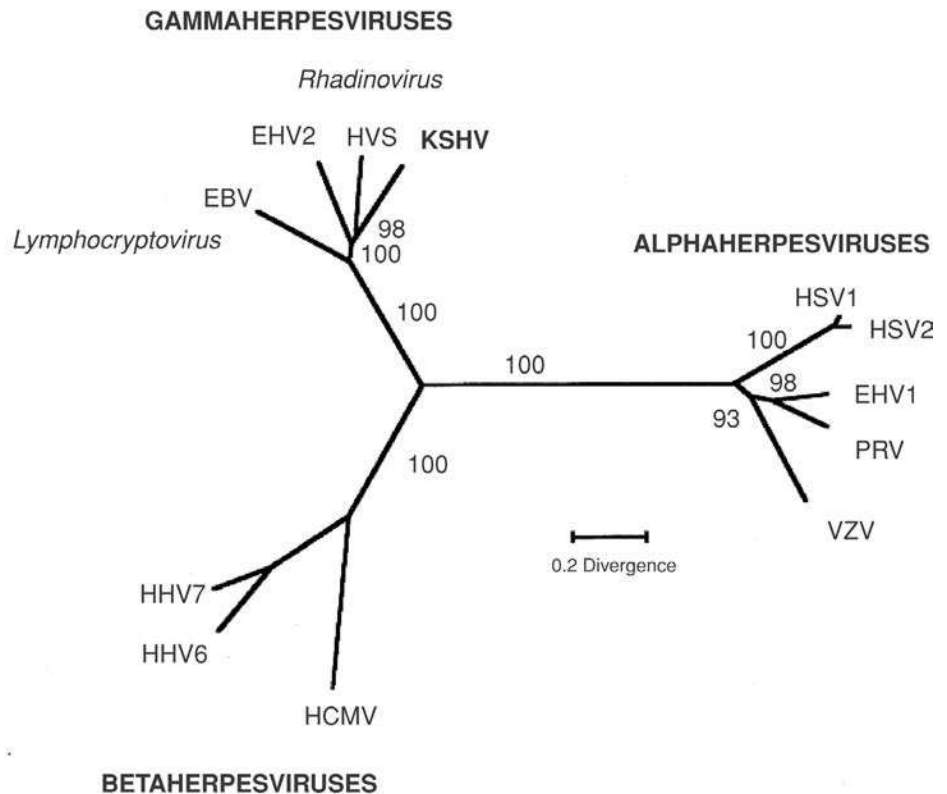
The KSHV genomic sequence confirmed the classification of the virus as a member of the lymphotropic (or γ -) herpesviruses (Fig. 65.1). Lymphotropic herpesviruses can be further divided into two groups: the $\gamma 1$ or *lymphocryptoviruses* (which includes EBV and its simian relatives) and the $\gamma 2$ or *rhadinoviruses*. KSHV falls squarely into the latter category,⁴⁹⁷ which also includes the prototype primate virus, *Herpesvirus saimiri* (HVS). With the identification of additional primate rhadinoviruses, it is now evident that rhadinoviruses are represented by two different lineages, based on their biological properties and genomic organization: One lineage is represented by KSHV and a primate virus named *retroperitoneal fibromatosis herpesvirus* (RFHV), and a second group is represented by HVS and rhesus monkey rhadinovirus (RRV) (reviewed in 56).

VIRION STRUCTURE

Structurally similar to virions of other herpesviruses, KSHV virions exhibit an electron-dense nucleocapsid surrounded by a lipid bilayer envelope as visualized by electron microscopy. In between the capsid and envelope is a morphologically amorphous but highly organized proteinaceous layer called the *tegument*. The envelope is studded with viral glycoproteins—for example, glycoprotein gB is thought to engage cell surface receptors and participate directly in viral entry. The virion contains six other glycoproteins, including open reading frame (ORF) 22 (gH), ORF39 (gM), ORF47 (gL), ORF53 (gN), ORF68, and K8.1.⁷³⁸ Little is known regarding the architecture of the tegument. However, multiple proteins have been identified that localize to this region.^{40,738} KSHV tegument proteins share the common feature of being delivered to the target cell along with the genome upon primary infection and may thus contribute to early events in the replication cycle even before the synthesis of immediate-early (IE) proteins. This is also the case for the 11 viral messenger RNAs (mRNAs) that are incorporated in the tegument layer.³⁸ These virion mRNAs are derived from viral transcripts expressed in the prior lytic cycle and are incorporated into the tegument during the process of budding and envelopment. It is likely that one or more mRNAs are selectively incorporated into the virus particle.^{38,40} How such targeted recruitment might occur is unknown.

Several abundant host cytoplasmic proteins such as Hsp90 and pyruvate kinase are also found in the virions.⁴⁰ The KSHV capsid, like its HSV and HCMV counterparts, is icosahedral. A significant amount of work has been done to characterize the KSHV capsid structure in terms of its three-dimensional architecture and polypeptide composition.^{495,656,714} Cryo-electron microscopy reconstruction has revealed that the icosahedral capsid is symmetric ($T = 16$) with 20 triangular faces. The capsomers are composed of hexamers and pentamers of the major capsid protein (MCP; encoded by ORF25). Each capsid contains 150 hexons and 12 pentons, which are interconnected by 320 triplexes that are heterotrimer structures comprised of

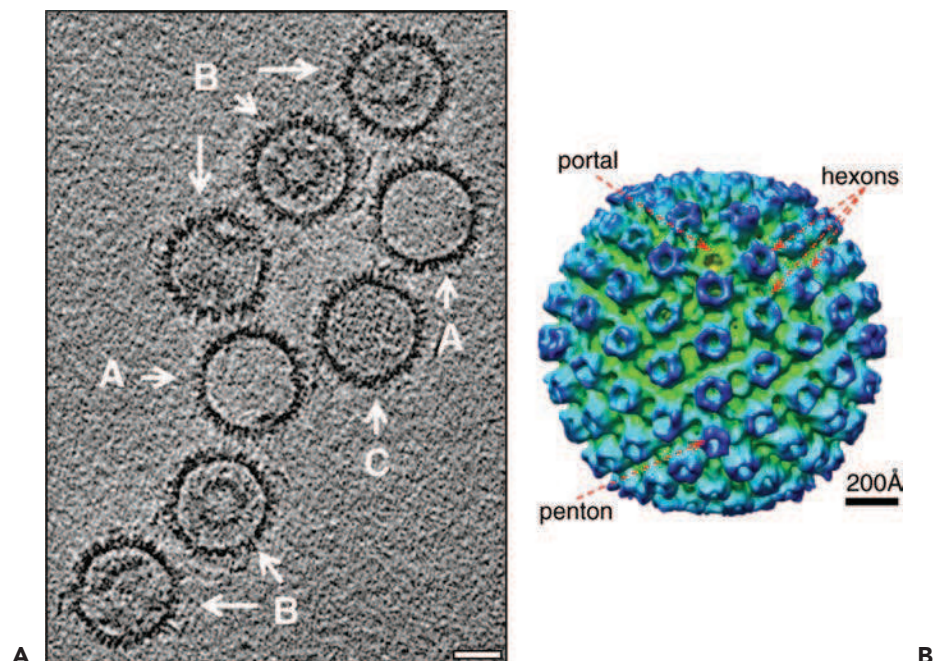
FIGURE 65.1. A radial phylogenetic tree was constructed based on comparisons of the herpesviral DNA polymerase proteins from 12 primate herpesviruses showing the relationships between members of the alpha-, beta- and gamma-herpesvirus subfamilies. Kaposi's sarcoma-associated herpesvirus (KSHV) is the first human rhadinovirus, the prototype member of which is *Herpesvirus saimiri*. Rhesus lymphocryptovirus (rhLCV) is a simian herpesvirus that shows high sequence relatedness to Epstein-Barr virus. Rhesus monkey rhadinovirus and retroperitoneal fibromatosis herpesvirus are simian viruses that show high sequence relatedness to KSHV. The phylogenetic tree was constructed using the default parameters in the Multalin program with a Blossum62 matrix (<http://multalin.toulouse.inra.fr/multalin/>).



ORF62 (one copy) and ORF26 (two copies). The capsomeric hexons form the capsid edges and faces, whereas the pentons are located at each capsid vertex (Fig. 65.2). There are three types of capsid structures as identified by electron microscopy: an empty capsid shell (A capsid), a capsid containing scaffolding protein (B capsid), and a capsid encapsidating the viral genome (C capsid)^{173,174,495} (see Fig. 65.2). For the most part, the KSHV capsid

resembles the features of the more well-studied herpes simplex virus type 1 (HSV-1) capsid, with the exception that the KSHV hexons lack the presence of a VP26 subunit that is seen bound to the HSV-1 hexon subunits, and the KSHV triplexes appear smaller and less elongated than those of HSV-1.⁷¹⁴ The viral linear genomic DNA of 165 to 170 kb⁵⁶³ is located inside the capsid.

FIGURE 65.2. Kaposi's sarcoma-associated herpesvirus (KSHV) A-, B-, and C-capsid. **A:** Central slice of a tomogram showing the KSHV A-, B-, and C-capsid. **B:** Radially colored surface representation of an average KSHV capsid showing the characteristic herpesvirus capsomers, including an "umbilical" portal, 11 pentons, and 150 hexons. (From Deng B, O'Connor CM, Kedes DH, et al. Cryo-electron tomography of Kaposi's sarcoma-associated herpesvirus capsids reveals dynamic scaffolding structures essential to capsid assembly and maturation. *J Struct Biol* 2008;161(3):419–427, with permission.)



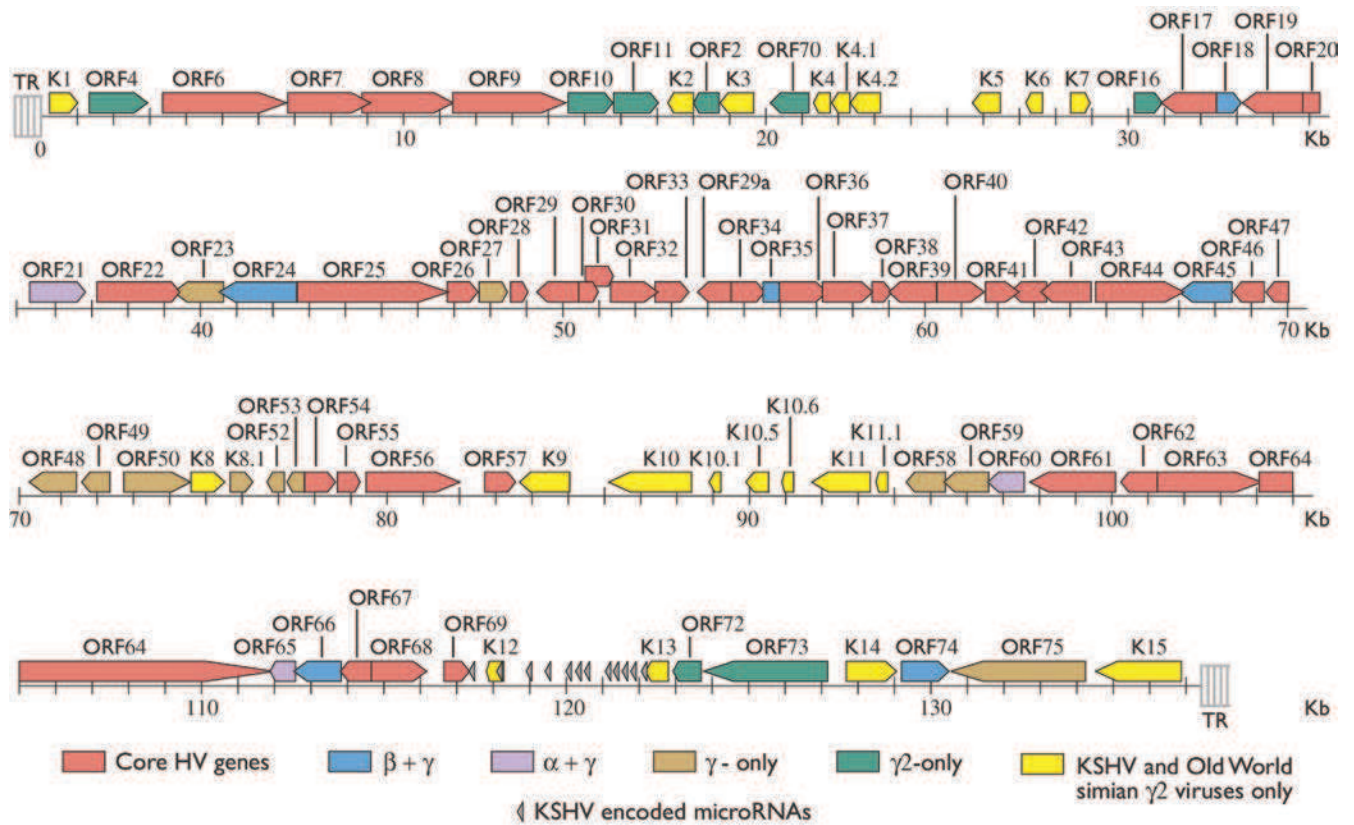


FIGURE 65.3. The Kaposi's sarcoma-associated herpesvirus (KSHV; human herpesvirus 8) genome map. Blocks of conserved genes between KSHV and related α , β , and γ herpesviruses are indicated by the same color. Arrows indicate open reading frames, with the direction of the arrow indicating the direction of translation.

KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS GENOME: STRUCTURE AND ORGANIZATION

The KSHV genome is a double-stranded linear DNA that is 165 to 170 kb long.⁵⁷¹ The genome is comprised of a central unique region of approximately 145 kb encoding the viral genes.⁵⁷¹ The central region is flanked by highly GC-rich direct terminal repeats (TRs) (Fig. 65.3). Each TR unit is 85% GC rich and 801 bp long. Commonly, viral isolates contain 20 to 25 kb of TR DNA per genome; however, the exact number of TRs present at each terminus differs.³⁵⁸

Comparison of the KSHV genome sequence with other herpesviruses reveals blocks of highly conserved genes that are common to all herpesviruses (see Fig. 65.3). These are typically genes that encode for proteins involved in viral replication or proteins that encode for structural components of the virions. In addition, there is a group of genes that show homology to the gammaherpesvirus ($\gamma 1$ and $\gamma 2$) lineage and those that are only common to the $\gamma 2$ lineage. Finally, there is a group of genes that is only encoded by KSHV and its simian relatives but not present in other herpesviruses from nonprimate species—for example, KSHV viral interleukin 6 (vIL-6) and viral interferon regulatory factors (vIRFs) (see Fig. 65.3).

The KSHV ORFs are numbered in consecutive order from left (ORF1) to right (ORF75). KSHV contains numerous

ORFs that are not conserved in other herpesviruses, and these are interspersed among the conserved herpesvirus genes. The KSHV unique ORFs are given “K” designations—ORFs K1 through K15—numbered from left to right (see Fig. 65.3). Some of the K ORFs encode for more than one protein owing to alternative splicing and/or alternative translational initiation sites. Many of the K ORFs encode signaling molecules and proteins that are homologs of cellular genes (e.g., vIL-6 and vIRFs).^{496,571} The KSHV genome also encodes for many small noncoding RNAs, including a set of microRNAs (miRNAs)^{87,496,531,579} and polyadenylated nuclear RNA (PAN), a noncoding lytic 1.1-kb nuclear RNA.^{148,642,735,736}

Genetic Analysis of Kaposi's Sarcoma-Associated Herpesvirus

Construction of mutant viruses using homologous recombination has revolutionized the study of replication and pathogenesis of herpesviruses. Two methods have been used to generate recombinant KSHVs. In earlier work, PEL were transfected with a targeting vector encoding the viral gene of interest, disrupted by a green fluorescent protein (GFP) cassette and selectable marker. Stable GFP-positive cells were selected, followed by viral reactivation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and supernatants were then tested for GFP transduction capacity.⁶⁷⁰ Zhou et al⁷³⁷ were the first to use BACmid technology to construct a KSHV BACmid. The entire KSHV

genome was cloned into a bacterial artificial chromosome (BAC), at a nonessential locus in the genome, with the BAC plasmid containing GFP and a selectable marker. The recombinant BAC was introduced into permissive cells, along with an expression vector for the key lytic protein, ORF50/replication and transcription activator (RTA); alternatively, the cells were treated with TPA, and reactivated supernatant virus harvested and amplified.⁷³⁷ Subsequently, this recombinant KSHV-BAC was then used for homologous recombination in *Escherichia coli* to make recombinant viruses. In principle, any nonessential gene can be rendered inactive using this protocol; for essential genes, *in trans* complementation is required to rescue the virus. Several groups have used BAC technology to make recombinant viruses. Recombinant viruses deleted for the following genes have already been constructed in the literature: gB, gpK8.1, ORF45, ORF50 (RTA), K8 (bZip), ORF56, ORF57, vFLIP, and ORF73 (latency-associated nuclear antigen [LANA]).^{170,272,323,346,373,429,688,721,725,741} In addition, replication-competent KSHV viruses with markers (GFP and RFP) to track latent (GFP) and lytic (RFP) infection have been constructed⁶⁷¹ and are highly beneficial for studies of viral infectivity.

VIRAL LIFE CYCLE

Viral Binding and Entry

KSHV is able to infect multiple cell types *in vitro* and *in vivo*. KSHV binds to and enters various human cell lines and primary cells in culture, including transformed B cells, primary B cells, 293 human embryonic kidney cells, THP-1 monocytic cells, myeloid and plasmacytoid dendritic cells (DCs), primary monocytes, human foreskin fibroblasts (HFFs), HeLa epithelial cells, and endothelial cells. KSHV can also infect monkey cells such as Vero and CV-1, hamster cells such as BHK-21 and CHO cells, and mouse cells *in vitro*.^{10,39,248,296,305,315,331,335,344,357,464,492,555,556,562,697,698}

KSHV encodes for multiple envelope glycoproteins; however, only three of these—gB, gH, and gL—are necessary to mediate membrane fusion.⁵²⁹ Attachment of the virion to the cell is thought to be mediated by several host receptor proteins as described later and highlighted in Figure 65.4.

Heparan Sulfate

Several glycoproteins encoded by KSHV have been shown to bind heparan sulfate present on the surface of the cell. These include KSHV gB, gH, ORF4, and gpK8.1A.^{12,52,270,490,676} The fact that the virus encodes four proteins that bind to heparan sulfate highlights the importance of this protein for attachment. Binding to heparan sulfate may contribute to efficiency of infection, because soluble heparan competes and hinders binding and impairs (but does not eliminate) infectivity. Thus, similar to HSV, the role of heparan sulfate binding helps to concentrate virus particles on the cell surface, thereby increasing their effective local concentration for interactions with cell surface receptors.

Integrins

Chandran and colleagues^{11,12,676} were the first to identify an integrin binding motif—the RGD (Arg-Gly-Asp)—motif, contained in the KSHV gB glycoprotein, and showed that it contributes to KSHV binding and entry. A twofold reduction in KSHV infection of endothelial and fibroblast cells was observed upon treatment with RGD peptides or antibodies

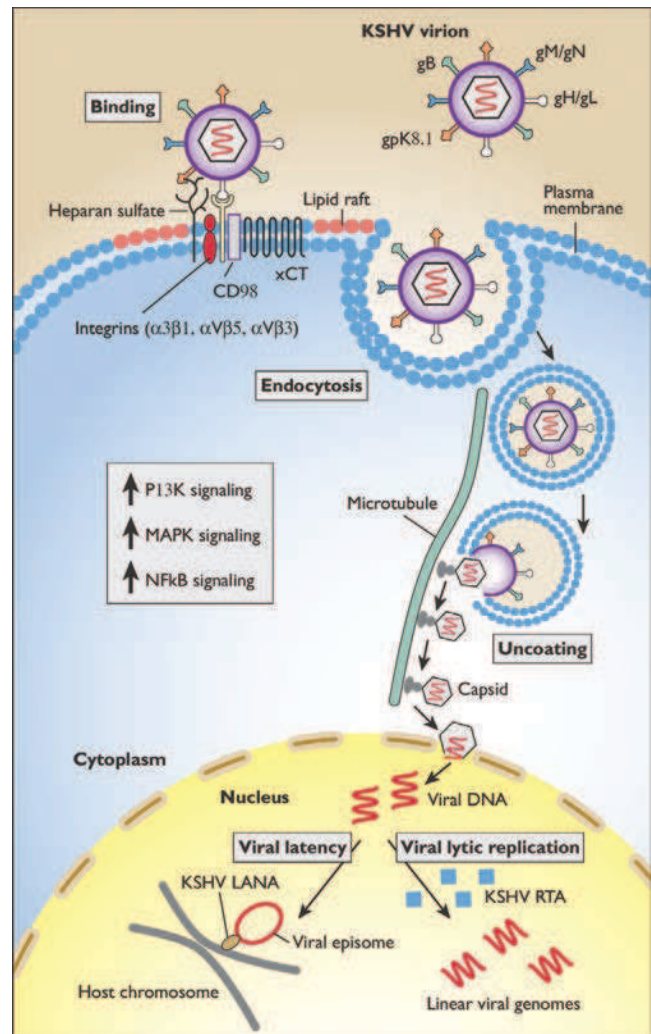


FIGURE 65.4. Kaposi's sarcoma-associated herpesvirus (KSHV) entry. Depicted are virion binding, attachment, and entry into the host cell. The different stages of KSHV entry are highlighted. Once the viral genome enters the nucleus, the decision to enter the latent or lytic phase of the life cycle is made.

against RGD-gB peptide.¹¹ Conversely, blocking of integrins using anti-integrin $\alpha3$ or $\beta1$ antibodies also prevented KSHV entry.¹¹ Subsequent studies have shown that $\alpha V\beta3$ and $\alpha V\beta5$ integrins also play roles in KSHV entry of adherent target cells.²³¹ Although anti-integrin antibodies reduced binding of KSHV to the cells, binding was not completely blocked suggesting that heparan sulfate is also required.

DC-SIGN

Dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN; CD209) is expressed on activated B cells, DCs, and macrophages. KSHV is thought to bind DC-SIGN because KSHV infection can be blocked by an anti-DC-SIGN monoclonal antibody and soluble DC-SIGN.^{555,556} However, similar to the situation with the integrins mentioned earlier, these agents did not completely prevent KSHV binding to the cell, suggesting that additional binding partners may exist (e.g., heparan sulfate) (see Fig. 65.4).

The incomplete inhibition of viral entry by the integrin blocking antibodies supports the notion that although integrins play an important role in viral entry, other host proteins may also be involved in KSHV entry.

xCT

xCT is a 12-transmembrane glutamate/cysteine exchange transporter protein that has also been proposed to be a KSHV entry receptor.³¹⁶ xCT is part of the cell surface CD98 (4F2 antigen) complex containing a common glycosylated heavy chain and a group of 45-kDa light chains; the xCT molecule is one of the light chains. Expression of recombinant xCT protein in cells that are not permissive for infection restores permissivity to KSHV infection. Additionally, antibodies against xCT block infection.³¹⁶ Generally, xCT exists as a heterodimer with CD98, and this complex can associate with cell surface integrins (e.g., $\alpha 3 \beta 1$). Collectively, these studies demonstrate the interactions of KSHV with closely linked cellular receptor proteins (e.g., heparan sulfate, integrins, and xCT) in different cell types⁶⁶² (see Fig. 65.4). Additionally, there are likely to be many different entry pathways *in vivo*. Of note, the KSHV-encoded miRNAs were reported to up-regulate xCT expression in macrophages and endothelial cells through miR-K12-11 suppression of the transcriptional repressor BACH-1.^{546,614} The up-regulation of xCT by the KSHV miRNAs increased cell permissiveness for KSHV infection and protected infected cells from death induced by reactive nitrogen species.⁵⁴⁶ Most recently, the ephrin receptor tyrosine kinase A2 (EphA2) was shown to be a cellular receptor for KSHV.^{111a,270a}

Mechanisms of Viral Entry

Endocytosis is the predominant mode of KSHV entry *in vitro* for both adherent and nonadherent target cells.^{10,116,550,660} KSHV utilizes clathrin-mediated endocytosis and a low pH environment for its infectious entry into human fibroblast (HFF) cells.¹⁰ Electron microscopic observation of infected BJAB (KSHV- and EBV-negative B lymphoma) cells demonstrated virus entry via large, as yet uncharacterized, vesicles.¹² Studies using agents blocking acidification of endosomes suggested a low pH-dependent endocytic infectious entry pathway in human embryonic kidney epithelial²⁹³ cells. Studies also suggested endocytosis as the mode of KSHV entry in activated primary human B cells.⁵⁵⁵ KSHV induces actin reorganization and enters human microvascular dermal endothelial cells (HMVEC) and umbilical vein endothelial (HUVEC) cells via macropinocytosis.⁵⁵⁰ In these cells, KSHV also induced membrane blebbing⁶⁶⁰ in a c-Cbl-dependent manner. Unlike entry in endothelial cells, KSHV enters human monocytic THP-1 cells via clathrin/caveolin-mediated endocytosis.³³¹

Binding of the virus to the cell not only promotes viral entry but also triggers host cell signaling cascades that may modulate the cellular microenvironment to enhance virus infection. For example, binding of the cell surface integrins by KSHV triggers phosphorylation and activation of focal adhesion kinase (FAK). This activates several signaling molecules, including PI3K, Src, Rho guanosine triphosphatases (GTPases; RhoA, Rac, and Cdc42), and diaphanous 2 (Dia2). Downstream effector molecules of these signaling moieties are also consequently activated and include Akt, Ezrin, protein kinase C (PKC), MEK, extracellular signal-regulated kinase (ERK) 1/2, NF- κ B, and p38 mitogen-activating protein (MAP)

kinase.^{11,345,490,491,518,551,572,599,600,663,720,728} (A more detailed review of KSHV binding and triggering of signaling pathways can be found in reference 116.) Signal transduction does not need active viral replication, because ultraviolet (UV)-inactivated virions also elicit signaling, and signaling is inhibited by soluble $\alpha 3 \beta 1$ integrin peptides. Inhibitors of PI3K, PKC- ζ , MEK, and ERK can reduce KSHV infectivity.⁵⁹⁹ FAK-PI3K activation induced by KSHV binding also activates Rho GTPase and induces cytoskeletal rearrangements.^{599,600}

Following virion binding, cellular kinases are activated, leading to the recruitment of signaling molecules to the plasma membrane. These signaling proteins then rapidly translocate to clathrin, caveolae, and other vesicles.⁴⁵⁹ Src-mediated phosphorylation of clathrin induces the translocation of clathrin to the plasma membrane, where it interacts with AP2, Eps15, and dynamin.⁴⁵⁹ Src-initiated phosphorylation also triggers the assembly of the plasma membrane-associated Ras activation complex, leading to the downstream activation of Rho and Rab GTPases by Ras and PI3K. The Rho and Rab GTPases play critical roles in the formation and movement of endocytic vesicles.²⁷¹ These various KSHV-induced signaling events are summarized as follows:^{11,345,490,491,518,551,572,599,600,663,720,728}

1. KSHV binding to integrin triggers FAK activation, which appears to be critical for viral entry.^{345,600}
2. The activation of FAK and Pyk2 leads to the activation of the Src kinase family, which subsequently activates PI3K and Rho GTPases. Inhibitors of PI3K reduced viral entry, suggesting that PI3K's activation of Rac, Rho, Cdc42, and Rab5 GTPases are critical for viral entry, because they induce actin reorganization and provide the mechanical force required for endosome formation and trafficking. Induction of the downstream modulator Akt kinase early during the entry process may be beneficial for triggering a pro-survival environment in the infected cell.^{345,551,600}
3. PI3K and Rho GTPase activation leads to cytoskeletal rearrangements and the formation of structures such as lamellipodia (Rac), stress fibers (RhoA), and filopodia (Cdc42). This allows for microtubule acetylation and the thickening of microtubule bundle. This process is also essential for delivery of the viral capsids to the nuclear membrane via the microtubules.^{491,663}
4. KSHV also activates the ERK1/2 signaling pathway and the NF- κ B pathway. These pathways are thought to be important for viral gene expression.^{490,492,518,599,720}

The viral capsids traverse down the microtubules toward the nuclear membrane. Consistent with this description, microtubule-depolymerizing agents block infection.⁴⁹¹ It is thought that the capsid then injects the viral genomic DNA into the nucleus through the pore complex. Once the viral DNA enters the nucleus, the virus has a choice to enter either the lytic phase of its life cycle or the latent phase (see Fig. 65.4). During latency, the genome is circularized and subsequently is organized into chromatin through the assembly of cellular histones, thereby making it a suitable template for host RNA polymerase II transcription. During lytic replication, the virus is in a linear state amenable for viral DNA replication.

Innate Immune Response Following Viral Entry

Toll-like receptors (TLRs) are the first line of defense against invading pathogens. They recognize pathogen-associated

molecular patterns (PAMPs) present on invading pathogens and trigger signaling cascades, which lead to the activation of type I interferon (IFN), NF- κ B, and many other proinflammatory cytokines. Some TLRs are expressed on the cell surface (e.g., TLR2 and TLR4), whereas others are expressed in the endosome (e.g., TLRs 3, 7, 8, and 9).⁸ TLRs 3, 7, and 8 are activated by RNA. TLR3 recognizes both single-stranded RNA and double-stranded RNA, whereas TLR7 and TLR8 recognize single-stranded RNA. It is important to note that different cell types have distinct TLR expression profiles; hence, TLR recognition of an invading pathogen depends on the cell type being infected.

During infection of primary human monocytes, KSHV activates TLR3, resulting in the up-regulation of TLR3 expression (both mRNA and protein are increased) and its downstream mediators, including IFN- β 1 and the chemokine CXCL10 (also referred to as IP-10).⁶⁹⁷ As mentioned previously, KSHV is known to be endocytosed in monocytes, and during this process, it is possible that the viral RNA transcripts present in the tegument of the virus trigger TLR3 activation. In human plasmacytoid dendritic cells (pDCs), which are the chief IFN-producing cells in the body, KSHV infection triggers the activation of TLR9, a TLR whose agonist is CpG DNA.⁶⁹⁸ Thus, in pDCs, TLR9 is likely recognizing KSHV viral DNA. Unlike monocytes, human pDCs do not express TLR3 and only express TLR7 and TLR9. Finally, TLR4 appears to be important for recognition of KSHV in endothelial cells, as the virus can down-regulate TLR4 expression on endothelial cells.³⁵⁶ Both vIRF1 and viral G protein-coupled receptor (vGPCR) are involved in TLR4 down-regulation.³⁵⁶

TLRs also play a role in a very different context—for example, during reactivation from latency. Stimulation of TLR7/8 in KSHV latently infected PEL cells, either by synthesized agonist (poly-Uridine) or by infection with a biologically relevant pathogen (vesicular stomatitis virus—a known activator of TLR7 and -8), led to viral reactivation and progeny virion production.²⁵⁰ This represents a new role for TLR stimulation in the context of KSHV infection (which differs from the role that TLRs play during primary infection) and suggests a possible physiologic stimulus that can lead to viral reactivation *in vivo*.²⁵⁰

OVERVIEW OF THE LYTIC AND LATENT PHASES OF THE KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS LIFE CYCLE

Following successful primary infection, KSHV can establish either a lytic or a latent state in the infected cell. Subsequent to initial viral infection, the host cell microenvironment is thought to govern the decision of which life cycle phase the virus enters. The default program appears to be latency. The kinetics of lytic versus latent gene expression during primary infection was examined in KSHV-infected endothelial cells and fibroblasts.³⁴⁴ ORF50/RTA levels were detected 2 hours postinfection but declined thereafter. Conversely, low levels of LANA were seen at first; however, expression increased substantially 48 to 72 hours postinfection. The latent genes were expressed continually, whereas lytic viral gene expression declined.³⁴⁴ Thus, concurrent expression of lytic and latent

genes occurs immediately following viral infection but only latent gene expression persists. It is thought that the minimal or marginal expression of lytic genes, many of which play key roles in immune evasion, cell proliferation, and prevention of apoptosis, could be necessary for the establishment and maintenance of latent infection *in vivo*.^{254,344}

Latency is the default pathway in most, if not all, experimental situations, and no virions are produced during this stage. During latency, viral gene expression is limited to only a subset of viral genes. The latent viral genome in the nucleus is replicated as an episome by host cellular DNA polymerase, and the replicated genomes are evenly distributed to the daughter cells. Although latently infected cells do not make virus, they maintain the viral genome and thus retain the potential for virus replication and virion production. Upon different exogenous stimuli, latently infected cells can undergo reactivation, a process that switches the virus from latency back into lytic replication mode. Physiologic stimuli during KSHV infection are not known; however, in tissue culture, several molecules can reactivate virus, including phorbol esters, histone deacetylase (HDAC) inhibitors (e.g., butyrate), TLR agonist stimulation, natural products from endemic regions, calcium ionophores, and IFN- γ .^{53,122,250,463,467,477,564,702,731,749} Additionally, cell differentiation has also been shown to induce KSHV lytic replication.^{311,597}

During lytic infection, the entire viral genome is expressed in a temporally regulated transcriptional cascade. Similar to other herpesviruses, the first set of genes that are expressed are known as the IE genes, which encode for transcriptional factors and regulatory proteins. The key lytic switch regulator is encoded by ORF50. ORF50 encodes for a protein referred to as RTA. Expression of RTA is sufficient to initiate the lytic replication cycle. The second temporal class of mRNAs encode for the delayed-early (DE) genes. The function of DE genes is to ready the cell for viral DNA replication and viral protein production. The DE genes encode for proteins (enzymes, co-factors) that are involved in viral DNA replication and proteins that affect nucleotide precursor pools and host transcription, as well as proteins involved in immune evasion. Once DE expression is under way, lytic cycle DNA replication commences in the nucleus. The objective of viral replication is to replicate the viral genomic DNA and produce many copies of the viral genomic DNA per cell. Subsequent to the onset of replication, the late (L) genes are synthesized. L genes are those that encode for viral structural proteins. These proteins are made after viral DNA replication because they are required in vast quantities. Late proteins are made after viral replication as they often need to be transcribed from genomic templates that have accumulated to a high enough copy number following viral genome replication.

After all three classes of IE, DE, and L genes are transcribed, virus assembly takes place in the nucleus. Newly replicated viral genomes are incorporated into newly synthesized viral capsids, which subsequently acquire their teguments by a poorly defined process. The capsids subsequently bud through host membranes in a series of steps to acquire a host-derived membranous envelope containing KSHV glycoproteins. Finally, viral progeny are released from the host cell. A more detailed view of KSHV latency and lytic life cycles is described later.

The transition to latency is not seamless. Following the first 12 hours postinfection, virus-infected cells display both lytic and latent gene expression.^{5,344} The lytic transcript profile is nontem-

poral in that different classes of IE, DE, and L genes are expressed simultaneously.^{5,344} It is possible that at least some of these lytic transcripts are delivered to the cell via the incoming virion tegument, which contains 11 viral lytic mRNAs that are assimilated into the virion during assembly.³⁸ Alternatively, some of these transcripts could represent genuine transcription using the host cell machinery. Some^{40,362} but not all⁷³⁸ studies have detected small amounts of the lytic switch protein ORF50/RTA in virions, which may contribute to this process. However, why the presence of RTA does not trigger the full lytic cascade is unknown and may depend on the host cell environment and the lack of sufficient amounts of cellular proteins required for lytic replication. Additionally, the level of viral DNA chromatinization also influences the ability of the virus to reactivate. Virion encapsidated viral genomes do not appear to harbor bound histones unlike the viral episomes found in latently infected cells.^{415,632} Several lytic transcripts represent inhibitors of apoptosis and viral mediators of immune evasion.³⁴⁴ At 24 hours postinfection, lytic gene expression declines, resulting in only latent gene transcription in the infected cell.

Approximately 24 to 48 hours postinfection, the classical latency program is established in nearly all infected cells, except for a small percentage (1%–3%) of cells that enter the lytic cycle. The frequency with which lytic infection ensues following *de novo* infection is related to the multiplicity of infection (MOI) and the cell type. For example, a high MOI in primary endothelial cells favors early progression into the lytic cycle. However, even under such circumstances, lytic replication tends to subside a few days postinfection, and the latency program dominates.

Our knowledge of the KSHV latent program comes from the study of PEL cell lines. These cells were originally isolated from PEL patients and subsequently propagated in cell culture.¹⁰⁷ PEL stably maintain latent KSHV genomes, and they express latent viral proteins in most of the cells. Although it is likely that latent gene expression in these cultured PEL cell lines is not representative of true latency *in vivo* per se, for the most part, mRNA transcripts identified in PEL are detected in other contexts (e.g., cell lines infected with KSHV *in vitro* as well as primary KS tumors).¹⁸¹

KSHV gene expression is complicated by the fact that a clear demarcation of latent versus lytic genes cannot always be made. For example, some genes are said to be expressed during latency (e.g., LANA), whereas other genes are expressed only during lytic replication (e.g., RTA/ORF50). However, although RTA is only expressed during the lytic cycle, LANA expression is observed during latency and lytic replication, suggesting a gray area in which some latent genes continue to be expressed at low levels during lytic replication, whereas other latent genes are expressed during latency and then highly up-regulated during lytic replication.

It is widely accepted that LANA, vFLIP, vCyclin, kaposins A, B, and C, and vIRF3 (aka LANA-2) are latent genes in PEL cells,^{179,567} as are the 12 pre-microRNAs (pre-miRNAs).^{87,579} However, there has been considerable debate about whether additional genes might also be expressed in latently infected cells, and several groups have shown that additional genes are expressed under some latent conditions. There is a class of genes that is expressed at low levels during latency but is highly up-regulated during lytic infection (e.g., vIL-6, K1)^{67,68,93,118,521,587,628,679} and K15 (Dr. Thomas Schulz, personal communication). Originally,

these genes were classified as purely lytic genes; however, more sensitive techniques indicate that these genes are also expressed at low levels under conditions in which there is no lytic gene expression.^{67,68,118,679} Additionally, the K1 promoter is active in epithelial, endothelial, and B cells in the absence of the lytic transactivator ORF50/RTA,^{67,68,118,679} lending credence to the fact that K1 is transcribed under latent conditions. A recent publication used tightly latent cell lines that displayed very low levels of spontaneous lytic reactivation to perform array-based transcript profiling and limiting dilution reverse transcription polymerase chain reaction (RT-PCR) in these cells. They found that both K1 and K2/vIL-6 genes were expressed under latent conditions.¹¹⁸ (This array was not able to analyze K15, which is another viral gene whose expression during latency has been controversial.) These results confirm earlier studies suggesting that K1 and vIL-6 could be viral latent genes.^{67,130,679} For K1, one compounding factor is that the protein is highly variable, thus antibodies against K1 from one strain may not readily recognize K1 from another strain, making protein expression analysis more difficult.^{67,130,679} It is also worth mentioning that in EBV, genes at the same genomic positions as K1 and K15 have been shown to be expressed during EBV types II and III latency and encode transforming abilities, raising the possibility of different latency patterns occurring during the KSHV viral life cycle.

In PEL, KSHV reactivation from latency is not 100%. During reactivation, low levels of latent gene expression can be detected in the background of lytic gene expression^{206,306,587} owing to the fact that it is usually not possible to reactivate all of the latent cells. Thus, there is always a background of latency. Finally, some viral gene products (e.g., vIRF3/LANA-2) are expressed in latent PEL but are not detected in latent KS spindle cells,⁵⁶⁷ whereas vIRF1 has been detected in latently infected KS cells but not in latently infected PEL.¹⁸¹ In EBV, at least three latent transcriptional programs have been described. Hence, the fact that KSHV also has a complex gene expression pattern should not be surprising.

In the following section, we first review the general patterns of latent and/or lytic transcription and then review the functions of these viral proteins.

Latent Phase Latent Transcripts

In PEL cells, the major latency transcript arises from a single genomic region, located between the K12 and Orf74 ORFs. At least two latent promoters are active, each of which initiate synthesis of a group of transcripts (Fig. 65.5). The first transcripts discovered originated from the LANA promoter, located just upstream of the LANA gene (ORF73).^{179,588,646} This promoter appears to be constitutively active. In transgenic mice, the LANA promoter drove LacZ expression mostly in B cells, although a few epithelial cells also demonstrated LANA expression.^{207,309} LANA promoter activity can be enhanced by exogenous expression of LANA, suggesting autoregulation of this transcript *in vivo*.³¹⁰ Additionally, there exists a second promoter element (just upstream of the latent LANA promoter) that up-regulates LANA transcription in the presence of increased RTA expression.^{362,447,630} There also exists a third latency promoter embedded within the LANA-vFLIP transcription unit that drives the vCyclin and vFLIP transcript, as well as the Kaposin transcript.^{382,526} All of these promoters also drive transcription of the viral miRNAs that are located in an

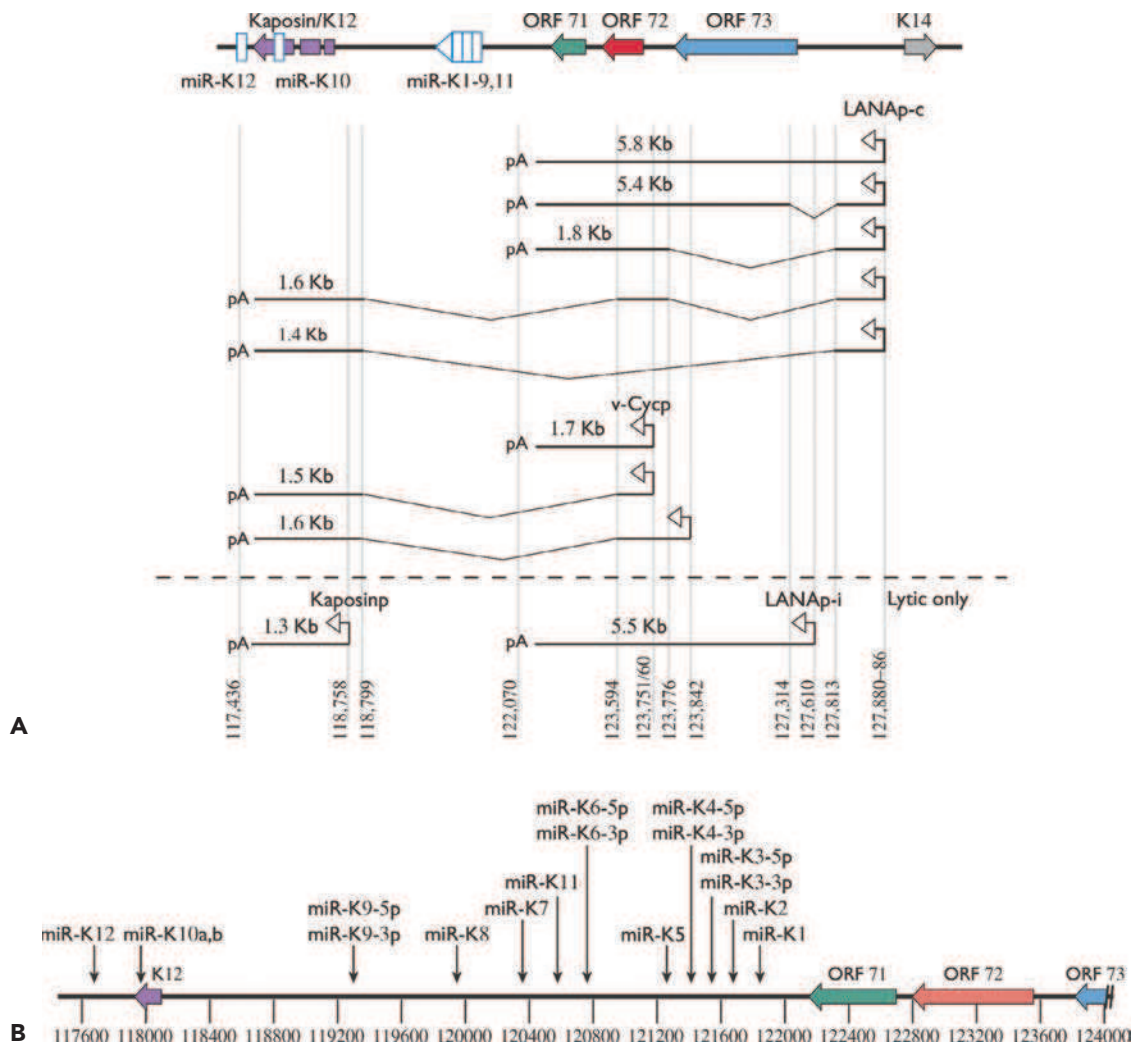


FIGURE 65.5. Map of known transcripts emanating from the main latency region of Kaposi's sarcoma-associated herpesvirus (KSHV). **A:** Top line shows map of the major genomic open reading frames (ORFs) (arrows), as well as the positions of most of the known microRNAs (miRNAs). Two major latency promoters exist: one 5' to ORF73 and the other just 5' to ORF72; each of these can generate the indicated spliced messenger RNA (mRNA) isoforms. Additionally, a lytic cycle promoter drives transcription of a 1.3-kb kaposin (K12) transcript. All mRNAs are polyadenylated at the indicated positions. (Modified from Yuan Y, Renne R. DNA tumor viruses. In: Damania B, Pipas J, eds. *Organization and Expression of the Kaposi's Sarcoma-associated Herpesvirus Genome*. New York: Springer Press; 2009:469–493.) **B:** A magnified view of the positions in the latency transcripts that encode the viral miRNAs.

intron obtained by read-through of the vFLIP-distal polyA-element and subsequent splicing.^{86,579}

This is a complex region of the viral genome because it has a nested arrangement of divergent promoters of latency genes (LANA, vCyclin, vFLIP, viral miRNAs) and lytic genes (vGPCR and K14). Thus, this region has a complex structure and intricate regulation so that lytic transcripts are suppressed during latent gene expression. A recent study showed that CTCF, a cellular protein that functions at chromatin boundaries, and cohesins, proteins that mediate sister chromatin cohesion, also bind to a specific site within the KSHV major latency control region.⁶³³ KSHV genes regulated by the CTCF-cohesin complex are under cell cycle control, and mutation of the CTCF binding sites abolished cell cycle-regulated transcription.³¹⁷ The cohesins interact with CTCF in mid-S phase

and repress CTCF-regulated genes in a cell cycle-dependent manner. Deletion of the CTCF-cohesin binding site in the KSHV genome caused an inhibition of cell growth as well as viral genome instability. Thus, CTCF and cohesins may play a critical role in regulating the cell cycle control of viral gene expression during latency.^{317,633}

The LANA promoter primarily drives transcription of three genes—ORF73 (LANA), ORF72 (vCyclin), and ORF71 (vFLIP), which utilizes both alternative splicing and internal translational initiation.¹⁷⁹ Figure 65.5 depicts the spliced mRNAs generated from this locus; all of these spliced transcripts are polyadenylated at a common site downstream of ORF71. The ORFs 73-72-71 tricistronic mRNA exists as an unspliced transcript, and another transcript with a small splice in its 5' untranslated region (UTR). An even more highly

expressed transcript is a spliced bicistronic transcript comprised of ORFs 72 and 71. This mRNA encodes both vCyclin (ORF72) and vFLIP (ORF71). vCyclin is translated by conventional cap-dependent initiation, whereas vFLIP (ORF71 or K13) translation is thought to occur through the use of an internal ribosome entry site (IRES) element located within the ORF of ORF72.^{50,255,411}

The second set of major latent transcripts in this region encodes for proteins from the kaposin locus (see Fig. 65.5). All mRNA transcripts originating from this locus are polyadenylated at a common site downstream of ORF K12. These transcripts include both ORF K12 and the upstream direct repeat (DR) sequences DR1 and DR2. When these mRNAs are translated, they generate kaposins A, B, and C proteins.⁵⁷³ Several latent promoters regulate the initiation of transcripts from this region. One transcript maps upstream of ORF72 and is spliced to a region just upstream of the direct repeats in latent PEL.^{86,382,526} Extensive transcript mapping has identified multiple spliced latent transcripts (all capable of expressing kaposins). Some of these transcripts originate from the LANA promoter through alternative splicing that removes ORFs 71 through 73^{86,526} (see Fig. 65.5). The spliced kaposin transcripts also encode for many of the viral miRNAs.^{86,87,257,531,579} Most of these miRNAs map to the region upstream of the kaposin DRs, which is essentially the intron of the latent kaposin pre-mRNAs. Other miRNAs map to the mature kaposin transcript (see Fig. 65.5). The miRNAs are derived by Drosha-mediated processing.

Finally, a separate promoter drives transcription of an unspliced mRNA (1.3 kb long) starting from a region upstream to the DR2 region⁵⁷³ (see Fig. 65.5). This mRNA transcript is made during lytic replication because its promoter contains RTA binding sites.¹²⁴ This results in the expression of high levels of kaposin proteins during lytic replication.⁶²⁹

Kaposi's Sarcoma–Associated Herpesvirus Latent Proteins

Latency-associated Nuclear Antigen

LANA, the product of ORF73,^{325,327,552} is a large and multifunctional protein that is present in the nucleus of latently infected cells. LANA exhibits characteristic punctate foci in the nucleus when examined by immunofluorescence and immunohistochemistry assays. LANA is highly immunogenic, and antibodies against LANA were the first utilized serologic markers of KSHV infection. LANA-specific CD8 T-cell responses have also been observed.³⁸¹ LANA has been detected in PEL and KS tumor cells *in vivo*, in B cells in MCD, and in every latent cell type infected *in vitro*.¹⁹¹ LANA has three major domains: an N-terminal domain involved in chromatin attachment and corepressor recruitment, a central region composed of highly acidic amino acid repeats, and a C-terminal basic region involved in DNA binding and oligomerization. LANA interacts with many viral and cellular proteins to exert its function. The binding sites for proteins that bind LANA is illustrated in Figure 65.6.

The best-characterized function of LANA is its involvement in the establishment and maintenance of the nuclear latent viral genome.^{28,152} The episomal viral genomes established in the nucleus during latency are replicated by the host DNA polymerase during cell proliferation in order to be

passed on to the daughter cells. LANA plays many roles in this latent replicative process. It directly binds DNA sequences within the TRs that contain the latent origin of replication^{29,153,229,230} and induces a bend.⁷¹⁰ The LANA–TR interaction initiates semiconservative replication by recruitment of the host DNA replication machinery to the viral episome.^{256,291,399} Each 801-bp-long TR contains a high- and a low-affinity LANA binding site (LBS1 and LBS2, respectively). LBS1 and LBS2 are separated by 22 nucleotides. There is a GC-rich replication element that lies upstream of LBS1 and LBS2. Deletion of this element hinders origin activity,^{293,665} suggesting that the replication element is the site for binding of cellular proteins involved in latent replication. In sum, LANA and the TR sequences recruit the host cell origin recognition complex (ORC) and mini-chromosome maintenance (MCM) proteins.^{399,632,664} LANA's C-terminal DNA binding domain binds the TRs at two sites which helps to recruit ORC and MCM.^{229,291,632,664} Although the C-terminal domain of LANA is sufficient for latent replication, the N-terminal chromosomal binding domain enhances latent replication, suggesting that replication and maintenance are interlinked.^{31,291}

TRs undergo reorganization in late G₁/S phase when replication licensing and initiation occur.⁶³² The structure-specific recognition protein 1 (SSRP1) and telomeric repeat binding factor 2 (TRF2) bind to LANA and the TR region²⁹² to facilitate viral episomal replication during latency.²⁹² The LANA–TR interactions are similar to the EBV EBNA-1–oriP complex, although LANA does not show significant sequence homology to EBNA-1, and the EBV oriP sequence is very different from the LANA binding elements of the TR. Yet, the LANA C-terminal DNA binding domain shows some similarity to EBNA-1 in terms of predicted secondary and tertiary structural motifs.²⁵⁶ Viral genomes are replicated once per cell cycle in synchrony with replication of the cellular genome.⁶⁶⁵

Semiconservative replication is not sufficient to explain the establishment of stable KSHV latency. A mechanism must exist in proliferating cells to ensure the proper segregation of replicated KSHV genomes to the daughter cells. Here, too, LANA plays a critical role. Transfected plasmids with KSHV TR elements joined to a drug-resistance selectable marker produced stable colonies at a higher frequency when LANA was present than in its absence.^{28,152} Such colonies display stably maintained circular genomes over subsequent generations, as is seen in PEL cultures. In interphase cells, viral genomes appear to be localized to LANA-containing dots or punctae. In mitotic cells, LANA and the viral episome co-localize to mitotic chromosomes. Thus, LANA can bind to mitotic chromosomes through its N-terminal domain.⁵³⁶ These data are suggestive of a model in which the N-terminus of LANA binds to the mitotic chromosome and tethers the viral genome (bound via its C-terminus) to this structure.^{32,347,718,729} This allows for the segregation of the tethered latent episomes to both daughter cells following mitosis. Tethering occurs through multiple protein–protein interactions, including binding to histones H2A–H2B, H1, centromeric protein F (CENP-F), budding uninhibited by benzimidazole 1 (Bub1), Brd4, and Brd2/RING3.^{32,152,347,451,539,672,718,729} Genetic evidence for this mechanism comes from the observation that a KSHV recombinant virus with a disrupted LANA gene does not generate a stable latent episome following primary infection.⁷²⁵

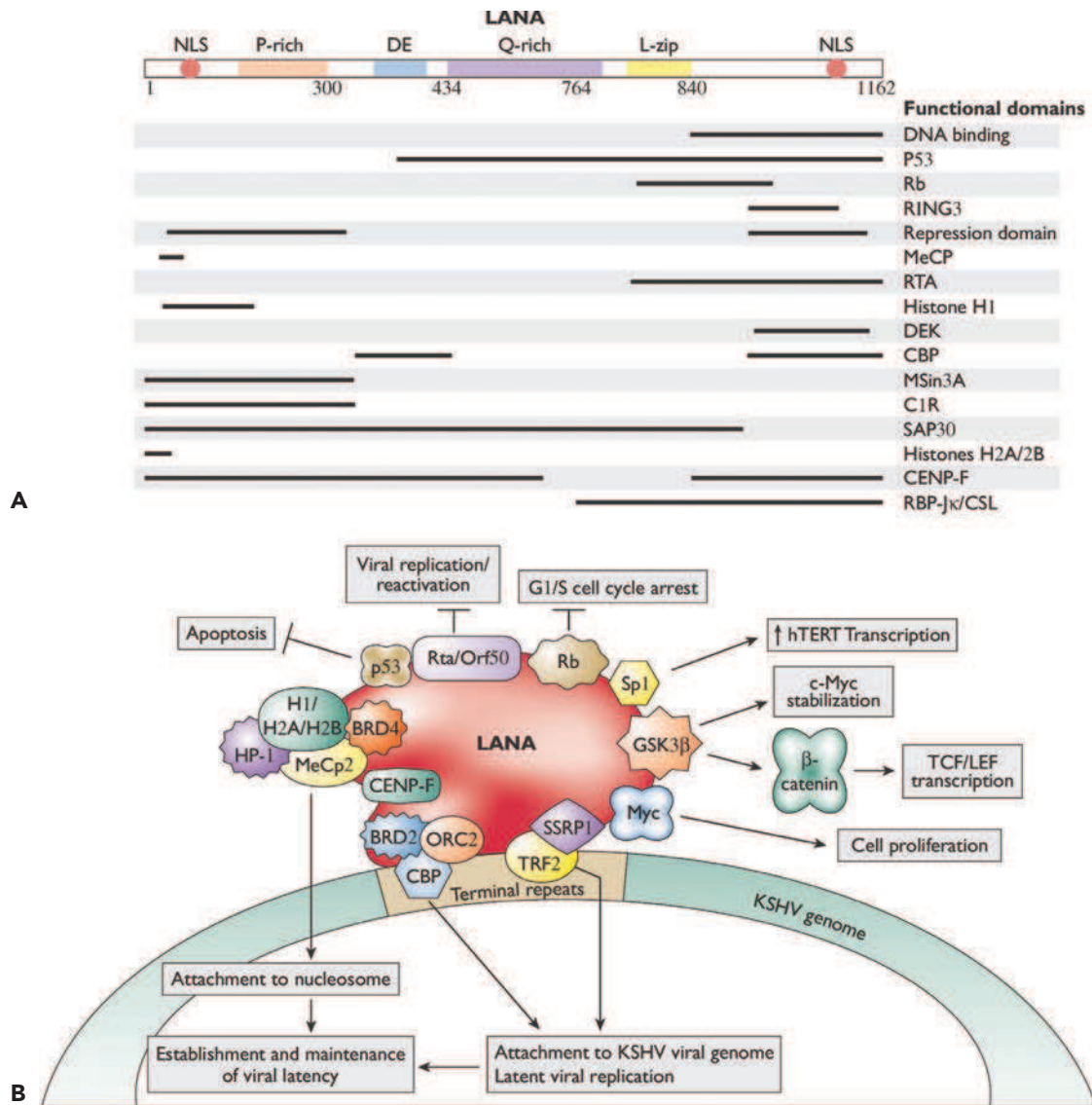


FIGURE 65.6. Latency-associated nuclear antigen (LANA) domains and interaction partners. A: Schematic depiction of LANA domains. Bars indicate regions implicated in the indicated interactions or functions. **B:** Functional consequences of the interaction of LANA with other cellular and viral proteins. NLS, nuclear localization signal; P-rich, proline-rich region; Q-rich, glutamine rich region; L-zip, leucine zipper; DE, aspartic acid/glutamic acid rich repeats.

The plasmid replication and segregation functions of LANA are linked because point mutations in the N-terminus chromosome binding region of LANA are defective in the LANA plasmid replication assay.³⁹⁴ Additionally, the minimal LANA sequence required for plasmid DNA replication is comprised of both the N-terminal chromosome binding domain and the C-terminal oriP binding domain.³⁹⁷ Taken together, these findings give rise to the model that plasmid DNA replication occurs in association with host chromatin.

Viral episome maintenance may be more complex, however. In cells harboring the KSHV genome that do not make use of drug-resistance marker inserted in the viral genome, LANA, while necessary for plasmid maintenance, is not always sufficient. When LANA expression plasmids and the TRs are introduced into cells, the resulting episomes are very unstable

and rapidly lost during the process of cell division.²⁵⁴ This suggests that viral latency requires the *in vivo* environment.^{254,481} In certain cell lines, it has been shown that small subpopulations of cells do exhibit stable genomes and enter permanent latency similar to the case in PEL tumor cell lines.¹³⁴ In these cases, the viral genome undergoes epigenetic changes that enable it to be stably maintained in the cell.²⁵⁴

Curiously, the KSHV genome also contains a *cis*-acting DNA element within the long unique region that can initiate and support latent replication of plasmids lacking LANA binding sequences or a eukaryotic replication origin. The replication machinery proteins, ORC2 and MCM3, associate with this sequence and are required for replication of this element. Thus, KSHV can initiate latent replication of its genome in the absence of LANA or any other viral protein.⁶⁶⁶

Aside from its function in maintenance of the KSHV latent genome, LANA is also involved in the dysregulation of cell growth and survival. Transgenic mice expressing LANA from the latent promoter display splenic follicular hyperplasia and increased GC formation²⁰⁷ as well as an increased response to antigen stimulation.⁶⁰⁹ Some of the older animals developed B-cell lymphomas. The slow onset of lymphomagenesis suggests that LANA itself may not be sufficient for transformation and requires additional genetic changes once LANA drives the expansion of B cells, which is likely the result of LANA-mediated proliferation or LANA's ability to augment cell survival.⁶⁹¹

There have been many biochemical studies looking at LANA interactions with cellular proteins.^{135a,135b,323a} It has been found that LANA binds p53, and LANA-expressing cells display less activation of p53-dependent reporter genes and also are more resistant to p53-dependent apoptosis induced by irradiation and so forth.²¹⁵ At the same time, most PEL respond to p53 activating DNA damaging agents.⁵³⁰ LANA expression in endothelial cells extends their life span, although this does not lead to immortalization.⁶⁹¹

LANA can also bind the tumor suppressor Rb.⁵⁴⁹ This binding functionally inactivates Rb, resulting in increased E2F-dependent reporter gene activation in the presence of LANA. LANA can cooperate with Ras in 3T3 cell transformation, similar to the case with SV40 large T and small t antigens. However, in KSHV-infected tumors, this inactivation of Rb may be incomplete. For example, some PEL cell lines display a loss of p16^{INK4a}, a cdk inhibitor.⁵³⁸ Cdk inhibitor is a cellular cyclin D/cdk6 inhibitor, which in turn phosphorylates and thereby inactivates Rb. Thus, p16^{INK4a} loss should induce cell proliferation. This suggests that despite LANA expression, Rb function is not completely inactivated in PEL. Consistent with this hypothesis, when p16^{INK4a} expression is restored, PEL cells are rendered sensitive to growth inhibition. These observations suggest that additional mechanisms may be necessary to completely eradicate the function of Rb in the infected cell.

LANA can also interact with GSK-3 β , a kinase that phosphorylates and inactivates β -catenin by targeting it for ubiquitin-mediated degradation.²¹⁷ LANA binding to GSK-3 β induces the kinase to move from the cytosol to the nucleus, allowing for cytoplasmic accumulation of β -catenin. Cytosolic β -catenin can then interact with the transcription factor LEF, which relocates it to the nucleus, where this complex up-regulates proliferative genes (e.g., cyclin D and c-Myc). The correlation between LANA expression and β -catenin up-regulation in PEL is inhibited by RNA interference (RNAi)-mediated knockdown of LANA expression.²¹⁷ Additionally, LANA expression in other cell types induces S-phase entry.²¹⁷ LANA has also been reported to enhance the stability of the c-Myc protein through its effects on GSK-3 β nuclear localization. In LANA-expressing cells, inactivation of nuclear GSK-3 β inhibited c-Myc phosphorylation at threonine 58 and contributed to c-Myc stabilization by decreasing c-Myc ubiquitination.^{80,407}

LANA also impacts host gene expression through binding of multiple components of the transcriptional machinery. Gene expression profiling of LANA-positive B cells identified many host genes whose expression is dysregulated by LANA.⁵⁶¹ LANA can transcriptionally modulate genes in the Rb/E2F pathway^{14,561,709} (Fig. 65.7). Although LANA can

activate transcription of some cellular genes,^{14,561,709} LANA primarily mediates repression of transcription. LANA inhibits expression of TR-linked reporter genes, and when fused to the DNA binding domain of GAL4, LANA does hamper transcription of GAL4-dependent reporters.^{229,348,594} This effect is partly owing to the recruitment of the mSin3 co-repressor complex through binding to its SAP30 subunit.³⁴⁸ LANA also binds the co-activator CBP to repress transcription by inhibiting its histone acetyl transferase (HAT) activity.³⁹⁵ LANA binds and inhibits the activation function of ATF4,³⁹⁸ a sequence-specific transcriptional activator. Finally, LANA interacts with RBP-J κ (also referred to as CSL) and is targeted to RBP-J κ sites in the ORF50 promoter,³⁶¹ where it again mediates repression. Further, LANA can inhibit the activated intracellular domain of notch (ICN)-mediated transactivation of ORF50, thereby preventing lytic reactivation.³⁶³

The physiologic role of LANA-mediated repression might affect cell survival and/or proliferation. It may function in the repression of viral lytic cycle genes, thereby allowing for establishment and maintenance of viral latency. This likely also applies to the many other nuclear proteins that can bind to LANA—for example, the methyl CpG binding protein (MeCP2), the bromodomain-containing RING3 protein 2 (Brd2), nuclear matrix proteins, and heterochromatin protein 1.^{347,395,396,451,539}

vCyclin

As described previously, the KSHV latency transcripts also encode two other proteins in addition to LANA. ORF72 encodes vCyclin, which shows homology to cellular cyclin D. The vCyclin transcript is made by splicing out the LANA gene, yielding a bicistronic ORF72 + 71 transcript whose 5' gene encodes vCyclin (see Fig. 65.5). Similar to its cellular homolog, vCyclin can bind and activate cdk6.¹²⁶ However, it differs from host cyclin D in that it is less active on cdk4, and although both can induce cdk6-mediated Rb phosphorylation, the viral cyclin can also activate phosphorylation of p27, histone H1, nucleophosmin (NPM), Id-2, and cdc25a.^{157,242,386} NPM is a nuclear phosphoprotein and a histone chaperone involved in transcription and chromatin organization. Phosphorylation of NPM by the vCyclin/cdk6 complex allows NPM to interact with LANA and regulate viral latency.⁵⁸⁵ Exogenous vCyclin expression can promote S-phase entry in 3T3 cells and also overcome Rb-mediated cell cycle arrest triggered by cdk inhibitors⁶⁴⁴ (Fig. 65.7). Overall, the vCyclin/cdk6 complex is less sensitive to inhibition by cdk inhibitory proteins such as p27, p21, and p16. Inactivation of p27 is mediated by phosphorylation.⁵⁸⁶ Moreover, p27 phosphorylation by vCyclin-cdk6 targets it for degradation, further impairing p27 control of cdk6.^{196,435} vCyclin can also interact with cdk9, leading to increased phosphorylation of p53 at serine 33, which results in cell cycle arrest.¹²³ Consistent with this report, overexpression of vCyclin in multiple cell types induces apoptosis.⁵⁰⁸

However there are some discrepancies. For example, although vCyclin overexpression destabilizes p27, PEL routinely display high levels of p27 expression.¹⁰⁰ Establishing stable cell lines expressing vCyclin has been difficult because such cells often undergo apoptosis, especially if they display high levels of cdk6.⁵⁰⁸

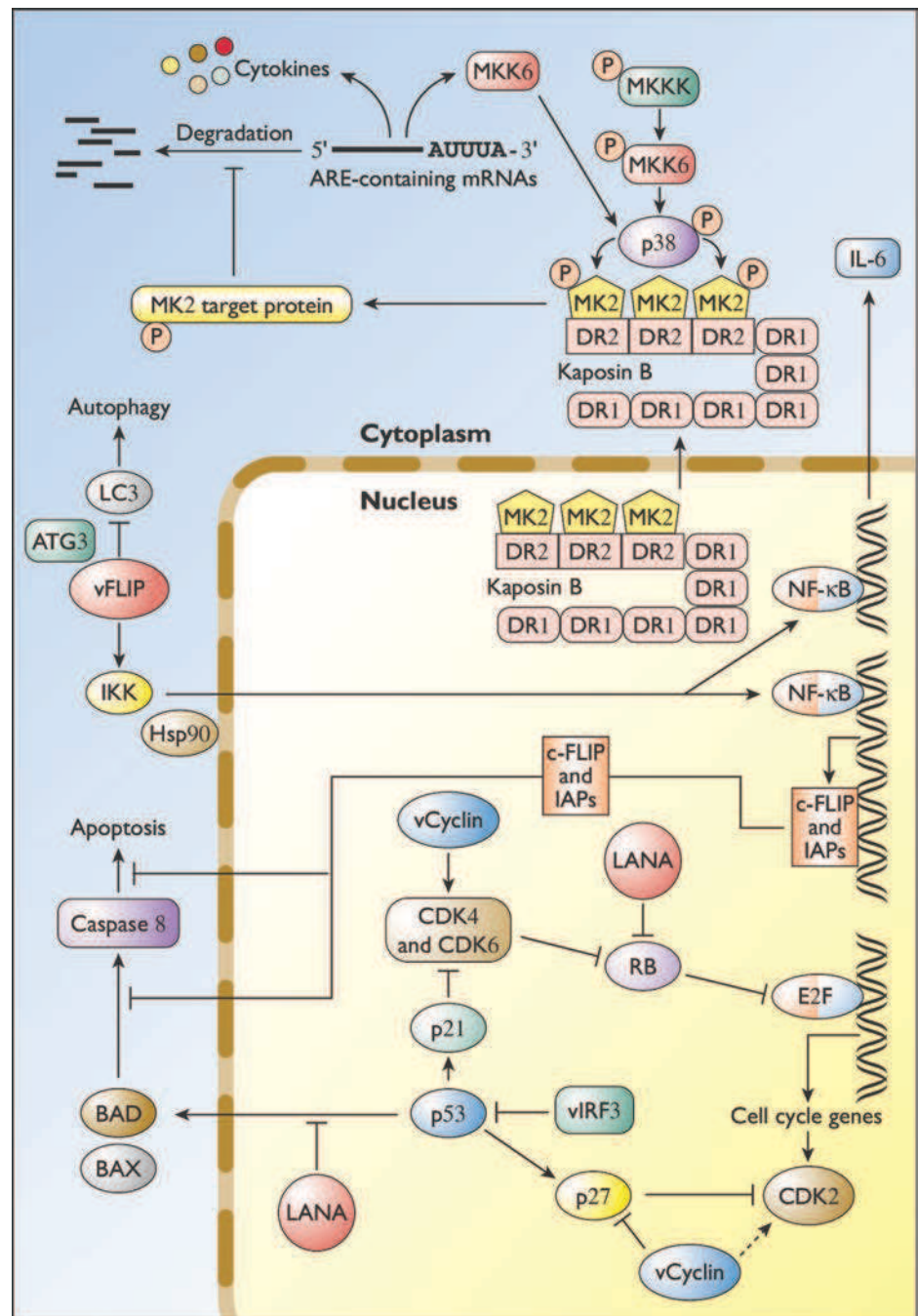


FIGURE 65.7. Functions and interactions of latent viral proteins. Latency-associated nuclear antigen (LANA), vCyclin, vFLIP, kaposin, and vIRF-3/LANA-2 act in concert to inhibit apoptosis and enhance cell survival and proliferative loops.

vCyclin-induced apoptosis is linked to the inactivation of the antiapoptotic factor bcl2⁵⁰⁹ owing to its phosphorylation by vCyclin/cdk6. Thus, the low levels of vCyclin protein expression during latency likely represent one way the virus avoids triggering cellular apoptosis. Alternatively, another viral protein might ablate this apoptotic effect. Along this vein, it is of interest that Verschuren et al^{667,668} demonstrated that loss of p53 allows for cell survival in the presence of increased levels of vCyclin. Moreover, when vCyclin was expressed in B cells in transgenic mice, lymphomas developed only in animals deleted for p53.^{667,668} This suggests that the functional inactivation of p53 by LANA during latency (or by vIRF3; see later discus-

sion) might unmask the oncogenic potential of vCyclin. The antiapoptotic effects of vFLIP may also alleviate vCyclin-induced toxicity.

A different line of transgenic mice was made in which vCyclin was expressed in only the lymphatic endothelium through the use of the vascular endothelial growth factor (VEGF)-R3 promoter. These mice displayed severe lymphatic dysfunction and developed chylous ascites. The reason for this is unknown but might reflect the toxicity of vCyclin overexpression.⁶³⁸ Indeed, in human dermal microvascular endothelial cells, vCyclin induces replicative stress leading to the development of senescence and activation of the DNA damage response.³⁴²

vFLIP

KSHV vFLIP, the product of ORF71, also referred to as K13, is the viral homolog of cellular FLIP (FLICE [FADD-like interleukin-1 beta-converting enzyme, now called caspase-8] inhibitory protein). KSHV vFLIP is composed of two death effector domains (DEDs) one right next to the other, which mediate homotypic protein–protein interactions with other DED-containing proteins. DEDs are present in the adaptor protein FADD, which binds the death receptor FasR. Initiator caspases, including caspase-8 and -10 in humans, also contain two tandem DEDs, and caspase-8 is a critical player in Fas-mediated death induction. Among the cellular FLIPs, the two predominant forms are the long form (FLIP_L) and the short form (FLIP_S). vFLIP is structurally more similar to FLIP_S, as they both lack a caspase-like domain present in FLIP_L. The long form includes a caspase-like domain, whereas the short form lacks this domain. After FasR is bound by Fas ligand present outside the cell, FasR binds FADD, which in turn recruits and activates procaspase-8, via interactions between the DED domains of FADD and the caspase, generating the death-inducing signaling complex (DISC). Caspase activation subsequently triggers the apoptotic cell death program. This process can be blocked by competitive binding by the cellular FLIP proteins.

Virally encoded FLIPs were first discovered in poxviruses and other herpesviruses, where they blunted Fas-mediated killing by preventing caspase-8 recruitment to the DISC.⁶⁵⁰ Procaspase-8 can also be bound and inhibited. Several early studies have supported this mechanism for KSHV vFLIP.^{30,43,183} However, the ability of vFLIP to block Fas-mediated killing has been questioned¹⁴¹; consistently observed in all studies is the observation that KSHV vFLIP up-regulates antiapoptotic transcription factor NF- κ B^{128,258,450,639,640} and that vFLIP can be found bound to NEMO (also referred to as IKK γ) in PEL cells.^{208,259,408} This complex activates IKK, leading to I κ B phosphorylation and the release of active p65-p50 NF- κ B heterodimers. In addition, vFLIP also increases accumulation of the p52 subunit of NF- κ B, indicating that vFLIP can activate the alternate pathway of NF- κ B, which involves the proteolysis of p100 to p52.⁴⁴⁸ These findings are concordant with the observations that (a) PEL exhibit high levels of NF- κ B activity, and inhibition of this pathway induces cell death,³³⁰ and (b) small interfering (siRNA)-mediated knockdown of vFLIP promotes apoptosis in PEL.^{243,258} It therefore appears that the key role of vFLIP during PEL latency is the activation of an antiapoptotic program through the up-regulation of NF- κ B activity. Notably, among the cellular genes that are clearly regulated by NF- κ B, and therefore by vFLIP, are the cellular inhibitor of apoptosis 1 and 2 proteins, as well as cellular FLIP,²⁵⁸ making studies to determine whether vFLIP can inhibit FAS-mediated apoptosis complicated, as the effect might be direct or indirect. Two different transgenic mouse models for vFLIP have been made. In one of these, where vFLIP was expressed in all B and T cells, mice showed enhanced responses to mitogenic stimuli and, not surprisingly, an increased incidence of lymphoma in a small proportion of mice, although in a much higher proportion when bred with c-Myc transgenic mice.^{7,141} In the second report, a conditional knock-in system was used to express vFLIP in all B cells, or specifically in GC B cells.³⁰ These mice developed lymphadenopathy with increased numbers of lambda light chain–expressing plasmablasts, reminiscent of

MCD, as well as tumors of B-cell origin with high frequency, albeit with an abnormal immunophenotype and histiocytic transdifferentiation. Consistent with these observations, treatment of PEL cell lines with Bay11-7082, an NF- κ B inhibitor, results in apoptosis *in vitro* and tumor responses in a mouse model of PEL.³²⁹ Furthermore, the proteasome inhibitor bortezomib blocks I κ B proteolysis, impairs NF- κ B activation, and triggers apoptosis of PEL cell lines^{4,16,449} and in a xenograft mouse model using freshly isolated human PEL cells into the peritoneal cavities of NOD/SCID mice without *in vitro* cell growth. These data suggest that this drug might have therapeutic efficacy in PEL.⁵⁸⁹ A single case report has documented a good response of a patient treated with bortezomib in combination with pegylated liposomal doxorubicin and rituximab.⁶⁰⁵

We have discussed the antiapoptotic effects of NF- κ B, which likely provides the selective force for maintaining KSHV vFLIP. However, NF- κ B is a key positive regulator of inflammatory responses.³⁸⁸ It seems paradoxical that a latency program in herpesviruses, designed for cryptic persistence, would call attention to itself by activating inflammation. However, vFLIP has additional effects, which may at least partially explain this paradox. In addition to the antiapoptotic function that is thought to be mediated mainly by NF- κ B–regulated genes, vFLIP has been shown to protect cells from autophagy by binding to Atg3.^{373,378a} In endothelial cells, it is required for the spindling seen after infection with KSHV.²⁵² It also contributes to prevention of detachment-induced apoptosis (anoikis) in endothelial cells.¹⁹⁵ Moreover, NF- κ B activation may not be the only function of KSHV vFLIP. The protein also binds to RIP and TRAF2 upstream of IKK, and TRAF binding results in JNK activation.^{17,259} Finally, NF- κ B activation by vFLIP may play other roles in KSHV biology.⁷⁷ It has also been shown that activation of NF- κ B hinders lytic gene expression and inhibition of NF- κ B activation induces lytic reactivation. Thus, vFLIP may play an important role in maintaining stable KSHV latency.

Kaposins

The locus for kaposin was initially identified by a screen for genes that were expressed during latency in PEL cell cultures.⁷³⁶ *In situ* hybridization using probes for kaposin mRNA revealed that kaposin is expressed at low levels in both uninduced body cavity–based lymphoma (BCBL)-1 cells and to some degree in latent KS spindle cells.^{629,636} Like several other latent loci, kaposin is also highly up-regulated during lytic replication.⁵⁷³

Kaposin mRNA encodes for three proteins owing to differential translation initiation⁵⁷³ (Fig. 65.8). At their 3' end, all kaposin messages contain a small ORF (ORF K12) encoding a highly hydrophobic 60 amino acid polypeptide called *kaposin A*. Kaposin A is found in intracellular membranes and the plasma membrane.⁶⁵² It is capable of transforming Rat-1 fibroblasts, and the resulting cell lines form tumors (fibrosarcomas) in nude mice.⁴⁸⁰ Kaposin A binds cytohesin-1, a guanine nucleotide exchange factor (GEF) for ARF GTPases, and a regulator of cell adhesion mediated by integrins. This interaction allows for GTP binding to ARF1. Because expression of a cytohesin-1 mutant deficient in guanine nucleotide exchange reverses transformation,³³⁶ this activity is likely to be important for transformation. Immediately upstream of the K12 ORF is a series of tandemly repeated 23-nucleotide, GC-rich elements known as DR1 and DR2. This region lacks AUG codons;

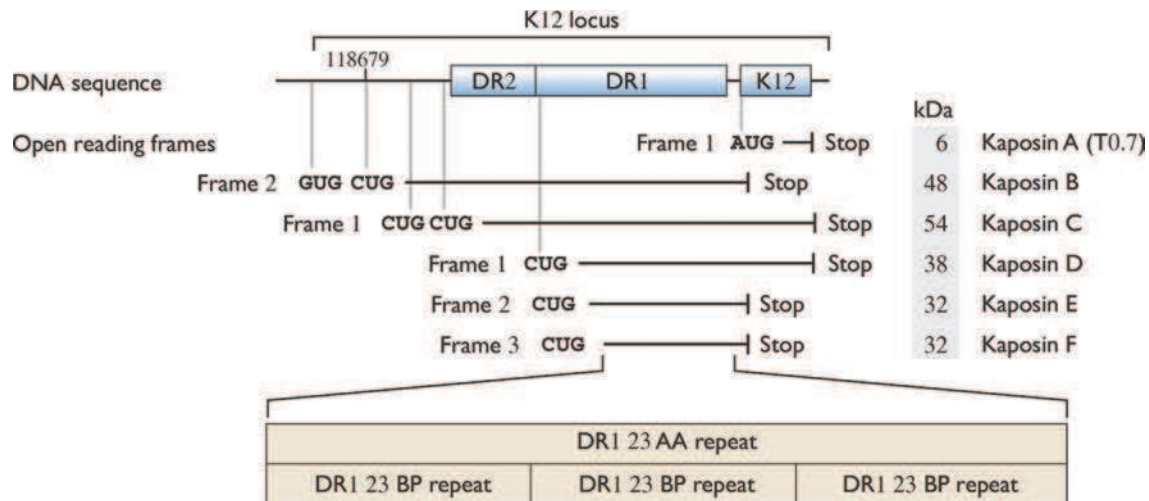


FIGURE 65.8. The coding capacity of the kaposin locus. **Top line:** Coding organization of kaposin messenger RNA. **Middle panel:** Protein products predicted from translation of each of the several CUG codons of kaposin. Kaposins A, B, and C are products of this locus. It is unclear as to whether the lower molecular weight species listed here as kaposins D, E, and F, which are regularly observed in infected cells, are in fact translated from the indicated CUG (as shown) or represent proteolytic end products of the larger kaposins.

however, multiple proteins are generated from this region by initiation at variant CUG codons. One of these initiates upstream of the DR2 repeat cluster and terminates after the DR1 repeats and encodes kaposin B, a nuclear protein that lacks the ORF K12 polypeptide region because of a stop codon in the reading frame. Another protein, kaposin C, similarly begins 5' to DR2 but in an alternative reading frame compared to kaposin B. Translation proceeds through the DR regions and ORF K12. This generates a transmembrane protein that contains the amino acid repeats (encoded by the DRs) fused to K12. Although the kaposins B and C DR sequences are in different frames, they share a common segment of repetitive sequence. Translation of an individual repeat induces the ribosome to enter the next repeat one nucleotide out of frame; after traversing three such repetitive sequences, the ribosome reverts back to the initial frame (see Fig. 65.8). Thus, the repeat regions of kaposin B and C share two sets of common 23 amino acid repeats, flanked on both sides by sequences unique to each protein. Consequently, kaposin C is partly a membrane-bound kaposin B isoform.

Kaposin B can bind and activate MAP kinase-associated protein kinase 2 (MK2) as well as the upstream kinase, p38 MAP kinase.⁴⁵⁷ MK2 is the major target of p38, which is a stress-inducible kinase. MK2 regulates the stability of cytoplasmic mRNAs that contain AU-rich elements (AREs) in their 3' UTRs. AREs are found in many cytokine mRNA transcripts (e.g., interleukin [IL]-1, -3, -4, -6, tumor necrosis factor [TNF]- α , and GM-CSF), as well as other growth factors (e.g., VEGF) and oncogenes (e.g., c-Myc). ARE mRNAs are highly unstable; however, activation of the MK2/p38 kinases inhibits their turnover and thereby enhances the levels of these cytokine mRNAs.^{343,707} Thus, by up-regulating MK2, kaposin B prolongs the half-lives of ARE-bearing reporter transcripts and increases cytokine production⁴⁵⁷ (see Fig. 65.7). Both the DR1 and DR2 repeat regions are essential for kaposin B's func-

tion.⁴⁵⁶ It was recently shown that kaposin B also enhanced stability of PROX1, a regulator of lymphatic endothelial differentiation that is up-regulated upon KSHV infection of endothelial cells. The PROX1 mRNA transcript also contains an ARE, and kaposin B enhances its stability.⁷²⁷

K10.5/vIRF3/LANA2

The KSHV genome has four genes that encode proteins of the IRF family. The family of IRFs are cellular transcription factors, several members of which (e.g., IRF3 and IRF7) transcriptionally activate type I IFNs and IFN-stimulated genes. The viral IRF homologs (vIRFs) are encoded by ORFs K9 through K11 (see Fig. 65.3), suggestive of the fact that they arose by duplication of one or more ancestral genes, plausibly obtained from the cellular genome. All vIRFs are lytic proteins, except for vIRF-3 (ORF K10.5), which encodes a nuclear protein that is expressed in latent PEL but not in KS spindle cells.^{181,419,567} For this reason, vIRF3 is also called *latency-associated nuclear antigen 2* or LANA-2.

vIRF3 expression inhibits the IRF3/7-mediated induction of the IFN- α 4 promoter in murine cells and hinders the ability of these cells to mount an IFN response to RNA virus infection.⁴¹⁹ vIRF3-expressing cells have decreased activation of protein kinase R (PKR), another activator of the IFN response.²⁰⁵ KSHV vIRF3 can also bind and inactivate the function of cellular IRF7.³¹³ This leads to the suppression of IFN- α -related activities.³¹³

vIRF3 can interact with a suppressor of c-Myc activity, MM-1 α , thereby sequestering it from interacting with and inhibiting the function of c-Myc.⁴¹⁸ Furthermore, vIRF3 also inhibits cellular IRF5 function,⁷⁰³ preventing its ability to induce p53-independent apoptosis and p21-mediated cell cycle arrest.⁷⁰³ In concordance, vIRF3 has also been shown to bind and inactivate p53.⁵⁶⁷ This vIRF3 function may function in prolonging the life span of KSHV-infected B cells, particularly

in the context of vCyclin expression.⁶⁶⁸ Indeed, central to its expression in PEL, a critical finding was that vIRF3 expression was required for PEL survival.⁷⁰⁴ Knockdown of vIRF3 expression in PEL hampered cell proliferation and increased activity of caspase-3 and -7.⁷⁰⁴ Additionally, these effects were seen even in the context of dually infected EBV- and KSHV-positive PEL lines.⁷⁰⁴ In endothelial cells, vIRF3 appears to play a role in angiogenesis. Hypoxia-inducible factor-1 (HIF-1), a regulator of angiogenesis, is composed of an oxygen-sensitive α subunit and a constitutively expressed β subunit. vIRF3 can bind the HIF-1 α subunit to stabilize it under normoxic conditions and increase the expression of VEGF and angiogenesis in endothelial cells.⁶⁰³ Thus, vIRF3 appears to play a key role in cell survival and angiogenesis in the context of viral latency, and perhaps angiogenesis in KS lesions (see Fig. 65.7).

Viral microRNAs

miRNAs are 21 to 23 nucleotide long RNAs that regulate gene expression via base pairing to their mRNA targets. miRNAs are generated from the cleavage of larger transcripts named *pri-miRNAs* via a processing pathway involving a ribonuclease (RNase) III-like endonuclease named *Drosha* located in the nucleus. These *pri-miRNAs* contain an imperfect inverted repeat that is cleaved by *Drosha* into a 65-nucleotide hairpin called a *pre-miRNA*, which is then transported to the cytoplasm where it is recognized by another RNase III-like endonuclease named *Dicer*. *Dicer* cleaves off the loop of the hairpin, thereby generating a partial duplex RNA molecule, which can bind to its target mRNAs. One strand of the RNA duplex is transferred to a cytoplasmic complex named *RNA-induced silencing complex* (RISC). In general, if base pairing of the miRNA to the target is perfect, then RISC cleaves the target mRNA. However, if base pairing of the miRNA to the target is imperfect, then RISC modulates translational repression of the target mRNA either through inhibiting translational initiation or elongation, or deadenylation (reviewed in 613).

Following the seminal discovery of viral miRNAs,⁵³¹ KSHV was found to encode 12 *pre-miRNAs* that arose from transcripts in the latency region^{87,257,531,579} (see Fig. 65.5). The miRNAs derived from these hairpins are designated as miR-K1 through miR-K12. Some of these *pre-miRNAs* (e.g. miR-K3, 4, 6, 9) donate each of the two arms of the hairpin to RISC, thereby giving rise to two mature miRNAs from each *pre-miRNA*. Each member of the pair is referred to as “5p” or “3p” to indicate its origin from the 5′ or 3′ segment of the hairpin. Additionally, the miR-K10 miRNA undergoes RNA editing at a single site in the seed sequence generating two different miRNAs (miR-K10a and miR-K10b). Thus, although there are 12 *pre-miRNA* transcripts arising from the KSHV genome, these lead to the generation of 17 mature miRNAs. These miRNAs are present in KS and PEL.^{504,506} There is strong sequence conservation in the genomic sequences that encode for these miRNAs, and only a subset of *pre-miRNAs* displayed single nucleotide polymorphisms (SNPs) that could affect their biogenesis.⁴³⁸

There has been a flurry of papers identifying putative functions for the KSHV miRNAs since their discovery. miR-K11 was found to contain an identical seed sequence to that of cellular miRNA miR-155.^{245,614} Viral miR-K11 was found to suppress known targets of cellular miR-155, including the transcriptional repressor BACH1, the proapoptotic protein

BIRC4BP/XAF1, and the cell cycle regulator FOS.^{245,614} Using a transcriptional profiling approach, another group identified that the target for miR-K5 was a proapoptotic Bcl2-associated factor named *BCLAF1*. Moreover, additional KSHV miRNAs including miRs K9, K10a, and K10b were also found to target BCLAF1. Repression of BCLAF1 expression by the KSHV miRNAs was found to be important for cell survival to apoptotic stimuli,⁷⁴⁴ as was the down-regulation of tumor necrosis factor–like weak inducer of apoptosis (TWEAK) receptor, a proapoptotic protein that is targeted by miR-K12-10a (also known as miR-K10a).² miR-K1 has been shown to down-regulate p21, a protein that induces cell cycle arrest,²⁴⁴ and also to target I κ B α .³⁷⁸ Moreover, miR-K12-3 and miR-K12-7 (also referred to as miR-K3 and miR-K7, respectively) down-modulate C/EBP β p20 (LIP), an isoform of C/EBP that represses IL-6 and IL-10 expression. This results in up-regulation of these cytokines in cells from the myeloid lineage.⁵⁴⁷ Curiously, miR-K12-7 (also referred to as miR-K7) has also been shown to down-regulate the stress-induced natural killer (NK) cell ligand MICB⁴⁸⁶ as a mode of immune evasion from the innate immune system. Thus, a single viral miRNA (i.e., miR-K12-7) appears to target different mRNA transcripts to ensure virus survival within the infected cell. One study also looked at gene expression profiling in cells stably expressing all KSHV-encoded miRNAs.⁵⁸⁰ They found that 81 genes were changed in the presence of the viral miRNAs. One of these proteins is the tumor suppressor protein, thrombospondin 1 (THBS1), whose protein levels were decreased over 10-fold.⁵⁸⁰ Reduced THBS1 expression in the presence of viral miRNAs led to decreased transforming growth factor (TGF)- β activity, suggesting that the viral miRNAs contribute to tumorigenesis and angiogenesis.⁵⁸⁰

It is known that KS is comprised of poorly differentiated endothelial cells, expressing markers of both lymphatic endothelial cells (LECs) and blood vessel endothelial cells (BECs). KSHV-encoded miRNAs appear to contribute to transcriptional reprogramming by silencing the cellular transcription factor musculoaponeurotic fibrosarcoma oncogene homolog (MAF).²⁷³ MAF is expressed in LECs but not in BECs, where it functions as a transcriptional repressor of BEC-specific genes to maintain the differentiation status of LECs. KSHV-infected LECs show an up-regulation of BEC-specific genes through miRNA-dependent down-regulation of MAF.²⁷³ Additional approaches have used more sophisticated techniques to identify miRNA targets on a global scale—for example, immunoprecipitation of Argonaute (Ago) protein-containing RISC followed by microarray analysis (RIP-Chip).^{184,244a,292a} In summary, KSHV-encoded miRNAs can influence the differentiation status of a cell in a manner similar to certain cellular miRNAs, and this suggests that the viral miRNAs may contribute to the development of viral malignancies via reprogramming the differentiation pathway of the infected cell.

In addition to their roles in cell survival and oncogenesis, the KSHV viral miRNAs also play a key role in regulating the lytic-latent switch. Several studies have examined the role of viral miRNAs in controlling this switch, and although all of these studies find that the miRNAs enhance latency and suppress lytic replication, the mechanism by which this occurs is different in each case. For example, miR-K9* has been reported to target the 3′ UTR of the ORF50 transcript, thereby enhancing latency and preventing viral reactivation.⁴⁴ Consistent with

this finding, a KSHV BAC with 10 of the 12 viral miRNAs deleted displayed elevated levels of viral lytic genes, including ORF50.⁴¹⁴ However, this group reported that another miRNA, miR-K12-5 (also referred to as miR-K5), could repress the ORF50 mRNA transcript.⁴¹⁴ Additionally, miR-K12-4-5p (also referred to as miR-K4-5p) was found to target Rbl-like protein 2 (Rbl2), a known repressor of DNA methyl transferase 3a and 3b (DNMT3a/b). miR-K12-4-5p reduced Rbl2 protein expression and increased DNMT1, -3a, and -3b mRNA levels to regulate genome-wide epigenetic reprogramming.⁴¹⁴ In further confirmation of the role of the viral miRNAs in suppressing lytic replication, deletion of 14 miRNAs from the KSHV genome also significantly enhanced viral lytic replication as a result of reduced NF- κ B activity.³⁷⁸ Finally, yet another group found that miR-K3 can enhance viral latency by inhibiting expression of nuclear factor I/B, a transcription factor that can activate the ORF50 promoter.⁴¹²

Genes Expressed at Low Levels During Latency but Highly Up-regulated During Lytic Replication

As mentioned earlier, K1 and vIL-6 were initially considered to be lytic proteins; however, several publications now demonstrate that their transcripts are expressed at low levels during latency and are highly up-regulated during lytic replication.^{67,93,118,521,628,679}

A recent publication used tightly latent cell lines that displayed very low levels of spontaneous lytic reactivation to perform array-based transcript profiling and limiting dilution RT-PCR in these cells. They found that both K1 and K2/vIL-6 genes were expressed under latent conditions.¹¹⁸ Additionally, the K1 promoter is active in multiple cell types in the absence of ORF50/RTA protein.^{67,68,118,679} It is likely that owing to their potent signaling properties, both K1 and vIL-6 need to be expressed at low levels in infected cells.

K1

ORF K1 is situated at the left-hand end of the genome and encodes for a type I transmembrane protein, whose signaling function is analogous to the B-cell antigen receptor complex. K1 is found in the endoplasmic reticulum and also on the plasma membrane. It contains a highly variable, glycosylated,^{502,751} N-terminal ectodomain, a transmembrane region, and a cytoplasmic tail that sports an immunoreceptor tyrosine-based activation motif (ITAM).³⁷⁰ This structure, also found in B-cell receptor (BCR) and T-cell receptor (TCR) signaling complexes, contains two tyrosine residues separated by characteristic spacing. Activation of the BCR by antibody binding results in phosphorylation of tyrosines by Src kinases. Phosphorylation creates binding sites for Syk kinase, which upon activation mediates downstream signaling, including PLC γ activation, calcium release and NFATc activation, among other events. The K1 protein signals in a similar fashion; however, its activity appears to be constitutive because it signals in the absence of a ligand.^{360,370} Although it is possible that the K1 ectodomain binds to a ubiquitously expressed cell surface protein, constitutive activation is more likely due to self-oligomerization mediated by cysteine residues in the K1 ectodomain. K1 aggregation leads to ITAM phosphorylation, Syk kinase recruitment, and increased NFATc and AP-1 activity similar to what occurs upon BCR activation. Moreover, the activated K1 tail can interact with other signaling proteins besides Syk, including PI3-kinase (via its p85 regu-

latory subunit), Lyn, RAS-GAP, PLC- γ 2, vav, and c-Cbl.^{360,368,654} PI3K activation results in phosphorylation and activation of Akt kinase.⁶⁵⁴ Activated Akt phosphorylates the forkhead (now known as FOXO) transcription factors, sequestering them in the cytoplasm (Fig. 65.9). Because FOXOs induce apoptosis, this phosphorylation is an antiapoptotic event, and K1 expressing cells exhibit greater resistance to apoptosis induced by both Fas ligand and the expression of FOXO proteins.⁶⁵⁴ K1's effect on apoptosis may also be linked to its ability to bind to chaperones. K1 was shown to interact with heat shock protein 90-beta (Hsp-90beta) and endoplasmic reticulum-associated Hsp40 (Erdj3/DnaJB11) by tandem affinity purification.⁶⁹⁵ K1 expression and antiapoptotic function highly depends on Hsp90 and Hsp40/Erdj3, because knockdown of these proteins, as well as pharmacologic inhibitors of Hsp90, dramatically reduces K1 expression.⁶⁹⁵ Furthermore, in B cells, K1 expression hinders the surface transport of the BCR,³⁶⁷ and this may be a means by which KSHV puts the infected B cell under the control of the virus rather than the control of the BCR. In this manner, stimulation of BCR signaling does not jeopardize the viral life cycle. K1 signaling function in B cells has been shown to be regulated by its endocytosis, and these two events are intertwined.⁶⁵³

K1 is capable of transforming rodent fibroblasts and substituting for the STP oncogene in the context of HVS-mediated transformation of T cells.³⁷¹ Lymphomas and sarcomas are known to develop in K1 transgenic mice,⁵⁴⁴ and the lymphomas show activated Lyn kinase⁵⁴³ and are refractory to Fas-mediated apoptosis.⁶⁸² In epithelial and endothelial cells, K1 expression has been reported to induce the secretion of angiogenic factors, including VEGF and matrix metalloproteinase-9.⁶⁸⁰ K1 can activate the PI3K/Akt/mTOR pathway in endothelial cells and can immortalize and extend the life span of primary HUVEC in culture⁶⁷⁹ (see Fig. 65.9). K1 ITAM expression was needed to activate both the VEGF/VEGFR-2 and the PI3K/Akt signaling pathways in these cells. Thus, K1 appears to be important in KSHV-associated tumorigenesis and angiogenesis.

In the context of lytic replication, the ITAM of K1 has been shown to augment lytic reactivation from latency.³⁵⁹ However, others have shown that the K1 ITAM is needed to suppress TPA-mediated viral reactivation in the context of PEL cells, and this suppression may contribute to the establishment and/or maintenance of KSHV latency *in vivo*.³⁶⁹ These differences may be explained by the experimental conditions used in each model system.

vIL-6

Cellular human IL-6 (hIL-6) signals through a heterodimeric receptor composed of a signaling subunit (gp130) and a high-affinity ligand-binding subunit (gp80/IL-6R α). Following hIL-6 binding to this receptor complex, STATs 1 and 3 are activated. The signaling mechanism of the viral homolog vIL-6 differs from hIL-6 in that it does not need the gp80 subunit; rather, it binds directly to gp130 to trigger signal transduction (Fig. 65.10). This enables vIL-6 to act on a wider spectrum of cells than its human homolog.⁷² Although vIL-6 does not need gp80 to activate signaling, it can still interact with this subunit of the receptor complex, which appears to modulate vIL-6's activity.^{290,383,384}

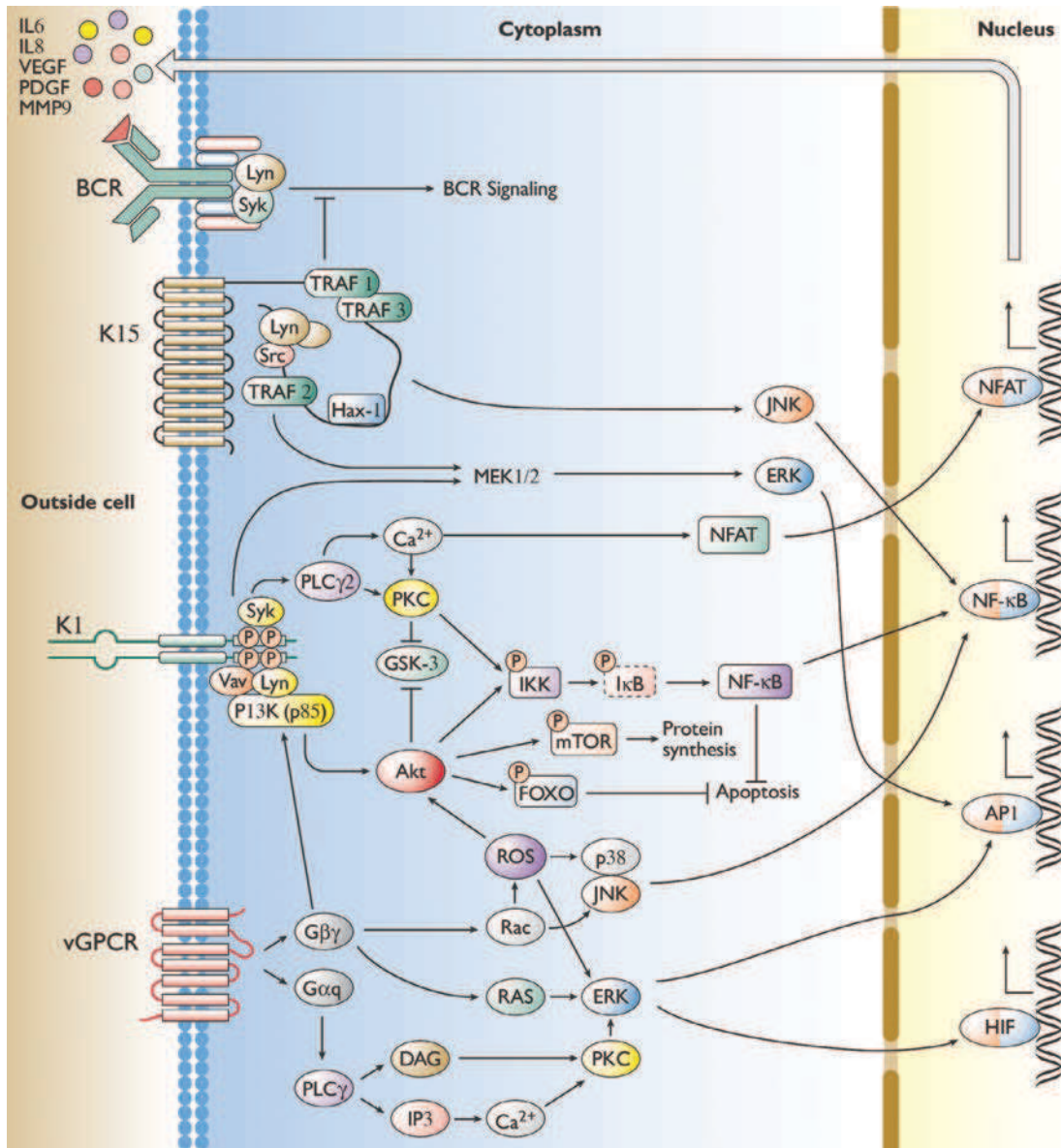


FIGURE 65.9. Signal transduction pathways activated by Kaposi's sarcoma-associated herpesvirus encoded transmembrane proteins K1, K15, and viral G protein-coupled receptor (vGPCR). Activation of the PI3K and mitogen-activated protein (MAP) kinase pathways by viral signaling proteins leads to enhanced production of cytokines and growth factors, cell proliferation, and inhibition of cell death.

IL-6 promotes B cell differentiation to plasma cells and also impairs B-cell apoptosis in response to apoptotic-inducing signals. vIL-6 shares the antiapoptotic activities of cellular IL-6 on B cells (including PEL cells),³¹² thus implicating vIL-6 in PEL and MCD pathogenesis.^{510,522}

Ectopic expression of vIL-6 in immortalized mouse fibroblast lines is linked to increased angiogenesis in the fibrosarcomas when these lines are transplanted into nude mice.¹⁹ Increased angiogenesis owing to VEGF induction by vIL-6 is an activity shared with cellular IL-6.^{19,146,406} In PEL, vIL-6 expression was shown to be selectively activated by IFN- α in the absence of full lytic replication. vIL-6 was also shown to blunt IFN signaling in these cells. In endothelial cells, vIL-6 expression caused an induction

of angiopoietin 2, a proangiogenic and lymphangiogenic factor.⁶⁶¹

Several groups have shown that vIL-6 can be localized to the endoplasmic reticulum,^{129,130,460} where it still retains "intracrine" signaling activity by interacting with gp130 intracellularly. The endoplasmic reticulum-resident chaperone protein calnexin plays a role in endoplasmic reticulum localization of vIL-6.¹²⁹ Intracellular vIL-6 appears to be functional in the autocrine promotion of proliferation and survival of PEL. Additionally, secreted vIL-6 is completely N-glycosylated, and this glycosylation is required for optimal conformation of the protein and optimal signaling.^{167,168,460}

Although a vIL-6 deletion mutant of KSHV did not affect viral latency or reactivation,¹³⁵ knockdown of vIL-6 expression

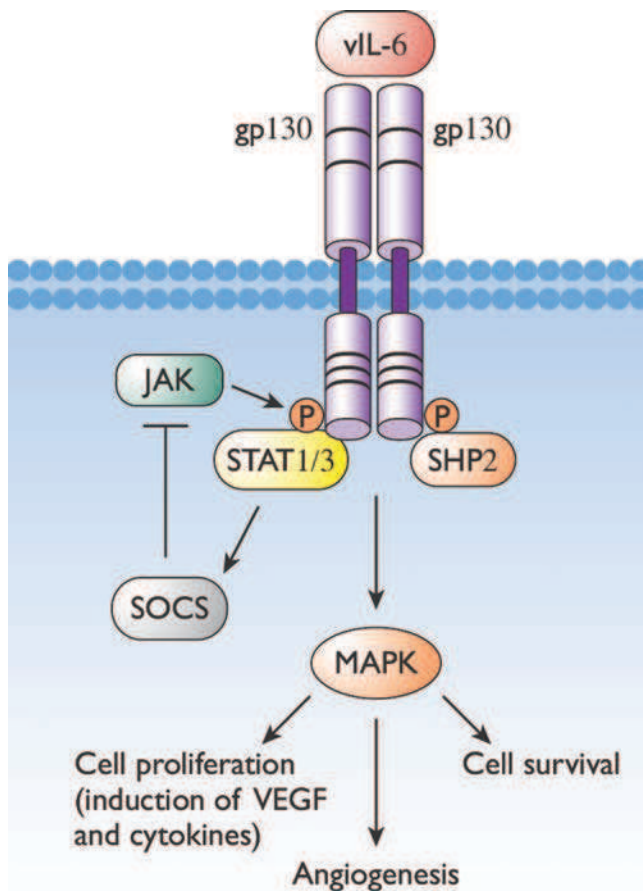


FIGURE 65.10. Kaposi's sarcoma-associated herpesvirus viral interleukin 6 (vIL-6) activates interleukin 6 receptor signaling by directly binding and activating the gp130 subunits of the IL-6 receptor complex. This leads to increased cell survival, proliferation, and induction of proangiogenic factors.

in PEL cells led to markedly reduced cell growth,¹³⁰ suggesting that vIL-6 likely plays an important role in PEL proliferation. Taken altogether, vIL-6 can contribute to KSHV pathogenesis through an autocrine manner during latency by augmenting survival and proliferation (see Fig. 65.10). During lytic replication, vIL-6 may function in a paracrine manner to promote survival of uninfected cells.

Lytic Phase

The default pathway for KSHV is latency. Hence, lytic infection, in cell culture, is most often studied by using compounds to induce reactivation from latency. In PEL cell lines, this is achieved by treatment with phorbol esters (TPA) or HDAC inhibitors (sodium butyrate, valproic acid), which will induce lytic reactivation in approximately 30% of PEL as judged by lytic protein expression. Transfected RTA expression plasmids or chemically induced RTA can also be used to induce more uniform reactivation.⁴⁸⁹ When immunofluorescence assays (IFAs) for late lytic proteins (e.g., ORF K8.1) are utilized, only half of the cells are positive, even at very late times. Thus, not every entry into lytic infection is completed successfully, and abortive lytic infection occurs some or most of the time. We

currently do not comprehend why many of these reactivated PEL cells fail to enter the lytic cycle when treated with HDAC inhibitors or TPA, and why only a fraction of cells in culture undergo lytic replication. One possibility is that many cells do not support the transmission of the inducing signal, or that the signal itself is ineffective or inhibitory in cells that do not support replication. Additionally, when PEL cell lines are cultured for many weeks in tissue culture, they lose efficient inducibility by TPA and HDAC inhibitors. Similarly, passage of stable PEL cell lines with drugs such as hygromycin and neomycin also hinders induction by TPA and HDAC inhibitors. Again, although we are not clear on why this happens, it is important to work with cells at low passage.

In addition to PEL, other latently infected cell lines, including 293, Vero, HFFs, and TIME cells,³⁵⁷ can be induced, whereas other lines (e.g., HeLa, SLK) into which a KSHV genome has been transfected are only transiently inducible by chemical means.^{15,39} However, inducible or forced expression of the lytic switch protein, RTA, can induce reactivation in a much wider array of lines than chemical activation.^{39,483,489} Finally, it was recently shown that the touselike kinases (TLK) suppress KSHV reactivation in B cells.^{178a}

Epigenetic Regulation of the Latent-Lytic Switch

Recently, several groups reported the presence of genome-wide epigenetic marks on the viral episome and their involvement in the regulation of the switch between latency and lytic replication.^{262,414,655} Histone modifications associated with the KSHV latent genome are involved in the regulation of latency, and also control the temporal and sequential expression of genes during the viral lytic life cycle. Activating histone modifications include acetylated H3 (AcH3) and H3K4me3, whereas repressive histone modifications include H3K9me3 and H3K27me3. The latent viral genome contains both activating and repressive histone modifications. These marks are mutually exclusive across the latent genome.^{262,655} For example, under latent conditions, the genomic region encoding the lytic gene ORF50/RTA contained activating H3K4me3 and repressive H3K27me3 marks that rapidly changed to increasing AcH3 and H3K4me3 activating modifications and decreasing H3K27me3 modifications upon viral reactivation.^{262,655} Additionally, latency-specific histone modification patterns were established following *de novo* infection, and there was rapid and widespread deposition of H3K27me3 marks across latent genomes.²⁶² H3K27me3 is a bivalent modification that can repress transcription despite the simultaneous presence of activating marks on the genome.²⁶² It was also observed that EZH2 (the H3K27me3 histone methyltransferase) was present on the entire KSHV genome during latency but was dissociated from the genome during ORF50-mediated reactivation. EZH2 inhibition or knockdown and expression of H3K27me3-specific histone demethylases all induced the lytic life cycle in PEL.⁶⁵⁵ Chromatin immunoprecipitation (ChIP) assays revealed that KSHV genomes are highly methylated at CpG residues (except for the LANA promoter [131]), leading to the establishment of characteristic global DNA methylation patterns. These types of studies are still in their infancy; however, thus far they have revealed that the viral genome is epigenetically modified in the latent state so that transcription is generally repressed. At the same time, parts of the genome also contain activating histone modification, and the virus may

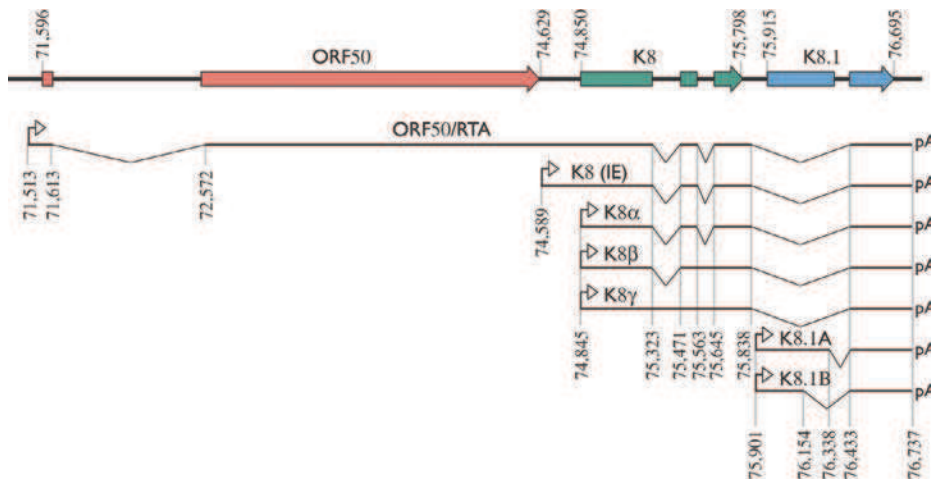


FIGURE 65.11. Map of known transcripts emanating from the main lytic region of Kaposi's sarcoma-associated herpesvirus. Top line shows a map of the major genomic open reading frames (ORFs) (arrows). ORF50, K8, and K8.1 are encoded on a polycistronic transcript. All messenger RNAs are polyadenylated at the indicated positions. (Modified from Yuan Y, Renne R. DNA tumor viruses. In: Damania B, Pipas J, eds. *Organization and Expression of the Kaposi's Sarcoma-associated Herpesvirus Genome*. New York: Springer Press; 2009:469–493.)

therefore be poised to enter the lytic cycle once reactivation is triggered. Additionally, ORF50/RTA lytic transcription is actively repressed by LANA during latency, which is relieved upon lysine acetylation of the LANA protein.⁴¹³

Immediate-Early Gene Expression

A single viral protein named RTA, encoded by ORF50, controls reactivation from latency and the induction of lytic replication. RTA is the key lytic switch protein as deduced from two types of experiments: (a) expression of ectopic RTA by itself is sufficient to induce reactivation from latency^{246,423,641}; and (b) knocking out ORF50 function, either by dominant-negative ORF50 mutants⁴²² or by removal of ORF50 from the viral genome,⁷²¹ prevents reactivation from latency. Thus, ORF50/RTA is the critical protein required for the induction of lytic replication. Several experiments show that (a) ORF50 is the first transcript to appear following chemical induction with TPA of PEL,^{423,643} (b) the RTA/ORF50 promoter is inducible by TPA, and (c) lytic induction is linked to demethylation of the ORF50 promoter.^{131,415} ORF50 is a canonical IE protein, as it is transcribed in the presence of cycloheximide.^{596,641,739} ORF50 is part of a polycistronic transcript encoding for both K8 and K8.1 genes (Fig. 65.11).

ORF50/RTA

ORF50/RTA is a viral transcription factor. Its sequence and function is highly conserved among the rhadinoviruses.¹⁶¹ KSHV RTA has an amino-terminal DNA binding domain and a carboxy-terminal activation domain. A fusion of the ORF50 activation domain with a GAL4 DNA binding domain results in robust activation of GAL4 responsive promoter elements. ORF50 is phosphorylated on its C-terminal activation domain during viral replication. Moreover, phosphatase-treated ORF50 runs at a higher mobility than its predicted molecular weight, suggesting that additional posttranslational modifications must exist.⁴²² ORF50 forms tetramers and higher-order multimers in solution and the amino-terminal proline-rich, leucine heptapeptide repeat (LR) of ORF50 is necessary, but not sufficient, for oligomerization. Mutagenesis of this LR region revealed that ORF50 tetramers are sufficient for transactivation and viral reactivation and that ORF50 mutants are unable to form tetramers

but can still form higher-order multimers and are either non-functional or reduced in function.⁷⁸

ORF50 has been shown to bind to at least 19 sites in the KSHV viral genome.¹³³ These include regions of promoters, introns, and exons of KSHV genes, including ORF8, K4.1, K5, PAN, ORF16, ORF29, ORF45, ORF50, K8, K10.1, ORF59, K12, LANA, K14/vGPCR, and K15, the two origins of lytic replication OriLyt-L and OriLyt-R, and the miRNA cluster.¹³³ ORF50 expression leads to a strong activation of DE promoters (8- to 300-fold depending on the DE promoter). The highest activation by ORF50 is of the PAN (nut-1) promoter, which drives the transcription of a very abundant non-coding PAN RNA. Several mutagenesis experiments demonstrated that the ORF50-responsive site in the PAN promoter is the sequence AAATGGGTGGCTAACCTGTC-CAAAA upstream of the PAN RNA cap site. ORF50 has been shown to bind to this site *in vitro*.^{124,622} A similar sequence is located just upstream of the lytic kaposin transcript.^{573,623} Many other replication and transcription activator response elements (RREs) were identified using promoter-reporter co-transfection assays in the promoters of DE viral genes (e.g., tk, ORF57/MTA (mRNA transcript accumulation), K8/RAP, K2, K9, K14, DBP)^{132,175,176,308,389,391,421,683,690,694} and in one late promoter (gB).⁷⁴³ ORF50 can also up-regulate its own promoter.⁵⁷⁶ Many of the RREs share little sequence homology to the PAN/kaposin site or to each other. The RREs display low-affinity interactions with purified ORF50 protein in *in vitro* ORF50 binding assays.^{392,623,743} Although ORF50 binds to these sites with low affinity, many of these sites are very strongly induced by ORF50 in transient transfection cell-based assays (e.g., the ORF57/MTA and K14 promoters).⁶³⁰

Direct DNA binding by ORF50 is not the sole mechanism by which this protein can transcriptionally activate promoters. Two-hybrid screens of ORF50 binding cellular partners revealed that ORF50 can efficiently bind to the transcription factor, RBP-Jκ³⁸⁹ (also called CBF-1 or CSL). Indeed, RBP-Jκ recognition sites are found in many promoters of ORF50-responsive genes (e.g., K8/RAP, tk, PAN, ORF57/MTA, K14, LANA, and gB).^{389,391,447,686,690,743} In many of these promoters, RBP-Jκ sites lie adjacent to low-affinity ORF50 binding sites, suggesting that cooperative interactions may exist to enhance or stabilize the binding of ORF50 at these sites. Even reporter

genes that harbor synthetic RBP-J κ elements can be strongly activated by ORF50. This suggests that in addition to binding promoters through its sequence-specific DNA binding activity, ORF50 is also targeted to promoters through protein–protein interactions with RBP-J κ (as well as other transcription factors) bound at the promoter. RBP-J κ deleted fibroblasts are not supportive of ORF50-transactivation of many reporter genes, and KSHV-infected fibroblasts show reduced reactivation when ORF50 plasmid is transfected into these cells.³⁹⁰

RBP-J κ protein normally functions as a transcriptional repressor. ORF50 binding to RBP-J κ converts it from a repressor to an activator via ORF50's activation domain. In uninfected cells, RBP-J κ -directed repression is controlled by the Notch pathway through extracellular ligands that activate the transmembrane receptor protein Notch. Activation of Notch leads to the cleavage of its juxtamembrane domain, liberating its intracellular domain. The Notch intracellular domain moves to the nucleus, where it can bind RBP-J κ , converting it from a repressor of transcription to an activator of transcription. This phenomenon is similar to what happens with RBP-J κ and ORF50. Thus, KSHV has co-opted the Notch-RBP-J κ pathway to transactivate ORF50-responsive, RBP-J κ binding site containing promoters.

In EBV latency, two viral proteins—EBNA2 and EBNA 3C—also target RBP-J κ .^{251,568,675} Interestingly, ORF50 binds to RBP-J κ at two sites, and one of these is identical to the site bound by the Notch intracellular domain and EBNA2. The human Notch intracellular domain that constitutively activates RBP-J κ was expressed in PEL, and gene expression profiling showed that this domain robustly induced expression of several lytic viral genes but could not evoke the full repertoire of lytic viral gene expression normally induced by ORF50 expression.¹¹⁹ These results indicate that the control of viral gene expression by cellular Notch signal transduction only partially overlaps with ORF50,¹¹⁹ suggesting that RTA does more than merely mimic Notch and that some RBP-J κ elements in the viral genome have completely diverged from Notch signaling and now only respond to the viral transactivator (D. Dittmer, personal communication). This result is not surprising given that ORF50 has additional mechanisms of promoter targeting besides RBP-J κ sites (see later discussion). However, these results suggest that certain lytic genes can be activated in an ORF50-independent manner and under conditions when ORF50 is not expressed.

ORF50 can also interact with the transcription factor C/EBP α in the K8/RAP, ORF57/MTA and PAN promoters,^{683,684} and with Oct-1 in the ORF50 promoter.⁵⁷⁶ These interactions are important for ORF50 recruitment to these sites. Additionally, the binding of ORF50 to both Oct-1 and the K8 promoter leads to transactivation and viral reactivation, and the ORF50/Oct-1 interaction is necessary for optimal KSHV reactivation.¹⁰² Oct-1 also appears to mediate ORF50's interaction with cellular HMGB1 protein to up-regulate its own promoter.²⁷⁷ ORF50 can interact with STAT-3, inducing it to move to the nucleus to activate STAT-responsive genes.²⁶⁸ ORF50 can also interact with the protein MGC2663.⁶⁸¹ ORF50 binds proteins involved in histone acetylation (CBP and HDAC 1),²⁶⁷ the chromatin remodeling complex, SWI/SNF and the TRAP/Mediator complex, which enables interaction of RNA pol II with many transcription factors.²⁶⁶ Interactions with histone modeling proteins occur via the C-terminal activation domain of ORF50. Mutational studies show that

blocking these interactions prevents both transcriptional activation and lytic induction, confirming their importance for ORF50 function.

The spliced isoform of plasma cell transcription factor X box binding protein 1 (XBP-1s) can regulate the latency to lytic switch for KSHV.^{706,730} XBP-1s is normally absent in PEL; however, the induction of endoplasmic reticulum stress or hypoxia leads to production of XBP-1s and induction of the lytic cycle.^{159,730} XBP-1s binds to ORF50 and activates the ORF50 promoter in a synergistic manner.⁷⁰⁶ This interaction links lytic reactivation to plasma cell differentiation, which may be a mechanism whereby virus is produced in sites where immunoglobulin production is occurring, which include lymphoid tissues in the oral mucosa allowing virus to be shed into saliva.⁷⁰⁶

Although most research has focused on ORF50 transactivation, several reports show that ORF50 can also bind transcriptional repressors. As mentioned earlier, ORF50 binds HDAC1, resulting in repression of ORF50-mediated transactivation.²⁶⁷ ORF50 also binds to and is inhibited by poly(ADP-ribose) polymerase 1 (PARP-1) and Ste20-like kinase hKFC.²⁶⁹ It has also been shown that ORF50 can bind a cellular zinc-finger protein named K-RBP (or KSHV RTA binding protein). K-RBP is a transcriptional repressor that was shown to repress ORF50-mediated transactivation in a HDAC-independent manner.⁷²³ K-RBP could also suppress ORF50-mediated lytic reactivation. In a subsequent study, it was found that ORF50 could induce ubiquitin-mediated degradation of K-RBP, and ORF50 mutants that were defective in mediating K-RBP degradation were not as transcriptionally active as wild-type ORF50.⁷²⁴ Interestingly, the HSV-1 ICP0 transactivator and the HCMV pp71 transactivator also induce degradation of transcriptional repressors, suggesting that this may be a common mechanism by which many herpesviral lytic transactivators function.⁷²⁴ Additionally, transducin-like enhancer of split 2 (TLE2) is another repressor that interacts with ORF50 and inhibits its transactivation activity and ability to induce the lytic life cycle.²⁸² TLE2 appears to interact with the Pro-rich domain of ORF50, which is the same site that interacts with RBP-J κ .²⁸²

Finally, ORF50 has been shown to target IRF7 through ubiquitin-mediated degradation. The degradation is mediated through ORF50's recruitment of E3 ubiquitin ligase RAUL, which normally imparts K48-linked ubiquitins on IRF3 and IRF7.^{732,733} A summary of ORF50's interactions and functions is shown in Figure 65.12.

Other Immediate-Early Genes

Detailing the kinetics of viral gene expression in the presence of cycloheximide led to the identification of several other IE genes.^{592,739} Additional IE mRNAs encode for the products of ORF45 and K4.2, as well as a 4.5-kb RNA with partial complementarity to ORF29. (Note: Some reports refer to ORF45 and K8 as DE genes). Moreover, several transcripts antisense to ORF50 have been identified; however, their biological function is not known.^{423,739} The K8 gene has two promoters, one of which is activated in the IE phase and a second that is activated as a DE promoter through a ORF50-responsive RBP-J κ site in the promoter.⁶⁸⁶ Interestingly, the gene for KSHV ORF57/MTA, the homolog of HSV ICP27, is transcribed in the DE phase. The IE ORF45 protein functions to evade host innate immunity (discussed later).

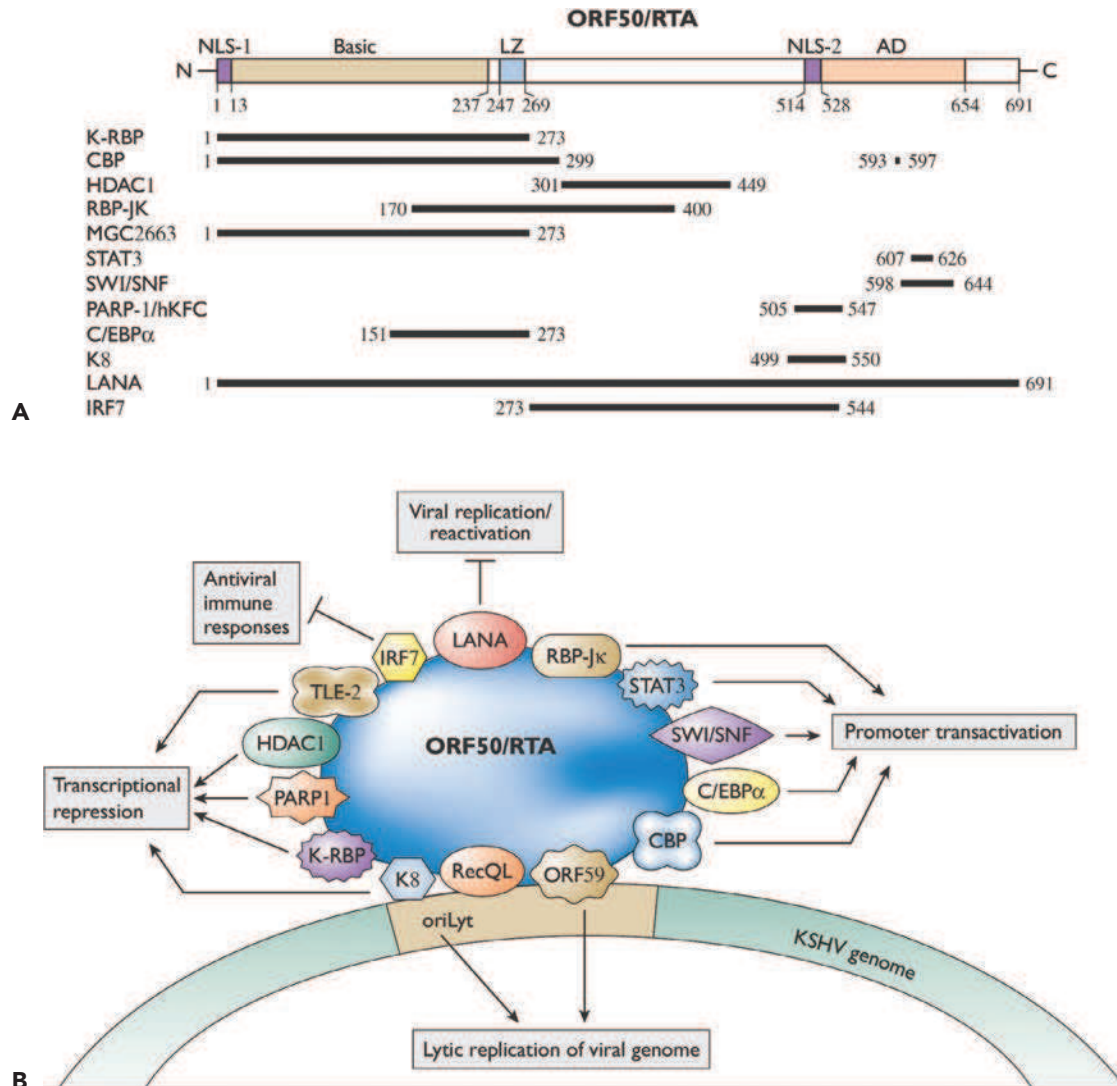


FIGURE 65.12. Open reading frame 50 (ORF50)/replication and transcription factor (RTA) domains and interaction partners. **A:** Schematic depiction of ORF50 domains. Bars indicate domains implicated in the noted interactions and functions. **B:** Functional consequences of the interaction of ORF50 with other cellular and viral proteins. NLS, nuclear localization signal (NLS-1 and -2); Basic, basic region; LZ, leucine zipper; AD, activation domain.

K8/RAP

ORF K8 is a DE protein that is a member of the bZIP family of proteins, including EBV Zta and cellular transcription factors c-jun and c-fos. K8 is also referred to as replication-associated protein (RAP) or K-bZIP. ORF K8 and EBV Zta share some sequence similarity,^{253,403} although unlike EBV Zta, K8 does not induce viral reactivation of KSHV or enhance ORF50-mediated reactivation from latency.⁵⁴⁰ K8 has two promoters, one which is expressed during the IE phase and the other during the DE phase.⁶⁸⁶ Several spliced transcripts emanate from the DE promoter⁴⁰³; however, the major protein product, K8, is obtained from the fully spliced mRNA.⁵⁴⁰

Unlike ORF50, K8 does not appear to affect promoter activity to a great extent. Yet together with other proteins, K8 contributes to KSHV lytic replication and gene expression. For example, it binds to cellular bZIP proteins such as C/EBP-α, a

transactivator that is involved in the induction of differentiation and cell cycle arrest.^{712,713} K8 binding to C/EBP-α prolongs the half-life of C/EBP-α, thereby augmenting its function. The K8 promoter itself contains C/EBP-α binding sites, and there is a positive feedback loop for both K8 and C/EBP-α during viral replication.^{683,684} Further, because ORF50 also binds C/EBP-α, ORF50-driven transactivation of K8 is increased by K8. Stabilization of C/EBP-α also helps ORF50 activate other viral lytic promoters with C/EBP-α binding sites, such as the ORF50 and ORF57 promoters.^{683,684} Although K8 augments C/EBP-α function, K8's effect on most other transcription factors appears to be of a repressive nature. For example, K8 hinders CBP²⁹⁵ transactivation, and direct binding of K8 to ORF50 hinders its transactivation function on some promoters (e.g., ORF57 and ORF50 promoters), although PAN promoter activity is not affected.^{299,301,393} K8

can bind Ubc9, the SUMO conjugase protein leading to its own SUMOylation but perhaps also to SUMOylation of other transcription factors that interact with K8, although ORF50 is not SUMOylated in the presence of K8.²⁹⁹

K8 induces a prominent G1 arrest. Many herpesviruses exhibit cell cycle arrest during lytic replication, including KSHV.^{302,712} This is thought to occur in order to prevent cellular DNA synthesis from ensuing and thereby reduces the competition for energy and nucleotide pools. K8 induces cell cycle arrest by enhancing C/EBP- α accumulation through inhibition of E2F expression and increased expression of the cdk inhibitor p21nd.⁷¹³ Moreover, K8 can directly bind cdk2 complexed to cyclins A and E, and this interaction leads to impaired kinase activity. Interestingly, K8 can be phosphorylated by cdk2⁵⁴⁰ as well as KSHV ORF36, a viral protein kinase.³⁰⁰ Curiously, K8 binds and basally activates the IFN- β promoter, thereby preventing IRF3 binding to the promoter and further IFN- β activation upon Sendai virus infection.³⁷⁶ Additionally, K8 also prevents the activation of IFN- α responsive genes (e.g., 2',5'-OAS) and interferon-sensitive gene (ISG) 15.³⁷⁵

Finally, K8 also plays important roles in viral lytic replication, as it is thought to bind the lytic replication origins of KSHV. These roles will be discussed later in the Lytic Viral Replication section.

Delayed-Early Gene Expression

KSHV proteins belonging to the DE class are genes whose transcription is sensitive to the effects of cycloheximide, because their expression depends on the transactivation of their promoters by IE proteins. However, their expression is independent of viral DNA synthesis inhibitors. Many DE proteins (e.g., DNA polymerase, thymidine kinase, ribonucleotide reductase) have enzymatic functions or serve as accessory proteins (single-stranded DNA binding protein, polymerase processivity factor, etc). The DE proteins prepare the infected cell for viral DNA replication. Other DE proteins are involved in immune evasion (K3/MIR1, K5/MIR2, and K14), host shutoff (ORF37/SOX), nuclear-cytoplasmic transport of viral RNAs (ORF57), or modulation of signal transduction (vGPCR, K1, K15). At least two noncoding RNAs have been identified as DE transcripts: the PAN RNA^{148,642,735,736} and a small transcript originating from one lytic replication origin.⁶⁸⁷ Please note that the term *delayed-early* is borrowed from the alpha herpesvirus literature and may not be as strictly applicable to the various environments that trigger KSHV reactivation. In most cases, the time delay is minimal.

ORF57/MTA

ORF57, also known as MTA, is a posttranscriptional regulator and a homolog of HSV ICP27 and EBV ORF57/MTA proteins. HSV ICP27 promotes the nuclear-cytoplasmic transport of unspliced mRNAs, inhibits RNA splicing, and modulates late mRNA polyadenylation.⁵⁸³ ORF57 moves between the nucleus and cytoplasm⁴⁵ and can also enable the cytosolic accumulation of unspliced mRNAs.^{264,332}

ORF57 interacts with Aly/REF, an export factor, to promote nuclear mRNA export.⁴³¹ ORF57 binds to intronless viral mRNAs and functions to recruit the human TREX (hTREX) complex (through interactions with the export adapter protein Aly), but not the exon-junction complex

(EJC), and assemble a viral ribonucleoprotein particle known as vRNP.⁶⁹ Aly subsequently binds the DEAD-box protein UAP56, which recruits the remaining hTREX components to the complex.⁶⁹ It has also been shown that ORF57 associates with PYM, a protein that recruits the 48S preinitiation complex to newly exported mRNAs through binding EJC. The ORF57-PYM interaction allows this complex to associate with intronless KSHV transcripts to initiate translation of viral mRNAs.⁷⁰

Unlike ICP27, ORF57 does not hinder the expression of intron-containing transcripts,³³² because unlike HSV, many lytic cycle KSHV genes are efficiently spliced (e.g., K8, K14, K15, ORF50, ORF57, the vIRFs, ORF29). During lytic replication, ORF57 partially co-localizes with the cellular splicing machinery in nuclear speckles and assembles into spliceosomal complexes in association with viral pre-mRNAs and essential splicing components. ORF57 binds Sm protein and interacts with small nuclear RNAs. Thus, ORF57 appears to function as a novel factor in the spliceosome-mediated splicing of viral RNA transcripts.⁴³⁰

ORF57 can bind ORF50⁴³¹ and augment ORF50's transactivation function on several viral promoters.^{332,517}

ORF57 can also promote the accumulation of nuclear PAN RNA^{148,332} in an export-independent manner.⁴⁹⁸ It can also bind PAN RNA and protect it from degradation by cellular factors. ORF57 binds PAN RNA directly at an ORF57-responsive element (ORE), thereby protecting it from the cellular RNA decay pathway.⁵⁷⁴ Another mechanism by which ORF57 has been shown to stabilize mRNAs is through inhibition of miRNA modulation of viral transcripts.³¹⁸ The vIL-6 transcript contains an MRE (ORF57/MTA-responsive element) analogous to the ORE reported previously.⁵⁷⁴ Binding of ORF57 to the MREs in vIL-6 stabilized its message and enhanced its translation.³¹⁸ One of the MRE sites was also a binding site for miR-1293, and hence, ORF57 competes with miR-1293 for binding to vIL-6, thereby preventing its RISC-mediated degradation. (ORF57 also interacts with a miR-608 binding site in the cellular human IL-6 transcript and prevents miR-608 repression of this message as well).³¹⁸ Finally, ORF57 interacts directly with the RBM15, thereby stabilizing ORF59 RNA transcript in the cytoplasm.⁴²⁸ A genome-wide analysis of viral RNA targets of ORF57 performed by a UV-cross-linking and immunoprecipitation (CLIP) assay revealed 11 viral transcripts that were targeted by ORF57, including vIL-6.³¹⁸

Additionally, two groups have shown that the disruption of ORF57 in a KSHV BACmid resulted in the lack of expression of several lytic genes, including ORF57, ORF59, K8 α , K8.1, and PAN RNA during the viral lytic cycle and yielded no virion production.^{272,429} Recently, it has been reported that ORF57 is cleaved by caspase-7 during reactivation. The cleaved ORF57 was hampered in aiding the expression of viral lytic genes, suggesting that caspase-7 displays antiviral activity against KSHV lytic proteins.⁴²⁸

Lytic Viral Replication

The DE phase is followed by lytic cycle DNA replication. Similar to the case with other herpesviruses, multisubunit complexes containing the core replication machinery are directed to a *cis*-acting replication origin called *oriLyt* by an ori-specific DNA binding protein. Replication complexes are nuclear substructures where viral replication occurs and these are in proximity to host ND10 domains that are often reorganized as replication progresses.

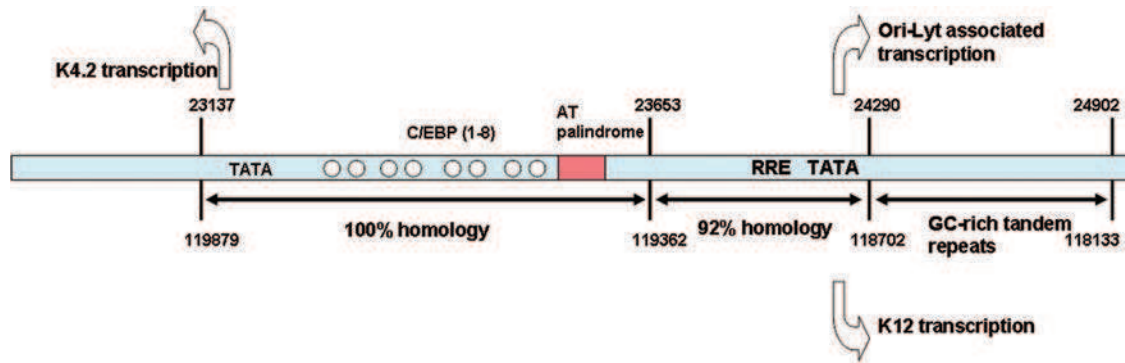


FIGURE 65.13. The structure of Kaposi's sarcoma-associated herpesvirus lytic replication origins. OriLyt-R and OriLyt-L are superimposed to reveal their commonalities. The *central bar* depicts important common sequence features. *Arrows above the bar* depict the transcripts from this region in OriLyt-L; *arrows below the bar* denote RNAs transcribed in oriLyt-R. Numbers refer to nucleotide sequence of each region. Degree of conservation of each domain between the two origins is indicated by percent homology. (Modified from Wang Y, Li H, Chan MY, et al. Kaposi's sarcoma-associated herpesvirus ori-Lyt-dependent DNA replication: cis-acting requirements for replication and ori-Lyt-associated RNA transcription. *J Virol* 2004;78(16):8615–8629.)

Replication utilizes a rolling circle mechanism, whereby linear genomes are processed and packaged into nascent capsids.

KSHV encodes six catalytic and accessory proteins similar to other herpesviruses, including the viral DNA polymerase (POL, encoded by ORF 9), helicase (HEL, ORF 44), polymerase processivity factor (PPF, ORF 59), primase (PRI, ORF 56), primase-associated factor (PAF, ORF 40/41), and single strand binding protein (SSB, ORF 6). When co-expressed in cells, these proteins form stable multisubunit nuclear structures.⁷¹¹ The KSHV genome contains two oriLyt regions: the left-hand origin (oriLyt L) is situated between ORFs K4.2 and K5, and the right-hand element (oriLyt R) lies between ORFs K12 and 71.^{24,401} These elements promote semiconservative replication that is inhibited by viral DNA polymerase inhibitors (e.g., phosphonoacetic acid). These two approximately 1.7-kb regions share striking sequence homology with one another; the right-hand element seems to be an imperfect inverted repeat of the left-hand element. Each lytic origin is comprised of two subdomains: (a) a 1.1-kb region with multiple AT-rich palindrome sequences, TATA boxes, and transcription factor binding sites and (b) an adjacent region (~600 bp) comprised of multiple GC-rich tandem repeats. Genetic studies have identified four critical elements required for oriLyt L function. These include (a) a high-affinity ORF50 binding site and nearby TATA box, (b) four pairs of C/EBP α sites, (c) an 18-bp AT-rich palindrome, and (d) a 32-bp sequence of unknown function.^{24,687} The RRE and TATA box are components of a promoter that drives expression of a 1.4-kb transcript. Similar features are also present in oriLyt R, where the RRE and TATA element drive expression of kaposin mRNA.⁵⁷³ These similarities are schematized in Figure 65.13, in which oriLyt L and oriLyt R have been superimposed to highlight their commonalities. Among these motifs, C/EBP α binding sites, and RREs function as key *cis*-acting elements.⁶⁸⁹

The oriLyt regions can bind K8 indirectly through the C/EBP α sites.⁴⁰¹ The 18-bp AT-rich palindrome is thought to be the place where initial strand unwinding occurs during the start of DNA replication. ORF50 activates the RRE-containing oriLyt promoter by binding to it and initiating transcription across the GC-rich tandem repeats.⁶⁸⁹ The pre-replication complex, of which ORF50 is a component, is recruited to

oriLyt through the ORF50-RRE and the K8-C/EBP interactions with the oriLyt.⁶⁸⁹

Consistent with its function as a repressor of viral genes, a K8 deleted KSHV BAC displayed an enhanced growth phenotype in terms of virion production and the expression of lytic genes in the context of ORF50/RTA induction.³²³ However, reactivation by chemical induction (TPA and sodium butyrate) yielded no virion production and an aberrant lytic profile compared to wild-type KSHV BAC.³²³ Curiously, immunofluorescence staining revealed that in the absence of K8 there was a disruption of LANA subcellular localization, suggesting that K8 can influence LANA localization.³²³ However, another group showed that a different K8 deleted KSHV BAC displayed no difference in overall viral gene expression during lytic reactivation with TPA in epithelial cells, fibroblasts, and endothelial cells. However, the K8 mutant virus-infected cells displayed lower copy numbers of latent KSHV genomes compared to wild-type KSHV-infected cells. These data suggest two possibilities: (a) K8 may be involved in abortive viral replication (leading to viral genome replication but not virion production), or (b) K8 may be involved in the maintenance of latent viral genomes⁶⁸⁸ through modulation of LANA function, as discussed earlier.^{323,688} The former is likely to be the case because it was recently shown that LANA represses origin-dependent lytic DNA replication in a dose-dependent manner and that this suppression was overcome by increasing amounts of K8.⁵⁶⁹ Additionally, LANA alone was shown to interact with oriLyt by ChIP, implying that the inhibition of lytic replication by LANA was mediated by direct binding.⁵⁶⁹ This suggests a model where LANA interacts with K8 to negatively modulate the switch from latency to lytic replication. Conversely, K8 positively modulates lytic replication by relieving the LANA-mediated suppression of lytic replication. This was also seen in the context of PEL cells where a direct correlation of K8 expression and lytic gene expression was observed.³⁷⁵

Late Gene Expression

As with other herpesviruses, late gene transcription starts subsequent to the onset of DNA replication. The transcription of

many (but not all) late genes is hindered by viral DNA synthesis inhibitors (e.g., phosphonoacetic acid, ganciclovir, foscarnet, cidofovir). Most late genes encode for structural proteins (e.g., capsid and envelope proteins).^{206,306,416,587} Late promoter containing plasmids are not properly transactivated when introduced into reactivated PEL cells, despite the fact that the late promoters in these infected cells are successfully transactivated from the viral genomes undergoing replication.¹²¹ Thus, late templates must either be actively replicating or have undergone a chromatin structure change that depends on viral replication. Consistent with this, when late promoter plasmids are transfected into reactivated PEL, regulation of late genes is partially restored when the reporter gene in question is linked in *cis* to an oriLyt element.⁶⁴⁸

A late gene, ORF19, was found to be regulated by ORF50 through a RBP-J κ site,⁵²⁸ which corroborated earlier findings that gB, another late gene, contained RRE elements in its promoter.⁷⁴³ This suggests that ORF50/RTA can regulate all three transcriptional phases of the lytic program.

FUNCTIONS OF LYTIC VIRAL PROTEINS

Modulation of Antigen Presentation by K3/MIR1 and K5/MIR2

The observation that diseases associated with KSHV infection are substantially more severe in hosts with T-cell impairment (e.g., AIDS) indicates that T cells are critical for the control of viral infection. Immune surveillance and clearance by cytotoxic T (CTL) cells is likely an important selective pressure in driving KSHV evolution with the host; therefore, it is no surprise that KSHV possesses an arsenal of proteins that evade the host's immune system.

The infected cell usually presents antigenic peptides from the virus in complex with class I major histocompatibility complex (MHC I) to CTLs. Two proteins (ORFs K3 and K5) prevent MHC I display.¹⁵¹ K3 and K5 encode for proteins referred to as modulator of immune recognition 1 and 2 (MIR1 and MIR2, respectively); K5/MIR2 down-regulates only human leukocyte antigens (HLA) A and B, whereas K3/MIR1 down-regulates all four HLA allotypes (A, B, C, and E)^{298,634} (Fig. 65.14).

K3 and K5 are transmembrane proteins that resemble each other, suggesting that they may have arisen from duplication of an ancestral gene. Each protein contains an amino-terminal variant RING finger with cysteine and histidine residues that vaguely resemble those found in PHD domain proteins. The zinc finger structure is on the cytosolic face of the membrane.⁵⁸¹ The KSHV MIR proteins show functional similarity to the cellular MARCH protein family. MARCH proteins are transmembrane ubiquitin ligases that ubiquitinate and target cellular glycoproteins for lysosomal destruction³⁵ (Fig. 65.14).

The MIR proteins reside in the endoplasmic reticulum membrane and augment the endocytosis of MHC I chains that reach the cell surface^{151,298} through MIR-mediated ubiquitination.^{285,581} Ubiquitination directed these chains to the endosomes and subsequently the multivesicular body (MVB), a sorting organelle from which the MHC I chains can be targeted for lysosomal degradation.^{285,410,461,581} Interestingly, K3 induces lysine-63-linked polyubiquitination (instead of K48-linked polyubiquitination) of MHC class I chains, which leads to endocytosis and endolysosomal degradation.¹⁸⁹ This

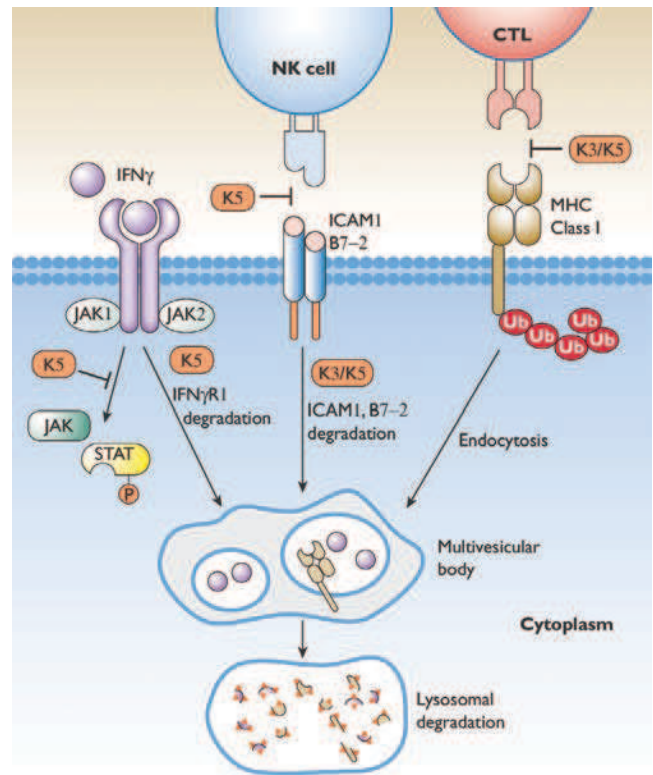


FIGURE 65.14. Ubiquitination and degradation of cell receptors by K3/MIR1 and K5/MIR2. The MIRs are able to induce the ubiquitination and degradation of several of these receptors, including major histocompatibility complex class I, intercellular adhesion molecule 1 (ICAM-1), B7-2, and interferon gamma receptor 1 (IFN- γ R1).

lysine-63-linked polyubiquitination requires the activities of UbcH5b/c- and Ubc13-conjugating enzymes.¹⁸⁹

The MIR RING finger recruits E2 ubiquitin conjugases. K3 and K5 target recognition maps to the transmembrane regions of both effector and target molecules.⁵⁸¹ Subsequently, transfer of the ubiquitin chain to lysines in the cytoplasmic tail can occur. Strikingly, K3/MIR1 can also ubiquitinate cytosolic cysteine residues.⁸⁵

The MIRs can also down-regulate CD1d, a MHC-related protein that presents lipids and glycolipids to classical and non-classical NK T cells, which respond with the release of IFN.⁴⁰⁴ This observation suggests that CD1d-restricted T cells likely play a role in host immunity against KSHV; however, the lipid presentation and antiviral immunity is not well understood. K5/MIR2 (but not K3) also down-regulates ICAM-1 and the co-stimulatory molecule B7-2 (CD86).^{150,297} These two proteins, which are present on antigen presenting cells, can bind and activate CD4-positive T cells. K5-mediated down-regulation of these proteins reduces helper T cell co-stimulation¹⁵⁰ and impairs susceptibility to NK cell cytotoxicity.²⁹⁷ K3 and K5 also down-regulate the interferon gamma receptor 1 (IFN- γ R1),³⁸⁷ thereby preventing it from binding IFN- γ released from activated T and NK cells. KSHV K5 reduces cell surface expression of the NKG2D ligands MHC class I-related chain A (MICA), MICB, and activation-induced C-type lectin (AICL), which is a ligand for NKP80. K5-mediated ubiquitination directs

internalization but not degradation of MICA, resulting in a decrease in NK cell-mediated killing.⁶⁴⁹

Although K3 and K5 appear to be functionally homologous in most cases, there are instances when K5 appears to encode additional functions not performed by K3. For instance, unlike K3, which induces ubiquitin-dependent endocytosis and degradation, K5 can induce the down-regulation of its targets through multiple pathways, including endocytosis-dependent and -independent, as well as ubiquitination-dependent and -independent pathways.⁴⁶² K5 induced the degradation of non-classical MHC I HFE via a polyubiquitination-mediated ESCRT1/TSG101-dependent pathway.⁵⁶⁶ A proteomic screen performed with K5 revealed that it also targets bone marrow stromal antigen 2 (BST-2, CD316), also known as tetherin, activated leukocyte cell adhesion molecule (ALCAM, CD166) and syntaxin-4.³⁶

Independent of its role in immune modulation, K5 has also been implicated in vascular permeability during lytic infection by targeting a junctional endothelial protein—vascular endothelial (VE)-cadherin for ubiquitin-mediated degradation, thereby preventing endothelial cell adhesion. α -, β -, and γ -catenins were also targeted for proteosomal destruction.⁴³⁶ Additionally, in monocytes, K5 expression increased aerobic glycolysis and lactate production in monocytes through activation of receptor tyrosine kinase receptors such as Flt-3 and PDGF- β .³²¹ These signaling and metabolic changes mediated by K5 imply that it potentially plays a role in KSHV-associated oncogenesis. However, because K5 is a lytic protein, its ability to play a role in oncogenesis has certain limitations, which are discussed in the KSHV pathogenesis section later in this chapter.

Although K5 is a lytic protein and only expressed in 1% to 5% of spontaneously reactivated PEL, *de novo* infection of naive cells resulted in down-modulation of the K5 targets in most cells. The down-regulation of MHC I and ICAM-1 correlated with intracellular KSHV load, indicating that low levels of K5 is sufficient for global down-regulation of these markers during primary KSHV infection.^{6,651}

Interferon Evasion by Viral Interferon Regulatory Factors

Several KSHV lytic proteins are involved in preventing the activation and function of type I IFNs that are produced in response to infection. As described earlier, IFNs are induced by PAMPs, which are recognized by innate immune sensors such as TLRs and RIG-I-like receptors (RLRs; e.g., RIG-I and MDA-5). Activation of TLRs and RLRs leads to the activation of cellular IRFs (e.g., IRF3 and IRF7) depending on which TLR is stimulated, type I IFN (IFN- α and IFN- β), and inflammatory cytokines. IFN- α/β released from the cell can bind to IFN- α and IFN- β receptors present on surrounding cells. This signal is transduced to the nucleus, thereby stimulating the transcription of multiple ISGs, which include known antiviral effectors (e.g., OAS, Mx, and PKR) as well as the IRFs themselves.

IFN- α/β binding to IFN receptor leads to the phosphorylation and activation of STAT1 and STAT2. The activated STATs then bind to IRF9 (p48) and relocate to the nucleus to induce transcription from ISG promoters. Both the IFN- α/β and the ISG promoters contain IRF binding sites for many cellular IRFs.

The KSHV genome encodes four different homologs of cellular IRFs from a single locus, which suggests that they arose from an ancestral gene through gene duplication followed by divergence. vIRF-1, -2, -3, and -4 were named based on their order of discovery and not by their homology to a particular cellular IRF. vIRF-3 is a latent gene, as discussed previously.

The remaining vIRFs (vIRF-1, -2, and -4) are mostly expressed during the lytic cycle. Although vIRF-1 is a lytic gene,^{81,747} it was found to be transcribed in latently infected KS cells as well.^{132,181} vIRF-1, -2, and -3 do not share the DNA binding domain of the cellular IRFs and hence do not bind to IRF binding sites in ISG and type I IFN promoters. However, these three vIRFs hinder the function of cellular IRFs, thereby suppressing IFN production (Fig. 65.15). Moreover, the vIRFs have also been shown to differentially modulate TLR3-mediated activation of IFN.^{303a} Please note that vIRF-4 has not yet been shown to affect IFN signaling.

vIRF-1 (encoded by ORF K9) is the most well studied vIRF and can inhibit IFN production in response to Sendai virus infection.^{226,747} vIRF-1 dimerizes with cellular IRF1 and IRF3, which prevents the cellular IRFs from transactivating the IFN promoters, although DNA binding does not appear to be affected. Inhibition of IRF function is thought to occur through vIRF-1's ability to bind and sequester co-activator CBP/p300 from cellular IRFs thereby inhibiting its HAT activity.^{81,385,402} vIRF-1's ability to prevent apoptosis and evade immune responses is likely to greatly contribute to KSHV pathogenesis.

Studies have also demonstrated the ability of vIRF-2 to impair IFN-dependent transcription. In the context of type I IFN, vIRF-2 inhibited ISG56 activation,²¹⁸ cellular IRF1- and IRF3-mediated transcriptional activation,^{81,218} IFN- β promoter activity in response to double-stranded RNA and IRF3 transfection,²¹ and ISRE transactivation mediated by the IFN- γ family members IL-28A or IL-29.²¹⁸ vIRF-2 was unable to inhibit IRF7-mediated transactivation of ISG promoters, as well as the IFN- γ promoter (pGAS) in response to IFN- γ treatment.²¹⁸

Similar to vIRF-1 and vIRF-2, vIRF-3 has been shown to inhibit transactivation of the IFN- $\alpha 4$ and IFN- $\alpha 6$ promoter upon Sendai virus infection as well as in the presence of exogenous IRF3 and IRF7.^{313,419} vIRF-3 could also inhibit IFN- γ -mediated activation of the GAS promoter.⁴¹⁷ Furthermore, vIRF-3 expression led to the reduction of endogenous IFN- $\alpha 1$, - $\alpha 4$, and - $\alpha 6$ mRNA levels upon Sendai infection³¹³ and the production of IFN following infection with Newcastle disease virus.⁴¹⁹

In addition to their ability to inhibit IFN, the vIRFs have also been shown to prevent apoptosis. vIRF-1 was shown to relocalize the proapoptotic protein Bim, a negative regulator of KSHV replication, to the nucleus.¹⁴⁰ This nuclear sequestration of Bim prevents apoptosis during viral replication, thereby leading to increased viral production during the lytic phase.¹⁴⁰ Furthermore, vIRF-1 expression in mouse fibroblasts can induce cellular transformation, which is probably due to the fact that vIRF-1 can also bind p53 and inhibit apoptosis.^{226,488} vIRF-1 co-precipitates with p53 and inhibits p53-driven transcription and targets (e.g., p21 and Bax).^{488,598} Expression of vIRF-1 can also lead to a decrease in total p53 levels owing to vIRF-1 directed ubiquitination and degradation of p53.⁶⁰⁴ This activity required the p53 E3

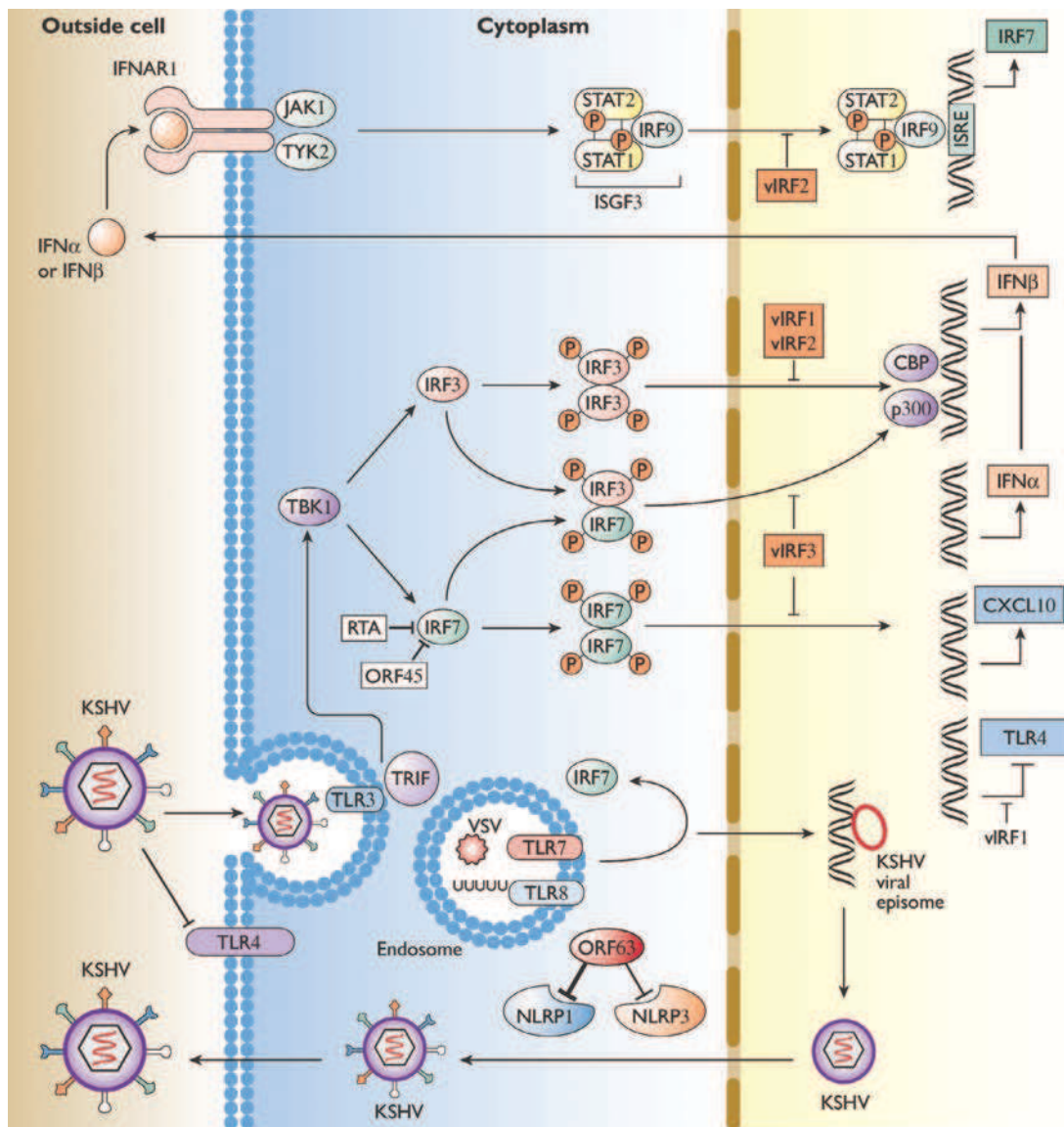


FIGURE 65.15. Innate immune evasion by Kaposi's sarcoma-associated herpesvirus (KSHV) viral interferon regulatory factors (vIRFs), open reading frame (ORF) 50/replication and transcription factor (RTA), ORF45, and ORF63. KSHV entry triggers activation of toll-like receptor (TLR) 3 in monocytes and TLR4 in endothelial cells. Furthermore, binding of latently infected B cells by TLR7/8 agonists leads to reactivation of KSHV from latency. The vIRFs block interferon activation by host cells. ORF50/RTA targets IRF7 for degradation, whereas ORF45 inhibits IRF7 function. ORF63 inhibits activation of the NLRP1 and NLRP3 innate immune sensors.

ligase MDM2. Inhibition of p53-mediated apoptosis may be a mechanism through which KSHV is able to establish latency. In addition to vIRF-1, vIRF-3 and vIRF-4 also interfere with p53 signaling. Like vIRF-1, vIRF-3 was shown to interact with p53 *in vitro* and reduce p53 reporter activity.⁵⁶⁷ vIRF-3 also decreased apoptosis and activation of caspase-8 mediated by p53.⁵⁶⁷ Finally, vIRF-4 interferes with the p53 pathway by interacting with MDM2 to increase MDM2 protein levels³⁷² through the inhibition of MDM2 autoubiquitination and prevention of MDM2 proteasomal degradation.³⁷² Interestingly, both vIRF-1 and vIRF-4 induce MDM2-mediated p53 degradation, but through dif-

ferent mechanisms. A detailed review of the function of the four vIRFs is provided elsewhere.³⁰³

Two other KSHV proteins, which are not homologs of cellular IRFs, can also block IRF action. ORF50 was shown to induce IRF7 degradation as described earlier. Additionally, the IE protein ORF45 can interact with IRF7 and prevent its phosphorylation and nuclear translocation⁷⁴⁰ (Fig. 65.15). ORF45 interacts with an inhibitory domain in IRF7,⁵⁹⁰ which keeps IRF7 in a closed inhibitory state. Because ORF45 is a tegument protein,⁴⁰⁶ in theory it should be able to hinder type I IFN activation pretty soon after viral infection. A KSHV recombinant virus deleted for ORF45 exhibited lower viral

replicative capacity compared to wild-type virus.^{741,742} ORF45 has also been shown to interact with p90 ribosomal S6 kinases (RSKs), RSK1 and RSK2, and strongly stimulates their kinase activities leading to ERK activation.^{353,354} ORF45 interacts with seven in absentia homolog (SIAH), an E3 ubiquitin ligase,¹ and KIF3A, a kinesin-2 motor protein that transports cargo along microtubules to the cell membrane. ORF45 principally mediated the docking of entire viral capsid-tegument complexes onto the cargo-binding domain of KIF3A.⁵⁹¹

Overall, KSHV encodes a battery of viral proteins dedicated to the ablation of IRF activation, and their function is testimony to the significant role that innate immunity plays in the viral life cycle (see Fig. 65.15).

Modulation of Inflammatory Pathways

In addition to the vIRF proteins, KSHV also encodes for virally encoded CC chemokines: vCCL-1 (formerly known as v-MIP-I, the product of ORF K6), vCCL-2 (vMIP-II, from ORF K4), and vCCL-3 (v-MIP-III, from ORF K4.1).⁵⁰¹ Although chemokines are generally positive mediators of immune and inflammatory responses, the receptor preferences for the KSHV-encoded chemokines are consistent with their function as inhibitors of inflammation in response to viral infection. The CD4 T-cell subset of Th1 cells shape antiviral cytotoxic immune responses by promoting IL-2, TNF- α , and IFN- γ induction, which can activate macrophages and promote the development of CD8+ CTL responses. Th2 cells, on the other hand, promote induction of IL-4, IL-5, IL-6, IL-10, and IL-13 in order to elicit B-cell differentiation and humoral immunity (as well as allergic-type responses). Th2 cells also polarize immunity away from cytotoxic responses regulated by Th1 cells. The chief chemokine receptor on Th1 cells is CCR5, whereas Th2 cells express CCR3, CCR4, and CCR8 receptors. KSHV vCCL-1 signals through CCR8, vCCL-2 signals through CCR8 and CCR3, and vCCL-3 signals through CCR4.^{199,500,635} In summary, the KSHV chemokines activate chemokine receptors that are distributed on Th2 cells. Moreover, vCCL-2 can interact with other chemokine receptors including CCR1, CCR2, CCR5, CXCR1, CXCR2, and CXCR4. However, binding of vCCL-2 to these receptors does not activate signaling but rather inhibits signal transduction by these receptor proteins in the presence of their chemokine ligands. For example, binding of RANTES and MIP-1 α to activate CCR5 is inhibited by low amounts of vCCL-2. Thus, vCCL-2 appears to promote Th2 responses and also inhibit Th1 responses (Fig. 65.16). Consistent with this observation, KS lesions contain more Th2 T cells (CCR3+) than Th1 T cells (CCR5+).⁶⁹³ Moreover, treatment of lymphocytic choriomeningitis virus-infected mice with vCCL-2 inhibits CD8 T-cell migration into the lesion and impairs local inflammation. vCCL-2 treatment also hinders CTL-mediated rejection of experimental corneal and cardiac allografts.^{165,535}

Apart from their anti-inflammatory properties, the viral chemokines can also promote angiogenic responses and induce VEGF from PEL.^{57,406} Additionally, the vCCLs can promote cell survival in an autocrine and paracrine manner—the vCCLs inhibit the expression of the proapoptotic protein Bim during KSHV lytic replication.¹³⁹

KSHV K14 encodes a glycoprotein of the immunoglobulin (Ig) superfamily that resembles cellular CD200 (also known as OX2). CD200 is expressed on many cell types (B, T, and

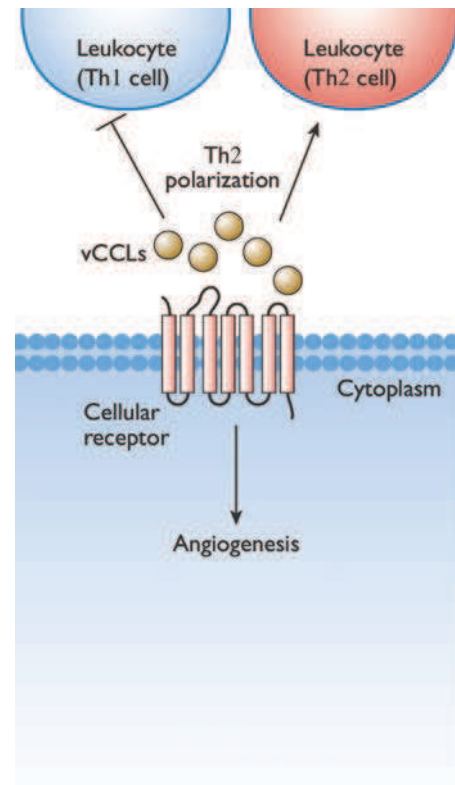


FIGURE 65.16. Kaposi's sarcoma–associated herpesvirus encoded viral chemokines (vCCLs) block Th1 responses and augment Th2 responses. These viral chemokines have also been shown to activate angiogenic pathways.

endothelial cells), and its receptor (CD200R) is located on cells of the myeloid lineage (basophils, neutrophils, and monocyte-macrophages). Cellular CD200 is a negative regulator of inflammation and myeloid cells, and CD200 knockout mice have an increase in monocytes and macrophages, as well as macrophage and microglial activation, and increased susceptibility to experimentally induced autoimmune disease.²⁸⁷ Soluble recombinant KSHV K14/vOX2 protein binds to CD200R.²¹⁴ There is some discrepancy in the literature regarding the inflammatory nature of K14,¹⁴² whereas other studies found that K14/vOX2 suppresses markers of myeloid activation, including suppression of TNF- α production by activated macrophages, decreased MCP-1 and IL-8 production by a monocytic cell line, reduced oxidative burst in primary neutrophils triggered with a phagocytic stimulus, and a blunted release of histamine from activated basophils.^{214,565} One study suggests that the function of K14 may depend on the cell type and activation state. In human primary monocyte-derived macrophages, K14/vOX2 induced proinflammatory cytokines and displayed higher phagocytic activity with respect to mock cells. However, the presence of K14 in MDMs undergoing IFN- γ activation led to a down-modulation of cytokine production and phagocytic activity.⁵⁷⁸

A recent report showed that KSHV encoded a viral protein, ORF63, with homology to the nucleotide-binding and

oligomerization, leucine-rich repeat (NLR) family of proteins.²⁴⁹ NLRs mediate antiviral immune responses to many different invading viruses. Some NLRs form a large molecular structure called an *inflammasome* upon activation. The inflammasome contains oligomerized NLRs, apoptotic-associated speck-like protein (ASC), and procaspase-1. Activation of this complex results in the autocatalytic processing of procaspase-1 to caspase-1. Activated caspase-1 subsequently cleaves the precursors of proinflammatory cytokines pro-IL-1 β and pro-IL-18 to their biologically active forms IL-1 β and IL-18, respectively.³¹⁹ ORF63 is a tegument protein and hence is present in the incoming virion. ORF63 could bind and inhibit the association of the NLR family member NLRP1 with procaspase-1 and prevent the processing of procaspase-1 and subsequent processing of pro-IL-1 β and pro-IL-18²⁴⁹ (see Fig. 65.15). ORF63 inhibition of NLRP1 was found to be important during reactivation from latency and primary infection.²⁴⁹

Transmembrane Signaling Proteins

K15

The coding regions adjacent to the KSHV TRs encode two signaling proteins: K1 and K15. K1 (described earlier) is located at the left-hand side of the genome, and at the right end of the genome lies ORF K15, which encodes for another transmembrane signaling protein. Expression of K15 is highly increased during lytic growth, and its expression during latency has been controversial. It was initially named latency-associated membrane protein (LAMP)²⁴¹ and is expressed at low levels during latency⁶⁰¹ (Dr. Thomas Schulz, personal communication). K15 mRNAs originate from alternatively spliced transcripts that include eight or fewer exons encoding up to 12 transmembrane domains, and a cytosolic tail.^{138,241} All spliced variants encode the carboxy-terminal tail linked to varying numbers of transmembrane domains. The full-length proteins contains eight exons (12 transmembrane). All K15 protein variants are found on intracellular membranes and the plasma membrane. A subset of K15 in the plasma membrane is associated with lipid rafts.⁷³ The K15 cytoplasmic region can be tyrosine phosphorylated and appears to inhibit BCR signal transduction.¹³⁸ The cytosolic tail contains a SH2 binding motif (YEEVL), and the Y is phosphorylated *in vivo* by the Src family of kinases. The cytoplasmic domain contains an SH3-binding region, and another motif (YASIL) represents an endocytosis signal and a potential site of tyrosine phosphorylation.

The cytoplasmic tail also contains a TRAF binding site (ATQPTDD). K15 binds TRAFs 1, 2, and 3 and constitutively activates NF- κ B, JNK and ERK, and MAP kinase signaling pathways, leading to up-regulation of transcription factor AP-1.^{73,241} In this manner, K15 is capable of inducing the expression of an array of cytokines and chemokines, including IL-8, IL-6, CCL20, CCL2, CXCL3, and IL-1 α/β , Dscr1, and Cox-2⁷⁴ (see Fig. 65.9).

Activation of MAP kinase signaling might be important for mediating lytic replication, because MAP kinase activation is necessary for full lytic induction and reactivation from latency.^{145,518,599,719} K15 also interacts with the protein HAX-1, an antiapoptotic factor, suggesting another mechanism (besides NF- κ B induction) by which K15 contributes to enhanced cell survival.⁶⁰¹ K15 can interact with Lyn and Hck kinases.⁵³⁴ Thus, K15 appears to contribute to KSHV

pathogenesis through the up-regulation of multiple inflammatory cytokines and the activation of multiple signaling pathways (see Fig. 65.9).

It is interesting to note that K1 and K15 occupy analogous positions in the KSHV genome to those occupied by LMP1 and LMP2A in the EBV genome (see Fig. 65.3). However, it appears that certain cytoplasmic motifs and functions may have been interchanged between these viral proteins. For example, although K1 is in the same genomic location as EBV LMP1, it contains an ITAM similar to EBV LMP2A and not EBV LMP1. On the other hand, K15 is similar to EBV LMP2A and can inhibit BCR signaling.^{138,466} Thus, generally speaking, K1 and K15 perform similar functions in concert to those of LMP1 and LMP2A combined. However, although LMP1 and LMP2A are expressed in some EBV latency programs, distinct latency programs for KSHV have not been described.

Viral G-protein Coupled Receptor

In addition to K1 and K15, discussed earlier, KSHV encodes a third transmembrane signaling molecule encoded by ORF74, a vGPCR. It is a member of the family of seven transmembrane G protein coupled chemokine receptors.¹¹¹ Expression of this protein depends on ORF50 and is restricted to the lytic cycle.^{137,333} The protein displays constitutive signaling activity in the absence of ligand, although its activity can be stimulated further by chemokines (e.g., GRO- α) and down-regulated by chemokines (e.g., CXCL10/IP10).^{22,234} The resulting signaling activates the PI3K/Akt/mTOR and p38 pathways,^{94,473,617,619,620,621} and the NF- κ B activity,^{439,595} angiogenesis, and cell proliferation^{26,27,274} (see Fig. 65.9). Indeed, vGPCR-deleted recombinant viruses are impaired for viral replication, and vGPCR's function in lytic replication depends on its ability to signal through G α_q and activate MAP kinase signaling.⁵⁸²

Owing to this potent signaling activity, vGPCR activates expression of many host genes.⁵⁴¹ Among these are Rac1⁴⁷⁵ and VEGF^{27,621} a critical mediator of vascular permeability and angiogenesis. Further, expression of vGPCR in mice in several different cell lineages generates focal angioproliferative lesions that have some features in common with KS.^{263,472,722} A notable commonality among two of the models where expression was evaluated at the cellular level is that vGPCR was expressed only in a small proportion of the cells in the lesions, which has led to the belief that in KS lesions, expression by a few lytic cells can induce pathology through paracrine mechanisms.¹⁰⁹ Because vGPCR can be linked to both proliferation and angiogenesis, it may be a key element in KS pathogenesis.^{26,621} Additionally, siRNA suppression of vGPCR in the context of the whole viral genome also inhibited angiogenesis and tumorigenicity.⁴⁸¹ Interestingly, transgenic mice that express the constitutively active form of the small GTPase Rac1 develop KS-like lesions.⁴²⁶ Transcription profiling showed many consistencies between KS tumors and the Rac1 tumors. The tumorigenesis seen in the Rac1 model depended on activation of Akt signaling, ROS-induced proliferation, and hypoxia-inducible factor 1- α (HIF-1 α). Furthermore, inhibitors of ROS reduced tumorigenesis in this model.⁴²⁶ The contribution of lytic genes to oncogenesis is further discussed in the Kaposi's Sarcoma-associated Herpesvirus Pathogenesis section.

Host Shutoff

Many previous attempts to examine the effects of lytic replication on host gene expression in KSHV were hindered by the low frequency of lytic replication in chemically induced PEL lines. However, systems now exist where lytic reactivation is seen in most cultured cells.^{483,671} This is typically performed by infecting cultured cells with KSHV at a high MOI, after which cells are allowed to establish latency; these cells are subsequently superinfected at a high MOI using baculoviral or adenoviral vectors expressing ORF50. This induces the lytic cycle in most cells in culture. Under these conditions, most lytically infected cells display some degree of inhibition of host gene expression 10 to 12 hours postinfection.²⁴⁰ The principal block appears to be at the level of host mRNA accumulation.^{239,240} This phenomenon can be attributed to a single protein encoded by ORF37.

KSHV ORF37 encodes for a DE protein called *shutoff exonuclease* (SOX). Homologs of SOX exist in other herpesviruses as well. In HSV, the SOX homolog is not involved in host shutoff (which instead is mediated by ICP27 and VHS). The KSHV SOX protein retains DNA exonuclease activity similar to its HSV counterpart; however, this function can be genetically separated from its function in host RNA shutoff.²³⁹ Importantly, a small subset of host mRNAs escape SOX-mediated turnover.²⁴⁰ Cellular IL-6 message is one of these transcripts. Mapping studies have shown that the 3' UTR of IL-6 mRNA contains elements that allow it to escape from SOX-induced degradation. The length of polyadenylated tails is now emerging as a distinct marker of mRNA fate, and differences from the canonical length led to either degradation or nuclear retention. KSHV SOX stimulates RNA turnover through polyadenylation-linked mRNA turnover.³⁷⁴ Inhibition of poly(A) tail formation blocks ORF37/SOX activity, and transcripts in SOX-expressing cells exhibit extended poly(A) polymerase II generated poly(A) tails. The poly(A) binding proteins (PABPs) were also shown to be essential for SOX function.³⁷⁴ Exactly how selectivity for host mRNAs is achieved by SOX, while viral transcripts are spared, remains to be elucidated.

KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS PATHOGENESIS

In this section, we review the cellular and molecular mechanisms that underlie KSHV's relationship with its associated cancers.

Molecular Pathogenesis of Kaposi's Sarcoma

Histologically, KS tumors are highly complex, unlike most classical cancers that arise as a clonal outgrowth of a single cell type.^{559,560} This makes KS different from traditional forms of cancer, where the malignant cell is of a clear, distinct type. In KS, the chief proliferating cell is called the *spindle cell*, which is named after its spindle-like shape. As mentioned earlier, the spindle cells are of endothelial lineage, because they express many markers of endothelial origin, including factor XIII, CD31, CD34, CD36, En-4, and PAL-E.^{18,203,204,284,559} However, some spindle cells lack the presence of factor VIII, which is a marker of differentiated vascular endothelium. Spindle

cells also display heterogeneity in marker expression.⁵⁵⁹ For example, some spindle cells in KS biopsies stain for factor VIII, whereas other cells stain for smooth muscle α -actin. This has prompted suspicion that spindle cells might arise from mesenchymal precursors that form vascular and smooth muscle cells.⁴⁰⁹ Another possibility is that the cells arise from lymphatic endothelium.⁴¹ This hypothesis is concordant with the observation that molecular markers of lymphatic endothelium (e.g., VEGF-C, VEGF-R3, LYVE-1, and podoplanin) are also regularly expressed by KS spindle cells.^{437,615,696}

Gene expression profiling studies of infected endothelial cells^{103,273,288,677} found that latent viral infection reprograms expression of endothelial markers, inducing the vascular endothelium to express lymphatic markers such as PROX1, whereas the lymphatic endothelium displays a more vascular-like profile. These studies indicate that examination of just one endothelial marker does not provide clues to the lineage of the infected cells. On the basis of miRNA profiling, it has been shown that there exist multiple distinct stages of endothelial cell reprogramming by KSHV, which is most significantly associated with the down-regulation of mir-221.⁵⁰⁴ In view of the stage-wise reprogramming, which endothelial cell type represents the actual progenitor of the KS spindle cell is still unclear; it is still possible that multiple types of endothelial cells could give rise to the KS spindle cell following viral infection. The resultant KS spindle cell may be shaped as much by the viral infection as by the microenvironment. Recently, KSHV infection of endothelial cells has also been shown to induce transcriptional reprogramming of lymphatic endothelial cells to mesenchymal cells through endothelial-to-mesenchymal transition (EndMT).^{135c}

KSHV-infected endothelial cells up-regulate several signal transduction pathways, including the PI3K/Akt/mTOR pathway and JAK2/STAT3 pathway.^{478,678} STAT3 activation was mediated by gp130 receptor, a common receptor of the IL-6 cytokine family. Both Akt and STAT3 were necessary for KSHV-induced lymphatic reprogramming of endothelial cells.⁴⁷⁸

In addition to spindle cells, KS lesions also contain infiltrating T cells, B cells, monocytes, and aberrant slit-like neovascular spaces. The neovascular spaces are lined with both infected and uninfected endothelial cells.¹⁹¹ They do not contain pericyte or smooth muscle cells and are very susceptible to leakage and rupture. Because of these features, KS lesions often exhibit hemorrhage, with erythrophagocytosis by inflammatory cells in the interstitial spaces. The KSHV-infected endothelial cells display increased vascular permeability. Lytic infection is associated with VE-cadherin degradation^{194,436,545} attributed to the actions of K5 and vGPCR, whereas latent infection is associated with increased Rac1 activation and ROS generation leading to disruption of endothelial junctions owing to the phosphorylation of VE-cadherin.²⁶¹ Thus, the histologic appearance of KS probably results from at least four different processes: proliferation, inflammation, reprogramming, and angiogenesis. These processes are intertwined but distinguishable.

KS can occur in various sites. It is frequently localized to the skin, and in the sporadic classical form, it may be restricted to the lower extremities. In the skin, the dermis is involved, with sparing of the overlying epidermis.⁵²¹ Although progression is not quite linear, and patients may present with lesions in all stages, in general terms, cutaneous KS is thought to begin as a *patch* lesion, with histologically demonstrable spindle cells

that are not necessarily the predominant cell type; inflammatory cells and neovascular elements are also abundant at this stage. The lesion may progress through a plaque-like stage to the *nodular* stage, with the proportion of spindle cells becoming progressively larger as the lesion progresses. By the nodular stage, spindle cells fill up most of the lesion. The spindle cell progressively dominates during the course of KS and is therefore considered to be the key tumor cell.

In immunocompetent adults, the classical form is frequently indolent, and afflicted individuals have long survival times. However, in immunocompromised hosts, KS is highly aggressive, with widespread involvement of lymphoreticular structures and multiple organs such as the gastrointestinal tract, the lung, and skin. Pulmonary KS has a grave prognosis because it is often accompanied by death from respiratory failure. Analysis of KS spindle cell clonality has revealed that KS lesions are either oligo- or polyclonal.^{169,192,238,314} Although clonality is observed in some KS lesions,⁵⁴⁸ the lesion is generally thought to be oligo- or polyclonal throughout much of its development, including progression to macroscopically visible KS. One striking feature is how frequently the individual lesions in a KS patient appear to have arisen independently. This is different than that observed in most classical cancers and underscores the uniqueness of KS. This scenario is reminiscent of that seen with EBV-driven post-transplant lymphoproliferative disorders, where lesions can be polyclonal, showing a possible progression to monoclonality, and when multiple, they may be of different clonal origin in a single patient.^{112,337} Cellular pre-miRNA profiling of KSHV-infected endothelial cells and KS tumors revealed that as cells infected with KSHV transitioned from immortalization to fully tumorigenic, there was a loss of mir-221 expression and a gain of mir-15 expression.⁵⁰⁴ Expression of mir-140 and the KSHV viral miRNAs increased proportionally with the degree of transformation.⁵⁰⁴ Additionally, down-regulation of multiple tumor suppressor miRNAs was observed in KS and PEL.⁵⁰⁶ Because the tumor suppressor proteins themselves are, for the most part not mutated in these tumors, regulation of their expression in KS and PEL highlights a novel, alternative mechanism of transformation.⁵⁰⁶

KS spindle cells only exhibit a few of the hallmarks of transformation. KS cells highly depend on growth factors and cytokines when cultured *in vitro*.^{203,204,465} KS cells also do not display genetic instability, although this may reflect the nature of viral cancers, which tend to show less genetic instability (e.g., p53 mutations) in general.¹⁵⁶ KS spindle cells are diploid and do not display microsatellite instability, although certain advanced AIDS-KS tumors can display these characteristics.⁴² The KS spindle cells depend on cytokines and growth factors (perhaps from infiltrating inflammatory cells) in their microenvironment for their proliferation; however, they themselves can generate angiogenic and proinflammatory factors to recruit additional blood vessels to the site of the infected lesion. Thus, KS highly depends on its microenvironment for growth and survival.^{203,204}

Role of Hypoxia in Kaposi's Sarcoma Biology

One descriptive observation involving KS was that it often (but not always) develops in the lower extremities, such as the feet, where tissue may be less oxygenated. A seminal finding revealed

that low oxygen conditions (i.e., hypoxia) leads to the reactivation of KSHV replication from latently infected PEL.¹⁶² Hypoxia augmented vIL-6 secretion, as did several compounds that increase the levels of HIF-1. Hypoxia also increased expression of the viral kinase KSHV ORF36 and the thymidine kinase encoded by ORF21.¹⁶³ HIF is a master regulator of angiogenesis and is activated posttranscriptionally by hypoxia, leading to increased protein stability of HIF-1 α and/or HIF-2 α . Consistent with this hypothesis, the KSHV genome contains several hypoxia response elements (HREs).²⁷⁵ The ORF50/Rta and the ORF34 promoters contain HREs, which under hypoxic conditions are activated by HIF-1 α and/or HIF-2 α .²⁷⁵ As mentioned earlier, XBP-1s can reactivate KSHV from latency, and it has been reported that hypoxia also induces active XBP-1s.¹⁵⁹

Additionally, in the context of primary KSHV infection, there is an increase in HIF activity under normoxic conditions.¹⁰⁴ Both HIF-1 α and HIF-2 α proteins are elevated in KSHV-infected cells.¹⁰⁴

Finally, KSHV infection of B and endothelial cells has also been shown to increase aerobic glycolysis leading to lactate secretion, i.e. the Warburg effect, and to augment fatty acid synthesis (FAS). Inhibitors of glycolysis and FAS enhance cell death of KSHV-infected cells.^{49a,171,171a}

Molecular Pathogenesis of Primary Effusion Lymphoma and Multicentric Castleman's Disease

Gene expression profiling demonstrated that PEL display a distinct profile from all other non-Hodgkin's lymphoma (NHL) that is related neither to GC nor to memory B cells.³³⁴ The mRNA profile of PEL was defined as plasmablastic because it showed features of both immunoblasts (similar to EBV-transformed lymphoblastoid cells) and plasma cells (similar to multiple myeloma cells).³³⁴ Another group reported that the stage of B-cell development represented by PEL closely resembles that of malignant plasma cells.³⁰⁷ The unfolded protein response (UPR) is partially activated in PEL (e.g. XBP-1), and genes overexpressed in PEL include those involved in inflammation, cell adhesion, and invasion.³⁰⁷ KSHV-infected PEL have also been found to up-regulate the PI3K/Akt/mTOR signaling pathway,^{49,610,657} as well as higher levels of B-Raf and angiogenic VEGF-A as compared to other lymphoma subtypes. MEK inhibition reduced VEGF-A expression in PEL.⁹ Genome-wide comparative genome hybridization (CGH) found consistent deletions in fragile sites that encode the FHIT and WWOX tumor suppressor genes.⁵⁷⁰ In terms of viral gene expression, most of the PEL cells express latent genes (e.g., LANA, vCyclin, vFLIP, vIRF-3).^{206,489} However, between 1% and 5% of PEL spontaneously reactivate in culture and display lytic gene expression.

MCD is associated with both latent and lytic infection. In MCD, latent (LANA) and lytic (ORF59, K8, and K10) viral proteins are expressed.^{322,521} One protein that is constitutively expressed in a significant proportion of KSHV-infected cells in MCD is vIL-6, which as described earlier is expressed during latency and is highly up-regulated during lytic infection.^{114,322,511,521} It is thought that expression of lytic proteins contributes extensively to the pathobiology of MCD.

Kaposi's Sarcoma—Associated Herpesvirus Gene Expression in Kaposi's Sarcoma and Primary Effusion Lymphoma

KSHV viral DNA is present in all KS tumors, regardless of type of KS or disease stage. As mentioned earlier, KSHV specifically targets the spindle cell compartment within the tumor.^{59,191,629} However, when passaged *in vitro*, the spindle cells generally lose the viral genome. It has been reported that monocytes in the lesion can also be infected by KSHV^{55,514} as well as some epithelial cells.^{211,521,558,629} *In situ* hybridization for latent mRNA transcripts suggest that most KS spindle cells are latently infected; yet, a small subpopulation (<1%–2%) expresses markers of lytic infection.^{179,191,322,629,636} With the exception of vIRF-3, all latent genes expressed in PEL are also found as latent genes in KS.⁵⁶⁷

Similar to EBV, it is thought that the latency program drives tumorigenesis. Early (patch) lesions of KS have only a small proportion of cells. Fewer than 10% of spindle-like cells are LANA-positive, whereas in more progressed lesions, a variable but higher proportion of spindle cells are latently infected.^{191,629} A study that included quantification of the LANA-positive cells in KS lesion tissues using automated image analysis showed that the percentage of LANA-positive cells ranged from 0.49% to 15% of the total number of cells in KS lesions.³⁴¹ This study was done in the context of a clinical trial of KS patients in the United States who were receiving antiretroviral therapy, raising the possibility that earlier studies, which reported the presence of KSHV in most spindle cells in advanced lesions, were seeing a larger proportion of infected cells in patients with more severe immunodeficiency. The *in vitro* properties of the latent proteins (e.g., the inhibition of p53 and Rb by LANA, the induction of NF- κ B by vFLIP, the stimulation of cdk6 by vCyclin, and the up-regulation of β -catenin by LANA) are commensurate with transformation, growth advantage, and survival. However, it is important to note that these functions were identified in experiments involving overexpression of individual viral proteins. When most transformed cell lines are infected with KSHV, the resulting latent infection does not greatly impact their biology.^{39,357,670} However, when KSHV infects primary (or immortalized) human endothelial cells, the virus replicates for the first few days (usually with significant CPE) and spreads through the monolayer.^{143,227} Over the next 7 to 10 days postinfection, lytic replication subsides and most cells become latently infected. The resulting LANA-positive cells exhibit a change in morphology (i.e., elongation to a spindle morphology) similar to that seen in KS tumors *in vivo*. In most cases, the primary cells are not immortalized by KSHV,^{143,227,647} although some experimental conditions have resulted in a marked delay of senescence.²¹⁰ Similarly, most KSHV-positive primary endothelial cells cannot form tumors in nude mice or grow in soft agar. However, An et al¹⁵ did isolate a KSHV-infected immortalized endothelial line (TIVE) in which KSHV-infected cells were tumorigenic and grew out many weeks following infection. These KSHV-infected TIVE cells do give rise to tumors in nude mice.¹⁵ However, this cell line appears to represent the exception to the rule rather than the norm (R. Renne, personal communication).

The above results may simply reflect the paucity of culture conditions to successfully propagate KSHV-infected endothelial cells. Indeed, a similar situation is observed in EBV where epithelial cells like nasopharyngeal carcinoma (NPC) cells lose

the EBV genome during passage in tissue culture.¹⁸² This is despite the fact that EBV-infected B lymphoma cells do not lose the virus during similar passage, as is the analogous situation with KSHV-infected B lymphoma cells. Thus, in contrast to B lymphoproliferative cells, it is generally not possible to establish cell lines from either NPC or KS that retain the EBV and KSHV episomes, respectively, during passage in tissue culture. However, *in vivo*, every KS and NPC tumor cell retains the viral genome.

Early studies showed that KSHV infection of primary B cells *in vitro* is inefficient and, in contrast to EBV, does not lead to immortalization.⁴⁶⁴ Infection efficiency was enhanced when primary B cells were activated and expressed DC-SIGN.⁵⁵⁵ When KSHV DNA was transfected into BJAB B cells, the virus was maintained as an episome and could establish latency with a low level (1%–5%) of underlying spontaneous lytic replication.¹³⁴ The latent viral gene profile was similar to that observed in PEL, and the virus could be reactivated to produce infectious progeny virions.¹³⁴ *In vivo*, it appears that KSHV can infect B cells and *in vitro* infection of primary tonsillar B cells has shown that activated T cells help to promote and stabilize latency^{482,484} and that these infected B cells predominantly express lambda light chain.²⁷⁹

Unprecedented Role for Viral Lytic Proteins in Kaposi's Sarcoma—Associated Herpesvirus Oncogenesis

The latency program has traditionally been associated with tumorigenesis (as is the case with EBV). Hence, it was surprising that independent evidence from clinical studies suggested that both lytic replication and latency play an important role in KS development. In the case of patients with advanced AIDS, the administration of ganciclovir (GCV)—a drug that blocks lytic but not latent KSHV infection—prevented the onset of new KS tumors.⁴⁴⁰ This is a striking result because these patients were infected with HIV and KSHV for many years; the fact that the administration of GCV blocks tumor appearance suggests that viral reactivation may be necessary to support KS development. Further, patients with KS typically display elevated levels of circulating KSHV viral load in the blood.^{89,90,95,200,542} However, treatment with two other antiviral drugs, namely oral valgancyclovir and cidofovir, failed to affect already established KS lesions.^{349,405} One could speculate that dependence of KSHV lytic genes exists during the initial stages of lesion formation. Subsequently, fully tumorigenic spindle cell clones may emerge, in which host cell mutations lead to independence of KSHV lytic proteins.

How Might Lytic Replication Contribute to Kaposi's Sarcoma Tumor Development?

The lytic and latent phases of the viral life cycle go hand in hand. Without lytic proteins, the virus cannot replicate, survive in the human population, or exert paracrine effects on neighboring uninfected cells and the tumor microenvironment. Without latent proteins, the virus cannot establish latency and persist for the lifetime of the host. Thus, these two phases of the viral life cycle are interdependent on one another, and the cumulative pathogenesis seen with KSHV infection is a contribution of both lytic and latent genes. Although there is no firm answer to the question of how lytic genes contribute to

KS tumor development, several (nonexclusive) possibilities can be considered:

1. Latent KSHV infection is not clearly immortalizing *in vitro* using current culture conditions, which distinguishes it from EBV. If spindle cells do not proliferate indefinitely, then for a tumor mass to grow, KSHV-positive cells that die must be replaced by newly infected cells. The source of such cells could be generated from *de novo* infection of endothelial cells with virus produced from lytically replicating cells.
2. When recently infected cells bearing latent KSHV genomes undergo sustained proliferation *in vitro*, they lose their viral genome.²⁵⁴ This is supported by the consistent observation that when infected KS spindle cells are explanted from clinical specimens and placed in culture under conditions that favor their proliferation and expansion, they rapidly lose their viral episomes but retain their transformed nature.^{13,178,209} This instability is likely the cause of the relative clinical indolence seen with KS, because cells that divide and proliferate aggressively may lose their viral latent episomes and thus their virally bestowed survival advantage. Thus, lytic replication may be continuously needed for KS development to infect and restore latent infection to spindle cells that have lost their viral genomes. Some experimental evidence indicates that selective pressures exist *in vivo* to retain the KSHV genome, and these are different from those of explanted KSHV-infected cells in culture.^{15,481}
3. Another way that lytic KSHV replication may contribute to KS pathogenesis is through activation of three processes required for KS development: proliferation, inflammation, and angiogenesis. Because fully lytically infected cells die, they are not able to contribute to the proliferative component of the disease. However, in the context of abortive lytic replication, the lytically infected cells can produce paracrine factors that support the inflammatory and angiogenic nature of the lesion. As described earlier, KSHV encodes multiple secreted signaling proteins expressed during the lytic cycle—examples include the three viral CC chemokines and vGPCR. The viral chemokines exhibit chemotactic properties for both Th2 cells^{626,635} and endothelial cells.²⁷⁶ Polarization of local T cells away from Th1 responses might inhibit antitumor cytotoxic responses, whereas the effect of viral chemokines on endothelial chemotaxis suggests that KS lesions are likely to recruit more endothelial cells for latent or lytic infection. Indeed, the application of the viral chemokines to chick chorioallantoic membranes stimulates angiogenesis.^{57,635} Moreover, vGPCR is a potent signaling protein, as discussed previously, and is capable of activating prosurvival, proangiogenic, and proliferative cellular pathways.
4. Finally, it is also possible that some lytic proteins are expressed outside of the traditional lytic cycle—if not during the canonical latent phase, then perhaps in alternative latent phases, which may occur in response to intra- or extracellular signals or microenvironment cues, which allows a specific lytic gene to be expressed in the absence of other lytic genes.

Conversely, one conundrum is the ability of a subset of latent proteins to up-regulate inflammatory cytokines. Several latent proteins are linked to the induction of inflammatory responses, including vFLIP, K15, and kaposin B, as described

earlier. Cytokines play important roles in KS and MCD pathogenesis, and it is likely that these viral proteins induce cytokine production in KS and MCD. Although understandable from a pathogenesis standpoint, from an evolutionary point of view, it appears contradictory that KSHV would embed such proinflammatory biology in the heart of its latency program. In contrast, many viruses encode proteins that prevent inflammatory responses or evade those they cannot ablate.⁴²⁰ This conundrum remains unresolved. It is possible that cytokines are important to maintain latency in naturally infected cells *in vivo*, or for sustaining the viability of such latent cells. Alternatively, an inflammatory microenvironment may help to recruit KSHV-permissive cells to sites of viral infection, thereby facilitating viral spread from cells undergoing lytic reactivation. The fact that some of the latent viral proteins also possess antiapoptotic activity suggests that these viral proteins acting in concert may negate the negative effects of proinflammatory cytokines, whereas the positive effects of these cytokines (e.g., recruitment of target cells) are retained. In addition, there is increasing experimental evidence suggesting that expression of lytic genes (at least vGPCR) in small subsets of cells can augment the proliferation or tumorigenicity of cells expressing latent genes (such as vFLIP and vCyclin).^{472,474}

The identity of the origin of the KSHV-infected tumor cells remains unresolved. One report suggested that bone marrow precursors serve as a reservoir for KSHV.⁵²⁴ Primary mesenchymal stem cells (MSCs) in human bone marrow were shown to be susceptible to KSHV in tissue culture.⁵²⁴ Human CD34+ hematopoietic progenitor cells (HPCs) were also subjected to KSHV infection and differentiated *in vitro*.⁷¹⁶ Infection of CD34+ HPCs with a recombinant GFP-containing KSHV followed by reconstitution with KSHV-infected CD34+ HPCs showed that the virus could be detected in human CD14+ and CD19+ cells recovered from NOD/SCID mouse bone marrow and spleen following infection.⁷¹⁶ These results suggest that KSHV can establish persistence in CD34+ HPCs, and this may serve as a KSHV reservoir to allow the virus to be disseminated to B cells and monocytes after differentiation.⁷¹⁶

EXPERIMENTAL SYSTEMS FOR STUDYING KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

Animal Models

KSHV does not establish persistent infection in mice, hence humanized mouse models have been used. Early studies showed that C.B-17 SCID mice implanted with human fetal thymus and liver grafts (SCID-hu Thy/Liv mice) were susceptible to KSHV infection, with an early phase of lytic replication and subsequent sustained latency.¹⁸⁰ Viral gene expression in CD19+ B lymphocytes was observed, and infection was inhibited by ganciclovir. However, no disease was evident in the infected animals.¹⁸⁰ In another humanized model, NOD/SCID mice with functional human hematopoietic tissue grafts (NOD/SCID-hu mice) were also injected with KSHV. A portion of these animals generated human KSHV-specific antibodies. Antiviral treatment of the NOD/SCID-hu mice with ganciclovir led to prolonged but reversible suppression of KSHV DNA and RNA levels, suggesting that KSHV can

establish latent infection *in vivo*.⁵²³ Human CD34+ cells have been infected *in vitro* and followed in immunodeficient mice, also without any disease.⁷¹⁶ Another reported model of KS was the use of engrafted human skin in SCID mice, into which KSHV was injected. Eighty percent of these mice developed erythematous and angiogenic lesions; however, none developed tumors or locally invasive disease.²¹²

Viruses related to KSHV from other species (e.g., murine herpesvirus 68 [MHV68], RRV, and HVS) are used as experimental models of KSHV pathogenesis (reviewed in 37,160). However, *in vivo*, KSHV infection is restricted to the human population, and no natural infection of any other species has been identified. KSHV is not successfully transmitted in small animal experimental hosts. Implantation of PEL into immunodeficient mice leads to tumor engraftment but not to spread of virus or infection in murine tissues.^{58,533} As described previously, SCID/hu mice transplanted with human lymphoid tissues display infected human B cells within the implant; however, there is no spread to the surrounding mouse tissue, and no disease state developed in these animals.¹⁸⁰

A recent study has shown that KSHV can be successfully transmitted to New World primates, specifically common marmosets (*Callithrix jacchus*).¹²⁰ Infected common marmosets displayed antibody reactivity to KSHV, and LANA protein and KSHV genomic DNA could be detected in PBMCs from these animals, indicating that KSHV could establish persistent infection in these animals.¹²⁰ One KSHV-infected animal developed a KS-like skin lesion consisting of spindle cells and infiltrating leukocytes.¹²⁰

Xenograft models have been very useful for the study of pathogenesis and to test various potential therapies. Xenograft models of PEL have been reported by several laboratories,^{49,58,329,575,610} and PEL cell lines expressing firefly luciferase have allowed *in vivo* tracking of tumor growth (BC-3-NFκB-luc).³²⁹ Xenograft models of KS have also been reported; however, these have used cell lines such as SLK, KS Y-1, and KS-IMM, which were obtained from KS patients but lacked KSHV.^{209,284,557,584} Other cell implantation models include injection of human endothelial cells that have been infected with KSHV *in vitro*¹⁵ and mouse bone marrow cells that have been transduced with a KSHV BAC.⁴⁸¹ These models have provided interesting biological insights and are particularly important because they assess the effect in the context of the entire virus. A related system that has been used to evaluate the interaction of cells expressing a lytic gene (vGPCR) and cells expressing latent genes (vFLIP and vCYC) is the implantation in mice of endothelial cells transduced to express viral genes.⁴⁷² These important studies have confirmed that KSHV can produce vascular tumors, that vGPCR is a critical viral gene for this process, and that there is a selective pressure *in vivo* for viral maintenance.

Cell Culture Models of Infection

To determine parameters of KSHV tropism and transmission, many different cell culture systems have been developed as experimental models to examine these events. The first successful cultivation of cells containing KSHV utilized PEL from AIDS patients with advanced disease.^{110,563} These cells grew as ascites tumors in the KS patients and were readily established in cell culture. A panel of these lines has now been established from both EBV-positive and EBV-negative cases of PEL.^{23,58,92,99,110,219,340} These cell lines have complex, hyperdiploid

karyotypes with multiple structural abnormalities, a few of which are recurrent (trisomy 7, trisomy 12, and aberrations in the proximal long arm of chromosome 1 [1q]).^{186,570,705} However, in general, they do not contain structural alterations in common genes involved in lymphomagenesis (such as cMyc, p52, RAS).⁴⁸⁷ PEL cells predominantly harbor latent KSHV episomes⁵⁶³ with only a handful of latent viral genes being expressed, and virus is not produced. However, in most experimental systems, 1% to 5% of PEL cells display lytic replication with the viral genome being expressed in a temporally regulated fashion, resulting in viral DNA replication and yielding infectious virions as described earlier. The viral titers are quite low but can be greatly increased by the addition of chemical inducers (e.g., phorbol esters) or the HDAC inhibitors (e.g., sodium butyrate and valproic acid).^{467,468,564,602} Chemical inducers reactivate 15% to 30% of latent PEL that begin to exhibit lytic gene expression. Virions isolated from PEL have been utilized for structural studies of the virus particle^{38,40,173,174,495,656,714,738} and for studies of infection studies *in vitro* and animal infectivity studies *in vivo*. These cell lines have also been utilized for serologic assays.

Although cell lines established from patients with advanced KS do exist (SLK, KS Y-1, and KS-IMM), these lack the KSHV genome and are therefore not useful for virological studies. Therefore, immortalized endothelial cell lines have been generated that contain the KSHV genome. Although these in general do not result in stable viral infection, a few cell lines have been generated that do appear to maintain the virus and grow indefinitely. The first such system used dermal microvascular endothelial cells immortalized with human papillomavirus E6 and E7 prior to infection with KSHV.⁴⁷⁹ Another important cell culture system was generated by infecting telomerized endothelial cells (TIVE) to generate long-term cultures (TIVE-LTC).¹⁵ Recently, a new KSHV-infected cell line named KSHV-iSLK and expressing an inducible RTA gene, has been shown to be efficiently induced by doxycycline. These cells seem to produce increased quantities of infectious KSHV virions.⁴⁸³

Most KSHV-infected cells *in vitro* result in viral latency and not lytic replication. These include common cell lines (e.g., HeLa and HEK293).^{39,670} Human cell lines of fibroblastic, endothelial, and epithelial lineages and rodent cell lines (3T3 and BHK) can all support latent infection *in vitro*.^{39,143,213,357,479,577,647,670} Thus, the entry machinery for KSHV is likely distributed across different cell types, and infection in culture is highly amenable and often does not parallel the pattern of infection seen *in vivo*. Established cell lines (e.g., HEK293, TIME, Vero, HFF) that are readily infected can also be induced to reactivate by the same chemical stimuli mentioned earlier for PEL (e.g., TPA or sodium butyrate).

Oral Infection Model

KSHV has been shown to infect epithelial cells, including those from the oral cavity.^{193,311,597,669} Indeed, KSHV has been detected in the saliva of KSHV seropositive individuals^{54,339,355,525} and is also known to be transmitted orally to children from KSHV-positive breast-feeding mothers.^{71,166,432,454} Tissue culture-based infection of oral keratinocyte raft cultures with a recombinant KSHV virus resulted in latent infection in the basal layer; however, when these keratinocytes differentiated, lytic replication was induced and virions were produced at the epithelial surface.³¹¹ Interestingly, early keratinocyte

differentiation did not result in lytic replication in cells attached to a substratum and only occurred in the suprabasal cell layer.⁵⁹⁷ Consistent with this model of adherence-dependent latency, keratinocytes that were differentiated in a suspension activated lytic KSHV.⁵⁹⁷ It appears that the engagement of integrins on the KSHV-infected cell surface plays a key role in maintaining latency and preventing reactivation. *In vivo*, it is likely that virus present in oral epithelial cells is subsequently transmitted to tonsillar B cells where the virus can establish lifelong latency.

Recently, it was shown that KSHV can infect both T and B cells from primary tonsillar explant cultures.^{279,482,484} T cells, however, do not support lytic replication (they display abortive replication); B cells spontaneously produce substantial amounts of infectious virus. When mixed cultures of B cells and activated T cells were exposed to KSHV virus, production was not seen unless the T cells were removed from the cultures or the culture was spiked with immune suppressants. Thus, it appears that oropharyngeal activated T cells recognize ligands on KSHV-infected B cells and suppress lytic replication thereby promoting latency.^{279,482,484} Furthermore, when human tonsillar cells were infected with KSHV and analyzed using multispectral imaging

flow cytometry (MIFC), LANA expression was mostly seen in cells that expressed the lambda light chain of the BCR.²⁷⁹ The KSHV-infected B cells displayed increased expression of Ki67 and IgM and IL-6 receptor. They proliferated and also displayed blast morphology. All of these characteristics are similar to those seen with MCD, which are IgM lambda-expressing plasmablasts.²⁷⁹ Thus, it is likely that oral transmission of KSHV leads to latent infection of a subset of tonsillar IgM lambda-expressing B cells than can expand and resemble plasmablasts.

Taken altogether, these observations suggest several possibilities through which KSHV could establish infection in endothelial cells and B cells *in vivo* (a putative model is shown in Fig. 65.17):

1. It is possible that the virus first infects the oral epithelium from which it is shed into the saliva and can then subsequently infect either endothelial cells or B cells in the oral submucosa.^{193,482,597}
2. It is equally possible that the virus can directly infect B cells (and plausibly endothelial cells) in the oral submucosa through a break in the mucosal epithelium, as described previously for KSHV infection of tonsillar B cells.⁴⁸²

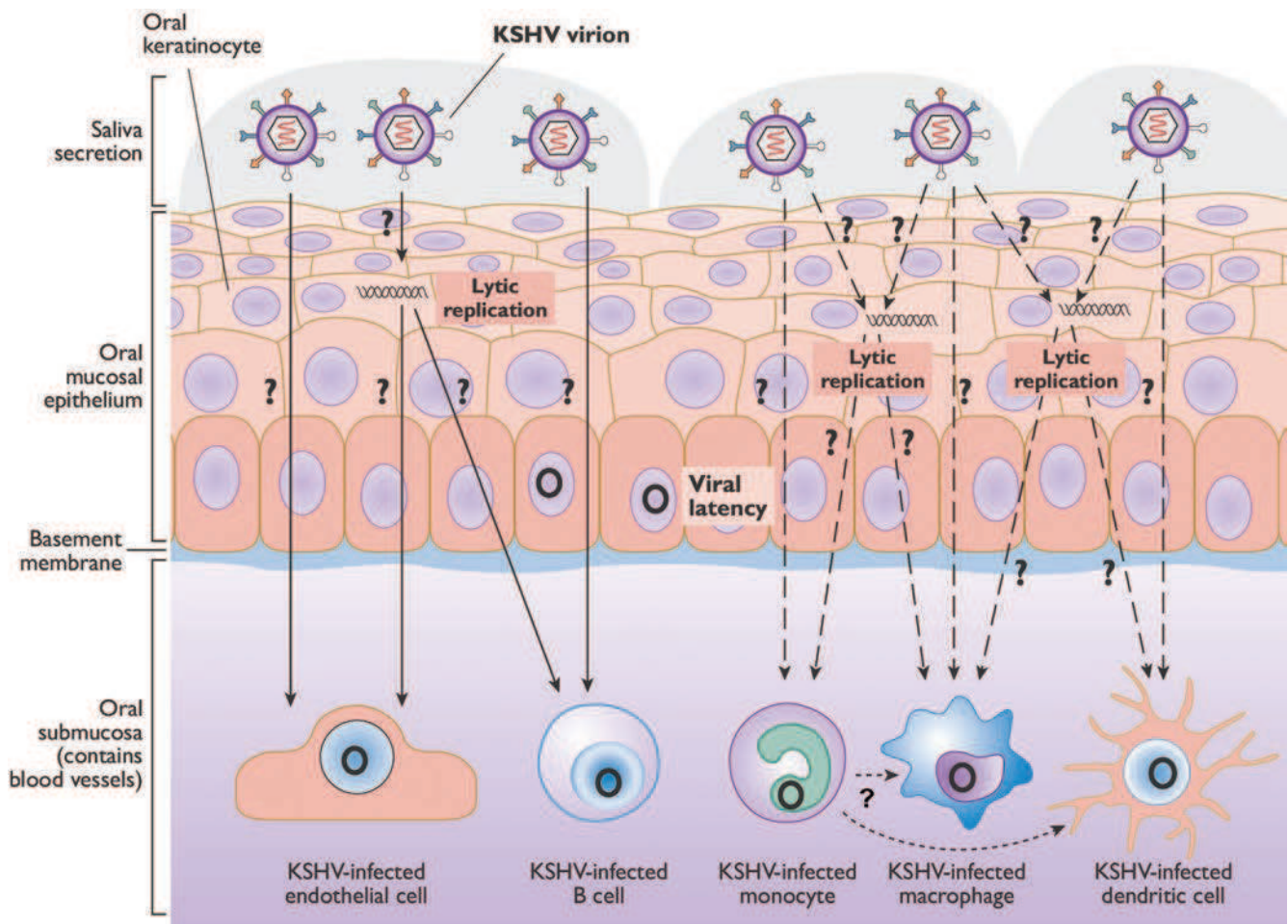


FIGURE 65.17. Hypothetical model of Kaposi's sarcoma-associated herpesvirus (KSHV) infection in the oral cavity. This model is based on various tissue culture studies and correlative measures of viral loads in different compartments *in vivo*. Several possible scenarios are depicted to indicate how KSHV establishes latency in B cells and endothelial cells following primary infection.

- Because KSHV-infected monocytes and macrophages have been detected *in vivo*,^{55,514,612} it is also conceivable that KSHV can directly infect these cells in the oral submucosa or indirectly through oral keratinocytes.
- Although KSHV has not been detected in DCs *in vivo*, it can infect these cells *in vitro*,^{283,556} which raises the possibility that these cells might also be directly or indirectly infected by KSHV in the oral submucosa.

It is important to note that the model described above is hypothetical and is based on different tissue culture studies and correlative measures of viral loads in various compartments *in vivo*. However, it provides some insight into how KSHV can establish latency in B cells and endothelial cells *in vivo*.

EPIDEMIOLOGY

Age

KSHV infection can occur at any age, beginning in early childhood. Reports of children with KS in endemic regions first indicated that viral transmission of KSHV can occur during childhood.⁶¹⁶ Studies evaluating the age in which viral transmission occurs have been possible in subequatorial Africa, where prevalence of KSHV is highest. These studies have largely shown that infection occurs at an early age, arguing for nonsexual transmission. The presence of antibodies to KSHV increases gradually with age,^{62,82,84,235,425,453,455,537} with as many

as 15% of children under 2 years of age having been exposed to KSHV in endemic areas. Closely following puberty, seroprevalence of KSHV continues to rise through early to mid adulthood, which is a pattern suggestive of inefficient heterosexual transmission,^{25,198} with close to half of the population being seropositive by age 50 years⁸³ (Fig. 65.18). There is a strong familial association, with a higher likelihood of a child being positive if the mother or a sibling is positive for the virus. A large study (>2,500 participants) was recently conducted to address whether sociodemographic, behavioral, and biological factors were associated with transmission in rural Uganda. This study confirmed that in this setting, infection is mostly acquired from intrafamilial contacts in childhood and that it continues into adulthood, most likely through nonsexual routes.⁸⁴ As many studies from different areas used variable methodology to assess the seroprevalence, analysis using a single method was conducted to determine the presence of antibodies among a cohort of more than 6,000 people from four sub-Saharan countries in Africa. This study showed that in addition to age, other factors impacted on the seropositivity to KSHV. These included higher rates of infection occurring in rural areas, geographic variation (highest seroprevalence in West Nile, Uganda, of 70% and lowest in Nigeria of 23%), and gender (higher in men than in women).⁵³² This study also nicely confirmed that variations in KSHV seropositivity parallel the cumulative incidence of KS (as shown in Fig. 65.18, which is based on a compilation of multiple studies). Regional variation was also shown in a study evaluating seroprevalence

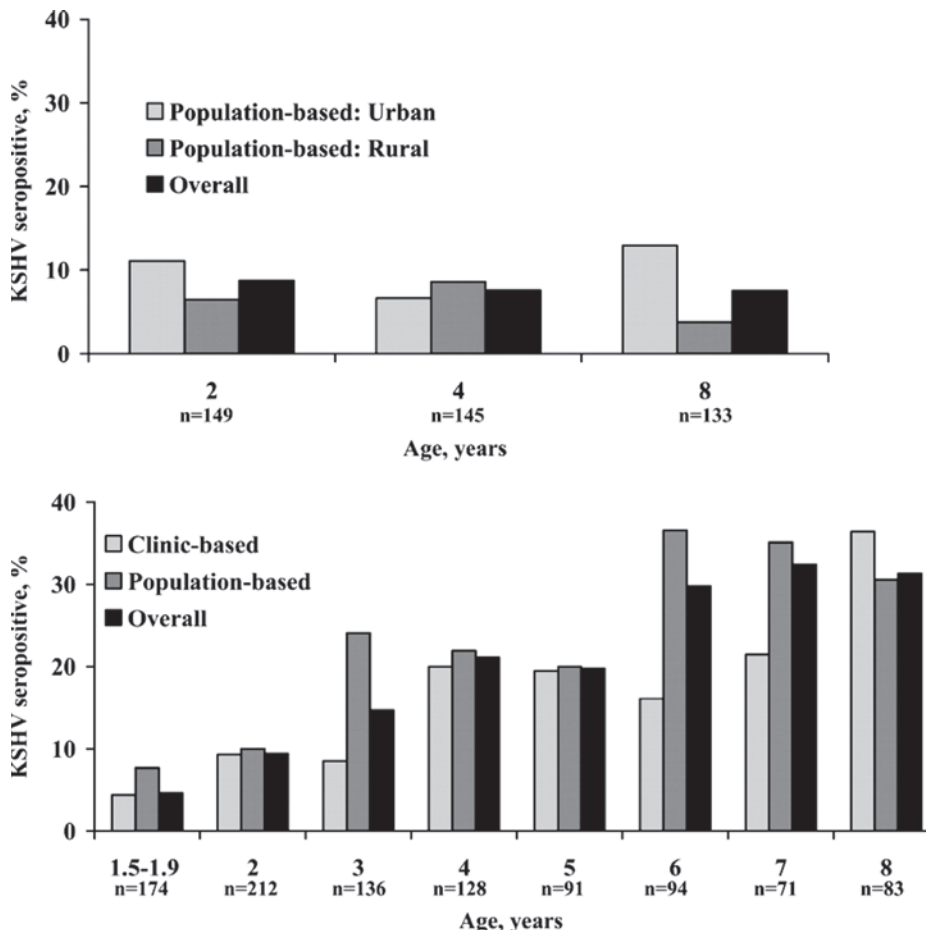


FIGURE 65.18. Kaposi's sarcoma–associated herpesvirus seroprevalence among children in South Africa (top) and Uganda (bottom) by age. The n values underneath bars refer to the overall number of children in each age group. (From Butler LM, Dorsey G, Hladik W, et al. Kaposi sarcoma-associated herpesvirus (KSHV) seroprevalence in population-based samples of African children: evidence for at least 2 patterns of KSHV transmission. *J Infect Dis* 2009;200:430–438, by permission of Oxford University Press.)

in China, where higher rates were found in the Uyghur, Mongolian, and Kazar ethnic groups, in which most of the cases of KS occur, as compared to the Han people.⁶⁸⁵

Routes of Transmission

KSHV has been found in maternal saliva and in breast milk in endemic areas, arguing for an oral/salivary route of transmission in African children.^{71,166,432,454} Analysis of a cohort of Italian patients with classic KS and their first-degree relatives showed that infection was significantly higher than in healthy controls. These relatives, which included spouses' siblings and offspring, were found to contain viral DNA in their saliva, indicating that close contact contributes to the spread of KSHV and that this spread can occur via nonsexual routes, although sexual transmission in the spouses could not be excluded.⁴³⁴

In contrast to endemic areas (Africa and the Mediterranean Basin), in western Europe and the United States, which are zones of low prevalence (seropositivity 1%–7%), infection is generally acquired sexually; prepubertal children are uninfected, whereas infection rates rise in adulthood, coinciding with sexual activity. Sexual transmission occurs efficiently among men who have sex with men, where seroprevalence can range from 25% to 60%.^{127,228,326,445,608,618} In this group, there is a clear linkage to infection and the number of sexual partners.⁴⁴² Seroprevalence rates tend to be lower among women than men,³²⁴ although rates of infection among female attendees of sexually transmitted disease clinics and commercial sex workers are elevated,^{96,164,304,326} suggesting that heterosexual transmission to females occurs. The finding that virus is consistently detected in saliva,^{525,669} and only sometimes in genital secretions,^{88,177,525,527} favors an important role for salivary virus-mediated sexual transmission.

The possibility of blood-borne transmission through blood transfusion has been witnessed, and there is evidence that it may

occur, albeit rarely. One prospective observational cohort study of transfusion recipients was conducted in Uganda, where the seroprevalence among blood donors was very high, leukocyte reduction was not used, and blood storage time was usually short. In this study, an estimated 12 of the 425 patients who received KSHV-seropositive blood were infected by transfusion. Other studies have confirmed that in some settings, transfusion may lead to acquisition of KSHV, and a couple of case reports have documented transfusion-associated KS.^{3,515,685} In contrast, a study in the United States that evaluated a historical cohort of participants in the Transfusion-Transmitted Viruses Study showed that there was no statistically significant difference in KSHV seroconversion in the transfusion and nontransfusion groups.⁹⁷ The question of whether organ transplants should be screened has also been raised. Although the incidence of KS is highly increased in organ transplant recipients, this is mostly attributable to reactivation as a consequence of immunosuppressive therapy. However, molecular studies have documented that the KSHV originated from the donor in some instances.⁴²⁴ Thus, some investigators have advocated for screening for organ transplantation and for blood transfusion in endemic areas; however, there are no formal guidelines.

Morbidity and Mortality

The major disease caused by KSHV is KS, and the incidence of this disease globally varies widely (Fig. 65.19). However, KS is now one of the most common cancers in many subequatorial African countries, where it is associated with significant morbidity and mortality,⁶¹¹ both of which increase when individuals are co-infected with HIV. KS incidence is 1 in 100,000 in the general population, whereas in HIV-infected individuals, the incidence is around 1 in 20,^{222,699} increasing to almost 1 in 3 in HIV-infected gay men prior to the introduction of highly active antiretroviral treatment (HAART).⁴⁶ KSHV can be detected in



FIGURE 65.19. Cutaneous lesions of Kaposi's sarcoma (KS). Upper left: Leg of human immunodeficiency virus (HIV)-negative patient with patch lesions. Lower left: Multiple nodules in arm of an individual with acquired immunodeficiency syndrome. Right: HIV-infected individual with extensive involvement of lower extremities by KS. (Courtesy of Dr. Susan Krown.)

PBMCs of HIV-infected individuals with KS.⁶⁹⁹ Individuals who acquire KSHV infection with pre-existing HIV infection have a significantly higher risk of developing KS; almost one in two develops KS, indicating that in this setting, KSHV is one of the most oncogenic viruses in humans.⁴⁴⁴ This evidence suggests that an already damaged immune system fosters a higher KSHV load, with subsequent KS development. Geographic areas where KS was endemic before the AIDS epidemic have seen a dramatic increase in the incidence of KS. In countries such as Uganda and Zimbabwe, the incidence of KS has increased about 20-fold, such that it is now the leading cancer in men and the second leading cancer in women. One study estimated close to 60,000 cases in sub-Saharan Africa, with an estimated age-adjusted survival for KS of only 12%.⁵¹⁹ Although the incidence of AIDS-KS in the Western world has declined since the widespread implementation of HAART, it remains increased compared to the pre-AIDS era and now occurs in the presence of high CD4 counts and low HIV viral load.^{350,452} For example, KS rates in the San Francisco area for white men were 0.5 per 100 000 people per year in 1973 (pre-AIDS), more than 30 during 1987–1991 (pre-HAART), and then declined to 2.8 in 1998 (post-HAART).¹⁹⁷

Origin and Spread of Epidemics

KS has been grouped into four major epidemiologic forms²²²: classic KS affecting elderly men of Mediterranean or eastern European Jewish ancestry; endemic KS, existing in parts of central and eastern Africa, described long before the HIV pandemic and often affecting children with disseminated lymphadenopa-

thy^{48,507,616}; iatrogenic KS, developing in immunosuppressed individuals, such as after an organ transplant or for autoimmune disorders⁶⁰⁶; and epidemic or AIDS-KS, a major AIDS-defining malignancy. In the Western world, AIDS-KS predominantly affects HIV-infected gay men. However, in Africa, since the spread of HIV, epidemic KS has become more common in both genders, with a lowering of the male to female ratio.⁶⁷³ The incidence of childhood KS has risen more than 40-fold in the era of AIDS in endemic regions.⁷⁴⁶

Lymphadenopathies linked to KSHV infection, namely PEL and MCD, are much more common in HIV-infected individuals. Although MCD has been reported in Africa, KSHV-associated lymphomas appear to be extraordinarily rare in regions that are endemic for KSHV, even in individuals with HIV infection.²⁰¹ However, it is possible that these cases are currently under-reported.

Prevalence and Seroepidemiology

Strong epidemiologic studies suggest that KSHV infection is absolutely required for the development of KS. Studies that utilized the measurement of antiviral antibodies demonstrated that these antibodies are made during primary infection. However, because primary infection gives way to latent infection in the host, seropositivity is a marker for not only past exposure but also the presence of ongoing viral latent infection.

Like the disease incidence, the prevalence varies globally (Fig. 65.20). Individuals with KS have higher anti-KSHV antibody titers than individuals without KS; thus, the overall seroprevalence may be underestimated. However, despite this

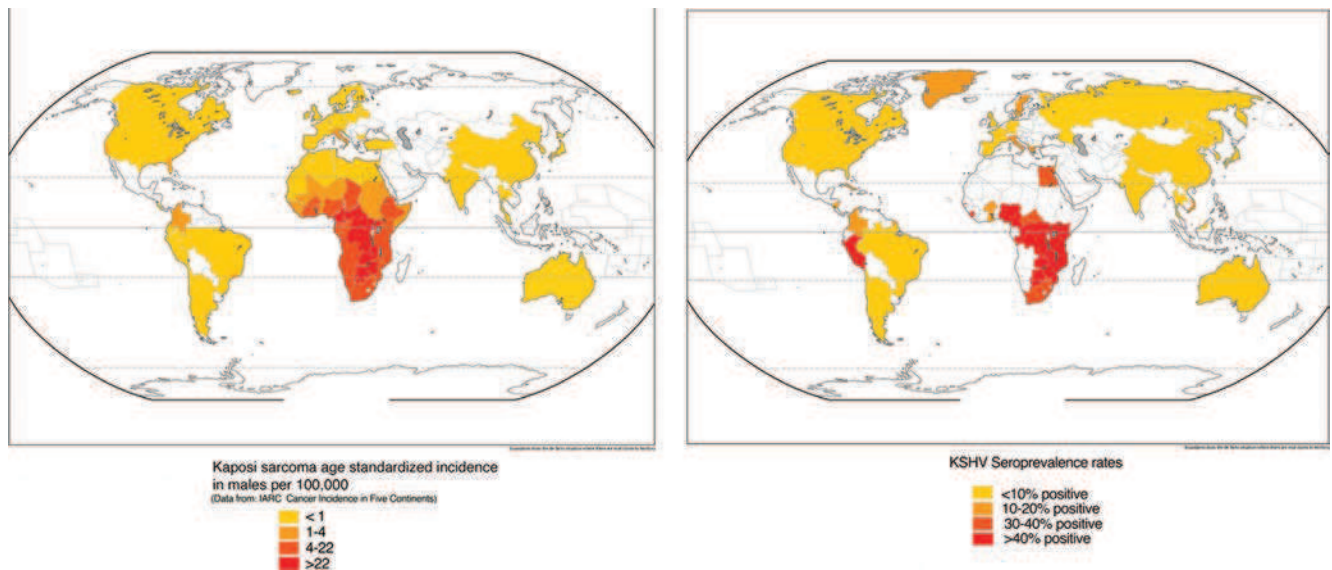


FIGURE 65.20. Geographic prevalence of Kaposi's sarcoma (KS) and seroprevalence of KS-associated herpesvirus. The standardized incidence of KS is depicted for males, and data were obtained from the International Agency for Research on Cancer's Cancer Incidence in Five Continents series.¹⁵⁸ The incidence provided for the United States is an average; however, in states including California, New York, Georgia, and the District of Columbia, it can be as high as 6 per 100,000 among some subpopulations, being particularly high among African Americans. According to the National Cancer Institute's Surveillance Epidemiology and End Results (14 registries), the overall incidence of KS in the United States is as follows: non-Hispanic whites, 0.8; white Hispanics, 1.4; blacks, 2.4. In Italy, incidence also varies by region, ranging from 0.2 to 2.2. Incidences of KS in Africa are from Globocan 2002.⁵²⁰ Seroprevalence rates were compiled from multiple publications; when different values from the same country were reported, an average was taken. Values represent those in the general population, usually blood donors. Cohorts comprising human immunodeficiency virus–infected individuals were excluded in the depiction.

caveat and some regional exceptions, there is a strong concordance between KSHV seroprevalence rates and incidence of KS (see Fig. 65.20). Different tests have been used to determine seroprevalence, and these are variably reliable, with some assays overestimating, and others underestimating, the true prevalence. Therefore, some studies are difficult to interpret. Because KSHV encodes multiple latent and lytic antigenic proteins,¹¹⁷ assays have variably targeted these antigens. The earlier studies utilized immunofluorescence using latently infected PEL cells, or PEL cells treated with TPA to induce lytic replication.^{228,326,380,608} Newer-generation methods rely on enzyme-linked immunosorbent assay (ELISA) using recombinant proteins or peptides, most frequently LANA, which is the major latently expressed antigen, or the lytic antigens K8.1 and ORF65. Usually, testing for both LANA and at least one lytic antigen is encouraged, because infected subjects may have detectable antibodies to one or the other^{51,469} and develop seroreactivity to these antigens at different times.

Nearly all KS patients are seropositive for KSHV with current testing techniques. Prospective studies suggest that KSHV infection predates AIDS-KS development and is a strong predictor of KS risk. Fifty percent of men infected with both HIV and KSHV, who received no effective treatment for either agent, developed KS within 10 years.⁴⁴² The association of KSHV infection with KS is also evident from the fact that KSHV viral genomic DNA and viral antigens are present in all cases of HIV-positive or HIV-negative KS.^{294,476} These data indicate that KSHV is the etiologic agent of KS and that KSHV is the *sine qua non* of KS—patients who do not have KSHV infection will not develop KS. Utilization of ganciclovir as a treatment for AIDS patients at high risk for KS reduces KS incidence and further bolsters this conclusion.⁴⁴⁰

Geographic Distribution and Genetic Diversity

KSHV infection is found at variable rates worldwide, with rates lowest in western Europe and America, and highest in Africa.^{164,441,593,701} In Europe, however, KSHV is more prevalent in the Mediterranean region (e.g., Italy and Spain).^{223,700} KSHV isolates from different regions of the world show differences that help to explain KSHV evolution and its relationship with its human host. As described in Figure 65.3, large blocks of KSHV sequence are highly conserved and do not exhibit much variation from different isolates. However, some genomic regions in the virus display remarkable sequence variability, which makes them useful as markers for strain diversity and to track epidemiologic spread. These include regions surrounding the internal sequence repeats and the coding regions directly adjacent to the left- and right-hand TRs—namely, ORFs K1 and K15, respectively. The biochemical functions of these transmembrane proteins were discussed earlier, and here we discuss their sequence variability in the context of KSHV evolution.

ORF K1 is a transmembrane protein whose N-terminal cysteine-rich ectodomain is highly variable; amino acid differences in K1 between geographically disparate isolates range from 30% to 60% of the total residues in the K1 ectodomain^{751,752} (see Fig. 65.20). Importantly, the cysteine residues, N-linked glycosylation sites, and signaling motifs in the cytoplasmic tail are not changed, which reflects the important nature of these amino acids for K1 function. A vast degree of sequence variation occurs in the hypervariable regions of K1,

named V1 and V2, in which amino acids surge to 60%. It is assumed that host immune pressure drives this variability; however, another hypothesis is that this diversity might have been driven by the need to recognize highly polymorphic cell surface proteins.⁷⁵⁰ K1 sequence variation allows for the separation of KSHV isolates into four major subtypes: A, B, C, and D (Fig. 65.21). (These subgroup identifications are also supported by sequence variations in other genomic loci).^{750,751} It is important to note that the viral strains are stable in a given individual, as different samplings recovered from a single patient taken at various time points often yield identical sequences. The A and C subgroups are viral isolates from KSHV-infected subjects in Europe, the United States, the Middle East, and Asia; subgroup B strains are viral isolates from sub-Saharan Africa; and type D strains are found primarily in Australia, southern Asia, and the Pacific islands. These data indicate that KSHV is an ancient human infectious agent that entered the human population at or around the time that modern humans arose in Africa in the early Pleistocene Age.²⁸⁰ The diversification of the main branches (A/C, B, and D) is thought to occur from isolation and founder effects associated with proposed migrations of humans out of Africa: the first (~100,000 years ago) disseminating subtype B through sub-Saharan Africa, the second (~60,000 years ago) spreading subtype D to southern Asia and onto Australia and the Pacific islands, and the third (~35,000 years ago) carrying subgroups A and C to Europe and northern Asia and onto the Americas.⁷⁵⁰ Alternative interpretations of these findings exist as well but seem less likely.⁴⁵⁸ The clustering of subtypes with ethnic and geographic populations also implies that the virus is primarily transmitted in a familial pattern (e.g., vertically from parent to child and horizontally among siblings), with wider horizontal transmission being less efficient. Of course, in the context of the AIDS epidemic, viral spread occurred by a predominantly sexual route.

There is additional sequence diversity at the right-most end of the viral coding region—the K15 coding sequence. Here, the A and C subgroups (and only rare B isolates) display two allelic variants, termed P (for prototype) and M (for minority). The K15 alleles show large sequence differences that sometimes can extend into the adjacent *ORF75* gene (and sometimes up to 20 kb from the right-hand TRs).^{542,750} A functional *ORF K15* gene is preserved in both P and M alleles. The P and M K15 isolates only share 33% amino acid identity. This extensive sequence divergence is consistent with the notion that the allelic variants were generated from recombination events with an unknown progenitor herpesviral genome during the course of evolution. The fact that both M and P alleles exist among KSHV subgroups A and C suggests that this recombination event likely occurred after the first two waves of emigration from Africa, thus allowing for dissemination of these alleles during subsequent emigration to the Middle East and northern Europe.⁷⁵¹ More recently, additional alleles (N and Q) at the right-hand end of the genome have been identified.⁷⁵⁰

The impact of the different strains in pathogenesis has been analyzed in different defined geographic areas, mostly based on K1 sequencing. One study in Brazil showed a significant predominance of A genotypes in KS lesions from HIV-positive patients, whereas C genotypes were found mostly in the HIV-negative setting.⁵⁵³ In China, subtype A was significantly

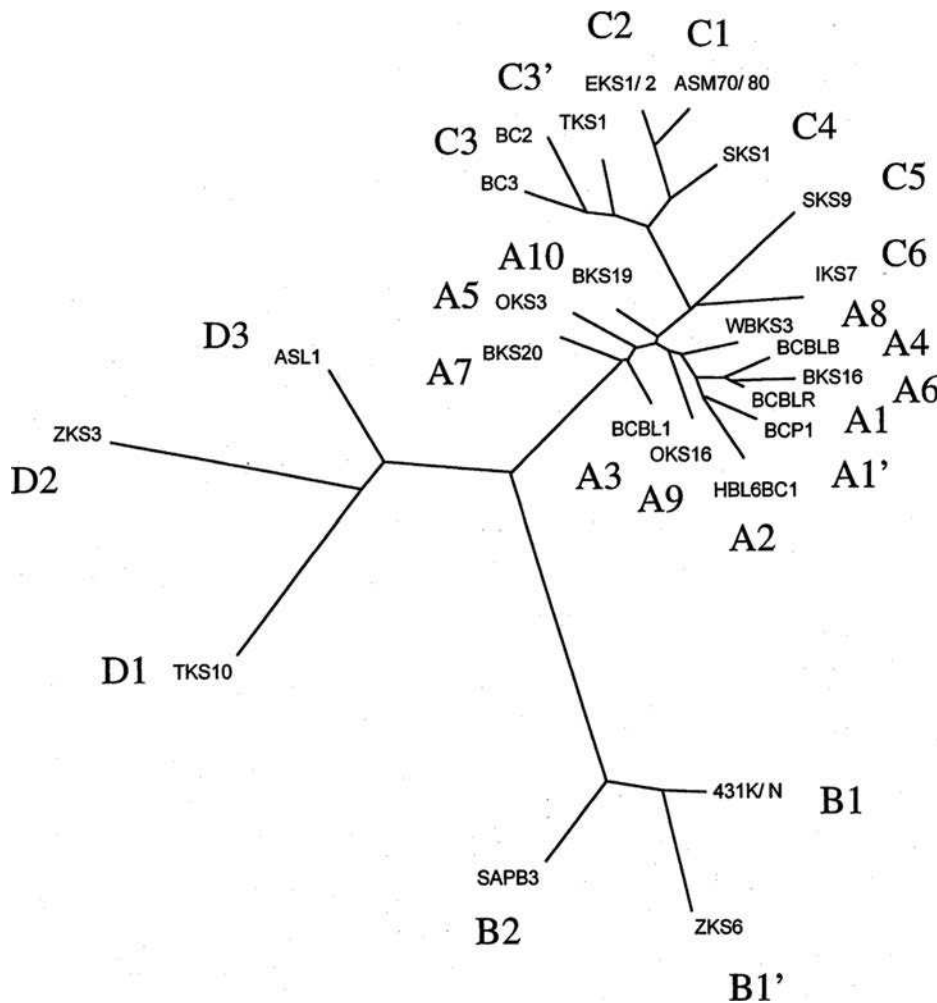


FIGURE 65.21. Clades of Kaposi's sarcoma-associated herpesvirus based on open reading frame K1 sequence differences. A and C clades cluster together and are typically found in Europe and the United States. B clade isolates are from sub-Saharan Africa, and D clades are from Australia and the Pacific Islands. The subgroups within each clade are denoted by numbers following the clade letter (e.g., C1-6). Many of the isolates shown here are from commonly used primary effusion lymphoma cell lines, including BC-1, BC-3, and BCP-1. (From Zong JC, Ciufo DM, Alcendor DJ, et al. High-level variability in the ORF-K1 membrane protein gene at the left end of the Kaposi's sarcoma-associated herpesvirus genome defines four major virus subtypes and multiple variants or clades in different human populations. *J Virol* 1999;73(5): 4156–4170, with permission.)

more frequent in mucosal KS lesions than subtype C.⁷³⁴ One study in Italy showed that patients with rapid progression of KS and higher blood viral loads contained type A KSHV, whereas C type was mainly present in slowly progressing patients.⁴³³

Co-Factors

Although KSHV infection is necessary for KS to develop, it is not sufficient and co-factors have been identified. Globally, the presence of antibodies to KSHV is much more common than KS, indicating that on average only about one in ten thousand infected people get the disease (see Fig. 65.20). Clearly, the most important co-factor is HIV infection. It is not yet evident if immunodeficiency itself is the main determinant of KS or whether HIV has a more direct role. Individuals with iatrogenic immunosuppression, particularly renal transplant patients, also have an increased risk for KS, although not as great as that seen with HIV infection. This may reflect differences in KSHV infection rates, rather than HIV-specific causes, or differences in immune dysfunction; however, a direct role for HIV as a co-factor has not been excluded.

Potential contributions from host genetic factors have been reported, including genetic polymorphisms of inflammatory and immune-response genes. Classic KS risk is associated with diplotypes of interleukin-8 receptor β (IL8RB), IL-13,⁷⁶

and certain HLA haplotypes.^{154,185} Transplant KS risk is associated with an IL-6 promoter polymorphism,²³² and genotypes of Fc γ RIIIA may influence the development of KS in HIV-infected men.³⁷⁷ Thus far, these association studies have only shown a relatively small overall increased risk and have not included very large patient cohorts.

Environmental exposures have also been evaluated and found to have an effect on KSHV seroprevalence or incidence of KS. These include regional variations associated with certain volcanic soils, arthropod bites, and living in rural areas.^{532,553,734,745}

These data suggest that common host genetic variants, in addition to co-infections, environmental factors, timing, and possibly routes of infection, all contribute to the oncogenic outcome of KSHV infection.

CLINICAL DISEASE

Kaposi's Sarcoma

Clinical Features

Moritz Kaposi first described five patients with a hemorrhagic sarcoma of the skin and mucous membranes, but also involving internal organs, as appreciated during postmortem

examination.³²⁰ He described an aggressive tumor; however, this form was eventually recognized to be a rare, frequently indolent disease confined to the skin, mostly occurring in older men of Mediterranean and eastern European origin and referred to as sporadic or classic KS. An endemic form of KS was recognized and described by the early 1960s, occurring in sub-Saharan Africa.⁴²⁷ Although African KS was histologically indistinguishable from the sporadic form, it tended to be more aggressive, and a lymphadenopathic form was documented to occur in children.⁶¹⁶ Subsequently, patients receiving immunosuppressive agents, such as solid organ transplant recipients, were also found to develop KS, which has been designated as iatrogenic KS.²⁷⁸ With the advent of AIDS, KS was found to occur with greatly increased frequency, therefore referred to as epidemic KS. In the context of HIV infection, KS was reported to have a much more aggressive clinical behavior, with frequent dissemination to internal organs.²¹⁶

KS can have a variable clinical presentation, ranging from limited to fulminant disease, where generally it tends to be more indolent in sporadic KS and more aggressive in Africans and in individuals with AIDS. However, in all epidemiologic subtypes, there is a range and overlap in clinical presentation (with the exception of pediatric KS that only occurs in Africa, with very rare exceptions). Cutaneous lesions are most common in the lower extremities, face, and genitalia but can appear anywhere¹³⁶ (see Fig. 65.19). In the context of HIV infection, the appearance of cutaneous KS lesions in visible areas may reveal AIDS and carry a heavy psychosocial burden because of existing stigma. Cutaneous KS lesions are typically multifocal and have the appearance of papules, patches, plaques, or nodules, and there can be fungating lesions and tumors. Among these, the patch lesions are considered to be of an early stage, and other forms are considered more advanced. However, whereas these definitions are useful as dermatologic descriptors, in practice these distinctions frequently do not reflect disease progression; several forms can appear simultaneously. There can be patch-type lesions comprising large areas or nodules with limited disease. Lymphedema may be extensive. KS can also involve noncutaneous sites. Particularly common is involvement of the oral cavity, which can be the initial site of presentation. KS can also affect practically all internal organs, most frequently the gastrointestinal tract, lungs, and lymph nodes. In children, lymph node involvement signifies a poor prognosis subset, whereas in adults, focal lymph node involvement is quite common and does not signify the same poor prognosis as other visceral disease.⁴⁸⁵ A clinical classification scheme was proposed for AIDS-related KS by the AIDS Clinical Trials Group (ACTG) Oncology Committee.^{351,352} This staging system incorporates extent of tumor, where T0 (good risk) is when KS is confined to skin and/or lymph nodes and/or minimal oral disease (i.e., non-nodular KS confined to the palate), and T1 (poor risk) is when there is any of the following: tumor-associated edema or ulceration, extensive oral KS, gastrointestinal KS, or KS in other non-nodal viscera. Other criteria in this classification are severity of immunodeficiency (I0 and I1 with a cutoff of <200 CD4 cells per cubic millimeter) and presence of systemic symptoms. This classification was validated prospectively in the pre-HAART era³⁵²; the study found that the combination of poor immune response and tumor stage most accurately predicted survival. However, more recently, a prospective evaluation of the ACTG staging system conducted in the HAART era showed that the

combination of poor tumor stage and systemic illness identified patients with unfavorable prognosis.⁴⁹⁴

Early lesions can be mistaken clinically for purpura, hematomas, hemangiomas, and dermatofibromas, thus a biopsy is necessary, especially when lesions that are not entirely typical of KS occur. It is important to rule out mimics, such as bacillary angiomatosis and angiosarcoma when KS is associated with systemic symptoms. Other diseases that can be confused with KS clinically include lymphangioma, hemangioendothelioma, hemangiopericytoma, and pyogenic granuloma.

Laboratory Diagnosis

Diagnostic confirmation of KS is done through histopathology. At present there are no blood tests for this disease, mostly because viremia is highly variable, and seropositivity does not necessarily indicate a diagnosis of KS. The histologic features of KS are essentially indistinguishable in the four clinical-epidemiologic forms. In skin, it affects the dermis, and similar to the clinical appearance of cutaneous KS, there is a range in the cellularity and microscopic appearance of the lesions. Lesions are characterized by the presence of jagged, thin-walled, and poorly formed dilated vascular spaces with large endothelial cells that may protrude into the lumen (Fig. 65.22). Between these vascular spaces is a spindle cell proliferation that can range from sparse to significant, sometimes forming sheets and extending irregularly in various directions. These spindle cells are considered to be the KS tumor cells. The elongated nuclei vary in size, and some are atypical, thus resembling a fibrosarcoma. However, the presence of narrow irregular, angulated slits containing erythrocytes among the spindle-shaped cells is a distinguishing feature of KS. These can be intertwined with normal vascular channels as well as lymphatic channels, some of which may show considerable cystic dilatation. There is a variable but consistently present inflammatory mononuclear cell infiltrate composed of lymphocytes, plasma cells, and some macrophages. Red blood cells and hemosiderin pigment are frequently seen, often extravasated between the spindle cells. Small granules of intracytoplasmic or extracellular hyalin material may be identified. Granules of hemosiderin can also be seen within the spindle-shaped cells as a result of phagocytosis.^{79,144} Confirmation of a diagnosis of KS is most commonly done by immunohistochemistry in formalin-fixed paraffin embedded tissues using commercially available monoclonal antibodies to the KSHV LANA (ORF73). Because this protein is essential for viral episome maintenance, it is expressed by all KSHV-infected cells, at least in theory. Using immunohistochemistry for LANA, KSHV is detected in a variable proportion of the spindle cells and endothelial cells lining the vascular spaces (see Fig. 65.22). KSHV can also be detected by PCR in KS biopsies.

Clinical Course

The clinical course of disease can be variable. It can be stable, have marked exacerbations, or even regress, especially in post-transplant patients where immunosuppressive therapy can be adjusted and in HIV-infected individuals who begin antiretroviral therapy. The latter is likely owing to the improvement of immune reconstitution against KSHV,⁶¹ and possibly also attributable to decreased circulating HIV-associated proangiogenic and inflammatory cytokines.²²¹ Interestingly, in some patients, KS may progress dramatically, and even be fatal, upon treatment with antiretrovirals owing to an immune reconstitution

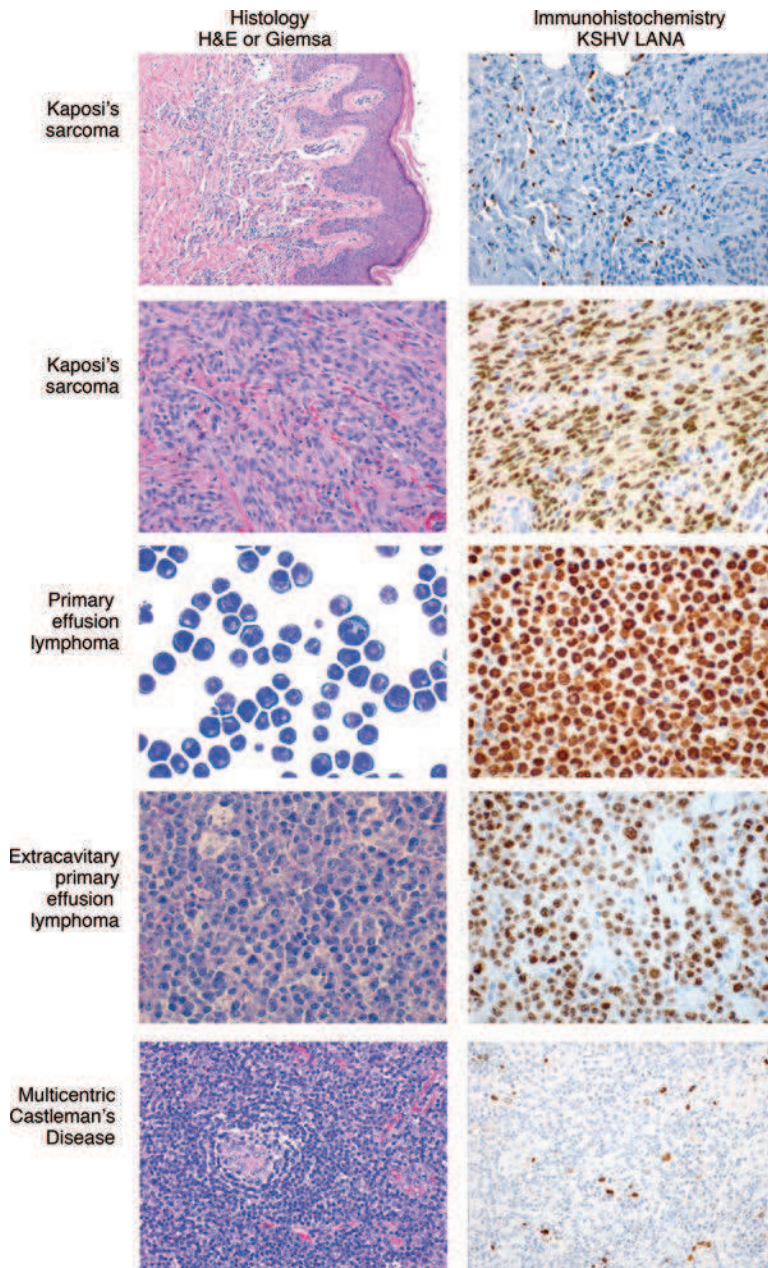


FIGURE 65.22. Histologic features of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). Skin biopsies of two different patients with KS are shown, illustrating the spindle cell proliferation and presence of irregular vascular spaces. **Left columns** are hematoxylin and eosin (H&E) staining of formalin-fixed, paraffin-embedded tissues, and the **right column** shows immunohistochemical (IHC) staining for latency-associated nuclear antigen (LANA), demonstrating broad variability in the number of infected cells. The third row shows a cytospin preparation stained with Giemsa of a PEL on the left, and IHC for LANA on the right, performed on a PEL cell block. The fourth row shows H&E staining of a biopsy from a patient with a non-Hodgkin's lymphoma that was positive for KSHV LANA (**right**), therefore subclassified as extracavitary PEL. The lower row exemplifies a case of MCD, with scattered LANA-positive cells. Original magnification, left column rows 1–2: 20X and 40X, rows 3–4: 60X, row 5: 20X; right column rows 1–2 and 5: 40X, rows 3–4: 60X.

inflammatory syndrome (IRIS).^{64,147,155,379} Onset of IRIS-associated KS flare usually occurs between 3 weeks and 3 months following immunologic and virologic response to antiretrovirals. Early systemic chemotherapy can be effective in suppressing IRIS-associated flares.³⁷⁹

Treatment

HAART has reduced the incidence of AIDS-KS in developed countries and can induce AIDS-KS regression in some individuals. HIV protease inhibitors have been shown to display direct antitumor activity⁴⁷¹; however, non-protease-inhibitors containing antiretroviral combinations also induce KS regression.⁶⁶ However, KS remains a clinical problem with only half of patients achieving complete resolution even with successful antiretroviral therapy.⁴⁹⁹ In addition to HAART, isolated lesions are treated

with radiotherapy and, rarely, surgery. Systemic chemotherapy is useful for the treatment of disseminated disease. Options include doxorubicin, vincristine, bleomycin, and etoposide, as single agents or in combination, as well as paclitaxel and liposomal formulations of doxorubicin or daunorubicin. However, although these treatment modalities can be of great benefit, none is considered curative. Complete regression of cutaneous KS was reported in patients with transplant-associated KS when the immunosuppressive therapy was switched to rapamycin.^{91,627} Good response rates have been confirmed by other investigators in most patients with transplant KS (cutaneous and visceral) when rapamycin was used as the immunosuppressive regimen.^{265,366,748} Data are limited with regard to treatment with rapamycin in patients with KS in other risk groups²⁶⁰; thus, it is too early to know with certainty if the effects are owing to changes in immune response to KSHV

as a result of changing the immunosuppressive therapy or to direct effects on the KSHV-infected lesional cells. These possibilities are not mutually exclusive, and experimental evidence may indicate that both mechanisms contribute to these responses: (a) tumor regression, achieved by switch to rapamycin in two posttransplant KS liver recipients, was shown to correlate with the recovery and the maintenance of both CD8 and CD4 memory responses against KSHV lytic (K8.1) and latent (ORF73) antigens, although one patient who failed to recover CD4 responses to KSHV showed only partial remission³⁴; and (b) KSHV activates the mTOR pathway,^{619,678} and rapamycin is toxic to PEL cells.⁶¹⁰

Primary Effusion Lymphoma

Soon after the discovery of KSHV in KS, viral sequences were also identified in a unique type of lymphoma,¹⁰⁷ referred to as primary effusion lymphoma (PEL) or body cavity based lymphoma (BCBL), which led to the designation of the first cell lines as BC or BCBL. The first reported cases of malignant lymphoma occurring as body cavity effusions were described before the discovery of KSHV as AIDS-associated lymphohematopoietic neoplasms containing EBV and displaying an indeterminate immunophenotype.³³⁸ In this report of three cases, two were lymphomatous effusions, and a B-cell lineage was demonstrated by using DNA-based assays. Subsequent studies recognized that these lymphomatous effusions occurred relatively frequently in HIV-infected individuals.^{247,674} However, they were thought of as unusual AIDS-related lymphomas, and it only became clear that they represent a quite distinct clinicopathologic entity with the recognition of the presence of KSHV within them.⁴⁸⁷ As described earlier, the presence of KSHV in this subset of lymphomas allowed the development of cell lines that have been used as a tool for its propagation, as well as for serologic assays and viral purification. PEL contain many KSHV genomes, ranging from 40 to 80 copies per cell, a feature that has facilitated analysis of this virus.

PEL possess quite distinctive clinicopathologic features. Most importantly, they commonly present as lymphomatous effusions in body cavities, which are usually the pleural, peritoneal, or pericardial cavities, but can also occur in unusual sites, such as an artificial cavity related to the capsule of a breast implant or cerebrospinal fluid.^{470,575} Although they are more common in HIV-positive males, HIV-negative men and women can also develop PEL.^{487,575} Because some KSHV-negative lymphomas, such as Burkitt's lymphoma, can involve body cavities as lymphomatous effusions and KSHV-positive lymphomas can present as solid tissue masses, diagnostic criteria for PEL have been proposed.^{108,487,554} These criteria include immunoblastic-anaplastic large cell morphology (see Fig. 65.22), null cell phenotype (including the lack of B-cell-associated antigen and Ig expression in most cases), and B-cell genotype. The expression of CD138/syndecan-1²²⁰ and hypermutation of the Ig genes⁴⁴⁶ indicated that PEL are at a preterminal stage of B-cell differentiation. This assumption was confirmed by gene expression profiling of PEL,^{307,334,516} as these studies found that PEL resembled plasma cells and had a profile between multiple myeloma and EBV-associated immunoblastic lymphoma.

Lymphomas containing KSHV can also arise with the first presentation as a solid tissue mass with nodal or extranodal involvement, similar to other AIDS-related NHLs. Although some of these patients subsequently develop a lymphomatous effusion, others apparently do not. These cases may be diag-

nosed as diffuse large cell, immunoblastic, or anaplastic large cell lymphomas, in which the presence of KSHV in practically all tumor cells has been demonstrated^{101,113,172,202} (see Fig. 65.22). Most of these are immunoblastic in appearance and have a high mitotic rate and variable amounts of apoptotic debris. Because these lymphomas have some of the defining features of PEL, including a similar morphology, lack expression of B-cell antigens and Ig, and are frequently co-infected with EBV, they have been classified as a variant of PEL, referred to as either extracavitary PEL or solid PEL.

The almost invariable presence of KSHV in PEL suggests that it is necessary for the development of this disease. However, such as in the case of KS, infection by KSHV is necessary but clearly not sufficient, as PEL are very rare tumors, even in populations with high KSHV seroprevalence, accounting for about 3% of AIDS-related lymphomas and 0.4% of all AIDS unrelated diffuse large cell NHLs. Therefore, it is evident that KSHV infection represents only one of several events involved in the development of PEL. Another co-factor appears to be EBV, because many PEL contain both viral genomes. Analysis of the pattern of EBV gene expression in PEL revealed that only EBNA1 was expressed, corresponding to type I latency,^{289,645} and expression of at least one EBV miRNA (BART2) has been shown.^{505,717} PEL lack structural alterations in most cellular-transforming genes frequently involved in lymphomagenesis, with the possible exception of mutations in the regulatory region of BCL-6 (the relevance of which is unclear because PEL do not express BCL-6).^{98,487} The *p53* and *PTEN* genes are rarely mutated.⁵³⁰ Given the limited number of PELs, genome-wide evaluations have limited statistical power. Nevertheless, two fragile site tumor suppressor genes, *FHIT* and *WWOX*, were found deleted in most PEL cell lines.⁵⁷⁰ These observations suggest that KSHV is a critical element for the development of PEL, which is supported by the essential role of several latent viral genes in this disease.

Survival with conventional chemotherapy is very poor, with a median survival of approximately 6 months.^{487,607} Nevertheless, there are case reports of good outcomes with antiviral therapy using bortezomib or rituximab^{60,236,286,400,512,605} or pleurodesis with bleomycin.⁷²⁶

Multicentric Castleman's Disease

Castleman's disease is an atypical lymphoproliferative disorder that encompasses various clinicopathologic entities. There have been pathologic and clinical classifications, which overlap partially. Two distinct histopathologic subtypes were described prior to the identification of KSHV: the more common hyaline vascular type and the plasma cell type.³²⁸ Clinically, Castleman's disease can be localized, or the patient may have multiple enlarged lymph nodes, therefore called *multicentric Castleman's disease*. Approximately 90% of patients with MCD have the plasma cell type morphology. Systemic constitutional symptoms in MCD include fevers, malaise, wasting, hypoalbuminemia, cytopenias, and hyponatremia.⁶⁹² Soon after the discovery of KSHV, an association of this virus with MCD was described.⁶²⁵ There is a strong risk for these patients to develop malignancies, most commonly KS and NHL. Approximately half of MCD cases from immunocompetent individuals have detectable KSHV, whereas in individuals with HIV, almost all patients with MCD have KSHV in involved lymph nodes.

The presence of both KS and MCD in a single lymph node is not uncommon in HIV-positive patients. Notably, MCD, also called *multicentric angiofollicular hyperplasia* in early publications,

is characterized histologically by a vascular proliferation that is reminiscent of KS. Although KSHV has been reported in MCD with both hyaline vascular and plasma cell morphology,³⁶⁴ most cases have closer resemblance to the plasma cell type of MCD. One report indicated that the KSHV-positive cases showed the highest intensity of angiosclerosis and GC and perifollicular vascular proliferation, whereas plasmacytosis was found to be less pronounced than in the KSHV-negative cases of the plasma cell type.⁶³⁷ KSHV-positive cases most likely represent a distinct morphologic variant—resembling more the plasma cell type but additionally showing the presence of larger cells in the mantle zones, which have been called plasmablasts. These contain KSHV and are positive by LANA staining.¹⁹⁰ One study showed that KSHV-positive endothelial cells can also be found in MCD lymph nodes in both HIV-positive and -negative patients.⁷⁵ KSHV-infected B cells can be numerous, coalesce, and form microlymphomas or frank lymphomas. KSHV-infected plasmablasts are B cells that are monotypic but polyclonal, almost invariably expressing IgM lambda.¹⁸⁸ These infected cells are different from PEL cells in terms of B-cell differentiation in that they lack somatic hypermutation of the Ig genes, they express cytoplasmic Ig but lack expression of CD138, and variably express CD27.^{114,188} Therefore, it appears that even if both PEL and MCD originate from KSHV-infected pre-terminally differentiated B cells, infected cells in MCD arise from extrafollicular B cells that did not undergo a GC reaction, whereas PEL may originate from cells that have traversed the GC.

Lymphomas arising in KSHV-associated MCD have been described mainly as occurring in HIV-positive patients¹⁹⁰ and have also been called *plasmablastic lymphomas*. However, there is a type of lymphoma—also referred to as plasmablastic lymphoma and frequently occurring in the oral cavity—that is associated with EBV infection, lacking KSHV. Thus, the formal terminology for the KSHV-associated variant should be “lymphoma arising in KSHV-associated MCD” according to the World Health Organization (WHO) classification.⁵⁵⁴ These lymphomas differ from PEL in several ways. Lymphomas arising in KSHV-associated MCD are EBV negative, do not contain mutations in the Ig genes, and are thought to arise from naive IgM lambda-expressing B cells rather than terminally differentiated B cells. A different rare entity has also been reported, called *germinotropic lymphoproliferative disorder*, in which GC B cells are co-infected with EBV and KSHV.¹⁸⁷

Systemic symptoms in MCD are believed to be a result of production of excess cytokines—in particular, IL-6—and probably both the cellular and viral versions of this cytokine contribute to pathogenesis. vIL-6 has been demonstrated to be expressed in MCD in scattered plasmablasts surrounding the lymphoid follicles.^{93,522,628} Lytic antigens are also expressed more frequently in KSHV-infected cells in MCD than in other disorders associated with this virus, indicating that lytic viral replication may be a feature of MCD.³²² It is possible that MCD falls in the spectrum of KSHV-associated lymphadenopathies, as KSHV-infected cells can be found in lymph node biopsies in patients with HIV-associated lymphadenopathy that lack all the defining pathologic characteristics of MCD.¹¹⁵ Furthermore, an inflammatory syndrome with symptoms very similar to those of MCD has been described in patients with HIV and KS but without a pathologic diagnosis of MCD,⁶⁵⁹ and high serum levels of vIL-6 have been shown in similar patients, suggesting that at least some of the symptomatology of MCD is attributable to this viral cytokine.^{281,511,659}

Median survival of HIV-associated MCD in the pre-HAART era was approximately 14 months.⁵¹³ Various approaches have been used for the treatment of patients with MCD, including cytotoxic chemotherapy, with variable success.⁶³ Evidence of lytic viral expression has led to testing of ganciclovir or other antiviral therapy, with mixed results.^{106,149} The best responses have been obtained with the advent of two different antibody therapies. A humanized antihuman IL-6 receptor monoclonal antibody (tocilizumab) has been reported to result in clinical responses in two studies of patients in Japan; however, the patients did not have AIDS, and only two patients were positive for KSHV.^{503,624} Larger studies have used the humanized monoclonal anti-CD20 antibody rituximab, which has resulted in successful treatment with responses in approximately 70% of patients.^{47,65,149,233} The mechanism of action is not very clear, because KSHV-infected cells in MCD frequently lack expression of CD20.^{114,493} The use of rituximab monotherapy for good performance status patients without organ involvement, and rituximab with chemotherapy for more aggressive disease has been recommended.⁶³ A recent report demonstrated that targeting lytic proteins ORF21 and ORF36 with valganciclovir and zidovudine, respectively, showed very promising results.⁶⁵⁸

PREVENTION AND CONTROL

Prevention of KSHV infection is challenging, especially in endemic areas where the prevalence rate is high. Transmission occurs mainly through saliva within families. Immunization would be ideal; however, to date, no candidate vaccines have been developed. As with EBV, it is difficult to use live attenuated viruses because of the establishment of persistent infection and the potential danger of malignancy development.⁷¹⁵ So far, the clearly successful approach in individuals with HIV has been the use of antiretroviral therapy, which has led to a marked decrease in the incidence of KS, at least in Western countries. This decrease in the incidence of KS is believed to be attributable to improvement in immune function, although a direct role on KS or KSHV has also been proposed.²²⁵ Several reports have shown regression of AIDS-KS following protease inhibitor-based therapy, suggesting that this approach may be better for patients with AIDS and a high risk of KS than the use of nucleoside or nonnucleoside reverse transcriptase inhibitors.^{365,443,708} However, one relatively small prospective study found that both regimens with or without protease inhibitors can decrease KSHV viremia and result in clinical improvement of KS, which occurred more commonly in patients who had undetectable or very low HIV loads.²³⁷ Because a direct effect of HIV infection in KS pathogenesis has been proposed (e.g., owing to paracrine angiogenic effects of Tat), it could be argued that control of HIV infection itself may be partly responsible for the beneficial effects of antiretroviral therapy.^{20,33} Nevertheless, there is evidence that protease inhibitors have a direct role in KSHV. In particular, nelfinavir has been found to inhibit KSHV replication using a recombinant virus assay at concentrations that are achieved in plasma with standard oral dosing.²²⁴ This observation warrants prospective preventive trials of antiretroviral regimens that include nelfinavir in individuals with HIV infection with a high risk of KS, such as those in endemic areas.

Prevention of KS may also be possible in high-risk individuals using inhibitors of lytic replication, such as valganciclovir,

which has been found to reduce KSHV shedding.¹⁰⁵ One study, in which ganciclovir was used for the treatment of CMV retinitis in patients with HIV, showed that the incidence of KS was markedly reduced by 75%.⁴⁴⁰ Although oral valganciclovir did not show beneficial effects on HIV-negative patients with established KS, it appears that compounds that inhibit KSHV lytic replication may have a role in preventing the spread of KSHV and the development of KS.

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Poxviridae

Classification

Virion Structure

- Morphology
- Chemical Composition
- Genome
- Polypeptide Components

Cell Entry

- General Considerations
- MV Entry
- EV Entry
- Superinfection Inhibition
- Cell Entry and Signaling Receptors

Gene Expression

- Programmed Expression of POXV Genes
- Regulation of Early-Stage Transcription
- Early-Stage Promoters and Termination Signals
- Enzymes and Factors for Early-Stage Transcription
- Regulation of Intermediate-Stage Transcription
- Intermediate-Stage Promoters
- Enzymes and Factors for Intermediate-Stage Transcription
- Regulation of Late-Stage Transcription
- Late-Stage Promoters and RNA Processing Signal
- Enzymes and Factors for Late-Stage Transcription

DNA Replication

- General Features
- DNA Replication Models
- Enzymes Involved in DNA Precursor Metabolism
- Viral Proteins Involved in DNA Replication
- Concatemer Resolution
- Homologous Recombination

Virion Assembly

- Overview
- Formation of the Crescent and IV Membrane
- Association of Core Proteins With IVs
- Genome Packaging
- Packaging of the Transcription Apparatus
- Removal of the D13 Scaffold From IVs
- Proteolytic Processing of Virion Proteins
- Intramolecular Disulfide Bond Pathway
- Occlusion of MVs

Formation of the EV

- Overview
- Wrapping
- Intracellular Movement and Exocytosis
- Actin Tail Formation

Virus–Host Interactions

- Tropism and Host Range
- Effects on the Cytoskeleton
- Effects on Host Macromolecular Synthesis
- Interactions With the Ubiquitin-Proteasome System
- Stimulatory Effects on Cell Pathways

Viral Defense Molecules

- Overview
- Extracellular Defense Proteins
- Intracellular Defense Proteins

Adaptive Immune Response to Poxviruses

Antivirals

Expression Vectors

The *Poxviridae* comprise a fascinating family of complex DNA viruses that replicate entirely in the cytoplasm of vertebrate or invertebrate cells. Two members of the family, variola virus (VARV) and molluscum contagiosum virus (MOCV), are obligate human pathogens, and others, such as monkeypox virus (MPXV), can be transmitted from animal hosts to humans and cause severe disease. VARV was the cause of smallpox, a once common and highly lethal disease that altered human history. Smallpox was eradicated in 1977 through a dedicated effort spearheaded by the World Health Organization, nearly two centuries after the introduction of highly effective prophylactic inoculations with cowpox virus (CPXV) and vaccinia virus (VACV). In addition to obvious public health value, vaccination contributed to present concepts of infectious disease and immunity. From a historical perspective, VACV was the first animal virus seen microscopically, grown in tissue culture, accurately titered, physically purified, and chemically analyzed. Moreover, a once prevalent view of virus particles as packets of nucleic acid was revised following the discovery of RNA synthetic activity in purified poxvirus (POXV) virions. This finding sparked investigations that led to the discovery of transcriptase and reverse transcriptase activities in RNA viruses and to the elucidation of structural features of viral and eukaryotic messenger RNA (mRNA), including the 5' cap and 3' poly(A) tail. Recombinant DNA technology

eliminated obstacles to working with such large viruses, and considerable progress has been made in elucidating the cycle of virus replication. Discoveries of virus-encoded proteins that affect cell growth and modulate immune defense mechanisms continue to provide new insights into virus–host relationships. In addition, the development of POXVs as live recombinant expression vectors provides a powerful tool for immunologists and biochemists as well as an alternative approach to the development of vaccines against a variety of infectious agents and cancer.

CLASSIFICATION

Distinguishing properties of the family *Poxviridae* include a cytoplasmic site of replication and a large complex enveloped virion, which contains enzymes that synthesize and modify mRNA and a genome composed of a single linear double-stranded DNA molecule of 130 to 300 kilobase pairs (kbp)

with a hairpin loop at each end. POXVs are divided into the subfamilies *Chordopoxvirinae* and *Entomopoxvirinae*, based on vertebrate and insect host range (Table 66.1). DNA sequencing and bioinformatic analysis confirm the genetic relationship between the POXV subfamilies and further suggest that POXVs are distantly related to *Asfarviridae*, *Ascoviridae*, *Iridoviridae*, *Phycodnaviridae*, *Mimiviridae*, and *Marseillvirus*.³⁹⁸

The subfamily *Chordopoxvirinae* consists of nine genera—*Avipoxvirus* (10 species), *Capripoxvirus* (3 species), *Cervidopoxvirus* (1 species), *Leporipoxvirus* (4 species), *Molluscipoxvirus* (1 species), *Orthopoxvirus* (9 species), *Parapoxvirus* (4 species), *Suipoxvirus* (1 species), and *Yatapoxvirus* (2 species)—and 2 unassigned species (Table 66.1). Members of the same genus are genetically and antigenically related and have a similar morphology and host range. The orthopoxviruses (OPXVs) have been studied most intensively. The species names frequently refer to the host from which it was first isolated rather than the important reservoir species (e.g., rodents are reservoirs for

TABLE 66.1 Family *Poxviridae*

Subfamily	Genus	Species ^a	Features
Chordopoxvirinae	<i>Avipoxvirus</i>	<i>canarypox</i> , <i>fowlpox</i> ^b (FWPV), <i>juncopox</i> , <i>mynahpox</i> , <i>pigeonpox</i> , <i>psittacinepox</i> , <i>quailpox</i> , <i>sparrowpox</i> , <i>starlingpox</i> , <i>turkeypox</i>	DNA ~270 kbp, G + C ~35%, birds, arthropod transmission
	<i>Cervidopoxvirus</i>	<i>deerpox</i>	DNA ~170 kbp
	<i>Capripoxvirus</i>	<i>goatpox</i> , <i>lumpy skin disease</i> , <i>sheeppox</i> ^b	DNA ~150 kbp, ungulates, arthropod transmission
	<i>Leporipoxvirus</i>	<i>hare fibroma</i> , <i>myxoma</i> , ^b <i>rabbit fibroma</i> , <i>squirrel fibroma</i>	DNA ~160 kbp, G + C ~40%, leporids and squirrels
	<i>Molluscipoxvirus</i>	<i>molluscum contagiosum</i>	DNA ~190 kbp, G + C ~60%, human host, localized tumors, contact spread
	<i>Orthopoxvirus</i>	<i>camelpox</i> (CMLV), <i>cowpox</i> (CPXV), <i>ectromelia</i> (ECTV), <i>monkeypox</i> (MPXV), <i>raccoonpox</i> , <i>skunkpox</i> , <i>taterapox</i> (TATV), <i>vaccinia</i> ^b (VACV), <i>variola</i> (VARV), <i>volepox</i>	DNA ~190 kbp, G + C ~36%, wide to narrow host range, <i>variola</i> (smallpox), <i>vaccinia</i> (smallpox vaccine)
	<i>Parapoxvirus</i>	<i>bovine papular stomatitis</i> , <i>orf</i> , ^b <i>parapoxvirus of red deer</i> , <i>pseudocowpox</i>	DNA ~140 kbp, G + C ~64%
	<i>Suipoxvirus</i>	<i>swinepox</i>	DNA ~150 kbp, narrow host range
	<i>Yatapoxvirus</i>	<i>tanapox</i> , <i>yaba monkey tumor</i> ^b	DNA ~140 kbp, G + C ~33%, primates and ? rodents
	<i>Unassigned</i>	<i>squirrel poxvirus</i> <i>crocodile poxvirus</i>	DNA ~160 kbp, G + C ~66% DNA ~190 kbp, G + C ~61%
Entomopoxvirinae	<i>Alphaentomopoxvirus</i>	<i>Anomala cuprea</i> , <i>Aphodius tasmaniae</i> , <i>Demodema boranensis</i> , <i>Dermolepida albohirtum</i> , <i>Figulus subleavis</i> , <i>Geotrupes sylvaticus</i> , <i>Melolontha melolontha</i> ^b	DNA ~260–370 kbp, <i>Coleoptera</i>
	<i>Betaentomopoxvirus</i>	<i>Acrobasis zelleri</i> , <i>Amsacta moorei</i> , ^b <i>Arphia conspersa</i> , <i>Choristoneura biennis</i> , <i>C. conflicta</i> , <i>C. diversuma</i> , <i>C. fumiferana</i> , <i>Chorizagrotis auxiliars</i> , <i>Heliothis armiger</i> , <i>Locusta migratoria</i> , <i>Oedaleus senigalensis</i> , <i>Operophtera brumata</i> , <i>Schistocera gregaria</i>	DNA ~232 kbp, G + C ~18%, <i>Lepidoptera</i> and <i>Orthoptera</i>
	<i>Gammaentomopoxvirus</i>	<i>Aedes aegypti</i> , <i>Camptochironomus tentans</i> , <i>Chironomus attenuatus</i> , <i>C. luridus</i> , ^b <i>C. plumosus</i> , <i>Goeldichironomus haloprasimus</i>	DNA ~250–380 kbp, <i>Diptera</i>
	<i>Unassigned</i>	<i>Diachasmimorpha</i> , <i>Melanoplus sanguinipes</i>	

^aFour-letter abbreviations of species used in text appear in parentheses.

^bPrototypal member.

CPXV and MPXV). VACV, the prototype OPXV, has been propagated as the smallpox vaccine for 200 years and apparently no longer exists in its original host,⁶¹ although vaccine-derived strains are currently circulating in cattle in Brazil and occasionally infect humans.⁷⁰³ DNA sequencing reveals that genes common to camelpox virus (CMLV), CPXV, ectromelia virus (ECTV), MPXV, taterapox (TATV), VACV, and VARV are greater than 90% identical, whereas OPXVs indigenous to the Americas (e.g., raccoonpox, skunkpox, and volepox virus) exhibit greater genetic divergence.²³⁴ CPXV contains all genes present in other OPXVs, suggesting that it resembles the ancestral member of this genus, whereas the other species have lost some genes. Recent analyses suggest that more than one species of OPXV have been grouped under the CPXV species.¹²⁷ The entomopoxviruses are divided into three genera—*Alphaentomopoxvirus* (7 species), *Betaentomopoxvirus* (13 species), and *Gammaentomopoxvirus* (6 species)—and 2 unassigned species based on the insect host of isolation (Table 66.1).

VIRION STRUCTURE

Morphology

The virions of POXVs are large compared to most other well-studied animal viruses and are just discernible by light microscopy. Nevertheless, higher magnification provided by electron microscopy is needed to resolve ultrastructure. The basic infectious form of POXV is the mature virion (MV). As will be

discussed later, an extracellular enveloped (EV) form of VACV consists essentially of an MV enclosed by an additional membrane. Cryo-preparation, which avoids drying artifacts, has been combined with electron tomography to provide a 3-D reconstruction of VACV MVs (Fig. 66.1)¹⁶⁶ that is consistent with classical descriptions derived from thin sections of fixed and dehydrated samples (Fig. 66.2).¹⁷⁸ Reconstructed images of virions have a barrel shape with dimensions of $\sim 360 \times 270 \times 250$ nm. The thickness (5 to 6 nm) and density of the outer layer are consistent with one lipid membrane bilayer, the outside of which appears corrugated because of irregular protrusions extending 3 to 5 nm. The internal structure of the virion is complex, consisting of a dumbbell-shaped core and lateral bodies between the concavities and the outer membrane. The core wall seems to be composed of two layers with an overall thickness of 18 to 19 nm. The inner layer appears continuous, except for a small number of channels, and has a diameter and density consistent with a lipid membrane. The outer layer has a palisade structure made of T-shaped spikes (8 nm in length and 5 nm wide) that are anchored in the putative lower membrane. Freeze fracture and deep-etch electron microscopy confirmed the outer structure of the virion as consisting of a single membrane bilayer with surface corrugations and the palisade structure of the core wall but did not reveal an internal core membrane and questioned its existence.³¹¹ The ultrastructure of the core is not resolved by cryoelectron tomography but appears to consist of two phases: the denser layer just under the core wall has a fiber-like morphology suggesting nucleoprotein. Cylindrical elements that may take an S-shape or more complex flower-like structures, presumably representing nucleoprotein, were visualized by other means within POXV cores.^{349,582} Proteins have not yet been localized to structural features of virions.

Chemical Composition

The mass of a VACV MV is 9.5 fg.²⁹⁴ The principal components of the MV are protein, lipid, and DNA, accounting for 90%,

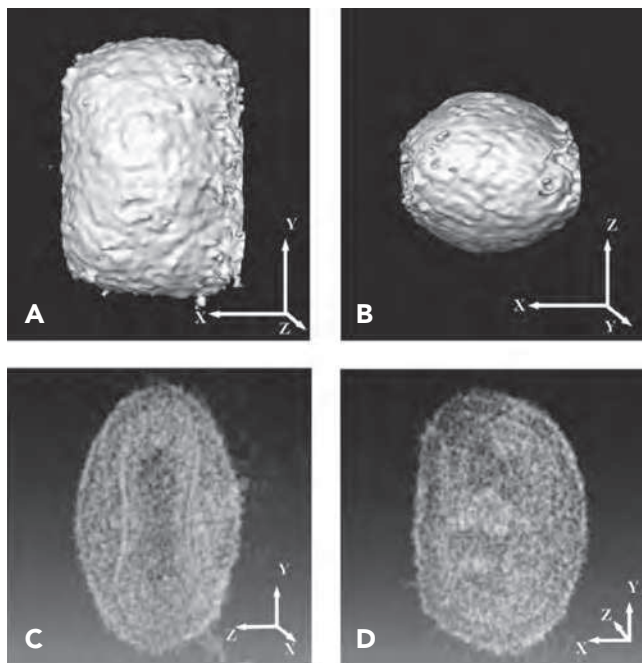


FIGURE 66.1. Reconstructions of vaccinia virus (VACV) mature virion (MV). **A, B:** Volumetric representations highlighting the outer shape and size of the virions in two orthogonal views along perpendicular axes. **C, D:** Translucent representations showing the complex internal structure of the core. In one orientation (**C**), the dumbbell shape of the core is seen. (From Cyrklaff M, Risco C, Fernandez JJ, et al. Cryo-electron tomography of vaccinia virus. *Proc Natl Acad Sci U S A* 2005;102:2772–2777. Copyright 2005 National Academy of Sciences, U.S.A.)

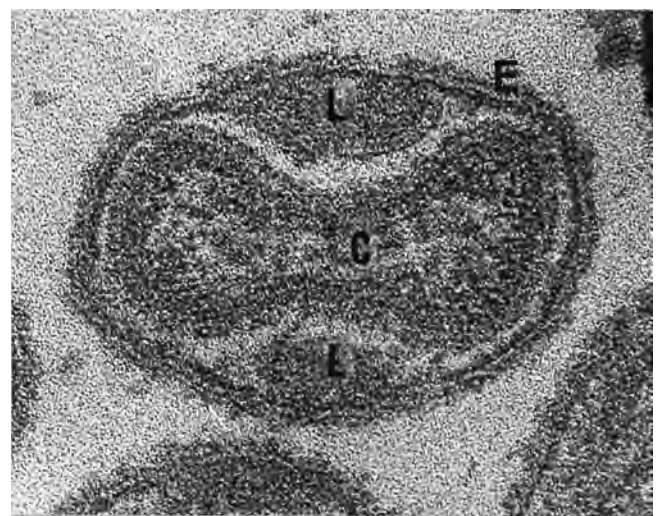


FIGURE 66.2. Electron microscopic image of a thin-sectioned intracellular vaccinia virus (VACV) mature virion (MV) showing core (C), lateral bodies (L), and external membrane (E). (From Pogo BGT, Dales S. Two deoxyribonuclease activities within purified vaccinia virus. *Proc Natl Acad Sci U S A* 1969;63:820–827.)

5%, and 3.2%, respectively, of the dry weight.⁸⁷⁴ In contrast, about one-third of fowlpox virus (FWPV) virions is lipid.⁴⁴¹ The lipid components of VACV MVs are predominantly cholesterol and phospholipids,^{150,718,735} whereas FWPV virions also contain squalene and cholesterol esters.⁴⁴¹ Carbohydrate is present in the VACV EV as a constituent of glycoproteins. Spermine and spermidine⁴¹⁶ and trace amounts of RNA⁶³⁰ also have been found in VACV MVs, but their significance is uncertain.

Genome

POXVs have linear double-stranded DNA genomes that vary from 140 kbp in parapoxviruses to 300 kbp in some avipoxviruses. POXVs have inverted terminal repetitions (ITRs), which consist of identical but oppositely oriented sequences at the two ends of the genome.²⁶⁶ The ITRs include an A+T-rich, incompletely base-paired, hairpin loop that connects the two DNA strands⁵³; a highly conserved region of less than 100 bp that contains sequences required for the resolution of replicating concatemeric forms of DNA^{203,482}; variable length sets of short, tandemly repeated sequences⁸⁴³; and up to several open reading frames (ORFs). The ends of the VACV genome are depicted in Figure 66.3. The ITRs are variable in length due to deletions, repetitions, and transpositions.

Complete genome sequences have been reported for at least one member of each *Chordopoxvirus* genus and two *Entomopoxvirus* genera. Nearly 100 genes are conserved in all chordopoxviruses and about half of these can be recognized in entomopoxviruses.⁷⁸⁷ Based on this, several generalizations can be made: genes are largely nonoverlapping, tend to occur

in blocks pointing toward the nearer end of the genome, are usually located in the central region if highly conserved and concerned with essential replication functions, and are usually located in the end regions if variable and concerned with host interactions. An ORF map of the VACV genome is shown in Figure 66.4. The arrangement of the central genes is remarkably similar in all chordopoxviruses. A convention for naming VACV ORFs, originating prior to sequencing the entire genome and used for the complete sequence of the Copenhagen strain of VACV, consists of using the HindIII restriction endonuclease DNA fragment letter, followed by the ORF number (from left to right except for HindIII C) within the fragment, and L or R, depending on the direction of transcription. Polypeptide names correspond to gene names, except that L or R is dropped. In contrast, ORFs have been numbered successively from one end of the genome to the other in most subsequent complete POXV genome sequences; nevertheless, the old letter designations have been retained as common names for homologs to provide continuity in the literature. The ORF number of the Western Reserve (WR) strain of VACV, which has been used for the majority of biochemical and genetic studies, is included in tables.

Polypeptide Components

Consistent with their size and complex structure, POXV virions contain a multitude of polypeptides.¹⁵⁶ Approximately 80 virus-encoded polypeptides have been identified by mass spectrometry,^{147,613,870} and 30 have been localized near the exterior of purified MVs by surface-specific labeling, sensitivity to

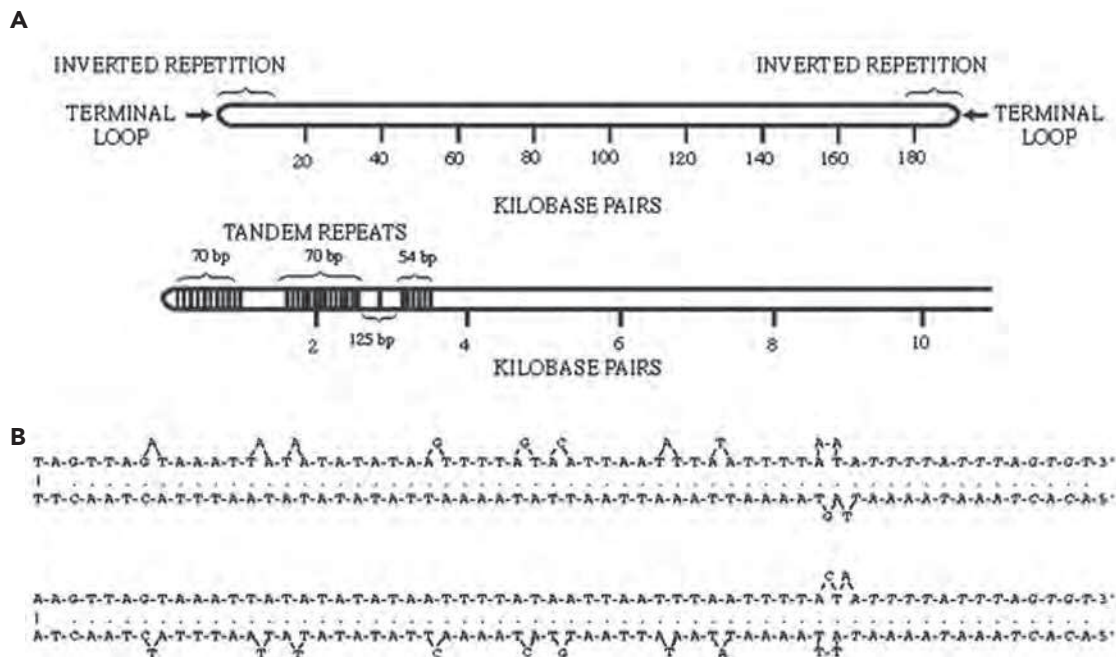


FIGURE 66.3. Structural features of vaccinia virus (VACV) genome. **A:** Representation of the entire linear double-stranded DNA genome and an expansion of the 10,000-bp inverted terminal repetition. (**A** from Moss B, Winters E, Jones EV. Replication of vaccinia virus. In: Cozzarelli N, ed. *Mechanics of DNA Replication and Recombination*. New York: A. Liss, 1983:449–461.) **B:** Nucleotide sequences of the inverted and complementary forms of the terminal loops. (**B** from Baroudy BM, Venkatesan S, Moss B. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. *Cell* 1982;28:315–324.)

TABLE 66.2 VACV MV Membrane-Associated Proteins

Gene ^a		kD	T ^b	C ^c	TM ^d	Properties	References
COP	WR						
Attachment							
A26L	149	58	L		—	Binds laminin, associated with A27	141
A27L	150	13	I		—	Binds heparan, associated with A17, N	148
D8L	113	35	I		N	Binds chondroitin, N	332
H3L	101	38	I	P	C	Binds heparan, N	426
Entry							
A16L	136	43	I	P	C	EFC; paralog G9, J5	550
A21L	140	14	L	P	N	EFC	767
A28L	151	16	L	P	N	EFC, N	679
F9L	048	24	L	P	C	EFC associated	100
G3L	079	13	L	P	N	EFC	364,777
G9R	087	39	L	P	C	EFC; paralog A16, J5	549
H2R	100	22	L	P	N	EFC	677
I2L	071	8	L	C	C	EFC?	539
J5L	097	15	L	P	C	EFC; paralog A16, G9	678
L1R	088	27	L	P	C	EFC associated, N	77
L5R	092	15	L	P	C	EFC	766
O3L	069.5	4	I	C	N	EFC	654
REDOX							
A2.5L	121	9	L	C	—	S-S to E10, G4	673
E10R	066	11	L	P	—	ERV/ALR family, S-S to A2.5	680,681
G4L	081	14	I	P	—	Thioredoxin family, S-S to A2.5	829,830
Morphogenesis							
A9L	128	12	L	P	N	Post-IV	869
A13L	132	8	L	C	N	Post-IV, N	784
A14L	133	10	L	C	N, C	Pre-IV, associated with A17	480,629,772
A17L	137	23	L	C	N, C	Pre-IV, associated with A14, N	625,847
D13L	118	62	I	P	—	Pre-IV, lattice scaffold, associated with A17	78,750
Virulence							
A14.5L	134	6	L	C	N, C	Virulence	76
F14.5L	53.5	6	I		N	Cell adhesion, virulence	363
I5L	074	9	I	C	N, C	Virulence	723

^aCopenhagen (COP) or Western Reserve (WR) designation.

^bTime of synthesis: intermediate (I) or late (L).

^cConservation in all POXV (P) or chordopoxviruses (C).

^dN-terminal (N) and/or C-terminal (C)

VACV, vaccinia virus; MV, mature virion; N, known target of neutralizing antibody; TM, transmembrane domain; EFC, entry–fusion complex; IV, immature virion.

proteases, extraction with nonionic detergents, or reactivity with virus neutralizing antibodies. The surface proteins can be divided structurally into those with transmembrane domains and those without and functionally into those required for attachment, entry, disulfide bond formation, morphogenesis, and virulence (Table 66.2). None of the MV membrane proteins are glycosylated, which could have implications for the derivation of the viral membrane.

Virus cores can be produced *in vitro* by treatment of MVs with a nonionic detergent and reducing agent.²³¹ Nearly 50 polypeptides, distinct from those in the membrane fraction, have been identified in such cores. Of these, about 30 are enzymes, of which at least half are directly involved in early mRNA biosynthesis (Table 66.3); the nonenzymatic proteins may be primarily involved in morphogenesis and structure

(Table 66.4). The F17R, L4R, A3L, and A10L ORFs encode particularly abundant core proteins.

Evidence for a distinct extracellular form of VACV came from vaccine-related studies. Antibodies to inactivated MVs did not protect rodents against an OPXV infection as well as antibodies to live virus or inactivated EVs.⁹² Furthermore, there were differences in the relative neutralizing abilities of the different types of sera. Subsequent studies showed that the EV has an additional membrane and consequently a lower buoyant density.⁶²² The additional membrane contains several unique glycosylated proteins (A33, A34, A56, B5, K2) and one non-glycosylated (F13) (Table 66.5). Of the six EV membrane proteins, only A34 and F13 are conserved in all chordopoxviruses. Although EVs are usually considered as MVs with an additional membrane, that is not precisely true. There is evidence

TABLE 66.3 VACV Core-Associated Enzymes and Transcription Factors

Enzyme/Factor	Gene	WR #	kD	T ^a	C ^b	Comments	References
RNA polymerase						Multisubunit	52
RPO 147	J6R	098	147	E	P	RPB1 homolog	107
RPO 132	A24R	144	132	E	P	RPB2 homolog	17
RPO 35	A29L	152	35	E	P	No cell homolog	19
RPO 30	E4L	060	30	E	C	SI1 elongation factor homolog	5
RPO 22	J4R	096	22	E	C	RPB5 homolog	107
RPO 19	A5R	124	19	E	P	RPB6 homolog	10
RPO 18	D7R	112	18	E	P	RPB7 homolog	7
RPO 7	G5.5R	083	7	E	C	RPB10 homolog	18
RAP94	H4L	102	94	L	P	RNA pol associated, early transcription	9
Early transcription factor (VETF)						Promoter binding, DNA-depend ATPase	109
Large subunit	A7L	126	82	L	P	Interacts with promoter core	131,271
Small subunit	D6R	111	74	I	P	Helicase motif	103,271
Capping enzyme						RNA triphosphatase, guanylyltransferase, guanine-7-methyltransferase	455
Large subunit	D1R	106	82	E	P	Catalytic activities	503
Small subunit	D12L	117	33	E	P	Stimulates methyltransferase	543
Cap 2'-methyltransferase	J3R	039	39	E	P	Ribose methyltransferase; also poly(A) polymerase subunit	662
Poly(A) polymerase						Adds As to 3' ends of mRNAs	513
Large subunit	E1L	057	55	E	P	Catalytic activity	269
Small subunit	J3R	039	39	E	P	Stimulatory activity; also methyltransferase	269
Nucleoside triphosphate phosphohydrolase I (NPH I)	D11	116	72	I	P	DNA-dependent ATPase	557
Nucleoside triphosphate phosphohydrolase II (NPH II)	I8R	077	77	I	P	DNA/RNA-dependent NTPase, helicase	557
DNA topoisomerase	H6R	104	37	L	P	Sequence-specific nicking, early transcription	702
DNA helicase	A18R	138	57	E	P	DNA-dependent ATPase	704
Holiday junction resolvase	A22R	142	22	I	P	RuvC homolog; resolves concatemer junctions	262
DNA packaging ATPase	A32L	155	31	I	P	Related to phage enzyme	130
Protein kinase 1	B1R	183	34	E	—	Serine/threonine	428
Protein kinase 2	F10L	049	52	L	P	Serine/threonine	427
Protein phosphatase	H1L	099	34	I	—	Tyr/Ser	289
Protease 1	I7L	076	49	L	P	Putative cysteine protease	115
Protease 2	G1L	078	68	L	P	Putative metalloprotease	24
Glutaredoxin	O2L	069	12	I	—	Cofactor ribonucleotide reductase	8
DNA nicking-joining	K4L	035	49	I	—	Nonessential, role unknown	232
Superoxide dismutase	A45R	171	14	L	—	Nonessential, inactive catalytic site, role unknown	15

^aTime of synthesis: early (E), intermediate (I), or late (L).

^bConservation in all POXV (P) or chordopoxviruses (C).

VACV, vaccinia virus.

that MVs contain at least two surface proteins, A26 and A25, that are absent from EVs.⁷⁸³

CELL ENTRY

General Considerations

Investigations into mechanisms used by POXVs to enter cells have been complicated by the existence of two infectious forms: MVs, which have a single outer membrane, and EVs, which have an additional membrane with a different protein composition.⁵⁰⁷ Although the MV is the more abundant form, the EV

is specialized for cell-to-cell spread as will be discussed later. Following cell attachment, however, the EV-specific membrane is discarded and the underlying MV membrane fuses with the cell.⁴¹⁹ The next two sections summarize current understanding of MV and EV entry.

MV Entry

Enveloped viruses typically enter cells by fusion with the plasma membrane or following endocytosis. VACV is versatile in that MVs use both entry mechanisms (Fig. 66.5A, B), perhaps accounting for their ability to enter most cells.^{28,176} Endosomal acidification promotes entry of the virus core into

TABLE 66.4 VACV Core-Associated Nonenzymatic Proteins

Gene ^a		kD	T ^b	C ^c	Properties	References
COP	WR					
A3L	122	73	I	P	4b precursor, I7-dependent cleavage, morphogenesis	510,637,794
A4L	123	31	E	C	F10-dependent phosphorylation, morphogenesis	163,619,836
A10L	129	102	L	P	4a precursor, I7-dependent cleavage, morphogenesis	510,796,842
A12L	131	20	I	C	I7-dependent cleavage, morphogenesis	863
A15L	135	11	I	C	7-protein complex, morphogenesis	748
A30L	153	9	I	C	7-protein complex, morphogenesis	479,754
D2L	107	17	L	C	7-protein complex, morphogenesis	748
D3R	108	28	L	C	7-protein complex, morphogenesis	748
E8R	064	32	I	C	ts mutant virion decreased transcription	378
E11L	067	15	I		ts mutant noninfectious virions	812
F17R	065	11	E	C	Phosphoprotein, morphogenesis	572,842,883
G5R	082	50	E	P	FEN1-like nuclease, DNA recombination	675
G7L	085	42	L	C	7-protein complex, morphogenesis	479,749
H5R	103	22	E	C	Interacts with A20, transcription, morphogenesis	66,80,207,401,463
I6L	075	43	I	C	Telomere binding, DNA packaging	206,287
J1R	093	18	I	C	7-protein complex, morphogenesis	140,142
L3L	090	41	L	P	ts virions defective in core transcription	614
L4R	091	28	I	P	Single-strand DNA/RNA binding, core transcription, I7-depend cleavage	23,62,833

^aCopenhagen (COP) or Western Reserve (WR) designation.

^bTime of synthesis: early (E), intermediate (I), or late (L).

^cConservation in all POXV (P) or chordopoxviruses (C).

VACV, vaccinia virus.

TABLE 66.5 VACV WV and EV Membrane Proteins

Gene	WR #	kD	T ^a	C ^b	TM ^c	Properties	References
A33R	156	21	E*		N, T2	EV, glycosylated, phosphorylated, associated with A36, actin tail formation	632,848
A34R	157	20	I	Ch	N, T2	EV, N-gly, lectin-like, associated with A36 and B5, actin tail formation, EV release	85,640,846
A36R	159	25	E*		N, T1b	WV only; phosphorylated; associated with A33, A34, cellular Nck, and kinesin; actin tail formation and microtubule movement	657,792,849
A56R	181	35	E*		C, T1	EV; hemagglutinin; glycosylated; associated with K2, EFC proteins, and C3; prevents syncytia and superinfection	671,689,779,806
B5R	187	35	E*		C, T1	EV; glycosylated; associated with A34, A36, and F13; WV formation	308,816
F12L	051	73	E*	Ch	—	WV only, microtubule movement	791,876
F13L	052	42	I	Ch	—	EV, palmitylated, phospholipase domain, associated with B5, WV formation	83,661
K2L	033	42	I		—	EV, signal peptide, associated with A56, prevents syncytia and superinfection	779,805,806
E2L	058	86	?	Ch	—		

^aTime of synthesis: early plus intermediate or late (E*), intermediate (I).

^bConserved in all chordopoxviruses (Ch).

^cTransmembrane domain: N-terminal (N), C-terminal (C), type 1 (T1), type 2 (T2).

VACV, vaccinia virus; WV, wrapped virion; EV, extracellular enveloped virion.

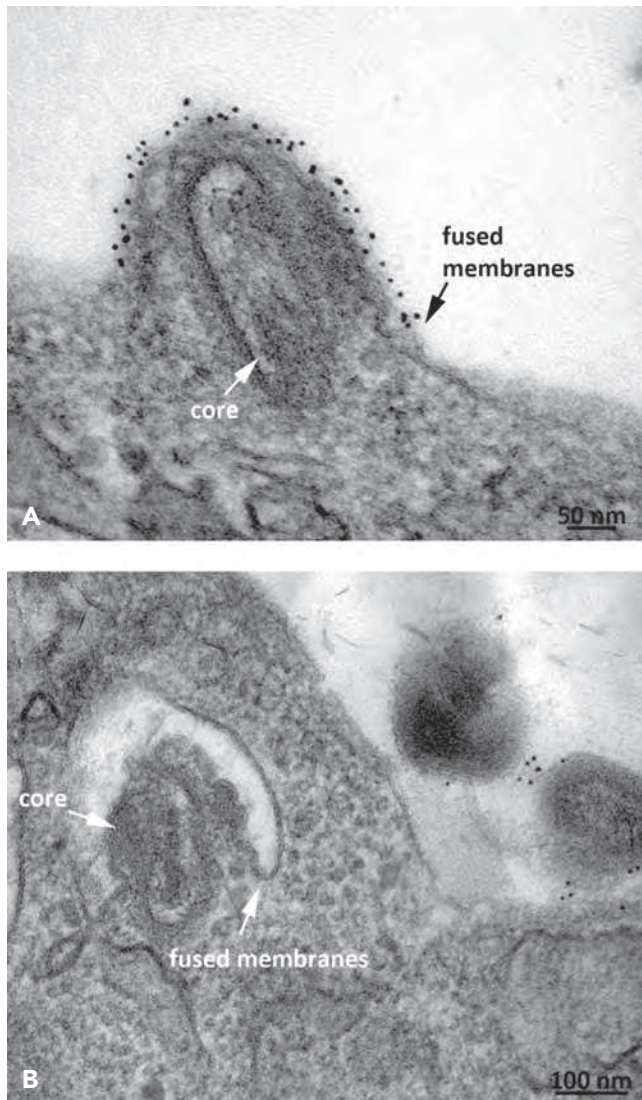


FIGURE 66.5. Vaccinia virus (VACV) mature virion (MV) fusing with the plasma (A) or endosomal (B) membrane. VACV MVs were deposited on the cell surface by spinoculation and incubated at 37°C for 30 minutes. The unpermeabilized cell was incubated with antibody to the D8 membrane protein and labeled with protein A conjugated to gold spheres. The gold particles demonstrate that the viral membrane has fused with the plasma membrane in panel A; the core is within the cytoplasm. (Courtesy of A. Weisberg.)

the cytoplasm following actin-dependent macropinocytosis or fluid phase uptake of the large virus particles.^{339,478,504,768,769} There is considerable VACV strain variation regarding the preference for the alternative entry pathways,⁷⁰ which may depend in part on the A25 and A26 proteins.^{133b} Initial studies indicate that myxoma virus also enters cells through a low pH endocytic pathway.⁸⁰¹ VACV enters primary cultures of well-differentiated human airway epithelial cells through the basolateral surface and exits through the apical surface.⁸⁰⁰

Many enveloped viruses contain one or two proteins that mediate attachment as well as fusion with cell membranes. VACV is exceptional in encoding at least 4 proteins for attachment and 12 dedicated to entry (Table 66.2). Cell surface glycosaminoglycans and laminin can mediate MV attachment.

The protein D8 binds chondroitin sulfate,³³² A27 and H3 bind heparan sulfate^{148,331,426,798} and A26 binds laminin.¹⁴¹ Of these attachment proteins, only D8 and H3 have transmembrane domains. A26 and A27 interact with each other and the A17 transmembrane protein anchors the latter.^{325,626} Individually, the attachment proteins are not essential and heparin appears to have a greater competitive inhibitory effect on strains that preferentially enter by a neutral pH mechanism.⁷⁰

Fusion of VACV with cell membranes was demonstrated many years ago,²²³ but the participating viral proteins were identified only recently. The number of viral proteins required for entry is unprecedented. The 12 proteins implicated thus far are listed in Table 66.2. Most or all of the proteins are components of the entry fusion complex (EFC) or associated with it.⁶⁷⁸ The proteins are each dedicated to entry, because the assembly of normal-looking virions that are unable to initiate an infection occurs despite their repression. Their conservation in all POXVs strongly suggests a common entry mechanism. Three of the proteins, A16, G9, and J5, are related in sequence and were apparently derived from a common gene early in POXV evolution. The entry proteins vary in size from 4 to 43 kD, are nonglycosylated, and do not resemble either type 1 or type 2 fusion proteins of other viruses. Except for G3, I2, and O3, the entry proteins contain conserved intramolecular disulfide bonds that are formed by the cytoplasmic redox system, which is also encoded by all POXVs and will be described later. The EFC proteins are not required for attachment of virions to cells, but most are needed for the initial fusion of viral and cellular membranes.⁴¹⁴ The L1 protein has been reported to bind to cell surfaces and block virus entry.²⁵⁵ Nevertheless, the mechanism of fusion and the function of individual proteins remain to be determined. The anatomy of the complex has not yet been solved, although some interacting partners have been identified.^{535,806,844} The VACV A17 and A27 proteins have also been implicated in membrane fusion,³⁹⁴ but there is no genetic evidence that their activities are required for entry.

EV Entry

Efficient cell-to-cell spread of VACV is mediated largely by EVs that adhere to the cell surface at the ends of long, mobile, finger-like projections that are formed by actin polymerization, as illustrated in Figure 66.6.^{84,737} Deletion of the *A33R*, *A34R*, or *A36R* gene (Table 66.5) prevents actin tail formation and reduces the efficiency of virus spread.^{640,846} However, mutations of the A33, A34, or A36 proteins, which cause enhanced release of EV, can overcome a deficiency in actin tail formation resulting in rapid spread.^{84,383}

For technical reasons, most entry studies have been carried out with free EVs rather than the biologically important cell-associated form. However, the EV membrane is fragile and it is difficult to be sure that the membrane is intact in the majority of particles. Available information suggests that free EVs are taken up by endocytosis much like MVs.⁶⁶⁰ The entry proteins described earlier are required for cell-to-cell spread of EVs and for low pH-induced syncytia formation, supporting the idea that only the MV membrane is fusogenic. Consequently, removal of the external EV membrane with exposure of the MV membrane precedes the fusion step because the entry/fusion proteins are located in the latter. Disruption of the outer EV membrane within endosomes was suggested by sensitivity to lysosomotropic agents³⁵²; in addition, electron micrographs

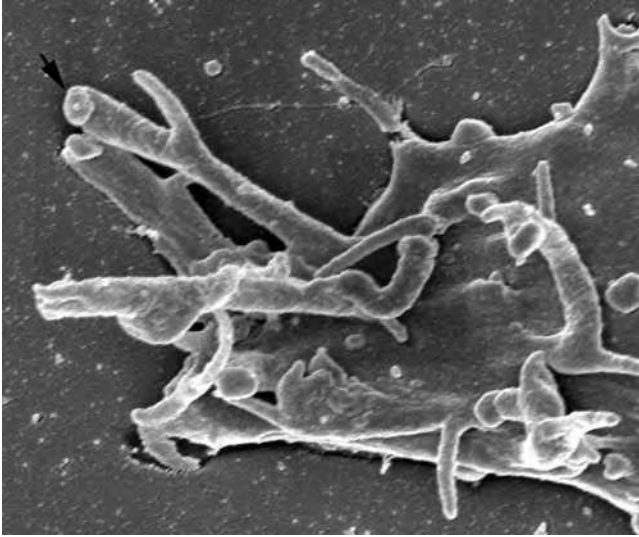


FIGURE 66.6. Scanning electron microscopy of a cell infected with vaccinia virus (VACV). Cells were infected with VACV for 20 hours and then fixed and coated with palladium gold. The arrow points to an extracellular enveloped virion (EV) at the tip of a virus-induced actin-containing microvillus. (Courtesy of A. Weisberg.)

of MVs fusing with the plasma membrane beneath a disrupted EV wrapper have been obtained.⁴¹⁹ Disruption of the EV wrapper at the plasma membrane is dependent on polyanionic molecules or low pH and requires the membrane glycoproteins A34 and B5, which may explain the low infectivity of EVs lacking the A34 protein.^{419,621} The fusion of infected cells to each other, mediated by short exposure to low pH, may mimic the endocytic path by disrupting the outer membrane of virus particles on the cell surface.^{223,277} Mutations of the A56 hemagglutinin or the K2 glycoprotein results in fusion of adjacent infected cells without the need for a low pH trigger.^{671,778}

Superinfection Inhibition

The spread of EVs to uninfected cells may be enhanced by a repulsion mechanism that prevents superinfection of cells that express the A33 and A36 proteins on the plasma membrane and generates new actin protrusions.²¹⁸ Another mechanism operates to prevent fusion of MVs with infected cells: the A56-K2 heterodimer interacts with the A16 and G9 subunits of the EFC.^{780,805,806} There are likely additional processes that inhibit superinfection.¹⁴⁴

Cell Entry and Signaling Receptors

Interaction of the MV with the cell surface triggers a signaling cascade that results in membrane rearrangements and the formation of actin and ezrin-containing protrusions that seem to envelope the virus and are important for virus entry and replication.^{21,189,437,460,478} Further studies suggest that signaling and actin rearrangements are needed for the initial step of fusion of viral and cellular membranes.⁴¹⁴ The lipid composition of the cell membrane is important for entry as depletion of cholesterol inhibits virus penetration.¹⁴⁹ However, specific protein receptors that are required for the penetration step have not yet been identified and could vary with cell type. Proposals

that the epidermal growth factor receptor⁴⁵¹ and chemokine receptors⁴¹² serve as portals of entry for POXVs have not been confirmed.^{344,460} Infectivity of VACV can be increased with phosphatidylserine,³⁵⁴ and an apoptotic mimicry model with a role for a specific phosphatidylserine receptor has been suggested based on reconstitution of delipidated virus.⁴⁷⁸ However, the stereoisomer of phosphatidylserine and other phospholipids not known to signal apoptotic uptake can also reconstitute infectivity, indicating that the putative receptor has broad specificity or that the phospholipids have a different role in enhancement of infectivity.⁴¹³

GENE EXPRESSION

Programmed Expression of POXV Genes

Studies primarily with VACV have led to an understanding of the gene expression program, which occurs exclusively in the cytoplasm: (a) a complete early transcription system is present within the core of virus particles, providing a mechanism for the synthesis of viral early mRNAs soon after infection; (b) the early mRNAs encode enzymes and factors needed for synthesis of viral DNA and for transcription of the intermediate class of genes; (c) the intermediate gene transcripts encode enzymes and factors for late gene expression; and (d) the products of the late genes include the early transcription factors, which are packaged with RNA polymerase and other enzymes in progeny virions (Fig. 66.7). Most studies refer to posttranscriptional gene products as late without distinguishing between intermediate and late classes. However, progress in resolving the intermediate and late gene classes has been made recently,⁸⁶⁷ allowing the construction of the transcription map in Figure 66.4. POXV transcription has been reviewed recently.¹⁰⁴

Regulation of Early-Stage Transcription

Infectious POXV particles contain a transcription system that synthesizes mRNAs that are capped, methylated, and polyadenylated.^{375,376,525,822} A large number of virus-encoded enzymes and factors that are directly involved in the synthesis and modification of mRNA including the multisubunit DNA-dependent RNA polymerase, RNA polymerase-associated protein of 94 kD (RAP94), early transcription factor (VETF), capping and methylating enzymes, poly(A) polymerase, nucleotide phosphohydrolase I (NPH I), and topoisomerase are packaged in the virus particle (Table 66.3). Additional enzymes may have roles in virus assembly, protein processing, or DNA packaging as discussed later.

Following entry into the cytoplasm, virus cores are transported on microtubules to sites of transcription^{128,446} and mRNA synthesis is detected within 20 minutes (Fig. 66.8).⁴¹ RNA/DNA hybridization studies revealed that about half of the vaccinia virus genome is transcribed prior to DNA replication,^{88,556} and genome tiling and deep RNA sequencing have corroborated this.^{30,864,865} Although early transcripts can be divided into two groups based on kinetic cluster analysis, the significance of this is unclear as both are expressed in the presence of protein synthesis inhibitors and therefore can be classified as immediate-early.⁸⁶⁵ The genomic arrangement of early genes is shown in Figure 66.4. The cessation of early gene expression coincides with disruption of the virus core, a process known as uncoating. Electron microscopic images suggest that the nucleoprotein complex

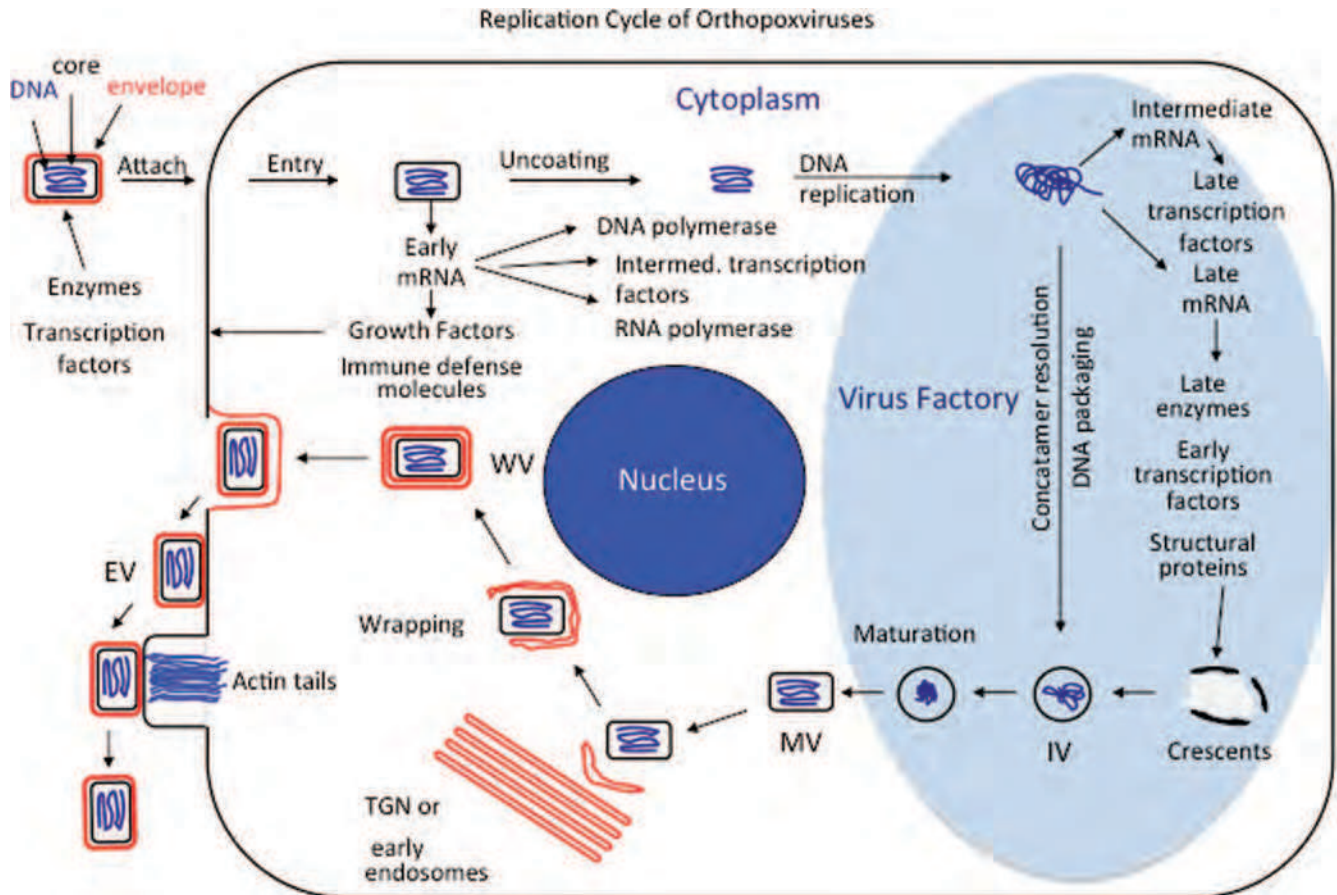


FIGURE 66.7. The replication cycle of vaccinia virus (VACV). Virions, containing a double-stranded DNA genome, enzymes, and transcription factors, attach to cells and fuse with the cell membrane, releasing cores into the cytoplasm. The cores synthesize early messenger RNAs (mRNAs) that are translated into a variety of proteins, including growth factors, immune defense molecules, enzymes, and factors for DNA replication and intermediate transcription. Uncoating occurs and the DNA is replicated to form concatemeric molecules. Intermediate genes in the progeny DNA are transcribed and the mRNAs are translated to form late transcription factors. The late genes are transcribed and the mRNAs are translated to form virion structural proteins, enzymes, and early transcription factors. Assembly begins with the formation of discrete membrane structures. The concatemeric DNA intermediates are resolved into unit genomes and packaged in immature virions. Maturation proceeds to the formation of infectious intracellular mature virions (MVs). The virions are wrapped by modified *trans*-Golgi and endosomal cisternae, and the wrapped virions (WVs) are transported to the periphery of the cell via microtubules. Fusion of the wrapped virions with the plasma membrane results in release of extracellular enveloped virions (EVs).

passes out through breaches in the core wall.¹⁷⁵ If uncoating is prevented by protein synthesis inhibitors,³⁶⁹ early mRNA synthesis is increased and prolonged,^{41,850} suggesting that under normal conditions core disassembly leads to the disruption or disorder of the early transcription apparatus. A putative uncoating protein has been purified but not genetically identified.⁵⁷³

The rapid decline of steady-state early mRNA levels (Fig. 66.8) is due to an enhanced rate of mRNA degradation as well as to cessation of transcription.^{546,668} Degradation of mRNAs is enhanced by the products of the D9R and D10R ORFs,^{564,695} which are decapping enzymes.^{562,565} Presumably, the decapped mRNAs are rapidly destroyed by cellular 5' exonuclease activity. Rapid mRNA turnover may be a mechanism for eliminating cellular mRNA and also viral mRNA at the end of early and intermediate stages.

Early-Stage Promoters and Termination Signals

Transcription of early genes is determined by an A/T-rich sequence upstream of the RNA start site. Saturation mutagenesis of a VACV early promoter defined a critical core region, from -13 to -25, in which many single-base substitutions have a drastic effect on expression.¹⁸⁴ The consensus core sequence derived from an analysis of the region upstream of all annotated early start sites⁸⁶⁴ is consistent with the mutagenesis studies (Fig. 66.9). Transcription initiation usually occurs with a purine, predominantly 12 to 17 nucleotides downstream of the core region. The core motif, however, is present at numerous locations in the genome and appears to be required but not sufficient for transcription initiation. Further analysis correlated a high AT content in the spacer region between the

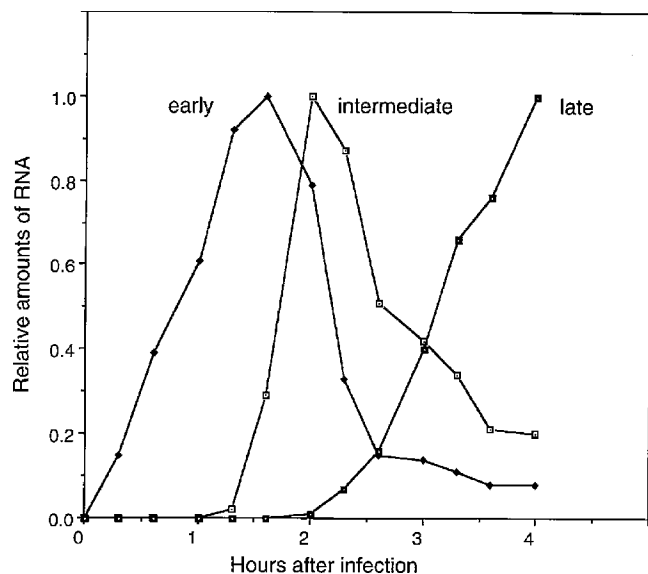


FIGURE 66.8. Steady-state levels of representative early-, intermediate-, and late-stage messenger RNAs (mRNAs) in vaccinia virus (VACV)-infected cells. Total RNA was isolated from infected HeLa cells at various times after infection and hybridized to antisense RNA probes specific for the 5' ends of mRNAs encoded by the C11R (early), G8R (intermediate), or F17R (late) open reading frames (ORFs).⁴¹ After ribonuclease digestion, the protected probe fragments were analyzed by polyacrylamide gel electrophoresis and the radioactivity quantified. The numbers were normalized to the peak value in each case.

core motif and the RNA start site with active transcription.⁸⁶⁴ Nevertheless, there appear to be many more transcription start sites than annotated ORFs, possibly allowing for the generation of new proteins during evolution. Promoter motifs are conserved between POXV genera, explaining an old phenomenon called nongenetic reactivation, which consists of rescue of a heat-inactivated POXV by co-infection with a second poxvirus belonging to a different genus.^{251,301} Thus, the heat-killed POXV provides the template and the second POXV provides the enzymes for transcription.

The 3' ends of VACV early mRNAs frequently occur 20 to 50 bp downstream of the sequence TTTTNT (abbreviated as T5NT).⁸⁷³ However, termination is actually mediated by U5NU in RNA.⁷⁰¹ T5NT sequences are present near the ends of most but not all viral early genes. When absent, the mRNA tail may extend through the next early gene downstream. *In vivo* studies suggested that the efficiency of termination following a single T5NT is about 80%,²²⁸ although in some cases it is less probably because of RNA secondary structure.^{421,439} T5NT sequences have been noted near the ends of putative early genes of other POXV genera,^{3,122,674} suggesting a similar role in termination. Nevertheless, a VACV genome-wide analysis of the 3' ends of early mRNAs revealed that only about two-thirds had a T5NT sequence within 100 nucleotides upstream, suggesting an additional termination mechanism.⁸⁶⁴ A pyrimidine-rich sequence in the coding strand up to position -25 relative to the polyadenylation site was found regardless of the presence of T5NT, suggesting that it might facilitate termination.

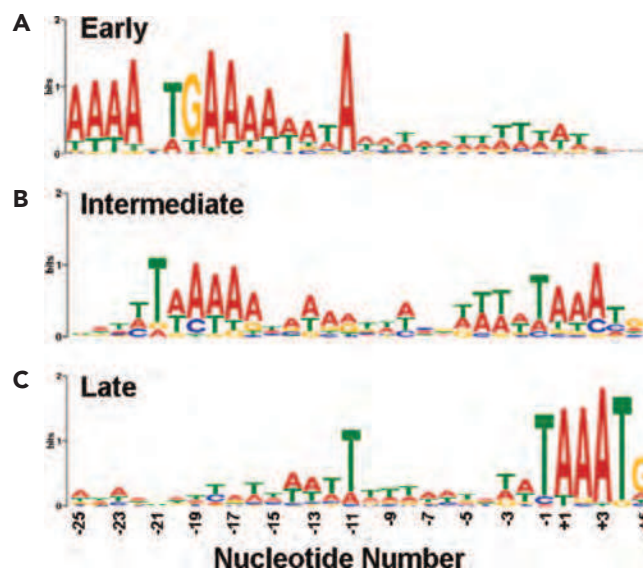


FIGURE 66.9. Motif logos of vaccinia virus (VACV) promoters. Motifs represent early genes (A), intermediate genes (B), and late genes (C); +1 represents the RNA start site. (From Yang Z, Reynolds SE, Martens CA, et al. Expression profiling of the intermediate and late stages of poxvirus replication. *J Virol* 2011;85:9899–9908.)

Enzymes and Factors for Early-Stage Transcription

Soluble extracts of VACV virions can transcribe an early promoter template *in vitro* to generate properly initiated and terminated mRNAs⁶³¹ and therefore provide a source of materials for characterization of the relevant enzymes and factors listed in Table 66.3. The virion RNA polymerase is eukaryotic-like with regard to its size and subunit complexity.⁵² The subunits, ranging from 7 to 147 kD, are encoded by eight viral genes and, except for RPO30 and RPO35, are homologous to those of cellular RNA polymerases, suggesting similar functions.³⁹¹ The RPO30 subunit is approximately 23% identical in sequence to eukaryotic transcription elongation factor SII (TFIIS).

The 94-kD polypeptide known as RAP94, encoded by the *H4L* gene, is associated with RNA polymerase in VACV virions and is specifically required for transcription of early promoter templates.^{6,9,210} RAP94 interacts with VETF, the VACV early transcription factor.⁸⁶⁶ Synthesis of RAP94 occurs late, at the time of virion assembly, consistent with its role as a virion-associated early transcription factor. In contrast, the genes encoding the core RNA polymerase subunits have early promoters because they are also needed for intermediate and late transcription.

Transcriptional activity can be reconstituted *in vitro* with RNA polymerase and VETF, a heterodimer of 82- and 70-kD subunits.^{103,109,271} VETF, like RAP94, is synthesized only at late times after infection. The protein binds to the core region of early promoters and to DNA downstream of the RNA start site, thereby altering the conformation of the DNA.^{105,131} Single nucleotide substitutions in the core sequence of the promoter that decrease transcription also abrogate specific DNA binding.⁸⁷² A DNA-dependent adenosine triphosphatase (ATPase) activity associated with the small subunit of VETF is not required for

promoter binding but is essential for transcription, apparently via a promoter clearance mechanism.^{101,106,424} Complexes of VETF and RNA polymerase have been detected, suggesting that VETF may recruit RNA polymerase to the promoter.^{40,108,423} The elongation complex has a 3' RNase activity that permits resumption of transcription by stalled polymerase.²⁹⁶ The latter RNase activity is similar to one exhibited by eukaryotic RNA polymerase II in the presence of TFIIS, a VACV RPO30 homolog.

A complex of RNA polymerase, RAP94, VETF, capping enzyme (sometimes called VTF when referring to its termination role), and NPH I can accurately terminate as well as initiate transcription on DNA templates containing an early promoter and T5NT sequence.^{108,873} Although RNA polymerase and VETF can reconstitute the transcription initiation and elongation activities, capping enzyme and NPH I are needed to release nascent mRNA containing a U5NU sequence from the transcription complex in an adenosine triphosphate (ATP)-dependent reaction.^{145,146,209,583,698,701} The physical interaction of NPH I with RAP94 may explain the specificity of this termination system for early transcripts.^{494,584} NPH I also serves as a polymerase elongation factor to facilitate read-through of intrinsic pause sites.²⁰⁹

Early viral transcripts made *in vivo* or *in vitro* are capped^{87,822} and polyadenylated³⁷⁵ like eukaryotic mRNAs. RNAs synthesized by virus cores contain a cap I structure that consists of a terminal 7-methylguanosine connected via a triphosphate bridge to a 2'-O-methylribonucleoside. The N⁷-methylguanosine component of the cap is required for mRNA stability and for binding of VACV mRNA to ribosomes,⁵²⁷ whereas ribose methylation may have a role in subverting the activity of type I interferon (IFN).⁸⁸⁶ Capping occurs during transcription when the nascent RNA chains are approximately 30 nucleotides long.^{295,823} The steps in cap formation are (a) removal of the terminal phosphate of the triphosphate end of the nascent RNA to form a pp(5')N-terminus, (b) transfer of a guanosine monophosphate (GMP) residue from guanosine triphosphate (GTP) to form G(5')ppp(5')N-, (c) transfer of a methyl group from S-adenosylmethionine to produce m⁷G(5')ppp(5')N-, and (d) transfer of a second methyl group to form m⁷G(5')ppp(5')Nm. The first three reactions are catalyzed by the virus-encoded 127-kD capping enzyme heterodimer.^{453,454,799}

The fourth step in cap formation, ribose methylation of the penultimate nucleoside, is mediated by a separate viral enzyme.⁵¹ The capping enzyme large subunit forms a covalent lysyl-GMP intermediate.^{542,699} The RNA triphosphatase and guanylyltransferase activities reside in an N-terminal segment of the large subunit,^{293,528,700} whereas a complex of the C-terminal part of the large subunit and the small subunit contains the N⁷-methyltransferase (N⁷-MTase) activity.^{312,450} The nucleoside 2'-MTase is a 39-kD protein that exists as a monomeric species and has an additional role as the VP39 subunit of poly(A) polymerase.⁶⁶² High-resolution x-ray crystal structures of the nucleoside 2'-MTase complexed to its methyl donor and mRNA cap have been obtained.^{319,320} Viral mRNAs synthesized *in vivo* have additional base and ribose methylations that are catalyzed by cellular enzymes.⁸⁷ Phenotypic analysis of a temperature-sensitive mutant in the large subunit of the capping enzyme supports its multifunctional roles.⁶⁸⁷

The enzyme that catalyzes poly(A) tail formation is a heterodimer of virus-encoded 55- and 39-kD subunits called VP55 and VP39, respectively.^{269,511,520} VP55 binds to uridylate

sequences near the end of the RNA chain and catalyzes the processive addition of 30 to 35 adenylate residues before changing to a slow and nonprocessive mechanism.^{208,273,871} VP39 binds poly(A) and stimulates VP55 to semiprocessively add additional adenylate residues.²⁷² Thus, VP39, which is present in an excess over VP55, serves as a processivity factor for the poly(A) polymerase as well as a MTase. The two activities are independent because mutated forms of VP39 that lack MTase retain adenylyltransferase stimulatory activity.^{663,688} Genetic and biochemical studies indicate an additional role of VP39 as an RNA elongation factor for intermediate and late RNA synthesis to be discussed later.^{418,854} The finding that the capping enzyme/termination factor and the ribose MTase/poly(A) polymerase processivity factor function at the 5' and 3' ends of the mRNA is intriguing. The association of such apparently disparate functions in the same enzymes may provide a specific advantage or represent an economical use of proteins.

The minimal components for synthesis of correctly initiated, terminated, capped, and polyadenylated mRNAs were defined by *in vitro* reconstitution assays. However, several additional proteins are needed within the confines of the virus core. These include the DNA topoisomerase,¹⁷⁰ NPH II,²⁸⁶ the H1 serine/tyrosine phosphatase,⁴³⁶ the L4 DNA-RNA-binding protein,⁸³³ and the L3⁶¹⁴ and E8³⁷⁸ proteins of unknown function.

Regulation of Intermediate-Stage Transcription

Amino acid labeling^{514,574} and transcription^{41,803} studies indicated the existence of an intermediate class of genes that are expressed after DNA replication but before expression of the late genes (Fig. 66.8). The rapid decline in intermediate mRNAs results from shutoff of transcription coupled with rapid mRNA turnover. Analysis of some intermediate mRNAs by gel electrophoresis indicated diffuse bands, equal to and longer than the coding regions, suggesting preferred sites of 3' end formation that do not correlate with early gene transcriptional termination signals.⁴¹ The original 5 VACV genes belonging to the intermediate class^{384,803} have been expanded to 53.⁸⁶⁷ Three of the intermediate genes (*A1L*, *A2L*, and *G8R*) encode late-stage transcription factors,³⁸⁴ and others include DNA binding/packaging and core-associated proteins.

The DNA replication requirement for intermediate gene expression may result from the inaccessibility of the genome within the infecting particle to newly synthesized transcription factors. This hypothesis is consistent with transfection experiments showing that DNA isolated from purified virus particles can serve as a template for intermediate and late transcription in the absence of DNA replication.³⁸⁴

Intermediate-Stage Promoters

Mutagenesis of intermediate promoters indicated two important regions: a 14-bp core element separated by 10 or 11 bp from a 4-bp initiator element.^{42,392} The intermediate core resembles that of early promoters in A/T richness but differs in specific sequence. The tetranucleotide TAAA serves as an initiator element of intermediate promoters. A consensus (Fig. 66.9) has been derived from an analysis of the sequences preceding recently identified intermediate ORFs.⁸⁶⁷ Intermediate-stage RNAs are initiated within the AAA triplet of the coding strand, but as discussed later, they contain additional A residues incorporated by a polymerase slippage mechanism.

TABLE 66.6 Viral Stage-Specific Transcriptional Regulatory Factors

Stage	Factor	Gene ^a		kD	T ^b	C ^c	References
		COP	WR				
Early	VETF	A7L	126	82	L	P	271
		D6R	111	74	L	P	271
Intermediate	RAP94	H4L	102	94	L	P	9
	VITF-1	E4L	060	30	E	C	635
	VITF-3	A8R	127	34	E	C	649
		A23R	143	45	E	P	649
		Cap enzyme	D1R	106	E	P	802
Late	VLTF-1	D12L	117	33	E	P	802
		G8R	086	30	I	C	384
	VLTF-2	A1L	119	17	I	P	384
	VLTF-3	A2L	120	26	I	P	384
	VLTF-4	H5R	103	22	E	C	401

^aCopenhagen (COP) or Western Reserve (WR) designation.

^bTime of synthesis: early (E), intermediate (I), or late (L).

^cConservation in all POXV (P) or chordopoxviruses (C).

Enzymes and Factors for Intermediate-Stage Transcription

Intermediate promoter templates can be transcribed by extracts prepared from cells infected with VACV in the presence of an inhibitor of DNA replication.⁸⁰³ Further studies indicated a role for both viral and cellular proteins. The viral proteins include RNA polymerase; the capping enzyme, which acts by a mechanism that does not involve cap formation^{304,802}; VITF-1, which is encoded by the gene for RPO30, a viral RNA polymerase subunit with homology to eukaryotic transcription elongation factor TFIIS⁶³⁵; and VITF-3, a heterodimer composed of polypeptides encoded by the *A8R* and *A23R* genes⁶⁴⁹ (Table 66.6). Unlike the early transcription factor VETF, neither VITF-1 nor VITF-3 has ATPase activities or exhibits demonstrable sequence-specific DNA binding. A cellular component, called VITF-2, was found to be necessary to complement *in vitro* transcription.⁶³⁶ Recently, both natural and recombinant forms of two cellular proteins, Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137), which form a complex, were individually shown to fulfill the requirement for VITF-2 in an *in vitro* complementation assay.³⁸⁰ Their role *in vivo* is suggested by the accumulation of both G3BP and p137 at sites of transcription in viral factories (Fig. 66.10). There is evidence from *in vivo* experiments employing RNA interference (RNAi) and dominant negative inhibitors that the cellular TATA-binding protein has a positive role in VACV intermediate and late gene expression and that YY1 has a negative role by binding to promoter and initiator elements, respectively.^{392,393} The reliance on cellular proteins is surprising because POXVs encode their own RNA polymerase and other transcription factors.

Genetic studies provide evidence for virus-encoded positive and negative regulators of intermediate and/or late transcription elongation. To summarize an interesting story,^{155,471,553} mutations in the *A18R* gene increase transcriptional read-through of downstream gene sequences, whereas mutations of the *G2R* gene decrease mRNA length and *G2R* mutants can rescue *A18R* mut

ants.^{79,157,858} Physical interactions between the proteins encoded by *G2R*, *A18R*, and *H5R* (a late transcription factor) were demonstrated by a variety of methods.^{80,463} Additional biochemical studies suggest that the *A18R* helicase is a transcript release factor and acts in conjunction with an unidentified cellular protein.⁴¹⁰ Genetic approaches also led to the identification of the product of the *J3R* gene, which also serves as the cap 2'-O-MTase and as a subunit of the poly(A) polymerase, as an mRNA elongation factor.^{418,854}

Regulation of Late-Stage Transcription

The transcription of late genes quickly follows that of intermediate genes and persists till the end of the virus life cycle (Fig. 66.8). The half-life of late mRNAs has been estimated to be 30 minutes or less,^{546,668} emphasizing the need for continued high-level transcription. A recent study distinguished intermediate and late genes and identified 38 belong to the latter class and encoding many morphogenesis and mature virion membrane proteins, including those involved in entry, in addition to early transcription factors.⁸⁶⁷ Although distributed throughout the genome, the late-stage genes cluster in the central region (Fig. 66.4).

Late-Stage Promoters and RNA Processing Signal

Late-stage promoters consist of a core sequence of about 20 bp with some consecutive T residues, separated by a region of about 6 bp from a highly conserved TAAAT element within which transcription initiates.¹⁸⁵ A synthetic promoter with exclusively T residues forming the core sequence was stronger than natural late promoters tested, and any mutations of TAAAT severely decreased transcription. A consensus promoter (Fig. 66.9) derived from the sequence upstream of late ORFs is consistent with the mutational analysis.⁸⁶⁷ A G or A usually follows the late promoter TAAAT sequence: in the former case, the TAAATG transcription initiation sequence and the ATG translation initiation codon overlap. The seeming absence of an untranslated

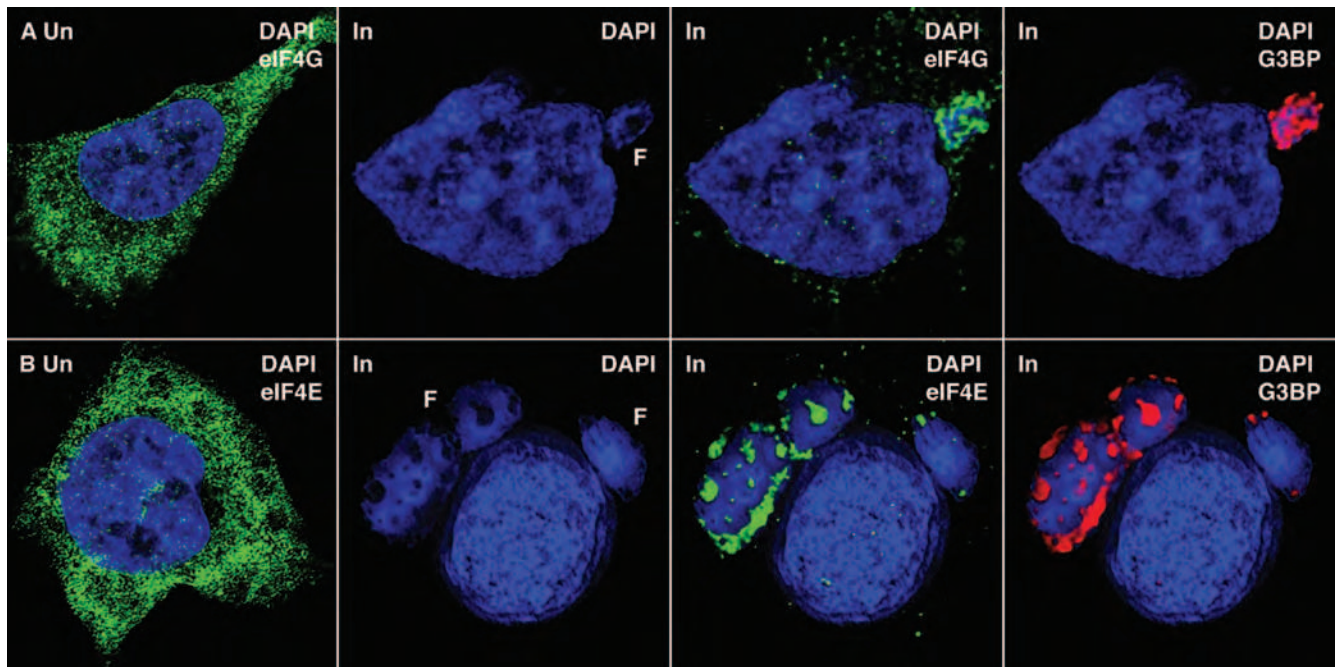


FIGURE 66.10. Localization of translation initiation factors within virus factories. **A:** Uninfected (Un) and vaccinia virus (VACV)-infected (In) HeLa cells were stained with antibody to the elF4G translation factor (green) and to DNA with DAPI (blue). The infected cells were also stained with antibody to cellular G3BP, which co-localizes with viral RNA in the factory. F, viral factory. **B:** Same as panel **A** except that antibody to the elF4E translation initiation factor (green) was used instead of antibody to elF4G. (From Katsafanas GC, Moss B. Colocalization of transcription and translation within cytoplasmic poxvirus factories coordinates viral expression and subjugates host functions. *Cell Host Microbe* 2007;2:221–228.)

RNA leader in this situation was puzzling, until it was found that late mRNAs have a 5′ capped, heterogeneous-length, poly(A) leader sequence formed by RNA polymerase slippage.^{567,667} Poly(A) leaders are also present on mRNAs of certain early genes that have a TAAAT initiation site^{7,356,864} as well as on intermediate mRNAs,⁴¹ suggesting that slippage on an AAA sequence is an intrinsic property of the viral RNA polymerase. The 5′ poly(A) may facilitate stability and translation.^{72,691}

Most postreplicative transcripts are long and heterogeneous and lack defined 3′ ends.^{159,445} The early termination signal is not recognized even though T5NT is frequently present within the coding region of postreplicative genes. Terminal heterogeneity, combined with transcription from both DNA strands, causes overlapping of transcripts, explaining their ability to self-anneal or anneal with early transcripts to form ribonuclease-resistant hybrids.^{89,152,797} Possibly, the VACV-encoded double-stranded RNA-binding protein^{133a} or RNA helicase⁶⁹⁷ prevents deleterious effects of the double-stranded RNA. Alternatively, the 5′ poly(A) leader could compensate for the complementary RNA by providing a single-stranded binding site for initiation factors and the 40s ribosomal subunit, which would then move unimpeded by antisense RNA to the first AUG where ribosome assembly and translation occur.

Exceptions to the general 3′ heterogeneity of late mRNAs occur.²⁵ The CPXV late mRNA encoding the A-type inclusion protein has a 3′ end corresponding to a precise site in the DNA template. The DNA sequence at this position encodes an RNA *cis*-acting signal for RNA 3′ end formation that can function independently of either the nature of the promoter or the RNA

polymerase responsible for generating the primary RNA.³²⁷ Cleavage also generates the 3′ end of F17R transcripts,¹⁶⁷ and endoribonuclease activity associated with the product of the *H5R* gene can cleave this RNA,¹⁶⁸ which is then polyadenylated. The number of late mRNAs that are processed in this manner remains to be determined. Therefore, POXVs employ at least two mechanisms of RNA 3′ end formation. The first, operative at early times in viral replication, terminates transcription downstream of an RNA signal, whereas the second, operative at late times, involves RNA site-specific cleavage.

Enzymes and Factors for Late-Stage Transcription

The viral RNA polymerase catalyzes late mRNA synthesis in conjunction with specific factors. Late transcription factors (Table 66.6) were identified by the systematic screening of cloned viral DNA fragments: open reading frames A1L, A2L, and G8R were necessary and sufficient for transactivation of a transfected late promoter reporter gene in VACV-infected cells that were blocked in DNA replication.³⁸⁴ An intermediate promoter regulates each of these late transcription factor genes, which is consistent with a cascade model of regulation. *In vitro* studies confirmed that the products of the *G8R*, *A1L*, and *A2L* genes are VACV late transcription factors,^{342,386,566,851,852} and they have been named VLTF-1, VLTF-2, and VLTF-3, respectively. Temperature-sensitive and repressible mutations of A1L and G8R, respectively, block late gene expression under nonpermissive conditions.^{126,884} Both A1 and A2 can bind zinc,³⁸⁵ and yeast two-hybrid studies indicate interactions between G8 with itself and with A1.⁴⁶³ Computational analysis suggests

that G8 has a PCNA-like sliding clamp motif.¹⁷⁴ *In vitro* studies indicated that the product of the early *H5R* gene, named VLTF-4, stimulated late transcription severalfold,⁴⁰¹ and interactions between H5 and other late transcription factors have been described.²⁰⁵ H5 also appears to have a role in transcript elongation.⁸⁰ *In vitro* transcription assays suggest a role for a host factor originally called VLTF-X and shown to consist of the heterogeneous nuclear ribonucleoproteins A2/B1 and RBM3.^{292,853} As noted above, there is evidence that the cellular TATA-binding protein has a positive role in VACV intermediate and late gene expression and that YY1 has a negative role by binding to promoter and initiator elements.^{392,393}

DNA REPLICATION

General Features

POXV DNA replication takes place in the cytoplasm and can occur even in enucleated cells.^{575,593} Discrete cytoplasmic foci of replication, termed *factory areas*, are discerned by light and electron microscopic autoradiography.^{121,302} Factories visualized with a dye that stains double-stranded DNA are shown in Figure 66.10 (which also shows the redistribution of translation initiation factors discussed later and the localization of the G3BP, which is involved in intermediate transcription as discussed earlier). Each factory can be initiated by a single virion, although factories merge together with time.^{379,429} Replicating DNA may be associated with endoplasmic reticulum (ER) membranes.⁶⁶⁵ The time of onset of DNA synthesis varies with different members of the POXV family and to some extent with the multiplicity of infection and cell type. DNA replication begins 1 to 2 hours after infection of growing cells synchronously infected with VACV and results in the generation of about 10,000 genome copies per cell, of which half are ultimately packaged into virions.^{370,643} Studies depending on thymidine incorporation instead of hybridization for DNA quantification generally underestimated the length of the replication period, possibly because of a decline in the activity of the viral thymidine kinase (TK) used for incorporation of the radioactive precursor via the salvage pathway.

Efforts to locate a specific POXV origin of replication using a plasmid assay in transfected cells were unsuccessful; surprisingly, any circular DNA replicates in cells infected with Shope fibroma virus (SFV)²⁰³ or VACV.⁴⁸⁷ Further studies demonstrated that origin-independent plasmid replication occurs in virus factory areas and requires each protein needed for genome replication, confirming the specificity of this activity.¹⁹³ The absence of any stimulatory effect of VACV DNA sequences led to speculation that POXVs, unlike nuclear DNA viruses, do not require specific replication origins. Using another transfection assay, in which the template was a linear DNA molecule containing VACV hairpin ends, a specific enhancing effect of the terminal 200 bp of the viral genome was found.²²⁶ The mechanism of enhancement is uncertain, however, and this region contains the concatemer resolution sequence (to be described later).

DNA Replication Models

The unique terminal structure of the POXV genome,⁵³ evidence that nicking and initiation occur near the ends of the molecule,^{588,590} and the presence of junction fragments^{54,522} and high-molecular-weight DNA^{201,486} suggested a model similar

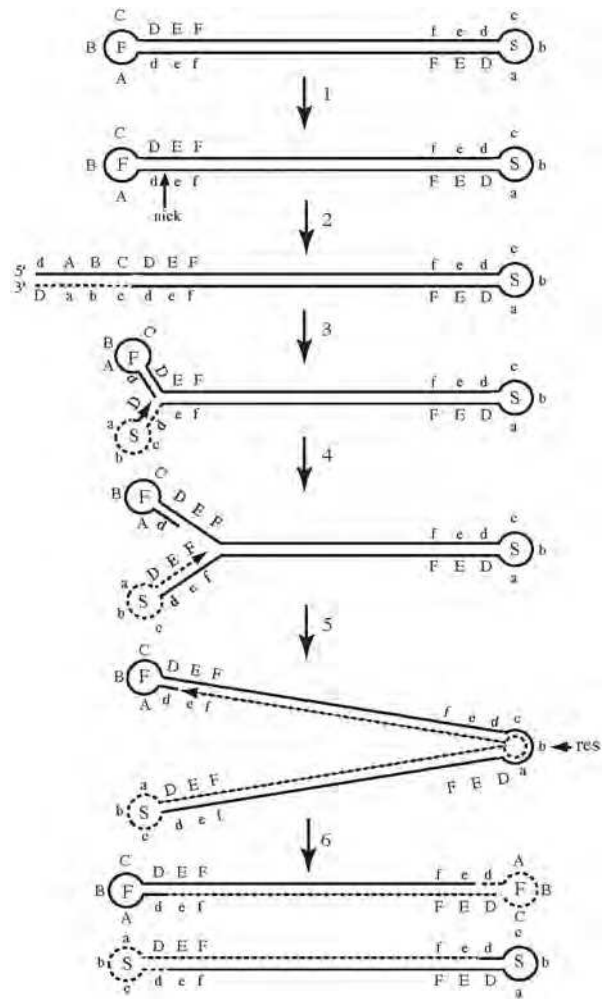


FIGURE 66.11. Self-priming model of poxvirus DNA replication. F and S within the hairpin loops refer to the fast and slow electrophoretic mobilities of DNA fragments containing inverted and complementary hairpin sequences as shown in Figure 66.3. Dashed lines indicate newly synthesized DNA with arrowheads at the 3' OH ends. Complementary sequences are depicted by upper- and lowercase letters. The resolution site (res) within the concatemer junction is indicated. (Adapted from Moss B, Winters E, Jones EV. Replication of vaccinia virus. In: Cozzarelli N, ed. *Mechanics of DNA Replication and Recombination*. New York: A. Liss, 1983:449–461.)

to the rolling hairpin strand displacement mechanism proposed for replication of single-stranded parvovirus DNA.⁷⁵⁸ As depicted in Figure 66.11, a hypothetical nick occurring at one or both ends of the genome provides a free 3' end for priming replication. The replicated DNA strand then folds back on itself and the replication complex copies the remainder of the genome. Concatemer junctions form after replication through the hairpin; very large branched concatemers can arise by initiating new rounds of replication before resolution occurs. After the onset of late-stage transcription, unit-length genomes are resolved and the incompletely base-paired terminal loops, with inverted and complementary sequences, are regenerated. Despite the attractiveness of this model, neither the site

of nicking nor an essential nicking enzyme needed to initiate DNA replication has been identified.

A more conventional model posits RNA priming and semidiscontinuous DNA synthesis at replication forks. Early reports of VACV DNA covalently linked to RNA and the chasing of short DNA into larger molecules suggested lagging strand synthesis.^{238–240,589} A major difficulty with the discontinuous model, however, had been that a virus-encoded RNA primase had not been identified and the virus-encoded DNA ligase was not essential. However, as described later, a VACV-encoded primase was recently discovered and it was found that a cellular DNA ligase substitutes for the viral ligase in the absence of the latter. In addition, recombination-dependent DNA replication involving invasion of duplex DNA by the 3' OH end of single-stranded DNA as described for phage T4⁴⁰³ could account for branched DNA molecules. The aforementioned models are not mutually exclusive and it is possible that POXVs use multiple mechanisms to achieve replication of their large genomes.

Enzymes Involved in DNA Precursor Metabolism

Many POXVs encode enzymes involved in the synthesis of deoxyribonucleotides, evidently to enhance DNA replication in cells with suboptimal precursor pools, whereas others lack some or all of these enzymes. In OPXVs, these enzymes include a TK,^{39,329} thymidylate kinase,⁷¹³ ribonucleotide reductase,^{705,761} and deoxyuridine triphosphatase (dUTPase).¹⁰² In addition, there is an incomplete guanylate kinase, raising the possibility that an intact form might be found in some other POXV.⁷¹² Leporipoxviruses are missing genes encoding the large subunit of ribonucleotide reductase, thymidylate kinase, and remnants of the guanylate kinase,^{122,835} and MOCV is missing all of them, perhaps contributing to its limited host range.⁶⁷⁴ FWPV encodes a protein related to human deoxycytidine kinase that is not present in the other POXVs.³⁹⁶ Entomopoxvirus *Melanoplus sanguinipes* lacks all of the previously described POXV genes involved in nucleotide metabolism but has a thymidylate synthetase homolog.³ However, a homologous TK gene is present in other entomopoxviruses.^{288,443}

The TKs encoded by POXVs are 20 to 25 kD and related in sequence to corresponding eukaryotic enzymes (35% to 70% amino acid identity) but not to the pyrimidine kinase of herpesviruses. The structure of VACV TK is very similar to human TK, but there are subtle differences in the association with deoxythymidine triphosphate (dTTP) that might be exploited for antivirals.²³³ The TK gene is regulated by an early promoter, as befits its role in increasing precursors for DNA replication.⁸²⁴ The VACV enzyme exists as a tetramer, has ATP and Mg²⁺ binding domains, and is susceptible to feedback inhibition by deoxythymidine diphosphate (dTDP) or dTTP.⁸¹ Although the TK gene is not required for virus growth in tissue culture cells, deletion mutants are severely attenuated *in vivo*.¹¹³

Thymidylate kinase catalyzes the next step in thymidine monophosphate (TMP) metabolism. The VACV gene encodes a 23-kD protein that can complement *Saccharomyces cerevisiae* mutants deficient in the homologous enzyme.³⁴³ The protein is expressed early in infection and is not required for virus replication in tissue culture. VACV thymidylate kinase has a 42% identity with its human homolog but differs in substantial ways and has broader substrate specificity.^{31,120}

The synthesis of ribonucleotide reductase, an enzyme that converts ribonucleoside diphosphates to deoxyribonucleoside

diphosphates, is induced soon after VACV infection.⁷⁰⁷ The small catalytic subunit and the large regulatory subunit closely resemble their eukaryotic counterparts structurally (70% to 80% identity) and functionally.^{328,706} Catalytic activity is inhibited by hydroxyurea preventing DNA replication, and drug-resistant mutants generate direct tandem repeats of the gene encoding the catalytic subunit.⁷⁰⁹ Mutation of the large subunit prevents induced ribonucleotide reductase activity in tissue culture cells without affecting replication.¹³⁹ However, the mutant virus was mildly attenuated in a mouse model. In contrast to OPXVs, many chordopoxviruses only encode the large subunit, which complexes with the cellular small subunit to form active ribonucleotide reductase.²⁶⁰

The hydrolysis of deoxyuridine triphosphate (dUTP) by the VACV dUTPase provides deoxyuridine monophosphate (dUMP), an intermediate in the biosynthesis of TTP, and might also minimize dUTP incorporation into DNA.¹⁰² The protein is synthesized early in infection⁷⁰⁸ and is nonessential for virus replication in dividing cells.⁵⁷⁹ The dUTPase is more important in quiescent cells, particularly in the absence of the viral uracil DNA glycosylase (UDG).¹⁹² The structure of VACV dUTPase is very similar to the human homolog, although there are subtle differences that might be exploited in drug design.⁶⁴⁴

Viral Proteins Involved in DNA Replication

Several complementation groups of temperature-sensitive (ts) mutants that express VACV early proteins are impaired in DNA synthesis,⁴¹¹ and multiple proteins constitute the replication complex. One complementation group contains mutations in the DNA polymerase, which has a mass of about 110 kD, an associated 3' exonuclease activity, and homology with other eukaryotic and viral DNA polymerases.^{132,229,464} Certain codon substitutions confer resistance to inhibitors of DNA synthesis, providing information regarding the active site of the polymerase.^{199,229,755,756,771}

A second DNA[−] complementation group maps to the *D5R* gene, which encodes a 90-kD protein with a nucleic acid-independent nucleoside triphosphatase activity.^{243,244,245,638} A motif present in the archaeo-eukaryotic primase superfamily is present in the N-terminal domain of D5 and its orthologs,³⁶² and primase activity has been demonstrated.^{191,195} Moreover, mutations of D5 in either the nucleoside triphosphatase (NTPase)⁹⁵ or the primase¹⁹¹ active site are unable to complement a conditional lethal ts mutant.

A third DNA[−] complementation group maps to the B1R ORF, which encodes a 35-kD serine/threonine protein kinase that is expressed early in infection and packaged in virions.^{49,428,612} Human homologs of B1 can complement the replication defect of a ts VACV mutant defective in B1 kinase activity.^{96,537,540} A recent discovery is that a cellular cytoplasmic protein called barrier to autointegration factor, or BAF, acts as a potent inhibitor of POXV DNA replication unless its DNA-binding activity is blocked by B1-mediated phosphorylation.^{541,832} This activity of the B1 kinase can explain its requirement for VACV DNA replication. The B1 kinase may have additional substrates involved in immune defense.^{647,648} An early protein encoded by the *H5R* gene is a substrate for the B1 kinase,⁶⁶ and interactions between these proteins was demonstrated by the yeast two-hybrid system.⁴⁶³ H5 appears to be a multifunctional protein with roles in DNA replication, transcription, mRNA processing, and morphogenesis.^{80,162,168,169,401}

A fourth VACV ts mutant impaired in DNA replication maps to the D4R ORF.⁴⁸⁹ A mutant with a deleted *D4R* gene could be propagated in a transfected cell line stably transfected with D4R.³²³ Both the D4R ORF and its SFV homolog encode functional uracil DNA glycosylases (UDGs).^{740,788} Because these enzymes function in DNA repair by removing uracil residues that have been introduced into DNA, either through misincorporation of dUTP or through the deamination of cytosine, a DNA⁻ phenotype was surprising. However, mutagenesis studies demonstrated that the requirement for UDG is independent of its glycosylase activity, suggesting that it functioned as part of the replication complex.¹⁹⁴ Nevertheless, VACV mutants with enzymatically inactive UDG are attenuated in mice, indicating that the repair function is beneficial, consistent with the preservation of the catalytic site in all POXV orthologs. The sequence identity of POXV UDG with non-POXV homologs is only about 20% and differs in secondary and tertiary structure.⁶⁶⁴

A role for the VACV 49-kD A20 protein, for which non-poxvirus homologs could not be found, was first suggested by a yeast two-hybrid analysis that demonstrated an interaction with the DNA replication proteins D4 and D5 as well as H5.⁴⁶³ Additional yeast two-hybrid studies demonstrated that non-overlapping regions of A20 bind D4, D5, and H5, suggesting that these proteins can interact simultaneously to form a multicomponent complex.³⁵⁹ The interaction of these proteins in infected cells was confirmed by co-immunoprecipitation.³⁵⁹ A role for A20 in VACV DNA replication was shown by targeted mutagenesis^{360,595} and evidence that the complex of A20 and D4 with DNA polymerase imparts processivity.⁷³⁴

The VACV *I3L* gene encodes a 34-kD phosphoprotein that is expressed early in infection, forms octameric complexes on single-stranded DNA,^{183,623,774} and is found in punctate cytoplasmic inclusions containing parental DNA.^{221,825} The inability to isolate a deletion mutant suggests that it is essential for VACV replication.⁶²³

VACV encodes a functional ATP-dependent DNA ligase that is not essential for replication in tissue culture, although it imparts sensitivity to DNA damaging agents and is important for virulence.^{153,387} The ability of VACV to replicate in the absence of the viral DNA ligase depends on cellular DNA ligase I, which is recruited to the viral DNA factories in the cytoplasm.⁵⁵⁸ Thus, viral or cellular ligase activity appears to be required for VACV replication. Furthermore, replication of ligase-deficient VACV was greatly reduced and delayed in resting primary cells, correlating with initial low levels of ligase I and subsequent viral induction and localization of ligase I in virus factories.⁵⁵⁸ By encoding its own ligase, VACV can “jump-start” DNA synthesis in resting cells and enhance replication.

Photolyases, which protect DNA from ultraviolet radiation by excision of cyclobutane pyrimidine dimers, are encoded by avipoxviruses and leporipoxviruses as well as entomopoxviruses.^{71,533,730,731} This enzyme is likely important for POXVs that are transmitted through the environment rather than from animal to animal.

Concatemer Resolution

The replication of the POXV genome involves the formation of concatemers and their resolution into unit-length molecules.^{54,522} The concatemer junction consists of a precise duplex copy of the hairpin loop present at the ends of mature DNA

genomes.⁴⁸³ Circular plasmids containing VACV⁴⁸⁵ or SFV²⁰⁴ concatemer junctions are converted into linear molecules with hairpin termini when transfected into POXV-infected cells. Using this assay, the structural and sequence requirements for resolution of concatemer junctions were determined by site-directed mutagenesis.^{202,467,482,484} The minimal requirement for resolution is two copies of the sequence T₆-N₇₋₉-T/C-A₃-T/A present in an inverted repeat orientation on either side of an extended double-stranded copy of the hairpin loop. Interestingly, the resolution sequence encompasses a functional promoter, suggesting that resolution may be linked to transcription.^{334,739} The sequence of the intervening region, destined to form the hairpin loop, is not highly conserved but must be palindromic and less than 200 bp long.

Concatemer resolution occurs rapidly, as junctions do not accumulate unless postreplicative gene expression is inhibited.^{201,486} DNA incapable of entering a pulse-field gel also accumulates under the latter conditions, suggesting multiply branched DNA molecules. Studies with drugs and mutants indicate that concatemer resolution occurs independently of virus assembly.

The concatemer junction contains an inverted repetition, which in supercoiled plasmids can form a cruciform structure resembling a four-way Holliday junction (HJ) recombination intermediate.^{213,483} This suggested that the putative concatemer-resolving enzyme would be an HJ resolvase. Bioinformatic analyses led to the discovery of motifs and structural elements that are critical for activity of *Escherichia coli* RuvC HJ resolvase in ORFs that are conserved in all POXV genomes.²⁶² Moreover, the RuvC homolog encoded by the VACV *A22R* gene was expressed as a recombinant protein in *E. coli* and found to specifically cleave HJs.²⁶² Like RuvC, the A22 protein is a dimer in solution and when bound to HJ structures, but exhibits less cleavage sequence specificity.²⁶⁴ The quaternary structure and cleavage specificity of the HJ have been explored extensively.^{165,264} A VACV-inducible A22 null mutant was defective in processing concatemers into unit-length genomes with hairpin ends under nonpermissive conditions, indicating that the enzyme is required for resolution, although additional proteins may also be involved in determining site specificity.²⁶³

Type I DNA topoisomerases form a covalent link with DNA and relieve supercoils during replication, transcription, recombination, and other activities. POXVs encode a type I topoisomerase that has served as a model for this class of enzymes.^{686,702,862} The POXV topoisomerase is unusual in that it exhibits some sequence specificity.⁶⁹⁶ *In vitro*, the topoisomerase can carry out a variety of reactions including strand transfer, transesterification, recombination, and cleavage and ligation of a variety of DNA structures *in vitro* including an HJ.^{554,672} The late promoter of the topoisomerase gene is consistent with a role in early transcription or DNA processing *in vivo*. Characterization of a VACV topoisomerase deletion mutant that forms very small plaques indicated that the enzyme participates in early transcription within the virus core and that subsequent DNA synthesis and concatemer resolution occurred.¹⁷⁰

Homologous Recombination

Recombination occurs actively within POXV-infected cells²⁴⁹ and has apparently occurred naturally between SFV, which produces benign fibromas in rabbits, and myxoma virus (MYXV), the agent of myxomatosis, to form malignant rabbit fibroma

virus,⁸⁶ and between individual capripoxviruses.²⁷⁰ Recombination between the terminal sequences of POXV DNA may explain variations in the number of tandem repeats as well as translocations and mirror image deletions.^{237,466,515,523} Most extraordinarily, field and vaccine strains of FWPV carry a near-full-length and apparently infectious integrated avian retrovirus genome.³¹⁰

Recombination can also occur between viral genomic DNA and transfected subgenomic DNA fragments, and this has been exploited to map and construct mutations and to insert genes for expression.⁵⁰⁹ Viral genomes rapidly eliminate direct repeats with the formation of intra- and intermolecular recombination products.⁴⁵ Single- and double-crossover products resulting from recombination between transfected plasmids and viral genomes⁷²⁹ and inter- and intramolecular plasmid or bacteriophage DNA recombinants^{242,561} have been detected in POXV-infected cells.

The mechanism of recombination has not been fully elucidated. Recombination does not require postreplicative gene products, and there appears to be a strong connection between recombination and replication.^{481,834} There is evidence that the DNA polymerase participates directly in recombination and that the 3' exonuclease and DNA joining activities are involved.^{259,299,300} The protein encoded by the *G5R* gene, which is conserved in all POXVs and expressed early in infection, belongs to the FEN1 family of *exo-/endonucleases*.^{173,361} A VACV G5 deletion mutant is severely impaired and the yield of infectious virus is reduced by two orders of magnitude.⁶⁷⁵ The mutant virions contain an apparently normal complement of proteins but appear spherical rather than brick shaped and contain little or no DNA. Although the amount of viral DNA produced in the absence of G5 is similar to that made by wild-type virus, the mean size is approximately one-fourth of the genome length. Experiments with transfected plasmids show that G5 was required for double-strand break repair by homologous recombination, suggesting a similar role during VACV genome replication.⁶⁷⁵ Double-strand breaks commonly occur at replication forks, consistent with the semidiscontinuous model of POXV DNA replication.

The chordopoxviruses, except for avipoxviruses, encode homologs of serine recombinases.¹ However, only the crocodile POXV contains all amino acids that make up the catalytic active site, and deletion of the VACV F16 homolog had no effect on replication in cultured cells.⁶⁷⁶

VIRION ASSEMBLY

Overview

The assembly of VACV is outlined within Figure 66.7, and a thin-section transmission electron microscopic image of a cell infected with VACV and enlarged pictures of virions at successive stages of assembly and egress are shown in Figures 66.12 and 66.13, respectively. The initial stages of virion assembly occur in the circumscribed, granular, electron-dense areas of the cytoplasm containing viral DNA called virus factories. The first morphologically distinct structures are crescent membranes and circular immature virions (IVs) that represent sections through cupules and spheres. These structures consist of a membrane with spicules on the convex surface and granular material in the concavity. Deep-etch electron microscopy revealed that the

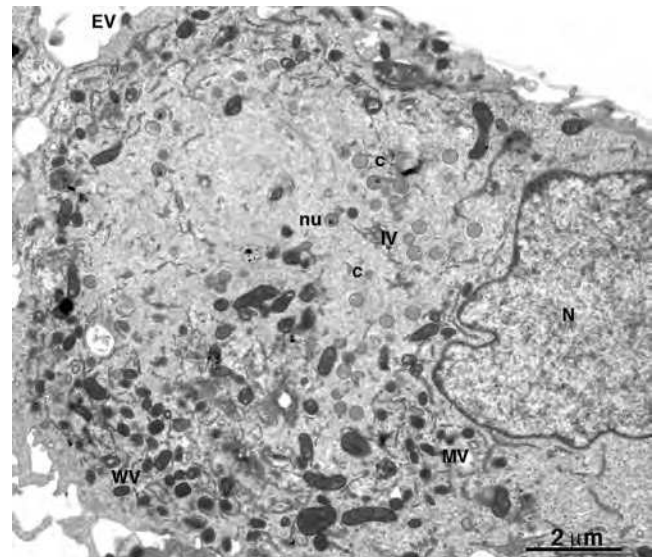


FIGURE 66.12. Transmission electron microscopy of cell infected with vaccinia virus (VACV). N, cell nucleus; c, viral crescent; IV, immature virion; nu, DNA nucleoid within IV; MV, mature virion; WV, wrapped virion; EV, extracellular enveloped virion. (Image kindly provided by A. Weisberg.)

spicule layer is actually a honeycomb lattice viewed on edge (Fig. 66.14).^{311,750} A dense nucleoprotein mass containing the genome enters the immature envelope (Fig. 66.13) before it is completely sealed,⁵⁰¹ apparently in association with a membrane.⁶⁶⁵ Next, the spherical IV is converted to an oval or brick-shaped particle, with a concomitant loss of the lattice scaffold and internal reorganization (Fig. 66.13). The MV is the most abundant infectious form of VACV, although it can only be released by cell lysis. VACV assembly and morphogenesis is the subject of a recent review.¹⁵⁶

Formation of the Crescent and IV Membrane

The mechanism of IV formation is incompletely understood. Microscopic images showing crescents open to the cytoplasm and with no apparent connection to cellular organelles led to the idea that the viral membrane forms *de novo*.¹⁷⁷ Such an open architecture, however, goes against current thoughts that stable membranes must have sealed ends and arise from pre-existing membranes. An alternative hypothesis proposed that the IV membrane is a flattened cisterna derived from a cellular organelle and hence is a double membrane. While some images seem to support two membranes,^{283,620,718} others clearly show a single bilayer with a spicule or lattice protein coating.^{177,188,284,303,322,501} and the group originally promoting a double-membrane has accepted the single-membrane structure.¹⁴³ Moreover, freeze fracture studies provide no evidence for more than one bilayer.³¹¹

In the absence of unambiguous images depicting crescent membranes in continuity with membranes of cellular organelles, other approaches to determine their origin have been pursued. The evidence seems to favor the ER, although the intermediate compartment between the ER and Golgi apparatus (ERGIC) has been suggested. Antibodies to viral membrane proteins co-localize with markers of the ERGIC^{404,620,642,718} and an

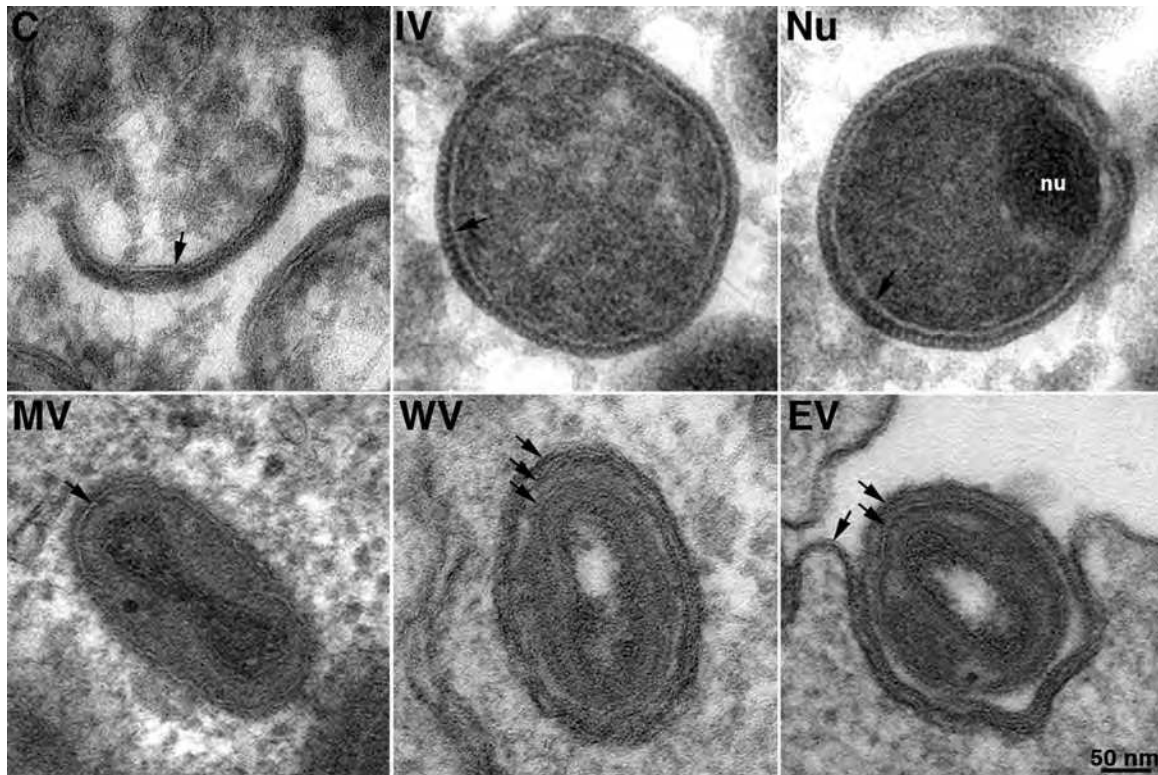


FIGURE 66.13. Stages of vaccinia virus (VACV) morphogenesis. Infected cells were cryosectioned and viewed by transmission electron microscopy. In the panels showing crescent stage (C), immature virion (IV), immature virion with nucleoid (Nu), and mature virion (MV), the arrow points to the single outer membrane. Note the presence of the thick “spicule layer” comprising the D13 lattice on the outside of the crescent and IVs. In the panel showing the wrapped virion (WV), the inner arrow points to the MV membrane and the two outer arrows to the double wrapping membrane. In the panel showing the extracellular enveloped virion (EV), one arrow points to the outer wrapping membrane that has fused with the plasma membrane; the other arrows point to the MV membrane and remaining EV wrapper. (Courtesy of A. Weisberg.)

inhibitory effect of the protein kinase inhibitor H89 on viral morphogenesis was interpreted as due to blocking ER exit.⁵⁹⁶ However, blockade of the COPII ER exit pathway by dominant negative inhibitors of the Sar1 GTPase³⁴⁵ and the drug brefeldin A⁷⁸² have no effect on formation of IVs or MVs, suggesting that the ERGIC is not a necessary source of the IV membrane. In addition, MV membrane proteins have been shown to associate with the ER although the opposite (i.e., association of ER proteins with IV membranes) has not,³⁴⁸ and none of the viral proteins incorporated into purified MVs have a signature of ER translocation, such as signal peptide cleavage or glycosylation. However, a heterologous signal peptide fused to the N-terminal region of a VACV membrane protein was cleaved and the truncated protein localized in IVs and MVs, providing evidence for a functional pathway between the ER and viral membranes.³⁴⁸

Several proteins required for the formation of crescents and IVs have been identified. The formation of masses of electron-dense “viroplasm” resembling the interior of IVs with few or no crescent membranes represents a characteristic phenotype of cells infected with F10 kinase,^{770,811} H5,²⁰⁷ G5,¹⁷¹ A11,⁶¹⁶ H7,⁶⁵⁵ and L2⁴⁵⁸ conditional lethal mutants. H5 and G5 have other roles, so their action in crescent formation may be indirect. In contrast to the others, L2 is made early in infection, has a

transmembrane domain, associates predominantly with the ER, and can be found near the growing edge of crescents.⁴⁵⁹ F10 phosphorylates the A14 and A17 IV membrane proteins^{75,211} and the kinase activity is required for its function in morphogenesis.^{596,751} A11 is phosphorylated but not by the F10 kinase.⁶¹⁶

When synthesis of the A17 or A14 protein is repressed, small vesicles accumulate, though they differ in appearance, and crescent formation is reduced or abrogated.^{628,629,772,847} The related phenotypes are consistent with evidence that the two proteins interact with each other.^{75,480,628} Whether these vesicles represent true intermediates that accumulate due to the absence of A17 or A14 or represent aberrant structures is unknown.

In the presence of the antibiotic rifampicin, irregular viral membranes that lack the honeycomb lattice accumulate (Fig. 66.15).^{512,529} Within minutes after removal of the drug, the single-membrane bilayer becomes coated with the protein forming the honeycomb and assumes a crescent shape even in the presence of inhibitors of RNA or protein synthesis, suggesting that rifampicin directly interferes with assembly.²⁸⁴ The gene responsible for resistance of mutant viruses to rifampicin was mapped to the D13L ORE,^{43,135,757b} which encodes a 65-kD protein that rapidly associates with the rifampicin membranes after drug removal.^{490,719,795} When expression of the D13 is repressed, morphogenesis of the viral envelope is blocked at

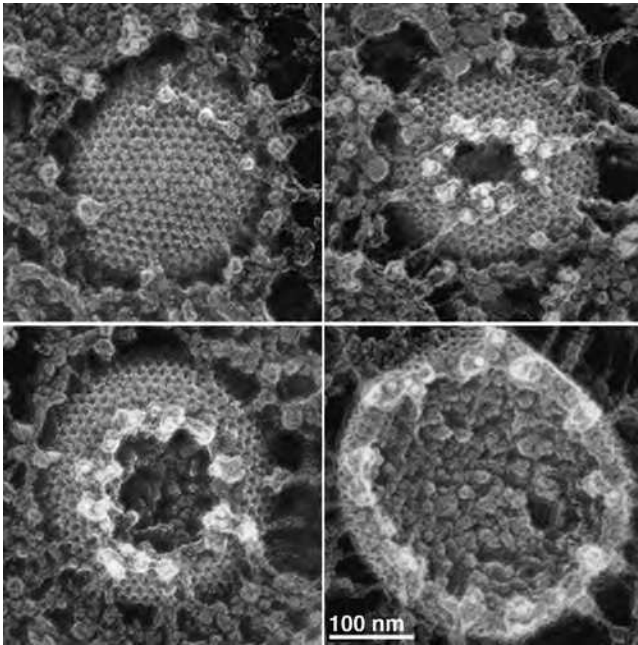


FIGURE 66.14. Deep-etch electron microscopy of immunogold-labeled immature virions (IVs). Thawed cryosections of IVs were labeled with antibody to the D13 scaffold protein and protein A conjugated to gold spheres and then were processed for deep-etch electron microscopy. IVs that were uncut or cut at various depths off their equator are shown. The gold particles look white as a result of contrast reversal. (From Szajner P, Weisberg AS, Lebowitz J, et al. External scaffold of spherical immature poxvirus particles is made of protein trimers, forming a honeycomb lattice. *J Cell Biol* 2005;170:971–981.)

the same stage as occurs with rifampicin.⁸⁸² The 65-kD protein forms trimers that assemble to form the honeycomb lattice scaffold, which gives the IVs a spherical shape.⁷⁵⁰ Deep-etch electron microscopy of immunogold-labeled sections at various depths through IVs is shown in Figure 66.14. Note that this technique only labels the cut surfaces of the IVs. The D13 protein interacts with the N-terminal region of A17 to form the scaffold.⁷⁸ The retention of a capsid-like structure around the immature form of POXVs suggests that their morphogenesis, like embryogenesis, recapitulates evolution to the present MV form.⁷⁵⁰ Structural studies confirm the predicted similarity of D13 with the capsids of other viruses and phage.^{37,350,351}

Association of Core Proteins With IVs

The dense viroplasm that is engulfed during the extension of the crescent into the IV contains unprocessed core proteins. Insight into the association of the core proteins with the crescent membrane has been obtained through use of conditional-lethal VACV mutants. Seven core proteins, A15, A30, D2, D3, F10, G7, and J1, are each required for the association of crescent membranes with the granular viroplasm.^{142,748,749,752–754} These seven proteins form a complex that is presumably linked to one or more membrane proteins. Similar phenotypes occur when expression of the F17 (also called F18), A10, and E6 core proteins are repressed,^{94,307,615,624,883} and the former has also been found in association with the complex.⁸³¹

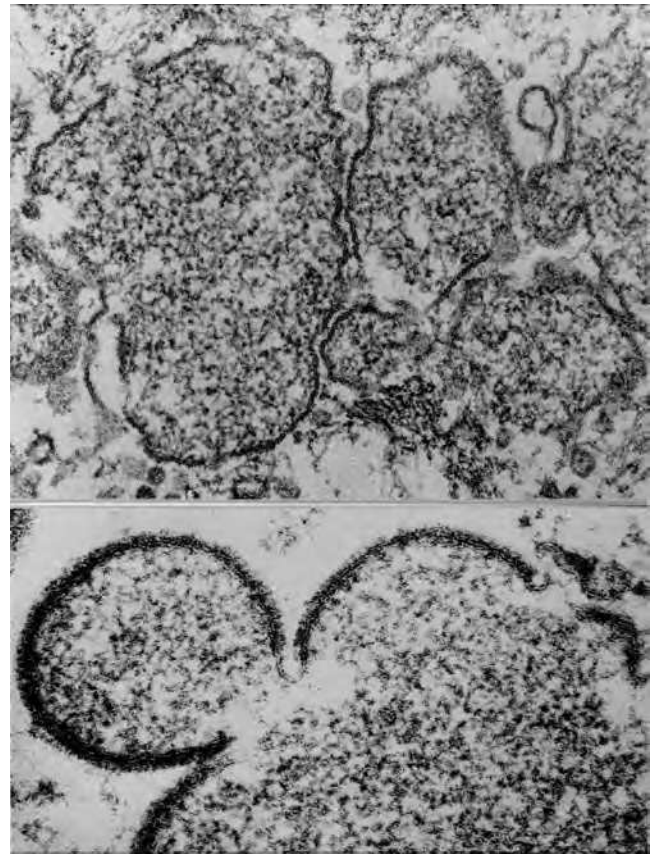


FIGURE 66.15. Electron micrographs of thin sections of cell infected with vaccinia virus (VACV) in the presence of rifampicin. Upper panel: HeLa cells were fixed and sectioned at 8 hours after infection in the presence of rifampicin. Lower panel: Cells were fixed and sectioned at 10 minutes after removal of rifampicin. Note the thin uncoated single membrane bilayer loop connecting the more rigid crescent-like membranes that have acquired the "spicule" layer. (From Grimley PM, Rosenblum EN, Mims SJ, et al. Interruption by rifampin of an early stage in vaccinia virus morphogenesis: accumulation of membranes which are precursors of virus envelopes. *J Virol* 1970;6:519–533.)

Genome Packaging

Electron micrographs show DNA nucleoids entering IVs before their complete closure.⁵⁰⁰ When morphogenesis is interrupted at a pre-IV stage, the viral DNA concatemers are processed normally and mature DNA accumulates in large crystalloid structures.²⁸⁴ The ultrastructure of these crystalloids is similar to that of nucleoids in normal IVs. Although concatemer resolution occurs in the absence of viral morphogenesis, the opposite is not true; for example, DNA processing is required for morphogenesis.²⁶³ A DNA packaging role for the protein encoded by the *A32L* gene was predicted because of sequence similarity to the products of gene *I* of filamentous single-strand DNA bacteriophages and to the *IVa2* gene of adenovirus, both of which are ATPases involved in DNA packaging.³⁹⁷ This hypothesis was confirmed by the finding that repression of A32 synthesis blocked VACV genome packaging.¹³⁰ DNA packaging also fails to occur under nonpermissive conditions in cells infected with a ts mutant that mapped to the I6 telomere-binding protein

of VACV.^{206,287} Mutation of a second telomere-binding protein encoded by the *I1* gene, however, causes a defect at a later stage of morphogenesis.^{287,390} Conditional lethal mutants of the A13 protein, which is a component of the virion membrane, also display a defect in genome packaging, and DNA crystalloids associated with membrane accumulate.⁷⁸⁴ Exactly how the membrane protein, telomere-binding protein, and ATPase enable genome entry is unknown.

Packaging of the Transcription Apparatus

Like other core proteins, the transcription enzymes and factors are enclosed within the IVs. Morphologically normal but non-infectious virus particles form when synthesis of RAP94, the RNA polymerase-associated protein required for transcription of early genes, is repressed.⁸⁸¹ These particles contain structural proteins as well as the early transcription factor VETF, but lack the viral RNA polymerase, poly(A) polymerase, capping enzyme, topoisomerase, NPH I, and NPH II. Such a specific effect could be explained by the presence of a multienzyme complex that associates with promoter-bound VETF through RAP94. Physical association of RAP94 with NPH I⁴⁹⁴ and VETF⁸⁶⁶ has been demonstrated. A reduced amount of RNA polymerase in virions formed with a ts mutation in VETF⁸⁸¹ is also consistent with the latter model. Repression of synthesis of VETF, however, leads to a more severe defect with accumulation of immature virus particles.^{337,338}

Removal of the D13 Scaffold From IVs

The remarkable transition of the spherical IV to the more compact and barrel-shaped MV involves loss of the D13 scaffold (Fig. 66.13).^{311,497,719} Disassembly of the D13 scaffold correlates with processing of A17 by the I7 protease; when I7 expression is repressed, D13 is retained on aberrant virus particles.⁷⁸ Disassembly of the scaffold and transition to MVs fail to occur when expression of A9 is repressed⁸⁶⁹ and with a ts A6L mutant at the nonpermissive temperature.⁴⁷⁵ There is also some accumulation of IVs and delayed formation of MVs when the *H3L* gene is deleted.^{172,426}

Proteolytic Processing of Virion Proteins

At least one membrane protein (A17) and five core proteins (A3, A10, A12, G7, L4) undergo proteolytic processing. Processing of A17 depends on formation of the membrane component of the IV,⁶¹⁶ whereas processing of core proteins fails to occur if a later step in morphogenesis is blocked.^{381,510} In each case, cleavage occurs at the consensus sequence AG↓X.⁷⁹⁴ The protease responsible for cleavage of the aforementioned proteins appears to be the product of the *I7L* gene based on the phenotype of ts mutants^{374,492} and conserved cysteine protease active site mutants.^{23,115,116} Nevertheless, I7 protease activity has not yet been demonstrated *in vitro*. The product of the *G1L* gene contains a motif HXXEH that is found in some metalloproteases. Conditional lethal G1 mutants are blocked in morphogenesis, but processing of the known membrane and core proteins is not affected.^{24,306} Nevertheless, mutagenesis of the putative active site abrogates the ability of G1 to complement a null mutant. The G1 protein itself appears to be proteolytically processed, and one hypothesis is that it mediates self-cleavage.

Intramolecular Disulfide Bond Pathway

The cytoplasmic domains of some MV membrane proteins contain intramolecular disulfide bonds. Because disulfide bonds usually form in the ER, this raised the possibility that POXVs encode novel oxidoreductases. Indeed, the three viral proteins A2.5, E10, and G4 form a unique cytoplasmic disulfide bond pathway.^{673,681,682,829} The atomic structure of G4 has been solved; the protein crystallized as a dimer that buried the Cys-X-X-Cys active site, which could protect the reactive disulfides from reduction in the cytoplasm.^{35,741} Repression of any one of the three redox proteins results in a block in virion maturation. Thus far, nine viral membrane proteins, all involved in virus entry, have intramolecular disulfides formed by the cytoplasmic redox system (Table 66.2). No evidence that this pathway is involved in the formation of disulfide bonds of other membrane proteins or core proteins has been obtained. Both the entry proteins and the redox system are conserved in all POXVs, suggesting their co-development. The POXV redox system is the only one known to operate on the cytoplasmic side of a membrane.

Occlusion of MVs

The MVs of some chordopoxviruses (e.g., CPXV, ECTV, racoonpox virus, and FWPV) become occluded in a dense protein matrix within the cytoplasm. These have been referred to as A-type inclusions (ATIs) to differentiate them from the sites of virus replication and assembly, which are sometimes called B-type inclusions instead of factories.³⁷⁷ Presumably, ATIs are released following degeneration of infected cells and protect the enclosed MVs from the environment. The major ATI protein of CPXV is a 160-kD myristylated species that may represent up to 4% of the total cell protein at late times after infection.^{258,452,568} Some CPXV mutants form inclusions without virions, implicating a role for an MV-specific protein called 4C,^{690,783} which is encoded by the *A26L* gene of VACV.⁴⁶⁸ The A26 protein has a bridging role between the ATI protein and the A27 protein, which is tethered to MVs through interaction with the A17 protein.³²⁶ Although VACV does not form ATIs, a truncated homologous protein is encoded by some strains^{20,186} and is also associated with the A26 protein.³²⁵ Interestingly, neither the A26 nor the A25 protein is present in EVs.⁷⁸³

Entomopoxvirus virions are also occluded.⁷³ Following ingestion by a larval host, infectious particles may be released in the alkaline pH of the gut. The sequences of homologous occlusion proteins, called spheroidin or spherulin, have been deduced from the ORFs of several entomopoxviruses.^{3,50,297,298,650} These proteins are cysteine rich, have a mass of approximately 100 kD, and lack homology to fusolin, the abundant 50-kD spindle-body protein of entomopoxviruses,¹⁷⁹ the A-type inclusion protein of chordopoxviruses, or the polyhedrin protein of baculoviruses.

FORMATION OF THE EV

Overview

The EV is an extracellular form of POXV that consists essentially of an MV with an additional membrane that is important for virus dissemination.^{92,570} For OPXVs, the extra membrane is frequently formed through an intracellular MV wrapping process followed by exocytosis,^{353,501,571} although direct budding of the MV at the plasma membrane can also

occur.^{316,472,776} In contrast, FWPV exits the cell predominantly by budding.⁹¹ There have been several reviews on various aspects of this process.^{714,813,814}

Wrapping

Some MVs are wrapped by a pair of additional membranes (Figs. 66.12 and 66.13) derived from virus-modified *trans*-Golgi or endosomal cisternae^{313,659,765} that are important for intracellular movement and EV formation. Nine viral proteins have been associated with either the wrapping or EV membrane: A33,⁶³⁴ A34,²²⁷ A36,⁵⁶⁰ A56 hemagglutinin,⁶⁸⁹ K2,⁷⁷⁹ B5,^{235,358} E2,^{219,222} F12,⁸⁸⁰ and F13³¹⁷ (Table 66.5). Of these, A36 and F12 are associated exclusively with the outermost membrane and are not retained in the EV. With the exception of E2, F12, K2, and F13, the proteins have a transmembrane anchor. Association of F13 with the wrapping membrane requires palmitoylation of two cysteines.^{285,313} F12 has been reported to associate with E2²¹⁹ and A36.³⁶⁸ Complex interactions between the A33, A34, and B5 proteins determine the composition of the EV membrane.^{97,230,576,578} A33 has unique C-type lectin domains that could interact with other viral or cell proteins.^{406,742}

Deletion of any of the genes encoding wrapped virion (WV) or EV membrane proteins, except A56 and the associated K2 protein, results in a small plaque phenotype. A56 and K2 deletion mutants have a syncytial phenotype that may be explained by the abrogation of the role of A56 in binding to the EFC.⁸⁰⁵ Studies with mutant VACVs indicate that several proteins are required for efficient wrapping and that severe effects are caused by repression or deletion of the A27 MV protein,^{627,815} the F13 protein,⁸³ and the B5 glycoprotein.^{236,845} The F13 protein is required for Golgi membrane localization of B5³⁴⁷ and has a putative phospholipase motif that is essential for its role in wrapping⁶³³ and induction of post-Golgi vesicles.³⁴⁶ The role of E2 is less defined.^{219,222}

Intracellular Movement and Exocytosis

It has been estimated that it would take a particle as large as a POXV about 5 to 6 hours to move 10 μ M by diffusion,⁷¹⁷ suggesting the importance of an active, directed process. The movement of MVs to the wrapping sites depends on microtubules, but the viral attachment protein is not known. There is some disagreement as to whether the A27 MV protein is required for movement as well as for wrapping *per se*.^{646,815} Long-range intracellular movement of WVs occurs on microtubules^{267,321,618,818,819} rather than by actin polymerization as originally proposed.¹⁶⁴ The A36 and the F12 proteins appear to have roles in microtubular movement. The A36 protein interacts with the cargo binding domain of the light chain of kinesin, a microtubule motor protein.^{366,817} The F12 protein has a structural similarity to the light chain of kinesin and has a motor-binding motif important for virion export.^{309,502}

When the WVs arrive near the periphery of the cell, their migration through the dense cortical actin is facilitated by the action of the F11 protein in preventing RhoA signaling.^{27,161} The outer of the two wrapping membranes fuses with the plasma membrane to liberate the EV with one more membrane than an MV (Fig. 66.13). The externalization of VACV resembles vesicle exocytosis, but the detailed mechanism has not been determined. A role of the ESCRT machinery has been suggested.³²⁴

Only a minority of the externalized virus is found in the medium, whereas most adhere to the cell surface. The ratio of

adherent to released virions varies in different VACV strains,⁵⁶⁹ and in one case was due to a single amino acid difference in the putative lectin binding domain of the A34 membrane protein.⁸⁵ Mutations in the *A33R* and *B5R* genes also enhance release³⁸² and the host Abl-family tyrosine kinases and host phosphoinositide 5-phosphatase SHIP2 are involved.^{470,608} On cell monolayers the adherent EVs can mediate efficient cell-to-cell spread due to actin tails discussed later, whereas the released EVs provide long-range dissemination.⁸⁴

Actin Tail Formation

The efficient cell-to-cell spread of EVs on the cell surface depends on their location at the tip of motile, actin-containing microvilli, which have been visualized by fluorescence and electron microscopy^{164,314,737} (Fig. 66.6). The proteins encoded by the *A33R*, *A34R*, or *A36R* genes are required for the formation of the actin-containing microvilli, and their absence results in a small plaque phenotype.^{632,645,846,849} Nucleation of the actin tails depends on tyrosine phosphorylation of the A36 protein of OPXVs and functional homologs of other POXVs by Src or Abl family kinases.^{220,256,608,609} The phosphorylated A36 protein interacts with the adaptor protein Nck, which results in the recruitment of the Ena/VASP family member N-WASP to the site of actin assembly.^{256,657}

VIRUS–HOST INTERACTIONS

Tropism and Host Range

POXVs frequently have a narrow host range that supports their survival in the wild, although they may occasionally infect and cause disease in other species. Indeed, the eradication of smallpox could not have succeeded if there were a wild animal reservoir. Occasionally, infection of an unnatural host (e.g., the European rabbit by MYXV)²⁵⁰ results in a high mortality. Tropism and pathogenicity are frequently based on the genus- and species-specific properties of defense genes rather than the essential ones conserved in all or most POXVs. This is reflected in the broader species specificity for cultured cells than whole animals. Where restriction occurs, it is usually at a postentry step.⁴⁶⁵ An interesting example of tropism is the ability to infect primary mouse fibroblasts and mice by MYXV only if the IFN response is abrogated.⁸⁰⁹ Under certain nonpermissive conditions of orthopoxvirus infection, viral macromolecular synthesis may be inhibited. Examples are the abortive VACV infection of *Drosophila* cells restricted at the stage of viral DNA replication⁶⁹ and Chinese hamster ovary cells restricted at the stage of intermediate protein synthesis, which can be overcome by the CPXV gene encoding a 77-kD protein.^{330,333,599,726} In addition, VACV K1L deletion mutants are blocked at the stage of early protein synthesis in rabbit RK13 cells^{274,580,600,744} and VACV double K1L and C7L mutants exhibit a similar defect in human cells. Some of the host range effects may be mediated in part by IFN effectors such as protein kinase R (PKR).^{473,837} Host range genes have also been identified in other POXV genera.^{55,435,474}

Effects on the Cytoskeleton

Infection of tissue culture cells with VACV and other orthopoxviruses results in profound morphologic changes consisting of rounding, cell–cell dissociation, and migration that are dependent on viral early gene expression and long-branched

projections that are dependent on late gene expression.³² These cytopathic effects involve alterations in microtubules, actin, and intermediate filaments.^{82,252,488,586,658} The VACV-induced cell motility requires F11 protein-mediated inhibition of RhoA signaling.^{499,789}

Effects on Host Macromolecular Synthesis

The rapid and profound reduction in cell protein synthesis following VACV infection can be seen by labeling cells with radioactive amino acids at various times after infection and analyzing the labeled proteins by gel electrophoresis.^{241,514} Several factors may contribute to the switch from host to viral protein synthesis, and the relative contribution of each factor may depend on the virus multiplicity, cell type, and time of analysis. Inhibition of host protein synthesis frequently remains incomplete until after DNA replication, suggesting a major role of a viral intermediate or late protein. The primary factor in the shutoff of host protein synthesis is a profound reduction in cellular mRNA. After several hours, most of the mRNA present in the cytoplasm of infected cells is viral^{88,158,865} due to accelerated degradation of host species^{67,617,668} coupled with active virus transcription and inhibition of host RNA synthesis and transport.^{67,573,594,668,839} The accelerated degradation of host and viral mRNAs is initiated by the expression of the viral decapping enzymes D9 and D10^{563,564,565,695,724,725} and presumably completed by host cell exonuclease activity.

VACV also hijacks cellular translation initiation factors to expression sites within cytoplasmic factories (Fig. 66.10) and thereby contributing to the suppression of cell protein synthesis.³⁷⁹ The abundance of the cellular translational repressor 4E binding protein decreases during VACV infection, enhancing translation of viral mRNAs.⁸⁰⁷ At high virus multiplicities inhibition of host protein synthesis can occur in the absence of viral gene expression, implicating a virion protein.^{462,506,581} Other studies suggested that small poly(A)-containing RNA molecules are involved.^{33,34,119}

VACV also inhibits nuclear DNA replication.³⁷² This effect may be indirect, although a role for VACV-encoded deoxyribonucleases was suggested.²¹²

Interactions With the Ubiquitin-Proteasome System

Ubiquitination is a posttranslational modification that regulates cellular processes by several mechanisms including protein degradation. Studies with inhibitors suggested that orthopoxviruses require the ubiquitin proteasome system for uncoating cores and DNA replication.^{653,760} A few POXVs encode ubiquitin-like genes, but many more encode modulators of the ubiquitin-proteasome system, including E3 ubiquitin ligases,^{291,340,449,536} ankyrin/F-box-like proteins,^{367,477,720,721,727} and BTB-kelch proteins^{44,63,257,395,826,838} as reviewed.⁵⁹ Some of the ankyrin/F-box-like proteins interact with the skp1 component of the SCF ubiquitin ligase complex to exploit the cell proteasome machinery. Although many of these modulators of ubiquitin are required for virulence, the functions of only a few have been discovered.

Stimulatory Effects on Cell Pathways

To enhance their replication, POXVs may stimulate certain host pathways (e.g., mitogen-activated protein kinase [MAPK] and phosphoinositide 3-kinase [PI3K]/Akt)^{21,716,810} and some,

such as FWPV,¹³⁶ SFV,⁶⁹⁴ Yaba virus,⁵⁴⁴ and MOCV,⁵⁹² induce hyperplasia and tumors. In the case of orthopoxviruses and leporipoxviruses, the hyperplastic effect is due to secretion of a homolog of epidermal growth factor, which binds to ErbB receptors and enhances virulence.^{111,112,134,430,552,738,781} A gene encoding a mitogenic polypeptide with homology to mammalian vascular endothelial growth factor is present in the ORFV genome and may account for the extensive vascularization of lesions and virulence.^{656,841}

VIRAL DEFENSE MOLECULES

Overview

The first line of host defense against viruses consists of nonspecific, innate mechanisms involving IFNs and other cytokines, signaling pathways, complement activation, and natural killer cells; subsequently, specific cytotoxic T cells and antibodies become important. However, POXVs encode multiple proteins that help evade innate and adaptive immune responses. Some inhibitory proteins are secreted from infected cells and resemble host cytokines or soluble immune regulators (virokines)⁴⁰⁰ or cellular receptors that have lost their transmembrane anchor sequences and sequester ligands (viroceptors),⁷⁸⁵ while others interfere with intracellular signaling or effector pathways. In contrast to the highly conserved mechanisms of entry, gene expression, and genome replication that are employed by all POXVs, there is considerable diversity in POXV defense genes, which are adapted to specific hosts. Many of these genes were acquired from their hosts and retain recognizable motifs despite extensive modification, whereas others may represent acquisition of new functions by proteins. Several review articles have been published recently^{36,493,577,585,821} and updates can be anticipated in this fast-moving field.

Extracellular Defense Proteins

Complement Regulatory Protein

VACV and other orthopoxviruses encode a secreted complement control protein (VCP), consisting largely of tandem, inexact copies of a 60-amino acid short consensus repeat found in cellular complement regulatory proteins, that inhibits the classical and alternative pathways of complement activation by binding and inactivating C4B and C3B and accelerating the decay of C3 convertases.^{4,399,400,469,524,641} Although VCP is secreted, some of it is retained on the surface of infected cells by binding to heparin-like molecules²⁶¹ and to the VACV A56 protein.^{200,275,804} Both the VACV and ECTV complement control proteins contribute to virulence.^{276,357} Interestingly, the VARV ortholog of VCP is more potent than the VACV version in inhibiting human complement,^{431,432,639} and strain variation in the MPXV ortholog may contribute to differences in virulence.^{425,433} Although the VACV B5 EV membrane protein also has short consensus repeats, there is no evidence that it has complement inhibitory activity. Host proteins incorporated into the EV membrane could also inhibit complement activation.⁷⁹³

IFN-Binding Proteins

Type I IFN inhibitors encoded by VACV and other orthopoxviruses are present in the supernatants and on the surface of cells and are important for virulence.^{151,190,746,860} Glycosaminoglycans help retain the IFN-binding proteins (BPs) to the cell

surface.⁴⁹⁸ The VACV B18 and orthologs are immunoglobulin (Ig) family proteins with limited sequence similarity to regions of the IL-1 receptor family but not to the ligand-binding subunits of IFN receptors. Nevertheless, these proteins neutralize IFN- α , - β , and - ω depending to some extent on the animal species. Myxoma virus encodes a protein important for virulence that is related in sequence to VACV B18 but is smaller and membrane bound and apparently cannot bind IFN.⁵⁶

Proteins that bind to type II IFNs are secreted by POXVs of several different genera. The IFN- γ BPs of MYXV and orthopoxviruses are related in sequence to the extracellular domain of the IFN- γ receptor.^{13,519,545,786} Deletion of the MYXV and VACV IFN- γ BP attenuate disease in rabbits.^{518,732,747} Because of the low affinity of the VACV IFN- γ BP for mouse IFN- γ compared to human IFN- γ , this cytokine would have a more profound inhibitory effect on VACV in mouse compared to humans, which may be important for interpreting disease models.⁷⁷³ The type I IFN inhibitor encoded by Yaba monkey tumor virus also inhibits type III IFN.³⁴¹

IL-18 BPs

IL-18 induces IFN- γ and acts in synergy with IL-12 to induce natural killer (NK)-cell activation, T-cell activation, and T-helper cell 1 (Th1) response polarization. MOCV, OPXVs, Yaba monkey tumor virus, and some other POXVs encode secreted proteins that are homologous to the mammalian IL-18 BP and which bind and inactivate IL-18.^{90,476,534,715,855,856} Despite limited sequence identity, similar amino acids of the human and viral residues interact with IL-18.⁴⁰⁵ The MOCV IL-18 BP is secreted as a full-length form that binds cell surface glycosaminoglycans through the C-terminal tail and a furin-cleaved form with only the IL-18 binding domain.⁸⁵⁷ ECTV and VACV IL-18 BP deletion mutants exhibit decreased virulence compared with wild-type virus.^{90,606,745}

Soluble Tumor Necrosis Factor BPs

Tumor necrosis factors (TNFs) bind to their cognate receptors and induce pro-inflammatory responses or death of virus-infected cells. Leporipoxviruses,^{666,710,785,861} OPXVs,^{335,438,604,651} and yatapoxviruses¹¹⁰ encode from one to four soluble TNF receptor homologs with varying binding specificities. The VACV CrmE protein shares structural features with mammalian type 2 TNF receptors,²⁸⁰ whereas the tanapox and Yaba-like virus TNF BPs have sequence similarity with major histocompatibility class (MHC) class I molecules.^{110,597,868} CPXV and ECTV also encode a soluble homolog of CD30, another member of the TNF receptor family, which inhibits interaction of CD30 with its ligand.^{555,652}

Soluble IL-1 β Receptor Homologs

IL-1 β mediates a broad-spectrum response to virus infection by binding to a high-affinity cell receptor and triggering a signal transduction pathway. OPXVs encode homologs of the IL-1 receptor that bind IL-1 β .^{11,12,728} Depending on the route of inoculation, deletion of the gene can reduce or enhance virulence. Frameshift mutations in the VARV homolog preclude synthesis of an active protein.

Chemokine Inhibitors

A principal role of chemokines is to coordinate the activation and migration of leukocytes to sites of virus infection. POXVs

encode chemokine-binding proteins and mimics presumably to interfere with the chemokine response. Secreted proteins that bind to a variety of CC chemokines but lack sequence similarity to chemokine receptors are encoded by OPXVs, leporipoxviruses, and parapoxviruses and prevent activation and chemotaxis of leukocytes.^{14,114,125,279,417,607,669,670,711} The structures of several POXV chemokine BPs have been solved and the binding sites determined.^{29,38,877}

Several POXVs encode homologs of the chemokine receptor CCR8. The Yaba-like disease virus protein is inserted into the plasma membrane, binds human CCL1, and activates signal-regulated kinases.⁵³¹ A recombinant VACV expressing the Yaba-like disease virus CCR8 homolog was attenuated in mice.⁵³⁰

Chemokine homologs have been found in MOCV and FWPV. The MOCV-encoded homolog was predicted to be a chemokine antagonist based on the absence of the conserved N-terminal region⁶⁷⁴ and was found to bind specifically to the CCR8 chemokine receptor and to competitively inhibit the binding of I-309, the natural ligand.⁴⁴⁰ Other reports, based mainly on chemotaxis assays, suggested a broader activity.^{180,198,402}

Other Cytokine-Binding Proteins and Homologs

Some parapoxviruses encode a secreted protein that binds granulocyte-macrophage colony-stimulating factor and IL-2.^{196,197} ORFV encodes an IL-10 homolog with a high degree of identity to animal IL-10 molecules.^{253,254,355} The ORFV IL-10 suppresses macrophage activation and exerts an immunosuppressive effect.⁸⁴⁰ Some chordopoxviruses secrete a semaphorin homolog with pro-inflammatory properties that interacts with plexin C1.^{2,154,265,434}

Secreted NK-cell Inhibitor

NK cells have an important role in combating OPXV infections.^{248,559} CPXV and MPXV encode a secreted class I-like protein that acts as a competitive antagonist of the NKG2D-activating receptor and inhibits NKG2D-dependent killing by NK cells.¹²³

Intracellular Defense Proteins

Inhibitors of Cell Pattern Recognition Receptor Signaling Pathways

Cell pattern recognition receptors (PRRs) can detect a variety of viral components including cytoplasmic DNA, single-stranded RNA with triphosphate ends, double-stranded RNA, and viral proteins. The PRRs include Toll-like receptors (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLRs), and cytoplasmic DNA sensors (DAI, AIM2, and RNA polymerase III). After ligand binding, the TLRs dimerize and recruit cytosolic TIR domain-containing adapter proteins MyD88, MAL, TRIF, TRAM, and SARM, which then activate signaling complexes containing TRAF and IRAK protein pathways, leading to activation of kinases that result in translocation of transcription factors nuclear factor- κ B (NF- κ B), ATF2/c-Jun, and IFN regulatory factors (IRF) that promote expression of pro-inflammatory cytokine genes and IFN. RNA is sensed by TLR3, 7, and 8; RIG-I; and MDA-5.

TLR2, 3, 4, 8, and 9 have been reported to recognize POXV infections. VACV proteins A46, A52, B14, K7, and N1 and homologs in other POXVs have Bcl-2-like structures

and interfere with the TLR signaling pathway at different levels.^{26,60,93,129,137,138,160,216,278,281,282,305,365,373,447,456,457,547,733} DNA sensing of POXVs may occur through at least two pathways: the AIM2 inflammasome⁶⁰¹ and RNA polymerase III.⁷⁹⁰ The VACV E3 protein interferes with the latter mechanism by binding to double-stranded RNA.

NF- κ B Signal Transduction Pathways

The NF- κ B complex comprises a family of dimeric transcription factors that have a central role in responding to viral infections and mediating an inflammatory response. NF- κ B exists in an inactive complex with I κ B α in the cytoplasm until the latter is phosphorylated by I κ kinase (IKK) complex, polyubiquitinated, and then degraded. POXVs can trigger NF- κ B activation but use a variety of mechanisms to suppress this. Modified VACV Ankara, a highly attenuated strain of VACV used as a safe smallpox vaccine and as a vector for other vaccines, has suffered multiple deletions and lost the ability to prevent NF- κ B activation due to double-stranded RNA triggering of PKR in some cells.^{442,548,692} Restoration of the *N1L* or *M2L* genes prevents induction of NF- κ B by the PKR or extracellular signal-regulated kinase 2 (ERK2) pathway, respectively.^{268,315,442,837}

The VACV B14 protein and the nonhomologous ORFV 024 protein prevent NF- κ B signaling by binding to the IKK complex and preventing phosphorylation of I κ B α .^{68,138,214} The VACV A52 Bcl-2-like protein prevents NF- κ B signaling by preventing signaling by Toll-like receptors TRAF6 and IRAK2.⁴⁴⁷

Many OPXVs, other than VACV, encode ankyrin-repeat proteins that bind directly to NF- κ B and prevent the signaling pathway.^{495,496} The nonhomologous ORFV 121 gene encodes a viral NF- κ B inhibitor that also binds to and inhibits the phosphorylation and nuclear translocation of NF- κ B.²¹⁵ MC160, a second death effector domain (DED)-containing protein encoded by MOCV, inhibits TNF- α -induced NF- κ B activation by association with Hsp90 to increase IKK1 degradation and by interaction with procaspase-8.⁵³⁸ Myxoma virus MO13 protein binds NF- κ B.⁵⁹⁸

Inhibitors of IFN Pathways

POXVs encode proteins that prevent signaling and function of IFN response factors. The VACV dual-specificity H1 phosphatase inhibits phosphorylation of the Stat1 and Stat2 transcription factors and blocks type I and II IFN-mediated immune responses.^{448,532} Myxoma virus inhibits type I IFN by blocking the activity of Janus kinase type 2, which is upstream of Stat.⁸⁰⁸ OPXVs encode a double-stranded RNA-binding protein (e.g., VACV E3) that prevents activation of the IFN response PKR, which inhibits translation initiation.^{133a} Loss of E3L can also lead to PKR-dependent activation of IFN regulatory factor 3 and mitogen-activated protein kinases.^{878,879} E3L deletion mutants show host range restriction and enhanced apoptosis, RNA degradation, and IFN sensitivity.^{64,389} The C-terminal end of E3 contains the double-stranded RNA binding domain and the N-terminal segment contains a Z-DNA binding domain important for virulence.⁴⁰⁹ Some POXVs also encode a nonfunctional homolog of the translation initiation factor eIF-2 α (e.g., VACV K3), which inhibits eIF-2 α phosphorylation by PKR.^{181,182} Deletion of K3L increases the IFN sensitivity of VACV.^{65,415} The E3 and K3 proteins have overlapping roles in blocking IFN action.

Apoptosis Inhibitors

POXVs encode a variety of proteins that inhibit apoptosis by different mechanisms to prevent activation of the extrinsic pathway (e.g., TNF, Fas ligand) or intrinsic pathway mediated by mitochondria. Cytokine response modifier A (crmA), a viral member of the serine protease inhibitor (serpin) superfamily initially described as an inhibitor of IL-1 β converting enzyme caspase 1,⁶⁰² blocks apoptosis activated through Fas and TNF receptors^{217,491,762} and potentially inhibits caspase-8, the apical caspase in these pathways.⁸⁸⁵ CrmA deletion mutants replicate normally in most cell lines without inducing apoptosis but do induce apoptosis in a pig kidney cell line.^{444,603} The effect on virulence caused by deletion of CrmA or related Serine protease inhibitor 2 (SPI 2) has varied in different animal models.^{388,764}

MOCV encodes two FLIP proteins with DEDs.⁶⁷⁴ DEDs are also present in cellular FADD and procaspase-8 and mediate their interaction following the binding of TNF or FasL to their receptors. One of the MOCV DED proteins, MC159, has been shown to bind FADD and protect transfected cells against death effector filament formation and apoptosis induced by Fas and other members of the TNF receptor superfamily.^{74,336,422,526,763,775}

Some POXVs encode Bcl2-like proteins (e.g., VACV F1, myxoma m11, ORFV 125, FPV 039, deerpox virus 022) that prevent cytochrome c release from mitochondria by preventing Bak and Bax activation.^{46–48,124,225,246,407,408,551,591,736,759,820,827,828,875} F1 is also a caspase 9 inhibitor, indicating that it is a multifunctional apoptosis inhibitor.⁸⁷⁵

Several POXV proteins prevent apoptosis by additional mechanisms. GAAP (Golgi antiapoptotic protein) is encoded by CMLV, some strains of VACV, and eukaryotes and can inhibit apoptosis mediated by extrinsic and intrinsic pathways.²⁹⁰ The ECTV p28 protein contains a RING finger motif that is required for virulence in mice,⁶⁸³ acts upstream of caspase-3 to block UV-induced apoptosis,⁹⁸ and has been shown to be an E3 ubiquitin ligase.³⁴⁰ Overexpression of the SFV ring finger protein N1 can also block apoptosis induced by UV light.⁹⁹ MOCV encodes a selenocysteine-containing protein, MC66, that is homologous to cellular glutathione peroxidase and can protect cells against the cytotoxic effects of hydrogen peroxide, which could be produced by inflammatory cells, and UV irradiation.⁶⁹³ A putative glutathione peroxidase is also encoded by FWPV.² The MYXV M-T4 localizes to the ER of infected cells and is required for virulence and to prevent induction of apoptosis in a rabbit CD4+ T-cell line or primary rabbit lymphocytes.^{58,318} The MYXV M-T5 protein has a single ankyrin motif, is related to OPXV host range genes, and is required to prevent apoptosis in a rabbit CD4+ T-cell line.⁵¹⁷ The VACV E3 protein was described earlier as a double-stranded RNA-binding protein that protects against the action of PKR and provides resistance to the action of IFN. E3L deletion mutants induced apoptosis in HeLa cells through failure to inhibit PKR.^{389,420}

Inhibitors of Antigen Presentation

CPXV encodes two proteins that down-regulate MHC class I presentation by distinct mechanisms. CPXV203 retains fully assembled MHC class I by exploiting the KDEL-mediated ER retention pathway, whereas CPXV12 binds to the peptide-loading complex and inhibits peptide loading on MHC class I molecules.^{117,118} The VACV A35 protein inhibits MHC class II antigen presentation by an unknown mechanism.^{610,611}

Additional POXV Defense Proteins

The VACV β -hydroxysteroid dehydrogenase contributes to virulence by inhibiting an effective inflammatory response.⁶⁰⁵ The mechanism of action of many other POXV proteins that are not required for replication in cell culture but reduce virulence in animal models remain to be determined.^{57,224,247,684,722,723}

ADAPTIVE IMMUNE RESPONSE TO POXVIRUSES

Adaptive humoral and cell-mediated immune responses are important in clearing poxvirus infections, and knowledge in this area helps to understand the efficacy of vaccines and the design of new ones. Not surprisingly, given their large size and complexity, there are numerous antigens and consequently protective antibodies are directed toward both the MV and EV forms. As in other areas, most information has been obtained for OPXVs, and this has been the subject of several recent reviews.^{508,521,685,859}

ANTIVIRALS

In view of the large number of viral proteins involved in cytoplasmic replication, there are many potential targets for antivirals and many have been investigated over the years.¹⁸⁷ Two potent antivirals have reached the stage of clinical testing. Cidofovir is an acyclic nucleoside analog that is incorporated into the growing DNA strand and inhibits the 5' to 3' chain extension and 3' to 5' exonuclease activities of VACV DNA polymerase.²² The other antiviral is ST-246, which inhibits the wrapping of MVs and formation of EVs by targeting the F13 protein.³⁷¹

EXPRESSION VECTORS

Several attributes of POXVs have led to their extensive use as expression vectors.⁵⁰⁵ These include relative ease of formation and isolation of recombinant viruses, capacity for large amounts of DNA, relatively high expression, and wide host range. Expression has been achieved either by using poxvirus promoters or by employing bacteriophage RNA polymerases and cognate promoters. Recombinant viruses have been used for the synthesis of proteins *in vivo* or *in vitro* and as vaccine candidates to prevent infectious disease and treat cancer.⁴⁶¹ In particular, there has been a recent emphasis on attenuated strains of VACV such as modified VACV Ankara (MVA)⁷⁴³ and NYVAC^{757a} and the combination with DNA priming.¹⁶

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Poxviruses

Orthopoxviruses

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- Infectious Agent
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- Systemic Disease Pathogenesis
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Parapoxvirus

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Molluscum Contagiosum

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Yatapoxviruses

- History
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Other Poxviruses

- Orthopoxviruses
- Capripoxviruses
- Suipoxvirus
- Leporipoxvirus
- Avipoxvirus

are *Orthopoxvirus*, *Parapoxvirus*, *Yatapoxvirus*, and *Molluscipoxvirus*. The majority of human pathogenic poxvirus infections are zoonoses; only variola virus and molluscum contagiosum virus are sole human pathogens. The appearance and general structure of poxvirus virions is depicted in Figure 67.1.

ORTHOPOXVIRUSES

History

The orthopoxviruses are perhaps best known for their most notorious member, variola virus, the causative agent of human smallpox. Smallpox was a febrile rash illness with a variable mortality rate extending to 30% to 40% in some outbreaks. The disease was understood to be a sole human pathogen; extensive experimental studies infecting closely related species failed to produce a viral infection that could be sustained in other host species. Recognition that an infectious agent that caused lesions on the skin and mucosal surfaces on cows and their caretakers could be used to prevent smallpox infection was published by Jenner in 1798.⁹⁴ The viruses cowpox, and later, vaccinia were used in the 19th and 20th centuries to prevent smallpox infection and led to the term “vaccination.” Previously, material derived from convalescent smallpox patients, usually scab material, was used either as an intranasal or percutaneous preparation (termed inoculation) to prevent smallpox in humans.⁵⁹ Ultimately, this, in part, led to our early understanding of the genus *Orthopoxvirus*, where at least cowpox virus was used to cross-protect against smallpox.¹⁰ In addition, the unsuccessful use of infectious material from “spurious” lesions¹²⁸ for smallpox vaccinations, which were later recognized to be of parapoxvirus origin (termed paravaccinia or pseudocowpox), led to the recognition of this distinct group of poxviruses. Vaccinia virus, however, is the most comprehensively studied of the orthopoxviruses.

The history of smallpox and its eradication program has been chronicled in detail,⁵⁹ and is available from the World Health Organization (WHO) website. Perhaps the earliest, most reliable accounts of smallpox are from China in the fourth century AD: contained in writings that distinguish the disease from measles, report that smallpox was endemic in the region, and report the disease was introduced from the west in 48 AD.⁵⁹ Early practices from the 10th to the 18th centuries to control disease included intranasal insufflation with powdered smallpox scabs, quarantine, and cutaneous inoculation with pulverized smallpox scabs. With the recognition that vaccination with cowpox or vaccinia would diminish iatrogenic cases, improvements in disease control were seen. By 1953, smallpox had been eliminated from Europe. The development of freeze-dried vaccine preparations permitted vaccination in

The family *Poxviridae* is extensive, with member species infecting insects (*Entomopoxvirinae*) and vertebrates (*Chordopoxvirinae*). This chapter focuses on vertebrate poxviruses (Table 67.1). Genera of poxviruses with species described to cause human illness

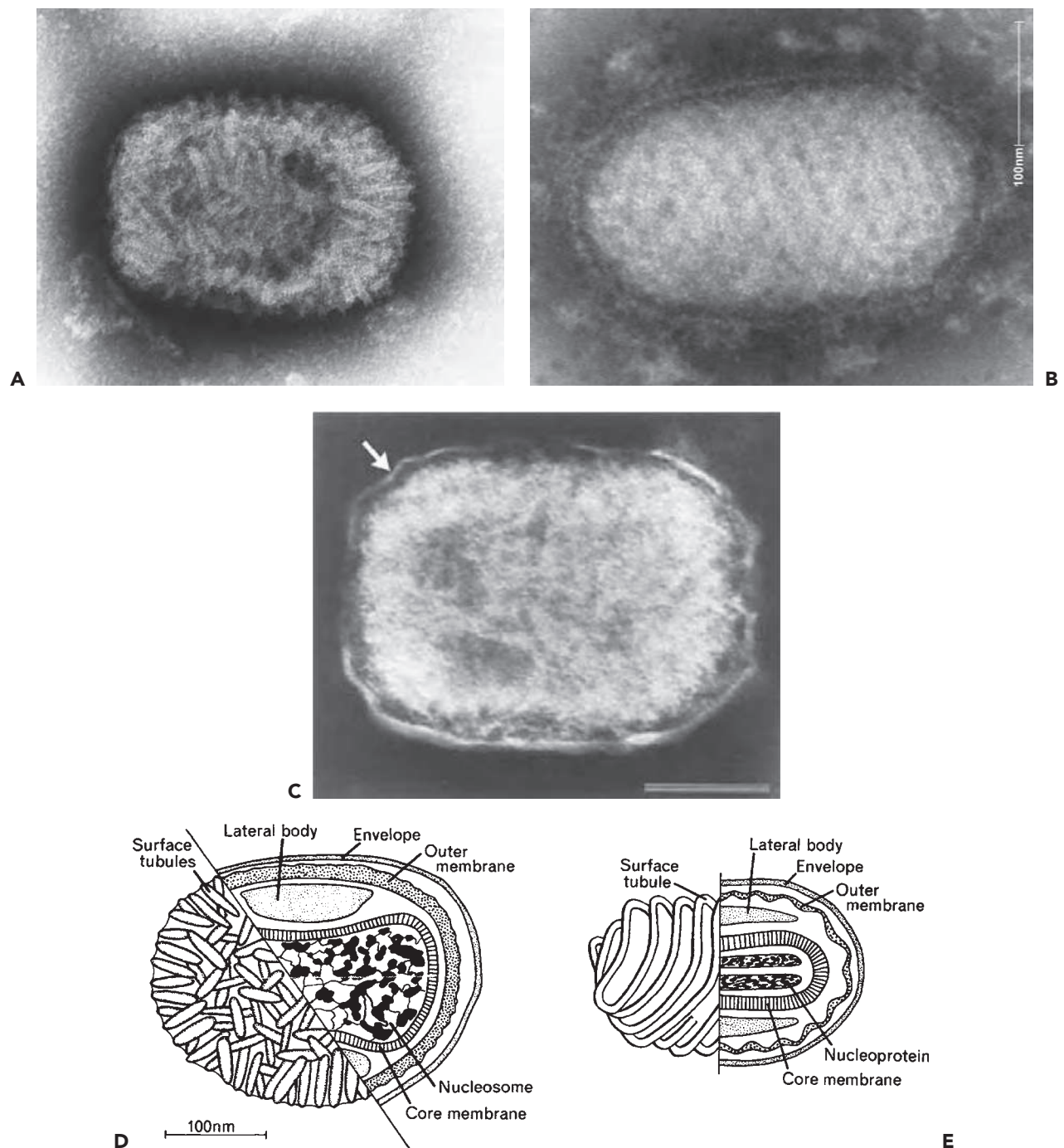


FIGURE 67.1. Negative stain electron microscopic appearance of orthopoxviruses (A), parapoxviruses (B), and yatapoxviruses (C). Bars indicate 100 nm. The structure of the yatapoxvirus and molluscipoxvirus virions is similar to that of the orthopoxvirus virion. As seen in **C**, clinical material from the yatapoxvirus tanapox often contains virion forms with enveloped virions (extracellular enveloped virions [EEVs]; see *arrow*). Knobby, brick-shaped virion forms, consistent with mature virions (MVs), are more often seen in clinical material from orthopoxvirus infections. Parapoxviruses (**B**) are ovoid and smaller, with a distinctive criss-cross filament pattern derived from the wrapping of a surface tubule. **D**: Schematic drawing of a brick-shaped virion typical of an orthopoxvirus. Viral genome DNA and several proteins within the core are organized as a nucleosome. The core has a 9-nm-thick membrane with a regular subunit structure. By negative stain techniques, the virion core assumes a dumbbell shape with large lateral bodies occupying the concavities. The core and lateral bodies are enclosed within a lipoprotein structure, the outer membrane, which is 12-nm thick. The outer membrane surface appears to consist of irregularly shaped tubules that produce the knobby or mulberrylike M form seen in **A**. The majority of infectious virions remain cell associated; others acquire an additional envelope composed of host cellular membrane and virus-specific proteins. These associate on the cell surface to spread to neighboring cells or are released as EEVs to allow more distant spread. **E**: Schematic drawing of parapoxvirus virion showing wrapping pattern of the outer protein tubule, which appears to wrap around the virus. (**A** courtesy of Centers for Disease Control and Prevention, Atlanta, GA. **B** courtesy of A. Likos. **C** from Dhar AD, Werchniak AE, Li Y, et al. Tanapox infection in a college student. *N Engl J Med* 2004;350:361–366, with permission of the Massachusetts Medical Society. **D** and **E**: With kind permission from Springer Science+Business Media, from Fenner F, Nakano JN. Poxviridae: the poxviruses. In: Lenette EH, Halonen P, Murphy FA, eds. *The Laboratory Diagnosis of Infectious Diseases: Principles and Practice*. Vol II. Viral, Rickettsial and Chlamydial Diseases. New York, NY: Springer-Verlag; 1988:177–210, copyright 1988.)

TABLE 67.1 Host Range and Geographic Distribution of Genera and Unclassified Members of the Subfamily *Chordopoxvirinae*

Genus and species	Reservoir host	Geographic distribution	Other infected hosts
<i>Orthopoxvirus</i>			
Camelpox virus	Camels	Africa, Asia	Nil
Cowpox virus	Bank voles <i>Clethrionomys glareolus</i> , long-tailed field mouse <i>Apodemus sylvaticus</i>	Europe, western Asia	Cats, cattle, humans, zoo animals
Ectromelia virus	Rodents	Europe	Nil
Horsepox virus	?	Central Asia	Horses
Monkeypox virus	? Unknown—likely rodent	Western and central Africa	Monkeys, zoo animals, humans, prairie dogs
Raccoonpox virus	Raccoons	(Eastern) United States	Nil
Skunkpox virus	Skunks	(Western) United States	Nil
Taterapox virus	Gerbils (<i>Tatera kempi</i>)	Western Africa	Nil
Uasin Gishu virus	?	Eastern Africa	Horses
Vaccinia virus	?	?	Humans, rabbits, cows, river buffaloes (<i>Bubalis</i>)
Variola virus	Humans	Eradicated (formerly worldwide)	Nil
Volepox virus	California vole (<i>Microtus californicus</i>)	Western United States	Nil
<i>Parapoxvirus</i>			
Ausdyk virus	Camels	Africa, Asia	Nil
Bovine popular stomatitis virus	Cattle (beef)	Worldwide	Humans
Orf virus	Sheep, goats	Worldwide	Ruminants, humans
Pseudocowpox virus	Cattle (dairy)	Worldwide	Humans
Red deer poxvirus	Red deer	New Zealand	Nil
Seal parapoxvirus	Seals	Worldwide	Humans
<i>Capripoxvirus</i>			
Sheeppoxvirus	Sheep	Asia, Africa	Nil
Goatpox virus	Goats	Asia, Africa	Nil
Lumpyskin disease virus	? African cape buffalo	Africa	Cattle (<i>Bos taurus</i> , <i>Bos indicus</i>)
<i>Suipoxvirus</i>			
Swinepox virus	Swine	Worldwide	Nil
<i>Leporipoxvirus</i>			
Myxoma virus	<i>Sylvilagus brasiliensis</i> <i>Sylvilagus bachmani</i>	South America, Western United States	<i>Oryctolagus</i> , other leporids
Fibroma virus	<i>Sylvilagus floridanus</i>	Eastern United States	<i>Oryctolagus</i>
Hare Fibroma virus	<i>Lepus capensis</i>	Europe	Nil
Squirrel fibroma virus	<i>Sciurus carolinensis</i>	Eastern United States	? Woodchuck
Western squirrel fibroma virus	<i>Sciurus griseus</i>	California	Nil
<i>Yatapoxvirus</i>			
Tanapox virus		Eastern and central Africa	Humans
Yabapoxvirus	? Primates	Western Africa	Humans
<i>Molluscipoxvirus</i>			
Molluscum contagiosum virus	Humans	Worldwide	Nil
Unclassified			
Crocodilepox	Crocodiles	Australia, South Africa	Nil
	Caimans	Florida	Nil
Macropod poxvirus	Kangaroos	Australia	Nil
	Quokkas		
Cotia poxvirus	? Mice	Brazil	Nil
Squirrelpox virus	Grey squirrel	? Europe	<i>Sciurus vulgaris</i> (red squirrel)
Deerpox virus	? <i>Odocoileus hemionus</i> (North American mule deer)	North America	<i>Odocoileus hemionus</i> (North American mule deer) ³³
Mucocutaneous disease			
Avipoxvirus			
Many species	Birds	Worldwide	

areas outside the cold chain. Largely through the global efforts in design and implementation of WHO-sponsored vaccination programs, the disease smallpox was declared eradicated in 1980 by the WHO. The last naturally occurring cases of smallpox occurred in the Indian subcontinent in 1975 and in the Horn of Africa in 1977. Remaining stocks of variola virus were subsequently moved to two designated WHO Collaborating Centers for Smallpox and Other Poxvirus Infections with maximum containment laboratory facilities. The dismantling of population-based vaccination programs began in 1980. The eradication of smallpox and the discontinuation of vaccination programs likely contribute to the increase in observations of other human orthopoxvirus infections since 1980. In addition, it has enhanced the concerns that if unknown stocks of variola virus exist and were to be used, that the effects on our current population would be profound.

Infectious Agent

By electron microscopy, the orthopoxviruses are large brick-shaped particles; as with all other poxviruses, the virus replicates and matures within the cytoplasm of the host cell. The viral steps involved in entry, fusion, morphogenesis, and release of virus are described in Chapter 66, as well as in a number of detailed reviews.^{145,172} By light microscopy, cytopathic effects in monkey kidney cell lines are apparent and profound. Cell rounding and detachment from neighbor cells is followed by formation of elongated cellular lamellipodia¹⁸⁰; ultimately, cells detach from the substrate. Similar to other poxvirus genomes, the nuclear material is a covalently closed double-stranded genome. Genome length ranges from 170 to 240 kilobase (kb) pairs and its composition is 34% G+C. The central 90 kbp of the central region is highly conserved and contains virion structural genes and enzymes responsible for its DNA replication and messenger RNA (mRNA) transcription; the terminal regions are varied across species and encode genes involved in the host–pathogen interaction. In addition to nucleic acid–based techniques, classification of orthopoxviral species has also been accomplished based on various biological properties (Table 67.2). These include the presence (or absence) of acidophilic-type inclusion (ATI) bodies, and cytoplasmic structures con-

taining collections of virions, and seen in cowpox, ectromelia, raccoonpox, volepox, and skunkpox infections. However, as additional sequence information becomes available, classification of viruses is becoming more complex. For instance, phylogenetic analysis of strains within species was initially supportive for at least two clades of cowpox virus.⁷⁰ A recent analysis, using additional cowpox virus genomic information is supportive of up to five genetic groupings of cowpox virus, and suggests that these groupings may be significant to the species taxa level.³⁰ Current information is supportive of two clades of monkeypox virus^{39,127} and two or three clades of variola virus.^{55,124} The majority of the orthopoxviruses appear to have a broad host range, although the spectrum of disease may vary, depending on what host is infected. This is exemplified by cowpox, which causes discrete localized lesions on the udders of cows but appears to cause a systemic disease in cats. The exception is variola virus, which as we understand the virus, was a sole pathogen of humans and humans appeared to be the sole host. A review on the cellular host range of poxviruses is available.¹³⁹

Pathogenesis and Pathology

Orthopoxvirus infections can be classified as systemic or localized (at the site of virus entry) illnesses. Generalized disease usually manifests with rash. The type of infection that results is dependent on the species of orthopoxvirus, the route of entry, and the genus/species of susceptible animal and its immune status. For this chapter, infections of humans and models to understand infections of normal host humans are largely described. In some cases, understanding of the natural pathogenesis is limited due to the lack of data on the disease pathogenesis in its natural host. The most obvious example is variola. Although clinical descriptions of disease are well notated from early medical writings, the specifics of human pathogenesis are largely derived from inferences evaluating other (e.g., ectromelia, which causes mousepox infection in mice, rabbitpox in rabbits, and monkeypox in nonhuman primates) orthopoxvirus virus species infections of various animal models.^{28,36,65,103} Other respiratory challenge animal models of orthopoxvirus disease are the intranasal vaccinia and intranasal or aerosol cowpox virus challenges of mice.^{79,135} These appear to primarily

TABLE 67.2 Biologic Properties of Orthopoxviruses that can Infect Humans

Property	Variola	Monkeypox	Vaccinia	Cowpox
Host range	Narrow	Broad	Broad	Broad
Pocks of the chorioallantoic membrane (CAM)	Small, opaque, white	Small, opaque, hemorrhagic	Strains vary; large, opaque, white or hemorrhagic	Large, hemorrhagic
Ceiling growth temperature on CAM (°C)	37.5–38.5	39	41	40
Rabbit skin lesion	Small transient, nontransmissible	Indurated, hemorrhagic	Strains vary; indurated nodule, sometimes hemorrhagic	Large, indurated hemorrhagic
Lethality for:				
Mice	Low	High	High to very high	Variable
Chick embryo	Low	Medium	Very high	High
A-type inclusion bodies	No	No	No	Yes
B-type inclusion bodies	Yes	Yes	Yes	Yes
Genome DNA size (kbp)	186	191	192	220

cause upper and lower respiratory infection; virus antigen predominates in the sinuses, intranasal passages, trachea, airways, and lungs. Some evidence of viremic spread is suggested by occasional lesions in the skin or bone marrow.

Nonetheless, the use of animal models of systemic or localized disease, in combination, will be useful in determining efficacy of various therapeutic and/or prophylactic disease mitigation and prevention strategies. Intranasal or aerosol challenge models have been developed using many of the orthopoxviruses in different animal species to represent a respiratory route of infection. Different routes of exposure result in different pathogenesises, involve some different sets of viral proteins, and provide information relevant to understanding disease acquired via contact versus respiratory routes of transmission.¹⁹⁹ Contact transmission has been classically modeled in the footpad model of infection of mouse species with ectromelia,⁵⁶ and more recently, in the dermal ear model with vaccinia, which also provides a model for understanding the immune response to multiple puncture immunization in eliciting (vaccination) protection against smallpox. Models of specific disease manifestations in various immunocompromised hosts have also been developed in order to evaluate the effect of various host factors in both disease pathogenesis and response to, containment of, and clearance of pathogen.

Systemic Disease Pathogenesis

Entry

The cellular receptor used by poxviruses for entry into their host cells is unknown. Member viruses have a broad tropism for entry into tissue culture cells; usually it is the later steps that inhibit completion of the viral maturation process to produce infectious progeny and affect “host range.”^{139,156} A summary of *in vitro* research on *in vitro* (orthopoxvirus) viral entry and fusion mechanisms is available.¹⁴⁵ The portal of entry for orthopoxvirus infection is usually considered to be percutaneous (via contact) or the respiratory tract. Oral routes of exposure have also been reported if very large doses of ectromelia virus are administered¹⁵⁸ to mice; this has been proposed to be a potential route of exposure for cats to cowpox, and squirrels to monkeypox.⁶¹ However, investigations associated around a laboratory-associated outbreak in a mouse colony have suggested that the natural route of transmission is through abrasions in the skin after exposure to infected animals or contaminated fomites.²⁰³ The route of infection also affects the disease course. Inoculation of variola, when unsuccessful as a form of disease prevention, caused iatrogenic disease, with a compressed time from infection to illness with respect to that seen with respiratory acquired disease. Transmission of human smallpox is thought to occur via an upper respiratory route of infection, which is consistent with the typical time frame of disease; infection via contact with contaminated fomites has also been observed. The exposure route/portal of entry for human monkeypox infection may be either percutaneous or upper respiratory; the clinical disease course varies with route of infection.¹⁶⁸ To model a respiratory route of exposure, a variety of aerosol challenges with various orthopoxviruses have been used to infect various animal species; these may produce initial stages of disease that occur in lower portions of the respiratory tract than seen in natural human infection. An alternative exposure route has been intranasal challenges.^{65,73,90,135,170,207} For one North American orthopoxvirus, volepox, mechanical arthropod vec-

tors have been suggested as a route of transmission to California voles¹⁶⁷; this has not been observed in other orthopoxviruses, although it is a route of transmission, and portal of entry, for fowlpox and *Capripoxvirus* and *Leporipoxvirus* species. Importantly, in rabbitpox virus challenge studies, natural respiratory/airborne transmission appeared to occur over 12 feet.²⁰⁷

Site of Primary Replication

When the skin is the portal of entry, studies with ectromelia have suggested the virus needs to be introduced to the dermis if systemic infection is to eventually result.¹⁷¹ Virus replicates in the Malpighian layer of the epidermis, and dermal infection consisted of fibroblasts and histiocytes; the latter were likely responsible for subsequent movement of virus to the lymphatics to initiate the course of events required for systemic illness.

When the respiratory tract has been used as portal of entry, in experimental aerosol infections, likely dependent on particle size, a combination of upper and lower respiratory tract infections has occurred. Viral replication occurred in alveolar macrophages and small bronchioles, and then moved to regional pulmonary lymph nodes. Epidemiologic understanding of the natural human infections with variola virus to cause smallpox suggests that the common method of transmission was via a respiratory route. Primary infection is believed to have occurred within the mucosal surfaces of the nasopharyngeal tract, and then move to the regional lymphatics by extrapolation from that seen with ectromelia and rabbitpox virus studies. A recent review of aerosolized rabbitpox virus infection in rabbits is available.²⁰²

Virus Spread

Our understanding of orthopoxvirus spread after initial infection and spread to regional lymphatics comes again from studies of ectromelia in mice, rabbitpox in rabbits, and monkeypox in nonhuman primates. The use of fluorescent-antibody stains of fixed tissues and titers of infectious virus in organs at various time points in disease has provided the majority of this information. More recently, bioimaging studies have also been applied.¹³² Virus moves from the regional lymphatics to the bloodstream to cause a primary viremia, and then multiplies in the spleen, liver, bone marrow, and other reticuloendothelial organs. After this, a second viremic period ensues, followed by seeding of distant sites, specifically the skin, and generation of the characteristic generalized rash. Virus particles are mostly cell associated during viremic phases of disease. In ectromelia, the virus replication in the macrophages of the liver spreads to the parenchymal cells of the liver causing necrosis; similar pronounced replication and effect of virus is found in spleen and bone marrow. In certain inbred mouse species, the disease is so pronounced at this stage that mortality ensues prior to development of rash.²⁸

Experimental challenge studies of monkeypox infection, which clinically resembles smallpox more than mousepox, have been done in several species of animals, including nonhuman primates, and have demonstrated infectivity and pathogenesis through a number of routes of infection, including aerosol, intranasal, or parenteral administrations. Recent reviews of this literature are available.^{36,89} The pathological changes associated with aerosol challenge of cynomolgus monkeys have been described.²¹³ The time course of disease, and related mortality,

is related to the virus challenge dose.¹⁴⁷ Initially, there is regional lymph node uptake and replication of virus in lymphoid organs, followed by viremia. An eruption begins, with lesions forming macules, papules, vesicles, pustules, and crusts to scar formation from days 6 to 23 of infection. Areas of inflammation and cell necrosis have been seen in tonsils, lymph nodes, digestive tract, testes, ovaries, kidneys, liver, and lungs. Epithelial degeneration, necrosis, and intracytoplasmic bodies have been seen in skin and mucous membranes.

Use of high doses of variola in nonhuman primate challenges, via parenteral route of administration, produced a severe illness with hemorrhagic manifestations of disease.⁹³ Apoptosis and a dysregulated cytokine response are believed to be responsible for what has previously been referred to as the “toxemia” of smallpox.

Pathology

Cytoplasmic inclusion bodies are a typical histopathological feature of orthopoxvirus infections. Two morphologies (Fig. 67.2) are manifest: A-type inclusion bodies, where virions are clustered within an intracytoplasmic structure (Fig. 67.2D), or B-type inclusions (Guarneri bodies), which are perinuclear and contain the viroplasm and maturing viral particles. Not all orthopoxviruses form A-type inclusions; a longer predicted form of the A-type protein (130 to 160 kD vs. 90 kD) appears to be required. In cowpox, the A-type inclusion is termed a Downie body; in ectromelia, it is termed a Marchal body. The North American orthopoxviruses also form A-type inclusions.

In humans, it is the rash that has been extensively studied during smallpox disease pathogenesis. The viral lesion primarily develops in the epidermis, although early changes of capillary dilation, endothelial cell swelling, and perivascular cuffing with lymphocytes, macrophages, plasma cells, and eosinophils are seen in the papillary layer of the dermis prior to development of the rash lesion. Subsequently, within the epidermis, the cells of the Malpighian layer swell and vacuolate to undergo ballooning degeneration. B-type inclusions could be seen in the cytoplasm. The cytoplasm continues to enlarge, loss of nuclear material is noted, and coalescence of vacuoles via cell rupture creates reticulating degeneration of the middle and upper layers of the stratum spinosum. In the next stages, the vesicle is formed. Cells of the lower stratum spinosum and basal layer exhibit nuclear condensation and nuclei fragment. The cavity of the vesicle (later the pustule) thus develops adjacent to the dermis, permitting the “deep-seated” feel of the smallpox pustular lesions. The cavity retains some cellular remnants that create a multiloculated appearance, also adding to the firmness of the lesion. When polymorphonuclear cells move into the cavity, pustulation occurs. High titers of virus are found within the lesions.²³ In mucosal surfaces, the absence of a horny layer allowed the necrosis caused by proliferation of virus within the epithelium to create ulcers and led to liberation of large quantities of virus into the oropharynx.¹⁸³

Evaluation of other organs in human smallpox has been done in select autopsies from cases where death was attributed to ordinary or hemorrhagic forms of smallpox.²³ Some of the observations from autopsies of ordinary smallpox case deaths have been that, unlike ectromelia, liver and spleen do not show as extensive evidence of viral replication and necrosis that is seen in ectromelia infection of susceptible mice. In general, mild pathological changes are seen in the lungs. This is in contrast to

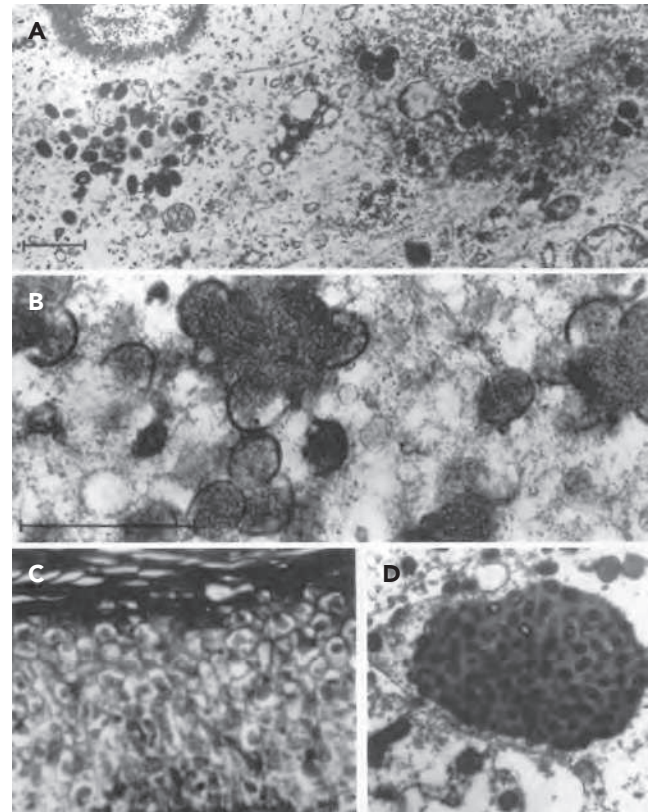


FIGURE 67.2. Cytoplasmic inclusion bodies produced by poxviruses. **A** and **B**: Electron micrographs of viral cytoplasmic factories that manifest by light microscopy as B-type inclusions; these structures are basophilic by hematoxylin-and-eosin staining. **A**: Rounded structures, the immature virions (IVs) are seen on the right, and the nonenveloped intracellular virions (IMVs) are seen on the left. **B**: A higher-power magnification demonstrating the IVs and the earlier crescent-shaped progenitor forms. **C** and **D**: A-type inclusion bodies (ATIs) visualized by light microscopy (**C**) and electron microscopy (**D**). **C**: Hematoxylin-and-eosin staining of mouse skin demonstrating eosinophilic intracytoplasmic structures in the skin of a mouse infected with ectromelia. **D**: Electron micrograph of mature cowpox virions within an ATI. (**A** from Payne LG, Kristensohn K. Mechanism of vaccinia virus release and its specific inhibition by N1-isonicotinoyl-N 2-3-methyl-4-chlorobenzoylhydrazine. *J Virol* 1979;32:614–622. **B** from Stern W, Pogo BG, Dales S. Biogenesis of poxviruses: analysis of the morphogenetic sequence using a conditional lethal mutant defective in envelopes self-assembly. *PNAS USA* 1977;74:2162–2166. **C** from Fenner F. Mousepox. In: Foster HL, Small JD, Fox JG eds. *The mouse in biomedical research*. San Diego, Calif: Academic Press; 1982:209–230, copyright 1982. **D** courtesy of Y. Ichihashi.)

the more severe lung pathology seen in apparent cowpox infection of captive felines, which caused death and was associated with severe bronchopneumonia.¹³⁴ Similarly pulmonary disease has been seen in respiratory rabbitpox infection of rabbits, where in addition to subpleural nodules focal areas of consolidation were noted.⁶⁸

The pathology of the localized lesions of cowpox in humans has been studied. The lesions are characterized by a marked inflammatory and erythematous response.¹² The bulk of the lesion is caused by hypertrophy and proliferation of the basal cell layer of the epidermis, together with massive inflammatory

infiltration. Infection usually spreads into follicles, and typical A-type inclusions are usually seen.

Immune Response: Primary Infection

Early responses to virus infection are the production of interferons, nitric oxide, and elicitation of natural killer (NK) and macrophage cellular functions prior to the development of adaptive, antigen-directed B-cell and T-cell responses. Both complement pathways are also activated by the host in response to virus infection. Studies of ectromelia virus infection in various mice deficient in one or more immune response pathways demonstrate the importance of interferon γ (IFN- γ), granzymes A and B, perforin, and CD4 and CD8 T cells in the clearance of primary infection. Antibody is also essential.^{37,38,155} Additional studies in other orthopoxviruses demonstrate successful clearance of a primary orthopoxvirus infection to be dependent on the adequate production of IFN- γ and Th1 cytokines¹⁶³ to promote an effective cytotoxic T-lymphocyte (CTL) effector response. Experiments where high levels of interleukin 4 (IL-4) are produced early in infection, expressed from recombinant challenge poxvirus, show delayed or inhibited ability of the host to clear the recombinant poxvirus.^{6,92,190} In secondary infection, as in the circumstance of infection post vaccination with a heterologous orthopoxvirus, antibody alone may be sufficient.¹⁵⁴ This latter finding has also been demonstrated in vaccine provided protection for monkeypox infection.⁵⁴

Macrophages and NK cells are involved in early responses to infection and are used for clearance of orthopoxvirus infections.^{57,102} The secreted IFNs are needed for containment of primary orthopoxvirus infections.^{132,175} The cellular secretion of tumor necrosis factor is another host response to orthopoxvirus infection. Viral infection can induce apoptosis, and the poxviruses have a number of strategies to inhibit apoptosis, which are also involved in host range, that have been reviewed²⁰⁵ and are described in Chapter 66.

Studies designed to better define the pathogenesis of adverse effects of current replicative vaccinia used as smallpox vaccine in individuals with atopic dermatitis (AD) provide additional information about the early steps in viral pathogenesis and the normal and aberrant host innate immune responses, as well as factors important in containment of viral infection to produce localized disease. In AD, there is an abundant Th2 response.¹⁵ Human skin explant models have been developed to understand eczema vaccinatum, the dissemination of vaccinia virus in individuals with atopic dermatitis.⁸⁴ In human atopic dermatitis skin, an abundance of Th2 cytokines IL-4 and IL-13 (with respect to normal skin) diminish the effectiveness of an innate immune response to vaccinia virus, in a signal transducer and activator of transcription (STAT)-6-dependent manner, allowing increased replication of virus. In normal skin, viral replication is inhibited by cathelicidins. In human keratinocytes infected by vaccinia, the cathelicidin LL-37 is downregulated through the effects of IL-4 and IL-13.⁸⁵

As mentioned previously with regard to apoptosis, the orthopoxviruses express a number of proteins that have been shown *in vitro* or *in vivo* to evade or modulate the host's response to orthopoxvirus infection. There are a number of reviews that summarize this work,^{3,62,99,186,210} and the specific effects of these proteins may have different manifestations, depending on the route of challenge or the model of infection used.^{2,199} One strategy that the orthopoxviruses have evolved is soluble secreted

proteins to bind the host cytokines, complement C3b and C4b, chemokines, interferons, and their receptors. These include viral proteins that bind tumor necrosis factor (TNF), IL-1 β , and IFN- γ via secretion of an ortholog of the mammalian protein that lacks its intermembranous and signaling portions. The orthopoxviral proteins that bind the type I interferons, as well as those that bind IL-18 (which regulates IFN- γ) and the chemokines, have apparently evolved to develop a structure distinct from the mammalian receptors for these immune response proteins; the IL-18 binding proteins are similar to the mammalian IL-18 regulatory binding proteins.

The orthopoxviruses have strategies to inhibit intracellular antiviral effects of the IFNs. All orthopoxviruses sequenced to date have a homolog of the vaccinia E3L protein, and most have a homolog of the K3L protein. E3L homologs bind double-stranded RNA (dsRNA) and have at least two functions: one is to inactivate the IFN-induced dsRNA-dependent protein kinase (PKR), and the other is to inactivate 2'5'-oligoadenylate synthetase. K3L mimics eIF-2 α , which prevents eIF-2 α phosphorylation and inhibits autophosphorylation (activation) of PKR. E3L inhibition of PKR also interferes with its proapoptotic functions^{13,109,122} to affect cellular host range of vaccinia virus. A number of orthopoxvirus proteins have also been identified that interfere in various intracellular steps of the IL-1R and toll like receptor (TLR) signaling pathways, ultimately regulating the nuclear factor kappa B (NF- κ B) pathway¹⁴⁴ and IFN regulatory factors (IRFs). Two vaccinia proteins appear to act early in the pathway and target multiple nonoverlapping TLR pathways; A52R and A46R both block NF- κ B activation. A vaccinia virus with A52R deleted appears attenuated *in vivo*. A46R inhibits TIR domain containing adapter-inducing interferon β (TRIF)-dependent signaling to inhibit IRF3 activation and TRIF-dependent gene induction.¹⁹² TRIF may have an additional role in inhibition of vaccinia replication in the macrophage.⁸² The product of the vaccinia A52 open-reading frame (ORF) has the ability to block NF- κ B activation via a number of pathways. Additional vaccinia proteins (K1L and N1L) block specific steps further downstream in the signaling pathway for NF- κ B activation.^{51,191}

The orthopoxviruses also express proteins that interfere with various steps in apoptosis.¹⁶⁰ The vaccinia F1L protein interferes with mitochondrial components of the apoptotic cascade²⁰⁴ through interaction with Bak. The serpin, SPI-2 (vaccinia) or crmA (cowpox), inhibits caspase 1 and 8; the latter effect protects target cells against CTL lysis induced by the Fas pathway.

The host-pathogen relationship of orthopoxviruses is complex and involves many host and viral factors. Host responses to pathogenic and nonpathogenic orthopoxvirus infections examined using microarray techniques^{25,72} provide additional information regarding complex host-pathogen interactions. In variola parenterally infected nonhuman primates, which models aspects of the second viremia and subsequent disease course in severe forms of smallpox,⁹³ peripheral blood mononuclear cell transcripts showed an increase in interferon-associated gene transcripts not evident in animals that rapidly succumbed to infection.¹⁷⁹ There was also a significant upregulation of Ig gene transcripts. Overall, there were minimal TNF- α and NF- κ B responses.

Immune Response: Vaccination

The protective effects of smallpox vaccination and correlates of protection remain an area of active investigation.⁶² Vaccinia

strains provided an effective strategy for use as a live viral vaccine to prevent smallpox and to facilitate disease eradication. Smallpox disease was eradicated 25 years ago, at a time when the current advances in immunology were not available to dissect the mechanisms of protection. Although some studies demonstrated high levels of neutralizing antibody after vaccination or smallpox infection were protective,¹⁸¹ other studies have demonstrated the level of CD8+ T-cell memory correlates with protection from subsequent viral disease.²⁰⁸ A number of studies have demonstrated long-lived Ig and T-cell responses to smallpox vaccination^{77,78}; individuals vaccinated in childhood demonstrate long-lived B-cell memory response, and a longer duration of CD4 T-cell memory than CD8 T-cell memory,^{4,5,42,50} which may be important for long-term B-cell memory.

However previous remote childhood smallpox vaccination was not completely protective against acquisition of the related disease monkeypox when it was introduced in the United States in 2003.¹⁰¹ Studies with ectromelia virus infection in mice demonstrate the requirement of a virus-neutralizing antibody response, even in the absence of CD8+ T cells, in order to control a pathogenic virus challenge after immunization.

Release from Host

For smallpox, data on virus shedding show infectious virus in the oropharyngeal secretions, with lesser amounts in the urine and conjunctivae.¹⁸² Levels of virus in oropharyngeal secretions were highest just after rash onset between 3 to 4 days post fever onset, the clinical manifestation of the secondary viremia; however, virus could be detected up to 2 weeks post fever onset in cases with more pronounced rash (confluent disease). Virus could be detected in urine over approximately the same time period during illness. In localized orthopoxvirus infections, such as vaccination with vaccinia virus (smallpox vaccination), virus can be found at the lesion until the scab separates.¹¹⁴

Epidemiology

Of the orthopoxviruses that infect humans, only variola is not a zoonotic infection. Interhuman transmission of variola virus occurred through the inhalation of large airborne respiratory droplets, containing infectious variola virus, from infected persons. Transmission usually required prolonged face-to-face or other close contact, although airborne transmission over longer distances had been reported.⁵⁹ Transmission via fomites or contact with the infectious material from the rash also occurred. Aggregate data, collected during the smallpox eradication campaign, suggest a secondary attack rate of 58.4% in unvaccinated close or household contacts, and a secondary attack rate of 3.8% in previously vaccinated close or household contacts.⁵⁹ Case fatality rates for variola major varied with the type of disease manifest, but aggregate rates of 10% to 30% in various outbreaks have been recorded. Severity of disease correlated with rash burden and was also more severe in children and pregnant women. Variola alastrim minor is a variant of variola with a case fatality rate of <1%, but manifest with apparent similar epidemiologic features of human-to-human transmission.

Monkeypox has a more complex epidemiology; two genetically discrete virus clades have been described,^{39,127} with apparent distinct clinical and epidemiologic parameters. Identification of human infections, in Western and Central Africa, was first made in 1970. Investigations in the Congo Basin country Zaire, now the Democratic Republic of Congo (DRC),

demonstrated that human-to-human transmission of monkeypox was less than that of smallpox. The secondary attack rate in unvaccinated contacts of monkeypox cases were calculated to be 9.3% versus 37% to 88% for smallpox.⁹⁶ Prior smallpox vaccination (3- to 19 years previously) appeared to be 85% protective at preventing disease acquisition in contacts and also ameliorated disease severity. Overall, although the majority of cases identified acquired disease from a presumed animal exposure(s), only 28% of cases were ascribed to person-to-person transmission. A case fatality rate of ~10% was observed in unvaccinated persons; the majority of fatalities and severest disease manifestations was observed in children younger than the age of 5. Serosurveys suggested subclinical infection may have occurred in up to 28% of close contacts of monkeypox patients in some communities, which may have contributed to the rarity of sustained generations of human-to-human transmission in household and other close contact situations.

Because the majority of cases were believed to have acquired disease through animal exposures, case-control studies were attempted to determine the source of infection. These were not definitive because the population appeared to have multiple daily contacts with the same animals in settlements, forests, or cleared agricultural areas. Among primary cases, recent close contact via hunting, skinning, killing, cooking, or playing with carcasses was identified to *Cercopithecus*, *Colobus*, and *Cercocebus* primate, *Cricetomys* terrestrial rodent, and *Funisciurus* and *Heliosciurus* squirrel species. Ecologic studies, usually using convenience samples of animals collected in areas surrounding human cases in West and Central Africa, demonstrated orthopoxvirus and sometimes monkeypox-specific seroprevalence in various members of these species, but these were not reported for *Cricetomys* species. This work has been comprehensively reviewed.⁹⁶ Virus was only found in one euthanized, moribund squirrel species *Funisciurus anerythrus*. The prevailing hypothesis was that squirrel species were the likely reservoir of disease. Near the end of the 1980s, disease surveillance waned after modeling studies based on the epidemiologic observations of secondary attack rates from human-to-human exposure suggested that limited number of transmission events were feasible, even with low population immunity provided by waning immunization rates in the populations of Central Africa. The virus had not, therefore, adapted to survive solely through human infection and would not manifest with the same human-to-human transmission dynamics of smallpox. This work also led to the recommendations to not continue routine smallpox vaccination in individuals considered at risk for monkeypox infection.

A re-emergence of disease was noted in 1996 in the DRC; a salient observation from a series of investigations was that more cases were derived from secondary human-to-human contact (88%) than seen in 1981 to 1986 investigations (28%). This was, in part, attributed to a larger population of humans fully susceptible to disease because of the cessation of routine smallpox vaccination in 1980 after smallpox eradication. Another observation was that disease epidemiology showed more cases in the older child/young adult population.⁸⁸ Disease mortality (1%) was observed to be lower than previously seen in 1981 to 1986. This may have been due to a smaller demographic of very young children (ages 0–4 years) being infected, may have been due to technical limitations on the investigations, or may have been due to the inclusion of chickenpox cases in

the case definition. Whereas previous ecologic serosurveys of animal populations had implicated tree squirrels as having significant orthopoxvirus seroreactivity, these investigations were the first to show orthopoxvirus seroprevalence in terrestrial rodents (*Cricetomys emini*) and in one domestic pig (*Sus scrofa*) sampled. Outbreaks of monkeypox, some with apparent high interhuman transmissibility,¹²⁰ and ongoing surveillance documents an increased incidence of monkeypox in regions of the Congo Basin.¹⁶⁹

Monkeypox virus was introduced to the United States in 2003 via a consignment of wild-captured animals from the West African country of Ghana. The virus was identified as belonging to a distinct clade of monkeypox, which included previous West African monkeypox isolates and isolates derived from earlier outbreaks in primate colonies. Detailed comparison of the clinical and epidemiologic characteristics of the U.S. cases (imported from West Africa) with DRC cases from 1981 to 1986 demonstrated significant differences in human disease manifestation¹²⁷; pronounced rash and more severe illness were seen in the Congo Basin cases. Controlling for age and vaccination status, disease severity remained more extreme in the Congo Basin than in the U.S. case patients. Monkeypox-related mortality and human-to-human transmission were only seen in the Congo Basin. These data are suggestive of at least two populations of monkeypox viruses being maintained in Africa.

Cowpox

Cowpox is a virus found in Europe and Asia that is maintained in rodents; in Britain, these are bank voles and woodmice (Table 67.1).^{16,31,35} Human infection is a zoonosis. The domestic cat is one common source of human infection,¹⁷ and this probably explains the occurrence of cases in children; 26% of 54 cases identified between 1969 and 1993 were in children younger than 12 years.¹² Most feline and human cases occur between July and October, with only occasional cases between January and June. Human cases occur in which no source is traced, but despite detailed investigations, only three human cases in Britain since 1968 have been traced to a bovine source, and no case of bovine cowpox has been detected since 1976.

Cowpox virus has a wide host range, and an interesting finding has been the occurrence of cowpox in a variety of captive exotic species in European zoos. Susceptible animals have included cheetahs, lions, anteaters, rhinoceros, elephants, and okapi, and infection has occasionally been transmitted to animal handlers.^{11,158,159} Recent outbreaks of human infections with cowpox virus in Europe have been traced to rat populations used in the pet trade.²⁹

Vaccinia

The origin of vaccinia is unknown, and no natural host for the virus is known. However, a number of vaccinia variants have been described. Buffalopox has been described in India; human infection results from contact with infected animals. It is not known whether buffaloes are a reservoir or whether other wildlife is involved, as with cowpox. Similarly, there have been increasing reports of vaccinia viruses infecting cattle handlers in Brazil. Cantagalo virus has been classified as a vaccinia variant, but other similar nonidentical vaccinia-like viruses have also been reported in the region.^{44,47,48,123,197} Studies have begun to investigate the disease ecology of these viruses.¹

Clinical Features

Variola: Smallpox

Naturally acquired variola virus infection caused a systemic febrile rash illness; the course of clinical features is schematically diagrammed in Figure 67.3. For ordinary smallpox, the most common clinical presentation, after an asymptomatic incubation period of 10 to 14 days (range 7 to 17 days), was fever, which quickly rose to about 103°F, sometimes with dermal petechiae. Associated constitutional symptoms included backache, headache, vomiting, and prostration. Within a day or two after incubation, a systemic rash appeared that was characteristically centrifugally distributed (i.e., lesions were present in greater numbers on the oral mucosa, face, and extremities than on the trunk). Lesions commonly manifested on the palms and soles. The rash lesions were initially macular and then advanced to the papular stage, enlarging and progressing to a vesicle by day 4 to 5 and a pustule by day 7; lesions were encrusted and

FIGURE 67.3. The clinical development of discrete ordinary smallpox. After respiratory droplet exposure and infection, an asymptomatic incubation period of 7 to 17 days precedes prodromal symptoms of high fever and malaise. Subsequently, rash develops 2 to 4 days after the prodrome. The fever diminishes at the onset of rash development. The characteristics of smallpox and chickenpox rash are contrasted.

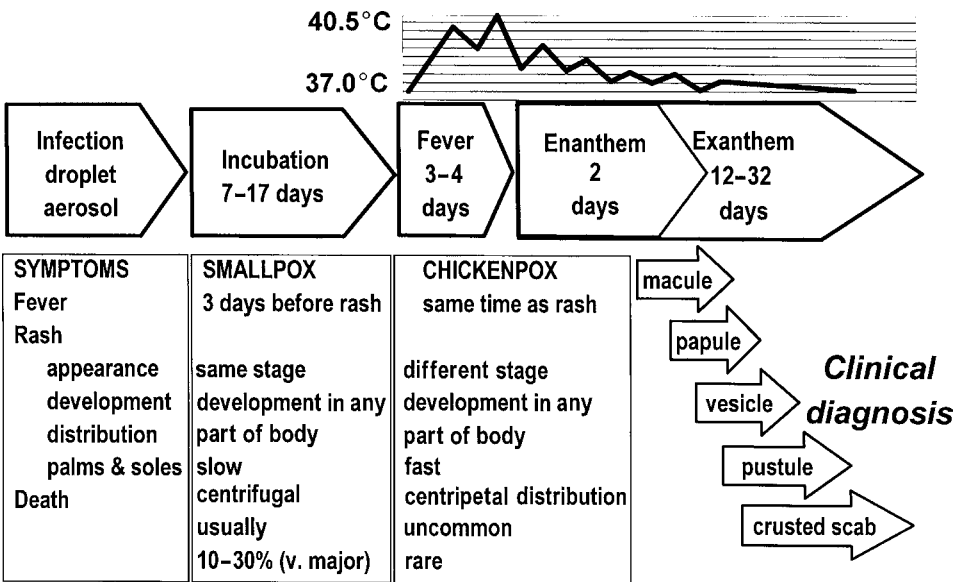




FIGURE 67.4. The rash of smallpox. The smallpox recognition card used during the Intensified Smallpox Eradication Programme of the World Health Organization, showing the nature and distribution of the rash of ordinary smallpox. (From Fenner F, Henderson DA, Arita I, et al. *Smallpox and its Eradication*. World Health Organization, Geneva, 1988, with permission.)

scabby by day 14 and then sloughed off. Skin lesions were deep seated and in the same stage of development in any one area of the body. Images of the classic presentation of ordinary smallpox are depicted in Figure 67.4. Milder and more severe forms of the rash were also documented. Less severe manifestations (modified smallpox or *variola sine eruptione*) occurred in some vaccinated individuals, whereas hemorrhagic or flatpox types of smallpox are believed to have developed as a result of an impaired immune response of patients.

Variola major smallpox was differentiated into four main clinical types: (a) ordinary smallpox (~90% of cases) produced viremia, fever, prostration, and rash; mortality rates were generally proportionate with the extent of rash and ranged, using the WHO classification, from less than 10% for ordinary discrete to 50% to 75% for the rarer ordinary confluent presentation; (b) (vaccine) modified smallpox (5% of cases) produced a mild prodrome with few skin lesions in previously vaccinated people and had a mortality rate of less than 10%; (c) flat smallpox (5% of cases) produced slowly developing focal lesions with generalized infection and had an approximate fatality rate of 80%; and (d) hemorrhagic smallpox (<1% of cases) induced bleeding into the skin and the mucous membranes and was invariably fatal within a week of onset. Notably, prior vaccination did not appear to protect from mortality associated with

the more severe flat or hemorrhagic forms of disease. A discrete type of the ordinary form, with typical febrile prodrome and rash, resulted from *alastrim variola minor* infection.⁵⁹

The WHO established a system for classifying smallpox case types on the basis of disease presentation and rash burden. The hemorrhagic and flat types have been briefly described previously. The ordinary type was subgrouped into three categories, which were based on the extent of rash on the face and the body: confluent, semiconfluent, and discrete. In ordinary confluent disease, no area of skin was visible between vesiculopustular rash lesions on the trunk or the face. In ordinary semiconfluent and discrete disease, patches of normal skin were visible between rash lesions on the trunk and face, respectively. Disease modified by vaccine presented with sparse numbers of lesions. Survival from infection conferred lifelong immunity.

Monkeypox

In humans, clinical disease is believed to result from either respiratory, percutaneous, or permucosal exposures. Classical descriptions from the active surveillance program in Zaire, now the DRC, have historically provided most of our understanding of human monkeypox. Following an incubation period of 7 to 17 days (mean 12 days) a prodrome of fever, headache, backache, and fatigue begins. The cutaneous eruption evolves



FIGURE 67.5. Human monkeypox. The day 8 appearance of monkeypox rash on a 7-year-old girl from the Democratic Republic of Congo. Prominent lymphadenopathy is apparent at the angle of the chin and in the inguinal areas. (From Breman JG, Kalisa R, Steniowski MV, et al. Human monkeypox 1970–1979. *Bull WHO* 1980;58:165–182.)

similar to that of smallpox. Lesions evolve in the same stage in any one part of the body from macules, papules, vesicles, pustules, and then crust and scar. This is apparently more common in unvaccinated individuals⁹⁶; in previously vaccinated individuals, pleomorphic presentation of rash lesions was observed. Following resolution of the rash, hypopigmentation is followed by hyperpigmentation of the scarred lesions. Pronounced lymphadenopathy clinically distinguishes monkeypox from smallpox. Lymphadenopathy in the facial area: cervical, postauricular, submandibular, and inguinal can be quite pronounced. Images of the typical presentation of human monkeypox in the DRC are in Figure 67.5.

Vaccinia Virus

Multiple-puncture vaccinia virus infection, using a bifurcated needle, is currently used in the smallpox vaccination regimen used for laboratory workers using orthopoxviruses¹⁷⁷ and public health care personnel and military in the United States. Most commonly, the infection progresses through a standard course of events from vesicle to pustule (Fig. 67.6). However, of all vaccines used today, the smallpox vaccine, comprising live replicative vaccinia virus, has one of the highest rates of adverse events.⁴¹ Major complications include progressive vaccinia, eczema vaccinatum, generalized vaccinia, postvaccinial encephalitis, accidental infection, and carditis (Fig. 67.7).

Progressive vaccinia, previously called vaccinia necrosum or vaccinia gangrenosum, is a rare and often fatal vaccine complication in persons with severe deficiencies of cellular immunity.²⁴ In 1968 in the United States, there were five cases among 6 million primary vaccinees and six cases among 8.6 million revaccinated persons¹¹⁶; 4 of these 11 patients died. Progressive vaccinia is characterized by progressive, often painless growth and spread of the vaccine virus beyond the inoculation site, often leading to necrosis, sometimes with metastases to other body sites.⁶⁴ The possibility of progressive vaccinia should be considered if the vaccination site lesion continues to progress and expand without apparent healing more than 15 days after vaccination.¹⁵⁰ Initially, limited or no inflammation is present at the inoculation site and histopathological examination shows an absence of inflammatory cells.¹⁰⁴

Eczema vaccinatum (EV) can occur in people with a history of atopic dermatitis (eczema), irrespective of disease severity or activity. This complication is the clinical result of local spread or dissemination from the primary vaccination site in such persons or the result of the skin of an individual with atopic dermatitis contacting the unscabbed vaccination site of another person.^{64,149} A localized or generalized papular, vesicular, or pustular rash anywhere on the body or localized to previous eczematous lesions is the clinical presentation of eczema vaccinatum. Systemic illness with fever, malaise, and lymphadenopathy may occur. In the national U.S. surveillance of smallpox vaccination that was done in 1968, there were 66 cases (no deaths) of eczema vaccinatum among 14.5 million vaccinees (4.6 cases per million) and 60 cases (one death)

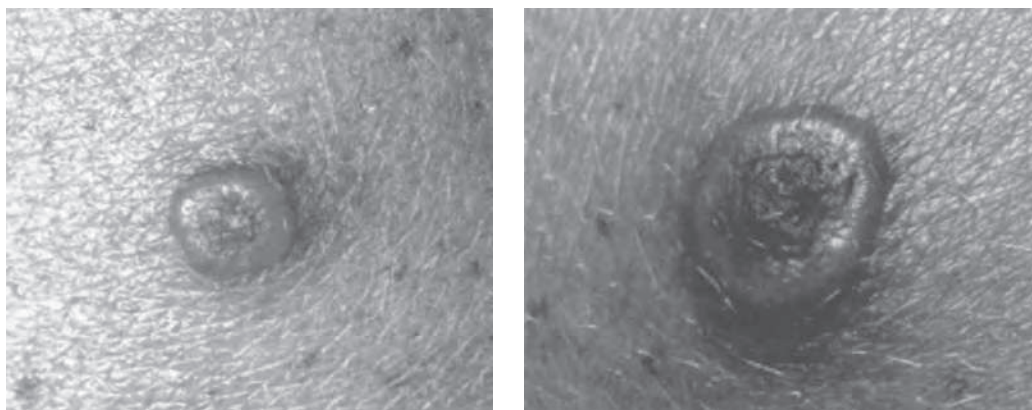


FIGURE 67.6. Primary response to vaccination: typical vesiculopustular response, maximal at 8 to 11 days. Left: Day 7; right: day 11. (Courtesy of J. R. L. Forsyth.)



FIGURE 67.7. Severe complications of vaccination. **A:** Eczema vaccinatum in the unvaccinated contact of a vaccinated sibling. **B:** Progressive vaccinia, previously known as vaccinia necrosum, in a child with a congenital defect in cell-mediated immunity. **C:** Generalized vaccinia 10 days after vaccination. **D:** Ocular vaccinia. (From Fenner F, Henderson DA, Arita I, et al. *Smallpox and its Eradication*. World Health Organization, Geneva, 1988, with permission.)

among their several million contacts.¹¹⁶ In one study, early vaccinia immune globulin (VIG) administration reduced mortality from 30% to 40% to 7%.¹⁰⁶

Generalized vaccinia is a nonspecific term that is used to describe a vesicular rash that develops after vaccination. Excluding dissemination associated with eczema vaccinatum and progressive vaccinia, it has been extremely rare to document virus in these lesions.¹⁰⁵ True generalized vaccinia is believed to represent the end product of viremic spread of virus, and no predisposing factors have been identified. Generalized vaccinia was estimated to occur in about 242 of every 1 million primary vaccinations¹¹⁶; likely many of the historic reports also included allergic and inflammatory responses to the vaccine.²⁰⁰

Postvaccination encephalomyelitis (PVEM) is a rare but serious complication that usually occurs in primary vaccinees. The frequency of its occurrence differed widely from country to country and with the strain of vaccinia virus used in the vaccine. The incidence of PVEM was lower with the New York City Board of Health (NYCBOH) vaccinia virus strain (the U.S. vaccine strain) than with the strain used in other countries.⁵⁹ No

predisposing factors for PVEM are known, although host factors are believed to be important; the pathophysiology of PVEM is not well understood. Patients have variably displayed clinical and diagnostic features suggestive of a postimmunization demyelinating encephalomyelitis or direct viral invasion of the nervous system. This postvaccination reaction typically occurs 11 to 15 days following vaccination. Symptoms of PVEM include fever, headache, vomiting, confusion, delirium, disorientation, restlessness, drowsiness or lethargy, seizures, and coma. The cerebral spinal fluid can demonstrate elevated pressure, but generally has a normal cell count and chemistry profile.¹⁹³ Infants younger than 2 years old can also develop a rare, postvaccination encephalopathy (PVE) similar to PVEM. Acute onset of PVE occurs earlier in the postvaccination period (6–10 days after vaccination), presents with the same symptoms as PVEM, and may also include hemiplegia and aphasia. Diagnosis of PVE or PVEM is one of exclusion because there are no specific diagnostic tests to confirm the diagnosis of this complication and there are many other infectious and toxic etiologies that can result in similar clinical symptoms. Accidental infection occurs when virus from the vaccination site is transferred to another site or to another person via intimate skin contact. This usually occurs in primary vaccinees rather than revaccinees. Accidental self-inoculation, which most commonly occurs on the face, mouth, lips, or genitalia, is usually not serious and requires no specific treatment. Inoculation of the eye or eyelid is more serious and can be sight threatening if not evaluated and treated appropriately. In the 5 years between 1963 and 1968, ocular vaccinia was observed in 348 persons; 259 were vaccinees, and 66 were contacts of vaccinees. Of these, 22 had evidence of corneal involvement and 11 experienced permanent defects.^{116,178}

Cardiac adverse events had not been reported before 2003 in any person vaccinated with the NYCBOH strain. Myocarditis and pericarditis were documented in 18 of 230,734 primary vaccinees immunized with the NYCBOH strain in 2002 and 2003.⁷⁶ Arrhythmias and myocardial ischemia have also been described recently, but the association with vaccination is not as clear.

Vaccinia viruses in the Indian subcontinent (buffalopox) and in Brazil (Cantagalo virus and related strains Aracatuba virus, Belo Horizonte virus, Passatempo virus, and others) have been described to cause human infection in handlers of buffalo or cattle.⁴⁸ Up to 10 lesions have been described on the hands or arms of the human handlers; fever, lymphadenopathy, backache, and fatigue have also been associated symptoms. Transmission is believed to occur via unprotected contact with active lesions present on animal teats and udders. Interhuman transmission of buffalopox to family members has been reported to occur via contact.¹¹³

Cowpox

Cowpox also causes localized lesions in humans (Fig. 67.8D), and lesions in cows are typically found on their teats and udders (Fig. 67.8A). Most information is available from a detailed analysis of 54 human cases investigated during 1969 to 1993.¹² Lesions are generally restricted to the hands and face, and most patients (72%) have only one lesion. Multiple lesions may be caused by multiple primary inoculations, autoinoculation, and rarely by lymphatic or viremic spread. Occasionally, a very severe infection, and death, may occur usually in immunosuppressed individuals.

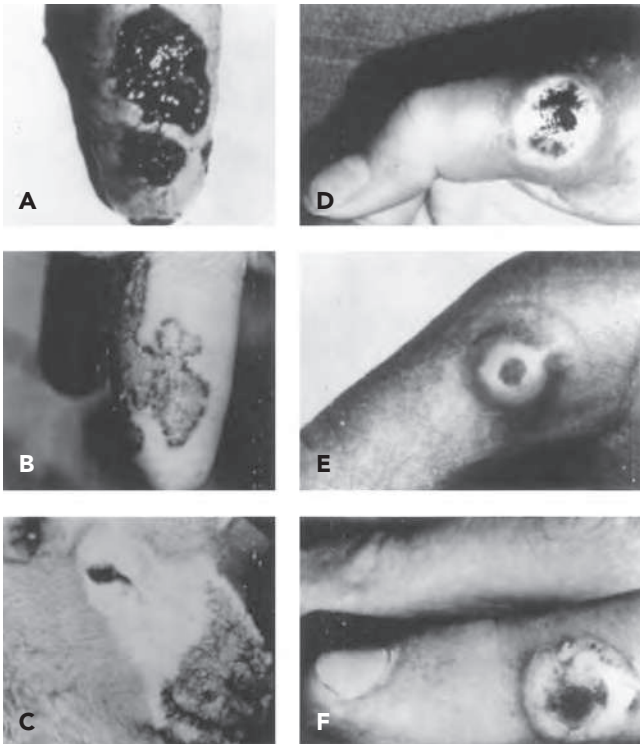


FIGURE 67.8. Cowpox, pseudocowpox, and orf virus infections of animals (A–C) and the lesions caused by these viruses on the digits of humans (D–F). A: Cowpox ulcer on teat of cow, 7 days after onset of symptoms. B: Pseudocowpox (milker's nodule) virus infection of cow teat. C: Orf virus scabby mouth in a lamb. D: Cowpox. E: Milker's nodule. F: Orf. (A and B courtesy of E. P. J. Gibbs; C courtesy of A. J. Robinson; D courtesy of A. D. McNae; E and F courtesy of J. Nagington.)

The lesion passes through macular, papular, vesicular, and pustular stages before forming a hard black crust. The lesion is usually quite painful, and erythema and edema are common at the late vesicular and pustular stages (Fig. 67.8A). There is usually lymphadenitis, fever, and general malaise, often referred to as “flulike.” These features are usually severe in children, and absence from school or work is common; 16 of 54 patients (30%) were hospitalized. Most cases take 6 to 8 weeks to recover; in some cases, it may take up to 12 weeks. Scarring is usually permanent.

Diagnosis

Differentiating Smallpox from Other Diseases and Conditions

Prior to its eradication, smallpox as a clinical entity was relatively easy to recognize, but other exanthematous illnesses were mistaken for this disease. For example, the rash of severe chickenpox, caused by varicella-zoster virus, was often misdiagnosed as that of smallpox. However, chickenpox produces a centripetally distributed rash and rarely appears on the palms and soles. In addition, in the case of chickenpox, prodromal fever and systemic manifestations are mild, if they manifest at all; the lesions are superficial in nature; and lesions in different developmental stages may present in the same area of the body. Other diseases and conditions that could be confused

with vesicular-stage smallpox include monkeypox, generalized vaccinia, disseminated herpes zoster, disseminated herpes simplex virus infection, drug reactions (eruptions), erythema multiforme, enteroviral infections, scabies, insect bites, impetigo, and molluscum contagiosum. Diseases confused with hemorrhagic smallpox included acute leukemia, meningococcemia, and idiopathic thrombocytopenic purpura. The Centers for Disease Control and Prevention (CDC), in collaboration with numerous professional organizations, has developed an algorithm for evaluating patients for smallpox. The algorithm assists in differential diagnoses of the vesiculopustular stage of rash. The algorithm and additional laboratory testing information are available at www.bt.cdc.gov/agent/smallpox/diagnosis/evalposter.asp and www.bt.cdc.gov/agent/smallpox/diagnosis/rashtestingprotocol.asp. An evaluation of the algorithm has been published.¹⁸⁹

Laboratory

A variety of diagnostic strategies are available for the laboratory diagnosis of orthopoxvirus infections. Negative stain electron microscopy cannot differentiate the virions of orthopoxvirus from those of yatapox or molluscipox, but the clinical and epidemiologic information will likely aid interpretation. Some of the classic methods for distinguishing orthopoxvirus species are depicted in Table 67.2. Protein-based diagnostics can be used to detect the genus, but for the most part cannot identify individual species.⁴⁵ An IgM capture serologic assay may provide a useful tool for assessing orthopoxvirus disease incidence¹⁰⁰ and may provide utility in assessing infectious etiologies of encephalitic symptoms associated with orthopoxviruses.¹⁸⁷

The majority of tests that can differentiate orthopoxvirus species, however, rely on nucleic acid testing. Identification of orthopoxvirus species can be accomplished through morphologic observations of virus pock growth on chorioallantoic membrane of embryonated eggs. A number of nucleic acid test strategies have been developed and published; the majority are polymerase chain reaction (PCR) based.^{115,125,126,153,185}

Prevention and Control

Vaccination has been the historic mechanism for control of interhuman transmissible orthopoxvirus infections. The successful eradication of smallpox, declared in 1980 by the WHO, was largely due to the effective use of vaccinia vaccine strains worldwide in an extensive immunization program. Vaccination could also be used postexposure to provide protection; greatest protection was afforded if provided within days of contact to a patient,^{136,173} and if the contact had previously been vaccinated.⁸⁰ For laboratory workers, vaccination is recommended in some countries¹⁷⁷ for those using any orthopoxvirus capable of causing disease in humans; in others, only for those working with the more human pathogenic orthopoxviruses. Because of the adverse events associated with the use of current vaccinia virus vaccine strains, evaluation of vaccination regimens using attenuated vaccinia strains^{107,138,143} or subunit vaccines have been undertaken.

Recent work has also focused on the development of antiorthopoxvirus therapeutic strategies. Compounds with direct antiviral effects, as well as others that inhibit viral use of cellular functions, have been evaluated and demonstrate efficacy in various animal model systems of orthopoxvirus challenge.^{21,86,108,117,162,165,166} At least two antiviral agents, with different mechanisms of antiviral action, are moving into

advanced stages of research and development for orthopoxvirus treatment. A DNA polymerase inhibitor, cidofovir, and an orally bioavailable derivative CMX-001™ of the parent compound has activity against orthopoxviruses in tissue culture, and in treatment of various orthopoxvirus-animal model challenges. ST-246 (Tecoviramat™), a novel compound that functions to inhibit later stages of viral maturation and egress has been shown effective against orthopoxviruses in similar studies. These products have been used investigationally, with vaccinia immune globulin, for the treatment of severe vaccinia infections related to vaccination.^{33,34,201}

PARAPOXVIRUS

History

Parapoxvirus infections are widespread, worldwide in sheep, goats, and cattle. Human infections from these sources are a common occupational hazard for those in contact with infected animals or, less commonly, fomites. The current International Committee on Taxonomy of Viruses (ICTV)—recognized members of the genus *Parapoxvirus* are orf virus (ORFV), bovine papular stomatitis virus (BPSV), pseudocowpoxvirus (PCPV), and parapoxvirus of red deer in New Zealand (PVNZ). BPSV and PCPV are maintained in cattle, whereas ORFV is maintained in sheep and goats, but all three are zoonoses. A squirrel parapoxvirus had been described by electron microscopic morphology as a parapoxvirus, but genome sequencing suggests it is an unclassified poxvirus.¹⁹⁴ Distinct parapoxvirus infections of, and viral species from, red deer in New Zealand^{83,173} have been characterized. PVNZ has not been observed to infect humans. In the United States, a novel deer-associated parapoxvirus was characterized as the cause of cutaneous infections in two hunters. The viruses appear related to pseudocowpoxviruses.¹⁷⁶ Other tentative members of the genus are camel contagious ecthyma virus or chamois contagious ecthyma virus, which causes a disease initially called Ausdyk or camel contagious ecthyma in camels; this may also be the virus responsible for cases of “camelpox” described in humans in the Middle East.⁹⁷ Another tentative member is seal parapoxvirus.^{14,198} A poxvirus infection of reindeer¹¹⁰ has been classified as an orf virus and demonstrated the ability of orf to cross species barriers.

Human infection with ORFV is termed orf. Most commonly, the disease is transmitted from sheep or goats. The disease in animals is alternately referred to as orf, contagious pustular dermatitis, contagious ecthyma, or scabby mouth. Disease in sheep was recognized as early as 1787; the earliest known report of human disease was made in 1879. Human infection with PCPV is commonly called milker’s nodule. The disease is transmitted from dairy cattle, and is referred to as paravaccinia or pseudocowpox in these animals. Notably, this disease was not formally distinguished from that caused by the orthopoxvirus cowpox until the end of the nineteenth century, although Jenner did recognize “spurious” forms of cowpox on cow teats, described in the late 1700s as “pustular sores” lacking “bluish or livid tint”.⁹⁴ Human disease with BPSV is termed *bovine papular stomatitis* and is transmitted from beef cattle; the disease name when virus infects cattle is the same. Initial descriptions of disease in beef cattle were published in 1884; transmission of infection, causing illness in humans, was described in 1953.¹⁵² Subsequent reports demonstrated humans could transmit disease

to cattle.³² Human disease with seal parapoxvirus is called sealpox and is transmitted from seals. Human infection of a seal handler was first reported in 1987⁸¹; the virus was first detected in seals and sea lions in 1969.²⁰⁹

Infectious Agent

By negative stain electron microscopy, parapoxviruses appear ovoid with a criss-cross surface appearance (Fig. 67.1), generated by the genus’ characteristic outer spiral tubule structure. The particles of orf virus are 260-nm long by 160-nm wide; pseudocowpox virions appear a bit larger 300 nm by 190 nm. The viruses obtained from clinical materials grow most readily on primary ovine or bovine cell culture systems and do not grow in embryonated eggs. A few strains have been laboratory adapted to grow on VERO cells or MRC-5 cells; this has been associated with genome changes and attenuation of the virus.

The separation of the parapoxviruses into at least four distinct groups has been based on natural host range, pathology, and nucleic acid methods, including restriction endonuclease, DNA/DNA hybridization, and, most recently, partial or full gene or genome sequence analyses.^{49,142,194} As with other poxviruses, the latter studies have shown that the parapoxviruses share extensive homology between central regions of their genomes but much lower levels of relatedness within the genome termini. The high G+C content of parapoxvirus DNA is in contrast to most other poxviruses (except for molluscipoxvirus) and suggests that a significant genetic divergence from other genera of this family has occurred. DNA sequencing of the genome of two parapoxviruses, species BPSV and ORFV, has allowed a detailed comparison with the fully sequenced genomes of other chordopoxviridae. These studies have provided a genetic map of ORFV and BPSV, and revealed that within the central core of the genome, 88 ORF are conserved with the other sequenced chordopoxviridae.⁴⁹ This conservation is not maintained in the genome termini where insertions, deletions, and translocations have occurred. Studies have demonstrated a great degree of diversity within species via comparison of three complete orf genome sequences.¹⁴²

Pathogenesis and Pathology

Most of the work on the pathogenesis of parapoxvirus infections comes from studies of orf and paravaccinia viruses. Most animal models have used orf virus in sheep; however, there is detailed literature on the pathologic appearance of human orf lesions. Infection occurs via cuts and scratches and remains localized. After entry through nonintact epithelium, virus replicates in the regenerating epidermal keratinocytes.¹⁴⁰ Lesions are produced by hypertrophy and proliferation of epidermal cells, often marked, and leukocyte infiltration. Histologic examination shows many small multilocular vesicles within the dermis; true macrovesicles rarely occur.^{98,211} In the first 2 weeks, the epidermis shows ballooning degeneration in the keratinocytes, and the dermis shows newly formed thin-walled blood vessels with an inflammatory infiltrate. As the infection progresses, in weeks 2 to 3, the intraepidermal vesicles described previously are seen in some cases, with intraepidermal bullous lesions in others. At this point, keratotic material could be visualized on the skin surface. Viral particles were only visualized in the epidermis and were present in the greatest numbers in the first 2 weeks after infection.⁹⁸

The immune response in natural human infection has been investigated.²¹¹ There is a vigorous but short-lived cell-mediated

response and a relatively poor and short-lived humeral response. This is consistent with the occurrence of second infections in 8% to 12% of individuals.^{174,211} Studies of the immune response to orf infection in sheep have been reviewed.⁷⁴ Immunohistochemical studies of lesions demonstrate large numbers of neutrophils, lymphocytes (T and B), and dendritic cells surrounding virus-infected epidermal cells.⁵ In comparison of primary versus reinfection lesions, the appearance of CD8+ T cells and B cells are later in primary infection than in reinfection; CD4+ T cells are the predominant cell present in either lesion. Virus replication is decreased in reinfection lesions with respect to that seen in primary lesions. Studies of cytokine response demonstrated γ -IFN mRNA+ cells, and some TNF- α mRNA+ cells in reinfection, but not in primary infection. Cellular depletion studies¹³⁰ have suggested that CD4+ T cells, IFN- γ , some CD8+ T-cell responses, and some humeral components are necessary for host resolution of infection. Passive antibody transfer, however, does not appear to protect young animals from infection,²⁷ nor does there appear to be a predominant neutralizing antibody response to orf infection.⁴³

The parapoxvirus genomes are predicted to express several proteins involved in the host–pathogen interaction; the majority of those studied have been in the context of orf virus. Orf virus expresses a homolog of the mammalian vascular endothelial growth factor (VEGF), which has been demonstrated to be a viral virulence factor. The absence of the orf protein produces an attenuated virus¹⁸⁴; it is hypothesized that the viral protein stimulates keratinocyte growth likely to provide cell substrate for viral replication; in addition, it inhibits apoptosis.¹⁸⁴ The product of a viral gene, OVIFNR, a homolog of vaccinia E3L, prevents inhibition of host protein synthesis to permit virus utilization of the protein synthetic apparatus. The mechanism for this is protein binding to dsRNA formed during viral infection, preventing PKR kinase activation and subsequent eIF-2 phosphorylation. Other virally encoded proteins are postulated to evade or modulate the host immune response. These include proteins that inhibit granulocyte-macrophage colony-stimulating factor (GM-CSF) and mimic IL-10. *In vitro* studies implicate the orf expressed IL-10 homolog in impairment of an effective immunologic memory response, and is a virulence factor.^{63,119} A chemokine binding protein expressed by the virus can interfere with recruitment of dendritic cells to the site of orf infection.¹¹⁸

Epidemiology

Human infections with parapoxviruses are an occupational hazard of farm workers, abattoir workers, veterinary surgeons, and students, and so on. It is most common in the lambing and calving seasons, and more commonly reported in sheep workers than in cattle workers; this probably reflects differences in animal husbandry. Of 191 cases with a known source surveyed from 1978 to 1995, 84% had an ovine source and 16% were from cattle. During the same period, 32 cases occurred in abattoir workers.¹¹

Factors responsible for ongoing transmission have been attributed both to the environmental stability of orf virus in scab material (although infectivity wanes over time) and to the manifestation of chronic infections in some animals.¹²⁹ There is no known latency state for this or any other poxvirus. Transmission of virus to humans and to animals is via direct contact with lesions or with fomites.

Clinical Features

The progressive stages of human infection have been described in detail,^{98,121,211} and illustrations have also been provided⁶⁶ (Fig. 67.8B, C, E, and F). Lesions start as erythematous papules and progress to a “target” stage. This, seen 1 to 2 weeks after infection, has a red center surrounded by a white halo and an outer inflamed halo. The lesions progress to a nodular and then papillomatous stage, which often has a “weeping” surface. In some patients, lesions may enlarge and persist for some weeks before resolving, and may cause some concern. The lesion resolves via a crusting stage, which may last some weeks. Very large granulomatous lesions can occur, usually in immunocompromised individuals.

Most patients have only one lesion, but multiple primary lesions may occur. Systemic reaction is relatively uncommon, and the lesion is often not particularly painful. Lymphadenopathy is present in some patients, and lymphangitis is also observed but is relatively uncommon. *Erythema multiforme* was reported in one-third of patients reported in one case series.⁹⁸ However, because many ordinary uncomplicated cases of orf go unreported, the actual incidence of *E. multiforme* is probably low.

Diagnosis Differential

Differential diagnosis of parapoxvirus lesions can include ecthyma gangrenosum (a pseudomonas infection of immunocompromised persons), cutaneous anthrax, erysipeloid, and tularemia or tumor. Clinical diagnosis of uncomplicated cases in patients with a known animal contact should not cause difficulties but has been confused with anthrax. However, farmworkers and so on recognize the infection and tend not to seek medical attention for routine cases. Consequently, a disproportionately large number of reported cases have no known contact with infected animals. Of approximately 500 cases surveyed from 1978 to 1995, some 45% had no such contact. Clinical diagnosis of such cases, particularly if severe or prolonged, may be difficult. In particular, large weeping granulomatous or papillomatous lesions may be misdiagnosed as malignancies.

Laboratory

With negative stain electron microscopy, virions with the characteristic morphology of parapoxviruses are usually easily seen in lesion extracts, and this provides a rapid, certain diagnosis of the genus. The virus can be grown in cell culture, but this is not attempted routinely in laboratories that do not maintain primary ovine or bovine cultures.

A number of methods derived from limited or complete genomic analysis have been used for nucleic acid detection (PCR) and laboratory diagnosis of infection.^{91,125,196} Species-specific and species-generic protein-based diagnostics for parapoxviruses have also been developed.⁴³

Prevention and Control

Most workers at risk get infected at some stage, and reinfection is not uncommon. Individuals should take care not to spread infection by autoinoculation or to contacts, including animals. The vaccine used to control orf in sheep is fully virulent and has caused human infection. Work is underway to create live attenuated viruses that can be used as vaccines. Treatment options

are limited; anecdotal reports have described the use of topical cidofovir,¹³⁷ or topical imiquimod. Other options may be topical formulations of interferon-modulating compounds such as imiquimod.

MOLLUSCUM CONTAGIOSUM

Molluscum contagiosum virus causes a disease characterized by one or more benign, self-limited skin “tumor(s)” or papular eruption(s), occurs worldwide, and is regarded as a specific human infection.²⁰ There is no evidence of disease transmission between humans and other animals, although lesions resembling molluscum and containing pox virions have been detected in species other than humans (e.g., horses, chimpanzees).

Infectious Agent

Four subtypes of molluscum contagiosum, characterized by restriction endonuclease digests, have been described.¹⁴⁶ Disease presentation by all subtypes appears to be similar. The genome of molluscum contagiosum virus subtype I has been sequenced¹⁸⁸ and encodes a number of novel gene products involved in its pathogenesis and evasion of the immune system, including, among others, an IL-18 binding protein and apoptosis inhibitors. Attempts at propagating the virus in tissue culture have been unsuccessful.

Pathogenesis and Pathology

Molluscum contagiosum does not cause systemic disease and remains localized at the site of inoculation. Molluscum contagiosum lesions have long been known to have a distinctive pathology. Description of the characteristic molluscum bodies, or Henderson-Patterson bodies, was made in 1841 by Henderson and Patterson. A good review of the histopathological features of the infection is available¹⁶¹ (Fig. 67.9A). The onset of infection occurs when virus begins replicating, extending upward, in the lower layers of the epidermis.¹⁵⁷ The asymptomatic incubation period is variable but can be protracted. As the infection progresses, the epidermis hypertrophies and

extends down into the underlying dermal strata. Characteristic inclusions (molluscum bodies) are formed in the prickle cell layer and gradually enlarge as the cells age and migrate to the surface. These cells are replaced by hyperplasia of the basal cell layer; few to no viral structures are seen in the stratum basale. The structure of the basement membrane remains intact; the hypertrophied epidermal cells, with their cytoplasm occupied by a large acidophilic granular mass (i.e., the molluscum body), project above the skin to appear as a tumor. Little to no inflammatory infiltrate is seen until late in disease, just prior to natural resolution of the lesion.

Clinical Features

Molluscum infection occurs as a result of molluscum contagiosum coming in contact with nonintact skin. The characteristic lesion begins as a small papule and, when mature, is a discrete, 2- to 5-mm diameter, smooth, dome-shaped, pearly or flesh-colored nodule, which is often umbilicated (Fig. 67.9B). A cheesy, off-white or yellowish material is easily expressed from lesions. There are usually 1 to 20 lesions, but there may occasionally be hundreds. Because of multiple simultaneous infections or mechanical spread, the lesions may become confluent along the line of a scratch, and satellite lesions are sometimes seen.

In children, molluscum lesions occur mainly on the trunk and proximal extremities, and in adults, they tend to occur on the trunk, pubic area, and thighs; however, in all cases, infection can be transmitted to other parts by autoinoculation. In males infected with human immunodeficiency virus (HIV), molluscum lesions appear to occur along the beard line, and with persons with infections involving the face, there have been reports of ocular involvement. Individual lesions last for about 2 months, but the disease usually lasts 6 to 9 months. More severe and prolonged infection tends to occur in individuals with impaired cell-mediated immunity, including persons with HIV infection.⁶⁶

Diagnosis

The clinical appearance of molluscum lesions in normal cases is generally sufficiently characteristic to permit clinical diagnosis. Brick-shaped virions can usually be seen in large numbers if the cheesy material expressed from the lesion is examined by negative-stain transmission electron microscopy. The virus has not been cultured in standard tissue culture systems. The characteristic histopathology of these lesions is diagnostic, but PCR methods have also been described for use in identifying molluscum contagiosum.^{125,195}

Epidemiology

Molluscum contagiosum virus occurs worldwide, and increasing reports of the disease have paralleled the number of reported cases of AIDS. Traditional modes of transmission are associated with mild skin trauma such as abrasions, direct contact with a lesion, and, in some cases, fomites (e.g., shared towels); however, there is increasing evidence that the disease is sexually transmitted and that genital lesions are more common than lesions elsewhere on the body.²⁰ The disease presents a significant concern for individuals whose children are in day care or school situations, where potential transmission to other children may exist. Secondary spread of lesions may occur by autoinoculation (excoriation of primary lesions and spread to areas of normal skin) and shaving. No known animal reservoir exists.

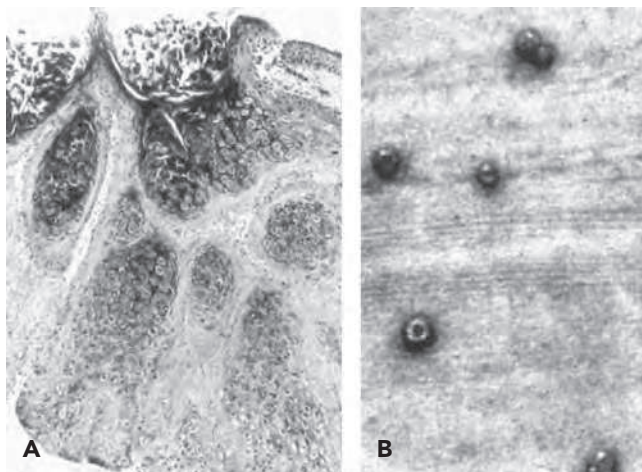


FIGURE 67.9. Molluscum contagiosum. **A:** Section of a skin lesion visualized by hematoxylin-and-eosin staining. **B:** Lesions of molluscum contagiosum in a human. (Courtesy of D. Lowy.)

Prevention and Control

The covering of lesions and the use of proper hand hygiene after contact with lesions should prevent transmission in most situations.

Molluscum contagiosum infection is benign and recovery is usually spontaneous, but treatment may be sought for cosmetic reasons, particularly for facial or multiple lesions. Various treatments have been tried, including cryotherapy, mechanical curettage, and chemical treatments, such as podophyllin/podofilox, cantharidin, iodine, and tretinoin. Irritation has been a side effect of many of the chemical treatments. Topical application of a 3% Cidofovir antiviral cream or suspension has been reported to be beneficial, as has the use of potentially immune-modulating cimetidine or topical imiquimod therapy.⁴⁰ The absence of well-controlled trials makes it difficult to assess the efficacy of various therapeutic regimens. For individuals with AIDS and molluscum, the use of highly active antiretroviral therapy, with improvement of CD4 cell counts, appears to be efficacious.

YATAPOXVIRUSES

History

Human infection with tanapox virus, a species of the genus *Yatapoxvirus*, was first recognized in the Tana River area of Kenya in 1957 and was best characterized during post-smallpox eradication surveillance efforts. An account of 264 laboratory-confirmed cases from Zaire (now the DRC), with color illustrations, is available,⁹⁵ as is information on the virus itself.¹¹² The genome of the virus has been sequenced. Yaba-like disease of monkeys is caused by the same virus that causes tanapox in humans,⁵³ and it was responsible for outbreaks in three primate centers where animal handlers were infected. There have been a few reports of human disease outside Africa that have been published and illustrate the need to consider poxvirus etiologies of illness in travelers returning from, and emigrants from, areas where the virus is endemic.⁵⁰

Yaba monkey tumor virus (YMTV), the other member of the genus, was recovered from rhesus monkeys in Nigeria.¹⁵¹ Although no naturally occurring human infections have been reported, the virus does cause disease if injected subcutaneously or intradermally in humans or nonhuman primates.⁶⁷

Pathogenesis and Pathology

The pathogenesis of tanapox or Yaba-like disease is incompletely understood. The clinical features, described here, are

suggestive of some systemic phase of disease. The ultimate skin lesion shows epidermal thickening with marked ballooning degeneration; eosinophilic inclusions are often visible. YMTV causes localized histiocytic “tumors” at the site of inoculation. After inoculation into the dermis, an accumulation of proliferating spindle-shaped cells begins about 1 week later. This continues for 4 to 6 weeks, resulting in the formation of a “tumor.” The lesion can become superinfected with bacteria. Virus remains cell associated, and the lesion appears to regress as mononuclear cells move from the periphery into the lesion.¹⁵¹

The genomes of both species encode a novel TNF-binding protein, distinct from the receptor homolog expressed in orthopoxviruses. Studies of the protein expressed from tanapox demonstrate its high affinity for human, not murine or rabbit, TNF.^{26,186} This and other unique immune-modulatory proteins expressed by one or both members of this genus likely affect their pathogenesis.

Epidemiology

Tanapox virus is restricted to Africa, principally to Kenya and the DRC, and is thought to have a simian reservoir.⁵² Cases of direct primate-to-human transmission, via a break in skin, have been described in animal handlers, although such cases appear to be extremely rare.⁷⁵ Several factors have led to speculation that an insect or arthropod intermediary may be involved in transmission of tanapox virus to humans. Persons confirmed to have tanapox infection have denied contact with nonhuman primates but have reported arthropod and culicine mosquito bites prior to infection; in patients who developed multiple lesions, there was no evidence that the virus had been spread mechanically.⁹⁵ Furthermore, the seasonal variation of human tanapox infections follows the activity of local arthropod populations. No human-to-human transmission has been reported.

Clinical Features

Tanapox infection begins with a short febrile (38°C to 39°C) illness of 2 to 4 days that is sometimes accompanied by headache, backache, or prostration. The eruption of a lesion is often heralded by pruritus at the site of the outbreak. The lesion appears as a hyperpigmented macule, often with central elevation, and the macule then evolves to a papule, with palpable induration. Fever and systemic symptoms wane as the lesion manifests. The papule then becomes more “pocklike” but contains no fluid; umbilication or the formation of a pseudocrust has been reported at this stage. Typically, the papule evolves into a firm, deep-seated, elevated nodule (Fig. 67.10A). At the end of the

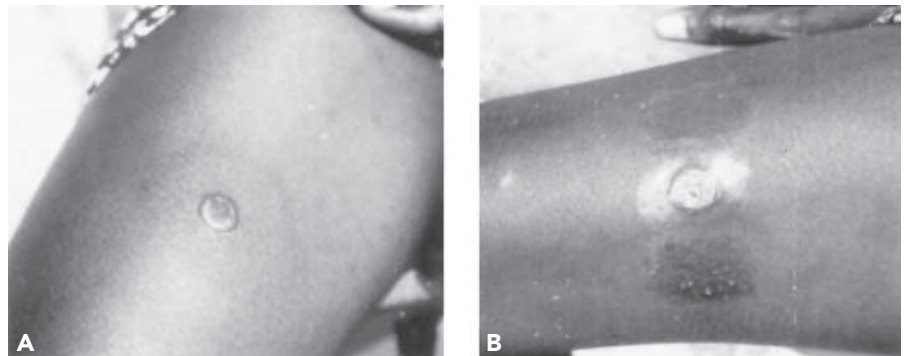


FIGURE 67.10. Lesions of tanapox in a Congolese youth. **A:** Day 10 of rash. **B:** Day 31 of rash. (Courtesy of Z. Jezek.)

first week, the lesion is surrounded by erythema and indurated skin. Regional lymphangitis is common at this stage. During the next stage, lesions either ulcerate or become larger nodules (up to 2 cm in diameter) (Fig. 67.10B). In the African series, the maximum lesion size was usually reached within 2 weeks and then the local inflammatory response began to wane, and the lesion began to granulate. Resolution of lesions occurred within 6 weeks.⁹⁵

Most cases (78% in one series) involve a solitary nodule; however, as many as 10 lesions on one individual have been described. Most lesions (72%) occur on the lower extremities, and the fewest occur in the face and areas normally covered by clothing. Infection appears to confer lifelong immunity.⁹⁵

Diagnosis

The limited geographic distribution of tanapox virus and the patient's travel history should be considered in the diagnosis of tanapox infection. Unique clinical features that allow for the differentiation of tanapox from other orthopoxvirus infections are the nodular nature of the rash lesion, the paucity of lesions, the benign disease course, and the protracted course of rash resolution. The solid nodular/ulcerated lesions are larger and develop more slowly than those of monkeypox, but they are smaller and develop more rapidly than those of tropical ulcers.

Tanapox virus can be detected by electron microscopy, and the virions usually appear enveloped, but this finding would not exclude the possibility of infection with other morphologically similar brick-shaped poxviruses; nucleic acid testing^{50,214} on lesion extract could be used for that purpose. Tanapox virus grows in a number of cell lines (e.g., owl monkey kidney, Vero, MRC-5, BSC-1) but not on chorioallantoic membrane.

OTHER POXVIRUSES

This section discusses poxviruses of veterinary importance or of other interest.

Orthopoxviruses

Camelpox

Camelpox virus has a host range that is apparently restricted to *Camelus dromedarius*. The illness it causes is a systemic disease with generalized rash in dromedary camels. The disease is enzootic in camels. Geographically, the virus can be found anywhere in the native geographic distribution of the host in Asia and Africa. Case fatality rates as high as 28% in outbreaks have been noted,⁹⁷ young animals are more susceptible to severe disease manifestations, and extensive chains of transmission between camels could be documented. Another study in Somalia evaluated 295 cases of camelpox in 1,052 camels. The case fatality rate was highest in animals up to 1 year of age (13.5%), and only 2.8% in animals 4 years of age. The genome sequence shows a high degree of similarity with that of variola virus⁷¹; despite this and the clinical similarity of camelpox disease in camels with smallpox in humans, camelpox is generally not considered to be a zoonotic disease of humans.⁹⁷ A recent report, from India, attributes the localized lesions on the hands of camel handlers to a camelpox-like virus.¹⁸ Although virus was not isolated from the human lesions, genetic characterization of amplicons from one human lesion was similar to that of amplicons obtained from the camels' camelpox virus isolates.

Additional surveillance is needed to understand the significance of these observations.

Camelpox disease has been experimentally studied using intradermal inoculation. By day 5, papules appear at the primary inoculation site, and then form vesicles and pustules and crust by days 9 to 10. In camels, a generalized rash manifests between days 9 and 11, which is preceded by fever. The rash is most pronounced on the mouth, nose, eyes, and oral cavity. Fewer lesions are seen on the extremities. The illness can last for 2 to 5 weeks. Diagnostics historically involved evaluation of characteristic morphology on chorioallantoic membrane, in addition to electron microscopy and nucleic acid techniques. Nucleic acid PCR-based techniques are increasingly used.¹⁸

Prevention and control have been reported through the use of live-attenuated camelpox viruses.²⁰⁶ The sequences of these vaccine virus strains are not yet available, but genome restriction maps show similarity to the pathogenic strains.

Ectromelia

Ectromelia was first described in 1930.¹³³ The reservoir or natural hosts have been minimally characterized; virus has been reported from wild rodents.⁶⁹ Our epidemiologic understanding of this pathogen comes from studies of outbreaks of disease in captive mouse colonies; fundamental biological and genetic studies of this pathogen have provided a detailed understanding of factors involved in the host–pathogen interaction.

At least two forms of clinical “natural” disease may manifest. A rapidly fatal form that results in extensive necrosis of the liver and spleen and death occurs shortly after symptom onset; no rash is seen. Some animals do survive, however, and, if this is the case, a generalized rash can be seen (Fig. 67.11A). Characteristic organ pathology is imaged in Fig. 67.11B and C. A more chronic form with ulcerating lesions of the feet, tail, and snout is seen in other animals. C57Bl/6 and AKR mice are highly resistant, whereas Balb/c and other inbred species are far more susceptible. Much is known about the host genetic determinants for disease presentation, and this has been reviewed.⁵⁶

Taterapox

This virus has been isolated once in the West African country of Dahomey, now known as Benin. The virus was isolated from an apparently healthy gerbil (*Tatera kempi*) in 1968, at the time of a human smallpox outbreak. Identification as an orthopoxvirus was accomplished after liver and spleen homogenates used to inoculate suckling mouse brain induced illness, and infectivity was blocked via administration of hyperimmune vaccinia mouse ascitic fluid. The virus had biological properties most similar to those of variola alastrim minor. The sequence of the virus is closely related to that of variola.⁵⁵ Neither 1 nonhuman primate nor 12 gerbils showed signs of viral dissemination in organs after parenteral or intracerebral inoculation.¹³¹ This suggests that this orthopoxvirus has evolved to have a “successful” parasitic relationship and further suggests that other “nonpathogenic” orthopoxviruses exist.

North American Orthopoxviruses

Raccoonpox, skunkpox, and volepox are the North American orthopoxviruses. In general, these too have been isolated from fairly asymptomatic animals or animals with localized lesions. The viruses have been propagated in monkey kidney cell lines; A-type inclusions are evident, whereas full genome sequence

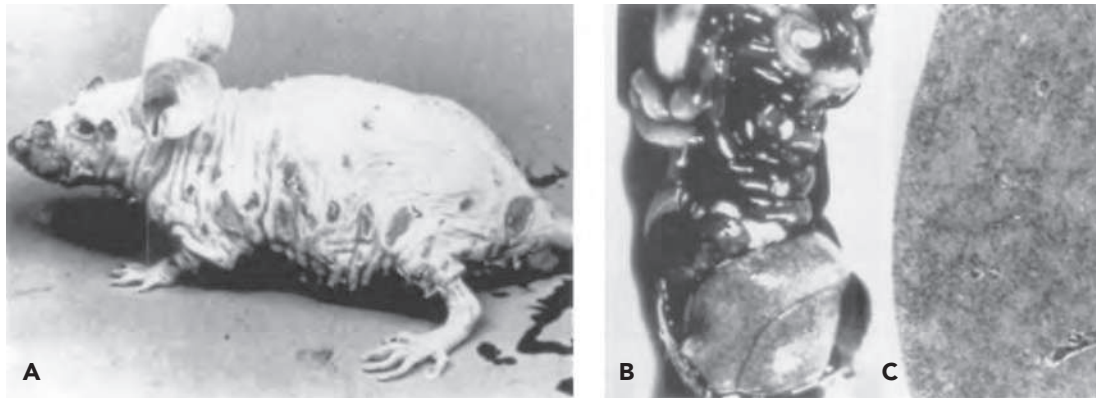


FIGURE 67.11. Lesions of mousepox in mice. **A:** The generalized rash in a susceptible hairless nonathymic mouse. **B:** Swelling and necrosis of liver and spleen and hemorrhagic intestine in a susceptible mouse that died prior to appearance of rash. **C:** Histologic appearance of necrotic liver. (A courtesy of Zentralinstitut für Versuchstiere, Hanover, Germany; B and C from Allen AM, Clarke GL, Ganaway JR, et al. Pathology and diagnosis of mousepox. *Lab Anim Sci* 1981;31:599–608.)

information is not yet available. Raccoonpox was identified in the upper respiratory tissues of 2 of 92 healthy-appearing raccoons trapped in Maryland. Twenty-two of these animals appeared to have orthopoxvirus seroreactivity.¹¹¹ Volepox was isolated from a skin lesion on a vole (*Microtus californicus*) in the San Francisco Bay Area of California; serosurveys of voles suggested the virus was enzootic. Experimental infection of seronegative voles demonstrated development of a localized lesion and antiorthopoxvirus seroconversion after challenge via footpad or tail inoculation.^{167,209}

Capripoxviruses

The capripoxviruses are somewhat larger, by electron microscopy, than other poxviruses with dimensions of $300 \times 270 \times 200$ nm. A number of strains of each species have been sequenced; the genome is ~154 kbp. Three species of capripoxvirus have been categorized. The diseases sheeppox, goatpox, and lumpyskin are caused by the viruses of similar names (Table 67.1). Representative strains of these viruses have been sequenced.

Lumpyskin disease was first identified in 1929 in Zambia; the virus infection has largely remained restricted to the African continent¹⁴⁸ but has emerged in the Middle East. Illness consistent with sheeppox is recorded as early as 2 AD.¹⁶⁴ Reports of goatpox were first made in Norway in 1879. Sheeppox and goatpox diseases are considered to have a significant impact on domesticated animals. Geographically, they currently cause disease in Southwest Asia, on the Indian subcontinent, and in central and northern Africa. There is increasing concern for the agricultural and economic impact of this emerging infectious disease.⁷

Transmission of lumpyskin disease is mechanical through arthropod bites; virus has been detected in two species of biting flies, and one of these, the stable fly (*Stomoxys calcitrans*), has been shown capable of transmitting disease.¹⁴¹ The reservoir host is postulated to be the African Cape Buffalo. Experimentally infected young buffalo and wildebeest failed to show disease or a rise in antibody titers after infection.²¹² However, LSDV seroprevalence in wild-caught buffalo has been low.⁴⁶ Within susceptible species, *Bos taurus* and *Bos indicus* mortality

is usually 1% to 2%. Transmission of sheeppox and goatpox within domestic flocks is considered to be airborne¹⁶⁴; additional evidence supports mechanical arthropod transmission by *S. calcitrans*. Scabs contain infectious material for months permitting fomite transmission. Sheeppox and goatpox are notifiable diseases in many countries. The spread of disease within a flock can infect up to 75% of the animals. Morbidity and mortality is highest in young animals and in lactating females, and overall mortality rates of 10% to 58% are reported.

Lumpyskin disease primarily affects species *Bos taurus* and *Bos indicus* and presents with a febrile prodrome followed, 10 days later, by cutaneous manifestations and lymphadenopathy. The skin lesions initially appear as raised nodules in the dermis and epidermis, which subsequently ulcerate and can become superinfected. In the oropharynx and mucosal surfaces, the nodules appear yellow-gray. Edema of the extremities and lymphadenitis are associated features. Recovery is slow, lasting up to 1 month.

Generally, sheeppox and goatpox are host (sheep and goat, respectively) specific, although clinical disease symptoms have been reported in both species during some outbreaks. An asymptomatic incubation period of 1 to 2 weeks is followed by febrile prodrome (up to 108°F) of mucosal swelling of the eyelids and mucosal discharge from the nose. Decreased appetite and occasionally arched back stance is noted. One to 2 days following prodromal symptoms, generalized appearance of ~1-cm lesions over the corpus develop (Fig. 67.12A and B), which progress through macular, papular, vesicular, and pustular stages; these are most obvious on areas with the least amount of hair. The lesions persist for 3 to 4 weeks and eventually scab over. Generalization of rash is more pronounced in young animals ages 4 to 5 months than it is in adult animals. Oral lesions ulcerate, and, in some animals, lesions in the lower respiratory tract are seen.

Diagnosis of capripoxvirus infections is often made on a clinical basis; differential diagnosis of lumpyskin disease includes consideration of bovine herpesvirus 2. Control strategies have largely used vaccination. Sheeppox and goatpox viruses can be grown in primary culture from ovine bovine or caprine sources. Cytoplasmic inclusion bodies are characteristic. Additional laboratory diagnostics, including enzyme-linked

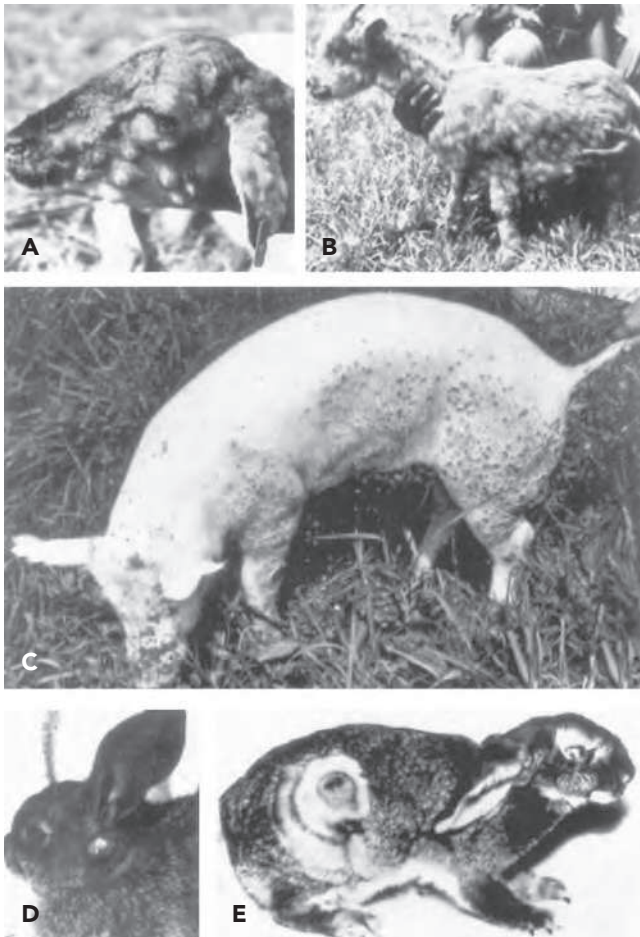


FIGURE 67.12. Infections of domestic animals with poxviruses of other genera. Capripoxvirus infection in sheep (A) (sheepox) and in goats (B) (goatpox). C: Swinepox infection demonstrating generalized lesions. Myxoma virus in its native host the Californian rabbit *Sylvilagus bachmani* (D) and in the European rabbit *Oryctolagus cuniculi* (E). (A–D from Robinson AJ, Kerr PJ Poxvirus Infections in Williams ES, Baker IK eds. *Infectious Diseases of Wild Animals*, 3rd edition Ames, Iowa: Iowa State University Press 2001:179–201, with permission. A and B courtesy of M. Bonniwell; C courtesy of R. Miller; D courtesy of D. C. Regnery.)

immunosorbent assay (ELISA) and PCR-based techniques, are available and have been reviewed.¹⁶⁴

Prevention and control of capripoxvirus disease involve vaccination strategies. For lumpyskin diseases, strategies have included the use of sheepox or goatpox, attenuated by tissue culture passage, or an attenuated strain of lumpyskin disease virus (strain Neethling). A review of the epidemiology and vaccination control issues is available.⁸⁷ Control of sheepox and goatpox disease has been attempted with live-attenuated and inactivated viral vaccines. However, inactivated vaccines provide only short-term protection, and the safety of live-attenuated vaccines is questionable; they can cause generalized disease and death in some animals.

Suipoxvirus

Suipoxvirus is an episodic viral disease of pigs with worldwide distribution; the swinepox genome has been sequenced. The

virus is currently the sole member of the *Suipoxvirus* genus and is phylogenetically most similar to species of the *Capripoxvirus* genus. Clinical disease can be heralded by low-grade fever, followed by papules, which over the course of 1 to 2 days per stage of development, progress to vesicles and pustules with umbilication (Fig. 67.12C). Crusting and scabbing of lesions ensues over the following 7 days. The duration of clinical disease can be 3 weeks. Mechanical transmission of disease between pigs occurs via the pig louse (*Haematopinus suis*). Disease control is provided by appropriate hygienic animal husbandry practices and control of the louse population.

Leporipoxvirus

The Leporipoxvirus virions are brick shaped, with a size of $300 \times 250 \times 200$ nm. Genome size is ~160 kbp and is 40% G+C in content. The viruses can infect multiple tissue culture cell lines. Myxoma virus is an obligate rabbit pathogen that demonstrates features that typify a general understanding of the distinction between reservoir host species and nonreservoir-susceptible species. Viral infection results in a localized “fibroma” infection in its reservoir species (Fig. 67.12D); the South American rabbit (*Sylvilagus brasiliensis*); causes a systemic, lethal disease in nonreservoir European rabbits (*Oryctolagus cuniculus*); and has coevolved with this new host. After intentional introduction of myxoma to control feral rabbit populations in Europe and Australia (the susceptible nonreservoir hosts), initial success of the program resulted in 99% mortality; however, milder virus variants emerged, and the surviving rabbits became more resistant to the effects of infection.⁶⁰

In species susceptible to generalized disease, the illness proceeds along an aggressive course. Early clinical signs of illness are blepharoconjunctivitis and swelling of the muzzle and anogenital regions; symptoms are fever and listlessness. Within 48 hours of development of clinical signs and symptoms, animals can die; if they survive past this time point, the classic myxomas, subcutaneous swellings, develop (Fig. 67.12E). The disease is mechanically transmitted by arthropod vector bites or experimentally by intradermal injection; it can also be transmitted by droplets.

The pathogenesis in nonreservoir species follows deposition of virus into the dermis. Virus replicates in major histocompatibility complex (MHC)-II dendritic-like cells and moves, within cells, to the draining lymph node within 24 hours to replicate in the lymph node,¹⁹ and then disseminates to the epidermis of the skin, lung, testis, and spleen. Virus from the primary lesion also moves to epidermis. A number of myxoma genes have been characterized to affect virulence and disease pathogenesis, usually via inflammatory and immune response modulation, and have been reviewed.^{8,9,215} Proteins are expressed that modulate TNF, γ -IFN, and chemokines; bind CC chemokines; and inhibit apoptosis.

The fibroma viruses cause small, localized tumors—the fibroma. The host range (Table 67.1) is restricted. To date, rabbit fibroma, squirrel fibroma, and hare fibroma diseases are described, caused by rabbit fibroma (also called Shope fibroma), squirrel fibroma, and hare fibroma viruses, respectively. These have been reviewed.⁹

Control and prevention of disease is facilitated through immunization. Vaccination and protection of rabbits from myxomatosis can be provided using a related species, fibroma virus, or through the use of attenuated myxoma viruses.

Avipoxvirus

The virions of the *Avipoxvirus* genus are brick shaped, with dimensions of $330 \times 280 \times 200$ nm. The genome size is one of the largest, about 300 kbp. There are a large number of avipoxviruses, mostly described by the avian species where infection has been evident to cause illness. The viruses do not cause illness or true replicative infection in mammalian hosts or mammalian cell lines but are able to present antigen to the immune system. For this reason, some have become important research vaccine vehicles. Currently, the ICTV classifies a number of avipoxvirus species: fowlpox virus, canarypox virus, juncopox virus, mynahpox virus, psittacinepox virus, quailpox virus, sparrowpox virus, starlingpox virus, turkeypox virus, and pigeonpox virus. Unclassified members are penguinpox virus, peacockpox virus, and crowpox virus.

The disease fowlpox has been best characterized. Two forms of disease manifest, and it is likely that the different manifestations are due to different routes of exposure. The first to be described manifests with largely cutaneous manifestations on the comb, wattles, and around the beak; other lesions can be evident on the limbs and cloacae. Most likely, disease results via the mechanical transmission of virus from a biting arthropod to the fowl. The lesions that develop are nodular and yellowish, and can coalesce and present a nidus for secondary bacterial infection. Lesions usually resolve in 3 weeks. Avian species with large combs are more susceptible to this form of disease than those with small combs. The other form of fowlpox has also been referred to as “diphtheric” disease. Transmission is likely via respiratory droplets between members of a flock in proximity. The infectious lesions present in the mucosal surfaces of the airway, and can coalesce to create a pseudomembrane and cause death by asphyxiation. Mortality is usually low in healthy flocks, but in flocks under stress, such as those that are egg laying, mortality can range to 50%.²²

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Hepadnaviruses

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Perspective

Acknowledgments

HISTORY

Highly transmissible liver disease has been known for several thousand years. A major cause is hepatitis A virus (HAV), a picornavirus that infects the liver and is shed in feces. Evidence for a distinct form of hepatitis, transmitted from blood and body fluids, began appearing in the nineteenth and early twentieth centuries. This second form was finally accepted following outbreaks of hepatitis after vaccination for measles, mumps, and yellow fever in the 1930s and 1940s. These vaccines all contained convalescent serum or plasma, or human serum added as a “stabilizer,” which inadvertently contained an infectious agent. Plasma, blood transfusions, and repeated use of nonsterile needles were also identified as causes of hepatitis outbreaks, and the disease was shown to have a viral etiology (reviewed in 28,268,815). Originally identified as hepatitis B or serum hepatitis, distinct from the disease caused by hepatitis A virus, this newly recognized entity was later discovered to be two separate diseases. Once tests were available for hepatitis B virus (HBV), a unique DNA virus discovered during the 1960s, it became clear that there was a second form of serum hepatitis, thereafter called nonA nonB hepatitis. A virus with structural similarities to flaviviruses was identified in the late 1980s as the major cause of nonA nonB hepatitis, and named hepatitis C virus (HCV).^{11,60,122,123,128}

The discovery of HBV came by an indirect route. To identify and track genetic differences in human populations, Blumberg and colleagues were using sera from multiply transfused individuals as sources of antibody to human serum proteins. The idea was that these sera would contain antibodies that bound to proteins differing in sequence from those of the transfusion recipients. During the course of these studies a new antigen, named “Australia antigen,” was identified in serum from an Australian Aborigine.⁴⁷ Because this antigen was found to be common in leukemia patients and in Down syndrome patients, who have a high risk of leukemia, it was hypothesized that the antigen predicted leukemia risk. However, a Down syndrome patient initially negative for Australia antigen was observed to seroconvert, and seroconversion was correlated with a mild case of hepatitis. At about the same time, a member of Blumberg’s laboratory experienced a mild case of hepatitis following contact with contaminated material, again with the appearance of Australia antigen in the blood.^{48,50}

The Australia antigen was quickly associated with serum hepatitis in a wider group, including a significant fraction of post-transfusion hepatitis cases.⁵¹⁰ At the time, post-transfusion hepatitis occurred in at least 10% to 30% of multiply transfused individuals.^{9,10,339,340,346,558,631} Screening blood banks for

contaminated blood (Australia antigen-positive) resulted in an approximately twofold decline in the incidence of post-transfusion hepatitis. The remaining cases were mostly due to HCV. For this discovery Blumberg received the Nobel Prize in Medicine in 1976.⁴⁶

The ability to carry out retrospective studies with assays for Australia antigen confirmed a long-held suspicion that HBV was responsible for a chronic hepatitis leading to cirrhosis and liver cancer in many parts of the world.^{49,215,428,479,484,560,638,644,673,696,710,738,741,742} The Australia antigen, purified from the serum of infected individuals, also proved to be an effective vaccine, with greater than 90% efficacy in inducing an antibody response in adults. However, universal vaccination still remains a goal rather than accomplished fact. The World Health Organization (WHO) estimates there are now 400,000,000 individuals worldwide who are chronically infected with HBV, 25% of whom will die of chronic liver disease or hepatocellular carcinoma.

Electron microscopic (EM) studies revealed that Australia antigen is carried by spherical particles, with a diameter of

~22 nm, and to a lesser extent, by ~22-nm, rod-like particles (Fig. 68.1). Sera contain a much smaller amount of spherical virus particles with a diameter of approximately 42 nm, termed the Dane particle.¹⁴⁹ Australia antigen is a component not just of the 22-nm particles but also of the virus envelope.²⁵⁸ Treatment with nonionic detergent releases a spherical particle from virus, the viral capsid, with a diameter of approximately 27 nm. Robinson and colleagues showed that the capsids contain a circular viral DNA of about 3000 base pairs (bp), as well as an endogenous DNA polymerase activity that synthesizes virus DNA when virions are treated with nonionic detergent and incubated in the presence of dNTPs.^{314,585–587} Summers showed that the circular conformation is maintained by a short cohesive overlap between the 5' ends of the two DNA strands and that the circle is only partially double stranded, one strand being incomplete. This incomplete strand is extended and the single-strand gap is at least partially filled in by the endogenous DNA polymerase.⁶⁷⁴

The endogenous DNA polymerase activity facilitated the discovery of several HBV-like viruses (Fig. 68.2) including woodchuck hepatitis virus (WHV) in eastern woodchucks (*Marmota monax*),^{646,677,678,695,727} duck hepatitis B virus (DHBV) in domestic ducks in China (Summers, personal communication; 749, 807) and the United States,⁴⁴⁵ and ground squirrel hepatitis virus (GSHV) in Beechey

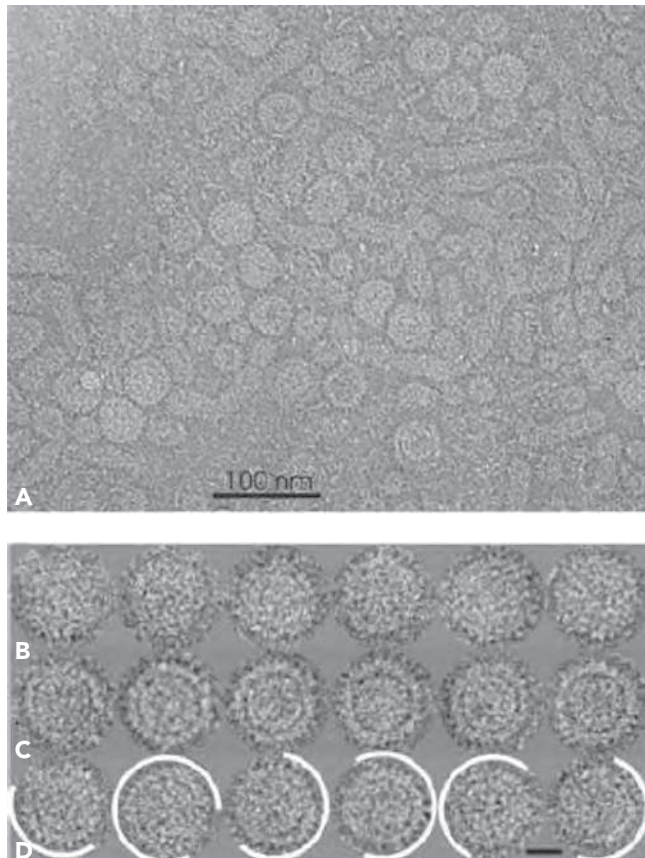


FIGURE 68.1. Cryo-electron microscopy of viral particles from a chronically infected patient. **A:** 42-nm Dane particles, and 22-nm filamentous and spherical subviral particles are seen. **B** and **C:** Dane particles with compact and gapped morphology, respectively. **D:** Particles with mixed morphology. Gapped areas are delineated in white. (Adapted from Seitz S, Urban S, Antoni C, et al. Cryo-electron microscopy of hepatitis B virions reveals variability in envelope capsid interactions. *EMBO J* 2007;26:4160–4167, with permission.)

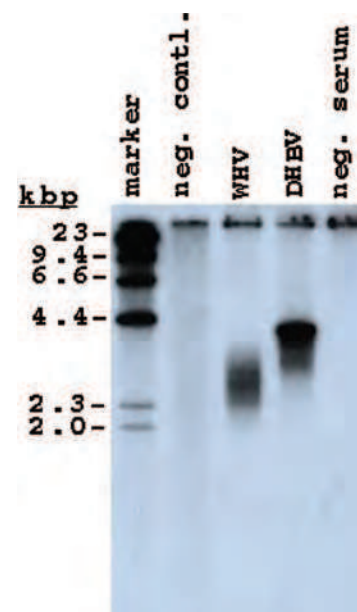


FIGURE 68.2. Detection of hepatitis-B like viruses using an endogenous DNA polymerase assay. Serum samples from a woodchuck and duck were centrifuged to pellet virus. The pellet was suspended in a DNA polymerase reaction cocktail containing radiolabeled nucleotides and incubated at 37°C. SDS-pronase was then added, and after digestion at 37°C to free DNA from protein, the products were subjected to gel electrophoresis in 1.5% agarose. Radiolabeled DNA was detected by autoradiography. The marker is bacteriophage lambda DNA digested with the restriction endonuclease Hind III. Woodchuck hepatitis virus (WHV) DNA migrates faster than duck hepatitis B virus (DHBV) DNA because the incomplete strand of WHV was only partially filled in by the endogenous DNA polymerase reaction.

ground squirrels (*Spermophilus beecheyi*).⁴³⁵ Hepatitis B-like viruses closely related to the original isolates were later identified in Richardson's (*Spermophilus richardsonii*),^{467,694} and arctic ground squirrels (*Spermophilus parryi kennicotti*),⁶⁹⁹ ducks,^{236,430,728} wild mallards,¹³⁵ geese,^{98,236} cranes,⁵⁵⁷ storks,⁵⁶⁵ and herons,⁶⁵¹ but the three original nonprimate animal models—particularly the woodchuck and the domestic duck—have been the mainstay of hepatitis B research for the past 30 years.

HBV itself is found in all apes, including chimpanzees, gorillas, orangutans, and gibbons.^{280,358,422,500,584,601,658,685,707,740,753,816} These isolates are closely related in sequence to human HBV and human isolates were shown to infect chimpanzees and gibbon apes.^{20–22,39,40,157,270,615} At present, these primate isolates are considered subtypes of HBV rather than distinct species. Only the chimpanzee has seen significant use as an experimental model, though for ethical reasons as well as cost its use has been limited. A primate virus closely related to HBV has also been isolated in the New World from the woolly monkey.^{357,356} This virus, woolly monkey hepatitis B virus (WMHV), differs in host range from HBV and has been designated the prototype for a new species of hepatitis B-like virus.¹⁸²

CLASSIFICATION OF VIRUSES WITHIN THE HEPADNAVIRUS FAMILY

All of these hepatitis viruses share remarkable similarities in genome organization and replication strategy and, with the *Spumaviridae* (foamy viruses) (see Chapter 52), are the only DNA viruses of animals known to replicate their DNA by reverse transcription of a viral RNA. Collectively, the hepatitis B-like viruses are assigned to the family *Hepadnaviridae* (hepatitis DNA virus), for which (human) HBV is the prototype. This family contains two genera, the orthohepadnaviruses, infecting mammals, and the avihepadnaviruses, infecting birds.¹⁸² A maximum sequence divergence of about 40% is found among the orthohepadnaviruses,^{207,618} compared to about 20% among avihepadnaviruses.²³⁶ Designation of the *Hepadnaviridae* as a new family of viruses is based on the extremely small size of the viral genomes (3–3.3 kbp), the novel arrangement of open reading frames, and the unique replication strategy, differing almost completely from other viruses replicating by reverse transcription. Assignment to two genera is based on the strong DNA sequence similarities among all orthohepadnaviruses, and all avihepadnaviruses, but an almost complete lack of homology between the two groups (Fig. 68.3).

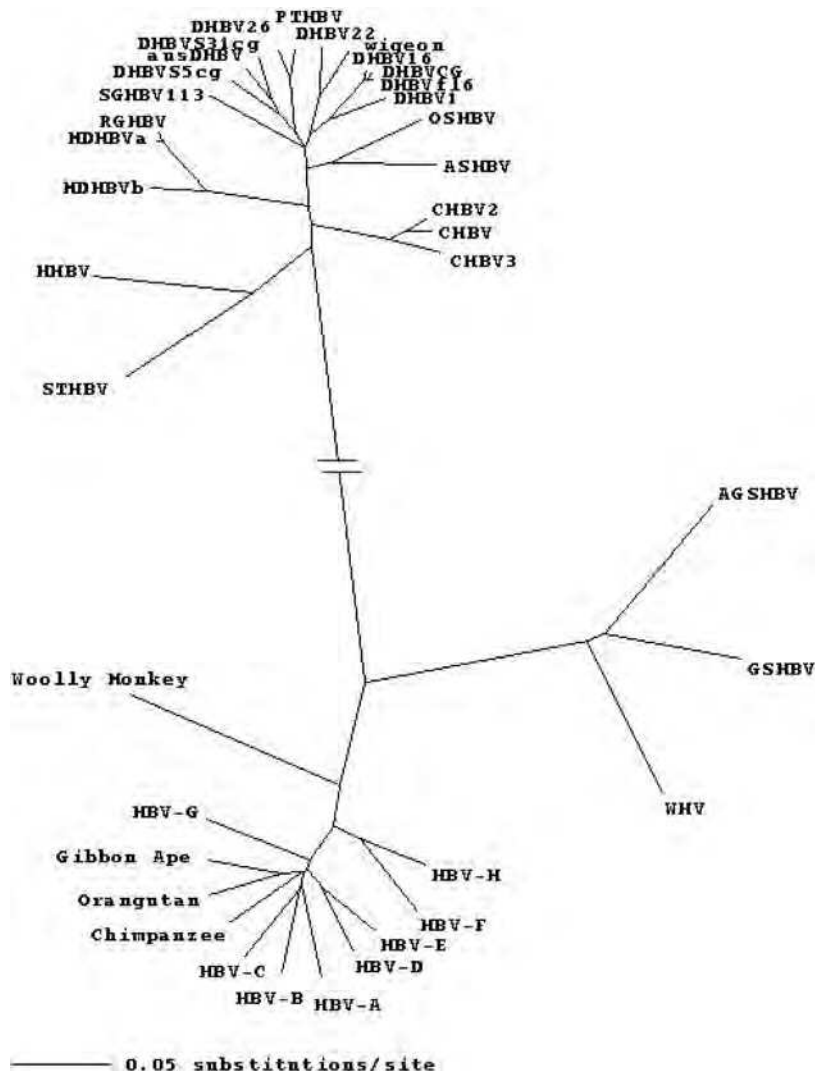


FIGURE 68.3. Phylogenetic tree of avi- (top) and ortho- (bottom) hepadnaviruses. A dendrogram file constructed using ClustalX was displayed by Treeview.

Assignment to separate species within the two genera has been based primarily on differences in viral host range, which has also been associated with differences in sequence. Two species have been assigned in the avihepadnavirus group, DHBV and heron hepatitis B virus (HHBV). Most newer isolates are as yet unassigned.^{182,236} Where sequence and host range data are available, orthohepadnavirus species have also been assigned. These species include HBV, WHV, GSHV, and WMHV.

With the availability of polymerase chain reaction (PCR)-based assays, numerous studies have been performed to gain information on the number and geographic distribution of HBV genotypes and naturally occurring HBV mutants infecting humans. Eight HBV genotypes, A to H, have been identified,^{15,206,499} with isolates belonging to different genotypes showing pairwise differences greater than 8% and less than 17%. A ninth genotype, I, has been proposed but remains controversial as the divergence is about or slightly less than 8%, with a close relationship of half the I genome to genotype C HBV.^{515,793} An isolate possibly defining a tenth genotype, J, has also been described.⁶⁸⁹ Distinct genotypes have also been found in great apes. Different genotypes tend to have distinct geographic distributions and possibly distinct clinical manifestations (Fig. 68.3).

VIRION STRUCTURE

HBV is a spherical virus with an outer diameter of approximately 42 nm (Fig. 68.1). The inner shell of the virus has a diameter of ~22 nm and is made up of 120 dimers of the core protein. The dimers form the icosahedral capsid with a triangulation number $T = 4$. A small fraction of capsids consists of only 90 dimers with a triangulation number $T = 3$.^{55,132,140,167,772} It is not known whether virions with the smaller capsids are infectious or represent

dead-end products caused by an aberrant assembly process. The capsid is covered with a lipoprotein membrane made up of three forms of the viral envelope protein, large (L), middle (M) and small (S) (Fig. 68.4), acquired together with host lipids during budding into multivesicular bodies (Fig. 68.5). The L, M, and S proteins are present in the virus envelope at a ratio of about 1:1:4.²⁵⁸ A model based on the analyses of virions and capsids by electron cryomicroscopy (cryoEM) predicts that $T = 4$ capsids carry 180 dimers formed by envelope proteins.^{167,621} The proportions of homo- and heterodimers is unknown. Notably, virions from patient sera exhibit morphologic variation: they appear either as compact or as gapped particles, which differ in the distance between the capsid and membrane⁶²¹ (Fig. 68.1). Capsids contain a single copy of the partially double-stranded DNA (dsDNA) genome, which is covalently linked to the viral reverse transcriptase (RT) at the 5' end of the complete strand (Fig. 68.6). RT provides the endogenous DNA polymerase activity, discussed earlier^{314,674} (Figs. 68.2 and 68.6). There is also evidence for the presence of cellular proteins including one or more serine kinases within the virus.⁷ The virus has a buoyant density of 1.24 to 1.26 g cm⁻³ in CsCl and an $s_{20,w}$ of 280S. The titers of HBV can vary significantly among patients, ranging up to 10¹⁰ per ml in blood.

As noted earlier, HBV infections also lead to the production of noninfectious subviral particles. The 22-nm spheres can reach titers as high as 10¹² per ml and represent the most abundant particle released into the blood from infected liver cells. CryoEM studies of isometric particles isolated from sera of transgenic mice revealed that they have an octahedral symmetry, different from the icosahedral structure of Dane particles.³²⁵ These spheres are composed of 48 dimer subunits that assemble into two classes of particles that differ in size, presumably caused by the heterogeneity of the subunits. Spheres contain M and S proteins at a ratio of about 1:2 and only trace amounts

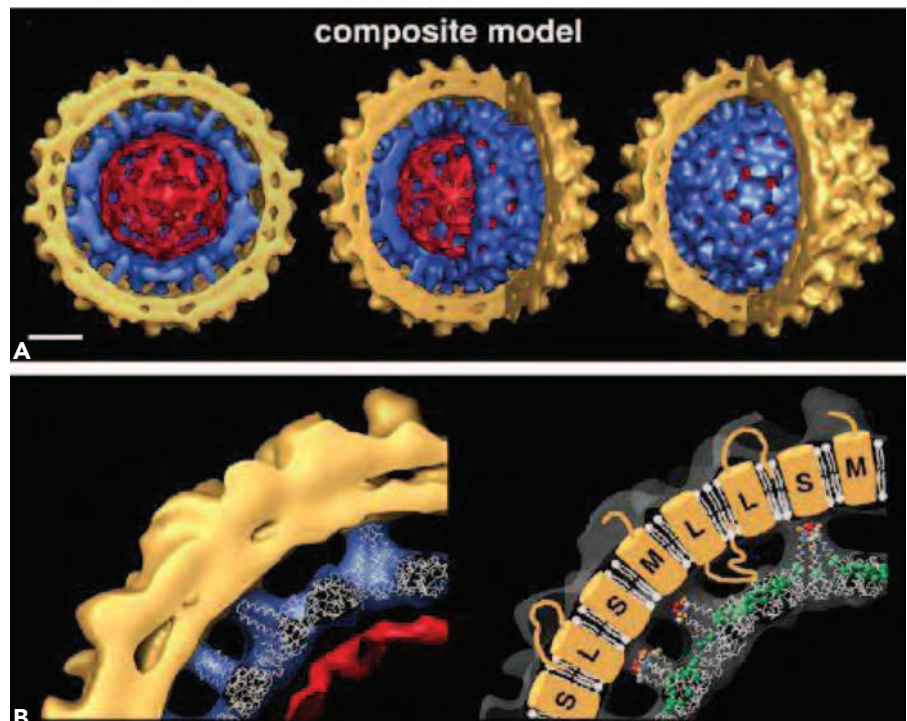


FIGURE 68.4. Model of HBV virions. A: HBV virion with a $T = 4$ icosahedral capsid (blue) with 120 spikes and an outer envelope with protein projections. **B:** X-ray crystal structure of a capsid docked into the cryo-electron microscopy density map of the virion capsid (left). S, M, and L refer to the three envelope proteins described in the text. Amino acids around the base of the spikes in core proteins, which are important for envelopment of core particles, are shown in green.^{370,522} (From Dryden KA, Wieland SF, Whitten-Bauer C, et al. Native hepatitis B virions and capsids visualized by electron cryomicroscopy. *Mol Cell* 2006;22:843–850, with permission.)

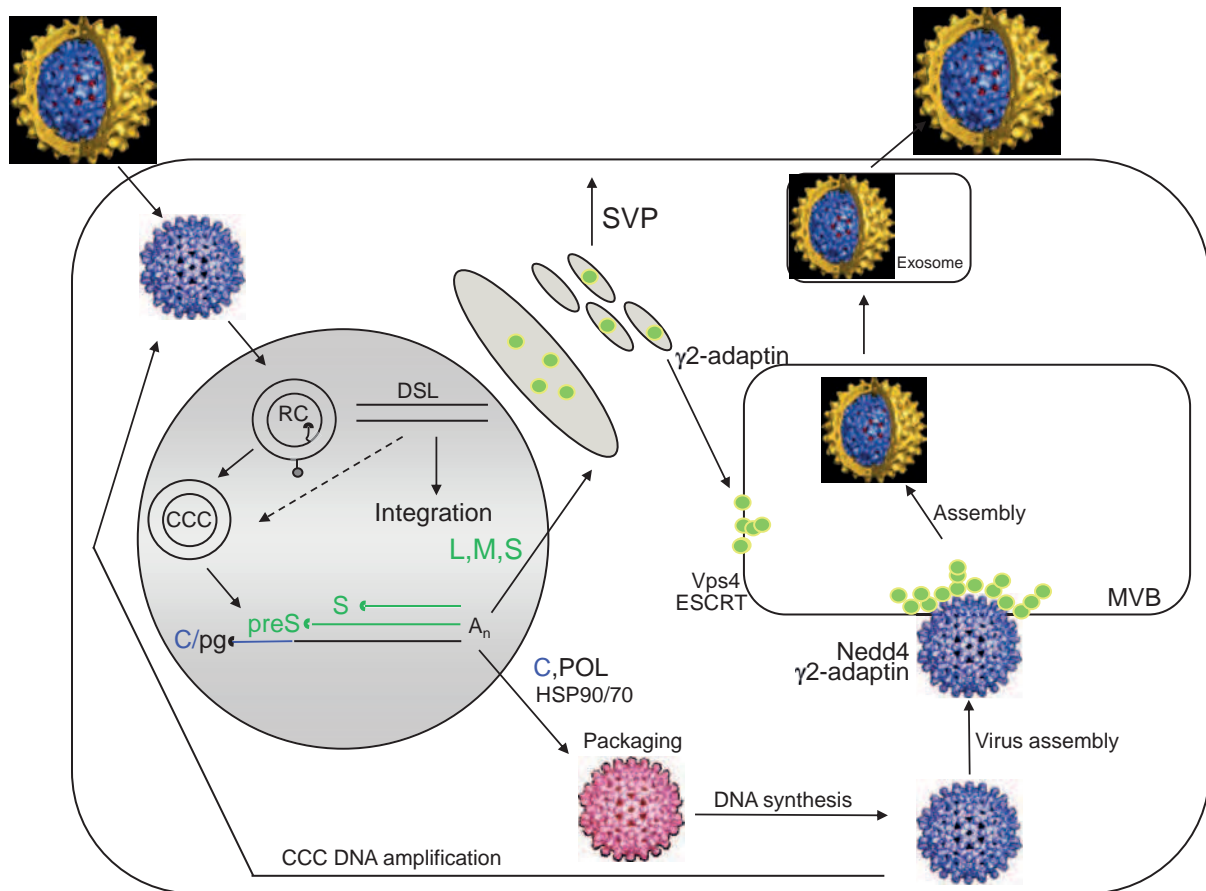


FIGURE 68.5. Model for the life cycle of hepadnaviruses, as described in the text.⁵²² Envelope proteins are shown in *green*, DNA-containing capsids in *blue*, and RNA-containing capsids in *red*. Early in infection, when envelope protein concentrations are low, capsids enter the CCC DNA amplification pathway. Envelope proteins enter the endoplasmic reticulum and assemble into subviral particles (SVP) or transfer to MVBs where virion assembly is believed to occur. Mature virions might exit cells through exosomes (for details and references see the text).

of L.²⁵⁹ The rod-like particles (tubes, filamentous particles) contain approximately equal amounts of M and L. Their surface exhibits spike-like features composed of homo- and heterodimers of L, M, and S proteins which, like virus, are in the ratio 1:1:4, with a diameter of about 22 nm.⁶³⁵ However, in contrast to octahedral isometric particles, tubes isolated from patient sera do not have an ordered structure.⁶³⁵ Subviral particles contain 40% lipid and sugar by mass and have a buoyant density of 1.18 g cm⁻³ in CsCl. Their exact role in the HBV life cycle is not known. One possibility is that, by adsorbing virus-neutralizing antibodies, they facilitate virus spread and maintenance in the host.

GENOME STRUCTURE AND ORGANIZATION

The structure of the HBV genome and organization of open reading frames on viral DNA is shown in Figure 68.6. All of the ORFs are in the same direction (clockwise in this illustration), defining minus and plus strands of viral DNA. Within virions, minus-strand DNA is complete and spans the entire genome, in contrast to plus strands, which extend to

about two-thirds of the genome length and have variable 3' ends.^{420,674} In this regard, avihepadnaviruses differ from orthohepadnaviruses because they normally extend plus strands almost all the way to the location of the modified 5' end.⁴⁰⁰ The primers of both plus- and minus-strand DNA synthesis remain attached throughout virus maturation. Minus strands are covalently linked to the viral reverse transcriptase through a phosphotyrosine bond. Plus strands contain a short RNA oligomer derived from the 5' end of pregenome (pg) RNA, the template for minus-strand DNA synthesis. Minus strands exhibit a small 8- to 9-nucleotide-long terminal redundancy, termed *r*, which is required for the formation of relaxed circular (RC) DNA during plus-strand DNA synthesis.^{400,617,764} A small fraction (5%–20%) of virus contains double-stranded linear (DSL) DNA in lieu of RC DNA, a consequence of *in situ* priming of plus-strand DNA synthesis.⁶⁵⁷ Virions with DSL DNA are infectious, but can lose important sequences from their ends during initiation of infection and appear, therefore, to play only a minor role in hepadnavirus replication.^{782,784}

The genetic organization of HBV is complex. The genome contains four promoters, two enhancer elements, and a single polyadenylation signal to regulate transcription of viral RNAs.

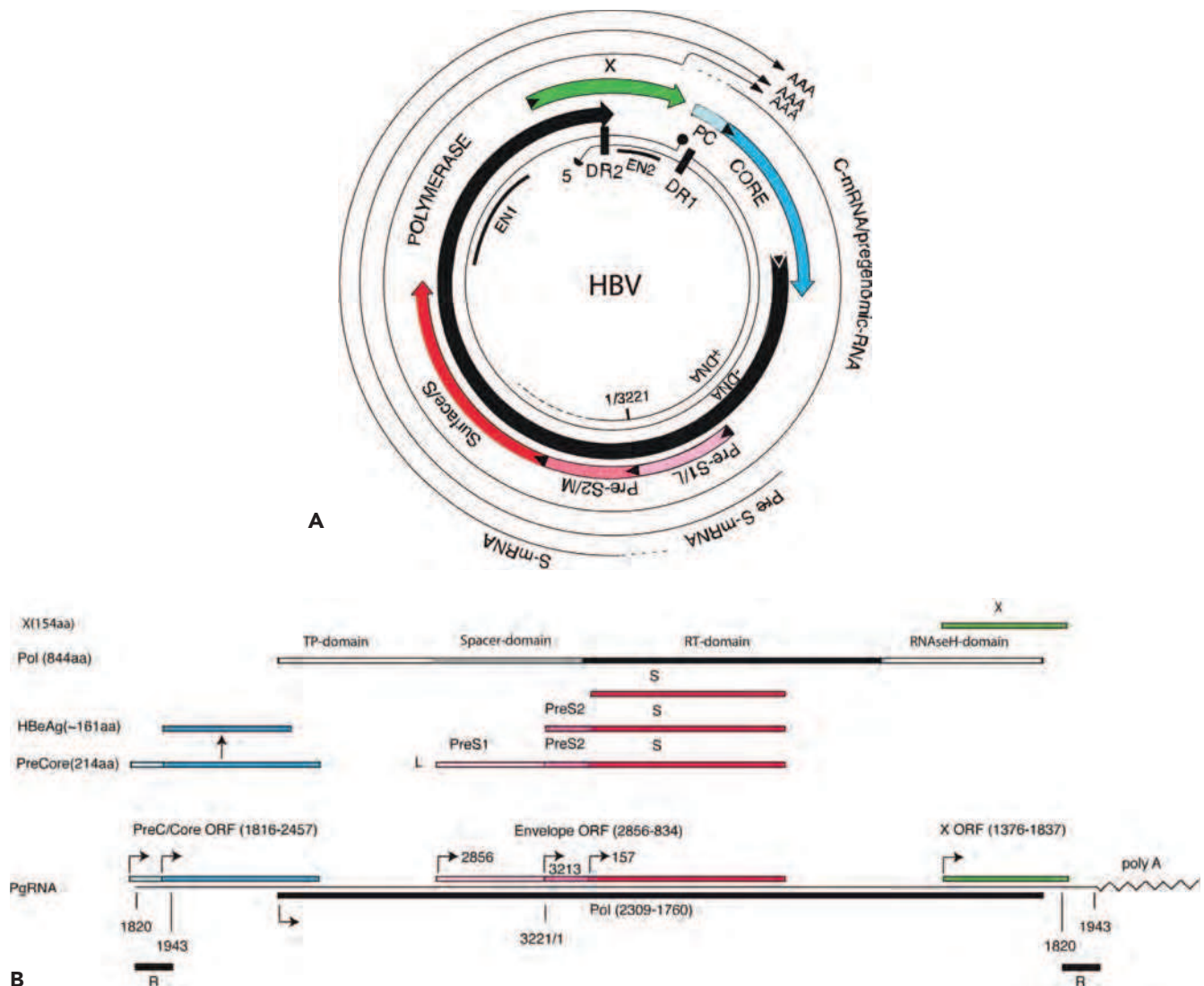


FIGURE 68.6. Genome structure and organization. The relaxed-circular DNA genome of HBV with a complete minus strand and incomplete plus strand is shown in **A** (inner circle), along with the major mRNAs, all of which end at a common polyadenylation signal located in the core open reading frame. All open reading frames have a clockwise direction. The single-stranded gap in the plus strand is filled in by the viral RT, which is covalently attached to the 5' end of minus-strand DNA. The proteins produced from each open reading frame are illustrated in **B**, using pgRNA, a terminally redundant mRNA that is reverse transcribed to produce viral DNA, as a map reference. Map coordinates are from the sequence reported by Valenzuela et al.⁷³⁵ (accession number X02763), with numbering from a unique EcoRI restriction endonuclease site. R, large terminal redundancy; pgRNA, pregenomic RNA; DR, direct repeat; EN, enhancer, PRE, post-transcriptional regulatory element.

In addition, there are four open reading frames and several *cis*-acting signals required for viral DNA replication (Fig. 68.6). All viral transcripts are encoded by the minus strand, and are capped and polyadenylated. Transcription regulatory regions are present within open reading frames and are active following the transport of the genome into the cell nucleus, where it is converted into a covalently closed circular DNA form, called CCC DNA.

The major transcripts that are detected by Northern blot analyses of HBV-infected livers are 3.5 kb, 2.4 kb and 2.1 kb in length and termed pre-C/C, pre-S and S messenger RNAs (mRNAs), respectively.^{90,175} In addition, a minor transcript,

X mRNA, about 0.7 kb, has occasionally been detected in infected tissues and more consistently in cells transfected with subgenomic HBV DNA.^{221,599,717} The existence of an X mRNA would provide the most plausible mechanism for the translation of this gene, although internal initiation of translation from pre-C/C RNAs, pre-S or S mRNAs cannot be excluded as alternative mechanisms. Avihepadnaviruses express three major transcripts analogous in length to the three major mRNAs of mammalian hepadnaviruses.⁷⁸ All hepadnavirus transcripts share a common 3' end created by a polyadenylation signal located in the core gene. Fine mapping of the 3.5-kb preC/C mRNAs revealed three different 5' ends bracketing the initiation codon

of the pre-C gene, indicating that translation of the overlapping pre-C and core genes occurs from separate transcripts (Fig. 68.6).^{90,175} The two longer RNAs, beginning upstream of the pre-C initiation codon, are referred to as pre-core (pre-C) mRNAs and the shorter, beginning downstream, is pregenome RNA. PgRNA is the template for the translation of the core and RT proteins and, as the name indicates, is also the template for viral DNA synthesis via reverse transcription. In contrast, the function of the pre-C mRNAs appears to be limited to the translation of the pre-C gene. As with the large, terminally redundant mRNAs, S mRNAs also have heterogeneous 5' ends flanking the initiation codon of pre-S2 and, hence permitting the translation of either the M or S protein.^{89,656} The third major transcript, pre-S, has a unique 5' end and supports the translation of L. The 5' end of X mRNA is heterogeneous.⁷⁷⁵ In addition to the four promoters, two enhancers, EN1 and EN2, regulate transcription of the viral RNAs (Fig. 68.6).

The core protein is a cytoplasmic, basic phosphoprotein with a molecular weight (MW) of 21 kd that assembles into subviral capsids. Early on, its antigenicity was recognized and diagnostic assays to monitor ongoing or resolved infections were developed. The pre-C protein is best known by its serologic name: e-antigen, or HBeAg. Although the pre-C gene includes the entire core protein open reading frame and upstream coding sequences, the polypeptide is shorter than the core protein due to posttranslational processing, and has a MW of only 15 kd. The mature pre-C protein exhibits distinct antigenic properties from the core protein. Pre-C does not play a role in viral replication, but might exert a role in the regulation of the immune response against HBV, particularly against the core protein.¹⁰⁹ HBeAg is an important diagnostic tool that can be used to determine the status of ongoing HBV infections.

The *pol* gene encodes the viral DNA polymerase, which is the sole enzyme encoded by hepadnaviruses. It consists of three functional domains and a hinge region, known as the “spacer,” and has an MW of about 90 kd. The N-terminus encodes the terminal protein (TP) domain, which acts as the primer for minus-strand DNA synthesis. The C-terminal region encodes the reverse transcriptase and RNaseH (RT) domains.

The PreS/S genes overlap the hinge region and RT domains of the *pol* gene, albeit in a different reading frame. They encode three integral transmembrane envelope glycoproteins with distinct N-terminal domains. The shortest, the S protein, is the most abundant envelope protein in virions and in the subviral spheres and rods. It contains the major antigenic determinants of Australia antigen, which led to the discovery of HBV and provided the reagent for the development of diagnostic tools for the detection of HBV infections and of vaccines against HBV infections.⁴⁶ A fraction of S protein is modified by asparagine (N)-linked oligosaccharides, increasing the MW of the protein from 24 to 27 kd.^{437,543} The 31-kd M protein represents a larger form of HBsAg with a 55 amino acid N-terminal, glycosylated extension, referred to as PreS2, translated from an in-frame initiation codon. It represents about 10–15% of total envelope proteins in infected cells. A specific function for this protein is not yet known, as it is not essential for virion assembly.⁷³ Recent studies suggest that the 55 amino acids that distinguish M from S may serve primarily a spacer function between S and the PreS1 domain of L.⁴⁹³

The 42 kd L protein is a myristoylated polypeptide translated from the first initiation codon of the PreS/S open reading

frame.⁵³⁹ The extra amino acids at the N-terminus, relative to M, define the PreS1 domain. Although it represents only 1% to 2% of total surface proteins in infected cells, L is enriched in virions, where it represents approximately 17% of the envelope proteins.²⁵⁸ In contrast, subviral particles are primarily composed of M and S proteins. Consistent with its distribution, L protein provides the primary ligand for the viral receptor. In contrast to the mammalian hepadnaviruses, avihepadnaviruses encode only two nonglycosylated envelope proteins, corresponding to L and S of HBV. DHBV subviral particles are pleomorphic, roughly spherical, with diameters ranging from 35 to 60 nm,⁴⁴⁵ as compared to the ~40-nm DHBV virion.

The smallest viral gene, found exclusively in mammalian hepadnaviruses, encodes HBx or X with a MW of 17 kd. DHBV has been reported to express an X-like protein,⁹⁹ but a functional significance for this ORF was not supported by *in vivo* studies.⁴⁵⁵ The X gene overlaps, in a different reading frame, the C-terminal portion of the polymerase and two transcriptional control elements, EN2 and core promoter. X is predominantly a soluble cytoplasmic protein with a short half-life in the range of 15 to 20 minutes.^{147,148,166,260,605} However, it has also been found associated with the cytoskeleton³⁶³ and in the nucleus.^{54,166,260} Modifications of the protein (phosphorylation, acetylation) have been observed under selected cell-culture conditions,^{378,732} but not yet in infected liver tissue. Except for the spacer region in the polymerase, X is the least conserved hepadnavirus protein. While X is required for efficient infection *in vivo*,^{106,804,814} its exact role in the viral life cycle is not known. Since the report that HBx has transcription factor-like activity,^{649,726} experiments in tissue culture cells revealed many other functions of HBx that will be summarized later in this chapter.

STAGES OF REPLICATION

Infection of Hepatocytes

The mechanisms by which HBV and other hepadnaviruses infect hepatocytes are still not well understood. Efforts to investigate this problem have been hampered by the lack of widely available cell lines that are permissive for infection. As a consequence, studies have been limited to the use of primary hepatocyte cultures (PHC) that typically remain susceptible to infection for only a few days following their preparation from liver tissue,^{8,225,503,725} or to a cell line that, under extreme culture conditions, becomes susceptible to HBV.²²⁶

Investigations into the identification of envelope components that play a role in infection revealed that the PreS1 domain of L has a critical role. The most compelling results stem from genetic experiments with chimeric envelope proteins between closely related hepadnaviruses that exhibit different host-range specificity, such as DHBV and its close relative, heron hepatitis B virus (HHBV), or HBV and WMHBV.^{124,292} These studies revealed that the specificity of these viruses for their cognate hepatocytes segregates with the N-terminal half of the PreS1 domain. Consistent with these observations, infectivity of DHBV and HBV can be neutralized by anti-PreS1 antibodies,^{114,354} and infection of hepatocytes can be blocked by peptides homologous to portions of the PreS1 region of the L protein.^{224,542,731} A study with hepatitis delta virus (HDV, Chapter 69), a viroid-like satellite of HBV that requires HBV envelope proteins to infect hepatocytes, provided evidence that

a second determinant, mapping to the external hydrophilic loop of the S protein is required for infectivity.²⁹⁸ More recent work with HBV confirmed this finding. It remains unclear how this determinant functions during infection of hepatocytes.^{44,600}

Infection of duck hepatocytes by DHBV has been intensely studied. It is a slow process that takes place over a period of at least 16 hours.^{564,682} Internalization rather than binding to the receptor appears to be the rate-limiting step because the latter can rapidly occur at 4°C. An estimated 10⁴ receptors with high-affinity binding sites for DHBV are present on primary cultures of duck hepatocytes.^{326,562}

Infection by DHBV and HBV is most likely pH independent because it can occur in the presence of lysosomotropic agents or after pretreatment of virus at low pH.^{246,328,580} Moreover, for DHBV, infection may depend on a conserved peptide translocation motif (TLM), which is located near the N-terminus of preS1.⁶⁶² TLM might become functional following cleavage of envelope proteins on virus particles by one or several proteases present in endosomes. Curiously, the corresponding TLM motif in HBV is not required for infection,⁴⁴ suggesting that avian and mammalian hepadnaviruses might differ in their mechanisms of infection.

Several cellular and serum proteins that bind to HBV, DHBV, subviral HBsAg particles or recombinant envelope components have been identified since the mid-1980s as possible virus receptors.^{77,152,199,261,454,486,487,551,598,691,715,716,769} Many of these studies were of a descriptive nature and yielded only preliminary evidence of a role for the respective protein in viral entry. Exceptions are studies on a receptor candidate for DHBV, carboxypeptidase D (CPD). CPD was originally identified as a cell glycoprotein that binds DHBV particles and recombinant L protein.^{347,348} The DHBV-CPD interaction occurs in a species-specific manner, requires the PreS1-specific domain of the envelope protein, and is inhibited by PreS1 specific neutralizing antibodies.^{347,712} Many of the characteristics of CPD are consistent with its proposed role as a viral receptor. It is a type I transmembrane protein that cycles between the *trans*-Golgi network and the plasma membrane, and provides a high-affinity binding site for L within one of its three extracellular domains (domain C).^{63,650,734} This domain does not exhibit enzymatic activity. Both soluble CPD and antibodies against domain C can block infection of primary duck hepatocytes.^{733,734} Interestingly, CPD is downregulated in DHBV infected hepatocytes, which could contribute to the resistance of hepatocytes to superinfection.⁶² Such resistance could also be explained by other factors, including blocking of receptors by viral envelope proteins produced in the infected cell.⁷⁴⁵ However, while the evidence for a role of CPD in DHBV infections is compelling, proof for its role as a receptor is still lacking. The fact that CPD is expressed in cells that are nonpermissive for DHBV infections indicates that additional cell components must participate in the formation of a functional receptor complex and explains why transfection of cells with recombinant CPD does not confer susceptibility to DHBV infections, although particle internalization may occur.⁷¹¹ Glycine decarboxylase has been shown to bind truncated L protein and might represent a tissue-specific co-factor that plays a role for establishment of DHBV infections following the binding of virus particles to their cell-surface receptor(s).^{387,388} More recently, heparan sulfate proteoglycans have been invoked as low-affinity receptors for HBV that “capture” virus particles to facilitate binding to the high-

affinity, tissue-specific receptor(s).³⁷⁹ Moreover, evidence has been obtained for a role of caveolin-1 in entry of HBV in a tissue culture system.⁴²⁴ Finally, a recent report provided very strong evidence for a role of sodium taurocholate cotransporting polypeptide (NTCP, SCL10A1) as a receptor for HBV.^{777a}

After entry and uncoating of virus, capsids must be transported to the cell nucleus.³¹⁰ Experiments with capsids produced in hepatoma cells and in *Escherichia coli* provided evidence for a model in which core particles migrate along microtubules to the nuclear periphery. From there, capsids enter the nuclear basket in an importin α/β -dependent process and bind to nucleoporin 153.^{568,612} The model predicts that capsids disintegrate within the nuclear pore complex and release RC DNA into the nucleus,⁵⁶⁹ where it is converted into CCC DNA, the template for transcription of the viral RNAs.

Regulation of Transcription and Translation

Under physiologic conditions, CCC DNA is associated with histones and other proteins to form a mini-chromosome.^{51,52,488} Orthohepadnaviruses contain four promoters that control the transcription of the 3.5-kb preC and pgRNAs and the subgenomic 2.4-kb, 2.1-kb, and 0.8-kb RNAs, PreS1, S and X, respectively (Fig. 68.6). All promoters, except for preS1, lack a TATA box and hence produce transcripts with heterogeneous 5' ends, which in the case of the S and preC/C promoters encode distinct proteins: M and S, and pre-C and core/pol, respectively. The possibility that the PreC/C promoter actually consists of two distinct promoter elements has been suggested by genetic experiments and by naturally occurring mutants that fail to express HBeAg.^{76,205,508,603,798}

A single polyadenylation signal located in the core gene regulates the formation of the 3' ends of all four transcripts.⁶³⁹ In the case of Pre-C/C RNAs, RNA polymerase II bypasses the poly A signal once, leading to the formation of terminally redundant transcripts (Fig. 68.6). Sequences located close to the 5' end of the transcript play a role in the suppression of premature polyadenylation during the first passage by RNA polymerase II.⁵⁹⁷

The two enhancers, EN1, located upstream of the X region and EN2, overlapping the pre-C/C promoter, regulate the transcription of the four promoters.^{625,785} Consistent with the hepatotropic nature of hepadnaviruses, all transcriptional regulatory elements of HBV, except for the S promoter, contain binding sites for liver-enriched transcription factors (Fig. 68.7; for a more comprehensive description, see 341,604,624). For instance, the PreS1 promoter contains binding sites for the liver-enriched factors HNF1 and HNF3.^{134,240,413,573}

EN1, a highly complex enhancer less than 300 nucleotides in length, harbors binding sites for liver-enriched factors HNF1, HNF3, and C/EBP.^{108,514,719} In addition, the pre-C/C promoter and both enhancers contain binding sites for nuclear receptors (NRs) including HNF4 α , retinoid X receptor alpha (RXR α), peroxisome proliferator-activated receptor alpha (PPAR α), the chicken ovalbumin upstream promoter transcription factors (COUP-TF) 1 and 2, and others (reviewed in 341). Note that a clear separation between the binding sites on the pre-C/C promoter and En2 is not possible because of the overlap between the two elements. Ectopic expression of RXR α and PPAR α in NIH3T3 cells can induce the expression of pgRNA and the accumulation of HBV replication intermediates that otherwise are not produced in these cells, underscoring the significance of NRs in the control of viral gene expression.^{281,688,797} Curiously,

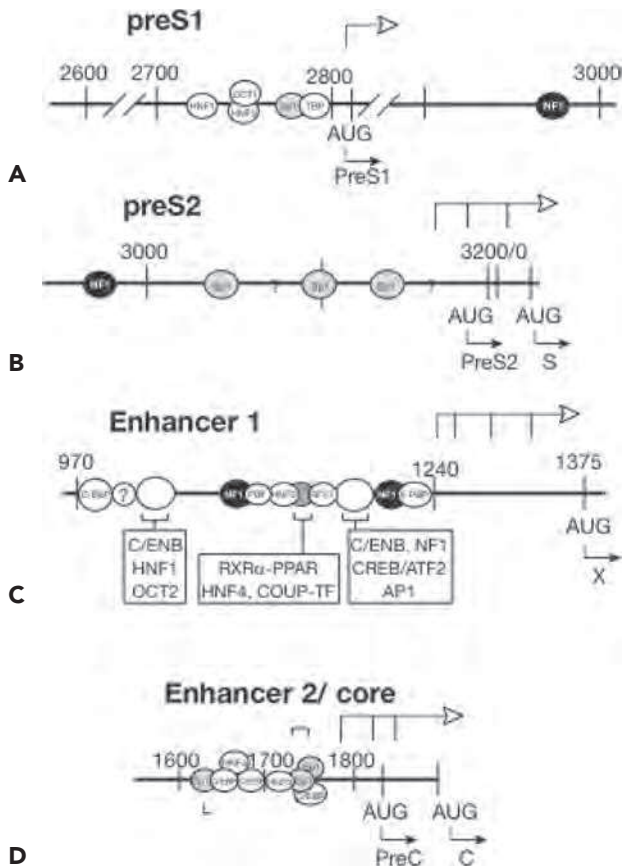


FIGURE 68.7. Transcription factor binding sites. The figure shows the binding sites for transcription factors on the preS1 and preS2 promoters (**A**, **B**), enhancer 1 and the X promoter (**C**) and enhancer 2 and the pre-core/core promoter (**D**) (adapted from [341]). Transcription start sites are indicated with arrows. For details see text. (**D** adapted from Kosovsky MJ, Qadry I, Siddiqui A. The regulation of hepatitis B virus gene expression: an overview of the *cis*- and *trans*-acting components. In: R. Koshy R, Caselman WH, eds. Hepatitis B virus: molecular mechanism in disease and novel strategies for antiviral therapy. London: Imperial College Press, 1998.)

substantial differences in the organization of the transcriptional control elements seem to exist among mammalian hepadnaviruses. For example, WHV does not bear an element corresponding to HBV enhancer EN1.¹⁵⁹

The exact mechanism by which these factors regulate transcription from CCC DNA is not well understood and major unresolved questions remain about the regulation of transcription from CCC DNA under physiologic conditions. For example, does transcription from all three major promoters occur simultaneously from the same copy of CCC DNA or does CCC DNA differentiate and support transcription from a subset of the four promoters? Because hepatocytes contain several copies of CCC DNA, it is possible that each CCC DNA undergoes a developmental process that leads to the inactivation of all but one promoter. Such a model would predict that the first CCC DNA molecule, derived from virion DNA, produces pgRNA, sufficient for initiation of viral DNA synthesis, and that CCC DNA derived from the intracellular CCC DNA amplification process (see Amplification and Stability of CCC DNA section) supports the transcription of either

pre-C, pg, X, or envelope mRNAs. For instance, COUP-TF1 was shown to suppress transcription of PreC/C transcripts in cell culture and might play such a role *in vivo*.⁷⁹⁷ Also, the viral core and X proteins could be involved in differentiation of CCC DNA. DHBV core proteins co-localize with pgRNA in infected hepatocytes and might play a role in the regulation of pgRNA synthesis.^{421,613} Moreover, HBx was shown to bind the HBV CCC DNA minichromosome and to act as a transactivator of the viral promoters.³⁶

Major mammalian and avian hepadnavirus transcripts are unspliced. In HBV and WHV, transport of unspliced viral RNA from the nucleus to the cytoplasm is regulated by a posttranscriptional *cis* regulatory element (PRE) on viral RNAs.^{164,165,283,288} PRE co-localizes with EN1 and a portion of the X gene. Avian hepadnaviruses appear to lack PRE. Instead, they contain positive and negative effectors of transcription (pet, net) that regulate the synthesis of pgRNA.²⁸⁴ Pet spans a 60-nucleotide-long sequence near the 5' end of pgRNA, whereas net is located downstream of the polyadenylation signal. Pet prevents net-induced termination of transcription of nascent pregenomes during the first passage of the RNA polymerase through the polyadenylation site. Like PRE, pet acts in an orientation-specific fashion, but its mode of action is unknown.

Evidence for the presence of a spliced transcript has been obtained with DHBV.⁵⁰² It contains a short sequence from the 5' end of pgRNA fused to PreS mRNA. The exact role of this transcript, if any, for viral replication is unclear. The observation of a spliced HBV transcript, this time encoding a fusion of S and pol, was made in chronically infected patients^{429,648,698} but, again, its functional significance is unknown.

Translation of HBV proteins is controlled by initiation codons located closest to the 5' end of the relevant mRNA. An exception is the polymerase protein. It is translated from an internal AUG codon on pgRNA located at the beginning of the *pol* gene (Fig. 68.6). Although many other viruses (i.e., picornaviruses, hepaciviruses) control internal initiation of translation with internal ribosome entry sites (IRES), hepadnaviruses do not have an IRES. Moreover, *pol* is not translated by a mechanism depending on a plus one frameshift from the core to the polymerase gene, as it has been described for certain retroviruses,²⁹⁵ since stop codons or frame shift mutations placed upstream of the initiation site have no effect on translation.^{95,608} One model, consistent with some experimental data, predicts that a small fraction of ribosomes recruited at the 5' end of pgRNA bypass the core AUG codon and scan the transcript until they reach the initiation AUG of the *pol* ORF.^{193,401} Another, more recent, model suggests that ribosomes bind at the 5' end but are then shunted to the initiation codon for *pol* without scanning the intervening codons.⁶²³ Whatever the correct mechanism, core and polymerase polypeptides accumulate at a constant ratio, which is believed to be in the order of 200 to 300 to 1.

Viral Proteins

CORE

The core protein of HBV is a 183- to 185-aa-long polypeptide of MW 21 kd with an arginine-rich "protamine" domain located at its C-terminus (residues 150–183) (Fig. 68.8). Avihepadnaviruses encode core proteins that are ~80 aa longer than HBV core, with similar properties except for an approximately 45-aa-long

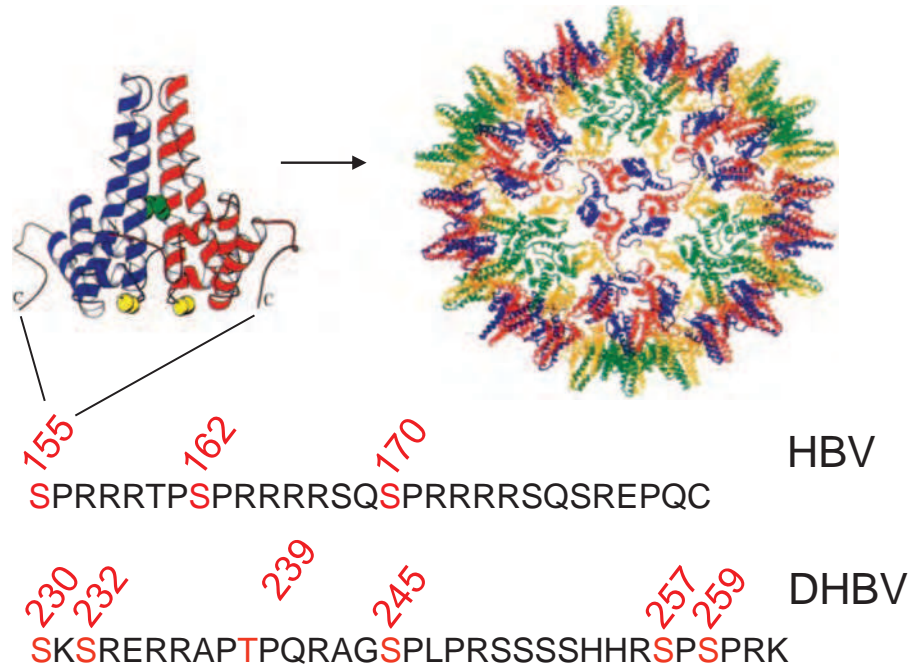


FIGURE 68.8. Structures of the HBV capsid. The figure shows the dimeric structure of core proteins with Cys-61 (green) forming the disulfide bridge between the monomers and amino acid sequences of arginine-rich C-terminal domains of HBV and DHBV. The figure also shows the arrangement of the dimers in icosahedral capsids. (From Wynne SA, Crowther RA, Leslie AG. The crystal structure of the human hepatitis B virus capsid. *Mol Cell* 1999;3:771–780, with permission.)

insertion in the central domain of the polypeptide that forms the “spike” characteristic of viral capsids, and additional amino acids in the arginine-rich carboxy terminus.⁶⁵ CryoEM and X-ray crystallography helped reveal the structure of capsids produced in *Escherichia coli*.^{55,132,772} The folding of the core polypeptide chain is primarily α -helical and, unlike other viral capsid proteins, lacks β -sheets (Fig. 68.8). Two juxtaposed alpha helices ($\alpha 3$, $\alpha 4$) connected by a loop represent the central domain of the monomeric structure. Dimerization leads to the formation of a 4-helix bundle that assumes the shape of an inverted T, where the stem constitutes the dimer interface linked by a disulfide bridge and forms the spikes on the surface of capsids. The tips of the arms form the contact points, primarily located in $\alpha 5$, for the polymerization of the dimers into capsids. During an infection, the majority of capsids are assembled from 120 dimer subunits into a T = 4 structure. Capsids used for the structural studies were comprised of core proteins lacking about 30 aa from the C-terminus including serine phosphorylation sites and, as a consequence, exhibited an increased fraction of particles consisting of 90 dimers with T = 3. A structural analysis of the peptide that links the shell-forming core domain with the C-terminal region was consistent with a model predicting that the C-terminal “pro-tamine” domain provides a mobile platform for viral DNA synthesis inside capsids.⁷⁵⁷ Nevertheless, a conformational change at the exterior surface of capsids could still occur as a result of DNA replication in the interior, and provide a signal for the assembly of cores with envelope components, a step known to depend on DNA synthesis.^{534,671}

The C-terminal domain (CTD) contains three serine phosphorylation sites that are located at the beginning of arginine-rich motifs harboring nuclear localization signals.^{172,311,355,386,392,786} One or several cellular enzymes must mediate the phosphorylation because none of the viral proteins exhibit kinase activity. In cellular extracts the SR protein-specific kinases 1 and 2 (SRPK1, SRPK2) associate with cores and phosphorylate the three serine residues in the SPRRR motif.¹⁵¹

In addition, several kinases, including cyclin-dependent kinase 2 (Cdc2), protein kinase C, and a 46-kd serine kinase can phosphorylate cores *in vitro*.^{213,312,319} However, which kinases play a role in the HBV life cycle is still unknown. Moreover, it is not certain which kinase represents the protein kinase activity associated with Dane particles, which was first described more than 30 years ago.⁷ Experiments with DHBV revealed that DNA replication is accompanied by the gradual dephosphorylation in the core protein, which might contribute to a reorganization of the C-terminus.^{29,535,561,794,795} As with DHBV, all steps of HBV DNA synthesis may be regulated by serine phosphorylation in the core protein, indicating that this mechanism is shared by all members of the *Hepadnaviridae*.^{355,385,456} Finally, genetic studies demonstrated that the arginine clusters in the SPRRR motifs play a role in packaging of pregenomic RNA, and in DNA replication and perhaps in the recruitment of SRPK1 and SRPK2.³⁸⁵ Although the exact role of the charged arginine residues in DNA replication is not known, they might play a role in regulating the spatial organization of pregenomic RNA and minus-strand DNA to facilitate viral DNA synthesis.³⁷⁰

As mentioned previously, a second product derived from the core region is pre-core or HBeAg. It is translated from pre-C mRNA with 5' ends located a few nucleotides upstream of the first AUG in the pre-C/core open reading frame (Fig. 68.6). A signal sequence directs the translation of HBeAg to ER membranes. As a consequence, the protein enters the secretory pathway, where it undergoes a second cleavage event that removes about 34 aa from its C-terminus, before it is secreted from infected cells as a 15-kd protein.^{74,210,316,518,520,655,806} Expression of pre-core is not required for the establishment of productive infections in experimentally infected woodchucks and ducks.^{92,107} Consistent with these observations, HBV mutants that are defective for HBeAg production have been detected in patients with chronic infections.⁶⁸ One possible role for this protein could be to transiently suppress the immune response to the virus, thereby increasing

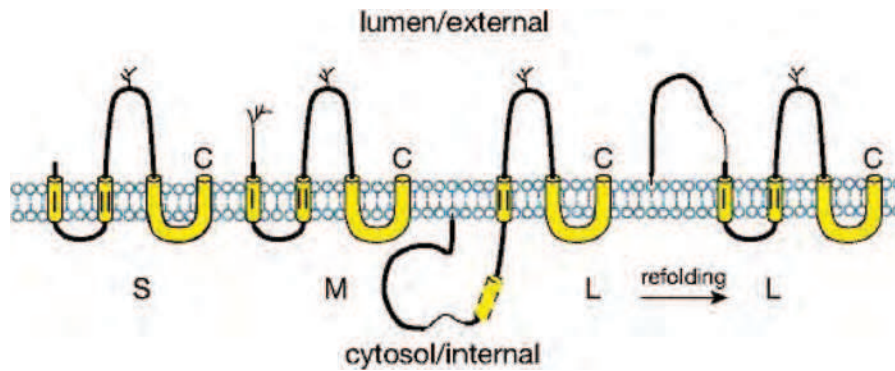


FIGURE 68.9. Proposed membrane topology of HBV envelope proteins. The orientation of the transmembrane domains (TM) is identical in S and M and the refolded L proteins. The external hydrophilic domain of S between amino acids 99 and 168 is illustrated at the left, along with the “a” determinant and the glycosylation site (*branched structure*) at asparagine 146. All proteins share a hydrophobic C-terminal domain that is embedded in membranes. M bears a second glycosylation site at its N-terminal extension (*thin line*). In the primary product of L, TM I is located in the cytosol where the N terminus is attached to membranes by the modification with myristic acid (for details see text). (Adapted from Bruss V. Envelopment of the hepatitis B virus nucleocapsid. *Virus Res* 2004;106:199–209.)

the frequency of chronic infections, which might explain the conservation of this gene. It is assumed that this function is no longer required once a chronic infection is established.¹⁰⁸

ENVELOPE

The three envelope proteins, L, M and S, encoded by the mammalian hepadnaviruses have two principal functions: (a) they provide the protein components of the virus envelope and (b) they assemble into aggregates that are secreted as subviral particles. L and M differ from S in their N-terminal regions (Figs. 68.6 and 68.9). As a consequence of the common S region, the three proteins share several features: they contain two topogenic signal I and II that determine their orientation in lipid bilayers, a hydrophobic C-terminal region that is most likely embedded in ER membranes and a common N-glycosylation site (Asn-146) in S. Like a conventional signal sequence, signal I is located at the N-terminus of S, but is not proteolytically processed. Signal II is a hydrophobic domain acting as a stop-transfer sequence and a signal sequence. As a result, the two hydrophobic domains form a hairpin structure with a cytosolic loop.^{170,171} The presence of a third hydrophobic domain invokes the theoretical possibility of two additional transmembrane passages. In addition to the common glycosylation site located downstream from signal II, the M protein harbors a second N-glycosylation site (Asn-4) near its N-terminus.²⁵⁸ This site is also present in L, but not used, because of the cytoplasmic location of the preS2 domain of L. Some HBV genotypes contain O-glycans (Thr-37) in the preS2 domain of both L and M proteins.⁶¹¹ L and M proteins contain modified N-termini. L carries a myristate group at Gly-2,⁵³⁹ whereas M is N-terminally acetylated.⁶¹⁰ The role of M in HBV replication is not yet understood, because this protein is not required for the production of Dane particles.¹⁸⁵ In avihepadnaviruses, L-proteins are also myristoylated, but not glycosylated.⁵⁶³ Instead they become phosphorylated in the preS1 domain.²²³

A major feature of L is that it exists in two conformations that differ in the localization of the N-terminal domain

(Fig. 68.9). In the first, the N-terminus, including signal I, is located in the cytosol,^{75,516,556} where it is required for binding of capsids and for the assembly of virions. In the second, the N-terminus is present in the ER lumen and, as a consequence, exposed on the surface of viral particles where it plays a role in the infection of hepatocytes.⁵⁵⁶ The conformational change is facilitated by interactions of L with the molecular chaperones Hsc70/Hsp40 and BiP.³⁵³ However, the details of the mechanism regulating this step are not yet understood. The major determinants for infectivity of HBV are located in the N-terminal 55 amino acids of L (44, 371, reviewed in 218). Evidence for this conclusion is derived from genetic experiments with mutant envelope proteins. In addition, peptides spanning different regions of the envelope proteins have been useful in mapping domains critical for infection. Experiments with peptides demonstrated the importance of N-terminal myristoylation. Myristoylated peptides are much more potent inhibitors of HBV infection than peptides with normal N-termini.^{23,219,224,542} A second region overlapping with the so-called “a” determinant or antigenic loop located between transmembrane domain II and the hydrophobic C-terminus of S contains a second infectivity determinant.^{367,600} However, the function of this determinant in infection is not yet known.

Following integration into membranes, envelope proteins form intermediates that include homo- and heterodimers stabilized by covalent disulfide bridges between different cysteine residues in the S domain, and subsequently assemble into either subviral or Dane particles^{290,637} (Fig. 68.5). S and M proteins contain the necessary signals for the export process because they can be secreted independently. In contrast, when synthesized in the absence of the other two envelope proteins, L is retained in membranes, suggesting that it carries a retention signal that prevents, in the absence of S and M, the export process controlled by its S domain.^{113,538}

In addition to their roles as envelope proteins, L and M can activate, *in trans*, the transcription from selected promoters in transfected cells.^{88,322} This function was initially described

for naturally occurring mutants of these proteins with C terminal truncations. Because truncated forms of L and M were initially identified in tissues from chronically infected patients, it has been speculated that they contribute to the development of hepatocellular carcinoma (HCC). Later work showed that the complete L protein could also transactivate selected promoters.^{190,262,263} This may be indirectly related to the fact that accumulation of L protein in the ER can lead to ER stress and consequently increase expression of M and S.^{287,773} This presumably facilitates L secretion and relieves ER stress. Thus, the transactivating potential of the HBV envelope proteins may ultimately reflect adaptations to facilitate survival of infected hepatocytes and might, but only in the long term, lead to transformation of rare infected hepatocytes.³⁵²

REVERSE TRANSCRIPTASE

Hepadnaviral polymerases have an approximate MW of 90 kd and consist of three functional domains: the terminal protein (TP) required for the priming of minus-strand DNA synthesis, and the reverse transcriptase (RT) and RNaseH for DNA synthesis and degradation of pgRNA. A spacer (hinge) separates the TP from the other two domains (Fig. 68.10). The spacer region appears to have no other function than to provide a flexible connection between the TP and RT domains.^{27,94} As will be discussed later in this chapter, RT is the target for all currently approved antiviral therapies with the exception of interferon.

Unlike retroviral RT, hepadnaviral polymerases are strictly template specific, which is a direct consequence of the mechanism for the activation of the enzyme. It requires binding of the polypeptide to the packaging signal, termed epsilon, located at the 5' end of pgRNA (a second copy of epsilon is located near the 3' end, but does not serve as a binding site for Pol; Fig. 68.11B). As is discussed later in this chapter, binding of the polymerase to epsilon leads to the priming of reverse transcription from a tyrosine residue (Y65) in the TP domain.^{362,747,758} This results in the formation of a covalent link between the polymerase and the nascent viral minus strand. However, this priming activity of Pol also requires cellular factors, explaining why early attempts to demonstrate polymerase activity with Pol expressed in bacteria were not successful, although similar strategies yield functional retroviral polymerases.⁶⁸⁶ Enzymatically active polymerase was first produced with the DHBV enzyme translated in rabbit reticulocyte lysates, and led to the discovery that the interaction between the polymerase and epsilon RNA is dependent on chaperones including heat shock proteins 90 (Hsp90), Hsp70, and p23.^{276,278,654} Consistent with these observations, both DHBV and HBV replication are sensitive to geldanamycin and its derivatives, which bind to the N-terminal ATP binding domain of Hsp90.^{275,278} Similarly, novobiocin, which binds to the C-terminus of Hsp90, inhibits HBV replication.²⁷⁵ Moreover, Hsp90, p23, and three additional chaperones—Hsp70, Hsp40, and Hop—can activate DHBV

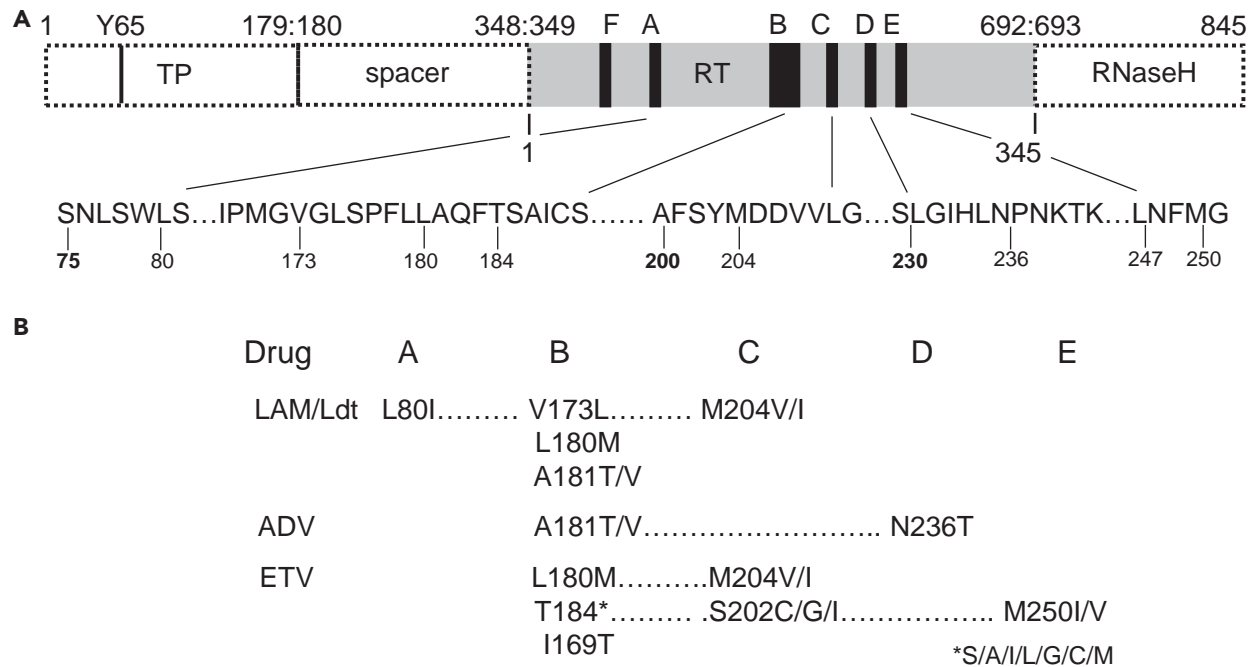


FIGURE 68.10. Physical organization of the HBV polymerase and resistance to nucleoside analogs. A: Domains of the pol protein. The polymerase contains three main functional domains, the terminal protein domain (TP), the reverse transcriptase (RT) domain, and the RNaseH domain (A). Functional domains within the RT have been assigned based on structural modeling using the crystal structure of the HIV RT as a guide.^{150a} By this analogy, domains A, C and D would appear to be involved in deoxynucleotide binding and polymerization. The B domain is thought to participate in template binding and the E domain in binding of the primer strand. **B:** Resistance mutations. The figure shows the location of amino acid mutations that confer resistance to lamivudine (LAM) and telbivudine (Ldt), adefovir (ADV) and entecavir (ETC). (Adapted from Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009;137:1593–1608.)

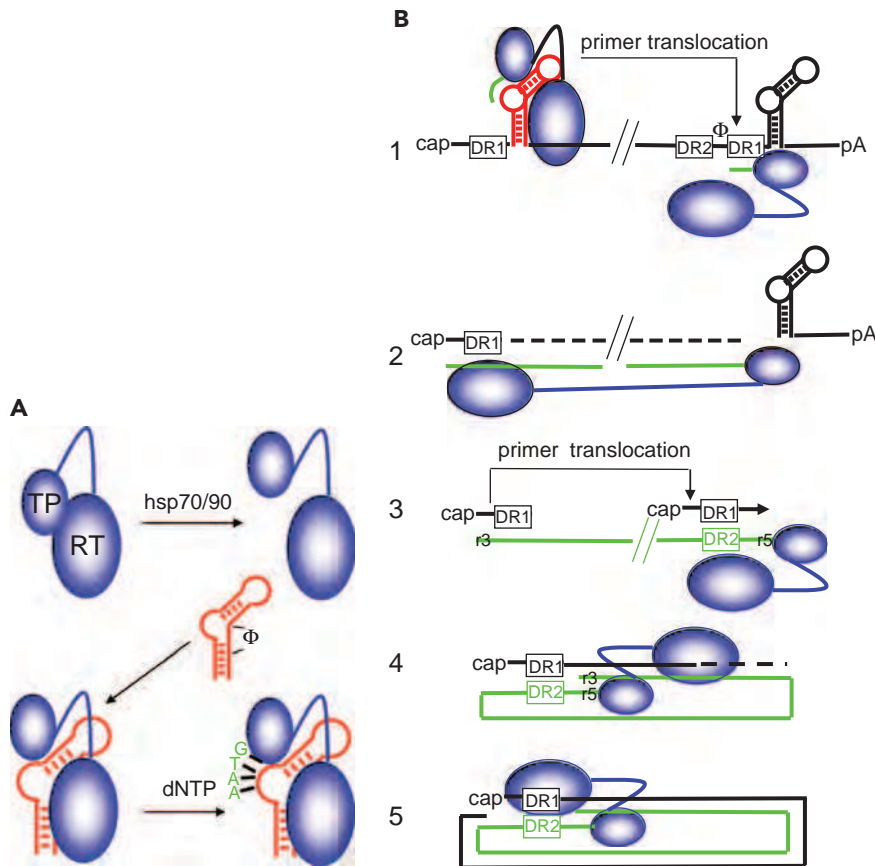


FIGURE 68.11. Genome replication. **A:** Binding of epsilon RNA to the TP and RT domains of the polymerase facilitated by chaperones (hsp70/90) and initiation of reverse transcription at the bulge of epsilon RNA as described in the text. Phi (Φ) depicts the RNA sequence on epsilon proposed to be required for circularization of pgRNA (see **B**). **B:** The figure depicts five important steps in viral DNA synthesis: 1) Transfer of the DNA primer from epsilon to DR1 near the 3' end of pgRNA; 2) Elongation of minus-strand DNA and digestion of pgRNA by RNaseH of the polymerase; 3) Transfer of the capped RNA primer from DR1 to DR2 and synthesis of plus-strand DNA to the 5' end of minus-strand DNA; 4) Template switch of the nascent plus strand with the help of the small terminal redundancies r5 and r3, resulting in circularization of the genome; 5) Completion of plus-strand DNA as described in the text. The figure was not drawn to scale.

and HBV polymerases to bind to epsilon RNA *in vitro* and, in the case of the DHBV polymerase, restore the protein-priming activity.^{275,277} With HBV, the protein-priming activity has been demonstrated in insect cells,³⁶¹ but so far not in bacteria or the reticulocyte lysate system. With DHBV enzymatically active polymerase can also be expressed in yeast.⁶⁹⁰

The function of the chaperones is not completely understood, because structural information about the polymerases of hepadnaviruses is lacking. It is possible that they stabilize an energetically unfavorable conformation of the polymerase and, in this way, facilitate the binding of polymerase with epsilon RNA^{84,653} (Fig. 68.11A). Consistent with such a model is the observation that DHBV polymerases with deletions of the RNaseH domain can exhibit protein-priming activities in the absence of cellular factors.⁷⁵⁰ Thus, these domains might hold the polymerase in a “closed” conformation that, in the absence of chaperones, prevents interaction with the packaging signal.

It is likely that a single polymerase polypeptide catalyzes one complete round of DNA replication because assembly of the polymerase into capsids depends on its interaction with epsilon sequences on pgRNA, which would indicate that polymerase and RNA templates are present at equimolar amounts in subviral particles.^{25,803} Consistent with such a model, experiments meant to quantify polymerase levels in HBV capsids revealed a molar ratio of ~0.7 polymerase molecules per virion DNA.^{25,26}

HBx

X is an enigmatic protein of the orthohepadnaviruses that is required for efficient infection and replication *in vivo*.^{106,415,804,814}

Expression of this polypeptide has been assessed in the livers and primary hepatocytes from WHV-infected woodchucks where it can accumulate to 40,000 to 80,000 copies per cell in the cytoplasm.¹⁴⁸ In contrast, in hepatoma cell lines HBx can be detected in both the cytoplasm and the nucleus.¹⁶⁶ However, the exact role of X activity has been difficult to elucidate because the protein interacts with many cellular factors, including the proteasome, and its activity varies depending on the cell lines used for a study (for more detailed reviews see 57, 475).

Originally, HBx was identified as a relatively weak transactivator for promoters with NF- κ B, AP-1, AP-2, c/EBP, ATF/CREB, or NFAT binding sites in tissue culture cells. Because HBx does not bind directly to DNA, it regulates transcription either directly by binding to transcription factors and chromatin (e.g., on CCC DNA), or indirectly through activation of signal transduction pathways in the cytoplasm (Fig. 68.12).^{36,57,594,649,726} For example, it has been reported that HBx enhances the binding affinity of CREB, a member of the basic leucine zipper family, for the CREB/ATF2 binding site in HBV enhancer I (Fig. 68.7).^{427,765} Other reports provided evidence for binding of HBx to p53,⁷⁵¹ the RNA polymerase subunit RPB5⁶⁸¹ and the DNA helicase components, ERCC2 and ERCC3 of TFIIH.⁷⁵¹ Whether *in vivo* HBx is essential for transcription of viral RNAs or regulation of cellular genes required for the viral life cycle is difficult to assess. Experiments with hydrodynamically injected mice demonstrated that nuclear (but not cytoplasmic) HBx acts as a transactivator of the viral promoters and, by inference, might exert this activity in the natural life cycle of the virus.^{320,321}

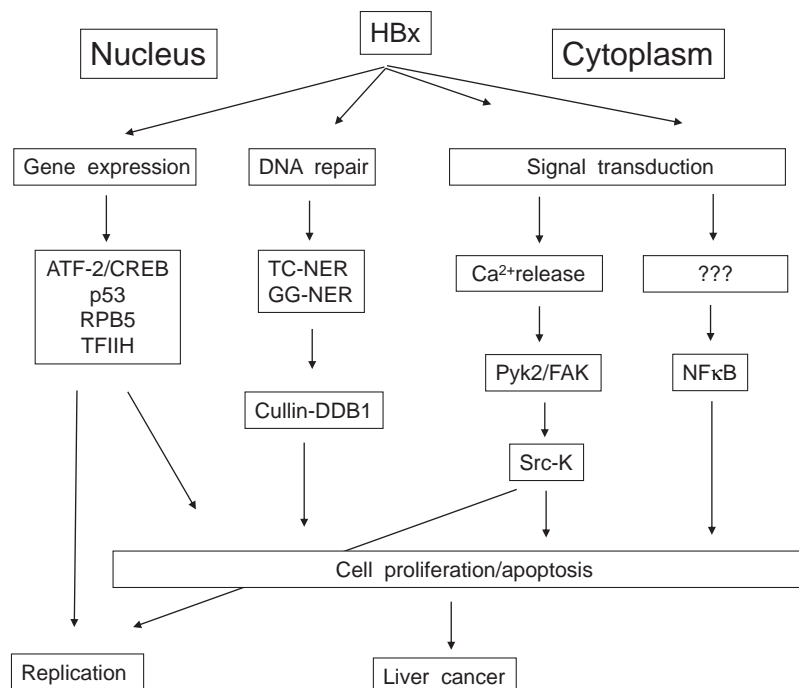


FIGURE 68.12. Model for HBx functions. The figure depicts three major proposed activities of HBx: regulation of gene expression, inhibition of DNA repair and activation of several signal transduction pathways described in the text.

Investigations on the cytoplasmic activity of HBx led to a model predicting that expression of HBx induces the release of calcium ions (Ca^{2+}) into the cytosol, either from the ER or from mitochondria.⁵⁸ The transient influx of Ca^{2+} then activates the nonreceptor tyrosine kinases proline-rich tyrosine kinase 2 (Pyk2) and focal adhesion kinase (FAK), which in turn activate the Src family kinases (Src-K) (Fig. 68.12). The latter are known to activate the downstream Ras-Raf-MAP kinase signal transduction pathways. In HepG2 cells, where X expression can significantly stimulate viral DNA synthesis, drugs that chelate intracellular Ca^{2+} or prevent activation of Src-K can inhibit HBV replication.⁵⁶ Conversely, compounds known to induce the efflux of Ca^{2+} into the cytosol can increase the replication levels of HBx negative mutants. Nevertheless, it needs to be determined whether expression of HBx in natural infections triggers the transient Ca^{2+} fluxes that have been observed in transfected hepatoma cell lines, and how this activity is involved in chronic infection of liver cells *in vivo*. Notably, the effects on viral replication appear to be independent of the Ras pathway, suggesting that Ras-dependent activation of transcription is not required. One possibility is that X-activated pathways play a direct role in viral replication by regulating the phosphorylation of the viral core protein.⁴⁵⁶ While a model invoking a role for HBx in the release of Ca^{2+} is consistent with many activities attributed to this polypeptide, including activation of transcription, cell-cycle control, and apoptosis, it cannot explain all the features of the protein, most notably, the reported activation of NF- κ B.^{116,383,416,666}

Several groups reported that expression of X proteins from both HBV and WHV can inhibit nucleotide excision repair (NER) through binding to DNA damage-binding protein 1 (DDB1),^{35,301,375,642} though it remains unclear if inhibition is specific to global or transcription-coupled NER, or to both. More recent studies provided evidence for an HBx-DDB1 complex binding to cullin4A-RING (CUL4A) ubiquitin ligase. This association is mediated by DDB1 acting as

an adaptor protein.⁵⁴⁴ Structural data has revealed an α -helical motif at position 88 to 100 in HBx that binds to the BPA-BPC domains of DDB1,³⁹⁰ and validated the HBx-CUL4A interaction.³⁹¹ Thus, HBx might act as a bridge linking the CUL4A-DDB1 complex to a substrate that is then ubiquitinated by the CUL4A-RING ubiquitin ligase. The substrate for the CUL4A-DDB1-HBx complex is not known. Notably, the V proteins of simian virus 5 and human parainfluenzavirus type 2 can redirect the CUL4A-DDB1 ligase to STAT proteins, promoting polyubiquitination and degradation of these transcription factors.^{160,729} Hence, it is possible that HBx plays a role in inhibiting innate immune pathways. Results from recent work indicated that HBx can inhibit dsDNA-induced activation of the RIG-I pathway.⁷⁸⁶ Alternatively, the protein might play a role in regulating a cellular pathway required for a specific step in viral life cycle, such as the formation of CCC DNA. In addition to its role in HBV replication, HBx might also play a role in the development of liver cancer, as discussed later in this chapter.

Identification of the physiologic role of HBx in viral replication and pathogenesis remains one of the most challenging problems in HBV biology. The lack of mouse models or hepatocyte cell lines permissive for HBV infections has stymied efforts to investigate relevant HBx-dependent host-virus interactions that play a role in viral replication during natural infections. Nevertheless, many important properties of HBx have been uncovered with available experimental systems. It is certain that HBx is required for viral replication *in vivo* and that HBx expression may effect signal transduction and other gene expression pathways that alter the physiology of hepatocytes in a fashion that promotes viral replication and persistence. The use of primary hepatocyte cultures in lieu of immortalized or transformed cell lines might be required to unravel the true function of HBx. For example, recent studies with primary cultures provided evidence for a role of HBx in promoting progression of the cell cycle from G0 to G1.³⁸⁶

Replication of Genomic Nucleic Acid

FORMATION OF CCC DNA

The first step in the hepadnavirus replication cycle is the conversion of genomic RC DNA into CCC DNA (Fig. 68.5). Although the details of the mechanisms responsible for this step are not understood, a comparison of the two DNA forms can be used to establish a model for CCC DNA formation. It predicts that the polymerase and one of the terminally redundant segments on the minus strand, termed *r*, are removed prior to the ligation of the two ends. Similarly, a capped RNA oligomer present at the 5' end of plus-strand DNA must be removed and the incomplete plus strands extended before the ends can be joined. Whether the viral DNA polymerase or a cellular polymerase elongates the 3' end of plus strands is not known. Reverse genetic experiments with DHBV suggested that an endonuclease removes the 5' end of minus strand DNA prior to CCC DNA formation, leading to the formation of a protein-free RC DNA, which can be detected in tissue culture cells.^{209,235,330,647} Most likely, cellular DNA repair enzymes are responsible for the conversion of RC to CCC DNA. Consistent with this view, CCC DNA formation following infection of primary hepatocyte cultures with DHBV or WHV is not blocked by inhibitors of the viral polymerase.^{198,470} Whatever the exact mechanism of CCC DNA synthesis, it must be extremely efficient, because in natural infections virions have a specific infectivity close to one.^{16,305}

PACKAGING OF pgRNA

pgRNA (C mRNA) has dual functions in viral replication. It acts both as the mRNA for the translation of the core and polymerase polypeptides and as the template for genome replication. The transition from the first to the second function is triggered by the binding of the polymerase to the packaging signal, epsilon, at the 5' end of the mRNA (Fig. 68.11A). In turn, this reaction creates a signal for the assembly of this ribonucleoprotein complex (RNP) into capsids. Polymerases preferentially bind to their own mRNA, possibly while translation is still ongoing, which increases the chance for packaging and replication of biologically intact pgRNA.^{26,265,285} The interaction between the polymerase and epsilon RNA requires cellular chaperones, as described earlier. While structurally intact polymerase is required for RNA packaging, DNA polymerization activity *per se* is not required for this process, indicating that the RNP can induce assembly without a requirement for DNA synthesis.^{24,94,111,265,596} Nevertheless, as will be discussed, packaging and initiation of reverse transcription are intimately linked events.

Based on secondary structure predictions, epsilon contains two inverted repeats that can fold into an RNA hairpin with a basal and apical stem that are separated by a bulge (Fig. 68.11). The upper stem is capped by a loop.^{33,189,279,307} RNA footprint analysis of free and bound epsilon RNA suggested that binding of the polymerase could induce a single-stranded conformation in the upper stem.³⁴ Again, it should be noted that due to a terminal redundancy (*R*), epsilon RNA is present at both ends of pgRNA. However, genetic experiments showed that only the 5' copy provides a binding site for the polymerase and is required for packaging.^{307,327,546} Consistent with these results, evidence has been obtained that the nearby cap structure on pgRNA may play a role in RNA packaging. These results also evoked the possibility that the polymerase might interact with translation factors to induce a transition from translation to replication.³⁰⁰

Interestingly, epsilon maps upstream of the AUG for core, and evidence has been obtained that translation of pre-C RNA, with passage of 80S ribosomes through epsilon, prevents its packaging.⁴⁸³

In contrast to the mammalian hepadnaviruses, packaging in the avian viruses requires, in addition to epsilon, a second pregenome sequence, termed region II, located about 900 nucleotides downstream of epsilon and spanning approximately 300 nucleotides.^{83,266,517} The role of this downstream sequence in RNA packaging is not yet understood.

In addition to the chaperones required for RNA packaging described previously, the human cytidine deaminase APOBEC3G is incorporated into viral particles through binding to the viral reverse transcriptase.^{490,491} Ectopic expression of several members of the APOBEC family of proteins inhibits HBV replication.⁵⁹² Notably, unlike the inhibition by APOBEC of retrovirus and retroposon replication,⁵⁹³ inhibition of HBV was not dependent on the catalytic activity of the deaminase. Instead, it appears that the protein inhibits virus replication during an early step of minus-strand DNA synthesis, perhaps by binding to viral RNA or the polymerase.⁴⁹⁰

MINUS-STRAND DNA SYNTHESIS

The first step in minus-strand DNA synthesis is the priming reaction that leads to the formation of a covalent link between a tyrosine residue in the TP domain of the polymerase and dGMP (Fig. 68.11A).^{359,747,758,813} The template for this protein-priming reaction is a C residue located in the bulge of epsilon. Although *in vitro* priming can occur immediately following the binding of the RT to epsilon in the absence of core protein, the exact sequence of events in infected cells is not known. Thus, priming could occur prior to, during, or after the assembly of capsids. To complete the priming reaction the polymerase copies the next two or three nucleotides from the bulge of epsilon. As a consequence of this mechanism, the polymerase remains covalently linked to the 5' end of minus-strand DNA during all subsequent steps of viral DNA synthesis, virus assembly, and release.^{214,468,469}

To continue DNA synthesis, the 3- to 4-nt DNA oligomer is transferred to the 3' end of pgRNA, where it anneals with a complementary sequence motif located in a 10- to 12-nucleotide-long region known as DR1 (Fig. 68.11B, step 1).⁷⁴⁶ However, the 3- to 4-nucleotide acceptor site by itself is too short to specify the transfer to DR1. Additional sequences on pgRNA are necessary to control the translocation of the DNA primer to DR1. The selection of the natural site is most likely facilitated by the structural arrangement of pgRNA in the capsid. The acceptor site and epsilon RNA must be held in close physical proximity to facilitate the transfer of the 3- to 4-nt oligomer across the pregenome. Indeed, a short *cis*-acting element, termed phi (Fig. 68.11A), located upstream of the acceptor site at DR1 is required for accurate minus-strand DNA synthesis from DR1 in HBV, but not DHBV.^{2,431,633,687} Phi can base-pair with the 5' region of epsilon RNA, thereby stabilizing the predicted structural conformation of pgRNA required for the transfer of the short minus strand. Following the translocation reaction, minus-strand DNA synthesis continues all the way to the 5' end of the RNA template (step 2). During this reaction, pgRNA is degraded by the RNaseH activity present near the C-terminus of the polymerase.^{110,570,671} Due to the location of DR1 within the terminal redundancy

on the pregenome, the completed minus-strand DNA bears the short terminal redundancy, *r*. As described below, *r* plays a role in the circularization of the viral genome.^{400,617,764}

PLUS-STRAND DNA SYNTHESIS

Plus-strand DNA is primed by an 18-nucleotide-long, capped RNA oligomer derived from the 5' end of pgRNA. The oligomer contains a complete copy of DR1 and represents a product of the RNaseH activity of the polymerase (Fig. 68.11B, step 3).^{399,410} As a consequence of this priming mechanism, plus-strand DNA synthesis can only begin after minus-strand DNA synthesis is complete. To prime plus-strand DNA synthesis, the RNA oligomer must first translocate to and anneal with DR2, located near the 5' end of the minus-strand DNA and identical in sequence to DR1.^{399,617,657,764} As expected, mutations that disrupt the homology between DR1 and DR2 block the formation of RC DNA and instead favor an *in situ* DNA priming reaction from the nontranslocated primer, leading to the formation of double-stranded linear (dsl) DNA.^{130,657,783} DSL DNA is produced even under natural conditions, albeit with a low frequency of about 5% to 20% of RC DNA (Fig. 68.5).

The mechanism responsible for the transfer of the RNA primer to DR2 is not completely understood. The most likely scenario is that the regions encompassing DR1 and DR2 on minus-strand DNA are juxtaposed to facilitate the transfer of the primer from DR1 to DR2. Studies with DHBV and HBV revealed the presence of three sequence motifs on the minus strands, which have the potential to form short duplexes that might stabilize a secondary structure required for plus-strand primer translocation.^{241,256,384,408,473} Mutations that would be expected to disrupt the formation of these duplexes inhibited RC DNA, but not dsl DNA synthesis.^{384,407,408} In addition, capsid proteins might impose certain structural constraints on minus strands and thereby play a role in primer transfer.

Following the priming reaction at DR2, plus-strand DNA synthesis ensues until it reaches the 5' end of minus-strand DNA. At this point, a template switch (i.e., circularization) is required for the continuation of DNA synthesis (Fig. 68.11B, step 4). The template switch is facilitated by the aforementioned terminally redundant sequences, *r*, in minus-strand DNA. The structural requirements for this reaction must be complicated, because the polymerase attached to the 5' end of the minus strand accommodates both ends of the minus-strand DNA in close proximity. In spite of the expected steric constraints, the polymerase can copy the entire *r*5 region, including the dGMP residue that is covalently linked to the RT, and then induce the necessary template switch to *r*3.⁴⁰⁹ As with priming at DR2, this recombination event also depends on the formation of small duplexes on distant sites in minus-strand DNA, indicating that the two critical steps in plus-strand DNA synthesis might be controlled by the same mechanism.^{255,472}

In mammalian hepadnaviruses, plus-strand DNA synthesis is incomplete and reaches approximately half the genome length prior to virion formation.^{585,671} The cause and significance of the premature termination of plus-strand synthesis remains obscure. Perhaps the arrest in DNA synthesis is caused by steric factors imposed by the capsid and by the polymerase itself. For instance, capsids of HBV assembled from core proteins with truncated C-termini accumulate virion DNA with a greater deficiency in plus-strand synthesis, and a shift to *in situ* priming of plus-strand elongation⁴⁸²; with DHBV, capsids assembled

with core proteins with truncated C-termini are also defective in plus-strand elongation.⁷⁹⁴ Thus, capsid structure can influence elongation of plus-strand DNA. In addition, the coating of capsids with envelope proteins probably leads to the depletion of the dNTP pool prior to the completion of DNA synthesis. This latter possibility is supported by the fact that plus-strand DNA can be extended in an *in vitro* reaction in the presence of precursor dNTPs and nonionic detergents that disrupt the viral envelope^{314,671} (Fig. 68.2). In contrast to orthohepadnaviruses, plus-strand DNA synthesis in wild-type avihepadnaviruses is virtually complete,⁴⁰⁰ except that the polymerase does not displace the RNA primer from DR2, so DR2 has to be copied prior to CCC DNA formation.

AMPLIFICATION AND STABILITY OF CCC DNA

The fate of DNA-containing capsids in the cytoplasm of infected cells is twofold: The particles either enter the cell nucleus and release RC DNA or assemble with envelope proteins into virions and enter the secretory pathway (Fig. 68.5). The first pathway amplifies the copy number of CCC DNA.^{724,770} Using DHBV, CCC DNA amplification in cultures of nondividing hepatocytes was shown to occur early in an infection when the cytoplasmic concentration of viral envelope proteins is still low.^{675,676} The final, average CCC DNA copy number per nucleus *in vivo* is usually between 1 and 50.^{306,308,466,802} The route and mechanism of transport of capsids to the nucleus are unknown. Transport of newly made capsids from the cytoplasm to the nucleus does not require envelope proteins because CCC DNA amplification occurs when hepatocytes are infected with viral mutants that are unable to synthesize these proteins.^{675,676} Instead, signals generated on capsids during their maturation might play a role in retrograde transport.

A critical issue with important implications for antiviral therapies with inhibitors of viral DNA replication is whether CCC DNA has a half-life and, therefore, whether ongoing CCC DNA synthesis is required to maintain a steady state within a nondividing cell. An early study¹²⁶ addressed CCC DNA stability with a BUDR pulse/chase protocol using primary, nondividing cultures of hepatocytes derived from ducks infected with DHBV. CCC DNA labeled in a pulse chase appeared completely stable. However, if BUDR was instead added later and the fate of unlabeled CCC DNA present prior to BUDR addition was followed, a shorter half-life of 3 to 5 days was observed. This discrepancy is not yet understood. In another study, CCC DNA stability in the presence of reverse transcription inhibitors was analyzed following infection of primary woodchuck hepatocyte cultures with WHV. These experiments suggested a half-life greater than 30 days in nondividing hepatocytes.^{470,809} CCC DNA also appeared to survive mitosis in primary woodchuck hepatocyte cultures that were treated with adefovir dipivoxil to block viral DNA synthesis.¹⁴⁵ A fourth study took a different approach, examining CCC DNA stability in chicken hepatoma cells expressing DHBV from an inducible promoter.²³⁸ This study, like the earlier study in duck hepatocytes, reported a short CCC DNA half-life: ~2 days. Moreover, it suggested that CCC DNA could survive cell division and partition to daughter cells and thus, that in the absence of new viral DNA synthesis, CCC DNA would be gradually lost from cells through dilution, as also demonstrated in a chimeric mouse model with implanted human hepatocytes.⁴¹⁹ A fifth study demonstrated a CCC DNA half-life of ~14 days in HepG2 cells transduced with HBV using

a baculovirus vector.¹ Given the possibility that cell loss might have contributed to CCC DNA loss in the LMH and HepG2 cell-line experiments, published studies seem to suggest, on balance, that CCC DNA is stable in nondividing cells in culture, and perhaps in dividing cultures as well.^{145,238} Some,^{5,418,809} but not all^{759,771} *in vivo* studies of antiviral therapy with nucleoside analogs that inhibit viral DNA synthesis suggest that CCC DNA may be stable in the chronically infected liver and survive through mitosis. The idea that CCC DNA stability is high is also supported by studies of competition in the fully infected liver between strains of DHBV with different replication rates. Competition between the strains essentially stops in the fully infected adult liver, where cell turnover is low, again suggesting that CCC DNA has a lower turnover rate in infected cells.⁸⁰¹

Virus Assembly and Release

Assembly of hepadnaviruses is still a poorly understood process. Assembly of Dane particles occurs in at least two distinct steps: the formation of capsids that contain pgRNA and RT and the formation of enveloped virus particles that contain the viral DNA genome. During DNA synthesis, capsids gain the ability to interact with envelope proteins.^{290,531} (Fig. 68.5). The N-terminal domain of L plays an important role in this interaction because M and S proteins alone cannot support translocation of capsids across membranes.³⁸¹ Recent reports provided evidence for a mechanism in which DNA containing core particles assemble with envelope proteins at membranes of multivesicular bodies (MVPs).^{323,754} This model is supported by data demonstrating a requirement for vacuolar protein sorting proteins AIP1 and VPS4B in assembly and release of virus (Dane) particles.⁷⁵⁴ In contrast, formation and secretion of SVPs occur through the ER-Golgi compartment and do not depend on MVPs. Other factors reported to interact with capsids and envelope protein that could play a role in assembly include the ubiquitin ligase Nedd4,⁵⁹⁵ gamma2-adaptin, a clathrin adaptor protein,²⁵⁴ and thioredoxin-related transmembrane protein 2,⁷⁰⁸ a protein involved in clathrin-mediated endocytosis and, hence, the early endocytic pathway. The observed interaction of capsids with Nedd4 is particularly interesting because members of the Nedd4 family of ubiquitin ligases play a role in linking capsid proteins of certain retroviruses and RNA viruses with components of the ESCRT (endocytic sorting complexes required for transport) machinery that sorts cargo proteins into the luminal vesicles of MVPs and facilitates budding.²⁰⁰

PATHOGENESIS, PATHOLOGY, AND EPIDEMIOLOGY

Entry into the Host

HBV is found at high titers, sometimes up to 10^{10} Dane particles per ml, in the blood of infected individuals. Thus, the main routes of infection involve exposure to blood or blood-derived products, such as during childbirth from an HBV-positive mother, blood transfusion, or other potential sources of percutaneous exposure, including sexual intercourse.³⁷⁷

Perinatal Infections

The greatest sources of infection worldwide are from infected mothers to newborns, or among very young children. The risk

of vertical transmission varies depending on geographic regions. In Asia, the rate of perinatal transmission from infected mothers is as high as 90%, because many of the pregnant women that are chronically infected have high titers of circulating HBV. In infants infected by HBV, the rate of chronicity reaches 90%⁶⁵⁹ without vaccination. In North America, Western Europe, and Africa, the risk of vertical transmission from chronically infected mothers is about 10% without any preventative therapy. This lower risk is consistent with reports that infected mothers in these locales usually have a low viral load. These different viral loads are likely a result of different natural histories of chronic infection in the different locales, with infections at the time of birth that become chronic, leading to a more persistent high-level viremia than infections later in life. That is, women infected at birth would have a higher viremia as they aged and would be more likely to pass the infections to the neonate than women infected later during the first few years of life. Thus, the high rate of chronicity in Africa appears mostly due to horizontal spread to young children from playmates and adults involved in their care, rather than directly from infected mothers during birth.

Additional Routes of Transmission: Blood Transfusion, Intravenous Drug Use, Sexual Transmission and Nosocomial Infections

The risk of HBV transmission by blood transfusion has decreased dramatically since the early 1970s because of the exclusion of paid donors and the introduction of serologic screening of volunteer blood donors for serum HBsAg and anti-HBc immunoglobulins. In the United States, the risk of HBV transmission via blood products is now one out of 63,000 transfusions, down from 15% in the 1960s.^{12,220} The current incidence may be attributed to the failure to identify infected blood donors because of the serologic window during the incubation period following infection, the presence of some rare HBsAg variants that are not detected by the serologic assay for HBsAg, particularly when concurrent testing for anti-HBc is not performed, and the problem of so-called occult HBV infections, in which neither HBsAg or anti-HBc are detected.

In contrast to blood transfusion, percutaneous infection of young people and adults via intravenous drug use, tattoos, acupuncture, ear piercing, sharing razors, and other avenues, remain as major modes of HBV transmission. Sexual transmission still represents 40% of the new cases of acute hepatitis B in many developed countries,^{12,220} while the role of intravenous drug use seems to be decreasing with time, currently representing 6% to 10% of new cases. HBV can also be transmitted by accidental needle stick in the healthcare setting.^{12,220} Nosocomial transmission represents approximately 10% of the new cases of HBV infection, usually as the consequence of invasive treatment or diagnostic procedures. The risk of accidental transmission by percutaneous route is estimated to be 30% from highly viremic patients. Transmission from healthcare worker to patients may also occur.²⁵³ Other cases of nosocomial transmission have been reported in hemodialysis centers, and in the setting of organ transplantation, even from donors who only have anti-HBc antibodies. When found alone, anti-HBc antibodies are usually a marker of a past infection from which an individual has recovered. HBV infection of the liver graft recipient, presumably virus reactivation in the donor liver, is observed in more than 50% of cases when the donor has

antibodies to HBcAg but no other serologic markers of HBV infection.¹⁵³ As will be discussed later, this is consistent with other studies indicating that residual amounts of HBV remain for years or decades after clearance of transient infections. Horizontal transmission can be observed among institutionalized persons via close bodily contact, leading to HBV infection through minor skin breaks and mucous membranes.⁷³⁷

In brief, high-risk groups for HBV infection include healthcare workers, especially surgeons and physicians working in hemodialysis, oncology, or AIDS units; laboratory workers in contact with blood or human fluids; institutionalized handicapped persons, their attendants, and family; patients requiring frequent blood product transfusions in countries where blood screening procedures are inadequate; patients on hemodialysis; patients with organ transplantation; intravenous drug users; men who have sex with men; and promiscuous heterosexuals.

The Liver and Its Response to HBV Infection

The main cellular target of HBV is the hepatocyte, which in humans is the only cell type convincingly shown to replicate the virus. However, the belief that other cells replicate the virus in humans has persisted, despite a lack of conclusive evidence.

The liver has a central role in synthesizing plasma proteins, storing and metabolizing glycogen as a source of energy, removing dead and dying cells from the blood stream, and detoxifying

harmful chemicals, among other things. Structurally, the liver is comprised of microscopic lobules into which blood enters from the hepatic artery and portal vein, which are situated in a region known as the portal triad, and exits via the hepatic vein. The lobule itself is not an anatomically defined structure but a region arbitrarily defined by the positioning of the portal tracts and central vein. The structure of a small portion of a lobule is illustrated in a two-dimensional view in Figure 68.13. The parenchymal cell of the lobule, comprising 60% to 70% of liver cell mass, is the hepatocyte. Other cells include bile ductule epithelial cells, sinusoidal endothelial cells, hepatic stellate cells (Ito cells), and Kupffer cells, the resident liver macrophages. In addition to the portal vein and the hepatic artery, the triad also contains lymphatics as well as the bile duct, through which bile, produced by hepatocytes during breakdown of bilirubin, is exported to the gall bladder and small intestine. In contrast to blood, which flows away from the portal tracts to the central vein, lymph⁵⁰⁷ and bile flow towards the tracts. Lymph flows through the space of Disse, between hepatocytes and the overlying endothelial cell layer, and bile is excreted into and flows via channels (canaliculi) formed at the interface of adjacent hepatocytes. Bile enters the bile ductules through a specialized structure known as the Canal of Hering. Destruction of large numbers of hepatocytes during immune clearance of hepatitis virus infections leads in some patients to jaundice (icterus) due

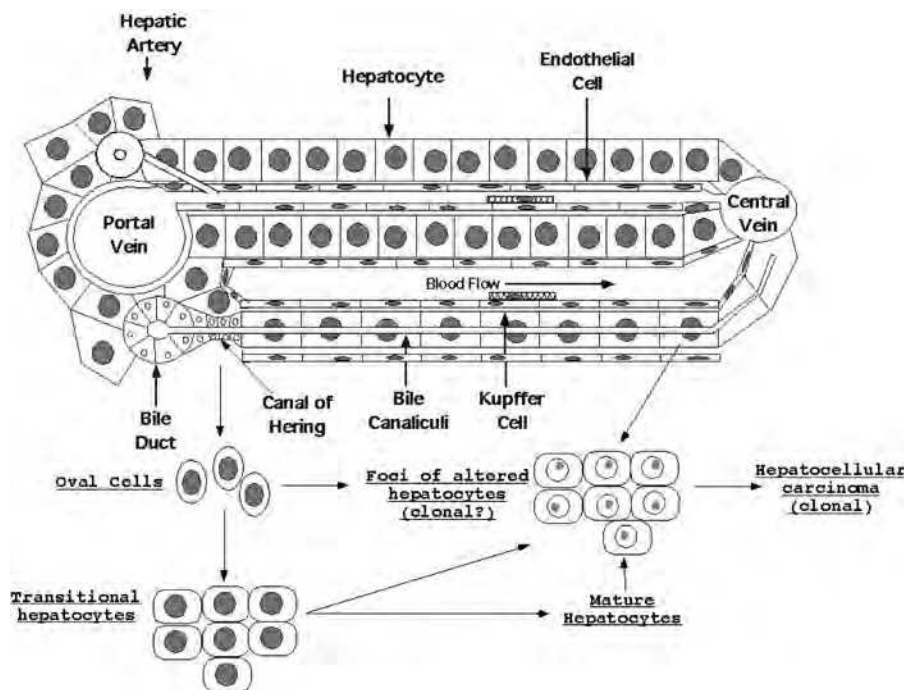


FIGURE 68.13. Structure of the liver lobule. Two-dimensional representation of a small portion of a liver lobule with various cell types present (hepatic stellate cells, localized between sinusoidal endothelial cells and hepatocytes, within the space of Disse, are not shown). Blood enters the lobule from the hepatic artery and portal vein, and flows through the sinusoids bounded by plates of hepatocytes, exiting at the central vein. Hepatocytes produce bile, which is released into bile canaliculi, small channels formed where the apical surfaces of hepatocytes make contact, flows to the canals of Hering and then to bile ductules. From there it flows to larger ducts and exits the liver. The origin of hepatic progenitor cells, which normally only appear during certain conditions of acute and chronic liver injury, is also illustrated. The exact location of progenitor cells in the healthy liver is uncertain, with different lines of evidence pointing to either bile duct epithelium or cells in the canals of Hering. In the actual lobule, many plates of hepatocytes connect the portal triad to the central vein, though only two are shown here.

to a buildup of bilirubin in the blood, producing a yellowing of the skin, eyes, and mucous membranes.

Two liver cell types, hepatocytes and bile duct epithelial cells, differentiate from a common precursor during embryonic development.⁶³⁴ In the duck both cell types are targets of hepadnavirus infection.^{192,249,373,494} One early study⁴⁵ suggested this was also true in humans, though later reports have not yet confirmed this observation. Other liver cell types do not appear to be infected.

Hepatocytes are long lived, with a half-life exceeding 6 months under normal conditions, and a correspondingly low proliferation rate.^{227,342,423,622,739,766} Though hepatocytes are a self-renewing population in the normal liver, under conditions of severe injury or where hepatocyte proliferation is blocked—for instance by a toxic chemical—facultative progenitor cells, considered to be located in the Canal of Hering, may give rise to oval cells that proliferate and ultimately differentiate into hepatocytes.^{143,180,183,640,705,706} Progenitor cells are also found in the bone marrow,⁴⁸⁹ though their quantitative contribution to hepatocyte replacement and relationship to progenitor cells attributed to the Canals of Hering is unclear. Hepatocyte replacement in response to killing of infected hepatocytes by antiviral cytotoxic T cells (CTL) during acute, transient infections appears to be primarily through division of other hepatocytes.^{306,308,670} Replacement from progenitor cells, with the appearance of oval cells in the lobule, is more evident during late stages of chronic infections, by which time the liver may be highly damaged,^{203,272,589} but hepatocyte proliferation also occurs, and the relative contribution of the two pathways to hepatocyte replacement during late phases of chronic infections has not been determined.

When the liver is injured through killing of hepatocytes, hepatic stellate cells, located in the space of Disse, will respond by producing collagen fibers.^{448,545} During chronic infections the persistent injury due to CTL killing of hepatocytes leads to persistent deposition of collagen, building up fibrous tissue that can evolve to cirrhosis, a condition that distorts the lobular structure, disrupts normal blood flow through the liver, and can lead to death due to liver failure. The progression to cirrhosis may be interrupted, and even reversed, if the infection is controlled by antiviral therapies.^{396,524,581,588,788}

A number of studies suggest that the liver can regulate or at least protect itself against the host immune response. First, in some species including rats, pigs and mice, liver transplantation between allogeneic animals induces tolerance to grafts of other tissues from the same donor that would normally be rejected, suggesting that the liver has immunoregulatory properties, possibly attributable to hepatic dendritic cells.^{702,704} Second, a number of different cell types in the liver appear to have the ability to present antigen in a suboptimal context, in some cases leading to immune tolerance or a weak immune response.^{139,703} Third, the immune response to a number of human viruses that appear to productively infect only hepatocytes—including HAV, HBV, HCV, and hepatitis E virus (HEV) in humans—only becomes robust enough to induce high levels of cell death and virus clearance 4 to 8 weeks after infection, during which time the entire hepatocyte population may become infected. A similar pattern has been seen following DHBV infection of ducks and WHV infection of woodchucks. These observations suggest that scanning of hepatocytes by the immune system is low, and has been attributed to low expression of major

histocompatibility class I (MHC I) genes and poor access of circulating lymphocytes to hepatocytes, despite the occurrence of fenestrations in the liver endothelial cells. The possibility that liver cells other than hepatocytes may induce at least partial tolerance to viral antigens could also contribute to the prolonged course of transient and chronic infections.

Other Sites of Hepadnavirus Infection

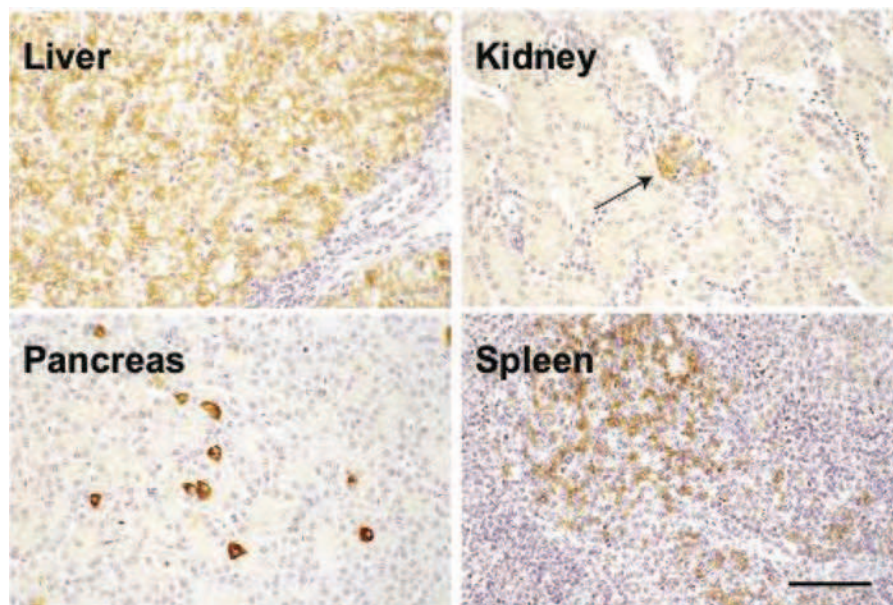
The best evidence for replication in cells other than hepatocytes comes from studies of ducks infected with DHBV. Replication in the extrahepatic sites has been observed during chronic DHBV infections established *in ovo*^{163,251} or following inoculation of young ducklings.^{192,201,302,304,305,441} CCC DNA and typical DHBV DNA replication intermediates, with abundant single-stranded DNA (ssDNA), are found not only in liver, but also in pancreas and kidney of chronically infected ducks,^{201,249,271,304,683} as well as in the yolk sac during embryonic development.⁶⁸⁴ Viral DNA also accumulates in the spleen, due to passive accumulation of virus by follicular dendritic cells³⁰⁴; evidence for DNA replication intermediates⁶⁸³ and CCC DNA²³⁵ in the spleen has also been reported, though DNA replication intermediates were not observed in the latter study. The site of DNA replication in the kidney appears to be the proximal tubular epithelium, though infection of glomeruli is also suggested (Fig. 68.14).^{201,249,304} Viral DNA replication in the pancreas appears restricted to a small subset (~1%) of exocrine cells but a majority of endocrine cells,^{142,176,201,249,248,304} which in ducks are localized to alpha and beta islets. Infection of bile duct epithelial cells of the liver also occurs^{249,494} and studies with primary cell cultures suggest that these are sites of DHBV reproduction *in vivo*.³⁷³

Extrahepatic infection has also been studied in chronically infected woodchucks. Gel electrophoresis and Southern blot analysis demonstrated typical viral DNA replication intermediates in the liver and, at an approximately 10-fold lower level, in the spleen. Though typical replicative intermediates were not demonstrated at other sites, ~1,000-fold lower amounts of RNA and total episomal viral DNA than in the liver have been reported in kidney, pancreas, thymus, bone marrow, testes, and ovary,^{336,337,506} suggesting possible infection; in addition, some cells in these latter tissues appeared to contain viral nucleic acids by *in situ* hybridization. It remains unclear, however, if these observations of low levels of viral nucleic acids, outside the woodchuck liver, reflect actual infection or passive accumulation. The most convincing evidence of extrahepatic infection in the woodchuck was obtained from studies with PBLs of WHV-infected woodchucks. Although PBLs did not replicate WHV *in vivo*, they produced typical viral DNA replication intermediates and released virus when stimulated with lipopolysaccharide *in vitro*, and thus appear to be latently infected *in vivo*.^{333,335}

Early immunohistochemical studies of human tissues other than the liver suggested that, as in the duck, exocrine and endocrine cells of the pancreas may be infected.^{632,789} Evidence for DNA replication intermediates in the spleen was also described for humans¹⁵⁸ and chimpanzees,³⁹⁷ though we are unaware of any recent follow-up studies. Evidence for infection,^{91,267,364,398,527,528,550,590,627} gene transcription, and viral replication in peripheral blood mononuclear cells has been reported.^{19,59,242,497} Data in support of infection in bone marrow, as evidenced by the presence of HBsAg and 3 kbp RC DNA, but not DNA replication intermediates, has also

FIGURE 68.14. Sites of virus replication in the DHBV-infected duck.

Tissue sections of liver, kidney, pancreas (exocrine) and spleen, immunoperoxidase stained using an antibody to the DHBV surface antigen (*brown stain*), and counterstained with hematoxylin. Virtually all hepatocytes are infected in the liver. Glomerular infection is illustrated in the kidney (*arrow*). Infection occurs in a few percent of exocrine cells in the pancreas. The reason that some but not all exocrine cells in the pancreas are targets of infection is unknown. Accumulation of viral surface antigen is found in follicular dendritic cells in the spleen but is not believed to represent actual infection. Bar = 50 μ M. (Courtesy of Dr. Allison Jilbert, Institute of Medical and Veterinary Sciences, Adelaide, Australia.)



been presented.^{549,591} The idea that any of these observations reflect actual infection of blood cells, however, has been challenged by a study that failed to detect CCC DNA in these cells. This suggests instead that viral DNA and antigen signals—and, by inference, virus replication reported in these studies—arose from observations of passively adsorbed virus.³³¹ This interpretation has itself been challenged⁶⁶³; the issue remains unresolved.

As independent support for the idea that some human PBMCs are infected, it has been proposed that latently or actively infected blood cells are the source of HBV in post-transfusion hepatitis cases attributed to blood donors with a history of transient HBV infection.⁴⁶¹ However, small amounts of circulating virus originating in other donor tissues, including the liver, have not been ruled out as a cause.^{369,574,790,791} Thus, at present it seems clear that virus, or at least viral DNA, can persist in a variety of tissues of a recovered individual even though the liver is no longer overtly infected. This was shown convincingly in studies in which HBV carriers in liver failure were xenotransplanted with baboon liver, which is resistant to HBV infection.³⁶⁰ Virus DNA persisted in extrahepatic sites for the 2-month follow-up. Whether this persistence reflects active virus replication remains unclear. The question of whether PBMCs or any other site outside the liver of HBV carriers are actually infected, or simply accumulate HBV that was produced in the liver, remains elusive.

Immune Response to Hepadnavirus Infections

HBV infection, replication, and virus shedding are not cytopathic to hepatocytes. This may be another reason the immune response to infected cells is slow to evolve, often not appearing until weeks after essentially every hepatocyte is infected,^{306,308,701,760} which may take weeks to months depending on the infecting viral dose. The result of this massive infection is that a strong immune reaction, with killing of large numbers of hepatocytes, is typically seen during the clearance phase of an infection.⁶⁷⁰ About 95% of immunocompetent adults are able to mount such a response, resolving the infection within

a few weeks once clearance begins. Unfortunately, about 5% of adults and most children under the age of one year are unable to resolve the infection (Figs. 68.15 and 68.16). Instead, chronic hepatitis B with ongoing liver injury may occur, mediated by a T-cell immune response targeting hepatocytes that express viral antigens.^{41,117,208} Chronic infections, with persistence of readily detectable levels of serum virus and surface antigen particles, are generally life long. In chronically infected patients, a certain degree of T-cell function defect is reported, which in turn may be responsible for viral persistence. It is suggested to result from T-cell exhaustion by high antigen concentrations, which promotes HBV-specific T-cell dysfunction by affecting phenotype and function of peripheral and intrahepatic T cells.^{150,188}

Nonetheless, virus titers in the blood often decline during the course of a chronic infection, from as high as 10^{10} per ml to as little as a few hundred per ml. The cause of this decline has not been determined. One possibility is that antiviral cytokines produced during chronic hepatitis suppress virus replication,⁴⁶⁰ as also seems to occur during the clearance phase of transient infections.^{308,670,701,760,762} Another possibility, not exclusive of the first, is that killing of infected cells by antiviral CTLs selects for the survival of hepatocytes that have lost the ability to support high levels of virus replication.⁴⁴²

Results of placebo-controlled trials of antiviral agents suggest that approximately 5% of patients each year experience a spontaneous virus clearance and enter the remission/inactive hepatitis phase.^{162,349,433,787} However, unlike clearance of a transient infection, the serum of a chronic HBV carrier, undergoing spontaneous remission, generally remains HBsAg positive, most likely because many hepatocytes contain integrated viral DNA with an intact S coding region.

Immune Response in Transient Infections

The mechanisms of clearance of transient infections have been investigated in detail in clinical studies as well as through the use of experimental models. Viral clearance appears to require the coordinated action of several components of the immune response^{229,446,670,762,781}. Possibly the innate immune response

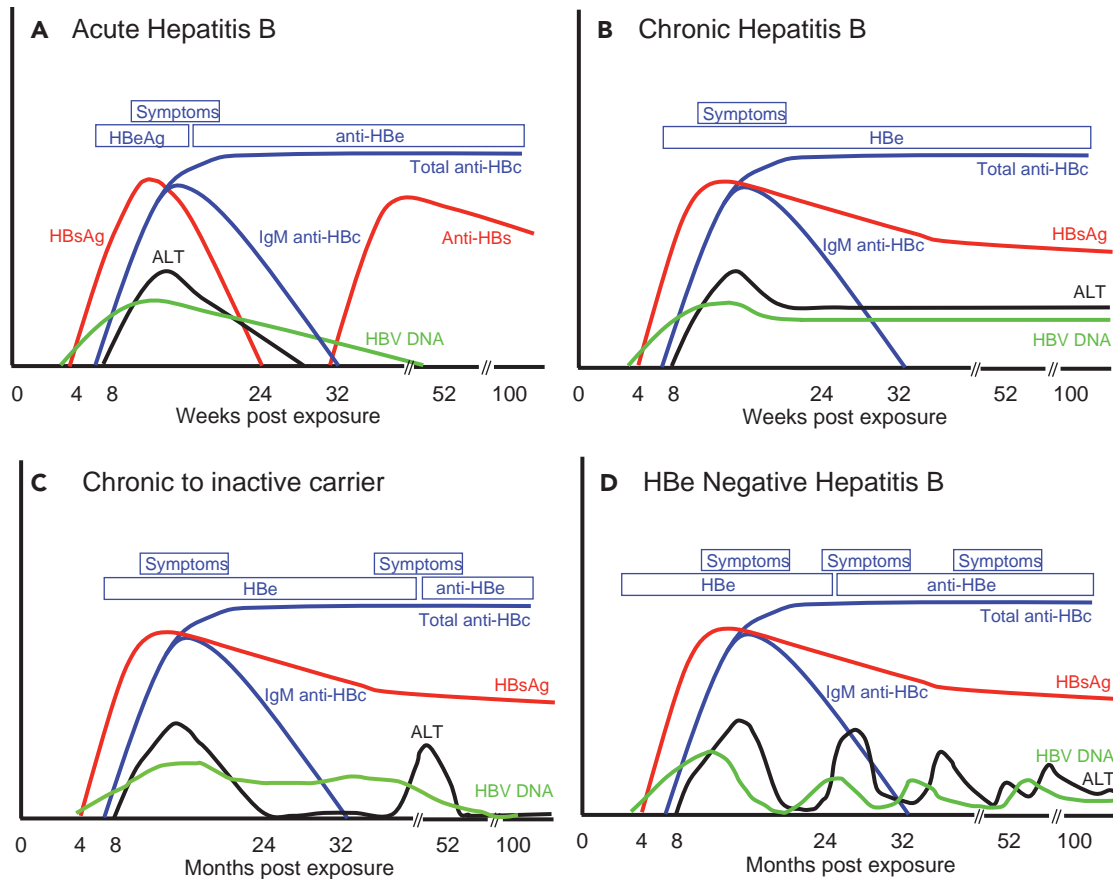


FIGURE 68.15. Serological profiles of HBV infections. **A:** Laboratory diagnosis of acute hepatitis B. Acute hepatitis is diagnosed in patients with jaundice or fatigue and high ALT levels. Evidence of acute HBV infection is obtained by the detection of HBsAg and anti-HBc IgM antibody. Viral replication is authenticated by the detection of serum HBeAg and HBV DNA. Resolution of infection is accompanied by the decline in viral load, seroconversion to anti-HBe, and normalization of ALT levels. Subsequently, HBs seroconversion is observed and represents a serologic marker of the cure of the infection. **B:** Laboratory diagnosis of chronic hepatitis B associated with wild-type virus infection. Chronic HBV infection is diagnosed by the persistence of serum HBsAg and markers of viral replication (i.e., serum HBeAg and HBV DNA). Depending on the phase of disease, ALT levels may be normal or elevated. **C:** Laboratory diagnosis of transition of chronic hepatitis B to the inactive carrier state. HBe seroconversion is associated with the loss of serum HBeAg, the appearance of anti-HBeAb, the decline of serum HBV DNA levels, and the normalization of ALT levels. A flare in ALT levels sometimes precedes it, suggesting that HBe seroconversion may be associated with an immune-mediated hepatocyte lysis. HBe seroconversion is associated with an improved clinical outcome and the patients are classified inactive carriers. **D:** Laboratory diagnosis of HBeAg-negative chronic hepatitis. In some patients, pre-C mutants are selected at the time of HBe seroconversion, and the disease continues to progress with a typical fluctuation of serum HBV DNA and ALT levels. This stage of chronic hepatitis B, which is frequent in the Mediterranean basin, is usually referred to as HBeAg-negative chronic hepatitis B.

mediated by NK and NKT cells, though a study of experimental HBV infection of chimpanzees suggests that HBV infection, *per se*, may not induce an innate response in the liver⁷⁶⁰ (however, see 169, 187); a cytotoxic response by CD8 positive cells recognizing infected hepatocytes expressing viral antigens; a noncytolytic response whereby the CD8 positive cells and other, nonantigen-specific, cells recruited to the sites of inflammation⁶⁴¹ produce TH1 cytokines such as IFN- γ , tumor necrosis factor alpha (TNF- α), and interleukin 12 (IL-12) that exhibit a direct antiviral effect, suppressing virus reproduction in the infected liver; the production of neutralizing antibodies to prevent infection of recovered, or newly differentiated, hepatocytes, by residual virions; cell lysis and apoptosis and compensatory proliferation to generate new uninfected cells that will repopulate the liver.

While there is a consensus on the occurrence and presumed importance of different effectors, their precise role and the order of events is still debated. For instance, antibodies to the viral envelope (anti-HBs) are often not detectable until after virus clearance from the liver, so their requirement during the resolution phase of transient infections, if any, has remained obscure.^{186,308,553} The possibility that antibodies are responsible for disappearance of virus from the serum as clearance from the liver begins has not been directly ruled out, as even low levels of antibody might be enough to bind all circulating virus and facilitate removal from the serum. However, a more attractive hypothesis is that IFN- γ and/or other cytokines released by CTLs, CD4+ T cells, and macrophages are sufficient to rapidly shutdown viral DNA synthesis and, consequently, virus

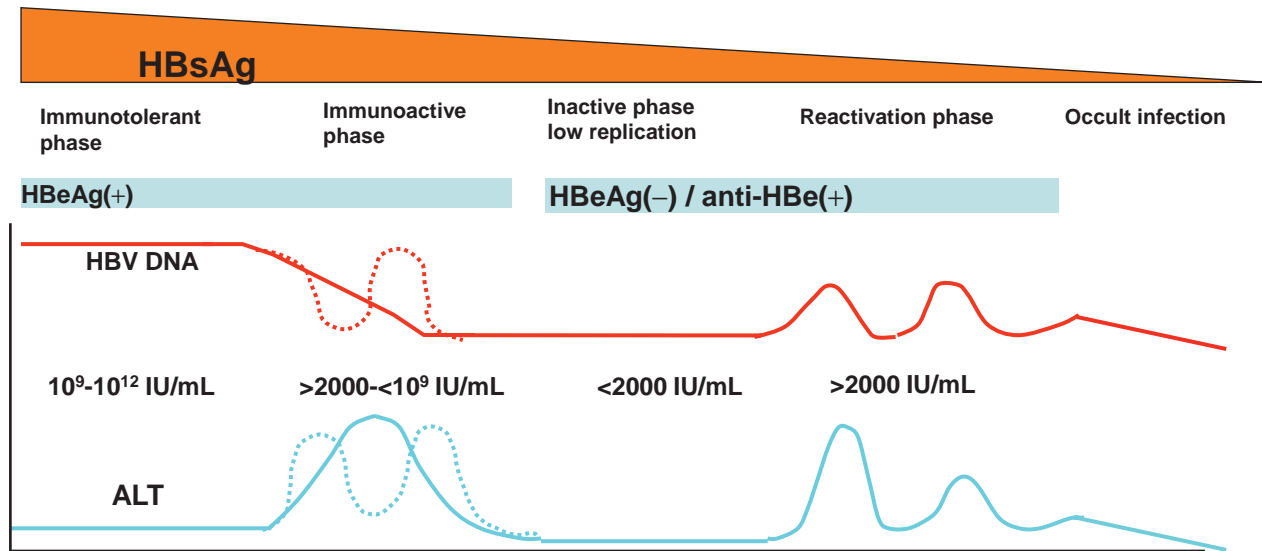


FIGURE 68.16. Natural history of hepatitis B. Following acute infection, approximately 95% of adults and 5% to 10% of newborns recover, while the remainder evolve towards chronic viral infection. Depending on the vigor of the immune response, chronic infection can follow several phases including immune tolerance, chronic hepatitis (immune clearance phase), the inactive carrier state, and a reactivation phase. These phases may not be stable over time, and patients may evolve from one phase to the other. The major risk of chronic hepatitis is the evolution towards cirrhosis and hepatocellular carcinoma (Fig. 68.17).

production by blocking assembly of DNA replication complexes (viral capsids).^{238,521,526,582,613,614,761}

In HBV transgenic mice that produce virus in hepatocytes, a major role for cytokine mediated, noncytolytic processes in the clearance of viral replication intermediates from the cytoplasm—including DNA replication intermediates, mRNAs, and proteins—has been demonstrated.²²⁹ However, the interpretation of these findings is complicated by the fact that clearance occurs within a few days of induction of an antiviral response (e.g., by adaptive transfer of HBsAg-specific CTLs) and without the need for large amounts of cell death, whereas clearance in infected hosts is relatively slow and involves significant amounts of hepatocyte destruction.^{239,446,670,762} Moreover, the mice used in these studies did not produce CCC DNA, the nuclear species that maintains the infection.

Results of a subsequent study with transgenic mice that do produce CCC DNA suggested that this DNA is sensitive to noncytolytic purging by antiviral cytokines,¹³ though the contribution of hepatocyte death in this transgenic line is unclear.⁵⁵² Studies of acute infection of chimpanzees have also been interpreted to argue the importance of noncytolytic clearance of HBV DNA replication intermediates and CCC DNA but, again, interpretation of the CCC DNA decline is unclear because of the difficulty of quantifying hepatocyte destruction throughout the course of viral clearance.^{233,701,762}

One study, using the woodchuck model of HBV, showed that a substantial fraction of hepatocytes in the recovered liver originated from hepatocytes that were infected, indicating a mechanism for curing these infected cells.⁶⁷⁰ This and another study⁴⁴⁶ also showed that a substantial amount of hepatocyte turnover occurred during resolution of a transient infection. However, it still remains unclear if this hepatocyte turnover, together with proliferation of the remaining hepatocytes to restore liver mass, were sufficient to completely eliminate CCC DNA, even assuming it does not survive mitosis. In brief, a role

for cytokines in the shutdown of virus replication appears settled, but their direct role, if any, in destruction of CCC DNA, is undetermined.

Loss of CCC DNA is not induced in cell culture by cytokines (IFN- α , IFN- γ , TNF- α) that are able to induce a block to assembly of DNA replication intermediates.^{613,614,808} Moreover, CCC DNA appears to be stable, with no evidence for a short half-life in the infected liver. Thus, several competing scenarios for CCC DNA loss have emerged. One theory is that CCC DNA degradation within infected cells occurs during the clearance phase of an infection by a process that has not yet been reproduced in cell cultures. Another theory is that CCC DNA is only lost through cell death. Assuming dying hepatocytes are replaced by division of surviving hepatocytes, and new CCC DNA synthesis is blocked, CCC DNA would be distributed to daughter cells during mitosis and diluted out through multiple rounds of division of those hepatocytes that escaped the antiviral CTLs.⁴⁴⁶ A third theory, related to the second, –is that CCC DNA is lost during mitosis. A fourth is that during resolution of infection the dying hepatocytes are replaced, not by division of mature hepatocytes, but through proliferation of uninfected progenitor cells (Fig. 68.13). However, the last possibility appears to be ruled out by observations that infections can resolve without extensive proliferation of hepatocyte progenitor cells and that a substantial fraction of hepatocytes at recovery are derived from hepatocytes that had been infected.⁶⁷⁰ Current evidence does not allow a clear distinction of the other possibilities. The concept of noncytotoxic loss of CCC DNA is attractive, but unproven.

Clinical Manifestations of Chronic HBV Infections and Decisions to Treat

The natural history of chronic hepatitis B (CHB) can be schematically divided into five phases, which are not necessarily sequential (Fig. 68.16).^{178,412} The “immune tolerant” phase is characterized by HBeAg positivity, reflected by high titers of

serum HBV DNA (>2,000 IU/mL, 1IU=5 viruses), normal levels of aminotransferases, and mild or no liver necroinflammation. During this phase, the rate of spontaneous HBeAg loss is very low. This first phase is more frequent and more prolonged in subjects infected perinatally or in the first years of life. Because of high levels of viremia, these patients are highly contagious. The “immune reactive phase” is characterized by HBeAg positivity, lower titers of serum HBV DNA (but >2,000 IU/mL), increased or fluctuating levels of aminotransferases, moderate or severe liver necroinflammation, and more rapid progression of fibrosis compared to the previous phase. This phase may last from several weeks to several years. In addition, the rate of spontaneous HBeAg loss is enhanced, but may occur at a late stage when fibrosis has already developed. This phase may occur after many years of immune tolerance and is more frequently reached in subjects infected during adulthood. 3) The “inactive HBV carrier state” may follow seroconversion from HBeAg to anti-HBe antibody. It is characterized by very low or undetectable serum HBV DNA levels and normal aminotransferases. As a result of immunologic control of the infection, this state confers a more favorable long-term outcome with a lower risk of cirrhosis or HCC. HBsAg loss and seroconversion to anti-HBs antibody may occur spontaneously, usually after several years with persistently undetectable HBV DNA. “HBeAg-negative CHB” may also follow seroconversion from HBeAg to anti-HBe antibody during the immune reactive phase. It is characterized by periodic reactivation with a pattern of fluctuating levels of HBV DNA and aminotransferases and active hepatitis. As discussed in the section Serology of HBV infections, these patients are HBeAg negative, and circulate HBV unable to express or expressing low levels of HBeAg. HBeAg-negative CHB is associated with low rates of prolonged spontaneous disease remission. It is important and sometimes difficult to distinguish true inactive HBV carriers from patients with active HBeAg-negative CHB who, at the time of examination, are in a phase of spontaneous remission. In the “HBsAg-negative phase” after HBsAg loss, low-level HBV replication may persist with detectable HBV DNA in the liver. Generally, HBV DNA is not detectable in the serum and anti-HBc antibodies with or without anti-HBs are detectable. HBsAg loss is associated with improvement of the outcome with reduced risk of cirrhosis, hepatic decompensation, and HCC.

Current clinical guidelines recommend against antiviral treatment during the immune tolerance phase because patients have no, or very minimal, liver disease (inflammation and/or fibrosis) that can be detected by histologic examination of liver biopsies.^{178,412} That is, treatment is costly and the risk of disease progression is considered minimal so long as ALT levels remain within the normal range. Also, the results of therapeutic trials with IFN- α , or with early nucleoside analog inhibitors of the viral DNA polymerase, showed that patients with high HBV DNA load and normal ALT levels have almost no chance of HBe seroconversion or sustained virologic response as a result of treatment.^{178,412} Nonetheless, it remains unclear if subsequent disease progression, particularly to HCC, is dependent on unapparent injury that occurred in the immune tolerance phase. It is possible that treatment guidelines will be revised, as more effective antiviral drugs have become available.

With the current generation of antiviral drugs and treatment guidelines, antiviral therapy is likely to lead to HBe seroconversion and long-term viral suppression/control by the immune system only in patients in the immune clearance

phase (e.g., ~30% vs. a spontaneous rate of ~5%).^{125,787} This on-therapy transition is usually associated with HBeAg seroconversion, in which HBeAg antigen disappears, anti-HBe antibodies appear, and only low levels of viral DNA (<2,000 IU per ml) persist in the serum. HBsAg usually remains at high levels in the serum. Continued production of HBsAg is thought to be mostly due to expression from viral DNAs that have integrated at random sites in host DNA or from persisting low levels of CCC DNA.⁷⁵⁹ Liver histology usually reveals remission of liver disease activity, and arrest of fibrosis/cirrhosis progression as long as viral load remains low and ALT levels remain normal.^{102,161} Even without this immune clearance during antiviral therapy, suppression of virus replication by antiviral therapy may slow or prevent the cumulative liver damage that can otherwise occur during the immune clearance phase of infection, whether or not overt symptoms are present.^{100,178,297,350,351,365,412,434} (Fig. 68.16).

A more favorable outcome of chronic infection is associated with clearance of HBsAg and the appearance of anti-HBsAb. Virologically, this is more like clearance of transient infections, since anti-HBsAb will help prevent virus rebound. As with clearance of transient infections, HBV DNA may still be detected in serum, though generally at very low titers (<10 IU per ml) and in the liver by the presence of low levels of CCC DNA.^{452,759} Also, as with transient infections, such infections may reactivate with immunosuppression. Whether, in the absence of reactivation, residual virus replication contributes to disease progression seems unlikely. Still, while fibrosis and cirrhosis do not appear to progress, these patients remain at significant risk of developing HCC.⁵⁷¹

Emergence of HBV Variants During a Chronic Infection

HBV is under selective pressure from the immune system and HBV variants may emerge over the course of a chronic infection.²³⁴ These variants are generally assumed to facilitate immune evasion in some way and, as might be expected, have been detected throughout the viral genome. Because both B- and T-cell epitopes may be mutated, it is unclear if emergence of these variants occurs through virus spread or selective survival of hepatocytes infected by the mutants, or both. In the setting of the natural history of the disease, the most studied of these mutants are those that no longer express HBeAg. These variants harbor mutations in the pre-core region and/or in the basic core promoter. When this happens, infections are characterized by presence of HBsAg and absence of HBeAg, and HBV DNA levels that are fluctuating, but usually are >2,000 IU per ml. ALT levels may also be elevated and/or fluctuating over time.^{53,245} (Fig. 68.15D). Like HBeAg-positive infections, this form of chronic hepatitis B is associated with a high risk of liver fibrosis, cirrhosis, and HCC.^{53,222,245} Unlike HBeAg-positive infections, treatment endpoints are more difficult to define, since HBeAg seroconversion (see above) cannot be used as a surrogate marker for immune control of virus replication; therefore sustained, therapeutic suppression of HBV replication is the primary objective in this situation.

CLINICAL ASSESSMENT OF HBV INFECTIONS

Laboratory Diagnosis of Infection

Persistence of HBsAg in the blood circulation for more than 6 months is an indication of chronic infection. Loss of HBsAg,

on the other hand, is an indication of almost complete elimination of virus. Antibodies directed against HBsAg are used as a marker of protection against HBV. HBeAg is a serologic marker of active viral replication. Loss of circulating HBeAg and appearance of anti-HBe antibodies (HBeAg seroconversion) may indicate the end of active viral replication and the beginning of clinical resolution of both transient and chronic infections. HBcAg, representing the capsid protein, is highly immunogenic and the appearance in the circulation of anti-HBc IgM is usually the first immunologic sign of a transient HBV infection. Typical serologic profiles of chronic HBV infections are shown in Figures 68.15 and 68.16.

The most rigorous way of determining the presence of circulating virus is to assay for viral DNA. The lower threshold for risk of active hepatitis and liver damage is considered to be around 10^4 viral genome equivalent per ml, equivalent to $\sim 2,000$ IU/mL.^{82,178,125,403} Figure 68.16 shows the evolution of viral load during the natural history of infection and underlines the necessity of highly sensitive and quantitative assays to monitor viral load in the different phases of the disease. In patients who have lost HBsAg from serum and may have an occult infection, virus titers are usually very low, and thus can escape detection even by PCR-based assays.⁵⁷¹

Serology of HBV Infections

The development of quantitative assays for HBsAg detection revealed that chronically infected patients, as well as those at the peak of a transient infection, can have HBsAg titers of $>10^{12}$ particles per ml, or >500 μ g of protein per ml.⁸¹² Clinical assays can detect as little as 0.2 ng (or 0.05 IU/mL) of HBsAg per ml of serum, providing a highly sensitive assay for HBV infection. HBsAg levels in serum were found to correlate with HBV DNA levels during transient infections, but this correlation breaks down during the course of a chronic infection.^{299,492}

Mutations in the S gene, particularly a region known as the “a” determinant (Fig. 68.9), can lead to loss of recognition by the antibodies used in commercial assays.⁸⁵ Major improvement of these commercial assays has been made recently to detect the main S gene mutants. These HBV mutations can emerge primarily in response to therapy, for instance during passive antibody therapy with high titer immunoglobulins, and after HBsAg vaccination of young, HBV-exposed children. The existence of these rare mutant viruses suggests that HBsAg-negative patients with hepatitis of unknown etiology should be tested with a secondary assay, e.g., viral genome detection. Prospective blood donors should also be tested with secondary assays when HBsAg assays yield negative results.

A number of secondary assays are available. IgG reactive to HBcAg is a marker of past or ongoing infection and is therefore found in both resolved infections and in chronically infected individuals. Anti-HBc IgM is typically found during transient infections and may be detected, at low levels, during acute exacerbation of chronic infections^{243,537,812} (Fig. 68.16). Alternatively, HBeAg is generally found in the blood of transiently infected individuals, as well as many chronically infected individuals when they are in the high viral replication phase, particularly the immune tolerant and early immune clearance phases.

Seroconversion to anti-HBe antibody generally occurs during resolution of transient infections.^{32,478,718} As noted, loss

of HBeAg often occurs as well during the course of chronic infections, sometimes but not always with the concurrent appearance of anti-HBe antibodies. Loss of HBeAg from serum, even in the absence of anti-HBe antibodies, usually reflects a decline in the amount of virus in the liver. Thus, the loss of HBeAg in chronically infected individuals, either with or without the concomitant appearance of anti-HBeAg, may signal a major drop in virus titers and a reduction in disease activity as patients enter a remission phase. However, in other patients it may be associated with an ongoing disease activity because it simply reflects immune selection of virus that has lost the capacity to produce HBeAg. This loss, as already noted, is typically due either to stop codon mutations near the 5' end of the pre-C gene or to mutations in the basic pre-C/C promoter (Fig. 68.7).²⁴⁵ Thus, HBe seroconversion is often, though not always, an indication that virus production has either ceased or significantly abated.

Finally, sensitive real-time PCR assays for quantification of viral DNA in serum are available with a cut off as low as approximately 30 to 50 viral genome copies per mL, or ~ 10 IU/mL.^{115,700} The combination of serologic markers, including quantification of HBsAg and HBV DNA in serum, has improved the diagnosis of inactive carriers, which usually have a low viral load (HBsAg $<1,000$ IU/mL and HBV DNA $<2,000$ IU/mL).⁶⁷ In patients who have lost HBsAg in serum after resolution of a transient infection or during the course of chronic HBV infection [i.e., who are HBsAg (–) and anti-HBc Ab (+), either with or without anti-HBs] detection of serum HBV DNA by sensitive PCR assays may reflect the persistence of infected hepatocytes after the clinical resolution of the disease⁵⁷¹ (Fig. 68.16).

HBV Genotype and Infection Outcomes

As discussed, HBV has been divided into eight genotypes, A through H⁴⁹⁸ (Fig. 68.3). Genotype A is found in Western and Northern Europe, as well as in Africa. Some studies suggest that it may be associated with a better response rate to IFN therapy for chronic hepatitis.⁷⁹ Genotypes B and C are found in Asia. Clinical studies have suggested an association of genotype C with a more severe chronic liver disease and a poorer response rate to IFN therapy.^{313,796} Genotype D is observed mainly in the Mediterranean basin and is associated with a higher prevalence of pre-C mutants in chronically infected individuals (i.e., the patients are HBeAg negative)²²² as well as a lower response rate to IFN.⁷⁹ Genotype E is mainly observed in Africa, while genotype F has been identified in South America and Polynesia. Genotype G has been identified in chronic carriers living in France and the United States, and is often found as a co-infection with genotype A.⁶⁶⁵ Finally, genotype H has been described in Amerindians from Central America.

Because of RNA folding constraints placed on the epsilon region of pgRNA, pre-C stop codon mutations that abolish pre-C protein synthesis and, hence, HBeAg production, would also abolish virus replication in some HBV genotypes. This restricts their occurrence to compatible HBV genotypes or by the requirement of compensatory mutations. In practice, these mutations are mainly observed in patients chronically infected with HBV genotype D.³⁸⁹ Patients infected with other HBV genotypes may also present with HBeAg-negative chronic hepatitis B, in these cases mainly caused by mutations in the pre-C/C promoter.⁵⁰⁸ Mutations in the pre-C/C promoter are not HBV genotype restricted.^{222,402,525}

Occult HBV Infections

A form of hepatitis that has generated a lot of debate is so-called occult HBV infection.^{61,81,103,571} Occult infection is characterized by the detection of HBV DNA by PCR in the serum and/or the liver of patients in whom serum HBsAg assays give negative results. Occult HBV infection can be observed in up to 30% of patients with liver disease of unknown etiology. It was shown that the HBV genome of these occult infections is replication competent in tissue culture and that viral suppression observed *in vivo* may be due to epigenetic control of intrahepatic CCC DNA.⁵⁴⁷ It is unclear whether in some or perhaps all such patients the occult infection is actually a serologically cured infection (e.g., 369,463,533,790,791), or whether it is actually the cause of the ongoing liver disease. Occult HBV infections have been reported in a significant proportion of HBsAg-negative patients with HCC.^{61,529,548} However, even here it is possible that a chronic infection had spontaneously resolved after the progression to liver cancer was already fixed, but before appearance of the neoplasm. Thus, it is unclear if occult infections represent a distinct class of infection or fit into the categories discussed earlier. Additional confusion may arise because some infections characterized as occult simply escaped detection due to mutations in the S gene that prevented detection by the serologic assay for HBsAg that was employed. In these patients, in contrast to those so-called occult infections in which a more broadly reactive HBsAg assay failed to detect the infection, HBV DNA titers may be very high.⁵⁷¹

Diagnosis of Acute Hepatitis B

Acute hepatitis, which may occur during resolution of a transient infection or during a hepatic flare in chronic infection, is diagnosed in patients with jaundice or fatigue and high ALT levels. Evidence of HBV involvement is obtained by the detection of HBsAg and anti-HBc IgM. However, other factors need to be considered. For instance, co-infection with hepatitis delta virus (HDV) is evaluated at this stage by assaying for serum HDsAg, anti-HDV IgM, and HDV RNA. Other causes of acute viral hepatitis should be tested including acute hepatitis C virus (HCV) infections, acute HAV, acute HEV, and other rare cases of acute HSV or CMV infections. If all factors suggest involvement of HBV, transient infection is indicated by disappearance of HBsAg in the weeks immediately following the flare of acute liver disease (Fig. 68.15A). It should be

noted, again, that an acute flare on chronic infection can be preceded by months of high-titer viremia but little or no liver disease.

Diagnosis of Chronic Hepatitis B

Chronic HBV infections are usually asymptomatic for most of their course, although patients may complain of fatigue or arthralgias. The diagnosis of chronic hepatitis B is usually made by the detection of serum HBsAg and elevation in ALT levels (Fig. 68.15B).^{178,412} Nausea, loss of appetite, and jaundice may occur during acute hepatic flares (e.g., during the immune clearance phase). However, a hepatic flare may be completely asymptomatic and diagnosed only by a close monitoring of HBV DNA and ALT levels. It is also important to note that an acute rise in ALT levels and other symptoms of acute hepatitis may not always be due to HBV infection. It is necessary to rule out superinfection by HAV/HEV, HCV, or HDV, especially in patients with a high risk for new infection, such as intravenous drug users or patients requiring transfusions. Other causes of chronic liver diseases should be excluded.

PREVENTION AND CONTROL

Antiviral Therapy

Seven therapies have received approval for the treatment of CHB, including interferon alpha (IFN- α), pegylated IFN- α , and several nucleoside(tide) analogs (NUCs), which are viral polymerase inhibitors. They belong to one of three structural groups: L-nucleosides (lamivudine and telbivudine), alkyl phosphonates (adefovir dipivoxil and tenofovir disoproxil fumarate) and D-cyclopentanes (entecavir).

As discussed earlier, antiviral therapy is currently recommended only for patients with active liver disease who have a significant risk of liver disease progression in the short term, and not for patients that do not have elevated ALTs and/or histologic evidence of inflammatory liver disease.^{178,412} To a certain extent this has been mediated by the limited efficacy of antivirals such as lamivudine, to which resistance rapidly develops (Table 68.1). The higher barrier to entecavir and tenofovir resistance may lead to a change in treatment guidelines to include patients who still appear histologically normal or with minimal liver damage. Irrespective of the possible benefits of

TABLE 68.1 Incidence of Drug Resistance During Antiviral Therapy

Drug and patient population	Resistance at year of therapy expressed as percentage of patients					
	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6
Lamivudine	23	46	55	71	80	—
Telbivudine, HBeAg-Pos	4.4	21	—	—	—	—
Telbivudine, HBeAg-Neg	2.7	8.6	—	—	—	—
Adefovir, HBeAg-Neg	0	3	6	18	29	—
Adefovir (LAM-resistant)	Up to 20	—	—	—	—	—
Tenofovir	0	0	0	0	—	—
Entecavir (naïve)	0.2	0.5	1.2	1.2	1.2	1.2
Entecavir (LAM resistant)	6	15	36	46	51	57

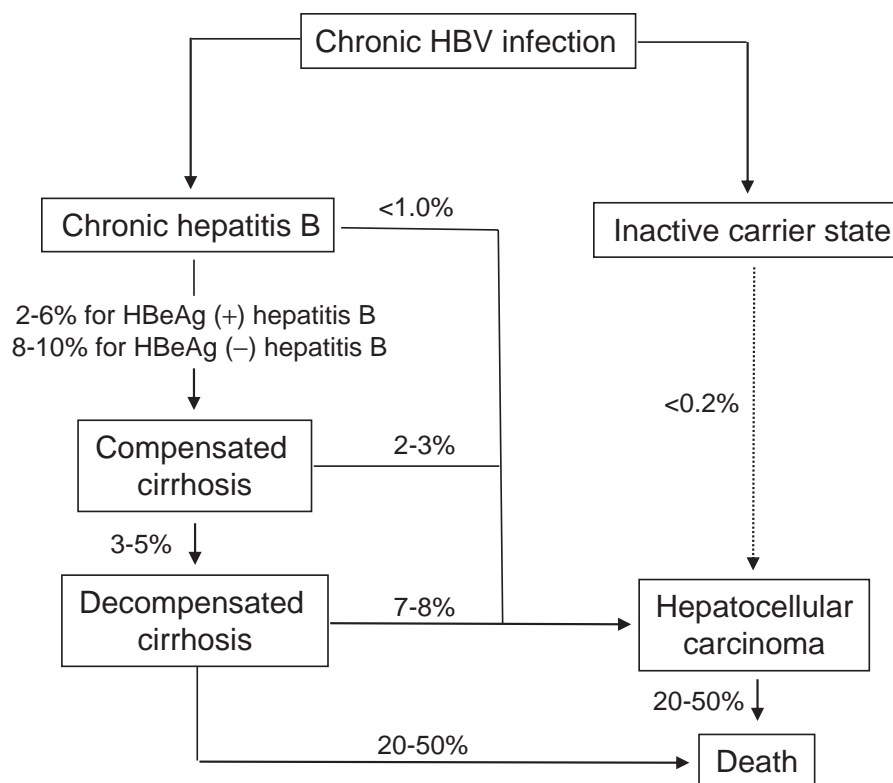


FIGURE 68.17. Pathology of chronic HBV infection. Progression of chronic HBV infection through different clinical states. Estimated annual rates (%) are indicated. (From Ou JH, Rutter WJ. Regulation of secretion of the hepatitis B virus major surface antigen by the preS-1 protein. *J Virol* 1987;61:782–786, with permission.)

starting antiviral therapy earlier, there are major reasons for not delaying therapy once active liver disease is diagnosed: if HBV replication and elevation of ALT levels are ongoing, there is a proven risk of progression to cirrhosis and hepatocellular carcinoma (Fig. 68.17).^{396,575,779,799} Antiviral therapy, particularly with NUCs, decreases viral load, normalizes ALT levels, induces a remission of the disease,⁸¹⁰ and reduces the progression of cirrhosis and HCC.^{104,291,396}

Interferon (IFN) Therapy

The principle immunologic treatment for hepatitis B is IFN- α , which has been used since the 1980s. Although IFN- α has a direct antiviral effect, suppressing HBV replication, its major effect is considered immunologic, inducing and/or facilitating a hepatic flare that resolves the infection and establishes immunologic control that prevents virus rebound. This treatment only has lasting effects in patients with immunologically active liver disease, augmenting an already active host response against the virus.

In HBeAg-positive chronic hepatitis B, treatment with standard IFN- α or its pegylated form leads to HBeAg seroconversion, loss of viral DNA, and clinical remission in ~20–30% of patients.^{297,365,495,767} Clinical benefit appears to be long term in these responding patients, including a decreased risk of progression to cirrhosis and hepatocellular carcinoma (HCC). HBsAg loss may occur several years after treatment cessation. However, IFN- α treatment is not particularly well tolerated, provoking a “flu-like” syndrome in many individuals. In addition, IFN- α needs to be injected subcutaneously for 6 to 12 months, and thus causes additional discomfort to the patient. It is, however, the only medication that can be administered with a finite duration.

Nucleoside Analog Therapy

NUC therapy represents the primary choice for treatment of chronic HBV. The effect of treatment with inhibitors of viral DNA synthesis is to stop viral DNA replication within hepatocytes and prevent release of infectious particles into the bloodstream. The half-life of circulating particles is relatively short, around 24 hours, and the decline in virus titers predictable.⁵⁰¹ The half-life of DNA replication intermediates has not been studied in great detail, but is probably a few days. However, the intrahepatic viral CCC DNA that maintains the cellular infections and thus, infected hepatocytes, is cleared much slower, with a half-life in the liver of a few days to several months according to the strength of the host immune response. Therefore, NUC therapies necessitate long-term treatment.^{501,720,759} CCC DNA loss is believed to reflect, primarily, the death of infected hepatocytes rather than intracellular instability, at least in the context of chronic infections.^{196,675,670,672,809} NUCs that inhibit HBV DNA synthesis thus prevent the replacement of CCC DNA, as some infected hepatocytes die and others proliferate to maintain liver cell number.⁸⁰⁹ Unfortunately, but not unexpectedly in view of its history in the treatment of HIV infections, one of the major problems of long-term NUC therapy is the emergence of drug-resistant virus (Table 68.1) The rate of emergence of the mutants depends on their fitness to spread to hepatocytes that have lost the HBV and on the rate of destruction of infected hepatocytes.^{405,811}

Endpoints of Therapy

Therapy must reduce HBV DNA to as low a level as possible, ideally below the lower limit of detection of real-time PCR assays (10–15 IU/ml), to ensure a degree of virologic suppression that will then lead to biochemical remission, histologic

improvement, and prevention of complications. IFN- α - or NUC therapy-induced HBV DNA reduction below 2,000 IU/ml is associated with disease remission. Sustained virus suppression increases the chance of HBe seroconversion in HBeAg-positive patients and the additional possibility of HBsAg loss in both HBeAg-positive and HBeAg-negative patients.¹⁷⁸

Results with Current Therapies

The antiviral efficacy of the currently approved therapies, in terms of viral suppression and clinical endpoints, has been assessed in several studies.^{100,350,365,434} Comparisons between studies are limited due to differences in patient characteristics, baseline HBV DNA levels, study design, and methodologies used to quantify HBV DNA.

In clinical trials, lamivudine achieved higher levels of viral suppression than pegylated IFN- α following 1 year of therapy; however, sustained response rates during the 24-week off-treatment follow-up period were superior for IFN.^{297,365} Furthermore, treatment with pegylated IFN has been associated with improved serologic responses such as HBsAg and HBeAg seroconversion several years after treatment cessation. Moreover, IFN does not induce any form of resistant virus and has been shown to reduce lamivudine resistance during combination therapy.^{297,365}

Resistance to NUCs is complicated by the fact that resistance mutations to one antiviral drug may be at least partially effective against another.⁸¹¹ Thus, resistance to a new drug can emerge more rapidly in patients previously treated with another drug than in treatment-naïve patients (Fig. 68.10).

Cumulative annual incidences of resistance in CHB patients on a single NUC, with no prior history of NUC therapy, are shown in Table 68.1. Lamivudine resistance occurs frequently and is observed in up to 80% of patients treated for 5 years.⁴¹¹ Telbivudine resistance is slower to emerge, but rates are substantial, with 25% of HBeAg-positive and 11% of HBeAg-negative patients experiencing virologic breakthrough due to resistance after 2 years of treatment.³⁹³ Among adefovir-treated patients, the cumulative incidence of resistance over 5 years has been reported at 29% for HBeAg-negative patients and 42% for HBeAg-positive patients.^{244,432} No tenofovir resistance has been observed after 4 years of treatment.⁶⁴⁵ Long-term studies of entecavir monotherapy in nucleoside-naïve patients have demonstrated that resistance remains low through 6 years of therapy, at 1% to 2%.¹⁰¹ In brief, current studies indicate that entecavir and tenofovir are the most potent of the currently available NUC antivirals.

The consequences of developing resistance to NUCs have been well documented. Patients treated with lamivudine or adefovir who develop virologic breakthrough of drug-resistant mutants frequently experience acute exacerbations of disease (ALT elevations) and more rapid progression to acute liver failure or HCC.^{396,411} Furthermore, the emergence of drug resistance and the subsequent need for rescue therapy increases the risk of development of multidrug-resistant HBV (Fig. 68.10). For example, entecavir is effective in patients with adefovir resistance and patients with prior lamivudine treatment who had not developed resistance, but not in patients with proven lamivudine resistance.⁵⁷⁶ Similarly, tenofovir has also been shown to be effective for patients with lamivudine resistance and an incomplete response to adefovir, but not necessarily for patients with adefovir resistance.^{38,530} In case of treatment failure, adaptation

of therapy should be made as early as possible using a drug that does not share the same cross-resistance profile.⁸¹¹

Nucleoside Analog Therapy of Difficult-to-Treat Patients

In patients with end-stage cirrhosis and little liver reserve, IFN treatment is not recommended because it increases the risk of liver failure due to immune killing of the remaining functional hepatocytes. In contrast, NUC therapy can benefit these patients, with control of viral replication, accompanied by significantly improved liver functions.^{394,395}

In liver transplant recipients with HBV recurrence in the liver graft, IFN is also contraindicated because of the risk of inducing liver graft rejection. Both tenofovir and entecavir have shown a clinical benefit that seems to be superior to that obtained with earlier NUCs.^{127,536,607} The prevention of HBV recurrence after transplantation relies at present on pretransplantation antiviral therapy with NUCs to decrease viral load, and posttransplantation hepatitis B immunoglobulin (HBIG) and NUC administration to prevent HBV recurrence. With such prophylactic protocols, the risk of HBV recurrence has decreased from 20% to 30% to below 5%.

Other Drugs in Clinical Development

Currently, there are only a few new treatment strategies in clinical evaluation. These include the combination of IFN with NUCs (tenofovir and entecavir) with high barrier to resistance, as well as the combination of NUCs with immunomodulation strategies (i.e., IL-7 with or without vaccine therapy). Other drugs targeting viral entry, nucleoside assembly, or viral morphogenesis are undergoing preclinical evaluation.

VACCINES

Current Vaccines

Vaccines containing S alone are produced by processing of HBsAg purified from plasma of HBV carriers as well as from the yeast *Saccharomyces cerevisiae* expressing S from recombinant DNA. In Europe an M/S recombinant vaccine, containing both the S and M envelope proteins expressed in a Chinese hamster ovary cell line, is also available. Both vaccines contain the immunodominant “a” determinant of HBsAg in S.

These vaccines are administered in the deltoid muscle and are highly immunogenic, inducing a protective anti-HBs antibody titer (>10 IU per ml) in more than 95% of children or young adults.^{12,366,736} Two schedules of administration are approved: 3 initial injections at 1-month intervals and a booster at 12 months; or 2 initial injections 1 month apart followed by a booster at 6 months. About 5% of those vaccinated fail to respond with development of antibodies to the vaccine. Several factors seem responsible, including genetically determined nonresponsiveness, age over 40 years, high body mass index, and immunosuppression.^{12,366,736} Vaccination is associated with rare side effects, most commonly pain or soreness at the injection site. Neurologic disorders such as multiple sclerosis, Guillain-Barré syndrome, and transverse myelitis have not been causally linked to the HBV vaccine.^{17,131}

The efficacy of protection against HBV infection has been established in large clinical studies of high-risk populations such

as homosexual men, healthcare workers, and infants born to HBsAg-positive mothers.^{12,366,736} Protection was evident when anti-HBs Ab titers were above 10 IU per ml. Several years after vaccination, the anti-HBs Ab titer may decline to undetectable levels but immunity persists, suggesting the existence of an immunologic memory.^{12,366,736} Based on these results, a booster is not recommended in healthy individuals who are not constantly exposed to HBV infection.

A universal vaccination program has been recommended by the WHO with the goal of eradicating HBV. In Taiwan and Italy, mass vaccination of infants has not only decreased the incidence of HBV infection and the prevalence of chronic carriers, but also the incidence of HCC.^{96,97,105,664} In addition to implementation of a universal vaccination program for young children, it will be important to perform antenatal screening of pregnant women for HBV infection and to administer vaccine and HBIG to neonates of HBV-positive mothers.^{12,366,736} Catch-up vaccination of 11- to 12-year-old children, high-risk adolescents and adults—along with HBV screening and vaccination of household contacts of chronic carriers and immigrants to hyperendemic regions—will also be important. Finally, resources need to be in place for HBIG and vaccine administration after contaminated needle-stick injury in unprotected individuals.

Based on the difficulties of treating chronic HBV infection—including high cost, the emergence of drug-resistant mutants, and the risk of development of HCC—the efficacy of prevention through vaccine is a strong incentive for the continuation of the mass vaccination program worldwide.⁴⁴⁷

Vaccine Escape Mutants

Vaccine escape mutants have been reported in follow-up studies of individuals receiving HBsAg vaccines composed of the S protein. Vaccinated individuals typically mount a very strong antibody response to the immunodominant “a” determinant (Fig. 68.9) located in the exposed hydrophilic domain of S between amino acids 99 and 168.^{171,540,660} The “a” determinant itself has been mapped from amino acids 124 to 147,^{18,42,66,559,755} though some authors have extended the domain a few amino acids upstream and/or downstream. Two loops bounded by disulfide bridges are inferred by some authors to exist between cys124 and cys137, and cys 139 and cys147, but other pairings have also been suggested.¹¹² The “a” determinant is highly conserved and is found in all genotypes of HBV.⁴⁹⁸ Vaccine escape represents a distinct phenomenon, in which a vaccinated individual develops chronic HBV infection and is seropositive for HBsAg despite the presence of antibodies to the group-specific “a” determinant of HBsAg that were induced by the vaccine. This is possible because HBsAg present in the serum of these patients lacks the group-specific “a” determinant, generally as a result of missense mutations in the region of S that encodes amino acids 124 to 147 (Fig. 68.9).

Most studies of vaccine escape have involved at-risk children and infants who were either vaccinated or received vaccine plus HBIG, which is strongly reactive to the “a” determinant of HBsAg. For instance, in the 1980s a follow-up study of childhood vaccination in Italy revealed vaccinees who became HBsAg positive despite the presence of a strong antibody response to HBsAg. The incidence was rare, involving about 2% of the children of HBsAg-positive mothers, or with HBsAg-positive family contacts.^{87,800} More detailed analysis of virus from one patient

revealed a point mutation encoding a G145R substitution at amino acid 145 of the S protein.⁸⁷ Analysis with monoclonal antibodies to the “a” determinant of wild-type HBV revealed a loss of binding to the mutant HBsAg.^{87,756} Subsequent studies in other populations with high HBV endemicity confirmed the rare occurrence of vaccine escape mutants in children, including the glyc145arg substitution in “a,” other substitutions or insertions in “a,” and mutations within the external hydrophilic domain, but outside of “a.”^{86,191,273,286,317,374,509,512,513,776} Vaccine escape variants have also been detected in unvaccinated children and adults with circulating virus as well as anti-HBs,^{273,776} possibly reflecting past or concurrent infection with wild-type and mutant virus. One concern is that these mutant viruses will become an increasing fraction of the pool of HBV carriers and an increasing risk for vaccinated individuals,²⁷³ though recent studies suggest this scenario may not be correct.²⁷⁴

ANIMAL MODELS OF HBV INFECTIONS

Chimpanzee

Higher primates, including the chimpanzee, are susceptible to HBV. The chimpanzee in particular has been employed periodically to study virus transmission, the host response, and vaccination. Viral hepatitis was known to occur in captive chimpanzees even prior to the discovery of HBV, and early studies showed the presence of Australia antigen in the serum of a fraction of captive animals, including evidence of chronic infections.⁴⁵¹ Chronic infections are associated with persistent liver inflammation.^{173,343,636} Subsequent studies showed that chimpanzees were susceptible to experimental infection with HBV^{21,39,212} and were protected from infection by the vaccine comprised of HBsAg purified from virus carriers.^{80,344,449,566} Chimpanzees were also used to demonstrate the efficacy of recombinant vaccines comprised of surface antigen particles produced in yeast,^{43,204,606} and have been used, for example, to test other approaches to preventative or therapeutic vaccination, including peptide antigens,^{293,485} virus core protein,^{294,477} and virus vectors.^{414,471,523} Recent experiments demonstrated vaccine escape of lamivudine-resistant HBV variants, due to the fact that mutations creating drug resistance mutated the overlapping S gene, confirming observations made in vaccinated infants and liver transplant recipients who experienced breakthrough infections.³⁰⁹

In other areas it was shown, shortly after the first cloning of the HBV genome, that cloned DNA was infectious in chimpanzees,⁷⁶³ as was HBV produced by cultures of human^{4,679} and rodent cells,⁶³⁰ and that transient infection in chimpanzees followed a similar course to transient infection in humans.^{21,39,40,270} However, for ethical reasons, as well as cost, use of the chimpanzee to address questions about the biology and immunology of infections has been limited. Early studies suggested that HBV was able to replicate in the spleen to a limited extent,³⁹⁷ though follow-up studies to determine if the observations reflected actual replication or passive accumulation of virus remain to be done. More recent studies have demonstrated the key roles of CD4 and CD8 T cells in the clearance of transient HBV infections,^{16,701,762} the apparent lack of induction of innate immune responses by the primary infection,⁷⁶⁰ and the existence of clonal hepatocyte proliferation in chronic liver disease.⁴⁴⁴ The chimpanzee has not so far proven to be a model for

HCC, as only a few cases in HBV-infected chimpanzees have been reported, and only in chimpanzees co-infected with either HDV or HCV.⁵⁵⁴ It is possible that the failure to detect HCC reflects the limited numbers of chimpanzees that have been subjected to long-term studies.

Woodchuck and Ground Squirrel

WHV was discovered in woodchucks housed in the Penrose Laboratory of the Philadelphia Zoo,⁶⁷⁷ and quickly found to be prevalent in woodchucks trapped in the mid-Atlantic states,⁷²⁷ but not in woodchucks trapped in upstate New York.^{133,695,768} The correlation seen in early studies between chronic WHV infection and HCC⁶⁷⁷ reinforced the conclusion from concurrent studies of human populations^{49,450,560} suggesting that chronic hepadnavirus infection was a cause of liver cancer. Investigations with captive woodchucks showed that WHV induced HCC in essentially 100% of neonatally infected woodchucks within 2 to 4 years, and in 20% of those that resolved the infection after neonatal inoculation.^{338,692,693}

Shortly after the discovery of WHV, a related virus, ground squirrel hepatitis virus (GSHV) was discovered in California (Beechey) ground squirrels.⁴³⁵ Like WHV, GSHV causes HCC in chronically infected animals. However, the course of infection is milder, the onset of HCC is delayed beyond 4 years, and the incidence is lower—around 50%.^{141,436,438} GSHV infection also runs a milder course than WHV when inoculated into woodchucks and, again, the onset of HCC is delayed, suggesting that the difference in pathogenesis between WHV and GSHV in their natural hosts might be a property of the viruses rather than the hosts.⁶¹⁶

In recent years, more research has been done with the woodchuck than the ground squirrel, primarily because a colony of woodchucks at Cornell University was established and extensively used for preclinical evaluation of antiviral compounds,⁶⁹⁵ and because uninfected and chronically infected woodchucks were available from commercial sources. Similar to findings with human HBV carriers treated with lamivudine, treatment of carrier woodchucks with nucleoside analog inhibitors of WHV replication—clevudine or entecavir—appears to delay the onset of HCC.^{129,334} A current deficiency of the woodchuck model, aside from the technical difficulties in managing a large nondomesticated animal in a laboratory setting, is the lack of reagents available to study woodchuck gene expression, including antibodies to characterize the immune response to transient and chronic infections. Attempts to correct this deficiency^{3,217,239,457,458,462,778} are still at an early stage.

Ducks

The domestic duck was the first avian species discovered to be infected with an HBV-like virus,^{445,749,807} DHBV, and the first host in which virus replication was studied in detail. The reason is that DHBV is widely distributed in domestic flocks, chronically infecting 10% or more of the birds. Therefore, infected animals were readily available for research. Moreover, the infection is generally apathogenic because spread is predominantly vertical rather than horizontal.^{445,504} DHBV replicates in the yolk sac⁶⁸⁴ and rapidly spreads to the developing embryo, with replication detected in the liver as early as 4 to 5 days of embryogenesis⁷³⁰ (ducks hatch at 28 days). As a result, the congenitally infected ducks are immunotolerant to the virus and do not develop significant cellular or humoral immune responses

or liver disease due to their infection, despite the fact that all hepatocytes are producing virus, and sera and liver contain high titers of virus and virus replication intermediates, respectively.

DHBV infection of domestic ducks shares many similarities with orthohepadnavirus infections. There are some notable differences, however. First, perinatal and congenital DHBV infections do not appear to lead to chronic liver disease, cirrhosis, or HCC. A high incidence of HCC has been reported in domestic ducks in the People's Republic of China, but is likely due to aflatoxin exposure rather than DHBV infection.¹⁶⁸ The lack of HCC due solely to DHBV infection may reflect the fact that chronic infection in ducks is usually established under conditions in which the immune system does not respond to the infection, enforcing the idea that liver disease is the result of the host antiviral response. It needs to be kept in mind, however, that prospective studies of more than 3 to 4 years have not been carried out, and this does not extend to near the potential life span of these ducks, which probably is in excess of 10 years. Thus, cirrhosis and HCC developing later in life would have been missed. One reason these long-term studies have not been pursued is that ducks develop a chronic liver disease that is unrelated to DHBV infection.⁴⁴³ Secondary amyloid disease of the liver,^{136,137,579} a condition characterized by the aberrant, extracellular deposition of fibrils of serum amyloid A protein, a secretory protein produced by hepatocytes, often appears in 50% or more of the ducks in a cohort by 6 to 9 months of age, and eventually replaces the cellular population of the liver by a proteinaceous mass,²³⁷ leading to liver failure.

A second feature distinguishes DHBV infection from typical orthohepadnavirus infections. Following inoculation of adult chimpanzees with HBV or woodchucks with WHV, a viremia of 4 to 8 weeks can develop before the infection begins to resolve. Inoculation of adult ducks or ducklings more than about 3 weeks of age leads to the rapid appearance of virus neutralizing antibodies, so that a viremia is never detected beyond a few days postinfection, and the number of infected hepatocytes is largely determined by the amount of virus in the inoculum.^{302,306} This observation reinforces the notion that maintenance of DHBV in nature is largely dependent on *in ovo* transmission.

Viral DNA replication intermediates (viral capsids) in infected duck liver are easy to isolate and study^{250,671} because, in contrast to mammalian hosts, ducks infected *in ovo* do not contain large amounts of antibodies that can bind to and aggregate the capsids. As discussed earlier, several steps in viral DNA replication, including reverse transcription and priming of plus- and minus-strand DNA synthesis, were first characterized using liver and sera from DHBV infected ducks.^{399,400,468,469,671}

Primary duck hepatocyte cultures have also played a key role in our current understanding of hepadnavirus replication, facilitating analysis of early steps in the virus life cycle, including the finding that CCC DNA copy number increases early in infection⁷²⁵; that copy number amplification, at least for DHBV, is negatively regulated by viral envelope proteins^{675,676}; that overproduction of CCC DNA is cytopathic³⁸⁰; and that CCC DNA is produced via reverse transcription rather than semi-conservative DNA synthesis.⁷²⁴ Many of these experiments have not yet been possible with the orthohepadnaviruses.

The duck model has also been used to characterize transient and chronic infections and antiviral therapies. Early studies with DHBV showed that a hepadnavirus could be cleared even after

infection of the entire hepatocyte population,³⁰⁶ ruling out the possibility that chronic infection occurred simply because the immune response did not control virus spread before the entire hepatocyte population was infected. Subsequent experiments revealed the very high specific infectivity of DHBV and, by inference, other hepadnaviruses, showing that nearly 100% of virions could be infectious,³⁰⁵ as recently shown with HBV.¹⁶ Analysis of ducks that were chronically infected via congenital transmission or by inoculation at birth, and therefore mounted either no response or a minimal immune response to the infection, provided some of the early evidence that hepadnavirus infections were nonpathogenic.^{249,303} Like the woodchuck, the duck model has also been extensively used in preclinical trials of investigational antiviral drugs.^{5,6,154,192,196,197–198,202,264,289,318,345,368,372,417,418,440,465,474,496,505,511,620,626,652,680,709,721,722,743,752}

Transgenic Mice

Transgenic mice have been developed to study the consequences of liver-specific expression of the entire virus^{14,121,181,232,269} as well as individual gene products. Individual products that have been examined include HBsAg,⁴⁶⁴ core,²³¹ L,¹¹⁹ S^{120,555} and X.^{324,332,376,774,792}

One of the earliest studies with HBV transgenic mice involved strains that produced viral envelope proteins. Of the three envelope proteins, S is the most abundant and L the least, reflecting in part the relatively weak PreS promoter in comparison to the S promoter.²⁵⁸ When transcription of the PreS mRNA was under the control of a relatively weak, nonviral promoter, and M and S under control of the S promoter, HBsAg was produced and secreted into the blood stream, just as in a natural infection. However, when a stronger promoter was used to direct synthesis of PreS mRNA, a very different result was obtained. Secretion of HBsAg was blocked and, instead, HBsAg accumulated in the ER, leading to the proliferation of intracellular membranes to give the hepatocytes a ground-glass appearance.¹¹⁸ This lesion had been previously observed in human liver chronically infected with HBV and is characterized by the accumulation of the 22-nm diameter, rod-like form of surface antigen in the cisternae of the ER.²¹¹ Cell-culture studies confirmed that ER retention was due to overexpression of L compared to S.^{453,519}

Analysis of transgenic mice overexpressing L revealed morphologic distortion of hepatocytes, inflammation, and a high rate of apoptosis, leading ultimately to selective outgrowth of hepatocytes that no longer expressed or had lost the transgene, as well as to HCC.^{119,138} In contrast to these mice, transgenic mice expressing the entire viral genome from birth do not develop either chronic liver disease or HCC, though antibody to HBsAg is sometimes found in the serum. HBV is constitutively produced in hepatocytes of these mice from a greater than unit length transgene that includes EN1 and EN2 and the preC/C promoter at the 5' end, and extends through the HBV polyadenylation signal at the 3' end²³² (Fig. 68.6). Most hepatocytes in mice express viral proteins, but only centrilobular hepatocytes (i.e., located near the central vein) produce enough core antigen to assemble functional DNA replication complexes.²³² Virus is released from these cells but for unknown reasons, CCC DNA is not formed unless the mice are also deficient for HNF1 α .⁵⁷²

Transgenic mice have been extensively used to examine how the immune system might clear an HBV infection. This work has provided convincing evidence that replicating viral

DNA, viral proteins, and mRNAs can be cleared rapidly from the mouse liver by antiviral cytokines.^{228,230,760} Indeed, when these mice were injected with CTLs reactive to HBsAg, DNA replication intermediates were nearly gone within 24 hours, possibly through a block in assembly and proteasome-dependent degradation.^{230,583}

The rapidity with which replicative intermediates disappear from the liver of the transgenic mice following cytokine induction does not have an evident parallel in the resolution of transient hepadnavirus infections. Nonetheless, findings with the transgenic mice highlight and possibly illuminate a critical issue in the resolution of HBV infections. Following recovery from a transient infection, how is it that the liver is populated by hepatocytes, or the progeny of hepatocytes, that at one time had been infected⁶⁷⁰?

Chimeric Mice

About 20 years ago, Brinster and colleagues created transgenic mice that express the serine protease urokinase-type plasminogen activator (uPA) in hepatocytes beginning around the time of birth.⁶⁰² More than half die with intrahepatic bleeding and those that survive either lose or turn off uPA expression within a subpopulation of hepatocytes that then repopulate the liver. Syngeneic hepatocytes, or in a severe combined immunodeficiency (SCID) background, xenogeneic (rat) hepatocytes, inoculated within a week or two of birth, also expanded to repopulate the liver.^{577,578} These early observations suggested that hepatocyte repopulation might also be achieved with human or woodchuck hepatocytes. Mouse livers repopulated with human hepatocytes were shown to be susceptible to HBV¹⁴⁴ and HCV,⁴⁵⁹ and livers repopulated with woodchuck hepatocytes to WHV.⁵⁴¹ An advantage of chimeric mice is the long-term maintenance of transplanted hepatocytes, particularly human hepatocytes. Utilization of this system has been limited because of the technical challenge of acquiring and transplanting human hepatocytes, which is done by injection of hepatocytes into the spleen, from where they migrate to the liver.⁵⁷⁷

In spite of these difficulties, several studies have relied on this model. For example, the requirement for HBx in establishing viremia has been reproduced with the chimeric mouse model.⁷²³ Hence, it can be concluded that X is required for the HBV life cycle even in the absence of a functional immune system. The model has also been useful for studies on the dynamics of HBV infections with genotype G, which is dependent on co-infection with another genotype, commonly genotype A.⁶⁶⁹ Finally, experiments with uPA/SCID mice demonstrated that prolonged HBV replication in the absence of an adaptive immune response can induce pathology similar to fibrosing cholestatic hepatitis observed in immunosuppressed carriers.⁴⁶⁰

Hydrodynamic Infection

A number of years ago, efficient transfection of the liver by DNA or RNA was demonstrated using hydrodynamic injection, which involves inoculation of a hypotonic solution of RNA or DNA via the tail vein.^{93,406} This has been used successfully to establish transient expression of HBV in mouse liver⁷⁸⁰ and to study effectors of virus clearance in this model system (e.g., 781).

Tupaia

The search for a small mammal susceptible to HBV infection led to the discovery that tree shrews (*Tupaia belangeri chinensis*)

can be experimentally infected with HBV.⁷⁷⁷ Similar to acute, self-limited hepatitis B in humans, HBsAg was rapidly cleared from the serum of Tupaia, followed by seroconversion to anti-HBe and anti-HBs.⁷⁴⁴ Moreover, primary hepatocytes isolated from livers of Tupaia are susceptible to HBV infections.^{329,744} The model is useful for investigations on receptor-mediated uptake of HBV (i.e., 216).^{64,219,379} Primary Tupaia hepatocytes were also used for the construction of chimeric mouse livers that could be infected with HBV.^{146,419} Finally, Tupaia was used for studies on the pathogenesis of clinical HBV isolates from patients with fulminant hepatitis.³⁰ These studies raised the possibility that in some cases the virus is directly cytotoxic.

HEPATOCELLULAR CARCINOMA

Epidemiology

About 5 years after the discovery of HBV, Sherlock and colleagues looked for a link between HBV infection and HCC.⁶²⁹ In a cohort of 17 chronically infected males, five presented with HCC. Definitive support for the hypothesis that HBV plays a primary role in the development of HCC came from epidemiologic studies,^{450,560} and particularly from a prospective study by Beasley and colleagues, who followed more than 22,000 Taiwanese men.³¹ The relative lifetime risk for HBsAg-positive males was found to be about 20 compared to uninfected individuals.¹⁷⁹ As mentioned before, the strongest correlation between chronic hepadnavirus infection and HCC was found in WHV-infected woodchucks: almost every animal develops liver cancer within 2 to 3 years postinfection.^{338,677} In humans, liver cancer usually develops after several decades of infection, and is often associated with cirrhosis of the liver. Consistent with the distribution of chronic HBV infections around the globe, HCC is one of the two or three most common malignant neoplasms in people living in China, Taiwan, and southeast Asia, as well as in sub-Saharan Africa.

The realization that HBV is a major carcinogen has spurred intense efforts to unravel the mechanism responsible for the development of HCC. The details of this process are still not understood; there is still uncertainty about the cells from which HCCs arise. The vast majority of tumors contain integrated HBV DNA and, since hepatocytes are the only liver cells clearly susceptible to HBV, it is assumed that most tumors arise by dedifferentiation of mature hepatocytes. This also seems reasonable since hepatocytes constitute a mostly self-renewing population of long-lived cells ($t_{1/2}$ of 6 months or more) that can undergo extensive proliferation to maintain liver mass during periods of chronic or acute hepatocyte destruction. It is also possible that some tumors arise from undifferentiated progenitor cells present in the liver. Present findings are consistent with the concept that the majority of tumors arise from hepatocytes.

In any case, the field has been driven by two hypotheses, one positing a direct effect of the virus in cancer development, the other predicting that HCC formation is a consequence of persistent liver injury caused by the immune response against infected hepatocytes, attributing an indirect role to the virus. In practice, both may contribute to HCC. The former hypothesis appears dependent on the assumption

that HCCs arise from hepatocytes, whereas the latter does not require this assumption.

Viral Factors in HCC

Evidence for a direct role of HBV in human HCC was fueled by the observation that DNA from HCC contained integrated HBV DNA and that tumors were clonal with respect to the viral integration site.¹⁷⁷ This discovery was consistent with a model of insertional mutagenesis found by retrovirologists, in which activation of proto-oncogenes was caused by nearby provirus integration.^{257,532} However, efforts to identify proto-oncogenes adjacent to integrated HBV DNA did not yield clear candidates,⁴⁷⁶ save for a few isolated cases, in which integration sites were found adjacent to or within coding regions of candidate proto-oncogenes including cyclin A, *erb-A*, or retinoic acid receptor beta.^{155,156,748} It is still possible, though unproven, that HBV DNA alters the expression of genes that are distant from the integration site,⁴⁷⁶ perhaps by altering the structure of chromosomes.^{71,628}

Similar investigations of tumors from WHV-infected woodchucks revealed very different results. The majority of these HCCs overexpress N-myc2, a functional pseudogene of N-myc1^{195,567} that is not normally expressed in the liver.^{195,252,296} C-myc and N-myc1 are more rarely overexpressed in woodchuck HCCs. N-myc1 or C-myc overexpression in woodchuck HCCs is typically associated with nearby integration of WHV DNA, while overexpression of N-myc2 may be due either to nearby integration^{195,252} or to integration in the distal *b3n*^{69,70} or *win*¹⁹⁴ loci.

The majority of ground squirrel HCCs overexpress C-myc.^{252,714} However, C-myc overexpression in the ground squirrel does not correlate with nearby integration of GSHV DNA, though it sometimes correlates with copy number amplification of the C-myc locus. Interestingly, the pattern of DNA integration and myc activation in HCCs that arise in woodchucks infected with GSHV is more typical of that seen in ground squirrels infected with GSHV. This suggests that N-myc activation in WHV-infected woodchucks characterizes WHV infections rather than hepadnavirus infections of the woodchuck *per se*. Transcriptional activation of N-myc and C-Myc have not been reported to characterize human HCCs, suggesting that myc activation may play a more central role in rodent than primate HCCs.⁷¹⁴ However, this does not preclude the possibility that genes regulated by myc play a central role in human HCC.³¹⁵

Alternative models, consistent with a direct role of HBV in human HCC, posit that one or several viral gene products are oncogenic. Although the long latency period observed for HCC development indicates that HBV gene products cannot transform hepatocytes in a single hit event, the hypothesis gained momentum with the discovery that transgenic mice expressing HBx developed HCC.³²⁴ The success of this model depended on the selection of the mouse strain, CD1, known to develop spontaneous hepatomas at an increased rate compared to other mouse strains, and high expression levels of the transgene that are not observed during natural infections.³³² It thus appears that X promotes, but does not necessarily initiate, carcinogenesis. In agreement with this notion, an HBx transgene did not promote HCC in another genetic background but, interestingly, these mice were more susceptible to HCC induction by diethylnitrosamine (DEN) than nontransgenic

controls,⁶⁴³ which was subsequently attributed to increased proliferation of altered hepatocytes in the absence of elevated host gene mutation rates.⁴²⁵ In addition, an HBx transgene accelerated development of HCC in C-myc transgenic mice.⁶⁹⁷ Moreover, many cell-culture studies have demonstrated effects of HBx on the cell cycle (e.g., 37,619,713) and on cell viability (e.g., 35,382,404). Conflicting results emerged from these studies, some suggesting that HBx activates cell proliferation and others suggesting that it is cytostatic, or even induces apoptosis (reviewed in 57). Therefore, it must be concluded that the outcome of experiments with HBx are strongly influenced by the selection of cell lines and other experimental conditions.

Nevertheless, HBx may contribute to a multistep hepatocyte transformation process by activating certain signal transduction pathways via its capacity to transactivate at least some viral and host genes or by binding to host proteins, as discussed earlier, such as the DNA repair protein DDB1 or even the proteasome subunit XAPC7, or p53.^{174,184,282,375} It is important, in this regard, to note that HBx is not always expressed in HCCs,⁶⁶⁸ implying that it may not be needed to maintain the transformed state. In addition, subsequent data suggested that the role of HBV gene products in carcinogenesis may be more complex: mice transgenic for the entire viral genome exhibit an increased sensitivity to DEN-mediated HCC that is not dependent on HBx expression.⁸⁰⁵ Consistent with the latter observations, other studies described below suggested that viral envelope proteins are oncogenic. In summary, despite a wealth of information about biological activities of HBx, it has so far not been possible to obtain proof for a direct role of this protein in HCC development.

The discovery of the transactivation function of truncated M envelope proteins also supported a viral oncogene hypothesis (i.e., 609). It should be noted that truncated M proteins are not produced by wild-type HBV. Overexpression of the truncated M protein, with the help of the albumin promoter in transgenic mice, activated the c-Raf/Erk2 signal transduction pathway and in turn increased hepatocyte proliferation.²⁶² These mice developed very small liver tumors by 1 to 2 years of age. Whether these and other observations with model systems bear any relevance to HCC development in humans remains a matter of debate. Interestingly, Su and colleagues have suggested that L protein mutants are responsible for development of a class of preneoplastic lesions consisting of ground-glass hepatocytes (see Cellular Factors in HCC section).⁶⁶⁷ In addition, the intact L protein also has been shown to transactivate viral and host genes.^{190,287,661,773}

Cellular Factors in HCC

Models explaining HCC development in HBV patients entirely via indirect mechanisms take into consideration a hallmark of HBV infections, death, and compensatory regeneration of hepatocytes. Hepatocytes, as noted earlier, constitute a mostly self-renewing population except in conditions of severe liver injury. Progenitor cell proliferation may also be seen late in infection, especially in tumor-bearing and cirrhotic livers. While hepatocyte death and compensatory proliferation to maintain liver mass process are inconsequential on a sporadic basis, this liver injury can cause substantial fibrosis and cirrhosis of the liver under persistent conditions such as those observed in chronic hepatitis B. It is noteworthy that alcohol abuse, storage diseases, and chronic HCV infections have the

same effects, and all are risk factors for HCC. That is, independent of the cause, chronic liver disease is associated with an increased risk of HCC.

The importance of chronic liver injury in HCC has been documented in many studies with mouse models. For instance, transgenic mice expressing levels of the HBV L-protein that are toxic to hepatocytes developed HCC.¹¹⁹ It was suggested that a contributing factor in emergence of HCC in this model was free radicals generated by the large number of macrophages activated in response to the high rate of apoptosis, leading to extensive hepatocyte DNA damage. The idea that oxidative DNA damage was occurring was supported by the detection of deoxyguanosine adducts on DNA isolated from the livers of these mice.²⁴⁷ These data support the notion that HCC is not just due to the effects of L overexpression on the hepatocytes, leading to a high level of hepatocyte death and compensatory proliferation, but also to hepatocyte DNA damage attributable to free radical formation by activated macrophages.⁴³⁹

In a perhaps more relevant model of the events occurring during chronic HBV infections, studies were carried out with transgenic mice expressing low levels of L that did not cause any apparent liver disease. When the immune system of these mice was ablated and replaced with bone marrow cells from syngeneic nontransgenic mice, the resulting immune response led to liver cell injury and HCC.⁴⁸⁰ Moreover, inoculation of the mice with anti-fas ligand antibodies reduced the incidence of HCC, establishing a correlation between the rate of hepatocyte death, compensatory proliferation, and development of HCC.⁴⁸¹ The significance of this relationship for liver cancer has been further emphasized with a novel transgenic mouse model permitting controlled activation of the latent transcription factor NF- κ B.⁴²⁶ Mice deficient for NF- κ B activation in hepatocytes exhibited increased hepatocellular death and compensatory proliferation after DEN treatment compared to normal mice that correlated with an increased rate of HCC development.

In summary, the details of HBV-induced liver cancer remain elusive. We can predict that the combination of alterations in hepatocyte physiology caused on one side by viral gene expression and on the other by changes in the micro-environment of hepatocytes due to inflammation results in the proliferation and eventual transformation of hepatocytes. Viral mechanisms that could contribute to hepatocyte transformation include changes in gene expression caused by HBx, truncated M (or L), and integrated HBV DNA, along with inhibition of DNA repair pathways. Cellular mechanisms include hepatocyte killing by CTLs, induction of DNA damage via reactive oxygen (and nitrogen) radicals produced by macrophages, and expression of cytokines that in conjunction with viral gene expression provide a proliferative stimulus to hepatocytes.

PERSPECTIVE

HBV biology is multifaceted and touches on many areas in the basic and clinical sciences. Among them are liver biology, immunology, virology, and antiviral therapy. During the past three decades we have witnessed major progress in all areas. Investigations on viral DNA replication have uncovered the mechanism for HBV persistence in the infected liver, which is based on continuous rounds of CCC DNA amplification. Studies in animal models have provided insights into the

course of natural infections and into the process of viral clearance. The revelation that clearance depends on a strong CTC response that can eliminate infected hepatocytes explains why current nucleoside-based antiviral therapies can attenuate, but not cure, chronic HBV infections.

In spite of great progress, many gaps in knowledge remain to be filled. For instance, the receptors for hepadnavirus entry into hepatocytes need to be further investigated. The mechanisms for the disassembly of viral capsids, delivery of the genome into the cell nucleus, and formations of CCC DNA need to be clarified. Although a wealth of new information about the possible function of the X protein has accumulated, we still do not know how this small polypeptide regulates viral transcription and replication in hepatocytes during the course of an infection. For the development of antiviral therapies that can cure infections, it will be imperative to gain a better understanding of the immune response against HBV and the possibility of augmenting this response to cure chronic infections. Moreover, the role of cytokines in the resolution of acute infections needs to be investigated. Although CCC DNA does not appear to be a target for the action of cytokines, do they protect regenerated hepatocytes from *de novo* infection? If so, it would explain why infected and uninfected hepatocytes can coexist during the clearance phase. Finally, there are large gaps in our understanding of the role of HBV in hepatocarcinogenesis, the major burden associated with chronic HBV infections. Does the virus play a secondary role (i.e., by causing an increase in hepatocyte death and compensatory proliferation) or does it act as a tumor promoter in a multistep transformation process, as has been documented for colon cancer? Or does HCC in HBV carriers arise from hepatocyte progenitor cells or from differentiated hepatocytes? These and other questions will have to be addressed to complete the picture of HBV biology that has come only partially into focus over the past 30 years.

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Hepatitis D (Delta) Virus



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HISTORY

In the mid 1970s, Mario Rizzetto, a gastroenterologist in Turin, Italy, detected a previously unrecognized nuclear antigen in hepatocytes of patients with chronic type B hepatitis. The antigen resembled hepatitis B core antigen (HBcAg) in its association with hepatitis B virus (HBV) infection, although it rarely coexisted with HBcAg (Fig. 69.1). The antigen was initially interpreted as a novel hepatitis B-specific antigen; it was called *delta antigen* (HDAg) and its antibody, *antidelta*.¹³⁵ By 1980, transmission experiments in chimpanzees confirmed that HDAg was a component of a transmissible pathogen that was defective and required co-infection with HBV for its replication.¹³⁶ A radioimmunoassay for antidelta was developed¹³⁹ and showed a high prevalence of antidelta among hemophiliacs and drug users.¹³⁸ Hepatitis associated with HDAg was found to be highly endemic in Italy, especially in the south. By the early 1980s, HDAg and a small RNA were shown to be internal components of a virus-like particle enveloped with hepatitis B surface antigens (HBsAg), the envelope proteins of HBV.¹³⁷ The virus-like delta agent was shown to be associated with the most severe forms of acute and chronic hepatitis in many HBsAg-positive patients.¹⁶⁰ Thus, in some cases, severe disease thought to be caused by HBV was actually the result of concurrent infection with the delta agent. By 1983, evidence for infection with the delta agent had been found on every populated continent.¹³⁴ A year later, in Venezuela, it was found to be the cause of severe hepatitis among indigenous populations not frequently exposed to blood. Subsequently, its importance in South America was extended to include etiologic associations with traditionally severe hepatitis in Colombia (hepatitis de Sierra Nevada de Santa Marta) and Brazil (Labrea black fever), as well as severe hepatitis in Peru and indigenous populations of Ecuador.¹⁰⁵ By 1983, the delta agent was considered sufficiently unique and medically important to be designated a distinct hepatitis virus—hepatitis delta virus (HDV)—and the disease it caused was designated delta or type D hepatitis. Cloning and sequencing of the HDV genome in 1986 confirmed its uniqueness among animal viruses.¹⁸⁰ Other advances in the

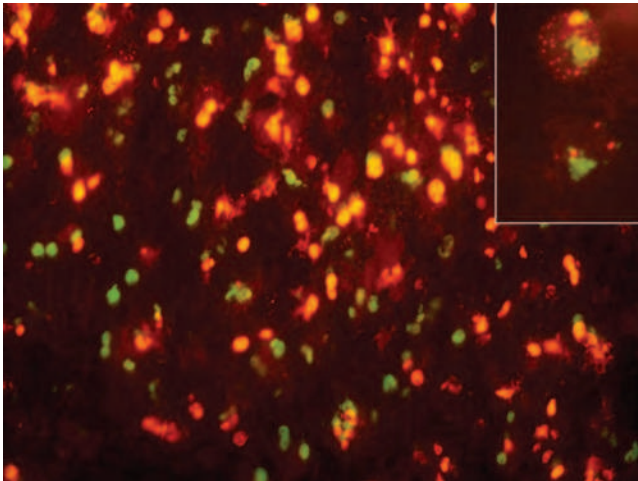


FIGURE 69.1. Immunofluorescent staining of a liver biopsy from a hepatitis D virus-infected patient. Hepatitis B virus core antigen (red) is present mainly in the cytoplasm, whereas delta antigen (green) is mainly in the nuclei of hepatocytes, although only rarely coexisting with core. (Courtesy of Dr. Mario Rizzetto.)

molecular biology of HDV have revealed novel pathways; these and the frequently devastating consequences of HDV infection drive further research on this cause of acute and chronic liver disease, despite its diminishing medical importance, in part because of the success of vaccination programs against hepatitis B.⁶⁶ Thus, a chance observation by an inquisitive gastroenterologist resulted in the discovery of one of the most interesting and unusual of human pathogens.

INFECTIOUS AGENT

Classification

HDV is the only member of its own separate genus: *Deltavirus*.⁸⁹

Virion Structure

Figure 69.2 is an image of partially purified HDV particles obtained by electron microscopy (EM) after negative staining.⁷⁵ Particles are roughly spherical and somewhat heterogeneous in size (36 ± 4 nm in diameter).^{75,78} No cryo-EM images or reconstructions are yet available.

Figure 69.3 includes a representation of the HBV envelope proteins L, M, and S. They share a C-terminus, and about 50% of each undergoes site-specific, N-linked glycosylation. In HBV-infected cells, they are used to assemble HBV DNA-containing infectious particles. Also produced is a great excess of empty subviral particles (SVPs), typically 25 nm diameter spheres and 22 nm diameter filaments (as also seen in Fig. 69.2). When HDV genome replication is occurring in such a cell, the HDV RNA-containing particles can be assembled. Such particles will or will not be infectious depending on the presence of the HBV L protein. Within HDV RNA-containing particles is a nucleocapsid: a ribonucleoprotein complex of genomic RNA and 70 to 220 copies of HDAg. By extrapolation from HBV studies, the lipid-containing envelope of HDV contains about 100 copies of the HBV envelope proteins L, M, and S.

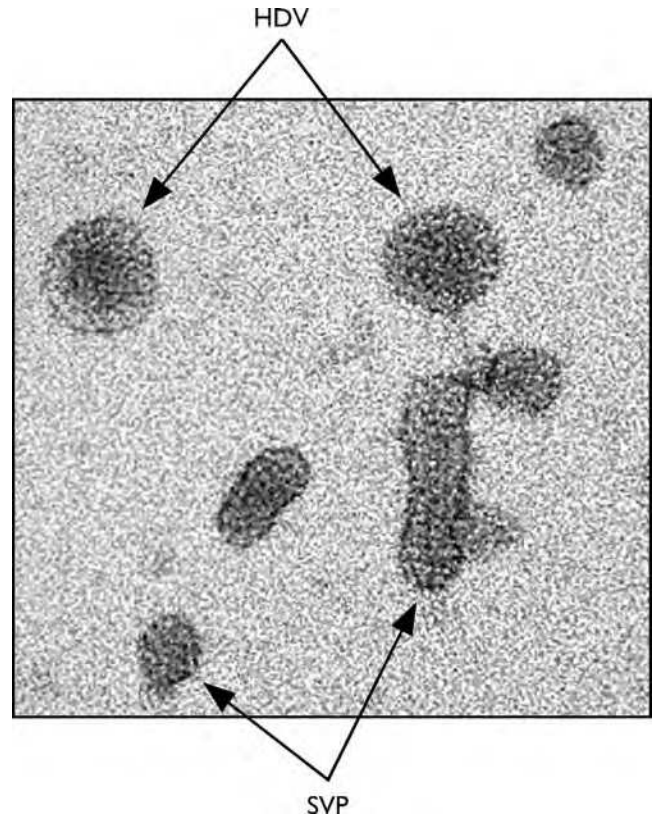


FIGURE 69.2. Electron microscope examination of assembled hepatitis D virus (HDV). Virus was assembled in Huh7 cells transfected with one plasmid to initiate HDV genome replication and another to express the three envelope proteins of hepatitis B virus (HBV). Thus, there are no infectious HBV particles present. Particles were affinity purified and negatively stained for electron microscopy. HDV particles ranged from 36 ± 4 nm diameter. However, still present are subviral particles: 25 nm diameter spheres and 22 nm diameter filaments. (Adapted from Gudima S, He Y, Meier A, et al. Assembly of hepatitis delta virus: particle characterization, including the ability to infect primary human hepatocytes. *J Virol* 2007;81:3608–3617.)

Genome Structure and Organization

As indicated in Figure 69.4, the RNA genome is a single-stranded RNA with a circular conformation that can fold on itself to form an unbranched rod-like structure with about 74% base pairing.^{92,180} Most full-length HDV sequences are in the range of 1,672 to 1,697 nucleotides.¹⁴² During replication, genomic RNA increases to around 300,000 copies per cell.³⁹ Also detected are fivefold lower amounts of an exact complementary RNA, the antigenome. This RNA encodes the sole viral protein, HDAg. Thus, the HDV genome is considered as negative stranded.

Both genome and antigenome contain a ribozyme. *In vitro*, these ribozymes, in the presence of divalent metal ions, cleave at a specific site via a trans-esterification to produce a 5'-OH and 2', 3'-cyclic monophosphate.^{60,93} The cleavage sites are at the 5'-edge of 85-nucleotide ribozyme domains. Crystal structures have been reported for these domains both before and after cleavage.^{61,88} Comparison of pre- and post-cleavage states reveals a conformational switch and details of the catalytic mechanism. Recently, these structures have been

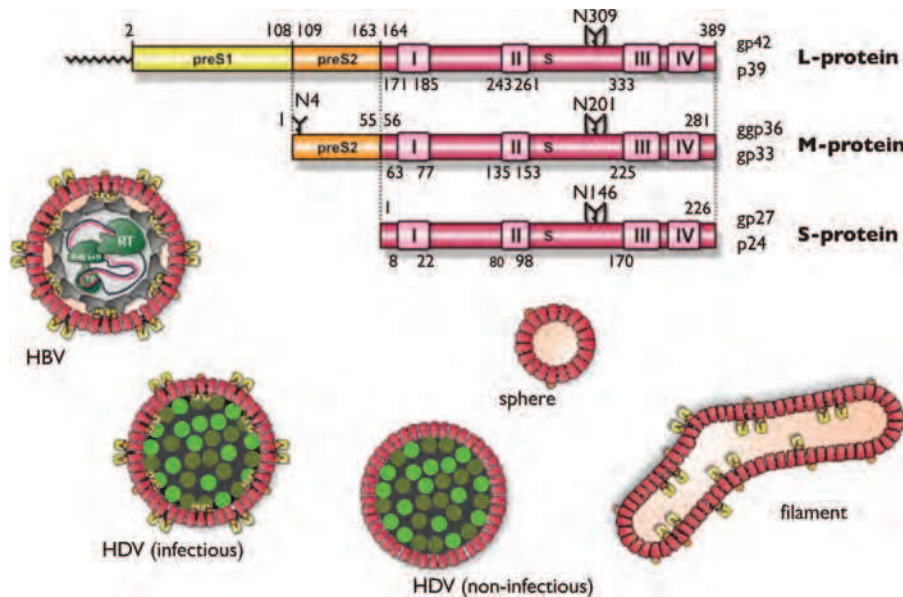


FIGURE 69.3. Hepatitis B virus (HBV) envelope proteins and particle assembly. At top are the three envelope proteins of HBV—L, M, and S—which share a common C-terminus, with four transmembrane domains (I–IV). The presence or absence of the N-linked carbohydrate chain leads to two different sizes, as indicated by electrophoretic mobility. M has a unique domain pre-S2 relative to S. Similarly, L has a unique domain pre-S1, relative to M. As indicated at bottom, the serum from HBV-infected individuals contains the infectious DNA-containing HBV as well as empty subviral particles, 25 nm diameter spheres, and 22 nm diameter filaments. The serum of an individual infected with hepatitis D virus (HDV) also contains the infectious and noninfectious forms of HDV. Although delta antigen (*green*) and genomic HDV RNA can be assembled using only the HBV S protein, the particles are noninfectious, because HDV, like HBV, requires the pre-S1 domain of L to be infectious. (Adapted from an unpublished figure provided by Dr. Stephan Urban.)

used in genome-wide searches for HDV-like ribozymes. Many have been found and confirmed as being expressed as RNAs and of having ribozyme activity.¹⁸³ Such information may assist in understanding the origin of HDV.¹⁶⁷

Stages of Replication

Attachment and Entry

The only natural susceptible cells are hepatocytes. HDV will infect cultures of primary human hepatocytes (PHHs)⁷⁵ and the HepaRG cell line.^{74,164,176} Attachment is via cell surface glycosaminoglycans^{100,154} and is increased more than 15-fold by 4% to 5% polyethylene glycol.^{8,75,164} After attachment, virus entry is relatively slow.^{171,176} For example, after 3 hours of exposure of PHH to HDV, more than 50% of the virus is still on the cell surface and susceptible to entry inhibitors, such as a peptide corresponding to part of the pre-S1 domain at the N-terminus of HBV L.¹⁵⁵ Antibodies to this region can block infectivity.¹⁷⁶ This supports other evidence that HBV L must be present on HDV and HBV for the particles to be infectious. While many host entry receptors have previously been proposed¹⁷⁶ an important new study demonstrates that sodium taurocholate cotransporting peptide is a functional receptor for both HBV and HDV.^{197a} Although HDV genomic RNA and multiple copies of HDAg are present within virions, there is no evidence for organization into a specific nucleocapsid structure of RNA within a proteinaceous shell. In contrast, RNA might be wrapped around multimers of HDAg.³

Transcription

In the Baltimore classification system,⁶³ the HDV RNA genome is single stranded and of negative polarity. However,

unlike other RNA viruses of negative polarity, HDV does not encode its own RNA polymerase. It somehow redirects the host RNA polymerase II, which normally uses DNA templates, to copy the HDV RNAs.^{72,169} Some studies suggest that replication involves redirection of an additional host polymerase, maybe RNA polymerase I.¹⁷⁴

Translation

The exact complement of the HDV genome, the antigenome, contains the coding region for HDAg. However, translation is considered to be from a third and less abundant RNA that has features of a typical host messenger RNA (mRNA). It is linear, with a 5'-cap and a 3'-polyadenylation (see Fig. 69.4).

There are actually two forms of HDAg translated. First, S-HDAg is typically 195 amino acids in length. It is essential for the accumulation of HDV RNAs during replication.³⁷ Five reasons, some or all of which may be correct, have been proposed to explain the basis for this essentiality.¹⁷⁰ S-HDAg is too small to be a polymerase; however, some studies suggest that it might help to redirect a host polymerase in RNA-directed transcription.¹⁹⁶

During replication, there is posttranscriptional RNA editing of HDV RNA by a class of enzymes referred to as ADAR, or adenosine deaminases acting on RNA. An important and specific target is on antigenomic RNA at what corresponds to the middle of the amber termination codon UAG for S-HDAg.²⁵ Targeting to this site involves the rod-like folding of the RNA. Deamination to inosine leads to mis-transcription so that mRNAs arise with UGG that codes for tryptophan and allows translation of a 19 amino acid longer form of HDAg

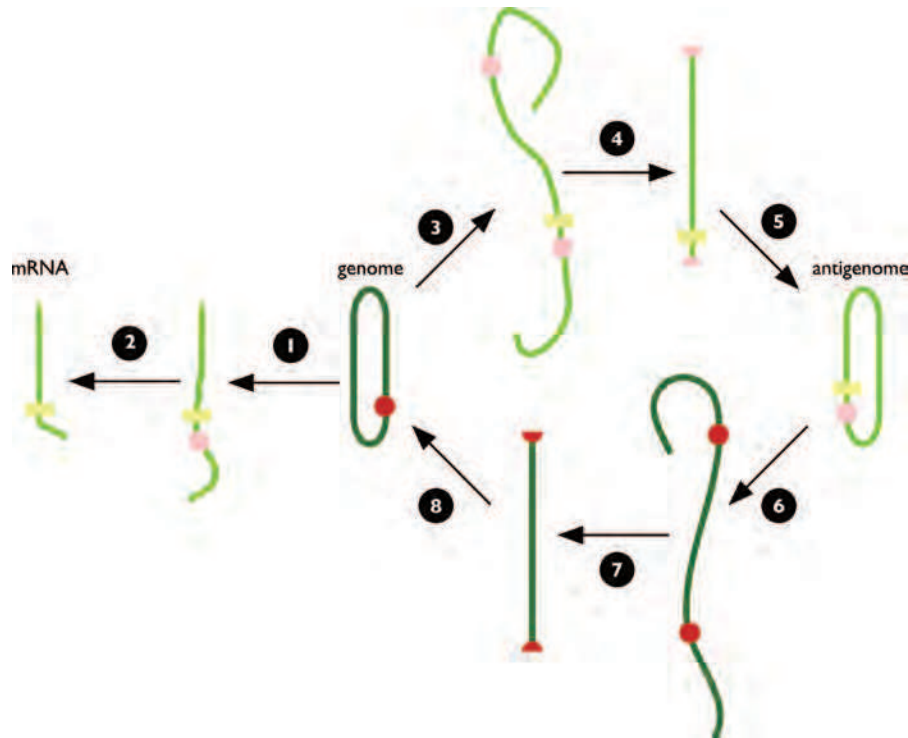


FIGURE 69.4. The three processed RNAs of hepatitis D virus and a double-rolling circle model for replication. The *genome* is a 1,700 nucleotide, single-stranded RNA. It is circular in conformation and can fold on itself, with about 74% base pairing, to form an unbranched rod-like structure. This is the RNA assembled into virions. In infected cells, there exists not only this genome but an RNA that is its exact complement, the *antigenome*. The antigenome contains the open reading frame for the small delta antigen; however, the actual template for this antigen is a messenger RNA (mRNA) that is less than full length, linear in conformation with a 5'-cap and 3'-polyadenylation. The polyadenylation signal is indicated in *light green*. Both the genome and antigenome contain site-specific ribozymes, with sites indicated in *red* and *pink*, respectively. Steps 1 through 8 present a model of RNA-directed replication. **1–2:** The genome RNA acts as the template for transcripts that are processed to become mRNA. **3:** The genome also acts as template for transcripts that proceed more than once around the template. **4–5:** Such multimeric, linear RNAs have at least two copies of the antigenomic ribozyme and can be cleaved to release unit-length linear RNA, which in turn is ligated to produce new circular antigenomic RNA. **6–8:** Similarly, the new antigenomic RNA can act as the template for the transcription of multimeric, linear RNA, which is processed to produce new genomic RNA circles. (Adapted from Taylor JM. Hepatitis delta virus. *Virology* 2006;344:71–76.)

(L-HDAg) that does not support HDV RNA accumulation. It is a dominant negative inhibitor of accumulation supported by S-HDAg.³⁷ Furthermore, four amino acids from the new C-terminus is a unique cysteine that acts as the target for farnesylation. After this modification occurs, L-HDAg can interact with HBV envelope proteins and thereby support the assembly of new virus particles.⁶⁹

S-HDAg and L-HDAg are highly basic proteins with electrophoretic mobilities of 24 and 27 kDa, respectively.^{10,13} They share 195 amino acids of N-terminal sequence and, as

represented in Figure 69.5, have three common features.^{10,13} First, near the N-terminus is a domain essential for the formation of dimers. A crystal structure of this region indicates an antiparallel coiled-coil interaction.²⁰² Second, a bipartite domain facilitates nuclear localization,^{36,194} although just one part is sufficient to transport a fusion protein.⁴ Finally, a bipartite domain with arginine-rich motifs allows HDAg to bind to viral RNAs. Early studies *in vitro* indicated specific recognition of the rod-like structures of genomic and antigenome RNA,³⁸ whereas recent studies find no specificity for binding to RNA

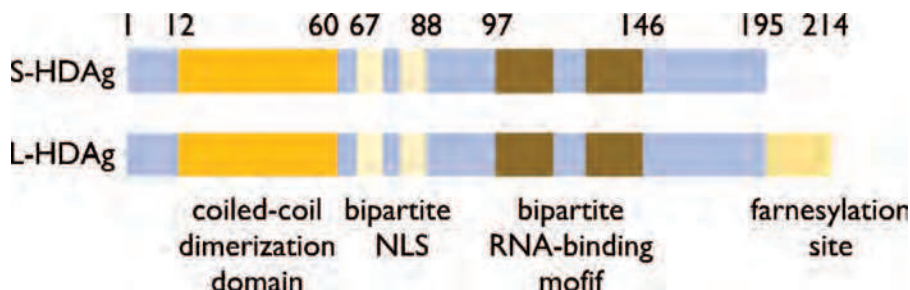


FIGURE 69.5. Features of delta antigens S and L. They differ only in the unique carboxyl-terminal extension of delta antigen L, which includes a site for an essential farnesylation. They share many features, as shown. *Numbers* indicate amino acid position.

versus DNA, single- versus double stranded, relative to rod-like RNAs.³

HDAg can undergo posttranslational modifications: serine phosphorylation, arginine methylation, lysine acetylation, and lysine sumoylation.^{82,175} Some of these sites have been mapped hoping to determine whether the modifications confer ability to contribute to one or more aspects of viral RNA transcription and accumulation.¹⁷⁵ A concern for such implications is that the extent of such modifications at a particular site can be low.

Attempts have been made without success to determine a structure for full-length S-HDAg. This failure agrees with other studies that indicate a high level of intrinsic disorder.³ Typical of disordered proteins, HDAg readily forms higher-order multimers *in vitro*.³

Replication of Genomic RNA

Transcription takes place in the nucleus. Nuclei isolated from infected cells can continue transcription of both genomic and antigenomic RNAs in a manner that depends on the functionality of host RNA polymerase II.³¹ Some studies invoke the need for a second host polymerase, possibly polymerase I.¹⁷⁴ S-HDAg might facilitate redirection of the host polymerase(s) or the ability to achieve elongation of transcripts on HDV RNA templates.¹⁹⁵ Binding of S-HDAg to RNA polymerase II has been reported.^{23,196} However, an *in vitro* system achieving reconstitution of HDV RNA-directed transcription, with initiation and extensive elongation of nascent RNA, has yet to be established.

Figure 69.3 is a model of HDV replication adapted from studies of plant viroids.⁶⁵ Viroids have similarities to HDV. Their RNA genomes are single stranded, often circular, often with ribozymes, and with transcription in all cases via redirection of host RNA polymerases.¹⁶⁷ Many viroids redirect a plant RNA polymerase II.⁶⁵ Viroids are smaller than HDV, with genomes of 250 to 400 nucleotides. They do not encode proteins and do not have helper viruses.

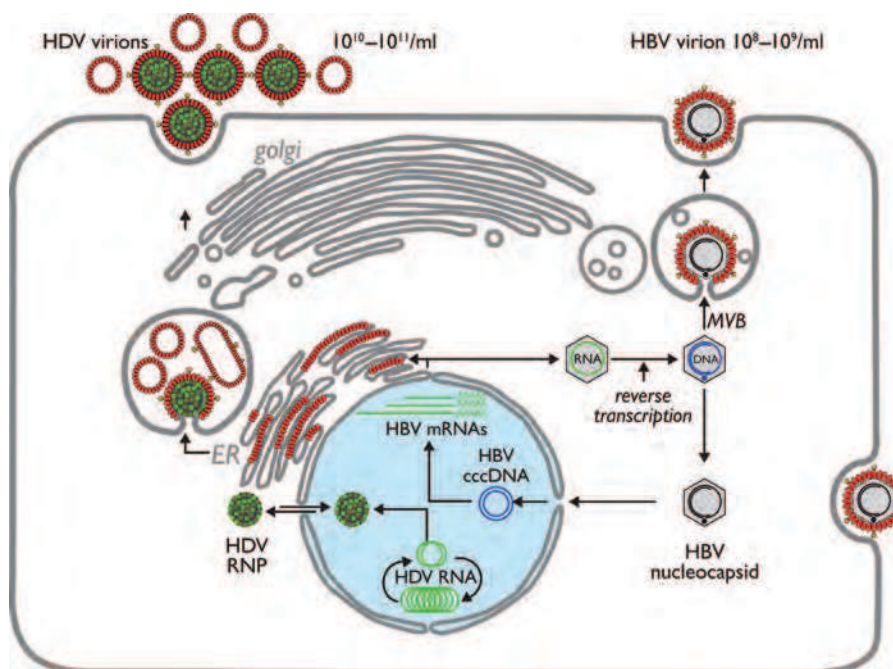
The model is referred to as a double-rolling circle because both genomic and antigenomic RNAs are circular templates. Transcription can produce RNAs that are even multiples of the unit-length. These can be processed to unit-length by the genomic and antigenomic ribozymes. Such cleaved RNAs then adopt the rod-like folding to facilitate ligation to make new RNA circles. Ligation may be achieved by reversal of the ribozyme¹⁵⁶ or by action of a host RNA ligase.¹³² The model is modified relative to that used for viroids to allow for the synthesis of HDAg mRNA. This RNA transcript has a unique initiation site and is recognized by the host polyadenylation machinery via a polyadenylation signal sequence. The sites for initiation of multimeric RNAs that go on to be processed genomes and antigenomes have yet to be determined. *In vitro* studies have shown that the ends of the rod-like folding for genomic and antigenomic RNA will bind polymerase II.⁷¹ However, they will also bind polymerase I and polymerase III.⁷³

HDV replication produces millions of HDAg per cell.³⁰ More than 100 host proteins are identified as interactors with HDAg. This includes several subunits of polymerase II and other proteins that are essential for HDV RNA accumulation.²³

Assembly and Release

HBV envelope proteins L, M, and S are used for HDV assembly. In nature, HDV can only be released from a cell that has been co-infected with HBV. It remains to be determined whether there is some specific order for this co-infection—that is, does HDV enter before or after HBV? HBV assembly is highly inefficient, leading to a huge excess (1,000–100,000-fold) of empty spheres and filaments (see Figs. 69.2 and 69.4). As represented in Figure 69.6, HDV exploits the excess of these SVPs. HDV nucleocapsids can be assembled using only HBV S, although such particles are not infectious. It is only when relatively small amounts of L are also present that the particles are infectious.¹⁶⁵ M is not essential for assembly or infectivity.¹⁶⁶ Assembly uses envelope proteins already associated with

FIGURE 69.6. Assembly of particles in a cell infected with both hepatitis D virus (HDV) and hepatitis B virus (HBV). Although HDV and HBV are assembled with the same HBV envelope proteins, the mechanisms of assembly and release may be quite different. HDV might assemble like subviral particles, using a secretory pathway. In contrast, HBV seems to use the multivesicular body for release. (Adapted from an unpublished figure provided by Dr. Camille Sureau.)



membranes of the endoplasmic reticulum and/or Golgi apparatus; however, HBV infectious particles and SVPs are assembled and released from cells by distinct pathways with separate host factor requirements.¹⁸² HBV uses the compartment known as the multivesicular body, whereas SVPs are assembled using a secretory pathway. Camille Sureau explains HDV assembly as using a secretory pathway, an interpretation fitting well with observations that HDV titers in the serum of infected patients can reach 10^{11} per milliliter, which is much higher than the maximum of 10^9 observed for HBV.

L-HDAg is essential for HDV assembly. Experimentally, it can be assembled and released by HBV envelope proteins in the absence of HDV RNAs.¹⁴⁷ It is not yet understood how when HDV replication is occurring, that it is only the genomic RNA assembled.

HEPATITIS DELTA VIRUS HETEROGENEITY AND VIRAL GENOTYPES

HDV is characterized by a high degree of genetic heterogeneity. Estimates of the rate of mutation fall between 3×10^{-2} and 3×10^{-3} base substitutions per genome site per year.⁹⁹ HDV circulates within a single infected host as a quasispecies.⁵¹ Eight

distinct HDV genotypes have been proposed⁴⁹ (Fig. 69.7 and eBook supplementary information). The genetic heterogeneity of HDV is not uniformly distributed over the viral genome; the most conserved region is within the ribozymes.¹³⁰ Genotype 1 has a worldwide distribution, whereas the remaining genotypes appear to be more geographically restricted (see Fig. 65.7). Genotypes 2 and 4 are found predominantly in the Far East, and genotype 3 is found exclusively in South America. Genotypes 5 through 8 have been exclusively found in West and Central Africa. In Taiwan, genotypes 1 and 4 overlap, and chimeric forms have been detected in co-infected patients.^{181,192} Such *in vivo* recombination, presumably via a template-switching mechanism,³⁴ is a unique phenomenon for a virus whose replication depends on a host RNA polymerase.

HOST RANGE AND EXPERIMENTAL INFECTION IN ANIMALS

In nature, HDV infection has been found only in humans. The host range for productive replication of HDV is limited to those species that support the replication of a hepadnavirus capable of supplying the essential helper function—the surface antigen envelope. These include the chimpanzee (HBV)¹³⁶ and

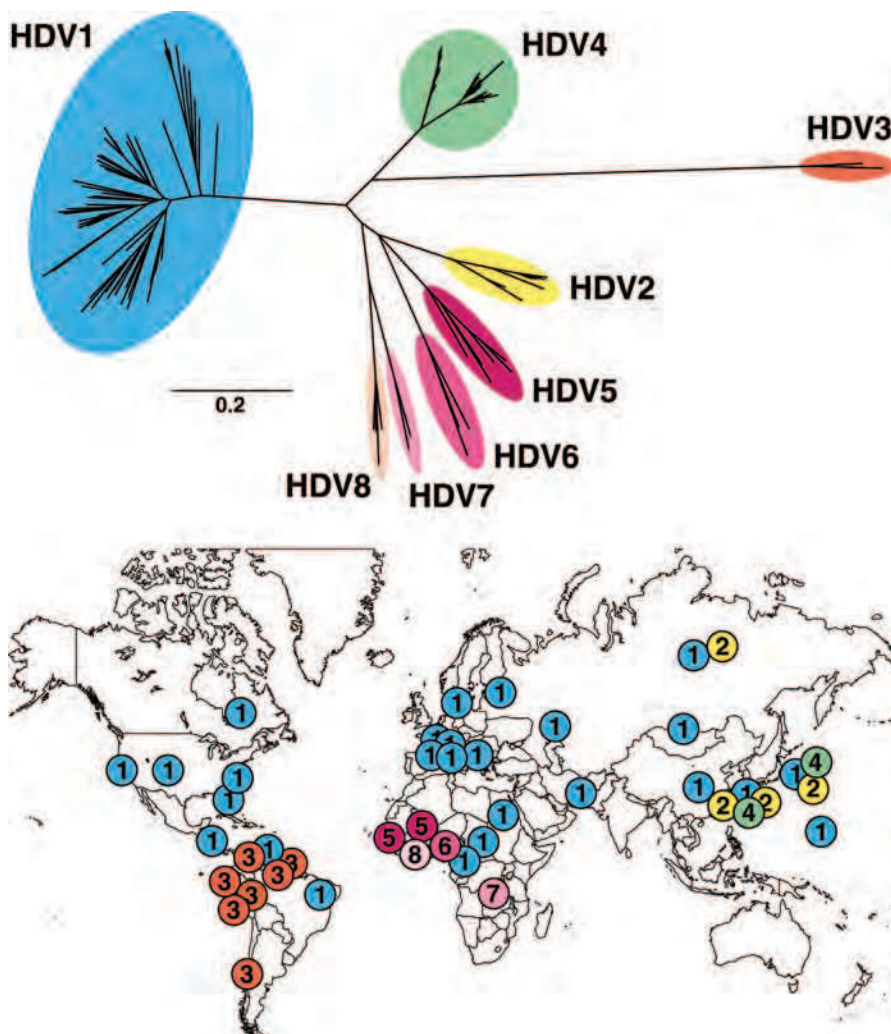


FIGURE 69.7. Geographic distribution of hepatitis delta virus (HDV) genotypes. Bayesian phylogenetic reconstruction based on full-length HDV sequence retrieved from databases (March 2011, $n = 167$). Alignment was performed using Clustal X. MCMC bayesian analyses were conducted in parallel for three million generations using MrBayes until an average standard deviation of split frequencies of 0.025. The first 50% of trees were burn in and the final display obtained with FigTree in the radial option. Selected full-length sequence isolates were kept for clarity. Circles on the map correspond approximately to geographic origin of selected isolates, identified by genotype. The horizontal bar indicates the percentage expected substitution per position. (Courtesy of Dr. Paul Deny).



the eastern woodchuck (woodchuck hepatitis virus [WHV]).¹²⁴ Infection of chimpanzees mimics that of humans, and much of our understanding of HDV infection is based on studies in this experimental model; both co-infection (simultaneous infection with HBV and HDV) and superinfection (infection of an HBsAg-positive subject) have been documented.¹²⁹ Co-infection results in moderately severe hepatitis consisting of a unimodal or bimodal elevation of serum alanine aminotransferase (ALT) activity and associated hepatitis (see eBook supplementary information). Co-infections of HDV and HBV usually result in acute, self-limiting hepatitis D. Superinfection of HBV-carrier chimpanzees with HDV usually results in a more severe acute hepatitis, which progresses to chronicity in about 50% of superinfected animals.¹⁰⁹ The liver appears to be the only organ involved in HDV infection of chimpanzees. Hepatitis D in chimpanzees is generally more severe than other types of human viral hepatitis in this animal model.

HDV of human origin also infects woodchucks (*Marmota monax*) that are infected with WHV, causing acute or chronic hepatitis in a high proportion of animals.¹²⁴ Hepatitis D in woodchucks is similar to that seen in chimpanzees; the liver is the only organ involved. Replication of HDV in woodchucks depends on the presence of replicating WHV and replacing the HBsAg envelope of HBV with the WHV envelope (WHsAg).

Several efforts have been made to develop a mouse model of HDV infection. A limited infection was achieved by intravenous inoculation of HDV or HDV complementary DNA (cDNA) or HDV RNA, resulting in a single round of replication of the HDV genome in hepatocytes.^{33,112} In the absence of a helper hepadnavirus, no release of complete virus and no secondary infection can occur. To develop models that may be more physiologically relevant, mice have been modified to contain human hepatocytes.¹⁴ A high degree of human hepatocyte chimerism (up to 95%) has recently been achieved using *Fab^{-/-}Rag2^{-/-}Il2rg^{-/-}* mice, which were shown to sustain the growth of both HBV and hepatitis C virus (HCV).¹² Efforts to develop transgenic mouse models of HDV RNA replication have been successful, resulting in HDsAg expression in multiple tissues, particularly in skeletal muscle, although no pathologic changes were observed in any tissue, including the liver, supporting the hypothesis that HDV is not directly cytopathic.¹²³ Although these models have expanded the possibilities to study HDV infection, their relevance to pathogenesis remains to be established.

PATHOGENESIS AND PATHOLOGY

Entry Into the Host and Site of Primary Replication

In accordance with the dependence of HDV on HBV, natural infection with HDV follows the same parenteral route of transmission of the helper HBV. In natural and experimental infections, the only infected cell is the hepatocyte. A recent study has demonstrated that sodium taurocholate polypeptide is a functional receptor on hepatocytes for both HBV and HDV.^{197a} If one circumvents the viral entry process by transfection, the HDV genome can replicate in virtually any animal cell. Thus, the restriction of HDV (and HBV) is at the level of virus entry. HDV is assembled in infected hepatocytes and released into the blood. Using the duck hepatitis B virus (DHBV) model, it has

been proposed that the virus first reaches the liver sinusoids, where it is uptaken by liver sinusoidal endothelial cells to be actively transported to the underlying hepatocytes.¹⁸ Whether this model also applies to HDV remains to be established.

Mechanisms of Liver Damage

The pathogenesis of hepatitis D is still poorly understood. HDV is a highly pathogenic virus that causes the most severe forms of acute and chronic viral hepatitis. Controversy exists, however, regarding the relative role played by the direct pathogenic effects of HDV versus immune-mediated liver damage. A direct cytopathic effect of the small form of the delta antigen, S-HDAs, expressed *in vitro* at high levels in the absence of replication of the HDV genome has been reported.⁴³ In other *in vitro* models, replication of the HDV genome was shown to interfere with the ability of transfected cells to expand and form clones¹⁷⁹ and, more recently, to cause cell cycle arrest and cell death.³⁰ These *in vitro* observations are not supported, however, by (a) the presence of intact hepatocytes containing HDAs in human liver biopsies,¹¹⁰ (b) the lack of liver injury documented in liver grafts expressing HDAs in the presence of low levels of HBV replication,¹¹⁹ (c) the lack of liver pathology in a transgenic mouse model expressing both the small and large forms of HDAs,¹²³ and (d) the observation that some HDV-infected cell lines can survive *in vitro* for at least several cell divisions.¹¹ Thus, these data argue against a direct cytopathic effect of HDV and support the notion that *in vivo* damage to infected hepatocytes is immunologically mediated. As with the other hepatitis viruses, peak HDV replication precedes the peak histopathologic changes. Viral replication is often markedly diminished at the time of the peak of pathology when the adaptive humoral and cellular immune responses have begun.

Liver Fibrogenesis and Hepatocarcinogenesis

The mechanisms whereby HDV induces the most rapidly progressive form of liver fibrosis are unknown. L-HDAs may play a role in HDV-related fibrogenesis by regulating transforming growth factor beta (TGF- β)-induced intracellular signaling.⁴² Of note, studies by microarray technology have shown distinct profiles of gene expression in HDV-related cirrhosis compared to that associated with other hepatitis viruses (P. Farci et al, unpublished data).

Although hepatocellular carcinoma (HCC) develops in a proportion of patients with HDV infection, it is still uncertain whether this virus possesses inherent oncogenic properties. Recent data have shown that HDV replication epigenetically enhances the expression of clusterin—a protein that plays a role in tumorigenesis and is highly expressed in HCC—leading to decreased sensitivity to adriamycin.¹⁰¹ Moreover, expression of L-HDAs was shown to increase tumor necrosis factor alpha (TNF- α)-induced NF κ B activation via direct interaction with the transcription factor TRAF2.¹²¹ The relevance of these observations to hepatocarcinogenesis in HDV-infected individuals remains to be established.

IMMUNE RESPONSE

Innate Immunity

The interaction between the virus and the host immune response is key in determining the outcome of viral infections.

Studies of gene expression profiling in weekly liver biopsies of experimentally infected chimpanzees have provided evidence that HBV does not induce expression of intrahepatic genes during entry and expansion, suggesting that it behaves as a stealth virus early in the infection;¹⁸⁸ nevertheless, recovery occurs in more than 90% of cases. By contrast, HCV induces a strong early innate immune response but seems to be resistant to the antiviral effects of the innate immunity, resulting in chronic infection in up to 80% of the infected individuals.¹⁶² Recent studies in chronic HBsAg-positive chimpanzees superinfected with HDV reveal that HDV, like HCV, induces a strong innate immune response, even though the infection in this setting becomes chronic in about 90% of cases (R. H. Purcell, unpublished data). In this respect, *in vitro* data using a hepatoma cell line have documented an inhibitory effect of HDV on interferon alpha (IFN- α)-elicited intracellular signaling, mediated by blockade of Tyk2 activation, resulting in selective impairment of nuclear translocation of STAT1 and STAT2.¹²⁷ Conversely, up-regulation of the interferon-inducible gene *MxA* by L-HDAg, resulting in HBV repression, has been reported in another study.¹⁸⁹ Thus, HDV seems to induce a robust innate response; however, as in HCV, this is ineffective in viral clearance.

Adaptive Immunity

HDV elicits specific antibodies of immunoglobulin G (IgG) and immunoglobulin M (IgM) class against HDAg, which are typically detected during the acute and chronic phase of infection¹⁴⁰ (Fig. 69.8). These antibodies do not have neutralizing activity because they are directed against the HDAg, the only protein encoded by the HDV genome, which is located in the interior of the virion. Because both HBV and HDV share the same envelope, HBsAg, antibodies to HBsAg (anti-HBs) confer protective immunity to both viruses.

The contribution of cell-mediated immune response in the defense against HDV, as well as in the pathogenesis of HDV disease, remains largely undefined.¹⁸⁵ The limited available data suggest that the strength of the host HDAg-specific T-cell response in peripheral blood of patients with chronic hepatitis D correlates with the degree of control of HDV infection.^{83,117} More recently, the frequency of antigen-specific cytotoxic CD4⁺ T cells was found to be higher in patients with HDV infection than in those with HBV or HCV infection.⁷

ROLE OF HEPATITIS B AND D VIRUS GENOTYPES IN PATHOGENESIS

The role of the various HDV and HBV genotypes in the pathogenesis and severity of hepatitis D has been an area of active investigation. Genotype 1, the most prevalent worldwide, has been linked with a broad spectrum of disease severity.¹⁴⁰ Genotypes 2 and 4 have been associated with milder forms of the disease,^{163,191} whereas genotype 3 has been linked with outbreaks of severe and fulminant hepatitis in northern South America.^{26,70,105} Although initially associated almost exclusively with HBV genotype F,²⁷ HDV genotype 3 has since been found in patients with HBV genotypes A and D, although less frequently,⁷⁰ suggesting that HBV genotype F is not essential for the development of fulminant hepatitis D. Further studies are needed to elucidate whether major biological differences exist among HDV genotypes, including the recently described African genotypes, in terms of viral replication, packaging, infectivity, transmissibility, and pathogenicity, as well as the role of the HBV genotypes and the variability of the host immune responses.

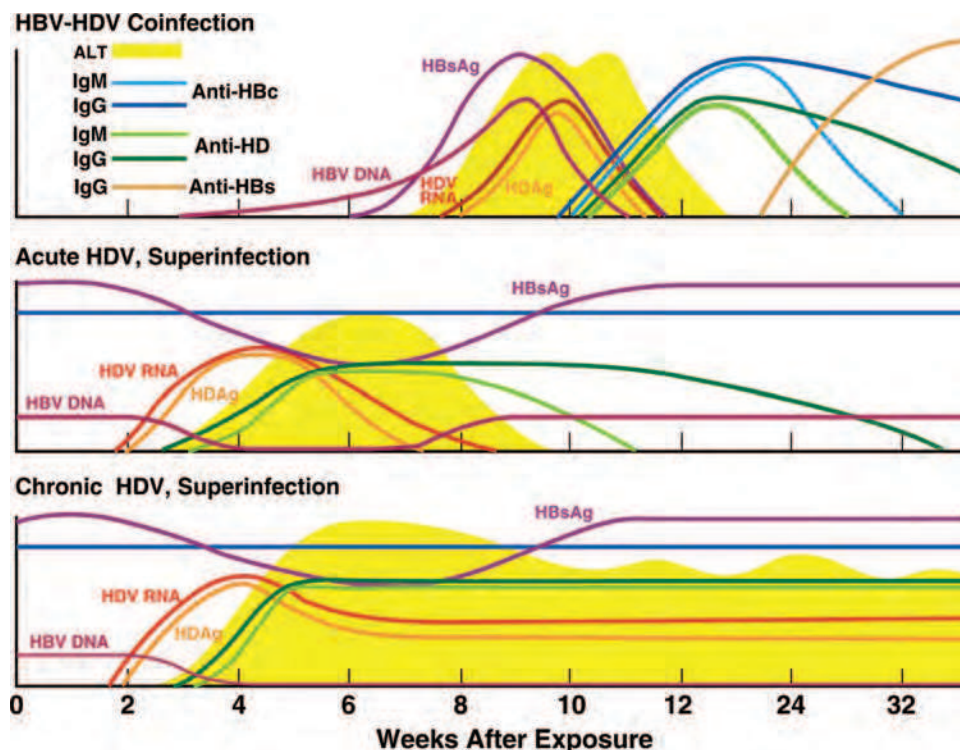


FIGURE 69.8. The serologic patterns of type D hepatitis: co-infection and superinfection. **Top:** Coexistent acute hepatitis B and hepatitis D. **Middle:** Acute hepatitis D superimposed on a chronic hepatitis B virus infection. **Bottom:** Acute hepatitis D progressing to chronic hepatitis, superimposed on a chronic hepatitis B virus infection.

LIVER PATHOLOGY

Direct pathologic changes in hepatitis D are limited to the liver—the only organ in which HDV is known to replicate. Histologic changes consist of hepatocellular necrosis and inflammation typical of acute or chronic viral hepatitis. No distinguishing features of the liver histology differentiate hepatitis D from other forms of viral hepatitis, except that the former tends to be more severe and that elements of both acute and chronic hepatitis may coexist if acute HDV infection is superimposed on chronic HBV infection. In acute hepatitis D, inflammatory cells, primarily lymphocytes and Kupffer cells, are prominent in the parenchyma and portal areas. Hepatocytes may be swollen and undergoing eosinophilic necrosis. Pathologic changes are often patchy and focal, except in more severe cases, when confluent, submassive, or massive necrosis is seen. Inflammation and hepatocellular necrosis are also typical of chronic hepatitis D, although inflammation is largely portal, predominantly periportal, with aggregates of lymphocytes and Kupffer cells (Fig. 69.9A). Necrosis of hepatocytes is usually evident, and its distribution is typically periportal. The degree of periportal necrosis (interface hepatitis) is more prominent than in the other forms of chronic viral hepatitis and is often accompanied by active cirrhosis (see Fig. 69.9B). Once cirrhosis is established, HCC may eventually develop in some cases (see Fig. 69.9C). HDAg is commonly detected in the hepatocyte nuclei (see Fig. 69.9D).

A histologic lesion peculiar to infection with HDV genotype 3 in northern South America, especially in the Amazon Basin, is a microvesicular steatosis that gives rise to a characteristic alteration of the hepatocytes (morula cells).¹²⁶ Similar cells, however, have been seen in severe hepatitis D elsewhere, including Africa and Italy.¹⁴⁰ Only nonspecific EM changes have been observed in natural and experimental HDV infections (see eBook supplementary information).

CLINICAL FEATURES AND NATURAL HISTORY

Acute Hepatitis D: Co-infection and Superinfection

Because HDV infection has an absolute requirement for an associated HBV infection, acute disease occurs as one of two patterns, depending on the prior HBsAg status of the HDV-infected subject: co-infection and superinfection. Simultaneous infection of a susceptible individual with HBV and HDV (co-infection pattern) results in both acute type B hepatitis and acute type D hepatitis. The clinical expression of acute hepatitis D acquired by co-infection with HBV may range from mild to severe or even fulminant hepatitis, the most severe form of acute disease, which is a rare sequela of acute viral hepatitis but is approximately ten times more common in hepatitis D than in the other types of viral hepatitis. The mortality rate of fulminant hepatitis was approximately 80% prior to the advent of orthotopic liver transplantation.

Co-infection of HBV and HDV usually results in an acute self-limited hepatitis that is clinically indistinguishable from the typical acute hepatitis B (see eBook supplementary information). The outcome is a complete recovery, as typically seen in acute hepatitis B; in only 2% of cases, it may progress to chronicity.²⁴

HDV infection of an individual chronically infected with HBV (superinfection pattern) causes a generally severe acute hepatitis with a relatively short incubation period that may run to a fulminant course. In this setting, the pre-existing HBsAg status provides the biological background for the full expression of the virulence of HDV. The acute hepatitis in the superinfection pattern is usually marked by an overt clinical illness and jaundice. Clinically, it may present as an exacerbation of a pre-existing chronic hepatitis B leading to liver decompensation or as a new hepatitis in a previously asymptomatic HBsAg carrier. If the HBsAg state is unknown, it may be misdiagnosed as acute

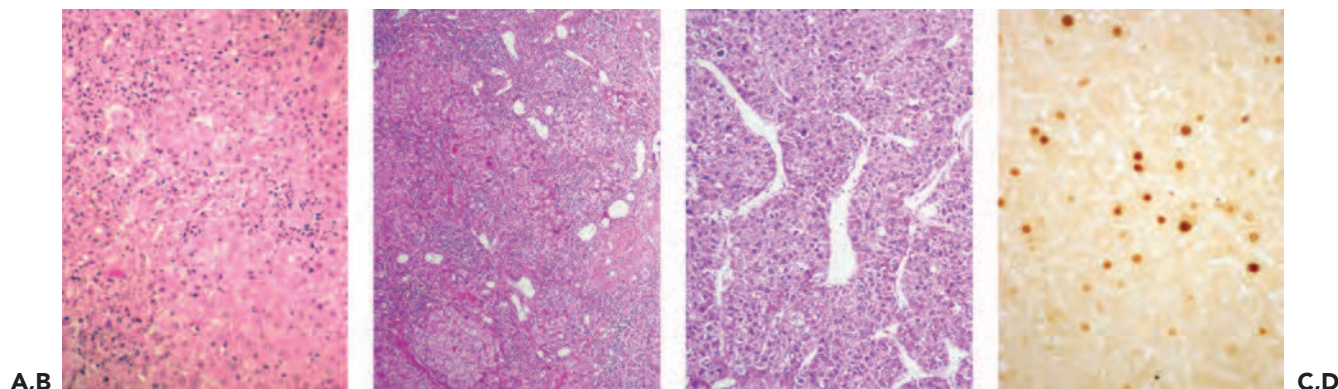


FIGURE 69.9. Histologic stages of hepatitis D. Chronic hepatitis D showing marked periportal (interphase) and lobular inflammatory activity (**A**). Aggregates of Kupffer cells and lymphocytes are seen around hydropic and necrotic hepatocytes. (Hematoxylin and eosin [H&E] $\times 200$). (Courtesy of Dr. Sugantha Govindarajan). During the progression of chronic hepatitis D, active cirrhosis develops in a high proportion of patients (**B**) (H&E $\times 100$). Hepatitis D virus–related cirrhosis is characterized by a marked septal and periseptal inflammation associated with hepatocytolysis. Once cirrhosis has become established, hepatocellular carcinoma may develop in some patients (**C**) (H&E $\times 200$) (Panels B and C courtesy of Dr. David Kleiner). In (**D**), immunoperoxidase staining shows hepatitis delta antigen within the nuclei of hepatocytes (H&E $\times 200$). (Courtesy of Dr. Sugantha Govindarajan; adapted from Farci P. Hepatitis D. In: Dooley JS, Lok ASF, Burroughs AK, et al, eds. *Sherlock's Diseases of the Liver and Biliary System*. 12th ed. Indianapolis: Wiley-Blackwell; 2011:393–405.)

hepatitis B.⁵⁸ Superinfection with HDV is often associated with fulminant hepatitis.

Because the HBsAg state permits the continuous replication of HDV, superinfection of acute hepatitis D leads to chronic hepatitis D in more than 90% of cases.²⁴ A subacute, rapidly progressive form of HDV superinfection has been seen in HBsAg carriers among the Yucpa Indians, an indigenous population of Venezuela.¹²⁶ Such patients often die within 18 months of disease onset.

Chronic Hepatitis D

Chronic viral hepatitis, a frequent sequela of hepatitis D acquired through the superinfection pattern, is associated with the most severe and progressive form of chronic liver disease, often leading to cirrhosis. Unlike other forms of chronic viral hepatitis, chronic hepatitis D is initiated in about half the patients by a clinically apparent acute hepatitis, which likely represents the time of HDV superinfection. The clinical presentation of chronic hepatitis D is variable and shows no distinctive clinical features, except for a higher frequency of splenomegaly. It may be asymptomatic and discovered incidentally, or it can present with general symptoms such as fatigue, malaise, anorexia, and right upper quadrant discomfort, or with the complications of cirrhosis. Typically, patients with chronic hepatitis D exhibit persistently high levels of serum ALT and aspartate aminotransferase (AST), which tend to decrease with the progression of the disease to the end-stage liver cirrhosis. Patients with chronic hepatitis D are usually positive for IgG and IgM antibodies to HDV and show high levels of HDV viremia, although the viral load does not seem to correlate with the severity of the disease.²⁰¹ The production and serum titer of HDV are correlated with the amount of HBsAg in serum in both *in vitro* and *in vivo* studies.¹⁵⁷ Although most chronically HDV-infected patients are positive for antibodies to hepatitis B e-antigen (HBeAg) with little or undetectable HBV DNA levels in serum,¹⁴⁹ a minority of them are HBeAg positive with high levels of HBV DNA replication, a pattern most frequently seen in intravenous drug addicts.¹⁴⁰ The usual suppressive effect of HDV on HBV replication suggests that the liver damage is caused by the ongoing HDV infection and not by the accompanying HBV infection, as further demonstrated by the lack of correlation between HBV replication levels and ALT levels. Chronic hepatitis D can be associated with several autoimmune manifestations, including the presence of autoantibodies against microsomal membranes of the liver and kidney (LKM-3), human thymocytes, and nuclear lamin C.¹¹⁸

In addition to the typical acute and chronic hepatitis D, a third form of HDV infection has been described in the liver transplantation setting. In this unconventional form, defined by some authors as *latent infection*,¹¹⁹ markers of HDV infection are detectable in the absence of markers of HBV infection, suggesting that HDV might replicate at low levels in the absence of the helper function of HBV. However, subsequent analysis with sensitive molecular assays has documented low-level HBV replication coincident with HDV replication, thus reaffirming the dependence of HDV on the HBV helper function and the absence of true latency.¹⁵⁸ Nevertheless, the temporal sequence of HBV and HDV reinfections in transplanted patients remains to be determined.

Natural History

HDV is a highly pathogenic virus that causes the most severe and rapidly progressive form of viral hepatitis at all ages. Studies on the natural history of chronic hepatitis D have shown that cirrhosis develops in 70% to 80% of patients within 5 to 10 years¹⁴¹ and at a younger age compared to hepatitis B–associated cirrhosis.⁵⁹ The risk of developing cirrhosis is about threefold higher in HDV-infected patients compared to those infected with HBV alone.⁵⁹ Once established, HDV-related cirrhosis can be a stable disease compatible with a good quality of life for another decade, although later in the course a high proportion of patients die of liver decompensation or HCC unless they receive liver transplantation.¹⁴⁵ The lack of large prospective studies on the course of chronic hepatitis D makes it difficult to define the proportion of patients who develop each of these long-term complications. Most of the data are inferred from retrospective studies. In two recent longitudinal studies conducted in Italy,^{116,144} liver decompensation—not HCC—was the dominant complication of HDV cirrhosis, developing in 29% to 33% of cirrhotic patients, whereas HCC was observed in 13% to 25%. The estimated annual incidence rates were 2.5% and 2.7% for liver decompensation and 1% to 2.8% for HCC.^{116,144} The association of HDV with HCC has been demonstrated; however, the data on the risk of HCC in HDV-infected patients are not univocal. Whereas in some studies HDV appeared to be a promotion factor of HCC,^{77,178} with a threefold increased risk compared to patients with HBV cirrhosis alone,⁵⁹ in other studies HDV did not appear to increase the risk of HCC over HBV.^{19,44} Recent epidemiological studies in Mongolia, where the incidence of HCC is one of the highest in the world, showed the presence of markers of HBV and HDV infection in 25% of patients with HCC.¹²⁰ A high prevalence of HDV markers (20%) was also documented in patients with HCC in Turkey.¹⁷⁷ Whether HCC is the result of the underlying cirrhosis (a known risk factor for HCC), a direct carcinogenic effect of HDV, or the cumulative carcinogenic effect of HBV and HDV remains to be established.

Although HDV infection is usually associated with severe and progressive liver disease, a few studies have documented a high proportion of individuals with antibodies specific for HDV antigen (anti-HD) without evidence of liver disease.^{5,77} In patients with chronic HDV infection, spontaneous clearance of HBsAg is a rare event, although it may occur either spontaneously^{19,114} or following successful interferon therapy.^{37,116} Clearance of HBsAg is often associated with an improvement of HDV-associated disease, unless it takes place after HDV disease has progressed to advanced stages. Overall, these data suggest that the natural history of chronic hepatitis D varies widely.

The clinical picture of HDV disease worldwide is evolving as a consequence of a changing epidemiologic trend, which has led over the past two decades to a significant decline in the incidence of HDV infection in developed countries, especially in southern Europe.⁶⁶ This reduction has resulted in a dramatic decline in the frequency of new and florid forms of hepatitis D in Europe, with a preponderance of long-standing infections in patients who survived the impact of hepatitis D at the time of the HDV epidemic in the 1980s, who have either advanced cirrhosis or, in a minority, an indolent, nonprogressive disease.¹⁴⁵ Over the past few years, however, new florid HDV cases are on the rise again in western Europe as a

result of immigration from areas of the world where HDV is endemic.^{44,67,97,184}

Interaction of Hepatitis D Virus with Other Hepatitis Viruses

The interaction of HDV with other hepatitis viruses is complex and may lead to different patterns of viral interference. Despite the critical dependence of HDV on HBsAg for its assembly and transmission, a series of studies in chimpanzees¹²⁹ and humans^{79,149,201} have demonstrated that HDV suppresses the replication of HBV. Most HDV-infected patients are anti-HBe positive and have low to undetectable levels of serum HBV DNA, although studies performed with sensitive molecular assays have suggested that serum HBV DNA and HDV RNA levels tend to fluctuate over time in individual patients.^{131,153} By contrast, the serum levels of HBsAg are usually high despite the suppression of HBV DNA and are not significantly different from those that are HBV monoinfected.^{79,201} In a recent study,¹²² the intrahepatic levels of relaxed-circular HBV DNA and covalently closed circular HBV DNA (cccDNA) were shown to be approximately 2-log lower in HDV-infected than in HBV-monoinfected patients, in parallel with approximately 5-log lower levels of serum HBV DNA; however, the concentrations of HBsAg in serum were similar in the two groups, with higher levels of intrahepatic pre-S/SRNA and serum HBsAg amounts per cccDNA molecule in HDV-positive patients, showing that HBV replication was reduced, whereas the synthesis of envelope proteins was not. These results illustrate the “smart” selective mode of HDV-mediated HBV suppression, with maintenance of the capacity to synthesize large amounts of envelope proteins that are necessary for HDV assembly and transmission.

In patients with triple infection with HBV, HCV, and HDV, most reports have documented a suppressive effect of HDV on both HBV and HCV,^{6,85,148} whereas studies from Taiwan have demonstrated a suppressive role of HCV on HBV and HDV.^{102,103} Co-infection with human immunodeficiency virus (HIV) does not seem to influence the natural history of chronic hepatitis D, although the clinical prognosis of hepatitis D in HIV-infected patients is worse when associated with hepatitis C.²⁰ The prevalence of cirrhosis in HIV-infected patients is higher in HBV/HCV/HDV co-infection (67%) compared to HBV/HCV co-infection (42%) or to infection with HBV alone (6%), emphasizing the highly pathogenic effects of HDV.²⁸ In HIV-infected patients, the antibody response to HDV may be more elusive as a consequence of immune suppression, leading to the reappearance of serum HDsAg in some patients.¹⁴³ Also in the HIV setting, HDV was shown to exert a suppressive role on both HBV and HCV.¹⁷

EPIDEMIOLOGY

Age

HDV infects all age groups, although perinatal HDV transmission (with HBV) is not common in regions of the world, such as Asia, where perinatal transmission of HBV is common. Hepatitis D affects children in the Amazon Basin and has occurred among children with hemophilia in industrialized countries, especially before blood was screened for HBV. It also occurs among young adults over most of its geographic distribution

and especially among young users of illicit drugs in industrialized countries.

Morbidity and Mortality

The ratio of clinical to subclinical cases of HDV co-infection and superinfection is unknown; however, the general severity of both forms of infection suggests that most cases are clinically important. The chronicity rate is 2% for co-infection and 90% for superinfection.²⁴ The mortality rate of hepatitis D is 2% to 20%—ten times higher than the rate for hepatitis B, the next most severe type of viral hepatitis.

Epidemics

The epidemiology of HDV, which is principally transmitted by exposure to blood, is not conducive to epidemics in the classic sense. Contamination of lots of clotting factors with HDV, as well as with HBV, HCV, and HIV, in past years was responsible, however, for the high incidence of hepatitis among recipients of these products and the high prevalence of serologic evidence of infection.^{146,172} Epidemics of severe hepatitis D have occurred among drug addicts, with prolonged epidemics leading to high cumulative attack rates. Outbreaks of severe hepatitis D have occurred in various regions of northern South America, especially the Amazon Basin, often involving fragile indigenous Amerind populations, such as the Waorani,¹⁰⁵ in Brazil,⁷⁰ as well as in southeastern Russia,⁶⁴ Mongolia,¹⁷³ and Greenland.¹⁶

Prevalence and Seroepidemiology

Seroprevalence studies of anti-HD in HBsAg-positive individuals have shown a worldwide, but nonuniform, distribution (Fig. 69.10). It has been estimated that 15 million HBsAg carriers are also infected with HDV, although these estimates are inaccurate because seroprevalence studies on blood donors or other healthy groups are not representative of the true prevalence because HDV-infected individuals are more likely to be sick than healthy.¹³³ Areas of high prevalence include the Mediterranean Basin, eastern Europe, the Middle East, central and northern Asia, East and Central Africa, the Amazon Basin, and certain islands of the Pacific. Hepatitis D is less common in eastern Asia, although there are regional exceptions. High-risk populations in industrialized countries include users of illicit parenteral drugs and (formerly) individuals exposed to blood or blood products. Infection with HDV is less frequent than infection with HBV or HIV among sexually promiscuous homosexual and heterosexual groups, suggesting that it is not typically a sexually transmitted disease; however, such transmission does occur, as shown by the high prevalence of HDV among prostitutes.^{47,193}

Over the past two decades, the introduction of universal HBV vaccination programs, the improvement in hygiene and socioeconomic conditions, and campaigns for the prevention of acquired immunodeficiency syndrome (AIDS) have led to a significant decline in the prevalence of HDV in industrialized countries, especially in Europe.¹³³ In Italy, the rate of antibody prevalence to HDV in HBsAg carriers decreased from 24.6% in 1983 to 8.3% in 1997,⁶⁶ and significant declines have been reported in Spain,¹⁰⁸ Turkey,⁴⁸ and Taiwan.⁸⁴ This trend, however, is not generalized, as an increased prevalence of HDV was recently found among injection drug users with chronic HBV infection in some cities, such as Baltimore, Maryland, with a

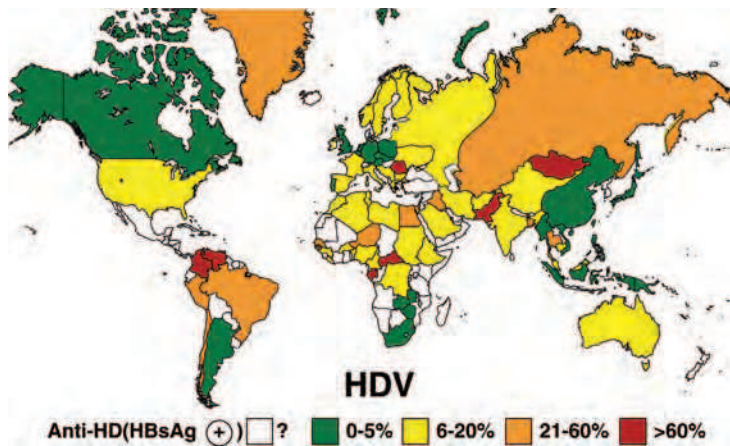


FIGURE 69.10. The worldwide distribution of hepatitis delta virus infection as measured by the prevalence of anti-HD in hepatitis B surface antigen (HBsAg)-positive individuals with acute or chronic hepatitis.

prevalence of 50% in 2005–2006 compared to 29% in 1988–1989.⁹¹ Efforts should be made to expand HBV vaccination coverage to high-risk populations.⁸⁰ In addition, screening of all HBsAg carriers for HDV should be performed and is often neglected, even in the industrialized world.^{44,80}

Despite the overall decline of HDV infection in the industrialized world, immigration from high-endemicity areas is posing a new threat—particularly in Europe, where in the past decade, it has halted the declining trend in the incidence of new cases of HDV infection. In Italy,¹⁶¹ England,⁴⁴ and Germany,¹⁸⁴ the prevalence of anti-HD has not further diminished after 1997, with stable rates ranging from 8% to 14% of HBsAg-positive subjects, mostly confined to immigrants from Romania, Albania, southern and eastern Europe, Africa, and the Middle East. In France, the infection is even increasing in the population of African immigrants.⁹⁷ Thus, the reservoir of HDV in Europe is sustained by two different pools of HDV-infected individuals: one composed by aging individuals who survived the HDV epidemic in the 1970s and 1980s, and the other by young individuals who migrated to Europe from HDV-endemic areas.¹³³

Along with the decline of HDV in developed countries, new foci of HDV infection have emerged in other regions of the world, such as southeastern Russia,⁶⁴ Okinawa, northern India, and Albania.¹³³ Thus, HDV remains a major public health problem in many developing countries where HBV vaccination has not been implemented. Recently, it has been reported that the levels of HDV endemicity are particularly high in Pakistan, Mongolia, Iran, India, Gabon, Vietnam, Tajikistan, Tunisia, Mauritania, and Turkmenistan.^{1,133}

Immunity

Second cases of hepatitis D have not been reported, suggesting that some form of immunity after acute, self-limiting infection exists; this may involve cellular immune mechanisms. HBV-carrier chimpanzees superinfected with HDV were resistant to rechallenge with HDV 6 months later.¹²⁹ Similar chimpanzees rechallenged 3 to 5 years after initial HDV infection were protected or developed mild transient hepatitis. All of the chimpanzees were anti-HD positive at the time of challenge, although most lacked antibodies to the envelope of HDV (i.e., HBsAg). In some of these chimpanzees, however, HDV might still have been replicating at low levels at the time of rechallenge,¹¹¹ and protection by interference rather than immunity could not be ruled out.

Some experimental evidence in woodchucks suggests that immunity to epitopes of the internal HDAg of HDV modifies the course of HDV infection.⁴⁵ Other studies, however, have failed to demonstrate protection.⁶²

Many viruses have developed mechanisms to subvert one or more functions of the host's innate immune mechanism. One study indicated that HDV replication can interfere with interferon signaling.¹²⁷ The HDV mRNA can be attacked by exogenously provided small interfering RNA (siRNA).³⁵ The genome and antigenome are not susceptible to siRNA, however, nor are they cleaved by the enzyme dicer.^{32,35}

DIAGNOSIS

Differential Diagnosis

Hepatitis D should be considered in individuals who are HBsAg positive or who have evidence of recent HBV infection, especially if they have been exposed to blood or blood products, or if they are members of a high-risk population or have lived in a high-risk environment (see later discussion). Other conditions to be differentiated from hepatitis D include co-infection of hepatitis A virus or HCV with HBV, superinfection of an HBsAg carrier with one of these two viruses, or hepatitis E virus, and exacerbation of chronic HBV infection, with disease recurrence.

Laboratory Diagnosis

Co-infection

The diagnosis of HBV/HDV co-infection is based on the simultaneous presence of serologic markers of primary HBV and HDV infection. The typical serologic course of acute HDV co-infection is shown in Figure 69.8. HBsAg, HBeAg, and HBV DNA appear in serum during the incubation period in a pattern characteristic of acute hepatitis B. Both IgM and IgG antibodies to HBcAg appear coincident with the onset of clinical disease; evidence of active viral replication often disappears by the peak of illness. Antibodies to HBsAg appear later, during convalescence. The diagnosis of acute hepatitis D is made on the basis of serologic and molecular tests for this virus. Antibodies to HDAg (anti-HD) develop late in the acute phase of infection and may be present only transiently and in low titer. Assays for IgM anti-HD⁵⁵ and for virion-associated

HDV RNA^{98,197} or HDAg in serum¹⁵⁹ are the most reliable markers of acute HDV infection in the presence of high-titer IgM anti-HBc. In the co-infection pattern, all markers of viral replication disappear in early convalescence, and IgM anti-HD and even IgG anti-HD disappear within months to years after recovery. Loss of IgM anti-HD confirms the resolution of acute hepatitis D, whereas persistence predicts chronicity.

Superinfection

Superinfection of a chronic HBsAg carrier with HDV usually results in persistent HDV infection. A typical serologic pattern for such hepatitis is shown in Figure 69.8. The absence of IgM anti-HBc distinguishes superinfection from co-infection. HDV viremia becomes detectable during the incubation phase and is followed by the appearance of IgM anti-HD and IgG anti-HD during the acute phase. Markers of HBV replication are usually suppressed during the acute phase and may be difficult to demonstrate without comparison with pre-HDV levels or in subjects who are anti-HBe–positive HBsAg carriers but lack active HBV replication at the time of superinfection. Progression to chronicity is associated with high and persisting levels of IgM and IgG anti-HD. These antibodies persist, as do positive tests for serum virion-associated RNA and intrahepatic HDV RNA and HDAg. In addition to its diagnostic value, testing for IgM anti-HD also provides prognostic information, because its decrease and disappearance predicts impending resolution of chronic HDV disease, either spontaneous or induced by interferon therapy.¹⁵

Persisting viremia is associated with chronic HDV infection.^{159,201} The increasing use of molecular methods, such as polymerase chain reaction (PCR) in its standard or quantitative (real-time) formats, to assess viremia in the laboratory diagnosis of clinical disease¹⁰⁷ has overcome the limitations of serologic detection of HDAg caused by antigen sequestration in immune complexes. Molecular tests are crucial for the early diagnosis of HDV infection, before antibody seroconversion. Quantitative PCR may be particularly useful for monitoring the efficacy of antiviral therapy,^{98,106} as well as for investigating the molecular events during acute and chronic hepatitis D.^{159,197} Because of the genetic heterogeneity of HDV, it is important to select primers from highly conserved regions among the eight major genotypes. Primers derived from the ribozymes—the most conserved regions of the HDV genome—are able to detect HDV RNA from all HDV genotypes.⁹⁸ The HDV genotype can be determined by restriction fragment length polymorphism analysis of PCR-amplification products, by sequencing, or, on liver biopsies, by immunohistochemical staining using genotype-specific antibodies.^{81,159}

TREATMENT

Currently, there is no specific treatment for hepatitis D, which remains one of the most difficult liver diseases to treat. Although several antiviral agents have been tried, the only drug of proven benefit for the treatment of chronic hepatitis D is IFN- α .¹¹⁵ The fact that HDV lacks its own viral polymerase, combined with its unusual replicative cycle and its high pathogenic potential, make this virus a difficult target for antiviral therapy.⁵⁴ Specific HDV inhibitors have not yet been developed, and specific HBV inhibitors have little or no effect on HDV replication because

they do not attain a marked suppression of HBsAg expression. Oral antiviral agents that potently inhibit HBV replication, including lamivudine,^{94,115,190} famciclovir,¹⁹⁸ and adefovir,¹⁸⁶ failed to show any efficacy on HDV viremia or liver disease activity in patients with chronic hepatitis D. Data for other more potent antiviral agents presently used for the treatment of chronic hepatitis B, such as entecavir, telbivudine, and tenofovir, are lacking. Immunomodulators (e.g., steroids, thymosin, levamisole, thymic humoral factor- $\gamma 2$) and other antiviral agents (e.g., acyclovir or ribavirin) were also shown to be ineffective.¹¹⁵

Acute Hepatitis D

There is no evidence for benefit of IFN- α in acute hepatitis D,¹¹⁵ including fulminant hepatitis D.¹⁵² Patients with acute hepatitis D should be closely monitored to allow early detection of progression to fulminant hepatitis, for which liver transplantation remains the only valid therapeutic option.

Chronic Hepatitis D

Interferon Alfa Monotherapy

IFN- α was first employed to treat chronic hepatitis D in the mid 1980s and still remains the only licensed drug for the treatment of this disease. Its efficacy is related to the dose and duration of therapy. The usual regimen is 9 million units three times a week or 5 million units daily by injection for at least 1 year.^{56,68} In a controlled trial in Italy, long-term treatment with a high dose of IFN- α (9 million units three times a week for 12 months) led to ALT normalization in 50% of patients 6 months after cessation of therapy.⁵⁶ The biochemical response correlated with a significant decrease in the level of viremia but not with a loss of HDV RNA as measured by sensitive PCR.⁵⁷ Overall, a 1-year course of standard IFN- α at high doses was associated with only a 10% to 20% chance of sustained HDV clearance (Fig. 69.11) and a 10% chance of HBsAg clearance.^{9,96} Patients co-infected with HIV or HCV respond poorly to IFN- α treatment.^{128,187} The results have been disappointing also in children.^{46,50}

Several strategies have been explored to improve the efficacy of IFN- α , most notably a longer duration of treatment.^{95,199} However, most patients still fail to clear the virus.¹¹⁵ Moreover, these regimens are poorly tolerated. Side effects are common during IFN- α treatment; therefore, continuing medical monitoring is essential for the early detection and management of medical and psychiatric complications.⁶⁸

The rationale for treatment is to alter the natural history of chronic hepatitis D, yet very few studies have investigated the long-term effects of IFN- α treatment. A prospective controlled trial of 36 patients followed for up to 20 years provided evidence that the beneficial effects of IFN- α extend well beyond the termination of therapy.⁵⁷ High doses of IFN- α for 12 months significantly improved the long-term clinical outcome and survival of patients with chronic hepatitis D⁵⁷ (Fig. 69.12A). Reversion of advanced hepatic fibrosis occurred in some patients with an initial diagnosis of active cirrhosis (see Fig. 69.12B,C).

Pegylated Interferon Monotherapy

Following the superior results obtained with pegylated IFN- α (Peg-IFN- α) in chronic hepatitis B and C, the efficacy and safety of Peg-IFN- α was recently evaluated in three small studies in which Peg-IFN- $\alpha 2b$ was given at a dose of 1.5 $\mu\text{g/kg}$ for a period of 12 to 72 months.^{29,52,113} These pilot studies showed a

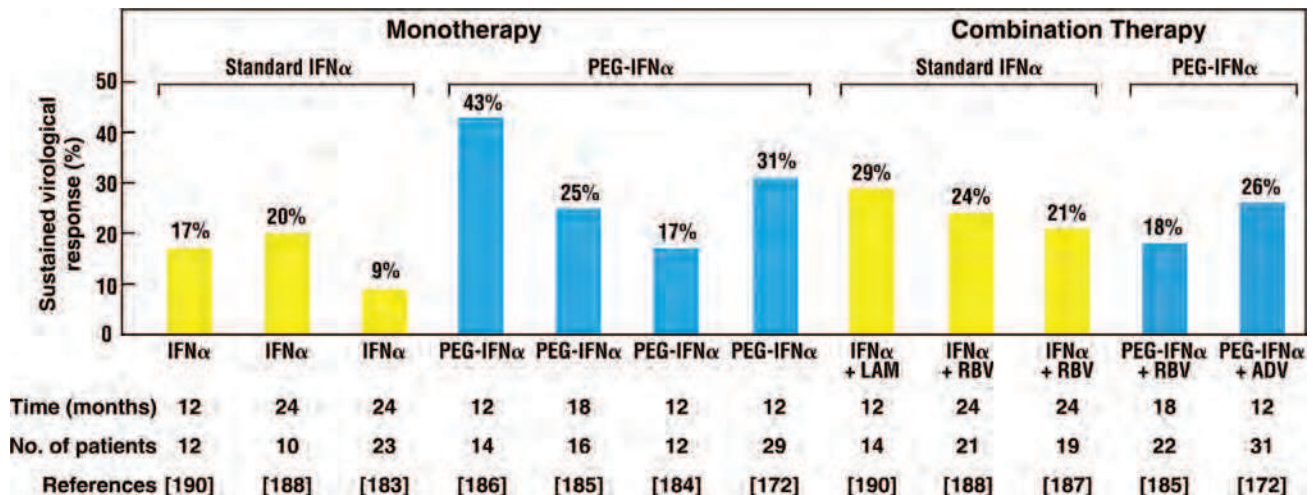


FIGURE 69.11. Rate of sustained virological response to standard interferon (IFN- α) or pegylated interferon (Peg-IFN- α), alone or in combination with lamivudine (LAM), ribavirin (RBV), or adefovir (ADV), in clinical trials in patients with chronic hepatitis D. Hepatitis D virus RNA was measured in serum using polymerase chain reaction assays with a sensitivity ranging from 10 to 1000 genome copies per milliliter. (Adapted from Farci P. Hepatitis D. In: Dooley JS, Lok ASF, Burroughs AK, et al, eds. *Sherlock's Diseases of the Liver and Biliary System*. 12th ed. Indianapolis: Wiley-Blackwell; 2011:393–405.)

sustained virological response, as measured by a sensitive PCR assay, 6 months after the end of treatment in 17% to 43% of patients with chronic hepatitis D (see Fig. 69.11). Differences in disease duration and liver histology (at baseline) on entry into the trial may explain the different rates of virological response achieved in these small studies. Similar results were recently obtained in the largest randomized trial so far conducted,¹⁸⁶ in which 31 patients received 180 μ g of Peg-IFN- α 2a weekly, leading to a sustained virological response in 31% of patients 6 months after the end of treatment (see Fig. 69.11). Although the number and size of studies with Peg-IFN- α are still limited,

they indicate that in both IFN-naïve patients and in previous nonresponders to standard IFN- α , Peg-IFN- α is well tolerated and more effective than standard IFN- α in chronic hepatitis D. The side effects are similar to those observed with standard IFN- α .

Combination Therapy with Conventional or Pegylated Interferon

A few small-size studies have evaluated the effects of combination therapy with standard IFN- α or Peg-IFN- α (see Fig. 69.11). The addition of ribavirin^{76,87} or lamivudine^{22,200} over

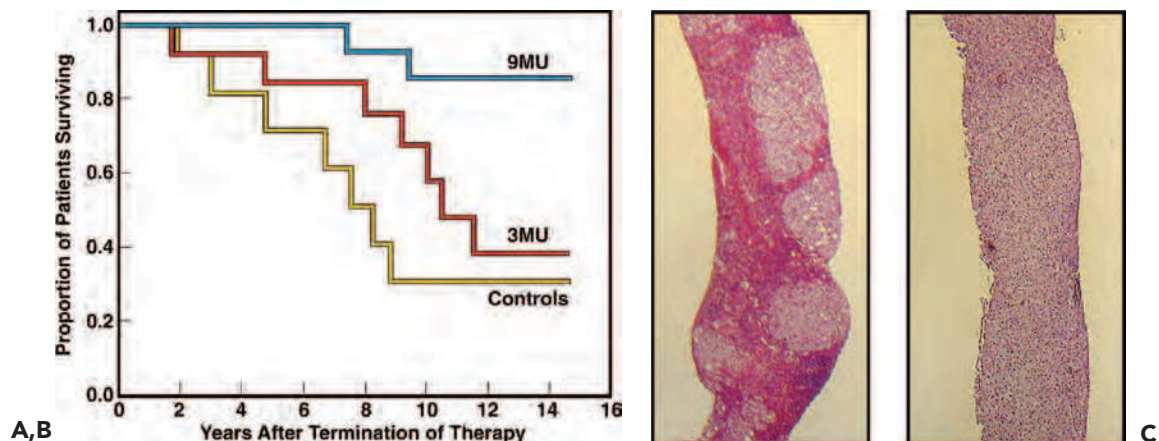


FIGURE 69.12. Interferon treatments. **A:** Cumulative survival among untreated patients and patients treated with 9 or 3 million units of interferon. Survival was significantly longer in the high-dose group than in the low-dose or control groups. No significant difference was seen between the low-dose and control groups. **B, C:** Photomicrographs of liver biopsy specimens obtained from a patient with chronic hepatitis D before (**B**) and 13 years after (**C**) completion of treatment with 9 million units of interferon. The patient had active micronodular cirrhosis before therapy. Inflammatory activity and fibrosis were not detected in the posttreatment biopsy. Similarly, biochemical and clinical evidence of hepatitis was markedly improved following therapy (Adapted from Farci P, Roskams T, Chessa L, et al. Long-term benefit of interferon alpha therapy of chronic hepatitis D: regression of advanced hepatic fibrosis. *Gastroenterology* 2004;126:1740–1749.)

monotherapy with standard IFN- α did not improve the biochemical or the virological response as did combination therapy of Peg-IFN- α with ribavirin¹¹³ or adefovir.¹⁸⁶ Clearance of HBsAg occurred in one case treated with Peg-IFN- α 2b monotherapy²⁹ and in two cases treated with Peg-IFN- α 2a plus adefovir.¹⁸⁶

Predictors of Response to Therapy

There are no clinical or virological features that predict which patients are likely to respond to therapy. However, as documented with standard IFN- α , patients in the early stage of chronic hepatitis D respond better to Peg-IFN- α than those with cirrhosis, underscoring the importance of an early diagnosis and treatment in the management of chronic hepatitis D. Albeit limited in size, recent studies have highlighted the importance of HDV RNA kinetics studies, which provided evidence that a negative PCR for HDV RNA within 6 months of therapy was the best predictor of a sustained virological response to Peg-IFN- α , although in a minority of cases it did not differentiate between responders and relapsers.^{29,52}

Current Recommendations

Peg-IFN- α should be the first choice for the treatment of chronic hepatitis D. It should be offered to all interferon-naïve patients, as well as to previous nonresponders to standard interferon, with well-compensated HDV chronic liver disease, whereas it is contraindicated in patients with advanced, decompensated, liver disease, for which liver transplantation remains the only therapeutic choice. Treatment should be started as soon as the diagnosis is made before cirrhosis develops in order to obtain the best chance of achieving a virological response. Therapy should be continued for at least 1 year before a patient is considered a nonresponder. Monitoring the HDV RNA levels offers clinicians the possibility of tailoring treatment schedules, which will be an important step forward for improving the rate of sustained virological response in chronic hepatitis D.⁵³ The development of commercial assays for the qualitative and quantitative detection of serum HDV RNA is a high priority. Quantification of serum HBsAg levels provides an additional tool for monitoring antiviral therapy.¹⁰⁴ Continuing clinical monitoring is mandatory for the early detection of the side effects induced by interferon treatment.

Future Perspectives for Therapy

Although the results with Peg-IFN- α are better than with standard interferon, the rate of relapse is still high, and a high proportion of patients still do not respond. The reasons for such poor response are unknown, although a recent *in vitro* study suggests that HDV may impair the IFN- α stimulated JAK-STAT signaling pathway.¹²⁷ Treatment of chronic hepatitis D remains unsatisfactory, emphasizing the need for improving the efficacy of interferon therapy as well as for identifying, through innovative molecular approaches, novel agents that target the life cycle of HDV. One of the primary targets is HBsAg, whose production and integrity is critical for the assembly, secretion, and entry of HDV.^{2,86,90,157} Of greater promise is a novel class of prenylation inhibitors, which block an essential step in the assembly of the HDV particles.⁶⁹ Recently, using an experimental transgenic mouse model for HBV that supports high levels of HDV replication, prenylation inhibitors were shown to be highly effective in clearing HDV viremia.¹⁴

Another strategy is the use of synthetic peptides corresponding to part of the HBsAg-L pre-S1 domain, which would inhibit entry of both HBV and HDV presumably through specific interference with a cellular receptor.¹⁵⁵ Additional molecular strategies include the use of ribozymes,⁴¹ antisense oligonucleotides,⁴⁰ and siRNAs³⁵; however, at present, the potential clinical applicability of these approaches appears remote.

Liver Transplantation

Orthotopic liver transplantation represents the only therapeutic choice for treating fulminant hepatitis and end-stage chronic hepatitis D. The results have been better than the disappointing results obtained with chronic HBV infection.¹⁵⁰ Survival after liver transplantation is better in HDV than in HBV or HCV infection. Markers of continuing HDV replication are sometimes found in transplant recipients shortly after transplantation; however, liver disease does not recur unless significant HBV replication occurs.¹⁵⁸ Reinfection is followed by relapse of hepatitis in only 50% of cases, and the hepatitis is usually milder than the original disease. Recurrence of HBV in patients with both HBV and HDV infections is less frequent than in patients with HBV alone and can be prevented by the continuous administration of anti-HBs immunoglobulins¹⁵¹ and lamivudine.²¹ Following the introduction of anti-HBs immunoglobulin prophylaxis, the risk of reinfection with HDV has dropped to 10% and that of reinfection with HBV to 30%. Furthermore, the addition of lamivudine to anti-HBs immunoglobulins before and after liver transplantation has virtually abolished the risk of HDV reinfection.²¹

PREVENTION AND CONTROL

Situations in which an opportunity exists for repeated exposure to HBV constitute the major risks for co-infection or superinfection with HDV. The current policy of excluding blood donors with serologic markers of HBV infection has markedly diminished the chances of HDV transmission by blood transfusion. Similarly, screening of donors to large plasma pools used for the fractionation of blood products has diminished the risk of HBV or HDV infection from such preparations; however, the inadvertent introduction of a single unit of plasma positive for HDV and HBV could contaminate the whole lot because of the high infectivity titers of these two viruses in blood.¹²⁵ Practices designed to minimize the transmission of HBV, HCV, and HIV among drug addicts will also diminish the transmission of HDV to the extent that they are implemented. Implementation of better hygiene, especially control of weeping skin lesions (thought to be involved in the transmission of HDV in some primitive settings), may help to control the spread of HDV and other blood-borne viruses.

Immunoprophylaxis against HDV is achieved by vaccination against HBV, because HDV uses the envelope protein of HBV and has no detectable unique surface antigens.¹³⁶ Hepatitis B vaccines are highly effective in preventing HDV infection and its sequelae. Public health measures to interdict the transmission of blood-borne pathogens, combined with aggressive HBV vaccination programs at the national level, offer the best hope for the control of hepatitis D. Some high-risk populations, however, are among the most difficult to reach with such programs. For example, as with other infectious diseases, the

possibility of vaccination against HBV in developing countries is remote, thereby providing through the high circulation of HBV the biological foundation for the spread of HDV, which therefore remains a major cause of morbidity and mortality in developing countries.

PERSPECTIVES

More than 300 million people in the world who are chronically infected with HBV are also at risk of contracting hepatitis D. These HBsAg carriers are refractory to vaccination with current HBsAg vaccines. Therefore, public health control measures depend on strategies that limit exposure to HDV through high-risk behavior. Future opportunities for prevention of HDV transmission to HBV carriers may include the development of HDV-specific vaccines; however, for the most part, limiting the burden of HDV disease will depend on the aggressive pursuit of HBV control. The implementation of universal HBV vaccination in the industrialized world has resulted in a significant decline in the prevalence of HDV, although immigration from endemic areas poses a threat of HDV resurgence. HDV is still endemic in many developing countries. Thus, more challenges need to be faced in the decades ahead to reduce the global impact of HDV infection in developing countries, where there is an urgent need for political interventions to alleviate poverty and underdevelopment.

HDV is unique among animal viruses, and despite advancement in the molecular biology of this virus, many questions remain in understanding its unusual replicative cycle. The tools provided by the postgenomic era are likely to provide a better understanding of the molecular pathogenesis of hepatitis D, including the role of HDV in hepatocarcinogenesis, and the interaction between HDV and HBV in order to identify novel therapeutic agents that target the life cycle of HDV. Finally, the difficulties in eradicating HDV globally should stimulate a more vibrant research in the different aspects of HDV, because this virus still poses many challenges to both virologists and clinicians.

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INFECTIOUS AGENT

Hepatitis E was not recognized as a distinct clinical entity until 1980, when sensitive and specific tests for antibody to hepatitis A virus (anti-HAV) were first applied to the study of epidemic water-borne hepatitis in India.^{79,167} Such disease had long been thought to be caused by hepatitis A virus (HAV), based on the epidemiologic and clinical similarities between these epidemics and well-characterized epidemics of hepatitis A occurring elsewhere. However, these epidemics were found not to be hepatitis A.

The first direct experimental evidence for the existence of an additional water-borne hepatitis agent was reported in 1983 by Balayan et al,¹⁶ who described the successful fecal–oral transmission of hepatitis to a volunteer from patients with hepatitis A–like disease in Central Asia. The volunteer developed severe clinical hepatitis 36 days after ingestion of a fecal suspension from the patients. Virus-like particles (VLPs) of 27 to 30 nm in diameter were detected by immuno-electron microscopy (IEM) in the feces collected from the volunteer between 28 and 45 days after exposure. Balayan et al¹⁶ also reported the transmission of hepatitis to cynomolgus monkeys inoculated with the fecal sample and the recovery of VLPs from their feces.

This form of non-A, non-B hepatitis was named epidemic or enterically transmitted non-A, non-B (ENANB) hepatitis, and the agent of this disease was subsequently found to be the

major cause of sporadic hepatitis cases in regions where the epidemic form was known to exist.^{11,81,125} IEM and transmission to primates (see later discussion) remained the only means of studying ENANB hepatitis until 1990, when Reyes et al¹³⁹ succeeded in cloning and sequencing a part of the virus genome. Many complete sequences of the viral genome are now known, and the agent has been renamed hepatitis E virus (HEV).^{137,139}

Classification

Based on genome organization and superficial structural morphology, HEV was initially classified in the family *Caliciviridae*; however, further sequence analyses showed a closer, although distant, relationship to rubella virus, alphaviruses of the *Togaviridae* family, and plant furoviruses.⁸⁵ Therefore, a new family *Hepeviridae* and genus *Hepevirus* were created with HEV as the sole member¹¹¹ (Fig. 70.1). Genotypes differ from each other by 25% or more and have a characteristic geographic distribution. Currently, there are four recognized genotypes of HEV that infect mammals: genotype 1 (Burmese-like Asian strains), genotype 2 (a Mexican and a few South African strains), genotype 3 (animal strains mainly from swine worldwide and from sporadic human cases in industrialized countries), and genotype 4 (swine strains and sporadic human cases in Asia)^{12,110,132} (Table 70.1). Genotypes 1 and 2 infect only humans and nonhuman primates. Genotypes 3 and 4 infect humans and primates as well; however, their main host is swine but have occasionally been isolated from deer and rabbits, and once from a mongoose.^{49,119,127,177} Isolations from other mammals have been reported but not yet confirmed. All four genotypes have the same serotype.^{41,96,136}

An HEV-like virus isolated from chickens exists as three or more genotypes, shares only 50% nucleotide identity with mammalian strains, and has not been assigned to a genus.^{55,58,102,111,128} Similarly, an unclassified virus isolated from rats shares nearly 55% sequence identity with genotypes 1 through 4 and approximately 50% with avian strains.^{65,66} Additional strains undoubtedly will be isolated from other species.

VIRION STRUCTURE

Morphology

HEV is a nonenveloped, spherical particle, approximately 30 to 34 nm in diameter,¹⁶ with an indefinite surface substructure that is slightly less pronounced than that of the Norwalk agent (a calicivirus) but distinguishable from the smooth, featureless surface of HAV (a picornavirus) (Fig. 70.2). On the basis of morphology, however, HEV cannot be reliably distinguished from other “small round viruses” found in feces. Through the

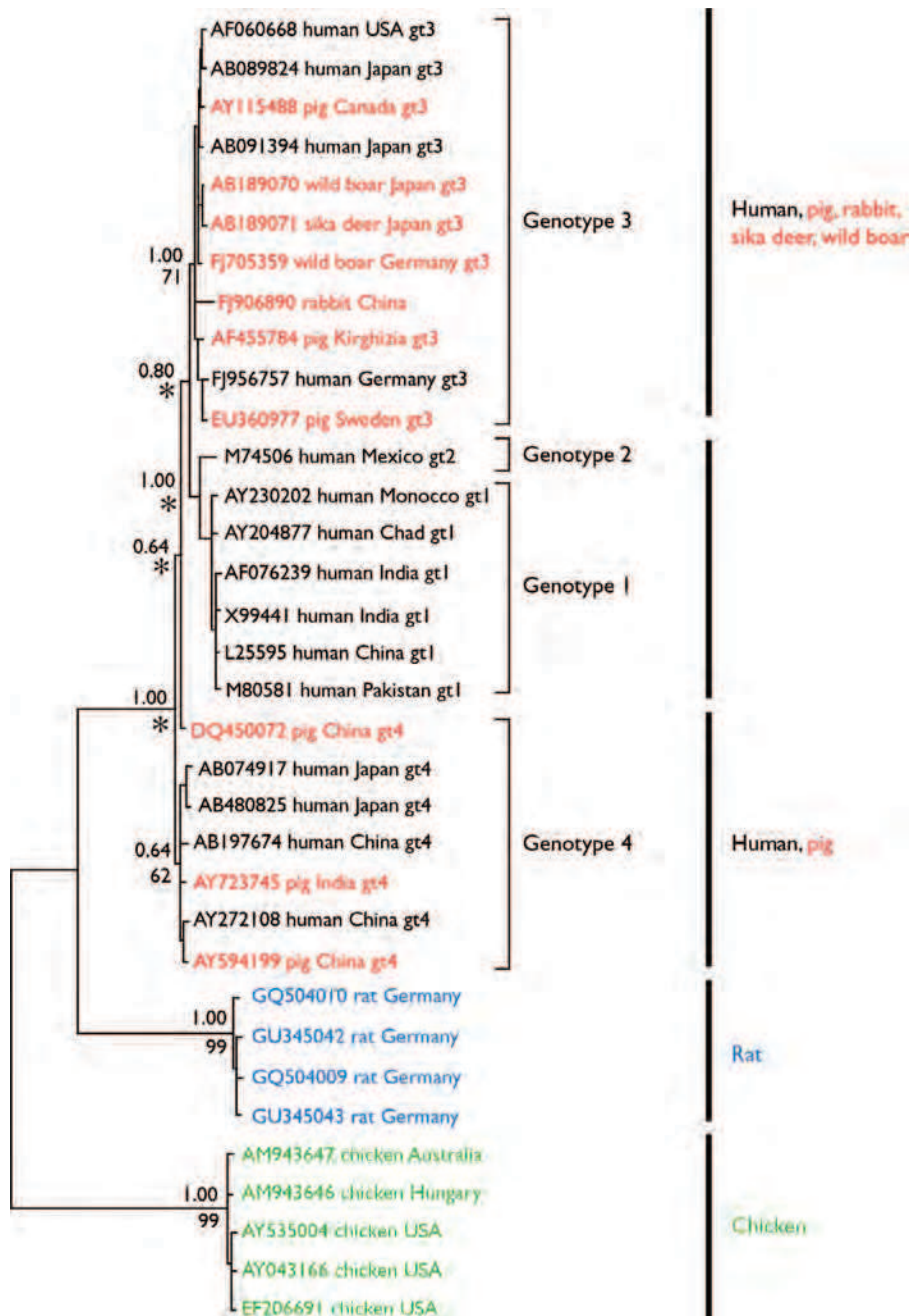


FIGURE 70.1. Phylogenetic tree of hepatitis E virus (HEV) partial nucleotide sequences (1,576 nucleotides). Robustness of nodes is given for Bayesian (above branches) and neighbor-joining (below branches) algorithms. The *asterisk* indicates that neighbor-joining algorithms suggest a closer phylogenetic relationship between genotypes 3 and 4, with genotype 1 basal to these. *Scale bar* indicates phylogenetic distances in nucleotide substitutions per site. The four recognized mammalian genotypes (1–4) are depicted in *black* for human strains and *red* for animal strains; rat HEV sequences, comprising a putative new genotype, are shown in *blue*. Avian HEV strains, comprising a putative new genus, are shown in *green*. (Reprinted with permission from John R, Heckel G, Plenge-Bönig A, et al. Novel hepatitis E virus genotype in Norway rats, Germany. *Emerg Infect Dis* 2010;16(9):1452–1455.)

TABLE 70.1 Taxonomy and Host Range

Genotype	Natural host	Other susceptible hosts
1	Human	Nonhuman primates
2	Human	Nonhuman primates
3	Swine	Human, nonhuman primates, deer (sika, red, roe), mongoose
4	Swine	Human, nonhuman primates, cattle (?), sheep (?)
Related to 3	Rabbit	Pig
New Genotype?	Rat	(?)
New Genus?	Chicken	Turkey

techniques of cryo-electron microscopy and crystallography, considerable progress has been made in understanding the structure underlying the morphology of HEV. The principal, if not only, capsid protein of HEV, open reading frame (ORF) 2 (normally 660 amino acids [aa] in length), when truncated (aa 112–608), forms homodimers that can further assemble into 27-nm VLPs with a $T = 1$ icosahedral symmetry consisting of 30 dimers. ORF2, somewhat less truncated (aa 14–608), can form virus-size (34-nm) VLPs with a $T = 3$ structure consisting of 90 dimers that mimic the virus and encapsidate RNA (Fig. 70.3). The assembly and morphology of HEV resemble those of other small nonenveloped viruses, especially small plant viruses.^{53,94,169,170}

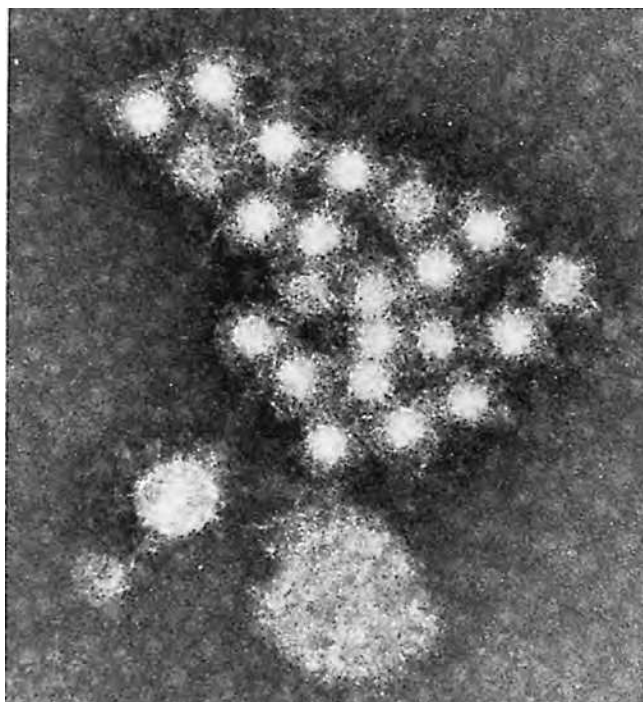


FIGURE 70.2. Antibody-coated hepatitis E virus particles recovered from the stool of a patient with hepatitis E in Mexico. Immunoelectron microscopy ($\times 223,000$). (Courtesy of D. Bradley.)

Chemistry

The buoyant density of hepatitis E viral antigen or virus particles in feces is reported to be 1.35 g/cm^3 ¹⁷ and 1.39 to 1.40 g/cm^3 ⁴⁴ in cesium chloride (CsCl), 1.29 g/cm^3 in potassium tartrate or glycerol, and 1.27 to 1.28 g/mL in sucrose.^{150,151} Although the virus in feces is not enveloped, the density in serum was 1.15 to 1.16 g/mL in sucrose owing to an uncharacterized association with lipids.¹⁵¹ The sedimentation coefficient of HEV is 183S. Survival of HEV in the intestinal tract suggests that the virus is relatively stable to acid and mild alkaline conditions. HEV is more heat labile than is HAV.³⁸ Although not specifically tested, HEV is likely to be inactivated by the same agents that inactivate HAV, and chlorination is suggested to inactivate HEV in water.²³ HEV contains an RNA genome enclosed within a capsid that is probably composed of a single viral protein; however, insufficient virus has been available for purification and direct chemical analysis.¹⁵²

GENOME STRUCTURE AND ORGANIZATION

The genome of HEV mammalian genotypes 1 through 4 is a single-stranded, positive sense, polyadenylated RNA molecule of approximately 7.2 kb in length, excluding the poly(A)¹⁵² (Fig. 70.4). All three coding frames are utilized. A subgenomic, bicistronic messenger RNA (mRNA) is synthesized following infection.^{52,63} The genome consists of a $5'$ noncoding region of approximately 26 nucleotides that is capped with m⁷G at its $5'$ end,^{63,68,99} followed by ORF1 of approximately $5,100$ nucleotides. Deletions or insertions within a hypervariable region (HVR) of ORF1 account for most size differences. ORF2

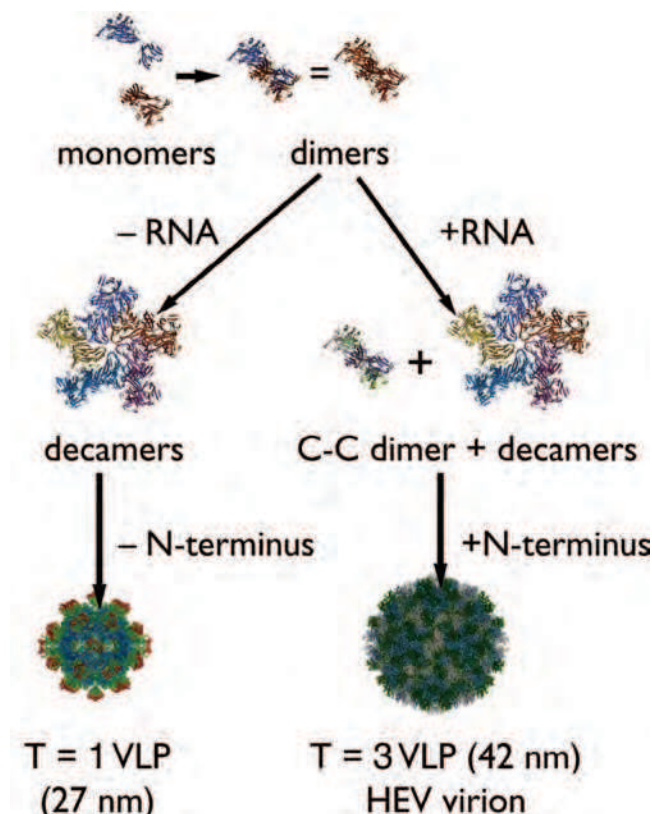


FIGURE 70.3. Proposed assembly scheme for hepatitis E virus (HEV) particles. Identical monomers and dimers are shown at the top: colors are added to aid visualization. Truncated HEV capsid protein (aa 112–608) forms A-B dimers that assemble into intermediate decamers and ultimately form small, empty, virus-like particles with $T = 1$ icosahedral symmetry. A slightly larger truncated HEV capsid protein (aa 14–608) similarly forms dimers and decamers that then interact with slightly differently oriented dimers (C–C) to form larger virus-like particles with a $T = 3$ icosahedral lattice. These larger particles can encapsidate RNA. (Courtesy of L. Xing and H. Cheng.)

begins in coding frame 2, which is 38 nucleotides $3'$ of the termination codon of ORF1, and consists of $1,977$ to $1,980$ nucleotides. A 65 - to 74 -nucleotide $3'$ noncoding region is contiguous with the termination of ORF2 and is terminated by a $3'$ stretch of 150 to 200 or more adenosine residues. ORF3, which is 348 to 351 nucleotides in length, extends 325 to 328 nucleotides into ORF2 in coding frame 3.

Avian HEV genomes have a similar organization but are 6.6 kb in length,^{57,111} whereas rat HEV has a genome that is 6.9 kb in length and has a similar arrangement of ORFs 1 through 3 plus three more possible ORFs.⁶⁵

Several ORF1 recombinant polypeptides have enzymatic activities. Methyltransferase and guanylyltransferase activities (MT) responsible for capping the viral genome are encoded within the $5'$ half of ORF1.⁹⁹ The RNA-dependent RNA polymerase (RdRp) responsible for replicating the genome and producing a subgenomic mRNA^{52,63} is encoded within the $3'$ half of ORF1.⁶ The upstream helicase domain exhibits RNA $5'$ triphosphatase, nucleoside triphosphatase (NTPase), and RNA unwinding activity and belongs to helicase superfamily 1.^{75,76}

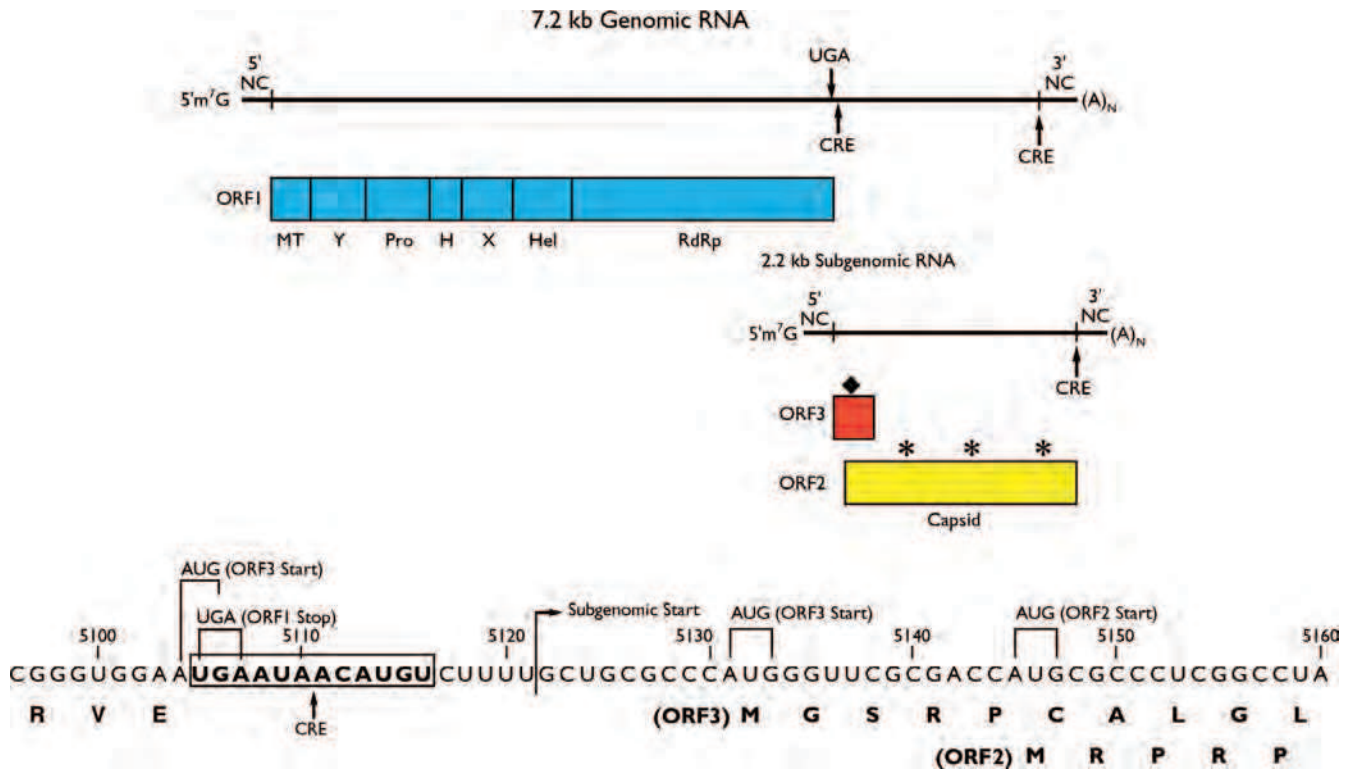


FIGURE 70.4. Organization of the hepatitis E virus genome. The approximately 7.2-kb genome serves as messenger RNA (mRNA) and encodes three open reading frames (ORFs). The nonstructural proteins required for viral RNA synthesis are encoded within the approximately 5,100-kb ORF1 (see text for details). A bicistronic subgenomic mRNA of 2.2 kb encodes the ORF2 capsid protein, and the overlapping ORF3 encodes a small immunogenic protein proposed to have multiple functions. The *diamond* indicates a putative phosphorylation site on ORF3 protein, and the *asterisks* indicate three putative glycosylation sites on the ORF2 protein. The sequence of the genomic mRNA spanning the ORF1 translation stop codon and the methionine initiation codons for ORFs 2 and 3 are shown. The subgenomic mRNA starts at nucleotide 5,122 and thus does not include the two upstream methionine codons (AUG) in ORF3, which begins at nucleotide 5,104. A boxed 12-nucleotide-long *cis*-reacting element (CRE) controls subgenomic mRNA synthesis. MT, methyltransferase/guanylyl transferase; Hel, helicase; RdRp, RNA-dependent RNA polymerase; Y, Pro, H, and X domains, function not demonstrated. (From Graff J, Torian U, Nguyen H, et al. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. *J Virol* 2006;80(12):5919–5926.)

Conserved motifs found in proteins of other viruses suggest that ORF1 also encodes the following⁸⁵:

1. A Y domain, a sequence of unknown function found in certain other viruses, including rubella virus
2. A papain-like cysteine protease found predominantly in alphaviruses and rubella virus
3. A proline-rich hinge region that is thought to impart flexibility and that contains a region of hypervariable sequence
4. An X or “macro” domain of unknown function that flanks papain-like protease domains in the polyproteins of other positive-strand RNA viruses.⁷

However, none of the latter activities has been demonstrated to date.

ORF2 and ORF3 proteins are encoded by the same bicistronic subgenomic RNA^{52,63} (see Fig. 70.4). ORF2 capsid protein contains a typical signal sequence near its 5′ end, immediately followed by a region rich in arginine and highly basic in charge, that may be involved in the encapsidation of the genomic transcript.^{147,152} The ORF2-encoded protein contains potential

glycosylation sites that are glycosylated *in vitro*; however, it has not been established that these are functional *in vivo*.^{157,174}

Because the subgenomic RNA that encodes ORF3 protein (see the Stages of Replication section) has a truncated reading frame 3, ORF3 protein contains 114 to 115 aa rather than the 123 to 124 predicted by the genomic RNA sequence.^{52,61,63} ORF3 protein is bound to virus particles in serum,¹⁵¹ as well as those produced in cell culture,^{40,151} and is required for virus release from cultured cells^{40,117,172} and for infection *in vivo*.^{51,61} However, ORF3 protein is not required for genome replication, viral assembly, or infection of hepatoma cells³⁹ *in vitro* and is not found on virus in feces.^{40,151} When overexpressed *in vitro*, ORF3 fractionates with the cellular cytoskeleton¹⁷⁵ and is phosphorylated, although phosphorylation is not required for viability *in vivo*.⁵¹ *In vitro*, overexpressed ORF3 does many things, suggesting that it may have regulatory functions (reviewed in reference 7). It interacts with nonglycosylated ORF2,¹⁶⁰ expedites the export of α 1-microglobulin and its precursor from hepatocytes¹⁶¹ and binds to Src homology 3 domains, and activates mitogen-activated protein kinase.⁸⁶ In addition, the HEV

genome contains at least two *cis*-reactive elements (CREs). One CRE overlaps the 3' end of ORF2 and the 3' noncoding region and is essential for replication⁴²; the second CRE may be the promoter for synthesis of the 2.2-kb subgenomic mRNA.^{22,51,52}

STAGES OF REPLICATION

HEV proves to be exceedingly difficult to culture. Tanaka et al.¹⁵⁴ reported the first propagation of a genotype 3 strain, isolated from a patient during the acute phase, and later reported the propagation of a genotype 4 strain, from a patient with fulminant hepatitis E.¹⁵⁵ In both cases, virus in feces was serially passaged in two human cell lines, A549 lung carcinoma and PLC/PRF/5 hepatoma. Another genotype 3 strain, isolated from a chronically infected patient, was propagated in the human HepG2/C3A hepatoma cell line; this cultured virus differed from the other two in that it infected swine cells as well as human cells and contained a large portion (174 nucleotides) of a human gene sequence inserted into the HVR.¹⁴⁵ The effect of this insertion on host range or disease manifestation is unknown; however, similar recombinants have since been isolated. The critical factor for achieving growth in cell culture was the titer of the virus inoculum^{124,149,150}; most likely, greater quasispecies diversity provides a variant with a constellation of mutations that permits propagation in cultured cells.

Much of the molecular biology of HEV is still unknown, and many steps are inferred from studies of other viruses (Fig. 70.5). A high-affinity cell receptor has not been identified; however, heparan sulfate proteoglycans may serve as a low-affinity receptor to promote initial contact.⁶⁹ The virus enters cells by an unknown mechanism. Heat shock protein 90 is implicated in the intracellular transport of the virus capsid early in infection.¹⁷⁸ The virus replication cycle is confined to the cytoplasm, and once the viral RNA is released from the capsid, ORF1 is immediately translated via cellular mechanisms that recognize capped mRNA.^{42,68,99} Transfected, capped genomes of HEV are able to replicate *in vitro*^{51,58,60,171} and cause infection *in vivo*.^{42,57,60} It is unclear if, or how, ORF1 protein is cleaved into subunits.

The newly translated RNA polymerase, guanylyltransferase, methyltransferase, and helicase/RNA triphosphatase activities of ORF1 protein would all be involved in RNA synthesis; it is not known if other regions of ORF1 protein participate. RNA synthesis probably occurs on the intracellular membranes.¹³⁸ The polyadenosine tract and stem-loop structures at the 3' end of the virion RNA mediate binding of recombinant polymerase to template RNA.⁶

The negative-strand replicative-intermediate would be synthesized first and serve as a template for capped genomic-sized mRNA and a capped bicistronic subgenomic mRNA that encodes ORF2 and ORF3 proteins.^{52,63} Twelve conserved nucleotides and a double stem-loop structure control subgenomic RNA synthesis.^{22,52} There is a temporal separation, and alternating cycles of negative- and positive sense RNA synthesis with the highest level of negative sense RNA (1,000-fold lower than positive sense) present at approximately 8 hours posttransfection; negative sense subgenomic RNA has not been detected.¹⁶³

ORF2 and ORF3 proteins are translated from two very closely spaced AUG codons on the same subgenomic mRNA; it is not clear how the relative translation of the two proteins is regulated.^{52,145} ORF2 capsid protein probably self-assembles into

virus particles in a reaction requiring viral RNA¹⁶⁹ (see the Morphology section). ORF2 protein in infectious virions has not been biochemically characterized; thus, although ORF2 protein contains a signal-like sequence at its 5' end and three internal sites that are glycosylated *in vitro*, it is not known if they function as such *in vivo*. The site of virus assembly is unknown.

Virions in serum^{124,150} or released into the medium of cultured cells are associated with lipids (uncharacterized) and ORF3 protein; they have a lower buoyant density than virions in feces and can be immune precipitated with anti-ORF3 if treated with nonionic detergent to disperse the lipids.^{40,172} Surprisingly, most virions in culture medium are not immune precipitated with antibodies to ORF2, unlike fecal virions. Therefore, virions produced in cell culture contain ORF2 protein that is masked or requires a maturation step. There are discrepant reports on whether ORF2 protein is more accessible to antibody when ORF3 protein is eliminated by mutagenesis, and on lipid association with intracellular virions and virions in feces^{40,172}. It has been suggested that detergents and proteases in bile and the intestine might remove these components.¹²⁴

ORF3 protein is not required for the production of infectious virions; however, it is important for virus exit from cells. ORF3 protein deletion mutants exit cultured cells inefficiently.^{40,172} Exit from the cells depends on an intact PXXP motif in ORF3 protein, which is conserved among all mammalian strains.^{40,117} Mutants with an altered PXXP motif are indistinguishable from ORF3 null mutants.^{40,117} The PXXP motif is believed to mediate binding to a host protein, perhaps Tsg101 in the vacuolar protein sorting pathway.^{7,172} Neither ORF3 null nor PXXP mutants are able to infect rhesus macaques.^{40,51}

PATHOGENESIS AND PATHOLOGY

The pathogenesis of hepatitis E is poorly understood. Some speculative conclusions can be drawn, however, from an analysis of the clinical and serologic events of a typical HEV infection (Fig. 70.6). Entry of the virus into the host is believed to be primarily by the oral route. The incubation period ranges from 2 weeks to 2 months. The site of primary replication has not been identified, although it is presumed to be in the intestinal tract. It is not clear how the virus reaches the liver, but it is presumably via the portal vein. It replicates in the cytoplasm of hepatocytes⁸⁷ and is released into the bile and blood by mechanisms that are not understood. Based on limited studies of oral infection in volunteers, viremia and fecal shedding are first detected about 3 weeks after exposure and about a week before onset of disease.^{16,27} Liver enzyme values peak about 7 to 8 weeks post exposure. Viremia may diminish at that time; however, fecal shedding may continue for days to weeks.

As in hepatitis A, specific immunoglobulin M (IgM) and immunoglobulin G (IgG) immune responses occur early in the disease, usually about the time of onset of clinical illness and peak of liver enzymes (see Fig. 70.6). IgM anti-HEV disappears after several months; IgG anti-HEV persists but at relatively rapidly decreasing levels shortly after infection.²¹ Anti-HEV can be detected for years after infection.⁸² It is not known whether such antibody eventually disappears or remains at low but detectable levels. Anti-HEV of the immunoglobulin A (IgA) class has also been detected in the serum of naturally infected individuals.²⁶ The significance of such antibody is unknown.

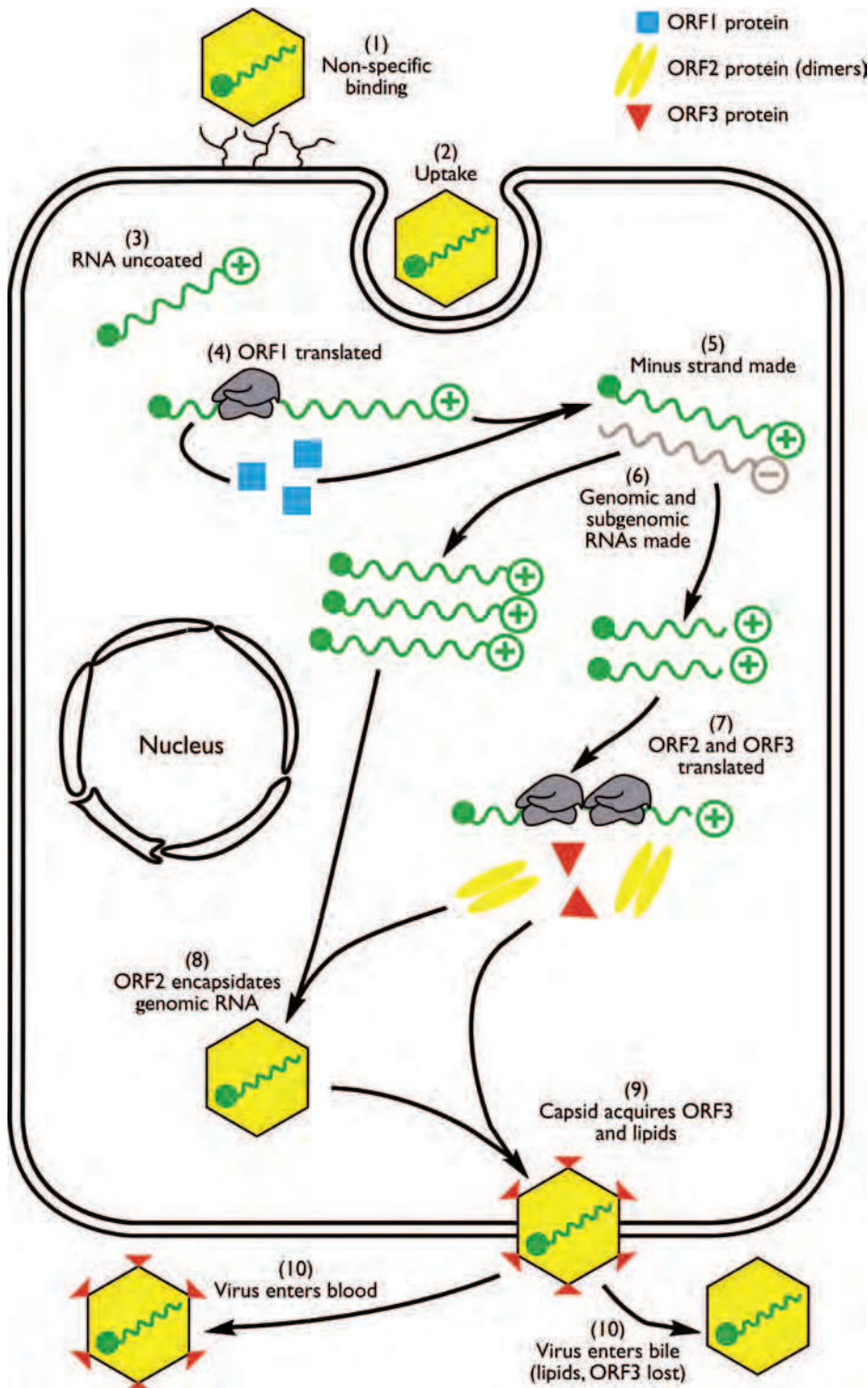
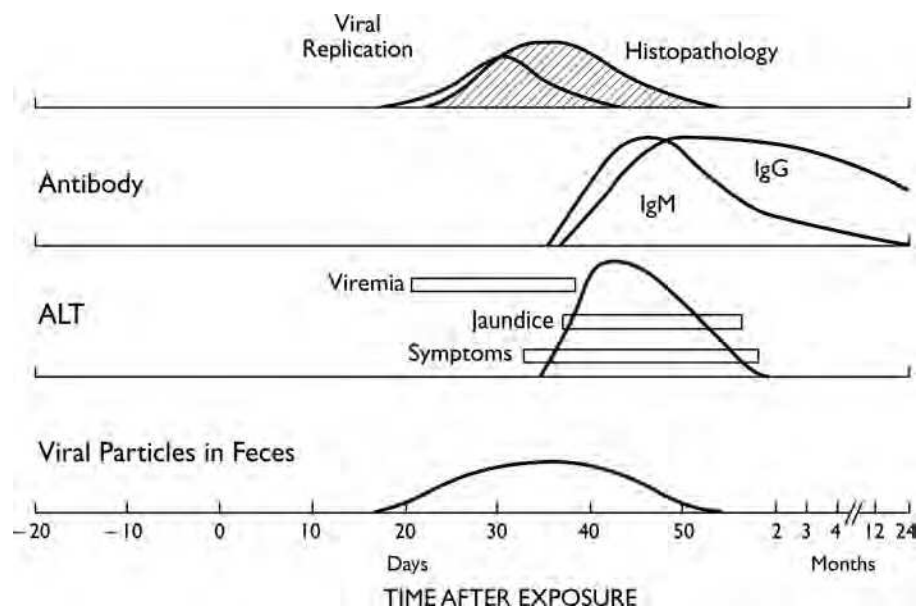


FIGURE 70.5. Hepatitis E virus (HEV) replication cycle. The diagram presents the most likely scenario for HEV replication based on current knowledge from studies of HEV and other plus-strand RNA viruses. **1:** Ingested virus is transported to the liver, where it binds nonspecifically to heparan surface proteoglycans. **2, 3:** The virus binds to a specific unidentified receptor (**2**) and is delivered to the cytoplasm where the plus strand genomic RNA is released from the capsid (**3**). **4:** Open reading frame (ORF) 1 is immediately translated to generate the nonstructural proteins required for viral RNA synthesis. **5, 6:** The newly synthesized polymerase proteins make a minus-strand copy of the genomic RNA (**5**), which serves as the template for amplification of the plus-strand genomic messenger RNA (mRNA) and for production of a bicistronic subgenomic mRNA by the polymerase proteins (**6**); both the genomic and subgenomic RNAs are capped; however, the 5' status of the minus strand is unknown. **7:** Both ORF2 and ORF3 proteins are translated from the same subgenomic mRNA. **8, 9:** ORF2 protein encapsidates the genomic mRNA (**8**), and ORF3 protein and lipids associate with the capsid (**9**). **10:** The virus is transported out of the cell and enters either the bloodstream or the bile duct; ORF3 and lipids remain on virus in the blood but are removed from virions by the time they are excreted in feces.

Virus found in the feces of infected humans or animals is presumed to be the primary source of infectious virus in the environment. Because large quantities of HEV have been found in the bile of experimentally infected primates, it is assumed that most virus in the intestinal tract originates in the liver.¹³⁹ In this respect, hepatitis E is similar to hepatitis A.

The severity of HEV infections is, on average, somewhat greater than the severity of HAV infections. Mortality of hepatitis E has varied in different reports but has been as high as 1%, compared to 0.2% for hepatitis A.¹³⁵ More important, however, is the severity of hepatitis E in pregnant women.^{1,23,64,78,84,137} The mortality of hepatitis E in pregnancy increases with each

FIGURE 70.6. Diagrammatic illustration of the clinical and serologic events in a typical case of acute hepatitis E. Antibody pattern depicted as measured by enzyme-linked immunosorbent assay (ELISA). Viremia and fecal shedding patterns are based on polymerase chain reaction (PCR) data. ALT, alanine aminotransferase. (Modified from Purcell RH, Hoofnagle JH, Ticehurst J, et al. Hepatitis viruses. In: Schmidt NJ, Emmons RW, eds. *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*. 6th ed. Washington, DC: American Public Health Association; 1989:957–1065.)



succeeding trimester and may reach 20%. In contrast, none of the other four recognized hepatitis viruses causes such severe hepatitis in pregnancy.¹¹⁵ The reason for the excessive mortality of hepatitis E in pregnancy is unknown, although a high viral load and abnormalities of progesterone signaling pathways have been suggested.^{20,64,74} HEV is an uncommon cause of fulminant hepatitis in nonpregnant individuals.^{90,123} Individuals with chronic liver disease of other etiologies are also at higher risk of severe, life-threatening hepatitis E.^{77,88,176} The hepatitis E mortality in such patients may be as high as 30%.

Although most HEV infections are self-limiting and resolve without sequelae, a significant proportion (>50%) of infected organ transplant patients or those with other types of immunosuppression may develop chronic infection that can progress to chronic hepatitis and cirrhosis.^{33,72,73,130}

Histologic changes in the liver of patients with hepatitis E include focal necrosis with minimal infiltration and no localization to a particular zone of the lobule. Modest inflammation consisting predominantly of Kupffer cells and polymorphonuclear leukocytes is seen, and the focal lesions resemble drug-associated toxic hepatitis. Cholestatic hepatitis is often present, characterized by ballooning hepatocytes, cytoplasmic cholestasis, and focal cytolytic necrosis. An unusual *pseudoglandular* alteration of the hepatocyte plates has also been recognized in some epidemics and sporadic cases (Fig. 70.7). The discrepancy between the time of appearance of viral replication in the liver and histopathologic and biochemical evidence of hepatitis suggests that HEV is not cytopathic and that the pathogenesis of hepatitis E is immunologically mediated. Alterations of both innate and adaptive immunity during acute infection have been reported; however, the mechanisms of pathogenesis are poorly understood.^{118,143,146,173}

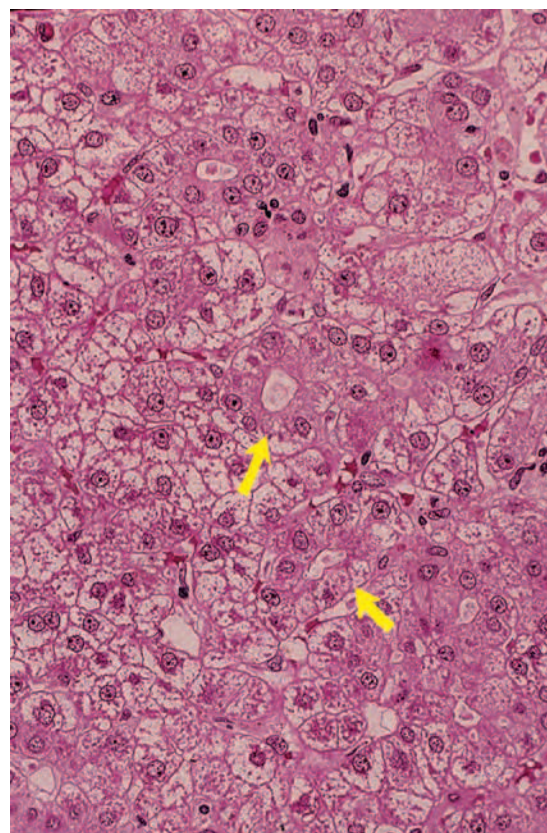


FIGURE 70.7. Liver biopsy from a case of acute hepatitis E, Pakistan (hematoxylin and eosin [H&E] ×100). Acinar transformation ("pseudoglandular" alterations) is indicated by arrows, and intraluminal cholestasis is depicted. Hepatocytes demonstrate "ballooning" and degeneration. (Courtesy of M. Sjogren.) (Reprinted from Purcell RH, Emerson SU. Hepatitis E. In: Mandel GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases*. 6th ed. Philadelphia: Elsevier; 2005:2204–2217.)

EPIDEMIOLOGY

Hepatitis with clinical and epidemiologic characteristics of hepatitis E (peak clinical attack rate in young adults, high rate of

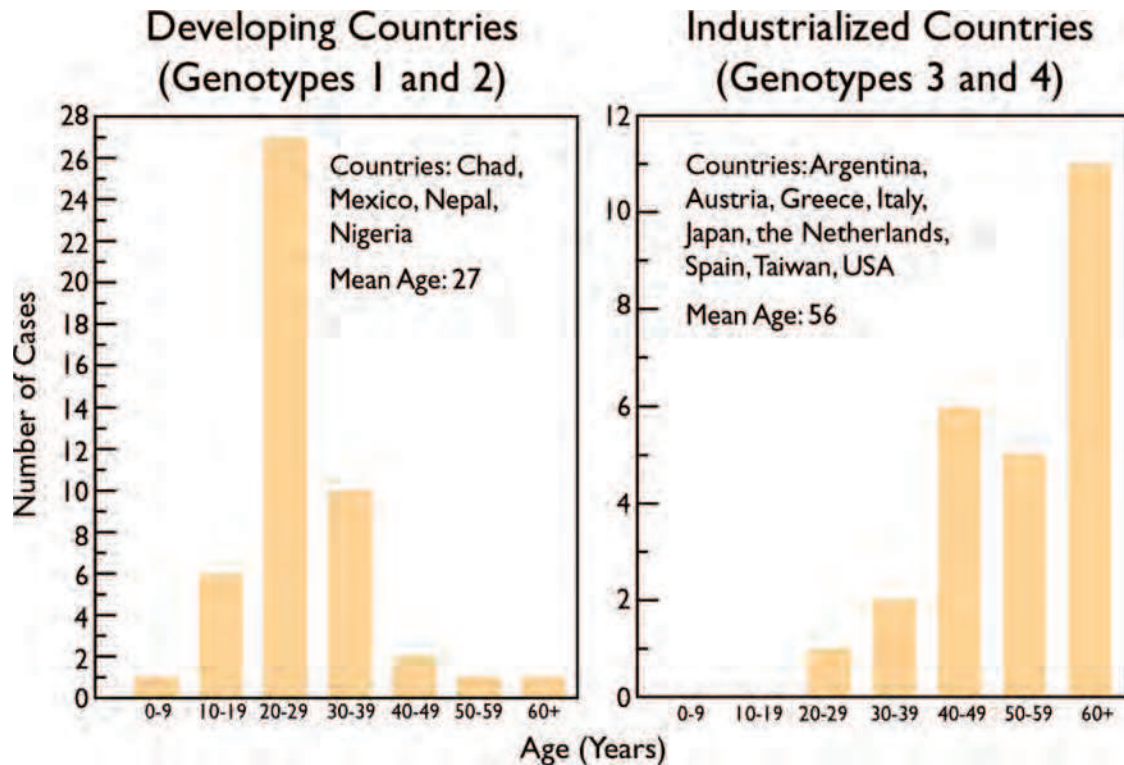


FIGURE 70.8. The age-related clinical attack rate of hepatitis E differs by genotype. Endemic disease associated with hepatitis (HEV) infections of genotype 1 or 2 in developing countries peak in the 20- to 29-year age group. This is similar to attack rates from HEV epidemics in the area. In contrast, clinical hepatitis E associated with infections of HEV genotype 3 or 4, often in industrialized countries, peaks in those 60 years of age or older. (Reprinted from Purcell RH, Emerson SU. Hepatitis E: an emerging awareness of an old disease. *J Hepatol* 2008;48:494–503.)

fulminant disease in pregnancy, epidemics of water-borne disease) has been reported in Southeast and Central Asia, the Middle East, Africa, and North America (Mexico).⁴ In 2004, a large outbreak among internally displaced persons in Darfur, Sudan, resulted in an unusually high case-fatality ratio of 18% among hospitalized patients, with pregnant women accounting for almost half of the deaths.^{19a} Epidemics in most of these regions have been confirmed serologically as hepatitis E, as has sporadic hepatitis occurring in many of the same regions.¹³¹ In fact, HEV is believed to be one of the most common causes of sporadic hepatitis in regions where the virus is endemic.

The age-specific clinical attack rate in developing countries, with its peak among young adults, is striking^{79,164,167} (Fig. 70.8). Clinical disease, however, has been found with similar frequency in all age groups in some epidemics¹¹⁶; clinical hepatitis E in children has also been reported.^{9,37,50,116} A male preponderance has been observed in some, but not all, epidemics. With the exception of a few food-borne epidemics, most serologically confirmed epidemics of hepatitis E have been associated with fecally contaminated water. In the 2004 Darfur outbreak, chlorinated water was implicated as one source of HEV.^{52a} Cases of probable sporadic hepatitis E have been linked epidemiologically to consumption of raw or uncooked shellfish²⁴ or, more commonly, of undercooked pork, offal, or wild game.^{32,105,107,108,126,127,133,148,153} The epidemiologic risk factors associated with most sporadic cases of hepatitis E have been difficult to identify. Person-to-person

spread from cases to contacts has been reported; however, this appears to be relatively uncommon and, in fact, has been questioned.^{3,23,37,80,120,156} Evidence for a higher risk of infection with HEV among healthcare professionals is not convincing,¹²² although transmission via exposure to blood has been documented in regions where the virus is endemic and, rarely, in industrialized countries.^{2,15,48,83,104,168} Infants of mothers with hepatitis E are also at risk of being infected perinatally.⁸⁹ HEV RNA has been detected in colostrum from infected mothers; however, there is no epidemiologic evidence that suggests transmission to offspring by nursing.²⁸ Travel to an area where HEV is endemic has been a risk factor in several cases,^{25,46} and one hospital-related outbreak has been reported.¹⁴⁰

The recent development of sensitive serologic tests for infection with HEV has permitted a more complete analysis of the worldwide distribution and seroprevalence of HEV infection. Surprisingly, with a few exceptions,⁴⁵ the prevalence of IgG antibody to HEV in suspected or documented endemic regions has been much lower than expected, and the prevalence of such antibody in nonendemic regions has been much higher than anticipated¹³¹ (Fig. 70.9). For instance, anti-HEV seroprevalence in the U.S. population was estimated to be 21% even though hepatitis E was rarely reported.^{89a} Except in epidemic settings¹¹⁶ and limited other instances,^{5,45} the prevalence of anti-HEV in infants and children has also been low.^{14,92,97,116} The greatest increase in prevalence of anti-HEV has occurred in young adults—the age group at highest risk of clinical

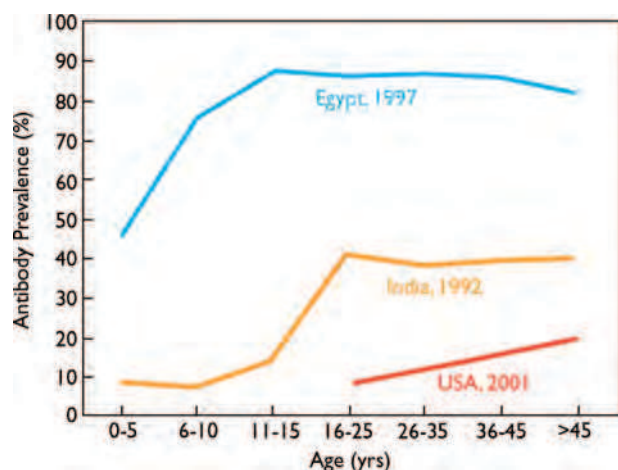


FIGURE 70.9. Age-specific prevalence of anti-hepatitis E virus (HEV) in different regions. The prevalence of anti-HEV in India and the peak age of antibody acquisition (16–25 years) is lower and later, respectively, than expected for a fecally transmitted virus. In contrast, the prevalence of anti-HEV in the United States is higher than expected, given the rare diagnosis of hepatitis E. Finally, the prevalence of anti-HEV in Egypt is particularly surprising because, in contrast to India, water-borne epidemics of hepatitis E are virtually never reported. All of the anti-HEV enzyme-linked immunosorbent assay (ELISA) tests were performed with the same assay. (Reprinted from Purcell RH, Emerson SU. Hepatitis E: an emerging awareness of an old disease. *J Hepatol* 2008;48:494–503.)

disease in developing countries^{14,92,97} (see Fig. 70.9). Some years ago, virtually identical age-specific anti-HEV and anti-HAV patterns were detected in the same population in India over a decade, suggesting that endemic exposure was continuing.¹⁴ Since then, there is evidence that both viral infections are diminishing in the population, especially among those of higher socioeconomic status, and similar changes appear to be occurring in other developing countries. In industrialized countries, there is invariably a higher prevalence of antibody among older individuals, suggesting a cohort effect signaling diminished exposure to the virus in more recent times. Indeed, such a cohort effect was recently demonstrated in Denmark²⁹ (Fig. 70.10). Thus, in some settings, the epidemiologic characteristics of HEV appear to differ significantly from those of viruses such as HAV, which are readily transmitted by the fecal–oral route. These may be reflections of the differences in amount and duration of viral shedding, relative stability of the viruses to environmental factors, and the differential role of zoonotic exposure.

The impact of HEV infections in various wild and domesticated animals on the epidemiology of hepatitis E in humans is the subject of much debate (see Table 70.1). The close genetic relationship between swine HEV strains and strains of HEV isolated from humans living in the same geographic regions suggests that transmission from swine to humans can occur (Figs. 70.11 and 70.12). This hypothesis is strengthened by the demonstration of HEV (transmissible in one instance) in commercial pig livers in several countries, the recovery of virtually identical HEV sequences from hepatitis E cases and food they had eaten, and a higher prevalence of anti-HEV in those who have had greater contact with swine and swine products.^{108,127,133}

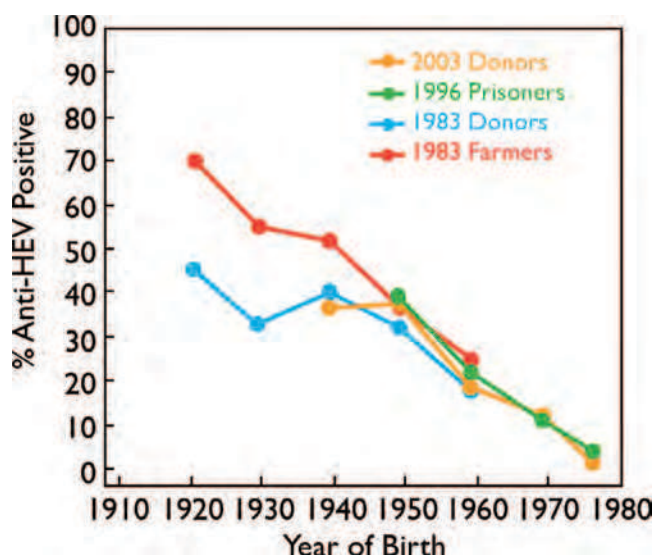


FIGURE 70.10. Prevalence of anti-hepatitis E virus (HEV) in Danish blood donors, farmers, and prisoners who were bled at different times (1983–2003). The data are plotted by year of birth and are interpreted to demonstrate the Cohort Effect, in which individuals born early in the 20th century were more likely to be exposed to HEV than those born later in the century. Similar patterns of antibody to hepatitis A virus were also found, suggesting that both viruses were more common at an earlier time. (From Christensen PB, Engle RE, Hjort C, et al. Time trend of the prevalence of hepatitis E antibodies among farmers and blood donors: a potential zoonosis in Denmark. *Clin Infect Dis* 2008;47(8):1026–1031.)

Indeed, cases of hepatitis E in industrialized countries that are associated with infection by swine-like strains occur in older individuals or those with impaired immunity (see Fig. 70.8), and such infections with strains that can replicate in swine (genotypes 3 and 4) tend to be less severe than those caused by exclusively human strains (genotypes 1 and 2). Thus, in developing countries where human and swine strains coexist, the more virulent human strains predominate clinically. However, in industrialized countries where human strains cannot sustain themselves in the environment, swine strains are the predominant strains associated with disease (Fig. 70.13). However, zoonotic transmission of HEV from swine to humans cannot explain endemic cases of hepatitis E that occur in regions where swine are uncommon and not eaten for religious or other reasons. The recent recovery of genotype 4 HEV from cattle and sheep in China suggests that these important components of the human food chain (along with goats) might be important vectors of transmission, although this has yet to be demonstrated.^{56,165} Neither avian HEV nor rat HEV is thought to be transmissible to humans, because neither is transmissible to rhesus monkeys—a sensitive host for the four principal mammalian HEV genotypes and a surrogate of man.^{113,134}

Molecular approaches to the epidemiology of HEV hold some promise. Reverse transcription polymerase chain reaction (RT-PCR) has been useful for confirming the results of serologic tests and for estimating the duration of infectivity of individual cases.²⁷ It is less sensitive, however, than serologic tests for diagnosis of acute hepatitis E and of limited value for molecular-epidemiologic studies because of the relatively short

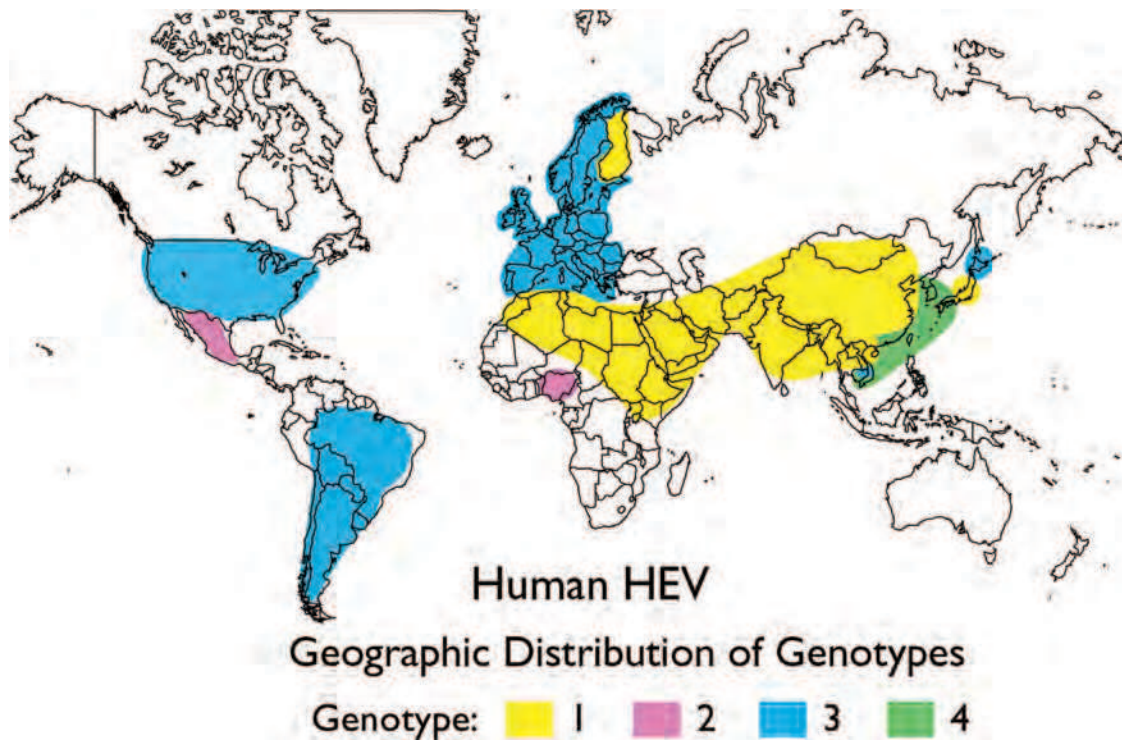


FIGURE 70.11. Each of the four genotypes of hepatitis E virus that infect humans has a distinct—and in some cases, overlapping—geographic distribution. Uncolored areas lack sufficient information. (Reprinted from Purcell RH, Emerson SU. Hepatitis E: an emerging awareness of an old disease. *J Hepatol* 2008;48:494–503.)

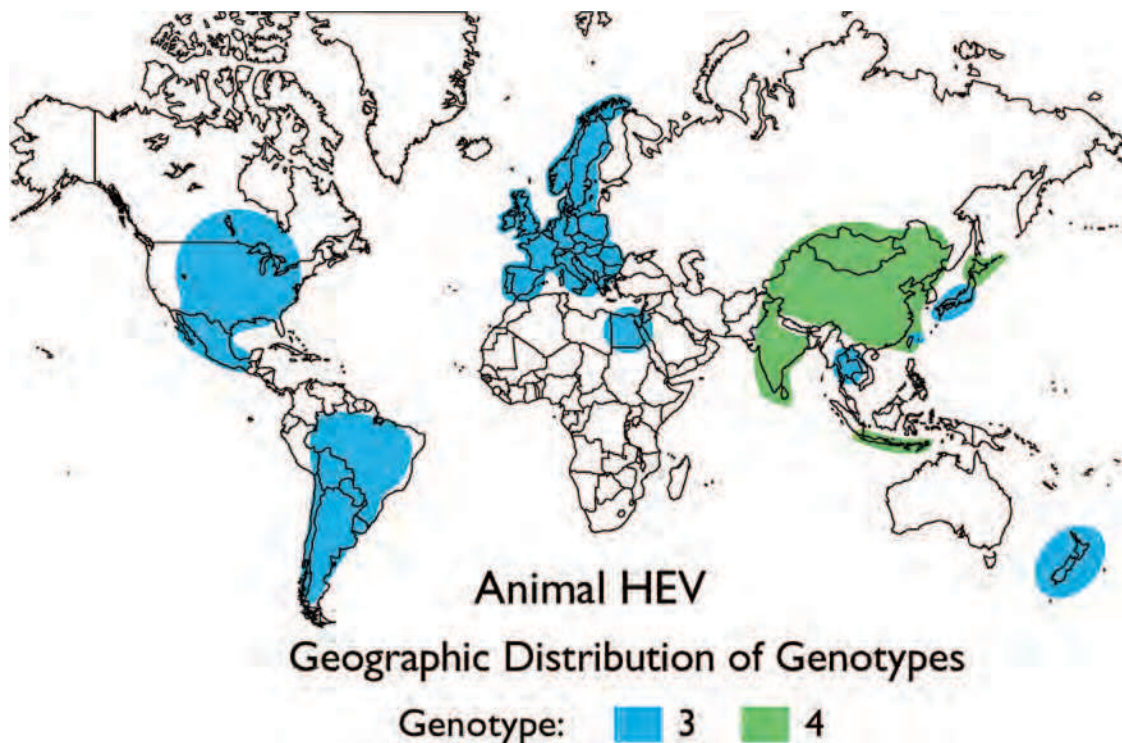


FIGURE 70.12. Hepatitis E virus genotypes 3 and 4, which infect both humans and swine, have been recovered from pigs (and occasionally other animals) in regions that roughly parallel distribution of these viruses in human infections. However, there are exceptions (see Figure 70.13). (Reprinted from Purcell RH, Emerson SU. Hepatitis E: an emerging awareness of an old disease. *J Hepatol* 2008;48:494–503.)

HEV Infections: Developing vs Industrialized Countries

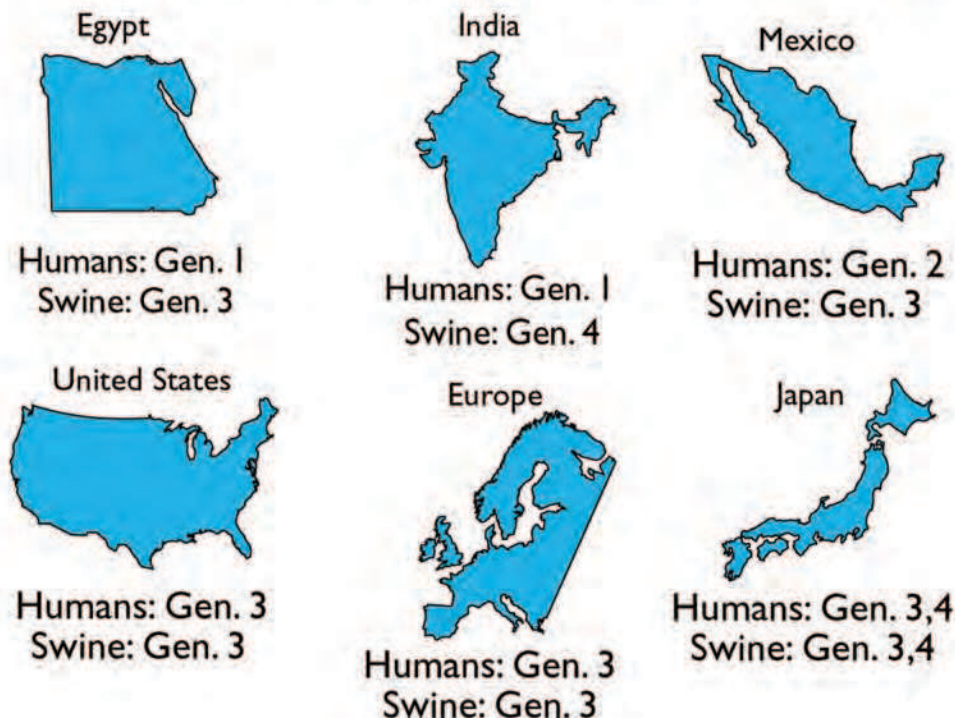


FIGURE 70.13. In many countries where human hepatitis E virus (HEV) genotypes 1 or 2 are endemic, these viruses are responsible for the most human clinical infections, even though swine HEV genotypes 3 or 4 are endemic in local swine herds. In regions where the human HEV genotypes cannot persist, presumably for reasons of sanitation, swine HEV strains occasionally cause human disease, probably by ingestion or other contact with infected pigs.

duration of viremia and fecal shedding. Its main value in this regard has been the identification of HEV genotypes and their relationship to specific epidemics and geographic locations. In addition, RT-PCR has been used to detect HEV in contaminated water and sewage.^{30,91,129,162}

CLINICAL FEATURES AND COURSE

Individual cases of hepatitis E cannot be differentiated from other cases of hepatitis on the basis of clinical presentation.¹³⁷ In fact, clinical features are of limited usefulness in the diagnosis of acute hepatitis caused by any of the recognized viruses.¹³⁵ Sensitive serologic tests have revealed that not all HEV infections are clinically apparent.²¹ In epidemics, however, most patients will experience jaundice, anorexia, and hepatomegaly. Approximately half of the patients will experience abdominal pain and tenderness, nausea and vomiting, and fever. Sequelae and extrahepatic manifestations of infection include various neurologic manifestations, including inflammatory polyradiculopathy, Guillain-Barré syndrome, encephalitis, and various neuropathies and myopathies.^{47,71,98,101} These have been diagnosed in patients with acute, self-limiting hepatitis E as well as in immunosuppressed patients with chronic infection. HEV has also been detected in the cerebrospinal fluid of some of these patients.⁷¹ Most reports of neurologic sequelae have been in European patients infected with genotype 3, presumably via

zoonotic exposure. In Asian pregnant patients with severe hepatitis E, excess fetal wastage and premature deliveries with a high infant mortality rate have been reported.

DIAGNOSIS

Hepatitis E should be suspected in outbreaks of water-borne hepatitis occurring among citizens of developing countries, especially if the disease is more severe in pregnant women. In the United States and Europe, hepatitis E should be suspected in a patient with hepatitis who has recently returned from an endemic region. In addition, hepatitis E has been reported with increasing frequency in individuals who have not traveled to an endemic region. These cases, principally from Europe but also from other regions, including the United States and Japan, may be associated with eating undercooked pork or wild game or other contact with potentially infected animals. As with all types of viral hepatitis, serologic tests are necessary to establish a definite diagnosis. Specific tests for IgM and IgG antibodies to HEV have been developed and are commercially available in Europe, Asia, and elsewhere, but not in the United States, where the diagnosis of hepatitis E remains an experimental procedure. Current tests are capable of detecting IgM anti-HEV in up to 90% of acute infections if a serum sample is obtained 1 to 4 weeks after the onset of disease.^{21,43,50,116} IgM anti-HEV reaches peak titers during the first 4 weeks after the onset of hepatitis,^{10,21} and by

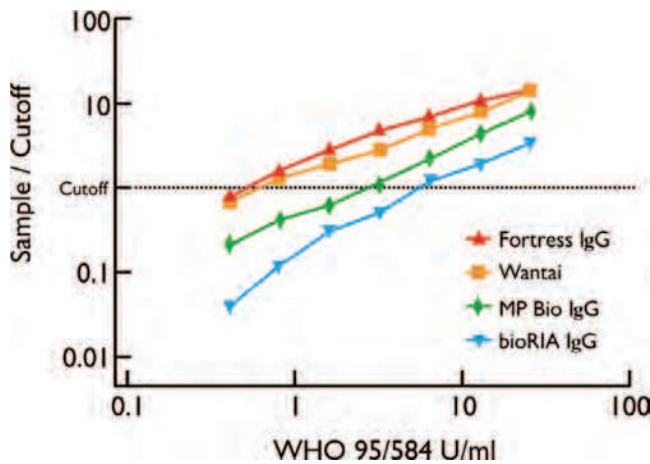


FIGURE 70.14. Variation in sensitivity of commercial tests for detecting immunoglobulin G anti-hepatitis E virus (HEV) when tested against World Health Organization Anti-HEV Standard 95/584. The titer of the standard differed by 24-fold when tested with the most and least sensitive tests. These tests are licensed and available in several countries; however, no test is licensed and commercially available in the United States. Consequently, it is thought that at least some cases of hepatitis E remain undiagnosed.

3 months after the onset of disease, IgM is no longer detectable in 50% or more of patients with hepatitis E.^{10,16}

A rising titer of IgG anti-HEV is also diagnostic. IgG anti-HEV peaks in titer between 2 and 4 weeks after onset of hepatitis and diminishes relatively rapidly thereafter.^{10,21,35} It is not clear how long IgG anti-HEV persists at detectable levels. Serologic tests continue to vary greatly in sensitivity and specificity^{19,36,103} (Fig. 70.14).

Molecular approaches (nested RT-PCR, real-time polymerase chain reaction [PCR]) are useful for detecting HEV in blood and feces during the acute phase of infection. They have contributed to the identification of new genetic variants of HEV^{114,141}; however, the sensitivity of the technique depends on a proper match between the HEV strain and the PCR primers.^{18,141} More broadly reactive RT-PCR primers that can detect most, if not all, mammalian HEV strains have recently been described.⁶⁶ Thus, serologic and molecular approaches to diagnosis are complementary.⁵⁹

TREATMENT

No specific treatment exists for acute hepatitis E. Both interferon alpha and ribavirin have been used successfully to treat chronic HEV infections.^{54,70,73,100} Aggressive management of fulminant hepatitis E may reduce the mortality of this complication in pregnant women.⁶²

Access to a clean water supply and cooking meats to a temperature that inactivates HEV should, in theory, greatly decrease the incidence of HEV infections. HEV is only partially inactivated at 56°C.^{38,154}

Interdiction

Improved personal and public hygiene appears to have resulted in a reduction in the incidence of HEV infections in industrialized

countries and more recently in developing countries; however, paradoxically, more cases of hepatitis E are being reported now than previously, probably because of improving diagnostic tests, better surveillance, and a higher index of suspicion. Hepatitis occurring in Europe before the 20th century, which was believed at that time to have been hepatitis A, had the epidemiologic characteristics of hepatitis E.³¹ Thus, HEV—more labile and shed in lower titers than HAV—may have largely disappeared from more industrialized countries in the recent past, just as HAV is diminishing in importance in these countries; seroepidemiologic studies of HEV and HAV in Denmark are consistent with this interpretation.²⁹ Regions where HAV is still highly endemic may, however, also harbor endemic HEV.^{8,14,92} It is worth noting that this *labile* virus was still infectious when recovered from sewage in Spain, a country in which the virus is not highly endemic.¹²⁹ The high prevalence of anti-HEV in domestic and wild animals, even in industrialized countries, and the epidemiologic association between hepatitis E and ingestion of undercooked pork and wild game are evidence that zoonotic spread of HEV can continue in such regions, despite improved sanitation.^{13,34,93,109,112,121,166}

IMMUNOPROPHYLAXIS

Attempts to prevent or modify hepatitis E by the administration of normal immune globulin manufactured from source plasma obtained in the areas where HEV is endemic have been unsuccessful^{23,67,80} or uncertain.¹⁵⁸ Because most of these studies were performed before the development of sensitive and quantitative tests for anti-HEV, the lots of globulin employed were not tested for the presence or titer of anti-HEV. Because even populations in regions where HEV is endemic have a relatively low prevalence and titer of anti-HEV (see earlier discussion), it is perhaps not surprising that unselected lots of normal immune globulin may not contain protective quantities of anti-HEV. Limited experimental studies in primates suggest that passively acquired anti-HEV (convalescent or monoclonal) modifies but may not prevent HEV infection.^{142,159}

Vaccine

Neutralization epitopes for the capsid protein are conformational and encompass approximately 150 aa (aa 458–607) of this 660 aa protein.^{106,179} This region of the capsid protein also contains elements that are essential for homodimer formation and assembly into VLPs, both associated with eliciting neutralizing antibodies.^{94–96} Homodimers, the smallest units that can interact with neutralizing antibodies and stimulate protection, spontaneously form VLPs that share the antigenic characteristics of homodimers^{94,169,170} (see Fig. 70.3). Both the homodimer and VLP forms of the truncated capsid antigens, whether expressed from baculovirus or *Escherichia coli*, have been highly antigenic and suitable for use as vaccines. The four genotypes of HEV that infect humans constitute a single serotype, and challenge studies in monkeys demonstrated protection across genotypes.^{136,137} Vaccines for the prevention of hepatitis E are not commercially available. Candidate recombinant hepatitis E vaccines, expressed from the capsid gene of HEV in *E. coli* or from baculovirus in insect cells, however, have been shown to be highly protective in clinical trials^{144,180} (Table 70.2).

TABLE 70.2 Candidate Hepatitis E Vaccines

Vaccine manufacturer	Antigen (genotype 1)	Expressed from	Dose	HEV genotype in vaccinated population	Efficacy (95% CI)
GlaxoSmithKline (Belgium)	Recombinant capsid protein (aa 112–607)	Baculovirus in insect cells	20 µg with alum	1	95.5% (89–99)
Xiamen Innovax Biotech (China)	Recombinant capsid protein (aa 368–606)	<i>E coli</i>	30 µg with alum	4	100% (72–100)

T-cell responses in hepatitis E have not been extensively characterized. However, a recently developed interferon gamma Elispot assay that was sensitive and quantitative should prove useful because it correlated strongly with anti-HEV results from human subjects and detected specific cell-mediated immune responses to HEV in convalescent chimpanzees 3 to 4 years after experimental infection.¹⁴³

PERSPECTIVES

Considerable progress has been made in understanding HEV since the virus was first identified in 1983. Four genotypes have been characterized and more are being discovered, their worldwide distribution is being determined, and two recombinant vaccines have completed clinical trials and have been shown to be highly protective. HEV has been recovered from numerous species of animals, and the zoonotic transmission of HEV has been documented.

In contrast to these advances, the lack of a reliable cell culture system had inhibited studies of the molecular biology of this virus; however, this is changing, as new cell culture systems and new sources of virus (e.g., from chronic infections) have recently become available.

Future studies are expected to determine whether (or which) animal reservoirs are the most important sources of human infection. The hypothesis that circulating naturally avirulent strains are common in some countries needs to be examined, as does the possibility that frequent contact with low levels of virus protects against disease. We need to know why and how the virus causes uniquely high mortality in pregnant women. Also, a commercially available licensed test is desperately needed in the United States so that cases of idiopathic hepatitis might be diagnosed.

In summary, many questions concerning the biology, pathogenesis, epidemiology, and immunology of HEV remain unanswered, and the tools to answer these questions are just now becoming available.

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Mimivirus, Other Viruses Infecting Phagocytic Protists, and Their Virophages

Mimiviridae

Acanthamoeba Polyphaga Mimivirus
Acanthamoeba Castellani Mamavirus
Cafeteria Roenbergensis Virus

Marseilleviridae

Marseillevirus
 Lausannevirus

Virophages: Viruses Infecting Mimiviridae

Definition
 Sputnik
 Mavirus

Epidemiology of Giant Viruses Associated with Phagocytic Protists

Ecology of Giant Viruses with a Sympatric Lifestyle in Phagocytic Protists: Amoebae as Genitors of Giant Viruses with Mosaic Gene Repertoires

Pathogenicity

Laboratory Diagnosis

Prevention and Control

Giant Viruses Associated with Protists Challenge the Definition of Viruses and Lead to Proposal of the Fourth Domain Theory

Conclusion

The serendipitous discovery in 2003 of *Acanthamoeba polyphaga mimivirus* led to groundbreaking findings and, subsequently, fed several debates regarding the evolution, origin, and definition of viruses.^{42,76,113,114} Although several uncommonly large viruses had already been discovered since the 1960s, the Mimivirus capsid was larger than those known earlier, and the Mimivirus gene content was broader than that of any other known virus. In addition, several genes encoding proteins involved in amino acid, carbohydrate, or lipid metabolism, among which some had never been previously found in viruses, are present in the Mimivirus genome. Taken together, Mimivirus changed our vision of the viral world. The size and gene repertoire of Mimivirus deeply challenged the canonical definition of viruses, which had historically been considered as small “ultra-filterable” entities. Indeed, viruses were mostly defined on the basis of negative criteria, including the fact that

they were not retained by a porcelain filter and were not visible using light microscopy, in contrast with bacteria.^{6,57,84,115} Moreover, viruses were removed from the tree of life, which contains bacteria, archaea, and eukaryotes,^{96,142} based on the absence of genes shared with members of these three recognized domains of life. Furthermore, the increased knowledge of giant viruses reactivated the debate about whether or not viruses are alive.^{41,96,114}

Following Mimivirus, other giant viruses have been recovered from *Acanthamoeba* spp., including Mamavirus, a new strain of Mimivirus, and Marseillevirus and Lausannevirus, which are close relatives.^{15,28,78,133} In addition, another giant virus has been described in *Cafeteria roenbergensis*, a marine dinoflagellate protist, but was nonetheless classified within the family *Mimiviridae*.³⁹ Moreover, based on new high-throughput protocols for amoebal culture, La Scola et al⁷⁷ identified 19 new giant viruses infecting *Acanthamoeba* spp., among which several can be classified within the family *Mimiviridae*. Furthermore, viruses infecting Mimivirus, Mamavirus, and *Cafeteria roenbergensis* virus were also discovered, which led to the concept of “virophages” based on functional similarity with bacteriophages.⁷⁸

MIMIVIRIDAE

Acanthamoeba Polyphaga Mimivirus

History and Discovery

Mimivirus was discovered in water collected from a cooling tower in Bradford (England) through the use of culture on amoebae.⁷⁶ Mimivirus was part of a collection of pathogens isolated by Dr. Tim Rowbotham by culturing on the amoeba *Acanthamoeba polyphaga* to investigate a pneumonia outbreak.¹¹⁵ Several bacteria that are pathogenic in amoebae were identified by this approach, but no other agent could be identified. Only the use of electron microscopy to understand the mechanism by which this agent resisted protocols that were used to amplify and detect its nucleic acid led to the discovery of Mimivirus. Notwithstanding, Mimivirus was visible by optical microscopy and appeared as a gram-positive coccus after staining.^{76,115} Therefore, it was surprising to find viral particles using electron microscopy of a size greater than that of two dozen bacteria, which led to consideration initially and for several months of these particles as bacteria, not viruses. Later, the genome of Mimivirus was sequenced and, congruently with the particle size, was revealed as the largest sequenced viral genome.¹¹³

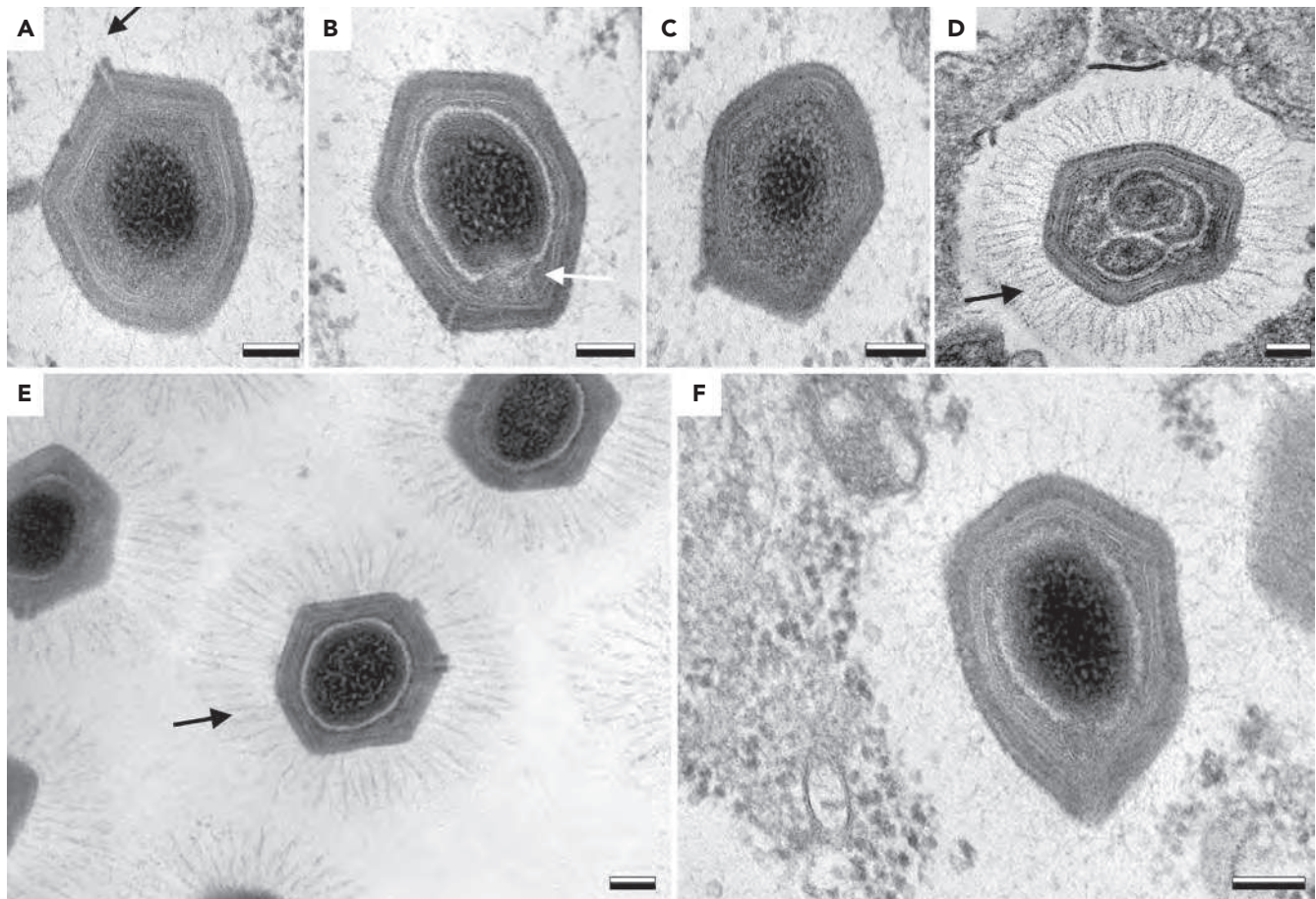


FIGURE 71.1. Electron microscopy of Mimivirus particles. **A:** Arrow shows a membrane conduit formed by the star-shaped structure sliced in its center. **B:** Arrow shows the concave face of the nucleocapsid and the low-density space beneath the starfish-associated vertex. **D, E:** Arrows show Mimivirus fibrils. Scale bars indicate 100 nm.

Structure

GENERAL SHAPE

The Mimivirus structure was studied through different approaches, that is, by means of traditional transmission electron microscopy (TEM) of thin sections, scanning electron microscopy (SEM), cryoelectron microscopy (cryo-EM), electron tomography, x-ray crystallography, atomic force microscopy (AFM), and x-ray laser.^{64,74,75,122,144,145,149} Taken together, these studies revealed a complex structure for a virus as well as structural relationships to other viruses including, for instance, phycodnaviruses (e.g., *Paramecium bursaria chlorella* virus 1 [PBCV-1])¹³⁵ and the infectious bursal disease virus.^{11,64} The Mimivirus virion size is ~750 nm, as demonstrated using EM (Fig. 71.1).^{75,76} Along with making Mimivirus the largest known virus thus far at time of its discovery, its huge size is of the same order of magnitude as intracellular bacteria, such as *Rickettsia conorii* and *Tropheryma whipplei*, and it is even greater than that of very small bacteria, such as *Ureaplasma urealyticum*.^{76,130} EM and cryo-EM revealed that Mimivirus has an icosahedral shape with a vertex-to-vertex diameter of ~500 nm (Fig. 71.2).^{64,76,144} However, the shape of the virion does not display ideal icosahedral symmetry due to the presence of a starfish-shaped structure at one icosahedral fivefold vertex (Figs. 71.2 and 71.3).⁶⁴ The five faces supporting the

starfish are inclined by ~5% compared to what would correspond to an ideal icosahedral capsid.¹⁴⁵ Only one fivefold symmetry axis does exist, which passes through this special vertex.

OUTER FIBERS

Fibers ~120 to 140 nm in length and ~1.4 nm in diameter are present on almost all of the surface of the viral capsid (Fig. 71.1D, E; Fig. 71.2B). The fibers are closely packed and compose a dense layer.^{64,75} These fibers are extensively glycosylated, peptidoglycan likely protecting them from proteolysis.⁷⁵ This is suggested by AFM images, by resistance of fibers to proteases unless they have been previously treated with lysozyme, and by the initial observation that Mimivirus was gram positive after staining.^{76,115,145} These fibers are suspected to be helicoidal and polar.⁷⁵ They were also suspected to be composed of collagen,^{113,144} but this hypothesis has since been ruled out.⁷⁵ The fibers seem to be fabricated apart from the viral particle and to be attached to the viral particle at a late stage during the viral assembly pathway.^{129,149} Successive rings of density separated by 20 to 50 nm have been observed on the fibers after partial treatment with bromelain, which appear to be different segments.¹⁴⁵ At their distal ends, outer fibers are linked to a small globular shape that has an estimated size of 3.5×5 nm.^{75,145} Multiple surface fibers, possibly in groups of three or four, may

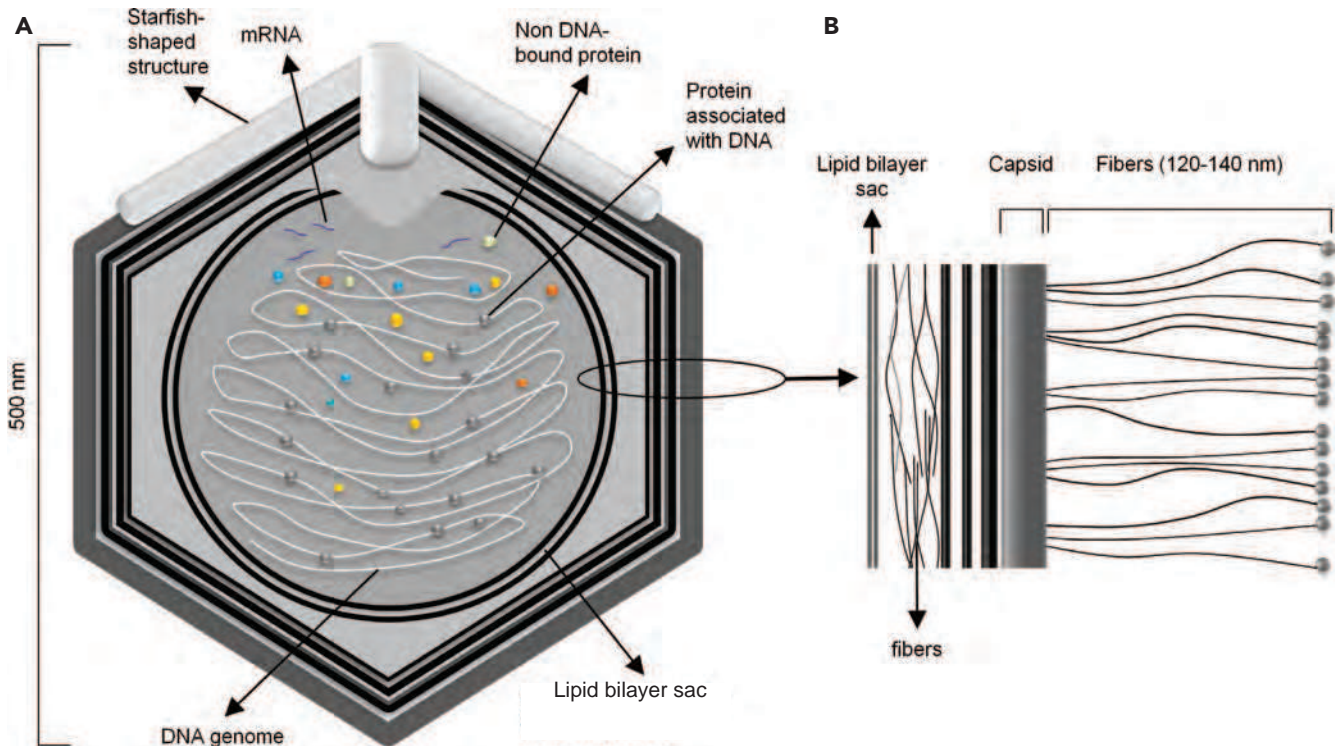


FIGURE 71.2. Schematic of the structure of Mimivirus particles. **A:** The whole viral particle. **B:** The outer fibers, the capsid, and the membrane of the sac that contains the DNA genome. (Adapted from 64.)

be attached at their proximal extremity to a disc-shaped anchor protein or to a capsomer.^{75,145} Indeed, an anchor layer seems to connect the fibers to the capsid lattice. It appears to be composed of proteins arranged in a closely packed array of likely common structures that protrude from the capsid surface by ~3 to 4 nm.⁷⁵ The anchor protein may be the capsomer itself, corresponding to large extended loops that would protrude

from the double jelly-roll structure of the major capsid protein (MCP).¹⁴⁴ Alternatively, the anchor structure may be composed of other proteins that lay upon the capsid shell.

CAPSID

The Mimivirus MCP has a double jelly-roll fold,⁶⁴ as in other large double-stranded DNA (dsDNA) viruses, including

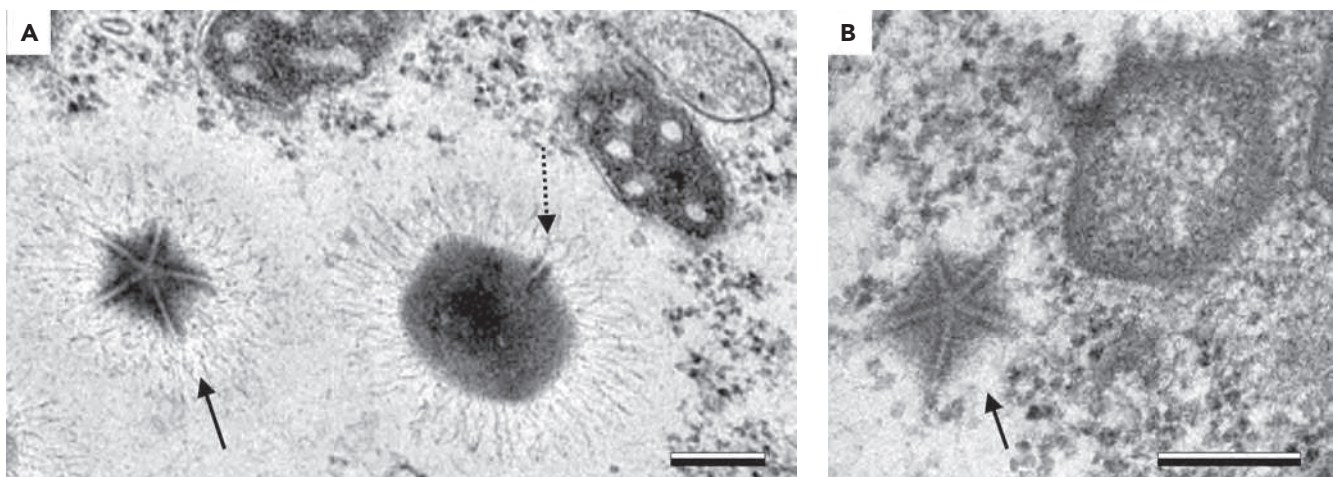


FIGURE 71.3. Electron microscopy showing vertices of Mimivirus particles. Star-shaped structures (indicated by solid arrows) as visualized by transmission electron microscopy. The dashed arrow in **A** shows a star-shaped structure sliced in its center, forming a membrane conduit. Scale bars indicate 200 nm.

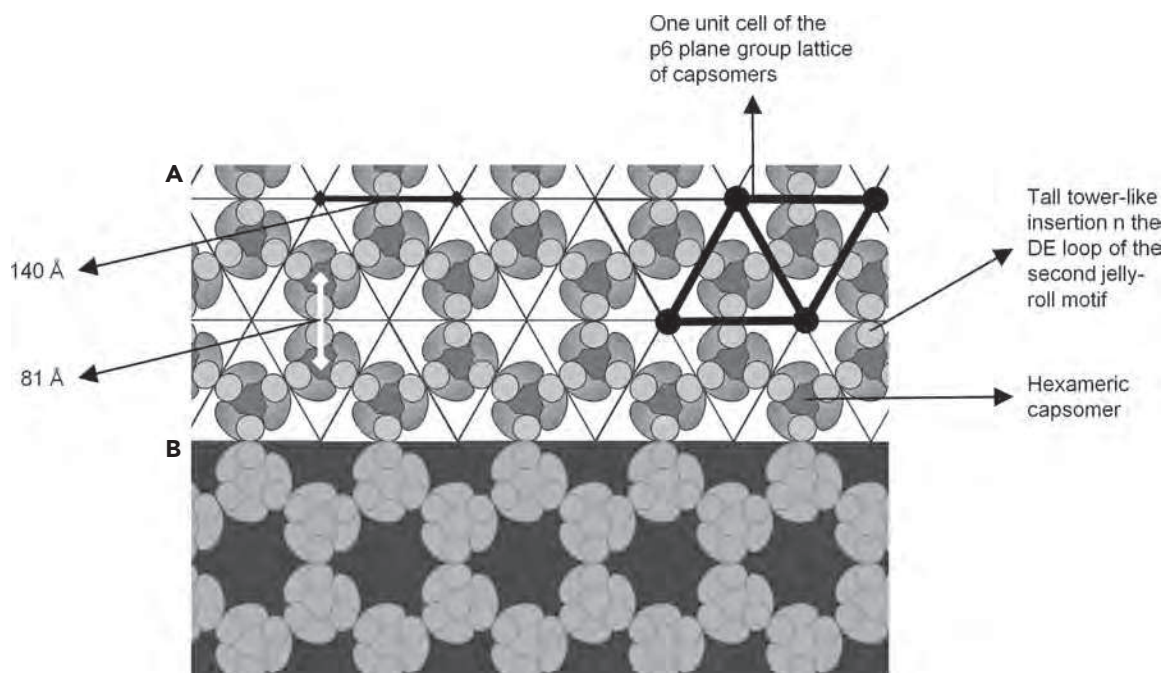


FIGURE 71.4. Schematic of capsomer arrangement in the Mimivirus capsid. A: Arrangement of capsomers with p6 symmetry in the Mimivirus capsids. **B:** Honeycomb array appearance composed by alternating hexagonal rings of capsomers and depressions. (Adapted from 145.)

PBCV-1, *Sulfolobus* turreted icosahedral virus, adenoviruses, and bacteriophages PRD1 and PM2.^{9,145} The L425 protein is the most abundant capsid protein in Mimivirus, and it shares substantial amino acid sequence similarity (31%) with the Vp54 protein of PBCV-1.¹⁴⁴ Capsomers are hexameric and are composed of three monomers of the MCP, each of which is formed by two consecutive jelly-roll folds (Fig. 71.4A).¹⁴⁵ In Mimivirus, an insertion in the DE loop of the second jelly-roll motif is particularly long (190 amino acids). These amino acids form a tall tower-like insertion, which is similar to that found in adenoviruses.¹⁴⁵ The Mimivirus capsid has a p6 plane organization and a 2T/3 estimated number of jelly-rolls; the distance between capsomers is 81 Å (Fig. 71.4A).¹⁴⁵ Depressions separated by 14 nm and organized in a hexagonal array are present on the viral surface, as determined by cryo-EM and AFM (Fig. 71.4B).¹⁴⁵ Six capsomers arranged with p6 symmetry surround each depression. This arrangement has the appearance of a honeycomb with alternating hexagonal rings and depressions. The triangulation number (T) of the Mimivirus capsid can only be estimated due to the insufficient resolution of the hexagonal lattice on the viral surface, and it is within the range of 972 to 1,200.⁶⁴ It was observed that each equilateral triangular face contains 19 rows of depressions, which are parallel to the edge and comprise one less depression from one row to another away from the edge.¹⁴⁵ Kuznetsov et al⁷⁵ pointed out that the Mimivirus MCPs may interact in a very flexible manner with their neighbors, or they may have a broad range of conformations, to provide such a continuous smooth aspect to the icosahedral surface. In fact, the capsid seems to have three layers of dense matter, consisting of a ~7-nm-thick protein shell that surrounds two successive 4-nm-thick lipid membranes separated by 6 nm (Fig. 71.2).¹⁴⁴

Thus, the inner capsid surface differs from the outer surface and is suspected to be coated with a lipid membrane associated with proteins or a layer of proteins.^{75,149} The length of the edges of the capsid is estimated to be ~250 nm. Thus, the interior volume may be $\sim 3.4 \times 10^7 \text{ nm}^3$ with only two-thirds of this space filled with the DNA genome, proteins associated with the genome, and the membrane sac.⁷⁵

INNER LAYERS AND NUCLEOCAPSID

The presence of an inner lipid membrane, which surrounds the central core, is shared with other nucleocytoplasmic large DNA viruses (NCLDVs), including phycodnaviruses and African swine fever virus.^{144,149} Beneath the capsid and the two electron-dense layers, at a distance of 300 to 500 Å from the outer capsid, is a lipid bilayer sac that surrounds the genome (Fig. 71.2).^{75,145} The nucleocapsid forms the central electron-dense core of the Mimivirus. It displays a large depression that faces the starfish-associated vertex and is separated from it by a low-density space constituting a cavity that is hypothesized to contain enzymes required for infection (Figs. 71.1 and 71.2).^{144,145} The nucleocapsid is held within the virus in such a manner that the depression constantly faces the special vertex and that the distance between the outer capsid and the sac surrounding the genome remains stable. It has been observed that ~1.2-nm-diameter internal fibers with a 7-nm periodicity are present in the space between the inner capsid surface and the sac containing the DNA, and they might support this architecture (Fig. 71.2).^{75,144} These fibers are long and flexible, and they may be extended arrangements of coiled-coil protein polymers, similar to those composing keratin, myosin, or kinesin.^{54,74} In contrast, however, they are unlikely to be constituted by successive protein subunits or to be polysaccharides or nucleic

acids.^{74,75} These fibers may be glycosylated, at least over some lengths, and were occasionally associated with toroidal complexes, which are presumed to be enzyme complexes that may modify the fibers by synthesizing and then attaching oligosaccharides.⁷⁴ Notably, these fibers appear capable of producing ribbons via a multiple-strand side arrangement as well as thick cables by twisting about one another.⁷⁴

The Mimivirus DNA chromosome exists in a sac whose diameter is estimated to be ~340 nm, corresponding to an approximate spherical volume of $2.1 \times 10^7 \text{ nm}^3$.⁷⁵ DNA shed by experimentally pressing viral particles appears as single strands with many turns and loops. Various proteins that are associated with the genome and that differ in both size and shape can be observed (Fig. 71.2). Based on the length of the DNA genome, its packing density is $\sim 0.06 \text{ nm}^3/\text{bp}$.^{75,113} This density is on the same order of magnitude as that estimated for the vaccinia virus⁷³ but is more than 10 times lower than for bacteriophages lambda and P22.⁷⁵ Nevertheless, Mimivirus and vaccinia virus DNA are associated with proteins, whereas the genomes of the two latter bacteriophages are not.

STARFISH-SHAPED STRUCTURE

Each Mimivirus harbors a starfish structure at one icosahedral fivefold vertex (Figs. 71.2, 71.3, and 71.5A). The arms of this starfish nearly extend to the five nearest surrounding vertices (Figs. 71.2 and 71.5).^{64,75} They are ~200 to 250 nm in length, ~50 nm in width, and ~40 nm in thickness.^{75,149} These arms are integrated into the capsid network, protruding above the capsid surface by ~20 nm, and they do not lie on capsid proteins but display a structural continuity with the capsid network on their edges.⁷⁵ The composition of these starfish arms remains unknown. They are thought to be constituted of proteins that are different from the MCP because they are devoid of hexagonal arrays of depressions.¹⁴⁵ Nevertheless, they suffer no damage when exposed to cysteine and serine proteases.⁷⁵ The starfish is not coated with fibers or with a layer of anchoring proteins, and it therefore forms a starfish-shaped depression on the surface of viral particles that harbor fibers.¹⁴⁹ The outer shell at this location has a partially open configuration that is sealed by the underlying inner shell (Fig. 71.5). The starfish-shaped structure may be a gate for the internal viral content to move to the amoebal cytoplasm.¹⁴⁹ Special vertices allowing viral genome exit have also been described in other large dsDNA viruses, including PRD1,⁵¹ as well as herpesviruses and tailed bacteriophages.^{3,62,145}

UNCOMMON MORPHOLOGICAL ASPECTS OF MIMIVIRUS

Various uncommon morphological aspects have been observed for Mimivirus particles, including some showing uncondensed core material.¹³⁰ Some of these observations are suggested to correspond to immature or defective particles. Moreover, La Scola et al.⁷⁸ reported atypical morphological features for Mamavirus, another strain of Mimivirus, when infected with the Sputnik virophage (see the section on the Sputnik virophage) (Fig. 71.6).

Genomics

GENOME SIZE AND SHAPE

The Mimivirus genome was described in 2004.¹¹³ It is a ds, linear DNA molecule of ~1.18 megabase pairs, as attested by restriction digests and pulse-field gel electrophoresis (Fig. 71.7A).¹¹³ This genome became therefore the largest among viral genomes, being even larger than the genomes of several parasitic bacteria.⁶⁶ The Mimivirus chromosome is AT rich (72%). It has been hypothesized that the Mimivirus genome may adopt a circular conformation by means of pairing between inverted repeats ~600 nucleotides (nt) in length; due to location of these repeats toward the extremities of the genome, the circular chromosome would have two tails, a short one (~250 nt) and a longer one (~22,500 nt).^{20,113}

GENOME ORGANIZATION AND ANNOTATION

A total of 1,262 putative open reading frames (ORFs) were first identified in the Mimivirus genome, of which 911 have been predicted to encode proteins while the DNA also exhibited 6 tRNA (Fig. 71.7B).¹¹³ The DNA molecule has therefore a coding density of 90.5% and a mean intergenic space of 157 nt.¹¹³ Noteworthy, the Mimivirus genome harbors ~2.5 times as many genes as *Mycoplasma genitalium* or the archaeon *Nanoarchaeon equitans*.⁶⁶ The predicted genes are evenly distributed on both strands, with 450 and 461 ORFs located on negative and positive strands, respectively; they tend to be located far from a hypothetical origin of replication.¹¹³

A total of 298 ORFs have been annotated as having a functional attribute, which corresponded to approximately one-quarter of the predicted gene content. This proportion is low compared to that of the genomes of small bacteria and archaea, which is ~70%.⁴⁶ Among the Mimivirus ORFs, 194 could be assigned to 108 clusters of orthologous groups of proteins (COGs)¹¹³ corresponding to 17 functional categories (COGs are groups of three or more proteins present in

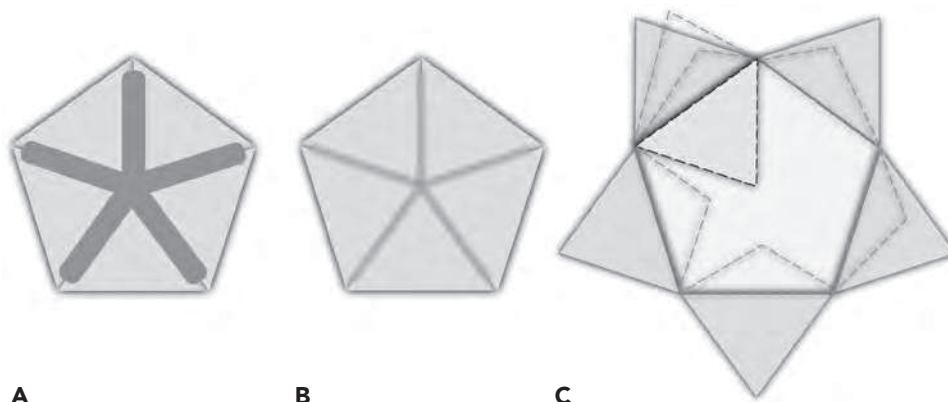


FIGURE 71.5. Schematic of the star-shaped structure and the opening of the five triangular faces of the capsid at the special vertex. The special star-shaped vertex is covered by the starfish structure arms (A), which detach from the viral particle (B) before the five triangular faces of the capsid underneath open outward (C).

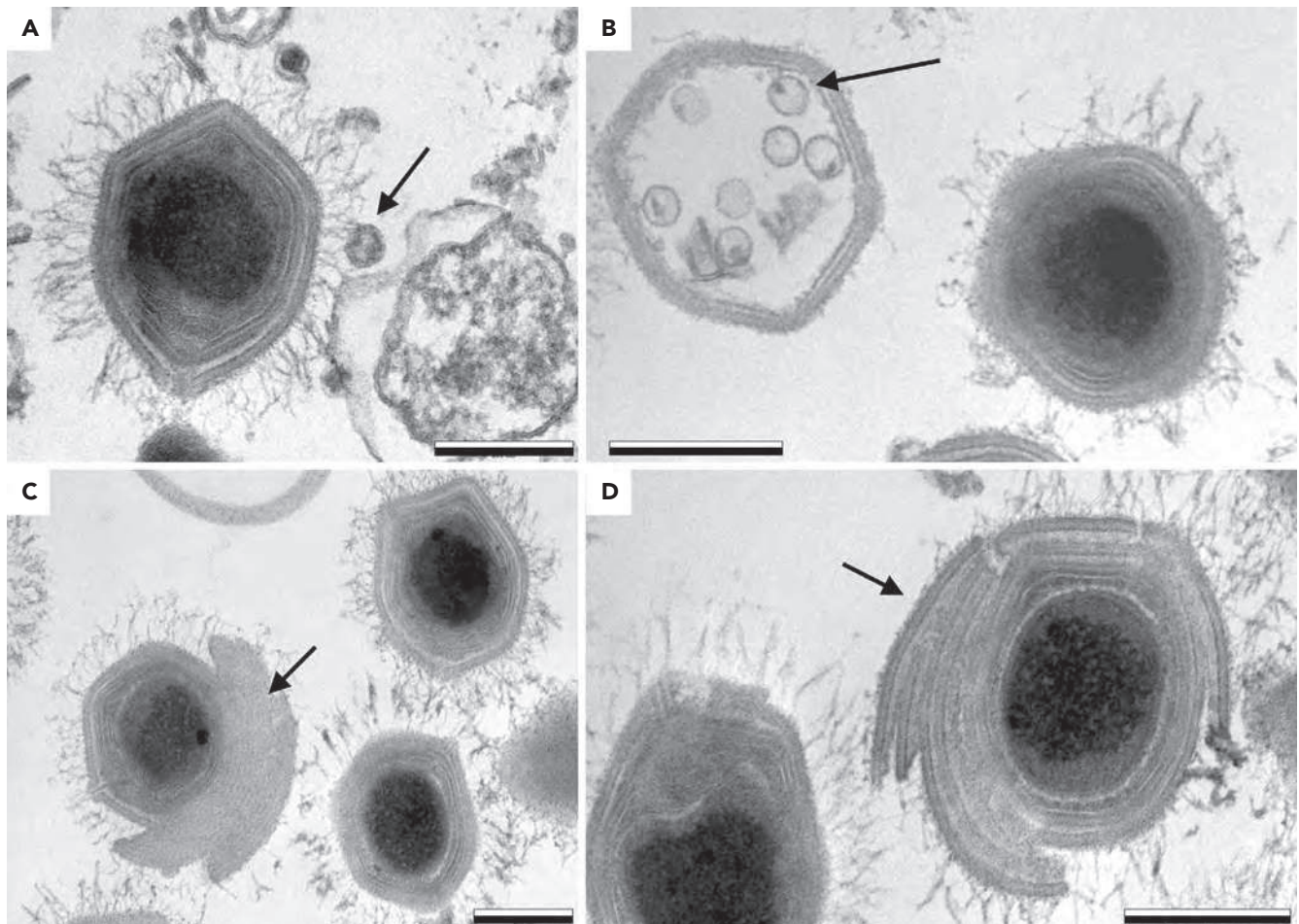


FIGURE 71.6. Electron microscopy of Mamavirus particles in co-infection with the Sputnik virophage (at H16 post-amoeba infection). **A:** Arrows show Sputnik particles associated with Mamavirus fibrils. **B:** Arrows show a Sputnik virophage particle inside a Mamavirus particle. **C, D:** Arrows show extramembrane layers in Mamavirus particles. Scale bars indicate 200 nm.

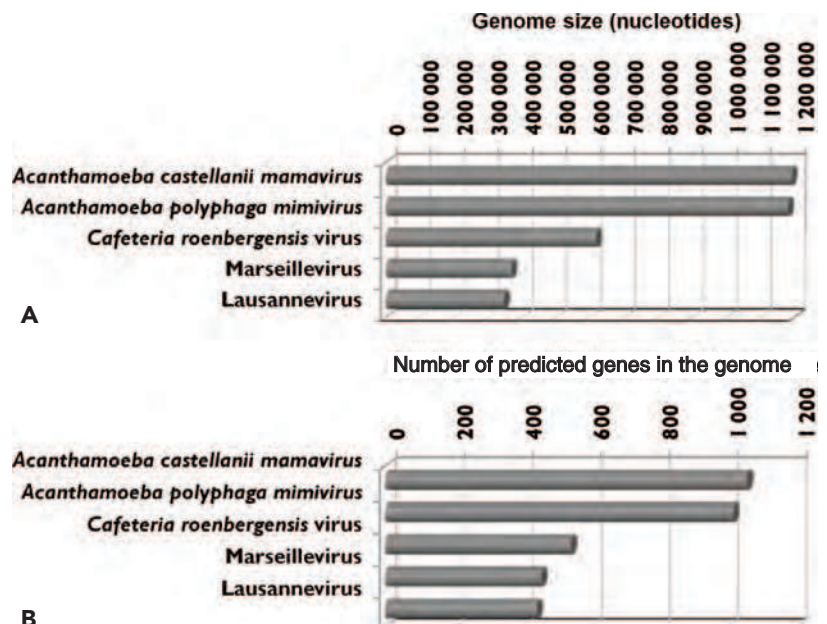


FIGURE 71.7. Genome size (**A**) and number of predicted protein-encoding genes (**B**) in giant viruses associated with phagocytic protists.

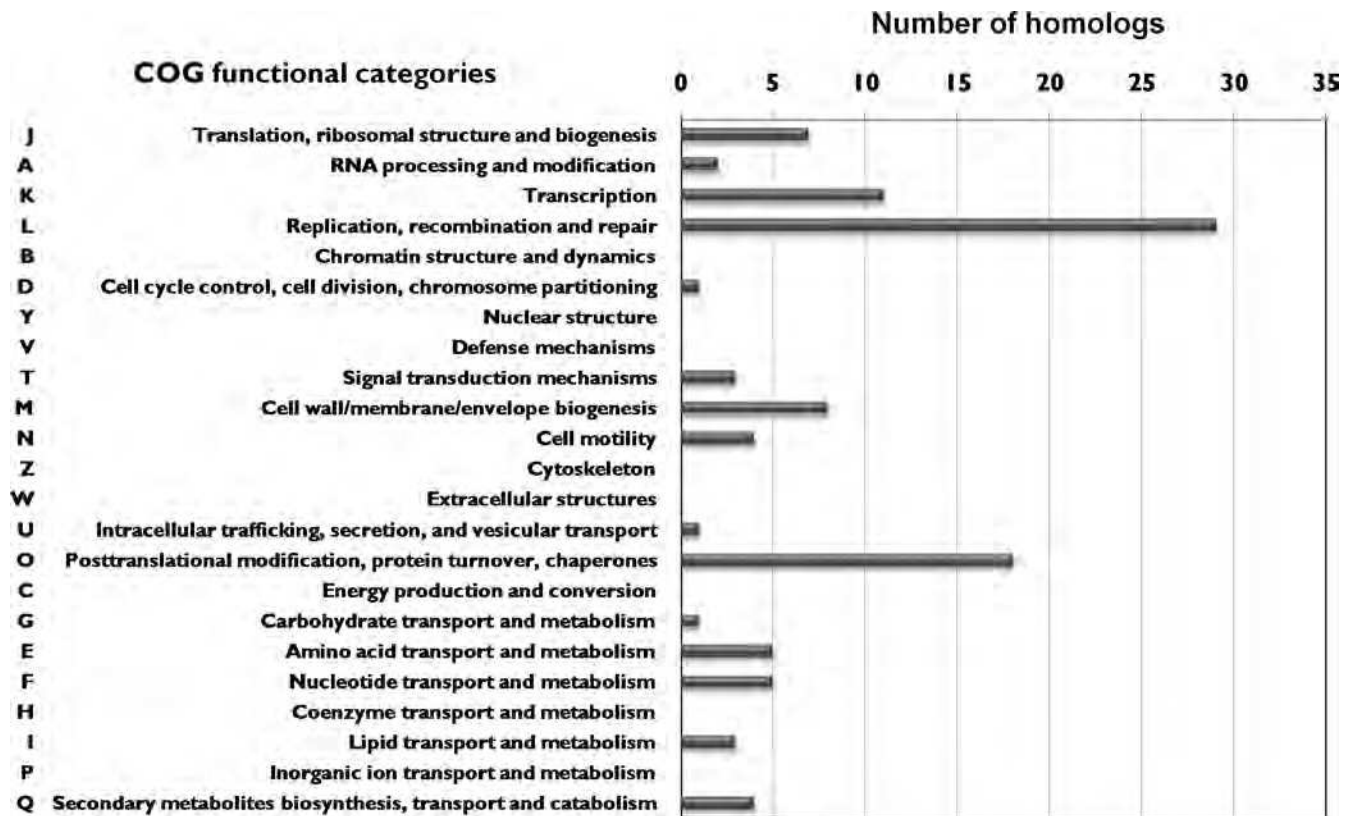


FIGURE 71.8. Distribution by functional categories of Mimivirus genes homologous to clusters of orthologous groups of proteins.

distant genomes and more similar to each other than to any other proteins within the same genome) (Fig. 71.8).¹³¹ In 2010, the Mimivirus genome was resequenced using SOLID ultra deep genome and transcriptome sequencing, then reannotated by Legendre et al.^{81,82} (GenBank accession no.: NC_014649.1). The new version of the genome has a length of 1,181,549 bp and was predicted to harbor 1,018 genes consisting of 979 genes that putatively encode proteins, 33 noncoding RNAs, and 6 transfer RNA (tRNA). Additionally, comparison of the Mamavirus and Mimivirus genomes enabled the amendment of the annotation for 159 ORFs (16%) of this new version of the Mimivirus gene content, which includes helicases and primases, kinases, endo-/exonucleases, methyltransferase, and adenosine triphosphatase (ATPase)/guanosine triphosphatase (GTPase).²⁸ The Mimivirus genes can be classified into several groups including those composed by the core genes, the duplicated genes, the genes transferred horizontally, and the ORFans.²⁶ Several studies have attested that horizontal gene transfer (HGT) and gene duplication have contributed considerably to the gene content of Mimivirus.²⁶

GENOMIC-BASED CLASSIFICATION

Mimivirus has been classified within the group of NCLDV, on the basis of comparative genomics and the definition of genes shared by all the viruses and composing a core genome (Fig. 71.9).^{58,59} The NCLDV group was conceived in 2001 by Iyer et al.⁵⁸ Originally, it consisted of four families of viruses, *Poxviridae*, *Asfarviridae*, *Iridoviridae*, and *Phycodnaviridae*,

which share 9 genes (including three viral hallmark genes) found in all of the viral families (group I) and 22 additional genes found in at least 3 of the 4 viral families (group II and III) (Fig. 71.10). In 2006, Iyer et al.⁵⁹ examined the Mimivirus genome and several other genomes newly available for three of the previously defined NCLDV families, and they tentatively defined the gene complement of the ancestral NCLDV and found that it harbored at least 41 core genes. Finally, Yutin et al.¹⁴⁸ identified 1,445 NCLDV COGs, so-called NCVGs, including 177 represented in more than one NCLDV family and 5 including proteins from all 45 analyzed viruses (Fig. 71.11).¹⁴⁸ Additionally, they used a maximum-likelihood reconstruction of the evolution of NCLDVs to define a set of 47 conserved genes that were probably present in the genome of the NCLDV common ancestor.

GENE REPERTOIRE

Core Genes. The core genes of the NCLDVs have been classified from the most to the least evolutionarily conserved, as class I when found in all lineages, as class II when missing in some species albeit being present in all lineages, and as class III when missing in one lineage (Fig. 71.10).¹¹³ The Mimivirus genome encodes homologs for the 9 NCLDV core genes of group I, for 6 out of 8 core genes of group II, and for 11 out of 14 core genes of group III (Fig. 71.12A).

Horizontally Transferred Genes. HGT refers to the exchange of genes between unrelated organisms and is a major factor of evolution in microbiology.^{50,60,65} Because of the high frequency

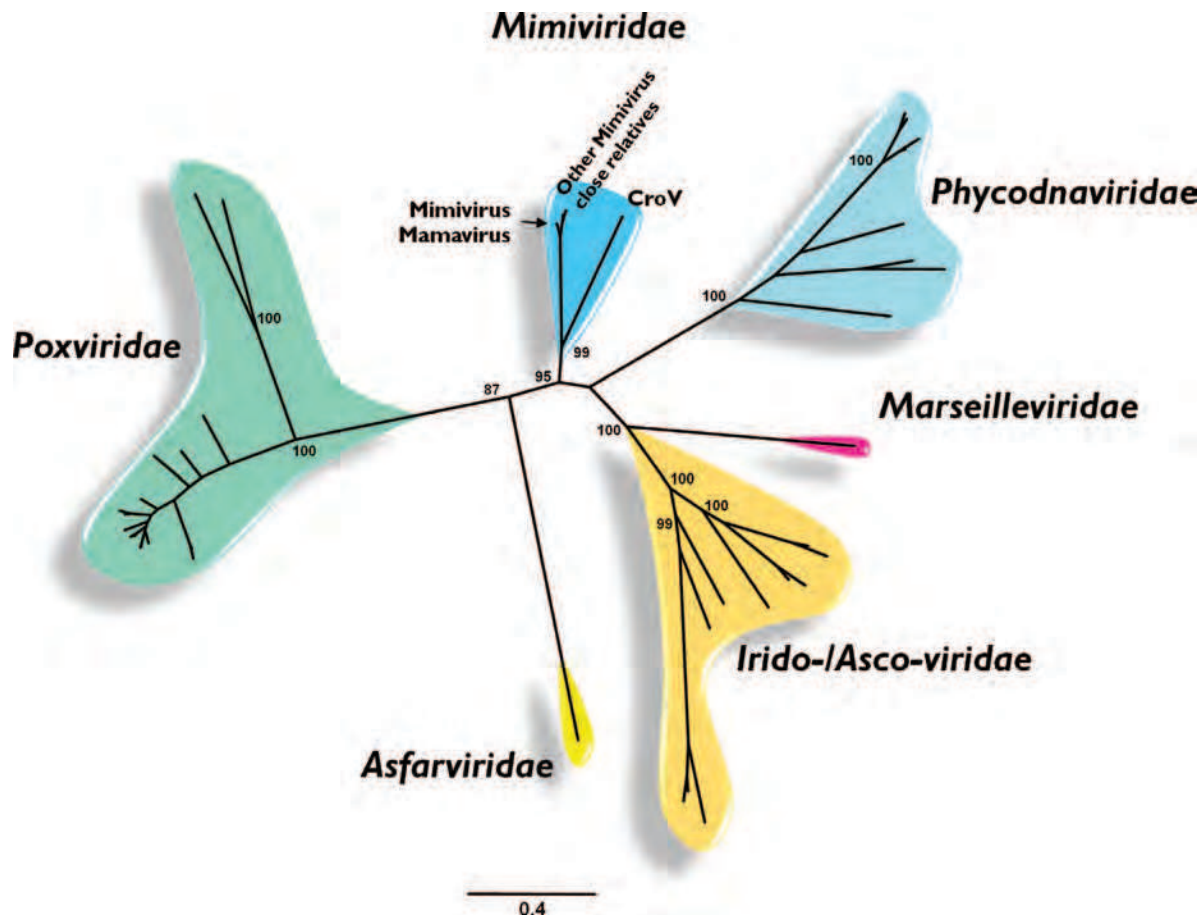


FIGURE 71.9. Phylogeny reconstruction of nucleocytoplasmic large DNA viruses based on four concatenated core genes. Bayesian phylogenetic tree was constructed from a cured concatenated alignment of four universal nucleocytoplasmic large DNA virus clusters of orthologous groups of proteins (NCVOGs) (496 conserved positions): primase-helicase (NCVOG0023), DNA polymerase (NCVOG0038), packaging adenosine triphosphatase (ATPase) (NCVOG0249), and A2L-like transcription factor (NCVOG0262). Bayesian posterior probabilities are mentioned near branches as a percentage and are used as confidence values of tree branches. Only probabilities at major nodes are shown. Scale bar represents the number of estimated changes per position for a unit of branch length. Other Mimivirus close relatives are among those cited in.⁷⁷ (Adapted from 25.)

of HGT, phylogenetic boundaries can be blurred in some cases.^{29,68} Regarding viruses, HGT has had a major impact on the shaping of the genomes of bacteriophages, and HGT between bacteriophages and their bacterial hosts is considered as a major engine of the evolution of bacteria.¹⁸ Among the proteins encoded by herpesviruses, which are also large DNA viruses, many are assumed to have been acquired from their host to mimic or block normal cellular functions.^{86,87,93}

Ogata et al¹⁰³ found that among the 363 Mimivirus ORFs that exhibit recognizable homologs in other organisms, 8.3% of ORFs likely originated from recent HGT. Later, Filée et al³⁶ reported unambiguously identifying 8.6% of the Mimivirus genes (78 of 96 bacterial-like genes) as having a bacterial origin. These genes of putative bacterial origin show a bias toward DNA replication and repair (20% protein) and cell envelope (12.5% protein) in COGs functional categories (Fig. 71.12D).³⁶ Filée et al³⁴ noticed that among NCLDV, only Mimivirus and Chlorella phycodnaviruses acquired more than 2% of their genes from bacteria, the highest proportion

being in Mimivirus. In contrast, the Mimivirus genome seems to contain the lowest proportion (0.8%) of genes acquired from the eukaryotic host among the NCLDVs, this proportion being about one-third to one-tenth of that observed in poxviruses. Moreira and Brochier-Armanet⁹⁴ specifically studied a set of 198 Mimivirus proteins that were previously assigned to COGs.¹¹³ Clear homologs were retrieved for 126 of these ORFs, and the most abundant sets of ORFs ($n = 47$ ORFs, 37%) were composed of ORFs present only in eukaryotes and bacteria, followed by ORFs present in all three domains ($n = 29$, 23%); 21 ORFs (17%) were found only in eukaryotes. Lastly, less than 10% of the 126 Mimivirus ORFs were found only in bacteria ($n = 12$), in bacteria and archaea ($n = 9$), and in archaea and eukaryotes ($n = 8$). Moreover, phylogenetic analysis inferred a eukaryotic, archaeal, bacterial, and viral origin for 60, 1, 29, and 4 of the 126 Mimivirus ORFs, respectively; approximately 10% of ORFs for which a eukaryotic origin was inferred appeared to be acquired from amoebae (Fig. 71.12D). A close phylogenetic relationship was identified

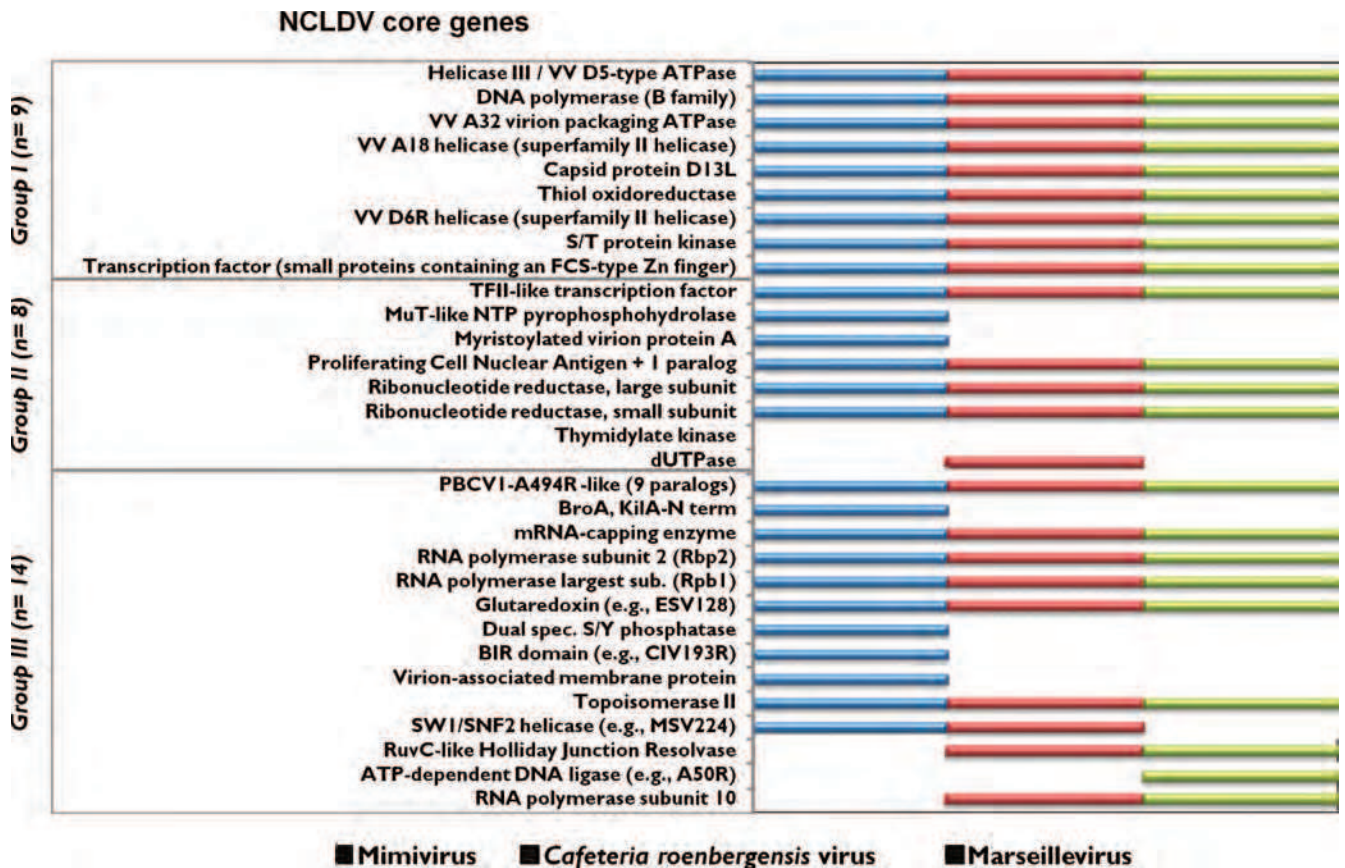


FIGURE 71.10. Presence/absence of NCLDV core genes of classes I, II, and III in the gene repertoire of Mimivirus, *Cafeteria roenbergensis virus*, and Marseillevirus.

for some Mimivirus ORFs with eukaryotes unrelated to the *Amoebozoa* that belong to the *Heterolobosea* (including *Naegleria*) and to the *Kinetoplastida* (comprising *Trypanosoma* and *Leishmania* as members). Noteworthy, Forterre⁴² reviewed phylogenies reported by Moreira and Brochier-Armanet⁹⁴ and did not agree with their interpretation. Indeed, he concluded that 32, 34, and 21 Mimivirus proteins are of bacterial, eukaryotic, and viral origin, respectively, whereas Moreira and Brochier-Armanet concluded that 29, 60, and 4 Mimivirus proteins are of bacterial, eukaryotic, and viral origin, respectively (Fig. 71.12D). These differences highlight that inferring an origin for genes putatively involved in HGT can be tricky.

Filée et al³⁶ analyzed the distribution of bacteria-like genes in Mimivirus and identified three consecutive co-inherited ORFs encoding a transaminase sugar, a glycosyltransferase, and a protein of unknown function. The synteny for these three ORFs with genes in the *Clostridium acetobutylicum* genome suggests their transfer as a contiguous block. Moreover, Filée et al³⁶ noted that Mimivirus genes of putative bacterial origin tend to be positioned in the first and last 250 kilobases (kb) of the genome, whereas NCLDV core genes and genes of eukaryotic origin tend to be located in the central fragment of the genome. Remarkably, it was proposed for other larger eukaryotic DNA viruses that viral core genes inherited vertically might be located in the central segment of the genome, while genes

involved in HGT with cellular hosts or other viruses might be positioned at the extremities of the genome,¹²³ which were identified as highly recombinogenic regions in poxviruses.⁸⁸ In addition, Filée et al³⁶ detected numerous mobile genetic elements (MGEs) in the Mimivirus genome, although these were previously considered specific to prokaryotes. These MGEs include apparently intact insertion sequences (ISs), regarded as major agents of HGT in prokaryotes.⁴⁵ ISs have not been identified in another eukaryotic viral genome, except in the *Ectocarpus silicosus* virus 1 genome. Those detected in the Mimivirus genome seem to be full-length copies and belong to the IS607 family. They are embedded within areas of adjacent bacterial-like genes or ORFans, which supports their possible co-inheritance with bacterial genes. In addition, the Mimivirus genome contains numerous homing endonucleases, including two HNH homing endonucleases that are found principally in bacteriophage genomes.³⁶ Thus, HGT involving different sources appears to have fashioned substantially the genome of Mimivirus, as for some other NCLDVs.^{34,35,59} In contrast, NCLDVs with host cells that do not prey on bacteria, as algae or metazoa, contain a lower proportion of genes putatively acquired from bacteria in their genomes.^{34,110} This suggests that living sympatrically with bacteria in amoeba may provide a biological niche appropriate for the exchange of DNA. This concept is developed later specifically in another part of this

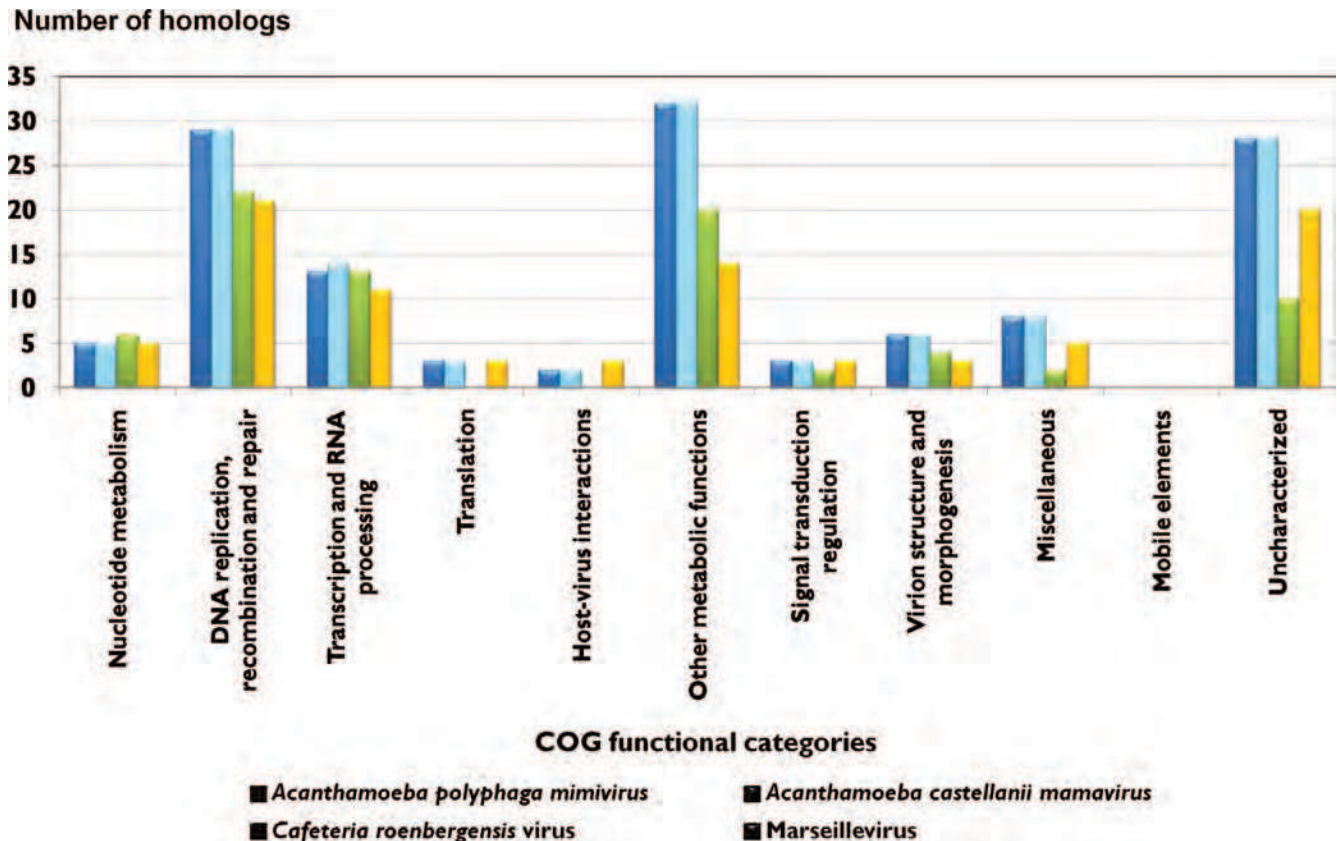


FIGURE 71.11. Distribution by functional categories of predicted proteins identified in protist-associated giant viruses that are homologous to the 177 nucleocytoplasmic large DNA virus clusters of orthologous groups of proteins (NCVOGs) found in at least 2 nucleocytoplasmic large DNA virus (NCLDV) families. (Data used for this figure are from Yutin N, Wolf YI, Raoult D, et al. Eukaryotic large nucleocytoplasmic DNA viruses: clusters of orthologous genes and reconstruction of viral genome evolution. *Virology* 2009;6:223.)

chapter (Ecology of Giant Viruses Associated With Phagocytic Protists: Amoebae as Genitors and Reservoirs of Giant Viruses). The remarkably extensive and diverse gene content of amoeba-associated giant viruses may provide them several benefits. For instance, *Legionella pneumophila* has acquired by HGT eukaryotic genes involved in a variety of cell functions, including two serine/threonine protein kinases,¹⁶ which have been shown to inhibit phagosome–lysosome fusion in several pathogens.¹³⁸ It was hypothesized that three serine/threonine protein kinases and a Ras GTPase identified in the Mimivirus genome could be involved into the regulation of the amoeba cycle by Mimivirus.⁹⁴

Duplicated Genes. Genome and gene duplication lead to the emergence of new genes.^{56,150} Duplicated genes can diverge considerably; they can lose their functionality and degenerate into pseudogenes, or alternatively, they can evolve toward sub- or neo-functionalization. Suhre¹²⁶ determined that both genome and gene duplications have contributed importantly to the structure and large size of the Mimivirus genome. Mimivirus appears to be the NCLDV member with the greatest proportion of duplicated genes in its gene repertoire, this proportion being lower than 15% for most of the other NCLDVs.³⁴ First, Suhre

reported data that suggested the occurrence of a segmental duplication involving a ~200,000-nt-long 5' end fragment of the Mimivirus genome; this duplication may have been followed by a rearrangement around the center of the chromosome fraction. Second, Suhre determined that about one-third of the Mimivirus genome consists of duplicated genes (from 26% to 35% of its genes, depending on whether the e-value cutoff for PSI-BLAST search is 10^{-25} or 10^{-10} , respectively) (Fig. 71.12A). This rate is of the same order of magnitude as frequencies determined for members of the *Archaea*, *Bacteria*, and *Eukarya* domains of life.¹²⁶ Suhre also showed that a log-linear correlation exists for Mimivirus and other DNA viruses between the number of predicted genes and the number of paralogs in the genome. Regarding Mimivirus, the maximum number of duplications is 11. Duplicated genes are inserted about two times more frequently in parallel orientation (in reference to the coding direction of the matching ORF) as in antiparallel orientation. In addition, they are not evenly distributed along the genome: 79% of those in *cis* orientation compared to 39% of those in *trans* orientation are located in the same half of the genome. Among the largest paralogous gene families, several display homology with proteins that may play a role in interactions between the Mimivirus and its

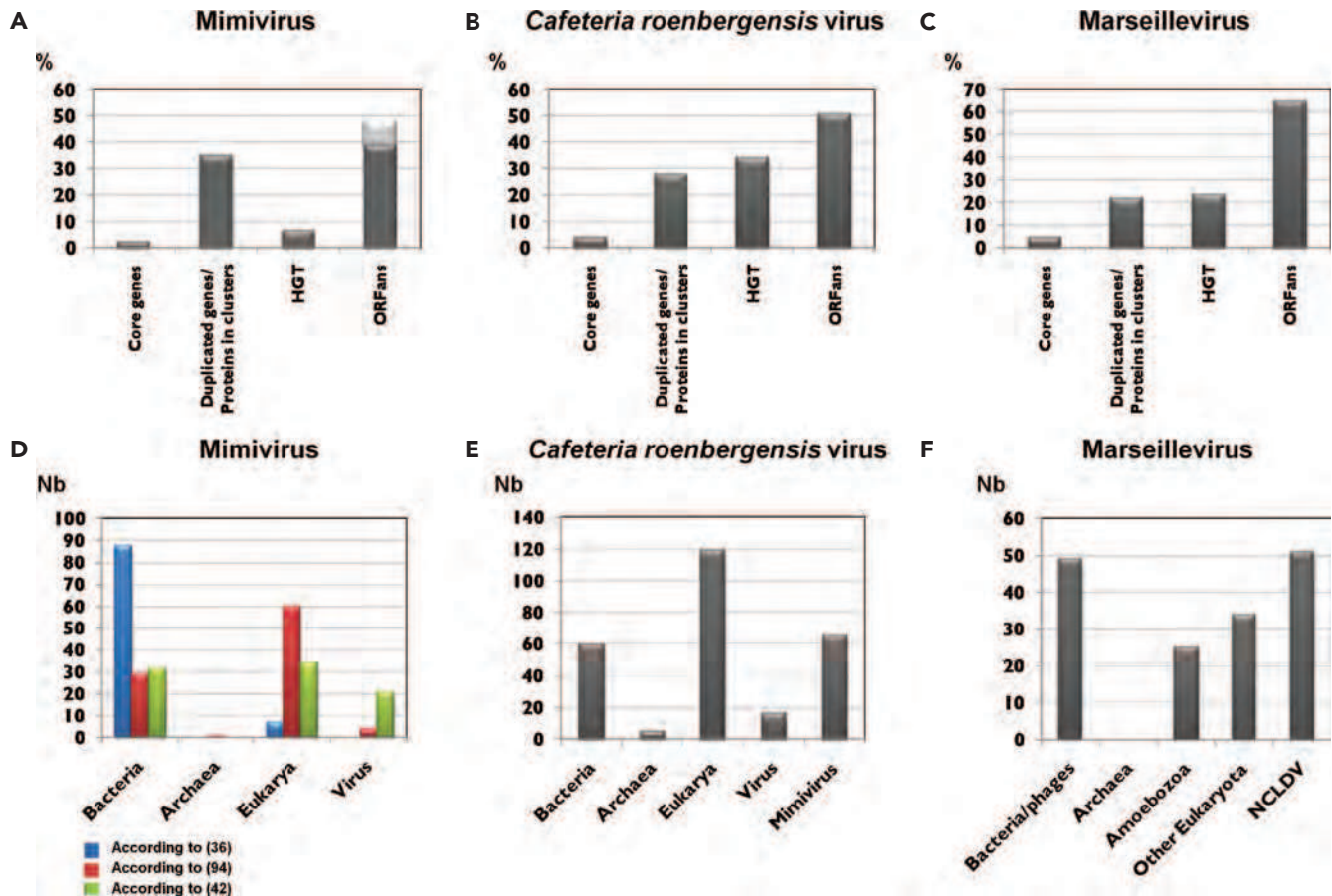


FIGURE 71.12. Distribution of open reading frames (ORFs) identified in the genomes of Mimivirus (A,D), *Cafeteria roenbergensis* virus (B,E), and Marseillevirus (C,F), according to categories of genes (A–C) or inferred origin (D–F).

amoebal host. The largest paralogous gene family is composed by ankyrin repeat-containing proteins, which mediate protein–protein interactions.⁸³ The second-largest family is composed of predicted proteins of unknown function with the BTB/POZ domain that mediates homo- or heterodimeric dimerization. Another family of paralogs includes proteins with several FNIP repeats; an N-terminal domain matches the Pfam F-box domain that is a receptor for ubiquitination targets, while several ubiquitin-conjugating enzymes are concurrently detected in the Mimivirus genome. Another paralogous family is composed of proteins with collagen triple helix repeats.

ORFans. ORFans refer to genes that lack homolog in a sequence database.³⁸ This means that when a BLAST search is performed against all sequence databases, those genes were not found in any other organism. As a comparison, it was recently assessed that the proportion of ORFans among the NCLDV genomes varied considerably, ranging from 2.8% for *Paramecium bursaria chlorella* virus NY-2A (PBCV-NY2A) to 75.2% for *Emiliania huxleyi* virus 86 (EhV-86)¹³; besides, this proportion was ~30% and ~9% in viral and bacterial genomes, respectively.³⁸ In Mimivirus, the number of ORFans was 474 (48.1% of the gene repertoire) when determined against the National Center for Biotechnology Information (NCBI) RefSeq protein sequence database (Fig. 71.12A). Among these ORFs, 63 (6.9% of the whole gene repertoire) were defined

as meta-ORFans, because they were found to have the closest matches to environmental sequences.¹³

Mimivirus Specific Genes. A remarkable and unexpected feature of the Mimivirus gene repertoire was the identification of several predicted proteins associated with protein synthesis. Indeed, viruses are known as devoid of such genes and as obliged to rely on the host cell protein translation apparatus.¹¹⁴ Before the Mimivirus discovery, tRNA-like genes were known to exist in dsDNA viruses including bacteriophages, herpes virus 4, or chlorella viruses, and an elongation factor gene was also found in chlorella viruses.¹¹³ However, the Mimivirus expanded considerably the set of viral genes associated with protein translation.¹¹³ Indeed, the identification of four amino acyl-tRNA synthetases (a cysteinyl-, an arginyl-, a tyrosyl-, and a methionyl-tRNA synthetase; messenger RNA (mRNA) encoding the three first proteins are packaged within the viral particle), a tRNA-modifying enzyme, three translation initiation factors (4E, SUI1, IF-4A), and a peptide release factor (eRF1) were unique to the Mimivirus. Moreover, several findings indicated that these proteins may have an important role in viral protein translation and assembly. In addition, six tRNA-like genes were identified. Also, an unusual and large set of genes encoding homologous proteins to enzymes involved in different repair pathways of DNA damages due to oxidation, methylation, alkylation, UV light, and nucleotide mismatch

was identified. These genes include some never described before in dsDNA viruses: two encoding formamidopyrimidine-DNA glycosylase, a UV-damage endonuclease, a 6-O-methylguanine DNA methyltransferase, and a protein that belongs to the MutS protein family associated with DNA mismatch repair and recombination. Mimivirus is also the first virus with a gene encoding a topoisomerase IA; additionally, genes encoding topoisomerases IB and IIA, previously found in NCLDV, are also present in the Mimivirus genome. Thus, topoisomerases of type IA, IIA, and IIB are concurrently encoded by Mimivirus genes, which is rarely encountered among microorganisms.¹¹³ Besides, two Mimivirus genes uniquely encode chaperones of the HSP70 family, and three other ORFs encode proteins with a DnaJ domain that associate with HSP70 family proteins. Mimivirus is also the first virus to encode a putative peptidyl-prolyl *cis-trans* isomerase of the cyclophilin family, which accelerates protein folding. As with other large dsDNA viruses, the Mimivirus genome has genes involved in various metabolic pathways such as nucleotide synthesis, amino acid metabolism, lipid metabolism, and polysaccharide metabolism. Among these proteins, some are unique in viruses, especially those intervening in the glutamine metabolism. In addition, glycosyltransferases that may be involved in posttranslational modifications of proteins were detected.

Intein and Introns. An intein has been detected in the family B DNA polymerase of Mimivirus.^{105,113} Inteins are segments of proteins that catalyze self-splicing at the protein level. Mimivirus is one of the few eukaryotic viruses that has been found to harbor an intein, and this intein is most likely to be functional.^{105,130} In addition, six introns were identified in the Mimivirus genome, one in the DNA-directed RNA polymerase (II) subunit 1, three in the DNA-directed RNA polymerase subunit 2, and two in the MCP.^{2,113} Proteomic analysis showed that intron in the β -subunit of the RNA polymerase is excised.¹¹⁷

ADDITIONAL FEATURES

The Mimivirus genes can be classified as early, intermediate, and late according to three major temporal classes of expression, as determined by mRNA deep sequencing.⁸¹ An early promoter (AAAATTGA sequence, unique to Mimivirus) found in front of 74% of the Mimivirus ORFs classified in the early class versus 6% of those classified in the late class, and late promoters found in 24% of the genes classified in the late class versus less than 3.5% of those classified in the early or intermediate classes were successively predicted.^{81,127} In addition, Mimivirus genes were found to be expressed as polyadenylated transcripts.¹⁷ A large majority (greater than 80%) of the analyzed mature mRNA 3' ends were found to contain palindromic sequences promoting the perfect pairing of 13 or more successive nucleotides into hairpin-like structures. The nature and the stringency of the hairpin rule defining the location of polyadenylation sites in Mimivirus mRNA are unique to this virus; in addition, this signal appears to be absent in the amoebal host.¹⁷

The codon usage of Mimivirus and its amoebal host, *Acanthamoeba* spp., is almost the opposite.¹¹³ Indeed, the proteome of Mimivirus exhibits a substantial positive bias for residues encoded by AT-rich codons, with isoleucine, asparagine, and tyrosine, for instance, being twice as frequent in Mimivirus as in *Acanthamoeba* proteins. Besides, it has been demonstrated that genome signatures of Mimivirus, and those of large viruses, tend to deviate from the genome signatures of their hosts.⁹⁸

MIMIVIRUS PROTEIN COMPONENTS

The composition of purified isolated virions by capillary liquid chromatography-tandem mass spectrometry LC-MS/MS, two-dimensional (2D) gel electrophoresis, and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry revealed that 137 proteins are packaged into the Mimivirus particles.^{22,117} The most abundant group of proteins associated with the Mimivirus particles is constituted by proteins of unknown function ($n = 65$ in first analysis), 69% of them being ORFans. Apart from this set of proteins, enzymes and factors involved in transcription compose the largest functional category of these proteins.¹¹⁷ They include four transcription factors, mRNA guanylyltransferase, two helicases, and the five subunits of a DNA-directed RNA polymerase. The third largest group of encapsidated proteins contains gene products associated with oxidative pathways. Other proteins are involved in protein/lipid modifications, DNA topology and repair, and particle structure, including the capsid protein and the major core protein. Further analysis of these proteomic data identified additional proteins including the B-type DNA polymerase and several ankyrin repeat-containing proteins as encapsidated into Mimivirus particles. A total of four among the nine class I core genes were detected in the virions, including the major capsid protein.²² Moreover, analysis of 2D gels suggested posttranslational modifications for proteins encapsidated within Mimivirus particles, including glycosylation and, possibly, cleavage and maturation.¹¹⁷

Besides, some of the Mimivirus proteins were expressed and their function was studied in some cases. The methionyl-tRNA synthetase and the tyrosyl-tRNA synthetase were expressed in *Escherichia coli* and their enzymatic function could be characterized.²² The function of other Mimivirus proteins was also studied, including that of a topoisomerase IB,⁷ a nicotinamide adenine dinucleotide (NAD⁺)-dependent DNA ligase,⁸ a mitochondrial carrier,⁹² human endonuclease VIII-like proteins,⁴ a nucleoside diphosphate kinase,⁶¹ a uridine 5'-diphosphate (UDP)-D-glucose-4,6-dehydratase, and a bifunctional UDP-4-keto-6-deoxy-D-glucose epimerase/reductase involved in L-rhamnose production.¹⁰⁷

Life Cycle

VIRAL ENTRY

Mimivirus is an obligate intracellular pathogen. It was initially isolated by culture on *Acanthamoeba polyphaga*, which was an efficient fortuitous strategy for the discovery of several amoebal pathogens.^{76,115} The virus can be propagated on *Acanthamoeba* spp., including *A. castellanii*, *A. polyphaga*, and *A. mauritanensis*. Several primary or established cell lines from invertebrate and vertebrate animals were tested for their ability to support Mimivirus infection and replication but without success.^{49,130} Thus, Mimivirus appears to have a narrow spectrum of host cells, which could indicate a high specificity for target cells. Otherwise, this virus would be able to propagate from amoeba to other eukaryotic cells.¹³⁰ However, Mimivirus is internalized by different professional phagocytes, including human myeloid cells, as circulating monocytes or monocyte-derived macrophages.⁴⁹

The 120- to 140-nm-long external fibrils may be involved in the attachment of Mimivirus to the amoeba. Specifically, the protein heads of the outer fibers are likely involved in the attachment of the virus to the surface of the amoeba.⁷⁵ Peptidoglycans present at the outer surface of Mimivirus particles may

promote phagocytosis by amoebae. In addition, amoebae are capable of phagocytosis of any particle larger than $0.5\ \mu\text{m}$, a size range that includes the giant Mimivirus.^{71,110} Ghigo et al.⁴⁹ demonstrated that Mimivirus enters the amoebal cell, which is a professional phagocyte, through phagocytosis, whereas this virus cannot enter nonprofessional phagocytes. Mimivirus is the first virus described to enter cells by canonical phagocytosis. Such a phagocytic-like process for entry of viruses into their host cell was previously only suggested for herpes simplex virus infection of fibroblasts, which are not professional phagocytes.²⁴ Ghigo et al.⁴⁹ studied Mimivirus uptake by macrophages and detected viral uptake after 1 to 2 hours using real-time polymerase chain reaction (PCR) with plaque-forming unit (PFU)-to-cell ratios ranging from 10:1 to 200:1; uptake plateaued after 8 hours and was maintained for 24 hours.⁴⁹ Notably, in these experiments conducted on RAW 264.7 macrophages, Mimivirus was cytopathogenic, and viral particles released by macrophages replicated within amoebae, indicating that a productive cycle of Mimivirus replication occurred. The engulfment of Mimiviral particles requires F-actin but does not occur through macropinocytosis.⁴⁹ Protrusions of the cytoplasmic membrane at the virus entry site were demonstrated by F-actin labeling and ultrastructural analysis. Mimivirus activates phosphatidylinositol kinases (PIK3) and downstream phagocytosis effectors. Dynamin-II was shown to promote phagosome formation by membrane extension during the engulfment of the viral

particle. In contrast, neither clathrin-dependant endocytic routes nor degradative endosome-mediated endocytosis was found to be involved in Mimivirus entry mechanisms in these experiments. Involvement of a caveolin-independent lipid raft mechanism could not be excluded. Strikingly, cellular events associated with Mimivirus internalization are very similar when observed in macrophages and in amoebae.^{49,129} This observation is congruent with the knowledge that several microorganisms, including *Legionella pneumophila*, *Parachlamydia*, *Coxiella burnetii*, and *Cryptococcus neoformans*, are resistant to amoebae and macrophage pathogens.^{49,52,124}

VIRAL FACTORY AND RELEASE OF THE PROGENY

In the initial description by La Scola et al.⁷⁶ of the Mimivirus replication cycle using confocal microscopy and Mimivirus-specific monoclonal antibodies, rare phagocytized Mimivirus particles were detected within the amoeba cytoplasm at $T = 0$ hour. Then, an eclipse phase, typical of a viral replication cycle, occurred and no particle was visible at $T = 4$ hours. At $T = 8$ hours, Mimivirus particles appeared within amoebae while an increasing number of amoeba cells were infected with Mimivirus. Finally, at $T = 20$ hours, viral particles were seen in ghost amoebae (Figs. 71.13, 71.14, and 71.15). The localization of the Mimivirus replication cycle has been debated. Suzan-Monti and colleagues^{129,130} observations indicate that Mimivirus DNA might be transported to the nucleus following viral

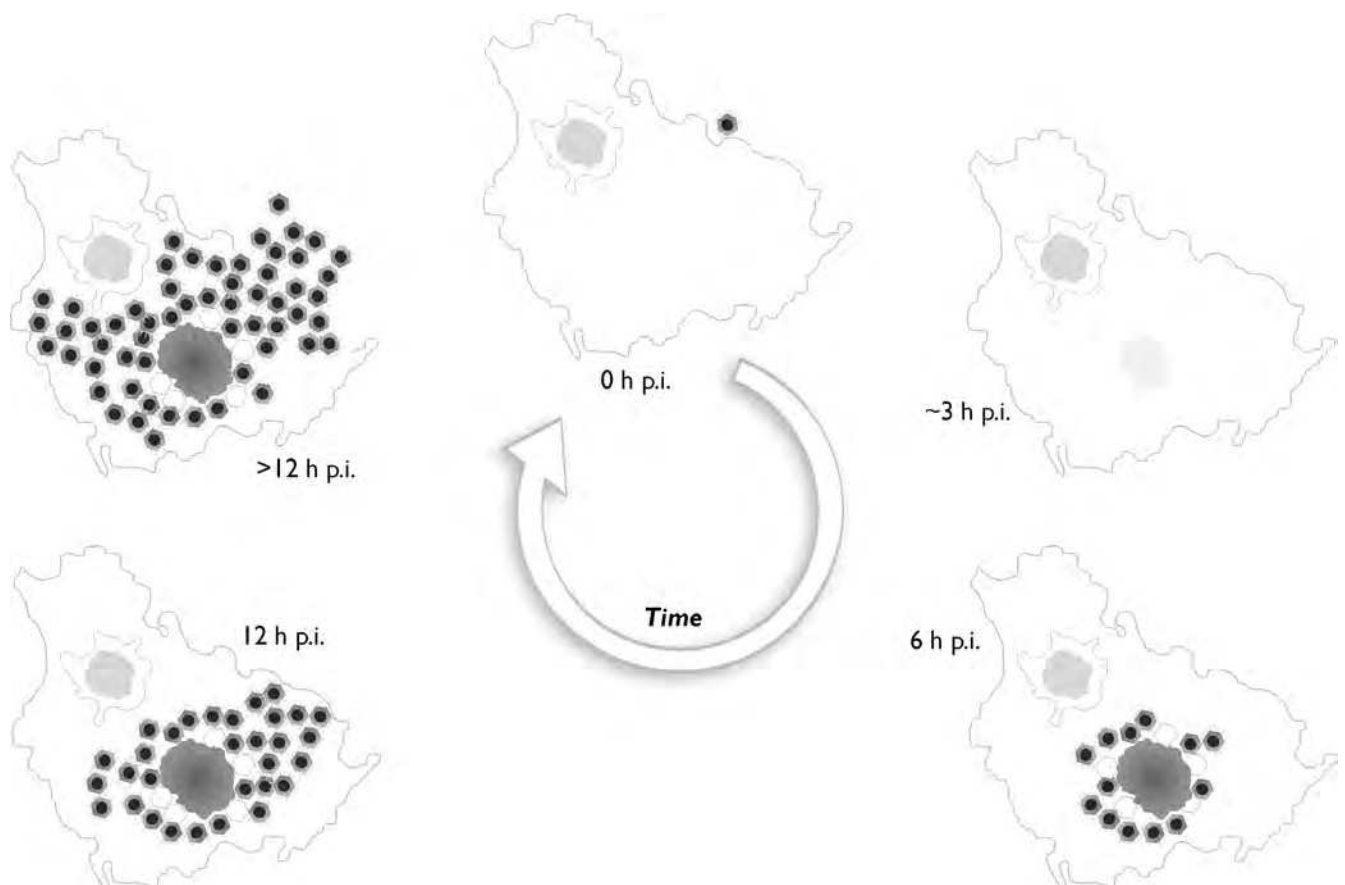


FIGURE 71.13. Schematic representing the Mimivirus replication cycle.

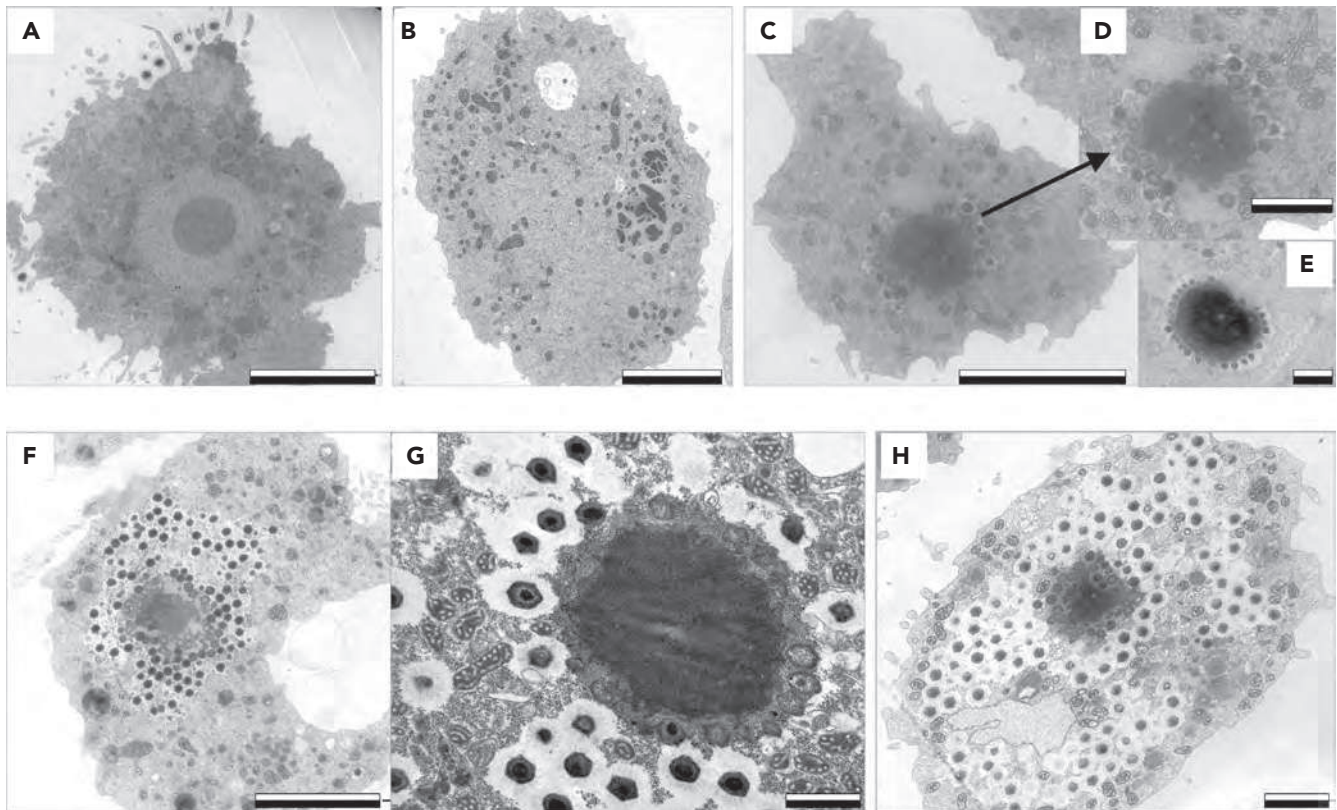


FIGURE 71.14. Electron microscopy of different stages of Mimivirus replication in *Acanthamoeba*. **A:** Hour (H) 0 postinfection. **B:** H3 postinfection. **C–E:** H6 postinfection. **F, G:** H12 postinfection. **H:** H16 postinfection. **D, E, and G** show the Mimivirus factory. **A–C, F:** Scale bars indicate 5 μm . **D, E, H:** Scale bars indicate 2 μm . **G:** Scale bar indicates 200 nm.

decapsidation, while the experiments by Mutsafi et al¹⁰⁰ suggest that the Mimivirus replication cycle occurs exclusively in the cytoplasm. In the study conducted by Suzan-Monti et al,¹²⁹ Mimivirus could be observed following entry into the amoeba within phagocytic vacuoles. Empty particles with an opened vertex were seen at this stage, with several viruses present in the same vacuole in some cases. At 4 hours postinfection, the internal viral membrane extrudes, sheds the viral DNA genome, and fuses with the membrane of the phagocytic vacuole, while outer Mimivirus structures remain intact. Subsequently, a cytoplasmic viral factory (VF) appears and is surrounded by mitochondria. VFs are structural and functional elements associated with the replication of nucleic acids and with a massive production of virions.¹⁰² Compared to the VFs observed for other viruses, Mimivirus VFs display some similarities and major differences, including with that of other NCLDV.¹²⁹ Notably, no membrane surrounds the Mimivirus VF. Around 5 hours postinfection, the cytoplasmic area occupied by the nuclei decreases by 50%, while the cytoplasmic viral factory area increases by 50%. Then, between 5 and 8 hours postinfection, the cytoplasmic VF increases in size, and new viral particles can be observed at its periphery at the end of this period. Thereafter, the VF continues to expand to occupy an estimated area of 250 μm^2 at 12 hours postinfection, representing ~42% of the amoebal cytoplasmic surface. Most of the infected amoebae are lysed at 24 hours postinfection, and amoebal lysis represents the most probable mode of virion release. Otherwise, the absence of an outer

membrane suggests that Mimivirus exit from amoeba does not occur via budding at the cytoplasmic membrane.¹³⁰ Notably, Mimivirus appears to have a very rapid lytic effect on amoebae, more rapid than that observed for other amoebae-resistant pathogens.⁵² Three zones were described by Suzan-Monti et al¹²⁹ within the cytoplasmic VFs of Mimivirus: an inner heterogeneous structure with dense inclusions, which corresponds to the replication center, where condensation of the viral DNA appears to initiate; an intermediate zone where viral assembly is thought to occur; and a peripheral zone where new viral particles gain their outer fibrils. From the center to the outside, capsid shells are assembled and then filled with DNA through an open portal (Figs. 71.15 and 71.16). Suzan-Monti et al¹²⁹ studied the localization of Mimivirus DNA and the kinetics of the VF and viral DNA formation using direct fluorescent staining with diamidino-2-phenylindole (DAPI), which preferentially stains dsDNA with a high AT proportion.¹²⁹ Overall, the total fluorescence intensity within the cell increased sevenfold between 0 and 8 hours postinfection, which represents a doubling time of 2.7 hours. Viral replication appeared to be composed of two successive phases with distinct localizations: from 0 through 3 hours postinfection, the mean fluorescence intensity statistically significantly increases in the nucleus; then, from 4 through 8 hours postinfection, the mean fluorescence intensity significantly increases within the cytoplasmic VF. Stained DNA dots are seen in the amoeba nucleus at 1 hour postinfection, and at 4 hours postinfection, a heterogeneous

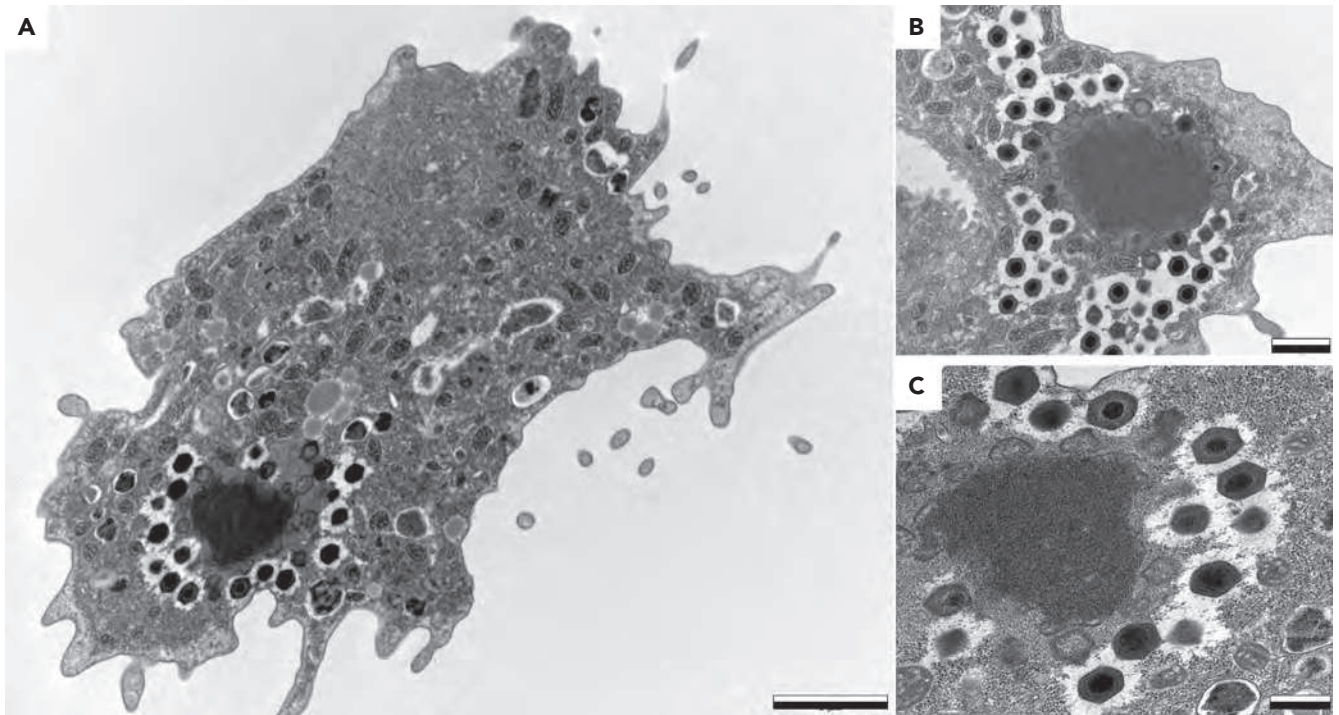


FIGURE 71.15. **A:** Electron microscopy of Mimivirus replication in *Acanthamoeba*. **B, C:** Details of a Mimivirus factory surrounded by newly formed viral particles at 12 hours postinfection of amoebae. Scale bars indicate 2 (**A**), 1 (**B**), and 0.5 (**C**) μm .

structure appears outside of the nucleus, which corresponds to the formation of the core of the cytoplasmic VF. This core becomes homogeneous between 4 and 12 hours postinfection, while viral production is observed from 7 hours postinfection. Finally, the VF reaches a heterogeneous degenerative stage. Taken together, these observations led to the proposal that Mimivirus DNA first enters into the nucleus of infected amoebae, possibly for a first round of replication, and then the Mimivirus DNA exits from the nucleus and moves to the cell

cytoplasm. At 3 hours postinfection, the viral DNA forms the replication center of a cytoplasmic VF, which is the major site of the DNA genome replication.¹²⁹

Mutsaers et al¹⁰⁰ used diverse imaging techniques and proposed a different scenario than Suzan-Monti and colleagues for the replication cycle of Mimivirus, which is entirely cytoplasmic, as demonstrated for poxviruses. These authors claim that this scenario is compatible with difficulties that may be encountered for the transport of the huge viral DNA

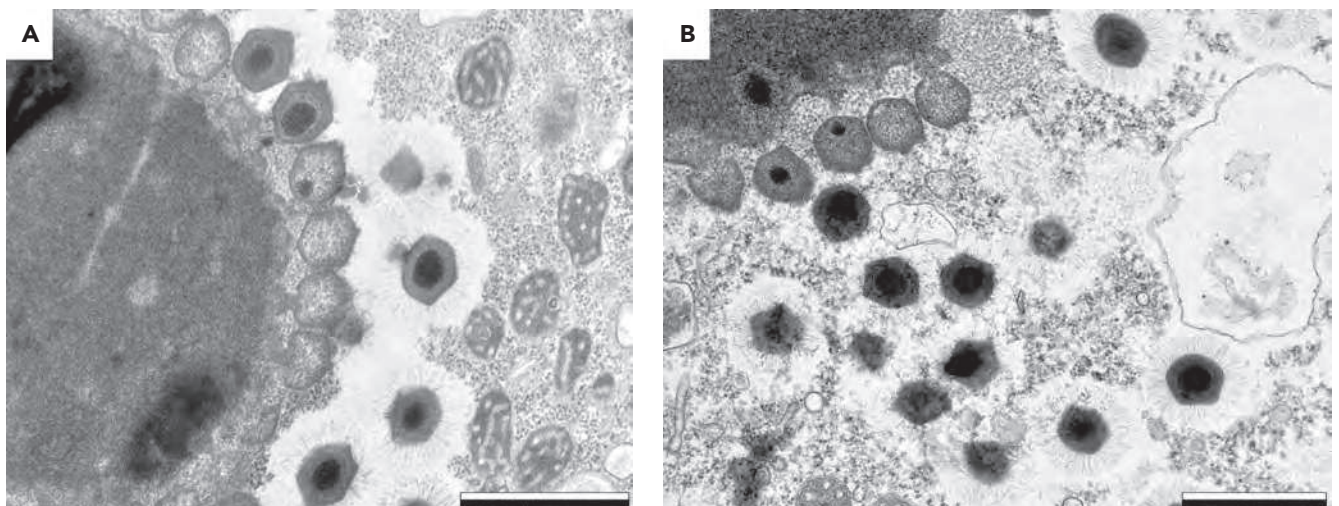


FIGURE 71.16. Electron microscopy of Mimivirus factory and newly formed Mimivirus particles in an amoeba 12 hours postinfection. Scale bars indicate 1 μm .

genome into the nucleus then back into the cytoplasm. According to Mutsafi et al, Mimivirus particles are detected within amoebal phagosomes at 2 hours postinfection, and free spherical particles and spheres surrounded by replicating DNA are seen at 2 to 3 hours postinfection. The genome release is accompanied by DNA decondensation and the initiation of massive DNA replication. In addition, early transcription appears to initiate in the viral cores during DNA release and shortly after their exit from Mimivirus particles.¹⁰⁰ Then, the newly synthesized mRNAs accumulate at discrete cytoplasmic sites adjacent to but in close opposition to DNA replication sites. Notably, according to the observations of these authors, each Mimivirus core seeds one replication factory at the site where the viral DNA genome is released. Subsequently, fusion of several expanding VFs can eventually occur, which may lead to a single VF at the late stage of infection.^{100,149} In this scenario, replication is not necessarily entirely independent of the amoebal nucleus because it may involve nuclear factors as previously shown for the vaccinia virus.^{100,106} Viral proteins may be partially or fully synthesized or grouped in the replication center, while no cellular host proteins seem to be incorporated into the virus particles, as demonstrated by proteomics.¹¹⁷ It remains largely unknown if and by which mechanisms cellular gene expression is modulated and the cell machinery is used to participate in the formation of Mimivirus virion progeny.

DNA RELEASE AND PACKAGING

The star-shaped structure, which was designated as the star-gate by Zauberman et al,¹⁴⁹ is suspected to mediate a large-scale opening in the capsid that allows the protrusion of the inner membrane of the Mimivirus. Using AFM, Kuznetsov et al⁷⁵ observed that the arms of the star-gate detach from the viral particle, allowing the five triangular faces of the capsid underneath to open outward with the icosahedral edges folding as hinges (Fig. 71.5). This event is thought to precede the fusion of the viral membrane sac with the phagosomal membranes, promoting the formation of a membrane tube for the release of the viral genome. Thus, it was proposed that the DNA genome may be released into the amoebal cytoplasm enclosed within a vesicle. Such a process would allow the long Mimivirus genome to be translocated across the amoebal host cytoplasm and to be protected against nucleases. In addition, Zauberman et al¹⁴⁹ observed multiple lysosomes undergoing fusion with the phagosomes, and they hypothesized that these lysosomes possibly act to open the viral capsid. Renesto et al¹¹⁷ hypothesized that the lipolytic and proteolytic enzymes encapsidated in the Mimivirus particles may act to allow the virus to access the amoebal cytoplasm. Thereafter, ejection of the Mimivirus DNA from the viral particle, DNA repair, and early transcription may be mediated by the two topoisomerases, IA and IB, and the DNA repair machinery components. The presence of immediate early gene transcripts is suggested by the detection in the virions of transcripts encoding three of the core genes (including the DNA polymerase, the capsid protein, and the TFII-like transcription factor), three of the four amino-acyl tRNA synthetases, and four ORFan genes.¹³⁰ These transcripts may be required for the first steps of the Mimivirus replicative cycle. According to Zauberman et al,¹⁴⁹ Mimivirus DNA packaging into procapsids appears to occur at a vertex or, alternatively, through an opening located at an icosahedral face in

~60% of observations and that spans the outer and inner capsid shells and the internal membrane, as assessed by electron tomography and volume reconstruction analysis.¹⁴⁹ The aperture is cone shaped and has a diameter of 35 nm at the outer shell and of 25 nm at the inner shell. This opening is sealed after the completion of DNA packaging into Mimivirus capsids (Fig. 71.3). However, no evidence was found in other studies that an alternate portal exists for DNA packaging, and observations have led to the hypothesis that the Mimivirus DNA may be released and incorporated into the viral capsid by the starfish-shaped aperture.

DNA REPLICATION

The mechanism of Mimivirus DNA replication is unknown. The Mimivirus DNA may assume a circular shape, as deduced from the existence of an inverted repetition of a sequence that is ~600 nucleotides in length at both extremities of the linear dsDNA genome (at positions 22,515 and 1,180,529 of the chromosome, respectively).^{22,113} This repeated inverted sequence may allow DNA to take a Q shape with a short and a long tail.¹¹³ Notably, the ~22,500-nt-long region that may constitute the long tail region harbors a set of seven genes encoding proteins involved in DNA replication and binding.²² An origin of DNA replication is hypothesized to be located near position 400,000 based on a slope reversal of the cumulative A+C excess along the genome.¹¹³ Indeed, such a sequence bias has been related to the location of the origin of replication in bacteria.⁴³ Nevertheless, this hypothesis remains uncertain for Mimivirus.

TRANSCRIPTION OF MIMIVIRUS GENES

The transcriptional activity of Mimivirus genes was studied using deep mRNA sequencing and provides additional hints to the aforementioned structural analysis to elucidate the Mimivirus replication cycle.⁸¹ At $T = -15$ minutes of infection, ~90% of the transcripts correspond to *Acanthamoeba* genes. At $T = 0$, more than 50% of transcripts correspond to Mimivirus genes, while ~50% of the host transcripts correspond to mitochondrial genes. At $T = 1.5$ hours, Mimivirus transcripts decrease to ~50% of the transcriptional activity, and after T is greater than 3 hours, they become predominant. Three major patterns are observed in the transcriptional activity of Mimivirus-infected amoebae, each accounting for one-third of this activity: from the entry of Mimivirus to $T = 3$ hours, from $T = 3$ to 6 hours, and from $T = 6$ through 12 hours. Mimivirus transcripts detected during these three periods are classified as early, intermediate, and late, respectively, and this classification is congruent with that obtained using hierarchical clustering and with the presence of early or late promoters.^{81,127} Nonetheless, substantial variability can be observed within each of the three classes regarding the level of transcriptional activity and its timing. Early-expressed Mimivirus genes are enriched with genes of unknown function and include three of the four amino-acyl tRNA synthetases. Interestingly, hypothetical proteins represent 85% of the most abundant transcripts and are particularly abundant in the class of early expressed genes suspected to be critical for the replication cycle. Intermediate Mimivirus genes include a large proportion of genes involved in DNA replication. Finally, late-expressed genes comprised genes involved in the structure of the viral particle, including the MCP. In addition, most of the genes encoding the transcription apparatus belong to this class, and their abundance peaks at $T = 6$ hours. All but one of these proteins are

found within the virions.^{22,117} Most of the genes involved in the biosynthesis of the outer layer of the viral particle and in some of the posttranslational protein modifications are also expressed after T = 6 hours. Regarding the component of the translation apparatus, most are expressed at a medium or high level and exhibit an early or late expression pattern.⁸¹

Acanthamoeba Castellanii Mamavirus

The existence of another strain of Mimivirus was first reported in 2008 by La Scola et al.⁷⁸ This virus was named Mamavirus due to initial observations suggesting a particle size slightly larger than that of Mimivirus. However, both giant viruses were later shown to be phenotypically indiscernible. Of note, the sputnik virophage was discovered in amoebal cultures co-infected with Mamavirus (Fig. 71.6).⁷⁸ *Acanthamoeba castellanii mamavirus* was recovered by inoculating *Acanthamoeba* culture with water collected from a cooling tower in Paris, France. The Mamavirus genome was revealed to be larger (by ~10,000 nt) than that of Mimivirus; it is 1,191,693 nt in length (Table 71.1; Fig. 71.7A).^{28,78} In addition, this genome harbors 1,023 predicted protein-coding genes, which are evenly distributed on the direct and reverse DNA strands (Fig. 71.7B). Besides, 30 non-coding RNAs and the 6 same tRNAs found in the Mimivirus genome were identified.²⁸ A total of 879 Mamavirus protein-coding genes were identified as *bona fide* orthologous genes to Mimivirus genes, the mean amino acid identity being 98.3% for those genes. Seven orthologs have different positions in the Mamavirus and Mimivirus genomes. Overall, the two genomes are highly similar with ~99% nucleotide identity in their alignable regions, which represent nearly the entire length of these genomes. Notably, the Mamavirus genome has an extra 5' terminal fragment that is ~13,000 nt in length and contains disrupted duplicated genes. Conversely, Mimivirus contains a 3' terminal segment that is ~900 nt in length and has no counterpart in Mamavirus. In total, 75 ORFs are differentially present in the Mamavirus versus Mimivirus genome; 29 Mimivirus ORFs and 46 Mamavirus ORFs are partially or completely missing in the counterpart genome. A small regulatory subunit of polyA polymerase is present only in Mamavirus; homologs for this predicted protein could be only identified in some unicellular eukaryotes and in poxviruses.

Cafeteria Roenbergensis Virus

In 2011, the genome of a giant virus recovered from the coastal waters of Texas in the 1990s was described. This virus, with a 300-nm-diameter capsid, was named *Cafeteria roenbergensis* virus (CroV) because it infects a marine heterotrophic flagellate, *Cafeteria roenbergensis*, a phagotrophic protist that grazes on bacteria and viruses and is widespread in marine environments (Table 71.1).³⁹

CroV has a linear dsDNA genome that is ~730 kb in length; the portion of the chromosome that was sequenced was 618 kb with flanking regions described as highly repetitive (Fig. 71.7A). The genome is AT rich with 77% A+T content. CroV appears to be a *bona fide* Mimiviridae member based on the analysis of its DNA polymerase B and phylogeny reconstruction from four universal NCVOGs (primase-helicase, DNA polymerase, packaging adenosine triphosphatase [ATPase], and A2L-like transcription factor).^{25,39}

A total of 544 putative protein-coding sequences have been predicted in the 618-kb sequenced segment of the CroV

chromosome; coding capacity is 90.1% (Fig. 71.7A). In addition to the nine NCLDV core genes of class I, five and nine NCLDV core genes of classes II and III, respectively, are present (Fig. 71.7B). Among the predicted ORFs, 267 (49%) display significant similarities to sequences in the NCBI GenBank database (Fig. 71.12B), while 134 (23%) have been assigned to 1 or more COGs. A putative function can be assigned to 32% of the ORFs, and some of these have never previously been reported in viruses. Proteins involved in DNA replication, recombination, and repair are the most highly represented (37 ORFs), followed by those involved in posttranslational modifications and protein turnover and classified as chaperones (27 ORFs); those involved in transcription (17 ORFs); and those involved in translation, ribosome structure, and biogenesis (13 ORFs). Fischer et al.³⁹ found that at least 172 CroV genes belong to an NCVOG, while 32% of the CroV genes display significant similarity with a Mimivirus gene (Fig. 71.11).³⁹ The distribution of top BLASTp hits against the NCBI nonredundant database for the 544 ORFs demonstrates affiliation with eukaryotes in 22% of cases, bacteria in 11% of cases, archaea in 1% of cases, Mimivirus in 12% of cases, other viruses in 3% of cases, and no hits for 51% of the ORFs (Fig. 71.12E). Paralogous genes are more frequent at the ends of the genome.²⁵ Several ORFs are predicted to be involved in protein synthesis, including an isoleucyl-tRNA synthetase, putative homologs of eukaryotic translation initiation factors, and two tRNA-modifying enzymes. In addition, 22 tRNA genes have been identified, which are clustered around position 510,000 in the genome in a 2.8-kb segment. Other proteins are involved in DNA repair, including some associated with DNA mismatch repair and alkylated DNA repair and others composing a complete base excision repair pathway as well as two photolyases. One of the photolyases is the first viral homolog identified for a class I photolyase, and the second is a homolog of a Mimivirus sequence. CroV also harbors numerous ORFs encoding transcriptional machinery, including a protein highly similar to an ELP3-like histone acetyltransferase that has not been previously identified in viruses and that may modulate the genome condensation state or be involved in the replication and packaging of the CroV genome. In addition, three DNA topoisomerases of types IA, IB, and IIA were identified. Besides, 51% of the CroV ORFs are ORFans, as they found no significant hit against the NCBI nonredundant database.³⁹

A 22-amino acid-long leucine-rich repeat, similar to the FNIP/IP22 repeat and also found in Mimivirus, was detected in at least 28 ORFs and is present with greater than 400 copies in the CroV chromosome. Overall, ~5% of the CroV genome corresponds to repetitive elements. Additionally, several genes are predicted to encode proteins of the ubiquitin pathway, which may represent a defense strategy against hosts in NCLDVs. Strikingly, a 38-kb genomic fragment was identified within the CroV genome between positions 264,800 and 302,500 that is highly AT rich (80.6% A+T) and contains no early or late promoters that have been identified within the other parts of the genome. This region is proposed to be the result of large-scale HGT from bacteria (Fig. 71.12B). It is composed of 34 ORFs, among which 14 are most related to bacterial proteins. Some genes are predicted to encode sugar-modifying enzymes, including glycosyltransferases, while others may be involved in carbohydrate metabolism, including enzymes for the biosynthesis of 3-deoxy-D-manno-octulosonate (KDO), which is

TABLE 71.1 Main Features of Giant Viruses Associated to Protists and their Virophages, Whose Genome is Available in the NCBI Genbank Genome Database

Family	Name	Isolation source and location	Particle size (nm)	Genome topology	Genome size (nt)	GenBank accession no. (date of creation)	GC content (%)	% Coding	Number of genes	Number of protein-coding genes	Number of structural transfer RNA
<i>Mimiviridae</i>	<i>Acanthamoeba castellanii</i> mamavirus	Freshwater, cooling tower, Paris, France	~750	Linear	1,191,693	JF801956 (22/10/2011)	28	N/A	1 059	1,023	6
	<i>Acanthamoeba polyphaga</i> mimivirus	Freshwater, cooling tower, Bradford, UK	~750	Linear	1,181,549	NC_014649 (12/11/2010)	27	88	1,018	979	6
	<i>Cafeteria roenbergensis</i> virus	Marine coastal water, Texas, US	~300	Linear	617,453	NC_014637 (01/11/2010)	23	90	544	544	22
<i>Marseilleviridae</i>	Marseillevirus	Freshwater, cooling tower, Paris, France	~250	Circular	368,454	NC_013756 (25/01/2010)	44	83	457	428	0
	Lausannevirus	Freshwater, Seine River, France	190–220	Circular	346,754	NC_015326 (01/04/2011)	42	92	450	450	0
<i>Unclassified</i>	Sputnik virophage	Freshwater, cooling tower, Paris, France	~74	Circular	18,343	NC_011132 (09/08/2008)	27	79	21	21	0
	Mavirus virophage	Marine coastal water, Texas, US	~60	Circular	19,063	NC_015230 (19/03/2011)	30	93	20	20	0

NCBI, National Center for Biotechnology Information.

an essential component of the lipopolysaccharide layer (LPS). Thus, this protein links lipid A to polysaccharides in gram-negative bacteria.¹⁰⁹ Four ORFs also contain an intein. One of these inteins is the only previously reported from a virus in the DNA-dependant RNA polymerase II subunit 2 with that found in the *Emiliana huxleyi* virus 163; another intein is the unique case detected in a DNA topoisomerase gene. A perfectly or nearly perfectly conserved motif (AAAAATTGA) was identified as an early promoter for 23% to 35% of the ORFs ($n = 127$ to 191); this motif is highly similar to that found in Mimivirus (AAAATTGA). In addition, a putative late promoter was identified for 124 genes classified as “late,” but this late promoter is not related to that described in Mimivirus.⁸¹ Microarray analysis revealed that 63% of studied genes are expressed, though it has been hypothesized that nearly all CroV genes are expressed. In experiments by Fischer et al.,³⁹ CroV gene expression comprised an early phase (0 to 3 hours postinfection) and a late phase (6 hours postinfection or later). Most of the predicted proteins involved in DNA replication and transcription belong to the early class, whereas predicted structural proteins belong to the late class.

MARSEILLEVIRIDAE

Marseillevirus

Marseillevirus has been isolated by co-cultivating *A. polyphaga* with water collected in 2007 from a cooling tower in Paris, France.¹⁵ This virus has an icosahedral shape and a diameter of ~250 nm (Fig. 71.17; Table 71.1). The capsid shell has a thickness of ~10 nm, and 12-nm-long fibers with globular ends are present at the viral surface. A membrane may surround the nucleocapsid, which is separated from the capsid shell by a gap of ~52 nm. It has been observed in amoebal culture that Marseillevirus enters the amoeba 30 to 60 minutes postinfection. Later, a VF appears close to the nucleus of the amoeba. Capsid assembly and viral genome encapsidation

are observed simultaneously in these VFs, leading to mature and immature Marseillevirus particles. The replication cycle is complete 5 hours postinfection, which is a short period of time. Additionally, the morphology of the host cell nucleus changes considerably between 30 minutes and 2.5 hours postinfection, which suggests that the replication cycle may involve the nucleus.¹²⁹

Marseillevirus was classified within the NCLDV group based on the finding of all group I core genes from this grouping (Fig. 71.10). The Marseillevirus genome is a circular, dsDNA molecule that is 368,454 bp in length (Fig. 71.7A). The Marseillevirus genome, with a G+C content of 44.7%, is less AT rich than that of *Mimiviridae*. A total of 457 ORFs are predicted to encode proteins whose sizes range from 50 to 1,537 amino acids (Fig. 71.7B). The coding sequence represents 89.3% of the chromosome. The ORFs are evenly distributed on the positive and negative strands (224 and 233, respectively). Significant matches with sequence databases were obtained and/or conserved domains were identified for 188 proteins, which represent 41% of the putative genes. In addition, significant similarity was found for 163 ORFs by searching the Global Ocean Survey (GOS) environmental database. As in other NCLDVs, the proportion of the Marseillevirus gene content that belongs to the NCLDVs' core gene set is very low (4.8%). Indeed, 22 ORFs belong to the 31 previously defined NCLDV class I to III core gene sets (Fig. 71.10).⁵⁸ Additionally, six Marseillevirus ORFs correspond to universal NCLDV proteins, and 17 are shared with Mimivirus/Mamavirus (though they are absent in the other NCLDVs). Phylogenetic analysis based on universal NCLDV proteins indicates that Marseillevirus represents the first member of a new putative viral family of NCLDVs, the *Marseilleviridae*. Among the NCLDVs, Marseillevirus branches deeply with iridoviruses/ascoviruses.^{15,148} In contrast, comparison of the NCLDV gene repertoires groups Marseillevirus with Mimivirus and Mamavirus. Besides, 17 proteins were identified as present in Mimivirus but absent in other NCLDVs.¹⁵

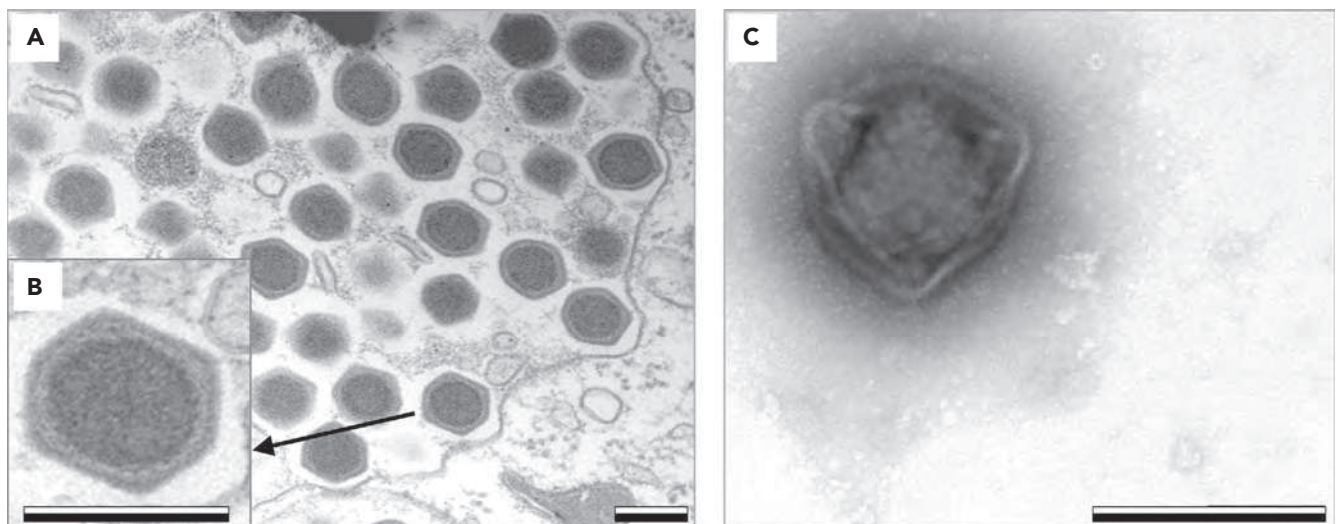


FIGURE 71.17. Electron microscopy of Marseillevirus particles. A, B (detail of A): Transmission electron microscopy images of Marseillevirus particles in the cytoplasm of an infected amoeba. C: A negative staining electron microscopy image of a Marseillevirus particle. Scale bars indicate 200 nm.

The analysis of the *Marseillevirus* genome highlighted its mosaicism and the role of amoeba as an opportune biological niche for gene acquisition and exchange between sympatric bacteria, viruses, and their amoebal hosts (Fig. 71.12C, F).¹⁵ Thus, the findings of Boyer et al¹⁵ indicate that numerous *Marseillevirus* ORFs were derived through HGT from many different sources. Among the *Marseillevirus* gene content, 59, 57, 70, and 2 predicted proteins exhibit the highest sequence similarity to viral, bacterial, eukaryotic, and archaeal homologs, respectively. Among the 80 *Marseillevirus* ORFs with putative amoebal homologs, the ORF that has the closest similarity to the *Marseillevirus* ORF is from *Acanthamoeba* in eight cases. On the basis of phylogenetic analyses, the *Marseillevirus* genome contains 51 genes (11%) of probable NCLDV origin, 49 (11%) of probable bacterial or bacteriophage origin, and 85 (19%) of probable eukaryotic origin. For 22 proteins, a phylogenetic relationship encompasses Mimivirus, *Marseillevirus*, and *Acanthamoeba*. Notably, in several cases, it was inferred by Boyer et al¹⁵ that related genes were likely acquired from independent sources by Mimivirus and *Marseillevirus*, suggesting that HGT may be a common event. Additionally, origins inferred for the *Marseillevirus* proteins display a tendency for being related to their function. Thus, bacterial or bacteriophage origin is inferred for many of the proteins involved in defense and repair, including nucleases. Eukaryotic and bacterial origins are inferred for many proteins involved in metabolic functions and lipid modification or degradation. Finally, eukaryotic origin is inferred primarily for proteins involved in signal transduction.

Comparative analysis of the *Marseillevirus* protein sequences identified 28 clusters of proteins representing a total of 121 proteins (22% of the ORFs).¹⁵ The largest protein family is composed of 20 proteins containing bacterial-like membrane occupation and recognition nexus (MORN) repeat domains; these domains are described to mediate membrane–membrane or membrane–cytoskeleton interactions.⁵³ Three predicted *Marseillevirus* proteins are homologous to histone-like proteins not previously identified in NCLDVs, and they have been detected in the viral particle, suggesting that they may be involved in condensation of the viral DNA before it is packaged. Additionally, the *Marseillevirus* genome carries the largest number of serine/threonine protein kinases for a virus ($n = 15$) and a large set of ubiquitin system proteins, which suggests an important potential for signaling. In addition, 20 proteins are involved in interactions between membranes or with the cytoskeleton, and 10 encode bacteriophage HNH endonucleases and restriction-like endonucleases that typically reside in mobile selfish genetic elements. A total of 49 proteins were identified in purified virions. These proteins comprise NCLDV core proteins, including the capsid protein; proteins likely involved in the early steps of viral replication (e.g., an early transcription factor); and other structural proteins. Ten of the 49 proteins were glycosylated, and 19 were phosphorylated, indicating posttranslational modification of *Marseillevirus* proteins. In addition, as for Mimivirus, several *Marseillevirus* RNAs are likely packaged within *Marseillevirus* particles, including transcripts encoding the capsid protein, DNA polymerase, D6R helicase, and TFII-like transcription factor.

Lausannevirus

Lausannevirus was isolated using amoebal co-culture from freshwater collected in 2005 from the Seine River, France.¹³³

When cultured on amoebae, viral particles were observed 30 minutes postinfection, and then an eclipse phase occurred that lasted until 2 hours postinfection. Vesicles filled with viruses and viral particles free in the cytoplasm are observed from 4 hours postinfection. Viruses fill all amoebae at 8 hours postinfection, and amoebal lysis is observed 16 hours postinfection. The Lausannevirus host range appears to be restricted to *Acanthamoeba* spp.

Lausannevirus is a close relative of *Marseillevirus* and can be classified within the putative family *Marseilleviridae*. The Lausannevirus genome is 346,754 bp in length and has a G+C content of 42.9% (Table 71.1).¹³³ It is either a linear DNA molecule with terminal repeats or a circular molecule. This genome harbors 450 ORFs that cover 93% of the genome and have a mean length of 716 bp. A total of 332 proteins (73.8% of the putative proteome) display significant similarity to proteins in the NCBI nonredundant sequence database, among which 320 (71.1%) have a *Marseillevirus* protein as the best BLASTp hit. Homologs were identified in Lausannevirus for all NCLDV core genes detected in *Marseillevirus*. Notably, the comparison of the Lausannevirus genome to that of *Marseillevirus* shows a 150-kb region with poor synteny that is enriched in hypothetical proteins followed by a 200-kb region with a higher co-linearity and slight enrichment for NCLDV core genes. The largest viral families in Lausannevirus correspond to MORN repeat-containing proteins, endonucleases, and serine/threonine protein kinases. As in *Marseillevirus*, three histone-like proteins were identified in Lausannevirus, which may form histone doublets, be functional, and be involved in interactions with the viral DNA. Additionally, Thomas et al¹³³ reported that a deoxyuridine triphosphate nucleotidohydrolase (dUTPase) is present but truncated in *Marseillevirus*, but it is nearly complete in Lausannevirus. In addition, differences were underscored regarding the presence or absence of blocks composing an endonuclease domain within the ribonucleotide reductase intein present in Lausannevirus and *Marseillevirus*.

VIROPHAGES: VIRUSES INFECTING MIMIVIRIDAE

Definition

The virophage concept was coined in 2008 by La Scola et al⁷⁸ when reporting the discovery of icosahedral viral particles 50 nm in size in the amoebal cytoplasm of infected cells and inside the Mamaviruses factories (Figs. 71.6 and 71.18).⁷⁸ This small virus, named Sputnik, can only multiply within *Acanthamoeba* spp. co-infected with Mamavirus or Mimivirus. Indeed, Sputnik and the giant virus both replicate in the VF, but differences were noted regarding the location and kinetics of their replication. Sputnik is produced earlier than Mamavirus, while co-infection with Sputnik and Mamavirus is associated with a 70% decrease in the yield of infective Mamavirus and a threefold decrease in lysis of *Acanthamoeba*, as assessed 1 day postinfection. Concurrently, a significant increase in the production of Mamavirus virions with morphological anomalies is observed (Fig. 71.6C, D), which includes an increase in the capsid thickness (in 11% of virions instead of 1% in the absence of Sputnik co-infection) that reached sixfold the usual thickness in some viral particles. Besides, Sputnik was observed in a small fraction of the Mamavirus particles (Fig. 71.6B). Taken together, these observations indicate that

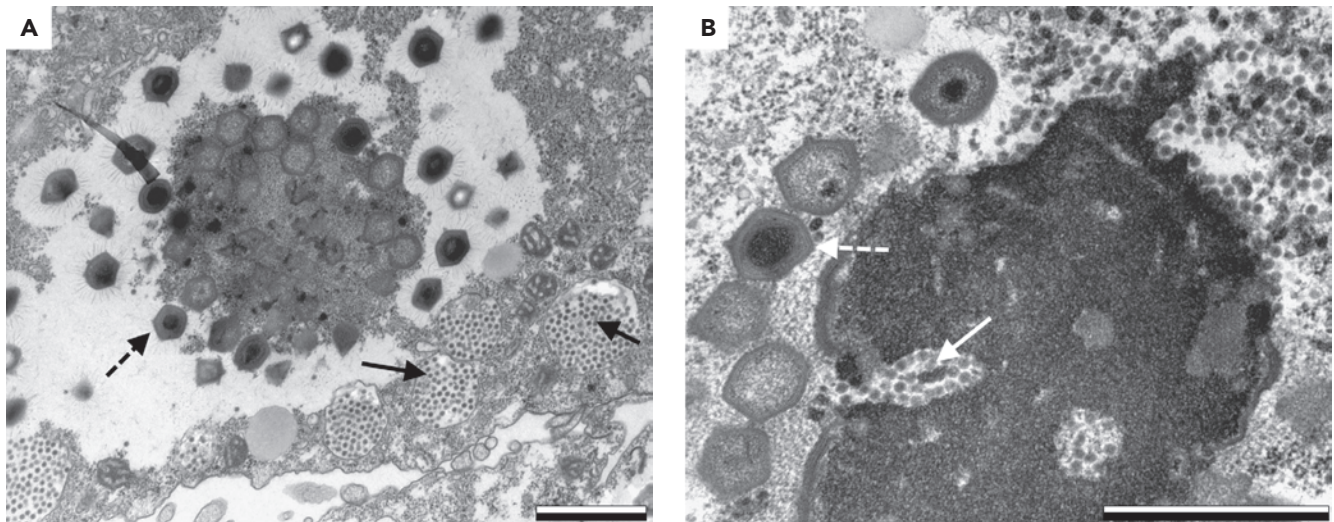


FIGURE 71.18. Electron microscopy of viral factories during co-infection of *Acanthamoeba* with Mamavirus and Sputnik (at 16 hours after infection). Solid arrows show Sputnik particles; dashed arrows show Mamavirus particles. **A** and **B** represent two different magnifications. Scale bars indicate 1 μm .

Sputnik considerably affects the replicating cycle of its host virus and highlight functional analogies for Sputnik with bacteriophages, which led to the name virophage. Sputnik appears as a virophage rather than a satellite virus. Thus, in addition to the aforementioned features, it was not observed in the absence of Mamavirus or Mimivirus and requires these giant viruses for its replication. In addition, the replicative cycle of Sputnik occurs entirely within the viral factory of these giant viruses, no signal having been detected within the amoebal nucleus.³¹ Sputnik particles were usually observed at one side of VFs in the absence of Mamavirus/Mimivirus particles, or both viruses were seen separately at different sides of the VFs.³¹ Moreover, 16 Mimivirus-like putative hairpin structures in the Sputnik genome, which were recently found to correspond to polyadenylation signals and specifically used by the Mimivirus-encoded transcription machinery, were identified in the Sputnik genome; thus, their presence suggests the late expression of Sputnik genes in Mimivirus VFs and coincides with the rarity of the AAAATTGA early Mimivirus promoter.^{17,21,127}

The existence of virophages was confirmed in 2011 by the identification of Mavirus, a virophage infecting CroV.⁴⁰ Moreover, a third virophage, named Organic Lake virophage (OLV), which preys on viruses infecting phototrophic algae, was recovered from Organic Lake, a hypersaline meromictic lake in Antarctica, and described in 2011. The detection of another virophage associated with a Mimivirus-like virus was reported by La Scola et al.⁷⁷ These findings suggest that virophages may be common in ecological niches of protist-associated giant viruses.

Sputnik

The Sputnik particle is ~ 74 nm (71 to 84 nm according to symmetry axis) in size (Figs. 71.6 and 71.18).¹²⁸ Its structure was analyzed by cryo-EM three-dimensional reconstruction. The major capsid protein is organized into a hexagonal lattice, the triangulation number being 27. The capsomer structure is trimeric. The thickness of pseudohexameric capsomers is

~ 7.5 nm, and the distance between the centers of adjacent capsomers is ~ 7.5 nm. Mushroom-like fibers are present at the surface. They may help to stabilize capsomers or may be involved in entry into host cells; they may be flexible. These fibers are 5.5 nm in length, have a triangular head, and protrude from the center of pseudohexameric but not pentameric capsomers. Instead, centers of pentameric capsomers harbor a cavity that may represent a gate for DNA release or packaging, as in bacteriophages. The major capsid protein of Sputnik is likely to have a double jelly-roll fold as in viruses of the PRD-1-adenovirus lineage, and the crystal structure of PBCV-1 MCP was fitted into the cryo-EM map of Sputnik.¹²⁸ Notwithstanding, no sequence similarity was found for the MCP, neither with that of these latter viruses nor with the Mimivirus MCP. Besides, Sputnik and Mimivirus MCP have a similar length (595 vs. 591 amino acids, respectively). The capsid shell surrounds two layers, likely a lipid bilayer, which have a total thickness of 4 nm and may harbor a transmembrane protein. Around 2.5 nm underneath this putative membrane is the packaged DNA; its packaging density is ~ 1.966 angstrom³/bp. In proteomic analyses, the Sputnik ORF20, which corresponds to the putative capsid protein, was the most abundant; two other minor virion proteins (ORF 8 and 19) were concurrently detected.

Sputnik has a circular dsDNA genome that is 18,343 bp in size with an organization that is typical of viral genomes, that is, with a tight ORF arrangement (Table 71.1).⁷⁸ This genome is AT rich (73%) as observed in mimiviruses. The Sputnik genome was likely not integrated into the Mamavirus genome; noteworthy, no read was found that overlapped both genomes. A total of 21 genes predicted to encode proteins of 88 to 779 amino acids were identified. Among them, 14 are ORFans, whereas the 7 remaining ORFs have various putative origins including eukaryotic, bacterial, viral or plasmid, and environmental origins (Fig. 71.19). Sputnik appears an unknown family of viruses based on comparative analyses. Thus, the Sputnik DNA is a particularly chimerical genome, which suggests its involvement in HGT. Furthermore, such

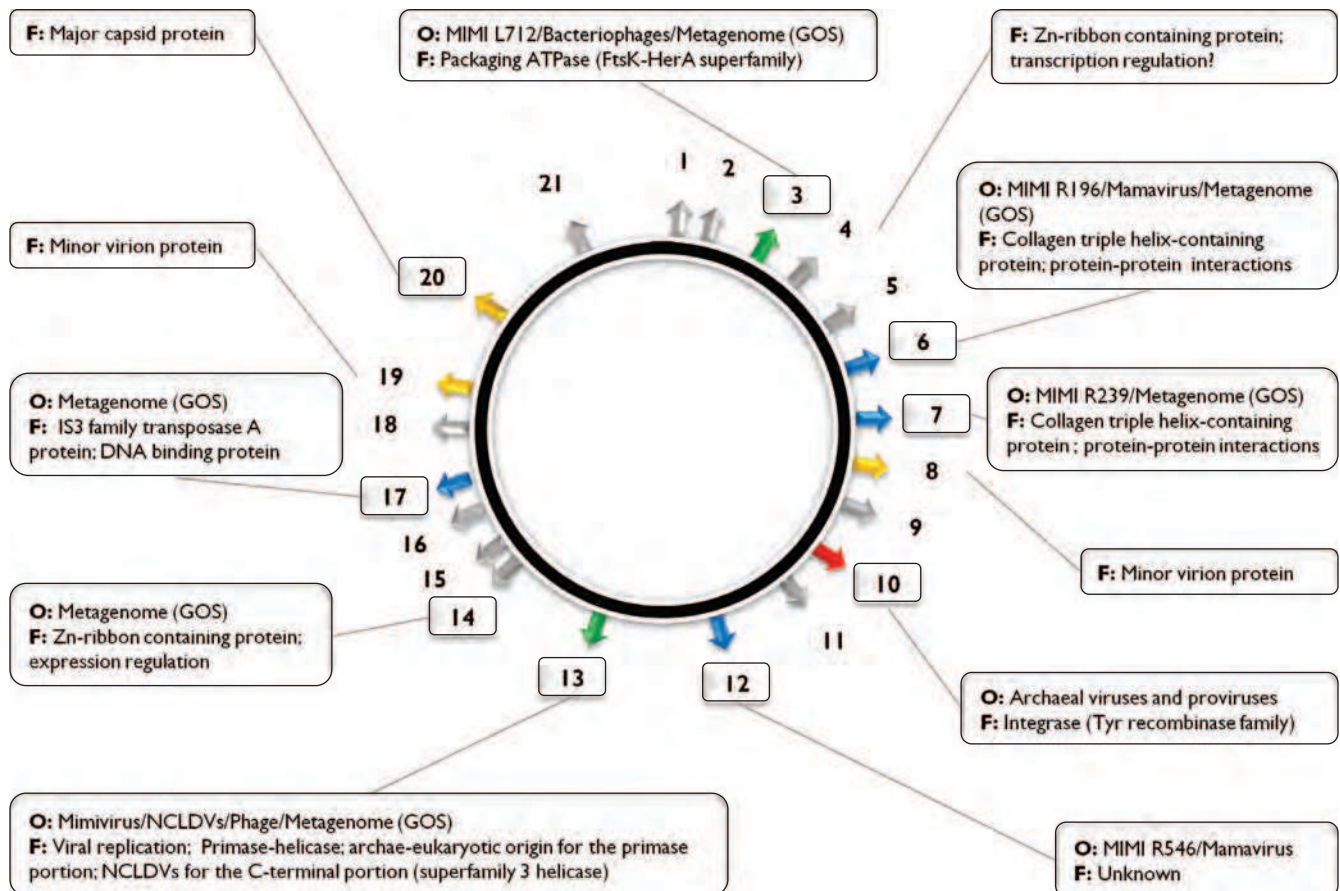


FIGURE 71.19. Sputnik genome annotation. Sputnik open reading frames (ORFs) are represented by their number; F, predictive function, activity, protein family; O, closest homolog in the National Center for Biotechnology Information (NCBI) GenBank protein sequence database (nr) or the Global Ocean Survey (GOS) data set, putative origin.

a chimeric gene repertoire and the presence of putative integrase and transposase led to the hypothesis that this virophage might represent a vehicle of genes. Noteworthy, viral RNAs corresponding to all but one Sputnik ORF (ORF 17) were detected inside the Sputnik virions; nevertheless, this ORF was detected together with all other ORFs at 4 hours postinfection during the replication cycle.³¹

Sputnik might adhere to Mamavirus/Mimivirus fibrils, which would allow its entry into amoeba, in the same endocytic vacuole as Mamavirus/Mimivirus.³¹ Sputnik has been indeed frequently observed in association with or trapped inside the surface fibers of Mamavirus.³¹ In addition, Sputnik replication was not detected during co-infection with Marseillevirus, which is devoid of fibers, and with a bald form of Mimivirus.¹² Mimivirus protein R135 might be involved in the adhesion of Sputnik to Mimivirus/Mamavirus because it is one of the three proteins recently detected by two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry from purified Mimivirus fibers,¹² and this protein was also identified from purified Sputnik particles.⁷⁸

Mavirus

A virophage, named Mavirus (for Maverick virus), was identified in association with the recently described CroV.⁴⁰ As

Sputnik, it reduces host cell lysis and production of its associated giant virus, in the absence of which it is not able to replicate. Viral particle size is about 60 nm. Mavirus has a 19,063-bp circular dsDNA genome, with an A+T content of 69.7% (Table 71.1). This genome was predicted to encode for 20 proteins. A nearly perfect inverted repeat of ~50 bp, which may adopt a hairpin structure with a 15-nt loop, was identified between ORFs 20 and 1. The mean size of Mavirus ORFs is 883 nucleotides, corresponding to a coding density of 92.6%; a conserved promoter motif located in the intergenic region and that resembles a CroV putative late promoter motif was found to precede all 20 ORFs. This finding suggests that Mavirus gene expression is ruled by the CroV transcription apparatus during the late stages of its replication cycle.

Similar genome length and structure and a close evolutionary relationship were highlighted by Fischer and Suttle⁴⁰ between Mavirus and the self-synthesizing *Maverick* or *Polinton* transposable elements (MP TEs), a type of DNA transposons with a length of 9 to 22 kb that encode up to 20 proteins. Mavirus and MP TEs share indeed seven homologous proteins, including a rve-superfamily retroviral integrase that harbors a conserved chromo domain, which was found to be duplicated in several chromatin-binding proteins and may be associated with positive or negative regulation of transcription,⁷⁰ and

a protein-primed DNA polymerase B; the highest similarity for these two genes was found with sequences from the *Polysphondylium pallidum* genome. In addition, a superfamily 3 helicase and the MCP predicted in Mavirus were found sporadically encoded by MP TEs. Besides, four Mavirus predicted proteins (20% of the gene content) have homologs in Sputnik: these putative proteins are a capsid protein, a DNA-pumping ATPase, a cysteine protease, and a GIY-YIG endonuclease/Zn ribbon containing protein. On the basis of aforementioned data, Fischer and Suttle hypothesized that MP TEs may derive from a Mavirus ancestor; alternatively, but less likely, an escaped MP TE may be at the origin of Mavirus. Moreover, Mavirus was highlighted to increase the survival of *Cafeteria roenbergensis*, the host for this virophage and the giant CroV. This suggests that Mavirus might confer a selective advantage to the cell population suffering from CroV infection. This is potentially true for all virophages and was recently emphasized for OLV, a third virophage found to control dynamics of arctic algal host-virus populations.¹⁴⁷

EPIDEMIOLOGY OF GIANT VIRUSES ASSOCIATED WITH PHAGOCYtic PROTISTS

To date, the epidemiology of giant viruses associated with phagocytic protists and their virophages is largely unknown. A growing body of data indicate that mimiviruses and marseilleviruses may be ubiquitous in the biosphere. Regarding direct evidence through viral isolation, Mimivirus and Mamavirus were notably recovered in two different countries, the United Kingdom and France, respectively, from water collected in cooling towers.^{76,78} In addition, CroV was recovered in the United States in marine water from a different host than that of the two former viruses. Indeed, *Cafeteria roenbergensis* is a heterotrophic dinoflagellate that is widespread in marine water and belongs to the *Chromalveolata*, a phylum that is phylogenetically distant from *Acanthamoeba* spp., which are the hosts of mimiviruses and marseilleviruses.³⁹ Interestingly, viruses may regulate populations of *C. roenbergensis*.⁸⁵ Moreover, 19 other giant viruses, whose capsid sizes range from 150 to 600 nm, have been recovered from various water and soil samples using culture on amoebae.⁷⁷ Among these viruses, at least five were related to mimiviruses by means of phylogenetic analysis of the B-family DNA polymerase and MALDI-TOF mass spectrometry. Strikingly, these 19 giant viruses were recovered by inoculating 105 environmental samples using modified protocols for amoebal culture, which corresponds to a ~20% isolation yield. Recently, Mimivirus-like particles were retrospectively observed by light microscopy within *Acanthamoeba* spp. in final-stage conventionally treated sewage sludge from a wastewater treatment plant in the West Midlands, United Kingdom.⁴⁷ The infected *Acanthamoeba* spp. culture could not be resuscitated, and only sewage sludge samples collected later were tested for Mimivirus by PCR with negative results. Nonetheless, the initial observation of a high density of viral-like particles suggests that *Acanthamoeba* spp. that survive sewage treatment can disseminate Mimivirus- or other giant virus-infected amoeba to agricultural land and surface waters.

Additionally, bioinformatic analyses performed on metagenomes recovered from marine samples suggest that microalgae and modern sponges might be hosts for unidentified members

of the family *Mimiviridae*.^{48,91,120} Indeed, sequences similar to those of Mimivirus were identified in databases of sequences recovered from water samples collected in the Sargasso Sea and during the GOS (Global Ocean Sampling) expedition. Regarding this latter study, DNA polymerase fragments corresponding to Mimivirus relatives were the most frequently recovered sequences in 86% of sample sites, after those of bacteriophages. Kristensen et al.⁷² also found hits for Mimivirus sequences against marine metagenomes. In addition, two sequences from a viral metagenome library from water recovered 200 m deep in Monterey Bay, California, were recently found to have a BLAST hit with Mimivirus.¹²⁵ Regardless, the results of these metagenomic analyses should be interpreted with caution with respect to the question of the prevalence of giant viruses. Thus, since Ivanovsky's experiment, viruses have not been considered to be visible by light microscopy.¹¹⁵ This paradigm has led to a considerable bias in recent studies of viral metagenomics, which have filtered the samples prior to analysis.^{1,33,134} Such procedures inevitably prevent the detection of viruses larger than the pores of the filters used (i.e., 0.2 to 0.45 μm).^{32,134,141,151}

ECOLOGY OF GIANT VIRUSES WITH A SYMPATRIC LIFESTYLE IN PHAGOCYtic PROTISTS: AMOEBAE AS GENITORS OF GIANT VIRUSES WITH MOSAIC GENE REPERTOIRES

The tremendous size and composition of giant viruses that infect protists has led to a fundamental revision of our vision of viruses and has been linked to their sympatric lifestyle, consisting of their replication and survival in a community with other viruses and bacteria (Fig. 71.20).¹¹⁰ Despite differences in their epidemiology and host, it is notable to observe several common features for the genomic organization of mimiviruses and marseilleviruses.²⁵ Thus, comparative genomics of the currently identified giant viruses of protists underscores a substantial level of plasticity and various origins of their gene content, in accordance with the recently proposed theory that the evolution of life resembles the shape of a rhizome more so than a tree.¹¹¹ Overall, the genomes of giant viruses are mosaics containing genes of eukaryotic, bacterial, and/or archaeal origin and are substantially reshaped by horizontal gene transfer, by gene duplications, and possibly through recombination.^{15,36,94,126} The lifestyle of giant viruses, replicating and surviving in phagocytic protists, likely explains the remarkable diversity of origins for their repertoire of genes, as these phagocytic protists are genitors of chimeric viruses.^{15,39,110} *Acanthamoeba* spp., the host cells of Mimivirus, Mamavirus, Marseillevirus, and Lausannevirus, are free-living amoebae that are ubiquitous in the environment, including the air, soil, and water,¹¹⁹ as well as wild phagocytes that ingest any particle greater than 0.5 μm .¹¹⁰ They harbor, transiently or for long periods, a variety of intracellular bacteria, including *Proteobacteria*, *Firmicutes*, *Chlamydia*, *Bacteroidetes*, and *Actinobacteria*, as well as giant viruses.^{55,110} *Cafeteria roenbergensis*, the CroV host, and *Acanthamoeba polyphaga* and *castellanii* are phylogenetically distantly related, but *Cafeteria roenbergensis* feeds on bacteria and viruses.^{39,85} Giant viruses infecting phagotrophic protists live sympatrically within their host cell with numerous other bacteria and

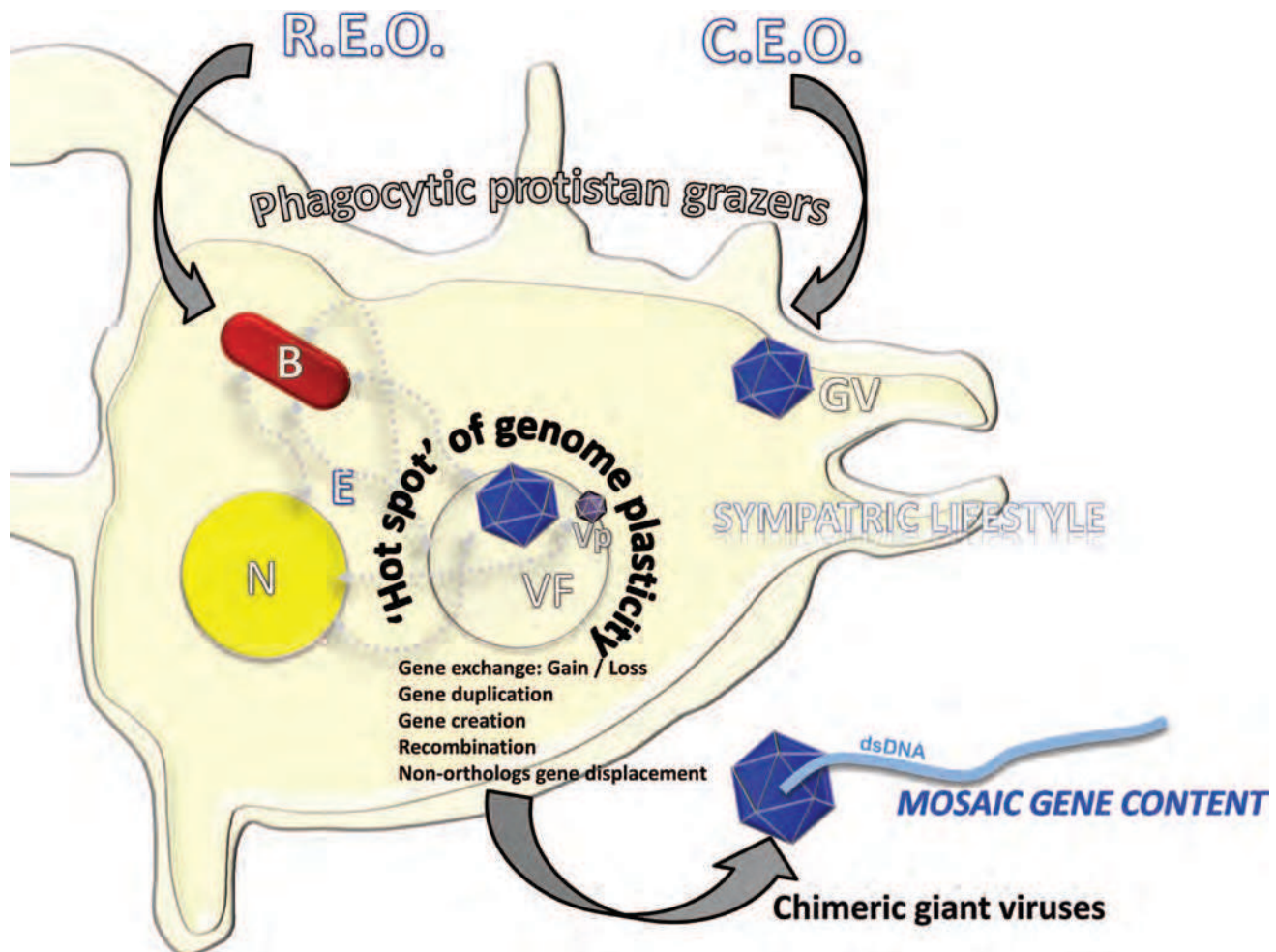


FIGURE 71.20. Schematic of the sympatric lifestyle of giant viruses in phagocytic protists. B, bacteria; C.E.O., capsid-encoding organism; E, eukaryote; GV, giant viruses; N, nucleus; R.E.O., ribosome-encoding organism; VF, viral factory; Vp, virophage. Dashed arrows represent gene transfers.

viruses, which enables them to exchange genes. As an illustration, *Legionella drancourtii*, a *Parachlamydia* strain, and Marseillevirus were observed concurrently within *A. castellanii* following experimental co-infection.¹⁵ Phagocytic protists may even become ribo-viro cells, where genes from ribosome-encoding organisms (REOs) or capsid-encoding organisms (CEOs) can be exchanged, possibly including exchange by virophages when they are present (Fig. 71.20).^{15,42,78,114} Thus, gene foraging by such bacteria and viruses with a sympatric lifestyle is possible due to the promiscuity between their replicating genomes and that of their host. As an illustration, two Mimivirus proteins, corresponding to a Zn-dependent alcohol dehydrogenase and an outer membrane lipoprotein, seem to have homologs in common amoebal inhabitants, *Legionella pneumophila* and *Campylobacter* spp., respectively.⁹⁴ In such arenas as phagocytic protistan grazers, viruses and bacteria must struggle for life, and they may require a tremendous gene armory to resist their phagocytic host and outcompete or at least survive in competition with other viruses and bacteria.¹¹⁰ In contrast, for other obligate intracellular bacteria that live allopatrically in other eukaryotic cells, the capacity to acquire foreign

genes is limited. It has been noted that obligate intra-amoebal parasites (i.e., bacteria and viruses) exhibit larger genomes than their intracellular relatives,¹¹⁰ including some in which genome reduction was described.¹²¹ A statistically significant correlation was found between lifestyle and genome size for NCLDV; having a sympatric lifestyle is positively correlated with carrying a larger genome.¹¹⁰ For instance, the *Legionella drancourtii* genome is larger than that sequenced from *Legionella pneumophila* strains,¹⁰⁴ and the genome of *Rickettsia bellii* is the largest among *Rickettsia* species.⁹⁰ Among the NCLDVs, mimiviruses have the largest genomes.¹¹⁰ Strikingly, a relationship between genome size and sympatric lifestyle was shown experimentally for Mimivirus. Thus, subculturing this virus 150 times in a germ-free amoebal host was associated with a dramatic reduction of its genome (~16%).¹² Interestingly, in this study, the gene loss was associated with the emergence of phenotypically different viruses that lacked surface fibers and whose VFs differed morphologically compared to wild-type viruses.

One possible mechanism for HGT between viral and bacterial genomes within phagocytic protists may involve

recombination. Filée et al³⁶ hypothesized that gene acquisition in Mimivirus may resemble recombination-primed replication processes that have been described in the bacteriophage T4⁹⁷ and that allow the introduction of foreign DNA by a splice or patch mechanism. Filée et al also underscored the high frequency of homologous recombination during the replication cycle of poxviruses with the requirement of low-sequence identity^{139,146} and in phycodnaviruses.¹³² Processes allowing strand exchange, such as those observed in phage lambda, *Escherichia coli*, or herpesviruses, which can occur in the absence of replication and involve a DNA-binding protein and an exonuclease, may represent another mechanism for DNA exchange.^{36,101,118} Alternatively, it has been proposed that recombination may be promoted by an enzyme similar to the topoisomerase IB of vaccinia virus. Indeed, a DNA topoisomerase IB encoded by the Mimivirus genome has been described and characterized.^{7,36}

PATHOGENICITY

Acanthamoeba can infect a large spectrum of mammals, including humans,⁸⁹ and have been considered as “Trojan horses”.⁵ Most of the bacteria that survive and multiply in amoebae are indeed human pathogens.⁵² The question of the Mimivirus pathogenicity has focused initially on the capability of this virus to cause pneumonia. Indeed, water-associated bacteria that are amoebae-resistant pathogens are involved in both community- and hospital-acquired pneumonia.⁷⁹ In addition, pneumonia remains undiagnosed in 20% to 50% of cases, and the spectrum of causative agents in pneumonia is incompletely recognized.^{27,79} Six clinical studies have assessed whether Mimivirus is associated with pneumonia, and the results are summarized in Table 71.2. In these studies, either serology or PCR was used, with serology being the most commonly used strategy, to search for indirect evidence of Mimivirus infections.

Overall, several findings argue for Mimivirus pathogenicity in humans or mice. Mimivirus is capable of infecting macrophages through phagocytosis, similar to what can be observed in *Acanthamoeba* spp., and infection leads to productive replication.⁴⁹ This may represent a pathway to pathogenicity. Alternatively, the outstanding Mimivirus genome size and gene armory suggests a possible adaptation to an extended range of hosts.²⁶ Nevertheless, Mimivirus does not efficiently replicate in co-culture with any other mammalian cells.¹¹⁵ Additionally, Mimivirus induces pneumonia in experimentally inoculated mice.⁶³ In all pneumonic mice, Mimivirus was cultured from the lung tissues, Mimivirus antigens were detected in the lung tissues, or both.⁶³ In humans, seroconversion to Mimivirus was observed in several patients presenting with pneumonia.⁷⁹ In addition, seroprevalence to Mimivirus was significantly higher in pneumonia patients than in controls,⁷⁹ the presence of antibodies to Mimivirus was associated with a poorer outcome in mechanically ventilated patients in intensive care units,¹³⁶ and rehospitalization after discharge was significantly associated with antibodies to Mimivirus.⁷⁹ Moreover, Mimivirus serology was positive in a patient with pneumonia and comprised reactivities against 23 different specific Mimivirus proteins, including 4 without known homologs.¹¹⁶ Nevertheless, cross-reactivities may explain some of the positive serologies to Mimivirus.¹⁰⁸

Studies have failed to isolate Mimivirus from patients with pneumonia. Furthermore, Mimivirus DNA could be amplified from a single patient with unexplained pneumonia.⁷⁹ The absence of additional cases of Mimivirus DNA amplification from patients presenting pneumonia may be related to infection with mimiviruses genetic variants.¹³⁷ Notably, one sample from a pediatric patient suffering from acute diarrhea was found to contain five sequence reads that displayed 47% to 52% amino acid identity to endonuclease genes in Mimivirus, but these sequences also possessed nearly similar levels of identity to bacterial and phage genomes, and phylogenetic analysis did not identify their precise origin.³⁷ However, Senegalvirus, a new giant virus associated with amoebae and that is a close relative to Marseillevirus, was recently identified in the feces from a young, healthy Senegalese man, which represents the first isolation of such a giant virus isolated from a human (GenBank accession no. HQ008533.1, HQ008532.1, HQ008531.1).

LABORATORY DIAGNOSIS

To date, diagnostic tools that aim to identify giant viruses associated with protists or their virophages in clinical samples have only been developed by a few teams and targeted the Mimivirus. Serological testing has been developed and implemented in the lab where this virus was discovered.²⁷ Seroprevalence studies were conducted to test the association of pneumonia with Mimivirus infections (Table 71.2). Cross-reactivity with other antigens besides the Mimivirus proteins was identified.¹⁰⁸ PCR assays have also been described by two teams for the detection of Mimivirus DNA in clinical respiratory samples.¹³⁷ In recent studies based on alignment of partial genomic sequences, it has been demonstrated that molecular diagnosis of Mimivirus or other giant viruses of protists should likely deal with the potential genetic diversity of these viruses.^{27,30,137} The recent and ongoing discovery of additional mimiviruses, marseilleviruses, and virophages confirms that considerable genetic diversity exists between viruses included within the same family. In addition, the gene content can vary substantially.^{15,39,78} Nonetheless, the availability of these new genomes might be influential for the design of more reliable molecular tests. Recently, La Scola et al⁷⁷ tentatively characterized giant viruses recovered from amoebal cultures using MALDI-TOF mass spectrometry.⁷⁷ This technology provided congruent results with the phylogenetic analysis of the B-family DNA polymerase (DNA PolB) and is a promising method to enable the preliminary identification and characterization of giant viruses associated with amoebae. Additionally, EM remains a powerful tool for the diagnosis of various giant viruses.

PREVENTION AND CONTROL

The question of the prevention and control of infections with protist-associated giant viruses may be considered premature because, at the present time, no pathology is strongly associated with them. However, it should be noted that culture of these large viruses has underscored their high resistance to several chemical compounds and physical treatments, and their sensitivity to antimicrobials is largely unknown. In addition, their intra-amoebal lifestyle may protect them from various agents.

TABLE 71.2 Summary of Clinical Studies that Have Tested Pneumonia Patients for Mimivirus Infections

Reference	Year(s) of sample	Country	Population size	Subgroup size	Main characteristics of patients	Respiratory sample types	Serology	PCR
La Scola et al, 2005 ⁷⁹	1985–1997	Canada	887	121	Adults; ambulatory/community-acquired pneumonia patients, Nova Scotia	N.t.	36 positive (9.7%)	N.t.
				255	Adults; hospitalized patients for community-acquired pneumonia, multiple centers across Canada			
				511	Adults; healthy control subjects, Nova Scotia	N.t.		
	2003–2004	France	129	32	Adults; ICU-acquired pneumonia patients, 1-year survey	BAL	5 positive samples out of 26 (19.2%) All negative	1 positive sample out of 32 (3.1%) N.t.
				50	Adults; controls (patients tested for anti- <i>Rickettsia</i> spp. antibodies)	N.t.		
				21	Adults; intubated control patients in ICU who did not present pneumonia	BAL		
Berger et al, 2006 ¹⁰	2003	France	157		Adults; ICU pneumonia patients (pneumonia was community acquired or ventilator associated)	N.t.	7 cases with high level of evidence and 7 additional cases with low level of evidence Positive	N.t.
Raoult et al, 2006 ¹¹⁶	2004	France	1		38-year-old laboratory technician	N.t.		N.t.
Vincent et al, 2009 ¹³⁶	2006–2008	France	300		Adults; ventilated patients in ICU with a suspicion of a ventilator-associated pneumonia and a positive serology for Mimivirus (cases)	N.t.	59 positive (19.7%)	N.t.
Larcher et al, 2006 ⁸⁰	2005–2006	Austria	214		Children hospitalized for respiratory tract infections; 209 nonimmunocompromised; 6-month survey during fall and winter seasons	NP aspirate samples	N.t.	All negative
Dare et al, 2008 ³⁰	2000–2001	Urban US	496	124	Children <5 years old; community-acquired pneumonia cases	Nasal swabs	N.t.	All negative
	2003–2004	Rural Thailand		120	Adults, children; community-acquired pneumonia cases	NP swabs	N.t.	All negative
	2002–2004	US		71	Geriatric; nosocomially acquired pneumonia outbreak, retirement centers	Nasal or oro-/NP swabs	N.t.	All negative
		US		5	Adults, children; community-acquired pneumonia outbreak (familial cluster)	Lower respiratory samples	N.t.	All negative
	2001–2003	US		87	Adults; bone marrow transplant recipients	NP aspirates, nasal wash, or NP swabs	N.t.	All negative
	2002–2003	Canada		89	Adults; lung transplant recipients	NP swabs	N.t.	All negative

N.t., not tested; BAL, bronchoalveolar lavage; NP, nasopharyngeal.

Thus, based on current knowledge, it is reasonable that Mimivirus and its relatives should be considered as biosafety class 2 pathogens. Notably, the case of a technical worker was reported who manipulated high titers of Mimivirus and later presented pneumonia and seroconversion to this virus without other identified causes for his clinical symptoms.¹¹⁶

GIANT VIRUSES ASSOCIATED WITH PROTISTS CHALLENGE THE DEFINITION OF VIRUSES AND LEAD TO PROPOSAL OF THE FOURTH DOMAIN THEORY

Giant viruses infecting phagocytic protists differ in many aspects from other canonical viruses and strongly challenge the vision of viral entities conveyed by Lwoff's classification.⁸⁴ Mimiviruses, the largest giant viruses to date, have a capsid diameter of ~500 nm, which is not in accordance with the historical concept of viruses as small, ultra-filterable entities.^{6,114,115} In addition, mimiviruses have large genomes that encode more than 1,000 predicted proteins.¹¹³ Moreover, Mimivirus was shown to harbor mRNA,^{113,117} which contradicts the criteria of Lwoff's⁸⁴ viral classification that viruses only possess one type of nucleic acid. Besides, the mimiviruses genomes encode proteins involved in translation, which represents a unique feature among viruses; they also encode tRNAs. Likewise, mimiviruses can themselves be infected by viruses. This was initially described by La Scola et al.,⁷⁸ who coined the name virophage, with Mamavirus,⁷⁸ and three years later by Fischer et al.³⁹ with CroV.

In addition to revealing unique features for viruses, the genomes of mimiviruses and marseilleviruses have been very influential in considering giant viruses as possible major ancestors in the early stages of the evolution of life.^{14,42,69,113,114,148} Viruses have been excluded from the tripartite putative tree of life composed of *Eukarya*, *Bacteria*, and *Archaea* because they lack ribosomes and, more generally, genes shared with members of these three canonical domains. The hypothesis that Mimivirus may compose a fourth domain of life was therefore groundbreaking.¹¹³ This assumption relies on the phylogeny of some of the Mimivirus genes that are shared with members of the three canonical domains of life. Two genes, which encode the tyrosyl tRNA synthetase (TyrRS) and the universal DNA replication clamp loader protein (RFC), have been particularly analyzed, and the interpretation of their phylogeny has fostered discussion about whether or not Mimivirus and, more generally, the NCLDV as a whole constitute a new branch in the tree of life. Some consider that the branching of Mimivirus as a domain on its own is an artifact.^{23,42,67,95,96,112–114} A major point of discussion is that horizontal gene transfer and nonorthologous gene displacements that occurred during the evolution of NCLDVs might considerably impair phylogenetic reconstructions.^{29,68,148} Furthermore, the interpretation of tree topologies may be complicated because there is no evidence of the viral ancestors that may have infected extinct proto-eukaryotes.¹⁹

Besides, it was pointed out that some interpretations of tree topologies suggesting the absence of a fourth domain might have been influenced by the paradigm that viruses are gene robbers and have acquired many of their genes from the

three cellular domains.⁴² Thus, when reinterpreting the phylogeny reconstructions of Moreira and Brochier-Armanet,⁹⁴ Forterre⁴² concluded that 32 instead of 60 Mimivirus proteins have a putative eukaryotic origin, and 21 instead of 4 proteins have a putative viral origin. In 2010, Boyer et al.¹⁴ performed a phylogenetic analysis of genes involved in nucleotide metabolism and DNA processing, which are present in cellular organisms and NCLDVs. They identified that several of these genes, including those encoding the DNA-dependent RNA polymerase (RNAP II), transcription factor IIb, flap endonuclease, and the processing factor proliferating cell nuclear antigen, support the monophyly of the NCLDVs, their ancient origin, and the existence of four domains of life, with the NCLDVs composing the fourth domain.¹⁴ These findings were criticized, based on the assumption that long-branch attraction and compositional heterogeneity might jeopardize the topology of phylogenetic trees.¹⁴⁰ Nonetheless, several other arguments suggest that mimiviruses, marseilleviruses, and other NCLDVs have an ancient origin dating back to the proto-eukaryotes and compose a fourth domain of life.^{14,42} For instance, Suhre¹²⁶ found that the proportion of duplicated genes in Mimivirus is similar to that observed in *Eukarya*, *Bacteria*, and *Archaea*, which is compatible with the deep rooting of this giant virus on the tree of life. In 2009, Yutin et al.¹⁴⁸ updated the core NCLDV gene set on the basis of constructing COGs with an extended number of NCLDV genomes, including those of the Mamavirus and the Marseillevirus. They identified 1,445 NCLDV COGs, so-called NCVOGs, including 177 represented in more than one NCLDV family and five including proteins from all 45 analyzed viruses. Yutin et al. were able to delineate, by means of maximum-likelihood reconstruction, a set of 47 conserved genes that were likely harbored by the genome of the NCLDV common ancestor and proposed that this ancestor may have been a giant virus itself.¹⁴⁸ Additionally, it was highlighted that NCLDVs infect various hosts that belong to the three canonical domains of life, and cross-mapping of the NCLDV and host eukaryotic trees demonstrated that some members of a same NCLDV branch are related to eukaryotic organisms belonging to different supergroups.⁶⁹ These findings suggest that the ancestral NCLDV was involved in early events of eukaryotic evolution, and the hypothesis of a "Big Bang-like" event for the origin of the NCLDVs was proposed by Yutin et al.¹⁴⁸ Moreover, by searching metagenomic data to identify novel deep branches on the tree of life, Wu et al.¹⁴³ found several such branches in the *recA* and *rpoB* gene families, two marker genes widely used for phylogeny reconstructions, and concluded that some may correspond to uncharacterized viruses. Alternatively, they may represent assembly artifacts, recombinants, or paralogs, but these hypotheses are considered unlikely. Importantly, Boyer et al.¹⁴ provided an additional argument in favor of the existence of a fourth domain of life composed of the NCLDVs. Indeed, in addition to phylogenetic reconstructions based on putative orthologous sets of some of the informational genes, they performed hierarchical clustering that relied on the presence/absence patterns in each of the genomes of NCLDVs, *Eukarya*, *Archaea*, and *Bacteria* of the genes assigned to COGs involved in information storage and processing. The topology of the dendrogram tree for this gene content conspicuously showed that a clade composed of the NCLDVs was distinct from three other clades corresponding to *Eukarya*, *Bacteria*, and *Archaea*, the organization of these three latter clades being

congruent with that of the ribosomal phylogenetic trees. Taken together, the aforementioned data support the hypothesis that the mimiviruses, the marseilleviruses, and other NCLDV share a common origin and emerged as deep roots from the rhizome of life, carrying a core genome as ancient as those of *Eukarya*, *Bacteria*, and *Archaea*.^{14,111}

CONCLUSION

Mimivirus and other giant viruses recovered from phagocytic protists considerably broadened the diversity within the viral world and changed our vision of viruses. Future studies should be valuable to gain increased understanding of the epidemiology of these giant viruses in the environment and in clinics, as well as to more accurately define their history and role in the evolution of life.

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Plant Viruses

History and Importance of Plant Virology
Bromoviridae Family and *Tobamovirus* and *Tymovirus* Genera
***Tombusviridae*, *Luteoviridae*, *Sobemovirus*, and *Umbravirus* Genera**
Potyviridae
Cell-to-Cell Movement of Plant Viruses
Vector Transmission of Plant Viruses
Viroids and Satellite RNAs
Host Defenses and Viral Countermeasures
Future Perspectives
Epilogue

HISTORY AND IMPORTANCE OF PLANT VIROLOGY

There are far more plant viruses known than all vertebrate viruses combined.¹⁶⁷ This is not surprising given the vastly greater number of plant species than vertebrate species. Instead of attempting to cover all plant viruses in the limited space of this chapter, we provide examples of special importance to the whole field of virology, highlight the relevance to biomedical virology, and discuss features that are unique to plant viruses. For complete descriptions of all plant viruses we refer the reader to the International Committee on Taxonomy Viruses report on virus taxonomy¹⁶⁷ or the ICTV web page (www.ictvonline.org). For a comprehensive textbook on the entire field of plant virology we recommend *Matthews' Plant Virology*.¹⁴¹

History of Plant Viruses as Models for Understanding All Viruses

In the history of virology, investigations of plant viruses have a long and distinguished legacy in telling us what viruses are and how they work (Table 72.1). Many fundamental discoveries on the nature of viruses were made first in studies of Tobacco mosaic virus (TMV).²⁹² The first descriptions of a virus as an infectious agent ("*contagium vivum fluidum*") capable of passing through a porcelain filter that blocked all bacteria, were of TMV^{21,144} (Fig. 72.1). Although some virologists credit Dmitri Ivanovsky with the first discovery of TMV as a filterable agent in 1892,^{200,310} he did not specify or claim that the agent was distinct from other infectious agents such as bacteria. Therefore, many virologists credit Martinus Beijerinck, who performed a series of conclusive experiments in 1898 showing transmission

properties of the filterable agent that caused tobacco mosaic disease,²¹ as the first to discover and conclude that a virus is a new type of infectious agent.^{31,372} This discovery barely preceded the description of foot and mouth disease virus, which was also described in 1898.¹⁹³

The first discoveries of the chemical nature of viruses also resulted from studies of TMV. In 1935, W.M. Stanley found that TMV was (mostly) crystalline protein.³⁰⁹ Initially he detected no carbohydrate or phosphorus and ruled out a nucleic acid component. (TMV particles are 95% protein by mass.) In 1936, F.C. Bawden, N.W. Pirie, J.D. Bernal, and I. Fankuchen detected the carbohydrate and phosphorus and considered the possibility of RNA as a key component of the virion.²⁰ They correctly determined that the agent consisted of long, narrow particles or fibers owing to physical properties of the virus in solution. One demonstration of the anisotropy of the TMV solution was birefringence visible upon gentle agitation by a goldfish swimming in a dilute solution of sap from infected plants (Fig. 72.2A).²⁰ Stanley was awarded the Nobel Prize in Chemistry in 1946 for "purification of enzymes and virus proteins in a pure form." Subsequently, TMV was the first virus on which x-ray crystallography²³ and electron microscopy¹⁶¹ were performed, revealing the infectious agent to consist of surprisingly discrete, symmetrical, highly structured, rod-shaped particles (Fig. 72.2B) (reviewed in [292]). In 1955, Heinz Frankel-Conrat showed, astonishingly at the time, that merely by mixing TMV coat protein subunits and RNA, an intact virion could self-assemble⁹⁷ (Fig. 72.3A). More recently, the first transgenic resistance to a eukaryotic virus was pathogen-derived resistance to TMV, in which expression of the coat protein dramatically delayed and reduced infection by TMV.²⁴⁷

Other plant viruses also played leading roles in virology. In the late 1970s, the first atomic resolution crystal structures of icosahedral virions were solved for Tomato bushy stunt virus (TBSV) and Southern bean mosaic virus (SBMV)^{1,132} (Fig. 72.3B). This proved to be broadly relevant, as the same basic structural rules apply to icosahedral viruses of plants and animals.¹³¹ The first template-specific cell-free replication extracts for positive strand eukaryotic RNA viruses were obtained from Brome mosaic virus (BMV)-infected plants,¹³⁰ enabling dissection of the structure of the origin of replication of a positive-strand RNA virus.⁸¹ In addition, infectious transcripts from a cloned viral genome of a eukaryotic positive-sense RNA virus were obtained for BMV⁴ before any animal virus. Small self-cleaving ribozymes were first discovered in plant viral satellite RNAs,^{95,96,249} paving the way for investigation of hepatitis delta virus RNA, which replicates as a rolling circle with self-cleaving ribozymes like plant satellite RNAs (See Chapter 69), and for construction of site-specific messenger

TABLE 72.1 Major Discoveries Made in Plant Virology Before Similar Discoveries or Methods Were Made in, or Applied to, Mammalian Virology or Biomedicine^a

First discovery	Virus	Year	Reference
Virus is filterable agent	TMV	1892	144
Virus is novel filterable agent and not bacteria	TMV	1898	21
Chemical nature of a virus: crystalline “protein”	TMV	1935	309
Virus is protein with small amount of carbohydrate and phosphorus	TMV	1936	20
Electron microscopy of virus particles	TMV	1939	161
X-ray crystallography of virus particles	TMV, TBSV	1941	23
Self-assembly of virion	TMV	1955	97
Naked viral RNA can be infectious	TMV	1956	114
Natural, infectious, circular subviral RNA (viroid)	PSTVd	1971	73
Complete genome sequence of a eukaryotic pathogen	PSTVd	1978	123
Atomic resolution structure of an icosahedral virus	TBSV	1978	132
Infectious transcripts from a cloned genome of a eukaryotic RNA virus	BMV	1984	4
Mechanism of subgenomic mRNA synthesis by a positive strand RNA virus	BMV	1985	212
Genetically engineered resistance to a pathogen	TMV	1986	247
Gene-targeted ribozymes	STobRV	1988	134
Transgenic RNAi-mediated virus resistance.	TEV	1993	187
Ribosome shunting	CaMV	1993	100
Natural RNA-mediated virus resistance	TBRV	1997	267
Viral suppressor of RNAi-mediated resistance.	TEV	1998	7,159
Small RNAs mediate RNAi	PVX	1999	129
Atomic resolution structure of a silencing suppressor	TBSV	2003	369

^aTMV, Tobacco mosaic virus; TBSV, Tomato bushy stunt virus; PSTVd, Potato spindle tuber viroid; BMV, Brome mosaic virus; STobRV, Satellite tobacco ringspot virus; TEV, Tobacco etch virus; CaMV, Cauliflower mosaic virus; TBRV, Tomato black ring virus; PVX, Potato virus X.

AMSTERDAM.

Royal Academy of Sciences, November 26.—Prof. Van de Sande Bakhuyzen in the chair.—Prof. Beijerinck, on a contagium vivum fluidum, causing the spot-disease of tobacco leaves. This disease, also known as the mosaic disease of tobacco leaves, may be inoculated into healthy plants by injecting into the stem, near a bud, sap pressed from infected plants. The active virus passes completely through the pores of very dense porcelain, and can even penetrate into agar by diffusion; therefore it cannot be a contagium fixum in the usual sense, but it must be fluid. Out of the tobacco plant it cannot be made to multiply; but in the dividing tissues of the leaf-rudiments and the meristems of the buds it multiplies freely and over a great extent. A very small drop of the porcelain filtrate can render all the leaves of the infected plant entirely covered with spots, and the sap of these leaves would be sufficient for the contagion of an unlimited number of healthy plants. The virus is destroyed by boiling at so low a degree as 90° C.—Prof.

FIGURE 72.1. First report of a “contagium fluidum vivum” as a virus, that is, an infectious agent that could pass through a porcelain filter that removed all bacteria. This report on the Netherlands Academy of Sciences meeting appeared in *Nature* in 1898.¹¹ (Reprinted with permission from Macmillan Publishers, Ltd: *Nature* 1898;59:216).

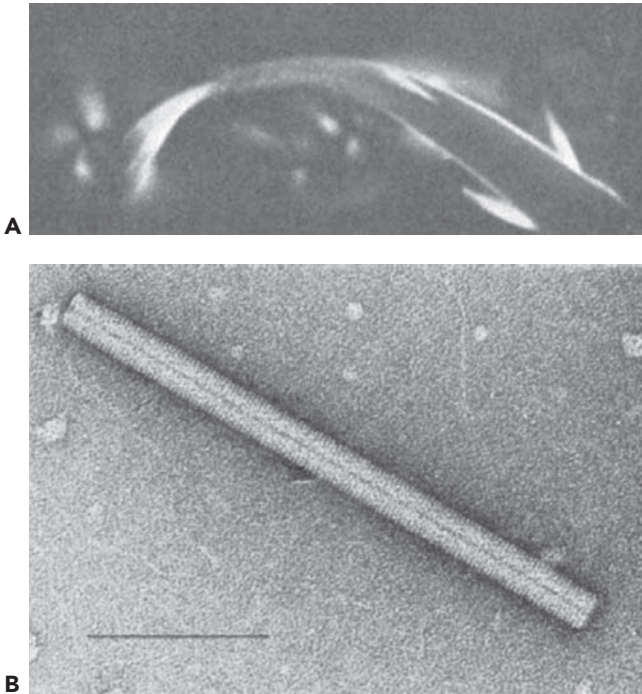


FIGURE 72.2. Characterization of TMV particles. **A:** Shimmering currents generated by a goldfish swimming in a dilute solution of sap from infected plants, reveal birefringence indicating anisotropy of the particles. (Reprinted with permission from Macmillan Publishers, Ltd: Bawden et al. *Nature* 1936;138:1051–1055.²⁰ **B:** Electron micrograph of tobacco mosaic virus (TMV) virion. (**B** reprinted with permission of University of North Carolina.)

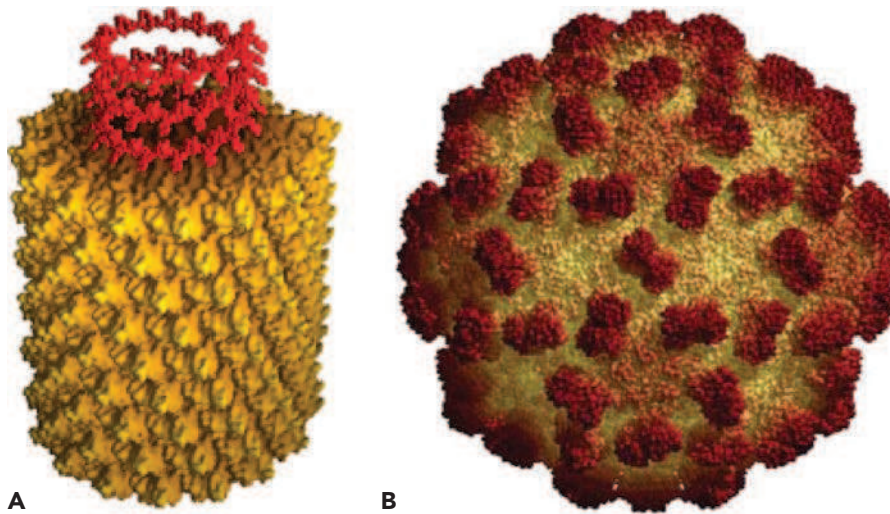


FIGURE 72.3. High-resolution images of plant virus particles. **A:** End of partially assembled tobacco mosaic virus (TMV) virion, showing helical arrangement of coat protein (CP) subunits (gold) and RNA (red) protruding from the interior. Diameter = 18 nm, CP subunits/turn = 16.3 axial rise/residue = 0.14 nm, pitch = 2.3 nm. PDB ID: 1vtm. **B:** Tomato bushy stunt virus, T=3 virion, diameter = 30 nm. PDB ID: 2tbv. (Courtesy of Jean Yves-Sgro, Institute for Molecular Virology, University of Wisconsin.)

RNA (mRNA)-cleaving ribozymes,¹³⁴ useful tools for knocking out specific genes.

Studying virus replication in yeast to identify the roles of nearly all host proteins in a high throughput manner has been used to great advantage for the study of plant viruses.^{171,237} In the late 1990s and early 2000s, the existence of RNA-mediated silencing⁸⁷ in innate immunity and the roles of small RNAs in the RNA interference (RNAi) process,¹²⁹ as well as the occurrence of numerous viral suppressors of RNAi (VSRs),^{159,344} were pioneered in studies of plant viruses. Because discoveries in plant virus molecular mechanisms often precede those in animal and human viruses, it is the astute virologist who pays at least passing attention to the plant virus literature.

A reason for the above pioneering discoveries, despite the relatively few resources devoted to plant virus research relative to human virus research, may be that plant viruses are usually easier to work with than vertebrate viruses. They often accumulate to levels in the host that are orders of magnitude greater than those of animal viruses. Many plant viruses are very easy to transmit: simply grinding leaves and rubbing the sap onto other plants usually suffices for efficient inoculation. Local lesion assays on leaves of certain hosts serve as the equivalent of plaque assays in mammalian cell culture. Plants are much less expensive to grow than animals, and regulatory hurdles are fewer because no plant viruses infect mammals. Therefore, in addition to plant pathologists seeking to control disease, researchers who simply want to understand what a virus is and how it works, have been drawn to plant virology.

Economic Importance of Plant Viruses

In addition to the value of plant viruses as model systems, of course many plant viruses have a significant effect on human welfare by reducing crop yield. Recently a list of the “top ten” plant viruses that are either models (but not necessarily major pathogens) or are pathogens of economic impact was compiled.²⁹¹ In Table 72.2, we present a longer list of viruses of significant economic impact. However, it too is incomplete, as many viruses have high impact only in localized areas or occur only sporadically, but cause severe devastation when they strike.

A widespread virus of major economic impact is Tomato spotted wilt virus (TSWV). It infects more than 1,200 plant species and has caused major epidemics in all continents in tomato, pepper, tobacco, peanut, lettuce, potato, and many ornamental species.²³⁸ (Plant viruses are named for the first plant species in which they were discovered, and for the symptoms induced in that particular host–virus interaction. Most plant viruses infect many host species, and symptoms can vary depending on the strain of virus, genotype of host plant, or environment in which the plant is grown.) Cucumber mosaic virus (CMV) infects more than 1,000 plant species.²²⁶ In the presence of a satellite RNA, CMV devastated tomato crops in Europe.¹⁵⁷ Barley yellow dwarf virus and its cousin Cereal yellow dwarf virus are the most economically important viruses of wheat, barley, and oats, especially in Asia, North Africa, South America, and Australia.¹⁸⁸ Potato virus Y (PVY), the type member of the largest plant virus family, the *Potyviridae*, is a major problem in potato. Recently the N strain of PVY has been of particular concern in North America. Citrus tristeza virus led to the loss of more than 100 million trees on the widely used sour orange rootstock worldwide.²²⁵ Loss of sour orange rootstock was a major problem owing to its desirable agronomic qualities. Geminiviruses, which have twin icosahedral particles, unlike any animal virus, devastate dozens of crops and fruits in the tropics. In India viruses in the *Begomovirus* genus of the *Geminiviridae* threaten the livelihoods of small farmers. African cassava mosaic virus, another begomovirus; Groundnut rosette disease, resulting from co-infection of Groundnut rosette virus and Groundnut rosette assistant virus, and a satellite RNA³¹⁸; Rice yellow mottle virus¹⁶⁹; and Sweet potato feathery mottle virus all have caused crop failure and contributed directly to hunger and poverty in subsistence farms in Africa. Then there are viroids, small circular noncoding RNAs unlike any infectious agent known outside of the plant kingdom. The cadang-cadang viroid destroyed more than 30 million coconut palm trees in the Philippines in the 1980s.²⁶⁴ This is just a smattering of examples of economic impact of plant viruses. Economic importance of other viruses is discussed throughout this chapter. Finally, it is important to bear in mind that many viral infections go undiagnosed, dragging down yield without causing obvious symptoms.

TABLE 72.2 Some Economically Important Plant Viruses and Viroids^a

Virus	Economic hosts	Geographic location
African cassava mosaic virus	Cassava	Sub-Saharan Africa
Barley yellow dwarf and cereal yellow dwarf viruses	Wheat, barley, oat, rye, maize, rice	Worldwide
Beet necrotic yellow vein virus	Sugar beet	Europe, North America
Cacao swollen shoot virus	Cacao	West Africa
Cadang-cadang viroid	Coconut palm	Philippines
Citrus tristeza virus	Citrus, esp. sour orange rootstock	Americas, Mediterranean, Australia
Cotton leaf curl virus	Cotton, tomato, chili, beans, sunflower, sesame, soybean, cowpeas, eggplant, sun kukra, and citrus species	Africa, Nigeria, Sudan, Tanzania, Philippines and Pakistan.
Cucumber mosaic virus	>1000 host species, especially, tomato, banana, pepper, cucurbits, legumes	Worldwide
East African cassava mosaic virus	Cassava	Sub-Saharan Africa
Groundnut rosette + Ground nut rosette assistor virus + satellite RNA co-infection	Groundnut (peanut)	Sub-Saharan Africa
Lettuce mosaic virus	Lettuce, other Asteraceae	Worldwide
Papaya ringspot virus	Papaya	Hawaii, SE Asia
Pepino mosaic virus	Tomato	Worldwide
Plum pox virus	Stone fruits	Worldwide
Potato leafroll virus	Potato, tobacco, tomato	Worldwide
Potato spindle tuber viroid	Potato	Worldwide
Potato virus Y	Potato, tomato, pepper, tobacco	Worldwide
Prunus necrotic ring spot virus	Stone fruits	Worldwide
Rice yellow mottle virus	Rice	Sub-Saharan Africa
Rice tungro bacilliform + rice tungro spherical virus co-infection	Rice	Asia
Soybean mosaic potyvirus	Soybean	Worldwide
Sweet potato feathery mottle virus + sweet potato chlorotic stunt virus co-infection	Sweet potato	East Africa, Peru, Israel, USA
Tobacco mosaic virus	Tobacco, greenhouse tomatoes and hundreds of other vegetables	Worldwide
Tomato spotted wilt virus	>1000 host species, especially tomato, peanut, pepper, pineapple and tobacco	Worldwide in temperate regions
Turnip mosaic virus	Oilseed rape, other brassicas, lettuce, pea	Worldwide
Zucchini yellow mosaic virus	Squashes	Worldwide

^aSee also ref. 291.

Beneficial Applications of Plant Viruses

Plant viruses, or sequences from them, have been used, and are being modified and exploited for beneficial purposes. Some viruses confer desirable ornamental symptoms. In 17th century Holland, the delicate striping in flowers caused by Tulip breaking virus led to huge demand for virus-infected tulips in a social phenomenon deemed “tulipmania” (Fig. 72.4). Vast sums greater than the annual salary of most wealthy merchants were promised for individual virus-infected tulip bulbs, until the tulip economic “bubble” burst, leading to a historic price collapse in 1636.¹¹⁸

In modern times, plant viruses are used as expression vectors to produce massive amounts of protein in plant cells, often for human or veterinary pharmaceutical purposes.²⁰³ Gene expression control sequences, such as the 35S RNA promoter from Cauliflower mosaic virus,^{162,233} and the omega translation enhancer sequence from TMV,¹⁰⁵ are used widely in genetically engineered plants. In the last decade, the development of many plant viruses as virus-induced gene silencing (VIGS) vectors

has revolutionized plant genetics by providing a quantum leap in the speed with which geneticists can knock down expression of, and thus determine function of, any plant gene of interest, including essential genes that would be lethal if knocked out by other means.^{18,45,79,375} Plant viral suppressors of silencing are used to boost expression of transgenes in plants.³⁴⁵ Plant virus particles engineered to display epitopes from animal or human pathogens show great potential for improved, inexpensive vaccines.^{222,311} Plant virus particles also are being modified and exploited to function as drug delivery vehicles,¹⁷⁰ tiny vessels for nanomedicine,²⁰⁴ and as components of rechargeable batteries.⁵³

How Plant Viruses Differ from Animal Viruses

Obviously plant viruses undergo the same basic processes of entry, disassembly, gene expression, replication, and encapsidation as animal viruses. However, the nature of the host has led to some important differences. Unlike animal cells, each plant cell is surrounded by a dense cell wall consisting of



FIGURE 72.4. Tulips infected with Tulip breaking virus, a desirable infection in 17th century Holland. (Copyright Fitzwilliam Museum, University of Cambridge.)

cellulose, pectins, lignins, and other material impenetrable to a virion. However, plant cells are connected by small openings called plasmodesmata through which small molecules, proteins, and sometimes RNAs pass.^{198,199} Plasmodesmata are roughly analogous to gap junctions in vertebrate cells, but are perhaps more similar to recently discovered tunneling nanotubes in animal cells.¹⁰³ To infect the whole plant, plant viruses encode one or more movement proteins (MPs) that facilitate cell-to-cell movement of viral RNA or whole virions through the plasmodesmata. Plants lack an open vascular system. Sugars, hormones, and certain proteins and RNAs are transported through the phloem. Unlike the open tubes formed by blood vessels, the phloem transport system consists of cells connected via sieve elements. For viruses to travel “long distance” (from leaf to leaf) they have adapted ways to move from one phloem cell to the next through the sieve elements.⁸⁹

Plants have a much more developed RNA-based innate immune system than mammals, which rely on other innate defenses, such as protein kinase RNA-activated (PKR) or retinoic acid-inducible protein I (RIG-I) that are absent or reduced in plants. Hence all plant viruses appear to encode viral suppressors of RNAi (VSRs), proteins that inhibit the RNA-based gene silencing system in plants⁷¹ (see section Host Defenses

and Viral Countermeasures). Plants are sessile, so they rely more on insect vectors for plant-to-plant transmission. Therefore, most plant viruses are “arboviruses” (see section Vector Transmission of Plant Viruses).

Plant viruses are smaller and simpler than most animal viruses (Fig. 72.5). The largest plant virus genome is about 20 kb, but most plant virus genomes are 10 kb or smaller. The small size may be required for cell-to-cell movement through the plasmodesmata. Many plant viruses form flexuous rod-shaped virions that are rare among animal viruses, or rigid rods, such as TMV, a morphology not known among animal viruses. There are no plant viruses with large DNA genomes (>10 kb) or with linear DNA genomes. Plant retroviruses are not known to exist, but plant pararetroviruses and plant retrotransposons are well characterized. No plant viruses are known to integrate their genome into the host genome as part of the replication cycle, but an infectious copy of banana streak pararetrovirus has integrated into the banana genome.¹¹⁰ Although normally silenced, the expression of the integrated viral genome can be activated by vegetative propagation methods, creating infections that have become a major problem in banana breeding programs.⁶³

Many plant viruses have multipartite, positive-sense RNA genomes, which are absent among mammalian viruses. Interestingly, many insect viruses resemble plant viruses in this way. A few plant viruses may have escaped from (or co-evolved with) the mammalian virus world, as some are in the same family or order as mammalian viruses. These include members of the *Bunyaviridae* (e.g., TSWV), *Rhabdoviridae* (e.g., Lettuce infectious yellows virus), *Reoviridae* (e.g., Fiji disease virus of sugarcane), and *Picornavirales* (e.g., Cowpea mosaic virus).¹⁷⁶ They represent a minority of all plant viruses, and the plant rhabdoviruses and bunyaviruses are the only enveloped plant viruses. Because of the similarities of these viruses to animal viruses that are discussed elsewhere in this volume, this chapter focuses primarily on the plant viruses that do not fall into the same families as animal viruses.

BROMOVIRIDAE FAMILY AND TOBAMOVIRUS AND TYMOVIRUS GENERA

The *Bromoviridae* family includes one of the best-characterized RNA viruses of eukaryotes, BMV (genus *Bromovirus*), as well as some of the most widespread and economically important plant viruses, such as Cucumber mosaic virus (CMV, genus *Cucumovirus*) and Alfalfa mosaic virus (AIMV, genus *Ilarvirus*). TMV, for which genus *Tobamovirus* is named, is an extremely efficiently transmitted pathogen of tobacco and tomato, and thus is often a problem, especially in greenhouses.

Unlike positive-sense RNA viruses of vertebrates, viruses in the *Bromoviridae* and many other positive-sense plant viruses have multipartite genomes (Fig. 72.6). The genome of *Bromoviridae* consists of three RNAs, each of which is packaged in a separate icosahedral virion. A fourth, subgenomic RNA (sgRNA), representing the 3' end of RNA 3, is co-encapsidated with RNA 3. An exception in particle structure is genus *Ilarvirus*, in which the virion is bacilliform and the length is proportional to the length of the encapsidated RNA.²⁷ With the possible exception of ilarviruses (including AIMV) all of the RNAs of the *Bromoviridae* feature a transfer RNA (tRNA)-like structure (TLS) at the 3' end. These are discussed in detail later in this section.

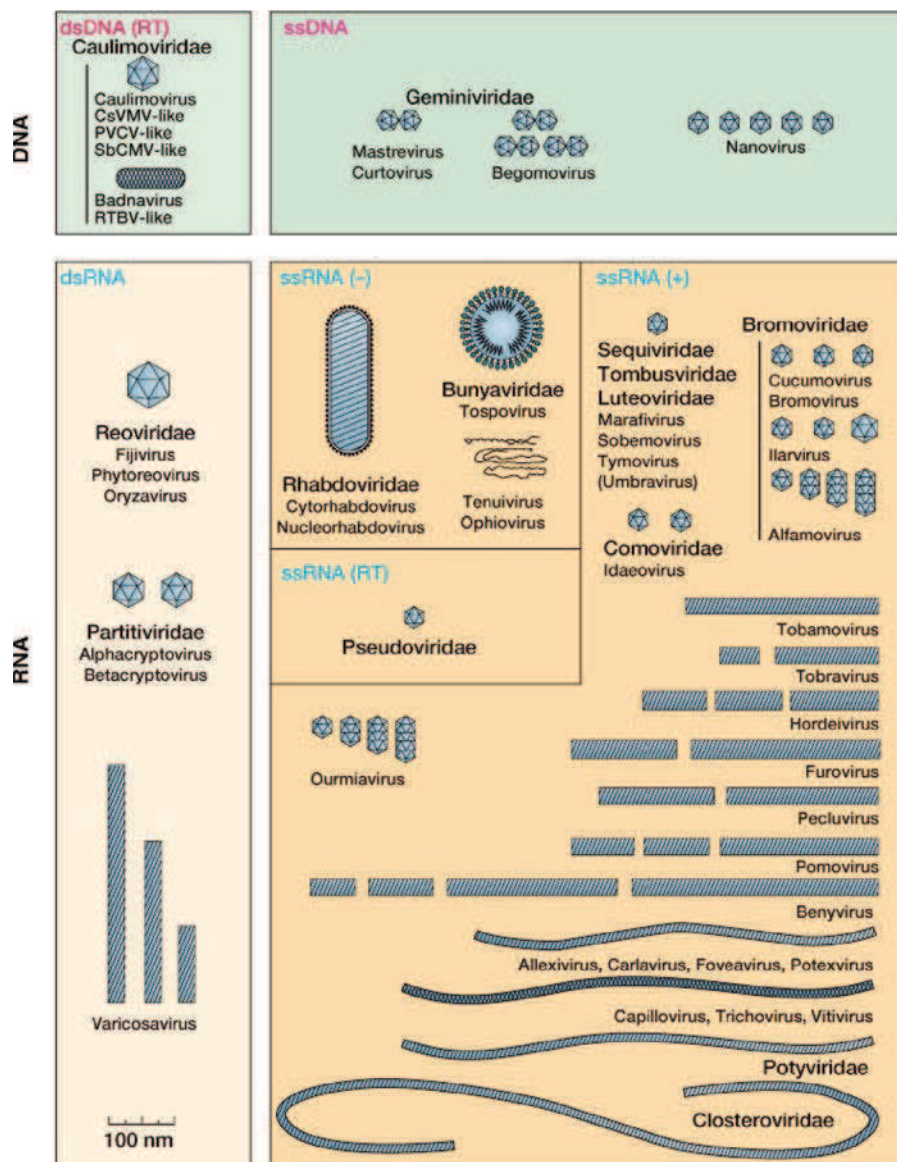


FIGURE 72.5. Plant viruses arranged by virion structure. (From King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. Philadelphia: Elsevier; 2012.)

In the *Bromoviridae*, RNAs 1 and 2 are each about 3-kb long.²⁷⁸ RNA 1 encodes an open reading frame (ORF) that includes a methyl transferase domain for capping viral RNAs and an RNA helicase domain. The virus encodes its own capping enzyme because the entire replication cycle takes place outside of the nucleus where capping of host mRNA occurs. The ORF that occupies most of RNA 2 encodes the viral RNA-dependent RNA polymerase (RdRp). A small ORF (2b) that overlaps with the 3' end of ORF 2 in the cucumo and ilarviruses encodes a viral suppressor of RNA-mediated silencing (VSR), a key innate immune response in plants³⁷ (discussed in the Host Responses section of this chapter).

The coding region of the ~1.7-kb RNA 3 is split into two nonoverlapping ORFs (Fig. 72.6). ORF 3 in the 5' end encodes the cell-to-cell movement protein. The 3' end of RNA 3 encodes the coat protein (CP) in ORF 4. ORF 4 is translated from RNA 4, an sgRNA derived from the 3' end of RNA 3. It is 3'-co-terminal with RNA 3, but is truncated so that the 5' end of

RNA 4 is just upstream of the CP ORF start codon. This permits highly efficient translation of CP from monocistronic RNA 4 by conventional ribosomal scanning from the 5' end of the RNA.

Genus *Tobamovirus* is in the *Virgaviridae* family, which is similar to the *Bromoviridae* in (a) the sequences of the replication genes,¹³⁵ (b) expression mechanisms of 3'-proximal ORFs via 3'-co-terminal sgRNAs, and (c) presence of a tRNA-like structure at the 3' end of the genome (Fig. 72.7). Tobamoviruses differ from *Bromoviridae* in that (a) they have a monopartite genome, (b) the polymerase ORF is expressed via translational readthrough of the stop codon of the upstream ORF that encodes the methyl transferase and helicase functions, (c) the movement protein is translated from a subgenomic mRNA, (d) sgRNAs are not encapsidated, and (e) the virions are helical rods. Genomes of viruses in genus *Alphavirus* of the *Togaviridae* resemble tobamoviruses as well.⁵ The replication genes have significant sequence homology, the RdRp is expressed via in-frame readthrough, and the structural proteins are translated via 3'-co-terminal subgenomic

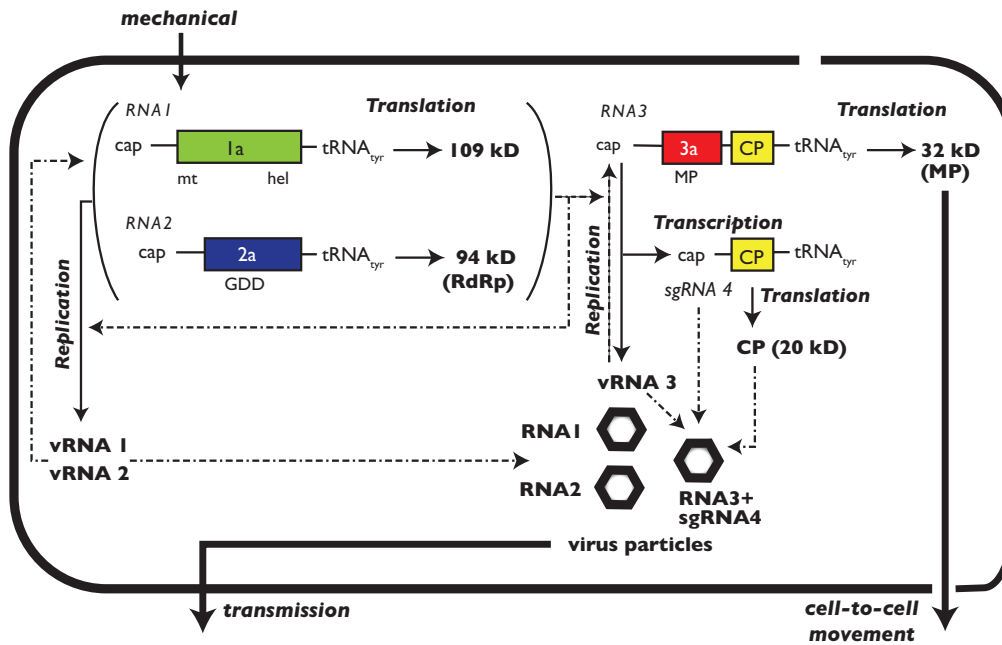


FIGURE 72.6. Replication strategy of Brome mosaic virus. (Modified from: Lazarowitz S. Plant Viruses. In: Knipe DM, Howley PM, eds. *Fields Virology* 5th ed. 2000).

RNA (Fig. 72.8). However, alphaviral RNAs lack 3' TLSs, and the genomes and virions are more complex than those of plant viruses.

Tymoviruses (*Tymoviridae*) are not closely related to the tobamo and bromoviruses, but they share the presence of a TLS

at the 3' end. The movement protein is encoded in an ORF that overlaps a long section of the RdRp ORF at the 5' end of the genome (Fig. 72.9). Like the bromoviruses and tobamoviruses, and many other plant viruses, the CP is translated from a 3'-co-terminal sgRNA.

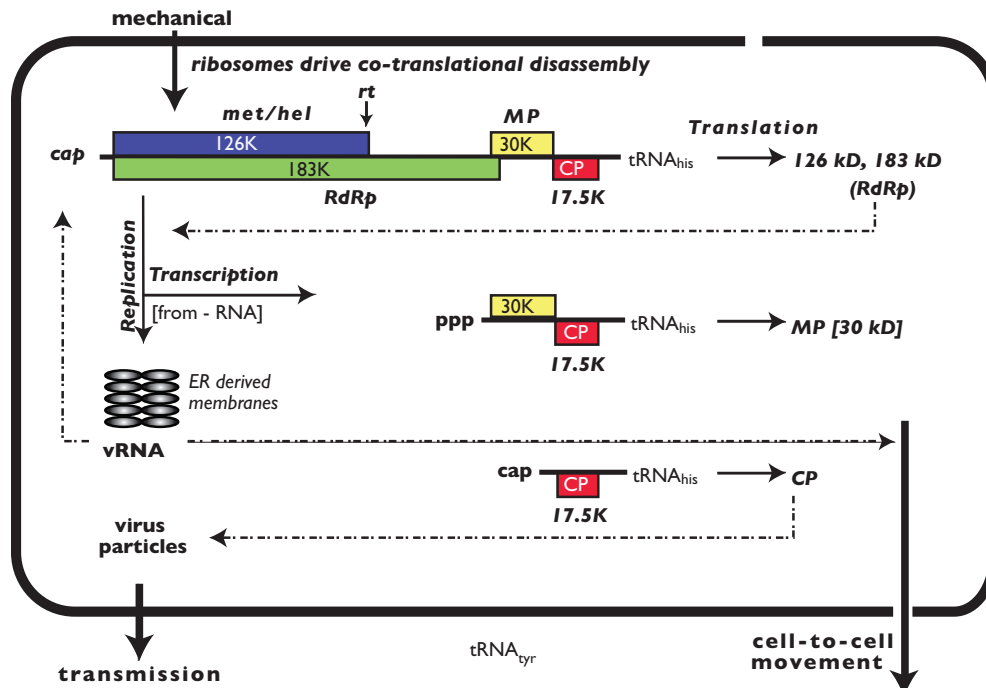


FIGURE 72.7. Replication strategy of Tobacco mosaic virus. (Modified from: Lazarowitz S. Plant Viruses. In: Knipe DM, Howley PM, eds. *Fields Virology* 5th ed. 2000).

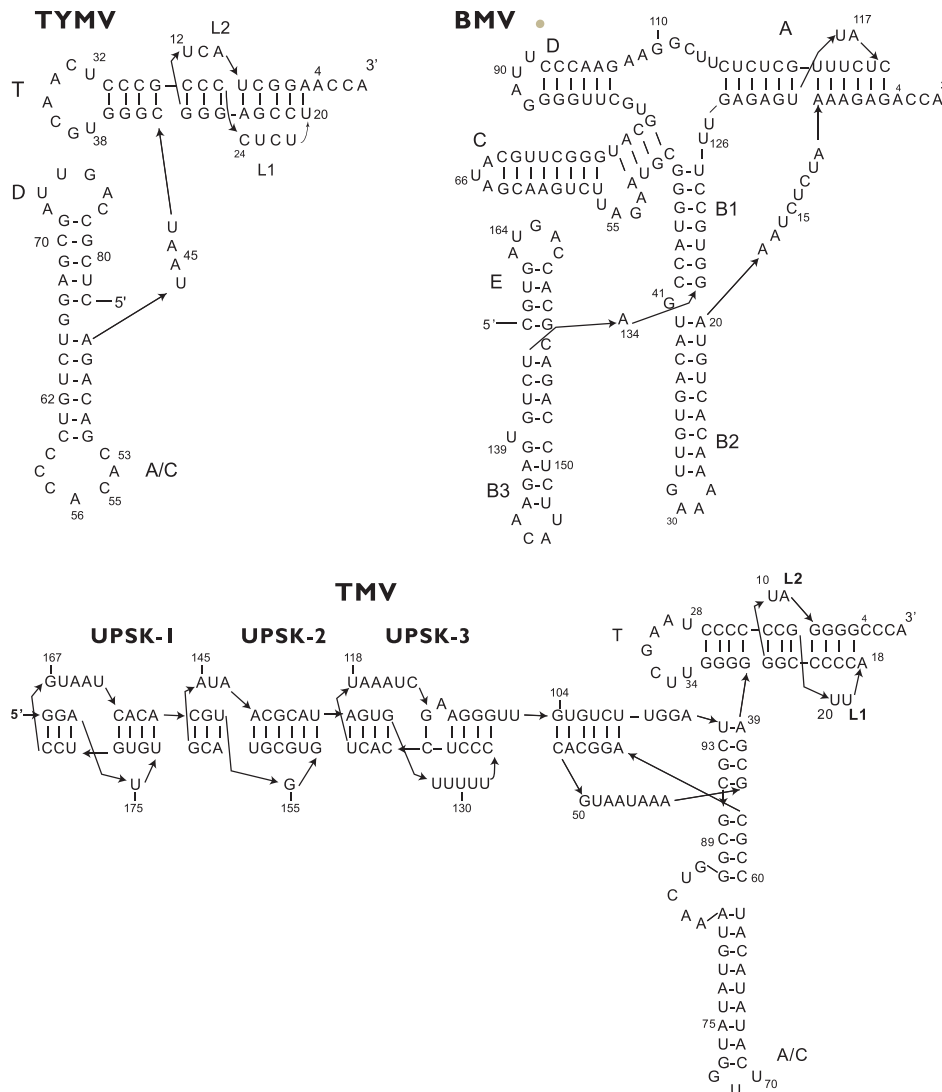


FIGURE 72.10. Transfer RNA (tRNA)-like structures.⁸⁰ Numbering is from the 3' end of the genome. Arrows indicate phosphodiester bonds distorted to allow depiction of structure in two dimensions. UPSK, upstream pseudoknot.

that could not support viral RNA synthesis but which were otherwise normal.¹⁴³ This revealed many new and sometimes surprising host factors required for RNA virus replication.

Host proteins involved in the BMV replicase include a subunit of translation initiation factor eIF3,²⁵⁵ and the Lsm1-7 complex.⁷⁴ The Lsm1-7 complex normally regulates mRNA decay. In BMV RNAs it binds the TLS and A-rich internal sequences and regulates both replication and translation, leading to the proposal that it regulates the switch between translation and replication,¹⁰² processes that are incompatible on the same RNA template.¹⁰⁷ This is also facilitated by the BMV 1a protein that binds the replication element (RE) stem-loop in each viral RNA, bringing the RNA into replication vesicles.²³²

Using electron microscopy and tomography, Ahlquist and colleagues showed that BMV replicates on perinuclear endoplasmic reticulum (ER) membranes.²⁷¹ Invaginations form vesicles that serve as replication factories (Fig. 72.11). Each vesicle is formed by, and lined with, hundreds of copies of the multifunctional 1a protein, and contains 10 to 20 copies of the RdRp (2a protein) and just a few copies of positive- and negative-strand RNA.²⁹⁴ The hypothesized mechanism is that the RdRp copies

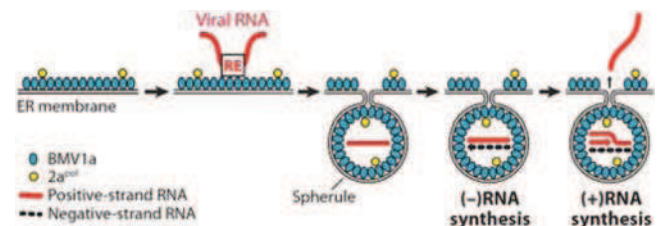


FIGURE 72.11. Model for assembly and function of the Brome mosaic virus (BMV) RNA replication complex. BMV1a accumulates on perinuclear endoplasmic reticulum (ER) membranes, recruits 2a^{pol}, binds the replication element (RE) in genomic RNA (red), and incorporates both into the interior of a spherule. This serves as an isolated compartment free of ribosomes or RNA-based antiviral systems. Newly synthesized RNAs are ejected through the neck-like opening in the ER. (Reprinted with permission from den Boon JA, Ahlquist P. Organelle-like membrane compartmentalization of positive-strand RNA virus replication factories. *Ann Rev Microbiology*. 2010;64:241–256.)

viral RNA, ejecting the positive strand out through the neck of the vesicle as the RNA is synthesized. The vesicle keeps the RNA (in particular the double-stranded replicative intermediate) sequestered from antiviral defenses, such as virus-induced RNA silencing induced by double-stranded RNA (dsRNA), and also from the ribosomes, which would block replication by translating the positive strand. The positive strands ejected from the vesicles would be available for (a) translation of more viral proteins, (b) serving as template for additional replication in another vesicle, (c) transport to neighboring cells, or (d) encapsidation. This general strategy of RNA synthesis in a membranous vesicle lined with a viral RNA binding, helicase-like protein, and containing low numbers of RdRp and template RNA may apply to all viruses in the alphavirus-like superfamily of viruses.^{3,70} This underscores the value of a plant virus as a model for understanding mechanisms of human virus replication.

tRNA-like Structures

TLSs are widespread among plant viruses, but apparently absent in animal viruses. In addition to the *Bromoviridae*, plant viruses in the *Tobamovirus* (e.g., TMV), *Tymovirus* (e.g., Turnip yellow mosaic virus, TYMV), and four other genera also have a TLS at the 3' end of the genome; see reference 80 for an excellent review. All TLSs differ from tRNA by having a pseudoknotted acceptor stem (see Fig. 72.10) instead of a simple helix. The TYMV TLS is the simplest and most tRNA-like of the TLSs, whereas the BMV TLS is the most complex, containing extra stem-loops unlike anything in tRNA. The TLS plays a plethora of roles in virus infection, which vary between viruses. Three biochemical properties that TLSs share with tRNAs are: (a) they can be aminoacylated with a specific amino acid, (b) the aminoacylated form binds translation elongation factor eEF1A•GTP complex, and (c) they serve as a substrate for CTP:ATP:tRNA nucleotidyltransferase (CCA-adding enzyme), which adds the 3'-terminal CCA sequence to tRNAs.⁸⁰

The role of aminoacylation is not to donate the amino acid to a growing polypeptide as in normal translation. For TYMV, it was reported, using only cell-free extracts, that the valine with which the TLS is acylated is donated as the first amino acid in a novel translation initiation mechanism.¹⁶ However, this was shown not to be the case *in vitro* or in plant cells for TYMV²⁰⁵ or for other TLS-containing viruses.^{17,280} Aminoacylation does enhance translation.^{104,206} It may functionally replace the role of the poly(A) tail in stimulating translation initiation. In the case of TMV, a pseudoknot repeat domain upstream of the TLS (see Fig. 72.10) plays this role,¹⁰⁶ and aminoacylation is not required.

Owing to its presence at the 3' end, the TLS is also the origin of minus-strand synthesis.⁵⁰ RNA synthesis begins by incorporation of a guanosine monophosphate complementary to the penultimate base of the genome, that is, the middle base of the CCA at the 3' end.^{211,304} For BMV and TYMV, it has been shown that the sequences and structures within the TLS recognized and required by the replicase differ from those required for aminoacylation (and hence eEF1A binding).^{42,67,83} Binding of eEF1A to the aminoacylated RNA inhibits minus-strand synthesis, possibly by blocking the replicase from interacting with the CCA terminus. This may shut off minus-strand synthesis to favor plus-strand synthesis, which initiates at the 3' end of the minus strand that has no TLS. This would favor generation of the high ratio of (+) to (−) strand RNA in these positive-strand RNA viruses. It may also allow the viral RNA to be translated

but not replicated, two functions that are presumed to be incompatible on the same RNA molecule. However, a mechanism must also exist to allow replicase to outcompete eEF1A and the tRNA synthetase for access to the 3' end.

One tRNA mimicry function that has an obvious role in RNA replication is substrate for the CCA-adding enzyme. Because minus-strand synthesis initiates at the penultimate base, the terminal A must be added in a nontemplated fashion. The CCA-adding enzyme provides this function because it requires no template to add the CCA to the 3' end of tRNA. Mutations that prevent CCA addition block replication, demonstrating that the CCA-adding enzyme serves as a telomerase and the CCA is the telomere.²⁶⁵ This function extends to the tobamoviruses, which lack a TLS but have a tRNA-acceptor arm mimic, which allows it to be a substrate for CCA addition.³³²

Finally, the TLS is required for encapsidation of BMV RNAs,⁵⁶ but not all other TLS-containing viruses.^{9,54} The role of the TLS in packaging viral genomes can be difficult to discern due to the co-replicative nature of RNA packaging.^{10,54} In summary, TLSs play multiple roles for each virus, and the roles and requirements often differ between viruses.

TOMBUSVIRIDAE, LUTEOVIRIDAE, SOBEMOVIRUS, AND UMBRAVIRUS GENERA

The *Tombusviridae* family encompasses many viruses in nine genera. These viruses are grouped into the same family by having (a) related RdRp's; (b) an icosahedral, T = 3 virion; (c) an uncapped, unmodified, nonpolyadenylated positive-sense genomic RNA; and (d) a 4- to 6-kb genome which, with one exception, is monopartite. The exception is the *Dianthovirus* genus, which has two genomic RNAs. The Tombusvirid genome encodes a highly expressed ORF (ORF 1) fused to the RdRp ORF via readthrough of a leaky stop codon in most cases, or a -1 ribosomal frameshift in the dianthoviruses (Fig. 72.12). Because readthrough and frameshifting are rare events, the ORF 1 product is far more abundant than the RdRp, the amino terminus of which is encoded by ORF 1. These are the only proteins needed for RNA replication.³⁵⁸ The downstream genes encode one or more movement proteins, the coat protein, and a suppressor of RNA-mediated silencing (VSR). These ORFs are translated from subgenomic (sg) mRNAs generated during virus infection.¹⁵² Specialized translation events such as ribosome initiation at more than one start codon (via leaky scanning) are necessary for translation of some of these downstream ORFs, depending on the genus. The sgRNAs are not encapsidated, and they are generated during the process of RNA replication.

The function of the protein encoded in ORF 1 is best characterized for TBSV, the type member of the *Tombusvirus* genus. This protein, (33 kD P33 in TBSV), is an RNA-binding protein that binds a specific stem-loop in TBSV RNA.²⁴⁶ About 1% to 5% of these proteins contain the RdRp fused to the C terminus via ribosomal readthrough of the ORF 1 stop codon, making it a 92-kD protein. This is the only way the RdRp is translated. This relationship of a highly abundant RNA-binding protein (RBP), a few copies of which contain the viral RdRp by virtue of the longer C terminus, resembles that of the gag-pol protein of yeast LA virus, most retroviruses, and the protein products of RNAs 1 and 2 of the *Bromoviridae*. In the latter case, rather than a translational recoding event, the

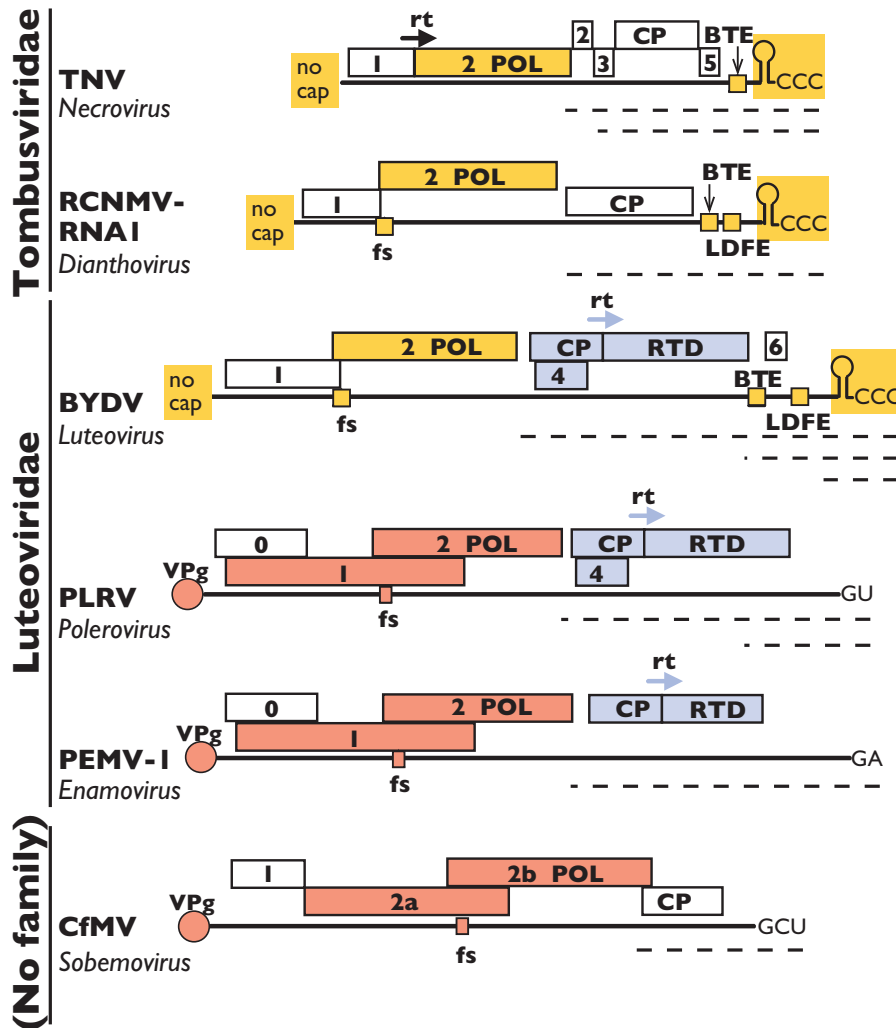


FIGURE 72.12. Relationships of representative *Tombusviridae*, *Luteoviridae*, and *Sobemovirus*.²¹⁵ Common features of different genera are highlighted in the same color. The “Luteoviridae block” shared only among *Luteoviridae* is in blue. rt, stop codon readthrough site; fs, ribosomal frameshift site; BTE, BYDV-like cap-independent translation element; LDFF, long-distance frameshift element required for frameshifting at the fs site 4-kb upstream. Dashed lines, subgenomic RNAs. VPg, genome-linked protein; RTD, readthrough domain.

RBP and RdRp are translated from separate RNAs. It has been speculated that the P33 forms the replication vesicle, like the 1a protein of BMV or gag of LA virus, and that this contains just one or two copies of the RdRp sequence to allow efficient replication of genomes inside the replication vesicle.

Genome Replication

The roles of viral and cellular components in TBSV replication have been determined to an amazingly advanced level quite rapidly due to the herculean efforts of the lab of Peter Nagy. Like Ahlquist did with BMV, Nagy developed a yeast replication system for TBSV. He used the system for rapid screening of yeast knockout lines or inducible knock-down lines representing a total of 95% of yeast genes to reveal genes that were required for, or in some cases inhibited, TBSV RNA replication.²²⁷ In addition, yeast protein arrays were screened to identify host proteins that bind viral proteins or viral RNAs, and finally, mass spectrometry identified host proteins in purified viral replicase.

At least 254 yeast proteins play a role in TBSV RNA synthesis.²²⁷ The proteins represent almost all types of function in a cell, including protein synthesis (e.g., translation elongation factor eEF1A), protein metabolism (e.g., ubiquitination proteins), RNA metabolism (e.g., XRN1), general metabolism

(e.g., glyceraldehyde 3-phosphate dehydrogenase, GAPDH), lipid metabolism (e.g., sterol synthesis enzymes), stress (e.g., heat-shock protein 70, Hsp70), and membrane-associated proteins (e.g., PMR1, a $\text{Ca}^{2+}/\text{Mn}^{2+}$ pump).

Unlike the previous work with BMV, the roles of many of the proteins identified in yeast screens were confirmed in planta. Knockdown of the *GAPDH* gene in *N. benthamiana*, application of a pharmacological inhibitor of Hsp70, or silencing or chemical inhibition of sterol synthesis genes, all drastically reduced TBSV replication in plants and in protoplasts.³⁴⁷ A cell-free replicase extract capable of synthesizing positive-sense RNA was isolated from yeast, and its components were characterized. This revealed the requirement for eEF1A in replicase assembly and that Hsp70 is an integral component of the replicase complex.²⁴⁵ Combining all of the information gleaned from the above approaches led to the model shown in Fig. 72.13. Translation of the viral genome leads to production of a large amount of p33 from ORF 1, and—via readthrough of the ORF 1 stop codon—p92. p92 binds a specific hairpin loop containing an essential C C bulge in the genomic RNA, and with many copies of p33, brings the RNA to the peroxisome membrane. This assembly is facilitated by eEF1A, peroxisomal localization protein PEX19, and the essential replicase component, Hsp70.³⁴⁷

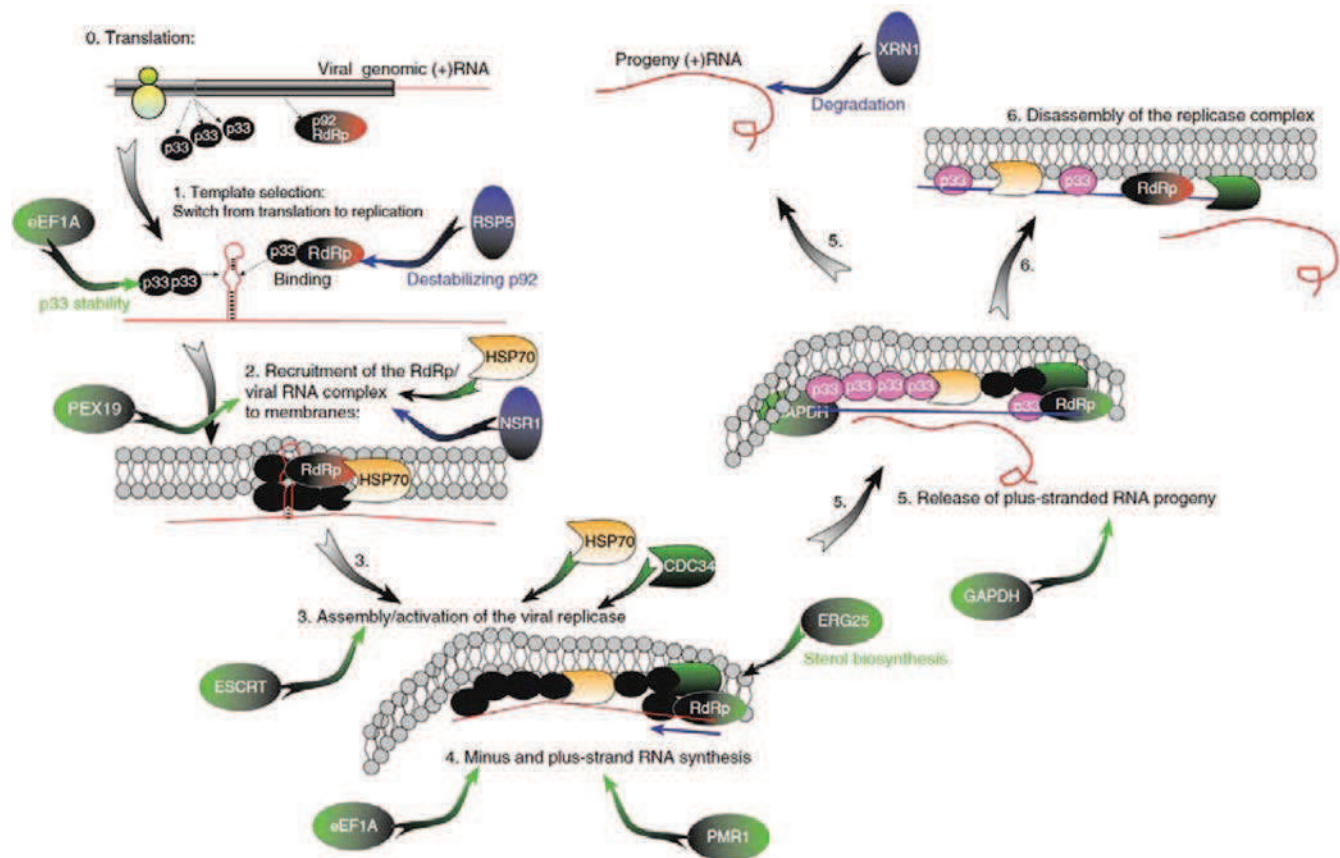


FIGURE 72.13. Model of Tomato bushy stunt virus (TBSV) replication. Host factors shown in green circles and green arrows are required, whereas factors in blue circles and blue arrows inhibit TBSV replication. Hsp70 is colored uniquely due to the essential role of Hsp70 in several steps in TBSV replication. Active and inactive (phosphorylated) viral p33 proteins are shown as black or pink circles, respectively. Active and inactive forms of p92 are shown as green and red circles, respectively. (Reprinted with permission from Nagy PD, Pogany J. Global genomics and proteomics approaches to identify host factors as targets to induce resistance against tomato bushy stunt virus. *Advances in Virus Research* 2010;76:123–177.)

The functional replicase is assembled or activated on the membrane by endosomal sorting complex required for transport (ESCRT) proteins, CDC34 and HSP70. Membranes are altered by sterols, and by forming invaginations and replication vesicles. eEF1A and PMR1, the $\text{Ca}^{2+}/\text{Mn}^{2+}$ pump, facilitate RNA synthesis.¹⁴⁶ Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) favors positive-strand synthesis, possibly by facilitating its release from the replicase complex. Phosphorylation of p33/p92 inhibits their RNA-binding function, leading to disassembly of the replicase complex. Some progeny RNA is degraded by XRN1,²⁹⁶ exonuclease which is not surprising given that the viral RNA has an unmodified 5' end. Apparently secondary structure at the 5' end, or sheer numbers of viral RNAs generated during replication, prevents total degradation of viral RNA by XRN1. Many of the events and host enzymes were shown to apply to mammalian virus replication. Therefore, this extremely tractable yeast-plant system to investigate TBSV replication may have relevance to many stages of human RNA virus replication.

Luteoviridae and Genus Umbravirus

Two virus genera that are not in the *Tombusviridae*, but which have all of the above properties, including polymerase genes and

cis-acting replication and translational control signals highly homologous to those of the *Tombusviridae*, are *Luteovirus* and *Umbravirus* (see Fig. 72.12). Genus *Luteovirus* has been assigned to the *Luteoviridae* family based on the property that its members are transmitted by aphids in a circulative, persistent manner (see vector transmission), unlike any *Tombusvirid*. The coat protein is similar to that of some *Tombusviridae*, except that some (perhaps 5% to 10%) of the CP subunits in the virion contain a ~50-kD C-terminal extension necessary for the unique aphid transmission properties.³⁵ This extension is added by in-frame readthrough of the CP ORF stop codon.³⁹ Luteoviruses (i.e., genus *Luteovirus* members) have all the replication and gene expression features of *Tombusviridae*, and differ only in the CP, movement protein, and possibly silencing suppressor (which is unidentified to date). The genera within *Tombusviridae* vary in these same properties. Thus it has been proposed that genus *Luteovirus* of the *Luteoviridae* could be reassigned to the *Tombusviridae*.²¹⁵

The *Luteoviridae* family comprises three genera: *Luteovirus*, *Polerovirus*, and *Enamovirus*. They include major pathogens such as the Barley yellow dwarf viruses (genus *Luteovirus*) and Cereal yellow dwarf viruses (genus *Polerovirus*) that afflict wheat, barley, and oats wherever these crops are grown; Potato

leafroll polerovirus in potato (worldwide); Beet mild yellowing virus (despite its name, a major problem in European sugar beet); and Pea enation mosaic virus (PEMV, a complex of the sole *Enamovirus* and an *Umbravirus*), which causes major losses periodically in peas and lentils from the Pacific Northwest to the Middle East.⁶⁶

Poleroviruses and the single-member genus *Enamovirus* (Pea enation mosaic virus 1, PEMV1) are distinct from genus *Luteovirus* and the *Tombusviridae*. The RdRp sequence is more closely related to those of viruses in genus *Sobemovirus* (no family assigned) than it is to genus *Luteovirus* or any *Tombusviridae* (see Fig. 72.12). In addition, it contains a genome-linked protein (VPg) attached covalently to the 5' end of its genomic RNA. There are no close similarities in translational control signals to those shared among genus *Luteovirus* and some of the *Tombusviridae*. The only features shared closely among the three *Luteoviridae* genera and which distinguish them from *Tombusviridae* and sobemoviruses are that they are persistently transmitted by aphids, and with the exception of PEMV, they are confined to the phloem and not mechanically transmissible. These properties are conferred by the CP-RTD protein in the virion¹²¹ and probably by the movement protein, which is encoded by an ORF embedded within the CP ORF, in a different reading frame. Unlike the replication genes and *cis*-acting signals, these three genes have higher homology within the *Luteoviridae* than they do with viruses outside the family.²¹⁵ To summarize the relationships, viruses in the genus *Luteovirus* appear to be *Tombusviridae* that acquired a “*Luteoviridae* block” of ORFs: the CP-RTD and overlapping MP ORF. Polero and enamoviruses resemble sobemoviruses that acquired the same *Luteoviridae* block. How the *Luteoviridae* block first evolved is unknown.

Umbraviruses are unusual in that they encode no coat protein. Instead, they rely on co-infection with a specific partner virus in either the *Polerovirus* or *Enamovirus* genus of the *Luteoviridae* family, for encapsidation and transmission to other plants by aphids.³¹⁶ Umbraviruses encode their own polymerase, enabling them to replicate in, and infect, plants on their own. Therefore, they are not satellite viruses. However, satellite RNAs of umbraviruses exist and play a role in disease. Groundnut rosette umbravirus, in combination with a true satellite RNA, and the mild polerovirus, Groundnut rosette assistant virus, together form a tripartite complex that leads to devastating losses of groundnuts (peanuts) in sub-Saharan Africa.^{276,317}

Although they lack a CP and do not form virions on their own, umbraviruses form a ribonucleoprotein (RNP) complex containing the viral genome and the protein encoded by ORF 3. Formation of this structure requires movement of the ORF 3 protein through the Cajal bodies to the nucleolus.¹⁶⁶ There, the ORF 3 protein interacts with fibrillarin, which leads to formation of nuclease-resistant RNPs capable of long-distance movement in the phloem.¹⁶⁵ Although not a virion, the RNase-resistant nature of the RNP suggests it functionally performs some of the role of a virion for movement within, but not between, plants.

Gene Expression

CAP-INDEPENDENT TRANSLATION

The RNAs of most plant viruses lack a 5' cap structure, which is essential for translation of host genes. Instead, uncapped plant viral RNAs contain a cap-independent translation element (CITE). Some of these elements may behave like an animal virus internal ribosome entry site (IRES) upstream of

the initiation codon. However, *Tombusviridae*, luteoviruses, umbraviruses, and *Nepovirinae* contain CITEs in the 3' UTR of their genomes, and, other than in the *Nepovirinae*,¹⁵⁸ the 3' CITEs do not confer internal ribosome entry.²¹⁶ These are unlike any known animal virus translation element.

3' CITEs fall into about seven different classes, based on their sequence and structural features (Fig. 72.14A). These elements appear to stimulate translation by binding a surface of the translation initiation factor complex, eIF4F, with high affinity. eIF4F is a rate-limiting, highly regulated, heterodimeric initiation factor. In plants, it has two subunits, the cap-binding protein, eIF4E, and the multifunctional scaffolding protein, eIF4G. eIF4E normally binds the 5' cap, bringing eIF4G to the mRNA. eIF4G binds numerous other initiation factors that ultimately recruit the 40S ribosomal subunit, and it facilitates scanning of the ribosome to the start codon.¹⁴⁷ The 3' CITE of Barley yellow dwarf virus (BYDV) and related viruses (BYDV-like translation enhancer, BTE) binds eIF4G with high affinity and does not bind or require eIF4E.³²² In contrast, the 3' CITE of Panicum mosaic virus (PTE) and certain other *Tombusviridae* and one umbravirus, has a structure that is completely different from that of BTE, and it binds eIF4E with high affinity.³⁵¹ This was surprising because eIF4E was known to bind only capped RNAs, owing to the requirement for the m⁷GpppN (cap structure) at the 5' end to fit in the cap-binding pocket of eIF4E. The 3' translation enhancer domain (TED) of Satellite tobacco necrosis virus (STNV)¹¹¹ and the I-shaped 3' CITEs of Maize necrotic streak virus (MNeSV) and Melon necrotic spot virus (MNSV)²³⁰ bind eIF4F, but not to either individual subunit with high affinity. Genetic evidence supports an interaction between the I-shaped CITE and eIF4E.³²⁵ The T-shaped structure (TSS) of Turnip crinkle virus (TCV) RNA has been shown to bind directly to the yeast 60S ribosomal subunit.³⁷⁸ All of the above CITEs are in genomes of the *Tombusviridae*, with the exception of those in the related *Luteovirus* and *Umbravirus* genera. Interestingly, the class of 3' CITE associated with a particular virus does not always correlate with the classification of the virus. Finally, the nepoviruses, which are completely unrelated to the *Tombusviridae*, being in the *Picornavirales* order, also have a 3' CITE in their genomes. It differs from the others by acting in conjunction with a 5' UTR to confer IRES function.¹⁵⁸

The location of the CITE in the 3' UTR raises the question of how it facilitates ribosome entry at the 5' end of the genome. In most cases, this is achieved by long-distance base pairing between the 3' CITE, or an adjacent sequence, and the 5' UTR.^{90,124} One model for this mechanism is that the 3' CITE binds eIF4F, and simultaneously base pairs to the 5' UTR, placing eIF4F in the proximity of the 5' terminus to which it recruits the 40S ribosomal subunit (Fig. 72.14B).²⁶³ An alternative possibility is that the factors and ribosomes are all recruited to the 3' CITE, which then delivers the ribosome to the 5' UTR by long-distance base pairing. In TCV RNA, it is proposed that the 60S subunit bound to the TSS interacts with the 40S subunit at the 5' end, thus circularizing the viral mRNA.³¹²

The location of the CITE in the 3' UTR may provide an elegant mechanism to regulate the switch from translation of the first viral RNA molecule that enters the cell to replication (Fig. 72.15). Circularization and translation take place in the absence of viral proteins and would be the first event as the RNA is released from the first invading virion. Once synthesized from the pioneer viral RNA, the RdRp would initiate negative-strand

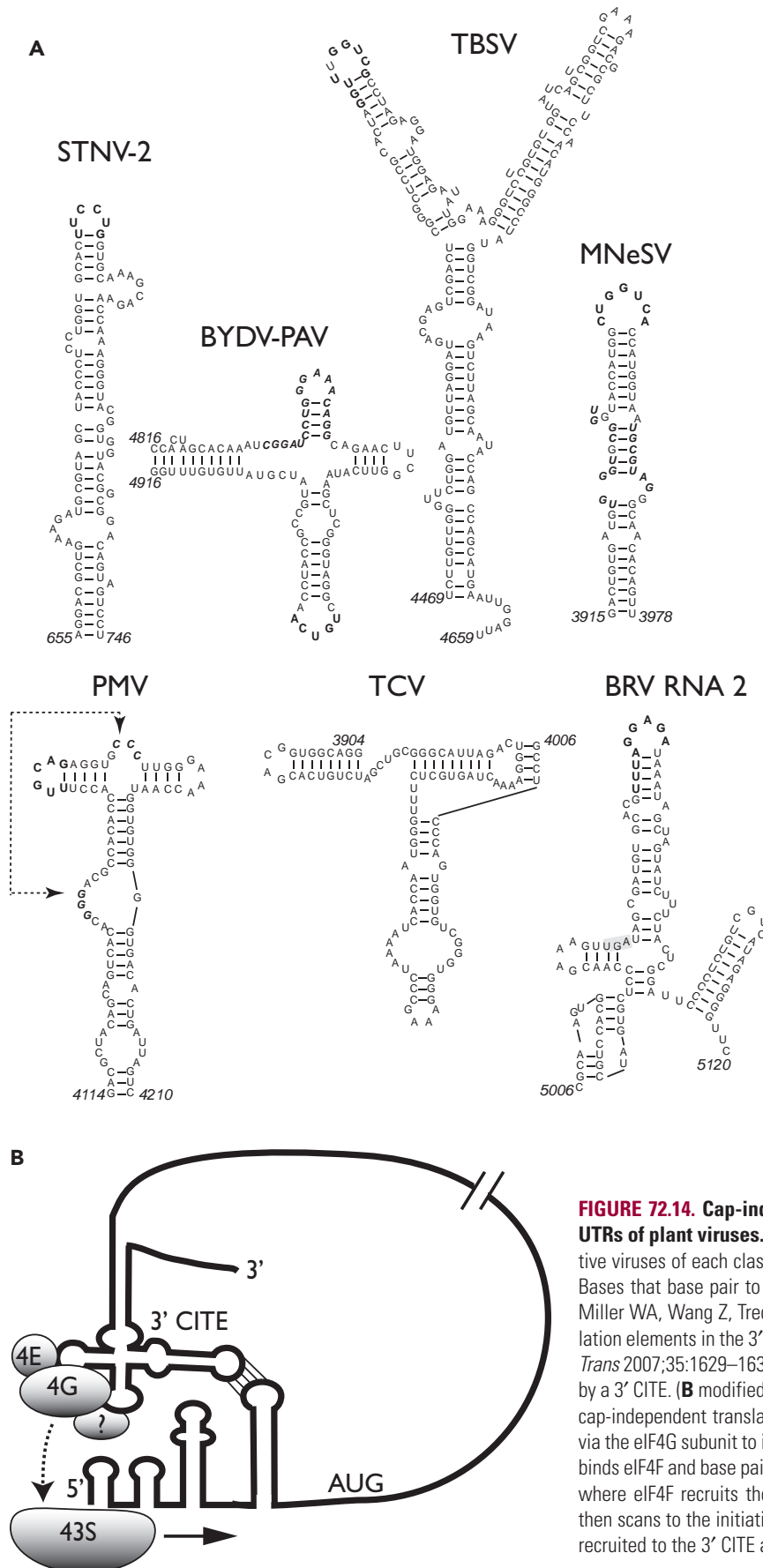


FIGURE 72.14. Cap-independent translation elements (CITES) in the 3' UTRs of plant viruses. **A:** Secondary structures of elements from representative viruses of each class of element. Required, conserved bases are in *italics*. Bases that base pair to the 5' UTR are in *bold*. (**A** adapted and updated from Miller WA, Wang Z, Treder K. The amazing diversity of cap-independent translation elements in the 3'-untranslated regions of plant viral RNAs. *Biochem Soc Trans* 2007;35:1629–1633.) **B:** Model for cap-independent translation mediated by a 3' CITE. (**B** modified from Treder K, Pettit Kneller EL, Allen EM, et al. The 3' cap-independent translation element of Barley yellow dwarf virus binds eIF4F via the eIF4G subunit to initiate translation. *RNA* 2008;14:134–147.) The 3' CITE binds eIF4F and base pairs to the 5' UTR, placing eIF4F in proximity of the 5' end where eIF4F recruits the 43S ribosomal subunit preinitiation complex, which then scans to the initiation codon (AUG). Alternatively, the 43S subunit may be recruited to the 3' CITE and delivered to the 5' end by base pairing.

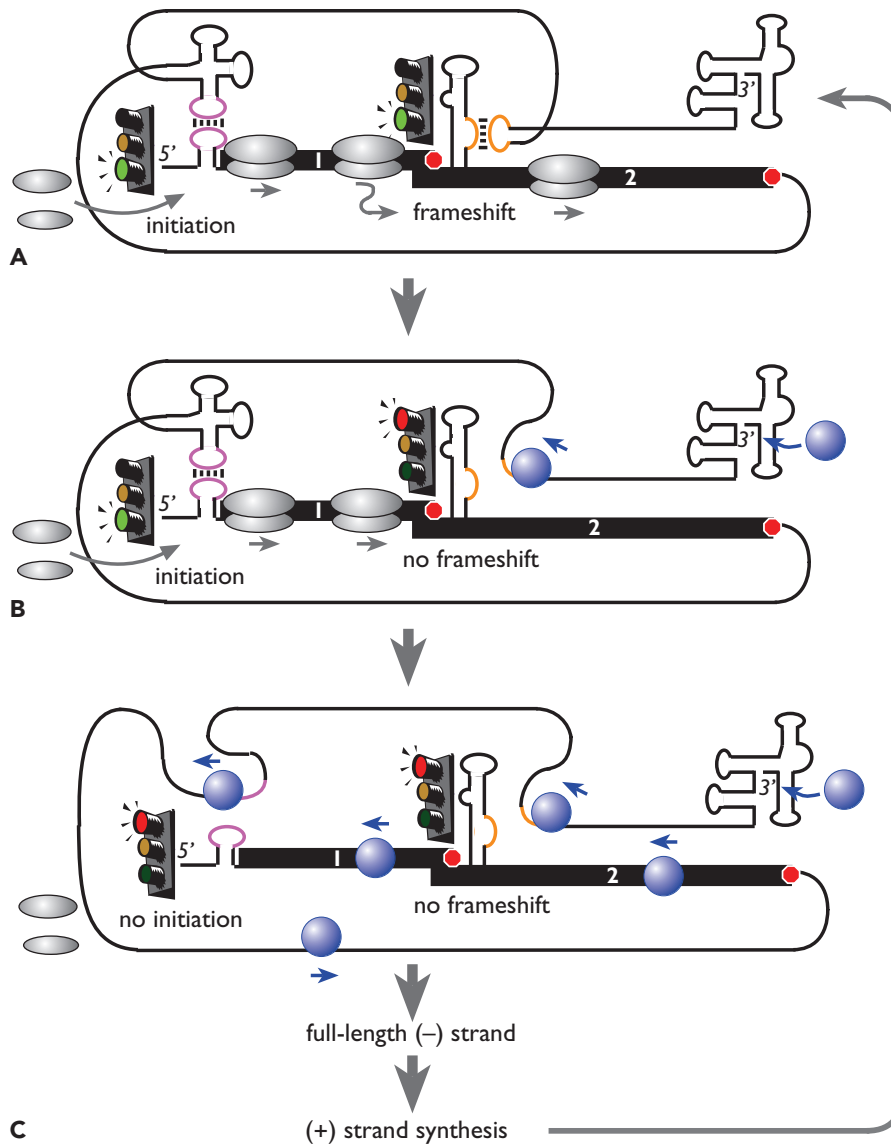


FIGURE 72.15. Model for shut-off of translation to permit replication of Barley yellow dwarf virus (BYDV).²¹⁷ **A.** Long-distance base pairing allows for translation initiation (magenta) and frameshifting (yellow). **B.** The RdRp produced by translation (blue sphere) copies viral RNA starting from the 3' end. This disrupts the upstream frameshifting and initiation events, freeing the RNA of ribosomes to permit complete genome replication (**C**). This potential mechanism of initiation shut-off by the RdRp in the 3' UTR applies to all viruses with a 3' CITE. The frameshift shut-off applies to luteoviruses and dianthoviruses, which have a long-distance frameshift element in the 3' UTR.

synthesis at the 3' end of the genome and begin copying in the 5' direction. If it encountered ribosomes still translating the viral RNA, the RdRp would be unable to proceed and replication would be blocked.¹⁰⁷ Instead, upon encountering the CITE in the 3' untranslated region, the RdRp could disrupt this structure, prevent both eIF4F binding and long-distance base pairing, thus shutting off translation initiation at the 5' end, 4 kb upstream, long before the RdRp would encounter a ribosome. This would free the genome from ribosomes and permit full-length negative-strand synthesis. The luteoviruses and dianthoviruses also harbor a sequence in the 3' UTR required for the frameshift event that is located at the start of the RdRp ORF about 3 to 4 kb upstream (see Fig. 72.12). The RdRp could disrupt this interaction as well (Fig. 72.15).

TRANSLATION OF POLYCISTRONIC PARARETROVIRUS mRNAs

Plant pararetroviruses in the *Caulimoviridae* and *Badnaviridae* families express multiple genes from a large polycistronic mRNA by two complicated translation initiation mechanisms: ribosome

shunting and ribosome reinitiation.²⁸² Multiple large ORFs are translated from the 35S pregenomic RNA of CaMV, the best characterized plant pararetrovirus. Translation initiates at the 5' end via a ribosome shunt mechanism.¹⁰⁰ The highly structured 5' leader contains several small ORFs (sORFs). The 40S ribosomal subunit binds in a normal cap-dependent mechanism and scans to the first sORF. The six codon sORF is translated, and termination at the sORF stop codon takes place immediately upstream of a large stem-loop structure. The 40S subunit then jumps across the structured region, skipping several sORFs in the process, and resumes scanning until it reaches the start codon of the first large ORF, ORF VII where translation reinitiates.²⁸¹ After this mechanism was discovered in CaMV, a very similar shunting mechanism was discovered in translation of adenovirus late mRNAs, demonstrating unexpected relevance of this plant virus mechanism to mammalian virology.³⁷¹

The ORFs downstream of ORF VII (I-V, or III-V, depending on splicing of the mRNA; Fig. 72.16) are translated by a different re-initiation mechanism mediated by the

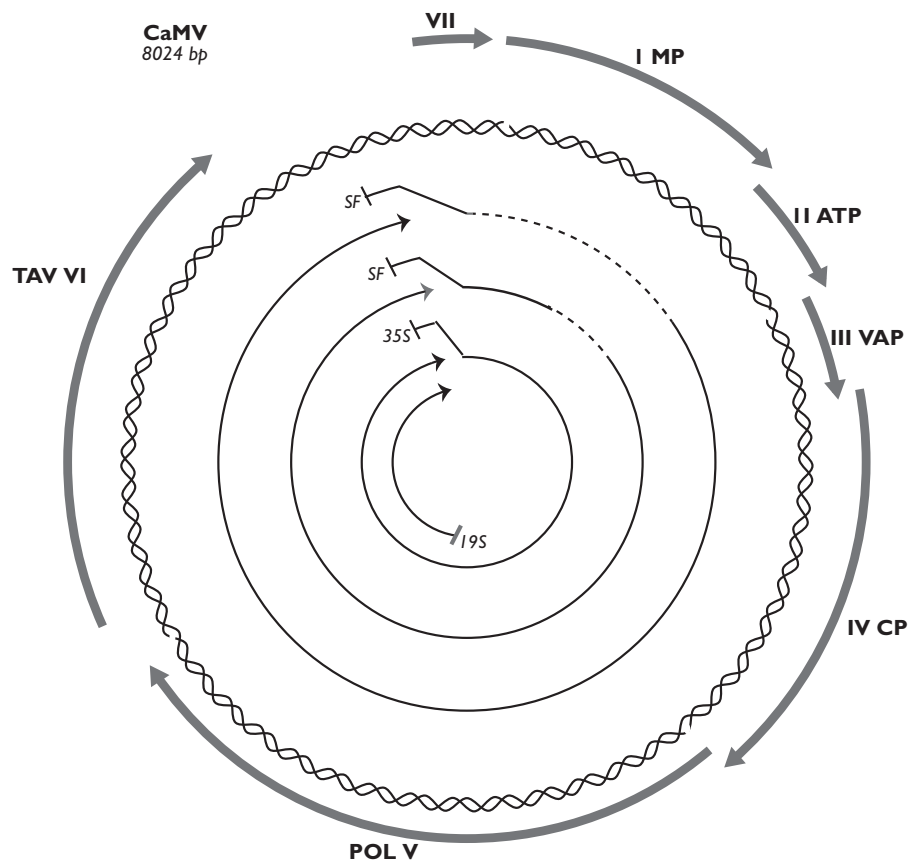


FIGURE 72.16. Genome organization of Cauliflower mosaic virus (CaMV).

Nicked, circular, double-stranded genomic DNA is indicated. Interior circular arrows indicate the most abundant transcripts, with spliced out regions as *dashed lines*. Functions of numbered open-reading frames (ORFs): MP, movement protein; ATP, aphid transmission protein (P2 in Fig. 72.25); VAP, virion-associated protein (P3 in Fig. 72.15); CP, coat protein; POL, RNA-dependent DNA polymerase; TAV, trans-activator of translation.

viral *trans*-activator (TAV) protein, encoded by ORF VI on its own separate mRNA.^{28,239} TAV binds 60S ribosomal subunit proteins L13 and L18 and also translation initiation factor eIF3 which brings the 43S ribosomal ternary complex to the mRNA in normal translation.²³⁹ TAV also recruits the protein kinase target-of-rapamycin (TOR) that becomes hyperactivated leading to phosphorylation of a host factor called reinitiation supporting protein (RISP).²⁸⁸ Normally, when the 40S subunit reaches the start codon, eIF3 is released upon 60S-subunit joining. But TAV, eIF3, phosphorylated TOR, and phosphorylated RISP remain bound to the ribosome during translation elongation and after termination. Presence of these proteins keeps the 40S subunit on the mRNA, and facilitates re-recruitment of the ternary complex and scanning to the next AUG codon where the 60S subunit is recruited and re-initiation takes place.²⁸⁸ This reveals new and complex interactions of ribosomes with mRNAs and new roles for host proteins in translation not known previously.

SUBGENOMIC RNA SYNTHESIS

A key step in gene expression of many positive-strand RNA viruses is RNA-templated transcription of subgenomic mRNAs (sgRNAs).^{214,314} These are usually 3' co-terminal RNAs that permit translation of downstream ORFs of a polycistronic genomic RNA. Examples in the animal kingdom include viruses in the *Nidovirales* order and in the *Togaviridae* family. A much larger portion of the plant virus world generates sgRNAs, including viruses in the *Bromoviridae* (BMV), *Tymoviridae*, *Luteoviridae*, *Tombusviridae*, *Virgaviridae*, *Closteroviridae*, *Flexiviridae* families, and the *Tobamovirus*, *Carlavirus*, *Benyvirus*, *Sobemovirus*, and *Umbravirus*

genera. These viruses have evolved more than one way to generate sgRNAs, and they don't always function as mRNAs.

BMV generates a sgRNA (RNA 4) from the 800 bases at the 3' end of bicistronic RNA 4 (see Fig. 72.6). RNA 4 is the mRNA for the CP. A template-dependent replicase extract, primed with full-length negative-strand genomic RNA extract was used to demonstrate that RNA 4 is generated by internal initiation of the RdRp at a specific promoter sequence in the negative strand.²¹² It then copies the negative strand until it reaches the 5' end of the template (Fig. 72.17). The RNA structure recognized by the RdRp consists of a core promoter sequence at nts -19 to -1 relative to the initiation site (+1), which contains a short hairpin loop.³⁰⁰ This is preceded (in the 3' direction on the negative strand template) by a 17 nt poly(U) tract (poly(A) in the positive strand) and an A/U-rich tract (Fig. 72.18) that are required for maximal sgRNA transcription.^{314,360} The hairpin may form only upon binding of the replicase in an induced fit process.³⁰¹ It is not needed for replicase binding but is required for sgRNA synthesis. CMV (*Bromoviridae*) uses a very similar promoter,¹⁵⁶ and unrelated TMV and the *Togaviridae* family of animal viruses also generate sgRNAs by internal initiation at promoters containing key primary and secondary structural specificity elements (reviewed in (214,314)).

Viruses in the *Tombusviridae* generate sgRNAs by premature termination (PT) of negative-strand RNA synthesis from the full-length genomic RNA (gRNA) template.¹⁵² In this case, the viral polymerase encounters a highly structured region in the positive-strand template, apparently causing it to terminate negative strand synthesis (see Fig. 72.17). The 3' end of the

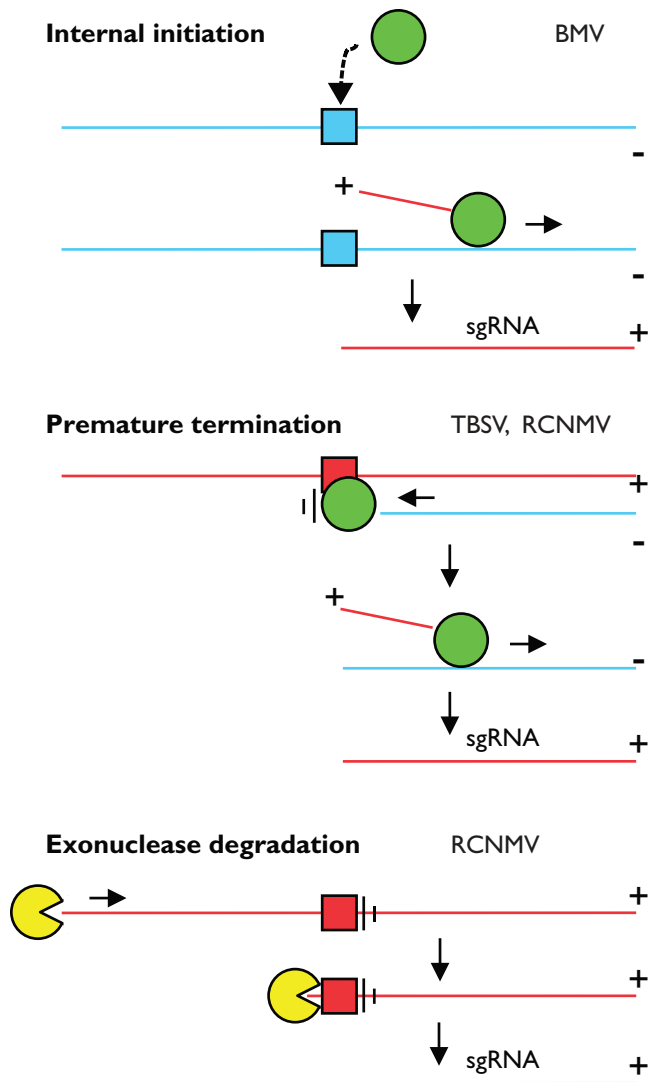


FIGURE 72.17. Mechanisms of plant virus subgenomic RNA synthesis. Green sphere, RNA-dependent RNA polymerase (RdRp); blue box, promoter in minus strand; red box, termination signal in plus strand; yellow sphere: exonuclease. **A:** RdRp initiates internally at the promoter in the full-length (–) strand.²¹² **B:** RdRp terminates early during (–) strand synthesis off the genomic RNA template.¹⁸⁴ **C:** An exonuclease degrades genomic RNA from the 5' end until it reaches a structure that blocks further degradation.¹⁴⁵ No RNA replication is needed in this mechanism.

resulting negative strand contains a short promoter sequence resembling the sequence at the 3' end of full-length gRNA. Presumably this is recognized by the replicase that then makes multiple copies of positive-strand RNA by end-to-end copying of the truncated negative strand. This PT mechanism was first indicated by the requirement for a *trans*-acting RNA to synthesize RCNMV sgRNA. The 3' co-terminal CP mRNA is generated from RCNMV RNA 1. Sit et al.³⁰⁵ showed that a loop in RCNMV RNA 2 must base pair to a sequence immediately upstream of the sgRNA start site in RNA 1, in order to generate the sgRNA (see Fig. 72.18). It appears that the viral polymerase terminates negative-strand synthesis when it encounters this bimolecular base paired region. This results

in a 3'-truncated negative strand that serves as a template for positive-sense sgRNA synthesis. By elegantly designed mutations that disrupt and restore base pairing in the positive but not the negative strand (through selective use of G-U base pairs, whose complements, C and A, do not pair in the opposite strand), the White lab demonstrated that, in TBSV and related viruses, long-distance base pairing in *cis* forms a stable structure in the positive strand just upstream of the 5' end of each sgRNA. This base pairing is required for sgRNA synthesis by PT³⁴⁸ (see Fig. 72.18). Various other viruses known or thought to produce sgRNAs by PT are discussed in reference 314.

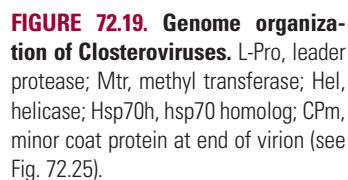
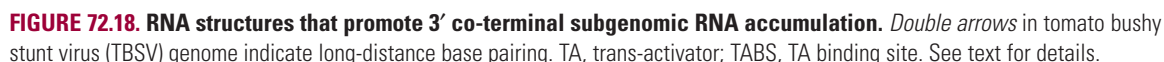
RCNMV generates a second sgRNA, spanning only the 3' UTR of RNA 1, by a nonreplicative method. This sgRNA, called SR1f, is generated by a host 5'-to-3' exonuclease¹⁴⁵ (see Fig. 72.17). This resembles the mechanism and genome location (3' UTR) of the sfRNA of flaviviruses.²⁴⁴

Some viruses generate 5' co-terminal subgenomic RNAs that lack the 3' end of the genomic RNA. A 5' co-terminal sgRNA, encoding ORF 3 from BMV RNA 3 terminates with a poly(A) tail derived from the intergenic oligo(A) tract in BMV RNA 3.³⁶¹ It may serve as a more efficient mRNA for the BMV 3a movement protein than full-length RNA 3. Viruses in the *Closteroviridae*, which resemble *Nidovirales* in genome organization but are not classified in *Nidovirales*, produce many 3' co-terminal sgRNAs (Fig. 72.19). Like the *Toroviridae* family, and unlike *Coronaviridae* and *Arteriviridae* (all families in *Nidovirales*), the 3' co-terminal sgRNAs of *Closteroviridae* do not contain sequences derived from the 5' end of the genome.³³⁹ Unlike any known nidovirus, Citrus tristeza closterovirus generates two 5' co-terminal sgRNAs with 3' truncations, as well as the expected nested 3' co-terminal sgRNAs that serve as mRNAs for the downstream ORFs. One of the 5' sgRNAs is produced by premature termination (PT) at a stem-loop structure,¹¹⁹ the other by the process of assembly by the minor coat protein.¹²⁰ The roles of these RNAs in infection are unknown. They are not necessary for virus replication in protoplasts.¹¹⁹

In addition to serving as mRNAs, some sgRNAs serve as *trans*-regulators of viral, and possibly host, gene expression. The SR1f RNA of RCNMV and sgRNA2 of BYDV both correspond to the 3' UTR and inhibit translation *in trans*.^{145,297} Because both of these sgRNAs contain a BTE that binds the eIF4G subunit of the limiting translation initiation factor eIF4F with high affinity,³²² they probably inhibit translation by sequestering this key translation initiation factor. This binding allows sgRNA2 to tilt the balance of translation of the two BYDV mRNAs, genomic RNA (gRNA) and sgRNA1, in favor of the latter,²⁹⁷ which is less dependent on eIF4G owing to its less structured 5' UTR (Fig. 72.20). Because the 3' UTR sgRNAs of RCNMV and BYDV also inhibit translation of capped, non-viral mRNAs,^{145,297} it is possible that they globally inhibit host translation, a phenomenon that has not been reported in plant virus infections.

POTYVIRIDAE

About 30% of all plant viruses are in the *Potyviridae* family. *Potyviridae* fall into six genera, with the vast majority of the members in the *Potyvirus* genus. With the exception of genus *Bymovirus*, viruses in *Potyviridae* have one genomic RNA. In the bymoviruses, the same coding capacity is split into two



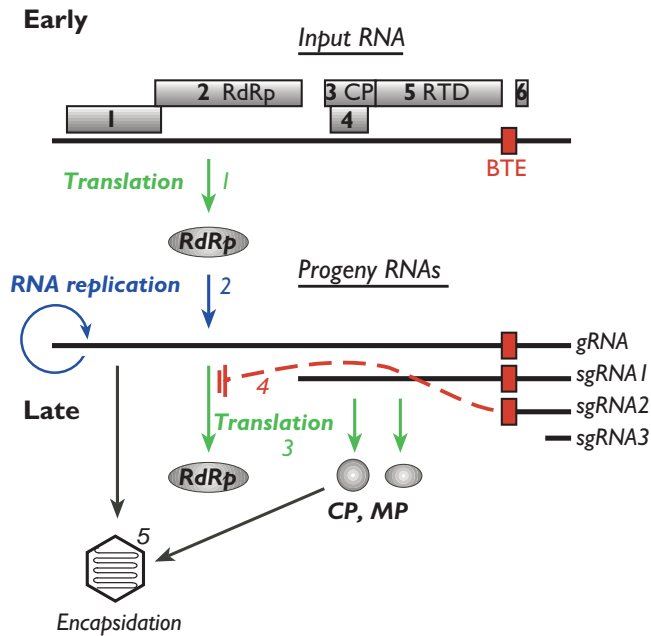


FIGURE 72.20. Regulation *in trans* of Barley yellow dwarf virus (BYDV) translation by sgRNA2.²⁹⁷ 1, open-reading frames (ORFs) 1 and 2 are translated from invading genomic RNA to generate the RNA-dependent RNA polymerase (RdRp). 2, replication by the RdRp generates more copies of genomic RNA (gRNA) and three subgenomic RNAs (sgRNA1–3). 3, translation of sgRNA1 produces the coat protein (CP) and movement protein (MP), the former then packages gRNA. 4, simultaneous with step 3, abundant sgRNA2 selectively inhibits translation of gRNA, freeing it for more replication and for encapsidation, and favoring translation of sgRNA1 to produce late functional proteins, such as the CP.

RNAs. Potyviruses employ the highly successful gene expression strategy used by the *Picornavirales*, and many other positive-strand RNA viruses, that is, the genome encodes one large ORF, which is translated as a polyprotein. This polyprotein is co-translationally and posttranslationally cleaved by viral proteases in a controlled manner to produce the functional proteins at the appropriate levels and timing necessary for a productive infection (Fig. 72.21).

Potyviridae also resemble *Picornavirales* in the sequence of the RdRp, and due to the presence of a genome-linked protein (VPg) at the 5' end and a poly(A) tail at the 3' end. However, they differ from the *Picornavirales* because, for example, (a) the virion is a flexuous rod rather than an icosahedron, (b) the coat protein is encoded at the 3' end of the polyprotein ORF rather than the 5' end, (c) the VPg has no homology to the VPg of picornaviruses and probably has different function, and (d) many of the viral proteins have no orthologs in the *Picornavirales*. The inability to infect animals does not necessarily exclude a virus from the *Picornavirales*, as the *Secoviridae* family of plant viruses is a member of the *Picornavirales* order (Fig. 72.22).

Functions of Potyviral Proteins

The N-terminal protein (P1) of the polyprotein is a serine protease that cleaves itself from the adjacent protein HC-Pro. In genus *Ipomovirus*, which lacks HC-Pro, P1 is a suppressor of antiviral silencing.^{207,330} In genus potyvirus, HC-Pro is a powerful silencing suppressor⁷ and has many other functions. HC-Pro functions include: (a) a papain-like cysteine protease that rapidly cleaves the junction with the downstream protein P3,⁴⁸ (b) the helper component required for aphid transmission, serving as the “glue” to attach the potyvirus particle to the interior wall of the aphid stylet,^{13,22,24,25} (c) a role in RNA amplification and systemic movement,^{160,277} (d) a powerful

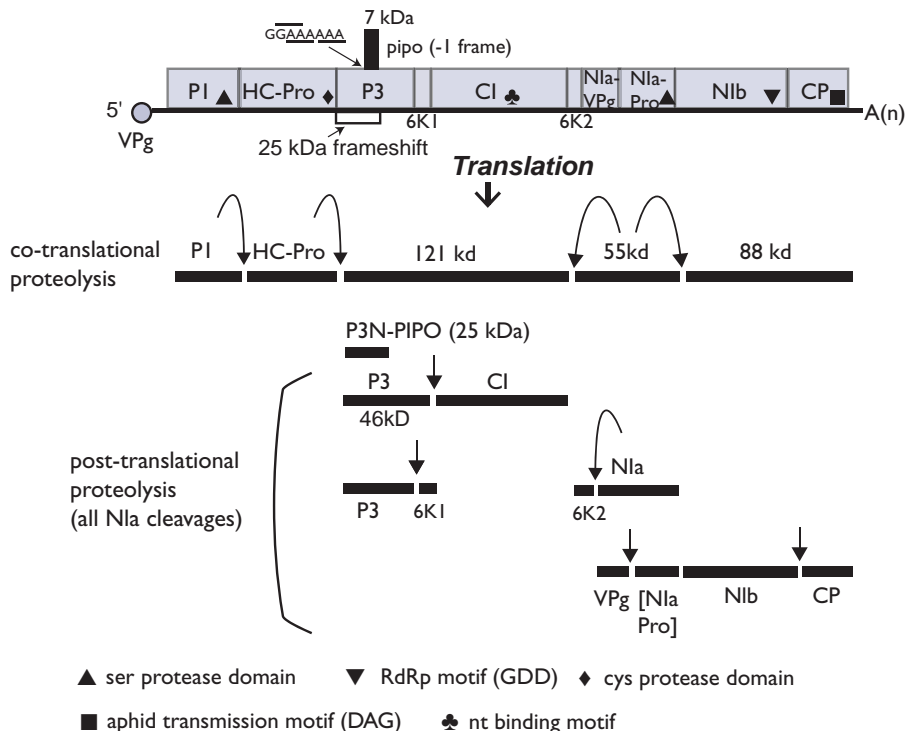


FIGURE 72.21. Genome organization of a potyvirus and proteolytic processing of the polyprotein. P3N-PIPO is a trans-frame protein with the N terminus of P3 and the C-terminus derived from overlapping open-reading frame (ORF), pipo. Frameshift is predicted at the GGAAAAA motif at the 5' end of the pipo ORF. Downward-pointing solid arrows indicate proteolytic cleavage sites. Curved arrows show self-cleavage. (Adapted and updated from Lazarowitz S. Plant Viruses. In: Knipe DM, Howley PM, eds. *Fields Virology* 5th ed. 2000).

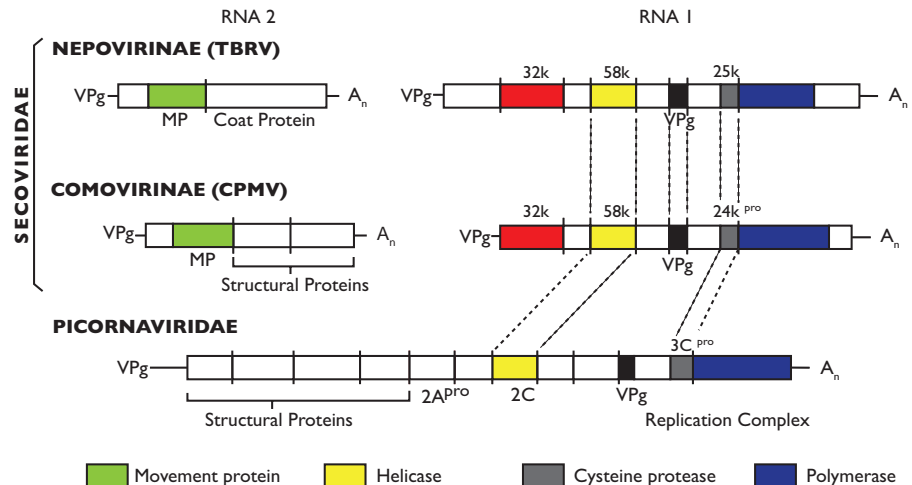


FIGURE 72.22. Sequence similarities between plant and animal *Picornavirales*. Colored regions indicate regions of high sequence homology.

suppressor of host gene silencing¹⁵⁹ that binds the small interfering RNA,¹⁷² and (e) a component of amorphous cytoplasmic inclusion bodies in cells infected by certain potyviruses, such as Papaya ringspot virus.²⁷³

P3 is the least understood potyviral protein. It has been identified as a symptom and avirulence determinant. Point mutations in Turnip mosaic virus (TuMV) P3 affect ability to infect certain Brassica species,¹⁵⁰ and in P3 of Soybean mosaic virus (SMV) allow infection of normally resistant soybean lines,¹²⁸ but its biochemical function is unknown. A small (60 to 100 codon) ORF, *pipo*, overlaps with the middle portion of the P3 coding region⁵⁷ (Fig. 72.21). It appears to be expressed as a trans-frame fusion with the N-terminal half of P3, to form a protein called P3N-PIPO. Mutations in *pipo* impair potyvirus infectivity on whole plants but permit replication in individual cells, suggesting it plays a role in cell-to-cell movement of the virus.^{55,355} P3N-PIPO interacts with the potyviral cylindrical inclusion (CI) protein and localizes to the plasmodesmata, consistent with a role in virus movement.³⁵⁴ The 6-kD protein immediately downstream of P3, 6K1, usually remains part of P3 due to delayed or incomplete proteolytic cleavage at the P3-6K1 junction, and includes a pathogenicity determinant in Plum pox virus (PPV).²⁸³ Therefore, three P3-derived proteins exist in infected plants, all with the same N-terminus but different C termini: P3N-PIPO, P3, and P3-6K1.

The CI protein is the main component of striking pinwheel or cylindrical structures in the cytoplasm and an ATP-dependent RNA helicase required for RNA synthesis.⁴⁹ It appears to facilitate cell-to-cell movement with help from the coat protein (CP)⁴⁹ as it localizes to, and increases size exclusion limit of, the plasmodesmata, which are necessary for movement of the viral RNP between cells.^{275,277,354} As might be expected of a protein with such crucial functions, CI protein is also a symptom determinant and mutations in the CI coding region can allow potyviruses to break resistance.^{2,374}

The 6-kD 6K2 protein plays a key role in transporting the replication machinery from the ER, where it is translated, to the chloroplast membrane where replication takes place in vesicles.^{272,285,320} This process involves COPII-mediated transport to the Golgi apparatus from which it is transported to the chloroplast membrane via actin filaments.³⁵³ NIa is cleaved into two proteins, the 21-kD genome-linked protein (VPg)

and a 27-kD protease (molecular weights are those of Tobacco etch virus, TEV). The VPg (a) binds eukaryotic translation initiation factor 4E (eIF4E),^{12,177,178,362} (b) serves as the primer for viral RNA synthesis via uridylation of a specific tyrosine residue by the RdRp (NIb),^{8,251} (c) contains a nuclear localization signal (NLS),^{261,284} (d) is associated with membranes, as expected for a replication protein,¹²⁶ (e) is necessary for cell-to-cell movement,²⁸⁶ (f) contributes to suppression of gene silencing,²⁶² and (g) controls phloem loading,²⁶² and (h) affects disease symptoms and resistance breaking.^{2,12,163}

Nuclear inclusion protein, NIb, is the viral RdRp. NIb interacts with NIa in order to catalyze uridylation of the VPg, which presumably primes RNA synthesis in a manner similar to the VPg of picornaviruses.²⁵¹ The role of NIb in the nucleus is unknown, as RNA replication takes place in cytoplasmic membranes.^{59,353}

In addition to forming the virion, the CP also has been implicated in cell-to-cell movement. An aspartate-alanine-glycine (DAG) motif on the surface of the CP is required for aphid transmission, as it binds the HC-Pro protein, which provides the “glue” to attach the virion to the aphid stylet (see details under vector transmission of plant viruses).

VPg-Translation Factor Interactions and Host Resistance

We will expand on the role of the interaction of the VPg with eIF4E because it appears to be the step that is inhibited in most natural resistance genes to potyviruses. About one-half of known plant virus resistance genes are recessive.³²⁴ The sequenced recessive resistance genes against potyviruses encode alleles of the cap-binding translation initiation factor, eIF4E, or the related protein, eIFiso4E.^{274,324} These mutant factors confer resistance because they do not bind the VPg efficiently.^{177,370} An obvious role of VPg binding to eIF4E may be to substitute for the role of the 5' cap in recruiting the ribosome to potyviral genomic RNA for translation. Even when present in trans (not covalently attached to viral RNA), VPg enhanced affinity of eIF4F for TEV RNA and increased translation efficiency.¹⁶⁴ However, TEV RNA translates efficiently in the absence of the VPg, via an IRES³⁷³ that interacts with eIF4G.²⁶⁸ Moreover, some mutations in eIF4E that render it unable to initiate translation, still allow it to facilitate replication of Lettuce mosaic potyvirus¹¹³ or Pea seed-borne mosaic virus (PSbMV) potyvirus.¹² Thus, the

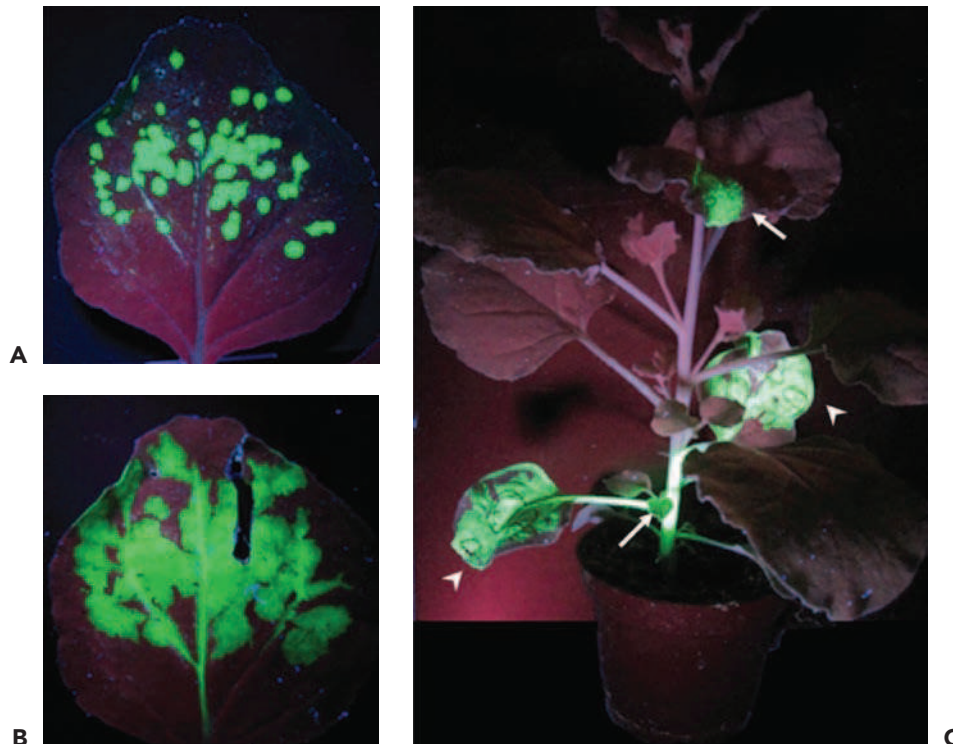


FIGURE 72.23. Use of green fluorescent protein (GFP)-tagged virus to monitor plant virus movement in whole plants. Plants were inoculated with tobacco mosaic virus (TMV) engineered to express GFP. **Panels A–C** indicate increasing times post inoculation. *Arrowheads*, inoculated leaves; *arrows*, systemically infected, non-inoculated leaves. Plants are shown under UV light. Chloroplasts in uninfected tissue fluoresce red. (Reprinted with permission from Niehl A, Heinlein M. Cellular pathways for viral transport through plasmodesmata. *Protoplasma* 2011;248:75–99.)

eIF4E-VPg interaction must play other, more essential roles in virus infection than translation stimulation.

CELL-TO-CELL MOVEMENT OF PLANT VIRUSES

Plant virus infections initiate at one or a few cells, from which the virus must spread systemically throughout the plant (Fig. 72.23). However, the plant cell wall is an excellent barrier to infection by all pathogens. Even if the cell wall is penetrated,

for example by an aphid stylet (below), to permit virus entry, the infection often remains confined to a single cell and is aborted because the virus cannot escape. But plant cells must remain in contact with each other and they do so via intercellular tunnels through the cell wall called plasmodesmata. It is this connection that viruses exploit to move from cell to cell. The walls of the plasmodesmata are lined with the plasma membrane, which is contiguous between neighboring cells. The endoplasmic reticulum, which spans the plasmodesmata in the form of the desmotubule, and the cytoplasm are also contiguous from one cell to the next (Fig. 72.24A). Plasmodesmata

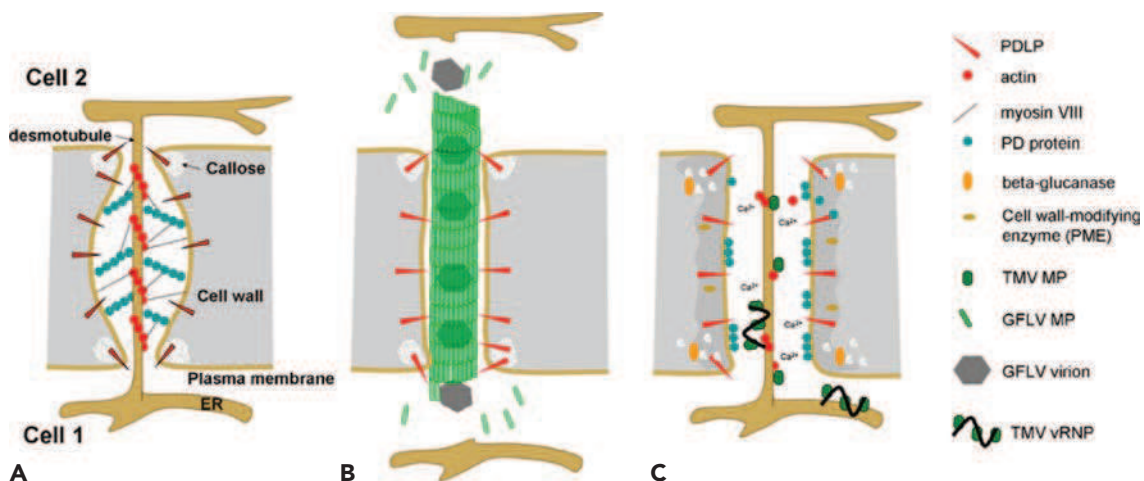


FIGURE 72.24. Diagrams of two different mechanisms of plant virus movement to new cells. **A:** A normal plasmodesma. **B:** A plasmodesma containing an MP tubule from the tubule-expressing virus, GFLV. **C:** Movement of nontubule-forming virus tobacco mosaic virus (TMV).²³¹ Proteins involved in virus-induced plasmodesmata modification are indicated at right. (Reprinted with permission from Niehl A, Heinlein M. Cellular pathways for viral transport through plasmodesmata. *Protoplasma* 2011;248:75–99.)

allow movement of small molecules between cells by diffusion. Normally, the size exclusion limit (SEL) is too small to permit macromolecules such as nucleic acids or proteins to fit through plasmodesmata, but mRNAs encoding certain non-cell-autonomous proteins are transported selectively through plasmodesmata.^{198,199} Plant viruses encode movement proteins (MPs) that increase the size exclusion limit and specifically transport the viral RNP through the plasmodesmata to adjacent cells.

Many different viral cell-to-cell movement mechanisms have evolved. (See 232,327,338 for excellent reviews.) Viral MPs either leave plasmodesmata intact but modify it to permit viral RNP transport, or they obliterate much of the plasmodesmatal structure by formation of a tubule, through which intact virions move. A partial list of virus movement mechanisms includes: (a) the 30K protein of TMV and related viruses, (b) the triple gene block of Potex-, Hordei-, Carmo-, and other virus genera,³³⁷ (c) the quintuple gene block of *Closteroviridae*, (d) coordinated nuclear shuttle and cell-to-cell movement by two proteins of *Geminiviridae*, and (e) tubule formation, by diverse RNA viruses in the *Secoviridae*, *Tospoviridae*, *Ilarviridae* and *Bromoviridae*, and DNA viruses in the *Caulimoviridae* and *Badnaviridae* families.

The “30K-type” movement protein (MP) found in TMV is a single-stranded nucleic acid-binding protein⁵⁸ that increases the SEL of plasmodesmata³⁶³ and interacts with microtubules that may direct the viral genome to the plasmodesmata¹³⁶ (Fig. 72.24C). Interactions with calreticulin, pectin methylesterases, and DnaJ chaperones are also thought to participate in transport of the MP and its viral genome cargo to the plasmodesmata.³²⁶ The plasmodesmal pore size is increased by interaction of MP with an ankyrin protein that decreases callose levels around the neck of the plasmodesma.³²⁷ Citovsky and colleagues propose that the diameter of the plasmodesma is gated by callose.³²⁷ Lower levels of callose would increase the SEL and permit virus movement through the plasmodesma. MP also increases the levels of callose-degrading enzyme beta-glucanase and cell wall-degrading enzyme pectin methylesterase in the plasmodesmata, both of which are expected to increase the SEL. These processes may be regulated by calcium ion concentration²³¹ either via regulation of signaling pathways or by direct effect on beta glucanase activity. Increases in calcium levels have been shown to decrease SEL.¹³⁹

Potexviruses, hordeiviruses, pomoviruses, pecluviruses, benyviruses, and carmoviruses use a “triple gene block” suite of three proteins, TGBp1, TPBP2, and TGBp3, to facilitate cell-to-cell movement. TGBp1 is about 25 kD and is translated from a subgenomic mRNA. Like the TMV MP, TGBp1 increases the SEL, and binds and transports the viral RNA throughout the plasmodesmata. TGBp1 of potexviruses can move between cells on its own, whereas TGBp1 of the other genera listed above requires TGBp2 and TGBp3. TGBp2 and TGBp3 are also necessary for cell-to-cell movement but they do not move between cells. TGBp2 (~12 kD) and TGBp3 (~7 kD) are translated from overlapping ORFs on the same subgenomic RNA. The TGBp3 ORF requires leaky scanning for translation and thus accumulates to much lower levels than TGBp2. Both proteins associate with virus-induced vesicles in the ER and deliver the TGBp1-viral RNA RNP to the plasmodesmata.³³⁷ Although the TGB proteins appear similar in size and expression mechanism, their functions are quite diverse among the different viruses that use the triple gene block strategy. Like TMV MPs, TGB MPs reach the plasmodesmata via

the microtubules, and some potexvirus TGBps increase levels of beta glucanase in the plasmodesmata.³³⁷

Geminiviruses (*Geminiviridae*) have circular single-stranded DNA genomes and twin icosahedral (geminata) particles. Most geminiviruses fall in genus *Begomovirus*. Begomoviruses have two genomic DNAs of 2.5 to 2.8 kb. The other geminivirus genera have only one genomic DNA of the same size. Begomoviruses express two proteins that move the genome intracellularly and intercellularly.¹⁷⁵ After genome replication in the nucleus, the nuclear shuttle protein (NSP) moves the new genome copies out of the nucleus. In the cytoplasm, the movement protein (MP) binds the NSP-DNA complex and transports it through the plasmodesmata in ER-derived tubules (not MP-derived tubules) to adjacent cells. The NSP then delivers the viral DNA to the nucleus of the new cell for more rounds of replication.¹⁷⁵

The MP of Cabbage leaf curl virus (CaLCuV) binds the plant synaptotagmin protein, SYTA.¹⁷⁹ Virus movement was delayed and restricted in SYTA knockout lines of *Arabidopsis*, and overexpression of dominant negative mutants of SYTA inhibited movement of CaLCuV as well as TMV, indicating that TMV may also require SYTA for movement.¹⁷⁹ Confocal laser scanning microscopy of GFP-tagged SYTA mutants indicated that SYTA regulates endocytosis and endosome recycling. Therefore, the MPs of begomoviruses and tobamoviruses appear to transport their DNA and RNA viral genomes, respectively, to the plasmodesmata by an endocytic recycling pathway.¹⁷⁹ Synaptotagmins are regulated by calcium ion. This supports a common theme of calcium regulation of proteins associated with plant viral cell-to-cell movement.

Tubule-forming MPs form a hollow shaft through the plasmodesma, eliminating the desmotubule and disrupting the intercellular ER connection (Fig. 72.24B). These MPs bind viral RNA weakly, if at all, necessitating movement of the viral genome in the encapsidated form.²³¹ The MP of Grapevine fanleaf virus (GFLV) is delivered to the plasmodesma via the secretory pathway.¹⁷⁴ The MPs of GFLV, an RNA virus, and CaMV, a DNA virus, form tubules by interacting with plasmodesmata-localized proteins (PDLPs). Any one of three different PDLPs is necessary and sufficient for efficient tubule formation.⁶ *Arabidopsis* plants harboring knockouts of all three PDLPs reduced GFLV tubule formation by about 50% and slowed spread of infection by GFLV or CaMV. However, both viruses still managed to infect and move at significant levels, indicating that movement, albeit at a slower rate, can take place in the absence of the PDLPs.⁶

Finally, as with so many other examples in this review, understanding of plasmodesmal transport of macromolecules pioneered in plant virology may provide valuable insight into mammalian cell biology. Relatively recently, intercellular trafficking connections called tunneling nanotubes have been discovered in animal cells.¹⁰³ They may share many functions and mechanisms with plasmodesmata.

VECTOR TRANSMISSION OF PLANT VIRUSES

Because plants are sessile organisms, plant viruses are more dependent than animal viruses on vectors for transmission from host to host.¹²² Few plant viruses are transmitted vertically through seed, and for those few seed-transmitted viruses, seed transmission is a rare stochastic event. The large majority

of plant viruses rely on aphids, whiteflies, or leafhoppers for plant-to-plant transmission.^{137,228} All of these insects are in the order *Hemiptera*, and are sap-sucking insects that feed on the vascular system of the plant. Thrips (order *Thysanoptera*), especially *Frankliniella occidentalis*, are important vectors because they transmit TSWV, a bunyavirus with a vast host range and worldwide distribution.³⁵⁹ Beetles, mites, nematodes, fungi, and humans also serve as vectors of some important plant viruses. Humans, via agricultural equipment, are the only known vectors of most viroids. Therefore, it has been speculated that viroid diseases did not exist before agriculture.

Here we will focus on hemipterans, the mosquitoes of the plant world. Hemipterans feed directly on the phloem, using the feeding structure called the stylet. When a hemipteran lands on a plant, it inserts the stylet between plant cells until it penetrates the phloem cells, where it spends its life feeding on the sugar-rich, protein-poor plant sap. Aphids usually lack wings, and reproduce parthenogenetically. Therefore, during the summer aphids accumulate clonally to huge numbers and all are female. When populations reach high levels or are stressed, new progeny will be winged and fly to neighboring plants, or reach the high altitude winds on which they can travel hundreds of kilometers.³⁷⁷ Therefore, certain aphid-transmitted plant viruses can be transmitted long distances depending on weather patterns.

Aphids and other hemipterans transmit viruses by a variety of mechanisms.³⁴ These have been termed nonpersistent, semipersistent, and persistent.^{122,137,228,229} Nonpersistently transmitted

viruses are acquired rapidly and are carried by the vector for only a period of minutes or hours. However, this is sufficient for such viruses to spread efficiently in the field. Potyviruses and the unrelated CMV are nonpersistently transmitted. Virions of potyviruses and CMV adhere to the distal end of the aphid stylet in order to be transmitted (Fig. 72.25).

Potyviruses rely on the multifunctional HC-Pro protein (HC is the abbreviation for helper component for aphid transmission) to act as a bridge or glue to bind the virion to the aphid stylet. HC-Pro is a nonstructural protein, acquired by the aphid from the infected cell.³²¹ The N-terminus of HC-Pro, including a conserved stylet-binding motif, KITC, is required for binding of PVY HC-Pro to the stylet, but not to the virion.²⁴ Overlay binding assays have revealed different putative receptor proteins in the aphid stylet that interact with HC-Pro. These include a group of four aphid cuticular proteins⁷⁸ and ribosomal protein S2 (RPS2).⁹² RPS2 has greater than 60% sequence identity to the RPS2/laminin receptor in humans, a known receptor for diverse mammalian viruses and prions. Lysine to glutamate mutations in the stylet binding motif of HC-Pro prevented binding to the cuticle proteins and to RPS2, supporting a specific role for these proteins in aphid transmission.⁹² On the CP side of the interaction, a highly conserved motif, aspartate-alanine-glycine (DAG) in the potyviral CP is required for aphid transmission of most potyviruses,¹⁴ and is required for HC-Pro-binding to the virion.²⁵

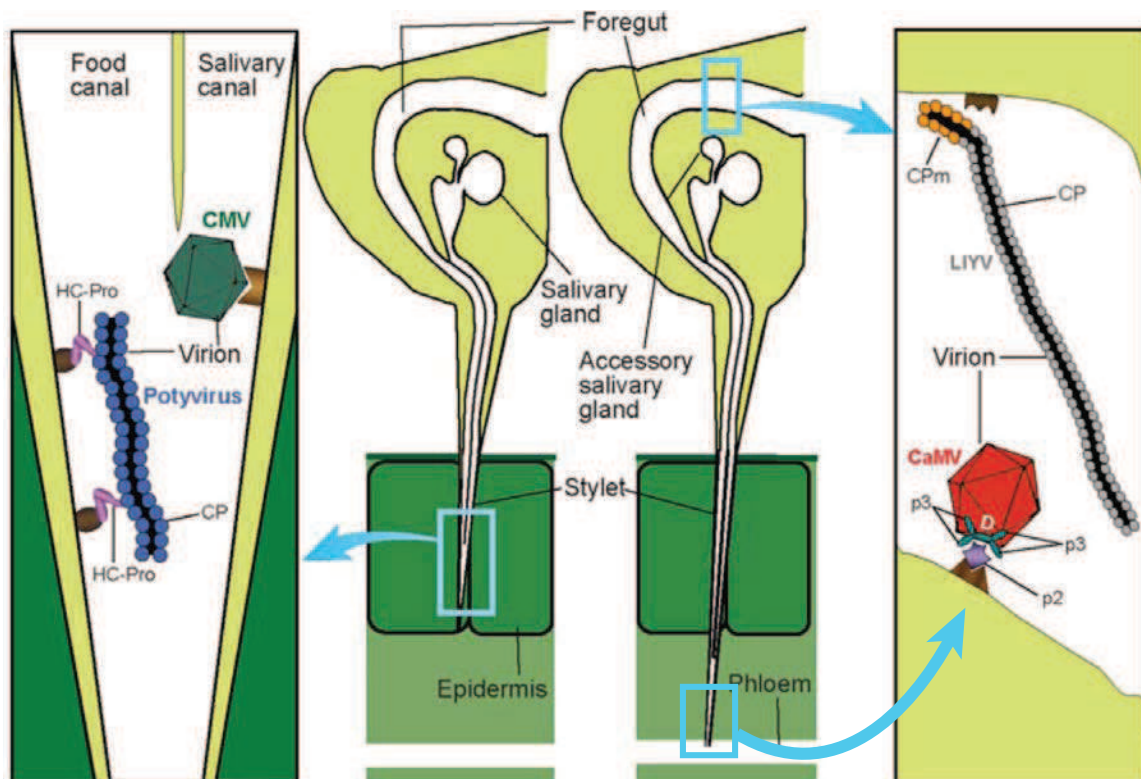


FIGURE 72.25. Nonpersistent (left) and semipersistent (right) transmission of viruses by aphids. Semipersistent transmission of CaMV was thought to involve binding of the virion (via nonstructural proteins P2 and p3) to the foregut, but p2 was shown to bind only to the common duct at the tip of the stylet. (Reprinted with permission and modified from Ng JC, Falk BW. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annu Rev Phytopathology* 2006;44:183–212.)

In contrast to potyviruses, CMV requires no helper component for aphid transmission (Fig. 72.25). The virion binds the stylet directly. A highly acidic, aspartate-glutamate-rich tract in the β H- β I loop of the capsid protein is required for aphid transmission.¹⁹⁰ This may be the site that binds the stylet. To our knowledge, a candidate host binding site or receptor has not been identified for CMV.

Semipersistently transmitted viruses are acquired within minutes to hours of feeding and are retained by the vector for hours to days. Examples of semipersistently transmitted viruses include CaMV (*Caulimoviridae*) and Lettuce infectious yellows virus (LIYV, *Closteroviridae*) (Fig. 72.25). CaMV relies on two nonstructural proteins to attach to the stylet. These include the viral protein P3, which dimerizes to form the digitation domain. Viral protein P2 forms a bridge between the P3-virion complex and the wall of the aphid stylet. Although semipersistently transmitted viruses were thought to bind the foregut, Uzest et al.³²⁹ showed that the P2 protein of CaMV binds only to the extreme tip of the stylet in the common duct, in a previously undescribed anatomical structure they call the acrostyle.³²⁸ Therefore the P3-virion complex is found only there. Other semipersistently transmitted viruses, such as LIYV, bind more proximally in the stylet and foregut. Like other closteroviruses, the long flexuous rod-shaped virion of LIYV contains a few copies of a minor coat protein (CPm), located only at one end of the flexuous rod-shaped virion. This CPm is thought to bind directly to a receptor in the foregut of its whitefly vector.²²⁸ In summary, the helper component mechanism or the direct virion binding mechanism of attachment of the virion to the aphid mouthparts can apply to either semipersistent or nonpersistent transmission strategy.

Persistent transmission can be either circulative, in which the virus circulates through, but does not replicate in, the vector, or propagative, in which the virus replicates in the vector. Circulative transmission is far more common, as viruses in the *Luteoviridae*, and large *Geminiviridae* families are transmitted in this manner by aphids¹²¹ and whiteflies,^{153,223} respectively. These are unlike mammalian arboviruses, all of which replicate in their vectors. In fact, the few plant viruses that undergo propagative transmission, closely resemble mammalian arboviruses. For example, TSWV, which replicates in its thrips vector,⁶⁵ is a member of the *Bunyaviridae* family. Plant reoviruses⁹⁹ and rhabdoviruses²⁶⁹ also replicate in their insect vectors. In contrast, the *Luteoviridae* and *Geminiviridae* are distinct families with no close relatives in the animal virus world.

Circulative transmission is best characterized in the luteoviruses. Luteovirus virions, which accumulate only in the phloem, are acquired by the feeding aphid and transported into the aphid hindgut, or occasionally foregut, depending on the virus, without being digested. They cross into epithelial cells by receptor-mediated endocytosis in clathrin-coated vesicles and are then ejected into the hemolymph (blood stream) where they circulate¹¹⁵ (Fig. 72.26). They then enter the accessory salivary gland through another endocytosis process.¹¹⁶ Upon feeding, the aphid salivates, ejecting virions, along with saliva, into the phloem cells.

The *Luteoviridae* virion consists of a standard T = 3 icosahedron similar to those of *Tombusviridae* or *Sobemoviridae*. However, unlike those viruses, a small fraction of the *Luteoviridae* CP subunits contain a long C-terminal extension that is translated by readthrough of the CP stop codon³⁹ (see Fig. 72.12). This readthrough domain (RTD) is essential for aphid transmission.³⁵ Its biochemical function is unclear. Viruses lacking the RTD can

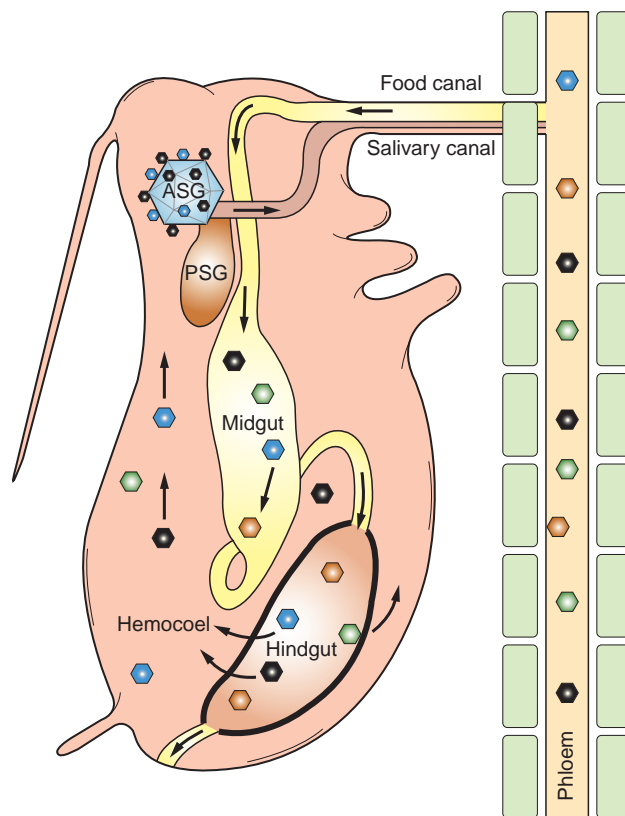


FIGURE 72.26. Movement of persistently transmitted luteovirus through the aphid. Arrows indicate path of virions from phloem in plant through the digestive tract and open circulatory system, into the accessory salivary gland (ASG) and out through the salivary canal when aphid feeds on a different plant. Purple hexagon: efficiently transmitted luteovirus particle; blue hexagon, inefficiently transmitted luteovirus particle; green hexagon, nontransmitted luteovirus particle enters the hemolymph but not the ASG; orange hexagon, nonluteovirus particle remains in digestive tract until it is ejected in aphid honeydew. (Modified and reprinted from: Power AG, Gray SM. Aphid transmission of barley yellow dwarf viruses: interactions between viruses, vectors, and host plants. In: D'Arcy CJ, Burnett PA, eds. *Barley yellow dwarf: 40 years of progress*. APS Press, St. Paul, 1995;259–291.)

still replicate in the plant and assemble virions. These virions can even be taken up into the aphid hemolymph after aphids are fed the RTD-lacking virions,²⁷⁰ but the virions are not transmitted by the aphid to other plants.³⁵

The RTD was shown to bind, *in vitro*, to a GroEL-like chaperonin protein called symbionin, which is synthesized in massive amounts by endosymbiotic bacteria (*Buchnera* spp.) in aphids.³³³ This interaction was reported to stabilize the virion in the hemolymph.²⁷⁰ However, the binding does not resemble normal interaction of groEL with proteins,¹³⁸ and recently, the bacteria that produce symbionin were shown to be confined in sealed organelles in the aphid, rendering symbionin inaccessible to virions in the aphid hemolymph.³³ These observations shed doubt on a role for symbionin in aphid transmission. The receptors in the gut epithelium and the accessory salivary gland membranes, to which luteovirus virions are thought to attach, have not been identified. However, a combination

of genetics and biochemical approaches suggests a luciferase and a cyclophilin as potential luteovirus receptors on the accessory salivary gland.³⁶⁸ They play key roles in determining aphid specificity of transmission of Cereal yellow dwarf virus.³⁶⁸

Over 100 viruses in genus *Begomovirus* (*Geminiviridae*) are transmitted in a circulative manner by a single whitefly species: *Bemisia tabaci*.¹⁵³ Therefore, *B. tabaci* is probably the most economically important vector species in all of agriculture, worldwide.²²³ The general mechanism of circulative transmission of *Geminiviridae* is thought to follow a similar process as that of the *Luteoviridae*, but the twinned geminivirus particles lack the readthrough domain required for luteovirus transmission. In contrast to the *Geminiviridae*, *Luteoviridae* are transmitted by dozens, if not hundreds, of aphid species, and the relationship between virus and aphid can be highly specific. For example, different BYDV and CYDV viruses were first differentiated by their preferred aphid vector.¹²¹ Even different biotypes of one aphid species can have very different efficiencies of transmitting a particular BYDV or CYDV isolate.³⁶⁸

VIROIDS AND SATELLITE RNAs

Viroids

Viroids are noncoding, single-stranded, circular RNAs that are unique to plants. For an excellent history of the discovery of these remarkable infectious agents, see the review by T.O. Diener.⁷² Viroids range from about 220 to 400 nt long, and most fold into rod-shaped secondary structures with small single-stranded regions, whereas some form branched structures (Fig. 72.27). They encode no proteins and are not packaged in virions. Viroids fall into two families, the *Pospiviroidae* (named for Potato spindle tuber viroid, PSTVd) and the *Avsunviroidae* (named for Avocado sunblotch viroid, ASBVd). The former replicate in the nucleus, whereas the latter replicate in the chloroplast. Both RNAs replicate by a rolling circle mechanism that generates multimeric progeny RNAs, which are cleaved and ligated to generate new circular viroids⁹⁴ (Fig. 72.28).

Viroids rely entirely on host proteins for replication and movement in the plant.⁷⁵ DNA-dependent RNA polymerase II is “fooled” into recognizing RNA of the *Pospiviroidae* and

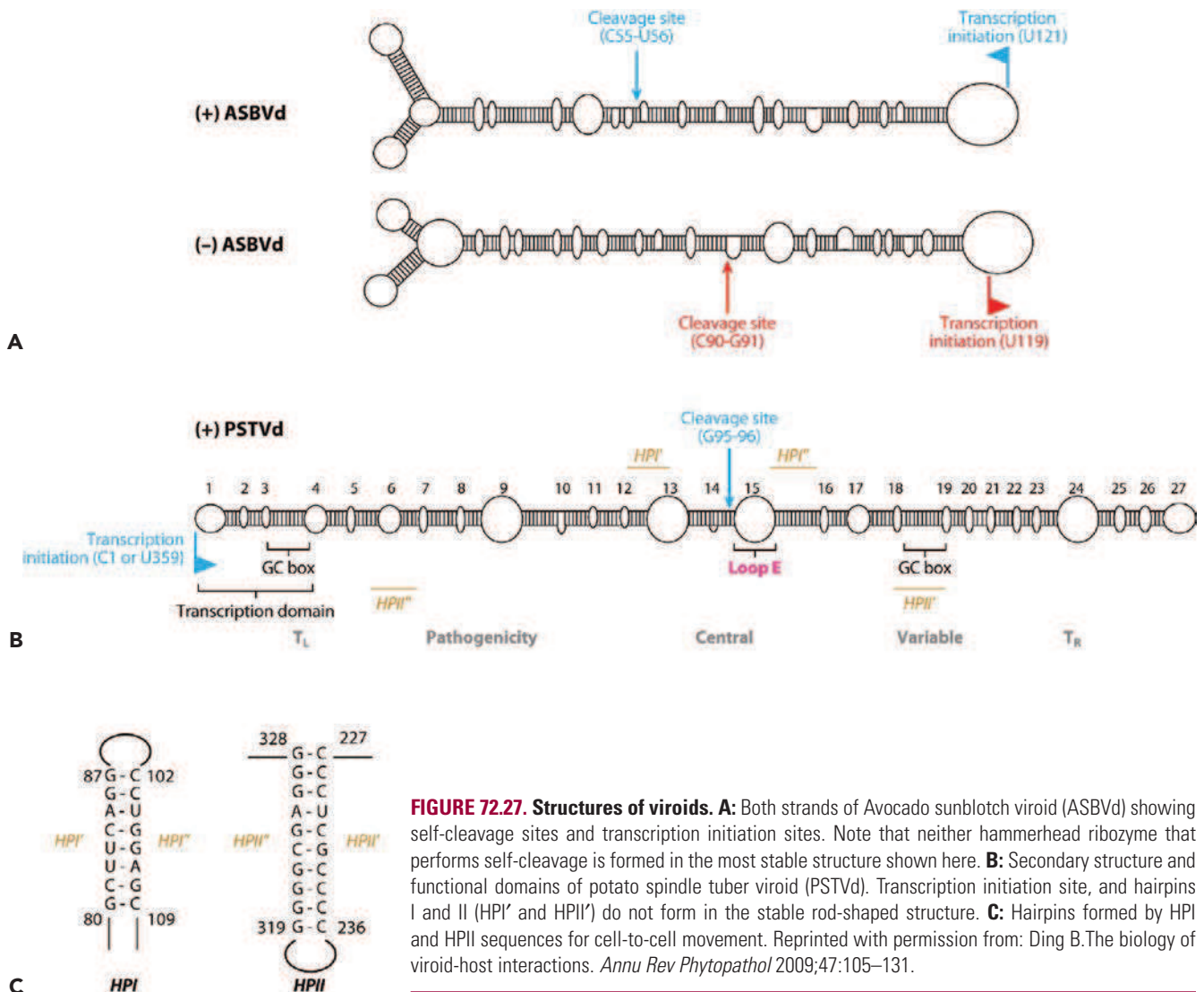


FIGURE 72.27. Structures of viroids. **A:** Both strands of Avocado sunblotch viroid (ASBVd) showing self-cleavage sites and transcription initiation sites. Note that neither hammerhead ribozyme that performs self-cleavage is formed in the most stable structure shown here. **B:** Secondary structure and functional domains of potato spindle tuber viroid (PSTVd). Transcription initiation site, and hairpins I and II (HPI' and HPII') do not form in the stable rod-shaped structure. **C:** Hairpins formed by HPI and HPII sequences for cell-to-cell movement. Reprinted with permission from: Ding B. The biology of viroid-host interactions. *Annu Rev Phytopathol* 2009;47:105–131.

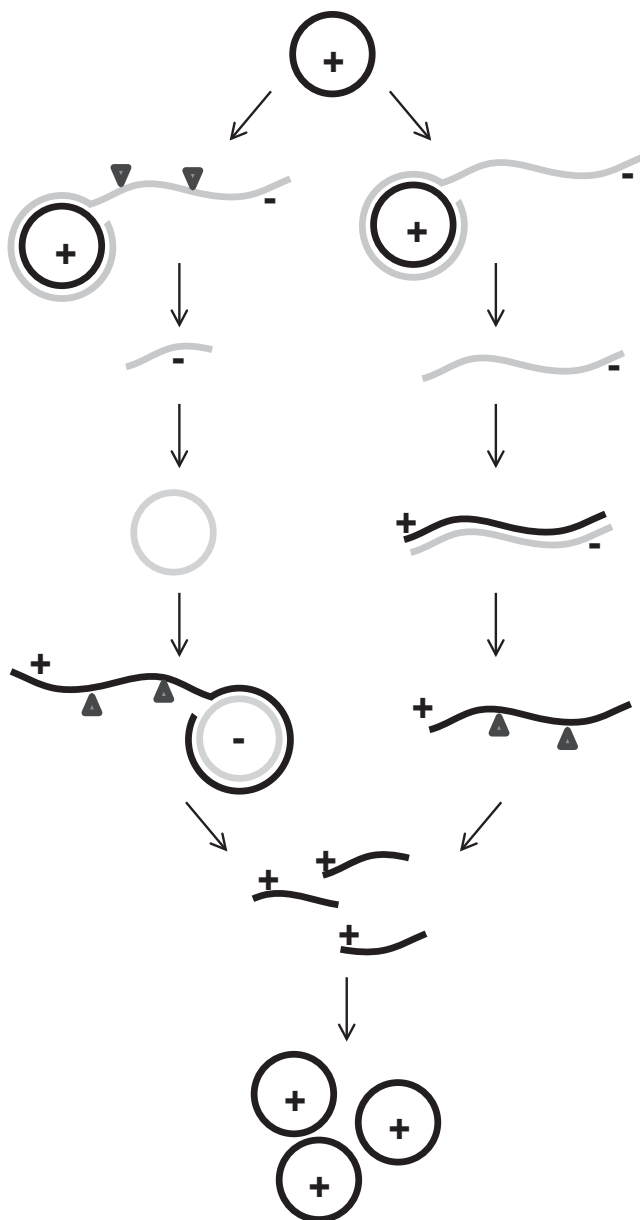


FIGURE 72.28. Rolling circle replication mechanism of viroids and some satellite RNAs. Infectious strand is defined as + strand (dark line). Triangles indicate cleavage sites. **Left column:** symmetrical replication mechanism in which multimeric minus-strand generated by rolling circle copying of plus strand is cleaved into monomers and circularized prior to serving as template for plus-strand synthesis. **Right column:** asymmetrical replication mechanism in which minus strand remains uncleaved and the multimeric form serves as template for plus-strand synthesis.

serves as the viroid replicase.²⁵⁶ A specific domain in the viroid RNA serves as origin of replication.¹⁶⁸ A different domain, loop 6, is required for cell-to-cell movement³¹⁵ (see Fig. 72.27). *Positiviridae* require an RNase III-type enzyme for cleavage and an as-yet unidentified ligase, other than tRNA ligase, for ligation.¹⁰⁹ In contrast, the *Avsunviridae* contain hammerhead ribozymes in both strands that self-cleave the multimers into monomers.⁹³ It is important to note that viroids and satellite

RNAs form metastable structures: the same sequence folds to form different structures, each of which has a different function, at different stages of the replication cycle (see Fig. 72.27). For example, two hairpin loops, not formed in the native viroid rod, are essential for PSTVd replication.²⁹³

Satellite RNAs

In contrast to viroids, satellite RNAs and satellite viruses depend on a helper virus for replication and cell-to-cell movement. Satellite viruses encode their own coat protein gene, distinct from the helper virus. For example Satellite tobacco necrosis virus (STNV) encodes a CP that packages only the 1260 nt STNV RNA in a T = 1 virion, whereas the helper virus, TNV, which replicates both its own 5-kb RNA and STNV RNA, packages only TNV RNA in a T = 3 virion made of TNV and not STNV CP subunits.¹⁵⁴ Satellite tobacco mosaic virus differs even more from its helper by forming a spherical virion, whereas TMV, of course, forms rods.³³¹

Satellite RNAs, in contrast to satellite viruses, do not encode CP. They rely on the helper virus for replication and encapsidation. Satellite RNAs come in two varieties: linear-only RNAs, and viroid-like RNAs that replicate via the rolling circle mechanism (Fig. 72.28). The viroid-like satellite RNAs have no ORFs, but some linear satellite RNAs encode non-structural proteins. For example, the satellite RNA of Bamboo mosaic virus encodes its own movement protein.³³⁸ In contrast, the linear satellite CMV RNA encodes no proteins.

Satellite CMV RNA was discovered by its severe effect on symptoms caused by CMV.³³¹ In 1972, the tomato crop in Alsace was decimated by a CMV outbreak, with far more severe symptoms than usual. The culprit turned out to be a satellite RNA associated with CMV, originally called CARNA 5, now called satellite CMV RNA.¹⁵⁷ Interestingly, this same virus-satellite combination that is lethal in tomato, attenuates disease symptoms of the helper virus in most hosts (Fig. 72.29).³⁵² The simplest explanation for attenuation is that, like a defective interfering (DI) RNA, satellite RNA competes for the helper virus' replication machinery, reducing levels of the helper virus. However, recently it has been observed that the high levels of satellite RNA induce a strong antiviral RNAi response, which would attenuate virus replication and disease symptoms.¹⁴⁰ In contrast, the Y-satellite RNA of CMV causes bright yellowing symptoms in tobacco, because a small RNA generated from it by the host RNAi machinery, fortuitously has homology to the chlorophyll biosynthetic gene, *ChlI*. This results in host degradation of its own *ChlI* gene, which in turn causes bright yellow symptoms.^{101,299,306} Consistent with this observation, plants transformed with a viral silencing suppressor that reduced the host antiviral response, showed much milder symptoms upon infection with CMV and satellite RNA.³⁴⁶

Like viroids, viroid-like satellite RNAs replicate by a rolling circle mechanism, but replication takes place in the cytoplasm and is carried out by the helper virus' RNA-dependent RNA polymerase. The RdRp presumably recognizes an origin of replication, then it copies the circular "positive" strand RNA (the strand that is encapsidated), to make multimeric negative strands. In some satellites, this multimer serves directly as template for synthesis of multimeric positive strands⁶⁴ (see Fig. 72.28, right). In other satellites, the multimeric negative-strand self-cleaves at either a hammerhead or a hairpin ribozyme, and then is circularized by ribozyme or host

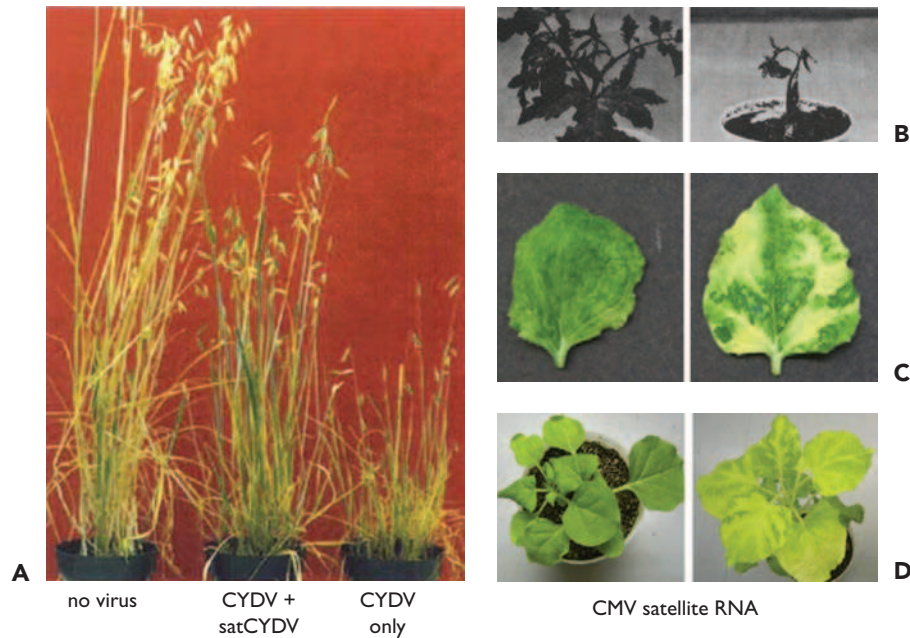


FIGURE 72.29. Effect of satellite RNAs on virus disease symptoms. **A:** Oat plants 3 weeks after inoculation with indicated virus-satellite RNA combination. (A reprinted with permission from Rasochova L, Miller WA. Satellite RNA of barley yellow dwarf-RPV virus reduces accumulation of RPV helper virus RNA and attenuates RPV symptoms in oats. *Molecular Plant-Microbe Interactions* 1996;9:646–650.) **B:** Tomato plants inoculated with ***CMV alone (left), or with CMV + original CARNA 5 satellite RNA (right). (B reprinted with permission from Waterworth HE, Kaper JM, Tousignant ME. CARNA 5, the small cucumber mosaic virus-dependent replicating RNA, regulates disease expression. *Science*. 1979;204:845–847.) **C:** *Nicotiana benthamiana* leaves infected with Cucumber mosaic virus (CMV) alone (left) or CMV + Y-sat RNA. **D:** *N. benthamiana* plants infected with CMV + Y-sat mutated to lack sequence homology with *ChII* RNA (left), or with CMV + wild-type Y-sat RNA (right). (C reprinted with permission from Shimura H, Pantaleo V, Ishihara T, et al. A viral satellite RNA induces yellow symptoms on tobacco by targeting a gene involved in chlorophyll biosynthesis using the RNA silencing machinery. *PLoS Pathogens* 2011;7:e1002021.) Top, tomato; middle, pepper; bottom, maize. Notice that CMV satellite RNA greatly exacerbates symptoms in tomato, but attenuates symptoms in pepper and maize.

ligase activity⁴⁰ (see Fig. 72.28, left). This circular negative strand then serves as template for production of multimeric positive strands. The positive-strand multimers self-cleave at a hammerhead ribozyme site. Some are then ligated into circles before packaging in the virion along with helper virus genomic RNA. In others, the linear cleaved RNA is encapsidated, with monomers being most abundant, but linear dimers, trimers, and so on, are also present.²¹³ These RNAs are circularized upon disassembly of the virion as it invades the cell. Like viroids, the viroid-like satellite RNAs form different structures to perform different functions throughout the replication cycle.^{51,307} Like linear satellite RNAs, viroid-like satellite RNAs usually reduce symptoms caused by the helper virus. As a result, transgenic expression of satellite Tobacco ringspot virus RNA can serve as a resistance gene.¹¹²

Satellite RNAs differ from defective interfering (DI) RNAs in that DI RNAs contain sequences derived from the helper virus, whereas satellite RNAs have no sequence homology to the helper.³⁰² Because DI RNAs harbor the replicase recognition site(s) from the helper virus,³⁵⁷ it is obvious how they compete with helper viral RNA for the RdRp. It is less obvious how the helper virus RdRp recognizes the satellite RNA, which bears no sequence or obvious structural similarity to the helper. It may be that this difference allows the satellite to be an even more efficient template than the helper virus RNA.

Understanding viroid and satellite RNA replication has led to a better understanding of hepatitis δ virus (HDV) RNA, which was discovered more recently.⁹⁴ Like many viroids, HDV RNA is replicated by host DNA-dependent RNA polymerase and uses a rolling circle replication mechanism that is dependent on ribozyme self-cleavage. It differs in that it is much larger than the plant satellites and viroids and it encodes a protein, delta antigen, in its negative strand. HDV has been proposed to be a fusion of a viroid and an mRNA,³⁶ which would be the first example of a plant virus or viroid providing some of the starting material for evolution of a human pathogen.

HOST DEFENSES AND VIRAL COUNTERMEASURES

The responses of host plants to viruses are determined by the interactions of viral proteins and nucleic acids with host factors as well as host surveillance systems that protect plant cells against virus infection. Resistance to viruses can be determined by specific recognition that is mediated by dominant resistance (R) genes, by recessive resistance genes that include defective host factors, or by RNA silencing. The recessive resistance mediated by alleles of *eIF4E* and *eIFiso4E* was discussed in the section on potyviruses. Here we focus on dominant resistance genes and RNA silencing.

Dominant Resistance Genes

Dominant, monogenic virus resistance traits mediate defense against specific strains of a virus species, an entire virus species, or several virus species within a genus. The *N* gene of tobacco is an example of an *R* gene with broad specificity, because it confers resistance to all tobamoviruses except the Ob strain of TMV.²³⁵ *R* gene-mediated resistance is frequently accompanied by (a) programmed cell death, (b) induction of reactive oxygen species, (c) induction of defense-related proteins, (d) accumulation of the phytohormone salicylic acid, (e) accumulation of defense-related secondary metabolites, and (f) fortification of cell walls. These concomitant events are known collectively as the hypersensitive response (HR) that is accompanied by the appearance of macroscopic necrotic lesions (local lesions) at the site of inoculation. Another form of induced resistance to viruses is extreme resistance. It is also mediated by *R* genes, but the plants do not display local lesions and remain symptomless. Genes that confer extreme resistance prevent virus replication and movement in initially infected cells. A virus strain that is recognized by an *R* gene is said to be avirulent. In many cases, it has been possible to map the particular amino acids in viral proteins that determine recognition by *R* genes. This is typically accomplished by exchanging domains of viral strains that differ in their abilities to infect a plant carrying the *R* gene. Frequently, a single protein such as replicase, movement protein, VSR, or coat protein is the avirulence determinant.^{47,308} However, the avirulence determinant may be more complex for some resistance traits. For example, the HC-Pro and P3 proteins of Soybean mosaic virus are both involved in recognition by *Rsv1* in soybean.^{85,127,128,356}

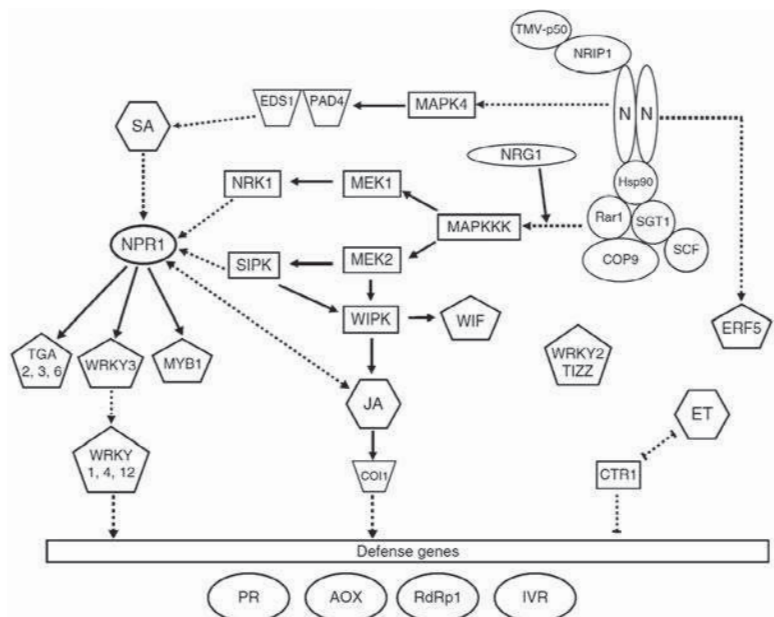
The dominant-resistance genes that mediate HR or extreme resistance and have been cloned fall into a large class known as nucleotide binding site leucine-rich repeat proteins (NB-LRR).^{47,98,308} These proteins can be further subdivided into the TIR-NB-LRR and CC-NB-LRR subclasses based on the sequences at their amino termini. The TIR-NB-LRR proteins have similarity to the cytoplasmic domains of the Toll and

Interleukin-1 receptors, whereas amino termini of CC-NB-LRR proteins are predicted to have a coiled-coil structure. The complete mechanism by which these proteins signal is not understood. The most work has been done with the *N* protein (a TIR-NB-LRR) that recognizes the TMV RdRp (Fig. 72.30), and the *Rx1* protein (a CC-NB-LRR) that recognizes the PVX coat protein.

There are two basic models for how plant *R* proteins recognize pathogens, including viruses. The ligand-receptor model posits that the *R* protein, and the component of the virus that is recognized, interact directly. Alternatively, in the guard model, the *R* protein monitors the status of a host protein that interacts with—or is a target of—a viral protein. Therefore, the *R* protein indirectly detects the presence and activity of the viral protein. The guard model has been demonstrated for the *N*-TMV interaction and the *Rx1*-PVX interaction. The TIR domain of *N* interacts with a pre-recognition complex containing a chloroplast protein named NRIP1 (N receptor-interacting protein) and the TMV replicase.^{43,46} Subsequently, *N* may oligomerize via its TIR domain,²¹⁰ and then localize to the nucleus.^{43,46} Localization to the nucleus is required for *N*-mediated resistance,⁴³ suggesting that activated *N* reprograms transcription.²³⁶ *N* function also involves the interaction of its LRR domain with HSP90, which recruits the co-chaperones RAR1 and SGT1.^{191,197,242} RAR1 and SGT1 in turn recruit the COP9 signalosome.¹⁹² Recognition of the NRIP1-TMV replicase interaction by *N* activates downstream signaling networks that require the phytohormones salicylic acid (SA), jasmonic acid, and ethylene, as well as numerous signal transduction genes including NPR1, MAP kinase cascades, and transcription factors such as WRKY, TGA, and MYB⁴⁷ (see Fig. 72.30).

The CP of PVX is the elicitor of *Rx*-mediated resistance, but its recognition appears to be indirect.²¹⁹ Recognition of PVX CP occurs through the C-terminal region of the LRR domain of *Rx*,²⁵⁸ and this recognition specificity can be altered and extended by mutations within the LRR domain.⁹¹ Intramolecular interactions between CC and NBS-LRR domains or CC-NBS and LRR domains correlate with an inactive *Rx*

FIGURE 72.30. Model of a virus resistance signaling pathway based on the *N* gene for TMV resistance from tobacco. *N* recognizes the complex of the chloroplast protein NRIP1 bound to the TMV-p50 elicitor portion of the viral replicase protein. The recognition activates a signaling network that is regulated by phytohormones including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). This network results in increased transcription of defense genes. For details, see text and.⁴⁷ (From Carr JP, Lewsey MG, Palukaitis P. Signaling in induced resistance. *Adv Virus Res* 2010;76:57–121, with permission.)



conformation. In the presence of CP elicitor these interactions are disrupted, providing evidence that Rx is activated when interactions between its C-terminus and LRR are abolished.^{257,258} Rx, like N and other NB-LRR proteins, also requires HPS90, RAR1, and SGT1 for its function.

In host–virus interactions that produce HR locally, there is also typically induction of a secondary defense response in other noninoculated parts of the plant known as systemic acquired resistance (SAR).^{47,308} SAR increases resistance to the pathogen that initially triggered HR and enhances broad-spectrum resistance to a variety of viruses, bacteria, and fungi. SAR is caused by increased accumulation of SA in systemic tissues.³⁴⁰ SA is transported systemically as methyl salicylate (MeSA), which is demethylated in the target leaves, leading to increased levels of SA systemically.²⁴⁰ The accumulation of SA in concert with lipid-based signals activates defenses in the systemic tissues.^{189,341} SA induces accumulation of reactive oxygen species and expression of a set of pathogenesis-related (PR) defense proteins. Currently there is little understanding of how SA-dependent responses mediate antiviral resistance. However, some components of antiviral silencing discussed

below, such as RNA-dependent RNA polymerase 1, are induced by SA, suggesting that SA-mediated defenses and antiviral RNA silencing pathways intersect.⁴⁷ This idea is supported by the finding that the CMV-2b proteins, which suppresses RNA silencing (discussed below), also interferes with SA-mediated defenses in Arabidopsis.¹⁵¹

PTGS—An Inducible, Adaptable Innate Immune Response

One of the major functions of the RNA silencing system in plants and other eukaryotes is to protect cells against foreign, nucleic acid–based invaders including RNA viruses, DNA viruses, and viroids.^{61,76,77,298,303} The importance of RNA silencing in antiviral defense is punctuated by the fact that most, if not all, plant viruses encode suppressors of RNA silencing (VSRs) that function by a variety of different mechanisms.^{44,61,76,77,298}

Key components of the plant antiviral RNA silencing system are Dicer-like (DCL), Argonaute (AGO), and RNA-dependent RNA polymerase (RdRp) proteins (Fig. 72.31). These proteins function in concert to produce and amplify virus-derived small RNAs (vsRNAs) in order to direct degradation or modification

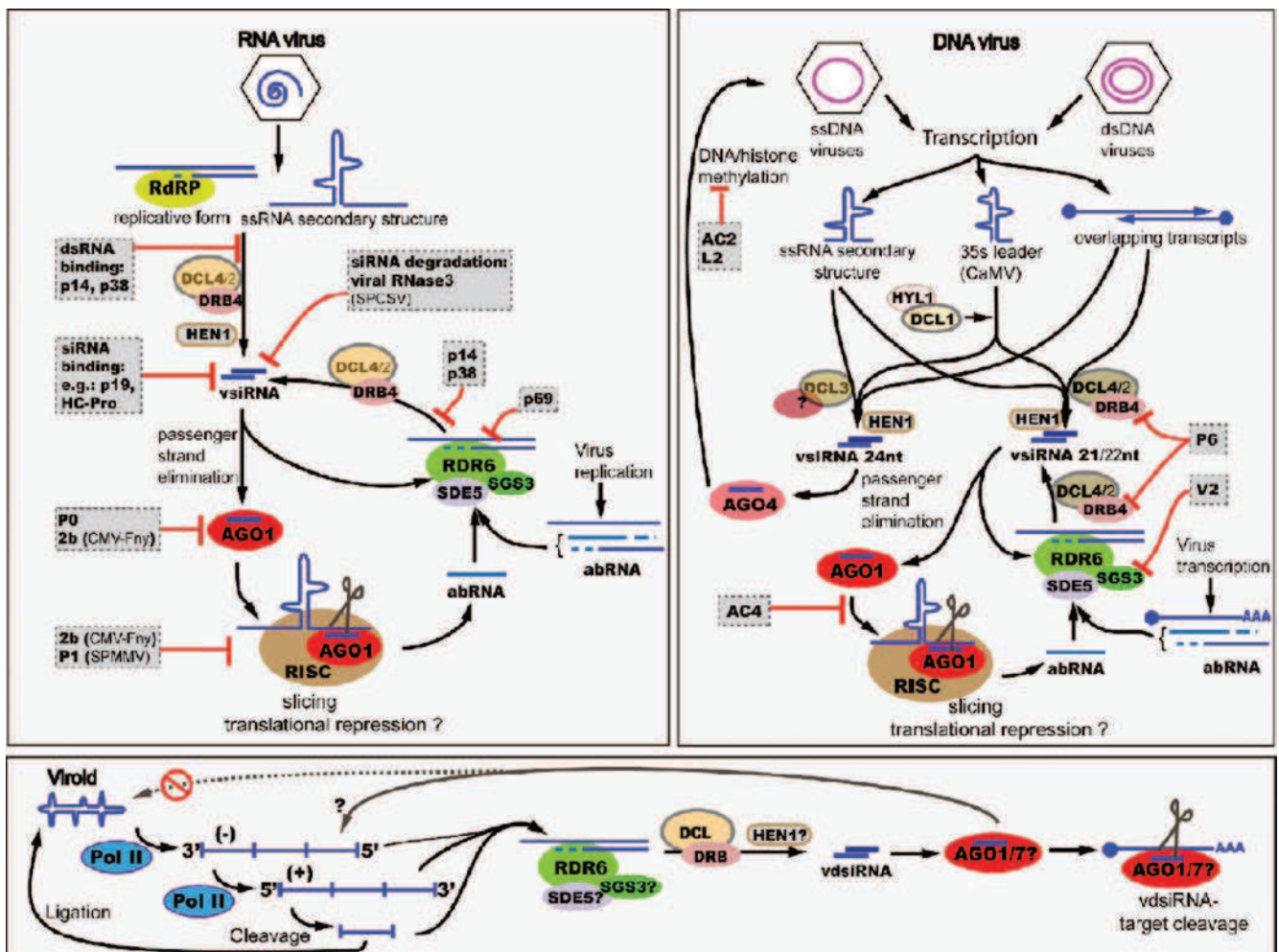


FIGURE 72.31. Antiviral RNA silencing pathways directed against viruses and viroids. Silencing suppressors and their mechanisms are shown in gray boxes. The red bars indicate the points in the silencing pathways in which they interfere. For details, see text. (From Csorba T, Pantaleo V, Burgyn J. RNA silencing: an antiviral mechanism. *Adv Virus Res* 2009;75:35–71, with permission.)

of nucleic acid–based pathogens or inhibit their translation. Unlike the genetic resistance described above, antiviral RNA silencing is adaptive because RNA silencing is initiated by dsRNA, irrespective of origin and sequence.

The mechanisms of antiviral RNA silencing have been investigated in most detail in the model plant, *Arabidopsis thaliana*. *Arabidopsis* encodes four DCL enzymes (DCL1 – 4) that possess ribonuclease III activity. DCL1 processes dsRNA into 21 nt fragments, and is primarily responsible for processing microRNAs from their precursor RNAs encoded by the host. DCL1 also processes dsRNAs produced in the nucleus by DNA viruses, such as CaMV (e.g., 221). DCL3 processes dsRNA derived from heterochromatic regions of the genome or from DNA viruses into 24 nt fragments that direct methylation of homologous DNA sequences. DCL2 and DCL4 process dsRNA into 22 nt and 21 nt fragments, respectively, and these enzymes participate in antiviral silencing directed against plus-stranded RNA viruses. DCL4 functions in the primary role in this pathway, whereas DCL2 serves in an accessory role. Loss of function of both DCL2 and DCL4 is necessary to abrogate antiviral silencing. Interestingly, 24 nt vsRNAs generated by DCL3 accumulate during plus-strand RNA virus infection, but DCL3 alone is not sufficient for antiviral defense.¹⁰⁸ Antiviral silencing against plus-strand RNA viruses initiates when dsRNAs produced during replication, gene expression, or regions of duplex RNA secondary structure are cleaved by DCL2 and DCL4 to produce populations of 21 and 22 nt vsRNAs in the infected cells.

Deep sequencing revealed vsRNAs distributed across entire viral genomes. However, some regions of viral genomes are “hotspots” from which disproportionate numbers of vsRNAs are derived. These hotspots correlate with regions of secondary structure in the viral genomes.^{221,313} However the correlation is not perfect,²⁵³ leading to a hypothesis that template specificities of RdRps may be associated with some hotspots.

The *Arabidopsis* genome encodes 10 AGO proteins, of which AGO1, AGO2, and AGO7 have been implicated in antiviral silencing.^{133,148,224,254,289,349} AGO proteins function downstream of DCL proteins by using the sRNA products as guides to direct both endonucleolytic cleavage and translation inhibition of RNA species containing homologous sequences, as well as methylation of homologous DNA sequences.^{142,336} AGOs are the catalytic components of the RNA-induced silencing complexes (RISCs). AGO proteins incorporate one of the strands from the sRNAs generated by the DCL enzymes, whereas the other strand is degraded. The incorporated sRNA then serves as a guide to direct the RISC to targets containing complementary sequences including viral genomes and mRNAs.^{61,76,77,298,303} In most cases, the endonuclease activity in the piwi domain of AGO slices the target RNA of viral origin. Cleaved viral RNAs are degraded rapidly by cellular exonucleases. Therefore, RISC is programmable, adaptable, exquisitely specific, and serves as a second layer of antiviral RNA silencing defense.

RdRps provide plant cells with the ability to amplify vsRNAs, which is needed for effective antiviral silencing.²⁵³ In antiviral defense, RdRp levels increase during viral infection^{151,365} and direct synthesis of nascent double-stranded RNA (dsRNA) using templates of viral origin. These dsRNAs are then processed by the DCL enzymes to generate additional vsRNAs that can be bound by AGO1 to direct specific

degradation or modification of viral templates. The *Arabidopsis* genome encodes six RdRps, and of these, RDR1 and RDR6 function mainly in antiviral defense.^{108,252,253,350} *rdr1 rdr6* double mutants support systemic infections by mutant viruses that lack VSR activity such as CMVΔ2b and TuMV-AS9, which cannot infect wild-type plants.^{108,350} Current evidence indicates that RDR1 and RDR6 interact synergistically, because the *rdr1 rdr6* double mutant leads to much higher levels of CMVΔ2b genomic RNA than either single mutant.³⁵⁰ The activity of RDR1 is required for high levels of TuMV and CMV vsRNAs, because these are drastically reduced in *rdr1* mutants. However, *rdr1 rdr6* double mutants are much more susceptible to TuMV-AS9 or CMVΔ2b than the single mutants.^{108,350} The explanation for this is that RDR1 and RDR6 have only partially redundant functions, and they appear to have different biases in their template preferences. RDR1 is involved in producing vsRNAs derived from RNAs 1, 2, and 3 of the CMV genome, but RDR6 is biased toward production of vsRNAs from RNA 3 and the subgenomic RNA 4. In addition, RDR1 is biased toward production of vsRNAs from the 5′ ends of CMV RNAs, whereas RDR6 has a bias toward the 3′ end. These template preferences would be advantageous, for example, if a VSR suppressed RDR1 function, leaving the plant with a functional RDR6 to limit viral infection.

DNA viruses are also targets of antiviral RNA silencing pathways.^{61,76,77,260,298,303,334} vsRNAs corresponding to the pararetrovirus CaMV and geminiviruses such as Cabbage leaf curl virus (CaLCuV) are readily detectable in infected plants.^{26,52,220} The biogenesis of vsRNAs from CaMV involves all four DCLs, and the 21, 22, and 24 nt classes of vsRNAs are found in CaMV-infected plants.^{26,220} Interestingly, in *dcl1*, partial loss-of-function mutants, accumulation of all three size classes is reduced suggesting that DCL processing of CaMV transcripts is hierarchical and facilitated by DCL1. DCL1 produces CaMV vsRNAs by processing the highly base-paired stem-loop region of the 35S leader of CaMV to produce 21 nt vsRNAs, which may improve accessibility of this structure to the other DCLs. Geminivirus vsRNAs are also 21, 22, and 24 nt, but their biogenesis does not depend on DCL1. Accumulation of 21 nt vsRNAs from CaLCuV is abolished in *dcl4* loss-of-function mutants.²⁶ Secondary structures within the geminivirus transcripts as well as regions of overlapping complementarity between the 3′ termini of convergent transcripts provide dsRNA substrates on which DCL2 – DCL4 act.³³⁴ Although geminivirus transcripts are posttranscriptionally targeted, their DNA genomes are the target of transcriptional gene silencing (TGS). The 24 nt vsRNAs bound to AGO4 direct methylation of complementary viral DNA sequences cause TGS. AGO4 mutants are deficient in RNA-directed DNA methylation and as a result they are hypersusceptible to geminiviruses.²⁵⁹

Viral Suppressors of RNA Silencing (VSRs)

Although the innate plant defense systems are effective, it is clear that viruses have evolved diverse strategies to counteract plant defenses (see Fig. 72.31). The ability of a virus to suppress defenses in a particular host species or group of related species is one factor that enables systemic infection. The best-characterized counter defense deployed by viruses is suppression of RNA silencing by VSRs.^{44,61,76,77,298} VSRs are typically identified based on their ability to prevent the initiation of

RNA silencing or their disruption of the maintenance of established RNA silencing of marker genes such as GFP. Most RNA and DNA plant viruses encode at least one VSR with some viruses in the *Closteroviridae*, such as Citrus tristeza virus (Closterovirus) and Tomato chlorosis virus (Crinivirus), encoding as many as three VSRs.¹⁹⁶ An extensive list of VSRs of plant DNA and RNA viruses is provided in reference 61. In general, VSRs are not conserved in sequence across broad taxonomic boundaries, and they employ different strategies to interfere with the RNA silencing pathway. However, a few major themes have emerged for mechanisms of VSR function including (a) the targeting of silencing-related RNAs and (b) the interaction of VSRs with silencing-related host proteins.⁶¹ These mechanisms inhibit viral RNA sensing and dicing, prevent RISC assembly, or interfere with epigenetic modification of viral genomes.⁴⁴

TARGETING SILENCING-RELATED RNAs

Sequestration of vsRNAs is the most widely used mechanism for suppression of RNA silencing for three major reasons. The first is that vsRNA sequestration prevents assembly of functional RISC. Second, vsRNA sequestration can prevent the proper processing and maturation of vsRNAs. Finally, sequestration prevents vsRNA mobility that allows vsRNAs to move ahead of the virus to establish an antiviral state in cells neighboring infected cells. The best characterized example of a VSR that sequesters vsRNAs is the P19 protein of tombusviruses. P19 functions as a homodimer that binds to the 19-bp dsRNA region of small RNA duplexes in a sequence-independent fashion. X-ray crystallography of P19 bound to small RNA duplexes demonstrated that it is a molecular caliper that specifically measures and binds to small RNA duplexes that would be produced by the primary antiviral dicing enzyme DCL4^{335,369} (Fig. 72.32). This binding prevents virus-derived small RNAs from being loaded into RISC where they would direct cleavage of the viral RNAs from which they were derived. VSRs from at least 11 plant virus genera have been demonstrated to have the ability to bind sRNAs.⁶¹ Sequestration of vsRNAs has also been associated with inhibition of 2' O-methylation of sRNAs in the cases of tombusvirus p19, potyvirus HC-Pro, and Cr-TMV p122.^{60,84,195,342} Methylation by the HEN1 (HUA ENHANCER1) methyltransferase is necessary for the maturation of sRNAs, and thus, nonmethylated vsRNAs are expected to accumulate at reduced levels. A final interesting example

of targeting silencing-related RNAs is the RNase3 protein of Sweet potato chlorotic stunt virus. This protein has an RNase III activity that cleaves 21, 22, or 24 bp products of DCLs into 14-bp fragments that cannot function in RNA silencing.⁶²

INTERACTION OF VSRs WITH SILENCING-RELATED PROTEINS

AGO proteins are frequent targets of VSRs, through either direct or indirect interactions, and there is evidence that DCLs can be targets as well.^{68,125,208,209} The P0 protein of Potato leaf roll virus is essential for infection; it interacts with the PAZ domain and other nearby regions of AGO proteins. Through its F-box motif, P0 recruits SCF E3 ubiquitin ligase proteins, which promote the degradation of the bound AGO.^{19,30,241} The 2b protein of CMV strain Fny interacts with AGO1, and seems to directly inhibit its activity.³⁷⁶ It is important to note that the 2b protein of CMV strain TAV has been shown to bind sRNAs,²⁶⁶ suggesting that the 2b proteins of CMV strains can function by different mechanisms. The P1 protein of Sweet potato mottle virus (*Ipomovirus*) and the p38 coat protein of TCV represent another strategy for targeting AGO. These VSRs were both found to contain (glycine tryptophan) GW motifs that mediate binding to AGO proteins.^{15,117} Interestingly, the presence of GW motifs appears to represent a form of molecular mimicry, because host-encoded proteins containing GW motifs are involved in assembly of functional RISC. Inhibition of AGO1 by TCV p38 was further shown to disrupt DCL homeostasis: DCL1 becomes overexpressed leading to decreased expression of DCL3 and DCL4, which is the primary antiviral DCL in Arabidopsis.¹⁵

In addition to serving as the translational activator (TAV) for translation reinitiation, CaMV P6 is a VSR that directly targets vsRNA processing.^{125,194} When P6 enters the nucleus, it interacts with dsRNA-binding protein 4 (DRB4). DRB4 is required for DCL4 activity, so P6 causes loss of this key antiviral silencing factor. VSRs of geminiviruses interfere with epigenetic modification of their genomes,²⁶⁰ which is interesting because methylation of geminivirus genomes inhibits their replication.^{38,88} The AL2 and L2 proteins of the geminiviruses Tomato golden mosaic virus and Beet curly top virus, respectively, are VSRs that interfere with the methylation cycle in order to promote viral replication. These VSRs bind and inactivate adenosine kinase (ADK), which is needed for the efficient production of S-adenosyl methionine (SAM), the donor of methyl groups on DNA and histones⁴¹ (Fig. 72.33).



FIGURE 72.32. Stereo view of the structure of a co-crystal of P19 with a 21 nt small interfering RNA (siRNA) at 1.85 Å resolution. P19 forms a dimer indicated in magenta and purple. Note the alpha helices that serve as calipers by framing the ends of the RNA helix via tryptophan residues that stack with the terminal bases of the RNA. (From Ye K, Malinina L, Patel DJ. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature*. 2003;426:874–878, with permission.)

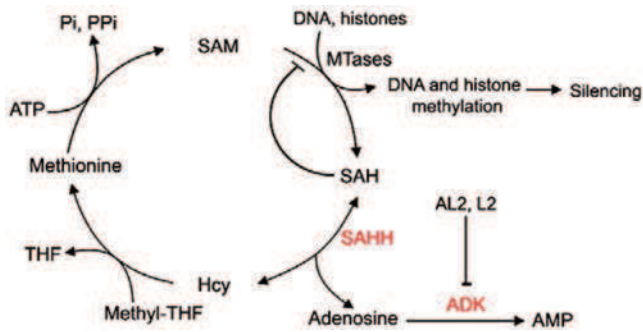


FIGURE 72.33. Inhibition of the methyl cycle by the AL2 and L2 viral suppressors of RNA (VSRs) of geminiviruses. (From Bujarski JJ, Dreher TW, Hall TC. Deletions in the 3'-terminal tRNA-like structure of brome mosaic virus RNA differentially affect aminoacylation and replication in vitro. *Proc Natl Acad Sci U S A* 1985;82:5636–5640, with permission.)

Although many VSRs may function autonomously, some VSRs require host factors for their silencing suppressor activity. The potyvirus VSR HC-Pro and TCV p38 were both shown to require an ethylene-inducible host transcription factor named RAV2.⁸⁶ Among the *Arabidopsis* genes found to be upregulated by RAV2 in the presence of HC-Pro were *FRY1* and *CML38*, two endogenous suppressors of RNA silencing. These results suggest that suppression of RNA silencing by HC-Pro could involve the upregulation of a set of host genes that antagonize antiviral silencing defenses. It has also been postulated that the geminivirus VSR AL2 induces the expression of host genes that negatively regulate RNA-silencing pathways.³²³ This function of AL2 is distinct from its role in inhibiting the methyl cycle and suggests that this protein promotes viral infection by interfering with different silencing pathways.

The ability of VSRs to interfere with host RNA-silencing systems has many detrimental effects upon hosts in addition to the promotion of virus infection. The presence of ectopically expressed VSRs in transgenic plants can phenocopy many of the symptoms of viral infections, and many VSRs were known previously to be pathogenicity determinants.³⁴³ Inhibition of DCL or AGO by VSRs can interfere with the biogenesis and function of endogenous siRNA species, such as miRNAs and trans-acting siRNAs (ta-siRNAs), which regulate many aspects of plant growth and development, as well as responses to the environment.²⁹⁸ Expression of three VSRs—HC-Pro, P19, and pecluvirus P15—causes misregulation of transcription factor AUXIN RESPONSE FACTOR 8 (ARF8).¹⁴⁹ *ARF8* expression is regulated posttranscriptionally by AGO1 via miR167 during the normal course of plant growth and development. Developmental defects in the transgenic plants were alleviated when an *arf8* loss-of-function mutation was introduced into the VSR-expressing lines demonstrating that misregulation of *ARF8* is responsible for symptom-like phenotypes induced by these VSRs. In addition, *arf8* mutants supported TuMV accumulation similar to wild-type plants, but they did not develop symptoms similar to wild-type plants. Therefore, virus accumulation can be uncoupled from symptoms, and misregulation of genes such as *ARF8* have apparently neutral effects on virus titer in experimentally inoculated plants. It is important to note, however, that VSRs do not account for all the symptoms observed

in systemically infected plants. For example, in this *ARF8* study, the authors noted that the *arf8* mutation did not alleviate chlorosis induced by TuMV. Furthermore, when *dcl2 dcl4* double mutants are infected by TuMV-AS9 carrying a mutation in HC-pro or by CMVΔ2b mutants that lack VSR activity, they still display stunted growth phenotypes.^{77,108} These observations provide strong evidence that viral factors other than VSRs also contribute to symptoms.¹⁸⁰

The discovery and intense study of antiviral silencing has led to some important technological advances that have aided plant research and control of viral diseases. The ability of viruses to trigger the RNA-silencing pathway has been exploited extensively for gene function analysis by the method of virus-induced gene silencing (VIGS).¹⁸ VIGS provides a way to transiently and specifically knock down the expression of host genes (Fig. 72.34). Recent studies suggest that it may also be possible to exploit VIGS to alter host gene expression in a heritable fashion.^{155,295} Plant viruses engineered as VIGS vectors by carrying inserts homologous to plant genes silence the homologous plant gene as systemic infection occurs.²⁵⁰ This happens because sRNAs corresponding to the plant gene are generated as the plant attempts to defend itself against the recombinant virus. These sRNA are incorporated into RISC where they direct cleavage of the recombinant viral RNA as well as the host mRNA corresponding to the homologous sequences in the virus. The antiviral RNA silencing pathway can also be primed to protect plants against nucleic acid-based invaders. The expression of inverted repeat constructs or artificial microRNAs corresponding to viral sequences can be used to effectively engineer resistance against plant viruses.³⁰³ Indeed studies investigating pathogen-derived resistance through generation of transgenic plants carrying portions of viral genomes provided early clues about the roles of RNA silencing pathways in eukaryotes.^{185–187}



FIGURE 72.34. Virus-induced gene silencing (VIGS) of *Phytoene desaturase* (PDS) in soybean. A *Bean pod mottle virus* vector carrying a 300-bp fragment of *PDS* was used to infect soybean plants. The soybean leaves appear white, because *PDS* is needed to produce carotenoid pigments that protect chloroplasts from photobleaching.

FUTURE PERSPECTIVES

Virus–Host Interactions

Although some molecular details of plant virus structure and replication are among the best characterized of all viruses, much remains to be learned, of course. We end by discussing a few areas of plant virus research that are moving rapidly and promise to open new vistas in our understanding of plant viruses. In general, the host side of the virus–host interaction is much more complex than the virus with its small genome and handful of proteins. For example, although cis-acting viral translational control signals are well studied, the interactions with host components and their effect on host translation in general are not well known. These host components include translation initiation factors,³¹⁹ translation elongation factors, ribosomal proteins,¹⁸² and regulatory kinases such as TOR and S6K1.^{288,367} The fact that host proteins involved with translation have been associated with viral replicases also implicates them in viral replication.^{183,366} More detailed understanding of these interactions will help to resolve the important problem of how viral RNAs are able to outcompete host mRNAs to successfully parasitize ribosomes for translation yet eventually serve as templates for viral replication.

Innate Immunity Signaling Pathways

Many details of host resistance mechanisms have been uncovered, but important outstanding questions remain. Antiviral silencing pathways are known, but the complexity of some of the gene families involved, such as the AGO family, allows for specialization and subfunctionalization of the participating proteins. Additional work is needed to firmly establish the functions of the various gene family members in antiviral silencing. As we move away from model organisms and into crop species that have much more complicated genomes, we may discover more interesting nuances in the functions of proteins involved in antiviral silencing. The many VSRs that have been identified offer critical reagents with which to dissect the antiviral silencing pathways.

The signaling pathways activated by viral resistance genes have been studied in great detail. However, we still lack a clear understanding of the biochemical details of recognition and early signaling events. The complexity of and our incomplete understanding of plant defense responses has also complicated efforts to understand the molecular basis for how a plant becomes resistant to a virus after defenses are activated by *R* genes. What specific response or group of responses that occur during hypersensitive cell death actually inhibit further viral infection? There are interesting links between *R* gene–mediated resistance and antiviral silencing that merit further exploration such as the induction of genes involved in RNA silencing⁴⁷ and the recent discovery that expression of the *N* gene encoding TMV resistance in tobacco is regulated by miRNAs.¹⁸¹

Plant Virus Cell Biology

The cell biology of viral processes in plants has long lagged behind that of animal viruses. However, rapid progress is being made in our understanding of the structure and localization of virus replication vesicles (replication factories), and in intracellular and intercellular trafficking of the viral genome from these replication factories to the plasmodesmata and into the neighboring cell.^{173,231} This has been achieved through electron microscopy and tomography to reconstruct three-dimensional images of the perinuclear endoplasmic reticulum, for example.⁶⁹ In addition,

the use of multiple fluorescent proteins fused to host proteins with known subcellular localization sites and bimolecular fluorescence complementation assays have enabled the interactions of host and viral proteins and their trafficking to be unveiled.^{32,353} These and other systems biology approaches to characterize entire virus–host protein interaction networks^{182,218,227} are becoming the norm for modern plant virology. With the application of these and other new techniques such as VIGS and next-generation sequencing, we are indeed—as noted by Karen-Beth Scholthof—“at the dawn of mechanistic plant virology studies”.²⁹⁰

Plant Virus Ecology

Finally, a completely different emerging field of research is the role of plant viruses in ecosystems.³⁶⁴ In this burgeoning research area centered on the effects of pathogens on wild (nonagricultural) ecosystems, plant viruses serve as model organisms.^{202,248} In particular, several ecology laboratories are investigating the role of BYDV and CYDV in the “battle” between perennial native grasses and invading annuals. It appears that wild oats (weeds) imported from Europe have taken over the grasslands of California in part by their ability to accumulate virus, transmit it to the native perennials (via aphids), and then shed the virus by dying in the winter, leaving virus-free seed to sprout each spring. The uninfected progeny oats then outcompete the native perennials, which remain infected from year to year, reducing growth rate, and rendering them less fit.^{29,201}

The advent of next-generation sequencing has revolutionized plant virus ecology by allowing massively parallel, high-throughput genome sequencing of all viruses in an entire ecosystem.²⁷⁹ These “ecogenomics” techniques have revealed plant virus populations in Costa Rican forests spanning a wide range of ecosystems,^{279,364} and in native tall grass prairies in the central United States.²⁸⁷ As these environmental virus sequencing projects continue, it is likely that the next few years will see a many-fold increase in the number of known plant viruses.

EPILOGUE

The authors realize that many viruses and major areas of plant virology were not included in this review, as this chapter was not intended to be a comprehensive summary of all plant virology. Our intent has been to provide representative examples of plant viruses and plant viral functions. We hope that the reader has come away with an understanding of plant virus genome structure and function, and vector and host interactions that provides insight on viral mechanisms in general, including those relevant to research on vertebrate viruses. Moreover, this review is intended to spotlight major differences between plant and animal viruses, owing to the fundamental differences between plant and animals, and to celebrate the diversity of life at all levels including that of the virus.

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Insect Viruses

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IMPACT OF INSECT VIRUSES

Insects comprise well over half of the existing animals on earth. They include an estimated six to ten million species and represent some of the most diverse and successful animals on the planet. Accordingly, the RNA and DNA viruses infecting insects are also highly diverse. It is likely that these viral pathogens have existed as long as the insects themselves. They therefore have evolved along with their hosts for more than 350 million years. Insect viruses inflict disease and mortality in their hosts. Certain insect viruses are also serious pathogens of vertebrate animals, humans included. Thus, the insect viruses have long been of medical concern, agricultural relevance, and scientific interest to humankind. Indeed, much of the early research on insect viruses focused on their impact on human health and their applied use in insect control strategies. As molecular studies of insect viruses advanced, it was quickly appreciated that these viruses provide important tools for investigating fundamental problems in molecular and cellular biology and have direct application to the field of biotechnology.

Molecular and Cell Biology, Virus Interactions, and Virus Evolution

Biochemical and genetic studies on the multiplication and host pathogenesis by insect viruses have contributed enormously to our understanding of molecular and cellular biology, virus structure and assembly, virus–host interactions, and disease states.

These advances are due in a large part to the diversity of insect viruses as well as the availability and convenience of cell cultures or live insects in which to propagate them. Such systems, when combined with the ease with which insect viruses can be engineered or genetically manipulated, provide remarkably high yields of virus particles or virus-encoded macromolecules for studies of gene structure and function. There are also distinct experimental advantages to the use of many insect hosts, including their relatively simple genomes that have been sequenced and annotated. Compared to vertebrates, insects exhibit limited genetic redundancy, a property that has simplified studies on host–virus interaction pathways. Finally, there exists a striking conservation of genes and genetic pathways between insects and mammals. Thus, discoveries with insects and their viruses have led to important insights for all animals, humans included. In a striking example, the identification of inhibitor-of-apoptosis (IAP) genes in the insect baculoviruses led to the discovery of IAP homologs in mammals and subsequently established the critical role of IAPs in cell survival, normal development, virus pathogenesis, and human diseases that include cancer and neurodegeneration (reviewed by 186,235,280,298,326). The baculoviruses encode other suppressors of apoptosis that have revealed novel mechanisms for the regulation of apoptosis and have provided important insight into the role of apoptosis in insects and mammals (see later discussion).⁵⁶

There are numerous examples by which insect viruses have advanced fundamentals of biology. The insect cypoviruses of *Reoviridae* were the first to reveal the critical 7-methyl GpppN cap structure on eukaryotic mRNAs.¹⁰² Cricket paralysis virus of the family *Dicistroviridae* has provided insight into the function and structure of internal ribosomal entry sites and novel mechanisms for initiation of protein synthesis.¹⁴⁴ The nodaviruses revealed the first example of an animal virus–encoded suppressor of host RNA silencing (also known as RNA interference [RNAi]).¹⁹⁶ Other novel RNAi suppressors have recently been identified in the dicistroviruses. Finally, the polydnaviruses have disclosed a remarkable symbiotic relationship between virus and host that involves virus-mediated immune suppression.^{20,302,345} Recent polydnavirus discoveries are reworking the definition of a virus. Insect viruses have collectively provided key understanding in numerous aspects of cell biology, host–virus interactions, and pathogenesis.

Insect viruses have also provided unique insight into virus evolution. Due to the close association of insects with plants and other animals, natural exchange and mixing of viruses is accelerated. Insect viruses within the families *Poxviridae*, *Rhabdoviridae*, *Reoviridae*, *Picornaviridae*, and *Parvoviridae* have vertebrate counterparts, leading to speculation that viruses of higher animals arose from insect virus ancestors. Certain insect

viruses have co-evolved with unrelated insect virus families. The striking conservation of specific genes among diverse insect virus families—including baculoviruses, entomopoxviruses, orthomyxoviruses, ascoviruses, and iridoviruses, as well as the newly discovered relationship between polydnaviruses and nudiviruses—is consistent with co-evolution of invertebrate viruses. Certain DNA viruses of insects also accommodate host-derived transposons and retroid elements, which are especially abundant in arthropods. Stable retention of such mobile genetic elements facilitates virus evolution and likely contributes to the exchange of genes between a virus and its host organism.²⁷⁸

This chapter describes those viruses that multiply primarily or exclusively in insects: baculoviruses, polydnaviruses, ascoviruses, nudiviruses, nodaviruses, tetraviruses, and the dicistroviruses. Viruses such as the arboviruses, in which insects are used as temporary hosts or vectors during transmission, are described in other chapters. Insect virus families such as the entomopoxviruses, iridescent viruses, densoviruses, and cypoviruses have been described elsewhere.^{4,220}

Expression Vectors for Foreign Genes

Among eukaryotic viruses, the insect viruses are among the best known for their use as vectors for foreign gene expression because of their productivity, efficiency, and utility. The most widely used vectors are those derived from the helper-independent baculoviruses (reviewed by 49,159,167,175,260,321). This widespread application for these large DNA insect viruses is the direct result of intense molecular studies on their genetics, biochemistry, and multiplication strategies; it represents a striking example of the utility of insect viruses for basic and applied research. The remarkable strength of very late baculoviral promoters, the high level of virus multiplication, the extraordinary capacity for protein synthesis in cultured insect cells, and the ability to maintain large foreign DNA inserts are the principle factors that contribute to the efficiency and popularity of baculoviruses as eukaryotic expression vectors. The most used baculovirus expression vector system is derived from *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), a nuclear-replicating DNA virus that can generate virus-specific macromolecules comprising well over one-third of the mass of the infected cell. Thousands of different heterologous proteins have been expressed and subsequently studied by using the AcMNPV system since its original development in the early 1980s by the independent laboratories of Max Summers and Lois Miller.^{258,294}

Baculovirus vectors have been improved significantly for speed of recombinant virus generation, purification, and production of a large variety of protein types, including cytoplasmic, nuclear, secreted, and membrane-bound polypeptides (in order of abundance). Methods for recombinant vector production are now efficient and routine. Strategies for insertion and simultaneous expression of multiple genes or for temporally regulated, promoter-specific expression are available. Since post-translational modifications and biological activity of the resulting protein products often mimic or parallel that of mammalian cells, these vectors have provided abundant material for applications in diagnostics, therapeutics, and vaccine development, as well as general studies on protein structure and function. Like other expression systems, however, baculovirus vectors have advantages and disadvantages that depend on the type of protein to be produced.^{159,321} Insect cell lines have also been improved

by adaptation to serum-free media and specific engineering for glycosylation of protein products that mimics that of mammalian cells.¹⁵⁹ Live insect larvae have also been used as effective and convenient large-scale bioreactors for the production of high levels of many important proteins for academic and commercial purposes. *Bombyx mori* nucleopolyhedrovirus (BmNPV) has been used as an efficient vector for protein production in silkworms (reviewed in 167). For antigen preparation, the baculoviruses have also been used to express heterologous peptides, usually as fusions in their surface envelope.^{175,260,321}

The promoters for certain immediate early baculovirus genes are highly active in the absence of virus proteins.^{178,181,267,313} As a result, several of these baculovirus promoters, including those for IE1 and IE2, have been used extensively for high-level gene expression in diverse lepidopteran and dipteran insects by transient transfections or the generation of stable cell lines.⁷⁸ This approach allows long term, high-level expression in the absence of virus vectors.

It is noteworthy that other insect viruses, including the entomopoxviruses,²⁵⁹ densoviruses, and alphaviruses, have also been exploited as expression vectors. Likewise, flock house virus of the RNA virus family *Nodaviridae* has been used to express foreign epitopes on the surface of its isometric virion particle (reviewed by 327).

Gene Transduction, Delivery, and Gene Therapy

As a result of recent advances, the baculovirus AcMNPV has been used effectively as a gene transfer vector for a surprising variety of vertebrate cells, including those from mammals (reviewed by 49,339). Although baculoviruses can enter mammalian cells, they do not multiply or cause widespread cell death. Thus, when inserted into the baculovirus genome under control of appropriate mammalian transcriptional signals (promoters and enhancers), foreign genes are readily transduced and expressed after inoculation of mammalian cell lines. Expression is transient and levels vary depending on the protein. Interestingly, early AcMNPV genes are expressed, including the immediate early proteins IE1 and IE2 (see later discussion) and those viral genes that respond to these potent transcriptional activators.⁴⁹ The mechanism of virus entry is unknown, likely differing for each mammalian cell or tissue type. Nonetheless, the AcMNPV envelope fusion protein GP64 (see later discussion) is required. GP64 has been used to facilitate entry of pseudotyped envelope viruses into mammalian cells.^{124,246,292}

Because of its lack of multiplication in mammalian cells, its low cytotoxicity, the capacity for foreign gene expression, and its high transduction efficiency, AcMNPV has also been evaluated as a gene therapy vector. Applications have included its use in cancer therapy, regenerative medicine, vaccine vectors, and gene product ablation by RNA interference.^{49,339} Only preclinical trials have been conducted to date. In the case of baculovirus-mediated cancer therapeutics, tested approaches include virus-mediated expression of tumor-specific antigens for anticancer vaccines, introduction of functional tumor suppressors (e.g., P53) or proapoptotic genes, and the delivery and expression of suicide genes that confer tumor-specific susceptibility to select toxins or chemotherapeutic drugs. It is expected that these novel therapeutic approaches that exploit insect viruses will receive more attention in the future. AcMNPV vectors have already found use in the production of adenovirus gene therapy reagents.²⁹⁵

Biopesticide Viruses for Insect Control

While comprising over 80% of the existing animal species on earth, many insects present serious biomedical adversity for humans and cause significant agricultural and forest damage on a worldwide scale. Thus, it is not surprising that throughout history humankind has sought to control insects. In nature, insect populations are held in balance by predation, parasitism, and infection by microbial pathogens of viral and fungal origin. Of these diverse factors, viruses have numerous practical advantages for deployment as insect control agents and already represent viable components in current integrated pest management strategies.^{44,305}

In nature, viruses cause widespread epizootics and morbidity in insects, especially within dense populations. Spontaneous epizootics by viruses have occurred frequently in agricultural and forest settings (reviewed by 277). Nonetheless, dependence on epizootics for insect control has been impractical due to the unacceptable levels of crop damage that occur before the pest population declines. Thus, much effort has been invested in the development of viral biopesticides for field application in agriculture. Several viruses have already been used as effective, safe, and highly specific biocontrol agents that have the added benefit of reducing dependence on broad-range, toxic chemical insecticides. Of these viruses, the DNA baculoviruses have received the most attention due to their relative safety in the environment, their insect-specific virulence, the capacity for large-scale production, and the availability of effective formulations for application in agricultural and forest settings.

In one of the most successful applications of viral pesticides, the baculovirus AgMNPV is currently used to control the soybean looper on a large scale in Brazil.^{156,225,305} Other baculoviruses are used in Europe to control the codling moth, a common pest of fruit crops. In 1975, the Environmental Protection Agency registered the first baculovirus as an insecticide in the United States. While other baculoviruses have also been registered, their implementation has been pursued mostly by US agencies.^{25,225} Commercial development of virus insecticides has been sporadic due in part to restricted markets and limitations in their efficacy. Nonetheless, because the baculoviruses are amenable to genetic engineering, progress has been made to improve insecticidal efficacy by the insertion of insect-specific toxin or hormone genes.^{156,305} Carefully controlled field tests of genetically improved baculoviruses have been conducted in several countries, including the United States and United Kingdom.^{31,156,225} It is expected that viral insecticides will have an expanded role in integrated pest management programs as insects acquire resistance to chemical pesticides and environmental concerns increase. This practical application should expand with continued discovery and characterization of other virus families (ascoviruses, iridoviruses, tetraviruses, entomopoxviruses, nodaviruses, dicistroviruses, and cytopoviruses) that are potent pathogens of insects.

CLASSIFICATION OF INSECT VIRUSES

Insects are susceptible to highly diverse families of DNA and RNA viruses (Table 73.1). While some viruses are unique to arthropods, many members of insect virus families have counterparts among vertebrate viruses. Interestingly, insect-specific viruses also have novel properties that are common among insect virus families. A striking example is the use of an occluded virus particle for virus transmission between insect

hosts. This morphologic feature is conserved among diverse virus families, including the baculoviruses, entomopoxviruses, cytopoviruses (reoviridae), and possibly the nudiviruses.

DESCRIPTION OF INSECT VIRUS FAMILIES

Baculoviridae

Classification of Baculoviruses

Members of the family *Baculoviridae* have a large, circular genome of double-stranded DNA (dsDNA) that is packaged into an enveloped, rod-shaped virion.³¹¹ The family name is derived from the Latin term “baculum,” meaning stick or staff, to describe the shape of the virion. These viruses are characterized by their two morphologically distinct forms of infectious particles: occluded virus (OV), comprised of enveloped virions embedded within a crystalline matrix of protein; and budded virus (BV), comprised of a single virion enveloped by a plasma-derived membrane. The family consists of four genera. The alphabaculoviruses and betabaculoviruses infect lepidopteran insects (moths and butterflies), whereas the gammabaculoviruses and deltabaculoviruses infect hymenopteran insects (wasps and sawflies) and dipteran insects (mosquitoes), respectively (reviewed in 277). The alpha- and betabaculoviruses include the nucleopolyhedroviruses (NPVs) and granuloviruses (GV), respectively; these two virus groups are the best characterized of *Baculoviridae*. For GVs, a single virion is embedded within large ovoid cylindrical-shaped OV particles, which collectively appear as “granules” under light microscopy. By contrast, the polyhedral-shaped OV particle (polyhedra) of the NPVs has numerous (>20) virions. The OV are produced and accumulate in the nucleus of NPV-infected cells, whereas in GV-infected cells, intracellular OV assemble after the nuclear membrane disintegrates.

Baculoviruses infect well over 500 different species of insects. To date, virus isolates have only been identified in arthropods, mostly from insects of the order Lepidoptera. The NPV type species is *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), which was isolated originally from and named after the alfalfa looper *Autographa californica*. The GV type species is *Cydia pomonella* granulovirus (CpGV), isolated from the codling moth *Cydia pomonella*. Baculoviruses usually have distinct and relatively narrow host ranges that are limited to insects within a single genus or family. However, some baculoviruses, such as AcMNPV, have broad host ranges that include >40 lepidopteran species.

In the next sections, the molecular biology and biochemical mechanisms of baculovirus multiplication are described, comparing these unique viruses to the DNA viruses of vertebrates and highlighting the properties of baculoviruses that have been exploited for important applications in biotechnology. Recent reviews describe additional details of baculovirus biology, host and cellular interactions, evolution, host range, pathology, and vector applications.^{29,49,57,61,63,134,141,156,159,175,250,260,277,316,321}

Baculovirus Structure: OV and BV

Baculoviruses have a biphasic multiplication cycle in which BV is produced before OV (reviewed by 277). Both virus forms (Fig. 73.1) are infectious and contain one or more rod-shaped nucleocapsids (30–60 nm in diameter, 250–300 nm long). The nucleocapsid contains a single covalently closed circle of double-stranded genomic DNA that is associated with a 6.9-kDa, protamine-like core protein.³³⁷ The outside proteins of the

TABLE 73.1 Virus Families Infecting Invertebrates

Characteristics	Virus family	Genus	Representative member
dsDNA, enveloped	Baculoviridae	Nucleopolyhedrovirus (NPV)	<i>Autographa californica</i> multiple NPV (AcMNPV)
		Granulovirus (GV)	<i>Cydia pomonella</i> GV (CpGV)
	Polydnaviridae	Ichnovirus	<i>Campoletis sonorensis</i> ichnovirus (CsIV)
		Bracovirus	<i>Cotesia melanoscela</i> bracovirus (CcBV)
	Ascoviridae	Ascovirus	<i>Spodoptera frugiperda</i> ascovirus 1a (SfAV-1a)
dsDNA, nonenveloped	Nudiviruses ^a	Unclassified	<i>Heliothus zea</i> virus 1 (HzV-1) ^a
			<i>Oryctes rhinoceros</i> virus ^a
	Poxviridae	Alphaentomopoxvirus	<i>Melolontha melolontha</i> entomopoxvirus
		Betaentomopoxvirus	<i>Amsacta moorei</i> entomopoxvirus "L"
		Gammaentomopoxvirus	<i>Chironomus luridus</i> entomopoxvirus
ssDNA, nonenveloped	Iridoviridae	Iridovirus	Invertebrate iridescent virus 6 (IIV-6)
		Chloriridovirus	Invertebrate iridescent virus 3 (IIV-3)
	Parvoviridae	Densovirus	<i>Junonia coenia</i> densovirus
		Pefudensovirus	<i>Periplaneta fuliginosa</i> densovirus
		Brevidensovirus	<i>Aedes aegypti</i> densovirus
dsRNA, nonenveloped		Iteravirus	<i>Bombyx mori</i> densovirus
	Reoviridae	Orbivirus	Bluetongue virus
		Cypovirus	Cypovirus 1 (i.e., BmCPV)
		Coltivirus	Colorado tick fever virus
		Seadornavirus	Banna virus
ssRNA, enveloped		Idnoreovirus	Idnoreovirus 1
		Fijivirus	Fiji disease virus
		Phtoreovirus	Wound tumor virus
		Oryzavirus	Rice ragged stunt virus
	Birnaviridae	Entomobirnavirus	<i>Drosophila</i> X virus
Retrotransposons	Togaviridae	Alphavirus	Sindbis virus
	Flaviviridae	Flavivirus	Yellow fever virus
	Rhabdoviridae	Vesiculovirus	Vesicular stomatitis Indiana virus
	Bunyaviridae	Orthobunyavirus	Bunyamwera virus
		Phlebovirus	Rift Valley fever virus
ssRNA, nonenveloped		Nairovirus	Dugbe virus
	Metaviridae	Errantivirus	<i>Drosophila melanogaster</i> gypsy virus
		Semotivirus	<i>Bombyx mori</i> Pao virus
	Nodaviridae	Alphanodavirus	Nodamura virus, Flock House Virus
	Tetraviridae	Betatetravirus	<i>Nudaurelia capensis</i> β virus
		Omegatetravirus	<i>Nudaurelia capensis</i> ω virus
	Dicistroviridae	Cripavirus	Cricket paralysis virus
			<i>Drosophila</i> C virus
	Iflavirus	Iflavirus	Infectious flacherie virus (IFV)

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

^a*Heliothus zea* virus 1 and *Oryctes rhinoceros* virus (nudiviruses) were previously classified as members of Baculoviridae but were revised by the International Committee on Taxonomy of Viruses (ICTV) to "unclassified".

nucleocapsid interact to form ring-like subunits stacked every 4.5 nm; these include one major ~40-kDa capsid protein plus additional minor proteins.³³⁸ The apical end of the virion has a distinct structure consisting of several unique proteins, suggesting a specialized function.³²⁸ One of these virion end-structure proteins is pp78/83, a Wiskott-Aldrich syndrome (WASP)-like protein that facilitates nuclear actin assembly and is essential for production of progeny virus.¹⁰⁸

An especially distinctive feature of the baculoviruses is the large size (0.15–15 μ m diameter) and polyhedral or oval shape of their OV particles (Fig. 73.1). The OV mediates virus transmission between insect larvae, which is the developmental stage most susceptible to baculovirus infection. Upon inges-

tion, the OV protein matrix dissolves in the alkaline pH of the larva's midgut to release the occlusion-derived virions (ODV) that initiate infection of the midgut epithelium. The crystalline matrix of the OV is comprised primarily of a single ~29-kDa protein, designated either polyhedrin or granulins for the NPVs and GVs, respectively; these two proteins are closely related. The ODV of NPVs (Figs. 73.1A and 73.1B) are embedded in the matrix either as single nucleocapsids (SNPVs) or multiple nucleocapsids (MNPVs) that are surrounded by a single membrane envelope. This nuclear-derived envelope contains virus-encoded proteins that are responsible for binding to midgut epithelial cells, the initial site of infection.²⁷⁷ The membrane envelope is synthesized *de novo* or represents a modified form

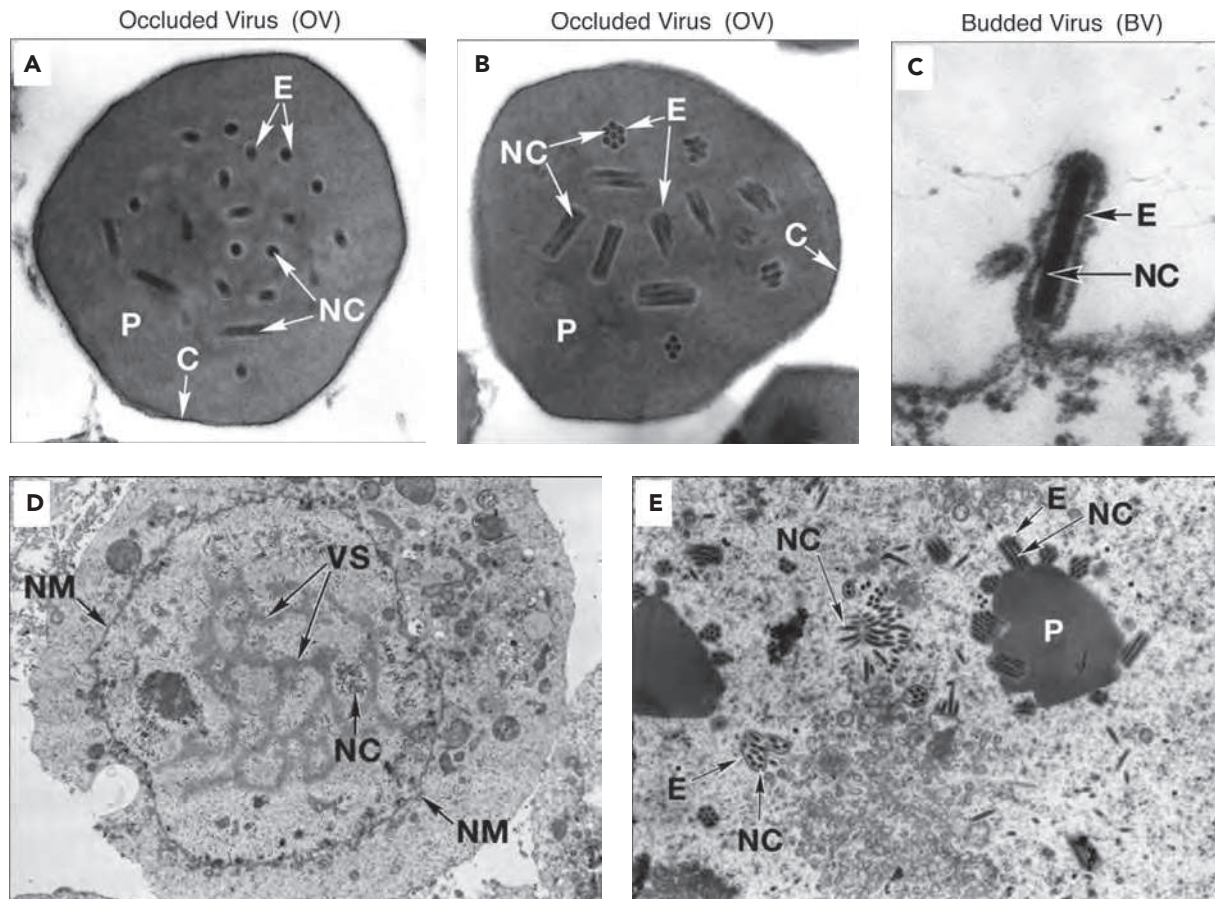


FIGURE 73.1. Nucleopolyhedrovirus OV and BV particles. **A:** Cross-section of an OV particle from a single-capsid NPV (SNPV). Single nucleocapsids (NC) are enveloped within a unit membrane (E) and embedded within a matrix of polyhedrin protein (P). The carbohydrate-rich calyx (C) covers the external surface of the particle, which is ~2 to 3 μm in diameter. **B:** Cross-section of an OV particle from a multi-capsid NPV (MNPV). The membrane envelopes (E) contain multiple nucleocapsids and are embedded within the polyhedrin matrix (P) of the particle. **C:** Budded virus (BV) particle during budding. The BV contains a single nucleocapsid (NC) (~30 \times 250 nm) surrounded by a virus glycoprotein-containing envelope derived from the plasma membrane of the NPV-infected cell. **D:** Thin section of a typical cell in the late phase of MNPV infection. The virogenic stroma (VS) and associated nucleocapsids (NC) are generated within the infected nucleus still contained by the nuclear membrane (NM). **E:** Enlarged thin section of an MNPV-infected cell nucleus early during the very late, occlusion-specific phase. Newly assembled nucleocapsids (NC) associate with intranuclear membranes, are enveloped (E), and embedded within the polyhedrin matrix (P). (Electron micrographs courtesy of Dr. Malcolm J. Fraser, Jr., University of Notre Dame.)

of the nuclear membrane.³⁵ On their surface, the mature OV particle has an additional covering of protein and carbohydrate known as the polyhedron envelope, or calyx.

The BV form is produced prior to OV during infection. BV is responsible for cell-to-cell transmission within the infected insect. It is the infectious particle for inoculating cultured cells, thus is the form used for baculovirus vectors. The BV consists of a nucleocapsid surrounded by a loose-fitting membrane envelope that is acquired by budding from the plasma membrane of the host cell (Fig. 73.1C); it is therefore distinct from nucleocapsids embedded within OV. The envelope fusion protein (EFP) is the most abundant viral protein associated with the BV membrane. The NPVs are subdivided on their EFP type (reviewed by 277). The EFP of group I NPVs (i.e., AcMNPV, OpMNPV) is GP64, whereas the group II NPVs (i.e., LdMNPV, SeMNPV) contain an F protein. GP64 is the best characterized of the baculovirus EFPs. It is a class III transmembrane glycoprotein that is required for efficient virus budding and BV

infectivity.²²³ BV can be neutralized by GP64-specific antiserum.^{330,363} GP64 likely interacts with the host-cell receptor or membrane components (which are still undefined in insects) and mediates membrane fusion upon activation by low pH in the endosome. Prefusion cleavage is not required. Detailed molecular studies^{199,200,363} and the postfusion crystal structure¹⁶³ have revealed that GP64 is a stalk-like, cross-linked trimer with an internal peptide domain required for fusion. However, the exact mechanism of GP64-mediated membrane fusion remains unclear. Because GP64 is essential for virus entry of mammalian cells during baculovirus-mediated gene transduction and is used to facilitate entry of a broad range of enveloped viruses into mammalian cells by using pseudotype strategies,^{49,124,246,292} protein GP64 represents an active area of research.

Baculovirus Genome Structure

The baculovirus genome is a large (80–180 kbp) covalently closed, supercoiled circle of dsDNA. To date, the complete

nucleotide sequence of >50 baculoviruses, including viruses from each genus, has been reported. This abundance of genetic information has provided important clues regarding baculovirus functions, evolution, and host interactions.^{138,141} About 30 genes have been identified as core genes that are shared by all baculoviruses and confer the distinctiveness to this unique family of insect viruses (reviewed by 134,140,141,277,315). Certain conserved genes are essential for baculovirus transcription (early and late phases), viral DNA synthesis, viral DNA packaging, and assembly of the OV and BV particles. Other core genes contribute specifically to baculovirus host range and the virus's unique life cycle within the insects. Not surprisingly, some of these genes are nonessential and thus dispensable for virus multiplication in cell culture.

AcMNPV is the best-studied baculovirus. Its 134-kbp DNA genome consists almost entirely of unique sequences that encode ~150 open reading frames (ORFs).^{6,277} The ORFs are closely spaced on both DNA strands with no apparent organization with respect to temporal expression. Early, late, and very late genes are interspersed throughout the genome. Most ORFs are separated by only 2 to 200 nucleotides. These intergenic regions have a high adenosine/thymidine content and function as promoter or termination sequences. In addition, the translational termination codon (usually UAA) often overlaps the polyadenylation signal, AAUAAA. Multiple examples of overlapping ORFs exist. Interestingly, there are few introns in baculovirus genomes. A rare example of splicing involves the nonessential *ie-0* gene and yields protein IE0 (see later discussion), which is nearly identical to transcriptional activator IE1.⁵²

AcMNPV RNA transcripts often initiate from within or extend through adjacent genes, a phenomenon especially prevalent among viral RNAs late in infection.^{95,177,208} Thus, numerous genes are transcribed into bi- or multi-cistronic RNAs wherein only the upstream ORF is preferentially translated. The biologic implication of this arrangement is unknown. There are also multiple examples of the transcription of overlapping and in some cases, antisense RNAs. Thus, the interspersed temporal classes of RNA transcripts may have functional or regulatory significance. For example, activation of the very late promoter of *polh*, the gene encoding the major structural protein (polyhedrin) of OV, downregulates overlapping, antisense RNAs initiated downstream of *polh*.²⁴⁴

A distinguishing feature of the baculovirus genome is the presence of multiple copies of interspersed repetitive sequences ranging in size from 30 bp to 800 bp that are designated homologous regions or *hrs*.^{62,117} The *hrs* act as transcriptional enhancers^{117,121} and probably function as origins of viral DNA replication.^{171,254} AcMNPV contains eight *hrs*, which account for 3% to 4% of the genome. The *hrs* have a complex modular organization consisting of ~60-bp repeats, each with a highly conserved 28-bp imperfect palindrome (28-mer). The 28-mer is the minimal sequence required for orientation- and position-independent enhancement of promoter activity and DNA replication.^{192,274} The *hrs* bind host- and virus-specific proteins. In particular, the transcriptional activator IE1 binds as a dimer to the palindromic 28-mer in an interaction that is required for IE1-mediated stimulation of enhancer activity.^{176,192,275} Deletion of a single *hr* (either *hr1*, *hr1a*, *hr2*, *hr3*, *hr4a*, *hr4b*, or *hr5*) from the AcMNPV genome reduces transcription of proximal genes, but has no significant effect on viral DNA replication or virus multiplication.^{41,274} Thus, individual *hrs* are dispensable

and no single region is absolutely required for virus multiplication in cell culture. The functional significance of multiple origin elements remains unclear. Nonetheless, the novel distribution and repetition of the *hrs* or *hr*-like elements within multiple baculoviruses suggest that these sequences play a critical role in the virus life cycle.

Stages of Baculovirus Replication

PRIMARY AND SECONDARY INFECTION

Baculovirus multiplication in the insect larva is divided into primary and secondary stages to describe the initial infection of larval midgut cells and the subsequent infection of secondary tissues, respectively (Fig. 73.2). Although virus binding to the host cell initiates infection in both stages, aspects of primary and secondary infections differ. Secondary infection in the larva most closely resembles that of cultured cells. Most of the details of AcMNPV replication (see later discussion) have been obtained through studies of high-multiplicity infection of cultured SF21 (or clonal SF9) cells, a convenient cell line derived from the nocturnal moth *Spodoptera frugiperda* (Lepidoptera; noctuidae). It should be appreciated that the timing of replication events often varies between viruses and insect hosts.

ATTACHMENT, ENDOCYTOSIS, AND UNCOATING

Budded virus (BV) of AcMNPV enters the host cell (Fig. 73.2) by what appears to be receptor-mediated endocytosis (reviewed by 277). The specific host-cell receptor(s) or lipid molecules are unknown. The major glycoprotein of BV, EFP GP64 (see earlier discussion) is required for receptor interaction and envelope fusion of the endosomal membrane. After release within the cytoplasm, nucleocapsids migrate to the nucleus in a process that coincides with virus-induced actin polymerization.^{47,108} Nucleocapsids interact end-on with the nuclear pore, enter the nucleus, and uncoat their genome. Phosphorylation of the 6.9K protamine-like core protein by a virus-encapsidated protein kinase promotes DNA release.^{337,350} These processes are rapid since new viral RNA transcripts can be detected within 15 min after inoculation.^{52,122,152,180,230,312}

EARLY PHASE REPLICATION

By definition, the early replication phase precedes virus DNA replication. During this period that extends to 6 hours, baculoviruses express genes encoding transcriptional activators, a virus-specific RNA polymerase activity, DNA replication factors, apoptotic suppressors, and other critical factors. These early gene products prepare the cell for the enormous burden imposed by the synthesis of viral nucleic acid and structural components that can constitute more than a third of the cell's mass (Fig. 73.3A). The level and timing of early and late gene expression is orchestrated to insure proper temporal assembly of infectious BV and OV. Early viral gene products also block cell-cycle progression, causing infected cells to accumulate in S and G₂/M phases.^{34,155} In addition, filamentous actin and microtubules are redistributed, causing a dramatic rearrangement of the host-cell cytoskeleton that is manifested by nuclear hypertrophy and cell rounding. These changes are characteristic of the early stages of baculovirus infection.^{47,108,279}

Baculovirus gene expression involves a highly coordinated cascade of gene activation events that is regulated primarily at the level of transcription (reviewed by 94,277). Transcription of strictly defined early genes usually peaks between 6 and 12 hours

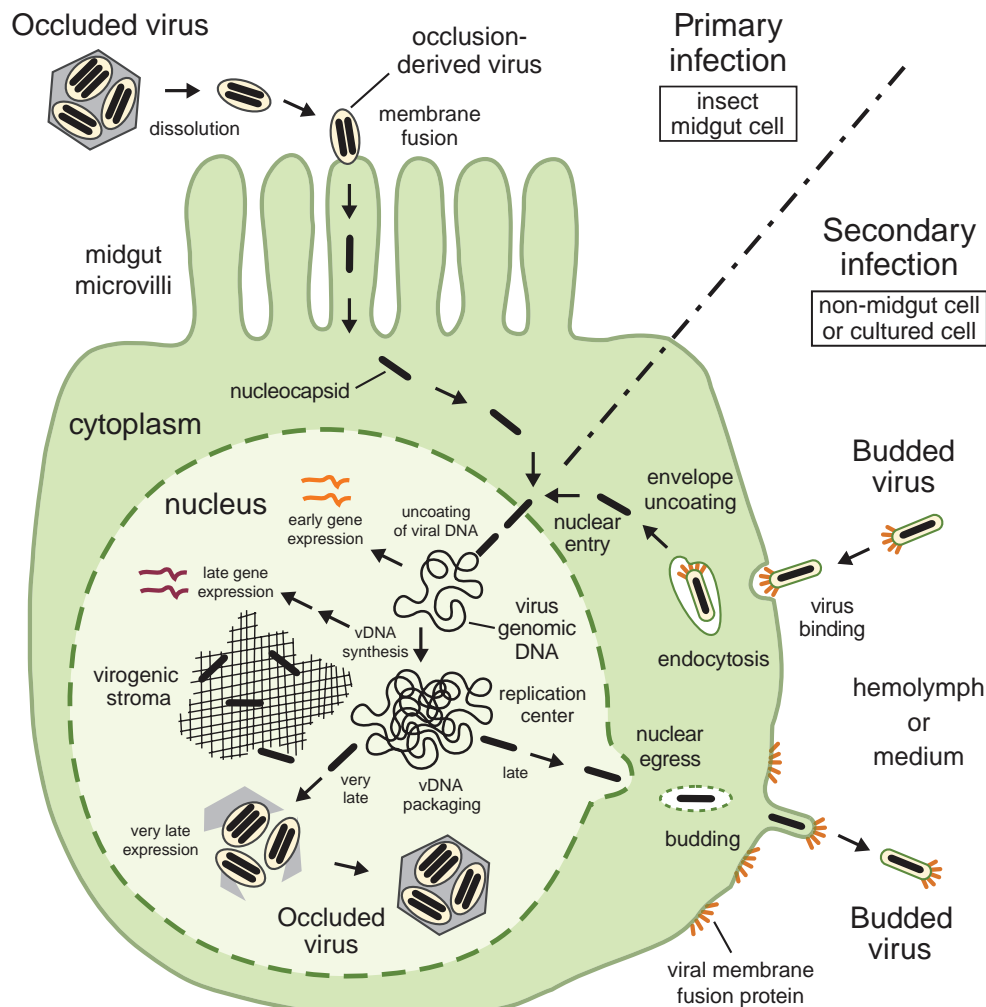


FIGURE 73.2. Baculovirus multiplication cycle. Except for the virus particle and the route of its entry, most steps in the baculovirus infection cycle are identical for an insect midgut epithelial cell (primary infection) or a subsequently infected nonmidgut cell (secondary infection). The steps for BV infection of a permissive cultured cell are the same as that for a secondary infection. Upon infection of a midgut cell by OV (top), the polyhedrin matrix of larval-ingested OV dissolves in the midgut lumen to release occlusion-derived virions that cross the peritrophic membrane and gain access to the columnar epithelial cells. These enveloped virions fuse with the microvilli membranes, releasing nucleocapsids into the cytoplasm for nuclear entry or transport to the basal membrane side of the midgut cell for budding into the hemocoel or tracheal epithelium. In secondary infections (right), enveloped BV in the infected larva's hemolymph or growth medium from infected cell culture attaches to unidentified cell-surface receptors and enters by endocytosis. As the endosome acidifies, the viral and endosomal envelopes fuse to release nucleocapsids into the cytoplasm. Occlusion-derived or BV-derived nucleocapsids both proceed to the nucleus, where they interact with the nuclear pore. The viral DNA genome is released by uncoating and the early phase of transcription and gene expression begins. Once the appropriate early gene products appear, viral DNA is synthesized in association with the newly formed virogenic stroma. Late gene expression occurs after DNA replication and produces structural components for the nucleocapsid, which then package just-replicated viral genomic DNA. During this late phase, newly assembled nucleocapsids exit the nucleus, migrate to the plasma membrane, associate with an envelope fusion protein, and exit the cell by budding to yield infectious BV. By contrast, during the very late phase, nucleocapsids are retained in the nucleus, acquire a nuclear-derived envelope, and become embedded within a crystalline matrix of polyhedrin to yield OV particles. Mature OV are released by virus-mediated cell lysis. (Drs. Nadine Dalrymple and David Taggart, University of Wisconsin-Madison, contributed to this illustration.)

after infection and declines thereafter as late viral transcription accelerates. During this early time, at least a portion of the incoming viral DNA genome adopts a nucleosome-like structure.³⁵¹ Early viral transcription is mediated by the host's RNA pol II.^{26,115,146,153,267} Early promoters resemble host RNA pol II promoters, complete with a consensus TATA element located ~30 bp upstream from the RNA start site.^{27,71,119,179,203,267,312} A tetranucleotide CAGT motif positioned at the RNA start site

is common to many early promoters and functions as a transcriptional initiator element.^{26,268} These core promoter elements also cooperate with upstream activating elements or more distal transcription enhancers (including the *hrs*) to interact with sequence-specific transcription activators. The *cis*-acting transcriptional control elements for multiple AcMNPV early genes have been defined, including those for *ie-1*, *gp64* EFP, *pp31*, *he65*, *p143*, and *p35*. Many early promoters are highly

responsive to virus-encoded transactivators, especially the multifunctional protein IE1 (see later discussion). As expected, host factors also participate in early transcription events.^{106,168,179,272} Remarkably, purified viral genomic DNA devoid of protein is infectious. Thus, host factors are sufficient to initiate infection, albeit at a rate slower than that of infectious virus particles. This feature is exploited in the generation of the baculovirus expression vectors.^{159,321}

Immediate early protein IE1 is the principal early trans-regulator of many baculoviruses. This nuclear phosphoprotein is highly conserved among the lepidopteran baculoviruses and is essential for virus multiplication by virtue of its transcriptional and DNA replication activities.^{253,286,300,307} IE1 transactivates early baculovirus promoters and contributes to a critical but unknown virus DNA replication function.^{27,119,120,178,182,203,230,252,253,273,307,312} Typical of transcriptional activators of DNA viruses, IE1 has a modular structure (Fig. 73.4). The N-terminal third consists of a potent transactivation domain that is largely acidic.^{176,276,293} A small stretch of residues at the extreme N terminus of IE1 is required for viral DNA replication.^{253,307} Furthermore, this N-terminal replication domain contributes to IE1 phosphorylation. Potential phosphorylation sites within a highly conserved cdc/cyclin B kinase-like motif (TPXR/H) within the replication domain are required for viral DNA replication and virus multiplication but not transactivation.³⁰⁷ Thus, phosphorylation may serve as a molecular switch to ensure proper division of IE1's DNA replication and transcriptional duties in a temporally controlled manner. The responsible kinase, either viral or host, is unknown. Also within its transactivation domain (Fig. 73.4), *AcMNPV* IE1 contains a highly conserved patch of basic residues (designated basic domain I) that is required for DNA binding.²⁴² This function of IE1 is essential for virus multiplication.³⁰⁶ IE1 binds as a dimer to the repetitive 28-bp palindromes that constitute the *hrrs*; IE1 binding is required for optimal transcriptional

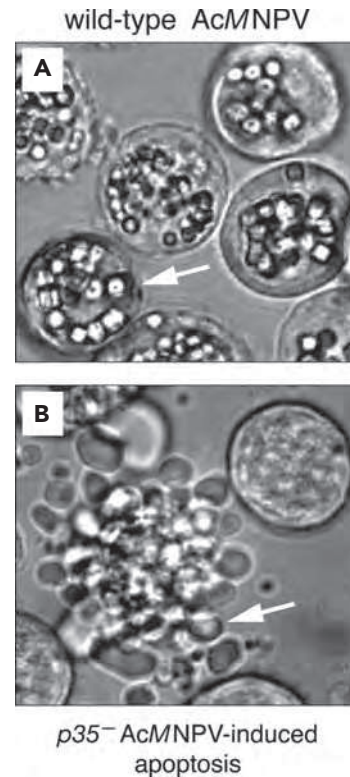


FIGURE 73.3. Fates of baculovirus-infected cells. **A:** Wild-type *AcMNPV* infection of *Spodoptera frugiperda* SF21 cells. Upon successful inhibition of apoptosis by viral P35, the highly refractile OV particles (arrow) accumulate in the hypertrophied nucleus of each cell by 48 hours after infection. **B:** Late stages of apoptosis triggered by an *AcMNPV* *p35*-deletion mutant. When virus-induced apoptosis is not suppressed, SF21 cells undergo widespread apoptosis that includes the formation of membrane-enveloped apoptotic bodies (arrow). Magnification, 200 \times . (Micrographs courtesy of Gulam Manji, University of Wisconsin—Madison.)

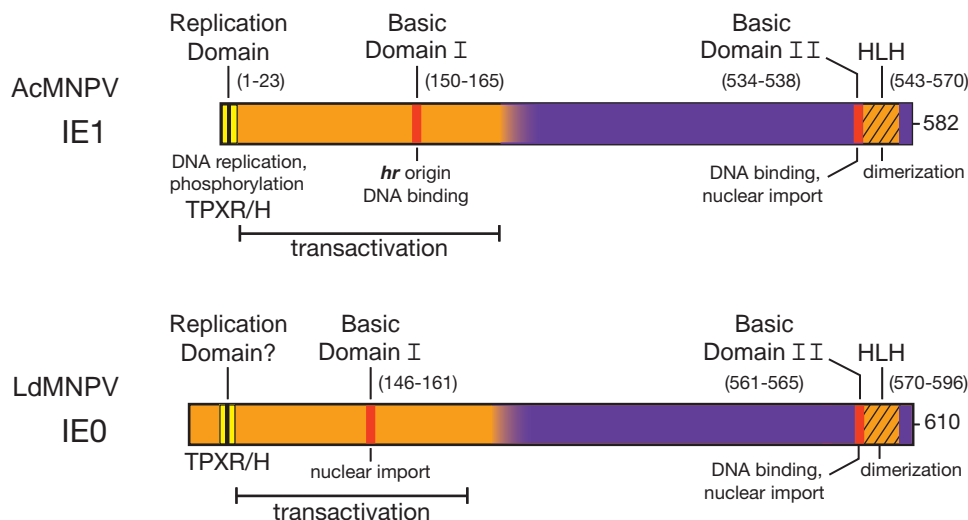


FIGURE 73.4. Structure of baculovirus transactivator IE1. NPV immediate early IE1 is a potent transcriptional activator essential for virus gene expression, DNA replication, and virus propagation. It has a conserved modular organization consisting of an N-terminal transactivation domain and a C-terminal helix-loop-helix (HLH) domain required for dimerization and nuclear import. The small basic domains (I or II) are required for DNA binding. IE1 from *AcMNPV* and *LdMNPV* share structural organizations but have very divergent sequences. Both possess N-terminal TPXR/H motifs required for DNA replication and phosphorylation³⁰⁷ but differ with respect to the function of their basic domains⁶⁸; *AcMNPV* basic domain I is required for *hrr* DNA binding; the same domain in *LdMNPV* IE1 is required for nuclear import.

enhancer function of the *hrs*.^{117,121,176,192,230,274,275} The IE1 C terminus possesses a domain that also contributes to DNA binding (Fig. 73.4). Here, IE1 contains an oligomerization domain and a novel nuclear localization signal.^{240,241} IE1 dimerization is required for nuclear import. Once in the nucleus, IE1 interacts with the *hrs* of the virus DNA genome as suggested by co-localization of IE1 and DNA replication factors and the requirement of basic domain I for virus multiplication.^{228,238,306} As indicated by its requirement in plasmid replication assays and synthesis of virus DNA in infected cells (see later discussion), IE1 also contributes to viral DNA replication.^{253,271,273,307} However, the exact replicative functions of IE1 during infection are still unclear.

Baculoviruses encode additional transcription activators, including IE0, IE2, and PE38. Although these proteins are conserved among baculoviruses, the understanding of their roles during infection is limited. Derived by RNA splicing, IE0 is identical to IE1 except for an additional 54 residues, encoded by *exon 0*, at its N terminus. IE0 has redundant regulatory activities with IE1 and is dispensable for virus replication.^{52,178,206,255,275,300} IE2 is a 47-kDa leucine zipper, RING finger–containing transactivator.^{40,313,361} AcMNPV mutants containing loss-of-function *ie-2* alleles are viable, but exhibit delays in virus replication.²⁶² Upon overexpression in plasmid-transfected cells, *ie-2* causes cell-cycle arrest,²⁶³ but the significance of this activity is unknown. IE2 and the transregulator PE38 both localize to punctate nuclear

structures in transfected cells.¹⁸¹ PE38 is a promoter-specific transactivator with a leucine zipper and RING finger.^{180,203,252} AcMNPV *pe38*-deletion mutants exhibit reduced levels of DNA synthesis and BV production, but are viable.²¹⁷

LATE PHASE REPLICATION

The late phase of the baculovirus life cycle starts with viral DNA replication, which begins about 6 hours after AcMNPV infection. The synthesis of new genomic DNA and the expression of late structural genes are necessary for the production of BV, the infectious form generated during this period. Late gene expression is dependent on viral DNA replication. This dependence is illustrated by inhibiting viral DNA synthesis through use of a temperature-sensitive (*ts*) mutant in the essential helicase *p143* gene (see later discussion) that subsequently blocks late gene expression and eliminates BV production.¹⁰⁹ Likewise, RNA silencing (RNAi)–mediated ablation of viral DNA replication factors prevents AcMNPV DNA synthesis, blocks late viral transcription, and prohibits the characteristic shut-off of host protein synthesis (see later discussion).²⁸⁵ The molecular basis for the coupling of late gene expression to DNA replication is unknown, but is common among large DNA viruses.

At least six baculovirus genes, which include *p143*, *ie-1*, *lef-1*, *lef-2*, *lef-3*, and *dna pol*, are required for DNA replication (Table 73.2). These genes were originally identified using

TABLE 73.2 Baculovirus DNA Replication and Late Expression Factors

Virus gene	Essential ^a	DNA replication	Late expression	Function/homology	Virus mutants
<i>ie-1</i>	Yes	+	+	Transcription transactivator, DNA origin binding protein	<i>tsB821</i>
<i>ie-2</i>		+	+	Transcription transactivator, cell line–specific cell cycle regulator	
<i>p47</i>		–	+	Late RNA pol component	<i>ts317</i>
<i>p143</i>		+	+	Helicase, DNA binding, ATP binding motif	<i>ts8</i>
<i>dnapol</i>	Yes	+	+	DNA polymerase, 3'→5' exonuclease	
<i>pp31</i>		–	+	Virogenic stroma association	
<i>p35</i>	No	+	+	Apoptosis suppressor	
				Caspase inhibitor	
<i>lef-1</i>		+	+	Primase homology, LEF2 association	
<i>lef-2</i>		+	+	Primase-associated protein?	VLD1
				Late, very late expression	
<i>lef-3</i>		+	+	ssDNA binding, p143 association	
<i>lef-4</i>		–	+	Late RNA pol component, guanyl transferase, 5' triphosphatase	<i>ts538</i>
<i>lef-5</i>		+	+	Transcription initiation with TFIIIS homology (Zn ribbon)	
<i>lef-6</i>		+	+	Vaccinia virus RNA pol homology	
<i>lef-7</i>	No	+	+	Cell line–specific DNA replication	vlef-7-AG
<i>lef-8</i>		–	+	Late RNA pol component	<i>tsS1</i>
<i>lef-9</i>		–	+	Late RNA pol component	
<i>lef-10</i>		–	+	Unknown	
<i>lef-11</i>	Yes	–	+	Unknown	
<i>lef-12</i>	No	–	+	Cell line–specific	
<i>pe38</i>	No	+	+	Transactivator of <i>p143</i>	
<i>an</i>	Yes	–	–	5'→3' exo- and endonuclease viral DNA maturation or packaging	
<i>hcf-1</i>		+	+	Cell line–specific, late and very late expression	
<i>vlf-1</i>	Yes	–	–	Very late expression	<i>tsB837</i>
				Integrase/resolvase	
				DNA replication	

^aGene is designated “essential” as determined by direct deletion or replacement within the AcMNPV genome.

transient DNA replication assays conducted in cultured cells with plasmids containing a viral *hr* enhancer/origin.^{169,205} Replication factors include a DNA binding helicase (p143), a putative primase (LEF-1), a primase-associated protein (LEF-2), a single-stranded DNA (ssDNA) binding protein (LEF-3), and a virus-encoded DNA polymerase. The essential function of these replication genes during infection has been confirmed through the analysis of conditionally lethal mutants and deletion mutants generated by using *Escherichia coli*–replicating bacmid technology (Table 73.2). Besides its transcriptional activities, IE1 promotes origin (*hr*)-specific plasmid DNA replication.^{169,205,271} A stretch of 23 residues at the IE1 N terminus is required for this activity.^{253,307} IE1's capacity to act as a DNA origin (*hr*)-binding protein and to stimulate homologous recombination suggests that it has a direct role in DNA replication,^{65,192,275} but its specific functions are still unclear. The *dna pol* gene³²⁰ is also essential for AcMNPV replication as demonstrated by the loss of virus viability upon its deletion.³²⁴ Other genes—including *ie-2*, *lef-7*, *pe38*, and *p35*—directly or indirectly stimulate replication in transient replication assays (reviewed in 204). Viral factors localize to centers of viral DNA replication within the virogenic stroma that forms within the nucleus (see later discussion).²³⁸ As these replication centers expand, host chromatin becomes marginalized to the inner edge of the nucleus; viral factors IE1, LEF-3, and helicase P143 in the presence of a viral *hr* element is sufficient for this marginalization.²²⁸

The precise mechanisms of baculovirus DNA replication are poorly understood. The *hr* repeats are likely origins of viral DNA replication,^{171,192,254,274} but this function has not been demonstrated formally. Non-*hr* sequences have also been implicated as genome replication origins.^{170,191,354} During infection, multiple unit-length genome fragments and concatamers of viral genomes have been detected.^{191,247} Although these potential replicative forms are consistent with a rolling-circle mechanism, no clear mechanism for DNA replication has emerged from various studies. Experimental evidence also suggests that recombination-dependent DNA replication contributes to the generation of baculovirus genomic DNA (reviewed by 277). For example, all baculoviruses encode an alkaline nuclease (*AN*) gene with sequence similarity to that of the phage lambda *red* exonuclease, which is involved in phage DNA recombination. Deletion of the alkaline nuclease gene *AN* from AcMNPV causes loss of virus viability even though viral DNA synthesis is normal.²³⁹ *AN* contributes to production of longer-than-genome length DNA fragments in infected cells, a property consistent with a role in recombination. The endonuclease and 5'→3' exonuclease activity of the alkaline nuclease may also participate in viral DNA maturation and packaging. Newly replicated, unit-length circles of DNA are packaged into individual nucleocapsids both within the virogenic stroma. Early in infection, one or more of the processes associated with viral DNA replication is sufficient to trigger DNA damage response and apoptosis.^{150,222,285} Baculovirus-encoded apoptotic suppressors (see later discussion) are necessary to block premature cell death caused by apoptosis and to allow completion of the virus's life cycle. Recent studies suggest that the host's DNA damage response is necessary for optimum viral DNA replication.^{150,222} For example, pharmacologic inhibition or RNAi-mediated ablation of the host kinases that signal the DNA damage response reduce AcMNPV DNA replication and severely restrict virus production. Thus,

baculoviruses likely modify specific aspects of the host's DNA damage response for their own benefit.²²²

Nineteen baculovirus genes are required for late gene expression (Table 73.2), as first determined for AcMNPV.^{197,205,271} These genes, designated late expression factors (*lefs*), were identified by their ability to support high-level expression of a reporter gene placed under control of a virus late promoter and *cis*-linked to an *hr* origin/enhancer element.²⁵² Due to the link between DNA replication and late gene expression, several genes required for late gene expression (*ie-1*, *p143*, *lef-1*, *lef-2*, *lef-3*, and *dnapol*) are essential DNA replication factors (Table 73.2). Because they are dispensable for late gene expression in certain cell lines, multiple *lefs* (*ie-2*, *lef-7*, and *hcf-1*) confer tissue or species specificity. In addition, a variety of viral genes contribute to host range, including helicase *p143* and host range factor-1 (*hrf-1*).^{48,64,164} Several *lefs* function directly or indirectly at the level of transcription or mRNA stabilization (Table 73.2). Of these genes, four (*p47*, *lef-4*, *lef-8*, and *lef-9*) encode components of the baculovirus RNA polymerase¹²³ that is specific for late and very late gene expression (see later discussion). The roles of transcription-specific *lefs* during infection have been characterized by using conditionally lethal mutations (Table 73.2).

The baculoviruses are unique among nuclear-replicating DNA viruses in that they switch to a novel RNA polymerase for late and very late transcription (reviewed by 251,277). The virus-encoded RNA polymerase retains specificity for late virus promoters *in vitro* and is distinguished from other host RNA polymerases by its resistance to α -amanitin and tagetitoxin, which are inhibitors of RNA pol II and III, respectively.^{100,107,152,357} The late RNA polymerase complex includes virus proteins p47, LEF-4, LEF-8, and LEF-9 as well as other proteins.^{16,123} The viral-encoded polymerase recognizes and initiates transcription within a nucleotide motif (A/G/T) TAAG that functions as the primary sequence element for late and very late promoter activity. All genes encoding proteins that are synthesized in abundance during the late phase (e.g., major capsid protein vp39, 6.9K basic core protein, gp64 EFP) possess this late TAAG motif. Fewer than 18 nucleotides surrounding this initiation site are sufficient to direct high-level late gene expression.²²⁴ Some late genes, including the *vp39* capsid gene, have multiple TAAG motifs, each of which serve as an independent RNA start site.³¹⁷ This redundancy boosts production of gene products needed in abundance for virion assembly. It is noteworthy that some early genes (*p35*, *gp64*, *pp31*, and *ie-1*) are also transcribed late in infection. Late transcription of these genes is often mediated by late TAAG motifs that overlap the early promoter elements.^{27,119,230,268}

VERY LATE PHASE REPLICATION

The final, very late stage of baculovirus infection is characterized by the hyperexpression of occlusion-specific genes and the production of mature OV from 18 through 76 hours after infection or until cell lysis (Figs. 73.3A and 73.5). The very late phase is marked by a dramatic increase in transcription of *polh*, which encodes the major matrix protein polyhedrin of OV. The very late hyperexpression of *polh* and its nonessential nature for BV production form the basis of the baculovirus expression vector system (see earlier discussion). Foreign genes are commonly placed under control of the *polh* promoter replacing the *polh* ORF to obtain high levels of expression very late in infection of cultured cells (recently reviewed by 159,260,321). The promoter

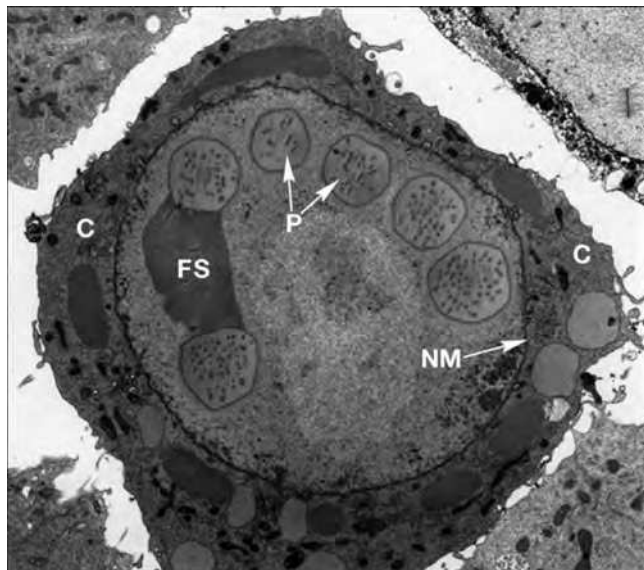


FIGURE 73.5. The very late occlusion stage of AcMNPV infection. Multiple OV particles (P) form on the inside edge of the hypertrophied nucleus near the inner nuclear membrane (NM). Dense fibrillar structures (FS) often form within the nucleus. The characteristic swelling of the nucleus forces the densely packed cytoplasm (C) to the cell's edge. (Electron micrograph courtesy of Dr. Peter Faulkner and Dr. Gregory Williams, Queens University.)

for the very late, hyperexpressed *p10* gene (see later discussion) has also been used.

The baculovirus-encoded RNA polymerase also mediates very late gene transcription. The promoters of the hypertranscribed genes (*polh* and *p10*) contain the late TAAG motif and are positively regulated by the 50 nucleotides constituting the noncoding leader of their gene (reviewed by 251,277). This region is responsible for the very late burst of transcriptional activity that distinguishes very late genes from late genes. These “burst” sequences may interact with activation factors responsible for the stimulation of transcription at this late time. Host and virus-specific factors likely participate in transcription of very late genes.^{251,277} Indeed, the baculovirus very late factor-1 (VLF-1) is a candidate for a positive activation.²¹² VLF-1 forms a complex with the burst sequences and stimulates very late transcription.^{221,360} Interestingly, VLF-1 is an essential protein (Table 73.2); in the absence of VLF-1, very late gene expression is compromised and viral DNA accumulation and packaging is reduced.³²³ Thus, VLF-1 may also contribute to viral DNA replication and nucleocapsid assembly.

p10 is the other very late, hyperexpressed gene of baculoviruses. It encodes a small 10-kDa coiled-coil domain protein that associates with expansive fibrillar structures (Fig. 73.5), which assemble in the nucleus and cytoplasm late in infection.²³² P10 is a nonessential protein that may disrupt the nuclear membrane or stabilize OV particles.^{329,349}

VIRUS ASSEMBLY, BUDDING, AND OCCLUSION

Nucleocapsids assemble during the late and very late phases of infection. New virus particles appear at the edge of the stromal matte located in the virogenic stroma (Fig. 73.1D), a poorly understood viral nucleoprotein structure centered within the nucleus. Consisting of DNA, RNA, and virus protein, the virogenic stroma is the likely site of viral DNA replication, conden-

sation, and encapsulation.^{90,362} As infection proceeds, the viral replication center (virogenic stroma) expands and host chromatin becomes marginalized to the nuclear periphery. This process involves the combined activities of the *ie-1*, *lef-3*, and *p143* genes and requires the presence of an *hr* element.²²⁸ Baculovirus phosphoprotein pp31 is a principal factor associated with the virogenic stroma; it binds DNA by a phosphorylation-dependent mechanism, but its exact function is unclear.^{36,118} pp31 appears to stimulate viral transcription rather than regulating viral DNA replication.³⁵⁹ Newly assembled nucleocapsids exit the nucleus by budding or transport through nuclear pores (Fig. 73.2) (reviewed in 277). Nuclear actin plays a critical role in the maturation of these nucleocapsids.^{47,108,237} The nuclear envelope is removed during transport to the plasma membrane. By interacting end-on with the plasma membrane, single nucleocapsids bud from the cell surface (Fig. 73.1C) and acquire the envelope fusion protein EFP GP64.²⁴⁵ The rate of BV release from AcMNPV-infected cells increases exponentially from 10 to 20 hours, then declines through 36 hours.¹⁹⁰

Late in infection, OV particles assemble and accumulate in the nucleus of infected cells (Figs. 73.3A and 73.5). For AcMNPV, OV production begins about 18 to 24 hours after infection and accelerates as production of BV declines. Not surprisingly, OV assembly involves multiple virus genes and events, including virus modification of nuclear membranes and vigorous import of polyhedrin into the nucleus (reviewed by 277). Nucleocapsids align along newly assembled membrane segments in the nucleus and subsequently acquire an envelope prior to encasement by polyhedrin (Fig. 73.1E). Studies of the generation of this nuclear membrane envelope and nuclear import of critical membrane proteins have revealed new facets to integral membrane protein trafficking.³³ OV formation is first observed on the inside edge of the nucleus (Fig. 73.5). Time-lapse video microscopy has suggested that the number of OV per cell is established early, and with time each particle increases in size.²¹⁰ Maturation of the OV includes deposition of the polyhedron envelope (calyx) over the particle's surface. Finally, OV are released upon disintegration of the nucleus and loss of plasma membrane integrity very late in infection.

Disease Progression in Insects

Horizontal transmission of baculoviruses is mediated by the OV, the form of the virus that is most stable and resistant to environmental degradation. Primary infection occurs in the midgut epithelium (Fig. 73.2) of the insect larva (reviewed in 29,277). Infection is initiated by larval ingestion of virus-contaminated food. Upon entry in the midgut lumen, the OV particles dissolve in response to the alkaline environment and release occlusion-derived virions (ODV). Passage of virus through the peritrophic membrane (Fig. 73.2) is facilitated by virus-encoded proteases (called enhancins), first discovered in the GV.¹⁹³ ODV subsequently bind to the brush border membrane of columnar epithelial cells and enter by direct fusion with the plasma membranes (Fig. 73.2). Infection proceeds by pathways comparable to that in cultured cells with the interesting exception that newly synthesized EFP GP64 is transported unidirectionally to the basal membrane side of the midgut cells (reviewed by 29,250). This targeting of GP64 allows nucleocapsid budding at the basal lamina and promotes rapid access of enveloped virus to the hemocoel and tracheal epithelium for systemic spread. Reduction of early GP64 synthesis selectively lowered the *per os* virulence of an AcMNPV mutant,³⁴³ suggesting an important role for this

directional transport. The production of OV in these midgut cells is rare for lepidopteran hosts. However, multiplication of certain baculoviruses, including mosquito NPVs, is restricted to the midgut. Thus, OV is produced there.²⁹

BV first generated in the midgut is next spread through infection of hemocytes (blood cells) and the epithelial cells that line the tracheal network.^{29,250} Interestingly, the lepidopteran baculoviruses encode *vfgf*, which bears sequence similarity to fibroblast growth factor. Recent studies with AcMNPV²¹⁴ suggest that *vfgf* activity in infected midgut cells activates extracellular metalloproteases that permeabilize the basal lamina, attracts susceptible tracheal cells, and allows BV to rapidly gain access to the trachea network and hemocoel. This secondary infection affects virtually all internal tissues of highly susceptible larva. Within several days, the larva is converted into a milky white liquid, consisting mostly of OV particles. This process, referred to as “melting” or “liquification,” is facilitated by a virus-encoded chitinase and a cathepsin, which cooperate to breakdown the larval cuticle (skin) that includes chitin.^{136,145} The activation and secretion of these enzymes is carefully regulated so as not to prematurely destroy infected cells or the larva before virus production is maximized.¹⁴⁵ A typical AcMNPV infection produces over 10⁸ OV particles, a yield that constitutes >10% of the dry weight of the insect. Upon rupture of the cuticular exoskeleton, OV are liberated into the environment. Liquification represents the successful conclusion to the baculovirus life cycle as the release of OV is required for virus spread through the host population. Nonetheless, there is limited evidence that baculoviruses establish persistent infections in some hosts.²⁷⁷

Baculovirus-Mediated Alterations of the Host

Besides causing mortality, baculoviruses can exert dramatic effects within their host. At the cellular level, infection induces major changes in the cytoskeleton and nucleus of infected cells (see later discussion). Transcription of most host genes is significantly reduced in AcMNPV-infected cells.^{243,281} Nonetheless, certain host proteins used by the virus are upregulated, including heat shock proteins, membrane trafficking proteins, and TATA-binding proteins.^{270,281} In general, host-protein synthesis declines rapidly after the onset of baculovirus DNA synthesis. When AcMNPV DNA replication is blocked, virus-mediated arrest of host-protein synthesis is prevented.²⁸⁵ Conversely, when AcMNPV late gene expression is selectively blocked, arrest of host-protein synthesis is unaffected. Because vDNA replication also triggers AcMNPV-induced apoptosis (see later discussion), the shutoff of host-protein synthesis and apoptosis may be caused by the same host-cell response to viral DNA synthesis. The biochemical mechanisms involved are not yet understood.

Remarkably, the baculoviruses also exert control of their hosts at the physiologic and behavioral level as a means to enhance virus production and dissemination (reviewed by 232,316). For example, baculovirus-infected larvae often display a classical behavior of wandering that involves climbing to the top of vegetation, where they succumb and liquefy while hanging from a leaf or branch. This strategy facilitates virus dispersal by wind and rain. A baculovirus-encoded protein tyrosine phosphatase gene (*ptp*) contributes to this virus-induced behavior.¹⁶⁶ Uninfected caterpillars in late stages of development also wander. Because this behavior is controlled by a cellular *ptp*, it is postulated that baculoviruses acquired a host ancestral *ptp* because of the conferred selective advantage for dissemination. In another striking example, baculoviruses block

development of their larval hosts by controlling the activity of ecdysone, the steroid hormone that triggers molting.²³³ The baculovirus *egt* gene encodes an ecdysteroid UDP-glucose/galactose transferase that is secreted from infected cells and transfers glucose or galactose to these ecdysteroids, thereby inhibiting uptake of the hormone by cells. Virus-encoded *egt* blocks larval molting (reviewed by 232,316), which is correlated with increased virus production; indeed, *egt* deletion mutants cause early mortality and yield less virus.²³⁴ The increased speed-of-kill of *egt* deletion mutants has already been adapted for the generation of engineered baculoviruses for improved insecticidal activity.¹⁵⁶ Interestingly, the inhibition of molting by baculovirus *egt* is also associated with enhanced wandering (climbing) of gypsy moth (*Lymantria dispar*) caterpillars upon infection with *Lymantria dispar* NPV.¹⁴⁷ The extent of this effect depends on virus dose and the developmental stage at the time of inoculation.

Baculovirus Manipulation of Apoptosis

The amazing replicative success of baculoviruses often depends on their capacity to suppress host-cell apoptosis, a dynamic suicide response that can function as an effective antiviral defense. As such, the baculoviruses have provided a wealth of information on the mechanisms by which viruses impede host apoptosis (also referred to as programmed cell death) to facilitate their multiplication (reviewed in 18,56,61). Moreover, these viruses have revealed highly conserved genes that regulate apoptosis in insects and mammals alike. Apoptosis is critical for many normal processes in animals, including development, differentiation, immune function, and elimination of damaged, oncogenic, or pathogen-infected cells. Thus, its misregulation is associated with many disease states (reviewed by references 101,332). By virtue of the discovery of multiple baculovirus genes that function as potent suppressors of apoptosis in phylogenetically diverse organisms, these viruses have provided valuable insight and important tools for studying the pathways by which apoptosis is either suppressed or amplified in human diseases and how apoptosis can be manipulated in treatment regimens.

Baculovirus mutants that lack suppressors of apoptosis trigger widespread, species-specific cell death.^{58,59,67,75,142,165,213} In some cases, apoptotic death approaches 95% of the infected cells within a 24-hour period, a response level that is unmatched by mammalian viruses. Baculovirus-induced apoptosis is characterized by premature cell death that includes activation of host pro-death components, fragmentation of host and viral DNA, and dismemberment of the cell into extracellular vesicles called apoptotic bodies (Fig. 73.3B). Accordingly, apoptosis severely limits production of virus. In the case of AcMNPV mutants deficient in apoptosis suppressors, virus yields are reduced as much as 10,000-fold in cultured cells.^{59,142} Moreover, the infectivity of these mutants in certain permissive insects (e.g., *Spodoptera frugiperda*) is 25- to 1,000-fold lower than that of wild-type virus.⁵⁹ This reduced infectivity is correlated with increased apoptosis in larvae and reduced virus spread.⁵⁵ Thus, apoptosis impedes baculovirus multiplication and can function as an effective antiviral defense in insects.

Cultured insect cells have proven invaluable in studies of baculovirus-induced apoptosis. In particular, lepidopteran *Spodoptera frugiperda* (SF21) cells and dipteran *Drosophila melanogaster* (DL-1 or S2) cells are now used as model systems for these studies and those of invertebrate apoptosis.^{43,58,59,149,187–189,210,227,285,286,290} Many of the cellular components comprising the apoptotic

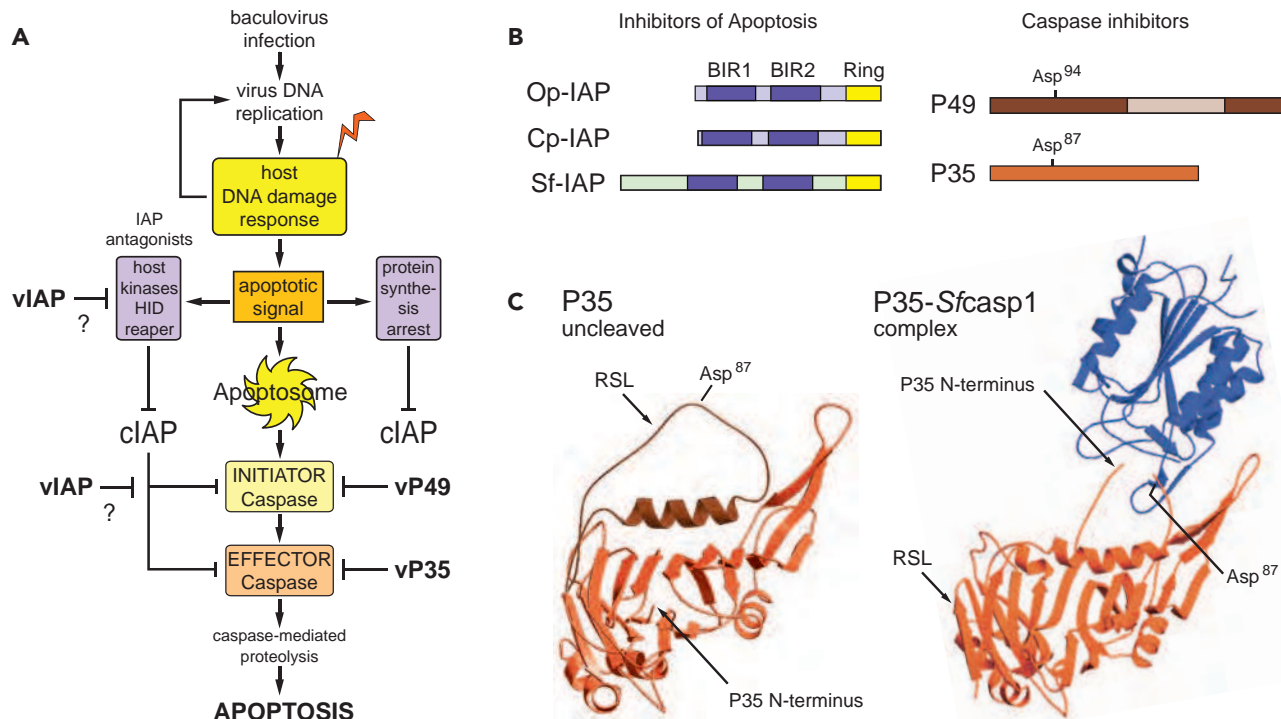


FIGURE 73.6. Baculoviruses and apoptosis. **A:** Model pathway for virus-induced apoptosis. AcMNPV triggers widespread apoptosis of model *Spodoptera frugiperda* (order Lepidoptera) cells due to events associated with viral DNA replication. Such replication triggers a DNA damage response, which produces an apoptotic signal that depletes host-cell IAP (cIAP). Loss of cIAP activates initiator and effector caspases, which if uninhibited cause proteolysis and apoptotic cell death. Virus-induced cIAP antagonists may be prodeath factors, like HID and reaper, activated host kinases, or the arrest of protein synthesis that accompanies baculovirus infection. Virus-encoded apoptotic suppressors produced early in infection prevent apoptosis. P35 inhibits active caspases, including *Sf*-caspase-1, whereas P49 inhibits an activated initiator caspase. Baculovirus-encoded IAPs block procaspase activation at an unknown (?) step upstream of P49. **B:** Baculovirus apoptotic regulators. The IAPs contain two BIR motifs and a C-terminal Ring finger motif. The viral IAPs, Op-IAP and Cp-IAP, closely resemble SfIAP, a cellular IAP from *S. frugiperda*. Caspase inhibitors P49 and P35 are 49% identical and contain aspartate residues (Asp⁹⁴ and Asp⁸⁷) required for caspase cleavage. P49 contains a 120-residue insertion (cross-hatched) unrelated to P35. **C:** Caspase inhibition by baculovirus P35. In uncleaved P35 (*left*), the prominent reactive site loop (RSL), with the Asp⁸⁷ caspase cleavage site at the apex, is solvent exposed while the N terminus is buried. During cleavage, P35 undergoes a dramatic conformation change (*right*) in which the RSL folds backward and the N-terminus is liberated so that it enters the active site of the targeted caspase, forming a stable, inhibited complex.

pathways of these cells have been identified (Fig. 73.6A). Moreover, these cells are sensitive to diverse apoptotic stimuli, including that caused by viruses, DNA-damaging agents, transcription and protein synthesis inhibitors, calcium ionophores, phosphatase inhibitors, and others. Nonetheless, baculovirus-permissive cell lines refractory to virus-induced apoptosis have been identified. For example, cultured *Trichoplusia ni* TN368 cells fail to undergo apoptosis upon infection with AcMNPV mutants that cause apoptosis of SF21 cells.^{59,142} The infectivity of these mutants in *T. ni* larvae is comparable to that of apoptosis-suppressing wild-type virus. The molecular basis of this interesting resistance to virus-induced apoptosis is unknown.

BACULOVIRUS-ENCODED APOPTOTIC SUPPRESSORS

To counteract the host suicide response and thus promote multiplication, baculoviruses have evolved novel apoptotic suppressors. These viral proteins regulate apoptosis at different steps in

the death pathway (Fig. 73.6A) and in a surprising diversity of organisms. Among different baculovirus species, two mechanistically distinct apoptotic suppressors have been identified: the caspase inhibitors (P35 and P49) and the inhibitor-of-apoptosis (IAP) proteins (Fig. 73.6B). The discovery of cellular IAPs with structural and biochemical properties comparable to those of viral IAPs suggests that baculoviruses originally acquired these apoptotic regulators from their insect hosts.^{43,151,289} It is likely that the same is true for the baculovirus-encoded caspase inhibitors, but no cellular homologs have yet been identified. The strong selective advantage conferred to baculoviruses by expression of an apoptotic suppressor, like P35,¹⁹⁴ is consistent with the acquisition and maintenance of such host genes. Interestingly, a single functional regulator per virus appears sufficient. For example, AcMNPV encodes one copy of the *p35* gene along with two nonfunctional *iap* genes.^{60,113} By contrast, OpMNPV encodes a single functional *iap* (Op-*iap3*), three nonfunctional *iaps*, and no *p35*.^{24,213}

Baculovirus P35 is a potent caspase inhibitor, often considered a universal suppressor of apoptosis due to its function in phylogenetically diverse organisms. First discovered in AcMNPV,⁵⁸ p35 prevents programmed cell death in plants, nematodes, flies, moths, and mammals (human cells included), among others, when expressed ectopically.^{18,56} Accounting for its widespread effectiveness, P35 (35 kDa) is a broad-specificity substrate-inhibitor of the effector caspases, which are the proteases that execute the cleavages, that dismember the apoptotic cell (reviewed by 39,101). P35 is cleaved by its target caspase at a requisite aspartate residue (Asp⁸⁷) and forms a stable, stoichiometric complex with it.^{17,37,364} The 2.2 Å crystal structure of P35 reveals that the Asp⁸⁷ cleavage site is located at the apex of a novel solvent-exposed reactive site loop (Fig. 73.6C), the orientation of which is critical to P35's anticaspase activity.^{86,366} Caspase-mediated cleavage induces a dramatic conformational change in the reactive site loop that stabilizes the covalent link between P35 and the caspase (Fig. 73.6C) in a novel mechanism for substrate-protease inhibition.^{69,358} Baculovirus P49 is a related substrate-inhibitor of the caspases. First identified in *Spodoptera littoralis* NPV,⁷⁴ P49 exhibits 49% amino acid identity with P35 (Fig. 73.6B) and likewise possesses a reactive site loop that is cleaved at a requisite aspartate residue (Asp⁹⁴) by the participating caspase. Interestingly, P49 targets apical initiator caspases in permissive insect cells. Thus, P49 inhibits the caspase(s) that proteolytically cleave and activate the effector caspases that are inhibited by P35.^{126,157,257,367} This target specificity is conferred in part by the cleavage site residues of P49.^{126,367} Thus, baculoviruses have evolved multiple caspase inhibitors with different specificities. A functional P35-like caspase inhibitor is also encoded by the insect entomopoxviruses.²¹⁵

The baculovirus IAPs were the first discovered members of the larger IAP family resident in diverse organisms and their viruses. The cellular IAPs are highly conserved regulators of cell fate that function during cell stress, immunity, tumorigenesis, and pathogen invasion (reviewed in 101,127,137,298,326). In insects, these short-lived (~30-minute half-life) cellular proteins regulate caspase activation and activity. The IAPs are defined by the presence of one or more essential baculovirus IAP repeat (BIR) domains (~80 residues), consisting of a conserved Zn²⁺-coordinating arrangement of Cys and His residues (CCHC). Many IAPs also possess a critical C-terminal RING domain, which functions as an E3-ubiquitin ligase.³²⁶ The antiapoptotic activity of some, but not all, IAPs is derived from the ability of the BIRs to bind directly to and neutralize initiator and effector caspases through ubiquitination by the RING. During apoptosis initiated by certain signals, the BIRs also interact with proapoptotic IAP binding motif (IBM) proteins, which bind to and dissociate the IAP-caspase complex to liberate active caspases and execute apoptosis.^{137,298,326}

Several insect virus families, including the baculoviruses, entomopoxviruses, iridoviruses, African swine fever virus, and the unclassified nudiviruses, encode functional and nonfunctional IAPs.^{18,56} Of these, the baculovirus IAPs are the best characterized. The viral IAPs share many structural and biochemical features with the cellular IAPs.^{43,151} However, despite these striking similarities and their potent antiapoptotic activity, the molecular mechanism by which baculovirus IAPs prevent apoptosis in insects is largely unknown. Current evidence suggests that viral IAPs use a mechanism that does not involve

direct inhibition of active caspases. The best-studied viral IAP is Op-IAP3 from *Orgyia pseudotsugata* NPV.^{18,24,56} This small, 268-residue IAP contains two BIRs and a C-terminal RING (Fig. 73.6B); each of these motifs is required for antiapoptotic activity.^{149,334,352} Upon its overexpression, Op-IAP3 blocks apoptosis triggered by various signals in cells from certain insects and mammals, suggesting that a broad and possibly conserved mechanism is involved.⁵⁶ In baculovirus-infected *Spodoptera* cells, Op-IAP3 functions upstream of baculovirus caspase inhibitors P49 and P35, thus likely inhibits initiator caspase activation.^{211,367} Op-IAP3 blocks the proteolytic activation of *Sf*-caspase-1 by an upstream initiator caspase.^{188,211,288} Nonetheless, in contrast to host insect IAPs, Op-IAP3 fails to bind and inhibit active caspases.³⁵³ The Op-IAP3 BIRs interact with *Drosophila* proapoptotic IBM proteins, including Reaper, Hid, and Grim.^{18,56,137,333,335} However, the relevance of these interactions is unknown since Op-IAP3 fails to block apoptosis in *Drosophila*.^{353,367} Interestingly, the Op-IAP3 BIRs mediate homo-oligomerization, which is also necessary for functionality of lepidopteran cellular IAP.^{43,149} Nonetheless, the target(s) and molecular mechanism by which the baculovirus IAPs function remains unresolved.

BACULOVIRUS-INDUCED SIGNALING OF APOPTOSIS

The signal for host-cell apoptosis originates early in the baculovirus life cycle. A critical link between virus DNA replication and the initiation of apoptosis has been established. Host caspase activation is one of the earliest events in the apoptotic cell. Caspase-mediated cleavage of viral caspase inhibitors P35 and P49 is first detected when viral DNA synthesis begins from 6 to 9 hours after infection with AcMNPV.^{17,367} Furthermore, P35 must be synthesized early in infection before viral DNA replication to effectively block virus-induced apoptosis.¹⁴³ Likewise, proteolytic activation of the proform of *Sf*-caspase-1, the principal executioner caspase in permissive *Spodoptera frugiperda* cells, coincides with the initiation of virus DNA replication.^{89,187,188,211,288} Thus, apoptotic signaling occurs before or during early viral DNA replication. Studies using viral DNA polymerase inhibitors and conditional AcMNPV mutants defective for viral DNA synthesis indicated that viral DNA replication or viral late gene expression was required to trigger apoptosis.^{60,187} Subsequent studies using RNA silencing for selective ablation of AcMNPV factors required either for viral DNA replication or for late gene expression demonstrated that viral DNA synthesis or events involving DNA replication, but not late gene expression, are required to trigger apoptosis.^{285,286} Thus, like vertebrate DNA viruses, baculovirus DNA replication events are sufficient to trigger the apoptotic response.

Recent studies have uncovered important facets by which baculovirus DNA replication triggers apoptosis. In invertebrates, the short-lived cellular IAPs are central regulators of apoptosis. In cell lines of dipteran and lepidopteran origin, a threshold level of IAP is necessary to block constitutive signaling towards apoptosis. Thus, when intracellular IAP drops below this level, caspases are activated and apoptosis ensues. Coincidental with the beginning of viral DNA synthesis, AcMNPV causes a dramatic loss of cellular IAP that forces caspase activation in lepidopteran and dipteran cell lines.³²⁵ This loss is independent of caspase activity and is conferred by residues within the N-terminal leader of the cellular IAP (e.g., SfIAP) and the ubiquitin ligase activity encoded by its C-terminal

RING domain.^{43,325} The AcMNPV *lef3* involved in viral DNA replication, but not those involved in late gene expression, are required for the virus-induced loss of cellular IAP. Thus, viral DNA replication likely triggers a host-cell response that depletes cellular IAP and executes apoptosis. The replication of vertebrate virus DNA can induce the host's DNA damage response, which triggers apoptosis (reviewed by 201,348). Thus, it is possible that baculovirus DNA replication is interpreted as DNA damage, which is a potent cause of apoptosis in insects as well. Indeed, AcMNPV DNA replication induces a DNA damage response in lepidopteran and dipteran cell lines.^{150,222} Additional study is required to determine the link between the DNA damage response and cellular IAP depletion in baculovirus-infected cells.

Host Transposons

The baculoviruses are one of the few families of animal viruses that accommodate the frequent and spontaneous insertion of host-derived transposable elements.⁹³ By virtue of their replication in the transposon-rich insect cell nucleus and the flexibility with respect to size of their DNA genome, baculoviruses are highly vulnerable to transposon insertion. This novel property has led to the identification of new families of transposons, which have been further developed as vectors for stable transformation of insects (see later discussion). Moreover, it is likely that baculovirus evolution has been significantly influenced by arthropod transposons, which represent potent insertional mutagens that generate genetic diversity and provide new genes to recipient viruses.^{130,277}

Baculovirus mutants with transposon insertions are often distinguished by altered plaque morphology, including the “few polyhedra” (FP) phenotype in which reduced nuclear accumulation of OV facilitates mutant identification by light microscopy. Several FP-causing lepidopteran transposons, including PiggyBac, IFP2.2, IFP1.6, TFP3, and Hitchhiker, have been identified by their insertion within or near the 25K protein gene (Ac61) of AcMNPV.^{13–15,42,91,92} Loss-of-function Ac61 mutants have reduced polyhedrin gene transcription and limited intranuclear envelopment of nucleocapsids that thereby lowers OV accumulation^{14,32,135}; the exact function of the highly conserved 25K protein is unknown. The most prevalent baculovirus-associated transposons possess terminal inverted repeats flanking a transposase gene with specificity for the sequence TTAA. For example, PiggyBac is a small (2.47-kb) autonomous transposon identified in the moth *T. ni*.⁹¹ It was the first nondrosophilid transposon used for stable germline transformation.¹²⁹ PiggyBac and other related transposons have now been used for gene transfer in Lepidoptera, Diptera, Coleoptera, and Hymenoptera, and most importantly in insect species (i.e., mosquitoes) of medical and agricultural significance for which P-element transformation has not been possible (reviewed by 130,231).

The retrotransposon TED (transposable element D) was also discovered as an insertion within AcMNPV.^{93,96,216,219} TED is a 7.5-kb, middle-repetitive retrotransposon that is actively transcribed within the AcMNPV-permissive host *T. ni*. Classified as an errantivirus (family *Metaviridae*), TED has a retrovirus-like organization with *gag*, *pol*, and *env* genes that are flanked by long terminal repeats. It is closely related to errantiviruses of other lepidopteran and dipteran (*Drosophila*) insects.^{96,216} Like a functional retrovirus, TED assembles virus-like gag-containing

particles, complete with protease and reverse transcriptase activities.^{128,195} The TED *env* gene also encodes a membrane-associated glycoprotein with properties expected of a retroviral envelope protein.²⁴⁸ Interestingly, the *env* gene of TED and other errantiviruses bear a striking similarity to the baculovirus Group II envelope fusion (F) proteins. Thus, it has been suggested that lepidopteran errantiviruses acquired their *env* gene from baculoviruses through an ancient integration/excision event.^{209,256} Not ruled out is the alternative possibility that baculoviruses have acquired *env* (F) genes through errantivirus insertion, as is the case for the AcMNPV insertion mutant-carrying TED.^{96,219} Nevertheless, the novel association between baculoviruses and active transposons has also led to speculation that virus-mediated shuttling of mobile genetic elements between insect species occurs in nature.^{93,277}

Polydnaviridae

Polydnavirus Structure and Classification

The polydnaviruses are large DNA viruses that are distinguished by their obligate and mutualistic association with their hosts, the endoparasitic wasps (Order Hymenoptera). The family *Polydnaviridae* derives its name from the taxonomically characteristic “polydisperse” DNAs that comprise the multisegmented genome of these novel viruses (reviewed by 20,185,302,344). Polydnavirus virions contain numerous double-stranded, supercoiled DNA molecules with sizes ranging from 2 to >30 kbp. The endoparasitic wasps that carry polydnaviruses transfer these virions to susceptible host caterpillars during oviposition (egg laying) in such a way that virus-encoded virulence genes compromise the caterpillar's immune response, facilitating development of the young wasp larva (see later discussion). Remarkably, virus multiplication and particle assembly occurs only in the wasp, not in the parasitized larva. Because virion DNA is generated from proviral DNA that is integrated within the host wasp genome, the polydnaviruses are transmitted vertically. The proviral DNA is also polydispersed within the wasp genome, where it is amplified and selected DNAs are packaged as circular segments within the virion.^{20,302} Recent sequence analyses of viral RNA transcripts expressed in ovaries (the site of virus replication) of polydnavirus-carrying endoparasitic wasps^{19,331} have revealed an unexpected relatedness between the polydnaviruses and the nudiviruses, which are large DNA viruses with an autonomous life cycle in insects (see later discussion). This possible relationship and the unique life cycle of the polydnaviruses have stimulated recent debate as to whether they fit the true definition of a virus.^{20,84,302}

The best-characterized polydnaviruses are from endoparasitic wasps. These predatory insects lay their eggs, accompanied by virus, in the body of a living insect larva, where the parasitic eggs hatch and the wasp's offspring develop. Polydnaviruses have been isolated from the *Ichneumonidae* and *Braconidae* families of wasps, and are correspondingly divided into two genera: ichtnoviruses and bracoviruses.^{20,185,302,345} The virus particles of these genera are morphologically distinct. The ichtnovirus virions are ellipsoid, with a uniform size of approximately 90 × 300 nm (Fig. 73.7); they resemble virions of the insect ascoviruses (see later discussion). The ichtnovirus nucleocapsid is enveloped by two unit membranes, one derived from the nucleus during virion morphogenesis and the other by budding from the plasma membrane of the host cell. In contrast, bracovirus virions consist of enveloped rod-shaped nucleocapsids with a uniform diameter (35–40 nm) and variable length (30–200 nm);

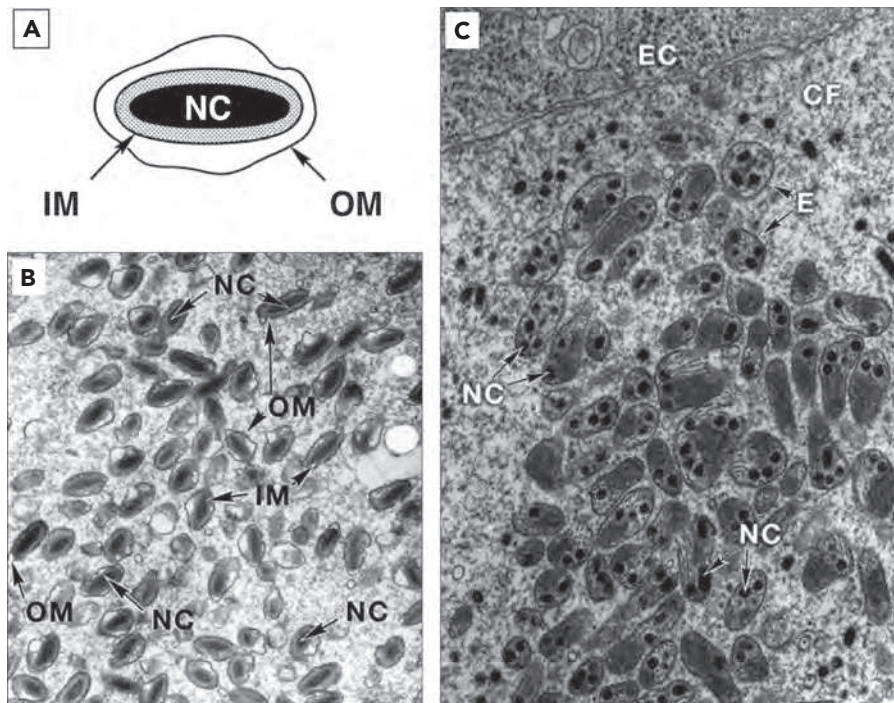


FIGURE 73.7. Morphologic features of polydnviruses. **A:** Schematic of an ichnovirus particle. An inner (IM) and outer (OM) membrane surrounds the nucleocapsid (NC). **B:** Electron micrograph of an ichnovirus. Inner and outer membranes envelope the lenticular nucleocapsids (NC) of uniform size. **C:** Electron micrograph of bracovirus in the calyx fluid (CF) of a female parasitoid wasp. A single membrane envelope (E) surrounds multiple nucleocapsids (NC) viewed end-on or laterally. An epithelial cell (EC) of the calyx is shown. (Micrographs courtesy of Dr. Donald B. Stoltz, Dalhousie University. Modified from Stoltz DB, Vinson SB. Viruses and parasitism in insects. *Adv Virus Res* 1979;24:125–171.)

they resemble the nucleocapsids of baculovirus and nudiviruses. Bracoviruses have a single membrane envelope, which is derived from the nucleus and surrounds one or more nucleocapsids (Fig. 73.7). Polydnvirus virions contain twenty or more different polypeptides associated with the nucleocapsid and the membrane envelope. These structural proteins are produced in the wasp through the expression of proviral DNA.^{20,185,302,345}

Polydnvirus Genome Organization

The polydnvirus genome is unique among animal viruses. It consists of an interrelated assortment of covalently closed, dsDNA circles that exist in multiple topologies that include superhelical and relaxed circular forms. The complete genomes of several bracoviruses and ichnoviruses have been sequenced to date. These and other studies have revealed that virion DNAs (designated by letters) are nonequimolar and polymorphic in length (reviewed by 185,302,345). For example, the 568-kbp genome of *Cotesia congregata* bracovirus (CcBV) consists of 30 dsDNAs ranging from 10 to >30 kbp.⁷⁷ Although each segment encodes at least one gene, a majority of the CcBV genome is comprised of non-coding DNA, in contrast to that of most autonomous DNA viruses. Not surprisingly, the aggregate size of polydnvirus genomes varies greatly and can be as large 600 kbp.³⁰² Many polydnvirus genes contain introns and thus resemble host genes. In the case of the ichnovirus prototype, *Campoletis sonorensis* polydnvirus (CsIV), the encapsidated 247-kbp genome is comprised of 24 nonredundant circular DNA segments.³⁴⁶ However, the CsIV genome also contains multiple redundant segments and a large number of repetitive elements. These higher-copy-number segments may provide increased expression of genes encoded therein. Each CsIV genome segment encodes at least one gene, but also contains portions that are homologous to other segments. Several of the ichnovirus segments are likely derived by recombination from larger segments and are thus described as

“nested” segments.^{185,302,345} This phenomenon also increases the copy number of certain virulence genes.

Until recently, the origin of the polydnviruses has remained an enigma. Nucleotide sequence analyses indicated that encapsidated DNA genomes of the bracoviruses and the ichnoviruses lack many genes that are characteristic of known virus families, including those genes involved in DNA replication.^{20,302} Instead, the virion-packaged DNAs encode several novel gene families that contribute to the pathology and immunomodulation by these viruses in the parasitized host (see later discussion). Thus, in the absence of any virus multiplication outside the wasp itself (i.e., in the parasitized larva), replicative genes appear dispensable. The recent surprise concerning the origin of polydnviruses, specifically the bracoviruses, is the finding that provirus genes expressed in the wasp ovaries include a set of 22 genes that are most closely related to those encoded by the autonomous nudiviruses that also infect lepidopteran caterpillars.¹⁹ Of these bracovirus genes, 14 individual genes exhibit sequence similarity to those baculovirus genes that contribute to virus late gene transcription, viral DNA packaging, particle assembly, and membrane envelopment of virions. However, there is no evidence for expression of those genes known to contribute to baculovirus DNA replication, such as the *lef* encoding DNA helicase, DNA polymerase, or DNA binding proteins. These findings and others support the notion that the polydnviruses evolved from an ancestral nudivirus that had established a persistent infection in the braconid wasps.^{20,302} In contrast, similar sequence analyses of nonencapsidated ichnovirus structural genes that are expressed in ovaries of ichneumonid wasps³³¹ failed to identify any relationships with nudivirus genes. Thus, the ichnoviruses may have evolved from a yet-to-be-identified ancestral virus unrelated to the bracoviruses.

Proviral DNA of the polydnviruses is present as integrated segments that are clustered at different loci of the wasp

genome; thus, the provirus is inherited in a Mendelian fashion. The mechanisms by which the polydnavirus genome is amplified, excised, and packaged into virus particles are poorly understood.^{185,302,345} Evidence exists that proviral DNA segments within the wasp genome are amplified before excision and encapsulation. Where the relationship between virion and proviral DNA is known, the largest or “master” DNA segments within the wasp genome are flanked by direct repeats. For example, the 16-kbp integrated W segment of CsIV is flanked by 1.2-kb repeats.^{66,185} Within the W segment, additional 350-bp repeats generate nested segments R and M, apparently by homologous recombination during amplification in the female wasp. In the wasp genome, CsIV segment B is flanked by 59-bp direct repeats that are removed during excision and circularization.⁸⁸ This finding and others are consistent with a mechanism in which CsIV proviral segments are excised from the wasp genome, possibly by recombination, prior to amplification.¹¹⁴ The presence of chromosomal sites lacking proviral

DNA in tissues that accommodate viral replication is consistent with this later mechanism.¹¹⁴

Polydnavirus Functions in the Wasp's Life Cycle

Polydnaviruses play a critical role in the life cycle of endoparasitic wasps. Moreover, these integrated viruses depend on the wasp for their survival. Thus, the mutualistic interaction between virus and wasp is a central determinant of the genetic composition and multiplication strategy of these novel viruses. Polydnaviruses multiply in the oviducts of female wasps as a normal step in the development of the reproductive tract (Fig. 73.8). Virus multiplication is under hormonal control and initiates during development of the wasp as a pupa.^{185,302,345} Virus DNA production, encapsidation, and virion assembly occurs primarily in the nuclei of epithelial calyx cells of the wasp's oviducts (Fig. 73.8). In mature females, the virions are released from the calyx cells into the oviduct lumen, either by budding (ichnoviruses) or by cell lysis (bracoviruses). Polydnavirus particles accumulate to high

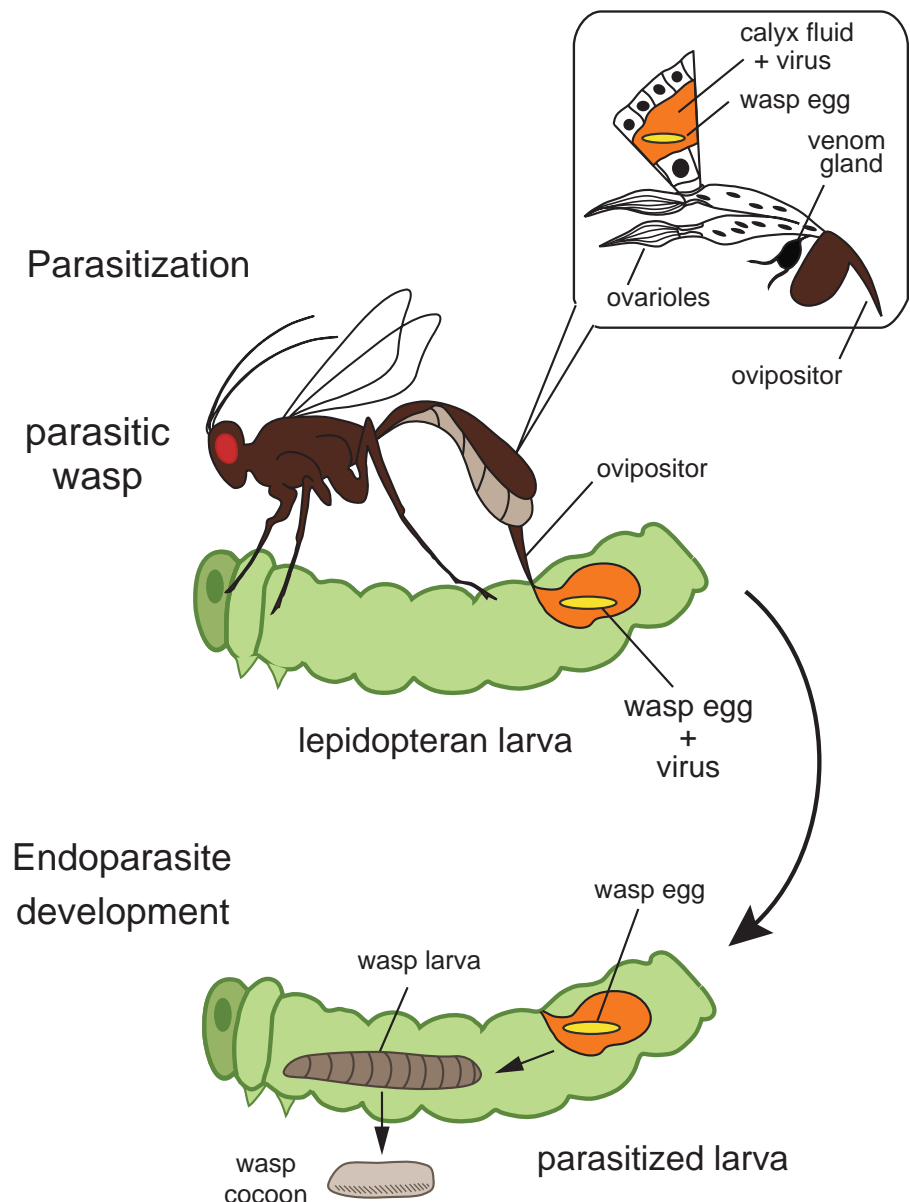


FIGURE 73.8. Polydnavirus transmission.

Polydnavirus particles form in the epithelial cells of adult female ovaries and are released into the calyx fluid (*top inset*) where they accumulate to high densities. In the first step of parasitization (*top*), the wasp egg is injected along with virus and venom into a lepidopteran larva during oviposition. During endoparasitic development (*bottom*), polydnavirus-encoded gene products suppress the larval immune response allowing the wasp egg to develop. After parasitization, the wasp larva spins a cocoon, pupates, and emerges as an adult to repeat the process. (Courtesy of Dr. Bruce A. Webb, University of Kentucky; modified with permission.)

density in the calyx fluid (Fig. 73.7C) and are deposited along with the egg and other wasp secretions in the target larva during oviposition. Virus gene expression in the wasp differs from that in parasitized caterpillar hosts.^{28,185,302,314} Despite the presence of high concentrations of virions, polydnnaviruses cause no obvious pathology in provirus-carrying wasps. The predominant form of viral DNA in males is the integrated provirus.

Polydnnavirus Modulation of Larval Responses During Parasitization

Upon oviposition, the female wasp delivers her egg accompanied by virus to the recipient caterpillar (Fig. 73.8). The caterpillar normally mounts an immune response that encapsulates and destroys the intruding egg in a process mediated in part by hemocytes of the larva's circulatory system. However, in the case of polydnnavirus-carrying ichneumonid and braconid wasps, the virus suppresses encapsulation and other aspects of the larva's immune response, which allows proper development of the wasp's offspring. In experiments in which the egg is artificially injected into the larva in the absence of virus, the egg is destroyed, demonstrating a definitive role for virus-mediated abrogation of the larva's immune response.⁷⁶ Thus, the relationship between polydnnavirus and wasp is mutualistic: the wasp egg requires the virus for protection from the immune response of the recipient larva and the virus requires survival and proper development of the wasp for its own perpetuation.

The most obvious physiologic effect of the polydnnaviruses is in the parasitized insect host, in which larval immunity and development are altered.^{185,302} Neither virus replication nor virus assembly occurs in parasitized larva. However, virion DNA is long lived and viral RNA transcription occurs throughout parasitization, principally in larval hemocytes and fatbody (the equivalent of the vertebrate liver). In caterpillars, the primary immune response to parasitization is encapsulation of the wasp egg and includes direct attachment of larval hemocytes in layers. Polydnnaviruses suppress encapsulation by altering the physiology of the capsule-forming hemocytes (granulocytes and plasmatocytes).^{302,345} Some polydnnavirus gene products are secreted and may affect surrounding hemocytes and tissues. In the case of *Microplitis demolitor* bracovirus (MdBV), virus-induced apoptosis kills a subclass of hemocytes that mediate the encapsulation response.³⁰³ Nonetheless, some hemocytes survive to express polydnnavirus genes that alter their function.

Polydnnaviruses carry the genes for suppression of the caterpillar's immune response.^{185,302,345} Multiple gene families contribute to immunomodulation. This repetition and variation of related pathogenicity genes may enhance expression levels or facilitate tissue targeting during parasitization.²⁸ One such gene family expressed during parasitization includes the viral ankyrin (*vankyrin*) genes, which encode factors with ankyrin repeat domains belonging to the I κ B family of proteins.^{184,318} Two *vankyrin* genes of MdBV exhibit normal I κ B activity by inhibiting NF- κ B responsive genes when exogenously expressed in *Drosophila* cells.³¹⁸ Suggestive of their importance in immunomodulation, *vankyrin* genes are encoded by both the ichneumonviruses and the bracoviruses. NF- κ B is an important regulator of insect genes encoding antimicrobial peptides and factors involved in cellular immunity (reviewed by 61,302). Thus, the I κ B-like activity of the *vankyrins* could disrupt NF- κ B activation of these immune response genes in the parasitized larva. Polydnnaviruses also carry a family of genes (*ptp*) that encode tyrosine

phosphatase-like proteins (PTPs). Cellular PTPs regulate receptor-mediated signal transduction pathways, suggesting that the virus-encoded PTPs function similarly to downregulate the immune response. Two bracovirus *ptps* are involved in suppression of encapsulation and phagocytosis.^{266,304} Polydnnaviruses pathogenesis genes can also have either proapoptotic or antiapoptotic effects on lepidopteran cells,^{79,304} suggesting that virus-mediated control of apoptosis contributes to immunosuppression. Other polydnnavirus-encoded immunomodulator genes, including those predicted to encode epidermal growth factor-related proteins, cysteine-rich motif proteins, vinnexin, and *rep* proteins, have been identified.^{77,185,302,345}

Ascoviridae

Classification and Structure of Ascoviruses

Ascoviridae is a family of large DNA viruses that primarily infect insects of the order Lepidoptera (moths and butterflies) and cause a slow, fatal disease.⁸³ The name ascovirus is derived from the Greek word *askós* (sac), which describes the large membrane-bound vesicles that are associated with virus development. The formation of these virion-containing vesicles represents the family's distinguishing feature⁸¹ and is critical to the virus's dissemination strategy that involves endoparasitic wasps (see later discussion). The ascoviruses are one of the newest DNA virus families to be discovered, with only five different species identified to date. Depending on the species, ascovirus particles are reniform (kidney-like) or bacilliform in shape (Fig. 73.9A). The virions (~400 × 130 nm) have an electron-dense core surrounded by two unit membrane envelopes (Fig. 73.9B). At least 21 different proteins, ranging in size from 9 to 120 kDa, are present in virions of the prototype ascovirus, *Spodoptera frugiperda* ascovirus 1a (SfAV).³⁰⁸ The ascovirus genome is a double-stranded circle of DNA ranging in size from ~116 to 186 kbp, depending on the species (reviewed in 83). Nucleotide sequences indicate that the ascovirus family is unique but exhibits some relatedness to the invertebrate iridoviruses.²²

Stages of Ascovirus Replication

The first visible sign of ascovirus infection is hypertrophy of the cell and its nucleus; the infected cell's diameter can increase up to 10-fold.⁸² The nucleus subsequently fragments and the infected cell is divided into 20 to 30 membrane-bound vesicles containing assembling virions (Fig. 73.9C). This process involves extensive invagination of the plasma membrane and mimics that which occurs during apoptosis. Once virus-containing vesicles dissociate from the infected cell, they are released into the larva's hemolymph. The larva's basement membranes are then disrupted and the hemocoel fills with ~10⁸ vesicles per ml, which gives the hemolymph a distinguishing milky white color.⁸¹ These vesicles with their associated virus are subsequently picked up during the egg-laying process by the ovipositor of an endoparasitic female wasp.

Interestingly, a host-like effector caspase encoded by the ascovirus SfAV likely contributes to this unusual vesicle formation during infection.²¹ Thus, the ascoviruses may have exploited host-cell apoptosis as a means to expedite virus transmission by endoparasitic wasps (see later discussion). *Heliothis virescens* ascovirus (HvAV) also encodes a caspase, but multiplication in cell lines fails to induce comparable vesicle formation.³ Thus, the role of ascovirus caspases or that of the host's caspases during larval infection remains to be clarified. The

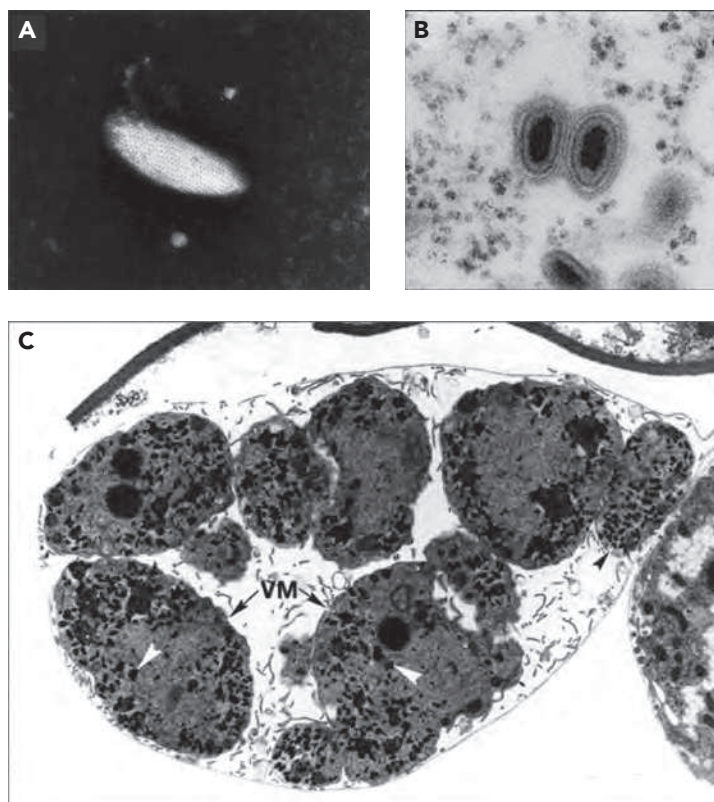


FIGURE 73.9. Ascoviruses. **A:** A single nucleocapsid of *Trichoplusia ni* ascovirus. The electron micrograph shows that the surface of the kidney-shaped particle has a reticular texture. **B:** Cross-section of two *Scotogramma* ascovirus virions. A multi-layered membrane surrounds the nucleocapsid. **C:** Intracellular vesicles formed during ascovirus infection. Dark-staining bodies are membrane-bound vesicles (VM) that contain *Autographa precationis* ascovirus particles (white arrowheads). The VM are bordered by a basement membrane. (**A** courtesy of Dr. Brian A. Federici, University of California–Riverside, and Dr. J. J. Hamm, USDA–ARS, Tifton, GA; **B** modified from Federici BA. A new type of insect pathogen in larvae of the Clover cutworm *Scotogramma trifolii*. *J Invertebr Pathol* 1982;40:41–54; and **C** modified from Federici BA, Hamm JJ, Styer EL. Ascoviridae. In: Adams JR, Bonami JR, eds. *Atlas of Invertebrate Viruses*. Boca Raton, FL: CRC Press 1991:339–349.)

ascoviruses encode several lipid metabolism enzymes, which may also contribute to vesicle production.⁸³

To date, ascovirus replication mechanisms are poorly understood. Multiplication in lepidopteran cell cultures has revealed possible roles for viral and cellular microRNAs in regulation of HvAV (reviewed by 5). Interestingly, HvAV and other ascoviruses encode an RNase III gene, which exhibits nuclease activity for dsRNA and appears to be essential for virus multiplication.¹⁵⁴ Encoded by few other viruses, RNase III proteins play an important role in RNA silencing.

Ascovirus Transmission, Disease, and Host Range

In nature, the principal transmission route of ascoviruses involves endoparasitic wasps, which carry virus-containing vesicles on their ovipositor as a result of egg laying. In the laboratory, ascovirus inoculation is accomplished by pricking the host larva with a needle bearing virus-containing vesicles.⁸³ A dose of 10 larva-derived vesicles is sufficient for 90% infection. The viruses cause a slow, chronic infection in larvae or pupae. The disease causes reduced feeding, developmental arrest, and death within 2 to 6 weeks. There is little obvious pathology during the early stages of infection. Some larvae exhibit the characteristic milky discoloration. The host range of ascoviruses may include only a few closely related insect species, mostly of the order Lepidoptera (reviewed in 83). However, the lack of obvious pathology early in infection may mean that the host range is broader than that suggested by entomologic surveys. Of the ascoviruses studied to date, *Diadromus pulchellus* ascovirus (DpAV) multiplies both in its lepidopteran hosts and at low levels in the reproductive tract of the ichneumonid wasp *D. pulchellus*. A mutualistic relationship between

DpAV, the most divergent of the ascoviruses, and its wasp vector is suspected.^{23,83}

Nudiviruses

Classification, Structure, and Genomics of the Nudiviruses

Historically, the nudiviruses were thought to be nonoccluded baculoviruses because of their common characteristics, including rod-shaped nucleocapsids, a circular, dsDNA genome, and nuclear replication.³⁸ However, nucleotide sequence analyses indicated that the nudiviruses are only distantly related to the baculoviruses (reviewed by 342). Thus, a new genus *Nudivirus* has been proposed to accommodate these novel insect pathogens. Of the five species characterized to date, these viruses have been isolated from most of the major insect orders, suggesting an ancient co-evolution of the nudiviruses and their insect hosts.

Varying in size from 97 to 230 kbp, the nudivirus DNA genome is packaged into a nucleocapsid that is surrounded by a membrane envelope acquired within the nucleus and one obtained upon budding from the plasma membrane. As determined from the four nudiviruses sequenced to date, a core of 33 genes is conserved. Of these genes, 20 exhibit sequence similarity with baculovirus genes that are involved in transcription, DNA replication, and nucleocapsid composition or assembly.³⁴² Nudivirus replication mechanisms are poorly understood, thus the functions of these similar genes are unknown. Although existing as nonoccluded viruses, certain nudiviruses also possess a gene related to polyhedrin or granulin used by baculoviruses for occlusion. It is interesting to speculate that this occlusion-like gene is the remnant of a common ancestor of the nudiviruses and baculoviruses.

Nudivirus Latency and Transmission

Originally isolated from a *Heliothis zea* (corn earworm) cell line, HzV-1 is one of the best-characterized nudiviruses. Its genome is a large (~228 kbp) circle of dsDNA⁵⁰ that has limited sequence similarity to certain baculovirus genes, confirming its very distant relationship to *Baculoviridae*. A hallmark of HzV-1 is its capacity to establish persistent or latent infections.³⁸ HzV-1 persistence involves stable integration of the genomic DNA and low-level, spontaneous replication of virus that can cause lytic infection of naive cells.²⁰² The establishment of viral persistence or latency is associated with the presence of a single 2.9-kb HzV-1 RNA transcript, designated the persistency-associated transcript (PAT1).⁴⁶ PAT1 is a noncoding RNA that is the only HzV-1 RNA detected during latency. Recent evidence suggests that PAT1 suppresses the HzV-1 *hhi-1* gene by a mechanism that involves the generation of microRNAs that target and destabilize *hhi-1* RNA transcripts.³⁵⁶ Transcription of HzV-1 *hhi-1* gene occurs early in infection and mediates reactivation from latency, thereby contributing to a productive infection.³⁵⁵ Consequently, HzV-1 may use microRNAs derived from its single latency transcript in an RNA interference mechanism that is required for establishment of latency.³⁵⁶ Thus, HzV-1 will serve as a useful model for mechanistic studies of insect virus latency.

Oryctes virus is another notable nudivirus. Distinguished by its unique tail-like structure protruding from one end, this 128-kb DNA virus shares many genes with other nudiviruses and certain replication, transcription, and nucleocapsid genes with the

baculoviruses.³⁴¹ Oryctes virus causes fatal infections of the rhinoceros beetle *Oryctes rhinoceros*, which is a serious pest species of palm trees in Southeast Asia and the Pacific region.¹⁵⁸ Consequently, this virus is a very effective biologic control agent used in coconut and palm oil plantations of the area. Virus is transmitted by contaminated food sources or mating. Typical of most nudiviruses, little is known about its replication mechanisms.

Nodaviridae

Classification and Host Range of Nodaviruses

Members of the family *Nodaviridae* are small RNA viruses with a single-stranded, positive-sense genome consisting of two RNAs. The bipartite genome is packaged within a single, non-enveloped virion (~30 nm dia) with T = 3 icosahedral symmetry (reviewed in 10,12,327). The genome organization and replication strategy of the nodaviruses is among the simplest of known viruses (Fig. 73.10). As such, the nodaviruses are powerful models for exploring strategies of virus replication, virion assembly, host interactions, and RNA silencing.

The nodaviruses are divided into two genera, the alphaviruses and betanodaviruses, which infect insects and fish, respectively. The family name originated from Nodamura virus, which was first discovered in mosquitoes near the Japanese village of Nodamura.²⁸² The best-studied insect nodaviruses include flock house virus (FHV), black beetle virus (BBV), Nodamura virus (NoV), and Wuhan nodavirus (WhNV). These viruses were isolated from the grass grub *Costelytra zealandica* (Coleoptera) in New Zealand; the scarab beetle *Heteronychia arator*

Nodavirus genome organization

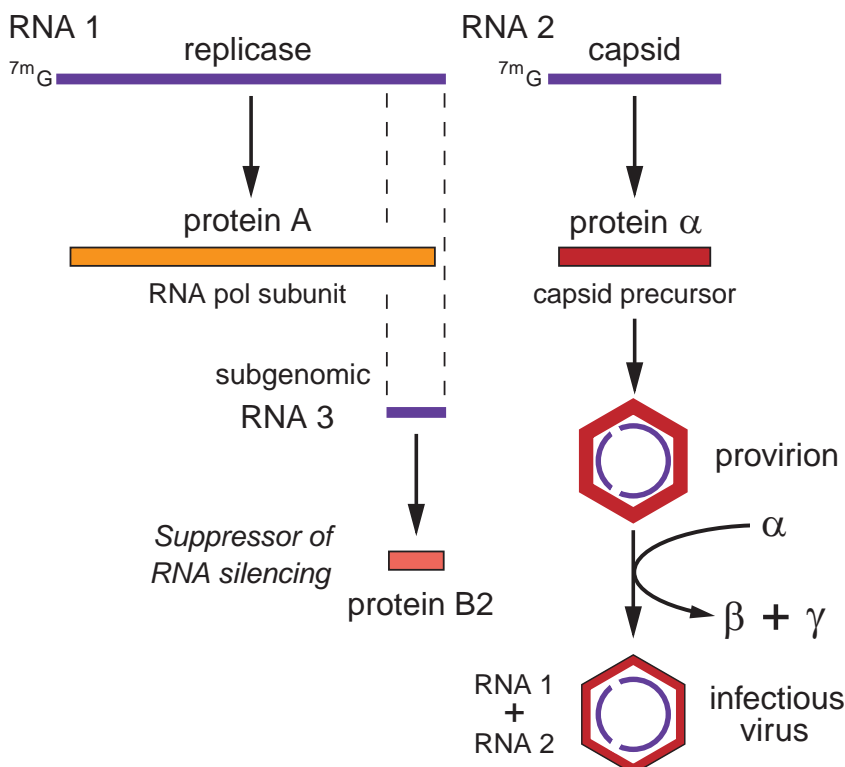


FIGURE 73.10. Nodavirus genome organization and assembly. Positive-sense genomic RNAs 1 and 2 encode the replicase (protein A) and capsid precursor protein (protein α), respectively. RNA 1 also generates subgenomic RNA 3, which encodes protein B2, a suppressor of host-cell RNA silencing. Assembled from capsid precursor α , provirions undergo a maturation cleavage producing capsid proteins β and γ . Infectious virions contain an equimolar ratio of genomic RNA 1 and 2.

(Coleoptera), also in New Zealand; the mosquito *Culex tritaeniorhynchus* (Diptera) in Japan; and the small cabbage white butterfly *Pieris rapae* (Lepidoptera) in China, respectively.³²⁷ Most members of the alphanodavirus genus were isolated from insects of Australasia. Other alphanodaviruses include Pariacoto virus (PaV), New Zealand Virus, Boolarra virus, and Manawatu virus. NoV is the only nodavirus with the capacity to multiply in vertebrates.¹⁰ Nonetheless, upon transfection of their genomic RNA, many nodaviruses can replicate in cells from highly diverse organisms, including mammals, nematodes, plants, and the yeast *Saccharomyces cerevisiae*.^{7,207,261,287}

Nodavirus Virion Structure

The nodaviruses have provided enormous insight into icosahedral virus assembly and maturation. The structures of BBV, FHV, NoV, and PaV have been determined at atomic resolution (see images at <http://virology.wisc.edu/virusworld/> or <http://viprdb.scripps.edu/>),^{12,87,148,309,365} Each virion contains 180 copies of the same capsid protein (Fig. 73.10A). Sixty triangular units, consisting of three similarly folded capsid proteins, form an icosahedral lattice with T = 3 symmetry.^{160,283} Three capsid proteins form a prominent peak at the quasi-threefold axis. At the twofold axes, conserved C-terminal residues of the capsid protein make contact with duplexed genomic RNA that is highly ordered and stabilize the capsid protein interface.⁸⁷ The crystal and cryomicroscopy structures of nodaviruses, including that of PaV and FHV, reveal a dodecahedral cage of ordered dsRNA (Fig. 73.11B) that constitutes 35% (1,500 nucleotides) of the ssRNA genome.^{309,319} The RNAs interact extensively with the basic N-terminal region of the capsid protein as well. However, the ordered RNA structure does not require a specific nucleotide sequence to form. Interestingly, C-terminal residues of the capsid protein are required for RNA encapsulation.³²⁷ At the fivefold axis, the capsid proteins form a pentameric helical bundle that has been proposed to participate in release of the genomic RNA upon virus penetration into the host cell's cytosol.⁵¹

The nodavirus provirion is assembled from 180 copies of the ~43-kDa capsid precursor α . During virion maturation (Fig. 73.10), α is cleaved very near its C terminus to generate capsid proteins β and γ .^{99,105} For FHV, this autocatalytic post-assembly cleavage occurs between residues Asn³⁶³ and Ala³⁶⁴, which are located on the inside surface of the virion. This cleavage event is required for infectivity and virion stability.^{105,284} Either this maturation cleavage or the liberated γ peptide may also contribute to virus uncoating and release of the genomic RNAs into the cytosol (see later discussion).

Nodavirus Genome Organization

The nodavirus genome consists of two single-stranded, messenger-active RNAs, designated RNA 1 and 2, that are 7-methyl GpppN capped, but not polyadenylated (Fig. 73.10). The 3' ends of both RNAs have an unknown structure or unidentified factor attached. RNA 1 (~3.1 kb) encodes the functions required for RNA transcription and replication, whereas RNA 2 (~1.4 kb) encodes the coat protein. Both RNAs are required for infectivity and are packaged within the same virion.³²⁷ Functions of the genomic RNAs were determined by using heterologous expression systems, which include baculovirus vectors, vaccinia virus vectors, transformed yeast, and transfected *Drosophila* cells. RNA 1 carries all the information necessary for autonomous replication.¹⁰⁴ It encodes at least two polypeptides:

proteins A and B2 (Fig. 73.10). Protein A (~112 kDa) is an RNA-dependent RNA polymerase (RdRp) that is required for virus RNA replication.^{9,104} Encoded by the 3' end of RNA 1, protein B2 (~12 kDa) is translated from subgenomic RNA 3 (Fig. 73.10), which is abundant in infected cells, but not packaged into virions.^{97,116} Protein B2 is a suppressor of host-mediated RNA silencing (see later discussion). Nodavirus genomic RNA 2 encodes the capsid precursor protein α (Fig. 73.10). Thus, the functions necessary for genome replication (RNA 1) and virus transmission (RNA 2) are segregated onto different genetic elements.

Stages of Nodavirus Replication

FHV, the best-studied nodavirus, and its close relative BBV multiply prolifically in *Drosophila melanogaster* cell lines,³²⁷ providing a highly tractable system in which to dissect virus replication. It is likely that FHV enters the host cell by receptor-mediated endocytosis. However, the *Drosophila* receptor remains to be identified. The acidity of the endosome triggers an irreversible exposure or release of the capsid γ peptides, which disrupts the endosomal membrane and facilitates release of the genomic RNAs into the cytoplasm.³³⁶ Required for membrane penetration, the lipophilic γ peptide is generated by autoproteolytic cleavage of capsid protein precursor α within the assembled virion.^{11,236} Subsequent viral multiplicative events occur in the cytoplasm. Uncoated genomic RNA 1 is translated to produce the protein A-containing RdRp. Newly synthesized viral RNAs are detected within 2 hours after inoculation and continue to accumulate through 20 hours. By then viral RNA represents up to 50% of the total cellular RNA, an astounding level considering that it is comparable to that of ribosomal RNAs.^{98,99,105} Protein A-mediated viral RNA replication occurs on the outer mitochondrial membranes. In particular, replication occurs within newly formed 50-nm diameter membrane-bound spherical structures (spherules) that are comparable to those formed during replication of other positive-strand RNA viruses.²¹⁸ Protein A and replicating FHV RNA are required for spherule formation; proteins B2 and capsid proteins are dispensable.¹⁷³ It is estimated that within each spherule there are three RNA replication intermediates and an average of 100 membrane-associated protein A molecules, which self-interact.¹⁷² A single neck-like portal, which is large enough to accommodate messenger-active RNAs, connects these virus-induced spherules to the outside cytoplasm. Mitochondrial phospholipids and cellular chaperones are critical for FHV RNA replication in transfected yeast cultures, thus are also likely important for virus multiplication.^{299,347}

During infection, nodavirus RNA synthesis is temporally regulated. Late in infection, the replication of genomic RNA 1 and 2 is coupled, which ensures production of an equal molar ratio of both RNAs required for virion packaging. However, early in infection, subgenomic RNA3, which produces B2 to suppress the host RNAi response, transactivates RNA2 replication.⁸ As the level of RNA2 increases, it represses RNA3 production¹⁰⁴ through a feedback inhibitory mechanism.³²⁷ Not surprisingly, nodaviruses generate their plus-stranded genomic RNAs through (–) RNA intermediates. *Cis*-acting sequences required for plus- and minus-strand RNA transcription and RNA 3 suppression have been identified.¹⁰ The FHV RNA 1 nucleotides that direct synthesis of this self-replicating RNA and its recruitment to the mitochondrial membranes have also been defined.³²²

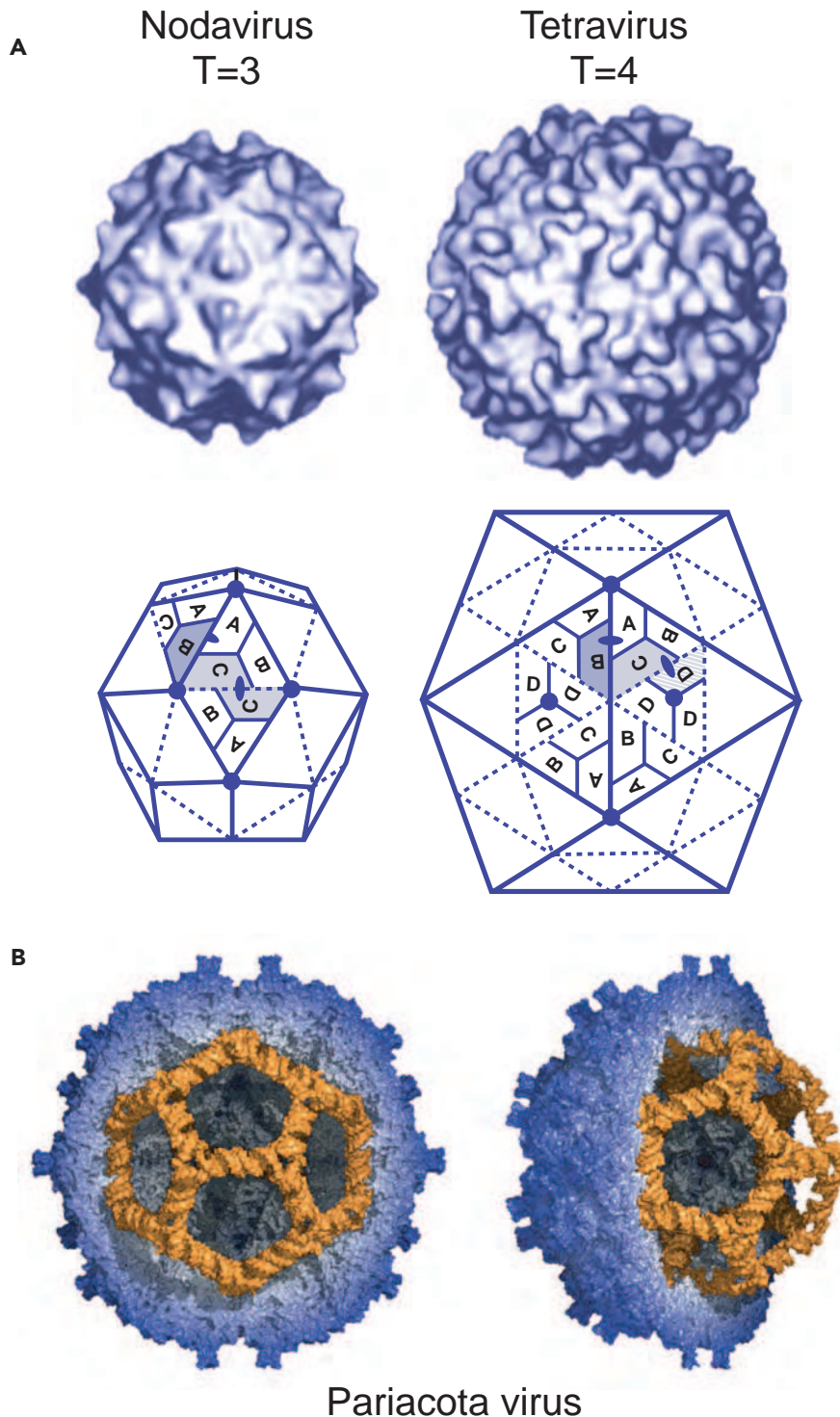


FIGURE 73.11. Comparison of nodavirus and tetravirus structures. **A, top:** Image reconstructions of a T = 3 nodavirus (flock house virus) and a T = 4 tetravirus (*Nudaurelia capensis* ω virus). The average particle diameter for the nodaviruses and tetraviruses is 320Å and 415Å, respectively. **A, bottom:** Subunit arrangement of the nodavirus (T = 3) and the tetravirus (T = 4) virions. Each subunit is indicated by a trapezoid. Nodaviruses contain 60 subunits of three similarly folded capsid proteins (A, B, and C) that form a prominent peak at the quasi-threefold axis (*top image*). Tetraviruses contain 240 capsid protein subunits (A, B, C, and D), in which 12 subunits comprise each triangular face of the virion. (See images at <http://virology.wisc.edu/virusworld/> or <http://viperdb.scripps.edu/>.) **B:** Arrangement of genomic RNAs within the virion of the nodavirus Pariacoto virus. The symmetrically arranged shell of helical RNA (*lighter*) contacts capsid proteins on the inside surface of the virion (*darker*). (Adapted with permission from Dr. John E. Johnson, Scripps Research Institute, La Jolla, CA. Images of Pariacoto virus courtesy of Jean-Yves Sgro, Institute for Molecular Virology, University of Wisconsin–Madison.)

Translation of nodavirus RNA 2 yields abundant levels of the capsid precursor α , which is cleaved during capsid assembly (Fig. 73.10). Despite equal intracellular levels of RNA 1 and 2, synthesis of capsid protein α predominates late in infection as a direct result of the ability of RNA 2 to outcompete RNA 1 for rate-limiting translation factors.⁹⁸ As capsid precursor α accumulates, an equimolar ratio of RNA 1 and 2 are packaged within the nodavirus provirion; the mechanism ensuring this

stoichiometry is unknown.¹⁸³ Before cell lysis, extraordinary yields of assembled particles (2 mg per 10^8 cells) accumulate in the cytosol of the infected cell and extensive crystalline arrays in the cytoplasm are often observed. Owing to the segmented genome, reassortment of genomic RNAs has been detected upon mixed infections of cultured cells. Moreover, nodaviruses can generate defective interfering particles and can establish persistent infections in cell cultures.^{162,327}

Permissive *Drosophila* cells respond to nodavirus FHV infection by undergoing caspase-dependent apoptosis.²⁹⁰ Both alphaviruses and betanodaviruses have the capacity to trigger apoptosis during multiplication.^{125,290} FHV-mediated depletion of the endogenous inhibitor-of-apoptosis DIAP1 triggers apoptosis in *Drosophila*, as it does during baculovirus-induced apoptosis in these cells.^{290,325} Because host caspases are not required for nodavirus assembly or maturation, the process of apoptosis and the resulting membrane-protected vesicles may facilitate virus release and subsequent dissemination to tissues within the host insect.

Nodavirus Suppression of Host RNA Silencing

Nodaviruses have also provided important understanding of the molecular mechanisms by which RNA viruses trigger the antiviral RNA silencing response in host cells and how viruses suppress the response through the activity of virus-encoded RNA-binding proteins (reviewed by 2,72,327). RNA silencing—also known as RNA interference (RNAi)—is a critically effective antiviral defense in invertebrates, vertebrates, and plants. Capable of targeting and destroying RNAs from viruses and transposons, RNAi may be a primordial form of innate immunity.⁷⁰ Nodavirus protein B2 functions to suppress RNA silencing that is mounted by the host cell in response to viral RNA replicative intermediates.¹⁹⁶ B2 of FHV was the first discovered example of an RNAi suppressor from an animal virus. B2 enhances FHV multiplication and virus-mediated killing in cultured cells as well as adult *Drosophila* flies.^{103,161,196,340} Demonstrating the effectiveness of the RNAi response in insects, mutant flies with defective RNAi components are more susceptible to killing by FHV.^{103,340} Interestingly, Nodamura virus B2, which grows in both vertebrates and invertebrates, can suppress the RNAi response in both.^{196,198}

The anti-RNAi function of nodavirus B2 is attributed to its sequence-independent dsRNA binding activity.^{2,327} B2 binds to dsRNA (probably dsRNA replicative intermediates generated during virus RNA synthesis) and prevents cleavage by the RNAase III-like endonucleases (Dicer) of the RNAi pathway. Thus, B2 suppresses the generation of small interfering RNAs that are complementary to viral RNAs and used to degrade viral mRNA. The crystal structure of FHV B2 in complex with dsRNA shows B2 as a small four-helix polypeptide that binds as a dimer to a single face of duplex RNA in a sequence-independent manner.⁴⁵ Nodamura virus B2 has a similar protein fold despite its divergent sequence.¹⁷⁴ It is likely that multiple copies of nodavirus B2 bind to viral dsRNAs, protecting them from cleavage by Dicer and blocking incorporation into the RNAi silencing complexes.^{45,269} It will be of interest to ascertain where in the infected cell B2's dsRNA binding activity is localized relative to the nodavirus RNA-replicating spherules on the mitochondrial membranes.

Tetraviridae

Classification of Tetraviruses

The tetraviruses are small RNA viruses that multiply exclusively in lepidopteran insects (moths and butterflies). They contain a single-stranded, positive-sense RNA genome that is packaged into a nonenveloped, icosahedral virion. The family name *Tetraviridae* (“tetra” from Greek *tettares*, meaning “four”) is derived from the virion's T = 4 symmetry, which is the only known T = 4 symmetry among nonenveloped viruses (reviewed in 73,160). A major contribution of these viruses has been their use as models for defining molecular mechanisms of

assembly and maturation of icosahedral viruses. The family is divided into *betatetravirus* and *omegatetravirus* genera, in which *Nudaurelia capensis* β virus (N β V) and *Nudaurelia capensis* ω virus (N ω V) are the prototype species, respectively.¹³³ The tetraviruses *Euprosteria elaeasa* virus and *Thosea asigna* virus are under consideration by the ICTV as members of a new family *Permutotetraviridae* to account for their closer relationship with the RNA viruses of *Birnaviridae*.

Tetravirus Structure

The T = 4 structural features of the tetraviruses (Fig. 73.11) is indicated by cryo-EM reconstructions and X-ray crystallography, including that of N ω V and Providence virus (PrV), a betatetravirus.^{226,297} These studies (reviewed by 296) revealed that the virion contains 240 capsid protein subunits. Twelve chemically identical capsid proteins comprise each triangular face of the virion. The T = 4 capsid arrangement is readily distinguished from the T = 3 symmetry of the smaller nodaviruses that have 60 fewer capsid proteins per particle (Fig. 73.11). Remarkably, the tertiary structures of the capsid proteins of both virus families are very similar. Moreover, the N ω V capsid precursor is cleaved after Asn⁵⁷⁰ near the C terminus in a postassembly mechanism that generates capsid protein β and a smaller peptide γ in a pattern analogous to the required maturation cleavage of nodavirus capsid precursors (see previous discussion). In the nodaviruses, surface exposure of the lipophilic γ peptide can disrupt membranes,^{11,236} suggesting a similar role for tetravirus membrane penetration. The capsid precursor of PrV is also autocatalytically cleaved to produce a larger β capsid protein and a smaller C-terminal γ peptide. However, the PrV γ peptide has a structure and function unlike that of N ω V.²⁹⁷ Instead, the PrV γ peptide contributes to genomic RNA binding within the virion particle, like the nodavirus γ peptide. The striking similarities between the PrV and nodavirus peptides suggest that PrV is more closely related to the T = 3 nodaviruses, indicating that the tetraviruses and nodaviruses may have evolved from a common ancestor.^{160,297}

Tetravirus Genome Organization

The tetravirus RNA genome is either mono- or bipartite. To date, the genomes of four betatetraviruses have been sequenced (reviewed by 73). The single, monopartite RNAs range in size from 5.6 to 6.6 kb, contain a 7-methyl GpppN cap structure at the 5' end, and possess a unique tRNA-like structure at the 3' end.^{1,132} The monopartite genome of betatetravirus N β V¹¹² has two large ORFs. The 5' proximal ORF encodes a 214-kDa, RNA-dependent, replicase-like protein with motifs characteristic of a methyltransferase, nucleotide-binding helicase, and an RNA polymerase. As expected, the RNA replicase-containing polypeptides of the tetraviruses are proteolytically processed.⁷³ The 3' proximal N β V ORF encodes the 66-kDa capsid protein. The capsid ORF overlaps the replicase ORF but uses a different (+1) reading frame. It is therefore likely that the capsid is translated from a 2.5-kb subgenomic RNA¹¹²—*Thosea asigna* virus (TaV) synthesizes a 2.5-kb subgenomic RNA that encodes the capsid protein.²⁶⁴

The omegatetraviruses have a bipartite RNA genome (reviewed by 73). The bipartite genome of omegatetravirus *Helicoverpa armigera* stunt virus (HaSV) contains RNA1 (5,300 bases), which encodes the RNA replicase and smaller proteins, and RNA2 (2,478 bases), which encodes the 71-kDa HaSV

capsid protein and a smaller protein (p17) of unknown function.^{111,132} Typical of the tetraviruses, the HaSV capsid precursor is cleaved near its C terminus to generate an ~62-kDa β protein and a smaller 7-kDa γ peptide during assembly. It is unknown whether the omegatetraviruses co-package both genomic RNAs into a single virion.

Tetravirus Replication, Transmission, and Pathology

Tetraviruses multiply in the cytoplasm of infected cells whereupon crystalline arrays of virus particles accumulate within vesicles.¹³⁹ However, a paucity of permissive cell lines has hindered studies on replicative mechanisms. Such studies have been limited to ectopic expression of individual virus genes in heterologous cell lines.²⁹¹ PrV multiplies (albeit poorly) in lepidopteran cell lines, and newly synthesized genomic and subgenomic RNAs—as well as virus-encoded capsid proteins—are detected.^{265,297}

The tetraviruses are the only known RNA viruses restricted to insects, specifically those within the order Lepidoptera. Larval infection can be initiated by the ingestion of virus-contaminated foods.¹³¹ Virus multiplication is often restricted to the foregut and midgut cells, even upon injection of infectious virus into the hemolymph of larvae. Virus-mediated killing of midgut cells may account for the stunting pathology associated with HaSV. Susceptible insect larvae exhibit a wide range of pathogenic effects that include rapid death, an extended wasting disease, or a delay in pupation.⁷³ Death occurs within 4 to 7 days, usually accompanied by discoloration, flaccidity, and sometimes liquefaction. Tetravirus infections occur sporadically in natural populations. Thus, ecological aspects of virus spread and the existence of virus reservoirs are poorly understood. Nevertheless, these insect viruses are natural biopesticides that have been observed to control certain insects; several viruses, including *Darna trima* virus and *Thosea asigna* virus have been successfully applied as biopesticides in oil palm plantation settings.¹¹⁰

Dicistroviridae

Classification and Structure

Members of *Dicistroviridae* are small, nonenveloped RNA viruses named for the unusual bicistronic arrangement of their single genomic RNA (Table 73.1). Although these icosahedral insect viruses resemble the vertebrate picornaviruses, they form a taxonomically distinct family.^{30,53,144} The best-characterized dicistrovirus is Cricket paralysis virus (CrPV), the type species within the *Cripavirus* genus of *Dicistroviridae*. Other cripaviruses include the well-studied dipteran *Drosophila* C virus, hemipteran (aphids and leafhoppers) *Rhopalosiphum padi* virus, and hymenopteran (bees and wasps) black queen cell virus. The dicistroviruses, including Israeli acute paralysis virus, have received increased attention recently due to their association with colony collapse disorder in honeybees.³⁰

The physicochemical properties of the cripavirus virion are strikingly similar to those of the vertebrate picornaviruses. The icosahedral virion (< 40 nm diameter, $\rho = 1.35\text{--}1.37$ g/ml) contains four capsid proteins (VP1, VP2, VP3, and VP4) that are arranged in a pseudo T = 3 lattice. The 2.4 Å resolution crystal structure of CrPV shows a picornavirus-like capsid conformation and morphology.³¹⁰ However, CrPV lacks the deep depression (canyon) at the fivefold axis or a VP1 pocket that distinguishes the enteroviruses. Also unlike the picornaviruses, CrPV VP4 residues are located between VP2 and VP3. The proteolytic processing of VP0 \rightarrow VP3 + VP4 appears to be autocatalytic. Thus, cripavirus virion assembly may more closely resemble that of the insect nodaviruses and tetraviruses than the vertebrate picornaviruses.³¹⁰

Genome Organization and Novel Translation Initiation

Dicistroviruses possess a single-stranded, messenger-sense RNA genome about ~9 kb in length. The genome is distinguished by its bicistronic organization of two large ORFs (Fig. 73.12). Upon translation, each ORF produces two different

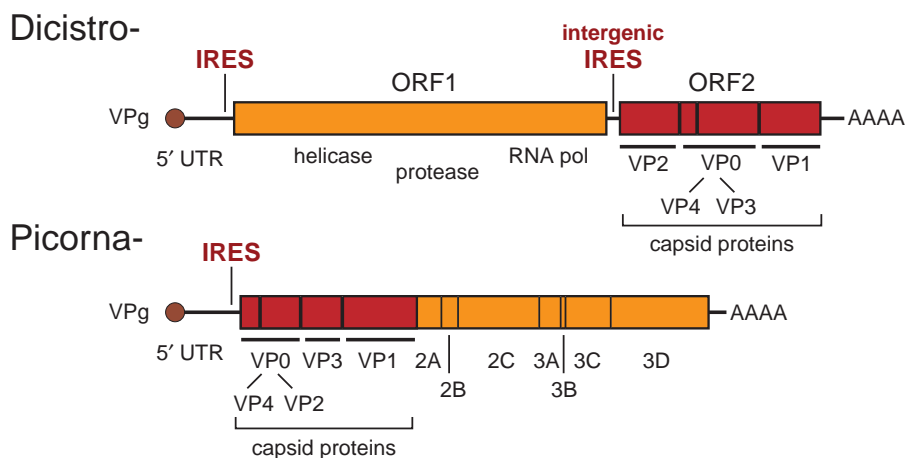


FIGURE 73.12. Genome organization of the dicistroviruses. The single-stranded RNA genome of the dicistroviruses, including CrPV and DCV, possess two open reading frames (ORF1 and ORF2) separated by an ~200 nucleotide spacer. An internal ribosome entry site (IRES) precedes both ORFs. ORF1 encodes virus replicative functions (helicase, protease, and RNA-dependent RNA polymerase), whereas ORF2 encodes capsid proteins VP0, VP1, and VP2. VP3 and VP4 are generated by processing of precursor VP0.³¹⁰ The small protein VPg and an untranslated region (5' UTR) are located at the 5' end of the genomic RNA. By contrast, the vertebrate picornavirus genome possesses a single ORF; it must therefore produce equimolar levels capsid proteins and replicative functions from the 5' and 3' ends of the genome, respectively.

polyproteins, which are proteolytically processed (reviewed by 30,144). Preceded by a 5' UTR, ORF1 encodes proteins with predicted RNA helicase, picornavirus 3C-like protease, and RNA-dependent RNA polymerase activities. In addition, the N-terminal end of ORF1 of CrPV and DCV encodes novel RNAi suppressors.²²⁹ These suppressors act by distinct mechanisms, but represent a common function encoded by insect viruses, including the nodaviruses. ORF2 is preceded by an intergenic UTR (~200 nucleotides) and encodes the capsid proteins. Thus, unlike the picornaviruses, the capsid proteins and nonstructural replication proteins are encoded by the 3' and 5' halves of the genome, respectively (Fig. 73.12). To date, dicistrovirus replication mechanisms are poorly understood. However, full-length infectious clones, now available for *Rhopalosiphum padi* virus,²⁴⁹ will expedite such studies.

During infection, the dicistrovirus capsid proteins are produced in great excess compared to the nonstructural proteins despite being encoded by the 3' half of the messenger-active genome. Accounting for this difference, an internal ribosome entry site (IRES) regulates translational initiation from each of the two ORFs. Characterized from CrPV, DCV, *Rhopalosiphum padi* virus, and *Plautia stali* intestine virus, these structures are common features of the *Dicistroviridae* and have provided novel insight into mechanisms of translational initiation (reviewed by 144). The use of an IRES avoids the need for a 5' 7-methyl GpppN cap on viral mRNAs, thereby allowing the virus to escape host- or virus-mediated shutdown of cap-dependent protein synthesis during infection. Thus, insects share these antiviral mechanisms with vertebrates. The IRES preceding ORF2 is particularly interesting in that, unlike a typical picornavirus IRES, this dicistrovirus IRES does not require any host initiation factors.¹⁴⁴ Rather, the IRES within the intergenic UTR (Fig. 73.12) assembles an 80S ribosome in the absence of initiator Met-tRNA_i or canonical initiation factors to initiate protein synthesis from the second triplet (GCU), which is positioned within the A site of the ribosome. This novel mechanism for IRES-mediated translation initiation involves a pseudoknot structure within the IRES that substitutes for required interactions between Met-tRNA_i and the P-site initiator codon.¹⁴⁴ Thus, the intergenic dicistrovirus IRES has facilitated important understanding of the molecular mechanisms of IRES function.

Dicistrovirus Prevalence and Transmission

The dicistroviruses are widely disseminated in animal populations (reviewed by 30,144). CrPV was originally isolated from Australian crickets *Teleogryllus oceanicus* and *T. commodus*; its name is derived by its capacity to cause paralysis of the hind limbs of early-instar cricket nymphs. Interestingly, CrPV has one of the widest experimental host ranges of any of the invertebrate viruses and can infect diverse species of multiple insect orders, including those of Diptera, Lepidoptera, Orthoptera, Hymenoptera, and Hemiptera.⁵⁴ Experimental inoculation by injection leads to virus multiplication and death of multiple insect species. CrPV transmission can also occur *per os* through contaminated food sources. In contrast to CrPV, DCV has a more restricted host range. DCV has been isolated from wild or laboratory strains of *Drosophila melanogaster*.⁵⁴ It can be transmitted *per os* to adult flies or larvae and cause death. In addition, DCV is transmitted horizontally between infected and uninfected adults. Thus, the transmission routes and pathology of dicistroviruses among insects is highly variable.

PERSPECTIVES

Considering the abundance and diversity of insect life on earth, it is likely that only a small fraction of the potential viruses that infect insects have been discovered thus far. Nonetheless, studies of the viral pathogens characterized to date have revealed novel mechanisms for virus replication, have identified unique viral genes with functions important in human disease (cancer, neurodegeneration, immunodeficiency), and have provided highly useful applications for biotechnology, insect pest management, and gene therapy. It is fully expected that further studies of the insect viruses will uncover fundamental aspects to host–virus interactions, including novel strategies by which these prolific viruses modify and exploit host processes to enhance multiplication and evade antiviral responses. On the basis that many of these responses are conserved in mammals, such studies will provide important insight into the mechanisms of antiviral defenses and virus pathogenicity in humans. In light of the expansion of the natural range of many arthropod vectors, an important but poorly understood area that will have major impact is the molecular interaction of arboviruses, including dengue virus and West Nile virus, with their insect (mosquito) vectors and how such interactions contribute to transmission to human hosts. In as much as the contributions of insect viruses to basic biology and biotechnology have been enormous and that insect viruses continue to plague humankind, there is a critical need for continued studies at the molecular level and adequate funding to support such investigations.

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Viruses and Prions of Yeasts, Fungi, and Unicellular Eukaryotes

Double-Stranded RNA Viruses

L-A Virus of *Saccharomyces cerevisiae*, Type
Species of the *Totiviridae*
Partitiviridae

Single-Stranded RNA Replicons

Viruses Reducing Virulence of *Cryphonectria parasitica*
A Reovirus of *Cryphonectria*
20S RNA and 23S RNA
Cryphonectria parasitica Mitochondrial Replicon
NB631 Double-Stranded RNA

Retroviruses (Retroelements)

Retroviruses, Retrotransposons, Retroposons,
and Retrointrons
Structure of Ty and Other Retroelements
Replication Cycle of *Saccharomyces cerevisiae*
Ty Elements
Host Limitations on Ty Transposition
Efficiency
Schizosaccharomyces pombe Retroelements
Candida albicans Plasmid Retrotransposon and
LINE Elements
Summary of Retroelements

DNA Viruses: *Chlorella* Viruses

Prions of *Saccharomyces* and *Podospora*

[URE3]—A Transmissible Amyloidosis
of Ure2p
[PSI]—A Transmissible Amyloidosis
of Sup35p
[PIN+]—A Prion that Generates Other
Prions
[Het-s]—A Prion of *Podospora* Responsible for a
Normal Function
Prion Variants
Structures of Yeast Prion Amyloids Explain Variant
Propagation
HET-s Prion Amyloid Structure
[URE3] and [PSI] are Diseases of Yeast
Chaperone Involvement in Prion
Propagation

Perspectives

Leishmania Double-Stranded RNA Viruses
Giardia lamblia Virus
Trichomonas Virus and Host Phenotypic Variation



Prions, retroviruses, double-stranded RNA (dsRNA) viruses, single-stranded RNA (ssRNA) viruses, and double-stranded DNA (dsDNA) viruses have all been found in simple eukaryote hosts, and the experimental advantages of these systems has made some of them the most thoroughly characterized of their classes. This is an overview of these infectious entities and their unique aspects, with some emphasis on recent developments.

All of the viruses, prions, and retrotransposons (or retroviruses) of yeast and fungi have wholly intracellular life cycles. These viruses have forsaken the extracellular route of transmission, being passed from cell to cell either vertically or by cytoplasmic mixing, such as occurs in mating, or in hyphal anastomosis (fusion) of filamentous fungi. They resemble, in this regard, the plant cryptoviruses and the intracisternal A type particles (retroelements) and LINEs (retroposons) of mammals. We have argued that the high frequency of mating and hyphal fusion of fungi in nature makes an extracellular phase dispensable for these viruses. Indeed, many wild *Saccharomyces cerevisiae* carry the RNA viruses,²⁶³ and it is difficult to find strains of *S. cerevisiae* that lack the Ty retrotransposons.³⁹⁰ Nor is the direct cell-to-cell route of infection completely neglected by mammalian viruses—those of the herpes group and human immunodeficiency virus being prime examples.

Recently, sequences of many yeast genomes have revealed occasional (diverged) copies of the yeast L-A viral and M₂ killer toxin genomes.^{136,350} Interestingly, the integrated viral sequences from distantly related yeasts were less diverged than their hosts, indicating horizontal transfer.³⁵⁰ The means by which this transfer occurs is not yet clear.

The largely intracellular mode of transmission means that these viruses must balance the need to spread and propagate against consideration for the viability of their host. This is clearly seen in the Ty elements, which tend to target sites that can tolerate an insertion. The Ty elements, particularly Ty3, regulate their transcription such that most transposition

occurs on mating. This design optimizes the chances of infecting the potentially unoccupied genome of the mating partner. The copy numbers of several of the RNA replicons (L-A dsRNA, L-BC dsRNA, and 20S RNA) are repressed by the host Ski proteins acting in translation,^{242,294,321,388} messenger RNA (mRNA) stability¹⁸⁵ and positive strand stability.^{139a,b} Furthermore, the fact that most of these elements are widespread means that the hosts have been selected for the ability to protect themselves. Thus, the host blocks Ty1 retrotransposition at several levels: translation, virus particle and DNA stability, structure of the chromatin target, and by antisense RNA.^{26,75,180,195,224,230,245,286}

Most of the viruses and prions discussed in this chapter can best be viewed as selfish RNA or DNA (or selfish protein); however, the killer satellite dsRNAs clearly benefit their hosts (and promote the L-A helper virus) by allowing them to kill competitors. The [Het-s] prion of *Podospora* is unique among prions described to date in being necessary for a normal cellular function—namely, heterokaryon incompatibility.⁷⁹

Yeast and fungal prions have led the way in proving the prion concept, defining the structure of prion amyloids and in relating those structures to prion biology and heritability. Moreover, the study of yeast prions has explored myriad chaperones and other host components affecting prion generation and propagation.

It is striking that many of the viruses and retroelements discussed use some form of ribosomal frameshifting to make a Gag-Pol fusion protein. This is not surprising for the retroelements but was not expected for the dsRNA genomes. Ribosomal frameshifting (in contrast to splicing or RNA editing) provides a mechanism for viruses whose mRNA is their genome to obtain two proteins with overlapping sequence from one mRNA without producing mutant genomes.¹⁸³ Other translational tricks used include read-through of stop codons and internal ribosome entry.

The use of *S. cerevisiae* as a model host for viruses of plants and insects promises to facilitate the understanding of these viruses^{187,261,285} but will not be dealt with here. Surprisingly, a mammalian defense against retroviruses—the APOBEC3 cytidine deaminases that are preferentially incorporated into retrovirions—are also incorporated into Ty1 particles and block retrotransposition by deaminating the viral genome.¹¹²

DOUBLE-STRANDED RNA VIRUSES

Fungal dsRNA viruses were first detected in 1948 as “La France” disease of cultivated mushrooms in Pennsylvania, and its study led to the first description of fungal viruses in 1962. The antiviral agents helenine and statolon, discovered in *Penicillium* in the 1950s, later proved to be fungal virus dsRNA that was inducing interferon production (reviewed in 45).

The dsRNA viruses are found in many fungal species (Table 74.1) and include the single-segment *Totiviridae*, the oligosegmented *Partitiviridae*, the potyvirus-like *Hypoviridae* that limit the virulence for chestnut trees of the chestnut blight fungus *Cryphonectria parasitica*, and reoviruses of the same species.

L-A Virus of *Saccharomyces cerevisiae*, Type Species of the *Totiviridae*

Some strains of *S. cerevisiae* secrete a protein toxin that is lethal to other strains but to which they are themselves immune²³⁷ (Fig. 74.1). This killer character of some strains is inherited as a non-Mendelian genetic element, and its study led to the discovery of the L-A dsRNA virus and its satellites M₁, M₂, M₂₈, and so on, each M encoding a different toxin-immunity specificity.

L-A Virion Structure

The icosahedral structures of L-A and the *Ustilago* virus P4 are unusual in that they combine T = 1 symmetry with 120 coat protein molecules per particle^{58,131,262} (Fig. 74.2). This same symmetry has been found in the cores of all other dsRNA viruses examined, including reovirus, rotavirus, bluetongue virus, aquareovirus, and bacteriophage ϕ 6 (see Chapters 44, 45, and 75). Because sequence data suggest that different dsRNA viruses each arose independently from different ssRNA viruses,²¹³ rather than one being descended from each other, their common structure suggests that there is something about this structure that is well adapted to the intraviral replication pattern of dsRNA viruses.

The combination of 120 subunits and T = 1 icosahedral symmetry implies that individual coat protein monomers can be in either of two environments. This expectation has been verified as subtly different morphology of the nonequivalent Gag protomers²⁶² (see Fig. 74.2). The packing of the dsRNA within the particles is less dense than is typical for dsDNA viruses, possibly reflecting the fact (see later discussion) that in both replication and transcription, the genome must translocate sequentially past the RNA-dependent RNA polymerase immobilized on the particle wall, and this requires more space than is required by the static DNA genomes.⁵⁰ The L-A virions also have 10- to 15-Å pores, which allow entry of nucleotides and exit of positive-strand transcripts but do not allow the dsRNA genome to exit or degradative enzymes to enter.²⁶² A trench on the outer virion surface contains His154 of Gag, the site of the mRNA decapping activity, and several nearby residues essential for this activity have been identified.³⁴⁸

L-A Genome Structure

The L-A positive strand has two overlapping open reading frames (ORFs)¹⁸³ (Fig. 74.3A). They are *gag*, encoding the 76-kd major coat protein, and *pol*, encoding the 100-kd Pol domain of the Gag-Pol fusion protein.^{143,183} The *pol* ORF includes the consensus amino acid sequence patterns typical of viral RNA-dependent RNA polymerases. Three *in vitro* ssRNA binding domains have also been localized to Pol,^{143,291,292} the central one cryptic unless an adjacent inhibitory region is deleted (see Fig. 74.3A). Pol residues 67 to 213 are necessary for packaging of the viral positive strands.^{141,292} Neither 5' cap structure nor 3' poly(A) has been found on either strand of genomic L-A dsRNA or M₁ dsRNA⁴³; however, there is, at each 3' end, an uncoded base that can be either A or G.³⁸

The M dsRNAs each encode, in their 5' portion, a prepro-toxin protein^{33,99,233,320} (see Fig. 74.3B). The M₁ positive strand has an internal encoded polyA region whose length shows frequent clonal variation, presumably owing to transcriptase stuttering,³³⁴ and a substantial 3' region that encodes no protein but contains essential *cis* sites.^{127,139,158,311,328} Sites on the L-A

TABLE 74.1 Double-Stranded RNA, Single-Stranded RNA, and Double-Stranded DNA Viruses of Simple Eukaryotes

Virus	Host species	kb	Features (reference)
dsRNA viruses			
<i>Totiviridae</i>			One segment, Gag-Pol, T = 1 icosahedral
L-A	<i>Saccharomyces cerevisiae</i>	4.6	Type species
M ₁ , M ₂ , M ₂₈	<i>S. cerevisiae</i>	1.6–1.8	Satellites of L-A; killer toxins (378)
L-BC	<i>S. cerevisiae</i>	4.6	(378)
Hv190S	<i>Helminthosporium victoriae</i>	4.5	Coat protein phosphorylated; internal translation initiation (159)
P1-H, P4-H, P6-H	<i>Ustilago maydis</i>	2.6–6.1	Killer toxin (212)
Af V-S, AfV-F	<i>Aspergillus foetidus</i>		(44)
AnV-S, AnV-S	<i>Aspergillus niger</i>		(44)
YIV	<i>Yarrowia lipolytica</i>		(165)
LRV	<i>Leishmania braziliensis</i>	5.28	(340)
GLV	<i>Giardia lamblia</i>	6.27	Infectious; transformation (372)
TVV	<i>Trichomonas vaginalis</i>	4.6	(200)
	<i>Zygosaccharomyces bailii</i>	4.0, 2.9, 1.9	Killer toxin (287)
	<i>Eimeria</i>		(324)
	<i>Blastomyces dermatitidis</i>	5.0	(211)
<i>Partitiviridae</i>			Two segments
	<i>Atkinsonella hypoxylon</i>	2.2, 2.1, 1.8	(271)
	<i>Penicillium chrysogenum</i>		(45)
	<i>Penicillium stoloniferum</i>		(45)
	<i>Agaricus bisporus</i>		(45)
	<i>Rhizoctonia solani</i>	2.2, 2.0	(135)
<i>Hypoviridae</i>			
CHV1-EP713	<i>Cryphonectria parasitica</i>		Hypovirulence-associated virus
<i>Reoviridae</i>			
	<i>C. parasitica</i>	1–3 (11 segments)	Hypovirulence-associated virus (174)
ssRNA replicons			
<i>Narnaviridae</i>			Naked RNA viruses
20S RNA	<i>S. cerevisiae</i>	2.5	(129)
23S RNA	<i>S. cerevisiae</i>	2.9	(126)
NB631 RNA	<i>C. parasitica</i>	2.7	(345)
dsDNA viruses			
PBCV-1 and many others	<i>Chlorella</i>	333	(365)

dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; dsDNA, double-stranded DNA; PBCV, *Paramecium bursaria Chlorella* virus.

and M₁ positive strands necessary for packaging and negative-strand synthesis have been determined (see later discussion and Fig. 74.3), and some limits have been set on the possible transcription signals.

Stages in the Replication Cycle

The L-A replication cycle and the closely related cycle of its satellites are shown in Figure 74.4.^{131,140} L-A dsRNA-containing viral particles synthesize positive ssRNA in a conservative reaction,^{140,173} and these new positive strands are then extruded from the particle.¹⁴⁰ There, they serve as mRNA for the production of the Gag and the Gag-Pol fusion proteins. These proteins then assemble with a viral positive strand to form new particles. The newly assembled viral particles carry out the synthesis of negative strands on the positive-strand template to form dsRNA and complete the cycle.¹⁴⁰

In addition to their appropriating viral proteins from L-A, the replication cycle of the satellite dsRNAs, M₁, S (deletion

mutants of M₁), and X (a 530 base pair [bp] deletion mutant of L-A) are similar to that of L-A itself except that they replicate more than once within the viral particle until it is full (see Fig. 74.4). Only a single positive ssRNA is packaged per particle,¹³⁹ thus L-A and M are separately encapsidated. Because the particle capacity is determined by the structure of Gag to be sufficient to accommodate one full-length L-A dsRNA molecule, positive-strand transcripts of the smaller M and X dsRNAs are often retained within the particles, where they are converted to new dsRNA molecules. All new positive strands are extruded from the full particles.^{130,131} This headful replication mechanism contrasts with the headful packaging mechanism of many DNA bacteriophage.^{130,131} This implies that extrusion of the transcripts is a mechanical consequence of the head being full rather than an active process. Both positive and negative strands are synthesized within the viral particles, but at different points in the cycle; therefore, the replication is conservative, intraviral, and asynchronous, and it fills the capsid (headful mechanism).



FIGURE 74.1. The killer phenomenon of *Saccharomyces cerevisiae*.

A sensitive strain of yeast was spread as a lawn, and streaks of a killer strain (**above**) or a nonkiller strain (**below**) were applied. After 2 days of incubation, the lawn of the sensitive strain has not grown in a zone around the killer strain. The secreted protein killer toxin, and immunity to the toxin, are encoded by M_1 , a satellite double-stranded RNA (dsRNA) of the L-A dsRNA virus.

Transcription Reaction (Positive-Strand Synthesis)

As in the *Reoviridae*, the transcription reaction for L-A is conservative,^{140,391} resulting in the overall process of viral replication being conservative.²⁶⁹ However, in *Aspergillus foetidus* slow virus and *Penicillium stoloniferum* slow virus,⁴⁶ dsRNA transcription is semiconservative. The difference between conservative and semiconservative reactions concerns whether or not there is re-pairing of the template negative strand with the parental positive strand that was (in either case) displaced during the synthesis.

Treatment of L-A virus particles with very low ionic strength results in their rupture and release of the dsRNA. These opened empty particles can be reisolated free of RNA and carry out a dsRNA template-dependent reaction that is, like the *in vivo* reaction, conservative.¹⁴⁵ This reaction is highly template specific, using only L-A, M, and X dsRNAs (all physiologic templates), but not L-BC, p6, or rotavirus dsRNAs.¹⁴⁵ Because X dsRNA retains only 25 bp of the end of L-A from which the transcription reaction starts,¹³⁰ the signal recognized by the transcriptase is most likely within this region, perhaps within the terminal 6 bp that are in common among L-A, M_1 , and M_2 .^{169,356} The template-dependent transcription reaction requires very high concentrations of polyethylene glycol (20%), suggesting that the transcriptase has a low affinity for the dsRNA template.¹⁴⁵ Because the dsRNA is normally formed within and stays inside the viral particles, the RNA polymerase sees a very high effective concentration and need not have a particularly high affinity.

Translation

The translation apparatus is a prime battleground for the fight between an RNA virus and its host. For example, interferon acts primarily by blocking viral translation. Picornaviruses cleave a cap-binding protein inactivating host translation

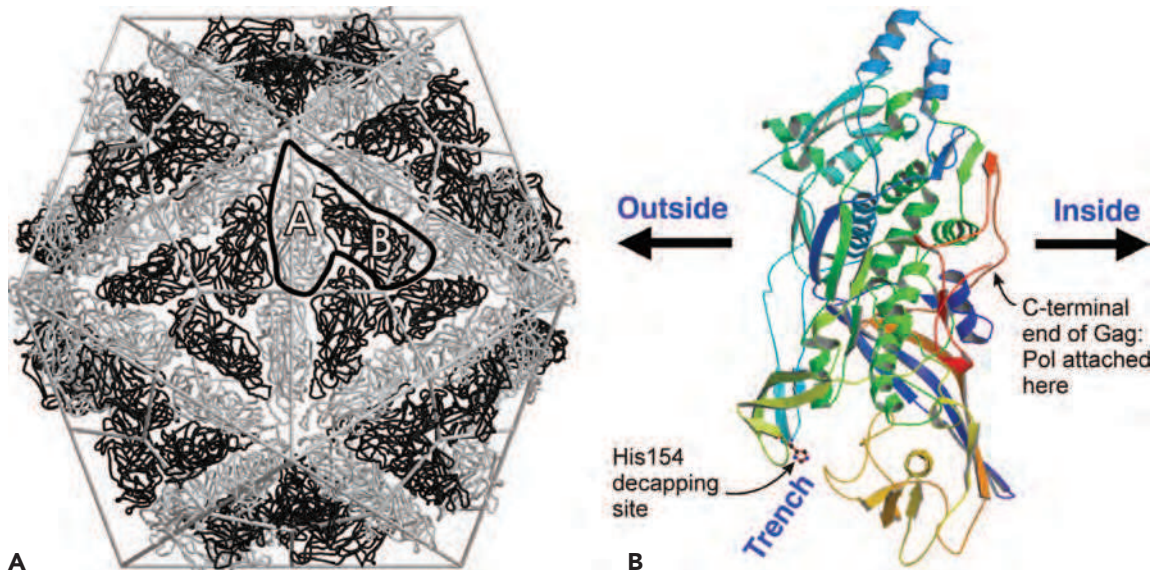


FIGURE 74.2. L-A double-stranded RNA virus capsid at 3.4-Å resolution from X-ray crystallography.^{262,348} **A:** Overall organization of the L-A capsid. Identical subunits occupy two different environments, with “A” subunits (blue) contacting the two- and five-fold axes and “B” subunits contacting the threefold axes. **B:** A single A subunit is shown with the trench on the outside surface including His154, the site of the decapping activity.

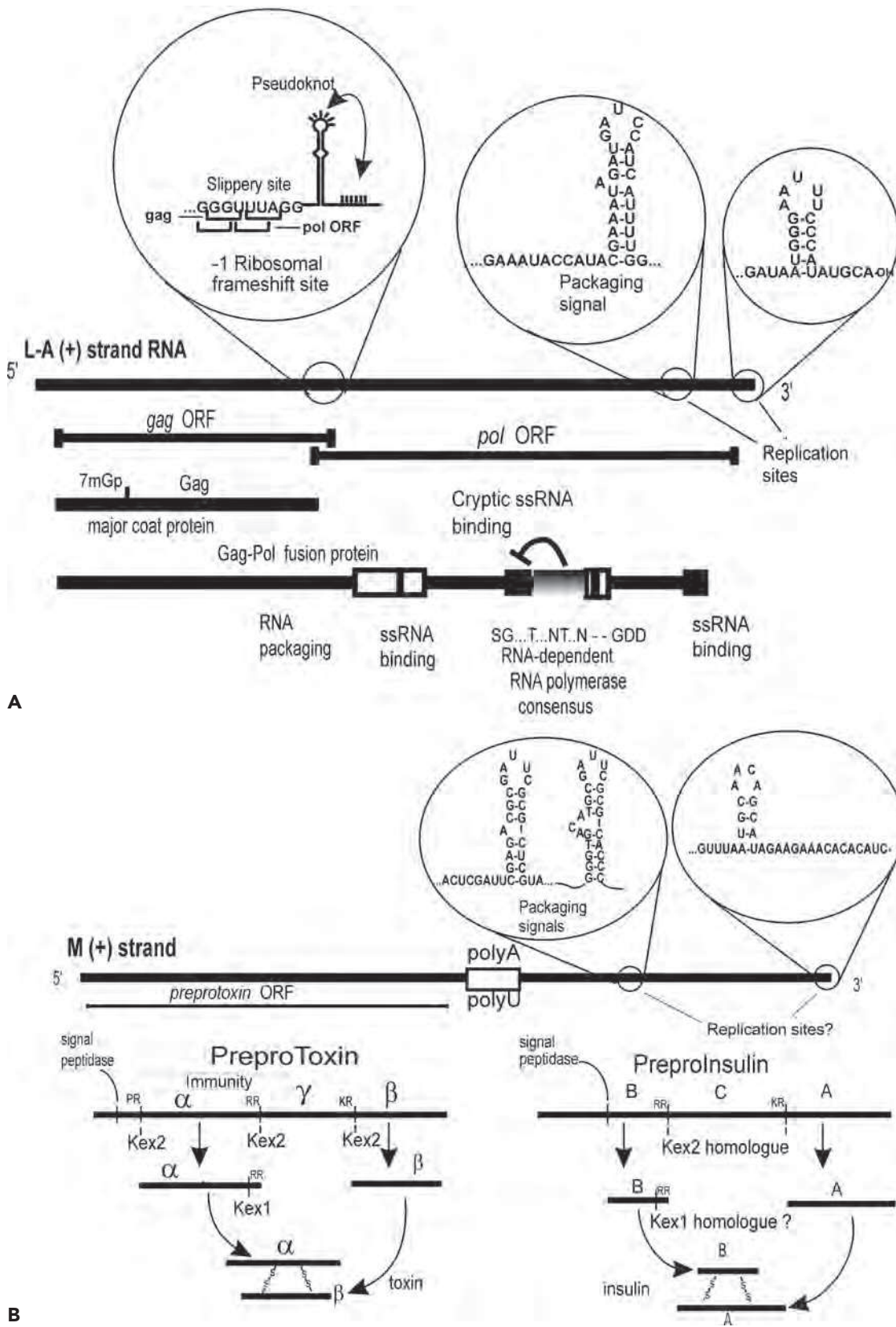


FIGURE 74.3. A: Sites and encoded proteins of the L-A positive strand. The sites responsible for -1 ribosomal frameshifting, positive single-stranded RNA packaging, and replication are shown. The pseudoknot makes the ribosome pause over the slippery site, where the transfer RNAs can unpair from the messenger RNA and re-pair in the -1 frame with correct base pairing of the nonwobble bases. Functional domains in the Pol region are also indicated. 7mGp indicates the cap-binding site at His154 of Gag. **B:** Coding and *cis* sites of M1 positive strand. The analogous and homologous processing of the K1 preprotoxin encoded by M1 is compared to that of preproinsulin.

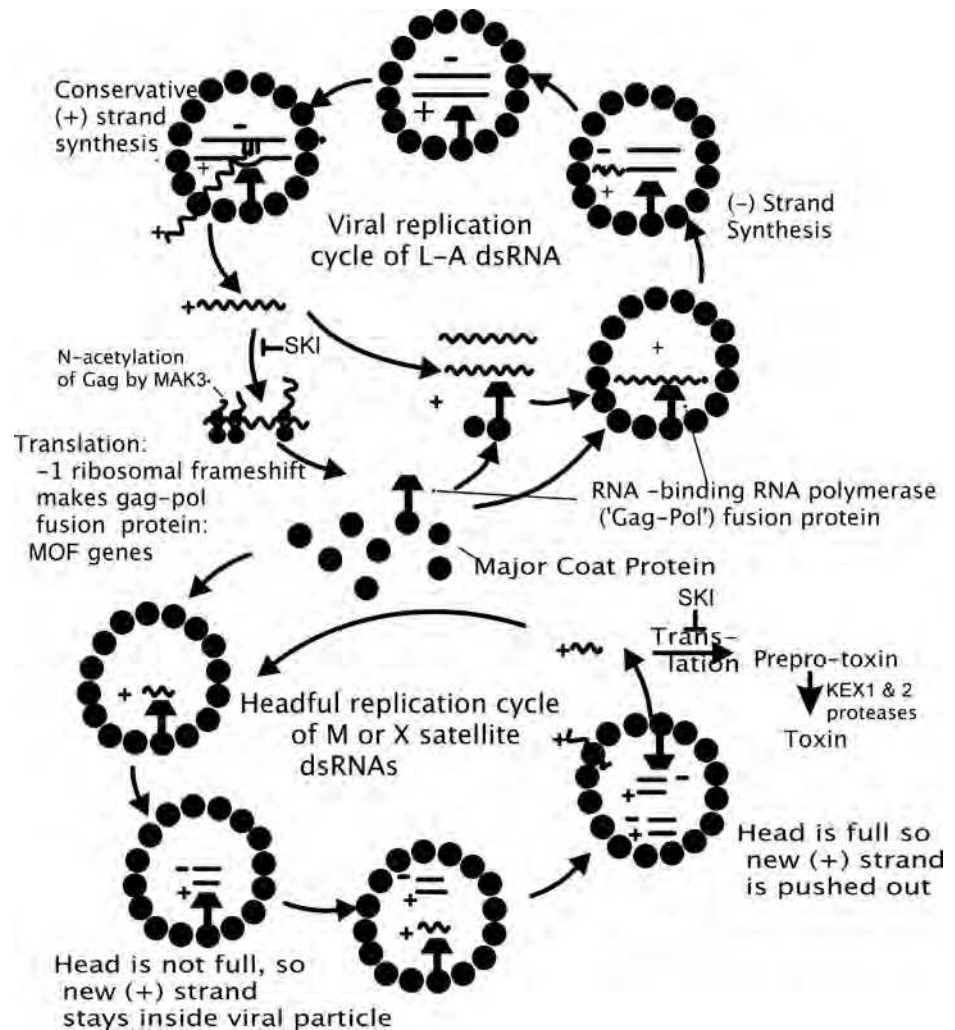


FIGURE 74.4. Replication cycles of L-A virus and its satellite double-stranded RNAs (dsRNAs), M and X. The packaging region of the Pol domain of Gag-Pol binds to the packaging site of positive strands as the Gag domain associates with other Gag molecules. This ensures packaging of viral positive strands.^{141,143} L-A particles have only one dsRNA molecule per particle. However, the smaller M or X dsRNAs replicate within the viral coat until they fill the head.^{130,131}

(Chapter 16), and influenza virus steals caps from cellular mRNAs (Chapter 40). RNAi also blocks translation of target mRNAs or promotes their degradation. Studies of the L-A virus and its satellites likewise suggest that translation of viral proteins is a critical event determining the balance between virus and host.

ribosomal frameshifting

The *gag* and *pol* ORFs of L-A overlap by 130 nucleotides, and the Pol protein is expressed only as a fusion protein with the major coat protein, Gag^{143,183} (see Fig. 74.3A). The mechanism of formation of this Gag-Pol fusion protein is a -1 ribosomal frameshift,¹⁰¹ very similar to those described in Rous sarcoma virus, many mammalian retroviruses, coronaviruses, and several plant viruses (184; reviewed in 15,40).

The frequency of -1 ribosomal frameshifts is determined by the “slippery site,” a sequence of the form X XXY YYZ, where the *gag* reading frame is shown, and by a downstream pseudoknot (see Fig. 74.3A). The slippery site allows the transfer RNAs (tRNAs) reading XXY and YYZ to move back one base on the mRNA and still have their nonwobble bases correctly paired.¹⁸⁴ X can be any base, although Y can be only A or U, probably because the frequency with which unpairing of the tRNAs from the 0 frame codons (XXY and YYZ) is also

important, and because A site pairing is stronger than P site pairing.^{39,101} Z can be any base but G, suggesting that specific tRNAs are more able to frameshift than others.⁵² The RNA pseudoknot, just downstream of the slippery site, slows ribosomal movement at this point.^{39,101} The location of this secondary structure is particularly critical,³⁹ and a pseudoknot is far more effective in promoting frameshifting than a simple stem loop of the same overall melting energy. The pseudoknot, more than the simple stem loop, should halt melting of the stem at a unique point because the rotation of the stem is limited by the downstream stem. This precisely positions the ribosome with the slippery site in the A and P sites.²⁸²

The efficiency of -1 frameshifting, and thus the ratio of Gag-Pol fusion protein to Gag protein produced, is critical for viral propagation,¹⁰⁴ as is the efficiency of +1 frameshifting for Ty1 retrotransposition frequency (see later discussion). A two-fold change away from L-A’s normal 1.9% efficiency results in failure to propagate the M₁ satellite dsRNA. L-A propagation is less sensitive; however, the antibiotics sparsomycin, which increases ribosomal frameshifting, and anisomycin, which has the opposite effect, can cure L-A.¹⁰² The Gag-Pol to Gag ratio is likely to be important for viral assembly. Excess Gag-Pol (high frameshift efficiency) may result in starting too many particles and winding up with too little Gag to complete any of

them. In contrast, excess Gag might result in particles closing before the packaging domain of Pol has had a chance to find a viral positive strand.¹⁰²

Host genes affecting the efficiency of –1 frameshifting are referred to as *MOF* (maintenance of frame).^{104,105} The differential effects of specific host mutations on frameshifting at specific slippery sites suggests that drugs similarly affecting frameshifting might target specific viruses.¹⁰⁵ *MOF9* is the 5S ribosomal RNA (rRNA), showing a role for this 60S subunit component in maintenance of reading frame.¹⁰³ *MOF6* is *RPD3* encoding a histone deacetylase; the *mof6-1* allele affects rRNA processing in such a way that ribosomal frameshifting is increased.²⁵⁴ Ribosomal frameshifting is also used in some mammalian genes, such as *Edr* (embryonal carcinoma differentiation regulated),³³¹ and various yeast genes affected by a frameshifting site have been identified.^{23,168} Whereas viral ribosomal frameshifting produces two viral proteins in a desired ratio, that of yeast cellular genes increases mRNA instability by diverting translating ribosomes to termination codons in another frame and inducing nonsense-mediated decay.²³

The 190S virus of *Helminthosporium victoriae* (Hv190SV) is a totivirus that does not make a coat protein–RNA polymerase fusion protein and does not use ribosomal frameshifting in its gene expression. Instead, the termination codon for the coat protein overlaps with the initiation codon for the RNA polymerase, the sequence being...GGA CAA **TG** AGT G....¹⁸¹ The RNA polymerase is detected only as a separate protein, and it is apparently translated by the occasional reinitiation of ribosomes that have just terminated at the end of the coat protein part of the mRNA.

60S SUBUNITS—CRITICAL TO VIRAL PROPAGATION

Mutations in any of 20 chromosomal genes (called *MAK* [maintenance of killer]) that result in diminished levels of free 60S ribosomal subunits also produce loss of M dsRNA and decreased copy number of L-A dsRNA.^{116,272} Mutations diminishing the supply of free 40S subunits generally had no such effect.²⁷² The deficiency of 60S subunits results in selectively decreased rates of translation of mRNAs which, like L-A mRNA, lack 3' poly(A).¹¹⁶ These *mak* mutations are suppressed by the *ski* mutations that derepress translation of non-poly(A) mRNAs.^{242,358}

GAG DECAPPING ACTIVITY—NECESSARY FOR M EXPRESSION

The *SKI1/XRN1* gene was first detected as superkiller mutants,³⁵⁷ and overexpression of Ski1p can cure the L-A virus.¹²⁸ The L-A and L-BC Gag proteins each covalently bind the 5' cap from any RNA *in vitro*.²⁷ L-A Gag covalently attaches ^{7m}GMP from the cap to His154 in a reaction that requires only Mg²⁺.²⁸ Modification of His154 destroys Gag's ability to covalently bind cap, resulting in loss of killer toxin expression from M₁ without affecting propagation of the M₁ satellite virus.²⁸ Mutation of the *SKI1/XRN1* gene encoding the 5' to 3' exoribonuclease, specific for uncapped RNAs and responsible for the major mRNA decay pathway,¹⁷⁹ results in restoration of expression of killer toxin despite mutation of His154 of Gag.²⁴² It has been proposed that the virus decaps cellular mRNAs to decoy the Ski1p exoribonuclease from degrading the capless viral mRNA.²⁴² However, recent evidence shows that L-A virions can transfer the cap structure to nascent L-A mRNAs.^{139a,b}

SKI ANTIVIRAL SYSTEM BLOCKS TRANSLATION OF VIRAL [NON-POLY(A)] MESSENGER RNA

The sole essential function of four of the *SKI* genes of *S. cerevisiae* is the repression of viral copy number, particularly control of M.^{24,25,294,335,358} The Ski proteins repress three unrelated viral systems: L-A and its satellites, L-BC (a dsRNA virus), and the ssRNA replicon referred to as 20S RNA.^{14,246,358} Detailed studies of *SKI2* suggested that the system acts by limiting translation of viral mRNA.^{242,388} Because none of these cytoplasmic replicons has either 5' caps or 3' poly(A) structures, it was speculated that the *SKI* system recognized the absence of one or both of these structures.³⁸⁸ That *SKI2* also represses translation of RNA polymerase I transcripts, probably lacking cap and/or poly(A), supports this idea.³⁸⁸

Electroporation of mRNAs into spheroplasts shows that the Ski2, Ski3, Ski6, Ski7, and Ski8 proteins inhibit the translation of mRNA specifically if it lacks a 3' poly(A) structure.^{24,25,242} Kinetic studies indicate that both the initial rates and the duration of translation are affected by the Ski proteins. The Ski proteins also affect a minor mRNA degradation pathway.¹⁸⁵

Among the *SKI* genes, only *SKI6* is essential for growth in the absence of M dsRNA.^{24,25,290,335,388} Ski2p has helicase motifs, a glycine-arginine-rich domain, and it is highly homologous to two mammalian genes.³⁸⁸ Ski3p has the TPR amino acid repeat pattern,²⁹⁰ whereas Ski8p has a different sequence repeat first identified in β -transducin.³³⁵ Ski2p, Ski3p, and Ski8p form a cytoplasmic complex.⁴¹ Ski6p is homologous to RNase PH, a tRNA-processing enzyme,²⁴ and it is part of a nuclear complex of exoribonucleases that has a role in rRNA processing.²⁵⁶ The *ski6* mutants produce abnormal 60S ribosomal subunits and show abnormalities in processing of 5.8S rRNA.²⁴ Ski7p has homology to EF1- α , an elongation factor, suggesting that it is involved in the translation process.²⁵

SLH1 is a yeast homolog of *SKI2*, and like *ski2 Δ* , *slh1 Δ* has elevated L-A copy number.²⁴¹ The *ski2 Δ slh1 Δ* double mutants show dramatically increased L-A, L-BC, and M₁ dsRNA copy numbers but grow normally in the absence of the L-A and M₁ viruses.³²¹ Remarkably, the *ski2 Δ slh1 Δ* cells show the same *in vivo* translation kinetics of non-poly(A) mRNA as they do for poly(A)+ mRNA, despite the presence of normal amounts of competing poly(A)+ mRNA.³²¹ Thus, the translation apparatus is indifferent to the 3' poly(A) structure except for the action of the Ski proteins and Slh1p. This suggests that one role of the eukaryote 3' poly(A), like that of Ski2p and Slh1p, is to allow cells to distinguish their own mRNAs from those of invading viral genomes.

Posttranslational Modification

KEX1 AND KEX2 PROTEASES AND DISCOVERY OF PROHORMONE PROTEASES

The *kex1* and *kex2* mutants were first isolated because of their inability to produce the “killer” toxin encoded by the M₁ satellite dsRNA (*KEX*, from killer expression).³⁸⁴ The *kex2* mutants have a defect in mating specific to cells of the α mating type, and *kex2/kex2* homozygous diploids are defective for sporulation.²²⁶ The α -specific mating defect was partially explained by the finding that the cells failed to secrete the α pheromone,²²⁶ a peptide that prepares cells of the opposite (a) mating type for mating by arresting them in the G1 phase of the cell cycle. The failure to secrete killer toxin and a pheromone was explained

by the finding that *KEX2* encodes a protease that cleaves C-terminal to pairs of basic amino acid residues,¹⁹⁰ and that *KEX1* encodes a carboxypeptidase that can remove the pair of basic amino acids.^{76,106} These were the cleavages needed to convert the toxin and α pheromone proproteins to their mature forms (see Fig. 74.3B).

The specificities of the Kex proteases are the same as those needed to process preproinsulin (see Fig. 74.3B), pre-pro-opiomelanocortin, and other mammalian prohormones; however, the enzymes responsible for these maturation cleavages had been elusive. Several genes and enzymes with homology to Kex2p were identified, and they are indeed involved in these prohormone processing steps (reviewed in 301).

The Kex2p-homologous enzymes are also involved in the proteolytic processing of some mammalian viral proteins. Mutant CHO cells resistant to Sindbis virus and Newcastle disease virus were made sensitive by expression of the Kex2p homolog, mouse furin, or by expression of Kex2p itself.²⁵⁷

N-ACETYLATION OF GAG BY MAK3P—NECESSARY FOR ASSEMBLY

MAK3, *MAK10*, and *MAK31* are necessary for the propagation of the L-A dsRNA virus. Mak3p is homologous to *N*-acetyltransferases,³⁵⁴ and the *N*-acetylation of the L-A Gag protein is lost in a *mak3* mutant host, resulting in failure of viral assembly.³⁵⁵ Mak3p recognizes the N-terminal four amino acid residues of Gag.³⁵³ Like L-A, the major coat proteins of Rous sarcoma virus, tobacco mosaic virus, turnip yellow mosaic virus, alfalfa mosaic virus, and potato X virus are N-terminally acetylated.

The only three host proteins known to be necessary for L-A propagation, Mak3p, Mak10p, and Mak31p,³⁸¹ form a complex,²⁹⁵ indicating that Mak10p and Mak31p are also involved in *N*-acetylation of the coat protein. In addition to its action on Gag, the Mak3p acetylase also is responsible for modification of the $\alpha 5$ and $\alpha 6$ proteasome subunits,²⁰⁵ and both *mak3* and *mak10* mutants grow poorly on ethanol or glycerol.^{100,225,354}

Viral Assembly

The headful replication mechanism implies that the coat protein determines the structure of the head, not the genome. This is typical of isometric viruses. In fact, expression of the Gag protein alone produces empty particles that are morphologically indistinguishable from normal L-A virions.¹⁴¹ Normal L-A particles have a $T = 1$ icosahedral structure with an asymmetric unit consisting of a dimer of Gag (262; see preceding discussion). Each particle has only one or two Gag-Pol fusion proteins (perhaps as a dimer). The requirement for the Mak3p-catalyzed *N*-acetylation of Gag for assembly suggests that the Mak3/10/31 complex is involved in this process.

The existence of a Gag-Pol fusion protein, the ssRNA binding activity of its Pol domain, and the fact that positive ssRNA is the species encapsidated to form new viral particles led to a model of assembly and packaging that has been supported by subsequent findings¹⁴³ (see Fig. 74.4). The Pol domain of the Gag-Pol fusion protein recognizes and binds to a packaging site on the viral positive strands. Then (or concomitantly), the Gag domain of the fusion protein associates with the free Gag protein. This leads to encapsidation of a single

positive strand per particle if there is only one Gag-Pol fusion protein (or one dimer) per particle.

In vivo, Pol residues 67 to 213 of the Gag-Pol fusion protein are necessary for packaging of positive strands but not for assembly of morphologically normal viral particles.^{141,292} One of the three ssRNA binding domains of Pol is located within this region and is necessary for the packaging²⁹² (see Fig. 74.3A).

The *packaging site* recognized is an internal stem-loop sequence about 400 nucleotides from the 3' end with an A residue bulging from the 5' side of the stem¹³⁹ (see Fig. 74.3A). Binding requires the stem structure but not the sequence of the stem. In contrast, the sequence of the loop is important. The protruding A residue must be present and must be an A.¹³⁹ A similar site, similarly located on the M₁ positive strand, was found¹³⁹ by examining the predicted folded structure of the M₁ sequence,¹⁵⁸ and another was found by studying sequences involved in exclusion of M₁.³²⁸ Either the L-A or M₁ stem loops are sufficient for binding; however, the addition of 10 bp from the 5' side of either one improves the binding substantially.^{139,328} Both the L-A and M₁ sites contain direct repeat sequences whose significance has not yet been determined. A site with the same structure has also been found in a similar location in the M₂₈ satellite dsRNA.³¹⁹

The L-A and M₁ sites can each serve as a portable packaging signal *in vivo*, directing packaging of heterologous transcripts by L-A virus or by proteins produced from the L-A complementary DNA (cDNA) clone.^{139,141} The heterologous transcripts were packaged alone in viral particles, confirming the prediction of the headful replication model that a single positive strand is initially packaged per particle.

Replication (Negative-Strand Synthesis)

Newly assembled viral particles contain an L-A positive strand and are capable of converting it to the dsRNA form when supplied with nucleoside triphosphates.¹⁴⁰ The particles formed in this reaction have all the properties of mature L-A particles and can carry out the transcription reaction.

The opened empty particles (see earlier discussion), when supplied with viral positive strands, Mg²⁺, nucleoside triphosphates, and a low concentration of polyethylene glycol, carry out negative-strand synthesis to form dsRNA.¹⁴⁶ Only L-A, M, or X positive-strand templates are active in this reaction, supporting the notion that it accurately reflects the *in vivo* reaction.

A maximally active template requires both sequences at the 3' end of the L-A (or X) positive strand and internal sequences (the internal replication enhancer) overlapping the packaging signal.¹³⁰ The 3' end of L-A positive strands has a stem loop whose structure is necessary for template activity. Whereas the sequence of the loop and that of the 3' terminal 4 nucleotides are important, that of the stem is not. Despite the requirements for these structures and sequences in the context of L-A, the 3' terminal 33 nucleotides of M₁'s positive strand can substitute for the L-A 3' end, although there is little or no similarity between the two sequences¹³⁰ (see Fig. 74.3). The internal replication enhancer and the 3' end site must be bound together for optimal template activity in the replication reaction.¹⁴⁴ However, they need not be covalently attached and can simply be hydrogen bonded. This suggests that the RNA polymerase binds first to the internal site and is thus brought close to the 3' end site where polymerization is to begin.

Because the RNA-dependent RNA polymerase consensus domains defined by Kamer and Argos¹⁹⁴ have since been found in essentially all positive ssRNA and dsRNA viruses examined, the detailed mutagenesis of the most highly conserved of these regions was carried out using the L-A cDNA clone.²⁹³ This has defined the extent of the domains necessary for the propagation of the M₁ satellite dsRNA. Interestingly, homologous regions from reovirus or Sindbis virus RNA polymerases could partially substitute for that of L-A.²⁹³

Other Factors Affecting L-A, M, and L-BC Replication

Mutation of either *POR1*, encoding the major mitochondrial outer membrane porin, or *NUC1*, encoding the major mitochondrial nuclease, results in derepression of L-A copy number.^{100,232} A similar effect results from mutation of *scs1/lbc2*,¹⁵⁶ encoding a subunit of serine palmitoyltransferase. *MKT1* is necessary for M₂ propagation but not for M₁.³⁷⁹ Several natural variants of L-A have been described based on their interactions with *MKT1*, M₂ dsRNA, and *mak* mutations affecting levels of free 60S ribosomal subunits; however, the mechanisms of these interactions have not yet been determined (reviewed in 381).

Partitiviridae

The *Partitiviridae* have a bipartite genome with the two segments separately encapsidated in particles containing the proteins encoded by both segments.⁴⁴ This group includes viruses of the filamentous fungi *Penicillium* and *Aspergillus* and the mushroom *Agaricus*, as well as many other fungi. It

also includes a large group of plant cryptoviruses,^{29,266} whose genome is dsRNA and whose biology is much like that of the systems discussed in detail here.

Strain 2H of *Atkinsonella hypoxylon* has three dsRNA segments of 2,180, 2,135, and 1,790 nucleotides. Segment 1 encodes an RNA-dependent RNA polymerase, and segment 2 encodes a 74-kd protein that may be the major coat protein.²⁷¹ The third segment does not appear to encode a protein.²⁷¹

SINGLE-STRANDED RNA REPLICONS

Viruses Reducing Virulence of *Cryphonectria parasitica*

The American chestnut tree was devastated by the accidental introduction, in 1905, of the pathogenic fungus *C. parasitica*, along with an oriental variety of the chestnut tree (reviewed in 175,270). The fungus virtually eliminated the upper parts of the chestnut trees in the eastern United States, although the root systems were not killed and shoots continued to emerge, only to have their growth limited by the reattack of the fungus. *C. parasitica* had a similar effect in Europe; however, in the 1950s, the emergence of fungal strains in Italy with markedly reduced virulence to the trees was noted. These “hypovirulent” strains (Fig. 74.5), later also found in Michigan, could transmit their hypovirulence by hyphal anastomosis to virulent strains, and this was used successfully for the biologic control of chestnut blight in Europe. Hypovirulent strains also show decreased asexual spore formation, decreased production of

Chestnut tree infected with *Cryphonectria parasitica*:

not carrying hypovirus

carrying hypovirus

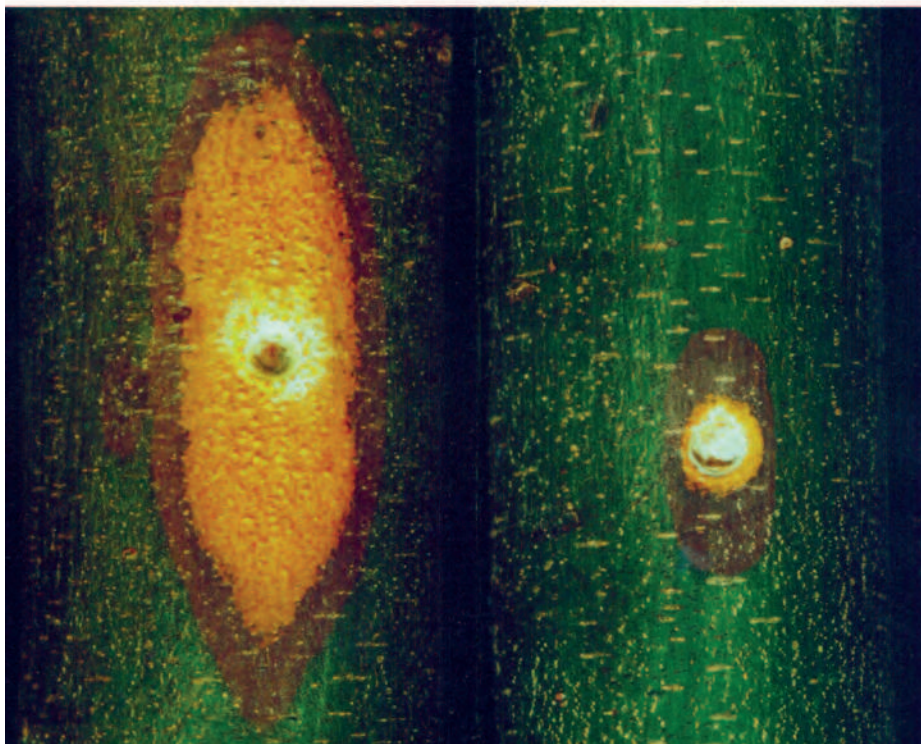


FIGURE 74.5. Virulent (uninfected, right) and hypovirulent (virus-infected, left) *Cryphonectria parasitica* (chestnut blight fungus) inoculated into chestnut trees. Infection of the fungus by any of several hypoviruses attenuates its virulence toward the trees. (Courtesy of Dr. Donald Nuss.)

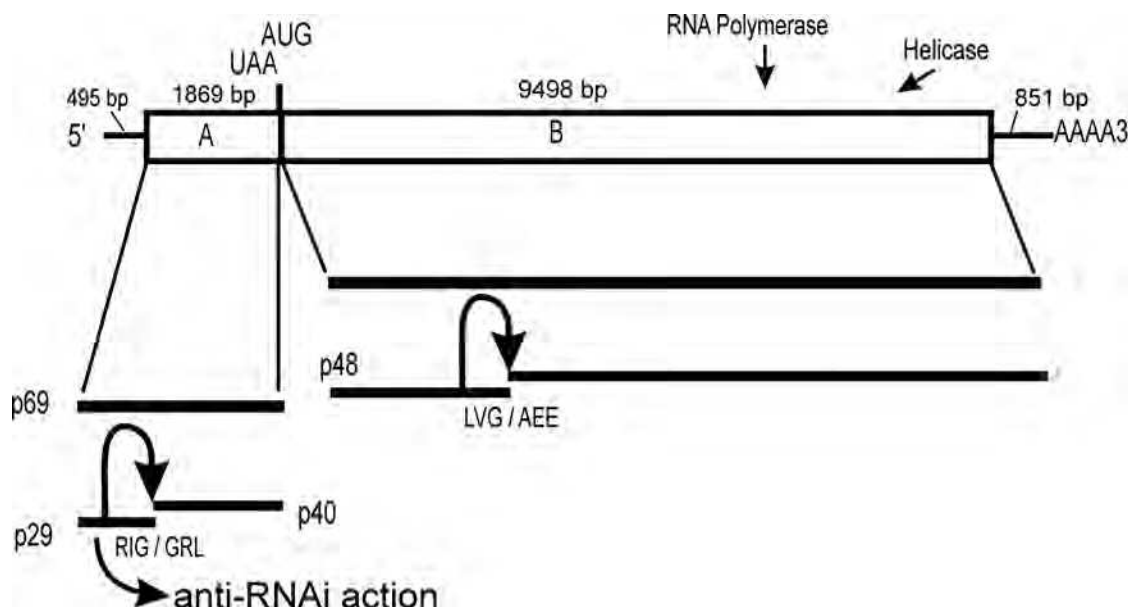


FIGURE 74.6. Coding information and protein processing of the *Cryphonectria parasitica* virus, L. The sites of action of the p29 and p48 proteases are indicated. This virus is now designated CHV1-EP713, the type member of the *Hypoviridae*. (Adapted from Nuss DL. Hypovirulence: mycoviruses at the fungal-plant interface. *Nat Rev Microbiol* 2005;3:632–642.)

laccase (a phenol oxidase possibly involved in pathogenesis), and reduction in pigment formation (reviewed in 270).

The cytoplasmically inherited factor that reduces the pathogenicity of the fungus and produces these hypovirulence-associated traits is any of several apparently unrelated dsRNA replicons called *hypoviruses* (8,90,253; reviewed in 270). The hypoviruses include those related to the positive-strand RNA plant potyviruses, single-stranded mitochondria-associated viruses, and true reoviruses.

Genome Structure

The most thoroughly studied hypovirulence-producing virus is CHV1-EP713, the L-dsRNA of hypovirulent *C. parasitica* strain EP713, a 12,712-bp molecule whose positive strand has two long ORFs: ORFA and ORFB³²⁵ (Fig. 74.6). Each ORF encodes a papain-like cysteine protease in its N-terminal portion that self-cleaves the primary translation products at least once^{70,326} (see Fig. 74.6). The presence of related papain-like proteases and helicase domains, as well as RNA-dependent RNA polymerase motifs, indicates that L-dsRNA of *C. parasitica* is related to the potyviruses, a group of positive ssRNA viruses of plants.²¹⁴

ORFA and ORFB overlap by a single nucleotide, with the UAA termination codon of the first overlapping with the AUG codon of the second,³²⁵ resulting in a reinitiation mechanism for the expression of ORFB.¹⁶⁷ The translation of ORFA also poses an interesting problem, as the 5' noncoding region contains six short ORFs.³²⁵ Whether ribosomes initiate internally as in the case of picornaviruses (see Chapter 16) or use these tiny ORFs for regulatory purposes, as in the *GCN4* gene of *S. cerevisiae*,¹⁷⁶ is not yet clear.

Virus Replication in Intracellular Vesicles

Unlike other mycoviruses, most of the *C. parasitica* dsRNAs described to date are not associated with virus particles. Rather, they are found in intracellular vesicles,^{107,170} with an RNA

polymerase activity producing both ssRNA and dsRNA.¹³² The vesicles have markers typical of the *trans*-Golgi network.¹⁸⁶ This situation is reminiscent of the membrane association of *in vivo* RNA synthesis of many positive-strand RNA viruses, supporting the notion that this virus is related to the positive-strand RNA potyviruses.

RNA Interference Antiviral System of *Cryphonectria parasitica*

The chestnut blight fungus has an RNA interference (RNAi)-based antiviral system that limits the effects of infection by the hypovirus CHV1-EP713.^{323,342,405} *C. parasitica* has two homologs (*dcl1* and *dcl2*) of Dicer, the dsRNA-specific endoribonuclease that produces 21- to 24-bp fragments from larger dsRNAs (viral replicative forms or hairpin molecules), and four homologs (*agl1* to *agl4*) of Argonaute, the protein that brings these dsRNA fragments to the complex that “slices” or blocks translation of target RNAs. The *Cryphonectria* antiviral system involves only *dcl2* and *agl2*. Hypovirus infection induces transcription of both *agl2* and *dcl2*, with the induction of *dcl2* transcription dependent on *agl2*.³⁴² This antiviral response is muted by the viral p29 protein,^{270,322,342} a papain-like protease homologous to the potyvirus suppressor of RNA silencing.¹⁹⁶ Mutation of *dcl2* or *agl2* also prevents accumulation of defective interfering viral particles and blocks viral recombination.^{342,404} Moreover, dsRNA virus-based vectors are more stable in these mutants.^{342,404}

In *Aspergillus nidulans*, an RNAi system has also been defined. This system acts to limit virus replication, and a virus suppressing the system has also been described.³²² *S. cerevisiae* lacks the RNAi system, but the closely related *Saccharomyces castellii* has it.^{109a} Expressing Dicer and Argonaut from *S. castellii* in killer *S. cerevisiae* results in loss of M dsRNA and L-A (but not L-BC). The presence of RNAi systems in yeast species correlates with the absence of killer systems, leading the

authors to propose that the advantage of having a killer system resulted in loss of the RNAi system.^{109a}

Virus Induction of Hypovirulence

CHV1-EP713 has very specific effects on certain genes. For example, the laccase gene encodes a phenol oxidase believed to be involved in pathogenesis. Transcription of the laccase gene is repressed by CHV1-EP713 infection,^{67,296} an effect that can be produced by expression of just the p29 protease encoded by part of ORFA.⁶⁸ Although p29 is sufficient to alter fungal phenotypes, it is neither necessary nor sufficient to cause hypovirulence.⁸² Deletion of p29 from the virus results in decreased induction of the hypovirulence-associated traits but no decrease in hypovirulence itself. The symptom-determining domain of p29 has now been localized to the interval Phe25 to Gln73 of p29.³⁴⁴ The effect of CHV1-EP713 on laccase appears to be transmitted by an influence on the inositol triphosphate–calcium signal transduction system of the fungus, possibly explaining the multiple phenotypic effects of hypovirulence.²²² The CHV1-EP713 hypovirus also up-regulates the cppk1 protein kinase and prevents activation of the HOG pathway mitogen-activated protein (MAP) kinases resulting in hypersensitivity to osmotic stress.^{204,273}

Cryparin, a hydrophobic cell surface protein, is also reduced in hypovirulent strains, an effect mediated at transcription.⁴⁰³ Another gene, *Vir2*, was isolated based on its decreased transcription in a hypovirulent strain. Although no effect was seen on virulence itself, deletion of this gene partially mimics some of the hypovirulent-associated traits, with decreased asexual sporulation and fruiting body formation and impaired mating ability.⁴⁰² The hypovirulence-associated traits are thus caused by specific effects of viral gene products, rather than by the presence of a replicating dsRNA.

G protein signaling is also implicated in the induction of hypovirulence by CHV1-EP713. Hypovirus infection leads to decreased expression of the host Gia (an inhibitory subunit of G protein) and a concomitant increase in cAMP levels.^{56,66} Moreover, disruption of *cpg-1*, the gene for Gia, results (in an uninfected strain) in elevated cAMP levels, total loss of virulence, and the development of the same associated phenotypes seen on virus-induced hypovirulence.¹⁵⁰

Infectious Complementary DNA Clones and Biological Control of Chestnut Blight

Introduction of complete cDNA clones of L-dsRNA under control of the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase promoter resulted in both a complete hypovirulence phenotype of the fungus and the launching of the RNA replicon in a form transmissible to other strains.⁶⁹ In addition to its usefulness in studying the mechanism of the effects of CHV1-EP713 dsRNA on the cell, this infectious cDNA clone method is an important advance in attempts to control chestnut blight. Although artificial inoculation of trees with hypovirulent fungal strains in Europe resulted in spread of the hypovirulence dsRNAs to virulent strains and control of the blight, this approach has not succeeded in the United States. CHV RNA spreads by fusion between fungal cells, a process called *hyphal anastomosis*, but not by sexual crosses.⁷ Hyphal anastomosis requires that strains have identical alleles at several different loci determining compatibility. The number of compatibility groups is apparently much greater in the United

States, limiting the spread of the hypovirulence dsRNA. In fact, limiting the spread of debilitating viruses is the presumed function of this heterokaryon incompatibility system (see the section, [Het-s]—a Prion of *Podospora* Responsible for a Normal Function).

In contrast, infectious cDNA incorporated into the *C. parasitica* genome, while generating dsRNA replicons, will also naturally spread to other mating and vegetative compatibility groups through sexual transmission.^{69,270} It is thus expected to be more effective in the biological control of chestnut blight than the natural virus, and early results support this expectation (reviewed in 270).

Recently, the cDNA clone has also been used to introduce CHV1-EP713 into other plant-pathogenic fungi of the order *Diaporthales*.³¹⁵ Infection produced both phenotypic changes and a decrease of virulence in these heterologous hosts.

Reovirus of *Cryphonectria*

Two strains of *C. parasitica* have been found carrying reovirus-like elements associated with hypovirulence.^{120,277} Strains C18 and 9B21 each have 11 dsRNA segments ranging in size from about 1 to about 3 kb and associated with 60-nm virus particles, unlike the vesicles in which the potyvirus-like *Hypoviridae* are found. These 11 segments are present in equimolar amounts, and transmission studies show that either all or none of the segments are transmitted, suggesting that they are parts of a single viral genome. The C18 and 9B21 genomes are not closely related, and sequencing shows that 9B21 is related to the *Coltivirus* group (e.g., Colorado tick fever virus).³⁴⁵ As has been shown for the L-A dsRNA virus system with the killer trait,¹¹⁹ these reovirus particles can be introduced into *Cryphonectria* spheroplasts and transmit the hypovirulence traits.¹⁷⁴ This system adds new dimensions to the study of both hypovirulence and the *Reoviridae*.

20S RNA and 23S RNA

In 1971, a stable species of RNA, intermediate in size between 18S and 25S rRNAs, dubbed 20S RNA, was found to appear specifically in cells exposed to the condition used to induce meiosis and sporulation—namely, when acetate is supplied as the carbon source in the absence of a nitrogen source.¹⁹³ The ability to produce 20S RNA was then found to be inherited as a non-Mendelian genetic element, distinct from other known elements.¹⁵⁷ It was not connected with meiosis, except that the same culture conditions are used to induce both.¹⁵⁷ 20S RNA was finally proven to be an independent RNA replicon whose copy number is inducible in acetate.²⁴⁶ Its sequence shows that its 2,500 nucleotides encode a single 95-kd protein with some similarities to the RNA-dependent RNA polymerases of RNA phages and RNA viruses.^{247,302} W dsRNA, a minor species inducible by growth at high temperature,³⁷⁷ proved to have the same sequence as 20S RNA.^{247,302} Electron micrographs of purified 20S RNA showed about 50% circular molecules²⁴⁶; however, biochemical experiments indicate that the RNA itself is not circular.³⁰³ The terminal sequences of 20S RNA were shown to be 5'GGGGC GCCCC-OH3',³⁰⁴ suggesting that a circular structure may have been formed by hydrogen bonding.

20S RNA is cytoplasmic and lacks a coat protein³⁸⁷ but is found in a 1:1 complex with its encoded 91-kd RNA polymerase,¹⁵¹ and these complexes synthesize 20S RNA and

its complementary strand.¹⁵² An infectious clone of 20S RNA has now been developed and used to show the importance of terminal nucleotides.¹²⁹ The RNA polymerase binds to three sites on 20S RNA, at the 5' end, the 3' end, and in the middle of the sequence, and all three sites are important for propagation of the genome.¹³⁸ Intermediates in replication are positive and negative single strands, not dsRNA.¹⁴²

T dsRNA was discovered as a minor species of dsRNA easily detected in cells lacking L-A and L-BC, and shown to be like W dsRNA, an independent replicon inducible by growth of cells at high temperature.³⁷⁷ T has the same sequence as 23S ssRNA.¹²⁵ 23S RNA has substantial homology with 20S RNA and likewise appears to encode an RNA-dependent RNA polymerase,¹²⁵ with which it is associated in extracts.¹²⁴ 23S and 20S are closely related independent replicons, much like L-A and L-BC.¹²⁹ An infectious cDNA clone of 23S RNA has been developed and used to study the terminal sequence requirements for replication.¹²⁶

The copy numbers of 20S RNA and 23S RNA are controlled by media conditions, requiring acetate as the carbon source and the absence of a nitrogen source for their 10,000-fold induction.^{124,193,246} Both are also induced by growing cells at 37°C, and at least 20S is repressed by the *SKI* system.^{246,377} The *SKI* effect apparently reflects the absence of 3' poly(A) on 20S mRNA (see earlier discussion).

Cryphonectria parasitica Mitochondrial Replicon NB631 Double-Stranded RNA

Polashock and Hillman²⁸⁴ have described a mitochondrial dsRNA species in *C. parasitica* strain NB631. If one assumes the mitochondrial genetic code (UGA=Trp), then the NB631 dsRNA has a single long ORF that encodes an RNA-dependent RNA polymerase. This RNA polymerase is most closely

TABLE 74.2 Groups of Retroelements

Element	<i>env</i>	LTRs	RT	Examples
Retrovirus	+	+	+	RSV, HIV
Retrotransposon	—	+	+	Ty1-5
Retroposon	—	—	+	LINEs, Tad
Retrointron	—	—	+	Intron al1 of <i>COX1</i>

LTR, long terminal repeat; RT, reverse transcriptase; RSV, Rous sarcoma virus; HIV, human immunodeficiency virus.

related to those of 20S and 23S RNAs, and all are more closely related to RNA phage such as Q β , than to RNA viruses of higher organisms.^{283,302} The degree of similarity is such that these elements must be very close relatives. Nevertheless, 20S RNA and 23S RNA are primarily ssRNA replicons, whereas NB631 has been identified as a dsRNA element.

NB631 dsRNA is transferred by hyphal anastomosis, like other *C. parasitica* RNA replicons; however, unlike those, and other viruses of filamentous fungi, it is also efficiently transmitted by meiotic spore formation if the female parent had the virus.²⁸³

RETROVIRUSES (RETROELEMENTS)

Retroviruses, Retrotransposons, Retroposons, and Retrointrons

Retroelements (Table 74.2) all share their use of reverse transcriptase in their propagation. The retrotransposons of fungi and parasitic microorganisms (Table 74.3) resemble

TABLE 74.3 Retrotransposons of Simple Eukaryotes

Retrovirus (copy #)	Host species	LTRs	ϵ (unique)	Group ^a	Reference
Ty1, Ty2 (217, 34)	<i>Saccharomyces cerevisiae</i>	d-334–8 bp	5.2 kb	<i>copia</i>	31
Ty3 (41)	<i>S. cerevisiae</i>	s-340 bp	4.7	<i>gypsy</i>	171
Ty4 (32)	<i>S. cerevisiae</i>	t-371 bp	5.6	<i>copia</i>	171
Ty5 (7)	<i>S. cerevisiae</i>	245 bp		<i>copia</i>	368
Tf1, Tf2	<i>Schizosaccharomyces pombe</i>	349–358 bp	4.4	<i>gypsy</i>	228
DIRS-1	<i>Dictyostelium discoideum</i>	ITRs	4.2		49
DRE	<i>D. discoideum</i>	Complex			240
Tp1	<i>Physarum polycephalum</i>	277 bp	8.3	<i>copia</i>	310
Cft-1	<i>Cladosporium fulvum</i>	427 bp	6.1	<i>gypsy</i>	251
pCal	<i>Candida albicans</i>	280 bp	5.9	<i>copia</i>	248
Several	<i>Candida</i>	—		LINEs	162
Several	<i>Cryptococcus neoformans</i>	—		LINEs, <i>gypsy</i> , <i>copia</i>	163
CRE1	<i>Crithidia fasciculata</i>	—		LINEs	149
SLACS, CZAR	<i>Trypanosoma brucei</i> , <i>Trypanosoma cruzi</i>	—	6.7	LINEs	3
TOC1	<i>Chlamydomonas reinhardtii</i>	217, 237 bp	4.6		89
Tad	<i>Neurospora</i>	—	7.0	LINEs	208

^aBased on amino acid sequence homology and gene order, retrotransposons may be divided into those similar to the *copia* element or the *gypsy* element of *Drosophila*.⁸² Copy numbers of *S. cerevisiae* retrotransposons in the genome are from reference 154. LINE-like elements are retroposons, lacking LTRs.

LTRs, long terminal repeats; LINEs, long interspersed elements.

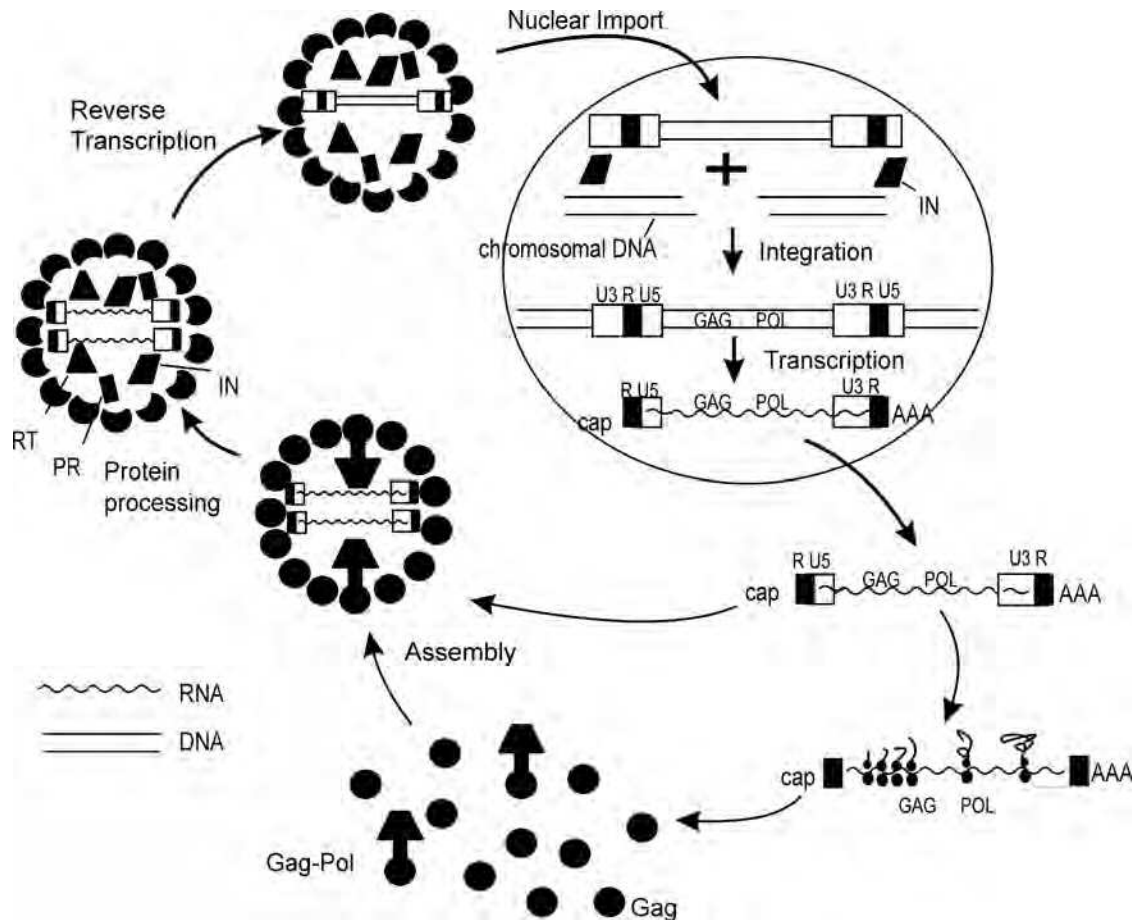


FIGURE 74.7. The Ty replication cycle. It is likely that all retrotransposons follow this cycle.

mammalian retroviruses in all essentials except for their lacking an *env* (envelope) gene and, in part for this reason, are restricted to propagation without leaving the intracellular environment (Fig. 74.7). Nonetheless, the frequency with which these cells mate in nature is so high that most of these elements are widely distributed in their respective species. The retrotransposons are one step further removed in that they lack the long terminal repeat (LTR) structure, and their transposition process differs from those of the other groups. These elements resemble the mammalian LINE elements but have the advantage of readily detectable transposition and facile host genetics for their study. For example, Tad, of *Neurospora*, can retrotranspose between nuclei of a heterokaryon.²⁰⁸ Excellent reviews of retrotransposons are available.^{32,153,227,313,368}

A group of reverse transcriptase encoding introns in mitochondrial DNA of *S. cerevisiae* has also been identified (199; reviewed in 220,221). These are introns aI1 and aI2 of the cytochrome oxidase subunit I (COX1) gene. These introns are capable of retrotransposition both into their normal location and into heterologous locations, both *in vitro*¹⁹⁹ and *in vivo*.²⁶⁰ These retroelements lack LTRs and have only a single ORF. Their properties suggest that introns may have begun as parasitic elements.

The retrotransposons are also divided based on amino acid sequence homologies and gene order in the *pol* domain

into the *copia*-like and the *gypsy*-like elements. *Copia* and *gypsy* are retroelements of *Drosophila*. Because Ty3 is more similar to *gypsy* than to Ty1, it is likely that Ty3 and Ty1 entered the yeast genomes at different times and that horizontal transfer of these elements occurs.

Structure of Tys and Other Retroelements

The Ty elements of *S. cerevisiae* each have LTRs of 245 to 371 bp, separated by a unique region of 4.7 to 5.6 kb³⁰ (Fig. 74.8). The major Ty RNA transcript begins within the 5' LTR (at base 241 from the 5' end in Ty1) and ends at base 289 of the 3' LTR, 45 bp from the 3' end of the element (see Fig. 74.8). This provides the basis for the conventional division of retroviral LTRs into the U3 region (present only at the 3' end of the Ty RNA but located at the 5' end of the LTR), the R region (repeated at both ends of Ty RNA), and the U5 region (present only near the 5' end of Ty RNA but at the 3' end of the LTR).

Ty1 through Ty4 all have two overlapping ORFs: TYA, corresponding to *gag*, and TYB, homologous to the *pol* of mammalian retroviruses. Like mammalian retroviruses and *gypsy*, Ty3 has the gene order protease (PR)–reverse transcriptase (RT)–RNase H (RH)–integrase (IN) in the *pol* ORF,¹⁷¹ whereas Ty1 and Ty2 have the *copia* order PR-IN-RT-RH.^{72,374}

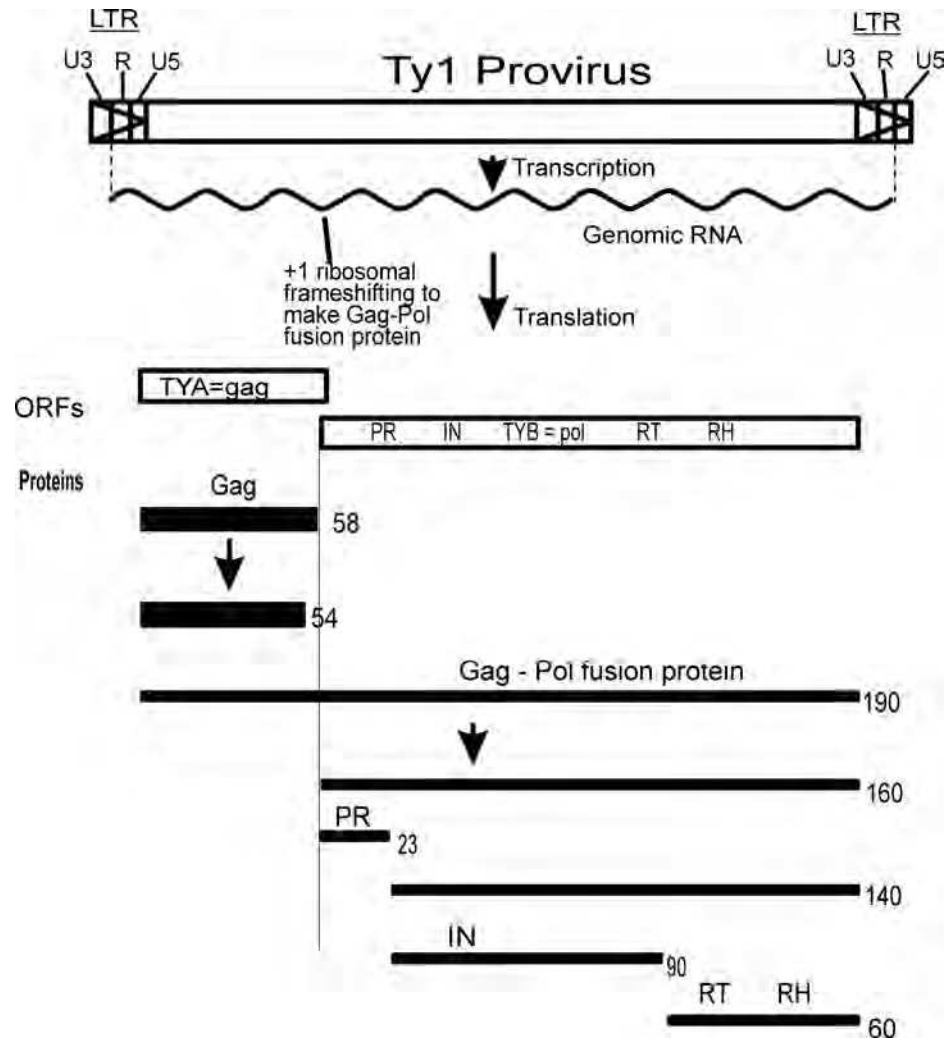


FIGURE 74.8. Genome structure and expression of Ty1. Gag and Gag-Pol are the primary translation products, which are processed by cleavage with the viral protease to form the proteins shown. (Adapted from Garfinkel DJ, Hedge A-M, Youngren SD, et al. Proteolytic processing of *pol-TYB* proteins from the yeast retrotransposon Ty1. *J Virol* 1991;65:4573–4581.)

Replication Cycle of *Saccharomyces cerevisiae* Ty Elements

The Ty replication cycle resembles that of mammalian retroviruses but begins and ends with the integrated form of the genome (see Fig. 74.7). Ty transcripts made with RNA polymerase II are translated to make the Gag and Gag-Pol fusion proteins. These proteins assemble, packaging the Ty RNA to make particles that are homologs of the core particles of retroviruses. The reverse transcriptase and RNase H make a dsDNA copy of the genome, and integrase inserts this into the genome, producing a short repeat of the chromosomal integration site.

Reverse Transcription

The reverse transcriptase and RNase H—homologous domains are present in the Ty1 particles as a 60-kD protein that is produced by proteolytic processing of the 190-kD Gag-Pol fusion protein¹⁵⁴ (see Fig. 74.8). Isolated Ty1 particles have reverse transcriptase that is active on either the endogenous Ty1 RNA or externally added templates. The Ty particles are also open to externally added enzymes, indicating that they are not impermeable shells but have pores. This porous structure may help explain how pseudogene formation can

occur.⁹⁷ Extraction of active polymerase requires that the C-terminal part of integrase be covalently attached, reflecting the likely cooperation of reverse transcriptase and integrase in virions.³⁸⁹

The mechanism of reverse transcription of Ty RNA is largely the same as that for mammalian retroviruses. The primer for reverse transcription is $tRNA_{i}^{Met}$, with ten nucleotides of the 3' acceptor stem complementary to a site on the Ty1 positive strand just 3' to the 5' LTR, called the negative primer binding site (–PBS).⁵⁴ This tRNA is specifically packaged in viral particles.⁵⁴ Interestingly, although the primer function of the initiator $tRNA_{i}^{Met}$ depends on its complementarity with the –PBS, its packaging is independent of this complementarity.⁵⁴ This indicates that the $tRNA_{i}^{Met}$ is recognized by some other component of the Ty virus particle and that it is probably not the acceptor stem of the tRNA that is recognized. Ty1 cDNA synthesis unexpectedly requires *DBR1*, encoding the enzyme that debranches the lariat structure produced by intron excision by cleaving the 2'-5' linkage at the branchpoint.⁵³ Dbr1p acts by cleaving a 2'->5' linkage between the ribonucleotide at the 5' end of Ty1 RNA and an internal Ty1 ribonucleotide.⁵⁹

Integration

Ty3 shows a tight specificity for target sites, integrating 16 or 17 bp upstream of the 5' ends of tRNA coding regions.⁵¹ The DRE element of *Dictyostelium discoideum* and the spliced leader-specific elements SLACS, CZAR, and CRE1 of trypanosomes have a similar integration target specificity.^{3,149,240} Other genes transcribed by RNA polymerase III, such as 5S rRNA and U6 small nuclear RNA, similarly are targets for Ty3.⁵¹ The basis for this site specificity is a specific interaction of Ty3 integrase with the PolIII transcription factor TFIIC¹⁰ and recognition of the TPB and Brf1 subunits of TFIIB.⁴⁰¹ Thus, the integration apparatus recognizes the transcription apparatus, not the transcription initiation site or the promoter itself.

Other Tys are capable of insertion at many different sites, with Ty1 producing a five-bp duplication of the target DNA¹³³; however, these sites tend to be within 750 bases of a tRNA gene or other RNA polymerase III-transcribed gene.²⁰³ Ty1 insertions in RNA pol II-transcribed genes tend to be in the 5' part of the *URA3*, *LYS2*, and *CAN1* genes,^{117,230,265} with control regions targeted far more often than the ORFs.

An *in vitro* integration system using Ty1 viral particles produced from an element carrying a copy of the *Escherichia coli supF* gene and, as target, λ DNA from a multiple amber mutant suppressible by *supF* shows that linear dsDNA substrates carrying the terminal 12 bp at each end of the LTR are sufficient for the integration reaction to occur at normal efficiency.¹¹⁸ There are also no nucleotide requirements. The substrate DNA must have 3' hydroxyls, suggesting that the reaction involves covalent attachment of these 3' ends to the target DNA. Unlike mammalian retroviruses, Ty1's IN does not remove two terminal bases in the process of integration,¹¹⁸ probably because in Ty1 the -PBS is immediately adjacent to the U5 part of the LTR. In fact, the purified IN protein is capable of carrying out an integration model reaction without other components of the viral particle.²⁵⁸

Expression

CONTROL OF TRANSCRIPTION

Transcription of Ty1, Ty2, Ty4, and Ty5 is inhibited in diploid cells by the action of the $\alpha 1/\alpha 2$ encoded by the *MAT* locus.⁷⁴ In the wild, the haploid phase for yeast is transient, and cells soon mate and spend most of their time as diploids. Is the 20 times higher transcription of Ty1 in haploids a preparation for mating and the possible opportunity to hop into a new genome?

Transcription of Ty3 and Ty5 are derepressed by exposure of cells to the mating pheromones.^{73,198,364} Ty3 transposition is, in fact, induced in mating cells, and Ty3 transposing from the genome of one mating partner to that of the other occurs at high rates.²⁰⁹ Here, the interpretation of preparation to transpos into the potentially Ty3-free genome of the mating partner seems clear.

Filamentous growth, associated with starvation, also mobilizes Ty as a result of elevated transcription produced by the Kss1 MAP kinase cascade.⁷⁵ Again, a stressful condition results in Ty mobility.

DNA damage, induced by ultraviolet irradiation or 4-nitroquinoline-1-oxide also induce Ty1 transposition by inducing transcription of the element.³⁷ Is this effect adaptive for Ty1 as a first step in finding a new home, or is it a consequence of a failure of the host anti-Ty system?

Retroelements all must cajole the cellular RNA polymerase II into transcribing their proviral form to make viral RNA. They are constrained, however, to place the controlling sequences inside the limits of the element. Whereas most mammalian retroviruses have enhancers in their LTRs upstream of the start site of transcription, Ty1 and Ty2 elements have major transcriptional control sequences downstream of both the transcript start site and the translation start site, most of them inside the unique region. Ty1 has two major downstream sites responsible for its haploid-specific transcription: the sterile-responsive element (SRE) binds Ste12p, and another site (block II or PRTF) binds Mcm1p.^{74,121}

EFFECT OF TY INSERTION ON CELLULAR GENES

Insertion of Ty elements into the control regions of cellular genes can activate, inactivate, or alter the control of the target gene (122,392; reviewed in 227). The Ty insertions often move the normal regulatory sequences 5 kb away from the target gene, eliminating the normal regulation. Insertions of Ty whose 5' end is close to the 5' end of the target gene impose the Ty transcriptional control on the target gene. This produces a divergent transcription of the Ty element and the target gene; however, both are under mating type control because of the effect of the Ty control region.

CHROMOSOMAL GENES REGULATING TY TRANSCRIPTION

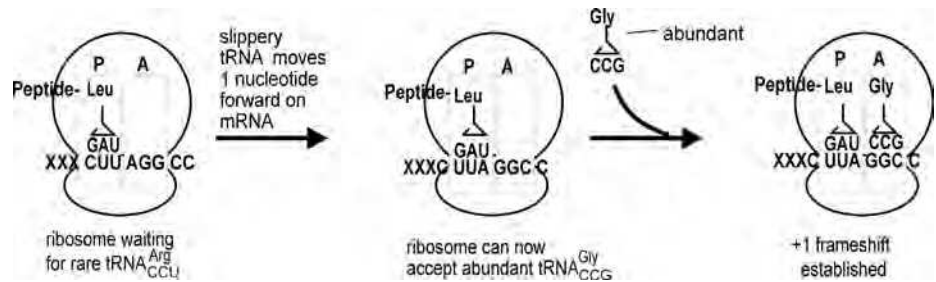
Mutant cellular genes that have come under control of Ty1 have been used extensively to investigate the cellular genes affecting the transcription of Ty1 itself.^{71,394,395} Second-site mutations (suppressors) that restore the normal expression of the target genes have defined a large group of genes, called *SPT*, that include the TATA binding factor TFIID, the genes encoding histones, and many general transcription factors with effects on many genes (e.g., *SNF2*, *SNF5*, *SNF6*, *GAL11*, *SIN1*). In addition, the control of the target genes by mating type and mating pheromones have led to studies of effects of the mating type and pheromone control pathway genes on Ty itself. For example, *STE12* controls Ty1 transcription, as do the genes upstream of *STE12* in the mating type control kinase cascade.¹²³

+1 RIBOSOMAL FRAMESHIFTING

Like mammalian retroviruses, Ty elements direct the synthesis of a Gag protein and a Gag-Pol fusion protein (see Fig. 74.9). For reasons that are not yet clear, each of the *Saccharomyces* Ty elements uses +1 ribosomal frameshifting, whereas animal retroviruses all use -1 frameshifting (or read-through of a terminator) to make Gag-Pol (reviewed in 15,339). As shown by studies of the L-A dsRNA virus, yeast can perform -1 ribosomal frameshifts by the same simultaneous slippage mechanism used by retroviruses.

The mechanisms of the +1 frameshifts in Ty1 or Ty2 (see Fig. 74.9) and Ty3 all involve the combination of starvation for a rare tRNA and an unusual tRNA able to perform the frameshift.^{22,134} In the case of Ty1, the slippery site on the mRNA is CUU-AGG-C. The ribosomes are slowed by the AGG codon at the ribosomal A site. This codon is recognized by a tRNA^{Arg} that is present in low abundance in yeast, thus it is referred to as a hungry codon. The tRNA^{Leu}, located at the P site while the A site is waiting for the AGG codon to be occupied, has as its anticodon UAG, and it is capable of pairing with either the 0 frame CUU codon or the +1 frame codon UUA.

FIGURE 74.9. Mechanism postulated for +1 ribosomal frameshifting of Ty1. Ty3 frameshifting involves the new tRNA pairing in the +1 frame without a shift of the peptidyl transfer RNA as for Ty1.³⁶⁷ (Adapted from Belcourt MF, Farabaugh PJ. Ribosomal frameshifting in the yeast retrotransposon Ty tRNAs induce slippage on a 7 nucleotide minimal site. *Cell* 1990;62:339–352).



When it slips into the +1 frame, the GGC codon can be easily recognized by an abundant tRNA^{Gly} species, and the ribosomes then continue in the +1 pol reading frame to make Gag-Pol fusion protein.²²

The efficiency of the Ty1 +1 ribosomal frameshift depends on the scarcity of the tRNA^{Arg} recognizing the AGG codon. Thus, artificially oversupplying this tRNA lowers frameshift efficiency²² and also lowers transposition frequency.³⁹⁷ Likewise, deletion of the gene for this tRNA^{Arg} increases the efficiency of frameshifting and lowers the frequency of transposition.¹⁹⁷ Like the similar experiments done with the L-A dsRNA virus of *S. cerevisiae*, these results suggest that drugs affecting frameshifting efficiency might be useful as antivirals.

The Ty3 frameshift site is GCG-AGU-U, and, like that of Ty1, it is based on a hungry codon in the ribosomal A site, namely AGU.¹³⁴ AGU is recognized by a low-abundance tRNA^{Ser}, thus the ribosome pauses at this point. However, the tRNA^{Ala}_{CCG} that decodes the GCG codon in the P site cannot slip +1 and re-pair. Rather, it is believed that the valine tRNA simply pairs out of frame.³⁶⁷ A downstream sequence may stimulate Ty3 frameshifting by interacting with rRNA.³³⁹

PROTEOLYTIC PROCESSING AND PHOSPHORYLATION

Ty1 Gag is expressed as a primary translation product of about 58 kd, most of which is processed by the Ty1 protease to form the 54-kd major particle protein.¹ After Pol is synthesized as a 190-kd Gag-Pol fusion protein, it is processed through several intermediates to form a 23-kd protease, the 90-kd integrase, and the 60-kd reverse transcriptase–RNase H¹⁵⁴ (see Fig. 74.8).

PACKAGING AND ASSEMBLY

The Ty1 RNA site determining packaging has been localized to within a 381-nucleotide region between nucleotides 239 and 620.³⁹⁹ The RNA structure recognized and the parts of Gag or Gag-Pol proteins that recognize this region have not yet been determined.

Ty5 and Ty1 mRNA and Gag localize in cytoplasmic particles (T-bodies) thought to be involved in viral particle assembly.^{21,55,238} Assembly of T-bodies requires components of the mRNA-processing bodies referred to as P-bodies (Dhh1p, Lsm1p, Pat1p); however, T-bodies are distinct from P-bodies in the case of Ty1^{55,111} but may be identical in the case of Ty3.²¹ Mutants in these P-body components are defective for retrotransposition, as well as for cDNA accumulation. Retrotransposition requires not only the P-body structure but also its RNA-degrading enzymatic activities, such as the decapping enzyme *Dcp1/2* and 5' to 3' exoribonuclease *Xrn1p/Ski1p*.^{55,111} Because these activities also degrade Ty1 antisense

RNA, it is possible that their effects on Ty1 are a mix of effects on packaging and effects on the antisense copy number control system.

Host Limitations on Ty Transposition Efficiency

Although normal cells have about 35 chromosomal copies of Ty1, most of which are probably transposition competent,⁸⁶ transposition is a relatively rare event, and the host has several levels of defense against Ty1 attack.²⁴⁹ In normal cells, approximately 1% of mRNA is Ty1 mRNA; however, Ty1 protein levels are low, in part a result of sequestration of Ty1 mRNA in P-body-related particles in an untranslated state.¹¹¹

Many studies of the mechanism of transposition have used a high-copy plasmid with a GAL1-promoted Ty1 carrying a marker (such as *HIS3* in the antisense orientation with an artificial intron in the sense orientation) to facilitate detection of transposition.⁸⁴ When Ty1 transposition is induced with such a plasmid, although Ty1 RNA is increased only a few fold over that derived from the normal chromosomal Ty1 copies, the frequency of transposition increases about 100-fold, suggesting a block to transposition that is saturable by Ty1 mRNA.⁸³ This blockade of Ty1 transposition is enhanced by an increase of Ty1 copy number.¹⁵⁵ Transposition is normally blocked at several levels, including transcription,¹⁸⁸ translation and processing of viral proteins,^{75,83} virus assembly, cDNA synthesis or stability, and chromatin structure (see later discussion). Many host genes have been identified whose mutation derepresses Ty1 transposition (reviewed in 227), analogous to the effect of ski mutations on RNA virus copy number (see earlier discussion). Recently, antisense RNA transcribed from Ty1 itself has been found to play an important role in copy number control.^{26,245} Antisense RNAs transcribed from the 5' end of the Gag ORF and incorporated into viral particles are necessary for this copy number control.^{26,245} The antisense RNAs decrease the level of Gag, integrase, and reverse transcriptase, and block cDNA synthesis, apparently acting at a posttranslational level.²⁴⁵ Mutation of components of the 5' to 3' RNA decay pathway, particularly the 5' to 3' exoribonucleases *xrn1* and *kem1*, result in elevated antisense RNA and decreased Ty1 retrotransposition.^{55,111}

Mutation of the cellular *rad6* gene, encoding a ubiquitin-conjugating enzyme, increases the frequency of Ty transposition at either *URA3* or *CAN1* and changes the distribution of insertions.²⁸⁰ This effect is not caused by altered Ty transcript levels and was seen even when retrotransposition from a GAL1-promoted Ty1 was studied. Rad6p alters chromatin structure by monoubiquitination of histone H2B on Lys123,³⁰⁰ suggesting that the Rad6p effect on Ty1 transposition is on the nature

of the target. Indeed, *rad6* mutations specifically affecting silencing have the same altered Ty1 transposition as the deletion.¹³⁷ Mutation of *CAC3*, encoding a subunit of chromatin assembly factor, and *HIR3*, a histone transcription regulation gene,²⁸⁶ also affects both the frequency of integration and the distribution of insertions by alterations of the structure of the target.¹⁸⁰

Mutation of the *FUS3* MAP kinase results in a more than 20-fold increased Ty1 transposition frequency.⁷⁵ Ty1 transcript levels and primary translation products were not affected by *fus3*; however, virus particle-associated processing products of Pol and Ty cDNA were each elevated 10-fold or more in the mutant. The Fus3 kinase is activated by exposure to the mating pheromones via a cascade of kinases; thus, this explains the inhibition of Ty1 transposition by exposure to mating pheromones.³⁹⁸ Although Fus3 may act indirectly to block Ty1 transposition, several observations suggest a direct action.⁷⁵ Gag is phosphorylated,²⁵² and this phosphorylation is increased concomitant with the inhibition of transposition that occurs when cells are treated with mating pheromone, which is known to activate Fus3.³⁹⁸ Gag has several consensus sites for Fus3 phosphorylation.

Mutations in more than 20 genes encoding DNA replication and repair components show derepression of Ty1 retrotransposition (reviewed in 249). For example, mutants in *SSL2* and *RAD3*, encoding excision-repair DNA helicases, show elevated retrotransposition,²²³ as do mutants in *RRM3*, encoding a helicase that promotes replication through DNA-protein complexes.⁸⁵ There is no elevation of Ty1 transcripts; however, the level of Ty1 cDNA is elevated.^{85,223,343} This group of mutations is known to activate the DNA damage checkpoint pathway or the DNA replication stress pathway, and their derepression of Ty1 transposition is prevented by mutation of elements of the checkpoint pathways, such as *rad53*, *rad9*, *rad24*, *mec1*, and *dun1*.⁸⁵ Thus, DNA damage or replication arrest from a host of sources, by activating the checkpoint pathways, turns on Ty1 retrotransposition. Although the mechanism of this effect is not yet clear, the best explanation appears to be that cellular conditions at the arrest point in the cell cycle are particularly favorable for transposition.²⁴⁹ There is no evidence that this derepression favors either the yeast host or Ty1.

Ty5 integration is concentrated in regions of heterochromatin, unlikely to be detrimental to the host.⁴⁰⁶ A nine-residue section of the Ty5 integrase determines association with Sir4p, a heterochromatin protein, and hence the site specificity.³⁹⁶ However, this interaction and the corresponding targeting to heterochromatin only occur properly when this targeting domain site is phosphorylated at S1095 by a host kinase.⁸⁷ Under stress conditions, this control system breaks down, the targeting domain is not fully phosphorylated, and integration is random, producing more serious mutations.⁸⁷

The variety of host defenses against Ty1 and Ty5 reflects the dangers inherent in its mobility. Although occasional favorable mutations can result, Ty1 copy number is inversely correlated with fitness.³⁹⁰

Schizosaccharomyces pombe Retroelements

The Tf1 and Tf2 elements of *Schizosaccharomyces pombe* are unusual in that a single ORF encodes both Gag and Pol.²²⁸ From the primary 140-kD translation product, the viral pro-

tease cleaves the proteins that form the viral particles.²²⁹ In view of the strict requirement for the ratio of Gag to Gag-Pol in Ty1^{197,397} and retroviruses, the assembly process in Tf1 and Tf2 must be significantly different. In log phase cells, the ratio of Gag to IN in particles is close to 1, whereas in stationary cells, it is quite high.⁹ Selective degradation of the non-Gag protein components alters the ratio after particle formation.⁹

The priming mechanism of Tf1 is also unique. Instead of tRNA priming as for other retrotransposons and retroviruses, Tf1 negative-strand DNA synthesis is primed by the first 11 nucleotides at the 5' end of the viral RNA, which is complementary to the primer binding site.²³¹ A hairpin is formed, which is nicked to separate the first 11 nucleotides from the 5' end of the RNA and give it a 3'OH that can serve as a primer for strong-stop DNA synthesis.

Although the *piwi* RNAi system controls retrotransposons in mammals and many other organisms, two chromatin-modifying protein-based systems have been identified in *S. pombe* that silence *Tf2* elements and *Tf2*-derived LTRs and prevent their recombination that could damage the genome.^{48,164} The HIRA complex, a nucleosome-assembly apparatus that includes Hip1, Slm9, and Hip3, is important for the assembly of silencing heterochromatin at various sites, including the *Tf2* retrotransposon.¹⁶⁴ Three homologs of human CENP-B—Abp1, Cbh1, and Cbh2—are involved in a different chromatin silence system that works by binding to *Tf1* and *Tf2* loci (and lone LTRs) and recruiting the histone deacetylases Clr6 and Clr3 to those sites.⁴⁸ In addition to the silencing produced by localized histone deacetylation, the CENP-B homologs draw together the *Tf* loci into intranuclear bodies, which may facilitate their silencing.⁴⁸

Candida albicans Plasmid Retrotransposon and LINE Elements

Most retrotransposons have minute amounts of cDNA present in normal strains; however, that of *Candida albicans* (pCal) produces 50 to 100 copies per cell of linear dsDNA in a particular strain of *Candida*.²⁴⁸ pCal is a member of the Ty1/*cop* group found in integrated form in most strains, and the basis for its high free levels is not yet known. pCal is also unique among retrotransposons in that it uses read-through of a stop codon to make its Gag-Pol fusion, as in murine leukemia virus.²⁴⁸

C. albicans also carries several families of non-LTR retrotransposons (LINEs), homologous to the mammalian L1 LINE elements.¹⁶² One of these elements, Zorro3, has been shown capable of transposition in *S. cerevisiae*.¹⁰⁹ Non-LTR retroelements have also been identified in *Cryptococcus neoformans*.¹⁶³

Summary of Retroelements

The mating type and pheromone control of Ty transcription is clearly adapted to maximize transposition activity at the time when a potentially unoccupied genome becomes available (mating), and to minimize potential damage to the host, whose health is indispensable for survival. Ty gets moving when the host's genome seems in danger, perhaps in an effort to find a safe haven. The host limits retrotransposition at several levels, apparently to prevent damage to its genome.

The many interesting questions about Ty elements include the following: Why do all Ty's use +1 frameshifting but

mammalian retroviruses use -1 frameshifting or termination read-through to make their Gag-Pol fusion proteins? How does RNA polymerase II know to stop in the 3' LTR but to keep going in the identical sequence in the 5' LTR? The bewildering array of retroelements continues to amaze.

DNA VIRUSES: *CHLORELLA* VIRUSES

Chlorella is a unicellular eukaryotic alga with a rigid cell wall and a single chloroplast (reviewed in 365,393,400). Most *Chlorella* species are free living; however, several, collectively referred to as zoochlorellae, live as endosymbionts (intracellular symbionts) of *Hydra* or *Paramecium*.

An attempt to isolate zoochlorellae free of their *Hydra* or *Paramecium* host often induces multiplication of a virus that grows and kills the zoochlorellae. These viruses are called *Hydra viridis Chlorella* virus (HVCV) or *Paramecium bursaria Chlorella* virus (PBCV). Hundreds of *Chlorella* viruses have been isolated directly from freshwater and are found throughout the world. They are grown on cultured zoochlorellae and form lytic plaques on agar plates.

Chlorella viruses are large (150–230 nm), polyhedral particles containing 5% to 10% lipid. The outer shell is a T = 169 icosahedron, surrounding an inner lipid layer. The dsDNA genome of PBCV-1, the best studied *Chlorella* virus, is 333 kb, one of the largest viruses known. PBCV-1 DNA is linear with (a) terminal inverted repeats of 2.2 kb, (b) terminal hairpin structures, and (c) shorter direct repeats within the inverted terminal repeats. All three characteristics are in common with the poxviruses, vaccinia virus, and African swine fever virus. Some other features are shared with the iridoviruses.

Chlorella virus DNA is heavily methylated, with 5-methylcytosine accounting for as much as 47% of C residues and 6-methyladenine for up to 37% of A residues. Surprisingly, the *Chlorella* viruses encode their own methylases. They also have been found to encode various restriction endonucleases, similar in properties and, in many cases, in specificity to bacterial type II restriction endonucleases. The variety of such methylases and restriction endonucleases has only begun to be explored; however, it is clear that a wide variety of specificities will be found.²⁶⁷ The function of these enzymes is completely unclear, and there is evidence against their being required for either degradation of host DNA or exclusion of other co-infecting viruses (reviewed in 267).

The 54-kd major capsid protein of PBCV-1 has a carbohydrate component of about 5 kd. Unexpectedly, the virus encodes glycosyl transferases and other products that determine the glycosylation of Vp54.³⁷³

The complete sequence of PBCV-1 showed that the 330,742-bp genome encodes enzymes for synthesis of hyaluronic acid, aspartate transcarbamylase (uracil biosynthesis), an EF-3 homolog (translation elongation factor), 10 tRNA genes, one with a small intron, genes with self-splicing type I introns, other genes with spliceosome-dependent introns, protein kinases, a potassium channel protein, and numerous enzymes involved in nucleic acid metabolism.

Large DNA viruses of marine brown algae have also been described (reviewed in 366). The giant *Mimivirus*, with the largest known viral genome, and infecting *Acanthamoeba*, is reviewed in Chapter 71.

PRIONS OF *SACCHAROMYCES* AND *PODOSPORA*

The concept of an infectious protein arose in studies of the transmissible spongiform encephalopathies (TSEs), such as scrapie of sheep, mad cow disease, and Creutzfeldt-Jakob disease of humans (see Chapter 76). Based on genetic evidence (Fig. 74.10), two non-Mendelian genetic elements of *S. cerevisiae*—[URE3] and [PSI]—were identified as prion (infectious protein) forms of Ure2p and Sup35p, respectively.³⁸⁰ This conclusion is supported by extensive further evidence, and both are transmissible amyloidoses, a remarkably close parallel to the mammalian disease. Two further amyloid-based prions—[Het-s] of the filamentous fungus *Podospora anserina*⁷⁹ and [PIN⁺] of *S. cerevisiae*⁹⁴—were next found and have been studied extensively. An infectious protein consisting of an enzyme whose active form is essential for activation of its own inactive precursor can also be an infectious protein, and two such cases have been described.^{202,299} Recently, several other yeast prions have been reported (Table 74.4).

[URE3]—A Transmissible Amyloidosis of Ure2p

When yeast has a good nitrogen source, such as ammonia, Ure2p, by binding the transcription factor Gln3p, shuts off transcription of genes encoding enzymes and transporters needed for the utilization of poor nitrogen sources, such as *DAL5*, encoding the allantoin transporter.⁷⁷ In 1971, Lacroute described a non-Mendelian genetic element of *S. cerevisiae* called [URE3], which allowed cells to take up ureidosuccinate despite the presence of a rich nitrogen source.²¹⁹ Ureidosuccinate is the product of aspartate transcarbamylase (*URA2*) in uracil biosynthesis. Because of chance resemblance to allantoin (a poor nitrogen source), it is taken up from the medium by Dal5p, and *DAL5* is repressed by ammonium via Ure2p action. Thus, [URE3] *ura2* strains can grow on ureidosuccinate in the presence of ammonia, whereas [ure-o] cells (lacking [URE3]) cannot. Fusing the *DAL5* promoter to the *ADE1* ORF allows assaying [URE3] by adenine prototrophy.³¹⁸

Genetic Evidence that [URE3] Is a Prion

Three lines of genetic evidence first showed that [URE3] is actually an altered form of Ure2p that has lost its activity in repressing nitrogen metabolic enzymes through Gln3p but has acquired the ability to convert the normal Ure2p to this altered form³⁸⁰ (see Fig. 74.10). First, [URE3] is efficiently cured by growth of cells on guanidine HCl; however, the curing is reversible in that [URE3] derivatives may again be selected from the cured strain.³⁸⁰ Second, overproduction of Ure2p leads to a 20- to 100-fold increase in the frequency with which [URE3] arises *de novo*.³⁸⁰ Third, Ure2p is required for propagation of the [URE3] nonchromosomal gene, but the phenotypes of *ure2Δ* and [URE3] are essentially identical.^{2,380} These three features are strongly predicted by the prion model, although not if [URE3] were a nucleic acid replicon—a virus or plasmid.³⁸⁰ These three properties, none of which were known for the mammalian TSEs, also served to identify [PSI⁺] as a prion form of Sup35p,³⁸⁰ [PIN⁺] as a prion of Rnq1p,⁹⁴ and other prions.

genetic properties of a yeast prion

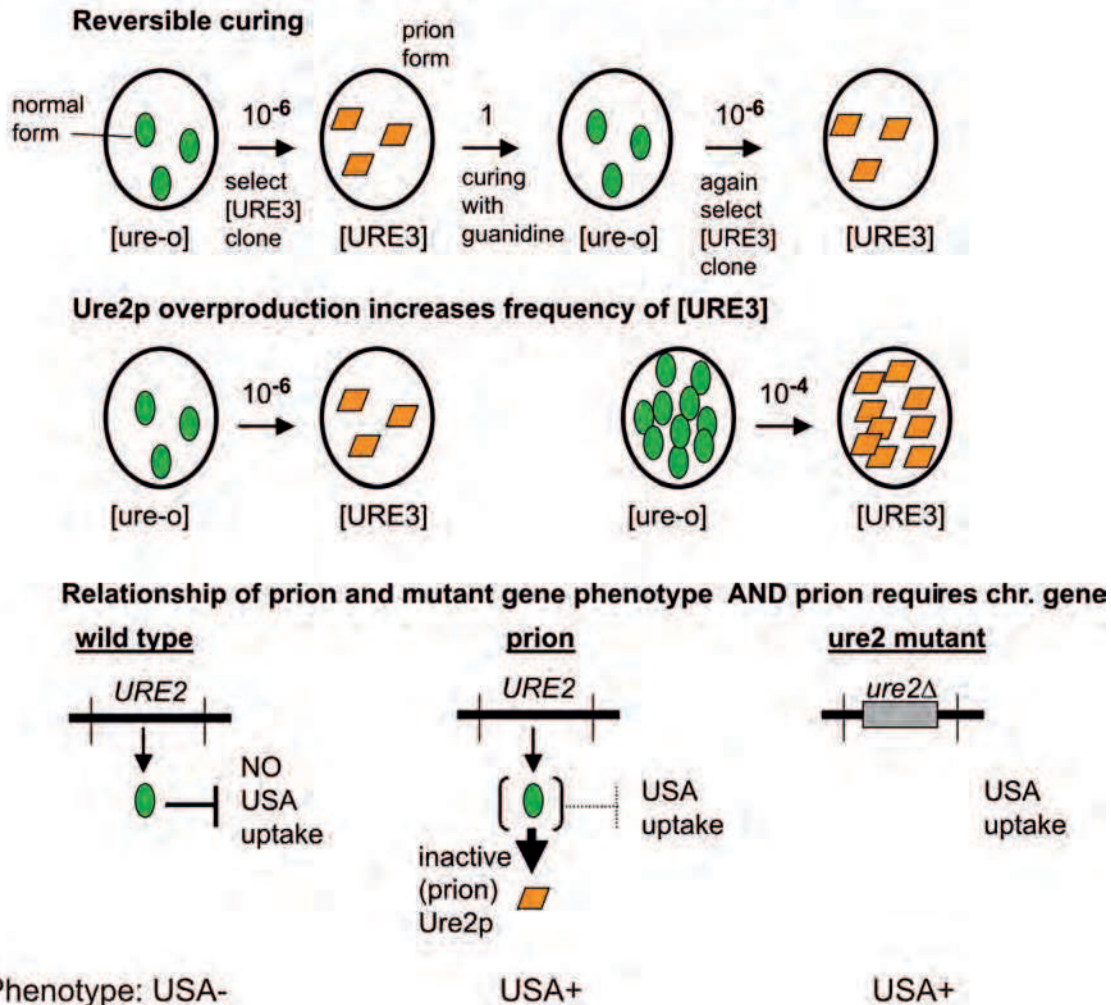


FIGURE 74.10. Three genetic criteria for a prion. These are unusual features, expected of a prion but not of a nucleic acid replicon. (Adapted from Wickner RB. [URE3] as an altered URE2 protein: evidence for a prion analog in *S. cerevisiae*. *Science* 1994;264:566–569.)

Ure2p is partially protease resistant in extracts of [URE3] strains, like the amyloid of PrP in brains of TSE-infected animals.²⁴⁴ Moreover, the generation and propagation of [URE3] was unaffected by the repression or derepression of nitrogen regulation, eliminating some alternative models. [URE3] truly does arise *de novo*, and it is the Ure2 protein, not the *URE2* mRNA or the copy number of the *URE2* gene, that induces [URE3] to arise.²⁴³

Domains of Ure2p

Ure2p is composed of a C-terminal functional domain, whose structure resembles glutathione-S-transferases (residues 95–354),^{34,244,363} and an unstructured N-terminal domain (residues 1–94).^{244,281} Ure2p^{90–354} is sufficient, if overexpressed, for nitrogen regulation but is rapidly degraded without the N-terminal residues.³²⁹ This stabilization domain is also the part of the molecule responsible for the prion change (the prion domain) and comprises roughly the N-terminal 65 residues

(244; Fig. 74.11). [URE3] is efficiently transmitted by cytoplasmic mixing to cells expressing only Ure2p^{1–65} from those expressing the full-length protein. The cells expressing only Ure2p^{1–65} stably propagate the prion and efficiently transmit it again to cells expressing the full-length Ure2p.²⁴³ On conversion to the infectious amyloid form, the C-terminal domain is largely unchanged in structure,^{11,17} and the prion domain (residues 1–65) assumes an extremely stable amyloid form.^{18,351} Residues 66 through 94 remain unstructured in the amyloid form and are called the *tether* because they attach the structured C-termini to the structured amyloid core.

Infectious Amyloid of Ure2p Is [URE3]

Ure2p–green fluorescent protein fusions are aggregated in [URE3] cells, indicating that aggregation is involved in the [URE3] change.¹¹⁴ The Ure2p prion domain synthetic peptide can form amyloid, and this domain also directs the conversion

whose infectivity is nearly as high as that of extracts. Infectivity resides entirely in the N-terminal prion domain, again showing its central role.³⁵

[PSI]—A Transmissible Amyloidosis of Sup35p

In 1965, Cox⁸⁰ described [PSI], a non-Mendelian genetic element of *S. cerevisiae* that increases the efficiency of nonsense suppression by classical tRNA suppressors. Like [URE3] and Ure2p, [PSI] is reversibly curable,^{234,333} overexpression of Sup35p increases the frequency with which [PSI] arises by 100-fold,^{60,96} the phenotype of [PSI] is like that of *sup35* mutants, and *SUP35* is necessary for propagation of [PSI].^{108,352} This logical parallel with [URE3] and Ure2p indicates that [PSI] is also a prion form of Sup35p.³⁸⁰

Infectious Amyloid of Sup35p

Sup35p is aggregated in [PSI+] strains but not in [psi-] strains,^{276,279} and the *in vitro* aggregation of native soluble Sup35p seeded by the prion form from [PSI+] cells will continue indefinitely as long as fresh native Sup35p is supplied.²⁷⁸ Further, synthetic prion domain peptide spontaneously forms amyloid *in vitro*.²⁰⁷ In addition, the full-length Sup35p, made in *E. coli*, forms β -sheet-rich filaments spontaneously when diluted out of denaturant, a process that can be accelerated specifically by an extract from [PSI+] cells.¹⁶⁰

Amyloid formed from recombinant Sup35p transmits [PSI+] to cells, inducing the appearance of several prion variants.^{206,346} Extracts of cells bearing a variant are infectious and transmit the variant carried by the strain from which the extract was made. Moreover, seeding soluble Sup35p with an extract amplifies amyloid transmitting that prion variant. Adjusting the conditions under which amyloid forms spontaneously strongly biases the variant encoded by the amyloid formed.³⁴⁶ These results are useful in studies of the structural differences between prion variants.

[PIN+]—A Prion that Generates Other Prions

Derkatch et al⁹⁵ found that whereas overproduction of Sup35p induces the high frequency appearance of [PSI+] in some strains, others did not show this effect. Their analysis showed that this effect was attributable to the presence of a nonchromosomal genetic element in the strains in which [PSI+] generation was inducible. The Rnq1 (rich in N and Q) protein showed a self-propagating aggregation,³³⁶ and indeed Rnq1 amyloid formation proved to be the basis of the [PIN+] nonchromosomal gene.⁹⁴ Overproduction of other N/Q rich proteins likewise has [PIN+]-like activity (without being themselves prions), as does the [URE3] prion.⁹⁴ Moreover, [PIN+] also modestly promotes *de novo* generation of [URE3].³⁶ [PIN+] probably promotes generation of prions by other N/Q rich proteins by rare cross-seeding.

Recombinant Rnq1p also forms amyloid *in vitro*,³³⁶ and this amyloid can infect yeast cells with the [PIN+] prion.²⁷⁵

[Het-s]—A Prion of *Podospora* Responsible for a Normal Function

When two colonies of the same strain of filamentous fungus meet, they fuse their cellular processes (called *hyphae*) in a process referred to as hyphal anastomosis or vegetative fusion or heterokaryon formation. This allows exchange of cytoplasm

Heterokaryon Incompatibility in *Podospora* requires the [Het-s] prion

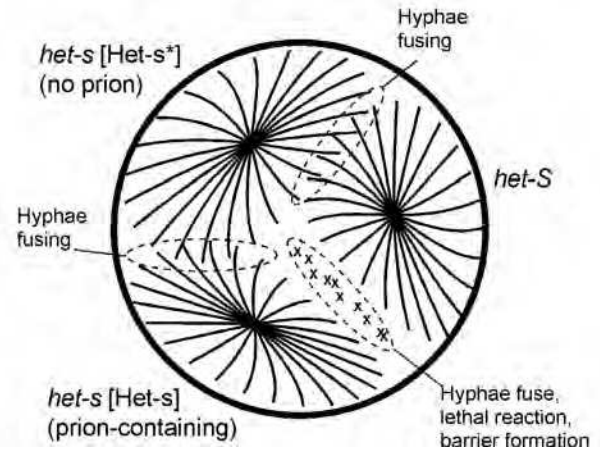


FIGURE 74.12. The [Het-s] prion of *Podospora anserina* is required for heterokaryon incompatibility. (Adapted from Coustou V, Deleu C, Saupe S, et al. The protein product of the *het-s* heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog. *Proc Natl Acad Sci U S A* 1997;94:9773–9778.)

and even nuclei and allows cooperation between the colonies in obtaining nutrients. However, a virus initially present in only one colony will spread throughout the other colony; thus, the process is tightly controlled, requiring identity of the two strains at 6 to 10 special chromosomal loci, called *het* loci in *Podospora anserina*. Fusion of two strains that differ at a *het* locus begins; however, it is quickly arrested with death of the few fused hyphae and formation of a barrier to further hyphal fusion. This is referred to as the heterokaryon incompatibility reaction (Fig. 74.12) (reviewed in 316).

One of the *het* genes of *Podospora anserina* is *het-s*, with alleles *het-s* and *het-S* encoding a 289-residue protein differing at 14 positions,³⁶¹ although the difference at residue 33 is sufficient to trigger the incompatibility reaction.⁹³

Rizet²⁹⁸ found that *het-s* strains could show the incompatibility reaction only if they also carried a non-Mendelian genetic element referred to as [Het-s]. [Het-s] could be “cured” from a male strain by mating; however, from the cured strain, [Het-s] cells arose at a frequency of about 10^{-7} .²⁰ This is the *reversible curing* criteria for a prion.

Coustou et al⁷⁹ have made the case that [Het-s] is a prion of the protein encoded by *het-s*. They showed that overproduction of the Het-s protein increased the frequency with which [Het-s] arose, and that the Het-s protein is relatively protease resistant in strains carrying [Het-s]. Moreover, deletion of the *het-s* gene makes a cell unable to propagate the [Het-s] non-Mendelian genetic element.⁷⁹

The prion domain of HET-s is the C-terminal residues 218 through 289, of which residues 228 through 289 are unstructured in the native form.¹³ The HET-s prion domain is not N/Q rich but forms amyloid filaments *in vitro* that are infectious for cells not carrying the prion.²³⁶ The demonstration that amyloid formed *in vitro* from recombinant HET-s protein was the first demonstration of its kind for any prion.²³⁶

Beyond its obvious interest as a new prion, [Het-s] is particularly important because it is the first case in which a prion appears to be responsible for a normal cellular function. Heterokaryon incompatibility is observed in most filamentous fungi, usually controlled by chromosomal genes that have no particularly unusual features. In this case, it requires the [Het-s] prion to be observed. This suggests that other prions may be found responsible for normal cellular functions.

However, another interpretation of [Het-s] is possible, based on a [Het-s]-based meiotic drive system. Mating a female *het-s* [Het-s] strain with a *het-S* strain results in death of *het-S* meiotic segregants that received [Het-s] cytoplasm.⁸⁸ Thus, one could view the [Het-s] prion as a device for the preferential inheritance of the *het-s* allele by a meiotic drive mechanism.

Prion Variants

Strains of the mammalian TSEs were described early on, characterized by different incubation period, symptoms and signs, and distributions of brain lesions.⁴² Independent isolates of [PSI⁺], [URE3], or [PIN⁺] may likewise have various intensity of phenotype or stability.^{36,96,318} These prion variants breed true, and extracts of a strain carrying a given variant of [PSI⁺] or [URE3] transmit almost exclusively that variant by transformation.^{35,206,346} It is clear that different [PSI⁺] prion variants have different amyloid structures,³⁴⁷ and one of the main challenges is to determine these structural differences (Fig. 74.13).

It is striking that only one variant of [Het-s] has been described. Because [Het-s] is either carrying out a function for the host or is a part of a meiotic drive system, it is in either case selected in evolution to be a prion. The protein sequence has presumably been selected to efficiently form the single prion variant that carries out this function.

Structures of Yeast Prion Amyloids Explain Variant Propagation

Ure2p and Sup35p prion domain amyloids have the cross- β -sheet structure typical of amyloids, meaning that the β -strands run perpendicular to the filament long axis.^{16,210,351} Infectious prion domain amyloids of recombinant Sup35p, Ure2p, and

Rnq1p each have a parallel in-register β -sheet structure based on solid-state nuclear magnetic resonance (NMR) studies,^{19,330,382} with identical residues in adjacent molecules aligned in a row along the long axis of the filament (Fig. 74.14). The mass per unit length of Ure2p, Sup35p, and Rnq1p prion domain filaments is in each case the one monomer per 4.7 Å expected for this structure.^{18,57,98} The diameters of the filaments^{207,351,382} show that these filaments must be multiply folded along the filament axis (see Fig. 74.14). This structure is like that found for amyloids of A β , amylin, α -synuclein, β 2-microglobulin, tau, and most other human pathologic amyloids (reviewed in 239,362).

The in-register parallel β -sheet structure is maintained by the positive interactions among identical aligned amino acid side chains—H-bonds between the amide side chains of glutamines or asparagines, H-bonds between the –OH groups of serine or threonine side chains, or hydrophobic interactions among hydrophobic amino acid side chains. These interactions demand that identical residues be aligned. The same interactions direct a monomer joining the end of the amyloid filament to assume the structure of the other molecules in the filament.³⁸⁵ Thus, although any of many different parallel

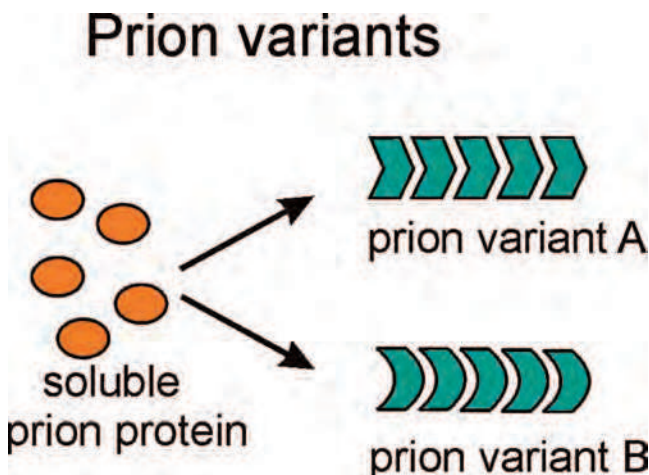


FIGURE 74.13. A single protein sequence can stably propagate any of several prion variants, each corresponding to a different amyloid structure.

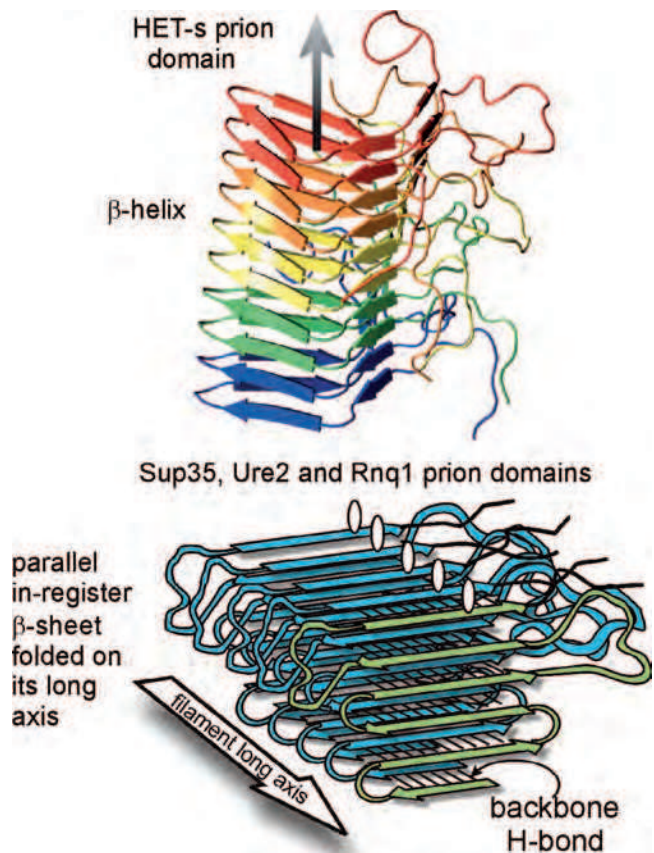


FIGURE 74.14. Structures of prion amyloids. **A:** Infectious amyloid of HET-s²¹⁸⁻²⁸⁹ has a two-turn β helix structure (Adapted from Wasmer C, Lange A, Van Melckebeke H, et al. Amyloid fibrils of the HET-s (218-279) prion form a beta solenoid with a triangular hydrophobic core. *Science* 2008;319:1523–1526). **B:** Infectious amyloid of Ure2p¹⁻⁶⁵, Sup35p¹⁻²⁵³, and Rnq1p¹⁵³⁻⁴⁰⁵ each have an in-register parallel beta sheet architecture.^{19,330,382}

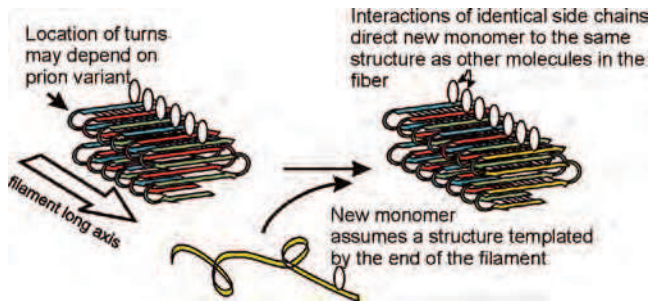


FIGURE 74.15. Prion amyloid structure can be templated by the structure of molecules in the filament. As a new monomer joins the filament interactions of side chains at the end of the filament with those of the new monomer force the new monomer to assume the same structure as the rest of the monomers in the filament. Different filaments may have different locations of the folds (different turn locations) and different extents of the β -sheet structure; however, this information is transmitted to new monomers joining the filament.^{383,385}

in-register structures may form at the time of filament initiation, the location of the folds of the sheet, and the extent of the β -sheet structure will be propagated as the filament is elongated by this mechanism (Fig. 74.15). This mechanism^{383,385} can explain how a protein can template its own conformation (just as DNA can template its own sequence), and thus how proteins can be genes.

HET-s Prion Amyloid Structure

Consistent with its forming only a single prion variant *in vivo*, the HET-s protein prion domain forms a very well defined amyloid structure *in vitro*. This unique structure thus produces very sharp cross peaks in solid-state NMR, making possible the determination of its detailed structure.^{297,375} This structure is a β helix, with two turns of the helix per HET-s molecule (see Fig. 74.14). Interestingly, the HET-s prion domain includes two imperfect direct repeat sequences, and these repeats are aligned in the β helix structure, producing an effect somewhat similar to the parallel in-register architecture of the yeast prions. How this structure is uniquely specified and how interaction of the HET-s amyloid and soluble HET-S produces cell death are important remaining problems.

[URE3] and [PSI] are Diseases of Yeast

Although mammalian prion infections are uniformly fatal, the yeast and fungal prions are often compatible with robust growth. This, in part, led to the suggestion that yeast prions could benefit their host, and it was reported that [PSI+] conferred resistance to high temperature or high ethanol stress.¹¹³ Further studies showed that this observation was not general, and that under most conditions tested, [PSI+] was a disadvantage to the host.³⁵⁹ Nonetheless, because in a minority of conditions [PSI+] appeared to be an advantage, although varying with the yeast strain, it was proposed that [PSI+] helps yeast evolve by allowing the yeast to resist stress until mutation or recombination can produce a stress-resistant genotype.³⁵⁹ Although these results were not consistently repeatable,²⁶⁴ the notion of beneficial yeast prions has gained some currency.³³² Because some Sup35 of some other species can form

prions,^{61,218,314} it was inferred that prion-forming ability is selected by evolution and must therefore benefit the host.³³² Similarly, the conservation of sequences in the prion domains of Sup35p and Ure2p was interpreted to mean that prion-forming ability was selected by evolution.¹⁷²

Several approaches have been taken to evaluate whether yeast prions are diseases or beneficial. Yeast prions are infectious and can be very stable. Moreover, they arise *de novo* in about one in 10^6 cells, thus no substantial population can be isolated from them. If they were advantageous to yeast, they would surely be often found in the wild. A survey of 70 wild yeast strains has failed to turn up any strains carrying either [URE3] or [PSI+], indicating that both are a net disadvantage to the host. In contrast, the 2 μ DNA plasmid, the L-BC dsRNA viruses, and the 20S and 23S ssRNA replicons were found in 38, 8, 14, and 1 strains, respectively, although none of these selfish infectious nucleic acids encode anything but proteins to promote their own duplication.²⁶³

The notion that conservation of prion-forming ability implies a benefit of prions to the host has been discredited by the finding that prion-forming ability of Ure2p and Sup35p is not conserved, even within *Saccharomyces*,^{115,312} and that sequence conservation is not needed for conserving prion-forming ability^{307,308}; therefore, sequences in the prion domains must be conserved for another reason.

Indeed, the prion domains of Sup35p and Ure2p each have normal nonprion functions. The N-terminal domain of Sup35p of yeast and humans interacts with the poly(A) binding protein and poly(A) shortening enzymes to assist in general mRNA turnover.^{78,147,177,178} The Ure2p prion domain protects the protein from rapid degradation and thus is necessary for proper nitrogen regulation.³²⁹ These domains are preserved for their normal functions, not for prion formation.

Finally, the premise that [PSI+] and [URE3] are generally not harmful to their host has been disproven by the finding that lethal or severely detrimental variants of [PSI+] are actually more common than the usually studied mild variants.²⁵⁰ These “killer [PSI]” variants probably inactivate nearly all of the essential Sup35p and are not found in the usual selection procedure. [URE3] variants that severely impair growth in strains whose growth is not slowed by *ure2 Δ* are also common²⁵⁰ and must be owing to a toxic effect of the Ure2p amyloid. It appears clear that the yeast prions [URE3] and [PSI+] are diseases, like their mammalian counterparts.

Chaperone Involvement in Prion Propagation

Chernoff found that either overproduction of Hsp104p or deletion of the *HSP104* gene resulted in the inability of cells to propagate [PSI]^{62,64} (Fig. 74.16). This finding is important because it at once supported the prion model for [PSI], it was the first proof of involvement of a chaperone in a prion phenomenon, and it provides a potential pathway to treatment of prion diseases. Hsp104 is necessary for the propagation of all known yeast prions except [ISP+].^{305,306} Normal levels of Hsp104 promote [PSI] stability by breaking large filaments into smaller ones, providing new seeds for propagation and infection.^{191,217,268,306,360} The mechanism of [PSI] curing by Hsp104 overproduction is unclear³⁰⁶; however, it apparently involves a different activity from that which produces disaggregation.¹⁸² Hsp70s and Hsp40s are also important for prion propagation, at least in part for their roles with Hsp104.^{6,63,189,192,216,327}

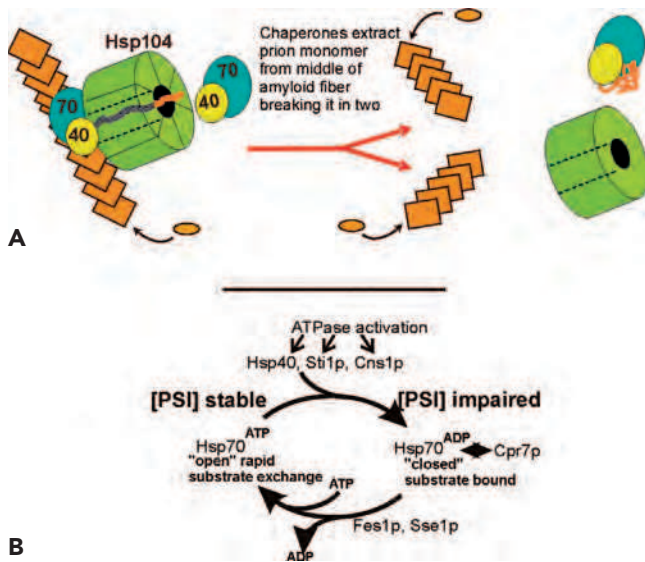


FIGURE 74.16. A: Role of chaperones Hsp104, Hsp70, and Hsp40 in propagation of the prions of *Saccharomyces cerevisiae* (Adapted from Masison DC, Kirkland PA, Sharma D. Influence of Hsp70s and their regulators on yeast prion propagation. *Prion* 2009;3:65–73.) Hsp104 is necessary for [PSI⁺] propagation^{62,64} and for other prions (see text) for breaking filaments to form new seeds,^{81,191,268} as are the cytoplasmic Hsp70s.¹⁹² Hsp40s are also critical for yeast prion propagation.^{259,337} Because Hsp104 cooperates with Hsp70 and Hsp40 in disaggregation,¹⁶¹ it is proposed that they cooperate in extracting monomers from filaments, thereby breaking the fibers.¹⁸² Overproduction of Hsp104 cures the [PSI⁺] prion by a different, but unknown, mechanism. Normal levels of Hsp104 are needed for [PSI⁺] propagation, because it has a role in breaking up aggregates to ensure that each daughter cell gets at least one aggregate. **B:** The bound ATP/ADP status of Hsp70 affects prion propagation as indicated (reviewed in 327).

Hsp90 co-chaperones are also important for prion propagation, although largely because of their interactions with Hsp70s.^{189,216,288}

Other cellular components also affect prion propagation. The ubiquitin system is important in curing of [PSI⁺] by overproduction of Hsp104,^{5,65} and the cytoskeletal protein Sla1p assists prion propagation.¹² Btn2 is an endosome-associated protein involved in protein sorting whose overproduction cures [URE3], apparently by collecting Ure2p aggregates at a single cellular site.²¹⁵

PERSPECTIVES

The discovery of prions of yeast and fungi resolved the debate over whether prions could exist at all and widened the scope of this concept, showing that it is not unique to a single mammalian protein. The yeast system is now being used to explore the mechanisms of amyloid and prion generation and propagation, and to devise approaches to the many heretofore intractable amyloid diseases. Likewise, studies of retroelements, RNA viruses, and DNA viruses of unicellular eukaryotes have provided extensive information about each virus group, with

much of it more easily obtained because of the facile genetics of these systems.

NOTE: See the e-Book for additional information on *Leishmania* dsRNA viruses, *Giardia lamblia* virus, and *Trichomonas* virus and host phenotypic variation.

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Bacteriophages

Brief History

Caudovirales: dsDNA Tailed Phages

Bacteriophage λ and the “Lambdoid” Phages

Bacteriophage T4

Other Tailed Phages

Bacteriophage HK97

Bacteriophage T7

Bacteriophage P22

Bacteriophages P2 and P4

Bacteriophage G

Bacteriophage Mu

Bacteriophages P1 and N15

Bacteriophage ϕ 29

Insights from Other Phage Families

Tectiviridae: dsDNA, Linear, with Proteins on 5' Ends

Microviridae: ssDNA, Circular

Inoviridae: ssDNA, Filamentous

Leviviridae: +ssRNA

Cystoviridae: dsRNA, Segmented

Corticoviridae: dsDNA, Circular

Plasmaviridae: Circular dsDNA

Evolution of Phages

Genome Mosaicism

Common Ancestry of Archaeal and Eukaryal Viruses with Phages

Phage Parts in Other Contexts

Pyocins

Gene Transfer Agents

Encapsulins

Type VI Secretion Systems

Host Interactions

Parasitic Interactions

DNA Replication

Antagonistic Interactions

Symbiotic Interactions

Phage Communities and Ecology

Acknowledgments

Bacteriophages—viruses that infect bacteria—collectively constitute a majority of organisms on planet Earth. Virions of the tailed double-stranded DNA (dsDNA) phages, which are the most easily identified in environmental samples, are present at roughly 10^7 /mL in coastal seawater and at comparably high numbers in numerous other environmental situations examined.¹⁷⁶ A rough calculation indicates that there are about 10^{31} individual tailed phage virions on the planet, a literally astronomical number, which means that if all the tailed phages were laid end to end, they would extend for 200 million light years into intergalactic space.⁷⁴ These numbers become even more impressive when we consider that most bacterial genomes contain from one to two dozen phage genomes as prophages.³⁰ Measurements of the persistence of phages in the environment suggest that the entire population of 10^{31} individual virions turns over every 4 to 5 days,¹⁹³ and this leads to the estimate that it takes roughly 10^{24} productive infections per second to maintain the population—speaking only of the tailed phages.⁷⁴

BRIEF HISTORY

Bacteriophages were discovered in 1915 and 1917, based on their ability to cause clearing (cell lysis) in bacterial lawns and to be propagated as infectious agents.^{47,180} The first ~20 years of research on phages was aimed at understanding the basis of these phenomena, and in the process these studies contributed to developing ideas about the nature of viruses more generally. There was also keen interest during this time in phage therapy, the idea of using phages as agents to combat bacterial infections in human patients.¹⁷³ The “modern” era of phage research began in about 1940 when a diverse group of physicists and biologists who shared an interest in understanding the nature of the gene began to use phages as their experimental model system in this quest. This approach was wildly successful, playing a dominant role in the development of the field of molecular biology and leading over a period of ~35 years to much of our current understanding of not only the nature of the gene but also the ways that the information in genes is expressed and how that expression is regulated. Along the way, work on phages also made fundamental contributions to the field of virology. Phage research during this time was concentrated on a small number of phages, with the result that we now have a very deep understanding of a handful of individual phages. A crowning achievement of this era of phage-dominated molecular biology was the development in the early 1970s of the methods of recombinant DNA. This made many of the overwhelming technical advantages of phages as experimental

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systems available for other organisms, including eukaryotes, and the center of gravity of molecular biology research shifted away from phages, though important basic research on phage life cycles has continued to the present. In recent years new themes have come to the fore in phage research that relate to natural phage populations and their effects on the environment and to phage evolution, including the influence of the phage population and its evolution on the evolution of their host bacteria.⁴⁹ These studies are inspired and made rational by the numbers cited in the first paragraph of this chapter, and they are made possible by the development of high-throughput sequencing technology and by the availability of the detailed knowledge of the biology of phages developed over the past 70 years.⁷² Studies on the assembly and structure of phage virions have had a central role in structural virology and have given insight into biological structure and assembly more generally.⁸⁸ Finally, throughout the history of phage biology there has been interest in the application of phages and their components to practical problems, and this has continued to the present, including but certainly not limited to a renewed interest in phage therapy.⁷¹

This chapter will first consider the main features of the life cycles of the different groups of phages that have been identified, essentially summarizing the detailed studies of individual “model” phages with an emphasis on what general principles of viral lifestyle are illustrated by each. For some of these, particularly the dsDNA tailed phages, there are enough examples of different phages in the group that we can begin to identify which features of the life cycles are universal among the group and which are either “optional” or accomplished by different mechanisms by different phages. We will then consider questions about phage populations and phage evolution—questions that are informed by the very large numbers cited in the first paragraph of this chapter.

Known bacteriophages have been divided into 10 families in the taxonomy of the International Committee on the Taxonomy of Viruses (ICTV)³ (Table 75.1). Three of these are somewhat arbitrary divisions (based on tail morphology) of the order *Caudovirales*, the dsDNA tailed phages. The other seven families each encompass a group of viruses with

common ancestry apparently shared among the group but not shared between the groups. In that phylogenetic sense the *Caudovirales* order and each of the other seven families can be regarded as parallel categories and roughly equivalent to the families defined for viruses with eukaryotic hosts. Nonetheless, the tailed phages (*Caudovirales*) are special in the sense that they are likely the numerically most abundant group of viruses on the planet and almost certainly the most genetically diverse. They are also the most extensively studied group of phages and so will occupy most of the space in what follows.

CAUDOVIRALES: dsDNA TAILED PHAGES

The dsDNA tailed phages are tremendously diverse genetically, to the extent that a majority of the protein sequences encoded in a newly determined phage genome sequence typically fail to make any matches in a database search.^{32,72} Genome sizes of known tailed phages range from a low of less than 19 kilobase pairs (kbp) up to just under 500 kbp, a range of more than 25-fold.⁷⁵ As will become evident, they are also very diverse in the specifics of their life cycles, including how they interact with the host cell. On the other hand, the similarities these viruses share across this range of diversity are so extensive as to leave no doubt that they share (distant) common ancestry, at least for the genetic features like the capsid protein genes that they all have.⁸⁰

The head of a tailed phage consists of a protein shell based on icosahedral symmetry that is usually a rather straightforward manifestation of the principles of capsid structure described by Caspar and Klug.³⁷ There is no lipid membrane. Replicated dsDNA is pumped into the empty protein shell through a 12-subunit grommet-like structure at one vertex of the icosahedral shell, known as the *portal*, or sometimes the *connector*. DNA is packaged in these viruses to an unusually high density, roughly the same as the density in a DNA crystal.¹⁰¹ Following DNA packaging, the portal, together with some adaptor proteins, serves as the attachment point for the tail.¹⁴⁷ The morphology of the tail is quite variable among different tailed phages, but it always has the same functions, namely, to attach to the surface of the host cell and serve as a conduit for delivering the DNA into the cytoplasm of the cell. The bulk of the virion protein is left on the outside of the cell after DNA injection, a circumstance that made possible the classical Hershey/Chase experiment⁸¹ that showed that the genetic material is DNA and not protein. Once the DNA arrives in the cytoplasm, the genetic diversity among different phages alluded to earlier is manifested as diversity in the details of their life cycles, and specific examples of the life cycles of a few well-studied phages are given later. At a fundamental level, these phages can be divided into “virulent” and “temperate” categories. Virulent phages always enter the lytic life cycle, in which infection always leads to production of progeny virions and their release by cell lysis, with cell death. Temperate phages, in contrast, make a “choice” soon after infection between lytic growth and entering the lysogenic cycle, in which gene expression from the phage DNA is largely repressed and the phage becomes a part of the genetic complement of the host cell—now called a *prophage*—and

TABLE 75.1 ICTV-Designated Phage Families

Family	Prototypes	Characteristics
<i>Siphoviridae</i>	λ	dsDNA, long, noncontractile tails
<i>Myoviridae</i>	T4	dsDNA, contractile tails
<i>Podoviridae</i>	T7	dsDNA, short, stubby tails
<i>Tectiviridae</i>	PRD1	dsDNA, linear 5' proteins, internal membrane
<i>Microviridae</i>	ϕ X174	ssDNA, circular, icosahedral
<i>Inoviridae</i>	M13	ssDNA, filamentous
<i>Leviviridae</i>	MS2, Q β	ssRNA, small icosahedral
<i>Cystoviridae</i>	ϕ 6	dsRNA, segmented, enveloped
<i>Corticoviridae</i>	PM2	dsDNA, circular, internal membrane
<i>Plasmaviridae</i>	L2	dsDNA, circular, enveloped

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

replicates as part of the cellular DNA without killing the cell for an indefinite number of generations.

In the following sections we consider in moderate detail the life cycles of two very well-studied tailed phages, λ and T4, both phages of *Escherichia coli* and possibly the most extensively studied viruses of any kind. These two have served as important model experimental systems since the 1940s and 1950s. They were the initial source of much of our understanding about the nature of tailed phages, and their numerous differences highlight the diversity seen among the well-studied tailed phages. There are numerous other tailed phages that have made similarly important contributions to our understanding, and we have chosen several of them to illustrate features of their life cycles that are not represented in the two phages that are discussed in detail. Some of the topics considered here have been the subjects of intensive study by multiple investigators for 50 years or more, and in such cases it may not be possible to cite all the relevant primary literature; if so, useful reviews will be cited that give access to the primary literature.

Bacteriophage λ and the “Lambdoid” Phages

The K12 strain of *E. coli* was isolated from a pediatric patient at Stanford University Hospital in 1922. Some 30 years later, in the course of bacterial genetics experiments with K12, Lederberg and Lederberg¹⁰⁵ discovered a temperate phage that was carried as a prophage in the K12 genome, which they named λ . Phage λ subsequently became the most extensively studied of a small number of temperate phages that were chosen for experimental investigation. Other phages that are similar to λ in gene organization and lifestyle, and which can form recombinants with λ at reasonably high frequency, have been

termed *lambdoid* phages.²⁶ Some of the lambdoid phages have been studied in their own right, and all of them and their hybrids with λ have had an important role in illuminating some of the most fundamental aspects of the temperate phage lifestyle.⁷⁷

Phage λ 's dsDNA genome is 48,503 bp in length (Fig. 75.1).^{78,164} The DNA molecule in the virion has its ends at discrete sequences, and there are 12 base single-stranded 5' extensions at the ends. The extensions are complementary in sequence, allowing the linear molecule to circularize by base pairing between these “cohesive ends,” which it does immediately upon being injected into the host cell. The two joined ends are then covalently joined by the host enzyme, DNA ligase.¹⁹⁵

The λ virion has an isometric head (capsid) with T = 7 icosahedral symmetry and a long noncontractile tail (Fig. 75.2). It infects its *E. coli* host by binding to the surface of the cell first through its long “side tail fibers,” which bind to the outer membrane protein OmpC, and then through an interaction between the host outer membrane protein, LamB, and the phage protein, gpJ, located at the tip of the tail.¹⁸⁹ Only this second interaction (gpJ:LamB) is essential for successful infection; the side tail fibers, which make adsorption kinetics faster, were lost through a mutation early in the laboratory history of λ .⁷⁸ (Note on nomenclature: in the phage literature, gpX refers to the protein encoded by gene X, with “gp” meaning “gene product” and *not* “glycoprotein.”) Details of the DNA injection process are not well understood but are thought to include a rearrangement of some of the proteins that make up the tail tip to facilitate passage across the cell envelope, a process for which there is experimental support in some other phages but not yet in λ . One thing that must

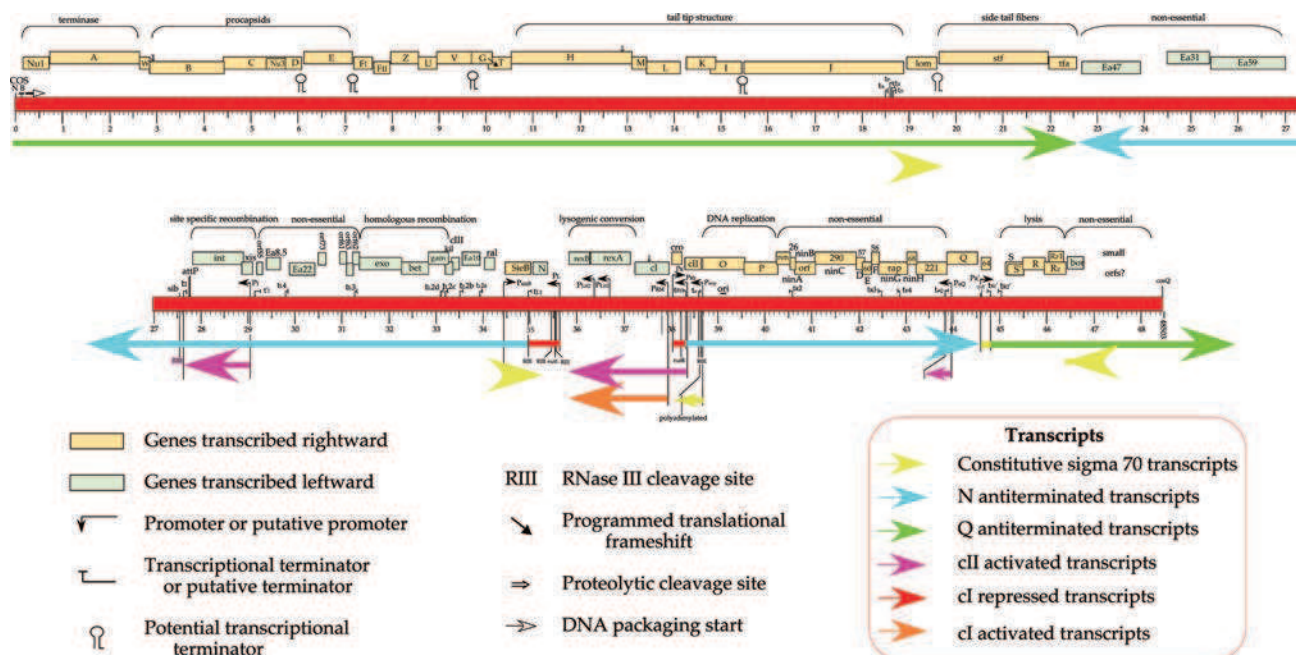


FIGURE 75.1. Map of the phage λ genome. λ DNA is represented as the red bar with genes shown as boxes above; functions are indicated.

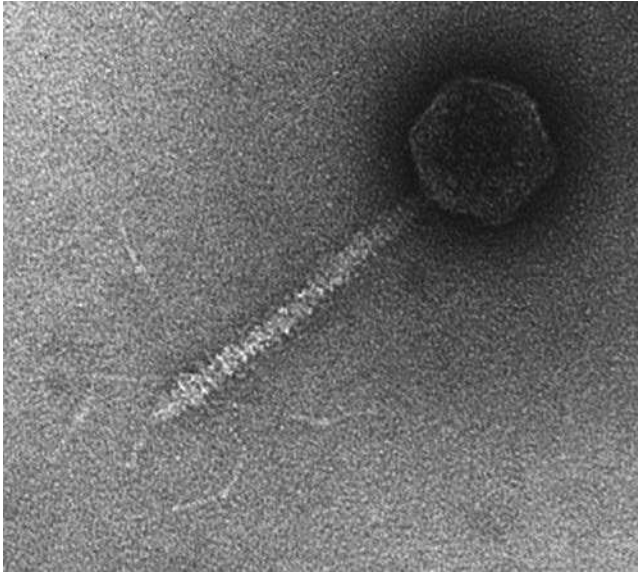


FIGURE 75.2. The bacteriophage λ virion. Electron micrograph of λ , negatively stained with uranyl acetate. Scale: the length of the tail, excluding fibers, is ~ 150 nm. (Courtesy of Robert Duda.)

happen before the DNA can pass out of the head, down the tail tube, and into the cell is that the tail length tape measure protein, gpH, a few copies of which occupy much of the lumen of the tail, must exit. There is biochemical evidence that gpH is injected into membrane vesicles if the membranes contain the LamB receptor protein,¹⁵⁸ and there is genetic evidence that gpH must interact with an inner membrane sugar transport protein, PtsM, in order for injection to be accomplished successfully.¹⁵⁷

Following DNA injection and circularization, λ makes a decision between the lytic and lysogenic life cycles. We first describe the lytic cycle, in which the phage programs the cell to produce a crop of progeny virions and releases them by cell lysis at the end of the growth cycle. Following that, we describe the lysogenic cycle, in which the expression of most phage genes is repressed and the phage DNA integrates into the continuity of the host chromosome as a prophage.

The Lytic Cycle

All transcription of λ genes is carried out by the host RNA polymerase, unmodified for immediate early transcription and altered in its properties by interaction with phage-encoded proteins for subsequent temporal classes of transcripts.⁶⁶ The genes are generally arranged in large operons, making it possible to regulate their transcription by regulating a small number of promoters. Upon DNA entry, RNA polymerase initiates transcription at the two divergent immediate early promoters, P_L and P_R , completing transcription of just one gene in each case, gene *N* and gene *cro*, respectively, before encountering a ρ -dependent terminator and stopping.¹³³ The N protein, gpN, is a transcription antitermination factor that renders polymerase insensitive to termination signals by forming a complex with polymerase, several host factors, and a specific signal near the start of

the nascent transcript called *nut*, for Nutilization. The RNA polymerase, altered in this fashion, now reads through the terminators and into early genes farther down the P_L and P_R controlled operons. The most relevant of these genes for the lytic cycle are the *O* and *P* genes, which mediate phage-specific DNA replication, and the *Q* gene, which has a central role in mediating late gene transcription, all transcribed from the P_R promoter, and three genes for recombination enzymes, transcribed from P_L .⁴⁵ An important feature of the *nut* sites is that they only act *in cis*—that is, gpN can cause antitermination only for a polymerase that starts from a promoter with a *nut* site near the beginning of the transcript, which in λ means only P_L and P_R . A consequence of this is that some transcribing polymerases can be insensitive to termination signals at the same time that others are susceptible to termination, depending on which promoter they started at. This differential sensitivity to termination signals figures in the regulation of the proteins responsible for integration and excision of the prophage, described later.

Transcription of the late genes starts about 10 minutes after the start of infection, from the P_R' promoter located downstream from the *Q* gene (Fig. 75.1). The P_R' promoter is a strong promoter, recognized by the unmodified host RNA polymerase, but the polymerase requires the action of the early phage protein gpQ to read through a strong pause site located soon after the promoter.¹⁵⁵ As with polymerase that has been acted on by gpN, polymerase acted on by gpQ is insensitive to termination signals and reads through the entire ~ 26 -kb late gene operon, which includes the lysis genes, head genes, and tail and tail fiber genes. Despite the fact that all of the late genes are transcribed equally, the molar amounts of the proteins produced by translation of the messenger RNA (mRNA) vary more than 1,000-fold due to differences in efficiency of translation initiation.³⁴ The amounts of the proteins produced appear to be appropriate to what is required for efficient assembly of virions.

Phage DNA replication is carried out by the host replication machinery, but that machinery is directed to the phage replication origin by two phage-encoded early proteins, gpO and gpP. gpO binds to the origin, four tandem imperfect 19-bp repeats within the gene *O* coding region, to form a compact complex to which gpP binds.¹⁸⁸ gpP recruits the host replication helicase, DnaB, to the complex; the rest of the host replication machinery joins; and gpO and gpP are removed by the host chaperones, DnaK, DnaJ, and GrpE, which allows the replication complex to start replication.¹⁴³ Replication is initially bidirectional, producing more unit-length circles by a “theta” mode of replication, but at 10 to 12 minutes after infection it shifts to a unidirectional rolling circle mode to produce the multigenome concatemer that is the DNA substrate for packaging into heads.

The heads and tails of phage λ are assembled separately and join to form virions after their assembly is complete.⁷³ Purified heads and tails will join efficiently *in vitro* to make infectious virions, implying that no additional factors are required for this step of assembly. Tail assembly starts with a complex of the proteins that will form the tip (head-distal) end of the tail. The initial complex is joined by a few copies of the *tape measure protein*, gpH, which extends the length of the tail as an α -helix and provides a template around which

the major tail protein, gpV, polymerizes as 32 stacked rings of 6 subunits each.⁹² This polymerization is thought to be mediated by two proteins, gpG and gpGT, which are essential for assembly but absent from the mature tail, and which are therefore regarded as tail assembly chaperones. The two chaperones are produced as the result of an inefficient translational frameshift between two overlapping open reading frames, an arrangement that is highly conserved among other long-tailed phages.¹⁹⁶ Following assembly of the tail tube, two adaptor proteins add to the top of the tail and it is ready to join to a head.

As with all other characterized tailed phages, λ head assembly first produces an empty protein shell—the prohead or procapsid—into which DNA is packaged. Four phage-encoded proteins participate in prohead assembly; the major capsid protein, gpE; the portal protein, gpB; the protease, gpC; and the scaffolding protein, gpNu3.³⁸ The details of how these proteins come together to assemble accurately into a $T = 7$ capsid are not entirely clear, but the assembly is thought to be nucleated by the portal, a 12-subunit grommet-like structure that occupies one vertex of the otherwise icosahedrally symmetric prohead.¹⁴⁷ Assembly of the major capsid (coat) protein requires the participation of similar numbers of the scaffolding protein. How the scaffolding protein contributes to this process remains the central mystery of how capsid assembly is achieved. In the absence of scaffolding protein, the capsid protein assembles into “monsters,” sheets, tubes, and misshapen spirals of capsid protein that have apparently lost the ability to insert pentamer vertices into the hexagonal lattice of capsid proteins with sufficient accuracy and regularity to make an isometric, icosahedrally symmetric structure.

Roughly 10 copies of the protease are assembled into the prohead, and these are responsible for cutting the scaffolding protein into fragments that are lost from the structure. The portal subunit is also proteolytically processed, with 22 amino acids removed from the N-terminus, but unlike what is observed in some phages, the major capsid protein is not processed. The protease itself participates in a curious “protein fusion” reaction, in which a portion of each protease molecule is joined covalently to a copy of the major capsid protein.¹²⁰ Following, or concomitant with, the proteolytic maturation of the prohead, DNA packaging commences. DNA is pumped into the procapsid through the ring of portal protein subunits by a phage-encoded, adenosine triphosphate (ATP) cleavage-powered DNA translocase similar to that of phages T4 and $\phi 29$ (see later). This molecular motor also contains the nuclease that cleaves *cos* sites to release the packaged DNA from the concatemer that is the substrate for packaging.

Progeny λ particles are released from the cell through a tightly controlled lysis system that ensures the concluding act of lytic growth occurs at the optimal time.¹⁸⁶ λ lysis requires the products of four genes, *S*, *R*, *Rz*, and *RzI*. gpR is the endolysin and cleaves the peptidoglycan layer in the cell wall, but it has no effect on the integrity of the cell until it has access to its substrate, which is prevented by the cytoplasmic membrane. Access, and the timing of lysis, is determined by the products of gene *S*, the 105-residue holin (S105), and the 107-residue antiholin that is translated from an initiation site two codons upstream of that used for S105. The ratio of S105 and S107

is critical for the timing of lysis, which occurs when S105 produces one or more large-sized holes in the membrane.⁵⁴ Rz and RzI form a *spannin* complex that spans the periplasm and leads to fusion of the inner and outer membranes to ensure efficient and complete lysis.¹⁵

Lysogenic Cycle

The lysogenic cycle starts the same way as the lytic cycle described earlier, with DNA injection and circularization, transcription from P_L and P_R , extension of that transcription by the action of the gpN antitermination protein, and the consequent expression of the early proteins. Among these early proteins are CII (“C-two”), transcribed from P_R , and CIII (“C-three”), transcribed from P_L .⁴⁵

The CII protein plays the central role in the “decision” between the lytic and lysogenic cycles. CII is a transcription factor that binds to and activates the leftward-pointing promoter P_{RE} (*promoter for repressor establishment*). The resulting transcription from P_{RE} goes backward through the *cro* gene and then forward through the leftward-oriented *cI* gene, causing production of the λ repressor, the CI (*C-one*) protein. When it is produced in sufficient quantity, the CI repressor shuts off further transcription from P_L and P_R by binding to the overlapping operators, O_L and O_R , respectively, and occluding the promoters from RNA polymerase binding. In this way repression is established and can persist for an indefinite number of bacterial generations.

The choice between the lytic and lysogenic cycles is determined by the level of CII protein in the infected cell—with enough CII, transcription from P_{RE} is activated sufficiently to produce enough CI repressor to establish repression, while with low levels of CII, too little CI repressor is made to stop transcription from P_L and P_R , and the lytic cycle goes forward as described earlier. The level of CII protein is determined in turn by environmental factors, principally the physiologic state of the infected cell. The readout of the state of the cell is the level of a cellular protease, FtsH, which degrades CII, and is modulated by HflKC and cyclic adenosine monophosphate (cAMP).⁹³ In this sense, CII is the sensor with which the phage assesses the state of the cell and decides between the lytic and lysogenic cycles. Another phage protein, CIII, biases the decision in favor of lysogeny by antagonizing the FtsH protease and disfavoring degradation of CII protein.⁹⁵ Finally, a high multiplicity of infection favors lysogeny; this is thought to be because a larger number of genomes in the cell means higher production of CII and CIII proteins. More recently, it has been shown that λ preferentially binds to the cell poles—especially at the low multiplicities of infection favoring lytic growth—where FtsH is also localized, suggesting there is a topologic component to the lytic/lysogenic decision.⁵⁸

Once lysogeny is established, expression of the CI repressor maintains the prophage state (Fig. 75.3). CI binds to two tripartite operator sites, O_L and O_R , to repress the early lytic promoters, P_L and P_R , respectively.¹⁰⁹ However, CI also plays an important role in regulating its own expression, acting as an activator of the promoter for repressor maintenance (P_{RM}) when bound to the O_{R1} and O_{R2} sites in O_R , and as a repressor when concentrations are sufficiently high to permit binding to the weaker site, O_{R3} . An important component to

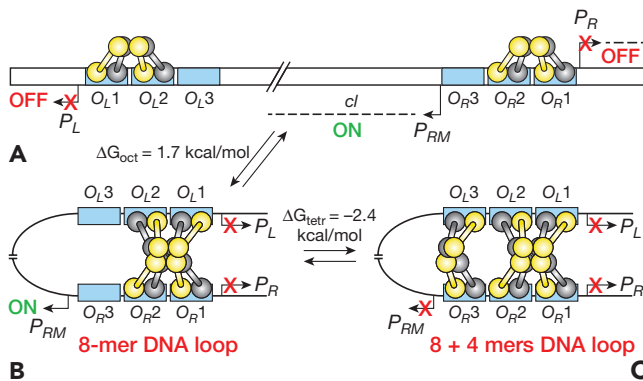


FIGURE 75.3. DNA looping by the λ repressor. Models of CI regulation by DNA looping. Detailed conformations of the structures are not known and maps are not drawn to scale. **A:** Promoters (P_R , P_L , and P_{RM}); operators O_L (O_L1 , O_L2 , O_L3) and O_R (O_R1 , O_R2 , O_R3) are in blue rectangles; CI dimers (one monomer is shown in yellow, the other in gray). The bent arrows show the transcription start points of promoters. The dashed line indicates transcripts from P_R , P_L , and P_{RM} . The cl gene is transcribed from P_{RM} . **B:** DNA looping and octamer formation (8-mer) by CI tetramer binding to $O_L1 \sim O_L2$ interacts with that at $O_R1 \sim O_R2$. **C:** Octamer and tetramer (12-mer) of CI binding to O_L and O_R . Red X means promoter is turned off. (From Lewis D, Le P, Zurla C, et al. Multilevel autoregulation of lambda repressor protein CI by DNA looping in vitro. *Proc Natl Acad Sci U S A* 2011; 108:14807–14812, with permission.)

repression is the formation of DNA loops formed by cooperative association of repressor tetramers bound at O_{L1} and O_{L2} , and at O_{R1} and O_{R2} ; this looping also stimulates activation of P_{RM} .⁵ Additional cooperative interactions between O_{R3} and O_{L3} enhance the repression of P_{RM} at high repressor concentrations.¹⁰⁹

The DNA replication genes of a λ prophage are repressed by the action of the CI repressor protein, and the prophage relies on the host replication functions for maintenance in the population as the cell grows and divides. To take advantage of these replication services, the prophage DNA must be integrated into (becomes part of) the host chromosome where it is replicated along with all the other host genes (Fig. 75.4). Integration is catalyzed by the phage-encoded integrase protein (Int), which carries out site-specific, reciprocal recombination between a site on the phage DNA, *attP* (attachment site, phage), and a site on the bacterial chromosome, *attB* (attachment site, bacterium). Because the phage DNA is at this point circular, having joined its cohesive ends immediately upon injection, the Int-mediated reciprocal recombination results in the insertion of a linear form of the phage DNA into the bacterial chromosome. Because the *attP* site, where the phage DNA is broken upon integration, is distant from the *cos* (cohesive ends) site, where the virion DNA was joined, the order of genes in the integrated prophage is a circular permutation of the gene order in the virion DNA. This mechanism of prophage integration, generally known as the *Campbell Model*, seems rather straightforward now; when it was proposed in 1962 by Allan Campbell (the author of this chapter in the previous edition), it was a major conceptual breakthrough.²⁷

The *attP* site has a 15-bp “core” sequence that is identical to the corresponding core sequence in *attB*, and it is within the core sequence that the strand exchanges of recombination take place.¹⁰² *AttP* extends on both sides of the core sequence and requires ~240 bp in total. These flanking sequences contain binding sites for Int; for integration host factor (IHF), a host-encoded DNA binding/bending protein; and for Xis and Fis (see later). The *attB* site is only 30 bp long, having fewer such binding sites flanking its core sequence.¹⁰² Integrative recombination takes place within a synaptic complex formed when an *attP*-intasome—containing Int and IHF—captures *attB* DNA.¹⁵³ The reaction proceeds with cleavage of the “top” strands of the two attachment sites by Int and reattaching them to the cut strand of the opposite *att* site to form a Holliday junction.¹⁰² The energy of the phosphodiester bond is preserved by two successive transesterifications, first to the active site tyrosine of the integrase and then to the recipient DNA strand, and no additional chemical energy is needed to drive the reaction. Following the top-strand exchange, the complex branch migrates by 7 bp, and bottom-strand exchange takes place by the same mechanism. The reaction product is an integrated prophage flanked by two hybrid attachment sites, *attL* and *attR*.

The lysogenic “cycle” is completed when, after an indefinite number of generations as a repressed prophage, the prophage enters the lytic cycle by the process known as induction. The proximate cause of induction is loss of repression. Induction occurs in about one cell in 10^4 to 10^6 in a culture of lysogenic cells under laboratory conditions (*spontaneous induction*), but it occurs in nearly 100% of the cells in the culture when they are activated by a moderate dose of ultraviolet light or similar DNA damaging agent. Such treatment causes high production of the cell’s RecA protein, which when bound to single-stranded DNA (ssDNA) causes the CI repressor to undergo an autoproteolytic cleavage. This cleavage separates the protein’s dimerization domain from its operator-binding domain,⁴⁶ and as monomers, the CI operator-binding domains do not have a high enough affinity for their operators to block transcription from P_L and P_R , and so the lytic cycle begins.

An essential part of λ prophage induction is the detachment of the prophage from the host chromosome, termed *excision*. Macroscopically, excision is the reverse of integration—that is, the *attL* and *attR* sites at the ends of the prophage undergo reciprocal recombination to release the circular phage DNA molecule. Excisive recombination requires two protein factors in addition to the Int and IHF needed for integration, namely, the host DNA binding/bending protein Fis and the phage-coded Xis (pronounced “excise”) protein. Together, these four proteins and the *attL* and *attR* sites form the excision complex.

Both integration and excision require the catalytic action of integrase, but whether the reaction goes in the direction of integration or excision depends on the absence or presence of Xis (Fig. 75.4). Accordingly, the amount of Xis is differentially regulated by a mechanism that senses whether or not the phage DNA is integrated; if integrated, both Int and Xis are made, allowing excision, and if not, only Int is made, allowing integration. The crucial regulatory site is a sequence named *sib*, located downstream from and across *attP* from the *int* and

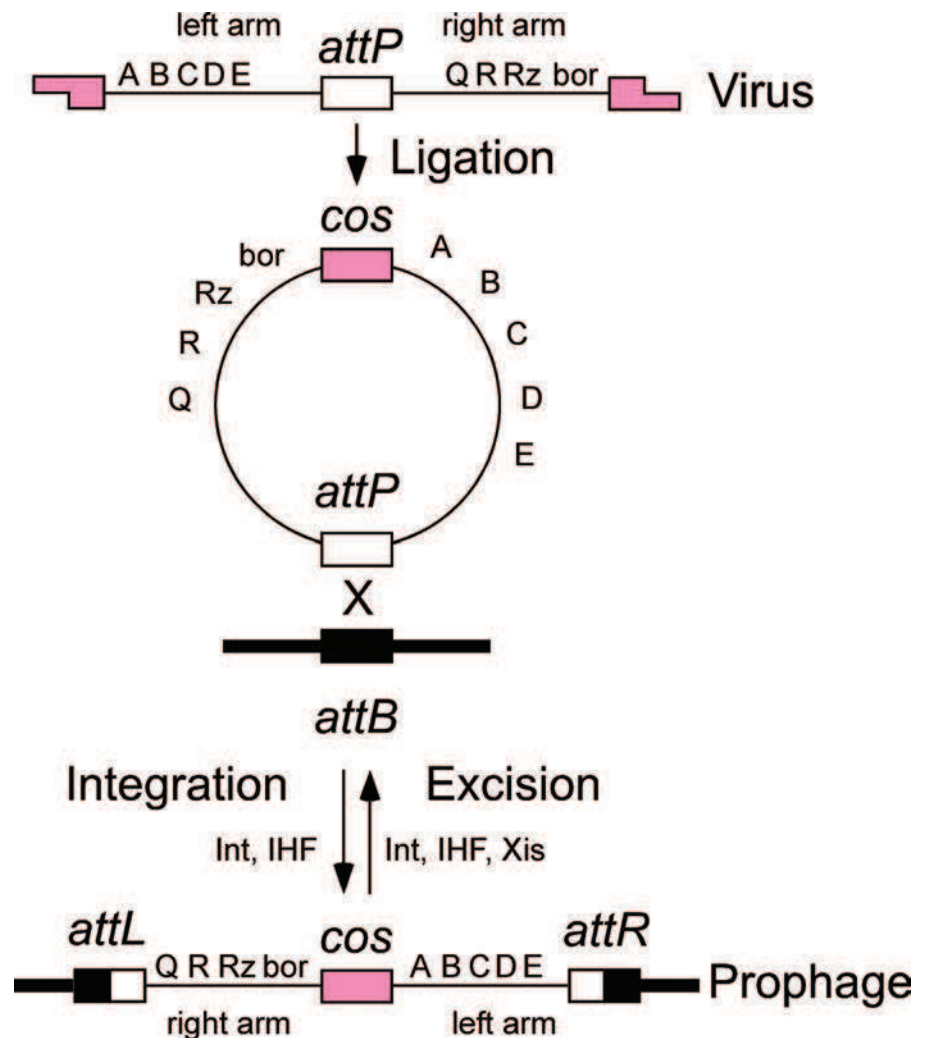


FIGURE 75.4. Insertion of λ DNA into the bacterial chromosome. λ virions contain linear DNA with short single-stranded cohesive ends that are ligated to circularize the genome following injection into the cell. *attP* is located near the center of the genome and the order of some of the genes (A, B, C, etc.) is shown. A prophage is formed by site-specific recombination between the phage attachment site (*attP*) and the bacteria attachment site (*attB*), which is catalyzed by the phage-encoded integrase but also requires integration host factor (IHF). Prophage excision occurs only in the presence of the phage-encoded Xis, which redirects integrase to recombine the attachment junctions *attL* and *attR* to re-create *attP* and *attB*. The virion structure and assembly genes are typically represented as forming the left arm (between *cos* and *attP*) in the viral genome but are permuted relative to the right arm (between *attP* and *cos*) in the prophage.

xis genes.¹¹⁷ *Sib* has the effect of destabilizing mRNA transcribed from P_L (antiterminated by gpN), which includes the sequences of both *xis* and *int*, but it does not destabilize mRNA from P_i , a CII-dependent promoter within the *xis* coding sequence that encodes only Int. Thus, when *sib* is downstream from *xis* and *int*—that is, when the phage DNA is not integrated—only Int protein can be made and so (provided sufficient CII is made to activate P_i and the phage is headed into the lysogenic cycle) integration ensues. In the integrated prophage, on the other hand, *sib* is no longer downstream from *xis* and *int*, as a result of the integrative recombination. When the prophage is induced, the transcript from P_L , encoding both Xis and Int, is not destabilized, so both proteins are made and excision occurs. Perhaps surprisingly, this elegant and efficient mechanism has been seen only in λ and a few of its closest relatives; there appears to be a great variety of different pathways for achieving the appropriate regulation of prophage integration and excision that have evolved in different temperate phages.

Bacteriophage T4

Bacteriophage T4 is one of seven “type” phages isolated by Delbrück in the 1930s and 1940s that he declared warranted further study.²⁵ It has received considerable attention from that time to the current day and continues to be a source

of new insights into virology, molecular biology, and structural biology. It is virulent and reproduces quickly (less than 30 minutes), and particles with prolate (i.e., elongated) heads and contractile tails contain a dsDNA genome of ~170 kb (Fig. 75.5). T4 is not alone in the biosphere, and many phages with similar features have been identified, including two other Delbrück “type” phages, T2 and T6, and phages of other *Proteobacteria* and of the *Cyanobacteria*. These contain genomes ranging from 160 kbp to 250 kbp and range from those with close DNA similarity to T4 to those much more distantly related. Although these relatives contain a “core genome” of about 12% to 15% of T4 that are typical of this group, they are otherwise mosaic with common segments interspersed with unrelated genes.¹⁴⁰ Although we will focus here on T4 as the most intensively studied of these, many of the other T4-like phages are likely to share much of its biology.

T4 Genome

The T4 genome contains a unique sequence of 169,903 bp, and virion genomes are circularly permuted with terminal redundancies of ~5,000 bp¹²⁴ (Fig. 75.5). It codes for 160 proteins with known functions, as well as about 120 open reading frames (ORFs) with unknown functions, and several small RNAs, including 8 transfer RNAs (tRNAs). Some parts of the

GENOMIC MAP OF BACTERIOPHAGE T4

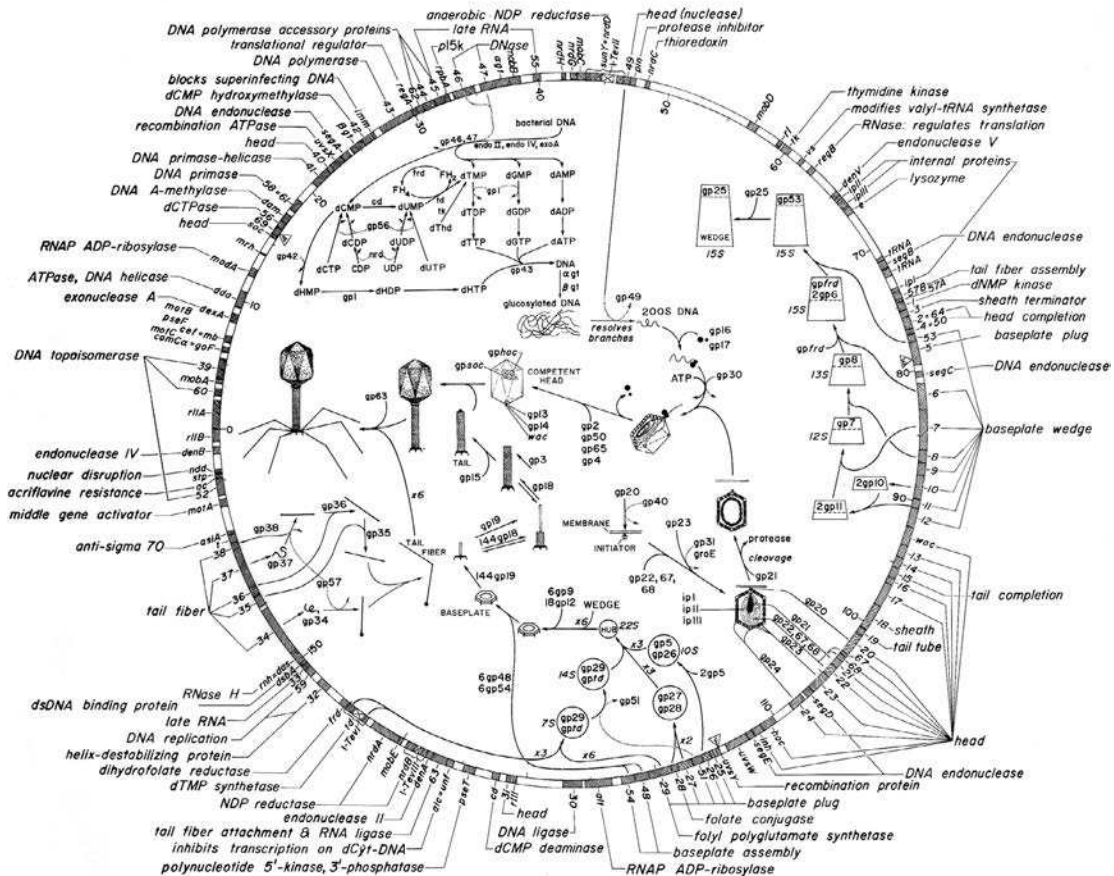


FIGURE 75.5. Genomic map of phage T4. The T4 genetic map is circular rather than linear because of the circular permutation of T4 phage chromosomes due to its headful packaging from long concatemers. The general structure of the map was established by standard genetic techniques using classical markers such as those affecting plaque morphology, as well as *amber* mutations that were used to identify many genes. This map shows many of the known and all of the essential genes, based on that classical mapping work and on genome sequence data; the small numbers just inside the circle show the coordinates of the genomic map in kilobase pairs. The names and functions of many genes and the grouping of genes into clusters of related function are shown on the periphery. The branched assembly map in the center shows how an efficient complex of enzymes is produced to make the deoxynucleotides and feed them directly into the T4 DNA polymerase complex (**upper left region**) as well as the branched virion assembly pathway, illustrating how components made in the various branches of the pathway merge to produce an infectious bacteriophage. The small numbers just inside the circle show the coordinates of the genomic map in kilobase pairs. This version of the T4 assembly genomic map is a reduction of a 3-foot-wide hand-drawn map representing an icon in bacteriophage biology. (Courtesy of B. Guttman and E. Kutter. Modified from Karam JD, Kreuzer KN, Hal DH. *The Molecular Biology of Bacteriophage T4*. Washington, DC: ASM Press, 1994, with permission.)

genome are predicted to be mobile, and there are at least 15 homing endonucleases, 3 of which are within introns.⁵⁹ T4 DNA is somewhat unusual in that it contains glucosylated hydroxymethylcytosine (hmC), which protects it from host restriction systems and distinguishes it from host DNA that can be degraded and utilized for phage replication.

Expression and Regulation

Expression of T4 genes follows an orderly series of temporally controlled patterns, all involving the use of the host RNA polymerase.⁸² However, a complex series of both covalent and noncovalent modifications to RNA polymerase are required to successfully orchestrate this transcriptional ballet. Transcription of the first T4 genes involves recognition of promoters resembling strong host promoters by σ^{70} -associated

host RNA polymerase, but two phage-encoded early products make important modifications to the RNA polymerase. Alt (a protein that is injected with the DNA into the infected cell) catalyzes adenosine diphosphate (ADP) ribosylation of one α subunit of RNA polymerase, improving the activity of certain T4 early promoters. Two other T4 proteins, ModA and ModB, also ADP-ribosylate RNA polymerase or proteins used in translation during early stages of infection. Another protein, Alc, leads RNA polymerase to selectively terminate transcription prematurely on the host dC-containing DNA while ignoring the viral hmC DNA.⁸² As a consequence of these events, transcription of T4 genes readily outcompetes that of the host, with a strong burst of activity within the first minute of infection. The functions of many of these early genes are not known, but two early products, MotA and AsiA, play important

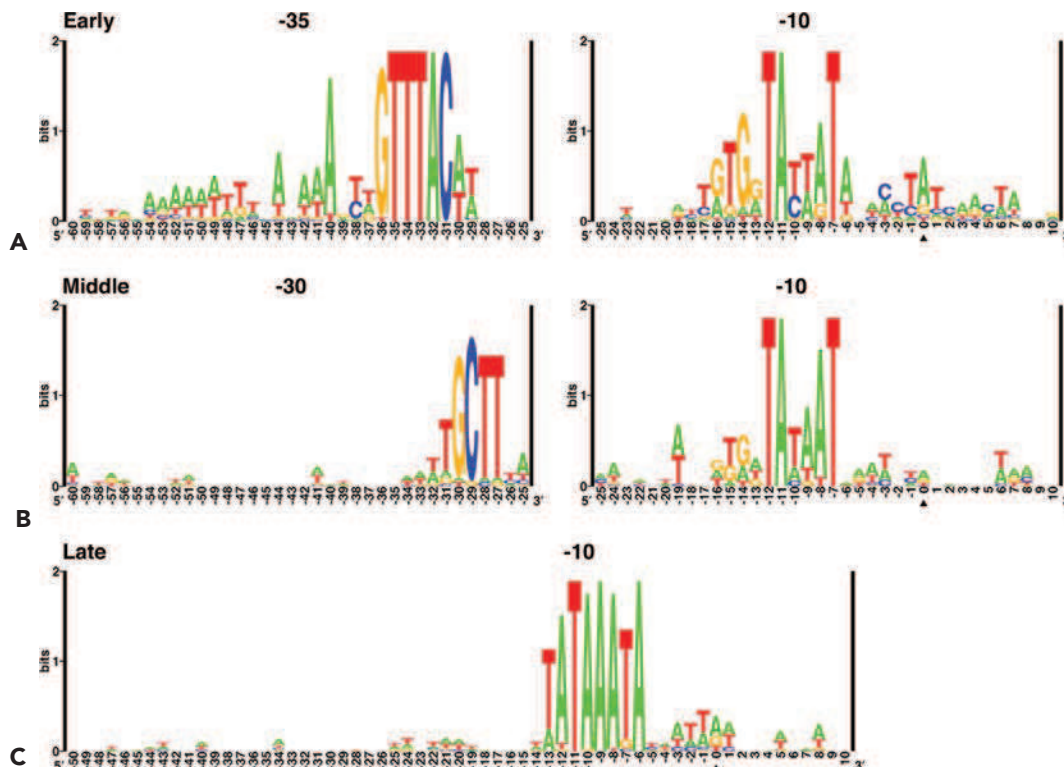


FIGURE 75.6. Logo of T4 promoters. Nearly all the sequences in each alignment have promoter activity, as demonstrated by primer extension, transcription from cloned DNA fragments, or RNA hybridization assays. Those promoters included whose start sites have not been mapped all precede a corresponding early, middle, or late gene and show significant similarity to the relevant promoter class. Sequences were independently aligned in the -10, -30, or -35 regions. The information content (Rs) is calculated in “bits” and is the sum of the Rs for each region (except for the late logo, which was calculated from the single alignment at -10). Alignments, logos, and Rs values were obtained as described elsewhere ((966) and E. Miller, T. Dean, and T. Schneider, unpublished data). The triangle marks the +1 transcription start site. **A:** Thirty-nine early promoters, Rs = 38.3 bits. **B:** Thirty middle promoters, Rs = 21.1 bits. **C:** Fifty late promoters, Rs = 16.2 bits. (From Miller ES, Kutter E, Mosig G, et al. Bacteriophage T4 genome. *Microbiol Mol Biol Rev* 2003;67:86–156, with permission from the American Society for Microbiology.)

roles in the transition from early to middle transcription. Many early T4 mRNAs also disappear quickly at the end of the early phase, largely through the action of RegB, an endoribonuclease that targets early gene transcripts.

MotA is a transcriptional activator that functions along with a co-activator, AsiA, to redirect transcription toward T4 middle promoters. The middle period genes primarily encode functions for DNA metabolism and DNA replication, including *denA* and *denB*, that encode the functions for degrading dC-containing host DNA. About 60 middle promoters have been identified, which contain a host-like -10 sequence (Fig. 75.6) and a MotA box at position -30. MotA binds somewhat weakly to its DNA site but associates strongly with σ^{70} . The co-activator AsiA plays an important role in the transition by also binding to the σ^{70} subunit of RNA polymerase and conformationally altering the helix-turn-helix DNA binding motif within region 4. As a consequence, recognition of promoter -35 sequences is interrupted (an “antisigma” activity), reducing transcription from both host and T4 early promoters. Thus, MotA and AsiA collaborate to effectively direct the host σ^{70} to initiate transcription at the unique promoters preceding T4 middle genes.

The transition from early to middle gene expression is also mediated by extension of early T4 transcripts, as many middle

genes lie immediately downstream of early genes (Fig. 75.6). Termination of the early transcripts likely occurs at Rho-dependent sites; however, readthrough or antitermination at these sites can give rise to some middle gene expression. However, the antitermination mechanism is unclear. The process could involve an as yet unidentified antiterminator factor (equivalent to λ N or Q; see earlier) or could simply be the consequence of transcription and translational coupling, such as seen when closely linked genes in bacterial operons overcome genetic polarity.⁸²

The transition from middle to late T4 gene expression requires the products of genes 33 and 55.⁶⁵ T4 gp55 is a highly divergent member of the σ^{70} group of proteins and associates with host RNA polymerase to redirect it toward late promoters, which are characterized by an eight-base pair TATA box (5'-TATAAATA-3') at the position where the -10 site is usually located (Fig. 75.6). There are about 50 related sites in the T4 genome, and many are used to direct late transcription. T4 gp33 binds to the flap tip of the RNA polymerase β subunit, which usually interacts with the region 4 of σ^{70} such that gp33 mimics this association. T4 gp33 does not recognize a specific DNA sequence (there is no -35 region in T4 late promoters), but it may function by diminishing nonspecific DNA interaction, thus increasing the specific affinity of gp55–RNA polymerase for T4 late promoters.⁶⁵

Interestingly, there is a close linkage between T4 late transcription and DNA replication. T4 gp45, the sliding clamp processivity factor for DNA replication, is strongly implicated in late transcription. It forms “split rings” that load onto DNA with a particular polarity (depending on how the DNA is nicked) and track along the DNA. T4 gp45 and the late transcription factor gp55 associate such that the sliding clamp facilitates the search for promoters. gp33 is also required for replication-activated transcription at T4 late promoters.

Studies on T4 gene expression also contributed many seminal findings in the area of postranscriptional control. These include the identification or discovery of translational stop codons, discovery of suppressor tRNAs, tRNA cleavage and subsequent repair by polynucleotide kinase and RNA ligase, protein-mediated translational repression, and group I autocatalytic introns (“ribozymes”). Details of these processes in T4 and T4-related phages are reviewed in Edgell et al.⁵⁹ and Uzan and Miller.¹⁸²

Genome Replication

Soon after T4 infection, synthesis of DenA and DenB targets host DNA for degradation to mononucleotides, followed by subsequent conversion of deoxycytidine monophosphate to deoxyhydroxymethylcytosine monophosphate. This is incorporated into T4 DNA during its replication, and the deoxyhydroxymethyl cytosines are subsequently modified by glucosyltransferases. A T4-encoded deoxycytidine-triphosphatase (dCTPase) keeps deoxycytidine-triphosphate (dCTP) levels low, so that dCTP does not get errantly incorporated into the newly synthesized T4 genomes. DNA replication occurs in two distinct modes, one that is origin dependent and one that is recombination dependent.

T4 DNA replication is bidirectional and initiates at as many as seven major origins of replication (*oriAi* through *oriG*) early in infection.⁹⁷ Initiation requires assembly of a replisome containing several T4 proteins (DNA polymerase, sliding clamp loader, sliding clamp, helicase, primase, and SSB) and synthesis of primers by the host RNA polymerase (to form R-loops). RNA primers can arise from the MotA-dependent promoters at the *ori* sites, and for the lagging strand, from the activity of the T4 primase, gp61. These primers are processed by a phage-encoded RNase H. Coordinated leading- and lagging-strand synthesis then proceeds through to the ends of the viral DNA, generating 3' extensions (because of the inability to prime synthesis at the extreme 5' ends of the genomes). Subsequent reprogramming of the host RNA polymerase specificity (see earlier) results in the cessation of this early mode of DNA replication, and a late-expressed protein, UvsW, actively unwinds the RNA–DNA hybrids used for initiation.⁵⁶

The second, late mode of T4 DNA replication is characterized by the dominant role played by homologous recombination and is known as recombination-dependent replication (RDR). The genome ends generated by early (origin-dependent) replication are good substrates and can engage in recombinational pairing with other (co-infecting) genomes or with the other end of the same genome because of the terminal redundancy. Genetic studies suggest that there are multiple pathways by which RDR can proceed, but the primary mode is the formation of D-loops by invasion of an ssDNA end into a DNA duplex, followed either by semiconservative synthesis or synthesis with branch migration.⁹⁷ Several T4 proteins are involved in RDR, including UvsX (a RecA-like protein), gp32 (ssDNA

binding protein), UvsY (which loads UvsX onto gp32-coated DNA), UvsW (a helicase involved in strand invasion and branch migration), gp59 (for loading the helicase), and the replisome complex. The structures of several of these components have been described.¹²⁹ Overall, multiple pathways involving several different phage-encoded enzymes ensure effective replication of the genome. These pathways do not function entirely independently of one another, and thus the T4 system has allowed detailed study of genome replication, recombination, and repair that is germane to both viral and cellular processes.

DNA Packaging

The products of T4 replication are branched, concatemeric networks of DNA in which individual molecules are not distinguishable.¹⁴⁶ Packaging of the DNA into capsids is initiated by the binding of the terminase small subunit protein gp16 to the viral DNA. The small terminase subunit binds as rings (probably 11-mers), and then the gp17 large terminase subunit associates with the protein–DNA complex. This complex associates with the 12-fold symmetric portal protein that sits at a position of one of the capsid pentamers (see later). This “packasome” also contains T4 gp49, a Holliday junction resolvase that removes DNA branches that are otherwise detrimental to packaging. gp17 has two important catalytic domains involved in DNA packaging: an N-terminal adenosine triphosphatase (ATPase) domain and a C-terminal endonuclease domain. Both functions are important for the translocation “motor” activity of the packaging system.

An attractive model for the packaging mechanism is the portal rotation model.⁷⁶ The symmetry mismatch between the 5-fold capsid and 12-fold portal means that only one subunit of each can align in any given register. Activation of the terminase ATPase activity could then lead to rotation of the portal and translocation of the DNA, as if a bolt were turning within a nut.¹⁴⁶ Although attractive, single-molecule experiments with the ϕ 29 packaging system (see later) argue against a portal rotation model,⁸⁶ other models include a terminase-driven packaging system in which cycles of ATP hydrolysis leads to DNA translocation.¹⁷⁴ Regardless of the mechanistic details, the T4 packaging motor is remarkably powerful when scaled to a macro level, being twice as powerful as a typical automobile engine.¹⁴⁶

Once packaging begins, the process continues until the capsid is full of densely packed dsDNA (~500 mg/mL)—typically about 103% of the unique genome content—at which point the nuclease activity of the large terminase subunit is triggered to cleave the DNA. Although the terminase–DNA complex dissociates from the filled capsid–portal complex, it may associate with another empty capsid to begin packaging of the DNA from the previous cleavage event. This *headful packaging* process can then continue for as many unit genome lengths present in the concatemer and is the basis for the circularly permuted, terminal redundancy found at the ends of the packaged genome in each virion.

Virion Structure and Assembly

The lunar-lander-shaped T4 virion is one of the most widely recognized icons of molecular biology, and its complex shape requires a correspondingly complex assembly strategy that uses premade subassemblies. These are capsids, tails, and tail fibers that are each made *via* independent pathways that do not merge until the subassemblies are joined together to make a complete virion after DNA packaging is complete. The general

outlines of T4 assembly were the result of decades of genetic, biochemical, and structural work. Our understanding of the structure and assembly of each of the subassemblies of the T4 virion has fortunately been enhanced in recent years through structural approaches, primarily cryoelectron microscopy (cryo-EM) and x-ray crystallography.^{63,106}

The mature T4 capsid is composed of 960 copies of the cleaved major capsid subunit, gp23*, and 55 copies of the cleaved capsid vertex protein, gp24*, both of which have the HK97-fold. The angular head is a $T = 13$, $Q = 21$ elongated icosahedron 86 nm wide by 120 nm long¹⁴⁶ and also contains about 1,000 molecules of proteolytically processed internal proteins. The capsid is made initially as a prohead composed of uncleaved proteins that are proteolytically processed only after all prohead components have been added, and after which the proteolytically processed proteins are denoted with a “*” (gp23 is converted to gp23*). Prohead assembly occurs at or around a scaffold core containing gp21 (protease), gp22, gp67, gp68 (scaffolding proteins), gpIP1, gpIP2, gpIP3, and gpalt (internal proteins) that forms atop a cell membrane-bound portal complex made of gp20, which later is the site of DNA packaging and tail addition. The prohead grows away from the membrane by simultaneous addition of capsid and core proteins (or in an alternative view, by addition of capsid proteins gp23 and gp24 around a preformed core) until assembly is halted by the formation of the distal cap when the correct length is reached. Assembly is also dependent on the host GroEL chaperone and the phage co-chaperonin T4 gp31 because their action is required for correct folding of the major capsid protein gp23. DNA packaging (see earlier) is accompanied by expansion of the prohead from its initial rounded shape into the angular prolate icosahedron form of the mature capsid. The DNA-filled head is completed by addition of specific proteins to the portal vertex and to the capsid surface. Proteins gp2 and gp4 are apparently added at the portal vertex, followed by neck proteins gp13 and gp14. T4 gp2 is thought to bind to the genome termini and protect the DNA from degradation by the host RecBCD nuclease after injection. Two *decoration proteins*, Hoc and Soc (155 and 870 copies, respectively), are also added to the outside of the shell. Hoc is located at the center of each hexameric gp23 capsomer, and six copies of Soc surround each capsomer, except for the vertices containing gp24. Both Hoc and Soc are dispensable, although Soc does confer additional stability to the capsid. Attached to the neck are also six trimeric whiskers of the Wac protein (also called fibrin), formed by extended coiled-coil structures.

A small set of T4 internal proteins (internal protein I [IPI], IPII, IPIII, and Alt) are specifically localized to the inside of the capsid where they are proteolytically cleaved at specific peptide bonds and subsequently injected into the host along with the DNA. These proteins contain a capsid targeting sequence that can be fused to other proteins such as GFP so as to target their introduction into the capsid.¹³⁰ The functions of some internal proteins are known; for example, IPI* protects the incoming DNA from an *E. coli* nuclease that is capable of digesting glucosylated hmC DNA (not all *E. coli* strains contain this activity, however).

Structure and assembly of the T4 tail is substantially complex, not only because of the larger number of protein components, but also because of the dynamic nature of the contractile tail¹⁰⁶ (Fig. 75.7). As with the capsid, a combination of cryo-

EM (Fig. 75.8) and crystallography have proven extremely informative in recent years, building on a wealth of genetic and biochemical experiments. The tail is composed of the baseplate, a tail sheath surrounding a tail tube, and long tail fibers; altogether it contains about 430 individual polypeptides, and tail assembly requires a total of 22 genes.

Assembly begins with the baseplate wedge, which is composed of seven different polypeptides (Fig. 75.8). The hub is independently assembled from gp5, gp27, and gp29 (the tail tape measure protein), which then joins with gp12, gp9, gp48, gp54, and six preassembled wedge components to form the baseplate, along with its short tail fibers (gp12). The tail tube (composed of 138 copies of gp19) assembles onto the baseplate, gp18 forms the tail sheath around the tube, and gp3 and gp15 cap the top of the tail. The long tail fibers are bent rods composed of trimers of gp34, gp36, and gp37 and a hinge composed of gp35. They are made by a separate pathway from the rest of the tail with the aid of gp38 and gp57A chaperones. Tail fibers normally attach to the baseplate only after heads and tails are joined and attach to the gp9 socket protein on the baseplate. Both the Wac whiskers and gp63, a protein that is also an RNA ligase, have been found to accelerate tail fiber addition. As a general rule, the assembly of the individual components of tails and tail fibers follows a strict order (with a few exceptions), such that omission of one component or protein halts assembly at the stage when that component is required.

Adsorption of T4 to *E. coli* involves recognition of the outer membrane-located OmpC receptor by the long tail fibers, specifically the C-terminal tip of gp37. This association results in a large conformational change in which the hexagonal-shaped baseplate opens up into a star shape, the sheath contracts, and the internal tube is pushed through the baseplate and through the cell envelope, with the lysozyme action of gp5 facilitating passage through the peptidoglycan layer. The tape measure protein gp29 is presumed to exit the tube so that DNA can be released from the capsid, through the tube, and into the cytoplasm of the cell. In this process, the baseplate and sheath essentially adopt two alternative configurations: a hexagonal baseplate with an extended sheath, and a “star” baseplate configuration with a contracted sheath. The transition from one to the other involves numerous dynamic changes, with perhaps the most prominent being the rotation of more than 100 degrees of the gp10–gp11 complex that attaches the side tail fibers to the baseplate. As a consequence, the short tail fibers unfold from under the baseplate and extend toward the cell surface. A movie of a reconstruction of the whole T4 injection process is available at <http://www.youtube.com/watch?v=41aqxcxsX2w>.

Antigen Display

Because Hoc and Soc are dispensable for the formation of mature virions, they provide an intriguing platform for display of antigens and other proteins on the outside of the head shell. Fusion proteins containing the gp23 association sequences of Hoc or Soc attached to an antigen of choice can be expressed during infection of a Hoc- and Soc-defective mutant of T4 to generate decorated particles. Alternatively, purified fusion proteins can be assembled onto *hoc*[−] *soc*[−] mature virions *in vitro*. The ability to add a large number of epitopes—up to 1,895—onto a single virion is a particularly attractive feature.¹¹⁰ These approaches have been successfully demonstrated for antigens

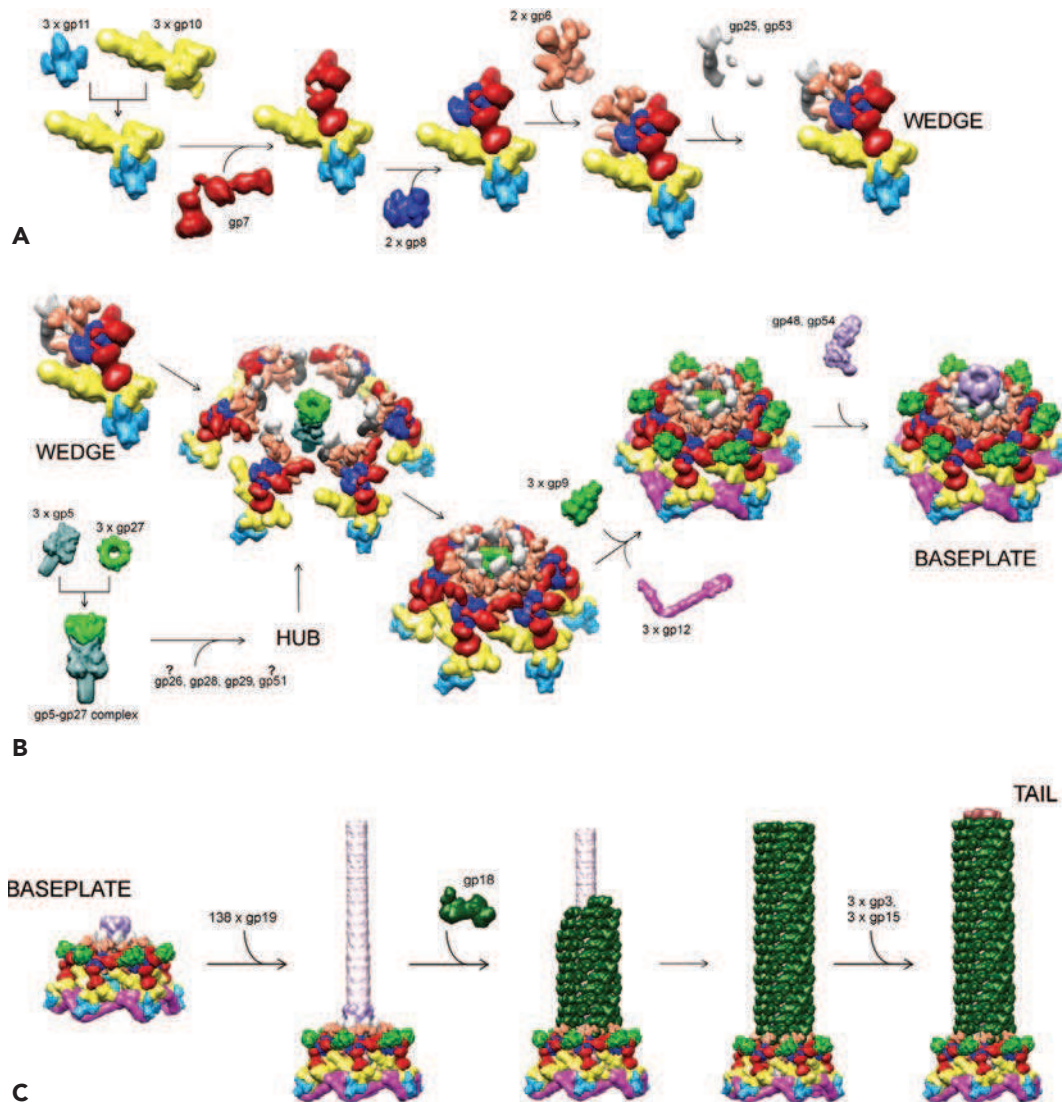


FIGURE 75.7. Assembly of the T4 tail. Assembly of the wedge (**A**), the baseplate (**B**), and the tail tube (**C**) with the sheath. (From Leiman PG, Arisaka F, van Raaij MJ, et al. Morphogenesis of the T4 tail and tail fibers. *Virology* 2010;7:355.)

of a variety of pathogens including human immunodeficiency virus (HIV), poliovirus, swine fever virus, foot-and-mouth virus, and *Neisseria meningitidis*.^{151,165} Some of these show considerable promise as vaccine candidates, including for protection against anthrax.¹³⁶

This approach for virion decoration has also been applied to the construction of small peptide libraries and using these to identify associating proteins. In one example, libraries of the T4 large terminase subunit were panned and a peptide identified that interacts with the late sigma factor, gp55, and subsequent analysis showed that gp55 plays a role in loading the terminase onto the viral genome.¹¹⁴

OTHER TAILED PHAGES

The *E. coli* phages T4 and λ are the most extensively studied individual phages of the $\sim 10^{31}$ on the planet, and they have shown the way over the past approximately seven decades to a

remarkable number of fundamental insights into how viruses earn their livings and interact with their hosts, not to mention revealing some of the most basic aspects of molecular biology. However, another lesson from studies of tailed phages is that they are astonishingly diverse, both in their genetic content and in the genetic and biochemical mechanisms they have developed. Here we present a small selection of other well-studied tailed phages, emphasizing only features of their life cycles that are not found in λ or T4.

Bacteriophage HK97

E. coli phage HK97 was a relatively unknown distant relative of phage λ , but it was selected for further study because it morphologically resembled λ but has a longer tail.⁷⁹ However, HK97 has become an important model system for capsid assembly and structure because of a relatively simple capsid assembly pathway that is experimentally amenable (Fig. 75.9A). HK97 requires only three proteins to direct capsid assembly of its T = 7 capsid: the portal protein, the protease, and the major capsid

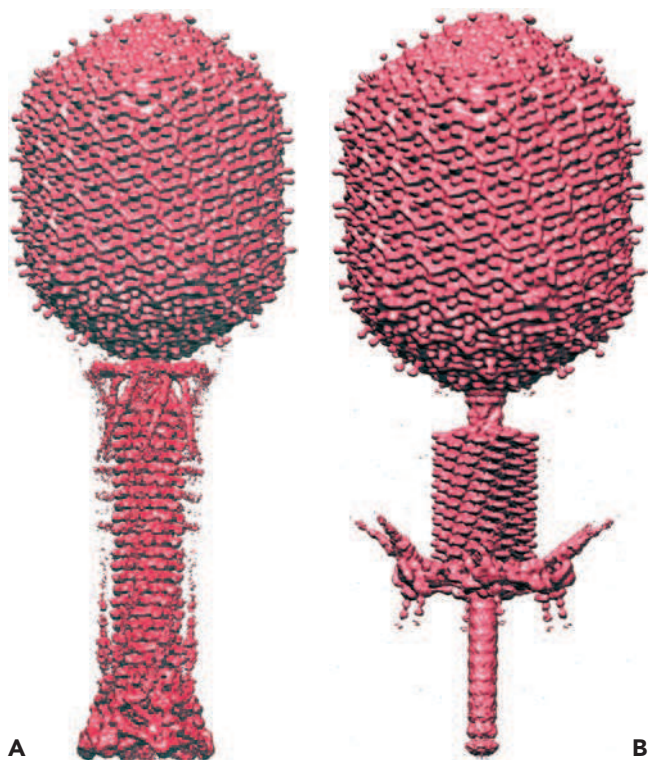


FIGURE 75.8. Cryoelectron microscopy (cryo-EM)-derived model of the T4 phage particle prior to **(A)** and upon **(B)** host cell attachment. Tail fibers are disordered in the cryo-EM structures, as they represent the average of many particles each having the fibers in slightly different conformations.

protein. Missing from this list is the scaffolding protein identified as a required assembly factor in all tailed phages studied previously. In HK97, the scaffolding protein role is played by an ~100 residue N-terminal extension of the major capsid protein called the delta domain. Like the scaffolding proteins of other phages, the delta domain is removed after assembly by the HK97 capsid maturation protease, and the digested pieces exit from the particle. This proteolysis makes the assembly essentially irreversible and results in a particle (prohead II) that is ready for DNA packaging; subsequent conformational changes lead to the expansion of the particle from the round prohead shape to the larger angular shape of the mature form, head II.⁴³

A particularly surprising aspect of HK97 virion assembly is how the mature capsid protein is stabilized, using covalent cross-links between the major capsid subunits.¹⁹¹ This is in contrast to phages that use *decoration proteins*, such as λ and T4 (as described earlier), or phages such as P22 and T7, which achieve their stable mature forms by conformational changes alone. The covalent bonds in HK97 act like rivets between the inside and outside of the capsid, forming between a lysine—part of a secondary structure element on the outside of the capsid, the “E-loop”—and an asparagine on an inner part of a separate subunit. The reaction occurs during the conformational changes of maturation, which brings the two cross-link residues into a reaction center containing a third residue, glutamic acid, which is required to “catalyze” the reaction

(Fig. 75.9C). There are 415 of these cross-links, which also change the overall topology of the capsid polypeptide chains into covalent circles that are interlocked in a way that resembles chain mail.¹⁹¹ This results in an unusually stable capsid.

All of the capsid assembly intermediates illustrated in Figure 75.9 have had their structures determined by cryo-EM, so there is a wealth of information about the conformation changes that occur, at increasingly higher resolutions as techniques improve.^{43,103} The very stable cross-linked HK97 mature capsid was the first of the tailed phage capsids to yield a structure by x-ray crystallography¹⁹¹ (other HK97 intermediates have also yielded x-ray structures since). The fold of the subunits found in the HK97 capsid (Fig. 75.9C), dubbed the HK97 fold because it was unique among known tertiary structures at the time, has since been found in virtually all other tailed phage capsid proteins that have been studied so far, and also in the inner part of herpesvirus nucleocapsids. The x-ray models of HK97 combined with lower-resolution cryo-EM density maps have enabled a fairly complete description of the later stages of HK97 maturation,^{79,103} and it is hoped that similar work on the earliest stages (Fig. 75.9B) will help complete the story and shed light on the mechanism of capsid size determination, which is still poorly understood.

Bacteriophage T7

E. coli phage T7 is one of the seven classical “type” virulent phages anointed by Max Delbrück as appropriate for study in the early days of phage molecular biology.¹²⁶ T7 is a strictly lytic phage with a short (~20-minute) life cycle; if T4 is the *Tyrannosaurus rex* of phages, T7 is the *Velocinaptor*. The genomic DNA in the T7 virion is not permuted, and all the genes are transcribed in the same direction. Upon infection, only the first 7 kbp of the “left” end of the DNA, corresponding to the upstream end of transcription, enter the cell initially. This segment of DNA contains three strong, tandem promoters recognized by the host RNA polymerase, leading to transcription and expression of a small number of genes. These include a protein that inactivates restriction enzymes but, most importantly, includes the phage RNA polymerase. This is the highly efficient single-subunit RNA polymerase that has been used to drive expression in many plasmid protein expression vectors. For T7, its job is to transcribe the remainder of the phage genes, starting from a phage-specific promoter near the end of the short sequence of DNA that was initially injected into the cell. Remarkably, entry of the remaining 83% of the genomic DNA into the cell is driven by transcription by the phage RNA polymerase; as the polymerase transcribes in the direction of the DNA still in the phage capsid, its translocation along the template pulls the remainder of the DNA into the cell.¹²⁷

In light of T7’s long-standing reputation as a quintessentially lytic phage, it was a surprise when a T7-like prophage (!) was found in the genome of a *Pseudomonas putida* strain.³⁰ The prophage has an essentially complete inventory and arrangement of T7 family genes, with an extra ~4 kbp of DNA at the right end of the genome encoding an integrase and at least three additional proteins of unknown function. Unlike in the better-studied prophages, the gene order of the T7-like prophage is not circularly permuted relative to the gene order in the virion DNA. Like many other prophages, the T7-like prophage is integrated into the middle of a tRNA gene and appears to have restored an intact tRNA gene with a copy of

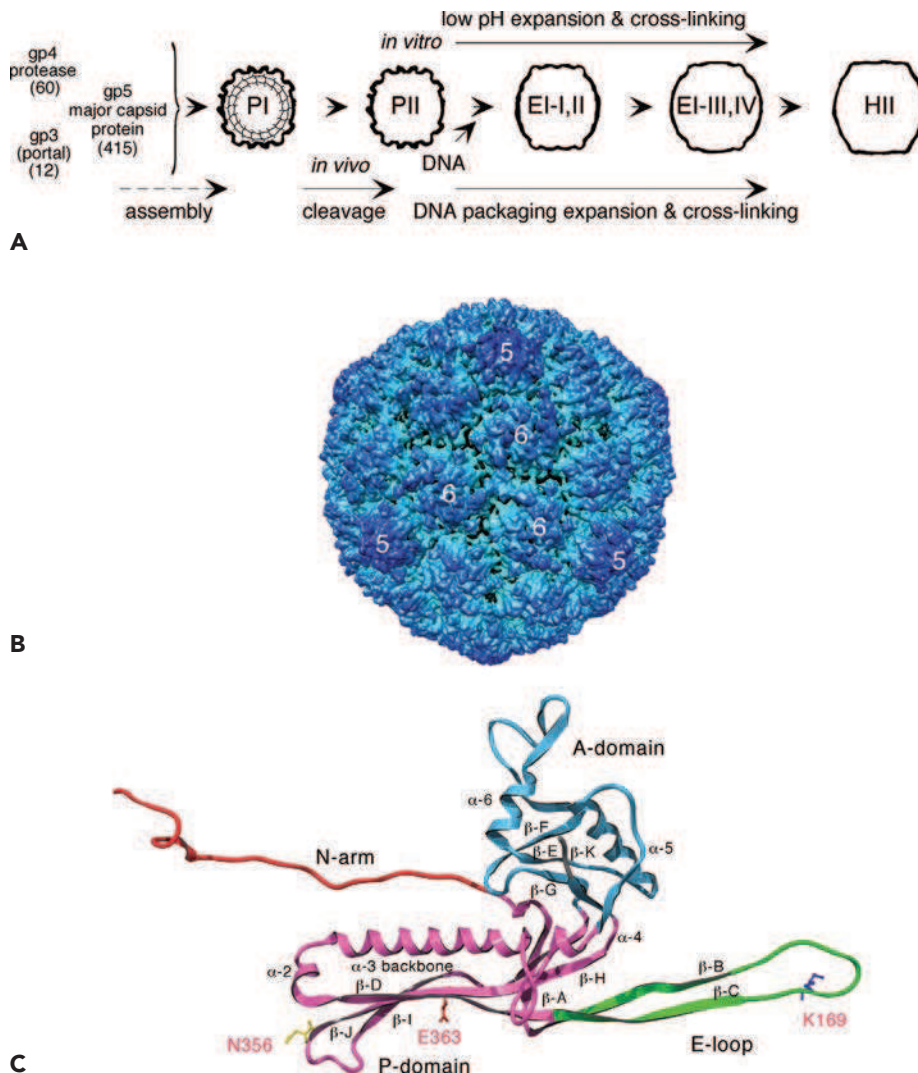


FIGURE 75.9. Assembly of HK97. **A:** PI is prohead I, the first intermediate, and PII is prohead II, which is the cleaved form after proteolysis. EI-I, EI-II, EI-III, and EI-IV are expansion intermediates, as detected in.¹⁰³ HIII is the mature HK97 capsid containing fully cross-linked major capsid protein. Numbers in parentheses on the left are copy numbers of proteins used. **B:** Cryoelectron microscopy reconstruction of HK97 prohead I with three hexons labeled with a 6 and three pentons labeled with a 5. (J. Conway, A. Steven, N. Cheng, R. Duda, and R. Hendrix, unpublished data.) **C:** Subunit F extracted from the head II structure (PDB ID 1OHG) showing major elements of the structure and the three residues involved in cross-link formation.

the downstream half of the tRNA gene encoded at one end of the prophage.

Bacteriophage P22

The genetic diversity of the lambdoid phages is legendary among virologists, and nowhere is this more clearly demonstrated than in their head assembly genes. There are currently nine types of lambdoid phages known whose head genes have diverged to the point where their major capsid protein amino acid sequences are not recognizably similar, and there are significant parallel variations in their head assembly mechanisms.³³ One of these nine types is exemplified by *Salmonella enterica* phage P22, a temperate phage with a 41,724-bp genome.¹³⁸ Its genes are organized and regulated like those of λ , but its virion has a very short tail (it is a member of the *Podoviridae* family; Table 75.1), and unlike λ it utilizes a headful packaging strategy. P22 was discovered at about the same time as λ , but it was immediately noticed that, unlike λ , P22 could transfer host genetic material from one *Salmonella* cell to another.²⁰⁰ This process was called *generalized transduction* because any host gene could be transferred in this way. It is now known that the P22 DNA packaging apparatus occasionally makes a mistake

and initiates packaging on the host chromosome instead of on its own phage DNA.¹¹⁸ P22 is a headful packaging phage, and the headful nuclease that cuts the packaged DNA from the long packaging substrate DNA has little sequence specificity. So, when a head is full of host DNA, the headful nuclease cuts the packaged DNA from the host chromosome, normal tails assemble on these heads, and a “virion” is produced, which is in all respects identical to a genuine P22 virion except that it contains a single ~43-kbp molecule of host DNA instead of the phage chromosome. Such a “transducing virion” can, like an authentic P22 virion, inject its host DNA payload into a susceptible *Salmonella* cell, but this DNA cannot replicate and does not encode P22 proteins. This would be a dead end of no consequence, except that the injected host DNA can replace the parallel portion of the new host’s chromosome by homologous recombination that is mediated by host cell proteins.¹¹⁸ Because P22 does not inject any toxic proteins with its DNA, this recipient cell can survive the experience, and if there are genetic differences between the original donor host and the new recipient host, these differences can be transferred from the donor cell to the recipient cell by this transduction process. Geneticists studying *Salmonella* soon found this to be an

extremely useful way of moving specific genetic alleles from one *Salmonella* strain to another, and small terminase subunit mutants of P22 were found that had error-prone DNA packaging machinery that made the process much more efficient.³¹ Soon generalized transducing phages had been isolated to perform this function for many bacterial species. For wild-type generalized transducing phages, virions with host DNA typically make up 0.1% to 2.0% of the total virions made during an infection, but the frequency of transduction of any given allele from one host to another is considerably lower than this, because only a small fraction (1% to 3%) of the transducing virions carry host DNA that contains any particular host DNA sequence. In addition, the efficiency of recombination of the injected host DNA with its resident homolog is much less than 100%.¹¹⁸ Nonetheless, successful transduction frequencies as low as one in a million recipient cells can be utilized in the laboratory, and transduction remains a common tool of bacterial genetics. Generalized transduction certainly also occurs in nature, where it is thought to be responsible for much of the genetic exchange that happens between bacteria in the wild.

P22 was also the first phage found to encode an *antirepressor* or protein that antagonizes its prophage repressor.¹⁷⁵ P22 antirepressor protein binds the P22 prophage repressor and stops it from binding to its operator. In this way the presence of antirepressor can initiate prophage induction or prevent establishment of lysogeny. Expression of the antirepressor gene is controlled by two other P22 transcriptional repressor genes (*mnt* and *arc*) and a small RNA gene (*sar*).¹¹¹ The Mnt repressor binds its operator, which overlaps the antirepressor gene's promoter, and keeps transcription from initiating there in the prophage state. However, if the Mnt repressor releases its operator, antirepressor protein is made, and it in turn inactivates the prophage repressor so that prophage induction ensues. The small *sar* RNA, which blocks translation of antirepressor mRNA, and both Mnt and Arc transcriptional repressors are all required to lower antirepressor synthesis to the very low level required for P22 to establish and maintain the lysogenic state. The *sar* RNA was one of the first untranslated regulatory RNAs to be discovered and characterized.¹¹¹ Antirepressors of several types are now known to be fairly commonly encoded by temperate phage genomes.

Some tailed phage virions utilize polysaccharide receptors to adsorb to the bacterial surface. P22 is such a phage, and it was instrumental in the discovery that such virion receptor-binding proteins (tailspikes) are usually also enzymes that cleave these surface polymers. P22 virions have 6 trimeric tailspikes and so have 18 polysaccharide binding/cleavage sites. The detailed role of this cleavage is not completely understood, but it appears to allow the virion to work its way down to the outer membrane surface.³⁵ Such tailspikes are very specific for the particular sugars that make up the polysaccharide chain, which means that these phages are very specific for particular bacterial subspecies, strains, or "serovars." P22, for example, only adsorbs to a small fraction of *Salmonella* cells, those whose O-antigen polysaccharides are built with a mannose-rhamnose-galactose repeat. This specificity of P22 tailspike binding can be used to identify its host bacterium, *S. enterica* serovar Typhimurium.¹⁷⁷ Phages that infect *Enterobacteriaceae* bacteria (the family that includes *Escherichia*, *Shigella*, *Salmonella*, *Serratia*, etc.) are known that carry similar tailspikes with at least 51 different polysaccharide specificities.³⁶ All these phages are most likely similarly

restricted to infecting a small subset of their host species, and the surface polysaccharide of their hosts could presumably be identified in a similar manner. Some phage virions carry more than one tailspike, and they can adsorb to and infect multiple hosts that have surface polysaccharides to which their different tailspikes bind.¹⁶⁷

The genetic diversity and mosaicism of the phage P22 virion assembly genes have been particularly well studied. These phages have been evolving for a very long time, and even in a phage group as narrowly defined as the P22-like lambdoid subgroup, the coat proteins have diverged *within* this group to the point that some pairs are less than 15% identical in amino acid sequence, apparently without genetic contact with coat genes from other tailed phage types.¹³⁵ Other proteins involved in recognition of DNA to be packaged and in receptor binding are even more variable (see earlier), and in these two cases there has been horizontal acquisition of some gene types from other more distantly related tailed phage groups.³⁶ In addition, there has been extensive genetic exchange among the various phages *within* the P22-like group, and in many cases parts of genes that encode protein domains rather than whole genes have been exchanged.

Bacteriophages P2 and P4

Bacteriophage P2 is the best-characterized member of a large family of temperate *Myoviridae* found in the genomes of γ -*Proteobacteria*.¹³² It was isolated in 1951 by Bertani¹⁶ from the oldest known lysogen, the Lisbonne and Carrère strain of *E. coli*, along with phages P1 and P3. One hallmark of P2-related phages is their lack of ultraviolet (UV) inducibility; unlike the lambdoid phages, repressors of P2-related phages are not cleaved in the presence of activated RecA.

P2 is among the smallest *Myoviridae*, with a genome of 33,593 bp; the virion DNA is linear and double stranded, with 19-bp cohesive ends. DNA replication proceeds by a modified rolling circle mechanism reminiscent of that used by single-stranded DNA phages such as ϕ X174, and like ϕ X174, P2 depends on the host *rep* helicase.¹³² Replication requires a *cis*-acting phage-encoded initiator protein, the A protein, that catalyzes cleavage at the origin and becomes covalently linked to the 5' end of the cleaved strand. It acts again at the end of replication to cleave the newly synthesized origin and rejoin the displaced old strand. The resulting products are monomeric double-stranded circles, an unusual substrate for packaging of double-stranded phage DNA but one that is required for the P2 packaging reaction.¹⁸

All P2 transcription is carried out by the host RNA polymerase. As in other temperate phages, early transcription initiates from a pair of divergent promoters encoding the lysogeny and replication functions. The P2 immunity repressor, C, differs from the majority of phage repressors in that it recognizes nonpalindromic direct repeats of DNA, even though it is structurally similar to repressors that bind to inverted repeats. P2 C regulates its own promoter and blocks expression of the rightward operon encoding the replication genes and *cox*, which encodes the repressor of the lysogenic promoter. P2 Cox is unusual in that it functions not only as the repressor of the lysogenic promoter but also as the recombination directionality factor for prophage excision.¹⁹⁹

Transcription of the P2 late genes requires the product of the phage *ogr* gene, a transcriptional activator that binds to a

conserved sequence about 55 bp upstream of the initiation sites for the four P2 late promoters.³⁹ Ogr was the earliest example of a transcription factor that interacts with the α subunit(s) of *E. coli* RNA polymerase. It is a small zinc-binding protein found almost exclusively among P2-related phages and their satellites, with a Cys₂Cys₂ motif essential for metal binding and activity.

Capsid assembly has been studied extensively in P2, largely in the context of its relationship as a helper phage for satellite phage P4, which is discussed in more detail later. P2 has also served as a model for bacteriophages with contractile tails. An exciting recent development was the demonstration that the bacterial membrane-penetrating protein, gpV, is an iron-binding spike.²⁰

P4 was discovered in the early 1960s by Erich Six and was initially characterized as a satellite bacteriophage dependent upon a P2 helper phage for lytic growth. Subsequent work has shown that P4 may be more appropriately considered to be an integrative plasmid that acquired the ability to exploit a helper phage for highly specialized horizontal transfer.¹³² In the absence of a helper genome, P4 can be maintained as an integrated prophage or as a multicopy plasmid. P4 replicates its DNA by θ mode in both the multicopy plasmid state and during the lytic cycle, and this replication is independent of helper phage functions. P4 immunity is regulated by a novel mechanism involving transcription termination caused by sequence-specific binding of a short stable immunity RNA. This immunity RNA is processed out of the same transcript that it regulates.

Exploitation of P2 by P4 can occur in several situations, including P4 infection of a P2 lysogen, P2 infection of a strain carrying P4 in either the immune-integrated or multicopy plasmid state, and co-infection by both phages. In each of these cases, P4 responds to the presence of the helper phage by activating functions that allow it to modify P2 gene expression appropriately. The nature and timing of the regulatory cross-talk depends on the infection conditions and appears to be designed to optimize P4 reproduction. One set of reciprocal regulatory interactions between P4 and P2 allows mutual derepression. The P4 Epsilon protein binds to the P2 immunity repressor and interferes directly with its function. Derepression of P4 by P2 requires a third activity of the P2 Cox protein, which stimulates transcription of the operon encoding the P4 replication functions from a promoter that bypasses the P4 immunity system. A second set of reciprocal interactions regulates late gene transcription. The two P4 late promoters are activated by P2 Ogr, the same protein required for P2 late transcription. One of the P4 late gene products, Delta, is an Ogr homolog that activates the same two P4 late promoters and the four P2 late promoters.⁸⁹ This mutual transactivation allows earlier expression of P4 late genes in the presence of a P2 helper. It also allows P4 to activate directly the transcription of the P2 morphogenetic genes required for packaging and lysis, bypassing their normal requirement for P2 replication.

The P4 lytic cycle requires all of the morphogenetic functions of the helper phage. A striking feature of the P2–P4 interaction is the redirection of the viral capsid assembly pathway. The 33-kb P2 genome is packaged into icosahedral, isometric T = 7 capsids with a 60-nm diameter. Satellite phage P4 packages its smaller (11,624 bp) genome into a T = 4 particle formed by the interaction of the P4-encoded size determina-

tion protein, Sid, with the P2 major capsid protein, gpN. The P2 scaffolding protein, gpO, is an internal scaffold required for assembly of the larger T = 7 P2 procapsids. Both P2 and P4 require gpO for capsid maturation; in addition to its role as a scaffold, it also acts as a protease for N-terminal processing of both gpN and itself. P4 Sid functions as an external scaffold, forming a size-restricting cage that influences the bending of the phage capsid protein at a flexible hinge region. Mature P4 virions have lost the external Sid scaffold but contain a P4-encoded decoration protein, Psu, that helps to stabilize the smaller capsid. The smaller P4-induced capsid cannot accommodate the P2 genome, leading to interference with helper phage growth.¹³²

Bacteriophage G

Bacillus megaterium phage G is the largest phage known and among the nonphage viruses is exceeded in size only by the Mimiviruses. The G genome is 497,513 bp long, with nearly 700 predicted protein genes and 17 tRNA genes.⁷⁵ Fewer than one-third of the predicted protein sequences match any sequences in the databases. The sequences with good database matches include some virion structural proteins, DNA replication functions, and a wide variety of metabolic enzymes. Compared to phages with smaller genomes, phage G has an unusually large number of apparent transcription factors and even has some translation components, including the tRNAs and an apparent tRNA synthetase, features that have also been reported for the very large eukaryotic Mimiviruses.

Bacteriophage Mu

E. coli phage Mu (*Mu* is short for “Mutator” and is not the Greek letter) is a temperate phage that gets its name from the fact that the prophage integrates at essentially random positions around the host chromosome, often inactivating genes into which it inserts.¹³⁴ Mu is a transposon as well as a bacteriophage, and when it grows lytically it replicates its DNA by replicative transposition, resulting in ~200 copies of the phage genome inserted around the host chromosome. These phage genomes are excised from the host DNA and packaged into procapsids in such a way that some flanking host DNA is included in the DNA in the virion— 50 to 150 bp on the left end and 1 to 2 kbp on the right end. In this sense the Mu genome is always inserted between flanking host sequences, whether it be in the virion particle, as a prophage, or replicating lytically.

Another striking and unusual feature of Mu, shared with only a minority of the extensive family of Mu-like phages, as well as with some members of the P1-like and P2-like phage families, that have been described is that it has two alternative types of tail fibers, which give it two alternative host ranges. It achieves this by having a 3-kb invertible segment of DNA (the “G segment”) containing sequences that encode two alternative C-terminal halves of the tail fiber, one on each end of the G segment.²² The orientation of the G segment in the genome determines which of these alternative C-terminal half-genes is fused to the half-gene located immediately adjacent to the upstream side of the G segment encoding the N-terminal half of the tail fiber. The N-terminal half of the tail fiber attaches the fiber to the phage tail and the C-terminal half contains the host cell receptor binding specificity, determining the host range. Inversion of the G segment is mediated by a DNA invertase,

Gin, encoded within the G segment. Gin is a member of a family of invertases, including the Hin invertase of *Salmonella*, which is responsible for flagellar antigen switching.

Bacteriophages P1 and N15

Just as phage Mu is both a bacteriophage and a transposon, *E. coli* phages P1 and N15 are both bacteriophages and plasmids. N15 is a temperate phage with a 46,363-bp genome that has many features in common with phage λ . P1 is a different type of temperate phage whose genome is considerably larger (93,601 bp). In the lysogenic state these two phages do not integrate their DNA into the host chromosome. The P1 and N15 prophages replicate as linear and circular plasmids, respectively.^{150,197} P1 is a generalized transducing phage and a classical tool of *E. coli* genetics,¹¹⁸ and it also played a critical early role in the discovery of restriction endonucleases (the P1 genome encodes such an enzyme), which in turn was critical in the early development of genetic engineering.⁴¹

The P1 and N15 prophage plasmids are both present in low copy number close to the number of bacterial chromosomes in the cell, and the study of P1 was important in our current understanding of how bacterial plasmids are partitioned into the two daughter cells during cell division.⁵⁷ Both phages encode two proteins, ParA and ParB, that are responsible for accurate plasmid segregation. ParB molecules bind to a centromere-like site(s) on the phage DNA to form a large macromolecular structure, and ParA protein is an ATPase that can form filaments and binds to ParB. The precise mechanism of control of plasmid partitioning by these proteins is not yet known, but it has been speculated that polymerization of ParA into filaments acts to push or pull the plasmid DNAs into the daughter cells.⁵⁷ In spite of the fact that P1 does not integrate its prophage into the host chromosome, it encodes a site-specific DNA recombinase, Cre protein, that performs several roles for P1, including aiding prophage plasmid segregation by resolving dimer circles (that form by homologous recombination between two monomers) into two monomer circles.¹⁹⁷ The Cre/*lox* recombination system has been applied to the genetic engineering of higher organisms with great success, where it is used to cause gene targeting or deletion to occur in response to a specific environmental signal or to occur only in a specific tissue.¹⁶⁶

Most bacterial DNAs are circular, so the fact that the N15 prophage plasmid is a linear dsDNA molecule is unusual. At the ends of this plasmid the two strands are joined covalently to form a closed hairpin at each end. The mechanism by which the N15 plasmid prophage is formed is interestingly analogous to the mechanism of λ prophage integration. The DNA in the N15 virion, like λ virion DNA, is linear dsDNA with 12 base ssDNA cohesive ends that join upon DNA injection to circularize the molecule. When the phage enters the lysogenic cycle, this circular form of the DNA is converted into the linear prophage form by integrase in the case of λ and by a phage-encoded enzyme known as protelomerase (“prokaryotic telomerase”) in the case of N15.¹⁴⁹ Protelomerase binds to a *tel* site adjacent to its gene, analogous to λ 's *attP*, and cuts both strands of the DNA. But instead of joining the cut ends to host DNA, it joins the 5' end of each strand to the 3' end of its complementary strand, thus forming the hairpin ends of the linear prophage plasmid.⁸⁵ Replication of the linear N15 plasmid then proceeds as normal semiconservative duplication by separation

of the two parental strands and synthesis of the complementary strand without breaking the parental DNA chain. This converts the linear molecule into a dsDNA head-to-head dimer circle, which is in turn resolved into two hairpin-ended linear molecules by action of the protelomerase at the two *tel* sites in the dimer circle.¹⁴⁹

Bacteriophage ϕ 29

The *Bacillus subtilis* bacteriophage ϕ 29 is a small, dsDNA lytic virus of the *Podoviridae* family. Although λ and T4 were being investigated intensively in the mid-1960s, ϕ 29 was chosen for study because it is small (~20 genes) yet shares some of the complexities of other larger phages, including a prolate (elongated) head and a complex, noncontractile tail.⁴ From the start, the study of ϕ 29 held the promise of understanding the function of every gene and of isolating and characterizing every intermediate of the assembly pathway. The system was deemed well suited to investigate complex questions such as the mechanism of form determination, the regulation of assembly *via* sequential protein and DNA conformational changes, and the mechanism of DNA packaging. ϕ 29 has since proven to be a rich model system, especially for the study of DNA replication and the DNA packaging phase of viral assembly.

The genome of ϕ 29 is a linear, unit-length (i.e., nonconcatemeric) chromosome of ~19.3 kbp.^{121,161} The ϕ 29 chromosome is unusual in that a phage terminal protein, gp3, is covalently bound to each 5' end of the DNA via a phosphoester bond. Terminal proteins are also found in the chromosomes of some other phages, such as PRD1 and the eukaryotic adenoviruses. These proteins are essential for DNA replication, which proceeds by a protein-primed mechanism. In ϕ 29, a specific serine residue of gp3 provides the free 3'-OH needed to initiate DNA replication. Replication is bidirectional, with the viral-encoded polymerase, gp2, forming a complex with a free gp3 at both ends of the DNA and then catalyzing covalent attachment of a 3'-deoxy-adenosine monophosphate (dAMP) to the terminal protein. The complex “slides back” to the first nucleotide, and DNA synthesis proceeds from this single priming event. This mechanism allows ϕ 29 to overcome the challenge of replicating the ends of linear DNA without loss of genetic information. The monomeric phage-encoded DNA polymerase is notable in that it has been shown to have exceptionally high processivity, and it is capable of both polymerization and template-strand displacement, precluding the need for a helicase to unwind the template DNA.

During dsDNA phage virion assembly, the genomic DNA is packaged into a preformed protein shell (prohead or procapsid).¹⁴⁷ This process is driven by an ATP-dependent molecular motor that transiently assembles at the unique portal vertex of the head. In most phages, the motor consists of the connector and a terminase complex composed of large and small subunits that house the ATPase/nuclease and DNA recognition functions, respectively. In ϕ 29, the covalently bound terminal proteins are also essential for packaging,¹²⁸ likely providing the role of the small terminase subunit. The ϕ 29 packaging ATPase is smaller (~60%) than other phage large terminase subunits, in part due to the lack of a required nuclease function as the packaging substrate is already unit length. Additionally, ϕ 29 is unique in that a small noncoding RNA is also an essential component of the motor. This 174 nucleotide prohead RNA (termed pRNA) forms a novel pentameric ring via intermolecular base

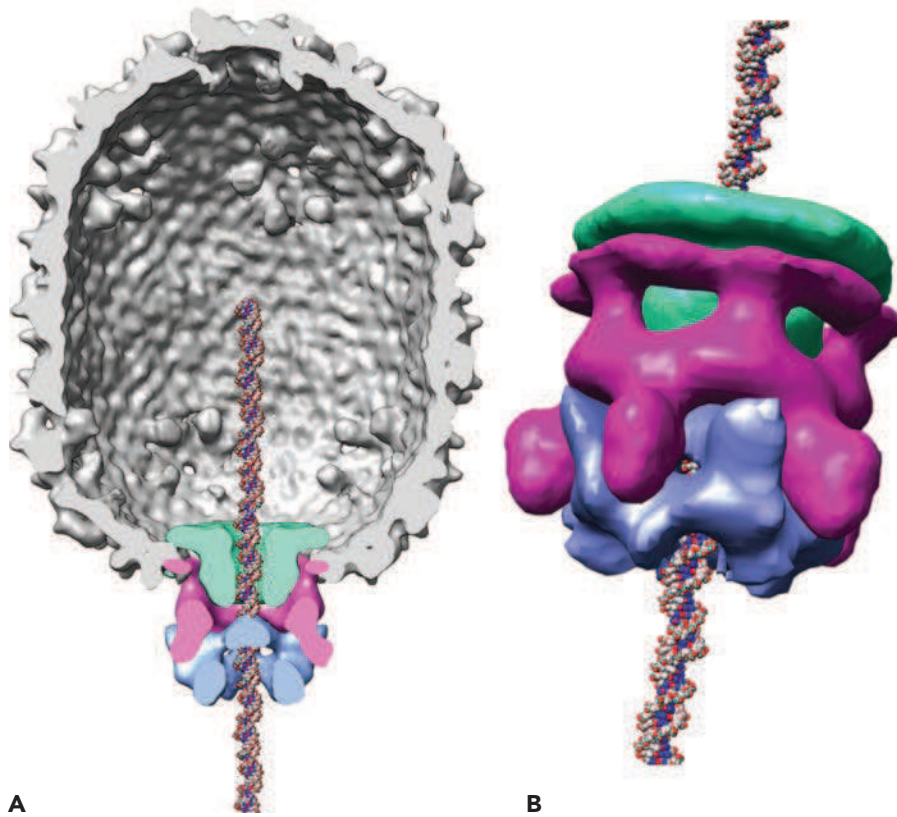


FIGURE 75.10. Packaging of ϕ 29 DNA. The ϕ 29 DNA packaging motor. **A:** Cutaway view of cryoelectron microscopy reconstruction of the prohead/motor complex showing the molecular envelopes of the motor components.¹²⁸ Gray is the capsid, green is the head–tail connector, magenta is the prohead RNA (pRNA) ring, and blue is the packaging ring adenosine triphosphatase (ATPase) gp16. A DNA model has been placed in the channel for reference. (**A** from Grimes S, Ma S, Gao J, et al. Role of phi29 connector channel loops in late-stage DNA packaging. *J Mol Biol* 2011;410:50–59, copyright 2011, with permission from Elsevier.) **B:** Close-up of the motor components. (**B** from Ding F, Lu C, Zhao W, et al. Structure and assembly of the essential RNA ring component of a viral DNA packaging motor. *Proc Natl Acad Sci U S A* 2011;108:7357–7362.)

pairing.¹²⁸ The pRNA ring serves to bridge the connector and the ATPase rings in the packaging motor.^{55,68} Because all the dsDNA phages are thought to employ similar packaging mechanisms, the functions of pRNA likely reside in subdomains of the larger motor proteins of other phages¹⁴⁷ (Fig. 75.10). Movement of DNA into the head is driven by the ATPase gp16. Like the analogous large terminase enzymes of other dsDNA phages, this ATPase ring is related to a large class of cellular ring ATPases that perform many substrate translocating functions.²³

A ring motor can work using a mechanism where each subunit works independently, or alternatively, two or more motor subunits can work together via a more complex and coordinated mechanism. Single-molecule laser-tweezers studies indicate that DNA translocation subunits are coordinated such that the ring loads with ATP while holding the DNA during a “dwell” phase, then hydrolyzes these ATPs to drive a “burst” of 10 base pairs into the head that is coupled to the release of inorganic phosphate from the motor. This high degree of coordination requires communication between motor components, possibly mediated through adjacent ATPase subunits, the pRNA ring to which it is bound, and/or the DNA substrate itself.

Studies of ϕ 29 and other phage motors have revealed that the packaging motor is powerful when compared to other biological molecular motors, generating forces over 65 piconewtons, which is approximately an order of magnitude stronger than skeletal myosin.^{147,168} In part this high-force-generating capacity is required to overcome the resistance encountered when compacting the highly charged and relatively stiff DNA to near-crystalline density inside the head.

INSIGHTS FROM OTHER PHAGE FAMILIES

Tectiviridae: dsDNA, Linear, with Proteins on 5' Ends

Tectiviruses, typified by *E. coli* phage PRD1, share with the tailed phages (*Caudovirales*) a linear dsDNA genome in the virion as well as a few general features of their infection strategy, but in many ways they are quite distinct. Perhaps the most prominent difference is that the tailless PRD1 virion contains a lipid membrane, located inside an icosahedral protein capsid and surrounding the viral DNA⁶⁷ (Fig. 75.11). Its linear DNA is 14,927 bp long, not permuted, with inverted terminal repeats at the ends. Thirty-seven genes have been identified that are arranged in five operons. Promoters for the two early operons are in the terminal repeats, pointing toward the middle of the DNA, and the three late transcripts fill the center of the map, all oriented in the same direction.⁶⁷ The 5' ends of the two DNA strands are covalently joined to a phage-coded protein that serves as the primer for DNA replication by a phage-coded polymerase.

The high resolution x-ray structure available of the entire virion reveals several noteworthy features of PRD1 structure.¹ The major capsid protein, also known as P3 or hexon protein, has two β -barrel domains, each structurally similar to the single β -barrel domains that are found in many virus capsids. P3 trimerizes into a “hexon” that has a nearly sixfold symmetric arrangement of β -barrel domains and occupies the position in the capsid lattice that would be occupied by a hexamer in a canonical Caspar-Klug icosahedral structure. A different protein occupies the icosahedral vertices as a pentamer. These

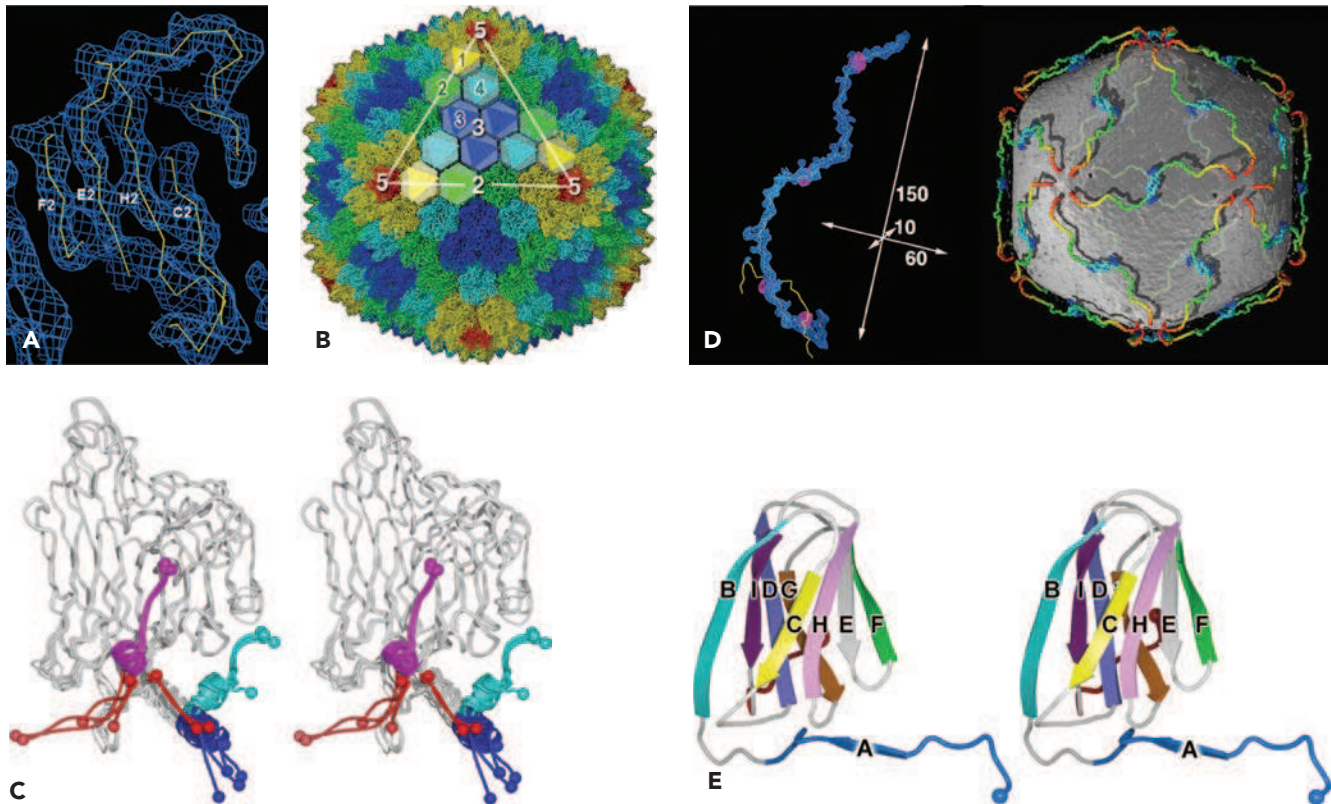


FIGURE 75.11. Architecture and structural components of bacteriophage PRD1. PRD1 is composed of four protein subunits P3, P16, P30, and P31, surrounding a membrane. P30 is a linearly extended protein that nucleates the formation of the icosahedral facets (composed of trimers of the major capsid protein, P3) and acts as a molecular tape measure, defining the size of the virus and cementing the facets together. Pentamers of P31 form the vertex base, interlocking with subunits of P3 and interacting with the membrane protein P16. **A:** Strands of the second of two jelly-roll domains in the PRD1 capsid subunit, P3. **B:** The building blocks of a facet (the triangular area defined by white lines). The icosahedral asymmetric unit contains 12 P3 subunits arranged as four trimers, with orientations represented by triangles labeled 1 to 4 and colored yellow, green, blue, and cyan, respectively (the underlying gray hexagons show the trimer morphology; numbers and symbols follow the adenovirus convention). P3 trimers outside the marked facet are colored similarly and shown as coil, as is protein P31, drawn in red. **C:** Stereo C α traces of the superposed 12 unique copies of major capsid subunit P3, showing switching at the N (blue and cyan) and C (red and magenta) termini. **D:** Protein P30 is shown on the left in royal blue, with the SeMet differences in magenta. Part of a twofold related P30 subunit is shown in yellow. Dimensions are in angstroms. On the right, 60 copies of P30 (colored blue through green to red from the N-terminus to the C-terminus) wrap around the electron density of the membrane. The small holes in the membrane close to the fivefold axes are the transmembrane helices of protein P16. **E:** Stereo view of P31. Jelly-roll strands are labeled B to G; the β -strand at the N-terminus is labeled A. N-terminal and C-terminal segments are colored blue and red, respectively. (From Abrescia NG, Cockburn JJ, Grimes JM, et al. Insights into assembly from structural analysis of bacteriophage PRD1. *Nature* 2004;432:68–74, with permission.)

hexons and pentons are arranged in a pseudo $T = 25$ structure. There are two additional proteins forming the spike structure at the fivefold symmetry position but displaying symmetry mismatch. The spikes are involved in receptor recognition. One of the 12 vertices is a portal for the DNA packaging process where the DNA enters an empty procapsid through the protein and membrane layers of the virion.¹⁷¹ The virus release utilizes similar mechanisms to those operating in tailed bacterial viruses where a holin protein makes an opening to the plasma membrane and a lytic enzyme digests the peptidoglycan layer leading to cell disruption and virus release.

Microviridae: ssDNA, Circular

The *Microviridae* are small viruses with icosahedral capsids that contain the genome in the form of a circular ssDNA molecule. They are typified by the *E. coli* phage ϕ X174, genome size 5,386 nucleotides, with which most of the experimental work with this group has been done.⁶⁰ A few tens of rather close relatives of ϕ X174 have been isolated on enterobacterial hosts, and these have been used in comparative experimental studies with ϕ X174 as well as in evolution studies.¹⁹⁰ A second small group of isolates infects hosts of the *Chlamydia*, *Bdellovibrio*, and *Spiroplasma* genera of bacterial hosts. This group of viruses shares general features with the ϕ X174 group but differs

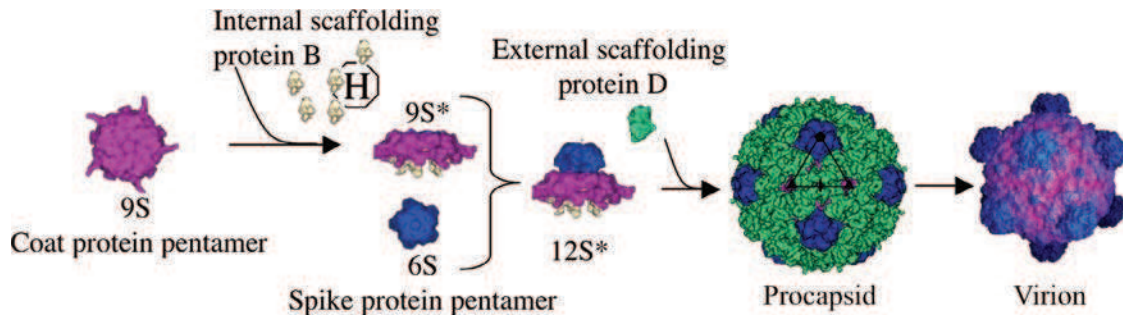


FIGURE 75.12. Assembly of ϕ X174 particles. The first identifiable assembly intermediates are pentamers of the viral coat F and major spike G proteins, the 9S and 6S particles, respectively, which can form in the absence of both scaffolding proteins. Five internal scaffolding B proteins bind to the underside of the 9S particle, yielding the 9S* intermediate. This interaction induces a conformational change that allows 9S*-6S particle associations, forming the 12S* intermediate. The internal scaffolding protein also facilitates the incorporation of the DNA pilot protein H. Two hundred and forty copies of the external scaffolding D proteins, most likely in the form of 60 tetramers, associate 12 12S* particles into the procapsid, a DNA-less particle. Both scaffolding proteins are removed from the procapsid when particles are filled with single-stranded DNA (ssDNA) during the last stages of the maturation.

in details of gene content and organization and of virion morphology; they are accordingly classified as a separate subfamily of the *Microviridae* family. Little has been known until recently about the abundance of the *Microviridae* in the environment. However, metagenomic studies of environmental samples have shown (when the sequencing technology employed can detect ssDNA) that *Microviridae* sequences are a significant component of the sequences in these samples that are recognizably of viral origin.¹⁷⁹

Infection by ϕ X174 begins with binding of the virion to the surface of the *E. coli* host, and electron micrographs of infecting virions show them at locations where the inner membrane and the cell wall of the host are closely apposed. The initial receptor on the host is a portion of the lipopolysaccharide, recognized by a glucose-binding site on the virion surface. Host range mutants of the phage map to a different part of the virion surface, suggesting that there is at least one more host receptor molecule. It is not known how the virion DNA crosses the cell envelope to enter the cytoplasm, but it appears that the virion proteins remain outside the cell.

The virion ssDNA is of the plus sense, so transcription to produce mRNA cannot start until replication of the DNA has produced the minus-strand template. In fact, the first stage of DNA replication, which converts the single-stranded circular virion DNA into a double-stranded circle, is carried out entirely by host replication proteins. The resulting double-stranded circle, called RF I (replicative form I), can serve as the template for transcription but also serves as the template for the second stage of replication, in which the dsDNA RF I circles are amplified to make more dsDNA circles, now called RF II.¹⁷² In addition to the host replication proteins required for converting virion DNA to RF I, this second stage of replication requires a phage-coded protein, the A protein, and an additional host protein, the *rep* helicase. The A protein binds to the replication origin, cuts the plus strand, and attaches the 5' end of the plus strand to itself by an ester bond. The *rep* helicase then unwinds the 5' end and replication proceeds by elongation of the free 3' end of the plus strand. This is the classical rolling circle form of DNA replication, first identified in

this phage. When the free single-stranded plus strand reaches unit length, the A protein cuts again and ligates the ends of the ssDNA to form an ssDNA circle. This ssDNA circle is identical to the virion DNA, and it is converted to the dsDNA circle, RF II, by the same host replication proteins that converted virion DNA to RF I.

The final stage of replication also uses a rolling circle mechanism initiated by the action of the phage-coded A protein, and the ssDNA plus-strand circles are produced as described for the second stage of replication. However, in this stage, replication takes place in a complex with the newly assembled procapsids. Formation of this complex is mediated by the phage-coded C protein. The ssDNA is apparently inserted into the procapsid as it is displaced from the rolling circle, with the packaging driven by the energy of replication (Fig. 75.12). Lysis involves a mechanism that alters the host *MraY* protein to interfere with cell wall synthesis.¹³

Inoviridae: ssDNA, Filamentous

Filamentous phages have striking morphologies, flexible long rods up to several micrometers in length, and diameters of only ~7 nm. These phages were first detected by Loeb¹¹² with phage f1, and later, M13 and fd were isolated, all of which are closely related and use *E. coli* as their host. Several other *Inoviridae* have been described with host bacteria other than *E. coli*,¹⁸⁷ but we will focus here on M13, perhaps the best studied of these phages.

M13 particles consist of a 6,407-base single-stranded DNA molecule that is helically coated by about 2,800 copies of a small (50 amino acids) major coat protein, gp8. The C-terminal sequence of gp8 is positively charged and interacts with the phosphate backbone of the DNA, stabilizing the phage particle. The tip of the phage has five copies each of gp7 and gp9, two minor coat proteins, whereas the base of the phage is made up of five copies each of gp3 and gp6 that are involved in adsorption to the host.

M13 adsorption to its host is quite distinct, is male specific, and uses *E. coli* F pili for attachment. The N2 domain of gp3 binds to the tip of the F pilus and induces a retraction signal in the pilus similar to that induced by a contact

with a female cell in bacterial conjugation. The F pilus retracts by sequential disassembly into the bacterial inner membrane²⁴ along with the phage that effectively extends the tip of the pilus. During this process the N1 domain of gp3 interacts with the periplasmic domain of the host TolA protein.¹¹³ As the coat proteins partition into the membrane bilayer upon pilus retraction, the single-stranded DNA is released into the cytoplasm where it is converted into double-stranded DNA by the host DNA polymerase.

Only 11 gene products are encoded by the M13 genome, most of which are involved in assembly of the virion. The major coat protein gp8 is synthesized as a “procoat” with a cleavable signal sequence and inserted into the inner membrane of *E. coli*. It is not a substrate of the signal recognition particle (SRP) and the Sec-translocase, but instead it requires the membrane insertase YidC for membrane localization.¹⁶² After insertion, the procoat protein is converted to mature coat protein by the leader peptidase of *E. coli*.¹⁰⁰ YidC is also involved in the membrane insertion of gp9.¹⁴² All the virion minor proteins, gp3, gp6, gp7, and gp9, are membrane proteins, as are gp1 and gp11.⁷⁰

Replication of M13 DNA proceeds by the rolling circle mechanism.⁸³ The endonuclease gp2 creates the free 5' ends that are required to form new single-stranded circles. These are first converted to dsDNA to increase the numbers of rolling circles, and later in infection, when the structural phage proteins are synthesized, the newly replicated ssDNA is covered by the phage-encoded ssDNA binding protein, gp5.

As early as 9 minutes postinfection, the first progeny are released from the host cells without lysis (Fig. 75.13). Virion assembly takes place at the host membrane, initiating with gp7 and gp9 to form the tip of the prospective virion. This likely

occurs within a transmembrane complex containing the membrane proteins gp1, gp11, and thioredoxin.¹⁴⁸ The ssDNA is then introduced into the assembly process by binding to the “packaging signal” in the M13 genome.¹⁶⁰ Oligomers of the major coat are then added to form a fivefold helical structure around the ssDNA that grows out from the cell surface.¹³¹ During this elongation process ssDNA moves through the membrane and is simultaneously covered by major coat protein subunits, replacing the bound gp5 molecules. Passage through the outer membrane is facilitated by a porin-like structure containing gp4.¹⁵⁹ Finally, the assembly process is terminated by attachment of gp3 and gp6 to the end of the phage. Thus, the length of the virion filament is determined by the length of the ssDNA genome.

The rate of phage production is constant with about six particles released per cell per minute,¹⁴¹ but because the particles are secreted, no host lysis occurs, and cell division continues, albeit at a reduced rate. Phage production ceases, however, as the cells enter the stationary phase. During an infection, a single cell can produce about 1,000 phage virions in 2 hours.

M13 has been of considerable biotechnical use, first for DNA sequencing and later in “phage display”.¹⁶⁹ Phage-display technology uses short peptides inserted into gp3 that are exposed on the surface of the phage and can be used as affinity probes. If a random sequence encoding the peptide is inserted into gene 3, the affinity of the best binding phages for a ligand can be selected. Applications in nanotechnology, in surface chemistry, and for medical and material science have shown the broad utility of this phage display technology.

The filamentous phage CTX ϕ phage of *Vibrio cholerae* is of considerable interest as it encodes the cholera toxin genes.¹⁸⁵

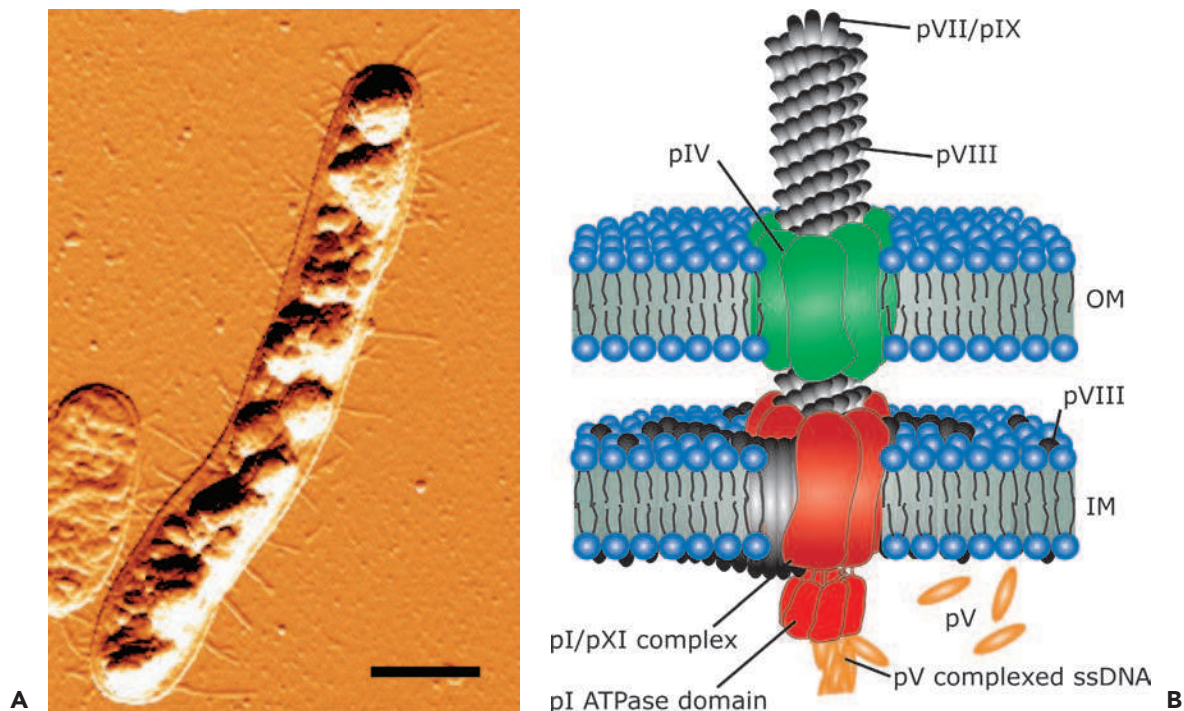


FIGURE 75.13. Secretion of M13. **A:** M13 phage secreted from *Escherichia coli* cells 9 minutes postinfection by atomic force microscopy. The bar represents 1 μm . **B:** Schematic model of M13 passing the inner membrane (IM) and outer membrane (OM) of the host envelope.

CTX ϕ phage encodes homologs of all M13 genes, except gene 4, which is replaced by the toxin genes *ctxA* and *ctxB*. Consequently, CTX ϕ phage uses the secretin EpsD in the outer membrane of *V. cholera* for traversing the outer membrane. To infect new host cells CTX ϕ phage uses a type IV pilus, the toxin co-regulated pilus (TCP), as a receptor. The pilus genes are localized in a pathogenicity island, which resembles elements of another filamentous prophage, VPI phage.⁹¹ CTX ϕ integrates into the chromosome of its host and CTX ϕ expression is regulated by the phage repressor, RstR. A second integrated filamentous satellite phage, called RS1, is involved in inducing CTX ϕ replication by binding of its antirepressor RstC to RstR.⁵⁰ The pathogenicity of *V. cholerae* is thus the consequence of a complex interplay between different filamentous phages.

Leviviridae: +ssRNA

The *Leviviridae* are the smallest of the known phages and the only ones with ssRNA in their virions.¹⁸⁴ The virion RNA is of the positive (i.e., mRNA) sense and encodes four genes, with functions that are described later. There are two slightly different varieties of these phages that have been well studied, one typified by phages MS2, R17, and others, with a genome length of ~3,500 bases, and a second typified by phage Q β , with a genome length of ~4,200 bases. These best-studied examples of this family of phages infect *E. coli*, but similar phages have been identified that infect a variety of other hosts, including gram-positive hosts, and virions of this family appear to be quite abundant in the environment.

The virion is a T = 3 icosahedral structure with 180 molecules of the “coat” protein and one copy of the “maturation” protein. The RNA is inside, and although it does not have the icosahedral symmetry of the protein capsid, a significant amount of RNA interacting with coat protein can be visualized in icosahedrally averaged cryo-EM structures of virions.⁹⁶ This indicates that there is significant order to the RNA within the virion. The coat protein has a fold that has not been seen in any other viruses, consisting of a β -sheet and a large α -helix. The subunits associate as dimers in which the α -helices are passed across the dimer twofold axis to link the two subunits by their α -helical “arms.” The dimer is the functional unit that assembles into the capsid.

Infection starts when the virion binds, through its maturation protein, to the side of the F pilus. The maturation protein becomes proteolytically cleaved and the RNA is released into an RNase-sensitive form, which transits to the cytoplasm of the cell. How this last step is accomplished is not clear, but it may involve retraction of the pilus. The maturation protein may have a role in guiding the RNA into the cytoplasm, but once it is there the RNA alone is sufficient to initiate the infectious cycle, as demonstrated by the fact that naked RNA can be electroporated into the cell to successfully initiate infection. The virion RNA and copies of it that are made subsequently serve as the mRNA for the virus. No subgenomic mRNA is made, yet there is substantial temporal and quantitative regulation of expression from the four genes. This is accomplished by a complex interaction of the extensive and dynamic secondary structure of the RNA with ribosomes and with the replicase.

The genes are arranged on the RNA in the order 5′–[maturation]–[coat]–[replicase]–3′. In MS2-type phages a fourth

gene, the lysis gene, overlaps both the end of the coat gene and the beginning of the replicase gene.⁶² When the RNA first enters the cell, its secondary structure allows productive binding of ribosomes only at the start of the coat gene. As ribosomes proceed through the coat gene they disrupt RNA secondary structure that is sequestering the start of the replicase gene, allowing other ribosomes to make replicase protein with delayed kinetics relative to coat. The phage-encoded replicase protein joins with three host proteins—translation factors EF-Tu and EF-Ts, and ribosomal protein S1—to constitute the four-subunit polymerase complex that copies the RNA. Replication is a conceptually simple synthesis of a (–) strand from a (+) strand template followed by synthesis of a (+) strand from a (–) strand template. Synthesis of (+) strands is more efficient than synthesis of (–) strands, in part because the polymerase has to compete with ribosomes for access to the (+) strands through a mechanism that prevents polymerase from synthesizing RNA in the 3′ to 5′ direction on the (+) strand template at the same time that ribosomes are synthesizing protein in the 5′ to 3′ direction. The translational start site for the maturation protein is always sequestered from ribosomes except for a brief period after that part of the mRNA is synthesized, before the secondary structure that hides the ribosome binding site forms. There is time for only about one ribosome to get on the mRNA before the secondary structure forms and the site is obscured; this means there is roughly one maturation protein made for every (+) strand RNA synthesized—just the ratio that is found in virions.

Progeny virions are assembled from 90 dimers of the coat protein, one molecule of the maturation protein, and one (+) strand RNA. Neither the coat protein nor the RNA will achieve their assembled configuration in the absence of the other. It has been shown that protein and RNA each interact with the other in ways that influence its conformation and assembly capabilities, in an intricate *pas de quatre-vingt-douze* leading to a mature infectious virion.¹¹ In phage MS2, the protein responsible for lysis is encoded by a gene that starts within the coding sequence of the coat gene, in a different reading frame. Ribosomes do not directly enter the start of the lysis gene; rather, the ribosomes that terminate at the coat gene termination codon remain associated with the mRNA, and a small fraction of them drift back by several nucleotides and initiate synthesis of the lysis protein.¹⁹⁸ Cell lysis occurs when the lysis protein has accumulated to a sufficient level, but the mechanism of lysis is unknown. In phage Q β the genes are organized slightly differently and there is no explicit lysis gene. Rather, lysis is caused by a portion of the maturation protein. In this case it has been shown that lysis is caused by inhibition of an enzyme in the cell wall synthesis pathway, MurA.¹⁴ This is similar to what is described earlier for the ϕ X174 E (lysis) protein, except that it targets a different step in the cell wall synthesis pathway.

At the time of the early studies of these phages, their virion RNA was the only prokaryotic mRNA available in pure, homogeneous form. As a result, it allowed the first view of what a translation start site looks like, as well as showing that some parts of an mRNA might not encode protein. In the time since, these viruses have provided extremely fruitful models for understanding how the structure and structural dynamics of RNA can influence and regulate biological processes of general importance, such as gene expression and virion assembly.

Cystoviridae: dsRNA, Segmented

Cystoviruses are unique phages that have a segmented genome composed of dsRNA and an envelope enclosing a viral nucleocapsid composed of two concentric protein shells and the genome.⁸ The viral envelope fuses with the outer membrane of the gram-negative host bacterium after receptor binding. The type member of the family *Cystoviridae* is *Pseudomonas* phage $\phi 6$, but other similar viruses have been isolated, all infecting gram-negative hosts, mainly in the *Pseudomonas* genus. The genome consists of three linear dsRNA molecules, 2,948, 4,063, and 6,374 bp in size, that encode 11 viral structural proteins and two nonstructural ones. The genes are clustered on the three genome segments according to their functions.¹⁴⁵ During the replication cycle the genome segments are converted into full-length polycistronic transcripts, each encoding four to five viral proteins. Noncoding regions at the 3' and 5' termini of the ssRNA molecules, flanking the internally located coding sequences, are approximately 370 and 310 nucleotides long, respectively. The sequence at the 5' end displays only limited homology (17 nucleotides identity) between the segments, while the untranslated regions at the 3' end are more similar to one another.¹²⁵

The innermost protein shell in the virion, the polymerase complex, is composed of four protein subunits. The major capsid protein P1 is arranged on an icosahedral T = 1 lattice so that a dimer of P1 occupies the asymmetric unit. Such capsid organization is also characteristic for the dsRNA viruses infecting eukaryotic hosts.¹⁴⁴ Hexamers of the packaging nucleoside triphosphatase (NTPase) occupy the fivefold symmetry positions, exterior to the P1 shell. The assembly co-factor P7 and the RNA-dependent RNA polymerase P2 are located within the interior of the P1 shell. The first detectable assembly intermediate formed during phage $\phi 6$ infection is an empty polymerase complex, procapsid.¹⁴⁵ The three genome segments are packaged into these capsids in single-stranded form. RNA encapsidation is dependent on the segment-specific packaging signals at the 5' termini of the viral ssRNA molecules and the activity of the packaging NTPase; it proceeds in an orderly fashion from the smallest to the largest genome segment.¹²⁵ The encapsidated ssRNA molecules are replicated within the polymerase complex into dsRNA form by the P2 polymerase utilizing a *de novo* initiation mechanism.¹⁸³ Efficient replication is dependent on 3' terminal replication signals. Following replication the polymerase produces new single-stranded mRNA molecules from these dsRNA segments using a semi-conservative strand displacement mechanism. During the encapsidation of the dsRNA genome the polymerase complex particle undergoes substantial conformation changes expanding the internal volume and making the particles compatible for nucleocapsid surface shell assembly.¹⁴⁵ The nucleocapsid surface shell follows T = 13 icosahedral symmetry and is made mainly of protein P8, which is essential during the virion entry to assist the polymerase complex particles through the host plasma membrane. The viral envelope containing four phage-encoded integral membrane proteins is derived from the host plasma membrane in a process that is dependent on the viral nonstructural protein P12. As a last step of the virion maturation, receptor-binding spike protein associates with the fusogenic protein embedded in the envelope.¹⁴⁵ The progeny virions are released from the infected cell by phage-induced host cell lysis.

Corticoviridae: dsDNA, Circular

Bacteriophage PM2 is the sole described member, and therefore the type organism, of the family *Corticoviridae*.⁶ The virion is icosahedral, with triangulation number 21. Its coat protein has the double β -barrel fold similar to that found in PRD1 (*Tectiviridae*). Consequently, also the virion architecture resembles that of PRD1 with an internal membrane.² The genome is represented in a tightly supercoiled circular dsDNA molecule of 10,079 bp, inside the membrane.¹¹⁶ Ten phage-encoded proteins have been identified as components of the virion: one making up the capsid shell and one forming spikes on the vertices of the capsid, and most of the others are membrane proteins. The lipids of the virion membrane are derived from the plasma membrane of the host cell, but curiously the relative amounts of the different lipids are significantly different from the average lipid composition of the host.¹⁰⁴

The host of PM2 is a marine bacterium of the genus *Pseudomonas*. Infection begins when the virion binds to a receptor on the cell surface leading to the release of the major coat protein and most possibly the virion membrane fuses with the host one.⁹⁴ Twenty-one protein-coding genes are inferred from the genome sequence, and the encoded proteins have been identified for 15 of them. The genes are organized into three operons. The *immediate early* operon has just three genes, is transcribed in the opposite direction to transcription of the other genes, and has sequence similarity to a region of a plasmid found in some *Pseudomonas* strains. The *early* operon encodes an apparent replication initiation protein and two transcription factors necessary for transcription of the *late* operon, which encodes the virion structural components. DNA replication is thought to occur by the rolling circle mechanism, based on EM views of replication intermediates, and this interpretation is bolstered by the similarity of the replication initiation protein to the corresponding protein of phages such as ϕ X174 (*Microviridae*), which is known to replicate its DNA by this mechanism. The mechanism of virion assembly is not well understood, but structures that appear to be membrane-enclosed DNA are seen associated with the cell membrane late in infection, and it is surmised that these are assembly intermediates around which the protein capsid assembles. In laboratory growth in rich medium, about 300 progeny virions/cell are released after a 60- to 70-minutes life cycle. The particles exit from the host utilizing a novel mechanism.⁹⁹ There is a new type of holin-like protein to permeabilize the plasma membrane, and PM2 seems to use a host-derived lytic enzyme to digest the peptidoglycan. There is also a viral protein that disrupts the outer membrane of the host, a function not described previously in phage release.

PM2 was the first bacteriophage shown to have a lipid membrane as part of the virion. It is noteworthy that PM2 was isolated in the mid-1960s yet no other examples of the family have been reported in the more than 40 years since. Very recently PM2-like sequences, which may be prophages, have been detected in the genomes of many marine bacteria.⁹⁸ This may mean that they are not that rare in the environment, and perhaps more plausibly it could mean that methods usually employed for isolating new phages fail to capture this group efficiently. This raises the question of how many novel groups of phages still await discovery.

Plasmaviridae: Circular dsDNA

The *Plasmaviridae* are a small family of phages in which the few known examples infect members of the bacterial genus

Acholeplasma, a member of the mycoplasma. Most of the work on the group has been done with phage L2. The genome of L2 is found in the virion as an 11,965-bp circular, supercoiled dsDNA molecule.¹¹⁵ Fifteen protein-coding genes have been deduced from the sequence, including at least four that encode components of the virion and one that encodes an integrase. The virions are small, quasispherical, somewhat pleomorphic structures, consisting of a membrane envelope surrounding the DNA. The locations of the viral proteins in the structure are not known.

Phage L2 has an unusual life cycle in which, instead of making a decision between lytic and lysogenic modes of growth, it does both every time it infects. That is, the first part of the life cycle is productive, producing virions that leave the infected cell by budding, without killing the cell. This is followed by the lysogenic phase in which virion production ceases and the phage DNA integrates into the host chromosome by a site-specific recombination event, apparently catalyzed by the phage integrase. Prophage-containing cells are immune to infection by homologous but not heterologous phages of the family, and lytic growth of the prophage can be induced by UV or mitomycin treatment. These observations are suggestive of a phage-encoded repressor analogous to those of phages like λ .

EVOLUTION OF PHAGES

Genome Mosaicism

How phages evolve has been a long-term interest in the field, and the scope and specificity of what can be learned have expanded greatly with the availability of high-throughput DNA sequencing. To somewhat oversimplify, phages have been used in two ways in evolutionary studies. In the first, phages are put into a controlled environment in the laboratory and followed by genome sequencing through various selections, competitions, population bottlenecks, and so forth. This approach has allowed rigorous testing of a number of different aspects of evolutionary theory and made it possible to elucidate specific pathways of adaptation, typically at the single nucleotide level, and in this way to illustrate individual evolutionary events that must take place in natural phage evolution. This work has been very valuable but has not necessarily represented most of the enormous and chaotic complexity of the natural environments where phages evolve nor the extraordinarily long times over which phage evolution is thought to have occurred, and it will not be discussed further here. The goal of understanding the mechanisms of evolution as actually practiced by phages has been more closely approached by isolating phages from the environment and sequencing and comparing their genomes.⁷² This approach has the limitation that the sampling of the population is extremely sparse and almost certainly biased, but it has nevertheless yielded a wealth of information about the structures of the genomes produced by phage evolution, and this has allowed inferences about the mechanisms by which they got there. In thinking about phage evolution it is helpful to keep in mind the numbers cited in the first paragraph of this chapter. For example, there are $\sim 10^{24}$ infections per second on a global scale for the tailed phages alone (see earlier), and each of those infections is an opportunity for genetic mischief. As we describe later, there is reason to believe that this has been going on for something like the past 10^{17} seconds. Most of the

discussion given later relates to the tailed phages, for which the most information is available; there is also some comparable work available for the *Microviridae*, which has produced similar but not identical conclusions about mechanisms in this different group of phages.

If the genomes of a closely related group of tailed phages are compared, they are seen to be genetic mosaics with respect to each other. That is, the sequences of two genomes may be nearly identical over the length of a few genes and then abruptly change so that they match less well or not at all.^{32,80} These sites are the “fossils” of nonhomologous recombination (i.e., recombination between two DNA sequences that are different from each other) in the ancestry of one of the phages, creating a “novel joint” in the genome such that two sequences are placed adjacent to each other that were not adjacent in either parental sequence.¹³⁷ This is the mechanism by which these phages are thought to achieve horizontal exchange of DNA sequences, and horizontal exchange is a central feature of the evolution of these phages. The locations of the novel joints are informative; in most cases they have recombined between genes or at gene boundaries. This was initially thought to suggest that recombination events occurred preferentially at gene boundaries.¹⁷⁵ However, closer examination of the data argues that nonhomologous recombination occurs promiscuously across the entire genome without site specificity, but that the vast majority of recombinants are eliminated by natural selection. The recombinants that survive are those that do not disrupt function, and most of these are at gene boundaries, or in other words, not within protein-coding regions. The exceptions that prove the rule are novel joints that fall within protein-coding regions but at positions corresponding to functional domains of the protein. The tyrosine integrases, tail fibers and tail-spikes, and DNA packaging proteins provide good examples of this. Opportunities for recombination between different phage genomes arise when a cell is infected simultaneously by two phages; however, a more frequent opportunity for recombination occurs when the infected cell carries one or more prophages, as is likely true for a great majority of cells.³⁰

Homologous recombination (i.e., between identical sequences) also certainly has a significant role in the reassortment of genes among genomes in that homologous recombination between identical sequences on different genomes can reassort the novel joints and dissimilar genes that flank the identical sequences. These identical sequences may be identical protein-coding sequences. In addition, there are scattered examples of identical stretches of sequence shared between intergenic regions, and more generally, intergenic regions may share more than random sequence similarity due to shared occurrence of transcription and translation signals, and this may bias recombination toward these locations.⁴⁰

There are apparent constraints beyond those described earlier on where recombination between genomes can occur (and survive natural selection to be seen by us). Thus, in the smaller tailed phages like λ and its relatives, there is little or no mosaicism seen within the group of procapsid assembly genes and usually also little mosaicism within the group of tail tip assembly genes. Similarly, genes for DNA binding proteins, for example, the λ *cI* repressor gene, “travel through evolution” together with the DNA sites their encoded protein binds. Such cases are thought to be explained by shared function of the genes that show this behavior. Thus, the proteins encoded by

the head genes of a phage like λ must each interact intimately with the other head proteins during head assembly and in the assembled structure, and they have co-evolved over time to maintain productive interactions among the group. Substitution of a homologous gene or genes from a phage that had followed a different path of mutation and co-evolution would in almost all cases be expected to produce a nonfunctional set of head genes and be counterselected, even though all the genes would be functional in their home genomes. A notable exception to such co-evolution is the parts of tail fiber and tailspike genes that bind bacterial receptors. They appear to have been horizontally exchanged many times among even very different phages. For example, there are members of the *Podoviridae*, *Siphoviridae*, and *Myoviridae* that, in spite of having very different life cycles, have nearly identical tailspikes that utilize the same host receptors. It seems that sometimes when a phage manages to obtain a new host specificity in this way, it can have an advantage, because it can enter an evolutionarily naive cell that is not prepared to defend itself against a phage that it has not “seen” before.

Common Ancestry of Archaeal and Eukaryal Viruses with Phages

Homology (shared ancestry) among viral genes can be inferred from similarity of their encoded amino acid sequences, as is done routinely, for example, for genes of mammalian genomes. However, this approach has turned out to be quite limited for viral sequences because the sequences of viral proteins that are thought on other grounds to be homologs have often diverged to the point that they can no longer be recognized as similar. This has meant that, while it is possible to construct phylogenetic trees based on protein sequences from closely related viruses, there is little hope of inferring the deep evolutionary history of viral proteins or their associated viruses based on sequence comparisons alone. Happily, this situation has changed somewhat with the advent of structural information about viral proteins—mostly virion structural proteins—in recent years.

The capsid *hexons* of bacteriophage PRD1 (*Tectiviridae*) are homotrimers of a protein subunit that has two domains, each of which is a β -barrel of the *jelly-roll* type.⁷ Each of the jelly-rolls occupies a position in the capsid lattice that would be occupied by a single capsid protein subunit in a classical Caspar-Klug structure. The high-resolution structure of the PRD1 hexon was a surprise when it was first determined because the aforementioned description applies equally well to the hexons of adenovirus.¹² The similarity extends to the protein folds of the two hexon proteins, and this has led to the inference that these two proteins may share common ancestry, despite the fact that their amino acid sequences no longer share any detectable similarity. The same double jelly-roll fold has been found subsequently in eukaryal viruses PBCV1 (*Phycodnaviridae*) and Mimivirus (*Mimiviridae*); *Sulfolobus* turreted icosahedral virus (STIV; *Rudiviridae*), which infects the hyperthermophilic archaeon *Sulfolobus*; and bacteriophage PM2, arguing that this diverse and sequence-dissimilar group of capsid proteins also shares in the PRD1 and adenovirus ancestry.

The tailed phages (*Caudovirales*) have a capsid protein with a very different fold, typified by that of *E. coli* phage HK97.¹⁹¹ Evidence to date indicates that this fold is shared across all of the tailed phages, including those that infect archaeal hosts,

often in the absence of detectable sequence similarity. It also appears that this same “HK97 fold” makes up the shell-forming domain of the herpesvirus capsid protein, arguing for a common ancestry with the tailed phage capsid proteins for the herpesvirus protein as well.⁸⁸ The case for common ancestry between tailed phage and herpesvirus capsid proteins is bolstered by shared mechanisms of capsid assembly and DNA packaging, including faint but convincing sequence similarity between the DNA-packaging proteins of phage T4 and herpes simplex virus. A third capsid protein fold reaching across host domains includes phage $\phi 6$ (*Cystoviridae*) and the reoviruses of plant and animal hosts. These viruses have double protein shells; the inner shell has the noncanonical $T = 2$ structure with a novel protein fold and the outer shell is a $T = 13$ shell of single jelly-rolls.¹⁴⁴ Together these observations lead to the view that these viruses define at least three viral lineages, or perhaps more properly three viral capsid lineages, whose hosts span all three domains of cellular life (Fig. 75.14). A simple (but not yet firmly established) interpretation is that viruses with the basic capsid characteristics of these contemporary viral lineages had already evolved by the time of the last common cellular ancestor of the cellular domains of life. There are of course viruses with icosahedral capsids but without any of the capsid protein folds of these three lineages, for example, the *Leviviridae* (small ssRNA genomes) among phages. The capsid protein folds associated with these other viruses are thus far associated only with hosts within one cellular domain, and it will be interesting to see how that situation changes as more viruses are identified and characterized. There are also other examples of virion protein structural similarities among very different virus types. For example, fibers that protrude from the icosahedral vertices of adenoviruses and tectiviruses, as well as fibers that extend from some phage tails, have knobs at the distal tips. The polypeptide folds of these three knobs are sufficiently similar to suggest a possible common ancestry.¹⁷

PHAGE PARTS IN OTHER CONTEXTS

Structures are occasionally found in bacterial or archaeal cells that bear a structural resemblance to virions—or parts of virions—of the tailed phages. For the examples given here, examination of the polypeptide folds of the components of the structures, and in most cases, comparisons of their amino acid sequences, makes a strong case for common ancestry shared by the virions of tailed phages and these “cellular” structures.

Pyocins

The pyocins are a class of high-molecular-weight bacteriocins encoded in the genomes of some *Pseudomonas* strains.¹²³ They have the morphology of phage tails, either contractile or not, and they kill sensitive cells after they are released from the cell that encodes them, apparently by making holes in the cell envelope and causing collapse of the cellular membrane potential. The genes encoding the pyocins have close sequence similarity to the tail gene operons of well-studied phages—*E. coli* phages P2 (contractile tail) and λ (noncontractile tail), respectively—in the well-characterized cases of the R2 and F2 pyocins of *P. aeruginosa*.¹²³ The high sequence similarity between the respective pyocin and phage tail operons bespeaks an evolutionarily recent common ancestry.

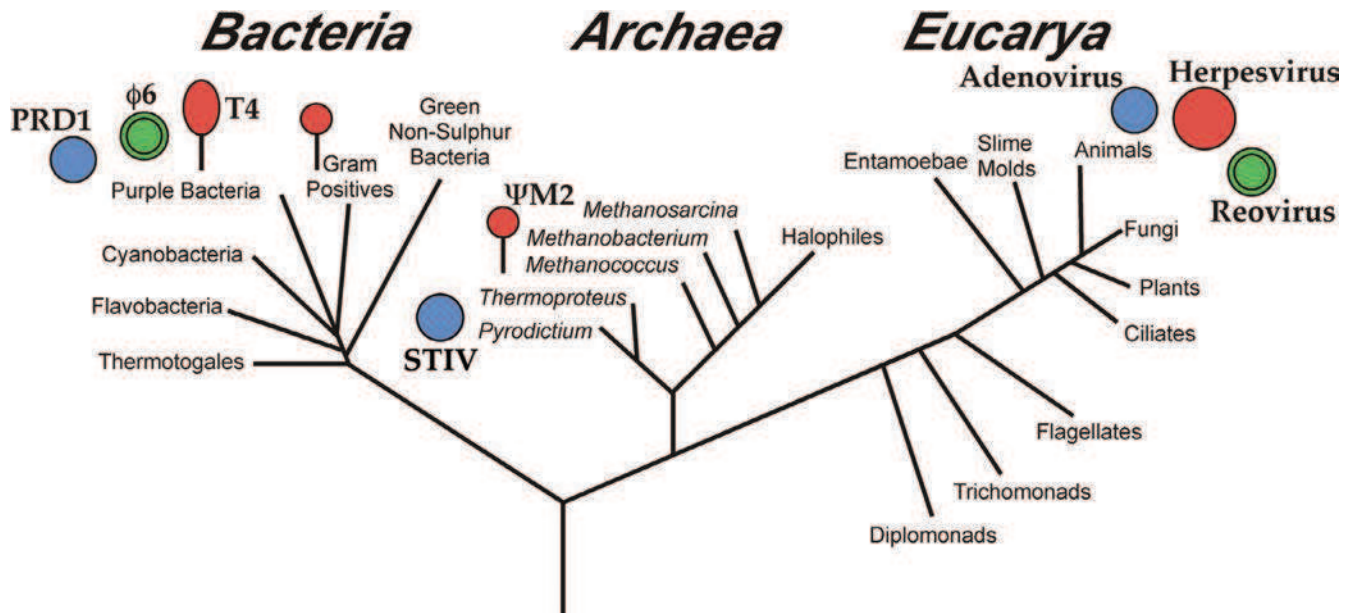


FIGURE 75.14. Deep evolutionary connections among viruses. The tree represents the tree of cellular life. The colored symbols represent the indicated viruses, placed near their cellular hosts. Shared color indicates shared “viral lineage”, as deduced from shared structural features of the virions.

Gene Transfer Agents

Gene transfer agents, or GTAs, were discovered as a generalized transducing activity in cultures of *Rhodobacter capsulatus*.¹⁰⁸ They were subsequently shown to be small particles with the morphology of a tailed phage that package short (4.5-kbp) pieces of DNA derived from apparently random locations around the *R. capsulatus* genome. GTA-like particles with similar properties have been identified in several other bacterial and archaeal species, but the *R. capsulatus* GTAs are the best characterized. The GTA particles are encoded by a ~14-kb-long operon of genes in the *R. capsulatus* genome. In both gene organization and sequence similarity, the genes of this operon strongly resemble the head and tail genes of a tailed phage with a noncontractile tail. The GTA operon bears some resemblance to a prophage, and it may well have derived from one, but it is not a conventional prophage that has suffered deletion of its nonvirion assembly genes for at least two reasons. First, the heads of the particles can only package the equivalent of about one-quarter of the genes that encode the particle. Second, expression of the GTA genes is under the control of a cellular two-component system that senses conditions outside the cell. It appears that the cell may have co-opted the GTA genes to serve its own purpose. What that purpose might be is not known, but a plausible possibility is that the GTA particles are genetic repair kits, sent from one cell to other cells in the population, to save the population from the ravages of Müller's ratchet.

Encapsulins

The encapsulins are a recently described group of subcellular shells found in both bacteria and archaea.⁴⁴ In cases where their function has been inferred, they enclose and sequester biochemical reactions that produce potentially harmful reactive intermediates. The shells are icosahedral structures, $T = 1$

or $T = 3$, made of protein subunits that bear clear homology to the phage HK97 family of capsid proteins, based both on close structural similarity and on a low but unequivocal level of amino acid sequence similarity. The genes encoding the encapsulin subunits are not associated with any other phage-like genes in the cellular genome.

Type VI Secretion Systems

Recent structural studies of the proteins of the needles of the *Pseudomonas* and *Edwardsiella* type VI secretion systems (T6SS) show that the major subunits of the needles have the same polypeptide fold as the tail tube protein of phage T4 (gp19), despite a lack of recognizable shared sequence.⁸⁷ This is taken to suggest a common ancestry for the two proteins and, by extension, possible common ancestry for the phage tail and the T6SS apparatus.¹⁰ A parallel study with the phage λ tail tube subunit, gpV, shows that it shares the gp19 fold, arguing that the common ancestry inferred for the tail tube of a contractile tailed phage and the T6SS needle extends to the noncontractile phage tails as well.¹³⁹ Recent structural studies have identified a long contractile structure, resembling an exceptionally long contractile phage tail, in cells indulging in type VI secretion¹⁰⁷ (Fig. 75.15). This structure assembles in the bacterial cell as it is preparing for secretion and disassembles after. It appears to drive the T6SS needle into the target cell by contraction of the sheath, just as a contractile phage tail drives the tail tube through the envelope of a target bacterium.²⁹

In all of these cases there seems little doubt that the structural proteins of phages and the corresponding proteins of phage-like structures in bacterial and archaeal cells share common ancestry. A simple and generally accepted explanation for this relationship is that a group of phage genes has been captured by the host cell, possibly from a resident prophage, and placed under a regulatory scheme that benefits the host. In this

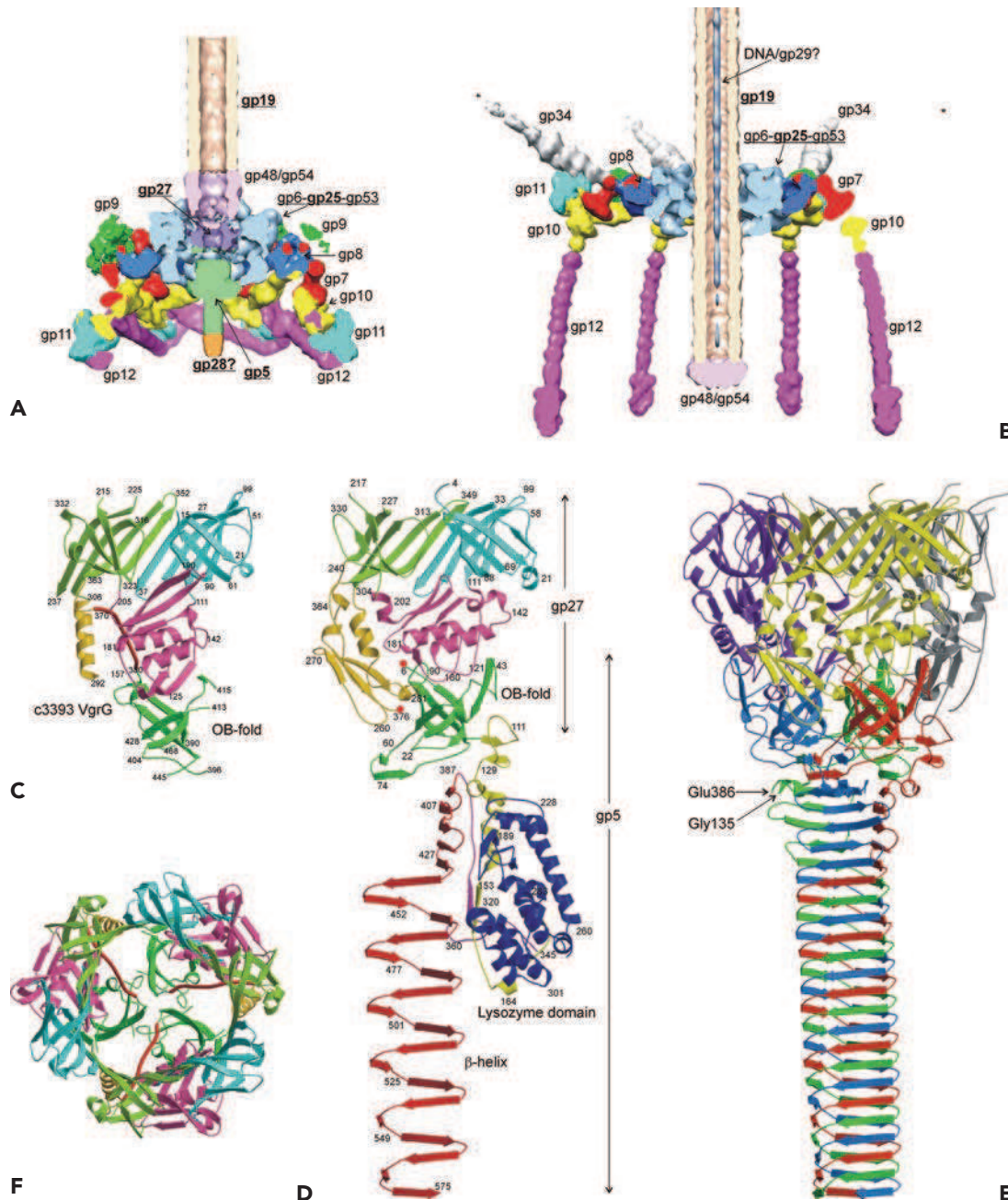


FIGURE 75.15. Structure of the bacteriophage T4 baseplate and comparison of the *Escherichia coli* CFT073 c3393 VgrG with its T4 homologs, gp5 and gp27. Cryoelectron microscopy (cryo-EM) reconstructions of the T4 baseplate before (A) and after (B) attachment to the host cell. Component proteins are labeled with their respective gene numbers. The T6SS protein homologs are highlighted in bold and underlined. C: The crystal structure of the c3393 VgrG. Different domains are colored in distinct colors. The gp27 tube domains are colored cyan and light green. The fragment of the polypeptide chain connecting the gp27 and gp5 modules is shown as a thick red tube. D: The structures of gp5 and gp27 monomers extracted from the (gp5)₃–(gp27)₃ complex. The terminal ends of the gp5 and gp27 polypeptide chains, which become fused in the VgrG structure, are highlighted with red dots. E: A model of the prototypical VgrG is created from the entire (gp5)₃–(gp27)₃ complex by removing the lysozyme domain. F: End-on view of the crystal structure of the c3393 VgrG trimer. (From Leiman PG, Basler M, Ramagopal UA, et al. Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proc Natl Acad Sci U S A* 2009;106:4154–4159, with permission.)

view, such acquisition of phage virion structural genes is simply an example of the more general phenomenon of cells acquiring “phage” genes and converting them over time into “cellular” genes. The difference in the case of the structural genes is that they are more easily recognized as being of phage origin, even after mutational drift has eliminated any detectable sequence similarity. However, despite the plausibility of tailed phages as the progenitors of the cellular structures enumerated earlier, there is no formal proof that the arrow might not go in the other direction—that is, that T6SS needles, for example, might be the ancestors of phage tails!

HOST INTERACTIONS

The essence of being a virus is interacting with the host, and these interactions influence both virus and host. We will consider some examples of interactions between phages and bacteria in three somewhat artificial and overlapping categories.

Parasitic Interactions

Phages hijack their hosts’ biochemical machinery, usually to the detriment of the host, and as in other areas of virology, understanding how these interactions work often tells as much about the cell as about the virus. The ways these interactions are carried out are as varied as the phages doing them; we give examples here of some of the better-studied cases.

DNA Replication

Some phages such as phage T4 encode all the proteins needed to replicate the phage genome. These form a complex that replicates the phage DNA with a preference for the modified cytosine nucleoside that T4 uses in place of cytosine. At the same time other phage-encoded enzymes are degrading the host DNA and recycling the nucleotides (in conjunction with some host nucleotide metabolism enzymes) for use by the phage replication machine. *Bacillus* tailed phage SPO1 has at least nine genes required for phage DNA replication but appears to be lacking some of the complete set of replication genes that a phage like T4 has.¹⁷⁰ SPO1 uses hydroxymethyl uracil in its DNA in place of thymine, and the phage encodes several enzymes with roles in depleting thymine pools and synthesizing hmU, and the phage-encoded DNA polymerase has an extra domain that is proposed to help it recognize the hmU-containing template. Many phages use the complete host replication machinery, synthesizing only the proteins needed to recruit the replication complex to the phage DNA template. An example is phage λ , which makes two replication-specific proteins, one of which (gpO) binds the phage replication origin and one (gpP) that recruits the host replication helicase, and through it the rest of the host replication complex, to the phage origin.¹⁷⁸ Unlike T4, λ does not degrade the host DNA, presumably because doing so would preclude the possibility of forming a lysogen. Phage ϕ X174 also uses the host replication machinery. However, unlike the host and unlike most other phages, it uses the host transcription RNA polymerase to lay down the RNA primers at the start of new DNA chains, rather than primase, the specialized RNA polymerase that usually carries out the priming function. For technical reasons, this peculiarity of ϕ X174 facilitated the original discovery that RNA primers have a role in DNA replication. The ssRNA

phage MS2 encodes a replicase protein, but to replicate the RNA it co-opts the activities of three cellular proteins (EF-Tu, EF-Ts, S1) that normally interact with RNA but to very different ends.

Transcription

Most DNA phages use the host RNA polymerase for some or all of their transcription. They often achieve temporal separation of different classes of transcripts by successive modifications of the host polymerase. For phage λ the transition from *immediate early* to *early* transcription is mediated by the immediate early antitermination protein gpN, which interacts with the nascent RNA transcript and the RNA polymerase to render the polymerase able to read through termination signals.¹⁵⁴ The transition from *early* to *late* transcription is mediated by another antitermination protein gpQ, acting by a different mechanism. Phage T4 makes covalent modifications to the host polymerase early in infection and adds a phage-specific sigma factor to the polymerase to effect the transition to late transcription.⁸² Phage T7 transcribes its early genes with the host RNA polymerase, but one of these genes encodes the phage RNA polymerase, which is responsible for subsequent transcription of phage genes. Another early gene of T7 encodes a kinase that inactivates host transcription by phosphorylation of the host RNA polymerase.¹²² A phage that flouts all the normal conventions for organizing transcription is *E. coli* phage N4. N4 packages a few copies of a *virion RNA polymerase* in the virion with the DNA, and they are injected into the cell along with the DNA.⁵² The virion RNA polymerase transcribes the early genes, some of which encode a second (phage-encoded) RNA polymerase that transcribes the middle genes. The middle genes include genes supporting phage DNA replication and also an ssDNA binding protein, which interacts with the late promoters in such a way that they can be read by the unmodified *E. coli* σ^{70} -RNA polymerase!

Translation

All phages, like all viruses, use the host translation apparatus to make their proteins. For the most part the phages appear to use it in unaltered form, though they do make use of some of the “special abilities” that ribosomes have beyond the textbook descriptions of translation. These include programmed translational frameshifts (many phages), a 50-nt ribosomal “hop” (phage T4), a few examples of regulation of gene expression at the level of translation (phage T4), and the extensive examples of regulation of translation described earlier in the section on the *Leviviridae* (the ssRNA phages).^{84,196} For many tailed phages, particularly (but not exclusively) those with larger genomes, there is suggestive evidence that they also make specific alterations to the host translation apparatus. The most frequently encountered support for this assertion is in the form of phage-encoded tRNAs. Although the majority of characterized phages do not encode any tRNAs, there are many that do. The numbers of apparent tRNAs per genome in this group range from 1 to greater than 30, with their genes usually arranged in one or more clusters on the genome. Some of these putative tRNAs give high scores in tRNA search programs trained on cellular tRNAs and some do not, and in only a few cases have they been tested and shown that they can actually function as tRNAs. It has been suggested that the purpose of the phage-encoded tRNAs is to adjust the tRNA pools in the infected cell

to better serve the needs of phage translation, but they may have other roles that are not yet identified. Several phages have been found to carry a copy of a transfer-messenger RNA (tmRNA), the RNA found in prokaryotes with features of both tRNA and mRNA and a role in releasing stalled ribosomes from mRNA. Finally, phage G of *Bacillus megaterium*, the phage with the largest genome known at 498 kb, has one aminoacyl tRNA synthetase.⁷⁵ This last feature is reminiscent of the very large Mimiviruses, which also carry aminoacyl tRNA synthetases for which the biological role also is not known.⁴² These observations taken together make a strong case that some phages modify the host translation machinery, but there is as yet little specific information about how or to what end they might actually do this.

Other Cellular Machinery

Phages rely on their hosts for energy metabolism. They also typically use the cellular biosynthetic machinery to make precursors for macromolecular synthesis, but a number of phages encode enzymes with roles in nucleotide metabolism. When these are present they typically act in conjunction with cellular enzymes of nucleotide metabolism. Two of the most easily recognized and frequently found nucleotide metabolism genes in phages encode ribonucleotide reductase and thymidylate synthetase. To date, there is no evidence of phages participating in amino acid biosynthesis, nor of phages incorporating noncanonical amino acids into their proteins. For phages with lipid membranes in their virions, the lipids appear in all cases to be derived from the cellular membrane.²

Phages also make use of host chaperone proteins, and in fact some of the most important of these were discovered because of their roles in the life cycles of phages λ and T4. The *E. coli* DnaK and DnaJ proteins (also known as Hsp70 and Hsp40) were found to be required for λ DNA replication, where they partially disassemble the replication initiation complex and allow the replication machinery to leave the origin and begin synthesis. GroEL and GroES (Cpn60 and Cpn10) are required for accurate assembly of phage λ heads, now understood as a consequence of their role in mediating correct folding of some of the head proteins. GroEL is also required for phage T4 head assembly because of its role in folding the major capsid protein gp23. The gp23 subunit is apparently slightly too big to fit into the GroE folding cavity with the GroES “lid” in place, and T4 encodes its own homolog of GroES, gp31, which leaves enough more space in the cavity for successful folding of gp23. We note that these two chaperones, now known as the hsp70 (DnaK) and hsp60 (groE) systems and known to be very important and present in virtually all organisms, were originally discovered in work designed to understand the molecular nature of these phage infections.

Phages interact in significant ways with cellular structures, particularly the cell wall and membranes, during cell entry and exit. The *Leviviridae* (ssRNA, icosahedral) and *Inoviridae* (ssDNA, filamentous) of *E. coli* bind to the sides and tip, respectively, of the F pilus, and they are thought to be brought to the surface of the cell for further interactions by retraction of the pilus. The tailed phages Cb13 and CbK of *Caulobacter crescentus* have helical tail fibers that wrap around the shaft of the bacterial flagellum.⁶⁹ This makes an interaction that is apparently able to slide along the helical flagellum, and when the flagellum rotates in the appropriate direction the phage is

screwed down to the surface of the cell where it interacts with a cell surface receptor.

Antagonistic Interactions

Phages and their bacterial hosts engage in ongoing “biological warfare” in which mutations arise in the bacterium that block successful lytic growth by the phage, which in turn creates a selective advantage for phages with mutations that overcome the block. The best-known examples are mutational changes that affect the binding between the virion and its receptor on the surface of the cell. In the tailed phages, a single amino acid change in the tail fiber protein can be sufficient to restore binding to a recalcitrant cellular receptor, but bigger changes occur as well. The parts of tail fiber genes that encode the receptor-binding part of the tail fiber often show evidence of recent horizontal exchange, and in some phages rapid changes of sequence, specifically in tail fibers, are programmed into the phage genome, as in the examples of phage Mu and phage BPP-1.^{119,163} The biological warfare continues after the phage has successfully gotten its genomic DNA or RNA into the cell. Many examples could be given; a particularly piquant example comes from the *prcC* gene of *E. coli*. The product, PrrC, is a nuclease that cuts in the anticodon loop of the cellular tRNA^{Lys}, inactivating it and so inhibiting protein synthesis in the cell.¹⁸² PrrC is ordinarily held in an inhibited form, but infection by phage T4 causes its release in active form, whereupon it cleaves this tRNA. The “purpose” of the cleavage is evidently to prevent the phage from making virions that could infect other cells in the population. However, T4 encodes two enzymes, polynucleotide kinase and RNA ligase, that counter this host defense by repairing the tRNA. Relatives of T4 that lack the repair enzymes are unable to grow on *E. coli* strains that carry *prcC*. A more widely distributed host defense is the CRISPR system, sometimes referred to as bacterial (and archaeal) adaptive immunity.⁵³ Cells with a CRISPR system have a mechanism to collect an array of short (~25-bp) DNA sequences derived from phages of past infections (which the ancestral cells evidently survived). When a phage that has an exact match to one of the sequences in the CRISPR array infects, it is inactivated by a mechanism that may have some features in common with the eukaryotic small interfering RNA (siRNA) mechanism. Phages can escape CRISPR surveillance if they acquire as little as a single nucleotide change in the sequence being recognized.

Symbiotic Interactions

When a phage enters the lysogenic cycle and becomes a prophage, most of the genes are repressed by the repressor protein. However, some phage-encoded proteins continue to be made from the repressed prophage—most obviously the repressor protein itself—and the list of other proteins expressed from an otherwise repressed prophage is growing.²¹ These proteins all have the potential to cause lysogenic conversion of their host cell, that is, a change in cell phenotype as a consequence of the protein's expression. In many cases the biological function of these lysogenic conversion genes is not known, but when it is, it can typically be construed as providing a selective benefit to the host cell in which the prophage resides. Thus, the phage λ prophage expresses the repressor and three other proteins, SieB, RexA, and RexB, each of which protects the cell from infection by certain other phages, and it also expresses two

proteins, Bor and Lom, which are thought to make the lysogenic cell better able to parasitize a mammalian host.⁹ Other such proteins are described later. It has been suggested that these lysogenic conversion genes do not provide a selective benefit to the phage directly but instead provide a selective benefit indirectly by providing a direct benefit to the lysogenic cell in which the prophage is being replicated. In other words, the lysogenic conversion protein is the rent the prophage pays to its host. It may also be that lysogeny provides a rather efficient pathway for the bacterium to acquire novel beneficial genetic functions. That is, as random deletions occur over time in the bacterial chromosome, any that remove one of the beneficial lysogenic conversion genes will be counterselected, whereas deletions that remove other parts of the prophage will be either neutral or selected. Over time only the beneficial genes will remain as part of the bacterial genome.

One of the best-studied groups of lysogenic conversion genes is the group of prophage genes known to encode toxins produced by bacterial pathogens.²⁸ The toxins of the human diseases botulism, diphtheria, cholera, and hemolytic uremic syndrome (HUS), among others, and the animal disease ovine foot rot, are encoded by prophage genes. The Shiga-like toxin encoded by *E. coli* 0157:H7, the causative agent of HUS, is of particular interest. This toxin is expressed at low levels from its own promoter, but the higher levels of toxin required to produce severe disease are only made when the prophage is induced and the toxin is produced under the control of the phage's strong late promoter.¹⁸¹ Some antibiotics cause induction of this prophage, and for this reason antibiotic treatment is contraindicated for this infection. The cholera toxin case is also noteworthy in that the toxin is carried on a prophage of a filamentous ssDNA phage of the *Inoviridae* family.⁵¹ All the other known examples involve prophages of the tailed dsDNA phages of the *Caudovirales* order.

Because studies of lysogenic conversion have been carried out by human biologists, it is not surprising that some of the best-studied examples of lysogenic conversion involve toxins of human pathogens. However, it is unquestionably true that there are prophages supplying selectively beneficial functions to their host bacteria (and host archaea) in virtually all of the ecological situations where these cells find themselves.

PHAGE COMMUNITIES AND ECOLOGY

Every gram of soil and sediment, every milliliter of water, and the surfaces and insides of plants and animals are hosts to extensive and diverse phage and bacterial communities. We now know that these environmental phage communities contain most of the uncharacterized genetic diversity on Earth. And because of their rapid killing of large numbers of bacteria, they strongly influence the flow of carbon, phosphorus, and other elements within the biosphere.

Metagenomic analyses of viral communities, also called viromes, have shown that 1 kg of marine sediment may contain over a million different phage genotypes (roughly equivalent to a species).¹⁹ Similarly, there are hundreds of thousands of viral species in the ocean and soils.⁶¹ Viromes from stool samples show that most of the genomic differences between individual humans are gut-associated phages.¹⁵² There are even phages in our blood.⁶⁴

Phage could potentially protect against pathogens by killing them, but phage may actually be the causes in others. For example, many bacterial pathogens are actually lysogens and their prophages carry the disease-causing genes. For example, the common mouth microbe *Streptococcus mitis* carries the prophage SM1. When induced, the phage SM1 virions carry modified tail fibers known as pblA and pblB, which “glue” *S. mitis* cells together and to platelets. If this happens in the blood, say, after tooth cleaning, then the phage–platelet–microbe blob can cause endocarditis. Scarily, the phage SM1 is induced by soy sauce and other more commonly ingested substances; eat up!¹⁹⁴

Bacterial communities in the environment are often controlled in a top-down manner; that is, there is more food available than the microbes use.^{48,192} This occurs because they are killed off by phage and other predators like unicellular protists. The continuous predation by phage leads to “kill the winner” dynamics, where one phage kills so much of a particular bacterial strain that another strain grows up in the unoccupied niche. This means that the dominant bacterial strain within an ecosystem will be constantly changing. Recent work has shown that kill-the-winner dynamics does not change the species of bacteria; rather, it causes cycling of different strains.¹⁵⁶

Our understanding of phage in the environment is in its infancy. At this point we know that there are almost unimaginable numbers of them, and they contain most of the uncharacterized genomic diversity on the planet. It is clear that to maintain the observed steady state, phages are influencing both the types and replication of bacteria, and it is also clear that there is an amazing, essentially unexplored world of phage diversity to be studied.

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Prions

Prions Are Infectious Proteins

Prions Differ from Viruses

Prion Disease Paradigm

Molecular Biology of Prion Proteins

- Expression of the PrP Gene
- On the Function of PrP^C
- Overexpression of Wild-Type PrP Transgenes
- PrP Gene Dosage Controls the Incubation Time
- PrP-Deficient Mice Resist Prion Infection
- Species Variations in the PrP Sequence
- N-Terminal Sequence Repeats

Structures of Prion Protein Isoforms

- Subcellular Localization of PrP^C and PrP^{Sc}
- High-Resolution Structures of Recombinant PrP
- Electron Crystallography of PrP^{Sc}
- X-Ray Fiber Diffraction of PrP 27–30 Amyloid

Prion Replication

- On the Mechanism of Prion Propagation
- In vitro* Production of PrP^{Sc}
- Prion Formation and Clearance
- Dominant-Negative Inhibition

Diagnosis of Prion Diseases

- Postmortem Tissue Diagnosis of Prions
- Antemortem Diagnosis
- Bovine Spongiform Encephalopathy Testing

Molecular Pathogenesis of Prion Diseases

- Apoptosis in Prion Diseases
- Dendritic Atrophy and Notch-1
- Neurotoxic PrP Molecules
- Transmembrane PrP Molecules

Sporadic Human Prion Diseases

Heritable Human Prion Diseases

- Gerstmann-Sträussler-Scheinker and Genetic Linkage
- Familial Creutzfeldt-Jakob Disease Caused by Octarepeat Inserts
- Familial Creutzfeldt-Jakob Disease in Libyan Jews
- Fatal Insomnia
- Human PrP Gene Polymorphisms

Infectious Human Prion Diseases

- Human Growth Hormone
- Variant Creutzfeldt-Jakob Disease
- Transmission of Variant Creutzfeldt-Jakob Disease
- Prions by Blood Transfusion

Strains of Prions

- Isolation of New Strains
- Interplay Between the Species and Strains of Prions

Prion Diseases of Animals

- PrP Polymorphisms in Sheep, Cattle, and Elk
- Bovine Spongiform Encephalopathy
- Compelling Evidence for Transmission of Bovine Prions to Humans
- Chronic Wasting Disease

Fungal Prions

- Some Differences Between Fungal and Mammalian Prions

Prevention and Therapeutics for Prion Diseases

- Prion Therapeutics
- Inducible Transgenes as a Model for Therapeutics
- Quinacrine and Other Acridine Derivatives
- Anti-PrP Antibodies
- Bioluminescence Imaging for Monitoring Drug Efficacy
- Inactivation of Prions

Conclusions

- Wider Spectrum of Neurodegenerative Diseases Caused by Prions

Acknowledgments

Prions are infectious proteins that have been identified in both mammals and fungi. That prions are composed solely of proteins makes them unprecedented infectious pathogens. Prion proteins can adopt at least two different conformations; prions multiply by forcing the precursor protein to adopt an alternative conformation. The replication of prions is a self-propagating process, in which the prion isoform imposes its structure on the nonprion, precursor form of the protein. Prion-mediated conformational changes can function as robust epigenetic switches that modify protein functions and cellular phenotypes as well as cause fatal diseases. Different conformations of proteins in the prion state encipher distinct strains and are prone to aggregation. In mammals, some prions accumulate to high levels in the central nervous system (CNS) where they cause dysfunction and fatal degeneration.

Both mammalian and fungal prions have been produced in cell-free systems. Synthetic prion protein (PrP) peptides

TABLE 76.1 The Prion Diseases Caused by the Prion Protein

Disease	Host	Mechanism of pathogenesis
Kuru	Fore people	Infection through ritualistic cannibalism
Iatrogenic CJD	Humans	Infection from prion-contaminated HGH, dura mater grafts, and so on
Variant CJD	Humans	Infection from bovine prions
Familial CJD	Humans	Germline mutations in PrP gene
GSS	Humans	Germline mutations in PrP gene
FFI	Humans	Germline mutations in PrP gene (D178N, M129)
Sporadic CJD	Humans	Somatic mutation or spontaneous conversion of PrP ^C into PrP ^{Sc}
sFI	Humans	Somatic mutation or spontaneous conversion of PrP ^C into PrP ^{Sc}
Scrapie	Sheep	Infection in genetically susceptible sheep
Bovine spongiform encephalopathy	Cattle	Infection with prion-contaminated MBM
Transmissible mink encephalopathy	Mink	Infection with prions from sheep or cattle
Chronic wasting disease	Mule deer, elk	Unknown
Feline spongiform encephalopathy	Cats	Infection with prion-contaminated bovine tissues or MBM
Exotic ungulate encephalopathy	Greater kudu, nyala, oryx	Infection with prion-contaminated MBM

CJD, Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; sFI, sporadic fatal insomnia; GSS, Gerstmann-Sträussler-Scheinker disease; HGH, human growth hormone; MBM, meat and bone meal.

and recombinant (rec) PrP fragments have been used to form mammalian prions, whereas prion domains that are rich in glutamine and asparagine have been used to form fungal prions.

Although the PrP prions causing disease in mammals are infectious in the sense that they spread from cell to cell as well as from host to host, some prions do not move between cells, as in the case of yeast prions, and still others do not spread from one host to another as in the case of tau prions. In yeast, prions are clearly transmissible from mother to daughter cells but not between nondividing cells. Recent studies have demonstrated that several different proteins, each of which becomes a prion, cause distinct neurodegenerative diseases.

In mammals, PrP prions cause a group of invariably fatal, neurodegenerative diseases. Prion diseases may present as genetic, infectious, or sporadic disorders, all of which involve modification of PrP. The tertiary structure of PrPs is profoundly altered as prions are formed, and as such, prion diseases represent disorders of protein conformation. Creutzfeldt-Jakob disease (CJD) generally presents as a progressive dementia in humans, whereas scrapie of sheep and bovine spongiform encephalopathy (BSE) usually manifest as ataxic illnesses (Table 76.1).

A common feature of prions is the conformational transition of a precursor protein from a monomer to an oligomeric form. Although few proteins undergo the structural changes that transform them into prions, the prion precursor generally is a monomeric protein, but some precursors are multimers, such as the cellular PrP (PrP^C) dimer or the α -synuclein tetramer.^{19,183,251} Once formed, prion oligomers appear to bind

to other precursor protein monomers or multimers and induce the conformational change, thereby creating a self-propagating process. PrP oligomers possess a high β -sheet content and are more stable than the precursors; prion oligomers polymerize into amyloid fibrils.^{280,311,315}

PRIONS ARE INFECTIOUS PROTEINS

Mammalian prions reproduce by recruiting PrP^C and stimulating its conversion into the disease-causing isoform (PrP^{Sc}) (Table 76.2). PrP^C has a high α -helical content and little β -sheet structure, whereas PrP^{Sc} has less α -helical structure and a high β -sheet content. Comparisons of secondary structures of PrP^C and PrP^{Sc} were performed on proteins purified from Syrian hamster (SHa) brains.²⁸⁰ Limited proteolysis of PrP^{Sc} produces PrP 27–30, which retains prion infectivity; under these conditions, PrP^C is completely hydrolyzed (Fig. 76.1).

Using nuclear magnetic resonance (NMR) spectroscopy, solution structures of rec SHa and mouse (Mo) PrPs produced in bacteria showed three α -helices denoted A, B, and C as well as two short β -strands.^{223,324} These recPrPs are thought to have structures similar to those of PrP^C.¹⁶⁰ The atomic structure of PrP^{Sc} has not been determined because of the insolubility of the protein. Electron crystallographic and x-ray diffraction studies of PrP 27–30 have been used to constrain computational models of PrP^{Sc}, suggesting it contains α -helix C and a portion of α -helix B, whereas α -helix A, the two β -strands, and the surrounding segments form a β -helix.^{146,434} Fiber diffraction studies of PrP^{Sc} and PrP 27–30 support the model with a

TABLE 76.2 Glossary of Prion Terminology

Term	Description
Prion	A proteinaceous infectious particle that lacks nucleic acid and can self-propagate.
PrP ^{Sc}	Abnormal, pathogenic isoform of the prion protein that causes illness. This protein is the only identifiable macromolecule in purified preparations of prions causing the diseases listed in Table 76.1.
PrP ^C	Cellular isoform of the prion protein.
PrP 27–30	N-terminally truncated PrP ^{Sc} , generated by digestion with proteinase K.
PRNP	Human PrP gene located on chromosome 20.
Prnp	Mouse PrP gene located on syntenic chromosome 2. <i>Prnp</i> controls the length of the prion incubation time and is congruent with the incubation time genes <i>Sinc</i> and <i>Prn-i</i> . PrP-deficient (<i>Prnp</i> ^{0/0}) mice are resistant to prion infection.
PrP amyloid	Fibril of PrP fragments derived from PrP ^{Sc} by proteolysis. Plaques containing PrP amyloid are found in the brains of some mammals with prion disease.
Prion rod	An amyloid polymer composed of PrP 27–30 molecules. Created by detergent extraction and limited proteolysis of PrP ^{Sc} .
Protein X	A hypothetical macromolecule that is believed to act as a molecular chaperone in facilitating the conversion of PrP ^C into PrP ^{Sc} .

β -helix described earlier.⁴³¹ The authors of a recent investigation argue that PrP^{Sc}(Δ GPI) formed from anchorless PrP expressed in transgenic (Tg) mice is composed entirely of β -strands based on findings from H-D exchange studies.³⁶⁷

Electron crystallography of two-dimensional (2D) crystals suggest a trimeric arrangement of PrP 27–30 molecules within a unit cell, and ionizing radiation inactivation analyses have suggested that the infectious monomer is a trimer of PrP^{Sc} molecules.^{20,146} Other studies have shown that prion infectivity may exist in a wide variety of sizes, presumably due to the hydrophobic properties of PrP^{Sc}.³¹³ To date, it has not been possible to solubilize native, infectious PrP^{Sc} except by dissolving the prions into liposomes.¹¹⁸ Purified prion rods composed of PrP 27–30 were fragmented by sonication into spherical particles measuring 9 to 29 nm in diameter without any loss of infectivity.²⁴⁷ A more recent study showed that infectious prions particles measuring 17 to 27 nm in diameter and consisting of 14 to 28 PrP^{Sc} molecules could be obtained by partial disaggregation using the detergent Na *n*-undecyl SO₄.³⁶⁵

Recombinant antibody fragments have been used to map the surfaces of PrP^C and PrP^{Sc}, and those results are consistent with the foregoing structural data.²⁹² The structural transition from an α -helical-rich or unstructured protein into a β -sheet-rich molecule seems to be the fundamental event underlying the formation of nascent prions.

Limited proteolysis truncates the N-terminus of PrP^{Sc} to produce PrP 27–30 consisting of the C-terminal ~142 amino acids (Table 76.2) (Fig. 76.2). PrP 27–30 polymerizes into

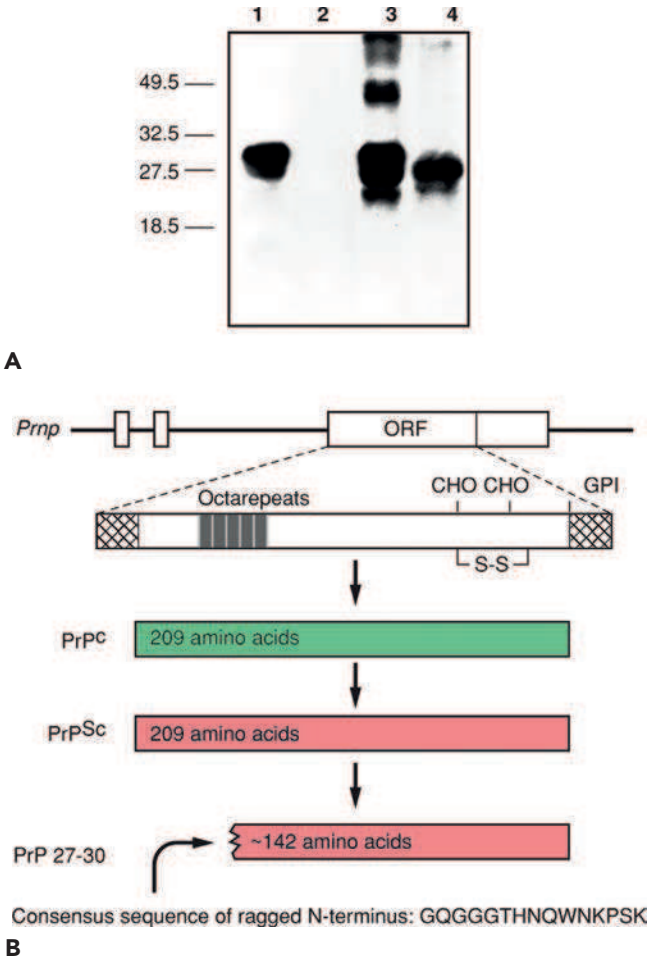


FIGURE 76.1. Prion protein isoforms. A: Western immunoblot of brain homogenates from uninfected (lanes 1 and 2) and prion-infected (lanes 3 and 4) Syrian hamsters. Samples in lanes 2 and 4 were digested with 50 $\mu\text{g mL}^{-1}$ of proteinase K for 30 minutes at 37°C. Cellular prion protein (PrP^C) in lanes 2 and 4 was completely hydrolyzed under these conditions, whereas approximately 67 amino acids were digested from the N-terminus of the disease-causing isoform (PrP^{Sc}) to generate PrP 27–30. After polyacrylamide gel electrophoresis (PAGE) and electrotransfer, the blot was developed with anti-SHaPrP R073 polyclonal rabbit antiserum.³⁵⁴ Molecular weight markers are depicted in kD. **B:** Diagram of the SHaPrP gene that encodes a protein of 254 amino acids. After processing of the N- and C-termini, both PrP^C and PrP^{Sc} consist of 209 residues. After limited proteolysis, the N-terminus of PrP^{Sc} is truncated to form PrP 27–30, which is composed of approximately 142 amino acids, the N-terminal sequence of which was determined by Edman degradation.

amyloid fibrils that are indistinguishable from fibrils found in amyloid plaques of the brains of mammals with prion disease.^{92,315} When full-length PrP^{Sc} was purified from SHa brain, only amorphous aggregates were seen by electron microscopy.²⁴⁸ Although limited digestion of purified PrP^{Sc} with proteinase K (PK) produced PrP 27–30, the ultrastructural appearance of the samples remained unchanged. Addition of a detergent, such as Sarkosyl, either before or after limited proteolysis, provoked the assembly of PrP 27–30 into rod-shaped structures with the ultrastructural and tinctorial properties of amyloid (Fig. 76.2).

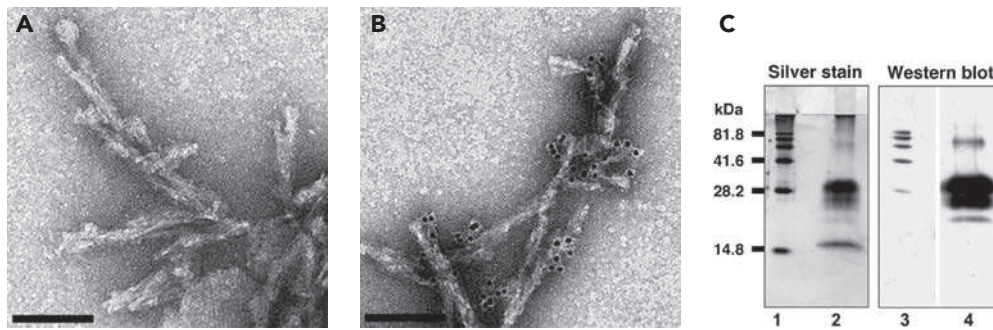


FIGURE 76.2. Electron micrographs of negatively stained and immunogold-labeled Syrian hamster (SHa) prion rods. A: Prion rods recovered from sucrose gradients were negatively stained with 2% uranyl acetate. The prion rods are indistinguishable from many purified amyloids. **B:** Prion rods pretreated with 3 M urea and then immunostained with F4-31 mAbs derivatized with 10-nm gold particles. Bars in **A** and **B** represent 100 nm. **C:** Prion rods are composed of the SHaPrP 27–30 protein shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). **Left Panel:** gel stained with silver. **Right panel:** Western blot developed with polyclonal anti-PrP rabbit antiserum W5517.

It is important to realize that the formation of PrP amyloid is a nonobligatory feature of the PrP prion diseases. Only ~10% of all cases of sporadic CJD (sCJD) have detectable PrP amyloid deposits.⁹¹ In Tg mice expressing both MoPrP and SHaPrP, amyloid plaques were found in those Tg mice inoculated with Sc237 hamster prions but not in the Tg mice inoculated with RML mouse prions.³¹⁶ In studies of non-Tg mice, amyloid deposition was found to depend on the strain of scrapie prion.⁴²

PRIONS DIFFER FROM VIRUSES

Prions differ from viruses in many respects. In contrast to viruses that possess an RNA or DNA genome, prions are devoid of a nucleic acid genome that directs the synthesis of their progeny. Many investigators argued for a nucleic acid genome within the infectious prion particle, whereas others contended for a small, noncoding polynucleotide of either foreign or cellular origin. No nucleic acid has been found, despite intensive searches using a wide variety of techniques and approaches.³³⁶

Prions are composed entirely of protein. In mammals, the most well-studied prions are composed of PrP^{Sc}, which is formed from PrP^C expressed normally in all mammals studied to date. PrP is encoded by a chromosomal gene, designated *PRNP* in humans and *Prnp* in mice. The PrP gene is located on the short arm of chromosome 20 in humans and the syntenic region of chromosome 2 in mice.³⁷⁰ That prions are composed entirely of protein is supported by numerous studies. The most convincing of these studies are those describing the creation of prion infectivity by modifying the conformation of a synthetic or recombinant polypeptide. Such studies have been reported in both mammals and fungi; they are reviewed in detail below.

In contrast to viruses, prions are nonimmunogenic. Mammals fail to mount an immune response to PrP^{Sc}. The lack of an immune response presumably reflects the sharing of epitopes by PrP^C and PrP^{Sc},²⁹² as well as tolerance to PrP^C.³¹² Because viral proteins are encoded by the genome of a foreign pathogen, these proteins often elicit a profound immune response, which is the basis for vaccination.

When prions and viruses are passed from one host species to another, the consequences are quite different. The passage of prions from one host to another is accompanied by the acquisition of a new PrP amino acid sequence encoded by the genome of the new host. The crossing of prions from one species to another is restricted by what has been called the “species barrier”.²⁸⁵ Prion replication is more likely to occur when the evolutionary relationship is close between the newly infected animal and the host in which the prions last replicated.³⁴⁸ However, some strains of prions seem to be exceptions to this rule. For example, variant CJD (vCJD) prions from humans replicate much more readily in Tg mice expressing bovine (Bo) PrP than in Tg mice expressing human (Hu) or chimeric human-mouse PrP.^{14,204,352}

Although strains of viruses are encoded in nucleic acid genomes, no information resides in polynucleotides for prion strains. The issue of prion strains posed a profound conundrum for many years. How could an infectious pathogen composed only of protein encipher biological information? This riddle was solved when prion strains with different physical properties were isolated.^{25,401} Subsequently, new strains of prions have been isolated either by passage through mice expressing artificial PrP transgenes or by forming synthetic prions from recPrP produced in *Escherichia coli*.^{221,291}

The dramatically different principles that govern prion biology from those underpinning the viral diseases are frequently misunderstood. This lack of understanding has led to some regrettable decisions of great economic, political, and possibly public health importance. For example, scrapie and BSE have different names, yet they are the same disease in two different species. Scrapie and BSE differ in only two respects: (a) the PrP sequence in sheep differs from that of cattle at seven or eight positions of 270 amino acids,¹⁴² which results in different PrP^{Sc} molecules; and (b) most scrapie strains of prions seem to differ from BSE strains.

PRION DISEASE PARADIGM

Despite some similarities between prion and viral illnesses, these disorders are quite different. Viral diseases are infectious

illnesses and, as such, begin with infection by exogenous virions. In contrast, most prion diseases are initiated from within the host by a mechanism that causes prions to arise spontaneously. Often the term “prion infection” is used synonymously with “prion disease” because once prions form spontaneously, they can be transferred to another host and, thus, are infectious.

The prion diseases are uniformly fatal. No human or animal has ever recovered from a prion disease once neurologic dysfunction is manifest. No host defenses are mounted in response to prion infection: No humoral immunity, no cellular immunity, and no interferons are elicited to the replicating prion.

In humans, the PrP prion diseases occur in three different forms: (a) sporadic, (b) inherited, and (c) infectious (Table 76.1). In the sporadic and inherited forms of prion disease, prions arise spontaneously. Although the incidence of sporadic prion disease is low (one to five cases per 10⁶ people), this is the most common form of prion disease, accounting for approximately 90% of all cases. The inherited forms of prion disease account for ~10% of all cases of prion disease. In contrast, the infectious forms of human prion disease account for <1% of all cases. Whether the infectious forms of human prion disease are underestimated and whether low levels of animal prions in the food supply are responsible for 10% to 20% of the sporadic cases are unknown.

Molecular genetic studies have been crucial in deciphering the novel features of the prion disease paradigm. In the sporadic form of prion disease, the sequence of the PrP gene is wild-type (wt), whereas in the inherited prion diseases, the sequence of the PrP gene harbors a nonconservative substitution or insertion. As noted previously, both the sporadic and inherited prion diseases arise spontaneously. In contrast, the infectious prion diseases result from exposure to an exogenous source of prions. Generally, the PrP genes of humans and animals infected with exogenous prions are wt (i.e., these genes do not carry a mutation).

Before the discovery that mutations in the PrP gene cause familial prion disease, geographic clusters of prion disease were believed to be due to common-source exposures to exogenous prions. For example, Libyan Jews with a very high incidence of CJD were believed to have contracted the disease by eating lightly cooked sheep brain.⁶ Molecular genetic investigations showed that every Libyan Jew developing prion disease carried a PrP gene mutation, resulting in an E-to-K substitution at position 200 (Table 76.3).^{139,166} Risk analysis studies revealed that every Libyan Jew carrying the E200K mutation would eventually develop prion disease if he or she did not otherwise die of some other illness.

Investigations of humans with PrP gene mutations were extended to Tg mice. Tg mice expressing high levels of MoPrP(P101L), the analogous mutation causing Gerstmann-Sträussler-Scheinker (GSS) disease in humans, developed neurodegeneration spontaneously.⁴⁰⁰ Extracts prepared from the brains of these mice transmitted disease after approximately 250 days to other Tg mice (designated Tg196) expressing low levels of MoPrP(P101L). Subsequently, a synthetic PrP peptide of 55 residues carrying the P101L mutation, designated MoPrP(89–143,P101L), was produced and inoculated into the Tg196 mice.¹⁸⁶ The Tg196 mice developed CNS dysfunction about 1 year after inoculation, and brain extracts from the ill mice were found to produce disease on serial passage.^{268,405}

TABLE 76.3 Examples of Human PrP Gene Mutations Found in the Inherited Prion Diseases

Inherited prion disease	PrP gene mutation
Gerstmann-Sträussler-Scheinker disease	P102L ^a
Gerstmann-Sträussler-Scheinker disease	A117V
Familial Creutzfeldt-Jakob disease	D178N, V129
Fatal familial insomnia	D178N, M129 ^a
Gerstmann-Sträussler-Scheinker disease	F198S ^a
Familial Creutzfeldt-Jakob disease	E200K ^a
Gerstmann-Sträussler-Scheinker disease	Q217R
Familial Creutzfeldt-Jakob disease	Octarepeat insert ^a

^aSignifies genetic linkage between the mutation and the inherited prion disease.

The MoPrP(89–143,P101L) peptide produced disease in the Tg196 mice only if it was folded into a β -rich conformation.

An approach similar to the one used in the studies with the MoPrP(89–143,P101L) peptide was employed with wt PrP. In these studies, wt MoPrP(89–230) was produced in *E. coli*, purified by chromatography, and polymerized into amyloid fibrils.^{77,79,220} The amyloid fibrils were injected into Tg mice expressing MoPrP(89–230) and produced neurodegeneration after approximately 500 days. Brain extracts from the ill Tg mice contained protease-resistant PrP and produced disease on subsequent passage into both wt and Tg mice.²²¹ Several investigators have developed alternative approaches that have also generated prions *de novo*.^{93,231} Taken together, these studies demonstrated that only PrP^C is required to generate prion infectivity and, as such, spontaneous forms of prion disease can occur in any mammal because PrP^C seems to be ubiquitous among this class of vertebrates.

Spontaneous prion disease contrasts with viral disorders, where exogenous infection is required, except in the case of latent retroviral genomes. For example, after infection with exogenous HIV, the virus may disappear, but often its RNA genome has been reverse-transcribed into DNA, and the DNA copies may remain dormant for years.

MOLECULAR BIOLOGY OF PRION PROTEINS

PrP is encoded by a chromosomal gene denoted *Prnp*, which is a member of the *Prn* gene family. The second member of this family to be identified is the *Prnd* gene that lies approximately 19 kb downstream from the PrP locus and encodes the doppel (Dpl) protein.²⁶¹ The respective genes that encode PrP and Dpl appear to represent ancient gene duplication that occurred prior to the speciation of mammals. The sequences are approximately 25% identical, but the structures of the two proteins are highly conserved²⁶¹ (Fig. 76.3). In contrast to PrP, which is expressed in many different tissues, Dpl expression is confined to the testis. Both Dpl and PrP are found on the surface of sperm, but their functions are unknown. In contrast to PrP-deficient (*Prnp*^{0/0}) mice, Dpl-deficient (*Prnd*^{0/0}) mice are sterile. The knockout of both PrP and Dpl genes resulted in a sterile phenotype.²⁷⁸ A third member of the *Prn* gene family is *Sprn*, which encodes the Shadoo (Sho) protein. Sho shares some structural features with

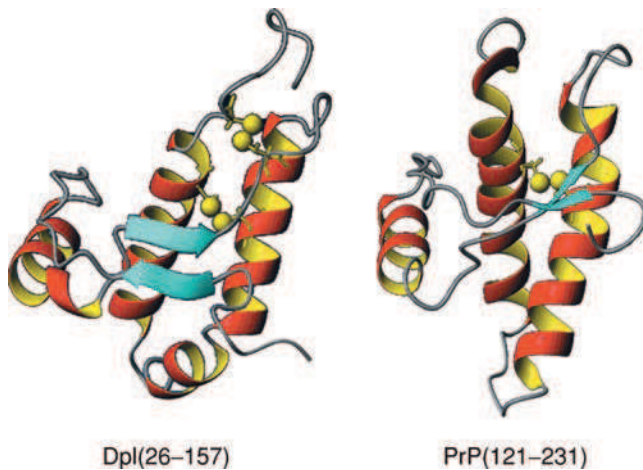


FIGURE 76.3. Comparison of the nuclear magnetic resonance (NMR) structures of mouse doppel (Dpl) protein and prion protein (PrP). Backbone topology of mouse Dpl(26-157)²⁵⁹ and mouse PrP(121-231).³²⁴ (Figure prepared by Jane Dyson and Peter Wright.)

PrP and/or Dpl, such as a glycosylphosphatidyl inositol (GPI) anchor, an N-linked complex oligosaccharide, a central hydrophobic tract, and N-terminal amino acid repeats.^{303,418} Notably, the level of Sho was inversely proportional to the level of PrP^{Sc} in the brains of prion-infected mice.⁴¹⁷

In two *Prnp*^{0/0} lines, Purkinje cell loss was accompanied by ataxia beginning at approximately 70 weeks of age.³³⁸ Crossing one of these *Prnp*^{0/0} lines with Tg mice overexpressing MoPrP rescued the ataxic phenotype. This conundrum was solved with the discovery of Dpl; it became clear that Dpl expression in the brains of *Prnp*^{0/0} mice provoked cerebellar degeneration.²⁶¹ The expression of Dpl in the CNS was due to intergenic splicing of the nontranslated exons of the PrP gene with the translated exon of Dpl. That Dpl expression is neurotoxic was demonstrated by construction of Tg mice expressing Dpl in the brain.^{262,329}

The entire ORF of all known mammalian and avian PrP genes resides within a single exon, which eliminates the possibility that PrP^{Sc} arises from alternative RNA splicing. The two exons of the SHaPrP gene are separated by a 10-kb intron; exon 1 encodes a portion of the 5' untranslated leader sequence. The PrP genes of Syrian hamster, mouse, sheep, and rat contain three exons, with exon 3 encoding the ORF and 3' untranslated region. The promoters of both the SHaPrP and MoPrP genes contain multiple copies of G-C-rich repeats and are devoid of TATA boxes. These G-C nonamers represent a motif that may function as a canonic binding site for the transcription factor Sp1.

Expression of the PrP Gene

Although PrP messenger RNA (mRNA) is constitutively expressed in the brains of adult animals,^{68,274} it is highly regulated during development. In the septum, levels of PrP mRNA and choline acetyltransferase were found to increase in parallel during development. In other brain regions, PrP gene expression occurs at an earlier age. *In situ* hybridization studies show that the highest levels of PrP mRNA are found in neurons.²¹⁰

Although PrP^C is expressed in many tissues,²⁷⁴ only a few cell types appear to support PrP^{Sc} formation: neurons, myo-

cytes, follicular dendritic cells, and B lymphocytes.^{30,196,318} Whether astrocytes support PrP^{Sc} formation is debatable. Curiously, PrP^{Sc} formation appears to be elevated in lymphocytes, where an inflammatory response has been provoked.¹⁵⁵ That PrP^{Sc} formation occurs in a restricted set of cells, despite widespread expression of PrP^C, would seem to argue that auxiliary molecules (e.g., protein X) feature in prion replication.

On the Function of PrP^C

Although the function of PrP^C has eluded definition for many years, an enlarging body of evidence is accumulating that argues in favor of a role for PrP^C in signal transduction. Disruption of the PrP gene in mice results in animals with normal development, a normal lifespan, and no obvious abnormality.^{46,236} *Prnp*^{0/0} mice remain healthy for more than 2 years. Acute suppression of PrP expression by addition of doxycycline to the drinking water of bigenic mice with an inducible PrP transgene under the control of the tetracycline promoter did not result in any untoward effects in the adult mice.^{334,406} Similarly, bigenic mice, in which neuronal PrP expression was terminated in adulthood, showed no discernible deficits.²³⁴

In one study, *Prnp*^{0/0} mice were found to have abnormal sleep/wake cycles and altered circadian rhythms.⁴⁰⁴ Other investigators reported attenuation of long-term potentiation (LTP) and several altered electrophysiologic parameters of the hippocampus in *Prnp*^{0/0} mice. Another study described deficits in hippocampal-dependent spatial learning and LTP in the dentate gyrus.⁸⁷

One approach to elucidating the function of PrP^C has been to identify interacting molecules.³⁴⁴ PrP^C binds copper,^{161,379} and five octapeptide repeats are responsible for the coordination of bivalent copper ions. PrP^C interacts or associates with a number of proteins, including bacterial HSP-60; the 37-kD/67-kD laminin receptor³²²; laminin; the Grb2 protein, which is central in many signal transduction pathways²⁶⁵; the lipid raft protein caveolin-1; and the neuronal cell adhesion molecule (N-CAM).³⁴⁵ Although these studies identified molecules interacting with PrP^C, they did not clarify its function.

The association of PrP with N-CAM on the surface of mouse neurons was found to result in recruitment of N-CAM to lipid rafts and activation of the p59Fyn kinase.³⁴¹ In an earlier study, binding of anti-PrP antibodies to the surface of 1C11 neuronal cells resulted in (a) activation of p59Fyn kinase,²⁶⁵ (b) production of NADPH oxidase-dependent reactive oxygen species, and (c) phosphorylation of the MAP extracellular regulated kinases 1/2 (ERK1/2).³⁴⁶

A role for PrP^C in axon growth was proposed after increased expression of PrP^C was detected on the surface of elongating retinal axons in hamsters,³³⁹ and a PrP-Fc fusion protein was shown to bind strongly to the granule cell layer of mouse cerebellum, suggesting the presence of one or more PrP^C-interacting ligands in this region. In studies that did not distinguish axons and dendrites, exposure of neonatal mouse cerebellar or hippocampal neurons to a PrP-Fc fusion protein enhanced the total length of neurites by a factor of ~1.5.⁶⁶ In a more recent study, the effect of PrP-Fc on neurite outgrowth in cultures of mouse neurons has been suggested to be mediated through its binding to N-CAM.³⁴¹

When purified SHa or Mo recPrP in an α -helical-rich conformation was added to the media of cultured rat fetal hippocampal neurons,¹⁸⁵ overnight exposure resulted in (a) a 2-fold increase in neurons with a differentiated axon, (b) a 14-fold increase in neurons with differentiated dendrites, (c) a

5-fold increase in axon length, and (d) the formation of extensive neuronal circuitry. Formation of synaptic-like contacts was increased by a factor of five after exposure to recPrP for 7 days. Neither the N-terminal nor C-terminal domains of recPrP or of Dpl enhanced the polarization of neurons. Inhibitors of PKC and Src kinases, including p59Fyn, blocked the effect of recPrP on axon elongation, whereas inhibitors of phosphatidylinositol 3-kinase showed partial inhibition, suggesting that signaling cascades involving these kinases are candidates for transduction of recPrP-mediated signals. The foregoing results argue that full-length PrP^C functions as a “growth factor” involved in the development of neuronal polarity.^{185,265}

Consistent with the foregoing results on neuronal cells, studies of T-lymphocyte activation also argue that PrP^C functions in signal transduction. Addition of anti-PrP antibodies to cultured T lymphocytes produced capping where PrP^C bound the reggie-1 and reggie-2 proteins.³⁸¹ PrP^C in association with the reggie (or flotillin) proteins clusters with p59Fyn and other kinases in caps where elevated phosphorylation of ERK1/2 was found.

Overexpression of Wild-Type PrP Transgenes

Mice expressing the wt SHaPrP transgene were inoculated with SHa prions. The abbreviated incubation times exhibited by these Tg mice demonstrated abrogation of the species barrier.³⁴⁸ The length of the incubation time after inoculation with SHa prions was inversely proportional to the level of SHaPrP^C in the brains of Tg(SHaPrP) mice.³¹⁶

Bioassays of brain extracts from clinically ill Tg(SHaPrP) mice inoculated with SHa prions revealed that SHa prions, but not Mo prions, were produced. Conversely, inoculation of Tg(SHaPrP) mice with Mo prions led to the synthesis of only Mo prions.

PrP Gene Dosage Controls the Incubation Time

Incubation times have been used to isolate prion strains inoculated into sheep, goats, mice, and hamsters.⁹⁸ The *Sinc* gene is a major determinant of incubation periods in mice. Once molecular clones of *Prnp* became available, a study showed that the incubation time locus, designated *Prn-i*, was found to be either congruent with or closely linked to *Prnp*.⁵⁴

Although the amino acid substitutions in PrP that distinguish *Prnp*^a from *Prnp*^b mice argued for the congruency of *Prnp* and *Prn-i*, experiments with *Prnp*^a mice expressing *Prnp*^b transgenes demonstrated a “paradoxical” shortening of incubation times. From studies of congenic and Tg mice expressing different numbers of the *a* and *b* alleles of *Prnp*, these findings were discovered not to be paradoxical but to result from increased PrP gene dosage.⁵³ When the RML isolate was inoculated into congenic and Tg mice, increasing the number of copies of the *a* allele was found to be the major determinant in reducing the incubation time; however, increasing the number of copies of the *b* allele also reduced the incubation time, but not to the same extent as that seen with the *a* allele. Gene targeting studies established that the *Prnp* gene controls the incubation time and, as such, is congruent with both *Prn-i* and *Sinc*.²⁶⁰

PrP-Deficient Mice Resist Prion Infection

Prnp^{0/0} mice inoculated with prions resist infection.^{45,312} *Prnp*^{0/0} mice were sacrificed 5, 60, 120, and 315 days after inoculation with RML prions, and brain extracts were bioassayed in Swiss CD-1 mice. Except for residual infectivity from the inoculum

detected at 5 days, all bioassay mice remained healthy. Introduction of a foreign PrP gene into *Prnp*^{0/0} mice renders the mice susceptible to the corresponding foreign prions.^{45,312,351,402}

Because *Prnp*^{0/0} mice do not express PrP^C, we reasoned that they might more readily produce α -PrP antibodies. *Prnp*^{0/0} mice immunized with Mo or SHa prion rods produced α -PrP antisera that bound MoPrP, SHaPrP, and HuPrP.³¹² That *Prnp*^{0/0} mice readily produce α -PrP antibodies is consistent with the hypothesis that mice are tolerant to PrP^C, and that PrP^C and PrP^{Sc} share many epitopes.

Species Variations in the PrP Sequence

PrP is posttranslationally processed to remove a 22-amino acid, N-terminal signal peptide. The C-terminal 120 amino acids contain two conserved disulfide-bonded cysteines and a sequence that beckons addition of a GPI anchor. Twenty-three residues are removed during the addition of this GPI moiety, which anchors the protein to the cell membrane.³⁷³ Contributing to the mass of the protein are two Asn side chains linked to large oligosaccharides with multiple structures that have been shown to be complex and diverse.¹⁰⁶ Although many species variants of PrP have now been sequenced, only the chicken sequence has been found to differ greatly from the human sequence. The alignment of the translated sequences from more than 40 PrP genes shows a striking degree of conservation between the mammalian sequences and is suggestive of the retention of some important function through evolution (Fig. 76.4). Cross-species conservation of PrP sequences makes it difficult to draw conclusions about the functional importance of many of the individual residues in the protein.

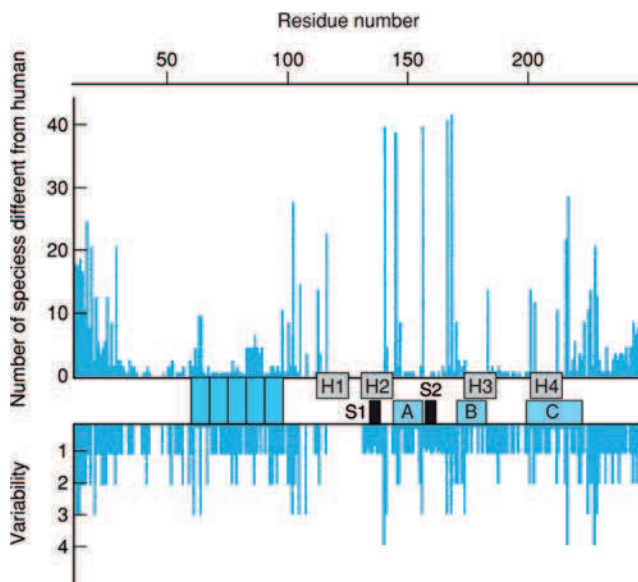


FIGURE 76.4. Species variations of PRNP. The x-axis represents the human prion protein (PrP) sequence with the five octarepeats and H1–H4 regions of putative secondary structure shown as well as the three α -helices A, B, and C and the two β -strands S1 and S2 as determined by nuclear magnetic resonance (NMR). Vertical bars above the axis indicate the number of species that differ from the human number of alternative amino acids at each position in the alignment. The vertical bars below the axis indicate variability for each PrP residue. (Data were compiled by Paul Bamorough and Fred E. Cohen.)

Notably, some investigators have found using low stringency analyses that zebrafish have candidate genes that encode proteins possessing many of the structural features of mammalian PrPs but relatively low sequence homology of ~20%.³²⁵ Two duplicated candidate piscine PrPs, designated PrP-1 and PrP-2, are expressed at high levels in the adult and developing brain. Knockdown of zebrafish PrP-1 prevented embryos from carrying out gastrulation and led to early developmental arrest. In contrast, PrP-2 depletion did not affect gastrulation but produced embryos with severely malformed heads and eyes. Remarkably, the PrP-1–arrested phenotype could be rescued not only by PrP-1, but also partially by PrP-2 and even mouse PrP mRNAs, strongly supporting the notion of functional homology between fish and mammalian PrPs.²³² PrPs have also been investigated from puffer fish and salmon.²³²

N-Terminal Sequence Repeats

The N-terminal domain of mammalian PrP contains five copies of a P(H/Q)GGG(G)WGQ octarepeat sequence, occasionally more, as in the case of one sequenced bovine allele, which has six copies. Although insertions of extra repeats have been found in patients with familial prion disease, naturally occurring deletions of single octarepeats do not appear to cause disease, and deletion of these repeats does not prevent PrP^C from undergoing a conformational transition to form PrP^{Sc}.^{110,386}

Using full-length recPrP, Cu²⁺ was found to bind with a much higher avidity than any other metal ion, but the concentration for half-maximal binding for Cu²⁺ was 14 μ M at pH 6.0, indicating a rather low affinity of PrP for Cu²⁺.³⁷⁹ At neutral pH, Cu²⁺–PrP complexes tended to form large aggregates and precipitate, so synthetic peptides containing the octarepeats were used to study the interaction of Cu²⁺ with PrP.¹⁶¹ When the PrP(58–91) peptide containing four octarepeats was studied, the binding of Cu²⁺ was found to be cooperative and highly pH dependent (Fig. 76.5). The midpoint of the pH dependence transition was pH 6.7, suggesting that the binding of Cu²⁺ occurred through the imidazole nitrogens of histidine residues. Because of these histidine residues, it was suggested that immobilized metal ion affinity chromatography (IMAC) might facilitate the purification of PrP^{Sc}; indeed, IMAC did prove to be useful in the purification of PrP^C.

In studies of full-length recPrP, Cu²⁺ was found to catalyze the oxidation of the histidine residues within the octarepeats. These findings argue that PrP may function as Cu²⁺-binding protein.³²¹ Interestingly, the copper-chelating reagent cuprizone administered to rodents causes spongiform degeneration resembling morphologically that induced by prions.²⁸⁶

STRUCTURES OF PRION PROTEIN ISOFORMS

When no chemical modifications differentiating PrP^C from PrP^{Sc} were found,³⁷⁴ the search for a conformational change that distinguishes the two PrP isoforms ensued. Fourier transform infrared (FTIR) and circular dichroism spectroscopy studies showed that PrP^C contains approximately 40% α -helix and little β -sheet, whereas PrP^{Sc} appeared to contain approximately 30% α -helix and 45% β -sheet.²⁸⁰ That the two PrP isoforms have the same amino acid sequence ran counter to the widely accepted view that the amino acid sequence specifies only one biologically active conformation of a protein.⁸ Like

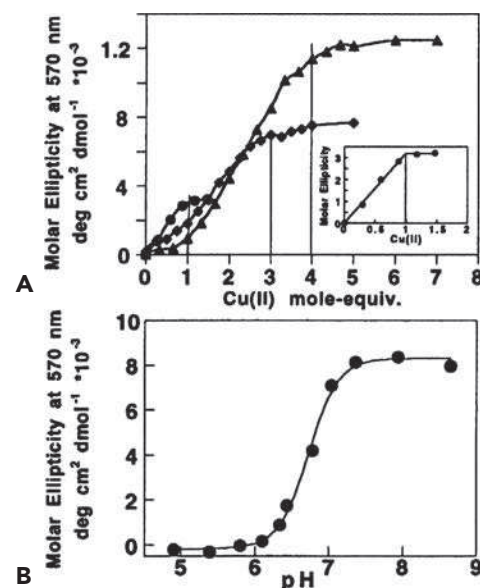


FIGURE 76.5. Binding of copper ions to synthetic peptides containing the octarepeats of the prion protein (PrP). **A:** Cu(II) binding curves: molar ellipticity at 570 nm with increasing amounts of Cu(II), pH 7.5. Filled circles, 2-His peptide, PrP(51–75) (0.34 mM). Filled diamonds, 3-His peptide, PrP(66–91) (0.021 mM). Filled triangles, 4-His peptide, PrP(58–91) (0.033 mM). **B:** pH dependence of the ellipticity at 570 nm for PrP(58–91). The pH dependence curve has been fitted to the following equation: $\Delta\epsilon_{\text{obs}} = [\Delta\epsilon_{\text{acid}}[H^+]^n + \Delta\epsilon_{\text{basal}}[H^+]Ka]/[H^+]^n + Ka$, in which n = Hill coefficient and Ka = acid dissociation constant for the transition. The midpoint of the transition is pH 6.7. (Reprinted from Viles JH, Cohen FE, Prusiner SB, et al. Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc Natl Acad Sci U S A* 1999;96:2042–2047, with permission; copyright © 1999 National Academy of Sciences, USA.)

PrP^{Sc}, PrP 27–30 has high β -sheet content,⁶³ which is consistent with the earlier finding that PrP 27–30 polymerizes into amyloid fibrils.³¹⁵ Recent H-D exchange experiments raise the possibility that PrP^{Sc} does not retain any α -helical structure³⁶⁷; the authors question the validity of earlier optical spectroscopy measurements.^{63,123,280}

Subcellular Localization of PrP^C and PrP^{Sc}

Prior to comparative studies on the structures of PrP^C and PrP^{Sc}, metabolic labeling studies showed that the acquisition of protease resistance in PrP^{Sc} is a posttranslational process.²⁹ In a search for chemical differences that would distinguish PrP^{Sc} from PrP^C, ethanolamine was identified in hydrolysates of PrP 27–30, which signaled the possibility that PrP might contain a GPI anchor.³⁷⁵ Both PrP isoforms were found to carry GPI anchors. PrP^C was found on the surface of cells, where it could be released by cleavage of the anchor. Subsequent studies showed that PrP^{Sc} formation occurs after PrP^C reaches the cell surface⁶¹ and localizes to caveolae-like domains (CLDs).^{144,396}

When PrP was directed to the cytoplasm of neurons in Tg mice, neurodegeneration was observed²²⁹; however, attempts to demonstrate the spontaneous formation of prion infectivity were unsuccessful. Although these Tg mice were initially heralded as a model for sCJD, their relevance remains unclear.

In other studies, the role of the GPI anchor that attaches PrP^C to the external surface of the plasma membrane³⁷⁵ was initially studied by expressing the mutant in scrapie-infected neuroblastoma (ScN2a) cells.³²⁶ In these scrapie-infected cells, anchorless PrP was converted into a protease-resistant molecule, arguing that it had acquired prion infectivity. In the brain, secreted forms of PrP^C lacking a GPI anchor are generated by sheddase cleavage of membrane-anchored PrP^C³⁹⁹; the physiological role of such unanchored PrP isoforms, termed PrP(Δ GPI), remains unclear. In earlier studies, Tg mice expressing low levels of PrP(Δ GPI) did not develop signs of spontaneous neurologic illness, but harbored large amyloidogenic PrP(Δ GPI) aggregates in the brain after exposure to prions.^{67,69} Mutations in human PrP leading to C-terminally truncated polypeptides lacking the GPI signal sequence, which cause the inherited prion disorder GSS, are known to result in the production of secreted, highly amyloidogenic forms of PrP.¹³¹ Other mutations that do not lead to truncated PrP but cause GSS, such as the F198S mutation, result in the production of anchorless PrP upon expression in cell culture.¹⁹¹ Importantly, GSS patients with mutations in PrP that result in the production of nearly full-length anchorless PrPs¹⁷⁶ suggest that anchorless PrP might be a cause of GSS-like neuropathology and clinical disease.

To investigate further the role of the GPI anchor in the biology of prions, several lines of Tg mice expressing various levels of PrP(Δ GPI) were established.³⁸⁰ The Tg mice expressing low levels of PrP(Δ GPI) remained healthy and exhibited little or no neuropathologic changes like those reported by others.⁶⁹ In contrast, neuronal expression of PrP(Δ GPI) at 1.7 \times levels compared to PrP^C expression in wt mice resulted in late-onset, spontaneous neurologic illness accompanied by a CNS amyloidosis resembling that found in GSS.³⁸⁰ This amyloidosis was transmissible to mice of the same line and to mice expressing wt GPI-anchored PrP^C. Mice expressing both PrP(Δ GPI) and wt PrP^C showed earlier onset of neurologic illness compared to those expressing only anchorless PrP. Neurologic illness was not hastened if Tg mice coexpressed PrP(Δ GPI) and N-terminally truncated PrP(Δ 23–88), arguing that the N-terminus has an important role in disease pathogenesis.³⁸⁰ These studies demonstrated that PrP(Δ GPI) spontaneously forms prions, and that membrane-bound wt PrP^C modulates the kinetics of PrP(Δ GPI) amyloid formation.

High-Resolution Structures of Recombinant PrP

Solution NMR structures of recSHaPrP(90–231), recSHaPrP(29–231), and recMoPrP(23–231) were determined after the proteins were expressed in *E. coli*, purified by gel filtration and reverse-phase chromatography, and refolded in α -helical-rich conformations similar to mammalian PrP^C (Fig. 76.6A). Circular dichroism measurements and ¹H-NMR spectra of bovine PrP^C were found to be consistent with results obtained with recombinant bovine PrP, arguing that the solution NMR structures are relevant for the structure of native PrP^C.¹⁶⁰ Most features of the structure of recSHaPrP (90–231) are similar to those reported earlier for the smaller recMoPrP(121–231) fragment.³²⁴ Like the shorter fragments, full-length PrP is also a three helix-bundle protein with two short antiparallel β -strands. Although the three helices form a globular C-terminal domain, the N-terminal domain is highly flexible and lacks identifiable secondary structure under the experimental condi-

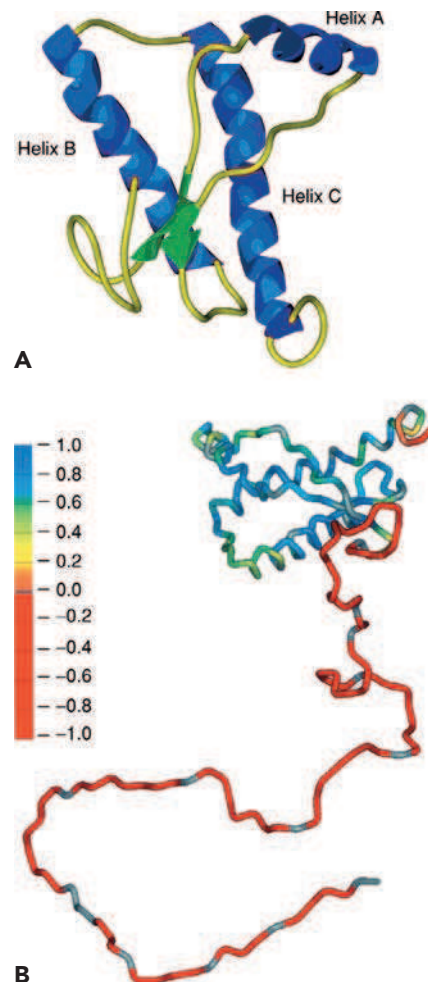


FIGURE 76.6. Structures of cellular prion protein (PrP^C). **A:** Nuclear magnetic resonance (NMR) structure of recSHaPrP(90–231). Presumably, the structure of the α -helical form of recSHaPrP(90–231) resembles that of PrP^C. recSHaPrP(90–231) is viewed from the interface where the disease-causing isoform (PrP^{Sc}) is believed to bind to PrP^C. α -Helix A, helix B, and helix C span residues 144–157, 172–193, and 200–227, respectively; residues 129–134 encompass strand S1 and residues 159–165 encompass strand S2. Residues 90 to 112 are not shown because marked conformational heterogeneity was found in this region, whereas residues 113 to 126 constitute the conserved hydrophobic region that also displays some structural plasticity.¹⁷⁵ **B:** Schematic diagram showing the flexibility of the polypeptide chain for PrP(29–231).¹⁰² The structure of the portion of the protein representing residues 90 to 231 was taken from the coordinates of PrP(90–231).¹⁷⁵ The remainder of the sequence was hand-built for illustration purposes only. The color scale corresponds to the heteronuclear [¹H]-¹⁵N NOE data: red for the lowest (most negative) values, where the polypeptide is most flexible, to blue for the highest (most positive) values in the most structured and rigid regions of the protein.¹⁰²

tions employed (Fig. 76.6B).¹⁰² Studies of recSHaPrP(29–231) indicate transient interactions between the C-terminal end of helix B and the highly flexible, N-terminal random coil containing the octarepeats (residues 29–125).¹⁰²

The solution NMR and x-ray structures of PrPs from 16 different species have been determined to be similar (Table 76.4). Moreover, the structures of MoDpl and HuDpl were

TABLE 76.4 List of Structures for PrP, Dpl, Ure2p, Sup35, HET-s, HET-S^a

Species	Protein	How	PDB accession codes	Reference
Mouse	rec PrP 23–231	solution NMR	data not released	(323)
Mouse	rec PrP 121–231	solution NMR	1AG2	(324)
Mouse	rec PrP 121–231	solution NMR	1XYX	(145)
Mouse	rec PrP 121–231, at 20°C	solution NMR	2L1H	none available
Mouse	rec PrP 121–231, at 37°C	solution NMR	2L39	none available
Mouse	rec PrP 121–231, V166A	solution NMR	2KFO	(72)
Mouse	rec PrP 121–231, D167S	solution NMR	2KU5	(293)
Mouse	rec PrP 121–231, D167S, N173K	solution NMR	2KU6	(293)
Mouse	rec PrP 121–231, Y169A	solution NMR	2L40	none available
Mouse	rec PrP 121–231, Y169G	solution NMR	2L1D	none available
Mouse	rec PrP 121–231, S170N	solution NMR	2K50	(73)
Mouse	rec PrP 121–231, S170N, N174T	solution NMR	1Y16	(145)
Mouse	rec PrP 121–231, N174T	solution NMR	1Y15	(145)
Mouse	rec PrP 121–231, F175A	solution NMR	2L1E	none available
Mouse	rec PrP 121–231, Y225A, Y226A	solution NMR	2KFM	(72)
Mouse	rec PrP 121–231, Y169A, Y225A, Y226A	solution NMR	2L1K	none available
Mouse	syn PrP 137–142	X-ray	3NVG	(12)
Mouse	syn PrP 137–143	X-ray	3NVH	(12)
Syrian hamster	rec PrP 29–231	solution NMR	data not released	(102)
Syrian hamster	rec PrP 90–231	solution NMR	2PRP, updated 1999 to 1B10	(175,223)
Syrian hamster	rec PrP 23–106, bound to pentosan polysulfate	solution NMR	2KKG	(398)
Syrian hamster	syn PrP 104–113 in complex with antibody 3F4	X-ray	1CU4	(188)
Syrian hamster	syn PrP 138–143	X-ray	3NVE	(12)
Human	rec PrP 23–230	solution NMR	1QLX, 1QLZ	(448)
Human	rec PrP 90–230	solution NMR	1QM0, 1QM1	(448)
Human	rec PrP 90–231	X-ray	1I4M	(199)
Human	rec PrP 90–231, D178N	solution NMR	2K1D	none available
Human	rec PrP 90–231, M129, D178N	X-ray	3HEQ	(218)
Human	rec PrP 90–231, V129, D178N	X-ray	3HJX	(218)
Human	rec PrP 90–231, M129, F198S	X-ray	3HES	(218)
Human	rec PrP 90–231, V129, F198S	X-ray	3HER	(218)
Human	rec PrP 90–231, M129, swapped dimer	X-ray	3HJ5	(218)
Human	rec PrP 90–231, V129, swapped dimer	X-ray	3HAF	(218)
Human	rec PrP 90–231, E200K	solution NMR	1FKC, 1F07	(449)
Human	rec PrP 90–231, M129, V210I	solution NMR	2LEJ	(27)
Human	rec PrP 90–231, M129, Q212P	solution NMR	2KUN	(170)
Human	rec PrP 119–231, in complex with antibody ICSM 18	X-ray	2W9E	(10)
Human	rec PrP 119–231, in complex with ICSM 18 Fab fragment	X-ray	2W9D	(10)
Human	rec PrP 121–230	solution NMR	1QM2, 1QM3	(448)
Human	rec PrP 121–230, at pH 7.0	solution NMR	1HJM, 1HJN	(52)
Human	rec PrP 121–230, M166V	solution NMR	1E1G, 1E1J	(50)
Human	rec PrP 121–230, S170N	solution NMR	1E1P, 1E1S	(50)
Human	rec PrP 121–230, R220K	solution NMR	1E1U, 1E1W	(50)
Human	rec PrP 121–230, M166C, E221C	solution NMR	1H0L	(447)
Human	rec PrP 125–227, V129	X-ray	3HAK	(218)
Human	syn PrP 127–132, M129	X-ray	3NHC	(11)
Human	syn PrP 127–132, V129	X-ray	3NHD	(11)
Human	syn PrP 138–143	X-ray	3NVF	(12)
Human	syn PrP 170–175	X-ray	2OL9	(342)
Human	syn PrP 173–195	solution NMR	2IV5	(327)

(continued)

TABLE 76.4 List of Structures for PrP, Dpl, Ure2p, Sup35, HET-s, HET-S^a (Continued)

Species	Protein	How	PDB accession codes	Reference
Human	syn PrP 173–195, D178N	solution NMR	2IV6	(327)
Human	syn PrP 180–195	solution NMR	2IV4	(327)
Cattle	rec PrP 23–230	solution NMR	1DX0, 1DX1	(225)
Cattle	rec PrP 121–230	solution NMR	1DWY, 1DWZ	(225)
Cattle	syn PrP 1–30, in membrane mimic	solution NMR	1SKH	(28)
Cattle	syn PrP 95–104, in complex with P-clone Fab fragment	X-ray	2HHO	(226)
Sheep	rec PrP 94–233, R151C	X-ray	1UW3	(151)
Sheep	rec PrP 114–234, ARR, VRQ, and ARQ variants, in complex with Vrq14 Fab fragment	X-ray	1TPX, 1TQB, 1TQC	(104)
Sheep	rec PrP 122–234, R168, H168	solution NMR	1Y2S, 1XYU	(228)
Sheep	rec PrP 167–234, I208A, H2H3 domain	solution NMR	2KTM	(2)
Sheep	syn PrP 138–158	solution NMR	1S4T	(250)
Sheep	syn PrP 142–167, Y155A	solution NMR	2RMV	(22)
Sheep	syn PrP 142–167, R156A	solution NMR	2RMW	(22)
Sheep	syn PrP 145–169+C	solution NMR	1G04	(208)
Sheep	syn PrP 145–169+C, in TFE	solution NMR	1M25	(208,250)
Pig	rec PrP 121–231	solution NMR	1XYQ	(228)
Elk	rec PrP 124–234	solution NMR	1XYW	(145)
Elk	syn PrP 173–178	X-ray	3FVA	(437)
Horse	rec PrP 106–216	solution NMR	2KU4	(293)
Dog	rec PrP 121–231	solution NMR	1XYK	(228)
Cat	rec PrP 121–231	solution NMR	1XYJ	(228)
Bank vole	rec PrP 121–231	solution NMR	2K56	(73)
Rabbit	rec PrP 91–228	solution NMR	2FJ3	(422)
Rabbit	rec PrP 91–228, S173N	solution NMR	2JOH	(422)
Rabbit	rec PrP 91–228, I214V	solution NMR	2JOM	(421)
Rabbit	rec PrP 126–230	X-ray	3079	(190)
Tammar wallaby	rec PrP 121–230	solution NMR	2KFL	(72)
Chicken	rec PrP 128–242	solution NMR	1U3M	(51)
Turtle	rec PrP 121–226	solution NMR	1U5L	(51)
Xenopus	rec PrP 90–222	solution NMR	1XU0	(51)
Most mammalian species	syn PHGGGWGQ	solution NMR	data not released	(443)
Most mammalian species	syn HGGGW, Cu ²⁺ coordinated	X-ray	PDB refused data, peptide too short	(48)
Most mammalian species	rec HGGGWGQP	solution NMR	10EH	(446)
Most mammalian species	rec (HGGGWGQP) ₃	solution NMR	10EI	(446)
Most mammalian species	syn YMLGSA	X-ray	3MD4, 3MD5	none available
Mouse	rec Dpl 26–157	solution NMR	1I17	(259)
Mouse	syn Dpl 1–30	solution NMR	1Z65	(281)
Human	rec Dpl 24–152	solution NMR	1LG4	(227)
<i>S. cerevisiae</i>	rec Ure2p 95–354	X-ray	1G6W, 1G6Y	(31)
<i>S. cerevisiae</i>	rec Ure2p 95–354 Glutathione complex	X-ray	1JZR, 1K0B, 1K0D	(32)
<i>S. cerevisiae</i>	rec Ure2p 95–354 S-P-Nitrobenzylglutathione complex	X-ray	1K0C	(32)
<i>S. cerevisiae</i>	rec Ure2p 95–354 S-Hexylglutathione complex	X-ray	1K0A	(32)
<i>S. cerevisiae</i>	rec Ure2p 97–354	X-ray	1HQ0	(409)
<i>S. cerevisiae</i>	syn Sup35 peptide GNNQQNY	X-ray	1YJP, 20MM	(269,342)
<i>S. cerevisiae</i>	syn Sup35 peptide NNNQQNY, with Zn acetate	X-ray	1YJO	(269)

TABLE 76.4 List of Structures for PrP, Dpl, Ure2p, Sup35, HET-s, HET-S^a (Continued)

Species	Protein	How	PDB accession codes	Reference
<i>S. cerevisiae</i>	syn Sup35 peptide NNQQ	X-ray	2ONX, 2OLX	(342)
<i>S. pombe</i>	rec Sup35 196–662	X-ray	1R5B	(201)
<i>S. pombe</i>	rec Sup35 196–662, in complex with GDP	X-ray	1R5N	(201)
<i>S. pombe</i>	rec Sup35 196–662, in complex with GMPPNP	X-ray	1R5O	(201)
<i>P. anserina</i>	rec HET-s 218–289	solid-state NMR	2RNM	(415)
<i>P. anserina</i>	rec HET-s 218–289, in its amyloid form	solid-state NMR	2KJ3	(411)
<i>P. anserina</i>	rec HET-s 218–289, in complex with Congo red dye	solid-state NMR	2LBU	(347)
<i>P. anserina</i>	rec HET-S 1–227, prion inhibiting N-terminal domain	X-ray	2WVN, 2WVO	(147)
<i>P. anserina</i>	rec HET-S 1–227, D23A, P33H, prion inhibiting N-terminal domain	X-ray	2WVQ	(147)

^aData compiled by Holger Wille. rec, recombinant; syn, synthetic.

found to be similar to that of MoPrP and HuPrP, respectively, even though the two proteins share only 25% sequence similarity (Fig. 76.3).^{227,259} The folded domains of PrP and Dpl have a small but significant sequence homology (16–18% identity) to the N-terminal, extracellular domain of members of the Zrt- and Irt-like family (ZIPs) of zinc transporters.³⁴³ This homology may also extend to secondary and tertiary structural elements of PrP, Dpl, and the respective ZIPs. This sequence homology and potential structural similarity may indicate an ancestral relationship between the ZIP proteins and PrP and its paralogs.

Electron Crystallography of PrP^{Sc}

Because the insolubility of PrP^{Sc} has frustrated structural studies employing x-ray crystallography or NMR spectroscopy, electron crystallography was used to characterize the structure of two infectious variants of PrP. Isomorphous, 2D crystals of PrP 27–30 and a miniprion (PrP^{Sc}106) were identified by negative-stain electron microscopy.^{433,434} Image processing allowed the extraction of limited structural information to 10- to 15-Å resolution.⁴³² Comparing projection maps of PrP 27–30 and PrP^{Sc}106, the 36-residue internal deletion of the miniprion was visualized, and the N-linked sugars were localized (Fig. 76.7). The dimensions of the monomer, and the locations of the deleted segment and sugars, were used as constraints in the construction of models for PrP^{Sc}.¹⁴⁶

From a study of β -folds observed in globular proteins, it was determined that if PrP^{Sc} follows a known protein fold, it likely adopts a β -sandwich or parallel β -helical architecture.¹⁴⁶ With increasing evidence for a parallel β -sheet organization in amyloids,¹⁸⁴ it was argued that the sequence of PrP is compatible with a parallel left-handed β -helical fold. Left-handed β -helices readily form trimers, providing a natural template for a trimeric model of PrP^{Sc} (Fig. 76.8A). This trimeric model accommodates the PrP sequence from residues 89–175 in a β -helical conformation with the C-terminus (residues 176–227) retaining the disulfide-linked α -helical conformation observed in PrP^C. In addition, the proposed model matches the structural constraints of the 2D crystals, positioning residues 41–176 and the N-linked sugars appropriately. The parallel left-handed β -helical model provides a coherent framework that is consistent

with the stacking of trimeric units of PrP 27–30 to form PrP amyloid fibrils and predicts a height of four β -strands (= 19.2 Å) per molecule of PrP 27–30 (Fig. 76.8B).

X-Ray Fiber Diffraction of PrP 27–30 Amyloid

The specific interaction between PrP 27–30 and some polyoxometalates (POMs) favors formation of either PrP 27–30 amyloid fibrils or 2D crystals depending on the charge density of the POM (Fig. 76.9).⁴³⁴ Preparations enriched for PrP 27–30 amyloid fibrils were particularly suited for structural analyses by x-ray fiber diffraction.

Diffraction patterns of PrP 27–30 derived from the natural RML prion isolate or synthetic MoSP1 prion preparation both showed diffraction signatures on the meridian based on an axial repeat distance of 19.2 Å (Fig. 76.10). Although the first-order diffraction signal (at 19.2 Å) was not observed, the second-, third-, and fourth-order diffraction signals (at 9.6 Å, 6.4 Å, and 4.8 Å; white and black arrows) were present. Together, these reflections indicated that the repeating unit along the axis of the PrP 27–30 amyloid fibrils consists of four β -strands per molecule in a cross- β configuration. The equator of the diffraction patterns is characterized by a series of reflections originating from lipids and detergents. The absence of a strong equatorial diffraction signature around 10 Å indicates that the structure of PrP 27–30 amyloid is unlike that of “classical” amyloids that contain a stacked β -sheet architecture.⁴³¹ Instead, the meridional and equatorial reflections are most consistent with a β -helical architecture as was proposed in earlier modeling attempts (Fig. 76.8).

PRION REPLICATION

Many factors have been identified that influence the rate of prion formation. Although prion formation is inversely related to the length of the incubation time, it is directly proportional to the level of PrP^C expression in the brain and the amount of PrP^{Sc} in the inoculum. Another factor governing prion formation is the PrP sequence: Prion replication is generally facilitated when the sequences of PrP^C and PrP^{Sc} are identical. The strain-

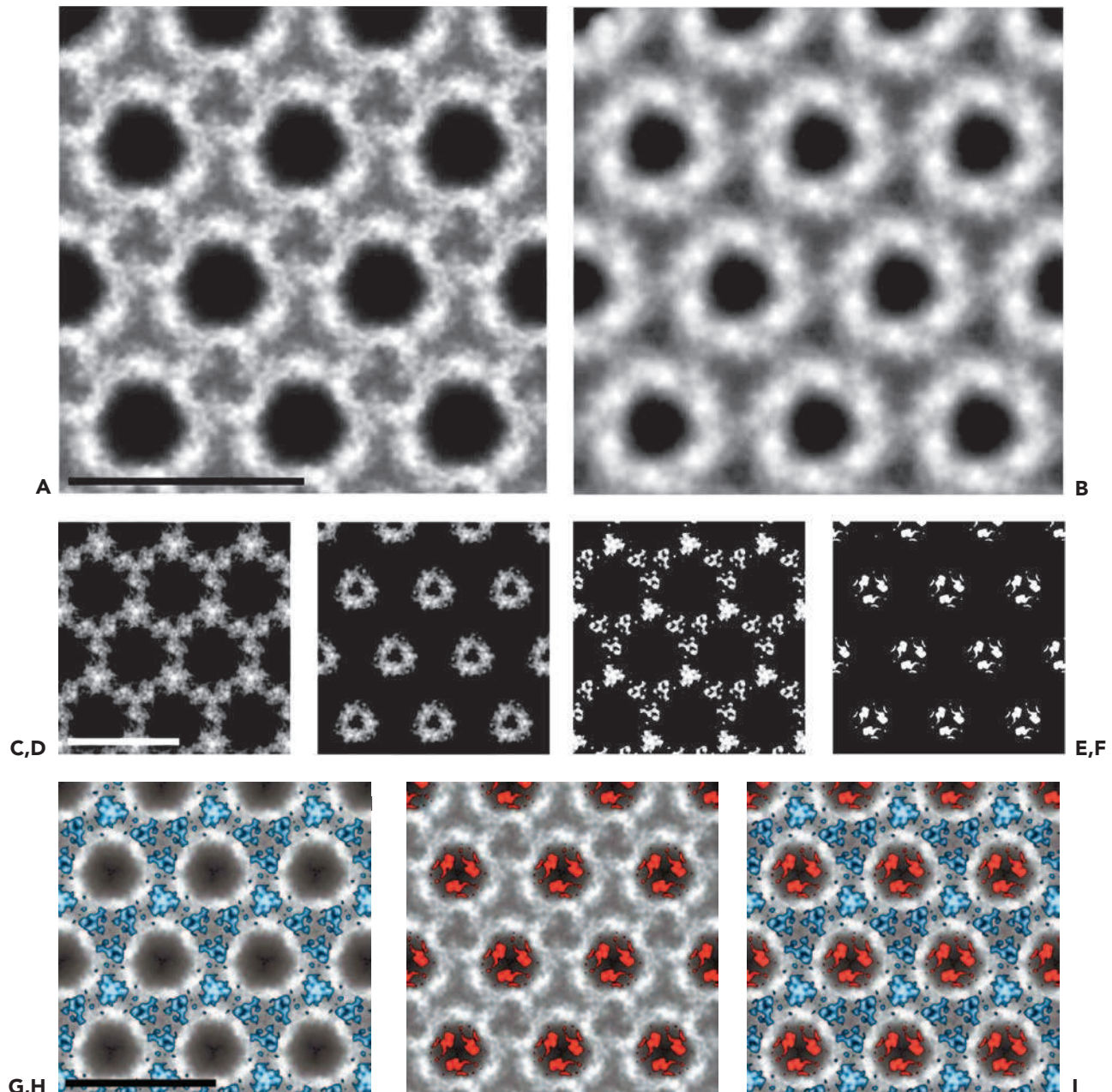


FIGURE 76.7. Electron crystallography images from two-dimensional (2D) crystals of two different infectious forms of the prion protein, denoted PrP 27-30 and PrP^{Sc}106. A and B: Crystallographic averages of PrP 27-30 and PrP^{Sc}106, respectively. **C:** Map of the difference of [(PrP 27-30) – (PrP^{Sc}106)]. **D:** Map of the difference of [(PrP^{Sc}106) – (PrP 27-30)]. **E and F:** Statistically significant differences (in white) between **A** and **B**, calculated by subtracting three times the standard error from the respective subtraction maps (**C** and **D**). **G:** The statistically significant difference from **panel E** overlaid onto the PrP 27-30 projection map (**A**) localizes the N-linked sugars. **H:** The statistically significant difference from **panel F** overlaid onto the PrP 27-30 projection map (**A**) localizes the internal deletion of PrP^{Sc}106. **I:** The statistically significant differences from **panels E** (blue) and **F** (red) overlaid onto the PrP 27-30 projection map. Bar in **A** represents 10 nm and also applies to panel **B**; bar in **C** represents 10 nm and also applies to panels **D–F**; bar in **G** represents 10 nm and also applies to panels **H–I**. (Reprinted from Wille H, Govaerts C, Borovinskiy A, et al. Electron crystallography of the scrapie prion protein complexed with heavy metals. *Arch Biochem Biophys* 2007;467:239–248, with permission; copyright © 2007 Elsevier.)

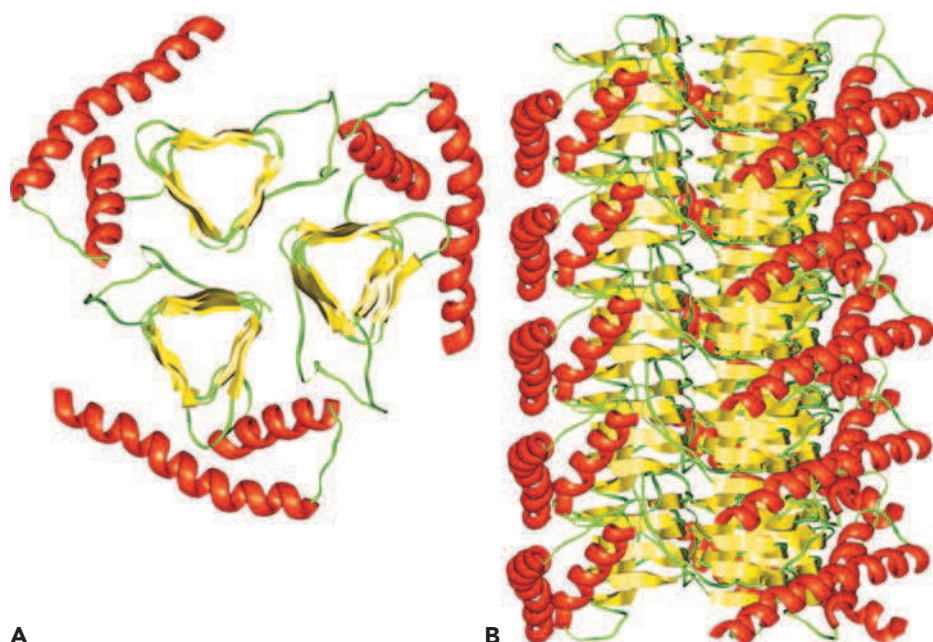


FIGURE 76.8. Left-handed β -helical models of PrP 27–30. **A:** Trimeric model of PrP 27–30 built by superimposing three monomeric models onto the coordinates of the C'_{α} s of the structure of trimeric carbonic anhydrase from *Methanosarcina thermophila* (PDBID1THJ). **B:** A model of the PrP 27–30 fiber constructed by stacking five trimeric discs. (Figure prepared by Cédric Govaerts.)

specific conformation of PrP^{Sc} is also a major determinant influencing the rate of prion formation. Some prion strains exhibit short incubation times, whereas others produce prolonged incubation periods. Some of these findings presumably reflect the stability of the PrP^{Sc} , the targeting of PrP^{Sc} to replication-competent cells, and the rate of PrP^{Sc} clearance.

Once infection in a cell is initiated and PrP^{Sc} production begins, the mechanism of prion propagation remains uncertain. If the assembly of PrP^{Sc} into a specific trimeric arrangement is slow, then a nucleation-polymerization (NP) formalism may be relevant.¹⁷⁷ In NP processes, nucleation is the rate-limiting step, whereas elongation, or polymerization, is facile.

These conditions are frequently observed in peptide models of aggregation⁵⁸; however, studies with ScN2a cells and Tg mice expressing foreign PrP genes suggest that prion propagation occurs through a different process.

Although the NP reaction has gained support from yeast prion studies, few data exist to argue in favor of such a mechanism for mammalian prions. Moreover, the chaperone Hsp104 has been shown to play an important role in the replication of the Sup35 yeast prions by fragmenting amyloid fibrils and thereby making more seeds available.³⁶⁰ It is noteworthy that yeast prions do not infect cells but rather are transmitted horizontally from cell to cell by cytoplasmic mixing during

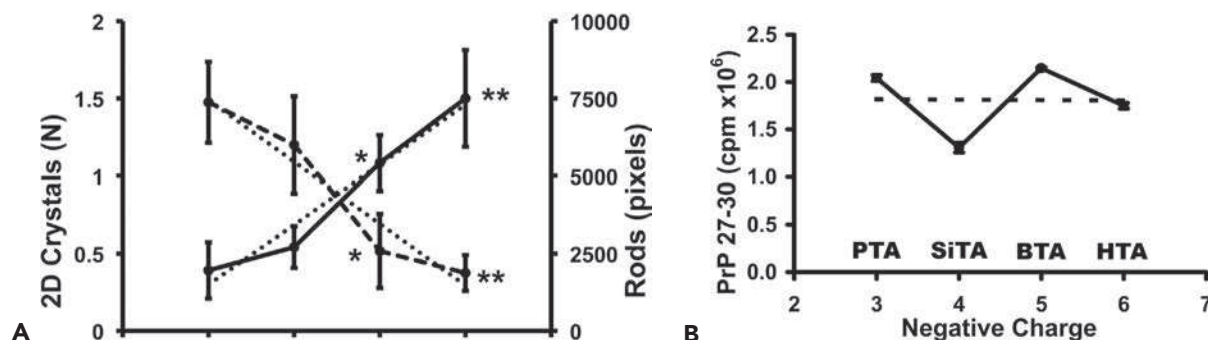


FIGURE 76.9. Inverse correlation between the quantities of prion rods and two-dimensional (2D) crystals in preparations with different polyoxometalates (POMs). **A:** With increasing charge density of Keggin-type POMs, the amount of prion rods decreased (dashed-line curve) as the amount of 2D crystals increased (solid curve), as determined by quantitative electron microscopy. The reductions in fibril content from phosphotungstic acid (PTA) to borotungstic acid (BTA) (factor ~2.9) and to metatungstate (HTA, $[\text{H}_2\text{W}_{12}\text{O}_{40}]^{6-}$) (factor ~3.9) were statistically significant, with $P = 0.015$ (*) and $P = 0.002$ (**), respectively. The correlation coefficient (r) for all four POMs is -0.97 (dotted line). The increases in 2D crystal content from PTA to BTA (factor ~4.0) and to HTA (factor ~4.3) were statistically significant, with $P = 0.009$ (*) and $P = 0.001$ (**), respectively. For all four POMs, $r = 0.93$. Because different methods of quantification were used for the prion rods and 2D crystals, different scales are shown. The error bars represent standard errors of the mean (SEMs). **B:** The negative charge of the Keggin-type POMs and the amount of precipitated PrP 27–30, as determined by conformation-dependent immunoassay (CDI), are not correlated ($r = -0.01$, dashed line). The error bars show the standard deviation. (Reprinted from Wille H, Shanmugam M, Murugesu M, et al. Surface charge of polyoxometalates modulates polymerization of the scrapie prion protein. *Proc Natl Acad Sci USA*. 2009;106:3740–3745, with permission; copyright © 2009 National Academy of Sciences, USA.)

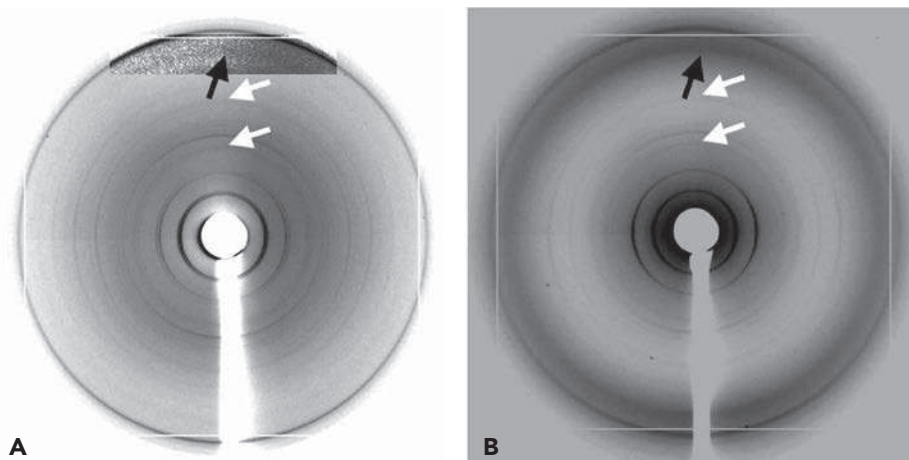


FIGURE 76.10. Fiber diffraction patterns from phosphotungstic acid (PTA)-precipitated mouse prions. Black arrows indicate cross- β meridional diffraction at close to 4.8-Å resolution. White arrows indicate second and third orders of meridional 19.2-Å diffraction. **A:** The RML prion strain passed in FVB mice and derived from the Chandler isolate. MoPrP 27–30 was purified by Sarkosyl extraction followed by PTA precipitation.⁴³⁴ Inset uses a different grayscale in order to show the weak, broad 4.8-Å diffraction. **B:** The MoSP1 synthetic prion strain derived from recPrP(89–230) amyloid, passed twice through Tg9949 mice and purified as in A.

ming.⁴²⁷ Yeast prions are also transferred from one cell to another by cytoplasmic mixing without transfer of nuclear material; this process is called cytoduction.

On the Mechanism of Prion Propagation

Studies of Tg mice expressing both Mo and SHa PrP^C demonstrated that the infecting prion interacts with the corresponding PrP^C to produce Mo or SHa prions.³¹⁶ These findings argued that PrP^{Sc} acts as a template and binds to the species-specific substrate PrP^C. The concept that PrP^{Sc} acts as a template in prion replication was extended when different prion strains were found to possess distinct conformations of PrP^{Sc}.^{25,221,291,401}

Results from many lines of investigation argue that auxiliary macromolecules feature in the formation of nascent PrP^{Sc} (Table 76.5), although some investigators continue to argue that prion replication occurs without any non-PrP factors. The

possibility that auxiliary proteins play a role in the multiplication of prions was first entertained when MoPrP^C was found to inhibit the formation of HuPrP^{Sc} in Tg mice expressing HuPrP^C (Table 76.6).⁴⁰² The inhibition of Hu prion replication by MoPrP^C was relieved by disruption of the MoPrP gene or by expression of a chimeric Mo-Hu PrP transgene, designated MHu2M. These findings, combined with those from cultured cell experiments, suggest that an auxiliary macromolecule, designated protein X, binds to PrP^C prior to the binding of PrP^{Sc}.¹⁸⁷

Mutagenesis studies showed that reversion of some human residues in the chimeric transgene of Tg(MHu2M) mice resulted in shorter incubation times after inoculation with human CJD prions. Although the incubation time for CJD prions in Tg(MHu2M)5378 mice was ~191 d, it was reduced almost 50% to ~106 d in Tg(MHu2M,M165V,E167Q)22372

TABLE 76.5 Some Arguments for Auxiliary Molecules Participating in Mammalian Prion Replication

1. PrP^{Sc} formation is restricted to a few cell types such as neurons, corneas, myocytes, and follicular dendritic cells (FDCs); yet, PrP^C expression is widespread.
2. Only a few cultured cell lines expressing PrP are permissive to prion infection, demonstrating that PrP expression is necessary but not sufficient for prion replication.
3. PrP^{Sc} formation is restricted to cholesterol-rich microdomains called rafts; PrP^{Sc} formation is prevented by cholesterol depletion.
4. Mouse PrP^C inhibited conversion of human PrP^C into human PrP^{Sc} but did not retard chimeric human-mouse PrP^C from being converted into chimeric PrP^{Sc} in mouse brain.
5. Reversion of 3 of 9 human residues to mouse in chimeric PrP^C reduced the incubation times progressively for human prions.
6. Substitution of a basic amino acid at any of 4 positions in the C-terminal mouse region of chimeric human-mouse PrP^C rendered PrP ineligible for conversion into PrP^{Sc}. Any of these basic substitutions made PrP act as a dominant negative.
7. Prion replication faithfully copied the conformation of the infecting PrP^{Sc} template. To copy different prion strains demands that the replication process possesses a mechanism to maintain the fidelity of prion reproduction.
8. Mammalian organ homogenates facilitated PrP^{Sc} formation by pulsed sonication, whereas those from flies, fungi, and bacteria did not.

TABLE 76.6 Selective Reversion of Human Residues to Mouse in Chimeric PrP Shortens the Incubation Time for CJD Prions^a

Transgene	MoPrP genotype	Incubation time (mean days \pm SEM) (n/n_0)
Non-Tg FVB	+/+	701 (1/10)
HuPrP(V129)	+/+	721 (1/10)
HuPrP(V129)	-/-	263 \pm 2 (6/6)
MHu2M	+/+	238 \pm 3 (8/8)
MHu2M	-/-	191 \pm 3 (10/10)
MHu2M(M165V,E167Q)	-/-	106 \pm 2 (13/13)
MHu2M(M111V,M165V,E167Q)	-/-	79 \pm 2 (15/15)

^aInocula from sCJD(MM1) case designated RG. All MHu2M transgenes encode M129. n , number of sick mice; n_0 , number of inoculated mice.

mice by reverting residues 165 and 167 from human to mouse in the chimeric transgene (Table 76.6).^{204,402} A further reduction of greater than 30% in the incubation time to ~77 d was accomplished by reverting one additional human residue at position 111 in Tg(MHu2M,M111V,M165V,E167Q)1014 mice.¹³⁴ These findings argue for a mouse-specific factor that participates in prion replication: when residues 111, 165, and 167 are mouse, the incubation time for RML prions is reduced more than 60% from 200 to 75 d. The results are consistent with the proposal that PrP^C interacts both with PrP^{Sc} and an auxiliary factor, for example, protein X, which would explain why replication of chimeric Hu/Mo prions is facilitated by replacement of some human residues with mouse. Obviously, some human residues are required in chimeric PrP^C so as to initiate efficiently the formation of nascent chimeric MHu2M(M111V,M165V,E167Q) PrP^{Sc}. It seems difficult to explain the foregoing findings solely on the basis of enhanced interactions between the chimeric PrP isoforms; these observations also make it difficult to argue against a mouse-specific, non-PrP factor such as a chaperone participating in prion replication.

Additional evidence for protein X emerged as it began to be appreciated that PrP^C is expressed in many tissues, but PrP^{Sc} appears to be limited to neurons, myocytes, and follicular dendritic cells (Table 76.5).^{30,155,196} The tissue-specific formation of prions contends that another protein with highly restricted expression participates in PrP^{Sc} formation. Moreover, only a few cultured cell lines expressing PrP have proven to be permissive to prion infection, demonstrating that PrP expression is necessary but not sufficient for prion replication.¹³⁵ This argument was extended by studies with mutant PrP that is ineligible for GPI addition.^{69,326} That anchorless PrP^{Sc} is formed more slowly than wt PrP^{Sc} in mouse brains⁶⁹ contends that PrP^{Sc} formation is confined to a particular subcellular compartment. Earlier studies showed that the GPI anchor directs PrP^C to cholesterol-rich rafts where it comes in contact with PrP^{Sc}^{144,396} and, likely, auxiliary macromolecules. If prion replication occurs without the aid of auxiliary proteins, then we would expect that altering the subcellular localization of PrP^C would not diminish prion replication.

Site-directed mutagenesis studies identified four residues in the C-terminal region of PrP^C that play a critical role in PrP^{Sc} formation. Substitution of a Lys or Arg at any of these residues rendered mutant PrP^C ineligible for conversion into PrP^{Sc}.¹⁸⁷ When mutant PrP was coexpressed with wt PrP, mutant PrP acted as a dominant negative, preventing the conversion of wt PrP^C into wt PrP^{Sc}.¹⁸⁷ The mechanism of dominant-negative inhibition was hypothesized to be the binding of mutant PrP^C to protein X, which prevents wt PrP^C from binding and, thus, being converted into wt PrP^{Sc} (Table 76.5). The findings in cultured cells were subsequently reproduced in Tg mice expressing either mutant PrP^C as well as both wt and mutant PrP^C.²⁹⁴

In vitro studies using pulsed sonication described below were used to search for protein X, also called factor X. Employing purified PrP^C and PrP^{Sc}, homogenates prepared from many different organs of diverse mammalian species facilitated the formation of protease-resistant PrP.¹ Subcellular fractionation showed that the non-PrP protein X was found predominantly located in the lipid raft fraction, the site of PrP^{Sc} formation.^{29,61,396} Importantly, no protein X activity was found in homogenates prepared from lower organisms including yeast, bacteria and flies.

In studies of prion strain interference, Dickinson and colleagues argued that a nonscrape agent factor governs the rate of multiplication.⁹⁷ To explain the prolongation of the incubation time for 22A strain in VM mice by prior inoculation with the 22C strain, they hypothesized that a limited number of “replication sites,” that is, factor X’s, existed. In other words, the affinity of the 22C prion strain for the replication sites was higher than that of 22A and thus, 22A replication was inhibited because 22A was unable to displace 22C from these sites. Recently, competition between the DY and HY strains of prions was studied by inoculating the prions into the sciatic nerve of Syrian hamsters.³⁵⁸ The investigators suggested that DY PrP^{Sc} prevented multiplication of HY PrP^{Sc} by sequestering either SHaPrP^C or occupying the prion “replication sites.”

***In vitro* Production of PrP^{Sc}**

Initial reports of the cell-free production of PrP^{Sc}-like molecules involved incubating an excess of PrP^{Sc} with PrP^C and measuring increases in protease-resistant PrP levels.⁶² The small increase in protease-resistant PrP derived from PrP^C did not permit meaningful measurements of prion infectivity. A modified system was used to investigate the need for non-PrP proteins in the suspension: after 48 h of incubation with a 10-fold molar excess of PrP^{Sc}, PrP^C acquired protease-resistance resembling PrP^{Sc}.³³² After partial purification of 139A or Me7 prions, the PrP^{Sc} was mixed with chimeric mouse-hamster (MHM2) PrP^C that was expressed in CHO cells. The formation of PrP^{Sc} was prion strain-specific based on the migration of PrP^{Sc} on Western blots. Although no formation of nascent PrP^{Sc} was observed under the same conditions using purified proteins without cell lysate, conversion was restored when PrP^C-depleted cell lysate was added to the purified proteins. The authors argue that these findings provide evidence for the participation of chaperone-like activity in the formation of PrP^{Sc}.

To increase the *in vitro* conversion of PrP^C to PrP^{Sc}, mixtures of brain homogenates from normal and prion-infected rodents were sonicated.³³¹ Repeated brief pulses of sonication were employed with 30- to 60-min intervals between them to prevent overheating; the procedure has been dubbed “protein

misfolding cyclic amplification,” or PMCA. Notably, PMCA has been found to produce prion infectivity in brain homogenates obtained from uninfected animals; this finding limits the value of PMCA as a diagnostic test for prion disease.^{18,93,96,413}

When PMCA was introduced, brain homogenate from prion-infected Syrian hamsters (SHa) were diluted 500-fold into brain homogenate from uninfected hamsters.³³¹ Subsequent studies employed purified PrP^C as substrate as well as purified PrP^{Sc} as the seeding template. PMCA was found to be sensitive to the PrP sequence as well as the source of homogenate^{55,56,271}; in addition, the extent of PrP^C glycosylation was found to influence amplification of PrP^{Sc}.²⁷² Cofactors such as lipids and polyanions like RNAs have also been found to modify the amplification of PrP^{Sc}.^{94,414}

Despite numerous studies extolling the virtues of PMCA, some caveats should be noted. PMCA results in uneven amplification of prions—from well to well and from experiment to experiment—which produces great variability²⁹⁸ and prevents the resulting data from being quantified. It is well documented that sonication produces an uneven distribution of energy, resulting in cavitation and high temperatures associated with cavitation¹¹¹ as well as the generation of free radicals.²³⁸ Protein conformation is sensitive to temperature denaturation, and free radicals may covalently alter proteins. Both cavitation and free-radical modification of proteins are stochastic processes and inherently difficult to control, potentially explaining the variability observed in PMCA experiments. In contrast, the denaturing agents urea and guanidine, used in the production of synthetic prions described below,^{77,79,220} result in comparatively even and well-defined denaturation of protein throughout the solution. In some cases, sonication initiates amyloid formation with proteins that are usually monomeric,³⁷⁶ indicating denaturation. In those studies, six different purified proteins showed increased aggregation and amyloid formation as a function of sonication time. Furthermore, such alterations in protein conformation may result in the degradation of the protein.⁴⁴¹ Degradation and denaturation may thus limit the usefulness of products produced by PMCA for structural studies.

That pulsed sonication promotes amyloid formation and the spontaneous formation of prion infectivity is consistent with other studies showing that both fungal and mammalian prion infectivity can be generated by polymerization of prion proteins into amyloids. In Tg mice, a 55-mer peptide carrying the P101L mutation was rendered infectious by increasing the β -structure^{186,405}; also, recPrP of wt sequence was rendered infectious by polymerization into amyloid fibrils.^{77,220} Similar results were obtained when the prion domain of the Sup35 yeast protein and the HET-s protein of the filamentous fungus *Podospora anserina* were polymerized into amyloid.^{230,371} More recently, altering the conditions of amyloid polymerization has been shown to produce different strains of both fungal and mammalian prions.^{77,195,395}

Prion Formation and Clearance

The accumulation of prions occurs when the rate of formation exceeds the rate of clearance. Bigenic mice were inoculated with RML prions and, after 56 days, they were given oral doxycycline, which suppressed PrP^C synthesis by approximately 95%. Under these conditions, the levels of PrP^{Sc} in the brains of bigenic mice were determined as a function of time.³³⁴ Such measurements allow calculation of the half-life ($t_{1/2}$) values of 1.5

days for both protease-sensitive and protease-resistant PrP^{Sc}. In contrast, a $t_{1/2}$ value of 18 hours was determined for PrP^C. In ScN2a cells, the $t_{1/2}$ value for PrP^C was 2.6 to 7 hours, while the $t_{1/2}$ for protease-resistant PrP^{Sc} was 30 hours.^{29,60}

Dominant-Negative Inhibition

As noted above, the discovery of dominant-negative inhibition of PrP^{Sc} gave additional support to the argument that an auxiliary protein participates in prion replication (Table 76.5). Substitution of a Hu residue at position 214 or 218 in MHM2 PrP prevented formation of MHM2 PrP^{Sc}.¹⁸⁷ The side chains of residues 214 and 218 protrude from the same surface of the C-terminal α -helix, forming a discontinuous epitope with residues 167 and 171 in an adjacent loop. When MHM2 PrP and MHM2 PrP(Q218K) were co-expressed, the conversion of MHM2 PrP^C into PrP^{Sc} was inhibited, arguing that MHM2 PrP(Q218K) was acting as a dominant negative. Like MHM2 PrP(Q218K), substitution of a basic residue at position 167 or 171 prevented PrP^{Sc} formation. Similar results were obtained when studies were performed with cells expressing MHM2 PrP(Q167R). Notably, the E219K substitution seems to render humans resistant to sCJD and Q171R renders sheep resistant to scrapie (Fig. 76.11).

Tg(MoPrP;Q167R)*Prnp*^{0/0} mice expressing mutant PrP at levels equal to non-Tg FVB mice were inoculated with prions and remained healthy for more than 550 days; immunoblots of brain homogenates and histologic analysis revealed no detectable protease-resistant PrP^{Sc}.²⁹⁴ Tg(MoPrP;Q167R)*Prnp*^{+/+} mice expressing both mutant and wt PrP exhibited neurologic dysfunction at ~450 days after inoculation; the brains of three of these mice that were sacrificed at 300 days revealed low levels of PrP^{Sc}, as well as numerous vacuoles and severe astrocytic gliosis.

In addition to studies with Tg(MoPrP;Q167R)*Prnp*^{+/+} mice, Tg(MoPrP;Q218K)*Prnp*^{+/+} mice demonstrate that PrP^{Sc} formation occurred slowly when dominant-negative and wt PrP are coexpressed. Expression of MoPrP(Q167R) at 1× or MoPrP(Q218K) at 16× in the absence of wt MoPrP failed to support replication of RML prions in these mice. Whether either of these dominant-negative PrPs in mice can support replication of another strain of prions such as 301V is unknown. The 301V strain was isolated from a case of BSE,¹¹⁴ and BSE prions are known to replicate in sheep expressing OvPrP(Q171R), in which ovine Q171R is equivalent to mouse Q167R.¹⁶³

DIAGNOSIS OF PRION DISEASES

The clinical diagnosis of human prion disease is often difficult until the patient shows profound signs of neurologic dysfunction.⁴²⁹ In humans with sCJD, the most common clinical presentation is a progressive dementia. Approximately 10% of sCJD patients present with a progressive ataxia.

It is widely accepted that the clinical diagnosis is provisional until a tissue diagnosis either confirms or rules out the clinical assessment. Prior to the availability of antibodies to PrP, a tissue diagnosis was generally made by histologic evaluation of neuropil vacuolation. Immunohistochemistry (IHC) using anti-GFAP antibodies in combination with hematoxylin-and-eosin staining preceded the use of anti-PrP antibody staining.

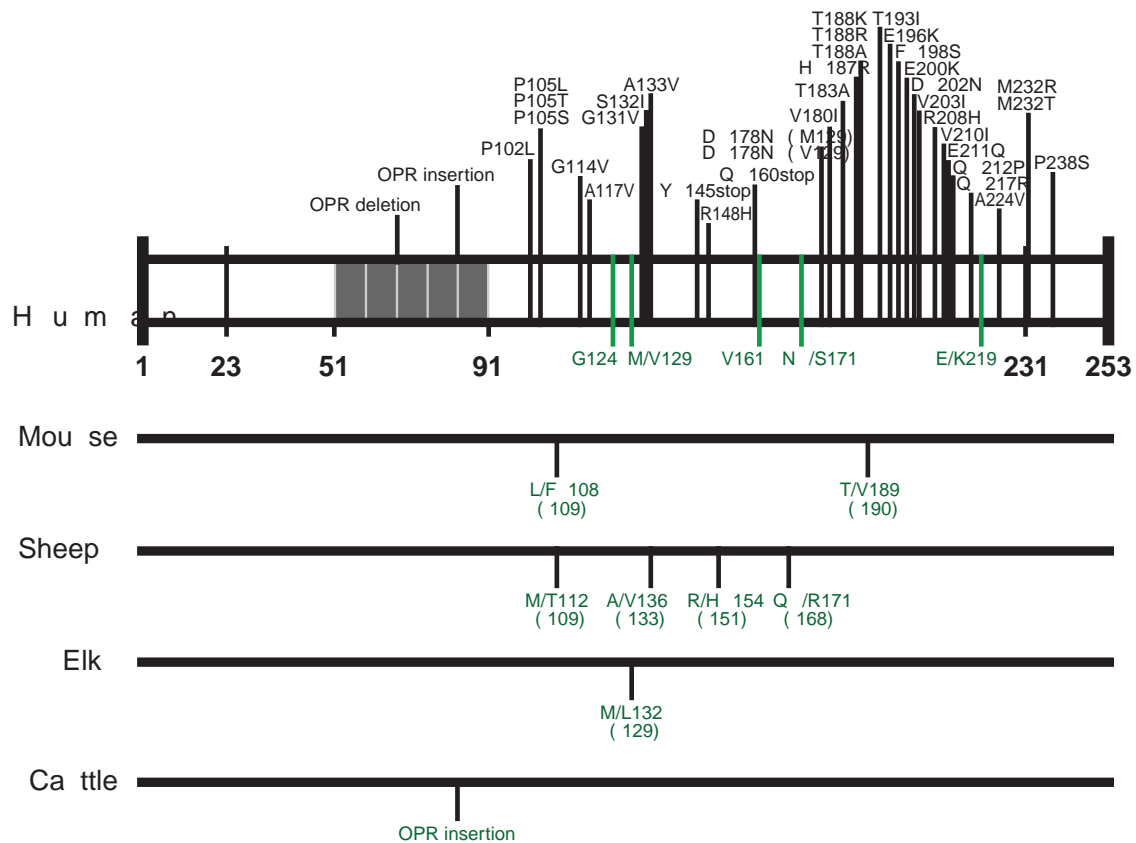


FIGURE 76.11. Mutations and polymorphisms of the prion protein gene. Above the line of the human sequence are mutations that cause inherited prion disease. Polymorphisms, some but not all of which are known to influence both the onset and the phenotype of disease, in the PrP genes of human, mouse, sheep, elk, and cattle are shown in green. Residue numbers in parentheses correspond to the human codons.

Postmortem Tissue Diagnosis of Prions

A study of the brains from eight clinically affected goats inoculated with the SSBP1 prion isolate challenged the accuracy of IHC.¹¹³ Thalamic samples taken from seven of eight goats with scrapie were positive for PrP^{Sc} by western blotting but negative by IHC. The eighth goat was negative by both western blotting and IHC. Consistent with these findings in goats, results of a study of humans who died of sCJD or familial CJD (fCJD) showed that IHC of formalin-fixed, paraffin-embedded human brain samples was substantially less sensitive than the conformation-dependent immunoassay (CDI).³³⁵

Concerned that limited PK digestion was hydrolyzing some or even most PrP^{Sc} in samples, the CDI was developed so as to not require PK digestion. The CDI revealed that as much as 95% of PrP^{Sc} is protease sensitive (sPrP^{Sc}), and thus, it was being destroyed during the limited proteolytic digestion used to hydrolyze PrP^C. Protease-sensitive PrP^{Sc} comprises 80% to 95% of the total PrP^{Sc} in the frontal lobes and in the white matter in CJD cases (Fig. 76.12).³³⁵

The CDI detected HuPrP^{Sc} with a sensitivity comparable to the bioassay for prion infectivity in Tg(MHu2M) mice (Fig. 76.13). The high sensitivity achieved by the CDI is due to several factors, including the use of phosphotungstic acid (PTA) that specifically precipitates sPrP^{Sc} and rPrP^{Sc}_{217,335} (Table 76.7). PTA has also been employed to increase the sen-

sitivity of western blots, enabling the detection of rPrP^{Sc} in human muscle and other peripheral tissues.^{136,412} A comparison between the CDI and western blotting on brain samples from sCJD and vCJD patients showed that the CDI was 50- to 100-fold more sensitive.²⁵⁷ In a recent study described below, a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) using a bioluminescence detection system was reported.¹⁰³

The CDI detected PrP^{Sc} in all regions of the brain that were examined in 24 sCJD and 3 fCJD(E200K) cases. Comparative analyses demonstrated that the CDI was superior to both histology and IHC. When 18 regions of 8 sCJD and 2 fCJD(E200K) brains were compared, it was discovered that both histology and IHC were unreliable diagnostic tools, except for samples from a few brain regions. In contrast, the CDI was a superb diagnostic procedure because it detected PrP^{Sc} in all 18 regions in 8 of 8 sCJD and 2 of 2 fCJD(E200K) cases.³³⁵

The CDI was also used to study GSS caused by the P102L mutation. In mice expressing the analogous mutant PrP transgene causing GSS in humans, the CDI detected high levels of sPrP^{Sc}(P101L) and low levels of rPrP^{Sc}(P101L) long before neurodegeneration and clinical symptoms were present.⁴⁰⁵ sPrP^{Sc}(P101L) and low concentrations of rPrP^{Sc}(P101L) previously escaped detection.

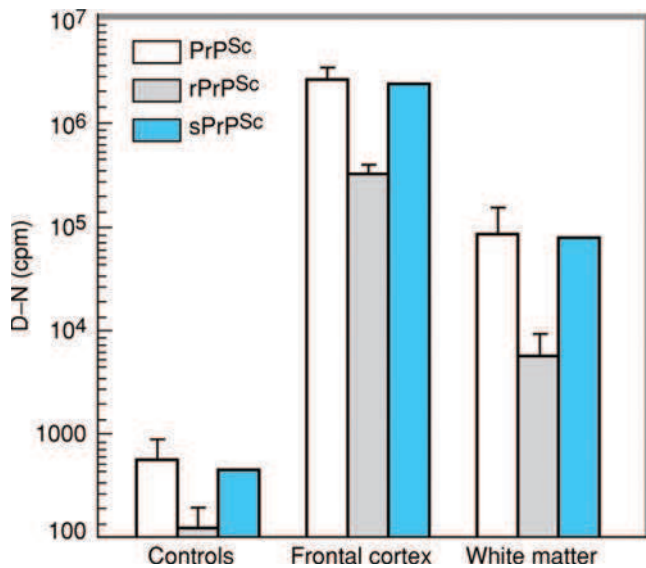


FIGURE 76.12. The most abundant disease-causing isoform (PrP^{Sc}) in the frontal cortex and white matter of sporadic Creutzfeldt-Jakob disease (sCJD) brains is protease-sensitive (blue bars, calculated from measurements of total PrP^{Sc} [white bars] and rPrP^{Sc} [gray bars]). Before measurement by the conformation-dependent immunoassay (CDI), undigested samples were phosphotungstic acid (PTA)-precipitated to measure total PrP^{Sc} or digested with 50 μ g per mL proteinase K at 37°C for 1 hour, followed by PTA precipitation to determine rPrP^{Sc}.³³³ The graphs show mean values \pm standard error of the mean (SEM) obtained from duplicate measurements of samples from the frontal cortex ($n = 19$) and white matter ($n = 12$) of sCJD-infected brains. (Reprinted from Safar JG, Geschwind MD, Deering C, et al. Diagnosis of human prion disease. *Proc Natl Acad Sci U S A* 2005;102:3501–3506, with permission; copyright © 2005 National Academy of Sciences, USA.)

Antemortem Diagnosis

Except for brain biopsy, no reliable antemortem test exists for prion disease. Although a variety of preliminary reports have described surrogate markers or PrP^{Sc} in blood, none of these markers has been developed into a reliable antemortem test.^{39,107} For example, the mRNA transcript of erythroid differentiation-related factor (EDRF) was reported to be diminished in the spleens of terminally ill mice inoculated with

scrapie prions.²⁵³ Attempts to show that EDRF is depressed in the blood of mice with scrapie or humans with CJD have been disappointing.

The stress protein 14-3-3 has been studied in the cerebrospinal fluid (CSF) of patients with CJD. Although some studies report that most sCJD patients show elevated levels of 14-3-3, other reports describe elevations in only ~50% of patients.¹²⁵ Patients with vCJD generally show no change in 14-3-3 levels in the CSF.⁴²⁹ Elevations in 14-3-3 CSF levels are nonspecific and have been found in stroke, herpes encephalitis, and several other neurologic disorders.

The detection of protease-resistant PrP in the urine of hamsters, cattle, and humans with prion disease was greeted with enthusiasm³⁵⁷; however, subsequent studies showed that the presumed immunostaining for PrP was an artifact.³⁵³ Immunoglobulin light chains or fragments of heavy chains were found to be detected by the secondary antibody used in the immunoassays.

A chemiluminescent capture assay for PrP^{Sc} in blood of humans with CJD was developed using a capture ELISA, where the detection was performed with the biotinylated primary mAb ICSM18. The amount of PrP^{Sc} bound to a solid phase was measured using NeutrAvidin-HRP that hydrolyzed a chemiluminescent substrate. Fifteen of 21 vCJD blood samples were positive using this assay, whereas 100 normal controls, 16 patients with probable sCJD, 11 patients with definite sCJD, and 42 patients from other neurodegenerative diseases (including 31 with Alzheimer's disease and 4 with frontotemporal dementia) were all negative.¹⁰³

Bovine Spongiform Encephalopathy Testing

In March 1996, 11 British teenagers and young adults were described to have died of a new form of prion disease, later labeled vCJD. In these young patients, the patterns of PrP^{Sc} deposition in the brain were markedly different from that found in typical CJD patients.^{90,429} The recognition that patients with vCJD were infected with BSE prions from cattle^{43,81,351} prompted the European Union to institute testing of all cattle older than 30 months of age at the time of slaughter. Currently, both western blotting and ELISA tests for rPrP^{Sc} are being used on brainstems from cattle. The CDI test, which measures both sPrP^{Sc} and rPrP^{Sc}, adapted to bovine brainstem has not been used routinely for testing cattle.

TABLE 76.7 Specificity of PrP^{Sc} Precipitation by NaMg PTA

Brain tissue ^a	n	CDI value	5% brain homogenate (cpm)	PTA pellet (cpm)	Fraction of PrP precipitated (%)
Uninoculated SHa	7	D	18,652 \pm 3,275	1,200 \pm 249	6.8 \pm 3.0
SHa inoculated with Sc237 prions	7	D	173,386 \pm 19,830	128,075 \pm 26,848	75.1 \pm 20.0
Uninoculated SHa	7	D-N	207 \pm 1,709	593 \pm 115	ND ^b
SHa inoculated with Sc237 prions	7	D-N	156,781 \pm 22,899	118,525 \pm 20,999	75.6 \pm 13.4

^aCDI values were obtained from denatured samples of Syrian hamster (SHa) brain, as described.²¹⁰ The values are the mean \pm SD calculated from seven independent experiments. PTA precipitation was performed without PK digestion, and the pellet was resuspended to the original volume and assayed in the direct CDI for PrP using Eu-labeled recHuM-P Fab.

^bThis percentage was not calculated due to frequent negative value of either the numerator or the denominator.

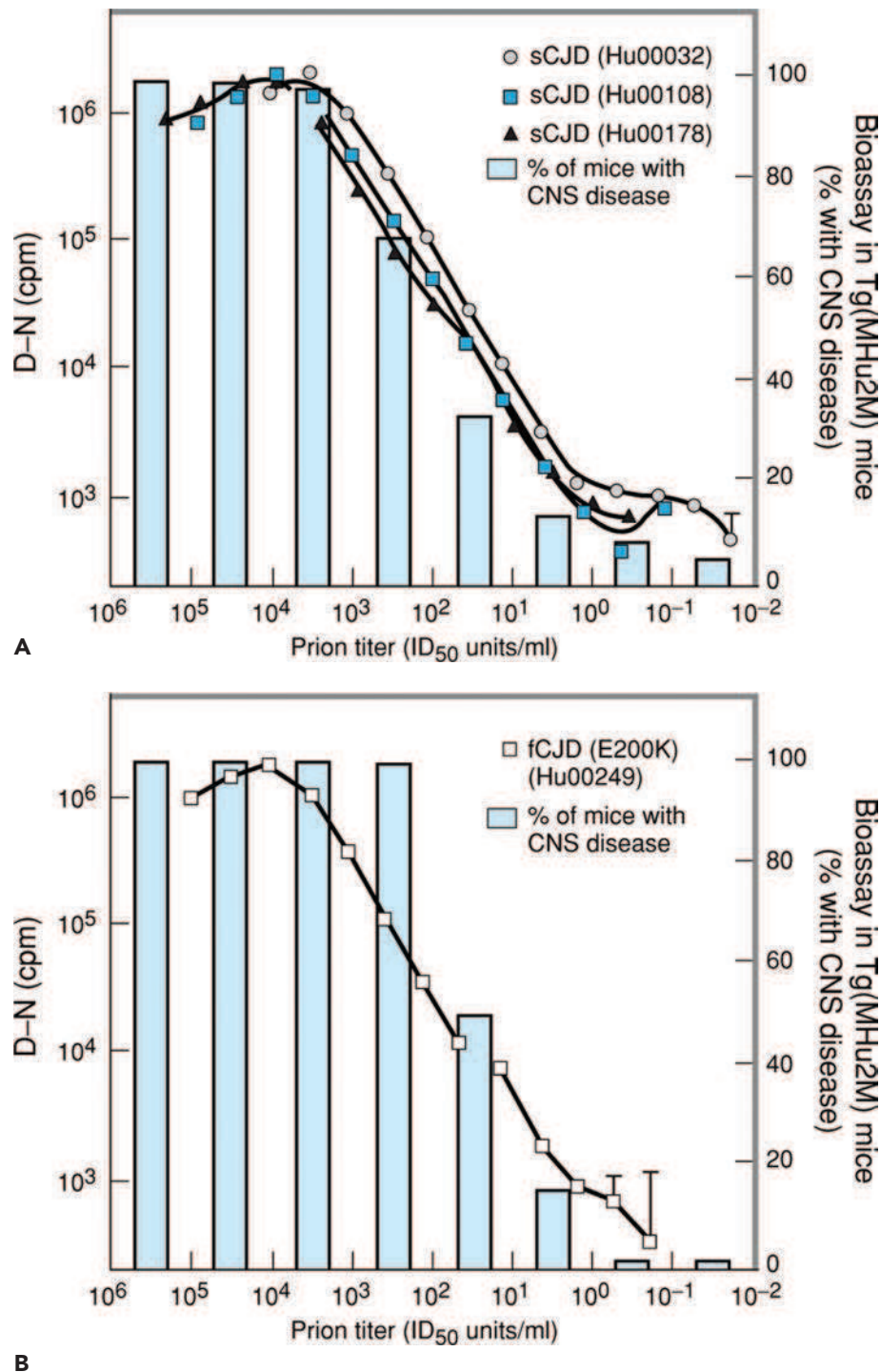


FIGURE 76.13. Correlation between the conformation-dependent immunoassay (CDI) and bioassay in Tg mice.

Sandwich CDI protocol for the detection of the disease-causing prion protein isoform (PrP^{Sc}) was compared to titration bioassays in Tg(MHu2M)5378/*Pmp*^{0/0} mice for three spontaneous Creutzfeldt-Jakob disease (sCJD) brains (**A**) and one familial CJD (fCJD) brain (**B**). Samples were precipitated with phosphotungstic acid (PTA) and digested with 2.5 μg per mL PK at 37°C for 1 hour. The MAR1 mAb²¹ was used for capture, and Eu-labeled 3F4 mAb was used for detection. The (D – N) values measured in counts per minute (cpm) are directly proportional to the concentration of PrP^{Sc} .^{333,337} Data points and bars represent the average \pm standard deviation (SD) obtained from three to four independent measurements. The cut-off (D – N) value of 1,789 cpm for this sandwich CDI protocol was calculated by [mean + 3(SD)] and determined from 100 brain samples obtained from patients who died from nonneurologic disease ($n = 6$), Alzheimer's disease ($n = 7$), and other neurologic diseases ($n = 5$). (Reprinted from Safar JG, Geschwind MD, Deering C, et al. Diagnosis of human prion disease. *Proc Natl Acad Sci U S A* 2005;102:3501–3506, with permission; copyright © 2005 National Academy of Sciences, USA.)

The identification of BSE-positive cattle in Canada and the United States has prompted increased surveillance in these countries, but the number of cattle tested remains less than 2% of the annual slaughter.³⁰⁶ Despite the small number of cattle being tested, new cases of BSE are still being found. These new cases are often attributed to tainted feed by agriculture authorities, who continue to think of prion diseases as being similar to infectious illnesses caused by viruses or bacteria. Some officials want to believe that BSE will disappear once the use of ruminant-derived feed ceases. Most cases of prion disease are likely to be sporadic. In Japan, ~4 million cattle were tested over 4 years, and ~20 cases of BSE have been identified. Two Japanese cows with BSE, one age 21 months and the other age 23 months, have been identified.⁴⁴² It seems likely that most or all of these young animals developed sporadic BSE.

Determining how early in the incubation period prions can be detected by bioassay is possible due to the construction of Tg(BoPrP)*Prnp*^{0/0} mice.^{49,352} Prior to the production of Tg(BoPrP)*Prnp*^{0/0} mice, cattle were used for bioassays of bovine prions. In a limited study using cattle bioassays, bovine prions were undetectable in the obex of the brainstem until 26 months after oral inoculation.⁴²⁰ In these studies, prion infectivity was detected much earlier in the lymphoid tissue of the distal ileum.

MOLECULAR PATHOGENESIS OF PRION DISEASES

In the prion diseases, PrP^{Sc} accumulates in the CNS and triggers presynaptic bouton degeneration, dendritic atrophy, vacuolation of neurons, and hypertrophy of astrocytes.¹⁷⁸ Kinetic studies following the development of prion disease have identified a stereotypical progression of neurodegeneration.³³ Following initiation of prion replication in a group of neurons, PrP^{Sc} spreads to other neurons and to other brain regions by anterograde transport to axon terminals. This is followed by presynaptic bouton degeneration and dendritic atrophy, and later by nerve cell death. Interestingly, neurodegeneration caused by prions requires PrP^C expression. When *Prnp*^{0/0} mice were implanted with neuronal grafts from wt mice and inoculated with prions, only the grafts exhibited neuropathology despite the deposition of PrP^{Sc} throughout the CNS.³⁷

Apoptosis in Prion Diseases

In both scrapie and CJD, cell death has been attributed to apoptosis. In one study, purified PrP^{Sc} was reported to cause the death of cultured cells.³⁰¹ In another study, PrP^{Sc} was shown to induce the release of Ca²⁺ from the endoplasmic reticulum (ER) and activate caspase-12.¹⁵⁷

Dendritic Atrophy and Notch-1

To investigate the molecular events that underlie dendritic atrophy, the expression of β -catenin, which initiates dendritic growth and maturation,⁴⁴⁴ and Notch-1, which inhibits their growth and maturation during neuronal development and causes regression of mature dendrites,³¹⁹ were studied. In neurons of prion-infected mice, increased levels of the Notch-1 intracellular domain (NICD) accompanied dendritic atrophy.¹⁷² Elevated levels of NICD were also found in ScN2a cells compared to uninfected (N2a) control cells. To determine if Notch-1 activation has a role in the shortening of neuronal

processes, ScN2a cells were transfected with a Notch-1 small interfering (si) RNA. The siRNA rescued the normal, long-neurite phenotype, suggesting that Notch-1 activation mediates dendritic atrophy in prion disease.

Neurotoxic PrP Molecules

It is important to distinguish between neurodegeneration caused by PrP^{Sc} and that produced by a toxic, mutant PrP molecule. For example, some PrP molecules with internal deletions²⁶⁷ and others with N-terminal truncations have been shown to be neurotoxic.³⁵⁹ In general, these PrP molecules are unable to support prion propagation. The smallest toxic PrP molecule synthesized to date is a 21-mer consisting of residues 106 to 126.¹¹² Recently, a 61-mer produced after deletion of residues 23 to 88 and 141 to 221 was found to be neurotoxic. Studies in Tg mice showed that expression of this 61-mer peptide caused ataxia. Neuropathologic examination found PrP accumulation in the CNS and neuronal apoptosis,³⁸⁵ but the disease could not be transmitted to wt rodents or Tg mice. To date, the smallest PrP molecule capable of supporting prion replication remains an N-terminally truncated PrP with an internal deletion, designated PrP106.³⁸⁴

The PrP paralog Dpl causes a toxic form of neurodegeneration and is unable to support prion replication. Both Dpl-induced neurodegeneration and that mediated by PrP carrying an N-terminal deletion from residues 32 to 134 were prevented by coexpression of wt PrP.^{262,359}

Transmembrane PrP Molecules

Based on cell-free translation studies, PrP^C seems able to be folded into three different forms: ^{Ntm}PrP, ^{Ctm}PrP, and ^{Sec}PrP.¹⁵⁴ ^{Ntm}PrP and ^{Ctm}PrP span the lipid bilayer once via a highly conserved hydrophobic region at the center of the molecule (residues 111–134) with either the N-terminus or C-terminus, respectively, on the extracytoplasmic side of the membrane. These two conformers are generated in small amounts (<10% of total PrP) as part of the normal biosynthesis of wt PrP in the ER. Mutations within or near the transmembrane domain increase the relative proportion of ^{Ctm}PrP to as much as 30% of total PrP.³⁷⁸ The ^{Ntm}PrP conformer spans the membrane with N-terminus in the ER lumen.

^{Ctm}PrP is hypothesized to be a key pathogenic intermediate in both familial and infectious prion diseases. Tg mice expressing PrP(A117V) or another ^{Ctm}PrP-favoring mutation, such as AV3 (with three alanine-to-valine substitutions at residues 113, 115, and 117), synthesize ^{Ctm}PrP in their brains and spontaneously develop neurologic dysfunction without detectable PrP^{Sc} or prion infectivity. The brain from a GSS patient with the PrP(A117V) mutation contained high levels of ^{Ctm}PrP.¹⁵⁴ In addition, a proteolytic fragment of ^{Ctm}PrP was found to be increased on the surface of cells expressing the P101L mutation.²⁵⁸ Taken together, these observations suggest that ^{Ctm}PrP may represent an alternative pathogenic form of PrP in some inherited prion diseases.

Subsequent studies showed that ^{Ctm}PrP has an uncleaved, N-terminal signal peptide.³⁷⁸ Substitution of a charged residue for a hydrophobic residue within the signal sequence (L9R) increased the proportion of ^{Ctm}PrP to ~50% during cell-free translation. Combining the L9R mutation with the AV3 mutation in the transmembrane domain resulted in a protein that was synthesized exclusively as ^{Ctm}PrP, in both cell-free translation reactions and transfected cells.³⁷⁸

SPORADIC HUMAN PRION DISEASES

In most patients with CJD, there is neither an infectious nor a heritable etiology. How prions arise in patients with sCJD is unknown. Various hypotheses include (a) horizontal transmission from humans or animals,¹²⁰ (b) somatic mutation of the *PRNP* ORF, (c) spontaneous conversion of PrP^C into PrP^{Sc}, and (d) the accumulation of PrP^{Sc}, which is normally present at undetectable levels. Numerous attempts to establish an infectious link between sCJD and a preexisting prion disease in animals or humans have been unrewarding.⁸⁶ Only in the case of vCJD has a link between prion-infected cattle and human prion disease been established.⁴²⁹

Studies demonstrating the formation of prions from the synthetic peptide PrP(89–143,P101L) and from recombinant PrP(89–230) in cell-free systems argue for the spontaneous generation of prions in mammals.⁷⁸ These findings provide an explanation for the sporadic forms of prion disease, for which extensive epidemiologic studies failed to identify an exogenous source of prions.⁸⁶ The spontaneous generation of prions from within a host is in stark contrast to viral infections, which can generally be traced to an exogenous source. Therefore, the cell-free generation of prions forces us to conclude that spontaneous conversion of PrP^C to PrP^{Sc} is a plausible explanation for sporadic prion disease in any mammal expressing PrP^C.

HERITABLE HUMAN PRION DISEASES

The recognition that 10% to 15% of CJD cases are familial (f) led to the suspicion that genetics plays a role in this disease. Subsequently, transmission of fCJD to apes and monkeys was reported.^{240,328} More than 40 different mutations of the PrP gene have been identified, of which 35 are point mutations and the remainder are octarepeat expansions or deletions (Fig. 76.11); see reviews.^{249,410} Five mutations have been genetically linked to inherited human prion diseases (Table 76.3). Virtually all cases of GSS and fatal familial insomnia (FFI) appear to be caused by germline mutations in the PrP gene. The brains of patients with inherited prion disease contain infectious prions that have been transmitted to experimental animals.

Gerstmann-Sträussler-Scheinker and Genetic Linkage

The transmissibility of GSS, a familial disease, to apes and monkeys was demonstrated at a time when most investigators believed that scrapie, CJD, and related disorders were caused by viruses.²⁴⁰ With the discovery that the P102L mutation of the human PrP gene was genetically linked to GSS, prion diseases were proposed to be both genetic and infectious disorders.¹⁶⁴ The P102L mutation was linked to the development of GSS with a logarithm of the odds (LOD) score exceeding 3, demonstrating a tight association between the altered genotype and disease phenotype.

Familial Creutzfeldt-Jakob Disease Caused by Octarepeat Inserts

An insertion of 144 bp containing six octarepeats at codon 53, in addition to the five that are normally present, was described in patients with CJD from four families residing in southern England.³⁰² Genealogic investigations showed that all four

families are related, arguing for a single founder born more than two centuries ago. The LOD score for this extended pedigree exceeds 11. Studies from several laboratories have demonstrated that inserts of two, four, five, six, seven, eight, or nine octarepeats, in addition to the normal five, are found in individuals with inherited CJD (Fig. 76.11). Tg mice expressing an expanded number of octarepeats develop neurodegeneration, but brain extracts have failed to transmit disease to inoculated recipients.⁷¹

To study the mechanism by which octarepeat expansions cause inherited prion disease, PrP peptides composed of residues 23–27 coupled to amino acids 60–91 comprising the four octarepeats (PHGGGWHQ) were studied by electron paramagnetic resonance (EPR) after being titrated with Cu²⁺ ions.¹³ The “wild-type” synthetic peptide with four octarepeats showed high Cu²⁺ occupancy, favoring the binding of one Cu²⁺ per His residue within an octarepeat as measured by EPR. Expanding the PrP peptide by adding up to four additional octarepeats did not alter the Cu²⁺ occupancy, but five or more additional octarepeats (totaling 9 or more repeats) shifted the Cu²⁺ binding from a high to a low occupancy state. This shift in Cu²⁺ occupancy correlates well with the age of onset of fCJD: for large octarepeat expansions resulting in nine or more repeats, the onset of CNS dysfunction was between ages 20 and 55.³⁷⁷ Eight or fewer octarepeats caused fCJD with an onset after age 55.

Familial Creutzfeldt-Jakob Disease in Libyan Jews

The unusually high incidence of CJD among Israeli Jews of Libyan origin was believed to be due to the consumption of lightly cooked sheep brain or eyeballs.¹⁸² Molecular genetic investigations revealed that Libyan and Tunisian Jews with fCJD have a point mutation at codon 200, resulting in a Glu → Lys substitution (Fig. 76.11).^{140,166} The E200K mutation has been genetically linked to fCJD, with an LOD score exceeding 3¹¹⁹; the same mutation has also been found in patients from many other families.¹⁴⁰ Life table analyses of carriers harboring the E200K mutation exhibit complete penetrance. Therefore, if the carriers live long enough, they will all eventually develop prion disease.

Fatal Insomnia

The D178N mutation has been linked to the development of FFI, with an LOD score exceeding 5. More than 30 families worldwide with FFI have been recorded.²⁰³ With the D178N pathogenic mutation, the amino acid at polymorphic residue 129 alters the clinical and neuropathologic phenotype. The D178N mutation combined with M129 results in FFI. In this disease, adults generally older than 50 years present with a progressive sleep disorder and usually die within 1 year. In their brains, deposition of PrP^{Sc} is confined largely within the anteroventral and the dorsal medial nuclei of the thalamus. In contrast, the same D178N mutation with V129 produces fCJD, in which the patients present with dementia and widespread deposition of PrP^{Sc} is found postmortem. The first family to be recognized with CJD was found to carry the D178N mutation.²⁰⁹

Tg mice expressing mutant PrP(D178N) had a normal lifespan, but old Tg mice exhibited cerebellar vacuolation and increased glial fibrillary acidic protein (GFAP) deposition in both

the thalamus and cerebellum.¹⁷⁴ No increased PrP deposition, as judged by IHC, was reported and no protease-resistant PrP was seen on western blots except when a 40-fold increase in protein was digested before gel electrophoresis. CNS dysfunction could be transmitted to mice overexpressing wt PrP^C after intracerebral inoculation, with incubation periods ranging between 300 and 500 days.

Human PrP Gene Polymorphisms

The Met/Val polymorphism at codon 129 (Fig. 76.11) appears to influence expression of the inherited, as well as the iatrogenic and sporadic, forms of prion disease.²⁷⁹ Most Caucasian patients with sCJD are homozygous for Met or Val at codon 129; this contrasts with the general Caucasian population among which 51% are Met/Val heterozygotes. In the Japanese population where the frequency of the Val allele is low, heterozygosity in Japanese sCJD patients was almost as frequent as in the general Japanese population.³⁹⁷

A second polymorphism resulting in an amino acid substitution at codon 219 (Glu/Lys) has been reported to occur with a frequency of approximately 12% in the Japanese population but not in Caucasians.¹⁹⁷ Studies of cultured cells and Tg mice expressing PrP(K219) argue that the Lys substitution produces dominant-negative inhibition. Presumably, PrP^C(K219) binds to protein X, which prevents PrP^C(E219) from being converted into PrP^{Sc}.^{187,294}

Two other polymorphisms have been identified. The third polymorphism results in an amino acid substitution at codon 171 (Asn/Ser),¹⁰⁹ which lies adjacent to the putative protein X binding site. The deletion of a single octarepeat (24 bp), which has been found in 2.5% of Caucasians, represents another polymorphism in the human PrP gene.²¹³ In another study of more than 700 individuals, this single octarepeat deletion was found in 1.0% of the population.

INFECTIOUS HUMAN PRION DISEASES

Prions from different sources have infected humans. Human prions have been transmitted to others both by ritualistic cannibalism and iatrogenic means. Kuru in the highlands of New Guinea was transmitted by ritualistic cannibalism, as people in the region attempted to immortalize their dead relatives by eating their brains.⁵ Iatrogenic transmissions include prion-tainted human growth hormone (HGH) and gonadotropin, dura mater grafts, and corneal transplants from people who died of CJD. In addition, CJD cases have been recorded after neurosurgical procedures in which ineffectively sterilized depth electrodes or instruments were used. As described below, vCJD resulted from the transmission of BSE prions through the ingestion of tainted beef products.⁴²⁸

Human Growth Hormone

More than 165 young adults have been diagnosed with iatrogenic CJD (iCJD) between 4 and 30 years after receiving HGH or gonadotropin from cadaveric pituitaries.^{255,429} The longest incubation periods (20–30 years) are similar to those associated with more recent cases of kuru.^{4,198} Since 1985, recombinant HGH produced in *E. coli* has been used in place of cadaveric HGH. With recombinant HGH, no cases of iCJD have been identified.

Variant Creutzfeldt-Jakob Disease

The first cases of vCJD in teenagers and young adults were identified in Great Britain in 1994.^{429,430} More than 215 teenagers and young adults have died of vCJD. The majority of vCJD patients presented with psychiatric symptoms including dysphoria, withdrawal, anxiety, insomnia, and loss of interest.⁴²⁹ Generally, neurologic deficits did not appear until at least 4 months later; these neurologic changes consisted of memory loss, paresthesias, sensory deficits, gait disturbances, and dysarthria.

As of March 2011, vCJD cases have been reported from the following countries: 175 from the United Kingdom; 25 from France; 5 from Spain; 4 from Ireland; 3 each from the United States and The Netherlands; 2 each from Portugal, Canada, and Italy; and one each from Japan, Taiwan, and Saudi Arabia. Two of the three U.S. cases, two of the four cases from Ireland, and the single cases from Canada and Japan were likely exposed to the BSE agent while residing in the United Kingdom. One of the 25 French cases may also have been infected in the United Kingdom. Although the average age of vCJD patients is 26 years, the youngest patient was 12 years old and the oldest was 74 years of age. The median duration of illness was 13 months, with the range from 6 to 69 months.

In addition to the young age of these patients, vCJD is characterized by numerous PrP amyloid plaques surrounded by a halo of intense spongiform degeneration in the brain.¹⁷¹ These unusual neuropathologic changes have not been seen in CJD cases in the United States, Australia, or Japan.^{64,171} Both macaques and marmosets developed neurologic disease several years after inoculation with bovine prions,¹⁶ but only the macaques exhibited numerous PrP plaques similar to those found in vCJD.²¹⁴

From both epidemiologic and experimental studies, evidence is now compelling that vCJD is the result of prions being transmitted from cattle with BSE to humans through consumption of contaminated beef products.

Transmission of Variant Creutzfeldt-Jakob Disease Prions by Blood Transfusion

vCJD has been identified in three patients who received blood transfusions from donors who later died of vCJD. In one case, the recipient was a 69-year-old man who was transfused 6.5 years before the onset of neurologic dysfunction.²²⁴ His PrP gene encoded Met/Met at position 129. Many details of the second case are not published, but the patient is known to have died of a nonneurologic disease.²⁸⁸ Although vCJD prions were found in the spleen and cervical lymph nodes of this patient, none were found in the brain. The patient's PrP gene encoded Met/Val at position 129 and, as such, is the first heterozygous codon 129 human identified with vCJD prions.

A glimpse of future vCJD cases caused by prion-tainted transfused blood may come from a survey of tissues collected during appendectomies and tonsillectomies. This U.K. survey reports that of the 12,674 appendectomy specimens examined, 3 were positive for PrP^{Sc} by IHC.¹⁵⁹ This finding argues that as many as 3,800 people in the United Kingdom may be replicating vCJD prions in their lymphoid tissues. Because IHC is considerably less sensitive than some ELISA-based assays, the number of Britons harboring vCJD prions in their lymphoid tissues may prove to be numerous.³³⁵ As noted above, 15 of 21 people in the United Kingdom who died of vCJD were found to have detectable PrP^{Sc} in their blood using a capture ELISA format.¹⁰³

STRAINS OF PRIONS

That goats with scrapie can manifest two different syndromes, one in which the goats became hyperactive and the other in which they became drowsy, raised the possibility that strains of prions might exist.²⁸⁷ Subsequent studies with mice documented the existence of multiple strains through measurements of incubation times and the distribution of vacuoles in the CNS.^{98,115}

Many investigators argued prion strain-specific information must be encoded in a small nucleic acid,⁴¹ but none was ever found.³³⁶ Others argued that this information must be enciphered within the structure of PrP^{Sc}.³⁰⁷ The first evidence supporting the hypothesis that strain-specific information is enciphered in PrP^{Sc} came from studies on prions causing transmissible mink encephalopathy (TME), which were passaged into Syrian hamsters. On serial passaging, two strains emerged: One strain (HY) produced hyperactivity in Syrian hamsters and the other (DY) was manifest as a drowsy syndrome, as was seen with scrapie strains in goats.²⁶ The HY strain is similar to the Sc237 prion strain with respect to protease resistance and sedimentation properties, whereas the DY strain shows minimal protease resistance and is less readily sedimented.²⁵

In subsequent binding protection studies, PrP^{Sc} from HY- or DY-infected hamster brains was mixed with labeled PrP lacking a GPI anchor that was produced in mammalian cells.²³ The labeled PrP bound to PrP^{Sc}, and the relative resistance to proteolytic digestion of the labeled PrP reflected that of PrP^{Sc}. Whether the protease resistance of the labeled PrP is the result of binding to PrP^{Sc} or is due to a change in the conformation of the labeled PrP itself remains to be determined.

Because the HY and DY strains emerged after passaging TME prions in Syrian hamsters, concern over the origin of these strains made the conclusions somewhat questionable. Such concerns were mollified by studies on the transmission of FFI and fCJD to Tg mice. Prions were generated *de novo* in patients harboring PrP gene mutations and propagated in mice expressing chimeric mouse-human (MHu2M) PrP. Brain homogenates of FFI and fCJD(E200K) patients transmitted disease to Tg(MHu2M) mice approximately 200 days after inoculation. The FFI inoculum induced formation of a 19-kD PrP^{Sc} fragment, as measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) after limited proteolysis and deglycosylation; in contrast, fCJD(E200K) produced a 21-kD PrP^{Sc} fragment.⁴⁰¹ On second passage, Tg(MHu2M) mice inoculated with FFI prions showed an incubation time of approximately 130 days and a 19-kD PrP^{Sc}

fragment, whereas those inoculated with fCJD(E200K) prions exhibited an incubation time of approximately 170 days and a 21-kD PrP^{Sc} fragment.³⁰⁹ These findings demonstrated that MHu2M PrP^{Sc} can exist in two different conformations, based on the sizes of the protease-resistant fragments, and argue that PrP^{Sc} acts as a template for the conversion of PrP^C into nascent PrP^{Sc}.

Studies with Tg mice also demonstrated that the prions causing BSE and vCJD are similar, but distinct from those responsible for sCJD, iCJD, and fCJD(E200K). Both BSE and vCJD prions transmitted to Tg mice expressing BoPrP after ~250 days, whereas mice expressing either HuPrP or MHu2M PrP developed disease after more than 500 days.^{158,206,352} Moreover, sCJD, iCJD, and fCJD(E200K) prions failed to transmit disease to Tg(BoPrP) mice after more than 500 days (Table 76.8).³⁵⁰

Isolation of New Strains

Many examples of new strains of prions arising upon passage into a host expressing a foreign PrP gene have been documented. Initially, this was observed when prions propagated in one species were passaged into another species.¹⁹⁴ New strains pathogenic to Syrian hamsters were obtained from prion strains that had previously replicated in mice after limiting dilution.³⁴⁹

Tg mice expressing MH2M PrP were inoculated with the two Syrian hamster prion strains, Sc237 and DY.²⁹¹ On first passage in Tg(MHM2)Prnp^{0/0} mice, the SHa(Sc237) prions exhibited prolonged incubation times, typical of a species barrier. On subsequent passage in Tg(MHM2)Prnp^{0/0} mice, the MH2M(Sc237) strain showed shortening of the incubation time. Moreover, the PrP^{Sc} of the MH2M(Sc237) strain exhibited structural properties differing from those of the parental SHa(Sc237) strain. Conversely, transmission of SHa(DY) prions to Tg(MHM2)Prnp^{0/0} mice did not encounter a species barrier, and the MH2M(DY) strain retained the conformational and phenotypic properties of SHa(DY).

Although cell-free models for mammalian prion propagation are still poorly understood, they have recently been applied to the study of prion strains. The highest yields for *in vitro* propagation have been achieved by seeding normal brain homogenate with prions and applying repeated rounds of sonication, that is, PMCA.⁵⁶ Although sonication introduces a series of problems, such as heterogeneous protein denaturation and high well-to-well variability in the generation of PrP^{Sc}, PMCA is currently the only cell-free method that converts PrP^C into an infectious form in substantial yields.

TABLE 76.8 Transmission of CJD Prions to Transgenic Mice

Prion inocula	Incubation period (mean days ± SEM; n/n ₀)		
	Tg(MHu2M)	Tg(MHu2M,M165V,E167Q)	Tg(BoPrP)
sCJD	197 ± 4 (24/24)	110 ± 2 (42/42)	>500 (58/58)
vCJD	605 ± 118 (6/14)	358 ± 17 (13/13)	267 ± 4 (80/80)
fCJD	170 ± 2 (10/10)	109 ± 3 (8/8)	>500 (2/36)
BSE	>500 (16/16)		240 ± 5 (41/41)

n, number of mice developing prion disease; n₀, number of mice inoculated.

In studies of the polymerization of recPrP into amyloid fibrils, several new approaches to the investigation of prion strains have emerged. Tg9949 mice, which overexpress MoPrP(89–231) and are not genetically predisposed to develop prion disease, were infected with recPrP (wt PrP residues 89–230) refolded into an amyloid conformation.²²⁰ The prion strain recovered from the brains of these mice was denoted MoSP1 and was transmissible to wt mice by serial passage.²²⁰ MoSP1 was readily distinguished from naturally occurring prions due to its high conformational stability.²²¹ During two subsequent rounds of serial passage, the incubation period (measured from inoculation to onset of neurological dysfunction) of MoSP1 isolates decreased from more than 500 days to 177 days in Tg9949 mice.²²² Strikingly, each shortening of the incubation period was accompanied by a decrease in the conformational stability of PrP^{Sc}.²²² No evidence suggesting that Tg9949 mice spontaneously generate prions was found, despite extensive experimentation, including repeated serial passage of three aged Tg9949 mouse brains as well as the examination of more than 50 Tg9949 brains by biochemical analysis and more than 100 Tg9949 brains by neuropathologic analysis.⁷⁷

Biochemical analysis of prions obtained from infected animals has given some insight into the structural variations that make up different strains. These variations include differences in glycosylation patterns, extent of protease resistance, electrophoretic mobility of proteolytic fragments, and conformational stability.^{24,129,333} However, the ability to modulate prion strain phenotypes purposefully by altering the conformation of PrP has only recently been demonstrated: recPrP folded into distinct amyloid conformations gave rise to distinguishable prion strains, with incubation periods that were dependent on the conformational stability of the recPrP amyloid.⁷⁷ By altering the conditions used to refold recPrP, amyloids with different conformational stabilities emerged. These amyloids were then inoculated into mice that overexpressed full-length

PrP at four-fold compared to wt levels. This resulted in prion strains with incubation periods and conformational stabilities that were correlated to the stability of the amyloid fibrils used to inoculate the mice (Fig. 76.14). The inability to infect wt mice directly with these preparations may reflect the low infectivity titers of such preparations or indicate that unidentified properties, in addition to conformational stability, modulate incubation period. Conflicting results using hamster prion strains with conformational stabilities that cover a much narrower range, compared to the synthetic prions studies, also support this notion.¹⁵ Nonetheless, the direct demonstration of the conformational basis of prion strain diversity provides further evidence that synthetic prions originate from the recPrP amyloid preparations, and not from the host or from contamination. If prions were arising spontaneously in the host, one would expect the strain properties to be independent of the amyloid properties. Exhaustive negative controls, including inoculation of the host mice with control solutions, biochemical and neuropathologic analysis of age-matched controls, and serial passage of aged brains from the host mice, also excluded spontaneous prion generation and contamination.⁷⁷

Recombinant PrP amyloids inoculated into Tg mice that overexpressed an N-terminally truncated PrP resulted in novel protease-sensitive, synthetic prions.⁷⁹ In contrast, many naturally occurring prions contain some fraction of PrP^{Sc} in a conformation that resists protease digestion (protease-resistant PrP^{Sc}, or rPrP^{Sc}).²⁴⁶ This observation has led to the idea that protease resistance is equated with prion infectivity and pathogenesis. However, many naturally occurring prion strains also contain PrP^{Sc} in a conformation that is sensitive to protease digestion (sPrP^{Sc}).^{121,333} The protease-sensitive, synthetic prion strains that were generated demonstrate that sPrP^{Sc} is transmissible and pathogenic, and can occur as a distinct entity from rPrP^{Sc}. Furthermore, repeated serial passage of these strains never resulted in the formation of rPrP^{Sc}, arguing that sPrP^{Sc} neither gives rise to nor results from rPrP^{Sc}.

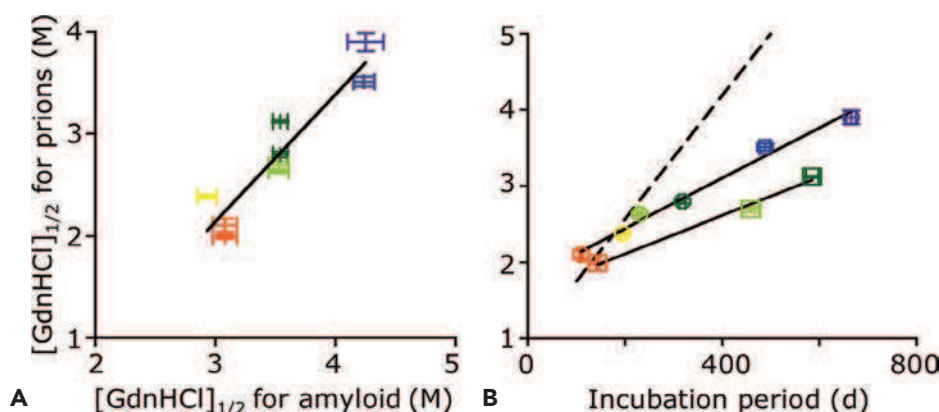


FIGURE 76.14. The properties of new synthetic prion strains are modulated by the conformational stability of amyloids used to generate them. GdnHCl_{1/2} values of synthetic prion strains directly correlated to the GdnHCl_{1/2} values of the respective amyloid preparations (**A**: $R = 0.94$, $n = 9$, $P = 0.0002$), as well as to incubation periods (**B**: dashed line from ref. 222) in Tg4053 mice (○; $R = 0.993$, $n = 6$, $P < 0.0001$) and FVB mice (□; $R = 0.994$, $n = 3$, $P = 0.07$). The synthetic prions strains inoculated were: MoSP5 (blue), MoSP6 (green), MoSP7 (orange), MoSP9 (yellow), MoSP12 (lime), MoSP13 (purple). (Reprinted from Colby DW, Giles K, Legname G, et al. Design and construction of diverse mammalian prion strains. *Proc Natl Acad Sci U S A* 2009;106:20417–20422, with permission; copyright © 2009 National Academy of Sciences, USA.)

Exposing amyloid fibers formed from recPrP of hamster sequence to heat in the presence of brain homogenate or albumin also led to the generation of a novel prion strain named SSLOW (for ‘Synthetic Strain Leading to OverWeight’).²³¹ Hamsters inoculated with amyloid fibers prepared in this way did not show symptoms of disease during 661 days of observation following inoculation, but were found to have low levels of PrP^{Sc} in their brains upon termination of the experiment. A serial transmission caused disease in more than 480 days. Biochemically, SSLOW had a conformational stability comparable to naturally occurring prion strains. However, the disease phenotype reported was unusual, in that it included weight gain and an unusually slow disease progression. Given the biochemical similarities between SSLOW and naturally occurring prions, the conformational basis of this unusual strain phenotype remains to be determined.

A central issue in the generation of prions from recombinant protein has been the presumed low titers of infectivity in the amyloid fibers formed,²⁴⁴ which results in long incubation times and often the inability to infect wt rodents directly. If long incubation times do in fact result from low titers, then this implies that synthetic prion preparations are heterogeneous mixtures containing many different conformations, and thus, limits their usefulness for determining the structure of PrP^{Sc}. Alternatively, long incubation times may indicate that amyloid fibers require maturation or further adaptation of their conformation in order to become fully infectious.

Several laboratories have succeeded in creating prion infectivity under conditions developed for PMCA.^{18,93,413} The initial report for the spontaneous generation of infectivity came in the form of unexpected amplification of prions in negative controls during studies that aimed to identify minimal components necessary for prion amplification *in vitro*.⁹³ The components required for amplification included polyanions in addition to PrP^C, which was accompanied by co-purified lipids. These results were verified by repeating the experiment in a laboratory that had never been used for prion research, reducing the potential for contamination. It was later shown that prions can be generated in a similar fashion using brain homogenate as the substrate, rather than minimal components, and that the newly created prion strain was distinct from a commonly used lab strain.¹⁸ Prions created in these studies using either PrP^C or normal brain homogenate had titers that were sufficient to infect hamsters with incubation periods of 113 to 168 days, whereas naturally occurring hamster prion strains can have incubation periods of 60–300 days.^{192,333}

Synthesis of highly infectious prions from recPrP has recently been reported using PMCA in the presence of lipids and RNA.⁴¹³ The infectivity of these preparations was said to be similar to that of naturally occurring strains, but these results remain unconfirmed despite the initial excitement surrounding this report.

Interplay Between the Species and Strains of Prions

The prion derives its PrP^{Sc} sequence, which encodes the species of the prion (Table 76.1), from the last mammal in which it was passed. Although the primary structure of PrP is likely to be the most important or even sole determinant of the tertiary structure of PrP^C, factors determining the conformation of nascent PrP^{Sc} are less well defined. Certainly, the PrP^{Sc} in

an inoculum functions as a template and, as such, it is important in determining the tertiary structure of nascent PrP^{Sc} molecules. One of the most instructive examples of the interplay between PrP^{Sc} sequence and conformation comes from transmission studies of BSE and vCJD prions to Tg mice (Table 76.8),³⁵⁰ some of which was reviewed previously. vCJD prions are composed of wt HuPrP^{Sc} that is folded into a conformation that favors binding to BoPrP^C over interactions with a variety of humanized transgene products, including HuPrP^C, MHu2M, and MHu2M(M165V,E167Q).^{14,204,350,352} Similarly, BSE prions display a greater affinity for BoPrP^C than for the humanized transgene products.^{14,350}

The foregoing studies demonstrate that the conformation of PrP^{Sc} can override differences in the PrP sequence between the infecting prion and host PrP^C. Such findings complicate the initially monastic view positing that the “species barrier” is the only determinant governing the transmission of prions from one species to another.²⁸⁵ It seems likely that defining the molecular language by which biological information is enciphered in the conformation of PrP^{Sc} will require determining the tertiary structures of PrP^{Sc} molecules isolated from many different strains.

PRION DISEASES OF ANIMALS

The prion diseases of animals include scrapie of sheep and goats, BSE, TME, chronic wasting disease (CWD) of mule deer and elk, feline spongiform encephalopathy, and exotic ungulate encephalopathy (Table 76.1).

PrP Polymorphisms in Sheep, Cattle, and Elk

In 1962, Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease that could be eradicated by proper breeding protocols.^{283,284} He considered its transmission by inoculation of importance primarily for laboratory studies and communicable infection of little consequence in nature. Other investigators viewed natural scrapie as an infectious disease and argued that host genetics only modulates susceptibility to an endemic infectious agent.⁹⁹

In sheep, PrP gene polymorphisms encoding amino acid substitutions at positions 136, 154, and 171 have been studied with respect to the incidence of scrapie (Fig. 76.11).^{76,141,212} Investigations of natural scrapie in the United States have shown that 85% of afflicted sheep are of the Suffolk breed. Only those Suffolk sheep homozygous for Gln at codon 171 developed scrapie, although healthy controls with Gln/Gln, Gln/Arg, and Arg/Arg genotypes were also found.^{76,143,273,423} These results argue that susceptibility to scrapie in Suffolk sheep is governed by the PrP codon 171 polymorphism; similarly, codon 171 as well as residue 136 in Cheviot sheep profoundly influences susceptibility to scrapie.¹⁶⁷

In contrast to sheep, different breeds of cattle have no specific PrP polymorphisms. The only polymorphism recorded in cattle is a variation in the number of octarepeats: most cattle, like humans, have five octarepeats but some have six. However, the presence of six octarepeats does not seem to be overrepresented in BSE.¹⁶⁸

In studies of CWD, the susceptibility of elk, but not deer, seems to be modulated by codon 132, which corresponds to

codon 129 in humans (Fig. 76.11). Elk with CWD consistently express Met/Met at position 132; no elk with CWD expressing leucine at this residue have been found.²⁷³

Bovine Spongiform Encephalopathy

Prion strains and the species barrier are of paramount importance in understanding the BSE epidemic in Britain, where it is estimated that between 1 and 2 million cattle were infected with prions.⁷ The mean incubation time for BSE is approximately 5 years. Therefore, most cattle did not manifest disease because they were slaughtered between 2 and 3 years of age. Nevertheless, more than 180,000 cattle, primarily dairy cows, died of BSE between 1986 and 2000.^{7,297} BSE is a massive common-source epidemic caused by meat and bone meal (MBM) fed primarily to dairy cows. MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high-protein nutritional supplement. In the late 1970s, the hydrocarbon-solvent extraction method used in the rendering of offal began to be abandoned, resulting in MBM with a much higher fat content.⁴²⁸ It is widely accepted that this change allowed scrapie prions from sheep to survive the rendering process and to be passed into cattle. Alternatively, bovine prions may have been present at low levels prior to modification of the rendering process and, with the processing change, survived in sufficient numbers to initiate the BSE epidemic when reintroduced into cattle through ingestion of MBM.²⁹⁷ Perhaps a particular conformation of BoPrP^{Sc} was selected for heat resistance during the rendering process and then reselected multiple times as cattle infected by ingesting prion-contaminated MBM were slaughtered and their offal rendered into more MBM. Against the latter hypothesis is the widespread geographic distribution of the initial 17 cases of BSE throughout England, which occurred almost simultaneously.⁴²⁸ It is noteworthy that brain extracts from cattle with BSE cause disease in cattle, sheep, mice, pigs, and mink after intracerebral inoculation.⁴¹⁹

In July 1988, the practice of feeding MBM to sheep and cattle was banned. Although statistical analyses demonstrate that the epidemic has disappeared as a result of this ruminant feed ban, reminiscent of the disappearance of kuru in the Fore people of New Guinea, it is unclear how many cases of BSE were due to infection and how many arose spontaneously.³⁰⁶ In 2003, there were 1,390 cases of BSE; how many were due to tainted feed is unknown. Although evidence of a preexisting prion disease of cattle, either in Great Britain or elsewhere, is scant, an outbreak of TME in Wisconsin has been cited as evidence for sporadic BSE.²³⁹ Moreover, the formation of synthetic prions from wt recPrP argues that any mammal expressing PrP^C can produce prions spontaneously.^{77,221}

Compelling Evidence for Transmission of Bovine Prions to Humans

The restricted geographic occurrence and chronology of vCJD raised the possibility that BSE prions were transmitted to humans. However, no set of dietary habits clearly distinguished vCJD patients from apparently healthy people, and there was no explanation for the predilection of vCJD for teenagers and young adults. It is noteworthy that epidemiologic studies since the mid-1970s failed to find evidence of transmission of sheep prions to humans.⁸⁶ Attempts to predict the future number of cases of vCJD, assuming exposure to bovine prions prior to

the offal ban, have been uninformative because so few cases of vCJD have occurred.¹³⁰

Studies of PrP^{Sc} from brains of patients who died of vCJD showed a PrP glycoform pattern different from those found for sCJD and iCJD,⁸¹ but the utility of measuring PrP glycoforms in trying to establish a relationship of BSE to vCJD has been questioned^{128,369} because PrP^{Sc} is formed after the protein is glycosylated and enzymatic deglycosylation of PrP^{Sc} requires denaturation.¹⁰⁶ Moreover, synthetic prions have been formed from unglycosylated recMoPrP(89–230), demonstrating that glycosylation is unnecessary for prion formation.^{77,220}

When non-Tg RIII mice were inoculated with human vCJD or bovine BSE prions, similar incubation times of approximately 310 days were observed.⁴³ This finding was used to argue for a relationship between vCJD and BSE. But such studies suffer from transmission of both BSE and vCJD prions to a heterologous host (i.e., non-Tg mice expressing MoPrP^C). Using Tg mice, it became possible to accumulate compelling evidence for the transmission of bovine prions to humans. BSE prions transmitted to Tg(BoPrP) mice after approximately 240 days but not to Tg mice expressing either HuPrP or MHu2M PrP (Table 76.8).³⁵² On second passage to Tg(BoPrP)Prnp^{0/0} mice, the incubation time was unaltered, demonstrating the complete absence of a species barrier. Similar to BSE prions, vCJD prions transmitted readily to Tg(BoPrP)Prnp^{0/0} mice, with a slightly longer incubation time of approximately 270 days (Table 76.8),³⁵² but poorly to Tg(HuPrP)¹⁵⁸ and Tg(MHu2M) mice.^{204,350} On second passage of vCJD prions to Tg(BoPrP)Prnp^{0/0} mice, the incubation time was reduced to approximately 225 days, demonstrating a small but expected species barrier. Moreover, sCJD, iCJD, and fCJD(E200K) prions failed to transmit disease to Tg(BoPrP)Prnp^{0/0} mice after more than 500 days.³⁵⁰ These findings argue that the strain-specific PrP^{Sc} conformations of BSE and vCJD prions are similar, despite substantial differences in the amino acid sequences of BoPrP and HuPrP. Clearly, the conformation of HuPrP^{Sc}(vCJD) makes these prions more readily transmissible to Tg(BoPrP)Prnp^{0/0} mice than to either Tg(HuPrP)Prnp^{0/0} or Tg(MHu2M)Prnp^{0/0} mice.³⁵⁰

Chronic Wasting Disease

Mule deer, white-tailed deer, and elk have been reported to develop CWD, which is the only prion disease known in free-ranging animals. CWD was first described in 1967 and reported to be a spongiform encephalopathy in 1978, based on histopathology in the brain. CWD has been found in the United States, Canada, and South Korea.

In the United States, CWD has been reported in Colorado, Wyoming, South Dakota, Nebraska, Oklahoma, Montana, New Mexico, Minnesota, Wisconsin, and New York. In captive cervid herds, up to 90% of mule deer have been reported to be positive for prions,⁴³⁶ and up to 60% of elk in Colorado and Wyoming develop CWD.⁴³⁵ Moreover, the incidence of CWD in cervids living in the wild has been estimated to be as high as 15%.²⁵⁴ The mode of transmission of CWD prions among mule deer, white-tailed deer, and elk is unresolved, but contamination of grass with prions in saliva or feces was hypothesized to be a likely source based on the high content of PrP^{Sc} in the intestinal lymphoid tissue of cervids with CWD.⁴³⁵ After feeding deer prions, we found that asymptomatic CWD-infected mule deer excrete CWD prions in their feces long before they develop clinical signs of prion

disease. Intracerebral inoculation of irradiated deer feces into Tg mice overexpressing cervid PrP revealed infectivity in 14 of 15 fecal samples collected from five deer at 7–11 months before the onset of neurological disease.³⁹² Although prion concentrations in deer feces were considerably lower than in brain tissue from the same deer collected at the end of the disease, the estimated total infectious dose excreted in feces by an infected deer over the entire incubation period may approximate the total contained in the brain.³⁹³ Prolonged fecal prion excretion by infected deer provides a plausible natural mechanism that explains the high incidence and efficient horizontal transmission of CWD within deer herds as well as prion transmission among other susceptible cervids. It is noteworthy that prions have also been detected in the saliva of cervids.^{152,242,270,394}

Brain homogenates from mule deer with CWD have transmitted disease to 4 of 13 cattle after intracerebral inoculation.¹⁵³ These findings are particularly important because there is great concern that CWD prions might be transmitted to cattle grazing in contaminated pastures. In addition, CWD has been transmitted to ferrets, mink, squirrel monkeys, goats, and mice after intracerebral inoculation⁴³⁵; however, only mule deer demonstrate efficient transmission of CWD prions by intracerebral inoculation. CWD has been transmitted from mule deer and elk to Tg mice expressing a cervid PrP gene.^{40,202,391} Such studies promise to increase substantially the understanding of prion disease in free-ranging animals. Whether CWD prions have been transmitted to deer hunters remains to be established.

FUNGAL PRIONS

Although prions were originally defined in the context of an infectious mammalian pathogen,³⁰⁸ the recognition that prions modify the metabolism of yeast has extended our understanding of these epigenetic elements. It is generally accepted that prions are elements that impart and propagate variability through multiple conformers of a normal, cellular protein. Nine prion determinants, [URE3], [PSI⁺], [PIN⁺]/[RNQ⁺], [MCA⁺], [SWI⁺], [OCT⁺], [MOT3⁺], [GAR⁺], and [ISP⁺] have been described in yeast^{70,425} and another prion determinant [HET-s] has been reported in the fungus *Podospora anserina*²³⁰ (see Chapter 22).

Some Differences Between Fungal and Mammalian Prions

A wealth of studies has now established the concept of prions in both mammals and fungi. Fungal prions do not form infectious particles and, as such, infection by exogenous prions does not generally occur. In contrast, prion infection in mammals can result from exposure to an exogenous inoculum and, like viruses, spread from one host cell to another. Fungal prions are produced in the cytoplasm, whereas mammalian PrP prions are produced within cholesterol-rich microdomains on the surface of cells, called rafts or CLDs.

In both fungi and mammals, synthetic prions have been formed from fragments of prion proteins that were forced to adopt β -sheet-rich conformations. These β -sheet-rich aggregates of prion protein fragments initiated prion replication when introduced into the appropriate fungal or mammalian host. Notably, different strains of both fungal and mammalian prions are distinct conformations of the particular prion pro-

tein. This finding necessitates a mechanism by which a distinct conformation can be templated and reproduced with a high degree of fidelity.

In the filamentous fungus *Podospora anserina*, the prion state of the HET-s protein initiates cell death through a process called heterokaryon incompatibility.¹⁴⁷ Filamentous fungi have several incompatibility loci that regulate the fusion of mycelium between genetically distinct individuals, one of these is the prion protein HET-s that has two-domains. The C-terminus is the prion domain (residues 218–289) that is required and sufficient for prion propagation; the N-terminus is the globular domain (residues 1–227) that specifies the incompatibility type. Solid-state NMR studies showed that the prion domain of HET-s in its fibrillar state forms a left-handed β -solenoid. Extended structural studies confirm six β -arcs, two salt bridges, and three asparagine ladders in the β -solenoid core.⁴¹¹ The β -solenoid is similar to the β -helix proposed for PrP^{Sc} based on EM crystallography studies and consistent with recent x-ray diffraction studies described above (see Fig. 76.10).

In yeast, the prion state is proposed to be functionally inert in the case of both [PSI⁺] and [URE3], and produces the same phenotype as inactivation of the maintenance gene. In contrast, prion diseases in mammals cannot be explained simply by the loss of function of PrP because ablation of the PrP gene does not produce neurodegeneration.^{46,87,234,334}

In studies of the rat hippocampus, a neuronal transcription factor called cytoplasmic polyadenylation element binding (CPEB) protein was found, which has an N-terminal region rich in Gln and Asn similar to the fungal prion domains. In *Aplysia*, the CPEB protein has been suggested to function as a prion in regulating protein synthesis in dendrites during long-term facilitation.³⁶² Interestingly, aggregated CPEB in the prion state appears to stimulate polyadenylation, whereas the monomer is much less active.³⁶¹ Undoubtedly, many more prions will be found throughout nature, some of which cause disease, whereas others function in the regulation of metabolism.

Similar to CPEB in that multimers are the biologically active form, the mitochondrial antiviral-signaling (MAVS) protein, which is required for innate immune defense against viruses, appears to be a prion.¹⁶² MAVS proteins form large aggregates after viral infection and these aggregates are potent activators of the IRF3 protein in the cytoplasm. MAVSs assemble into fibrous structures that convert endogenous MAVS proteins into more functional aggregates. This self-propagating process leads to the formation of nascent, biologically active MAVS aggregates and as such, MAVS is a prion. The MAVS aggregates produce a gain of function through a conformational switch that is highly efficient and tightly regulated by viral infection. Remarkably, *in vitro* incubation of retinoic acid-inducible gene I and mitochondria in the presence of K63 polyubiquitin chains efficiently converts endogenous MAVSs into functional MAVS prions.

PREVENTION AND THERAPEUTICS FOR PRION DISEASES

No therapeutics exist that halt or even slow neurodegeneration. The most effective drug that ameliorates the symptoms of a neurodegenerative disease is L-dopa, which was introduced in the mid-1960s.⁸⁵ Molecular genetics allows us to identify people at

risk for inherited neurodegenerative diseases decades before neurologic dysfunction is manifest, but no preventative therapeutics exist. Like the other inherited neurodegenerative diseases, the familial prion diseases are fully penetrant (see Fig. 76.11).^{65,372}

Prion Therapeutics

Various compounds have been proposed as potential therapeutics for treatment of prion diseases; these include polysulfated anions, dextrans, Congo red dye, oligonucleotides, and cyclic tetrapyrroles, all of which have been shown to increase survival time when given prior to prion infection in rodents, but not when administered a month or more after infection has been established.^{193,304,355}

In addition to studies in rodents, ScN2a cells chronically infected with scrapie prions have been used to identify a variety of compounds with antiprion activity,^{59,74,126,127,144,200,205,295,305,387,396} but none have been shown to be effective in halting prion diseases in either animals or humans except for N-(4-oxazol-5-yl-phenyl)-N'-pyridin-4-ylmethylene-hydrazine, referred to as Compound B.¹⁸⁹ Prolongation of the incubation time depended on the strain of prions as well as the time at which administration of Compound B was initiated. Moreover, the highly reactive phenylhydrazone structure of Compound B prevents it from becoming a drug.

Another approach has involved the search for small molecules that bind to PrP^C, the structure of which is known. *In silico* studies failed to identify any putative compounds likely to bind to PrP^C due to an absence of any exposed potential binding pockets. Despite these results, we developed several approaches to measure the binding of small compounds to purified recPrP.³⁰⁰ Isothermal titration calorimetry (ITC) was used to measure direct binding between the compounds and recPrP. We performed ITC with 16 confirmed antiprion compounds, but no substantial release of energy was detected for any of the compounds, suggesting that none interacted with recMoPrP(89–230). We next sought to measure the binding of compounds to recPrP by a thermal-denaturation upshift assay. The melting point of PrP was measured by circular dichroism (CD) in the presence of stabilizing and destabilizing agents. In accordance with the ITC results, none of the 16 compounds significantly increased the T_m of recMoPrP(89–230), suggesting that none of the compounds either stabilized or interacted with the folded domain of the protein.

Structure-based drug design based on dominant-negative inhibition of prion formation produced several lead compounds.²⁹⁵ Prion replication depends on protein–protein interactions, and a subset of these interactions gives rise to dominant-negative phenotypes produced by single residue substitutions.¹⁸⁷

A particularly interesting set of potential drugs is the branched polyamines, or dendrimers, which enhance the clearance of PrP^{Sc} from cells.^{128,387} Although these compounds cure cultured cells of prion infection, they have not been successfully deployed in mice because of difficulties in delivering such highly charged compounds into the CNS.

Inducible Transgenes as a Model for Therapeutics

Studies with inducible transgenes provide a model for prion therapeutics. Bigenic mice were constructed using a tetracycline-inducible PrP transgene system, in which doxycycline is capable

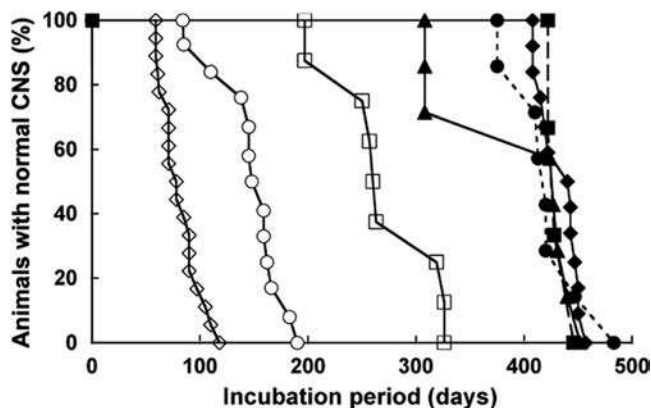


FIGURE 76.15. Both doxycycline dosage and levels of tetracycline-controlled transactivator (tTA) expression control the incubation time of RML prions in Tg(tTA:PrP)3 mice. Following the intracerebral inoculation of RML prions, Tg(tTA:PrP)3 mice (open circles), which express prion protein (PrP) at high levels in the brain, developed disease with an incubation period of approximately 149 days. Doubling the levels of PrP expression in Tg(tTA:PrP)3 mice (open diamonds) resulted in a shortening of the incubation period to 81 days, demonstrating that tTA controls PrP expression. Mice harboring only the target PrP transgene, designated Tg(tetO:PrP) mice (filled diamonds), became ill at ~433 days. The levels of PrP expression could be regulated in a dose-dependent manner by administration of doxycycline. Tg(tTA:PrP)3 mice treated with higher doses of doxycycline displayed longer incubation periods. To suppress cellular PrP (PrP^C) expression, animals received various concentrations of doxycycline in the drinking water beginning 7 days before intracerebral inoculation with RML prions. Bigenic Tg(tTA:PrP)3 mice were given doxycycline at concentrations of 0 (open circles), 0.002 (open squares), 0.02 (filled triangles), 0.2 (filled circles), and 2 (filled squares) mg ml⁻¹. (Reprinted, with permission, from Safar JG, DeArmond SJ, Kociba K, et al. Prion clearance in bigenic mice. *J Gen Virol* 2005;86:2913–2923, copyright © 2005 Society for General Microbiology.)

of suppressing approximately 95% of PrP expression. Bigenic mice inoculated with RML prions developed signs of disease approximately 80 days after inoculation, whereas bigenic mice maintained on oral doxycycline did not develop disease until approximately 450 days (Fig. 76.15).³³⁴ A dose-dependent relationship was found between the concentration of doxycycline in the drinking water and the prolongation of the incubation period: Higher doses of doxycycline produced longer incubation times. In earlier studies, the incubation times in prion-inoculated mice were prolonged by knockout of the PrP gene.^{233,312}

Studies with doxycycline argue that drugs diminishing the steady-state level of PrP^C are likely to be effective in treating prion disease. Such drugs might decrease transcription of the PrP gene or increase the degradation of PrP mRNA. Alternatively, an effective drug might diminish the synthesis of PrP^C or increase the clearance of PrP^C.

Another approach to controlling the expression of the PrP gene used the Cre/lox system to excise PrP-coding sequences in adult mice. Bigenic NFH-Cre/MloxP mice express PrP in neurons and nonneuronal cells until approximately 9 weeks of age, when they undergo Cre-mediated depletion of neuronal PrP^C.²³⁵ The NFH-Cre/MloxP mice were inoculated with RML scrapie prions at 3 to 4 weeks of age, and prion replication proceeded until Cre-mediated neuronal PrP^C depletion

occurred. At that time, early pathologic changes including spongiosis, gliosis, and PrP^{Sc} deposition were found. Over the next 4 weeks, these neuropathologic alterations resolved and the mice survived for more than 1 year despite the continued deposition of PrP^{Sc} in and adjacent to nonneuronal cells.

With *Prnp*^{+/-} mice that are heterozygous for ablation of the PrP gene, we and others found a marked prolongation of the incubation period after inoculation with RML prions.^{47,312} Although prion replication was somewhat slowed by a 50% reduction in PrP^C levels, the incubation time greatly lengthened. The cause of these longer incubation periods is unknown, but they may be due to changes in the location of the initial PrP^{Sc} deposition that occurs in *Prnp*^{+/-} mice.³¹² An alternative hypothesis suggests that the formation of some other form of PrP^{Sc} is uniquely delayed in *Prnp*^{+/-} mice, but no mechanism has been offered.³⁴⁰

Knockdown of PrP^C by RNAi⁴⁰³ resulted in a reduction of PrP^{Sc} in prion-infected cultured cells.⁸⁹ Lentivectors expressing PrP^C-specific short hairpin RNAs (shRNAs) that silence expression of *Prnp* were introduced into embryonic stem (ES) cells.²⁹⁶ Chimeric mice derived from lentivector-transduced ES cells expressed reduced levels of brain PrP^C by 15% to 70% depending on the degree of chimerism that ranged from 10% to 90%. The mean incubation period of the control mice was approximately 165 days, whereas those with less than 35% chimerism lived approximately 179 days and those with greater than 65% chimerism lived approximately 214 days. Extension of the incubation time by reducing PrP expression is consistent with studies using an inducible transgene controlled by doxycycline: the level of PrP expression was inversely proportional to the length of the incubation time.³³⁴

To explore the therapeutic potential of shRNAs, Tg mice at approximately 7 days of age were inoculated intracerebrally with RML prions and injected approximately 56 days later with lentivirus expressing an shRNA targeting PrP into the hippocampus bilaterally.⁴²⁶ In control mice not inoculated with prions, the PrP mRNA was reduced by approximately 75%. The prion-inoculated Tg mice that also received the lentivirus injections showed a modest, approximately 17-day prolongation of the incubation time, from 88 days to 105 days.

Quinacrine and Other Acridine Derivatives

Tricyclic derivatives of acridine exhibit half-maximal inhibition of PrP^{Sc} formation at effective concentrations (EC₅₀) between 0.3 and 3 μ M in cultured ScN2a cells.^{101,205} The EC₅₀ for chlorpromazine was 3 μ M, whereas quinacrine was 10 times more potent. Various 9-substituted, acridine-based analogs of quinacrine were synthesized, which demonstrated variable potencies similar to chlorpromazine and emphasized the importance of the side chain in mediating the inhibition of PrP^{Sc} formation.²⁰⁵ These studies showed that tricyclic compounds with an aliphatic side chain at the middle ring moiety constitute a new class of antiprion agents. Several bis-acridines proved to be 10 times more potent than quinacrine.²⁴³ However, the bis-acridines penetrated the blood–brain barrier (BBB) poorly and failed to extend the prion incubation time in mice.

Because quinacrine and chlorpromazine have been used for many years in humans as antimalarial and antipsychotic drugs, respectively, and are known to penetrate the BBB, such compounds became immediate candidates for the treatment of CJD and other human prion diseases.

Enthusiasm for quinacrine as an antiprion compound was considerable, since it was an U.S. Food and Drug Administration (FDA)–approved drug with a long history of administration to humans. Clinical trials of quinacrine in patients with CJD were initiated, since there were no effective drugs for the treatment of this uniformly fatal illness. Unfortunately, quinacrine was ineffective in treating humans dying of CJD⁸⁰ (M.G. Geschwind et al., unpublished data). Earlier studies demonstrated that quinacrine inefficiently penetrates the BBB, which could contribute to its lack of efficacy in mouse models of prion disease.^{17,82,124,445} Because quinacrine was found to be a substrate for P-glycoprotein (P-gp) multidrug resistance (Mdr1) transporters, we circumvented its poor BBB permeability by using Mdr1^{0/0} mice that are deficient in Mdr1a and Mdr1b genes encoding P-gps. Mdr1^{0/0} mice given oral quinacrine accumulated the drug in their brains up to 100 μ M without evidence of toxicity, a level approximately 100 times higher than that found in the brains of wt mice (Fig. 76.16¹²⁶). Moreover, brain levels of 100 μ M exceeded the EC₅₀ for quinacrine in ScN2a cells by more than 100-fold. Notably, an asymmetric carbon in the side chain of quinacrine creates two stereoisomers. (*S*)-quinacrine is two to three times more potent than the (*R*)-quinacrine enantiomer in cultured cells.³³⁰

Although PrP^{Sc} levels in the brains of prion-inoculated Mdr1^{0/0} mice decreased transiently upon the initiation of quinacrine treatment, the PrP^{Sc} levels rose after a couple of weeks despite the continued administration of quinacrine (Fig. 76.17¹²⁶). The transient depression of PrP^{Sc} levels in the Mdr1^{0/0} mice after initiating treatment with quinacrine did not result in a prolongation of the incubation time compared to untreated, control mice. A similar phenomenon was observed in cultured, prion-infected neuroblastoma cells: PrP^{Sc} levels initially decreased after quinacrine treatment and then rapidly recovered after 3 days of continuous treatment. The PrP^{Sc} that persisted in the brains of quinacrine-treated Mdr1^{0/0} mice showed a lower conformational stability and different immunofluorinities compared to that found in the brains of untreated controls. Because these altered physical properties were not maintained upon serial passage in Mdr1^{0/0} mice, we argued that quinacrine eliminates a specific subset of PrP^{Sc} conformers, resulting in the survival of drug-resistant prion conformations. The transient accumulation of this drug-resistant prion population provides a possible explanation for the lack of *in vivo* efficacy of quinacrine and other antiprion drugs.

Anti-PrP Antibodies

A panel of recombinant antibody fragments (recFabs), recognizing different epitopes on PrP, was studied with respect to inhibition of prion propagation in cultured ScN2a cells.²⁹⁰ Recombinant Fabs binding to PrP^C on the cell surface inhibited PrP^{Sc} formation in a dose-dependent manner. In ScN2a cells treated with the most potent recFab D18, prion replication was completely abolished and preexisting PrP^{Sc} rapidly cleared, suggesting that this antibody may cure established infection. The recFab D18 seem to recognize most of the PrP^C molecules on the cell surface, whereas other recFabs seem to bind fewer PrP^C molecules. In other studies, the monoclonal antibody, 6H4, which is believed to bind to the same region of PrP as recFab D18, was found to inhibit prion accumulation in ScN2a cells.¹⁰⁵

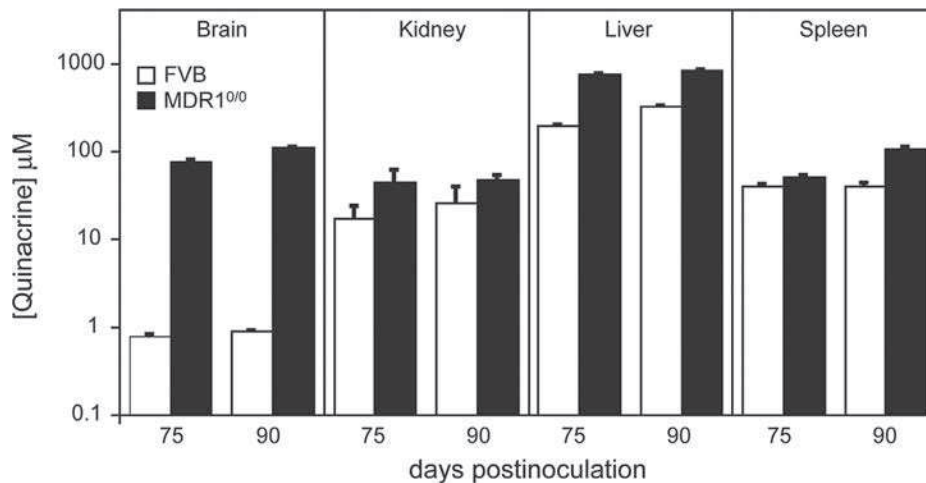


FIGURE 76.16. Accumulation of quinacrine in treated FVB and MDR^{0/0} mice. Quinacrine was given orally beginning 60 days postinoculation (dpi) for 30 d; the treated groups were designated FVB[60–90] and Mdr1^{0/0}[60–90]. The levels of quinacrine in the brains, kidneys, livers, and spleens of FVB[60–90] (white bars) and MDR^{0/0}[60–90] (black bars) were measured at 75 and 90 dpi by quantitative liquid chromatography tandem mass spectrometry and comparison to known standards. Error bars represent the standard error of the mean (n = 3). (Reprinted from Ghaemmaghami S, Ahn M, Lessard P, et al. Continuous quinacrine treatment results in the formation of drug-resistant prions. *PLoS Pathog* 2009;5:e1000673, with permission; copyright © 2009 Public Library of Science.)

Anti-PrP antibodies delivered systemically through transgenesis¹⁵⁶ or by injection^{364,425} were successful in blocking prion replication in mice inoculated intraperitoneally. The same anti-PrP antibodies were ineffective in treating prion infection initiated by intracerebral inoculation.

Whether antibodies or Fabs can be used for the prevention and treatment of prion diseases in humans or animals is unclear. Delivery of antibodies or Fabs to the CNS remains problematic due to the BBB. Notably, it seems unlikely that vaccination, which has been so effective in preventing many viral illnesses, will be a useful strategy for preventing or treating prion diseases.

Bioluminescence Imaging for Monitoring Drug Efficacy

Bioluminescence imaging (BLI) is based on the expression of a luciferase reporter gene driven by a promoter of interest. A sensitive CCD camera measures the luminescence signal following intraperitoneal (i.p.) injection of the substrate luciferin; light penetrates tissue and bone allowing *in vivo* monitoring. This technique allows mice to be repeatedly scanned throughout the disease process, enabling multiple data points to be collected on individual animals, each acting as its own control. Because

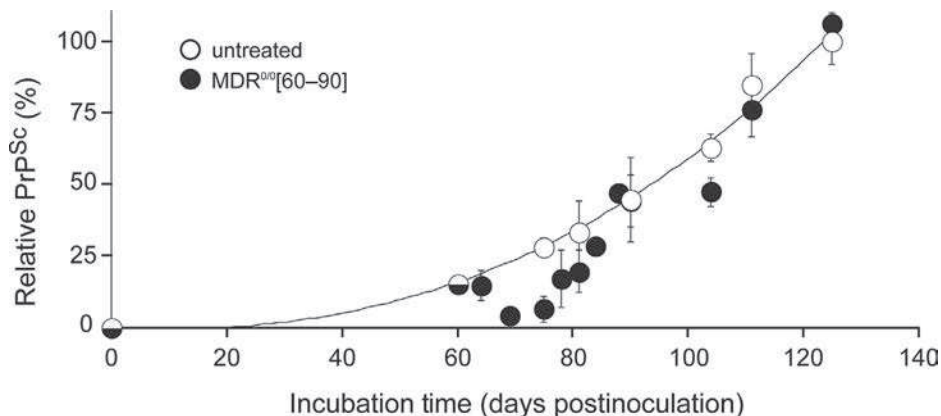


FIGURE 76.17. Levels of disease-causing prion protein isoform (PrP^{Sc}) in the brains of quinacrine-treated, RML-infected MDR^{0/0} mice. Quinacrine was given orally beginning 60 days postinoculation (dpi) for 30 days; the treated group was designated MDR^{0/0}[60–90]. The kinetics of PrP^{Sc} accumulation in the brains of untreated MDR^{0/0} and treated MDR^{0/0}[60–90] mice was analyzed quantitatively by enzyme-linked immunosorbent assay (ELISA). Error bars represent the standard error of the mean (n = 3). (Reprinted from Ghaemmaghami S, Ahn M, Lessard P, et al. Continuous quinacrine treatment results in the formation of drug-resistant prions. *PLoS Pathog* 2009;5:e1000673, with permission; copyright © 2009 Public Library of Science.)

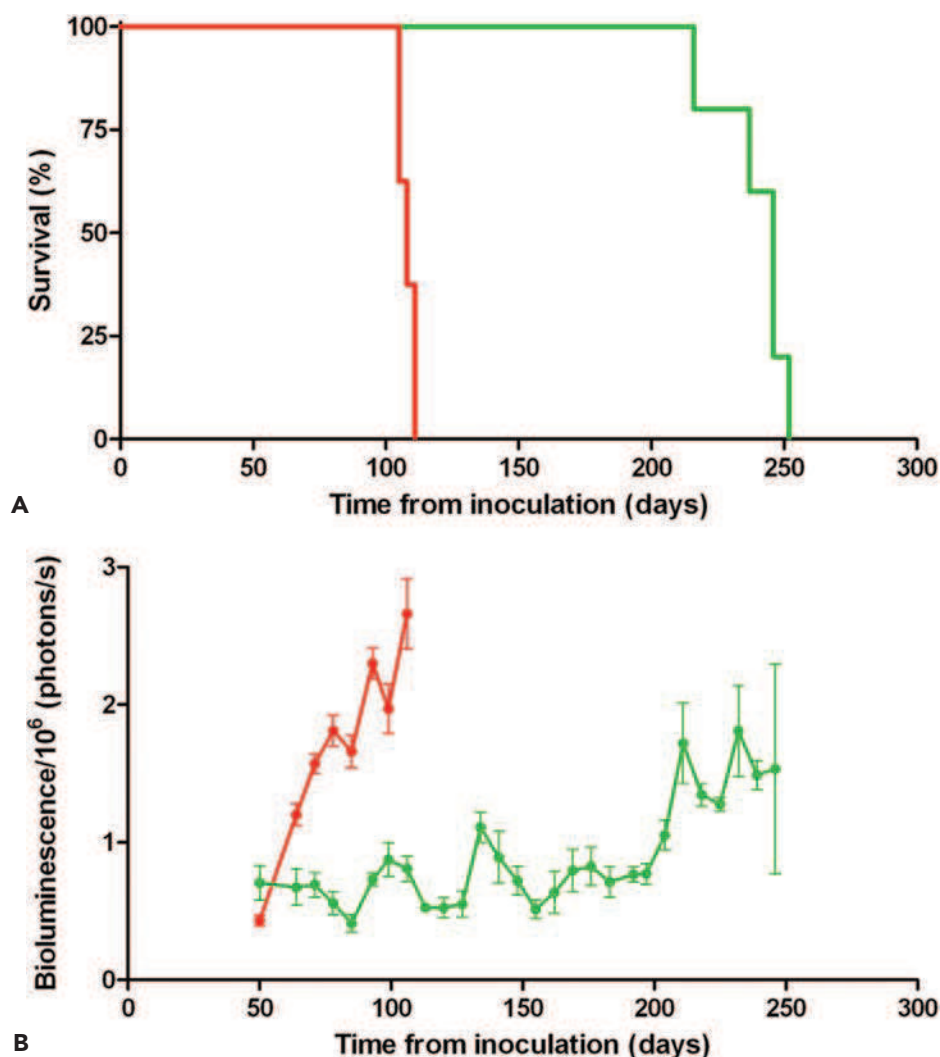


FIGURE 76.18. Bioluminescence imaging is predictive of drug efficacy. Tg(*Gfap-luc*)FVB mice were inoculated intracerebrally with RML prions; Compound B was given orally at 110 mg/kg/day in a liquid diet beginning on day 1. **A:** Kaplan-Meier survival plot and **B:** BLI signal of untreated (red) and Compound B-treated (green) mice.

GFAP is strongly upregulated in prion disease,²³⁷ we chose to use firefly luciferase (*luc*) driven by the *Gfap* promoter. Tg(*Gfap-luc*) mice⁴⁵¹ were inoculated with prions and monitored by BLI. Untreated mice develop clinical signs of prion disease at approximately 110 days post-inoculation (dpi) (Fig. 76.18), whereas the BLI signal starts to be upregulated at approximately 55 dpi (Fig. 76.18), reducing the time to disease diagnosis by approximately 50%.³⁹⁰

When we applied BLI to the assessment of compounds that extend the incubation time in mice inoculated with RML prions, we found that Compound B administration extended the survival of mice¹⁸⁹ and suppressed the bioluminescence signal. Mice were dosed continuously with Compound B starting immediately after inoculation, and the BLI signal was measured weekly. The BLI signal in untreated mice began to increase by approximately 60 dpi, whereas it remained suppressed in treated mice until shortly before the mice developed clinical signs of disease, at approximately 220 dpi (Fig. 76.18). Compound B efficacy was therefore detected by approximately 60 days, long before extension of survival could be measured by clinical observations. Equally important, BLI offers a procedure whereby multiple observations on a single animal can be obtained; this gives considerably more confi-

dence in the measurements than a single point of illness onset used in typical incubation-time studies. BLI offers a new tool in the development of therapeutics because it appears to be useful in predicting drug efficacy.

Inactivation of Prions

For many years, the scrapie agent was believed to be a slow-acting virus,³⁶³ but differences between the inactivation profiles of the scrapie agent and that of viruses provided the first clues that the agent was not a virus.^{3,5} Subsequent studies showed that as preparations from Syrian hamster brain were progressively enriched for scrapie infectivity, procedures that modified proteins were found to inactivate the samples, whereas those that modified nucleic acids had no effect.^{100,215,308,356} Protein denaturants played an important role in these studies, particularly in assessing the physical properties of prions during purification. Exposure to SDS, urea, or GdnHCl denatured PrP^{Sc}, resulting in diminished prion infectivity, susceptibility to proteolysis, and disaggregation of amyloid polymers composed of PrP 27–30.^{256,315} Prior to the studies with acidic SDS described below, sterilization of prions could be accomplished only with use of prolonged autoclaving at 134°C for 5 hours or exposure to strong alkali such as 2 N NaOH for several hours.³¹⁴

Prompted by the discovery showing that prions become protease-sensitive after exposure to branched polyamine dendrimers in weak acid,³⁸⁸ inactivation of prions by detergents such as SDS under weakly acidic conditions was investigated. SDS at pH values of less than 4.5 or greater than 10 rendered PrP^{Sc} susceptible to proteolytic digestion at room temperature.²⁸⁹ Exposure of Sc237 prions in hamster brain homogenates to 1% SDS and 0.5% acetic acid (AcOH) at room temperature reduced the prion titer by a factor of more than 10⁵.²⁸⁹ In addition to investigations of Sc237 prions, sCJD prions in human brain homogenates and bound to steel wires were studied; these prions harbored Met at codon 129 and a 21-kD protease-resistant fragment (designated type MM1). Infectivity bound to the wires was bioassayed by implanting the wires into the brains⁴⁵² of mice expressing a human–mouse chimeric transgene.²⁰⁴ sCJD prions in brain homogenates were more than approximately 10⁵-fold more resistant to inactivation than Sc237 prions in brain homogenates. Inactivation of sCJD(MM1) prions bound to wires was achieved by exposure to 1% SDS and 0.5% AcOH for 15 minutes at 121°C.²⁸⁹ Whether acidic SDS might form the basis for a noncorrosive disinfectant that is suitable for sterilization of surgical instruments and some invasive diagnostic devices remains to be determined.¹³³ Other approaches to inactivation of prions that might contaminate surgical, medical, dental, and ophthalmologic equipment include proprietary phenolic and alkaline cleaning solutions, as well as enzymatic cocktails.^{108,173,317}

CONCLUSIONS

Much of prion biology is unprecedented. As such, it is not surprising that some aspects of this emerging field have been embraced rather slowly. Although prion replication resembles viral replication superficially, the underlying principles are quite different. For example, in prion replication, the substrate is a host-encoded protein, PrP^C, that undergoes a conformational change to form PrP^{Sc}. In contrast, viruses harbor a DNA or RNA genome that directs the synthesis of most, if not all, of the viral proteins. Viral replication requires faithful copying of the viral genome followed by assembly of mature virions, each of which contains of a nucleic acid genome surrounded by a protein coat. In contrast, prions seem to be composed solely of PrP^{Sc} trimers.

When viruses pass from one species to another, they often replicate without any structural modification, whereas prions undergo a profound change. The prion adopts a new PrP sequence, which is encoded by the chromosomal PrP gene of the host. Changes in the PrP amino acid sequence can result in a restriction of transmission for some species while making the new prion permissive for others. In viruses, different properties exhibited by distinct strains are encoded in the viral genome, whereas in prions, strain-specific properties are enciphered in the conformation of PrP^{Sc}.

Whereas viral diseases are infectious illnesses caused by a foreign infectious agent, prion diseases may manifest as inherited or sporadic disorders as well as infectious maladies. The inherited and sporadic forms are caused by the spontaneous, endogenous generation of prions, whereas the infectious prion diseases are caused by exposure to exogenous prions.

Whether PrP^{Sc} in mammals represents a misfolded protein or an alternatively folded protein, as with yeast prions,

remains to be established. The concept that PrP^{Sc} might be an alternatively folded protein is appealing in some respects but puzzling in others. If PrP^{Sc} is an alternatively folded protein with some as-yet undefined function, then all mammals seem likely to be capable of producing small amounts of this isoform. Sufficiently low levels of PrP^{Sc} would prevent extracts prepared from uninoculated controls from causing disease in inoculated recipients after prolonged incubation times. Having considered such a scenario, we must next inquire about how distinct conformations of PrP^{Sc} encipher the properties of different prion strains.¹²⁹ How such conformational variations might affect the “normal function” of PrP^{Sc} is unknown.

Wider Spectrum of Neurodegenerative Diseases Caused by Prions

Over the last three decades, there has been a steady accumulation of evidence that each neurodegenerative disease is caused by a particular protein.^{310,311} As with the prion diseases caused by PrP^{Sc}, amyloid deposits in other neurodegenerative diseases were found to have the same protein as that identified by molecular genetic studies. For example, the amyloid fibrils in Alzheimer's disease (AD) were found to contain the A β peptide^{137,241} that is cut from the larger amyloid precursor protein (APP); familial AD was shown to be caused by mutations in APP.¹³⁸ Subsequently, Tg mice expressing mutant human APP were found to have amyloid plaques composed of the A β peptide.^{122,165} Those Tg mice expressing APP with the Swedish mutation were found to exhibit accelerated accumulation of amyloid plaques and astrocytic gliosis after inoculation with brain homogenates from old Tg mice expressing the Swedish mutation (Table 76.9).^{150,252,416} Recently, it was reported that Tg mice expressing wt human APP developed A β plaques after inoculation with homogenates prepared from the brains of human AD patients.²⁶³

In AD, neurofibrillary tangles (NFTs), which are composed of the tau protein, are abundant within neurons.^{38,148,207,219,438} Both the A β amyloid plaques and the NFTs begin in the entorhinal cortex and spread both rostrally and caudally.^{34,35} These findings suggest that A β prions move from one neuron to another as A β accumulates in the extracellular space to form plaques. Many experimental findings argue that oligomers of A β stimulate neurons to induce a structural transition in tau that leads to its aggregation and subsequent deposition in NFTs.²¹¹ Notably, A β dimers isolated from the cortex of AD patients induced tau hyperphosphorylation and neuritic degeneration in cultured cells.¹⁷⁹

The secondary but important role of NFTs in the pathogenesis of AD was resolved when mutations in the tau gene were found to cause heritable tauopathies including familial frontotemporal dementia (FTD), inherited progressive supranuclear palsy (PSP), and Pick's disease,¹⁶⁹ but not familial AD. Aggregates formed of truncated recombinant tau were shown to enter cells and seed the polymerization of endogenous tau.^{116,149} Self-propagating tau aggregates not only pass from cell to cell in culture but also in the brains of Tg mice.^{75,389} It seems plausible that aggregates are released into the extracellular space after a cell dies; the aggregates then break apart and infect other cells. The formation of tau prions is likely to be the central event in the tauopathies including the sporadic and familial FTDs, PSP, Pick's disease, and chronic traumatic encephalopathy (CTE).

TABLE 76.9 Neurodegenerative Diseases Caused by Prions. For Each of these Disorders, There Is Good Evidence in Animals and/or Cultured Cells for a Particular Protein Adopting an Alternative Conformation, Which Becomes Self-Propagating

Prion diseases	Precursor proteins	Prion forms	Protein deposits	Self-propagation in mammals	Self-propagation in cells
CJD/scrapie	PrP ^C	PrP ^{Sc}	PrP plaque	inoc wt & Tg mice	N2a, GT1
Alzheimer's	APP	A β	A β plaque	inoc Tg(Δ APP) mice	
Tauopathies (FTD, PSP, Pick's, CTE)	tau	tau aggregates	NFT, Pick bodies	inoc Tg(HuTau), inoc Tg(HuTau,P301S) & inducible Tg(HuTau, Δ K280) mice	C17.2, HEK293
Parkinson's	α -synuclein	SNCA aggregates	Lewy body	inoc Tg(HuSNCA,H53T) mice & Lewy bodies in fetal human grafts	
ALS	Δ SOD1	Δ SOD1 aggregates	Bunina body		N2a
Huntington's	Δ Htt	Δ Htt aggregates	Nuclear inclusion		Cos7

Studies of athletes participating in contact sports have shown that some individuals develop FTD after repeated traumatic brain injury (TBI).⁸⁴ In boxers, this illness has been called punch-drunk dementia as well as dementia pugilistica,⁸³ but in football players, a similar progressive neuropsychiatric disorder, in which numerous NFTs accumulate in the frontal lobes, is commonly referred to as CTE.^{245,277} Thousands of soldiers who served in the Iraq and/or Afghanistan wars have had posttraumatic stress disorder (PTSD) with neuropsychiatric symptoms indistinguishable from those found in FTD and CTE. A recent report of a 27-year-old male Marine with PTSD, who committed suicide, argues that concussions from shock waves from roadside bombs (improvised explosive devices) can initiate a tau prion-mediated process indistinguishable from football players with CTE.²⁷⁶

Recent findings implicate a prion-like spread of misfolded α -synuclein in Parkinson's disease.^{9,264,275} In Parkinson's disease, the aggregated forms of α -synuclein are thought to begin to accumulate in the peripheral nerves that innervate the gut and spread rostrally via retrograde axonal transport to the substantia nigra where they are toxic to dopamine neurons.³⁶ With α -synuclein (SNCA), a protein whose overexpression and misfolding causes Parkinson's disease,^{299,366} studies with Tg mice expressing mutant HuSNCA(A53T) showed marked acceleration of disease after inoculation with brain extracts from older Tg(HuSNCA,A53T)83 mice.^{132,264} The uninoculated Tg83 mice developed neurologic dysfunction at approximately 435 days of age, whereas those inoculated intracerebrally with 20 μ l of a brain homogenate prepared from a 2-month-old Tg83 mouse exhibited CNS dysfunction at approximately 359 days of age. When Tg83 mice were inoculated with homogenates of the brains of two 18-month-old Tg83 mice containing insoluble aggregates of phosphorylated mutant α -synuclein, the mice showed abbreviated median survival times of 182 days and 193 days. These results support the hypothesis that α -synuclein prions mediate the pathologic deposition of synuclein aggregates, which is accelerated by inocula prepared from brains of old Tg83 mice that contained diffuse perikaryal inclusions of mutant α -synuclein.

In cell culture, the formation of fibrils of rec α -synuclein was required for the protein to enter the cell. These fibrils were

internalized via endocytosis, whereas monomers diffused passively across the membrane.²¹⁶ Endocytosis of α -synuclein aggregates resulted in cell death³⁸³ and the release of toxic aggregates into the extracellular space where they infected other cells. Like tau, α -synuclein has also been shown to pass from cell to cell in both culture and the brain.⁹⁵

Aggregates composed of mutant SOD1(H46R) causing familial amyotrophic lateral sclerosis (ALS) were able to cross into N2a cells by macropinocytosis where they nucleated aggregation of cytosolic, mutant SOD1 protein.²⁶⁶ Once initiated, mutant SOD1 aggregation was found to be self-perpetuating. Transfer of mutant SOD1 aggregates from cell to cell did not require contacts between cells but depended on the extracellular release of the aggregates. These findings contend that SOD1 aggregates are prions and the aggregation is a self-propagating process. In addition to mutant SOD, mutations in two RNA-binding proteins, TDP-43 and FUS, have been identified in patients with familial ALS. As well as being involved in numerous events related to RNA metabolism, each form aggregates in neurons in ALS and FTD.⁴⁰⁸ Recent evidence also indicates that both TDP-43 and FUS contain prion-related domains rich in glutamine and asparagine residues, and in the case of TDP-43, this domain contains a substantial number of disease-causing mutations.^{117,181}

The prion-like spread of protein aggregates has been demonstrated for polyglutamine peptides,³²⁰ which represent a key sequence motif in the huntingtin protein. Additional polyglutamine expansions in the huntingtin protein cause Huntington's disease, with the number of repeats generally related to the age of onset. Aggregates of chemically synthesized polyglutamine peptides killed cultured cells after endocytosis and transport into the nucleus.⁴⁵⁰ Following uptake, aggregates initiate conformational conversion of endogenous polyglutamine-containing proteins, resulting in persistent protein misfolding.^{44,407}

The ability to model disease states using synthetic peptides and recombinant proteins refolded into pathogenic conformations has enlarged the spectrum of prion diseases and opened new avenues of investigation into the molecular mechanisms of degenerative diseases. For example, systemic amyloidosis caused by serum amyloid A (SAA) may be transmitted by a

prion-like mechanism, and amyloid fibers composed of synthetic peptides corresponding to fragments of the SAA protein were found to accelerate the disease process.^{57,180,368,424,439,440}

In summary, the field of prion biology still seems to be in its infancy. Although the number of prions identified in mammals (now at more than half a dozen) and in fungi (now more than 10) will undoubtedly continue to expand, we have no idea about prions in all the other phylogeny.^{44,78,88,424} Numerous attempts to identify auxiliary molecules that participate in the replication of mammalian prions have been unrewarding, to date. There seems to be little support for the outdated notion that a viral-like nucleic acid, which encodes for and directs the synthesis of progeny prions, will someday be found.⁷⁸

Defining a physiologic role for mammalian PrP prions has eluded investigators, but the MAVS prion seems to be required for innate immunity to RNA virus infection and the CPEB prion appears to modulate long-term memory in the invertebrate *Aplysia*. Studies of yeast prions have delineated plausible functions for several of these proteins, whereas investigations of the [HET-s] prion have defined its complex functions in preventing heterokaryon incompatibility in *Podospora anserina*.

The mammalian prions composed of PrP, A β , tau, α -synuclein, SOD1, and huntingtin all cause distinct neurodegenerative diseases. In each of these disorders, the mammalian prion proteins adopt a β -sheet-rich conformation and readily oligomerize. The oligomeric states of mammalian prions are thought to be the toxic forms, and assembly into larger polymers such as amyloid fibrils seems to be a mechanism for minimizing toxicity. To date, there is not a single medication that halts or even slows a neurodegenerative disease caused by prions. This dilemma may be a bellwether of the unique pathogenic mechanisms that feature in each of the prion diseases and of the urgent need to develop informative molecular diagnostics and effective antiprion therapeutics.

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